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A Fluorescence Enhancement Assay for Cellular DNA Damage

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SUMMARY

A fluorescence procedure is described for quantitative measurement of DNA damage in mammalian cells. The technique is based upon the time-dependent partial alkaline unwinding of cellular DNA followed by determination of duplex:total DNA ratios with bisbenzamide, which has a differential molar fluorescence with single-stranded and duplex DNA. The method is rapid, does not require radioactive labeling of DNA, and is sufficiently sensitive to detect damage induced with 100 rads of X-irradiation. This method is standardized with respect to the alkaline unwinding unit, Mn_0, and the unwinding constant, β. Results obtained with this new technique and with hydroxylapatite chromatography for physical separation of single- and double-stranded DNA were confirmatory. The utility of the technique was demonstrated by detection of dose-related damage with X-irradiation and a variety of antineoplastic agents in unlabeled murine leukemia cells.

INTRODUCTION

In 1973, Ahnstrom and Erixon (1) demonstrated that duplex mammalian DNA in dilute alkali undergoes a time-dependent transformation to the single-stranded form, and that this process is accelerated after introduction of DNA damage by X-irradiation. These observations provided the basis for sensitive assays of DNA damage developed by others. Rydberg (2), using hydroxylapatite columns at elevated (60°) temperatures to separate single-stranded from duplex DNA, established the theoretical background for the quantitation of DNA damage expressed by time-dependent alkaline denaturation procedures. Subsequently, we developed a hydroxylapatite batch procedure for rapid assay of large numbers of samples (3). A second means to quantitate the extent of alkaline denaturation was developed by Sheridan and Huang (4) with the bacterial enzyme S1 nuclease, which selectively degrades single-stranded DNA.

Bisbenzamide [2,2-(4-hydroxyphenyl)-6-benzimidazolyl-6-(1-methyl-4-piperazyl)-benzimidazol trihydrochloride], a fluorescent dye commonly used as a chromosome stain (5), complexes with DNA to form a stable, intensely fluorescent product; a less fluorescent product is formed with single-stranded DNA. We have developed a new method to quantitate mammalian DNA damage based upon this property of differential molar fluorescence. The assay is simple, rapid, and inexpensive to perform because of the absence of need for a radioactive DNA label. However, the assay is fully as sensitive and accurate as the hydroxylapatite or S1 nuclease procedures which require that cells be radioactively labeled for greatest accuracy of results. In this paper, we describe the technique and the direct assay of DNA damage in murine leukemia cells after exposure to a variety of antineoplastic agents and X-irradiation.

MATERIALS AND METHODS

Chemical, drug, and cell culture supplies. Bisbenzamide (Hoechst H33258) was obtained from American Hoechst Corporation (Somerville, N. J.). ACS scintillation cocktail and [2-^{14}C]thymidine (45 mCi/mmole) were purchased from Amersham/Searle (Arlington Heights, Ill.). Radioactive samples were counted in polyethylene scintillation vials (Packard Instrument Company, Downers Grove, Ill.) in a Packard Tri-Carb 3320 liquid scintillation spectrometer. Reagent-grade formamide was obtained from Eastman Kodak Company (Rochester, N. Y.), and hydroxylapatite (Bio-Gel HTP, DNA grade) was purchased from Bio-Rad Laboratories (Richmond, Calif.). Dialyzed fetal calf serum, RPMI 1640 medium, and penicillin-streptomycin solution (10,000 units of penicillin per milliliter, 10,000 units of streptomycin per milliliter) were obtained from Grand Island Biological Company (Grand Island, N. Y.). Antineoplastic agents were obtained from Dr. Harry B. Wood, Jr., Drug Development Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.) and from Profes-

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[1] Leukemia Society of America Scholar.

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sor F. Arcamone (Farmitalia, Milan, Italy). Salmon sperm DNA and miscellaneous reagents were obtained from Sigma Chemical Company (St. Louis, Mo.).

Cell lines and cell culture conditions. P388 murine leukemia cells were passaged weekly in DBA/2 mice (from the breeding colony of the Department of Experimental Therapeutics, Roswell Park Memorial Institute) by i.p. injection of 1×10^5 viable cells. Cells were harvested (4–7 days after passage) by peritoneal lavage with PBS[2] (0.01 M sodium phosphate-buffered (pH 7.4) 0.9% sodium chloride solution). The ascites cells in PBS were concentrated by centrifugation, erythrocytes were removed by osmotic lysis (10-sec exposure to distilled water followed by the addition of 20 volumes of PBS), and leukemia cells were counted (hemocytometer) and resuspended in PBS or RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin solution (standard growth medium). Cells in short-term tissue culture (1–8 hr) were maintained at 37° in a humidified (5% CO_2 atmosphere) incubator (Wedco, Silver Spring, Md.).

A tissue culture-adapted line of L1210 and CCRF-CEM human leukemia cells were maintained in continuous culture in standard growth medium. L1210 cells were labeled for 18 hr with [^{14}C]dThd (0.1 μCi/ml) for 18 hr, collected by centrifugation, washed two times in sterile PBS, and resuspended in fresh medium for 1 hr before use.

Irradiation procedure. P388 and L1210 leukemia cells were centrifuged, resuspended in PBS (1×10^6 cells/ml), and pipetted into glass scintillation vials, which were left uncapped. The cells on ice (air atmosphere) were irradiated with a General Electric Maxitron 300 therapy machine using settings of 20 mamp, 300 KVP, a 1/4 Cu filter, and a target-to-radiation source distance of 50 cm. The irradiation rate was 200 rads/min.

Bisbenzamide fluorescence enhancement assay. The fluorometric procedure for assay of DNA damage is outlined in Fig. 1. Cells suspended in PBS (1×10^6/ml) are pipetted (500 μl) [Oxford 500-μl manual pipetter (Oxford Laboratories, Forest City, Calif.)] into nine disposable (10 × 75 mm) borosilicate glass tubes (Kimble, Toledo, Ohio). To three of these tubes (Procedure A in Fig. 1) are added 1.00 ml of a solution made by the addition of equal volumes of 0.1 N NaOH and 0.1 N HCl, followed by the addition of 500 μl of buffer solution (0.16% sodium lauroyl sarcosinate, 0.2 M KPO_4, 0.04 M disodium EDTA, and 1.0 μg of bisbenzamide per milliliter, pH 7.4). The DNA in these tubes is sheared by complete immersion of a microsonicator tip into the cell lysate for 15 sec [(Branson Sonifier No. 185, Setting 4) (Branson Sonic Power Company, Danbury, Conn.)]. The cell sonication procedure should produce foaming in the tubes. If not, the tip should be withdrawn and reintroduced until foaming occurs. The DNA in these tubes remains in duplex form (i.e., F, the fraction of total DNA in duplex form, is equal to 1.0). To three tubes (Procedure B in Fig. 1), 500 μl of 0.1 N NaOH are added, followed 30 min later by neutralization with 0.1 N HCl (500 μl). During this carefully timed unwinding period the tubes must be kept in a dark and vibration-free area at relatively constant room temperature. Buffer (500 μl) is added to these tubes, and the lysate is sonicated as above. This set of triplicates contains DNA in partially denatured form, the extent of denaturation a function of the size of the unwinding unit and the amount of DNA damage present. Alkali (0.1 N NaOH, 500 μl) is added to the final set of three tubes (Procedure C in Fig. 1), the alkaline lysate is sonicated briefly (5 sec), and acid (500 μl, 0.1 N HCl) is added 30 min later, followed by the addition of buffer solution (500 μl) and resonication (15 sec). Because of extensive DNA fragmentation by sonication during the unwinding procedure in tubes marked *C*, conversion of native DNA to the single-stranded form is essentially complete (i.e., $F = 0$). All tubes are covered with Parafilm, and fluorometric readings are taken after the tubes have equilibrated at ambient temperature (if convenient, the next working day). Readings are taken of the solution in borosilicate tubes (it is unnecessary to transfer solutions to quartz fluorometry cuvettes) in an SPF 500 fluorometer (American Instrument Company, Silver Spring, Md.) operating in the ratio mode (excitation 353 nm, emission 451 nm, bandpass 5 nm).

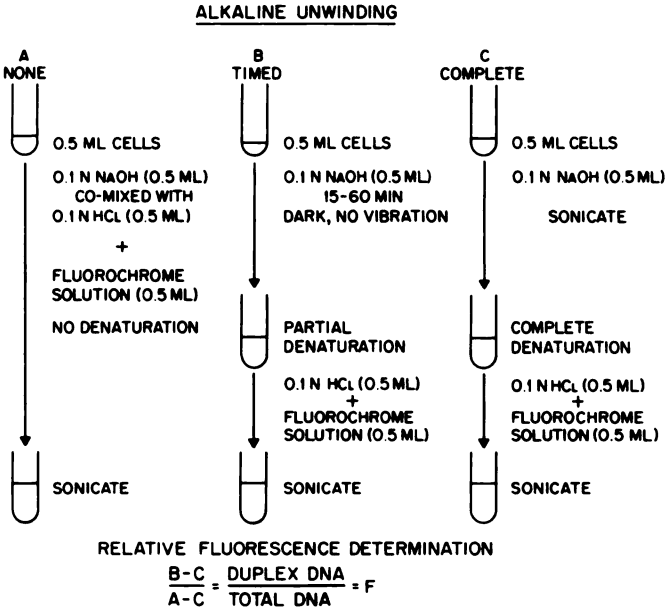

FIG. 1. *Bisbenzamide fluorescence enhancement assay*

Hydroxylapatite chromatography. Measurement of DNA damage in ^{14}CdThd-labeled L1210 cells was performed exactly as described previously (3).

Calculations and definitions. Rydberg (2) derived the relationship for strand separation of duplex DNA in alkali where lesions are introduced by ionizing irradiation (Eq. 1):

$$\ln F = \frac{\text{const}}{Mn} \cdot t^{\beta} \qquad (1)$$

where F is the fraction of double-stranded DNA remaining after alkaline denaturation for time t, Mn is the number-average molecular weight between unwinding points, and β is an unwinding constant less than 1, determined as previously described (2, 3).

The critical parameters of the fluorescence assay, the extent of fluorescence enhancement of DNA when $F =$

[2] The abbreviation used is; PBS, phosphate-buffered saline.

0 and $F = 1.0$, are internally defined by the assay for every sample analyzed. The amount of DNA in duplex form after partial alkaline denaturation (F_{exp} in Tube B) is determined by Eq. 2.

$$\frac{B - C}{A - C} = F \tag{2}$$

Mn_0, the size of the alkaline unwinding unit in untreated cells, may be estimated from irradiation data (3). As suggested by Kohn and Grimek-Ewig (6),

$$Mn_0 = \frac{\text{Mol wt DNA/cell}}{8 \text{ breaks/cell/rad} \cdot D37} \tag{3}$$

an apparent Mn_0 for each line can be determined.

The number of unwinding points (p) per alkaline unwinding unit of DNA after cell treatment is obtained from Eq. 4, where subscripts exp and 0 represent experimental and control samples, respectively.

$$Mn_0 \cdot \ln F_0 = Mn_{exp} \cdot \ln F_{exp} \tag{4}$$

and

$$p = \frac{\ln F_{exp}}{\ln F_0} \tag{5}$$

The number of breaks per alkaline unwinding units is:

$$n = p - 1 \tag{6}$$

If the Mn_0 for the cells under study has been determined, data may be expressed as n/mol wt DNA.

RESULTS

The bisbenzamide fluorescence enhancement technique for assay of mammalian DNA damage depends upon time-dependent alkaline denaturation of DNA for the expression of DNA lesions. Aliquots of cells are lysed for a precise time period (30 min). During this time the DNA unwinds slowly, starting at the ends of each strand or unwinding unit. The process is stopped by neutralization with acid, and the DNA is sheared to prevent reannealing. If the DNA is damaged, either by introduction of alkaline labile lesions or frank strand breakage, there is an increased number of unwinding points, and more single-stranded DNA will be formed during the unwinding period. Bisbenzamide, a fluorescent DNA stain which shows enhanced fluorescence in the presence of DNA, is used to estimate the amount of DNA present in duplex and single-stranded form.

The degree of fluorescence enhancement of bisbenzamide is a function of the amount of DNA present in the assay system. In the experiment depicted in Fig. 2, varying numbers of L1210 cells and CCRF-CEM cells, or amounts of duplex salmon sperm DNA, were mixed with buffer containing bisbenzamide (see Materials and Methods) and sonicated, and fluorescence was determined. As the data show, there was a strict linear relationship between fluorescence and cell number or DNA content.

The degree of fluorescence enhancement is due also to the state of the DNA (Fig. 3). DNA was isolated from CCRF-CEM leukemia cells, and a portion of the purified, duplex DNA was denatured to the single-stranded form

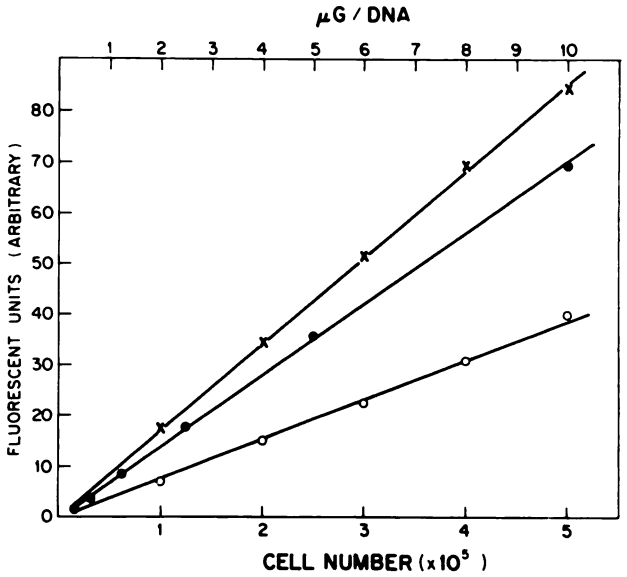

FIG. 2. *Fluorescence enhancement of bisbenzamide by purified and cellular DNA*

Varying numbers of CCRF-CEM leukemia cells (×), murine leukemia L1210 cells (○), and varying amounts of salmon sperm DNA (●) were mixed with a buffer solution containing bisbenzamide (Step A, Fig. 1) and sonicated, and fluorescence (arbitrary units) was determined.

by heat (immersion of DNA in a boiling water bath for 30 min and then plunge-cooling in an ice-water bath). The two forms of DNA were mixed to yield aliquots with varying ratios of single-stranded to duplex DNA, with total DNA kept constant. These aliquots were mixed with buffer containing bisbenzamide, and assayed fluorometrically. The aliquot containing duplex DNA ($F = 1.0$) yielded the greatest fluorescence, and less occurred with single-stranded DNA ($F = 0$). Between the two extremes a good linear relationship between fluorescence

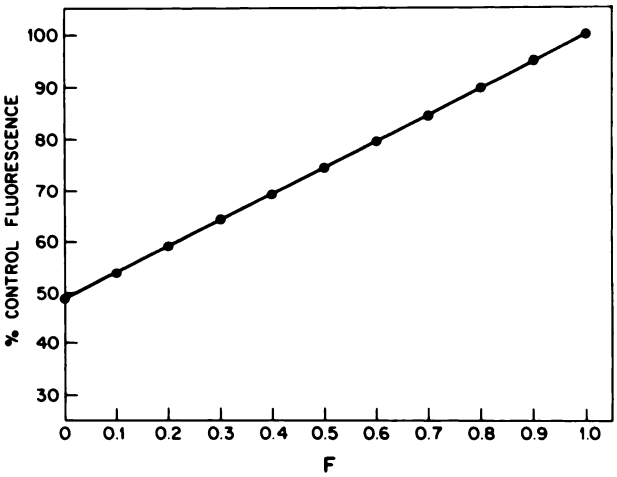

FIG. 3. *Fluorescence enhancement of bisbenzamide as a function of duplex:single-strand DNA ratio*

Purified duplex DNA from CCRF-CEM cells was mixed with single-stranded DNA from the same source to yield varying ratios of duplex DNA to total DNA (F). DNA samples (total DNA present kept constant) were mixed with buffer containing bisbenzamide and sonicated, and fluorescence was determined.

intensity and the fraction of total DNA in the duplex form was obtained ($r^2 > 0.999$).

A variety of assay conditions has been investigated, and, although not reported in detail, optimal conditions were selected for the assay. By way of example, Fig. 4 shows the relationship between fluorescence intensity of reagent blanks and cell lysates and the quantity of detergent (sodium lauroyl sarcosinate) present in buffer solutions. The addition of low levels of detergent increases the fluorescence enhancement of bisbenzamide by DNA (possibly by removal of proteins from DNA), but greatly increases reagent-blank fluorescence when present in high concentrations. In similar fashion, optimal conditions for pH, concentration of bisbenzamide, permissible salt concentrations, buffering capacity, sonication period and technique, and stability of reaction products were established.

Figure 5 shows the excitation and emission fluorescence spectra of CCRF-CEM leukemia cells treated by the technique shown in Fig. 1 for expression of DNA damage. Little overlap exists between the excitation and emission peaks. Tube A, which shows the greatest fluorescence, contains duplex DNA. Tube C, with single-stranded DNA, demonstrates the least, and Tube B, with partially alkaline-denatured DNA, contains an intermediate amount of fluorescent product, proportional to the amount of DNA remaining in duplex form.

P388 (from mice ascites) and L1210 (tissue-culture adapted) were used to calibrate the method. Washed P388 cells in PBS were exposed to varying amounts of X-irradiation (100–900 rads) and lysed in alkali for 15, 30, or 60 min (Fig. 6); F was then determined by fluorescence assay. As shown in Fig. 6, there is a log-linear relationship between irradiation exposure (rads) and the extent of alkaline denaturation (expressed as F). The data indicate the assay to be sufficiently sensitive to detect damage induced by less than 100 rads. Using Eq. 3, the number-average molecular weight (Mn_0) of the P388 alkaline unwinding unit was determined to be 7.75×10^8.

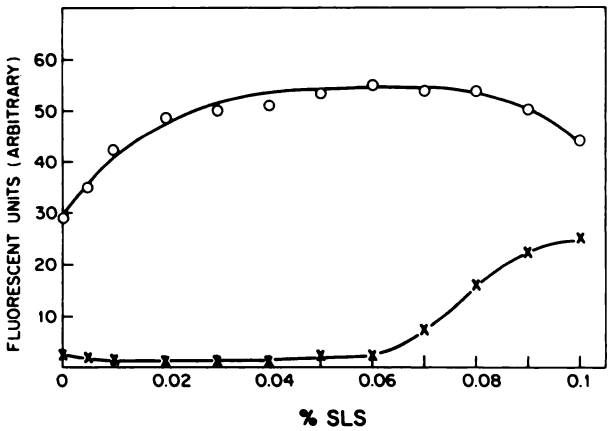

FIG. 4. *Effect of detergent concentration on fluorescence enhancement of bisbenzamide by cellular DNA*

L1210 cells (5×10^5) (○) were mixed with buffer solution containing varying amounts of sodium lauroyl sarcosinate and sonicated, and fluorescence was determined. Solutions without added cells (reagent blanks) (×) were similarly treated.

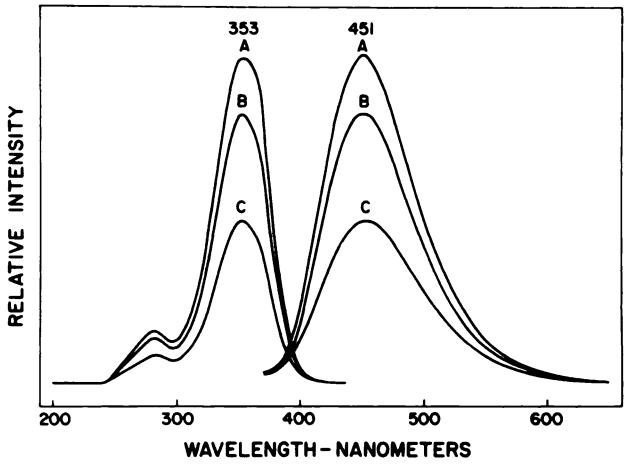

FIG. 5. *Excitation and emission fluorescence spectra of bisbenzamide complexed with cellular DNA*

Fluorescence spectra of Aliquots A, B, and C (see Fig. 1) were determined after lysis and sonication of CCRF-CEM cell samples.

^{14}C-Prelabeled L1210 cells were used in a comparison of this new technique, and the modified Rydberg procedure (3) for separation of single-stranded and duplex DNA on hydroxylapatite gels (Fig. 7). L1210 cells on ice (air atmosphere) were exposed to varying amounts of X-irradiation (100–1200 rads) and aliquots were lysed for fluorescence enhancement and hydroxylapatite analysis. Following determination of Mn_0 (See Eq. 3, Materials and Methods), data were converted to radiation-induced breaks per relative molecular mass of DNA ($n/10^9$ mol wt) and plotted against irradiation dose. As shown in Fig.

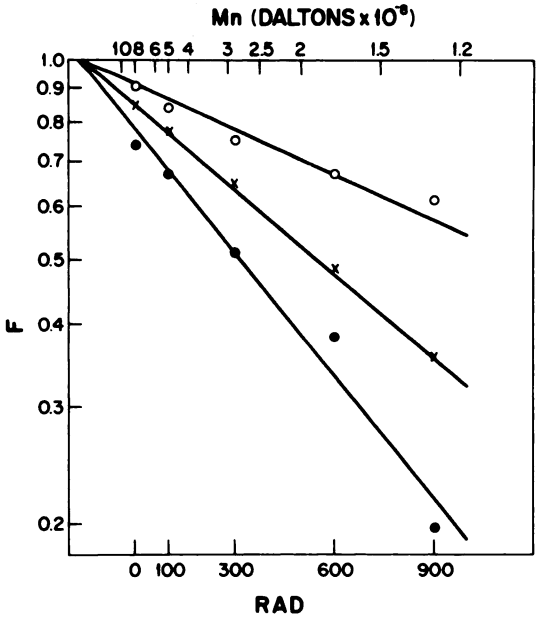

FIG. 6. *Fraction of DNA in duplex form after various doses of X-irradiation*

P388 murine leukemia cells were washed, irradiated on ice, and lysed (Step B, Fig. 1) for 15 min (○), 30 min (×), or 60 min (●). The fraction of duplex DNA (F) remaining was quantitiated by bisbenzamide fluorescence enhancement assay (Fig. 1). Molecular weight determinations were made by use of the Rydberg equation (Eg. 1, Materials and Methods).

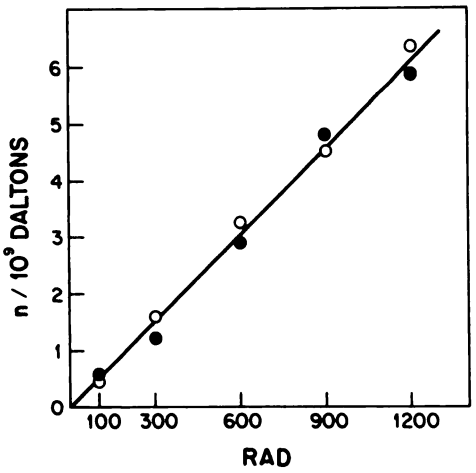

FIG. 7. *Comparison of results obtained by hydroxylapatite chromatography and fluorescence enhancement assay*

L1210 cells prelabeled with [^{14}C]dThd were irradiated on ice and denatured for 30 min in dilute alkali. Breaks per relative molecular mass of DNA ($n/10^9$) were determined by use of the Rydberg equation after determination of F by hydroxylapatite chromatography (●) and the bisbenzamide fluorescence enhancement assay (○).

7, data obtained by the two techniques are nearly identical ($r^2 > 0.99$).

The unwinding constant β was determined with L1210 cells. Cells were irradiated with 300 rads of X-irradiation and lysed for varying time periods (1–30 min). The fraction of total DNA in duplex form remaining after the limited periods of denaturation was determined by fluorescence enhancement analysis (Fig. 8). The fit of the straight line indicates that the Rydberg equation (Eq. 1) can indeed be applied to the data generated by this technique. The experimentally determined unwinding constant for this technique, 0.51, is close to the theoretical value (0.4–0.5) calculated by Rydberg (2).

In another series of experiments P388 ascites cells from DBA/2 mice were placed in short-term culture and exposed (2–4 hr) to varying concentrations of agents. The antineoplastic agents chosen, actinomycin D (7), 1,3-bis-(2-chloroethyl)-1-nitrosourea (8), daunorubicin (9), Adriamycin (10), carminomycin (10), bleomycin (11), 4′-(9-acridinylamino)-methansulfon-*m*-aniside (12), hycanthone (13), and neocarzinostatin (14), have been shown previously by ourselves and others to induce DNA damage in susceptible cells. Cells were harvested 4 hr after initial exposure to each agent and lysed, and DNA damage was assayed. Dose-response relationships were established for all of the agents tested, and are shown in Table 1.

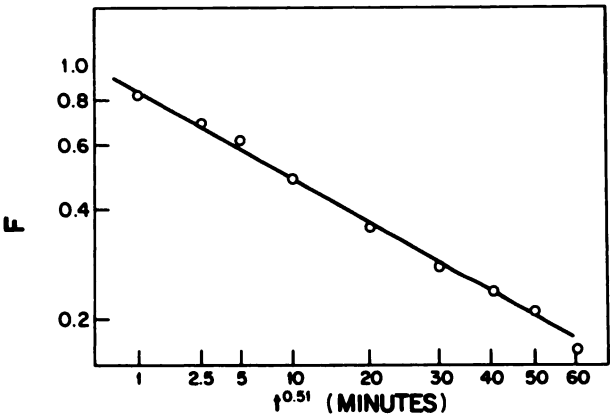

FIG. 8. *Kinetics of DNA strand separation determined by bisbenzamide fluorescence enhancement*

L1210 cells were irradiated (300 rads) on ice and denatured in alkali for varying time periods (1–60 min). The fraction of DNA in duplex form (F) remaining after denaturation was determined by fluorescence enhancement assay. The nonlinear abscissa shows unwinding time on a linear basis proportional to (time)$^{\beta}$.

DISCUSSION

Bisbenzamide fluorescence enhancement analysis of mammalian DNA damage can be used to measure low levels of DNA damage in unlabeled cells after exposure to a wide variety of DNA-reactive agents. Like other techniques which rely upon time-dependent partial alkaline denaturation for expression of DNA lesions (2–4, 6, 8), this assay method can determine only an average value for DNA damage. Unlike alkaline sucrose centrifugation techniques, it cannot distinguish between random and nonrandom forms of DNA damage. Each critical parameter for the assay and each experiment shown (e.g., β determination; calibration with irradiation; comparison with hydroxylapatite chromatography) were repeated with each cell line currently in use in our laboratory (L1210, P388, HeLa, CCRF-CEM), and all results were compatible with the experiments shown here.

Certain precautions must be exercised to assure reliable and reproducible results. Proper sonication technique is of critical importance. It was determined that foaming during sonication resulted in higher and more reliable fluorescence readings; the reason for this is unknown. Cells in Tubes B and C are lysed in alkali; this is not the case in Tube A, where the need for careful and complete sonication to lyse cells and bring about DNA dissolution is critical.

The fluorescent product formed is temperature-sensitive, and we have found that allowing development at room temperature overnight results in the most reliable fluorescence readings. The fluorescent product once formed, however, is quite stable; samples have been left on the benchtop exposed to normal room lighting for up to 8 weeks without any change in fluorescence. Furthermore, the complex is not photodissociated in the fluorometer.

We have found no advantage in siliconizing glassware prior to use, and no advantage was gained in transfer of lysates to conventional quartz cuvettes for fluorescence analysis. As in any fluorescence technique, however, scrupulously clean glassware and particle-free solutions should be used. All of the reagents used are stable at room temperature. A stock solution of bisbenzamide is made fresh every 2 weeks and is added to buffer the day of use.

As determined by others, bisbenzamide reacts specifically with DNA, and the degree of fluorescence enhancement is a function of adenine-thymine content (15). Enhancement of fluorescence by RNA relative to DNA has been reported to be below 1% (19). Recently a technique

TABLE 1

Effect of antineoplastic DNA-reactive agents on DNA integrity of P388 murine leukemia cells

P388 ascites cells freshly isolated from DBA/2N mice were incubated with varying concentrations of drugs for 2 or 4 hr, and DNA damage was assayed 4 hr after initial exposure to the various agents by the bisbenzamide fluorescence enhancement assay. For details see Materials and Methods.

Agent	Concentration	*F*	$n/10^9$ mol wt
	μg/ml		
Incubation for 2 hr			
BCNU[a]	0	0.74	—
	2.5	0.73	0.1
	5	0.74	—
	10	0.28	8.4
	20	0.06	21.8
Daunorubicin	0	0.73	—
	0.062	0.52	2.9
	0.125	0.45	4.2
	0.25	0.29	8.0
	0.5	0.06	21.6
Adriamycin	0	0.76	—
	0.62	0.51	3.5
	1.25	0.43	4.9
	2.5	0.36	6.5
	5.0	0.31	7.8
	10.0	0.22	10.7
Carminomycin	0	0.72	—
	0.062	0.66	0.8
	0.125	0.46	3.9
	0.25	0.42	4.7
	0.5	0.31	7.3
Bleomycin	0	0.79	—
	5.0	0.72	0.8
	10.0	0.69	1.2
	20.0	0.63	2.0
	40	0.57	2.8
Incubation for 4 hr			
Actinomycin D	0	0.67	—
	0.062	0.64	0.4
	0.125	0.55	1.7
	0.25	0.41	7.7
	0.5	0.22	9.6
	1.0	0.11	15.6
m-AMSA[b]	0	0.71	—
	0.031	0.50	3.0
	0.062	0.42	4.5
	0.125	0.34	6.4
	0.25	0.27	8.4
	0.50	0.20	11.0
Hycanthone	0	0.69	—
	3.1	0.51	2.6
	6.25	0.49	3.0
	12.5	0.30	7.2
	25	0.09	17.6
Neocarzinostatin	0	0.72	—
	0.156	0.63	1.2
	0.312	0.57	2.0
	0.625	0.50	3.2
	1.25	0.35	6.2
	2.50	0.17	12.5

[a] BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea.

[b] *m*-AMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-aniside.

was described for DNA quantitation with bisbenzamide (19). The total DNA content of cells in our assay can be determined with the aid of standard curves; however, we have found it necessary to employ internal DNA standards because of the quenching characteristics of certain cell lysates. Other agents with similar specificity for DNA (e.g., 4′,6-diamidino-2-phenylindole) can be used with this technique. However, the reaction conditions and reagent concentrations for the procedure have been optimized here only with bisbenzamide.

Certain DNA-reactive agents may interfere with the assay. Of the antineoplastic agents used in this study, only the anthracyclines, which bind strongly to DNA by intercalation and adlineation, appear to interfere with formation of the bisbenzamide-DNA complex. The alkaline procedures in Tubes B and C effectively destroy the tendency of the anthracyclines to interfere with bisbenzamide fluorescence enhancement, and fluorescence readings of treated cells in Tubes B and C are reliable. Such is not the case with Tube A, where lack of an alkaline environment allows the competitive binding to continue, yielding fluorescence readings lower than expected for duplex DNA. The problem is circumvented by calculation of the actual duplex:single-strand DNA fluorescent ratio in control cells and application of this derived factor to the calculation of an expected value for duplex DNA in treated groups. The possibility of similar interference with other agents may be easily ascertained by mixing equal amounts of the contents of Tubes A and C together. If no interference exists, the calculated value for $F = 0.50$ will be obtained experimentally.

REFERENCES

1. Ahnstrom, G, and K. Erixon. Radiation induced strand breakage in DNA from mammalian cells: strand separation in alkaline solution. *Int. J. Radiat. Biol.* **23**:285–289 (1973).
2. Rydberg, B. The rate of strand separation in alkali of DNA of irradiated mammalian cells. *Radiat. Res.* **61**:274–287 (1975).
3. Kanter, P. M. and H. S. Schwartz. A hydroxylapatite batch assay for quantitation of cellular DNA damage. *Anal. Biochem.* **97**:77–84 (1979).
4. Sheridan, R. B., and P. C. Huang. Single-strand breakage and repair in eukaryotic DNA as assayed by S1 nuclease. *Nucleic Acids Res.* **4**:299–318 (1977).
5. Hilwig, I., and A. Gropp. Staining of constitutive heterochromatin in mammalian chromosomes with a new fluorochrome. *Exp. Cell Res.* **75**:122–126 (1972).
6. Kohn, K. W., and R. A. Grimek-Ewig. Alkaline elution analysis, a new approach to the study of DNA single-strand interruptions in cells. *Cancer Res.* **33**:1849–1853 (1973).
7. Pater, M. M., and S. Mak. Actinomycin D induced breakage of human KB cell DNA. Nature (*Lond.*) **250**:786–788 (1974).
8. Ewig, R. A. G. and K. W. Kohn. DNA damage and repair in mouse leukemia L1210 cells treated with a nitrogen mustard, 1,3-bis-(2-chloroethyl)-1-nitrosourea, and other nitrosoureas. *Cancer Res.* **37**:2114–2122 (1977).
9. Schwartz, H. S. Some determinants of the therapeutic efficacy of actinomycin D (NSC-3053), adriamycin (NSC-123127), and daunorubicin (NSC-83142). *Cancer Chemother. Rep.* **58**:55–62 (1974).
10. Kanter, P. M., and H. S. Schwartz. Quantitative models for growth inhibition of human leukemia cells by antitumor anthracycline derivatives. *Cancer Res.* **39**:3661–3672 (1979).
11. Suzuki, H., K. Nagai, H. Yamaki, N. Tavoka, and H. Umezawa. On the mechanism of action of bleomycin scission of DNA strands *in vitro* and *in vivo*. *J. Antibiot.* **22**:446–448 (1969).
12. Furlong N. B., J. Sato, T. Brown, F. Chavez, and R. B. Nurlbert. Induction of limited DNA damage by the antitumor agent Cain's acridine. *Cancer Res.* **38**:1329–1335 (1978).
13. Bases, R., A. Leifer, H. Rozycki, C. Blake, and S. Neubort. Effects of Lucanthone on the sedimentation properties of DNA from HeLa cells. *Cancer Res.* **37**:2177–2181 (1977).
14. Hatayama, Y., and I. H. Goldberg. DNA damage and repair in relation to cell killing in neocarzinostatin-treated HeLa cells. *Biochim. Biophys. Acta* **563**:59–71 (1979).
15. Cesarone, C. F., C. Bolognesia, and L. Santi. Improved microfluorometric

DNA determination in biological material using 33258 Hoechst. *Anal. Biochem.* **100**:188–197 (1979).
16. Kanter, P. M., and H. S. Schwartz. Adriamycin-induced DNA damage in human leukemia cells. *Leukemia Res.* **3**:277–283 (1981).
17. Schwartz, H. S., and P. M. Kanter. DNA damage by anthracycline drugs in human leukemia cells. *Cancer Lett.* **13**:309–313 (1981).
18. Schwartz, H. S., H. D. Preisler, and P. M. Kanter. DNA damage in AML cells exposed to adriamycin: correlations with clinical response to therapy. *Leukemia Res.* **5**:363–366 (1981).
19. Brunk, C. F., K. C. Jones, and T. W. James. Assay for nanogram quantities of DNA in cellular homogenates. *Anal. Biochem.* **92**:497–500 (1979).

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