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ENTITLED Phototoxic Effects of Boldine and Chrysene,

Polycyclic Aromatic Hydrocarbons, on Bacterial Strains

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Phototoxic Effects of Boldine and Chrysene, Polycyclic Aromatic Hydrocarbons, on Bacterial Strains

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ABSTRACT

Catalase proficient *Escherichia coli* cells (*katF*$^+$) are sensitive to inactivation by chrysene and boldine in the presence of broad spectrum near-ultraviolet light (NUV; 320-400 nm). Catalase deficient cells (*katF*) appear to be no more sensitive to inactivation by NUV plus chrysene or boldine than with NUV alone. Testing under both aerobic and anaerobic conditions confirms the oxygen dependency of chrysene phototoxicity for *katF*$^+$ strains. A decrease in the transforming activity of *Haemophilus influenzae* DNA by simultaneous treatment with chrysene and NUV under aerobic conditions also suggests DNA is a lethal target although *E. coli* cells deficient in excision repair (*uvrA6*) do not exhibit increased sensitivity to such treatment. In contrast to chrysene plus NUV treatment, boldine plus NUV inactivates *H. influenzae* transforming DNA in a oxygen independent manner. Genes controlling carotenoid synthesis from genes cloned from *Erwinia herbicola* and expressed in *E. coli* HB101 offers protection against inactivation by NUV plus chrysene but not NUV plus boldine.

INTRODUCTION

The lethal and mutagenic effects of near-ultraviolet light (NUV) have been extensively researched and reviewed (18, 19, 24) and it has been proposed that NUV induced damage results from the production of reactive oxygen species including superoxide ions, singlet oxygen, and hydrogen peroxide (3, 6). NUV wavelengths are absorbed by specific endogenous photosensitizers such as porphyrins and flavins (29, 11). In addition, a multiplicity of molecules can serve as exogenous photosensitizers including polycyclic aromatic hydrocarbons and certain alkaloids. The toxicity of photosensitizer can be either oxygen dependent or independent reflecting basic differences in the mechanism(s) by which cells are damaged. To analyze the phototoxic

effects of these light-activated molecules at the molecular level, an array of *E. coli* K12 mutant strains were constructed that carry all possible combinations of the genes controlling DNA repair and catalase proficiency and deficiency (Table 1; 8, 10, 12, 20, 30, 34). Based on the response pattern of the variously marked *E. coli* strains, one can deduce the probable lethal target and possible role of reactive oxygen species in the attack. The *katF* allele has been shown to confer sensitivity to inactivation by NUV and H_2O_2 while the *uvrA6* allele leading to an absence of excision repair has no effect on the sensitivity of cells to inactivation by these two agents (21, 24). In addition to evaluating the mechanisms of phototoxicity, a revertible histidine allele, *his-1* (7), has been incorporated into the tester strains to allow for a preliminary evaluation of mutagenicity while ascertaining the nature of the phototoxicity (10, 26, 27).

E. coli HB101 strains carrying a cosmid and carrying the same cosmid with a cloned piece of *Erwinia herbicola* DNA encoding genes controlling the synthesis of carotenoid pigments (20) are used in the evaluation of phototoxicity since there is evidence that carotenoids protect a variety of organisms against photodynamic action by quenching triplet state photosensitizers and singlet oxygen ($^1O_2$; 2, 4, 5). However, quenching of $^1O_2$ may only occur under low oxygen tensions (1). More specifically, carotenoids have been found to protect against high fluences of NUV as well as against the photosensitizers α-terthienyl, harmine, and phenylheptatriyne (28). Also, dietary administration of carotenoids to hairless mice exhibiting induced porphyria can protect against porphyrin sensitization induced by either UVA or UVB (14, 15). Protection against photosensitized damage by carotenoids is suggestive of the involvement of $^1O_2$.

The ability of a particular photosensitizer to damage DNA can be evaluated using *Haemophilus influenzae* transforming DNA activity as the end point. Results from such experiments can be used to determine whether a photosensitizer could damage DNA *in vivo* if it were able to pass the barrier presented by the cell wall and membrane.

In this paper, the *E. coli* bacterial strains and transforming DNA were used to evaluate the phototoxicity and mutagenicity of the polycyclic aromatic hydrocarbons chrysene and boldine (Fig. 1).

MATERIALS AND METHODS

Chemicals Chrysene and boldine were donated by Jaques Kagan, Department of Chemistry, University of Illinois at Chicago. Hemin, 6-thioguanine (6TG), and β-nicotinamide adenine dinucleotide (DPN) were purchased from the Sigma Chemical Company (St. Louis, MO.).

Bacterial Strains The strains RT 7 - 10, 13, and 15 are all closely related having been derived ultimately from *E. coli* K12 strain AT713. Strains RT 7h - 10h, 13h, and 15h (24) were developed by P1 transduction of a Tn 10 insertion located next to the *his-4* allele in strain AB1157 (10). Strain RT 18h was similarily constructed from *E. coli* strain LF392 (25). The carotenoid producing strain HB101 pPL376 was constructed by cloning *Erwinia herbicola* DNA coding for genes controlling carotenoid synthesis into the cosmid pHC79 (20).

Bacterial Media Luria-Bertani (LB) complex medium contained 10 g tryptone (Difco), 5 g yeast extract (Difco), and 10 g NaCl per liter (16).

The semi-enriched minimal medium (SEM) used to assess survival consisted of minimal A medium (16) supplemented with the nutritional requirements of the individual strains and casamino acids (Difco; 0.2 ml of 20% solution per liter) and solidified with 1.2% agar (Difco). Arginine, lysine, and histidine are necessary for growth of the RT strains (7), while proline and leucine are required for the HB101 strains (28).

For the selection of 6TG^r forward mutants, 334 mg 6TG in 4 ml H_2O and 1 ml 10 M NaOH was added per liter of minimal media to a final concentration of 2mM. Glycerol was used as the carbon source.

The hemin solution for the transformation medium was prepared by adding 10 mg hemin and 10 mg l-histidine to 5 ml H_2O and shaking vigorously before adding 0.4 ml 100% triethanolamine and shaking again. An additional 4.6 ml of dH_2O was then added and shaken before heating at 60 °C for 10 min without shaking. The solution was stored in the refrigerator.

For the transformation assay, the liquid medium included 7.4 g brain heart infusion (Difco) and 210 ml H_2O and, after autoclaving, 1.5 ml hemin solution and 0.5 ml DPN solution (0.01 g ml^{-1}) was added. The solid medium contained 11 g brain heart infusion, 3 g Eugonbroth, and 5 g agar (Difco) in 410 ml H_2O to which was added 3 ml hemin solution , 1 ml DPN solution, and 0.5 ml streptomycin solution (0.2 g ml^{-1}, always made fresh) after autoclaving and cooling to 45 °C.

Transforming DNA The *H. influenzae* streptomycin resistant (*strep^r*) transforming DNA was ultimately derived from transforming DNA obtained from Dr. Jane Setlow. The DNA solution contained 1950 μg ml^{-1} of DNA and exhibited a 260/280 nm ratio of approximately 1.76 (26).

Near-Ultraviolet Radiation Source The broad spectrum NUV source consists of a bank of four lamps (GE40BLB, integral filter) with a range of emission from 300-425 nm with a maximum emission at 350 nm. The source was kept in a cold room at 10 °C to reduce heating effects. Measurement with a DRC-100X Digital Radiometer equipped with a DIX-365 sensor (Spectroline) shows the fluence rate to be 48 J sec^{-1} m^{-2} (26).

Preparation of the Cell Suspension Cells were grown at 37 °C with shaking in sidearm flasks (Bellco Glass, Inc.) containing 50 ml LB. Growth was monitored by measuring the change in absorbance with a Klett-Summerson colorimeter equipped with a green filter. A 5 ml sample of stationary phase cells was removed 2.5 h after entering the transition from exponential to stationary growth phase, washed three times with phosphate buffer (K-K; 0.067 M, pH 7.0), and diluted in cold buffer (ice bath temperature) to approximately $5x10^8$ cells ml^{-1}. Appropriately diluted chrysene dissolved in dimethylsulfoxide, or boldine in 95% EtOH, was then added to the cell suspension to produce a final concentration of 15 $\mu g\ ml^{-1}$ for chrysene or 10 $\mu g\ ml^{-1}$ for boldine. The cells were then treated with NUV and viability was determined by plating 0.1 ml samples of appropriate dilutions on each of three plates of SEM. Colony counts were made after 48-60 h incubation at 37 °C. The surviving fraction of cells following a certain NUV exposure in the presence of the phototoxin was calculated by dividing the sum of the surviving colonies by the sum of the colonies at time zero. Survival curves were then produced by plotting the time of NUV exposure versus the surviving fraction.

Mutagenicity of Phototoxins as Determined by his-4 Reversion Mutagenicity of the test compounds was assayed by plating 0.1 ml of the treated cell suspensions of the histidine dependent strains (*his-4*) following intervals of NUV exposure onto SEM without histidine and minimal medium supplemented with arginine and lysine, but lacking histidine or casamino acids. Those colonies arising on the supplemented minimal medium lacking both histidine and casamino acids therefore represented spontaneous mutants arising during culture growth while those arising on the SEM lacking only histidine represented both spontaneous (or "plate") mutants arising during

incubation of the cells on the plate and preexisting spontaneous mutants. Thus a rise in mutational frequency is assessed based on the following calculation: Δ mutational frequency = (t-p) - (s_0 x N/N_o) where t (total mutants) is the mean number of colonies growing on SEM without histidine at time, p is the number of plate mutants as determined by subtracting the mean number of preexisting spontaneous mutants on supplemented minimal medium lacking histidine and casamino acids at time zero (s_0) from the mean number of both preexisting spontaneous and plate mutants growing on SEM without histidine at time zero (total spontaneous mutants; s_t), s_0 is the mean number of preexisting spontaneous mutants at time zero, and N/N_0 is the surviving fraction of cells at the particular NUV exposure interval. Thus, if there is a positive change in the mutational frequency, the phototoxin is determined to be mutagenic.

Mutagenicity of Phototoxin as Determined by 6-thioguanine Forward Mutation Test compound mutagenicity was assayed by plating 0.1 ml of the treated RT18h cell suspension following intervals of NUV exposure onto SEM to determine the surviving fraction and onto 6TG containing medium to drtermine the number of spontaneous mutants. Mutagenicity of the chemical could then be determined by the following calculations: net induced mutants per plate = n - (s_t x N/N_0) where n is the number of mutants arising on 6TG containing medium at time , s_0 is the number of spontaneous mutants at time zero, and N/N_0 is the surviving cell fraction at time. Multiplying the net induced mutants per plate by 10 to account for the volume plated then results in the net number of mutants per ml which can then be divided by the number of surviving cells per ml to yield the net number of induced mutants in the surviving cells.

Phototoxin Oxygen Dependency For anaerobic conditions a 10 ml suspension of RT10h cells with the phototoxin was kept under a stream of nitrogen stringently purified by passing it through

a solution of 200 ml dH_2O, 80 g NaOH, 1 g pyrogallol which was made fresh for each experiment and through a solution of $CuSO_4$ (22). The entire nitrogen system was sterilized by autoclaving before the NaOH, pyrogallol solution was added. After holding the sample under anaerobic conditions for 30 min, it was then exposed to NUV while still under the nitrogen stream. Time points were taken with a pasteur pipet also filled with nitrogen. For aerobic conditions, the cuvette containing cells was exposed to a filtered stream of N_2 for 30 min prior to exposure to NUV. After 30 min irradiation, the sample was then removed from the NUV source and flushed with air for another 30 min before returning it to the NUV source and continuing to take samples over time.

Transformation The procedures used for transforming with *H. influnzae* are identical to those of Setlow *et al* (22) while the *H. influenzae* transforming DNA carrying the streptomycin resistance gene (*strep-r*) was prepared as described by Marmur (13).

RESULTS AND DISCUSSION

Inactivation of E coli RT Strains The inactivation kinetics of cells exposed to chrysene plus NUV was solely dependant on the catalase phenotype. Catalase deficient strains (*kat F⁻*); RT 1, RT 7h, RT 8h, and RT 13h; exhibited increased sensitivity to treatment, but were no more sensitive to inactivation than when exposed to NUV alone. On the other hand, catalase proficient strains; RT 3, RT 9h, RT 10h, and RT 15h (*kat F⁺*); were more sensitive to inactivation when exposed to chrysene plus NUV than with NUV alone. Sensitization to inactivation by chrysene plus NUV in the catalase proficient strains indicates lethal lesions produced by this treatment require the presence of a *functional* catalase. Among the catalase proficient strains, the DNA repair

deficient strains RT3 (*rec A 1*), RT9h (*uvrA6*) and RT 15h (*polA 1*) were no more sensitive to inactivation by chrysene plus NUV than was the DNA repair proficient strain (RT10h; Fig. 2). These results suggest chrysene probably does not form DNA adducts in the presence of NUV. However, DNA lesions formed by chrysene plus NUV that are not repaired by recombinational, excision or DNA polymerase I repair can not be excluded.

Although there is more scatter among the experiments involving boldine plus NUV, the response of the cells to this treatment is also dependent on the catalase phenotype and the results are equivalent to those obtained with chrysene plus NUV (Fig. 3). Again, strains lacking catalase (*kat F*); RT 1, RT 7h, RT 8h, and RT 13h; exhibited sensitivity of inactivation by boldine plus NUV that was indistinguishable from that seen with light alone, while the catalase proficient strains (*kat F*, RT 3, RT 9h, RT 10h, and RT 15h) were more sensitive to inactivation by boldine plus NUV as compared to light alone. Catalase proficient strains carrying *polA 1* or *uvrA6* mutations did not exhibit increased sensitivity to inactivation by boldine plus NUV indicating that the DNA repair functions controlled by these genes are not involved in the repair of lesions induced by this treatment (Fig. 3). Among the catalase deficient strains, there is some suggestion that the *recA 1* allele carried by strain RT1 may sensitize this strain to inactivation by boldine plus NUV. The variability in these experiments, however, makes this conclusion tentative and in need of verification.

The general conclusion from the experiments with these two chemicals (Fig. 2 and 3) is that phototoxic effects can only be observed in catalase proficient strains, a novel observation for studies with phototoxic molecules. That is, strains producing catalase display increased sensitivity with the addition of the chemical plus NUV as opposed to their sensitivity to NUV alone (31). Strains lacking catalase, on the other hand, do not show increased rates of inactivation upon treatment with the chemical plus NUV compared to exposure to NUV alone. Thus, it is postulated the target of these phototoxins or the oxygen radicals they produce is the *kat F* gene product itself,

which may be a novel sigma transcription factor (17), or one of the gene products under its control.

It was initially hypothesized the sensitivity to inactivation by NUV plus phototoxin was a result of the inactivation of catalase, the synthesis of which is controlled by the *katF* gene product. The postulation of catalase as a lethal target is in keeping with the knowledge that the enzyme has a porphyrin prosthetic group (23). It has been shown the *hemAB* mutation, which blocks the synthesis of δ-aminolevulinic acid (δ-ALA) required for the synthesis of porphyrins, confers resistance to inactivation by NUV alone when cells are grown in the absence of δ-ALA (29). Also, it has been shown that NUV rapidly inactivates bovine catalase *in vitro* and it was simultaneously reported that measuring catalase activity in the *oxyR1* strain shows NUV inactivates catalase *in vivo*. Exposure of bacterial alkaline phosphatase to NUV under the same conditions resulted in no decrease inactivity (9). However, preliminary spectrophometric data shows that inactivation of catalase by NUV *in vitro* is not increased by the presence of either phototoxin used in these investigations (32). Based on the spectrophotometer data and the results of this paper, the current suggestion is that *kat F*$^+$ strains are sensitized to inactivation by NUV plus boldine or chrysene because the phototoxins either affect a product(s) other than catalase controlled by the postulated sigma factor specified by the *kat F*$^+$ allele or the sigma factor itself.

Inactivation of E. coli HB101 Strains The HB101 pPL376 strain which expresses the *Erwinia* pigments displays a significantly reduced sensitivity to inactivation by chrysene plus NUV compared to the nonpigmented HB101 and HB101 pHC79 strains as well as to the RT *katF*$^+$ strains (Fig. 4). Expression of the pigments does not, however, protect these cells from inactivation by boldine plus NUV (Fig. 5). Furthermore, the observation has been made that prolonged treatment with boldine and NUV (60 min) causes approximately one third of the HB101

pPL376 cells plated to loose their pigmentation. The failure of such colonies to grow when streaked on medium containing ampicillin indicates plasmid loss. Their initial growth on the experimental plates is most likely the result of large numbers of cells being plated producing enough penicillinase to degrade all the ampicillin present, thus allowing amp^s cells to grow.

Mutagenicity When tested with strain RT10h, neither treatment with chrysene nor boldine and NUV was found to induce *his4* reversions detectable above the level accounted for by "plate mutants" (data not shown, 40). Nor did exposure to chrysene or boldine plus NUV induce 6TG resistant mutants with strain RT18h(data not shown).

Transformation To determine if either test compound plus NUV can produce inactivating lesions in DNA, either 10μg ml^{-1} boldine or 15μg ml^{-1} chrysene was added to purified *H. influenzae* transforming DNA and then exposed to NUV under either aerobic or anaerobic conditions. Treatment with chrysene was observed to inactivate transforming activity under both nitrogen and oxygen although inactivation was more pronounced under aerobic conditions. Boldine rapidly inactivated the transforming DNA without exhibiting oxygen dependence (Fig. 6). These results imply that DNA is a potential target for attack by both chrysene and boldine in the presence of NUV. Thus the fact that deficiencies in DNA repair (*recA1*, *uvrA6*, and *polA1*) do not sensitize *E. coli* cells to inactivation by either chrysene or boldine plus NUV (Fig. 2 and 3) taken together with the fact such treatment is not mutagenic may mean that these compounds are so hydrophobic they can not penetrate into the cells. Consequently, the only way to demonstrate potential effects on DNA is to use naked, transforming DNA. Therefore, if under particular circumstances the cells were permeablized, perhaps these compounds might be mutagenic in the presence of NUV.

Oxygen Dependency To test the oxygen requirement for phototoxicity of the chemicals, either 10 μg ml-1 boldine or 15 μg ml-1 chrysene was added to a cell suspension of RT 10h under aerobic and anaerobic conditions followed by exposure to NUV. Chrysene and boldine caused inactivation in both cases though it was again more pronounced under aerobic conditions (Fig. 7). Thus, it appears the phototoxicity of these chemicals is only partially dependant upon oxygen, maintaining some activity under anaerobic conditions. It is interesting to note that boldine exhibits some oxygen dependence when exposure involves whole cells while exhibiting oxygen independence when the target is transforming DNA. This suggests that the lethal target for whole cells is probably not the DNA, but rather some other target , most likely the membrane. That the membrane is an important lethal target for treatment of cells with boldine plus NUV would be consistent with the observation that this treatment is not detectably mutagenic.

CONCLUSIONS

The phototoxicity of both chrysene and boldine is dependant upon a catalase proficient phenotype, which suggests the target of inactivation by the phototoxin plus NUV is either the *kat F* gene product or one of the gene products, other than catalase, under its control. While DNA lesions caused by chrysene plus NUV can not be excluded, they are not apparent in this study. DNA adducts, however, do not appear to be formed by either phototoxin plus NUV since "classical" repair defects (*uvrA6, recA1, polA1*) do not sensitize cells to this treatment. Boldine plus NUV, on the other hand, may produce some DNA lesions as the *recA1* strain is marginally more sensitive to this treatment although the *uvrA6* and *polA1* strains are not. The presence of carotenoid pigments protects cells from inactivation by chrysene plus NUV but not by boldine plus

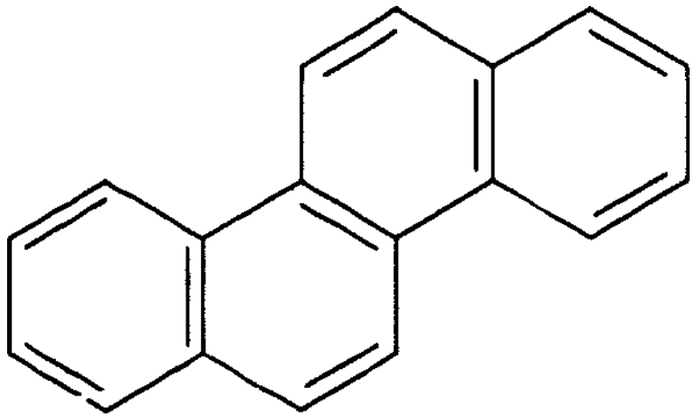
NUV. While chrysene and boldine inactivate transforming *Haemophilus* DNA under NUV, neither chemical exhibits pronounced effects on DNA repair mutants nor are they mutagenic. Therefore it is postulated the chemicals are too hydrophobic to enter the cell. Chrysene phototoxicity is partially oxygen dependent both *in vivo* and *in vitro*. Boldine phototoxicity is partially oxygen dependent only *in vivo*. Taking into account the nonmutagenicity boldine plus NUV, as well as the variable oxygen dependencies, suggests the membrane is an important lethal target for whole cells.

Table 1. Relevant genetic properties of *E. coli* tester strains.

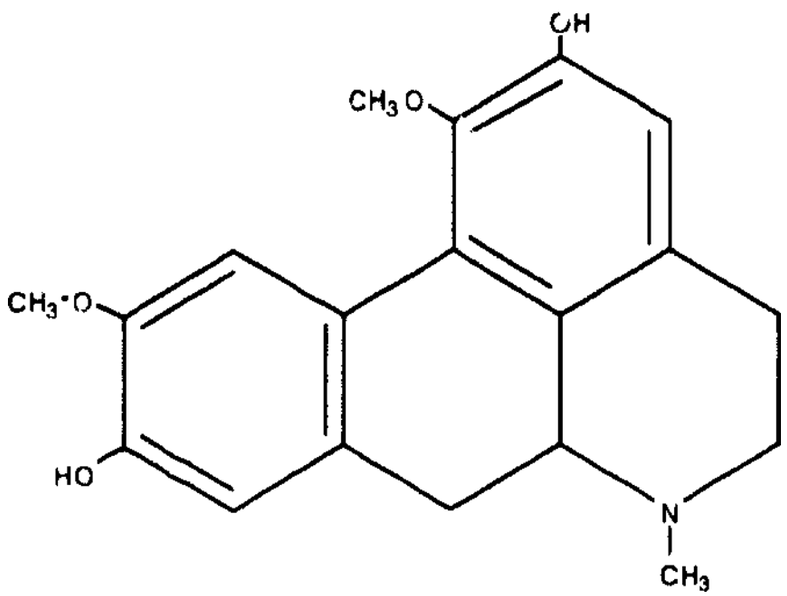
Strain	*recA1*	*uvrA6*	*polA1*	*katF*	Relevant Phenotype	Comments
RT1	-	+	+	-	Recombinational repair deficient, catalase deficient.	RT1 and RT3 test the role of recombinational repair in the reversal of damage induced by a particular phototoxin.
RT3	-	+	+	+	Recombinational repair deficient, catalase proficient.	
RT7h*	+	-	+	-	Excision repair deficient, catalase deficient.	RT7h - RT10h test the role of excision repair in the reversal of damage induced by a particular phototoxin.
RT8h	+	+	+	-	Catalase deficient.	
RT9h	+	-	+	+	Excision repair deficient.	
RT10h	+	+	+	+	Repair proficient (wild type for DNA repair).	
RT13h	+	+	-	-	DNA polymerase I deficient, catalase deficient.	RT13h and RT15h test the role of DNA polymerase I in the repair of damage induced by a particular phototoxin.
RT15h	+	+	-	+	DNA polymerase I deficient.	
RT18h	+	+	+	+	Repair proficient (wild type for DNA repair).	Derived from LE392 and used to test for mutations to histidine independent and 6TG^r mutations in a single experiment.
HB101	*recA13*	+	+	+	Recombinational repair deficient (alternate mutant allele of the *recA* gene).	HB101 is the recipient for the plasmid used to clone the genes controlling the synthesis of carotenoids.
HB101 pHC79	-	+	+	+	Same as HB101, but carrying the plasmid used to clone genes controlling carotenoid synthesis.	Tests the possible role of the plasmid alone in protecting against damage induced by a particular phototoxin.
HB101 pPL376	-	+	+	+	Same as HB101, but carrying the plasmid with genes controlling carotenoid synthesis.	Tests the possible role of carotenoids in protecting against damage induced by a particular phototoxin.
Haemophilus influenzae					Wild type, streptomycin sensitive	Used to test the biological (transforming) DNA activity following treatment with a particular phototoxin.

* The designation 'h' indicates that the strain carries the reversible *hsr-4* (ochre) allele (Kato *et al.*, 1977).

Figure 1



Chrysene



Boldine

Figure 2

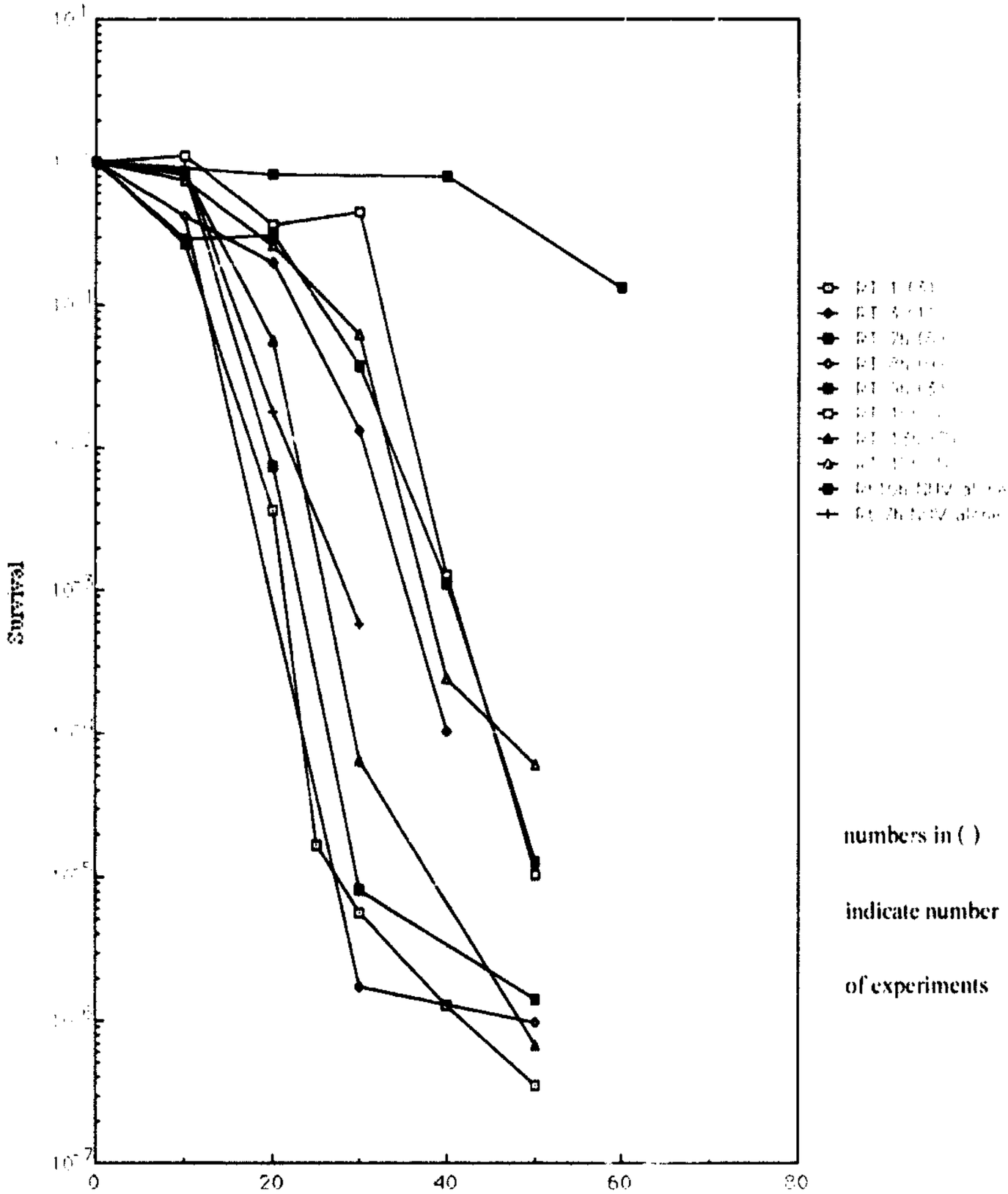
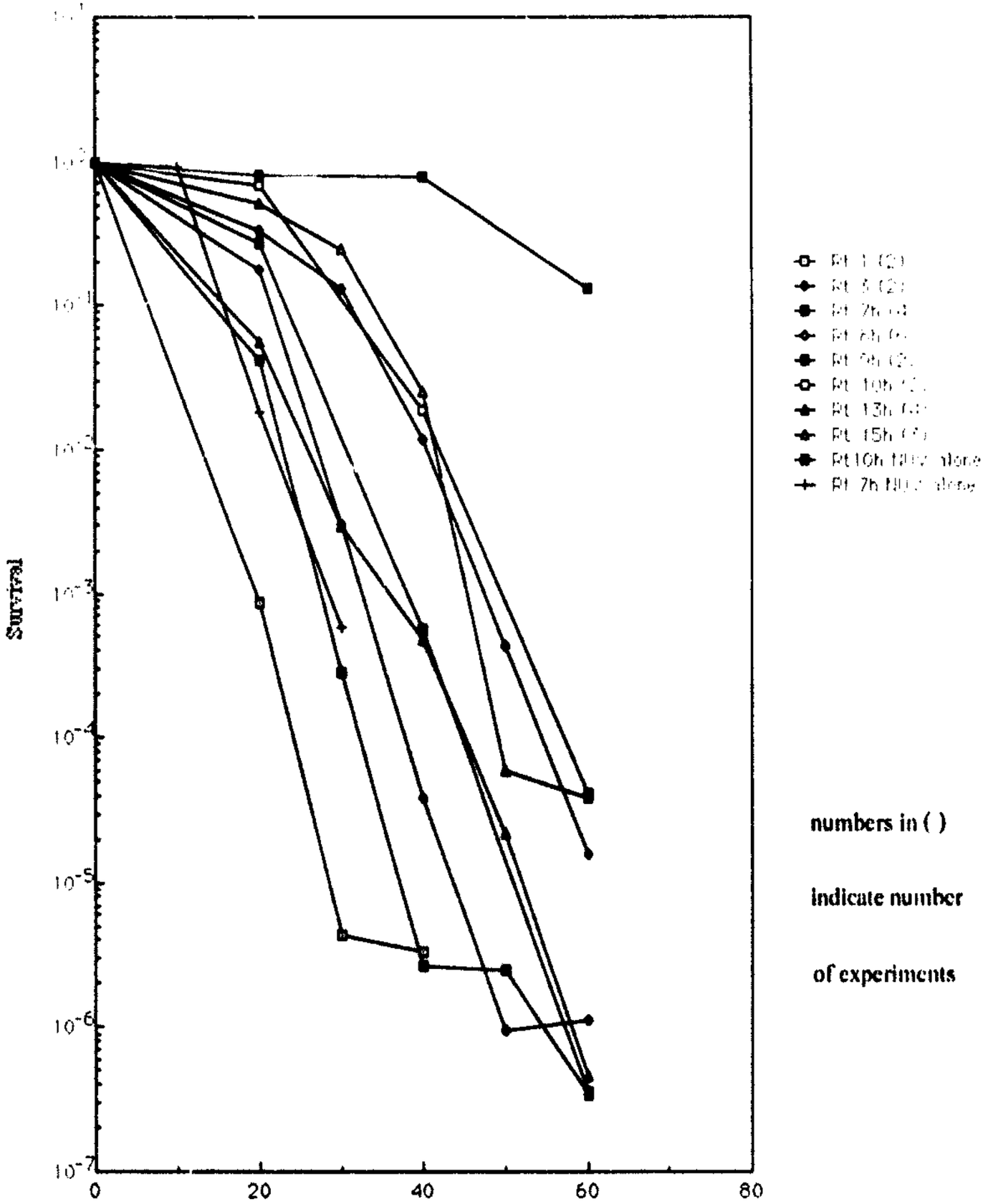

15 ug/ml CHRYSENE
Fraction
0
20
40
60
80
numbers in ( )
indicate number
of experiments
Time of NUV exposure (minutes)

Figure 3

10 ug/ml BOLDINE



Time of NUV exposure (minutes)

Figure 4

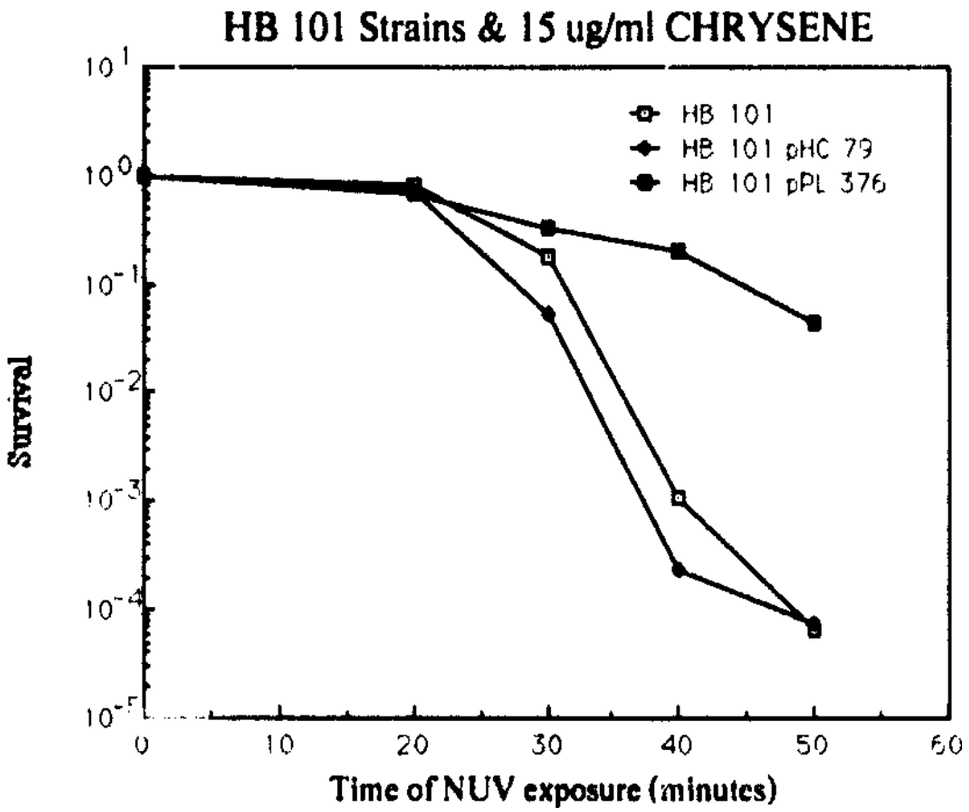


Figure 5

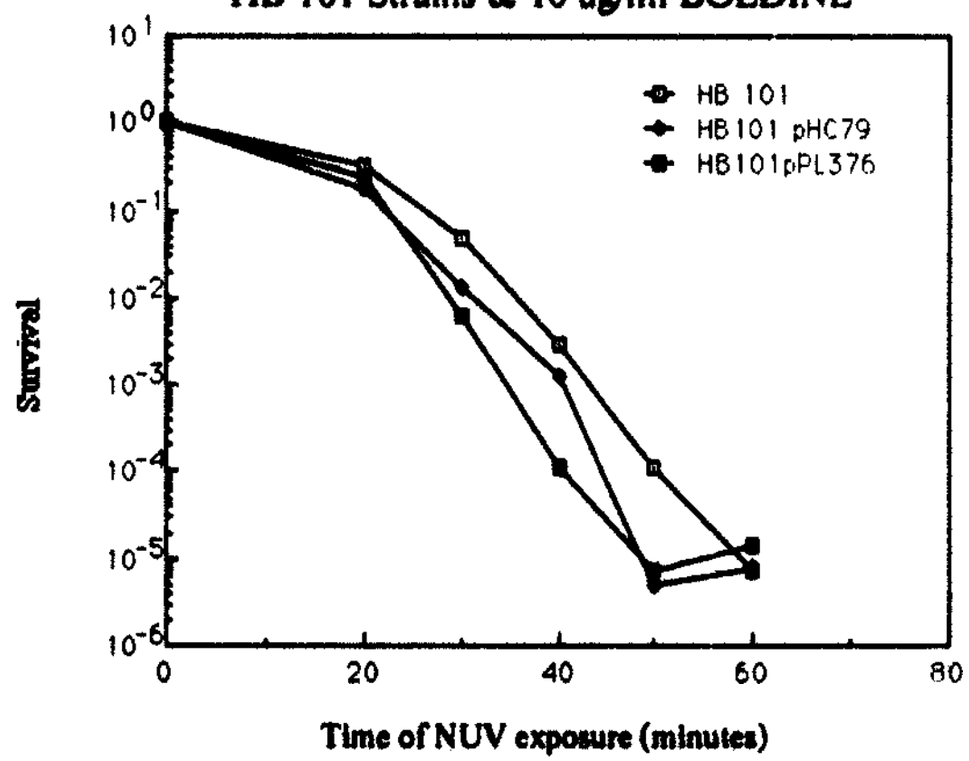


Figure 6

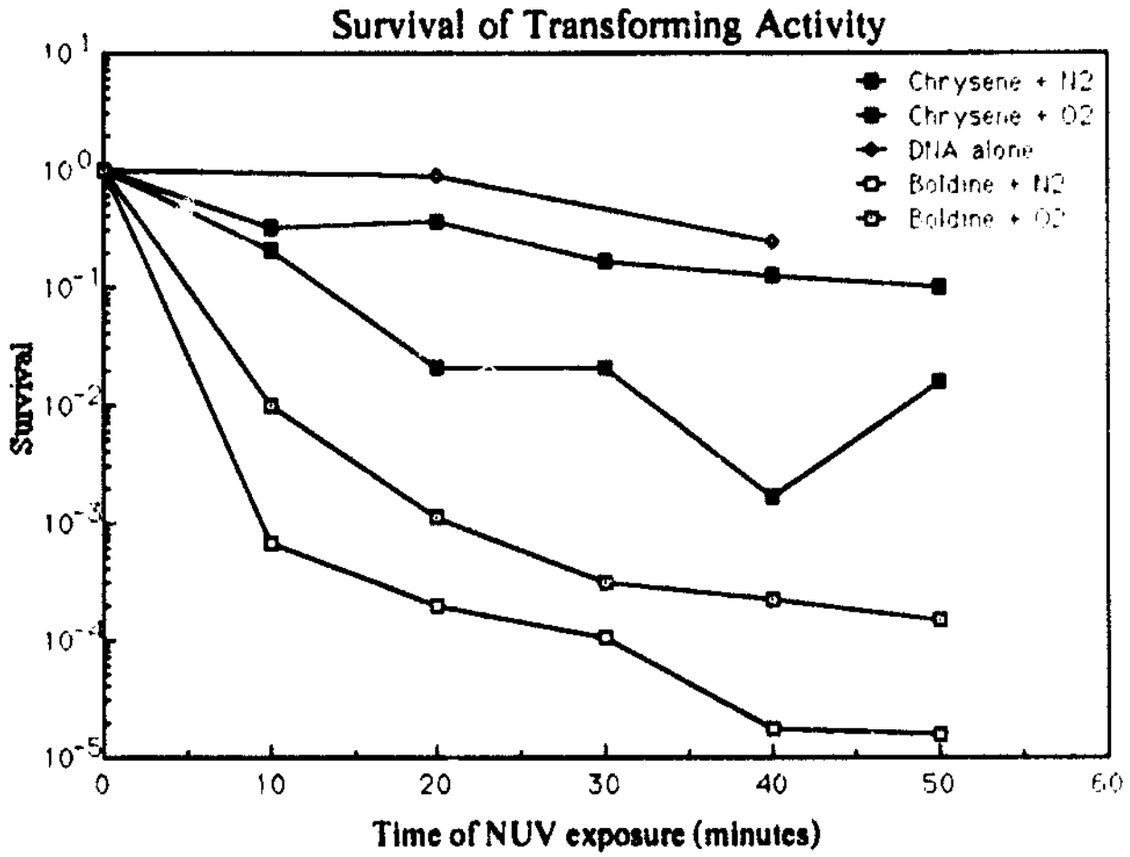
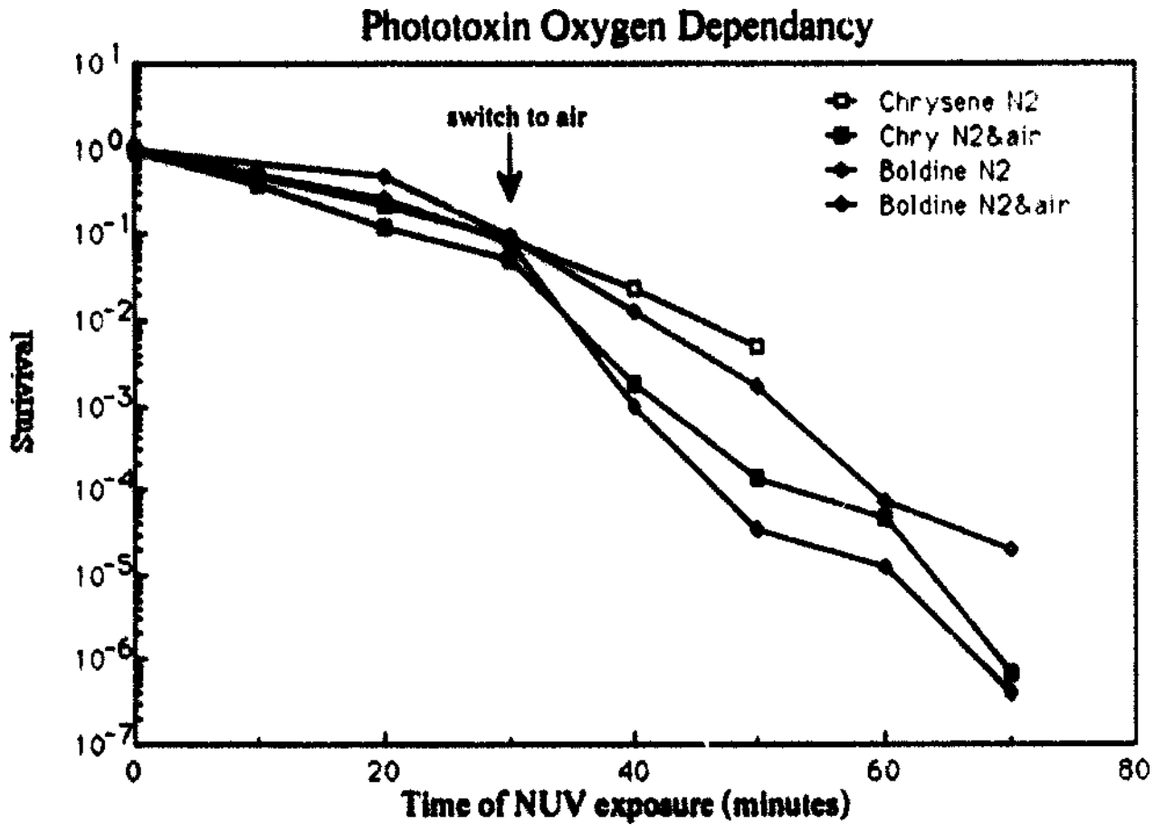


Figure 7



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