Volume 7 Number 3 1979 Nucleic Acids Research

X-ray induced DNA double strand break production and repair in mammalian cells as measured by neutral filter elution

Matthews O.Bradley[1]* and Kurt W.Kohn[2]

[1]Laboratory of Experimental Pathology, Human Tissue Studies Section, and [2]Laboratory of Molecular Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205, USA

Received 3 May 1979

ABSTRACT

This work presents a neutral filter elution method for detecting DNA double strand breaks in mouse L1210 cells after X-ray. The assay will detect the number of double strand breaks induced by as little as 1000 rad of X-ray. The rate of DNA elution through the filters under neutral conditions increases with X-ray dose. Certain conditions for deproteinization, pH, and filter type are shown to increase the assay's sensitivity. Hydrogen peroxide and Bleomycin also induce apparent DNA double strand breaks, although the ratios of double to single strand breaks vary from those produced by X-ray. The introduction of double strand cuts by HpA I restriction endonuclease in DNA lysed on filters results in a rapid rate of elution under neutral conditions, implying that the method can detect double strand breaks if they exist in the DNA. The eluted DNA bands with a double stranded DNA marker in cesium chloride. This evidence suggests that the assay detects DNA double strand breaks. L1210 cells are shown to rejoin most of the DNA double strand breaks induced by 5-10 krad of X-ray with a half-time of about 40 minutes.

INTRODUCTION

A voluminous literature exists on the production and repair of single strand breaks (SSBs) in mammalian cells, but the double strand break (DSB) literature is scanty. In part this disparity is due to the problems inherent in the technique of neutral sucrose sedimentation velocity determinations of DNA double strand molecular weight, the most common method for measuring DSBs in mammalian cells. Neutral gradients are often plagued by low sensitivity, anomalous sedimentation complexes, large quantitative variations between experiments, and sometimes a necessity for X-ray treatment of control cells. Regardless of these complications, the neutral gradient technique has shown that high doses of various radiation sources (α, γ, X, and electrons) will produce reductions in apparent double strand molecular weight (1-4 respectively). However, the evidence for rejoining of DSBs in mammalian cells is equivocal, with some workers finding repair (1,2) and others not (3,5).

© Information Retrieval Limited 1 Falconberg Court London W1V 5FG England

In the hopes of improving the existing methodology for measuring DSBs, we have developed a new technique based on the rate at which DNA double strands elute through a membrane filter under non-denaturing conditions. This approach, under alkaline conditions, has provided a sensitive measurement of DNA SSBs (6,7) and we have made certain modifications necessary for DSB detection. We report here the development of this technique and use it to measure the production and repair of DSBs in mouse L1210 cells after X-irradiation.

METHODS

Cell Culture and DNA Labeling

Mouse leukemia L1210 cells were grown as suspension cultures in RPMI 1630 medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum plus penicillin and streptomycin. Cultures were free of mycoplasma spp. as determined by Flow Laboratories. DNA was uniformly labeled in exponentially growing cells (doubling time, 12 hrs) with either [2-^{14}C]-thymidine (0.025-0.05 μCi/ml; 55 mCi/mmol) or [methyl-^{3}H]-thymidine (0.1-0.2 μCi/ml; 20 Ci/mmol plus 10^{-5} M unlabeled thymidine) for 20 hrs. At this time there were approximately 1 x 10^{6} cells/ml in exponential growth.

X-irradiation

Two-tenths to 0.5 ml (1.0 x 10^{6} cells/ml) of the ^{14}C-labeled cells in medium were chilled in plastic 15 ml centrifuge tubes in an ice water bath for approximately 1 hr before irradiation. These tubes were X-irradiated in an ice water bath with a Phillips therapeutic X-ray machine operated at 235 Kv, 15 mA with 0.5 mm copper and 0.25 mm aluminum filters. The dose rate from two opposing X-ray tubes was 632 rad·min^{-1}. The samples were irradiated in groups of 5 with each incremental dose added to the one before it. Chilled internal standard ^{3}H-labeled cells (0.2 to 0.5 x 10^{6}) that had been irradiated with either 5 or 10 krad were mixed with experimental cells. After X-ray the cells were held on ice until either lysis or incubation at 37° C for repair studies.

DSB Measurements

For the X-ray dose-response experiments, pre-mixed ^{3}H and ^{14}C-labeled cells were resuspended by vortexing and diluted with 15 ml of ice cold phosphate buffered saline (PBS) in the same tubes in which they were X-rayed. This cell suspension was pulled down by moderate vacuum onto a Uni-pore (Bio-Rad Laboratories) 25 mm 2.0 μ pore-size polycarbonate filter supported at the bottom of a solution reservoir. A second 15 ml of PBS was rinsed

through the tubes to remove any remaining cells and poured into the reservoir before all the previous solution had dripped through. Just before the last of the solution was through the filter, the vacuum was disconnected and the remainder flowed out by gravity so as to minimize damage to the cells. The pump tubing was connected to the outflow of the filter and the cells were immediately lysed with a room temperature solution of 3.0 ml 0.05 M Tris, 0.05 M glycine, 0.025 M Na_2 EDTA, 2% w/v sodium lauryl sulfate, adjusted to pH 9.6 with 10 N NaOH and containing 0.5 $mg{\cdot}ml^{-1}$ proteinase K freshly dissolved. After 8 samples had been lysed (approximately 15 min) the pump was started and run for 1 hr at 2.13 $ml{\cdot}hr^{-1}$. After 1 hr, 50 ml of the above solution without proteinase K was gently added on top of the remaining lysis solution. Fractions (90 min) were collected at the same pump speed for 15 hrs. The experiment was completed by pumping out the excess solution in the lines at the maximum pump speed and by removing the DNA left on the filter with a 1 hr treatment of the filter in a scintillation vial at 60° C in 0.4 ml of 1.0 N HCl followed by 1 hr at room temperature in 2.5 ml 0.4 M NaOH. The samples were counted in 10 ml of Aquasol containing 0.88% (v/v) glacial acetic acid.

Cesium Chloride Banding

L1210 cells were labeled with [2-^{14}C]-thymidine, X-rayed on ice with 10 krad, and eluted as described above. The fractions eluting between 1.5 and 3.0 hrs were combined and dialyzed against 0.05 M Tris-HCl, 0.005 M Na_2 EDTA, pH 7.0. A marker of heat denatured [^{3}H]-thymidine labeled DNA from L1210 cells was added to the eluted samples. Three grams of this sample plus 3.0 gms of 1 M sodium trichloroacetate (pH 8.0) were added to 7.28 gm of CsCl (Harshaw Chemical Co., Cleveland, Ohio, optical grade) and centrifuged for 46 hrs at 40,000 RPM (20° C) in a Beckman Type 65 angle rotor. We also ran separate gradients containing [^{14}C]-native and [^{3}H]-denatured DNA markers. Five-drop fractions were precipitated with 5% (w/v) trichloroacetic acid, 0.02 M sodium pyrophosphate. The precipitates were collected on filters (Millipore HAWP), air dried, and counted in 5 ml of Econofluor (New England Nuclear).

RESULTS

X-ray Induced Double Strand Breaks

In setting up the conditions for the DNA DSB assay, we attempted to optimize for X-ray sensitivity. A number of variables were found to be important in this optimization with the results shown in Fig. 1. Deproteinization: with less stringent deproteinization conditions than those

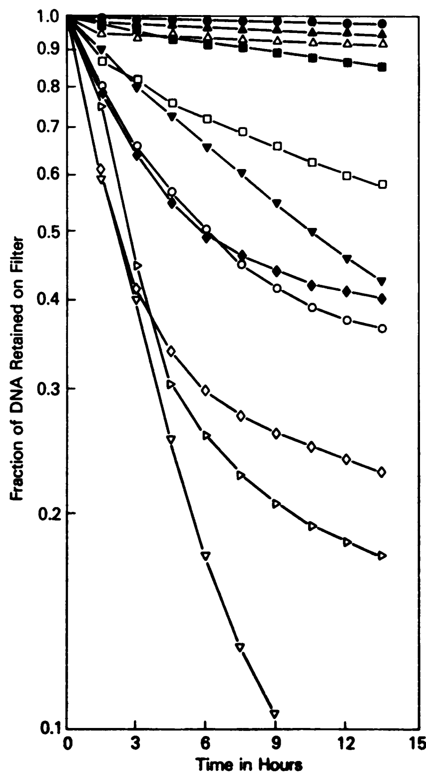

FIGURE 1: Variables affecting neutral elution after X-ray and hydrogen peroxide. The symbols corresponding to each experimental variable are as follows:

●, control

▲, H_2O_2, 100 μg·ml^{-1}, 2 hrs, 0° C, pH 9.6

■, H_2O_2, 400 μg·ml^{-1}, 2 hrs, 0° C, pH 9.6

▼, H_2O_2, 0.75 μg·ml^{-1}, 2 hrs, 0° C, pH 12.1

Δ, 7.5 krad, 2 M NaCl, 0.2% Sarkosyl, pH 10.2, no proteinase K in lysis

□, 7.5 krad, 2% sodium lauryl sulfate, pH 9.6, no proteinase K in lysis

o, 7.5 krad, 2% sodium lauryl sulfate, pH 9.6, 0.5 mg·ml^{-1} proteinase K in lysis

∇, 10 krad, pH 9.6, polycarbonate filter, 2-μm pore-size (Bio-Rad Laboratories)

◇, 10 krad, pH 9.6, polyvinylchloride filter, 2-μm pore-size (Millipore Corp.)

▷, 10 krad, pH 7.6, polycarbonate filter

◆, 10 krad, pH 5.6, polycarbonate filter

used here (2% SDS plus 0.5 $mg \cdot ml^{-1}$ proteinase K), the elution rate was not as sensitive to X-ray. For instance, a lysis solution containing 2 M NaCl, 0.02 M Na_2 EDTA, and 0.2% Sarkosyl (pH 10.2) produced a very small response to 7.5 krad of X-ray (Fig. 1, Δ). If proteinase K is omitted from the first hr of pumping, then the rate of elution is reduced (Fig. 1, compare ▭ to o). RNase did not alter the rate of elution under neutral conditions. Filters: the polycarbonate straight-through pore filters produced greater sensitivity to X-ray and straighter elution curves than did the polyvinylchloride type (Fig. 1, ∇ versus ◊). pH: the X-ray sensitivity was greater at pH 9.6 than at pH 7.6 or pH 5.6 (Fig. 1, ∇, ▷, ◆, respectively). DNA will remain double stranded at pH 9.6, so perhaps the higher pH is more effective because it removes more protein or other interfering cellular material.

Internal ^3H controls irradiated with 5 to 10 krad were included in many of the experiments. These controls showed that there is still some variation in the procedure although attention to the following conditions helped to reduce it. Temperature: the X-irradiated cells were kept at 1° C until lysis, which appeared to both improve assay reproducibility and to reduce rapidly eluting material from the first fraction. Cell Number: to help assure that the DNA strands of various sizes behave independently, it is important to keep the total cell number less than 0.5×10^6. When 10^6 or more cells are used, the elution rate of 5 krad internal standard ^3H cells is increased when the X-ray dose to the ^{14}C cells is more than 5 krad and reduced when it is less than 5 krad. This effect is eliminated at total cell numbers less than 0.5×10^6.

Figure 2 shows the dependence of DNA elution on X-ray dose for L1210 cells by the method described. Without X-ray there is very little elution. Such slow and reproducible untreated controls make detecting small effects much easier than if the controls are unstable. The rate of elution increases as a function of X-ray dose, with one krad producing an easily measurable response. In other experiments 0.3 krad has been detected.

On semi-logarithmic plots of the log DNA retention versus time, the rate of elution slows down indicating that portions of the DNA elute with different kinetics. With alkaline elution, where single strand DNA is examined, the curves appear to be straighter (6,7). The basis for this difference is not currently understood.

Figure 3 shows results from two separate experiments where the retention of DNA on the filter at both 4.5 hrs and 12 hrs is plotted as a function

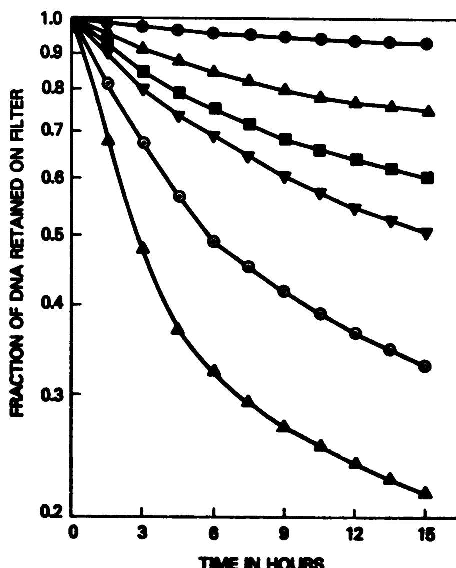

FIGURE 2: DSBs produced in L1210 cells by X-ray. The doses were: ●, no X-ray; ▲, 1 krad; ■, 3 krad; ▼, 5 krad; ●, 7 krad; ▲, 9 krad. The fraction of DNA retained on the filter is plotted as a function of time of elution.

of dose. A relationship exists between the amount of DNA eluted in a certain time and the dose of X-ray. This result is qualitatively similar whether the analysis is done at 4.5 or 12 hrs. These data are not precise enough yet to determine a functional relationship between dose and retention. The profiles appear to bend downwards, but with more data they might have another shape.

Double Strand Break Repair After X-ray

For measuring double strand break rejoining, the cells were prechilled and irradiated on ice as described in Methods. Repair was started by pipetting 5 ml of chilled, irradiated cells into a 150 ml bottle pre-warmed to 37° C in a water bath. Aliquots were removed as a function of time to prechilled 15 ml tubes on ice to stop repair. Fig. 4 shows the results of 5 different repair experiments after 5 to 10 krad of X-ray. The data were normalized as described in the legend to Fig. 4. The plot suggests that DSB repair is about 90% completed by 2 hrs and approaches 100% repair. If the data are plotted semi-logarithmically as [1.0-fraction repaired] versus time, than an apparent straight line is obtained with a t 1/2 for repair

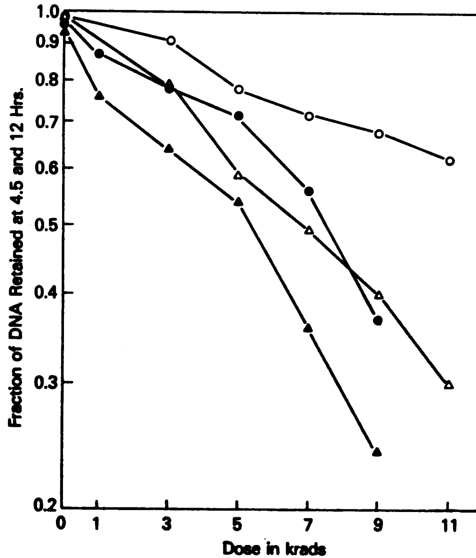

FIGURE 3. Semi-logarithmic plot of the fraction of the DNA retained on the filter at 4.5 and 12 hrs as a function of X-ray dose. Circles, 4.5 hrs of elution; triangles, 12 hrs of elution. The open symbols are from one experiment, the closed symbols from another.

of about 40 min (plot not shown). In contrast, Lehmann and Stevens (2) estimated a half-time of DSB rejoining of about 6 hrs in human fibroblasts after 50 krads using neutral sucrose gradients. We have no simple explanation for this difference, although a number of possibilities exist. First, we have measured rejoining of DSBs after only 5 and 10 krad, whereas Lehmann and Stevens used 50 krad, a dose that might partially inactivate repair systems. Second, neutral sucrose gradients may measure different parameters than neutral elution. Third, we have measured repair in malignant mouse leukemia cells grown in suspension, whereas they have used normal human fibroblasts grown attached to a substrate—different mammalian cells may repair DSBs at different rates. Fourth, repair of DSBs produced by X-ray as used here, may be quantitatively different than repair of DSBs produced by γ radiation as used by Lehmann and Stevens.

Evidence for DSBs

DNA denatures in alkali at a critical pH of about 11.6(8)—well above the pH of 9.6 used in this DSB assay. Therefore, the DNA eluting through the filters in this assay should be double-stranded based on pH considera-

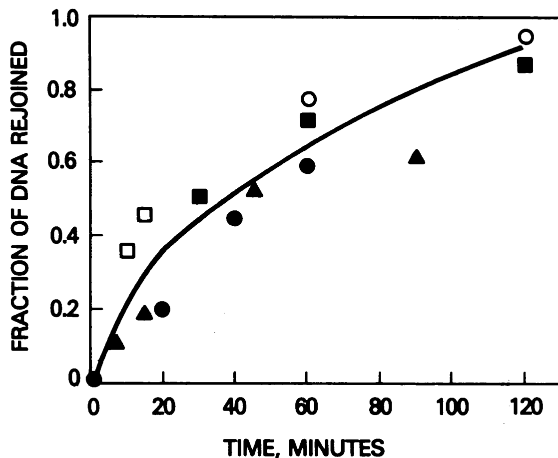

FIGURE 4: Rate of DSB repair in L1210. Five different repair experiments are shown. The doses used were: ■, □, 5 krad; o, 7.5 krad; ●, ▲, 10 krad. The fraction of DNA DSBs rejoined was calculated by measuring the log of the ratio of DNA retained on the filter at 4.5 hrs of the untreated to the unrepaired samples, i.e., log $\left[\frac{\text{fraction retained at 4.5 hrs, no X-ray}}{\text{fraction retained at 4.5 hrs, X-ray, no repair}}\right]$. This value was set equal to 1.0, and the fraction repaired calculated in the same way with the numerator being the fraction retained at 4.5 hrs with repair.

tion alone. However, this assumption deserves more stringent verification, which is what the following experiments were designed to provide.

Ratio of SSBs to DSBs Produced by H_2O_2, Bleomycin, and X-ray

If SSBs are randomly placed along the DNA, then there should be a certain number of apparent DSBs resulting from closely or oppositely placed SSBs in the complementary strands. Agents acting by different mechanisms to produce both DNA SSBs and DSBs would be expected to produce different rather than constant ratios of SSBs to DSBs. We tested this assumption by comparing the rates of elution produced by X-ray, H_2O_2, and Bleomycin under both alkaline and neutral conditions.

The rate of neutral elution after a given dose of X-ray is approximately 1/20 the rate of alkaline elution (compare the data in Fig. 2 with that previously published in Fig. 1 of Ref. 6). This ratio is similar to published estimates of the relative numbers of DSBs and SSBs produced by X-ray in mammalian cells (3,9).

Hydrogen peroxide at 0.75 $\mu g \cdot ml^{-1}$ for 2 hrs makes many SSBs in mammalian cells when assayed under alkaline conditions as shown in Fig. 1. When assayed

under neutral conditions, however, apparent breaks are detected only at much higher doses of H_2O_2 (100 to 400 $\mu g \cdot ml^{-1}$). Based on these data, the relative efficiency for SSB production by H_2O_2 is approximately 2000-fold higher than for DSB production. This difference shows that few DSBs are produced even though large numbers of SSBs are present in the DNA. Perhaps the relatively small number of apparent DSBs induced by H_2O_2 are due to closely spaced SSBs.

Bleomycin makes SSBs in L1210 DNA as measured previously by alkaline elution (10). As shown in Fig. 5, Bleomycin also induces DSBs as measured by neutral elution in L1210 DNA under similar experimental conditions. The shape and extent of the alkaline elution profile after 80 $\mu g \cdot ml^{-1}$ of Bleomycin for 1 hr is similar to the shape of the neutral elution profile after 400 $\mu g \cdot ml^{-1}$, implying that the ratio of SSBs to DSBs induced by Bleomycin is approximately 5 to 1.

Because these 3 agents gave different ratios of SSBs to DSBs there is not a constant quantitative relationship between the two lesions. This

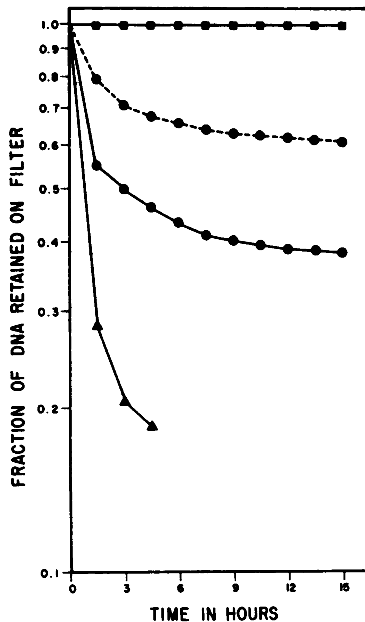

FIGURE 5: DSBs produced in L1210 cells by Bleomycin and HpA I restriction endonuclease. The symbols are: ■—■, control; ●- -●, 100 $\mu g \cdot ml^{-1}$ Bleomycin for 1 hr; ●—●, 400 $\mu g \cdot ml^{-1}$ Bleomycin for 1 hr; ▲- -▲, 83 units$\cdot ml^{-1}$ HpA I, 20 min incubation with DNA held on filters.

variation suggests that the DSBs are not formed only from closely spaced SSBs on complementary strands.

Double Strandedness of Eluted DNA

Although DNA should remain native at the pH of 9.6 used in these experiments, we wanted to be certain that the eluted DNA had not become single-stranded. We determined the strandedness of the DNA by equilibrium centrifugation in CsCl as described in Methods. Fig. 6 shows that the eluted DNA banded coincidentally with a double-stranded marker DNA, but banded distinctly from a heat-denatured single-stranded marker. Thus, the eluted DNA is predominately double-stranded.

HpA I Restriction of DNA on Filters

The HpA I restriction endonuclease makes blunt cuts (DSBs) in DNA at sites 5'...NGTT/pAACN...3'. We used this enzyme to further test whether the neutral filter elution method measures DSBs. Untreated cells were lysed on the filters as described in Methods and rinsed three times with 5 ml of 0.04 M Tris·HCl, 0.02 M Na_2 EDTA pH 7.0 to remove excess lysis solution. The DNA was then incubated for 20 min at 22° C with 4 ml of 83 units·ml^{-1}. HpA I in 0.01 M Tris·HCl, 0.01 M $MgCl_2$, 0.02 M KCl, pH 7.4. The enzyme

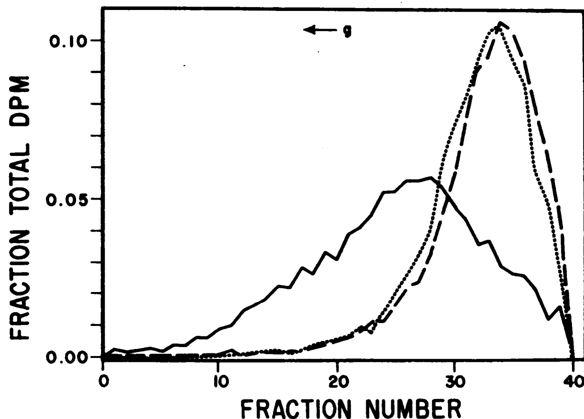

FIGURE 6: Isopynic centrifugation of eluted DNA. L1210 cells were labeled with [^{14}C]-thymidine, X-rayed with 10 krad, and eluted by the standard procedure. The DNA eluting between 1.5 and 3 hrs was combined, dialyzed and centrifuged to equilibrium. Density markers consisted of [^{3}H] and [^{14}C] L1210 DNA. Symbols: solid, [^{3}H] DNA, heat denatured marker; short dash, eluted [^{14}C] DNA; long dash [^{14}C] DNA, native marker. The denatured and eluted DNA were centrifuged in one tube; the native and denatured markers were centrifuged together in a second tube. The denatured peak is on the left in the direction of increasing density.

solution was removed and the DNA eluted as usual. The results in Fig. 5 show that the introduction of double strand cuts by HpA I restriction endonuclease results in a rapid rate of elution under neutral conditions. This finding shows that the assay will detect DSBs in DNA once they have been introduced.

DISCUSSION

Double strand breaks in DNA have been hypothesized to produce cell death (1,2,5), yet they have been little studied and are poorly defined. We will operationally define a DSB in DNA as closely or oppositely placed cuts in each of the two phosphodiester backbones of the duplex molecule that, under the conditions of any particular assay, cut the molecule into 2 pieces. The number of bases between 2 single cuts on opposite strands that result in a measured double strand cut is not known and may vary depending upon the assay conditions.

The neutral filter elution technique described here, appears to be a sensitive method for detecting DNA DSBs in mammalian cells, with at least 0.3 krad as a limit of detection. This sensitivity is much greater than that for results so far published with neutral gradients (1,2,5).

We have gathered the following evidence that the assay is measuring DSBs: (1) The pH we routinely use is 9.6, well below 11.6, the critical pH at which DNA begins to denature. (2) DNA eluting after 10 krad between 0 and 3 hrs bands in cesium chloride at the same density as an internal marker of native DNA (Fig. 6). (3) The ratio of DSBs to SSBs in this assay varies for different agents, and not as a simple function of the number of SSBs. For instance, Fig. 1 shows that H_2O_2 at the same concentration would produce approximately 2000 SSBs for each measured DSB. Bleomycin, on the other hand, produces approximately 5 SSBs to each DSB, while X-ray produces approximately 20 SSBs to each DSB. The 20 to 1 ratio for X-ray is similar to published estimates of the relative numbers of SSBs and DSBs produced by X-ray in mammalian cells (3,9), implying that both neutral elution and gradient methods are measuring quantitatively similar phenomena. (4) The introduction of double strand cuts by HpA I restriction endonuclease in DNA lysed on filters produces a rapid rate of elution under neutral conditions (Fig. 5). This finding suggests that the assay will detect DSBs in DNA once they have been introduced.

The rejoining of DSBs in mammalian cells has been a controversial issue for many years. Our work strongly supports the contention that double

strand rejoining occurs in mouse L1210 cells. Repair was seen in five independent experiments with similar kinetics and rejoining was about 90% completed by two hrs occurring with a half-time of 40 min. Given the apparent reproducibility and sensitivity of this assay, we expect to be able to examine a number of questions related to the biological consequences of DSBs in mammalian cells.

ACKNOWLEDGEMENTS

Thanks are given to Dr. Leonard C. Erickson for help with planning and performing the equilibrium CsCl centrifugations and to Jan Leliukas for typing the manuscript.

*Present address for correspondence and reprint requests: Merck Sharp & Dohme Research Laboratories, West Point, PA 19486, USA.

REFERENCES

1. Cole, A., Shonka, F., Corry, P. and Cooper, W. (1975) In: Molecular Mechanisms for the Repair of DNA, Setlow, R.B. and Hanawalt, P.C., Eds., pp. 665-676, Plenum Press, New York.
2. Lehmann, A.R. and Stevens, S. (1977) Biochim. Biophys, Acta 474, 49-60.
3. Lehmann, A.R. and Ormerod, M.G. (1970) Biochim. Biophys. Acta 217, 268-277.
4. Coquerelle, T., Bopp, A., Kessler, B. and Hagen, U. (1973) Int. J. Radiat. Biol. 24, 397-404.
5. Dugle, D.L., Gillespie, C.J. and Chapman, J.D. (1976) Proc. Natl. Acad. Sci. USA 73, 809-812.
6. Kohn, K.W., Erickson, L.C., Ewig, R.A.G., and Friedman, C.A. (1976) Biochem. 15, 4629-4637.
7. Bradley, M.O., Erickson, L.C, and Kohn, K.W. (1976) Mutation Res. 37, 279-292.
8. Vinograd, J., Lebowitz, J., Radloff, R., Watson, R. and Laipis, P. (1965) Proc. Natl. Acad. Sci. USA 53, 1104-1111.
9. Veatch, W. and Okada, S. (1969) Biophys. J. 9, 330-346.
10. Iqbal, Z.M., Kohn, K.W., Ewig, R.A.G., and Fornace, A.J. (1976) Cancer Research 36, 3834-3838.
11. Garfin, D.E. and Goodman, H.M. (1974) Biochem. Biophys. Res. Commun. 59, 108-116.