explain the high standard deviations completely, but we believe no significance can be attached to the somewhat wider scatter of values than is seen in the other measurements. The K values varied from 71.9 ± 4.1 g, corresponding to a 59-lb lean body weight for a 61-year-old female with an initial body weight of 137 lb, to 233.4 ± 5.3 g, corresponding to a 193-lb lean body weight for an 18-year-old male subject with an initial body weight of 227 lb. The average K value of all the 51 subjects prior to intiating the food-substitute therapy was 153.1 g, and at the end of the 8-week period it was 154.2 g. Since the final average K value is larger than the initial average value, there can be no statistical decrease in the average initial to final measurements. No statistical decrease means that the test statistic would accept the hypothesis of equality of the averages for the probability of rejecting a true hypothesis at least up to the 0.40 level. The lean body weight of each of the 51 subjects was thus considered to remain constant with the total weight loss resulting from loss of excess fat. A typical plot of the lean body weight and fat values for one of the male subjects over the period of the experiment is shown in Fig. 1.

These results confirm the findings reported by Berlin *et al.* (*6*), who studied the body composition of three obese subjects who showed weight losses of 65, 52, and 44 pounds when placed on a restricted calorie intake. Their method consisted of metabolic balance and body-water and body-density measurements. Nitrogen balance studies of obese subjects on weight-reducing regimens described by other authors (*7*) are also in agreement with these results. As cited by Berlin (*6*), a calculation of the density of the net tissue lost in his studies yields values which are not in good agreement with the density values previously reported (*8, 9*) for normal and overweight individuals on a weight-reduction regimen in which lean body mass was reported to be lost. Berlin indicates that data for the density of the tissue lost, when translated in terms of body composition, are in a large part misleading; the loss ought not be accredited to an anatomical entity for which there appears to be no evidence (*8*).

These results show the importance of relative measurements of body composition obtained by serial, in vivo determinations of potassium-40 (*10*).

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X-Ray Sensitivity and DNA Synthesis in Synchronous Populations of HeLa Cells

Abstract. *Inhibition with either 5-fluorodeoxyuridine or deoxyadenosine for specified periods during the division cycle of the HeLa S3 cell shows that the mid-interphase peak in sensitivity occurs just before DNA replication begins. Sensitivity subsequently decreases only after synthesis of DNA is resumed. One interpretation of the relation between fluctuations in sensitivity and in DNA synthesis is that the lethal radiation damage to these cells occurs in DNA.*

Sensitivity of HeLa S3 cells to 220 kev x-rays, as measured by loss of the capacity for sustained reproduction, fluctuates during the division cycle (*1*); synchronized populations display two peaks of sensitivity, one at mitosis and the second about midway through the cycle, 10 hours later (*2*). In this system about 60 percent of the cells have begun to synthesize DNA by the latter time (S phase cells), while the remainder are still in the postmitotic (G_1) phase of the DNA-synthetic cycle (*3*). It was not clear, therefore, whether maximal interphase sensitivity occurs before or after the inception of DNA synthesis. Furthermore, although we suggested (*2*) that the sensitivity fluctuations might be intimately related to the cyclic pattern of DNA synthesis, no evidence was adduced in support of any such relation. We have now measured the x-ray sensitivity of synchronous cell populations in which resolution was increased by inhibiting DNA synthesis for specified periods during the division cycle. Greatest sensitivity occurs just before the start of DNA duplication. In addition, the marked distortions which these treatments produce in the survival pattern correlate strongly with the changes induced in the DNA-synthetic cycle.

Replicate cultures, each containing roughly 10^5 selectively harvested mitotic HeLa S3 cells (*3*), were incubated in medium N16HHF (*4*) in plastic dishes, under the usual conditions. At appropriate times the growth medium was replaced with warmed medium containing either of two inhibitors of DNA synthesis: 5-fluorodeoxyuridine (FUdR) (*5*) at a concentration of $10^{-6}M$ (*6, 7*) and deoxyadenosine at $3.2 \times 10^{-5}M$ (or, in one experiment, $4.8 \times 10^{-4}M$) (*8*). All dishes in a given experiment were treated with inhibitor for the same period, irrespective of when they were irradiated. To reverse inhibition by deoxyadenosine, the medium was removed, the plates were rinsed once with Saline G (*4*), and fresh growth medium was added. Inhibition by FUdR was reversed with $10^{-5}M$ thymidine (*6*).

All operations were performed at 37°C, including irradiations which were carried out in single exposures of 300 rads (*9*). Cell survival after irradiation with this one dose is a satisfactory indicator of the changes in sensitivity that occur during the cell-division cycle (*2*). The shape of the dose-survival curves is not altered in the presence of FUdR (*10*) or deoxyadenosine. The criterion for cell survival was growth of a 50-cell colony within 10 days (*11*).

The rate of DNA synthesis was determined in normal synchronous populations and in those inhibited by deoxyadenosine by measuring the incorporation of C^{14}-labeled thymidine (*12*) during 30-minute periods (*3*). Inhibition

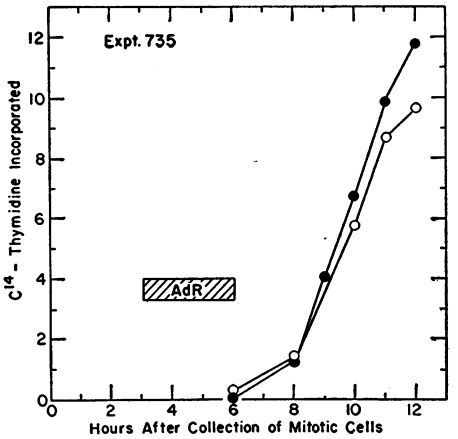

Fig 1. The solid circles show the rate (counts per minute) of DNA synthesis as measured by incorporation of C^{14}-thymidine during 30 min periods, in synchronous HeLa S3 cells treated with deoxyadenosine (AdR) during G_1 (bar). The rate of synthesis in untreated cultures is shown by the open circles.

of DNA synthesis by FUdR was measured by the diphenylamine method (*13*) in separate experiments with large numbers of randomly dividing cells. At the concentrations employed, the inhibitors reduce the rate of DNA synthesis essentially to zero. In addition, unirradiated control cultures, included in each experiment, showed that the drugs cause little or no cell killing under the conditions we used (*14*).

The specificity of these inhibitors of DNA synthesis in this system was investigated in several experiments. The results are summarized here. Incorporation of C^{14}-labeled uridine (*15*), a precursor of RNA, is not significantly affected by either FUdR or deoxyadenosine at the concentrations used except during the S phase, when incorporation is reduced up to 25 percent; a similar portion of the radioactivity is found in the DNA of uninhibited cultures (*3*). Incorporation of C^{14}-labeled leucine (*16*) into protein is not affected by FUdR (*17*).

Specific DNA inhibitors should not disturb the normal pattern of DNA replication in cells exposed only during G_1. Figure 1 shows that deoxyadenosine, when present from 3 to 6 hours after mitosis, does not significantly modify either the inception or the rate of DNA synthesis; no residual action of the drug is detected after its removal. Neither should these inhibitors affect events that normally occur during G_1; they might, however, modify the pattern of radiation sensitivity during this phase if the processes causing the progressive increase in sensitivity are related to DNA synthesis, as could be the case if x-irradiation damages DNA and the damage is partially repaired before replication begins (*2, 18*).

The occurrence of such unscheduled DNA synthesis in damaged chromosomes has been suggested by Taylor *et al.* (*19*). Figure 2 shows that the usual G_1 sensitivity increase occurs in the presence of FUdR. Sensitivity also increases progressively from 2 hours onward in the presence of deoxyadenosine (Fig. 4), but this agent sensitizes cells to radiation, so that the survival values are decreased by about 30 percent at all times. This sensitization has not been studied, except to show that the slope of the exponential portion of the dose-survival curve for random cultures is 10 percent larger in the presence of the drug. We conclude that the processes responsible for the increase in sensitivity from 2 to 10 hours do not include DNA synthesis during G_1, or any other process which might be affected by the inhibitors. If our suggestion is correct that the lethal damage from radiation occurs in DNA (see below), and if repair of radiation damaged DNA is blocked by FUdR (*19*), we can conclude further that no repair processes are involved in the G_1 sensitivity increase (compare *2, 18*).

Typical sensitivity changes in cells in which DNA synthesis is inhibited by FUdR are shown in Fig. 3. When added before the start, the S (lower bar; solid circles), FUdR abolishes the survival increase that normally begins 10 hours after mitosis (open circles); survival continues to decrease until 12 hours, when essentially all the cells have completed preparation for DNA synthesis (*3*), and then reaches a plateau; it rises only after the inhibitor is removed. This result resembles the decrease in x-ray sensitivity observed by Erikson and Szybalski (*10*) soon after the start of DNA synthesis in D98 cells in which synchronous growth has been initiated by addition of thymidine to FUdR-inhibited cultures. It is noteworthy that the minimum survival is about the same as that for mitotic cells, and that the highest survival observed in this experiment (at 24 hours) was some tenfold greater, corresponding to a mean lethal dose 3.1 times larger than that for the most sensitive cells (assuming exponential inactivation curves with extrapolation number of 2). This difference, about twice as large as observed previously (*2*), still may not represent the maximum fluctuation in

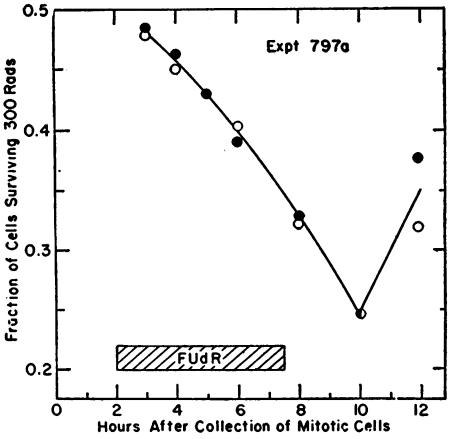

Fig. 2. Survival of synchronous HeLa S3 cells after irradiation with 300 rads, as a function of time after mitosis (zero hours), in the presence (solid circles) and absence (open circles) of FUdR during G_1 (bar).

sensitivity that occurs during the division cycle. When FUdR is added to a culture in which nearly all of the cells have entered S (upper bar; squares), the rise in the survival curve is interrupted until the drug is removed.

Because FUdR might possibly disturb the normal time course of those intermitotic events which determine sensitivity to x-rays and thereby produce a fortuitous correlation between the patterns of change in sensitivity and of DNA synthesis, a second inhibi-

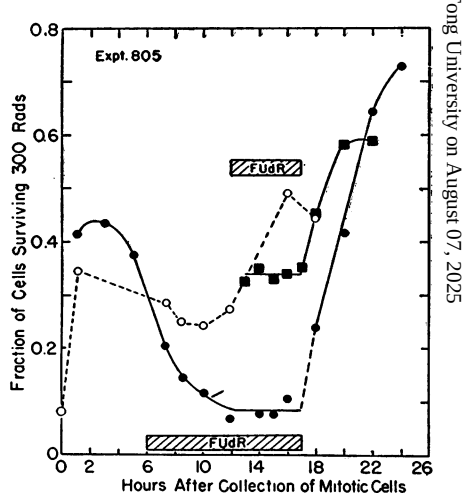

Fig. 3. Survival of synchronous HeLa S3 cells after irradiation with 300 rads in the presence of FUdR during all (solid circles; lower bar), or only the latter portion (squares; upper bar), of the normal S period. The dashed lines connecting the points for the untreated controls (open circles) do not accurately portray the normal sensitivity fluctuations, as survival values are lacking at several times. Typical sensitivity changes are better shown by the open circles in the upper curves of Fig. 4.

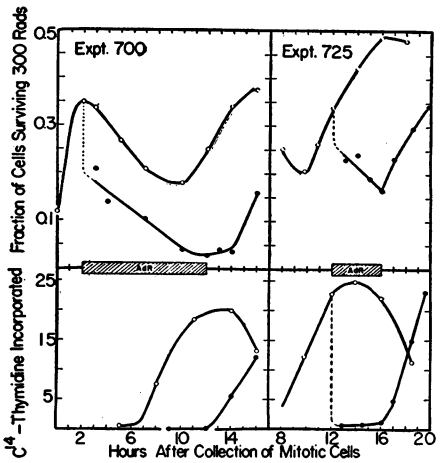

Fig. 4. Effect of AdR on the rate (counts per minute) of DNA synthesis in synchronous HeLa S3 cells, as measured by incorporation of C^{14}-TdR during 30-min periods (bottom curves), and on survival after exposure to x-rays (300 rad dose) (top curves). Deoxyadenosine concentration was $4.8 \times 10^{-4}M$ during G_1 and part of the normal S period in experiment 700; it was $3.2 \times 10^{-4}M$ during the normal S period in experiment 725. Open circles are control values. Vertical dashed lines in the upper curves indicate the drop in survival caused by deoxyadenosine sensitization.

tor of DNA synthesis, deoxyadenosine, which acts by a different mechanism (*8*), was tested. Substantially the same types of response were observed as with FUdR.

Figure 4 shows examples of the effect of deoxyadenosine on cultures virtually all of whose cells are in either G_1 (experiment 700) or S (experiment 725). The upper curves show survival after x-irradiation; the lower show the rate of DNA synthesis. Synthesis of DNA does not begin if deoxyadenosine is added during G_1 (experiment 700) and it stops if the inhibitor is added during S (experiment 725); it does not proceed until the inhibitor is removed (*20*). Correspondingly, survival falls immediately, presumably because of the aforementioned sensitization, and continues to fall until all cells have completed G_1 (experiment 700). (The continued drop in survival between 12 and 16 hours in cultures to which deoxyadenosine was added at 12 hours in experiment 725 is anomalous; a plateau would be expected, as in experiment 805.) Survival generally begins to increase as soon as DNA synthesis begins, but a considerable delay occurs in the survival response when deoxyadenosine is used as inhibitor and is present from early G_1 (experiment 700). The cause of the delay is not known; it may be related to the relatively slow rate of DNA synthesis observed after removal of the drug (*20*).

It was shown previously that x-irradiation of HeLa S3 cells in G_1 neither delays the inception nor decreases the rate of DNA synthesis (*2*) and that synthesis is inhibited only slightly when cells are exposed to lethal doses during S (*2, 21*). Thus the lethal action of x-rays on these cells cannot reasonably be attributed to gross failure of DNA to replicate. Yet it is clear from the present experiments that cells are most sensitive to x-rays just before the replication of DNA begins and that synthesis of DNA is accompanied by a decrease in sensitivity. Because the metabolic activity of irradiated HeLa S3 and several other strains of cells is maintained for long periods after exposure to lethal doses (*22, 23*), whereas reproductive structures and processes, including cell division, are extremely sensitive to radiation (*11, 23*), it has been postulated that it is the genetic apparatus of the cell, in particular its chromosomes (*11, 24*), whose damage leads to cell killing. In the context of this hypothesis, these results imply that some genetically related constituent undergoes structural or quantitative change during the cycle, either suffering more damage or being less susceptible to repair when irradiated just before DNA replication normally begins. This constituent remains sensitive to radiation insult until DNA synthesis is under way and then becomes more resistant as replication proceeds. DNA is a reasonable candidate for that role; it might, for example, dissociate from stabilizing protein or undergo incipient (*25*) or actual (*26*) strand separation before replication. Szybalski and co-workers (*27*) have already identified DNA as the constituent whose damage by x-rays leads to death in bacteria (*28*).

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14. Survival ranged from 75 to 96 percent of control values in five experiments in which FUdR was present for periods of 3.5 to 11 hours during G_1, S, or both, the lower values corresponding to the longer exposures. In five experiments with $3.2 \times 10^{-4}M$ deoxyadenosine, survival varied from 88 to 108 percent of controls, but at $4.8 \times 10^{-4}M$, survival was reduced to 69 percent.
15. 0.02 μc/ml; 33.9 c/mole; 30 min.
16. 0.05 μc/ml; 7.7 c/mole; 30 min.
17. These results agree with the report of Rueckert and Mueller (*7*) who found that no reduction of RNA or protein synthesis occurred for 24 hours after addition of FUdR to randomly dividing cultures. However, N. P. Salzman and E. D. Sebring [*Biochim. Biophys. Acta* **61**, 406 (1962)] have reported a substantial reduction within 20 hours in the presence of FUdR, which they attribute to complete inhibition of synthesis of that fraction of the RNA and protein contained in the nucleus. The discrepancies are unexplained.
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20. In general, the rate of DNA synthesis does not rise precipitously after removal of inhibitor, although occasionally it does increase faster than in controls; for example, in experiment 725 it did not achieve the rate which obtained at the time of deoxyadenosine addition until 3.6 hours after drug removal. In view of the enhanced synchronization of DNA synthesis presumably effected by the drug treatments applied (*7, 10*), the slow recovery is anomalous. It might possibly be caused by residual inhibitor remaining in the cells after removal of the drug from the medium, though the failure of treatment during G_1 to affect the rate during S (Fig. 1) makes this interpretation unattractive. Further study is necessary to determine if the acceleration is related to that found by G. C. Mueller, K. Kajiwara, E. Stubblefield, R. R. Rueckert, [*Cancer Res.* **22**, 1084 (1962)] following 20-hour inhibition of DNA synthesis by amethopterin.
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