SEDIMENTATION OF DNA RELEASED FROM CHINESE HAMSTER CELLS

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ABSTRACT Under high pH and high salt conditions, Chinese hamster cells lyse and release a DNA-containing material of large molecular weight. With increasing lysis time, a smaller material is resolved from the large one. Relative to T4 DNA, the smaller is estimated to be $\sim 2 \times 10^8$ daltons (number average). From a comparison of radiation data, the target size of the larger is about 15 times that of the smaller (probably a lower limit estimate). In addition to concentration of alkali, temperature, and time of lysis, the resolution of the smaller from the larger material is shown to be affected by other factors. Three of these are: fluorescent light exposure during lysis, X-irradiation before lysis, and incorporation of actinomycin D before lysis. All of these treatments result in degradation of the smaller molecules if large enough exposures are used. The sedimentation patterns of both DNA materials are strongly speed dependent. This probably results from changes in molecular conformation and concomitant increases in viscous drag with speed. The speed dependence differs qualitatively for the two materials, an observation which suggests that they differ in ways in addition to size.

INTRODUCTION

In the past, studies of the size, structural organization, replication, and segregation of eukaryotic DNA have been hampered by the lack of adequate techniques. Relative to sedimentation properties, this situation reflects, in part, the possibility that the DNA in the chromosomes of, say, mammalian cells is very large. In a Chinese hamster cell, there is about 7 $\mu\mu$g DNA (before DNA replication). Even in the smallest chromosome, if all the DNA is in a continuous piece it would have a molecular weight of $5\text{–}10 \times 10^{10}$ daltons. The longest chromosome could have seven times this amount. Just the matter of isolation of material of this length can present formidable problems.

The technique of extraction on top of a gradient solution offers in principle an approach which can minimize molecular damage. The first such attempt along these lines was that of McGrath and Williams (1966) and was applied to bacterial spheroplasts. They used an alkaline lysing layer on top of an alkaline, high salt, sucrose gradient to lyse spheroplasts and release the DNA. Since mammalian cells readily lyse in a high pH solution, and since histones can be separated from DNA under high salt conditions, a number of investigators concerned with eukaryotes have

been attracted to the technique of McGrath and Williams even though the high alkalinity implied that the DNA would be denatured.

This report describes the application of the alkaline sucrose gradient technique to Chinese hamster cells whose DNA was uniformly labeled with thymidine-^{3}H. It was found that, initially, a large material is released and that from this a smaller species is resolved in time. Resolution can be speeded by exposure to fluorescent light during lysis or before lysis by exposure to ionizing radiation or actinomycin D (see also Elkind and Kamper, 1970). Large enough doses of either form of radiation or of this drug lead to degradation. The molecular weight estimate of the smaller molecule, in comparison with T4 DNA, is $\sim 2 \times 10^8$ daltons (number average). Radiation target considerations lead to a size estimate for the larger material of 15 times greater, a minimum estimate which will be explained further.

More recently, it was also found—consistent with the observations made with *Escherichia coli* DNA in neutral gradients by I. Rubenstein and S. B. Leighton (personal communication)—that the sedimentation of both materials is strongly dependent on speed of rotation. The data support the possibility that the aforementioned materials differ in respects other than size alone. As a practical matter, the demonstration of an angular velocity dependence of sedimentation in alkaline gradients as well as neutral gradients indicates that, in general, caution must be observed in interpreting results obtained with large molecules ($\gtrsim 2 \times 10^8$ daltons). But as the data will show, the distortion of the pattern of sedimentation resulting from high speed affords a measure of resolution which can be used to advantage for some purposes.

MATERIALS AND METHODS

The procedure for growing cells in culture, preparing gradients, centrifugation, and many other details of the over-all experiment have been described already (Elkind and Kamper, 1970; Elkind et al., 1969). In the course of these studies, changes inevitably were made; these will be noted where appropriate.

The general format of the experiments was the following. Clonal derivatives of Chinese hamster cell line *V79-1* were used. (*V79-1* is our original designation [Elkind and Sutton, 1960]; some authors have referred to this line simply as V79). Cells were grown overnight in medium usually containing 0.16 μCi/ml of high specific activity thymidine-^{3}H but occasionally up to 0.25 μCi/ml ($\lesssim$10 Ci/mmole). Since these cells double in number in about 9 hr, overnight growth assured fairly uniform labeling of the DNA. No detectable effect on cell growth accompanied the incorporation of label. After labeling with thymidine-^{3}H, cells were rinsed and incubated at 37°C for 30 min in nonradioactive medium to allow for the completion of replicons only partially synthesized at the time the medium was changed. Usually trypsinization at ice temperature followed, although similar results were obtained when cells were suspended by scraping to remove them from the surfaces on which they had grown.

Irradiations

To irradiate cells before suspension, the medium was removed and the cells, attached in a Petri dish, were exposed to 50 kv X-rays at ~800 rad/min (for details see Elkind and Sutton,

1960). During and after exposure, the bottoms of the dishes were kept in contact with ice water to minimize repair processes. For visible light exposure, cells were suspended and placed on top of a gradient, and while lysing, cells were then either simply exposed to the light emitted from conventional ceiling-type fluorescent lamps (Fig. 2, intensity ~75 ft-candles) or were placed under two 15 w fluorescent lamps held in a conventional desk top reflector and positioned about 6 inches above the gradients (Fig. 1, ~500 ft-candles). In the former instance, tubes were capped with an inverted hollow plastic stopper to minimize evaporation, and in the latter evaporation was prevented while light transmission was assured by the use of a close-fitting cap, the top of which consisted of a glass cover slip. The exposures with visible light to be described were at room temperature, ~25°C; results qualitatively similar to those in Fig. 1 were obtained when the irradiations were performed in a cold room, 2–3°C.

Cell Lysis, Gradients, and Centrifugation

After the preparation of a cell suspension, a 0.025 ml aliquot was carefully placed on top of 0.25 ml of a lysing solution which in turn had been layered on 4.8 ml of a 5–20% sucrose gradient. The cell sample consisted of 3000–6000 cells, about 0.03–0.06 μg DNA. Both the lysing and the gradient solutions contained NaCl and NaOH; the total Na^+ molarity was kept close to 1.0 in all instances. During the course of the work, it was found necessary to increase the NaOH concentration in order to insure the maintenance of stock solutions at pH's greater than 12 (see figure legends for details). Qualitatively, it was found that lysis proceeded more rapidly in the more alkaline solutions. The chelator Na_2 EDTA was found to be essential; 0.01 M was used in the lysing solution and 0.003 M in the gradient solution.

Sedimentation was performed in the 5 ml polyallomer tubes which are standard for SW-39 and SW-50.1 rotors; an L2-50HV centrifuge was used (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). Temperature, speed, and time of sedimentation were varied and hence are noted in the legends. (Each run started with a 10–30 min period at 5000 rpm without evacuation to permit a more rapid cooling of the rotor to the selected operating temperature.) Following centrifugation, the contents of each tube were pumped off from the top and successive 10-drop fractions were collected on glass filters and dried. These were usually assayed for radioactivity directly (by liquid scintillation counting in a toluene-base counting fluid) since it was found that cold acid extraction had no qualitative effect on the results.

In the patterns to follow, radioactivity per sample is plotted normalized on a percentage basis to the total collected in 30 samples. In each instance, the percentage of radioactivity collected in 30 samples compared to the amount initially placed on the gradient in a 0.025 ml cell sample is indicated by P.Y., per cent yield. Generally, P.Y.'s of 70% or more were obtained indicating first, that the lysing conditions were adequate to release essentially all the DNA from the cells; second, that at most only a small amount of material attached to the walls of the tubes or was sedimented to the bottom; and third, that conclusions drawn relative to sedimentation properties apply essentially to all the DNA in the cells and not to a small, perhaps unrepresentative, proportion.

RESULTS

The Effect of Lysis Time and Visible Light

If cells are observed with the light microscope as they are added to the lysing solution, in the course of a few minutes most of the cells become undetectable even under

phase-contrast optics. When a small aliquot containing about 10^5 cells or more is layered on to the lysing solution, fairly promptly a gel-like substance is formed which contains the DNA. These qualitative observations are mentioned to indicate that while the alkaline, high salt lysing solution readily releases DNA, at least when the cell concentration is high, the material containing the DNA aggregates. The implication follows that even when low cell numbers are used, for a period after the start of lysis the sedimentation of the DNA-containing material should be very rapid. To minimize aggregation further when a 0.025 ml aliquot was pipetted onto a lysing solution, it was delivered in 5–6 small drops by gently touching the tip of a capillary pipette, onto which a small amount of suspension had been expelled, to different positions over the surface of the lysing solution. Even so, it was found that spinning at high speed for 60 min (36,000 rpm, 12–13°C) consistently drove all the radioactivity to the bottom of a tube if the lysis period was less than 30 min (at ~25°C). While a point-to-point distribution of the cell sample probably minimized nonspecific aggregation, it is likely that a degree of nonreproducibility resulted which was responsible in part for the somewhat variable sedimentation behavior of the material designated "the complex" in what follows.

Lysis periods between 30 and 60 min yielded patterns which were poorly reproducible with most of the radioactivity appearing in a number of sharp, often one-fraction, peaks in the lower half of the tube. For periods of 60 min and longer (~25°C), the patterns became progressively more reproducible and are typified by those traced by the open circles in Fig. 1. All of the observations to be described involved, therefore, at least a 60 min period for lysis although as will be noted, a 2–3°C ambient temperature during lysis was also used.

Starting then with cells treated at room temperature, Fig. 1 shows the results for lysis in the dark or light (~500 ft-candles) and for 60 min (upper panel) and 240 min (lower panel) before the start of centrifugation. Lysis for 60 min in the dark usually resulted in a bimodal pattern. To facilitate discussion at this juncture, the left peak in the upper panel will be referred to as the "complex" and the right peak as the "main peak"; these designations will be explained presently. Lysis for 240 min in the dark resulted in a trimodal pattern in this instance. (The peak at fraction 22, containing only 7% of the DNA, is only occasionally seen and, therefore, will not be discussed further. Also not to be discussed are the small peaks often found at the bottom of gradients which probably reflect a small amount of label dislodged from the bottom by the pumped-off solution.) The percentage of label in the main peak increased considerably because of the longer lysis time. Since the P.Y.'s are high, the patterns suggest that during lysis the DNA that would have sedimented in the complex was transferred to the main peak; further evidence of this is contained in later figures.

From the upper part of Fig. 1, it is clear that the effect of visible light during lysis is to accelerate the transition of the complex to the main peak. The result after 240 min suggests that now visible light has led to degradation since, at the expense of the

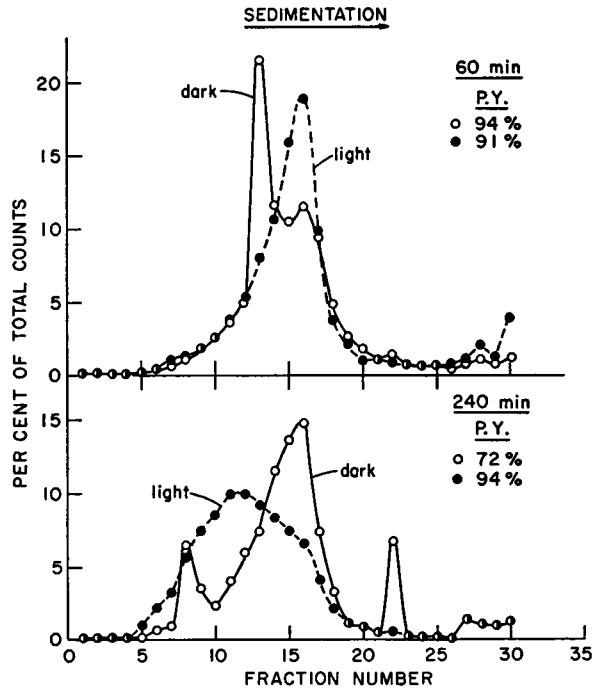

FIGURE 1 Sedimentation of labeled DNA material from unirradiated Chinese hamster cells (36,000 rpm, 60 min, ~12°C, SW-50.1 rotor). The periods indicated represent lysis times at ~25°C (lysing solution: 0.45 M NaOH, 0.55 M NaCl, 0.01 M Na_2EDTA). Visible light irradiation during lysis was with two 15 w fluorescent tubes positioned perpendicular to and at about 6 inches from the tops of the tubes; intensity about 500 ft-candles. All four patterns were obtained with cells from the same stock suspension. (P.Y. = per cent yield of radioactivity in 30 fractions relative to the radioactivity contained in the cell sample.)

material in the main peak, a broad peak formed in a region corresponding to smaller sedimentation values.

Taken together, these are the transitions which may be traced with the aid of Fig. 1. Shortly after cells are layered on an alkaline, high salt solution, the DNA-containing material is in large aggregates, judging at least from the fact that it sediments to the bottom under spinning conditions similar to those used for Fig. 1. By about 60 min (~25°C), essentially all the DNA is distributed along the gradient in two peaks. With time and/or light, DNA is transferred from a slower to a faster sedimenting peak; additional visible light exposure degrades the material in the latter peak. X-irradiation prior to cell lysis produces a similar degradative change, and since the radioenergetics are consistent with single-strand break formation (Elkind and Kamper, 1970), it is concluded that the degradation produced by light also reflects single-strand breaks. This by itself suggests that the transition *complex* → *main peak* brought about by light or lysis time results from the introduction of breaks, and therefore that the complex results from a larger structure than the main

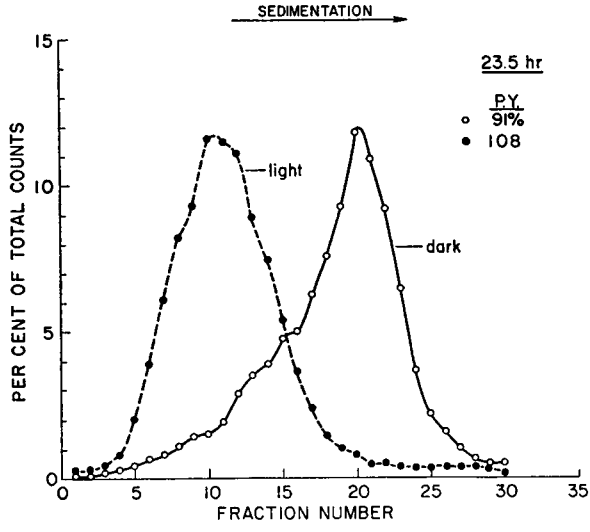

FIGURE 2 Sedimentation of labeled DNA material from Chinese hamster cells (36,000 rpm, 60 min, ~12°C, SW-50.1 rotor). Cells were lysed for 23.5 hr at ~25°C in the presence or absence of fluorescent light (~75 ft-candles). Lysing solution: 0.05 M NaOH, 0.95 M NaCl, and 0.01 M Na_2EDTA.

peak. Further evidence will be presented in support of this; it suffices to note here that if the main peak results from smaller molecules than those in the complex, the complex has either a greater viscous drag, a lesser density, or both.

The visible light doses used in Fig. 1 were fairly high (~500 ft-candles) in order that effects could be made clearly evident in the periods used. Also, the lysis solution contained 0.45 M NaOH. At 0.05 M NaOH, during the course of a few hours it was found that the intensity of conventional ceiling fluorescent lamps (~75 ft-candles) produced little effect. However, Fig. 2 shows that even normal room light can degrade the DNA from Chinese hamster cells if the period of lysis is long enough. Cells lysed in the dark for 23.5 hr yielded a pattern suggestive of only a small degree of alkaline degradation of the material in the main peak (note the bulge from fractions 11–16). Room light for the same period leads to marked degradation.

Complex Resolution and Strand Breakage by Actinomycin D

As already reported (Elkind and Kamper, 1970), and as additional results to be discussed will confirm (Figs. 7 and 8), small doses of X-radiation delivered to cells before lysis result in a dose-dependent resolution of the main peak. Fig. 3 shows that treatment with actinomycin D before lysis produces a similar result and that like radiation, this antibiotic can produce single-strand breaks.

To develop the points contained in Fig. 3, it is necessary first to recall that after a 60 min lysis period at room temperature, a substantial complex is still evident

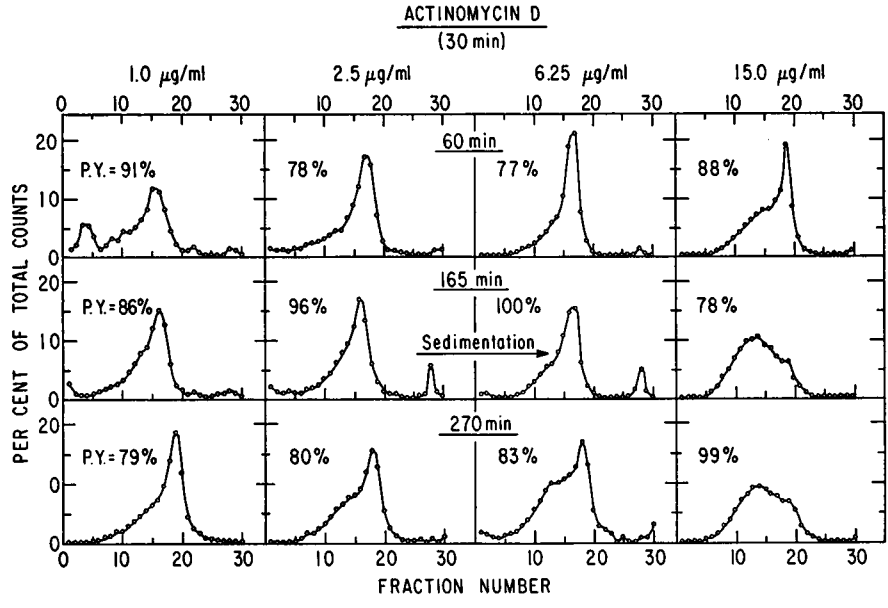

FIGURE 3 The effect of graded doses of actinomycin D on the alkaline sedimentation of labeled DNA material from Chinese hamster cells (38,000 rpm, 60 min, ~12°C, SW-39 rotor). In all instances, cells were incubated at 37°C for 30 min with the concentration of drug shown before suspension and lysis. Lysis conditions same as for Fig. 2. Sedimentation from left to right.

when cells are lysed in the dark (Fig. 1). Fig. 3 shows in the horizontal direction the effects of increasing concentrations of actinomycin D in the growth medium when cells are incubated with drug at 37°C for 30 min before suspension and lysis. In the vertical direction are shown the effects of increasing periods of lysis for each drug concentration. Even after an exposure to 1.0 μg/ml, the main peak is appreciably more resolved from the complex than it would be otherwise (see Fig. 1 to compare with lysis periods of 60 min, and Elkind and Kamper, 1970, for 165 min). With increasing lysis time, drug concentration, or both, the complex is first resolved and then a bulge on the left side of the main peak develops which, in analogy with X-irradiation, probably reflects single-strand breaks. Thus, as measured in this way actinomycin D and X-irradiation produce similar effects.

The foregoing qualitative similarity could not have been predicted simply on the basis of the bond-breaking property of ionizing radiation and the known binding ability of actinomycin D to DNA. By itself, this observation suggests similar actions of these two agents relative to cell function, a point already noted in regard to cell killing and inhibition of the repair of X-ray damage (Elkind et al., 1967; Elkind et al., 1968) and which will be elaborated upon later. However, viewed quantitatively, a difference has been noted in the actions of these agents in respect to those cells

which are in the majority in an asynchronous Chinese hamster cell population, i.e., cells undergoing DNA synthesis. While doses of drug in the measurable survival range (e.g., 80% to 8% survival; see Elkind et al., 1969) resolve the complex to approximately equal extents as do equal survival doses of X-rays, appreciably large doses are needed to degrade the material in the main peak by drug as compared with radiation action.

Sedimentation Relative to T4

The sedimentation of the main peak relative to bacteriophage T4 labeled with ^{32}P was measured, and from this its molecular weight was estimated. Fig. 4 shows the variation of peak position with time-at-speed. Although neither curve goes through the origin, shifting the curves to the right by the time-at-speed equivalent to the acceleration plus deceleration periods would bring the zero time intercepts of both curves closer, and these closer to the origin. Using the ratio of distance sedimented (at 60 min) raised to the 2.5 power (Studier, 1965) and a value for denatured T4 DNA of 6.7×10^7 daltons, the molecular weight at the mode of the main peak is estimated to be $\sim 2.5 \times 10^8$ daltons. Further discussion relative to the distribution of molecular weights in the main peak is presented later.

In addition to affording a size estimate, the data in Fig. 4 show that the sedimentation of the main peak is proportional to time-at-speed. Other results have shown that this property is essentially independent of the length of lysis. This is not true for the complex. To begin with, the position of the complex in a gradient pattern

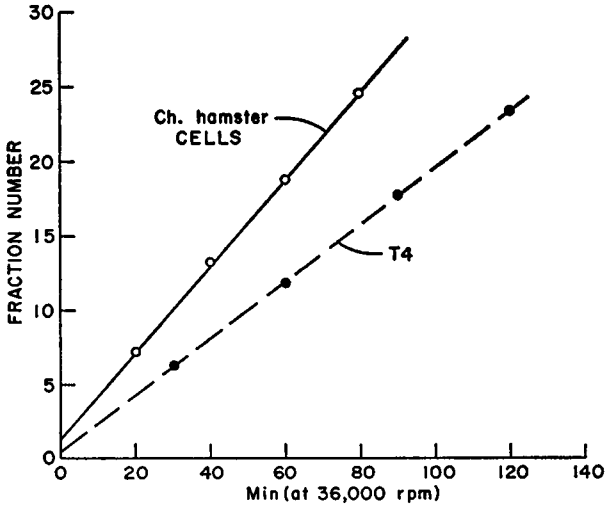

FIGURE 4 Variation of sedimentation distance with time of main peak DNA from Chinese hamster cells and DNA from bacteriophage T4. Lysis conditions for Chinese hamster cells and T4 were the same (see Fig. 1).

for a given lysis time, is not as reproducible as that of the main peak. Further, it depends on lysis time since the complex generally moves toward the meniscus from the main peak as the DNA in the latter is resolved from the former (e.g., Fig. 1). While it might appear from the foregoing that at least the material in the main peak sediments normally, it will be shown that both peaks represent atypical sedimentation.

Slow vs. High Speed Sedimentation of the Main Peak

Working with two-stranded DNA from *E. coli*, I. Rubenstein and S. B. Leighton (personal communication) observed a strong dependence of peak shape on angular velocity when using DNA estimated to be larger than $\sim 2 \times 10^8$ daltons. Although the observation of Rubenstein and Leighton referred to neutral gradients, it seemed possible that something similar might result with large molecules in alkaline gradients. The results to follow show that this is the case.

In Fig. 5, comparative patterns are shown for 36,000 rpm and 11,000 rpm. The spinning time at 11,000 rpm was lengthened to bring the mode value to essentially the same point in the gradient assuming the applicability of the usual *(angular velocity)*$^2 \times$ *time* relationship, $\omega^2 \times t$. Also, to restrict the experiment to the main peak, cells were lysed for 4 hr at room temperature which in this case was adequate to resolve the complex completely. For each dose, cell samples from the same suspension were used and the entire experiment was performed with a common stock of cells. (The values in parentheses locate the modes after accounting, on a linear basis, for variations in the total number of drops collected from gradient-to-gradient relative to an average of 300.)

Proceeding from 0 rad to 722 rad and then to 5780 rad by factors of two, it is clear that there is a major speed-dependent difference in peak shape and that this difference is lost with increasing dose. Apparently, after the largest dose, single strands are sufficiently small *not* to suffer significantly from speed effects judging from the fact that the bottom two patterns are almost identical. The upper panels in Fig. 5 show clearly that after smaller doses, those molecules which at low speed would sediment beyond about fraction 18 are retarded at high speed leading to a sharp, distorted peak. For unirradiated cells, the patterns are the same up to fractions 14–15 which means that a speed distortion does not set in significantly until molecular weights larger than $1–2 \times 10^8$ daltons are reached. For DNA from unirradiated cells, the weight average molecular weight of the distribution at 11,000 rpm (after smoothing the leading edge) is 12% larger (2.8×10^8 daltons) and the number average is 22% smaller (2.0×10^8 daltons) than the molecular weight of the mode value at 36,000 rpm (2.5×10^8 daltons). These estimates are based upon an assumed 2.5 power dependence of molecular weight on sedimentation (Studier, 1965).

In addition to the indication that in unirradiated cells a fairly broad range of molecular sizes is resolved from the complex, some further details in Fig. 5 should

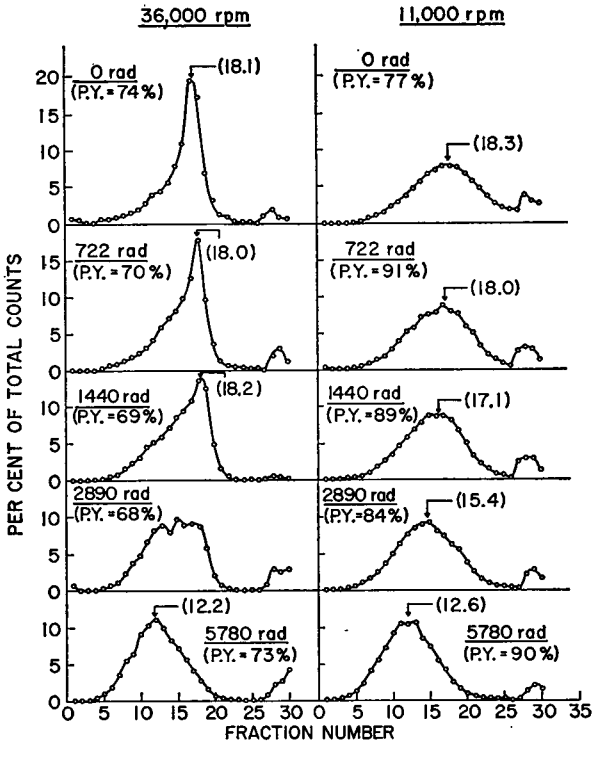

FIGURE 5 Comparative sedimentation patterns for labeled DNA material at 36,000 rpm (90 min, 2–3°C, SW-50.1 rotor) and 11,000 rpm (17.1 hr, 2–3°C, SW-50.1 rotor). The same cell suspensions were used for each pair of doses and cell lysis was at ~25°C for 4 hr in the dark (lysis as for Fig. 1). The numbers in parentheses are the locations of the modes after accounting, on the basis of a linear interpolation, for small differences in the total number of drops collected from each gradient since 10-drop samples were used throughout. Note that the lower temperature during sedimentation requires longer periods of sedimentation to bring main peaks to the same position as in earlier figures (see text). Sedimentation from left to right.

be noted. After 722 rad, the changes in peak shapes and positions are too small to be clear. In the 36,000 rpm case, a dose of 1440 rad results in a clearly different pattern consistent with our earlier findings relative to a shorter lysis time (Elkind and Kamper, 1970). In addition to a drop in the height of the peak at the mode, the peak clearly shows a bulge to the left. However, although there is a reduction in distance sedimented at the mode for the same preparation of cells in the 11,000 rpm case, the change is sufficiently small that such a pattern ordinarily might have been mistaken as one from unirradiated cells. Thus, it is apparent that while high speed distorts the shape of the main peak, the artificial sharpness that results permits a degree of resolution not otherwise obtainable. Ionizing radiation is expected to introduce breaks at random, and consequently after a given dose the larger molecules

suffer proportionately more hits. For unirradiated cells it is the large molecules that are compressed into the peak; when a dose is sufficient to hit a large enough fraction of these, a reduction in peak height results as well as a widening of the pattern in the direction of smaller molecular weights. Thus, two changes in the patterns result. In contrast, after irradiation and slow speed sedimentation, only a gradual shift of the pattern toward the meniscus results because the change in sedimentation does not involve a shift, to any appreciable extent, in the proportion of molecules suffering speed distortion. Essentially, therefore, the pattern is altered in only one respect and this has the effect of appearing to shift the distribution to the left with little change in its shape. The somewhat sharper distribution after 5780 rad reflects in part the narrowing which results from the smaller average distance of sedimentation.

In spite of the more limited resolution which accompanies slower speed sedimentation, from top to bottom the trends of the two sets of data in Fig. 5 are mutually consistent. As already noted, a dose greater than 722 rad is needed to lead to a clear change in pattern shape at high speed. While by itself the shift in the location of the mode at low speed is not impressive after 1440 rad, when compared with the higher and lower doses, it seems clear that at least 1000 rad are needed to produce a shift in the pattern. This is in agreement with our earlier estimate (Elkind and Kamper, 1970) and consistent with an average energy loss of $\sim$60 ev per radiation absorption event. From measurements with bacteriophage, it is known that about this energy is needed to produce a single-strand break; 10–20 times more is needed per double-strand break (Freifelder, 1966).

Thus, curve shape differences aside, the radioenergetics in Fig. 5 are mutually consistent within the limited resolution of measurements of this type. This consistency is fortuitous and comes about because the speed distortion of the sedimentation pattern starts to become appreciable at about the same range of molecular weights as the DNA in the main peak. The situation for the complex is different and is presented later.

Speed Dependence of Sedimentation Patterns

The results in Fig. 5 indicate that a reduction by a factor of 10 in centrifugal force converts a pattern whose half-width relative to the mode is $\sim$20% to one of $\sim$60% (unirradiated cells). Fig. 6 helps to illustrate the transition between these extremes in reference to the main peak. It also sketches out the speed dependence to be expected of the sedimentation of the complex.

Although the $\omega^2 \times t$ values for the two runs in Fig. 6 are almost equal, the mode values of the main peaks are not at the same positions, probably because a precooled rotor was used for the 18,000 rpm run and the cooling of the gradients was, therefore, more effective during the longer, slower speed centrifugation (see legend for conditions). Nevertheless, several points are clear. Starting with the results after

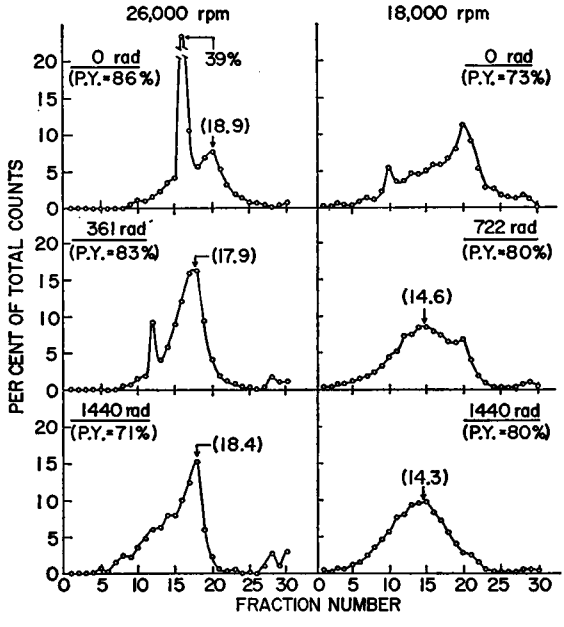

FIGURE 6 Sedimentation patterns at 26,000 and 18,000 rpm for labeled DNA material from Chinese hamster cells. Cells were lysed for 60 min, in the absence of light, as described for Fig. 1. The spinning conditions were (SW-50.1 rotor): 26,000 rpm, nonprecooled rotor, 147 min, ~1°C; and 18,000 rpm, precooled rotor, 323 min, ~0°C. Sedimentation from left to right.

1440 rad, we note, first, the similarities between the high and low speed curves and their counterparts in Fig. 5. Thus, while some distortion is still present at 26,000 rpm it appears to be essentially lost by 18,000 rpm. After 361 and 722 rad (middle panels, Fig. 6), the main peaks appear essentially the same as after the higher doses. (Note that the shape of the main peaks in Fig. 5 is invariant for doses up to ~1000 rad.) However, only a 60 min (~25°C) lysis period was used at 26,000 rpm and the complexes which are still evident after 361 rad and for unirradiated cells lie to the left of the main peak as in earlier figures. The small peak at fraction 20 after 722 rad, 18,000 rpm, might ordinarily be ignored as an adventitious "bump". That it probably represents a small amount of residual complex is suggested by the results for unirradiated cells at this speed. Here the main peak appears unresolved between two peaks and since we know that an appreciable percentage of the DNA should be in the complex after the lysis conditions used, it seems reasonable to infer that the complex has split and in this case (18,000 rpm) straddles the main peak.

The results in Fig. 6 show, therefore, that while 18,000 rpm is a low enough speed to eliminate most of the speed distortion of the main peak, this is not the case at 26,000 rpm. Further, the data suggest that the effect of speed on the complex is more dramatic than simply a change in the shape of the distribution. In what follows it

will be shown that, in addition to pattern shape, the relative positions of the complex and main peak are speed dependent and that to observe this a speed less than 18,000 rpm must be used.

The Temperature Dependence of Cell Lysis

The results in Fig. 6 suggest that sedimentations for extended periods at low speeds are required to study pattern distortion relative to the complex. In contrast to the main peak, the life time of the complex under alkaline, high salt conditions is relatively transient (e.g., Fig. 1). To minimize resolution effects during sedimentation, the temperature during centrifugation was reduced. The results in Figs. 5 and 6 already refer to centrifugations at reduced temperature, although cell lysis in those instances was at room temperature. To minimize further resolution of the complex before as well as during sedimentation, the temperature during lysis was reduced to 2–3°C and precooled rotors were used.

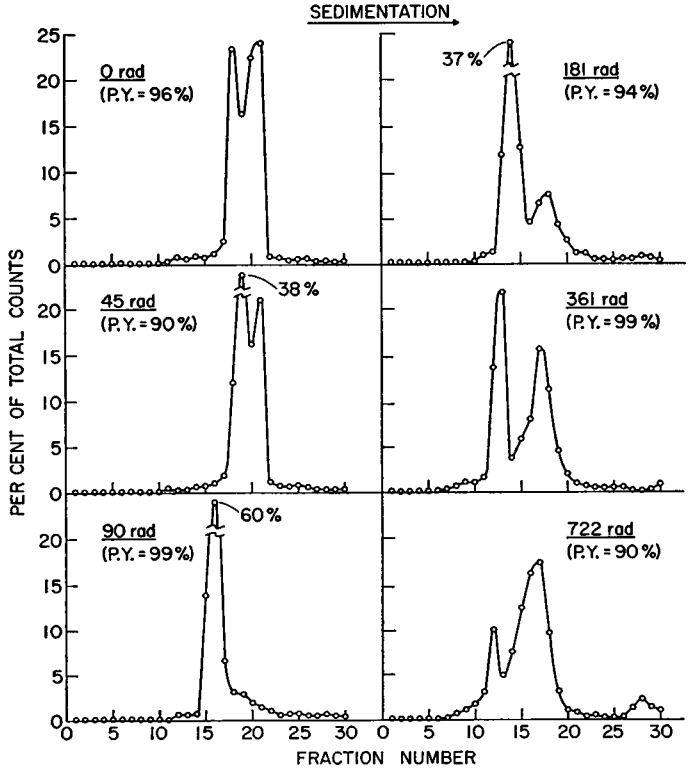

FIGURE 7 High speed sedimentation patterns of labeled DNA material from Chinese hamster cells. In contrast to earlier figures, lysis was at 2–3°C (60 min without light). Sedimentation: 36,000 rpm (SW-50.1 rotor), 90 min, ~1°C.

Fig. 7 shows a high speed run after cell lysis for 60 min at 2–3°C (in the dark). In contrast to the radiation dose dependence of the resolution of the complex at ~25°C already reported (Elkind and Kamper, 1970), and the degree of resolution of unirradiated cells after lysis for 60 min at ~25°C shown in Fig. 1, even after a dose of 45 rad, the complex in Fig. 7 remains essentially intact. After 90 rad, however, the pattern of the complex, while still extremely sharp, has moved sufficiently toward the meniscus so that this plus the radiation permits a small main peak to become evident at fractions 18 and 19. That this latter interpretation is very likely correct is clear from the remaining patterns. Dose increments of factors of two show a progressive loss of radioactivity from the complex, a concomitant increase in radioactivity in the main peak, and a clear but small shift in the complex toward the meniscus. This latter point by itself illustrates the atypical sedimentation of the complex since although the distributions after 90–722 rad show that almost all the radioactivity has been shifted to the main peak, the position of the complex is shifted only a small distance toward the meniscus.

In addition to demonstrating the reduced rate of resolution of the complex to be expected at reduced temperature, the results in Fig. 7 demonstrate two other points. First, as referred to earlier, small doses of X-irradiation and a fixed lysis time take the complex apart as do small doses of actinomycin D. Qualitatively, these resolutions are the same as obtained with the prolonged lysis of unirradiated cells. Second, the reduced temperature during sedimentation (e.g. Fig. 7 vs. Fig. 1) required a 50% increase in time-at-speed to bring the main peak to the same position. Aside from the longer running time, the qualitative features of the progressive effect of increasing doses of radiation on the sedimentation patterns remain the same.

Speed Dependence of Sedimentation of the Complex

The results in Fig. 6 suggest that the effect of reduced speed on the complex is at least to shift its position relative to the main peak. To examine this further, under low temperature conditions (to reduce the rate of resolution before and during centrifugation), the radiation dose dependence of the resolution of the complex was measured at 5000 rpm using a temperature of ~2°C during a 60 min lysis period as well as during the 16.25 hr of centrifugation.

Starting with the pattern after 1550 rad in Fig. 8—a dose the primary effect of which is to resolve the complex without a large amount of concomitant strand breakage—we see that the position of the main peak obeys the usual dependence on $\omega^2 \times t$. Although the peak appears sharp, the ratio of its half-width to mode fraction number is essentially the same as in Fig. 5 after a comparable dose and 11,000 rpm centrifugation. Having located the main peak, it can be identified more readily in the pattern for unirradiated cells. This is seen to be the very small bump at about fraction 4. 60 min of lysis at low temperature apparently results in only a small amount of radioactivity sedimenting to where the main peak would be expected.

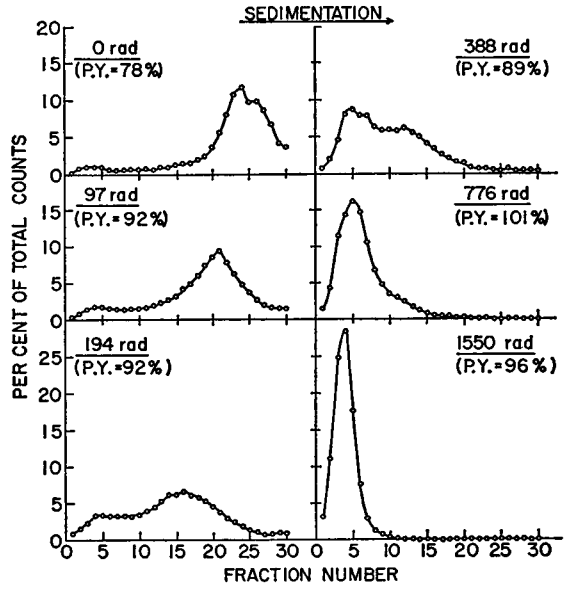

FIGURE 8 Low speed sedimentation of labeled DNA material from Chinese hamster cells. Lysis conditions same as for Fig. 7. Sedimentation: 5000 rpm (SW-50.1 rotor), 16.25 hr, ~2°C.

The more startling feature in the pattern of unirradiated cells in Fig. 8 is that almost all of the radioactivity is near the bottom of the tube. Since we have already accounted for the main peak, it must be concluded that the latter material is the complex. As shown in Fig. 7, the same lysis conditions led to a complex sedimenting as one or two sharp peaks in the vicinity of the main peak when the speed was 36,000 rpm. The pattern for control cells in Fig. 8 is still sharp (i.e., half-width to mode ratio) but its location is unexpected.

An examination of the remaining panels in Fig. 8 confirms the identifications made of the main peak and the complex in the light of the results in Fig. 7. With increasing doses of radiation, there is a progressive shift of radioactivity from the material near the bottom to that near the top of the tube. Further, the effect of the loss of label from the complex should be noted. Whereas with sedimentation at 36,000 rpm the shift in location of the complex is progressively toward the meniscus, the magnitude of the change is small. At 5000 rpm the change in location is marked. Taken together, the results in Figs. 7 and 8 suggest that although there is a smooth transition in the dose-dependent shift of material from the complex to the main peak at low speed, the complex is a DNA-containing material which differs in some essential way from that in the main peak. The results after 722 and 776 rad illustrate this point (Figs. 7 and 8, respectively). Although after 776 rad (Fig. 8) the complex blends smoothly into the leading edge of the main peak, after 722 rad (Fig. 7) it is

still a sharp peak on the trailing edge. Even though perhaps as little as 6% of the DNA remains in the complex after 722 rad (Fig. 7), it seems to suffer almost as much speed-dependent retardation as after a much smaller dose. This suggests that in contrast to the main peak, the complex may be a material or structure whose sedimenting ability is determined only in part by the amount of DNA in it. (Information about the composition of the complex will be contained in a separate report.)

Two control-type experiments were performed to test the possibility that just the longer sedimentation time was *the* factor responsible for the results in Fig. 8. In the first, cells were treated and sedimented as in Fig. 7 but gradients were stored overnight at 2–3°C before fractionation. The patterns were almost identical with those observed after prompt fractionation. In the second, cells were exposed to increasing doses of radiation and then lysed at 2–3°C overnight before sedimentation as in Fig. 7. For unirradiated cells it was clear that resolution of the complex had progressed to the point where a main peak was clearly identifiable; the resolution was comparable to that produced by 181 rad in Fig. 7. For irradiated cells. as expected, resolution of the complex was more extensive and some amount of degradation of the main peak was evident after doses of 700–800 rad. This was presumably because of base degradation at the high pH followed by strand scission, However, in all other respects, the patterns were essentially similar to those in Fig. 7.

It is hazardous to estimate the molecular weight of the complex in a manner similar to that used for the main peak. The obvious reason for this is the extent of the extrapolation of a relationship such as that specified by Studier (1965). An extrapolation would be of doubtful use also because, as noted above, the complex probably consists of more than just DNA. Although quite approximate, an estimate of relative size can be made using the principles of radiation target theory. From our prior report (Elkind and Kamper, 1970) and results such as those in Fig. 5, we know that a dose of $\sim$1500 rad is needed to produce a noticeable effect in the main peak, probably arising, as discussed earlier, from the production of single-strand breaks. In Fig. 8 we see that the sedimentation of the complex is affected by a dose of $\sim$100 rad. We may infer, therefore, that the complex is a target some 15 times larger, an estimate which probably is a lower limit. Besides the possibility that a dose lower than $\sim$100 rad might prove to be adequate to alter the complex, if it consists of material(s) in addition to DNA, a fraction of the hits scored by the radiation might be ineffective in altering sedimentation in a detectable way. Therefore, for a given degree of change in sedimentation a higher dose would be needed than otherwise, resulting in an underestimate of the target size.

DISCUSSION AND CONCLUSION

There are three principal points contained in the results presented. (*a*) When cells are lysed in a high pH, high salt solution, essentially all of the DNA emerges as part

of a material or large gross molecular weight—greater than $\sim 4 \times 10^9$ daltons—which is heterogeneous in composition. (*b*) The bonds that hold this complex together are labile under the conditions used. (*c*) From this material is resolved a smaller, more homogeneous and more stable, DNA-containing material whose number average molecular weight is 2×10^8 daltons. (Data on the chemical composition of both of these materials will be presented elsewhere.) The expected denaturing effect of high pH, the radioenergetics of break formation, and the observation (not reported here) that actinomycin D-^{3}H does not cosediment with main peak DNA labeled with thymidine-^{14}C, all support the view that the DNA is single-stranded in the latter material. However, a small amount of double-strandedness due perhaps to cross-linked regions cannot be ruled out nor small quantities of non-nucleic acid linkers or side chains.

The nonphysiological nature of the conditions used prompts the question of whether or not the DNA examined in this way has relevance to its organization and function in cells. While an unequivocal answer is not possible as yet, several points suggest that it does. Concerning main peak DNA, the size range involved is similar to that of replicons or small multiples of them (e.g. see Huberman and Riggs, 1968), that is from about 3×10^7 to 4×10^8 daltons (molecular weights at the half-width points of the number/molecular weight distribution) or from 30 to 200 μ assuming the foregoing molecular weights represent single strands. And as for the material in the complex, we have shown that in addition to the repair of breaks in main peak DNA, irradiated cells are able to repair damage to the complex (Elkind and Kamper, 1970). This means that those properties of the complex which may be examined by alkaline sedimentation are not simply a reflection of the conditions of cell lysis but depend upon the state of the DNA in the cell at the time of lysis. Further, relative to cell survival, actinomycin D acts as though it is equivalent to a dose of radiation (Elkind et al., 1967; Elkind et al., 1968); data presented here show that this is also the case relative to resolution of the complex. This plus other results on the ability of this drug to modify X-ray damage repair (to be reported elsewhere) further support the view that the complex reflects an organizational status within the cell fairly intimately related to integrated cell function, e.g., proliferation.

Whether or not the resolution of main peak DNA goes to completion during long enough periods of lysis or during shorter periods by the added action of visible light, or by small doses of X-rays or actinomycin D before shorter lysis periods, in all instances the main peak appears to have the same sedimentation properties. If, as expected, X-irradiation produces breaks at random, one reasonable possibility is that in all these instances resolution of the main peak is speeded by the production of breaks in the complex as a whole and not specifically in the DNA contained in it. For if the complex consists principally of DNA similar to main peak DNA but simply larger, increasing doses of radiation should result *only* in a progressive, smooth decrease in the average distance sedimented, and not in such a decrease plus the

appearance of main peak DNA at a relatively fixed position in the gradient much nearer the meniscus (e.g., Fig. 8). While some hits would be registered in the DNA in the complex, the radiation-induced speeding of the appearance of the main peak might reflect the opening of bonds in a structure which otherwise minimizes hydroxyl attack. However, in view of the discontinuity observed when sedimentation under alkaline conditions of intact duplex circles is compared with that of "nicked" circles (Bode and Kaiser, 1965; Gellert, 1967), an alternate possibility consistent with the foregoing is that the structure consists in part of catenated circles of duplex DNA in which a significant proportion of the hits are registered or some other structure which behaves similarly.

Whether or not the changes in sedimentation of the complex reflect mainly hits registered in non-DNA- or DNA-containing material, the results with actinomycin D contain some interesting suggestions. Actinomycin D is known to bind specifically and tightly to duplex DNA (Dingman and Sporn, 1965; Ebstein, 1967; Camargo and Plaut, 1967; Elkind et al., 1968). The drug is thought to intercalate between complementary base pairs leading to a small amount of untwisting for each molecule bound (e.g., see Waring, 1968). Even after exposure to concentrations which result in little cell killing, actinomycin D could have effects over extended regions of the DNA either because the additional strain produces nicks in circles leading to the interruption of continuity of the catenates, or because even a small amount of unwinding per intercalation results in appreciable angular displacements of long molecules. In either instance, while *short-range order* might not be significantly changed, *long-range order* could be. This illustrates how two agents as fundamentally dissimilar as ionizing radiation and a drug that intercalates could produce similar effects measured in respect to changes in sedimentation of the complex as well as cell killing. The preceding also is consistent with a quantitative dissimilarity between the actions of these agents relative to the production of single-strand breaks. The requirement for larger survival-equivalent doses of drug compared to X-rays to produce the same amount of strand breakage could result from the fact that duplex unwinding as such is not efficient in breaking strands in noncircular molecules of the size range of the DNA in the main peak, while the efficiency of bond breakage with X-irradiation is invariant per unit length of molecule. Lastly, the argument points up the notion that in addition to changes in short-range order in DNA (base changes, point mutations, or in general, changes which affect template action), mammalian cells may have their function significantly impaired if long-range order (e.g., catenate continuity and/or DNA in relation to nucleoprotein and membranes) is altered, thereby upsetting normal processes of replication and segregation. The effects of actinomycin D usually associated with the impairment of RNA synthesis may be of secondary importance as far as cell proliferation is concerned.

In addition to cell biological considerations, the results dealing with the speed dependence of sedimentation patterns have a number of implications. Among these

is the principal point that, under the conditions used, the configuration of single-stranded DNA apparently changes at high speed resulting in added viscous drag. While quantitation of the parametric dependencies of this effect is not pursued in this report, it is clear from the data that the putative increased, drag depends upon rotational speed and molecular weight. Further, the apparent qualitatively different speed effects for the materials in the main peak vs. the complex suggest that quantitatively at least the effect of rotational speed depends on the configuration of the molecules in question and possibly their association with materials other than DNA as well.

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REFERENCES

BODE, V. C., and A. D. KAISER. 1965. *J. Mol. Biol.* **14**:399.
CAMARGO, E. P., and W. PLAUT. 1967. *J. Cell Biol.* **35**:713.
DINGMAN, C. W., and M. D. SPORN. 1965. *Science (Washington).* **149**:1251.
EBSTEIN, S. 1967. *J. Cell Biol.* **35**:709.
ELKIND, M. M., and C. KAMPER. 1970. *Biophys. J.* **10**:237.
ELKIND, M. M., E. KANO, and H. SUTTON-GILBERT. 1969. *J. Cell Biol.* **42**:366.
ELKIND, M. M., K. SAKAMOTO, and C. KAMPER. 1968. *Cell Tissue Kinet.* **1**:209.
ELKIND, M. M., and H. SUTTON. 1960. *Radiat. Res.* **13**:556.
ELKIND, M. M., H. SUTTON-GILBERT, W. B. MOSES, and C. KAMPER. 1967. *Nature (London).* **214**:1088.
FRIEFELDER, D. 1966. *Radiat. Res.* **29**:329.
GELLET, M. 1967. *Proc. Nat. Acad. Sci. U.S.A.* **57**:148.
HUBERMAN, J. A., and A. D. RIGGS. 1968. *J. Mol. Biol.* **32**:327.
MCGRATH, R. A., and R. W. WILLIAMS. 1966. *Nature (London).* **212**:534.
STUDIER, F. W. 1965. *J. Mol. Biol.* **11**:373.
WARING, M. J. 1968. *Nature (London).* **219**:1320.