DOE/EV/04477-1

PARAMECIUM TETRAURELIA: PRESCREEN FOR HAZARDOUS AGENTS

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by

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ABSTRACT

The ciliated protozoan, paramecia, is proposed as a eukaryote model system for use in short-term screening assays for detection of mutagenic/carcinogenic and hazardous agents. The approach utilizes two assays: the mutagenic and photodynamic systems.

Mutagenesis has been successfully employed in prokaryotes for identification of potential carcinogens and can also be used for this purpose in paramecia. Cells are treated with the agent, starved to induce the self-fertilization process of autogamy, and the progeny examined for induced damage. The fraction of progeny surviving autogamy has been established by others as an index of lethal and detrimental mutations. Agents which cause significant reduction in progeny survival after autogamy are classified as DNA damaging agents. When activation of an agent to the mutagenic form is required, the Ames S-9 mammalian-microsome fraction is included. The survey of tested agents indicated that all known mutagens in paramecia are also known carcinogens.

In the photodynamic system, activity is defined as the biological response to a combination of light energy and chemical sensitizor induced by neither alone. In paramecia, photodynamic activity can be quantitated by the concentration of the agent and fluence required for cell immobilization. The photodynamic assay may be a rapid sensitive potential photomutagens and photocarcinogens. Agents identified as active in the photodynamic system can be further evaluated in other assays for light mediated

enhancement of mutagenesis or carcinogenesis. These light sensitizing agents, even if non-mutagenic or non-carcinogenic, represent an environmental hazard since such agents may interfere with the ecological balance in aqueous environments by killing some organisms exposed to sunlight.

INTRODUCTION

Paramecium and other unicellular eukaryote organisms occupy a strategically important level of organization, structure and function between prokaryote and multicellular organisms and as such provides an important research resource (Sonneborn, 1970). The wisest approach for detection of hazardous agents would seem to be the inclusion of biological indicators from some representatives among the three levels of organization for a battery of short-term assays to prescreen for hazardous agents. Paramecium tetraurelia contains two micronuclei and one macronucleus. The macronucleus contains 400 times as much DNA as the micronucleus, governs growth and cell division, and determines the phenotype of the organism during vegetative growth (Cummings, 1972; Jurand and Selman, 1974). The micronuclei are the repository of the genetic information for progeny after fertilization, and do not normally function in the phenotype determination of vegetatively dividing cells (Sonneborn, 1974). During fertilization, the micronuclei undergo meiosis, form haploid gamete nuclei, and only one is retained and is duplicated. During conjugation (cross-fertilization), mutual exchange of gametes occurs, while during autogamy (self-fertilization) the two identical gametes fuse to form the homozygous zygote nucleus or synkaryon. The zygote nucleus subsequently differentiates into the new micronuclei and macronucleus for progeny cells (for review see Beale, 1954; Sonneborn, 1970, 1974). The biology of Paramecium tetraurelia is exceptionally favorable for mutagenesis studies since autogamy results

in the homozygous condition of recessive mutations allowing immediate expression of the phenotype. Tetrahymena also offers potential for mutagenesis studies since "short circuit" matings and selection techniques provide recovery and immediate expression of any induced genetic damage (Bruns and Sanford, 1978). Prokaryote systems have been used successfully as detectors of carcinogens since carcinogens are mutagenic agents (Ames et al., 1973, 1975; McCann et al., 1975; McCann and Ames, 1976) and DNA damage has been implicated in carcinogenesis (Hart et al., 1977; Knudson, 1971). Paramecium will be considered as a eukaryote detector of mutagenic and DNA damaging agents (the two are not always the same) and for its predictive value in screening for carcinogens. The fraction of progeny which do not survive is an index of lethal or detrimental mutations (Kimball, 1949-1965; Kimball et al., 1951-1967) and can be utilized to quantitate the mutagenic potential of the agent. In addition, it is known that micronuclear susceptibility to mutagenic agents reaches a maximum just prior to the initiation of DNA synthesis possibly due to a loss of repair capacity at this time (Kimball, 1963). The utilization of synchronized cells at this sensitive period is equivalent to using repair deficient strains.

Three known carcinogens, ultraviolet irradiation (UV), methylmethane sulfonate and benzo(a)pyrene were tested. Studies of others have shown that photoreactivation repair monomerizes the UV induced cyclobutane dimers (DNA damage) but does not effect the other photoproducts (Setlow, 1966; Sutherland and Oliver, 1976; Sutherland et al., 1967, 1968). Therefore, differences in progeny survival after autogamy between cells receiving ultraviolet alone versus ultraviolet followed by photoreactivation allows quantitation of the biological effect of unrepaired versus

repaired DNA damage. Methylmethane sulfonate was chosen since other alkylating agents have been shown to be effective mutagens in paramecia (Kimball, 1965; Kimball and Perdue, 1967). Benzo(a)pyrene was selected because it is a common environmental pollutant. Polycyclic aromatic hydrocarbons are known to require metabolic activation to show mutagenesis (Ames et al., 1973, 1975; Miller and Miller, 1971; Garner et al., 1972). By using benzo(a)pyrene the value of Ames S-9 activation could be tested in the Paramecium tetraurelia system.

The photodynamic assay, in contrast to the mutagenesis assay cannot be considered a monitor of DNA damage since the mechanism(s) of action are unknown. Photodynamic action is a combination of light energy and chemical sensitizors to produce an effect induced by neither alone (Epstein et al., 1964). The major class of photodynamically active agents includes polycyclic aromatic hydrocarbons and certain dyes (Epstein et al., 1964; Imray and MacPhee, 1973 a,b,; Kelly-Garvert and Legator, 1973; Santamaria, 1972). Product wastes from coal and coke processes include polycyclic aromatic hydrocarbons and some are known carcinogens (Berenblum, 1974; Dundel and Dundel, 1966; NAS, 1972). Rapid detection of the hazardous agents could allow modifications in the developing processes to minimize the production of these compounds. Photodynamic activity has been demonstrated throughout the phylogenetic kingdom including Escherichia coli (Ellis et al., 1974) and Salmonella (Gutter et al., 1977; Ito, 1978), Paramecium (Epstein and Burroughs, 1962; Epstein et al., 1964), Neurospora (Malling et al., 1970), the brine shrimp, Artemia (Morgan and Warshawsky, 1977), and mammalian cells in culture (Morimura et al., 1964). Although Paramecium caudatum has been established as a valuable indicator of photodynamically active

compounds (Epstein et al., 1964), Paramecium tetraurelia was the preference for these studies since our work has been with this organism, and the bulk of background genetic and mutagenesis studies by others have been with this species (Sonneborn, 1950, 1970, 1974). The present study therefore evaluates the potential of the species as a photodynamic and mutagenic eukaryote unicellular indicator of environmental hazardous agents by utilizing known carcinogens and available data of others.

MATERIALS AND METHODS

Culture conditions: The standard culture conditions were used (Sonneborn, 1950). Cerophyl medium was inoculated 24 hours before use with Klebsiella aerogenes, incubated at 27°C and adjusted to pH 6.7. The cells used were stock 51, free of kappa particles, temperature insensitive, trichocyst discharge, and normal behavior.

Daily Isolations: The procedures for daily isolations have been described previously (Sonneborn, 1954; Smith-Sonneborn, 1979; Rodermel and Smith-Sonneborn, 1977). This technique allows determination of cellular age (the number of cell divisions which have elapsed since the origin of the clone at fertilization). Fertilization by autogamy is ascertained by observing 100% of a sample of cells with the nuclear changes characteristic of autogamy by fluorescence microscopy (Smith-Sonneborn, 1974). The unstained cells from that population are the progeny from autogamy.

Mutagenesis assay: The method used was developed by Kimball and co-workers (Kimball, 1963, 1965; Kimball and Perdue, 1967) and described here (see Fig. 1). Synchronized cells are obtained from a mass culture derived from a single cell and divided into control and treatment groups. At the most sensitive time for mutagenesis, 2.5 hours after cell division, 20 cells are placed in a microdrop, flooded with an appropriate dilution of a chemical agent for a given duration. Single-cell isolates were made after completion of treatment and each isolate was maintained as a cell lineage in daily isolation lines. Each lineage was screened for starved cells 100% in autogamy, for use

as the source of progeny from parent cells. Sixteen cells from each depression showing 100% autogamy were taken and placed as single isolates (clones) into fresh medium in depressions. The isolation of this number of autogamous specimens provides nearly full detection of recessive lethals and detrimentals anywhere in the four genomes in the two diploid micronuclei; the chance of missing a recessive lethal is $3/4^{16}$.

The clones were allowed to grow for 4 days and then each depression was observed under a dissecting microscope.

The clones were classified as follows: viable, cells which had cleared the medium of bacteria; detrimental or slow growers, cells which had not cleared the medium of bacteria; and lethal, cells which died or gave rise to only a few moribund cells (Sonneborn, 1970). The viable fraction from 16 progeny was scored for each parent from a given control or experimental group. The values for each group were pooled and an agent was considered a DNA damaging agent if the viable fraction was significantly lower than the control group. Significance was determined by placing confidence intervals on the difference of the two proportions using the normal approximation (Mendenhall, 1975). The variance of that difference is computed on the basis of a stratified random sample (Cochran, 1977) where such observation (surviving fraction) is a stratum and the sample size for each stratum is constant.

The survivors can also be tested for the presence of typical mutant phenotypes, i.e., temperature sensitives, behavioral mutants and trichocyst discharge mutants. Temperature sensitives are incapable of growth at higher temperatures (Sonneborn, 1970). Behavioral mutants do not exhibit the normal avoiding reaction of paramecia (Kung, 1971) and trichocyst mutants lack the ability to discharge trichocysts in picric acid (Pollack, 1975).

It should be cautioned that final classification of such phenotypes as "mutant", requires mating the mutant with wild type, and segregation of the types in the autogamous F_2 generation (Sonneborn, 1970).

Agents Used: Methylmethane sulfonate stock solution was diluted in culture medium containing Tris buffer, adjusted to pH 7.0 and added to cells in Dryl's salt solution (Dryl, 1959). Benzo(a)pyrene was dissolved in acetone, then diluted in culture medium and the suspension was added to the cells. The conditions for ultraviolet irradiation and photoreactivation have been reported previously (Smith-Sonneborn, 1971; Rodermel and Smith-Sonneborn, 1977). In all cases above, the cells were synchronized by selecting cells with the morphology of dividers using a micropipet under a dissecting microscope and treating at 2½ hours after division.

Photodynamic assay: A brief outline of the procedure is seen in Fig. 2. The agents were dissolved in either acetone or DMSO before dilution in culture medium. The cells are incubated 2 hours in the agent prior to 1 hour illumination with a Sylvania F40 BLB light at 4.4 cm. Photodynamic activity was defined in this study as 90% immobilization of 100 cells after 1 hour of exposure; weak positive was defined as 90% severely retarded or immobilized cells; and negative, 90% motile. Triplicate samples were assayed for each compound.

RESULTS

The significant reduction in progeny survival after autogamy, 13-19%, seen after ultraviolet radiation alone, was not found when ultraviolet irradiation was followed by photoreactivation, at 1800 and 2700 ergs of ultraviolet irradiation (Table 1). At higher ultraviolet doses, 3600 and 5400 ergs, the photoreactivation treatment did not restore the survival after autogamy to the untreated control level; the capacity for repair of all damage was exceeded. At appropriate ultraviolet doses however, the fraction of progeny surviving autogamy is an index of unrepaired DNA damage since repair by photoreactivation restores the survival to control values.

Methylmethane sulfonate (MMS) exhibited increased reduction of progeny survival after autogamy as dose increased (Table 2). The older clone, 90 fissions old, was more sensitive to the effect of the agent since the 10^{-4} dilution exhibited a significant effect only with these cells. The survivors of autogamy were also tested for other typical mutant phenotypes and temperature sensitives and swimming behavioral deviants were detected only in the MMS treated groups.

Benzo(a)pyrene showed significant reduction in survival when used in combination with Ames S-9 rat liver microsomal fraction; the S-9 fraction alone was not significantly different from the untreated controls, indicating that this carcinogen is effective in causing DNA damage after activation with the Ames fraction (representative data is seen in Table 3). The data further indicate that the microsomal fraction is useful in the paramecia system for agents which require

activation for mutagenesis. A linear increase in effect is not noted as concentration of benzo(a)pyrene increases; the optimum concentration appears to be 30 µg/ml. The S-9 concentration and cytotoxicity at 60 µg/ml could contribute to the observed results; i.e. increased S-9 may be required for higher benzo(a)pyrene concentrations and toxicity could kill parents before their progeny could be tested for lethal and recessive mutations.

Induction of mutagenesis by benzo(a)pyrene without an exogenously supplied activation system (S-9) has given variable results. Whereas benzo(a)pyrene was significantly different from controls in one experiment, no effect was noted in the other experiment (Table 3). This result is typical of our other attempts to show endogenous activation of benzo(a)pyrene as a mutagenic agent, indicating that our data at this time is insufficient to classify this agent as mutagenic unless S-9 is present.

Comparison of the agents tested in the P. tetraurelia mutagenesis assay in the present study and those investigated by others is seen in Table 4. All mutagenic agents are known carcinogens, all non-mutagenic agents, non-carcinogens.

In the photodynamic assay, only the polycyclic aromatic hydrocarbons were found to be active (Table 5). Other agents tested as negative include for example, methylmethane sulfonate (positive in the mutagenesis assay above), proflavin, benzene, phenol and ethylethane sulfonate. A comparison of our results with those of (Epstein et al., 1964) using P. caudatum and the Ames system (Table 6) indicate that with the limited comparison possible, the three agree completely with the exception of anthracene which is positive in both paramecia systems, but negative in

the Ames assay. The presensitization of P. tetraurelia by benzo(a)-pyrene to light is seen in Figure 3 and indicates this species is an indicator of photodynamically active agents. These cells are immobilized in 4 minutes of exposure to the black light bulb. The cells were also tested for the photodynamic response with sunlight, and immobilization was complete in 40 seconds when cells were preincubated in 20 µg/ml benzo(a)pyrene; sunlight alone showed no effect during this interval. The data is taken as evidence the P. tetraurelia is a sensitive detector of photodynamically active agents.

DISCUSSION

The mutagenesis assay has been used with a limited number of agents but all mutagenic agents are known carcinogens. The assay can be considered promising as a complimentary short-term monitor of DNA damaging agents. This eukaryote unicell offers rapid detection of induced recessive lethals and detrimentals since self-fertilization results in the immediate expression of these agents in this one generation.

The relation of fraction surviving after autogamy to the mean number of micronuclear mutations has been considered extensively (Kimball 1949, 1959, 1965; Kimball and Perdue, 1962, 1967), and transformation equations are available which allow comparison of fraction surviving after autogamy to an index of mutations. The threshold level of detrimental mutations required for detection and rate of increase of mutations as dose increases can be expected to vary with the agent used. Quantitative and qualitative differences in biological potency of the different agents can be determined. The mutagenesis assay proposed is to utilize only the fraction surviving autogamy as an index of mutations since extensive studies of Kimball and co-workers indicate the validity of this procedure (Kimball, 1949, 1959, 1962). In addition, the data reported here show unrepaired DNA damage reduces progeny survival after ultraviolet only, while repaired DNA damage, by photoreactivation can restore survival of progeny after autogamy to control values. These results mimic those of an earlier study by Kimball showing reversal of ultraviolet damage by photoreactivation (Kimball and Gaither, 1951). If other mutant phenotypes are used such as temperature sensitives,

behavioral mutants or trichocyst discharge mutants, such cells should be crossed with normals and then assayed for 1:1 segregation in the F_2 generation. The mating/segregation steps can be done but inclusion of these steps increases the complexity and time required for assay of potential mutagens.

All compounds tested in the Ames and Paramecium mutagenesis systems showed identical results except proflavin which was mutagenic in the prokaryotes and negative in paramecia (Table 4). It is questionable whether proflavin is weakly carcinogenic or non-carcinogenic (NCI, 1977). The complete correspondence of mutagenic with carcinogenic agents in the mutagenesis assay with the limited number of agents tested to date suggests that paramecia mutagenesis is very promising as a pre-screen for inclusion in battery testing of potential mutagenic/carcinogenic agents.

In the P. tetraurelia photodynamic assay, all compounds which were classified as carcinogens (NIOSH, 1976) were photodynamically active agents (Table 5). The carcinogen, anthracene, is negative in the Ames system but positive in the photodynamic assay (Table 6) suggesting that the photodynamic assay can identify some carcinogenic agents difficult to detect in the prokaryote system. In the P. caudatum photodynamic assay, 54% of the most active agents were known carcinogens and 22% were then classified as non-carcinogens (Epstein et al., 1964). We have reevaluated their data considering recent classification of agents and defining photodynamic activity as "agents which immobilize cells in 50 minutes of irradiation at μg/ml" to improve the predictive value of the assay. Their data then indicate that 93% (55/59) of the known carcinogens and 39% (35/90) of agents classified as non-carcinogens are photodynamically active (Table 7). The changes in classification include anthracene, which is a photocarcinogen (Heller, 1950; Blackburn and Taussig, 1975)

and 6-aminochyrsene, benz(e)acridine, dibenz(ac)anthracene, beno(k)-fluoranthene, benzo(ghi)fluoranthene, dibenz(aj)acridine and 6,8, dimehtylbenz(a)anthracene which are now listed as carcinogens (NIOSH, 1976). Eight photodynamically active agents have also been classified as neoplastic (NIOSH, 1976) which are classified here as non-carcinogens. The assay is more competitive as a predictive assay for carcinogenic agents with our modification, but there are more "false positives".

The classification of the compounds as photodynamically active or inactive should not be considered our final determination since our goal was to establish this species as an indicator of photodynamic activity. The assay needs to be used with larger numbers of agents, refined to achieve maximal sensitivity and reproducibility, and a standard definition of photodynamic activity established to include the maximal number of known carcinogens with minimal loss of specificity.

Both the mutagenic and photodynamic assays in Paramecium tetraurelia offer promise as eukaryote detectors of hazardous agents; the former identifies DNA damaging agents and the latter detects agents which presensitize cells and organisms to light illumination and its consequent effects on the cells. The mutagenesis assay may be applicable to a broad range of mutagenic-carcinogenic agents, while the photodynamic assay is useful for the polycyclic aromatic hydrocarbons and dyes. The duration of time required for the mutagenesis assay in paramecia is 7 days, and is relatively inexpensive, but requires knowledge of the biology and manipulation of the organism. The photodynamic assay requires only 3-5 hours, is extremely simple, inexpensive and requires minimal knowledge of the biology and manipulation of the organism.

A comparison of the mutagenesis and photodynamic assays within the eukaryote Paramecium, reveals opposing effects with black light; in the mutagenesis assay light is beneficial, while in the photodynamic assay, light is lethal. Illumination of cells with black light in the mutagenesis assay after treatment with ultraviolet, ameliorated the DNA damage induced by the agent. The beneficial effect has been shown to be due to the light stimulation of the photoreactivation enzyme which monomerizes the cyclobutane dimer damage in the DNA, i.e., black light activated a repair process (Setlow, 1966). In the photodynamic system the polycyclic aromatic hydrocarbon or sensitizing agent is "harmless" until activated to a damaging agent, and this activation can be provided by light. At higher doses, the combination is lethal, at sublethal doses possibly mutagenic or carcinogenic. The exact mechanisms responsible for photolethality have not been elucidated, but need not be due directly to DNA damage. The photodynamic action is related to an active oxygen species since its activity can be reduced in an oxygen free atmosphere and by scavengers of singlet oxygen (Blackburn and Taussig, 1975; Douzou, 1972; Paine, 1976; Jose, 1979). The endogenous activation of these agents in the dark requires oxygen and the mixed function-oxidase systems (Conney, 1967). Light may be stimulating the generation of oxygen, inducing the endogenous enzymes (Paine, 1976).

Evidence that DNA may be involved with some of the photodynamically active agents includes the following observations. Photodynamic activity was increased in prokaryotes defective in DNA repair (Imray and MacPhee, 1973) and benzo(a)pyrene, methylcholanthrene and anthracene exhibited increased efficiency of tumorigenesis when topical applications were augmented with illuminations at critical doses, fluence, and wavelengths. (Heller, 1950; Blackburn and Taussig, 1975; Clark, 1964).

Illumination alone was inactive. Anthracene becomes covalently bound to mammalian DNA in tissue culture cells when exposed to 365nm light. When the binding exceeds one hydrocarbon per 10^3 bases, lethality is observed. Light mediated enhancement of mutagenicity has been observed with methylene blue (Gutter, et al., 1977), toluidine blue (Ito, 1978), acridine orange (Imray and MacPhee, 1975) and the polycyclic aromatic hydrocarbon, chlorpromazine (Brendel, 1968; Kelly-Garvert and Legator, 1973; MacPhee and Imray, 1974).

The available evidence suggests that paramecia is valuable indicator of photodynamically active compounds and can be used as a prescreen for these agents. This short-term bioassay can be used in battery testing and as a bioassay for: 1) photodynamically active agents in air (Epstein et al., 1965) and water; 2) the bio-degredation of these agents in test ecosystems, and 3) identification of agents which should be tested in animal systems for light enhanced carcinogenicity. Photocarcinogens represent an important class since one of the most active agents, benzo(a)pyrene is a common environmental pollutant and has exhibited light enhanced carcinogenecity (Santamaria et al., 1966). Since photolethality is a phylogenetically universal process (Epstein et al., 1964; Gutter et al., 1977; Imray and MacPhee, 1975; Jose, 1979; Malling and Chu, 1979; Morimura et al., 1964; Morgan et al., 1977), active compounds could alter the ecological balance of the environment, selectively killing and/or mutagenizing the surface species which include the aerobic and phototropic organisms.

In summary, the mutagenesis assay provides a eukaryote unicellular assay at a level between the prokaryotes and multicellular organisms.

The photodynamic assay offers a rapid, inexpensive assay for photodynamically active agents which can then be screened for their mutagenic and carcinogenic potential in the presence of light. Environmental pollution of these agents can be rapidly detected by the paramecia assay both in air and water samples. Regardless of the mechanism by which these agents cause damage; these photodynamically active agents may represent a major environmental hazard.

ACKNOWLEDGMENTS

The authors wish to acknowledge the statistical consultant, T. Scavo, and the help of R. Palizzi in preparation of the manuscript, both from the University of Wyoming. The research was supported by DOE contract EE 77-5-0204477-A000.

Figure 1. Legend. The mutagenesis assay is outlined. The mass cultures are derived from the products of a single cell of known age. The genetically identical synchronized cells are placed in control or treatment groups with 20 cells per group. Each cell is then used to initiate a lineage by placing one cell per depression on day 1. The following day, a single cell is reisolated into a new depression with food. The cells not transferred, in back depressions, are a source of starved cells on day 3 or 4. Starvation induces autogamy. Depressions which show parent clones in 100% autogamy are used to obtain 16 single isolates, each then placed in separate depressions. After 4 days, the depressions are scored for the presence of viable clones. The frequency of viable clones after autogamy is an index of mutations or DNA damage present in the parent cells.

Fig. 1

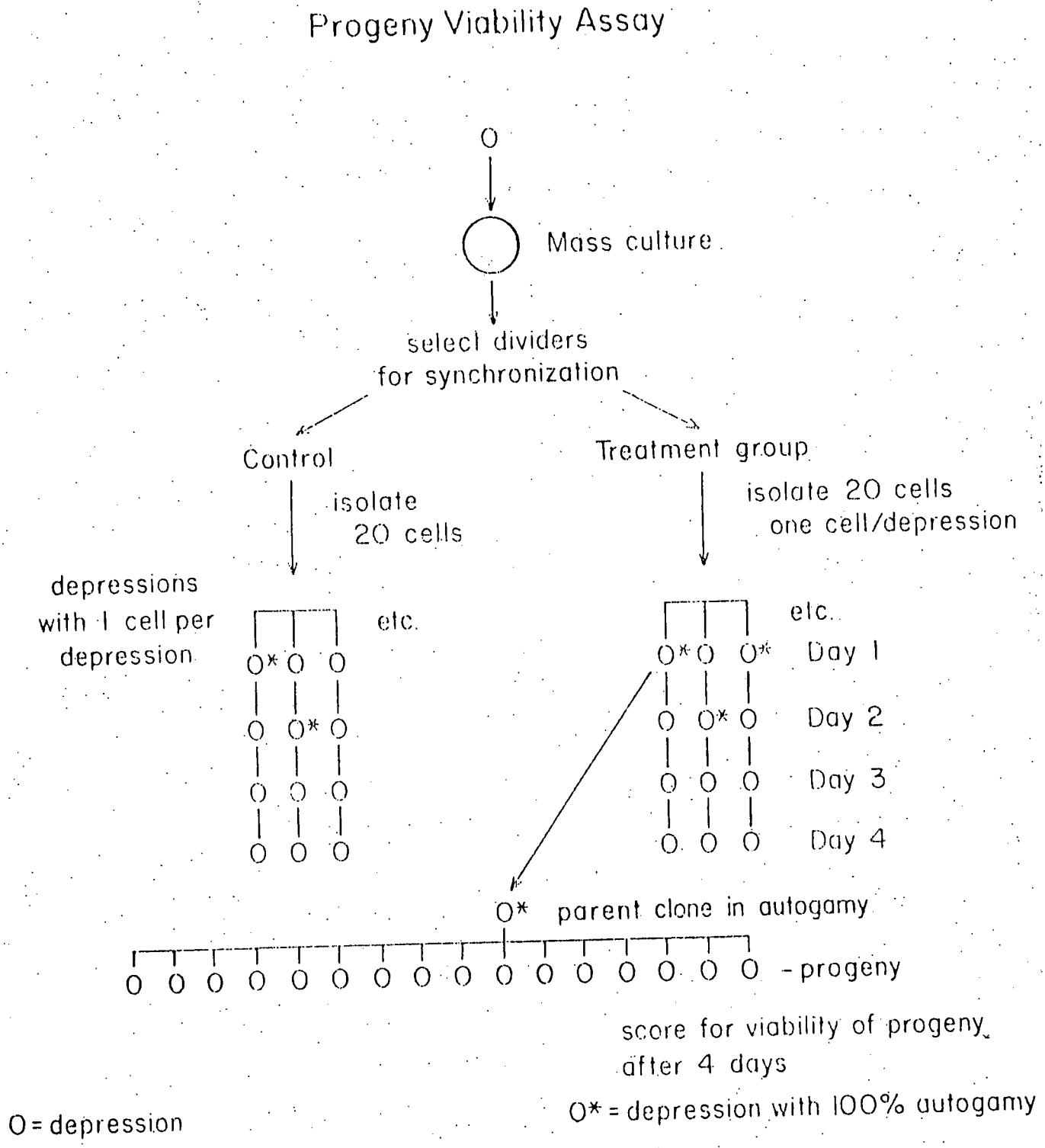
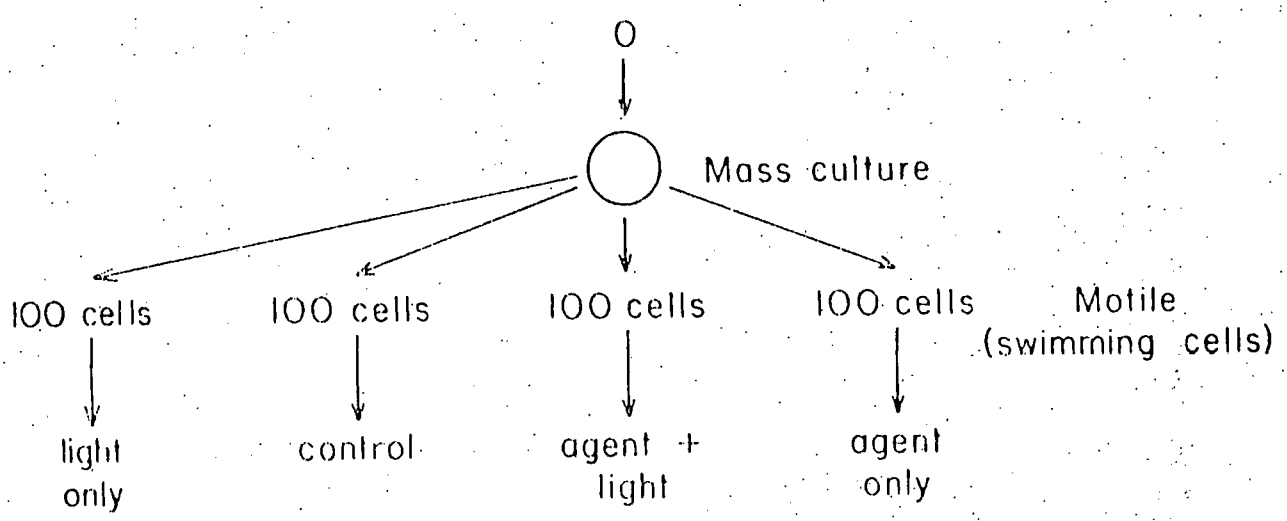

Progeny Viability Assay
Mass culture
select dividers
for synchronization
Control
isolate
20 cells
depressions
with 1 cell per
depression
etc.
Treatment group
isolate 20 cells
one cell/depression
etc.
Day 1
Day 2
Day 3
Day 4
parent clone in autogamy
- progeny
score for viability of progeny
after 4 days
O = depression
O* = depression with 100% autogamy

Figure 2. Legend. The progeny from a single cell of known age are used to initiate a mass culture. Samples of 100 cells are taken for the respective groups. The control group includes 100 cells which receive no treatment.

Fig. 2

Photodynamic Assay



\+ = Active, 90% immobilized

— = Inactive, 90% motile

w + = Weakly active, 90% severely retarded or immobilized

0 = single cell

Figure 3. Legend. Cells were pre-incubated for 17 hours in 20 µg/ml benzo(a)pyrene and then exposed to black light (Sylvania F40 BLB bulb at 4.4 cm) for various time intervals.

FIGURE 3

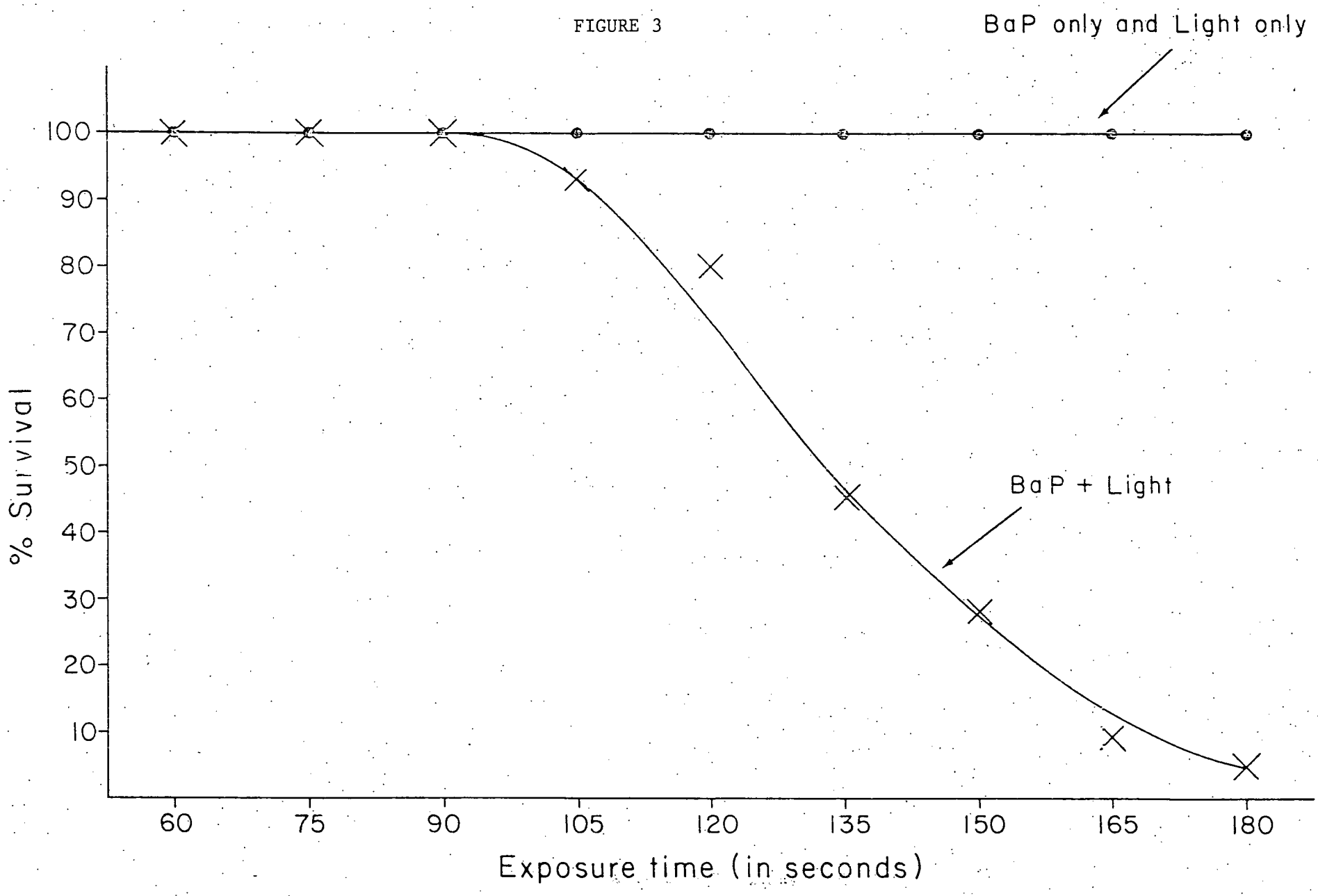

BaP only and Light only
BaP + Light
100
90
80
70
60
50
40
30
20
10
% Survival
60
75
90
105
120
135
150
165
180
Exposure time (in seconds)

Table 1. Effect of ultraviolet irradiation alone and when followed by photoreactivation.

UV Dose (ergs/mm^2)	Percent Survival after Autogamy		
	Ultraviolet only	Ultraviolet and Photoreactivation	Control
1800	19	97	97
2700	13	99	97
3600	13	92	97
4500	8	83	97
5400	2	72	97

Synchronized cells were treated 2½ hours after cell division. Data from three experiments was pooled and the surviving fraction among progeny from at least 6 treated parent cells was determined fo reach of the 11 groups, or 3,264 cells. Those values not underlined by the same line are significantly different with 95% confidence (Cochran, 1977; Mendenhall, 1975).

Table 2. Effect of Methylmethane Sulfonate on survival after autogamy.

Percent Survival				
	Methylmethane sulfonate dilution			Age
Control	10^{-4}	10^{-3}	10^{-2}	(fissions)
93	89	95	39	30
64	64	44	17	90

Synchronized cells were treated at 2½ hours into their interfission period. The values were obtained from pooling the surviving fraction among 16 progeny from at least 4 parent cells from each group or 544 cells. Those values not underlined by the same line are significantly different from one another with 95% confidence (Cochran, 1977; Mendenhall, 1975).

Table 3. Effect of benzo(a)pyrene/microsomal fraction on progeny survival after autogamy.

Percent Survival					
Benzo(a)pyrene/microsomes µg/ml			S-9	Benzo(a)pyrene µg/ml	Controls
30	60	15		30	
72	89	97	97	92	98
69	89	83	100	100	100

Synchronized cells were treated 2½ hours after cell division. In the two experiments above, the benzo(a)pyrene/microsomal group received .04 - .06 ml of the Ames S-9 fraction. The minimum number of treated cells from each group was 6 clones, with 16 progeny tested from each clone, or 1,824 cells.

Those values not underlined by the same line are significantly different at 95% confidence (Cochran, 1977; Mendenhall, 1975).

Table 4. Carcinogenic versus mutagenic agents in Paramecium.

Agent*	Classification	
	Mutagenic***	Carcinogenic****
Acridine mustard (36)	+	not listed
Benzo(a)pyrene (activated)**	+	+
Ethylmethane sulfonate (36)	toxic	+
Hydrogen peroxide (34)	-	-
Hydroxylamine (36)	-	-
N methyl N nitrosonitroguanidine (66)	+	+
Nitrogen mustard (21)	+	+
Methylmethane sulfonate**	+	+
Proflavin (36)	-	-
Triethylene melamine (36,41)	+	+
X-rays (39)	+	+
Alpha radiation (39)	+	+
Ultraviolet irradiation** (37,38)	+	+

*The number in parenthesis represent the reference used to classify the agent with respect to its mutagenic potential. + = mutagenic; - = non-mutagenic.

**The evidence is from the present study.

***In all studies mutagenic agents caused reduced progeny survival after autogamy. The non-mutagen hydrogen peroxide did not reduce progeny survival (Kimball, 1955).

****The carcinogenicity classification was from the suspected carcinogens List (NIOSH, 1976) for chemical agents and from Andrews (1974) for the physical agents. + = carcinogenic; - = non-carcinogenic.

Table 5. Photodynamic effect of agents with Paramecium tetraurelia.

Agent	Photodynamic response concentration 10µg/ml	100µg/ml	Carcinogenicity classification**
Anthracene	+	+	+
Benzo(a)pyrene	+	+	+
2,3 Benzophenanthrene	+	+	+
9,10 Benzophenanthrene	-	-	not listed
Chrysene	+w	not done	+
2-Methylanthracene	+	+	not listed
9-Methylanthracene	+	+	-
1-Methylpyrene	toxic		
Naphthacene	+w	+	not listed
Napthalene	-	-	-
Phenanthrene	-	-	-

*The photodynamic response was defined as follows: + = 90% of the cells were immobilized after 60 minutes of exposure to the black light bulb; +w = 90% of the cells showed severely retarded swimming or immobilization during the test interval; and - = 90% of the cells were motile after the treatment. In all cases the agent was added 2 hours prior to light exposure. Toxic agents were agents which showed an effect on swimming during the test interval in the absence of light.

**The classification of the agents was taken from the list of suspected carcinogens (NIOSH, 1976).

Table 6. Comparison of the photodynamic activity and mutagenicity in the Ames Assay.*

Compound	Ames Test*	P. caudatum**	P. tetraurelia***
Anthracene	-	+	+
Benzo(a)pyrene	+	+	+
Chrysene	+	+	+
Naphthalene	-	not done	-
Naphthacene	not done	+	+
Phenanthrene	-	-	-

*The data from the Ames test was from their published data (McCann et al., 1975) + = mutagenic, - = non-mutagenic.

**The P. caudatum data is from Epstein et al. (1964) + = photodynamically active; - = photodynamically inactive.

***The P. tetraurelia data is from the present study (Table 5).

Table 7. Photodynamic activity versus carcinogenicity.

Photodynamic Activity	Number of Carcinogenic Agents		Number of Non-carcinogenic Agents		Total
Active	55		35	=	90
Inactive	4		25	=	29
Total	59		60		119

The data was taken from the published results of Epstein et al., 1964. Since their data was published, eight of the agents which were photodynamically active have been reclassified as carcinogens. (See text for the definition of activity.)

REFERENCES

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

2. Ames, B.N., W.E. Durston, E. Yamasaki, and F.D. Lee, 1973 Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc. Natl. Acad Sci. U.S. 70: 2281-2285.

3. Andrews, H., 1974 Radiation biophysics. Prentice-Hall Inc., Englewood Cliffs, N.J.

4. Beale, G.H., 1954 The genetics of Paramecium aurelia. Cambridge University Press. Cambridge, England.

5. Berenblum, I., 1974 Carcinogenesis as a biological problem. Amer. Elsevier Publ. Co., N.Y.

6. Blackburn, G.M. and P.E. Taussig, 1975 The photocarcinogenicity of anthracene: photochemical binding to deoxyribonucleic acid in tissue culture. Biochem. J. 149: 289-292.

7. Brendel, M., 1968 Induction of mutation in phage T4 by extracellular treatment with methylene blue and visible light. Molec. Gen. Genet. 101: 111-115.

8. Bruns, P. J. and Y. M. Sanford, 1978 Mass isolation and fertility testing of temperature sensitive mutants in Tetrahymena. Proc. Natl. Acad Sci. U.S. 75: 3355-3358.

9. Clark, J.B., 1964 The effect of long ultraviolet radiation on the development of tumors induced by 20-methylcholanthrene. Cancer Res. 24: 207-211.

10. Cochran, W.G., 1977 Sampling techniques. p. 106. Wiley and Sons Inc., N.Y.

11. Conney, A.H., 1967 Pharmacological implications of microsomal enzyme production. Pharmacol. Rev. 19: 317-366.

12. Cummings, D.J., 1972 Isolation and partial characterization of macro and micronuclei from Paramecium aurelia. J. Cell Biol. 53: 105-115.

13. Douzou, P., 1972 Singlet oxygen as oxidizing intermediate and photodynamic action. pp. 37-47. In: Research progress in organic, biological, and medicinal chemistry, Vol. 3. Edited by U. Gallo and L. Santamaria. Amer. Elsevier Pub. Co., N.Y.

14. Dundel, P. and R. Dundel, 1966 Chemical carcinogenesis and molecular biology. p. 158. Inter-Science Pub. Co., N.Y.

15. Dryl, S., 1959 Antigenic transformation in Paramecium aurelia after homologous anti-serum treatment during autogamy and conjugation. J. Protozool. 6: 596.

16. Ellis, S.P., R.C. Smith, and W.C. Neely, 1974 The photosensitizing action of carcinogens I. The action of 2-naphthylamine on Escherichia coli K-12 and Paramecium caudatum. Can. J. Microbiol. 20: 125-129.

17. Epstein, S.S. and M. Burroughs, 1962 Some factors influencing the photodynamic response of Paramecium cuadatum to 3,4-benzpyrene. Nature 193: 337-338.

18. Epstein, S.S., M. Small, H.L. Falk, and N. Mantel, 1964 On the association between photodynamic and carcinogenic activities in polycyclic compounds. Cancer Res. 24:855-862.

19. Epstein, S.S., M. Small, E. Sawicki, and H.L. Falk, 1965 Photodynamic bioassay of polycyclic atmospheric pollutants. J. Air Pollut. Control Assoc. 15: 174-176.

20. Garner, R.C., E.C. Miller, and J.A. Miller, 1972 Liver microsomal metabolism of aflatoxin B_1 to a reactive derivative toxic to Salmonella typhimurium TA1530. Cancer Res. 32: 2058-2066.

21. Geckler, R.P., 1950 Genetic changes induced by exposure to nitrogen mustard and their inheritance in Paramecium aurelia variety 4. Genetics 35: 253-277.

22. Grover, P.L., A. Hewer, and P. Sims, 1972 Formation of K-region epoxides as microsomal metabolites of pyrene and benzo(a)pyrene. Biochem. Pharmacol. 21: 2713-2726.

23. Gutter, B., W.T. Speck, and H.S. Rosenkranz, 1977 A study of the photoinduced mutagenicity of methylene blue. Mut. Res. 44: 177-182.

24. Hart, R.W., R.B. Setlow, and A. Woodhead, 1977 Evidence that pyrimidine dimers give rise to tumors. Proc. Natl. Acad Sci. U.S. 74: 5574-5578.

25. Heller, W., 1950 Experimentelle Untersuchungen über den Lichtkrebs. Strahlentherapie 81: 387-410.

26. Imray, F.P. and D.G. MacPhee, 1973 The role of DNA polymerase I and the rec system in survival of bacteria and bacteriophages damaged by the photodynamic action of acridine orange. Molec. Gen. Genet. 123: 289-298.

27. Imray, F.P. and D.G. MacPhee, 1973 Induction of frameshifts and base-pair substitution by acridine orange plus visible light in bacteria. Mut. Res. 20: 433-435.

28. Imray, F.P. and D.G. MacPhee, 1975 Induction of base-pair substitution and frameshift mutations in wild-type and repair-deficient strains of Salmonella typhimurium by the photodynamic action of methylene blue. Mut. Res. 27: 299-306.

29. Ito, T., 1978 Cellular and subcellular mechanisms of photodynamic action: the $^1O_2$ hypothesis as a driving force in recent research. Photochem. and Photobiol. 28: 493-508.

30. Jose, J.D., 1979 Photomutagenesis by chlorinated phenothiazine tranquilizers. Proc. Natl. Acad Sci. U.S. 76: 469-472.

31. Jurand, A. and G. Selman, 1974 The anatomy of Paramecium aurelia. Macmillan Press, London.

32. Kelley-Garvert, F. and M.S. Legator, 1973 Photoactivation of chlopromazine: cytogenetic and mutagenic effects. Mut. Res. 21: 101-105.

33. Kimball, R.F., 1949 The induction of mutations in Paramecium aurelia by beta radiation. Genetics 34: 210-222.

34. Kimball, R.F., 1955 The role of hydrogen peroxide in the production of radiation damage in Paramecium. Ann. N.Y. Acad Sci. 59: 638-648.

35. Kimball, R.F., 1963 The relation of repair to differential radiosensitivity in the production of mutations in Paramecium. pp. 167-176. In: Repair from genetic radiation. Edited by F.H. Sobels. Pergamon Press, London.

36. Kimball, R.F., 1965 The induction of repairable premutational damage in Paramecium aurelia by the alkylating agent triethylene melamine. Mut. Res. 2: 413-425.

37. Kimball, R.F., 1969 Studies on mutations induced by ultraviolet radiation in Paramecium aurelia with special emphasis on photoreversal. Mut. Res. 8: 79-89.

38. Kimball, R.F., and N. Gaither, 1951 The influence of light upon the action of UV on Paramecium aurelia. J. Cell. and Comp. Physiol. 37: 211-233.

39. Kimball, R.F., N. Gaither, and S. Wilson, 1959 Reduction of mutations by post-irradiation treatment after ultraviolet and various kinds of ionizing radiations. Radiation Res. 10: 490-497.

40. Kimball, R.F. and S.W. Perdue, 1962 Studies on the refractory period for the induction of recessive lethal mutations by x-rays in Paramecium. Genetics 47: 1595-1607.

41. Kimball, R.F. and S.W. Perdue, 1967 Comparison of mutagenesis by x-rays and methylene melamine in Paramecium, with emphasis on the role of mitosis. Mut. Res. 4: 37-50.

42. Knudson, A.G., Jr., 1971 Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad Sci. U.S. 68: 820-823.

43. Kung, C., 1971 Genetic mutants with altered system of excitation in Paramecium aurelia II. Mutagenesis, screening and genetic analysis of the mutants. Genetics 69: 29-45.

44. MacPhee, D.G. and F.P. Imray, 1974 Mutagenesis by photoactivation of chlorpromazine, a tranquilizer of the phenothiazine group. Aust. J. Biol. Sci. 27: 231-234.

45. Malling, H.V. and E.H.Y. Chu, 1970 Carcinogenic and noncarcinogenic polycyclic hydrocarbons in Neurospora crassa and chinese hamster cells: their photodynamic effects. Cancer Res. 30: 1236-1240.

46. McCann J. and B.N. Ames, 1976 Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad Sci. U.S. 73: 950-954.

47. McCann, J., E. Choi, E. Yamasaki, and B.N. Ames, 1975 Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc. Natl. Acad Sci. U.S. 72: 5135-5139.

48. Mendenhall, W., 1975 Introduction to probability and statistics, 4th ed. p. 186. Duxbury Press, Belmont Ca.

49. Miller, E.C. and J.A. Miller, 1971 The mutagenicity of chemical carcinogens: correlations, problems and interpretations. pp. 83-119. In: Chemical mutagens, Vol. 1. Edited by A. Hollaender. Plenum Press, N.Y.

50. Morimura, Y., P. Kotin, and H. Falk, 1964 Photodynamic toxicity of polycyclic aromatic hydrocarbons in tissue culture Cancer Res. 24: 1249-1256.

51. Morgan, D. and P. Warshawsky, 1977 The photodynamic immobilization of Artemia salina nauplii by polycyclic aromatic hydrocarbons and its relationship to carcinogenic activity. Photochem. and Photobiol. 25: 39-46.

52. National Academy of Science, 1972 Particulate polycyclic organic matter.

53. NIOSH, 1976 Suspected carcinogens. U.S. Dept. of Health, Education, and Welfare, Center for Disease Control. HEW #77-149.

54. NCI Carcinogenesis Technical Report Series #5, 1977 Bioassay of proflavin for possible carcinogenicity. NIH Publication #77-805.

55. Paine, A.J., 1976 Induction of benzo(a)pyrene mono-oxygenase in liver cell culture by the photochemical generation of active oxygen species. Biochem. J. 158: 109-117.

56. Pollack, S., 1974 Mutations affecting trichocysts in Paramecium aurelia I. Morphology and description of the mutants. J. Protozool. 21: 352-362

57. Powers, E.L., Jr., 1955 Radiation effects in Paramecium. Annals N.Y. Acad Sci. 59: 619-637.

58. Santamaria, L., 1972 Further considerations on photodynamic action and carcinogenicity. pp. 271-684. In: Research progress in organic, biological, and medicinal chemistry, Vol. 3. Edited by U. Gallo and L. Santamaria. Amer. Elsevier Pub. Co., N.Y.

59. Santamaria, L., G.G. Giordano, M. Alfisi, and F. Cascione, 1966 Effects of light on 3,4-benzpyrene carcinogenesis. Nature 210: 824-825.

60. Setlow, J.K., 1966 The molecular basis of biological effects of ultraviolet radiation and photoreactivation. pp. 195-248. In: Current topics in radiation research, Vol. 2. Edited by M. Ebert and A. Howard. North-Holland Pub. Co., Amsterdam.

61. Smith-Sonneborn, J., 1971 Age-correlated sensitivity to ultraviolet radiation in Paramecium. Radiation Res. 46: 64-69.

62. Smith-Sonneborn, J., 1974 Acridine orange fluorescence: a temporary stain for paramecia. Stain Tech. 49: 77-80.

63. Smith-Sonneborn, J., 1979 DNA repair and longevity assurance in Paramecium tetraurelia. Science 203: 1115-1117.

64. Sonneborn, T.M., 1950 Methods in general biology and genetics of Paramecium aurelia. J. Exp. Zool. 113: 87-148.

65. Sonneborn, T.M., 1954 The relation of autogamy to senescence and rejuvenescence in Paramecium aurelia. J. Protozool. 1: 38-53.

66. Sonneborn, T.M., 1970 Methods in Paramecium research. pp. 241-594. In: Methods in cell physiology, Vol. 4. Edited by D. Prescott. Academic Press, N.Y.

67. Sonneborn, T.M., 1974 Paramecium aurelia. pp. 469-594. In: Handbook of genetics, Vol. 2. Edited by R.C. King. Plenum Press, N.Y.

68. Sutherland, B.M., W.L. Carrier, and R.B. Setlow, 1967 Photoreactivation in vivo of pyrimidine dimers in Paramecium DNA. Science 158: 1699-1700.

69. Sutherland, B.M., W.L. Carrier, and R.B. Setlow, 1968 Pyrimidine dimers in the DNA of Paramecium aurelia. Biophys. J. 8: 490-499.

70. Sutherland, B.M. and R. Oliver, 1976 Culture conditions affect photoreactivating enzyme levels in human fibroblasts. Biochem. Biophys. Acta 442: 358-367.

71. Rodermel, S. and J. Smith-Sonneborn, 1977 Age-correlated changes in expression of micronuclear damage and repair in Paramecium tetraurelia. Genetics 87: 259-274.