

α -Methyl ornithine, a potent competitive inhibitor of ornithine decarboxylase, blocks proliferation of rat hepatoma cells in culture

(putrescine/spermidine/DNA synthesis/hepatoma tissue culture cell)

PIERRE S. MAMONT*, PETER BÖHLEN, PETER P. MCCANN, PHILIPPE BEY, FRANCIS SCHUBERT†, AND CHANTAL TARDIF

Centre de Recherche Merrell International, 16, rue d'Ankara, 67000 Strasbourg, France; and † Institut de Botanique, Université Louis Pasteur, 67000 Strasbourg, France

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ABSTRACT A biphasic increase of putrescine concentration occurs in rat hepatoma tissue culture cells induced to proliferate. DL- α -Methyl ornithine, a competitive inhibitor of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.7) of hepatoma tissue culture cells, blocks the usual increases of putrescine and spermidine concentrations in these cells, and causes a rapid fall in the levels of putrescine which is followed by a striking decrease of spermidine. In parallel with the depletion of these amines, incorporation of [³H]thymidine into DNA and cell proliferation are inhibited. Addition of putrescine, spermidine, or spermine results in an immediate resumption of cell proliferation. Cell proliferation is also restored by L-ornithine presumably due to *in situ* competitive inhibition of ornithine decarboxylase. These findings in hepatoma tissue culture cells support the concept that polyamines play an essential function in the cell division processes.

The polyamines spermidine and spermine and their diamine precursor putrescine increase rapidly in many eukaryotic cell systems induced to proliferate (1-5). Recently, a direct linear correlation has been established between spermidine/spermine ratios, which reflect primarily the intracellular spermidine content and growth rates of tumor cells (6). However, there is as yet no conclusive evidence that increased polyamine synthesis is required for eukaryotic cell proliferation. One experimental approach to this problem is to specifically impair biosynthesis in a dividing cellular system.

Although in bacteria, certain polyamine-deficient cells have been obtained by selection of mutants (7, 8), polyamines could not be depleted in mammalian cells until the recent discovery of inhibitors of the polyamine biosynthetic pathway. α -Hydrazino-ornithine, a competitive inhibitor of both bacterial and mammalian ornithine decarboxylases (L-ornithine carboxy-lyase, EC 4.1.1.17) (9), inhibits putrescine accumulation in regenerating rat liver (10), in rat hepatoma tissue culture (HTC) cells (10), and in mouse parotid glands (11). Similarly, the increase of putrescine levels observed in lymphocytes stimulated by concanavalin A is blocked by α -methyl-ornithine (α -MeOrn) and α -hydrazino- α -methyl ornithine, two other competitive inhibitors of mammalian ornithine decarboxylase (12-14). Spermidine synthesis is also inhibited in rat liver and kidney (15) and in stimulated lymphocytes (16) by methylglyoxal bis-(guanyldrazone), a potent inhibitor of S-adenosyl methionine decarboxylase (17). However, no correlation has been reported between deple-

tion of polyamines and inhibition of proliferation of mammalian cells.

In growing HTC cells, a biphasic increase of ornithine decarboxylase with a concomitant increase of intracellular putrescine occurs after induction of cell proliferation (18). To determine whether this increase of putrescine is essential for cell division, we examined in the same cellular system the effect of α -MeOrn on polyamine biosynthesis and cell proliferation. In preliminary studies we found that α -MeOrn, described as a competitive inhibitor of rat liver and prostate ODC (13), is also an *in vitro* competitive inhibitor of HTC cell ornithine decarboxylase with a K_i value of 4×10^{-5} M. We now demonstrate that α -MeOrn depletes putrescine and spermidine concentrations in HTC cells and inhibits DNA replication and cell proliferation.

MATERIALS AND METHODS

Hepatoma tissue culture cells were grown in spinner culture to a high density (9×10^5 cells per ml) as previously described (19). Before each experiment they were diluted with fresh Swim's 77 medium supplemented either with 1 calf serum (Gibco) or 0.1% bovine-serum albumin (Sigma) to a final concentration of about 1.0×10^5 cells per ml. DL- α -Methyl ornithine hydrochloride, prepared in our laboratory

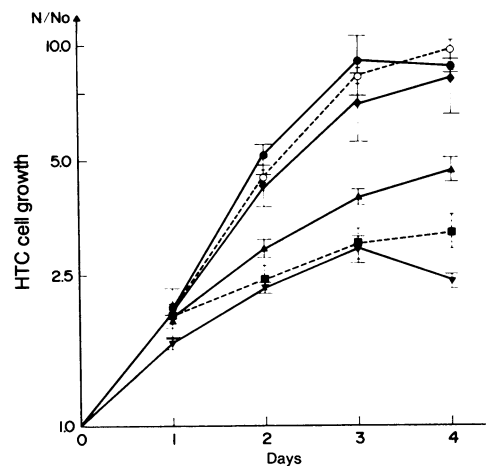


FIG. 1. Effect of α -MeOrn on HTC cell division. HTC cell spinner cultures (1×10^5 cells per ml) were incubated from time 0 in the absence (O) or in the presence of increasing concentrations of α -MeOrn: 0.1 mM (●), 1 mM (◆), 2.5 mM (▲), 5 mM (■), and 10 mM (▼). Results are expressed as the ratio N/No, where N = number of cells per ml at day 1, 2, 3, 4 and No = number of cells per ml at day 0, and are the means of at least three experiments \pm SEM.

Abbreviations: α -MeOrn, DL- α -methyl ornithine; HTC cells, hepatoma tissue culture cells.

* To whom correspondence should be addressed.

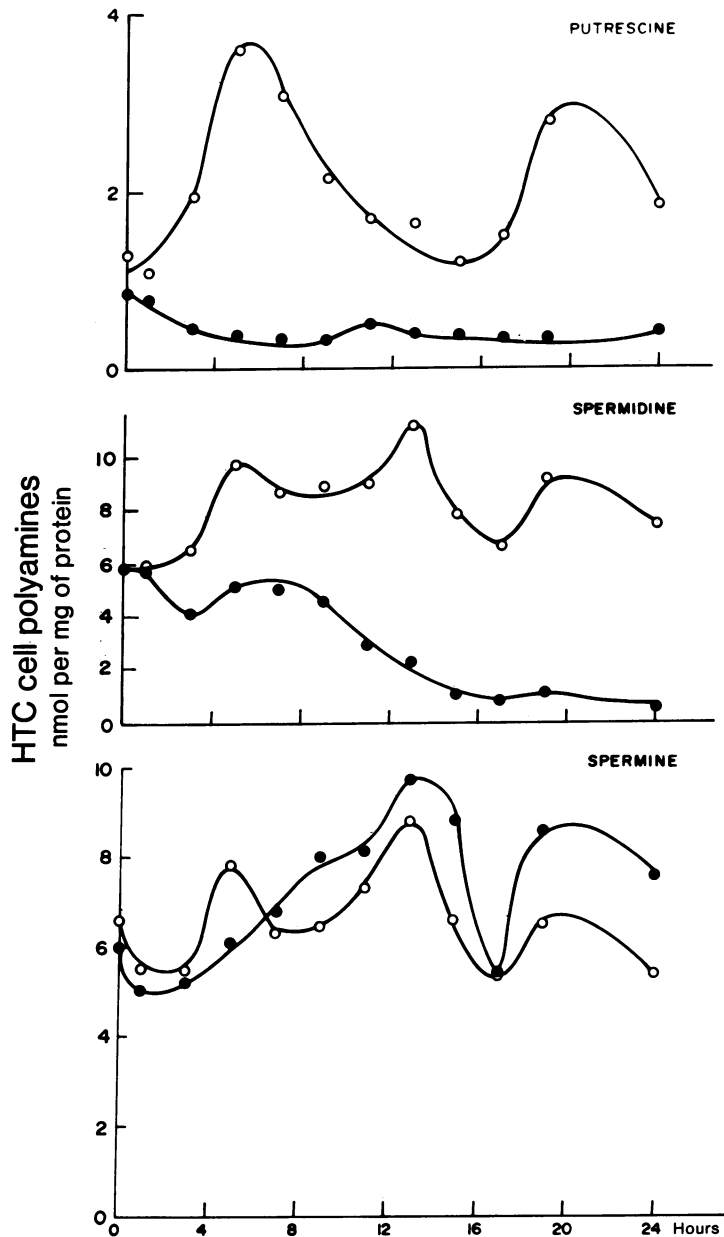


FIG. 2. Effect of α -MeOrn on HTC cell polyamine concentrations. HTC cell cultures (1×10^5 cells per ml) were incubated from time 0 in the presence (●) and absence (○) of 5 mM α -MeOrn. Results are expressed as nanomoles of polyamines per milligram of protein and are the averages of three determinations on a single sample.

(P. Bey, to be published), and polyamines (Sigma) were dissolved before use in phosphate-buffered saline and neutralized to pH 7.4. Cell growth was measured by cell counting in a hemocytometer and viability was estimated by counting cells excluding trypan blue.

For the determination of polyamine concentration, aliquots (4.5 – 9.0×10^6 cells) were collected by centrifugation and washed with cold phosphate-buffered saline. The cell pellet was resuspended in 0.8 ml of 0.2 M perchloric acid for 1 hr at 4° , and the perchloric acid extracts were then centrifuged. Polyamines were assayed in the supernatants by thin-layer chromatographic separation and fluorometric determination as their dansyl derivatives, as described by Seiler and Schmidt-Glenewinkel (20). For putrescine assays the chromatographic separation was modified according to Fleisher and Russell (21). Standard polyamine solutions were subjected to the same assay procedures for quantitation of the re-

sults. Small amounts (10% of the sample polyamine content) of [3 H]putrescine (425 mCi/mmol), spermidine (425 mCi/mmol), and spermine (1085 mCi/mmol) (Amersham), added to unknown and reference solutions before the chromatographic separation, served as internal standards and were used to correct the results for losses. Proteins were estimated by the fluorescamine assay of Böhlen *et al.* (22).

For DNA synthesis measurements, 10 ml aliquots of a suspension of cultured HTC cells were pulse-labeled for 1 hr in the presence of 2.5 μ Ci/mls of [*methyl*- 3 H]thymidine (0.7 Ci/mmol, Amersham). At the end of the labeling period, cells were collected as described above and lysed by addition of 1 ml of 0.1% sodium dodecyl sulfate. The amounts of trichloroacetic acid-soluble and insoluble radioactivities were estimated as previously described (23), except that the trichloroacetic acid-insoluble pellet was dissolved in 0.5 M NaOH and counted in 10 ml of scintillation fluid consisting

of 0.4% (wt/vol) "Omnifluor" (New England Nuclear) and 25% (vol/vol) Triton X-100 in toluene (Packard). The results, were expressed per mg of protein, estimated by the method of Lowry *et al.* (24).

RESULTS

Effect of ornithine decarboxylase inhibition on HTC cell replication and polyamine concentrations

When high-density HTC cell spinner cultures (9×10^5 cells per ml) were diluted 10 times by fresh Swim's 77 medium supplemented with 10% calf serum, cell proliferation was immediately induced. As shown in Fig. 1, control cultures exhibited a logarithmic phase of growth for at least 3 days with a population doubling time of about 24 hr. When α -MeOrn was added to the cultures immediately after dilution, cell growth slowed down significantly after a lag period of about 24 hr. The inhibition of cell growth reached a maximum at 5 mM α -MeOrn. At this concentration no difference in the percentage of dead cells was found between the inhibited and control cultures even after a 4-day incubation. L-Ornithine at 5 mM had no effect on cell growth (data not shown).

Biphasic increases of intracellular putrescine were observed after induction of HTC cell proliferation (Fig. 2). In the presence of 5 mM α -MeOrn, intracellular putrescine concentration did not increase (Fig. 2), but instead decreased rapidly to 50% of its initial level. α -MeOrn also prevented any increase in spermidine concentration (Fig. 2) and decreased it to about 10% of the zero-time concentration after a lag period of 7–8 hr. In contrast, spermine levels did not decline in the presence of α -MeOrn (Fig. 2).

Effect on DNA synthesis

HTC cell cultures were induced to proliferate in the presence or absence of α -MeOrn. [*Methyl*- ^3H]Thymidine incorporation was measured during a period of 36 hr (1.5 population doubling time). As illustrated in Fig. 3, two bursts of thymidine incorporation into DNA were observed in the control. In the presence of α -MeOrn, thymidine incorporation into DNA was similar to control cells during the first population doubling time period; afterwards a rapid decline to the basal value was observed in the presence of α -MeOrn. Thymidine incorporation into the acid-soluble pool was unaltered by the presence of α -MeOrn. The proportion of cells undergoing cell division, as measured by the appearance of mitotic figures in the presence of colcemid, was the same in both cultures (46%) during the first 24 hr. This proportion was decreased by 50% in the α -MeOrn-treated cells during the second doubling time period.

Reversion of HTC cell growth inhibition by polyamines and L-ornithine

If the inhibitory effect of α -MeOrn on HTC cell growth were the result of intracellular polyamine deficiency, then this effect might be reversed by the addition of polyamines. Low density (1×10^5 cells per ml) HTC cell cultures were deprived of serum during 36 hr to block the cells in the G₁ phase of the cell cycle (25). Then, cell proliferation was induced by the addition of 10% calf serum and the cultures were further incubated in the presence or absence of α -MeOrn (Fig. 4). The addition of 1 μM putrescine, spermidine, or spermine 1 or 2 days (data not shown for 2 days) after incubation with α -MeOrn resulted in immediate resumption of cell proliferation (Fig. 4B). In contrast, addition

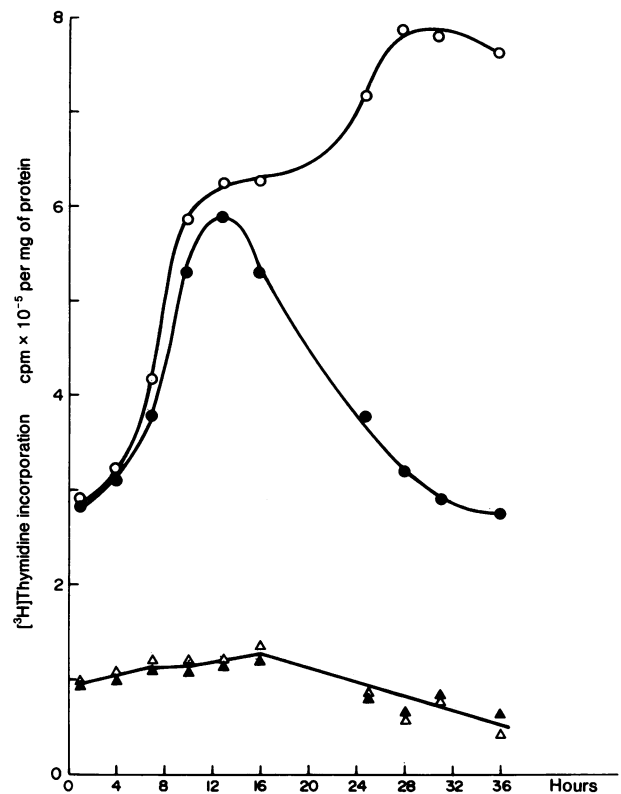


FIG. 3. Effect of α -MeOrn on [*methyl*- ^3H]thymidine incorporation. HTC cell cultures (1×10^5 cells per ml) were incubated from time 0 in the presence (●, ▲) or in the absence (O, Δ) of 5 mM α -MeOrn. At the indicated times, aliquots were pulse-labeled with [*methyl*- ^3H]thymidine, and determination of the trichloroacetic acid-soluble (Δ, ▲) and trichloroacetic acid-insoluble radioactivities (O, ●) were performed in duplicate; identical samples differed by no more than 10%.

of 1 μM cadaverine or 1,3-diaminopropane did not overcome the inhibitory effect of α -MeOrn (Fig. 4B). The addition of 0.1 or 1 mM L-ornithine to α -MeOrn-treated cultures prevented the inhibitory action on cell proliferation (Fig. 4A).

DISCUSSION

In many normal and neoplastic tissues, increases of polyamines are among the earliest events to follow initiation of cell proliferation (26, 27). If these two phenomena were causally related, the blockade of the increase of polyamine concentration by specific inhibition of ornithine decarboxylase, the rate-limiting enzyme of the polyamine biosynthetic pathway, might well lead to an inhibition of cell division. Using rat hepatoma tissue culture cells we found that α -MeOrn, an inhibitor of the decarboxylase enzyme, blocks DNA synthesis and cell proliferation.

In the presence of α -MeOrn, biphasic increases of putrescine were abolished and the immediate decline of the initial level of this amine was followed, after a lag period of 7–8 hr, by a marked decrease in spermidine concentration. These results suggest that α -MeOrn suppresses intracellular ornithine decarboxylase activity. In contrast, the intracellular spermine level was not apparently modified after 24 hr of incubation in the presence of α -MeOrn. A positive correlation between putrescine and spermidine depletion and inhibition of cell proliferation is suggested by the α -MeOrn-in-

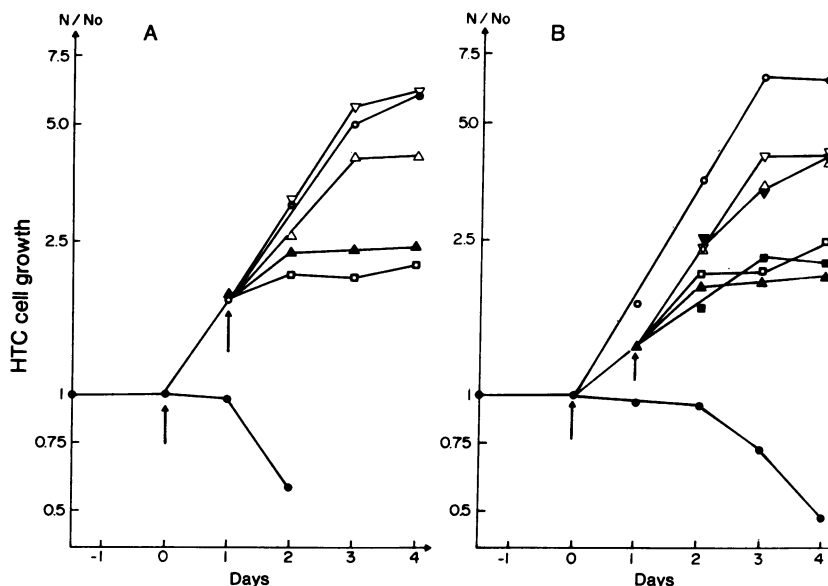


FIG. 4. Reversal of inhibition of HTC cell division by L-ornithine (A) and polyamines (B). HTC cell cultures (1×10^5 cells per ml) were deprived of serum by incubation in fresh Swim's 77 medium containing 0.1% bovine-serum albumin. At the indicated time (first arrow), 10% calf serum was added to the cultures and they were further incubated in the absence or presence of 5 mM α -MeOrn. At day 1 (second arrow) L-ornithine or 1 μ M polyamines were added to α -MeOrn-treated cultures. A: - serum (\bullet); + serum (O); + serum, + α -MeOrn (\blacktriangle); + serum, + α -MeOrn, + 0.01 mM L-ornithine (\square); + serum, + α -MeOrn, + 0.1 mM L-ornithine (\triangle); + serum, + α -MeOrn, + 1 mM L-ornithine (∇). B: - serum (\bullet); + serum (O); + serum, + α -MeOrn (\blacktriangle); + serum, + α -MeOrn, + 1 μ M cadaverine (\square); + serum, + α -MeOrn, + 1 μ M 1,3-diaminopropane (\blacksquare); + serum, + α -MeOrn, + putrescine (\triangle); + serum, + α -MeOrn, + spermidine (∇); + serum, + α -MeOrn, + spermine (\blacktriangledown).

duced depression of the incorporation of [3 H]thymidine into DNA.

When HTC cell cultures reach a saturation density (9×10^5 cells per ml) after 3 days of logarithmic growth, cellular proliferation stops. Upon dilution in fresh medium supplemented with serum, cells resume DNA synthesis with maximum [3 H]thymidine incorporation being observed at 12 hr. Previously we had estimated that, before dilution, stationary cells were primarily blocked in mid- G_1 (18). In the presence of α -MeOrn, thymidine incorporation into DNA and the percentage of cells undergoing mitosis were unaltered during the first doubling time period. Thereafter, the burst of thymidine incorporation, normally observed during the second doubling time period, was completely abolished by the presence of α -MeOrn. This finding suggests that the serum-induced early progression of cells from mid- G_1 through S phase and mitosis was not affected by blocking the accumulation of putrescine and spermidine. A possible interpretation of this finding is that stationary HTC cells had sufficient initial polyamine levels to allow one cycle of DNA synthesis. Indeed, the inhibition of the second burst of thymidine incorporation appears to be related to the decrease of initial level of putrescine and spermidine.

If depletion of putrescine and spermidine truly were the cause of inhibition of cell proliferation, then exogenously added polyamines might be expected to reinstate growth. Indeed, HTC cell proliferation was immediately restored by putrescine, spermidine, and spermine. Specificity of the reversing effect of these polyamines was suggested by the finding that neither cadaverine nor 1,3-diaminopropane, the higher and lower homologs of putrescine, could overcome the inhibition of cell proliferation. Reversion by spermine is the most surprising result because its endogenous level was not decreased by the presence of α -MeOrn. However, exogenously labeled spermine has been reported to be converted into spermidine in the rat (28), in Ehrlich ascites cells

(29), and in trout brain (30). Inhibition of HTC cell proliferation by α -MeOrn was also reversed by L-ornithine. This suggests *in situ* competitive inhibition of ornithine decarboxylase by α -MeOrn. It may also indicate a low intracellular pool of ornithine resulting from a low HTC cell arginase activity. Very low activities of arginase have been found in several transplantable hepatomas when compared to normal livers (31).

Some evidence reported in the literature appears to indicate that spermidine plays an essential function in DNA synthesis, e.g., stabilization of DNA folds (32), activation of DNA-dependent DNA polymerase (33), and stimulation of the replication of single-strand phage DNA (34). Our data cannot definitively exclude the participation of putrescine in DNA replication. However, we suspect that spermidine plays the major role in this macromolecular process because its level was maintained for at least 7–8 hr, which correlates with the initial maintenance of DNA synthesis. Furthermore, methylglyoxal *bis*-(guanylhydrazone), which blocks spermidine but not putrescine accumulation, appears to exert a specific inhibition on DNA replication in concanavalin A-stimulated lymphocytes (35, 36). The delay in the decrease of the spermidine level of HTC cells might explain the failure of Harik *et al.* (10) to demonstrate an inhibitory effect of α -hydrazino-ornithine on DNA synthesis during one doubling time period. In summary, our data suggest that in rat hepatoma cells, continued polyamine synthesis is necessary to maintain cell division processes.[†]

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[†] A recent paper of N. Relyea and R. R. Rando [(1975) *Biochem. Biophys. Res. Commun.* 67, 392–402] shows that *trans*-3-dehydro-DL-ornithine, a new potent competitive inhibitor of ornithine decarboxylase, blocks division of chick embryo muscle cells in culture.

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