IN VITRO
16(7):609-615; 1980
All rights reserved

SEMIAUTOMATED AUTORADIOGRAPHIC MEASUREMENT OF DNA REPAIR IN NORMAL AND XERODERMA PIGMENTOSUM CULTURED HUMAN FIBROBLASTS

KENNETH H. KRAEMER,[1] JOSEPH K. BUCHANAN, AND SHERMAN F. STINSON

Laboratory of Molecular Carcinogenesis (K. H. K., J. K. B.) and Tumor Pathology Branch (S. F. S.), National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

(Received August 16, 1979; accepted November 26, 1979)

SUMMARY

Assessment of DNA repair in cultured human fibroblasts by autoradiography may be facilitated by using semiautomated grain counting instruments. The instrument-determined number of autoradiographic grains per nucleus in cultured human skin fibroblasts was found to be linear in comparison to visual counts up to only 30 grains per nucleus. However, with two different instruments a greater range of linearity (to 100 to 120 grains per nucleus) was attained by measuring the grain surface area per nucleus. Semiautomated analysis of the grain surface area per nucleus yielded measurements of relative rates of unscheduled DNA synthesis after ultraviolet irradiation in xeroderma pigmentosum and normal human fibroblasts, which were reproducible and rapid.

Key words: autoradiography; DNA repair; automated grain counting; xeroderma pigmentosum fibroblasts.

INTRODUCTION

Microscopic counting of autoradiographic grains as used in measurements of DNA repair in cultured human fibroblasts (1) is a time consuming, tedious process. Several instruments have recently become available that may be used for semiautomated autoradiographic grain counting (2-6). These instruments can discriminate among objects projected on a television screen and can measure the number of objects and the surface area of the images. This report compares the usefulness of two alternative grain counting modes (object number and surface area) for measurement of DNA repair in cultured human fibroblasts.

MATERIALS AND METHODS

Cell culture, irradiation, and autoradiogram preparation. Details of human skin fibroblast cell culture, ultraviolet irradiation, and autoradiogram preparation were similar to those described previously (1). Normal (GM975 and GM1652) and xeroderma pigmentosum (XP) (GM435, GM544, and GM677) fibroblasts were obtained from the Human Genetic Mutant Cell Repository at the Institute for Medical Research, Camden, NJ. The cells were grown without antibiotics in Ham's F-12 medium with 5% fetal bovine serum in a 5% CO_2 incubator. Cover-slip cultures in 60-mm petri dishes were washed with Hanks' salt solution and preincubated for 1 hr at 37° C with 10 μCi methyl[^{3}H]thymidine ([^{3}H]TdR) (sp act 20 Ci/mmol; ICN Pharmaceuticals, Irvine, CA) in 1 ml salt solution without phenol red to obtain heavily labeled S phase cells. The cultures were then irradiated with 254 nm ultraviolet radiation from a germicidal lamp (G875, General Electric, Cleveland, OH) with intensity 0.2 W/m^2 as measured with a IL 770 Radiometer (International Light Co., Newburyport, MA). To measure repair in non-S phase cells, 2 ml of [^{3}H]TdR-containing salt solution was added and the cultures were placed in the incubator for 2 hr. After washing with salt solution, fixing in acetic acid—ethanol (1:3), and mounting on microscope

[1] To whom all correspondence should be addressed at National Cancer Institute, Building 37 Room 3E24, Bethesda, Maryland 20205.

slides, the autoradiograms were prepared by dipping in NTB-3 nuclear track emulsion (Eastman Kodak, Rochester, NY) diluted with equal volume deionized water at 42° C. The autoradiograms were exposed at 4° C for 7 days, developed in D-19 developer (Eastman Kodak) at 15° C for 3 min and lightly stained with Harris hematoxylin (Fisher Scientific, Pittsburgh, PA). All slides in a single experiment were developed simultaneously.

Semiautomated grain counting instruments. Instrument A was an Artek model 880 Macro/Micro Counting System (Artek Systems Corporation, Farmingdale, NY) consisting of a high resolution vidicon, separate television monitor, and electronic circuitry. It was used with a Leitz Dialux microscope with 100× objective magnification. This instrument is capable of analysis of image numbers and surface area only.

Instrument B was an Omnicon Pattern Analysis System (Bausch & Lomb, Rochester, NY), which consists of a high resolution vidicon, television monitor, electrical circuitry, and a programable 32K computer. It was used with a Zeiss Universal Research microscope with 200× objective magnification. This instrument is capable of sophisticated image analysis including feature, pattern, and shape recognition and analysis, and optical density measurements.

For either instrument the user places the slide on the microscope stage and adjusts the microscope so that the autoradiographic grains appear in sharp focus on the television monitor. The user then adjusts an electronic aperature so that the nucleus to be counted is framed on the monitor. A discriminator sensitivity adjustment is made to determine threshold limits of detection. The detector is sensitive to differences in shades of gray as projected on the monitor. The discriminator setting functions to define the limits of grayness that represent grains and to differentiate grain intensity from cell and background grayness. Those objects counted are automatically indicated by white "flags," or a white outline on the monitor. Pressing a button gives digital readout of the number of objects or the total grain surface area. The microscope slide is moved to focus on the next nucleus to be counted and the process repeated.

Results

Comparison of visual grain counts with those obtained with the semiautomated instruments using the object number mode appears in Fig. 1, *A* and *B*. The grains in the emulsion overlying 50 nuclei were counted on each of four cover slips. Normal and XP fibroblast cultures had received 0 or 12 J/m² ultraviolet radiation so that cells could be observed with a range of from 0 to more than 100 grains per non-S phase nucleus. The linear regression equation calculated by the method of least squares for all 200 nuclei had a coefficient of correlation (r) of 0.88 for Instrument A

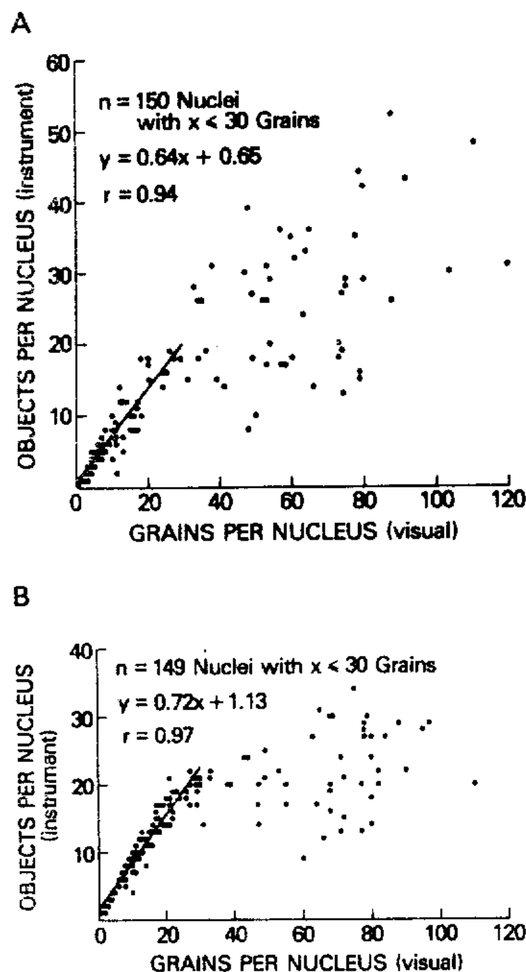

FIG. 1. Correlation of visual autoradiographic grain counts per nucleus with instrument counts of the same nuclei using the object number mode. Normal and XP fibroblasts were irradiated with 0 or 12 J/m² ultraviolet and autoradiograms prepared (*details in methods section*). Fifty nuclei from each of four cover slips in Experiment I were counted. Linear regression equations and coefficients of correlation (r) for those nuclei with 30 or fewer grains are indicated. *A*, Instrument A. *B*, Instrument B.

and 0.81 for Instrument B. When restricted to the 150 cells with 30 grains per nucleus or less, different lines were obtained with higher coefficients of correlation (0.94 and 0.97, respectively). Observation of the television monitor disclosed that with more than 30 grains per nucleus substantial overlapping of grains occurred and clusters of grains were flagged as single objects with either instrument resulting in poor correlation with visual counts.

The autoradiograms were evaluated further using the grain surface area measurement mode. Figure 2 illustrates the effect of varying the threshold sensitivity (discriminator setting) on grain surface area measurements of cells with different grain densities per nucleus. The curve for Instrument A (Fig. 2) was "U" shaped with a relatively broad minimum, which was flatter at low grain densities. The discriminator settings at which the upper and lower threshold indicators turn on and off are indicated. These are automatic sensors which, as demonstrated by Fig. 2*A*, give an indication of the range of readings that are on a relatively flat portion of the curve and that are thus rather insensitive to small changes in the threshold adjustment. In contrast (Fig. 2*B*) the sensitivity curve for Instrument B was only slightly concave. This instrument does not have automatic sensors and the sensitivity range where readings are made (indicated by *brackets*) was

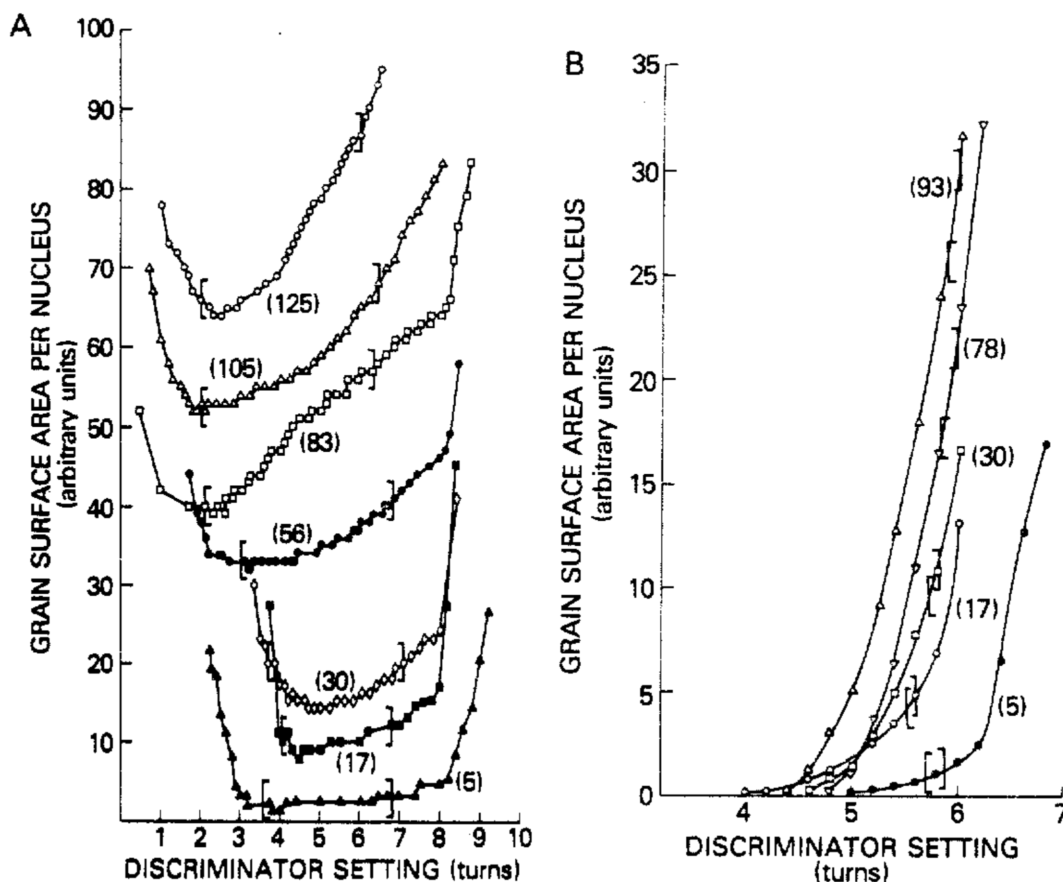

FIG. 2. Effect of changing the discriminator threshold setting on instrument readout using the grain surface area mode. Nuclei from Experiment II (with the number of visually counted grains indicated in parentheses) were focused and readings made at different discriminator settings. *A*, Instrument A. The brackets indicate settings at which the automatic upper and lower threshold indicators begin flashing on the television monitor. *B*, Instrument B. The brackets indicate settings where readings were made as governed by appearance of the white outline on the television monitor. (*Further details are in text.*)

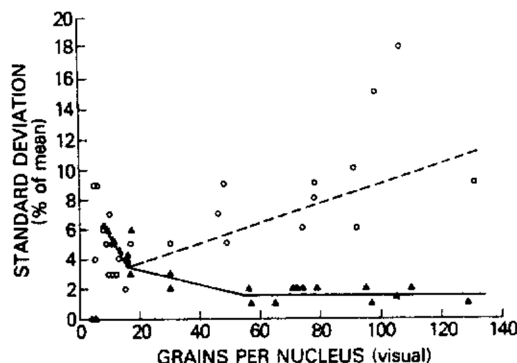

FIG. 3. Per cent standard deviation of grain surface area measurements as a function of the visually determined number of grains per nucleus. The grain surface area per nucleus was determined 10 times in succession for nuclei from Experiment II and the standard deviation expressed as a percent of the mean grain area per nucleus. ▲, Instrument A; ○, Instrument B. (Lines fitted by inspection.)

governed by the appearance of the white outline on the television monitor. This was on a steep portion of the curve where small changes in the discriminator setting would make large differences in the detected area.

The reproducibility of the sensitivity threshold adjustment process was assessed by counting the grain surface area of a cell 10 times. The percent standard deviation varied with the grain density for both instruments (Fig. 3). At low grain density (less than 10 grains/nucleus) both instruments had a higher percent standard deviation than with higher grain densities. This was probably because with small numbers the change of a single grain represents a large standard deviation. On Instrument A with increasing grain numbers the percent standard deviation progressively fell and above 40 grains per nucleus was constant at approximately 2%. With Instrument B, however, the percent standard deviation decreased to about 2% and then progressively rose to more than 10%. The rise probably reflects the very steep threshold sensitivity curves for cells with many grains per nucleus (Fig. 2*B*) resulting in increasing difficulty in reproducibly making threshold adjustments within the desired range.

The sensitivity threshold adjustment also compensates for differences in optical density due to factors such as uneven staining by discriminating between the gray intensity of grains and that of the nuclear background. When counting cells in succession, Instrument B was very sensitive to changes in background optical density and adjustments had to be made for each cell. In contrast, Instrument A was less sensitive and one adjustment was often suitable for many cells. Additional contrast between grains and stained nuclei could be attained by use of a red filter.

Figure 4, *A* and *B*, shows a comparison of visual grain counts with those data obtained with the semiautomated instruments using the grain surface area mode. The same 200 nuclei were counted as in Fig. 1. Calculation of the linear regression equations revealed linearity to 100 to 120

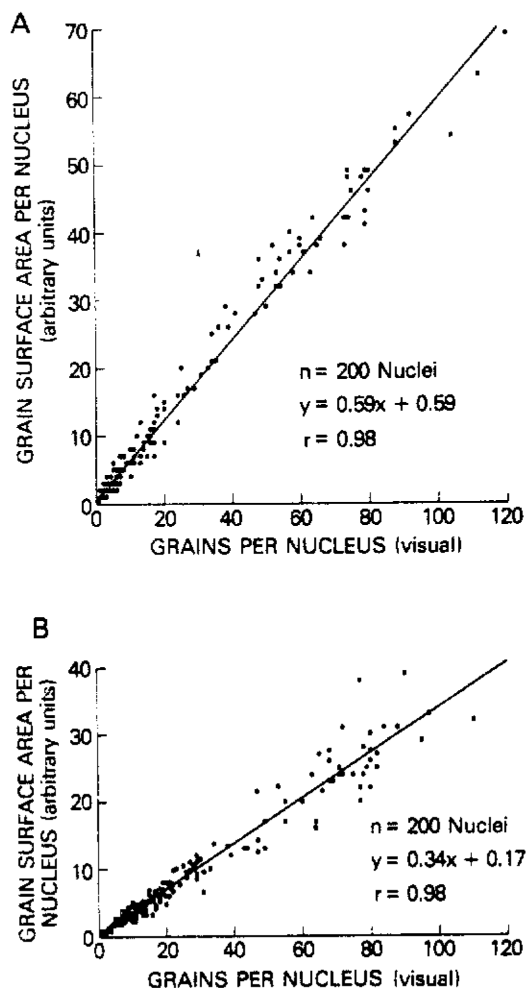

FIG. 4. Correlation of visual autoradiographic grain counts per nucleus with instrument counts of the same nuclei using the grain surface area mode. The same 200 nuclei from Experiment I were counted as in Fig. 1. Linear regression equations and coefficients of correlation (r) are indicated. *A*, Instrument A. *B*, Instrument B.

TABLE 1

COMPARISON OF LINEAR REGRESSION EQUATIONS AND COEFFICIENTS OF CORRELATION FOR NUCLEAR GRAINS IN DIFFERENT EXPERIMENTS ANALYZED IN GRAIN SURFACE AREA MODE

Experiment	Fibroblast Strain	Instrument	Linear Regression Equation[a]		
			Slope	Intercept	Coefficient of Correlation
I	Normal[b]	A	0.587[e]	1.022	0.99
		B	0.338[f]	0.046	0.98
I	XP[c]	A	0.614[e]	0.206	0.98
		B	0.365[f]	−0.116	0.95
II	Normal[d]	A	0.494[g]	2.369	0.99
		B	0.284[g]	−0.200	0.98

[a] The grains overlying 50 nuclei on each of 2 cover slips were counted visually and with the instrument in the grain surface area mode for each fibroblast strain. The linear regression equation of the relation between visual counts and instrument counts was determined by the method of least squares.

[b] GM975.

[c] GM435 (XP2NE complementation group D).

[d] GM1652.

[e] There was no significant difference between the slopes of the lines for the two strains in Experiment I. The test for coincidence indicates that the two lines are not significantly different ($P=0.18$).

[f] There was no significant difference between the slopes of the lines for the two strains in Experiment I. The test for coincidence indicates that the two lines are not significantly different ($P=0.18$).

[g] The slope of this line is significantly different from the slope of the corresponding lines in Experiment I ($P < 10^{-6}$).

grains per nucleus with a coefficient of correlation of 0.98 with each instrument. Separate slides within Experiment I had linear regression equations that were not significantly different in slope or intercept with both instruments (Table 1). Similar analysis of another experiment (II) also demonstrated high correlation ($r=0.98$) between the area measurement and the visual counts. However, the linear regression equation had a significantly different slope (Table 1). This probably reflects technical variations between experiments resulting in different average grain size in Experiment II.

Table 2 shows the use of Instrument A in the grain surface area mode to measure DNA repair after ultraviolet irradiation in autoradiograms from Experiments I and II. Normal fibroblasts and fibroblasts from XP patients with different DNA repair defects (1,7) were evaluated. Duplicate slides had similar nuclear grain surface area measurements. The two normal strains had similar rates of UV-induced unscheduled DNA synthesis. In experiment I XP complementation group A, C, and D strains had rates of UV-induced unscheduled DNA synthesis of less than 2, 19, and 35% of normal, respectively. In Experiment II the XP complementation group C strain had 21% of normal UV-induced unscheduled DNA synthesis. These rates are within the range previously reported for the different XP complementation group strains (1,7). Analysis of the 50 nuclei on each cover slip took approximately 25 min compared to an estimated 4 hr for visual counting.

DISCUSSION

Semiautomated grain counting is a reasonable approach to reducing the difficulties present in autoradiographic measurements of DNA repair in cultured cells. The counting was much more rapid than visual counts and, within limits, equally as accurate. Using our preparations of cultured human fibroblasts, those nuclei with 30 grains per nucleus or less had a high correlation between the number of objects counted and the visual counts ($r = 0.94$ to 0.97) (Fig. 1 *A* and *B*). However, with more than 30 grains per nucleus, with either instrument, multiple grains would touch each other and be counted as single objects with resulting poor correlation. In contrast, with smears of cultured sarcoma cells treated with hypotonic salt solution, Bunn et al. (2) found good correlation to more than 100 grains per nucleus using the number of objects mode. This difference can be explained by the fact that the sarcoma cell nuclei have two to four times the surface area of the human fibroblast nuclei (Kraemer, K. H.; Buchanan, J. K.; Shackney, S. E., *unpublished*) and that Bunn et al. (2) made adjustments in the instrument grain count to correct for grains observed to be overlapping.

Studies of DNA repair in human fibroblasts have demonstrated that XP complementation

TABLE 2

UNSCHEDULED DNA SYNTHESIS AFTER ULTRAVIOLET IRRADIATION IN NORMAL AND XERODERMA PIGMENTOSUM FIBROBLASTS

Experiment	Fibroblast Strain	Mean Grain Area/Nucleus UV Exposure[a]		UV-Induced Unscheduled DNA Synthesis	
		0	12 J/m²	Grain Area per Nucleus[b]	Fraction of Normal
					%
I	Normal[c]	1.78±0.42 2.30±0.62	29.8±3.18 32.0±2.42	28.89	100
I	Normal[d]	1.90±0.52 0.68±0.34	31.1±4.60 27.8±3.10	28.16	100
I	XP-A[e]	1.00±0.34 0.32±0.16	1.22±0.52 0.78±0.30	0.34	2
I	XP-C[f]	0.62±0.22 0.74±0.26	5.70±0.70 6.28±1.02	5.31	19
I	XP-D[g]	0.82±0.28 1.00±0.34	10.1±1.66 9.96±1.58	9.14	32
II	Normal[c]	1.04±0.64 0.58±0.28	23.2±2.40 22.9±2.52	22.26	100
II	Normal[d]	1.06±0.28 0.38±0.22	22.5±2.44 24.3±3.30	22.67	100
II	XP-C[f]	1.24±0.26 0.34±0.16	4.36±0.56 6.50±1.02	4.64	21

[a] The mean (± 2 SE) grain surface area per nucleus using Instrument A was determined in arbitrary units for 50 non-S phase nuclei on each cover slip. The instrument counts for duplicate cover slips for each treatment are indicated. Counts were determined using a red filter to maximize the contrast between the grains and the stained nuclei.

[b] Determined as the difference between the mean grain surface area per nucleus of the 100 irradiated nuclei (column 4) and the 100 unirradiated nuclei (column 3) evaluated for each cell strain.

[c] GM 975.

[d] GM 1652.

[e] GM 544 (XP16BR complementation group A).

[f] GM 677 (XP2BE complementation group C).

[g] GM 435 (XP2NE complementation group D).

groups may differ in rates of unscheduled DNA synthesis by less than 10% of normal (1). In order to detect reliably such small differences the normal controls must have approximately 100 grains per nucleus (1). Under these conditions use of the object number mode of the grain counting instruments would lead to large errors in the estimates of the number of grains in the control nuclei (Fig. 1, *A* and *B*). However, by using the grain surface area mode we found linearity with visual counts to approximately 100 to 120 grains per nucleus with a very high correlation coefficient using either instrument ($r=0.98$ to 0.99) (Fig. 4, *A* and *B*; Table 1) without need for adjustment for overlapping grains. Thus for most work with [^{3}H]thymidine we prefer to use the grain surface area mode for measuring DNA repair in human fibroblasts in culture. This approach might not be suitable for autoradiography with more energetic isotopes such as ^{14}C or ^{32}P. These isotopes produce relatively more irregular grains resulting from longer nonperpendicularly oriented tracks. Such grain areas might not correlate well with visual counts.

Both instruments were sensitive to changes in grain surface area (Table 1). This area may change from experiment to experiment due to variations in temperature or time of development or emulsion composition so that conversion of grain surface area to grain counts would depend on determination of a specific curve for each experiment. As a simpler alternative, differences in DNA repair within an experiment can be quantitated by direct comparison of grain surface areas without conversion to grain counts (Table 2). We found that this relative measure of DNA repair is reasonably constant between experiments (Table 2) so that reproducible differences in rates

of DNA repair of cultured human fibroblasts may be demonstrated.

References

1. Kraemer, K. H.; Coon, H. G.; Petinga, R. A.; Barrett, S. F.; Rahe, A. E.; Robbins, J. H. Genetic heterogeneity in xeroderma pigmentosum: Complementation groups and their relationship to DNA repair rates. Proc. Natl. Acad. Sci. U.S.A. 72: 59-63; 1975.
2. Bunn, P. A.; Ford, S. S.; Shackney, S. E. Rapid grain and cell counting for cell kinetic studies. Brief Communication. J. Natl. Cancer Inst. 59: 285-287; 1977.
3. Lipkin, L. E.; Lemkin, P.; Carman, G. Automated autoradiographic grain counting in human determined context. J. Histochem. Cytochem. 22: 755-765; 1971.
4. Prensky, W. Automated image analysis in autoradiography. Exp. Cell Res. 68: 388-394; 1971.
5. Preston, K.; Norgren, P. E. Automatic autoradiographic grain counting using the cellscan system. Ann. N.Y. Acad. Sci. 157: 393-399; 1969.
6. Wann, D. F.; Price, J. L.; Cowan, W. M.; Agulnek, M. A. An automated system for counting silver grains in autoradiographs. Brain Res. 81: 31-58; 1974.
7. Robbins, J. H.; Kraemer, K. H.; Lutzner, M. A.; Festoff, B. W.; Coon, H. G. Xeroderma pigmentosum: an inherited disease with sun sensitivity, multiple cuteneous neoplasms, and abnormal DNA repair. Ann. Intern. Med. 80: 221-248; 1974.

We thank Dr. Robert Tarone, Biometry Branch, National Cancer Institute, for assistance in the statistical analysis and Dr. Stanley Shackney, Clinical Pharmacology Branch, National Cancer Institute, for advice and encouragement.