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EFFECT OF HYDROXYUREA ON THE INCORPORATION OF THYMIDINE INTO EHRLICH ASCITES TUMOR CELLS*

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Abstract—The antitumor agent, hydroxyurea, was shown to reduce the rate of incorporation of thymidine-^{3}H into Ehrlich ascites tumor cells *in vitro*. A loss of previously incorporated thymidine-^{3}H was also observed. This loss was not accompanied by gross cellular disorganization. The action of hydroxyurea on the test system was found to differ from that of hydroxylamine.

IN AN earlier communication it was reported that the antitumor agent, hydroxyurea, inhibited the growth of *Pseudomonas* species.[1] Inhibition was accompanied by elongation of individual bacterial cells. Electron microscopy revealed that the nuclear material of treated cells was diminished or coalesced into electron dense particles. Alterations of the nucleic acid and ribonucleotide patterns were noted, the most consistent being a reduction of the DNA/RNA ratio. It was consequently suggested that the drug inhibits cell division through interference with DNA metabolism. No evidence was found that the effects on *Ps. aeruginosa* were mediated through the same mechanism proposed by Fishbein and Carbone,[2] who found acetohydroxamic acid in the sera of patients undergoing therapy with hydroxyurea. They concluded that the acetohydroxamic acid was one of the products of cleavage of acetyl CoA by hydroxylamine which was formed from hydroxyurea. Mohler[3] has recently demonstrated a reversal by thymidine of part of the growth-inhibiting and cytocidal effects of hydroxyurea on a Chinese hamster cell line *in vitro*; however, a similar phenomenon could not be shown with HeLa S3 cells as the test system.

To obtain more direct evidence of the extent to which DNA synthesis may be inhibited by hydroxyurea, the effects of the drug on incorporation of tritiated thymidine into ascites tumor cells have been studied. The following is a report of this work.

EXPERIMENTAL

Thymidine-methyl-^{3}H, with a specific activity of 6·70 c/mmole (New England Nuclear Corp.), was dissolved in saline so that 0·1 ml contained 5·0 μc. Aliquots were stored at −20° until needed. Hydroxyurea (m.p. 145–146°) was synthesized by Hynes Chemical Research Corp., Durham, N.C. Elemental analysis showed: C, 15·95%;

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H, 5·43%; N, 36·62%. Calculated for $CH_4N_2O_2$: C, 15·79%; H, 5·29%; N, 36·84%. Solutions of the drug were prepared daily in saline. Hydroxylamine hydrochloride (Fisher certified reagent) was dissolved in saline and adjusted to pH 7·4 immediately prior to use. Medium NCTC-109 was obtained from Microbiological Associates. The Ehrlich ascites tumor was originally provided by Dr. E. C. Horn, Duke University, and was maintained in Swiss mice purchased from Foster D. Snell, Inc., Baltimore Laboratories Division.

For studies of thymidine incorporation, ascites cells were withdrawn from mice 13 to 21 days after i.p. injections of the tumor. The cells were immediately centrifuged, the supernatant solution was removed, and the cell pellet was resuspended in 9 volumes of NCTC-109. This washing was repeated once, and the cells were finally suspended in 19 volumes of NCTC-109. In the initial experiments unstoppered conical centrifuge tubes containing 0·9 ml of cell suspension and 0·1 ml of hydroxyurea or saline were incubated with gentle agitation for 30 min at 37° prior to the addition of 0·1 ml of labeled thymidine solution. Hydrogen ion concentration remained virtually unchanged as judged by the indicator in NCTC-109. In the later experiments the labeled thymidine and hydroxyurea or saline were added to the cell suspension simultaneously. The tubes were incubated at 37° and at the indicated times metabolism was stopped by the addition of 1·0 ml of cold 3% trichloroacetic acid. The killed suspension was then centrifuged, the supernatant solution discarded, and the pellet washed three times with water. After the final washing the water was removed, and each cell pellet was suspended in 0·5 ml methanol. Two ml of 1·0 M Hyamine hydroxide was added to each suspension, and the mixture was allowed to stand overnight at room temperature to effect solution. This was then transferred quantitatively to a vial containing 10 ml phosphor solution, and the radioactivity was measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.).

Oxygen uptake of tumor cells was measured after washing the cells twice with Krebs-Ringer phosphate buffer, pH 7·4, and resuspending them 10% (v/v) in buffer. Each Warburg vessel contained 1·0 ml of cell suspension and 1·0 ml of glucose (22 μmoles/ml). Hydroxyurea solution or buffer (0·2 ml) was tipped in from the side arm, and the center well contained 0·2 ml 20% KOH. Measurements were made at 37° in air.

Specimens for electron microscopy were obtained by fixing the ascites tumor cells for 30 min in cold, veronal-acetate-buffered 1% OsO_4 containing 4·5% sucrose. The small clumps of cells were cut into small blocks and transferred to 1% uranyl acetate in 70% alcohol for 1 hr. After dehydration in a graded alcohol series the cells were embedded in Maraglas (Marblette Corp., Long Island, N.Y.). Sections were cut on a Porter Blum microtome (Ivan Sorvall, Inc.) and examined with an RCA EMU3-F electron microscope (50 kV accelerating voltage through a 25-μ objective aperture).

RESULTS

Figure 1 shows the effects of three concentrations of hydroxyurea on incorporation of thymidine into the ascites tumor cells. In this experiment the drug was added to the cell suspension 30 min before addition of thymidine. For the first 30 min after addition of thymidine, incorporation occurred but at a reduced rate. After this there was an actual loss of label upon further incubation. When thymidine and hydroxyurea

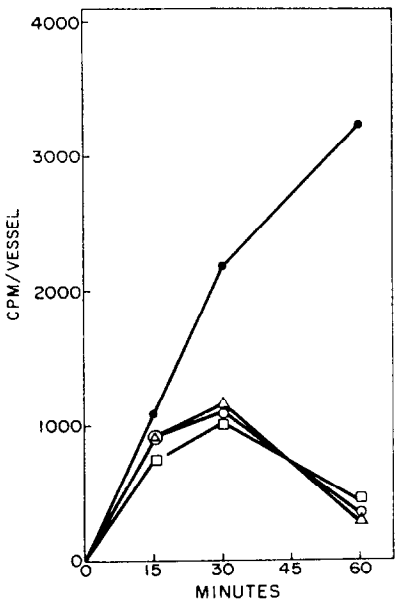

FIG. 1. Effect of hydroxyurea on incorporation of thymidine-^{3}H into Ehrlich ascites tumor cells. Hydroxyurea was added to the reaction vessels 30 min prior to the addition of thymidine-^{3}H; ●, ○, □, and △ represent 0, $1{\cdot}2 \times 10^{-4}$, $1{\cdot}2 \times 10^{-3}$, and $1{\cdot}2 \times 10^{-2}$ M hydroxyurea respectively.

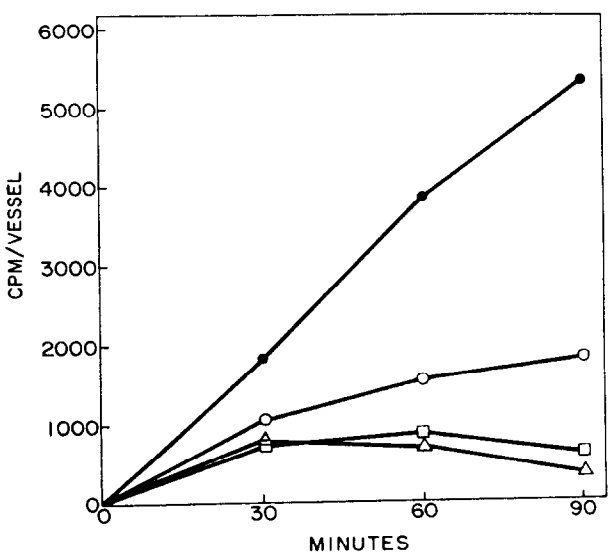

FIG. 2. Same conditions and symbols as in Fig. 1, except that hydroxyurea and thymidine-^{3}H were added simultaneously at zero time.

were added simultaneously, inhibition was also evident; however, the total amount incorporated in the presence of the drug was generally less, and the loss of label was not as marked (Fig. 2). Per cent inhibition by hydroxyurea did not vary a great deal over the 100-fold range of concentrations used.

To determine if the inhibitory pattern seen with hydroxyurea resembled that obtained with hydroxylamine, the latter was substituted for hydroxyurea at equimolar concentrations. Figure 3 shows three principal differences between the two compounds: (a) per cent inhibition obtained with $1{\cdot}2 \times 10^{-4}$ M hydroxylamine was much less than that obtained with the same concentration of hydroxyurea; (b) per cent inhibition was quite definitely a function of hydroxylamine concentration over the range tested; and (c) loss of label was not observed within the 90 min experimental period.

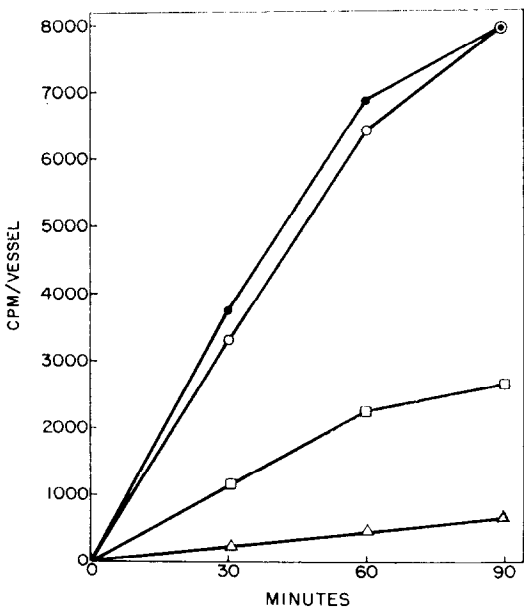

FIG. 3. Effect of hydroxylamine on incorporation of thymidine-^{3}H into Ehrlich ascites tumor cells. Hydroxylamine and thymidine-^{3}H were added simultaneously at zero time. Symbols represent same concentrations as in Figs. 1 and 2.

To determine if the loss of label in the presence of hydroxyurea was due to gross disruption and disintegration of the tumor cells, the capacity of the cells to consume oxygen in the presence of the drug was measured. Figure 4 shows no effect at a final hydroxyurea concentration of $1{\cdot}2 \times 10^{-2}$ M; lower concentrations gave similar curves.

A more direct estimate of the integrity of the drug-treated cells was obtained by electron microscopy. Figure 5 shows an ascites tumor cell fixed in OsO_4 immediately after removal from the mouse. This cell shows many of the characteristics of mouse sarcoma 37 cells described by Epstein.[4] A large nucleus with prominent nucleolus and homogeneous nucleoplasm was surrounded by cytoplasm of relatively low electron density. Mitochondria with sparse cristae and a matrix of low electron density were numerous. Smooth-surfaced vesicles and ribosomes constituted most of the cytoplasmic contents; lipid inclusions were seen occasionally. The surface of the cell displayed numerous projections. Cells which had been washed with NCTC-109 and incubated at 37° for 90 min prior to fixation, as for thymidine incorporation studies, revealed generally poorer preservation of the nuclei; cytoplasm of these cells was indistinguish-

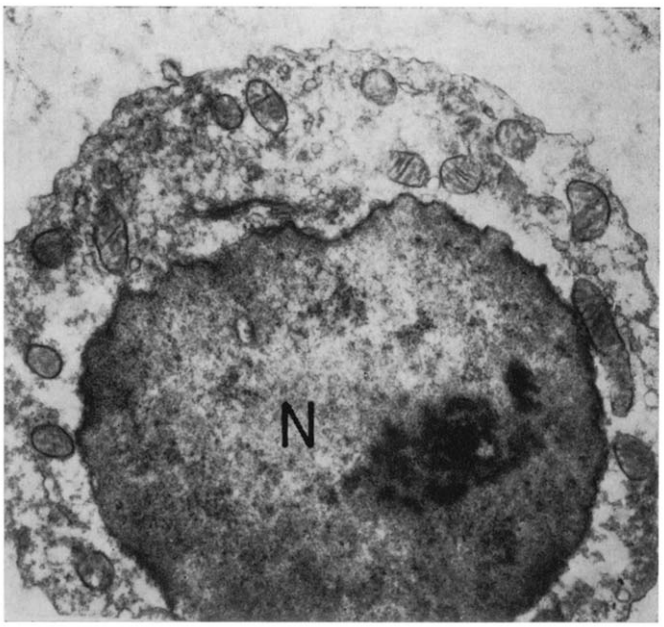

FIG. 5. Electron micrograph of Ehrlich ascites tumor cell fixed in OsO_4 immediately after removal from the mouse; 13,000.

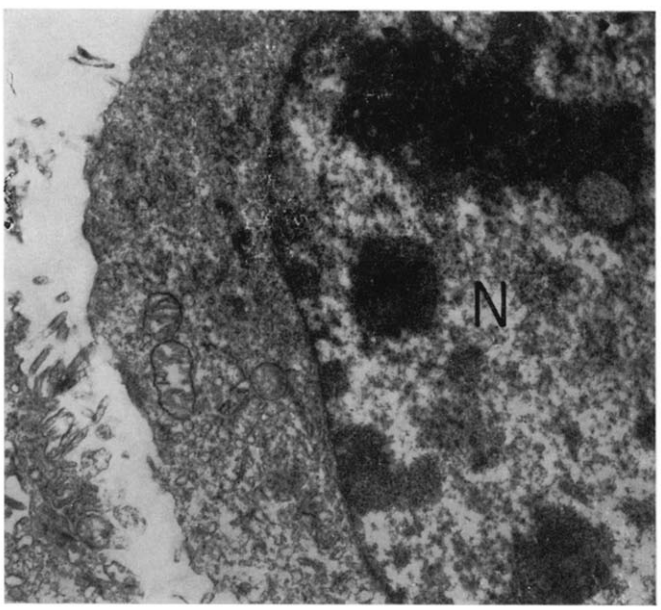

FIG. 6. Electron micrograph of Ehrlich ascites tumor cell fixed in OsO_4 after washing with NCTC-109 and incubating for 90 min in this medium; 13,000.

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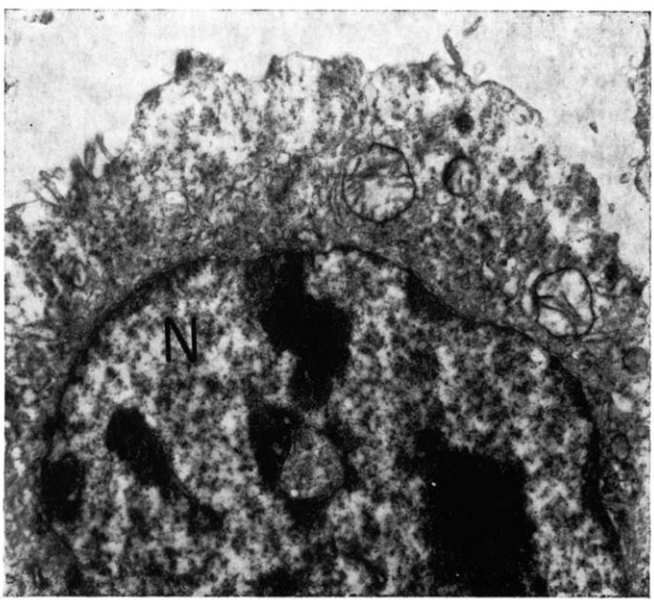

FIG. 7. Electron micrograph of Ehrlich ascites tumor cell fixed in OsO_4 after washing with NCTC-109 and incubating for 90 min in this medium containing $1{\cdot}2 \times 10^{-2}$ M hydroxyurea; × 13,000.

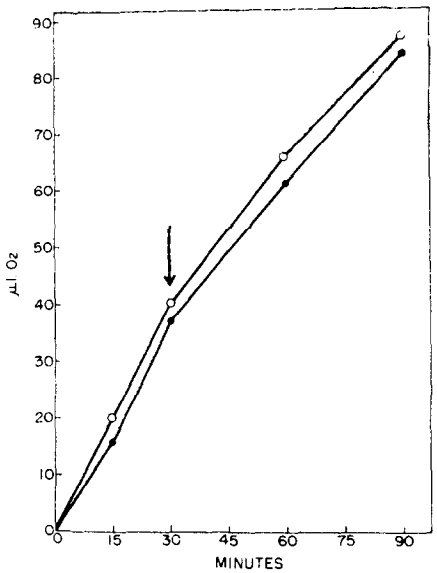

FIG. 4. Insensitivity to hydroxyurea of glucose oxidation by Ehrlich ascites tumor cells. Buffer was added from the side arm of the control vessel and hydroxyurea solution from that of the experimental vessel at the time indicated by the arrow; ●, control; ○, $1{\cdot}2 \times 10^{-2}$ M hydroxyurea.

able from that of freshly fixed cells. Nuclear changes consisted primarily of a loss of homogeneity of the nucleus (Fig. 6). Washed cells incubated under the same conditions, with the exception that hydroxyurea was added to a final concentration of $1{\cdot}2 \times 10^{-2}$ M, were indistinguishable from cells incubated without the drug (Fig. 7). No consistent differences from control cells were induced by the drug during this 90-min period, and no gross cellular disorganization was detected.

DISCUSSION

The data presented indicate that hydroxyurea, at concentrations equivalent to those obtained therapeutically *in vivo*,[5] reduces the rate of incorporation of thymidine into DNA of Ehrlich ascites tumor cells *in vitro*. Further, release of previously incorporated thymidine eventually occurs to a greater or lesser degree, a finding compatible with that of Bendich *et al.*,[6] who noted a marked drop in the viscosity and sedimentation coefficient of isolated mammalian and phage DNA in the presence of hydroxyurea. This release of thymidine is apparently not strictly a function of time of exposure of cells to the drug. In those experiments in which this phenomenon was best demonstrated (Fig. 1), the cells had been preincubated with hydroxyurea for 30 min prior to addition of thymidine-^{3}H; an uptake followed by a marked loss of label was shown 60 min after addition of thymidine-^{3}H. When thymidine-^{3}H and hydroxyurea were added simultaneously, the degree of loss of label was not as great after 90 min in any of the experiments (Fig. 2). The ratio of thymidine-^{3}H to unlabeled thymidine in NCTC-109 (information on composition of this medium was obtained from Dr. G. B. Gori, Microbiological Associates, Inc.) was only 0·021 in all cases. It appears that daily variations in age of cells used and slight differences in cell concentration may

determine the extent to which the previously incorporated thymidine is lost under the influence of the drug. The mechanism of this release is undoubtedly at a subtle level, since gross cellular disorganization by the drug could not be demonstrated either indirectly by measurement of glucose oxidation or directly by electron microscopic examination.

On the basis of inhibitory patterns, no evidence was obtained that the pharmacological action of hydroxyurea on the tumor cells depends upon its conversion to hydroxylamine. No loss of label was shown to occur up to 90 min with hydroxylamine (Fig. 3), but this phenomenon is apparently variable, as mentioned in the previous paragraph. However, the slight effect on thymidine incorporation induced by $1{\cdot}2 \times 10^{-4}$ M hydroxylamine implies that even complete conversion of hydroxyurea to hydroxylamine would not account for the inhibitory pattern obtained with hydroxyurea. Similarly, the dose–response relationships of the two compounds strongly suggest an action of hydroxyurea mediated in some way other than by a conversion to hydroxylamine. No evidence as yet exists to determine whether the present data represent an effect on thymidine incorporation and release or on its release alone.

ADDENDUM

Since this report was submitted for publication, two communications have appeared dealing with the mechanism of action of hydroxyurea. Philips *et al.*[7] found that in rats the drug causes severe disturbances in tissues having high rates of cell renewal, and Young *et al.*[8] have demonstrated that it inhibits uptake of thymidine by HeLa cells in monolayer culture. No effect on uptake of uridine or leucine could be demonstrated in the latter work. It thus appears rather well established that at least part of the carcinostatic action of hydroxyurea is mediated through interference with DNA metabolism.

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