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Indirect evidence for a strict negative control of *S*-adenosyl-L-methionine decarboxylase by spermidine in rat hepatoma cells

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1. Direct or indirect inhibitors of L-ornithine decarboxylase (EC 4.1.1.17), structurally related or unrelated to L-ornithine, including DL-α-difluoromethylornithine, α-methylornithine and 1,3-diaminopropane, used alone or in combination, decreased polyamine concentrations in rat hepatoma tissue culture (HTC) cells and increased *S*-adenosyl-L-methionine decarboxylase activity (EC 4.1.1.50). 2. Comparison of the catalytic properties of *S*-adenosyl-L-methionine from cells with elevated and normal activities revealed no apparent modification of the catalytic site as judged by affinity for the substrate, stimulation by di- and tri-amines and inhibition by methylglyoxal bis-(guanylhydrazone). 3. Actinomycin D and cycloheximide, an RNA and a protein-synthesis inhibitor respectively, blocked the increase of *S*-adenosyl-L-methionine decarboxylase activity elicited by α-difluoromethylornithine. In polyamine-depleted cells the apparent half-life of elevated *S*-adenosyl-L-methionine decarboxylase activity, determined by inhibition of protein synthesis, was 2.5-fold longer than in control cells. The present results suggest that elevation of *S*-adenosyl-L-methionine decarboxylase activity by α-difluoromethylornithine is due to stabilization of the enzyme. 4. Restoration of the normal intracellular putrescine content, by addition of putrescine to the medium of polyamine-deficient cells, transiently increased *S*-adenosyl-L-methionine decarboxylase activity. Thereafter, intracellular conversion of putrescine into spermidine was accompanied by inactivation of the enzyme at a rate that was similar to that found on addition of spermidine itself. No relationship between total intracellular spermine content and *S*-adenosyl-L-methionine decarboxylase activity could be established. 5. Addition of 1mM-1,3-diaminopropane to polyamine-deficient cells did not cause a decrease in the activity of *S*-adenosyl-L-methionine decarboxylase, whereas addition of 1,5-diaminopentane (cadaverine) did. 1,3-Diamino-*N*-(3-aminopropyl)propane did not accumulate in cells treated with α-difluoromethylornithine and 1,3-diaminopropane, whereas addition of 1,5-diaminopentane led to the accumulation of 1,5-diamino-*N*-(3-aminopropyl)pentane. 1,3-Diamino-*N*-(3-aminopropyl)propane (10μM) was as effective as spermidine in decreasing *S*-adenosyl-L-methionine decarboxylase activity. Thus effectiveness of a diamine in decreasing enzyme activity is related to its capability of being converted into a closely structurally related homologue of spermidine by spermidine synthase. 6. The spermidine site of action appears to be post-translational since (*a*) the spermidine-induced decrease of *S*-adenosyl-L-methionine activity was not prevented by actinomycin D and (*b*) spermidine in the presence of cycloheximide led to a synergistic inactivation of the enzyme with a decay rate that progressively approached control values. Altogether these results are indirect evidence for a strict negative control of *S*-adenosyl-L-methionine decarboxylase by spermidine and substantiate previous findings [Mamont, Duchesne, Grove & Tardif (1978) *Exp. Cell Res.* **115**, 387–393]. Spermidine appears to act on some processes involved in denaturation and/or degradation of the enzyme protein. Putrescine appears to decrease the rate of these processes. The physiological significance of the regulatory control of *S*-adenosyl-L-methionine decarboxylase is discussed.

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In mammalian cells, biosynthesis of the polyamines, putrescine, spermidine and spermine involves the sequential action of two decarboxylases and two aminopropyltransferases (Tabor & Tabor, 1976; Williams-Ashman & Canellakis, 1979). The first step is the decarboxylation of L-ornithine to putrescine catalysed by the pyridoxal phosphate-dependent enzyme L-ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) (Russell & Snyder, 1968). The second decarboxylase is *S*-adenosyl-L-methionine decarboxylase (*S*-adenosyl-L-methionine carboxy-lyase, EC 4.1.1.50), which generates an activated aminopropyl group needed to convert putrescine into spermidine and spermidine into spermine (Pegg & Williams-Ashman, 1969). That polyamine biosynthesis is a finely modulated and precisely timed process is now well documented (for a recent review see Maudsley, 1979).

In recent years, much effort has been directed towards the elucidation of mechanisms of regulation of L-ornithine decarboxylase, whereas less attention has been paid to the ways in which *S*-adenosyl-L-methionine decarboxylase is controlled. Apart from the fact that putrescine is a powerful activator *in vitro* of mammalian *S*-adenosyl-L-methionine decarboxylase (Pegg & Williams-Ashman, 1969; Williams-Ashman *et al.*, 1977) and that the enzyme appears to possess a very high turnover (Hannonen *et al.*, 1972; Fillingame & Morris, 1973; Pegg, 1979), little is known at present concerning the regulation of *S*-adenosyl-L-methionine decarboxylase gene expression. We have previously reported that DL-α-methylornithine and DL-α-difluoromethylornithine, a competitive (Abdel-Monem *et al.*, 1974) and an enzyme-activated irreversible inhibitor (Metcalf *et al.*, 1978) of L-ornithine decarboxylase respectively, lead to an increase in *S*-adenosylmethionine decarboxylase activity that correlates inversely with the depletion of intracellular putrescine and spermidine content in hepatoma tissue culture (HTC) cells and in HMOA cells, a putrescine- and spermidine-overproducing variant of this cell line (Mamont *et al.*, 1978*b*,*c*). These findings lead to the postulate that intracellular spermidine exerts a direct or indirect negative control in the expression of *S*-adenosyl-L-methionine decarboxylase. We report here further evidence in favour of this concept.

Experimental

Chemicals

Methylglyoxal bis(guanylhydrazone) {1,1'-[(methylethanediylidine)-dinitrilo]diguanidine} was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Actinomycin D, cycloheximide, emetine, *S*-adenosyl-L-methionine, putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrahydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), 1,5-diaminopentane dihydrochloride was from Fluka A.G. (Buchs, Switzerland). 1,3-Diaminopropane, 1,3-diamino-2-propanol (Fluka A.G.) and 1,3-diamino-*N*-(3-aminopropyl)propane (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) were transformed into their corresponding hydrochlorides before use. 1,5-Diamino-*N*-(3-aminopropyl)pentane was kindly donated by Dr. R. D. Westland (Warner–Lambert, Parke Davis, Ann Arbor, MI, U.S.A.). DL-α-Methylornithine, (+)-α-methylornithine, (−)-α-methylornithine and DL-α-difluoromethylornithine were synthesized in our Centre (Bey *et al.*, 1978; Metcalf *et al.*, 1978).

When added to the cell culture medium, compounds were dissolved in phosphate-buffered saline, and the solutions were adjusted to pH 7.4 with 10 mM-NaOH. Sterilization was obtained by filtration of these solutions through 0.22 μM pore-size membrane filters. *S*-Adenosyl-L-[1-^{14}C]methionine (sp. radioactivity 60 Ci/mol) and [5-^{3}H]uridine (sp. radioactivity 5 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks, U.K.). [1,5-^{14}C]Cadaverine (sp. radioactivity 106 Ci/mol) was obtained from New England Nuclear (Boston, MA, U.S.A.).

Cell culture

HTC cells, an established line derived from Morris rat hepatoma 7288 C (Thompson *et al.*, 1966), were routinely grown as a suspension culture in Swim's 77 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 16.6 mM-glucose, 5.9 mM-$NaHCO_3$, 50 mM-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}, 2 mM-glutamine and 10% (v/v) newborn calf serum (Hershko & Tomkins, 1971). Dialysed horse serum (Gibco) (10%, v/v) replaced newborn-calf serum in experiments with ornithine decarboxylase inhibitors and polyamines. Cell growth and viability were measured by cell counting in a haemocytometer in the presence of Trypan Blue.

S-Adenosyl-L-methionine decarboxylase assay

Cells (10^7) were harvested by centrifugation, washed with cold phosphate-buffered saline and sonicated in 0.7 ml of buffer consisting of 100 mM-sodium phosphate buffer, pH 7.2, 5 mM-dithiothreitol (Sigma Chemical Co.) and 0.1 mM-EDTA. Crude sonicated-cell extracts or the 100000 ***g*** supernatants were dialysed for 24 h against 100 vol. of the same buffer before assay. *S*-Adenosyl-L-methionine decarboxylase activity was determined by the procedure developed by Pegg & Williams-Ashman (1969). Standard assays contained 5 μmol of putrescine, 0.8 μmol of *S*-adenosyl-L-methionine, 1 μCi of radiolabelled *S*-adenosyl-L-methionine,

5μmol of dithiothreitol in 0.5ml of 100mM-sodium phosphate buffer, pH 7.2, and 0.5ml cell extracts. Proteins were determined by a modification (Ross & Schatz, 1973) of the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V, Sigma Chemical Co.) as standard.

Polyamine determinations

Cells were extracted with $HClO_4$ as previously described (Mamont *et al.*, 1978*a*). Measurements of polyamines were carried out with a Durrum D500 amino-acid analyser by the method of Marton & Lee (1975).

Uridine incorporation into RNA

Portions (10ml) of a suspension of cultured HTC cells were pulse-labelled for 30min in the presence of 2.4μCi of [5-^{3}H]uridine/ml (sp. radioactivity 0.8Ci/mmol). At the end of the labelling period, cells were collected by centrifugation, washed three times with cold phosphate-buffered saline, and precipitated by 10% (v/v) trichloroacetic acid. The trichloroacetic acid pellet was washed with 5% (v/v) trichloroacetic acid, ethanol/diethyl ether (3:1, v/v) and diethyl ether and was dissolved in 0.5M-NaOH at 37°C overnight. Radioactivity was determined in portions added to 10ml of scintillation fluid consisting of 0.4% (w/v) Omnifluor (New England Nuclear Boston, MA, U.S.A.) and 25% (v/v) Triton (Packard Instruments Co., Downers Grove, IL, U.S.A.) in toluene.

Results

Effects of ornithine decarboxylase inhibitors on intracellular polyamine content and S-adenosyl-L-methionine decarboxylase activity

α-*Difluoromethylornithine.* As illustrated in Fig. 1, dose-dependent depletion of HTC cell putrescine and spermidine content by α-difluoromethylornithine was accompanied by a dose-dependent increase in *S*-adenosyl-L-methionine decarboxylase activity, the maximum effect being observed at concentrations $\geqslant$100μM. The intracellular spermine content remained unmodified or was slightly increased (1.2-fold over control values).

(+)- *and* (−)-α-*methylornithine.* That the increase of *S*-adenosyl-L-methionine decarboxylase activity is related to the polyamine-depleting property of L-ornithine decarboxylase inhibitors was further demonstrated by taking advantage of the stereospecificity of enzyme catalysis. Thus (−)-α-methylornithine, which exhibits only 2% as much inhibitory activity *in vitro* against L-ornithine decarboxylase as the (+)-enantiomer (Bey *et al.*, 1978), decreased accumulation of putrescine and spermidine but did not completely deplete the cells of these amines (Fig. 2). As expected, *S*-adenosyl-L-methionine decarboxylase activity was not affected. In contrast, the active enantiomer (+)-α-methylornithine depleted putrescine and spermidine concentrations and increased *S*-adenosyl-L-methionine decarboxylase activity.

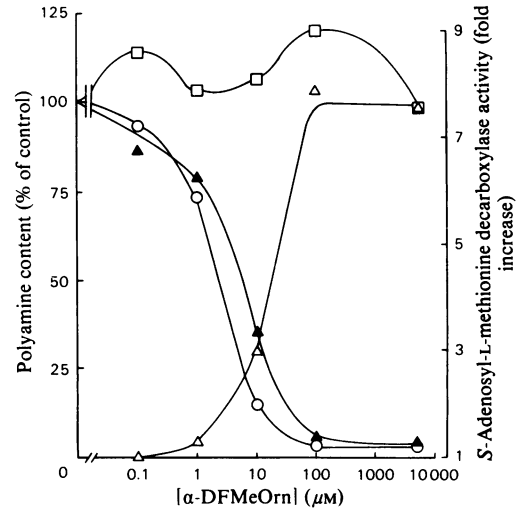

Fig. 1. *Dose-dependent effects of* α-*difluoromethylornithine* (α-*DFMeOrn*) *on S-adenosyl-L-methionine decarboxylase activity and intracellular polyamine content*

High-density cell cultures (8×10^5cells/ml) were diluted 8-fold by fresh medium supplemented with 10% (v/v) dialysed horse serum and were further incubated for 24h in the absence or in the presence of various α-difluoromethylornithine concentrations. At that time, *S*-adenosyl-L-methionine decarboxylase activities and intracellular polyamines were measured as described in the Experimental section. Results are means for two separate cultures and are expressed as fold increase relative to control *S*-adenosyl-L-methionine decarboxylase activity (1.1nmol/h per mg of protein) and as percentages of control values for putrescine (0.42nmol/10^6 cells), spermidine (2.65nmol/10^6 cells) and spermine (2.32nmol/10^6 cells). Symbols: △, *S*-adenosyl-L-methionine decarboxylase; ○, putrescine; ▲, spermidine; □, spermine.

1,3-Diaminopropane. 1,3-Diaminopropane, the lower homologue of putrescine, decreases L-ornithine decarboxylase activity in many cultured cell lines (McCann *et al.*, 1977; Heller *et al.*, 1978; Bethell & Pegg, 1979; Alhonen-Hongisto *et al.*, 1979) and decreases polyamine accumulation in Ehrlich ascites cells (Alhonen-Hongisto *et al.*, 1979). Addition of 1mM-1,3-diaminopropane to HTC cell cultures almost completely depleted putrescine and decreased the initial contents of spermidine and spermine by 53 and 45% respectively (Figs. 3*b*–3*d*). α-Difluoromethylornithine (5mM) completely dep-

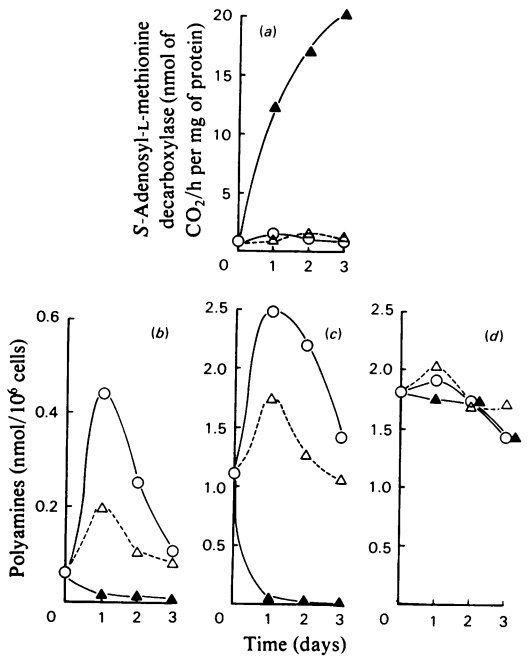

Fig. 2. *Effects of* (+)-α-*methylornithine and* (−)-α-*methylornithine on S-adenosyl-L-methionine decarboxylase activity and intracellular polyamine content*

High-density cell cultures were diluted by fresh medium supplemented with 10% newborn calf serum and were further incubated in the absence or in the presence of 2.5 mM-(+)-α-methylornithine or 2.5 mM-(−)-α-methylornithine. *S*-Adenosyl-L-methionine decarboxylase activities (*a*), putrescine (*b*), spermidine (*c*) and spermine (*d*) were measured at the indicated times. ○, Control; △, (−)-α-methylornithine; ▲, (+)-α-methylornithine.

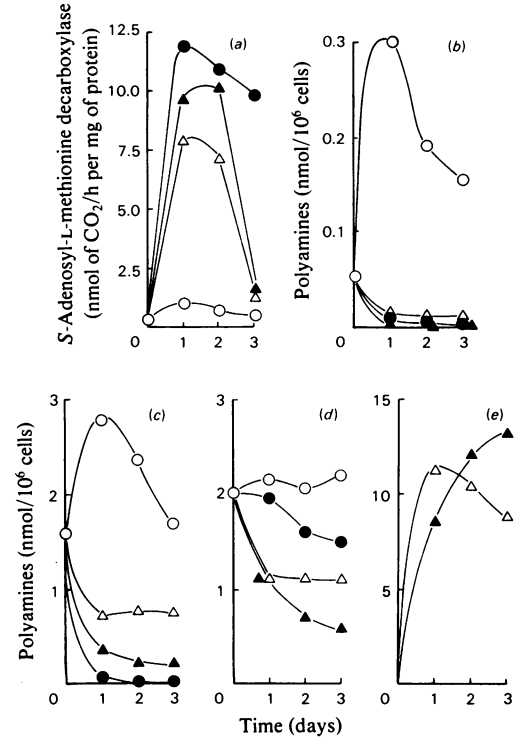

Fig. 3. *Effects of* α-*difluoromethylornithine and 1,3-diaminopropane, alone or in combination, on S-adenosyl-L-methionine decarboxylase activity and intracellular polyamine content*

High-density cell cultures were diluted as described in the legend to Fig. 1. Cells were further incubated in the absence or in the presence of 5 mM-α-difluoromethylornithine, 1 mM-1,3-diaminopropane or 1 mM-1,3-diaminopropane plus 5 mM-α-difluoromethylornithine. *S*-Adenosyl-L-methionine decarboxylase activities (*a*), putrescine (*b*), spermidine (*c*) and spermine (*d*) and intracellular 1,3-diaminopropane (*e*) were measured as described in the Experimental section. ○, Control; ●, α-difluoromethylornithine; △, 1,3-diaminopropane; ▲, α-difluoromethylornithine + 1,3-diaminopropane.

leted putrescine and spermidine and decreased by 25% the spermine content of the cell. As demonstrated, maintenance of high spermine content results from incomplete blockade of L-ornithine decarboxylase activity by the ornithine analogue (Mamont *et al.*, 1978*c*). Combination of 1,3-diaminopropane and α-difluoromethylornithine led to a slightly lower decrease in the spermidine content (77% of initial content) compared with α-difluoromethylornithine used alone (96% of initial content). At the same time spermine content was much more affected than when the amine or the ornithine analogue were used separately. These findings suggest that, in addition to its L-ornithine decarboxylase inhibitory activity, 1,3-diaminopropane may possibly affect *in situ* the spermine synthase reaction, confirming the previously reported inhibition of the enzyme *in vitro* (Hibasami & Pegg, 1978). Spermine depletion, observed after combination of 1,3-diaminopropane and α-difluoromethylornithine, appears to be due to dilution resulting from an increase cell number (Table 1). Cellular concentration of 1,3-diaminopropane ranged from 7 to 14 nmol/10^6 cells (Fig. 3*e*). Trace amounts (0.07–0.11 nmol/10^6 cells) of 1,3-diamino-*N*-(3-aminopropyl)propane accumulated intracellularly, especially when 1,3-diaminopropane was added in combination with α-difluoromethylornithine. This also confirms that 1,3-diaminopropane is a poor substrate for spermidine synthase (Hibasami & Pegg, 1978). When compared at days 1 and 2, *S*-adenosyl-L-methionine decarboxylase activity was inversely related to the spermidine-depleting

Table 1. *Effects of L-ornithine decarboxylase inhibitors on cell growth*
Experimental conditions refer to experiments illustrated in Figs. 2 and 3.

	10^{-5} × Cell number (cells/ml of cell culture)			
	Day 0	Day 1	Day 2	Day 3
Control (Fig. 2)	1.0	1.87	3.84	7.81
(+)-α-Methylornithine (2.5 mM)	1.0	1.92	2.40	2.89
(−)-α-Methylornithine (2.5 mM)	1.0	1.87	3.86	7.11
Control (Fig. 3)	1.06	2.18	5.13	8.31
DL-α-Difluoromethylornithine (5 mM)	1.06	2.17	2.77	3.52
1,3-Diaminopropane (1 mM)	1.06	2.20	5.07	6.73
DL-α-Difluoromethylornithine (5 mM) + 1,3-diaminopropane (1 mM)	1.06	2.16	4.73	6.45

efficiency of the L-ornithine decarboxylase inhibitors used alone or in combination (Fig. 3*a*). An obvious explanation for the rapid fall of enzyme activity between days 2 and 3 in cultures incubated in the presence of 1,3-diaminopropane alone or in combination with α-difluoromethylornithine cannot be furnished at the present time.

Effects of L-ornithine decarboxylase inhibitors on cell growth

As shown in Table 1, α-difluoromethylornithine decreased cell proliferation after a lag period corresponding to one generation time, confirming previous reports (Mamont *et al.*, 1978*a*,*b*,*c*, 1980). Similar anti-proliferative effects of DL-α-methylornithine, a competitive inhibitor of L-ornithine decarboxylase (Abdel-Monem *et al.*, 1974), have been previously documented (Mamont *et al.*, 1976, 1978*c*). That the anti-proliferative effects of these ornithine analogues were related to their ornithine decarboxylase-inhibitory activities and to the subsequent cellular putrescine and spermidine deficiency was further demonstrated by the fact that the (−)-enantiomer of α-methylornithine did not completely deplete the intracellular content of putrescine and spermidine (Figs. 2*b*–2*d*) and had no effect on cell growth in contrast with the active (+)-enantiomer (Table 1). 1,3-Diaminopropane, added alone, did not affect cell proliferation (Table 1), although polyamine accumulation was strikingly decreased, including that of spermine. Added in combination with α-difluoromethylornithine, the lower analogue of putrescine could overcome the inhibition of cellular proliferation by the ornithine analogue. As already discussed (Mamont *et al.*, 1978*c*) these findings suggest that in HTC cells, 1,3-diaminopropane can substitute for the usual endogenous polyamines as a growth promoter.

Effects of diamines, triamines and spermine on S-adenosyl-L-methionine decarboxylase activity of α-difluoromethylornithine-treated cells

If the increase in *S*-adenosyl-L-methionine decarboxylase activity was related to intracellular spermidine depletion, then this effect should be suppressed by the addition of spermidine or its precursor putrescine to the culture medium of α-difluoromethylornithine-treated cells.

As illustrated in Fig. 4, 1μM-putrescine, added to HTC cells pre-incubated for 24h in the presence of 5mM-α-difluoromethylornithine, transiently increased the residual intracellular putrescine content (2.5-fold) and stimulated *S*-adenosyl-L-methionine decarboxylase activity (1.5-fold) over α-difluoromethylornithine values. Thereafter, the putrescine content decreased. This decline was accompanied by a concomitant increase in the intracellular spermidine content. Inversely, a decrease of *S*-adenosyl-L-methionine decarboxylase activity occurred. A similar increase and subsequent decrease of *S*-adenosyl-L-methionine decarboxylase activity was observed using 10μM-putrescine (Fig. 5). Spermidine (1μM) immediately replenished the normal spermidine content of the cells with a concomitant decrease in *S*-adenosyl-L-methionine decarboxylase activity (Fig. 4). Spermine (1μM) affected neither the residual putrescine and spermidine content nor *S*-adenosyl-L-methionine decarboxylase activity (Fig. 4). However, higher spermine concentration (10μM) decreased *S*-adenosyl-L-methionine decarboxylase activity at a rate similar to 10μM-spermidine (Fig. 5).

A high *S*-adenosyl-L-methionine decarboxylase activity remained when 1mM-1,3-diaminopropane was added to α-difluoromethylornithine-treated cells (Fig. 5). In contrast, 10μM-1,3-diamino-*N*-(3-aminopropyl)propane, the lower homologue of spermidine, immediately decreased *S*-adenosyl-L-methionine decarboxylase activity with the same efficiency as spermidine.

1,5-Diaminopentane (cadaverine) (1mM), the higher homologue of putrescine, in contrast with 1,3-diaminopropane, decreased *S*-adenosyl-L-methionine decarboxylase activity, although at lower rates than spermidine (Fig. 5). When added in combination with α-difluoromethylornithine, 1,5-

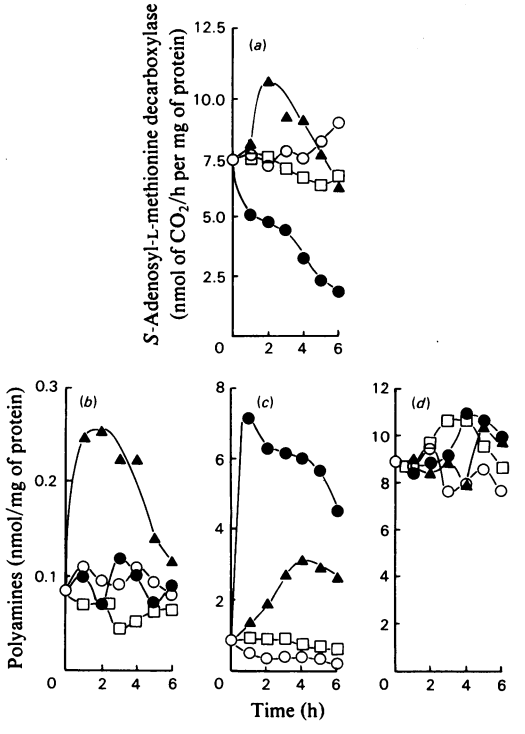

Fig. 4. *Effects of restoration of the intracellular polyamine content on S-adenosyl-L-methionine decarboxylase of α-difluoromethylornithine-treated cells*

A cell culture (1×10^5 cells/ml) was pre-incubated in fresh medium supplemented with 10% (v/v) horse serum and 5 mM-α-difluoromethylornithine for 24 h. At that time, 1 μM-putrescine, -spermidine or -spermine were added to portions of the cell culture. *S*-Adenosyl-L-methionine decarboxylase activities (*a*), putrescine (*b*), spermidine (*c*) and spermine (*c*) were measured as described in the Experimental section. ○, α-Difluoromethylornithine; ▲, α-difluoromethylornithine + putrescine; ●, α-difluoromethylornithine + spermidine; □, α-difluoromethylornithine + spermine.

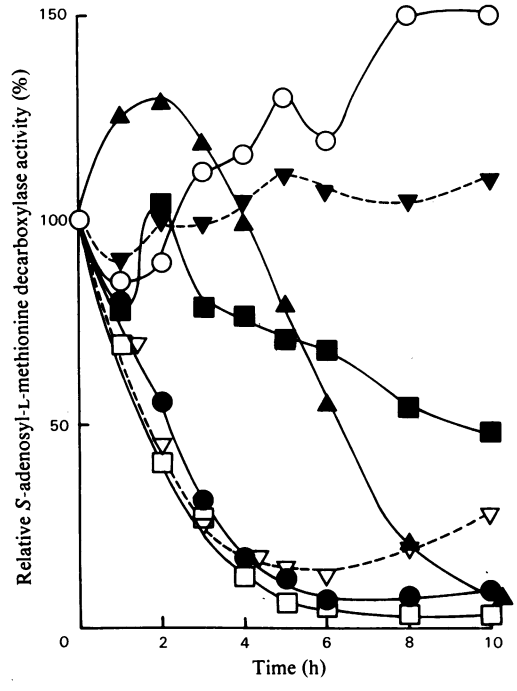

Fig. 5. *Effects of amine addition on S-adenosyl-L-methionine decarboxylase activity of α-difluoromethylornithine-treated cells*

Pre-incubation of cells with 5 mM-α-difluoromethylornithine was as described in Fig. 4. Putrescine (10 μM), spermidine (10 μM), spermine (10 μM), 1,3-diamino-*N*-(3-aminopropyl)propane (10 μM) or 1 mM-1,3-diaminopropane or -1,5-diaminopentane were added to portions of α-difluoromethylornithine-treated cells. *S*-Adenosyl-L-methionine decarboxylase activities are the means of two separate cultures and are expressed as percentages relative to *S*-adenosyl-L-methionine decarboxylase activity of α-difluoromethylornithine-treated cells measured at zero time (5.1 nmol/h per mg of protein). ○, α-Difluoromethylornithine; ▲, α-difluoromethylornithine + putrescine; ●, α-difluoromethylornithine + spermidine; □, α-difluoromethylornithine + spermine; ▽, α-difluoromethylornithine + 1,3-diamino-*N*-(3-aminopropyl)propane; ▼, α-difluoromethylornithine + 1,3-diaminopropane; ■, α-difluoromethylornithine + 1,5-diaminopentane.

diaminopentane reaches an intracellular content of 10 nmol per 10^6 cells and elicits the formation of two new amines with slightly lower and higher retention times than spermine using ion-exchange chromatography (Mamont *et al.*, 1978*c*). One of these amines was identified as 1,5-diamino-*N*-(3-aminopropyl)pentane by chromatographic comparison with an authentic sample and pulse-labelling of cells with radioactive cadaverine (results not shown). As much as 2 nmol of 1,5-diamino-*N*-(3-aminopropyl)pentane/10^6 cells accumulated intracellularly within 24 h of incubation of cells with 1 mM-1,5-diaminopentane. These findings which were recently confirmed in cultured Ehrlich ascites cells (Alhonen-Hongisto *et al.*, 1980), support the evidence *in vitro* for cadaverine being a substrate of spermidine synthase (Hibasami & Pegg, 1978).

Properties of S-adenosyl-L-methionine decarboxylase of α-difluoromethylornithine-treated cells

Various diamines stimulate mammalian *S*-adenosyl-L-methionine decarboxylase activity *in vitro* (Pegg & Williams-Ashman, 1969; Williams-Ashman & Schenone, 1972; Hannonen, 1975; Porta *et al.*, 1977). As shown in Table 2, 2 mM-putrescine,

Table 2. *Activation in vitro of S-adenosyl-L-methionine decarboxylase by amines*

HTC cell cultures were incubated for 24h in the absence or in the presence of 5mM-α-difluoromethylornithine. 100000*g* supernatants of cell extracts were prepared and dialysed overnight as described in the Experimental section. *S*-Adenosyl-L-methionine decarboxylase activities were measured in the presence or absence of 2mM-amine. Numbers in parentheses represent stimulation (fold).

Additions	*S*-Adenosyl-L-methionine decarboxylase (nmol/h per mg of protein)	
	Control	α-Difluoromethylornithine
None	0.41	2.20
1,3-Diaminopropane	1.13 (2.8)	6.50 (3.0)
1,3-Diaminopropan-2-ol	0.46 (1.1)	2.36 (1.1)
Putrescine	2.48 (6.0)	14.92 (6.8)
1,5-Diaminopentane	2.19 (5.3)	9.66 (4.4)
1,3-Diamino-*N*-(3-aminopropyl)propane	0.40 (1.0)	2.40 (1.1)
Spermidine	0.61 (1.5)	3.96 (1.8)
Spermine	0.43 (1.0)	2.28 (1.0)

1,5-diaminopentane, 1,3-diaminopropane and spermidine resulted in 6.0-, 5.3-, 2.8- and 1.5-fold increases over control *S*-adenosyl-L-methionine decarboxylase activity. These amines stimulated to the same degree *S*-adenosyl-L-methionine decarboxylase activity of cells treated by α-difluoromethylornithine. 1,3-Diaminopropan-2-ol, 1,3-diamino-*N*-(2-aminopropyl)propane and spermine neither activated nor inhibited either enzyme preparation.

Apparent K_m values for the substrate were not significantly different for both enzyme preparations in the absence of putrescine ($K_{m(Control)} = 242\mu M$; $K_{m(\alpha\text{-difluoromethylornithine-treated}} = 276\mu M$) or in the presence of putrescine ($K_{m(control)} = 31\mu M$; $K_{m(\alpha\text{-difluoromethylornithine-treated})} = 49\mu M$).

Furthermore, methylglyoxal bis(guanylhydrazone), a potent inhibitor of mammalian *S*-adenosyl-L-methionine decarboxylase (Williams-Ashman & Schenone, 1972), at concentrations of 1 to 10μM, similarly inactivated *S*-adenosyl-L-methionine decarboxylase of control and of α-difluoromethylornithine-treated cells in both the presence or absence of putrescine (results not shown).

Mechanisms of the increase of S-adenosyl-L-methionine decarboxylase activity by α-difluoromethylornithine

When extracts from cells treated with α-difluoromethylornithine were assayed for *S*-adenosyl-L-methionine decarboxylase activities in combination with extracts from untreated cells, no evidence for the presence in these extracts of inhibitors or activators of enzyme activities was found.

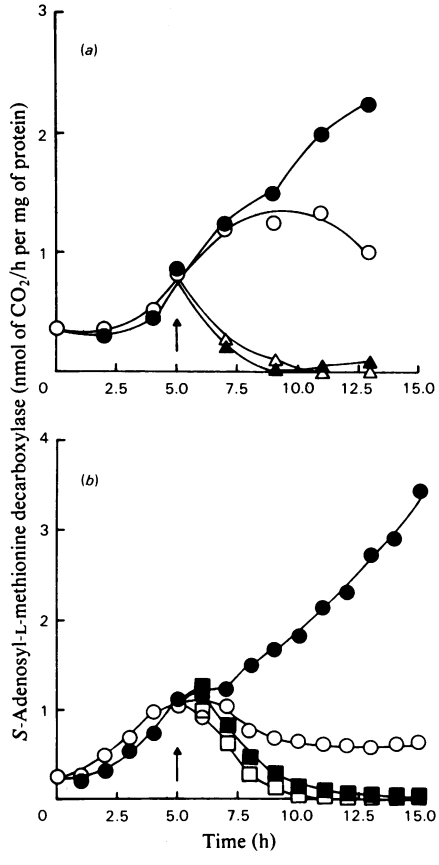

Fig. 6. *Effects of cycloheximide and actinomycin D on the increase of S-adenosyl-L-methionine decarboxylase activity by α-difluoromethylornithine*

High-density cell cultures were diluted as described in Fig. 1 and were further incubated in the absence or presence of 5mM-α-difluoromethylornithine. After 5h (arrow), portions received 200μM-cycloheximide (*a*) or 0.25μg of actinomycin D/ml (*b*). *S*-Adenosyl-L-methionine decarboxylase activities were determined at the indicated times as described in the Experimental section. ○, Control; ●, α-difluoromethylornithine; △, control + cycloheximide; ▲, α-difluoromethylornithine + cycloheximide; □, control + actinomycin; ■, α-difluoromethylornithine + actinomycin.

α-Difluoromethylornithine (5mM) increased *S*-adenosyl-L-methionine decarboxylase activity after a lag period of about 7–8h (Fig. 6). When added 5h after α-difluoromethylornithine, actinomycin D (0.25 μg/ml), a concentration that decreased [^{3}H]uridine incorporation into RNA by 79% within 40min (Table 3), blocked this increase (Fig. 6*b*). Cycloheximide (200μM) blocked elevation of *S*-adenosyl-

Table 3. *Effects of actinomycin D on RNA synthesis and S-adenosyl-L-methionine decarboxylase activity in α-difluoromethylornithine-treated and untreated cells*

HTC cell cultures (1.0×10^5 cells/ml) were pre-incubated in the absence or presence of 5 mM-α-difluoromethylornithine for 24 h. Portions were further incubated in the absence or presence of actinomycin D. After 10 min cells were pulse-labelled with [5-^{3}H]uridine for 30 min as described in the Experimental section. *S*-Adenosyl-L-methionine decarboxylase activities were determined 4 h after the addition of actinomycin D. Numbers in parentheses represent inhibition (% of control values).

	10^{-5} × Uridine incorporation (c.p.m./10^6 cells)		*S*-Adenosyl-L-methionine decarboxylase (nmol/h per mg of protein)	
Additions	Control	α-Difluoromethylornithine	Control	α-Difluoromethylornithine
None	6.35	3.97	1.13	5.22
Actinomycin (0.25 μg/ml)	1.32 (79.2)	1.09 (72.5)	0.23 (79.6)	4.06 (22.2)
Actinomycin (1.0 μg/ml)	0.43 (93.2)	0.43 (89.2)		
Actinomycin (2.5 μg/ml)	0.18 (97.2)	0.12 (97.0)		
Actinomycin (5.0 μg/ml)	0.09 (98.6)	0.06 (98.5)	0.15 (86.7)	3.34 (36.0)

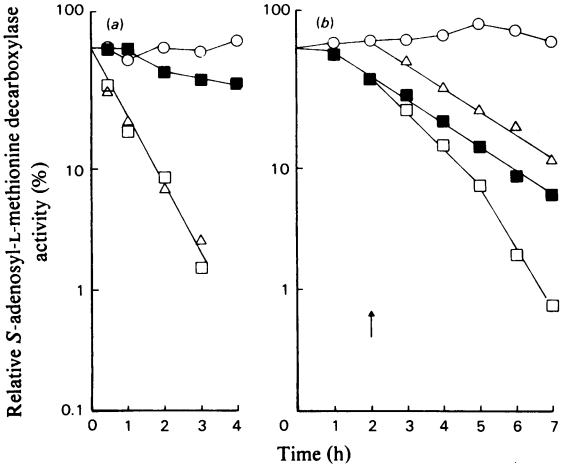

Fig. 7. *Effects of cycloheximide and spermidine, alone or in combination, on S-adenosyl-L-methionine decarboxylase activity of control and of α-difluoromethylornithine-treated cells*

Pre-incubation of cells in the absence or in the presence of α-difluoromethylornithine was as described in the legend to Fig. 4. (*a*) 10 μM-spermidine, 200 μM-cycloheximide or 10 μM-spermidine + 200 μM-cycloheximide were added to the medium of control cells; (*b*) α-difluoromethylornithine-treated cells were first incubated for 2 h in the absence or in the presence of 10 μM-spermidine and then with or without 200 μM-cycloheximide. *S*-Adenosyl-L-methionine decarboxylase activities are the means for two separate cultures and are expressed as percentages relative to time-zero values (control = 0.8 nmol/h per mg of protein; α-difluoromethylornithine = 6.7 nmol/h per mg of protein). Half-life values were calculated from the slope obtained by linear least-squares regression. ○, Control; ■, spermidine; △, cycloheximide; □, spermidine + cycloheximide.

L-methionine decarboxylase activity and caused a decrease in this activity at a rate that was similar for both control and α-difluoromethylornithine-treated cells (Fig. 6*a*). Thus increase of *S*-adenosyl-L-methionine decarboxylase activity appears to be dependent on RNA and protein synthesis *de novo*.

When added 24 h after α-difluoromethylornithine, actinomycin D (5 μg/ml) did not markedly affect *S*-adenosyl-L-methionine decarboxylase activity (43% decrease within 8 h) (Fig. 8*b*). At the same time, *S*-adenosyl-L-methionine decarboxylase activity of control cells decreased with a half-life of about 80 min (Fig. 8*a*). Similar differential effects on *S*-adenosyl-L-methionine decarboxylase activities were observed at actinomycin D concentrations ranging from 0.25 to 5 μg/ml (Table 3), although dose-dependent inhibition of [^{3}H]uridine incorporation into RNA by actinomycin D was equivalent for both α-difluoromethylornithine-treated and control cells (Table 3).

After 24 h incubation in the absence or presence of α-difluoromethylornithine, cycloheximide decreased *S*-adenosyl-L-methionine decarboxylase of control and α-difluoromethylornithine-treated cells with a half-life of 32 and 83 min respectively (Figs. 7*a* and 7*b*). Inhibition of [^{3}H]leucine incorporation into proteins by cycloheximide was identical for both control and α-difluoromethylornithine-treated cells (96–98% inhibition within 15 min). Emetine (1 μM), another inhibitor of protein synthesis (Grollman, 1966; Gupta & Siminovitch, 1976) inactivated control *S*-adenosyl-L-methionine decarboxylase at a slightly lower rate than cycloheximide (half-life, 42 min), although the extent of inhibition of [^{3}H]-leucine incorporation into HTC cell proteins was identical for both inhibitors. The increase of the half-life for *S*-adenosyl-L-methionine decarboxylase of α-difluoromethylornithine-treated over control

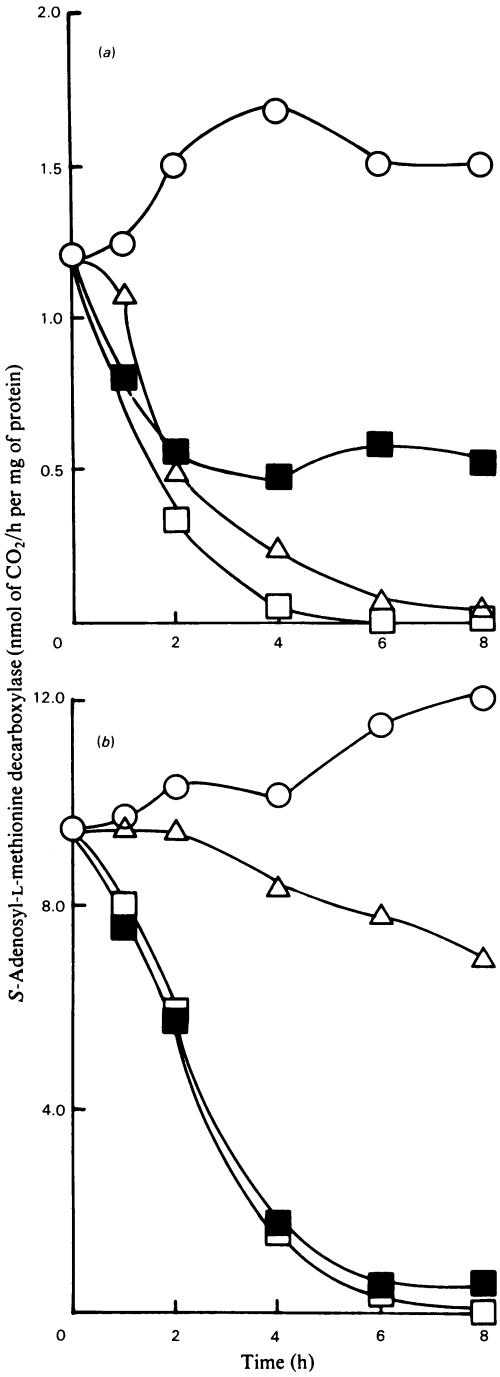

Fig. 8. *Effects of actinomycin D and spermidine, alone or in combination on S-adenosyl-L-methionine decarboxylase activity of control and of α-difluoromethylornithine-treated cells*

Pre-incubation of cells for 24h in the absence or in the presence of α-difluoromethylornithine was as described in the legend to Fig. 4. Spermidine + 5 μg of actinomycin D/ml were added at zero time to portions of control cells (*a*) and of α-difluoromethylornithine-treated cells (*b*). *S*-Adenosyl-L-methionine decarboxylase activities were measured as described in the Experimental section. (*a*) ○, control; ■, spermidine; △, actinomycin; □, spermidine + actinomycin. (*b*) ○, control (α-difluoromethylornithine); ■, spermidine; △, actinomycin; □, spermidine + actinomycin.

cells was, however, of the same order of magnitude as that found with cycloheximide (2.5-fold) (results not shown). The present results suggest that elevation of *S*-adenosyl-L-methionine decarboxylase activity by α-difluoromethylornithine is due to stabilization of the enzyme.

Site(s) of spermidine action

Whereas actinomycin D did not markedly affect *S*-adenosyl-L-methionine decarboxylase activity of α-difluoromethylornithine-treated cells (Fig. 8*b*), spermidine alone or in combination with actinomycin D decreased enzyme activity with a half-life of about 70 to 80min (Fig. 8*b*). This experiment argues in favour of a post-transcriptional level of action of spermidine.

Spermidine caused a 50% decrease of *S*-adenosyl-L-methionine decarboxylase activity within 4h (Figs. 7*a* and 8*a*), whereas cycloheximide inactivated the enzyme with a half-life of 32min (Fig. 7*a*). No significant additional inactivation was caused by the addition of spermidine and cycloheximide (Fig. 7*a*). In contrast, spermidine (10 μM) inactivated *S*-adenosyl-L-methionine decarboxylase of α-difluoromethylornithine-treated cells with a half-life that resembled that observed in the presence of cycloheximide (84min) (Fig. 7*b*). In combination with cycloheximide, spermidine led to a progressively increased rate of inactivation that approached the control value. Emetine in association with spermidine had similar synergistic effects on *S*-adenosyl-L-methionine decarboxylase activity of α-difluoromethylornithine-treated cells (results not shown). The present results suggest that spermidine acts on processes involved in denaturation and/or degradation of *S*-adenosyl-L-methionine decarboxylase and confirm the enhanced stabilization of the enzyme seen in spermidine-deficient cells.

Discussion

Comparison of *S*-adenosyl-L-methionine decarboxylase activities and spermidine contents in high- and low-*S*-adenosyl-L-methionine strains of *Escherichia coli* K12 with those in the wild-type has led Su & Cohen (1973) to suggest that *S*-adenosyl-L-methionine decarboxylase is regulated by spermidine. In eukaryotes, comparative studies of the effects of α-methylornithine and α-difluoromethylornithine in HTC cells and in its polyamine-over-

producing variant have also suggested an inverse relationship between intracellular spermidine contents and *S*-adenosyl-L-methionine decarboxylase activities (Mamont *et al.*, 1978*b*,*c*). Experimental evidence for a negative control exerted by spermidine on *S*-adenosyl-L-methionine decarboxylase expression is further substantiated in this report by using inhibitors of L-ornithine decarboxylase that are structurally related or unrelated to its substrate, which possess different modes of inhibitory activities and which produce different degrees of intracellular polyamine depletion. In all cases, the increase of *S*-adenosyl-L-methionine decarboxylase activity is inversely correlated with the degree of spermidine depletion. 1,3-Diaminopropan-2-ol, which decreases the putrescine and spermidine content of cultured Ehrlich ascites cells, also seems to elevate *S*-adenosyl-L-methionine decarboxylase activity (Alhonen-Hongisto *et al.*, 1980).

That increase of *S*-adenosyl-L-methionine decarboxylase activity is related to spermidine and not to putrescine depletion is further confirmed by experiments on restoration of the normal intracellular polyamine contents. Replenishment of intracellular putrescine content was accompanied by a further elevation of *S*-adenosyl-L-methionine decarboxylase activity and not by a decrease. The decline of enzyme activity observed thereafter most likely results from the conversion of putrescine into spermidine. Putrescine and spermidine added to phytohaemagglutinin-stimulated human lymphocytes have similar differential effects on *S*-adenosyl-L-methionine decarboxylase (Kay & Lindsay, 1973). A likely explanation for the further increase of *S*-adenosyl-L-methionine decarboxylase by putrescine may be the strong protection *in vitro* afforded by this amine against proteolytic digestion (Williams-Ashman *et al.*, 1977).

No apparent relationship between total spermine content and *S*-adenosyl-L-methionine decarboxylase activity could be established. Simultaneous addition of 1,3-diaminopropane and α-difluoromethylornithine to HTC cells or 1,3-diaminopropane to cells pre-incubated with α-difluoromethylornithine created no additive effects on *S*-adenosyl-L-methionine decarboxylase activities, although the spermine content was much more affected when these inhibitors were used in combination instead of alone (the present paper; Mamont *et al.*, 1980). Spermine (10 μM) added to spermidine-deficient cells was, however, as effective as spermidine in decreasing *S*-adenosyl-L-methionine decarboxylase activity. Spermine accumulates intracellularly as such with no subsequent conversion into spermidine (Mamont *et al.*, 1978*c*; P. S. Mamont & N. Seiler, unpublished work). It is not known whether the spermine effect results from the inactivation *in situ* of *S*-adenosyl-L-methionine decarboxylase activity, as has been reported *in vitro* for purified rat liver *S*-adenosyl-L-methionine decarboxylase (Sakai *et al.*, 1980*a*) or whether spermine can partly substitute for spermidine, as it does in antagonizing anti-proliferative effects of α-difluoromethylornithine (Mamont *et al.*, 1976, 1978*a*,*b*). 1,3-Diamino-*N*-(3-aminopropyl)-propane appears to substitute for spermidine. This may explain the inefficiency of 1,3-diaminopropane and the relative activity of 1,5-diaminopentane, since the former is a poor substrate of spermidine synthase and cannot be converted into the lower spermidine analogue, in contrast with 1,5-diaminopentane, which leads to intracellular accumulation of 1,5-diamino-*N*-(3-aminopropyl)pentane.

In summary the available information suggests that spermidine is a natural negative effector of *S*-adenosyl-L-methionine decarboxylase, whereas putrescine may exert opposite effects. This concept does not appear to be limited to HTC cell *S*-adenosyl-L-methionine decarboxylase, but seems to be of general significance. Administration of α-difluoromethylornithine, at concentrations that decrease putrescine and spermidine contents, increases *S*-adenosyl-L-methionine decarboxylase activity in EMT_6 sarcoma growing *in vivo* (Prakash *et al.*, 1980) and in mouse uterus during early gestation (Fozard *et al.*, 1980). In contrast, spermidine administration *in vivo* decreases rat diaphragm muscle *S*-adenosyl-L-methionine decarboxylase activity (Hopkins & Manchester, 1980).

Elucidation of the mechanism of the increase of *S*-adenosyl-L-methionine decarboxylase activity by L-ornithine decarboxylase inhibitors and determination of the site(s) of action of spermidine will not rely, in the future, solely on determinations of enzyme activities but on the use of sensitive techniques for the quantitative measurement of enzyme protein molecules. Recent advances in this domain seem to be promising (Demetriou *et al.*, 1978; Pegg, 1979; Sakai *et al.*, 1980*a*) and have begun to be exploited. Thus, the increase of mouse mammary *S*-adenosyl-L-methionine decarboxylase activity by α-methylornithine is due to an increased amount of enzyme protein as determined by antibody titration (Sakai *et al.*, 1980*b*). At present, the following indirect evidence also suggests that elevation of *S*-adenosyl-L-methionine decarboxylase activity by α-difluoromethylornithine is due to an increase of enzyme protein molecules. (1) Increase in *S*-adenosyl-L-methionine decarboxylase activity is dependent on RNA and protein synthesis. (2) No evidence for the presence in extracts, from cells treated by α-difluoromethylornithine or untreated, of inhibitors or activators of enzyme activity could be provided. (3) Increases in *S*-adenosyl-L-methionine activity in α-difluoromethylornithine-treated cells did not apparently result from modifications of the catalytic site as judged by the

comparison of the catalytic properties of both enzyme preparations. Furthermore, increased loss of CO_2 from *S*-adenosyl-L-methionine catalysed by α-difluoromethylornithine-treated cell extracts cannot be attributed to an increase in 'spurious' *S*-adenosyl-L-methionine decarboxylase activities, which is associated with *S*-adenosyl-L-methionine catabolism (Eloranta & Raina, 1978; Wilson *et al.*, 1979), because of its activation by putrescine and its inhibition by methylglyoxal bis(guanylhydrazone) in the presence or absence of the diamine. (4) The increase in the apparent half-life of *S*-adenosyl-L-methionine decarboxylase of α-difluoromethylornithine-treated cells appears to be one likely explanation for the effect of the L-ornithine decarboxylase inhibitor. Although doubts have sometimes been raised about the interpretation of results obtained with cycloheximide (Auricchio *et al.*, 1969; Hershko & Tomkins, 1971), stabilization of *S*-adenosyl-L-methionine decarboxylase of α-difluoromethylornithine-treated cells was also found with emetine, another inhibitor of protein synthesis. Moreover, at least for rat liver *S*-adenosyl-L-methionine decarboxylase, the loss of antigen, after cycloheximide administration *in vivo*, if not identical, is not markedly different (30% slower) from the rate of decay of enzyme activity (Pegg, 1979).

The conclusions regarding the site(s) of action of spermidine also depend on the specificity of action of the drug used to inhibit RNA and protein synthesis. Actinomycin D is known to have extranuclear effects including inhibition of protein degradation in step-down conditions (Kenney *et al.*, 1973; Auricchio *et al.*, 1969), activation or, on the contrary, inhibition of initiation of protein synthesis (Singer & Penman, 1972; Palmiter & Schimke, 1973) and disaggregation of polyribosomes (Steinberg *et al.*, 1975). Therefore, it would be premature to speculate on the mechanisms involved in the maintenance of *S*-adenosyl-L-methionine decarboxylase activity of α-difluoromethylornithine-treated cells in the presence of actinomycin D in contrast with the control enzyme. At least, differences in the extent of inhibition of total RNA synthesis were not observed and cannot contribute therefore to this phenomenon. Spermidine inactivation occurred similarly in the presence or absence of actinomycin D, suggesting that spermidine inhibition must occur at some post-transcriptional step. No direct experimental evidence for or against an effect of spermidine on *S*-adenosyl-L-methionine decarboxylase enzyme synthesis can be furnished at present. However, indirect evidence for a post-translational control is the significant synergistic inactivation that spermidine causes in the presence of cycloheximide or emetine and which tends to decrease the half-life of the enzyme activity to control values. This result suggests furthermore that the amine may possibly act on processes involved in denaturation and/or degradation of the enzyme protein and may confirm indirect evidence for stabilization of the enzyme in spermidine-deficient cells.

The physiological significance of the described regulatory control of *S*-adenosyl-L-methionine decarboxylase by spermidine is to contribute, in conjunction with the fine regulation of L-ornithine decarboxylase, to the overall maintenance of critical intracellular polyamine concentrations. These controls may be essential not only for optimal rates of metabolic processes to occur in precisely-timed sequences, but also to strictly couple spermine biosynthesis to DNA replication. Spermine reverses the anti-proliferative effects of α-difluoromethylornithine in a very narrow range of concentrations (Mamont *et al.*, 1978*c*). Furthermore, since biological transmethylations and the polyamine biosynthetic pathway share the common substrate *S*-adenosyl-L-methionine, the strict control of *S*-adenosyl-L-methionine decarboxylase may also prevent any unbalanced utilization of *S*-adenosyl-L-methionine for polyamine biosynthesis to the detriment of methylation reactions.

Note added in proof (received 22 February 1981)

A recent report (Alhonen-Hongisto, 1980), which appeared after the present paper was submitted, also concludes that polyamines regulate *S*-adenosyl-L-methionine decarboxylase in Ehrlich ascites carcinoma cells grown in culture.

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