STUDIES OF *EUGLENA* MUTANTS

STUDIES OF *EUGLENA* MUTANTS RESISTANT TO U.V. AND NITROSO COMPOUNDS

By

LEONARD ARNOLD MILLIS, B.Sc., M.Sc.

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

October, 1970

DOCTOR OF PHILOSOPHY (1970) McMASTER UNIVERSITY
(Molecular Biology) Hamilton, Ontario

TITLE: Studies of Euglena Mutants Resistant to U.V. and Nitroso Compounds

AUTHOR: Leonard Arnold Millis, B.Sc. (University of Alberta)
M.Sc. (University of Alberta)

SUPERVISOR: Dr. D. R. McCalla

NUMBER OF PAGES: vii, 80

SCOPE AND CONTENTS:

In order to compare the mode of action of mutagens which cause mass conversion of green Euglena to colorless forms lacking chloroplasts, mutants were selected either for resistance to U.V. light or for resistance to N-methyl-N-nitroso-p-toluenesulfonamide (MNTS). The U.V. resistant mutants selected showed strong resistance to U.V. but the slow growth of these mutants indicated that they probably carried multiple lesions. Some of the problems inherent in selection techniques were explored and discussed. A mutant selected for resistance to MNTS was found to have the ability to convert MNTS to an innocuous compound N-methyl-p-toluenesulfonamide. Experiments using the MNTS resistant strain provide information concerning the mode of action of MNTS and the related mutagen nitrosoguanidine.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I sincerely thank the following people for contributions to this thesis:

Dr. D. R. McCalla for his friendly guidance and encouragement throughout the research project and in the preparation of this manuscript,

Dr. I. Takahashi for his helpful discussions during the research programme, for helping with the analytical ultracentrifugation, and for critical reading of the manuscript,

Dr. A. Dingle for many useful suggestions throughout the research programme and for careful reading and criticism of the manuscript,

Mr. G. Cillis for drawing the figures,

Mrs. R. Millis for careful proof reading of the manuscript,

Miss J. Fink for careful editing and typing of the manuscript.

Financial support though fellowships from the National Research Council, and the National Cancer Institute is gratefully acknowledged.

INTRODUCTION

MUTATION

I. General Considerations

The term mutation was first introduced by De Vries in 1901 as part of a theory explaining the origin of new species (1). He defined mutation as any discontinuous heritable change which suddenly appears in any generation of a species. The term is used to describe changes not arising from a recombination of previously existing genetic material. The concept of mutation as introduced by De Vries is still used in basically the same form. Mutations can occur spontaneously or they can be induced, and can affect the genetic material at various levels as follows:

1. Whole genomes can be affected, i.e., the whole genome can be duplicated one or more times (polyploidy).

2. Whole chromosomes can be involved in the mutational event, i.e., one or more chromosomes may be deleted or added (aneuploidy).

3. Parts of chromosomes may be altered, e.g., large deletions, translocations, inversions.

4. Single genes may be changed. Nucleotides or nucleotide sequences could be deleted or added, changed, inverted or translocated.

5. Non-nuclear genetic determinants may be mutated, e.g., mitochondrial and plastid mutations.

De Vries and a number of other workers observed spontaneous mutations in both plants and animals. Muller (2) was successful in measuring the rate of spontaneous lethal mutations in Drosophila. De Vries (1) had suggested the possibility of artificially inducing mutations and Muller (3) in Drosophila and Stadler (4) in Hordeum vulgare (barley) successfully showed that X-rays were effective inducers of mutation. Following this, X-rays have been used to induce a variety of mutations in a wide spectrum of organisms.

The ability to induce mutations is a general property of ionizing radiations such as X-rays, γ-rays, α particles, and of U.V. light.

The first successful demonstration of chemical induction of mutation was in 1941 when Auerbach and Robson (5) discovered that β-β'-dichlorodiethylsulphide (mustard gas) was highly mutagenic in Drosophila. Independently Rapoport (6) showed that formaldehyde was also mutagenic in Drosophila. A vast number of chemicals have since been shown to be mutagenic in plants, animals, and microorganisms.

In De Vries' theory of evolution and in more modern theories, mutation is the basic source of variation upon which selection can act. Mutation may play an important role in certain human diseases such as cancer. The use of spontaneous and induced mutations has provided biologists with an extremely useful tool for genetic, biochemical, and other biological studies.

The mechanism by which mutations occur or can be induced is important to a more complete understanding of the origin, structure, and function of the genetic material.

II. Chloroplast Mutagenesis

This study is primarily concerned with an attempt to clarify the mechanisms by which mutational events occur.

The mutagenic system chosen is the chloroplast system of Euglena gracilis. Since the early part of this century, it has been noted that Euglena will, if grown in the dark, reversibly lose its chlorophyll. A number of irreversibly "colourless" strains of Euglena had also been selected and it was suggested that the wold colourless strains (Astasia) might have arisen from green autotrophs (see review by Pringsheim (7)). Provasoli et al. (8) in 1948 were able to induce apoplastidy (a permanent loss of chloroplasts) in Euglena gracilis var. bacillaris using streptomycin. They demonstrated that the "bleached" strains did not revert to the green form upon culture in the absence of the drug.

Since the first demonstration of chemical induction of apoplastidy in Euglena, a number of other treatments have been shown to have a similar effect. Exposure of Euglena to high temperatures (34-35°C) for several days (9), pyribenzamine (10) and other anti-histamines (11), erythromycin (12), kanamycin (13), O-methylthreonine (14), certain nitrofurans (15,16), certain methyl nitroso compounds (17,18), U.V. light (19,20,21), myxin (22), and other treatments have been shown to cause high numbers of apoplastidic cells in populations of Euglena.

A number of other mutagens can effect a loss of chloroplasts in Euglena, however, there seems to be a sharp distinction between those mutagens which cause "mass mutation" of the chloroplast system and those

which cause small amounts of bleaching. An effective "bleaching" agent is one which can transform essentially whole populations to the colourless form without killing an appreciable portion of the population of cells. Although the bleaching phenomenon is common to several species of Euglena (10), mass mutation of chloroplasts has not been reported in other organisms.

The Euglena chloroplast system provides:

(i) an opportunity to study mutation in the absence of killing,

(ii) a relatively simple assay system for the comparison of the effectiveness of certain mutagens,

(iii) a mutagenic system which might be employed to study chloroplast replication, development, and autonomy.

The phenomenon of mass mutation as seen in Euglena is in some respects similar to the mass induction of mitochondrial mutations in yeast (review by Ephrussi (23)). Certain yeasts can be transformed to the petite forms, which lack normal mitochondria, with a frequency approaching 1 (24,25). Since the demonstration that acriflavine was an effective inducer of mass mutation of mitochondria in yeasts (24), a number of physical and chemical agents including heat, X-rays, U.V. light, certain heavy metals, caffeine, camphor, allylglycine, various acridine derivatives as well as a number of other treatments have been found to be effective as agents of mass mutation in yeast (see review by Nagai et al. (26)).

Mass mutation contrasts sharply with classical examples of mutation induction. The frequency of mutation of most nuclear genes and the mutation frequency in prokaryotic cells is in general very low

relative to the degree of killing.

The end result of mutation, the change in phenotype, is expected to be the result of an alteration of the genetic material (usually DNA). Many mutagens have more than one detectable effect and possibly a number of undetectable effects on DNA. For example, methylating agents such as nitrosoguanidine are known to be capable of methylating guanine, cytosine and other components of DNA. Determination of specific chemical or physical effects of mutagens on DNA does not in itself indicate which, if any, of the alterations represent the mutagenic event. Most mutagens are fairly toxic under conditions used to induce mutation. Generally only a small fraction of the cells survive, with mutants representing only a small fraction of the survivors.

Experiments designed to study the chemical effects of mutagens on DNA often require doses considerably higher than would be tolerated by living organisms.

Similar if not more complicated problems exist when one is studying the earlier chemical events which might lead to mutation. Consequently, other biological and chemical information is necessary to decide which, if any, of the chemical effects of mutagens are important to mutation.

Although the same or similar molecular events may be involved in killing as in mutation, this may not be the case. Consequently, the more readily observed chemical effects of mutagens on cells might play an important role in killing, or represent relatively innocuous changes which have little to do with mutation. The rarity of detectable mutational events might reflect relatively rare chemical events.

The chloroplast system of Euglena, being extremely sensitive to mutation, provides some advantages over other systems with respect to the study of the chemical effects of mutagens. The advantage from this point of view may not be valid if the apparent sensitivity to mutation simply reflects a relatively high resistance to killing due to the fact that Euglena is probably a polyploid (likely octaploid (27)). If this is the case, then the chemical events leading to killing might represent the major chemical effects of the mutagen. The observation that primarily mutagenic events occur might not reflect the relative frequencies of the chemical events due to the redundancy of the nuclear gene.

The chloroplast system does, however, provide a relatively easily assayed mutagenic system and does provide an opportunity to study molecular events which may be more closely related to mutagenic than killing events.

The use of the Euglena chloroplast mutagenic system has been pursued in this study with two main goals in mind. 1. To help clarify the mode of action of certain mutagens, specifically MNTS, N-methyl-N-nitroso-p-toluenesulfonamide (MNTS), nitrosoguanidine (NG), and ultraviolet light (U.V.). 2. To determine what factors might contribute to the relatively extreme sensitivity of the chloroplasts to mutation.

The rationale for studying these particular mutagens will be presented following a discussion of some of the properties and effects of these mutagens.

BLEACHING AGENTS - MODE OF ACTION

I. U.V. Light

Ultraviolet light has been known to be mutagenic since the early work of Altenburg in 1934 (28). The chemical and physical effects of U.V. have been examined in considerable detail and only the relevant biochemical effects will be discussed here.

One of the significant discoveries which led to a better understanding of the mode of action of U.V. was the discovery that U.V. could reversibly induce thymine dimers in aqueous frozen solutions of thymine (29,30,31,32). This along with the discovery that thymine dimers could be detected in U.V. treated DNA (30,33,34) suggested a molecular reaction which might explain the killing and mutagenic effects of U.V. Thymine is more sensitive to dimerization than is cytosine. Thus the frequency of dimers in DNA is $\widehat{TT} > \widehat{TC} > \widehat{CC}$ (34). $\widehat{UT}$ and $\widehat{UU}$ dimers have been found as secondary products arising from deamination of cytosine containing dimers (34).

Many organisms have a highly effective repair system which utilizes visible light in the enzymatic splitting of pyrimidine dimers (photoreactivation) (35,36). Enzymes capable of *in vitro* destruction of pyrimidine dimers have been isolated from a variety of organisms (37). It is evident that the photoreactivating enzymes destroy pyrimidine dimers by monomerization in synthetic polynucleotides (38) and DNA (39). The *in vivo* photoactivated destruction of pyrimidine dimers, presumably by monomerization, has been detected in a number of organisms (37).

As well as having the ability to photoreactivate, many organisms are also able to undergo limited repair to U.V. damaged DNA under non-photoreactivating conditions (37). A number of mutants have been selected which show varying degrees of sensitivity to U.V. but little or no difference in the extent of pyrimidine dimers induced in DNA by U.V. (40,41,42). The pyrimidine dimers are removed from the DNA and appear in the soluble fraction of the cell (40,42). It is clear that dark repair is different from photoreactivation in that there seems to be little or no splitting of pyrimidine dimers during dark repair as is the case in photoreactivation.

Dark repair would seem to require that the regions of the DNA which possess pyrimidine dimers are removed from the DNA selectively from single strands so as to retain genetic integrity. One would then expect a resynthesis of the region removed using the parent homologous DNA strand as a template. This repair process is expected to require an endonuclease capable of making a single strand incision, an exonuclease, a polymerase, and a polynucleotide joining enzyme (37). Although the enzymes involved in dark repair have not been clearly identified, enzymes capable of catalysing the supposedly required reactions have been isolated and purified. Endonucleases capable of single strand incisions (43,44,45), exonucleases capable of degrading single strand regions of DNA (37) and polynucleotide joining enzymes (ligases) (46,47,48,49,50) have been characterized. That repair replication does occur is consistent with the observation that limited replication of small, scattered single strand regions of DNA occurs during the dark repair process (51,52,53).

The existence of photoreactivation and dark repair systems likely constitutes a protective advantage to the organism due to the abundance of U.V. in sunlight (36).

Although numerous studies have implicated the biological importance of pyrimidine dimers in killing and mutation, U.V. also has a number of other effects on nucleic acids. U.V. causes local denaturation (54), lowered thermal denaturation temperature (55,56), strand breakage (55,56), loss of transforming activity (56), and other chemical alterations of DNA including cytidine hydration (57). U.V. also affects proteins and is known to cross-link protein with DNA (58). The biological importance of these phenomena is at present unclear.

U.V. is of particular interest to this study due to the well documented effects of U.V. on the chloroplast system of Euglena.

U.V. causes a high degree of bleaching. The action spectrum of inactivation of chloroplast forming ability shows peaks at 260 mμ and 280 mμ suggesting a nucleoprotein as the receptive chromophore (59).

The bleaching of Euglena is highly photoreactivable, the most effective photoreactivation occurring in the near U.V. region of the visible spectrum (60). Photoreactivability is lost if cells are allowed to divide, but is retained in nondividing cells. There is no detectable photoreactivation in the green and red regions of the visible spectrum (500-700 mμ). Dark grown cells, possessing only proplastids and little or no chlorophyll, can form mature chloroplasts and synthesize chlorophyll when exposed to red light. It has been possible to demonstrate that proplastids can mature and chlorophyll

can be synthesized under nonphotoreactivating conditions in cells exposed to bleaching doses of U.V. When cells are grown in red light after U.V. treatment, no photoreactivation occurs and colonies arising from the exposed cells are bleached (61).

On the basis of multiplicity of inactivation sites, estimated at 30, the low degree of killing, the presence of sectored colonies in some experiments, and related information, it has been suggested that the targets in U.V. bleaching are cytoplasmic (61).

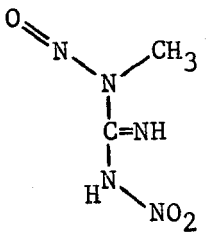
It was felt that nuclear ploidies of the order of 30 were unlikely whereas this number corresponded well to the number of proplastids seen by fluorescence microscopy in dark grown cells (59).

That the entities sensitive to U.V. are cytoplasmic has been demonstrated directly by Gibor and Granick (62). By using appropriate shading techniques they were able to irradiate selected areas of single cells with U.V. They demonstrated that bleaching occurred only if the cytoplasm or the whole cell was irradiated. Irradiation of the nucleus only did not bleach.

Chloroplasts are known to contain DNA (63,64,65). The chloroplast DNA has a relatively high thymine content. This has led to the idea that chloroplast DNA may be particularly sensitive to U.V. due to the presence of a relatively high frequency of adjacent thymine residues.

II. Nitrosoguanidine

Nitrosoguanidine (NG) was first synthesized in 1946 by McKay and Wright (66). The structure is given below:



NG is an effective mutagen (67,68,69,70), carcinogen (71,72), and shows weak antitumor (73,74) and antileukemic (75) activity in mice. NG is an effective bleaching agent in Euglena (18). NG has been shown to inhibit transforming DNA in vitro, as well as inhibiting growth, protein synthesis, RNA synthesis, and DNA synthesis in E. coli (76). NG also inhibits protein and nucleic acid synthesis in Euglena (77).

It has been suggested that the interference with protein synthesis in E. coli at pH 5 may be a major cause of lethality. The presence of streptomycin enhances the survival of NG treated streptomycin-resistant cells, suggesting that NG might interfere with reading of the genetic code (78).

NG has been considered to be an effective methylating agent since NG reacts with aniline to yield N-methylaniline; while under similar conditions diazomethane did not methylate aniline (79). When NG is treated with aqueous KOH, the product is diazomethane. McCalla and Reuvers (80) have shown that NG in neutral solutions reacts with proteins to give nitroguanido derivatives (e.g., lysine is converted to nitrohomoarginine). They have suggested that the reaction with protein accelerates the production of diazomethane.

Under acidic conditions NG is rapidly destroyed with N-methyl-N'-nitroguanidine being the principal stable product (81). The nitroso

group is apparently lost as nitrous acid (66). At high pH, NG yields diazomethane (82) and a mixture of other products as yet not clearly identified (81). Neither nitrous acid (18) nor diazomethane (83) bleach Euglena, which suggests that it is NG or other breakdown products that is responsible for bleaching effects. NG methylates DNA in vitro to give 7-methylguanine as the major product (84); however, there is little evidence to show that this is the mechanism involved in mutagenesis.

III. N-Methyl-N-nitroso-p-toluenesulfonamide

N-Methyl-N-nitroso-p-toluenesulfonamide (MNTS), like NG, is an effective bleaching agent in Euglena (85). MNTS is, however, reportedly non-mutagenic (86) and non-carcinogenic (87). MNTS, like NG, yields diazomethane under alkaline conditions (88). Under acidic conditions MNTS decomposes to give N-methyl-p-toluenesulfonamide in 50% yield and -p-toluenesulfonamide in 35% yield (81). The nitroso group is presumably lost as nitrous acid (81). The decomposition of NG and MNTS are somewhat analogous at high and low pH; however, they differ significantly in that NG is relatively unstable at high pH and relatively stable at low pH as compared to MNTS (81).

Although both MNTS and NG seem to behave similarly in some chemical reactions, they do differ in biological activity and certain chemical reactions. Schulz and McCalla (89) have found that MNTS and NG react differently with cysteine. The reaction of NG with cysteine yields diazomethane precursors while MNTS does not. This is particularly interesting since cysteine stimulates methylation of DNA by NG in vitro, while inhibiting methylation by MNTS.

PROTECTIVE AGENTS

Since this work includes a study of the *in vivo* breakdown of MNTS, it is relevant to review studies of protective agents and their effects on MNTS inactivation.

It has been known since the work of Barron in 1946 (90) that sulfhydryl containing enzymes such as phosphoglyceraldehyde dehydrogenase, adenosinetriphosphatase and succinoxidase are inactivated by X-rays, and that the effects of irradiation can be partially reversed by treatment with glutathione. Patt (91) showed that cysteine had a protective effect towards X-rays when injected into rats prior to exposure to lethal doses of irradiation. A number of protective agents have been discovered and sulfhydryl containing compounds are particularly effective in protection against X-rays (92).

Sulfhydryl containing compounds have also been found to have a protective effect against certain radiomimetic chemicals. This was first demonstrated by Weisberger and Heinle (93) in 1950 who showed that cysteine protects rats and mice against leucopenia if administered prior to treatment with nitrogen mustard. Brandt and Griffin found that rats and mice showed a 6- to 8-fold resistance to two nitrogen mustards when administered cysteine prior to exposure to the carcinogens (94). The effect was noted as a reduction in toxicity, although there was no noticeable reduction in the leucopenia effect of the drugs. Other amino acids and reducing agents such as methylene blue, glutathione, thioglycolic acid, and others had no apparent effect.

Weisberger *et al.* (95) found that only compounds with amino, sulfhydryl, and carboxyl groups in close apposition were effective

protective agents. The study involved the protection of rabbits to leucopenia after exposure to mustard gas. Compounds such as 2-cysteine, DL-homocysteine and glutathione were the most protective. Methylation of the SH group or the carbonyl, or the amino group was absent, protective activity was greatly reduced. It was suggested that it was unlikely that mustard gas decomposes in the presence of thiols, but rather that the thiols protected some vital substances in the cells.

McCalla (84), in a study of the methylation of DNA by NG and MNTS, has shown that the presence of cysteine inhibits methylation by MNTS, while increasing the methylating ability of NG. The reaction of cysteine with NG results in nitrogen gas evolution suggesting a diazomethane intermediate. On this basis it was suggested that cysteine "protects" by inactivation of MNTS, whereas the lack of protection of NG is the result of activation of NG by accelerating diazomethane production. Schoental and Rive (96) have shown that methylnitrosourea (MNU) and methylnitrosomethane react with cysteine to give a mixture of reaction products including some which are unstable. N_2 is evolved in the reaction suggesting diazomethane as an intermediate. Schulz and McCalla (89) have studied the reaction of MNTS and NG with cysteine. They found that the reaction of MNTS with cysteine results in the production of N-methyl-p-toluenesulfonamide (MTS) in 93% yield. Since MTS is relatively innocuous and is not a diazomethane precursor, it is presumed that the loss of methylating activity of MNTS is due to its conversion to MTS.

Under similar conditions nucleophilic attack on the imino carbon of NG produces a methylating agent. These results help to explain the increase in methylation by NG and the decrease of methylation by MNTS in the presence of cysteine and support the notion that protective agents might act by inactivating mutagens or carcinogens rather than directly protecting vital substances such as nucleic acids or proteins.

EXPERIMENTAL DESIGN

MNTS and NG, although chemically similar, differ in many respects. Some of these differences might be attributed to different stabilities at various pH values. It is of interest to see whether MNTS and NG both affect the Euglena chloroplast system in the same manner. With this in mind, mutants of Euglena were selected for resistance to MNTS with the aim of studying their cross resistance to other mutagens, particularly NG. McCalla (97) had previously isolated an NG resistant mutant which showed resistance to chloroplast inactivation but not to killing; however, this mutant seemed to lack any specific chemical resistance and its resistance was likely due to relatively low permeability. Its usefulness in comparing the mode of action of mutagens was limited.

It was felt that U.V. resistant mutants might also be useful in cross resistance studies, particularly since the effect of U.V. on the chloroplast system of Euglena is relatively well understood.

The mutant selection programme led to the isolation of mutants

which showed resistance to MNTS and little or no resistance to NG. The work of McCalla (84) suggested a difference in activity of MNTS and NG might be partially based on different inactivation properties of compounds such as cysteine. Schulz and McCalla (89) demonstrated that MTS was the principal product in the in vitro inactivation of MNTS by cysteine. It was felt that a study of the in vivo breakdown of MNTS to MTS in the resistant and normal strains might provide information about the basis of the resistance. As well, a knowledge of the in vivo breakdown of MNTS in resistant and normal strains would be useful in establishing whether breakdown products such as MTS play a role in bleaching and killing Euglena.

Breakdown studies were initiated in the wild type (strain Z) and a resistant strain of Euglena (strain MNTS 40A). These studies were carried out with MNTS by following the conversion of N-^{14}C-methyl-N-nitroso-p-toluenesulfonamide to N-^{14}C-methyl-p-toluene-sulfonamide. These two labeled compounds are easily extracted from whole cells with ether. Their separation and identification is relatively simple as will be described in detail in the next section.

In order to examine the relative effects of medium and living cells, breakdown of MNTS to MTS was studied independently in medium lacking cells and in log phase cultures. After establishing that the breakdown of MNTS appeared to be "catalysed" by living cells and not by medium from which cells had been harvested, and was enhanced in the mutant strain, the breakdown was studied in cells killed by sonication.

At this point it was evident that the next step in examining

the basis of the resistance in the mutant strain would be to try to identify the reactive species in the mutant strain responsible for inactivation. It was felt that the complexity of this problem necessarily eliminated further studies along these lines.

The role of MNTS and its breakdown in the living cell in relation to mutagenesis and killing in Euglena is the central theme of this thesis. The experiments mentioned above will be discussed in some detail.

MATERIALS AND METHODS

I. Cell Cultures

The organism used in these studies was Euglena gracilis strain Z originally obtained from Dr. S. H. Hutner. Unless otherwise indicated, all liquid cultures were essentially the pH 6.8 medium of Cramer and Myers (98) with changes as indicated in Table I. The carbon source used was 1% glucose, autoclaved separately. The pH of the medium was found to be close to pH 6.8 and the pH was not adjusted. The medium with glucose will be referred to as 6.8 medium.

Cultures on solid medium were on 6.8 medium with 1.5% bacto agar (Difco Laboratories, Detroit, Michigan) added as a gelling agent. Cultures were stored at 0-3°C on tryptic soy agar (Difco), (4%), for periods up to 6 months.

Cultures were grown at approximately 26°C under continuous lighting from cool white fluorescent lamps. Cultures to be grown in the dark were handled as follows: Flasks or tubes containing cultures were wrapped with foil and placed in a dark cupboard (approx. 26°C). Plates were placed in a light tight box and the box placed in a dark cupboard.

Cell concentration was determined by measuring absorbance at 750 mμ in a Spectronic 20 colorimeter (Bausch and Lomb, Rochester, New York). It was established by comparisons with a Coulter counter and by plating experiments that an optical density (O.D.) of 1.0 in a

TABLE I

Euglena growth medium, pH 6.8

Contents	Concentration (gm/ℓ)	
1. $(NH_4)_2HPO_4$	1.0	
2. KH_2PO_4	1.0	
3. $MgSO_4 \cdot 7H_2O$	0.4	(c.m. specify $MgSO_4$, 2 g/ℓ)
4. $Na_3C_6H_5O_7 \cdot 2H_2O$	0.5	(c.m. specify $Na_3C_6H_5O_7 \cdot HH_2O$, 0.8 g/ℓ)
5. $CaCl_2$	0.02	
6. glucose	0.1	
	(mg/ℓ)	
7. $Fe_2(SO_4)_3 \cdot 6H_2O$	3.0	
8. $MnCl_2 \cdot 4H_2O$	1.8	
9. $Co(NO_3)_2 \cdot 6H_2O$	1.3	
10. $ZnSO_4 \cdot 7H_2O$	0.4	
11. $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.2	(c.m. specify H_2MoO_4, 0.2 mg/ℓ)
12. $CuSO_4 \cdot 5H_2O$	0.02	
13. Thiamine	0.01	
14. Vitamin B_{12}	0.0005	
15. H_2O	1 ℓ	

Items 7 to 12 inclusive were added from a more concentrated mixed stock solution.

1 cm path length tube corresponded to approximately 1 x 10^6 cells/ml. The optical density for green cultures cells did not differ appreciably from that for bleached cultures of the same cell concentration. Dilutions, where required for plating experiments, were done in distilled water.

Unless otherwise indicated, all experiments utilizing potential growth medium or Euglena were carried out under sterile conditions. Medium, glassware, pipets, and other apparatus were sterilized by autoclaving at 121°C for 15 minutes.

II. Chemicals and Apparatus

Chemicals used in media and solutions were reagent grade or the purest available commercially. N-Methyl-N-nitroso-p-toluenesulfonamide (MNTS) and N-methyl-N'-nitro-N-nitrosoguanidine (NG) were obtained from Aldrich Chemical Company Inc., Milwaukee, Wis. N-Methyl-p-toluenesulfonamide was synthesized by A. Reuvers according to the method of De Boer and Backer (99). N-Methyl-^{14}C-N-nitroso-p-toluenesulfonamide (Specific Activity 3.32 mc/mM) was obtained from New England Nuclear, Boston, Mass. Radiochromatography indicated greater than 99% purity as determined in two solvent systems by New England Nuclear.

For ultraviolet irradiation experiments a model SL 2537 mineralight lamp (Ultra-Violet Products Inc., South Pasadena, California) was used. Aeration of cultures utilized laboratory compressed air, moistened by passing the air through water filled gas dispersion bottles. The air was passed through sterile cotton filters and then bubbled through 300 ml cultures in 500 ml erlenmeyer flasks.

Radioactivity was determined using a Mark I scintillation system (Nuclear-Chicago, Des Plaines, Illinois). Quench correction was made using the channel ratios method as described in the Mark I instruction manual. The solvent systems used are indicated in Tables II and III.

III. Selection of Resistant Strains

(a) U.V. Resistance

Euglena cultures were grown to an $O.D._{750}$ of approx. 0.20 in 6.8 medium lacking glucose and irradiated in 40 ml volumes in 2.6 cm diameter quartz test tubes, with stirring, at a distance of 14 cm for various periods of time in the dark. The suspensions were stirred and irradiated for various periods of time up to 135 minutes. After irradiation, the 40 ml cultures were introduced into 300 ml 6.8 medium lacking glucose and placed in the dark for 7 days. Following the dark period, the cultures were aerated in the light for periods up to 8 weeks to allow any green survivors, that could use CO_2 as a sole carbon source, to grow. Flasks containing green cultures were sampled and the samples plated on 6.8 medium + 1.5% agar. Several clones from each sample were transferred to 6.8 liquid medium and then plated again on agar and colonies were selected for further studies.

(b) MNTS Resistance

Selection for resistance to MNTS was based on the same principles as the U.V. selection. Cultures grown in 6.8 medium lacking glucose (300 ml in 500 ml erlenmeyer flasks) were treated with various doses of MNTS. The MNTS was introduced in 1 ml volumes

TABLE II

Toluene base scintillation liquid

	Contents	Quantity
1.	Toluene	1,000 ml
*2.	2,5-diphenyloxazole (PPO)	.4 gm
*3.	2-p-phenylenebis(5-phenyloxazole) (POPOP)	.05 gm

* Items 2 and 3 were added as a concentrated solution of PPO and POPOP in toluene (spectrafluor) (Amersham/Searle, Des Plaines, Illinois).

TABLE III

Dioxane-anisole base scintillation liquid

	Contents	Quantity
1.	dioxane	750 ml
2.	anisole	125 ml
3.	1,2-dimethoxyethane	125 ml
4.	PPO	12 gm
5.	POPOP	0.5 gm

of a suitably concentrated solution of MNTS in 95% ethanol. After addition of MNTS, the flasks were placed in the dark for 24 hours. The flasks were then aerated for periods up to 8 weeks or until green cultures appeared in the flasks.

Samples from flasks which turned green were plated and colonies selected and recloned as described above for selection of U.V. resistant mutants.

IV. Testing for U.V. Resistance

Strain Z and strains selected from cultures which survived high doses of irradiation were tested as follows. Cultures were grown to $O.D._{750} \approx 0.30$, diluted to 1000 cells/ml, and 0.2 ml volumes were spread on agar surfaces in petri dishes. The plates were irradiated for various time intervals at a distance of 21 cm from the surface of the agar to the filter of the lamp. The covers were removed during irradiation.

After irradiation, plates were placed in a dark box and this placed in a dark cupboard at room temperature for 4 days. Following the dark period, plates were placed in the light to allow cells to turn green and continue growing. When colonies reached a readily visible size (approximately 7 days), the plates were scanned and the numbers of green and bleached colonies recorded. A colony was considered green if any portion of the colony was green, i.e., sectored colonies were scored as green. Colony counting and classification were aided by the use of a low-powered binocular microscope.

V. Testing for MNTS Resistance

Colonies selected for resistance to MNTS were suspended and

grown to log phase in 6.8 medium. Two methods were used to compare the resistance of selected strains with strain Z.

(a) Gradient plate method (100)

The method involves making an agar surface which has a linear concentration gradient. This was done by pouring a layer of agar containing dissolved MNTS on the bottom of a square petri dish raised on one side as illustrated in (i), Figure 1. The agar was allowed to cool and the plate was then placed on a level surface and an overlying layer of agar without MNTS was poured ((ii), Figure 1). The overlying layer was then allowed to cool. The resulting plate was then considered to have a gradient of increasing concentrations from left to right after diffusion of MNTS took place ((iii), Figure 1).

Strain Z and selected strains were streaked on to the surface of the agar using small paint brushes ((iii), Figure 1). The plates were placed in the dark for 24 hours and then placed in the light until growth was sufficient to be easily seen without the aid of a microscope (usually about 7 to 10 days). Relative resistance was assumed if a strain grew considerably further along the concentration gradient than did strain X, or if a similar situation existed with respect to green colour (see IV, Figure 1).

The gradient plate method was used as a preliminary method of screening for MNTS resistant strains.

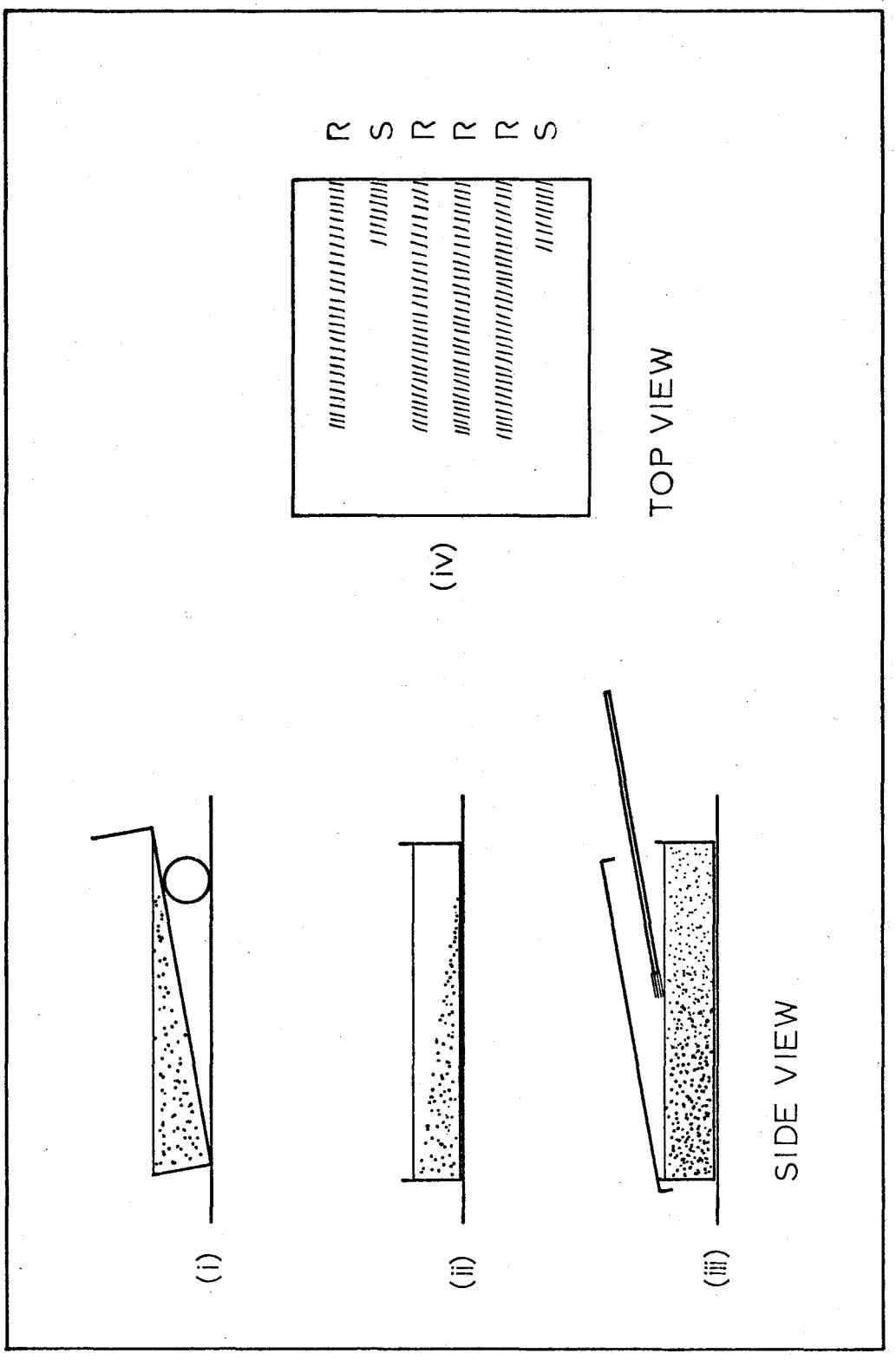
(b) Direct testing for MNTS resistance

Strains showing apparent resistance in the gradient testing were tested further by the exposure of cells to measured concentrations of MNTS and then plating the cells on agar. The technique

Figure 1. Illustration of gradient plate technique used to compare strains for MNTS resistance.

R = resistant

S = sensitive


R
S
R
R
R
S
TOP VIEW
(iv)
(i)
(ii)
(iii)
SIDE VIEW

involved growing cells to $O.D._{750} \approx 0.30$, and adding 1 ml of cell suspension to 4 ml 6.8 medium containing MNTS at appropriate concentrations. The medium also contained .04 ml ethanol (from the original solutions of MNTS made up in ethanol and subsequently diluted into 6.8 medium). The cultures containing cells and MNTS were then placed in the dark for 24 hours. Following this the cells were diluted appropriately, spread on agar plates, and placed in the light until colonies were readily visible. Colonies were counted and classified as green or bleached.

Cross resistance studies for NG resistance were as described for MNTS, except that concentrations differed in both gradient plate experiments and some direct experiments.

VI. Studies of Decomposition of MNTS

MNTS breakdown in Euglena cultures was studied in strain Z and strain 40A (an MNTS resistant strain which was selected) on a comparative basis. MNTS breakdown to N-methyl-p-toluenesulfonamide was followed in the two strains to determine whether the resistant strain had a more rapid decomposition rate. Cells were treated with ^{14}C MNTS in 6.8 medium in 100 ml volumes in the dark. In preliminary experiments 10 µg/ml MNTS was used along with the labeled MNTS to bring the effective concentration to approximately 10 µg/ml. This was done to bring the concentration of MNTS to a level at which strain Z was approximately 100% bleached, whereas strain 40A showed essentially no bleaching. Since this concentration might also inhibit growth over the short time periods of the experiment, later experiments were conducted with ^{14}C labeled MNTS without carrier added with

resulting concentrations being less than 1 μg/ml.

Cells were grown to $O.D._{750}$ 0.30 in 100 ml volumes of 6.8 medium. An attempt was made to do experiments on $O.D._{750}$ 0.30 cells with as little dilution as possible. In some cases adjustment of cell concentration was necessary. Cultures were used if $O.D._{750}$ was between 0.30 and 0.35 with adjustment to $O.D._{750}$ 0.30 by addition of fresh pH 6.8 medium. The cell density 0.30 was chosen since cells were approximately in mid-log phase at this density.

Cells treated with ^{14}C MNTS were treated in different ways depending on the aim of the experiment. In preliminary experiments cells were spun down, washed with 6.8 medium, resuspended and adjusted to O.D. 0.30 before treatment. ^{14}C MNTS was dissolved in 95% ethanol and added to the cell suspension in volumes of 1 ml per 100 ml suspension. The flasks were wrapped in aluminum foil to exclude light and were kept dark throughout the experiment.

At intervals, 5-ml samples were removed and shaken for one minute with an equal volume of ethyl ether in a small separatory funnel. The aqueous layer was sampled and 1 ml of the sample added to 10 ml dioxane: anisole base scintillation fluid for radioactive counting. The ether phase was sampled and suitably sized aliquots dried on 1" metricel filter (0.45 μ pore size) (Gelman Instrument Co., Ann Arbor, Michigan). The filters were placed in vials containing toluene base scintillation fluid (MNTS and MTS are soluble in this scintillation system) for radioactive counting. Quench correction was made using the channel ratios method. The ether phase was also processed further for MNTS and MTS determinations.

In order to determine the relative amounts of MNTS and MTS in the ether layer, 10 µl samples were chromatographed on thin layer plates (silica gel GF 254, E. Merck - supplied by Canadian Laboratory Supplies, Toronto). Approx. 1 µg MNTS and MTS were added as markers. The ascending method was used and the developing solvent was 3% methanol in benzene. Spots were visualized using a U.V. lamp.

In preliminary experiments the plates were scanned using a Packard Radiochromatogram Scanner Model 7200. The radioactivity in MNTS and MTS was estimated either by measuring the area under respective peaks or by cutting the peaks from the recording paper and weighing them. In later experiments, spots were removed from the plates by loosening the silica gel with a spatula and sucking the powder into scintillation vials containing 10 ml toluene base scintillation fluid. Radioactive counting was then done directly in a Nuclear Chicago Mark I scintillation counter.

VII. Decomposition of MNTS in 6.8 Medium

Measurement of MNTS breakdown in 6.8 medium was carried out by a method similar to the breakdown studies in whole cells described above. Instead of using a cell suspension, 100 ml of 6.8 medium was treated and extracted. Other procedures were identical to those described above.

VIII. MNTS Breakdown in Killed Cells

The breakdown of MNTS in killed cells was studied as described above except that cultures were first exposed to sonication before treatment with ^{14}C MNTS. Sonication was carried out using a Branson

Sonic Power Sonicator, Model S125, Branson Instruments, Inc., Danbury, Connecticut. The sonication was for three 60-second periods at maximum output of the sonicator. At this exposure no intact cells could be found upon microscopic observation. All cells appeared to be fragmented extensively.

IX. Nucleic Acid Labeling Experiments

In order to determine the relative labeling of nucleic acids in strains Z and 40A, cells were exposed to ^{14}C labeled MNTS, nucleic acids extracted and activities estimated as described below.

Cultures were grown to $O.D._{750}$ 0.30 or adjusted to that density (from $O.D._{750}$ 0.30-0.35) in 100 ml volumes and exposed to ^{14}C MNTS at a concentration of less than 1 μg/ml. The MNTS was introduced from a solution of ^{14}C MNTS in methanol (1 ml solution per 100 ml culture). After three hours, cells were centrifuged down and nucleic acids isolated by the method of Marmur (101) modified as described below. The solutions used in the extraction are given in Table IV.

TABLE IV

Solutions used in nucleic acid isolation

saline-EDTA	0.15 M NaCl, 0.1 M ethylenediamine-tetraacetate (EDTA), pH 8
sodium lauryl sulfate	25%
sodium perchlorate	5 M
chloroform-isoamyl alcohol	24:1 (v/v)
ribonuclease	0.2% Bovine pancrease Type 1-A Sigma Chemical Co., St. Louis. Heated to 80°C for 10 min to destroy any contaminating deoxyribonucleases.

Centrifuged cells were washed twice in saline-EDTA and suspended in 2 ml saline-EDTA in a conical 15 ml centrifuge tube. Sodium lauryl sulfate (0.2 ml) was added and the tube placed in an ice bath for 10 minutes. Sodium perchlorate (1 ml) solution was added and the mixture was shaken with chloroform-isoamyl alcohol for 5 minutes. The resulting mixture was centrifuged at low speeds in a clinical centrifuge until three distinct layers were evident (10 min). The lower chloroform-isoamyl alcohol layer and the middle protein layer were discarded. The upper aqueous layer was deproteinized twice more by shaking with chloroform-isoamyl alcohol.

The aqueous layer containing the nucleic acids was sampled for radioactive counting. Usually 50 µl was sufficient to give sufficient count rates. An estimation of nucleic acid content was based on optical density measurements taken on a model DB Beckman spectrophotometer at 260 mµ.

In order to estimate the radioactivity in DNA, the nucleic acid layer was treated with 50 µg/ml ribonuclease for 30 minutes at 60°C. The mixture was deproteinized twice with chloroform isoamyl alcohol. Following this, macromolecular DNA was separated from small fragments and mononucleotides using Sephadex G-25. This was achieved by adding approximately 0.5 ml volume of dry Sephadex to 1 ml of solution of nucleic acid components in a plastic centrifuge tube. A small puncture was made in the bottom of the plastic tube, and the plastic tube was placed inside a 15 ml conical centrifuge tube. After centrifugation at low speeds, some liquid was expelled through

the hole in the plastic tube (approximately 0.25 ml). This expelled liquid was diluted to 1 ml and again treated with Sephadex in the same manner. The DNA solution was sampled for radioactive counting and optical density measurements.

Samples for counting of radioactivity in total nucleic acids and in DNA were taken using disposable micropipets (Corning). The samples were drained on to the surface of millipore filters, allowed to dry, and counted in toluene base scintillation fluid.

X. Determination of radioactivity in 7-methylguanine

In order to determine the extent of formation of 7-methylguanine in strains Z and MNTS 40A, the following procedure was used. Cells in log phase ($O.D._{750}$ 0.30) were treated with ^{14}C-MNTS for 5 hours in the dark. Total nucleic acids and DNA were isolated as described in the previous section. The nucleic acids were hydrolysed in 1 N HCl at 100°C for 1 hr. The hydrolysate was chromatographed on Dowex 50-X2 in a 1 cm x 9 cm column using a 1.0 N to 2.0 N HCl gradient. Fractions were identified by absorption spectra and by chromatography of authentic standards on the same column. The desired fractions were evaporated to dryness, using a rotary evaporator, and resuspended in 0.5 ml 1 N HCl. The concentrated samples were neutralized with 1 N NaOH and counted in a dioxane base scintillation fluid. Counting efficiency was determined by the channel ratios method.

XI. Determination of cellular acid-soluble thiol concentration

Whole cells of strains Z and MNTS 40A were assayed for the presence of thiols according to the modification of Ellman's

procedure (102) described by Lawley and Thatcher (103). Approximately 6.2×10^6 cells (estimated from $O.D._{750}$) were centrifuged into a pellet in a conical centrifuge tube. The supernatant was removed and the cells suspended in 1 ml 5% trichloroacetic acid (T.C.A.). The cells were sedimented by centrifugation and 0.5 ml of the T.C.A. extract was added to 3.5 ml of a solution 5,5'-dithiobis-(2-nitrobenzoic acid) (200 µg/ml in 0.2 M sodium phosphate buffer; pH 7.6). The absorbance at 410 mµ was measured after 2 minutes and the thiol content determined from the value ε_{max} 410 = 1.36×10^4/mole/cm. The coloured reaction product is in a 1:1 molar ratio with the thiol (102).

XII. Analysis of chloroplast DNA

Cultures for DNA extraction were grown in 6.8 medium in 1500 ml volumes in low form erlenmeyer flasks. Cells in log phase were harvested by centrifugation and DNA isolated as described in section IX of this chapter with the following changes.

Centrifuged cells were washed twice with saline EDTA, and suspended in 10 ml saline-EDTA. Sodium lauryl sulfate (1 ml) was added and the mixture shaken with an equal volume of chloroform-isoamyl alcohol for 5 minutes. The deproteinization was repeated twice. Ethanol (2 volumes of 95%) was carefully added and the nucleic acids spooled out of the mixture as described by Marmur (101). The nucleic acids were dissolved in 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0 (S.S.C.) and then treated with 50 µg/ml ribonuclease. The mixture was deproteinized and the DNA precipitated with ethanol. The DNA was dissolved in S.S.C. and the concentration adjusted to

approx. 250 µg/ml.

For ultracentrifugation analysis the following mixture was prepared.

Englena DNA solution	0.22 ml	(approx. 50 µg DNA)
PBS1 DNA solution	0.01 ml	(approx. 2.0 µg DNA)
$CsCl_2$ solution (concentrated)	0.84 ml	
H_2O	0.01 ml	

The refractive index was adjusted to 1.400 ± .002 by addition of water or $CsCl_2$ solution. The refractive index was determined using a Bausch and Lomb No. 334558 refractometer.

The above preparation was centrifuged in a Beckman Model E analytical ultracentrifuge for 20 hours at 44,770 revolutions per minute at 20°C.

Photographs were taken using U.V. optics and prints made on Kodak Kodabromide paper.

RESULTS AND DISCUSSION

I. Selection for U.V. resistance

The irradiation exposures used for selection experiments corresponded to doses sufficient to kill almost 100% of the population. The doses were far greater than those required to bleach 100% of the cells. Typical killing and bleaching data are given in Table V and Figure 2 respectively. The data are based on irradiation of cells under the same conditions as irradiation for selection of resistant mutants.

TABLE V

Survival of *Euglena gracilis* strain Z

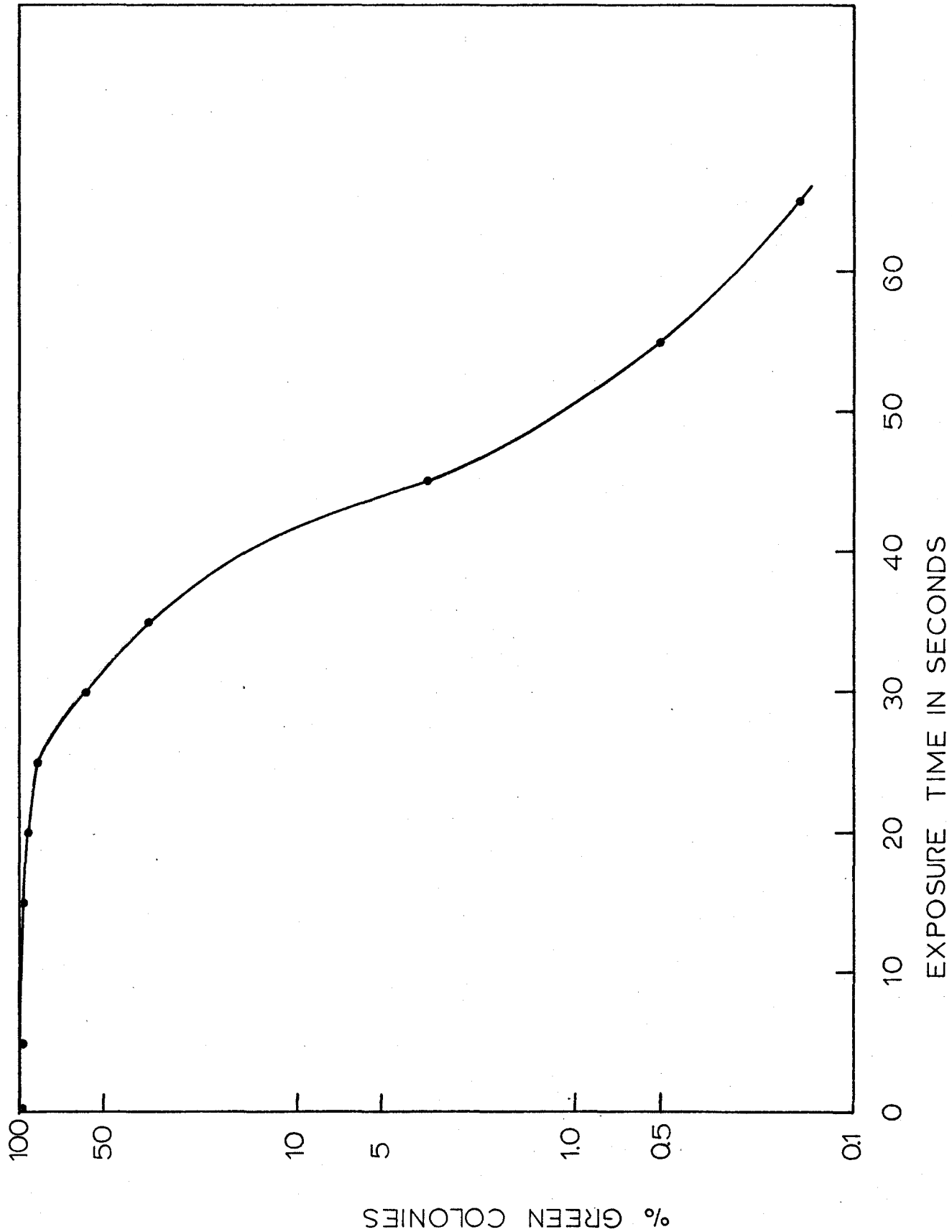
after high doses of U.V. irradiation

Dose in Minutes	No. of Plates	Total No. of Cells Plated	No. of Surviving Cells (colonies)	Surviving Fraction
0	10	1.5×10^6	*TNC	
5	10	1.5×10^6	TNC	
10	10	1.5×10^6	TNC	
20	9	1.35×10^6	46	0.33×10^{-4}
40	9	1.35×10^6	24	0.18×10^{-4}
70	9	1.35×10^6	4	0.29×10^{-5}
130	11	1.50×10^6	0	$< 0.67 \times 10^{-6}$
190	8	1.20×10^6	0	$< 0.83 \times 10^{-6}$

All colonies were bleached except for 0 dose.

*TNC = Too numerous to count.

Figure 2. U.V. bleaching curve for Euglena gracilis strain Z. Irradiation was carried out on a culture with $O.D._{750}$ of 0.20. Irradiation was in a quartz tube used for mutant selection. Points are values from a typical experiment with approximately 400 colonies examined per dose.


100
50
10
5
1.0
0.5
0.1
% GREEN COLONIES
0
10
20
30
40
50
60
EXPOSURE TIME IN SECONDS

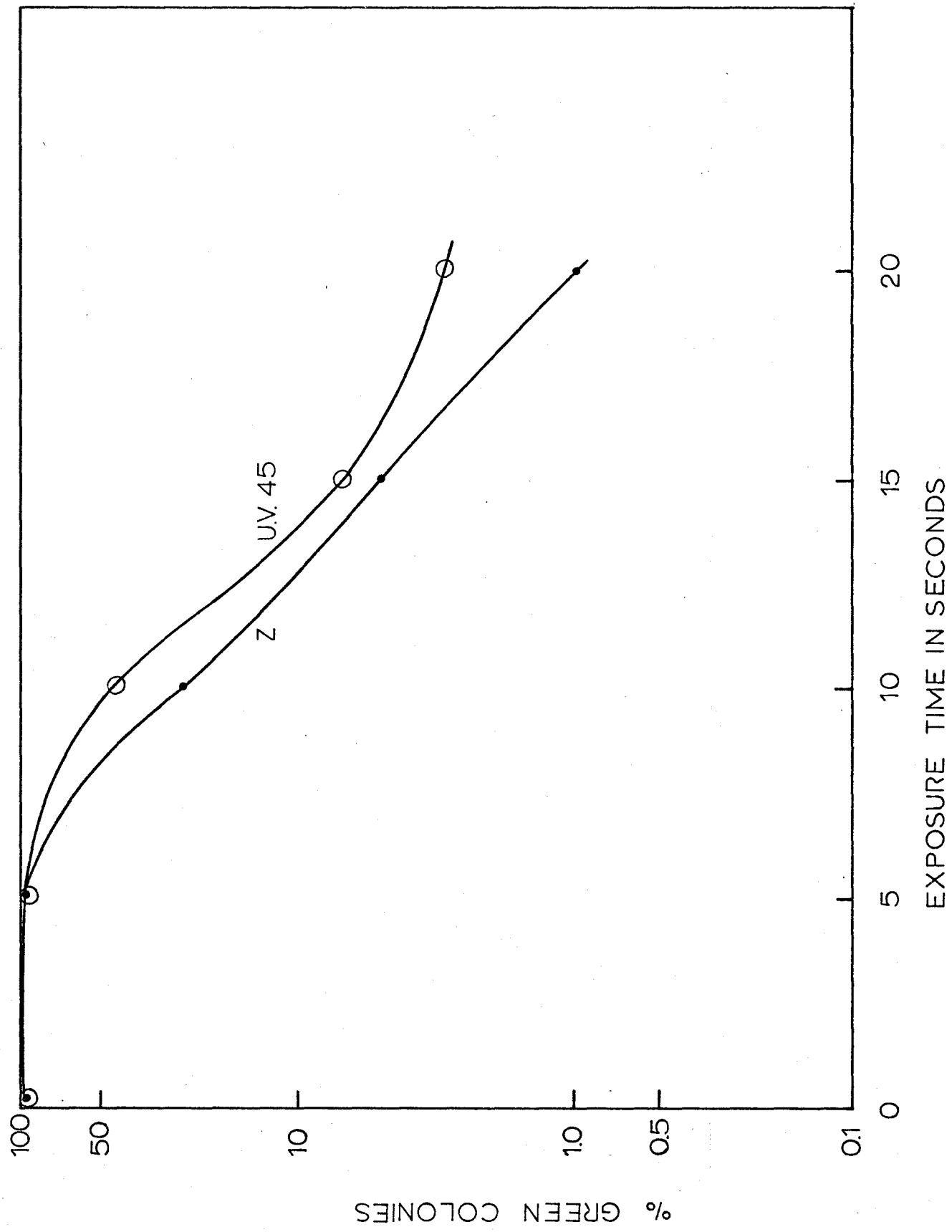
For doses up to 5 minutes, relatively little killing occurs. Table I shows that doses of 20 minutes or more cause greater than 99% killing. Chloroplast-forming ability is considerably more sensitive to U.V. light than is colony-forming ability (59). The survival of colony-forming ability and chloroplast-forming ability after high doses of U.V. will be discussed further following a discussion of mutants arising from these experiments.

Typical doses initially used to select for U.V. resistant mutants ranged from 10 minutes to 75 minutes. These irradiations were done in an open beaker. Cultures irradiated with doses up to 55 minutes turned green within 28 days. Cultures irradiated for 65 and 75 minutes did not turn green within 45 days and were discarded.

Clones were selected from the culture which turned green after 55 minutes U.V. and the clones were tested for U.V. resistance. Clones from strain U.V. 55 showed no special resistance to U.V. with respect to survival of chloroplast-forming ability. Strain U.V. 45 (selected from culture greening after 45 minutes of irradiation) showed a slight resistance to U.V. as depicted in Figure 3.

Strain U.V. 45 showed good growth characteristics (doubling time of 15 hours as compared to 12 hours for strain Z) and since strain U.V. 45 showed slight resistance to bleaching by U.V., sub-cultures of this strain were irradiated for 36, 48, 60, 69, 78, 88, 100, 112, 125, and 135 minutes (300 ml irradiated in 800 ml beakers, cell $O.D._{750}$, 0.30). In this particular experiment all flasks except the 78 and 135 min. exposures became contaminated before turning green.

Figure 3. U.V. bleaching curves for strains Z and U.V. 45. Cells were spread on agar surfaces before irradiation. Points represent an arithmetic average of duplicate experiments, with approximately 400 colonies per dose in each experiment.


% GREEN COLONIES
100
50
10
1.0
0.5
0.1
0
5
10
15
20
EXPOSURE TIME IN SECONDS
U.V. 45
Z

The cultures growing after radiation doses of 78 and 135 min. (strains U.V. 45-78 and U.V. 45-135 respectively) were both green.

Strain U.V. 45-78 did not grow as a green suspension but as a non-motile green mat of cells covering the bottom of the flask. This strain was cultured and stored on tryptic soy agar.

Two clones of U.V. 45-135 showed little or no resistance, relative to Z, and were not studied further.

II. Non-motile mutant strain U.V. 45-78

Microscopic observations of the non-motile strain indicated that the cells apparently lacked flagella. When cultures of strain U.V. 45-78 were left in 6.8 medium for periods up to 5 weeks, no motile cells were observed. In order to test strain U.V. 45-78 for resistance to U.V., colonies were inoculated from an agar surface into liquid 6.8 medium in a spinner flask. Under these conditions the non-motile strain grew as single cells in suspension. The growth rate was slow (doubling time of 21 hours as compared to 12 hrs for strain Z). The cells were then plated on 6.8 medium (solidified with agar) and irradiated. Figure 4 illustrates that strain U.V. 45-78 is approximately four-fold more resistant than strain Z with respect to chloroplast-forming ability (dose required for 50% bleached colonies is four times greater for strain U.V. 45-78).

Strain U.V. 45-78 occasionally gave rise to motile cells upon culture in spinner flasks. A test for U.V. resistance of a motile revertant strain (U.V. 45-78-A) indicated that it retained U.V. resistance (Figure 5). The data are not accurate enough to conclusively compare the resistance of strains U.V. 45-78 and U.V. 45-78-A.

Figure 4. Bleaching of strains Z and U.V. 45-78 by U.V. light. Cells were spread on agar surfaces before irradiation. Points represent an arithmetic average of duplicate experiments, with approximately 400 colonies per dose in each experiment.

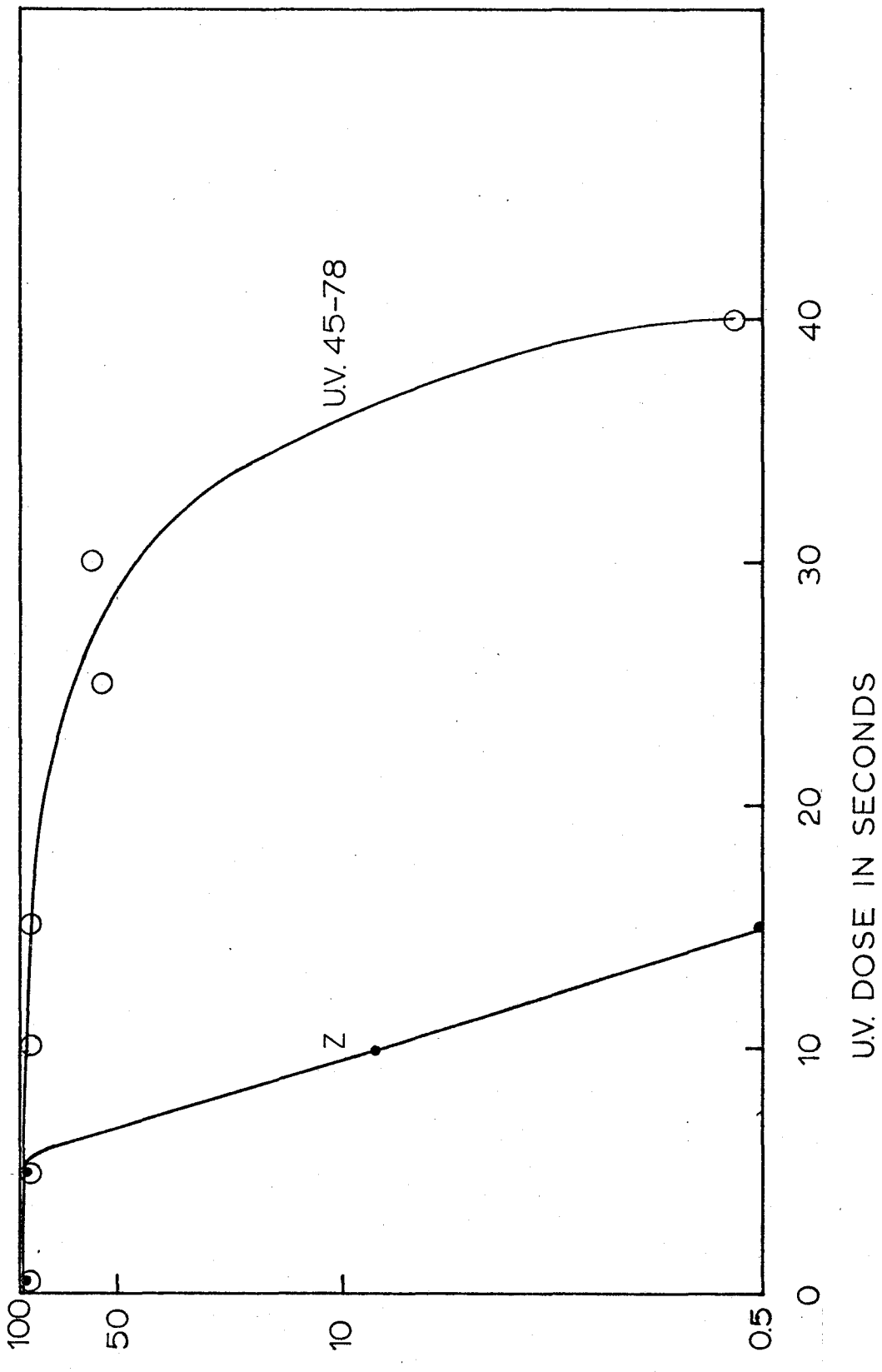
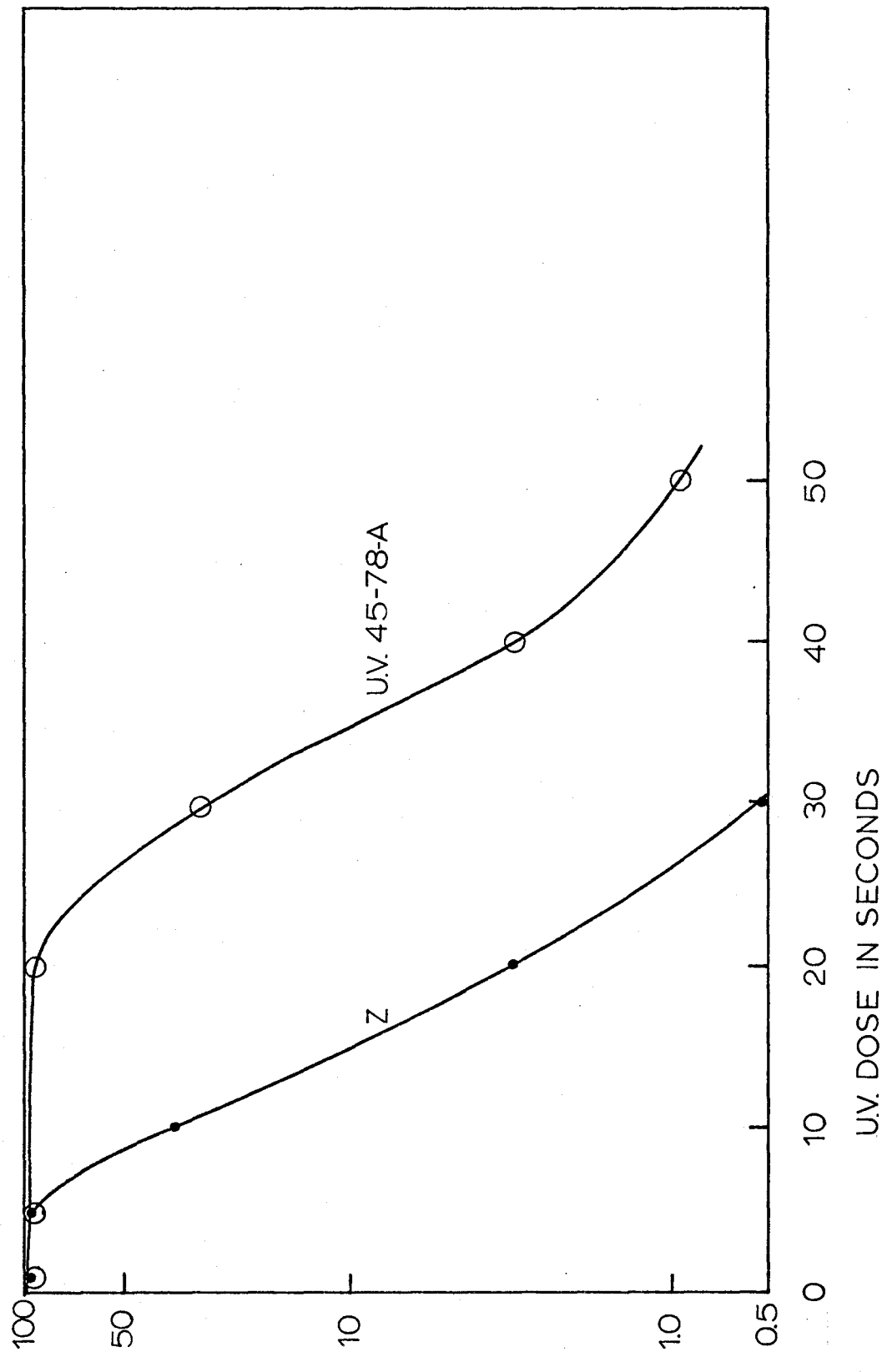

U.V. 45-78
Z
100
50
10
0.5
0
10
20
30
40
% GREEN COLONIES
U.V. DOSE IN SECONDS

Figure 5. Bleaching of strains Z and U.V. 45-78-A by U.V. light. Cells were spread on agar surfaces before irradiation. Points represent an arithmetic average of results from duplicate experiments with approximately 400 colonies per dose in each experiment.


% GREEN COLONIES
100
50
10
1.0
0.5
0
10
20
30
40
50
U.V. DOSE IN SECONDS
U.V. 45-78-A
Z

The mutants obtained which showed considerable resistance to U.V. (Strains U.V. 45-78 and U.V. 45-78-A) in preliminary experiments were considered to be of limited usefulness due to their relatively slow growth. Strain 45-78-A had a division time of 20 hours in 6.8 medium. On agar surfaces growth of strains U.V. 45-78 and U.V. 45-78-A was also noticeably slow.

The ability of cells to photoreactivate is lost when cells divide in the dark; however, if cells do not replicate in the dark, they remain photoreactivable (60). It is likely that at least some of the U.V. resistance shown by strains U.V. 45-78 and U.V. 45-78-A is due to photoreactivation after removal from the dark. The slow growth rate of these strains would complicate the interpretation of any cross-resistance studies. Consequently, resistance and cross-resistance studies with strains U.V. 45-78 and U.V. 45-78-A were not continued.

In the search for U.V. resistant mutants a contradiction became apparent. Green survivors were obtained at doses of U.V. at which the probability of any green survivors remaining is extremely low. This is based on the observation that very low doses are required to bleach 100% of the cells, as shown in Figure 2, and that the doses used should be sufficient to kill essentially entire populations of cells (see Table I). This presents the problem of chloroplast and cell survival at apparently unreasonably high doses. There are a number of factors which might lead to survival at high doses. One of these is shading. The earlier irradiations were

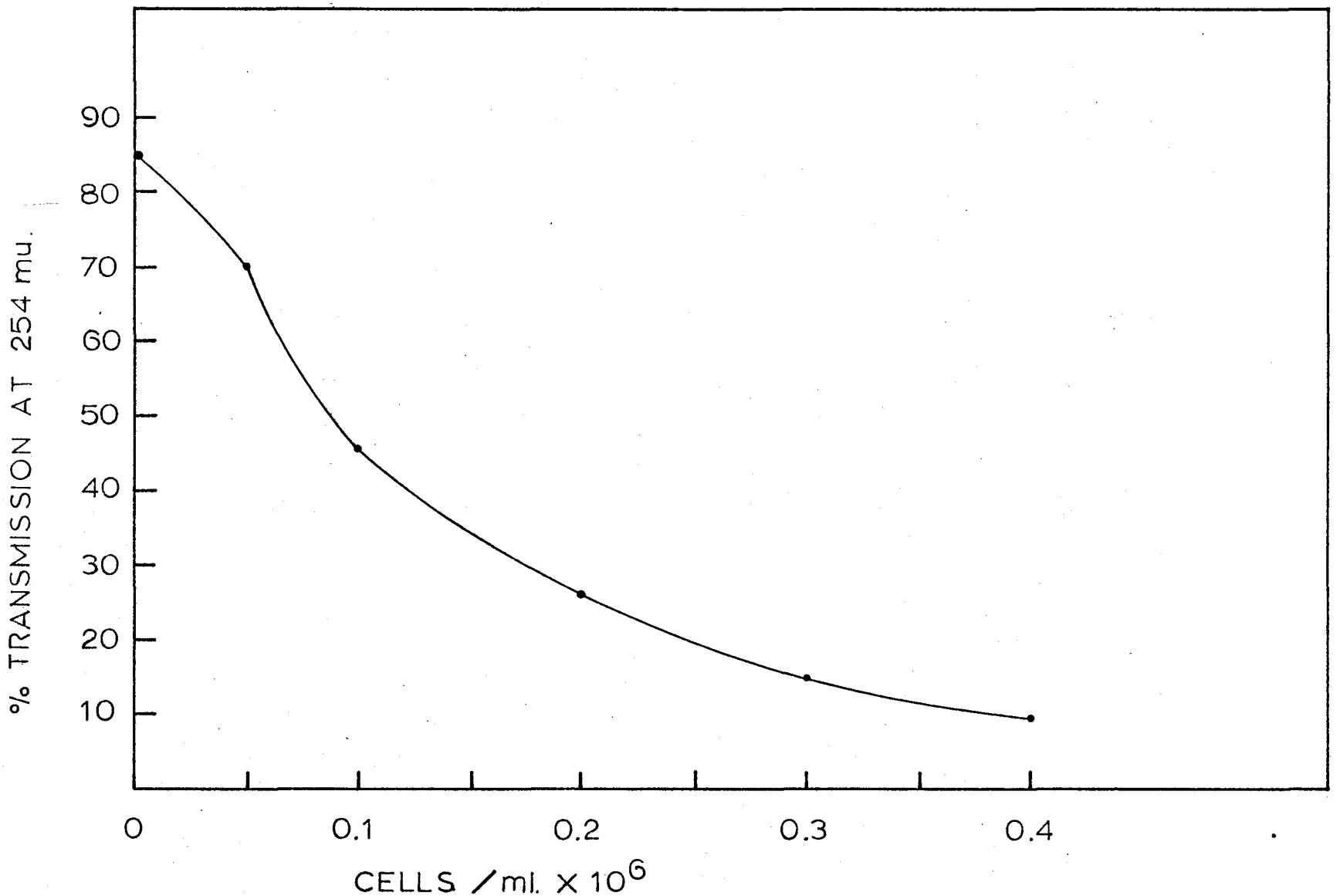
carried out in an open beaker with a liquid depth of approximately 4.5 cm. Although the cultures were stirred during irradiation, any cells adhering to the glass might obtain considerable shading due to absorption or reflection by other cells.

Later experiments utilized quartz tubes of 2.7 cm diameter and relatively low cell densities ($\sim 1.5 \times 10^5$ cells/ml). These cultures were also stirred during irradiation. The absorption of U.V. light by cells in 1 cm quartz cuvettes at various cell densities is indicated in Figure 6. The absorption of U.V. light by cells results in considerable shading of other cells; however, the bleaching and killing measured directly (Figure 1 and Table V) suggest that the doses in selection experiments were high enough to kill greater than 99% of the cells in spite of shading.

It has been shown that the decay of photoreactivability in Euglena with respect to chloroplast-forming ability depends on growth in the dark (60). Usually a period of 3 days in the dark is sufficient to prevent photoractivation. However, all cells of cultures treated with extremely high doses might not grow during the dark period of 7 days used in these experiments. Consequently, unexpected survivors might result from photoreactivation. This argument is somewhat negated by the results of Table I which indicate no green colonies even at relatively low doses and essentially no survivors at high doses.

On the basis that with extremely high doses of U.V. one might be selecting multiple mutants with resulting poor growth habits, an attempt was made to obtain green survivors after relatively low

Figure 6. Absorption of U.V. light (254 mμ) by Euglena gracilis strain Z at various cell concentrations. Absorbance was measured in 1 cm quartz cuvettes in a Beckman DB spectrophotometer. Cells were suspended in 6.8 medium and adjusted to the concentrations indicated. Water was used as a control (100% transmission).


% TRANSMISSION AT 254 mu.
90
80
70
60
50
40
30
20
10
0
0.1
0.2
0.3
0.4
CELLS / ml. X 10^6

doses of U.V. irradiation (up to 15 minutes irradiation) and to test those for U.V. resistance. Six strains isolated after low doses of U.V. grew well but showed no special resistance to bleaching by U.V.

Strain 45-78, which was non-motile, may provide useful material for the study of phototaxis. This strain possesses an eyespot but apparently lacks flagella. Since the phototactic response may depend on shading by the eyespot of a swelling at the base of the flagellum (104), this mutant may be useful for testing this hypothesis. The cells are motile only in the sense that they are able to "crawl" by contractile movements. If a suitable motility assay system could be developed for strain 45-78, it might prove useful for studies of phototaxis, particularly if used in conjunction with bleached cells lacking eyespots, but retaining flagella and bleached strains arising from strain U.V. 45-78 which might lack flagella and eyespots. Some furadantin (104), streptomycin (105), and heat (105) bleached strains are known to lack eyespots.

III. MNTS resistant strains

Selection for MNTS resistance was carried out at doses of 3, 6, 9, 12, 15 and 18 µg/ml. After eleven days aeration in the light, flasks containing 3 and 18 µg/ml MNTS contained green cultures. Clones were selected from the 18 µg/ml flask and the clones tested for MNTS resistance. Approximately 20 ml of the remaining culture were inoculated into flasks containing 6.8 medium (lacking glucose). These were used for further selection. The uncloned mixture was used in the event that if there was any diversity in the population with respect to MNTS resistance, the resistant cells could be

selected out.

The 18 µg/ml cultures were treated with 15, 18, 21, 24, 27, 31, 33, and 36 µg/ml MNTS. After five days of aeration, cultures exposed to 18, 27, and 31 µg/ml turned green. Clones derived from these cultures were designated as strains MNTS-18-18, MNTS 18-27, and MNTS 18-31 respectively. The culture from which strain 18-31 was obtained was used for further selection experiments. The exposures were to 30, 40 and 50 µg/ml MNTS. All cultures turned green and were sampled for cloning and testing.

On the basis of gradient plate tests, some strains showed obvious resistance to MNTS with respect to killing and chloroplast-forming ability. Table VI indicates which strains showed resistance. Strains designated A or B indicate separate clones from the MNTS selection indicated.

TABLE VI

Strains tested for MNTS resistance

Strain	Apparent resistance relative to Z
MNTS 18	-
MNTS 18-31	+ (slight)
MNTS 18-31-30A	+ (slight)
MNTS 18-31-30B	-
MNTS 18-31-40A (MNTS 40A)	+
MNTS 18-31-40B	-
MNTS 18-31-50A	-
MNTS 18-31-50B	-

Quantitative resistance tests were carried out by direct exposure of cells to MNTS in liquid medium, followed by plating and counting colonies. Strain MNTS 40A was at least 40-fold more resistant than Z to bleaching by MNTS, as indicated in Figure 7. As indicated in Figure 8, strain MNTS 40A was approximately 2.5-fold more resistant than strain Z with respect to colony-forming ability after exposure to MNTS.

In cross-resistance tests strain MNTS 40A was slightly more sensitive to NG than was Z, with respect to both killing and chloroplast-forming ability (Figures 9 and 10). Similarly, there was no apparent resistance of MNTS 40A to bleaching by U.V. light as shown in Figure 11.

The work of McCalla (84) indicated that the methylating ability of MNTS is decreased in the presence of cysteine, while that of NG is increased. Other thiols (and perhaps certain other nucleophilic compounds) also inactivate MNTS. The reactions of MNTS and NG with cysteine as proposed by Schulz and McCalla (89) are illustrated in Figure 12.

One possible explanation of the observation that strain MNTS 40A is resistant to MNTS but sensitive to NG is that MNTS is inactivated by thiols or other nucleophylic compounds more rapidly in strain MNTS 40A than in strain Z. If the resistance of MNTS 40A were due to other factors such as reduced permeability or a more efficient repair mechanism, one might expect some cross resistance to NG. Since Schulz and McCalla (89) demonstrated that N-methyl-p-toluenesulfonamide (MTS) was the only detectable breakdown product of

Figure 7. Bleaching of strains MNTS 40A and Z by MNTS.

a - Strain MNTS 40A

b - Strain Z

Points are actual values from three independent experiments with approximately 600 colonies per dose in each experiment.

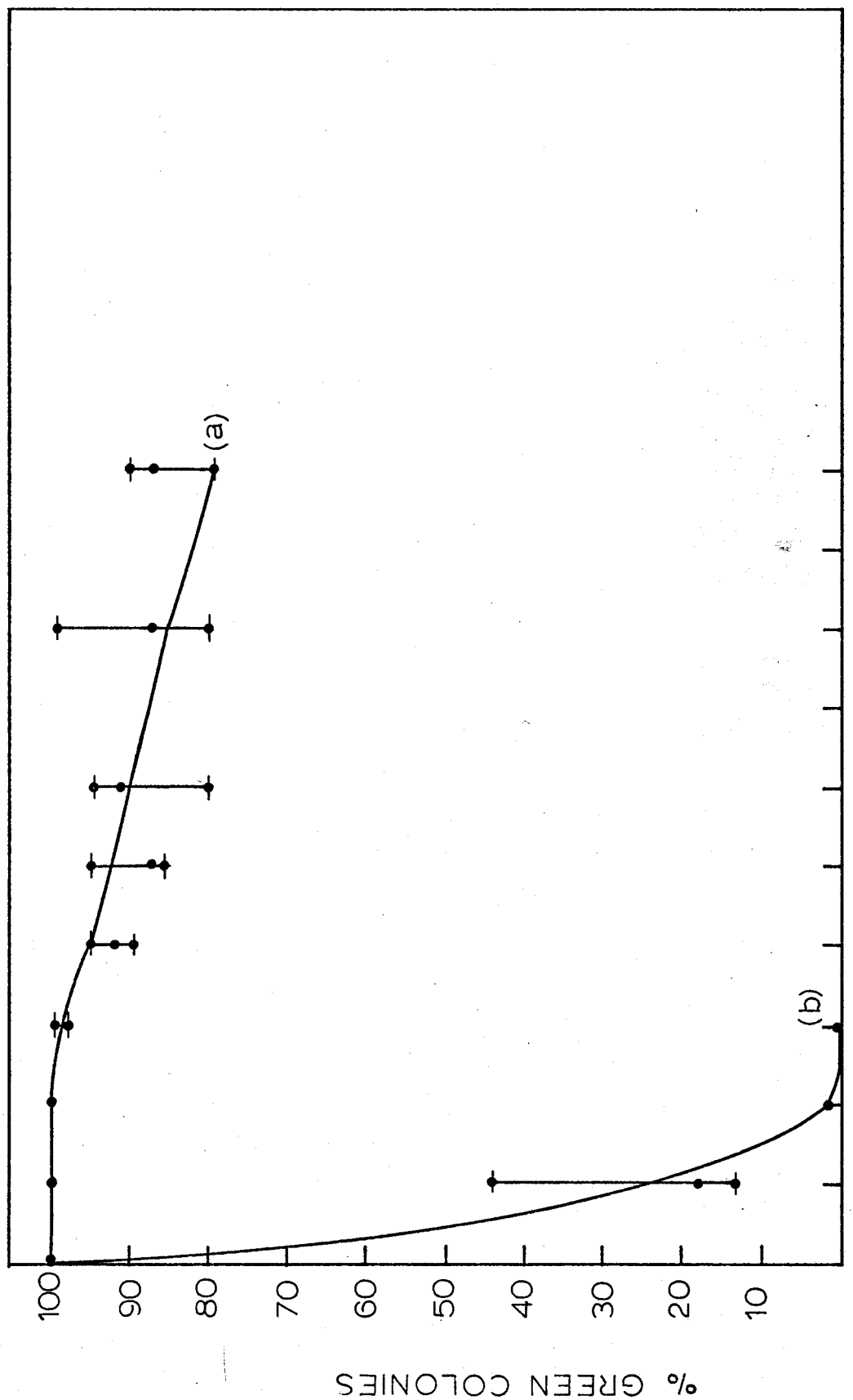

% GREEN COLONIES
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
DOSE OF MNTS IN µg. ml.
(a)
(b)

Figure 8. Killing of strains Z and MNTS 40A by MNTS.

a - Strain MNTS 40A

b - Strain Z

Points represent values from three independent experiments with approximately 600 colonies per dose in each experiment.

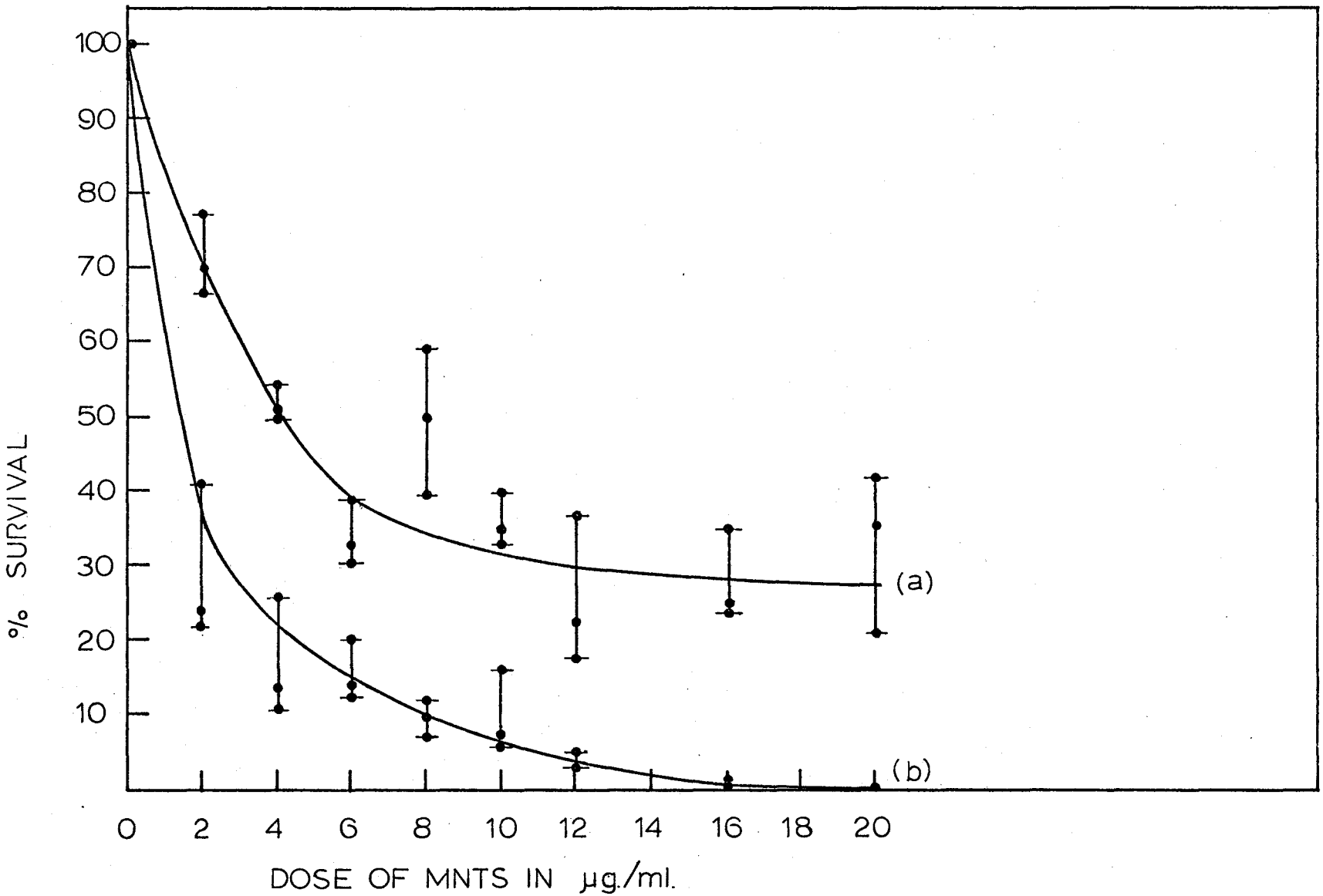

100
90
80
70
60
50
40
30
20
10
% SURVIVAL
(a)
(b)
0
2
4
6
8
10
12
14
16
18
20
DOSE OF MNTS IN µg./ml.

Figure 9. Bleaching of strains Z and MNTS 40A by NG.

Open circles - strain Z

Closed circles - strain MNTS 40A

Points represent values from three independent experiments with approximately 800 colonies per dose in each experiment.

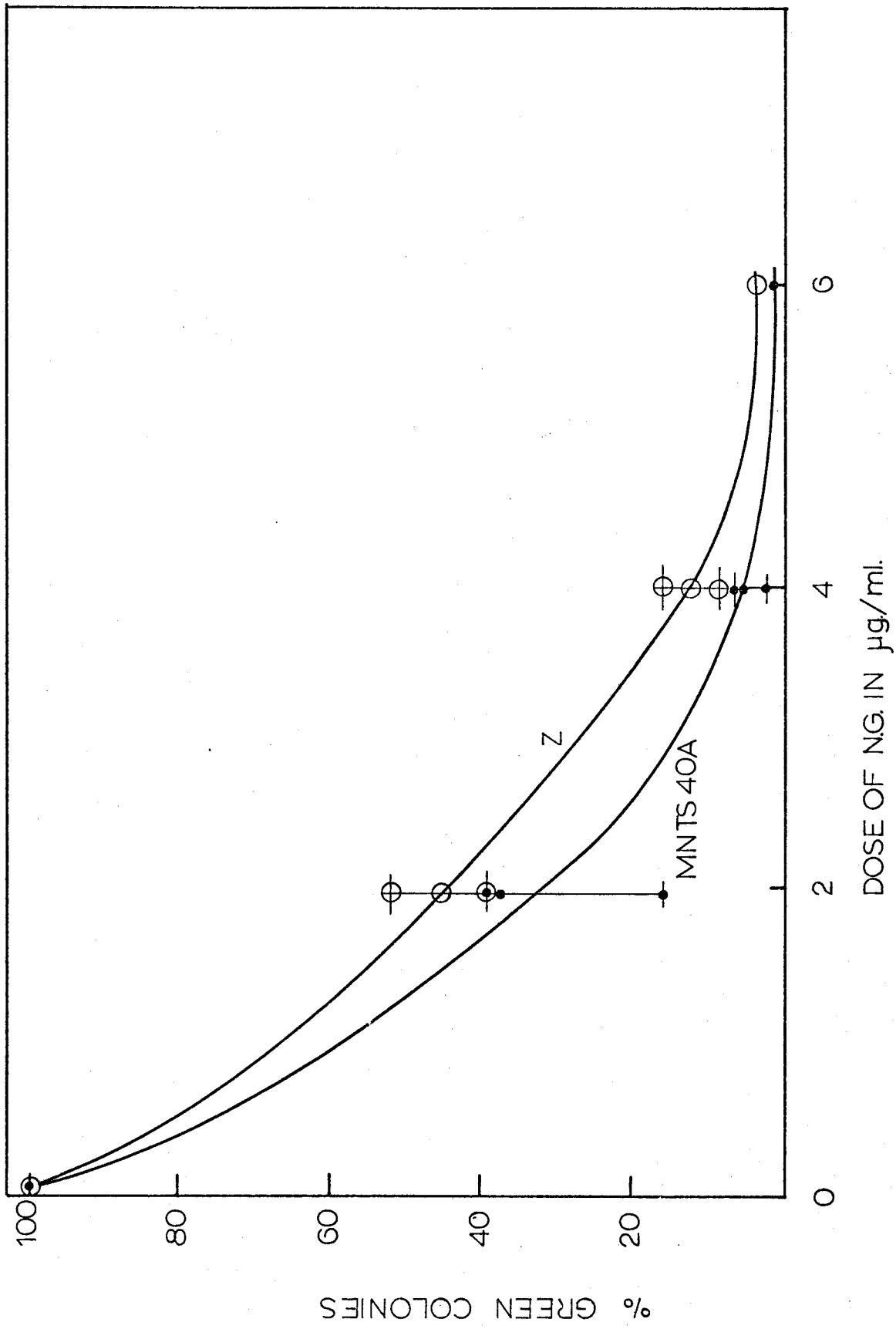

% GREEN COLONIES
100
80
60
40
20
0
2
4
6
DOSE OF N.G. IN µg./ml.
Z
MNTS 40A

Figure 10. Killing of strains Z and MNTS 40A by NG. Points represent values from three independent experiments with approximately 800 colonies per dose in each experiment.

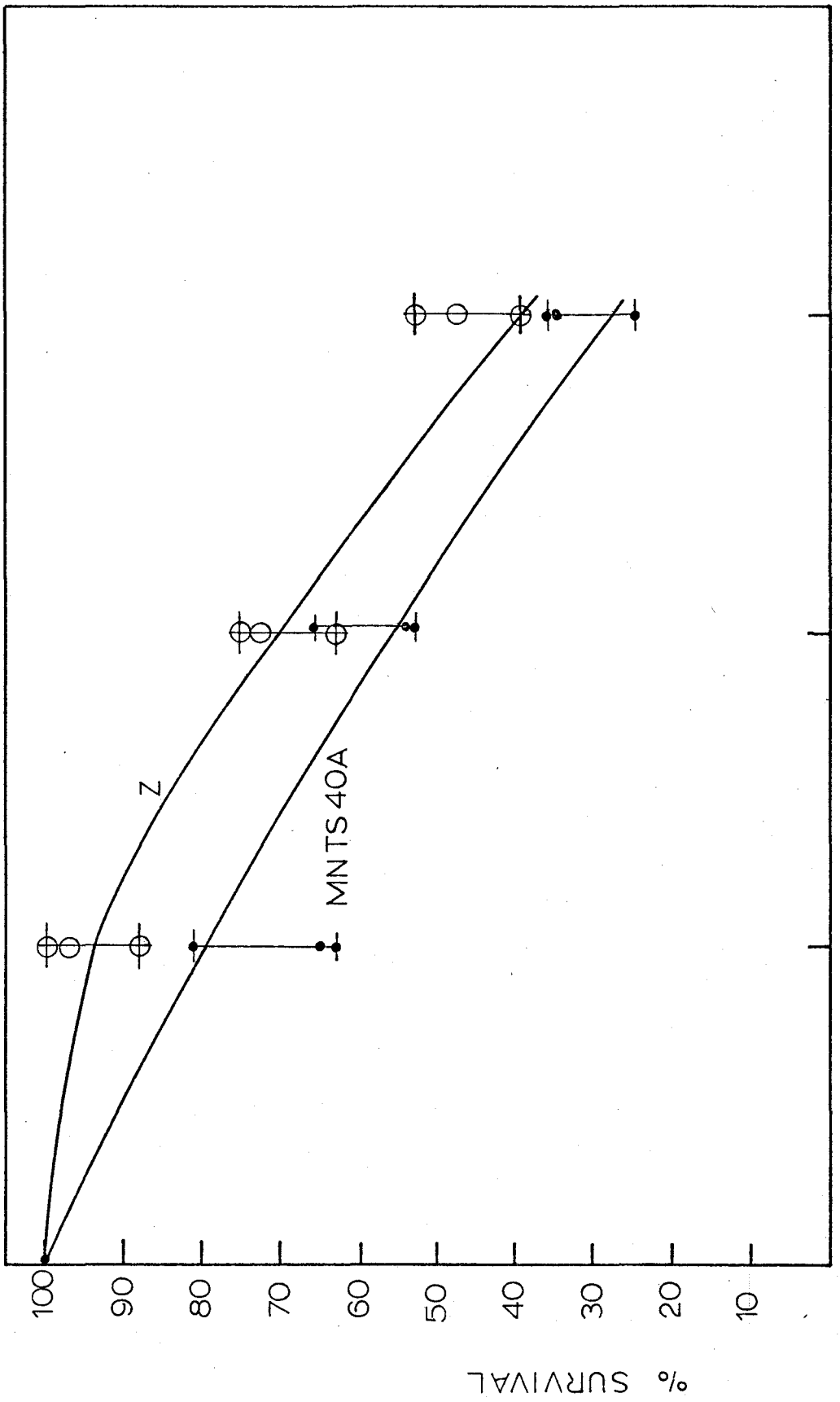

% SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
2
4
6
DOSE OF N.G. IN µg/ml
Z
MNTS 40A

Figure 11. Bleaching of strains Z and MNTS 40A by U.V. light. Points represent an arithmetic average of results from duplicate experiments with approximately 600 colonies per dose in each experiment. Cells were spread on agar surfaces before irradiation.

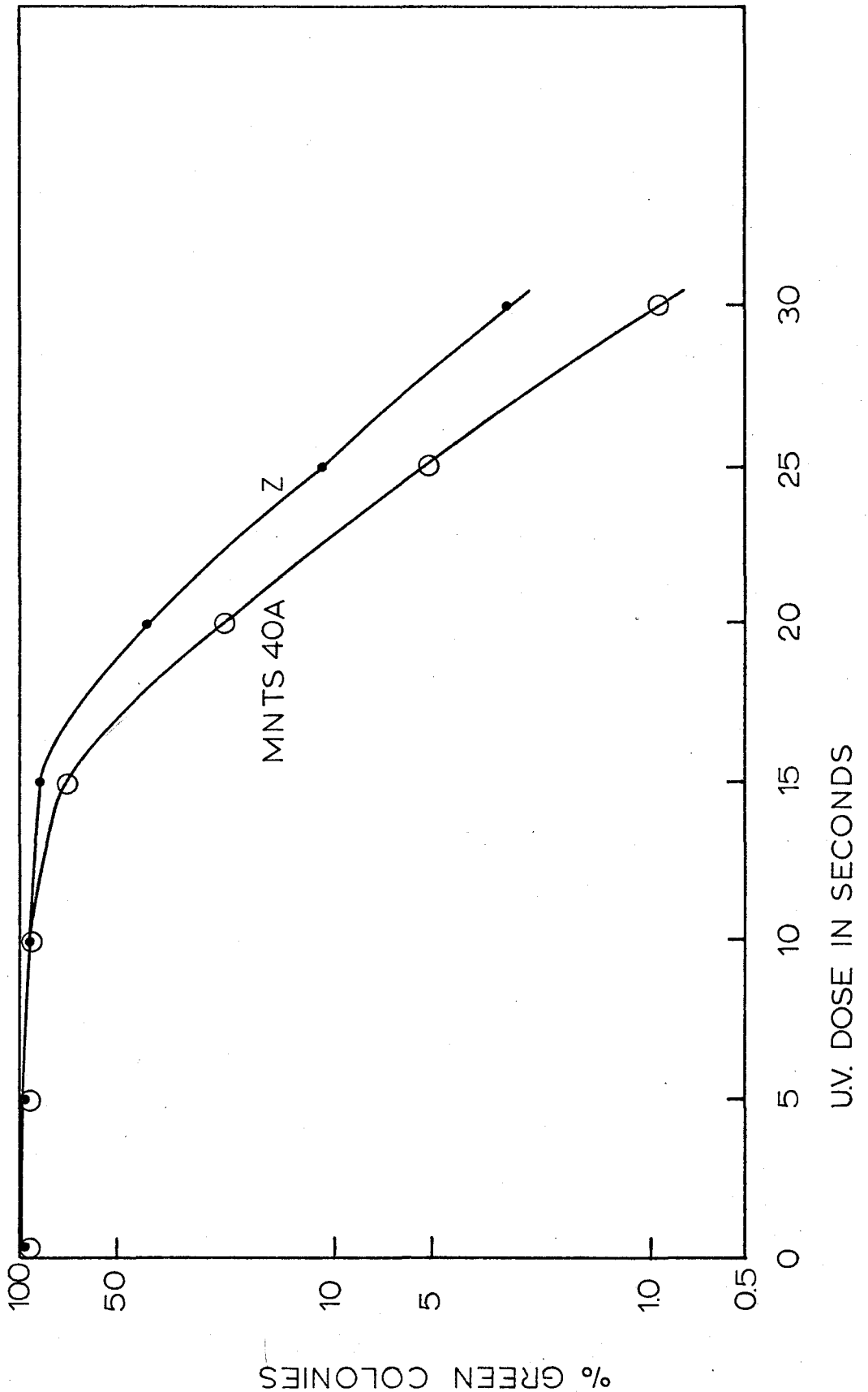


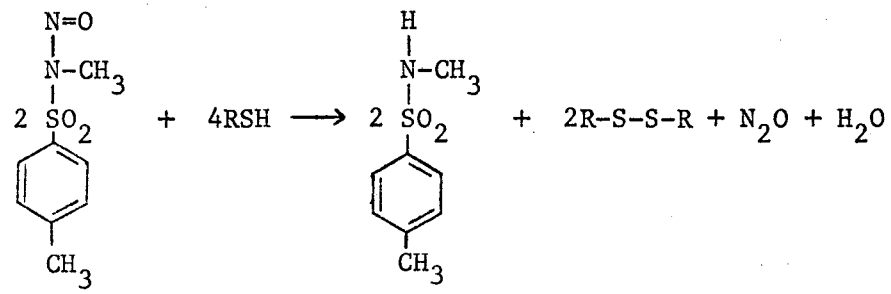
Figure 12. Reaction of MNTS and NG with cysteine (as proposed by Schulz and McCalla (89)).

A - Reaction of MNTS with cysteine

B - Reaction of NG with cysteine

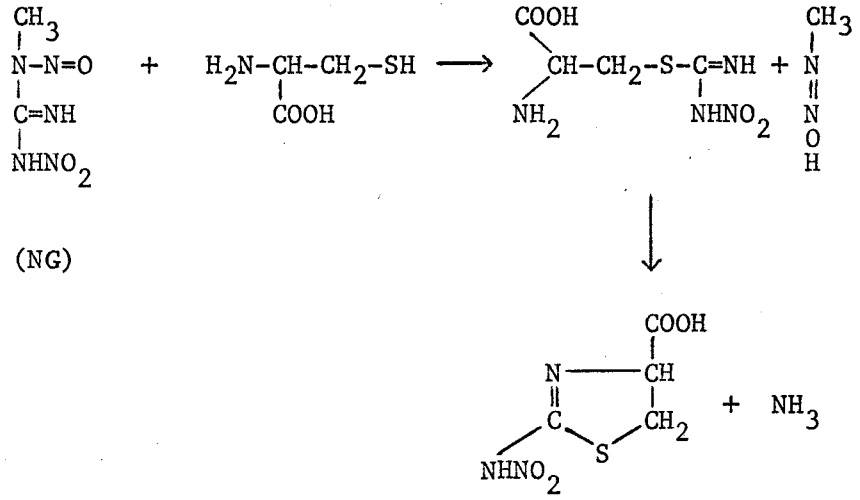
$$R = H_2N\text{-}\underset{\underset{COOH}{|}}{CH}\text{-}CH_2\text{-}$$

A.



(MNTS) (MTS)

B.



(NG)

MNTS in *in vitro* reactions with cysteine, and since ^{14}C methyl labeled MNTS was readily available, it seemed reasonable to attempt to compare the rates of *in vivo* conversion of MNTS to MTS in strains Z and 40A.

Initial experiments, carried out on washed cells treated with ^{14}C MNTS with 10 μg/ml cold MNTS added, showed a slightly more rapid breakdown of MNTS to MTS in strain MNTS 40A than in strain Z.

Determination of the breakdown rate was based on the assumption that essentially all the label would be present in MNTS or MTS. In these preliminary experiments extracted samples were concentrated by evaporation and radioactivity in MNTS and MTS estimated by scanning radiochromatograms. A sample of a chromatogram is shown in Figure 13.

Using the above technique, there was considerable variation in apparent breakdown rates within and between experiments; however, there was an indication that the breakdown rate was greater in strain MNTS 40A than in strain Z. In later experiments the technique was improved by eliminating the evaporation step. Cells were then extracted and a portion of the ether extract chromatographed. The chromatogram spots were then assayed for radioactivity by removing the spots and counting in liquid scintillation vials. This more direct analysis showed a significantly greater breakdown rate of MNTS in cultures of strain MNTS 40A than in cultures of strain Z. The time course of breakdown of MNTS in these strains is shown in Figure 14. The rate of breakdown of MNTS is greater at relatively high cell concentrations than at low cell concentrations as indicated in Figure 15.

In order to assess whether or not there is appreciable breakdown of MNTS by medium alone, breakdown experiments were carried

Figure 13. Radiochromatogram scan of an ether extract of strain Z exposed to ^{14}C-MNTS for 3 hours. The chart speed was 1 cm/min.

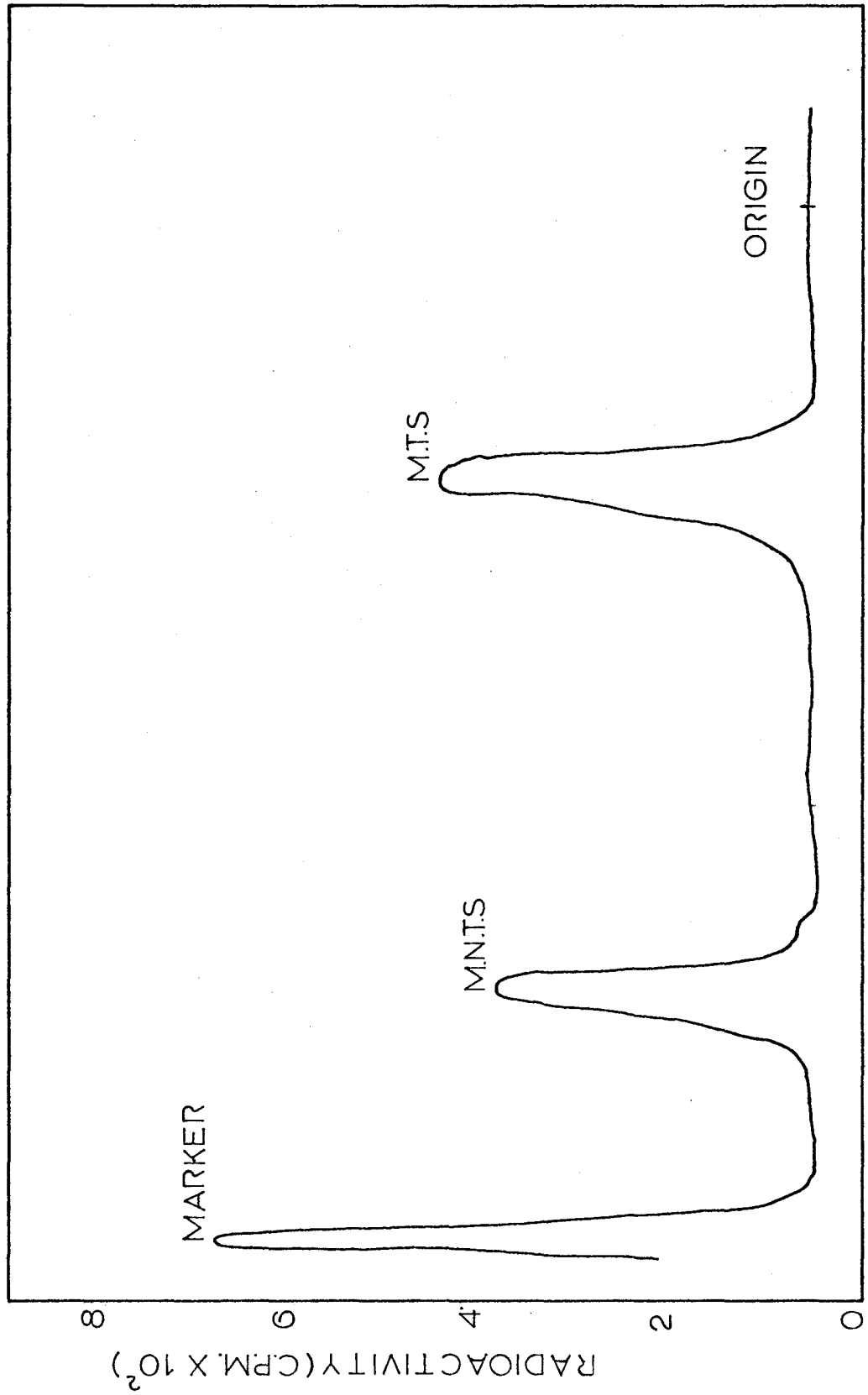

RADIOACTIVITY (C.P.M. X 10^2)
0
2
4
6
8
MARKER
M.N.T.S
M.T.S
ORIGIN

Figure 14. Breakdown of ^{14}C-MNTS in strains Z and MNTS-40A.

a - Medium from which cells were harvested (strains Z and MNTS-40A)

b - Sonicated cells (strain Z)

c - Sonicated cells (strain MNTS-40A)

d - Culture of strain Z, $O.D._{750}$ - 0.30

e - Culture of strain MNTS-40A, $O.D._{750}$ - 0.30

The points shown are values from two independent experiments. In curves a, b, and c, the duplicate values are essentially identical.

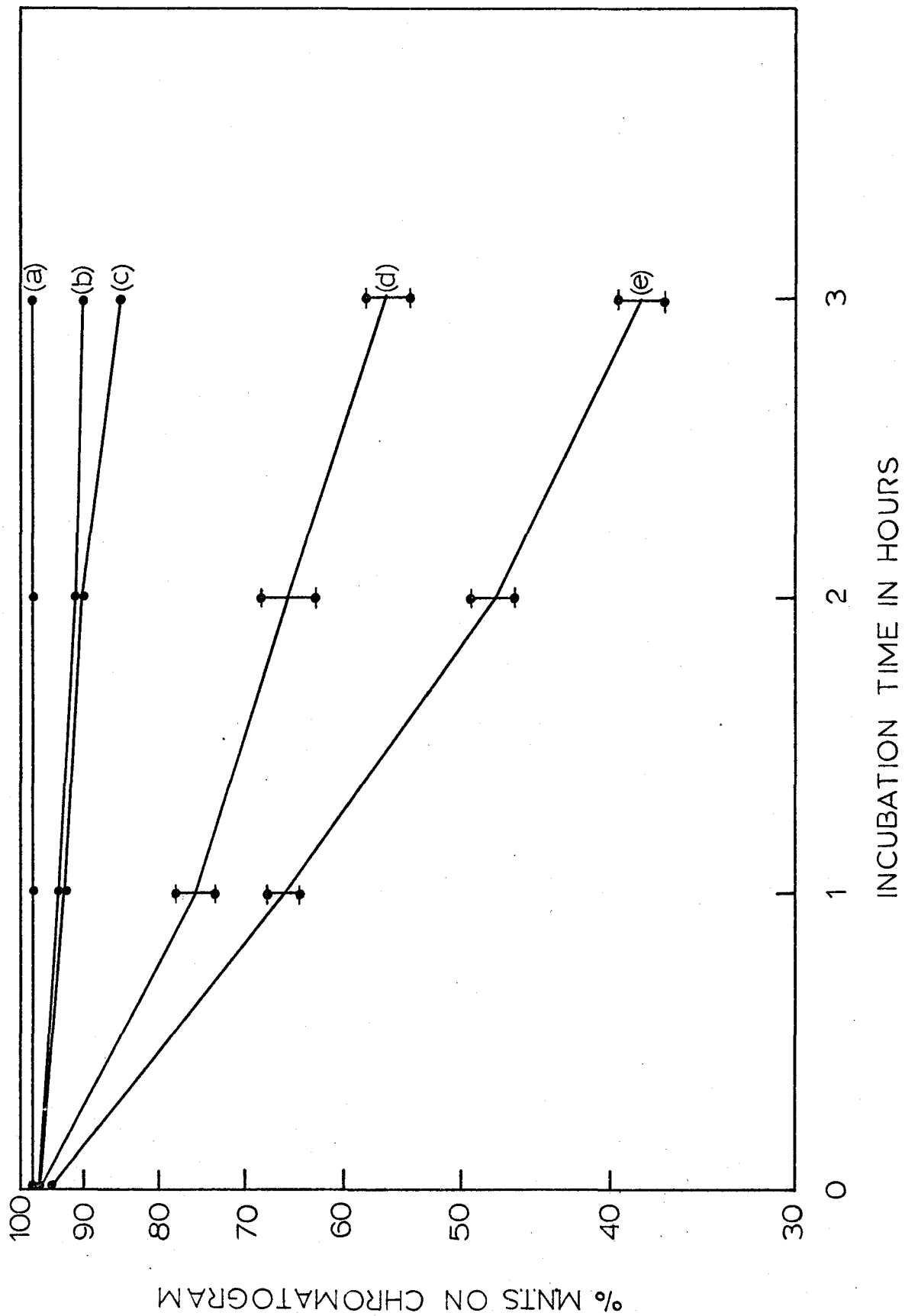

% MNTS ON CHROMATOGRAM
100
90
80
70
60
50
40
30
0
1
2
3
INCUBATION TIME IN HOURS
(a)
(b)
(c)
(d)
(e)

Figure 15. Breakdown of ^{14}C-MNTS in strains Z and MNTS 40A at two cell concentrations.

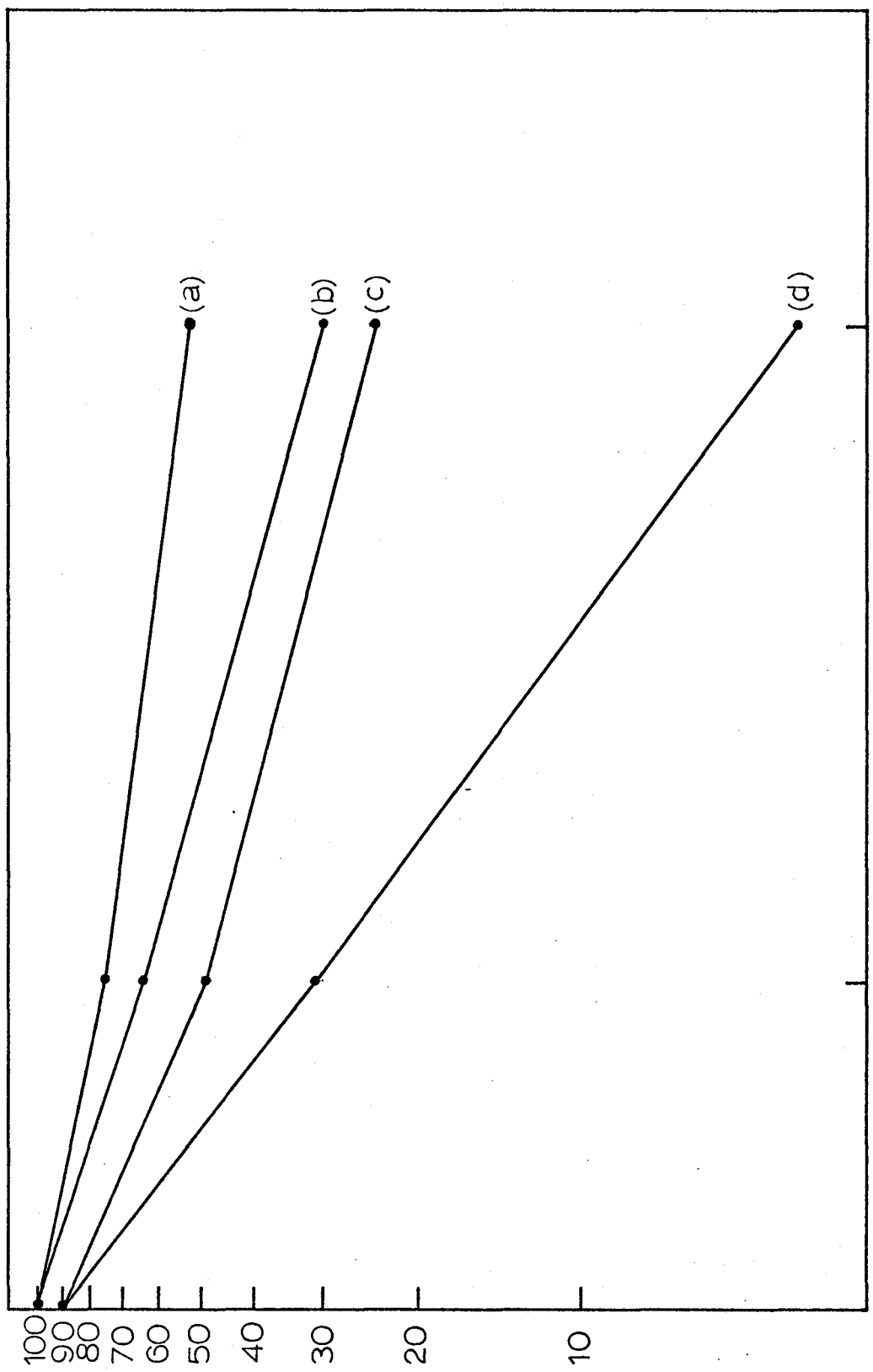
a - strain Z, $O.D._{750}$ 0.15

b - strain MNTS 40A, $O.D._{750}$ 0.15

c - strain Z, $O.D._{750}$ 0.45

d - strain MNTS 400, $O.D._{750}$ 0.45

Points are an arithmetic average of duplicate extractions from single experiments.


% MNTS ON CHROMATOGRAM
100
90
80
70
60
50
40
30
20
10
0
1
3
INCUBATION TIME IN HOURS
(a)
(b)
(c)
(d)

out on 6.8 medium lacking cells. Breakdown experiments were also carried out on cells ruptured by sonication. In 6.8 medium alone and in medium containing cells ruptured by sonication, the extent of decomposition of MNTS is very low over the time course studied (Figure 14).

In these experiments, it was possible to determine the efficiency of recovery of radioactivity as MNTS and MTS. For exposure times up to three hours essentially all of the initial radioactivity could be accounted for as ^{14}C-MNTS or ^{14}C-MTS (Table VII).

The high efficiency of recovery of radioactivity as ^{14}C-MNTS and ^{14}C-MTS indicates that MTS is the major breakdown product of MNTS in strains Z and MNTS 40A.

IV. Methylation of nucleic acids

The relative reaction of MNTS with nucleic acids in strains Z and MNTS 40A was undertaken to determine whether or not there was a reduction of methylation of nucleic acids in strain MNTS 40A as a consequence of its more rapid conversion to MTS. Table VIII shows the results of these experiments. The level of radioactivity in the total nucleic acids is 2.2 to 4.7 fold higher for strain Z than for strain MNTS 40A, after treatments with ^{14}C-MNTS. Similarly, there is 2.0 to 3.8 times as much radioactivity in the DNA of strain Z as in strain MNTS 40A under the same conditions.

In order to determine if methylation of bases in nucleic acids was greater in strain Z than in strain MNTS 40A, an analysis of 7-methylguanine content of nucleic acids of cultures treated with ^{14}C-MNTS was carried out. Chromatography of hydrolysed nucleic acids

TABLE VII

Recovery of radioactivity in ^{14}C-MNTS breakdown experiments

Strain	Time of extraction (hrs.)	Radioactivity before extraction (dpm/ml)	% of T_o activity	Radioactivity recovered as ^{14}C-MNTS and ^{14}C-MTS (dpm/ml of treated culture)	% Recovery of Radioactivity at T_o
Expt. A					
Z	0	27,600	100	26,700	96.7
"	1	28,200	102	26,500	96.2
"	2	26,900	97.5	23,800	86.5
"	3	27,100	98.2	27,600	100
MNTS 40A	0	26,200	100	26,500	101
"	1	28,000	107	28,300	108
"	2	28,100	107	28,200	108
"	3	26,700	102	27,600	94.9
Expt. B					
Z	0	26,800	100	27,400	102
"	1	24,700	92.2	26,100	97.5
"	2	25,200	94.0	24,900	98.8
"	3	-	-	15,900	59.4
MNTS 40A	0	26,900	100	28,600	102
"	1	27,200	101	26,400	98.0
"	2	26,300	97.8	26,500	105
"	3	26,600	99.0	26,900	100

TABLE VIII

Methylation of Nucleic Acids of strains Z and MNTS 40A by ^{14}C-MNTS

Treatment with ^{14}C-MNTS was for 5 hours. Methanol was present in the reaction mixture at a concentration of 1%

Experiment A

Total Nucleic Acids

strain	dpm/O.D.$_{260}$ unit (Sp. Act.)	
Z	41	$\frac{\text{Sp. Act. Z}}{\text{Sp. Act. MNTS 40A}} = 3.2$
MNTS 40A	13	

DNA

Z	69	$\frac{\text{Sp. Act. Z}}{\text{Sp. Act. MNTS 40A}} = 3.1$
MNTS 40A	22	

Experiment B

Total Nucleic Acids

strain	dpm/O.D.$_{260}$ unit (Sp. Act.)	
Z	23	$\frac{\text{Sp. Act. Z}}{\text{Sp. Act. MNTS 40A}} = 4.7$
MNTS 40A	4.9	

DNA

Z	17	$\frac{\text{Sp. Act. Z}}{\text{Sp. Act. MNTS 40A}} = 3.8$
MNTS 40A	4.5	

Experiment C

Total Nucleic Acids

strain	dpm/O.D. units (Sp. Act.)	
Z	39	$\frac{\text{Sp. Act. Z}}{\text{Sp. Act. MNTS 40A}} = 2.2$
MNTS 40A	17	

DNA

Z	62	$\frac{\text{Sp. Act. Z}}{\text{Sp. Act. MNTS 40A}} = 2.0$
MNTS 40A	31	

on Dowex-50 provides a simple method of isolating 7-methylguanine, the major detectable product of methylation by MNTS in in vitro (84) and in vivo experiments (106,107). Figure 16 shows an elution pattern for hydrolysed DNA plus added 7-methylguanine. The pattern is similar for hydrolysates of RNA or of total nucleic acids except for quantities and the composition of the first peak (UMP instead of TMP for RNA).

As indicated in Table IX, the 7-methylguanine fraction of hydrolysates of total nucleic acids and DNA of strains Z and MNTS 40A contains the bulk of the radioactivity recovered. The specific activity of 7-methylguanine in the total nucleic acids is 3.84 and 3.95 times as great in strain Z as in strain MNTS 40A. Similarly the specific activity of 7-methylguanine in the DNA is 4.99 and 9.42 times as great in strains Z as in strain MNTS 40A.

There is also a greater extent of labeling of other bases and nucleotides in strain Z than in strain MNTS 40A. This is likely a consequence of the slower breakdown of MNTS in strain Z than in strain MNTS 40A. Allen (106) has shown that in cells treated with ^{14}C-MNTS for four hours the majority of the radioactivity was found in guanine and adenine. This is probably due to the formation of ^{14}C-methanol from ^{14}C-MNTS via ^{14}C-diazomethane. Although methanol was added to cultures to reduce the effects of any ^{14}C-methanol arising from MNTS, some radioactivity might have become metabolically incorporated into nucleotides. Other minor methylated products such as 3-methylcytosine (chromatographs with cytosine), and 1-methyl-adenine (chromatographs with adenine) might also contribute to the

Figure 16. Elution pattern of a Dowex 50-X1 chromatograph of DNA hydrolysate (1 N HCl, 1 hr, 100°C) with authentic 7-methylguanine added. The chromatograph was run from 1.0 N to 2.0 N HCl. Relative absorption was determined using an L.K.B. 4701A ultraviolet absorptiometer.

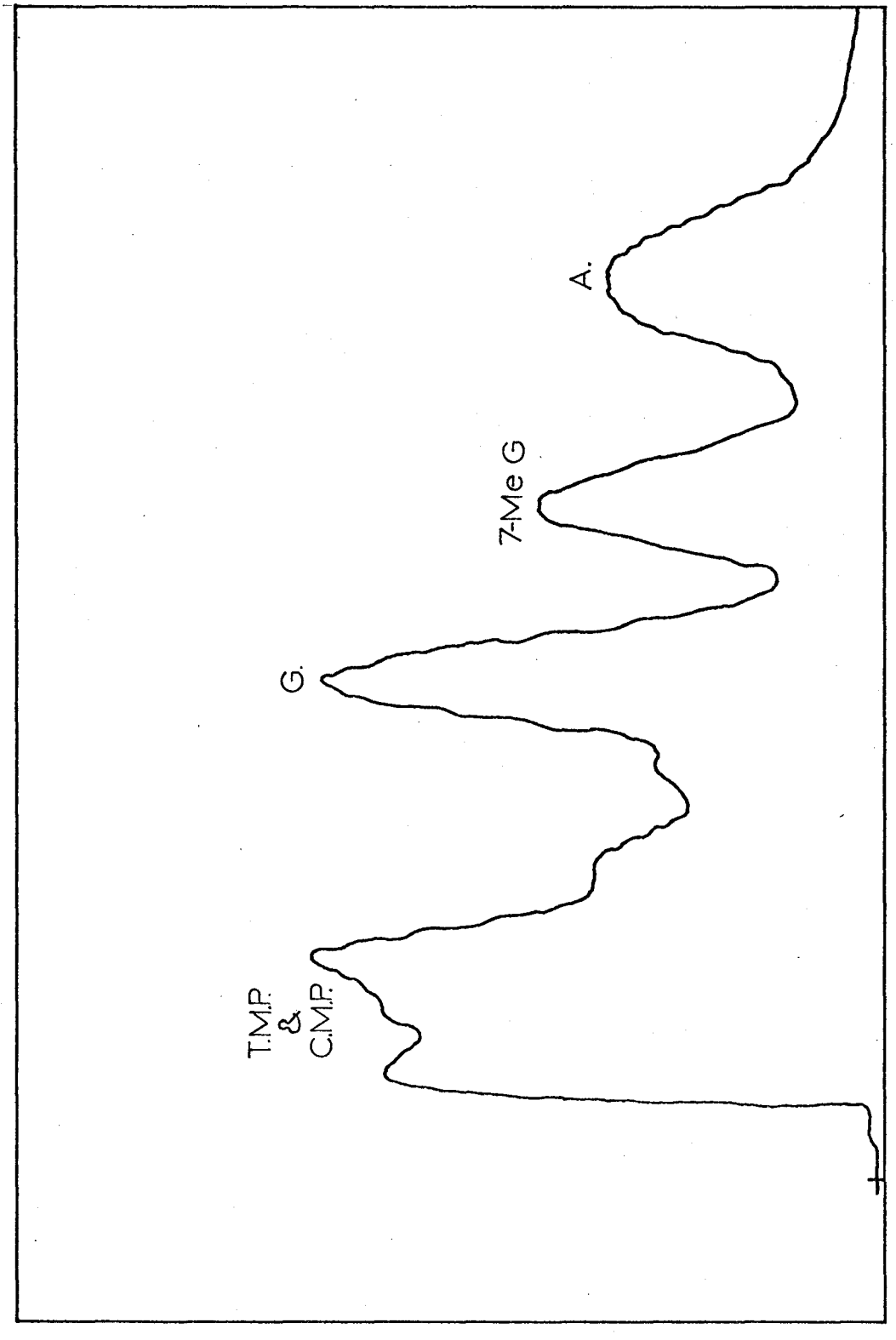

U.V. ABSORPTION (254mμ.)
T.M.P.
&
C.M.P.
G.
7-Me G
A.
1.0
INCREASING H.Cl. CONC.

TABLE IX

Radioactivity of fractions of hydrolysed nucleic acids from strains Z and MNTS 40A treated with ^{14}C-MNTS

Cultures were treated for 5 hours with ^{14}C-MNTS. The reaction mixture contained 1% methanol. After treatment, cells were hydrolysed in 1 N HCl (100°C for 1 hour) and chromatographed on Dowex 50-X2 using a 1.0 to 2.0 N HCl gradient.

Experiment A

1. Total Nucleic Acids

Strain	Fraction	Components	dpm/O.D.$_{260}$*
Z	A	CMP, TMP, UMP	146
"	B	G	66.5
"	C	7 MeG	618
"	D	A	340
MNTS 40A	A	CMP, TMP	34.0
"	B	G	23.0
"	C	7 MeG	161
"	D	A	50.6

$$\frac{7\ \text{MeG(Z)}}{7\ \text{MeG(MNTS 40A)}} = 3.84$$

* O.D.$_{260}$ refers to O.D.$_{260}$ of the DNA or nucleic acids before hydrolysis.

Table IX Cont'd...

TABLE IX (CONTINUED)

2. DNA

Strain	Fraction	Components	dpm/O.D.$_{260}$	
Z	A	CMP, TMP, UMP	90.6	
"	B	G	40.6	
"	C	7 MeG	426	
"	D	A	94.0	
MNTS 40A	A	CMP, TMP	15.3	
"	B	G	14.0	
"	C	7 MeG	85.3	
"	D	A	24.0	$\frac{7\text{ MeG(Z)}}{7\text{ MeG(MNTS 40A)}} = 4.99$

Experiment B

1. Total Nucleic Acids

Strain	Fraction	Components	dpm/O.D.$_{260}$	
Z	A	CMP, TMP, UMP	67.9	
"	B	G	49.5	
"	C	7 MeG	345.0	
"	D	A	80.9	
MNTS 40A	A	CMP, TMP	24.7	
"	B	G	16.2	
"	C	7 MeG	87.4	
"	D	A	18.0	$\frac{7\text{ MeG(Z)}}{7\text{ MeG(MNTS 40A)}} = 3.95$

Table IX Cont'd...

TABLE IX (CONTINUED)

2. DNA

Strain	Fraction	Components	dpm/O.D.$_{260}$
Z	A	CMP, TMP	52.5
"	B	G	38.9
"	C	7 MeG	390
"	D	A	75.0
MNTS 40A	A	CMP, TMP	13.0
"	B	G	5.71
"	C	7 MeG	41.4
"	D	A	8.49

$$\frac{7\ \text{MeG(Z)}}{7\ \text{MeG(MNTS 40A)}} = 9.42$$

radioactivity in the cytosine and adenine fractions. The 7-methylguanine containing fractions might also contain some 3-methyladenine in relatively small amounts but with a sufficiently high specific activity to increase radioactivity in this fraction.

V. Thiol content of Z and MNTS 40A

The conversion of MNTS to MTS is stimulated *in vitro* by the presence of cysteine (89). Since strain MNTS 40A converts MNTS to MTS more rapidly than does strain Z, one possibility is that the breakdown of MNTS *in vivo* is a consequence of reaction with thiols and that strain MNTS 40A contains a higher concentration of thiols than does strain Z. Table X shows that the acid soluble thiol content of log phase cells is 3.5 to 5.6 times greater in strain Z than in strain MNTS 40A.

TABLE X

Thiol Content of strains Z and MNTS 40A

	Strain	Thiol content (moles/cell)
Expt. A	Z	4.85×10^{-6}
	MNTS 40A	1.76×10^{-6}
Expt. B	Z	3.38×10^{-6}
	MNTS 40A	1.10×10^{-6}
Expt. C	Z	4.12×10^{-6}
	MNTS 40A	0.74×10^{-6}
Ave. of Expts.	Z	4.12×10^{-6}
A, B, and C	MNTS 40A	1.20×10^{-6}
Medium from which cells were harvested		0.00

Clearly, the relative concentrations of acid soluble thiols cannot account for the differences in resistance and MNTS breakdown between strains Z and MNTS 40A.

It is possible that any thiols which are not extracted by 5% trichloroacetic acid, or other nucleophiles might be involved in the conversion of MNTS to MTS in strains Z and MNTS 40A.

Although the mechanism of conversion of MNTS to MTS in Euglena has not been elucidated, it is clear that the conversion represents a reduction in the methylating ability of MNTS. MTS does not bleach or kill Euglena at doses up to 100 µg/ml (Table XI). Since an increase in the rate of conversion of MNTS to MTS is accompanied by a decrease in the mutagenic and killing effects of MNTS, it is evident that the conversion of MNTS to MTS represents an inactivation of MNTS with respect to lethality and bleaching in Euglena.

The loss of methylating ability which accompanies the conversion of MNTS to MTS is consistent with the hypothesis that methylation of DNA or DNA precursors is responsible for bleaching and killing in Euglena.

Although 7-methylguanine is the major detectable methylation product of nucleic acids treated with ^{14}C-MNTS (84,85,107), other minor methylation products may be more important than 7-methylguanine with respect to mutagenesis. Comparative studies of several alkylating agents indicate that there is good correlation between the presence of O-6-methylguanine and the extent of mutagenesis (103,108), whereas the correlation between 7-methylguanine production and mutagenesis is not as good. The assay for 7-methylguanine indicated in Table IX does not

TABLE XI

Effect of MTS on Euglena

Dose of MTS in µg/ml	No. Green Colonies	No. Bleached Colonies
0	1845	1
5	1890	1
10	1980	3
20	1820	6
50	1988	6
100	2062	6

The numbers given are the sum of values from three identical experiments. Treatment was in 6.8 medium.

clarify the role of 7-methylation in bleaching or killing, but serves only to illustrate the *in vivo* reduction of methylating ability at the level of DNA upon conversion of MNTS to MTS.

Relatively long exposure times are required for bleaching of *Euglena* by MNTS as indicated in Figure 17. Maximum bleaching is reached only after an exposure time of greater than thirty hours. The long exposure times required for bleaching may reflect a slow penetration of MNTS into the cells or accumulation of minor breakdown products other than MTS which might be involved in bleaching. A slow rate of reaction of MNTS with nucleic acids or other cellular constituents might also account for the long exposure times required for bleaching.

Since *Euglena* is bleached by a wide variety of mutagens, one might expect that different types of mutagens bleach *Euglena* by different mechanisms. MNTS and NG, both being good methylating agents, may bleach *Euglena* by a similar mechanism involving methylation. Spontaneous bleached mutants of *Euglena* and mutants bleached by U.V., streptomycin and heat have been found to lack detectable amounts of chloroplast DNA (109, 110). Likewise, in two mutants of *Euglena* bleached by MNTS and NG respectively, no chloroplast DNA was detected, as indicated in Figure 18. The technique used is not sensitive enough to indicate that chloroplast DNA is completely lacking, but may simply indicate a reduction of chloroplast DNA to a level undetectable by this technique. It has not been demonstrated that the level of chloroplast DNA in any bleached strains is less than one molecule per cell.

Figure 17. Extent of bleaching of Euglena gracilis strain Z by 10 µg/ml MNTS after various exposure times. Points are values from three independent experiments with approximately 600 colonies per dose in each experiment.

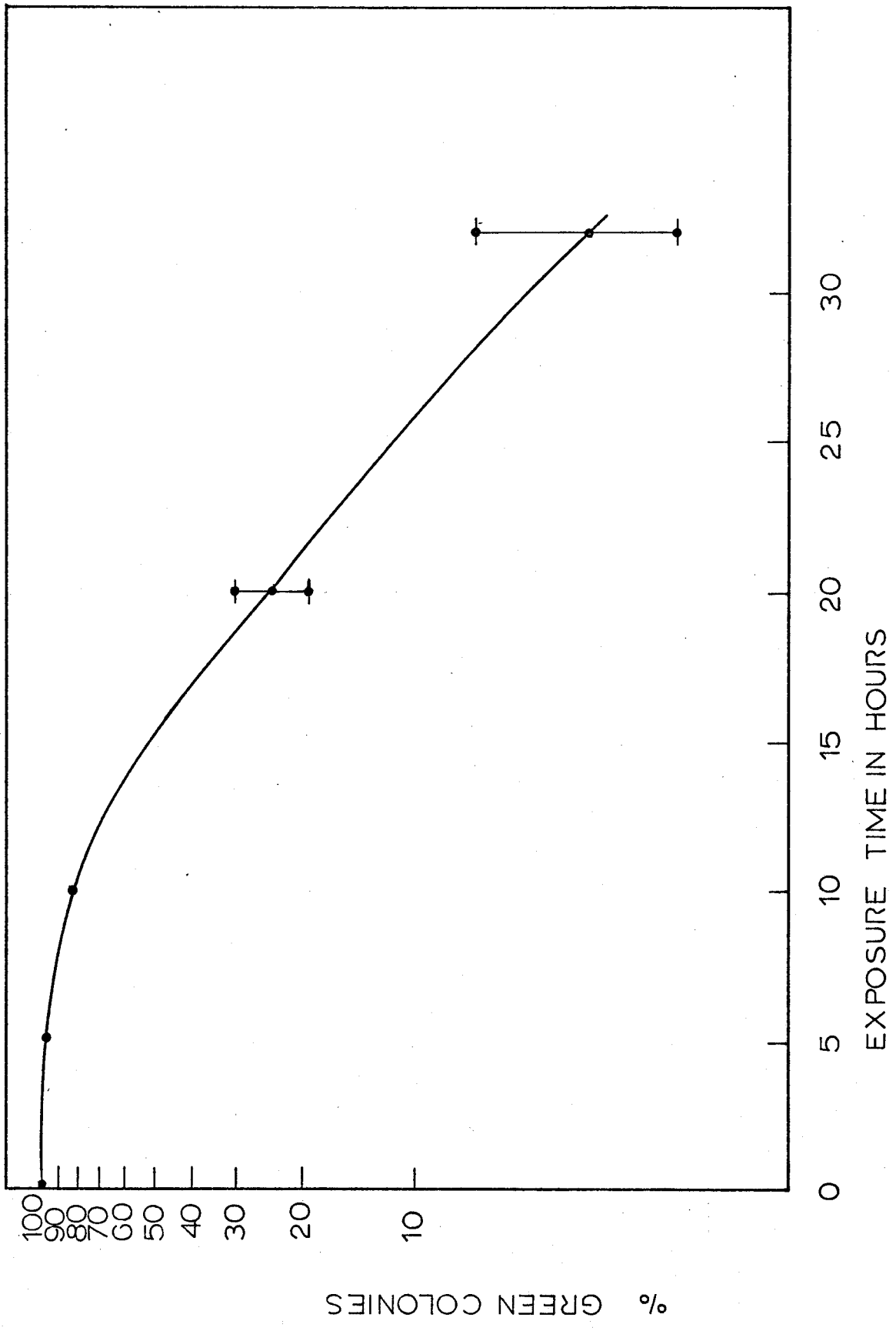

% GREEN COLONIES
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
EXPOSURE TIME IN HOURS

Figure 18. Ultraviolet photographs of DNA isolated from strain Z and from NG and MNTS bleached strains of Euglena.

(i) Strain Z

(ii) NG bleached strain

(iii) MNTS bleached strain

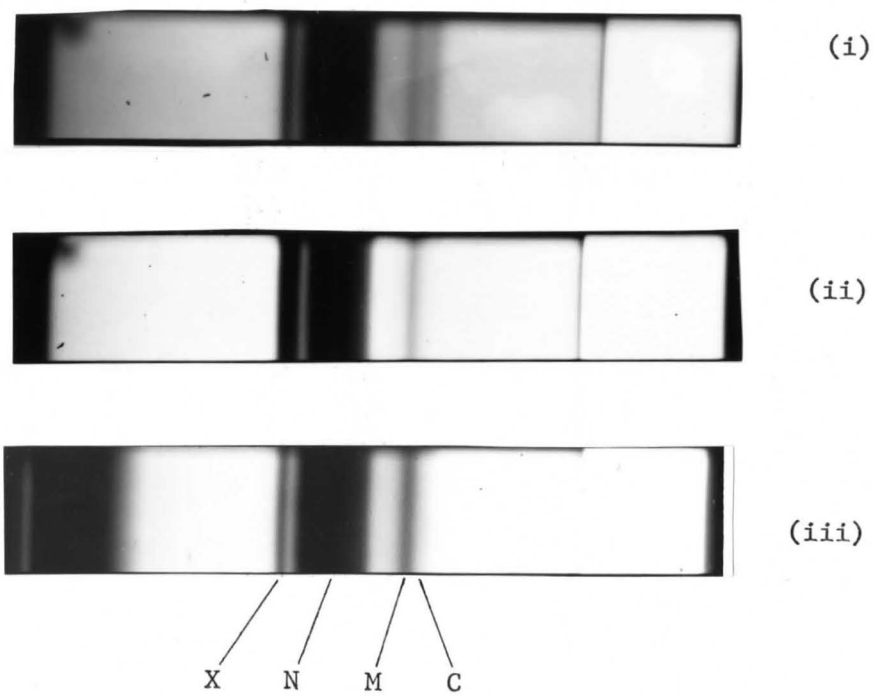
X - Marker DNA (PBS1)

N - Nuclear DNA

M - Mitochondrial DNA

C - Chloroplast DNA

The identification of bands is based on relative positions of the bands. The reported densities of nuclear, mitochondrial, and chloroplast DNA for Euglena are 1.707, 1.691, and 1.686 g/cm^3 (110).


(i)
(ii)
(iii)
X
N
M
C

The apparent absence of chloroplast DNA in bleached strains of Euglena is consistent with the hypothesis that bleaching agents act by preventing the replication of chloroplasts or chloroplast DNA, although the observation does little to clarify the mode of action of the mutagens concerned.

The experiments designed to select U.V. resistant strains of Euglena resulted in the selection of resistant mutants which had slow growth rates. This is likely a result of multiple lesions being induced in surviving cells at the doses used. The use of U.V. resistant mutants to study the mode of action of U.V. in bleaching is not a useful approach unless some better means of preventing multiple lesions in the DNA during the selection procedure is found.

The MNTS resistant strain (strain MNTS 40A) proved to be useful in clarifying the mode of action of MNTS in bleaching and killing Euglena. Further studies of the possible involvement of compounds other than acid soluble thiols might provide useful information concerning the mode of action of protective agents. Such studies might also help clarify the relative mechanisms of "activation" and "inactivation" of NG and MNTS. Such a study might be particularly profitable if MNTS resistant mutants could be selected on the basis of their ability to inactivate MNTS.

SUMMARY

The experiments carried out in an attempt to isolate mutants of Euglena which are resistant to the bleaching effects of U.V. light indicate that resistant mutants can be isolated, although such mutants probably carry multiple lesions and are, consequently, slow growing. The selection of strains carrying multiple lesions is likely a result of the polyploid nature of Euglena. It therefore becomes difficult to select any mutant without using doses of mutagens which cause extensive damage to the nuclear DNA.

The resistance of Euglena to killing by doses of U.V. which bleach essentially all of the cells might be explained on the basis of polyploidy. It would take a much greater dose to inactivate or damage multiple copies of any one gene in a polyploid than would be required to inactivate single molecules of DNA in each chloroplast. The relative sensitivities of the nucleus and the chloroplast would be comparable only if the ploidy of the chloroplast was as great or nearly as great as the nuclear ploidy. The actual ploidies of the nucleus and the chloroplasts are not known with any certainty.

The U.V. resistant mutants isolated were not considered to be useful for cross resistance studies.

Selection for N-methyl-p-toluenesulfonamide (MNTS) resistant mutants yielded a mutant (strain MNTS 40A) which had resistance to the bleaching and killing effects of MNTS and showed slight sensitivity

to killing and bleaching by N-methyl-N'-nitro-N-nitrosoguanidine (NG). The resistance of strain MNTS 40A was shown to be a consequence of the relatively rapid decomposition of MNTS to N-methyl-p-toluene-sulfonamide.

Exposure of strains Z and MNTS 40A to ^{14}C-MNTS showed that there was a greater methylation of nucleic acids in strain Z than in strain MNTS 40A.

The experiments provide evidence that MNTS bleaches and kills Euglena because of its ability to act as a methylating agent. Experiments indicate that MTS is the principal breakdown product in vivo and that MTS is innocuous in the bleaching and killing of Euglena.

REFERENCES

1. De Vries, H. The Mutation Theory. Translation by Farmer, J. B. and Darbishire, A. P. The Open Court Publ. Co., Chicago (1910).
2. Muller, H. J. Genetics 13, 279 (1928).
3. Muller, H. J. Science 66, 84 (1927).
4. Stadler, L. J. Science 68, 186 (1928).
5. Auerbach, C. and Robson, J. M. Proc. Royal Soc. Edinburg, B, 62, 271 (1947).
6. Rapoport, J. A. C.r. Acad. Sci. U.R.S.S. 54, 65 (1946).
7. Pringsheim, E. G. New Phytologist 47, 52 (1948).
8. Provasoli, L., Hutner, S. H. and Schatz, A. Society for Experimental Biology and Medicine Proceedings 69, 279 (1948).
9. Pringsheim, E. G. and Pringsheim, O. New Phytol. 51, 1 (1952).
10. Gross, J. A., Jahn, T. L. and Bernstein, E. J. Protozool. 2, 71 (1955).
11. Zahalsky, A. C. et al. Arch. Mikiobiol. 42, 46 (1962).
12. Ebringer, L. J. Protozool. 9, 373 (1962).
13. Ebringer, L. J. Antibiotics 15, 113 (1962).
14. Aaronson, S. and Bensky, B. J. Gen. Microbiol. 27, 75 (1962).
15. McCalla, D. R. Science 137, 225 (1962).
16. McCalla, D. R. J. Protozool. 12, 34 (1965).
17. McCalla, D. R. J. Protozool. 13, 472 (1966).
18. McCalla, D. R. Science 148, 497 (1965).

19. Pringsheim, E. Rev. Algol. (New Series) 4, 41 (1958).

20. De Deken-Grenson, M. Arch. Intern. Physiol. Biochem. 67, 506 (1959).

21. Lyman, H., Epstein, H. T. and Schiff, J. J. Protozool. 6, 264 (1959).

22. McCalla, D. R. and Baerg, W. J. Protozool. 16, 425 (1969).

23. Ephrussi, B. Nucleo-cytoplasmic relations in microorganisms. Oxford, Clarendon Press (1953).

24. Ephrussi, B. and Hottinguer, H. Nature 166, 956 (1950).

25. Marcovich, H. Annales Institut Pasteur 81, 452 (1951).

26. Nagai, S., Yanagishima, N. and Nagai, H. Bact. Rev. 25, 404 (1961).

27. Leedale, G. F. Nature 181, 502 (1958).

28. Altenburg, E. American Naturalist 68, 491 (1934).

29. Beukers, R. and Berends, W. Biochim. Biophys. Acta 41, 550 (1960).

30. Beukers, R. and Berends, W. Biochim. Biophys. Acta 49, 181 (1961).

31. Wang, S. Nature 190, 690 (1961).

32. Setlow, R. Biochim. Biophys. Acta 49, 237 (1961).

33. Beukers, R., Ijlstra, J. and Berends, W. Rec. Trav. Chim. 79, 101 (1960).

34. Setlow, R. B. and Carrier, W. L. J. Mol. Biol. 17, 237 (1966).

35. Wulff, D. L. and Rupert, C. S. Biochem. Biophys. Res. Commun. 7, 237 (1962).

36. Rupert, C. J. Cell. Comp. Physiol. 58, Suppl. 1, 57 (1961).

37. Setlow, R. Prog. Nuc. Acid Res. 8, 257 (1968).

38. Setlow, R., Carrier, W. and Bollum, F. Proc. Nat'l Acad. Sci. U.S. 53, 1111 (1965).

39. Cook, J. Photochem. and Photobiol. 6, 97 (1967).

40. Setlow, R. and Carrier, W. Proc. Nat'l Acad. Sci. U.S. 51, 226 (1964).

41. Setlow, R., Swenson, P. and Carrier, W. Science 142, 1464 (1963).

42. Boyce, R. and Howard-Flanders, P. Proc. Nat'l Acad. Sci. U.S. 51, 293 (1964).

43. Grossman, L. and Brown, D. in "Mutation as a cellular process". Wolstenholme, G. and O'Connor, M., eds., J. & A. Churchill, London.

44. Nakayama, H., Okubo, S., Sekiguchi, M. and Takagi, Y. Biochem. Biophys. Res. Commun. 27, 217 (1967).

45. Takagi, Y. et al. Cold Spring Harb. Symp. Quant. Biol. 33, 219 (1968).

46. Gellert, M. Proc. Nat'l Acad. Sci. U.S. 57, 148 (1967).

47. Olivera, B. and Lehman, I. Proc. Nat'l Acad. Sci. U.S. 57, 1700 (1967).

48. Zimmerman, S., Little, J., Oshinsky, C. and Gellert, M. Proc. Nat'l Acad. Sci. U.S. 57, 1841 (1967).

49. Gefter, M., Becker, A. and Hurwitz, J. Proc. Nat'l Acad. Sci. U.S. 58, 240 (1967).

50. Weiss, B. and Richardson, C. Proc. Nat'l Acad. Sci. U.S. 57, 1021 (1967).

51. Hanawalt, P. in "Photophysiology". A. C. Giese, ed., Vol. IV, p. 203. Academic Press, New York, 1968.

52. Pettijohn, D. and Hanawalt, P. Biochim. Biophys. Acta 72, 127 (1963).

53. Pettijohn, D. and Hanawalt, P. J. Mol. Biol. 9, 395 (1964).

54. Marmur, J. and Grossman, L. Proc. Nat'l Acad. Sci. U.S. 47, 778 (1961).

55. Cremonese, M. Biophys. J. 8, 153 (1968).

56. Marmur, J. et al. J. Cell. Comp. Physiol. 58, Suppl. 1, 33 (1961).

57. Setlow, R. and Carrier, W. Photochem. Photobiol. 2, 49 (1963).

58. Smith, K., Hodgkins, B. and O'leary, M. Biochim. Biophys. Acta 114, 1 (1966).

59. Lyman, H., Epstein, H. and Schiff, J. Biochim. Biophys. Acta 50, 301 (1961).

60. Schiff, J., Lyman, H. and Epstein, H. Biochim. Biophys. Acta 50, 310 (1961).

61. Schiff, J., Lyman, H. and Epstein, H. Biochim. Biophys. Acta 51, 340 (1961).

62. Gibor, A. and Granick, S. J. Cell. Biol. 15, 599 (1962).

63. Chun, E., Vaughan, M. and Rich, A. J. Mol. Biol. 7, 130 (1963).

64. Sager, R. and Ishida, M. Proc. Nat'l Acad. Sci. U.S. 50, 725 (1963).

65. Leff, J., Mandel, M., Epstein, H. and Schiff, J. Biochem. Biophys. Res. Commun. 13, 126 (1963).

66. McKay, A. F. and Wright, G. F. J. Am. Chem. Soc. 69, 3028 (1947).

67. Mandell, J. D. and Greenberg, J. Biochem. Biophys. Res. Commun. 3, 575 (1960).

68. Loprieno, N. and Clarke, C. H. Mutation Research 2, 312 (1965).

69. Müller, A. J. and Gichner, T. Nature 201, 1149 (1964).

70. Eisenstark, A., Eisenstark, R. and Van Sickle, R. Mutation Research 2, 1 (1965).

71. Schoental, R. Nature 208, 300 (1965).

72. Schoental, R. Nature 209, 726 (1966).

73. Leiter, J. and Schneiderman, M. A. Cancer Research 19, 31 (1959).

74. Greene, M. O. and Greenberg, J. Cancer Research 20, 1166 (1960).

75. Goldin, A., Venditti, J. M. and Kline, I. Cancer Research 19, 429 (1959).

76. Terawaki, A. and Greenberg, J. Biochim. Biophys. Acta 95, 170 (1965).

77. Allan, R. and McCalla, D. R. Can. J. Biochem. 45, 1357 (1967).

78. Cerdá-Olmedo, E. and Hanawalt, P. Biochim. Biophys. Acta 142, 450 (1967).

79. Henry, R. A. J. Am. Chem. Soc. 72, 3287 (1950).

80. McCalla, D. R. and Reuvers, A. Can. J. Biochem. 46, 1411 (1968).

81. McCalla, D. R., Reuvers, A. and Kitai, R. Can. J. Biochem. 46, 807 (1968).

82. McKay, A. J. Am. Chem. Soc. 70, 1974 (1948).

83. McCalla, D. R. J. Protozool. 14, 480 (1967).

84. McCalla, D. R. Biochim. Biophys. Acta 155, 114 (1968).

85. McCalla, D. R. J. Protozool. 13, 472 (1966).

86. Marquart, H., Zimmermann, F. and Schwaiter, R. Z. Vererbungsl. 95, 82 (1964).

87. Druckrey, H., Preussmann, R., Schmähl, D. and Müller, M. Naturwissenschaften 48, 165 (1961).

88. Backer, J. and de Boer, T. Proc. Koninkl. Ned. Akad. Wetenschup. 54B, 191 (1951).

89. Schulz, U. and McCalla, D. R. Can. J. Chem. 47, 2021 (1969).

90. Barron, E. S. G. Manhatten district declassified document #484. Oak Ridge, Atomic Energy Commission. Technical & Information Division, 1946, quoted by Barron, E. S., Dickman, S. R., Singer, T. P. and Muntz, J. A. in "Biological Effects of External X and Gamma Irradiation. Ed. Zirkle, R. E. McGraw-Hill Book Co., Inc. 1954. Part 1.

91. Patt, H. M., Tyree, E. B., Straube, R. L. and Smith, D. E. Science 110, 213 (1949).

92. Elson, L. A. Radiation and radiomimetic chemicals. Butterworths, London, pp. 101-105, 1963.

93. Weisberger, A. S. and Heinle, R. W. J. Lab. & Clin. Med. 36, 872 (1950).

94. Brandt, E. L. and Griffin, A. C. Cancer 4, 1030 (1951).

95. Weisberger, A. S., Heinle, R. W. and Levine, B. J. Clin. Investigation 31, 217 (1952).

96. Schoental, R. and Rive, D. Biochem. J. 97, 466 (1965).

97. McCalla, D. R. Biochim. Biophys. Acta 142, 219 (1967).

98. Cramer, M. and Myers, J. Arch. Mikrobiol. 17, 384 (1952).

99. DeBoer, T. and Backer, H. Rec. Trav. Chim. 73, 229 (1954).

100. Sybalsky, W. and Bryson, V. J. Bacteriol. 64, 489 (1952).

101. Marmur, J. J. Mol. Biol. 3, 208 (1961).

102. Ellman, G. Arch. Biochem. Biophys. 82, 70 (1959).

103. Lawley, P. and Thatcher, C. Biochem. J. 116, 693 (1970).

104. Diehn, B. and Tollin, G. Photochem, and Photophysiol. 5, 839 (1966).

105. Pringsheim, E. and Pringsheim, O. New Phytologist 51, 65 (1952).

106. Allan, R. K. Ph.D. Thesis, McMaster University, Hamilton Canada, (1967).

107. Olson, A. O. and McCalla, D. R. Biochem. Biophys. Acta 186, 229 (1969).

108. Loveless, A. and Hampton, C. Mutation Research 7, 1, (1969).

109. Ray, D. and Hanawalt, P. J. Mol. Biol. 9, 812 (1964).

110. Edelman, M., Schiff, J. and Epstein, H. J. Mol. Biol. 11, 769 (1965).