POTENT INHIBITION OF ORNITHINE DECARBOXYLASE BY β,γ UNSATURATED SUBSTRATE ANALOGS

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Summary

Trans-3-dehydro-D, L-ornithine and trans-1, 4-diamino-2-butene have been synthesized and shown to be potent competitive inhibitors of ornithine decarboxylase. The K_I's for trans-3-dehydro-L-ornithine and trans-1, 4-diamino-2-butene are 2.2 and 2.0 μM respectively. Both analogs bind much more tightly to the enzyme than either ornithine or putrescine. Studies of chick embryo muscle cells in culture show results consistent with reversible inhibition of division and/or fusion by addition of trans-3-dehydro-D, L-ornithine to the culture medium.

The frequent correlation of polyamine synthesis with rapid cell division and growth has been well documented (1, 2). Ornithine decarboxylase catalyzes the first step in polyamine synthesis in eukaryotic organisms. The rapid and marked response of this enzyme to various hormonal and other growth stimuli (3) suggests its possible importance as a regulatory enzyme. Thus, it would be of considerable interest to find a very specific inhibitor of this enzyme which might be used in vivo to test the role of polyamine synthesis in rapid cell growth.

Of the many compounds which have been tested, so far only two have been reported to show significant specific inhibition of this enzyme. α-Methyl D, L-ornithine is a fair competitive inhibitor of rat prostate ornithine decarboxylase (4), having an affinity more than 3 times that for L-ornithine, but it is only a very weak inhibitor of the rat liver enzyme (2). α-Hydrazino-ornithine shows strong inhibition of ornithine decarboxylase (4, 5), but this inhibition can be reversed by addition of pyridoxal 5'-phosphate, so the specificity of this inhibitor is questionable. Other PLP-requiring enzymes are also affected by this inhibitor, although to a lesser extent.

Previously, it was found that β,γ unsaturated amino acids can be profound

and irreversible inhibitors of certain pyridoxal phosphate linked enzymes (6). This report describes the synthesis and interactions of several β,γ unsaturated inhibitors of pyridoxal phosphate linked ornithine decarboxylase; one of these inhibitors appears promising for use in further in vivo studies to delineate the physiological roles of this enzyme and the polyamines.

Materials and Methods

Ornithine decarboxylase was prepared from the livers of rats which had been injected with thioacetamide, as described by Ono et al. (7). This enzyme had a specific activity of 0.04 - 0.06 nmoles CO_2/min - mg protein. Ornithine decarboxylase activity was assayed as described by Janne and Williams-Ashman (8). D, L-ornithine-[1-^{14}C] was purchased from New England Nuclear, as were all other radiochemicals used.

Pig kidney diamine oxidase was obtained from Sigma Chemical Co.; activity was measured using cadaverine-[^{14}C] (9).

Yeast S-adenosyl-L-methionine decarboxylase was prepared by the method of Janne et al. (10). Activity of this enzyme was assayed as described by Janne and Williams-Ashman (11). The use of this enzymatic method as a micro assay for putrescine, a potent activator of yeast S-adenosyl-L-methionine decarboxylase, has been described by Harik et al. (12). The 1, 4 diamino-trans-2-butene was tested and found to produce 40% the activation seen with putrescine over a considerable linear range.

L-Ornithine carbamyl transferase was assayed in crude homogenates of rat liver prepared as described by Weber and Singhal (13). The assay procedure used was that of Weber et al (14). For L-ornithine δ-transaminase, crude rat liver homogenates were prepared and assayed as described by Peraino and Pitot (15).

Trans-3-dehydro-D, L-ornithine was synthesized by a procedure similar to the one reported for vinyl glycine (16). Complete details of its synthesis and properties will be reported elsewhere. Trans 1, 4-diamino-2-butene was prepared by the method of Karlen (17).

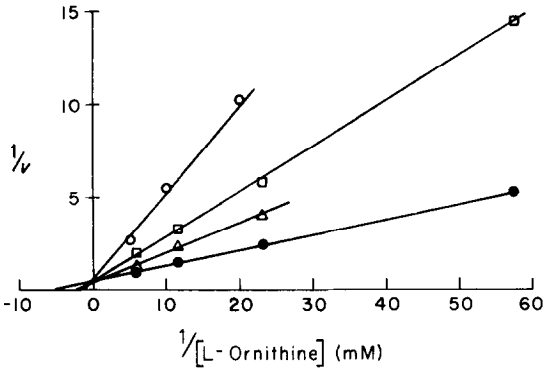


Fig. 1. Inhibition by dehydro-ornithine. The 1 ml assay mixtures each consisted of 0.3 units (1 unit = 1 nmole of CO_2/min) of enzyme in 0.035 M phosphate buffer, pH 7, containing 0.18 mM PLP and 7 mM dithiothreitol. The concentrations of L-ornithine-[carboxyl-^{14}C] were varied as indicated. Samples were incubated for 60 min at 37°, then stopped by injection of citric acid. The enzyme standard (●—●) is shown, as well as identical samples containing 5 μM (Δ—Δ), 10 μM (□—□), and 20 μM (o——o) dehydro-ornithine. The velocity (v) is expressed as nmoles CO_2 per 60 min per mg protein.

Results and Discussion

Inhibition of Ornithine Decarboxylase by trans-3-Dehydro-D, L-Ornithine

Trans-3-dehydro-D, L-ornithine (henceforth referred to as dehydro-ornithine) is an effective competive inhibitor of rat liver ornithine decarboxylase. The substrate-competitive inhibition observed with this analog is shown in Fig. 1. The K_I measured was 4.4 μM. However, in view of the strict stereospecificity of this enzyme, it is likely that only the L-isomer is active, and that the K_I for the pure L-isomer is 2.2 μM. The enzyme affinity for this analog is about 100 times greater than that for L-ornithine. No time-course was observed for inhibition by dehydro-ornithine, and thorough dialysis of the inhibited enzyme completely restored its original activity. Therefore, the possibility of irreversible inactivation can be ruled out. The inhibition by dehydro-ornithine is not competitive with pyridoxal phosphate (Fig. 2). In fact, high pyridoxal phosphate concentrations characteristically resulted in an additional slight inhibition.

Is Dehydro-Ornithine a Substrate for Ornithine Decarboxylase?

The question of the possible decarboxylation of dehydro-ornithine is of

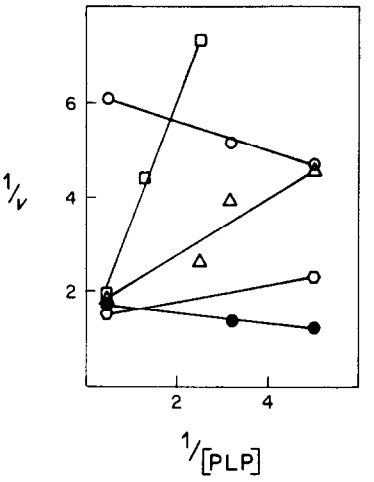


Fig. 2. Competition of pyridoxal phosphate (PLP) with various inhibitors. Assay mixtures were similar to those in Fig. 1, but each contained 0.1 mM L-ornithine-1-^{14}C, while concentrations of PLP were varied. Both the standard enzyme (●—●), and the sample which contained 20 μM trans-3-dehydro-ornithine (○—○) were slightly inhibited by the excess PLP. On the other hand, the inhibition by 10 μM 1, 4-diamino-trans-2-butene (△—△), 0.5 mM 1, 4-diamino-2-butyne (□—□), and 1.0 mM propargylamine (⬡—⬡) were reversed by excess PLP.

Table 1 Limits of possible decarboxylation of trans-3-dehydro-ornithine

Method	Detection limit*	Maximum possible 1,4-diamino-trans-2-butene**	Estimated diamine formation***	% of substrate activity excluded
electrophoretic isolation	5 nmoles	75 nmoles	0.75 nmoles	670%
diamine oxidase inhibition	0.5 nmoles	200 nmoles	3.0 nmoles	17%
S-adeno syl-methionine decarboxylase activation	0.1 nmoles	40 nmoles	1.5 nmoles	7%

* Minimum amount of 1, 4-diamino-trans-2-butene shown to be clearly detectable under experimental conditions employed.

**Based on total conversion of L-isomer of trans-3-dehydro-ornithine to diamine.

***Based on assumption that rate of analog decarboxylation is identical with that for ornithine.

interest because the product of this decarboxylation, trans 1, 4-diamino-2-butene, is also a highly potent competitive inhibitor of the enzyme. However, several approaches failed to measure any product formation. These approaches are summarized, along with their limits of detection, in Table I. Failure to detect any diamine formation by electrophoretic isolation was sufficient to establish that product inhibition was not the basis of the observed inhibition. The two enzymatic methods show that if there is any decarboxylation of this substrate analog, it occurs at a rate less than 10% that of the normal substrate decarboxylation.

The inhibition of diamine oxidase which was used as a sensitive assay for 1, 4-diamino-trans-2-butene should be mentioned. We have found that dog kidney diamine oxidase was very strongly inhibited by both 1, 4-diamino-trans-2-butene and 1, 4-diamino-2-butyne. This inhibition, in both cases, appeared to consist of both a competitive and a relatively irreversible component (not removed by gel filtration).

Inhibition of Ornithine Decarboxylase by trans-1, 4-Diamino-2-Butene

Despite the very weak competitive inhibition of ornithine decarboxylase by putrescine (7), 1, 4-diamino-trans-2-butene proved to be an extremely potent competitive inhibitor, as shown in Figure 3. The K_I measured in these experiments was 2 μM; this indicates an affinity more than 10,000 times that for putrescine. While this difference is very likely due, in part, to a preferential binding of the trans-conformer, this is not the complete explanation. Although this inhibition is strongly competitive with substrate, it can be reversed by very high concentrations (2 mM) of pyridoxal 5'-phosphate (Fig. 2). Also, 1, 4-diamino-2-butyne is a fairly good inhibitor, and even propargylamine shows significant inhibition. The inhibition observed in these cases is also reversed by pyridoxal 5'-phosphate. It is likely that the potent inhibition observed here with the unsaturated diamine results from prior complex formation between the amine and pyridoxal 5'-phosphate. These complexes are thought to be the actual competitive inhibitors.

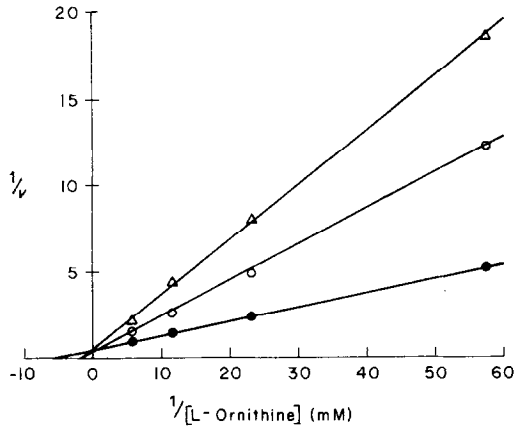
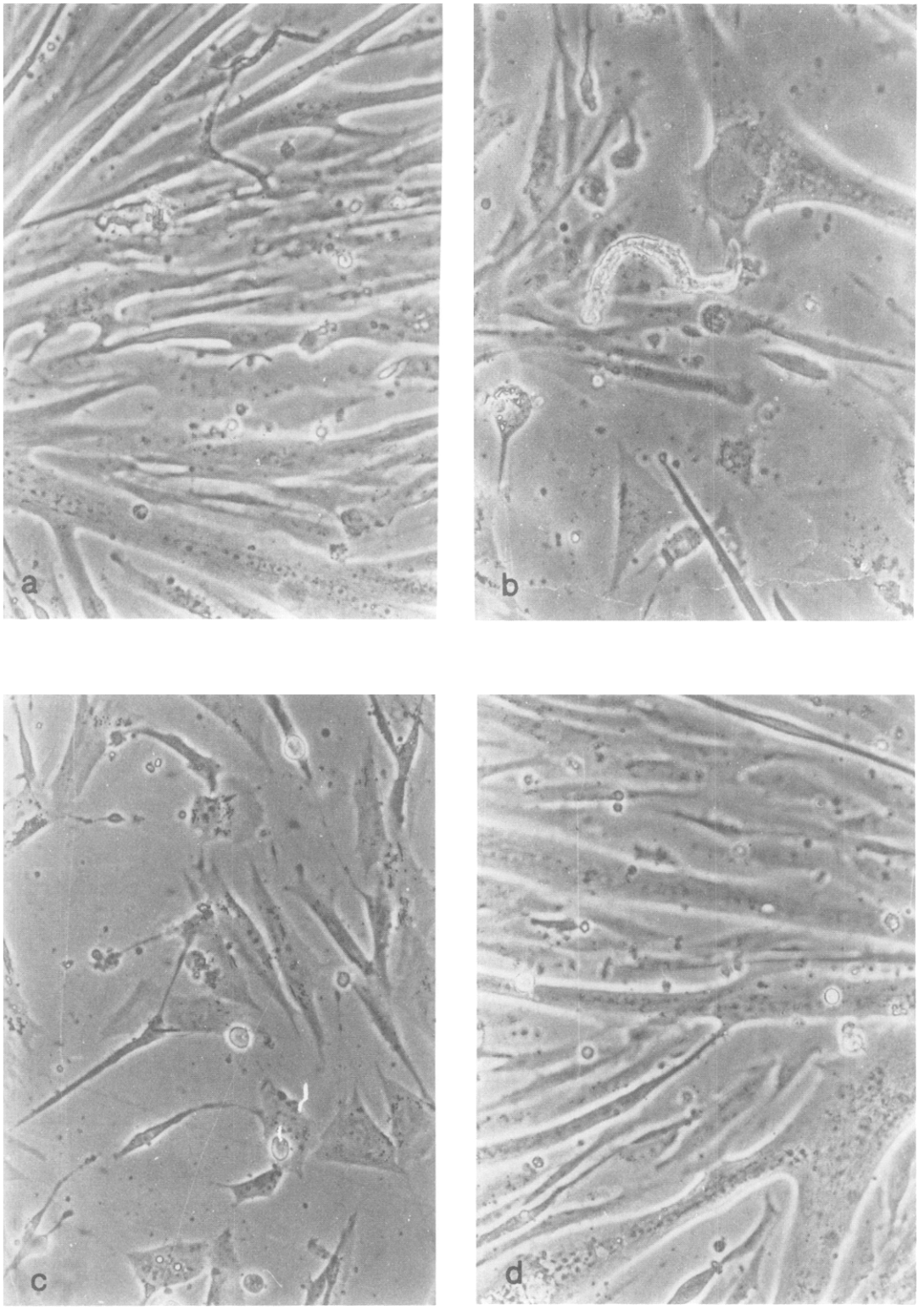


Fig. 3. Inhibition by 1, 4-diamino-trans-2-butene. These inhibition studies were done under the conditions described in Fig. 1. The standard enzyme (●—●), and samples containing 5 μM (o—o) and 10 μM (Δ—Δ), 1, 4-diamino-trans-2-butene.

Effect of Dehydro-Ornithine on Muscle Culture

A series of experiments was done to test the effect of trans-3-dehydro-D, L-ornithine on cultured 11-day chick embryo pectoral muscle cells. (It should be noted that these preparations also contained significant numbers of fibroblasts). This source is especially suitable since ornithine decarboxylase is known to be relatively high in chick embryo from days 3 to 11, declining from then until hatching, and it is highest in muscle (18). The ornithine decarboxylase from 11-day chick embryo pectoral muscle was found to be similar to that from rat liver both in its affinity for substrate (K_S=0.15 mM) and its affinity for trans-3-dehydro-D, L-ornithine (K_I=2.8 μM). No irreversible inhibition by this analog was apparent. Also, there was inhibition by 1, 4-diamino-trans-2-butene and 1, 4-diamino-2-butyne which was reversed by high PLP. Although addition of 1 mM 1, 4-diamino-trans-2-butene had no observable effect on the muscle cultures, a published report has since shown that putrescine does not readily diffuse in or out of cells (19), so this analog probably did not enter the cells.

When concentrations as low as 60 μM trans-3-dehydro-D, L-ornithine (30 μM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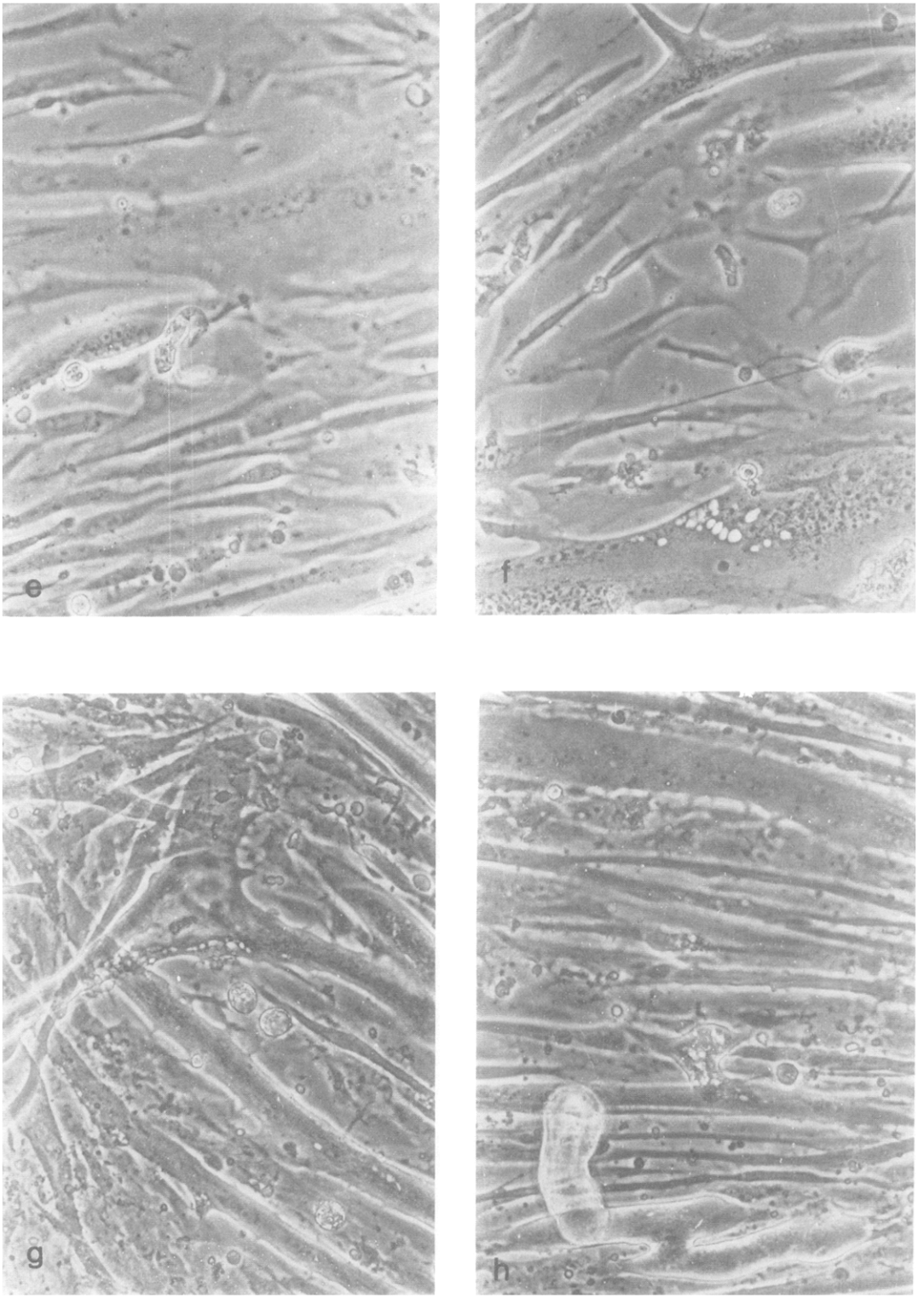
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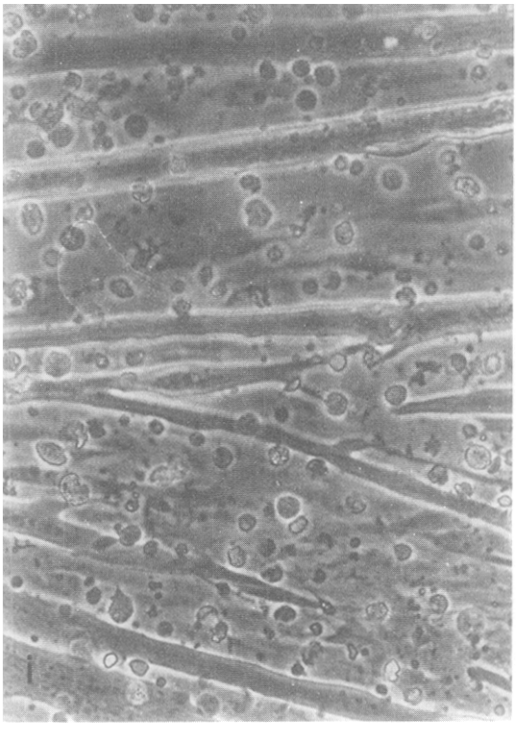
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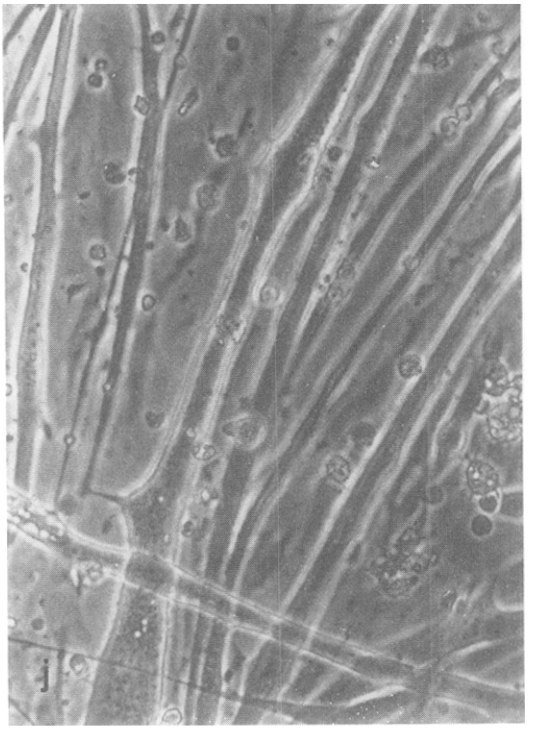
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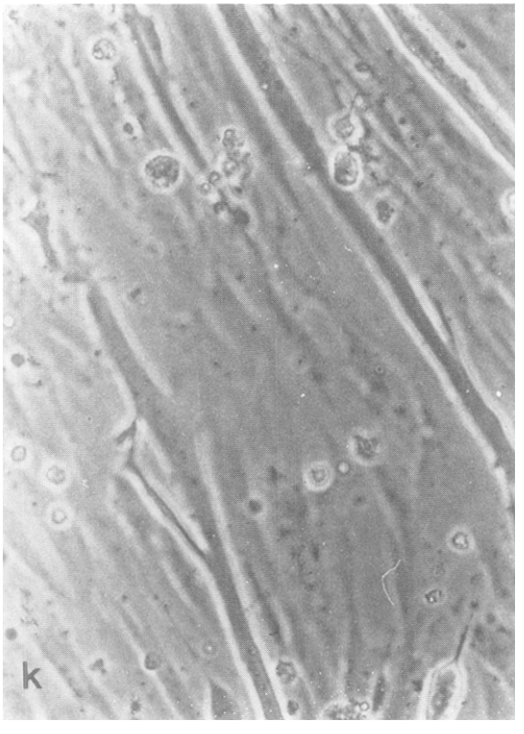
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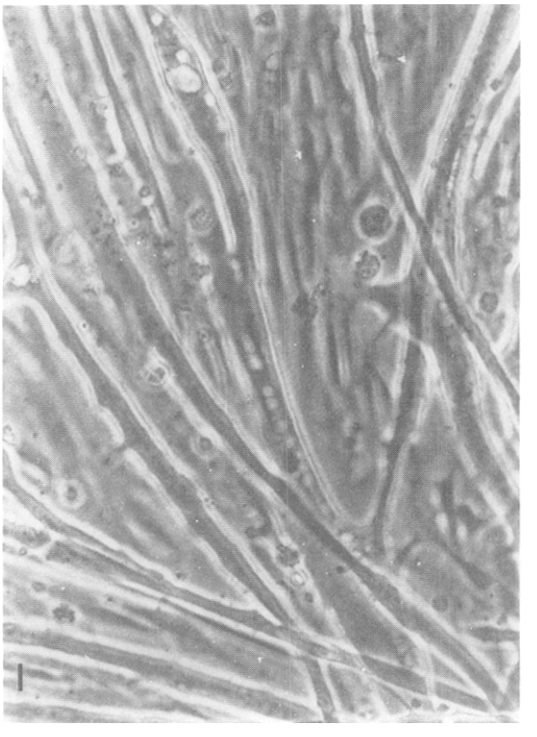
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L-analogue) were added at 3 - 5 hrs after plating of the muscles, a distinct effect was apparent within 48 hrs. The inhibited cell cultures had both fewer total cells, and particularly fewer differentiated fibers (Fig. 4). The difference became progressively more striking with continued incubation with inhibitor. As shown in Fig. 4, the inhibition was reversed somewhat by addition of a 40-fold excess (with respect to L-isomer) of L-ornithine. Unfortunately, higher concentrations of ornithine were somewhat toxic and so could not be used for further reversal. Several attempts failed to achieve an accurate estimation of diamine levels in these cultured cells, due to the small amounts of material available. Removal of inhibitor by replacement with fresh medium resulted in considerable recovery of the cells within about 48 hrs.

Assays of rat liver ornithine carbamyl transferase and ornithine α-transaminase showed that neither of these enzymes would be likely to be significantly inhibited by the concentrations of trans-3-dehydro-ornithine used (Table 2). Also, both of these enzymes are known to be extremely low or absent in muscle (15, 20). Thus, it is highly unlikely that effects on these other ornithine-

Table II Inhibition of ornithine-metabolizing enzymes

Enzyme (rat liver)	K_M for L-ornithine	L-ornithine	trans-3-dehydro-D, L-ornithine	Ratio of orn/analog	% inhibition
ornithine-carbamyl transferase	1.0 mM (12)	30 mM	3 mM	10	10
ornithine-δ-transaminase	7.0 mM (13)	100 mM	3 mM	33	15
ornithine decarboxylase	0.2 mM	10 mM	0.2 mM	50	50

Fig. 4. Reversible inhibition of muscle cell cultures by trans-3-dehydro-L-ornithine. Photos 1 a-f show the muscle cells on the 6th day in culture; a - control, b - 1.3 mM L-ornithine, c - 60 μM trans-3-dehydro-D, L-ornithine (analog), d - 60 μM analog + 1.3 mM L-ornithine, e - 120 μM analog, f - 120 μM analog + 1.3 mM L-ornithine. Photos 2 a-f show these same six plates 4 days after removal of inhibitor and ornithine from medium (10th day in culture).

metabolizing enzymes are responsible for the observed cellular inhibition. The results are all consistent with the reversible inhibition of division and/or fusion mediated in some manner by the reversible inhibition of ornithine decarboxylase by trans-3-dehydro-ornithine. While these results clearly require further investigation, it seems likely that dehydro-ornithine will be a fruitful tool for study of the elusive role of the polyamines in cell growth.

Acknowledgements

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