THE EFFECTS OF CAFFEINE ON THE UV-INDUCED FREQUENCY OF MUTATIONS IN CHINESE HAMSTER FIBROBLASTS

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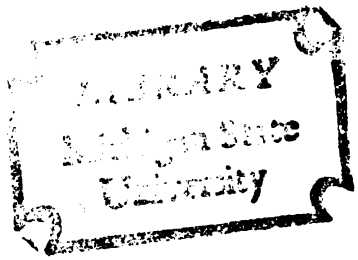

ABSTRACT

THE EFFECTS OF CAFFEINE ON THE
UV-INDUCED FREQUENCY OF MUTATIONS
IN CHINESE HAMSTER FIBROBLASTS

By

Constance Philipps

In an attempt to resolve a conflict in the literature (43, 44) as to the effects of caffeine (a presumed inhibitor of DNA repair) on the frequency of UV-induced mutations to 8-azaguanine resistance in V-79 Chinese hamster fibroblasts, a new approach was taken. Various concentrations of caffeine were tested for different intervals of time following UV-irradiation. The percentage of cells surviving was determined, as well as the frequency of mutants resistant to ouabain, using techniques of mammalian cell tissue culture.

Two different major effects on the induced frequency of mutations were observed depending on the time period caffeine was present. When caffeine was added immediately after UV-irradiation and removed about 48 hours later, just before addition of ouabain, an increase in the mutation frequency was observed. However, if the addition of caffeine was delayed for at least 48 hours or up to 240 hours and was left in the medium for the rest of the incubation

period a decrease in the frequency of mutations was observed. This seems to indicate that caffeine affects cells in other ways than only as a DNA repair inhibitor.

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Constance Philipps

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LIST OF ABBREVIATIONS

UV	ultraviolet light
hcr^+, hcr^-	host-cell reactivation, wild type(+) and mutant (-)
DNA	deoxyribonucleic acid
uvr^+, uvr^-	ultraviolet light resistance, wild type (+) and mutant (-)
3',5'-cAMP	cyclic adenosine monophosphate
3',5'-cGMP	cyclic guanosine monophosphate
A	adenine
T	thymine
RNA	ribonucleic acid
TdR	thymidine
BUdR	bromodeoxyuridine
^{3}H-TdR	tritiated thymidine
SV 40	simian virus 40
XP Group A	Xeroderma Pigmentosum Complementation Group A
$HGPRT^-$	hypoxanthine-guanine phosphoribosyl transferase deficient
MNNG	methyl-nitro-nitrosoguanidine
MMS	methyl methane sulfonate
DMM	2,5-dimethane sulfonoxy-hexane (dimethylmyleran)
MNUA or MNU	methyl-nitrosourea
SM	sulfur mustard (bis-β-chloroethylsulfide)

N-AcAAF	N-Acetoxy Acetyl Amino Fluorene
4-NQO	4-Nitroquinoline-1-Oxide
MMC or MC	Mitomycin C
Cis-Pt (II)	Cis Platinum II
Na^+/K^+ Atpase	Sodium Potassium Adenosine TriPhosphatase
CsCl	Cesium Chloride
$J/m^2/sec$	Joules/meter^2/second
G_1, G_2, S	growth 1, growth 2, and DNA synthesis phases of the cell cycle

PREFACE

> "I ca'n't believe that!" said Alice.
> "Ca'n't you?" the Queen said in a pitying tone. Try again: draw a long breath and shut your eyes."
> Alice laughed. "There's no use trying," she said: "One ca'n't believe impossible things."
> "I daresay you haven't had much practice," said the Queen. "When I was your age, I always did it for half-an-hour a day. Why, sometimes I've believed as many as six impossible things before breakfast. . . ."
>
> from Through the Looking Glass
> by Lewis Carroll

The above quote was discovered while browsing through a chemical genetics book by Bernard Strauss, who coincidentally, is one of the authors of a recently published report that presents a model of DNA repair to be discussed later in this thesis. The quote is included not to forewarn the reader of "impossible things," but to express an important change that has taken place in my thinking since this project was begun. I now more fully appreciate the position Einstein also spoke of, that nature may be impossible to understand by completely rational processes.

A review of the current literature of the effects of caffeine both *in vivo* and *in vitro*, emphasizing its many synergistic effects with other chemical and physical agents is next. A description of the rationale for this thesis follows the literature review.

LITERATURE REVIEW

Metabolism of Caffeine

When caffeine is added to living systems it is absorbed, distributed, and metabolized to different products at different rates depending on the species, and nature of organization of the system.

At the whole animal level, in mammals, caffeine is rapidly absorbed from the gastrointestinal tract, and is distributed in various tissues in proportion to their water content (1). It is rapidly and almost completely metabolized, its biological half-life being about three hours in both mouse and man (2). The main metabolite in the tissues of man is paraxanthine or 1,7-dimethylxanthine (3). The main urinary metabolites in man appear to be 1-methyluric acid and 1-methylxanthine (4); see Figure 1 for a diagram of these metabolic pathways.

At the cellular level, caffeine is rapidly metabolized in cultured human and mouse cells by demethylation (5). Within one to three hours of exposure to millimolar concentrations of labeled caffeine, more than 90% of the acid-soluble fraction of cells contains labeled products of metabolism and less than 10% is still caffeine. The

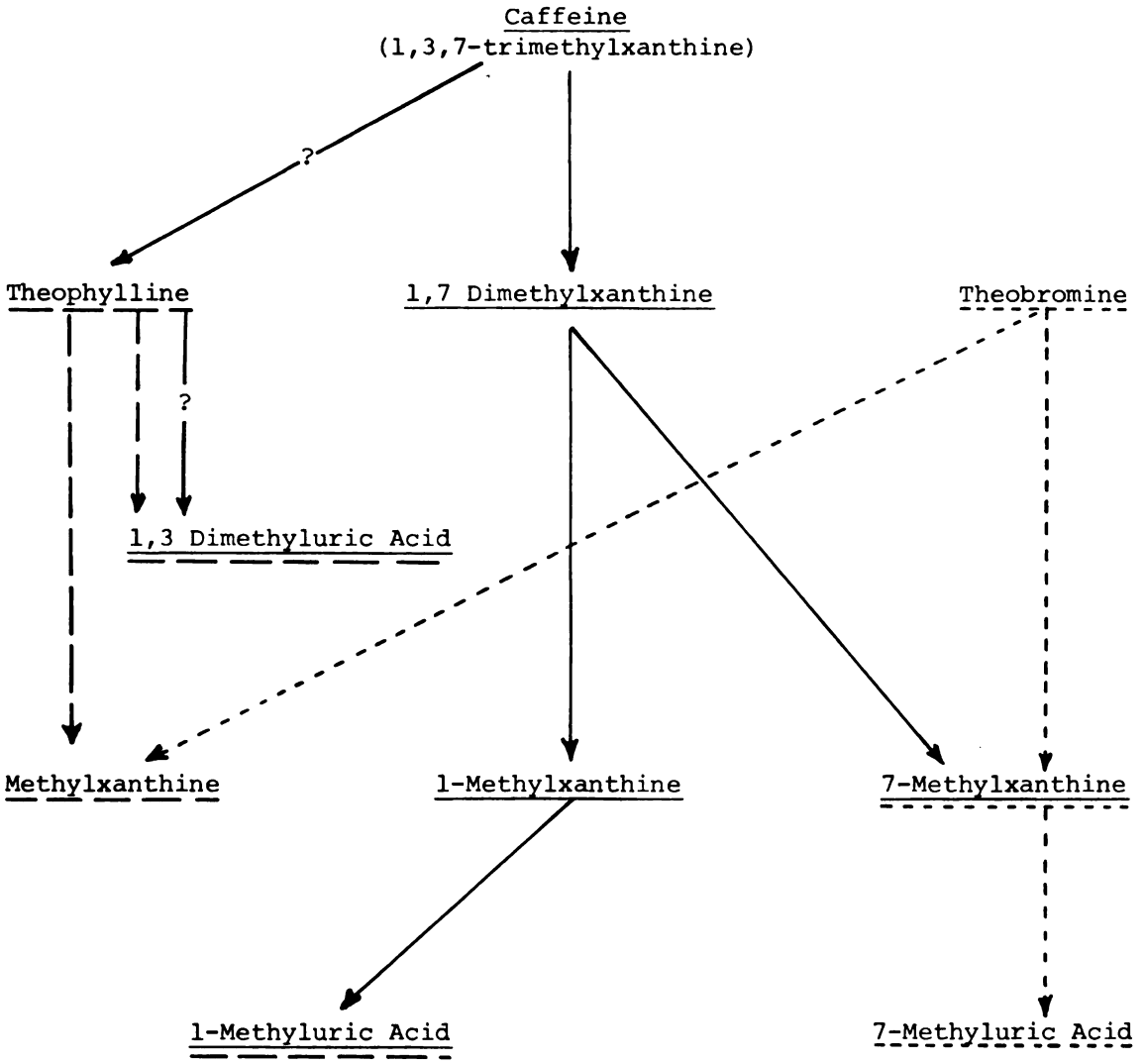


Figure 1. Metabolic degredation of caffeine in man (4).

absence of a label remaining on the caffeine metabolite prevented its identification.

Caffeine as an Inducer of Gene Mutations

In Escherichia coli, caffeine has been implicated as being a frameshift mutagen. Significant increases in lactose revertants (which have a frameshift mutation) have been recovered from cells grown in medium containing 1 mg/ml (5 mM) caffeine (6). Kihlman reviewed the following observations in his report of the effects of caffeine on genetic material. Increases in mutants resistant to bacteriophage T5 have been found in E. coli treated with 150 mg/l (0.75 mM) caffeine (7). (It should be noted that no appreciable cell killing was reported to occur at this concentration.) Adenosine and guanosine were found to inhibit this mutagenic effect (7), when present at a much lower concentration of 2 mg/l (10^{-3} mM). This mutagenic action of caffeine was found to require DNA synthesis, but not protein synthesis (8). Oxygen was necessary also for mutation production (7).

In eucaryots reports of gene mutations induced by caffeine are more rare. With prolonged treatments for five days or more, caffeine at high concentrations of 0.2% (10 mM), increased the frequency of physiological mutants (i.e., mutants unable to synthesize some factor necessary for normal growth) in the conidia of the fungus, Ophiostoma

multiannulatum (9). Two papers report the induction of sex-linked recessive lethals in Drosophila melanogaster by caffeine (10, 11), but others have been unable to confirm these results (12, 13, 14). Demonstration of the induction of gene mutations by caffeine in mammals has not been observed (15, 16).

Caffeine as a Producer of Chromosome Abberations

Caffeine has been described by Kihlman (7) as producing two major, different types of effects on chromosomes. The first, the "Ostertag effect," is observed only in cultured mammalian cells, and high concentrations of caffeine (10^{-2} M) must be present during S phase for the chromosome pulverization to occur (17, 18). The second effect, the "Kihlman effect," takes place in both animal and plant cells in vivo. Mammalian cells, in vitro also show this effect if their temperature is lowered 10°C. Using a 10^{-2} M concentration, subchromatid exchange leading to side arm bridges in mitosis is observed (7). Low levels of ATP inhibit this effect, and 8-substituted caffeine derivatives are more effective. The molecular nature of the lesion is not understood.

The frequency of chromosome loss and non-disjunction, due to caffeine treatment, have been studied in Drosophila

(14, 19), but the results are conflicting and the increases measured are not very large, being about two times the control.

The effects of caffeine on meiosis of mouse ova showed no production of numerical or structural chromosomal abnormalities although it did suppress entry of ova into *in vitro* meiosis, and prevented acute superovulation. Examination of the intrafollicular ova revealed persistance of the germinal vesicle stage (20).

Synergistic Effects of Caffeine

In Procaryots

At the biological level, 0.1% caffeine (5 mM) potentiated UV-induced killing in wild type strains (hcr^+, uvr^+) of *E. coli* (21, 22, 23, 24, 25). (See list of abbrevaiations used for an explanation of hcr and uvr.) The frequency of UV-induced mutations to streptomycin resistance and tryptophan revertants were maximally increased by caffeine following irradiation with low doses of UV (21, 24, 25, 26, 27, 28, 29). Neither of these synergistic effects were seen in hcr^- or uvr^- strains, which are deficient in excision repair (21).

At the molecular level, Sideropoulos and Shankel showed that the excision of UV-induced thymine dimers was inhibited by the presence of caffeine (17). If the addition

of caffeine was delayed for 40 minutes, no effect was seen (presumably since repair was completed). They suggested that caffeine prevents excision by binding to the "excising enzyme" since (1) photoreactivation was not inhibited by the presence of caffeine and (2) the addition of irradiated DNA did not inhibit the synergistic effects of caffeine. Harm, however, reports of an inhibition by caffeine of photo-enzymatic repair of UV-induced lesions, and suggested it was due to competition with the enzymes for the binding-sites (12).

Observations by Bendigkeit and Hanawalt as quoted in a review by Witkin, showed the rate of repair replication was inhibited by caffeine (25). It was suggested by others that perhaps caffeine inhibits the first single strand break in the DNA (30).

Wacker et al. showed that the addition of cAMP along with caffeine to the nutrient broth of E. coli $15T^-$ inhibited the synergistic reduction in survival induced by UV. The addition of cAMP alone did not have any effect on survival. Their conclusion was that both caffeine and cAMP were competing for the phosphodiester-like excision enzyme and that cAMP out-competed caffeine (31).

At the biological level, host-cell reactivation (hcr) of UV-irradiated phage (which is inferred to be a function of the host's excision repair enzymes) was

inhibited by caffeine in hcr^+ strains, which are wild type and thus can reactivate or repair irradiated phage, but not in hcr^- strains (22, 30, 32). Caffeine also inhibited mutation frequency decline (MFD) in E. coli (21). MFD is a decline in the number of mutations to prototrophy when cells are deprived of amino acids. It is hypothesized that the repression of the tRNA genes, following amino acid deprivation allows excision repair to be more accurate and thus result in fewer amber suppressor mutations.

Addition of caffeine at concentrations greater than 0.1% (5 mM) resulted in a lethal synergistic effect as well as a reduction in the number of mutations induced by UV in an hcr^- strain of E. coli (25). Also, recombination after conjugation was inhibited (25). Recombination was also inhibited in Bacillus subtilis by 1.5-3.0 mg/ml (7-15 mM) caffeine, as measured by a strong reduction in transformation and transfection (33).

Recently, McCulley and Johnson studied two excision deficient strains of E. coli, B and K-12. They found, with 2 mg/ml of caffeine, recombination during conjugation was inhibited only in the B strain, and post-replication repair, shown by alkaline sucrose gradient sedimentation profiles, was not inhibited in either strain (34).

In Eucaryots

Cell Death, Mutations, Chromosome Aberrations, Transformations, and Tumors

Eucaryotic microorganisms. The synergistic effects of caffeine with other agents is even more confusing in eucaryots than it is in procaryots. Observations in eucaryotic microorganisms indicate a potentiation of UV-induced cell death by caffeine, in both the slime mold, Physarum polycephalum (35) and in the yeast, Schizosaccharomyces pombe (36). In the yeast, caffeine had a potentiating effect on cell death in UV-irradiated wild type cells, and in mutants deficient in excision repair, but no effect was seen in mutants deficient in recombination. Others have found 0.2% (10 mM) caffeine inhibited UV- and MNNG-induced mutations to adenine requirement and back mutations to histidine independence (37). No effect of caffeine was seen in the spontaneously induced mutants. Caffeine also inhibited meiotic recombination in these yeast cells.

However, in the slime mold, Physarum polycephalum, caffeine was found to increase the mutagenic effect of UV (38). Caffeine treatment in a different species of slime mold, Dictyostelium discoideum, synergistically increased UV-induced mutants, as well as inducing mutants itself (39). Caffeine was present for 19 hours at 500 mg/ml (2 mM).

Caffeine did not increase the killing effect of UV in these cells, and all of the aggregateless mutant clones induced or potentiated by caffeine, reverted to a wild-type phenotype after a variable number of clonal reisolations.

Rodents. In mammalian cells, potentiation of cell killing induced by the following chemicals and physical agents: MMS, DMM, MNNG, MNUA, UV light, and SM, by caffeine was reported in Mouse L cells (40, 41, 42). A 2 mM concentration was used for 48 hours following chemical or physical damage. Similarly, caffeine potentiated cell death in Chinese hamster cells, induced either by UV light or MNU treatment (40, 43, 44, 45). The concentrations of caffeine that were used ranged from 0.25 to 1.0 mM. Caffeine synergistically potentiated cell killing in Syrian hamster cells due to N-AcAAF (46), in Mouse A31-714 (BALB/3T3-A31) cells due to 4-NQO (47), and in C3H2K cells treated with UV light (48). Caffeine, also, was found to have no effect on one line of Chinese hamster cells after UV-irradiation (49).

Caffeine is reported to both increase (43, 45) and decrease (40, 44) UV-induced and MNU-induced mutation frequencies to 6-thioguanine and 8-azaguanine resistance in V-79 Chinese hamster fibroblasts. It also decreased UV-induced mutants resistant to excess thymidine in mouse lymphoma cells (40). According to Fox, the decreased mutation frequencies measured were due to caffeine delaying the normal expression time in treated cells.

Other observations which indicate caffeine treatment somehow interacting with damaged DNA, include increases in MNU-induced chromosome aberrations (45), and inhibition of UV, 4-NQO, MMC-induced sister chromatid exchanges in Chinese hamster cells (50, 51). Kihlman (7) also reports of caffeine enhancement of induced chromosome aberrations in both plants and animals, and thoroughly investigated various environmental and genetic factors involved. Cell toxicity and chromosome damage due to Cis-Pt (II) was enhanced by caffeine in V-79 cells (52). This damage is amenable to post-replication repair, according to Van der Berg and Roberts. Weigle reactivation of UV-irradiated Herpes simplex virus in irradiated hamster kidney cells was reduced upon addition of caffeine (53).

Induced transformation frequencies were synergistically increased by caffeine in N-AcAAF treated Syrian hamster cells (46), decreased by caffeine in 4-NQO treated A-31 BALB mouse cells (47), and increased by caffeine in SV-40 and UV-treated C3H2K cells (48).

The tumor frequencies induced by 4-NQO in ICR/Jcl mice, by cigarette smoke condensates on mouse skin, and by UV-irradiated Swiss Carshalton mouse ears, were all reduced when animals were either injected with caffeine, or had it topically applied to their skin (54, 55, 56).

Human cells. More discrepancies seem to exist in results from human cells. Schroy and Todd (57) demonstrated caffeine potentiation of UV-induced cell death in five human epithelioid cell lines, including HeLa cells. The concentrations of caffeine they used were 1.0 and 3.0 mM. In two other reports it was shown that caffeine decreased survival in irradiated HeLa cells in one report by Arlett as quoted by Schroy and Todd (57), but Wilkinson (58) observed no effect on UV-induced cell death of two lines of HeLa in their lab.

On the chromosomal level, 1 mM caffeine synergistically enhanced the amount of chromatid breaks and exchanges in human lymphocytes treated with MMS, MC, and X-rays (59).

Many human cell studies on damaged cell survival and the influence of DNA repair have been done with the DNA repair mutants Xeroderma Pigmentosum (XP). When high concentrations (4 mM) of caffeine were used, for three days in agar, Rufus Day III (60) found a reduction in the plaque forming ability of UV-irradiated adenovirus 2 in normal human fibroblasts and in XP variants, but not in classical XP cells of complementation group A. XP variants have been shown to be defective in "post-replication" repair, but not excision-repair, whereas classical XP cells are defective in excision-repair (61). Both Arlett et al. (62) and

Maher et al. (63) report a synergistic decrease in survival of XP variants treated either with UV light or benz-α-pyrene when continuously fed 0.25-1.75 mM caffeine following carcinogen damage. No effects were seen in either normal human fibroblasts, or classical XP cells in these experiments. However, Fujiwara and Tatsumi recently showed that established XP cells (by SV 40 transformation) of Group A were sensitized by caffeine in terms of survival after UV irradiation (64).

Few studies have been done studying caffeine and mutagenesis in human cells, due to the lack of selection systems to quantitatively detect mutations in genes. Maher et al. (65) reported large increases in mutation frequencies to 8-azaguanine resistance in XP variants treated with UV followed by 0.75 mM caffeine treatment for the first three cell divisions following irridation.

Effect of Caffeine on DNA Synthesis

Lehmann (66) reports, from indirect alkaline sucrose sedimentation studies, that DNA is synthesized in smaller replicating units in mouse lymphoma cells (L5178Y) when grown in medium containing 1.6 mM caffeine. The overall rate of DNA synthesis, however, is not affected. Caffeine did not introduce strand breaks into preformed DNA, nor did it inhibit the rejoining of γ-ray induced strand breaks.

When DNA synthesis, measured by ^{3}H-TdR incorporation, was studied in chemically (MNU, SM) treated, Chinese hamster ovary cells, which were synchronized, 0.75 mM caffeine reduced the lag in the rate of synthesis, which usually is induced by these treatments (67). Unsynchronized UV-irradiated Chinese hamster V-79 cells showed a similar increase in the rate of ^{3}H-TdR incorporation if they were treated with 9 mM caffeine (unpublished results, Trosko). When treated with lower concentrations of caffeine very little effect on DNA synthesis in UV-irradiated V-79 cells was observed (68).

Domon and Rauth report of the effect of caffeine on Mouse L cells on cell progression and DNA synthesis (41, 69). Specifically, 2 mM caffeine alone caused small delays in the progression of G_1 cells to S phase and G_2 cells to mitosis, and increased the doubling time of such cells from 16 hours to 18 or 20 hours. Low exposures of UV irradiation (100 ergs/mm^2 or less) caused a specific block of irradiated cells through S phase. It was shown that the toxic effect of caffeine on irradiated Mouse L cells is well correlated with the mitotic delay of irradiated cells due to a block of S phase. Walters et al. (70) report that caffeine induced a state of G_1 arrest in exponentially growing Chinese hamster cells (CHO line). They also report of a reduction in the division delay

period following X-irradiation of caffeine treated cells. The X-ray induced reduction of phosphorylation of lysine rich histone F1 was less in caffeine treated cells than in untreated cells. Survival after X-ray irradiation was only slightly reduced in caffeine treated cells.

Excision Repair, Unscheduled DNA Synthesis, and Repair Replication

Trosko and Wilder have shown that the excision of UV-induced pyrimidine dimers in tissue culture cells of Drosophila was not sensitive to 1 mM caffeine treatment (71).

Cleaver found 1 mM caffeine to have no effect on repair replication or unscheduled DNA synthesis in UV-irradiated HeLa S3 cells (72).

Post-Replication Repair, Gap-Filling Repair, and Replicative Bypass Repair

Cleaver and Thomas (73) have shown from alkaline sucrose gradient sedimentation profiles, that 2 mM caffeine reduced the rate at which lower molecular weight DNA grew to higher molecular weight DNA after UV-irradiation in V-79 Chinese hamster cells, but not in the unirradiated control cells. This step is associated with semi-conservative DNA synthesis following UV irradiation.

Caffeine has also been reported to inhibit this shift in the molecular weight of DNA from V-79 cells that had been damaged with N-AcAAF (74), SM, and MNU (67).

Fujiwara (75) has shown caffeine (1, 2 mM) to inhibit gap-filling repair, measured by alkaline sucrose sedimentation studies in Mouse L5, but not in HeLa S3 cells. Although he showed a different line of HeLa-S3-91V were inhibited in post UV gap closure by 2 mM caffeine, as quoted by Schroy and Todd (57). In another paper, Fujiwara (76) reports that other metabolic inhibitors, such as actinomycin D, HU, Ara C, excess TdR, also inhibit post-replication repair to various extents. Cycloheximide allows cells to complete the gap-filling repair, and he concluded that a pre-existing polymerizing system is responsible for repair at earlier times up to 24 hours after UV irradiation without new protein synthesis. He used a high UV dose, 20 J/m^2 and a high concentration of caffeine, 2 mM. More recently, Fujiwara reports (64) that replicative bypass repair (which he considers a better description of "post-replication" repair than gap-filling) is dependent on the cell line as well as the cell type. He reports that transformed, established group A, XP cells were caffeine sensitive to inhibition of replication bypass, measured as BUdR incorporation following UV irradiation in the newly synthesized DNA on CsCl gradients. Unestablished XP group A cells were not caffeine sensitive.

Buhl and Regan (77) and Lehman et al. (78) have demonstrated that caffeine inhibited DNA chain elongation

and joining in UV-irradiated XP cells by 36 hours after irradiation. No effect of caffeine was seen in irradiated normal human cells or unirradiated cells.

Effects of Caffeine *in Vitro*

Chetsanga et al. (79) have demonstrated that caffeine (0.2 mM) enhanced the digestion of eucaryotic DNA from Mouse L cells by nuclease S_1 from Aspergillus orzae. This apparently was due to the denaturing effect (local unwinding) of caffeine on certain regions of the double helix, especially those rich in A and T bases, as demonstrated by a second, lowered melting temperature for the isolated DNA.

Caffeine has also been demonstrated to inhibit the elevation of DNA polymerase activity associated with damage to DNA by low doses of Mitomycin C (0.01 ug/ml) or aflatoxin B_1 (0.5 ug/ml) both in vivo and in vitro (80). Others have been unable to confirm this work by Wragg (81, 82).

A slight increase in DNA synthesis on extracted, irradiated DNA and isolated polymerases has also been reported (81).

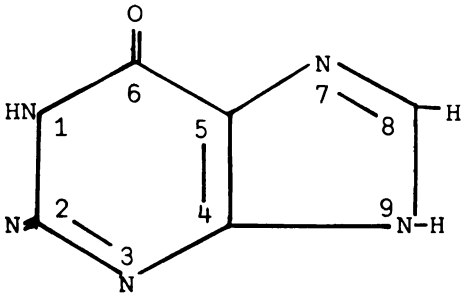
Caffeine increases the levels of cyclic 3'5' AMP by inhibiting phosphodiesterase enzymes in cells (83). Cyclic AMP has been implicated as mediating as a second messenger for various protein hormones and thus regulating gene expression (84).

Caffeine has also been shown to affect the transport of Ca^{++} across the sarcoplasmic reticulum (85); and to affect the transport of salicylic acid and other similar compounds across a simulated membrane approximating the conditions in the stomach (i.e., neutral buffer on one side [plasma pH] and a buffer of pH 3 on the other side [stomach pH]) (86).

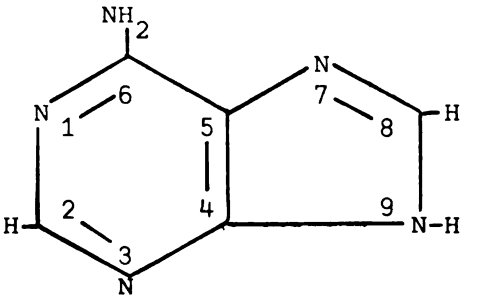
RATIONALE

Research interest in the effects of caffeine was initially stimulated due to the possibility that it might be a mutagen in man, since it is a purine, 1,3,7-trimethylxanthine (see Figure 2 for structures of purine analogues). Gene mutations were found to be induced by caffeine in procaryotic and eucaryotic microorganisms, but only with very high concentrations of caffeine treatment (9, 87) (about one hundred-fold more concentrated than the physiological level in the tissues of a heavy coffee drinker of ten cups/day). Peculiar chromosome aberrations, such as chromosome pulverization, were induced in higher eucaryotic systems, but again, only with very high concentrations of caffeine treatment (18, 88). Gene mutations induced by caffeine alone have not been observed in mammals, in either whole animals or in cells in culture (15, 16).

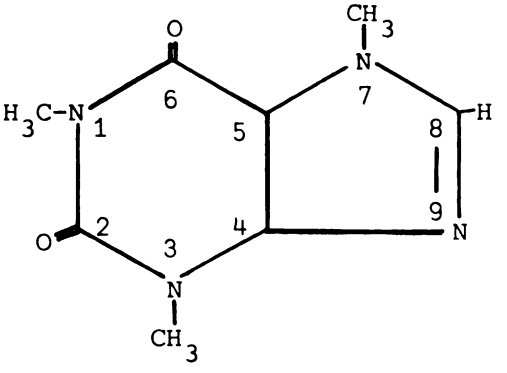
Data from UV-irradiated bacteria showed caffeine synergistically reduced the percent survival (21, 22, 23, 24) and increased the frequency of induced mutations (21, 24, 26, 27, 28, 29). Later, a reduction in the growth of "drug-resistant" tumors was shown when hamsters with cytoxan-resistant tumors being treated with cytoxan,



Guanine



Adenine



Caffeine

Figure 2. Structures of purines and caffeine.

nitrogen mustard, and X-rays were administered a 1.0% caffeine solution in their drinking water (89).

A surge of interest arose over these synergistic effects of caffeine. It seemed the effects were due to an inhibition of DNA repair mechanisms, especially when one considers that if caffeine treatment was delayed long enough no synergistic effects were observed. The concentrations of caffeine needed to produce these synergistic effects were ten-fold less than the mutation induction experiments required, but still much greater than physiological levels found in man.

Many reports, which seem to conflict, have accumulated in the literature with respect to these synergistic effects in mammals: reports of both increases and decreases in chemically or physically induced mutation frequencies, transformation frequencies, as well as negative results indicating no effect of caffeine at all in certain treated cells. Two reports which conflict describe increases (43) and decreases (44) in UV-induced mutation frequencies to 8-azaguanine resistance in V-79 Chinese hamster cells. Different interpretations regarding the number and type (i.e., error-free vs. error-prone) of DNA repair mechanisms and their sensitivity to caffeine resulted.

The specific purpose of this thesis was to resolve this conflict, and in general, to attempt to gain a better understanding of the mechanism of UV-induced mutagenesis in mammalian cells by using caffeine as a probe.

It was known that two important variables differed in the two reports mentioned above. One variable was the concentration of caffeine used, and the other was the duration of caffeine treatment. In bacteria different concentrations of post-UV treatment with caffeine, have been shown to inhibit different repair mechanisms (25). It also had been suggested that the presence of caffeine with the selection agent, 8-azaguanine, might be sensitizing mutant cells (38, 40).

Therefore, the question of what kind(s) of repair mechanism(s) is/are caffeine inhibiting was indirectly approached by studying changes in the frequency of UV-induced mutations, using various concentrations and time intervals of caffeine treatment. A different mutation system, measuring the frequency of resistance to ouabain, was used because of arguments questioning the nature of $HGPRT^-$ variants (90). Theoretically, both structural gene mutations and epigenetic repression of the DNA might produce an 8-azaguanine resistant cell. Whereas, with ouabain resistant mutants, conceivably only structural gene mutants would survive, since repression of the Na^+/K^+ ATPase would be fatal to the cell.

MATERIALS AND METHODS

Cell Culture

An aneuploid cell line, V-79, derived originally from the lung of a male Chinese hamster (Cricetulus griseus, 2N = 22) was used (91, 92). Cells were suspended in 10% dimethysulfoxide in phosphate buffered saline, sealed in glass ampules, and stored frozen in liquid nitrogen until needed for experimentation. Cell cultures were grown for experiments in monolayers on one side of glass prescription bottles in C-15 medium (described below). Incubation conditions were at 38°C, with 5% carbon dioxide and humid air. Cells were subcultured using 0.5% crystallized trypsin and heat (38°C), then diluted and seeded in medium.

In all experiments an appropriated number of cells were seeded in replicate 9 cm plastic petri plates (Falcon or Corning) and grown in deficient, D medium (described below), supplemented with antibiotics and 5% fetal calf serum.

Chemicals Used

C-15 medium is a modified Eagle's Minimum Essential Medium (MEM) (93), with Earle's Salts (Gibco, Grand Island,

N.Y.), supplemented with both essential and non-essential amino acids and vitamins as well as 1 mM sodium pyruvate, hypoxanthine, thymidine, uridine, and phenol red. The concentration of bicarbonate (HCO_3) is decreased to 15 g/10 l and 5% fetal calf serum is added before filtration. D medium is the same as C-15 except it is deficient for the nitrogenous bases, lacks phenol red and fetal calf serum, and the bicarbonate concentration is 10 g/10 l. Sterilization was done by millipore filtration with positive pressure. Medium was then stored at 0 to -20°C until use. Fetal calf serum (Gibco, Grand Island, N.Y.) was heat inactivated at 56°C for twenty minutes before usage. Antibiotics (Pfizer, Inc., New York, N.Y.), penicillin G (100 units/ml) and streptomycin sulfate (100 ug/ml) were prepared fresh, concentrated 1,000 times, and stored at 4°C until use. Caffeine (Nutritional Biochemicals Corp., Cleveland, Ohio) was prepared as a concentrated stock solution for most of the experiments (2 mM or 5 mM) in D medium and was sterilized by millipore filtration and stored frozen at 0 to -20°C. It was thawed and diluted to the desired concentration the day of use. In later experiments caffeine medium was prepared on the day of the experiments. Ouabain (Sigma Chemical Co., St. Louis, Mo.) was prepared in the desired concentration, 1 mM, dissolved in D medium and stored frozen at 0 to -20°C.

Cell Survival Assays

Caffeine Toxicity

To determine the toxicity of various concentrations of caffeine on Chinese hamster cells, 200 cells were plated in quadruplicate in medium containing 0 to 1.8 mM caffeine, and incubated for ten days. Colonies were rinsed in physiological saline (0.8%), fixed in 95% ethanol, stained with Giemsa, and counted. Plating efficiencies were determined from the average percentage of survivors in the four control plates, and the relative percentage of survivors were calculated.

Synergistic Effect of Caffeine with UV

To determine the percentage of cells treated with UV and caffeine that survived, a sufficient number of cells (so that approximately 200 colonies/plate would survive treatment) were seeded in triplicate plates for three and one-half hours before irradiation for attachment. With the medium removed from the plates the cells were exposed to ultraviolet light from a germicidal lamp (GE 15TW), positioned and calibrated with a Blak-ray UV meter, Model J-225 (UV Products, Inc., San Gabriel, Calif.) to deliver a flux of 1 $J/m^2/sec$. Medium containing caffeine was delivered immediately following irradiation, and cells were then incubated for ten days. (In the survival plates of the

mutation experiments, caffeine was present at the same time and for the same duration as in the mutation plates.) At ten days, the medium was decanted and colonies were rinsed, fixed, stained, and counted as described above. Plating efficiencies and survivals were calculated as already described.

In Vitro Mutation Assays

Mutagenicity of Caffeine

The effects of caffeine on mutagenesis were tested in an assay system involving forward mutations from ouabain sensitivity to resistance (94). Large numbers of cells (1×10^5) were seeded in thirty plates in medium containing caffeine. Forty-four to seventy-two hours later, the selective drug ouabain was introduced to cells with caffeine still present. By eleven days resistant colonies were visible, and counted, if there were at least thirty cells per colony. Plates were rescored to check for slow growing colonies, and false positives (variants that did not survive). Isolation of colonies, and rechecking the resistance to ouabain were done in 24-well plates. Survival plates were treated as described above.

Caffeine and UV Mutation Experiments

To determine the effects of caffeine on the frequency of UV-induced mutations, the same procedure was

followed as described above except that cells were allowed to attach for three and one-half hours before irradiation. Caffeine was then introduced at various times after irradiation for different durations. The optimum expression time, of 48 to 72 hours after irradiation had been systematically characterized in our lab (C. C. Chang, personal communication).

Reconstruction Experiments

To decide whether caffeine was selectively inhibiting the growth of ouabain resistant mutants, previously isolated resistant cells were seeded in quadruplicate plates with and without sensitive cells (to simulate survival and mutation conditions). They were allowed to attach for three and one-half hours in D medium, after which ouabain and caffeine were introduced to certain plates. After ten days, the colonies were rinsed, fixed, stained, and counted.

RESULTS

Toxicity and Survival

The highest nontoxic concentration of caffeine used in this study was determined to be 0.9 mM, from the toxicity curve in Figure 3.

Three concentrations of caffeine were chosen, 0.2, 0.5, and 0.9 mM, to analyze the dosage effects of caffeine on UV-induced cell killing. As shown in Figure 4, a greater synergistic reduction in survival was observed with a higher UV dose and/or caffeine concentration.

Mutation Assays

Cells treated with caffeine alone, and then assayed for mutations resistant to 1 mM ouabain, did not differ greatly from untreated cells. However, a decreasing trend is observed, and the mutation frequency of cells treated with 0.9 mM caffeine was decreased about four times as compared to the control, see Table 1.

Due to the extremely low survival of irradiated cells treated with 0.9 mM caffeine, 0.5 mM was the highest concentration used to analyze the mutagenic response of cells to post-irradiation treatment with caffeine.

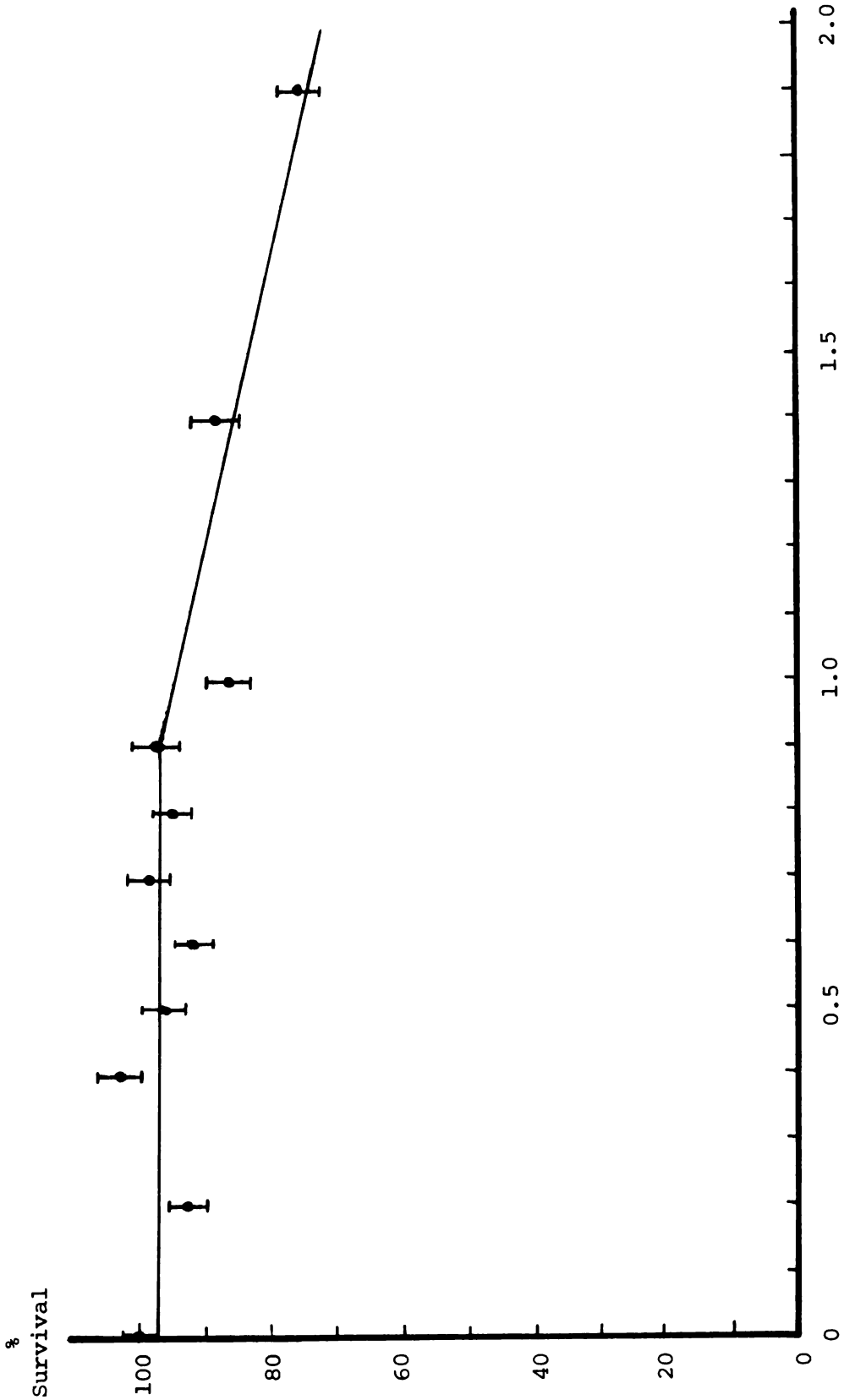


Figure 3. Caffeine toxicity curve.

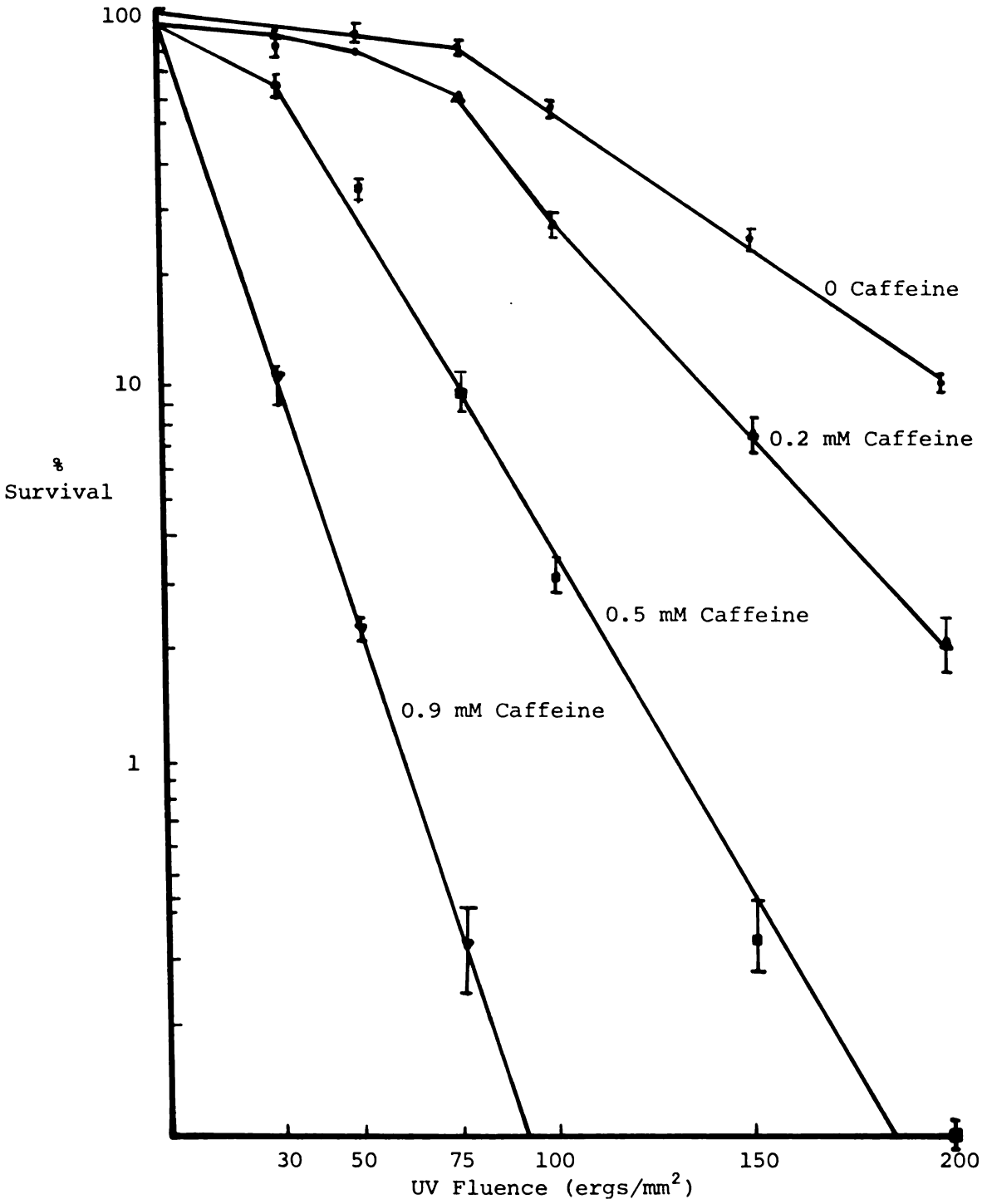


Figure 4. Dose-response curves using various concentrations of caffeine.

Table 1. Survival and frequency of spontaneous mutations from ouabain sensitivity to resistance after treatment with caffeine

Caffeine[a] (mM)	% Survival[b]	Mutation Frequency per 10^6 Survivors (No. of Mutants)
0[c]	100	4.8 (10)
0.2[c]	111	4.8 (11)
0.5[c]	108	4.0 (9)
0.9[c]	120	1.0 (3)

[a]Cells were plated in medium containing caffeine, and remained in it for entire incubation period.

[b]Plating efficiency of the control = 70%.

[c]Number of cells seeded and plates (9 cm) used: $30 \times 10^5/30$.

Observations from the three basic treatment periods studied are presented in Table 2.

In Experiment 1, caffeine, while present only during the mutation fixation and expression time (0-44 hours after UV treatment), increased the mutation frequency, three times with the 0.2 mM concentration and eleven times with 0.5 mM caffeine. Survival was reduced 30% with 0.2 mM caffeine and about 90% with 0.5 mM caffeine when compared to the control, which was only treated with UV.

Caffeine was present for the entire incubation period in Experiment 2, and the mutation frequency decreased two-fold with both concentrations used. Survival was reduced only 12% with 0.2 mM caffeine, and 60% with 0.5 mM caffeine.

To discover whether caffeine was influencing the expression of mutations, apart from its effects on DNA repair, the addition of caffeine was delayed until 44 hours after UV irradiation in Experiment 3. As would be predicted, cell killing was not affected, however a large decrease in mutation frequencies was measured; a three-fold decrease with 0.2 mM and a six-fold decrease with 0.5 mM. (It should be noted that a slight increase in cell survival of 12% and 8% was observed with this treatment.)

To be certain that the differences in the results of the three experiments described above were not due to

Table 2. Survival and frequency of mutations from ouabain sensitivity to resistance after UV and caffeine treatment

Experiment[a]	UV Fluence (J/m^2)	Caffeine (mM)	% Survival	Mutation Frequency per 10^6 Survivors (No. of Mutants)
1	0[c]	0	100	14 (46)
	7.0[d]	0	94	28 (105)
	7.0[e]	0.2	65	97 (296)
	7.0[f]	0.5	6.6	346 (137)
2	0[c]	0	100	1.4 (4)
	7.0[d]	0	86	27 (82)
	7.0[e]	0.2	74	16 (48)
	7.0[f]	0.5	26	16 (22)
3	0[c]	0	100	5.4 (13)
	7.0[d]	0	82	18 (42)
	7.0[d]	0.2	94	5.9 (16)
	7.0[d]	0.5	90	3.0 (8)

[a]Experiment 1 = caffeine was present during the expression period only, i.e., for 44 hours following UV irradiation.
Experiment 2 = caffeine was present for entire post-irradiation period, i.e., for approximately 240 hours.
Experiment 3 = caffeine was added 44 hours after UV irradiation, and was present for the remainder of the incubation period.

[b]Plating efficiency for controls: Experiment 1 = 112%; Experiment 2 = 96%; and Experiment 3 = 80%.

[c-f]Number of cells seeded and plates (9 cm) used: c = $30 \times 10^5/30$; d = $36 \times 10^5/18$; e = $42 \times 10^5/21$; f = $54 \times 10^5/18$.

differences in environmental conditions, such as pH, CO_2 levels, temperature, or humidity, all three treatments were grouped in one experiment, which was done two times. A slightly higher dose of UV was used to increase the induced number of mutants recovered. The results shown in Tables 3 and 4 are in fair agreement with respect to caffeine'e effect on the survival of irradiated cells. In each case survival is either reduced slightly less or is approximately equal to that in the first series of experiments. However, some noticeable differences were observed in the changes in the induced mutation frequencies, especially for the 0-48 hour treatment group. Both increases and decreases in the mutation frequencies were observed. The 0-240 treatment periods showed a reduction in the mutation frequency for the 0.2 mM treatment group in both experiments, and showed the mutation frequency of one group increase and the other decrease for the 0.5 mM treatment group in the two experiments. The results from the 42-240 hour treatment group agreed with the results of the previous experiment; both concentrations caused a reduction in the induced mutation frequencies. However, it should be noted that in Table 3 0.5 mM caffeine did not reduce the mutation frequency much more than 0.2 mM caffeine, and in Table 4 0.2 mM caffeine reduced the frequency to a greater extent than 0.5 mM caffeine.

Table 3. Survival and frequency of mutations from ouabain sensitivity to resistance after UV and caffeine treatment

UV Fluence (J/m^2)	Caffeine (mM)	Time Period Caffeine Present (hrs.)	% Survival[a]	Mutation Frequency per 10^6 Survivors (No. of Mutants)
0[b]	0	--	100	37.9 (117)
10.0[c]	0	--	70	76 (94)
10.0[d]	0.2	0[h]-48	48	55 (98)
10.0[e]	0.5	0-48	13	109 (78)
10.0[f]	0.2	0-240	51	45 (98)
10.0[g]	0.5	0-240	14	95 (85)
10.0[f]	0.2	42-240	65	32 (91)
10.0[f]	0.5	42-240	62	30 (81)

[a]Plating efficiency of the control = 103%.

[b-g]No. of cells seeded and plates (9 cm) used: b = 30 x 10 /30; c = 17 x 10^5/17; d = 36 x 10^5/18; e = 54 x 10^5/18; f = 42 x 10^5/21; g = 63 x 10^5/21.

[h]0 time is when cells were UV irradiated, caffeine was added immediately afterwards.

Table 4. Survival and frequency of mutations from ouabain sensitivity to resistance after UV and caffeine treatment

UV Fluence (J/m^2)	Caffeine (mM)	Time Period Caffeine Present (hrs.)	% Survival	Mutation Frequency per 10^6 Survivors (No. of Mutants)
0[b]	0	--	100	7.8 (12)
10.0[c]	0	--	42	101 (39)
10.0[d]	0.2	0[i]-48	30	119 (64)
10.0[e]	0.5	0-48	9	180 (55)
10.0[f]	0.2	0-240	29	41 (24)
10.0[g]	0.5	0-240	11	69 (29)
10.0[f]	0.2	48-240	43	19 (17)
10.0[h]	0.5	48-240	37	51 (40)

[a]Plating efficiency of the control = 102%.

[b-h]No of cells seeded and plates (9 cm) used: b = $150 \times 10^4/30$; c = $90 \times 10^4/18$; d = $18 \times 10^5/18$; e = $34 \times 10^5/17$; f = $20 \times 10^5/20$; g = $38 \times 10^5/19$; h = $19 \times 10^5/19$.

[i]0 is the time when cells were UV irradiated, caffeine was added immediately afterwards.

To further refine the effect of caffeine on mutagenesis in terms of time, thus separating the different effects on DNA repair and on expression of ouabain resistance, another experiment was done, the results of which are shown in Table 5. In agreement with Experiment 1 of Table 2, an increase (about two-fold) in mutation frequency was observed along with a 26% drop in survival when caffeine was present for only 24 hours after irradiation. The next 24 hour treatment period showed a very slight drop in the mutation frequency, although survival was reduced 13%. Post-expression time treatment with caffeine for twelve hours was sufficient to reduce the mutation frequency (more than two-fold). A longer and later treatment period of 72-240 hours reduced the induced mutation frequency five-fold. A downward trend was also observed in the spontaneous mutation frequency when continuously treated with caffeine.

Figure 5 represents the results of the first experiment done to determine the minimum time caffeine has to be present to increase the frequency of induced mutations. In this experiment, it was not until 18 hours of treatment that caffeine noticeably increased the mutation frequency (about three-fold); survival was reduced to 4% in this group. Surprisingly, a three-fold decrease was observed in the induced mutation frequency with the eight hour post-irradiation caffeine treatment.

Table 5. Survival and frequency of mutations from ouabain sensitivity to resistance after UV and caffeine treatment

UV Fluence (J/m^2)	Caffeine (mM)	Time Period Caffeine Present (hrs.)	% Survival[a]	Mutation Frequency per 10^5 Survivors (No. of Mutants)
0[b]	--	--	100	0.53 (6)
10[c]	--	--	28	38 (179)
10[d]	0.5	0-24	2	73 (67)
10[e]	0.5	24-48	15	33 (198)
10[f]	0.5	48-60	33	16 (173)
10[e]	0.5	60-72	41	16 (254)
10[g]	0.5	72-240	35	7.7 (110)
0[h]	0.5	0-240	110	0.19 (3)

[a]Plating efficiency of the control = 98%.

[b-h]No. of cells seeded and plates (9 cm) used: b = 115 x 10^4/22; c = 17 x 10^5/17; d = 51 x 10^5/17; e = 40 x 10^5/20; f = 34 x 10^5/17; g = 42 x 10^5/21; h = 15 x 10^5/30.

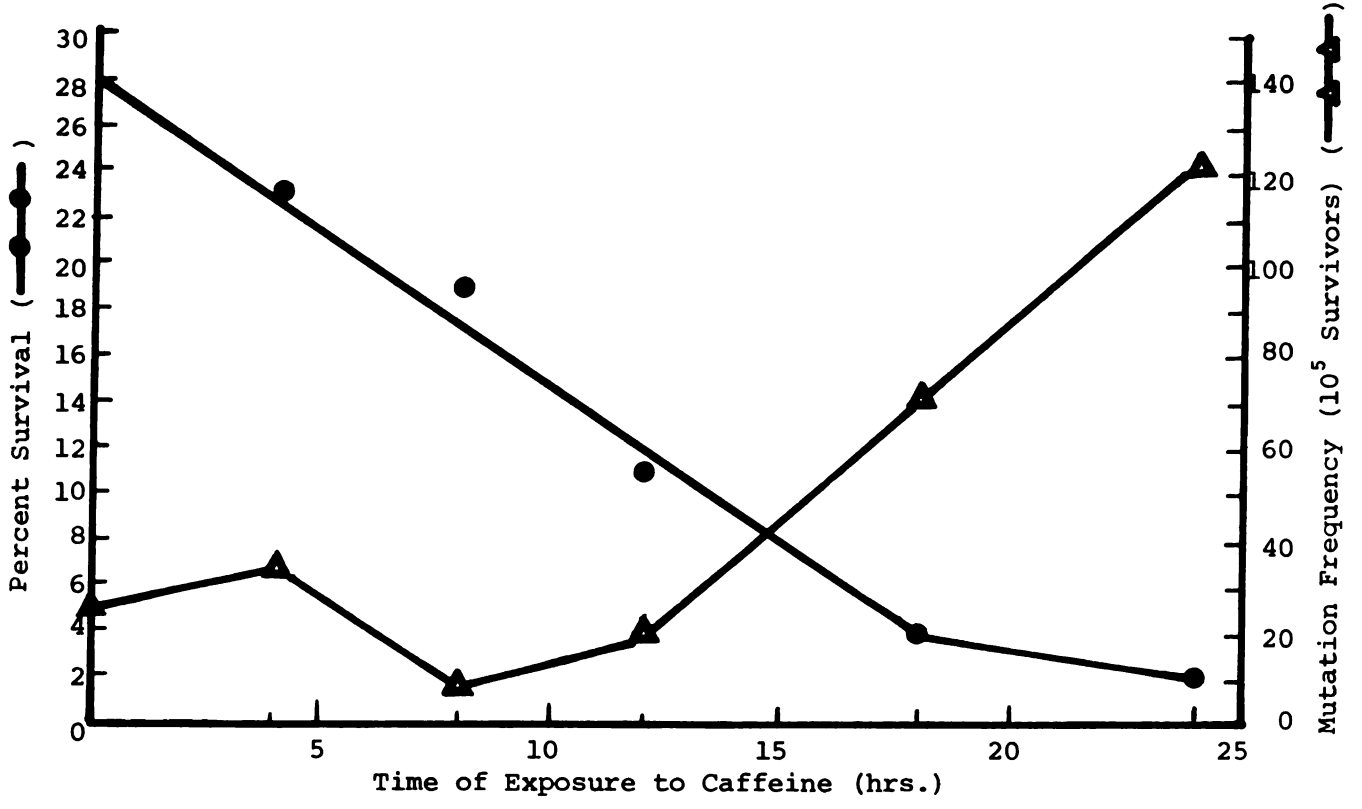


Figure 5. The effect of increasing periods of time of caffeine treatment following UV irradiation on both survival (●) and mutation frequency (▲).

Treatment of irradiated cells for four-hour periods gave more surprising results, as shown in Table 6. The first four-hour treatment group showed an increase in the induced mutation frequency of about two-fold, although no increase was observed in any of the following periods, including the 0-18 hour treatment group. Survival was reduced the most in the 12-16 hour group, when one compares the four-hour treatment groups.

It was noticed that short intervals of caffeine treatment (up to 24 hours) immediately after irridation increased the induced mutation frequency only when the survival was very low (less than 10%). Therefore, a UV-dose response of the mutation frequencies to the survival of cells treated with caffeine, mainly for 18 hours was done. A new cell culture from the frozen stock was used. Interestingly, the frequencies of mutations were not changed when treated with 0.5 mM caffeine either for 18 hours or for 24 hours, even at a low percent survival (5%), see Table 7.

To determine whether the reduction in mutation frequency observed with post expression time treatment with caffeine was due to ouabain resistant mutants being more sensitive to caffeine than wild-type cells, two reconstruction experiments were performed. The results of both experiments (only one is shown in Table 8) showed

Table 6. Survival and frequency of mutations from ouabain sensitivity to resistance after UV and caffeine treatment

UV Fluence (J/m^2)	Caffeine (mM)	Time Period Caffeine Present (hrs.)	% Survival[a]	Mutation Frequency per 10^6 Survivors (No. of Mutants)
10[b]	0	--	68	24 (16)
10[c]	0.5	0-4	58	67 (34)
10[d]	0.5	4-8	51	36 (17)
10[e]	0.5	8-12	42	32 (26)
10[e]	0.5	12-16	35	36 (25)
10[e]	0.5	16-20	44	38 (32)
10[d]	0.5	20-24	48	29 (13)
10[f]	0.5	0-18	22	27 (23)
10[d]	0.5	44-48	61	27 (15)

[a]Plating efficiency of the control = 108%.

[b-f]No. of cells seeded and plates (9 cm) used: b = $90 \times 10^4/18$; c = $80 \times 10^4/16$; d = $85 \times 10^4/17$; e = $18 \times 10^5/18$; f = $36 \times 10^5/18$.

Table 7. Survival and frequency of mutations from ouabain sensitivity to resistance after UV and caffeine treatment

UV Fluence (J/m^2)	Caffeine (mM)	Time Period Caffeine Present (hrs.)	% Survival[a]	Mutation Frequency per 10^5 Survivors (No. of Mutants)
0[b]	--	--	100	8.2 (93)
5[c]	--	--	99	17.4 (111)
7.5[d]	--	--	79	25.1 (136)
10.0[d]	--	--	58	28.4 (111)
15.0[e]	--	--	17	70.8 (109)
5.0[f]	0.5	0-18	70	20.6 (198)
7.5[g]	0.5	0-18	34	23.2 (167)
10.0[h]	0.5	0-18	17	30.9 (238)
15.0[i]	0.5	0-18	4	58.2 (256)
10.0[j]	0.5	0-24	17	26.0 (100)

[a]Plating efficiency of the control = 76%.

[b-j]No. of cells seeded and plates (9 cm) used: b = $150 \times 10^4/30$; c = $85 \times 10^4/17$; d = $90 \times 10^4/18$; e = $12 \times 10^5/12$; f = $18 \times 10^5/18$; g = $28 \times 10^5/14$; h = $60 \times 10^5/12$; i = $150 \times 10^5/15$; j = $30 \times 10^5/6$.

Table 8. The effect of caffeine on the colony formation of ouabain resistant and sensitive cells grown in medium with and without ouabain

A. Normal Medium--D

	Without Caffeine		With Caffeine[a]	
Cells/Plate	P.E.	Survival	P.E.	Survival
100 oua^s	129.5 ± 7.4[b]	100	132.0 ± 6.0	101.9
100 oua^r	75.3 ± 5.8	100	73.0 ± 5.4	97.0

B. Normal Medium Containing 1 mM Ouabain

	Without Caffeine	With Caffeine
	Average # Colonies	Average # Colonies
Cells/Plate	Plate	Plate
100 oua^r 1 x 10^5 oua^s	70.3 ± 1.7	87.6 ± 4.0
100 oua^r 4 x 10^4 oua^s	79.0 ± 7.2	72.5 ± 5.0
1 x 10^5 oua^s	0.0	0.0

[a] 0.9 mM caffeine was used.

[b] S.E.M.

no difference in the survival of ouabain-resistant (oua^r) cells over ouabain-sensitive (oua^s) cells in normal medium, either with or without caffeine.

To determine when newly induced mutant cells lose their sensitivity to caffeine, an experiment was constructed where 0.5 mM caffeine was not added until five or eight days after the addition of selective medium. The mutation frequency was reduced two-fold in the 5-21 day caffeine treatment group, and about one and one-half-fold in the 8-21 day treatment group; the results are shown in Table 9. Colonies were in a 16 cell stage (i.e., had divided about four times at five days).

Isolation of twelve random mutant colonies from each treatment group and testing them for sensitivity to caffeine showed no effect (results not shown).

Table 9. Survival and frequency of mutations from ouabain sensitivity to resistance after UV and caffeine treatment

UV Fluence (J/m^2)	Caffeine (mM)	Time Period Caffeine Present (hrs.)	% Survival[a]	Mutation Frequency per 10^5 Survivors (No. of Mutants)
0[b]	0	--	100	13 (14)
10[c]	0	--[g]	53	126 (374)
10[d]	0.5	120-528[g]	57	62 (236)
10[e]	0	--[g]	51	126 (250)
10[f]	0.5	192-528[g]	51	82 (148)

[a]Plating efficiency of the control = 70.6%.

[b-f]No. of cells seeded and plates (9 cm) used: b = 150 x 10^4/30; c = 80 x 10^4/18; d = 95 x 10^4/19; e = 55 x 10^4/11; f = 50 x 10^4/10.

[g]Caffeine was added 5 and 8 days (d and f), respectively, after the addition of ouabain, medium in the control plates (c and e) were also changed at these times.

DISCUSSION

The Synergistic Effect of Caffeine on UV-Induced Cell Death

Figure 4 confirms work done by others in different systems, such as mouse L cells (40, 41), normal human epithelial cells (57), XP variants (62, 63), transformed XP group A cells (64), certain HeLa cells (57, 64), as well as Chinese hamster V-79 cells (40, 43, 44). It also confirms the results obtained with different DNA damaging agents, such as N-AcAAF, EMS, MNNG, MNU as well as UV light in rodent cells (42, 45). The disappearance of the plateau on the survival curve of cells treated with caffeine following UV irradiation can be correlated with the well known inhibition of "post-replication" repair in certain mammalian cells by caffeine (67, 73, 74, 75). Caffeine has been reported to have no effect on the low level of repair synthesis (excision-repair) in mouse L cells (72). Chinese hamster cells also have low levels of repair synthesis, as measured by unscheduled DNA synthesis (unpublished results). The experiment described in Table 6, showed that most of the irradiated population of cells, which were not synchronized, were killed during the 12-16 hour post-irradiation caffeine

treatment period. This may be the time that most of the irradiated cell population is synthesizing DNA, and perhaps are dying due to the inhibition of caffeine sensitive repair mechanism associated with replication. Domon and Rauth (69) showed in synchronized mouse L cells that the toxic effect of caffeine on UV-irradiated cells was seen only if caffeine was present during the first S phase following irradiation. The significance of caffeine increasing the rate of normal DNA synthesis following UV irradiation (67) is not apparent presently.

Recent data from Fujiwara (64) cover differences in sensitivity to caffeine after UV-irradiation between cell types, both in terms of species and phenotype, with respect to the transformed state of the cell. It appears that some transformed cells (HeLa S3, XP 20S-Group A) are sensitive to caffeine after irradiation, although they are not when they are untransformed or normal. There also are reports of transformed cells which have no post-irradiation sensitivity to caffeine (HeLa [58], Chinese hamster V-79 [49]), as well as reports of normal human cells which have such a sensitivity (epithelial [57]). In conclusion, it is not clear what is occurring, except that certain factors in cells seem to alter the sensitivity of that cell to caffeine after UV-irradiation.

Similarly, as was noted in the results, there appeared to be a change or difference in the mutagenic response of cells which were more UV sensitive. This effect will be discussed further in the next section.

Effects of Caffeine on Induced Mutation Frequencies

It is clear that caffeine has a different effect on mutagenesis depending on when it is present. Immediately after irradiation it inhibits repair, which may be responsible for the decreased survival, and it seems to increase the frequency of induced mutations. There are two different possible explanations for this observed increase: (1) Mutation frequencies are calculated on the basis of the number of mutants per survivors. Therefore, if the number of survivors decrease, the mutation frequency can increase, even if the number of mutants observed for this specific UV dose and population of cells has not changed; or (2) the induced number of mutants actually is increased.

Possible ways of resolving whether reason number two is valid and which mechanism is correct would be the isolation of different Chinese hamster DNA repair mutants, each completely deficient in either excision-repair, post-replication repair, or error-prone repair. Testing these to determine the effect of caffeine on UV survival and mutagenesis would verify which repair mechanism is inhibited

by caffeine, and clarify how mutations arise from UV light. The most direct proof would be isolation and characterization of the DNA repair enzymes, and to study their activity with damaged DNA *in vitro* in the presence of caffeine. (Although, if the metabolism of caffeine *in vivo* is producing a repair inhibiting metabolic, this metabolite would have to be used instead of caffeine in the *in vitro* assay.) Both of these routes of repair mutant isolation and enzyme characterization presently are very difficult to accomplish. However, use of human repair mutants such as XP, which already exist, may be exploited at the present time.

Another way to determine when mutations are being produced would be to do mutation experiments on synchronized cells. It might be assumed that UV-induced lesions have different degrees of effect on the survival of cells at different times in the cell cycle. Using caffeine at different times after UV (before and after S phase) will verify when it influences the mutagenic effect.

In the results it was mentioned that a difference in the control level of survival of certain cells to the same dose of UV (10 J/m^2) was observed, see Tables 3, 4, 5, 6, 9, and Figure 4. Since their sensitivity seemed heightened, as shown by both survival and frequency of mutations induced, the possibility arose that a phenotypic difference in the cell population might exist. A new vial

of cells, from the same original population as the "changed cells" has initially produced similar results as the earlier populations of cells. Unfortunately, it will be difficult to test the hypothesis of a phenotypic difference since the "old cells" have not been maintained, and the only possible way to test it would be to continue to maintain the present cells for six months or longer, and determine if there is any increased sensitivity to UV and the production of mutations. A determination of the minimum time needed for caffeine to increase the induced frequency of mutations is underway.

The inhibition of caffeine on the recovery of mutants was seen consistently when caffeine was added to cells in the beginning or in the middle of the selective treatment. Twelve colonies of newly induced mutants (three weeks old) were not sensitive to caffeine either in D medium or medium containing ouabain, nor were mutants which had been used for reconstruction experiments more sensitive to caffeine than wild type cells.

This effect is not related to repair, although hypothetically there may be a similar basis for both observed effects (i.e. inhibition of repair and mutant recovery). The simplest explanation for this observed inhibition is the repression by caffeine of the mutant Na^+/K^+ ATPase gene, or of a regulatory gene controlling

Na^+/K^+ ATPase expression in newly induced mutants, thus inhibiting RNA transcription. It is plausible that the physical state of the DNA in newly induced mutants and "already expressed" mutants is different, and that the former is sensitive to "repression" by caffeine.

Before continuing to describe other levels at which caffeine may be inhibiting the recovery of mutants, it should be noted that caffeine is known to increase cyclic AMP concentrations by inhibiting the enzyme phosphodiesterase (83), and that the change in cAMP concentration may be responsible for inducing or inhibiting the expression of repair enzymes. Of course, first it must be demonstrated that cAMP is elevated in these cells after addition of caffeine, after irradiation, and later.

Caffeine may be decreasing recovery of ouabain resistant mutants by inhibiting (1) DNA replication of unexpressed mutants in ouabain; (2) RNA processing and translation of the mutant gene; (3) transport and assembly of the gene product; or (4) functioning of the mutant enzyme. It would be interesting to see if caffeine also inhibits the frequency of mutants of other genes, especially since resistance to ouabain is a membrane related phenomena and caffeine has been shown to affect the membrane transport of Ca^{++} in the sarcoplasmic reticulum (85).

The conflicting results of Trosko and Chu (44) and Arlett and Harcourt (43) seem in part resolved, since the two groups used caffeine for different periods of time. To complete an analysis, 0.5 mM and 1.0 mM caffeine should be added to UV treated cells after the proper expression time to determine its effect during selection with 8-azaguanine. One would expect based on my results and interpretation that caffeine would decrease the frequency of $HGPRT^-$ mutants induced (see concluding speculations for the mechanism).

Fox (40) also analyzed the differences between treatment procedures, and concluded that the mutation frequency was decreased due to a greater inhibition on the expression time of cells treated with caffeine for 0-200 hours. This seems not to be true in the ouabain system since the caffeine is added five days after ouabain treatment began (see Table 5), the mutation frequency still decreased. By this time if a cell has not expressed its mutation it has died due to ouabain toxicity (C. C. Chang, personal communication). She also concluded, based on her results, that caffeine was not inhibiting an error-prone repair mechanism. My results also might seem to indicate this to be true.

Concluding Speculations

The overall general aim of this research was to elucidate the mechanisms of mutagenesis in mammalian cells. It seems that in bacterial systems where the level of organization is much simpler, mutagenesis is still very complicated (see review by Witkin [95]). As mentioned in the literature review, the biochemical effects of caffeine are many, its metabolic products in different cells are not well identified and the effects of increases in its metabolic products, such as the purine pool and extra methyl groups are not well known. Therefore, a precise interpretation of the effects of caffeine on various biological systems at the molecular level is difficult due to many unknown variables changing simultaneously and thus the following remarks may be incomplete.

Evidence for a post-replication (gap-filling) mechanism of repair which occurs during or after the first round of replication following DNA damage has been critically evaluated as unsubstantial (96). Higgins et al. (97) have described and shown evidence for a "replication repair" mechanism which is error-free. From the results of this thesis, as well as others observations (40, 43, 44, 45), it seems reasonable to assume that caffeine might be exerting its biological effects on survival and mutations when present immediately after irradiation, by inhibiting

this repair mechanism. Molecularly, this might occur either by caffeine intercalating and preventing "branch migration," or by caffeine binding to the single strand ends and preventing the polymerization step. Cells which survive should have a higher ratio of error-prone to error-free repair, according to this hypothesis. The error-prone repair by which UV-lesions become mutations is apparently caffeine insensitive, and must be sought out in future experiments. It may be a constituitive mechanism (although the spontaneous rate of mutations would probably be very high), therefore the idea of an inducible "SOS" error-prone repair enzyme in mammalian systems, as found in E. coli (95), is appealing. In conclusion, it seems reasonable that the synergistic reduction in survival may be due to (1) huge deletions in DNA of cells that were not repaired; (2) cells not continuing to divide due to inhibition of DNA relication and (3) lethal mutations formed via error-prone repair. It is also possible that the equivocally increased mutation frequencies may be due to the increased ratio of error-prone to error-free repair.

The decreased mutation frequencies observed when caffeine is not added until after selection clearly appears after the repair process is completed. Trosko et al. (98) and Chang et al. (99) have presented a convincing scheme for an epigenetic mechanism of modifying mutation frequencies

via cAMP/cGMP concentrations based on data from phorbol ester and caffeine treated cells. Briefly, it appears that caffeine and phorbol seem to have diametrically opposite effects on cells in terms of "turning-on" or "turning-off" genes. As mentioned earlier, caffeine increases the level of cAMP; and phorbol ester increases the level of cGMP (100). One can therefore predict based on the observations of treatment with one agent what the results should be with the other. The only time when results seem inconsistent is immediately after UV, both phorbol ester and caffeine treatment produce increased mutation frequencies in the same mutation assays. But as described above, caffeine at least, if not phorbol ester, is having a distinct effect on inhibition of repair, which appears unrelated to cAMP at present. One experiment mentioned earlier in reference to resolving the conflict between Trosko et al. (44) and Arlett et al. (43) which might also be done to test this cAMP/cGMP theory is the addition of caffeine with 8-azaguanine to cells after repair is completed. It is predicted that the frequency of mutations should decrease if caffeine is "turning-on" these genes. Another interesting experiment might be to increase cAMP and cAMP either directly by adding them or a corresponding analogue (dibuteryl cAMP), or indirectly by using other agents which would alter the ratio of these two compounds in Chinese

hamster fibroblasts without interacting with mutagenesis directly on their own as caffeine does.

Long-range goals of understanding cancer and aging may be reached by gaining a better understanding of the process of mutagenesis (for a more detailed explanation see Trosko et al.) (98). Since DNA repair mutants of mammalian cells are not easily accessible presently, the use of caffeine as an inhibitor and modulator of biological processes provides a valuable way to gain new knowledge.

SUMMARY

Caffeine was found to have different effects on the frequency of UV induced mutations to ouabain resistance in Chinese hamster V-79 cells. The differences were dependent on the time after irradiation during which caffeine was present. When present immediately after irradiation for only 48 hours, survival was drastically reduced and mutation frequencies increased. But if 48 hours passed until the addition of caffeine after irradiation, no effect was seen on survival, and the recovery of mutants decreased. Possible explanations concerning DNA repair mechanisms and gene expression are discussed.

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