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COMPREHENSIVE REPORT

REPETITIOUS NATURE OF REPAIRED DNA IN MAMMALIAN CELLS

U.S.E.R.D.A. Contract No. E-(40-1)-4761

June 1, 1974 to February 1977

Submitted to:

Energy Research and Development Administration
Oak Ridge, Tennessee 37830

Submitted by:

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February 1977

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1041783

1. Main Research Accomplishments

The main research accomplishments we have achieved since the initiation of this project on June 1, 1974, are described in the attached manuscripts, entitled "Comparative DNA Repair and its Distribution in "Normal" and Cancerous Human Lymphoblastoid Cell Lines", and "Repetitious Distribution of DNA Synthesized Immediately and at Different Times after Ultraviolet Light Exposure of Mouse L-929 Cells."

The objective of the study, as described in our initial grant application, was to increase our knowledge of the molecular nature of the repair replication process in "normal" and cancerous mammalian cells maintained in tissue culture upon induction of repair by treating the cells with physical and chemical agents known to damage DNA. Our intention was to quantitate this repair as a function of dose and exposure time, and more significantly, to examine the repetitive nature of the DNA undergoing repair replication. We intended to investigate repair replication in a variety of cell lines, including mouse L-929 and human lymphocyte cells, normal human WI-38 diploid cells at different passages, normal human skin fibroblasts at early passage from biopsies from individuals of different ages, a disease-related human lymphocyte growing in culture (Kaplan's mononucleosis), a disease-related skin fibroblast (*Xeroderma pigmentosum*), and two human cancer cell lines, HeLa-229 and Burkitt's lymphoma. During the course of the studies described in the accompanying manuscripts, and also described in our annual Progress

1041784

Reports, we have found it appropriate to focus on several of these lines. Studies therefore have been performed with mouse L-929 cells, "normal" human WIL_2-A3 lymphoblastoid cells, the non-virus-producing RAJI line of Burkitt's lymphoma, the virus-producing EB-3 line of Burkitt's lymphoma, WI-38 cells of different passage number, and normal human skin fibroblasts from individuals of different ages. Studies are also being performed with the L5178Y mouse leukemic cell line.

In addition to the repair replication studies indicated above, preliminary work performed during the course of this project on the effect of ultraviolet light on DNA synthesis led us to complete the investigations described in the second manuscript listed above, in which we report on our study of the repetitious distribution of the DNA being synthesized immediately or at later times after UV-irradiation.

A. Lymphoblastoid Cell Studies

The DNA repair replication occurring in three human lymphoblastoid cell lines, measured using ^{3}H-BrUdR incorporation and alkali density gradient centrifugation procedures, has been compared. For the same UV exposure and repair replication labelling time, the extents of repair (dpm/μg) in a "normal" line, WIL_2-A3, and in the RAJI, a non-virus-producing line of Burkitt's lymphoma, were comparable; the extent of repair was less in EB-3 cells, a virus-producing line of Burkitt's lymphoma. Upon treatment with methyl methanesulfonate, at the same cell concentration

1041785

and with the same treatment-labelling medium, the extent of repair replication was again similar in the WIL_2-A3 and RAJI cells, and less in the EB-3 cells. The difference may be related to the similar population doubling times of the WIL_2-A3 and RAJI cells, and to the slower growth rate of the EB-3 cells, rather than to the extent and kind of viral information present.

When the repetitious distribution of the repair replicated DNA of the lymphoblastoid cells was examined using the DNA/DNA "CoT" technique, the UV-induced repair showed uniform distribution at low and intermediate CoTs. The MMS-induced repair replicated DNA showed divergence from the reassociation of semi-conservatively synthesized DNA at intermediate to high CoTs; i.e. for each CoT value, there was less reassociation of repair replication incorporated radioactivity than radioactivity incorporated during semi-conservative synthesis.

B. The Extent of Repair and its Distribution in Mouse L-929 Fibroblast Cells.

The extent of repair replication in mouse L-929 cells was observed to increase up to a maximum at a UV dose of approximately 75 ergs/mm^2. The extent of repair replication radioactivity decreased when the cells were exposed to UV light for more than 25 seconds at a dose rate of 9.4 ergs/mm^2/sec. When the extent of repair replication was measured using our standard H^3-BrUdR incorporation and alkali cesium chloride-cesium sulfate density gradient centrifugation procedure after a 16

second UV exposure at 9.4 ergs/mm^2/sec, we found the repair to continually increase over a 30-minute to 5 hour repair replication labelling interval.

When the repair replicated DNA isolated from UV-irradiated mouse L-929 cells was isolated and a kinetic experiment performed, the percent reassociation of the repair replicated radioactivity was very similar at each CoT value over the highly repetitive to intermediate range to semi-conservatively synthesized DNA.

C. DNA Synthesis Inhibition Studies; L-929 Cells.

We have observed, as has been described in the literature, that the ^{3}H-thymidine incorporation rate (dpm/μg DNA) in mouse L-929 cells decreases immediately after exposure to ultraviolet light. This decrease was initially dose dependent, but at exposures greater than 225 ergs/mm^2 appeared to be radio-resistant. We could not explain the radio-resistant incorporation by changes in the uptake of ^{3}H-thymidine into the acid soluble pool (dpm/μg DNA) of the cells. Since the radio-resistant incorporation amounted to approximately 35% of the control rate, the percentage which highly repetitious and intermediate repetitious DNA contributes to the total mouse genome, we performed DNA/DNA "CoT" studies with DNA labelled with ^{3}H-thymidine immediately after exposure of L-929 cells with a dose of UV resulting in radio-resistant incorporation. We hypothesized that only repetitious DNA was continuing to incorporate ^{3}H-thymidine at this dose. Our CoT studies, performed in the highly repetitive to intermediate range, did not show the rapid reassociation of radioactivity which would have confirmed this; i.e., the radioresistant synthesis was of DNA of all

1041787

classes of repetitious frequency. DNA synthesized at different times after UV exposure, during the period when post-replication repair is presumed to be occurring, was similarly labelled for short intervals and isolated. The DNA/DNA CoT studies showed that this DNA synthesis was also of all classes of repetitious frequency.

D. Repair Replication and Repetitious Distribution Studies Performed with WI-38 Human Diploid Fibroblast Cells

Two types of investigations have been performed involving WI-38 cells after UV and/or methyl methanesulfonate exposure. The first type of experiment is preliminary to a study of the repetitious distribution occurring immediately after damage by UV light or MMS treatment as compared to the repetitious distribution of the repair upon longer repair-labelling times. The second type of investigation is a study of the extent of repair and its distribution in the genome upon equivalent treatments of WI-38 cells at early-middle as compared to late passage.

(1) Since our intention is to compare the repetitious distribution of the DNA which is repair replicated immediately after UV exposure, and/or after MMS treatment, as compared to that repaired at later times, we have investigated the time course of the repair using a pulse repair-labelling procedure. For a UV dose of 30 seconds at 9.4 ergs/mm^2/sec, and an MMS treatment (5 mM) for one hour, we found that a one hour treatment label with MMS resulted in less repair replication radioactivity incorporated per μg of DNA than the repair

1041788

replication label incorporated during even the fourth hour post-UV exposure with this dose.

(2) Two experiments to study the extent of repair replication and its distribution in early-middle passage (passage 24) as compared to late passage (passage 46 or 48) WI-38 fibroblasts after UV-irradiation were initiated. In the first experiment, involving passage 24 and passage 46 cells, insufficient total radioactivity in an insufficient amount of DNA was isolated after the final alkaline cesium chloride-cesium sulfate density gradient centrifugation for CoT studies. The experiment was therefore repeated using passage 24 and passage 48 WI-38 cells. Both experiments indicated a slightly higher extent of repair in the late passage cells after equivalent UV exposure (150 ergs/mm^2). The passage 24 and passage 48 repair replication labelled DNA's isolated were then subjected to our DNA/DNA kinetic reaction experiments, over a CoT range extending from 2×10^{-4} to 10^1. This encompasses the range where highly repetitious and intermediate repetitious DNA would be reassociating. There does appear to be a difference in the percentage reassociation of repair replicated DNA as compared to semi-conservatively C^{14}-thymidine labelled DNA in the passage 24 cells, in contrast to the passage 48 cells, where we cannot see a significant difference. We were able to maintain the late passage cells in the experiment to passage 54.

1041789

E. Studies of the Repair Replication Labelling of Normal Human Skin Fibroblast Cultures from Individuals of Different Ages After UV Light Exposure.

Each of the cell lines employed in this study was observed to have its own characteristic growth rate and cell size. The number of cells which could be recovered per dish for each line were unknown to us prior to this experiment. We found that we were only able to recover DNA with sufficient repair replication radioactivity from the UV experiment after the first alkali cesium chloride-cesium sulfate density gradient. We are not prepared at this time to make any definitive statement as to the extent of repair in the human skin fibroblast cells isolated from individuals of different ages, even though the ages selected for subsequent study will be based on the preliminary data from this first experiment.

F. Alkaline Elution Technique

We have initiated studies of the application of the alkaline elution technique for detecting DNA single-strand breaks and cross-links so as to be able to study the relationship of DNA strand breakage and rejoining, cross-link formation, DNA repair replication, and L5178Y mutagensis. We will be employing the alkaline elution technique described by Kohn, et al. (1974, 1976). In performing these studies, we are using the L5178Y thymidine kinase heterozygote cells described by Clive and Spector (1975) and kindly donated to us by Dr. Clive.

1041790

We have performed a series of preliminary experiments, using ^{3}H-thymidine labelled UV-irradiated or nontreated control cells, so as to establish our confidence in the technique. We have compared such variables as the effects of room lighting on the elution (this must be performed in darkness), the effect of different molarities of H_4 EDTA on the elution (0.02 M H_4 EDTA has been chosen for future use), and on the reproducibility of elution when similarly treated cells are eluted from filters in separate filter holders.

2. Plans for Continuation of Present Objectives and New Objectives

A. Present Objectives

(1) Lymphoblastoid Cell Studies

We will investigate why the MMS induced repair replication radioactivity in WIL_2-A3 cells renatured to a lesser extent at intermediate and high CoT values then semi-conservatively synthesized DNA. To accomplish this we intend to treat WIL_2-A3 cells with MMS, but allow for extended repair to occur (e.g. 24 hours) before DNA isolation and kinetic study. Alternatively, we will investigate the use of an acid precipitation technique to see if the non-renatured repair replicated radioactivity is in intact DNA, or DNA which has been degraded to very small pieces (e.g., less than 20 nucleotides in length) during the extended high CoT incubation at 60°C. If this is the case, we will attempt high CoT incubations at low temperature in the presence of formamide.

(2) WI-38 Cell Studies

We have already repair replication labelled WI-38 cell DNA for 30 min and 3 hrs post-UV, and are preparing to isolate the DNA for kinetic study. WI-38 cells are being carried to late passage, and will be treated with UV and possibly MMS, so that our kinetic experiments comparing early to late passage repair replication can be repeated and carried out over the entire CoT range, including high CoTs.

(3) Skin Fibroblasts Cells from Donors of Different Ages

We will initiate cultures of skin fibroblasts from individuals of three ages (3 years, 31 years, and 92 years) so as to compare the extent of repair replication after equivalent UV-induced damage. We will investigate using a more rapid technique, described by Smith and Hanawalt (1976), for measuring repair replication radioactivity.

(4) Alkaline Elution Technique

We will continue our study of the application of this technique to the measurement of DNA single-strand breaks and cross-links in L5178Y mouse lymphoma cells heterozygous at the thymidine kinase locus.

B. New Objectives

The relationship of DNA synthesis, DNA damage, and its repair, will be investigated as related to survival and mutagenesis at the

1041792

thymidine kinase locus of L5178Y mouse lymphoma cells, with particular attention to events occurring during the post-treatment "expression time".

DNA synthesis will be indicated by measurements of effects on ^{3}H-TdR uptake and incorporation. Single-strand breaks and their rejoining, as well as cross-links, will be measured by the alkaline elution technique. Repair replication will be measured using an ^{3}H-BrUdR incorporation and alkali cesium chloride-cesium sulfate density gradient centrifugation procedure. The binding of radio-labelled mutagens to DNA, as a function of post-treatment incubation time, will be correlated with the above. Viability will be measured using eosin Y dye exclusion and soft agar cloning technique.

3. Bibliography

1. Meltz, M. L., Whittam, N. J., and Thornburg, W. H., 1977, Comparative DNA Repair and Its Distribution in "Normal" and Cancerous Human Lymphoblastoid Cell Lines, Submitted for publication.

2. Meltz, M. L., Whittam, N. J., and Thornburg, W. H., 1977, Repetitious Distribution of DNA Synthesized Immediately and at Different Times after Ultraviolet Light Exposure of Mouse L-929 Cells, Submitted for publication.

1041793

3. Kohn, K. W., Friedman, C. A., Ewig, R.A.G., and Iqbal, Z. M., 1974, DNA Chain Growth during Replication of Asynchronous L1210 Cells. Alkaline Elution of Large DNA Segments from Cells lysed on Filters. Biochemistry 13:4134.

4. Kohn, K. W., Erickson, L. C., Ewig, R.A.G., and Friedman, C.A., 1976, Fractionation of DNA from Mammalian Cells by alkaline Elution. Biochemistry 15:4629.

5. Clive, D., and Spector, J. A.F.S., 1975, Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Research 31:17.

6. Smith, C. A. and Hanawalt, P. C., 1976, Repair replication in human cells, Simplified determination utilizing hydroxyurea. Biochim. Biophys. Acta 432:336.

4. Significance of This Research Area

One of the significant questions remaining in the area of repair replication is what is its true significance in terms of cell survival and genetic alteration, after DNA is damaged by physical and chemical agents, considering that other types of DNA repair (e.g. rejoining, post-replication repair) are also occurring. While much information

1041794

has been accumulating in bacterial and mammalian cell systems, there is still a great need to relate DNA damage of all types and its repair to genetic changes, which include chromosome abervations, mutagensis, anc carcinogensis. This is especially important in the area of environmental health research, where some of these indicators are/or will be relied upon for assessing the relatve biohazard of a wide spectrum of environmental pollutants in rapid screening systems.

5. Present Division of Federal Research Support

A. USERDA Contract No. E-(40-1)-4761, "Repetitious nature of repaired DNA in mammalian cells." (June, 1974-May 31, 1977) Principal Investigator, 25% time. Level of funding for current active period, June 1, 1976-May 31, 1977: $32,045 (Direct Costs).

B. EPA Grant Project No. R803705, "*In vitro* assay of relative toxicity of diesel-engine exhausts," (July 7, 1975-July 6, 1977). Principal Investigator, 10% time. Level of funding for current active period, July 7, 1976-Jul6 6, 1977,: $79,324. (Direct Costs).

C. NCI Contract No. N01-CM-67075, "Conduct *in vitro* cell culture screening of new materials for cytotoxicity," (January 1, 1976-December 1, 1978) Principal Investigator, 20% time. Level of funding for current active period, January 1, 1977-December 31, 1977: $96,866. (Direct Costs).

D. General Research Support, 45% time; $10,000.

1041795

Amer. Soc. Cell Biol., Abst. #559, November, 1975. ORO-4761-3

"Repetititve Distribution of DNA in Mouse L-929 Cells Synthesized Immediately after Different Ultraviolet Light Exposures."

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The incorporation of ^{3}H-thymidine into acid precipitable DNA immediately after exposure of mouse L-929 cells to ultraviolet light decreases with increasing UV fluence. After exposures above 225 ergs/mm^{2}, the percentage incorporation in exposed as compared to unirradiated cell DNA appears to remain constant at a level of approximately 35%. Since this seemingly "radioresistant" percentage incorporation during the 30-minute labelling period corresponds to the percentage of mouse DNA comprising the highly repetitive and intermediate repetitive fractions, the DNA/DNA Cot technique was applied to the fractionation of semi-conservatively synthesized DNA from unirradiated cells, of DNA synthesized in cells exposed to a partially inhibitory dose, and of DNA synthesized in cells exposed to a dose resulting in "radioresistant" incorporation. In each case, the percentage renaturation of the incorporated radioactivity had the same values as DNA labelled for 24 hours in unexposed cells using ^{14}C-thymidine as DNA percursor. This indicates that there is no selective shutdown of synthesis of any particular repetitive class of DNA after ultraviolet light irradiation. The resistant incorporation may actually be due to increased uptake of ^{3}H-thymidine into the acid soluble pool during the labelling interval, while DNA synthesis is still continuing to decrease.

This work is supported by USERDA Contract No. AT-(40-1)-4761.

1041796

Radiat. Res. Soc., Abst. Be-7, 1975. ORO-4761-2

"Repair Replication and its Distribution in "Normal" and Cancerous Human Lymphoblastoid Cell Lines After Physical and Chemical Damage."

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A comparison will be presented of the ability of a presumptive "normal" human lymphoblastoid cell line, the WIL_2-A3 line, and the cancerous Burkitt's Lymphoma Raji line to repair damage to their DNA after ultraviolet light, alkylating agent (methyl methanesulfonate) and benz(α)pyrene treatment. The extent of repair after ultraviolet light induced damage appears slightly greater in the WIL_2-A3 line at a very high UV dose, as measured by dpm/μg of incorporated ^{3}H-BrUdR into preexisting DNA.

The benzo(α)pyrene, in contrast to MMS, presumably must be metabolized to an active form in order to damage DNA. The extent of repair replication occurring upon BaP treatment, in cells in which an attempt is made to pre-induce aryl hydrocarbon hydroxylase activity, will be reported. The repetitious distribution of repair replicated DNA (DNA/DNA Cot values) will be reported for the two cell lines.

This work is supported by U.S.A.E.C. Contract No. AT-(40-1)-4761.

1041797

Submitted For Publication, 1977

"COMPARATIVE DNA REPAIR AND ITS DISTRIBUTION IN "NORMAL" AND CANCEROUS HUMAN LYMPHOBLASTOID CELL LINES".[1,2]

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Running Title

DNA REPAIR IN HUMAN LYMPHOBLASTOID CELL LINES.

1041798

FOOTNOTES

1. Some of the data were presented at the Twenty-third Annual Meeting of the Radiation Research Society, Miami Beach, Florida, May 11-15, 1975, Abstract Be-7.

2. This work has been supported by the U.S.E.R.D.A., Contract No. AT-(40-1)-4761.

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5. The abbreviations used are: BrUdR, 5-bromodeoxyuridine; CoT, concentration X time; FUdR, 5-fluorodeoxyuridine; HU, hydroxyurea; MMS, methyl methanesulfonate; SSC, standard saline citrate:0.15 M sodium chloride, 0.015 M sodium citrate; UV, ultraviolet light.

1041799

ABSTRACT

The DNA repair replication occurring in three human lymphoblastoid cell lines, measured using ^{3}H-BrUdR incorporation and alkali density gradient centrifugation procedures, is compared. For the same UV exposure and repair replication labelling time, the extent of repair (dpm/μg) in a "normal" line, WIL_2-A3, and in the RAJI, a non-virus-producing line of Burkitt's lymphoma, is comparable, and the extent of repair is less in EB-3 cells, a virus-producing line of Burkitt's lymphoma. Upon treatment with methyl methanesulfonate, at the same cell concentration and with the same treatment-labelling medium, the extent of repair replication is again similar in the WIL_2-A3 and RAJI cells, and less in the EB-3 cells. The difference may be related to the similar population doubling times of the WIL_2-A3 and RAJI cells, and the slower growth rate of the EB-3 cells, rather than to the extent and kind of viral information present.

When the repetitious distribution of the repair replicated DNA of the lymphoblastoid cells is examined using the DNA/DNA "CoT" technique, the UV-induced repair shows uniform distribution at low and intermediate CoTs. The MMS-induced repair shows divergence from the reassociation of semi-conservatively synthesized DNA at intermediate to high CoTs.

INTRODUCTION

Deoxyribonucleic acid repair after ultraviolet light irradiation has been shown to occur in freshly isolated non-mitogen stimulated human

1041800

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 labelled cells, the number of grain counts, and the net disintegrations per minute 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. To expand on this investigation, we have studied 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, the RAJI cell line; and a virus-producing line the Burkitt's EB-3 cell line. 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 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 an alkylating agent, in particular, methyl methanesulfonate.

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In earlier studies, performed after UV-irradiation (4) and after chemical carcinogen treatments (5), the repetitious distribution of the repair replication radioactivity in the DNA of the genome was studied using the DNA/DNA CoT technique (6). The evidence in these investigations was that the repair replication was uniformly distributed throughout the DNA in the genome of the mammalian cells exposed. We have investigated whether or not the degree of viral involvement in the different lymphoblastoid cell lines has any effect on the distribution of the DNA repair occurring after UV-irradiation and MMS treatment.

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.); 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.).

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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, 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 cells and EB-3 Burkitt's lymphoma cells were obtained from the American Type Culture Collection.

Procedures for Cell Irradiation and Repair Replication Labelling. The repair replication protocol employed in the UV-irradiation studies is basically that previously described (4,7). 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 surfaces during the PBS washing and UV-irradiation steps which followed. At the end of the one-hour pre-incubation period, the cells in each flask were decanted into eight 50 ml Corning sterile conical tubes,

1041803

and centrifuged at 800 rpm for eight 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 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 ml plastic Optilux petri dishes (Falcon) for irradiation. Immediately after irradiating with a specially built UV-irradiation device (8) 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 labelling medium containing 1 μM FUdR, 1 mM hydroxyurea and 10 μCi/ml of ^{3}H-BrUdR (adjusted to 5 μM final BrUdr concentration). After three hours of repair replication labelling, 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 one 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 Labelling. The treatment and labelling protocols employed were basically those described above, with the exception that dextran-sulfate was not included in the experiment. Two types of experiments were performed;

1041804

in the first, the cells were pre-incubated 1 hour with BrUdR-FUdR medium, centrifuged, and resuspended in repair replication labelling 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 one hour. In the second type of experiments, the cells were incubated for different treatment-repair replication labelling times at one MMS concentration.

DNA Isolation Procedure. The procedure for DNA isolation has been described (7) 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 (9) 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 (10). 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.

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Modification of Repair Labelling Procedures for DNA to be Used in Kinetic Experiments. The experiments were performed essentially as described above, with the exception that the concentration of tritiated BrUdR in the repair labelling medium was at 50 μCi/ml (13-14 Ci/mmole in sterile aqueous solution).

Procedures for Kinetic Reactions. After the final cesium chloride-cesium sulfate alkali gradient centrifugation, the appropriate fractions were 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 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 combined DNA was then subjected to a series of steps, including: a) adding glycerol and shearing using a VirTis "60" homogenizer for 30 min at 50,000 rpm 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 Sorval SS-34 rotor, d) resuspension of the DNA in phosphate buffered-EDTA (0.12 M phosphate buffer, 0.002 M EDTA), e) measurement of the DNA concentration by radioactive counts (or by the spectrofluorometric procedure for high CoT values), f) denaturation of the samples in capillary or test tubes at the appropriate concentrations

1041806

by boiling in a hot water bath, 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 radioactive counting of the fractions in BBS-3 liquid scintillation solution. These methods have been derived from procedures previously described (11). Duplicates of each reaction were performed for each CoT point; the charts show the averages of these two values.

RESULTS

Cell Proliferation. The growth curves of the three human lymphoblastoid cell lines employed in this study, as determined by hemocytometer count, are shown in Chart 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; Extent of DNA Repair. The comparative repair replication radioactivity incorporated into the DNA of WIL_2-A3 and RAJI lymphoblastoid cell lines after different ultraviolet light exposures appears in Chart 2. The two cell lines were exposed and repair replication labelled with ^{3}H-BrUdR at 10 μCi/ml using the standard repair replication protocol described. The UV dose rate was 9.4 ergs/mm^2/sec. These experiments were performed on different days with different labelling solutions.

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In order to obtain definitive results for comparison of UV-induced repair between the different cell lines, we performed the experiment using cells from approximately the same regions of the growth curve for the three cell lines, incubating them at equivalent cell concentrations with the same labelling medium after one UV dose. The density gradient profiles resulting from this UV irradiation experiment appear in Chart 3. The results are tabulated in Table 1. From this table, we do not see any significant difference in the extent of repair replication occurring after ultraviolet light irradiation at this dose between the WIL_2-A3 and RAJI cell lines. On the other hand, the extent of repair occurring after a similar UV exposure and equivalent labelling time in the EB-3 cell line was lower.

Extent of DNA Repair in Human Lymphoblastoid Cells after Methyl Methane-Sulfonate Treatment. We first investigated the concentration-dependence of the incorporated repair replication radioactivity for the different lymphoblastoid cell lines upon exposure of the cells to the chemical mutagen methyl methanesulfonate. Typical first and second alkali cesium chloride-cesium sulfate gradient profiles for the DNA of WIL_2-A3 cells undergoing repair replication in the presence of different concentrations of MMS are shown in Chart 4. The concentrations employed were 0.1, 0.5, 1.0, 5.0, and 10.0 mM MMS. Control (untreated) cultures showed insignificant radioactivity in the normal density region. The results of these experiments, for WIL_2-A3 and RAJI cells, appear in Chart 5. It appeared from these curves, performed at different times with different MMS treatment-labelling solutions, that significant differences might exist between the extent of damage and repair upon MMS treatment in the WIL_2-A3 cells as compared to the RAJI cells. To investigate

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this, we selected from the above experiment 5 mM MMS as the concentration to be employed for determining the time course of repair replication in WIL_2-A3, RAJI, and EB-3 cells during MMS treatment.

The cells in each situation were obtained from the rapidly growing portion of the cell growth curve. The results of these experiments are shown in Chart 6. In contrast to the results of the concentration dependence experiment (Chart 5), the extent of repair replication in RAJI cell DNA for a given repair replication time and at the same concentration of MMS was much greater than for either WIL_2-A3 or EB-3 cells; the result also seemed to be in contradiction to the ultraviolet light results which indicated similar extents of repair after equivalent UV exposures. In order to resolve this inconsistency, we investigated the effect that the cell concentration would have on the extent of repair replication during treatment, and also the extent of repair occurring when the same number of cells were treated, but with the cells taken from different portions of the rapidly growing region of the cell growth curve. A schematic diagram of these experiments appears in Chart 7. The results appear in Table 2. From these experiments performed with RAJI cells, it would appear that neither the cell concentration during the treatment-labelling period, or the stage of the proliferating region from which the cells to be treated were initially taken, had a significant effect on the extent of DNA repair replication. The above discrepency could not be explained theoretically, and we therefore proceeded to perform a definitive experiment, wherein we treated cells from similar regions of the growth curve for the three cell lines at the same cell concentration with two MMS concentrations, and used the same treatment-labelling medium to treat each of the cell lines

1041809

at each MMS concentration. This protocol takes into consideration the fact that the alkylating agent might not be present at any time after preparation with the same level of activity in different media preparations containing serum; it is known that alkylating agents can have a relatively short half-life in solution. The first and second alkali cesium-chloride-cesium sulfate gradient profiles for these experiments appear in Charts 8 and 9. The results are summarized in Table 3. Based on this carefully performed comparative study, we see that the extent of repair occurring after equivalent treatment-labelling conditions is similar for the RAJI cell line and the WIL_2-A3 cell line, while the EB-3 cells again show, as after UV irradiation, a lower extent of repair replication at both MMS concentrations.

Repetitious Distribution Studies. Based on the possibility that viral information might not be uniformly distributed throughout the genome, but rather located in DNA sequences of a particular repetitious frequency, and hypothesizing that repair activity might be associated with this viral information, we investigated the repetitious distribution of the DNA undergoing repair replication in the three lymphoblastoid cell lines after UV light irradiation and in two of the three cell lines after MMS-induced damage.

In performing the kinetic DNA/DNA hybridization experiments described below, we always included a C^{14}-labelled, semi-conservatively synthesized (24 hr) DNA as an internal control. The control (untreated) CoT data plotted in Chart 10 are inclusive for almost all of the experiments performed over the course of this study; the straight line fit is a logarithmic curve of the form Y = a + b natural log X calculated on a Packard HP-9820A computer.

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Chart 11 shows the kinetic data obtained upon reassociation incubation of repair replicated DNA isolated from UV-irradiated EB-3 cells exposed to 16 seconds of ultraviolet light at 9.4 ergs/mm^2/sec and labelled with 50 μCi/ml ^{3}H-BrUdR. As can be seen over the low to intermediate CoT range of this study, the distribution of the ^{3}H-BrUdR labelled, repair replicated DNA is similar to that of the 24 hr C^{14}-thymidine labelled, semi-conservatively synthesized WIL$_2$-A3 DNA. In experiments performed using the repair replicated DNA isolated similarly from WIL$_2$-A3 and RAJI lymphoblastoid cells after a 16 second UV dose at 9.4 ergs/mm^2/sec, the ratio of reassociated tritium labelled DNA to reassociated C^{14}-labelled DNA was similar at the different CoT values studied over the low to intermediate CoT range (unpublished observation). The distribution of repair replicated radioactivitiy appears to be uniform under these conditions of exposure and labelling for all three cell lines.

Repetitious Distribution of Repair Replicated DNA After MMS-Induced Damage. The kinetic data obtained upon reassociation incubation of repair replicated DNA isolated from MMS-exposed WIL$_2$-A3 cells and RAJI lymphoblastoid cells (5 mM MMS) appear in Chart 12. In contrast to the data reported above for UV induced damage, there appears to be divergence between the extent of reassociation of ^{3}H-BrUdR labelled, repair replicated DNA and C^{14}-thymidine labelled, semi-conservatively synthesized DNA beginning in the low to intermediate CoT region.

When repair replicated DNA was obtained in a separate MMS (5 mM) treatment-labelling experiment, and subjected to reassociation at high CoT

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conditions (Chart 13), this divergence, with semi-conservatively synthesized DNA showing a greater precentage reassociation at each CoT point than the repair labelled DNA, appears to continue. These results were again obtained in another experiment (Chart 14), after another MMS (5 mM) treatment-labelling experiment. In this last experiment, the same DNA preparation was used over the entire CoT range.

DISCUSSION

Extent of Repair. Our original hypothesis was that the extent of repair replication in different human lymphoblastoid cell lines was somehow related to the presence of viral information in the genome, or to the cell's ability to produce virus, presuming equivalent amounts of DNA damage. The cell lines chosen for this investigation were the WIL_2-A3 cell line, isolated from a young boy with splenomegaly (12) (presumed normal), the RAJI line of Burkitt's lymphoma originally isolated by Pulvertaft (13), which has been shown to have Epstein-Barr viral information in the genome (14, 15), and the EB-3 line of Burkitt's lymphoma (16), which is a virus producer (17). Even though the WIL_2-A3 line is presumed normal, it is believed that the presence of at least some Epstein-Barr virus information is required in the genome for human lymphoblastoid cells to be capable of continuous *in vitro* growth (18).

While our results do indicate a difference in the extent of repair occurring in EB-3 cells after UV irradiation, as compared to WIL_2-A3 and RAJI cells, the situation is of such complexity that we cannot state that the difference is due to the capability for virus production of the EB-3

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cells. The lower extent of repair would in fact not be expected if we hypothesized that the additional viral information in the EB-3 cell would increase their repair capability. In fact, the simplest explanation for our results would be that the capability of repairing equivalent amounts of damage during an equivalent repair replication labelling interval presuming equivalent pool sizes and uptakes of radioactive precursor, is related to the cell growth rate. It has recently been shown that the rate of repair in lymphocytes is inhibited in proportion to the inhibition of the rate of DNA synthesis, as affected by different inhibitors (19). Support for such an explanation of our results would require a knowledge of the true DNA synthetic rate of the EB-3 cells, with their slower growth rate, and the DNA synthetic rates of the RAJI and WIL_2-A3 cells, with their faster population doubling times.

The results obtained upon MMS treatment and repair replication labelling of the DNA of the different cell lines, after treatment at two different MMS concentrations, would support the UV data in that we measure a lesser amount of repair replication in the EB-3 cells as compared to the WIL_2-A3 and RAJI cells. Such results are, however, subject to the additional complication that the molecular dosimetry necessary to verify identical extents of damage in the DNA of the different cell lines is not known.

Repetitious Distribution. The uniformity of repair replication of DNA damage has been previously demonstrated after treatment of other mammalian cells by both UV irradiation (4) and chemical carcinogens (5). The uniform

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distribution after UV irradiation has herein been confirmed in human lymphoblastoid cell lines, and appears independent of the degree of viral involvement in the cells. The results of our studies involving methyl methanesulfonate treatment present a different situation since, with increasing CoT, it appears that repair replication labelled DNA is reassociating to a lesser extent at each CoT value in proportionally different amounts as compared to the reassociation of semi-conservatively synthesized DNA. Because we are dealing with DNA which has been damaged by treatment with an alkylating agent, and because of the prolonged high (60°C) incubation temperatures employed in these studies, we cannot preclude the possibility at this time that the resultant lessening of reassociation is induced by some artifact of the incubation procedure which is affecting the reassociation ability of the alkali damaged, repair replicated DNA. The latter, in addition, probably still has unrepaired damage due to the alkylation process, probably including base damage. Additional investigations into resolving this problem are currently being performed.

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References

1. Evans, R. G., and Norman, A. Unscheduled Incorporation of Thymidine in Ultraviolet-Irradiated Human Lymphocytes. Radiat. Res., 36: 287-298, 1968.

2. Frey-Wettstein, M., Longmire, R., and Craddock, C. G. Deoxyribonucleic Acid (DNA) Repair Replication of Ultraviolet (UV) Irradiated Normal and Leukemic Leukocytes. J. Lab. Clin. Med., 74: 109-118, 1969.

3. Huang, A. T., Kremer, W. B., Laszlo J., and Setlow, R. B. DNA Repair in Human Leukemic Lymphoctyes. Nature (London) New Biol., 240: 114-116, 1972.

4. Meltz, M. L. and Painter, R. B. Distribution of Repair Replication in the HeLa Cell Genome. Int. J. Radiat. Biol., 23: 637-640, 1973.

5. Lieberman, M. W. and Poirier, M. C. Distribution of Deoxyribonucleic Acid Repair Synthesis among Repetitive and Unique Sequences in the Human Diploid Genome. Biochemistry, 13: 3018-3023, 1974.

6. Britten, R. J. and Kohne, D. E. Repeated Sequences in DNA. Science, 161: 529-540, 1968.

7. Meltz, M. L. DNA Repair in Baboon Alveolar Macrophages: A System for Assessing Biohazardous Materials. Environ. Res., 11: 359-366, 1976.

1041815

8. Steier, H. and Cleaver, J. E. Exposure Chamber for Quantitative Ultraviolet Photobiology. Lab. Pract., 18: 1295, 1969.

9. Gautschi, J. R., Young, B. R., and Painter, R. B. Evidence for DNA Repair Replication in Unirradiated Mammalian Cells - Is it an Artifact? Biochim. Biophys. Acta, 281: 324-328, 1972.

10. Hinegardner, R. T. An Improved Fluorometric Assay for DNA. Anal. Biochem., 39: 197-201, 1971.

11. Britten, R. J., Graham, D. E., and Neufeld, E. R. Analysis of Repeating DNA Sequences by Reassociation. Methods Enzymol. 29: 363-418, 1974.

12. Levy, J. A., Virolainen, M., and Defendi, V. Human Lymphoblastoid Lines from Lymph Node and Spleen. Cancer, 22: 517-524, 1968.

13. Pulvertaft, R. J. V. Cytology of Burkitt's Tumour (African Lymphoma). Lancet, 1: 238-240, 1964.

14. Zur Hausen, H. and Schulte-Holthausen, H. Presence of EB Virus Nucleic Acid Homology in a "Virus-Free" Line of Burkitt Tumour Cells. Nature (London), 227: 245-248, 1970.

15. Nonoyama, M. and Pagano, J. S. Detection of Epstein-Barr Viral Genome in Nonproductive Cells. Nature (London), New Biol., 233: 103-106, 1971.

1041816

16. Epstein, M. A. and Barr, Y. M. Cultivation In Vitro of Human Lymphoblasts from Burkitt's Malignant Lymphoma. Lancet, 1: 252-253, 1964.

17. Epstein, M. A. and Achong, B. G. The EB Virus. In: D. P. Burkitt and D. H. Wright (eds.), Burkitt's Lymphoma. pp. 231-248 London, Longman, 1970.

18. Nilsson, K., Klein, G., Henle, W., and Henle, G. The Establishment of Lymphoblastoid Lines from Adult and Fetal Human Lymphoid Tissue and its Dependence on EBV. Int. J. Cancer, 8: 443-450, 1971.

19. Cleaver, J. E. and Painter, R. B. Absence of Specificity in Inhibition of DNA Repair Replication by DNA-binding Agents, Cocarcinogens, and Steroids in Human Cells. Cancer Res., 35: 1773-1778, 1975.

1041817

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 Concentrations 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

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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

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Chart 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.

Chart 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.

Chart 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.

Chart 4. Typical first and second density gradient profiles obtained upon alkali cesium chloride-cesium sulfate density gradient centrifugation of DNA of WIL_2-A3 cells undergoing repair replication in the presence of different concentrations of MMS. Beginning with the upper left figure to the lower left figure; first gradient profiles obtained upon treatment with 0.1, 0.5, 1.0, 5.0, and 10.0 mM MMS.

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Beginning with the upper right figure to the lower right figure; second gradient profiles at the same concentration as adjacent figure.

Chart 5. 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.

Chart 6. 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.

Chart 7. 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.

Chart 8. Density gradient profiles of DNA obtained after MMS-induced repair replication at a concentration of 0.2 mM. Upper left and

1041822

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.

Chart 9. 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.

Chart 10. Plot of reassociation data for control C^{14}-thymidine labelled semi-conservatively synthesized WIL_2-A3 DNA from almost all of the CoT experiments performed for this project. The straight line is a logarithmic curve of the form Y = A + B natural log X, calculated on a Hewlitt-Packard HP-9820A computer.

Chart 11. Kinetic data obtained upon reassociation incubation of repair replicated DNA isolated from UV-irradiated EB-3 cells exposed to 16 seconds of UV at 9.4 ergs/mm^2/sec and labeled with ^{3}H-BrUdR (———), and of semi-conservatively synthesized DNA labelled with C^{14}-thymidine (- - -).

Chart 12. Upper chart: Kinetic data obtained upon reassociation incubation of repair replicated DNA isolated from MMS-exposed (5 mM) WIL_2-A3 cells, labelled with ^{3}H-BrUdR (———), and of semi-conservatively synthesized DNA labeled with C^{14}-thymidine (- - -). Lower chart:

1041823

Kinetic data obtained upon reassociation incubation of repair replicated DNA isolated from MMS-treated (5 mM) RAJI cells, labelled with ^{3}H-BrUdR (——), and semi-conservatively synthesized DNA labelled with C^{14}-thymidine (- - -).

Chart 13. Kinetic data obtained upon reassociation incubation at high CoT values of repair replicated DNA isolated from MMS-treated (5 mM) WIL_2-A3 cells, labelled with ^{3}H-BrUdR (——), and of semi-conservatively synthesized DNA labelled with C^{14}-thymidine (- - -).

Chart 14. Kinetic data obtained upon reassociation incubation of repair replicated DNA isolated from MMS-treated (5 mM) WIL_2-A3 cells, labelled with ^{3}H-BrUdR (——), and semi-conservatively synthesized DNA labelled with C^{14}-thymidine (- - -). DNA employed over the entire CoT range was from one experimental treatment.

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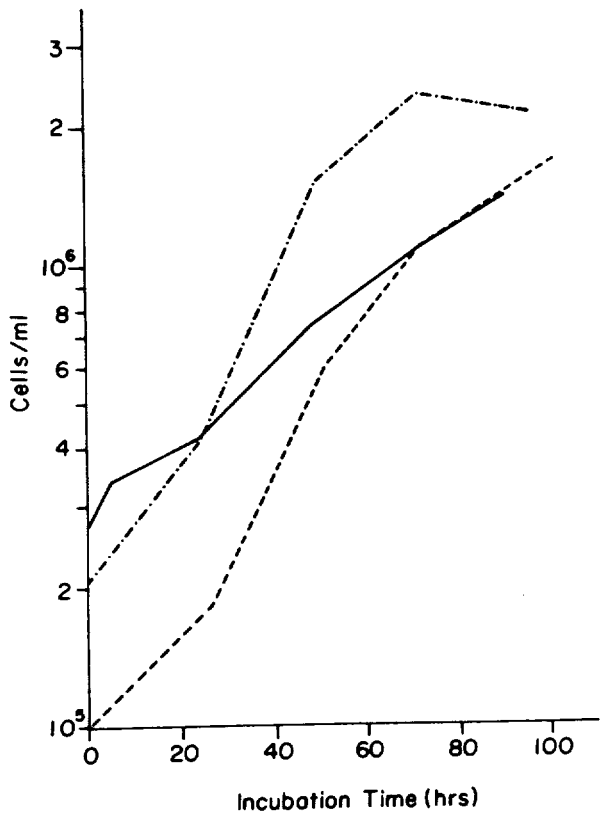

CHART 1

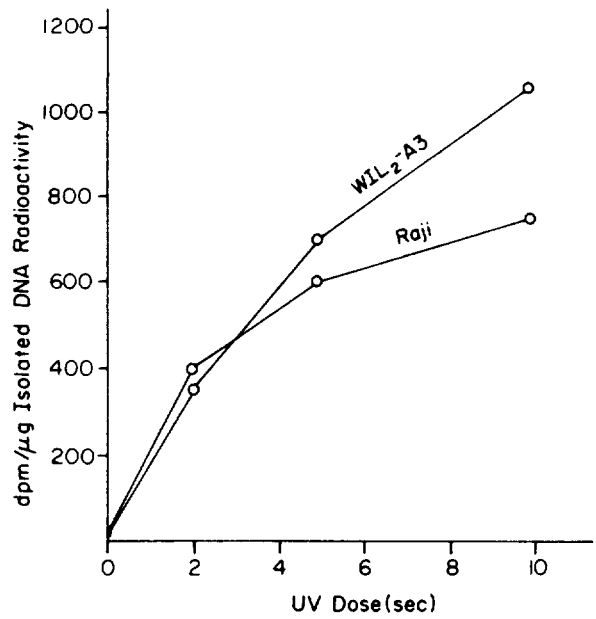

CHART 2

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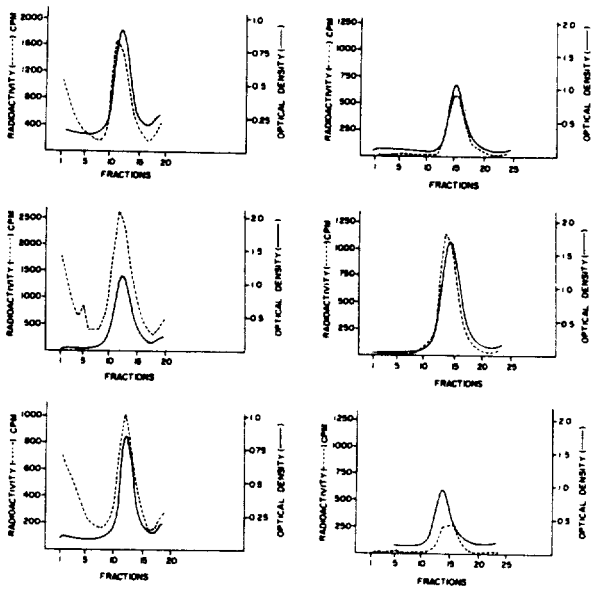

CHART 3

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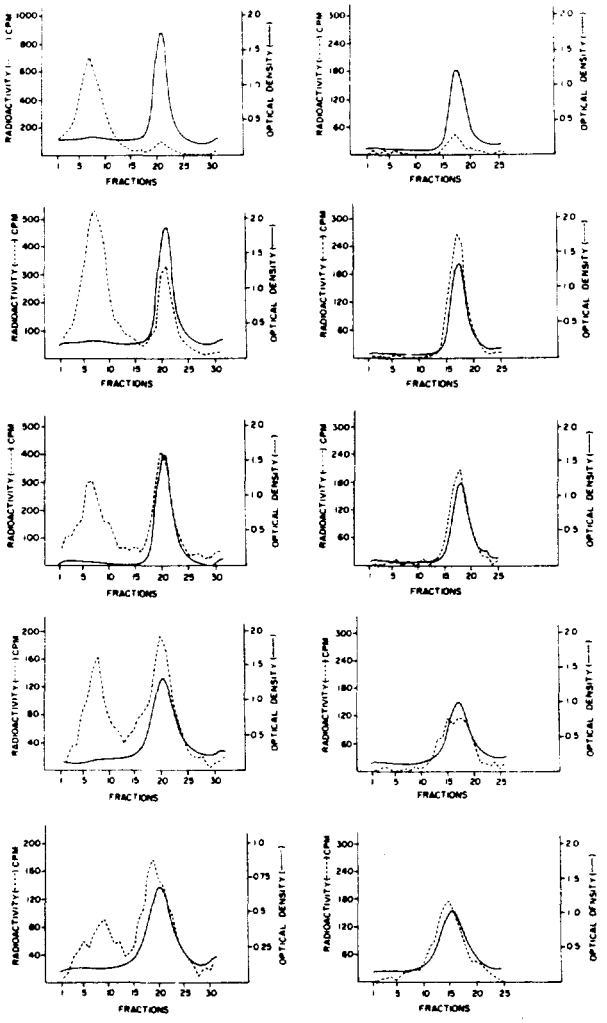

CHART 4

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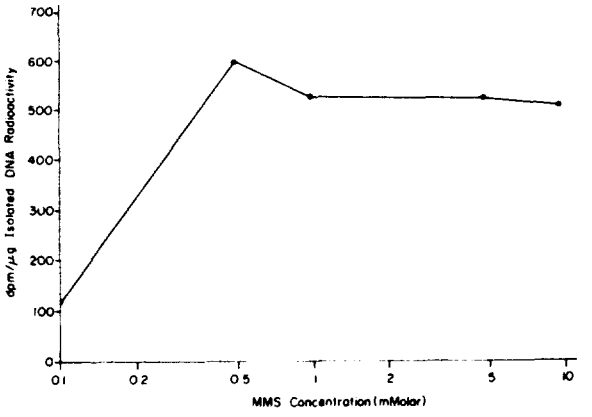

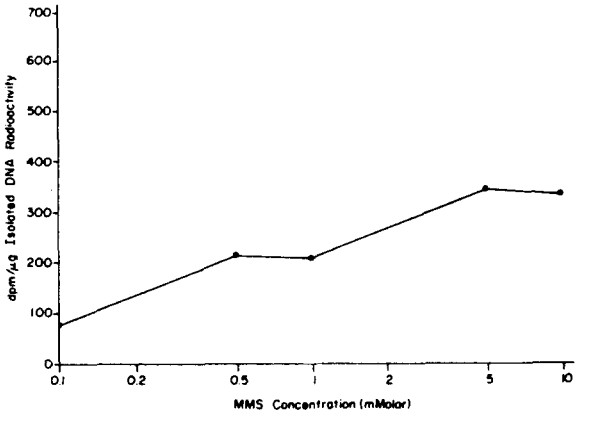

CHART 5

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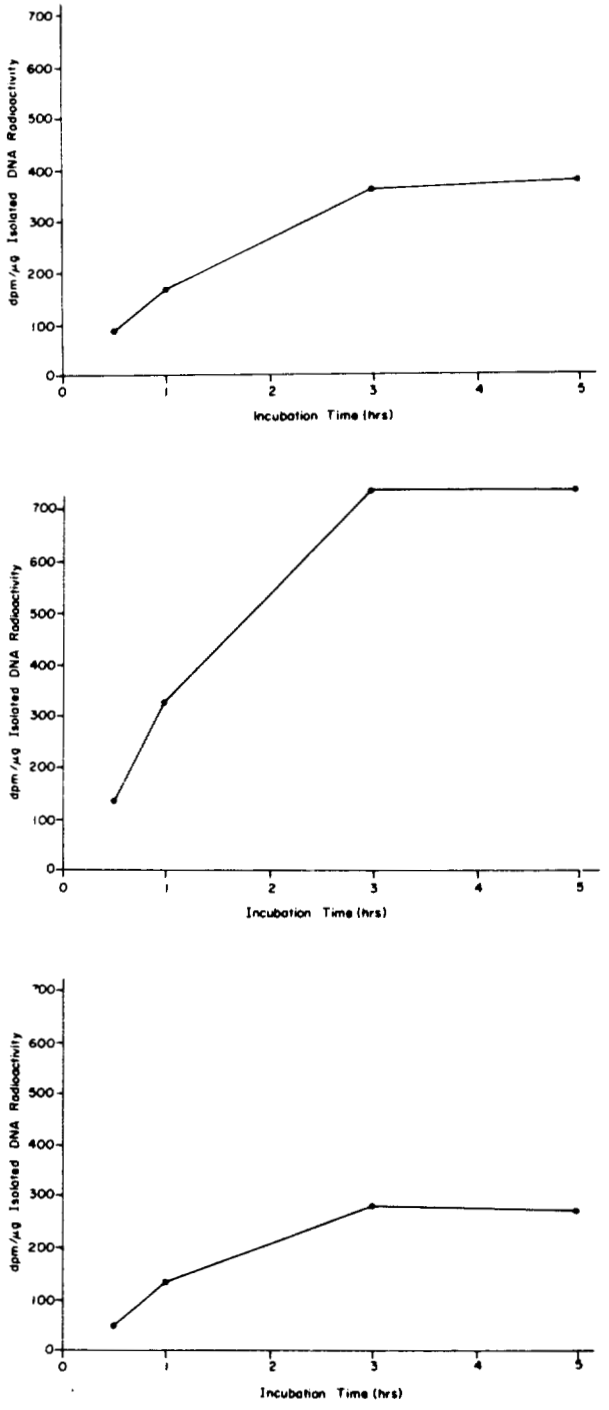

CHART 6

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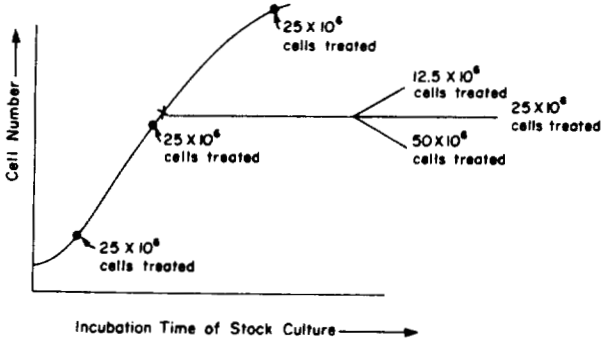

CHART 7

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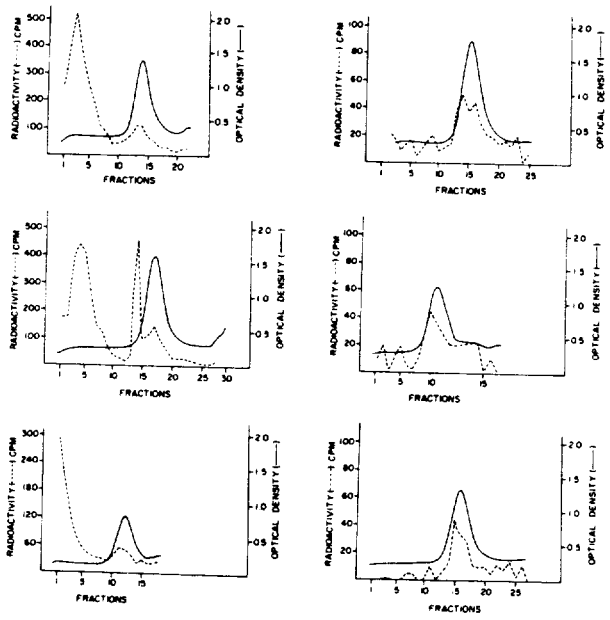

CHART 8

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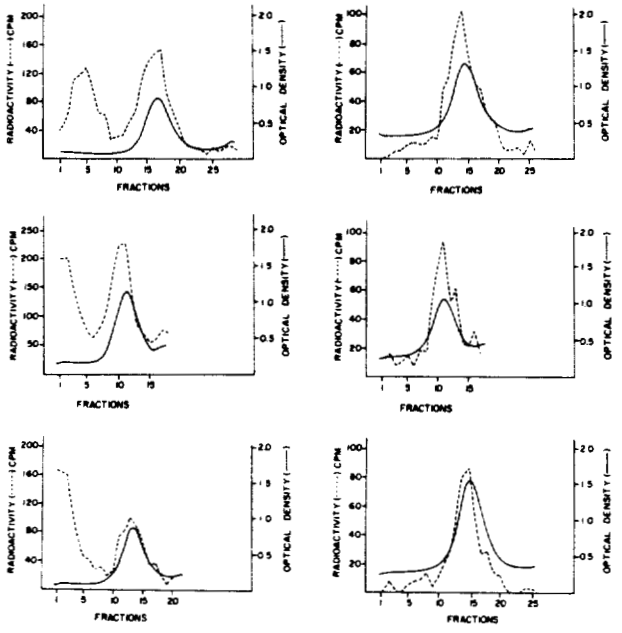

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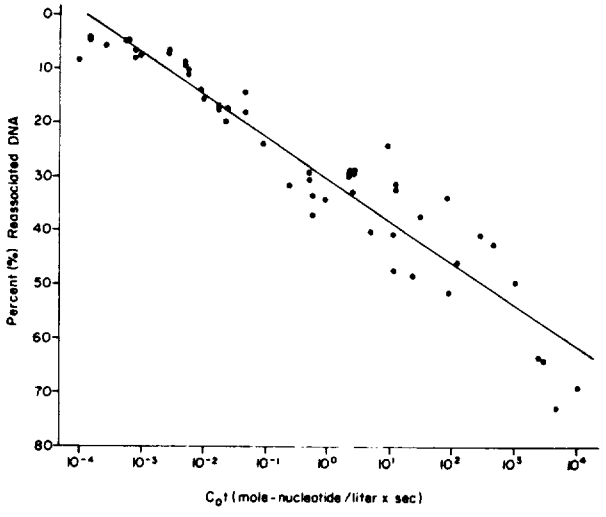

CHART 10

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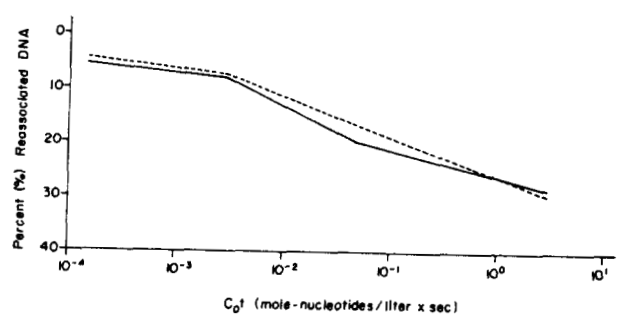

CHART 11

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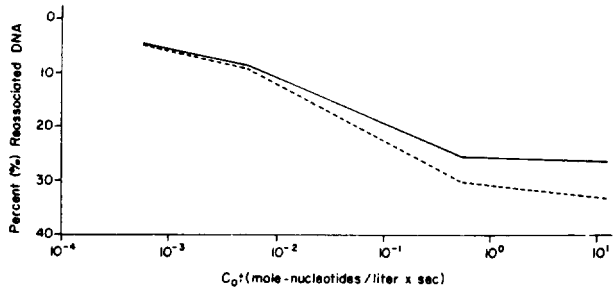

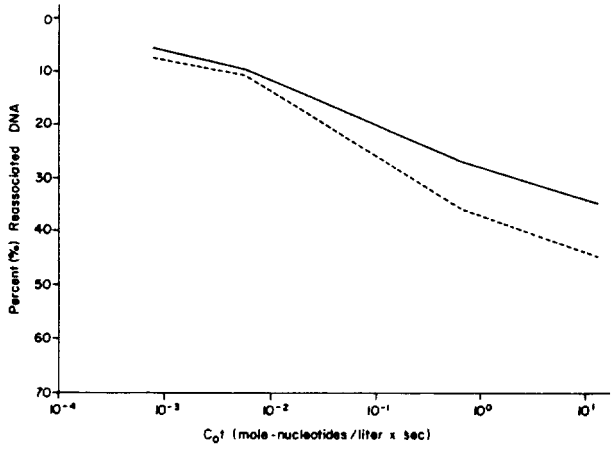

CHART 12

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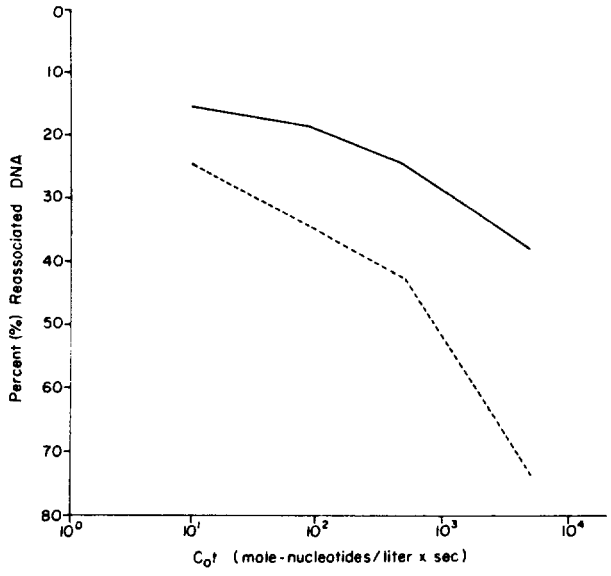

CHART 13

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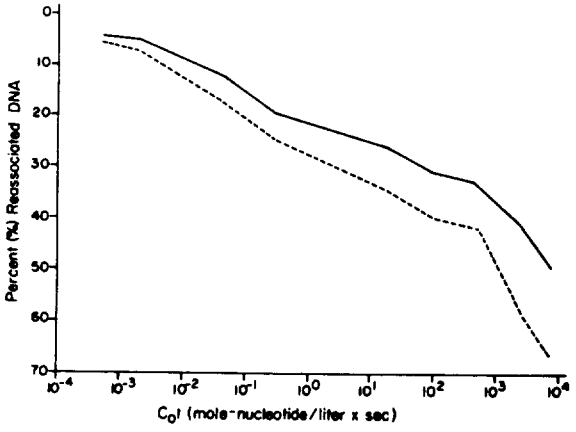

CHART 14

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Submitted For Publication, 1977

REPETITIOUS DISTRIBUTION OF DNA SYNTHESIZED IMMEDIATELY AND AT DIFFERENT TIMES AFTER ULTRAVIOLET LIGHT EXPOSURE OF MOUSE L-929 CELLS.

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Running Title

REPETITIVE DISTRIBUTION OF DNA SYNTHESIZED AFTER UV EXPOSURE.

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Footnotes

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1041840

Abstract

The DNA precursor ^{3}H-thymidine incorporation rate (dpm/μg DNA) in mouse L-929 cells decreases immediately after exposure to ultraviolet light. This decrease is initially dose dependent, but at exposures greater than 225 ergs/mm^{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). Since the radio-resistant incorporation amounted to approximately 35% of the control rate, the percentage which highly repetitious DNA contributes to the total mouse genome, we performed DNA/DNA "CoT" studies with DNA labelled with ^{3}H-thymidine immediately after exposure of L-929 cells with a dose of UV resulting in radio-resistant incorporation. We hypothesized that only repetitious DNA was continuing to incorporate ^{3}H-thymidine at this dose. Our CoT studies, performed in the highly repetitive to intermediate range, did not show the rapid reassociation of radioactivity which would have confirmed this; i.e., the radio-resistant synthesis is of DNA of all classes of repetitious frequency. DNA synthesized at different times after UV exposure, during the period when post-replication repair is presumed to be occurring, was similarly labelled for short intervals and isolated. The DNA/DNA CoT studies showed that this DNA synthesis was also of all classes of repetitious frequency.

1041841

Introduction

The inhibition of DNA synthesis immediately after ultraviolet (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; and 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 labelling intervals. Since this fraction is similar to the percentage of mouse DNA which is highly repetitious in terms of its sequence distribution using the DNA/DNA CoT technique (Britten and Kohne, 1968), we hypothesized that the residual synthesis might be due to continued synthesis of only one class of DNA, in particular highly repetitive DNA, and that the response in the cells after UV irradiation was a selective inhibition of intermediate and unique sequence DNA replication. We additionally posed the question as to whether DNA synthesized at different times after UV irradiation, i.e., during a period of time when post-replication repair is presumed to be occurring (Lehmann, 1972; and Fujiwara, 1972), 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-minute labelling interval, and the repetitious distribution of the DNA incorporating the precursor, are described below.

1041842

Materials and Methods

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 fungazone were also present in the medium. All materials were obtained from Gibco. 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.

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 saline A, aspirated, and then immediately exposed to ultraviolet light (9.4 ergs/mm^2/sec), using a specially built, ultraviolet light irradiation device (Steier and Cleaver, 1969). Immediately after irradiation, the cells were reincubated with warm complete medium containing 10 μCi/ml of tritiated thymidine (50.8 Ci/mM) adjusted to a final molarity of 5×10^{-6} M thymidine.

1041843

After incubation at 37°C for 30 minutes, the labelling 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 after aspiration by immersion in ethanol-dry ice.

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 minutes. The tube is then centrifuged for 15 minutes 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 hour to digest the ribonucleic acid. At the end of 1 hour, 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%

1041844

TCA. The tubes are allowed to stand in ice for 15 minutes. 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 minutes. After removal of the tube from the water bath, and allowing for 1 minute cooling at room temperature, the tube is immersed in ice for 15 minutes. 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 0.2 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 disintegrations per minute per microgram (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 for 36 hours at room temperature in a Beckman Spinco type 50 Ti rotor at 36,000 rpm in a Beckman L3-50 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

1041845

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 hours 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 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 technique (Heingardner, 1971).

For the DNA/DNA CoT studies, an appropriate concentration of DNA synthesized after irradiation (or control) and labelled with ^{3}H-TdR was mixed with an appropriate concentration of C^{14}-thymidine labelled, semi-conservatively synthesized L-929 DNA (24-hour 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, 0.002 M EDTA), e) measurement of DNA concentration by radioactive counts, f) denaturation of the samples in capillary 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

1041846

hydroxylapatite in columns to separate single-stranded from double-stranded DNA, and radioactive counting of the fractions in BBS-3 liquid scintillation solution. In performing these studies, we essentially followed the procedures described by Britten, Graham, and Neufeld (1974). All CoT reactions were performed in duplicate, and the value reported in the figures is the average.

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

Results and Discussion

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

1041847

residual resistant synthesis. A similar conclusion was reached by Cleaver (1965). For observing the sequence distribution of the ^{3}H-TdR labelled DNA synthesized after irradiation, exposure times were chosen which would provide labelled DNA from cells that had not been irradiated (0 sec), DNA which was labelled after an exposure which was half way down the dose response curve to the resistant level (12 sec), and an exposure which would result in labelling of the radio-resistant fraction (30 sec). The specific activities of the isolated DNA from two such experiments appear in Table 1. In performing the kinetic experiments, nonirradiated, 24 hour C^{14}-thymidine labelled, semi-conservatively synthesized DNA was included in each reaction mixture as an internal control. The experiments were performed over the low to intermediate CoT range, since we had hypothesized that the synthesis of only highly repetitive DNA was occurring in the radio-resistant region. This hypothesis would be verified if ^{3}H-TdR labelled DNA synthesized after 30 seconds of UV exposure were to show immediate renaturation at low CoT values as compared to the C^{14}-TdR labelled, semi-conservatively synthesized DNA. As can be seen in Fig. 2 for both of the experiments, our results do not indicate any such pattern; i.e., the UV radio-resistant incorporation is composed of DNA which is at least both highly and intermediate repetitive in frequency, and therefore also presumably unique in nature.

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 would presumably

1041848

be occurring (Lehmann, 1972; Fujiwara, 1972). For these experiments, the labelling medium contained 10 μCi/ml of high specific activity tritiated thymidine (52.6 Ci/mM). Rapidly proliferating cells in petri dishes were rinsed, exposed to UV at 9.4 ergs/mm^2 per second for 30 seconds, and then either immediately labelled for 20 minutes or reincubated for 1, 2, or 3 hours in fresh warm medium before addition of the labelling medium for 20 minutes. The cells were collected as described above. The delipidification and RNase digestion steps described above were not employed, but rather total nucleic acids were hydrolyzed with 10% TCA for determination of the acid precipitable ^{3}H-TdR radioactivity. The concentration of deoxyribonucleotides was determined using the diphenylamine reaction (Burton, 1956). The specific activities obtained as a function of post-irradiation time in hours appear in Fig. 3. A decreasing incorporation rate of precursor with increasing time post-irradiation is observed. The sequence distribution of DNA which was pulse-labelled at 0, 2, or 3 hours post-30-second UV exposure appears in Fig. 4. The distribution of the radioactivity appears to be uniform in the sequences of different repetitious character.

1041849

Acknowledgement

This work was supported by U.S.E.R.D.A. Contract No. AT-(40-1)-4761. The authors wish to acknowledge the assistance of Mr. Michael Mann in the hydrolysis experiments. These results were presented in part at the Annual Meeting of the American Society for Cell Biology, Abstract # 559, November, 1975.

1041850

References

1. Britten, R. J., D. E. Graham, and B. R. Neufeld (1974) Methods Enzymol. 29, 363-418.

2. Britten, R. J. and D. E. Kohne (1968) Science 161, 529-540.

3. Burton, K. (1956) Biochem. J. 62, 315-323.

4. Cleaver, J. E. (1965) Biochim. Biophys. Acta 108, 42-52.

5. Cleaver J. E. (1967) Radiat. Res. 30, 795-810.

6. Djordjevic, B. and L. J. Tolmach (1967) Radiat. Res. 32, 327-346.

7. Domon, M. and A. M. Rauth (1968) Radiat. Res. 35, 350-368.

8. Edenberg, H. J. (1976) Biophys. J. 16, 849-860.

9. Fleck, A. and H. N. Munro (1962) Biochim. Biophys. Acta 55, 571-583.

10. Fujiwara, Y. (1972) Exp. Cell Res. 75, 483-489.

11. Hinegardner, R. T. (1971) Anal. Biochem. 39, 197-201.

12. Klimek, M. and M. Valsinova (1966) Int. J. Radiat. Biol. 11, 329-337.

1041851

13. Lehmann, A. R. (1972) J. Mol. Biol. 66, 319-337.

14. Meltz, M. L. (1976) Environ. Res. 11, 359-366.

15. Meyn, R. E., R. R. Hewitt, L. F. Thomson, and R. M. Humphrey (1976) Biophys. J. 16, 517-525.

16. Rasumssen, R. E. and R. B. Painter (1964) Nature (London) 203, 1360-1362.

17. Schmidt, G. and S. J. Thannhauser (1945) J. Biol. Chem. 161, 83-89.

18. Steier, H. and J. E. Cleaver, (1969) Lab. Pract. 18, 1295.

1041852

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 minute pulse label after different UV-exposures. UV dose rate = 9.4 ergs/mm^{2}/sec. Upper figure; first experiment; lower figure; second experiment.

Figure 2. Kinetic data obtained upon reassociation incubation of labelled DNA isolated from UV-irradiated mouse L-929 cells after 30 minutes post-UV incubation with ^{3}H-thymidine at 10 μCi/ml; (——) 30 minute ^{3}H-thymidine pulse-labeled DNA; (----) 24-hour semi-conservatively synthesized C^{14}-thymidine labelled mouse L-929 DNA. Upper left to lower left figures, reassociation data for DNA isolated in Experiment 1 after 0 seconds, 12 seconds, or 30 seconds UV exposure; upper right to lower right, reassociation data for DNA isolated in Experiment 2 after 0 seconds, 12 seconds, or 30 seconds UV exposure.

Figure 3. Specific activity in dpm/μg of DNA isolated from mouse L-929 cells which were pulse-labelled for 20 minutes with ^{3}H-thymidine beginning at different times after a 30-second UV exposure.

Figure 4. Kinetic data obtained upon reassociation incubation of DNA pulse-labelled at 0, 2, or 3 hours post 30-second UV exposure. Upper curve: DNA pulse-labelled immediately after UV irradiation; middle curve: DNA pulse-labelled beginning 2 hours post exposure; lower curve: DNA pulse-labelled beginning 3 hours post exposure.

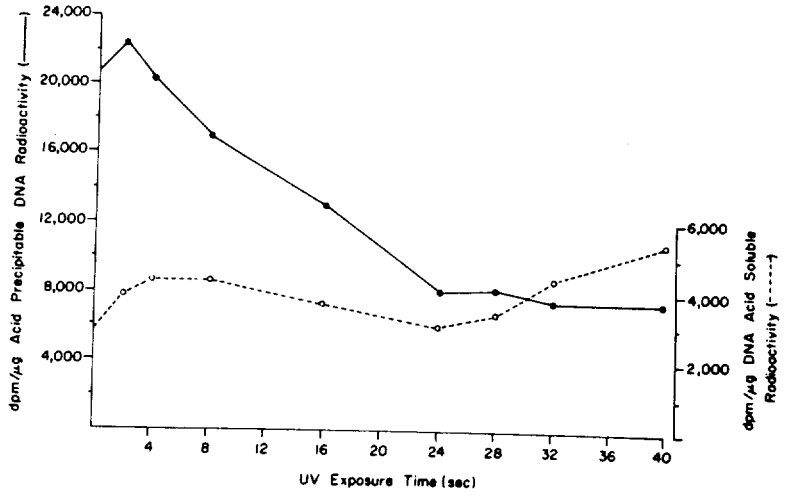
1041853

TABLE 1

Specific Activity (dpm/μg) of DNA Isolated From L-929 Mouse Fibroblast Cells, Labelled After Different UV Exposures.

UV Exposure Time (Secs)	Experiment 1		Experiment 2	
	dpm/μg	% of Control	dpm/μg	% of Control
0	42,991		54,651	
12	26,995	62.8	31,928	58.4
30	16,036	37.3	18,387	33.6

1041854



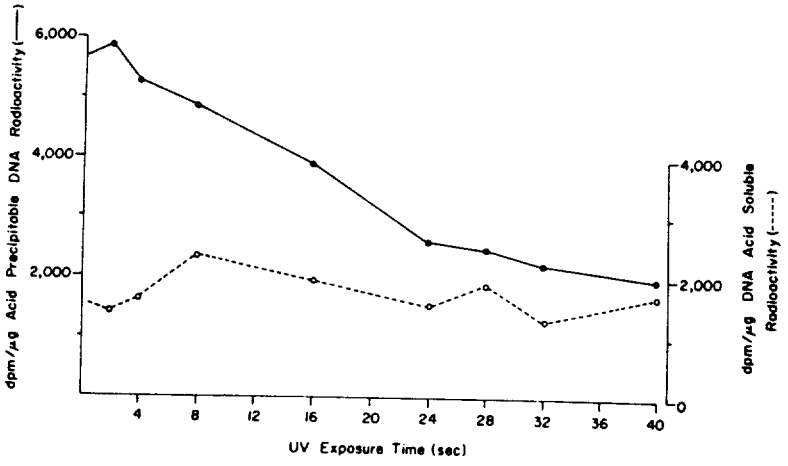


FIGURE 1

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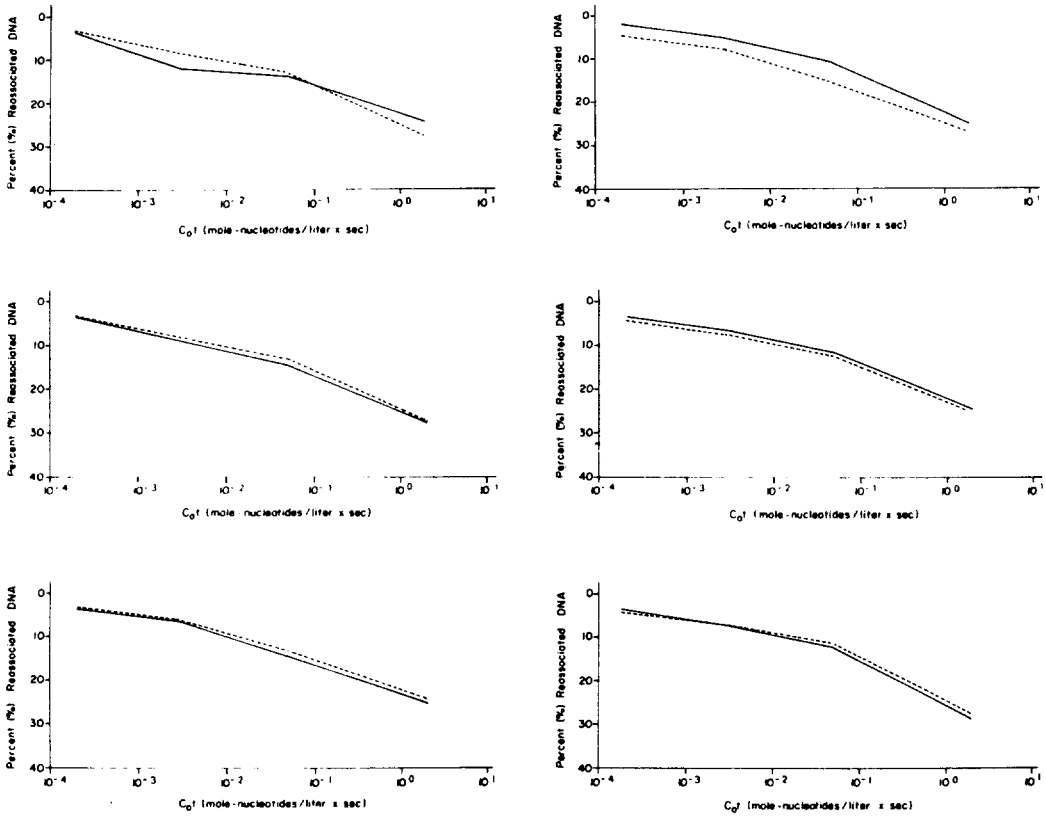

FIGURE 2

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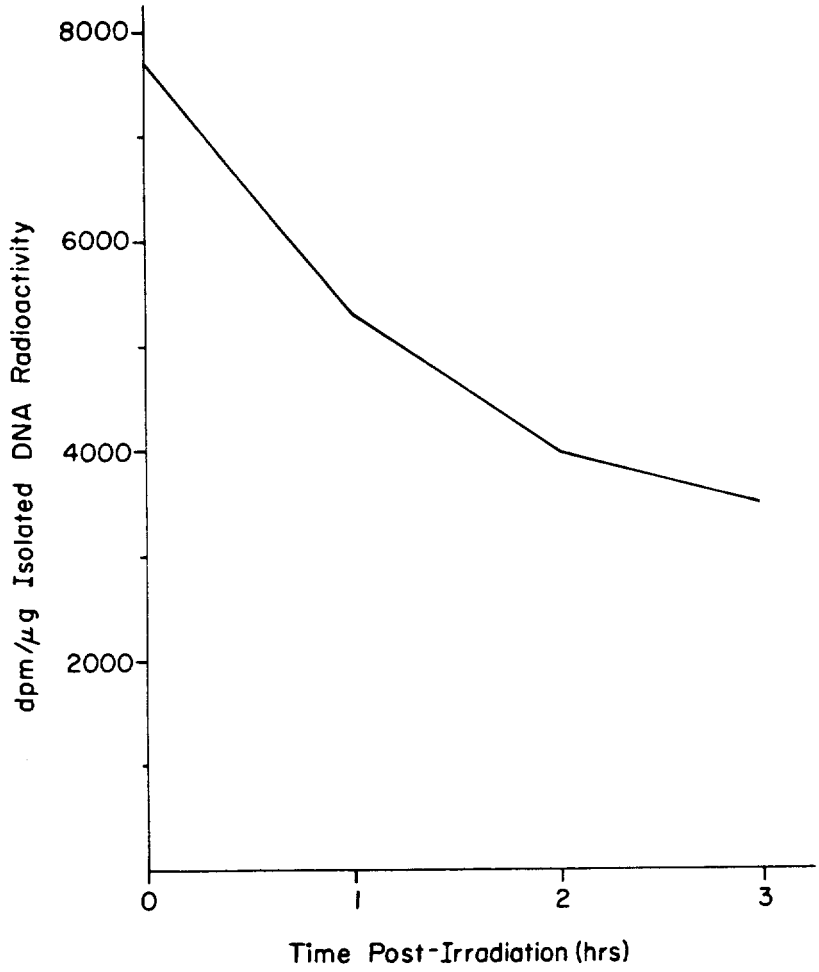

FIGURE 3

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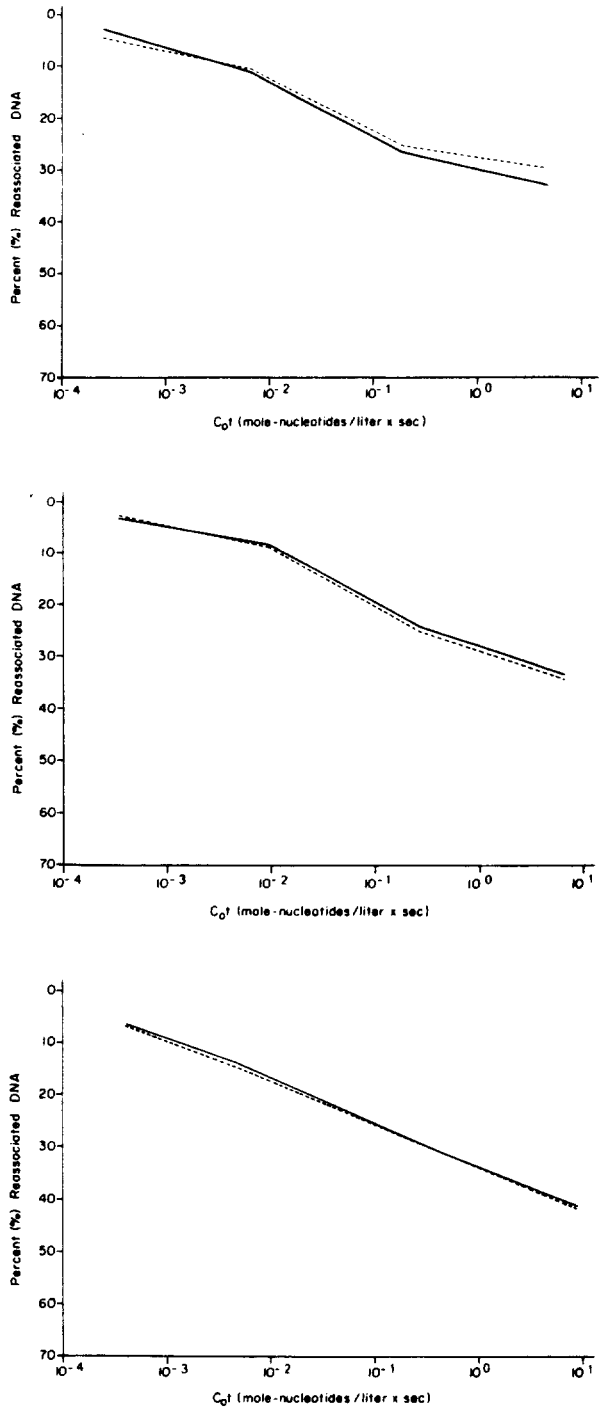

FIGURE 4

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