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THE REPETITIOUS NATURE OF REPAIRED DNA
IN MAMMALIAN CELLS

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RANDOM DISTRIBUTION OF HIGHLY REPETITIVE AND INTERMEDIATE FREQUENCY MOUSE L-929 CELL DNA SEQUENCES SYNTHESIZED AFTER UV LIGHT EXPOSURE

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Abstract—The DNA precursor ^{3}H-thymidine incorporation rate (dpm μg DNA) in mouse L-929 cells decreases immediately after exposure to UV light. This decrease is initially dose dependent, but at exposures greater than 22.5 J m^{-2} appears to be radio-resistant. This was not explained by measurements of uptake of ^{3}H-thymidine into the acid soluble pool (dpm μg DNA). The radio-resistant incorporation amounted to approximately 35% of the control rate. DNA reassociation ("C_0T") studies were performed with DNA labeled with ^{3}H-thymidine immediately after exposure of L-929 cells with a dose of UV resulting in radio-resistant incorporation to determine whether this radio-resistant incorporation was occurring in sequences of a particular repetitious frequency. These studies, performed in the highly repetitive to intermediate range, showed that the radio-resistant incorporation was occurring in DNA of all classes of repetitious frequency. DNA synthesized at different times after UV exposure, during the period when post-replication repair can occur, was similarly labeled for short intervals and isolated. The DNA reassociation studies showed that this DNA synthesis was also of all classes of repetitious frequency.

INTRODUCTION

The inhibition of DNA synthesis immediately after UV irradiation has been reported in several mammalian cell lines (Rasmussen and Painter, 1964; Cleaver, 1965; Klimek and Vlasinova, 1966; Cleaver, 1967; Djordjevic and Tolmach, 1967; Domon and Rauth, 1968; Meyn *et al.*, 1976; Edenberg, 1976). A characteristic phenomenon is the apparent presence of a component of tritiated thymidine incorporation which is radio-resistant; this component in mouse cells typically comprises approximately 35% of the control rate for short labeling intervals. On the basis of DNA reassociation ("C_0T") studies, Britten and Kohne (1968) have shown that mouse and other eukaryotic DNAs consist of unique, intermediate, and repetitive DNA sequences. Two possibilities were considered: that the radio-resistant incorporation was occurring uniformly in DNA sequences of all repetitious frequency classes or, alternatively, that the incorporation was partially or totally nonuniform, i.e. that it represented a continuation of synthesis of sequences of a given frequency, with synthesis of the remainder being partially or totally inhibited.

After having determined the extent of uptake and incorporation of DNA precursor, DNA reassociation studies were performed to determine the repetitious frequency of the radio-resistant DNA. Similar experiments were performed with DNA synthesized at different times after UV irradiation, i.e. during a period of time when post-replication repair can occur (Lehmann, 1972; Fujiwara, 1972), to determine whether this later synthesis was of a uniform distribution in terms of its sequence frequency. The extent of uptake of the DNA precursor tritiated thymidine (^{3}H-Tdr), its extent of incorporation during a 30 min labeling interval, and the repetitious distribution of the DNA incorporating the precursor, are described below.

MATERIALS AND METHODS

Materials. The mouse L-929 cells employed in these studies were obtained from the American Type Culture Collection. The cells were maintained in Earle's Minimal Essential Medium with 10% fetal calf serum, and supplemented with nonessential amino acids and additional L-glutamine. The antibiotics penicillin and streptomycin and the antimycotic fungizone were also present in the medium. All materials were obtained from Grand Island Biological Company, Grand Island, NY. The cells were maintained either in Falcon or Corning T-30 or T-75 sterile plastic tissue culture flasks containing 10 mℓ or 20 mℓ of medium, respectively. Flasks were gassed with 5% CO_2, 95% air, and maintained in a 37°C incubator. For UV irradiation experiments, an appropriate number of L-929 cells were distributed into Falcon 100 mm sterile plastic petri dishes, and the cells were incubated at 37°C in a Forma humidified CO_2 incubator at 5% CO_2.

Methods. In the two initial studies of uptake and incorporation after UV irradiation, the experimental protocol was as follows. Rapidly proliferating and nonconfluent cells in petri dishes were removed from the CO_2 incubator, washed two times with warm sterile pucks saline A, aspirated, and then immediately exposed to UV light (0.94 J m^{-2} s) using a specially built UV light irradiation device described by Steier and Cleaver (1969). The dose rate was measured at the exposed surface using a cali-

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brated Blak-ray J-225 shortwave UV intensity meter. Immediately after irradiation, the cells were reincubated in the CO_2 incubator with warm complete medium containing 10 μCi mℓ of tritiated thymidine (50.8 Ci mmole, methyl-^{3}H; New England Nuclear) adjusted to a final molarity of 5 μM thymidine. After incubation at 37°C for 30 min, the labeling medium was aspirated from the dishes, and the cells were washed two times with ice cold SSC (standard saline citrate: 0.15 M NaCl, 0.015 M sodium citrate). The cells were then scraped free using a rubber policeman into ice cold SSC, collected by centrifugation, and quick-frozen by immersion in ethanol–dry ice after aspiration.

For analysis of uptake and incorporation in these experiments, a modified Fleck and Munro (1962) and Schmidt and Thannhauser (1945) procedure was employed. In this procedure, the cell pellet is resuspended in 0.8 mℓ of ice cold SSC, to which is added 0.2 mℓ of 50% TCA. After mixing, the tube is immersed in an ice bath and allowed to stand for 30 min. The tube is then centrifuged for 15 min at 2500 rpm at 4°C in an International PR-J refrigerated centrifuge. The supernatant containing the acid soluble radioactivity is carefully removed to a tared tube, and the pellet is resuspended using a small volume of fresh ice cold 10% TCA. The tube is again centrifuged, and the supernatant is added to the initial acid soluble fraction. The pellet is then sequentially washed with ice cold 95% ethanol, ethanol:ether (3:1), and ether, and after aspiration, the residual ether is allowed to evaporate at room temperature. A small volume of 0.3 N KOH is added to each tube and the pellet resuspended. The tubes are then incubated at 37°C for 1 h to digest the ribonucleic acid. At the end of 1 h, the tubes are immediately chilled in an ice bath, the alkali is neutralized by addition of the appropriate volume of 1.0 N HCl, and the contents of the tubes are again adjusted to 10% TCA by addition of the appropriate volume of 50% TCA. The tubes are allowed to stand in ice for 15 min. After centrifugation, the ribonucleotides are removed, the pellet is washed with a small volume of ice cold 10% TCA, the latter is aspirated and then 0.8 mℓ of fresh ice cold 10% TCA is added to the pellet. After resuspending the pellet in the TCA, the DNA is hydrolyzed in a boiling water bath for 15 min. After removal of the tube from the water bath, and allowing for 1 min cooling at room temperature, the tube is immersed in ice for 15 min. The tube is then centrifuged and the supernatant containing the deoxyribonucleotides is transferred to a tared tube. The hydrolysis is repeated, and the second hydrolyzate is added to the first. A volume of 0.1 mℓ of the acid soluble and DNA nucleotide fractions are counted for radioactivity determination, and the DNA concentration is determined using the diphenylamine procedure described by Burton (1956). The incorporated radioactivity is reported as dpm μg of total DNA recovered. The radioactivity in the acid soluble fraction, after correction to total acid soluble radioactivity recovered and total DNA recovered, is also reported as dpm μg DNA.

For the kinetic studies requiring intact isolated DNA, the procedure for DNA isolation described by Meltz (1976) was employed. The isolated DNA was additionally purified by cesium chloride density gradient centrifugation (Flamm *et al.*, 1966) for 36 h at room temperature in a Beckman Spinco type 50 Ti rotor at 36,000 rpm in a Beckman 13-50 centrifuge. The gradients were collected by top collection using an Isco density gradient fractionator. Aliquots of each fraction were precipitated onto filters for radioactivity determination. The fractions containing the DNA peak were pooled based on both optical density and radioactivity determinations, which coincided. The appropriate fractions were pooled, and the cesium chloride solution was diluted with water. The DNA was then pelleted by centrifugation for 20 h at 42,000 rpm at 10°C in a Beckman L3-50 centrifuge. After slowly removing the supernatant by pipette, the pellet was resuspended in 1/10 SSC. The radioactivity of the final solution was determined by counting 0.2 mℓ aliquots in Beckman Bio-Solv BBS-3 counting solution, and correcting to disintegrations per minute by automatic computer correction using external standards. The DNA concentration was determined using a spectrofluorometric ("DABA") technique (Hinegardner, 1971).

For the DNA DNA C_0T studies, an appropriate concentration of DNA synthesized after irradiation (or control) and labeled with ^{3}H-TdR was mixed with an appropriate concentration of ^{14}C-thymidine labeled, semi-conservatively synthesized L-929 DNA (24 h label) and nonradioactive *E. coli* DNA. The combined DNA was then subjected to a series of steps, including: (a) addition of glycerol and shearing using a VirTis "60" homogenizer at 50,000 rpm for 30 min in ice, (b) precipitation with ethyl alcohol in the presence of sodium acetate, (c) pelleting of the DNA at 10,000 rpm for 20 min at 4°C in a Sorvall SS-34 rotor, (d) resuspension of the DNA in phosphate buffered-EDTA (0.12 M phosphate buffer, 2 mM EDTA), (e) measurement of DNA concentration by radioactive counts, (f) denaturation of the samples in capillary tubes or test tubes at the appropriate concentrations by boiling in hot water, (g) incubation of the samples for the appropriate times at 60°C, (h) freezing of the samples in acetone–dry ice until analysis, (i) fractionation of the samples on hydroxylapatite in columns to separate single-stranded from double-stranded DNA, and (j) radioactive counting of the fractions in BBS-3 liquid scintillation solution. In performing these studies, we essentially followed the procedures described by Britten *et al.* (1974). All C_0T reactions were performed in duplicate, and the value reported in the figures is the average.

For the kinetic studies, the UV irradiation, labeling, and cell recovery steps were as described above, with the exception that the cells were labeled with high specific activity ^{3}H-thymidine at 10 μCi mℓ without adjustment of the exogenous pool size by addition of nonradioactive thymidine.

RESULTS

The results of the two dose response experiments appear in Fig. 1. In both experiments, the presence of a UV radio-resistant region, similar to that previously reported, is apparent. It appeared from the first experiment that the UV resistant incorporation might be explained as the result of a decrease in rate of synthesis, with the same level of radioactivity being incorporated because of an increased amount of radioactivity in the acid soluble pool. On repeating the experiment, however, while the same radio-resistant region appeared, the acid soluble radioactivity did not show a similar increase in radioactivity. The issue remains unresolved, but it is not likely that the amount of radioactivity in the acid soluble pool is the responsible factor for the apparent residual resistant synthesis. A similar conclusion was reached by Cleaver (1965). For observing the sequence distribution of the ^{3}H-TdR labeled DNA synthesized after irradiation, exposure times were chosen which would provide labeled DNA from cells that had not been irradiated (0 s), DNA which was labeled after an exposure which was halfway down the dose response curve to the resistant level (12 s), and an exposure which would result in labeling of the radio-resistant fraction (30 s). The specific activity of the isolated DNA from two such experiments appears in Table 1. Four dishes were exposed at each dose in each

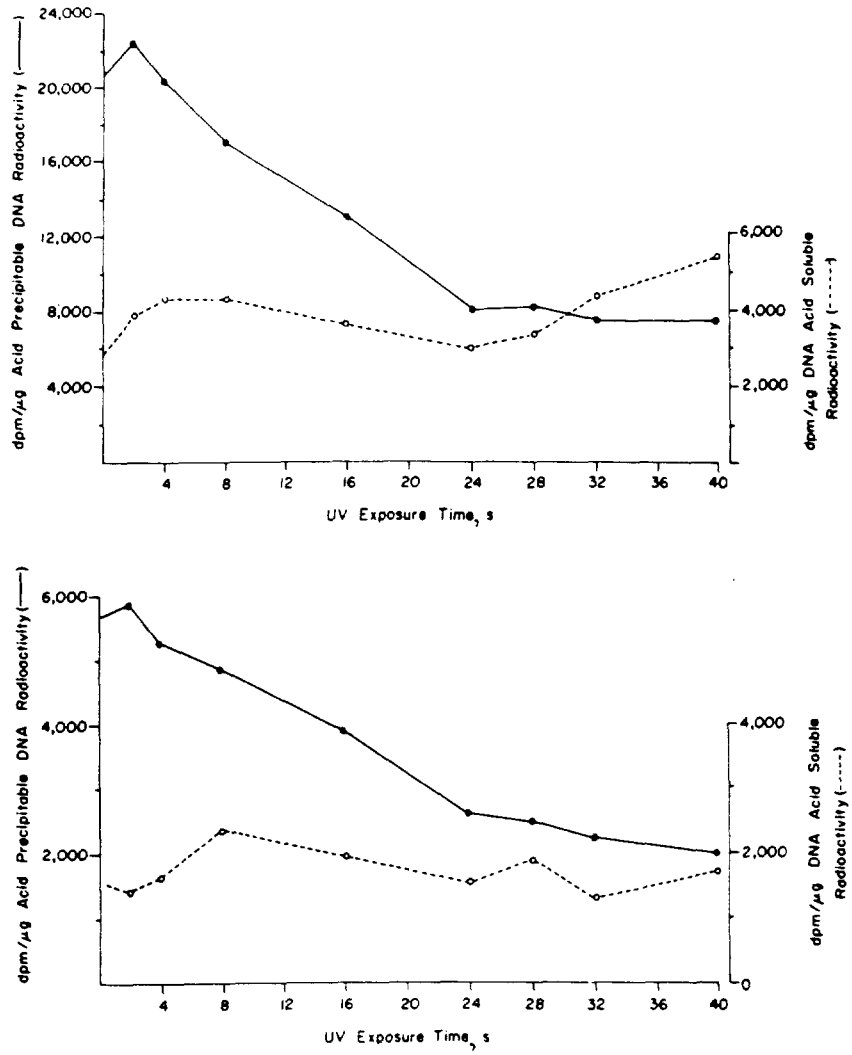

Figure 1. Uptake of ^{3}H-thymidine into the acid soluble pool and incorporation of ^{3}H-thymidine into acid-precipitable DNA in L-929 cells during a 30 min pulse label after different UV-exposures. UV dose rate = 0.94 J m^2 s. Upper figure: first experiment; lower figure: second experiment. In each experiment, one dish was exposed at each dose. The diphenylamine reaction was performed in duplicate and the results averaged for DNA concentration determination; the radioactivity concentration was the average of counts of duplicate samples.

experiment and the cells pooled. The isolated DNA concentration in each case was determined in duplicate using the DABA procedure. The radioactivity concentration was the average of duplicate samples. In performing the kinetic experiments, nonirradiated, 24 h ^{14}C-thymidine labeled, semi-conservatively synthesized DNA was included in each reaction mixture as an internal control. The experiments were performed over the low to intermediate C_0T range. As can be seen in Fig. 2 for both of the experiments, our results indicate that the UV radio-resistant incorporation is composed of DNA which is at least both highly and intermediately repetitive in frequency; unique DNA must therefore also have been synthesized.

Experiments were also performed to determine the extent of the incorporation and the repetitious distribution of DNA incorporating ^{3}H-TdR radioactivity at different times after UV irradiation. This is during the period of time when post-replication repair can occur (Lehmann, 1972; Fujiwara, 1972). For these experiments, the labeling medium contained 10 μCi m/ of high specific activity tritiated thymidine (52.6 Ci mmole). Rapidly proliferating cells in petri dishes were rinsed, exposed to UV at 0.94 J m^2 s for 30 s, and then either immediately labeled for 20 min

Table 1. Specific activity (dpm μg) of DNA isolated from L-929 mouse fibroblast cells, labeled after different UV exposures

UV exposure time (s)	Experiment 1 dpm μg	Experiment 1 % of Control	Experiment 2 dpm μg	Experiment 2 % of Control
0	42,991		54,561	
12	26,995	62.8	31,928	58.4
30	16,036	37.3	18,387	33.6

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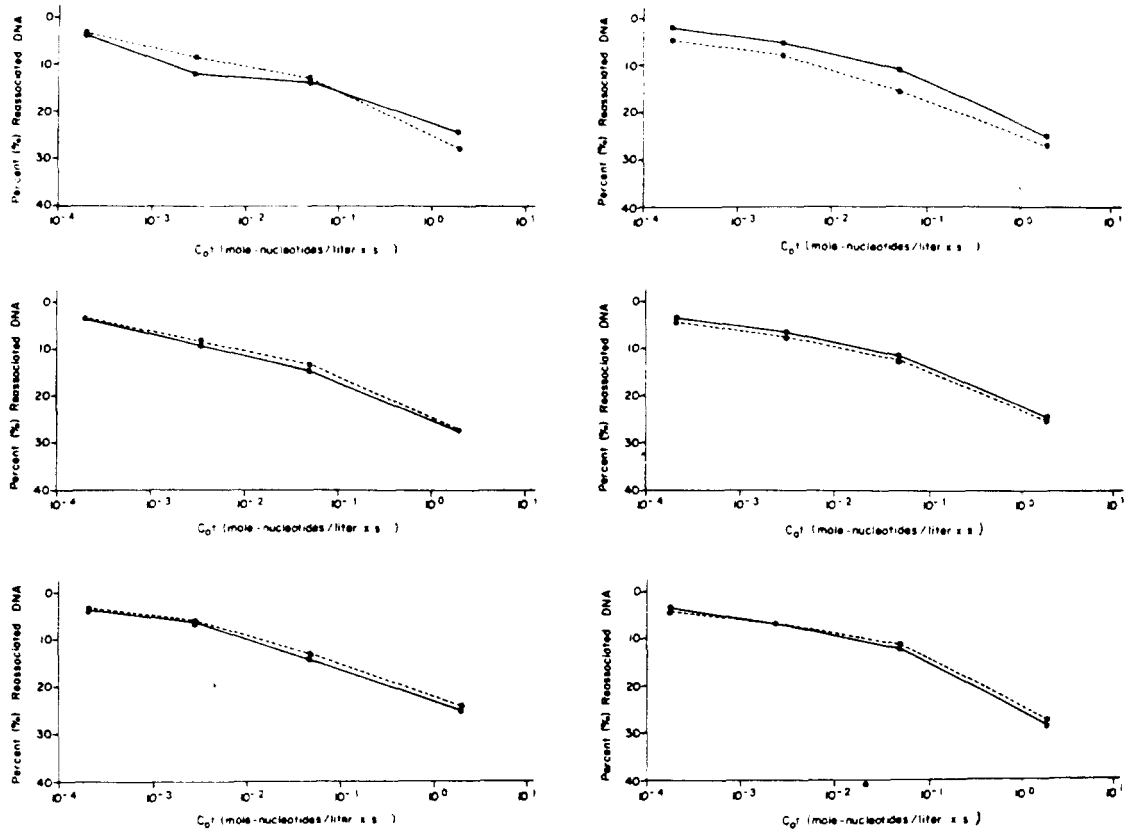

Figure 2. Kinetic data obtained upon reassociation incubation of labeled DNA from UV-irradiated mouse L-929 cells after 30 min post-UV incubation with ^{3}H-thymidine at 10 μCi/mℓ; (——) 30 min ^{3}H-thymidine pulse labeled DNA; (-----) 24 h semi-conservatively synthesized ^{14}C-thymidine labeled mouse L-929 DNA. Upper left to lower left figures, reassociation data isolated in Experiment 1 after 0 s, 12 s, or 30 s UV exposure; upper right to lower right, reassociation data for DNA isolated in Experiment 2 after 0 s, 12 s, or 30 s UV exposure. Each data point indicated is the average after incubation of duplicate samples. The results of the two experiments are presented independently since different C_0T values were employed in each experiment to obtain the resulting curves.

or reincubated for 1, 2, or 3 h in fresh warm medium before addition of the labeling medium for 20 min. The cells were collected as described above. The DNA was isolated and its concentration determined in duplicate using the DABA procedure. The specific activities obtained as a function of post-irradiation time in h appear in Fig. 3. A decreasing incorporation rate of precursor with increasing time post-irradiation was observed. The sequence distribution of DNA which was pulse-labeled at 0, 2, or 3 h post-30-s UV exposure appears in Fig. 4. The distribution of the radioactivity appears to be uniform in the sequences of different repetitious frequency.

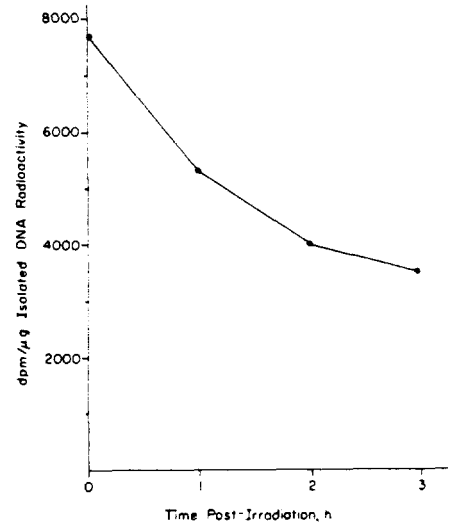

Figure 3. Specific activity in dpm μg of DNA isolated from mouse L-929 cells which were pulse-labeled for 20 min with ^{3}H-thymidine beginning at different times after a 30 s UV exposure. Four dishes were exposed and incubated for each post-irradiation incubation time and the cells pooled. The isolated DNA concentration was determined for each sample in duplicate using the DABA procedure. The radioactivity concentration was the average of counts of duplicate samples.

DISCUSSION

We have examined UV "radio-resistant" DNA and also DNA synthesized at various times after UV light exposure to determine whether the incorporation occurring was in DNA representative of the total mouse L-929 cell genome, or in DNA sequences of a particular repetitious frequency. This was accomplished by mixing ^{14}C-thymidine labeled DNA which was semi-conservatively synthesized in nonirradiated proliferating cells with DNA from UV irradiated and

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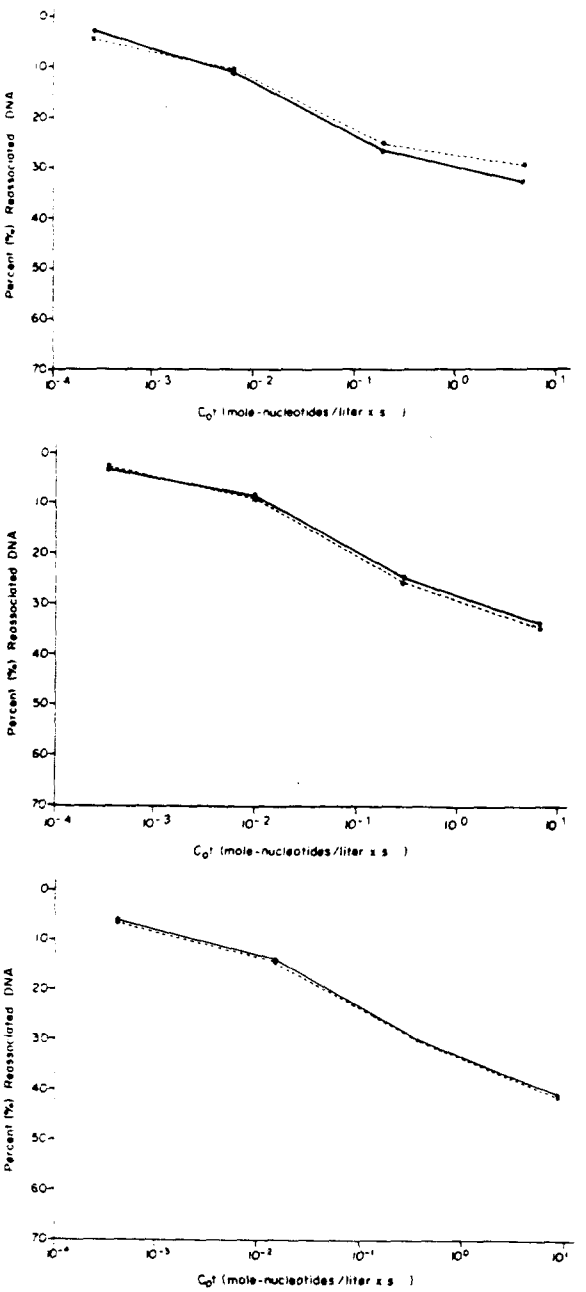

Figure 4. Kinetic data obtained upon reassociation incubation of DNA pulse-labeled at 0, 2, or 3 h post 30 s UV exposure. Upper curve: DNA pulse-labeled immediately after UV irradiation; middle curve: DNA pulse-labeled beginning 2 h post exposure; lower curve: DNA pulse-labeled beginning 3 h post exposure. Each data point indicated is the average incubation of duplicate samples.

^{3}H-thymidine pulse labeled cells, and then performing DNA DNA "C_0T" kinetic studies. If the sequences of a particular frequency were greatly enriched with the ^{3}H-thymidine, its reassociation would differ from the ^{14}C-thymidine labeled nonirradiated cell DNA, and would result in a C_0T curve corresponding more closely to that which would result if that particular class of DNA were separately isolated and then kinetically reacted (see fig. 13 in Britten and Smith, 1970; Davidson *et al.*, 1971). By the technique employed, the reassociation behavior of ^{3}H- and ^{14}C-labeled DNAs were indistinguishable from one another and resembled that of DNA from other mouse cell lines (Britten and Rake, 1967).

The proportion of DNA of the mouse genome reassociating at a C_0T of 10^0 is approximately 30% (Britten and Rake, 1967). The possibility that radioresistant DNA as well as that synthesized at different times after UV exposure is greatly enriched in the highly repetitive or other classes of DNA has been ruled out since a uniform distribution was observed in all frequency classes examined. It is interesting to note that repair replication, which contributes at least some portion of the incorporated radioactivity in these experiments, has also been shown to be uniformly distributed in the genome of mammalian cells (Meltz and Painter, 1973; Lieberman and Poirier, 1974). We have attempted in these experiments to employ UV irradiation as an inhibitor of synthesis of DNA of one class of repetitious frequency; our results show that such is not the case, and the question therefore remains as to whether any inhibitor can selectively inhibit DNA synthesis on a class frequency basis.

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Overall Objectives:

Several main objectives have been addressed in this project. The first objective was to determine appropriate conditions for performance of the repair replication procedure in three different human lymphoblastoid cell lines, one a presumptive "normal" cell line, and the other two Burkitt's lymphoma cell lines. In this study we compared the relative extents of repair in the three cell lines, which are distinguishable in terms of their degree of Epstein-Barr Virus involvement, while at the same time characterizing those treatment and repair labeling conditions which would result in maximum repair replication radioactivity incorporation into cell DNA upon treatment with either ultraviolet light or the alkylating agent and mutagen methyl methanesulfonate. This maximal radioactivity was essential for performance of the experiments described in objective 2 below. A summary of the preliminary studies of the comparative DNA repair in "normal" lymphoblastoid and Burkitt's lymphoma cell lines appears in Appendix I. The experiments included investigations of the dose or concentration dependence of repair, the dependence of amount of repair radioactivity incorporated on time of treatment and labeling, and comparisons at a selected UV dose and two MMS concentrations of the relative extent of incorporated repair radioactivity.

The second main objective was to investigate the distribution of repair replication radioactivity occurring in the "normal" WIL_2-A3 lymphoblastoid cell line, with the DNA being separable into highly repetitious, intermediate repetitious, and unique DNA sequences by use

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of the kinetic reaction of isolated and sheared DNA pieces in solution. This is known as the DNA/DNA Cot technique. If the damage caused by the UV or MMS was nonuniformly distributed in the genome, and repair occurred wherever that damage was located, or alternatively if the damage was randomly distributed, but the repair was not occurring uniformly in DNA of different degrees of repetitiousness, then the kinetic reaction of the DNA containing the repair radioactivity would show a different pattern of reassociation than DNA containing radioactivity incorporated by normal semi-conservative synthesis. The experiments performed to meet this objective appear in manuscript form in Appendix II (submitted for publication).

A third objective of this project was to determine if the DNA synthesized in mammalian cells immediately after UV irradiation, or alternatively at different times after UV irradiation, was representative of all classes of repetitious frequency, or alternatively if the irradiation had resulted in the inhibition of synthesis of one class of DNA as compared to other classes. Our initial investigation of DNA synthesis inhibition immediately after irradiation with UV light had indicated a radioresistant fraction of DNA synthesis, which corresponded as a proportion of total synthesis to the proportion of DNA in mouse cells which would be characterized as highly repetitious. The results of these experiments have been published, and appear in Appendix III.

The final objective of this project was to begin investigations of the extent of DNA single strand breakage as related to mutagenesis

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in the L5178Y mouse leukemic cell thymidine kinase locus forward mutation assay system. During the final stages of the project, a series of investigations were performed which allowed us to establish the alkaline elution technique for single strand break detection in our laboratory. The experiments performed are summarized in Appendix IV.

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Appendix I

Summary of Preliminary Studies of the

Comparative DNA Repair in "Normal" Lymphoblastoid and

Burkitt's Lymphoma Cell Lines

1041982

Abstract

The extent of DNA repair in three human lymphoblastoid cell lines after equivalent ultraviolet (UV) light exposure and after treatment with equivalent concentrations of the alkylating agent methyl methanesulfonate (MMS) have been examined. Two of the cell lines, the WIL_2-A3 cell line and the RAJI line of Burkitt's lymphoma, have similar growth rates in suspension, while the EB-3 line of Burkitt's lymphoma has a slower growth rate. WIL_2-A3 cells are presumptive "normal" cells, RAJI cells are known to contain EB-3 virus information in their genome, and EB-3 cells are able to produce the virus.

When careful attention is given to irradiating cells from approximately the same region of the growth curve, and then incubating them at equivalent cell concentrations with the same labeling medium after the same UV dose, the amounts of incorporated-repair radioactivity in WIL_2-A3 and RAJI cell DNA are similar, with that occurring in EB-3 cell DNA being slightly less.

When the three cell types (from similar regions of their growth curves) were treated with a 0.2 mM concentration of MMS, using the same treatment-labeling medium to treat each of the cell lines at the same time, the amount of incorporated repair radioactivity was highest in the WIL_2-A3 cell DNA, less in the RAJI, and still less in the EB-3 cell DNA. The 0.2 mM concentration is not in the plateau region of concentration dependence of repair. At a concentration of 5 mM, which is in the plateau

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region for MMS induced repair, the amount of repair replication radioactivity was similar in the WIL_2-A3 and RAJI cell lines, and less in the EB-3 cell line.

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Introduction

Deoxyribonucleic acid repair after ultraviolet light irradiation has been shown to occur in freshly isolated non-mitogen stimulated human peripheral lymphocytes (1,2). These studies, performed using autoradiographic and DNA acid precipitation techniques, did not indicate any significant difference occurring in the extent of DNA repair in fresh lymphocytes isolated from patients with chronic lymphocytic leukemia as compared to those from normal individuals after equivalent doses of UV irradiation. The parameters measured included the percent of labeled cells, the number of grain counts, and the net disintegrations per minute (dpm) per cell. In contrast to these earlier investigations, a more recent study (3) of the rate of repair in human chronic leukemic lymphocytes did indicate that there was an increased rate of repair, not explainable by alterations in pool size, in the lymphocytes of patients with chronic lymphocytic leukemia as compared to those of normal individuals. The measure of repair was by a determination of acid precipitable DNA radioactivity. The increased incorporation was also correlated with a faster removal of UV-induced dimers in the leukemic lymphocytes. These authors (3) suggested that the increased repair activity might be of viral origin. We wished to compare the extent of repair occurring in several continuous human lymphoblastoid cell lines, including a presumptive "normal" cell line, WIL_2-A3; a non-virus-producing line of Burkitt's lymphoma cells, RAJI; and a virus-producing line of Burkitt's lymphoma, EB-3. We raised the question as to whether different degrees of viral presence would have any effect on the ability of the cell to repair equivalent extents of damage

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induced by UV irradiation, and, in addition, whether equivalent amounts of repair would occur after treatment of the different cell lines with an equivalent concentration of the alkylating agent methyl methanesulfonate.

In performing these studies, we undertook a series of preliminary experiments with UV light exposure and MMS treatment so as to insure that appropriate conditions were employed in undertaking the definitive comparative experiments.

Materials and Methods

Chemical. The chemicals employed in this study included: cesium chloride, optical grade (Schwarz/Mann, Orangeburg, N.Y.); cesium sulphate, 99.99+% (Column One, Inc., Ann Arbor, MI.); 5-bromodeoxyuridine, A grade (Calbiochem., San Diego, CA.); 5-fluorodeoxyuridine (Hoffmann-La Roche, Inc., Nutley, N.J.); hydroxyurea, A grade (Calbiochem.); dextran sulfate, analytical, (Nutritional Biochemicals Corporation, Cleveland, OH); methyl methanesulfonate (Eastman, Rochester, N.Y.); Ribonuclease A, phosphate free (Worthington Biochemical Corp., Freehold, N.J.); Pronase (Calbiochem); and *E. coli* DNA (Worthington Biochemical Corp.).

Radiochemicals. The radiochemicals employed included: thymidine [methyl-^{14}C], 54-57 mCi/mmole; thymidine [2-^{14}C], 45 mCi/mmole; 5-bromo-2'-deoxyuridine [6-^{3}H], 12-15 Ci/mmole in 50% EtOH, or 13-14 Ci/mmole in sterile aqueous solution; all from Schwarz/Mann, Orangeburg, N.Y.

Cell Line Maintenance. The three lymphoblastoid cell lines employed in this study were all maintained in Minimal Essential Medium (Earle's) with 10% fetal calf serum supplemented with sodium pyruvate (1 mM), L-glutamine (4 mM), twice the normal amount of nonessential amino acids, and the antibiotics penicillin-streptomycin-fungazone (Grand Island Biological Company, Grand Island, NY). The cells were maintained in 125 or 250 ml capped tissue culture flasks on a New Brunswick rotatory shaker in a 37°C warm room. The WIL_2-A3 cells were generously supplied by Drs. Frank Dixon and Richard Lerner of Scripps Institute (La Jolla). The RAJI cells and EB-3 Burkitt's lymphoma cells were obtained from the American Type Culture Collection.

Procedures for Cell Irradiation and Repair Replication Labeling. The repair replication protocol employed in the UV-irradiation studies is basically that previously described (4,5). Capped 250 ml Erlenmeyer flasks were mass cultured with cells to a concentration of approximately 700,000 cells per ml in 50 ml. The medium in the flasks was adjusted to 1 μM FUdR, 5 μM BrUdR, and 5 μg/ml dextran sulfate. The inclusion of dextran sulfate was found necessary to prevent extensive loss of WIL_2-A3 cells by attachment to glass and plastic during the PBS washing and UV-irradiation steps which followed. At the end of the 1-hour preincubation period, the cells in each flask were decanted into eight 50 ml Corning sterile conical tubes, and centrifuged at 800 rpm for 8 minutes at room temperature in an International PR-J centrifuge. The supernatant was aspirated, and the cells in each tube were resuspended with 5 ml of PBS-dextran sulfate (5 μg/ml). The cells from each two tubes were combined, and again centrifuged. The wash solution was aspirated, and the cells, at a maximum number of

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3.5×10^7 per tube, were resuspended in 8 ml of PBS-dextran sulfate. For each dose (0, 2, 5, and 10 seconds) 2 ml aliquots of the above 8 ml were distributed into 100 mm plastic Optilux petri dishes (Falcon) for irradiation. Immediately after irradiating with a specially built UV-irradiation device (6) at 9.4 ergs per mm^2 per second, exposing four dishes at each dose, the cells were transferred by pipet back into a centrifuge tube, the dishes were washed with a fresh 4 ml aliquot of PBS-dextran sulfate, and the cell suspension was centrifuged, aspirated, and resuspended in 10 ml of labeling medium containing 1 μM FUdR, 1 mM hydroxyurea, and 10 μCi/ml of ^{3}H-BrUdR (adjusted to 5 μM final BrUdR concentration). After 3 hours of repair replication labeling, the cells were centrifuged, washed with fresh BrUdR-FUdR medium containing fetal calf serum (but without dextran sulfate), and finally resuspended in BrUdR-FUdR medium for 1 hour additional incubation. At the end of this incubation time, the cells were centrifuged, the medium aspirated, and the cells frozen by immersion in ethanol-dry ice.

Exposure of Lymphoblastoid Cell Lines to Methyl Methanesulfonate and Repair Replication Labeling. The treatment and labeling protocols employed were basically those described above, with the exception that dextran-sulfate was not included in the experiment. The cells were preincubated for 1 hour with BrUdR-FUdR medium, centrifuged, and resuspended in repair replication labeling medium containing MMS at the desired concentration. The cells were incubated for 3 hours on the rotatory shaker. They were then washed one time with BrUdR-FUdR medium and again incubated in BrUdR-FUdR medium for 1 hour.

DNA Isolation Procedure. The procedure for DNA isolation has been described (5) and includes cell homogenization, RNase digestion, pronase digestion, and de-proteinization steps.

Density Gradient Centrifugation Procedures. For these experiments, the density gradient procedure initially used (4) was modified (7) to employ two alkali density gradient centrifugations in cesium chloride-cesium sulfate at room temperature for 40 hours at 42,000 rpm in a Beckman-Spinco Type 50 Ti Rotor in a Beckman L3-50 centrifuge. After centrifugation, the alkali gradients were fractionated by bottom collection using an ISCO fraction collector with drop counter. The tubes containing the pre-existing, non-density shifted, repair replicated DNA were pooled, and centrifuged a second time using the alkali density gradient centrifugation procedure. The appropriate fractions were again pooled, the alkali neutralized by addition of HCl and the cesium chloride-cesium sulfate solution diluted with water. The DNA was then pelleted by centrifugation at 42,000 rpm at 10°C in a Beckman L3-50 centrifuge. After removing the supernatant, the pellet was resuspended in 1/10 x SSC. The DNA concentration was determined using a spectrofluorometric technique (8). The radioactivity of the final solution was determined by counting 0.2 ml aliquots in Beckman Bio-Solv BBS-3 counting solution, and correcting to dpm by automatic computer correction using external standards.

Results

Cell Proliferation. The growth curves of the three human lymphoblastoid cell lines employed in this study, as determined by hemocytometer

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count, are shown in Figure 1. As maintained in our laboratory, the WIL_2-A3 "normal" lymphoblastoid cells and the RAJI line of Burkitt's lymphoma cells have similar population doubling times in the exponential phase of growth; the EB-3 line of Burkitt's lymphoma cells appears to have a longer population doubling time.

UV Irradiation Studies. Preliminary dose response studies (separate experiments) were performed for WIL_2-A3 and RAJI cells. The results are presented together in Figure 2. The extent of incorporated repair radioactivity with increasing dose appears to be reaching a maximal level after 94 ergs/mm^2 in the RAJI cells; such a plateauing of repair radioactivity incorporation is typical for mammalian cells. A similar plateauing is indicated but not reached at 94 ergs/mm^2 for the WIL_2-A3 cells.

In order to obtain definitive results for comparison of UV-induced repair between the different lymphoblastoid cell lines, we performed the experiment using cells from approximately the same region of the growth curves of the three cell lines, incubating them at equivalent cell concentrations with the same labeling medium at a dose expected to be in the plateau region (150 ergs/mm^2). The results of this experiment are presented in Table 1; the data presented are for the pooled fractions of the isolated DNA from the second gradients which are shown in Figure 3. In this experiment, the extent of incorporated repair replication radioactivity are equivalent for the WIL_2-A3 and RAJI cells; the extent of incorporated repair radioactivity appears to be less in the EB-3 cells.

Extent of DNA Repair Replication in Human Lymphoblastoid Cells After Methyl Methanesulfonate Treatment. In preliminary experiments, we found

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that maximal repair replication labeling during a 3-hour treatment-labeling interval was reached at an MMS concentration of 5mM for both WIL_2-A3 and RAJI cells (Figure 4) and also that, at this concentration, the amount of repair replication radioactivity incorporated using our standard protocol reached a plateau at 3 hours in all three lymphoblastoid cell lines (Figure 5).

In order to insure that comparative cell line studies would prove valid, we investigated the effect that the cell concentration during treatment would have on the extent of repair replication, and also the effect that taking cells initially in different portions of the rapidly growing region of the cell growth curve would have on the extent of repair replication (Figure 6). The results, summarized in Table 2, A and B, indicate that treatment at a lower cell concentration (6.25×10^5 cells/ml) might result in a 10% higher extent of repair then treatment at a higher cell concentration (2.5×10^6 cells/ml); and that variation (as great as 16%) can occur in measurements made when cells are taken from different parts of the growth curve. The cells from the central region of the curve appear optimal for performing comparative experiments.

Based on these observations, a comparative experiment was performed, wherein cells from a similar region of the growth curves of the three cell lines were treated at the same cell concentration with two different MMS concentrations, using the same treatment-labeling medium to treat each of the cell lines at each MMS concentration at the same time. This protocol takes into consideration the fact that the alkylating agent might not be

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present at any time after preparation with the same level of activity in different media preparations. It is known that alkylating agents can have a relatively short half-life in solution. The density gradient profiles for the three cell lines after 5 mM treatment appear in Figure 7. The results appearing in Table 3 indicate that upon treatment with 0.2 mM MMS, the least amount of repair occurs in the EB-3 cells, with that occurring in the RAJI cells 20% greater, and that in the "normal" WIL_2-A3 cells 56% greater. The 0.2 mM concentration is not in the plateau region of concentration dependence for WIL_2-A3 or RAJI cells (Figure 4).

At 5 mM MMS, in the plateau region for WIL_2-A3 and RAJI cells, the extent of repair replication is similar in both cell lines, and approximately 2.5 times greater than in EB-3 cells.

Conclusion. It would appear from these experiments that even while variations in measurement of incorporated repair replication radioactivity are introduced experimentally, a consistent pattern of lower extent of repair occurs in human Burkitt's lymphoma EB-3 cells, as compared to presumptive "normal" lymphoblastoid cells (WIL_2-A3) and another Burkitt's lymphoblastoid line, RAJI after equivalent UV and chemical treatments. The presumption of "normality" for a lymphoblastoid cell line which proliferates in culture may not be justified when making comparisons of repair replication. The decreased extents of repair occurring in the EB-3 cells may be correlated with the decreased growth rate of the EB-3 cells in culture.

TABLE 1

Repair Radioactivity in the DNA of WIL_2-A3, RAJI and EB-3 Lymphoblastoid Cells After Exposure to 16 sec UV Irradiation (9.4 ergs/mm^2/sec).

Cell Line	dpm/μg Isolated DNA
WIL_2-A3	5999
RAJI	5920
EB-3	4980

TABLE 2

A. Repair Replication Radioactivity Resulting Upon Treating Different Cell Concentrations at the Same MMS Concentration (5 mM).

Cell Concentration (Cells/ml)	dpm/μg Isolated DNA
2,500,000	605
1,250,000	630
625,000	666

B. Repair Replication Radioactivity Resulting Upon Treating the Same Cell Concentration, with Cells Taken from Different Points in the Growth Curve (5 mM MMS).

Cell Concentration in Culture (Cells/ml)	dpm/μg Isolated DNA
1,192,800	612
718,200	712
276,784	635

TABLE 3

Comparative Repair Radioactivity in WIL_2-A3 (Normal), RAJI (Non-Virus Producer), and EB-3 (Virus Producer) Lymphoblastoid Cells Upon Treatment with MMS.

MMS Concentration (mMolar)	dpm/μg Isolated DNA		
	WIL_2-A3	RAJI	EB-3
0.2	303	233	194
5	657	683	272

Figure 1. Growth curves of human lymphoblastoid cell lines maintained in tissue culture. Cell concentrations determined by hemocytometer count. (———) EB-3 line of Burkitt's lymphoma cells; (- - -) RAJI line of Burkitt's lymphoma cell; (-·-·) WIL_2-A3 normal lymphoblastoid cells.

Figure 2. Comparative repair replication radioactivity in WIL_2-A3 and RAJI lymphoblastoid cell DNA after different UV exposures. The two cell lines were exposed and repair-replication labelled using our standard repair replication protocol. Dose rate = 9.4 ergs/mm^2/sec.

Figure 3. Density gradient profiles obtained upon first and second alkali cesium chloride-cesium sulfate density gradient centrifugations of DNA isolated from WIL_2-A3, RAJI or EB-3 cells after exposure to 16 seconds of UV light and labelled with equivalent concentrations of ^{3}H-BrUdr. Upper left and upper right profiles: first and second gradients for WIL_2-A3 DNA. Middle gradient profiles: RAJI cell DNA. Lower gradient profiles: EB-3 cell DNA.

Figure 4. MMS concentration-dependence of incorporated repair replication radioactivity determined using standard repair replication labelling and gradient centrifugation procedures. Upper figure; response of WIL_2-A3 cells. Lower figure; response of RAJI line of Burkitt's lymphoma.

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Figure 5. Repair replication radioactivity in isolated DNA determined as a function of repair replication treatment-labelling time. Cells treated with 5 mM MMS in the presence of ^{3}H-BrUdR. Top figure; WIL_2-A3 cells. Middle figure; RAJI cells. Lower figure; EB-3 cells.

Figure 6. Schematic diagram showing theoretical growth curves of lymphoblastoid cells. Cells were taken from different stages of the growth curve and treated at the same cell concentration, or obtained from one stage of the growth curve and treated at different cell concentrations, to determine the effects of these variables on the extent of repair replication upon treatment with methyl methanesulfonate.

Figure 7. Density gradient profiles of DNA obtained after MMS-induced repair replication at a concentration of 5 mM. Upper left and upper right gradient profiles: first and second gradients for WIL_2-A3 DNA. Middle gradient profiles: RAJI cell DNA. Lower gradient profiles: EB-3 cell DNA.

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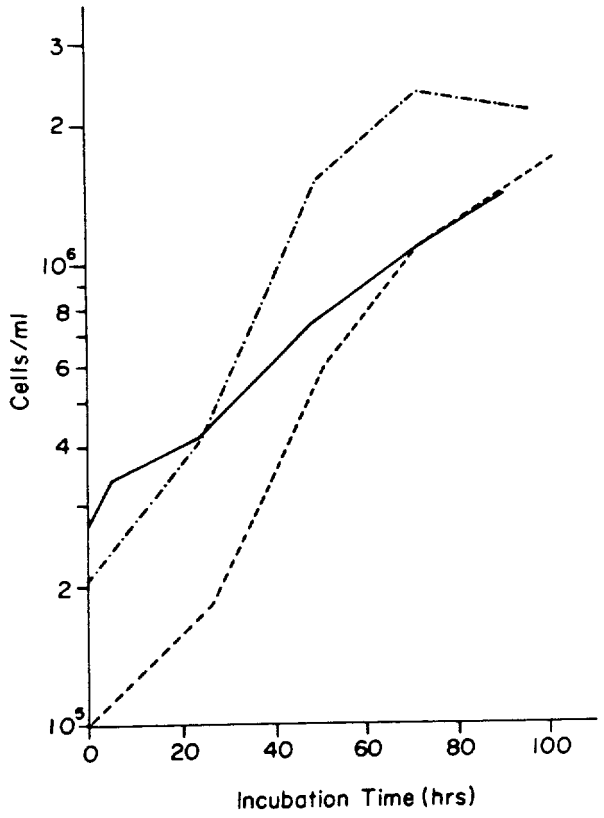

Figure 1

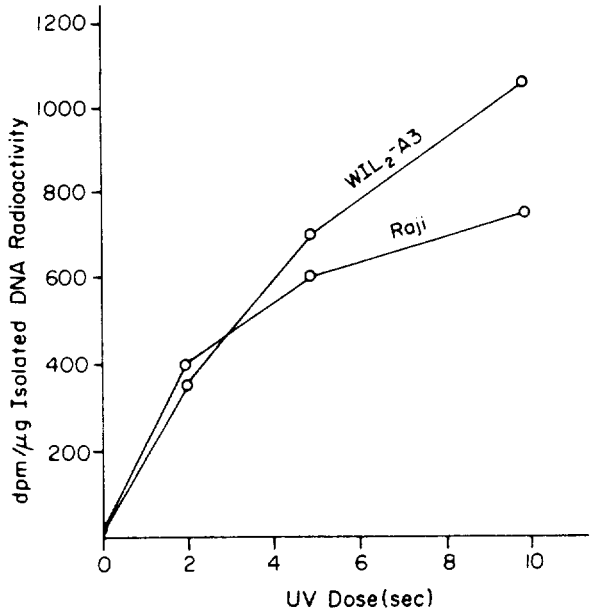
1200
1000
800
600
400
200
dpm/μg Isolated DNA Radioactivity
WIL2-A3
Raji
0
2
4
6
8
10
UV Dose(sec)

Figure 2

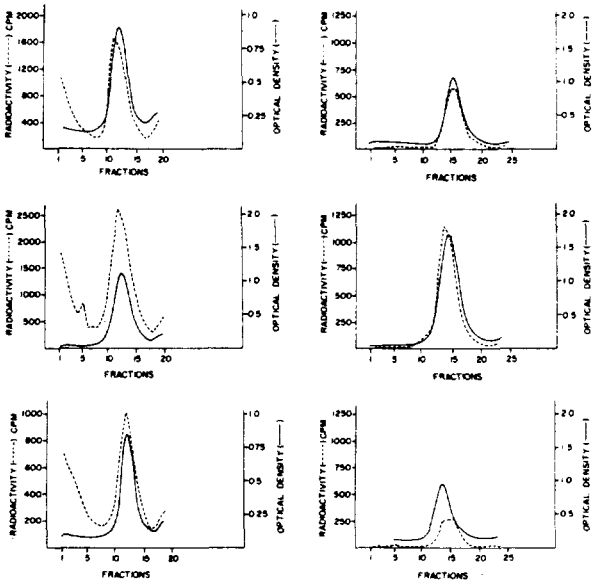

Figure 3

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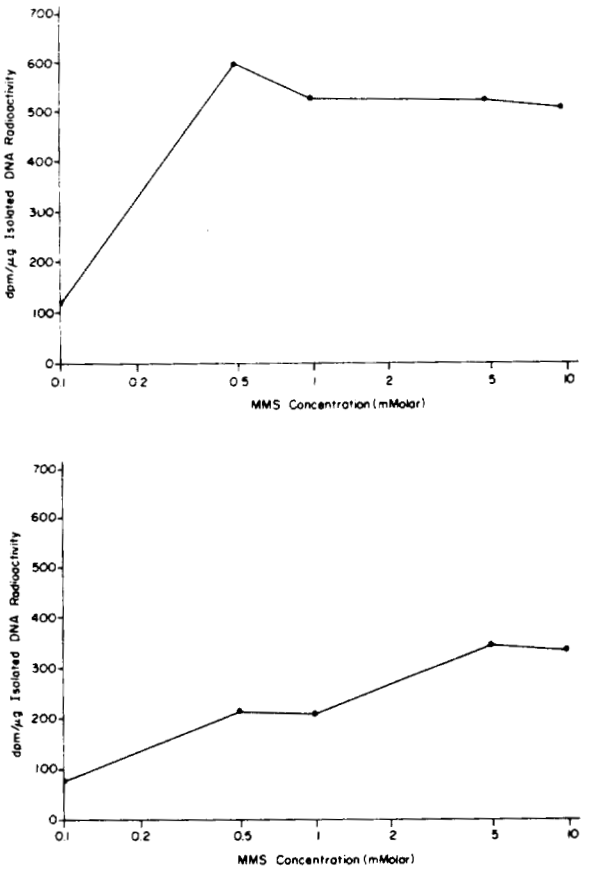
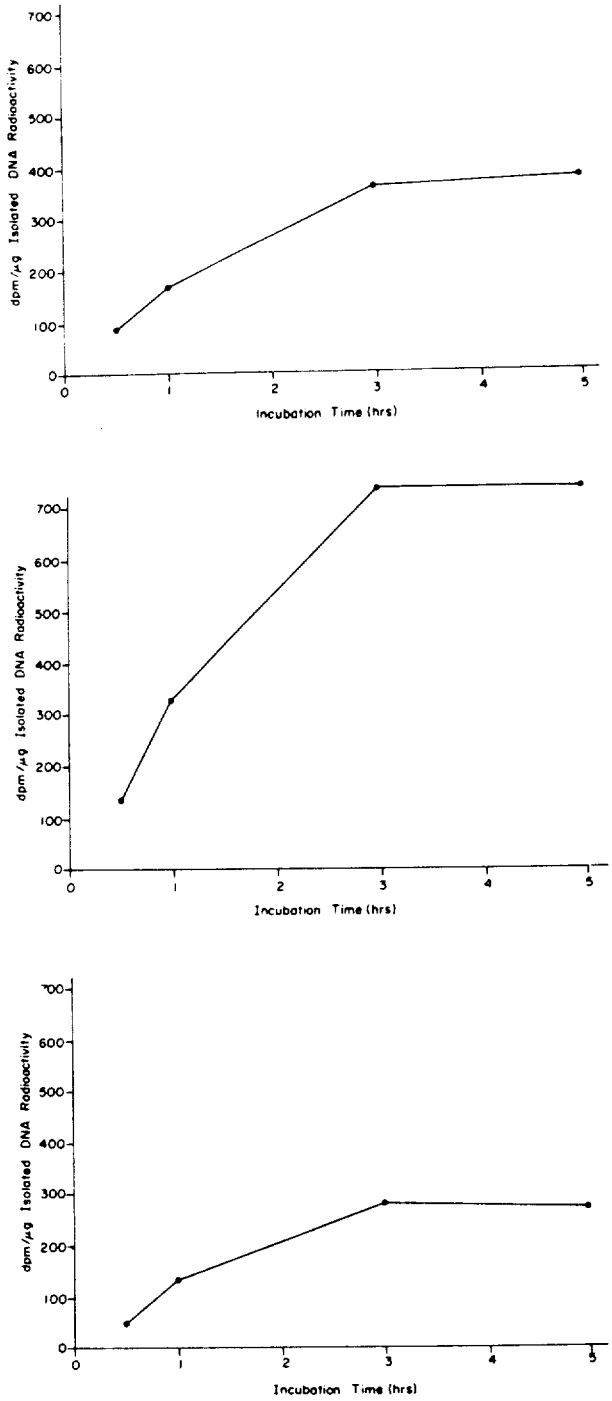

700
600
500
400
300
200
100
0
dpm/µg Isolated DNA Radioactivity
0.1
0.2
0.5
1
2
5
10
MMS Concentration (mMolar)
700
600
500
400
300
200
100
0
dpm/µg Isolated DNA Radioactivity
0.1
0.2
0.5
1
2
5
10
MMS Concentration (mMolar)

Figure 4


dpm/μg Isolated DNA Radioactivity
700
600
500
400
300
200
100
0
0
1
2
3
4
5
Incubation Time (hrs)
dpm/μg Isolated DNA Radioactivity
700
600
500
400
300
200
100
0
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1
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4
5
Incubation Time (hrs)
dpm/μg Isolated DNA Radioactivity
600
500
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200
100
0
0
1
2
3
4
5
Incubation Time (hrs)

Figure 5

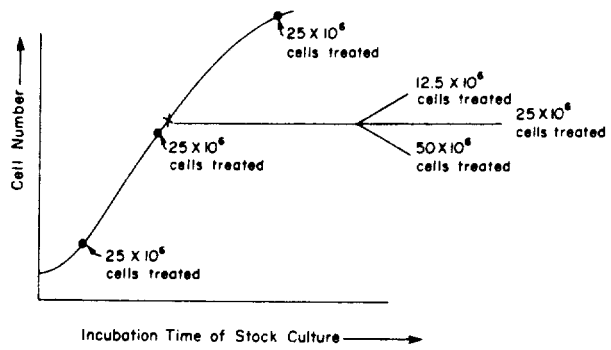
25 X 10⁶
cells treated
12.5 X 10⁶
cells treated
25 X 10⁶
cells treated
50 X 10⁶
cells treated
25 X 10⁶
cells treated
25 X 10⁶
cells treated
Cell Number
Incubation Time of Stock Culture

Figure 6

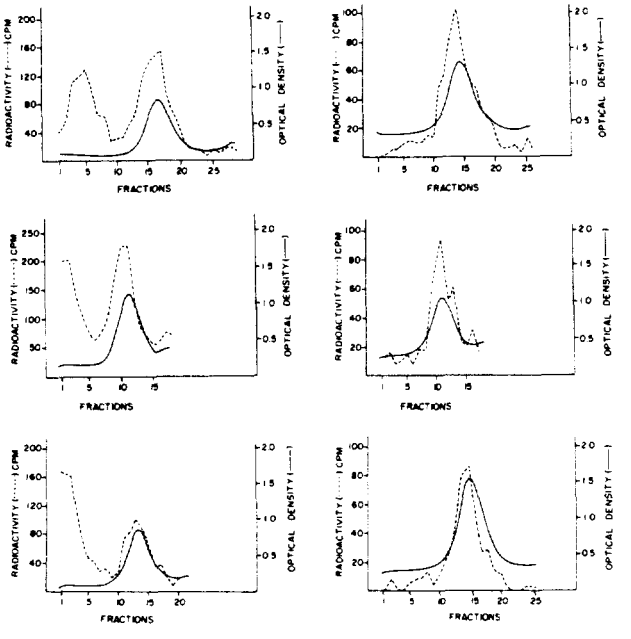

Figure 7

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Appendix II

1042007

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8/1/78

NON-UNIFORM REASSOCIATION OF HUMAN LYMPHOBLASTOID CELL DNA REPAIR REPLICATED FOLLOWING METHYL METHANESULFONATE TREATMENT

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Running Title:

Reassociation of Repaired DNA

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ABSTRACT

Meltz, M.L., Whittam, N.J., and Thornburg, W.H. Non-uniform Reassociation of Human Lymphoblastoid Cell DNA Repair Replicated Following Methyl Methanesulfonate Treatment. Radiation Res.

The reassociation rates of repair replicated DNA of human lymphoblastoid call lines have been examined using the DNA/DNA "Cot" technique. Previous studies have indicated that the repair replication occurring after ultraviolet light and a chemical carcinogen treatment of mammalian cells *in vitro* has been uniformly distributed throughout the genome.

Repair replication radioactivity in highly repetitive sequences of WIL_2-A3 cell DNA, whose incorporation was induced by methyl methanesulfonate treatment of the cells, reassociates as expected for a randomly distributed incorporation. However, the reassociation of repair radioactivity in sequences of fewer numbers of copies is less then expected for a random distribution, i.e. less than that occurring for semi-conservatively synthesized DNA of WIL_2-A3 cells co-incubated with the repair labeled DNA as an internal control.

This difference is not due to residual alkali label damage resulting from MMS treatment, since it was not observed when semi-conservatively labeled DNA from cells which had been treated with the mutagen MMS for the same time and at the same concentration as in the repair experiments was substituted for repair replicated DNA in the reassociation reactions.

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The observed difference could be due to an under representation of repair replication radioactivity in DNA sequences of lesser numbers of copies.

Key Words: DNA Repair, mutagen, reassociation, methyl methanesulfonate

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INTRODUCTION

The DNA/DNA "Cot" technique of Britten and Kohne (1) measures the rate of reassociation of isolated and denatured DNA in solution, and allows the characterization of DNA into classes based on this rate; those DNA molecules reassociating rapidly are designated highly repetitive DNA, those reassociating more slowly are called intermediate repetitive DNA, and those requiring extended incubations before reassociation occurs are designated unique DNA.

Since the initial damage of DNA caused by either UV light or chemical treatment may or may not be distributed proportionally (uniformly) in the three repetitive classes, and since the repair replication resulting after this damage occurs likewise may or may not be proportionally distributed in the three repetitive classes, the initial question which can be investigated is the nature of the distribution of the damage and its repair as reflected by the sequence distribution of the repair replication radioactivity in preexisting cell DNA. In previous studies, after ultraviolet light (2) and chemical carcinogen (3) induced repair, application of the Cot technique has indicated that this distribution is uniform.

Preliminary studies in our laboratory indicated that repair occurring during MMS treatment, in contrast to UV or N-acetoxy-2-acetylaminofluorene induced DNA repair, might show a non-uniform distribution; our investigation of this is described below.

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MATERIALS AND METHODS

Chemicals. The chemicals employed in this study included: hydroxylapatite Bio-Gel HTP (Bio-Rad Laboratories, Richmond, Ca.); ethylenediamine tetraacetic acid, disodium salt (as well as trisodium salt and free acid) (Sigma, St. Louis, Mo.); cesium chloride, optical grade (Schwarz/Mann, Orangeburg, N. Y.); cesium sulphate, 99.99+% (Column One, Inc., Ann Arbor, Mi.); 5-bromodeoxyuridine, A grade (Calbiochem., San Diego, Ca.); 5-fluorodeoxyuridine (Hoffmann-La Roche, Inc., Nutley, N. J.); hydroxyurea, A grade (Calbiochem.); methyl methanesulfonate (Eastman, Rochester, N. Y.); Ribonuclease A, phosphate free (Worthington Biochemical Corp., Freehold, N. J.); Pronase (Calbiochem.); and *E. coli* DNA (Worthington Biochemical Corp.).

Radiochemicals. The radiochemicals employed included: thymidine [methyl-^{14}C], 54-57 mCi/mmole; thymidine [2-^{14}C], 45 mCi/mmole; 5-bromo-2'-deoxyuridine [6-^{3}H], 13-14 Ci/mmole in sterile aqueous solution; all from Schwarz/Mann, Orangeburg, N. Y.

Cell Line Maintenance. The WIL_2-A3 human lymphoblastoid cell line employed in this study was maintained in Minimal Essential Medium (Earle's) with 10% fetal calf serum supplemented with sodium pyruvate (1 mM), L-glutamine (4 mM), twice the normal amount of nonessential amino acids, and the antibiotics penicillin-streptomycin-fungizone (Grand Island Biological Company, Grand Island, N. Y.). The cells were maintained in 125 or 250 ml capped tissue culture flasks on a New Brunswick rotatory shaker in a 37°C warm room. The WIL_2-A3 cells were generously supplied by Drs. Frank Dixon and Richard Lerner of Scripps Institute (La Jolla). The RAJI amd EB-3 lines of Burkitt's lymphoma, similarly miantained, were obtained from the American Type Culture Collection.

Procedures for Treatment and Repair Replication Labelling. The repair replication protocol employed is basically that previously described (2,4). Capped 250 ml Erlenmeyer flasks were mass cultured with cells to a concentration of approximately 700,000 cells per ml in 50 ml. The medium in the flasks was adjusted to 1 μM 5-fluorodeoxyuridine (FUdR) and 5 μM 5-bromodeoxyuridine (BrUdR) and incubated for 1 hr. At the end of this one-hour pre-incubation period, the cells were transferred into sterile conical centrifuge tubes (Corning), and centrifuged at 800 rpm for 8 minutes at room temperature in an International PR-J centrifuge. The supernatant was aspirated, and the cells were resuspended in 10 ml of repair replication labelling medium containing 5 mM methyl methanesulfonate (MMS), 1 μM FUdR, 1 mM hydroxyurea and 50 μCi/ml of ^{3}H-BrUdR (13-14 Ci/mmole in sterile aqueous solution). After 3 hours of treatment-labelling, the cells were centrifuged, washed by centrifugation with fresh medium containing BrUdR (5 μM) and FUdR (1 μM) and finally resuspended in this BrUdR-FUdR medium for 1 hour additional incubation. At the end of this incubation time, the cells were centrifuged, the medium aspirated, and the cells frozen by immersion in ethanol-dry ice.

Ultraviolet Light Irradiation Procedure. For the ultraviolet light irradiation experiment with EB-3 cells, the repair replication protocol described above was modified as follows. During the 1 hour pre-incubation with BrUdR and FUdR, 5 μg/ml of dextran sulfate was present. At the end of the 1 hour preincubation, the cells were washed two times by centrifugation with phosphate buffered saline containing 5 μg/ml dextran sulfate, and finally resuspended in the PBS-dextran sulfate. The dextran sulfate was present to reduce lymphoblastoid cell attachment to the plastic surfaces during the procedures. The suspended cells in 2 ml volumes were distributed into 100 mm plastic Optilux petri dishes (Falcon), and irradiation

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was performed in the specially built UV irradiation device (5). After irradiation, the cells were transferred into sterile tubes, centrifuged, and the PBS aspirated. The cells were then resuspended in repair replication labelling medium and subsequently treated as in the standard protocol.

DNA Isolation Procedure. The procedure for DNA isolation has been described (4) and includes cell homogenization, RNase digestion, pronase digestion, and de-proteinization steps.

Density Gradient Centrifugation Procedures. For these experiments, the density gradient procedure initially used (2) was modified (6) to employ two alkali density gradient centrifugations in cesium chloride-cesium sulfate at room temperature for 40 hours at 42,000 rpm in a Beckman-Spinco Type 50 Ti Rotor in a Beckman L3-50 centrifuge. After centrifugation, the alkali gradients were fractionated by bottom collection using an ISCO fraction collector with drop counter. The tubes containing the pre-existing, non-density shifted, repair replicated DNA were pooled, and centrifuged a second time using the alkali density gradient centrifugation procedure. The appropriate fractions were again pooled, pelleted, and resuspended as described below. The DNA concentration was determined using a spectrofluorometric technique (7). The radioactivity of the final solution was determined by counting 0.2 ml aliquots in Beckman Bio-Solv BBS-3 counting solution, and correcting to dpm by automatic computer correction using external standards.

Procedures for Kinetic Reactions. After the final cesium chloride-cesium sulfate alkali gradient centrifugation, the appropriate fractions were pooled,

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the alkali neutralized by addition of HCl, and the cesium chloride-cesium sulfate solution diluted with water. The DNA was then pelleted by centrifugation at 42,000 rpm at 10°C in a Beckman L3-50 centrifuge. After slowly removing the supernatant by pipet, the pellet was resuspended in 1/10 SSC. The radioactivity and concentration were determined as described above. An appropriate concentration of repair replicated DNA was mixed with an appropriate concentration of C^{14}-thymidine labelled, semi-conservatively synthesized WIL_2-A3 DNA (24-hour label). The latter was isolated as described above and further purified by one neutral pH cesium chloride density gradient centrifugation. For low Cots, non-radioactive E. coli DNA was added; for high Cots, non-radioactive WIL_2-A3 DNA was added. The subsequent steps for the kinetic reaction have been described (8).

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RESULTS

Typical profiles of first and second alkali cesium chloride-cesium sulfate density gradients of DNA isolated from WIL_2-A3 cells treated as described above with 5 mM MMS and repair replication labeled are shown in Figure 1. We have found in a series of preliminary experiments that the amount of repair radioactivity incorporated in both WIL_2-A3 and RAJI cells upon MMS treatment during a 3 hour treatment-labeling interval is maximal at a concentration of 5 mM. Also, the repair radioactivity incorporated in WIL_2-A3 cells during treatment with MMS at 5 mM reaches a near maximal level by 3 hours (unpublished observations). Experimental designs which allow recovery of a maximal repair replication radioactivity concentration (dpm/μg) were essential for satisfactory performance of the low Cot kinetic reactions.

Our initial kinetic studies, performed in the low to intermediate Cot range (6.2×10^{-4} - 1.4×10^{1}) with repair replicated DNA from 5 mM MMS treated WIL_2-A3 and RAJI cells appear in Figure 2. The lines represent the average of the reassociation values for samples incubated in duplicate. There appears to be an increasing divergence of the two curves with increasing Cot in this range; the reassociation of repair replication radioactivity appears to be less at each Cot point then radioactivity incorporated during semi-conservative synthesis.

These results can be compared to those of a kinetic experiment with DNA from ultraviolet light (UV) irradiated (16 sec of 9.4 ergs/mm^2/sec) EB-3 Burkitt's lymphoma cells which were repair replication labeled as described above for 3 hours immediately post-irradiation (Figure 3). There is no evidence after UV irradiation of delayed reassociation of the repair replication radioactivity

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in the low to intermediate Cot range (1.5×10^{-4} - 3×10^{0}), as compared to semi-conservatively synthesized DNA.

To further confirm and repeat the initial observations, an additional treatment-labeling experiment was performed with MMS treatment (5 mM) of WIL_2-A3 cells, and the kinetic reaction of the DNA with repair replication radioactivity performed from low to high Cot values (6.1×10^{-4} - 7.3×10^{3}), which includes the reassociation range of unique DNA sequences. As can be seen in Figure 4, there appears to be an increasing difference in reassociation of repair replication radioactivity with increasing Cot, the greatest different (17%) being at the highest Cot (7.25×10^{3}).

In establishing the validity of this difference, we were concerned with several parts of the protocol which might generate an artifactual result. We first explored the effect of two alkali cesium chloride-cesium sulfate gradients on the subsequent kinetic reaction of ^{3}H-thymidine labeled, semi-conservatively synthesized DNA, as compared to ^{14}C-thymidine labeled DNA which was also semi-conservatively synthesized, but subjected only to one neutral cesium chloride gradient. The results of the kinetic experiment (Figure 5) show differences in the two curves at individual Cot values, but the dispersive trend obvious in the MMS experiments is not apparent. We were further concerned that treatment with 5 mM MMS for 3 hours might result in extensive alkali or heat labile damage which could produce artifactual effects when the treated cell DNA was centrifuged in alkali gradients or incubated for long times at 60°C. We therefore prelabeled DNA with ^{3}H-thymidine by semi-conservative synthesis (21 hours incubation), and then treated the cells with 5 mM MMS for 3 hours. The DNA was then isolated, density gradient centrifuged two times in alkali cesium chloride-cesium sulfate,

and reacted according to the standard protocol. The results, which are shown in Figure 6, again do not indicate the increasing delay in reassociation observed in the MMS-repair replication experiments.

DISCUSSION

These experiments were undertaken to determine whether chemically caused DNA damage and the repair replication occurring in mammalian cells in response to that damage occurred randomly in DNA sequences of all repetitious frequencies, or whether a particular class(es) of sequences was either more susceptible to damage and/or more proficient (or deficient) in its repair. While such kinetic studies of repair replicated DNA after UV irradiation (2) and N-acetoxy-2-acetylaminofluorene treatment (3) of mammalian cells have shown uniform distribution, we have evidence in these experiments of a reduction in reassociation at intermediate to high Cots when single standed DNA sequences contain repair replication radioactivity, the incorporation of which was induced by treatment with a high concentration (5mM) of the mutagen MMS.

One possible explanation of these results is that disproportionately fewer sequences of lower repetition frequency have undergone repair; this in turn could have been caused by less extensive damage; these two phenomena are non-distinguishable by this technique. An alternative explanation could be that the repair sequences reassociate at the same rate, but the reassociated DNA is less stable during the subsequent fractionation of single stranded DNA from double stranded DNA. A necessary condition for reassociation of DNA is that the sequence of the bases in the repaired region in the DNA available for reassociation be "complementary enough" to allow suitable hydrogen bonding of the appropriate number of sequential bases during the kinetic reaction such that stable reassociation will occur. Further studies are necessary to determine the stability of duplex DNA containing MMS induced repair sequences.

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If we assume that the first explanation is correct, we must be concerned with any attempts to extrapolate to lower MMS concentrations, where such results would be more meaningful in terms of genetic alterations in mammalian cells. In the L5178Y heterozygote system (TK +/-) described by Clive (9) a 2 hour treatment of those cells in 10% serum with 0.5 mM MMS resulted in a surviving cell population at 48 hours after treatment of only 0.15%; the survival would have been less if the colony assay had been performed immediately after treatment (D. Clive, personal communication). We have found upon treating these L5178Y cells with 5 mM MMS in medium with 10% serum, and examining DNA single strand breakage using the alkaline elution technique of Kohn and co-workers (10,11), that massive single strand breakage is present immediately after treatment (unpublished observation). When we treat these cells for 2 hours with 1 mM MMS in medium with 3% serum, not only do we find extensive DNA damage, but the cell concentration of washed and reincubated cells continues to decrease over the 72 hour interval post-treatment (unpublished observation).

Our conclusion therefore, is that the treatment of a "normal" human lymphoblastoid cell line with a high concentration of an alkylating agent which is a known mutagen, methyl methanesulfonate, can cause a significant abnormal repair resulting in delayed kinetic reassociation of the DNA containing these repaired regions. Considering the extensive damage to DNA which results upon treating mammalian cells with MMS at high concentrations and the small differences in reassociation kinetics that we ultimately see, it is possible that the technique employed for these studies would not be sensitive enough to confirm such alterations at lower and genetically more interesting concentrations of this mutagen.

ACKNOWLEDGEMENT

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FIGURE LEGENDS

Figure 1. Density gradient profiles (alkaline cesium chloride-cesium sulfate) of DNA obtained from WIL_2-A3 cells repair replication labelled in the presence of 5 mM MMS. Upper figure: 1st gradient; lower figure: 2nd gradient.

Figure 2. Kinetic reassociation ("Cot") studies performed in the low to intermediate Cot range with repair replicated DNA from 5 mM MMS treated WIL_2-A3 cells (upper figure) and RAJI cells (lower figure). (□———□) DNA repair replication labelled with ^{3}H-BrUdR; (○- - - -○) DNA semi-conservatively labelled with ^{14}C-thymidine. Open ○ & □ average of replicate incubations; closed ● & ■, actual data points.

Figure 3. Kinetic reassociation study performed in the low to intermediate Cot range with repair replicated DNA from UV-irradiated EB-3 cells exposed to 16 sec of UV at 9.4 ergs/mm^2/sec. (□———□) DNA repair replication labelled with ^{3}H-BrUdR; (○- - - -○) DNA semi-conservatively labelled with ^{14}C-thymidine. Open ○ & □, average of replicate incubations; closed ● & ■, actual data points.

Figure 4. Kenetic reassociation study performed over the low to high Cot range with repair replicated DNA from 5 mM MMS treated WIL_2-A3 cells. (□———□) DNA repair replication labelled with ^{3}H-BrUdR; (○- - -○) DNA semi-conservatively labelled with ^{14}C-thymidine. Open ○ & □, average of replicate incubations; closed ● & ■, actual data points.

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Figure 5. Kinetic reassociation study performed with either ^{3}H-thymidine labelled (□———□) or ^{14}C-thymidine labelled (○- - - -○) semi-conservatively synthesized DNA. The former was subjected to two alkali cesium chloride-cesium sulfate density gradient centrifugations. Open ○ & □, average of replicate incubations; closed ● & ■, actual data points. ✕ indicates that data came from incubations with high concentration of WIL_2-A3 DNA, as compared to the points at similar Cot values from incubations with low concentrations of WIL_2-A3 DNA (overlap region).

Figure 6. Kinetic reassociation study performed with DNA of WIL_2-A3 cells which was semi-conservatively labelled for 21 hr with ^{3}H-thymidine, followed by a 3 hr treatment with 5 mM MMS. (□———□) ^{3}H-thymidine labelled and MMS treated DNA; (○- - - -○), DNA semi-conservatively labelled with ^{14}C-thymidine. Open ○ & □, average of replicate incubations; closed ● & ■, actual data points. ✕ indicates that data came from incubations with high concentration of WIL_2-A3 DNA, as compared to the points at similar Cot values from incubations with low concentrations of WIL_2-A3 DNA (overlap region).

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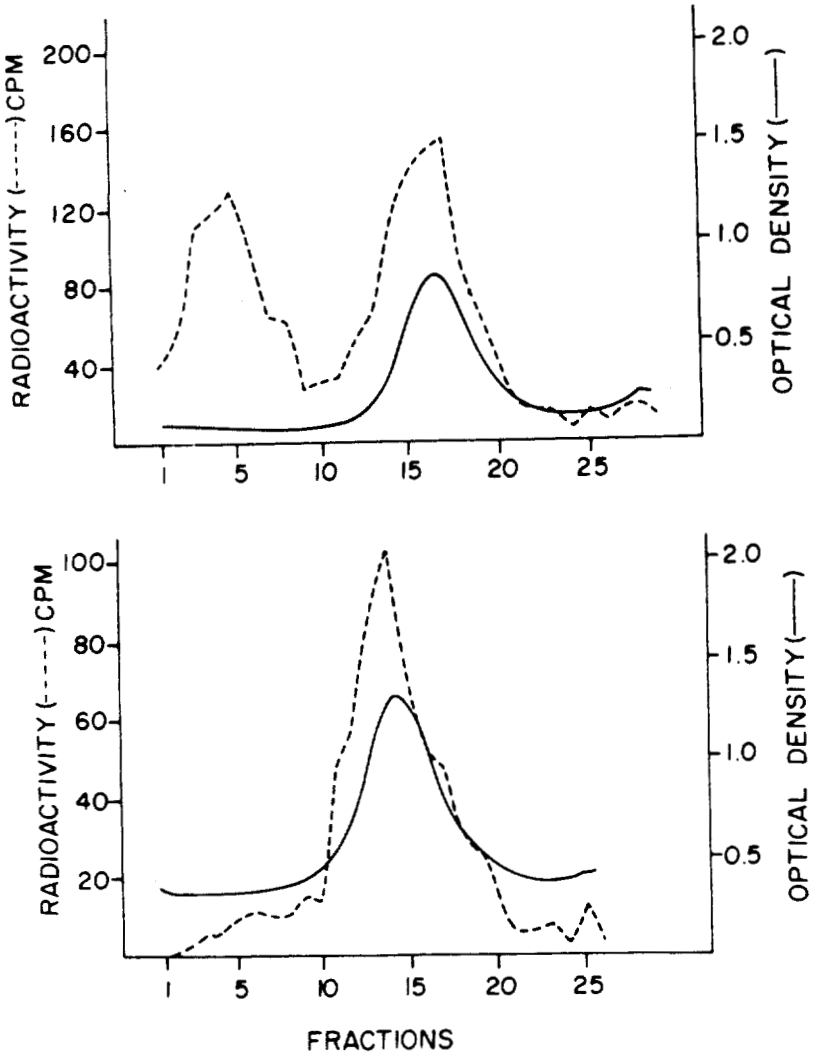
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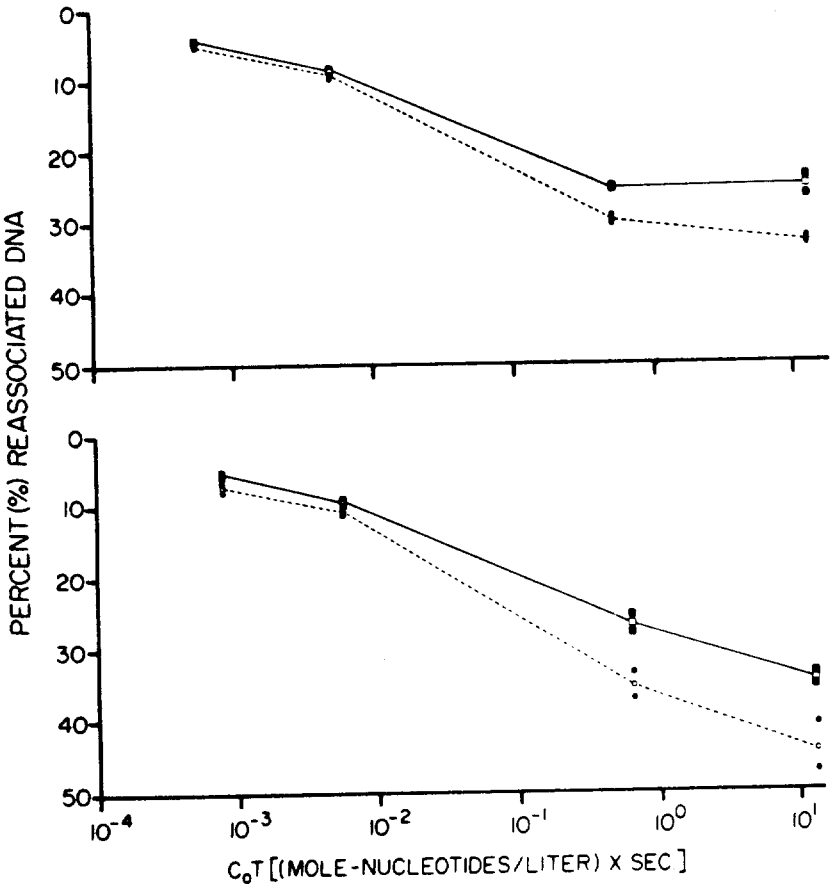
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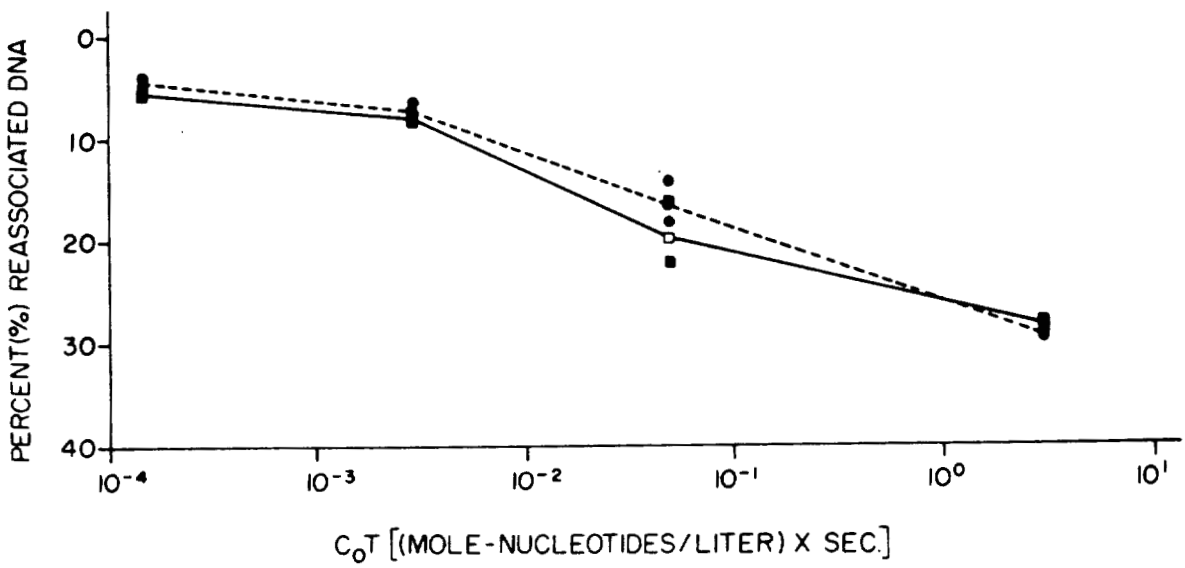
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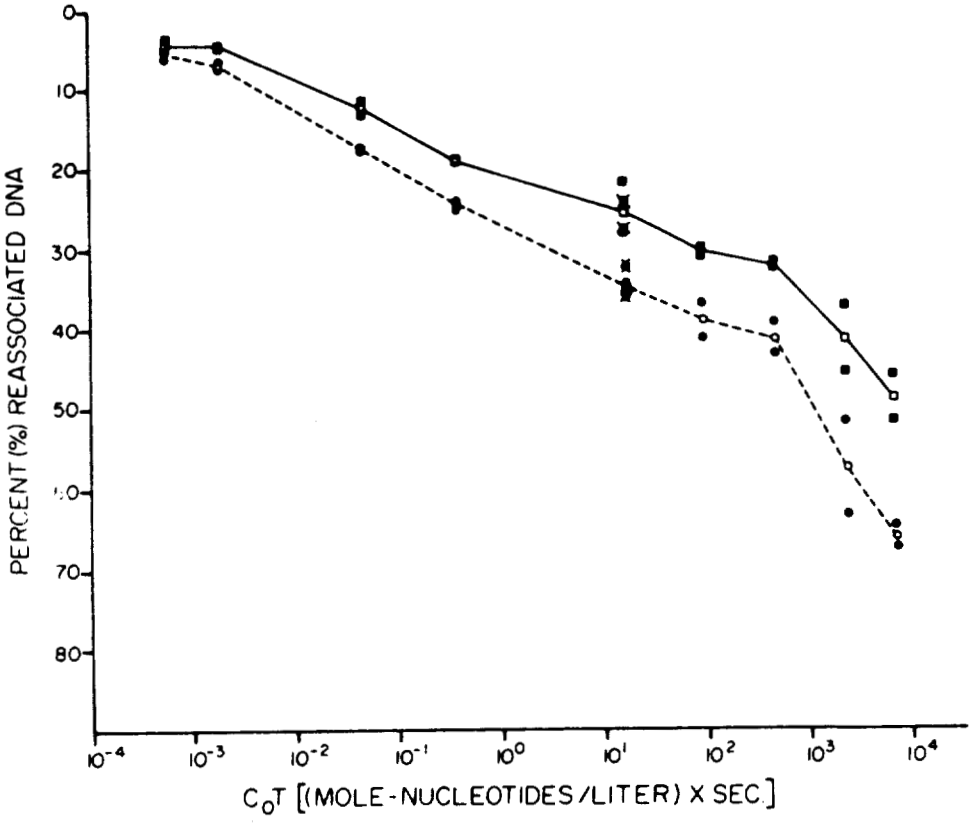
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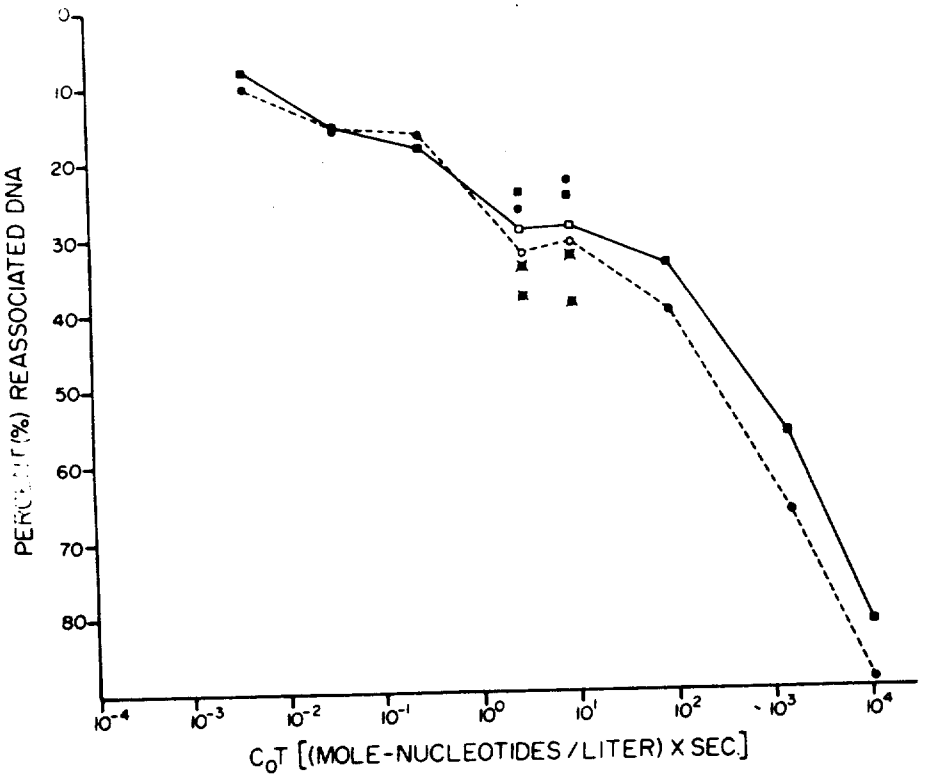
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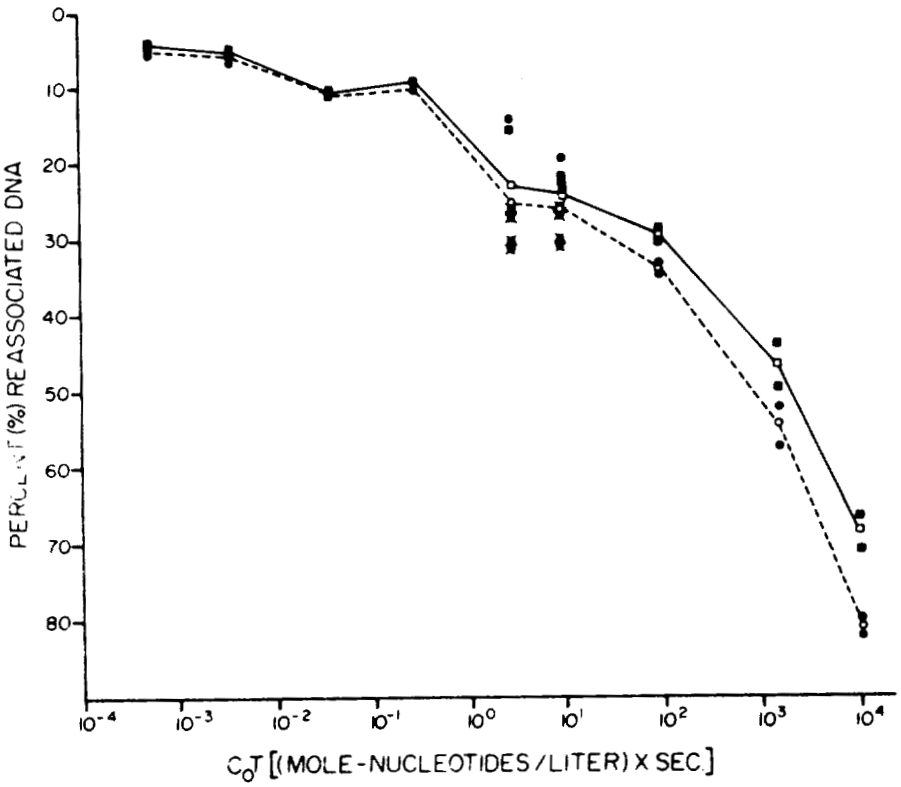
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C_0T [(MOLE-NUCLEOTIDES/LITER) X SEC.]

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Appendix III

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RANDOM DISTRIBUTION OF HIGHLY REPETITIVE AND INTERMEDIATE FREQUENCY MOUSE L-929 CELL DNA SEQUENCES SYNTHESIZED AFTER UV LIGHT EXPOSURE

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Abstract—The DNA precursor ^{3}H-thymidine incorporation rate (dpm/μg DNA) in mouse L-929 cells decreases immediately after exposure to UV light. This decrease is initially dose dependent, but at exposures greater than 22.5 J/m^2 appears to be radio-resistant. This was not explained by measurements of uptake of ^{3}H-thymidine into the acid soluble pool (dpm/μg DNA). The radio-resistant incorporation amounted to approximately 35% of the control rate. DNA reassociation ("C_0T") studies were performed with DNA labeled with ^{3}H-thymidine immediately after exposure of L-929 cells with a dose of UV resulting in radio-resistant incorporation to determine whether this radio-resistant incorporation was occurring in sequences of a particular repetitious frequency. These studies, performed in the highly repetitive to intermediate range, showed that the radio-resistant incorporation was occurring in DNA of all classes of repetitious frequency. DNA synthesized at different times after UV exposure, during the period when post-replication repair can occur, was similarly labeled for short intervals and isolated. The DNA reassociation studies showed that this DNA synthesis was also of all classes of repetitious frequency.

INTRODUCTION

The inhibition of DNA synthesis immediately after UV irradiation has been reported in several mammalian cell lines (Rasmussen and Painter, 1964; Cleaver, 1965; Klimek and Vlasinova, 1966; Cleaver, 1967; Djordjevic and Tolmach, 1967; Domon and Rauth, 1968; Meyn *et al.*, 1976; Edenberg, 1976). A characteristic phenomenon is the apparent presence of a component of tritiated thymidine incorporation which is radio-resistant; this component in mouse cells typically comprises approximately 35% of the control rate for short labeling intervals. On the basis of DNA reassociation ("C_0T") studies, Britten and Kohne (1968) have shown that mouse and other eukaryotic DNAs consist of unique, intermediate, and repetitive DNA sequences. Two possibilities were considered: that the radio-resistant incorporation was occurring uniformly in DNA sequences of all repetitious frequency classes or, alternatively, that the incorporation was partially or totally nonuniform, i.e. that it represented a continuation of synthesis of sequences of a given frequency, with synthesis of the remainder being partially or totally inhibited.

After having determined the extent of uptake and incorporation of DNA precursor, DNA reassociation studies were performed to determine the repetitious frequency of the radio-resistant DNA. Similar experiments were performed with DNA synthesized at different times after UV irradiation, i.e. during a period of time when post-replication repair can occur (Lehmann, 1972; Fujiwara, 1972), to determine whether this later synthesis was of a uniform distribution in terms of its sequence frequency. The extent of uptake of the DNA precursor tritiated thymidine (^{3}H-Tdr), its extent of incorporation during a 30 min labeling interval, and the repetitious distribution of the DNA incorporating the precursor, are described below.

MATERIALS AND METHODS

Materials. The mouse L-929 cells employed in these studies were obtained from the American Type Culture Collection. The cells were maintained in Earle's Minimal Essential Medium with 10% fetal calf serum, and supplemented with nonessential amino acids and additional L-glutamine. The antibiotics penicillin and streptomycin and the antimycotic fungizone were also present in the medium. All materials were obtained from Grand Island Biological Company, Grand Island, NY. The cells were maintained either in Falcon or Corning T-30 or T-75 sterile plastic tissue culture flasks containing 10 mℓ or 20 mℓ of medium, respectively. Flasks were gassed with 5% CO_2, 95% air, and maintained in a 37°C incubator. For UV irradiation experiments, an appropriate number of L-929 cells were distributed into Falcon 100 mm sterile plastic petri dishes, and the cells were incubated at 37°C in a Forma humidified CO_2 incubator at 5% CO_2.

Methods. In the two initial studies of uptake and incorporation after UV irradiation, the experimental protocol was as follows. Rapidly proliferating and nonconfluent cells in petri dishes were removed from the CO_2 incubator, washed two times with warm sterile pucks saline A, aspirated, and then immediately exposed to UV light (0.94 J/m^2/s) using a specially built UV light irradiation device described by Steier and Cleaver (1969). The dose rate was measured at the exposed surface using a cali-

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brated Blak-ray J-225 shortwave UV intensity meter. Immediately after irradiation, the cells were reincubated in the CO_2 incubator with warm complete medium containing 10 μCi/mℓ of tritiated thymidine (50.8 Ci/mmole, methyl-^{3}H; New England Nuclear) adjusted to a final molarity of 5 μM thymidine. After incubation at 37°C for 30 min, the labeling medium was aspirated from the dishes, and the cells were washed two times with ice cold SSC (standard saline citrate: 0.15 M NaCl, 0.015 M sodium citrate). The cells were then scraped free using a rubber policeman into ice cold SSC, collected by centrifugation, and quick-frozen by immersion in ethanol–dry ice after aspiration.

For analysis of uptake and incorporation in these experiments, a modified Fleck and Munro (1962) and Schmidt and Thannhauser (1945) procedure was employed. In this procedure, the cell pellet is resuspended in 0.8 mℓ of ice cold SSC, to which is added 0.2 mℓ of 50% TCA. After mixing, the tube is immersed in an ice bath and allowed to stand for 30 min. The tube is then centrifuged for 15 min at 2500 rpm at 4°C in an International PR-J refrigerated centrifuge. The supernatant containing the acid soluble radioactivity is carefully removed to a tared tube, and the pellet is resuspended using a small volume of fresh ice cold 10% TCA. The tube is again centrifuged, and the supernatant is added to the initial acid soluble fraction. The pellet is then sequentially washed with ice cold 95% ethanol, ethanol:ether (3:1), and ether, and after aspiration, the residual ether is allowed to evaporate at room temperature. A small volume of 0.3 N KOH is added to each tube and the pellet resuspended. The tubes are then incubated at 37°C for 1 h to digest the ribonucleic acid. At the end of 1 h, the tubes are immediately chilled in an ice bath, the alkali is neutralized by addition of the appropriate volume of 1.0 N HCl, and the contents of the tubes are again adjusted to 10% TCA by addition of the appropriate volume of 50% TCA. The tubes are allowed to stand in ice for 15 min. After centrifugation, the ribonucleotides are removed, the pellet is washed with a small volume of ice cold 10% TCA, the latter is aspirated and then 0.8 mℓ of fresh ice cold 10% TCA is added to the pellet. After resuspending the pellet in the TCA, the DNA is hydrolyzed in a boiling water bath for 15 min. After removal of the tube from the water bath, and allowing for 1 min cooling at room temperature, the tube is immersed in ice for 15 min. The tube is then centrifuged and the supernatant containing the deoxyribonucleotides is transferred to a tared tube. The hydrolysis is repeated, and the second hydrolyzate is added to the first. A volume of 0.1 mℓ of the acid soluble and DNA nucleotide fractions are counted for radioactivity determination, and the DNA concentration is determined using the diphenylamine procedure described by Burton (1956). The incorporated radioactivity is reported as dpm/μg of total DNA recovered. The radioactivity in the acid soluble fraction, after correction to total acid soluble radioactivity recovered and total DNA recovered, is also reported as dpm/μg DNA.

For the kinetic studies requiring intact isolated DNA, the procedure for DNA isolation described by Meltz (1976) was employed. The isolated DNA was additionally purified by cesium chloride density gradient centrifugation (Flamm *et al.*, 1966) for 36 h at room temperature in a Beckman Spinco type 50 Ti rotor at 36,000 rpm in a Beckman 13-50 centrifuge. The gradients were collected by top collection using an Isco density gradient fractionator. Aliquots of each fraction were precipitated onto filters for radioactivity determination. The fractions containing the DNA peak were pooled based on both optical density and radioactivity determinations, which coincided. The appropriate fractions were pooled, and the cesium chloride solution was diluted with water. The DNA was then pelleted by centrifugation for 20 h at 42,000 rpm at 10°C in a Beckman L3-50 centrifuge. After slowly removing the supernatant by pipette, the pellet was resuspended in 1/10 SSC. The radioactivity of the final solution was determined by counting 0.2 mℓ aliquots in Beckman Bio-Solv BBS-3 counting solution, and correcting to disintegrations per minute by automatic computer correction using external standards. The DNA concentration was determined using a spectrofluorometric ("DABA") technique (Hinegardner, 1971).

For the DNA/DNA C_0T studies, an appropriate concentration of DNA synthesized after irradiation (or control) and labeled with ^{3}H-TdR was mixed with an appropriate concentration of ^{14}C-thymidine labeled, semi-conservatively synthesized L-929 DNA (24 h label) and nonradioactive *E. coli* DNA. The combined DNA was then subjected to a series of steps, including: (a) addition of glycerol and shearing using a VirTis "60" homogenizer at 50,000 rpm for 30 min in ice, (b) precipitation with ethyl alcohol in the presence of sodium acetate, (c) pelleting of the DNA at 10,000 rpm for 20 min at 4°C in a Sorvall SS-34 rotor, (d) resuspension of the DNA in phosphate buffered-EDTA (0.12 M phosphate buffer, 2 mM EDTA), (e) measurement of DNA concentration by radioactive counts, (f) denaturation of the samples in capillary tubes or test tubes at the appropriate concentrations by boiling in hot water, (g) incubation of the samples for the appropriate times at 60°C, (h) freezing of the samples in acetone–dry ice until analysis, (i) fractionation of the samples on hydroxylapatite in columns to separate single-stranded from double-stranded DNA, and (j) radioactive counting of the fractions in BBS-3 liquid scintillation solution. In performing these studies, we essentially followed the procedures described by Britten *et al.* (1974). All C_0T reactions were performed in duplicate, and the value reported in the figures is the average.

For the kinetic studies, the UV irradiation, labeling, and cell recovery steps were as described above, with the exception that the cells were labeled with high specific activity ^{3}H-thymidine at 10 μCi/mℓ without adjustment of the exogenous pool size by addition of nonradioactive thymidine.

RESULTS

The results of the two dose response experiments appear in Fig. 1. In both experiments, the presence of a UV radio-resistant region, similar to that previously reported, is apparent. It appeared from the first experiment that the UV resistant incorporation might be explained as the result of a decrease in rate of synthesis, with the same level of radioactivity being incorporated because of an increased amount of radioactivity in the acid soluble pool. On repeating the experiment, however, while the same radio-resistant region appeared, the acid soluble radioactivity did not show a similar increase in radioactivity. The issue remains unresolved, but it is not likely that the amount of radioactivity in the acid soluble pool is the responsible factor for the apparent residual resistant synthesis. A similar conclusion was reached by Cleaver (1965). For observing the sequence distribution of the ^{3}H-TdR labeled DNA synthesized after irradiation, exposure times were chosen which would provide labeled DNA from cells that had not been irradiated (0 s), DNA which was labeled after an exposure which was halfway down the dose response curve to the resistant level (12 s), and an exposure which would result in labeling of the radio-resistant fraction (30 s). The specific activity of the isolated DNA from two such experiments appears in Table 1. Four dishes were exposed at each dose in each

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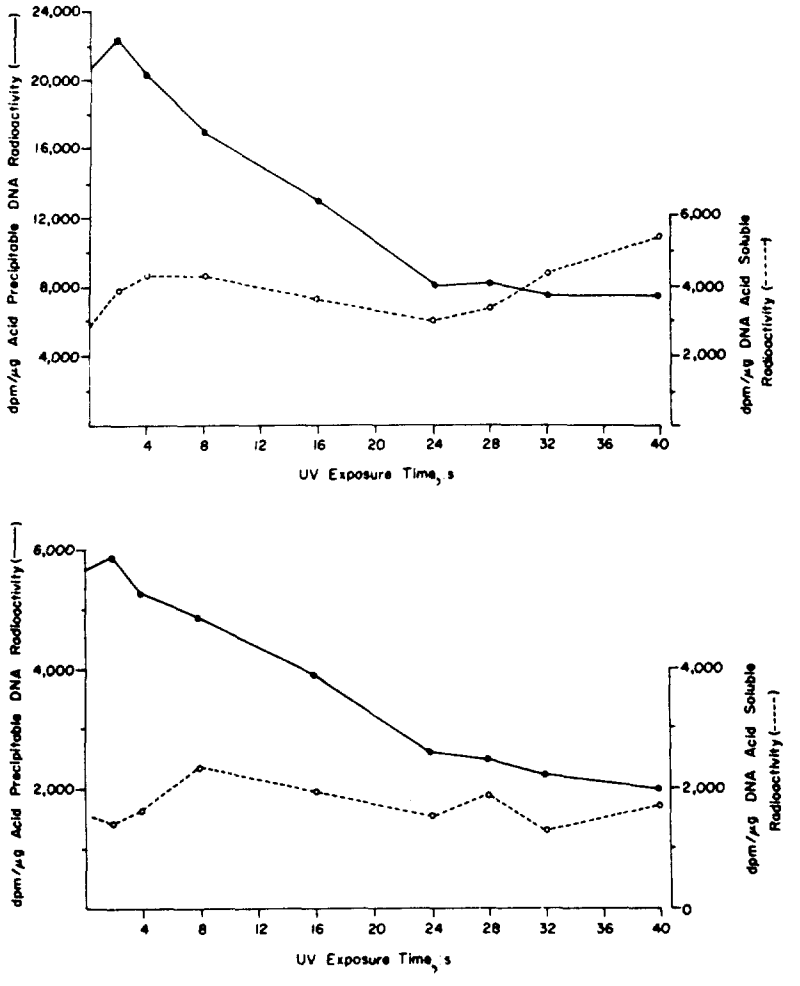

Figure 1. Uptake of ^{3}H-thymidine into the acid soluble pool and incorporation of ^{3}H-thymidine into acid-precipitable DNA in L-929 cells during a 30 min pulse label after different UV-exposures. UV dose rate = 0.94 J/m^2/s. Upper figure; first experiment; lower figure; second experiment. In each experiment, one dish was exposed at each dose. The diphenylamine reaction was performed in duplicate and the results averaged for DNA concentration determination; the radioactivity concentration was the average of counts of duplicate samples.

experiment and the cells pooled. The isolated DNA concentration in each case was determined in duplicate using the DABA procedure. The radioactivity concentration was the average of duplicate samples. In performing the kinetic experiments, nonirradiated, 24 h ^{14}C-thymidine labeled, semi-conservatively synthesized DNA was included in each reaction mixture as an internal control. The experiments were performed over the low to intermediate C_0T range. As can be seen in Fig. 2 for both of the experiments, our results indicate that the UV radio-resistant incorporation is composed of DNA which is at least both highly and intermediately repetitive in frequency; unique DNA must therefore also have been synthesized.

Experiments were also performed to determine the extent of the incorporation and the repetitious distribution of DNA incorporating ^{3}H-TdR radioactivity at different times after UV irradiation. This is during the period of time when post-replication repair can occur (Lehmann, 1972; Fujiwara, 1972). For these experiments, the labeling medium contained 10 μCi/mℓ of high specific activity tritiated thymidine (52.6 Ci/mmole). Rapidly proliferating cells in petri dishes were rinsed, exposed to UV at 0.94 J/m^2/s for 30 s, and then either immediately labeled for 20 min

Table 1. Specific activity (dpm/μg) of DNA isolated from L-929 mouse fibroblast cells, labeled after different UV exposures

UV exposure time (s)	Experiment I dpm/μg	Experiment I % of Control	Experiment 2 dpm/μg	Experiment 2 % of Control
0	42,991		54,561	
12	26,995	62.8	31,928	58.4
30	16,036	37.3	18,387	33.6

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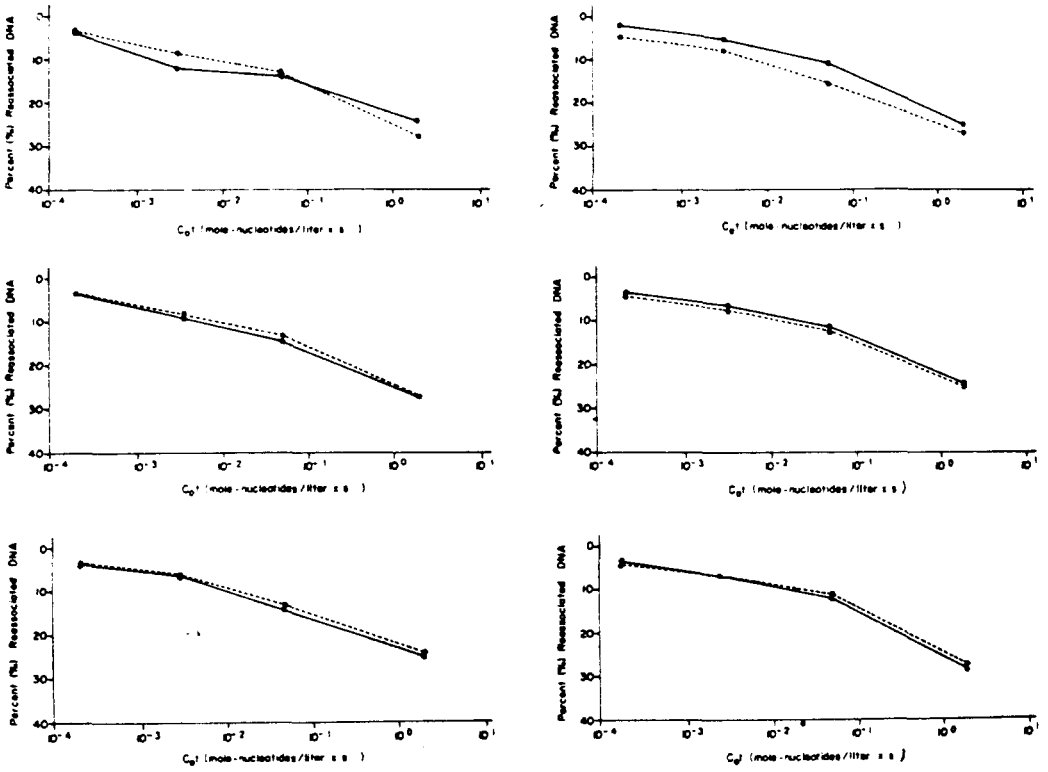

Figure 2. Kinetic data obtained upon reassociation incubation of labeled DNA from UV-irradiated mouse L-929 cells after 30 min post-UV incubation with ^{3}H-thymidine at 10 μCi/mℓ: (——) 30 min ^{3}H-thymidine pulse labeled DNA; (-----) 24 h semi-conservatively synthesized ^{14}C-thymidine labeled mouse L-929 DNA. Upper left to lower left figures, reassociation data isolated in Experiment 1 after 0 s, 12 s, or 30 s UV exposure; upper right to lower right, reassociation data for DNA isolated in Experiment 2 after 0 s, 12 s, or 30 s UV exposure. Each data point indicated is the average after incubation of duplicate samples. The results of the two experiments are presented independently since different C_0T values were employed in each experiment to obtain the resulting curves.

or reincubated for 1, 2, or 3 h in fresh warm medium before addition of the labeling medium for 20 min. The cells were collected as described above. The DNA was isolated and its concentration determined in duplicate using the DABA procedure. The specific activities obtained as a function of post-irradiation time in h appear in Fig. 3. A decreasing incorporation rate of precursor with increasing time post-irradiation was observed. The sequence distribution of DNA which was pulse-labeled at 0, 2, or 3 h post-30-s UV exposure appears in Fig. 4. The distribution of the radioactivity appears to be uniform in the sequences of different repetitious frequency.

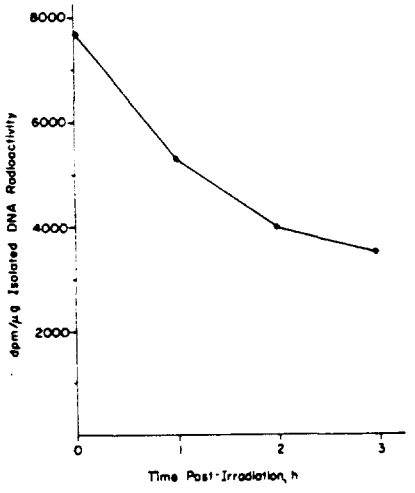

Figure 3. Specific activity in dpm/μg of DNA isolated from mouse L-929 cells which were pulse-labeled for 20 min with ^{3}H-thymidine beginning at different times after a 30 s UV exposure. Four dishes were exposed and incubated for each post-irradiation incubation time and the cells pooled. The isolated DNA concentration was determined for each sample in duplicate using the DABA procedure. The radioactivity concentration was the average of counts of duplicate samples.

DISCUSSION

We have examined UV "radio-resistant" DNA and also DNA synthesized at various times after UV light exposure to determine whether the incorporation occurring was in DNA representative of the total mouse L-929 cell genome, or in DNA sequences of a particular repetitious frequency. This was accomplished by mixing ^{14}C-thymidine labeled DNA which was semi-conservatively synthesized in nonirradiated proliferating cells with DNA from UV irradiated and

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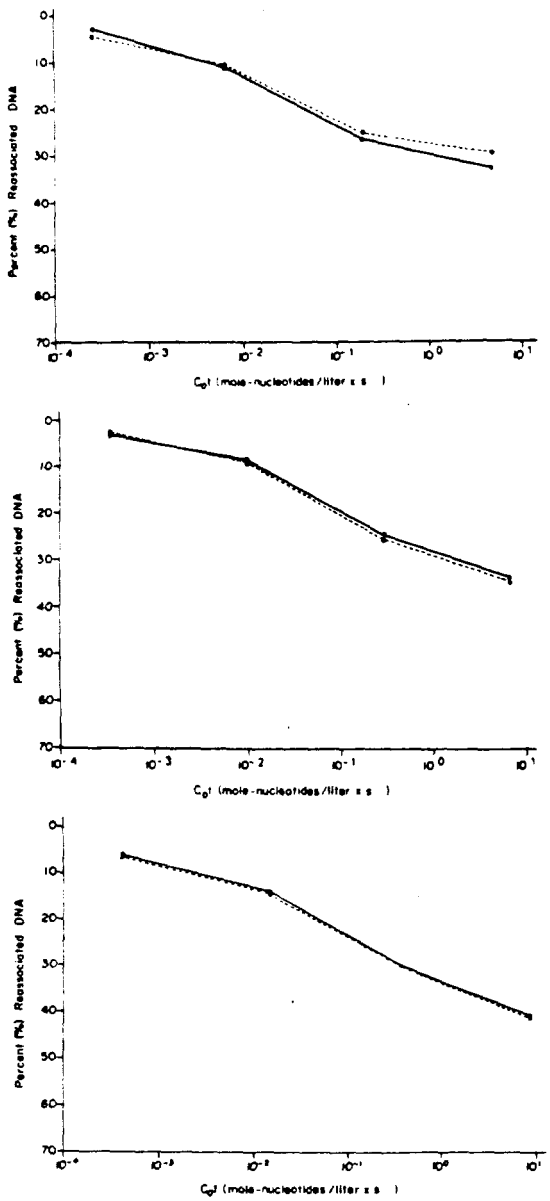

Figure 4. Kinetic data obtained upon reassociation incubation of DNA pulse-labeled at 0, 2, or 3 h post 30 s UV exposure. Upper curve: DNA pulse-labeled immediately after UV irradiation; middle curve: DNA pulse-labeled beginning 2 h post exposure; lower curve: DNA pulse-labeled beginning 3 h post exposure. Each data point indicated is the average incubation of duplicate samples.

^{3}H-thymidine pulse labeled cells, and then performing DNA DNA "C_0T" kinetic studies. If the sequences of a particular frequency were greatly enriched with the ^{3}H-thymidine, its reassociation would differ from the ^{14}C-thymidine labeled nonirradiated cell DNA, and would result in a C_0T curve corresponding more closely to that which would result if that particular class of DNA were separately isolated and then kinetically reacted (see fig. 13 in Britten and Smith, 1970; Davidson *et al.*, 1971). By the technique employed, the reassociation behavior of ^{3}H- and ^{14}C-labeled DNAs were indistinguishable from one another and resembled that of DNA from other mouse cell lines (Britten and Rake, 1967).

The proportion of DNA of the mouse genome reassociating at a C_0T of 10^0 is approximately 30% (Britten and Rake, 1967). The possibility that radioresistant DNA as well as that synthesized at different times after UV exposure is greatly enriched in the highly repetitive or other classes of DNA has been ruled out since a uniform distribution was observed in all frequency classes examined. It is interesting to note that repair replication, which contributes at least some portion of the incorporated radioactivity in these experiments, has also been shown to be uniformly distributed in the genome of mammalian cells (Meltz and Painter, 1973; Lieberman and Poirier, 1974). We have attempted in these experiments to employ UV irradiation as an inhibitor of synthesis of DNA of one class of repetitious frequency; our results show that such is not the case, and the question therefore remains as to whether any inhibitor can selectively inhibit DNA synthesis on a class frequency basis.

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Appendix IV

Preliminary DNA Single-Strand Breakage Studies in the L5178Y Cell Line

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Standard Protocol for Elution Only

The filter holder we have designed for these experiments is first filled through the outside inlet with ice-cold PBS. This is allowed to start dripping through the outlet tubing. When the PBS is moving freely, the tubing is clamped, and 10^6 cells in ice-cold PBS are layered on the PBS remaining in the syringe barrel; the free-flow is then allowed to continue into a tared flask. The syringe barrel is washed with two 8 ml aliquots of ice-cold PBS, which is also collected in tared flasks. As stated by Dr. Kohn (personal communication), it is necessary for the work to be done in a dark room to prevent photo-induced degradation of DNA. The washed cells are then lysed at room temperature with 5 ml of 0.2% Sarkosyl (Ciba-Geigy Corp., Ardsley, N.Y.), 2 M NaCl, 0.02 M Na_3EDTA, pH 10. This solution is allowed to flow freely into a new tared vial. The cells are then washed with 3 ml of 0.02 M Na_3EDTA, pH 10, which is collected in the same vial. After this wash, the outlet tubing is connected to a peristaltic pump. The alkaline eluting solution contains 0.02 M H_4EDTA and enough tetrapropylammonium hydroxide to make the solution pH 12.1. The filters are eluted with a constant pump speed of between 0.03 and 0.04 ml/min. Fractions are collected every 90 min in scintillation vials. At the end of the total collection time (21 hr), the speed of the pump is increased to quickly collect the remaining eluting solution from the filter holders. The filters are then removed from the filter holders and placed in scintillation counting vials. A volume of 0.4 ml of 1 N HCl is added to each filter-containing vial and the remaining DNA is hydrolyzed at 70°C for

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1 hr. After allowing the vials to cool to room temperature, 2.5 ml of 0.4 N NaOH is added and allowed to stand for 30 min. A volume of 12 ml of Aquasol (New England Nuclear Corp.) containing 7.5 ml acetic acid/l is then added to each vial. The filter holder is subsequently washed with 3 ml of 0.4 N NaOH three times, with the washings collected in three separate scintillation vials. The vials are then counted in a liquid scintillation counter with automatic computer correction to dpm, using external standardization.

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Summary of Experiments

Experiment 1

Comparison of the Alkaline Elution Curves for Radiolabeled DNA from Untreated L5178Y Cells which were Eluted from Two Different Filters.

Objective

The objective of this experiment was to determine the reproducibility of the alkaline elution procedure using two different filters for the elution of the same DNA.

Procedure

L5178Y cells were labeled for 18.5 hours with 0.5 μCi/ml ^{3}H-TdR. No chase was employed in this experiment. Immediately following the labeling, the cells were washed with cold PBS and subjected to the standard alkaline elution procedure; 1.0×10^{6} cells were placed on each of two filters.

Results and Conclusions

See Figure 1. The two filter holders gave reproducible results, with the percentage of the total dpm which remained on the filter at any time point never varying by more than 1% between the two filters.

Experiment 2

Alkaline Elution of DNA from L5178Y Cells Exposed to Co^{60} Radiation.

Objective

The objective of this experiment was to investigate the effect of dose of Co^{60} radiation on DNA single-strand breaks.

Procedure

The cells were incubated for 19.5 hours in medium containing 0.5 μCi/ml ^{3}H-thymidine. No chase was employed in this early experiment. After removing the radioactive medium, the cells were resuspended in 20 ml of fresh medium and chilled on ice. Cells (1.6×10^{6}) were distributed into specially prepared 15 ml screw-capped test tubes with a pellet of glass filling the round bottom. The tubes were placed on ice for transportation to and from the hot lab at the Southwest Research Institute.

The cells were irradiated for 0, 1, or 3 minutes at 130 rads/minute. After the cells were centrifuged, washed and resuspended in cold PBS, they were subjected to the alkaline elution procedure, with $4.5 - 5.5 \times 10^{5}$ cells placed on each filter.

Results and Conclusions

See Figure 2. The control shows a higher elution rate of the labeled DNA than is common for the controls of later experiments in which the cells

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were labeled with 0.05 μCi/ml ^{14}C-thymidine, and chased for longer times prior to treatment. With increasing dose there is a greater initial elution rate, with the 390 rad exposure being obviously different from the lower dose, which was also readily distinguishable from control.

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Experiment 3

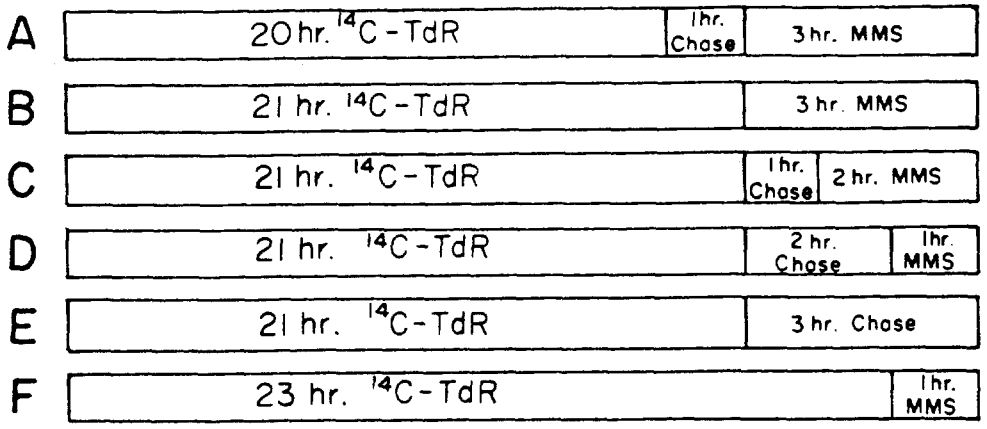
Alkaline Elution of DNA from L5178Y Cells Exposed to 1 mM Methyl Methanesulfonate (MMS) for Different Times. Effect of Chase after Labeling of DNA.

Objective

The objectives of this experiment were to compare the extent of DNA single-strand breakage occurring upon exposure of L5178Y cells to 1 mM MMS for 1 or for 3 hours, and to determine whether a chase in non-radioactive medium has any effect on the elution curve.

Procedure

L5178Y cells were labeled in 0.05 μCi/ml ^{14}C-TdR for 20 to 23 hours. After 20 hours of labeling, one sample of the cells was chased for 1 hour in non-radioactive medium, and then MMS was added to the cells to a final concentration of 1 mM MMS and incubated for 3 hours (A). After 21 hours of labeling, four samples of cells were resuspended in non-radioactive medium and incubated for 0, 1, or 2 hours, when they were made 1 mM MMS (B-D). The fourth sample was untreated (E). All four samples were incubated for a total of 3 hours of chase and treatment. After 23 hours of labeling, one sample of cells was resuspended in 1 mM MMS and incubated for 1 hour (F). At the end of the incubation time, all the cells were washed with cold PBS and subjected to the standard alkaline elution procedure.



Results and Conclusions

See Figure 3 and Figure 4. Whether the cells were treated with MMS for 1 hour or for 3 hours, those that were treated immediately following the labeling had a slower initial rate of elution than those which were treated after either a 1 hour chase in non-radioactive medium (3 hours of MMS treatment) or a 2-hour chase (1 hour of MMS treatment). Elution at later times of the labeled, chased, and treated cell DNA always lagged behind the non-chased treated cell DNA. At the 1 mM concentration tested, which at 1 hour has a high initial rate of elution, the 3-hour treatment did not result in an elution pattern readily distinguishable from the 1 hour pattern.

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Experiment 4

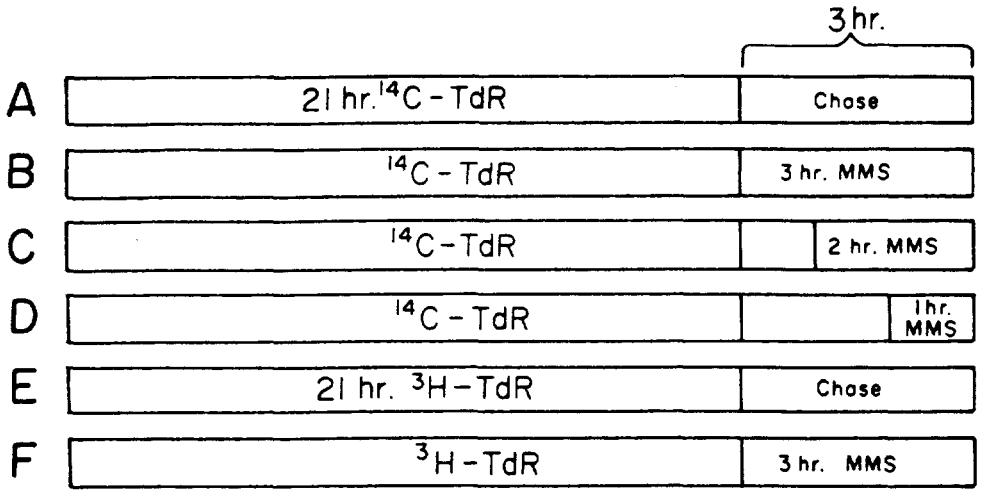
Alkaline Elution of DNA from L5178Y Cells Exposed to 5 mM Methyl Methanesulfonate (MMS) for Different Treatment Times: Comparison of Results when DNA is Labeled with ^{3}H- as Compared to ^{14}C-Thymidine.

Objective

The objectives of this experiment were to determine the effect of the time of exposure of cells to MMS (5 mM) on DNA single strand breakage, and also to compare the results of the alkaline elution when using ^{3}H-thymidine vs. ^{14}C-thymidine to label the cells. It was hypothesized that the ^{14}C-labeled cells might show less breakage than the ^{3}H-labeled cells.

Procedure

Two flasks of cells were labeled, one with 0.1 μCi/ml ^{3}H-TdR and one with 0.1 μCi ^{14}C-TdR, for 21 hours. At the end of the label, 5 ml aliquots of cells were centrifuged and resuspended in 5 ml warm medium in tubes. One tube of ^{14}C-labeled cells (A) and one of ^{3}H-labeled cells (E) were incubated for 3 hours in warm medium as controls. The other tubes were incubated also for a total time of 3 hours, with MMS (50 λ of 500 mM) being added to the ^{14}C-labeled cells at 0, 1 or 2 hours (B-D) after the beginning of the incubation and to the ^{3}H-labeled cells immediately after the beginning of the incubation (F). At the end of the 3 hours incubation, the cells were centrifuged, washed once with PBS, and subjected to the standard alkaline elution procedure.



Results

See Figure 5. At the specific activity employed (0.1 μCi/ml), the ^{3}H-thymidine control (E) showed a much slower elution rate than the ^{14}C-thymidine control. Both show a faster elution rate than consistently obtained in later experiments where the cells are labeled with ^{14}C-thymidine at both lower and higher specific activities (0.05 - 0.5 μCi/ml ^{14}C-thymidine), but where the chase time is increased prior to mutagen treatment.

The results show concentration dependence for 1- and 2-hour treatments at this concentration, while there is a distinct difference in the shape of the 3-hour curve as compared to the 1-hour and 2-hour curves. This different shape appears for both ^{14}C-labeled and ^{3}H-labeled cells.

Experiment 5

The Effect of the Concentration of H_4EDTA in the Eluting Solution on the Alkaline Elution of L5178Y DNA.

Objective

The objective of this experiment was to determine how the alkaline elution of DNA would be affected by lowering the concentration of H_4EDTA in the eluting solution from the previously used 0.04 M to 0.02 M.

Procedure

The alkaline solution used for eluting the DNA on the filters is prepared by weighing out the proper amount of H_4EDTA and adding to it enough tetrapropylammonium hydroxide to bring the pH of the solution to 12.1.

Rapidly proliferating L5178Y cells were labeled for 20 hours with 0.5 μCi/ml ^{3}H-TdR. The cells were washed immediately following the labeling with cold PBS and subjected to the standard alkaline eluting procedure, using 0.04 M H_4EDTA-tetrapropylammonium hydroxide on one filter and 0.02 M on the other.

Results and Conclusions

See Figure 6. Both molarities of eluting solution resulted in approximately the same rate of elution of DNA radioactivity during the first 7.5 hours of elution; after that time, the 0.04 M H_4EDTA solution caused slightly more rapid elution as compared to the 0.02 M H_4EDTA solution. Since the 0.02 M H_4EDTA solution resulted in a more slowly eluting curve for non-treated cells, and since the 0.02 M H_4EDTA solution would be less expensive, we have switched to the lower molarity solution for our experiments.

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Experiment 6

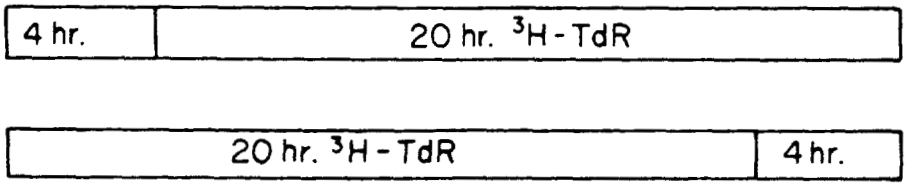
The Effect of a 4-Hour Chase after Labeling on the Alkaline Elution of DNA from L5178Y Cells.

Objective

The objective of the experiment was to determine whether chasing the cells in non-radioactive medium for 4 hours after a ^{3}H-TdR label would affect the elution of the DNA.

Procedure

L5178Y cells were resuspended in two glass culture tubes, one with 5 ml of non-radioactive medium, and the other with 5 ml of medium containing 0.5 μCi/ml ^{3}H-TdR. After 4 hours, the cells which were in fresh media were centrifuged, and resuspended in 5 ml of radioactivity containing medium. When the first set of cells had been labeled for 20 hours, they were centrifuged and resuspended in fresh, warm media for 4 hours. After the total 24 hours of incubation, both sets of cells were washed and subjected to the standard alkaline elution procedure.



Results and Conclusions

See Figure 7. During the first 9 hours of elution, the cells which had been chased for 4 hours showed no difference in rate of elution from the cells which had not been chased. The rate of elution of the DNA from the 4-hour chased cells did, however, became slower as compared to the non-chased cells between 9 and 21 hours.

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Experiment 7

The Effect of Chase Time (1 - 3 Hours) Following Radioactive Labeling on the Alkaline Elution of L5178Y DNA from Untreated Cells.

Objective

The objective of this experiment was to determine the effect which a 1-, 2-, or 3-hour chase following radioactive labeling has on the alkaline elution rate of DNA from untreated L5178Y cells.

Procedure

Rapidly proliferating L5178Y cells were labeled in 0.05 μCi/ml ^{14}C-TdR for 24 hours. At the end of the labeling period, the cells were centrifuged and resuspended in warm, fresh, non-radioactive medium and incubated for an additional 0, 1, 2, or 3 hours. After this chase period, the cells were washed with cold PBS and subjected to the standard alkaline elution procedure.

Results and Conclusions

See Figure 8. The rate of elution of the radioactivity is observed to decrease with increasing chase time. Based on this and the preceding experiment, the chase time was increased in later experiments to overnight incubations.

Experiment 8

The Effect of Specific Activity of the ^{14}C-TdR with Which the L5178Y Cells were Labeled on the Elution of the DNA of Untreated Cells.

Objective

The objective of this experiment was to determine whether the specific activity of the ^{14}C-TdR which is used to label the L5178Y cells prior to treatment could be raised from 0.05 μCi/ml to 0.2 μCi/ml without affecting the elution pattern.

Procedure

The L5178Y cells were labeled at 0.05, 0.2, and 0.5 μCi/ml ^{14}C-TdR for 24 hours. This was followed by an 18-hour chase, and an additional 2 hours of incubation in which 0.2 ml saline was added to the 10 ml of medium in which the cells were suspended (to mimic treatment). At the end of this time, the cells were washed and subjected to the standard alkaline elution procedure.

Results and Conclusions

See Figure 9. There was no significant difference in the alkaline elution patterns of the DNA from cells labeled with the different specific activities of ^{14}C-TdR.

Experiment 9

The Alkaline Elution of DNA from L5178Y Cells Treated with Methyl Methanesulfonate (MMS) at Different Concentrations. High Concentration Range vs. Low Concentration Range.

Objective

The objective of these experiments was to investigate the effect of MMS concentration on the production of single-strand breaks in DNA as determined by alkaline elution.

Procedure

L5178Y cells were labeled for 24 hours with 0.2 μCi/ml ^{14}C-TdR adjusted to a final molarity of 10^{-5} M TdR. The cells were then chased in fresh medium containing 10^{-5} M TdR for 16 hours. At the end of the chase, the cells were centrifuged, and resuspended in 10 ml of medium containing 3% serum. In a dark room, under red light, 0.2 ml volumes of MMS stock solutions in saline were added to give the desired final MMS concentrations. The cells were treated for 2 hours in 50 ml tubes while being rocked gently end to end on a rocker platform in the dark at 37°C. At the end of this time, they were washed and subjected to the standard alkaline elution procedure.

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Results and Conclusions

See Figure 10 and Figure 11. The first experiment was performed at MMS concentrations of 1, 5, and 10 mM (Figure 10). At all three treatment concentrations, over 75% of the radioactivity was eluted in the first 90 minutes and concentration dependence was not distinguishable. The experiment was therefore repeated at a lower concentration range, with final MMS concentrations of 0.01, 0.05, 0.1, 0.5, and 1.0 mM. This experiment demonstrated a very definite concentration dependence (Figure 11).

Experiment 10

The Alkaline Elution of DNA from L5178Y Cells Treated with Ethyl Methanesulfonate (EMS) at Different Concentrations.

Objective

The objective of this experiment is to investigate the effect of EMS concentration on the production of single-strand breaks in DNA as measured by alkaline elution.

Procedure

L5178Y cells were labeled for 24 hours with 0.05 μCi/ml of ^{14}C-thymidine. The final TdR molarity was adjusted to 10^{-5} M. The cells were then chased for 18 hours in fresh medium containing 10^{-5} M TdR. At the end of the chase, the cells were centrifuged and resuspended in 10 ml of medium with 10% serum. In a dark room under red light, 0.2 ml volumes of EMS stock solutions in saline were added to give the desired final EMS concentrations. The cells were treated in 50 ml stoppered Erlenmyer flasks at 37°C for 2 hours on the rotatory shaker. At this time, they were washed and subjected to the standard alkaline elution procedure.

Results and Conclusions

See Figure 12. An obvious concentration dependence of EMS induced DNA single strand breakage is evident.

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Conclusions

The alkaline elution procedure has been examined with relation to reproducibility, the appropriate specific activity of the DNA label, the appropriate radionucleotide (^{3}H- or ^{14}C-), the appropriate chase incubation time, and the appropriate molarity of the eluting solution.

We have found that reproducible and satisfactory results can be obtained for control (untreated cell) DNA elution rates if the cells are labeled for 24 hrs with 0.2 μCi/ml ^{14}C-thymidine, at a final thymidine concentration of 10^{-5} M, followed by 16-18 hr chase incubation in fresh medium with 10^{-5} M non-radioactive thymidine prior to treatment. All operations during and subsequent to treatment must be carried out under red light.

A lowering of the molarity of H_4EDTA in the eluting solution to 0.02 M was found to be advantageous.

Using these procedures, alkylating agent (MMS and EMS) induced DNA single-strand DNA breakage was readily detectable. The extent of breakage which can be indicated using the alkaline elution technique will "saturate" if the actual damage is too extensive.

FIGURE LEGENDS

Figure 1. Alkaline elution of DNA from L5178Y thymidine kinase locus heterozygote cells labeled for 18.5 hours with ^{3}H-thymidine, and eluted on filters in two different filter holders to compare the reproducibility of the procedure. (Δ——Δ) Filter 1; (O——O) Filter 2.

Figure 2. Alkaline elution of DNA from L5178Y cells labeled for 19.5 hours with ^{3}H-thymidine and irradiated with Co^{60} radiation at 130 rads/minute.

Figure 3. Alkaline elution of DNA from L5178Y cells which were labeled with 0.05 μCi/ml ^{14}C-thymidine and treated with 1 mM MMS for 1 hour. The cells were either labeled for 23 hours and then treated, or were labeled for 21 hours and treated following a 2-hour chase in nonradioactive medium. The control cells were labeled for 21 hours and then incubated in nonradioactive medium for 3 hours.

Figure 4. Alkaline elution of DNA from L5178Y cells which were labeled with 0.05 μCi/ml ^{14}C-thymidine and treated with 1 mM MMS for 3 hours. The cells were either labeled for 21 hours and then treated, or were labeled for 20 hours and treated following a 1-hour chase in nonradioactive medium. The control cells were labeled for 21 hours and then incubated in nonradioactive medium for 3-hours.

Figure 5. Alkaline elution of DNA from L5178Y cells which were labeled either with 0.1 μCi/ml ^{3}H-thymidine or 0.1 μCi/ml ^{14}C-thymidine, and exposed

to 5 mM MMS for 0, 1, 2 or 3 hours. The cells exposed for 1 hour or 2 hours were incubated in nonradioactive medium for 2 hours and 1 hour respectively, prior to treatment.

Figure 6. The effect of H_4EDTA concentration in the eluting solution on the alkaline elution of DNA from L5178Y cells. (O——O) 0.02 M H_4EDTA; (●——●) 0.04 M H_4EDTA.

Figure 7. The effect of a 4-hour chase in nonradioactive medium after labeling with 0.5 μCi/ml ^{3}H-TdR on the alkaline elution pattern of L5178Y DNA.

Figure 8. The effect of a 1-, 2-, or 3-hour chase in nonradioactive medium after labeling with 0.05 μCi/ml ^{14}C-TdR on the alkaline elution of L5178Y DNA.

Figure 9. The alkaline elution of L5178Y cells which were labeled with different specific activities of ^{14}C-thymidine. (Δ——Δ) 0.05 μCi/ml; (O——O) 0.2 μCi/ml; (●——●) 0.5 μCi/ml.

Figure 10. The alkaline elution of DNA from L5178Y cells which were exposed to MMS 0, 1, 5, and 10 mM for 2 hours in medium containing 3% serum. Label was for 24 hours with 0.2 μCi/ml ^{14}C-TdR (10 μM TdR) and chased for 16 hours (10 μM TdR) prior to treatment.

Figure 11. The alkaline elution of DNA from L5178Y cells which were exposed to MMS (0, 0.01, 0.05, 0.1, 0.5, and 1.0 mM) for 2 hours in medium containing 3% serum. Label was for 24 hours with 0.2 μCi/ml ^{14}C-TdR (10 μM TdR) and chased for 16 hours (10 μM TdR) prior to treatment.

Figure 12. The alkaline elution of DNA from L5178Y cells which were exposed to EMS (0, 1, 5, and 10 mM) for 2 hours in medium containing 10% serum. Label was for 24 hours with 0.05 μCi/ml ^{14}C-TdR (10 μM TdR) and chased for 18 hours (10 μM TdR) prior to treatment.

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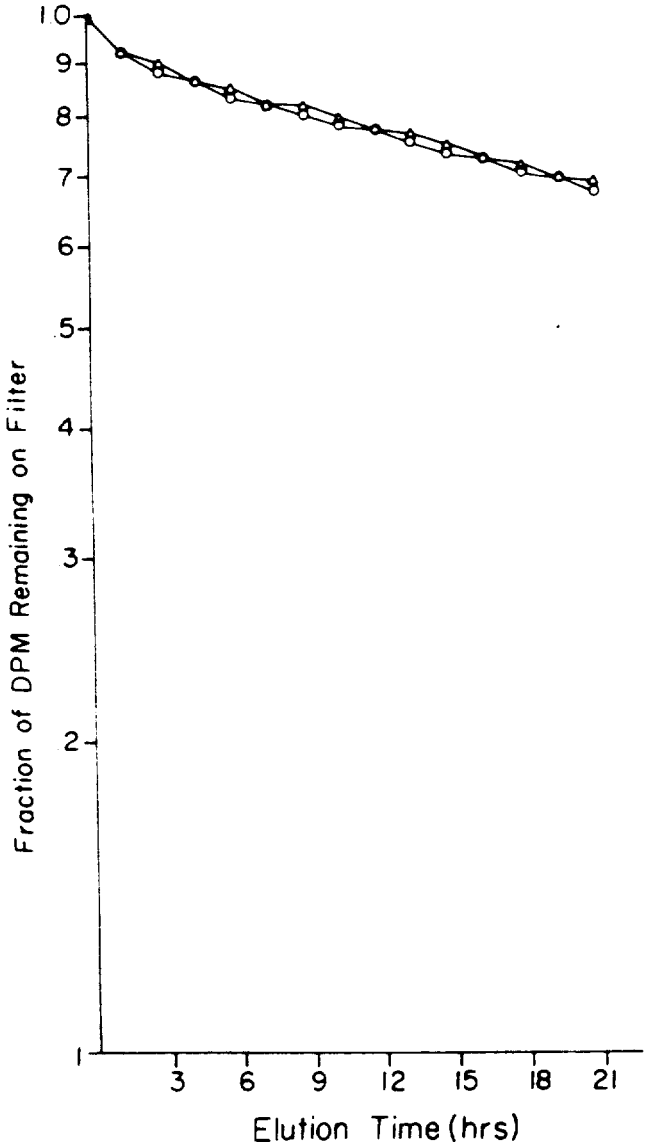
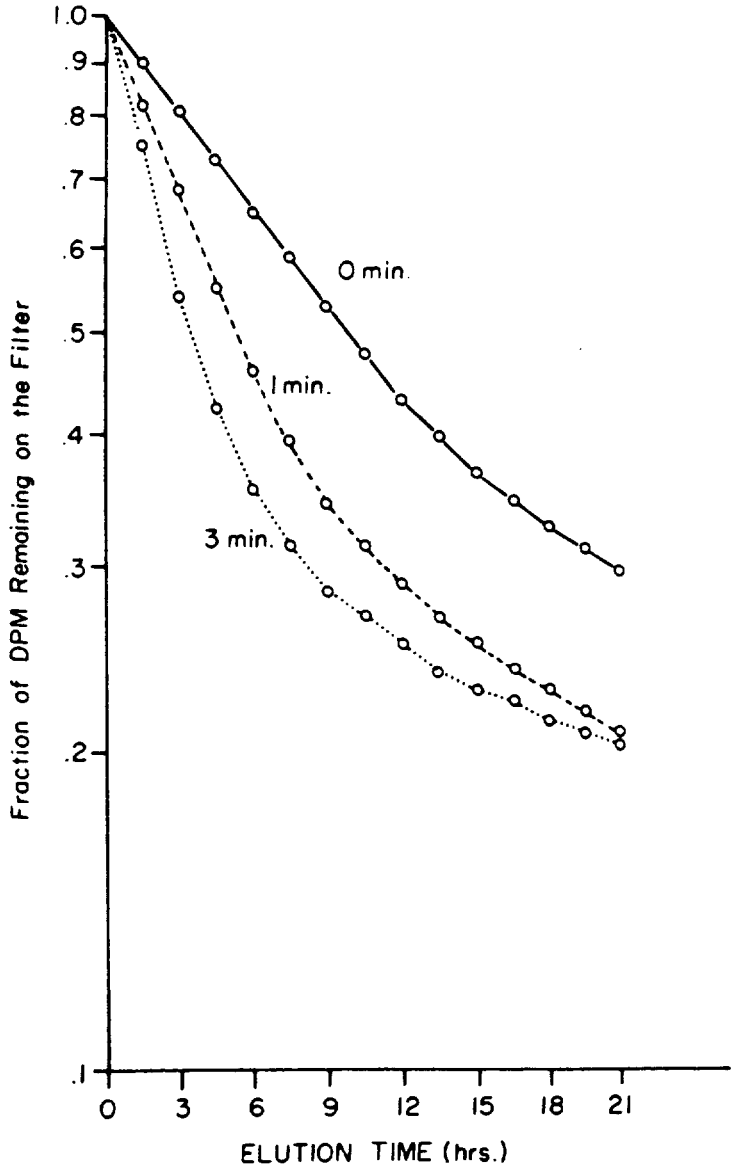

1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
Fraction of DPM Remaining on Filter
3
6
9
12
15
18
21
Elution Time (hrs)

Figure 1


1.0
.9
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.1
Fraction of DPM Remaining on the Filter
0 min.
1 min.
3 min.
0
3
6
9
12
15
18
21
ELUTION TIME (hrs.)

Figure 2

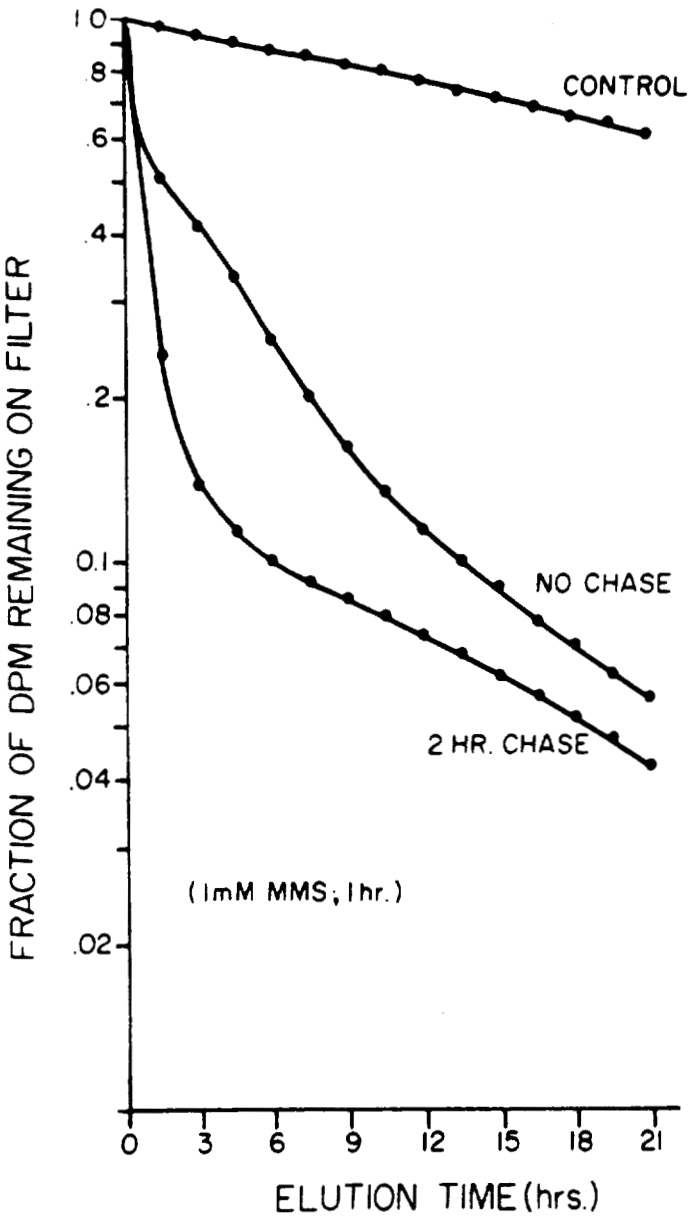

Figure 3

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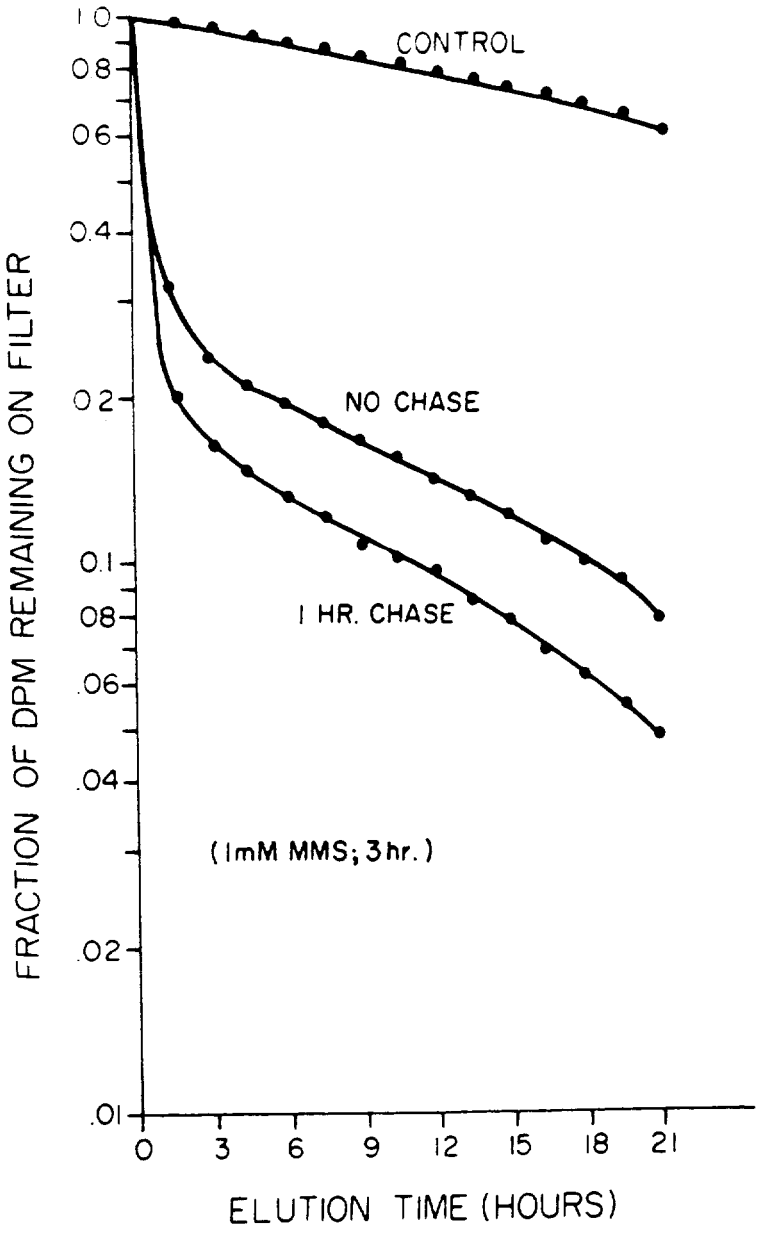
CONTROL
NO CHASE
1 HR. CHASE
(1mM MMS; 3hr.)
FRACTION OF DPM REMAINING ON FILTER
ELUTION TIME (HOURS)
1.0
0.8
0.6
0.4
0.2
0.1
0.8
.06
.04
.02
.01
0
3
6
9
12
15
18
21

Figure 4

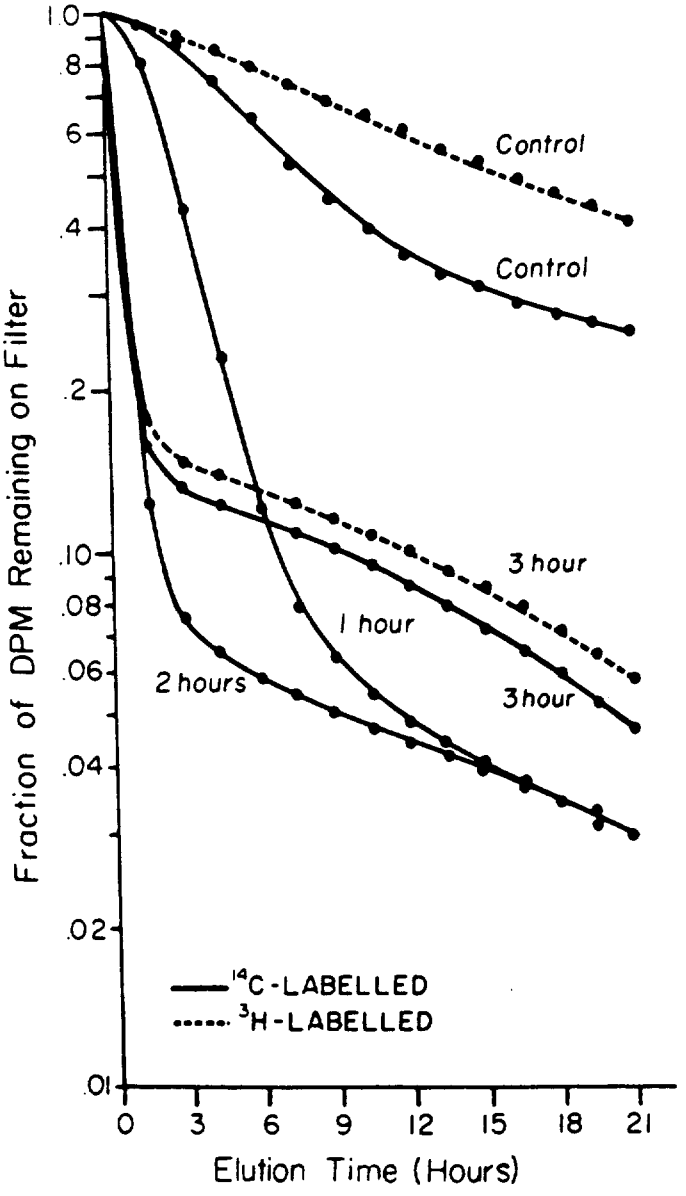
1.0
.8
.6
.4
.2
.10
.08
.06
.04
.02
.01
Fraction of DPM Remaining on Filter
Control
Control
3 hour
1 hour
2 hours
3hour
^{14}C-LABELLED
^{3}H-LABELLED
0
3
6
9
12
15
18
21
Elution Time (Hours)

Figure 5

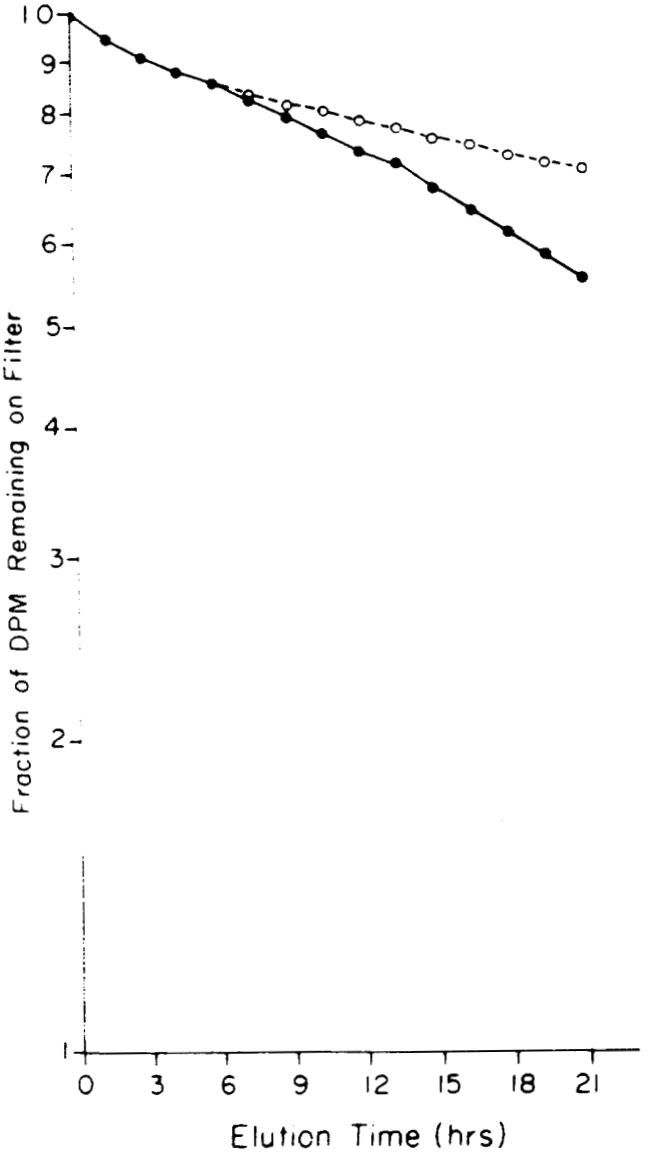

Figure 6

1042067

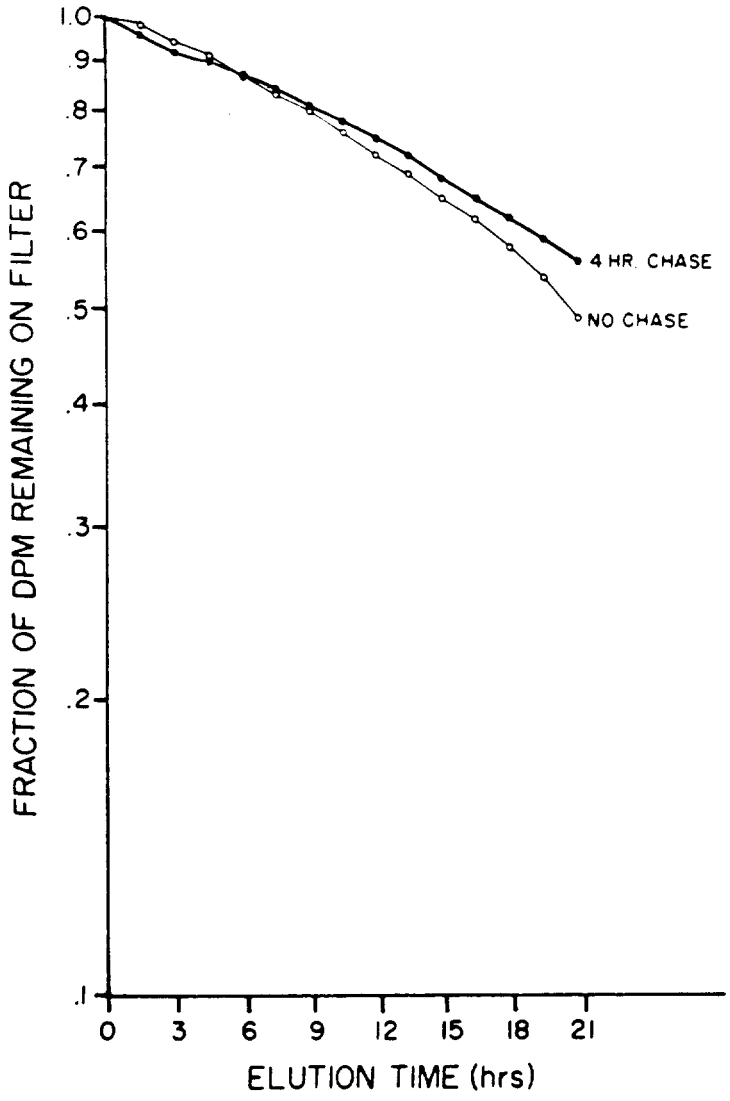
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
FRACTION OF DPM REMAINING ON FILTER
4 HR. CHASE
NO CHASE
0
3
6
9
12
15
18
21
ELUTION TIME (hrs)

Figure 7

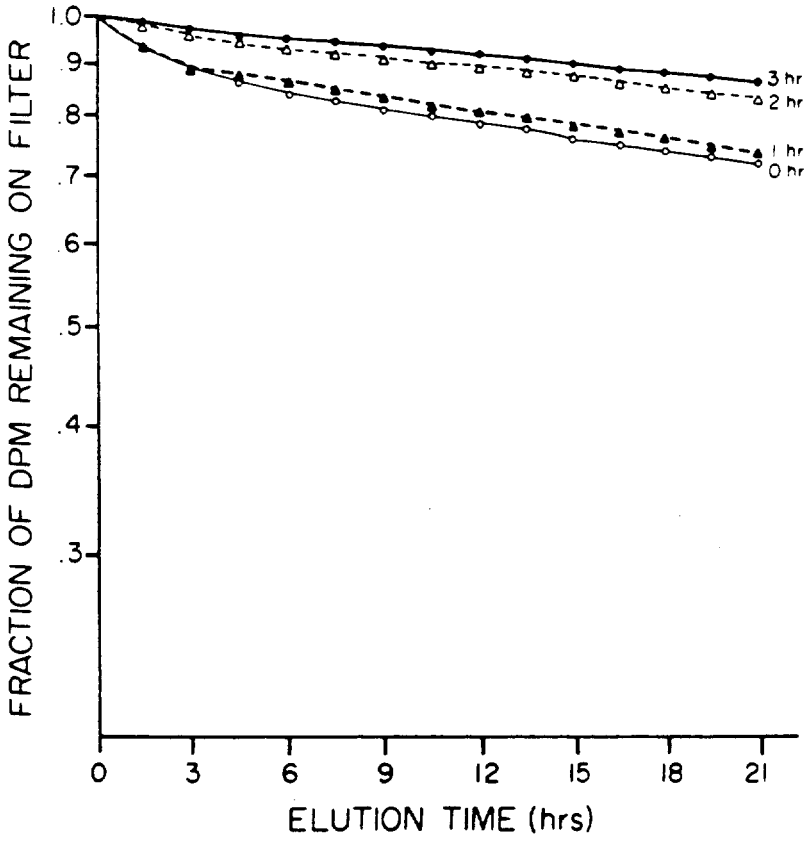

Figure 8

1042069

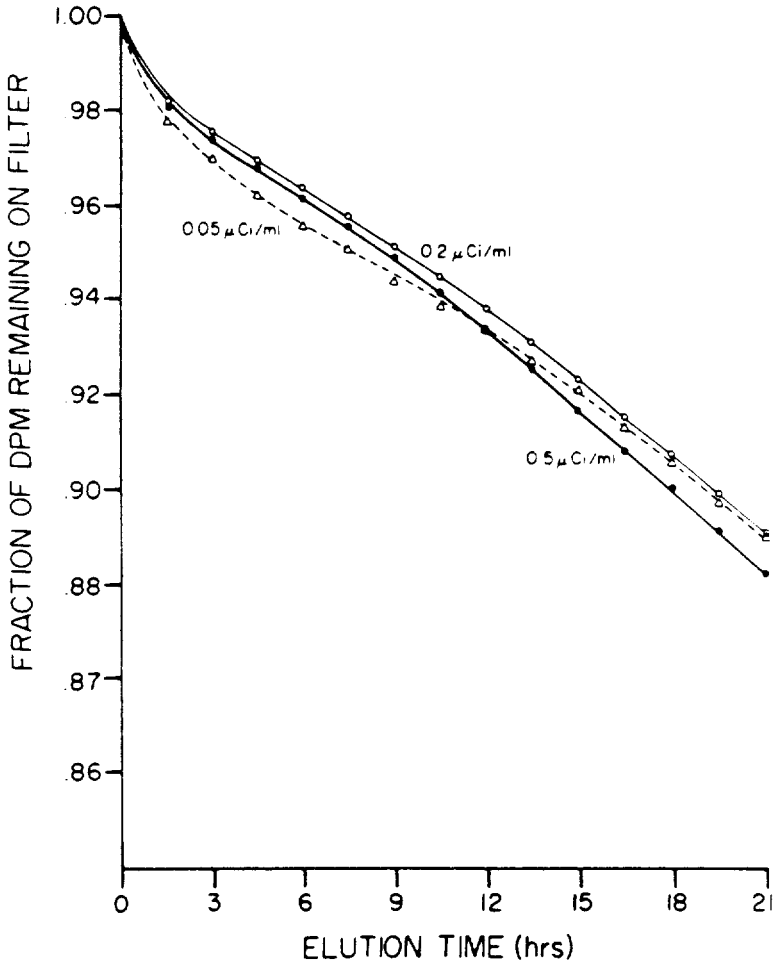
FRACTION OF DPM REMAINING ON FILTER
1.00
.98
.96
.94
.92
.90
.88
.87
.86
0.05 μCi/ml
0.2 μCi/ml
0.5 μCi/ml
0
3
6
9
12
15
18
21
ELUTION TIME (hrs)

Figure 9

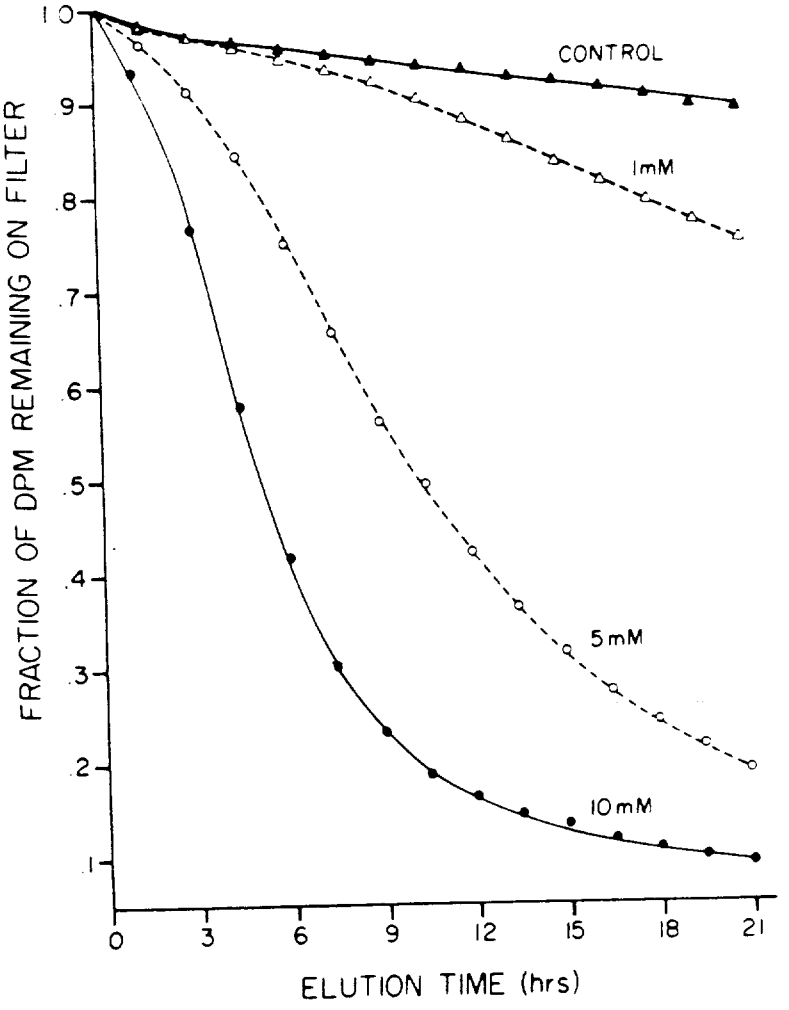

Figure 12

1042071