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HYDROXYUREA INHIBITS ODC INDUCTION, BUT NOT THE G_1 TO S PHASE TRANSITION

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SUMMARY: Chinese hamster ovary cells, selected in mitosis and plated into medium containing hydroxyurea, can progress through G_1 and enter S phase although bulk DNA synthesis is prevented. As the cells progress through G_1 in the presence of hydroxyurea, ornithine decarboxylase activity remains low while general protein synthesis appears unaffected. After hydroxyurea is removed, ornithine decarboxylase activity increases, but only after approximately 20% of the DNA has been replicated. These results suggest that ornithine decarboxylase induction is not essential for cellular progression into S phase but is required for the completion of DNA synthesis.

INTRODUCTION

ODC activity is the initial rate limiting step in polyamine biosynthesis (1) and is thought to be an important biochemical event regulating cell proliferation. The apparent half-life of ODC is on the order of minutes (2) and the enzyme activity is greatly increased in rapidly growing tissues (3). Embryonic tissues, some tumors and target organs all show a substantial increase in ODC after administration of a growth stimulus (4).

In continuously dividing cell cultures *in vitro*, ODC activity increases primarily during late G_1 and in the G_2 phases of the cell cycle (5,6). Inhibition of polyamine biosynthesis by methylglyoxal bis-(guanylhydrazone) (7,8,9) or by α-methylornithine (10) results in a substantial inhibition of DNA synthesis. Experiments by Heby *et al*, using Ehrlich ascites tumor cells, suggest that polyamine biosynthesis reflects the rate of DNA synthesis rather than the entry of cells into S phase (11). Inhibition of putrescine biosynthesis appears to reduce the rate of $[^3H]$dThd uptake without inhibiting the progression of cells from G_1 into S phase (12).

Abbreviations: ODC, L-ornithine decarboxylase (E.C. 4.1.1.17); CHO, Chinese hamster ovary; G_1 and G_2- pre- and post- DNA synthetic periods, respectively; S, DNA synthetic period; HU, hydroxyurea.

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In our experiments, we chose to examine independently the role(s) of ODC induction in the G_1 to S phase transition, and in S phase progression, using HU. This drug allow cellular progression into S phase but prevents bulk DNA synthesis by limiting the dATP pool (13,14). Our results demonstrate that ODC induction is temporally related to bulk DNA synthesis, but need not occur for cells to enter S phase.

MATERIALS AND METHODS

Cell Culture Techniques

CHO cells were grown as monolayer cultures as previously described (15). Under these conditions, the cell culture doubling time was 13-14 hours. Synchronous cell populations were obtained by selective detachment of mitotic cells (16). The mitotic index was $\geq$ 90% for all experiments.

Measurement of DNA Replication

Cells were grown for 24 hours in medium containing 0.15 µCi/ml [^{14}C] dThd (Schwarz Mann, 45 mCi/mmole) to uniformly label normal density DNA (1.70 g/cc). After mitotic selection and treatment with HU, cells were plated into medium containing 50 µg/ml 5-bromodeoxyuridine to provide a density label in newly replicated DNA (hybrid density 1.75 g/cc) and 0.1 µg/ml 5-fluorodeoxyuridine to inhibit de novo thymidylate synthesis. The isolation of DNA, banding in CsCl gradients and further manipulation are described elsewhere (17,18). DNA synthesis rates in figure 4 were measured by incubating the cells with [^{3}H] dThd (5.0 µCi/ml, Schwarz-Mann, 62 Ci/mmole) for 15 minutes. Cells were harvested by scraping and the amount of acid insoluble radioactivity measured. Each sample represents duplicate determinations of at least 2.0 x 10^6 cells.

Measurement of ODC

ODC activity was measured by the liberation of $^{14}CO_2$ from D,L-[1-^{14}C] ornithine (New England Nuclear, Boston, MA, 40-60 Ci/mmole). The specific activity was changed to 5.2 mCi/mmole by the addition of L-ornithine. Cells were sonicated in 0.05 M sodium potassium phosphate buffer, pH 7.2 containing 0.1 mM EDTA, 1.0 mM dithiothreitol and 20 µM pyridoxal phosphate. Two hundred micro liters of the cell homogenate were incubated for 30 minutes at 37°C in the presence of 0.5 µCi L-[1-^{14}C] ornithine. Minimum cell numbers were 5.0 x 10^6 cells per assay.

RESULTS

ODC Induction and DNA Synthesis During and After HU

Panel A of figure 1 shows the kinetics of DNA replication for cells, synchronized in mitosis, either progressing through the cell cycle in normal medium or moving through S phase following treatment with 2 mM HU for 13 hours. Panel B of figure 1 describes the patterns of ODC activity observed in control cells and cells during and after 2 mM HU treatment. In the control cells, a peak of ODC activity is observed approximately 4-5 hours after mitosis, before DNA synthesis has begun. An increase in ODC activity in these cells precedes DNA replication, with the enzyme activity then decreasing as

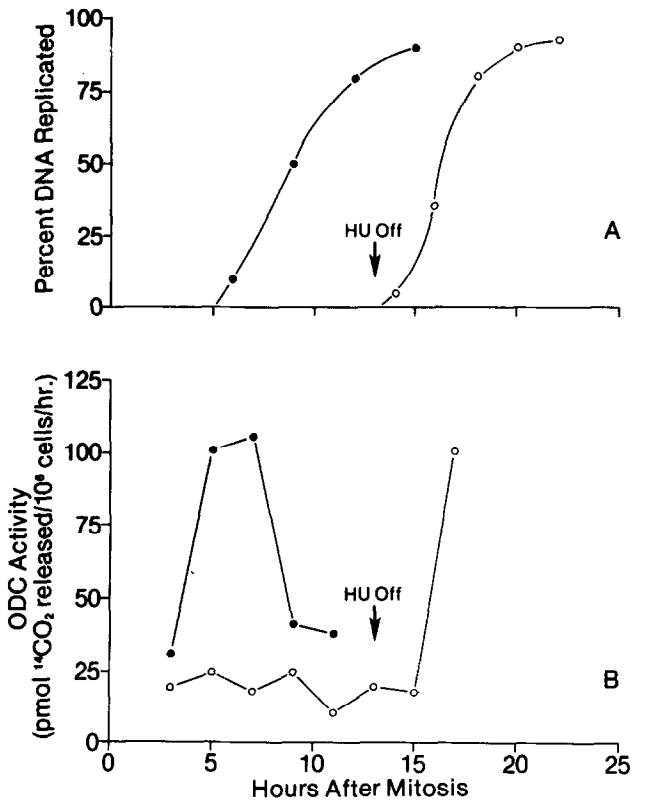

Figure 1, Panel A: Effect of 2 mM HU on DNA synthesis. CHO cells were selected in mitosis, and either allowed to progress through S phase (●-●) or incubated in 2 mM HU for 13 hours (O-O). Samples for CsCl density gradient analysis (as described in methods section) were collected at 1,3,5,7, and 9 hours after the HU was removed. Panel B: Effect of HU on ODC activity. CHO cells were selected in mitosis and allowed to traverse S phase (●-●) or incubated in 2 mM for 13 hours (O-O). ODC activity was measured at 2 hour intervals during the HU treatment and after the HU was removed.

cells move into S phase. In contrast, ODC activity remains low throughout G_1 phase in HU treated cells. These cells are able to initiate DNA synthesis (14) but are blocked from bulk DNA replication. ODC activity does increase after the HU is removed from the culture medium. This increase, however, does not occur until after DNA synthesis is initiated and at least 20% of the DNA has been replicated.

In order to investigate in more detail this increase in ODC activity after removal of HU, this enzyme activity was measured at 1/2, 1, 2, 3, 5, and 7 hours after drug removal and correlated with the amount of DNA replicated. The results, shown in figure 2, demonstrate that the increase in ODC activity parallels the amount of DNA replicated as these cells traverse S phase.

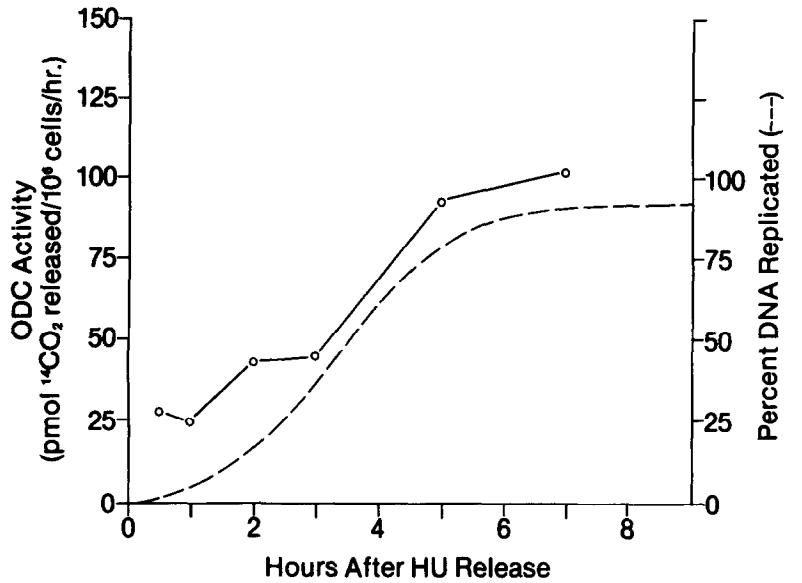

Figure 2: ODC activity after removal of 2 mM HU. CHO cells were selected in mitosis and incubated with 2 mM HU for 13 hours. ODC activity was measured after removal of the HU (O-O). The DNA replication pattern (---) in figure 2 is taken from figure 1, panel A for comparative purposes.

HU Inhibition of ODC Induction

During the CHO cell cycle, ODC activity increases at the G_1/S boundary primarily due to induction (5,6). The results presented here show that, in the presence of HU for 13 hours, no increase in ODC activity is observed. A concern is whether the 13 hour treatment is actually inhibiting induction of ODC or perhaps resulting in the synthesis of the ODC antizyme. Figure 3 demonstrates that HU added to synchronized cells 4 hours after mitosis can prevent the peak of ODC activity. These data show that HU can act within one hour to prevent this increase in ODC activity. It should be noted that the addition of HU does not completely inhibit existing ODC activity, only the induction.

General protein synthesis rates in the control and the HU treated cells were simil and HU did not inhibit ODC activity *in vitro* (data not shown).

Concentration and Time Dependence of HU Effects on ODC Activity and DNA Synthesis

Asynchronous populations of CHO cells were treated with varying concentrations of HU for 9 hours. Figure 4, panel A shows that DNA synthesis and ODC activity decrea

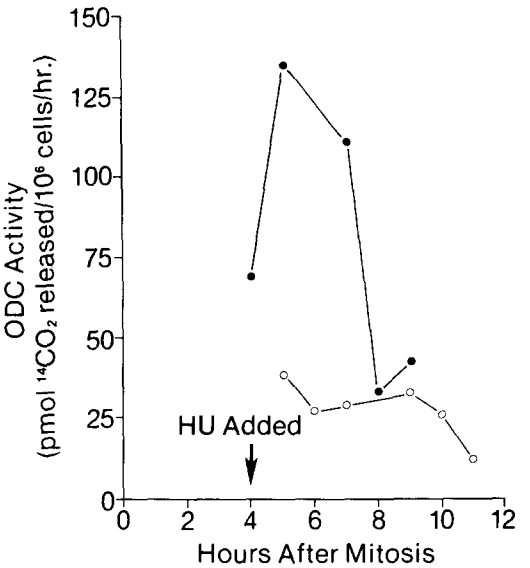

Figure 3: Effect of 2 mM HU on ODC activity. ODC activity was measured in CHO cells which had been selected in mitosis and either allowed to progress through S phase (●-●) or incubated with 2 mM HU (O-O). HU was added 4 hours after mitosis.

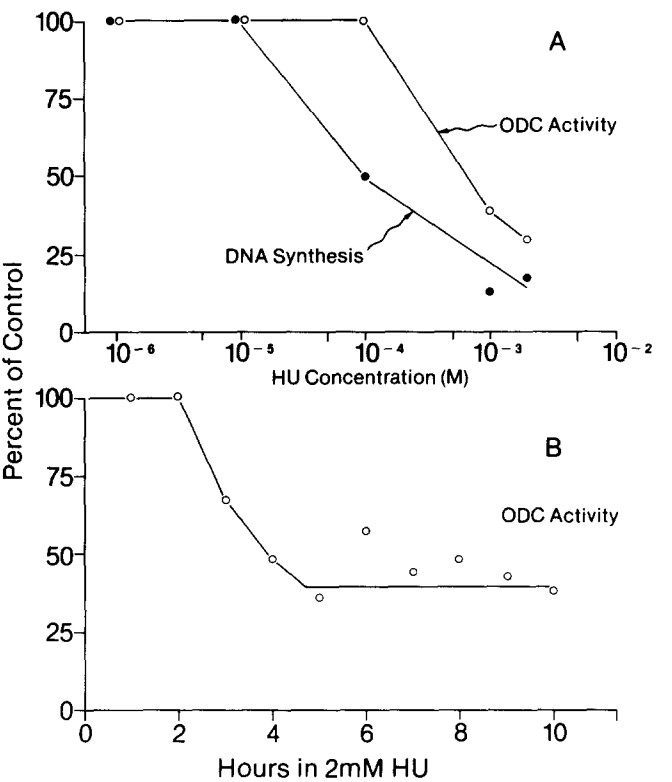

Figure 4, Panel A: Effect of various concentrations of HU on ODC activity and DNA synthesis. Asynchronous CHO cells were incubated in various concentrations of HU for 13 hours. ODC activity was measured (O-O) as well as [^{3}H] dThd incorporation (●-●). Panel B: Time course of 2 mM HU effect on ODC activity. Asynchronous CHO cells were incubated with 2 mM HU for various times and ODC activity was measured (O-O).

in a similar manner. At concentrations above 10^{-5}M, DNA synthesis is effected to a greater degree than ODC activity and may simply reflect the action of HU on ODC inductic rather than an effect on the enzyme itself. Panel B of figure 4 supports this result. These data demonstrate that HU has no effect on ODC activity in asynchronous cells for up to 2 hours of treatment. After 2 hours, the activity does decrease, probably due to accumulation of cells in cell cycle compartments where ODC induction should occur, and hence, would be prevented by HU.

DISCUSSION

ODC activity in G_1 traversing cells generally increases prior to DNA replication. The possibilities exist that ODC induction may, therefore, be functionally related to entry into, or maintenance of, S phase. In the presence of HU, cells are allowed to enter S phase but bulk DNA synthesis is prevented (14). Under these conditions, ODC is not induced. This enzyme activity remains low until HU is removed from the culture medium and, subsequently, greater than 20% of the DNA has replicated. These data show that ODC induction is not a strict requirement for cellular entry into S phase since a substantial amount of DNA can be synthesized without a previous increase in ODC activity.

The second possibility, that ODC activity is important for the maintenance of S phase, is supported by the observation that ODC activity increases in a similar manner to DNA replication after the HU is removed. Experiments are in progress to determine if dATP added to HU treated cells will rescue ODC activity, since such a treatment will rescue DNA synthesis (19).

We suspect that the inhibition of ODC induction by HU does not function through the ODC antizyme mechanism, since figure 3 showed that HU will prevent stimulation witl one hour. Since we know that the appearance of the ODC antizyme requires protein synt sis (20,21,22, 23), one would not expect such a rapid effect on the expression of the enzyme. In addition, if homogenates from HU treated cells are mixed with asynchronous cell extracts, ODC activity is not decreased (data not shown). This only indicates that no unbound ODC antizyme is present, since ODC activity is still detectable and any antizyme present would presumably be bound (20).

ODC induction may also be prevented by sensitive membrane mediated sites responding to extracellular diamines (20). HU is known to dimerize (24) and still inhibit ribonucleotide diphosphate reductase (25). However, the kinetics of ODC inactivation by HU do not correspond to the inactivation curve observed with spermine (data not shown). Concentrations of HU from 10^{-2}M to 10^{-6}M do not inhibit ODC activity when administered for 90 minutes whereas a spermine concentration of 10^{-6}M inhibits ODC activity to less than 50% of controls.

At this point, we have ruled out the decrease in ODC by HU as being due to a non-specific diamine effect mimicking the action of exogenous polyamines, appearance of the antizyme, a decrease in general protein synthesis, cell cytotoxicity or a direct inhibitory effect on the enzyme *in vitro*. Therefore, we suspect that HU acts to prevent ODC induction by a direct or indirect consequence of the already defined intracellular actions of HU, i.e., decreased DNA precursor pools or the scavenging of cations (26).

Acknowledgments

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