CELL KILLING AND MUTATION INDUCTION
ON CHINESE HAMSTER CELLS BY PHOTORADIATIONS

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ABSTRACT

The subject matter of this investigation concerns the killing and mutagenic effects induced by far-UV radiation and broad spectra of black, white and gold lights. Applying radiation directly on cells, far-UV is more effective than black light, and black light is more effective than white light in inducing proliferative death and in inducing resistance to 6-thioguanine (6-TG), ouabain and diphtheria toxin (DT). Gold light has no killing and mutagenic effects on CHO (Chinese hamster ovary) cells. Use of filters showed that a small percentage of shorter wavelengths in the far-UV region is responsible for most of the killing and mutagenic effects in the unfiltered broad spectra of black and white light.

Media irradiated with far-UV radiation does not produce cytotoxic photoproducts. However, media exposed to white or black light are toxic but not mutagenic to cells. Eighty percent of cells is killed by peroxide-types of toxic photoproducts and twenty percent by non-peroxide types. Tryptophan, tyrosin and riboflavin in media are responsible for the production of the toxic photoproducts. These three chemicals in aqueous solution individually or in combinations can produce photoproducts, with the highest level of phototoxic products produced in a solution containing all three chemicals.

The survival data of synchronous cells after the treatment of far-UV or unfiltered fluorescent lights show an age-response function. Cells in the G1/early S boundary are the most sensitive and cells in other parts of the cell cycle are more resistant. The cyclic sensitivity response disappears when synchronous cells are irradiated with black-light spectra with wavelengths shorter than 325 nm filtered.

Mutation induction of DT resistance is different from ouabain or 6-TG resistance for synchronous cells irradiated with far-UV and fluorescent lights. A distinctive DT resistance peak is noticeable in late G1 cells, 3-4 hours after mitotic shake-off. The cyclic response is not wavelength- or dose-dependent but the cyclic responses of 6-TG and ouabain resistance are. Mutations to 6-TG and ouabain resistance are slightly higher in early S period than in the remaining parts of the cell cycle for synchronous cells irradiated with moderate doses of far-UV or unfiltered broad spectra of black light. Synchronous cells irradiated by black light with wavelengths shorter than 300 nm excluded do not show slightly higher mutation frequencies in the early S period.

An optimal recovery of sublethal damage is achieved when the recovery period is 8 hours or longer and the conditioning dose is at the shoulder region of the dose-survival curve obtained in acute-dose experiments. During the recovery period, cells kept at room temperature (about 24°C), do not show recovery whereas those kept at 37°C do. Repair of sublethal damage in synchronous cells occurs throughout the entire cell cycle except for some uncertainty in G1 cells.

Mutation induction of 6-TG, ouabain or DT resistance is increased in the split-dose samples of the asynchronous and synchronous CHO cells after cells treated with far-UV or fluorescent lights. An increase of mutation induction of three loci occurs throughout the entire cell age cycle, with the highest increase in the early part of the cycle (from G1 to mid-S cells). CHO cells predominantly express an error-prone repair mechanism after photoirradiation.

INTRODUCTION

Molecular damages in cells induced by far-UV, black, white, and gold lights are selective and specific. With far-UV radiation at 254 nm, molecules absorbing energy at the 4.8 eV level will be affected, whereas with filtered fluorescent light, molecules absorbing energies at lower levels will be affected (see Appendix I for the relationship between energy and wavelength). Unfiltered broad spectra of white and black light have a low level of far-UV (Appendix 2). When such light is used to irradiate cells, many kinds of molecules are affected. However, cell death as a result of the radiation damage depends on the number and kinds of damaged molecules and the capability of the cell to repair these damaged molecules.

Cell killing, mutation induction and the capability of repairing damage following far-UV or near-UV irradiation have been compared in such varied systems as protozoa, yeast, bacteria and tissue cultured mammalian cells. The effects of far-UV and fluorescent light sources on CHO cells are compared in this study. Results with CHO cells agree with other cells in that cell killing and mutation induction are wavelength-dependent, with longer wavelengths being less effective. CHO cells can repair sublethal photoradiation damage, but in a departure from previous findings in other CHO cell lines, we have demonstrated that a synchronous cell population can repair sublethal damage outside of the S phase. In comparing mutation induction of 6-TG, ouabain- and DT-resistance in split-dose samples to those of acute-dose ones in asynchronous and synchronous cell populations,

higher mutation frequencies of all three loci have been found. It appears that an error-prone repair system is induced in CHO cells following photoirradiation.

Historical Perspective of Photoradiation Studies

UV radiation was discovered in 1803 by Ritter (Blum, 1955). A century later, its biological application was well-documented in the therapeutic treatment of human skin in Finsen's Light Institute in Copenhagen, where many microbial diseases were successfully cured (Dreyer, 1904; see review by Giese, 1964). The interpretation of far-UV effects on human skin was limited to empirical observation. Single cells were then used in the institute to obtain information to explain the far-UV effect. Much of our photoradiation knowledge indeed came from the cellular approach by employing protozoa, microorganisms and mammalian tissue-cultured cells.

The development of photoradiation, like ionizing radiation, can be chronologically described by three major periods. The first period emphasized the morphological changes of cells following irradiation. The second period was the application of mathematical models and statistical analysis to interpret radiation effects. The third period in recent years has been in the direction towards molecular radiation biology. Much of the prior work in the early photobiology had been with the bacterial systems. Later, using mammalian cells to examine similar effects, results were not always identical.

The first series of action spectra of bacterial cell killing by far-UV were reported in the 1920's by Gates (1928, 1929 and 1930).

The maximum killing effectiveness was found to be at about 260 nm, which was soon confirmed by others with the same or different bacterial strains (Wykoff, 1932; Hollaender and Dugger, 1936). Since these action spectra corresponded closely to the absorption spectra of DNA, DNA was suggested as the chromophore and target for bactericidal effects in far-UV radiation (see Appendix 3 for the definition of far-UV and near-UV).

The important role of DNA was further demonstrated in larger cells. By selectively damaging the nucleus or cytoplasm with UV microbeams, it was shown that if the nucleus containing DNA molecules was damaged, a much higher percentage of sea urchin eggs would not divide (Casperson, 1950). But the UV-microbeam absorption spectra of the eucaryotic cells peaks at 270 nm instead of 260 nm (Zirkle, 1956). The maximum effectiveness of eucaryotic cell killing obtained in the far-UV action spectra is also at 270 nm. The variation in the structure of the genetic unit between these two systems has been suggested to account for the absorption difference. The nuclear DNA of eucaryotic cells is histone coated in contrast to the naked DNA strands in most procaryotic cells.

When studying the maximum chromosomal damage induced by different far-UV wavelengths, Chu (1965) found it to be, coincidentally, at 270 nm. As for the types of damage, he reported that 260 nm light induced mostly "abberation-type" damages, whereas 280 nm light induced mostly "demolition-type" damages. When a protein blocking agent was present immediately after 280 nm-UV irradiation, the abberation-type

damages prevailed. The mechanisms and the protein component responsible for different kinds of chromosomal damage still remain unknown.

In the near-UV region, the action spectra for bacterial or mammalian cells exhibit a closer agreement with the absorption spectra of proteins than of DNA (Setlow and Boling, 1965). An oxygen dependence for cell lethality is observed in bacterial and mammalian cells, suggesting that the damage is related to a photodynamic process (Webb and Lorenz, 1970; Danpure and Tyrell, 1975, 1976). Webb and associates have recently demonstrated a significant difference in sensitivity after exposing an isogenic series of *E. coli* to a monochromatic wavelength of 365 nm; damage to the protein components of the repair systems is responsible for the sensitivity difference (Webb, 1977). If protein molecules are the target for near-UV radiation, repair of the damage would be expected to be more efficient and, consequently, cell death caused by protein injury would be less. A typical near-UV survival curve often possesses a broader shoulder as compared with the far-UV survival curve (Webb, 1977; Jacobson et al., 1978).

The shape of the survival curves on a semi-log plot has been traditionally described by a shoulder and an exponential region. Different mechanisms have been postulated to interpret these curves. Baker and Nanavitty (1929) proposed a cumulative action of a toxic substance following UV radiation to account for the steeper slopes at higher doses. The different shapes of survival curves, argued Gates

(1929), were due to heterogeneous cell populations with a variable degree of resistance to radiation. His view was supported by some workers (Rentschler et al., 1941). For nearly forty years, the target theory was singled out and favorably accepted by many others (Wykoff, 1932; Hollander and Clauss, 1936; Lea and Haines, 1940; Lea, 1947). The theory accounts for the exponential survival region as a single hit for cell killing and the shoulder region either as multiple hits required in one target or single hits in multiple targets for cell killing. The discoveries of excision repair in 1964 (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964) and recombination (or post-replication) repair in 1968 (Rupp and Howard-Flanders) in *E. coli* introduced a new way to interpret the survival curves. According to the repair model, cell survival depends on repair processes which account for the shoulder region of the survival curves. The strongest evidence to support this model has been from the isolation and characterization of the bacterial repair enzymes (Riazuddin and Grossman, 1977).

Tissue culture techniques in vitro was first reported in the literature in 1907 in Ross Harrison's frog tissue experiment in which the frog embryo tissue was transplanted in clots of lymph fluid. Under these conditions, the nerve fiber would live for about a week and in some cases, up to three weeks. Finding suitable ingredients to maintain cells in vitro has been a major task for almost five decades. After a trial-and-error search by many workers, the present use of synthetic media gradually developed. For the vigorous culture of mammalian cells, blood serum has to be used in the medium. Serum

proteins are vital for cell growth. Different batches of serum have different concentrations of serum proteins, and the only way to ensure a suitable batch for the best behavior of a particular cell line is through a serum test.

When the light microscope is used to observe the sequential events occurring in a living cell during its growth, the only noticeable event is during the mitotic period (M period) in which a single cell divides into two. This becomes the first identifiable period. Information regarding cellular changes during the interphase remained obscure until the availability of radioactively labeled compounds that provided a means to study macromolecular synthesis in the cell. Howard and Pelc (1953) observed that DNA synthesis occurs in only part of the interphase. Discovery of this discrete time for DNA synthesis led to a second identifiable period, designated as S phase. The periods before and after the S phase gaps without DNA synthesis are known as the G₁ and G₂ phase, respectively.

Structures and functions can be different during different parts of the cell age cycle. The adhesive property of the mitotic cell is different from the interphase cell. The latter flattens out and adheres firmly to the substrate whereas the former rounds up and is loosely attached. The nonadhesive characteristic of the M cell has provided a physical method to synchronize mammalian cells, namely by vigorously shaking the container in the presence of medium (Axelrod and McCulloch, 1958). Highly synchronized cells can be attained in this way. More recently, automatic synchronizers for shaking off cells have

been developed (Klevecz, 1975) and now are commercially available. Such a machine, when installed in a 37°C room, allows the shaken-off cells to continue growing immediately with little disturbance. The M cells collected by this method comprise less than 2 percent of the cell population per each shake-off.

A greater percentage of cells in S phase can be produced by using a DNA inhibitor such as hydroxyurea (Sinclair, 1965) or excess thymidine (Xeros, 1962; Bootsma et al., 1964). In order to collect a high percentage of S cells, cells must be in the medium containing hydroxyurea or excess thymidine for a long period of time (at least 8 hours). To prevent severe cytotoxicity in cells, the concentration applied may not result in complete cessation of DNA synthesis. As a rule, only partially synchronous S cells are produced. Another potential complication of using DNA antimetabolic drugs is the continuation of RNA, protein, and other intracellular syntheses during drug incubation. The S cells so obtained are generally enlarged and therefore may be different from S cells grown under normal condition.

Survival Characteristics in Mammalian Cells after Photoradiation

In constructing a survival curve for mammalian cells, the most commonly used endpoint is the ability to form a colony. After radiation exposure, if a cell can reproduce a colony of 50 cells or more during the time required normally for about 16 generations under growth conditions, the cell is considered to have survived the treatment. The killing effect, following the terms used by radiobiologists, may be divided into two categories, direct and indirect. To observe the direct effect, cells are often rinsed with clear buffer

solution before radiation exposures. An important point to be added here is that the indirect effect due to intracellular nutrient constituents is not distinguishable from the direct effect. The resulting survival curves after directly irradiating asynchronous cells with far-UV or fluorescent light generally possess a shoulder in a semi-log plot, with a broader shoulder region by the longer wavelength of fluorescent lights. Wang (1975) has reported that direct white-light irradiation of human cells with buffer has no toxic effect whereas such irradiation of cells with medium does. This led him to claim that, with a white light spectrum, indirect effect is the mode for killing human cells. Other mammalian cells, however, have been shown to be affected directly by white light (Bradley and Sharky, 1977; Hsie et al., 1977). Cells of different origin may respond differently to the exposure of white light. When gold light is employed, no toxic effects have been found for human (Wang, 1975) and CHO cells (Burki and Lam, 1977).

The direct effect of exposing synchronous cells to far-UV radiation varies in a complicated manner with the cell age at the time of irradiation and with the magnitude of the fluence applied. Following the treatment, the age-response curve indicates that the most sensitive period is at the late G1/early S boundary and the most resistant period is at the late S and G2 periods (Sinclair, and Morton, 1965). The ratio difference between the most sensitive and the most resistant periods is somewhat related to the fluence applied. At a relatively high fluence, corresponding to a survival level of 0.05 in asynchronous population, one order of magnitude difference has been reported (Burki et al., 1980).

Indirect effects can be studied by measuring the effects of photoirradiated chemicals or medium on cells. Medium irradiated by far-UV or near-UV is toxic and mutagenic to bacterial cells (Auerbach and Robson, 1943; Stone et al., 1947). The first demonstration of the toxic effect of medium previously irradiated by fluorescent light on mammalian cells was reported by Wang et al. (1974). They showed that photoactivated medium without riboflavin, tryptophan and tyrosin during irradiation did not affect cell viability, whereas medium with these three chemicals did. Therefore, they concluded, riboflavin, tryptophan and tyrosin are responsible for producing the photoproducts that kill cells in regular medium.

Riboflavin absorbs energy at 260 nm, 360 nm and 450 nm (Hodson and Norres, 1939), whereas tryptophan and tyrosin, like other aromatic amino acids, absorb at 280 nm. If these three chemicals are present in the solution, the activated riboflavin can transfer energy to tryptophan and tyrosin to form tryptophan/tyrosin photoproducts (Spikes and Livingston, 1969). One of the photoproducts has been identified as a complex tryptophan/tyrosin-peroxide (McCormick et al., 1976). Oxygen is required for the transferring reaction to take place, probably because oxygen potentiates free radical transfer and formation.

Photoproducts from irradiated media are stable (Yamafuji and Uchida, 1966; Rhaese and Freese, 1968). The cytotoxic effect has been found to be dose-dependent (Ananthaswamy and Eisenstark, 1977). In

analyzing data on lesions and cytotoxicity induced on human cells by photoactivated media, Ananthaswamy et al. (personal communication) discovered that peroxide-type photoproducts are responsible for the formation of all single-strand breaks but for only 40 percent of cell cytotoxicity. The other 60 percent killing is caused by non-peroxide photoproducts that do not produce DNA single-strand breaks. Part of their findings agree with results with monkey cells in which the peroxide type of photoproducts from photoactivated medium is completely responsible for the formation of single-strand breaks (Hoffman and Meheghini, 1979).

In the absence of riboflavin, some authors have reported finding peroxide photoproducts by near-UV irradiation of a solution containing tryptophan and tyrosin (Yoakum and Eisenstark, 1972; McCormick, 1975). At a lower amino-acid concentration, Hofferma and Meheghini (1979) found the same chemical solution had no killing effect on mammalian cells. In this study, we found that tryptophan and tyrosin in the absence of riboflavin indeed has killing effect on CHO cells. The effect is concentration-dependent and enhanced by the presence of riboflavin.

The mutagenic ability of phototoxic substances on mammalian cells has not been reported. In this study, we have carried out some experiments dealing with this subject and the finding, as seen later, is negative.

Post Photoradiation Effects

The most obvious effect of far-UV irradiation is the growth delay that has been related to the reduction of DNA synthesis rate (Rasmussen

and Painter, 1964; Klimek and Vlasinova, 1966), the reduction of the size of newly synthesized DNA and the repair of DNA (Rosenstein, 1981). At moderate doses, cells progress through S phase more slowly but cells in other parts of the cell cycle continue at normal rates until they arrive at the first S phase, when they too experience the delay (Cleaver, 1974). The S-phase delay induced by far-UV is dose-dependent, a more pronounced delay at a larger dose. After the delay, survivors resume growth at a nearly normal rate as measured by total cell numbers per dish or by average number of cells per colony (Cleaver, 1965; Bootsma and Humphrey, 1968; Domon and Rauth, 1968; Todd et al., 1969).

Growth delay following near-UV irradiation has been observed in HeLa cells (Klein and Edsall, 1967). Mechanisms and chromophores involved in this effect remain obscure. Several molecules have been speculated to be responsible for the longer-wavelength growth delay. Working with isolated rat cerebellar cells, Rounds and Olson (1967) have proposed that respiratory cytochromes are the photoinhibitors in longer wavelengths. Growth delay by near-UV or visible irradiation is thought to be caused by destruction of cytochromes and flavoproteins in algae and yeast (Epsel and Butler, 1970). Recently, Ballini et al. (1976) showed that 4-thiouracil was the chromophore leading to the near-UV induced growth delay in *E. coli* cells.

It is generally believed that far-UV induced damages in DNA leads to cell death. The major UV-induced lesions in mammalian cells have been identified as pyrimidine dimers (Jagger, 1967). Trosko et al.

(1965) estimated that there are about 5×10^6 dimers produced per Chinese hamster cell at a UV dose of 20 J/m^2 . Near-UV radiation from sunlamps and fluorescent lights also yields photodimers, but the formation efficiency is much less than that reported by far-UV at 254 nm (Zelle et al., 1980; Elkind et al., 1978). Following a moderate dose, cells can resume normal DNA synthesis with dimers along the parental DNA strands. In *E. coli* DNA synthesis stops at the dimer sites but resumes past them, leaving gaps and these gaps are filled in afterwards by the recombination repair system (Rupp, 1970).

There is considerable evidence that the dimer-formation, cell lethality, and mutation induction per unit dose in cells irradiated by far-UV are higher than those irradiated by near-UV. Elkind et al. (1978) and Zelle et al. (1980) have made calculations on mutation induction and lethality per unit to dimer formation and they have shown that both rates are much higher in near-UV radiation than those observed from far-UV radiation. Therefore, they proposed that the major lesions of near-UV are not dimers, but the lesion has not been identified.

Many types of non-dimer damage by far-UV radiation in DNA have been identified and were recently reviewed (Rahn, 1979). Photodimers can only partially explain the cellular disfunctions, and Rahn has suggested that DNA-protein links may be potentially important lesion for mammalian cells.

Recovery from sublethal damage was demonstrated by the studies of Elkind and Sutton, who initially performed split-dose experiments after cells were irradiated by x-ray (1959, 1960). Using this method, sublethal damage recovery was shown in far-UV damaged cells (Todd, 1973; Humphrey et al., 1970, 1971). Humphrey et al. (1970) showed that the recovery of asynchronous CHO cells occurred with a recovery period of 2-4 hours. Using partially synchronous cells, repair of UV sublethal damage was observed only when cells were in the first S phase after UV exposure (Domin and Rauth, 1969; Todd, 1973). This result led to the conclusion that the split-dose repair process bears no relation to excision repair. Data presented in this study, however, suggest that split-dose recovery occurs elsewhere than in the S phase, although it must be accompanied by cell progression. Since unscheduled DNA synthesis does not require cell progression, these two processes are probably not related.

Genetic Studies on Mammalian Culture Cells

The first mutagenesis experiments in mammalian cells were reported in 1968 by two groups, one by Chu and Malling and the other by Kao and Puck; in both studies, mutation induction of resistance to 8-AZ (8-azaguanine), a purine analog, was described. Since the beginning of somatic genetic studies, questions concerning the genetic or epigenetic nature of the purine-analog resistance were raised and the subject has been actively debated in the literature. Many experiments were designed to prove the genetic nature of HGPRT phenotypes (hypoxanthine-guanine-phosphoribosyl-transferase E.C.2.4.2.8).

Some criteria have been set by researchers in the field to determine if the observed phenotypic changes are inherited as genetic changes. Among the proposed criteria, the most detailed and the best outlined is that of Chu (1974) who listed the following: 1) Random occurrence; 2) retention of a stable phenotype in the absence of selection media; 3) induction with mutagens; 4) mutagenic specificity; 5) changes in activity and physiochemical properties of specific gene products; 6) conditional lethality; 7) interallelic complementation; 8) localization of a mutated gene in a specific chromosome region; 9) mitotic recombination; and 10) gene transfer between cells.

The way in which the somatic-cell genetic experiments are conducted can influence mutation results. In the first mutagenesis article, Chu and Malling (1968) found that some controllable factors such as cell density, expression time and the concentration of the selection agent must be controlled before the mutation frequency of any variants can be accurately measured. Producing consistency in mutation frequency requires, as suggested by Simmon (1974), the standardization of experimental procedures including freezing a large cell population, cloning cells, using preselected sera and identical methods to sub-culture. Some workers insist that, even under the same experimental conditions and procedures, mutation frequencies vary from experiment to experiment (Shapiro and Vashaver, 1973). Others observed that fluctuation of the spontaneous and induced mutation frequencies occur in the same direction - when the spontaneous mutation frequency is high, the induced mutation frequency is also high, and vice versa

(Arlett and Harcott, 1972). According to these authors, it is only meaningful to compare data in the same experiment. Most of the loci studied so far in mammalian cells require an expression period before cells can be challenged. How to handle the treated cells during this period has been a subject for argument. To account for all the mutants, Myhr and Dipalo (1975) have suggested that the cells not be subcultured during the expression period. If this is done, however, a low cell density must be plated and the resulting poor statistical sampling will increase uncertainty in the data analysis. Later Abdondandola et al. (1976) experimentally showed that mutation selection of HGPRT variants was not affected by subculturing but by the crowded conditions of the mutagenized cells.

HGPRT Mutants

The isolation of 8-AG resistant cells was initially conducted by Syzbaski in 1958, a decade before Chu and others used this drug for mutagenesis studies. The resistance to 8-AG, 6-TG or other purine analogs is related to malfunctioning HGPRT enzymes that can no longer incorporate potent purine analogs into the DNA. Mutant cells with these abnormal enzymes have to depend on de novo synthesis of the purines for their survival. Providing chemicals for or against de novo purine synthesis in the medium permits the forward or reverse selection of mutants. In the reverse selection, HAT medium (hypoxanthine-aminopterin-thymidine) is frequently employed where the de novo synthesis of purine is blocked by aminopterin. Any revertant arising from the mutant population can survive by utilizing hypoxanthine as the purine source.

Two studies were of particular distinction in assessing the epigenetic nature of the HGPRT system. With higher ploidy cells, Harris (1971, 1973) and Mezger-Freed (1972) obtained experimental data that were not consistent with an increase of HGPRT mutants in proportion to the number of chromosomes. They, therefore, stated that 8-AG resistance was due to changes in gene expression rather than in genetic information. Many workers in the field were not convinced by their explanation and many experiments were done to collect evidence to rebutte this suggestion. The preponderence of evidence now indicates that HGPRT mutants are caused at the genetic level. The evidence is as follows: 1) resistance to purine analog in various cell lines has been shown to be spontaneous and random by the fluctuation and/or the chi-square test (Chu et al., 1969; Morrow, 1970; Harris, 1971, 1973). 2) Mutation to purine analog resistance can be induced by alkylating agents (Chu and Malling, 1968; Goth-Goldstein and Burki, 1980), and by physical agents such as γ -ray or x-ray (Arlett and Potter, 1971; Bridges and Huckles, 1970), UV-radiation (Arlett and Harcott, 1972), near-UV radiation (Hsie et al., 1977; Bradley and Sharkey, 1977). 3) Mutagen specificity has also been reported (Sekiguchi et al., 1974; Burki et al., 1980; Goth-Goldstein and Burki, 1980). 4) The strongest evidence is that gene transfer via chromosomes of the HGPRT locus has been accomplished (McBride and Ozer, 1973; Willecke and Ruddle, 1975).

Expression Time and Metabolic Cooperation

About five days is needed to select 6-TG mutant colonies. This period is required for the expression of the newly arising poorly

functioning enzymes and for the dilution of the pre-existing wild-type HGPRT enzymes. Once the poorly functioning enzymes dominate in a cell, it then can be selected as a mutant. Characterization of altered HGPRT enzymes in mutant cells has shown that they possess various degrees of functional activities (Beaudet et al., 1970). A variant possessing immunologically active proteins but enzymatically inactive enzymes was isolated by Fenwick et al. (1977). Complementation tests indicate that HGPRT gene is interallelic, probably containing three cistrons (Sekiguchi et al., 1974). Point mutation has been suggested as the mutation type for HGPRT variants which were induced by alkylating agents.

The phenomenon of metabolic cooperation in a mixed population of wild-type and HGPRT mutants is well known (Subak-Sharper et al., 1969; Chu et al., 1968; Dancis et al., 1969). To obtain a better statistical sampling, it is a good practice to inoculate the highest possible number of cells during subculturing or challenging. But cell-to-cell contact permits molecules to exchange among the wild-type and HGPRT mutant cells (Cox et al., 1970; Fugimoto and Seegmiller, 1970). Exchange of molecules associated with wild-type HGPRT enzymes allows the mutant cells to be mistakenly eliminated during the mutant selection. The transmissible molecules have not yet been identified. Some have described it to be nucleotide corresponding to the HGPRT enzyme (Fugimoto and Seegmiller, 1970), and others have suggested that they are mRNA or even HGPRT enzymes themselves (Cox et al., 1970; Ashkenazi and Garlter, 1971). Whatever they are these molecules do not diffuse through the medium (Van Zeeland et al., 1972).

Gene Location of HGPRT

Locating the HGPRT gene position in chromosomes has been guided by clinical work on a human recessive and sex-linked genetic disorder, the Lesch-Nyham syndrome. Patients with this disease produce poorly functioning HGPRT enzymes (Rosenbloom et al., 1967). In humans, this gene is linked to glucose-6-phosphate-dehydrogenase (G-6PD) and phosphoglycerate kinase (PKG) in the long arm of the X-chromosome (Ricciuti and Ruddle, 1973). Westerveld et al. (1972) examined the gene multiplicity in conjunction with the enzymatic activity of these three genes in hamster cells and claimed that the three loci are also linked, presumably also on the long arm of the X-chromosome. It is, therefore, possible for variants to express this recessive gene because of the hemizygous nature of the sex chromosome markers.

Ouabain Locus

Ouabain is a lethal chemical for cells. It has a binding affinity towards $\text{Na}^+ \text{K}^+$ -activated-ATPase of the plasma membrane, thereby blocking the active transport system. The toxic effect of this action is potassium starvation in cells (Dunham and Hoffman, 1970). Other transport systems such as thymidine, uridine and amino acid uptakes are inhibited by ouabain, but not immediately. These delayed inhibitions eventually bring protein, RNA and DNA synthesis to a complete halt (Mayhew, 1971). Increase of potassium concentration in the medium can reduce ouabain toxicity because potassium and ouabain behave like competitive inhibitors.

Ouabain resistance is known to be a co-dominant trait (Baker et al., 1974). Cells resistant to ouabain are found to produce

altered ATPase that has lost its binding affinity towards ouabain. Fidelity of this mutant has been repeatedly reported from various laboratories (Baker et al., 1974; Chang et al., 1978; Burki et al., 1980; Goth-Goldstein and Burki, 1980). Observation of the phenotypic changes in ouabain resistant cells indicate that the alterations are of genetic nature. Baker et al. (1974), examined the selection of ouabain-mutant in conjunction with the effect of cell density over a large range. They have reported that ouabain-mutant recovery is not complicated by cell density. A lack of metabolic cooperation among ouabain mutants and wild-type cells in Chinese hamster cells has been reported by others (Chang et al., 1978).

Diphtheria Toxin Locus

Another marker that has been recently characterized in mammalian cells is the resistance to diphtheria toxin (DT). This toxin is a proenzyme, weighs about 63,000 daltons and contains two subunits, A and B polypeptides (Olsen et al., 1974; Collier, 1975; Collier and Kandel, 1977). These two subunits apparently play different roles in the action mode of DT. In an unknown manner, the B fragment first binds to the receptor sites of the cell membrane (Uchida et al., 1972) and then the A fragment penetrates into the cytoplasm (Boquet and Pappenheimer, 1976). After binding and penetration, toxin molecules react with elongation factor-2 (EF-2) and consequently, prevent protein synthesis from continuing. By this action cell death is inevitable.

DT resistance may derive from lesions at the cell membrane or at the EF-2 protein structure (Gupta and Simmovitch, 1978). Draper

et al. (1979) have characterized the phenotypes resulting from the alteration of EF-2 gene to be either a recessive or a co-dominant trait. The gene location for DT in Chinese hamster cells has been found to be in chromosome 2 (Roberts and Ruddle, 1980), but in human cells, it is associated with chromosome 5 (Creagen et al., 1975). DT-response is dependent on the species of cell origin; for instance, human cells are more sensitive to DT than mouse cells (Dendy and Harris, 1973). Since cell density does not affect the DT mutant recovery, there is no contact inhibition between DT mutants and the wild-type cells.

Mutation Effects by Photoradiation

Far-UV and unfiltered broad spectra of black or white lights are mutagenic to many kinds of mammalian cells. Inductions of 6-TG, ouabain and DT resistance by far-UV radiation have been reported (Hsie, 1975 a,b; Chang et al., 1978; Van Zeeland and Simmons, 1976; Burki et al., 1980). "Cool" and "daylight" white lamps, black lamps, and sunlamps are capable of inducing 6-TG resistance in mammalian cells (Bradley and Sharkey, 1977; Wang, 1975; Hsie et al., 1978; Jostes et al., 1977; Jacobson and Krell, 1979; Jacobson et al., 1981; Burki and Lam, 1977). The increase in mutation frequency obtained in radiation-treated cells is linear up to a certain dose-fluence level, then seems to remain the same or, in some cases, even decreases.

Riddle and Hsie (1978) have reported that in the most sensitive periods in cell killing of synchronous cells, there is a 6-TG mutation peak. Burki et al., (1980) have shown a slight increase of 6-TG

resistance in this period if the applied fluence is relatively high, i.e., at 17 J/m^2 . At moderate fluences, this age-response sensitivity is not detectable. Induction of ouabain resistance shows a slightly higher frequency in early S-cells (Burki et al., 1980). In response to DT resistance, cells are highly cell age-dependent. There is a distinctive single DT peak for the age of late G1 cells, and a low mutation frequency in the remainder of the cell cycle.

Rationale for the Present Studies

CHO-KK cells have been used in Burki's laboratory for several years and are relatively easy to grow. Procedures for inducing 6-TG and ouabain variants in this cell line were established by Burki and Aebersold (1976). Besides these two loci another was deemed of value for mutagenesis studies. Resistance to emetine was first tried, but preliminary results on this system were discouraging and it was not pursued. Fortunately, resistance to diphtheria toxin in Chinese hamster cells had just been characterized by Siminovitch's group (1978). The success of inducing DT mutants fulfilled the initial plan of using three loci for mutagenesis studies. In each experiment, mutagenized cells were challenged in medium containing DT, 6-TG and ouabain.

Of the various types of fluorescent light tested, only gold light did not induce toxic or mutagenic effects. Since the spectrum of this light is in the visible region, this indicates that longer wavelengths do not induce ill-effects in cells. An attempt was then made to ascertain which wavelengths in the white and black light spectra are

responsible for the killing and mutagenic effects. By using chemical and plastic filters, we demonstrated that wavelengths below 300 nm in the white and black spectra were important with respect to the ill-effects.

In the first attempt to investigate the capability of CHO-KK cells to repair damage caused by unfiltered broad spectrum of black light, we unexpectedly obtained a positive result with an asynchronous population as measured by split-dose recovery. When we repeated the experiment with white light, we also obtained a similar recovery pattern, as we did with far-UV radiation. With a few points in the synchronous experiments performed with black or white light, positive recovery patterns were seen. A more detailed study of synchronous cells was done with far-UV radiation. Summarized data from a few experiments, recovery was found throughout the entire cell cycle.

The present investigation on photoradiation effects on CHO-KK cells can be summarized in the following categories:

1. Fluence response curves for the induction of reproductive death and mutagenesis on asynchronous cells are determined by using far-UV at 254 nm, unfiltered broad spectra of gold, cool white and black lights, and broad spectra of black light with different filters (a) benzene-filtered (with wavelengths shorter than 276 nm filtered); (b) toluene-filtered (with wavelengths shorter than 280 nm filtered); (c) pyridine- or plastic-filtered (with wavelengths shorter than 300 nm filtered); and (d) acetone-filtered (with wavelengths shorter than 325 nm filtered); and by white fluorescent light spectra with

plastic filtered (with wavelengths shorter than 300 nm filtered);
plexiglass-filtered (with wavelengths shorter than 425 nm filtered).

2. Survival and mutagenesis studies of synchronous cells exposed to the above photoradiation.

3. Indirect effects from medium previously irradiated with fluorescent light on asynchronous cells.

4. Recovery of photoradiation damage measured from split-dose experiments of cells irradiated with far-UV, unfiltered white and black light, and plastic-filtered black light.

5. Repair of premutational damages from split photoradiation doses.

MATERIALS AND METHODS

Cell Line

The cell line used in these experiments was Chinese hamster ovary cells, which was obtained in 1976 from L. Kapp and R. Klevecz of the City of Hope Medical Center, Duarte, California. A large number of vials of stock cells, designated as CHO-KK in our laboratory, were frozen in that year in media containing 5 percent dimethyl sulfoxide (DMSO) and stored in liquid nitrogen at -196°C .

Periodically (about every 2-3 months), an ampule of cells was thawed and grown for 14 generations before use in experiments. By so doing, we hoped to minimize genetic and reproductive changes, and to maintain the characteristics of the cell line. These cells were sent to the Bioassay Systems Co. (Woburn, Mass.) to test for PPL0, and have been shown to be free of PPL0.

The CHO-KK cell line has a doubling time of 12 hours and its cell cycle consists of a G1 period of 4 hours, an S period of 6 hours and a G2 plus M period of 2 hours (Burki et al., 1980). It was chosen because its M cells could be shaken off mechanically by an automatic cell cycle analyzer. All synchronous cells in this study were obtained by being mechanically shaken-off of the M cells, except in one experiment where hydroxyurea was used to synchronize cells.

Cell Growth

The cells were grown in monolayers attached to the surface of a container. The cell line and cells from most asynchronous experiments were grown in plastic tissue culture flasks or dishes (Falcon or

Corning) in a humid CO₂ incubator at 37°C. To ensure that they were in logarithmic growth at all times, cells were detached every other day by 0.03 percent trypsin in saline A and seeded at cell numbers of 10⁶ or 2x10⁶ per 75 cm² or 150 cm² container, respectively in McCoy's 5A (Gibco) media, supplemented with 7.5 to 15 percent fetal calf serum (Flow Lab.), 1 percent glutamine, 100 units/ml of penicillin and streptomycin (both from Gibco) and 1mM HEPES buffer (Sigma).

In synchronous experiments, cells were transferred to a glass roller bottle at least 4 days prior to experiments. Cells in a tightly sealed roller bottle were kept on a rotating rack turning at 0.5 RPM in a 37°C walk in warm room (Barr Mfg. Co., Model No. ESLS-2363). The schedule for a complete synchronous experiment can be outlined as follows:

Day 1	Transfer cells to roller bottles.
Day 4	Seed 2 x 10 ⁷ cells/roller bottle.
Day 5	Set up synchronization apparatus (Talandic Research Co.) and start shake-off in the afternoon.
Day 6 AM	Use synchronous cells for experiment.
Day 8 and 11	Subculture cells to 2 x 10 ⁶ cells/roller bottle.
Day 14	Challenge cells with different drugs.
Day 22	Stain 6-TG and Ouabain plates.
Day 25	Stain DT challenged plates.

Synchronization Method and Synchronization Analysis

The cell cycle analyzer developed by Klevecz (Klevecz, 1975) and manufactured by Talandic Research Co. was employed for synchronizing

cells. CHO-KK cells were rounded up during the M phase when they could be shaken off readily. Keeping the machine in a 37°C room, the detached cells were then pumped into 75 cm² flasks where they divided, attached and proceeded through the cell cycle. The machine usually operated at the following setting: 54 min. at 0.5 RPM, 3 min. at 180 RPM, 2 min. for cell collection and 1 min. for media replacement in roller bottles. Under these conditions, the duration between shaken-offs was exactly one hour, which yielded about 2×10^6 G1 cells for each roller bottle with 10^8 cells. Thus, a continuous spectrum of synchronous cells at one hour interval spreading throughout the cycle was automatically collected.

Cell Volume Spectroscopy and FMF (Flow Microfluorimetry)

Cell volume spectroscopy and flow microfluorimetry (FMF) were used to analyze the degree of synchrony immediately after cells were irradiated with UV or fluorescent irradiation. As cells go through their cell cycle, more substances are made and their cell size increases. The cell volume can be used as one of the quickest ways to observe the success of the synchrony experiments. Routinely, Coulter Counter Model ZBI was used to examine the cell volume distribution, which could be output on the X-Y plot by the matched Coulter analyzer. Cell sizes in terms of relative channels were recorded for each synchronous sample. A good synchronous sample consists of cells in very few channels. In most of the synchronous experiments, we found there existed a linear relationship between the cell cycle age and the channel peaks.

The ratio of DNA content in G2 and G1 cells is almost 2. When a fluorescent dye is incorporated in DNA, the DNA content can be analyzed by an instrument recently developed by Steinkamp (Steinkamp et al., 1973). In Dr. Bartholomew's laboratory, this instrument is connected to a computer system so that data of many samples could be printed out in detail. The procedures for preparing cells for FMF, the fixation and staining methods as described by Hawkes and Bartholomew (1977) were rather simple. Cells were suspended in a fixative containing freshly mixed solution of 3 parts of 200 mM $MgCl_2$ and 1 part of alcohol. The cells could be stored in this fixative at refrigerator temperature for weeks. When the FMF instrument could be arranged, cells were collected by gentle centrifugation and stained for one hour with propidium iodine dye.

Fluorescent-Light Irradiation Facilities

The fluorescent-light irradiation apparatus consisted of an open metal box with eleven 48-inch fluorescer*-light fixtures mounted adjacent to each other overhead. Timing was done manually by turning the switches on and off. The adjustable shelves allowed us to vary the distance between the samples to be irradiated and the light source. Two layers of movable black cloth were surrounding the fixture to absorb scattered light rays and two or more small fans were placed at the end of the fluorescent tubes to cool them. The intensity varied less than 5 percent at the center part of the shelf, where twelve 75 cm^2 dishes or flasks could be irradiated at one time.

Fluorescent-Light Irradiation Procedures

In the experiments with asynchronous cells, cells were seeded at $1.5\text{--}2.0 \times 10^6$ cells/75 cm² dish, and allowed 3–3.5 hours for attachment. In the synchronization experiments, cell density was usually about 2×10^6 cells/75 cm² flask but the attachment time varied from one hour to twelve hours, depending on the cell cycle positions of the samples to be irradiated. All cells were washed twice with clear saline A and immersed in 5.0 ml of the same saline with 25 mM HEPES supplemented for irradiation exposures. If cells were irradiated through chemicals or plastic filters, all covers were removed, and in the case of flasks, the tops were removed inside the chemical hood by an electrically heated wire. A radiometer calibrated and manufactured by Yellow Spring Instrument Co. was used to determine the exposure fluences at the beginning and at the end of each experiment.

Immediately after the exposure, cells were trypsinized for survival test and the remaining were transferred to a container with prewarmed medium. In split-dose experiments, prewarmed medium was added between the two conditioning doses and kept in a 37°C incubator except where different treatment was indicated. After the second half of the dose was given, cells were then trypsinized for the survival test. All mutagenized cells were subcultured twice before they were challenged. Durations were 6 days for cells kept in plastic containers and 8 days for cells kept in roller bottles. This was ample time for treated cells to recover from photoradiation damage.

Radiation Temperature and pH Condition for Fluorescent Irradiation

Temperature and pH were controlled during fluorescent-light irradiation. Exposures at ice temperature were done with containers kept on a piece of flat metal that sat on top of a layer of ice. When cells were irradiated at a higher temperature, the containers were submerged in a water bath. The amount of water in the tray and its temperature was experimentally determined so that the heat induced by the fluorescent lights was equilibrated with the heat dissipated to the air. Throughout a six-hour experiment, a relatively constant temperature within 2°C could be achieved.

To slow pH changes, HEPES was added to saline A buffer. Under laboratory conditions, saline with 25 mM HEPES helped to stabilize pH for several hours.

Far UV Irradiation Facilities

The UV apparatus had three germicidal tubes that gave a maximum fluence rate of 2.1 J/m²/sec which was later changed to 1.5 J/m²/sec for samples irradiated on a fixed platform, about 8 cm from the light source. The predominant wavelength from the germicidal lamp was 254 nm. The exposures were controlled by an automatic shutter and timer. A black wooden box prevented exposure of the samples to room light during irradiation. Before and after exposure, cells were kept inside a styrofoam box.

Far-UV Irradiation Procedures

The irradiation procedures of far-UV were similar to those of the fluorescent light, except clear saline A without HEPES was used for

rinsing. During exposure, cells were irradiated without saline (only with a thin film of saline) at room temperature.

Chemical and Plastic Filters

Two types of filters, chemical and plastic, were employed to filter out the shorter wavelengths of the fluorescent-light spectrum. Using a 6-inch square quartz sheet for the bottom and four metal strips for the sides, several boxes were made to hold chemicals. Just before irradiation, 1 cm of benzene, toluene, pyridine or acetone was added and another 6-inch square quartz sheet was used as a top, sealed by electrician's tape. These procedures were done inside a chemical hood. The unit was then gently placed on two narrow metal strips between the light source and the sample as a filter. After the experiment the chemical in the container was discarded. The residual chemical was exhausted in the chemical hood.

The plastic filters were much easier to handle. They were cut to manageable sizes before placement on top of open dishes or flasks. The transmissions recorded for the chemicals and the plastics used in this study are presented later.

Colony-formation Techniques

Plating efficiency was determined by the ability of a single cell to form a colony. The cell number of 10^2 to 10^4 (depending on the fluences applied) were usually plated immediately after they were irradiated with fluorescent-light or far-UV treatments. Under normal growth conditions, surviving cells should grow to visible colonies in eight days. Colonies were then stained for 1 hour by adding 0.5 ml of

1 percent methylene blue to the media. Plates were allowed to air-dry before water rinsing once. Counting was done with the help of a 10X dissecting microscope.

Selection

Drug resistant mutants were also selected by the colony formation method. The cell numbers inoculated to determine mutation frequencies, however, were much higher than the numbers used for plating efficiency, i.e., 10^5 cells for 6-TG resistant selection, 10^6 cells for both ouabain and diphtheria toxin.

A minimum of five days was required for selecting 6-TG mutants, three days for ouabain and two days for DT mutants. For convenience, an expression time of six or eight days was chosen for all three loci and during this period, mutation induction of these loci was definitely at the maximum level. After cells were exposed to fluorescent light or UV, they were plated in 150 cm^2 plastic plates or flasks. During the subsequent subculturing, synchronous cells were seeded in large roller bottles, whereas asynchronous cells were still plated on 150-cm^2 plastic plates. In both cases, at least 2×10^6 cells were seeded. To maintain cells in exponential growth, cells in 150-cm^2 dishes had to be challenged on the second day but cells in the roller bottle did not have to be challenged until on the fourth day.

Selection Drugs

6-TG: A stock solution of 6-TG at a concentration of 60 mg/ml was dissolved in 1.0N NaOH. After filtering through a 0.45 Nalge filter, a fraction of the solution was further diluted to 6 mg/ml in sterilized

double distilled water. Aliquots of drug solution in both concentrations were stored in glass tubes, each containing only 1.0 ml. Freshly prepared 6-TG in media at a final concentration of 5 mg/ml was used to select 6-TG resistant mutants.

Ouabain: A stock solution of ouabain at a concentration of 90 mM in distilled water was prepared as follows. The stock solution was brought to a boiling point at about 100°C until ouabain crystals were completely dissolved. After it had been reduced to warm temperature (about 40°C) it was sterilized by Nalge filtration. Fractions of ouabain stock solution were stored in the refrigerator and at 0°C ouabain solubility were lower than at 100°C. Every time when ouabain stock was needed, it must be brought to the boiling point for dissolving ouabain crystals. Freshly prepared ouabain at a final concentration of 3.0 mM was used to select ouabain resistant mutants.

DT: When DT toxin was received, it was immediately diluted to 1000 LF/ml. Further dilutions of 100 LF/ml and 10 LF/ml were made in saline A buffer. 0.1 LF/ml or 1.0 LF/ml were the concentrations used to select DT resistant mutants.

RESULTS

Fluence-Rates of Fluorescent Lights

Fluence rates of fluorescent-light spectra were measured by a Yellow Spring radiometer. Measurements of black, white and gold light are shown in Fig. 1A, B and C, respectively. In the white or gold light, the fluence-rates of unfiltered broad spectra showed no detectable difference as the rate of the corresponding spectra with wavelengths shorter than 300 nm filtered (filtered by culture-dish plastic). Since cells irradiated with fluorescent lights were kept in saline or medium during irradiation, fluence-rates under these conditions were determined. Clear saline up to 0.4 mm thick (or 30 ml) did not change the rates in these two lights. Although medium reduced fluence-rates of white-light, it reduced not more than 5 percent for medium less than 0.13 mm thick (or 10 ml). The reduction of fluence-rates by medium in gold light was very little, less than 5 percent for medium of 0.53 mm thick (40 ml). The fluence rates of the black light spectra with wavelengths shorter than 300 nm filtered (filtered by culture-dish plastic) was about 80 percent as that measured with the unfiltered broad spectra. Both saline and medium reduced the black-light fluence-rates, with a bigger reduction with the latter. However, a 0.066 mm thick (5.0 ml) of clear saline or medium changed very little of the fluence-rate. Throughout this study, we assume that 5.0 ml saline or medium (the amount used during irradiation) did not reduce the fluence-rates of filtered or unfiltered broad spectra of all fluorescent lights.

Indirect Effect

Indirect effects were studied either by irradiating cells in the presence of medium or by incubating cells with the photoactivated medium for a short period of time (10-30 minutes). In the first case the reproductive death, as measured by the colony formation technique, was compared with cells irradiated in the presence of 0.066 mm thick layer of saline (or 5.0 ml) or a thin layer of saline (with saline aspirated off after rinsing). If indirect effect occurred, the survival would be lower for cells that irradiated in the presence of medium than for those irradiated in the presence of saline. Two sets of triplicates were prepared for each of the two fluences employed. Plating efficiency of one set of the triplicates was performed immediately after radiation exposure and of the other set was done 6 hours post-irradiation. In the latter set samples were kept in a solution similar to the regular medium during the waiting period. To achieve that, cells in a thin layer of saline during irradiation were replenished with 5.0 ml of fresh medium. Cells in medium were kept in the same medium and cells in 5.0 ml saline were added with an appropriate amount of 10X strength medium and fetal calf serum. All three plates were kept in a 37°C incubator.

When cells in saline (either in a thin layer of saline or 5.0 ml saline) were irradiated with fluorescent light, the survival levels were similar whether cells were plated immediately or 6 hours post-irradiation (Figs. 2A, 2B, 3A and 3B). Cells irradiated in the presence of medium were different. Cells plated 6 hours postirradiation had a much lower survival than those plated immediately. The

enhancement of cell killing indicated that cells irradiated with medium were damaged after they were incubated in this medium for 6 hours. Cells in saline or medium exposed to a high fluence of gold light showed a survival level similar to the control sample, whether cells were plated immediately or 6 hours after the exposure. With the two different fluences in the far-UV radiation tested, the survival levels were similar for cells in 5.0 ml saline or in a thin layer of saline during exposure whereas the survival was as high as the control for cells in the 5.0 ml regular medium. To observe far-UV induced damages, thorough rinsing with clear saline is necessary.

Photoirradiated media have a killing effect on wild-type cells. The severity of this effect is determined by the type of radiation, components in the medium (or solution), fluence, incubation time and cell density. When cells (300 or 10^5 cells per 75-cm^2 plate) were incubated for various lengths of time up to 12 hours with medium (McCoy's 5A) previously exposed to an extremely high far-UV fluence of $7.6 \times 10^3 \text{ J/m}^2$ (three orders of magnitude higher than the fluence applied for direct exposure), survival was similar to the control. But after 24 hours, a small percent of cells lost their reproductive ability (two experiments showed the survival level reduced by 9 percent and one by 15 percent). When the fluence was reduced to half ($3.9 \times 10^3 \text{ J/m}^2$), even a 24 hours incubation with this medium did not affect the survival.

Cells were sensitive to medium that had been irradiated with a fluence higher than $2 \times 10^4 \text{ J/m}^2$ of black light and $4 \times 10^4 \text{ J/m}^2$

of white light (Fig. 4). The toxic effect was slightly enhanced if wavelengths below 300 nm in the light spectra were included for irradiating the medium. A highly irradiated special medium containing no riboflavin, tryptophan and tyrosin was not toxic to CHO cells. This suggested that these three chemicals were responsible for the formation of toxic photoproducts that kill mammalian cells. To determine which one was most likely to produce toxic photoproducts, aqueous solutions of 0.20 ug/ml of riboflavin, 3.10 ug/ml of tryptophan or 26.2 ug/ml of tyrosin (concentrations identical to those found in McCoy's 5A medium), were irradiated by a black-light fluence of 9.0×10^4 J/m² individually, in a combination of two or in a combination of all three. Solutions containing 20 times the concentration of the two amino acids and 10 times the concentration of the riboflavin were also exposed to the same fluence and in the same combinations. Following irradiation, an appropriate amount of 10X strength medium, fetal calf serum and HEPES were added to make the solution similar to regular McCoy's 5A medium. Test of cell survival after cells had been incubated with this medium indicated that harmful photoproducts were produced (Table 1), with their highest level being produced when all three chemicals were in the solution. When only two chemicals were present, the toxic photoproducts were less efficiently produced. Riboflavin plus either amino acid produced more phototoxins. When the chemicals were irradiated separately and then combined, there was no enhancement in cell killing (Table 1).

At an extremely highly irradiated medium fifty percent of the cell population lost reproductive capability with an incubation of 10 minutes and ninety-five percent with an incubation of 30 minutes or longer (Fig. 5). When 1 unit/ml of catalase, which degrades peroxides, was added to the highly irradiated medium the effectiveness for cell killing was reduced to slightly less than 20 percent. This suggests that peroxide-type photoproducts were responsible for causing 80 percent of the cell death in CHO cells, when irradiated in this manner.

Effect of Cell Density

Cell densities of 0.3, 2, 4, 6 and $30 \times 10^4/\text{cm}^2$ were used to examine their effect in relation to cell irradiation (Fig. 6). Survival was done immediately after radiation exposure. These cell densities had no influence on cell survival when far-UV radiation was used. In gold-light radiation, survivals of cells in different densities were as high as the controls. Cell densities of up to $6 \times 10^3/\text{cm}^2$ resulted in similar survival levels for black or white light, but the highest cell density of $3 \times 10^5/\text{cm}^2$ had a higher survival, about 30 percent for the white and 45 percent for the black light. Cell densities of 0.3, 2, 4, 6 and $20 \times 10^4/\text{cm}^2$ were used to examine their effect on cell killing by photoactivated medium. The highest cell density of $2 \times 10^5/\text{cm}^2$ greatly reduced the killing effect of the highly irradiated medium to cells and other lower cell densities had no effect on cell survivals. Throughout this study, cell density of less than $2 \times 10^4/\text{cm}^2$ was chosen to seed cells for all radiation experiments.

Temperature Effect

The effect of temperature during fluorescent-light exposure was examined by keeping cells on top of an ice-bath ($0^{\circ}\text{--}4^{\circ}\text{C}$) or in a $26\text{--}28^{\circ}\text{C}$ water bath. When using the white-light spectra with wavelengths below 300 nm filtered (with plastic filtered), cells irradiated at $26\text{--}28^{\circ}\text{C}$ water bath had a higher survival than those irradiated at an ice-bath temperature. A higher survival level at a higher temperature was not observed in cells that were irradiated with the unfiltered broad spectra of white light or the black light spectra with or without a plastic filter (Fig. 7 and 8A).

Transmission of Different Filters

One-cm thick of benzene, toluene, pyridine or acetone as well as UVT plexiglass, UF-3 and the culture-dish plastic were used to study the effect of shorter wavelengths in fluorescent light spectra. The percentage transmission through a range of wavelengths of these filters was measured by a spectrophotometer (Superscan 3) and the data are plotted in Fig. 9. One-cm of benzene starts to transmit at 276 nm and reaches the highest transmission at 281 nm. Since it takes only 5 nm to reach its maximum transmission level from the starting transmission point, it is one of the best filters in this study. One-cm thick toluene starts to transmit light at 280 nm and reaches its peak value at 300 nm, with 50 percent transmission at 296 nm. One-cm thick pyridine blocks all wavelengths below 300 nm and transmits wavelengths beyond 318 nm, with 50 percent transmission at 315 nm. One-cm thick acetone shows the beginning and the maximum transmission at 325 nm and

332 nm, respectively. Among the plastic materials, UF-3 offers a better choice as a filter than the others tested. Its transmission starts at 425 nm and reaches a maximum at 435 nm, with 50 percent transmission at 430 nm. Plastic material from the culture dish is a poor filter because it takes 60 nm to reach to the maximum point from the starting transmission point of 300 nm. UVT plexiglass blocks all wavelengths below 250 nm and allows 60 percent transmission of wavelength at 280 nm or longer. Its 50 percent transmission is at 276 nm. Since the cut-off point of filters in this study cannot be expressed by a single wavelength, it is referred to as its beginning transmission wavelength.

Fluence Response of Cells to Photoradiations

A. Asynchronous Cells

Survivals of asynchronous cells after exposure to far-UV, gold, white or black light are shown in Figs. 6, 7, 8A, 8B and 10. These data were normalized to control plating efficiencies which were determined independently for each experiment. When the linear portion of the survival curves was obtainable, the least square equation was used for the drawing of the presented curves. Among the photoradiation tested, far-UV was the most efficient radiation to induce proliferative death in cells, followed by the unfiltered broad spectra of black light and white light. The gold light, with its wavelengths in the visible region, did not induce any cell killing.

To test the effect of shorter wavelengths in the black-light radiation, chemical and plastic filters were employed. One drawback of using chemical filters was that the toxic fumes quickly vaporized

into the air and refilling to the marked line in every 30 minutes was necessary. Because of the toxic chemical fumes in the room, each experiment was limited to about two hours and consequently, only partial survival curves were obtained in some cases. However, these partial survival curves serve the purpose to demonstrate that the proliferative death was predominately induced by wavelengths shorter than 300 nm in the fluorescent-light spectra.

Since a long exposure time was needed to observe the effect induced by the white light, chemical filters were not used. Data obtained from filters of culture-dish plastic or UF-3 plexiglas showed that the killing effect of this light spectra was also mainly due to the shorter wavelengths (Fig. 7 and 9). When the white-light spectra with wavelengths shorter than 440 nm filtered (UF-3 filtered), there was no killing effect on CHO cells at a magnitude several times higher than the highest fluence used in the unfiltered broad spectra.

B. Synchronous Cells

For each synchronous experiment, two sets of synchronous cells were routinely obtained, one for conducting experiments and the other for monitoring the degree of synchrony. Flow microfluorometry (FMF) and the Coulter Counter channel analyzer were methods used to test the degree of synchrony. The graphical representations of the peak channels of the FMF histogram and the size distribution of the Coulter Counter analyzer are shown in Figs. 11 and 12, respectively. From these data, the cycle time can be determined as follows: G1 - 4 hours, S period - 6 hours and G2 plus M - 2 hours (Burki et al., 1981). A

slight variation in the cycle time was frequently found, not only in different experiments but also in the two sets of synchronous cells of the same experiment.

The survival curves of synchronous cells were determined at one hour intervals throughout the cell cycle. The most sensitive period for far-UV radiation occurred approximately at the end of G1 and the beginning of S period. A greater fluence of 25 J/m^2 produced a higher survival difference between the most sensitive G1/early S cells and the most resistant late S/G2 cells (Fig. 13). A lower fluence of 9.0 J/m^2 gave a smaller difference.

A similar cyclic response pattern of survival curves was repeated for cells exposed to fluorescent-light spectra with or without wavelengths shorter than 300 nm (Figs. 14A and 14B). The maximum sensitivity in cell killing was found to be at a cycle age similar to that observed in cells irradiated with the far-UV radiation (at about 4 hours after the mitotic shake off). The trough in the vicinity of the sensitivity point became shallower if shorter wavelengths were blocked. When wavelengths were in the visible region, the plating efficiency throughout the entire cell cycle was as high as that of the control.

Recovery in Split-dose Experiments:

A. Asynchronous Cells

When cells were irradiated with two equally divided doses and allowed to have a recovery period between doses, survival was found to be higher than for cells that were given the same total dose all at

once. Survival curves of split-dose samples after irradiation by far-UV and unfiltered broad spectra of white or black light were plotted as a function of the duration between the two split doses (Fig. 15). The data indicate that cell survival increases linearly up to 10 hours and flattens for intervals longer than 10 hours. Experiments were carried out to test if deviations in the procedures of performing split-dose experiments were responsible for the increase of cell survival.

Cells in the split-dose experiments were allowed to grow for a period of time before the second half dose was delivered. To determine whether the growth time had any effect on cells responding to photoradiation, cells were seeded 3, 12 or 24 hours prior to the delivery of photoradiations. Results plotted in Fig. 16a are based on one experiment for each type of radiation. Plating efficiencies did not change for cells that had a growth time up to 24 hours before they were exposed to fluorescent light. For cells irradiated with far-UV, survivals had no detectable difference for growth periods between 3 and 12 hours, however, it was lower for cells that had a growth period of 24 hours. A repeat of the far-UV delayed irradiation confirmed that the survival was lower for cells that were allowed to grow for a longer growth period of 24 hours (Fig. 16A). Split-dose recovery (Fig. 15) reaches a maximum level within a recovery period of 10 hours and cells grown for this duration before giving radiation exposure had no effect on cell survival. In the case of a long recovery period (i.e., 24 hours or longer), the result of a lower survival induced by

the delayed UV irradiation would only underestimate the split-dose recovery. Therefore, the existence of a split-dose recovery period does not contribute to the increase of survivals in split-dose samples.

CHO-KK cells firmly adhere to substrates during their growth and dissociation of cells depends on trypsinization which involves medium removal and saline rinsing. Non-viable cells released into medium would be discarded. If the existence of a recovery period in the split-dose samples encouraged dead cells to release to the medium, an increase of survival would be expected. In the following we examined the number of photoirradiated cells released to the medium as a function of growth period after photoirradiation.

Three fluences of far-UV or black light that gave survival levels of about 70, 40 and 5 percent in the attached cell population were used. The percentage of floating cells was expressed by the number of floating cells in respect to the total cell number per plate. Since the percentage of floating cells from both radiations differed very little (less than 1 percent), it was reported without specified the radiation used. After eight hours postirradiation, the percentage of floating cells was about 0.001-0.05. This low level of floating cells was probably due to the rinsing steps involved in prior to irradiation. An increase to 1 percent of floating cells was found at 12 hours postirradiation and remained at the same level for 24 hours for cells that were given moderate fluences (40 percent or higher survival levels). If cells were given a high fluence (about 5 percent survival level), floating cells increased to 4 percent level after 24 hours incubation.

In parallel to counting floating cell numbers after irradiation, the colony formation potential of floating cells was determined at 8, 12 and 24 hours after far-UV and unfiltered broad spectra of black light radiation. Results were compared with the survival levels obtained from the floating cells in non-irradiated controls. The sham-control plating efficiency was 79 ± 9 percent from 8 to 24 hours. The survival of floating cell at 8, 12 and 24 hours postirradiation in samples that had 40 percent survival level of the attached cells was 55, 52 and 43 percent, respectively, for far-UV samples and 59, 61 and 42 percent for the black-light samples. Floating cells from the cultures that had 10 percent survival level of the attached cells consistently had the survival levels lower than 1 percent for the far-UV light and lower than 4 percent for the black-light samples. Cells in split-dose samples were given a moderate dose as the first conditioning fluence and then they were incubated for various times during the recovery period. The survival level of floating cells under these conditions was either slightly higher or similar to the attached cells. Therefore, floating cells did not contribute to the increase of survival in the split-dose samples. All of the control experiments tested here indicated that different procedures in split-dose experiments did not contribute to the split-dose recovery.

In the subsequent experiments, fluence curves of cell survival were obtained from cells that were given two evenly divided fluences of far-UV or fluorescent-light radiation and separated by a 6-hour recovery period. Survivals of acute-dose samples were plotted in the

same diagram for comparison (Fig. 17A, B and C). Two distinctive characteristics were observed. First, of the fluences tested, all of the split-dose samples have higher survivals than the acute-dose counterparts. Second, when cells were kept at room temperature during the recovery period, the survival recovery diminished. At room temperature, cell progression is greatly reduced (Jostes and Painter, 1981). It seems that photoradiation sublethal repair processes are coupled with some cell progression processes. In this study no attempt was made to identify these processes.

In multi-split dose experiments, fluences were given to cells in 4-6 aliquots in a manner such that the recovery period between the first and the last fluence was always 8 hours. Earlier results obtained in synchronous experiments showed that when the far-UV fluence was low, the sensitivity difference throughout the cell cycle was less (Fig. 13). In giving cells a smaller fluence each time in the multi-split dose experiments, we hope to minimize the cell age sensitivity response to photoradiation. In so doing, we are minimizing the cell age sensitivity response to take part in the split-dose recovery. A high far-UV fluence of 18 J/m^2 and 32 J/m^2 or a high fluence of unfiltered black light spectra of $6.75 \times 10^4 \text{ J/m}^2$ was employed (Table 2). The survival level of single-dose samples was less than 1 percent whereas the multi-dose samples were about 0.30 for the far-UV and 0.40 for the unfiltered black light. When one of the fractionated doses was in the exponential region of the single-dose survival curve, the overall survival level of the multi-dose samples immediately

dropped. Photoradiation damages can be readily repaired by CHO cells if they are not too severe.

B. Synchronous Cells

Recovery of split-dose samples can be best studied by using synchronous cells because the position in the cell cycle of the irradiated cells during radiation exposure can be determined. In the first set of synchronous experiments, late S/G2 cells (6.5, 7.5, 8.5, 9.5 and 10.5 or 7, 8, 9, 10 and 11 hours after mitotic shake-off) were exposed to the first conditioning dose of 9.0 J/m^2 of far-UV or $1.5 \times 10^4 \text{ J/m}^2$ of black-light radiation and 5 hours later the final half of the split-dose was given. Figure 19 demonstrates the average survival levels of four synchronous experiments for the former and only two for the latter. All of the survivals of the split-dose samples were higher than the acute-dose counterparts whether they were irradiated with far-UV or with black light.

In the next set of synchronous experiments, late G1/early S samples (2,3,4,5, and 6 hours after mitotic shake-off) were applied with a first conditioning dose of 6.0 J/m^2 or 9.0 J/m^2 of far-UV radiation. Six hours later the second half of the dose was applied. Figures 19 (A and B) and 20 (A and B) show that the split-dose samples have higher survival levels than the single-dose counterparts except with some fluctuation in the G1 cells (Fig. 20B). Two out of seven synchronous experiments showed no increase in the split-dose recovery for samples that received the first conditioning dose at 3 hours after the mitotic shake-off. The positive recovery of this sample in the other five experiments was as high as those found in the early-S-cell samples.

Split-dose samples with a recovery period of 24 hours in the synchronous cell population showed a higher recovery value than those with shorter recovery periods (Fig. 20B). One distinctive characteristic of split-dose samples was that the sensitive response of the late-G1/early S boundary cells and early S cells decreased. At a recovery period of 24 hours, survivals of split-dose samples flattened throughout the entire cell cycle.

By using a different synchronization method and a different cell line (M3-1), Todd et al. (1969) reported no immediate recovery after far-UV radiation. Following their synchronization method and protocols, we did an experiment with CHO-KK cells (Fig. 21). In this experiment, cells were grown in the presence of 1 mM hydroxyurea for 8 hours to achieve partial synchronization. The first conditioning dose was given 6 hours after cells were released from the drug, and the final half dose was followed at various intervals. A higher survival level of split-dose samples were observed for samples that had a recovery period of 6 hours or longer. However, the magnitude of the recovery was consistently lower than those reported earlier.

Three samples of different cell positions in late S and G2 cells (6, 9 and 11 hours prior mitotic shake-off) were irradiated with unfiltered broad spectra of white or black light. Five hours after the first conditioning dose, the final half dose was given. The survival of split-dose samples were 20-40 percent better than the acute-dose samples. In these samples, all of the fluence were given to the most resistant cell population of late S and G2 cells in acute-dose samples and only half of the fluence was given to these resistant cells in the

split-dose samples. The occurrence of cell progression after the first conditioning dose in the split-dose samples could only underestimate the split-dose recovery. Our preliminary results indicate that there was a recovery of synchronous cells at different positions in the later part of the cell cycle after irradiating with unfiltered fluorescent lights.

Study of Mutation Induction

Drug Toxicity Test

Appropriate concentrations of 6-TG, ouabain and DT for mutant selection were determined by observing the cytotoxic responses of cells to various concentrations of these drugs (Figs. 22A, B and C). Although medium containing 0.75 ug/ml 6-TG, 1 mM ouabain and 0.01 LF/ml DT was highly toxic to cells, a selection medium containing 5.0 ug/ml 6-TG, 3 mM ouabain or 0.1 LF/ml DT was used in our laboratory to ensure the selection of true drug or toxin-resistant mutants. In later studies, 1.0 LF/ml DT had to be used when cells, even the control were found to show many resistant colonies in 0.1 LF/ml DT selection medium. This insensitivity of cells toward DT could be caused by the loss of DT toxic activity or by a resistance change of the cell line. If the latter was the cause, a newly thawed cell would show less DT spontaneous mutants. When newly thawed cells were tested, they also exhibited many spontaneous mutants in 0.1 LF/ml selection medium. Therefore, it seemed that the loss of toxic activity in the DT drug itself after three years in storage might be the cause of this problem. In a newly acquired DT preparation, 1.0 LF/ml concentration had to be used. Thus, different batches of DT had various strengths of activity

towards a particular cell line and therefore, a suitable concentration for challenging cells had to be experimentally determined for each batch. This kind of variation among different batches of 6-TG or ouabain was not encountered.

Stability of Drugs and Toxin under Growth Conditions

A bottle of freshly prepared selection medium, containing 5.0 ug/ml of 6-TG, 3 mM ouabain or 0.1 LF/ml of DT was kept inside an incubator to test for their stability. Every two to three days, an aliquot of selection medium was taken out to challenge UV-mutagenized cells which had been irradiated 8 days prior to the challenge. Resistant colonies scored in the selection medium between freshly prepared one and the one kept in the incubator were compared (Table 3). Mutation induction of three loci for up to 27 days varied not more than 15 percent. Drugs and toxin in regular medium were stable under the incubation conditions during the selection period of the mutation experiments.

Metabolic Cooperation

A reconstruction method was used as one of the two methods to examine metabolic cooperation. This method involves the selection of a known number of mutant cells in the presence of a known number of wild-type cells. In this study, 200 mutants were allowed to grow in corresponding selection medium in the presence of 0.5, 1.0, 2.0, 5.0, 10 and 50×10^5 wild type cells in 75-cm² culture dish. Recovery of mutants that was expressed as the fraction of the plating efficiency of mutant cells grown in the selection medium without the presence of wild-type cells, was plotted as a function of the number of wild-type

cells (Fig. 23A). Recovery of 6-TG resistant cells was greatly influenced by the presence of wild-type cell at a cell number higher than 2.0×10^5 per 75-cm^2 dish whereas ouabain and DT resistant cells were not affected by the number of wild-type cells.

The other method to examine metabolic cooperation was to recover mutant cells by seeding various densities (5.0×10^4 to 5.0×10^6 cells per plate) of mutagenized cells. Mutation frequencies per viable cell were computed as a function of the number of cells plated (Fig. 23B). The highest 6TG mutation frequency was found when fewer than 1.12×10^5 cells were plated in a 75-cm^2 plastic plate. Mutation frequencies of ouabain and DT resistance showed no detectable change as a function of cell densities. Results from this and the previous reconstruction experiment indicated that metabolic cooperation occurred in 6-TG locus but not in ouabain and DT loci.

Recovery of mutant Cells after Growth in the Presence of Wild-type Cells

Two hundred mutant cells were mixed with 2×10^6 wild-type cells and allowed to grow in a 75-cm^2 petri dish in normal medium. Every other day after cells were trypsinized, 2×10^6 cells were set for subculturing and simultaneously an aliquot of cells was used to challenge, 10^5 cells for 6-TG and 10^6 cells for ouabain and DT mutation frequency tests. Mutant cells grown in the absence of wild-type cells were used as a control, which showed no detectable change in plating efficiency over a period of 32 days. This was not so for mutant cells grown in a mixed population of wild-type cells. Recovery

rates for all three loci gradually declined, with the fastest loss rate in DT mutants (Fig. 24). Mutagenized cells should thus be challenged in the shortest possible period. On the 8th day, the longest duration used for challenging mutagenized cells in this study, recovery rates for all three loci were better than 70 percent.

Expression Time

Far-UV- or fluorescent-light-mutagenized cells usually require a period of time to express 6-TG, ouabain or DT resistance. The period is probably needed for mutated enzymes to synthesize and/or for the already existing wild-type enzymes in cells to dilute out. When mutated enzymes have accumulated to control the enzymatic functioning, cells then can be selected as mutants. The expression time for 6-TG, ouabain and DT resistance was not the same (Fig. 25). Ouabain and DT resistance clones began to appear on day two after photoradiation treatment but the earliest 6-TG resistant clones appeared on day 3. It took 3 days for DT and 4 days for ouabain to reach to the maximal number of resistant clones and they remained at that level for about 20 days before tapering off. The frequency of 6-TG resistant clones increased dramatically from day 3 to day 4. By day 5, it reached the maximal level and remained at this level for about 26 days, after which it declined. Expression time observed from fluorescent-light irradiated cells agreed well with those observed from cells exposed to far-UV. Either six or eight days was chosen as the expression time throughout this study.

Mutation Induction by Irradiated Media in Wild-Type Cells

Media irradiated by fluorescent light was toxic, but not mutagenic to CHO-KK cells. Similar observation was found in media containing 1 ug/ml pure hydrogen peroxide (H_2O_2). It supported the suggestion that peroxide-type of phototoxic products was the major phototoxins produced by fluorescent lights.

Mutation Induction by Irradiating Cells Directly

A. Asynchronous Cells

Far-UV and fluorescent-light radiations induce 6-TG, ouabain and DT resistant mutants. The mutagenicity of far-UV is 3 to 4 orders more powerful than the unfiltered broad spectra of the white or black light (Fig. 26, 27 and 29). In the following, far-UV rays in the unfiltered broad spectra of fluorescent lights were shown to play an important role in inducing mutation resistance.

The influence of the shorter wavelengths in the black-light spectra was performed by chemical filters to exclude wavelengths below 276, 280, 290 and 300 nm and by using culture-dish plastic to exclude wavelengths below 300 nm. Only plastic filters were used to remove shorter wavelengths in the white-light spectra. When wavelengths below 300 nm in these two fluorescent-light spectra were removed, 6-TG and DT mutation induction rates decreased dramatically to less than 10 percent of the unfiltered-spectra values. The induction rate of ouabain mutation was decreased to about 50 percent level. If wavelengths shorter than 325 nm were removed (by acetone), ouabain mutation rate was reduced to less than 8 percent of the unfiltered value. This

clearly showed that majority of mutation was induced by shorter wavelengths in the unfiltered fluorescent lights.

When asynchronous cells were given two evenly divided doses and separated by a recovery period of different lengths, cells with a longer recovery period up to about 10 hours, showed a higher mutation rate (Figs. 30 and 31). In individual experiments, a fluctuation of 6-TG mutation induction rate was found and there was no distinctive pattern to this behaviour. Among the 13 asynchronous experiments performed with far-UV and unfiltered black-light radiation, only one experiment showed a good correlation between the increase of 6-TG mutation rates and the length of the recovery period. Ouabain mutation rates fluctuated in an individual split-dose experiment as well. However, the average of several experiments indicated that an increase of 6-TG and ouabain mutation rate was in proportion to the length of the recovery period up to 10 hours. The number of DT resistant clones was always in a good linear relationship between the mutation rate and the length of the recovery period, coincidentally, also for up to 10 hours after which mutation rate remained at the same level.

Fig. 32A and B are the fluence-mutation curves which were obtained by cells irradiated with two evenly divided doses of far-UV or unfiltered black light with a 6-hour recovery period. An increase of mutation induction was found in split-dose samples compared to their single-dose counter parts (Figs. 32A and 32B). Since 6-TG and ouabain mutation frequencies fluctuated in different experiments, there were points at which some of the curves showed close values between the

single- and split-dose samples. The DT mutant type was the only one that showed a consistently increasing pattern as compared with the single-dose values.

Survival and mutation were determined on split-dose samples with a long recovery period of 48 hours after treatment with the first conditioning dose of far-UV, black and white lights (unfiltered and plastic-filtered) (Table 4). The survivals increased dramatically. This is to be expected because some progenies in the population were not irradiated by the first conditioning dose. But the interesting aspect is that the mutations of three loci are higher than their single-dose counterparts, some by a factor of 2 or 3. This probably implies that split-dose recovery actually occurs and the repair mechanism is likely to be an error-prone repair one. These and previous data indicate that error-prone repair systems are induced in cells by the first conditioning dose of photoirradiation. When the subsequent fluences are applied to these cells, the induced error-prone repair systems are ready to repair photoirradiation damages.

B. Synchronous Cells

Induction of 6-TG, ouabain and DT mutation through the cell cycle with a 1-hour interval with 9.0 J/m^2 and 18 J/m^2 of far-UV is shown in Fig. 33. At 9.0 J/m^2 , cells produced a similar level of 6-TG and ouabain frequencies throughout the cell cycle. At 18 J/m^2 , late G1 and early S cells responded with slightly higher 6-TG mutation frequencies and early S cells yielded higher ouabain mutation frequencies. DT mutation induction was highly age-dependent at these

two doses. It had a predominant peak in G1 cells, approximately 3-4 hours after the mitotic shake-off. Cells irradiated in other parts of the cell cycle had relatively low DT mutation frequencies.

Induction of mutation in different positions of the cell cycle by black light with various shorter wavelengths filtered (plastic or chemical-filtered) and by unfiltered spectra is shown in Fig. 34. When cells were treated with unfiltered black light or with black light spectra with 276 nm excluded (1.0-cm benzene-filtered), early S cells had a slightly higher 6-TG or ouabain resistant frequency than the remaining parts of the cell cycle. A black-light spectrum excluding wavelengths shorter than 280 nm (toluene filtered), induced comparable levels of 6-TG and ouabain mutation resistant frequencies throughout the cell cycle. When cells were irradiated by black light (filtered or unfiltered), late G1 cells always had a predominant DT peak and cells in other parts of the cell cycle showed DT resistant frequencies similar to the spontaneous level.

6-TG, ouabain and DT mutation rates induced by unfiltered and plastic-filtered white light are presented in Fig. 35. The ouabain mutation frequencies oscillates throughout the cell cycle after synchronous cells were irradiated with unfiltered white light. The DT peak, similar to other photoradiations, occurs at 3-4 hours after the mitotic shake-off. Other parts of the cycle had very low DT mutation levels. In the plastic-filtered data, 6-TG and ouabain mutation induction was relatively flat throughout the cell cycle. Although the DT peak in cells irradiated with this spectrum was small, its position still centered between 3-4 hours after mitotic shake-off.

When a total fluence of 18 J/m^2 of far-UV was given to synchronous cells, 6-TG, ouabain and DT mutation induction throughout the entire cycle were higher in split-dose samples than in acute-dose samples (Fig. 36). The difference of 6-TG between these two samples was larger at late G1 early S cells and again at late S/early G2 boundary cells. The mutation frequencies of ouabain and DT between the split-dose and acute-dose behaved similarly (a large difference spreading from G1 to late S Cells). DT mutation induction was particularly interesting because it had a specific DT peak in G1 region for acute-dose samples in the synchronous population. By allowing sublethal repair after far-UV irradiation, cells other than G1 cells could produce DT resistancy.

Fidelity of 6-TG, Ouabain and DT Mutants

Fifty mutants of each mutation type were randomly chosen from differently challenged experiments involving various photoradiations. They were allowed to grow in regular medium for 30-35 days before being rechallenged with selection medium containing drugs at concentrations equal to or greater than that used for a routine challenge. Survivals were plotted against the concentration of the challenge drugs or toxin in Fig. 22A, B and C. The percentage of the total number of mutants following a certain survival curve is indicated along that curve in the figure. All mutants had a high survival at a concentration similar to the selection medium routinely used for selecting them.

DISCUSSION

Survival of Asynchronous Cells

The survival data obtained by the colony-formation technique indicate that the killing of CHO-KK cells by photoradiation is a function of wavelength and fluence. When a spectrum of white-light excluding wavelengths below 300 nm is used to irradiate cells, temperature becomes another factor that can affect cell survival. The survival is higher for cells that are kept at 26-28°C warm-water bath than at 0-4°C ice-bath temperature. The temperature effect on cell survival is not found in the black light with or without filters or with unfiltered broad spectrum of the white light.

Among radiations used in this study, far-UV has a predominate wavelength (254 nm) but fluorescent lights consist of a spectrum of wavelengths. Black and white lights contain an extremely low level of far-UV irradiance, a magnitude of 3 to 4 orders less than the wavelengths in the visible region (Appendix 3, G.E. Catalog, 1967; Jacobson et al., 1978). In many studies, a difference of this magnitude can be ignored. However, because the efficiencies for cell toxicity and mutagenicity by far-UV radiation is higher than fluorescent lights by about this magnitude, the low levels of far-UV in fluorescent lights cannot be ignored.

The fluence-survival response of asynchronous CHO-KK cells by far-UV radiation and by various filtered or unfiltered broad spectra of black, white or gold light are replotted in a log-log plot (Fig. 37) to provide a better representation of the fluence survival effect of

these photoradiations. A spectrum exclusively in the visible region, from the unfiltered gold light or UF3-filtered white light, at a dose up to $9.0 \times 10^5 \text{ J/m}^2$ produced no detectable effect on cell viability (one-fifth of this dose in unfiltered white light would result in less than 0.05 survival). When far-UV and/or near-UV rays are included in the white or black light spectra, the survival curves resemble the far-UV radiation at 254 nm, with a distinctive shoulder and an exponential region. If shorter wavelengths in these lamps are excluded, the low-fluence shoulder increases and the exponential portion, if obtainable, becomes less steep. The shape of survival curves, especially in the shoulder region, can offer a means to gauge the tolerance of cells toward a particular photoradiation. The broader shoulder means more damages must be accumulated before lethal effects occur. For more precise comparison, the sensitivity value, inactivation constant (k), and intercept value (n), if obtainable from the present survival curves are given in Table 5. As expected, when the broad spectrum of white light excluding wavelengths shorter than 425 nm, a high fluence of this spectrum is no longer toxic to cells. Jacobson et al. (1978) have reported that white-light at wavelengths above 388 nm is not toxic to cells. Our current results and those of Jacobson et al. indicate that mammalian cells, similar to bacteria are highly resistant to long-wavelength insults.

In comparing the toxic effects from various white-light spectra to those of black-light spectra the following conclusions can be drawn:

1. The killing effect by unfiltered broad spectra of black and white light is mainly due to the low level of longer UV rays rather from the near-UV rays (see Appendix 2 for the definitions for the terms far- and near-UV).

2. When wavelengths shorter than 300 nm are eliminated by a plastic filter, black light with the maximum peak at shorter wavelengths is more efficient in cell killing than white light. We should emphasize that culture-dish plastic is a poor filter because it transmits some wavelengths between 290 and 310 nm and cuts off at least 50 percent of the intensity of wavelengths between 310 and 350 nm. Chemical filters are generally better, but their toxicity and the necessity of constant refilling reduces their usefulness.

In general, temperature-dependent effects do not occur in procaryotic or eucaryotic cells during far-UV irradiation (Jagger, 1967, ch. 3). The higher survival from cells kept at 26-28°C during receiving the radiation exposure of white light with a spectrum excluding wavelengths shorter than 300 nm (plastic filtered) agrees in principal with Hollaender's findings in *E. coli* (1943). He observed that by using a broad spectrum of wavelengths above 350 nm, toxic effects became temperature-dependent at high fluences but not at low fluences. In the first analysis of our data, we thought the higher cell survival might have been caused by light scattering due to the water vapor in the air. The fact that cells irradiated at the warm-water bath temperature (26-28°C) have higher mutation frequencies rules out this simple explanation. If cells irradiated at the

warm-bath temperature were kept at a refrigerator temperature for 5-6 hours immediately after fluorescent-light irradiation, the survival fraction decreased. Some synergism in cell killing by longer-wavelength radiation combined with cold temperature occurs.

When clear McCoy's 5A medium without serum is irradiated with far-UV radiation it was not toxic to cells at moderate doses. However, if medium is irradiated with an extremely high fluence (an order of 10^3 J/m^2) and then incubated with CHO-KK cells for a long time (24 hours), a slightly lower survival was found. Although the major mercury (Hg) emission is at 254 nm, many weaker Hg lines at or above 300 nm are also available (Appendix 4). At an extremely high UV fluence, the low level of wavelengths longer than 254 nm play a role in inducing photoproducts. In order for this small amount of photoproducts to be effective, a long incubation time is required.

Cells were killed after incubating with medium that had been previously exposed to white or black light (Wang, 1975; Sloein and Wang, 1974). This cytotoxicity is a function of irradiation time of the medium and the incubation time of the cells in the irradiated medium (Fig. 4 and 5). When photoproducts are plentiful, such as after a long exposure time, the reaction of the irradiated medium to produce cell lethality is fast, in a matter of a few minutes (Fig. 5). Similar fast killing effects can also be demonstrated by medium supplied with 1 $\mu\text{g/ml}$ of H_2O_2 . Addition of 1 unit/ml of catalase to medium containing H_2O_2 effectively increases cell survival to the same level as the control. The same amount of catalase added to irradiated

medium rescued cell killing by about 80 percent. Eisenstart's group reported that with human cells, an increase of only 40 percent with the addition of 30 units of catalase to the irradiated MEM medium (personal communication with Ananthswamy). Therefore, peroxide type of photo-products is partially responsible for cell cytotoxicity in intact cells after media were photoactivated by light with wavelengths longer than in the far-UV region.

When a special medium containing no riboflavin, L-tryptophan or L-tyrosin was irradiated with a high fluence of fluorescent light, it had no toxic effect on cells. This result agrees with others that these three chemicals are responsible for producing toxic photoproducts in medium (Wang, 1974; Hoffman and Menghenini, 1979). The toxic action of ribofalvin, tyrosin or tryptophan was further demonstrable by a highly irradiated aqueous solution of chemicals in an individual or in a combined form. After irradiation, the photoirradiated aqueous chemical solution was used as a water base to dilute 10X strength medium. The degree of cell toxicity by the newly mixed medium depends on the chemical concentrations, the way of combining them, the irradiation time and the incubation time (Table 4). Partial effectiveness in killing cells can be achieved by irradiated chemicals individually or two at a time. When two chemicals were combined, riboflavin plus an amino acid produced more photoproducts than two amino acids combined, but photoproducts were formed at the highest level when all three chemical were present during the irradiation. These results indicate that riboflavin, tryptophan and tyrosin are capable of producing photoproducts, among which the presence of riboflavin is more important.

Split-dose experiments with recovery times up to 10 hours of asynchronous CHO-KK and CHO-9 cells after treatment with far-UV, white or black light showed a steady increase in cell viability. In the interval between 10 hours to 24 hours, split-dose survivals reached a plateau level. In contrast to our findings, earlier papers on far-UV split-dose experiments reported negative or inconclusive results (Han et al., 1964; Todd et al., 1969). Later Humphrey et al. (1970) demonstrated that there was an enhancement in cell survival for CHO asynchronous cells that were given a fractionated fluence of far-UV radiation with a recovery period of 2-4 hours. But they did not pursue the split-dose experiments with a recovery period longer than 4 hours. In this study and in the study of Humphrey et al., repair of photoradiation sublethal damage by CHO cells is evident with a recovery period as short as 2 hours. Our results further show that the magnitude of recovery increased with a longer recovery period for up to 10 hours. The similarity of the split-dose survival curves with different photoradiations suggests that the mechanisms of repairing sublethal damage induced by these radiations may be identical. Since the capacity for sublethal repair is strain specific (Humphrey et al., 1970), our data may be unique for this cell line.

Further positive recovery results have been obtained in our multi-split-dose experiments. Acute doses of 32 J/m^2 , $6.75 \times 10^4 \text{ J/m}^2$ and $1.8 \times 10^5 \text{ J/m}^2$ in far-UV, unfiltered black and white light, respectively, yield a survival less than 0.001. When fluences were given in three to six fractions with an interval of 8 hours between

the first and the last dose, a remarkable increase in survival fraction was observed, providing the magnitude of each conditioning dose applied was restricted to maintain a survival fraction of 0.40 or better in individual application (Table 2). If any fraction of multi-split dose is in the exponential region, for instance, above 15 J/m^2 , $3 \times 10^4 \text{ J/m}^2$ and 10^5 J/m^2 for far-UV, unfiltered black and unfiltered white light, respectively, the overall survival level immediately decreased. CHO-KK cells, undoubtedly, can repair sublethal damages induced by photoradiations.

The systems for repairing sublethal damages of photoradiations in Chinese hamster cells appear to be different from those for repair of x-ray induced sublethal damage. Systems for repair of sublethal damage induced by X-radiation can repair damages at room temperature at a rate comparable to that found at 37°C (Elkind et al., 1965; Jostes and Painter, 1980). After photoradiation, if cells are kept at room temperature during the recovery period, the survival levels of split-dose samples at best remain at the same level as the single-dose ones. At room temperature, cell progression through the cell cycle is suppressed (Jostes and Painter, 1980). But at 37°C cell progression in CHO cells continues. This suggests that the recovery observed in split-dose experiments with photoradiation is dependent on cell progression.

Response of Synchronous Cells to Photoradiation

Using reproductive death as an endpoint, synchronous cells respond to far-UV radiation with different sensitivities throughout the cell

cycle (Fig. 13). The most sensitive cells are in the G1-early S period and the most resistant cells are in the G2 period (Burki et al., 1981). This observation has been firmly established earlier by many others (Erikson and Szybalski, 1963; Sinclair and Morton, 1965; Rauth and Whitmore, 1966). Similar cyclic-sensitivity patterns are found in cells that are irradiated by black or white light (Fig. 14), implying that fluorescent light may trigger some mechanisms similar to the one induced by far-UV to cause cell death.

DNA is the critical target for far-UV radiation damage that causes cell death. The measurement of photodimer formation provides a means to estimate the extent of cell damage. Study of DNA damage in synchronous cells indicates that the most sensitive period is the S period and the most resistant period is G1 (Humphrey et al., 1963; Trosko et al., 1966; Stewart and Humphrey, 1966). Since the most sensitive period for reproductive death is in G1/early S period and the most resistant period is G2 period, the cyclic sensitivity of far-UV-induced DNA damage is not completely in accordance with the reproductive sensitivity. Therefore, reproductive death cannot be related to dimer formation alone.

DNA-protein adducts have recently been studied. It is important to note that the changes of cell-cycle sensitivity between reproductive death and the formation of DNA-protein adducts are identical (Todd and Han, 1976). With longer wavelengths in fluorescent-light radiation, protein molecules are likely to be the major target (Webb, 1977). If longer wavelengths of white and black lights share a common type of

photoradiation damage with far-UV radiation, DNA-protein adducts are a good candidate.

Split-dose survival of synchronous cells is higher than the single-dose counterpart throughout the entire cell-age cycle except with some uncertainty of the G1 cells (Fig. 19 and 20). Humphrey et al. (1970) have shown that the G1 cells of their CHO cells line has no recovery with incubation periods up to 3 hours. So far, split-dose data in the literature for synchronous cells all point to the same conclusion that cells only repair far-UV induced damages in the S period and require a recovery period of more than 6 hours for completion (Humphrey et al., 1970; Domon and Rauth, 1973; Todd, 1973). At least one of the split-dose samples in our data, however, shows that cells may repair UV sublethal damage outside of the S period. When cells receive the first half conditioning dose of far-UV at 10 or 10.5 hours after mitotic shake-off they are likely to be at G2 period. After the radiation treatment, G2 cells continue to progress at a normal growth rate. During the application of the second half dose at 5 hours later, most of the cell population is likely to be in G1 period. As seen in Fig. 19, a positive recovery was found in this split-dose sample. This indicates that repair UV sublethal damage may occur, other than in the S period.

To explain the contradictory finding, we compare the different synchronization method and different procedures for irradiating synchronous cells between this study and other studies. In the study of others, cells were synchronized by hydroxyurea. The first

conditioning dose was applied to the S-phase cells, then followed at various times by an equal second dose. The recovery becomes evident with a recovery period of 6 hours. Our asynchronous data show that the magnitude of recovery of split-dose samples remains at the same level for recovery periods longer than 10 hours. For the sake of argument, let us apply these data to their synchronous split-dose experiment: four hours after recovery occurs, the split-dose recovery becomes saturated. The apparent no recovery of survival after cells have progressed to the G2 period can be interpreted as indicating that the cells already have reached the recovery saturation point. We do not know the validity of this argument because different CHO cells may behave differently in the sublethal repair process (Humphrey et al., 1970). In any event, by applying the first conditioning dose to synchronous cells at various cell ages with a one-hour interval, cells of all periods are allowed to have the same recovery period. Any recovery through the cycle can be more accurately assessed. With this scheme, we have observed that cells can repair photoradiation damages throughout the entire cell cycle except with some fluctuation in G1 cells.

When the recovery period for synchronous split-dose samples is as long as 24 hours, the magnitude of recovery is even higher (Fig. 20B). We interpret this increase as a result of cell progression. The growth delay for cells that have been exposed to 9 J/m^2 of far-UV is about 16 hours (Fig. 18). After the delay, S-phase cells go through the S period at a nearly normal rate. Therefore, at about 24 hours, cells

frequencies in split- and acute-dose samples fluctuates among different experiments. This observation implied that an error-free repair system may simultaneously exist in cells which had been previously irradiated with far-UV radiation. Evidence supporting this idea comes from the study of Maher et al. (1980) who have shown that human cells can eliminate 6-TG mutation induction in cells that have been previously irradiated with far-UV radiation. This indicates that mammalian cells may possess error-free repair systems. Using HGPRT genes in Chinese hamster cells, others have demonstrated that the error-free processes are induced by X-radiation (Suzuki, 1977; Jostes and Painter, 1981). If error-free repair systems exist in cells and error-prone enzymatic repair systems are induced after cells have been irradiated with the first conditioning dose of photoradiation, these two processes are ready to compete for damages along the HGPRT gene when the second conditioning dose is given. Since some fluctuation were observed with ouabain mutation induction, perhaps, similar repair processes for ouabain locus exist. On the contrary, DT mutation induction consistently show that the rates are higher in the split-dose samples than their acute-dose counterparts. Therefore, our current data suggests that error-prone repair systems predominate in DT locus.

Mutation Induction in Synchronous Cells

Induction of 6-TG resistance during the cell cycle is a complicated function of wavelengths and fluences. When 17 J/m^2 of far-UV fluence was applied, higher mutation frequencies were found in G1 and in S cells than other parts of the cell cycle ages. More pronounced peaks in similar positions of the cell cycle have been reported by Ruddle and

Hsie (1978). At 9.0 J/m^2 , far-UV radiation produces similar levels of 6-TG mutation frequencies throughout the cell cycle (Fig. 33). Cells irradiated by the unfiltered broad spectrum of black light showed higher 6-TG mutation induction levels in the early S cells. If shorter wavelengths in the black-light spectrum are eliminated, this higher level of 6-TG mutation induction in the early S cells does not occur. At 17 J/m^2 , ouabain mutation induction has a slightly higher frequency in the early S cells. Similar patterns are shown with unfiltered black light or black light with wavelengths shorter than 276 nm excluded (benzene-filtered). The slightly higher ouabain mutation frequency is abolished if a black light spectrum with wavelengths shorter than 280 nm filtered (toulene-filtered) is used for irradiation. With unfiltered white light, there is an oscillating pattern for the ouabain mutation induction throughout the cell cycle. We have no explanation for this behavior. The slight increase of 6-TG and ouabain in the early S cells makes it difficult to conclude if there is a "hot spot" for these two loci. As seen later, it is different for DT locus.

A remarkable DT peak occurring in late G1 cells (3-4 hours after the mitotic shake-off) is induced by far-UV radiation and unfiltered and filtered black- or white-light radiation. As with asynchronous cells, the magnitude of the DT peak declines in the following order in the black-light radiation: Unfiltered broad spectra, spectra with wavelengths shorter than 276 nm filtered (benzene-filtered), spectra with wavelengths shorter than 280 nm filtered (toulene-filtered),

spectra with wavelengths shorter than 300 nm filtered (pyridine-filtered) and spectra with wavelengths shorter than 325 nm filtered (acetone-filtered). Although the magnitude of DT peak varies according to the wavelengths of the spectra, the position of the DT peak occurs at the same cell cycle position, which suggests that the induction of DT response from different photoradiation may be identical. The "hot spot" for DT mutation induction is at G1 cells.

Due to the incredible amount of work involved in experimentation for split-dose experiments in synchronous populations, mutation induction by split-dose samples concentrated only on far-UV radiation. With two experiments covering the entire cell cycle, the following conclusion can be made. DT resistant mutation frequency increases in the split-dose samples throughout the entire cell cycle, with the greatest difference over the acute-dose samples in the G1 through mid-S period and a smaller difference in the latter part of the cell cycle. The induction of 6-TG and ouabain mutations appears to be higher in split-dose than in single-dose samples throughout the entire cell cycle, with a larger difference in the G1-mid S cells than the difference in the remaining of cell cycle ages. The variation of mutation induction in the split-dose samples throughout the cell cycle may mean one or all of following three possibilities: 1) The induction of the error-prone processes vary during the cell cycle, with the level in G1 and early S periods being higher than in the late S and G2 periods. 2) The efficiency for error-prone repair is higher in G1 and early S cells than the latter part of cell cycle. 3) There are indeed "hot spots" for all three loci in the early part of the cell cycle.

FIGURE LEGENDS

Fig. 1. Fluence-rate with lights penetrating through a layer of culture-dish plastic plus various amounts of clear saline (open symbols) or media (solid symbols). (A) black light (fluence-rate of unfiltered spectra was $25 \text{ J/m}^2/\text{sec.}$) (B) White light (fluence rate of unfiltered spectra was $40 \text{ J/m}^2/\text{sec.}$) (C) Gold light (unfiltered spectra was $25 \text{ J/m}^2/\text{sec.}$).

Fig. 2. Survival fractions of asynchronous CHO-KK cells after irradiated with far-UV (circles) or black-light (Triangles). Cells during irradiation were in residual saline (Open symbols), in 5.0 ml clear saline (half-filled symbols) or in 5.0 ml regular medium (solid symbols). (A) Plating efficiency was determined immediately; (B) plating efficiency was determined 6 hours postirradiation.

Fig. 3. Survival fractions of asynchronous CHO-KK cells after irradiated with white light (Squares) or Gold light (Circles). Cells during irradiation were in residual saline (Open symbols), in 5.0 ml clear saline (half-filled symbols) or in 5.0 ml regular medium (solid symbols). (A) Plating efficiency was determined immediately; (b) plating efficiency was determined 6 hours postirradiation.

Fig. 4. Irradiated solution effect on cells: (O) medium irradiated by unfiltered light, (●) medium irradiated by fluorescent-light spectra with wavelengths shorter than 300 nm filtered (plastic filtered), (▲) special medium without tryptophen, tyrosin and riboflavin irradiated by fluorescent-light spectra with wavelengths shorter than 300 nm filtered and (Δ) saline A irradiated by unfiltered fluorescent light (A) was irradiated by white light except (X) irradiated by Gold light. (B) irradiated by black light.

Fig. 5. The killing effect by irradiated medium on cells with various incubation time. (A) White light with a fluence of $1.6 \times 10^5 \text{ J/m}^2$ and (B) Black light with a fluence of $9.0 \times 10^4 \text{ J/m}^2$.

Fig. 6. Cell density effect: (A) Cells of different densities were directly irradiated by (X) 10^5 J/m^2 of unfiltered Gold light, (●) $2 \times 10^4 \text{ J/m}^2$ of unfiltered black light, (O) $1.2 \times 10^5 \text{ J/m}^2$ of unfiltered white light and (▲) 12.6 J/m^2 of far-UV radiation. (B) Cells were incubated for 15 minutes in medium previously irradiated with (O) 10^5 J/m^2 of white light and (●) 10^4 J/m^2 of black light.

Fig. 7. Survival fraction of asynchronous CHO-KK cells irradiated with white light of (Circles) unfiltered spectra or (Triangles) spectra with wavelengths shorter than 300 nm filtered (plastic filtered). During the irradiation period, cells were kept at (Solid symbols) ice-bath temperature or (unfilled symbols) 26-28°C water-bath temperature.

Fig. 8A. Survival fraction of asynchronous CHO-KK cells irradiated with black light of (Circles) unfiltered spectra or (Triangles) spectra with wavelengths shorter than 300 nm filtered (plastic filtered). During the irradiation period, cells were kept at (solid symbols) 0°-4°C ice-bath temperature or (unfilled symbols) 26-28°C water-bath temperature.

Fig. 8B. Survival fraction of asynchronous CHO-KK cells exposed to black light with various 1-cm depth of chemical filters of (▲) no filter, (Δ) benzene (filtered off wavelengths shorter than 276 nm), (X) toluene (filtered off wavelengths shorter than 280 nm), (□) pyridine, filtered off wavelengths shorter than 300 nm and (○) acetone (filtered off wavelengths shorter than 325 nm). Each survival curve was determined by 4-6 experiments.

Fig. 9. Transmission curves of various filters measured by Superscan-3 spectrophotometer. (●) plexiglas UVF 0.16 mm, (▲) 1-cm depth benzene; (X) 1-cm toluene, (□) 1-cm pyridine; (Δ) culture plastic from Falco 75 cm² flask; (▼) culture plastic from Falcon petri dish cover; (○) 1-cm acetone and (■) plexiglas UF3.

Fig. 10. Survival response of asynchronous CHO-KK cells to far-UV radiation.

Fig. 11. FMF data analyzed from FMF histogram from a typical synchronous experiment.

Fig. 12. Peak channel of cell size measured by Coulter counter of a typical synchronous experiment after radiation treatment of (●) far-UV and (■) unfiltered black light.

Fig. 13. Survival fractions of synchronous CHO-KK cells after they were irradiated by far-UV at a fluence of (●) 9.0 (X), 17 and (Δ) 25 J/m².

Fig. 14A. Survival fraction of CHO-KK synchronous cells response to black light ($\times 10^4$ J/m²): (▲) unfiltered spectra at a fluence of 2.25; (○) spectra with wavelength shorter than 276 nm filtered (benzene-filtered) at a fluence of 2.16; (■) spectra with wavelengths shorter than 280 nm filtered (toluene-filtered) at a fluence of 2.28; (●) spectra with wavelengths shorter than 325 nm filtered (acetone-filtered) at a fluence of 3.06; (Δ) and (□) spectra with wavelengths shorter than 300 nm filtered (culture plastic filtered) at a fluence of 9.0 and 3.0, respectively.

Fig. 14B. Survival fraction of CHO-KK synchronous cells response to white light $\times 10^4$ J/m²: (▲) unfiltered spectra at a fluence of 14.4 (Δ) spectra with wavelengths shorter than 300 nm filtered (plastic-filtered) at a fluence of 23.8 (X) gold light at a fluence of 35.8.

Fig. 15. Split dose recovery as a function of recovery time after asynchronous CHO-KK cells were irradiated with photo-radiation. (X) far-UV at a total fluence of 18 J/m², (▲) unfiltered black light at a total fluence of 4.0×10^4

J/m^2 , (●) black light with wavelengths shorter than 300 nm filtered (plastic filtered) at a total fluence of $9.0 \times 10^4 \text{ J/m}^2$ and (Δ) unfiltered white light at a fluence of $1.44 \times 10^5 \text{ J/m}^2$.

Fig. 16A. Survival fraction of asynchronous cells with various incubation periods before exposing to photoradiations: (●) Far-UV radiation at a fluence of 9.0 J/m^2 , (Δ) unfiltered black light at a fluence of $2.25 \times 10^4 \text{ J/m}^2$, ($\blacktriangle$) black light spectra with wavelengths shorter than 300 nm filtered (culture-plastic filtered) at a fluence of $4.5 \times 10^4 \text{ J/m}^2$ and (○) unfiltered white light at a fluence of $7.2 \times 10^4 \text{ J/m}^2$.

Fig. 16B. Delayed response (cells seeded 24 hours before irradiation) and immediate response (seeded 3 hours before irradiation) of CHO-9 cells. Acute doses of far-UV (○) delayed and (Δ) immediate; Split doses of far-UV with a 24-hour recovery period; (●) delayed and ($\blacktriangle$) immediate response.

Fig. 17. Fluence curves of cells response to (solid symbols) single doses of photoradiation or (open symbols and X) split doses with a 6-hour recovery period at 2 different temperatures: (○ and Δ) at 37°C and (X) at room temperature of 24°C . (A) Far-UV radiation, (B) black light (circles) unfiltered and (triangles) spectra with wavelengths shorter than 300 nm filtered (C) unfiltered white light.

Fig. 18. Growth curves of asynchronous cells: A cell irradiated with far-UV (X) control, (O) at a fluence of 9J/m^2 and (●) at a fluence of 17J/m^2 . B. (Δ) Cells irradiated with unfiltered black light at a fluence of 10^4J/m^2 and (▲) at a fluence of $2 \times 10^4\text{J/m}^2$.

Fig. 19. Synchronous cells response to split dose and acute dose of (A) far-UV radiation of a total fluence of 18J/m^2 (X, O, and ▽) acute-doses (□, ▽ and O) split-doses. (B) Unfiltered black light at a total fluence of $3.0 \times 10^4\text{J/m}^2$. (▽ and ●) for acute dose, (O and ▽) split-dose. The recovery period was 6 hours in samples from 2-6 hours after mitotic shake-off and was 5 hours in samples from 6.5-12 hours after mitotic shake-off.

Fig. 20A. A typical experiment showing no recovery of the G1 cells. (●) synchronous cells response to acute doses of far-UV at 18J/m^2 ; (O) split-dose of the same total fluence with a recovery period of 6 hours for samples from 3.5-7.5 hours after mitotic shake-off and a recovery period of 5 hours for samples from 8.5 to 10.5 hours after mitotic shake-off.

Fig. 20B. Effect of recovery periods in split-dose recovery. (●) A single dose of far-UV of 18J/m^2 , same total dose given in 2 fractions with (X) a recovery period of 2 hours, (O) a recovery period of 6 hours and (□) a recovery period of 24 hours.

Fig. 21. Split-dose recovery after far-UV irradiation in 1 mm hydroxyurea-treated CHO cells. (●) Acute-doses of 17 J/m^2 were given at various times after cells were released from the drug. (○) Split-dose of a total dose of 17 J/m^2 were given as follows: the first conditioning dose of 8.5 J/m^2 was given after cells were released from hydroxyurea and the final conditioning dose was given at various times after Ho-urea removal.

Fig. 22. Sensitivity of (●) wild-type cells and (Δ , X, ○) mutant resistant cells: (A) 6-TG, (B) Ouabain and (C) DT.

Fig. 23A. A. Metabolic cooperation test. Recovery of mutant cells grown in presence of various number of wild-type cells. Mutant cells scored in the absence of wild-type cells were taken as 1. (●) 6-TG, (X) ouabain and (Δ) DT.

Fig. 23B. Recovery of mutation frequency with various number of mutagenized cells inoculated on the 8th day after far-UV irradiation. The highest mutation induction was normalized as one. (●) 6-TG, (X) ouabain and (Δ) DT.

Fig. 24. Recovery of mutant cells after grown with 2×10^5 wild-type cells for various periods. (X) ouabain, (●) 6-TG, (Δ) DT.

Fig. 25. Expression curves for 6-TG, ouabain and DT after cells were irradiated by (Δ) 17 J/m^2 of far-UV, (X) $2.25 \times 10^4 \text{ J/m}^2$ of unfiltered black light and (●) $1.1 \times 10^5 \text{ J/m}^2$ of unfiltered white light.

Fig. 26. Fluence mutation curves for asynchronous cells irradiated by far-UV (A) 6-TG, (B) Ouabain and (C) DT.

Fig. 27. Fluence mutation curves of asynchronous cells after cells were irradiated by (circles) unfiltered black light or (triangles) black light with wavelengths shorter than 300 nm filtered (plastic filtered). During radiation exposure, cells were kept at 0°-4°C (solid symbols) ice-bath temperature or (unfilled symbols) 26-28°C water-bath temperature. (A) 6-TG, (B) Ouabain and (C) DT.

Fig. 28. Fluence mutation curves of asynchronous cells by filtered-black light radiations. Symbols for filters are as follows: (o) no filter, (▲) 1-cm benzene, (□) 1-cm toluene, (x) 1-cm pyridine, and (Δ) 1-cm acetone. (A) 6-TG, (B) Ouabain and (C) DT.

Fig. 29. Fluence mutation curves of asynchronous cells after cells were irradiated by (circles) unfiltered white light or (triangles) white light with wavelengths shorter than 300 nm filtered (plastic filtered). One unit represents 5×10^4 and 10^5 J/m² for the unfiltered and filtered white light, respectively. During irradiation, cells were kept at (solid symbols) 0°-4°C ice-bath temperature and (unfilled symbols) 26-28°C water-bath temperature.

Fig. 30. Mutation induction as a function of recovery period after cells were irradiated with a total far-UV fluence of 18 J/m². (●) an average value of 6 experiments and (x) a typical experiment. (A) 6-TG, (B) Ouabain and (C) DT.

Fig. 31. Mutation induction as a function of recovery period after cells were irradiated with a total unfiltered black-light fluence of $4.0 \times 10^4 \text{ J/m}^2$. (●) an average value of 6 experiments and (X) a typical experiment. (A) 6-TG, (B) Ouabain and (C) DT.

Fig. 32A. Fluence mutation curve after cells were irradiated with far-UV (●) acute-dose or (X) split-dose with a 6-hour recovery period and during this period, cells were kept at 37°C .

Fig. 32B. Fluence mutation curve after cells were irradiated by unfiltered black light of (●) acute doses or (X) split doses with a 6-hour recovery period and during this period cells were kept at 37°C .

Fig. 33. Induction of mutation in different parts of the cell cycle after cells were irradiated by a far-UV fluence of (●) 9.0 J/m^2 or (X) 18 J/m^2 . (A) 6-TG, (B) Ouabain and (C) DT.

Fig. 34. Mutation induction of synchronous cells by black light. (○) unfiltered light at a fluence of $2.25 \times 10^4 \text{ J/m}^2$, (▲) with wavelengths shorter than 276 nm filtered off (benzene-filtered) at a fluence of $2.16 \times 10^4 \text{ J/m}^2$, (□) with wavelengths shorter than 280 nm filtered (toluene filtered) at a fluence of $2.08 \times 10^4 \text{ J/m}^2$, (X) with wavelengths shorter than 300 nm filtered off (pyridine filtered) at a fluence of $2.08 \times 10^4 \text{ J/m}^2$, (Δ) wavelengths shorter than 325 nm filtered off (acetone filtered) at a fluence of $1.98 \times 10^4 \text{ J/m}^2$. (A) 6-TG, (B) Ouabain and (C) DT.

Fig. 35. Mutation induction of synchronous cells by (●) unfiltered white light at a fluence of $1.08 \times 10^5 \text{ J/m}^2$ or (X) white light with wavelengths shorter than 300 nm filtered (plastic filtered) at a fluence of $2.88 \times 10^5 \text{ J/m}^2$. (A) 6-TG, (B) Ouabain and (C) DT.

Fig. 36. Mutation induction of synchronous cells by far-UV radiation at a fluence of 18 J/m^2 . (●) fluence was given all at once and (X) fluence was given two evenly divided portions with a recovery period of either 6 hours (samples taken 6 hours after mitotic shake-off) or 5 hours (samples taken 5 hours after mitotic shake-off). Cells were kept in darkness during the recovery period.

Fig. 37. Log-log plot of survival curves of far-UV, unfiltered white and gold light. (●) Far-UV, (Δ) black light, white light and (X) gold light.

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Table 1. Irradiated Solutions* on Non-Irradiated Cells

Solution	Incubation Time	Plating Efficiencies	Solution	Incubation Time	Plating Effect
1 x Riboflavin	10 mins	0.99	10 x Riboflavin	10 mins	0.78
	15 mins	0.74		15 mins	0.46
	30 mins	0.63		30 mins	0.38
1 x Tryptophan	10 mins	0.96	20 x Tryptophan	10 mins	0.64
	15 mins	0.97		15 mins	0.60
	30 mins	0.53		30 mins	0.77
1 x Tyrosin	10 mins	1.0	20 x Tyrosin	10 mins	0.87
	15 mins	0.89		15 mins	0.80
	30 mins	0.78		30 mins	0.87
1 x (Arg+Try+Tyr)	10 mins	0.61	10x Rib+Arg+Tyr	10 mins	0.77
	15 mins	0.39		15 mins	0.74
	30 mins	0.26		30 mins	0.2
1 x (Arg + Try)	10 mins	0.86	10 Rib + Tr.	10 mins	0.91
	15 mins	0.73		15 mins	0.79
	30 mins	0.64		30 mins	0.79
1 x (Try + Tyr)	10 mins	0.90	20 x (Try + Tyr)	10 mins	0.96
	20 mins	0.91		25 mins	0.91
	30 mins	0		30 mins	0.79
Medium (regular)	10 mins	0.54	Saline A	Saline A	
	15 mins	0.73		10 mins	0.91
	30 mins	0.21		15 mins	*
				30 mins	0.05
2.5 ml of 1xRib	30 mins	0.74			
1 x Try + 1 x Tyr					
(Combining after each chemical being irradiated separately)					

Table 2. Results of Multi-Split Dose Experiments.

Dose Given	0 Day Survival Fraction
Single far-UV dose of 22.5 J/m^2	0.03
18.0 J/m^2 , 6 Hrs, 3.0 J/m^2 , 2 Hr., 1.5 J/m^2	0.06
15.0 J/m^2 , 5 Hrs, 1.5 J/m^2 , 1 Hr, 1.5 J/m^2 , 1 Hr, 1.5 J/m^2	0.11
11.25 J/m^2 , 8 Hr, 11.25 J/m^2	0.20
11.25 J/m^2 , 4 Hr, 3.75 J/m^2 , 2 Hr, 3.7 J/m^2 , 3.75 J/m^2	0.28
3.75 J/m^2 , 4 Hr, 3.75 J/m^2 , 2 Hr, 3.75 J/m^2 , 2 Hr, 2 Hr, 11.25 J/m^2	0.29
7.5 J/m^2 , 2 Hr, 7.5 J/m^2 , 6 Hr, 7.5 J/m^2	0.29
4.5 J/m^2 , 3 Hr, 3.0 J/m^2 , 6 Hr, 15 J/m^2	0.18
4.5 J/m^2 , 2 Hr, 7.5 J/m^2 , 6 Hr, 10.5 J/m^2	0.34
3.0 J/m^2 , 1 Hr, 3.0 J/m^2 , 1 Hr, 3.0 J/m^2 , 4 Hr, 3.0 J/m^2 , 1 Hr, 3.0 J/m^2 , 2 Hr, 6.0 J/m^2	0.33
11.25 J/m^2 single dose	0.42
Single far-UV dose of 32 J/m^2	0.0001
6 J/m^2 2 Hr, 5 J/m^2 2 Hr, 10 J/m^2 , 4 Hr, 10 J/m^2	0.28
6 J/m^2 , 2 Hr, 1 J/m^2 , 2 Hr, 6 J/m^2 , 2 Hr, 6 J/m^2 1 Hr, 4 J/m^2 2 Hr, 4 J/m^2	0.31
Black light (unfiltered) $1 \times 10^4 \text{ J/m}^2$	
Single dose of 16.75×10^4	0.0002
$1.35 \times 10^4 \text{ J/m}^2$, 2 Hr; $1.35 \times 10^4 \text{ J/m}^2$, 2 Hr $1.35 \times 10^4 \text{ J/m}^2$, 2 Hr; $1.35 \times 10^4 \text{ J/m}^2$, 2 Hr. $1.35 \times 10^4 \text{ J/m}^2$	0.286
$22.5 \times 10^4 \text{ J/m}^2$, 4 Hr, $2.25 \times 10^4 \text{ J/m}^2$, 4 Hr., $2.25 \times 10^4 \text{ J/m}^2$	0.26
$3.0 \times 10^4 \text{ J/m}^2$, 8 Hr; $3.75 \times 10^4 \text{ J/m}^2$	0.004

Table 3. Inc. selection medium selection medium kept on a 37°C incubator.

	Ratio of DT Mut.		Ratio of Deletion Mut.		Ratio of DT Mut. Inc. Select. Med. Fresh Select. Med.
	dtf Mutation Per 10 ⁵ /viable cells	{Inc. Selection med., Fresh Select. medium}	Deletion Mut. 10 ⁵ /v.c.	DT Mut. 10 ⁶ /v.c.	
Inc. Med. (Day 0)	31.8		11.5	21.6	
Inc. Med. (Day 2)	29.0	2.1	16.1	25.0	+11.9
Fresh Sele. Med	31.3		15.6	28.4	
Inc. Med. (Day 4)	27.5	+1.8	27.8	21.5	-10
Fresh Select. Med	28.6		15.6	19.1	
Inc. Med. (Day 6)	34.7	-14	19.1	26.9	14.5
Fresh Sele. Med	30.5		17.0	23.5	
Inc. Med. (Day 9)	24.7	9.1	25.1	28.9	+12
Fresh Select. Med	22.6		28.0	33.0	
Inc. Med. (Day 11)	29.3	+1.14	11.7	24.7	-12.3
Fresh Select. Med	26.2		15.0	22.2	
Inc. Med. (Day 13)	19.8	12.0	14.4	18.7	-10.5
Fresh Select. Med	21.4		10.7	20.9	
Inc. Med. (Day 16)	35.4	10.4	19.6	30.2	-11.4
Fresh Select. Med	39.5		17.5	22.1	
Inc. Med. (Day 18)	33.5	13.7	10.8	25.6	+4.1
Fresh Select. Med	38.6		11.7	26.7	
Inc. Med. (Day 20)	21.3	17.6	16.4	22.2	-14.9
Fresh Select. Med	18.1		14.5	18.9	
Inc. Med. (Day 23)					
Fresh Select. Med	CONTAMINATION				
Inc. Med. (Day 25)	25.7	+1.7	1.6	19.6	12.1
Fresh Select. Med	26.0		17.6	16.4	
Inc. Med. (Day 27)	14.7	17.4	16.1	19.4	+1.9
Fresh Select. Med	14.1		16.1	16.6	

Table 4. A Recovery Period of 4H Rec. Between 2 Evenly Divided Split Doses.

Type of Radiation and Dose-Fluence		4 Day		5 Split 5 Acute	6 IG ^R	6TG ^R Split 6TG Acute	Diabain ^R	Ova Split Ova Acute	DT ^R	DT Split DT Acute
		Survival fraction								
Far UV 12 J/m ²	Acute	0.26			22.5		19.3		28.2	
	Split	0.22	2.7		53.8	1.9	22.3	1.15	87.7	3.1
Far UV 18 J/m ²	Acute	0.06			15.2		29.6		41.2	
	Split	0.38	6.1		61.7	1.75	58.7	1.98	8.1	2.4
Black light (No Filter) 3 x 10 ⁴ J/m ²	Acute	0.13			42.5		38.6		33.5	
	Split	0.43	3.1		98.2	2.3	79.7	2.06	59.0	1.76
Black light Plastic Filter 9 x 10 ⁴ J/m ²	Acute	0.05			17.5		45.6		32.	
	Split	0.76	4.2		4.78	0.96	21.8	1.69	8.1	1.17
White light plastic Cover 1.2 x 10 ⁵ J/m ²	Acute	0.54			6.96		1.4		2.4	
	Split	1.0	1.85		7.33	1.05	22.5	1.56	3.4	1.40

Table 5.

Type of Radiation	Wavelength (nm)	Slope $\times 10^{-11}$	$\frac{1}{\lambda^2}$	n	$(n_1 - n_2) \text{nm}$	$\text{nm} = \frac{0.9}{n - 1.7}$
Far UV	254	8.29	1.51×10^2	4.2	16.7	1.18
Black fluorescent No filters ice temp.	Incoherent	-0.614	$1.58 \times 10^3 \text{ J/m}^2$	4.17	2.64	
Black fluorescent Benzene filters	(starting at 275 nm)	-0.111	$9.0 \times 10^3 \text{ J/m}^2$	1.36	2.79	0.31
Black light plastic filter	298 nm	-0.071	$14.01 \times 10^3 \text{ J/m}^2$	2.0	9.7	0.69
White fluorescent	ice temp	0.129	$8.4 \times 10^4 \text{ J/m}^2$	2.14	23	1.22
No filters	norm temp	0.53	$1.09 \times 10^4 \text{ J/m}^2$	1.4	2.1	1.22
	ice temp	0.011	$30.3 \times 10^4 \text{ J/m}^2$	5.6	52.1	1.72
	Warm temp	0.0098	$352.1 \times 10^4 \text{ J/m}^2$	1.06	31.4	0.06

APPENDIX 1

Relationship of Wavelength of Photoradiation
and Energy Level of Photons

The relation between the energy level of a photon and the frequency is:

$$E = h\nu$$

where E is the energy of the photon, ν is the frequency in cycles per second of the photoradiation, and h is Planck's constant (4.136×10^{-15} eV-sec). The relationship between wavelength and frequency is

$$\nu = c/\lambda$$

where λ is the wavelength in centimeters and c is the velocity of light which is 2.998×10^8 m/sec in vacuum. With proper units we have

$$E = \frac{12400 \text{ eV-Å}}{\lambda(\text{Å})} \text{ eV}$$

APPENDIX 2

In this study, wavelengths for far-UV, near UV and visible regions are as follows:

far-UV	200-310
near-UV	310-390
visible	390-780

APPENDIX 3

Energy output of cool white, black, gold and other fluorescent lights published by General Electric Co.

Spectral irradiance measured by Jacobson and associates (1979).

(A) germicidal ultraviolet lamp. (B) GE F15T8 black light and
(C) Sylvania F15 T8 cool white lamp. Fluorescent-light tubes from the
same company but different lengths (48 inches vs. 15 inches) were used
in our study.

Compare the spectral irradiance output from germicidal lamp (G), black light (BL) and white light (CW) for every 10 nm wavelengths in the region of 150-400 nm). Reproduced from Jacobson et al. (1979).

Lamp	250-260 nm	260-270 nm	270-280 nm	280-290 nm	290-300 nm	300-310 nm	310-320 nm	320-330 nm
G	2.3×10^{-5}	5.0×10^{-8}	2.2×10^{-8}	3.6×10^{-8}	8.1×10^{-8}	5.2×10^{-8}	4.6×10^{-7}	2.9×10^{-8}
BL	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	1.4×10^{-8}	7.0×10^{-8}	4.0×10^{-7}	6.5×10^{-7}
CW	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1.0 \times 10^{-9}$	9.5×10^{-9}	7.0×10^{-8}	4.0×10^{-7}	6.5×10^{-7}

Lamp	330-340 nm	340-350 nm	350-360 nm	360-370 nm	370-380 nm	380-390 nm	390-400 nm
G	2.9×10^{-8}	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$	3.5×10^{-7}	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$
BL	1.2×10^{-6}	1.8×10^{-6}	2.3×10^{-6}	2.8×10^{-6}	2.4×10^{-6}	2.0×10^{-6}	1.7×10^{-6}
CW	4.0×10^{-8}	3.5×10^{-8}	5.8×10^{-8}	4.5×10^{-7}	1.2×10^{-7}	1.7×10^{-7}	2.3×10^{-7}

Appendix 4

Energy levels of mercury. Note the different units used in this diagram ($\text{\AA}$) and in our presentation (nm). $1 \text{ nm} = 10\text{\AA}$.

XBL819-4216

Figure 1.

Figure 2.

XBL8211-4224

Figure 3.

XBL8211-4223

XBL 819-4213

Figure 4.

XBL819-4212

Figure 5.

x80819-4211

Figure 6.

Figure 7.

XBL 819-4210

Figure 8A.

XBL 819-4209

Figure 8B.

XBL819-4208

xBL819-4207

Figure 9.

XBL819-4206

Figure 10.

Figure 11.

XBL819-4205

XBL819 - 420 2

Figure 12.

Figure 13.

XBL819-4201

XBL819-4204

Figure 14.

Figure 15.

XBL 819-4200

Figure 16A.

XBL813-4199

Figure 16B.

XBL819-4198

Figure 17A.

XBL819-4197

Figure 17B.

XB L819-4203

Hours after photoradiation
Figure 18.

XB L 819-4196

Figure 19.

XBL 819-4195

Figure 20A.

XBL819-4193

Figure 20B.

XBL 819-4194

Figure 21.

XBL8211-4233

Figure 22A.

xBLB19 4217

Figure 22B.

xBL619-4218

Figure 22C.

Figure 23A.

XBL819-4192

Figure 23B.

XBL819-4190

Figure 24.

Figure 25.

XBL819-4189

XBL819-4188

Figure 26.

Figure 27.

XBL819-4187

Figure 28.

XBL 819-4186

Figure 29.

XBL619-4185

Figure 30.

XBL819-4184

Figure 31.

XBL 819-4183

Fig. 1 (cont.)

xBL819-4182

Figure 32B.

XBL 819-4181

Figure 33.

Figure 34.

XBL 819-4179

Figure 35.

XBL 819-4178

Figure 36.

XBL 819-4177

JBL 819-4220

Dose fluences

Figure 37.