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Dark Repair Of Saccharomyces Cerevisiae Mitochondrial Dna And Its Proposed Role In Ethidium Mutagenesis.

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DARK REPAIR OF SACCHAROMYCES CEREVISIAE MITOCHONDRIAL DNA AND ITS PROPOSED ROLE IN ETHIDIUM MUTAGENESIS

by

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A DISSERTATION

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DEDICATION

To Edward Cameron Hixon, for his endless patience and continuous encouragement without which this dissertation would not have been possible.

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LIST OF ABBREVIATIONS

CPK	Corey-Pauling-Koltun Molecular Models.
EDTA	Ethylenediamine tetraacetic acid
SSC	Saline citrate, 0.15 M sodium chloride in 0.015 M sodium citrate, pH 7.
UV	Ultraviolet irradiation.
YEP	Yeast extract peptone, 1% yeast extract, 2% peptone, 1.5% agar.

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INTRODUCTION

Is mitochondrial DNA subject to a process of dark repair? This question, although studied in some detail, has not been answered conclusively. Much of the evidence presented has been fragmented and contradictory. The purpose of this research was to demonstrate the dark repair of ultraviolet damage in Saccharomyces cerevisiae mitochondrial DNA. It was also hoped that previous conflicting ideas about the dark repair of cytoplasmic DNA could be explained. An interest in ethidium bromide evolved because several investigators have implied that mitochondrial DNA mutagenesis by ethidium bromide might be linked to a process of limited DNA synthesis, such as dark repair. Results from experiments designed to pursue this idea have suggested that mutagenesis of mitochondrial DNA by ethidium bromide may proceed by a two step process; first, a direct damage to DNA by ethidium invoking repair, and second an inhibition of the repair process by ethidium to result in degradation of the DNA.

Repair of mitochondrial DNA must be an important factor in cell survival because mitochondria are indispensible cell organelles in aerobic eucaryotes, responsible for the production of vital cellular energy through the process of oxidative phosphorylation. They are able to maintain a semi-autonomous role within the cell because of a separate genetic system contained within them. Cytoplasmic genes were first

suggested by Correns (1909) and Baur (1909) after observing traits in plants which did not obey the classic rules of Mendelian segregation and inheritance. Further evidence came from Bresslau and Scremin (1924) with the demonstration of Feulgen-positive material inside the Kinetoplast of Trypanosomes. With the advent of electron microscopy, filaments within the matrix areas of mitochondria were identified as DNA (Meyer, Musacchio and Mendonen, 1958, Clark and Wallace, 1960). Later radioautography was used to demonstrate an uptake of radioactive nucleotides into mitochondrial DNA (Stone and Miller, 1965; Parsons and Rustad, 1968). Following the demonstration by Rolfe and Meselson (1959) and Sueoka, Marmur and Doty (1959) that the buoyant density of DNA varies linearly with the guanine-cytosine content, and the use of isopyknic cesium chloride gradients, cellular DNA from many cell types could be separated into two fractions, nuclear and mitochondrial (Sager and Ishida, 1963; Chun, Vaughan and Rich, 1963). Since the early 1960's mitochondrial DNA from many different sources has been characterized according to bouyant density, size, circularity, etc., (Borst, 1972). Furthermore, it is now known that mitochondrial DNA contains information for a set of t-RNAs and ribosomal RNAs which function in an autonomous protein synthesizing system within the cell organelle (Kuntzel, 1971). The information for some inner membrane proteins is also thought to reside within mitochondrial DNA, e.g., three of the six polypeptide subunits of cytochrome oxidase (Mason et al., 1972), one of the three subunits of cytochrome b (Weiss, 1972), four out of nine polypeptides which

form the mitochondrial ATPase complex (Tzagoloff, Rubin and Sierra, 1973), and one protein known to be contained within the small subunits of the mitochondrial ribosome (Groot, Reijnders and Grivell, 1972). Since the size of mitochondrial DNA limits the amount of genetic information contained within it, many enzymes and inner membrane proteins which make up and maintain the organelle are known to be coded in nuclear DNA, e.g., mitochondrial DNA polymerase (Ch'ih and Kalf, 1969), RNA polymerase (Barath and Kuntzel, 1972 a,b) and most ribosomal proteins (Davey, Yu and Linnane, 1969). The precise assembly of all of this information points to an extremely complex interrelationship which describes the co-habitation of mitochondrial and nuclear DNA within the cell. This relationship is even more complicated because it is dynamic in nature, and subject to important feedback controls via repression and derepression to allow for cellular adaptations to changing energy requirements.

Because of the importance of sustaining viable mitochondria, it seems entirely logical that the cell would provide for the continuation and protection of the mitochondrial genome just as it is known that mechanisms exist for the repair of environmentally derived damage to nuclear DNA. Damage to DNA may come from a variety of sources (ultraviolet irradiation, x-rays, γ-rays, alkylating agents, and thermal damage), each source producing a characteristic type of repairable lesion. Although some understanding of repair processes for nuclear DNA has been accomplished,

many questions remain unanswered in this area. DNA repair mechanisms to date may be classified into two categories, light and dark repair (Berndt, 1973; Fox and Lajtha, 1973). Light repair, often called photoreactivation, has been observed in a large variety of cell types. In this repair process a photoreactivating enzyme, acting by energy transfer from visible light, is able to cause the monomerization or reversal of thymine dimers induced by ultraviolet irradiation.

Dark repair may be subclassified into excision repair and post-replication repair. Excision repair is currently the best understood mechanism of DNA repair (Kushner et al., 1971), proceeding in a four step fashion, each step involving a specific repair enzyme. First, an endonuclease is able to detect a distortion in DNA and acts to form a "nick" near the damaged base. Next an exonuclease proceeds to degrade the DNA splitting off the modified base within a small oligonucleotide which is further cleaved into the free nucleotides. To fill in the gap left by this action, a DNA repair polymerase inserts the proper bases into the gap using as a template the undamaged strand of DNA adjacent to the gap. Finally a ligase joins the new DNA to the old DNA strand through the formation of a phosphodiester bond. Excision repair of DNA appears to occur in all cell types from Escherichia coli to man.

Post-replication repair includes recombination repair and a gap-filling process involving de novo synthesis of new DNA. Recombination repair as described by Rupp et al., (1971) for Escherichia coli consists

of the semi-conservative replication of new DNA up to and beyond the sites of damage along the DNA template leaving gaps in the newly synthesized DNA. To complete the information and fill in the gaps a recombination process takes place whereby old DNA from sister strands is incorporated into the new DNA. Another type of post-replication repair has been observed by Lehmann (1972) in which these gaps are filled not by the inclusion of information from pre-existing sister strands of DNA, but from de novo synthesis of new DNA derived from the information present in undamaged areas of the genome. The detailed mechanisms of post-replication repair are not yet understood or characterized.

The idea that mitochondrial DNA must be subject to repair in a fashion similar to nuclear DNA was first investigated by Pittman, Ranganathan and Wilson (1959). These workers observed the photoreactivation of ultraviolet damage in yeast mitochondrial DNA. They used as a model for study the induction of the petite mutation in Saccharomyces cerevisiae. The yeast cell has been the favored model for studies of mitochondrial DNA repair because as a facultative anaerobe it is able to survive with or without functioning mitochondria. Even though the mitochondrial DNA of a yeast cell may be rendered as nonsense, missing, or irreparably damaged, resulting in inoperable mitochondria, the cell is still able to survive in the form of a petite (rho^-) mutant.

These mutants were first called petites by Ephrussi (1953) because of the small colonies they produced on solid medium in

comparison to the colonies produced by normal yeast cells. The petite phenotype may also arise from a nuclear mutation. Since the two (cytoplasmic and segregational petites) cannot be distinguished without genetic characterizations, no distinction has been made in the data presented here. However, the number of petites induced by ultraviolet irradiation may still be used to reflect mitochondrial DNA damage and repair since segregational petites have been shown to comprise a small percentage of the total petite population (Raut, 1954). The reversal of the petite characteristic back to the normal (grande) cell type is a simple test therefore of repair of damage to mitochondrial DNA.

Petite colonies may be readily detected by their small size and by a dye overlay in agar onto visible colonies on solid medium using the dye 2,3,5-triphenyltetrazolium chloride. In the presence of this compound normal cells within colonies are able to reduce the dye to a colored (red) derivative affording easy detection of normal colonies. Petite colonies which have non-functioning mitochondria cannot reduce the dye and remain colorless so that colonies may be scored on agar plates as normal, petite, or sectored (a mixture of both cell types). To demonstrate repair by photoreactivation Pittman, Ranganathan and Wilson(1959) showed that yeast which had been irradiated with ultraviolet light followed by an exposure to visible light produced fewer petites per survivors on solid medium than cell populations exposed to the same dose of ultraviolet light and permitted to grow into colonies

in the dark.

The question as to whether dark repair occurs in mitochondrial DNA has been answered in part by Maroudas and Wilkie (1968) and Moustacchi and Enteric (1970). Maroudas and Wilkie reported that the number of petites at a low dosage of ultraviolet light was reduced after a split dose (time for repair between doses) vs a continuous dose. Only one dose, however, was investigated. Moustacchi and Enteric reported that after ultraviolet irradiation followed by a period of liquid holding of diploid yeast in non-nutritive media, an increase in the number of petites per survivors resulted upon the delayed plating of the yeast. This effect, although the opposite of Maroudas and Wilkie's finding, was explained on the basis of a degradative process of repair which predominated over the resynthesis step. Further work by Moustacchi (1971) with ultraviolet sensitive mutants derived from defects in chromosomal genes, showed that any dark repair of yeast mitochondrial DNA would of necessity involve enzymes coded from both mitochondrial and nuclear DNA. Moreover, Westergaard, Marcker and Keiding (1970) and Westergaard and Lindberg (1972) demonstrated the induction by ultraviolet light of a mitochondrial DNA polymerase in Tetrahymena. Such a polymerase may function as a repair enzyme.

This dissertation initially describes a series of experiments performed to gain more substantial evidence for the dark repair of ultraviolet damage to Saccharomyces cerevisiae mitochondrial DNA. This investigation was complicated by the fact that both

mitochondrial and nuclear DNA are vulnerable to photochemical damage. For this reason it was difficult to distinguish between the effects on these two targets. The repair of nuclear DNA was determined by the overall fraction of survival in a cell population because total cell survival depends only on the status of nuclear DNA in Saccharomyces. Mitochondrial DNA damage was evaluated by scoring from the total number of survivors following ultraviolet irradiation those colonies which appeared as pure clones of petites and those which were sectored. These experiments provided further evidence of the dark repair of mitochondrial DNA and clarified the conflicting results that have been reported previously by other workers.

Studies of specific drug effects on dark repair should contribute to an understanding of the molecular mechanism of the repair process. It has been suggested that ethidium bromide derived mutagenesis of mitochondrial DNA results from an involvement with mitochondrial DNA repair (Hollenberg and Borst, 1971; Mahler and Perlman, 1972). Ethidium bromide will convert an entire yeast population into whole colony petites (colorless colonies on tetrazolium overlay) at drug concentrations non-toxic for cell survival and replication. In contrast, ultraviolet irradiation is a very inefficient inducer of the petite mutation requiring a highly lethal dose to produce greater than 30% petites. Ultraviolet light is also potentially lethal for all cellular DNAs while ethidium is thought to exhibit a selectivity for cytoplasmic DNA

due to the production of petite mutants in yeast (Slonimski, Perrodin and Croft, 1968), the elimination of resistance factors in bacteria (Bouanchaud, Scavizzi and Chabbert, 1968), and its effectiveness in therapeutic applications for the treatment of parasitic infections derived from the protozoan family Trypanosomatidae (Morales et al., 1972, and Srivastava and Ahluwalia, 1973). All of these events are assumed to result from ethidium-DNA interactions. However, this may be an over-simplification of the mechanism, because ethidium bromide is also known to bind to mitochondrial membranes through a co-operative interaction (Azzi and Santato, 1971; Perlman and Mahler b 1971),and recent observations indicate that mitochondrial DNA may have important attachment sites on the mitochondrial membrane (Nass, 1969 a,b).

It has long been known that ethidium binds to nucleic acids through a reversible process called intercalation (Waring, 1965, 1970; LePecq and Paoletti, 1967). Such binding results in an increase in the internucleotide distance for all DNAs and a subsequent change in the winding number of circular superhelical DNA (Smith, Jordan and Vinograd, 1971). This reversible binding has, however, never appeared capable of initiating all the complicated effects of ethidium on cytoplasmic DNA mutagenesis. Recent observations by Mahler and Bastos (1974) have indicated that in yeast a small number of the total ethidium molecules within the cell may be metabolized to a product which binds

covalently to mitochondrial DNA. With this added information, a possible mechanism for mutagenesis involving mitochondrial DNA repair appeared probable, because any modification of bases within the DNA would signal the repair enzymes into action.

Any previous understanding of the mechanism of mutagenesis by ethidium bromide has been difficult because of the multiple number of events within mitochondria influenced by ethidium, i.e., the inhibition of mitochondrial DNA and RNA synthesis (South and Mahler, 1968; Meyer and Simpson, 1969; Fukuhara and Kujawa, 1970; Goldring et al., 1970; Perlman and Mahler, 1971a; Luha, Sarcoe and Whittaker, 1971; Nagley and Linnane, 1972), inner membrane structural alterations (Gitler, Rubalcava, and Coswell, 1969; Mahler and Perlman, 1972), and the eventual degradation of mitochondrial DNA after prolonged exposure to the drug (Goldring et al., 1970). The primary mutagenic event has been hard to discern from all of these effects. Nevertheless, the premise that DNA repair is involved in the mutagenic process could be tested. Earlier experiments demonstrated that ultraviolet damage to cytoplasmic DNA is subject to a dark repair process. If the ethidium induction of the petite mutation is involved with the inhibition of dark repair it was thought that a combination of ultraviolet light and ethidium bromide might be expected to produce petite mutants more efficiently in yeast. When these two mutagens were combined experimentally to produce the petite mutation, the number of petite mutants induced was far greater than a linear

combination of those petites induced separately by each agent. Photoreactivation or dark repair of ultraviolet damage prior to exposure to ethidium reduced the apparent synergism seen when these two agents were combined without the benefit of photoreactivation or time for dark repair. These results suggested a model for ethidium mutagenesis in yeast which could be employed to explain the synergism observed with ultraviolet light and ethidium bromide as well as the observed effects of ethidium alone on mitochondrial DNA culminating in the petite mutation. This proposed "magnification" mutation model states that mutagenesis by ethidium is a two-fold process beginning with the covalent binding of ethidium onto cytoplasmic DNA. This binding, similar to that of known alkylating agents, invokes the action of dark repair enzymes to replace the modified bases within the DNA. The repair process is, however, inhibited at some step by the additional reversible binding of ethidium to DNA resulting in degradation of the DNA and the petite phenotype.

A good biological model proposed to explain an observed phenomenon is one that can be tested by further experimentation to either reinforce its validity or to discover that the model is wrong and must be modified or discarded. The model proposed for ethidium mutagenesis suggested that the covalent binding of ethidium to cytoplasmic DNA "triggered" the mutation process. To test this idea a light sensitive analog of ethidium was synthesized which could be photolyzed to a product which bound

covalently onto cytoplasmic DNA. This analog, 3(8)-amino-8(3)-azido-5-ethyl-6-phenyl phenanthridinium chloride, was shown to bind reversibly to DNA and to produce petite mutants in yeast in the dark at concentrations similar to the parent compound. However, in the presence of light the analog was photolyzed *in vivo* to a reactive nitrene which attached covalently onto cytoplasmic DNA.* This enhanced number of ethidium molecules bound to cytoplasmic DNA resulted in an analogous enhancement of the number of petite mutants induced.

In addition to the use of the ethidium analog for testing a model of ethidium mutagenesis, this azide is the first use of a photoaffinity label for DNA engineered from a drug known to bind to DNA in a reversible manner. The development of other drug analogs should serve as useful tools in studies of drug binding and mechanisms of drug actions.

MATERIALS AND METHODS

Strain and Growth Conditions

A derivative of ATCC # 4098 Saccharomyces cerevisiae var. ellipsoideus was used in all experiments. Cultures were grown in a shaking water bath at 30°C in 1% yeast extract, 2% bacto-peptone, 1% glucose and 1% pyruvate for 24-25 hours (early stationary phase) using an initial inoculum of 10^6 cells per ml. At early stationary phase minimum cell budding occurred and no cell clusters were seen.

Ultraviolet Irradiation and Liquid Holding Recovery

Cells in stationary phase were harvested and washed three times at room temperature with .067 M potassium phosphate buffer pH 7.0 and suspended in buffer to a concentration of 10^3 or 10^4 colony forming units per ml. Suspensions (20 ml) were irradiated in an open petri dish placed under a Gates Raymaster Lamp with filter, emitting radiation of 15 ergs/mm^2/sec at 254 nm. The emitted dose was calibrated by the photohydrolysis of uranyl nitrate and oxalic acid (Daniels et al., 1962). Immediately after irradiation samples were diluted in buffer and plated in subdued light while the remaining suspension at each dose was placed in a covered 50 ml flask on a shaking water bath in the dark at 30°C for liquid holding. After the liquid holding period, samples were again withdrawn, diluted and plated. Dilutions were adjusted to produce approximately two hundred colonies per plate.

Cell suspensions were spread onto 1% yeast extract, 2% bacto-peptone, 1.5% agar (YEP) and either 1% glucose or 1% pyruvate as the carbon source. The introduction of 1% pyruvate as the sole carbon source was to select for the growth of grande colonies only. The use of a differential plating technique to select for grandes was first introduced by Ogur and St. John (1956) in which lactate was used as the selective carbon source. For these experiments the selectivity of pyruvate was demonstrated by plating suspensions of thirty-three whole colony petites that had been identified from plates overlaid with tetrazolium chloride agar. Inoculation of petites onto plates containing 1% glucose or 1% pyruvate resulted in growth only on plates containing 1% glucose as the carbon source. Thus, only those yeast cells with viable mitochondria could utilize pyruvate and grow into visible colonies on 1% pyruvate plates. For liquid holding in caffeine, 10 ml of each suspension was added to a flask containing 12 mg of dry caffeine crystals to obtain a final concentration of 1.2 mg/ml caffeine. Solution of the crystals occurred immediately upon addition of liquid.

Plates containing either 1% glucose or 1% pyruvate were incubated at 30°C for two days or three days, respectively. In each experiment every dose level and two unirradiated controls were plated in triplicate and the counts were averaged. All plates were scored for the number of grande, sectored and whole colony petites. The detection of petites was done according to the

tetrazolium overlay procedure as described by Ogur, St. John and Nagai (1957). The average percentage of spontaneous petites, always < 1%, which occurred among the unirradiated control colonies was subtracted from each set of data in which the percentage of petites is reported. The average increase in the number of colony forming units in the unirradiated controls after a period of liquid holding was approximately 5%. Each experiment discussed is one of at least three similar experiments.

Petite Induction with Ultraviolet Irradiation and Ethidium Bromide

Stationary phase yeast were washed and suspended in phosphate buffer to a concentration of 10^3 colony forming units per ml. Ten ml samples were added to an open petri dish for irradiation. Irradiated solutions were then transferred to covered 50 ml flasks and ethidium bromide was added to the specified concentrations shown in Figs. 6 and 7. Samples were held in the dark on a shaking water bath 24 hours and then spread onto YEP. For photoreactivation, spread plates were placed under a GE fluorescent light (30 watts) two hours prior to incubation at 30°C, two days. Petite colonies were scored using the tetrazolium overlay procedure. Only solid white colonies were scored as petites. Sectored colonies were counted with normal colonies.

Preparation and Isolation of Mitochondrial DNA on Cesium Chloride Gradients

Preferential radioactive labeling of mitochondrial DNA in the absence of protein synthesis was performed using the procedure of Grossman, Goldring and Marmur (1969). For each sample an inoculum

of 10^6 cells per ml was grown for 5 hours at 30°C with shaking in 20 ml of yeast nitrogen base, 1% glucose, followed by an additional 20 min. incubation with cycloheximide at 200 μg/ml. To each sample 200 μc uracil-6-H^3 was added and after two hours of labeling, the cells were washed twice in growth medium containing 50 μg/ml of cold uracil. Samples were then washed twice in phosphate buffer, irradiated and incubated 24 hours with shaking at 30°C in the dark with ethidium bromide concentrations shown in Fig. 8. After this holding period, cells were converted into spheroplasts using the procedure described by Duell, Inoue and Utter (1964). Yeast from each sample were washed in buffer and suspended in 5 ml of 20% mannitol, 0.1 M citrate phosphate buffer pH 5.8, .03 M mercaptoethanol and .001 M EDTA for one hour at 30°C, followed by the addition of 0.3 ml glusulase and continued incubation. Cells were monitored for spheroplast formation by periodic checks of suspensions under a light microscope. Spheroplasts were centrifuged at 5,000 g to remove the glusulase, washed twice with 18% mannitol, and lysed by the addition of 2 ml of saline citrate (SSC, 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7) containing 1% of the detergent Sarcosyl. To the lysed suspension pronase was added (0.1 ml of 1 mg/ml in SSC) followed by incubation at 37°C for 30 minutes. Cellular proteins were removed by two extractions with chloroform:isoamyl alcohol (24:1). RNA was digested by the addition of RNAase (0.1 ml of 1 mg/ml in SSC) and 0.05 ml RNAase T_1 (1,000 units) and incubation at 37°C for 30 minutes. Both enzyme preparations

were preincubated for 10 minutes at 90°C to remove any DNAase activity. DNA samples were dialyzed 48 hours at 4°C in SSC.

Cesium chloride solutions were prepared by the addition of each sample (6 ml in SSC) to a cellulose nitrate tube containing 7.210 g cesium chloride, ρ = 1.690. Solutions were centrifuged in a type 65 fixed angle rotor in a Beckman L2-65B preparative ultracentrifuge for 60 hours at 35,000 rpm, 20°C. Fractions (52 per sample) were collected and counted for radioactivity in Aquasol in a Beckman liquid scintillation counter. Buoyant densities of fractions were determined by reading the refractive index of a control set of fractions on an Abbe refractometer.

Synthesis of 3(8)-amino-8(3)-azido-5-ethyl-6-phenyl Phenanthridinium Chloride and Irradiation to the Light Product

The synthesis of the azido analog of ethidium and its irradiation to a light product was performed by Dr. William E. White of the Laboratory of Molecular Biology. These experimental procedures are included because they have not yet been published and they are necessary to demonstrate the behavior of the analog in the light.

Ethidium bromide (0.3 g, .76 mmol) was dissolved in about 40 ml of water with the aid of 0.4 ml of concentrated HCl. After cooling the solution to 5°C in an ice bath, NaN_3 (0.11 g, 1.7 mmol) was added followed immediately by the slow addition of $NaNO_2$ (0.13 g, 1.9 mmol). After ten minutes the pH was raised to 11.5 causing precipitation of the product. After filtering, the

precipitate was dissolved in water by lowering the pH to 7, and reprecipitated at pH 9.5 to remove any unreacted ethidium.

Assuming that the recovered product was the 6-hydroxyl derivative as shown in Fig. 1 with a MW of 357 the yield was 0.20 g (74%): UV Max (0.1 M Tris, pH 7.3) 283 nm (ε = 4.86 x 10^4); vis (0.1 M Tris pH 7.3) 432 nm (ε = 6.25 x 10^3); ir (KCl disk) 3400 (OH + NH), 3080, 2980, 2860 (C-H), 2130 (N_3) 1610, 1480 (C=C), 1290, 1250, 1160, 700. Because of the photosensitivity of the azide, its photoproduct was used for elemental analysis (see below).

Formation of the diazonium salt followed by nucleophilic substitution by the azide afforded an easy conversion from ethidium salts to the monoazide. Originally a large excess of ethidium was used to minimize disubstitution but since no diazide was found the conditions reported here were chosen to increase the yield and minimize the presence of unreacted ethidium. With a ten fold excess of NO_2^- in a strongly acidic solution, a product was found which may have been the diazide, but it was not characterized further.

The CPK molecular models of ethidium indicate that the lone phenyl ring at C6 must be perpendicular to the phenanthridine ring because of steric hinderance imposed by H7 and the ethyl group. The electronic spectrum of ethidium which resembles that of the phenanthridine nucleus (UV Atlas, 1966) if an allowance is made for the amino group (Brittain, George and

Wells, 1970), further supports the hypothesis that the orbitals of the phenyl ring are orthogonal to those of the fused ring system and therefore contribute no resonance stability to the transition state leading to the diazonium ion. It thus appears likely that inductive effects must dictate the position of the azide substitution. The benzene ring at C6 should stabilize a positive charge at C8 while the positive nitrogen at position 5 should destabilize substitution at C3. The monoazide has been assigned tentatively as the 3-amino-8-azido.

The monoazide from ethidium dissolved readily in water upon addition of HCl. This solution was irradiated for four days with a GE fluorescent (30 watts) lamp and then with an ultraviolet source until the absorption at 2100 cm^{-1} had disappeared. A noticeable color change of the azide occurred within a few minutes when solutions were left exposed to overhead room lights. After evaporation to dryness the product was recrystallized twice from methanol. Based on a molecular weight of 374 for the dichloride, the spectroscopic constants were: λ max (water), 287 nm (ε = 1.82 x 10^4) and 487 nm (ε = 5.03 x 10^3). The ir spectrum showed absorptions at 3340 (N-H), 3100 (C-H), 1610 (C-C), 1480, 1440, 1400, 700 cm^{-1}. Elemental analysis calculated for $C_{21}H_{21}N_3OCl_2$: C, 62.59; H, 5.26; N, 10.44; and Cl, 17.62%. Found were: C, 62.43; H, 4.88; N, 10.50; Cl, 17.92%.

In addition to the principal product a small amount of less

soluble material was found which had essentially the same ir spectrum and was attributed to dimer formation by addition of nitrene to either the azide or the hydroxylamine. The proposed pathway for azide synthesis and photolysis is shown in Fig. 1.

Drug Binding In Vitro, Dialysis and Gel Filtration

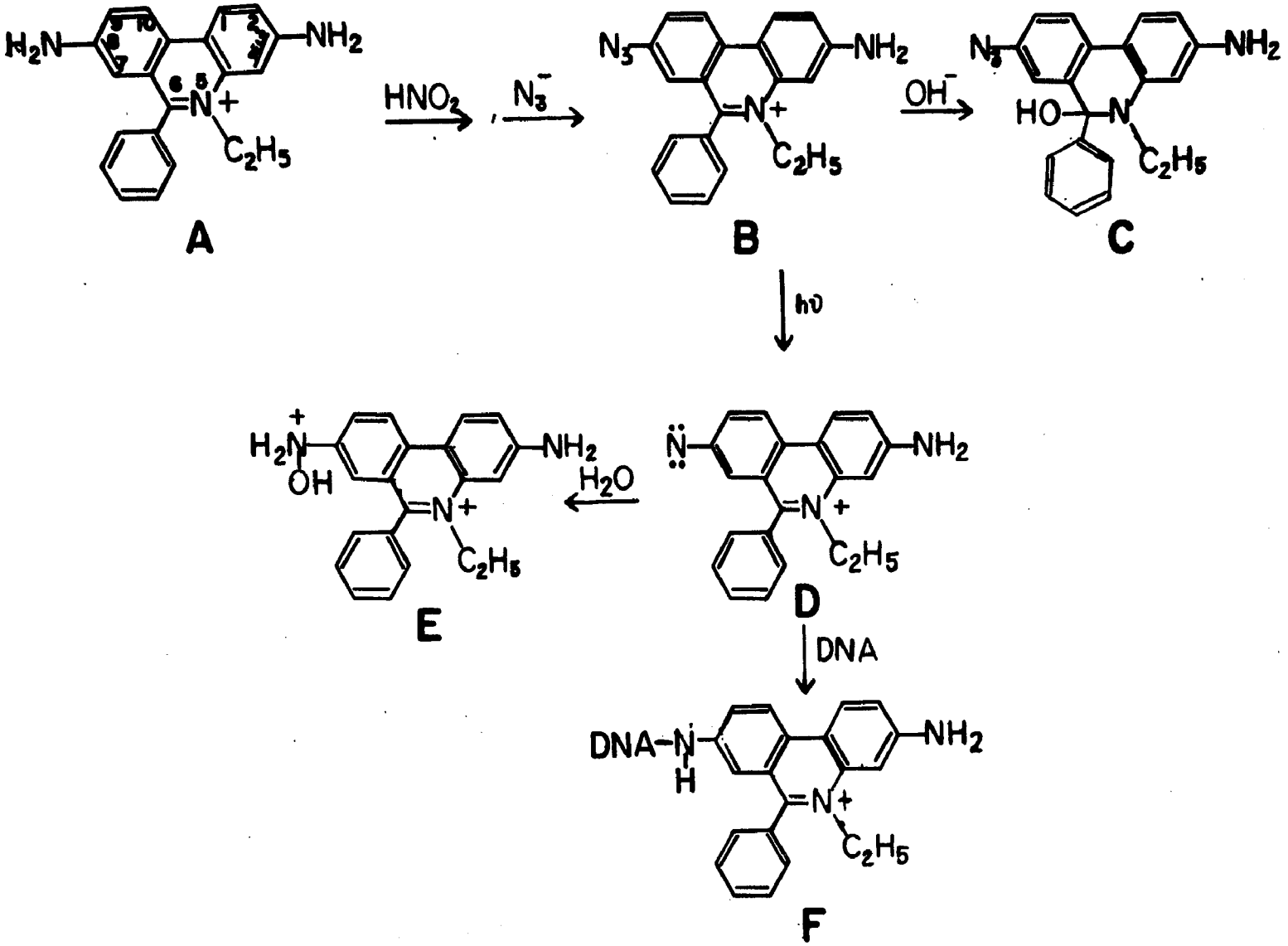
To 1.3×10^{-3} M calf thymus DNA in SSC the azide or ethidium bromide was added to a final concentration of 8.3×10^{-5} M. One set of samples was exposed to a GE daylight fluorescent lamp (30 watts) two hours prior to dialysis in the light. Another was carefully protected from light throughout the dialysis. Dialysis was against Dowex 50 (2% v/v), 1 M NaCl, .01 M Tris and .001 M EDTA for 72 hours (Smith, Jordan and Vinograd, 1971). DNA with bound drug was passed through a Sephadex G 15 column with a void volume of 5 ml. The eluent was .01 M NaOH. One ml fractions from the column were collected and read for absorbance at 260 nm and 490 nm.

Petite Induction with 3(8)-amino-8(3)-azido-5-ethyl-6-phenyl-Phenanthridinium Chloride

Stationary phase yeast were harvested, washed in phosphate buffer and resuspended in buffer to a concentration of 10^3 colony forming units per ml. To each suspension of cells in a 50 ml flask the azide, or the azide light product was added to the concentrations shown in Figs. 11 and 12. The light product was prepared by irradiation of the analog for two hours under visible light in aqueous solution. Flasks containing

FIGURE 1

Formation of 3(8)-amino-8(3)-azido-5-ethyl-6-phenyl-phenanthridinium chloride (B) from ethidium (A). Addition of base forms precipitate (C). Irradiation of (B) generates nitrene (D) which may react with water to form the hydroxylamine (E) or attach to DNA (F).


A
B
C
D
E
F
HNO2
N3−
OH−
hν
H2O
DNA
DNA–N
H
NH2
N3
HO
OH
C2H5

cell suspensions and drug were placed on a shaking water bath at 30°C for 24 hours. After this time yeast were spread onto YEP. Dilutions were adjusted to produce approximately 200 colonies per plate. Spread plates following exposure to the azide or the light product were further subdivided into two groups. The first group was placed under a GE fluorescent lamp (30 watts) for two hours before incubation in the dark at 30°C for two days, while the second group was carefully protected from the light during plating and placed in the dark incubator without the period of light exposure.

Drug Binding In Vivo

Four liters of Saccharomyces were grown in a shaking water bath to stationary phase as described previously. Cells were harvested, washed, and suspended in phosphate buffer. Half of the cells (18 g wet weight) were suspended in one liter of buffer and azide was added to a concentration of 10^{-4} M with continuous stirring in the dark. After a three hour incubation, the cell suspension containing the azide was exposed to overhead lighting plus two fluorescent lamps (30 watts each), one on each side of the cylinder containing the cell suspension plus azide. After an exposure time of 15 hours, yeast were again collected by centrifugation and washed in buffer.

Spheroplasts were prepared in the manner previously described, and lysed in 10 ml of 1% Sarcosyl. DNA was extracted as described previously and dialyzed in 0.2 M sodium phosphate

buffer, pH 6.8,overnight.

Chromatography of Yeast DNA on Hydroxyapatite

The hydroxyapatite separation of yeast nuclear and mitochondrial DNA was performed using the procedure described by Bernardi (1971) and Fukuhara (1969). Solutions of DNA were loaded onto a column of hydroxyapatite (2 x 20 cm) equilibrated with 0.2 M phosphate buffer, pH 6.8. The elution of nucleic acids was monitored using an ISCO fraction collector equipped with a continuous flow cell ultraviolet absorption spectrophotometer. Three ml fractions were collected at a flow rate of approximately 50 ml per hour. Contaminating RNA was removed during a preliminary wash with 0.2 M sodium phosphate buffer. Cellular DNA was then eluted with a linear gradient (250 ml + 250 ml) of sodium phosphate buffer (0.2 M - 0.45 M) pH 6.8.

Thermal Profiles of Yeast DNA

Melting transition profiles of yeast nuclear and mitochondrial DNA with and without the attached analog were performed on a Gilford recording spectrophotometer with a programmed temperature control attachment. Absorbance measurements were corrected for solvent expansion at higher temperatures (Mandel and Marmur, 1968).

TABLE I
MATERIALS AND SOURCE OF SUPPLY

Materials	Source
Aquasol	New England Nuclear
Bacto-dextrose	Difco Laboratories
Bacto-peptone	Difco Laboratories
Bacto Yeast Extract	Difco Laboratories
Caffeine	Eastman Organic Chemicals
Cesium chloride	Gallard Schlesinger, Mfg. Corp.
Chloroform	Matheson, Coleman and Bell
Cycloheximide	Sigma Chemical Co.
Deoxyribonucleic acid from calf thymus Type I	Sigma Chemical Co.
Dowex 50W - X4	J. T. Baker Chemical Co.
Ethylenediamine tetraacetic acid disodium salt (EDTA)	Sigma Chemical Co.
Ethidium Bromide	Calbiochem
Glusulase	Endo Laboratories
Hydroxyapatite Bio Gel HTP	Bio-rad Laboratories
Isoamyl Alcohol	Fisher Scientific Co.
Mannitol	J. T. Baker Chemical Co.
Mercaptoethanol	Eastman Organic Chemicals
Oxalic Acid	Allied Chemical Corp.
Pronase B Grade	Calbiochem
Pyruvic acid	Sigma Chemical Co.
Ribonuclease, bovine pancreas	Worthington Biochemical Corp.
Ribonuclease T_1 from Aspergillus oryzae	Sigma Chemical Co.

TABLE I (Con't)

Materials	Source
Sarcosyl NL-30	K and K Laboratories
Sephadex G 15	Pharmacia Fine Chemicals Inc.
Sodium Azide	Eastman Organic Chemicals
Sodium Nitrite	J. T. Baker Chemical Co.
1,3,5-Triphenyltetrazolium Chloride	Sigma Chemical Co.
Uracil-6-H^3 26.2 curies/mM	New England Nuclear
Uracil	Sigma Chemical Co.
Uranyl Nitrate	J. T. Baker Chemical Co.

RESULTS AND DISCUSSION

Evidence for the Repair of Ultraviolet Damage in Mitochondrial DNA

Recovery at high dose levels -

The effects of immediate and delayed plating on the survival of yeast following ultraviolet irradiation are shown in Fig. 2. An increased survival due to delayed plating in this experiment is consistent with DNA repair but does not distinguish between nuclear and mitochondrial events. The effects of ultraviolet damage to mitochondrial DNA may be observed by the subsequent induction of petite mutants. In all the experiments discussed here, the % mutants refers to the percent petites present expressed as the percent of the unirradiated control colonies. The control population did not vary significantly during the liquid holding period. Other workers have often expressed the induction of petites as the number of petites per survivors. Such an expression involves the quotient of two possible variables, the number of petites induced and the number of survivors at each dose level. This treatment may become confusing when the survival and mutation data are compared after immediate and delayed plating, because of the large fluctuations which may occur in the numbers of survivors and petites in the two experiments. In dosage ranges where the total cell survival approaches 100% after immediate and delayed plating, these two expressions of % mutants do not vary considerably, and in the discussion here the same conclusions may be drawn from either treatment of the data. However, an

expression of the number of petites per unirradiated control colonies which in fact is the survival fraction of petites appears to be a more straightforward treatment of the data in these experiments.

Fig. 3 shows that the production of petites when expressed as the log of % mutants has a linear relationship to the ultraviolet dose. A theoretical extrapolation of these data indicates that 100% petites plus sectored colonies and petites only could be achieved at approximately 840 ergs/mm^2 and 975 ergs/mm^2, respectively, provided that complicating killing and repair effects could be set aside. The extrapolation therefore, indicates the dose beyond which sufficient potential lethality may be inflicted to induce 100% petites. It does not state that 100% petites should be expected experimentally at these doses for two reasons. First, at dose levels $\geq$ 1,000 ergs/mm^2 less than one cell in a hundred survives the irradiated dose. It is probable that those cells which do survive have either received a lower dose from the random distribution of doses which occur throughout the cell population, or that the cell is very efficient in the repair of ultraviolet damage, both nuclear and cytoplasmic. In either case the percentage of petites appearing is not a true sampling of the actual petites induced because greater than 99% of the original population is not available for evaluation. Second, the survival curve upon immediate plating (Fig. 2) exhibits a repair shoulder which

indicates that some repair of ultraviolet damage is possible between the time of immediate plating and cell replication. Assuming that cytoplasmic DNA is also subject to repair one would expect fewer petites than predicted from the extrapolated data after immediate plating. Having clarified this point, in spite of these limitations, the purpose of the extrapolation in Fig. 3 is to obtain a reasonable estimate of the dosage beyond which significant damage occurred to mitochondrial DNA. From the combined data in Figs. 2 and 3, it can be concluded that irradiation $\geq$ 1,500 ergs/mm^2 produced considerable lethal damage to both mitochondrial and nuclear DNA. Thus, any increase in the number of grande colonies at high dose levels after liquid holding should demonstrate repair of ultraviolet damage in both nuclear and mitochondrial DNA, since repair of all cellular DNA must be accomplished to achieve a normal colony state. In this instance a grande colony is defined as a clone of cells resulting from the ability of a parent cell or cells to replicate through the utilization of pyruvate as a carbon source, or from the ability of the colony to appear as a non-petite after tetrazolium overlay on YEP 1% glucose plates.

To test for an increase in the survival of grandes at high ultraviolet doses, parallel liquid holding experiments were performed in which yeast were spread onto two sets of plates, YEP with 1% glucose and YEP with 1% pyruvate. Grandes and petites were scored on plates containing 1% glucose by the use of the

tetrazolium overlay procedure. An increase in survival after delayed plating at each dose level (Fig. 2) resulted from an increase in petites and grandes. The percent contribution from each colony type to produce an increase in the total number of colonies after liquid holding recovery was calculated using the data from three identical experiments. As shown in Table 2, there was a net increase at high dose levels in the number of grande colonies after liquid holding. Although an increased contribution to survival was also seen from the numbers of petites and sectored colonies, the only valid measure here for repair of both cytoplasmic and nuclear DNA was the increase in grandes. The experiments in Fig. 4 indicate that the number of grande colonies on 1% pyruvate medium after ultraviolet irradiation was increased by delayed plating. Since Figs. 2 and 3 showed that both nuclear and mitochondrial damage would be expected at the higher dose ranges, it is presumed that the production of additional grande colonies observed on this selective medium reflected both mitochondrial and nuclear repair. The overall survival was somewhat better on pyruvate than on glucose perhaps because a slower growth rate permitted more time for repair.

Recovery at low doses-

A more direct proof of the repair of mitochondrial DNA is the reduction in the total number of petites and sectored colonies after liquid holding. This phenomenon can be observed at low dose levels

in which total cell survival remains high. This approach was used by Pittman, Ranganathan and Wilson (1959) to demonstrate the photorepair of cytoplasmic DNA. Fig. 5 shows experiments in which the effects of liquid holding were studied on the production of petites by low dose ultraviolet irradiation. The number of whole colony petites was plotted as the percent of the number of unirradiated control colonies on immediate and delayed plating. As shown, there was an overall decrease in this number after delayed plating. It is important to note that the data when expressed as petites/unirradiated control revealed a maximum in the % Petites which thereafter declined sharply. This decline is due to the large increase in cell killing above 525 ergs/mm^2 (see Fig. 2). Therefore, although the actual proportion of petites increased greatly among the survivors at each increased dose level, the total number of petites surviving was reduced. By focusing on an area of the survival curve where the total survival remains high, meaningful fluctuations in the total number of petites could be observed. Furthermore, although the total cell survival increases slightly after twenty-four hours of liquid holding at 525 ergs/mm^2, a net decrease in the number of whole colony petites occurred. A similar decline in the number of whole colony petites plus sectored colonies was seen. However, since the exact scoring of sectored colonies was often doubtful and whole colony petites were easily identified (completely white colonies after tetrazolium overlay) all data shown in Fig. 5

represent the percent of whole colony petites. Using the data from three similar experiments a calculation was made using the Student t to test the significance of the decrease in the number of petites due to liquid holding for doses up to 525 ergs/mm^2. Although the percentage decrease is small it is highly reliable (see Table 3).

Caffeine has been shown to inhibit the liquid holding recovery of stationary phase yeast (Moustacchi and Enteric, 1970) and to have only a slight effect on the liquid holding recovery of irradiated dividing cells. Thus, it has been suggested that caffeine inhibits primarily the excision repair of irradiated DNA in yeast. A similar repair inhibition by caffeine in yeast mitochondrial DNA should result in an increase of petite mutants after ultraviolet irradiation and subsequent liquid holding in caffeine. To verify further that the decrease in the population of petites after liquid holding was due to repair of cytoplasmic DNA, a survival experiment was done with and without the presence of caffeine in the liquid holding cell suspension. Caffeine reduced the total cell survival after twenty-four hours of liquid holding due to the inhibition of nuclear DNA repair. In an unirradiated control with caffeine no petites were induced except for a low level (<1%) of spontaneous petites which also appeared in controls without caffeine. A marked decrease in the number of petites did not occur when caffeine was added to the liquid holding buffer. This seems to demonstrate more clearly

that the reduction in the number of petites at low ultraviolet doses after liquid holding without caffeine may be attributed to the repair of mitochondrial DNA.

Interpretation of survival data -

The data presented here may be divided into two areas: high and low levels of cell survival. In the high dose area the recovery of cells after liquid holding involves a large population of cells. For example, at 1875 ergs/mm^2 in Fig. 2 the increase in cell survival after twenty-four hours of liquid holding showed a thirty-fold increase in survival over that observed with immediate plating. Combining the data in Figs. 2 and 3 one might conclude that lethal damage to all cellular DNA occurs at high ultraviolet dose levels. If only nuclear DNA in a cell is repaired, then new colonies after liquid holding would appear as petites; however, if only mitochondrial DNA is repaired the cells will not grow because nuclear DNA survival is a requisite for cell survival. It is not surprising, therefore, that an increase in the number of petites may be seen after liquid holding at high doses as shown by our data (Table 2) and by Moustacchi and Enteric (1970), due to the repair of extensive nuclear damage. However, this says very little about DNA repair in mitochondria. At high doses it appears to be more significant to determine if an increase in the number of normal colonies results after liquid holding which testifies to the fact that both systems have undergone dark repair of DNA. Such an increase was demonstrated in these

experiments after delayed plating on 1% glucose and 1% pyruvate media.

In the low dose area the range of cells surviving after twenty-four hours of liquid holding is much more restricted. The changes in cell survival on delayed plating are much less dramatic so that fluctuations in the number of petites induced are easier to interpret. Hence, the decrease seen in the overall number of petites at twenty-four hours of liquid holding must point to a shift in the population of survivors from petites to grandes. It is not likely that this decrease in petites is due to their selective cell death during the period of liquid holding, because we are examining a population fluctuation within the shoulder region of the survival curve where very little killing is evident. The effect of caffeine in preventing this decrease after liquid holding is further evidence against cell death since this agent is presumed to act as a repair inhibitor.

This reduction in the number of petites induced at low doses and the overall increase in the number of grandes at high doses of ultraviolet light appear to conflict with an earlier interpretation by Moustacchi and Enteric (1970) which states that the repair process may aggravate lesions resulting overall increases in the number of petites after photochemical damage. This explanation evolved due to the observed increases in the number of petites per survivors at high doses after liquid holding. Similar results are indicated in Table 2 for the experiments reported

here. However, this increase in petites following lethal doses and liquid holding may only point to those cells in which only nuclear repair was completed. Because of the difficulty in interpreting the survival data after highly lethal doses, the demonstration of an increase in grandes following liquid holding at the same dose levels seems to more clearly demonstrate mitochondrial DNA repair, because the increase in the number of normal cells after liquid holding indicates repair of both nuclear and mitochondrial DNA.

It is presumed that the dark repair which occurs during the liquid holding period is similar to the process of excision repair which takes place in nuclear DNA (Patrick, Haynes and Uretz, 1964). This assumption is supported by the fact that caffeine, thought to be an inhibitor of excision repair in yeast (Moustacchi and Enteric, 1970), prevents the repair of cytoplasmic DNA during liquid holding. However, after the yeast are spread onto nutrient agar, following the period of liquid holding, a post-replication repair mechanism may also contribute to the total repair of cytoplasmic DNA. Thomas and Wilkie (1968) have demonstrated with the use of drug resistance markers that mitochondrial DNA undergoes recombination. These recombination mechanisms may play a role in mitochondrial post-replication DNA repair.

TABLE 2

The percentage contribution to the increase in cell survival from immediate to delayed plating on 1% glucose YEP. Delayed plating followed a period of liquid holding 24 hours in phosphate buffer, pH 7.0, 30°C. Each percentage increase is the average increase calculated from three experiments.

Ultraviolet Dose ergs/mm2	% Increase Due to Petites + Sectored Colonies	% Increase Due to Grandes
375	0	100
750	1	99
1125	17	83
1500	27	73
1875	27	73

TABLE 3

Calculation of the Student t to determine the significance of the decrease in % Mutants (whole colony petites) at low doses of ultraviolet irradiation from immediate to delayed plating. A typical induction curve showing a decrease in % Mutants after delayed plating is shown in Fig. 5.

Ultraviolet Dose ergs/mm^2	Student t df = 2	P*
300	1.6	<.30
375	5.2	<.05
450	20.6	<.01
525	9.9	<.01

* P is the probability of having this t value by chance.

FIGURE 2

The total survival of all colony types of Saccharomyces cerevisiae on 1% glucose YEP after ultraviolet irradiation followed by ● immediate plating, and ▲ delayed plating after 24 hours of liquid holding in .067 phosphate buffer, pH 7.0, 30°C.

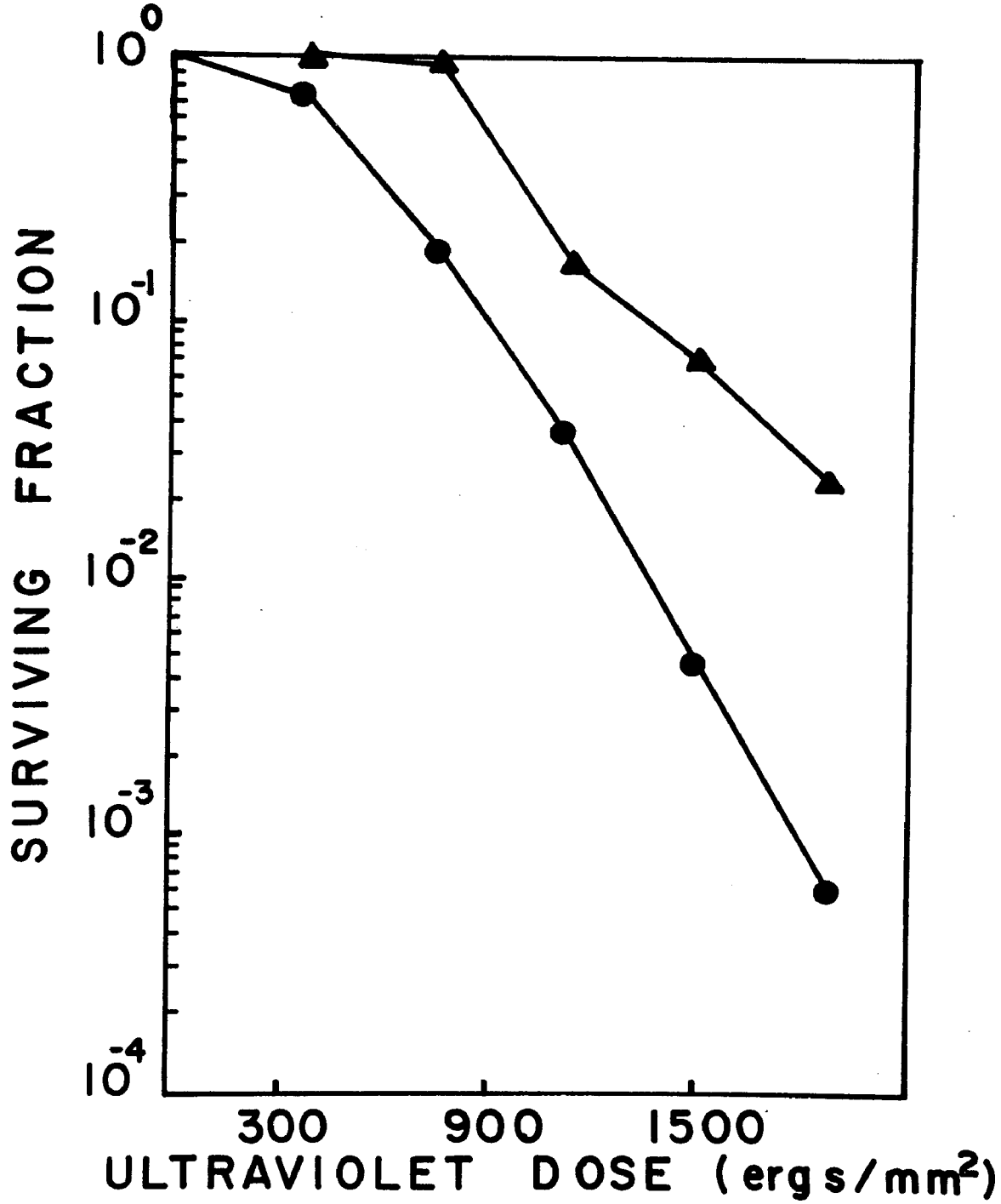

SURVIVING FRACTION
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
300
900
1500
ULTRAVIOLET DOSE (ergs/mm^2)

FIGURE 3

The induction of petite mutants by ultraviolet light followed by immediate plating onto 1% glucose YEP: ● whole colony petites plus sectored colonies, ▲ whole colony petites. An extrapolation to 100% Mutants is achieved at 840 ergs/mm^2 for whole colony petites plus sectored colonies, and 975 ergs/mm^2 for whole colony petites.

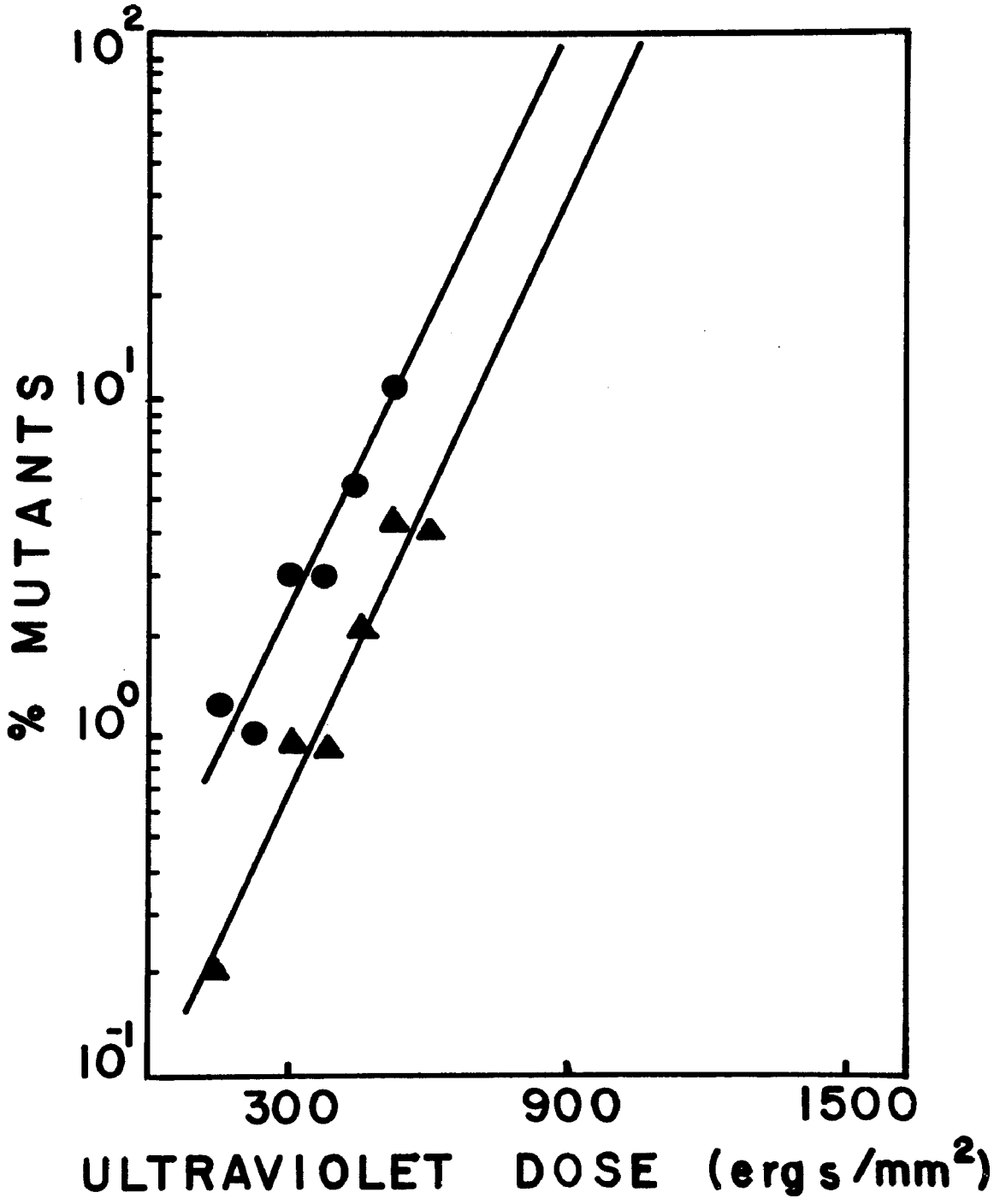

10²
10¹
10⁰
10⁻¹
% MUTANTS
300
900
1500
ULTRAVIOLET DOSE (ergs/mm²)

FIGURE 4

The selective survival of grandes on pyruvate YEP after ultraviolet irradiation followed by ● immediate plating, and ▲ delayed plating after 24 hours of liquid holding in .067 phosphate buffer, pH 7.0, 30°C. An increase in survival at $\geq$ 1500 ergs/mm^2 after delayed plating indicates dark repair of both nuclear and mitochondrial DNA.

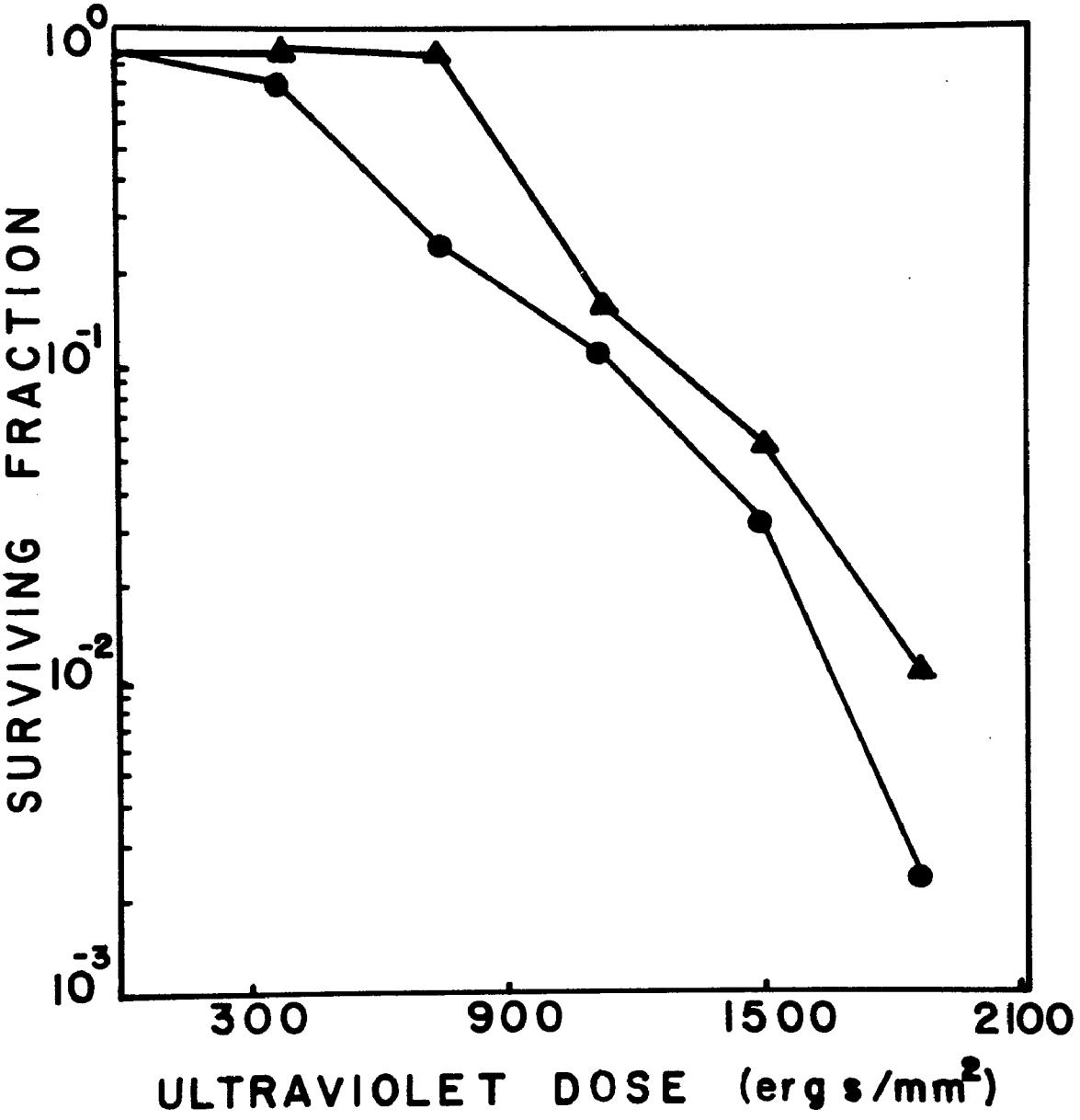
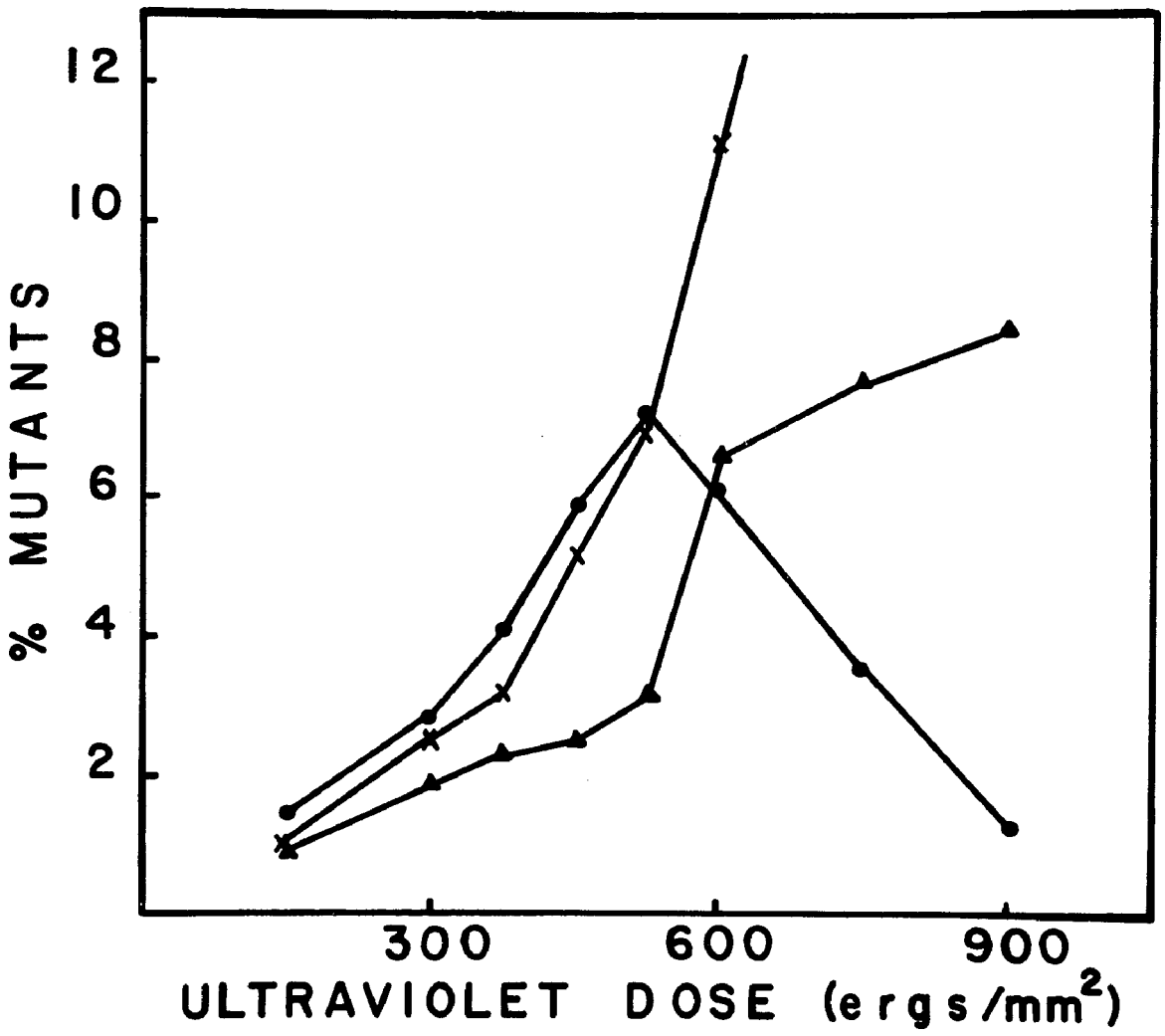

SURVIVING FRACTION
10^0
10^{-1}
10^{-2}
10^{-3}
300
900
1500
2100
ULTRAVIOLET DOSE (ergs/mm^2)

FIGURE 5

The induction of whole colony petites at low doses of ultraviolet light: ● immediate plating; ▲ delayed plating after 24 hours of liquid holding in phosphate buffer, pH 7.0, 30°C; X delayed plating after 24 hours of liquid holding as above with the addition of 1.2 mg/ml of caffeine. A decrease in the number of petites after delayed plating up to 525 ergs/mm^2 suggests dark repair of mitochondrial DNA. The lack of a marked decrease in % Mutants after a period of liquid holding in caffeine indicates an inhibition of mitochondrial DNA repair by caffeine.


12
10
8
6
4
2
% MUTANTS
300
600
900
ULTRAVIOLET DOSE (ergs/mm²)

Petite Induction by Ultraviolet Light and Ethidium Bromide

Hollenberg and Borst (1971) first suggested that mutagenesis of mitochondrial DNA by ethidium bromide might require the process of DNA repair. In experiments to discover conditions which might prevent petite induction by ethidium bromide they demonstrated that repressed yeast growing in 9% glucose were immune to mutagenesis by ethidium. Yeast growing at high glucose concentrations (glucose repression) have fewer visible mitochondria within them and decreased concentrations of mitochondrial enzymes (Polakis, Bartley and Meek, 1964; Reilly and Sherman, 1965; Jayaraman et al., 1966). Nevertheless, repressed yeast contain as much mitochondrial DNA as nonrepressed yeast (Fukuhara, 1969). Borst pointed out that since normal replication of mitochondrial DNA could take place in repressed yeast in the presence of ethidium bromide, some additional process like DNA repair or recombination might take part in ethidium mutagenesis. While this speculation may now require some modifications (see below) the idea that ethidium might influence dark repair prompted the experiments which follow.

Ethidium belongs to a group of aromatic drugs known to bind reversibly to DNA (Waring, 1965, 1970). The acridines which also bind reversibly to DNA are effective inducers of the petite mutation in yeast (Ephrussi and Hottinguer, 1950). The reversible binding of ethidium and acridines to bacterial DNA is thought to be related to their inhibition of dark repair of ultraviolet

damage in these procaryotes (Lankford and Yielding, 1971; Lankford, 1973). It was postulated that if ethidium mutagenesis was incurred by repair inhibition, the induction of petite mutants by ultraviolet irradiation should be enhanced by the presence of ethidium bromide. This prediction was tested experimentally.

The number of petite mutants induced by exposure of yeast to both ethidium bromide and ultraviolet irradiation was far greater than that predicted from an independent combination of both effects (Figs. 6 and 7). This synergism was observed by holding either mutagen at a constant dosage while the other was progressively increased. In both situations, petite induction was possible with a minimum of cell killing. In each experiment in Figs. 6 and 7 cell survival was always greater than 90%.

This enhanced production of petites following a combination of ultraviolet light and ethidium bromide was reduced if the ultraviolet damage was reversed by photoreactivation, or if time was allowed for dark repair prior to the addition of ethidium bromide. Photoreactivation proved to be most effective at low percentages of petite induction (less than 50%, Fig. 7). For petite populations greater than 90%, photoreactivation was not successful, suggesting that the petite characteristic had been irreversibly fixed during the liquid holding period following drug addition and ultraviolet damage. When cells were irradiated, and held for twenty-four hours in buffer without drug (time for dark repair) and than spread

onto agar plates containing ethidium bromide, no synergism could be seen when compared to petite populations induced by the growth of unirradiated cells on ethidium bromide plates (Fig. 6). The percentage of induced whole colony petites was less for cells grown on ethidium bromide than for cells held in solutions of the same drug concentration twenty-four hours before growth on drug free medium. However, the number of sectored colonies on ethidium bromide plates was very large, while cells held in drug prior to plating in the absence of drug evidenced very few sectored colonies. This again suggested a fixation of the petite mutation during the period of liquid holding in drug. Moreover, if yeast were irradiated, held for twenty-four hours in buffer (time for dark repair), and then held for an additional twenty-four hours in the presence of drug before plating onto drug free medium the ratio of petites per survivors was less than the number induced when both mutagens were added together (data not shown).

The co-operative effect of ethidium bromide and ultraviolet light in producing petite mutants was accompanied by an enhanced degradation of mitochondrial DNA over that seen with each mutagen separately. Fig.8 depicts the radioactivity of nuclear and mitochondrial DNA from cesium chloride gradients of cellular DNA samples treated <u>in vivo</u> with ultraviolet light and ethidium bromide either separately or in combination. While the nuclear DNA remained unchanged, a synergism in the process of mitochondrial DNA degradation could be seen which paralleled the results of

enhanced petite production with a combination of ultraviolet light and ethidium bromide as shown in Fig. 6.

The production of petite mutants by a combination of ethidium bromide and ultraviolet light observed in these experiments suggests that some common factor might operate on both kinds of DNA damage to magnify the total mutagenic effect. It was shown that the process of mutation appeared to be fixed during the liquid holding in which starved cells were exposed to drug immediately following ultraviolet irradiation, and that this fixation was correlated with mitochondrial DNA degradation. As previously predicted, the common factor which caused the observed synergism was thought to be the repair inhibition of ultraviolet damage by ethidium bromide. While this provided a plausible explanation for the enhanced number of petites produced by a combination of both agents, it appeared inadequate to explain the extremely large synergistic effect observed or the mechanism of ethidium mutagenesis per se. Ethidium bromide alone is an extremely efficient inducer of the petite mutation at concentrations non-toxic for cell survival and growth. Repair inhibition in the absence of any outside factor to invoke repair did not appear probable to explain mutagenesis by the drug.

Aside from the aspect of repair inhibition, some workers have previously suggested that the presence of ethidium bromide seemed to provoke repair enzymes into action. Westergaard,

Marcker and Keiding (1970) observed the presence of a mitochondrial "repair" polymerase in Tetrahymena after treatment with ethidium bromide. Paoletti, Couder and Guerineau (1972) observed increased nuclease action in mitochondrial preparations in the presence of ethidium bromide. A recent communication by Mahler and Bastos (1974) may serve to explain these effects. In experiments using radioactive labelled (C^{14}) ethidium bromide they demonstrated that a small number of ethidium molecules were metabolized to a product which became linked covalently to mitochondrial DNA. This covalent binding to mitochondrial DNA produces potential repair sites along the DNA and may provide the signal which provokes the repair enzymes into action.

A Proposed Model for Ethidium Mutagenesis

This new information coupled with the observed synergism between ultraviolet light and ethidium bromide may be combined to suggest a reasonable model for ethidium mutagenesis. The proposed model invokes a combination of both modes of binding by ethidium to mitochondrial DNA, reversible and non-reversible (covalent). The covalent binding to DNA and/or a distortion of the DNA-membrane complex probably is subject to the action of repair enzymes. Since these enzymes operate on DNA (e.g., incision, excision, synthesis, joining) they are potentially inhibited by the reversible binding of ethidium to DNA or the DNA-membrane complex. Such inhibition of repair by ethidium could lead to the eventual degradation of mitochondrial DNA

and thereby to the resulting petite mutation. Ethidium may therefore cause the petite mutation by a two-step "magnification" process, i.e., a direct damage which is subject to repair enzymes and at the same time inhibition of these repair processes by the presence of the reversibly bound drug.

By employing this model one is able to interpret the synergism between ultraviolet light and ethidium bromide. The damage generated by ultraviolet light within DNA, like that produced by ethidium, is subject to repair processes. In this model, the reversible binding of ethidium would inhibit repair for every lesion regardless of its origin, so that the mutagenic potential of each damaged site would be enhanced. This model may also explain the observation by Hollenberg and Borst (1971) that glucose repressed yeast were not affected by ethidium bromide. This could perhaps be explained by the absence of the enzyme in repressed yeast which activates ethidium to a product which binds covalently to DNA. Without this initial step of the mutation model, mutagenesis would not occur. Mahler and Bastos (1974) have also indicated that in some cell lines the ability to metabolize ethidium to an activated product parallels their sensitivity to the drug with regard to DNA degradation.

This model for petite mutation by ethidium bromide does not conflict with previous observations by Mahler and Perlman (1972) which point to an important involvement of the inner membrane with mitochondrial DNA and ethidium during the mutation process.

This membrane-ethidium-DNA complex may be involved with the process of repair inhibition, because a similar type of complex occurs with acridines (Kellerman, Biggs, and Linnane, 1969) which also inhibit DNA repair (Lankford and Yielding, 1971; Lankford, 1973) in addition to binding reversibly to DNA.

FIGURE 6

Petite induction at 525 ergs/mm^2 UV and increasing concentrations of ethidium bromide.

● - Induction of petites by UV followed by 24 hrs in buffer with ethidium bromide and plating on 1.5% agar, 1% yeast extract, 2% bacto-peptone and 1% glucose.

X - Induction of petites as - ● - except for 2 hrs of photoreactivation under a 30 watt GE fluorescent light after plating.

▲ - Induction of petites by 24 hrs of exposure to ethidium bromide in buffer, no UV.

o - Induction of petites by UV followed by 24 hrs in buffer and growth on media above with the addition of ethidium bromide.

□ - Induction of petites by growth on the media above containing ethidium bromide, no UV.

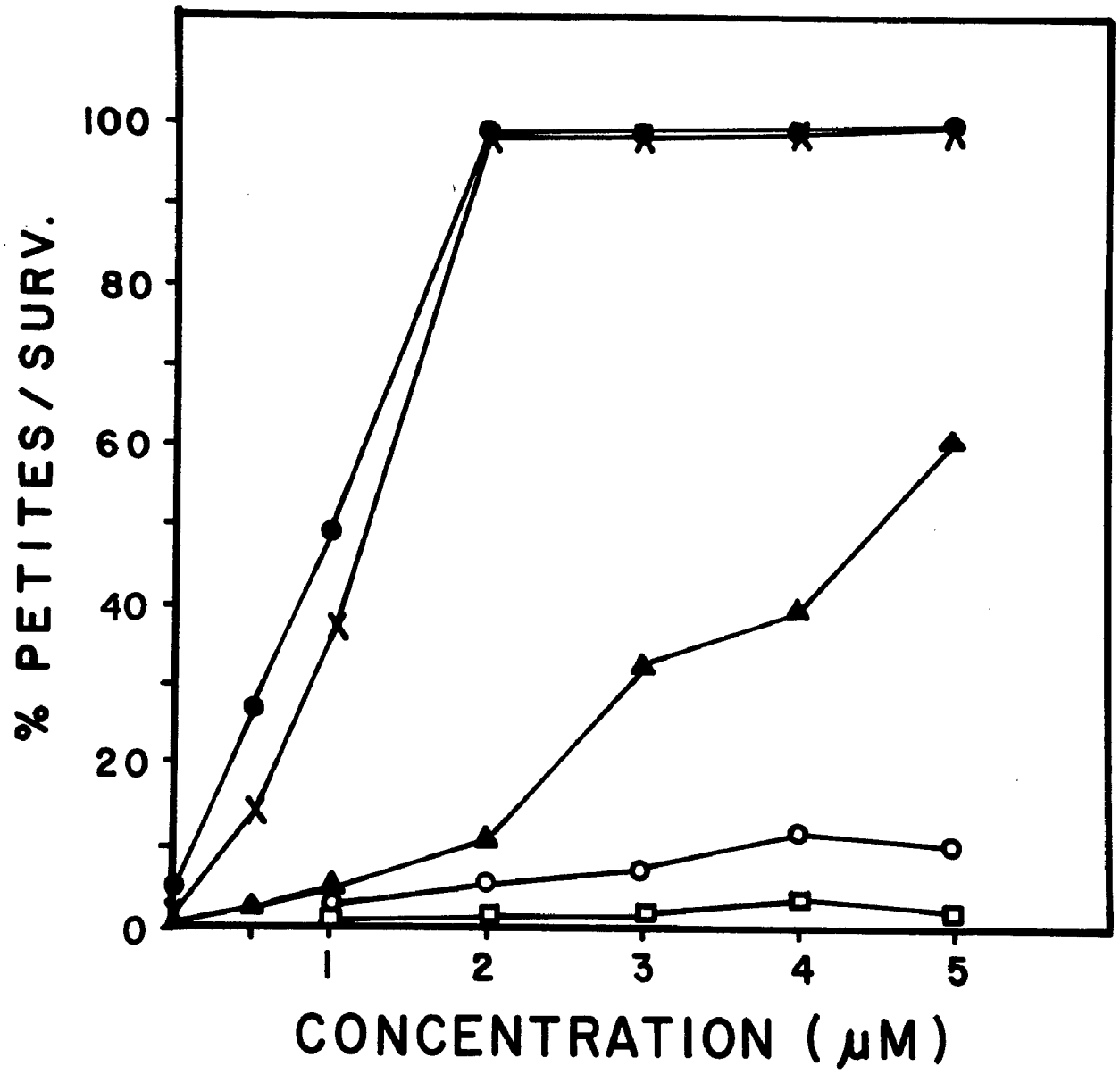

% PETITES / SURV.
100
80
60
40
20
0
1
2
3
4
5
CONCENTRATION (μM)

FIGURE 7

Petite induction at 1 μM ethidium bromide with increasing doses of UV.

● - Induction of petites by UV followed by exposure to 1 μM of ethidium bromide in buffer 24 hours and plating as in Fig. 6.

✘ - Induction of petites as - ● - except for 2 hours of photoreactivation after plating.

▲ - Induction of petites by UV only, no ethidium bromide present.

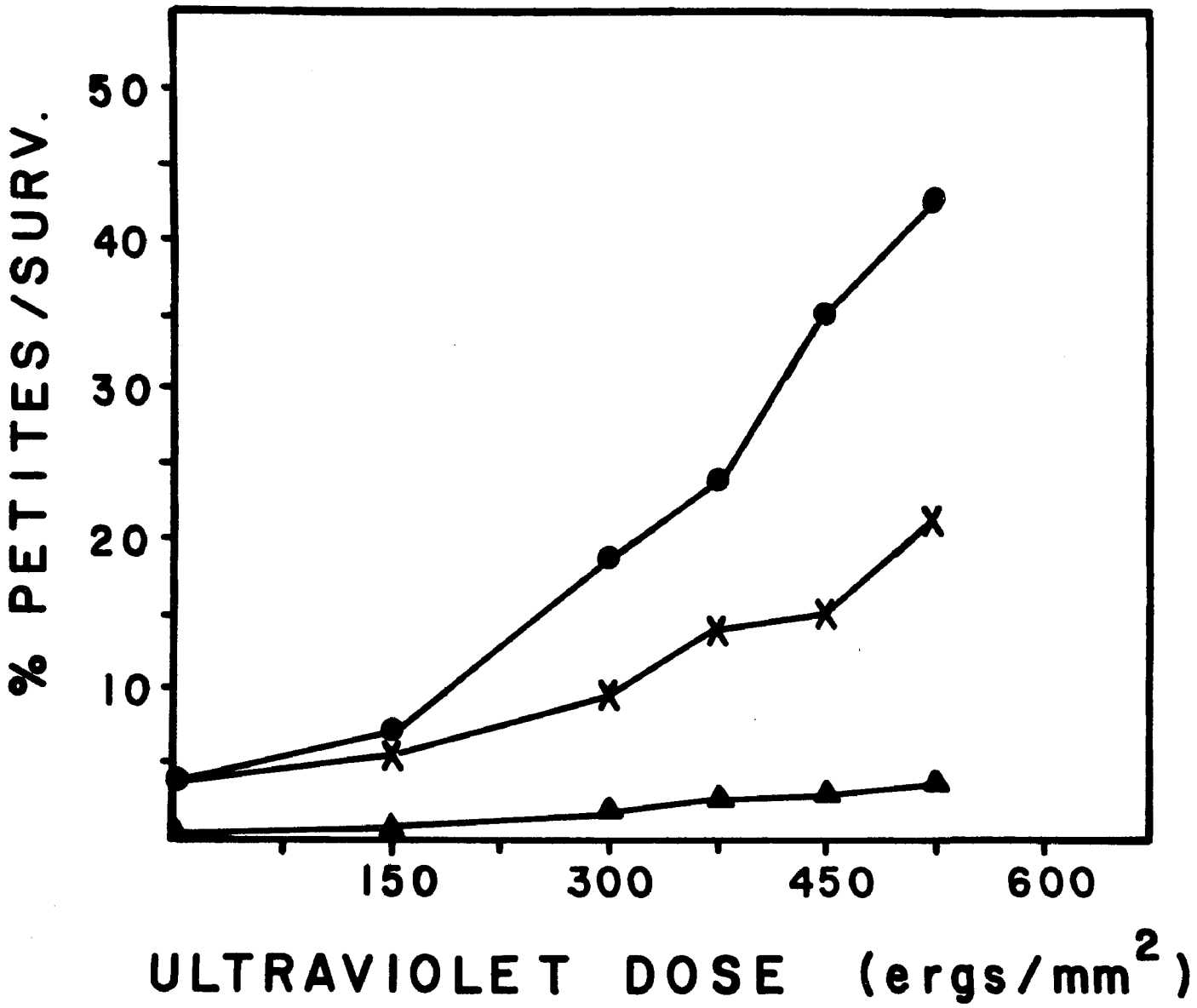
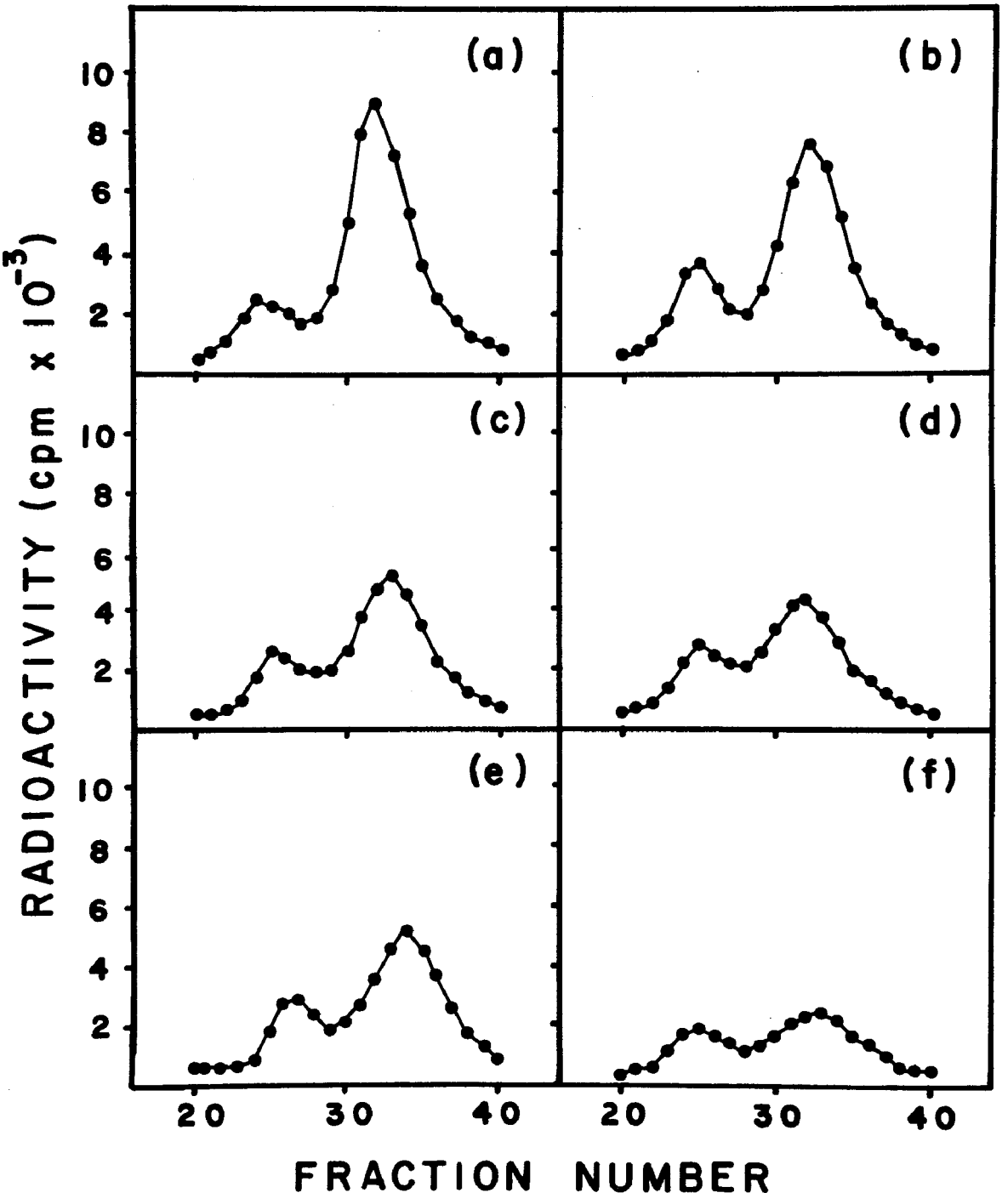

% PETITES/SURV.
50
40
30
20
10
150
300
450
600
ULTRAVIOLET DOSE (ergs/mm²)

FIGURE 8

Residual CPM (H^3) of prelabeled yeast nuclear and mitochondrial DNA separated on cesium chloride gradients after:

(a) A 24 hour period of liquid holding in buffer, the control sample.
(b) UV (525 ergs/mm^2) and liquid holding in buffer.
(c) Liquid holding in 2 μM ethidium bromide in buffer.
(d) UV (525 ergs/mm^2) and liquid holding in 2 μM ethidium bromide in buffer.
(e) Liquid holding in 5 μM ethidium bromide in buffer.
(f) UV (525 ergs/mm^2) and liquid holding in 5 μM ethidium bromide in buffer.

Nuclear DNA (fractions 20-30), mitochondrial DNA (fractions 30-40).


(a)
(b)
(c)
(d)
(e)
(f)
10
8
6
4
2
20
30
40
RADIOACTIVITY (cpm x 10^{-3})
FRACTION NUMBER

A Selective Covalent Binding of an Ethidium Analog to Mitochondrial DNA

The "magnification" mutation model for ethidium mutagenesis is an attempt to employ the known consequences for each kind of binding into a theoretical model that can be tested experimentally. The combination of ultraviolet light and ethidium bromide produced a synergistic effect probably because of repair inhibition by ethidium bromide of both types of DNA damage. However, the process proposed to "trigger" mutagenesis by ethidium is the covalent binding of ethidium to cytoplasmic DNA. The photosensitive derivative of ethidium bromide, 3(8)-amino-8(3)-azido-5-ethyl-6-phenyl phenanthridinium chloride, was prepared in order to covalently attach an ethidium analog to DNA by photolysis. A possible enhancement of the petite mutation by an increased number of covalently attached "ethidium-like" molecules to cytoplasmic DNA was expected. The azide was first characterized according to its ability to bind reversibly (in the dark) and nonreversibly (in the light) to DNA.

Azide binding to DNA -

The binding of the azido analog of ethidium to DNA was compared to that of ethidium by monitoring the effect of added DNA on the drug spectrum. A shift in the wavelength of maximum absorption, characteristic for drug binding was easily seen with both agents (Figs. 9 and 10). This is consistent with the prediction that the planar structure which is conserved in both the azide and the reactive nitrene intermediate should offer no interference to binding. Under the conditions of this spectral scanning there

was no measurable loss of the azide due to photodecomposition.

In vitro covalent attachment to DNA -

The reversibility of binding was tested directly for ethidium, the azide analog of ethidium and the light activated product of the azide. Calf thymus DNA mixed with ethidium bromide showed no retention of the drug whether or not the mixture was exposed to light 2 hours before dialysis in the dark for 72 hours. Similarly the ethidium azide was not retained on DNA when the mixture was kept in the dark or when the drug was photolyzed prior to its addition to DNA. However, the sample containing the DNA plus the light sensitive analog retained a visible pink color when it was exposed to visible light for 2 hours prior to dialysis. This pink sample was added to a Sephadex G 15 column and eluted with .01 M sodium hydroxide. DNA appeared in the first fraction after the void volume. Maximum absorption at 260 nm and 490 nm was present in this same fraction. The retention of the light activated ethidium analog in the DNA solution following 72 hours of dialysis indicated a stronger binding than that seen with ethidium bromide in vitro. The additional finding that the analog remained bound after the DNA was denatured at pH 12, and passed through a gel filtration column, strongly suggests that the light activated product was bound covalently to DNA.

Petite induction -

Ethidium produces petite mutants in Saccharomyces, presumably

due to its selectivity for mitochondrial DNA (Slonimski, Perrodin and Croft, 1968). The azide analog was compared with the parent compound for its ability to induce petites both in the dark and in the light in order to evaluate the effects of its reversible and nonreversible binding on the mutation process. As shown in Fig. 11 the ethidium azide analog, like the parent compound, induced petites in the dark at concentrations approaching 10^{-5} M. However, irradiation of the cultures with visible light following addition of the azide produced a large enhancement of its mutagenicity while the parent compound was unaffected by such treatment. For example, a concentration of the azide of 4.7×10^{-6} M produced 3% petites in the dark and 70% following irradiation. There were also large differences between cell killing in the light and in the dark (Fig. 12). While the azide plus light was able to induce greater than 90% petites at concentrations which did not cause a large amount of cell killing, increased concentrations in the light were highly toxic. The induction of petites in the dark by the azide produced practically no cell deaths. These data imply that petite induction by the analog follows a more effective route in the light than in the dark. Petite production by the azide in the light is likely due to light activation to the nitrene intermediate in situ, resulting in covalent attachment of the drug to cytoplasmic DNA. This drug addition to DNA probably results in an impass for replication and transcription, unless repair of the lesion is possible. It

is likely that petite induction by the azide in the dark results from a binding of the analog to DNA in a fashion similar to that known for ethidium bromide.

The differences in cell killing in the light and the dark may be interpreted according to these two mechanisms. Petite induction by ethidium bromide in this strain of Saccharomyces is practically identical to that of the azide in the dark with regard to concentration range and high cell viability (Fig. 6). The increased toxicity of the azide in the presence of light is not unlike the high toxic trend known for most arylakylating agents.

The azido ethidium light product produced by irradiation prior to addition to the culture was not as efficient in the induction of petites as the reactive azide. An increased production of petites by the light product (presumably the hydroxylamine) in the additional presence of light suggests that a residual amount of the unreacted azide was still present in the light product solution. Although the hydroxylamine derivative was least efficient in producing petites, it was extremely toxic to the cells at increased concentrations. This may explain part of the toxic effect of the azide in the presence of light, since any excess azide would probably react with the aqueous environment to produce the toxic hydroxylamine derivative. The toxicity of the hydroxylamine coupled with the covalent binding of the reacted azide to DNA would both contribute to the cell killing effect of the azide in the presence of light.

Since the azido ethidium analog appears to induce petites in the dark at similar concentrations and high cell viability like that of the parent compound, its enhanced mutagenesis in the light is attributed to the increased number of covalently bound molecules within the DNA. This increased ability of the azide to induce the petite mutation in the presence of light is consistent with the first step of the "magnification" mutation model which requires a covalent binding of ethidium to DNA. A residual amount of the azide plus the light product bound to DNA would provide for the inhibition of the repair of modified bases within the DNA.

Mahler and Bastos (1974) have demonstrated a preferential covalent binding of ethidium to cytoplasmic DNA. This special affinity could arise from a true selectivity of ethidium for mitochondrial DNA or because of an artificial concentration of ethidium within mitochondria due to an actual affinity to mitochondrial membranes. An alternate explanation may call for the preferential activation of ethidium to a product which forms a covalent attachment to DNA by an enzyme residing within the mitochondrial matrix or inner membrane.

Ethidium analog binding to yeast DNA -

The azido ethidium analog was allowed to bind to yeast DNA *in vivo* in the dark before photolysis and permanent attachment of the analog to the DNA. In order to identify the bound drug

following photolysis with the organism in culture, yeast DNA was separated by hydroxyapatite chromatography into nuclear and mitochondrial fractions (Fig. 12). Yeast DNA, following photolysis *in vivo* with the azido analog, was eluted at the same sodium phosphate molarities as untreated DNA. Proportional amounts of untreated nuclear and mitochondrial DNA were exposed to the analog in vitro. 260 nm/280 nm ratios were used to identify the drug since the azide compound and its derivatives absorb strongly at 283 nm. While the bound product may have a shift of λmax, a significant absorbance at 280 was attributed to the presence of the analog bound to DNA. The 260 nm/280 nm ratios were calculated for untreated DNA, and DNA with the ethidium analog bound *in vivo* and *in vitro*. Table 4 suggests that a larger amount of drug analog was bound *in vivo* to mitochondrial DNA because of the greater decrease in the 260 nm/280 nm ratio for mitochondrial than for nuclear DNA. This difference was not as significant when dye was bound *in vitro*.

Thermal transition profiles for each type of DNA are shown in Figs. 14 and 15. For untreated DNA the T_m's were 84.0 and 75.7 for nuclear and mitochondrial DNA, respectively. These results compare favorably with other published values for yeast DNA (Tewari, Jayaraman and Mahler, 1965). Attachment of the ethidium analog to DNA in vitro decreased the observed hyperchromicity due to melting presumably because of the contribution of the drug to the total absorption of the DNA

mixture at 260. Such an effect was also observed for mitochondrial but not for nuclear DNA isolated after treatment with the azide *in vivo*.

The 260 nm/280 nm ratios for drug bound *in vivo* and *in vitro* and a comparison of the thermal transition profiles for nuclear and mitochondrial DNA indicate that the azido ethidium analog binds preferentially to mitochondrial DNA prior to its covalent attachment by photolysis. No such preference is indicated *in vitro*, which seems to rule out a special affinity for cytoplasmic DNA because of its lower guanine-cytosine content or its possible circularity. A probable concentration of the analog within mitochondria because of an attraction to mitochondrial membranes and/or an enhanced binding to DNA due to enzyme activation plus photolysis may account for these results.

Although these experiments were concerned specifically with ethidium bromide, this technique of photoaffinity labeling should be applicable to a variety of agents which bind nucleic acids such as alkaloids, steroids, antibiotics, and other biologically effective compounds. A complete understanding of the molecular mechanism whereby these drugs exert their effect has not been acheived due to their reversibility of binding and the uncertainties about structural details of DNA in vivo. There is a great need therefore to target precisely the binding of drugs to DNA and to determine how this binding is coupled to specific biological effects. A selective means of covalently linking

such drug molecules to their binding sites subsequent to their usual mode of attachment should prove of extensive value in drug structure-function studies.

TABLE 4

Calculated 260 nm/280 nm ratios for yeast DNA. Nuclear and mitochondrial DNAs were separated on a hydroxyapatite column (Fig. 13) and dialyzed 48 hours in saline citrate buffer, pH 7. The absorbancies were determined at 260 and 280 nm for untreated DNA and DNA exposed to azide plus light *in vivo*. Samples of untreated DNA were then used for the *in vitro* drug binding. An aqueous solution of the azide (0.2 mg/ml) was added to DNA samples to give 1.46 µg azide/µg DNA and 13.2 µg azide/µg DNA. Solutions were then placed under a GE fluorescent light two hours and then dialyzed 48 hours in saline citrate. These samples were then read for absorbance at 260 nm and 280 nm.

DNA Sample	O.D. 260 nm	O.D. 280 nm	260 nm/ 280 nm	R/DNA	M-N
Nuclear Untreated	0.304	0.163	1.865	1	
Nuclear Dye - *In vivo*	0.352	0.198	1.777	0.953	
Nuclear 1.46 µg azide	0.366	0.245	1.494	0.801	
Nuclear 13.2 µg azide	0.588	0.560	1.050	0.563	
Mitochondria Untreated	0.173	0.097	1.784	1	
Mitochondria Dye - *In vivo*	0.130	0.084	1.548	0.863	-.085
Mitochondria 1.46 - µg azide	0.241	0.172	1.401	0.786	-.015
Mitochondria 1.32 µg azide	0.325	0.296	1.098	0.616	+.053

FIGURE 9

Effects of added DNA on the spectrum of ethidium bromide. Curve A in each panel represents free drug, 0.975 x 10^{-4} M in 0.1 Tris buffer pH 7.5, and curve B shows the effect of added calf thymus DNA, 1 x 10^{-3} M.

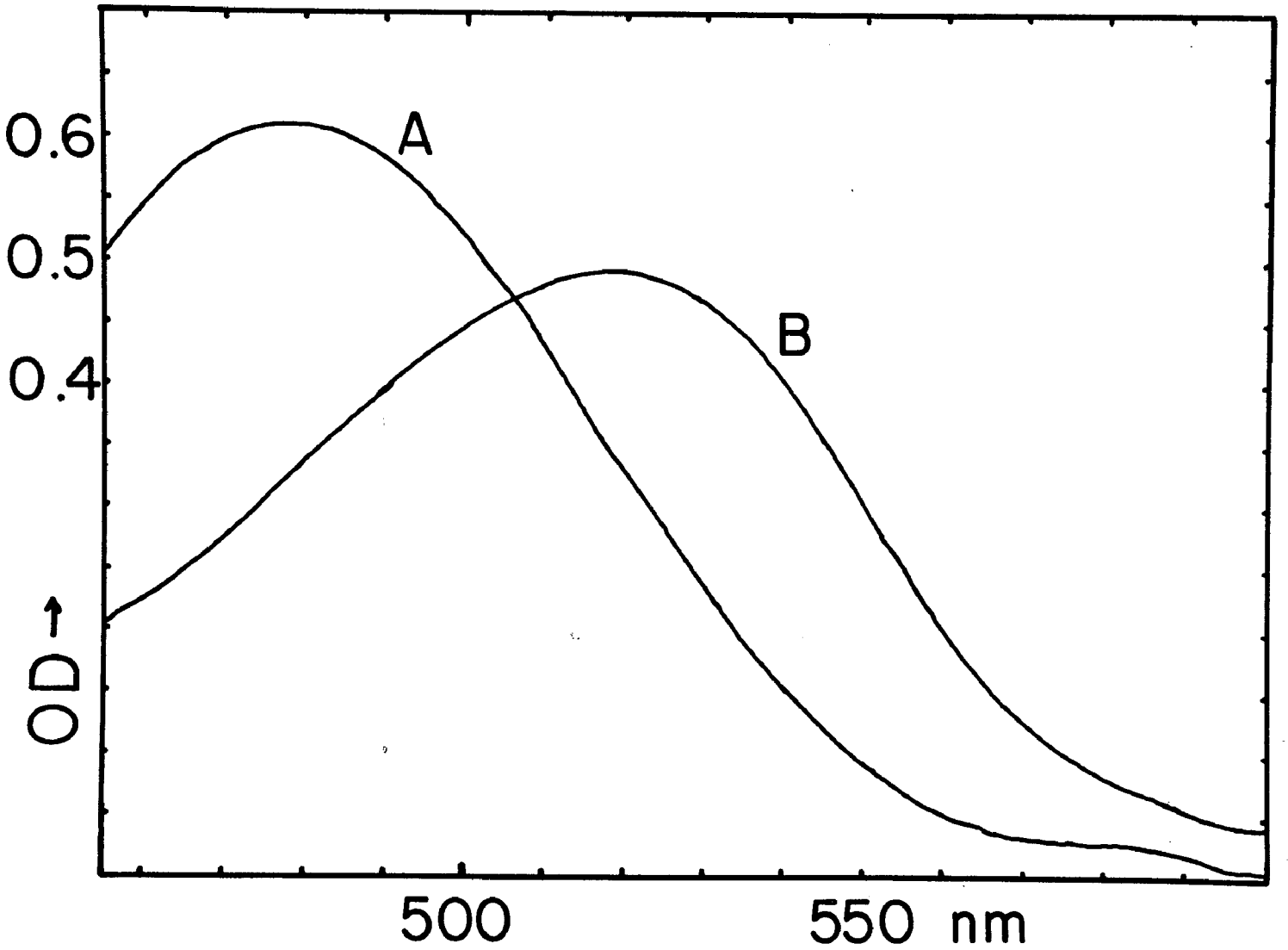

0.6
0.5
0.4
OD ↑
A
B
500
550 nm

FIGURE 10

Effects of added DNA on the spectrum of 3(8)-amino-8(3)-azido-5-ethyl-6-phenyl phenanthridinium chloride. Curve A and B are identical for drug concentration and treatment as in Figure 9.

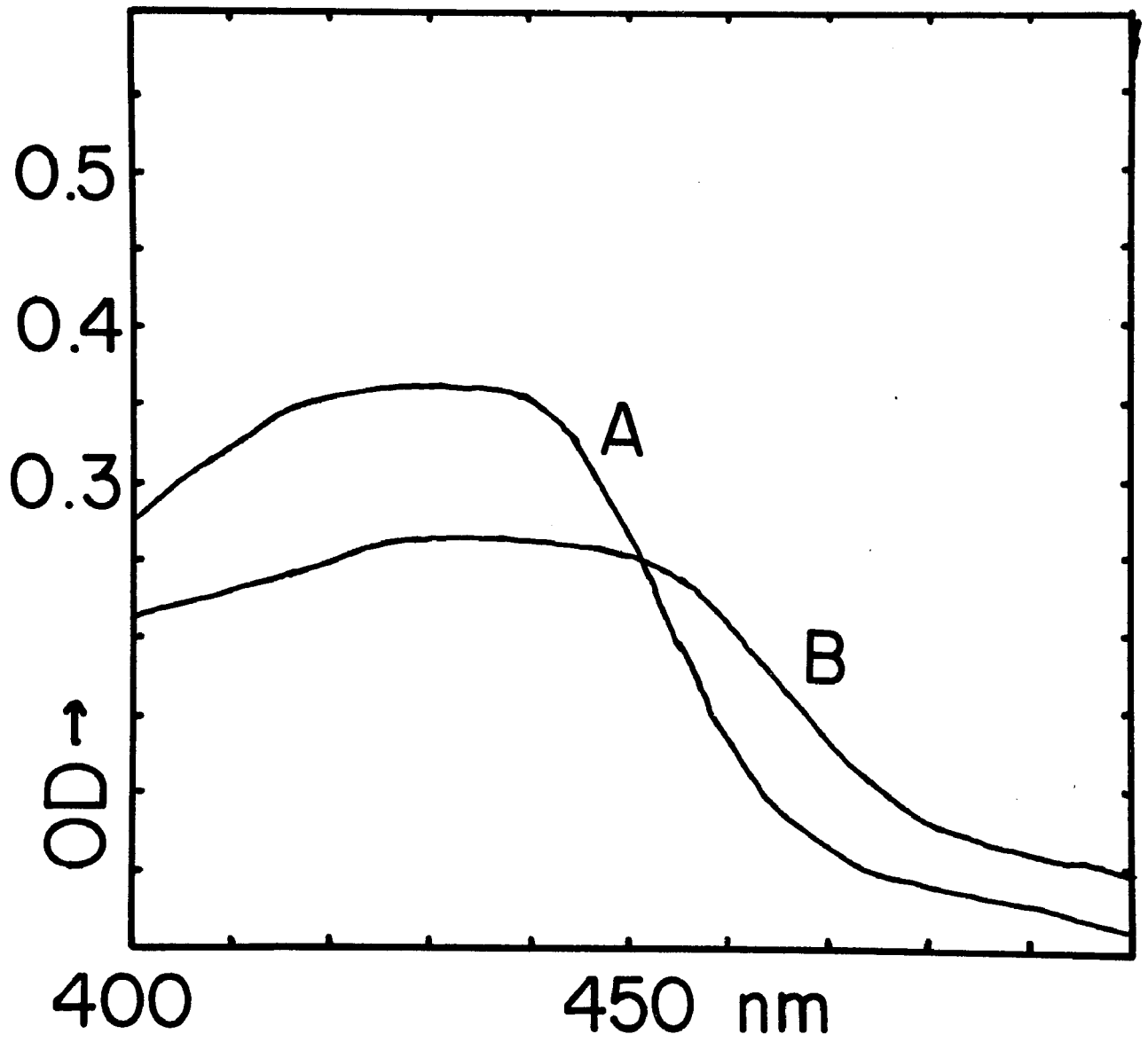

0.5
0.4
0.3
OD ↑
A
B
400
450 nm

FIGURE 11

Petite induction in yeast by light activation of the azido ethidium analog. Stationary phase yeast were washed in .067 M phosphate buffer, pH 7 and divided into two groups. To Group I. (○●) the azido ethidium analog was added to cell suspensions in 50 ml erlenmeyer flasks. To Group II. (△▲) the azido ethidium light product was added. The light product was prepared by the irradiation of an aqueous solution of the azide by a GE fluorescent lamp for two hours prior to the addition to cell suspensions. Samples with the axide or the azide light product were held in the dark at 30°C in a shaking water bath. After 24 hours, yeast were spread onto agar plates containing 1% glucose, 1% yeast-extract, and 2% bacto-peptone. Half of the plates from Group I and II (○△) were exposed to a GE fluorescent light two hours prior to incubation in the dark. The other half of Group I and II (●▲) were placed in the dark immediately after plating. Petites were scored after two days of growth in the dark at 30°C. Only colonies remaining completely colorless after tetrazolium overlay (Ogur, St. John and Nagai, 1957) were scored as petites. Sectored colonies were counted with normal colonies.

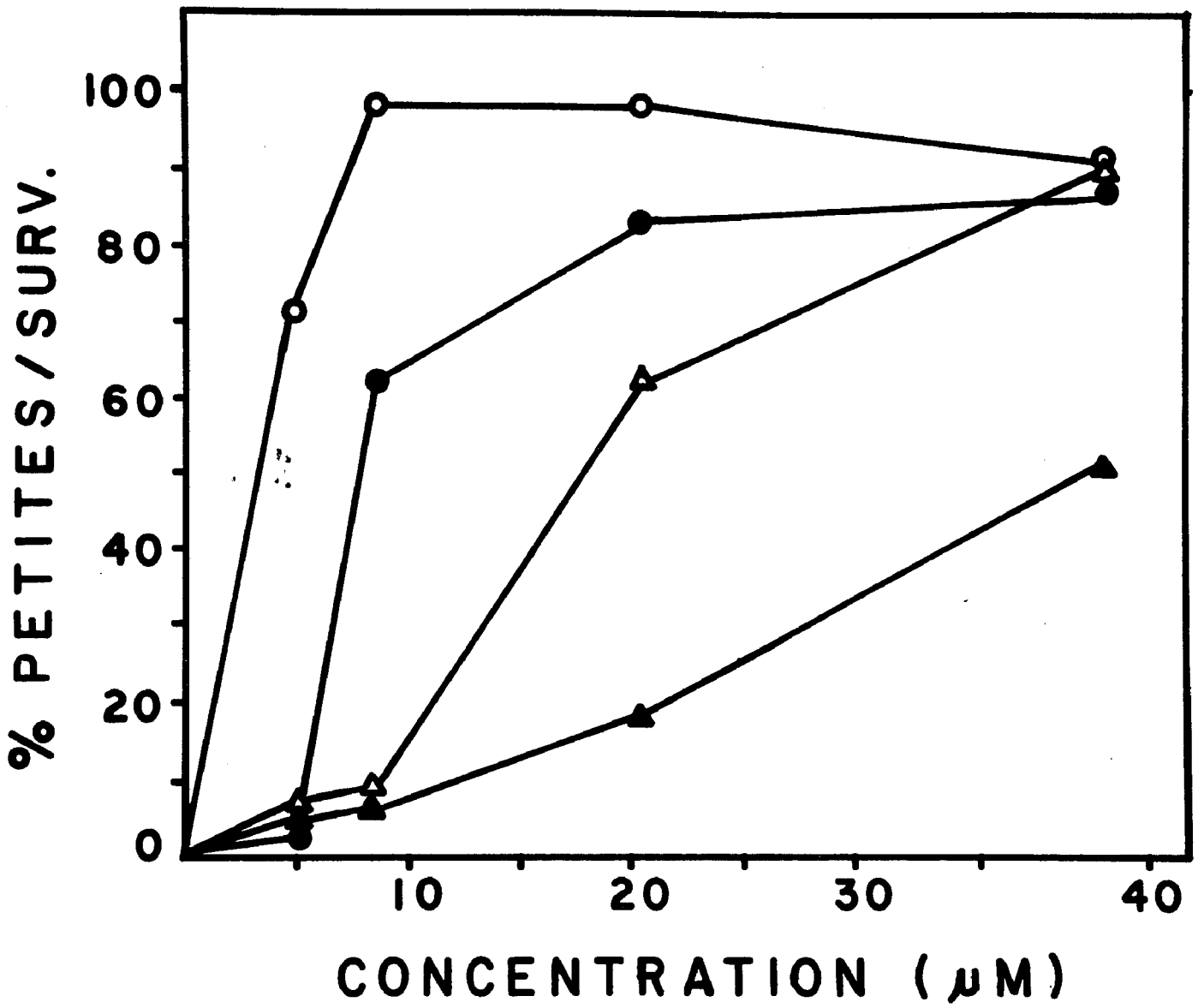

% PETITES/SURV.
100
80
60
40
20
0
10
20
30
40
CONCENTRATION (μM)

FIGURE 12

The survival of yeast following the conditions described in Fig. 11. Control populations were suspended in .067 M phosphate buffer without any drug addition, and handled in a fashion similar to those samples containing the azide or the azide light product.

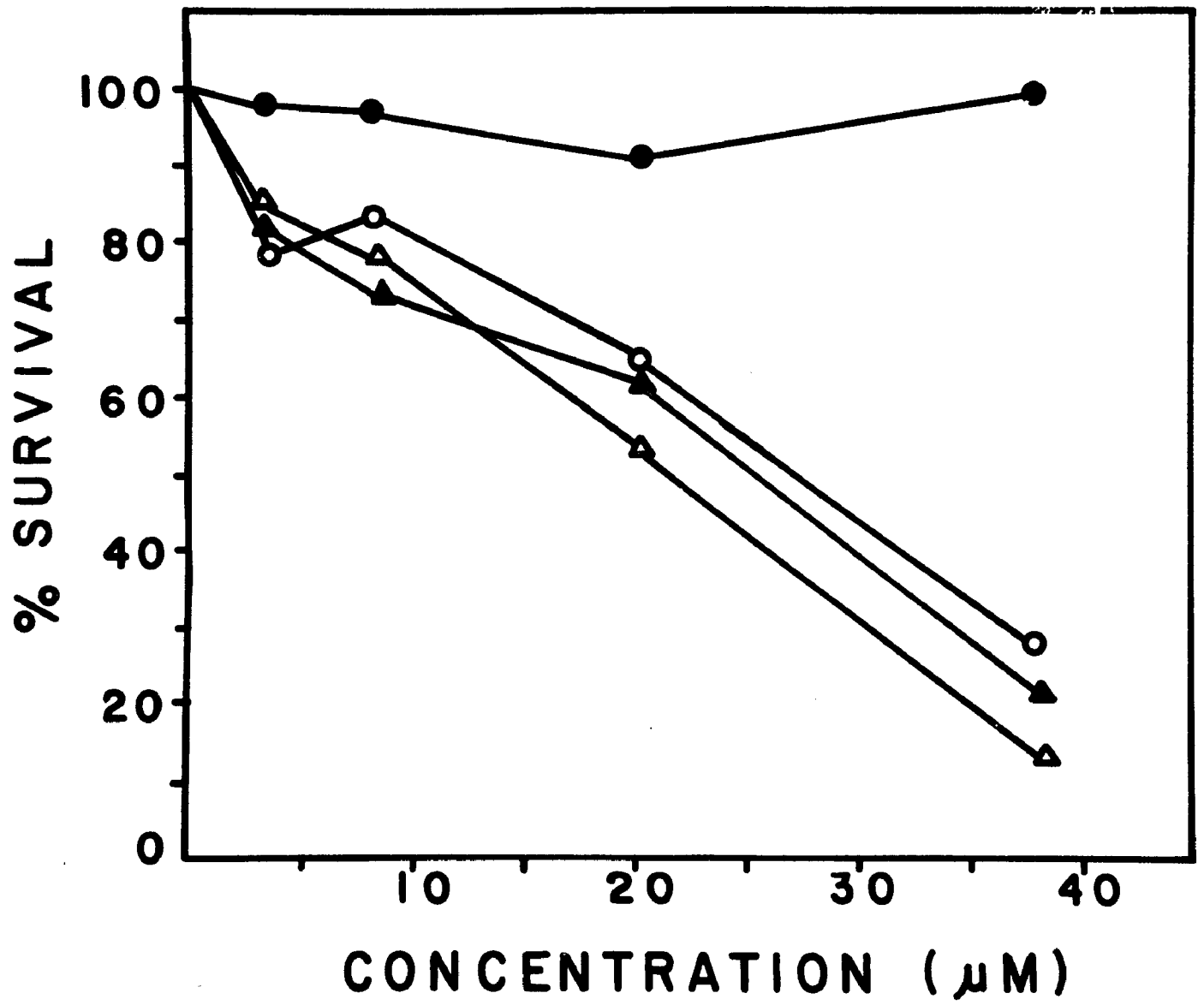

% SURVIVAL
100
80
60
40
20
0
10
20
30
40
CONCENTRATION (μM)

FIGURE 13

Separation of yeast nuclear and mitochondrial DNA by hydroxyapatite column chromatography. Elution was carried out with an increasing linear gradient (250 ml + 250 ml) of sodium phosphate buffer, pH 6.8 (0.2 - 0.45 M). Nuclear DNA was eluted at 0.275 M and mitochondrial DNA at 0.295 M.

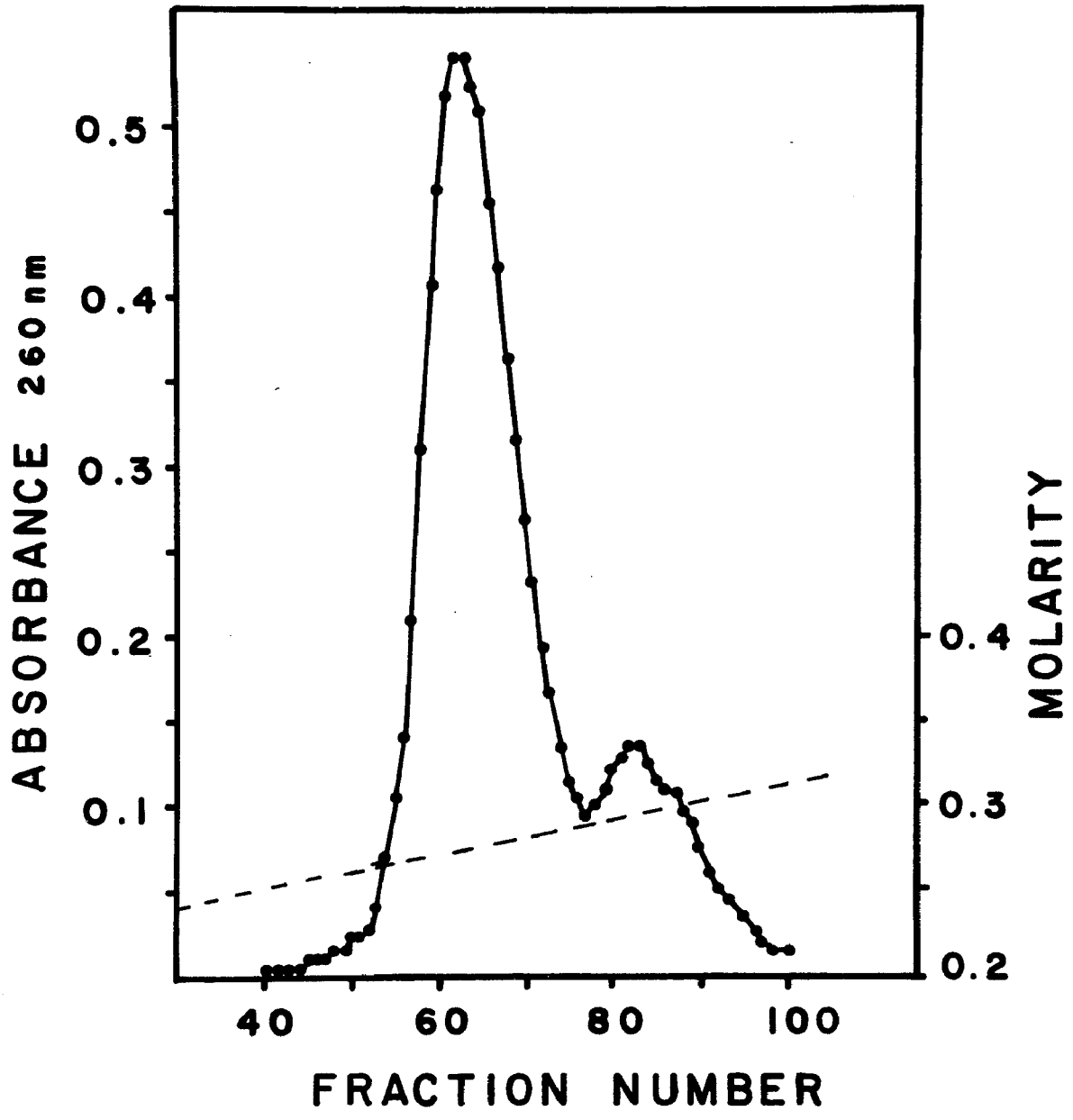

ABSORBANCE 260nm
0.5
0.4
0.3
0.2
0.1
MOLARITY
0.4
0.3
0.2
40
60
80
100
FRACTION NUMBER

FIGURE 14

Thermal transition profiles for yeast nuclear DNA. Nuclear DNA fractions collected from a hydroxyapatite column (Fig. 13) were pooled and dialyzed 48 hours in saline citrate buffer, pH 7, before melting. Nuclear DNA not exposed to the azide (—●—) had a sharp melting point (T_m) of 84.0°C with a hyperchromicity (h_{max}) 0.530. DNA from yeast exposed to the azide plus light in vivo (—●—) had a h_{max} = 0.572; addition of the azide (1.46 µg/ug DNA) in vitro (••●••) and subsequent exposure to light resulted in h_{max} = 0.459.

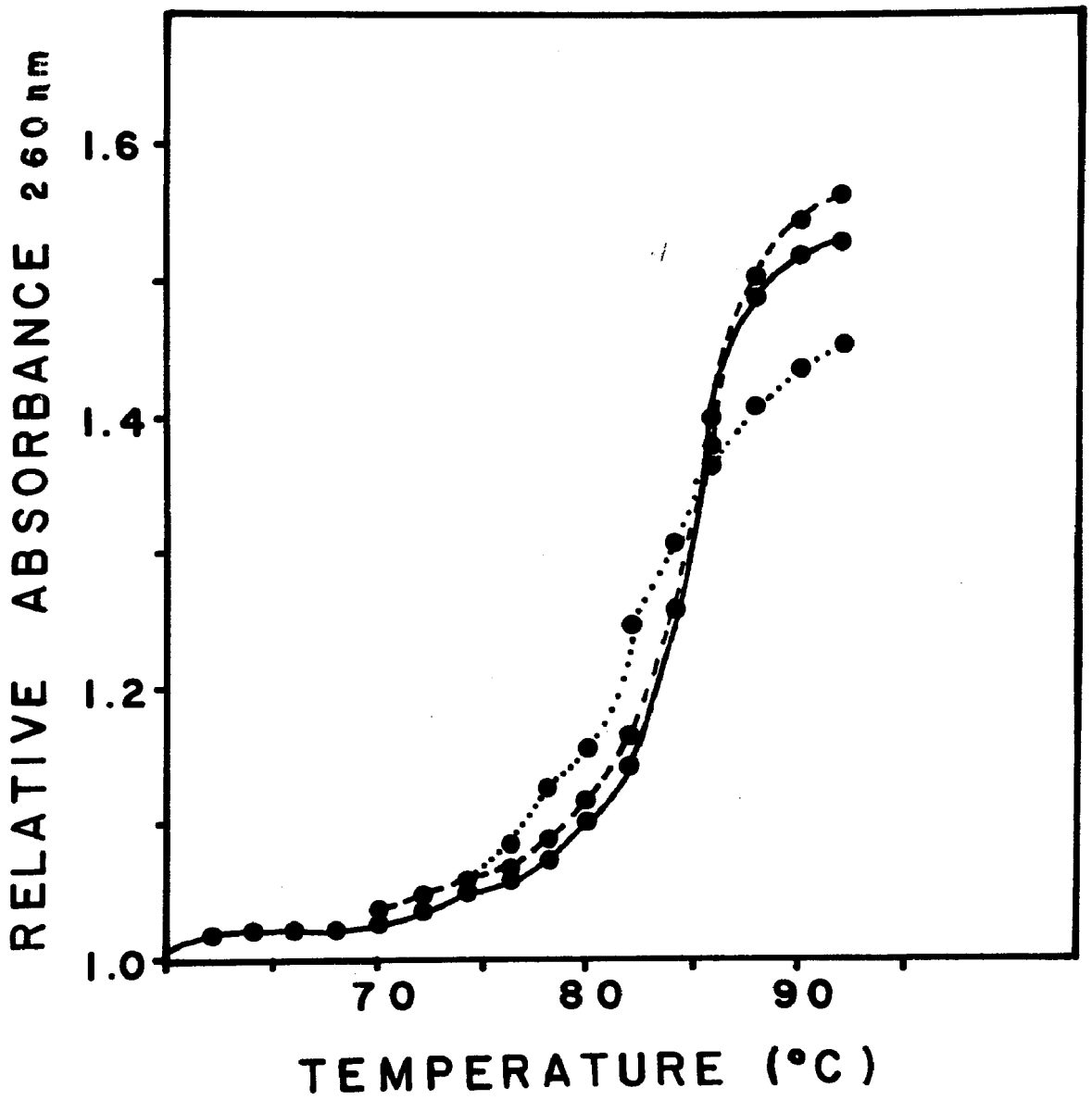
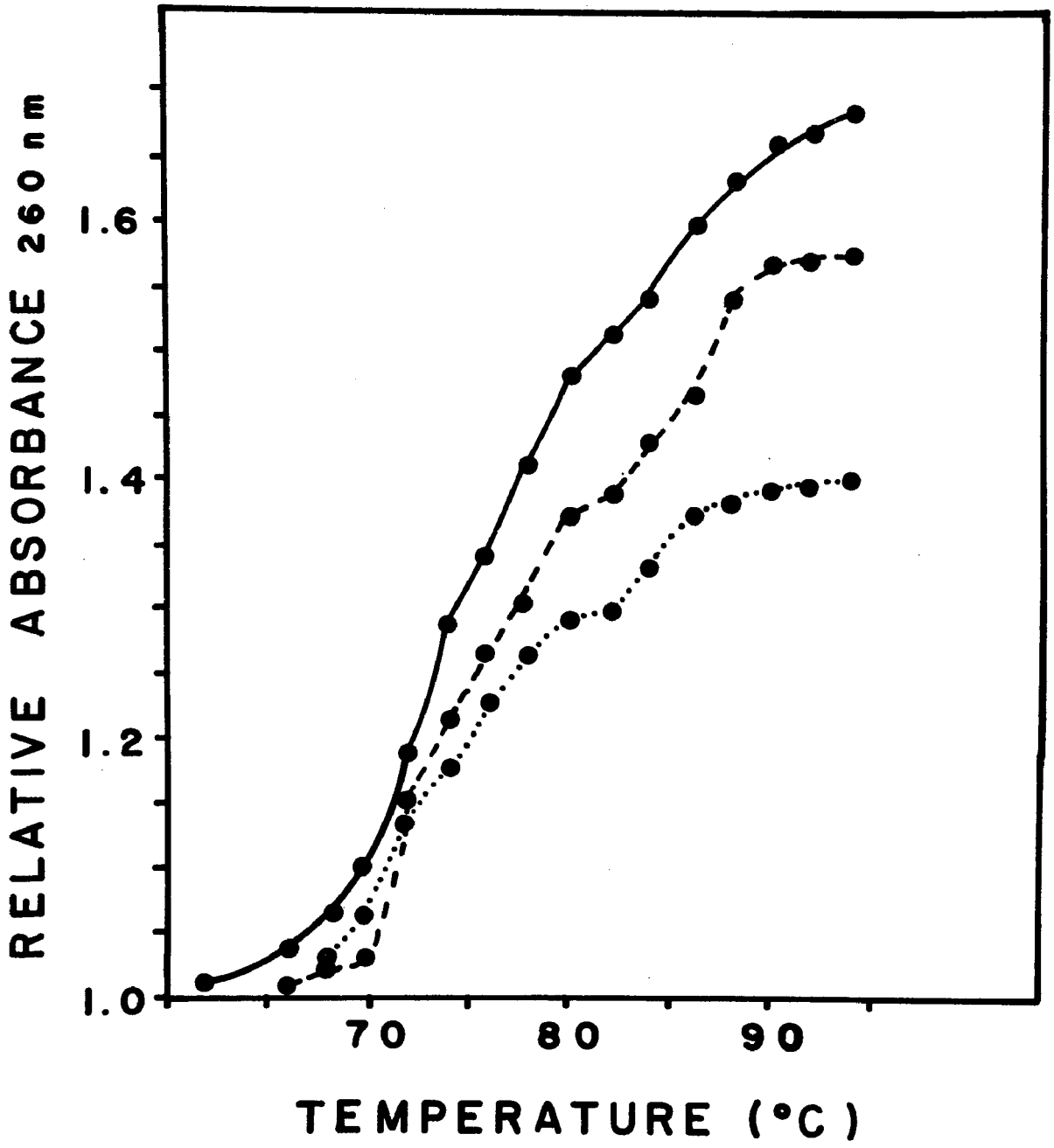

RELATIVE ABSORBANCE 260 nm
1.6
1.4
1.2
1.0
70
80
90
TEMPERATURE (°C)

FIGURE 15

Thermal transition profiles of yeast mitochondrial DNA in saline citrate buffer, pH 7. Mitochondrial DNA from yeast not exposed to the azide (—●—) had a melting point of 75.7°C with a hyperchromicity (h_{max}) of 0.691. Mitochondrial DNA from yeast exposed to the azide plus light in vivo (—●—) had an h_{max} = 0.573; addition of the dye (1.45 µg/ug DNA) in vitro (••●••) and subsequent exposure to light resulted in h_{max} = 0.393.


RELATIVE ABSORBANCE 260 nm
1.0
1.2
1.4
1.6
70
80
90
TEMPERATURE (°C)

CONCLUSIONS AND SPECULATIONS

This dissertation has presented evidence for the dark repair of ultraviolet damage to yeast mitochondrial DNA. The ultraviolet dose necessary to inflict significant damage to both nuclear and mitochondrial DNA was determined. Cell survival at these large doses of ultraviolet light was observed after immediate and delayed plating of yeast onto 1% pyruvate and 1% glucose media. In the very lethal dose ranges of irradiation an increase in the number of normal colonies appeared after a period of liquid holding and delayed plating. This increase, demonstrated separately on 1% glucose and 1% pyruvate medium suggested that the repair of both mitochondrial and nuclear DNA had occurred. After low doses of ultraviolet light an actual decrease in the number of petite survivors was seen after delayed plating, even though the total number of survivors increased. When a known repair inhibitor, caffeine, was added to the liquid holding buffer prior to the delayed plating of yeast, a marked decrease in the number of petites did not occur after delayed plating. Therefore, the decrease in the number of petite survivors after delayed plating following low doses of ultraviolet light was attributed to the repair of yeast mitochondrial DNA.

A model for ethidium bromide mutagenesis of mitochondrial DNA has also been proposed based on a direct effect of ethidium bromide plus its ability to inhibit mitochondrial DNA repair. Experiments were presented to demonstrate a synergistic effect of ultraviolet light and ethidium bromide to induce the petite mutation in yeast. Photo-

reactivation or dark repair of the ultraviolet damage reduced this synergism. The enhanced petite production by the two mutagens was also accompanied by increased degradation of mitochondrial DNA. The "magnification" mutation model for ethidium mutagenesis states that the covalent binding of an ethidium product to DNA "triggers" repair enzymes into action. However, the total repair process is inhibited by the reversible binding of ethidium to DNA resulting in DNA degradation and the petite mutation. An azido analog of ethidium was prepared which could be photolyzed to increase the number of bound ethidium derivated molecules onto DNA. This increased number of molecules bound covalently to DNA was correlated with an increase in petite production. These results were consistent with the idea that the "magnification" mutation model for ethidium mutagenesis is activated by the covalent binding of ethidium to the DNA.

The importance of mitochondrial DNA in aerobic eucaryotes has not been realized until recently, partly because knowledge of its presence is relatively new and because its complex relationship to nuclear DNA is only beginning to be unraveled. Since evidence has now been presented for the dark repair of mitochondrial DNA, the task presently at hand is to characterize the dark repair process in mitochondrial DNA on a molecular level. While it is assumed that the dark repair of mitochondrial DNA proceeds in a fashion similar to that known for nuclear DNA, a direct description of the repair process mechanism is yet to come. Moustacchi (1971) has isolated two mutants of Saccharomyces that are sensitive to ultraviolet irradiation because of a reduced ability to repair the cytoplasmic genome. Both produce a large number of petite mutants at low doses of irradiation. While the sensitivity in

uvs ρ 5 is inherited in a Mendelian fashion, uvs ρ 72 carries a mutation within cytoplasmic DNA. A characterization of dark repair in these ultraviolet sensitive mutants on a molecular level should indicate the missing repair enzyme in each case and its ultimate origin, nuclear or mitochondrial. Because of its difference in size and shape (organization from nuclear DNA, enzymes for mitochondrial DNA repair may be specifically engineered for mitochondrial DNA. An interesting model for study in this case would be Xeroderma Pigmentosum fibroblasts. These cells are unable to repair ultraviolet damage to nuclear DNA because they lack the ultraviolet specific endonuclease (Cleaver, 1969). A comparison of mitochondrial DNA repair in normal and Xeroderma fibroblasts should indicate whether or not the same endonuclease is absent for mitochondrial DNA repair.

The role of mitochondrial DNA in the survival of higher eucaryotes has been completely ignored. It is always assumed that cell death following irradiation or alkylation results from damage accumulated in nuclear DNA. Although mitochondrial DNA is duplicated by several copies residing within the cell, each is an extremely vulnerable target. In all differentiated cells mitochondrial DNA, unlike nuclear DNA, remains completely unmasked requiring all of its information to maintain a viable organelle. Since only a small amount of nuclear DNA is transcribed in each cell, its vulnerability to irradiation is not as great as is usually assumed. Todd (1968) has reported that mammalian cells that were irradiated and cloned on solid medium produced a variety of colony sizes. Many of these colonies were abnormally small and resembled petite mutants in yeast because of their reduced oxygen

comsumption. These small colonies were less resistant to further irradiation than cells from normal colonies. Todd's work would suggest that cell death from irradiation may depend upon both cellular DNAs and not completely upon nuclear DNA.

Irradiation (ultraviolet, x-rays, γ-rays) of nucleic acids results in an even distribution of damage to all cellular DNA. However, damage to DNA by alkylating agents could conceivably be concentrated in nuclear or in mitochondrial DNA. The demonstration by Mahler and Bastos (1974) that ethidium is activated to a product which binds covalently to mitochondrial DNA is the first example of an"alkylating" agent known to target specifically cytoplasmic DNA. Other drugs and environmental agents may be activated to target only nuclear or only mitochondrial DAN. The alkylation of cellular DNA and the eventual killing effect is always assumed to occur in the nuclear genome. The results with ethidium and the azido ethidium analog imply that other drugs may react preferentially with mitochondrial DNA. In these cases cell death may occur because of damage to mitochondrial DNA.

All environmental carcinogens that have been carefully studied are known to bind covalently to cellular nucleic acids via an electrophilic attack of the carcinogen onto a nucleophilic center on DNA (Miller and Miller, 1972). Many of these compounds are not carcinogenic until they have been altered within the cell to an active electrophile. The activation process may proceed by oxidation, addition of an active group or by cleavage to a reactive intermediate. The mitochondrial matrix and inner membrane contain a host of enzymes specific for the organelle. Many of these enzymes may be capable of activating a potential

carcinogen to its reactive intermediate. An activation of S-(1,2-dichlorovinyl)-L-cysteine to an active alkylating agent by isolated rat liver mitochondria (Stonard and Parker, 1971) points to the capability of these mitochondrial enzymes. It is known that cyclophosphamide, a nitrogen mustard, undergoes metabolic changes in the cellular mitochondrial fraction (Hill, Laster and Struck, 1972), but these metabolites have never been studied to pursue the possilbe alkylating intermediate of cyclophosphamide which may selectively attack mitochondrial DNA. This evidence suggests, therefore, that some carcinogens may specifically target cytoplasmic DNA and that resultant changes in mitochondrial DNA could theoretically lead to an eventual cell transformation. The idea that changes in cell respiration could be linked to the cancer state was first suggested by Warburg (1930), and more recently by Racker (1972). This proposal has long since been set aside by more elegant theories. Many tumors appear to have a characteristic pattern of energy metabolism utilizing energy primarily from glycolysis. Warburg proposed that cancer could result from a defect in the mitochondrial respiratory chain causing cell reversion to a primitive bioenergetic pathway of fermenting yeast. In light of new evidence regarding the presence of a mitochondrial genetic system that is closely interrelated to nuclear DNA, it seems that the proposal by Warburg should once again be examined.

Important feedback communications must exist between nuclear and mitochondrial DNA to provide for a constant cellular adaptation to changing energy requirements. Barath and Kuntzel (1972 a, b) have suggested that the synthesis of mitochondrial RNA polymerase may

be controlled by a regulatory protein synthesized within mitochondria and exported to the nucleus where it controls genes for mitochondrial enzymes. Moreover, the timing of mitochondrial DNA synthesis within the cell cycle and its turnover rate for different kinds of cells is a specific characteristic of each cell type and may be controlled by messages relayed between the two cellular genomes. An interesting aspect of mitochondrial DNA synthesis is the fact that mitochondrial DNA in resting cells continues to turnover (Goss, Getz and Rabinowitz, 1968). A possible explanation of mitochondrial involvement in transformation would involve mutations in cytoplasmic DNA which might lead to erroneous messages to nuclear DNA. The fact that resting cells may also be transformed may be explained by an active mitochondrial DNA turnover.

All of these speculations point to an increasing awareness of the importance of mitochondrial DNA to the welfare of the eucaryotic cell. Knowledge about the repair and maintenance of mitochondrial DNA will become invaluable as more information emerges regarding the intricate relationship which exists between nuclear and mitochondrial DNA.

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