Mode of Action of Mutagenic and Oncogenic Synthetic Quinoline N-Oxides

by

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ABSTRACT

The molecular events in cells exposed to mutagenic and oncogenic quinoline N-oxides were examined by analyzing single-strand breakage of DNA.

Secondary cultures of embryonic Syrian-hamster cells and a line of BHK-21 cells were used. The choice of nitroquinoline N-oxides afforded a series of water-soluble chemicals with three relative degrees of oncogenicity: highly oncogenic 4-nitroquinoline 1-oxide (4NQO), weakly oncogenic 3-methyl-4-nitroquinoline 1-oxide (3-methyl-4NQO), and the non-oncogenic 3-nitroquinoline 1-oxide (3NQO) and 8-nitroquinoline 1-oxide (8NQO). The detection of subtle alterations in DNA sedimentation velocity was greatly improved by a double-label procedure. Cells treated for 2 hours with the various nitroquinoline N-oxide compounds were prelabelled with 0.05 uCi/ml ^{14}C-thymidine for 24 hours. Untreated control cells were labelled with 0.25 uCi/ml ^{3}H-thymidine for 24 hours. Aliquots of quinoline N-oxide treated and untreated cells were mixed, layered on the alkaline sucrose gradient, centrifuged at 25,000 rpm for 300 minutes, and the amount of ^{14}C (treated) and ^{3}H(untreated) radioactivity in each fraction of the gradient was determined.

The alkaline sucrose gradient technique was modified in the following ways: (1) a PBS cell suspension was layered directly onto a 2% sucrose solution overlay, on the gradient, to reduce DNA-shearing forces believed previously encountered with a 0.5M NaOH overlay, (2) cell lysis and centrifugation were carried out at 4° rather than room temperature, and (3) the

number of cells layered per gradient was reduced to 12,000.

A correlation between the degree of oncogenicity of nitroquinoline N-oxides and their capacity to induce DNA single-strand breakage was indicated, although limited because of the low sensitivity of the sucrose gradient technique. Single-strand breaks of DNA occurred when cells were treated for 2 hours with 5×10^{-6}M 4NQO but were not detectable when exposed to 4NQO at a 1×10^{-6}M concentration or less. However, when cells were treated with the weakly oncogenic 3-methyl-4NQO (5×10^{-6}M) or the non-oncogenic 3NQO and 8NQO (5×10^{-6}M) there were either no single-strand breaks produced or the frequency was too small to be recognized.

The repair of single-strand breaks was measured by sampling 4NQO-exposed cells after various periods post-treatment and estimating the amount of ^{14}C(treated)-DNA (in percent of total ^{14}C counts) over and above the amount of ^{3}H(untreated)-DNA (in percent of total ^{3}H counts) occurring in the top half of each gradient. Substantial repair at 24 hours was indicated by less than 4% ^{14}C-DNA above ^{3}H-DNA as opposed to 29% ^{14}C-DNA above ^{3}H-DNA at 0 hours post-treatment incubation.

Caffeine (1,3,7-trimethylxanthine), added at a concentration of 4×10^{-3}M to cell cultures, greatly reduced DNA single-strand breaks induced by 4NQO (4×10^{-6}M).

Attempts were made to correlate the capacity of synthetic quinoline N-oxides to induce DNA single-strand breaks with their capacity to invoke charge-transfer complexes with one or more DNA nucleotides, and so this study suggests approaches by which biological phenomena can be interpreted, ultimately, in terms of relative electron charge densities of molecules.

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INTRODUCTION

The tremendous surge of research in molecular biology for the past decade has not only provided some conclusive biochemical mechanisms for the mutation of living cells, but also provoked elegant theoretical attempts to elucidate the broad concept of mutation[25,52,64,69]. Briefly, the contention is that mutations are changes in the hereditary material; and by convention include alterations in chromosome number and structure as well as gene or point mutations[64]. Perhaps the most significant aspect of mutation research, at present, is the need to understand neoplastic transformation of genetically complex mammalian cells in order to seek a solution to the problem of cancer.

Recent *in vitro* cytologic studies have demonstrated mutagenesis of cultured mammalian cells on exposure to X-radiation (and ultraviolet light)[2,19] and, also, unscheduled DNA synthesis occurring in mammalian cells after large doses of X-rays (indicative of DNA repair[6,7,47,49]). Furthermore, biochemical techniques have been used to show that polynucleotide chain breaks in the DNA of mammalian cells can be induced by ionizing radiation and intracellular rejoining of such single strand breaks takes place during incubation after irradiation[34] in the same manner as in microorganisms[20,39]. It is common knowledge that the photon energies of X-rays are at least 6.25 ev which is well above the chemical (covalent) bond energies in the DNA

molecule. All in all, it can therefore be proposed that upon high-energy irradiation of a mammalian cell, the DNA is in fact a target throughout which random photon-induced bond cleavages can occur which result in a certain amount of both single and double strand breaks[57]. This DNA damage can then be repaired or lead to any of countless phenotypic cell alterations, including cell death and neoplastic transformation[19].

Similarly, cytologic studies have shown that exposure to chemicals has resulted in mutagenesis of cultured mammalian cells[3], and unscheduled DNA synthesis after exposure to mustard gas[51] and methyl methanesulfonate[11]. Biochemical techniques, as in the classic work of Brookes and Lawley[30], have clarified the mode of action of such alkylating agents in showing that DNA is again a target, the alkylation of which causes single and double strand breaks[30-32]. Again, implication of DNA damage in mutagenesis is evident in that sub-lethal DNA breaks may be irrepairable and hence lead to permanent DNA compositional or stereochemical alterations[33].

The foregoing examples have briefly illustrated the obvious necessity of biochemical studies when dealing with a complex and varied mutagenic phenomenon like neoplastic transformation which, ultimately, has its origins at the molecular level.

The mutagenic and oncogenic synthetic quinoline N-oxides have been firmly established among the array of known carcinogens*

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* The term carcinogen will be used to mean any chemical which induces any form of neoplasia (not only carcinomas as the term correctly implies).

since the 1957 report of the potent carcinogenic action of 4-nitroquinoline 1-oxide (4NQO) by Nakahara and co-workers[44]. Laboratory studies with 4NQO (Figure 1) and its derivatives have been facilitated by two chemical properties of the compound.

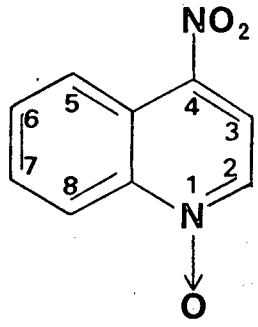

Figure 1. 4-Nitroquinoline 1-oxide (4NQO)

First, the particular heterocyclic structure of the quinolines is relatively easy to synthesize by established reaction sequences[24] and so a wide variety of structural analogs are readily available. Second, and most important, because of the highly polar N-oxide functionality, these compounds are all water-soluble[24], and consequently, their easy application to tissue culture systems facilitates rapid and reproducible cytologic and biochemical studies of the role of these compounds in mutagenesis.

In the past fourteen years, a veritable arsenal of biological activities of 4NQO has been collected. Not only do these reports illustrate the ability of 4NQO to be oncogenic _in vivo_[43,15] and to transform cells _in vitro_[45,54], but also to induce mutations[27,41], to inactivate phages[17], to destroy the transforming action of DNA[62,63],

to elicit chromosome aberrations[68], to induce mitotic irregularities[40], and to have carcinostatic activity[53]. DNA has been shown to be the target of 4NQO in mammalian cells *in vitro* by the study of Horikawa and co-workers[14]. The technique of alkaline sucrose density gradient centrifugation[39] of mammalian DNA was used to show a decrease in sedimentation velocity of Ehrlich ascites tumor cell DNA upon exposure to various concentrations of 4NQO and 4-hydroxyaminoquinoline 1-oxide (4HAQO, a reduction product of 4NQO thought to be a more potent carcinogen[56,10]). Moreover, an increase in sedimentation velocity of the mammalian DNA occurred upon incubation for various periods after 4NQO or 4HAQO treatment. In addition, unscheduled DNA synthesis occurred after 4NQO or 4HAQO treatment[14]. It was therefore suggested that 4NQO and 4HAQO cause single strand breaks in Ehrlich ascites tumor cell DNA *in vitro* and these cells are capable of repairing that damage[14].

However, the mode of action of 4NQO and its related compounds remained a mystery. The induction of single strand breaks in isolated DNA has been shown to occur with 4HAQO while 4NQO or 4-aminoquinoline 1-oxide (4AQO, the noncarcinogenic reduction product of 4HAQO) does not induce such a change in DNA[60]. This biochemical evidence was confusing as both 4HAQO and 4NQO could cause DNA breaks *in vitro*, with 4NQO being more active than 4HAQO[14].

In our laboratories, 4NQO and its derivatives were extensively used for cytologic studies of mammalian DNA repair[58,59], with repeated demonstrations of the correlation between the

degree of oncogenicity of the various derivatives and their capacity to evoke unscheduled DNA repair synthesis[59]. Secondly, projects were under way to show possible correlations between chromosomal aberrations and oncogenicity of 4NQO and its derivatives. Thirdly, projects were being carried out to demonstrate[12] mutagenesis of mammalian cells at the point mutation level, upon exposure to 4NQO and its derivatives. In order to accumulate further information regarding the kinetics of 4NQO-induced DNA repair, and to pursue the intricacies of the association between the degree of mutagenesis (neoplastic transformation) of mammalian cells and DNA repair synthesis, it became necessary to work at the molecular level. Therefore, it was decided to employ the technique of alkaline sucrose density gradient analysis of mammalian DNA[38,39] with all possible refinements of the existing sucrose gradient technique[14,39]. In this way, careful analysis of the sedimentation velocity of mammalian DNA after exposure to 4NQO and its derivatives, might cast some light on the puzzling mechanism by which these quinoline N-oxide compounds alter the DNA, and thereby induce the mutagenic phenomenon of neoplastic transformation.

MATERIALS AND METHODS

Cell cultures

Syrian-hamster embryos were cultivated as cell monolayers in Leighton tubes (without coverslips) in Eagle's minimal essential medium (MEM), supplemented with 10% fetal-calf serum and 1% antibiotic (penicillin, streptomycin). The cultures were incubated at 37° and only the first passage was used in these experiments. BHK-21 (clone 13) cells, an established line of Syrian-hamster cells[36], were obtained from Flow Laboratories, Maryland, and cultivated in the same manner. Sterile technique was used at all times up to the stage of sample harvesting.

Radioactive-label incorporation

Initially, cultures containing labelled DNA were prepared in a similar manner to that of Stich and San[58] in that cultures were incubated with ^{3}H-deoxythymidine (^{3}H-TdR, 17.9 Ci/mmole; Schwarz Bioresearch), at a final concentration of 10 uCi/ml, for 2 hours followed by a 4 hour incubation in fresh MEM without ^{3}H-TdR until a 2 hour carcinogen treatment (total chase*: 6 hours).

Both the concentration of ^{3}H-TdR and the pulse time were subsequently modified as follows: 4 uCi/ml for 2 hours and 3 hours; 1 uCi/ml for 2 hours and 3 hours; and, 0.25 uCi/ml for

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* The term 'chase' refers to the time allotted for incubation of cells, in normal MEM, after exposure to a radio-isotope in order to allow radioactive nucleoside to incorporate as uniformly as possible into newly synthesized DNA.

24-30 hours, 36 hours, and 48-60 hours. With the longer pulse times the ^{3}H-TdR solution (0.25 uCi/ml) was changed every 6 hours for the duration, and the chase time was reduced to 2-4 hours. The preferred labelling time was somewhat dependent on the duration of the particular cell growth cycle.

Cultures containing ^{14}C-labelled DNA were prepared by incubating the cultures with ^{14}C-deoxythymidine (^{14}C-TdR, 57 mCi/mmole; Amersham/Searle Corp.) at a final concentration of 0.05 uCi/ml[38] for the following pulse times: 24-30 hours (BHK-21 cells) and 36 hours (primary hamster embryo cells). The chase time was maintained at 2-4 hours.

Chemical treatment

Four quinoline N-oxide compounds, 4-nitroquinoline 1-oxide, 3-nitroquinoline 1-oxide, 8-nitroquinoline 1-oxide, and 3-methyl-4-nitroquinoline 1-oxide, were used. All compounds, with the exception of 4-nitroquinoline 1-oxide (Daiichi Pure Chemicals Co. Ltd., Tokyo), were supplied by Dr. Y. Kawazoe, National Cancer Centre Research Institute, Tokyo. Known weights of the compounds were dissolved in a minimum of 95% ethanol (final ethanol concentration: 0.004%) with gentle heating, diluted slowly with distilled, deionized water, and sterilized by filtering through membrane filters (0.22 u pore size, Millipore Ltd., Montreal). The resulting 10^{-3} molar stock solutions (10 or 100 ml volumes) were kept in the dark at 4° and checked monthly for stability by ultra-violet spectroscopy.

Aliquots of the stock solutions were diluted to the desired concentrations with MEM immediately prior to use. Before incubation with the chemical solutions for 2 hours, the cultures were washed once with aliquots of the same chemical solution. At the end of the 2 hour period the cultures were washed three times with MEM (no serum) and then harvested immediately or re-incubated with fresh MEM for repair studies. In a typical repair study, a 4×10^{-6}M 4NQO concentration was used to initiate DNA damage* and then cultures were usually sampled at 0, 2, 4, 8, 12, 16, and 24 hours after termination of chemical treatment.

The effect of the presence of caffeine (1,3,7-trimethylxanthine; Eastman Organic Chemicals, Rochester N.Y.) on the frequency of DNA breaks upon exposure to 4NQO was ascertained by adding a solution of 4×10^{-3}M caffeine and 4×10^{-6}M 4NQO in MEM, together, to prelabelled BHK-21 cell cultures for the usual 2 hour period. The cells were now washed and re-incubated in freshly prepared 4×10^{-3}M caffeine in MEM. Samples were harvested at similar intervals as in a typical repair study.

For a typical double-label analysis, only the ^{14}C-labelled culture was treated and these cells combined with ^{3}H-labelled untreated cells, from the corresponding control culture, upon harvesting. A corresponding ^{3}H-labelled control culture was maintained for each ^{14}C-labelled treated culture.

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* Throughout this study, the terms 'DNA damage', 'DNA breaks', and 'DNA pieces' will specifically refer to low molecular weight DNA single strands produced when double-stranded DNA, "nicked" by 4NQO (for example), is denatured on an alkaline sucrose density gradient. The Appendix illustrates this concept.

Sucrose density gradient analysis

For harvesting, each Leighton tube culture was first treated with 0.4 ml of 0.125% trypsin in MEM at 37° for 3-4 minutes, cells gently lifted from the glass surface with a makeshift rubber policeman (consisting of a small piece of Parafilm shaped on the end of a thin glass rod), and pipetted up and down to disperse aggregates. In the case of double-label analysis, ^{14}C-labelled treated cultures were combined with ^{3}H-labelled control cultures at this point. The cell suspensions were centrifuged at 1000xG for three minutes and remaining trypsin was removed by resuspending the cell pellets in cold phosphate bufferred saline (PBS, without Ca and Mg salts) with subsequent centrifugation. The final cell suspensions, in cold PBS, were counted on a hemacytometer and then diluted to give the appropriate number of cells in 0.1 ml of PBS.

The nitrocellulose centrifuge tubes for the SW-40 rotor (Beckmann Instruments, Inc., Palo Alto, California) contained 14.0 ml of a 10 to 30% linear sucrose density gradient made up in 0.3 M NaOH, 0.01 M EDTA, and 0.5 M NaCl. Prior to loading the cells, these gradients were allowed to sit at 4° for several hours to ensure temperature equilibrium. The gradients were overlaid with 0.8 ml of 2% sucrose in distilled, deionized water[38] (approximately 0.6 cm thick) on top of which was immediately placed 0.1 ml of the PBS cell suspension.

These loaded gradients were stored at 4° for 16-20 hours prior to centrifugation to allow for cell lysis and denaturation. Centrifuge tubes prepared in this manner were then centrifuged

in a Beckmann model L2-65B ultracentrifuge at 25,000 rpm and 4° for 300 minutes.

Immediately following centrifugation approximately 30-34 sequential fractions of 15 drops each were collected from the bottom of pierced tubes.

Radioactivity determination

Fractions were precipitated with 5% TCA in the presence of carrier albumin (approximately 50 ug/ml final concentration of protein serum albumin) and were collected on nitrocellulose filters (Millipore Ltd., Montreal) presoaked in 5% TCA and 10^{-3}M deoxythymidine. The filters were washed three times with 2 ml of 5% TCA, dried, and counted on a Packard liquid scintillation spectrometer (Model 3000).

RESULTS

I METHODOLOGICAL REFINEMENTS

(a) Reduction in Number of Cells per Gradient.

By pipetting cells directly onto a layer of 2% sucrose solution, on the alkaline sucrose gradient, it was hoped that the DNA would be released gently by hypotonic bursting of the cell membranes to give larger pieces of DNA than obtainable by previous techniques[38]. Semiquantitative or even comparative analysis of these larger DNA molecules seemed meaningless if there were enough layered cells to cause extensive aggregation of the DNA before, during, or after centrifugation. In other words, bands of radioactivity that would be essentially clumps of vastly heterogeneous DNA strands would be detected rather than the optimal single-stranded, individual DNA molecules. It was very important to know, therefore, any effect of cell numbers on the size of, and the shifting of the DNA bands in the alkaline sucrose gradient.

An experiment to assess the role of the number of cells layered was carried out using 0.1 ml aliquots of 100×10^3, 50×10^3, 25×10^3, 12×10^3, and 6×10^3 BHK-21 cells, prelabelled with a 2 hour pulse of 10 uCi/ml ^{3}H-TdR followed by a 6 hour chase, and layered on five alkaline sucrose gradients.

Figure 2 shows the marked effect of the number of layered cells on the sedimentation profile of Syrian hamster DNA (the quantity of DNA present being directly proportional to the acid-

insoluble deoxythymidine (TdR) radioactivity measured). With each aliquot of 1000 mammalian cells corresponding to approximately 0.01 ug DNA[9], the density of DNA* in each gradient (final volume of each: 14.3 ml) is estimated, from Figure 2(a) to 2(e), as: 0.071 ug/ml, 0.036 ug/ml, 0.018 ug/ml, 0.009 ug/ml, and 0.005 ug/ml.

For the utmost utility in analyzing for breaks in DNA exposed to various carcinogenic agents it is desirable to have a clearly defined sedimentation profile of untreated DNA. Figure 2(f) shows for comparison a typical 'parent peak' obtained by Whitmore and co-workers[38] using mouse L-cells. Because of the analogous procedure followed, a similar positioning of the parent DNA peak with little or no pelletted DNA was therefore expected. Hence, the only sedimentation profile concurring with Figure 2(f) is Fgure 2(d), obtained with 12,000 layered cells.

Only slight radioactivity, and no parent peak, was measured with 6,000 cells layered.

With 25,000 or more layered cells there are three noteworthy observations:

(1) There is a trend to increasing amounts of pelletted DNA in the lowest fractions. For example, there are 25,000 counts per minute (CPM) in the first fraction of the gradient in Figure 2(a) with 100,000 cells layered.

(2) There are two or more peaks in the region of fraction 5 to 15.

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* The term 'DNA' will hence refer to the DNA of mammalian cells.

Figure 2. Alkaline sucrose gradient sedimentation profiles of ^{3}H-DNA released from Syrian-hamster cells. The profiles are presented on the same scale for facile comparison and hence the level of counts of the lowest sediments, in the top left panels, extends beyond the range of the graphs. The cells were derived from the same culture bottle and suspended in PBS to give equal layering volumes (0.1 ml) containing the various cell numbers:

(a) 100,000 cells,

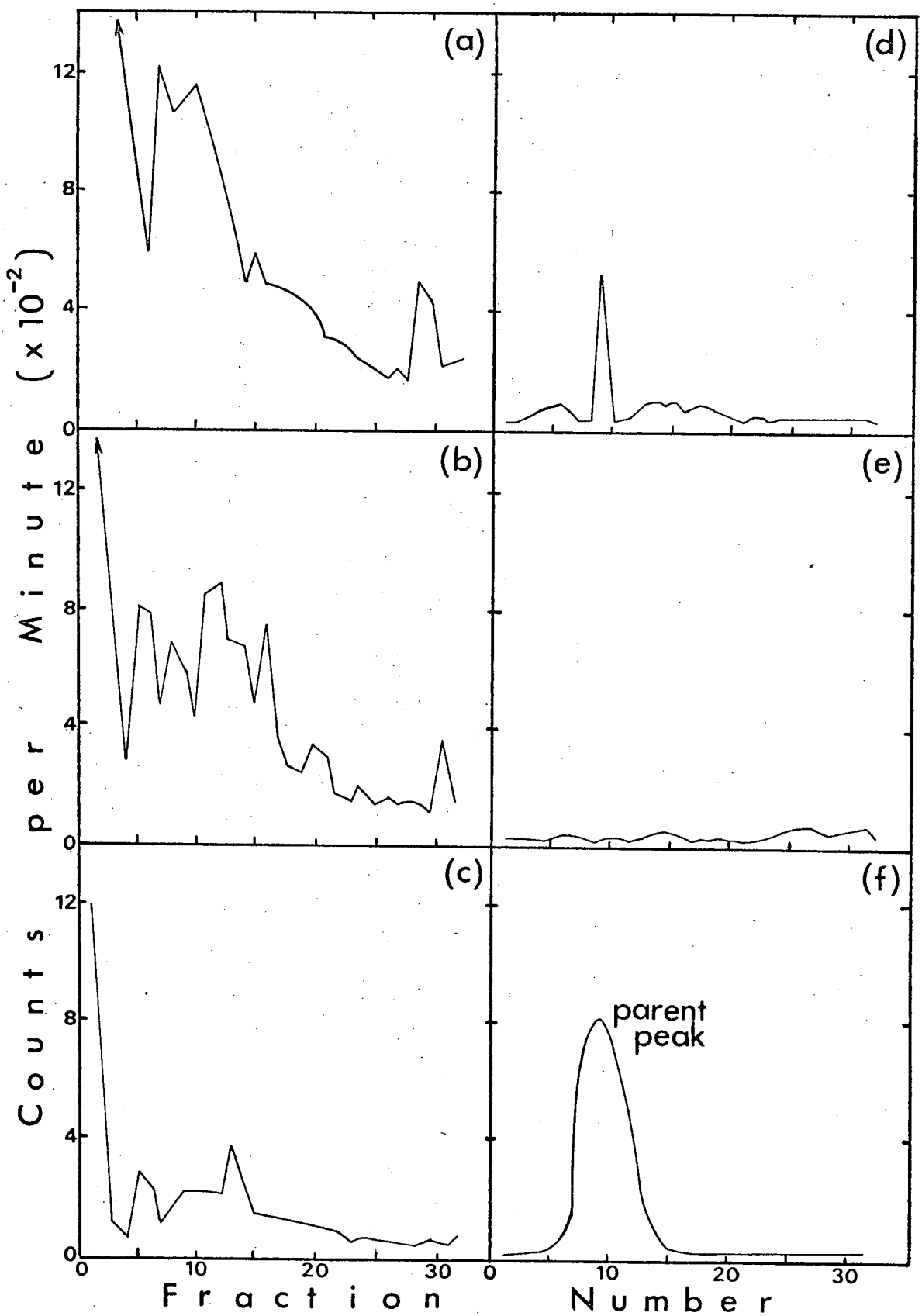
(b) 50,000 cells,

(c) 25,000 cells,

(d) 12,000 cells, and

(e) 6,000 cells.

(f) Parent peak of mouse L-cell DNA[38].


(a)
(b)
(c)
(d)
(e)
(f)
parent peak
Counts per Minute (x 10^-2)
0
4
8
12
0
10
20
30
Fraction Number

(3) There is a trend to increasing overlap of peaks of radioactivity (which is best imagined as increased intermingling of separate bands of DNA in the gradient tubes). This is illustrated by the progressive rise of the trough, at the region of fractions 4 to 6, as layered cell numbers increase.

(b) Reduction in Concentration of Radio-isotope.

It had been shown that proliferating BHK-21 cells could be killed on exposure to a ^{3}H-TdR concentration of 1 uCi/ml for less than 48 hours[29]. Since initial isotope doses, used to label the BHK-21 cells, were comparatively high (at 10 uCi/ml ^{3}H-TdR for 2 hours), it was desired to reduce possible internal cell damage due to excessive isotope decay. Moreover, it was of special interest to avoid DNA damage as any alteration in the molecular size could possibly be detected on the sucrose gradient, and so ruin the utility of the technique as a measuring tool for induced DNA breakage.

To determine the minimum radio-isotope dosage, the following trial runs were carried out using BHK-21 cells:

(a) 10 uCi/ml, 4 uCi/ml, and 1 uCi/ml ^{3}H-TdR, pulsed for 2 hours;

(b) 4 uCi/ml and 1 uCi/ml ^{3}H-TdR, pulsed for 3 hours;

(c) 0.25 uCi/ml ^{3}H-TdR, pulsed for 24 hours.

The criterion for deciding that sufficient label was in fact being incorporated was that bands of DNA in the sucrose gradient give fractions the peaks of which measure at least 50 to 75 CPM, after layering 12,000, and no more than 20,000 cells. The

liquid scintillation counter gives background counts in the neighbourhood of 25±5 CPM.

When trials were run with 4 uCi/ml and 1 uCi/ml ^{3}H-TdR doses, for the 2 hour pulse time, it was found that very little radioactivity could be measured. When the pulse time was increased to 3 hours, a 4 uCi/ml ^{3}H-TdR dose gave adequate label for reproducible results while a 1 uCi/ml ^{3}H-TdR dose did not. When the trend of increasing pulse time with decreasing dosage was extended to treating the cell cultures for 24 hours with 0.25 uCi/ml ^{3}H-TdR, adequate label was incorporated into BHK-21 cells (with a generation time of about 20 hours).

Similar trials were carried out on primary Syrian-hamster embryo cells (which have considerably longer generation times than BHK-21 cells) and it was found that adequate label was incorporated when the cultures were pulsed for 36 to 40 hours with 0.25 uCi/ml ^{3}H-TdR.

Carbon-14 labelling of BHK-21 cells was accomplished by exposing the cultures to 0.05 uCi/ml ^{14}C-TdR for 24 hours[38]. Primary cultures required a longer ^{14}C-TdR pulse time of 36 to 40 hours.

It is of interest to note that it was necessary to replenish the cell cultures with fresh radioactive medium at 6-8 hour intervals during the long pulse times.

II RELATIONSHIP OF DNA BREAKAGE TO 4NQO CONCENTRATION

Before embarking on extensive sucrose density gradient analyses of DNA from quinoline N-oxide treated mammalian cells,

it was realized that the choice of the correct concentration of 4NQO, with which to treat the cell cultures, was critical for two reasons. First, preliminary studies had been done in our laboratory to assess the toxicity of 4NQO. This was accomplished by measuring the survival of mammalian cells upon treatment with increasing doses of 4NQO. These studies showed approximately 10-15% cell survival with a $5x10^{-6}$M 4NQO concentration and no survival when this concentration was increased to $1x10^{-5}$M 4NQO. Therefore, it was not feasible to use concentrations of 4NQO greater than $5x10^{-6}$M in order to avoid complete death of the cell culture. Moreover, sucrose density gradient analysis of cells exposed to lethal concentrations of 4NQO could possibly measure DNA breakage due to enzyme digestion of dead-cell DNA rather than initial, live-cell DNA breakage. The second reason for a tactful choice of 4NQO concentration involved the possibility of choosing too low a concentration of 4NQO which could possibly cause too few DNA breaks to measure with the sucrose gradient technique.

An experiment was undertaken, therefore, to ascertain the relationship between DNA breakage and 4NQO concentration in order to find a practical concentration about mid-way between the two extremes. For reasons of cell survival the highest dose of 4NQO used was $5x10^{-6}$M, with the remaining doses tested decreasing to $1x10^{-6}$M, $5x10^{-7}$M, and $1x10^{-8}$M 4NQO. These carbon-14 prelabelled cell cultures (^{14}C-TdR) were harvested immediately after a 2 hour chemical treatment and each culture was combined with tritium prelabelled (^{3}H-TdR) cells which were

untreated. Figure 3 shows the profiles of the sedimented ^{3}H-DNA and ^{14}C-DNA.

There are two general observations to be noted from these profiles. First, the amount of lower molecular weight single strands of ^{14}C-DNA present in each gradient should be observed, as reflected by the amount of ^{14}C-DNA measured in the top half of each gradient (about fractions 16 or 17 to 32-35, depending on the total number of fractions)*. On this basis, Figure 3(a) shows a clean parent peak of ^{3}H-DNA and ^{14}C-DNA with no smaller pieces of ^{14}C-DNA evident. Figure 3(b) reflects the results of exposure to the most concentrated 4NQO dose used (5×10^{-6}M) and presents a well-defined profile of smaller ^{14}C-DNA pieces well removed from the more rapidly sedimenting parent ^{3}H-DNA peak at fraction 12. Smaller ^{14}C-DNA single strands are virtually absent from the profile in Figure 3(c), after 1×10^{-6} 4NQO treatment, but a slight reduction in molecular weight of the ^{14}C-DNA is evident from the shift of the parent ^{14}C-DNA peak from the parent ^{3}H-DNA peak at fraction 12. Further reduction of applied 4NQO concentration to 5×10^{-7}M gave a sedimentation profile which shows some smaller ^{14}C-DNA single strands present, as depicted in fractions 16-25 in Figure 3(d). There are virtually no smaller ^{14}C-DNA single strands measured in the profiles of Figures 3(e) and (f), after exposure to 1×10^{-7}M 4NQO and 1×10^{-8}M 4NQO, respectively.

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* Refer to the Appendix for a clear explanation of the significance of the slowly sedimenting DNA single strands.

Figure 3. Alkaline sucrose gradient sedimentation profiles of untreated ^{3}H-DNA (broken line) and treated ^{14}C-DNA (solid line). The cultures containing ^{14}C-DNA were exposed to a range of 4NQO concentrations for 2 hours:

(a) no treatment,

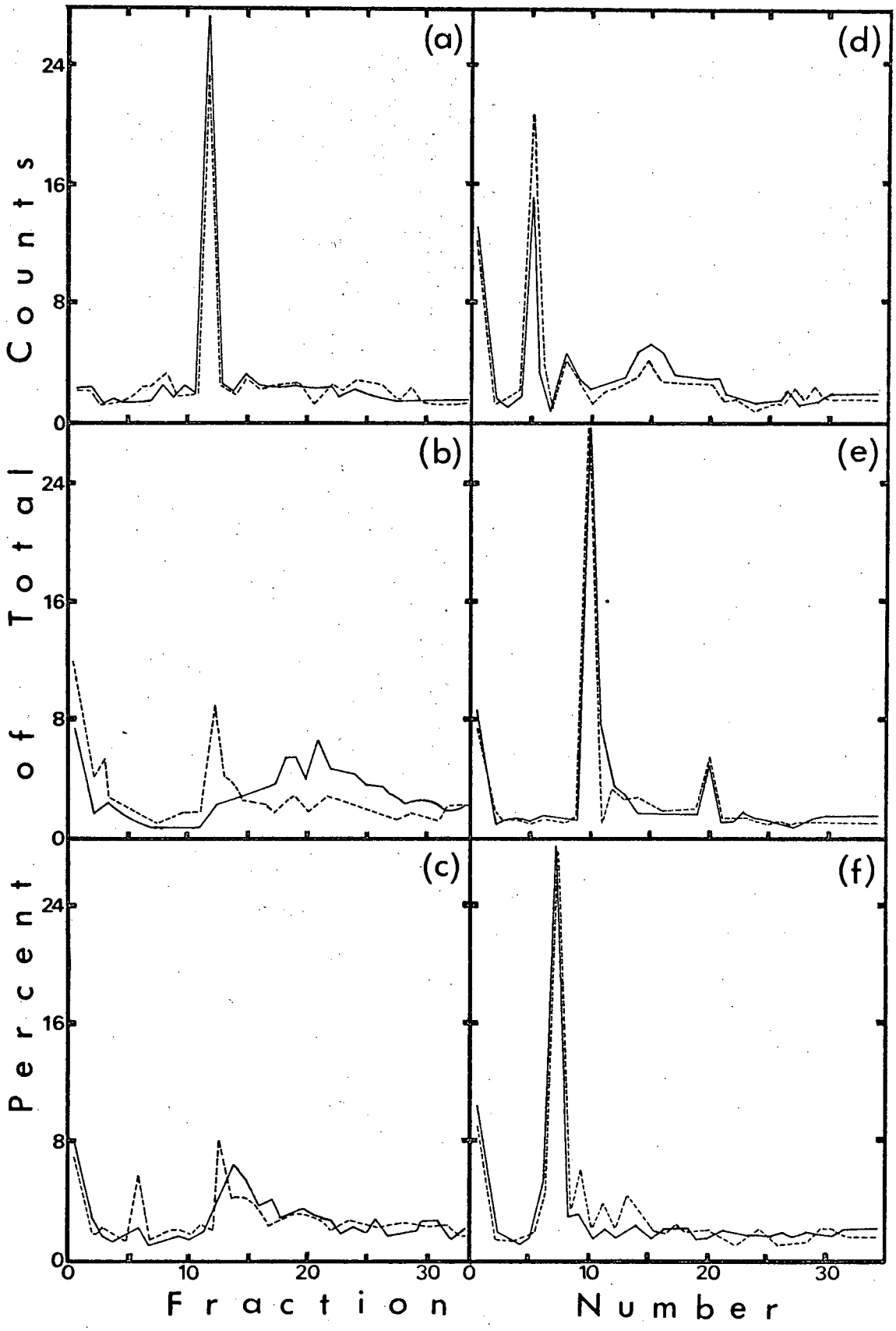
(b) 5×10^{-6}M,

(c) 1×10^{-6}M,

(d) 5×10^{-7}M,

(e) 1×10^{-7}M, and

(f) 1×10^{-8}M.


(a)
(b)
(c)
(d)
(e)
(f)
Percent of Total Counts
0
8
16
24
0
10
20
30
Fraction Number

Second, the shift of the superimposed ^{3}H-DNA and ^{14}C-DNA parent peaks in Figures 3(d) to (f) to more rapidly sedimenting, higher molecular weight values is worthy of note. Assuming the parent peak of untreated ^{3}H-DNA and of ^{14}C-DNA occurs, under these experimental conditions, at fraction 12, as evidenced by Figure 3(a), there is a drastic shift of these parent peaks to fractions 5 and 7 in Figure 3(d) and 3(f) respectively. Figure 3(e) shows a less obvious shift to fraction 10. It should be noted that these shifts occur after treatment with more dilute 4NQO concentrations (compared to, say, 1×10^{-6}M) and that the superimposed parent peaks in question are sharp and well-defined.

Therefore, to insure at least 15% cell survival, and still be able to induce enough small DNA single strands to be easily measured as ^{14}C-DNA radioactivity in the top half of the sucrose gradient, it was decided to apply 4NQO in concentrations of 4×10^{-6}M for subsequent repair studies.

III CORRELATION BETWEEN ONCOGENICITY OF QUINOLINE N-OXIDE COMPOUNDS AND EXTENT OF DNA BREAKAGE.

Stich and San[59] have recently indicated a link between oncogenicity of the synthetic quinoline N-oxides and the capacity of each to provoke DNA repair synthesis, as shown by autoradiographic measurement of the unscheduled uptake of ^{3}H-TdR. To check the possibility of parallelling this correlation at the molecular level, by using alkaline sucrose density gradient

analysis of induced DNA breakage, certain compounds were selected for trials on the basis of their relative oncogenic capacity. These compounds, their oncogenic capacity as revealed by *in vivo* studies by Nakahara and co-workers[23,44], and the degree of induced DNA repair synthesis[59], are shown in Table 1.

Derivatives	Oncogenicity	Repair Synthesis (Grains/Nucleus)		
		8×10^{-6}M	4×10^{-6}M	1×10^{-6}M
3NQO	none	0	0	0
4NQO	strong	101	66	51
8NQO	none	0	0	0
3-methyl-4NQO	weak	2	1	0

Table 1. Correlation between oncogenicity of some quinoline N-oxides and DNA repair synthesis induced by the compounds.

In order to avoid lethal concentrations it was decided to employ no higher a concentration than 5×10^{-6}M of any of the compounds. Moreover, cell survival had been ascertained for quinoline 1-oxide derivatives other than 4NQO, showing higher concentrations of these compounds to be toxic, though usually less toxic than 4NQO[59].

Two sets of experiments were run: one, using 5×10^{-6}M concentrations of these compounds; the other, using 1×10^{-6}M concentrations. Prelabelled BHK-21 cell cultures were exposed to the chemicals for 2 hours before harvesting and

immediately layering on alkaline sucrose gradients. Although the results reported are for BHK-21 cells, primary hamster embryo cells showed very similar behaviour.

Two groups of data are depicted in Figure 4. Figure 4(a) to 4(c) shows double-label sedimentation profiles of ^{14}C-DNA from cells exposed to 5×10^{-6}M 4NQO, 3-methyl-4NQO, and 3NQO, along with ^{3}H-DNA from untreated cells. Figure 4(d) and 4(e) is indicative of former sucrose gradient analyses prior to employing the 'double-label technique' and therefore a separate control (Figure 4(d)) is required for comparison with the profile (Figure 4(e)) of ^{3}H-DNA from cells treated with non-oncogenic 8NQO.

Again, cells treated with highly oncogenic 4NQO (5×10^{-6}M) give a well-defined sedimentation profile with abundant ^{14}C-DNA in the top half of the gradient. On the other hand, cells treated with weakly oncogenic 3-methyl-4NQO, and non-oncogenic 3NQO and 8NQO exhibit no measureable low molecular weight DNA radioactivity in the top half of the gradients.

A typical single-label experiment is depicted in Figure 5. Untreated cells give a sharp parent peak at fraction 17 in Figure 5(a) and this is comparable to the profile of ^{3}H-DNA from cells exposed to non-oncogenic 1×10^{-6}M 8NQO (figure 5(d)). Treatment with 1×10^{-6}M 4NQO annihilated the prominent parent peak at fraction 17 in Figure 5(b), caused a shift to higher sedimentation values with the major peak at fraction 13, and induced the sedimentation of some low molecular weight DNA radioactivity around fractions 22-30. Treatment with

Figure 4. Alkaline sucrose gradient sedimentation profiles of DNA exposed to higher concentrations of nitroquinoline N-oxide compounds.

Figure 4(a)-4(c). Sedimentation profiles of untreated ^{3}H-DNA (broken line) and treated ^{14}C-DNA (solid line), with 2 hour treatments as follows:

(a) 5×10^{-6}M 4NQO

(b) 5×10^{-6}M 3-methyl-4NQO, and

(c) 5×10^{-6}M 3NQO.

Figure 4(d) and 4(e). Sedimentation profiles derived from a single-label (^{3}H) study with 2 hour treatments as follows:

(d) no treatment, and

(e) 5×10^{-6}M 8NQO.

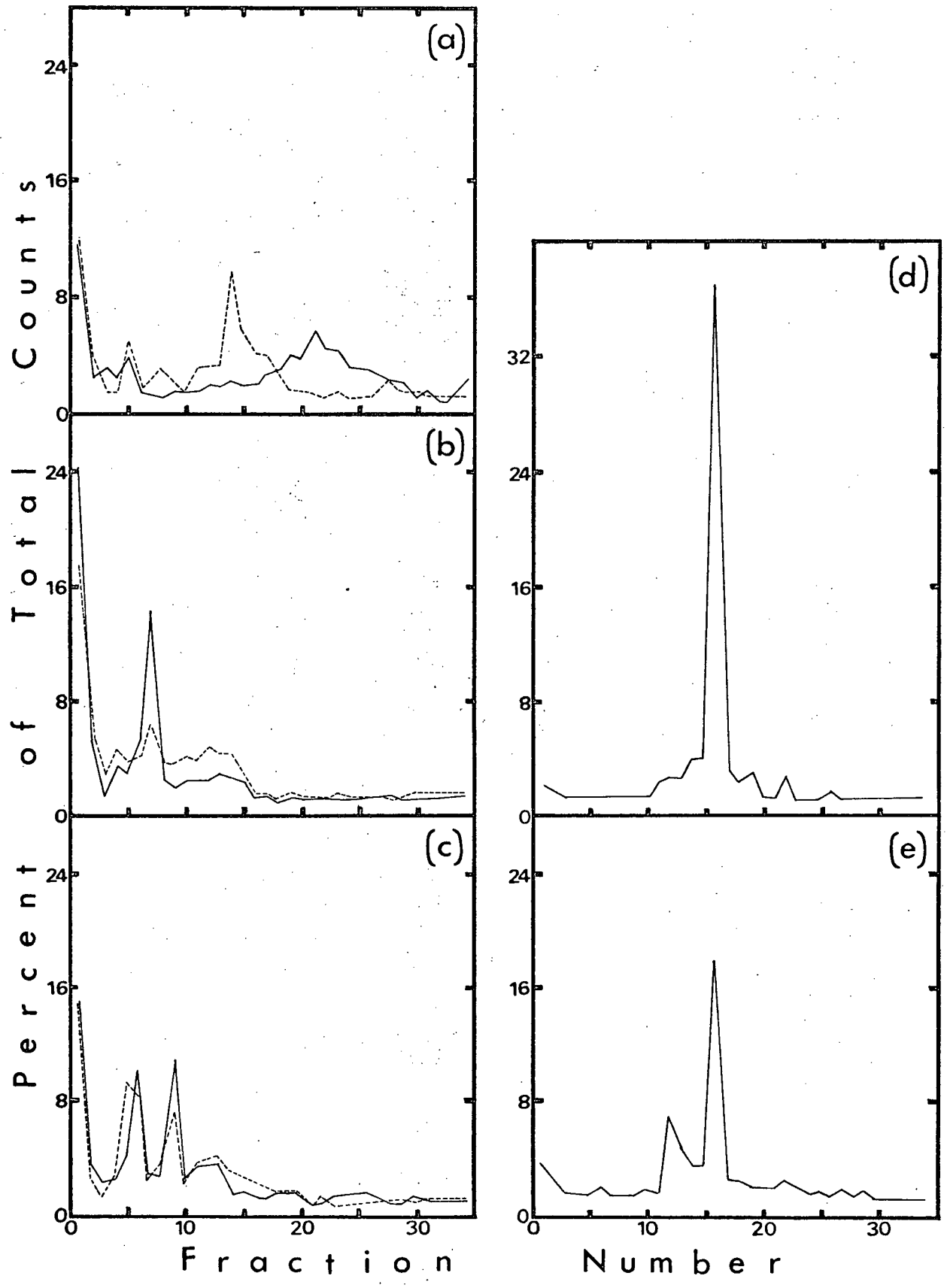

(a)
(b)
(c)
(d)
(e)
Percent of Total Counts
Fraction
Number
0
8
16
24
32
10
20
30

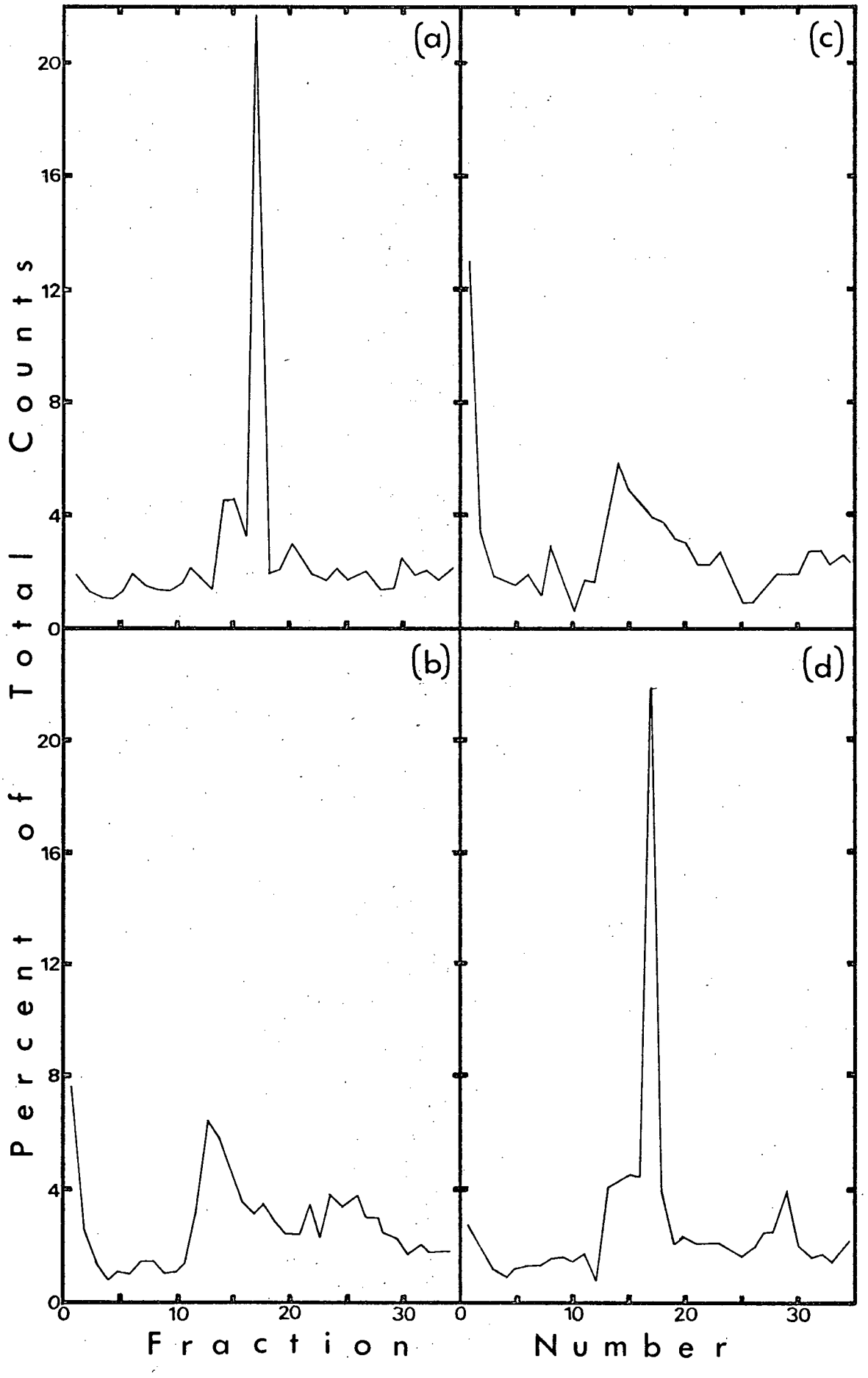
Figure 5. Alkaline sucrose gradient sedimentation profiles of ^{3}H-labelled DNA exposed to lower concentrations of nitroquinoline N-oxide compounds, with 2 hour treatments as follows:

(a) no treatment,

(b) 1×10^{-6}M 4NQO,

(c) 1×10^{-6}M 3-methyl-4NQO, and

(d) 1×10^{-6}M 8NQO.


(a)
(b)
(c)
(d)
Percent of Total Counts
0
4
8
12
16
20
0
10
20
30
Fraction Number

1×10^{-6}M 3-methyl-4NQO caused a similar small shift to higher sedimentation values with a peak at fraction 14 in Figure 5(c). No significant amount of low molecular weight DNA is suggested by the level of radioactivity in the top half of the gradient.

It therefore seems that the extent of DNA breakage, as measured by this alkaline sucrose density gradient technique, parallels the high, weak, and non-oncogenic property of the synthetic quinoline N-oxides to a limited extent. The highly oncogenic 4NQO does reproducibly produce low molecular weight DNA when added to cell cultures in doses as low as 1×10^{-6}M. On the other hand, the weakly oncogenic 3-methyl-4NQO does not produce any significant amount of low molecular weight DNA at concentrations of 5×10^{-6}M or 1×10^{-6}M, and the alteration of the parent DNA peak to slightly higher molecular weight is questionable and will be discussed further. The non-oncogenic 3NQO and 8NQO, at concentrations of $1\text{-}5 \times 10^{-6}$M, produce no detectable low molecular weight DNA radioactivity and no change in the parent DNA peak. A brief summary of these results appears in Table 2.

Derivative	Oncogenicity	Extent of DNA Breakage (1×10^{-6}M to 5×10^{-6}M concentrations)
4NQO	strong	high
3-methyl-4NQO	weak	not measurable
3NQO	none	not measurable
8NQO	none	not measurable

Table 2. Correlation between oncogenicity of some synthetic quinoline N-oxides and the extent of induced DNA breakage in mammalian cells.

The wide variance of such factors as solubility, membrane permeability, and toxicity, within the collection of synthetic quinoline N-oxides, emphasizes the limitations of these conclusions. The parallel between oncogenicity and extent of DNA breakage, at least for highly oncogenic versus weak and non-oncogenic compounds, will be complete only when all such synthetic quinoline N-oxides are tested.

IV REPAIR OF 4NQO-INDUCED DNA BREAKS.

In view of the recent refinements of the alkaline sucrose density gradient technique[38], as used in these studies, it seemed probable that this technique could clearly follow the kinetics of the repair of DNA breaks induced by the highly oncogenic 4NQO.

By comparing the relative amounts of low molecular weight DNA single strands present in mammalian cells* at various intervals after 4NQO treatment, information could be gained to help clarify a number of general questions concerned with the mode of action of 4NQO on mammalian cells, and the subsequent recovery of these cells from the induced damage:

(a) Does 4NQO induce DNA breaks during the initial application only (2 hours, in these experiments), or does DNA breakage continue for some time after removal of the medium containing the 4NQO?

(b) Do cells completely repair the induced DNA breakage*?

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* Refer to the Appendix to insure a clear interpretation of this concept.

(c) Is the kinetics of the repair process, when measured as described above, such that the rate of 'disappearance' of the small DNA single strands is continuous*?

(d) What are the theoretical limitations of this alkaline sucrose density gradient analysis with regard to conclusions concerning the mechanism of repair of 4NQO-induced DNA damage?

Two experiments were run in which prelabelled BHK-21 cells were exposed to 4×10^{-6}M 4NQO for 2 hours, washed three times with MEM, and then incubated at 37° with fresh MEM. Cultures were analyzed by the alkaline sucrose density gradient technique at various intervals after exposure to 4NQO.

Before analyzing the results, it would be advantageous to discuss the significant aspects of sedimentation profiles that could conceivably be used to gain information regarding the repair of 4NQO-induced DNA breakage. Figure 6 shows a schematic sedimentation profile of 4NQO treated DNA, obtained immediately after exposure to the oncogen. The parent DNA peak, having the higher sedimentation velocity, is represented to the left of the lower molecular weight DNA peak. As cultures are incubated to accommodate DNA repair, after exposure to 4NQO, the initial large amount of slowly sedimenting DNA radioactivity in the upper half of the gradient* would be expected to decrease as shown; simultaneously, an increase in the amount of DNA radioactivity in the parent peak, as shown in the lower half of the gradient, would be expected.

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* Refer to the Appendix to insure a clear interpretation of this concept.

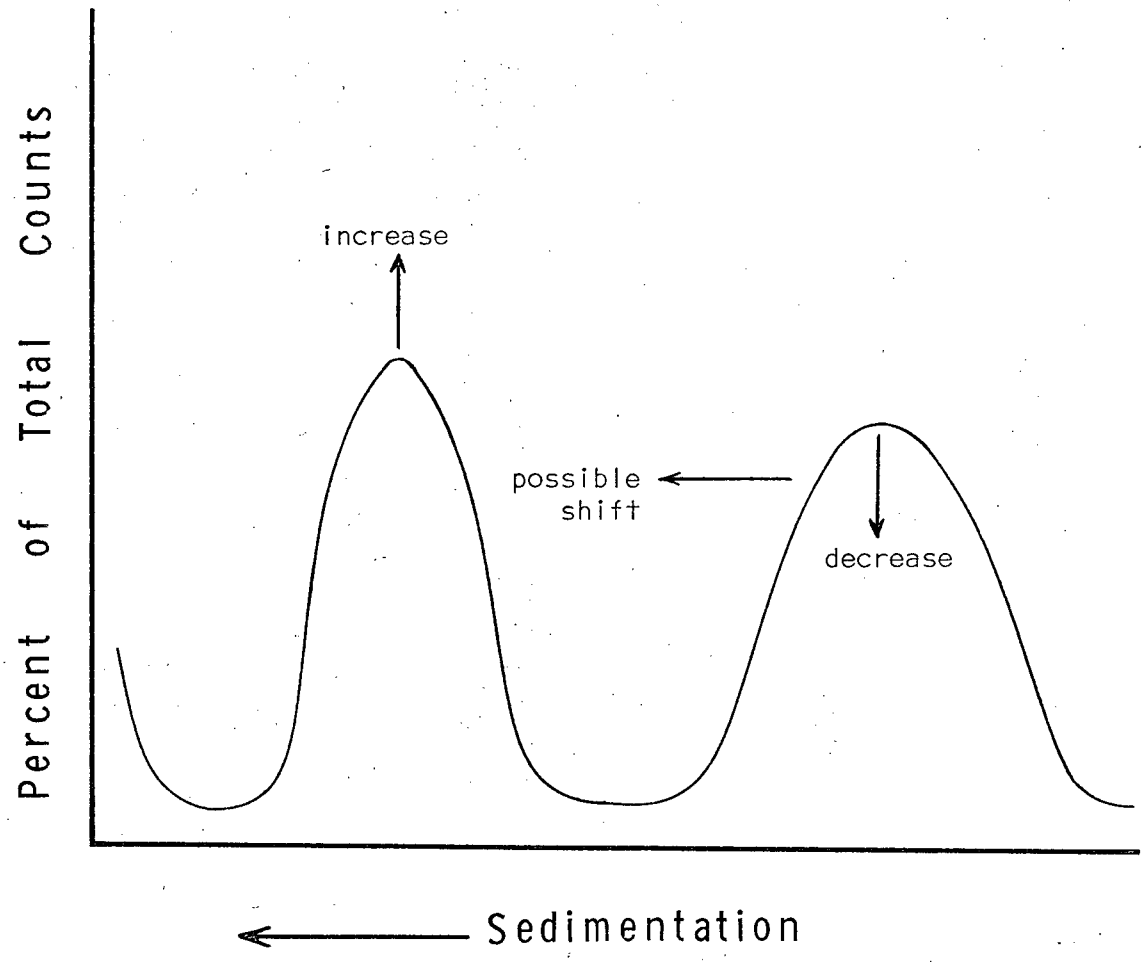


Figure 6. Schematic sedimentation profile of 4NQO-treated DNA illustrating the theoretical modifications of the profile that occur with repair of DNA breaks.

Typical experimental results are shown in Figure 7. In general, there appears to be two parent peaks of untreated ^{3}H-DNA with an occasional lack of definition occurring with no apparent trend (Figures 7(d), 7(f), 7(g)). There is a consistent low level of ^{3}H-DNA radioactivity in the low molecular weight region of all profiles. With regard to the parent peaks of 4NQO-treated ^{14}C-DNA, there is also an occasional lack of definition occurring simultaneously with the ill-defined ^{3}H-DNA parent peaks. The general lack of definition of parent DNA peaks does seem to parallel, at least in part, the increase in pelletted DNA radioactivity occurring in the first fractions. For example, in Figures 7(c) and 7(f), compare the relationship between the amount of DNA radioactivity in the first fractions with that in the parent peaks: the more the pelletted DNA, the less defined are the parent peaks.

Figures 7(a)-(d), in fractions 17 to 34 (approximately), show clearly the difference between the amount of low molecular weight untreated ^{3}H-DNA radioactivity and the amount of low molecular weight 4NQO-treated ^{14}C-DNA radioactivity. Careful scrutiny of Figures 7(e)-(g) will also show a difference, although less apparent.

Preliminary observation of these sequential sedimentation profiles therefore shows a trend of decreasing amounts of low molecular weight 4NQO-treated ^{14}C-DNA radioactivity, progressing from Figure 7(a) to 7(g). However, the expected opposing trend of increasing amounts of high molecular weight 4NQO-treated ^{14}C-DNA radioactivity is not apparent. The lack of definition of

Figure 7. Alkaline sucrose gradient sedimentation profiles of untreated ^{3}H-DNA (broken line) and 4×10^{-6}M 4NQO-treated ^{14}C-DNA (solid line), after incubation for the following post-treatment periods:

(a) 0 hours,

(b) 2 hours,

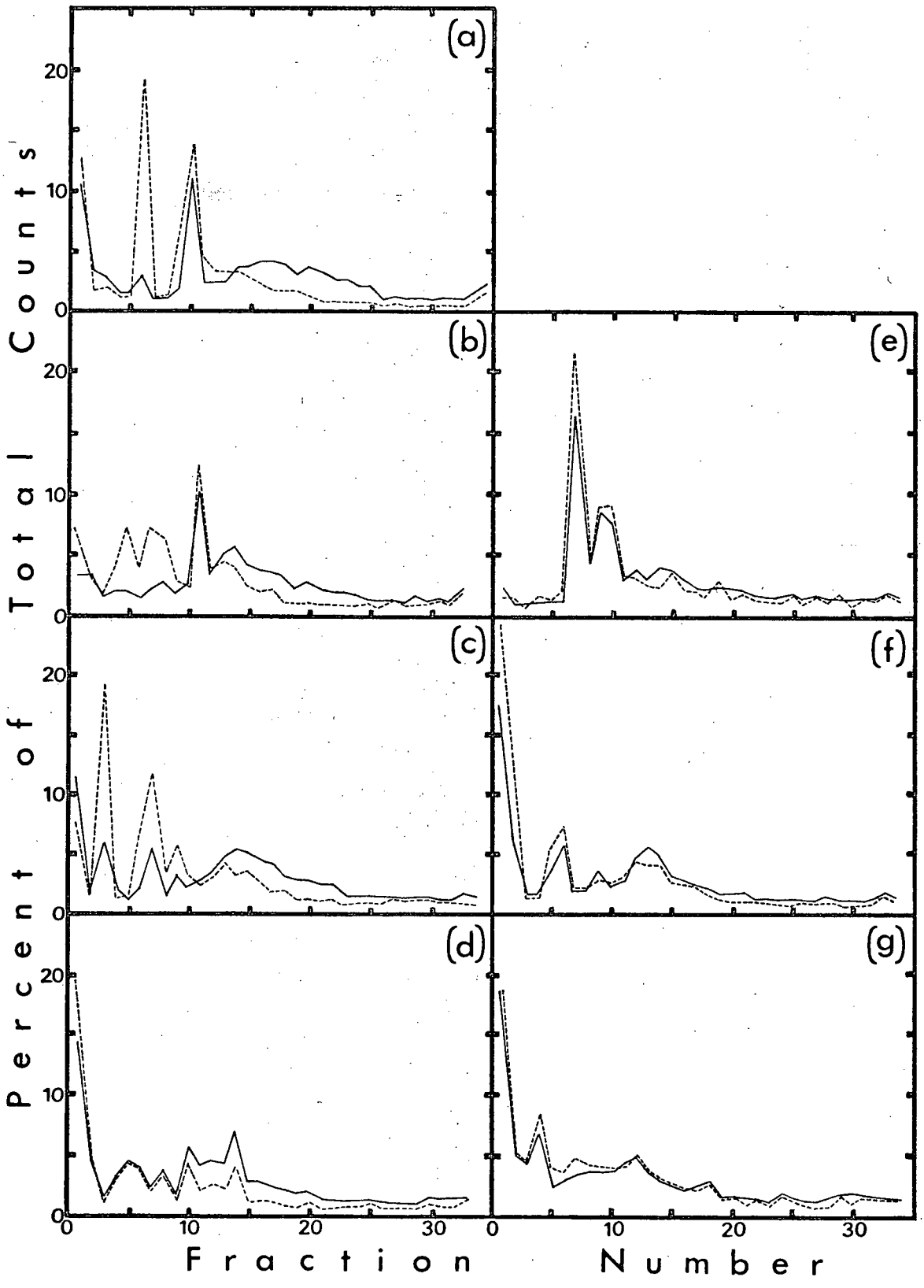
(c) 4 hours,

(d) 8 hours

(e) 12 hours,

(f) 16 hours, and

(g) 24 hours.


(a)
(b)
(c)
(d)
(e)
(f)
(g)
Percent of Total Counts
Fraction Number
0
10
20
30

the parent peaks is undoubtedly a prime factor in hindering the latter observation.

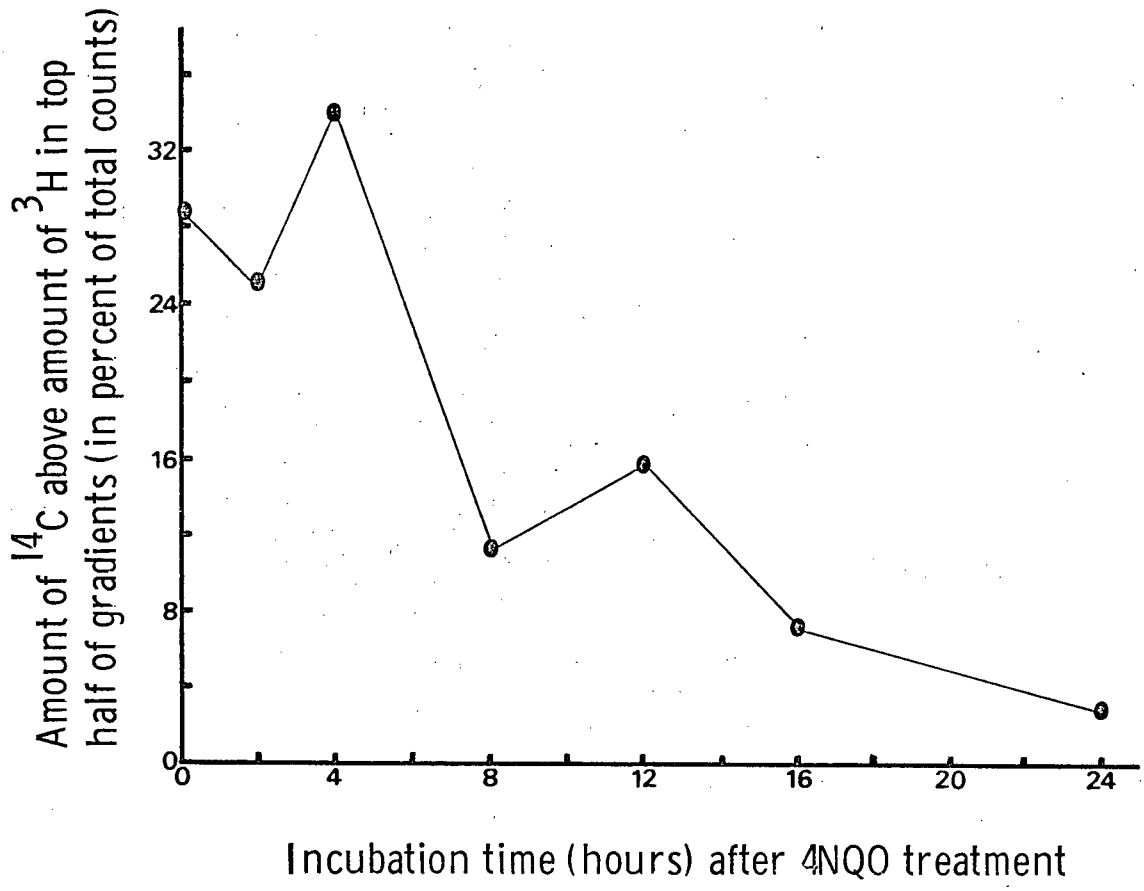
In order to establish a semiquantitative analysis of this DNA repair, the difference between the amount of slowly sedimenting 4NQO-treated ^{14}C-DNA radioactivity and the amount of slowly sedimenting untreated ^{3}H-DNA radioactivity was calculated for the top half of each gradient. The top half of each gradient (which is the right half of the sedimentation profile) was arbitrarily chosen as a measuring tool because that region appeared sufficiently removed from the region of the parent DNA peaks to assume that there would be no significant overlap of higher molecular weight sediment with lower molecular weight sediment.

The results of these computations, as calculated for the profiles in Figure 7, are plotted in Figure 8.

The trend to overall decreasing amounts of low molecular weight 4NQO-treated DNA is clear; and, the trend does not appear to be a smooth, continuous process but shows slight deviations at 4 hours and 12 hours after 4NQO treatment. Assuming that the existence of low molecular weight single-stranded DNA, in quantities measurable by this technique, is indicative of active DNA repair, it is therefore evident that DNA repair in this instance is practically complete after 24 hours incubation (with less than 4% ^{14}C-DNA radioactivity over and above the untreated ^{3}H-DNA radioactivity in the top half of the gradient).

Moreover, it is apparent that the gross DNA breakage, induced by 4NQO, occurs during the initial exposure to the on-

Figure 8. The amount (in percent of total counts) of 4NQO-treated ^{14}C-DNA, over and above the amount of untreated ^{3}H-DNA, in the top half of each gradient of Figure 7, is plotted against the duration of post-treatment incubation.


Amount of ^{14}C above amount of ^{3}H in top half of gradients (in percent of total counts)
32
24
16
8
0
0
4
8
12
16
20
24
Incubation time (hours) after 4NQO treatment

cogen and perhaps during the first 4 hours after removal of the 4NQO-containing medium, afterwhich the production of low molecular weight DNA single strands appears to decrease. Whether the actual event of the production of the DNA single-strand breaks occurs as a massive, quick process upon application of 4NQO, or as a more or less continuous process (for example, 4 hours or more), can only be speculated with the data available. Further such theoretical limitations of the sucrose gradient analysis described herein, with regard to conclusions concerning the mechanism of repair of 4NQO-induced DNA damage and the mode of action of 4NQO and derivatives, will be discussed further.

V EFFECT OF CAFFEINE ON 4NQO-INDUCED DNA BREAKAGE.

There have been a number of reports of the effect of caffeine on repair processes in mammalian cell cultures *in vitro*. Caffeine has been shown to inhibit the repair of UV light-induced lesions in mouse L-cell cultures[50], increase the number of X-ray induced chromosome breaks in leukocyte cultures[18,28], and inhibit the increase in polymerase activity, induced by Mitomycin-C, in leukocyte cultures[67].

By testing the effect of caffeine on the action of 4NQO on mammalian cells and on the subsequent repair, it was hoped to: (a) acquire some evidence of the mode of action of 4NQO, and (b) to analyze the synergistic effect of caffeine and 4NQO on mammalian cells. Because of the relative success of the alkaline sucrose density gradient analysis of DNA repair it

was therefore feasible to use this technique, in a similar manner, to analyze for the combined effects of 4NQO plus caffeine.

The maximum concentration of caffeine that did not show toxic effects on BHK-21 cell cultures was previously determined in our laboratory to be $4x10^{-3}M$[65]. Experiments were executed in which BHK-21 cell cultures were exposed to both $4x10^{-3}M$ caffeine and $4x10^{-6}M$ 4NQO, after which the cell cultures were incubated with $4x10^{-3}M$ caffeine in MEM. Cultures were then analyzed at time intervals analogous to those of the repair study described previously.

There are three significant features of the sedimentation profiles, shown in Figure 9, which typify the results obtained.

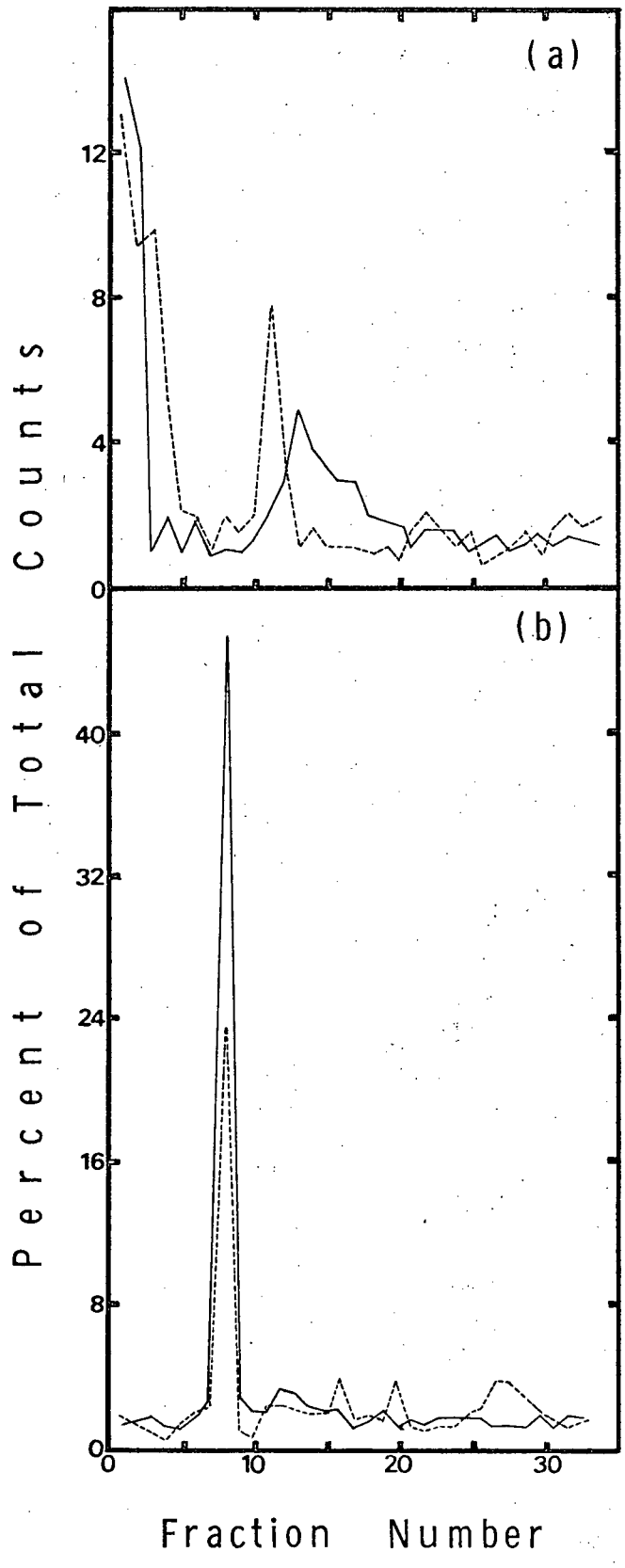
First, there is a substantial amount of pelletted DNA radioactivity in the profile of Figure 9(a) and virtually no pelletted DNA in the profile of Figure 9(b). It is of interest to note the 'shoulder' of ^{14}C-DNA radioactivity at fraction 2 and the peak of ^{3}H-DNA radioactivity at fraction 3 as these are suggestive of the parent peak of high sedimentation velocity that occurs, for example, in conjunction with a slower sedimenting parent peak in many of the profiles in Figure 7. The possible significance of this observation will be discussed further.

Second, there is a noticeable shift of the parent ^{14}C-DNA peak to fraction 13 from the parent ^{3}H-DNA peak at fraction 11. Moreover, the ^{14}C-DNA peak is considerably broader and extends to the lower molecular weight region of fraction 20 in contrast to the termination of the ^{3}H-DNA peak at fraction 13. This shifted, broad ^{14}C-DNA parent peak disappears by 2 hours as

Figure 9. Alkaline sucrose gradient sedimentation profiles of untreated ^{3}H-DNA (broken line) and treated ^{14}C-DNA (solid line), with treatments as follows:

(a) 4×10^{-6}M 4NQO plus 4×10^{-3}M caffeine, for 2 hours, and

(b) treatment as in (a) followed by 2 hours incubation in 4×10^{-3}M caffeine.


(a)
(b)
Percent of Total Counts
Fraction Number
0
4
8
12
16
24
32
40
10
20
30

evidenced by the sharp parent DNA profile in Figure 9(b). Hence, the size of the DNA single strands was apparently reduced a small amount by the combined exposure to both 4NQO and caffeine.

Third, and most prominent, is the virtual lack of treated ^{14}C-DNA radioactivity in the upper half of the gradients represented in Figure 9. Such analysis for low molecular weight DNA single strands in the upper half of these gradients allows a meaningful comparison to be made with the results obtained from 4NQO treatment alone (shown in Figure 7). The calculations tabulated in Table 3 give a more quantitative significance to this lack of relatively low molecular weight DNA single strands from 4NQO/caffeine-treated cells. The ratio of ^{3}H-DNA (untreated) to ^{14}C-DNA (treated), tabulated in Table 3, shows a striking difference depending on whether or not caffeine is present during the 4NQO treatment.

Calculations were not carried out on samples taken after longer periods of incubation because, on inspection, the sedimentation profiles were analogous to that in Figure 9(b) with virtually no ^{14}C-DNA radioactivity over and above the ^{3}H-DNA radioactivity in the low molecular weight region of the profiles.

Treatment	Period of Incubation	Total radioactivity in top half of the gradient (as percent of total counts)		Ratio
		^{3}H-DNA	^{14}C-DNA	^{3}H-DNA/^{14}C-DNA
(1) 4×10^{-6}M 4NQO plus 4×10^{-3}M caffeine	0 hours (Fig. 9(a))	26.3	24.7	1.1
	2 hours (Fig. 9(b))	29.6	19.7	1.5
(2) 4×10^{-6}M 4NQO	0 hours (Fig. 7(a))	16.9	35.7	0.47
	2 hours (Fig. 7(b))	18.2	33.0	0.55

Table 3. Total ^{3}H-DNA and ^{14}C-DNA radioactivities (expressed as a ratio ^{3}H-DNA/^{14}C-DNA) occurring in the top half of alkaline sucrose density gradients containing:

(1) untreated ^{3}H-DNA and 4NQO/caffeine-treated ^{14}C-DNA, and

(2) untreated ^{3}H-DNA and 4NQO-treated ^{14}C-DNA.

DISCUSSION

The analysis of the effects of carcinogens on mammalian cell systems has always been hampered by the inherent complexity of the mammalian cells themselves. It is therefore a rare event when a technique is developed that is capable of analyzing a molecular modification occurring in a mammalian cell with any facility and reasonable accuracy. The development of the alkaline sucrose gradient technique, by McGrath and Williams[39], for analysis of the molecular weight of mammalian cell DNA single strands was such a rare event. The modifications, by Whitmore and McBurney[38], of this technique brought about refinements that could possibly increase the overall applicability of the technique to analyses of more subtle modifications of the DNA rather than gross strand breakage.

The aim of these modifications, which largely consisted of a more gentle lysing system (hypotonic bursting of cells in a 2% sucrose solution overlay at 4°) than previously used[39] (alkaline disruption of cells in an alkaline overlay at 25°), was to isolate larger untreated DNA molecules and so make single strand break assays more sensitive.

In their studies, Whitmore and McBurney used their more sensitive single strand break assay to show, among other results, that prolonged exposure (18 hours) of mouse L-cells to 10 uCi/ml ^{3}H-TdR caused considerable reduction in the

sedimentation velocity of the parent DNA peak. This verified our suspicion of the possibility of measurable DNA damage occurring with exposure to high radio-isotope concentrations, and supported our choice of low radio-isotope concentrations for longer labelling periods. Moreover, it was reasonable to assume that a low dose of radioactive label given over a longer period of time would allow more uniform label distribution throughout the DNA molecule.

On the other hand, Whitmore and McBurney[38] found only slight changes in DNA sedimentation profile when the cell number loaded per gradient was varied from 10^4 to 10^6 cells. This observation apparently disagrees with our results but are, in fact, not strictly comparable. This is because of the fact that Whitmore and McBurney[38] used centrifuge tubes designed for the SW-27 rotor (Beckmann Instruments Inc., Palo Alto, California) and these are of about 36 ml capacity and of larger diameter than our 14 ml capacity centrifuge tubes designed to fit the SW-40 rotor (Beckmann Instruments). Therefore the volumes and relative dimensions of sedimenting bands of DNA are not likely to be the same in both cases and hence aggregation of DNA is likely to vary accordingly, depending on tube dimensions.

Suggestive of increased DNA aggregation with increasing cell numbers in our study (Figure 2) was the trend to increasing overlap of peaks of radioactivity (loss of resolution), the appearance of two or more parent peaks, and the increasing amount of pelletted DNA in the lowest fractions. Moreover, the reduction of cell number loaded per gradient from 12,000

to 6,000 did not reduce the ^{3}H-DNA radioactivity in the parent peak (520 CPM) to half, but simply annihilated the peak with slight radioactivity remaining in the top half of the gradient. This behaviour is strongly suggestive of the need for sufficient quantities of DNA to at least partially aggregate on the gradient and then sediment as a clump.

Analysis of all sedimentation profiles of this study has strongly suggested that the DNA aggregate may play a more involved role in alkaline sucrose density gradient analysis than was previously thought. Firstly, the implications of such DNA aggregates is obvious in the production of pelletted DNA: the more dense the aggregate, the faster it will sediment, and the more likely it will appear in the DNA pellet. A possible role of the number of cells layered per gradient in the formation of aggregates is suggested from the profiles of Figure 2 as the larger number of cells give greater amounts of DNA pellets which is perhaps due to the greater amount of DNA available to aggregate (in extreme cases of large numbers of cells layered per gradient this is the situation of 'overloading' the gradient). It is important to point out the fact that pelletted DNA is not observed in the sedimentation profiles obtained by Whitmore and McBurney[38]. The method of fraction collecting employed in their studies was to collect fractions from the top of the gradients while pumping 60% sucrose solution in the bottom of the gradients. Hence, the very bottom fraction of the gradient could not be routinely detected without washing the bottom of the centrifuge tube. In this

manner, when the bottom fraction of the gradient was collected, pelletted DNA radioactivity was rarely found[38]. In contrast, the fractions were collected from the bottom of the gradients in our studies and so the bottom fraction is easily collected first, and, in most cases, contained some pelletted DNA radioactivity. All in all, this discrepancy regarding pelletted DNA points out the fact that there may be more sensitive technical factors governing the reproducibility of this sucrose gradient technique than are initially apparent. Among these may be: size of centrifuge tube employed, layering technique, fraction collecting technique, cell line used, and cell treatment. Moreover, this discrepancy also emphasizes the need for caution when comparing sedimentation profiles obtained by different workers throughout the literature.

It is possible that the DNA aggregate might account for a number of seemingly anomalous parent peaks obtained in double-label experiments where untreated ^{3}H-DNA was centrifuged with chemically treated ^{14}C-DNA. Figure 10 clarifies the three general types of profiles frequently observed and a possible relationship of the two peaks observed is suggested by the vertical broken lines. The frequent reoccurrence of the two parent peaks, either singly or together, may suggest that there is a fairly consistent 'geometry' of the sedimenting aggregate such that its density is either that in Type I or that in Type III and only occasionally in between.

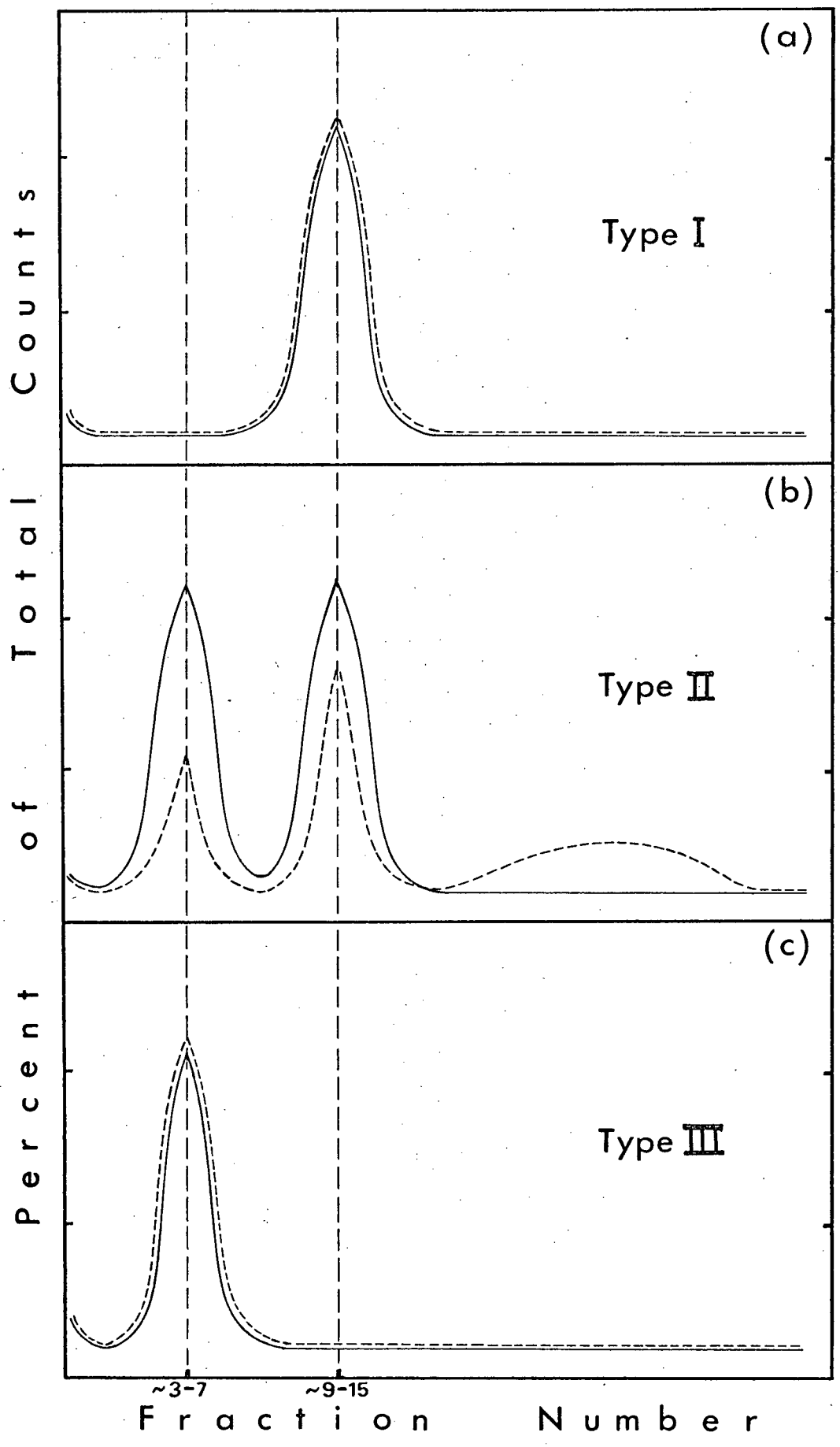
Figure 3(a) is representative of Type I. Type II is repeatedly observed to various degrees throughout these studies

Figure 10. Three general types of sedimentation profiles are shown schematically:

(a) Type I shows the most commonly obtained parent peak position. For example, Type I occurs when both untreated ^{3}H-DNA (solid line) and treated ^{14}C-DNA (broken line) are sedimented together; or, when any single-labelled untreated DNA sample is sedimented.

(b) Type II shows the second most commonly obtained parent peak positions. For example, Type II occurs most often when higher concentrations of 4NQO ($1\text{-}5\times10^{-6}$M) are used to treat ^{14}C-DNA (broken line) which is sedimented with untreated ^{3}H-DNA (solid line).

(c) Type III shows the least commonly obtained parent peak position. For example, Type III occurs most often when low concentrations of 4NQO (1×10^{-8}M to 5×10^{-7}M) are used to treat ^{14}C-DNA (broken line) which is sedimented with untreated ^{3}H-DNA (solid line).


(a)
Type I
(b)
Type II
(c)
Type III
Percent of Total Counts
~3-7
~9-15
Fraction Number

and especially in the profiles of Figure 7. Finally, and most significantly, Type III is observed in Figure 3(d)-(f), after exposure to low 4NQO concentrations, and in Figure 4(b) after exposure to weakly oncogenic 3-methyl-4NQO. It is proposed that the 4NQO, which is known to bind to DNA[16,62], not only induces DNA breakage in higher concentrations but also:

(1) affects the tertiary, and perhaps secondary, structure of the DNA such that the double-helical DNA molecules cannot unwind in the alkaline gradient to the same extent as they can when not exposed to 4NQO, resulting in a more rapidly sedimenting, denser DNA configuration; and,

(2) enhances DNA aggregation by perhaps intercalating between bases on separate molecules thereby binding them more closely, again resulting in a more rapidly sedimenting, denser DNA configuration. The latter proposal is supported by the observation that both the untreated ^{3}H-DNA and treated ^{14}C-DNA occur together, in a sharply defined parent peak, in the profiles of Figure 3(d)-(f) after exposure of only the ^{14}C-DNA cell cultures to low 4NQO concentrations. In other words, the 4NQO 'glues' the treated ^{14}C-DNA molecules together and then 'glues' the ^{3}H-DNA to the ^{14}C-DNA, during and/or after the 16-20 hour lysis period, to produce the denser aggregate of apparently higher molecular weight. The weakly oncogenic 3-methyl-4NQO may cause similar aggregation by a similar mechanism.

Elkind and Kamper[9] have provided support for this interpretation as they obtained similar shifts of parent peaks to

slightly higher sedimentation velocities with low X-ray doses (0-722 rad) on Chinese-hamster cells. These authors state that "it is hard to conceive of bond breakage leading to larger sedimentation velocities - ordinarily implying larger molecular weights - unless such breaks result in a change in conformation and/or density"[9].

This apparent enhancement of DNA aggregation may have some utility with regard to alkaline sucrose gradient analysis of single strand breaks induced by 4NQO. There is an apparent threshold dose of approximately 1×10^{-6}M 4NQO below which single strand breaks are no longer clearly evident with reproducible detection. Therefore, below this threshold concentration, it is possible in future studies with increased technical competence and subsequent higher reproducibility, to use such apparent increases in molecular weight as measuring tools for the effect of low 4NQO concentrations and perhaps for other compounds of similar DNA binding capabilities.

As much as this apparent increase in DNA molecular weight may be exploited to our advantage in analyzing for low concentrations of DNA-binding quinoline N-oxide compounds, the phenomenon, in all probability, forces severe disadvantages on future semi-quantitative analyses of the repair of 4NQO-induced DNA breakage. This is because of the fact that the strong binding of, say 4NQO[1], to DNA will possibly introduce anomalous sedimentation profiles of so-called 'repaired' DNA making it difficult to ascertain, with any reasonable accuracy, when the DNA has been repaired to

its original state.

Whether or not the sensitivity of this technique is sufficient to pick up DNA breaks induced by weakly oncogenic or non-oncogenic quinoline N-oxide compounds, only limited conclusions can be drawn from any observed correlation between frequency of DNA breakage and oncogenicity. It is very doubtful that any of these compounds have identical cell membrane permeabilities or solubilities in aqueous media and so, on this basis alone, one cannot draw detailed correlations between chemical structure of the carcinogens and extent of DNA damage. Such conclusions can only be drawn with careful reservation. Moreover, the correlation between oncogenicity of any compounds and frequency of DNA breakage, is severly hampered by the questionable extrapolation of *in vitro* data to *in vivo* systems and vice versa.

Therefore it can, at best, be said that highly oncogenic 4NQO does readily induce DNA breakage *in vitro*, weakly oncogenic 3-methyl-4NQO does so inconsistently, non-oncogenic 3NQO and 8NQO do not, and the implications of this correlation are merely suggestive of the possible role of DNA breakage in neoplastic transformation.

The actual mechanism by which 4NQO induces breaks in mammalian DNA is of prime consideration before any mechanistic conclusions can be drawn regarding the alkaline sucrose gradient analysis of the repair of 4NQO-induced DNA single strand breaks.

The following questions* can be raised concerning this phenomenon of 4NQO-induced DNA breaks and the repair of these breaks:

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* Refer to the Appendix to insure a clear interpretation of this discussion.

(1) Once the DNA single strands are excised, do they: (a) rejoin only after all gross DNA single strand breakage is complete; or, (b) rejoin simultaneously with continuing DNA breakage?

(2) Are these DNA single strand breaks produced by a rapid spontaneous chemical reaction between 4NQO and DNA, such that the DNA repair samples (taken up to 24 hours after 4NQO exposure) simply measure the kinetics of rejoining of the broken DNA single strands until the broken single strands are too few to be measured by the sucrose gradient technique?

(3) Are these small DNA single strands produced by an excision process mediated by one or more endonucleases and/or exonucleases which perhaps function by detecting DNA alterations resulting from the binding of 4NQO and then removing these alterations?

Firstly, the question of whether the rejoining of the DNA single strand scissions occurs (a) after the production of the scissions, or (b) simultaneously with the production of the scissions, could be answered by a careful correlation of autoradiographic results of unscheduled uptake of ^{3}H-TdR (incorporated into new DNA single strand portions used to 'patch' the DNA breaks), with alkaline sucrose density gradient results of the production of low molecular weight DNA single strands. Samples for each analysis would be taken at the same time intervals following exposure to 4NQO. Preliminary observations indicate that there is definitely an overlap of the excision and rejoining processes as, for example, there

is considerable unscheduled uptake of ^{3}H-TdR 2 hours after 4NQO treatment[29,30] (rejoining or 'patching' in progress), and considerable low molecular weight DNA single strands present at 2 hours and even moreso at 4 hours (excision of DNA single strands in progress; see Figure 8). However, only careful, refined experimentation will verify this observation and perhaps even semiquantify the kinetics of these processes.

The reduction in the frequency of 4NQO-induced DNA breaks that occurred when 4×10^{-3}M caffeine was added to the culture medium with 4NQO gave some suggestions, although indirect and speculative, as to how the 4NQO could possibly be breaking the DNA. These suggestions were arrived at by first compiling probable modes of action of caffeine as used in these studies.

Caffeine (1,3,7-trimethylxanthine; note Figure 11) is

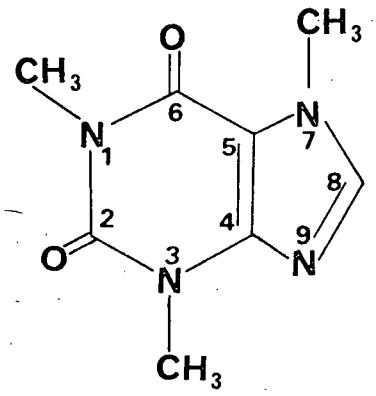


Figure 11. Caffeine (1,3,7-trimethylxanthine), a purine analog.

assumed to be sterically incapable of incorporation into DNA as a purine analog in most mammalian cell systems[1,65]. Although there are no clearcut results demonstrating the mutagenic effects

of caffeine on mammals, the synergistic effect of caffeine and UV-light was shown to produce more mutations than UV-light alone when caffeine was added to bacterial cultures immediately after UV treatment[35,66]. The fact that caffeine may inhibit the repair process was supported by research showing that caffeine directly affects the enzymes, of bacteria, that are responsible for repair[8,13,37,5 Moreover, and of the utmost significance, the caffeine effect on the repair processes of bacteria was also found in mammalian cell cultures, as caffeine was found to inhibit the repair of UV-light induced lesions in mouse L-cells[50]. Furthermore, possible enzyme inhibition was suggested by the fact that caffeine was able to inhibit the increase in polymerase activity, induced by Mitomycin-C, in leukocyte cultures[67].

Therefore, a possible mechanism of the caffeine-induced reduction in DNA single strand breaks, in these studies, could be that the caffeine is blocking the action of enzymes which are destined to remove portions of DNA that were in some way altered by the 4NQO. Perhaps caffeine, being a purine analog, is blocking the active site(s) of the enzyme(s) involved.

Alternatively, caffeine may in some way be competing with 4NQO for binding sites on the DNA and hence block the action of 4NQO in that manner. Additional experiments to assess the role of the time of application of caffeine in conjunction with 4NQO will cast some light on this interpretation of the results.

However, it is of interest to pursue the former suggestion that nuclease enzymes are required to mediate 4NQO-induced DNA single strand breaks as it is felt that this approach gives the

most informative molecular level explanation of the mode of action of 4NQO (and derivatives), as well as tying in neatly with previous studies. Moreover, Ikegami and co-workers add support to the involvement of enzymes in mediating 4NQO-induced DNA breaks: "In the case of 4NQO, the possibility of enzymatic removal of nucleotides modified by quinoline derivatives cannot be ruled out"[16].

It is well established, by biochemical studies, that 4NQO binds to DNA in vivo and mostly to the purine bases[16]. Karreman[21] was the first to develop a theory of the mode of binding by analyzing the relative π electron densities on each of the atoms of 4NQO and of the DNA bases. This was done by Hückel molecular orbital (HMO) computations and showed that the greatest π electron densities (symbolized as δ-), on 4NQO, occurred on the oxygen atoms of the nitro and N-oxide functionalities as shown in Figure 12:

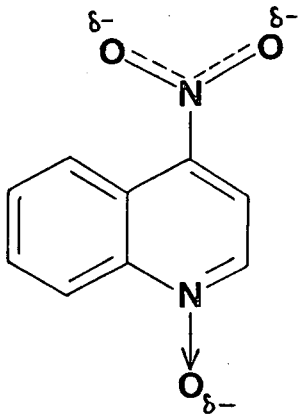


Figure 12. The oxygen atoms of 4NQO have the greatest π electron density (δ-) of all the atoms in the molecule.

Similarly, Karreman showed that the tautomer of adenine contains two nitrogen atoms that lack considerable π electron density (therefore symbolized as δ+) relative to the other atoms in the

molecule as shown in Figure 13:

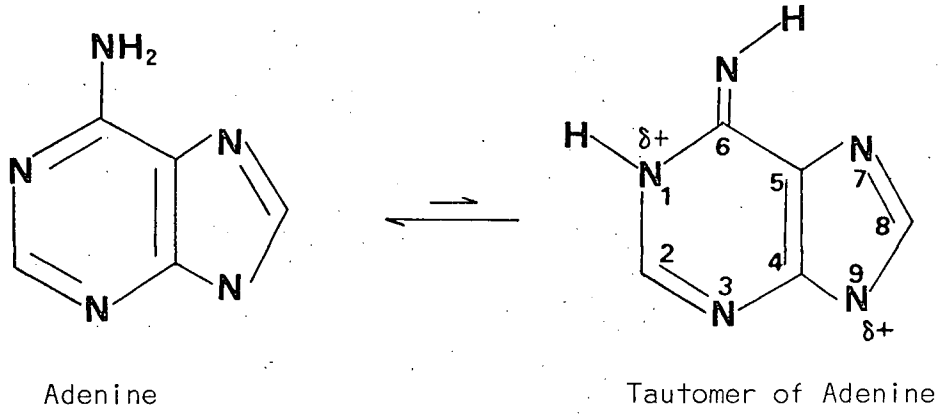


Figure 13. The N-1 and N-9 nitrogen atoms of the tautomer of adenine have the least π electron density (δ+) of all atoms in the molecule.

The δ- oxygen atoms of 4NQO and the δ+ nitrogen atoms of the tautomer of adenine are the same distance apart and so Karreman proposed that a charge-transfer complex between the 4NQO molecule and the tautomer of adenine may occur as shown in Figure 14:

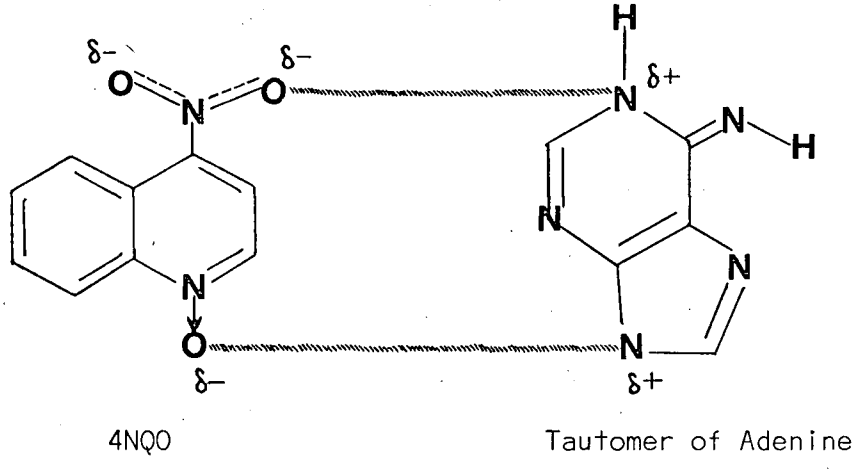
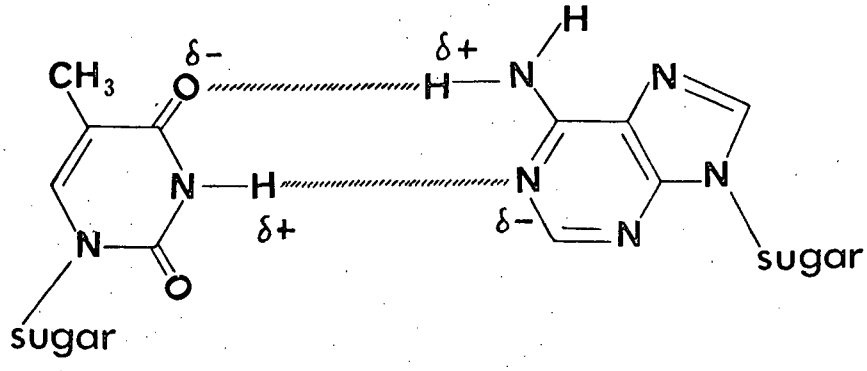


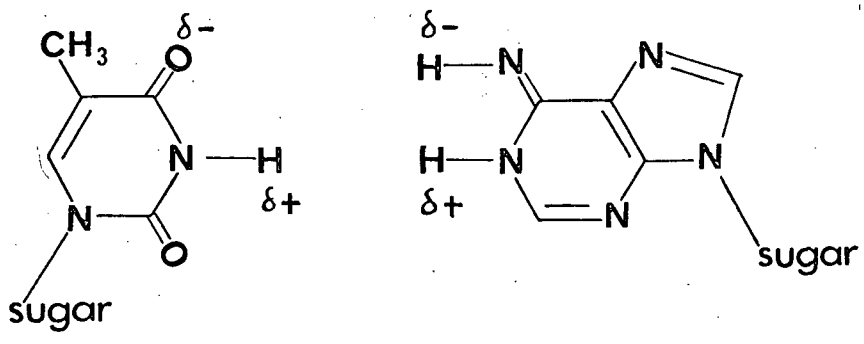
Figure 14. Schematic representation of the charge-transfer complex between 4NQO and the tautomer of adenine as proposed by Karreman[62].

Figure 15. 4NQO induces the formation of the tautomer of adenine and thereby disrupts the interstrand H-bonding.



Thymine Adenine

(a) Normal interstrand H-bonding between thymine and adenine.



Thymine Tautomer of Adenine (complexed to 4NQO, not shown)

(b) 4NQO-disrupted H-bonding.

The representation in Figure 14 can be somewhat misleading as to the probable *in vivo* stereochemistry of this complex. Since both of the complexing molecules are flat and rigid, it is likely that they are able to approach each other fairly close, one on top of the other, with little steric hindrance.

Karreman's proposal was that the stability of this complex would be such that the presence of 4NQO would enhance the formation of the otherwise electronically unstable tautomer of adenine. This abnormal electronic configuration of adenine would alter the electronic densities responsible for the normal interstrand hydrogen bonding to thymine. By studying a similar H-bonding situation with cytosine, rather than thymine, it can be seen why Karreman ultimately proposed that the generation of the adenine tautomer at the time of DNA replication would cause the biosynthesis of a cytosine opposite the adenine and so a point mutation would occur.

This idea of point mutation induction by 4NQO provided a mechanism for spontaneous mutagenesis as originally put forth by Watson and Crick in 1953[21], but really failed to explain the mode of DNA breakage by 4NQO and possibly similar derivatives. Figure 15 illustrates the annihilation of the interstrand hydrogen bonds between adenine (complexed with 4NQO) and thymine. The subsequent local denaturation of DNA, caused by 4NQO, and the probable stereochemical alteration in the sugar-phosphate back-bone of the DNA helix, are schematically illustrated in Figure 16:

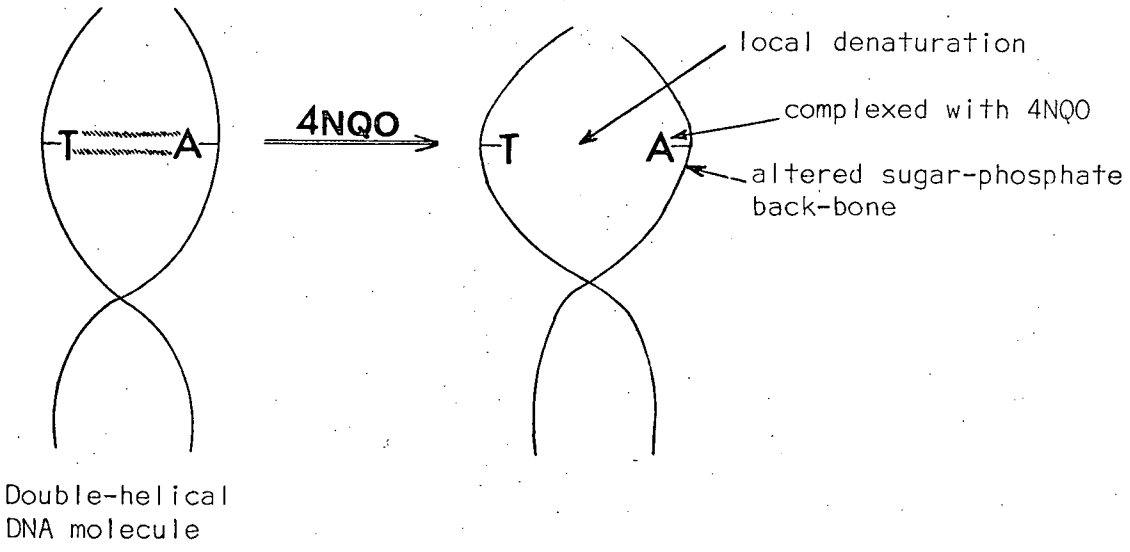


Figure 16. A schematic representation of the generation of a local denaturation by 4NQO.

It can therefore be proposed that the mammalian cell repair enzymes detect the local denaturation by 'reading':

(a) the altered DNA sugar-phosphate back-bone configuration, or

(b) the local denaturation, with regard to H-bond disruption, or

(c) the 4NQO-adenine complex, or

(d) any combination, or all, of the above.

The nuclease enzymes, by removing the altered nucleotides of DNA, thereby produce abundant single strand breaks in the DNA molecule which results in low molecular weight DNA single strands, which are produced and measured upon denaturation and sedimentation on the alkaline sucrose gradient. One can further hypothesize that a polymerase enzyme and a ligase enzyme may be required to replace the damaged nucleotides removed in this manner.

With this possible mode of DNA breakage by 4NQO, and similar quinoline N-oxide compounds for that matter, it seems likely that the actual DNA single strand breakage would occur mostly

during the initial application of 4NQO and perhaps in lesser amounts as the number of such 4NQO-bound DNA nucleotides decreases throughout the incubation period. Nevertheless, the kinetics of the proposed enzymatic excision of DNA single strands would likely be complicated by the existence of residual pools of 4NQO, or one or more of its active metabolites (for example, 4HAQO), within the cell.

Another similar model of the interaction of 4NQO with DNA was recently proposed by Paul and co-workers[48]. This theoretical model was derived in a manner, similar to that of Karreman[21], in which extended Hückel molecular orbital (EHMO) computations were performed on both carcinogenic and noncarcinogenic 4-nitroquinoline 1-oxides and related compounds. A model of charge-transfer complex formation between deoxyguanosine in DNA and 4NQO was constructed, based upon molecular orbital results and steric factors. This model is shown in Figure 17. Paul's model[48] allowed the prediction of the qualitative carcinogenicity of 26 derivatives of 4NQO and it was found that all correlations between molecular orbital properties and carcinogenicity were modified by one or more of the following structural factors:

(a) the absence of a 4-nitro group,

(b) the absence of a 1-oxide group, and

(c) the presence of bulky substituents in position 2 or 3 of the quinoline ring.

Any of the three factors resulted in either noncarcinogenicity or reduced carcinogenicity. It is interesting to note that these results are consistent with the correlations between

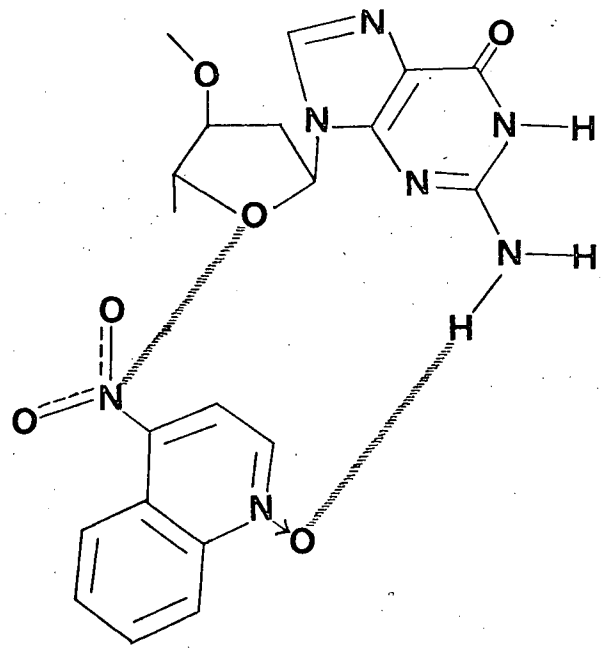


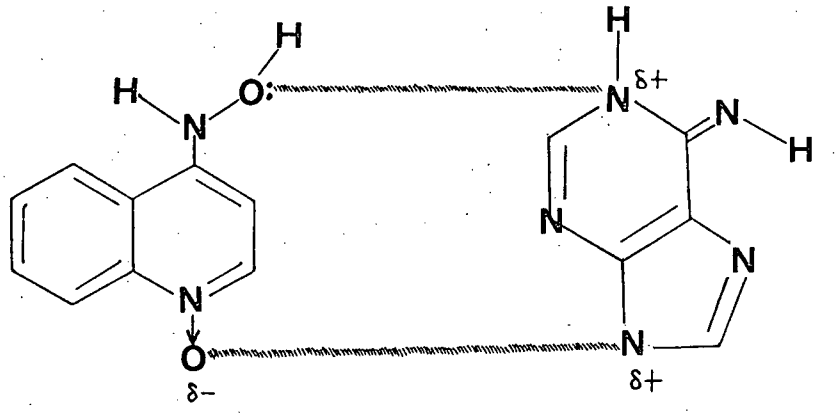
Figure 17. The postulated complex, between 4NQO and deoxyguanosine, by Paul and co-workers[48].

chemical structure and extent of DNA repair induction by these 4NQO derivatives, as found by Stich and San[59]. Moreover, with the necessary reservations in mind, the results obtained from the comparative analysis of the DNA breakage induced by 4NQO, 3-methyl-4NQO, 3NQO, 8NQO, as determined in these alkaline sucrose density gradient studies, also paralleled the above EHMO calculation results.

Of significance to the proposed mode of enzyme-mediated, 4NQO-induced, DNA breakage is the fact that the charge-transfer complex proposed by Paul and co-workers could also cause a local denaturation of the DNA helix and hence the repair enzymes (nucleases) may be evoked to remove the altered portions of the DNA molecule. All in all, the charge-transfer

complexes proposed by Karreman[21] and Paul and co-workers[48] may be only representative of a spectrum of various 4NQO-DNA charge-transfer complex possibilities - all of which may provoke enzyme-mediated removal of the altered DNA nucleotides.

A prime criticism of this proposed enzyme-mediated, 4NQO-induced breakage is that a metabolic reduction product of 4NQO, 4HAQO, may in fact be the proximal carcinogen[22,61]. It has been demonstrated that 4HAQO covalently bonds with DNA and apparently causes single-strand scissions of DNA[60]. However, the concept that the carcinogenic 4HAQO and related compounds interact with DNA by a mechanism other than covalent bond formation can be supported by several studies. Firstly, spectrophotometric studies have demonstrated that 4HAQO interacts with DNA in a manner similar to 4NQO[26] and probably involving an $n \rightarrow \pi^*$ interaction[46]. Figure 18 shows such an involvement of the nonbonding electrons of the oxygen of the hydroxyamino group. Note that the electrons are depicted as being donated to the $\delta+$ nitrogen of the π^* electron system of the tautomer of adenine (hence the term $n \rightarrow \pi^*$ interaction). Furthermore, the donation of the non-bonding electron pair of the oxygen could contribute to the stability of the tautomer of adenine. Again, this electronic alteration of the adenine π electron system, induced by 4HAQO in this case, would probably be sufficient to annihilate the interstrand H-bonding to thymine. Again, the repair enzymes may be implicated in removing the altered



4HAQO Tautomer of Adenine

Figure 18. The involvement of 4HAQO in a charge-transfer complex with the tautomer of adenine.

DNA fragment.

Secondly, 4HAQO, like 4NQO, has been shown to bind specifically with the purines, both at the nucleoside and polymer levels[26], and has also been shown to result in parallel base planes[42].

The foregoing evidence suggests a similarity in the interaction of 4HAQO and 4NQO with DNA. The proximal carcinogen does not, in fact, severely hamper the concept of enzyme involvement in the induction of DNA single strand breaks upon exposure to 4NQO and derivatives.

People with the homozygous recessive genetic disease, Xeroderma pigmentosum (XP) have a very high incidence of skin cancer[5]. It has been shown that XP fibroblasts do not release UV-induced thymine dimers from DNA and so do not repair photochemical damage[4]. It therefore seems that the XP fibroblasts lack an endonuclease enzyme which is the probable

means by which the altered DNA is removed, before the possible ligase involvement in the repair process. In our laboratory, Stich and San have recently shown that, unlike normal skin fibroblasts, XP fibroblasts exhibit very reduced unscheduled uptake of ^{3}H-TdR after 4NQO treatment and after UV-light treatment. This suggests that an endonuclease enzyme, similar to that involved in removing thymine dimers[4], may be required to remove 4NQO-altered DNA subunits.

Unscheduled uptake of ^{3}H-TdR, as a measure of DNA repair, is obviously dependent on the ligase activity in rejoining the 'nicks' in the DNA strands, and so it may be argued that XP fibroblasts show abnormally reduced ligase activity in the presence of 4NQO (although XP fibroblasts are capable of repairing X-ray induced DNA breaks, which requires a ligase[4]). Alkaline sucrose density gradient analysis of XP fibroblast DNA, after exposure to 4NQO and derivatives, would directly test the hypothesis that XP fibroblasts lack endonuclease activity, regardless of ligase activity. Moreover, the implication of enzyme involvement in removing 4NQO-induced DNA alterations of any mammalian cell would be substantiated.

It appears that the alkaline sucrose gradient detection of single strand breaks has parallelled cytologic studies, such as those of Stich and San[58,59], with regard to the mode of action of quinoline N-oxides. Although this new biochemical technique lacks such sensitivity as shown by autoradiographic methods[58,59] in reproducibly detecting DNA breaks induced by so-called weak oncogens, its utility is certainly commendable. Studies like those described, combined with the proposed studies on XP

fibroblasts, will perhaps broaden the overall concept of chemical carcinogenesis to include the role of nuclear repair enzymes in preventing chemical alterations of DNA, such as those suggested for 4NQO-treated DNA.

Attempts at correlating biological activities with specific chemical alterations, even down to the electronic level of charge-transfer reactions, will ultimately develop a specific molecular-level interpretation of the mutagenic phenomenon of neoplastic transformation.

SUMMARY

The aim of this study has been to reveal the mode of action of the mutagenic and oncogenic 4-nitroquinoline 1-oxide and its weakly or non-oncogenic derivatives. The main technical considerations, results, and conclusions are shortly summarized below:

1. The optimum labelling conditions for BHK-21 cells were: 0.05 uCi/ml ^{14}C-TdR and 0.25 uCi/ml ^{3}H-TdR for 24 hours. When diploid hamster embryonic cells were used the labelling time was increased to 36-40 hours.

2. The modifications of the alkaline sucrose gradient technique were: (1) layering the cell suspension directly onto a 2% sucrose overlay; and, (2) allowing cell lysis and centrifugation to occur at 4° rather than room temperature.

3. With the above technical modifications, the optimal number of cells layered per gradient was found to be 12,000.

4. A concentration of 5×10^{-6}M 4NQO was found to give the most reproducibly detected DNA single-strand breaks.

5. A limited correlation between the degree of oncogenicity of synthetic quinoline N-oxides and their capacity to induce DNA single-strand breaks was found. Highly oncogenic 4NQO readily induced DNA single-strand breaks whereas weakly oncogenic 3-methyl-4NQO or non-oncogenic 3NQO and 8NQO did not (within the limits of detection by this sucrose gradient technique).

6. A significant degree of repair of 4NQO-induced DNA single-strand breaks was found with repair almost complete at 24 hours.

7. DNA aggregation may be occurring in high proportions on the

sucrose gradients as evidenced by: (1) frequently occurring pelletted DNA; and, (2) frequent occurrence of 2 parent peaks.

8. 4NQO, known to bind to DNA, may be intercalating between bases of adjacent DNA strands and/or affecting the tertiary structure of DNA, thereby enhancing DNA aggregation on the gradient. This may, in future, be exploited in detecting very low concentrations of 4NQO.

9. A 4×10^{-3}M concentration of caffeine, present with 4×10^{-6}M 4NQO, was found to vastly reduce the extent of DNA single-strand breakage. This suggested that either: (1) the caffeine was competing favorably with 4NQO for DNA binding sites, or (2) the caffeine was reducing nuclease enzyme activity which may be involved in removing 4NQO-induced DNA alterations.

10. Karreman had shown that the electron densities of the individual atoms of 4NQO and adenine favored a charge-transfer complex between the two molecules.

11. This would stabilize the tautomer of adenine which, it is proposed, would annihilate the interstrand H-bonding to thymine. Such a local denaturation may be detected, and the altered fragment removed, by a nuclease enzyme(s).

12. The proposed proximate carcinogen, 4HAQO, could interact in a similar charge-transfer complex except that an $n \rightarrow \pi^*$ interaction would be involved as well as a $\pi \rightarrow \pi^*$ interaction as in the 4NQO-adenine charge-transfer complex.

13. Recent autoradiographic studies of DNA repair in Xeroderma pigmentosum (XP) cells have shown vastly reduced unscheduled uptake of ^{3}H-TdR, following exposure to 4NQO, which implies that

4NQO may require an endonuclease enzyme to mediate DNA breaks by removing 4NQO-altered nucleotides.

14. 4NQO can be tentatively categorized with UV-light as mutagenic agents requiring an endonuclease enzyme to remove the damaged DNA nucleotides. This is contrasted with agents such as X-rays and N-mustards which spontaneously break DNA even in XP cells.

15. 4NQO may cause neoplastic transformation because of: (1) altering a nucleotide such as adenosine, without removal of the alteration, and/or (2) causing too many breaks of the DNA single strands making repair impossible or incorrect.

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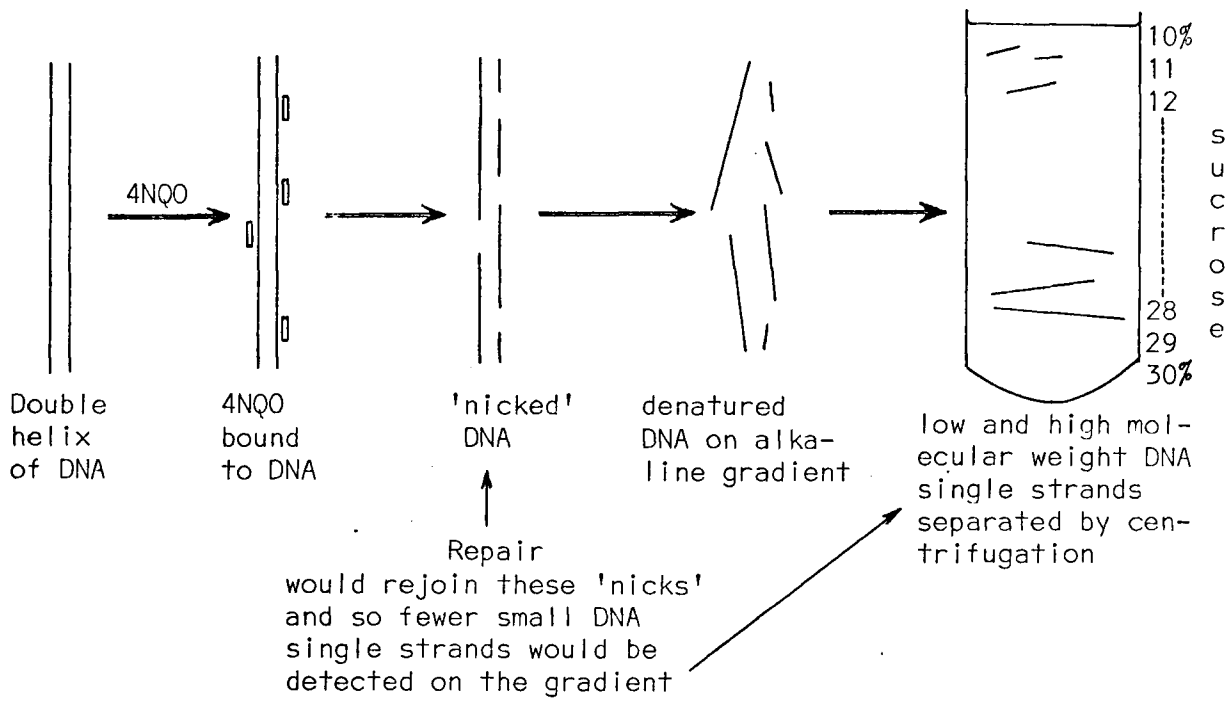
APPENDIX

A Schematic Representation of the 4NQO-induction of DNA Breaks, Subsequent Repair, and Alkaline Sucrose Gradient Analysis of the Phenomenon:



Hypothesis of Enzyme-mediated Breakage of 4NQO-altered DNA Subunits: