Fractionation of DNA from Mammalian Cells by Alkaline Elution†

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ABSTRACT: The method of alkaline elution provides a sensitive measure of DNA single-strand length distributions in mammalian cells and is applicable to a variety of problems concerning DNA damage, repair, and replication. The physical basis of the elution process was studied. The kinetics of elution above the alkaline transition pH were found to occur in two phases: an initial phase in which single-strand length is rate limiting, followed by a phase in which elution is accelerated due to the accumulation of alkali-induced strand breaks. The range of DNA single-strand lengths that can be discriminated by elution above the alkaline transition pH was estimated by calibration relative to the effects of x ray, and was found to be 5×10^8–10^{10} daltons. Shorter DNA strands elute within the pH transition zone, which extended from pH 11.3 to 11.7 when tetrapropylammonium hydroxide was used as base. This elution was relatively rapid, but was sharply limited by pH, according to the length of the strands: the length of the strands eluted increased with increasing pH. Alkaline elution was inhibited by treatment of cells with low concentrations of nitrogen mustard, a bifunctional alkylating known to cross-link DNA. On investigation of the possibility that DNA subclasses may differ in their elution behavior, satellite L strands were found to elute more slowly from cells exposed to a low dose of x ray than did the bulk DNA.

Size-distribution analysis of DNA single strands in cells is vital in studies of DNA damage and repair following radiation or cytotoxic drugs and in studies of DNA replication. Single-strand sizes up to $2–5 \times 10^8$ daltons can be measured by sedimentation in alkaline sucrose gradients (Lett et al., 1970; Elkind and Kamper, 1970; Elkind, 1971; Elkind and Chang-Liu, 1972; Ormerod and Lehmann, 1971; Ormerod and Stevens, 1971; McBurney et al., 1972; Cleaver, 1974a,b; Friedman et al., 1975). Above this size, however, no generally applicable method has been available. We have recently described a new method, based on the alkaline elution of DNA from cell lysates, which seemed to provide size measurements of extremely long DNA single strands (Kohn and Ewig, 1973; Kohn et al., 1974). We have obtained encouraging data upon applying this method to studies of DNA replication (Kohn et al., 1974), DNA repair following x ray (Kann et al., 1974; Bradley et al., 1976), DNA repair following ultraviolet irradiation (Fornace et al., 1976; Fornace and Kohn, 1976), DNA scission in cells treated with bleomycin (Iqbal et al., 1976; Kohn and Ewig, 1976), and DNA cross-linking in cells treated with nitrogen mustard or with chloroethylnitrosoureas (Ewig and Kohn, 1976). Aside from its analytic capabilities, the method has the advantage that it can readily be adapted to preparative applications, such as the estimation of the DNA single-strand lengths in which specific sequences are located. In the current work, we have examined some characteristics of the alkaline elution process, in order to elucidate the physical basis of the process and to explore its possible applications.

Methods

Cells. L1210 mouse leukemia cells were grown in suspension culture in RPMI 1630 medium supplemented with 20% fetal calf serum plus penicillin and streptomycin (Moore et al., 1966). Stock cultures were maintained in static bottles without antibiotics and were used to initiate suspension cultures at weekly intervals. Cultures were periodically tested for *Mycoplasma* and were free of contamination. Cultures used for experiments were in exponential growth with a doubling time of 12 h.

Uniform labeling of DNA was obtained with [2-^{14}C]thymidine (0.01–0.02 μCi/ml) or with [$^3H_3$]thymidine (0.1–0.2 μCi/ml, 10^{-5} M unlabeled thymidine added) for 20 h. Pulse labeling was with 0.1 μCi/ml of [2-^{14}C]thymidine or 2.5 μCi/ml of [$^3H_3$]thymidine for the indicated times.

X Ray. Cells were chilled in an ice bath and x irradiated while suspended in their growth medium in glass tubes packed in ice. X ray was delivered by two vertically opposed Westinghouse Quandrocondex x-ray units operated at 200 kV, 15 mA; 0.25-mm copper plus 0.55-mm aluminum filter. The dose exposure rate was 140 R/min for doses below 1000 R and 660 R/min for doses of 1000 R or higher.

Alkaline Elution. Unless otherwise specified, the standard procedure used was as follows (modified from Kohn et al., 1974). Cell suspensions were diluted 10–20-fold with cold phosphate-buffered saline, filtered onto a 25-mm diameter, 2-μm pore-size poly(vinyl chloride) filter (Millipore Corp., Bedford, Mass.), and washed several times with cold saline. The cells were lysed on the filter at room temperature with 5 ml of 0.2% sodium lauroylsarcosine (Sarkosyl, Ciba-Geigy Corp., Ardsley, N.Y.), 2 M NaCl, 0.02 M EDTA,[1] pH 10. The lysis solution was allowed to flow slowly through the filter without suction. The filters were then washed with 3 ml of 0.02 M Na_3EDTA, pH 10, and eluted in the dark with a solution consisting of 0.04 M H_4EDTA plus tetrapropylammonium hydroxide (Eastman Kodak Co., Rochester, N.Y., 10% in water) added in the amount required to give the desired pH (measured with a Beckman Type E glass electrode). The elution apparatus consisted of a modified filter funnel with output tubing passing through a peristaltic pump to a fraction collector (Kohn et al., 1974). The pumping rate was 0.03–0.06 ml/min. Fractions were collected at 90-min intervals and mixed with 3.3 volumes of Aquasol (New England Nuclear, Boston, Mass.) for scintillation counting. The chemiluminescence of Aquasol plus alkali was avoided by adding 0.3–0.7%

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[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

acetic acid. Radioactivity remaining on the filter and in the funnel after elution was determined as previously described (Kohn et al., 1974).

Sedimentation of DNA in Alkaline Sucrose Gradients. For sedimentation of DNA from whole cell lysates, 5×10^4 cells suspended in 0.2 ml of phosphate-buffered saline were mixed with 0.05 ml of 10 mg/ml of proteinase-K (EM Labs., Elmsford, New York), 5 mM $CaCl_2$, 2% sodium dodecyl sulfate, 1 mM EDTA, 0.01 M Tris buffer, pH 8.0, and 0.2 ml of 2% Sarkosyl, and incubated at 50 °C for 1 h (Dingman and Kakefuda, 1976). The lysates were then slowly pipetted onto preformed alkaline gradients and centrifuged at 20 °C in a Beckman SW40 Ti rotor for approximately 17 h at the speed indicated in each experiment. The gradients were 4–20% in sucrose and 0.1–0.3 M in NaOH, and contained 0.9 M NaCl and 0.05 M Na_2EDTA.

For sedimentation of eluted DNA, the elution apparatus was modified to minimize shear. The flow through the filter was controlled by pumping solution into the top of the funnel via an air-tight seal, and the outflow tubing, which was 2-mm in diameter, was placed at the surface of an alkaline sucrose gradient. The solution eluted during the desired time interval was pumped onto the gradient at a rate of 0.03–0.04 ml/min.

Molecular weight distributions and weight-average molecular weight ($\overline{M}_w$) were computed as described by Dingman (1972).

Number average molecular weight ($\overline{M}_n$) was determined from the relation for the length distribution expected from random strand scission (Lehmann and Ormerod, 1970a; Kohn et al., 1973): $\ln (F/l\Delta l) = pl + 2 \ln p$, where F is the fraction of the total DNA present in a given gradient fraction, l is the average strand length and Δl the range of strand lengths corresponding to that gradient fraction, and $p = 330/\overline{M}_n$ is the frequency of strand breaks. This relation predicts a linear dependence of $\ln (F/l\Delta l)$ vs. l with slope, $-p$, and intercept, $2 \ln p$. The observed sedimentation distributions exhibited linearity over the major parts of the distributions and the values of p, estimated from slope and intercept, were in close agreement. The advantage of this method of estimating p (or $\overline{M}_n$) is that it is relatively insensitive to errors due to material sedimenting near the top or bottom of the gradient.

Preparative Isopycnic Centrifugation. Eluted DNA was neutralized (to pH ~8) with 0.1 M Tris-HCl, 0.01 M Na_2EDTA, pH 7.0, and then dialyzed against 0.15 M NaCl, 0.015 M sodium citrate. Three milliliters of this solution was mixed with 1.5 ml of 0.2 M $NaHCO_3$, 0.1 M Na_2EDTA, pH 10.0, plus 1.5 ml of 1 M sodium trichloroacetate, 0.1 M $NaHCO_3$, 0.05 M EDTA, pH 10.0. Six grams of this solution was mixed with 7.28 g of CsCl (Harshaw Chemical Co., Cleveland, Ohio, optical grade) and centrifuged for 48 hr at 40 000 rpm at 25 °C in a Beckman Type 65 angle rotor. Five-drop fractions were precipitated with 5% trichloroacetic acid, 0.02 M sodium pyrophosphate. The precipitates were collected on filters (Millipore HAWP), air dried, and counted in 5 ml of Econofluor (New England Nuclear).

Analytical Isopycnic Centrifucation of Eluted DNA. Eluted solutions were neutralized with 0.2 volume of 0.07 M citric acid, 0.1 M Tris, 1.8 M NaCl, and filtered through nitrocellulose filters (Schleicher and Schuell, Keene, New Hampshire; Type B6, 25-mm diameter). All of the DNA adsorbed to the filter and was quantitatively extracted with 1.5 ml of 0.1 N NaOH, 1 mM EDTA. Six-tenths milliliter of this solution was mixed with 0.85 g of CsCl and the refractive index was adjusted to 1.4025. The solution was centrifuged at 44 000 rpm for 20 h at 23 °C in a Beckman Model E ultracentrifuge equipped with monochrometer and photoelectric scanner.

Results

In the alkaline elution procedure, cells are deposited on a filter, lysed with a detergent-containing solution, and then subjected to an alkaline solution which is slowly pumped through the filter (see Methods). The method is based on the observation that the rate of DNA release (elution) from the cell lysate at high pH is very sensitive to x irradiation of the cells (Kohn and Ewig 1973; Kohn et al., 1974). We address ourselves to the question of what determines the rate of DNA release. We will first review some general observations that indicate that elution rate is governed by the properties of the DNA itself. This will be followed by more quantitative data that indicate that elution generally requires DNA-strand separation and that the rate of elution depends on average single-strand size.

The procedure we use has the advantage that almost all of the non-DNA material of the cell can be removed before the analysis with alkali is begun. This is accomplished by the lysis solution (2 M NaCl, 0.2% Sarkosyl, 0.02 M EDTA, pH 8–10), the composition of which assures that most DNA–protein complexes are dissociated. Such cell lysates contained no remaining membrane structures upon microscopic examination using Nomarski interference optics. A further indication that the retention and release of DNA from filters are not governed by lipid-containing materials was the finding that addition of 0.2% sodium dodecyl sulfate to the eluting solution did not alter the kinetics of DNA elution from cells, whether or not x irradiated.

The extent of removal of protein was determined in cells that had been labeled with [^{3}H]leucine for 20 h. Approximately 90% of the labeled protein was removed by the lysis solution. In order to determine whether the residual protein remaining on the filter affects DNA elution, the filter was then treated with proteinase-K. By this means, it was possible to remove approximately 98% of the cell protein with little effect upon the subsequent elution of DNA from control or x-irradiated cells (Fornace and Kohn, 1976). Similarly, treatment with ribonuclease did not alter the kinetics of DNA elution.

The rate of DNA elution from irradiated or unirradiated cells was independent of the number of cells deposited on the filter in the range of $0.1–2.0 \times 10^6$ cells. The retention of DNA on the filter, therefore, does not depend on the aggregation or trapping of DNA strands in a large-scale gel.

If retention of DNA on the filter is dependent on the large size of the DNA strands, then elution should be sensitive to shear. This was found to be the case, both for the lysate, and for the DNA on the filter. When washed cells were gently lysed in solution, and the lysate then filtered, the fraction of the DNA trapped by the filter was markedly reduced if the lysate was first sheared, for example by inverting the tube containing the lysate several times prior to filtration.

The DNA on the filter also was noted to be highly susceptible to shearing. This was exhibited in the following ways. If at any time after lysis, the filter is disturbed, for example by gently lifting and immediately replacing it, there is striking and immediate increase in DNA elution. This may be due to DNA strands hanging down from the filter; such strands would be vulnerable to shear when the filter is moved. A spurious rapid elution of DNA also occurs if the flow rate is too rapid, or if air is sucked into the filter by continued pumping after the solution above the filter has been exhausted. (To avoid shearing

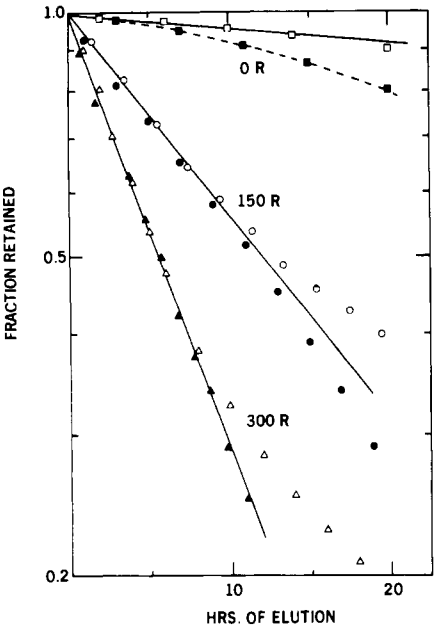

FIGURE 1: Kinetics of the early phase of elution. L1210 cells were labeled with [^{14}C]thymidine for 20 h and x irradiated with the indicated dose at 0 °C. Lysis was at pH 10.2, and elution at pH 11.96 (open symbols) or pH 12.82 (closed symbols).

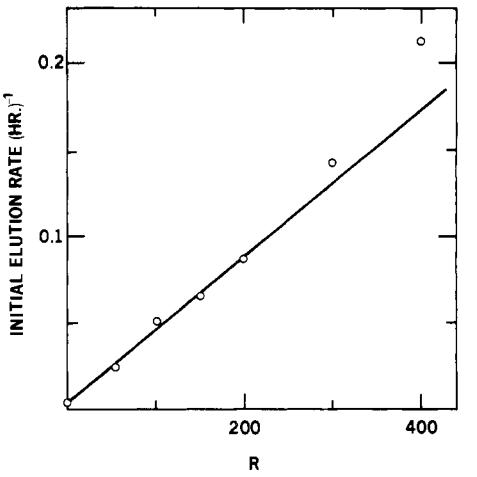

FIGURE 2: Dependence of the apparent initial rate constant of elution on x-ray dose. Data from experiments similar to those shown in Figure 1.

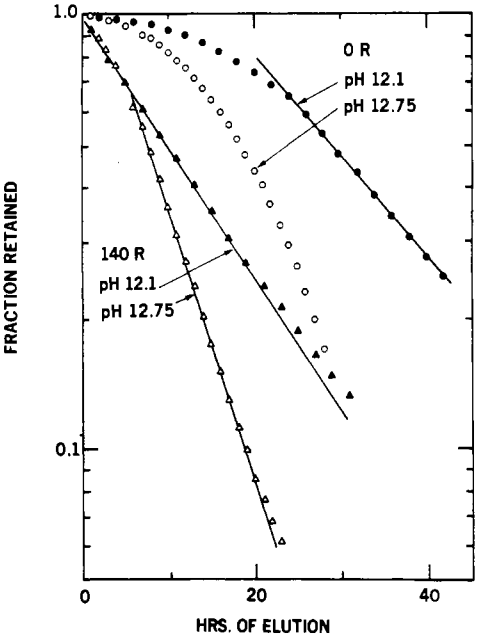

FIGURE 3: Elution patterns of DNA from unirradiated and x-irradiated L1210 cells. Cells were labeled with [^{14}C]thymidine for 20 h and some were irradiated with 140 R at 0 °C. 1.0×10^6 cells were filtered, lysed at pH 8.2, and eluted at pH 12.1 or 12.75.

the DNA, all air must be expelled from the dead-space below the filter before applying the cells).

The retention of DNA might be thought to depend on an interaction of the DNA with the filter material. To test this possibility, we compared various filter materials and found qualitatively similar effects using filters made of poly(vinyl chloride), cellulose triacetate, Teflon, or glass fiber. Furthermore, the results were essentially independent of pore size from 1 to 5 μm. Quantitative differences do exist, however, between different lots of even the same type of filter.

The elution of DNA was found to be markedly sensitive to exposure of the alkaline solution to room light. Even exposure to yellow light (Corning No. 3385 filter) increased the elution rate. The increase in elution rate in the presence of the yellow filter was about 30% as great as that produced by unobstructed fluorescent lighting under the same conditions. Light sensitivity of DNA in alkali has also been noted in alkaline sucrose gradient experiments (Elkind 1971; Friedman et al., 1975), indicating that DNA under alkaline conditions is susceptible to strand scission by visible light.

Kinetics of Elution. The kinetics of the alkaline elution of uniformly labeled DNA from mammalian cells was found to consist of two phases. In the initial phase, the rate is approximately first order with respect to time (Figure 1) and x-ray dose (Figure 2), and is independent of pH above pH 11.9 (Figure 1). In the later phase, the elution rate is accelerated and becomes dependent on pH and independent of x ray (Figure 3). These results suggest that the first phase of elution is rate limited by the size of the DNA strands resulting from strand breakage by x ray, whereas the later phase of elution is governed by strand breakage due to alkaline hydrolysis.

Quite different elution kinetics are exhibited by pulse-labeled cells (Figure 4). In comparison with the elution of fully labeled DNA from cells exposed to a low dose of x ray (dashed curve in Figure 4), the elution of pulse-labeled DNA exhibits an initial rapid component. The initial rapid elution represents newly synthesized DNA strands, since the amount of DNA eluting in this phase decreases with time after labeling. This phenomenon reflects the elongation of DNA strands, probably of replicon length, during replication (Kohn et al., 1974).

In order to better characterize the changes in DNA elution produced by low doses of x ray, x-irradiated [^{14}C]thymidine-labeled cells were mixed with [^{3}H]thymidine-labeled controls cells and the mixture was subjected to alkaline elution. The [^{14}C] remaining on the filter after various times of elution was plotted on a double-logarithmic scale against the ^{3}H remaining on the filter (Figure 5). The curves generated by the x-irradiated cells appear to be composed of two components: a rapidly eluting component, followed by a component that elutes with the same kinetics as DNA from control cells. The elution of a component having the same kinetics as undamaged DNA would have a slope parallel to that of control cells, shown by the dashed lines in Figure 5. Extrapolation of these parallel dashed lines back to the ordinate give estimates of fraction of the cell DNA that remains normal in elutability. The fraction

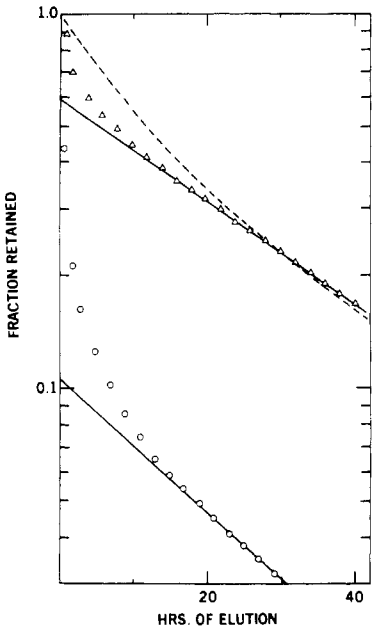

FIGURE 4: Elution patterns of newly synthesized DNA. L1210 cells were labeled with [^{14}C]thymidine for 20 min (○) or 2.3 h (△). After filtration, the cells were lysed at pH 9.9 and eluted at pH 12.1. The dashed curve represents the elution of uniformly labeled DNA from cells irradiated with 100 R and eluted under the same conditions.

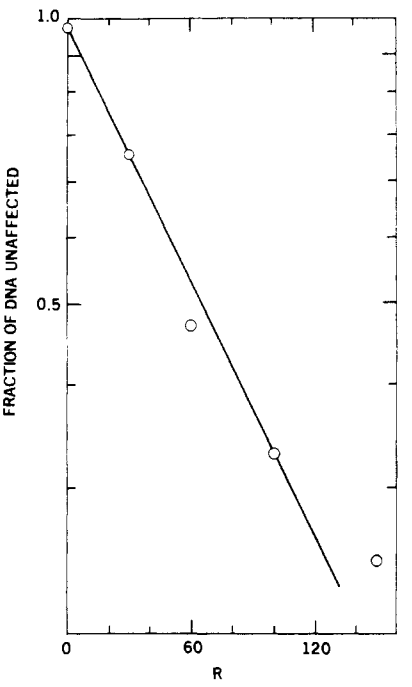

FIGURE 6: Fraction of DNA remaining unaffected as a function of x-ray dose. The final slopes of the elution curves in Figure 5 were extrapolated back to the vertical axis. The intercepts are taken as a measure of the fraction of DNA with elution unaffected.

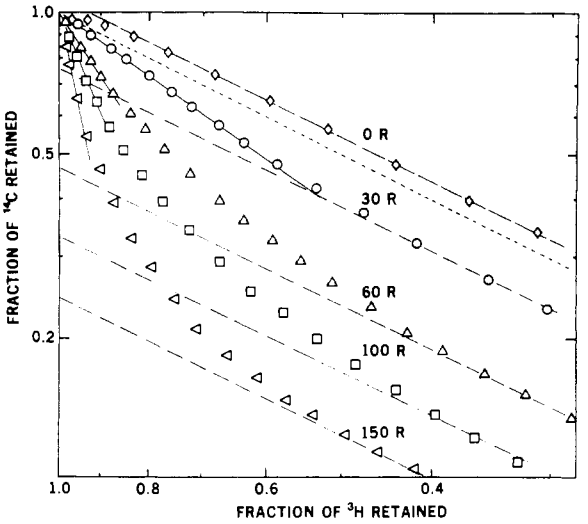

FIGURE 5: Normalized elution patterns from cells subjected to low doses of x ray. Cells labeled for 20 h with [^{14}C]thymidine were irradiated at 0 °C and mixed with unirradiated [^{3}H]cells. Lysis at pH 10.6; elution at pH 12.1. The fraction of the ^{14}C label retained on the filter is plotted against the fraction of ^{3}H label retained on the filter. The dashed line is theoretical for identity between ^{14}C and ^{3}H.

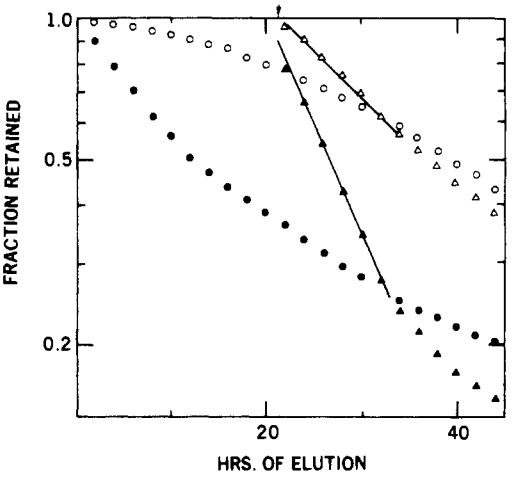

FIGURE 7: Effect of holding at pH 12.1 for 22 h on the kinetics of elution. Cells labeled with [^{14}C]- or [^{3}H]thymidine for 20 h were lysed at pH 9.9 and eluted at pH 12.1. The ^{3}H-labeled cells received 100 R at 0 °C (●, ▲) and were mixed with unirradiated ^{14}C-labeled cells (○, △). In one assay (△, ▲), the elution pump was turned off for 22 h beginning 1 h after application of the eluting solution; pumping was resumed at arrow. The other assay (○, ●) was carried out in the usual way.

of the DNA that remained normal decreased with x-ray dose approximately according to a first-order relation (Figure 6). The first-order relation suggests that a single x-ray event can convert a DNA segment from a normally eluting state to a state in which the elution is detectably more rapid. The D_0 dose in Figure 6 is 90 rad; this is the dose that gives an average of 1 hit/target, where the "target" is the minimum length of DNA strand that elutes at a normal rate.

Two hypotheses might be considered to account for the observed kinetics of elution; (1) the DNA is attached to nonelutable structures that are alkali sensitive and gradually release free DNA strands; (2) the rate of elution depends only on a property (such as length) of the DNA strands. These possibilities were tested by an interrupted-elution experiment, in which the pump was turned off after 1 h of elution and restarted 22 h later. If during this time, DNA strands were converted in a discrete step to an elutable form, then we would expect a portion of the DNA to emerge almost immediately from the filter. The result (Figure 7) showed that the 22-h holding time did increase the initial elution rate, but the rate was still first order and relatively slow compared, for example, to the rate of elution following high x-ray dose. The change in elutability of a given segment of DNA is, therefore, not an all-or-none change, and hypothesis 1 is excluded as the sole limit of DNA elutability.

TABLE I: Weight-Average Molecular Weight ($\overline{M}_w$) of DNA Single Strands Eluted at Various Times from X-Irradiated L1210 Cells.

Elution Time (h)	150 R ([^{14}C]DNA) Fraction of Total (dpm)	Cummulative Fraction of Total (dpm)	$\overline{M}_w \times 10^{-8}$	500 R ([^{3}H]DNA) Fraction of Total (dpm)	Cummulative Fraction of Total (dpm)	$\overline{M}_w \times 10^{-8}$
0-1	0.034	0.034	2.7	0.117	0.117	2.9
1-2	0.056	0.090	4.5	0.177	0.294	3.9
2-3	0.057	0.147	5.4	0.144	0.438	4.2
3-4	0.052	0.199	6.5	0.092	0.53	4.9
4-5	0.050	0.249	7.4	0.060	0.59	5.5

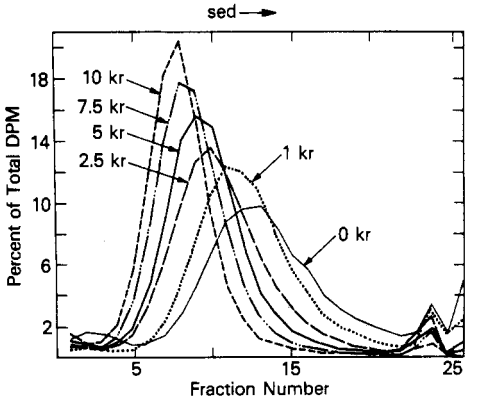

FIGURE 8: Alkaline sucrose gradient sedimentation of DNA from L1210 cells that were irradiated with the indicated doses of x-ray at 0 °C. Centrifugation was for 16.5 h at 9500 rpm at 20 °C ($\omega^2 t = 6.2 \times 10^{10}$ rad^2 s).

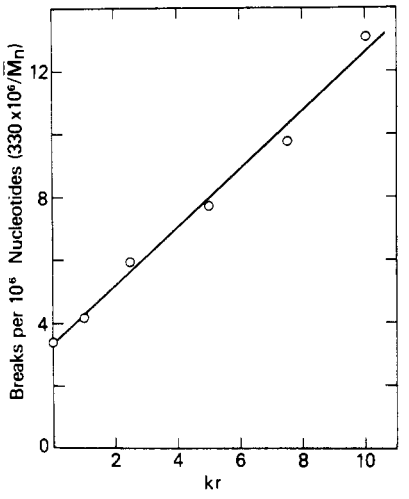

FIGURE 9: Frequency of DNA single-strand breaks as a function of x-ray dose, from experiment shown in Figure 7.

Efficiency for DNA Single-Strand Scission by X Ray. In order to relate the single-strand sizes present in x-irradiated cells with elution rates, we determined the efficiency for single-strand scission by x ray in L1210 cells. Cells exposed to various doses of x-ray at 0 °C were analyzed by alkaline sucrose gradient sedimentation (Figure 8). Number-average molecular weight ($\overline{M}_n$) was determined as described under Methods. The reciprocal of $\overline{M}_n$ is proportional to the frequency of single-strand breaks, which is plotted vs. x-ray dose in Figure 9. From the slope of this line, the frequency of single-strand breaks was found to be 2.7×10^{-12} rad^{-1} dalton^{-1} (0.90×10^{-9} single-strand breaks rad^{-1} nucleotide^{-1}). This is equivalent to an efficiency of 37 eV/break, in reasonable agreement with the value 44 eV/break reported by Lehmann and Ormerod (1970b) in another line of murine lymphoma cells. At a dose of 90 rad, which we had observed to produce an average of 1 hit/target observable in the elution assay, the number-average DNA single-strand length, thus, is 4.1×10^9 daltons $= 1.2 \times 10^7$ nucleotides.

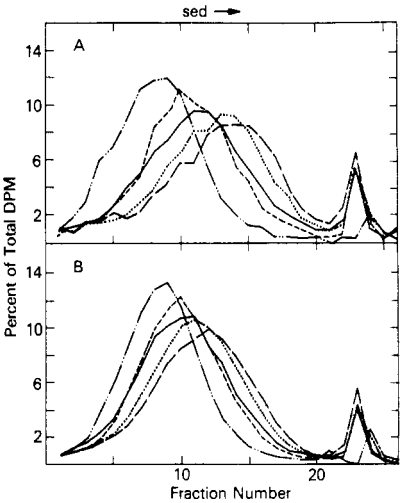

FIGURE 10: Alkaline sucrose gradient sedimentation of DNA eluted from x-irradiated L1210 cells. Cells labeled for 20 h with [^{14}C]thymidine and irradiated with 150 R were mixed wth cells labeled for 20 h with [^{3}H]-thymidine and irradiated with 500 R. The solutions eluted at successive time intervals were layered directly on alkaline sucrose gradients. (A) 150 R, [^{14}C]DNA; (B) 500 R, [^{3}H]DNA. Elution intervals: (- · · -) 0-1 h; (- - -) 1-2 h; (—) 2-3 h; (· · ·) 3-4 h; (– – –) 4-5 h. Curves are normalized to unit area. The fraction of DNA eluted in each time interval and the molecular weight averages are given in Table I. Centrifugation was for 17 h at 7500 rpm at 20 °C ($\omega^2 t = 4.003 \times 10^{10}$ rad^2 s).

Sedimentation Analysis of Eluted DNA. In order to determine whether the alkaline elution procedure fractionates DNA according to the single-strand length existing in the cell, we analyzed eluted DNA fractions by alkaline sucrose gradient sedimentation. Since the long DNA single strands that are eluted must be exceedingly fragile, experiments were designed so as to minimize shear (see Methods). The sedimentation patterns in Figure 10 were obtained from a mixture of [^{14}C]thymidine-labeled cells which had received 150 R and [^{3}H]thymidine-labeled cells which had received 500 R. Table I shows the molecular weight averages observed at various times of elution. The increase in average size of DNA with time of elution is clearly evident. At the earliest elution time, the sedimentation patterns of the [^{14}C]DNA (150 R) and [^{3}H]DNA (500 R) were similar. At later elution times, how-

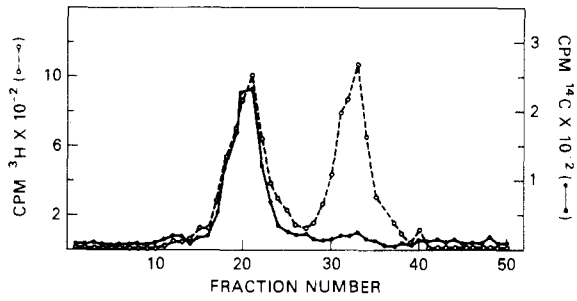

FIGURE 11: Isopycnic centrifugation of eluted DNA. Cells labeled for 20 h with [^{14}C]thymidine were eluted by the standard procedure. The DNA that eluted in the interval between 20 and 22 h was pumped directly into a neutralizing solution consisting of 0.1 M Tris-HCl, 0.01 M Na_2EDTA, pH 7.0. The neutralized solution was dialyzed against 0.15 M sodium chloride, 0.015 M sodium citrate. Density markers consisting of equal amounts of native and heat-denatured [^{3}H]DNA from L1210 cells were added and the sample was then centrifuged to equilibrium in CsCl. (●—●) Eluted [^{14}C]DNA; (○- - -○) mixture of native and denatured [^{3}H]DNA. Denatured peak is on the left, which is the direction of increasing density.

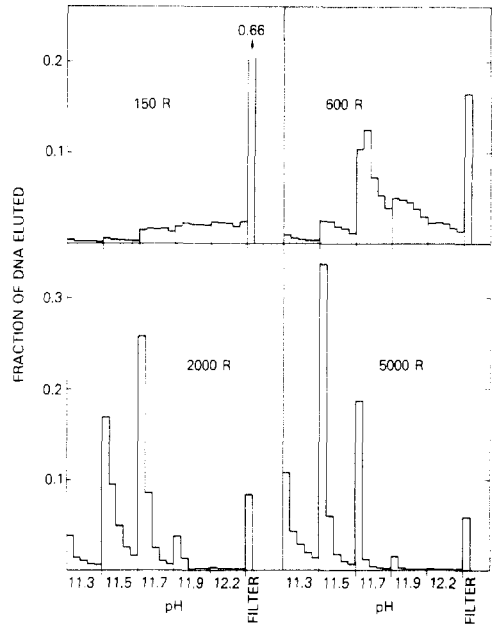

FIGURE 12: DNA elution in steps of increasing pH. L1210 cells were labeled for 20 h with [^{14}C]thymidine and subjected to various doses of x ray at 0 °C. The cells were lysed on filters in the usual way, and elution was carried out in steps of increasing pH. Five fractions were collected at each pH.

ever, the sedimentation of the [^{14}C]DNA was substantially faster. The sedimentation curves in Figure 10 have been normalized to unit area, so as to facilitate comparison of band shapes. As expected for the [^{3}H]DNA (500 rad), the total amount of DNA represented in each sedimentation pattern decreased markedly with elution time, while there was little change in the amount in the case of the [^{14}C]DNA (150 R) (Table I). The rapidly sedimenting component in the [^{14}C]-DNA (150 R), therefore, is much greater than that in the [^{3}H]DNA (500 R).

The results show that elution rate decreases as DNA single-strand length increases and the sizes of the eluted strands are of the expected order of magnitude. In the case of the [^{3}H]DNA (500 R), the sum of the [^{3}H]DNA in all gradients accounts for over half of the total [^{3}H]DNA (Table I). The weight-average molecular weight ($\overline{M}_w$) of the DNA eluted at the half-way point—i.e., when half of the total DNA has eluted—should approximate the weight-average molecular weight of all the DNA. The expected $\overline{M}_w$ for the total DNA can be determined from the value of $\overline{M}_n$ corresponding to 500 R—i.e., $\overline{M}_n = 0.73 \times 10^9$—and from the relation $\overline{M}_w = 2\,\overline{M}_n$ for a random distribution. Therefore, the expected $\overline{M}_w$ for 500 R is 1.46×10^9. The $\overline{M}_w$ found by sedimentation of the DNA collected near the median elution point (3–4-h collection in Table I) was 0.49×10^9. Hence, the observed size was about one-third of that expected. For molecules of this size, however, such a difference might arise either from strand breakage during elution and handling, or from a deviation from the assumed sedimentation behavior.

Single Strandedness of the Eluted DNA. DNA from cell lysates sedimented in alkaline sucrose gradients may remain double stranded, the two strands remaining entangled, although not hydrogen bonded (Simpson et al., 1973; Cleaver 1974c; Jolley and Ormerod 1974). In order to determine whether eluted DNA has completed strand separation, eluted solutions were slowly pumped into a neutralizing solution, using minimal shear conditions. The DNA was then centrifuged to equilibrium in CsCl, as described under Methods (Figure 11). Essentially all of the DNA was found to be denatured, regardless of whether the cells were irradiated or not, and regardless of the time of elution. Hence, the eluted DNA is almost entirely single stranded.

Size-Dependent Elution at the Transition pH. DNA strand separation in alkali occurs at a critical pH of about 11.6 (Vinograd et al., 1965). If DNA elution requires strand separation, we would expect to find a rapid transition from nonelutability to elutability as the pH is raised through the critical zone. This is shown in Figure 12. These elution experiments were conducted in the usual way except that the eluting solutions were applied in a sequence of increasing pH. Little or no DNA eluted below pH 11.3, unless the cells were grossly damaged. With cells exposed to 150 R of x ray, there was very little elution up to pH 11.5 (Figure 12). When the pH was then raised to 11.7, there was an abrupt increase in elution rate, which remained relatively constant despite further increases in pH. It should be noted that the elution rate remained nearly constant over the entire collection period at each pH. In contrast to this are the results with 2000 or 5000 R (Figure 12). Not only did elution after these doses occur at lower pH and in larger quantities, but the quantity of DNA eluting at each pH was limited, indicating that DNA from cells irradiated with these doses can be fractionated according to the pH of elution in the pH transition zone. By contrast, the larger DNA strands produced by lower doses of x ray elute too slowly to be fractionated in this way; their elution is limited by time instead of pH.

The pH-step fractionation of DNA in the transition zone was applied to pulse-labeled DNA (Figure 13C), and alkaline sedimentation analysis was carried out to determine whether the DNA strands are fractionated according to size. Although DNA from cells pulse labeled for 5 min elutes too quickly at pH 12.1 to show any resolution, a pH-step fractionation in the pH range 11.3–11.9 gave the resolution shown in Figure 13C. In a similar experiment, the solution eluted at each pH was pumped directly onto an alkaline sucrose gradient and centrifuged (Figure 14). The sedimentation patterns show that the pH fractionation was according to DNA strand size, with the smaller strands eluting at the lower pH.

The transition pH for DNA elution was found to depend on the nature of the cation in the elution buffer (Figure 13). With tetrapropylammonium cation the elution transition occurred

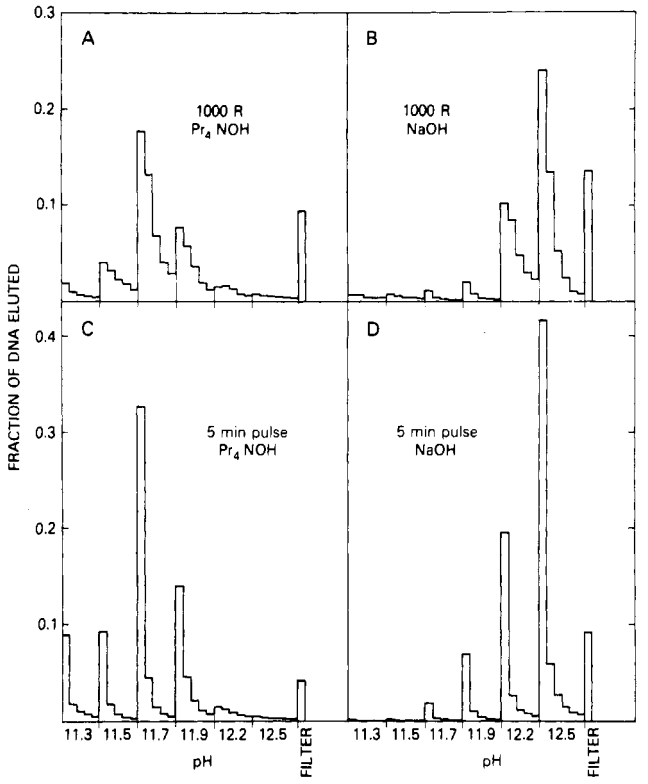

FIGURE 13: Cation effect on pH-step elution of DNA from x-irradiated or pulse-labeled cells. L1210 cells were labeled for 20 h with [^{14}C]thymidine (A,B) or for 5 min with [^{3}H]thymidine (C,D). The cells of panels A and B received 1000 R of x ray at 0 °C. The elution buffers consisted of tetrapropylammonium-EDTA (A,C) or NaOH-EDTA (B,D).

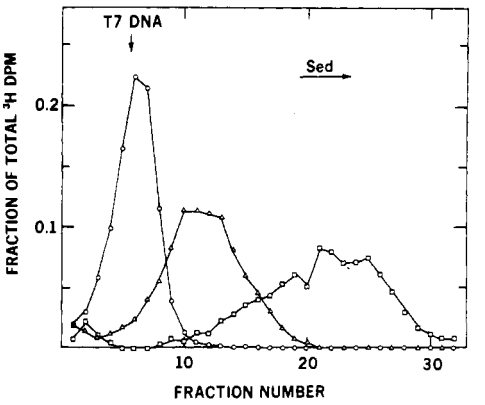

FIGURE 14: Alkaline sucrose gradient sedimentation of DNA fractions eluted at various pH values in an experiment similar to that shown in Figure 13C. (○) Eluted at pH 11.3 (16% of DNA); (△) eluted at pH 11.5 (13% of DNA); (□) eluted at pH 11.7 (23% of DNA). Approximately 3 ml of eluted solution was pumped at 0.05 ml/min onto a 5-20% sucrose gradient containing 0.1 M NaOH, 0.9 M NaCl, 0.01 M Na_2EDTA, and centrifuged at 10 000 rpm for 18 h ar 4 °C in a Beckman SW27 rotor. Arrow indicates the sedimentation of T7 reference DNA.

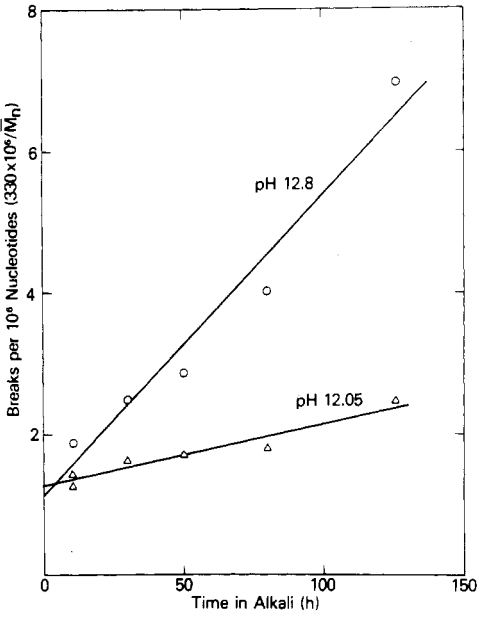

FIGURE 15: Formation of DNA single-strand breaks at pH 12.05 and 12.8 in tetrapropylammonium EDTA eluting buffers at 23-24 °C in the dark. DNA eluted from L1210 cells that had received 500 R was let stand in the eluting solution (pH 12.05 or 12.8) for various times and then analyzed by alkaline sedimentation.

at pH 11.3-11.9, whereas with sodium cation it was between pH 11.9 and 12.5. Thus, elution experiments can be carried out at lower pH using tetrapropylammonium hydroxide rather than using NaOH, thereby reducing the extent of concurrent alkali-induced scission of DNA chains.

Alkali-Induced Strand Scission. The sensitivity of the alkaline elution method for measurement of single-strand sizes in cells is limited by the late phase of elution in which elution accelerates and becomes dependent on pH (Figure 3). In order to determine whether this accelerated elution can be accounted for by alkali-induced strand scission, the rate of this process in our eluting solutions was determined by alkaline sedimentation (Figure 15). In this experiment, DNA eluted after exposure of cells to 500 R was allowed to stand for various times in the eluting solution at 23-24 °C in the dark and then sedimented in an alkaline sucrose gradient. DNA samples were transferred with minimal shear by slow pumping. The rates of production of single-strand breaks at pH 12.05 and 12.8 are shown in Figure 15. The rates were found to be 0.91×10^{-8} and 4.26×10^{-8} breaks per nucleotide per hour at pH 12.05 and 12.8, respectively. The ratio of the two rates approximates the ratio of hydroxide ion concentration at the two pH values and, thus, is consistent with an alkali-catalyzed process. These rates are sufficient to account for the observed acceleration in the late phase of elution.

Effect of DNA Cross-Linking. The elution of DNA may be limited by the ability of the strands to assume a "free" conformation. Any cross-linking of DNA strands to each other or to other large molecules in the lysate may inhibit the conversion of DNA strands to an elutable state. This effect is shown in Figure 16 for cells treated with nitrogen mustard (bis(chloroethylmethylamine, HN2), a known DNA cross-linking agent (Kohn et al., 1966). Cells were treated with nitrogen mustard and analyzed by elution at pH 12.2 (open symbols, Figure 16). Portions of the same treated cell suspensions were exposed to 300 R of x ray at 0 °C prior to elution (closed symbols). It is seen that, with or without x-ray treatment, the effect of nitrogen mustard was to retard elution. The effect is much more apparent, however, after x ray. DNA strands that would ordinarily elute due to strand breakage by x ray apparently are held back by cross-linking due to nitrogen mustard. Even in the absence of x ray, however, the late phase of elution is inhibited by cross-linking (Figure 16, open symbols).

Elution of Satellite DNA Strands. In order to determine whether DNA subpopulations may differ in their elution behavior, we compared the elution of satellite L strands with that of the bulk DNA. Unlabeled L1210 cells were irradiated with 150 R at 0 °C, in order to introduce a low frequency of DNA

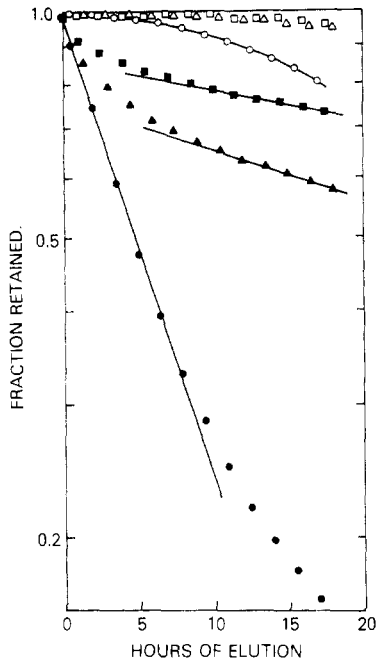

FIGURE 16: Effect of nitrogen mustard (HN2) on elutability of DNA from unirradiated (open symbols) and x-irradiated (closed symbols) L1210 cells. Cells were treated with 10^{-7} M HN2 for 1 h (△, ▲) or 3 h at 37 °C (□, ■). A portion of cells was then exposed to 300 R at 0 °C (closed symbols). (○, ●) No HN2. Elution was at pH 12.2.

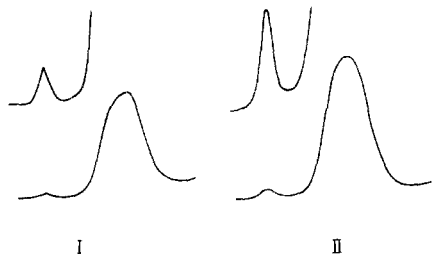

FIGURE 17: Equilibrium sedimentation patterns in alkaline CsCl of eluted L1210 DNA, showing satellite L band. Unlabeled cells exposed to 150 R were subjected to alkaline elution at pH 12.1. (I) DNA eluted between 0 and 6 h; (II) DNA eluted between 6 and 18 h. Lower and upper tracings obtained with photoelecric scanner gain set at 200 and 50 mV/cm, respectively.

breaks. The cells were then lysed on filters at pH 10 and eluted at pH 12.1 in the usual way. The solutions eluting in the intervals 0–6 and 6–18 h were collected, and the DNA was concentrated and centrifuged to equilibrium in alkaline CsCl, as described under Methods. The satellite L strands are clearly separated from the main-band DNA (Figure 17). Control experiments showed that the DNA concentration method did not cause any selective loss of DNA. The fraction of satellite L strands in the 0–6 and 6–18 h elution collections was found to be 1.0 and 2.2%, respectively, based on absorbance at 280 nm in alkaline CsCl (Figure 17). The results indicate that satellite L strands elute more slowly than the average for the total cell DNA. This suggests that differences may exist in the x-ray induced elutability of different parts of the mammalian genome.

Discussion

The alkaline elution phenomenon provides a sensitive measure of a physical effect of ionizing radiation on DNA in mammalian cells. Our results indicate that the rate of DNA elution, under appropriate conditions, is a measure of DNA single-strand size. The DNA elution rate probably depends on a property of the DNA itself, rather than on the association of DNA with some other cell constituent, since the characteristic elution phenomena can be demonstrated after nearly all of the non-DNA material of the cell has been removed from the filter. The kinetics of DNA elution from cells exposed to low doses of x ray were of the single-hit type, with a D_0 (dose producing an average of 1 hit/target) of 90 rad. This dose produced 1 single-strand break/1.3×10^7 nucleotides, estimated from the efficiency of production of single-strand breaks, which was determined to be 37 eV/breaks. This indicates that the intracellular DNA targets are at least 1.3×10^7 nucleotides (4×10^9 daltons) in size. Analysis of the size distribution of eluted DNA by alkaline sedimentation confirmed the supposition that elution rate depends on single-strand length.

Our observations do not exclude the possibility of non-DNA linkers, and no information is provided about conformation of the target. It is also possible that alkali-sensitive links would be counted as breaks.

Anhström and Erixon (1973) and Rydberg (1975) recently described a procedure for measurement of the effect of x ray on DNA in mammalian cells, based on the effect of single-strand breaks on the time required for unwinding of DNA double strands. The time required for unwinding increases with at least the square of the strand length (Freese and Freese 1963; Fixman 1973). The kinetics of alkaline elution also must involve the unwinding time, since the eluted DNA is almost entirely single stranded, and since elution requires a pH high enough to cause strand separation. The unwinding time, however, is probably not the major rate-limiting step in the elution kinetics. If it were, we would have expected that holding the lysate in alkali for a long period of time should have permitted complete unwinding of DNA strands, which then should have eluted almost instantly. Our findings contradict this expectation (Figure 7). The same reasoning argues against models based on attachment of DNA to unelutable structures, such as membrane fragments.

We have attempted to account for the observed elution kinetics after various doses of x ray by computer models. Although an adequate fit of theory to experiment has not yet been obtained, the introduction of time delays to represent unwinding time in all cases increased the deviation from the observed kinetics. Our current speculation is that the rate-limiting process involves the search for a molecular conformation that will allow a long DNA single strand to be pulled through a single filter pore. Since the strand dimensions are much larger than the filter pores, portions of a single strand may be pulled through many pores at the same time. Elution must require that the strand segment in one pore acquire enough dominance to pull the entire strand through.

DNA elution may be markedly reduced by the insertion of covalent cross-links, either between complementary strands or between DNA and protein. Nitrogen mustard, which produces both types of cross-links (Kohn et al., 1966; Klatt et al. 1969), clearly exhibited this effect (Figure 16). DNA elution measurements also have disclosed cross-linking of DNA in cells irradiated with ultraviolet light (Fornace and Kohn 1976); these cross-links involved protein, since the cross-linking effect was eliminated by proteolytic treatment of the filters. The alkaline elution method has also disclosed DNA cross-linking in cells treated with bis(chloroethylnitrosourea) (Ewig and Kohn 1976), and may be applicable to similar studies with other agents.

The cross-linking effect must be taken into account in studies of DNA single-strand scission by radiation or drugs. This can be done by measuring the increase in DNA elution produced by a test dose of x ray (Fornace et al., 1976; Fornace and Kohn 1976).

The limiting factor in the fractionation of extremely long strands by alkaline elution is the slow rate of strand scission caused by alkali. Alkali-induced strand scission becomes most significant for extremely long single-strands which elute very slowly and can be minimized by carrying out the elution at the lowest possible pH. The initial rate of elution of long strands was found to be independent of pH in the range of 11.9–12.8, when the cation is tetrapropylammonium. Under these conditions, the apparent first-order rate constant was inversely proportional to average single-strand length. This relationship holds as long as the elution rate remains independent of pH. Eventually, strand scission induced by alkali increases the elution rate, which then becomes dependent on pH and less dependent on initial strand length (Figure 3).

In the range of the pH transition (pH 11.3–11.7 in the case of tetrapropylammonium buffer), DNA strands smaller than about 5×10^8 daltons are eluted. The exact pH in this range critically limits the maximum size of strand that is elutable. Although strands in this size range can be separated by alkaline sedimentation, the elution procedure permits preparative fractionation of larger samples than can ordinarily be accommodated by sedimentation techniques.

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