both the phase and the period of the free-running rhythm. In conjunction, these results provide compelling support for the proposition that the circadian clock in the cockroach is in the optic lobes.

It is remarkable that the transplanted protocerebral tissue survives, undergoes functional regeneration, and conserves the period of the free-running oscillation so reliably. A question that arises is whether or not the pacemaker continues its motion between the time of its removal and the return of overt rhythmicity in the host. However, there was no clear indication that phase, as well as period, was conserved in the transplanted lobe (*19*). A positive result would have indicated that the oscillation had persisted; a negative result is open to a number of interpretations, some of which do not preclude the possibility that a circadian oscillation persists in the isolated optic lobe.

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10. Colonies of animals were maintained in light-tight boxes at constant temperature. Light-dark cycles (11 hours of light and 11 hours of darkness or 13 hours of light and 13 hours of darkness) were provided by a 4-W fluorescent light.
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13. Activity was monitored in Lucite cages with running wheels that activated event recorders (*5*). In the first experimental series, animals were separated in individual lighttight boxes; in the second experiment, the activity monitors were located in a single incubator. All experiments were run at 25° ± 0.5°C. Free-running periods were estimated by fitting (by eye) a line through activity onsets. With one exception, all period estimates are based on three or more weeks of data.
14. Surgery was performed with CO_2 anesthesia. Two three-sided cuticular flaps were cut in the head capsule which, when raised, exposed the brain. Fine scissors were used to cut the optic nerves and optic tract of each optic lobe. The lobes were removed from the head capsule and placed in physiological saline at room temperature while a second animal was similarly prepared. The lobes of the two animals were then exchanged, placed in close apposition to the midbrain of the host. Care was taken to maintain the orientation and handedness of the transplanted lobes. The cuticle was repositioned and sealed with wax.
15. Deciding the precise day on which the rhythm returns is subjective—the values given represent the extremes of the earliest and latest times a rhythm was judged to have been present after transplantation. The average number of days to return of a clear behavioral rhythm for all animals was 39 ± 8.9 (standard deviation) days.
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17. Unilateral optic lobe ablation or optic tract section consistently causes a slight increase in τ in animals raised in LD 12:12 ($\overline{\Delta\tau}$ = 0.21 ± 0.23 hours, N = 39) (*5*). Comparable small increases in τ resulting from unilateral optic tract section are found in animals raised in LD 11:11 ($\overline{\Delta\tau}$ = 0.27 ± 0.29 hours, N = 6) or in LD 13:13 ($\overline{\Delta\tau}$ = 0.16 ± 0.22, N = 5). The increase in τ after surgical interference of the optic lobe has been interpreted by use of a coupled oscillator model in which the pacemaker of each optic lobe has a period slightly longer than that of the coupled pair (*5, 6*). This may also explain the tendency for τ of the regenerated rhythm after optic lobe transplantation to be slightly longer (on the average by 0.2 ± 0.24 hour) than τ of the donor rhythm, if normally only one of the two lobes is successful in reestablishing the specific neural connections with the midbrain that are necessary to drive rhythmicity.
18. Brains were fixed overnight in an alcohol-formaldehyde solution, dehydrated, cleared, and embedded in paraffin. Serial sections (12 μm) were cut and stained in 1 percent methylene blue.
19. With the onset of activity as a phase reference point, the phase of the free-running rhythm was projected back to the day of surgery. There was no clear correlation between the rhythm phase and the phase of the donor, the phase of the host, or the time of surgery.
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Ornithine Decarboxylase: Essential in Proliferation but Not Differentiation of Human Promyelocytic Leukemia Cells

Abstract. *The ornithine decarboxylase inhibitor* DL-α-*difluoromethyl ornithine inhibited a proliferation-associated increase in ornithine decarboxylase activity in cultured human promyelocytic leukemia cells, resulting in a marked suppression of cell proliferation and subsequent cell loss. It also inhibited increases in ornithine decarboxylase activity associated with the phorbol ester–induced conversion of promyelocytic HL-60 cells to monocyte-like cells and the retinoic acid–induced conversion to granulocyte-like cells. However, the inhibition of ornithine decarboxylase activity did not prevent cellular differentiation. These results suggest that polyamine biosynthesis has a specific role in cell proliferation rather than in inducing differentiation that is not accompanied by proliferation. The data also demonstrate that cessation of proliferation in HL-60 cells is not necessarily associated with differentiation.*

The decarboxylation of ornithine by ornithine decarboxylase (ODC; E.C. 4.1.1.17) leads to the formation of putrescine and is the first rate-limiting step in polyamine biosynthesis. Marked increases in ODC activity and polyamine biosynthesis accompany the onset of proliferative events in most cell types studied (*1, 2*). In addition, certain differentiative processes, such as responses of target tissues to specific hormonal stimuli (*3*) and induced differentiation of Friend mouse erythroleukemia cells (*4*), have been associated with increased ODC activity. The importance of ODC in cell growth and differentiation was recently demonstrated in studies with DL-α-difluoromethyl ornithine (DFMO), an inhibitor of ODC (*5*). Inhibition of putrescine synthesis by DFMO arrests the growth of mouse L-1210 leukemia cells and rat hepatoma cells in culture (*6*) and leads to eventual death of human small-cell lung carcinoma in vitro (*7*). DFMO completely suppresses the sharp rise in uterine ODC activity accompanying murine embryogenesis, arresting embryonic development (*8*). Also, DFMO suppresses increases in ODC which accompany the maturation of intestinal mucosa and recovery from injury; both cellular processes are inhibited by the block in putrescine biosynthesis (*9*).

Increased ODC activity may thus play an important role in the initiation of both cellular proliferation and differentiation. It has been difficult, however, to evaluate the processes of proliferation and differentiation separately. Hence it has been unclear whether one or both cellular responses depend on polyamine biosynthesis. Recently, a cell system for studying these events separately was described (*10–12*). The human promyelocytic leukemia cell line HL-60 can be chemically induced to undergo terminal differentiation, probably without accompanying proliferation.

The cell line, derived from peripheral leukocytes from a woman with acute promyelocytic leukemia, contains predominantly leukemic promyelocytes (*13, 14*). HL-60 cells cease proliferation and "differentiate" morphologically and functionally toward granulocytes after the addition to the culture of such compounds as dimethyl sulfoxide, butyric acid, dimethylformamide (*10*), and retinoic acid (*11*). The cells can also be induced to differentiate along an alternate pathway, apparently toward monocytes, when treated with certain phorbol esters, especially 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (*12*). Thus, HL-60 cells are remarkable in their capacity to differentiate terminally despite

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Table 1. Effect of DFMO on TPA-induced differentiation of HL-60 cells into monocyte-like cells and on retinoic acid (RA)–induced differentiation of HL-60 cells into granulocyte-like cells. The culture conditions are as described in the legend to Fig. 1. Cells examined for morphology were stained with Wright-Giemsa. Adherence to plastic was determined microscopically. In the TPA studies, the macrophage-like cells were plated onto glass cover slips and, after becoming adherent, were incubated with 1-μm latex beads for 24 hours at 37°C. The fraction of cells completely engulfing three or more beads was then determined microscopically. In the RA studies, the granulocyte-like cells were centrifuged with latex beads at 600*g* and incubated for 45 minutes at 37°C. They were then separated on a Percoll gradient and the fraction of cells completely engulfing three or more beads was determined. For the nitroblue tetrazolium (NBT) reduction assay, 3×10^5 cells in 1 ml of saline were incubated with 1 ml of 0.2 percent NBT for 25 minutes at 37°C. The cells were centrifuged onto microscope slides, stained with Wright's, and examined microscopically for the presence of intracellular formazan deposits. Cellular polyamines 2 days after TPA or RA treatment were measured fluorometrically in acid extracts of cell sonicates (*9*); only the results for putrescine are shown, but spermidine and spermine showed similar increases with TPA or RA treatment and similar decreases with DFMO. The data are means ± standard errors for at least three separate studies.

Treatment	Cells with macrophagic morphology (%)	Cells with granulocytic morphology (%)	Phagocytic cells (%)	Adherent cells (%)	Cells with formazan deposits (%)	Putrescine (picomoles per 10^6 cells)
			TPA studies			
Control	< 15		3 ± 2	< 15		82 ± 12
DFMO	< 15		2 ± 1	< 15		43 ± 8
TPA	> 85		72 ± 5	> 85		245 ± 47
DFMO + TPA	> 85		64 ± 6	> 85		68 ± 7
			RA studies			
Control		< 15	3 ± 2		3 ± 1	87 ± 15
DFMO		< 15	2 ± 1		5 ± 3	52 ± 7
RA		> 85	72 ± 6		71 ± 6	289 ± 35
DFMO + RA		> 85	65 ± 7		68 ± 7	71 ± 9

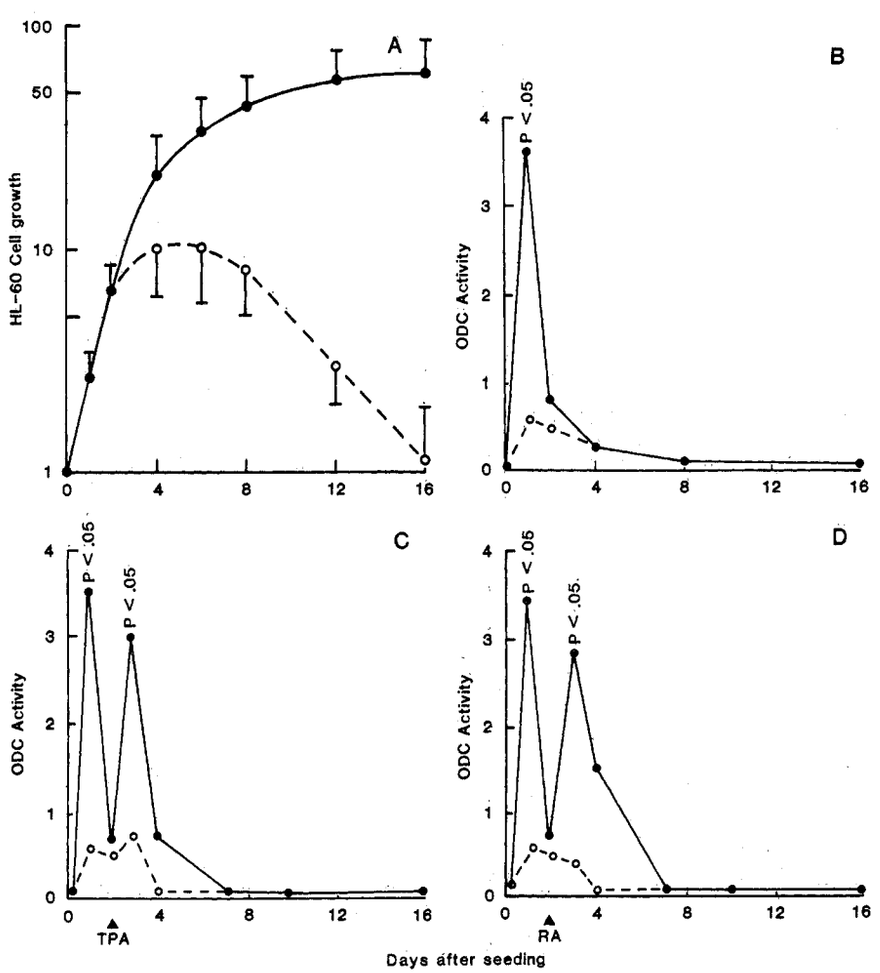

Fig. 1. Effect of DFMO on HL-60 cells in culture. (A) Cell number during proliferation. (B to D) ODC activity during (B) proliferation, (C) TPA-induced differentiation, and (D) retinoic acid–induced differentiation. Symbols: (●) untreated control cells; (○) cells treated with 5 m*M* DFMO continuously after seeding. Cell growth is expressed as N/N_0, where N_0 is the number of cells per milliliter on day 0 and N is the number per milliliter on subsequent days. ODC activity is expressed as nanomoles of substrate per hour per 10^6 cells. The data are means ± standard errors for at least four separate experiments; error bars are not drawn where the standard error is less than 10 percent of the mean. HL-60 cells were seeded at a density of 2×10^5 cells per millimeter in RPMI 1640 medium supplemented with 10 percent fetal calf serum and grown at 37°C with 5 percent CO_2. Viable cells (cells that excluded trypan blue) were counted in a hemocytometer. ODC activity was measured in cells disrupted by sonication by quantifying the $^{14}CO_2$ liberated after incubation with [^{14}C]ornithine (*9*).

their aggressive malignant growth potential in vitro and in vivo. Gallagher *et al.* (*14*) postulated, in this regard, the existence of a "dissociation of genetic control elements for replication and commitment to differentiation."

Huberman *et al.* (*15*) reported that polyamine levels increase with the induced differentiation of HL-60 cells and that the increase may be important for this process. They suggested that the mechanism for the increase might involve biochemical pathways other than the known ODC pathway (*15*). We thus studied the potential role of ODC and polyamine biosynthesis in the proliferation and induced differentiation of HL-60 cells. We report that increases in ODC activity accompany both processes and account for similar increases in polyamine concentrations. However, we also found that the increases are essential only for proliferation.

We cultured HL-60 cells as described in the legend to Fig. 1. During the early exponential growth phase there was a marked but transient increase in ODC activity (Fig. 1, A and B). Putrescine content increased threefold, spermidine threefold, and spermine twofold. The addition of 5 m*M* DFMO at the time of seeding abolished the increase in ODC activity and polyamine content and markedly inhibited proliferation (Fig. 1, A and B). Differences in cell number between control and DFMO-treated cultures could be noted by day 2; the treated cells thus ceased to proliferate after approximately two divisions. After proliferation ceased on day 5 there was an exponential loss of total cells (Fig. 1A). DFMO concentrations greater than 0.2 m*M* inhibited proliferation, with higher

doses achieving greater inhibition. As in our previous studies of human small-cell lung carcinoma (*7*), the inhibitory effect on HL-60 proliferation could be reversed by the simultaneous addition of 10 μM putrescine, the synthetic product of ODC. When added by itself, putrescine did not affect growth of the control cells. These results suggest that continued proliferation and survival of HL-60 cells in culture, like proliferation and survival of the lung carcinoma cells (*7*), are dependent on continued synthesis of polyamines through the ODC pathway.

The effects of DFMO on the growth of HL-60 cells were not associated with detectable cellular differentiation. All the DFMO-treated cells remaining in culture after cessation of proliferation are still promyelocytes, both morphologically and functionally (Table 1).

Next, we studied polyamine biosynthesis and chemically induced differentiation in HL-60 cultures. Two days after being seeded, HL-60 cells were treated with $1.6 \times 10^{-7} M$ TPA (*12*). The transient increase in ODC and polyamines accompanying the first 2 days of growth was followed 2 days after TPA addition by another transient increase in ODC activity (Fig. 1C) and by a threefold increase in the content of putrescine, spermidine, and spermine (Table 1). Two days after the TPA treatment, more than 85 percent of the cells had adhered to the plastic. They now resembled mature monocytes and were able to phagocytose latex beads (Table 1) (*12*). Whether DFMO was added on day 0 to abolish the increases in ODC activity and putrescine content accompanying growth onset and TPA treatment, or was added simultaneously with TPA to abolish only the second ODC and putrescine increase, there was no significant effect on the induced monocytic differentiation (Table 1). These results suggest (i) that DFMO inhibits HL-60 proliferation, but not by causing terminal differentiation of HL-60 cells to monocytes, and (ii) that TPA-induced monocytic differentiation of HL-60 cells is not dependent on ODC activity, despite the increases in ODC that occur when the cells are treated with TPA.

We then treated HL-60 cells with $10^{-7} M$ retinoic acid (*11*). By 2 days after treatment, ODC activity had increased (Fig. 1D), putrescine content had increased fourfold, and spermidine and spermine content had increased threefold (Table 1). After 5 days, more than 85 percent of the cells had acquired the features of mature granulocytes (Table 1). Again, whether 5 mM DFMO was added simultaneously with, or 2 days prior to, the retinoic acid, there was no significant effect on the induced granulocytic differentiation. Thus, as in TPA-induced monocytic differentiation, retinoic acid–induced granulocytic differentiation of HL-60 cells is not dependent on the increase in ODC activity.

Our results show that, in the HL-60 cell line, increased ODC activity and putrescine content are associated with the onset of cell proliferation and chemically induced differentiation. DFMO is effective in abolishing the increases in ODC associated with both processes, but only proliferation is suppressed; differentiation continues unhindered. There appear to be fundamental differences in the action of ODC in the proliferation and differentiation of HL-60 cells.

The polyamines have been proposed as being essential to the onset of cell proliferation (*1*, *2*)—an event which, of course, involves the initiation of DNA synthesis. Polyamine starvation interferes with the initiation of growth and the onset of DNA replication (*2*, *6*). Several investigators recently showed that TPA-induced monocytic differentiation of HL-60 cells is not dependent on DNA synthesis (*16*). Our results support this finding and suggest that the proliferative and differentiative responses of HL-60 cells in culture are unrelated.

It is not known why TPA and retinoic acid induces an increase in ODC if polyamine biosynthesis plays no role in the biological response of HL-60 cells to these agents. Perhaps the interaction of TPA with its membrane receptor (*17*) and of retinoic acid with its binding protein (*18*) initiate other elements of a target tissue response as well as a series of events in differentiation. Although this response may not be manifested biologically, some of its biochemical consequences, such as increased ODC, might be present. This postulation could be especially applicable to TPA, which in several cell types has a potent tumor promotion effect accompanied by increased ODC activity.

The use of DFMO to inhibit polyamine biosynthesis appears to be a potent tool for dissecting events in cellular differentiation which do and do not involve the need for concomitant proliferation. For example, failure of embryonic differentiation to occur in the presence of DFMO (*8*) and of intestinal mucosal maturation to proceed normally in the newborn rat (*9*) could be due to inhibition of the cell proliferation required for these events. Continued studies with DFMO in cell systems that model specific differentiative events may clarify the role of polyamines and cellular proliferation in differentiation.

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