SEDIMENTATION ANALYSIS OF DNA FROM IRRADIATED AND UNIRRADIATED L-CELLS

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ABSTRACT DNA, released from unirradiated mouse L-cells gently lysed in a thin layer of 2% sucrose on top of an alkaline sucrose gradient, was found to sediment in a narrow band with a sedimentation coefficient of about 500S. Exposure of cells to increasing doses of X-rays (89–712 rads) continuously reduced the DNA sedimentation velocity until, after about 890 rads, the DNA appeared in a narrow peak with a sedimentation coefficient of approximately 180S. As the dose given to cells was increased beyond 890 rads, the sedimentation coefficient of the DNA released continued to decrease and the sedimentation profiles now broadened in a manner consistent with the random production of single-strand breaks in the DNA. The DNA released from unirradiated cells (500S) is thought to be loosely aggregated and only partially single stranded. It is presumed that cells exposed to low doses of radiation release DNA with marked reductions in sedimentation coefficient because single-strand breaks produced in the DNA aid the alkaline denaturation process. By using the system to be described, it has been possible to demonstrate DNA repair (rejoining of X-ray–induced single-strand breaks) during postirradiation incubation of cells given doses as low as 400 rads.

INTRODUCTION

When the technique, pioneered by McGrath and Williams (1, 2), for velocity sedimentation of DNA in alkaline gradients has been applied to either bacterial or mammalian cells, the DNA released often appears to have a single-strand molecular weight of about 2–5 $\times$ 10^8 (1–13). After exposure of cells to large doses of radiation, it has been possible to demonstrate the formation of single-stranded breaks in DNA released from irradiated cells. It has also been possible to show that a large fraction of these breaks are rejoined during postirradiation incubation of cells; however, it is not known whether the repair phenomena seen after such large X-ray doses are characteristic of the cellular repair processes seen after the much lower doses normally used for viability studies. Also, since a single mammalian chromosome contains as much as 2 $\times$ 10^{11} daltons of DNA perhaps in a single molecule (14–18), the apparent regeneration of DNA of 2–5 $\times$ 10^8 daltons does not necessarily guarantee complete repair. For these reasons, it seemed desirable to in-

crease the sensitivity of the single-strand break assay by attempting to determine if single-stranded DNA of greater than 5×10^8 daltons could be isolated from mammalian cells.

The alkaline gradient technique employed by most investigators involves layering the cells directly onto an alkaline layer. Microscopic examination has shown that cell lysis occurs almost instantaneously upon contact with the alkali. It therefore seemed possible that the shear forces associated with loading of cells directly into alkali might cause mechanical breakage of large DNA molecules. It has also been shown that extensive DNA degradation occurs in alkaline gradients maintained at room temperature for extended periods before centrifugation (6, 11). In the course of conventional procedures, then, it seems possible that breakage of large DNA molecules might occur by mechanical forces and by alkaline hydrolysis. Observation of much smaller single-stranded DNA molecules could lead to an erroneous conclusion that the DNA in mammalian chromosomes was contained within small subunits (5, 11).

To determine if large pieces of single-stranded DNA might be released from mammalian cells, two modifications to the basic technique of McGrath and Williams were introduced. In an attempt to reduce alkali-induced degradation, gradients were maintained at 4°C during the cell lysis interval and during centrifugation. In order to minimize shear forces during cell disruption, a thin layer of neutral 2% sucrose was introduced to the top of the gradients. The L-cells layered onto the top of this hypotonic solution required approximately 20 min to approach the interface with the alkaline sucrose where lysis occurred. Under these conditions the cell disruption appeared to be much more gentle than when the cells were layered directly on alkaline solutions, and during lysis there was no turbulence associated with the loading pipette. After a 16–20 hr interval at 4°C followed by centrifugation, virtually all of the cellular DNA was found in a narrow band with a sedimentation coefficient of 450–550S when the 2% sucrose top layer was used, compared with sedimentation coefficients of approximately 200S observed when cells were layered into alkaline top layers.

The sedimentation coefficient of DNA released from cells irradiated with doses as low as 178 rads was significantly less than 500S; however, the sedimentation profiles of the irradiated DNA do not appear to be entirely consistent with those expected for randomly fragmented single-stranded DNA. This paper reports some attempts to characterize the 500S material and the effects of both small and large doses of radiation, with or without subsequent intervals for repair, on cellular DNA.

MATERIALS AND METHODS

Cell Culture

The cells used for these experiments are a subline of Earle's L-cells designated L60T (19). Suspension cultures were maintained in exponential growth in medium CMRL 1066 (20)

from which nucleosides and coenzymes were omitted. This medium was supplemented with 10% (v/v) bovine fetal serum (Flow Laboratories, Rockville, Md.) which had been previously dialyzed three times against 20 volumes of phosphate-buffered saline (PBS) (21). Under the conditions used, the cells had a generation time of approximately 16 hr.

For uniform labeling of DNA, cells were usually incubated in suspension culture for 18–30 hr in medium containing thymidine-^{14}C (TdR-^{14}C) (Amersham/Searle Corp., Arlington Heights, Ill.) at a concentration of 0.05 μCi/ml (specific activity 54 or 56 mCi/mM) along with an equal concentration of deoxycytidine (CdR). This low TdR-^{14}C concentration was found to give sufficient label in DNA for accurate counting statistics without altering cell viability.

In those experiments in which TdR-^{3}H was used to uniformly label DNA, the medium contained 0.25 μCi/ml TdR-^{3}H at 22.6 Ci/mM (Radiochemical Centre, Amersham). Proteins were labeled with leucine-^{3}H (Amersham/Searle) at a concentration of 10 μCi/ml and a specific activity of 3.3 mCi/mM in the medium. Lipids were labeled with choline-^{3}H (Amersham/Searle) at a concentration of 0.2 μCi/ml and at a final specific activity of 0.08 mCi/mM in the medium. In all cases labeling was carried out for at least 18 hr.

Cell Irradiation

Aliquots of labeled cells were centrifuged and resuspended in PBS at 4°C before irradiation at a dose rate of 890 rads/min (280 kv, 20 ma, half-value layer 1.2 mm Cu). After irradiation, the cells were again centrifuged at 4°C and the pellet resuspended in a Tris buffer at pH 7.4 (22). In repair experiments, the irradiated cells were pelleted, resuspended in warm medium, and incubated at 37°C for various times before being rechilled, repelleted, and resuspended in the Tris buffer.

Alkaline Sucrose Density Gradients

The nitrocellulose or polyallomer centrifuge tubes for the SW-27 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) contained 36 ml of a 10–30% linear sucrose density gradient made up in 0.3 N NaOH, 0.01 M ethylenediaminetetraacetate (EDTA), and 0.5 M NaCl. Before loading the cells, these gradients were allowed to sit at 4°C for several hours to ensure temperature equilibrium. The gradients were overlaid with a 2 ml layer of 2% sucrose in water (approximately 0.5 cm thick) on top of which was immediately placed 0.25 ml of Tris buffer containing 5–10 $\times$ 10^4 cells (approximately 1 μg of DNA [23]).

These loaded gradients were stored at 4°C for 16–20 hr before centrifugation to allow for DNA release and denaturation. Centrifuge tubes prepared in this fashion were placed in the SW-27 rotor and routinely centrifuged in a Beckman L2 ultracentrifuge at 23,000 rpm and 4°C for 150 or 360 min. After centrifugation, 1- or 2-ml fractions were collected from the top of the tube using an ISCO model D density gradient fractionator and a model 270 fraction collector (Instrumentation Specialties Co., Inc., Lincoln, Nebr.) by pumping 60% sucrose into the bottom of the tube at a rate of 2 ml/min.

Measurements of refractive index were used to confirm that the density of sucrose increased in a linear fashion down the gradient. Analysis of a gradient after centrifugation indicated that some diffusion had occurred with the result that the density of the top 7 of the 38 fractions diverged slightly from linearity with the rest of the gradient.

Radioactivity Determination

About 5 ml of 5% trichloroacetic acid (TCA) was added to each fraction from the gradient and the mixture drawn through a Whatman GF/C glass fiber filter (W. and R. Balston

Ltd., London, England). Each fraction tube was rinsed twice with 5% TCA and the filter washed with 10 ml of 95% ethanol. After drying, the filters were counted in a toluene-based liquid scintillation fluid. The total ^{14}C radioactivity put on each gradient was $1-3 \times 10^4$ cpm of which typically 60–80% was recovered.

Characterization of Gradients

The calculations of the sedimentation coefficient of L-cell DNA were based on a comparison with the sedimentation velocity of λ bacteriophage DNA which was assumed to have a sedimentation coefficient of 40.1S (24) in alkali. The sedimentation coefficient S was calculated by using the relation (25):

$$S = \beta \frac{D}{\text{rpm}^2 \times t}$$

where D is distance sedimented from the meniscus, rpm is the rotor speed, t is time, and β is a constant dependent on the gradient and centrifugation conditions which was determined by the sedimentation of λ bacteriophage DNA.

Cell Lysis

When only NaOH, EDTA, NaCl, and sucrose were present in the concentrations used in the gradients, it appeared under the phase-contrast microscope that some "membranous" cell material remained undissolved. The addition of 1% Sarkosyl (sodium lauroylsarcosinate) resulted in virtually complete dissolution of this material; however, Sarkosyl was not used in the gradients as it appeared to hasten DNA degradation.

The undissolved cellular material in centrifuged alkaline gradients was usually found in a "pellet" at the bottom of the centrifuge tube associated with some ^3H-labeled protein and lipid counts and small amounts of DNA-^{14}C. Occasionally, after centrifugation, some of this membranous material appeared to be trapped in the DNA peak (especially if the DNA was rapidly sedimenting) and created a small localized and visible white precipitate in the centrifuge tube near the position of the DNA peak. On a few occasions (usually after doses of about 1500 rads) this material remained near the top of the tube often associated with a small amount of DNA-^{14}C radioactivity.

RESULTS

DNA from unirradiated Cells

In order to determine whether large single-stranded DNA molecules might be released from L-cells, an attempt was made to minimize three possible sources of DNA fragmentation: (*a*) shear during lysis, (*b*) alkali attack, and (*c*) isotope decay. Fig. 1 *a* shows the sedimentation profile of DNA-^{14}C released from cells labeled with a low concentration of TdR-^{14}C and lysed "gently" in a layer of 2% sucrose in water which was sitting on top of an alkaline gradient held at 4°C. For the sake of comparison, Fig. 1 also shows the DNA sedimentation profiles obtained from cells layered directly onto an alkaline lysis layer (panel *b*), from cells lysed in a gradient held at room temperature for 7 hr (panel *c*), and from cells labeled with a

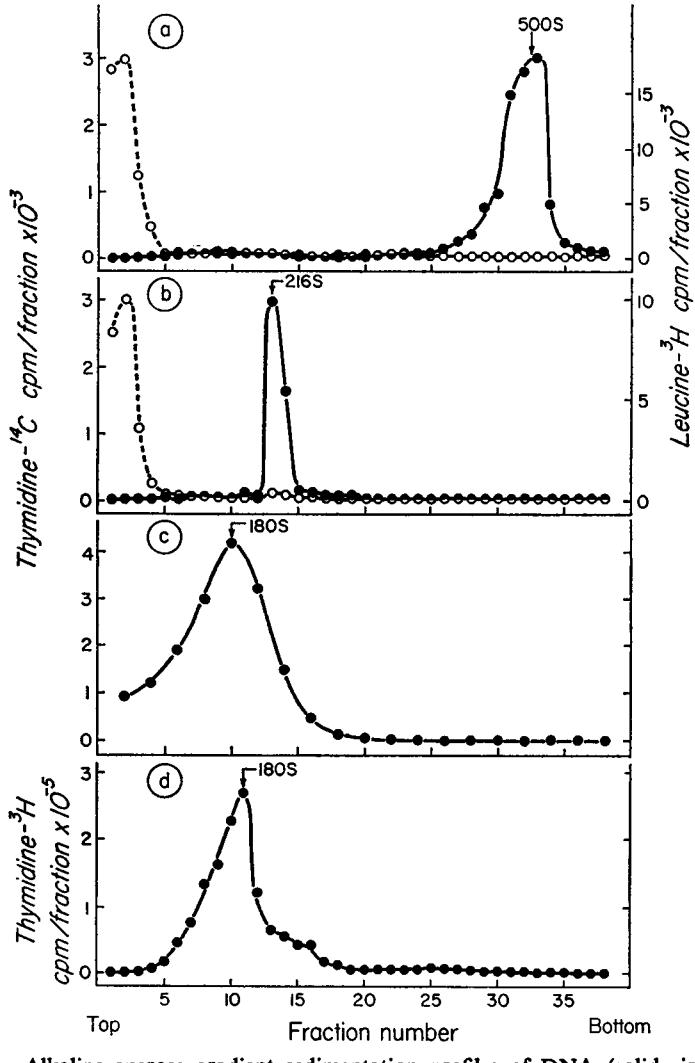

FIGURE 1 Alkaline sucrose gradient sedimentation profiles of DNA (solid circles) and protein (open circles) released from L-cells labeled and lysed under various conditions. In panel *a* the cells were labeled with TdR-^{14}C and leucine-^{3}H and loaded onto a gradient with a top layer (2 ml) of 2% sucrose. This gradient was maintained at 4°C for 18 hr before centrifugation. Panel *b* shows the DNA-^{14}C and protein-^{3}H profiles of the same cells as *a* loaded onto a gradient with a top layer (2 ml) of 0.5 N NaOH and 0.1 M EDTA. This gradient was also maintained at 4°C for 18 hr before centrifugation. Panel *c* shows the sedimentation profiles of DNA-^{14}C from cells loaded onto a 2 ml layer of 0.5 N NaOH and 0.1 M EDTA on top of a gradient maintained at 23°C for 7 hr before being placed at 4°C for the remaining 11 hr before centrifugation. The sedimentation profile in panel *d* was from cells labeled with TdR-^{3}H at a concentration of 10 μCi/ml before being layered onto a layer (2 ml) of 2% sucrose on top of a gradient kept at 4°C for the 18 hr before centrifugation. Centrifugation was for 150 min at 23,000 rpm and 4°C in the SW-27 rotor. Sedimentation is from left to right in these as in all profiles in this paper. The sedimentation coefficients have been corrected when appropriate for the fact that the DNA sedimentation starts from the interface of the 2% sucrose and the alkaline gradient, 2 ml below the top of the liquid in the centrifuge tube.

high concentration of TdR-^{3}H (panel *d*). The sedimentation coefficients of the DNA peaks were 500S, 216S, 180S, 180S, for tubes *a*, *b*, *c*, and *d* respectively. It would appear, then, that the DNA released from cells layered onto the 2% sucrose was much larger than that obtained under the other test conditions. This suggests that: (*a*) the DNA released from cells rapidly lysed (panel *b*) was fragmented presumably because of shear during the loading procedure, (*b*) the DNA released from cells lysed for 7 hr in a gradient held at room temperature (panel *c*) suffered alkali-induced breakage (longer lysis intervals at room temperature have been found to cause further degradation of this 180S DNA [6, 11]), and (*c*) DNA released from cells heavily labeled with TdR-^{3}H (panel *d*) was fragmented by the radioactive ^{3}H decay.

The DNA profiles shown in panels *a*, *b*, and *d* which were from cells loaded onto gradients maintained at 4°C were much sharper than the profile in panel *c* which was from cells loaded on a gradient which was later cooled from 23 to 4°C. Perhaps profiles *a*, *b*, and *d* were sharper because of a combination of aggregation and less diffusion at the lower temperature. The large half-width of the DNA peak in panel *c* was probably partly the result of convection during cooling of the gradient from room temperature to 4°C.

Because the DNA released from gently lysed cells (panel *a*) sedimented much faster than the DNA released from cells otherwise treated, the question may be asked as to whether this technique in fact produces single-stranded DNA free of protein and lipid contamination and without serious problems of macromolecular aggregation. In an attempt to determine the relative amounts of protein present in the DNA peaks, the cells whose DNA profiles are shown in panels *a* and *b* of Fig. 1 were also grown in leucine-^{3}H to label proteins. It may be seen from the ^{3}H profiles of panels *a* and *b* that less than 1% of the total radioactive protein label overlapped the DNA-^{14}C peak. This suggests that the DNA peak contained a maximum of 3% protein by weight. The balance of the labeled protein was found at the top of the gradient except for a small amount (about 5%) sometimes found in the bottom of the centrifuge tube. Whether the small amount of protein associated with the DNA peak represents material covalently bonded to DNA has yet to be established. Nevertheless, the fact that the amount of protein associated with the modified top layer was not greater than that associated with the DNA peak using the conventional lysis layer suggests that the more rapidly sedimenting DNA found with the 2% sucrose top layer is not due to nonspecific DNA-protein aggregation.

Experiments involving labeling of cell lipids with choline-^{3}H have indicated that less than 1% of the lipid label overlaps the DNA peak. It therefore seemed possible that the DNA released with the neutral top layer was in fact very large. It was also possible that this rapidly sedimenting DNA was an aggregate or a gel (4). More will be said about the nature of this DNA peak in a later section devoted to its characterization; however, for this characterization certain radiation data will be used and so a brief digression follows to discuss these experiments.

Sedimentation of DNA from Irradiated Cells

In order to determine the effect of radiation on the sedimentation properties of L-cell DNA, experiments were carried out in which cells were exposed to doses of X-rays ranging from 89 to 8900 rads before layering on the gradients. The resulting DNA sedimentation patterns are shown in Figs. 2 and 3. The DNA profiles in Fig. 2 indicate that after doses of between 89 and 712 rads, there was a progressive shift in the DNA pattern towards the meniscus with increasing dose and that a significant shift was produced by a dose as low as 178 rads. The secondary structures of the DNA profiles obtained after exposure of cells to 178 and 356 rads were not entirely reproducible, but there was a tendency in this and other similar experiments for doses in the range from 89 to 356 rads to produce DNA sedimentation patterns somewhat broader than either smaller or larger doses.

The DNA sedimentation profiles of Fig. 2 do not exhibit the shapes to be expected for randomly fragmented single-stranded DNA (26, 27); however, the DNA profiles shown in Fig. 3 were obtained from cells exposed to higher X-ray doses and are similar to those obtained by other investigators for this dose range (10, 11). As has been pointed out (11), DNA sedimentation patterns such as those of Fig. 3 are consistent with the interpretation that ionizing radiation induced random fragmentation of a uniform population of single-stranded DNA molecules each of molecular weight 5×10^8 daltons. Data presented elsewhere, however, suggest

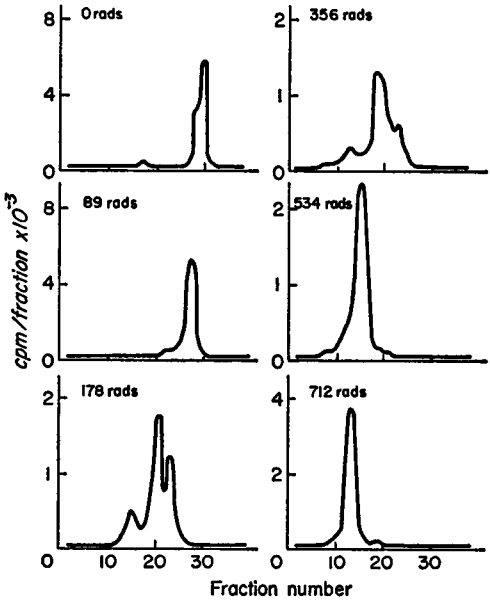

FIGURE 2 Alkaline sucrose gradient sedimentation profiles of DNA-^{14}C from cells irradiated with the doses given in the upper left of each panel. Except for cell irradiation, the procedure followed was that used to produce the profile in Fig. 1 *a*.

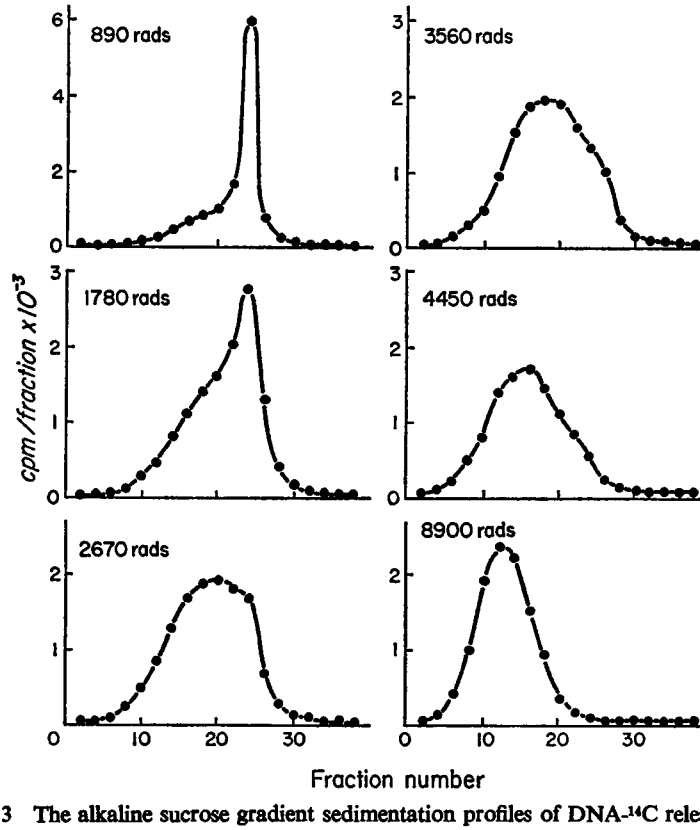

FIGURE 3 The alkaline sucrose gradient sedimentation profiles of DNA-^{14}C released from cells irradiated with the doses shown in the upper left of each panel. The procedure followed was the same as that used for Fig. 1 *a* except that centrifugation was for 360 min. Single-stranded DNA sedimenting to fraction 24 (the peak in the DNA profile from cells irradiated with 890 rads) would be expected to have a molecular weight of about 5×10^8.

that this interpretation may be invalid as the profiles appear to be the result of a sedimentation artifact (28).

Nature of the Unirradiated DNA

In this section an attempt will be made to answer some of the questions raised concerning the state of the DNA in the rapidly sedimenting 500S peak. This DNA has been shown to be relatively free of protein and lipid contamination and to be markedly reduced in sedimentation coefficient if cells are irradiated with low X-ray doses.

As a check on the sedimentation characteristics of the 500S material, gradients containing DNA from unirradiated cells were centrifuged for various lengths of time before fractionation. The sedimentation distance of the DNA was found to be directly proportional to the centrifugation time. Also, the sedimentation coeffi-

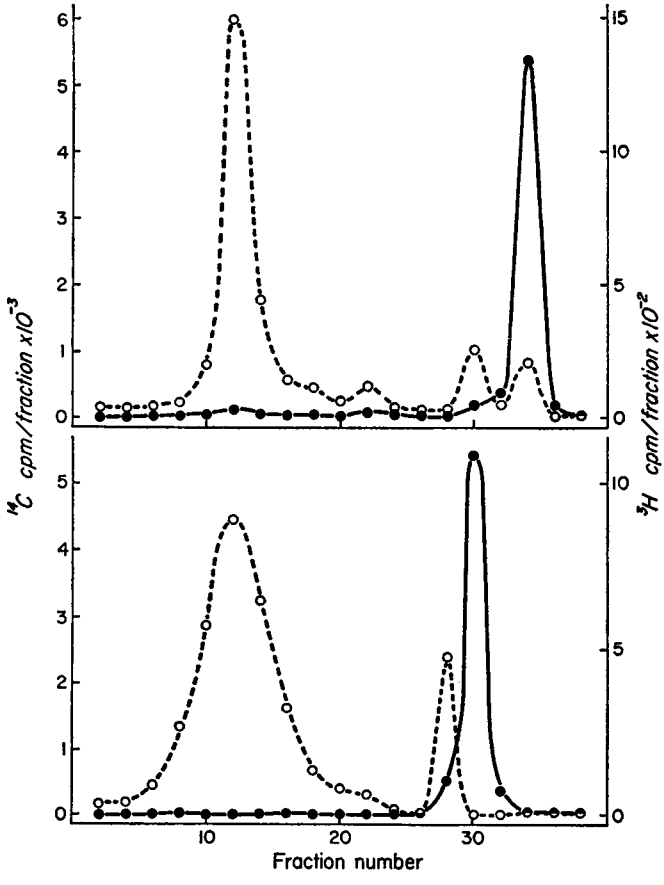

FIGURE 4 Sedimentation patterns of DNA from irradiated (open circles) and unirradiated (closed circles) cells mixed before loading onto the gradient described in Fig. 1 *a*. Unirradiated cells were labeled with TdR-^{14}C and were mixed with irradiated cells which had been labeled with TdR-^{3}H. Irradiated cells were exposed to 400 rads (upper panel) or 1335 rads (lower panel).

cient of the unirradiated DNA was found to be about 500S in centrifugation experiments carried out at 8000 rpm as well as at 23,000 rpm. This apparent lack of speed dependence is in contrast to that seen with single-stranded DNA (28, 29) but may only reflect the fact that sufficiently low speeds have not been used on the 500S material.

On the basis of Studier's relation (24) this rapidly sedimenting DNA, if it were single stranded, would have a molecular weight of 6–9 $\times$ 10^9 daltons. Two questions immediately arise in considering alkaline sedimentation of DNA molecules which may be up to 1 cm in length. First, is it possible for such a large molecule to sediment without becoming physically "tangled" with other molecules to form a

gel or aggregate (4)? Second, can such a large molecule denature completely in the time provided under the alkaline conditions used?

As a partial test for the presence of a DNA aggregate, two experiments were carried out. In the first, the cell number loaded per gradient was varied from 10^4 to 10^6 cells. Only slight changes in DNA sedimentation profiles were observed. The fact that the sedimentation profiles are not strongly dependent on DNA concentration suggests that nonspecific aggregation is not a serious problem.

As an independent and perhaps more critical test for aggregation, the second experiment was carried out as follows. Just before layering on the gradient, unirradiated cells labeled with TdR-^{14}C were mixed with the same number of TdR-^{3}H-labeled (at 0.25 μCi/ml) cells irradiated in one case with 400 rads and in the other with 1335 rads. As may be seen from Fig. 4, most of the irradiated DNA was found in a peak well separated from that of the unirradiated DNA; however, in both cases, some of the irradiated DNA seems to have sedimented in the unirradiated DNA peak. Similar low levels of intermixing were seen in other analogous experiments. It must be borne in mind that such a mixing experiment may only detect aggregation of DNA from different cells; it cannot be used to give information concerning aggregation of DNA molecules from the same cell. Nevertheless, the bulk of the molecules appear to have sedimented as independent particles, suggesting that the DNA was not in the form of a gel. The small amount of intermixing was perhaps due to a limited amount of physical entangling of the long DNA strands.

Regarding the question of denaturation of a DNA molecule to yield single-strand pieces of 6–9 $\times$ 10^9 daltons, it would seem improbable that such a process could be complete in 16 hr at 4°C under the alkaline conditions used (30, 31). Therefore it seems likely that the 500S material is composed of DNA in which the two strands have not unwound sufficiently to separate. Indirect evidence consistent with this hypothesis comes from the following experiment which is based on the assumption that single-strand breaks introduced into double-stranded DNA will increase unwinding of the DNA during the 16 hr exposure to alkali, whereas the same number of breaks introduced after the 16 hr exposure to alkali and immediately before centrifugation would not appreciably affect unwinding. In the experiment, the results of which are shown in Fig. 5, gradient *a* was loaded with cells exposed to 200 rads of ^{60}Co gamma rays and gradients *b*, *c*, and *d* were loaded with unirradiated cells. 2 hr after loading, by which time all cells would have lysed, the material in gradient *b* was irradiated with 200 rads. 16 hr after loading, and just before centrifugation, the material in gradient *c* was irradiated with 200 rads. Gradient *d* served as an unirradiated control. In all four cases the total exposure of cell material to the alkali was 16 hr at 4°C.

The DNA in gradients *a* and *b* sedimented at the same rate. This is consistent with the assumption that approximately as many nicks are produced in the DNA whether it is in the intact cell or in the gradient when irradiated, and because of this

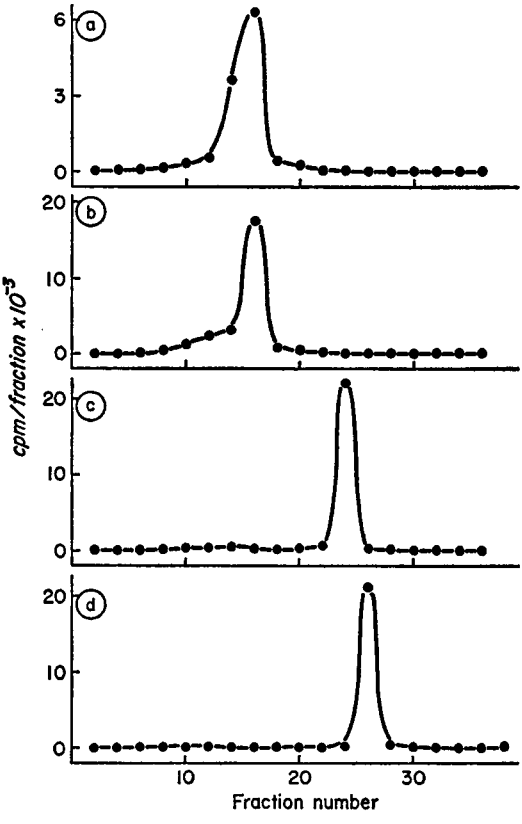

FIGURE 5 Sedimentation patterns of DNA-^{14}C released from L-cells which were (panel *a*) irradiated with 200 rads of ^{60}Co gamma rays (at a dose rate of 400 rads/min) before loading onto the 2% sucrose layer of the gradient, or (panels *b*, *c*, and *d*) unirradiated and layered onto 2% sucrose layers. The cellular material contained in centrifuge tube *b* was exposed to 200 rads 2 hr after loading whereas centrifuge tube *c* was given the same dose 16 hr after loading and just before centrifugation. Centrifuge tubes *a* and *d* were not irradiated. Centrifugation was for 150 min at 23,000 rpm and 4°C in the SW-27 rotor.

the DNA was unwound by the same amount during the 16 hr exposure to alkali. The fact that the DNA profile in gradient *c*, which was irradiated just before centrifugation, was intermediate between DNA profiles in gradients *b* and *d* was presumably because the time interval between nick formation and centrifugation was insufficient to allow appreciable unwinding about the radiation-induced nicks.

Rejoining of Single-Strand Breaks after Irradiation

It has been shown that the sedimentation coefficient of cell DNA may be markedly reduced by irradiation of cells before loading on the gradient with relatively low X-ray doses (Fig. 2). It is therefore possible to look for the rejoining of single-strand breaks in the DNA of cells incubated after low X-ray doses. Cells were

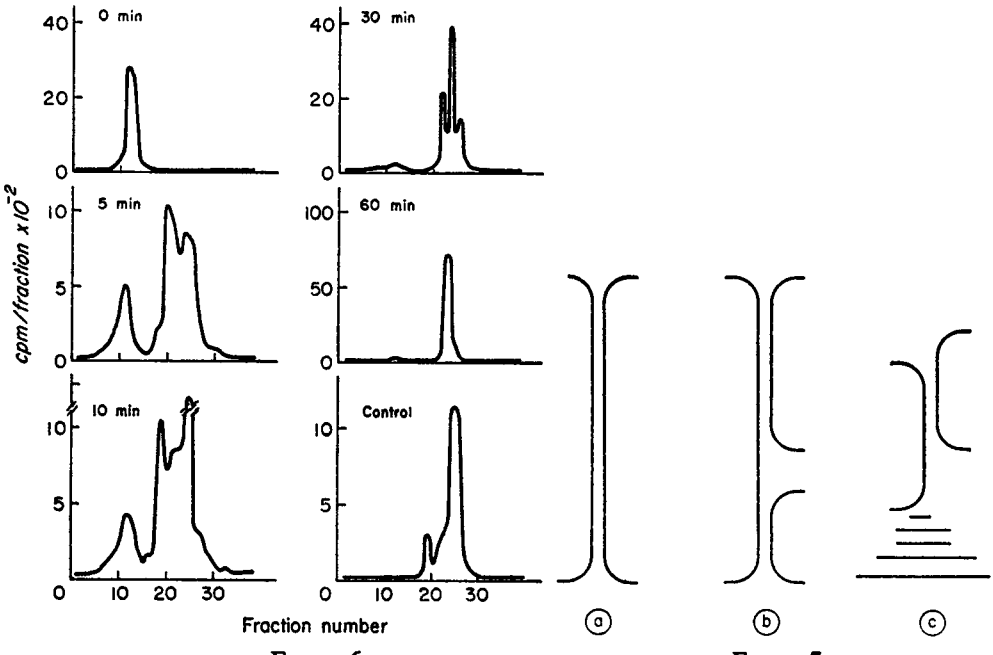

FIGURE 6

FIGURE 7

FIGURE 6 Sedimentation profiles of DNA-^{14}C from irradiated cells given postirradiation incubation. Cells were exposed to 400 rads and incubated at 37°C in growth medium for the interval recorded in the upper left of each panel. The control profile (lower right) was of DNA from unirradiated cells. Cell loading and centrifugation were as described in Fig. 1 *a*.

FIGURE 7 Model for the nature of cell DNA in alkaline sucrose gradients after 16 hr exposure to alkali at 4°C. Unirradiated DNA (*a*) is presumed to be unnicked and is thought to unwind only from the ends. Because of the alkaline conditions, the double-stranded region in the middle of this large molecule may not be hydrogen bonded but only intertwined. The DNA molecule *b* contains one single-strand break and unwinding may proceed from the break as well as from the end of the molecule. DNA molecules containing several single-strand breaks (*c*) denature almost completely into smaller single-stranded molecules.

irradiated with 40 rads at 4°C, a dose which corresponds to about 5% survival, and the sedimentation coefficient of the DNA released was found to be independent of the time (up to 1 hr) that cells were maintained in PBS at 4°C before loading on the gradient. If, however, these cells were incubated in growth medium at 37°C, an increase in sedimentation coefficient of DNA was found to occur. From the data of Fig. 6, it may be seen that single-strand break rejoining was virtually complete after 30 min incubation of cells. What was perhaps unexpected was the fact that DNA sedimented either in the light or heavy peak with very little indication of any DNA intermediates which would have sedimented in the region around fraction 15.

DISCUSSION

The gentle cell lysis modification of the technique of McGrath and Williams has been used to release, from L-cells, DNA with a sedimentation coefficient of about

500S. The fact that this DNA was very rapidly sedimenting suggested that either it was very large or was aggregated to form a rapidly sedimenting gel. Evidence against the latter possibility was provided by the experiment (Fig. 4) in which DNA from irradiated and unirradiated cells sedimented in two essentially distinct peaks with only a small amount of intermixing, probably the result of limited tangling of very large DNA molecules. The fact that the DNA sedimentation coefficient was markedly decreased by irradiation of cells with doses as low as 178 rads (Fig. 2) provided further evidence for the existence of large DNA molecules in the 500S peak.

Single-stranded DNA with a sedimentation coefficient of 500S would be expected to have a molecular weight in the region of 5×10^9–10^{10} (24). The denaturation kinetics of a double-stranded DNA molecule required to give such large single-stranded DNA, would suggest that complete denaturation of the molecule would not occur in 16 hr at 4°C even at pH 12 (30). Therefore it seems likely that the 500S DNA was not entirely single stranded. This assumption is supported by the results of Figs. 2 and 3. Fig. 3 shows that after doses greater than approximately 1000 rads the DNA released from irradiated cells behaved as though the radiation were inducing random single-strand breaks in double-stranded DNA molecules and that these double strands subsequently separated completely in the alkaline gradients. The DNA sedimentation patterns after doses in the region from 89 to 712 rads (Fig. 2), however, do not appear to be characteristic of randomly broken single-stranded DNA, suggesting that in this dose range the DNA may not be completely unwound in the alkaline gradients.

Certain assumptions concerning the mechanism of denaturation and the role of single-strand breaks in this process have been made in order that a molecular model may be proposed to account for the sedimentation behavior of DNA released from cells. The proposed forms of the DNA molecules as they exist in the alkaline gradients are shown in Fig. 7. It is postulated that the unirradiated and perhaps intact DNA molecule (*a*), which may be as large as 10^{10}–10^{11} daltons, may only be partially unwound after 16 hr of exposure to alkali at 4°C. It is assumed that the unwinding of strands occurs from the ends of the molecule (32). Exposure to alkali of this molecule containing a single-strand break (*b*) may give rise to localized unwinding about the nick (33) to produce single-stranded regions which are thought to reduce the sedimentation velocity of the large molecule.

This model is consistent with the results shown in Fig. 5 if it is further assumed that to produce an appreciable affect on the sedimentation coefficient, the unwinding about the single-strand break requires several hours of exposure to alkali. Higher doses of radiation (Fig. 7 *c*) provide more single-strand breaks in the DNA molecule from which unwinding may proceed with the result that: (*a*) a considerable amount of single-stranded DNA comes off the large molecule, and (*b*) the sedimentation coefficient of the remaining partially double-stranded DNA molecule is further reduced. After doses (greater than about 1000 rads) sufficient to induce a

large number of breaks, the DNA becomes completely denatured with a size distribution resulting from random degradation (34).

It is possible that by extending the time interval between cell loading and centrifugation, complete unwinding of unirradiated DNA could occur; however, no significant change in the DNA sedimentation pattern was observed for lysis times ranging between 12 and 48 hr.

The DNA profiles obtained from cells given postirradiation incubation (Fig. 6) are perhaps unexpected. The DNA released from cells given increasing incubation times appeared to flow from the light to the heavy peak with little evidence of DNA with intermediate sedimentation properties. The lack of an intermediate DNA component at times when both 180S and 500S components were present suggests that the rejoining of single-strand breaks may be a nonrandom process, i.e., repair of some molecules may be completed before the repair of others commences. It may also be noted from Fig. 6 that a small amount of 180S DNA was released from cells given 60 min of postirradiation incubation. It would appear, then, that a small fraction of breaks are rejoined very slowly in vivo.

Similar repair experiments were done with cells given a dose of 4450 rads. These indicated that the DNA profile from cells given 5 min of incubation resembled the profile obtained after 890 rads (Fig. 3) in the absence of a repair interval. Therefore, it would appear that most of the single-strand breaks were rejoined in the cells during the first 5 min of incubation; however, considerably longer incubation times (in the order of a few hours) were required before the cells released DNA which sedimented at 500S. In view of the fact that some bacteria are capable of rejoining double-strand breaks (35), it is tempting to speculate that the breaks which appear to be rejoined slowly in vivo are double-strand breaks.

The purpose of this investigation was to attempt to increase the size of the single-stranded DNA released from cells on alkaline gradients in order to increase the sensitivity of the single-strand break assay. The very large DNA obtained by use of the method described was not single stranded, but the sedimentation coefficient of this 500S DNA released from unirradiated L-cells could be markedly reduced if low doses of radiation were given to cells. A model is presented which explains the radiation data on the basis of the production of single-strand breaks in DNA. Therefore the technique appears to provide a sensitive assay for single-strand breaks produced in vivo and has been used to show rejoining of X-ray–induced single-strand breaks after doses as low as 400 rads (Fig. 6). It has also been used to obtain DNA profiles similar to those shown in Fig. 2 from cells exposed to ultraviolet radiation in the dose range from 200 to 1000 ergs/mm^2.

The authors are grateful to Doctors R. Sheinin and A. M. Rauth for assistance in the preparation of this manuscript, and Dr. A. Becker for provision of labeled λ bacteriophage.
The technical assistance of Miss M. McCulloch and Mr. O. Gray is also appreciated.

This work was supported by grants from the Medical Research Council and the National Cancer Institute of Canada.

Two of the authors, Mr. M. W. McBurney and Dr. F. L. Graham, were supported by fellowships from the National Research Council of Canada.

Received for publication 2 July 1971.

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