Hydroxyurea: Differential Lethal Effects on Cultured Mammalian Cells during the Cell Cycle

Abstract. *Hydroxyurea has a differential lethal effect on cultured Chinese-hamster cells that are at different stages in their cell cycle. Cells synthesizing DNA at the time of exposure to the drug are lethally damaged. Cells in the phase of growth preceding DNA synthesis (G_1) survive but are prevented from beginning DNA synthesis. Cells in the phase after DNA synthesis (G_2) survive and appear to progress until just before the beginning of the next period of DNA synthesis. This differential lethal and inhibitory effect of hydroxyurea may be useful for synchronizing asynchronous cell populations and explaining effects of the drug in human therapy.*

Hydroxyurea (*1*) has been under study as a chemotherapeutic agent (*2*) for cancer, and as a result considerable interest in its mode of action, both in vivo and in vitro, has developed. Several facts and suggestions concerning its behavior have been reported (*3*). Young and Hodas (*4*) found, in mammalian (HeLa) cells in culture, that hydroxyurea inhibited incorporation of thymidine into DNA and suggested that its action was a result of interference with ribonucleotide (diphosphate) reduction. This report is concerned with the use of hydroxyurea as an inhibitor of DNA synthesis in mammalian cells in culture and its lethal effects on these cells at different stages of their generation cycle.

Asynchronous cultures of Chinese-hamster lung cells (V79 line) (*5*) were grown in log phase on plastic petri dishes with EM-15 medium (*6*). Cultures were maintained at 37°C in a humid atmosphere of 2-percent CO_2 and air. Hydroxyurea (*7*) was prepared just before use; concentrations to yield $10^{-3}M$, $10^{-4}M$, and $10^{-5}M$ were each added to dishes of these cultures for ½, 3, and 6 hours. The medium containing the drug was then removed, the cultures were rinsed once with fresh medium, and the dishes were refilled with fresh medium. In one series, cells were "pulse-labeled" with tritiated thymidine (3 c/mmole, 0.3 μc/ml) for 15 minutes both during and after treatment with the drug. The plates were then fixed with acetic alcohol; liquid emulsion was added; and after exposure (2 weeks), development, and staining, the percentage of labeled cells was recorded (Table 1). In another group of plates, cells were detached from the plate and separated by using trypsin, and the resulting suspension was diluted and plated as single cells. Survival (reproductive integrity) was determined by scoring visible colonies after an incubation period of 10 days (Table 2). When no hydroxyurea was added the survival of cells, that is, the plating efficiency, was about 50 percent. At a concentration of $10^{-5}M$, hydroxyurea had essentially no effect either on the number of cells synthesizing DNA or on survival. At a concentration of $10^{-4}M$, hydroxyurea did not affect survival and caused only temporary partial reduction in the number of cells synthesizing DNA. At $10^{-3}M$, however, hydroxyurea completely inhibited cells from incorporating thymidine, the inhibition beginning as soon as ½ hour after exposure and continuing during 6 hours of exposure. Shortly after the drug was removed, the number of cells synthesizing DNA increased (Table 1, lower two columns). Survival was affected equally after 3 or 6 hours of exposure to $10^{-3}M$ hydroxyurea; about 60 percent of the cells that potentially could produce colonies failed to do so. These facts suggested that perhaps only cells actually synthesizing DNA at the time of exposure to hydroxyurea ($10^{-3}M$) were lethally damaged.

Experiments were then conducted with a synchronized population of Chinese-hamster cells, obtained by selecting cells at or near division (*8*). These populations were grown in the same medium and under the same conditions as the asynchronous cells. Hydroxyurea in amounts to yield 10^{-3} and $5 \times 10^{-4}M$ was added to groups of plates of synchronized cells at various times after inoculation. It was re-

Table 1. Percentage of Chinese-hamster cells pulse-labeled with tritiated thymidine during and after treatment with hydroxyurea.

Concentration of hydroxyurea (molar)	Percentage of cells pulse-labeled after exposure of			
	0.0 hours	0.5 hours	3.0 hours	6.0 hours
During treatment				
10^{-3}		2	1	2
10^{-4}		27	57	84
10^{-5}		55	70	73
None	74	74	64	74
One hour after removal				
10^{-3}			81	85
10^{-4}			88	90
10^{-5}			60	70
None			66	74

Table 2. Survival of Chinese-hamster cells after treatment with hydroxyurea.

Concentration of hydroxyurea (molar)	Percentage survival	
	3 hours of treatment	6 hours of treatment
10^{-3}	19	21
10^{-4}	51	48
10^{-5}	51	53
None	51	54

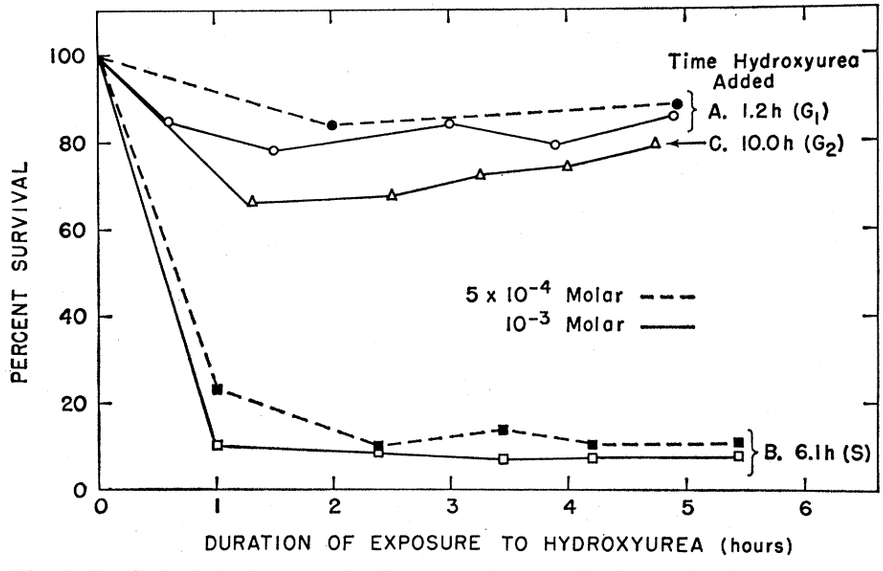

Fig. 1. Survival of G_1 (curves *A*), S (curves *B*), and G_2 (curve *C*) Chinese-hamster cells in culture after different lengths of exposure to hydroxyurea. Survival is relative to that of untreated cells; plating efficiency about 50 percent.

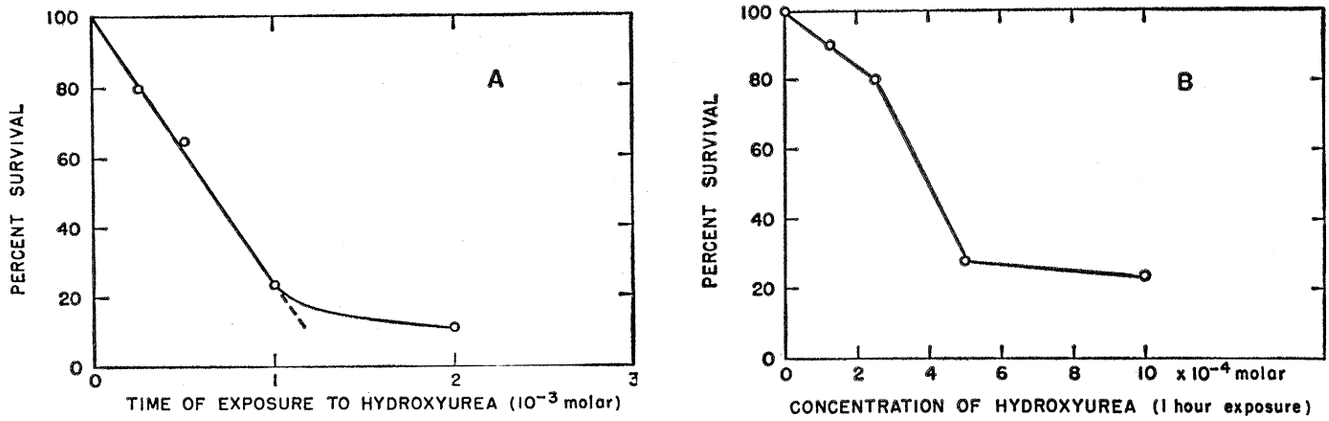

Fig. 2. Survival of Chinese-hamster *S* cells in culture after (*A*) different lengths of exposure to hydroxyurea ($10^{-3}M$) and (*B*) different concentrations of hydroxyurea for 1 hour. Survival is relative to that of untreated cells; plating efficiency about 60 percent.

moved after different intervals, by the method used earlier. When the cells had been incubated at 37°C for 1.2 hours after inoculation, most were in phase G_1, but about 15 percent of the cells were labeled by a 15-minute pulse of tritiated thymidine. The survival of these cells (Fig. 1, curves *A*) was lowered by about 15 to 20 percent after a 1-hour exposure to both concentrations of hydroxyurea. No more cells were killed by longer exposure to the drug. After incubation at 37°C for 6.1 hours after inoculation, about 90 percent of the cells incorporated tritiated thymidine and survival was lowered by about 90 percent with both concentrations of drug after about a 1-hour exposure (Fig. 1, curves *B*). Longer exposure did not reduce survival further.

In a later experiment (Fig. 1, curve *C*), $10^{-3}M$ hydroxyurea was added to synchronized cells 10 hours after inoculation, when many of the cells had reached the G_2 phase. About 35 percent of the cells were labeled with tritiated thymidine at this time, and survival was decreased by the same amount. In curve *C*, survival appears to increase with duration of exposure to the drug. This may be due to the fact that, in the process of removing the drug, some dividing cells, which are poorly attached, may become dislodged and be lost, while others reattach to form additional colonies. If this is so, the increase in survival is apparent rather than real, but it indicates that cells in the G_2 phase probably continue to grow (and thus divide) in the presence of hydroxyurea and presumably continue until they reach the beginning of the next synthesizing (*S*) period. Furthermore, hydroxyurea probably has no serious effects on dividing cells.

These experiments demonstrate clearly the selective, lethal action of hydroxyurea on cells synthesizing DNA. After an exposure of 1 hour or more to 5×10^{-4} or $10^{-3}M$ hydroxyurea, the proliferative capacity of cells synthesizing DNA is very effectively destroyed. Cells not synthesizing DNA, whether in G_1 or G_2, are not lethally damaged in 1 hour by either of these concentrations of hydroxyurea, nor by exposure to the drug for more than 5 hours. Such cells are, however, effectively blocked from entering DNA synthesis. This point will become more evident later.

The lethal effect of hydroxyurea on *S* cells was examined in synchronized cells incubated at 37°C for 6.0 hours after inoculation. Eighty-eight percent of the cells were labeled with tritiated thymidine at this time. The survival of cells after exposure to $10^{-3}M$ hydroxyurea declined almost linearly with time for up to about 1 hour (Fig. 2*A*). For 1-hour exposures, a concentration of $5 \times 10^{-4}M$ or greater was required to prevent *S* cells from proliferating (Fig. 2*B*). Examination, by methods other than cloning, of cell populations after hydroxyurea treatment indicates that *S* cells rendered nonproliferative by the drug nevertheless remain in the population for many hours.

The inhibitory effect of hydroxyurea on G_1 cells is shown in Fig. 3, in which

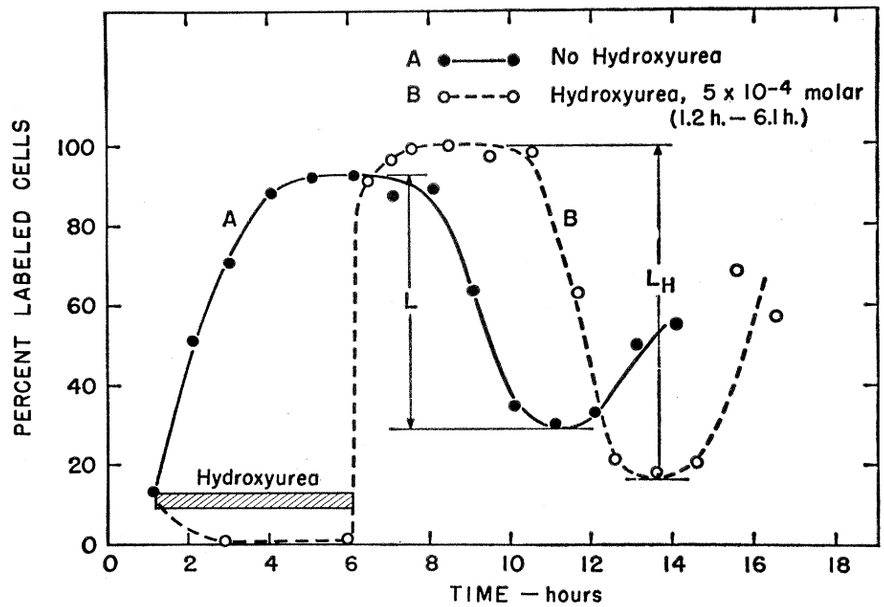

Fig. 3. Percentage of synchronized Chinese-hamster cells pulse-labeled with tritiated thymidine (15 min, 0.3 μc/ml, 3 c/m*M*) with time. Curve *A*, no hydroxyurea treatment; curve *B*, hydroxyurea, $5 \times 10^{-4}M$ for 5 hours starting at 1.2 hours (G_1 cells).

the progress of synchronized cells treated in G_1 with hydroxyurea for 5 hours (curve B) is compared with that of untreated cells (curve A). Passage of the treated cells into DNA synthesis was suppressed until the drug was removed. Almost immediately thereafter the percentage of cells labeled with tritiated thymidine rose to 100 percent. Thus even cells synthesizing DNA at the time hydroxyurea was added (and therefore destined not to form colonies) incorporated thymidine again when the drug was removed.

The data of Fig. 3 show clearly that the degree of synchrony in the cell population is markedly improved after hydroxyurea has been added and removed. Quantitatively, the labeling index of synchrony proposed by Sinclair and Morton (*8*) and indicated on curve A by L and curve B by L_H is improved from ~55 percent to ~83 percent. The spread of the S period is reduced from about 7½ hours to 6 hours.

These results indicate that hydroxyurea is likely to be an effective synchronizing agent for asynchronous populations, since the proliferative capacity of S cells can be destroyed while the remaining cells (about 40 percent in the case of hamster cells and higher percentages for cell lines such as HeLa and L, which have longer G_1 periods) merely pile up at the end of G_1. Thus, it is reasonable to expect better synchrony than is obtainable with inhibitors such as 5-fluorodeoxyuridine (FUDR) or amethopterin (*9*), which block S cells but may permit them to survive. The use of hydroxyurea may also have advantages over methods involving "suicide" with lethal amounts of tritiated thymidine (*10*). In the latter, S cells are killed but there is no inhibitory action resulting in accumulation of cells. Consequently, a relatively small portion of the population can be obtained in synchronized form. Any inhibitory drug such as hydroxyurea, however, has the inherent disadvantage that properties of surviving cells other than their proliferative capacity may be affected; the response of treated cells to other agents (such as x-rays) may not be the same as that of the untreated population. Experiments are in progress to test the possibilities of hydroxyurea as a synchronizing agent in asynchronous cells.

The lethal effect of hydroxyurea on S cells in vitro presumably has an important bearing on the behavior of the drug in vivo and may help to explain its antitumor action and the clinical complications of treatment in humans.

Furthermore, this selective action offers the prospect of damaging proliferative cells even more effectively by combining hydroxyurea with another agent [for example, in the case of hamster cells, perhaps with x-rays (*8*)] selectively effective in damaging cells at other stages of the generation cycle.

WARREN K. SINCLAIR
Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois

References and Notes

1. W. F. C. Dresler and R. Stein, *Ann. Chim.* **150**, 242 (1869).
2. W. G. Thurman, C. Bloedow, C. D. Howe, W. C. Levin, P. Davis, M. Lane, M. P. Sullivan, K. M. Griffith, *Cancer Chemotherapy Rept.* **29**, 103 (1963); and a group of papers by various authors in *Cancer Chemotherapy Rept.* **40**, 1–75 (1964).
3. W. N. Fishbein and P. P. Carbone, *Science* **142**, 1069 (1963); A. Bendich, E. Borenfreund, G. C. Korngold, M. Krim, *Federation Proc.* **22**, 582 (1963); E. Borenfreund, M. Krim, A. Bendich, *J. Natl. Cancer Inst.* **32**, 667 (1964); G. R. Gale, S. M. Kendall, H. H. McLain, S. DuBois, *Cancer Res.* **24**, 1012 (1964); H. S. Rosenkrantz and J. A. Levy, *Biochim. Biophys. Acta* **96**, 181 (1965); J. W. Yarbro, B. J. Kennedy, C. P. Barnum, *Proc. Natl. Acad. Sci. U.S.* **53**, 1033 (1965).
4. C. W. Young and S. Hodas, *Science* **146**, 1172 (1964).
5. W. K. Sinclair, *Radiation Res.* **21**, 584 (1964).
6. EM-15 medium is similar to HU-15 described by M. M. Elkind and H. Sutton, *ibid.* **13**, 556 (1960), but without NCTC 109.
7. Obtained from Miss Barbara Stearns, Squibb Institute for Medical Research. Now available also from Nutritional Biochemicals Corporation.
8. W. K. Sinclair and R. A. Morton, *Nature* **199**, 1158 (1963); ———, *Biophys. J.* **5**, 1 (1965).
9. R. R. Rueckert and G. C. Mueller, *Cancer Res.* **20**, 1584 (1960).
10. G. F. Whitmore, S. Gulyas, J. Botond, *Cellular Radiation Biology* (Williams and Wilkins, Baltimore, 1965), p. 423.
11. Work supported by the AEC. I acknowledge the assistance of R. A. Morton, Grace Racster, and C. R. Peri in these experiments.

7 October 1965

Visnagin: Biosynthesis and Isolation from Ammi visnagi Suspension Cultures

Abstract. *During an examination of* Ammi visnaga *Lam. suspension cultures for the biosynthesis of furanochromones and related medicinal compounds, visnagin was isolated in crystalline form and identified. Thus, certain medicinally important secondary plant metabolites may be produced in appreciable amounts by plant tissue cultures.*

A number of investigators have reported the biosynthesis of medicinal compounds by plant tissue cultures, but few have reported their isolation and identification (*1*). Among the medicinally important compounds produced by plant tissue cultures are substances similar to the digitalis glycosides from *Digitalis sp.* (*2*), nicotine from *Nicotiana tabacum* L. (*3*), tropane alkaloids from *Datura sp.* (*4*), reserpine from *Alstonia constricta* F. Muell. (*5*) and vinca alkaloids from *Catharanthus roseus* L. (*6*).

We now report the isolation and identification of the furanochromone, visnagin, from suspension cultures of *Ammi visnaga* Lam. (Umbelliferae). Furanochromones have a limited distribution in plants, being reported only in the genera *Ammi* (Umbelliferae) and *Eranthus* (Ranunculaceae) (*7*). Visnagin is present in the seeds of *A. visnaga* in a concentration of 0.045

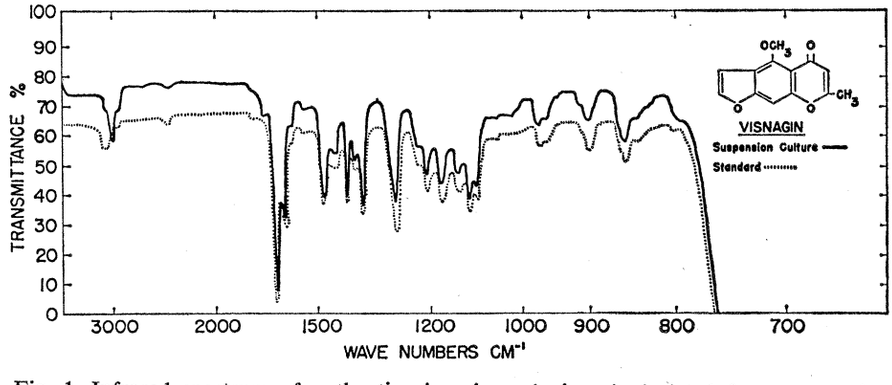

Fig. 1. Infrared spectrum of authentic visnagin and visnagin isolated from suspension cultures of *Ammi visnaga*.