THE EFFECT OF SEPARATED CHEMICAL CARCINOGEN TREATMENTS IN VITRO

by

Peggy Margaret Warren

B.Sc., University of British Columbia, 1972

A Thesis Submitted in Partial Fulfilment of the

Requirements for the Degree of

MASTER OF SCIENCE

in Zoology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

July, 1974

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Zoology

The University of British Columbia
Vancouver 8, Canada

Date July 18, 1974

ABSTRACT

Previously, *in vitro* studies of chemical carcinogens have been focussed on determining the effects of single, high doses; however, cells *in vivo* are exposed to varying low doses of numerous chemicals at varying intervals. Consequently, this study was initiated to investigate the effects of separated, low doses of a chemical carcinogen *in vitro*. Monolayer cultures of human skin fibroblasts were exposed to 4-Nitroquinoline 1-Oxide (4NQO) and were challenged at varying intervals (1 1/2, 2, 3, 5, 9 and 13 hours) with a second 4NQO treatment. To evaluate the effects, three end points were employed: DNA repair capacity, cell survival, and chromosome aberrations.

Following exposure to an initial, single dose of 4NQO, the time course of DNA repair synthesis (as measured by ^{3}HTdR incorporation) was determined. The peak of repair synthesis was evident in the second and third hour after addition of the carcinogen. DNA repair synthesis was virtually complete at 12 hours post-treatment.

When cells received a second 4NQO treatment within 3 hours of the first, the level of repair synthesis induced by this second dose was far below an expected value. With 9 hours incubation between treatments, repair synthesis after the second dose was at the expected level.

Replacement of the first 4NQO treatment with a UV treatment produced analogous results.

The clone forming capacity of cells exposed to split 4NQO treatments was investigated. A potentiation of effects was evident when

the two treatments were spaced less than 2 hours apart. With a 9 hour interval between treatments the cloning capacity was again at the expected value.

A direct proportionality between an increase in the frequency of chromosome aberrations and a reduction in the interval between treatments was observed. As the interval between treatments increased (up to 9 hours) the frequency of chromosome aberrations decreased.

The data indicate that when a second 4NQO treatment is applied close to the first, complete repair of the resultant damage does not occur. This absence of DNA repair may increase the carcinogenic potential of the chemical carcinogen.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. H. F. Stich for his interest and advice throughout this research, and to Mrs. Stich for her many words of encouragement.

I also wish to thank Lydia Chen, Dorothee Kieser and Brian Laishes for their stimulating discussions, Charlie Yoshizawa for her undying patience and excellent technical assistance, and especially Richard San for his numerous, helpful suggestions.

I am also grateful to Dr. R. L. Noble for providing the opportunity and the facilities to carry out this research project.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Both autoradiographic and biochemical data indicate that most carcinogens interact with nuclear DNA in such a manner that the DNA is altered[48]. This altered DNA then becomes a substrate upon which repair enzymes can act[53,67]. In spite of the abundance of research focussed on the problem, the question of how these interactions are related to carcinogenesis still remains unsolved. An understanding of these interactions may well provide an insight as to the mechanisms of chemical carcinogenesis.

Repair of DNA damage certainly plays a major role in restoring a cell to its normal functional state[28,33,36,53]. However, when one attempts to account for carcinogenesis, the amounts of residual, unrepaired DNA damage may be of even greater importance[12].

Treatment of human fibroblasts in culture with a highly carcinogenic compound will induce high levels of DNA repair synthesis[74], yet this does not necessarily prove that the normal activity of the cell will be restored. When large amounts of DNA damage are present, there is an increased possibility that unrepaired segments will remain or that imperfect repair will occur. If a cell enters into DNA replication with damaged and/or unrepaired DNA molecules and survives, it could become genetically altered and in turn acquire the potential to undergo neoplastic transformation[12,33].

Direct proof of this particular hypothesis has not yet been obtained, however an almost complete lack of DNA repair capacity has been correlated with tumorigenesis[8,9]. Patients with Xeroderma Pigmentosum, have an increased sensitivity to sunlight, resulting in numerous skin tumors in the exposed areas. The cells of these patients show a decreased capacity for repairing certain types of DNA damage [8,12,73,75], and it has been shown that the lack of an endonuclease activity is responsible for this effect[9,68].

Returning to normal human cells, if high levels of DNA damage (and concomitant DNA repair synthesis), imply increased carcinogenic potential, then one should be able to demonstrate a correlation between these two factors. This has proven to be the case[33]. Highly carcinogenic chemicals (as indicated by in vivo studies), elicit high levels of DNA repair synthesis (as shown by the unscheduled uptake of ^{3}HTdR), DNA breaks (as measured by alkaline sucrose gradients), decreased cell survival, and increased chromosome damage. On the other hand, chemicals with low carcinogenicity elicit little or no DNA repair synthesis, few DNA breaks, normal levels of cell survival (as compared to that of untreated controls), and few to none chromosome aberrations.

When screening for the carcinogenic potential of a chemical compound in vitro, these correlations are usually exploited. However, the major drawback of all such studies is that they have been employed to investigate only the effects of exposure to a single dose of one carcinogen. Furthermore, the doses used were so high that cell survival was negligible. Yet cells in vivo are usually exposed to varying low

doses of numerous chemicals at different intervals. The effect of such exposures on the cell and on the DNA repair mechanisms has not been investigated. Perhaps many of these chemicals act synergistically with the net result of increasing their carcinogenic potential. Since it has already been demonstrated *in vivo* that certain chemicals (named co-carcinogens), do possess the capacity to enhance the tumorigenic potential of a carcinogen[79], and since the ultimate goal in screening carcinogens *in vitro* is the determination of the total carcinogenic potential of a chemical compound; it is obvious that an *in vitro* assay system must be developed such that the effects of more than one carcinogen treatment can be carefully examined. The main objective of this study then, was to make an attempt at developing such a system.

Rather than examining the effects of various combinations of chemicals it was decided to investigate multiple dosages of one chemical. The system was designed to answer one major question: in what manner does a cell that is already in the process of repairing DNA damage respond to further damage, i.e. as the interval between treatments increases, what happens to the repair capacity: is there a period in which it is reduced and/or enhanced?

The chemical chosen for this study was 4-Nitroquinoline 1-Oxide (4NQO). This choice was made for two reasons. Firstly, its biological effects have been extensively studied; previous investigations have shown 4NQO to be highly oncogenic *in vivo*[35,51], mutagenic *in vitro*[22,50], and cell-transforming *in vitro*[64]. 4NQO also binds to DNA[39,41,44,47,77], elicits DNA repair synthesis[74], and produces chromosome aberrations[72].

Secondly, it is highly soluble in water, does not precipitate nor degrade readily when placed in medium[45].

To achieve the desired end point, cell cultures were exposed to a single 4NQO treatment, and were then challenged at varying intervals to a second dose of 4NQO. Levels of repair synthesis were determined as well as the effect on cell survival and chromosome aberrations.

MATERIALS AND METHODS

I. Cell Cultures

a) Media. For these studies the cells were maintained in two types of media; Eagles Minimal Essential Medium (MEM) (Grand Island Biological Co.) and MEM deficient in arginine (arginine deficient medium, ADM). Both ADM and MEM were routinely supplemented with the following:

1) Antibiotics: streptomycin sulfate, 29.6 µg/ml (General Biochemicals)

penicillin G, 204 units/ml (General Biochemicals)

kanamycin, 100 µg/ml ("GIBCO")

fungizone, 2.5 µg/ml ("GIBCO")

2) 1.8% sodium bicarbonate: 16 mls per 800 ml media

3) fetal calf serum ("GIBCO"): for stock cultures 15% fetal calf serum was added to MEM (15% MEM).

b) Cells. A skin punch biopsy was taken from a normal Caucasian female (23). Monolayer fibroblast cultures were derived from this biopsy and first to fifth transfer passages were used throughout these studies. Stock cultures were maintained in 15% MEM in 100 mm petri dishes (Falcon Plastics), and kept in a water saturated Co_2 incubator at 37°C.

II. Chemical Treatment

4-Nitroquinoline 1-Oxide (4NQO) was obtained from Daiichi Pure Chemical Co., Tokyo. Immediately prior to use 1.9 mg of 4NQO was dissolved in 0.4 ml ethanol, this was warmed slightly to ensure that the chemical was completely dissolved. To give a 10^{-3}M solution, 9.6 mls of ADM or MEM was added; serial dilutions were then made to obtain the desired concentrations. For the survival studies, cells were treated with 3 mls of the appropriate 4NQO solution in 2 1/2% MEM. For chromosome and DNA repair studies, the cells were treated with 1 ml of the chemical solution in 5% MEM or ADM. All treatments were for 1 hour unless otherwise stated. The chemical was removed by a sterile pipette attached to a suction device. After removal of the chemical the cells were washed twice with 2 mls of MEM or ADM (without fetal calf serum). Medium supplemented with fetal calf serum was added and the petri dishes returned to the CO_2 incubator.

III. UV Treatment

One aspect of the DNA repair studies involved treating the cells with UV irradiation. In this case a Sylvania germicidal lamp (G15T8) was used as the UV light source. At 20" it emitted a dose of 8 ergs/mm^2/sec as measured by a UV light meter (Ultraviolet Products, Inc.).

Cover slips containing cells for irradiation were dipped twice in sterile phosphate buffer saline (PBS) containing no phenol red, to

ensure complete removal of any UV absorbing material. For irradiation, cover slips were placed in an empty petri dish, and upon completion of irradiation were returned to a petri dish containing medium.

IV. Cell Survival Studies

1,600-2,000 cells were seeded into 60 mm petri dishes, and covered with 4 mls of 2 1/2% MEM and allowed to settle down as single cells for 16-20 hours. This low concentration of fetal calf serum was chosen in order to slow down the metabolic rate of the cells such that cell divisions would not occur during the course of the experiments. (The effect of 2 1/2% MEM on cell survival will be discussed more fully in the results.) The chemical treatments were in 2 1/2% MEM and the recovery period was also in 2 1/2% MEM. Once the chemical treatments were complete 15% MEM was added and the cells were allowed to divide and form colonies. When the clones had reached the 50-60 cell stage (approximately 7 days post-treatment) the preparations were fixed with Carnoy's solution (3:1 alcohol acetic acid); washed in 70% ethanol, and distilled water; air dried, and stained with a 2% aqueous solution of Toluidine Blue (Fischer Scientific Co.). The colonies were counted under a regular dissecting scope.

V. Chromosome Studies

Cells were seeded onto 20 mm sq coverslips (Corning) in 35 mm petri dishes and covered with 2 mls of 15% MEM. In order to obtain

well spread metaphase plates, the cells were used before they reached 80% confluency. The cells were treated twice with 4NQO in 5% MEM and allowed to recover between doses in 15% MEM. Once cell divisions were detected (by observation under an inverted microscope) 0.2 mls of a 0.01% solution of colchicine (BDH Chemicals, England) was added for 5 hours. The coverslips were then transferred to petri dishes containing 1% sodium citrate solution for 20 minutes. This hypotonic treatment causes the cells to swell, producing chromosomes that are well spread out and separated. The cells were then fixed with Carnoy's and air-dried. Once dry, they were stained for 5 minutes with 2% aceto-orcein, dehydrated through alcohol, butanol, butanol-xylol, xylol, and mounted on glass slides with Permount (Fischer Scientific Co.).

VI. Autoradiography

In order to distinguish between DNA repair replication and semiconservative DNA replication, only nuclei undergoing repair synthesis should become labelled. To achieve this, cells must be prevented from entering S-phase. This was accomplished by placing the cultures in ADM for 2 1/2 days, at which time approximately 90% of the cells are arrested at G_1.

Cells were seeded onto 20 mm sq coverslips in 35 mm petri dishes, and covered with 15% MEM. Upon reaching 80% confluency, the cells were put into 5% ADM. This was done by dipping the coverslips into two beakers of ADM (no serum), with subsequent transferral to new petri

dishes containing 2 mls of 5% ADM. The experiment was conducted 2 1/2-3 days later.

Tritiated thymidine (^{3}HTdR) was obtained from New England Nuclear (Chicago) and was diluted to a concentration of 10 μCi/ml in either 5% MEM or 5% ADM. The cells were pulsed with 1 ml of this solution for 2 hours, at which time the coverslips were moved from the petri dish and dipped in 3 changes of Hanks balanced salt solution to remove any excess ^{3}HTdR. They were then immersed in 1% sodium citrate for 15 minutes, fixed in Carnoy's, rinsed in 100% ethanol and air dried.

To facilitate handling, the coverslips (cell side up) were mounted on glass slides with melted paraffin. Excess Carnoy's was removed by passing the slides through a graded alcohol series, 95% EtOH, 70% EtOH, 20% EtOH (10 minutes each), 2 changes of distilled water, one change of PBS, two more changes distilled water (10 minutes each) and were then left to air dry.

The slides were coated with NTB3 emulsion (Kodak) (at 43°C), allowed to dry for 1 hour and then stored at 4°C in light-tight boxes for 2 weeks.

The autoradiograms were processed in Kodak D19 developer (3 minutes), Kodak fixer (10 minutes) and rinsed in running water for 1 hour. The cells were then stained with 2% orcein for 5 minutes, dehydrated through successive immersion in ethanol, butanol, butanol/xylol, xylol (2 minutes each) and mounted in Permount (by placing another coverslip over the exposed cells).

RESULTS

I. The Repair of 4NQO-induced DNA Damage

Prior to attempting any experiments with double 4NQO treatments, it was necessary to determine the time course of DNA repair synthesis after a single 4NQO treatment and to choose appropriate 4NQO concentrations and lengths of treatment for use in such experiments. A high 4NQO concentration may not be very toxic to cells when given in a single treatment but when given twice could become extremely toxic, and would most likely affect the levels of repair synthesis. Therefore it was important to select a concentration of 4NQO that would elicit a moderate, or even low level of DNA repair after a single treatment (thus implying moderate or low levels of DNA damage). Choosing an exposure time was of equal importance as it was essential to have very little repair synthesis taking place during the first chemical treatment in order that the second chemical treatment could be applied while repair of the initial damage was still proceeding.

An experiment was designed to investigate the repair of 4NQO-induced DNA damage, and the effect of different concentrations and different treatment times. Two concentrations of 4NQO were chosen; 5×10^{-7}M and 1×10^{-7}M. These had already been shown by Stich and San[72] to induce moderate and low levels of DNA repair synthesis respectively, after a 90 minute treatment time. The level of ^{3}HTdR incorporation

(seen as grains per nucleus) was used as the measure of DNA repair synthesis. The cells were maintained in ADM prior to and throughout the entire experiment.

Figure 1 outlines the protocol that was employed. Illustrated is a 60 minute 4NQO treatment (10^{-7}M or 5 x 10^{-7}M), the other treatment times were 30 and 90 minutes; all underwent subsequent incubation in ^{3}HTdR, 0, 2, 4, 8, 12 and 24 hours after removal of the carcinogen. All of the chemical solutions were applied at the same time, and this was designated as zero hour (for graphical purposes). To determine the amount of repair synthesis that was initiated during the chemical treatment, ^{3}HTdR was added simultaneously with 4NQO for 30, 60 or 90 minutes. A two hour ^{3}HTdR pulse was chosen for the remaining incubation periods and was later adopted for all subsequent repair experiments. This choice was made because the 4NQO concentrations that were employed did not elicit high levels of repair synthesis and an ^{3}HTdR pulse of 1 or 1 1/2 hours would have produced low grain counts, making the results difficult to interpret.

Three general observations can be made from these results (Figure 2); the peak of repair replication for all chemical treatments occurs in the first 4 hours following addition of the chemical, and then proceeds at a slightly lowered level for the next 6 hours; at 12 hours a low but significant uptake of ^{3}HTdR can be detected, and is still evident at 24 hours.

Upon examining the results more closely it is noticeable that during the 30 minute and 60 minute 4NQO treatments, the cells exhibit a very low level of ongoing repair synthesis, whereas in the next two

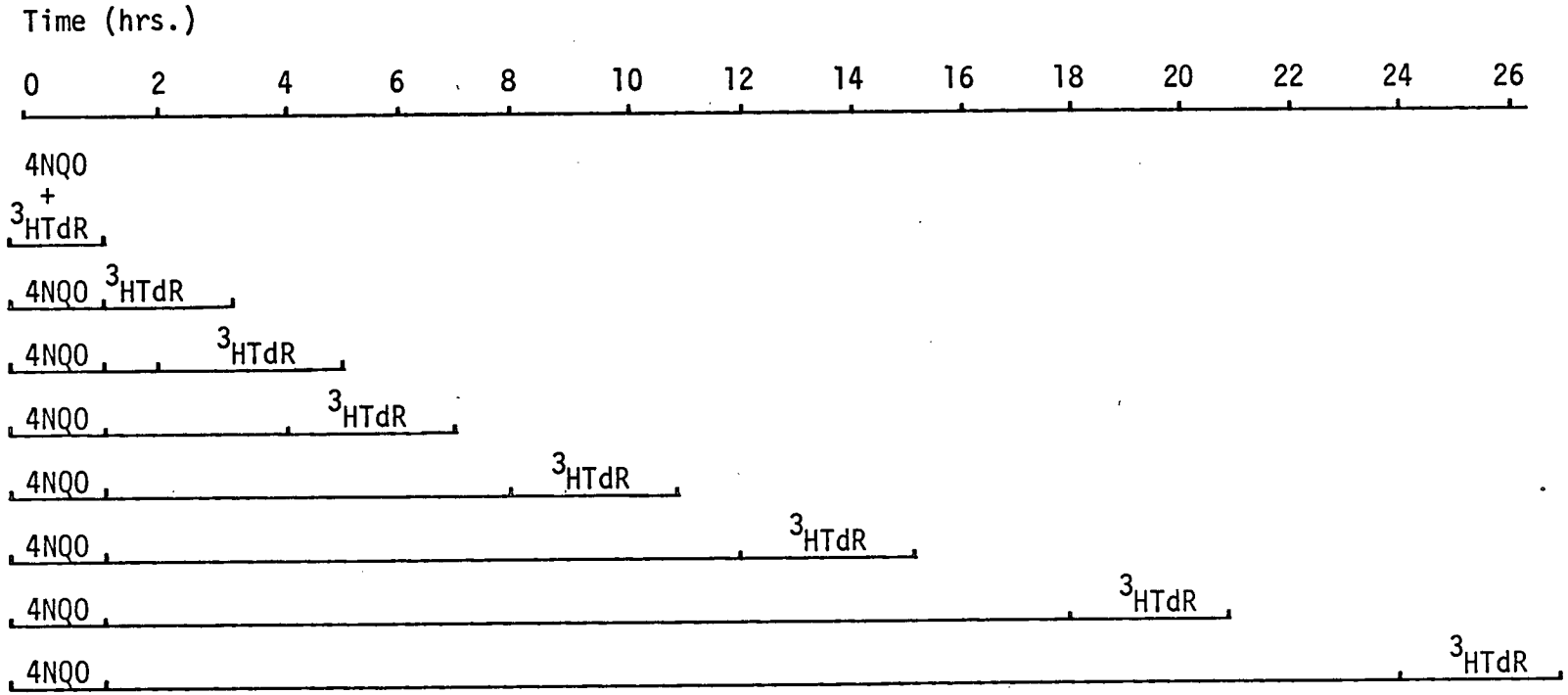


Figure 1: Experimental design. Time course of DNA repair synthesis following 4NQO-induced DNA damage.

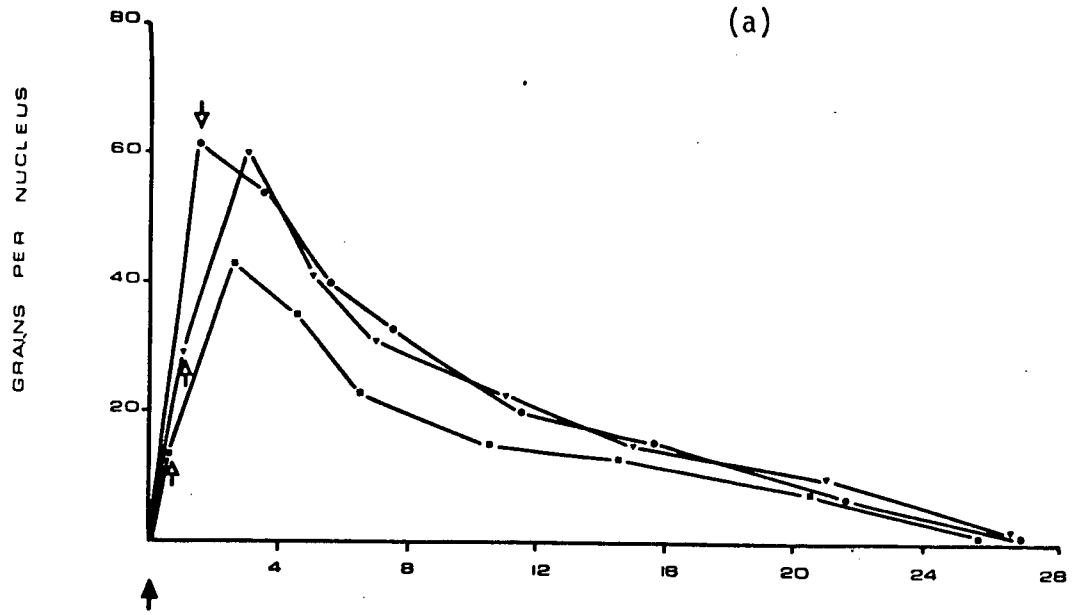
Figure 2: Repair of 4NQO-induced DNA damage. Effect of concentration and duration of exposure. Each point denotes the time when the sample was taken and the uptake of ^{3}HTdR over a two hour period prior to sampling (represented by grains per nucleus).

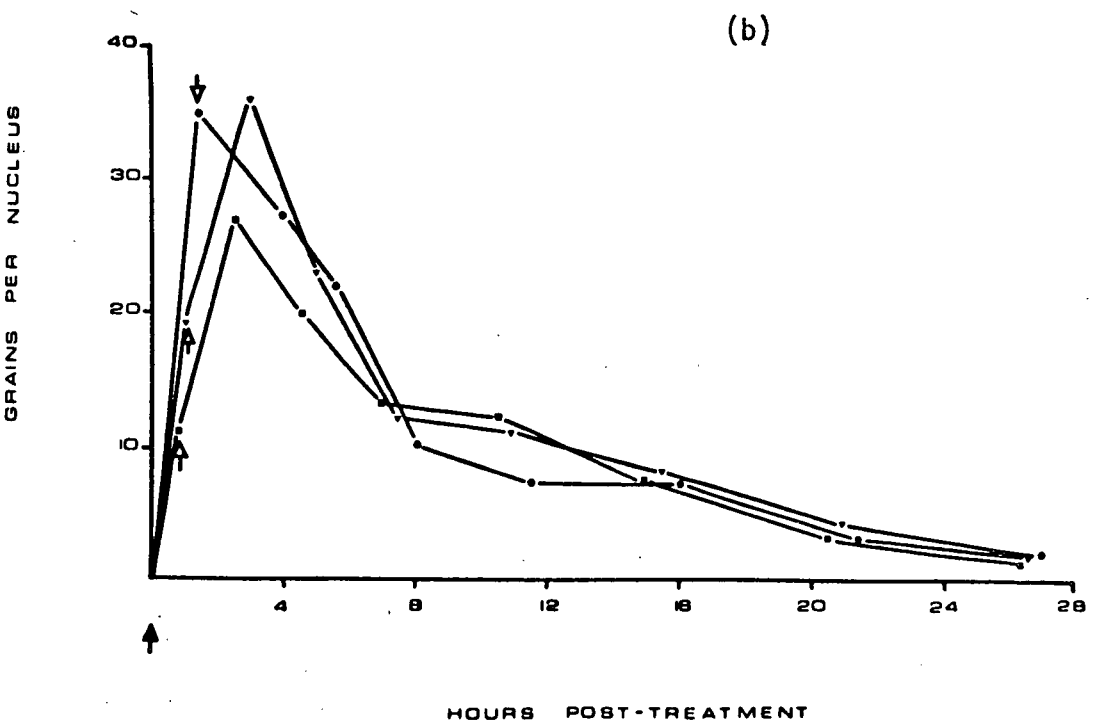
↓ 4NQO added

⇩ 4NQO removed

(a) 5×10^{-7}M 4NQO

(b) 1×10^{-7}M 4NQO


(a)
GRAINS PER NUCLEUS
80
60
40
20
4
8
12
16
20
24
28
HOURS POST-TREATMENT


(b)
GRAINS PER NUCLEUS
40
30
20
10
4
8
12
16
20
24
28
HOURS POST-TREATMENT

hour ^{3}HTdR pulse that follows, a high level of repair synthesis is obtained. In fact this is the peak of repair synthesis. However, in the cells exposed to a 90 minute 4NQO dose this is not the case, for the peak of repair is reached during the chemical treatment, only to drop slightly in the subsequent two hour ^{3}HTdR pulse. This seems to indicate that the peak of repair occurs in the second or third hour following addition of 4NQO.

Since both the 60 minute and the 90 minute 4NQO treatments elicited the same maximum level of repair synthesis, and since the peak of repair occurred after rather than during the 60 minute 4NQO treatment, it was decided to utilize a 60 minute 4NQO treatment (1×10^{-7}M and 5×10^{-7}M) for all subsequent experiments.

II. The Effect of Split 4NQO Treatments on DNA Repair Synthesis

Using the previous results as a guide, an attempt was made to ascertain the level of repair synthesis attained by cells exposed to a second 4NQO treatment whilst still recovering from a first treatment. Basically two problems were of interest here; first how does a cell at the peak of repair respond to a second treatment, and secondly how does a cell that has virtually completed repair synthesis respond.

In designing the experiment, an appropriate endpoint for comparing the data had to be chosen. Since it was known that the peak of repair occurred during the two hours immediately following removal of a single 60 minute 4NQO treatment, it was decided to measure the level of repair synthesis obtained in the two hours immediately following

removal of the second treatment and then compare these values. The experimental protocol outlined in Figure 3 was adopted. Cells were maintained in ADM throughout the experiment, treated initially with 10^{-7}M or 5 x 10^{-7}M 4NQO for 60 minutes, and then treated again 1 1/2, 2, 3, 5, 9 or 13 hours later. The two hour ^{3}HTdR pulse immediately followed removal of the second treatment. In order to determine the amount of repair synthesis induced by first treatment alone, controls were run for each recovery period. They were exposed to the initial 4NQO treatment only, allowed to recover whilst the remainder received a second treatment, and then pulsed with ^{3}HTdR at the same time as the "doubly-treated" sample.

The results, which are summarized in Figure 4, depict the actual levels of repair following recovery from a single treatment and after exposure to two treatments; and the total expected level of repair following two treatments. This expected value was determined by making the assumption that a cell should theoretically be able to repair a second chemical treatment as effectively as if it were the first, no matter when it is applied. In practise, the total expected level of repair synthesis obtained following the second treatment should be equal to the sum of the repair synthesis still proceeding after recovery from the first treatment plus the level of repair that is obtained immediately after removal of a single 4NQO treatment. In the histograms (Figure 4) the first column depicts the level of DNA repair immediately following removal of a single 4NQO treatment; this value is then added to the control (clear column in each set) to give the expected value (black column in each set). It should be clarified at this point that the control value

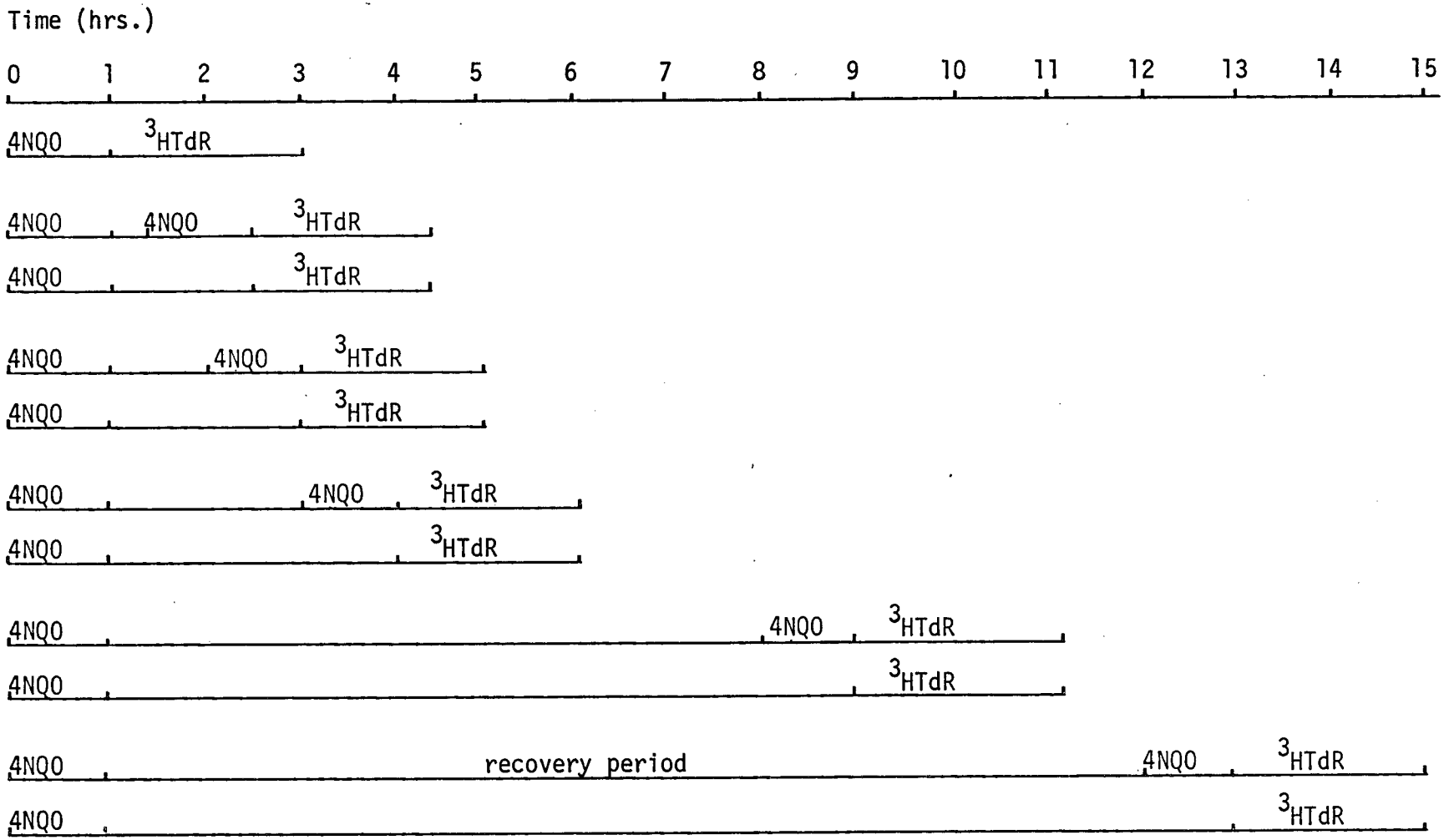


Figure 3: Experimental design. Variation of the intervals between split 4NQO treatments; effect on DNA repair synthesis.

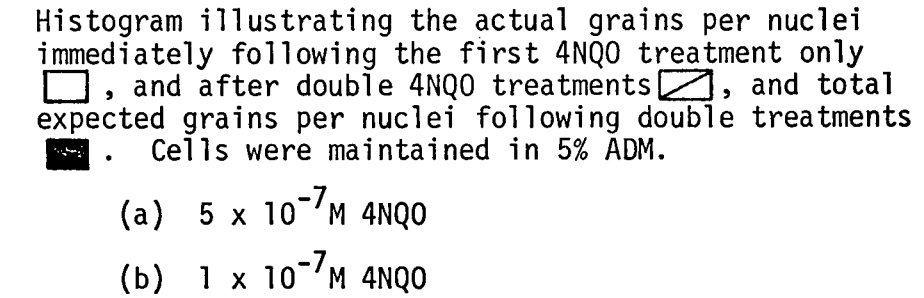
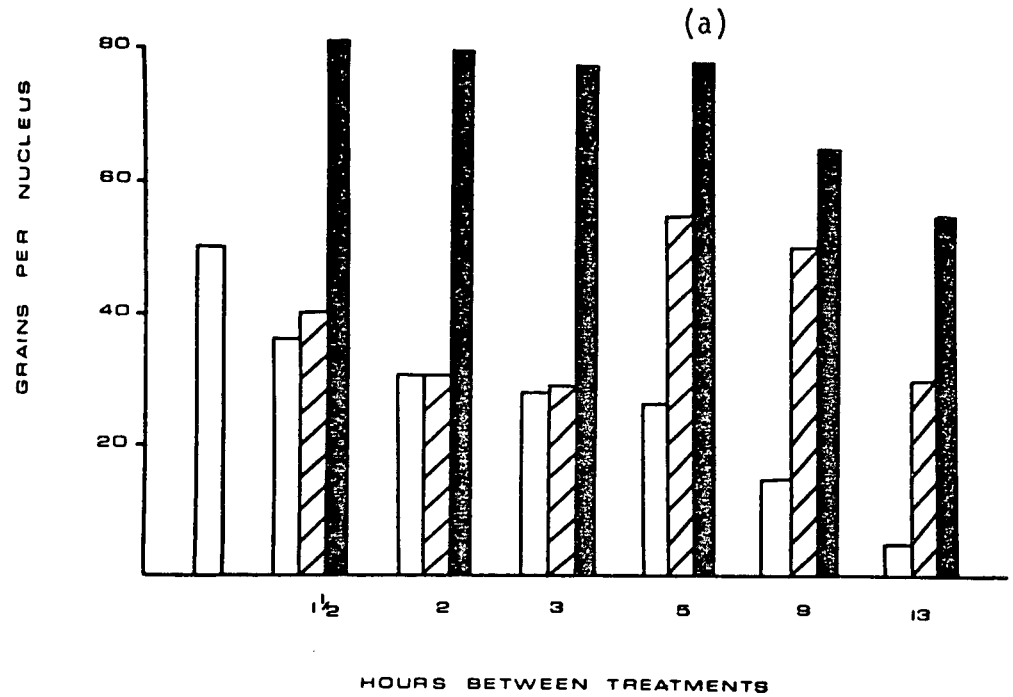
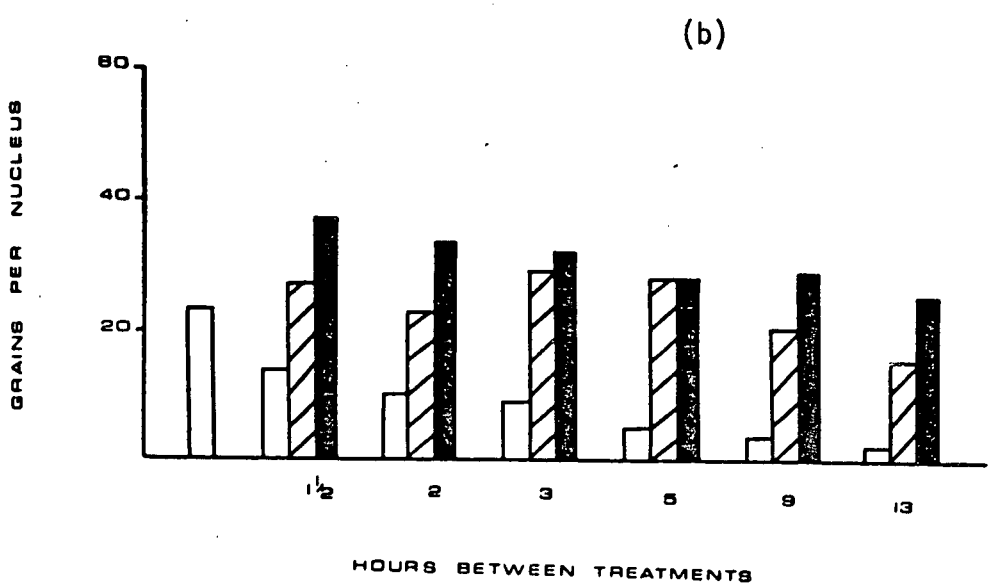


Figure 4: Histogram illustrating the actual grains per nuclei immediately following the first 4NQO treatment only □, and after double 4NQO treatments ▨, and total expected grains per nuclei following double treatments ■. Cells were maintained in 5% ADM.

(a) 5×10^{-7}M 4NQO

(b) 1×10^{-7}M 4NQO


(a)
GRAINS PER NUCLEUS
80
60
40
20
1½
2
3
5
9
13
HOURS BETWEEN TREATMENTS


(b)
GRAINS PER NUCLEUS
60
40
20
1½
2
3
5
9
13
HOURS BETWEEN TREATMENTS

does not indicate how much repair synthesis was actually taking place during the second 4NQO treatment (this can be obtained from Figure 2), it merely represents the DNA repair synthesis still proceeding after recovery from the first treatment. The purpose of the control was to serve as an aid in obtaining the expected value.

As illustrated in the results (Figure 4), if a second treatment is applied during the three hours immediately following addition of the first, the total level of repair is significantly lower than the expected. In fact there appears to be very little repair synthesis taking place on or above that for the first treatment. At the lower 4NQO concentration (Figure 4b), this effect is not as marked, and is only significantly below the expected for two hours post-treatment. After this period, the level of repair begins to approach the expected, and at the lower dosage reaches the expected at 5 hours post-treatment. However, at the higher concentration, the expected level is never attained; in fact at 13 hours the level is even further below the expected than it was at 5 hours. Such was the case for the lower concentration also.

This peculiar decrease in repair capacity was not predicted, since it seemed logical to assume that once the cells had regained the potential to repair the DNA damage inflicted by the second treatment, they would continue to do so.

To determine whether this drop in repair capacity may have been due to the arginine-deficient culture medium, an identical experiment, with a few modifications was designed. The cells were blocked as before in 5% ADM for 2 1/2 days, but 3 hours prior to the first 4NQO treatment,

the cells were returned to 5% MEM.* This 3 hour time period was chosen arbitrarily, to provide the cells with a chance to equilibrate. The rest of the experiment was then carried out in 5% MEM.

Comparison of the histograms for cells in ADM, with those in MEM reveal some differences (Figure 5). When the ADM blocked cells are placed in MEM briefly (3 hours) prior to 4NQO treatment, the two hour period is again noticeable during which the DNA repair level falls below expected values. However, this reduction in DNA repair synthesis is not as pronounced as in the case when the cells were maintained in ADM throughout the experiment. Furthermore, the second drop in repair capacity is not apparent in the MEM-maintained cells; at 12 hours the level of DNA repair following the second 4NQO treatment is identical to the expected.

There was no reason to suspect that this increase in repair capacity was the result of a concommitant rise in semiconservative DNA synthesis associated with cell division. The corresponding controls (single treatment) were carefully examined and did not contain any nuclei with high grain counts (indicative of DNA synthesis at S-phase). Furthermore Freed and Schatz[25] have shown that after removal of a block such as ADM the cells do not enter S-phase until 16 to 20 hours later. This is without a 4NQO treatment which tends to further slow down entry of cells into S-phase[44].

For comparative purposes, an experiment was run simultaneously to determine the course of repair after a single 4NQO treatment for cells in ADM and MEM. The protocol previously outlined in Figure 1

---

*such cells will be referred to as MEM-maintained cells.

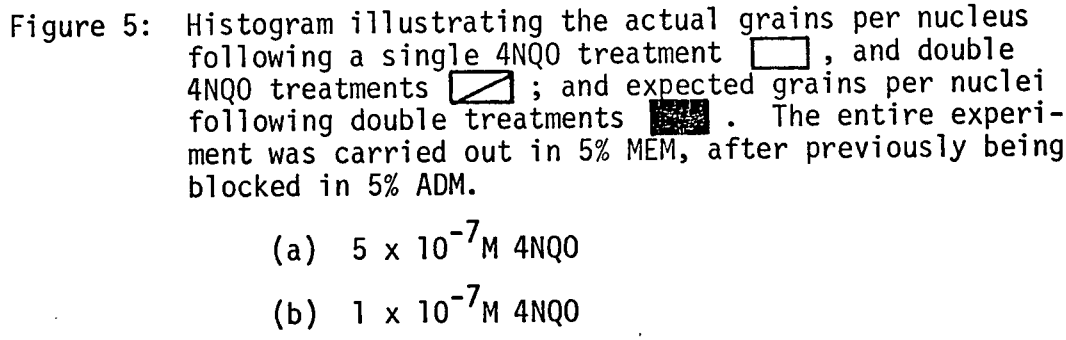
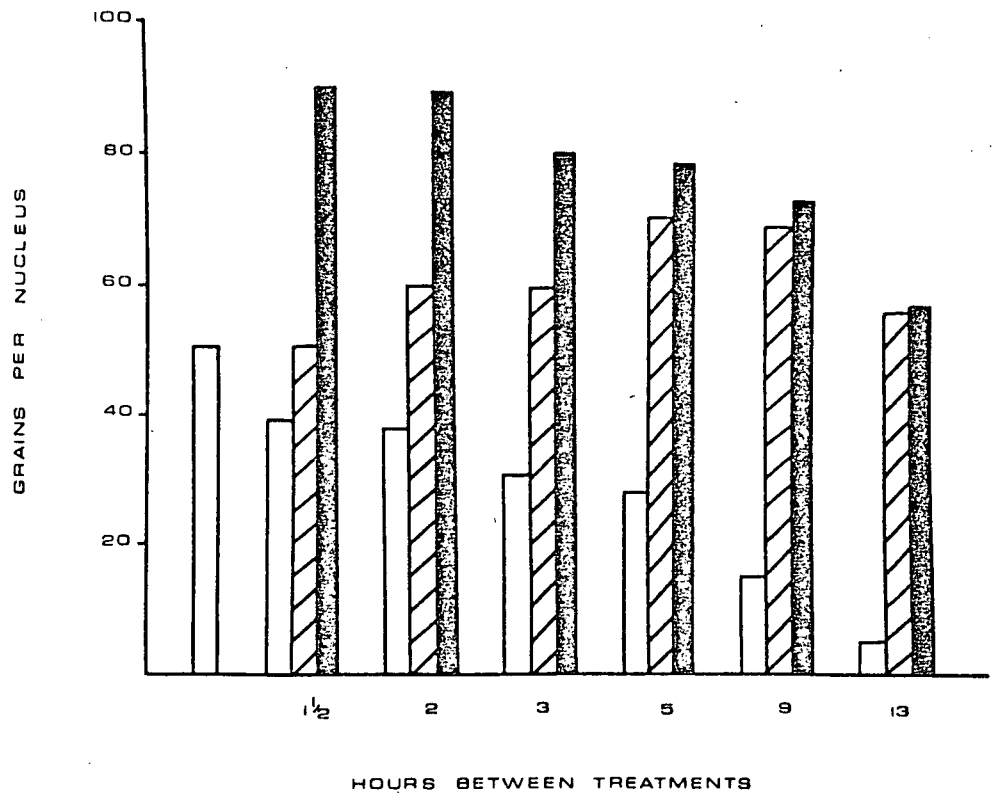


Figure 5: Histogram illustrating the actual grains per nucleus following a single 4NQO treatment ▭, and double 4NQO treatments ▨; and expected grains per nuclei following double treatments ▩. The entire experiment was carried out in 5% MEM, after previously being blocked in 5% ADM.

(a) 5×10^{-7}M 4NQO

(b) 1×10^{-7}M 4NQO

(a)


100
80
60
40
20
GRAINS PER NUCLEUS
1½
2
3
5
9
13
HOURS BETWEEN TREATMENTS

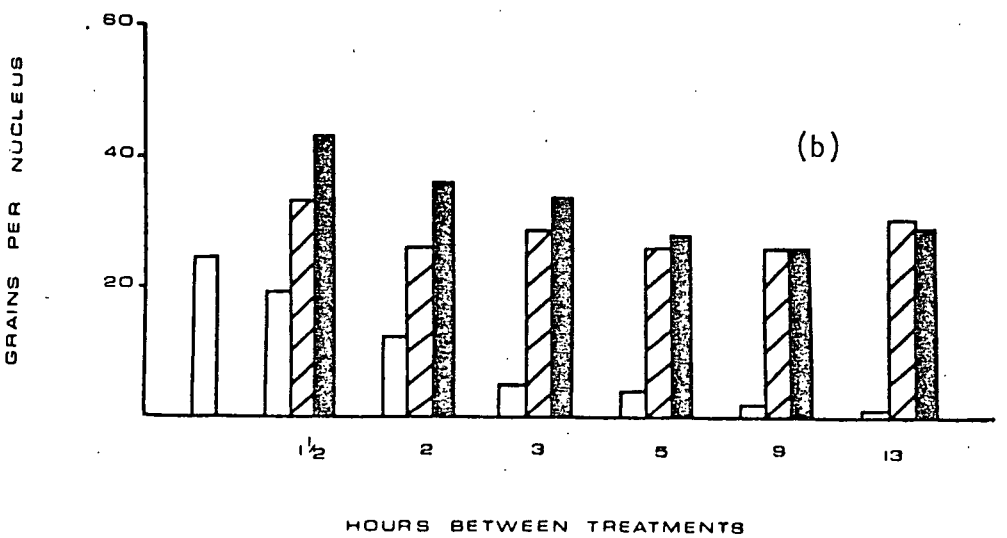
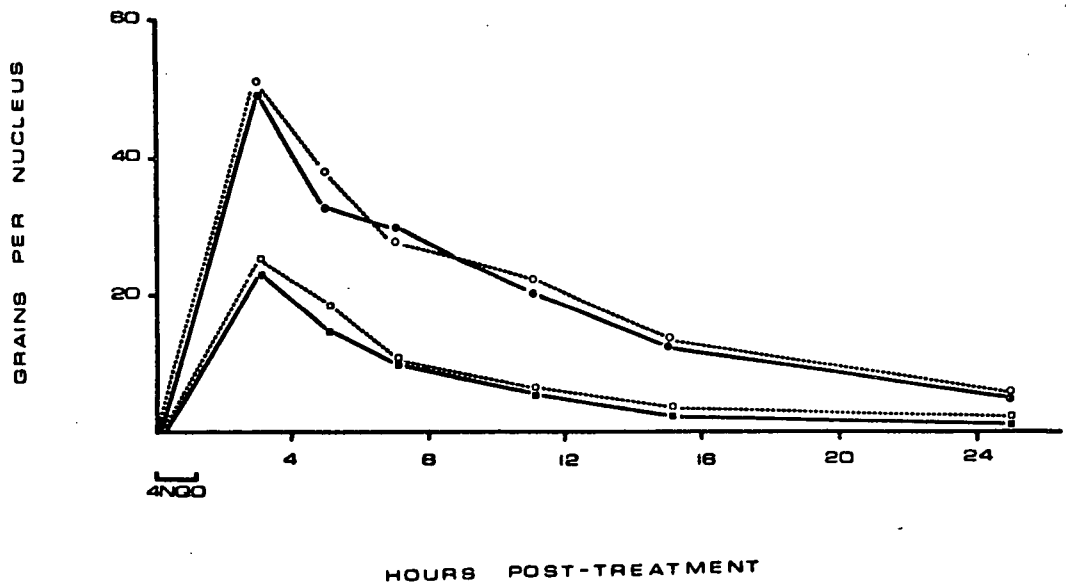

60
40
20
GRAINS PER NUCLEUS
(b)
1½
2
3
5
9
13
HOURS BETWEEN TREATMENTS

Figure 6: Time course of DNA repair synthesis following a single 4NQO treatment in either 5% ADM or 5% MEM. Each point denotes the time when the sample was taken; and the uptake of ^{3}HTdR over a two hour period prior to sampling (represented by grains per nucleus).

..... in 5% ADM
_____ in 5% MEM

(a) 5×10^{-7}M 4NQO ● ○

(b) 1×10^{-7}M 4NQO ■ □


GRAINS PER NUCLEUS
60
40
20
4
8
12
16
20
24
4N00
HOURS POST-TREATMENT

was used with the exception that only 1 hour treatments were employed, and that the experiment was run in 5% MEM as well as 5% ADM. The same chemical solution was used for both kinds of medium, with the final dilution being made in either 5% MEM or 5% ADM.

The results are summarized in Figure 6, and from this graph it can be concluded that repair of a single 4NQO treatment is not affected by the type of medium employed.

Returning to Figures 4 through 6 it is apparent then that cells in ADM are not able to repair a second set of DNA damage to the expected level, whereas cells in MEM can, even though both are capable of repairing the first treatment. Furthermore cells maintained in either medium possess a "sensitive" two hour period following chemically induced DNA damage, the sensitivity being more pronounced when cells are maintained in ADM.

III. The Repair of 4NQO-induced DNA Damage Following UV Irradiation

The question now arises as to whether DNA repair synthesis induced by other agents can produce the same results. For this purpose it was decided to replace the first 4NQO treatment with a UV treatment. Prior to running such an experiment, it was necessary to characterize the time-course of repair following a single UV exposure. The protocol used was basically identical to that used for a single 4NQO treatment (Figure 1) except that the cells were exposed to UV (designated as zero hour) and the experiment was run in both 5% ADM and 5% MEM.

Two dosages were used: 40 ergs/mm^2 and 20 ergs/mm^2 followed by two hour ^{3}HTdR pulses at 0, 2, 4, 8, 12 and 22 hours post-treatment.

The peak of repair was found to occur during the two hours immediately following UV treatment (Figure 7); this concurs with the time course of DNA repair synthesis after a single 4NQO treatment (Figure 2). After this initial peak of DNA repair synthesis, the level decreases markedly such that at 8 hours only a low but significant amount of repair synthesis is detectable, again in concordance with the time course of repair of 4NQO-induced DNA damage.

Both UV doses produced levels of repair synthesis that would serve as suitable replacements for the first 4NQO treatments (40 ergs/mm^2 for 5 x 10^{-7}M and 20 ergs/mm^2 for 10^{-7}M), and were therefore used for this set of experiments.

The experiment was run according to the protocol outlined in Figure 8, and was divided into two parts; half of the cells were maintained in 5% ADM for the entire experiment; the other half were placed in 5% MEM 3 hours prior to UV treatment, and remained in 5% MEM throughout the experiment. Corresponding controls were run as before and were exposed to the UV treatment only or the 4NQO treatment only.

The results are summarized in the histograms of Figures 9 and 10. Note that the first column in each graph represents the level of repair obtained immediately following a single one hour 4NQO treatment (5 x 10^{-7}M or 10^{-7}M). This value was used to calculate the expected level of repair (last column in each set). Theoretically all UV-treated cells should be capable of repairing the damage induced by a subsequent 4NQO dose (no matter when it is applied) to a level similar to that after a single 4NQO dose.

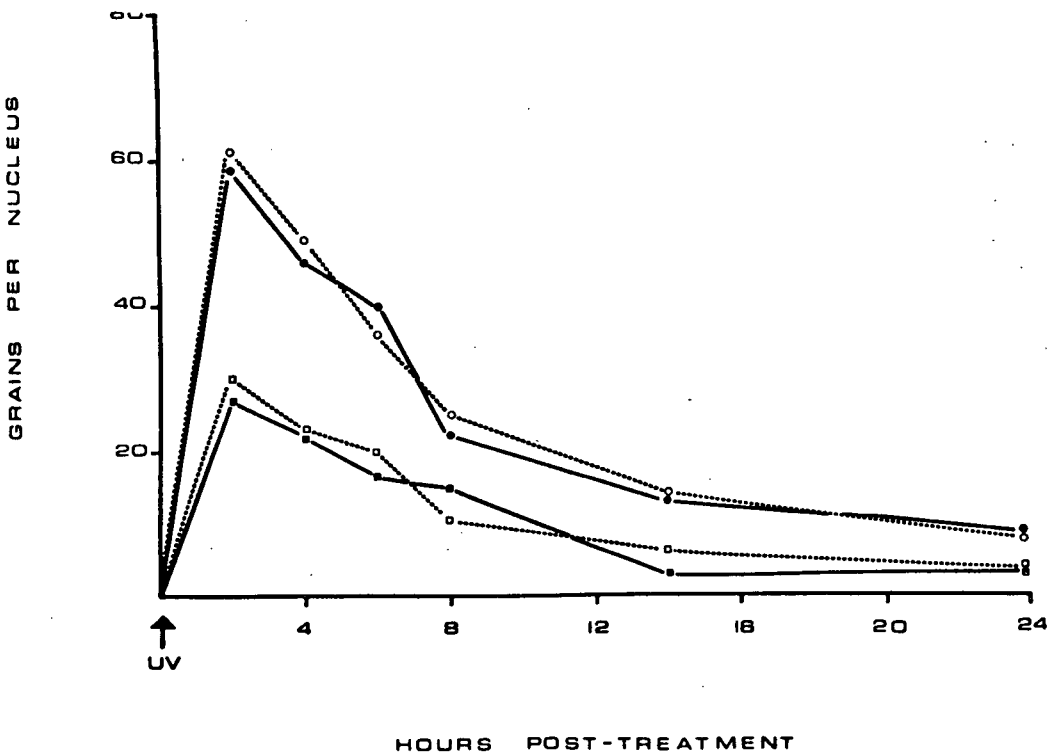
Figure 7: Time course of repair synthesis following UV-induced DNA damage. Each point denotes the time when the sample is taken; and the uptake of ^{3}HTdR over a two hour period prior to sampling (represented by grains per nucleus).

▪▪▪▪▪▪▪▪▪▪ in 5% ADM

——— in 5% MEM

(a) ○ ● 40 ergs/mm^2

(b) □ ■ 20 ergs/mm^2


GRAINS PER NUCLEUS
60
40
20
UV
4
8
12
16
20
24
HOURS POST-TREATMENT

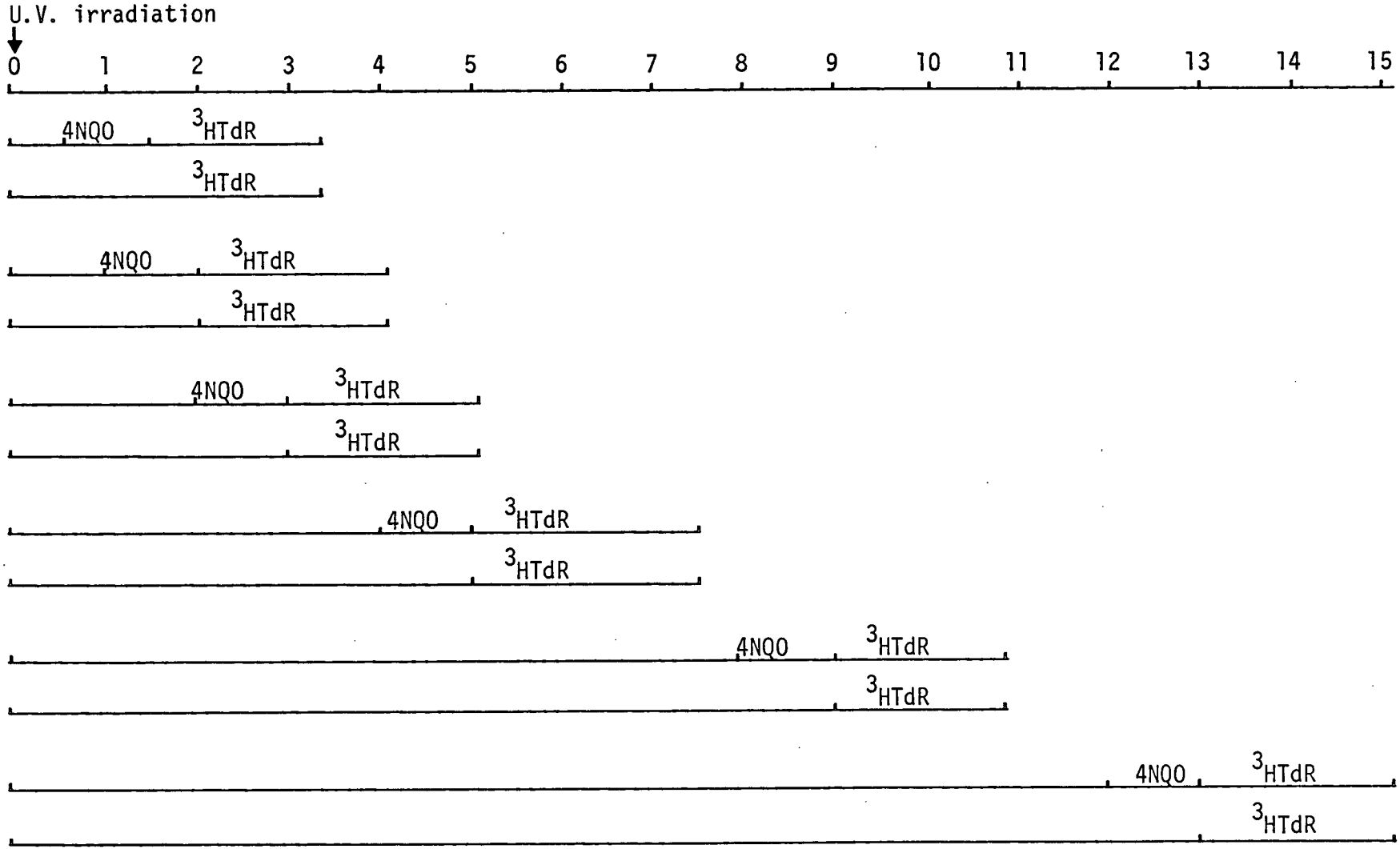
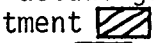
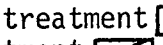
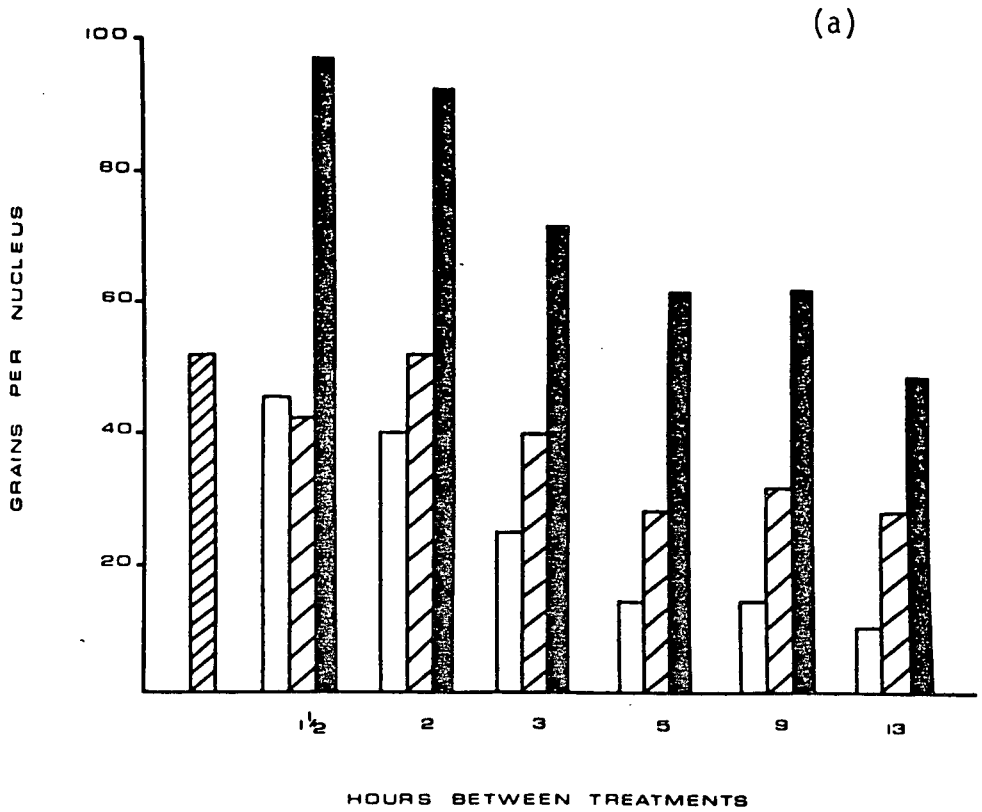


Figure 8: Experimental design. Variation of the intervals between UV irradiation and a subsequent 4NQO treatment; effect on DNA repair synthesis.

Figure 9: Histogram depicting actual grains per nucleus immediately following 4NQO treatment  , at varying periods of time after UV treatment  , and after UV treatment plus 4NQO treatment  ; expected grains per nucleus after treatment  . Experiment run in 5% ADM.

(a) 5×10^{-7}M 4NQO and 40 ergs/mm^2

(b) 1×10^{-7}M 4NQO and 20 ergs/mm^2


(a)
GRAINS PER NUCLEUS
100
80
60
40
20
1½
2
3
5
9
13
HOURS BETWEEN TREATMENTS

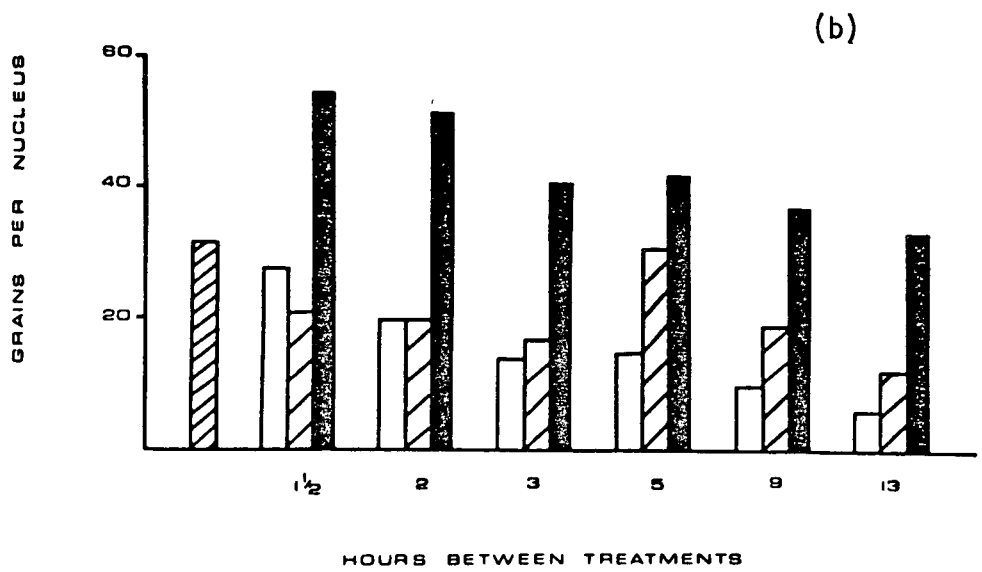
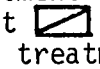
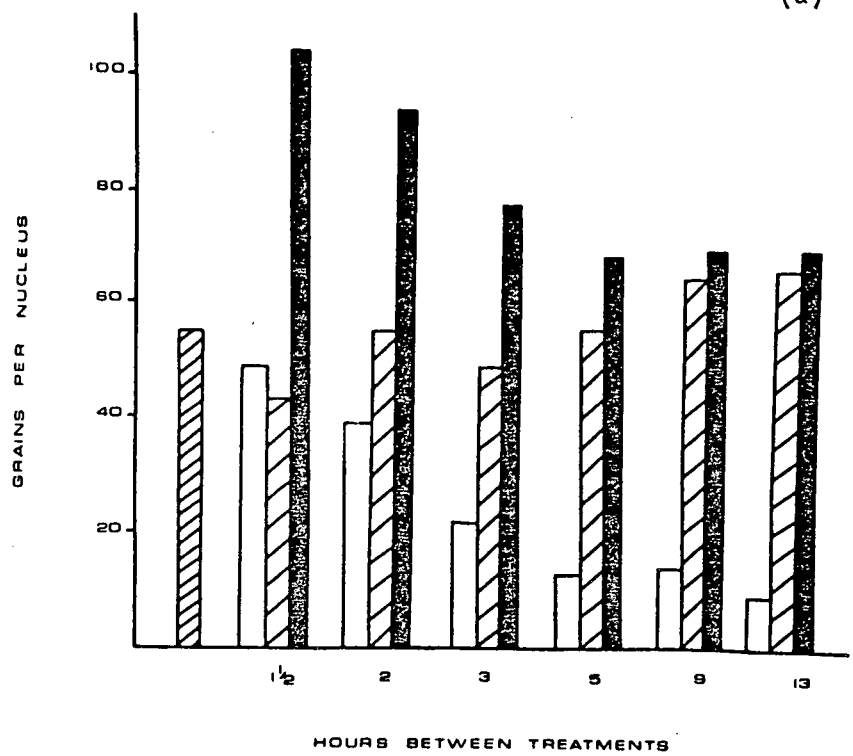

(b)
GRAINS PER NUCLEUS
80
40
20
1½
2
3
5
9
13
HOURS BETWEEN TREATMENTS

Figure 10: Histogram depicting actual grains per nucleus immediately following 4NQO treatment  , at varying periods of time after UV treatment  , and after UV treatment plus 4NQO treatment  ; expected grains per nucleus after UV plus 4NQO treatment  . Experiment was carried out in 5% MEM after previously being blocked in 5% ADM.

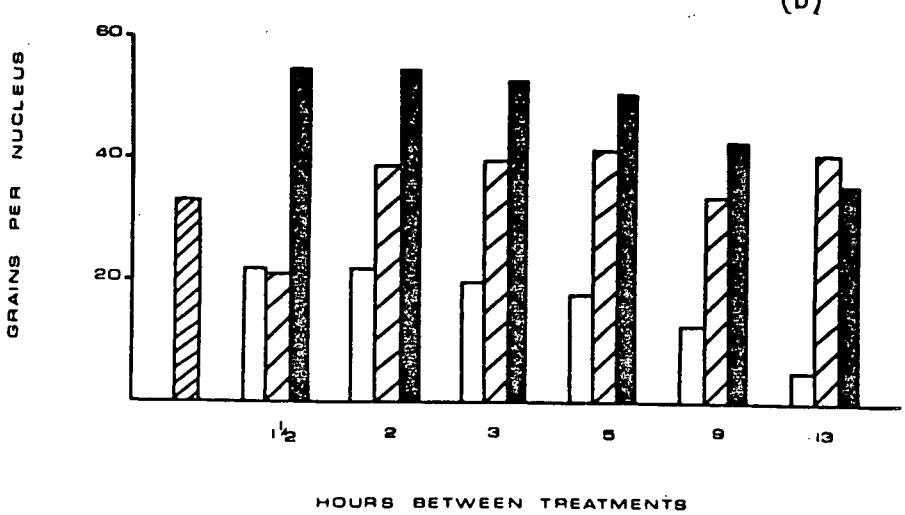
(a) 5×10^{-7}M 4NQO and 40 ergs/mm^2

(b) 1×10^{-7}M 4NQO and 20 ergs/mm^2

(a)


GRAINS PER NUCLEUS
100
80
60
40
20
1½
2
3
5
9
13
HOURS BETWEEN TREATMENTS

(b)


GRAINS PER NUCLEUS
60
40
20
1½
2
3
5
9
13
HOURS BETWEEN TREATMENTS

It is interesting that the results are very similar to those of the previous experiments. When the cells are maintained in ADM, DNA repair synthesis following the second dose (at either concentration) again fell below expected, and in this case (UV-4NQO combination) the decrease is even more pronounced than in split-dose 4NQO experiments. In fact at half an hour post-treatment repair of the UV damage appears to be slightly inhibited. Likewise, the ADM-maintained cells never totally regain the ability to repair the second 4NQO treatment (at both concentrations).

The pattern of repair for MEM-maintained cells does not change appreciably from before except that at both concentrations a slight inhibition occurs during the first hour. Again the cells in MEM are capable of repairing the second treatment to the expected level after an 8 hour recovery period. In fact the repair synthesis at this point is higher than expected.

These results imply then that the two hour "sensitive" period previously characterized is not peculiar to 4NQO treated cells and that the repair cycle induced by UV also has a similar temporal sequence. This observation indicates that repair of UV-induced damage may be affected to a greater extent by a subsequent 4NQO treatment than is repair of 4NQO-induced damage.

IV. Time Course of DNA Repair after a Second 4NQO Treatment

Interpretation of the aforementioned results is complicated by the fact that the choice of end point may not have been entirely appropriate (i.e. comparison of repair levels obtained in the two hour period immediately following removal of the second treatment). It is conceivable that the addition of a second 4NQO treatment close to the first, may initially produce a slight toxicity in the cells and thus delay the onset of repair synthesis. Consequently the peak level of repair would not be detected during the two hour ^{3}HTdR pulse. If this is the case, then the below expected DNA repair levels would merely reflect a lag in repair synthesis.

To clarify this point, periods of repair incubation following removal of the second dose were employed. In this manner, it would be possible to determine when the peak of repair was occurring.

Furthermore, since it has been shown that at 9 hours post-treatment, cells in MEM respond to a second treatment with a peak level of repair synthesis similar to that following a single treatment, it seemed logical to determine if the time course of repair after a second dose at 9 hours, also resembled that of a single dose.

In order to achieve both objectives it was necessary to run the experiment in 5% MEM. This decision was made because cells in ADM never seemed to regain the capacity for repairing DNA damage inflicted by the second treatment, and as a result the latter objective could never be attained.

For this experiment, cells were exposed to a second 4NQO treatment, 2, 3, 5 or 9 hours after addition of the first, and pulsed with ^{3}HTdR as outlined in Figure 11. One set of cells served as controls receiving the first treatment only, and were always pulsed simultaneously with samples given the double dose.

The results are summarized in Figure 12. In the graphs for "doubly-treated" cells the values are expressed as grains per nucleus resulting from the second treatment only. In these cases the level of repair for the "singly-treated" control was subtracted from the total level of DNA repair detected after the second treatment (for each repair incubation period).

A general observation can be made from these results; as the interval between treatments increases, the peak of repair increases and the overall pattern of repair more closely resembles that of the initial dose. It is interesting that if the second treatment is given 2 hours after the first, a peak in DNA repair synthesis does not occur at a later time. This would then rule out the existence of a lag period. Furthermore the results show that when a second treatment is given 9 hours after the first, the pattern of repair is basically the same as that elicited by a single treatment.

It seems reasonable to conclude then, that in the period immediately following induction of DNA damage by 4NQO, the application of a second 4NQO dose does not produce the expected response. Either this second dose does not induce further damage or if it does, for some reason DNA damage does not occur.

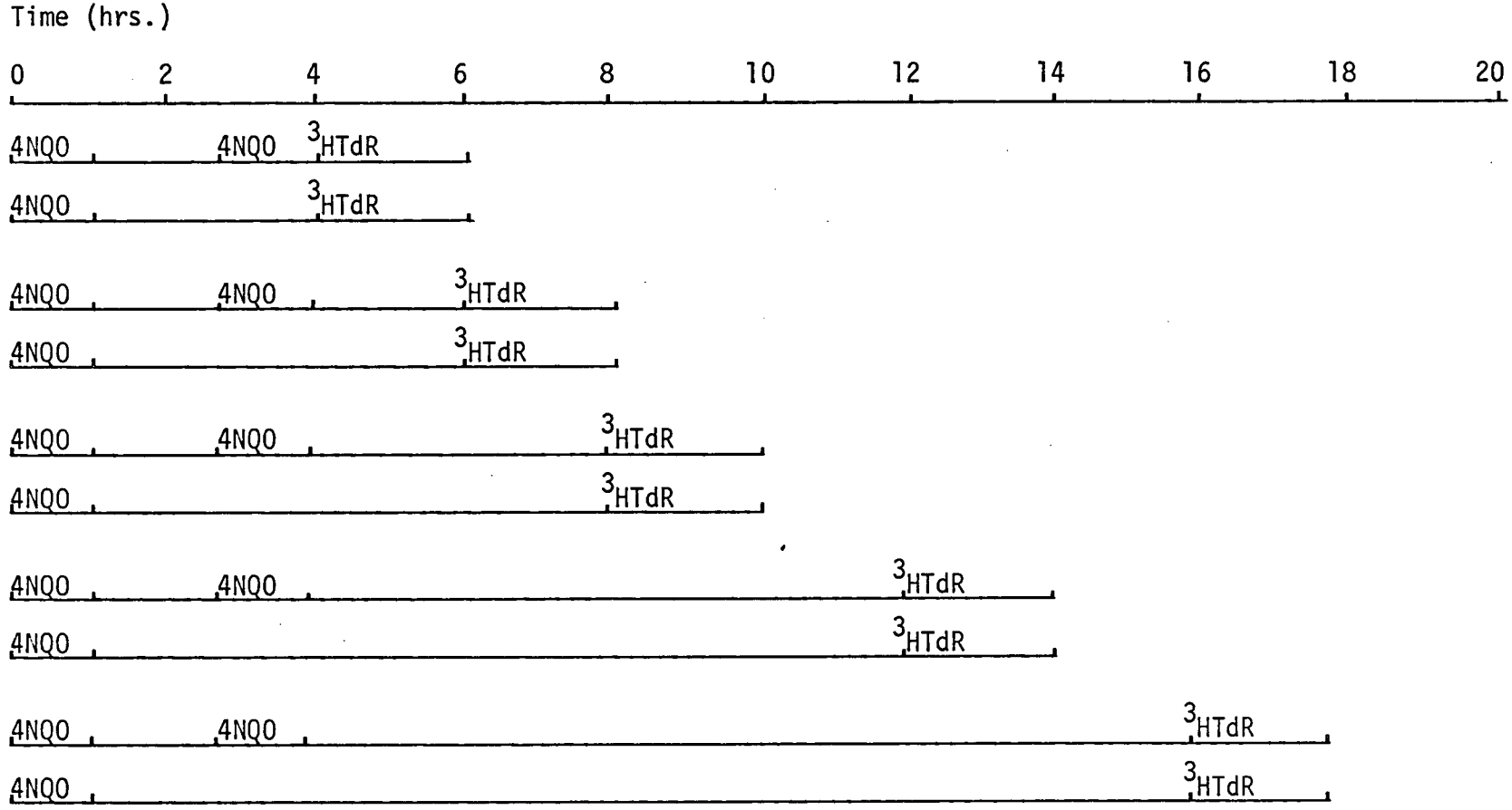


Figure 11: Experimental design. Time course of DNA repair synthesis following a second 4NQO treatment. Illustrated is the sequence of ^{3}HTdR pulses after removal of the second treatment given 3 hours after addition of the first. Other treatments were applied 2, 5 or 9 hours after the first and were followed by the same sequence of ^{3}HTdR pulses.

Figure 12: Time course of DNA repair synthesis following a second 4NQO treatment (5×10^{-7}M).

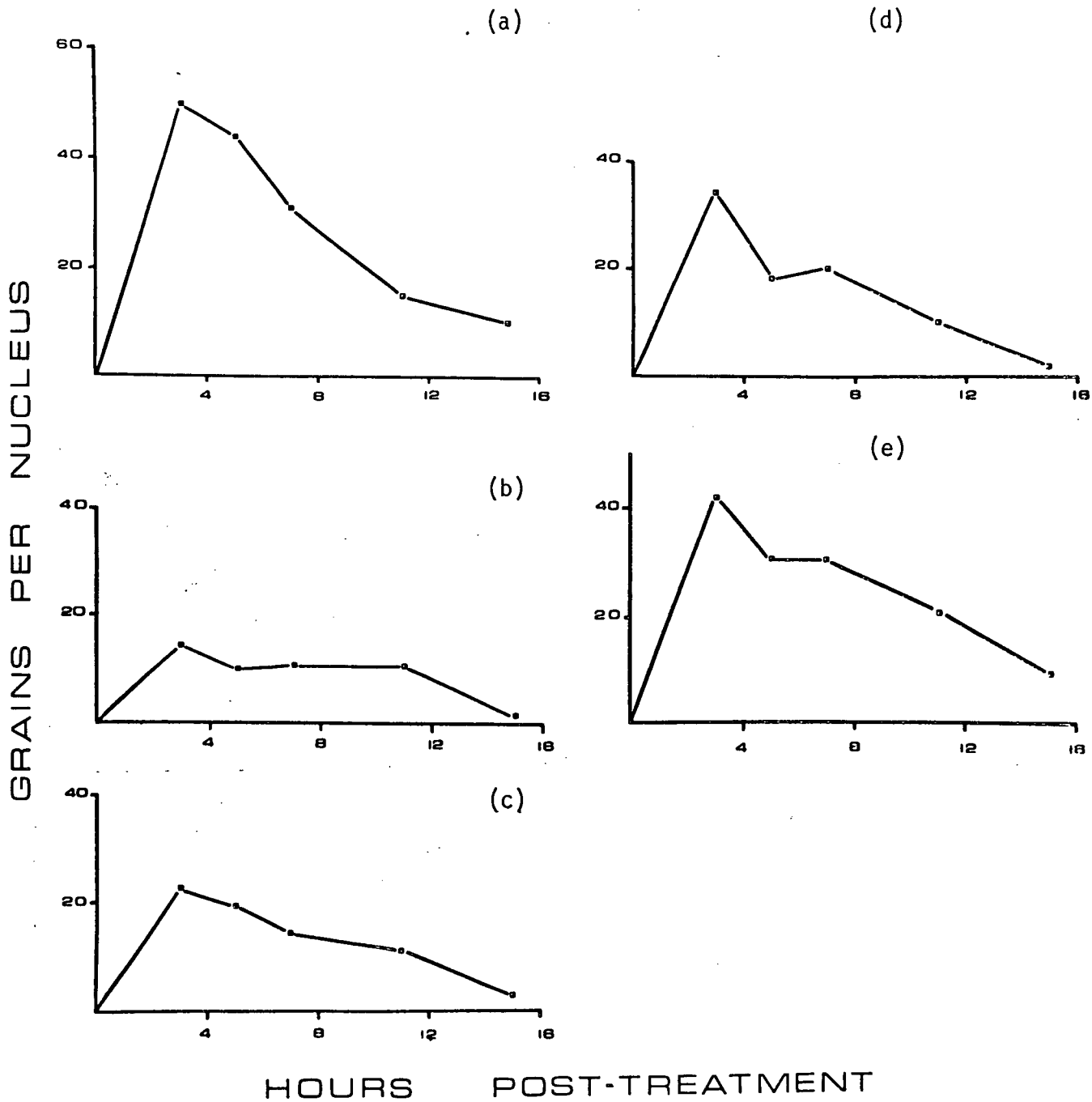
(a) initial treatment only

(b) 2 treatments 2 hours apart

(c) 2 treatments 3 hours apart

(d) 2 treatments 5 hours apart

(e) 2 treatments 9 hours apart


(a)
(b)
(c)
(d)
(e)
60
40
20
4
8
12
16
GRAINS PER NUCLEUS
HOURS POST-TREATMENT

V. Cell Survival and Chromosome Studies

In order to thoroughly characterize the carcinogenicity of a chemical compound, previous studies have employed cell survival and chromosome studies in addition to repair experiments [74,75]. Therefore it seemed logical that to complete this particular study the effect of split 4NQO treatments on cell survival and chromosome aberrations should be determined. If a period does exist in which a second 4NQO treatment does not induce further damage, then one would expect that in this period there would be no change in cell survival, or chromosome aberrations. On the other hand, if the damage does occur, yet is not being repaired, one would expect this to be reflected by changes in cell survival and in chromosome aberrations.

a) Cell Survival Studies

An experiment was designed to ascertain the effect of split 4NQO treatments on cell survival (Figure 13). However, before this experiment could be carried out, it was necessary to solve one technical problem. In most survival experiments, the cells are seeded into 15% MEM, allowed to settle down for 12-16 hours, exposed to a single chemical treatment, and then left to divide and form colonies. The cells do not have an opportunity to divide prior to treatment and as a result only single cells are exposed to the chemical. Each individual cell that survives will then divide to produce one colony. But as outlined in the experimental protocol (Figure 13), it was necessary to leave the cells for periods of up to 12 hours after exposure to the first dose

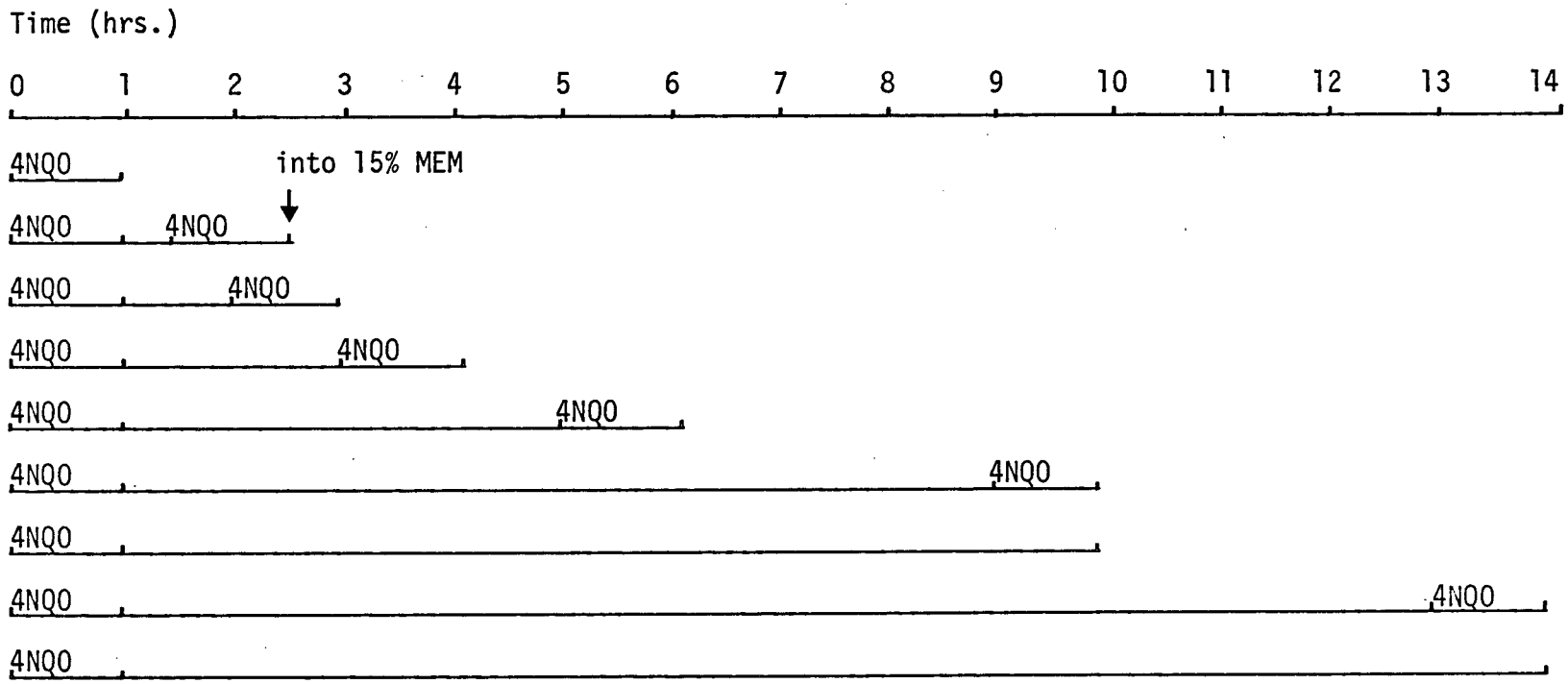


Figure 13. Experimental design. Variation of the intervals between split 4NQO treatments; effect on cell survival.

prior to application of the second treatment. During such a time period, cell divisions most likely will occur, and as a result two cells instead of one would receive the second treatment, invariably producing false results as only one cell need survive to form a colony.

It is apparent then that a method was required that would prevent the cells from dividing before completion of the experiment. Two alternatives seemed likely; firstly, seed the cells into 5% ADM, run the experiment in 5% ADM, and then return the cells to 15% MEM; or secondly, seed the cells into 2 1/2% MEM, run the experiment in 2 1/2% MEM, and then return the cells to 15% MEM. It was hoped that ADM would totally arrest the cells, and that 2 1/2% MEM would slow down cellular processes to a point, such that cell divisions would not occur during the course of the experiment. The first alternative proved not feasible; when the cells were seeded into 5% ADM they would not settle down and adhere to the petri dish. However, cells seeded into 2 1/2% MEM did attach to the petri dish.

Further experiments were then carried out to investigate the feasibility of utilizing cells seeded into 2 1/2% MEM for these particular survival studies. Cells left in 2 1/2% MEM for periods of time after they had adhered to the petri dish were not significantly affected in their ability to form colonies (Table 1). Close examination of the cells under the inverted microscope after 12 hours in 2 1/2% MEM revealed no cell divisions. Cells undergoing division can easily be detected as they become rounded whereas the others remain flat.

Table 1. Effect of 2 1/2% MEM on cell survival

	Hours in 2 1/2% MEM				
	0	2	4	8	12
Average number of colonies	75	70	71	72	73

Table 2. Effect of a 1 hour 4NQO treatment on cell survival

	4NQO concentration				
	5×10^{-8}M	10^{-8}M	5×10^{-9}M	10^{-9}M	5×10^{-10}M
% surviving colonies	0	9	68	81	90

Treatment of the cells for 1 hour with a range of 4NQO concentrations gave the results outlined in Table 2. Cells were seeded into 2 1/2% MEM, allowed to settle down, treated with chemical in 2 1/2% MEM and then immediately returned to 15% MEM. The number of surviving colonies was expressed as a percentage of the control (no 4NQO treatment). From these results, two concentrations were chosen for use in further experiments (5×10^{-9}M and 5×10^{-10}M). These concentrations were chosen because one dose allowed enough cells to survive so that after application of a second dose, a reasonable number of cells would still survive.

Finally cells seeded into 2 1/2% MEM and allowed to adhere to the petri dish, were assessed for their ability to repair 4NQO-induced DNA damage following 12 hours in 2 1/2% MEM; and their ability to repair 4NQO-induced DNA damage whilst maintained in 2 1/2% MEM for 12 hours. In the first case the cells were left in 2 1/2% MEM (after the initial settling down period) for 0, 2, 4, 8 or 12 hours prior to 4NQO treatment, treated and then placed in 15% MEM (Table 3). In the second case the cells were exposed to 4NQO (after settling down) and then allowed to recover in 2 1/2% MEM for 0, 2, 4, 8 or 12 hours, after which time they were returned to 15% MEM (Table 4). It is apparent from the results listed in Tables 3 and 4 that repair of 4NQO-induced DNA damage is not significantly affected by 2 1/2% MEM until 8 hours, at which time there is a small but significant drop in cell survival. It is interesting that the effect is the same whether or not the treatment was given before or after incubation in 2 1/2% MEM. This seems to imply that after prolonged periods in 2 1/2% MEM, a general toxicity occurs that is slightly enhanced by exposure to 4NQO.

The feasibility of employing this technique was then apparent, and only required inclusion of extra "singly-treated" controls. These extra controls would receive the first treatment only, were subsequently incubated in 2 1/2% MEM for 9 or 13 hours and then returned to 15% MEM along with the "doubly-treated" cultures. Other controls received the first treatment and were then placed immediately into 15% MEM.

As in the previous DNA repair experiments it was necessary to select a suitable end point for evaluating the results. Again an expected

Table 3. The effect on cell survival of incubation in 2 1/2% MEM, prior to a single 4NQO treatment

Hours in 2 1/2% MEM prior to treatment	% surviving colonies at concentrations	
	5×10^{-9}M	5×10^{-10}M
0	71	86
2	65	79
4	70	88
8	60	76
12	55	68

Table 4. The effect on cell survival of incubation in 2 1/2% MEM, following treatment with 4NQO

Hours in 2 1/2% MEM after treatment	% surviving colonies at concentrations	
	5×10^{-9}M	5×10^{-10}M
0	76	87
2	67	78
4	70	83
8	58	72
12	53	64

value was defined: if a single 4NQO treatment enables a certain percentage of cells to survive, then theoretically when a second treatment is applied to these surviving cells, the same percentage of these cells would be expected to survive. In other words if 68% of the cells survive the first dose, then 68% of these 68% should be expected to survive the second dose (i.e. 46%). In calculating the expected values at 9 and 13 hours, the controls for those time periods were employed in order to account for any loss of cells due to toxicity in 2 1/2% MEM.

The results outlined in Table 5 indicate a period in which there is a potentiation of the effects of the second dose. This occurs in the three hours immediately following addition of the first treatment, and correlates very closely with the results of the DNA repair experiments, in which the below expected repair levels are obtained in the same 3 hours.

Closer examination of the results reveal that after this period of decreased cell survival, the levels rise and reach a plateau at 9 hours. For both concentrations, the value attained is above the expected, though not the same; cells exposed to two doses of the lower concentration rise to a higher point above the expected value. The fact that cell survival does rise above the expected may simply reflect an incorrect choice of end point, or a capacity for increased survival in the cells that survive the first dose.

Nevertheless, it is obvious that as the repair of the first treatment nears completion, the cells regain their capacity for survival. It is also apparent that if two 4NQO treatments are given close together, DNA damage is induced by the second treatment, and furthermore that some of it is not being repaired.

Table 5. Effect of split 4NQO treatments on cell survival

[4NQO]	Time after first dose when second dose applied (hours)	% surviving colonies after second dose	Expected value (%)	Difference (%)
5×10^{-9}M	1 1/2 hrs.	8	50	-42
	2	6	50	-44
	3	34	50	-16
	5	30	50	-20
	9	40	30	+10
	13	37	32	+5
5×10^{-10}M	1 1/2 hrs.	55	79	-24
	2	51	79	-28
	3	80	79	+1
	5	92	79	+13
	9	58	44	+14
	13	61	38	+23

b) Chromosome Studies

Since in the previous experiments, a drop in cell survival was obtained when the two treatments were given close together, it would be interesting to ascertain if a simultaneous increase in chromosome aberrations also occurs. The experimental protocol outlined in Figure 14 was designed to investigate this. A 13 hour split treatment was not done for 2 reasons; firstly, the cells have more or less totally recovered in their ability to repair the second dose at 9 hours, and secondly the main interest of the experiment involved the 3 hours immediately following the first dose.

The cells were treated in 5% MEM, and allowed to recover in 15% MEM. Colchicine was added at 20 and 25 hours post-treatment (second dose).

The results are outlined in Table 6. A marked increase in chromosome aberrations is evident if the second treatment is given in the two hours following addition of the first. The effect is not as dramatic at the lower concentrations but is still evident. After five hours, the values approach normal levels. Again a correlation with the DNA repair experiments is obtained, and provides further evidence that DNA damage is not being repaired.

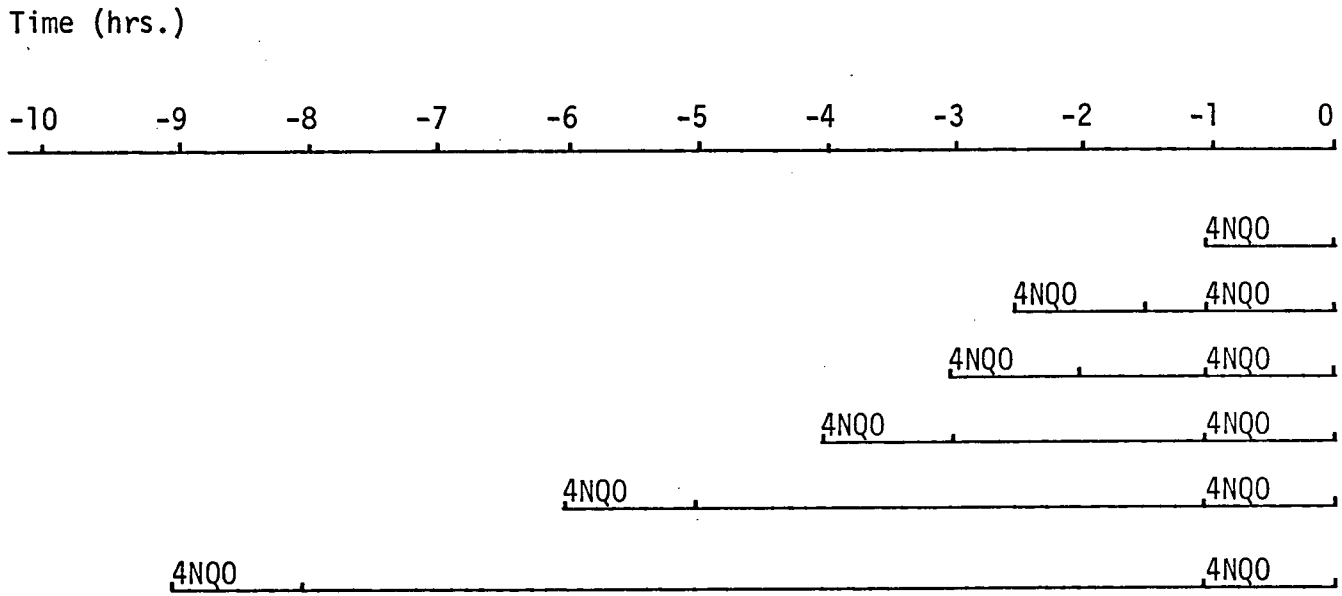


Figure 14: Experimental design. Variation of the intervals between split 4NQO treatments; effect on chromosome aberrations.

Table 6. Effect of split 4NQO treatments on chromosome aberrations

Time after first dose when second dose applied (hours)	Frequency of metaphase plates with chromosome aberrations at concentrations	
	5×10^{-7}M	10^{-7}M
1 1/2 hrs.	24	15
2	48	21
3	23	11
5	17	5
9	9	3
Single dose only	11	3

DISCUSSION

I. The DNA Repair Process

Before embarking on any discussion or interpretation of the results obtained in this study, it is essential that the underlying mechanisms of the DNA repair process be defined. The enzymes involved in excision-repair (also called dark repair, replication repair, unscheduled repair synthesis) have been well characterized in bacterial systems[6,32,36,57] and it is believed that a similar enzymatic process exists in mammalian cells[13,56,58]. The essential features of excision-repair in bacteria are outlined in Figure 15. Basically, the mechanism can be described as a cycle involving at least 4 enzymes and 5 defined activities:

a) UV irradiation results in the formation of pyrimidine dimers; each producing a localized structural distortion in the DNA helix.

b) These distortions serve as recognition sites for attack by an endonuclease; and a nick is established in the DNA close to the dimer on the 5' side.

c) DNA polymerase binds to the DNA at the nicked site and begins to synthesize new DNA using the opposite side of the DNA as a template[30].

d) An exonuclease cleaves the 3' side of the dimer releasing the damaged portion of the DNA; it is believed that the polymerase itself may have such an exonuclease activity[3,4,42].

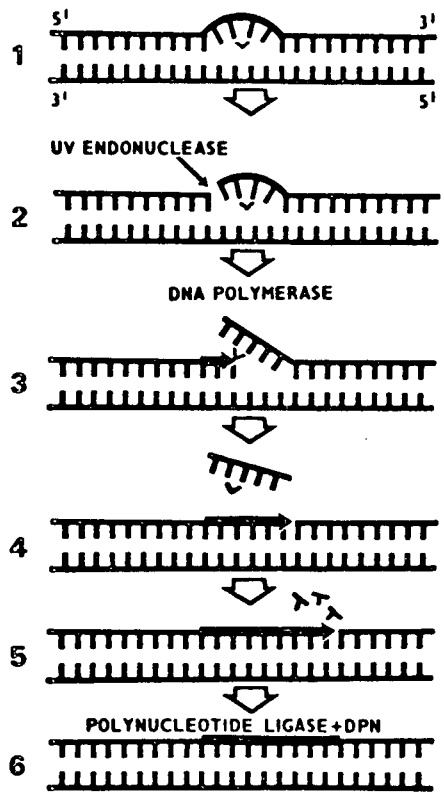


Figure 15. A model for DNA repair.
(From Molecular and Cellular Repair Processes.
Baltimore: John Hopkins, 1972, p. 15).

e) Simultaneous synthesis and degradation continues in the 5' to 3' direction until the ligase displaces the polymerase and seals the last bond.

The net result of this process is restoration of the functional integrity of the DNA.

The repair of both UV and 4NQO-induced DNA damage is postulated to be basically similar; this fact was further substantiated by the data obtained in the present study. However, the induction of damage is not identical. The structure of 4NQO is illustrated in Figure 16, and probably binds covalently to a purine base in the DNA[67,77]. As a result depurination of the DNA occurs followed by distortion of the sugar phosphate backbone. This distortion is the recognition site for the endonuclease. Removal of this distortion proceeds in the same manner as removal of pyrimidine dimers.

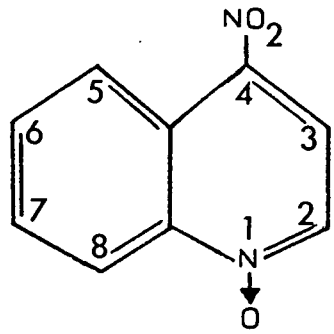


Figure 16. 4-Nitroquinoline 1-Oxide (4NQO).

II. Interpretation of ^{3}HTdR Incorporation Studies

The results of the DNA repair experiments can be summarized as follows:

1) DNA repair synthesis is below the expected levels when a second treatment is given within 3 hours of the first,

2) DNA repair synthesis reached the expected levels if the second treatment is applied as repair of the first approaches completion, and

3) analogous results when the first 4NQO treatment is replaced with UV irradiation.

Interpretation of this data was somewhat simplified by employing cell survival and chromosome studies. The results of these were:

1) a drop in cell survival and an increase in chromosome aberrations when the interval between doses is less than 3 hours;

2) a rise in cell survival and a drop in chromosome aberrations approaching the expected when the interval between doses is more than 3 hours.

Five interpretations of this data warrant consideration and will be discussed individually in the subsequent paragraphs.

The most logical explanation for the below-expected repair levels is that further damage did not ensue when the second dose was applied close to the first. This could have originated from any one of three conditions: the enzyme pools necessary for activation of the chemical were depleted, the DNA was already overloaded with damage, or the 4NQO was somehow prevented from entering the cell. Yet the data does not support any of these suggestions. The results of the chromosome studies indicate that damage is not being repaired, consequently if the second dose does not induce further damage, one would expect the level of repair synthesis following the second treatment

to be below the control level (single dose). But it is not; therefore, further damage must occur, though all of it is not necessarily repaired.

The next possibility is that the enzymes necessary for the repair process are being inhibited by the 4NQO, which in turn would be reflected by low repair levels. This does not seem very plausible because one would expect such an inhibition to occur after each 4NQO treatment. But it does not; low repair levels are only obtained following the second treatment and only when it is applied close to the first.

Furthermore, if UV-irradiated cells are treated with a chemical that is known to inhibit one or more of the enzymes involved in DNA repair (i.e. iodoacetate), the effect is very pronounced, with an almost total inhibition of repair synthesis[10]. This was not evident in the present study. Consequently, inhibition of the repair enzymes by 4NQO can be discounted.

The third interpretation is that the repair enzymes are being degraded after a single cycle of repair. As a result, when a second dose of 4NQO is given close to the first, the repair levels would be below the expected as not enough enzymes would be present. The repair capacity would be restored when synthesis of new enzymes increases, and would account for normal repair levels when the interval between the two treatments increases.

However, many observations disagree with such an interpretation; in the first place it implies that renewal of the enzyme pool would result in the damage being repaired at a later time. The results of

the time course of repair after a second dose do not verify this; if two doses are given close together the peak of repair occurs immediately after removal of the second treatment, it is not delayed. The results of the chromosome and survival studies also do not indicate that the damage is repaired at a later time.

Secondly, there is very little evidence to substantiate the concept that the degradation of the repair enzymes occurs after one cycle of repair. In fact most of the work indicates that the enzymes for repair are very stable, possess a long half-life and are not induced[29,60]. Experiments with puromycin and cycloheximide demonstrated that protein synthesis was not a prerequisite for DNA repair; the enzymes were already present in the cell in quantities capable of repairing most of DNA damage. Treatment of the cells with cycloheximide for up to 8 hours before exposure to UV did not alter the repair levels; even though protein synthesis had been almost totally blocked[5]. Cells left in cycloheximide for 20 hours prior to UV irradiation showed levels of repair replication that were still 65% of the untreated, irradiated cultures. In other studies, puromycin also had no effect on repair of X-ray induced damage[18,65].

In view of these arguments, it seems highly unlikely that the results of this study can be accounted for by degradation of the repair enzymes.

Alternatively, it is possible that the below expected levels of repair synthesis are artifacts of the technique employed. During the initial peak of repair synthesis, there is a limit to the number

of incisions that can occur at one time along the DNA[2,14]. This implies that even though many segments of the DNA are undergoing repair synthesis during the early stages of recovery, numerous sites will still contain damaged portions. If the DNA is subjected to further damage during this time, an alkylated base could occur close to a previously alkylated base (but as yet unrepaired). When such a DNA segment is finally repaired, theoretically more than one base could be removed per 100 nucleotides (excision repair involves the removal of approximately 100 nucleotides [11,17,59,61]). In this manner, a lower than expected grain count could ensue. If a second dose is given when most of the initial damage has been repaired, such an overlap would not occur, and consequently the expected number of grains would be obtained.

The major drawback of this explanation is that even though the grain counts are not up to the expected, the damage is still repaired; again this contradicts the results of the chromosome and cell survival studies. Moreover, current research does not indicate that more than one dimer is excised per region[21,59].

The final explanation concerns the possibility that the repair process itself is somehow being inhibited by the second 4NQO treatment. Since direct inhibition of the enzymes themselves has already been discounted, one of the few remaining solutions is that the ongoing repair process itself is being affected. This would be especially apparent when many sites along the DNA are being repaired (for instance, during the peak of repair). However, if direct inhibition of repair by the second dose is occurring, the levels obtained after this second

dose would be far below the controls. As this is not the case, it seems logical to conclude that induction of damage and inhibition of repair occur simultaneously upon application of the second dose.

It is not difficult to envisage how this inhibition would occur, for as each repair cycle progresses, many susceptible sites would be exposed. Binding of molecules at this time could easily take place in such a manner that continuation of the cycle is prevented. The enzymes themselves would remain unaffected, and would eventually fall off initiating a new cycle of repair synthesis at some other location. Implicit in this explanation is that the partially repaired segment of DNA is never repaired; the binding would have occurred in such a manner that movement of enzymes along the segment is permanently blocked.

The net result when the two treatments are applied close together would be inhibition of repair of the first set of damage; and induction and repair of a second set. Possibly the amount of damage produced by the second dose would be less than usual because some of the binding would have resulted in inhibition, not induction of damage. This combined effect would account for the below expected levels of repair. As repair of the first treatment nears completion, inhibition by the second dose would not be as evident, and normal levels of damage and consequently repair synthesis would be obtained.

If this is the case, then it suggests that the carcinogenicity of 4NQO may be due in part to its ability of slightly inhibiting damage induced by itself. Such an effect may not be as noticeable during a single treatment, because most likely a major portion of the binding

will have taken place before repair synthesis reaches its peak; therefore only a slight inhibition would take place. Accordingly, if a second dose is applied right when the peak of repair is in progress the effect may be very marked.

Inhibitors of DNA repair have been reported previously[10,16,27,40], and fall into two categories, those that bind to DNA, and those that show a wide spectrum of effects. The first group includes such compounds as acriflavine and chloroquine[10,27] which do not induce DNA repair synthesis but considerably inhibit the repair of UV damage. Acriflavine preferentially kills irradiated bacteria, and enhances UV mutagenesis, most likely by inhibiting excision of pyrimidine dimers[16,66]. In vivo, chloroquine and caffeine have been found to enhance tumorigenesis[79].

In the second group are such compounds as progesterone, testosterone, and diethylstilbestrol. These compounds do not bind to DNA and do not illicit DNA repair synthesis, but again they have a significant effect on DNA repair induced by UV[26,28]. Most of these compounds have been classified as co-carcinogens because they have the ability to enhance the tumorigenic action of other carcinogens[35]. It is believed that their target is some aspect of the repair process.

In view of such evidence it does not seem too unlikely that 4NQO could be inhibiting some aspect of the DNA repair process.

Discussion of the ^{3}HTdR incorporation studies would be somewhat incomplete if two additional aspects were not mentioned. The first concerns the data obtained with cells in 5% ADM. When a second treatment was given to these cells, the level of repair never reached the expected.

In fact as the intervals between the two doses increased, the level of repair following the second dose decreased. Identical experiments employing cells transferred to 5% MEM did not yield these results, implying that the effects was due to the lack of arginine in the medium.

Amino acid deprivation causes a fairly rapid cessation of semi-conservative DNA synthesis[5]. The mechanism behind this is not clear, for even though synthesis of new enzymes is blocked, enough enzymes are present to enable DNA synthesis to take place. It is believed that accurate DNA replication requires uninterrupted, co-ordinated, de novo synthesis of proteins[25,29,34]. Perhaps the DNA repair process is affected in the same manner. This seems highly unlikely for one would expect both treatments to be affected, not just the second.

A more logical explanation is that after prolonged incubation in ADM, the cells are not in an optimal physiological state, and consequently the toxicity of 4NQO is enhanced. Repair of the first treatment may be normal, but most likely after 8 hours incubation, the cells begin to die, and therefore cannot respond to additional chemically induced damage. When the two treatments are close together, the response of cells in ADM and MEM was almost, but not completely similar; for the cells in ADM showed a larger decline in levels of repair synthesis. This could reflect either cells in early stages of death or an effect due to arginine deficient medium. This phenomenon warrants further investigation.

The second result that must be mentioned is the slight inhibition of repair that occurred when UV exposure was closely followed by a 4NQO treatment. This was not evident if the first treatment involved exposure to 4NQO.

This observation may simply reflect the differences in the manner whereby damage was induced by 4NQO and UV; 4NQO treatment was for 1 hour, whereas UV treatment was for a few seconds. Even though the processes that are involved in the repair of these two types of damage are postulated to be the same[31,55,71,76], initiation of repair synthesis probably occurs at different times. As a result the two sets of data may not be truly comparable.

Returning to Figure 2, it can be noted that the peak of repair synthesis may already be in progress half an hour after removal of the 4NQO. But following UV treatment the peak of repair synthesis may only just be initiated half an hour after treatment. Consequently, the effect of a 4NQO treatment half an hour after a UV treatment may have a more profound effect, for it would be present when repair of UV-induced damage is at a maximum.

In retrospect, it is obvious that there are some limitations to the ^{3}HTdR incorporation experiments; it was not possible to determine exactly when, and for how long the peak of repair occurred, nor was it possible to define the peak of "sensitivity." Nevertheless, a foundation for further investigation has been established. Characterization of the two hour "sensitive" period in more detail should be attempted by spacing the two treatments 15, 30, 45, 60 and 90 minutes apart. Also addition of ^{3}HTdR simultaneously with the second 4NQO treatment, would perhaps give a better indication of when and to what extent inhibition may be occurring during the second treatment.

III. Split Doses and Cell Survival

Since treatment of cells in vitro with split doses of chemical carcinogens has not been reported before, many aspects of this study are unique. Consequently it is difficult to make any direct comparisons between the results of this work and those of others. Nevertheless, a few indirect comparisons can be made and are possibly relevant to this discussion.

The effects of split doses of UV and X-irradiation on cell survival has been reported and perhaps can be compared to the effects of split 4NQO treatments on cell survival that have been reported in this study.

Humphrey et al.[37,38] using a line of Chinese hamster cells detected an apparent increase in cytotoxicity (i.e. decrease in cell survival) when double doses of UV were separated by 30-120 minutes. However, the results were not interpreted in terms of potentiation but as a lack of repair in the cell line employed. The concomitant rise in survival after 2 hours was thought to be caused by selection of a radiation-resistant portion of the cell population. Such an interpretation was not warranted as a simultaneous study of repair synthesis was not attempted.

A more comprehensive study has recently been published[78], in which the effects of split UV treatments on both survival and mutation rates was investigated in a different line of Chinese hamster cells. A striking increase in the frequency of cytotoxicity and mutations was obtained when the intervals between doses were 15, 30, and 60 minutes.

At an interval of 3 hours, the mutation rate returned to the value expected, as did cytotoxicity at 5 hours. Both values reached a stable expected level after 9-12 hours. It was concluded that the second dose was somehow interfering with an aspect of the cell's repair process.

Analogous data was not reported when split doses of X-irradiation were employed. In these studies, if survival is plotted as a function of the time between exposures, a sharp rise in cell survival is obtained in the first two hours, and remains constant for the next 5 hours, to level off at a higher value after 10 hours[1,19,20]. No significant decrease in cell survival was observed. These results are not surprising though, because they most likely reflect the inherent differences between the kinds of damage and repair processes induced by UV and X-irradiation. UV damage produces pyrimidine dimers, induces a repair cycle that involves incorporation of approximately 100 new nucleotides and which is virtually complete at 6 hours[13,24,67]. On the other hand, the major product of X-ray damage is single-strand breaks[53], which induces a repair cycle involving incorporation of 2-3 nucleotides[23,54,56,70], and that is rapidly completed[43,49,65,69]. This repair does not require a rate-limiting cleavage step, only a small amount of synthesis prior to re-joining[9,15,52]. It is conceivable that when cells undergoing this particular repair are exposed to a second treatment, any effect that does occur is probably difficult to detect.

Two observations emerge; firstly, since the repair induced by UV damage is basically similar to the repair of damage induced by 4NQO, a legitimate correlation can perhaps be made between the results of the cell survival experiments in this present study and those reported above

involving split UV doses. In both cases a period of increased sensitivity is evident.

Secondly, since chemicals differ in the type of DNA damage they produce, eg. some bind to bases, whereas others produce single strand breaks[7,62]; and the types of DNA repair processes induced, it is very probable that the effects of split treatments on these two kinds of DNA repair synthesis also differ. Further investigation of this phenomenon should be the next step.

IV. Outlook

It was suggested previously that residual, unrepaired DNA damage may be of more importance than the levels of repair synthesis that take place upon treatment with a chemical carcinogen. Continuing on this assumption, if the repair of DNA damage is then also partially inhibited, the frequency of neoplastic transformation may be further increased.

In this study, exposure of cells to split treatments of a carcinogen revealed a potentiation of the effects when the doses were given close together, and was interpreted to reflect a lack of repair synthesis. If this is the case, then care must be exercised in evaluating experiments that involve only single doses of carcinogens, for the true carcinogenic potential of the chemical may not be evident.

In designing assay systems to be employed in screening for carcinogens, it is necessary to incorporate many exceptions in order that a "true" value can be obtained. Firstly, the existence of cell

lines that cannot repair certain types of DNA damage cannot be ignored; the addition of chemicals to these cells in some instances can produce a false negative result. To circumvent this difficulty thorough screening should employ more than one cell line. Secondly, some chemical compounds require metabolic activation before they can induce DNA damage[48]. Consequently, measures must be taken to allow for activation of these compounds; this usually involves the addition of a liver microsomal preparation simultaneously with the chemical[46].

It is obvious from the evidence presented in this study, that a more meaningful evaluation of the carcinogenic potential of a compound can be obtained by inclusion of the technique designed in this study, into future screening programs. Many carcinogens that previously have not illustrated any capacity to induce DNA damage[63], can be assessed using such techniques. Perhaps their carcinogenicity is due to their ability to alter the repair of DNA damage. Furthermore, chemicals that have been previously tested using only single doses, should be re-evaluated using double doses, for they may in fact have a greater carcinogenicity than previously imagined.

Additional research in this area should be directed at investigating the interactions of various combinations and sequences of different carcinogens, in vitro. These studies should increase our understanding of the processes involved in the repair of DNA damage and its role in chemical carcinogenesis.

SUMMARY

1. The primary objective of this study was to investigate the effects of repeated exposure to a chemical carcinogen, 4NQO, in human skin fibroblasts. Three end points were employed: DNA repair synthesis (as measured by ^{3}HTdR incorporation), cell survival and chromosome aberrations.

2. Following a single one hour treatment with 4NQO, the peak of repair synthesis was evident in the second and third hours after addition of the carcinogen. Repair synthesis was virtually complete at 12 hours post-treatment.

3. When cells were challenged with a second 4NQO treatment within 3 hours of the first, the level of repair synthesis induced by this second dose was below a defined expected value. Following 9 hours incubation between treatments, repair synthesis after the second dose had returned to the expected value.

4. Replacement of the first 4NQO treatment with a UV treatment produced analogous results.

5. Cell survival dropped significantly when the second dose was applied within one hour of the first. An increase in the frequency of chromosome aberrations was detected when the two treatments were given less than two hours apart. Both values had returned to expected levels when treatments were separated by more than 5 hours.

6. Evaluation of all three sets of data indicated that insufficient DNA repair synthesis was ocurring when the second treatment was applied whilst repair of the first was still in progress. As repair of the first treatment neared completion, this effect was no longer evident.

7. Several possible explanations were discussed and it was concluded that the second dose was possibly inhibiting the ongoing repair process.

8. Interpretation of this conclusion in terms of the increased carcinogenic potential of a chemical carcinogen was also presented.

REFERENCES

1. Arlett, C. F., and Potter, J. Mutation to 8-azaguanine resistance induced by γ-irradiation in Chinese hamster cell lines. Mutation Res., 13:59, 1971.

2. Ben-Hur, E., and Ben-Ishai, R. DNA repair in UV-irradiated Hela cells and its reversible inhibition by hydroxyurea. Photochem. Photobiol., 13:337, 1971.

3. Boyle, J. M. Genetic and physiological factors relating to excision repair in E. coli. In Molecular and Cellular Repair Processes. Baltimore: John Hopkins, 1972, pp. 14-33.

4. _____, Paterson, M. C., and Setlow, R. B. Excision-repair properties of an E. coli mutant deficient in DNA polymerase. Nature, 226:708, 1970.

5. Brown, R. F., Umeda, T., Takai, S., and Lieberman, I. The effect of inhibitors of protein synthesis on DNA formation in the liver. Biochim. Biophys. Acta., 209:49, 1969.

6. Cerda-Olmedo, E., and Hanawalt, P. C. Repair of DNA damaged by N-methyl-N-nitro-N-nitrosoguanidine in E. coli. Biochem. Biophys. Res. Commun., 22:268, 1967.

7. Clarkson, J. M. and Evans, H. J. Unscheduled DNA synthesis in human leucocytes after exposure to UV light, γ rays and chemical mutagens. Mutation Res., 14:413, 1972.

8. Cleaver, D. E. Defective repair replication of DNA in Xeroderma Pigmentosum. Nature, 218,652, 1968.

9. _____. Xeroderma Pigmentosum: A human disease in which an initial stage of DNA repair is defective. Proc. Natl. Acad. Sci. (USA), 63:428, 1969.

10. _____. Repair replication of mammalian cell DNA: effects of compounds that inhibit DNA synthesis or dark repair. Radiation Res., 37:334, 1969.

11. _____. Excision repair; our current knowledge based on human and cattle cells. In Molecular and Cellular Repair Processes. Baltimore: John Hopkins, 1972, pp. 195-211.

12. Cleaver, J. E. DNA repair with purines and pyrimidines in radiation and carcinogen-damaged normal and Xeroderma Pigmentosum human cells. Cancer Res., 33:362, 1973.

13. _____, and Painter, R. B. Evidence for repair replication of Hela cell DNA damaged by UV light. Biochim. Biophys. Acta., 161:552, 1968.

14. _____, Thomas, G. H., Trosko, J. E., and Lett, J. T. Excision repair in primary cultures of eukaryotic cells. Exptl. Cell Res., 74:67, 1972.

15. Dalyrymple, G. V., Sanders, J. L., Moss, A. J., Baker, M. L., and Wilkinson, K. P. Energy dependent nucleolytic processes are responsible for the production of many post-irradiation breaks in L cell DNA. Biochem. Biophys. Res. Commun., 36:284, 1969.

16. Davies, D. R. Acriflavine inhibition of dark repair and late generation death. Biochem. Biophys. Res. Commun., 23:652, 1966.

17. Edenberg, H., and Hanawalt, P. Size of repair patches in the DNA of UV-irradiated Hela cells. Biochim. Biophys. Acta., 272:361, 1972.

18. Elkind, M. M., Moses, W. B., and Sutton-Gilbert, H. Radiation response of mammalian cells grown in culture. Radiation Res., 31:156, 1967.

19. _____, and Sutton, H. X-ray damage and recovery in mammalian cells in culture. Nature, 184:1293, 1959.

20. _____, and _____. Radiation response of mammalian cells grown in culture. I. Repair of X-ray damage in surviving chinese hamster cells. Radiation Res., 13:556, 1960.

21. _____, and _____. The Radiobiology of Cultured Mammalian Cells. New York: Gordon and Breach, 1967, pp. 237-301.

22. Endo, H., Wada, A., Miura, K., Hidaka, Z., and Hiruki, C. Mutation in tobacco mosaic virus induced by a new carcinogen, 4-Nitroquinoline N-Oxide. Nature, 190:833, 1961.

23. Evans, R. G., and Norman, A. Radiation stimulated incorporation of thymidine into the DNA of human lymphocytes. Nature, 217: 455, 1968.

24. Evans, R. G., and Norman, A. Unscheduled incorporation of thymidine in UV-irradiated human lymphocytes. Radiation Res., 36:287, 1968.

25. Freed, J. J., and Schatz, S. A. Chromosome aberrations in cultured cells deprived of essential amino acids. Exp. Cell Res., 55: 393, 1969.

26. Gaudin, D., Gregg, R. S., and Yielding, K. L. DNA repair inhibition: A possible mechanism of action of co-carcinogens. Biochem. Biophys. Res. Commun., 45:630, 1971.

27. _____, _____, and _____. Inhibition of DNA repair replication by DNA binding drugs which sensitize cells to alkylating agents and X-rays. Proc. Soc. Exp. Biol. Med. 141:543, 1972.

28. _____, _____, and _____. Inhibition of DNA repair by cocarcinogens. Biochem. Biophys. Res. Commun., 48:945, 1972.

29. Gautschi, J. R., Young, B. R., and Cleaver, J. E. Repair of damaged DNA in the absence of protein synthesis in mammalian cells. Exp. Cell Res., 76:87, 1973.

30. Goulian, M. Some recent developments in DNA enzymology. Progr. Nucl. Acid Res. and Mol. Biol., 12:29, 1972.

31. Hahn, G. M., Yang, S., and Parker, V. Repair of sub-lethal damage and unscheduled DNA synthesis in mammalian cells treated with monofunctional alkylating agents. Nature, 220:1142, 1968.

32. Hanawalt, P. C., and Haynes, R. H. Repair replication of DNA in bacteria: irrelevance of chemical nature of base defect. Biochem. Biophys. Res. Commun., 19:462, 1965.

33. Heidelberger, C. Chemical oncogenesis in culture. Advan. Cancer Res., 18:317, 1973.

34. Horikawa, M., Fukuhara, M., Suzuki, F., Nikaido, O., and Sugahara, T. Comparative studies on induction and rejoining of DNA single-strand breaks by radiation and chemical carcinogens in mammalian cells, in vitro. Exp. Cell Res., 70:349, 1972.

35. Hoshino, H., Kawazoe, Y., and Fukuoka, F. Detection of potential weak carcinogens and procarcinogens. I. Effect of the derivatives of 4NQO on submanifestational dose of 4NQO. GANN, 60: 523, 1969.

36. Howard-Flanders, P. DNA repair. Annual Review Biochem. 37:175, 1968.

37. Humphrey, R. M., and Meyn, R. E. Recovery and repair in Chinese hamster cells following UV-irradiation. In Molecular and Cellular Repair Processes. Baltimore: John Hopkins, 1972, pp. 159-167.

38. _____, Sedita, B. A., and Meyn, R. E. Recovery of Chinese hamster cells from UV-irradiation damage. Int. J. Radiat. Biol., 18: 61, 1970.

39. Ikegami, S., Nemoto, N., Sato, S., and Sugimura, T. Binding of ^{14}C-labelled 4-Nitroquinoline 1-Oxide to DNA *in vivo*. Chem-Biol. Interact., 1:321, 1969.

40. Kann, H. E., Kohn, K. W., and Lyles, J. M. Inhibition of DNA repair by 1, 3 Bis (2 chloroethyl)-1-nitrosourea breakdown product, 2-chloroethyl isocyanate. Cancer Res., 34:398, 1974.

41. Kawazoe, Y., Huang, G., Araki, M., and Koga, C. Chemical binding of carcinogenic 4-Nitroquinoline 1-Oxide derivatives with DNA *in vivo* and *in vitro*. GANN, 63:161, 1972.

42. Kelly, R. B., Atkinson, M. R., Huberman, T. A., and Kornberg, A. Excision of thymine dimers and other mismatched sequences by DNA polymerase of *E. coli*. Nature, 224:495, 1969.

43. Kleijer, W. J., Lohman, P.H.M., Mulder, M. P., and Bootsma, D. Repair of X-ray damage in DNA of cultured cells from patients with Xeroderma Pigmentosum. Mutation Res., 9:517, 1970.

44. Kuroki, T., Ishizawa, J., and Sato, H. Studies on early events in *in vitro* carcinogenesis with 4-Nitroquinoline 1-Oxide. I. Effect on macromolecular synthesis and cell life cycle. GANN, 60:261, 1969.

45. Laishes, B. A. Unpublished Results.

46. _____, and Stich, H. F. Repair synthesis and sedimentation analysis of DNA of human cells exposed to dimethylnitrosamine and activated dimethylnitrosamine. Biochem. Biophys. Res. Commun., 52:827, 1973.

47. Matsushima, T., Kobuna, I., and Sugimura, T. *In Vivo* interaction of 4-Nitroquinoline 1-Oxide and its derivatives with DNA. Nature, 216:508, 1967.

48. Miller, J. A. Carcinogenesis by chemicals: an overview. Cancer Res., 30:559, 1970.

49. McGrath, R. A., and Williams, R. W. Reconstruction *in vivo* of irradiated *E. coli* DNA; the rejoining of broken pieces. Nature, 212:534, 1966.

50. Nagai, S. Production of respiration-deficient mutants in yeast by a carcinogen, 4 Nitroquinoline 1-Oxide. Mutation Res., 7: 333, 1969.

51. Nakahara, W., Fukuoka, F., and Sugimura, T. Carcinogenic action of 4-Nitroquinoline 1-Oxide. GANN 48:129, 1957.

52. Omerod, M. G., and Stevens, U. The rejoining of X-ray induced strand breaks in the DNA of a murine lymphoma cell. Biochim. Biophys. Acta., 232:72, 1971.

53. Painter, R. B. Repair of DNA in mammalian cells. Current Top. Radiat. Res. (Quart.), 7:45, 1970.

54. _____, and Cleaver, J. E. Repair replication in Hela cells after large doses of X-irradiation. Nature, 216:369, 1967.

55. _____, and _____. Repair replication, unscheduled DNA synthesis and the repair of mammalian DNA. Radiation Res., 37:451, 1969.

56. _____, and Young, B. R. Repair replication in mammalian cells after X-irradiation. Mutation Res., 14:225, 1972.

57. Pettijohn, D., and Hanawalt, P. Evidence for repair replication of UV-damaged DNA in bacteria. J. Mol. Biol., 9:395, 1964.

58. Rasmussen, D. E., and Painter, R. B. Evidence for repair of UV-damaged DNA in cultured mammalian cells. Nature, 203: 1360, 1964.

59. Regan, J. D., Setlow, R. B., and Ley, R. D. Normal and defective repair of damaged DNA in human cells: a sensitive assay utilizing the photolysis of bromodeoxyuridine. Proc. Natl. Acad. Sci. (USA), 68:708, 1971.

60. _____, Trosko, J. E., and Carrier, W. L. Evidence for excision of UV-induced pyrimidine dimers from the DNA of human cells in vitro. Biophysical J., 8:319, 1966.

61. Roberts, J. J. Repair of alkylated DNA in mammalian cells. In Molecular and Cellular Repair Processes. Baltimore: John Hopkins, 1972, pp. 226-238.

62. _____, Crathorn, A. R., and Brent, T. P. Repair of alkylated DNA in mammalian cells. Nature, 218:970, 1968.

63. San, R.H.C. Unpublished Results.

64. Sato, H., and Kuroki, T. Malignization in vitro of hamster embryonic cells by chemical carcinogens. Proc. Japan. Acad., 42:1211, 1966.

65. Sawada, S., and Okada, S. Rejoining of single-strand breaks of DNA in cultured mammalian cells. Radiation Res., 41:145, 1970.

66. Setlow, R. B. Physical changes and mutagenesis. J. Cell. Comp. Physiol. 64, supp 1:51, 1964.

67. Setlow, R. B. The photochemistry, photobiology, and repair of polynucleotides. Progr. Nucl. Acid Res. and Mol. Biol., 8: 257, 1968

68. _____, Regan, J. D., German, J., and Carrier, W. L. Evidence that Xeroderma Pigmentosum cells do not perform the first step in the repair of UV damage to their DNA. Proc. Natl. Acad. Sci. (USA), 64:1035, 1969.

69. Spiegler, P., and Norman, A. Kinetics of unscheduled DNA synthesis induced by ionizing radiation in human lymphocytes. Radiation Res., 39:400, 1969.

70. _____, and _____. Unscheduled DNA synthesis at low X-ray doses. Mutation Res., 10:379, 1970.

71. Stich, H. F., and Laishes, B. A. DNA repair and chemical carcinogens. Pathobiology Annual, 3:341, 1973.

72. _____, and San, R.H.C. DNA repair and chromatid anomalies in mammalian cells exposed to 4-Nitroquinoline 1-Oxide. Mutation Res., 10:389, 1970.

73. _____, and _____. Reduced DNA repair synthesis in Xeroderma Pigmentosum cells exposed to the oncogenic 4-Nitroquinoline 1-Oxide and 4-Hydroxyaminoquinoline 1-Oxide. Mutation Res., 13:279, 1971.

74. _____, _____, and Kawazoe, Y. DNA repair synthesis in mammalian cells exposed to a series of oncogenic and non-oncogenic derivatives of 4-Nitroquinoline 1-Oxide. Nature, 229:416, 1971.

75. _____, _____, and _____. Increased sensitivity of Xeroderma Pigmentosum cells to some chemical carcinogens and mutagens. Mutation Res., 17:127, 1973.

76. Strauss, B., Coyle, M., and Robbins, M. Alkylation damage and its repair. Cold Spring Harbor Symp. Quant. Biol., 33:277, 1968.

77. Tada, M., and Tada, M. Interaction of a carcinogen, 4-Nitroquinoline 1-Oxide with nucleic acids: chemical degradation of the adducts. Chem.-Biol. Interact., 3:225, 1971.

78. Thilly, W. G., and Heidelberger, C. Cytotoxicity and mutagenicity of UV-irradiation as a function of the interval between split doses in cultured chinese hamster cells. Mutation Res., 17: 287, 1973.

79. Yielding, K. L., and Gaudin, D. Enhancement of tumor response to X-ray and alkylating agents by the excision repair inhibitors, caffeine and chloroquine. 23rd Annual Symp. Fund Cancer Res., pp. 523-527, 1969.