On the Possibility That DNA Repair Is Related to Age in Non-Dividing Cells
(single-strand breaks/neurons/radiation)

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Communicated by Shields Warren, February 6, 1974

ABSTRACT
Possible age-related deterioration in the efficacy of DNA repair was investigated in a complement of non-dividing mammalian cells which is not replenished during the lifetime of the animal. Internal granular-layer neurons were extracted from the cerebella of beagle dogs, aged from 7 weeks to 13 years, following exposure in situ to 4700 rads of collimated 60Co gamma rays. The alkaline sucrose-gradient sedimentation profiles obtained from the DNA of those neurons after various post-irradiation periods in situ can be interpreted: (a) that there is not an age-associated decrease in the ability of the cells to rejoin the single-strand breaks induced by radiation, but (b) that there may be an age-associated decline in the size of the DNA-containing species which can be extracted from unirradiated cells. The latter effect may reflect normal aging of the cerebellum.

Despite outward differences, a majority of the current theories of aging rest upon the assumption that modification of genetic material is an essential factor in the aging process. There have been indications that structural alterations in mammalian chromatin, such as chromosomal aberrations (1-3) and ruptures of DNA strands (4, 5) accumulate with age in cell populations which usually undergo limited proliferation in situ. Both phenomena may represent expression of unrepaired DNA damage.

We believe that the repair of x-ray induced breaks in the DNA structure of mammalian cells in proliferative culture may be interpreted in terms of two generalized rejoining mechanisms (6-8). One of these mechanisms rejoins the strand breaks within DNA molecules thought to represent chromosomal subunits (7-9); the other restores the DNA structure found in the unirradiated cell (7, 8, 10-13). Since non-dividing cells, as exemplified by the retinal photoreceptor cells of the rabbit (14, 15) and the internal granular-layer neurons of the young beagle (13), also exhibit analogous repair mechanisms, a general rule could be that all normal mammalian cells possess such capacities. Exceptions to this rule, expressed as a loss of the ability to repair disruptions in the DNA structure, could then occur under certain special conditions. One such exception would seem to be represented by cells of certain prematurely senescent individuals (16). A second concerns mitotic populations of Chinese hamster ovary cells following extensive x-irradiation (8). A third could involve the expression of normal aging.

We chose to explore the third possibility with the internal granular-layer neurons of the cerebellum of the beagle because those neurons appear as a fully differentiated population of non-dividing cells at the birth of the animal (or very shortly thereafter) and are not replenished during its lifetime. The primary analytical approach explored the rejoicing of DNA strand breaks, produced in situ by a single dose of ionizing radiation, as a function of age. The use of a single γ-ray dose was imposed by the complexity of the rejoicing mechanisms which effectively contracted even a colony of a hundred beagles into a limited supply of animals of suitable ages. Similar considerations motivated the distribution of animals among control and experimental groups. The magnitude of the chosen γ-ray dose reflected detailed experiments with 7 week-old pups (13) which provide the base line for the present study.

METHODS AND MATERIALS
Cerebella of some hundred beagle dogs†, aged from 7 weeks to 13 years, were irradiated in situ with 4700 rads of collimated 60Co γ-rays from an Eldorado 8 teletherapy unit. Source decay imposed a 20% decline in dose rate from 235 rads/min during the experiments. At a suitable post-irradiation interval an animal was sacrificed, the cerebellum was removed, the internal granular-layer neurons were separated, and 2 to 3 X 10^7 cells were layered onto lytic zones of NaOH-EDTA over duplicate 10-30% alkaline sucrose gradients in titanium zonal rotors. After sedimentation the DNA fractions were collected and analyzed fluorometrically. All the above procedures have been fully documented (13, 17-20). Radioactively-labeled DNA markers obtained from Chinese hamster ovary cells which had been exposed to 1 krad of x-rays (14, 15, 21) were employed in all sedimentation runs with the DNA from animals older than 2 years.

RESULTS
Sedimentation. A detailed description of the rejoicing of radiation-induced strand breaks in the DNA of the internal granular-layer neurons in the cerebellum of the 7 week-old beagle has been published (13). The following resumé will provide the background for the present experiments.

When the neurons from unirradiated animals were lysed in alkaline EDTA the sucrose gradients contained DNA species with sedimentation coefficients up to 300 S (Fig. 1). A γ-ray dose of 4700 rads to those neurons in vitro resulted in a distribution of broken DNA molecules with a mean sedimentation coefficient of about 100 S and an experimentally detect-

† From the cesarian-derived controlled-access colony maintained at the Collaborative Radiological Health Laboratory, Colorado State University, which is supported by Public Health Service contract CPE-R-70-0001.
TABLE 1. Percent DNA in sedimentation profile greater than “subunit” size as a function of animal age and post-irradiation time

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Unirradiated</th>
<th>Time after 4700 rads of $^{60}$Co $\gamma$-rays (hr)</th>
<th>0.5</th>
<th>1.5</th>
<th>3-5</th>
<th>5-9</th>
<th>9-12</th>
<th>12-16</th>
<th>17-20</th>
<th>24</th>
<th>38-42</th>
<th>48</th>
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<td>57.4(2)*</td>
<td>0(4)</td>
<td>0(3)</td>
<td>3.2(1)</td>
<td>10.8(2)</td>
<td>19.7(7)</td>
<td>22.2(3)</td>
<td>35.4(1)</td>
<td>38.1(1)</td>
<td>51.5(2)</td>
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<tr>
<td>2</td>
<td>39.9(1)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>5.4(1)</td>
<td>8.7(2)</td>
<td>15.5(4)</td>
<td>24.5(3)</td>
<td>29.9(1)</td>
<td>24.8(2)</td>
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<td>3</td>
<td></td>
<td>22.5(2)</td>
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<tr>
<td>4†</td>
<td>38.0(3)</td>
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<td>0(1)</td>
<td></td>
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<td>24.4(4)</td>
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<td>13.4(2)</td>
<td>12.8(1)</td>
<td>21.4(1)</td>
<td>21.4(1)</td>
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<td>6</td>
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<td>0(1)</td>
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* Number of animals in parentheses; routinely two profiles per animal.
† Animals in the 4-year and 5-year age groups were males; all others were females.

Fig. 1. DNA sedimentation profiles from the internal granular-layer neurons of the cerebella of 7-week-old beagles. ——, unirradiated; ——, 90 min following 4700 rads of $^{60}$Co $\gamma$-rays.

Rejoining of $\gamma$-ray induced strand breaks seems to proceed as rapidly in old dogs as in young animals. The profile in Fig. 2a, which was obtained 90 min after irradiation of a 10-year-old dog, differs little from that obtained under similar circumstances from a 7-week old animal (Fig. 1). Faster-sedimenting DNA species began to appear in the profiles from older animals (Fig. 2b) after extended post-irradiation intervals, a behavior which again parallels the results from young animals (13). There was some evidence that a degradative process similar to that observed in the pup also occurred in 2-year-old dogs but at later times (9-12 hr). Consolidation of those indications was prevented by the restrictions in animal number so they will not be discussed further in this communication.

A complete record of our sedimentation experiments is contained in Table 1 where they are given in terms of the size distributions present in the sedimentation profiles. As in a number of other situations (7, 8, 15), the profiles shown by the solid lines in Figs. 1 and 2a seem to represent an intermediate stage in the rejoining process, because this stage is reached in

Fig. 2. DNA sedimentation profiles from the internal granular-layer neurons of aging beagles. (a) Age, 10 years; ——, 90 min following 4700 rads of $^{60}$Co $\gamma$-rays; ——, Chinese hamster ovary marker DNA profile. (b) Age, 13 years; ——, 72 hr following 4700 rads of $^{60}$Co $\gamma$-rays; ——, Chinese hamster ovary marker DNA profile.
fibroblasts would then have DNA.

Also, character would then have

whether the percentage of DNA

The cycle usually designated

in "blocked" post-mitotic stage of

the "165S subunit" until they reach

the proximity of the G1-S border (8).

Since the irradiated fibroblasts progressed from G1 into S, we were unable to
determine whether the onset of DNA replication synthesis was necessary for the restoration of the overall DNA structure.

Incorporation of radioactive precursors into the neuronal DNA in situ was impracticable, so 25 neuron preparations,

selected from the whole range of experimental conditions in Table 1, were analyzed by flow microfluorometry (FMF).

With this recently developed, elegant technique it is possible to determine the DNA content of single cells and hence the distribution of a cell population within the cell cycle (22, 23).

Our neuron preparations were analyzed by Dr. U. K. Ehmann with the FMF instrument at the University of California Laboratory at Los Alamos.

The FMF profiles from unirradiated neurons showed that at least 85% of the population contained a 2C (G1) DNA complement. The remaining 15% contained either a 4C (G2) DNA complement or were present as doublets (Fig. 3). There were no other indications that any of the cells were in the S or G2 phases of the cycle. Small, but variable, contamination by cytoplasmic fluorescence, as from residual axons, did not mar the resolution of the fluorometer since it appeared primarily in channels lower than those containing 2C DNA complements (Fig. 3). Within an experimental reproducibility of ±10%, identical spectra were obtained from all 25 preparations studied. The spectra in Fig. 3 were chosen to illustrate the maximum differences among the preparations. For completeness, we can also add that similar results were obtained from preparations of photoreceptor cells following in situ irradiation of rabbit retinas (14, 15).

So the FMF studies demonstrated that the cerebellar neurons were not stimulated to undergo detectable (>10%) DNA replication in our experiments and that extents of replication exceeding that figure were not required for the reconstruction of rapidly sedimenting DNA species.

DISCUSSION

Comprehensive interpretation of the alkaline sucrose-gradient sedimentation profiles from mammalian DNA is not possible at this time. But currently prevailing opinions will certainly allow that under the conditions of our experiments (and calibrations) molecules with sedimentation coefficients ≤165 S are probably single stranded, while values above that figure reflect an increasing probability of double-stranded configuration (9–11). We shall proceed on the reasonable assumption that an increase in sedimentation coefficient represents an increase in molecular size. The data from unirradiated animals recorded in Table 1 indicate that the size of the DNA which can be extracted from the internal granular-layer neurons of the beagle cerebellum, by our experimental procedures, may decrease with age. Also the results from lengthy post-irradiation periods suggest that for a given animal age, the DNA molecules broken by γ-irradiation can only be rejoined to the size of the DNA which can be extracted from unirradiated animals of that age. For example, following irradiation, a 10-year old animal does not seem to be able to restore molecular sizes to those found in unirradiated 7-week old animals.

In order to explore the kinetics of the rejoicing of radiation-induced DNA strand breaks as a function of age, the results in Table 1 must be corrected for the change in the molecular size of the DNA from unirradiated animals. This can be achieved by expressing the data obtained at post-irradiation intervals with animals of a given age in terms of the DNA distributions from unirradiated animals of the same age. When the results were calculated in that manner (not shown) it was found that the kinetics of rejoining of radiation-induced breaks were insensitive to age. Since the data in the unirradiated animal category are limited, the results in Table 1 can be utilized more fully with the assumption that the
72 hr post-irradiation interval also achieves the restoration of molecular size to control values. Calculations performed in that way also indicated that the rate of rejoining of radiation-induced strand breaks was insensitive to age. By inspection of Table 1 it is possible to discern a trend which indicates that the temporal appearance of fast-sedimenting DNA species suffers increasing delay with animal age. Although this trend is not substantiated by calculations performed in the above manner, it must remain a possibility.

Within the limitations of the present experiments we may conclude (a) that there is a finite possibility the DNA structure in the internal granular-layer neurons of the beagle cerebellum decreases in molecular size with age and (b) that the age of the animal does not affect the capacity of those cells to rejoin additional strand breaks induced by a single dose of γ-radiation. These conclusions are in general accord with the results of Price et al. (4) and Modak and Price (5). Moreover, if a chromosomal aberration is directly related to a disruption in DNA structure, then our results also concur with the findings of Curtis and his coworkers (1-3) concerning the accumulation of chromosomal aberrations with age.

Hayflick's demonstration of the senescence of proliferative cells in culture (24, 25) added another dimension to the aging phenomenon. Recently, Epstein et al. have shown that skin fibroblasts from a patient with a nonclassical Hutchinson-Gilford progeroid syndrome of accelerated aging have a decreased capacity to rejoin radiation-induced DNA strand breaks (16). Those data were collected from the 8th to the 10th generations of a culture which could not be carried beyond the 11th generation. The radiation response of those cells seems to be distinctly different from the population of non-dividing neurons which we have studied in situ. However, since the expected life-span of the beagles used in our studies was approximately 14 years we cannot rule out the possibility that an abrupt loss of repair capacity occurs in the final stages of the aging process. Furthermore, comparison of proliferative cell populations (and their life-spans in vitro) with non-dividing populations (and their life-spans in situ) would be decidedly tenuous at this time, because we would immediately be faced with the problem of whether the life-span of the whole animal is meaningful in terms of the true longevity potentials of its non-dividing cell populations. Clearly, if it is not, the neurons that we irradiated may not have even reached adolescence.

We wish to thank all the members of the Collaborative Radiological Health Laboratory, Colorado State University, for their fiscal, physical, intellectual, and moral support without which this project would not have been possible. Special acknowledgment in this respect goes to Drs. R. J. Garner, R. Pemister, D. Thake, and R. Thomsassen. Special acknowledgment must also be accorded by Dr. W. J. Tietz for providing the nucleus of the aging dog colony; fiscal support from Dr. M. R. Zelle was essential for its maintenance. Dr. D. F. Petersen kindly facilitated, and assisted in, the FMF experiments performed at Los Alamos. Centrifugation assistance was provided by C. T. Rankin, C. Frye, and F. Keng. This work was supported by the Department of Health, Education, and Welfare under NIH grant NS-08491 (NINDS) and Contract PR05458, by Union Carbide Subcontract no. 3080 from Oak Ridge National Laboratory (Dr. N. G. Anderson), and by the Beckman Instrument Co. (J. Finney).