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PROGRESS REPORT

February 1975

REPETITIOUS NATURE OF REPAIRED DNA IN MAMMALIAN CELLS

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June 1, 1974 - May 31, 1975

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REPETITIOUS NATURE OF REPAIRED DNA IN MAMMALIAN CELLS

Progress Report

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June 1, 1974 - May 31, 1975

PREPARED FOR THE U.S.E.R.D.A.

UNDER CONTRACT NO. AT-(40-1)-4761

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I. Scope of Investigation and Significant Results

The experimentation during the initial period of study has focused on three cell lines and three agents known to act as mutagens or carcinogens. The cell lines are the mouse L-929 fibroblast, the WIL_2-A3 human lymphoblastoid cell clone, and the Raji line of Burkitt's lymphoma. The agents are ultraviolet light, methyl methanesulfonate, a direct acting alkylating agent, and benz(α)pyrene. The latter was chosen for study because of its production during combustion of fuels for energy purposes, because it must be metabolized to an active chemical form before being carcinogenic, and because its metabolism is dependent on the presence of the enzyme aryl hydrocarbon hydroxylase.

A. L-929 Mouse Fibroblast Cell Line

The studies to date have been concerned with measuring two phenomena, (a) the effects of ultraviolet light on the levels of DNA precursor uptake and incorporation immediately after irradiation, and (b) the extent of DNA repair replication in units of dpm/μg of DNA as a function of UV dose and of time after exposure to a single dose.

The former study was initiated because of reports of resistant DNA synthesis immediately after X-ray exposure of L5178Y cells (1), and the realization that the radioactive DNA precursor incorporation after exposure of cells to UV light is a function of at least two components, (a) residual normal semi-conservative synthesis, and (b) repair replication. Our initial study was to determine whether a UV

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resistant DNA synthesis component (or rather ^{3}H-TdR incorporation component) was present.

As can be seen in Figure 1, after an exposure of L-929 cells to UV light (9.4 ergs/mm^2 sec) for 24 secs, the ^{3}H-TdR incorporated (dpm/μg DNA) plateaus, indicating a possible "radio-resistant" component. This component may actually be due to either one or a combination of several phenomena; i.e., (1) induced repair replication occurring at an unusually high rate (compared to the normal semi-conservative incorporation rate for such a short 30 min incubation time), or (2) due to an increased uptake of radioactive label (which appears to be the case at exposures greater than 24 secs; Figure 1), or (3) due to a radio-resistant component of DNA synthesis.

To further examine this component, L-929 cells were exposed in a second experiment to 0, 12, and 30 sec of UV light. In this experiment, instead of using a modified Fleck and Munroe (2) hydrolysis procedure as for the first experiment, the DNA was isolated using our standard isolation procedure of lysis with shearing in the presence of SDS, RNAse and pronase digestion, and deproteinization with chloroform:isoamyl alcohol. The DNA was further purified by CsCl density gradient centrifugation at neutral pH. The samples were fractionated by top collection in the Isco density gradient fractionator, pooled, and dialyzed. As can be seen in Table 1, the response in terms of percent of control incorporated radioactivity fter UV exposure in the two experiments was similar.

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Table 1

Percent of Control ^{3}H-Thymidine Incorporation into L-929 Cell DNA During 30 Min Pulse after UV Exposure

	Experiment 1		Experiment 2	
	Hydrolysis		DNA Isolation	
	dpm/μg DNA	Percent of Control	dpm/μg DNA	Percent of Control
Control	20586	100	42991	100
8 sec	16815	81.7		
12 sec			26995	62.8
16 sec	13076	63.5		
28 sec	8082	39.3		
30 sec			16036	37.3
32 sec	7384	35.9		

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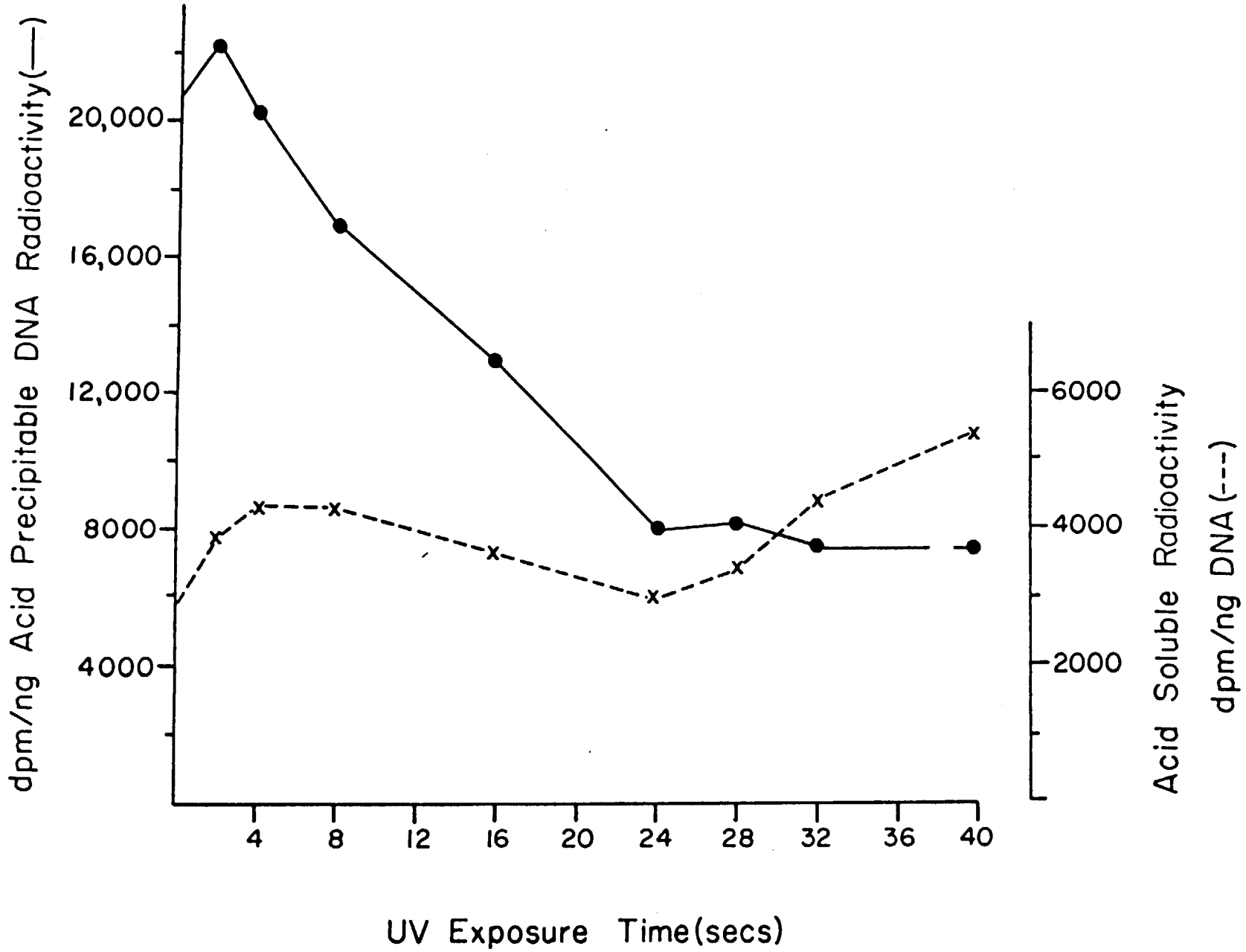
Fig. 1 L-929 Cell H-TdR Uptake and Incorporation After UV Irradiation



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The latter samples have been frozen, and will be examined for the distribution of DNA precursor incorporation into DNA sequences of different repetitive frequencies in March, 1975, using the improved DNA/DNA Cot procedures at low Cot values to be described below. I believe that this is the first time that this type of analysis is being applied to DNA precursor incorporation studies immediately after treatment with a DNA synthesis inhibiting agent; we will be examining similar events occurring as a result of chemical carcinogen treatment.

In preparation for the DNA/DNA Cot studies, we have labeled semi-conservatively synthesized DNA by incubating L-929 cells for 24 hrs with either 2.0 μCi/ml (5×10^{-6} M) ^{3}H-TdR or 0.5 μCi/ml ^{14}C-TdR (5×10^{-6} M). The DNA has been isolated from the cells, and further purified by CsCl (neutral pH) density gradient centrifugation. Bulk L-929 cell nonradioactive DNA has similarly been isolated and purified, and is currently being concentrated by centrifugation at 42,000 rpm for 16 hrs. This bulk DNA will be added to appropriate amounts of experimental radiolabeled L-929 cell DNA for shearing as described below.

Establishment of Conditions for Maximum Repair Replication Labeling of UV-Damaged L-929 Cell DNA.

1. Repair replication vs. UV dose.

In this experiment, repair replicated DNA was purified by 2 sequential CsCl (neutral pH) density gradient centrifugations, followed by one alkali CsCl - Cs_2SO_4 (4.8 gms CsCl + 1.0 gm Cs_2SO_4 + 4.5 ml DNA solution at 0.1 N NaOH) density gradient centrifugation. This was the

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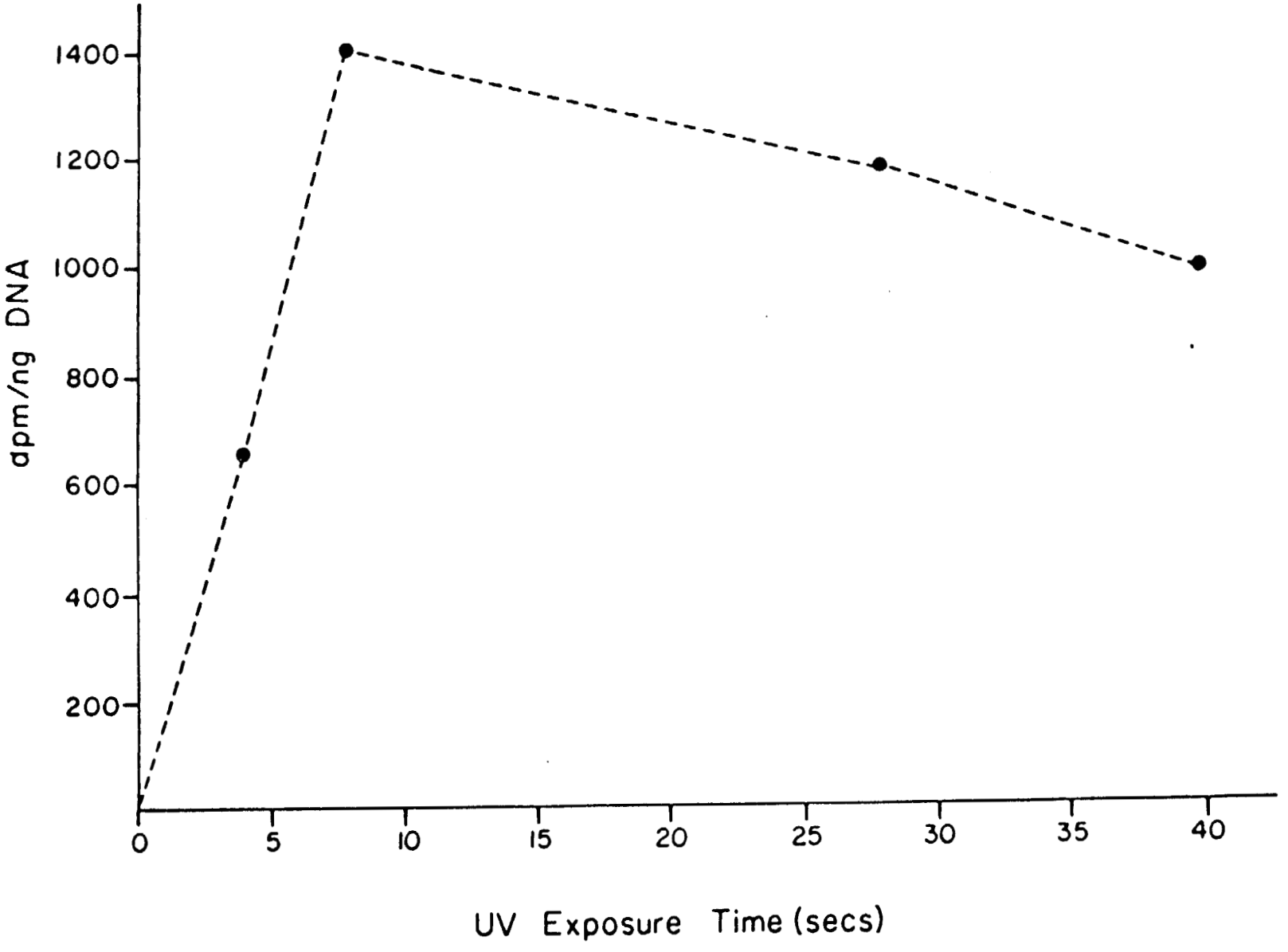
first repair replication experiment performed under this contract. The neutral CsCl gradients were collected by top collection using an Isco density gradient fractionator (0.3 ml fractions) and the alkali gradients were collected by bottom collection using a Buchler bottom collection device and Isco Golden Retreiver Fraction Collector (10 drop fractions). The needle in the Buchler apparatus had to be modified in our shop by welding the needle end of an Isco needle, with side holes, to the holder end of the Buchler needle, which was found to plug on puncturing the polyallomer tubes.

The results of this first experiment are presented in Figure 2. In this experiment, we followed a standard procedure for repair replication as follows: Preincubate for 1 hr with 10^{-6} M fluorodeoxyuridine (FUdR) and 5 x 10^{-6} M 5-bromodeoxyuridine (BrUdR); rinse the cells with warm saline A to remove residual serum, expose to UV light, add labeling media containing 20 μCi/ml ^{3}H-BrUdR (adjusted to 5 x 10^{-6} M BrUdR with nonradioactive BrUdR), 10^{-6} M FUdR, and 10^{-3} M hydroxyurea (HU), incubate for 3 hrs, wash with warm saline A to remove radiolabel, and reincubate for 1 hr with 10^{-6} M FUdR and 5 x 10^{-6} M BrUdR.

As can be seen in Figure 2, the repair replication occurring during 3 hrs appears to saturate at a dose of less than 10 sec (9.4 ergs/sec). The exact value was not determined in this first experiment due to collapse of the tube containing the 16 sec exposure time samples; we have chosen 16 sec, however, as a dose-time to obtain maximum repair replication radiolabeling.

Fig. 2 Repair Replication of L-929 Cell DNA as a Function of UV Dose



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2. Repair replication of L-929 cell DNA after 16 sec exposure as a function of radiolabeling time.

In this experiment, the times allowed for repair replication radiolabeling to occur were 30 min, 1 hr, 3 hr, and 5 hr. In Figure 3, we see that repair replication radiolabeling in terms of dpm/μg appears to continue to increase through the 5 hr time point. As can be seen in Table 2, the DNA yield (DNA recovered) from the same number of exposed dishes at each time point is significantly less at 3 and 5 hrs than at 30 min and 1 hr. Since the procedure used for radiolabeling includes a rinse of the plastic attached cells immediately after radiolabeling, and before a further 1 hr incubation with BrUdR-FUdR media, I am concerned that we may be losing cells which did not remain attached, and which did not undergo repair, thus raising the average dpm/μg of repair replicated DNA above the level which might otherwise have resulted had they not been removed from the final collected cell population. I have not explored this problem further, but rather decided to continue using a 5 hr radiolabeling interval after UV exposure in preparative experiments for obtaining maximally labeled repair replicated DNA for DNA/DNA Cot studies.

Preparative Experiments for DNA/DNA Cot Studies.

Mouse L-929 cells were exposed to 16 secs of UV light using the standard protocol, and incubated with 100 μCi/ml ^{3}H-BrUdR (at approximately 0.83 x 10^{-5} M BrUdR) for 5 hrs before rinsing and re-incubating. The DNA from these cells is being isolated and CsCl density gradient purified at the present time, and will be available by March 1 for DNA/DNA Cot studies.

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Fig. 3 Repair Replication of L-929 Cell DNA
After 16 sec. UV Exposure (9.4 ergs/mm²-sec)
As a Function of Repair Labeling Incubation Time

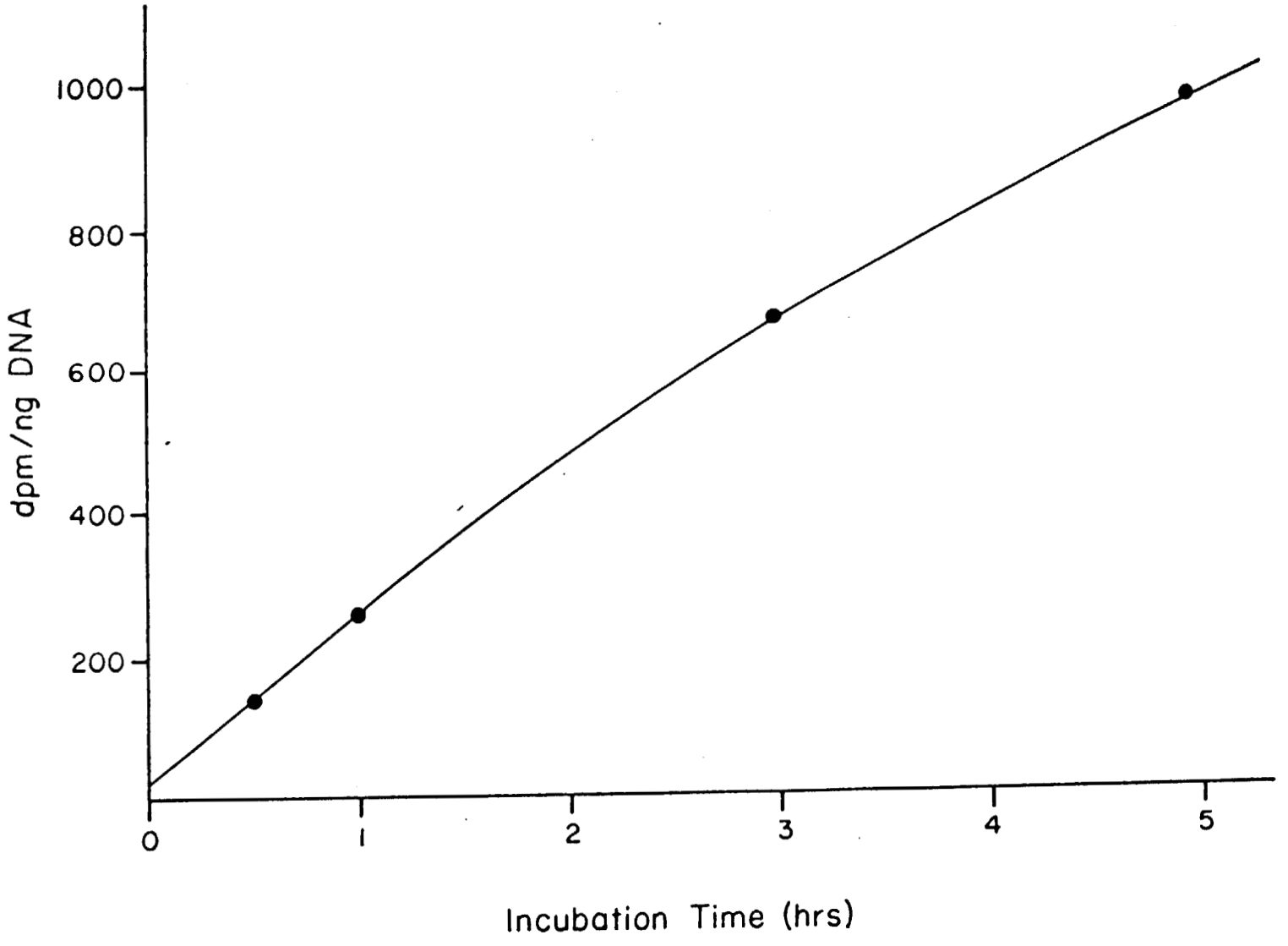

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Table 2

L-929 Cell DNA Repair Replication as a Function of Repair Labeling Time after UV Exposure (16 sec; 9.4 ergs/mm^2-sec)

Incubation Time (hrs)	dpm/μg	Total DNA Yield (μg)
0	28	42
0.5	141	47
1	255	44
3	663	18
5	964	15

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Because of the expense of ^{3}H-BrUdR, we have also attempted to radiolabel UV-induced (16 sec) repair replicated DNA with high specific activity ^{3}H-TdR and nonradioactive 5-BrUdR such that the total DNA precursor analog concentration is 10^{-5} M; the TdR:BrUdR ratio is approximately 1:9. In the published procedures, the ratio is usually 1:100; such a ratio would not be useful in our DNA/DNA Cot experiments requiring maximally labeled repair replicated DNA. The DNA from these cells is also currently being isolated and purified by CsCl density gradient centrifugation. It also will be available for DNA/DNA Cot studies in March.

As will be indicated below for the human cell lines studied, extensive repair replication of DNA occurred after a 1 hr incubation with the direct acting alkylating agent methyl methanesulfonate at a concentration of 10^{-3} M. We have therefore treated L-929 cells for 5 hrs with 10^{-3} M MMS in the presence of radiolabel (120 μCi/ml ^{3}H-BrUdR at 10^{-5} M) plus 1 mM HU and 10^{-6} M FUdR, this treatment period preceeded and followed by incubation with 10^{-6} M FUdR + 10^{-5} M BrUdR for 1 hr intervals. This DNA is currently being isolated and purified, and will be ready for DNA/DNA Cot studies in March.

The reason for the procedural change, i.e., the co-incubation with carcinogen and repair replication radiolabel, is our desire to measure all of the repair replication occurring upon carcinogen treatment, and not just the repair replication of residual damage left after a prior incubation with carcinogen. It must be mentioned that

in this experiment, the incubation bottle was placed on a slowly moving rocker platform in our warm room during the 5 hr incubation, and more than 50% of the cells had detached by 5 hrs. In order not to lose these cells, all of the cells were scraped free into solution, and washed by centrifugation. The resuspended cells were then returned to a new bottle for the 1 hr chase incubation with BrUdR-FUdR media. No substantial cell attachment was observed during this 1-hr interval.

As indicated above, a number of experimental samples have been prepared for DNA/DNA Cot studies, and these will be initiated in March and continue on a regular basis for the remainder of the contract period. As will be described later, we are making a substantial effort to maximize the specificity of the DNA/DNA Cot reaction to be used in this study, and these modifications will be described in the procedural modifications section.

B. Human Lymphoblastoid Cell Lines: "Normal" WIL_2-A3 and Burkitt's Lymphoma Raji Line

The WIL_2-A3 and Raji cell lines are maintained on the rotating shaker in our 37° C warm room in capped culture flasks in Eagles Minimal Essential Medium with 10% fetal calf serum supplemented with sodium pyruvate (1 mM), L-glutamine (4 mM), twice the normal amount of non-essential amino acids, and antibiotics. Growth curves for these cell lines are shown in Figures 4 and 5. As can be seen for both cell

Fig. 4 WIL_2-A3 Growth Curve:
Cell Concentration and (Percent Viability)

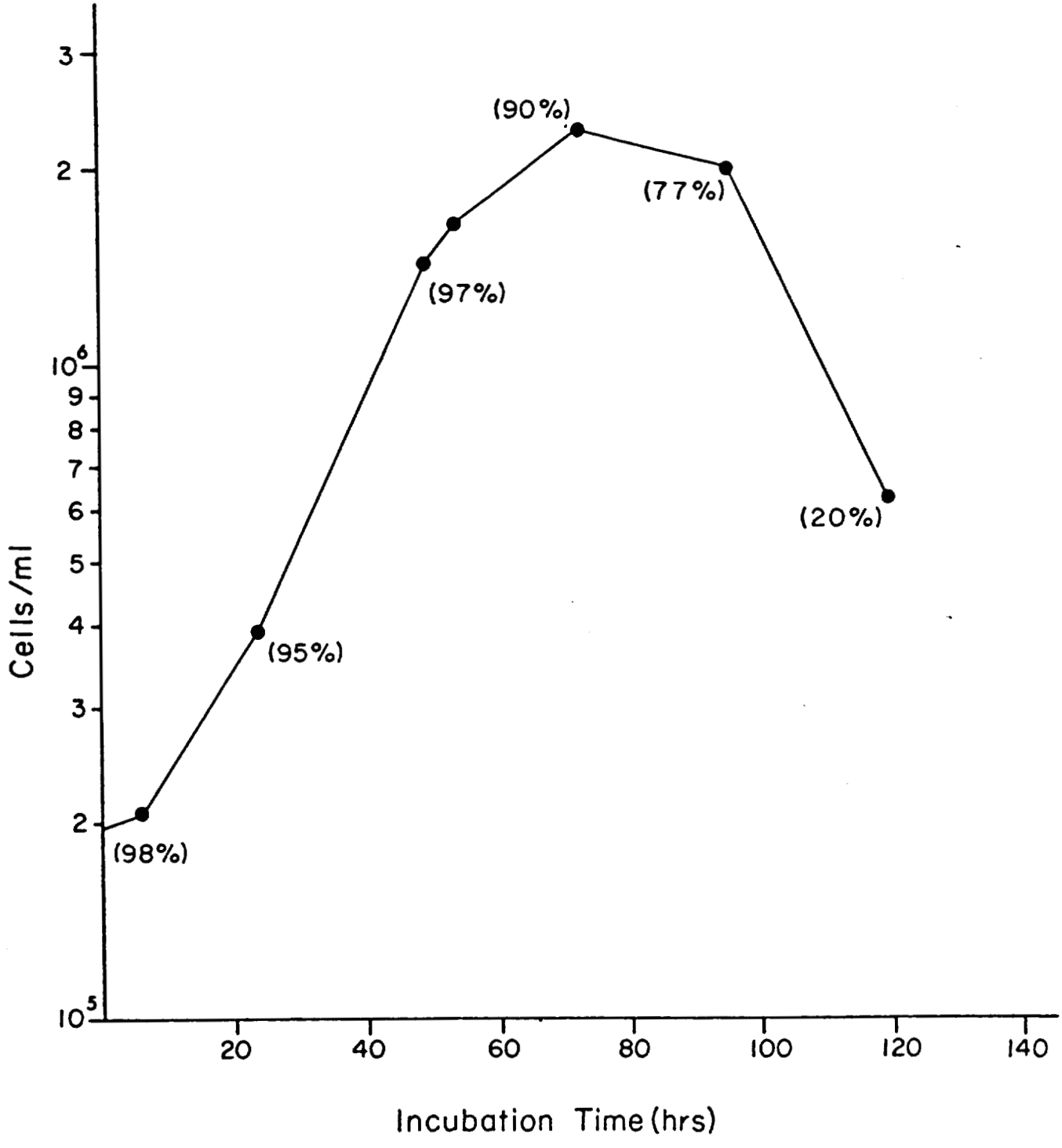

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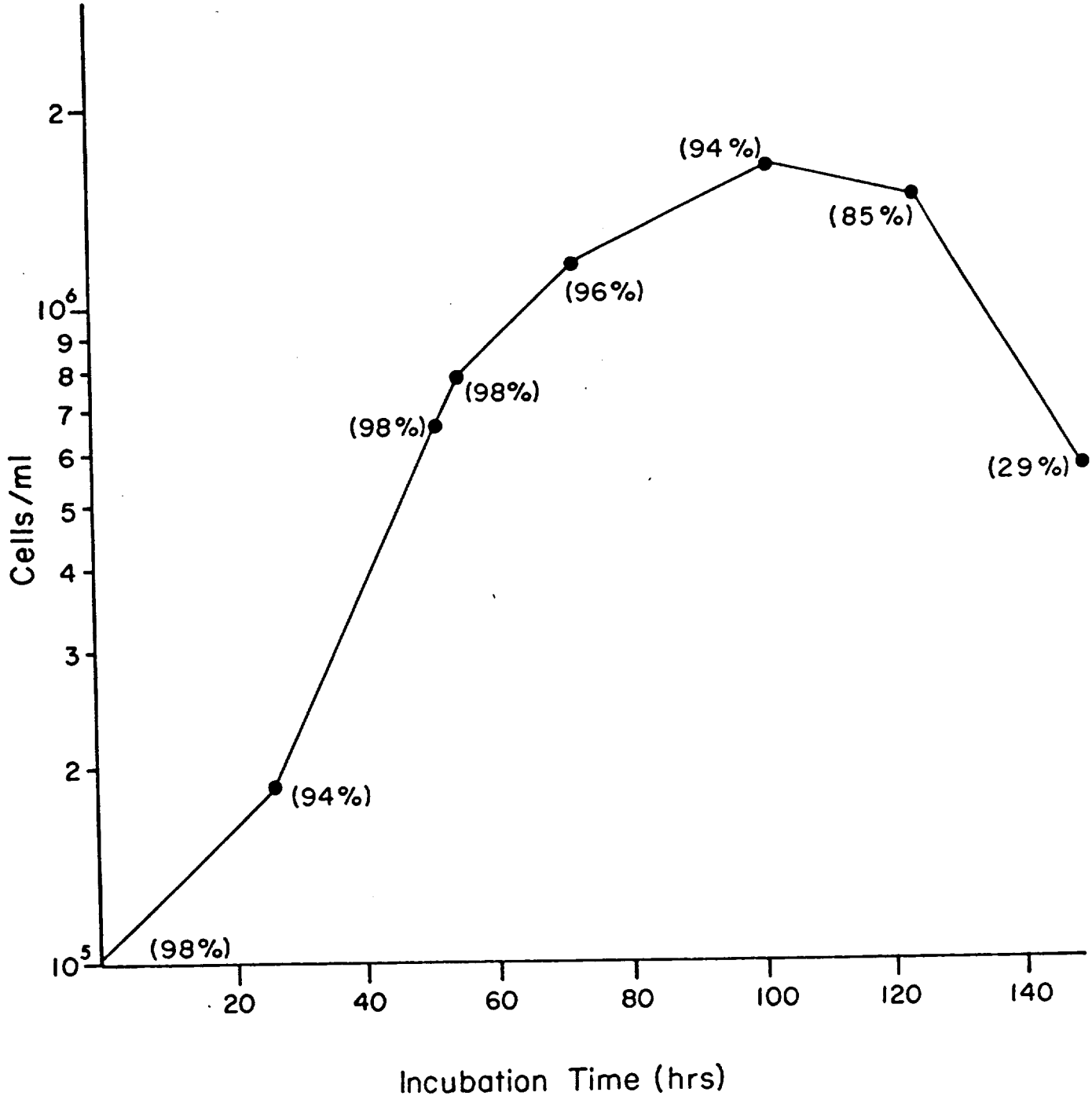
Fig. 5 RAJI Growth Curve:
Cell Concentration and (Percent Viability)



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lines, after the maximum cell number is achieved, there is rapid death of the cells, as measured by using dye exclusion (trypan blue), and also as indicated by the decreasing coulter count (decreased cell capacitance).

Addition of cell free-media prepared after cells were in this "death phase" to cells in logarithmic growth did not affect the proliferation of the latter.

In preparing to perform the experiments with ultraviolet light irradiation using the human cell lines, we discovered early in our investigation that the human WIL_2-A3 cells, on removal of serum before irradiation, rapidly and extensively attached to the walls of all glassware used during the washing procedure and exposure. This occurrence necessitated our initiating an extensive series of experiments to develop a protocol which would allow us to satisfactorily perform the UV study. This attachment loss did not occur with Raji cells. In our initial repair replication protocol for suspension cells, the cells were first centrifuged out of their growth media with negligible cell loss, and resuspended in BrUdR-FUdR preincubation media. After 1 hr incubation, they were again centrifuged from complete media so as to resuspend them in wash solution containing no fetal calf serum. As soon as this resuspension was performed, extensive cell loss by attachment to the centrifuge and pipette walls began, whether glass or plastic tubes or pipettes were employed. We initially attempted to solve the problem by testing a vaiety of salt solutions as the wash solution;

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these inlcuded (a) MEM without fetal calf serum, (b) Dulbecco's PBS (Ca^{++} and Mg^{++} free), (c) Dubecco's PBS (with Ca^{++} and Mg^{++}), (d) EBSS (Ca^{++} and Mg^{++} free), (e) EBSS (with Ca^{++} and Mg^{++}), (f) HBSS (with Ca^{++} and Mg^{++}), and (g) Spinner's salt solution with Earles salts and without fetal calf serum. With either plastic or glass labware, our results were unsatisfactory.

We then took advantage of a procedure described in the literature for growing HeLa cells, normally glass attached, in suspension by incubating them in the presence of 5 μg/ml dextran sulfate. We found that by using a modification of this procedure, i.e., preincubating in BudR-FUdR media with dextran sulfate for 1 hr and then washing in phosphate buffered saline (PBS) with dextran sulfate and suspending in the same for irradiation, we could obtain a cell recovery of approximately 50% at 85% viability for unirradiated cells as compared to extensive loss in the initial procedure.

We also investigated and found that incubation of the WIL_2-A3 cells in the presence of 5 μg/ml dextran sulfate resulted in a growth profile essentially the same as that seen in Figure 4; i.e., the dextran sulfate at 5 μg/ml was not detrimental to cell growth.

In the repair replication experiments with both the WIL_2-A3 cells and Raji cells described below, the dextran sulfate was present for both cell lines during the preincubation period, during the first washing, and during UV exposure. The same dextran sulfate procedure was not required for the Raji cells, but was employed for comparative purposes; dextran sulfate at 5 μg/ml did not affect the Raji growth curve.

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Repair Replication of WIL_2-A3 and Raji Burkitt's Lymphoma Cells after UV-Induced Damage.

The procedure for performing these experiments is essentially as follows:

Four 250 ml flasks were mass cultured with cells to a concentration of approximately 700,000 cells/ml in 50 ml. The media in the flasks was adjusted to 10^{-6} M FUdR, 5 x 10^{-6} M BrUdR, and 5 μg/ml dextran sulfate. At the end of the 1 hr preincubation period, the cells in each flask were decanted into two 50 ml conical tubes, and centrifuged at 800 rpm for 8 min at room temperature in an International PR-J centrifuge. The supernatant was aspirated, and the cells in each tube were suspended with 5 ml of PBS-dextran sulfate. 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 x 10^7 per tube, were resuspended in 8 ml of PBS-dextran. For each dose (0, 2, 5, and 10 sec) 2 ml aliquots of the above 8 ml were distributed into 100 mm plastic optilux dishes for irradiation. Immediately after irradiating the four dishes at each dose, the cells were transferred by pipette back into a centrifuge tube, the dishes were washed with a fresh 4 ml of dextran sulfate-PBS (this was added to the original suspension), centrifuged, aspirated, and resuspended in 10 ml of labeling medium containing 10^{-6} M FUdR, 1 mM HU, and 10 μCi/ml ^{3}H-BrUdR (adjusted to 5 x 10^{-6} M). After 3 hrs of repair replication labeling, the cells

Fig. 6 Repair Replication of WIL_2-A3 Cell DNA After UV Irradiation (9.4 ergs/mm^2-sec)

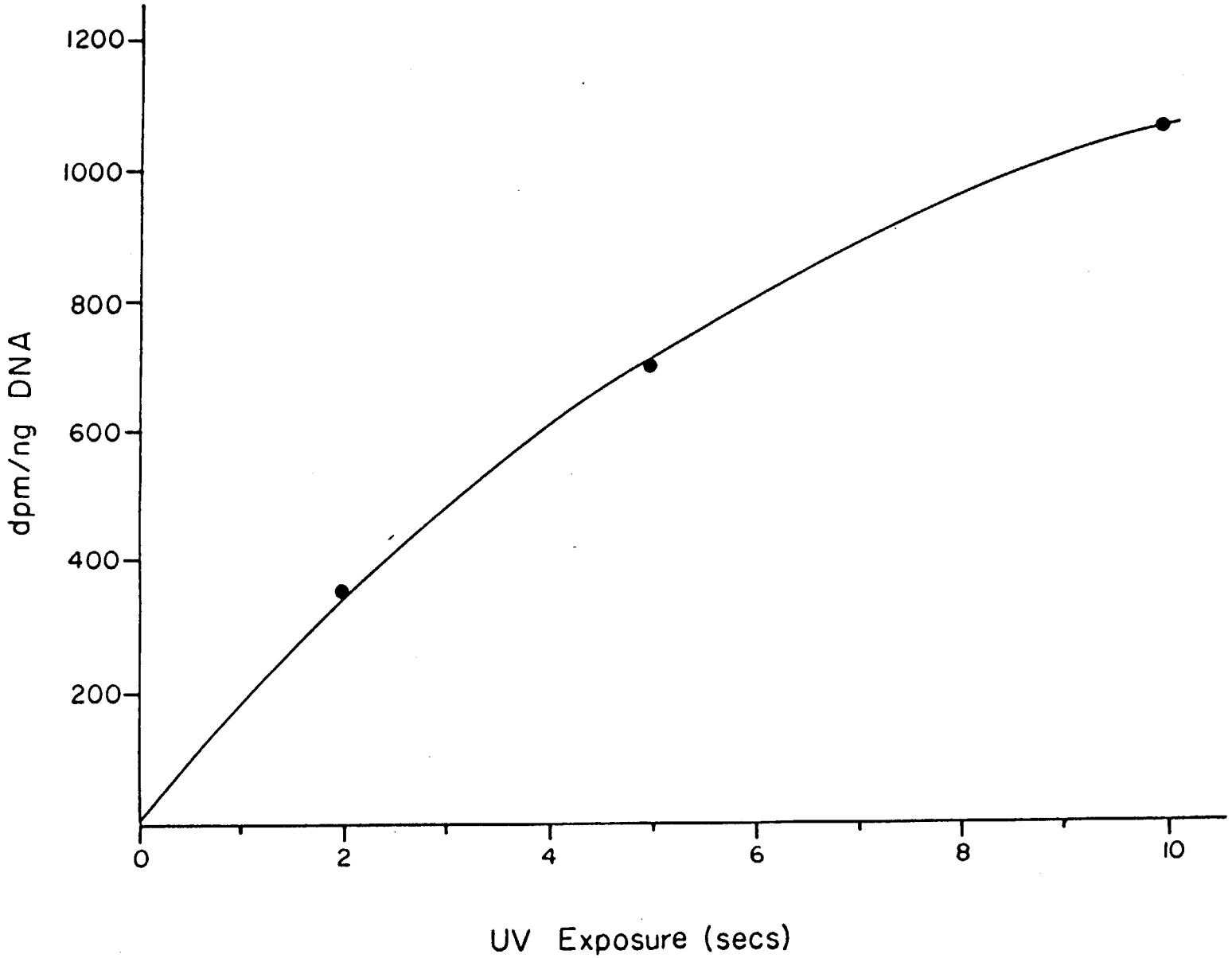

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Fig. 7 Repair Replication of RAJI Cell DNA After UV Irradiation (9.4 ergs/mm²-sec)

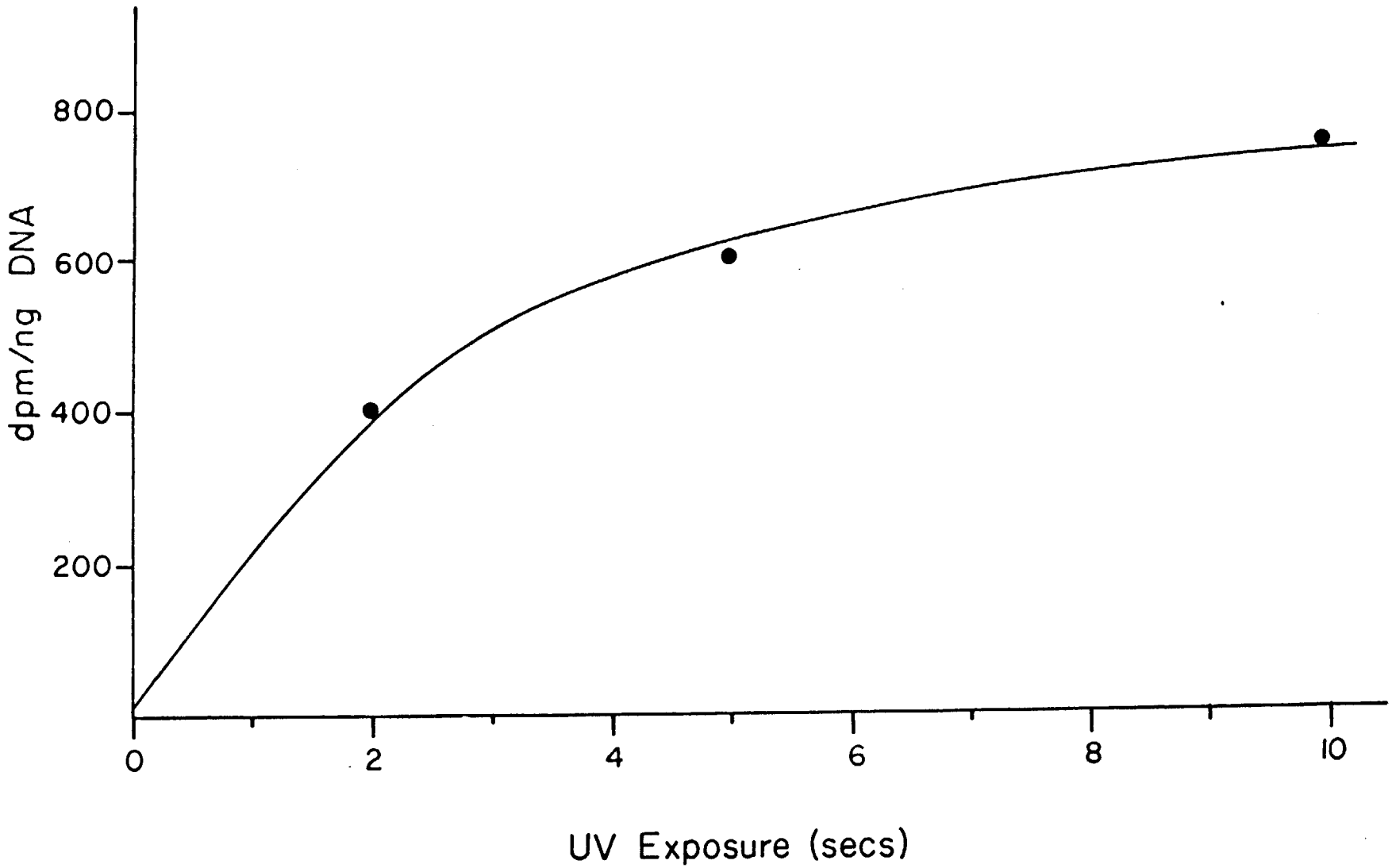

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Table 3

Repair Replication of Human Lymphoblastoid Cell DNA after UV Irradiation (9.4 ergs/mm^2-sec): Dose Dependence

Exposure Time (sec)	WIL_2-A3 dpm/μg	Raji dpm/μg
0	10	11
2	347	399
5	695	598
10	1059	746

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were centrifuged, washed with fresh BUdR-FUdR media containing fetal calf serum, and finally resuspended in BrUdR-FUdR media for a 1 hr incubation.

The experimental results are shown in Figures 6 and 7 and are summarized in Table 3. The values are similar to a degree which would indicate that any viral information present in the DNA of the non-virus producing Raji cell line is active in terms of its repair replication enzyme activities to no greater a degree than that in the "normal" WIL_2-A3 cell line, when the damage is produced by UV irradiation exposure. Damage by UV light would not be effected by differential masking of different DNA regions by chromosomal constituents such as histones and acidic proteins. In the remaining time of the current contract period, the UV study will be extended to the Burkitt's EB_3 cell line, which is a viral producer, to see if active viral production affects the extent of repair after UV damage.

Repair Replication of WIL_2-A3 and Raji Cell Lines after Acute Exposure (1 hr) to Methyl Methanesulfonate.

In these experiments, both the incubation medias and the wash media included fetal calf serum, so the difficulties with cell loss encountered in the UV experiments were avoided.

The procedure followed was very similar to that described above. The major difference was that after the preincubation for 1 hr in media containing 10^{-6} M FUdR and 5×10^{-6} M BrUdR, the cells were centrifuged and resuspended in BrUdR-FUdR media containing either 0, 10^{-3} M, 10^{-4} M,

Fig. 8 Repair Replication of Human Lymphoblastoid Cell DNA After Acute (1hr) Treatment With MMS

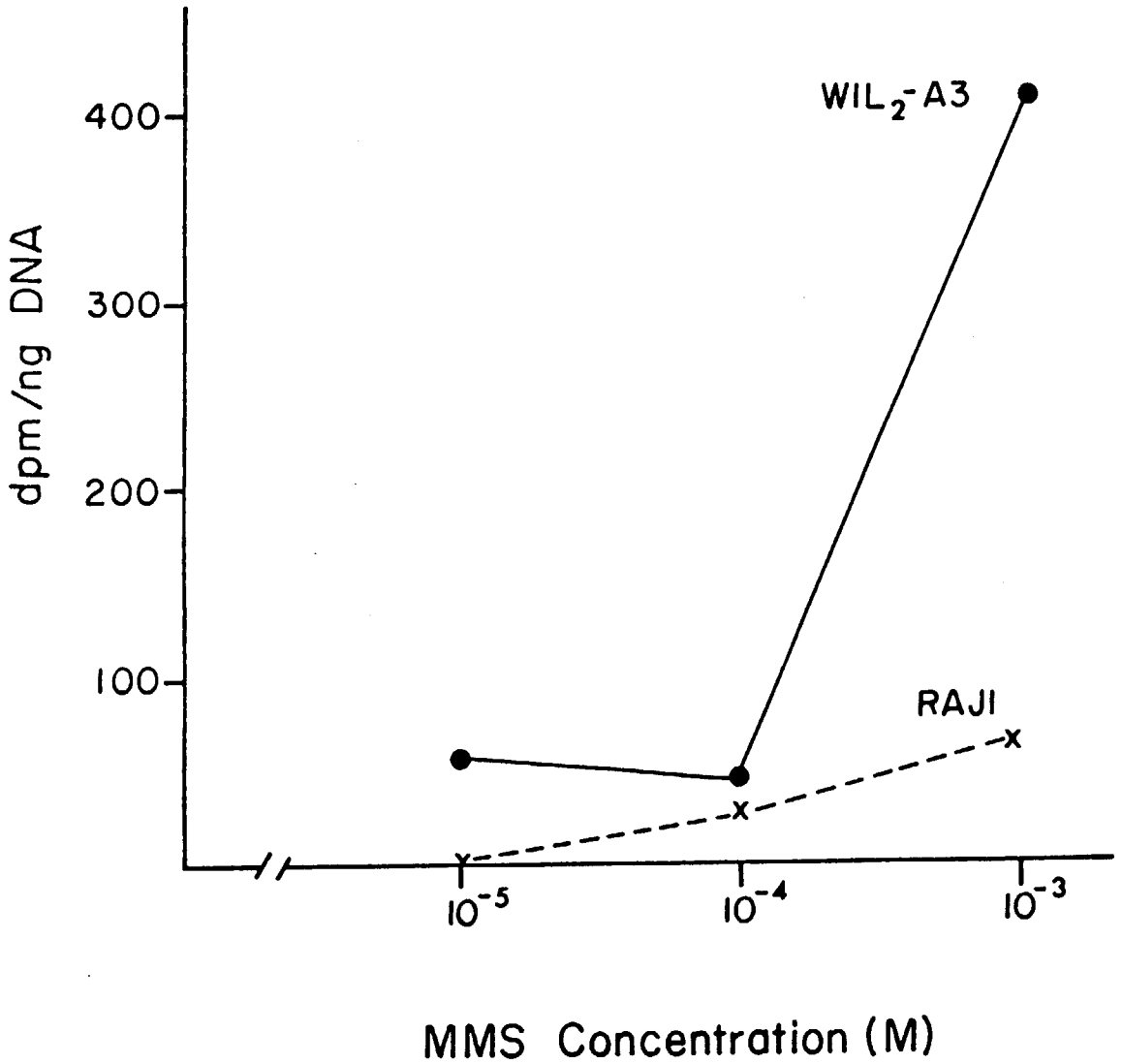

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Table 4

Repair Replication of Human Lymphoblastoid Cell DNA after Acute (1 hr) Treatment with Methyl Methanesulfonate (MMS): Concentration Dependence

MMS Concentration (Molarity)	WIL_2-A3 dpm/μg	Raji dpm/μg
Control	5	--
10^{-5} M	57	1
10^{-4} M	44	29
10^{-3} M	403	67

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or 10^{-5} M methyl methanesulfonate (MMS). After an addditional 1 hr of incubation, the cells were again centrifuged, washed one time in fresh media with fetal calf serum by centrifugation and aspiration, and then resuspended in repair replication labeling media for a 3 hr incubation. They were then washed one time and again incubated in BrUdR-FUdR media for one hr.

The results of these experiments are summarized in Figure 8 and in Table 4. These results indicate a possibly significant difference in the extent of repair replication occurring in dpm/μg between the WIL_2-A3 and the Raji lines at an MMS concentration of 10^{-3} M: We will be repeating these experiments in the 10^{-4} to 10^{-5} M MMS concentration range to verify this observation; the DNA yield of the 10^{-3} M WIL_2-A3 point was lower than expected. It must be remembered that the final repair replication observed in these experiments as performed is of DNA damage remaining at the end of the 1 hr acute treatment. This damage can be a function of a number of variables, including different entry rates of the chemical into the cell, different degrees of the chemical reaching the chromosomes in the cell because of different extents of competition for the MMS by cellular cytoplasmic and nuceoplasmic biochemicals, different extents of available DNA unprotected by histones, acidic proteins, chromosomal RNA, etc. Any further elucidation of these mechanisms will await our repetition of these experiments. The very fact that we have observed a difference after chemical treatment of the two cell lines, in contrast to our result after UV treatment, lends special interest to the DNA/DNA Cot studies we will be performing during the latter part of the 1st contract year

of this proposal with cells treated with 10^{-3} M MMS for extended time periods. It is also our intention to investigate if the repair replication of residual damage occurring after treatment with MMS is in any way different from repair replication occurring during exposure to the chemical agent; this will be accomplished by incubations with MMS in the presence of the repair replication labeling media.

Repair Replication in WIL_2-A3 and Raji Cells after Acute Treatment with Benzo(a)pyrene (BaP).

In these experiments, the repair replication protocol employed was essentially the same as described above for MMS treatment. One significant difference in the procedure is the preparation of the chemical containing medias; in the MMS study, the water soluble MMS was diluted in sterile saline before addition in equal aliquots to the BrUdR-FUdr media. In the BaP study, it is first necessary to dissolve the BaP in an organic solvent. For this purpose, we employed acetone, dissolving 5 mg of BaP (Sigma or Eastman Kodak) in 1 ml of glass distilled acetone. This was then added dropwise with swirling in our biohazard hood to 100 ml of BrUdR-FUdR media yielding a concentration of 50 μg/ml BaP. Serial dilutions of 1:10 in media were made to yield final BaP concentrations of 5 and 0.5 μg/ml. We repeatedly observed, upon addition of BaP in acetone, the immediate formation of a fine precipitate in the media; this was found not to be due to the acetone carrier, but rather to the BaP, the latter presumably interacting with serum protein. The results of our repair

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replication experiments for both the Raji cells, based on dpm/μg, and the WIL_2-A3 cells based on the gradient profiles (final results are awaiting repair of the spectrofluorimeter), indicate that no significant repair replication is occurring under the 1 hr acute treatment procedure of this experiment.

There are several possible reasons for this result: (a) The BaP is removed from solution in the fine precipitate, and cannot enter the cell at a high enough concentration to damage enough DNA so that repair can be detected. We will be investigating the extent of BaP remaining in solution during the course of our experiments using spectrofluorometric scanning techniques after removing the fine precipitate. We have already initiated, in conjunction with another project, a study to determine whether ethanol or DMSO would be a better solvent for the BaP in terms of reducing the amount of precipitate. (b) A very strong possibility is related to the fact that the BaP which does enter the cell must be metabolized to an active form before it can damage the DNA; i.e., the pre-carcinogen, benzo(a)pyrene, must be metabolized to an ultimate carcinogen, e.g., 3-hydroxybenzo(a)pyrene or 6-hydroxybenzo(a)pyrene, before it can damage the DNA. If this is the case, the rate of metabolism of BaP and therefore its DNA damaging potential would be dependent on the level of the enzyme aryl hydrocarbonhydroxylase, with the latter being present in human lymphocytes on a genetically determined basis. Since the level of AHH can be increased by induction, i.e., by treatment of cells with benz(α)anthracene or 3-methylcholanthrene, or by incubation

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with benzo(a)pyrene itself, it becomes possible to attempt to increase the amount of DNA damage and therefore repair in two ways; (1) Treat the cells with the pre-carcinogen benzo(a)pyrene in the presence of the repair replication label for an extended period of time, allowing repair radioactivity to slowly accumulate in the DNA over the entire incubation period, or (2) pre-incubate the cells with a lower concentration of an AHH inducer, for an extended period of time (1-2 days), before challenging for a shorter period of time (e.g., 5 hrs) with the higher concentration of BaP in the presence of the repair label. A preliminary experiment of this type, which included an 18 hr induction period with 3-methylcholanthrene followed by a 1 hr acute treatment with 50 μg/ml BaP has been attempted; the final results are not yet available. As previously indicated, we already plan to modify the experimental protocol from the acute treatment to allow more time for induction and metabolism.

C. Procedural Modifications

In our more recent experiments, we have not been performing neutral CsCl gradients in the purification of normal density repair replicated DNA, but instead have been performing two sequential alkali CsCl-Cs_2SO_4 gradients to remove any artifactually bound radioactivity (3). This procedure, which reduces our centrifugation time by 1/3 and CsCl utilization by 1/3, will be standard in our future work.

Additionally, we have found that while we obtain excellent recovery of native DNA after centrifugation in CsCl and dialysis, we are able to recover only approximately 33% of denatured DNA after each

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alkali $CsCl-Cs_2SO_4$ gradient centrifugation. We have determined that pelleting of the pooled $CsCl-Cs_2SO_4$ fractions containing normal density DNA upon diluting with distilled water, adjusting the pH to neutrality, and centrifuging at 40,000 rpm for 16 hrs also results in only approximately 30% recovery. Between the first and second alkali gradients, we therefore no longer intend to dialyze, but rather add the appropriate DNA containing pooled $CsCl-Cs_2SO_4$ solution directly to solution for the second alkali gradient. It is still necessary for us to dialyze after the second gradient, since we must determine the specific activity of the repair replicated DNA in dpm/μg before adding heterologous DNA for shearing and maximum recovery as described below.

In determining the DNA concentration, in μg/ml, we no longer rely on the colorimetric diphenylamine procedure, which is not appropriate for low DNA concentrations. Optical density readings are not satisfactory for alkali denatured and dialyzed DNA. Instead, we now regularly use the diaminobenzoic acid spectrofluorimetric technique (4), which is not affected by denaturation, and which can measure absolute DNA amounts as low as 0.1 μg.

Procedure for the Determination of Reassociation (Cot) Curves.

A new procedure has been developed for processing DNAs in preparation for the determination of their reassociation (Cot) patterns. In this procedure we are also striving to limit the number of manipulations of the DNA and thereby minimize losses. This is an essential

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requirement because of the limited quantities and often low specific activities of repair replicated DNAs.

As indicated above, the dialysis of the second alkali gradient fractions will result in a considerable loss of DNA; this step is still essential as the DNA specific activity must subsequently be determined. After this is done, the procedure is as follows: Two volumes of glycerol are added to the dialyzed experimental DNA, along with sufficient unlabeled carrier DNA to give a final concentration of approximately 200 μg/ml. In future experiments, we plan to split the original sample into two parts. In one part unrelated E. coli DNA will be used as carrier. This will prevent nonspecific adsorption to hydroxyapatite. To the second part unlabeled homologous DNA will be added in sufficient quantities to not only prevent adsorption, but also to elevate the DNA concentration to permit attainment of high Cot values.

In the second step of our procedure the mixture is homogenized in a small 5 ml virtis homogenizer flask for 30 min at 50,000 rpm to shear the mixture of labeled and unlabeled DNA. This procedure has been employed to reproducibily generate low molecular weight fragments of DNA. We have not measured the size distribution of sheared DNA in this laboratory but we plan to do so.

At the completion of shearing, the third and final preparative step prior to hydroxyapatite fractionation is performed. In this step two volumes of ethanol is added to the DNA mixture, along with .02

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volumes of acetate buffer. After standing for 30 min at 0-4° C, the precipitate is collected by centrifugation. Pellets of DNA are then resuspenced in 0.12 M phosphate buffer. In the initial test of the procedure a chelating agent was not included in the phosphate buffer in order to be able to monitor the absorbance profiles of hydroxyapatite columns. In subsequent experiments we shall either use 0.002 M EDTA as a chelator or pass the samples over chelex-100 in order to prevent erroneous Cot patterns due to contamination by trace metal ions. Most of the steps in this procedure, including shearing precipitation and metal ion removal are those recommended by R. J. Britten, D. E. Graham, and B. R. Neufeld (Methods in Enzymology, in press).

The DNA concentration of samples is again determined in order to calculate the specific activities of ^{14}C- and ^{3}H-labeled semi-conservative and repair replicated DNAs. Such a determination also permits us to calculate the overall recovery of DNA, from the processing of cesium fractions through the shearing and precipitation steps.

Samples at this point are ready for processing for HAP fractionation. Appropriate aliquots are taken for each point on the Cot curve. Those from the sample containing E. coli will be taken for low Cot points. Those from the samples containing unlabeled homologous DNA will be taken for high Cot points and sufficiently diluted to also provide the intermediate points. All point samples are then heated for 10 minutes in a boiling water bath. These are then immediately immersed in a 60° C bath for a sufficient time to reach the desired Cot value. Samples are then separated into single and double stranded fractions in order to

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determine the extent of reassociation. We have chosen to use the column method for fractionation rather than the batch method used previously. The column method may be utilized to considerable advantage. For one thing, the short period of time required to adsorb samples to HAP may be further decreased by applying pressure to the columns. Thus very low Cot values are attainable and differences between repair and semi-conservatively labeled DNA patterns in this region may be evaluated. To determine the length of time required to process the low Cot ("0 time") point and also to determine if the column arrangement was performing satisfactorily, two sample points were tested in which dpm and A_{260} of the column eluate fractions were measured. Table 5 indicates that a low Cot value of approximately 10^{-4} may be achieved because the time interval between removal from the boiling water bath and adsorption to the column is minimized to one or two minutes. Two other important features are illustrated by Table 5. First, even with high pressure elution, both radioactivity and absorbance decline to background after each wash. Secondly, a total of 10 ml is sufficient to elute the DNA with both 0.12 M PB and 0.40 M PB washes at this input. This indicates that in future experiments it will only be necessary to collect 10 ml aliquots of both washes for all points. Aliquots of these will then be counted to determine the total dpm in each fraction. To further test whether the columns were fractionating properly, L-929 DNA in the native form rather than reassociated DNA was loaded onto a column and exhaustively washed with 0.12 M PB and 0.40 M PB. Native DNA was recovered exclusively in the 0.40 M PB fraction, thus indicating that there is no overlapping of double

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Table 5

Fractionation of ^{3}H-TdR Labeled L-929 DNA by HAP Column Chromatography

Cot = $.68 \times 10^{-4}$

	0.12 MPB		0.40 MPB	
	A_{260}	dpm	A_{260}	dpm
1.	.030	0	000	0
2.	.177	185	.041	90
3.	2.492	2287	.446	425
4.	2.896	491	.104	96
5.	1.092	736	.025	58
6.	.409	261	.000	0
7.	.280	107	.000	0
8.	.150	64	.000	0
9.	.018	37	.000	0
10.	.000	28	.000	0

Cot = 0.05

	0.12 MPB		0.40 MPB	
	A_{260}	dpm	A_{260}	dpm
1.	.032	0	.002	0
2.	.180	119	.216	180
3.	2.159	1661	.807	674
4.	2.212	1086	.245	127
5.	.716	530	.073	43
6.	.233	168	.043	27
7.	.104	65	.013	18
8.	.052	26	.006	5
9.	.032	6	.004	0
10.	.018	3	.002	0

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stranded DNA in the single strand 0.12 M PB wash. Experiments are now in progress in which we shall determine patterns of reassociation from 10^{-4} to 10^{4} Cot values for semi-conservatively synthesized DNAs by the procedure outlined. Initial determinations for repair replicated DNAs will include values from 10^{-4} to 10^{2}, but ultimately we shall also strive to determine patterns over the full Cot range for those DNAs.

One of the major advantages of the column method over the batch method originally proposed is that when the amount of repair replication radioactivity (dpm/μg) is low, large volumes at low DNA concentrations can be used to obtain low Cot values.

These DNA/DNA Cot procedural changes are being performed with the collaboration of Dr. William Thornburg of the Southwest Foundation for Research and Education staff, using the column apparatus shown in the accompanying photograph.

II. Compliance with the Contract Requirements

The initial proposal listed nine aims to be accomplished over a three year period of study. As indicated above, we are well underway in terms of understanding and developing appropriate exposure protocols and radiolabeling conditions for increasing the sensitivity, in terms of the amount of repair replication radioactivity incorporated, of the three cell systems currently under study. The expansion of the study into other cell lines, either previously listed or pertinent to establishing more significant biological relationships, will be described below for the remainder of this contract period, and in the renewal proposal for subsequent study.

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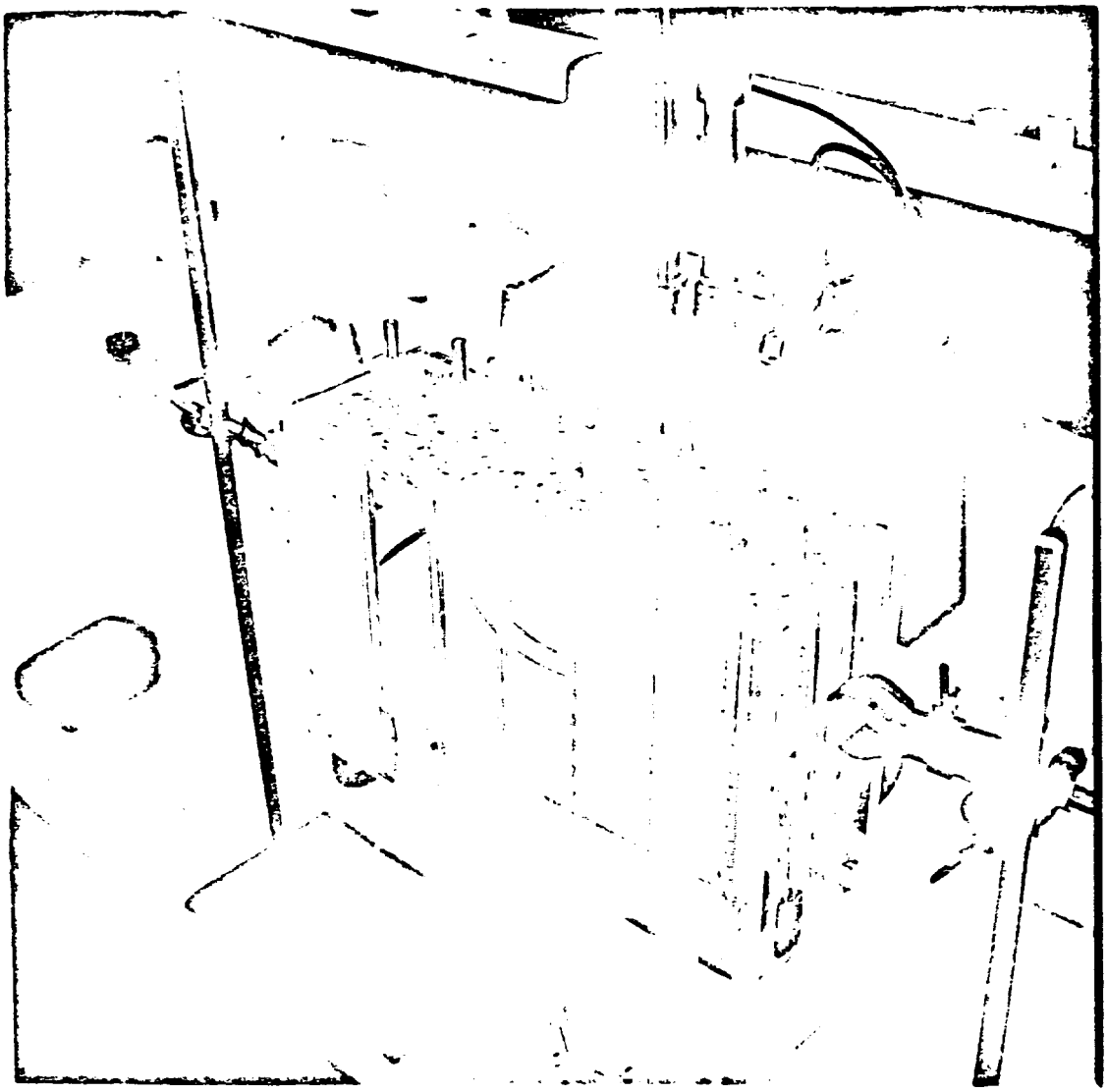

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The initial studies have also been limited in terms of the number of agents tested; as was indicated earlier; the three agents already under study have different characteristics relative to their entry into the cell and damage potential of DNA. The reasons for the choice of chemical carcinogens to be studied during the remainder of this contract term, and extended into the next, will be described below.

The preliminary information described above is to be reported at the May 1975 meeting of the Radiation Research Society (see attached abstract); the studies are now at a stage where DNA/DNA Cot studies of the repetitious distribution of repaired DNA will be determined.

III. Percentage Effort of Principal Investigator

Dr. Martin L. Meltz, Associate Foundation Scientist and Principal Investigator, is devoting 50% of his time to this project. Part of the remainder of his time has been applied to development in his laboratory of the L5178Y mutation assay system. As will be described in the associated renewal proposal, Dr. Meltz will begin studies of the repair replication occurring in this L5178Y cell line as it relates to mutation rate and survival after treatment with known mutagens.

IV. Probable Events during Remainder of the Current Contract Term

In addition to verification and expansion of the individual studies as described above, and performance of the DNA/DNA Cot studies related to these investigations, we will initiate studies of UV-induced repair replication

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in EB_3 Burkitt's lymphoma cells, WI-38 lung fibrobasts, and skin fibrobasts from individuals of different ages. The cell lines obtained will be initiated at two-month intervals, so as to allow initiation of repair studies without becoming overloaded with maintenance of cell lines. The development of the program, in terms of cell lines being studied, will have to extend into the second contract year.

The study of repair replication in EB_3 cells is of interest relative to the studies underway with the non-virus producing "normal" and Raji lymphoblastoid cell lines; the WI-38 lung fibroblasts are of importance, since they are of lung origin, relative to studies with benzo(a)pyrene, a common atmospheric pollutant, and the skin cells of people of different ages are of import relative to altered response to UV and chemical induced damage, especially in the latter case where metabolism might be involved.

Only one additional chemical is proposed for study during the remainder of this contract term; this is 4-nitroquinoline-1-oxide, which apparently requires metabolite activation before damaging DNA, but which, in contrast to benzo(a)pyrene, is apparently water soluble. Studies with other carcinogens will be deferred to the second year of this project (see renewal proposal for discussion of choice of carcinogens for study).

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CRL-7761-2

"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 benz(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.

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