MODIFICATION OF X-RAY-INDUCED KILLING OF HeLa S3 CELLS BY INHIBITORS OF DNA SYNTHESIS

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ABSTRACT After irradiation of HeLa S3 cells with 220 kv x-rays during G1, treatment with any of six inhibitors of DNA synthesis results in the progressive enhancement of cell killing (loss of colony-forming ability). Incubation with hydroxyurea, cytosine arabinoside, or hydroxylamine reduces survival five- to twentyfold in about 8 hr, following an x-ray dose of 400 rads. In contrast, treatment with 5-fluorodeoxyuridine, deoxyadenosine, or thymidine after this same dose reduces survival less than twofold during a comparable time interval. These differences occur at drug concentrations which reduce the rate of DNA synthesis by at least 95% (except in the case of hydroxylamine, which inhibits DNA synthesis to a smaller extent), but which kill no unirradiated cells during the treatment periods. When inhibition of DNA synthesis with either hydroxyurea or cytosine arabinoside is reversed by addition of appropriate precursors of DNA, the enhancement is abolished. With hydroxyurea, the rate of cell killing is dependent on the dose of x-rays previously administered, and the extent of enhancement seems to be related to the drug concentration. Imposition of a delay between irradiation and addition of hydroxyurea does not abolish the enhancement effect, but instead causes a proportional lag in its inception. Postirradiation treatment of S phase cells with either hydroxyurea or cytosine arabinoside also enhances killing. Furthermore, unlike early G1 cells, S cells (and, as shown previously, cells blocked at the G1-S transition) are sensitized by preirradiation exposure to hydroxyurea.

INTRODUCTION

The modification of x-ray–induced cell killing (loss of colony-forming ability) by appropriate preirradiation or postirradiation treatment has been observed in several in vitro mammalian cell systems. Although many of the treatments found to be

[1] The following abbreviations are used in this paper: FUdR, 5-fluorodeoxyuridine; HOU, hydroxyurea; CHI, cycloheximide; ara-C, cytosine arabinoside; HA, hydroxylamine; AdR, deoxyadenosine; TdR, thymidine; dCMP, deoxycytidine monophosphate; CdR, deoxycytidine; GdR, deoxyguanosine.

effective in modifying survival are nonspecific, e.g., exposure to suboptimal temperatures (Beer et al., 1963; Whitmore and Gulyas, 1967; Phillips and Tolmach, 1966), or fortification of the growth medium with various nutrients (Montes de Oca et al., 1963; Miletić et al., 1964; Horikawa et al., 1964), agents with more selective action have been described also. In particular, a number of compounds which interfere specifically with DNA synthesis have been shown to enhance the killing of HeLa S3 cells by x-rays (Phillips, 1965; Phillips and Tolmach, 1966). In the latter study, the modifying effects of two such inhibitors, 5-fluorodeoxyuridine (FUdR)[1] and hydroxyurea (HOU), were investigated using synchronous HeLa S3 cells. It was found that immediate postirradiation incubation of G1 or S phase cells with either of these drugs enhances cell killing as measured by colony-forming ability. It was also shown that, in contrast, similar postirradiation treatment with an inhibitor of protein synthesis, cycloheximide (CHI), during late G1 or early S, markedly increases survival. Further studies revealed that simultaneous incubation with both HOU and CHI results in scarcely any postirradiation response. In addition, the introduction of a delay between irradiation and addition of HOU appeared to indicate that the cells become increasingly insensitive to the drug.

On the basis of those findings, Phillips and Tolmach (1966) postulated that irradiation produces both lethal and potentially lethal damage, the postirradiation modification of survival representing a disturbance to one or the other of two competing processes which normally operate in the irradiated cell, whereby the potentially lethal damage is altered. One of these was pictured as a repair process, and the other as a process by which potentially lethal damage is fixed in the cell, resulting in cell death. According to this model, any agent, specific or nonspecific, which alters the relative rates of these two reactions will alter the observed survival. Thus, the action of HOU was attributed to interference with the repair process, and that of CHI, with the expression process. It may be noted that the distinction between lethal and potentially lethal damage is an operational one; that part of the damage which is modified, as determined from an alteration of cell survival, is termed potentially lethal.

Because study of these modifications of x-ray damage may lead to increased understanding of the mechanism of cell killing by ionizing radiations, we have undertaken further analysis of the phenomena. In particular, we have examined several additional inhibitors of DNA synthesis, in order to determine the generality of the behavior elicited by HOU and FUdR. These other inhibitors include cytosine arabinoside (ara-C) (Chu and Fischer, 1962), hydroxylamine (HA) (Young and Hodas, 1964), deoxyadenosine (AdR) (Overgaard-Hansen and Klenow, 1961), and thymidine (TdR) (Xeros, 1962; Galavazi et al., 1966). All the inhibitors studied enhance the killing of HeLa S3 cells irradiated in either G1 or S, though not with equal efficiencies. That is, while a strong correlation has been found between the level of inhibition of DNA synthesis and the amount of enhanced killing produced

with HOU, a heterogeneity in postirradiation response among the inhibitors has also been observed, at essentially the same high levels of DNA inhibition. In addition, it has been found that as much as a 12 hr delay can be imposed between irradiation and drug treatment without loss of sensitivity of the irradiated cells to the action of the drug.

MATERIALS AND METHODS

HeLa S3 cells were maintained in monolayer cultures in medium N16HHF by routine procedures described by Ham and Puck (1962). Synchronous cultures were obtained by the mitotic selection method developed by Terasima and Tolmach (1963 *a*) and modified by Phillips and Tolmach (1966). One of the modifications introduced involved the substitution of 5% fetal calf and 10% calf sera for the human and horse sera in N16HHF, this new medium being designated N16FCF. Progression of cultures through the generation cycle was monitored by measuring the rate of incorporation of ^{14}CTdR as a function of time after collection of mitotic cells.

Stock solutions of inhibitors were prepared at concentrations which permitted the addition of 0.01–0.02 ml per ml of growth medium to achieve the desired concentration. In most cases solutions were stored at −20°C; none were refrozen after thawing. Solutions of HA were prepared just before use, since they were found to be highly toxic to cells if prepared far in advance and stored at 5°C, indicating some conversion of the compound to a more toxic product during prolonged storage at this temperature.

Concentrations of the various inhibitors which reduce the rate of DNA synthesis to less than 5% of the control value were either chosen from previously reported work, or were established during the course of this study. FUdR was used at a concentration of 1 μM which had been shown to essentially abolish DNA synthesis (Terasima and Tolmach, 1963 *c*). The concentration of TdR employed, 2 mM, had been found to block DNA synthesis adequately (Xeros, 1962). HOU was used at a concentration of 2.5 mM, which had been found to be both completely inhibitory and nontoxic to HeLa cells in any part of the generation cycle (Pfeiffer and Tolmach, 1967). HA, as the hydrochloride, was used at a concentration (1.2 mM) which produced less than complete inhibition of DNA synthesis, because toxicity developed after only 6 hr of exposure to even this concentration.

Inhibition by all agents tested, except FUdR, was reversed by removing the medium (N16FCF) containing the inhibitor, rinsing the cells with 3 ml of fresh N16FCF, and replacing this finally with 5 ml of N16HHF. Inhibition by FUdR was reversed by adding TdR to a final concentration of 10^{-5} M. Experiments involving FUdR were run in N16HHF, since the fetal calf serum in N16FCF contains thymidine which overcomes the FUdR block. All additions and reversals were carried out at 38°C.

Two different assays were used for measuring the inhibition of DNA synthesis in synchronous cultures. In the first, the inhibitor was added to cultures in early G1 (3 hr after collection), and the continuous incorporation of ^{14}CTdR was monitored during the succeeding 9–10 hr. In the second, the inhibitor was added to cultures when they were at the peak rate of DNA synthesis (11–13 hr after collection), and the incorporation of ^{14}CTdR during a 20 min pulse was determined immediately after drug addition and 1 hr later. For inhibition of randomly dividing cultures, incorporation after continuous or pulse labeling was measured periodically for 3 hr.

Cell viability was assayed by colony formation, as described by Puck and Marcus (1956). In all experiments involving treatment with both radiation and inhibitors, control plates treated with inhibitors alone for comparable time periods were included. None of the inhibi-

tors used in this work produced measurable levels of toxicity over the time periods involved in the experiments presented.

Irradiations were carried out using a 220 kv, 15 ma constant potential x-ray therapy unit with added filtration of 0.25 mm Cu and 1.0 mm Al (half-value layer, 1.0 mm Cu). The dose rate, which was measured in each experiment, was about 83 rads/min. The irradiation chamber contained air with 4% CO_2, and was maintained at 37°C.

RESULTS

Postirradiation Treatment of Early G1 Cells with Various Inhibitors of DNA Synthesis

The effect of inhibitors of DNA synthesis on the survival of x-irradiated HeLa S3 cells was studied with six compounds: HOU, FUdR, AdR, TdR, ara-C, and HA. The concentrations chosen reduced the rate of DNA synthesis to less than 5% of the control rate, except in the case of HA, which was used at a concentration that reduced the rate to 20–40% of the control. Inhibition of DNA synthesis was measured

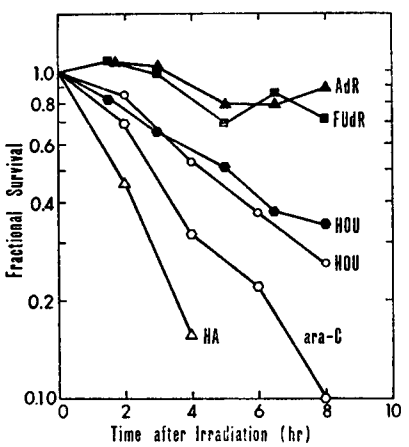

FIGURE 1 Postirradiation treatment of G1 cells with DNA inhibitors. The inhibitors were added to synchronous cultures 3.0 hr after collection of mitotic cells. The following inhibitors were used: 1 mM AdR (closed triangles), 1 μM FUdR (squares), 2.5 mM HOU (closed hexagons and open circles), 4 μM ara-C (open hexagons), 1.2 mM HA (open triangles). Irradiation of all samples with 400 rads was performed immediately after drug addition. At the times shown, the medium was changed to reverse the inhibition (in the case of FUdR, 10 μM thymidine was added to the fresh medium). Survival values have been normalized so that the surviving fraction after irradiation alone is 1.0. The open and closed symbols refer to 2 independent experiments, in which survival after irradiation alone was 0.128 and 0.08 respectively.

in each experiment involving HOU, AdR, ara-C, or HA, using either of the two methods described above. A standard x-ray dose of 400 rads was administered 3 hr after the collection of mitotic cells, i.e., in early G1, a relatively x-ray resistant portion of the generation cycle (Terasima and Tolmach, 1963 b). The inhibitors were usually added just before irradiation, but the results were not affected if the order was reversed.

Fig. 1 illustrates the decrease in survival exhibited by irradiated cells on treatment with 1 mM AdR, 1 μM FUdR, 2.5 mM HOU, 4 μM ara-C, or 1.2 mM HA; thymidine at high concentrations (2 mM) yields a survival curve similar to that for AdR and FUdR. As reported previously (Phillips and Tolmach, 1966), treatment with HOU causes a marked increase in the amount of cell killing brought about by a given dose of x-rays. Ara-C and HA are similarly quite active. The curves for AdR

and FUdR (as well as TdR), in contrast, are much less steep even though the rate of DNA synthesis is reduced to the same extent by all these agents except HA. In addition, it has been observed consistently that ara-C produces a more rapid decline in survival than does HOU, even when higher concentrations of HOU are used, and that HA is even more effective. Thus, although HA is the most active of the agents tested in enhancing cell killing by x-rays, it is the least effective inhibitor of DNA synthesis at the concentration used. (However, reduction of the HA concentration to 0.12 mM, at which DNA synthesis is not inhibited, results in no postirradiation enhancement).

The shapes of the curves in Fig. 1 are in some doubt because of interexperimental variability. For example, of the curves obtained in nine experiments carried out with FUdR, three appear to fall rapidly and reach a plateau at 60–80% of the control level, four exhibit shoulders preceding a drop, and two are more complex. Survival

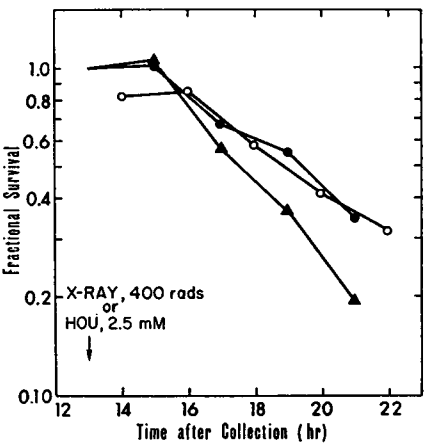

FIGURE 2 Enhancement of cell killing by HOU and ara-C in S phase cultures 13 hr after collection. The closed symbols represent the effects of postirradiation treatment with 4 μM ara-C (closed triangles) or 2.5 mM HOU (closed circles). Cells were irradiated with 400 rads 13 hr after plating. The drugs were added just before irradiation and inhibition was reversed at the times shown, by changing the medium. The open circles represent the effects of preirradiation treatment of 13 hr S phase cells with 2.5 mM HOU. At the indicated times triplicate samples were irradiated with 400 rads and the inhibitor was removed immediately afterward.

after 8 hr of treatment ranged from 47–87% of the control, with a mean of 66%. Similarly, while an exponential decrease in survival was observed in the majority of the experiments with HOU, others yielded curves of greater complexity. However, in nine experiments the range in survival after 8 hr of treatment was only 7–33% of the control, with a mean survival of 20%. Thus, in spite of this variability, it is clear that response to treatment with HOU was consistently much greater than with FUdR; neither is there any doubt as to the large quantitative differences among the other agents.

Postirradiation Treatment of S Phase Cells

It was shown previously (Phillips and Tolmach, 1966) that cells surviving irradiation in late G1 (8.2 hr after collection), like those irradiated in early G1, are progressively killed by treatment with HOU. In order to determine the effect of DNA

inhibition on cells irradiated when already synthesizing DNA, cells were examined in the middle of S (10–13 hr after collection). When such cells are irradiated and treated with HOU, killing is again enhanced (Fig. 2, solid circles), but the rate of killing is apparently less rapid than with G1 cells, and a lag of 1–2 hr is evident before the onset of killing. In all of these experiments, the same initial level of survival was achieved before treatment with HOU. Hence the different rates of killing brought about by HOU treatment after irradiation at these different times in the generation cycle would not appear merely to reflect phase-dependent variations in sensitivity to x-rays (Terasima and Tolmach, 1963 *b*). It may be recalled that unirradiated S phase HeLa S3 cells, unlike Chinese hamster cells (Sinclair, 1965), are not killed by 2.5 mM HOU during the treatment periods employed (Pfeiffer and Tolmach, 1967).

Treatment with ara-C also enhances the effect of x-radiation on S cells (Fig. 2, triangles). Again, the rate of killing is lower than that with early G1 cells, and ara-C is somewhat more active than HOU. AdR also is active on cells irradiated in S, yielding somewhat less than a twofold reduction of survival in 8 hr. Irradiated G2 cells have not been examined with any of these drugs.

Preirradiation Treatment of S Phase Cells with HOU

The scheme discussed in the Introduction implies that modification of survival is a postirradiation phenomenon. In apparent confirmation of this interpretation, it had been found that preirradiation treatment of G1 cells with HOU (or FUdR or CHI) does not sensitize these cells to x-rays. However, once cells have accumulated at the G1-S transition, further exposure to the drug prior to irradiation results in a progressive decrease in the fraction of cells which survive a given dose of x-rays (Phillips, 1965; Phillips and Tolmach, 1966). Thus preirradiation treatment with HOU can sensitize the cells at this stage of the generation cycle to the lethal effects of x-rays.[2] The effect is apparently absent with FUdR (Terasima and Tolmach, 1963 *c*). When the same treatment is carried out with cells already in S (12–13 hr after collection), sensitization is also observed. In comparison with untreated cells, survival is reduced by about 70% when cells are irradiated with 400 rads after 8 hr of exposure to HOU (Fig. 2, open circles). These preirradiation effects, while not predicted by the aforementioned model, are not in conflict with it; modification of the postulated repair or expression processes might be brought about by metabolic alterations effected prior to irradiation. The similarity of rates of killing by pre- and postirradiation treatment of S phase cells with HOU (Fig. 2, open and closed circles) may be noted in this connection.

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[2] A recent report by Terasima and Fujiwara (1966) to the effect that HeLa cells blocked at the G1-S transition by HOU do not undergo progressive sensitization to x-rays is difficult to evaluate because a fivefold lower concentration of the drug was used.

Correlation of Postirradiation Killing with Inhibition of DNA Synthesis: Concentration Dependence of Postirradiation Killing by HOU

It was reported by Phillips and Tolmach (1966) that prolonged treatment with HOU at a concentration of 0.0125 mM, which does not usually inhibit DNA synthesis in HeLa S3 cells, appeared to produce a small, delayed decrease in the survival of cells irradiated in early G1. It was suggested, therefore, that postirradiation enhancement

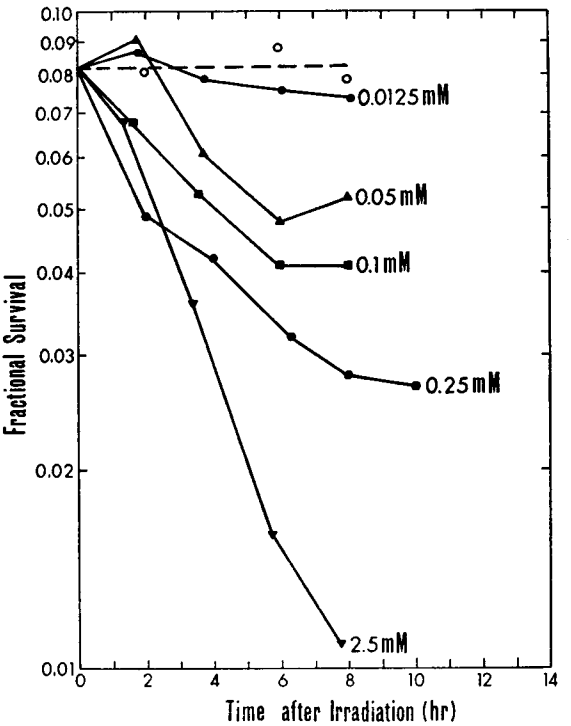

FIGURE 3 Concentration dependence of the postirradiation enhancement of cell killing by HOU. All plates were irradiated with 400 rads 3.0 hr after collection of mitotic cells. The following concentrations of HOU were added to each set of plates just before irradiation: 0.0125 mM (closed circles), 0.05 mM (triangles), 0.1 mM (squares), 0.25 mM (hexagons), 2.5 mM (inverted triangles). At the indicated times the medium was changed to reverse the inhibition. The untreated control plates were subjected to medium change at the times indicated by the open circles.

of cell killing by this compound might not arise entirely from its action as a DNA inhibitor. However, repeated experiments with a similar noninhibitory concentration have failed to confirm the activity of this low concentration of HOU. In fact, the concentration dependence of the postirradiation response to HOU is very similar to that of the inhibition of DNA synthesis.

Fig. 3 shows the dependence of cell killing on the concentration of HOU, over an 8 hr period following irradiation in early G1. The data do not permit accurate

comparison of the rates of killing, though they suggest that the rate depends on the drug concentration. It is clear, however, from this and other experiments, that at all concentrations of HOU tested except the highest, survival reaches a concentration-dependent plateau. While the particular curves presented here and in Fig. 1 for 2.5 mM HOU do not exhibit plateaus, in experiments in which treatment was continued for longer than 8 hr, survival eventually reached a constant level at 5–10% of that of untreated irradiated cells. When relatively high concentrations of HOU (1.0, 2.5, and 5.0 mM) were tested, at which inhibition of DNA synthesis is almost complete (97.1, 98.5 and 99.2%, respectively, in one experiment), the rate of killing was found to be essentially the same, and no plateaus were evident during the 8 hr period of observation.

The relation between postirradiation cell killing and inhibition of DNA synthesis is shown in Fig. 4, in which survival after 8 hr of treatment is taken as a measure

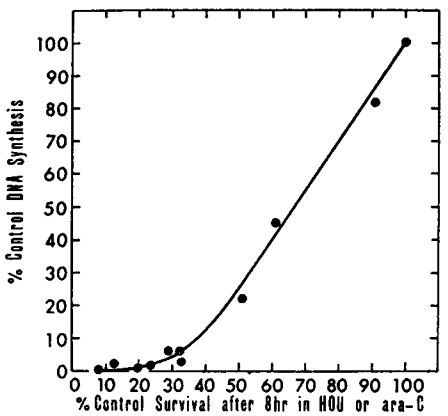

FIGURE 4 Relation between the enhancement of cell killing by HOU, and the level of inhibition of DNA synthesis. Survival after 8 hr of treatment with various concentrations of HOU is taken as a measure of the postirradiation enhancement effect. All the survival data are for cells irradiated with 400 rads in early G1.

of the postirradiation enhancement effect. Using this arbitrary measure of enhancement (which might not be entirely suitable at the higher concentrations), there appears to be a linear relation between inhibition and enhancement up to 95% inhibition. Moreover, the simultaneous addition of 2.5 mM HOU and the three nucleosides, AdR, CdR, and GdR, at concentrations established by Young et al. (1967) to be optimal for reversal of the inhibition, partially restores DNA synthesis and also eliminates the enhancement of cell killing produced by HOU (Table I).

Postirradiation enhancement of cell killing by ara-C is similarly dependent on drug concentration. Furthermore, supplementation of cultures containing 4 μM ara-C with 0.5 mM deoxycytidine monophosphate (dCMP), which partially restores DNA synthesis (Chu and Fischer, 1962; Kim and Eidinoff, 1964), abolishes postirradiation enhancement (Table I). It would appear, therefore, that inhibition of DNA synthesis is intimately related to the postirradiation enhancement effect. However, the quantitative discrepancies between the two phenomena that are apparent

when different inhibitors are used (Fig. 1) indicate that the relation is not simply one of cause and effect (see Discussion).

Dependence of the Rate of Postirradiation Killing on X-Ray Dose

Fig. 5 shows that the rate of cell killing in the presence of 2.5 mM HOU is strongly dependent on the x-ray dose. The decline in survival seems to be exponential at all doses except 650 rads, and at no dose except the latter do the curves plateau during 8 hr of treatment. (The significance of the plateau in the 650 rad curve is in doubt because of the small number of surviving cells scored. No plateau was observed

TABLE I

EFFECT OF REVERSAL OF HYDROXYUREA (HOU) AND CYTOSINE ARABINOSIDE (ARA-C) INHIBITION OF DNA SYNTHESIS ON POSTIRRADIATION KILLING OF EARLY G1 CELLS

Postirradiation treatment	Inhibition of DNA synthesis	Fractional survival (8 hr post-irradiation)
	%	
None	0	0.081
AdR + CdR + GdR*	46	0.136
HOU (2.5 mM)	99	0.023
HOU + AdR + CdR + GdR	88‡	0.146
None	0	0.117
dCMP (0.5 mM)	41	0.128
ARA-C (4 μM)	97	0.016
ARA-C + dCMP	77‡	0.117

* The concentrations of the nucleosides used were 0.1 mM AdR, 0.1 mM GdR (deoxyguanosine) and 1.0 μM CdR (deoxycytidine).

‡ If correction is made for the inhibition of ^{14}CTdR incorporation arising from the addition of AdR, CdR, and GdR alone, the inhibition of DNA synthesis by HOU in the presence of the 3 nucleosides is reduced to 42%. If a similar correction is made in the case of ara-C + dCMP, the inhibition is reduced to 36%.

during 8 hr in other experiments at this dose.) Moreover, in an experiment in which a dose of 300 rads was applied, no plateau was detected when treatment was prolonged to 17 hr; at that time survival was reduced by about 90%. Failure to detect a plateau does not, of course, mean that survival does not eventually reach some constant level. However, it might have been expected that at the lower doses, where less damage is sustained, the plateaus would become evident at earlier times and/or at higher survival levels, and therefore be detectable within the 8 hr observation period. These results are not easily reconciled with a radiation-dependent limitation on the extent of enhancement.

The slopes (t_0, the inverse of the first order rate constant) measured in this and

other experiments are plotted as a function of dose in Fig. 6. The dependence of rate on dose is certainly not linear, but the data do not permit characterization of the shape of the curve; it might be exponential.

Data like those of Fig. 5 may also be depicted as a series of conventional dose-survival curves measured after increasing times of treatment with HOU. In this form (Fig. 7), a progressive decrease in mean lethal dose from 110 rads immediately after irradiation, to 68 rads after 8 hr of treatment and 62 rads after 12 hr can be discerned. The extrapolation number does not seem to change greatly. If the shoulder on the dose-survival curve is taken to indicate the accumulation of sublethal damage (Elkind and Sutton, 1960), these results indicate that HOU does not interact with this kind of damage to bring about cell death. Were sublethal damage involved, it

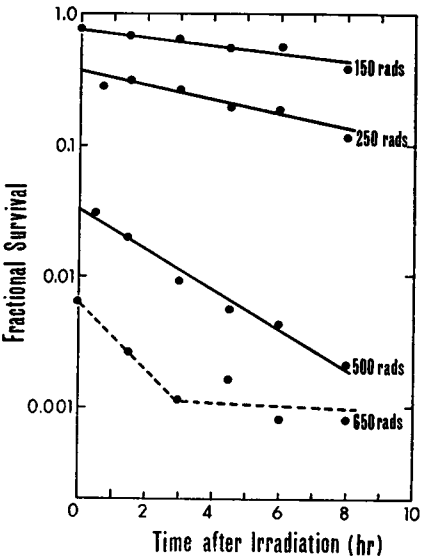

FIGURE 5 Dependence of the rate of postirradiation killing induced by HOU on x-ray dose. HOU (2.5 mM) was added to harvested cells 3 hr after plating. Irradiation at 150, 250, 500, or 650 rads was carried out immediately after drug addition. Each point represents survival at the time of reversal of the inhibition. The dashed line for 650 rads indicates uncertainty as to the shape of the curve.

would be expected that the rate of killing would be independent of dose, since at all but the lowest doses applied, survival falls within the exponential region of the survival curve, where all surviving cells should have sustained the same amount of sublethal damage.

Delayed Postirradiation Addition of HOU

Phillips and Tolmach (1966) reported that x-irradiated HeLa S3 cells become increasingly refractory to a 5 hr treatment with HOU, with increasing delay in addition of the drug following irradiation. This result agreed well with the hypothesis that potentially lethal damage is inflicted by x-irradiation (see Introduction). The data indicated that all potentially lethal damage is either repaired or expressed by 5 hr after irradiation. However, extension of the period of observation has now shown

that this is not the case: HOU has the capacity to kill cells surviving x-ray exposure when added as late as 12 hr after irradiation.

Fig. 8 shows that development of the effect can be observed, however, only if the cells are exposed to the drug for increased periods of time. In these experiments, HOU was added at times up to 12 hr after irradiation, and in the latter case killing was initiated only 5 hr after drug addition, i.e., about 17 hr after irradiation. Thus,

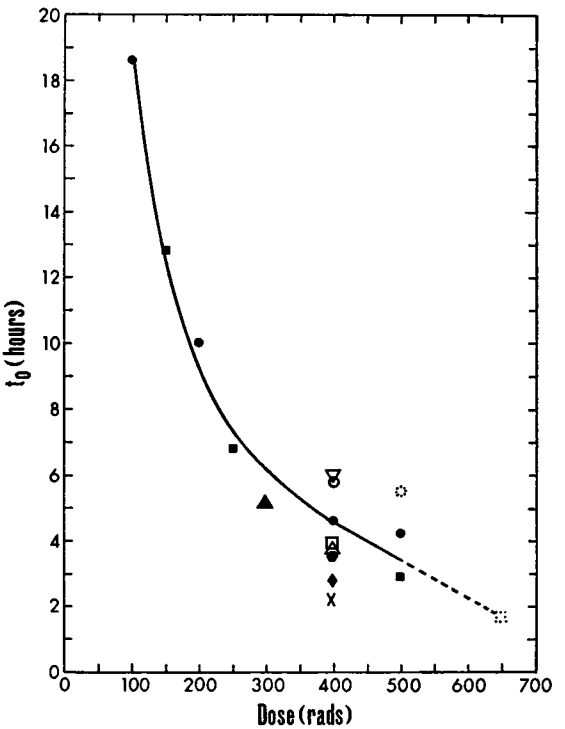

FIGURE 6 The slopes (t_0) of the curves appearing in Fig. 5 (and those calculated from several additional experiments) plotted as a function of x-ray dose. The parameter t_0 represents the time required to reduce survival to 37% of its value immediately after irradiation. Each symbol refers to a separate experiment. The dotted square at 650 rads is derived from the initial portion of the corresponding curve in Fig. 5. The dotted circle at 500 rads represents an alternative estimate of t_0, to that shown by the solid circle.

a delay in addition of HOU produces a roughly proportional lag in the inception of killing. This property of delayed expression explains the apparent decay of susceptibility to HOU as recorded by Phillips and Tolmach (1966). Fig. 9 depicts the relation between delay in HOU addition and the lag in expression of the cell killing effect, as determined from estimates of the intersection of the descending portions of the killing curves with the horizontal line representing the level of survival of untreated irradiated cells. The lag seems to be a direct function of delay, but it is not

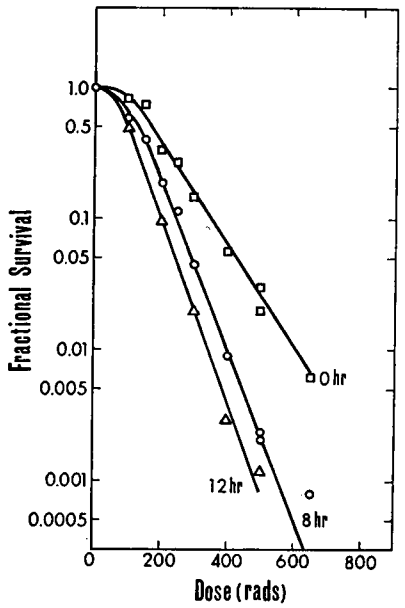

FIGURE 7 Dose-survival curves constructed from data of three experiments similar to and including that of Fig. 5, for untreated irradiated G1 cells (squares) and cells exposed to HOU (2.5 mM) for 8 hr (circles) or 12 hr (triangles).

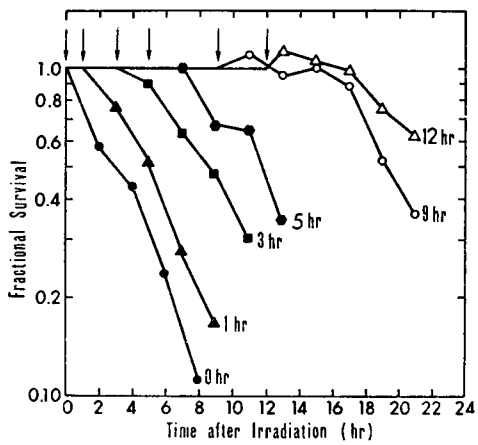

FIGURE 8 Delayed postirradiation addition of HOU. The cells were irradiated with 400 rads 3 hr after harvesting mitotic cells. HOU was added to each series of plates at 0 hr (closed circles), 1 hr (closed triangles), 3 hr (squares), 5 hr (hexagons), 9 hr (open circles), or 12 hr (open triangles) after irradiation (arrows). At the times indicated by each point, the inhibitor was removed. All survival values have been normalized so that the surviving fraction after irradiation alone is 1.0. The closed and open symbols represent two independent experiments in which survival after irradiation alone was 0.076 and 0.084, respectively.

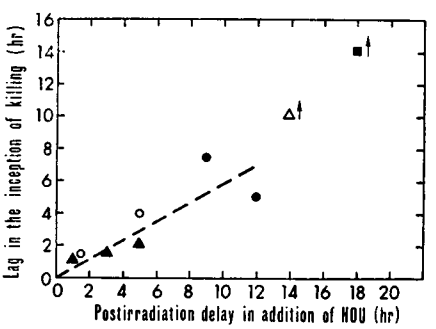

FIGURE 9 Relation between the delay in drug addition after irradiation, and the lag in the inception of cell killing produced by HOU. The estimation of the lag is discussed in the text. Each symbol refers to a separate experiment. some of the data from which the plotted values were obtained are presented in Fig. 8. The arrows indicate that the values for the lag after a 14 or 18 hr delay are minimal values; i.e., no killing by HOU was detected during the period of observation.

clear whether the dependence is linear with a proportionality factor of about 0.6, or if the slope increases. If it is linear, the effect would appear to cease after 12 hr. Similar results have been observed with ara-C.

DISCUSSION

All the compounds chosen for this study inhibit DNA synthesis; they also enhance the lethal effect of x-rays on HeLa S3 cells. They vary considerably, however, with respect to the latter property: at concentrations that inhibit DNA synthesis more than 95% (except for HA), FUdR, AdR, and TdR produce less than a twofold enhancement of killing after 8 hr of treatment, while HOU, ara-C, and HA produce a five- to twentyfold enhancement during the same period. This latter observation led us to examine more closely the relationship between inhibition of DNA synthesis and the enhancement of cell killing. All the results clearly indicate an intimate connection between the two phenomena. Thus, the enhancement of killing by HOU (and possibly ara-C) shows approximately the same concentration dependence as the inhibition of DNA synthesis (Fig. 4), and the enhancement produced by HOU or ara-C is eliminated upon partial reversal of their inhibition of DNA synthesis (Table I).

Although it would appear reasonable from these results to ascribe the action of these drugs on irradiated cells to their DNA inhibitory activity, i.e. to postulate that DNA synthesis normally occurs even during G1 in HeLa cells after x-irradiation and that such synthesis is concerned with the repair of potentially lethal damage (Phillips and Tolmach, 1966), unscheduled synthesis of DNA during G1 or G2 has been demonstrated only after exceedingly high doses of x-rays (Painter, 1967). Furthermore, the large quantitative differences among the inhibitors under conditions in which DNA synthesis is largely and equally (except for HA) inhibited speaks against the effect arising from inhibition of DNA synthesis per se. The particular modes of action of the drugs as inhibitors must play a large role. The way in which each of these compounds interferes with DNA synthesis is quite possibly unique, but only with FUdR is the mechanism of action reasonably well established; it irreversibly inhibits thymidylate synthetase, preventing the formation of thymidine monophosphate (Danneberg et al., 1958). The molecular effects of the other drugs are much less certain (Morris et al., 1963; Overgaard-Hansen and Klenow, 1961), and there is evidence that some of them may have multiple sites of action (Young et al., 1967; Silagi, 1965). Hence, it is not possible, at present, to attempt any correlation of the mode of action of these drugs as inhibitors with their activity in enhancing the killing effects of x-radiation. However, there would appear to be no obstacle to accommodation of these findings in the general model for potentially lethal damage.

HeLa cells in G1 seem to be killed more rapidly than S phase cells by postirradiation drug treatment. The preirradiation results of Phillips (1965) for cells in G1 and

our results for cells in S also indicate that the response to HOU is dependent on the cells' position in the generation cycle; early and mid-G1 cells are unaffected by preirradiation incubation with HOU, while cells in late G1 and S become sensitized to x-rays. Sinclair's findings with both unirradiated (1965, 1967 *a*) and irradiated (1967 *b*) Chinese hamster cells also indicate a phase-dependent response to HOU (in addition to its specific action on DNA synthesis). The significance of these phase-dependent responses is unknown.

Using Chinese hamster cells, Sinclair (1967 *b*) has recently shown that either pre- or postirradiation treatment of both G1 and S phase cells enhances cell killing by x-rays. The postirradiation effect is of a magnitude similar to that found in HeLa though it occurs more rapidly. Taking into account this cell line's very short G1 period, which probably is analogous to late G1 in HeLa cells, the two cell strains appear to respond in a remarkably similar fashion to treatment with HOU. Furthermore, Chinese hamster cells, like HeLa cells, appear to respond less to postirradiation treatment with TdR than with HOU (Sinclair, 1967 *b*).

This identity of behavior must be qualified somewhat since the preirradiation sensitization of Chinese hamster cells by HOU ceases after 4 hr and, in fact, is partially reversed upon continued incubation with the inhibitor (Sinclair, 1967 *b*), while the surviving fraction of HeLa cells continues to decline when inhibited cells are irradiated at progressively later times, for at least a 12 hr period following drug addition in early G1 (Phillips, 1965), or 8 hr following addition in S (Fig. 2).

The origin of the plateaus that are observed on prolonged treatment of irradiated cells with HOU (Fig. 3) remains obscure. Although in principle the plateaus could arise from contaminating cells from regions of the cycle which fail to respond to postirradiation treatment with HOU, e.g. parts of G2 or M, the survival level at the plateau (at 2.5 mM HOU, which completely blocks cell progression) would appear to require an amount of contamination greater than is calculated from measurement of the degree of synchronization (Terasima and Tolmach, 1963 *a*). Another possible cause of saturation of the effect—binding or modification of the HOU, or some other mode of detoxification of the inhibitory medium on prolonged incubation with cells—may also be dismissed in view of experiments which showed that medium containing either 2.5 or 0.25 mM HOU retains full inhibitory activity against DNA synthesis after 8 hr of preincubation with a similar number of HeLa cells (Table II). Alternatively, the extent of postirradiation enhancement could be limited by the amount of damage inflicted by the radiation, or by other factors. To test the former possibility, the rate of postirradiation killing was examined at a series of x-ray doses (Fig. 5). No plateaus were observed at even the lowest doses over an 8 hr period of treatment, but the rate of decline of survival was much slower than at higher doses, so that the plateau region may not have been reached during the period of observation. However, as mentioned above, when cells were irradiated with 300

rads and treated with HOU for 17 hr, no plateau was found even after 90% of the x-ray survivors were killed. This result suggests that the extent of enhancement of killing is not limited by the amount of damage sustained from irradiation, even though the rate of killing is dependent on this quantity.

The apparently exponential rate of cell killing, that is, the first order rate-limiting lethal interaction of HOU with the irradiated cell, might arise from any of a number of hypothetical processes. One such process, the passage of HOU through radiation-produced holes in a cellular membrane, is discussed below and is rejected. Another possibility might be the reaction of a radiation-produced substance with HOU. Such a substance need not be limited to small molecules; it could include activated sites of a macromolecule, such as polynucleotide chain ends exposed by single-strand breaks in DNA (Freifelder, 1965; McGrath and Williams, 1966).

TABLE II

EFFECT OF PREINCUBATION OF HOU-CONTAINING MEDIUM ON ITS INHIBITORY ACTIVITY

Concentration of HOU	Inhibition of DNA synthesis*	
	Fresh medium	Preincubated medium‡
mM	%	%
0	0	0
0.25	89	88
2.5	97	97

* Inhibition was measured by pulse labeling with ^{14}CTdR 2 hr after adding the medium to test cells.

‡ Medium containing HOU at the concentrations specified was preincubated for 8 hr with about 10^4 randomly dividing cells.

It is probable that the damage with which HOU interacts when it is added immediately after irradiation is closely related to the damage that remains susceptible to expression on treatment with HOU when the addition of the drug is delayed for an extended period after irradiation (Fig. 8). At present the cause of the lag in expression which accompanies the delayed addition of the drug, as well as the nature of the damage itself, remains unknown. One possibility—that the delay reflects a decrease in the heightened permeability of a cell membrane brought about by the irradiation (assuming that the dependence of killing on the HOU concentration within the nucleus is a threshold phenomenon)—would appear to be untenable for the following reasons: (*a*) DNA synthesis in unirradiated cells is immediately inhibited on addition of HOU, and the effect is rapidly reversed when the drug is removed (Pfeiffer and Tolmach, 1967); (*b*) the dependence of DNA synthesis on HOU concentration is not altered by x-irradiation (Table III).

It may be noted that loss of viability on treatment with HOU after irradiation with the lower x-ray doses (Fig. 5) reveals the presence of potentially lethal damage in a large fraction of the original cell population. For example, 40% of the starting population is killed by 8 hr of treatment with 2.5 mM HOU following a dose of 150 rads. Whatever the nature of the potentially lethal damage, the present findings show that it persists for at least 12 hr (or 17 hr, if we assume that it does not interact with the HOU until after the lag period). However, the progressive development of a lag in response with increasing delay in the initiation of treatment indicates that, in the context of the model for potentially lethal damage (Phillips and Tolmach, 1966), some change occurs in the nature of that damage, or in its ability to interact with HOU. Attempts to study that change by determining whether irradiated cells remain

TABLE III

EFFECT OF X-IRRADIATION ON THE INHIBITION OF DNA SYNTHESIS BY HOU (0.05 mM)

Treatment	Inhibition of DNA synthesis
	%
None	0
400 rads	25
HOU (0.05 mM)	23*
400 rads + HOU (0.05 mM)	25‡

Random cultures were exposed to 400 rads approximately 27 hr after plating. HOU was added just prior to irradiation. ^{14}CTdR was added to all plates immediately after irradiation. Cells were fixed at hourly intervals after treatment. The per cent inhibition of DNA synthesis was calculated from the slopes of the incorporation curves.

* The per cent inhibition of DNA synthesis was calculated with respect to the unirradiated control.

‡ The per cent inhibition of DNA synthesis was calculated with respect to the irradiated control.

susceptible to killing by HOU for even longer times have been frustrated by the onset of toxity to unirradiated cells after 18–20 hr incubation with HOU.

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Note Added in Proof. A recent paper of Elkind et al. (1967), concerning the relation between sublethal damage and potentially lethal damage (they refer to the latter as lethal damage) in Chinese hamster cells, presents the result of an experiment involving postirradiation treatment with actinomycin D that is at least superficially similar to the one reported here (Fig. 8) with HOU. However, actinomycin D is toxic to Chinese hamster cells, and the death of the radiation survivors that develops only slowly when initiation of drug treatment is delayed more than 6 hr is attributed by those workers to such toxicity. They interpret their results as indicating that irradiated cells become progressively less susceptible to the potentiating action of actinomycin D, the half-time for the loss of susceptibility being about 1.5 hr (cf. Phillips and Tolmach, 1966, Fig. 6), and hence that the time for repair of potentially lethal damage is close to that for repair of sublethal damage. They report also that the slope of the survival curve is changed only slightly by postirradiation treatment with actinomycin D, while the shoulder is reduced. They suggest that in S phase Chinese hamster cells, sublethal damage is qualitatively the same as potentially lethal damage. From the result presented here for HOU treatment of irradiated HeLa S3 cells (Fig. 7), we suggest the opposite (see also Phillips, 1965, pp. 61–68; Phillips and Tolmach, 1966, pp. 429–430).