Decreased protein-synthetic activity is an early consequence of spermidine depletion in rat hepatoma tissue-culture cells

Brian B. RUDKIN, Pierre S. MAMONT and Nikolaus SEILER*
Centre de Recherche Merrell International, 16 rue d'Ankara, 67084 Strasbourg Cedex, France

(Received 18 August 1983/Accepted 13 October 1983)

Hepatoma tissue-culture (HTC) cells were exposed to DL- α -difluoromethylornithine (DFMeOrn), a specific irreversible inhibitor of ornithine decarboxylase. Concomitantly with the decrease in spermidine, a decrease in the amount of ribosomes in polyribosomes was observed. Spermine concentrations remained essentially comparable with those in cells not exposed to this inhibitor. Exposure of putrescine- and spermidine-depleted HTC cells to spermidine or spermine rapidly reversed the effect of DFMeOrn on polyribosome profiles, whereas addition of putrescine to the cell culture medium had an effect only after its transformation into spermidine and spermine. The results show that the perturbation of polyribosome formation in DFMeOrn-treated HTC cells is due to spermidine deficiency and that a normal polyamine complement is required for optimal protein-synthetic activity in these cells. The results also indicate that protein synthesis is perturbed before DNA synthesis during depletion of putrescine and spermidine in HTC cells.

Specific depletion of intracellular polyamines, by inhibitors of polyamine-biosynthetic enzymes, has been widely used during the last decade as an approach to elucidating physiological roles of the natural polyamines putrescine, spermidine and spermine (for reviews, see Heby, 1981; Heby & Jänne, 1981; Pegg & McCann, 1982). Among the inhibitors, DFMeOrn has attracted much interest. DFMeOrn is an enzyme-activated irreversible inhibitor of ODC (Metcalf *et al.*, 1978). On the basis of mechanistic considerations (Bey, 1978), it was assumed that DFMeOrn specifically inhibited ODC. However, its specificity has only very recently been demonstrated unambiguously, since the compound associates irreversibly with a single protein (Pösö *et al.*, 1983). Therefore it can now be considered a suitable tool for the gradual highly specific depletion of cellular polyamines, and for the study of subtle biological consequences of polyamine deficiency in eukaryotes.

Although numerous studies *in vitro* indicate a role for polyamines in protein biosynthesis, relatively little information *in vivo* is available, particularly in eukaryotes (for references see Tabor & Tabor, 1976; Algranati & Goldemberg, 1977). In

order to address this problem in eukaryotes, we have used DFMeOrn for the exploration of early events during polyamine depletion in rat HTC cells. This cell line has been extensively studied with regard to consequences of ODC inhibition by substrate analogues such as α -methylornithine and DFMeOrn (Mamont *et al.*, 1978, 1980). Since changes in protein-synthetic activity of a cell are reflected in changes in the polyribosome pattern (Fan & Penman, 1970; Van Venrooij *et al.*, 1972), the evolution of polyribosome profiles during polyamine depletion was followed.

Materials and methods

Chemicals

Usual laboratory chemicals were from E. Merck (Darmstadt, Germany) or Baker Chemicals (Deventer, The Netherlands). Putrescine dihydrochloride, spermidine trihydrochloride, spermidine phosphate and spermine tetrahydrochloride were from Fluka (Buchs, Switzerland). Bovine serum albumin, cycloheximide and emetine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and 2-deoxy-D-ribose was from Serva (Heidelberg, Germany). Sucrose from sugar cane (analytical-reagent grade) was from BDH Chemicals Ltd. (Poole, Dorset, U.K.). DFMeOrn was synthesized in our Centre (Metcalf *et al.*, 1978). [*methyl*- ^3H]-

Abbreviations used: ODC, ornithine decarboxylase (EC 4.1.1.17); HTC cells, hepatoma tissue-culture cells; DFMeOrn, α -difluoromethylornithine.

* To whom correspondence should be sent.

Thymidine (20 μ Ci/mmol), L-[4,5- 3 H]leucine (60 Ci/mmol) and Aquasol-2 were from NEN Chemicals G.m.b.H. (Dreieich, Germany).

Tissue-culture materials (Swim's 77 medium, newborn-calf serum, dialysed horse serum, acetoorcein and Trypan Blue) were from Gibco (Grand Island, NY, U.S.A.).

Compounds to be added to cell cultures were dissolved in phosphate-buffered saline (137 mM-NaCl/6.4 mM- Na_2HPO_4 /6.4 mM- KH_2PO_4 /2.7 mM-KCl), and pH was adjusted to 7.4 with NaOH. Solutions were sterilized by filtration through 0.22 μ m-pore-size membrane filters (Millipore S.A., Molsheim, France).

Cell culture

HTC cells were used as described previously (Mamont *et al.*, 1981a).

Cell harvest and preparation of HClO_4 extracts

Cells were harvested by centrifugation (200g, 5 min), washed with sterile ice-cold phosphate-buffered saline, and frozen as pellets at -90°C until extraction. For extraction, 0.2 M- HClO_4 was added to the cell pellet to give a final volume of 1 ml, and the cells (0.4×10^7 – 1×10^7) were disrupted by sonication with a microtip (three 14 s pulses at 120 W, 20 kHz; Ultrasons-Annemasse S.A., Annemasse, France) on ice. After 1.5 h at 4°C the precipitate was pelleted by centrifugation (1500g, 20 min). The supernatants were stored at -90°C until analysis, and the pellets were immediately analysed for RNA, DNA and protein contents.

Determination of RNA, DNA and protein

These were determined in the HClO_4 -insoluble material. Separation and determination of RNA, DNA and protein were accomplished as described by Seiler & Schmidt-Glenewinkel (1975).

Incorporation of [methyl- 3 H]thymidine and [3 H]leucine

Samples of HTC-cell cultures (22 ml; 1×10^5 – 3×10^5 cells/ml) were incubated for 30 min in the presence of [methyl- 3 H]thymidine (1.4 μ Ci/ml; 0.18 Ci/mmol) or L-[4,5- 3 H]leucine (1.8 μ Ci/ml). At the end of the labelling period, 10 ml samples were centrifuged (200g, 5 min), washed twice in ice-cold phosphate-buffered saline and frozen at -90°C until analysis.

RNA, DNA and protein were separated by a modified Schmidt–Thannhauser procedure (Seiler & Schmidt-Glenewinkel, 1975). After hydrolysis, samples of the appropriate fraction were taken for determination of radioactivity and deoxyribose or protein content.

The leucine content of the medium and acid-

soluble cell extracts was determined by cation-exchange column chromatography (Grove *et al.*, 1982). The elution buffer was 0.2 M-sodium citrate, pH 3.70. For determination of specific radioactivity of leucine, the column effluent (in the absence of *o*-phthalaldehyde reagent) was collected in 0.1 ml fractions and radioactivity was measured after addition of 10 ml of Aquasol-2.

Polyamine analysis

Polyamines were determined in the HClO_4 cell extracts by using a published method (Seiler & Knödgen, 1980). Some of the analyses were performed by another version of reversed-phase ion-pair chromatography (Wagner *et al.*, 1982).

Determination of DFMeOrn and basic amino acids

The high-performance liquid-chromatographic technique is described in detail elsewhere (Seiler & Sarhan, 1983). Briefly, β -alanine, DFMeOrn, 4-aminobutyric acid, ornithine, histidine, lysine and arginine are separated on a cation-exchange column (Whatman Partisil SCX 25) by using a two-slope linear gradient composed of 0.2 M-acetic acid (buffer A) and 0.2 M-sodium acetate, pH 4.5 (buffer B). The amount of buffer B is increased by 0.5%/min for 18 min, then by 10%/min for 9 min, at which time the column is re-equilibrated at 4% buffer B for 10 min before the next run. The total time between analyses is 36 min. Detection is achieved by post-column derivative formation with *o*-phthalaldehyde/2-mercaptoethanol reagent as described by Roth & Hampai (1973), and continuous recording of fluorescence intensity at 455 nm (excitation at 345 nm).

Cell fractionation and sucrose-gradient analysis

All operations were performed on ice. Fresh or frozen cells (2×10^7 – 3×10^7) were suspended in a hypo-osmotic buffer (0.3–0.5 ml/ 10^7 cells) of the following composition: 10 mM-NaCl, 1.5 mM- MgCl_2 , 10 mM-Tris/HCl (pH 7.4), 0.05% Triton X-100 (Zylber & Penman, 1970; Perry & Kelley, 1968). After 15 min the suspensions were homogenized in a 5 ml Potter–Braun glass homogenizer with ten strokes of a motor-driven Teflon piston (approx. 1500 rev./min), then centrifuged at 12000g for 10 min.

The ribosome distribution patterns were obtained by layering 2 A_{260} units of the 12000g supernatant (100–150 μ l) on a 5 ml 10–30% (w/w) linear sucrose gradient and centrifuging at 150000g for 60 min at 0– 5°C (Beckman L5-75 Ultracentrifuge, SW 50.1 rotor, 40000 rev./min). The sucrose gradient was made in the high-salt buffer described by Zylber & Penman (1970), i.e. final concentrations 0.5 M-NaCl, 50 mM- MgCl_2 , 10 mM-Tris/HCl (pH 7.4). Gradients were analysed by

introducing a stainless-steel tube to the bottom, pumping (0.5 ml/min) the solutions through an 8 μ l flow cell (1 cm path length) and recording the A_{260} .

The high-salt gradient buffer was chosen after preliminary trials with a low- K^+ , low- Mg^{2+} buffer (0.1 M-KCl / 4 mM-magnesium acetate / 20 mM-Tris/HCl, pH 7.5). Under the low-salt conditions the 60S peak was almost non-existent, owing to formation of a dimer with a sedimentation coefficient around 90S. Another peak at 105S was also observed. This aggregation is apparently characteristic of rat liver (Martin *et al.*, 1971) and HTC-cell ribosomes (Scott *et al.*, 1972) at 0–4°C under these salt conditions. It can be prevented by raising the temperature during centrifugation or by increasing the salt and Mg^{2+} concentrations. We choose the latter approach.

For determination of ribosome distribution, a baseline was established from the A_{260} profile of blank gradients, which essentially corresponded to a horizontal line at 0.05 A_{260} unit (not shown in the

Figures). The areas under the monoribosomal (70–80S) and polyribosomal regions (≥ 90 S) were determined by cutting out and weighing the corresponding zones from A_{260} profiles after subtraction of the baseline. The proportion of ribosomes in polyribosomes was calculated by dividing the weight of the polyribosome region by the sum of the weights of the monoribosome (70–80S) and polyribosome regions.

Results

Effect of DFMeOrn on polyamine contents, growth, and accumulation of macromolecules in HTC cells

Figs. 1(a)–1(c) show how putrescine and polyamine contents changed during the first 12 h after HTC cells were stimulated to proliferate by dilution of high-density cultures into fresh medium. In the presence of 5 mM-DFMeOrn, putrescine content fell to approx. 10% of control values within the first 6 h (Fig. 1d). Spermidine decreased to

Fig. 1. Effect of 5 mM- α -difluoromethylornithine (DFMeOrn) on polyamine contents in HTC cells. Cells in high-density cultures (9×10^5 cells/ml) were stimulated to proliferate by resuspension at low density (1×10^5 – 1.5×10^5 cells/ml) in fresh medium containing dialysed horse serum in the absence or presence of 5 mM-DFMeOrn. Samples were taken at the specified times from DFMeOrn-treated and non-treated sister cultures for analysis of polyamine and deoxyribose contents. The data shown in panels (a)–(c) and (d) are compiled from respectively four and six separate experiments performed at overlapping times after dilution. (a)–(c): ●, control; ○, +5 mM-DFMeOrn; (d): ○, putrescine; △, spermidine; ●, spermine.

Table 1. α -Difluoromethylornithine (DFMeOrn), ornithine and total polyamine content of HTC cells after 24 h exposure to DFMeOrn or ornithine

HTC cells were stimulated to proliferate in the absence or presence of the indicated concentrations of DFMeOrn or ornithine as described in the legend of Fig. 1. After 24 h, samples were taken for determination of polyamine, basic amino acid and deoxyribose contents as described in the Materials and methods section. Values represent individual determinations on one or two samples from the same cell culture, except where mean values $\pm$ S.D. are given, in which case the number of separate experiments is indicated in parentheses.

Addition	Concn. (mM)	Content (pmol/nmol of deoxyribose)		
		DFMeOrn	Ornithine	Total polyamine
None		—	4.3 $\pm$ 0.1 (2)	570 $\pm$ 17 (5)
DFMeOrn	0.1	6	4.5	305
	1.0	62	4.3	259
	5.0	310 $\pm$ 18 (4)	3.2 $\pm$ 1.1 (4)	239 $\pm$ 20 (5)
Ornithine	5.0	—	346	619

approx. 20% of control values within 12 h, whereas spermine contents remained comparable in the presence and absence of DFMeOrn. DFMeOrn decreased the total polyamine content of HTC cells in a dose-dependent manner, with the maximum effect at 1 mM concentration (Table 1, and Mamont *et al.*, 1981a). No effect on cell growth was observed during the first 24 h (one doubling time). Thereafter, population doubling times were increased by approx. 60% at 0.1 mM- and by 100% at 1 mM- and 5 mM-DFMeOrn (Mamont *et al.*, 1978). Evaluation of the mitotic index after dilution showed that control and 5 mM-DFMeOrn-treated cells exhibited identical kinetics during the first 24 h. During the second mitotic wave, approx. 50% fewer mitotic figures were observed, and the peak occurred 2 h later in 5 mM-DFMeOrn-treated cultures. This indicated that the cell cycle time was increased. Similar effects have been observed when putrescine and spermidine were depleted with 5 mM- α -methylornithine (Mamont *et al.*, 1980). These observations indicate that the initial polyamine complement was sufficient to permit only one normal population doubling in the presence of 5 mM-DFMeOrn.

Protein and RNA contents increased slightly in control cells 24 h after the growth stimulus, but there was no significant increase in DNA content per cell. In the presence of 5 mM-DFMeOrn, neither the DNA nor RNA contents were significantly affected. Protein content, however, was consistently lower between 24 h and 48 h in DFMeOrn-treated cells as compared with controls (results not shown). These observations are in agreement with the fact that no effect on doubling of the cell population was evident during the first 24 h.

No detectable difference in the incorporation of [*methyl*- 3 H]thymidine into DNA was observed until after 12 h of exposure to 5 mM-DFMeOrn (Fig. 2). This result is in agreement with previously

Fig. 2. Effect of DFMeOrn on [*methyl*- 3 H]thymidine incorporation

Cells were stimulated to proliferate as described in the legend to Fig. 1. Samples of the cell suspensions were taken at the specified times and [*methyl*- 3 H]-thymidine incorporation was performed as described in the Materials and methods section. Points represent the average of duplicate determinations. DNA: ●, control; ○, + 5 mM-DFMeOrn; acid-soluble: ▲, control; △, + 5 mM-DFMeOrn.

reported findings in HTC cells, by using α -methylornithine, a competitive inhibitor of ODC (Mamont *et al.*, 1976).

Effect of polyamine depletion on polyribosome formation

Increased accumulation of ribosomes into polyribosomal structures is a characteristic of the positive pleiotypic response (Hershko *et al.*, 1971). Fig. 3 illustrates how ribosome distribution patterns changed during the first 45 h after dilution of the high-density cell culture in the absence or presence of 5 mM-DFMeOrn. The pattern at 5 min is typical of nutrient-deprived cells (Hogan &

Fig. 3. *Evolution of polyribosome profiles in HTC cells during depletion of putrescine and spermidine*
HTC cells were stimulated to proliferate in the absence or presence of 5 mM-DFMeOrn as described in the legend of Fig. 1. Samples of both cell cultures were taken for polyribosome analysis at various times after dilution, as described in the Materials and methods section. —, Control; ····, 5 mM-DFMeOrn.

Fig. 4. Effect of DFMeOrn on ribosome distribution in HTC cells

The percentage of ribosomes in polyribosomes was determined from the area under the monoribosome and polyribosome regions of the absorbance profiles as described in the Materials and methods section. Data are compiled from several independent experiments performed at various times after dilution. Where shown, bars represent ± 1 s.d., and the numbers of separate cultures analysed are indicated in parentheses. ●, Control; △, +0.1 mM-DFMeOrn; ■, +1 mM-DFMeOrn; ○, +5 mM-DFMeOrn.

Korner, 1968; Gelehrter & Tomkins, 1969; Van Venrooij *et al.*, 1972). The amount of polyribosomes increased during the first 6 h after growth stimulation whether or not DFMeOrn was present. Subsequently the polyribosome region gradually increased in control cells, whereas the monoribosome region decreased; the opposite was observed in the presence of DFMeOrn.

As illustrated in Fig. 4, the effect of DFMeOrn on the proportion of ribosomes in polyribosomes appears to be dose-dependent, the maximum effect being observed with 1 mM-DFMeOrn. The proportion of ribosomes in polyribosomes in putrescine- and spermidine-depleted cells reached a minimum value of $72.5 \pm 1.3\%$ (average $\pm$ s.d.) after 24 h in the presence of 1 mM- or 5 mM-DFMeOrn. This value is significantly lower than that observed in control cells at this time ($85.7 \pm 0.8\%$) ($P \leq 0.001$ by a two-tailed Student's *t* test). The decrease in the amount of ribosomes in polyribosomes in DFMeOrn-treated cells was accompanied by an increase in the monoribosome region, which was detectable as early as 6 h after dilution (see Fig. 3, 6h). The decrease in the proportion of ribosomes in polyribosomes paralleled the loss of spermidine

Fig. 5. Correlation between spermidine content and percentage of ribosomes in polyribosomes of HTC cells in the presence and absence of DFMeOrn

Both parameters were determined as described in the Materials and methods section. Results are compiled from eight independent experiments (77 gradients) performed at various times between 6 h and 24 h after dilution of HTC cells in the absence or presence of 0.1 mM-, 1 mM- or 5 mM-DFMeOrn and from two independent experiments where cells were treated with 5 mM-DFMeOrn for 24 h and then exposed to 1 μ M-putrescine for 4 h. The line was derived by linear regression analyses: $r^2 = 0.957$. ●, Control; ○, +DFMeOrn; △, +5 mM-DFMeOrn (24 h) + 1 μ M-putrescine.

(see Fig. 1d). Indeed, Fig. 5 shows that there is a high correlation ($r^2 = 0.957$) between the spermidine content and the amount of ribosomes in polyribosomes between 6 and 24 h after dilution in the presence and absence of DFMeOrn.

To estimate the extent to which the increase in protein-synthetic activity caused by re-feeding is decreased by depletion of putrescine and spermidine, we considered the amount of ribosomes in polyribosomes in high-density cells as the basal value. This value was approx. 60%, which is in close agreement with that reported for other cell lines (Van Venrooij *et al.*, 1972; Hogan & Korner, 1968; Stanners & Becker, 1971). Thus the increase in the amount of ribosomes in polyribosomes above the basal value, in cells exposed to 1 mM- and 5 mM-DFMeOrn for 24 h, represents approx. 50% of that observed in control cells. When [3 H]leucine incorporation was used for the determination of protein-synthetic activity, comparable results were obtained (Table 2); DFMeOrn-treated HTC cells showed a lower rate of [3 H]leucine incorporation than control cells at 9 h after dilution into fresh medium. By 24 h the rate of protein synthesis in DFMeOrn-treated cells represented approx. 40%

Table 2. *Effect of DFMeOrn on the rate of [³H]leucine incorporation into protein*

High-density cell cultures were stimulated to proliferate as described in the legend of Fig. 1. Samples were taken at the indicated times and processed as described in the Materials and methods section. The specific radioactivity of leucine in the acid-soluble fraction was determined and used for calculation of rates of incorporation into the protein fraction (data from individual determinations).

Time after dilution (h)	Rate of leucine incorporation (pmol/30 min per nmol of deoxyribose)		Increase above basal value (DFMeOrn-treated as % of control)
	Control	DFMeOrn	
0	40	40	100
3	80	75	88
6.5	105	112	111
9	107	89	73
12	120	80	50
24	97	63	40

of the increase above basal values observed in control cells (Table 2).

Since DFMeOrn is an analogue of ornithine, it was necessary to determine if comparable concentrations of the amino acid itself had an effect on ribosome distribution. When HTC cells were exposed to 5 mM-L-ornithine, it accumulated to amounts approx. 80-fold higher than normal. These amounts were comparable with those of DFMeOrn at the same external concentration (Table 1), yet no effect on polyribosome profiles was observed (results not shown). At 0.1 mM-DFMeOrn the cells accumulated this inhibitor to concentrations comparable with that of endogenous ornithine. Nevertheless, a significant decrease of polyamine content and of the proportion of ribosomes in polyribosomes was observed. These results suggest that the effect of DFMeOrn on ribosome distribution is related to its inhibitory properties towards ODC. Further proof that the observed effects are related to the depletion of spermidine was given by replenishment experiments.

Effect of exogenously added polyamines on polyribosome profiles of DFMeOrn-treated cells

The effect of DFMeOrn on HTC-cell growth can be reversed by addition of natural polyamines or related compounds to the culture medium (Mamont *et al.*, 1978). In our attempt to re-establish normal polyribosome profiles of DFMeOrn-treated cells, 1 μ M concentrations of putrescine and spermidine were chosen to prevent supranormal accumulation of polyamines, a condition that has been shown to induce polyamine catabolism in these cells (Mamont *et al.*, 1981*b*). A spermine concentration of 1 μ M was chosen because this is the optimum concentration for reversal of cell growth inhibition. Higher spermine concentrations are toxic for the cells (Mamont *et al.*, 1978).

When putrescine- and spermidine-depleted cells

were exposed to 1 μ M-spermidine, the cellular content of this polyamine specifically increased (Table 3). Spermidine re-accumulation was linear during the first 45 min, with a rate of approx. 4 pmol/min per nmol of deoxyribose. Spermine contents did not change during this period, nor was there any detectable degradation of spermidine to putrescine during this time. Intracellular DFMeOrn content did not change either (results not shown), in agreement with observations in fibroblasts (Erwin & Pegg, 1982).

Fig. 6 illustrates how ribosome distribution was affected during spermidine replenishment. As early as 30 min after addition of spermidine, the amount of ribosomes in polyribosomes was increased. Between 1 h and 4 h the profiles became comparable with those observed in control cells. The presence of spermidine in the hypo-osmotic buffer, in amounts equivalent to those observed in spermidine-replenished cells, had no significant effect on the polyribosome profile during the isolation of polyribosomes from DFMeOrn-treated cells.

When putrescine- and spermidine-depleted cells were exposed to 1 μ M-spermine, this polyamine accumulated to supranormal concentrations (Table 3) and caused an increase in polyribosomes. The amplitude and time course of this increase was comparable with that observed in the presence of 1 μ M-spermidine (results not shown).

When 1 μ M-putrescine was added to DFMeOrn-treated cell cultures, very little intracellular accumulation of the diamine was observed. After 1 h, no change in the polyribosome profile was detectable. At 4 h, there was a slight increase in polyribosomes (results not shown), comparable with that observed with spermidine at 30 min, which may be related to the conversion of putrescine into spermidine (Fig. 5) and spermine (Table 3).

Effect of cycloheximide on polyribosome profiles

No significant difference in total RNA content

Table 3. Polyamine content of putrescine- and spermidine-depleted HTC cells during exposure to exogenous polyamines High-density cell cultures were diluted into fresh medium in the presence of 5 mM-DFMeOrn as described in the legend of Fig. 1. After 24h, portions of the culture were further incubated in the absence or presence of 1 μ M-putrescine, -spermidine or -spermine. Samples were taken at the indicated times for determination of polyamine content as described in the Materials and methods section. Results are mean values $\pm$ S.D. from two to seven separate experiments. Putrescine contents were below 9 pmol/nmol of deoxyribose when putrescine was added to the culture medium and below the limit of detection (0.8 pmol/nmol of deoxyribose) under all other experimental conditions described in the Table. At 24h after dilution, polyamine contents (pmol/nmol of deoxyribose; means $\pm$ S.D. from seven experiments) in cells not exposed to DFMeOrn were: putrescine, 61 $\pm$ 22; spermidine, 221 $\pm$ 31; spermine, 256 $\pm$ 24. Abbreviation: n.a., not analysed.

Addition	Time after addition (h) ...	Spermidine (pmol/nmol of deoxyribose)				Spermine (pmol/nmol of deoxyribose)			
		0	0.5	1	4	0	0.5	1	4
None		14 $\pm$ 10	11	12 $\pm$ 9	5 $\pm$ 3	209 $\pm$ 21	198 $\pm$ 12	224 $\pm$ 18	204 $\pm$ 12
Putrescine		n.a.	n.a.	30 $\pm$ 10	38	n.a.	n.a.	217 $\pm$ 9	249 $\pm$ 4
Spermidine		n.a.	118 $\pm$ 5	196 $\pm$ 15	205 $\pm$ 20	n.a.	210 $\pm$ 14	215 $\pm$ 28	229 $\pm$ 18
Spermine		n.a.	12 $\pm$ 2	12	25 $\pm$ 5	n.a.	256 $\pm$ 4	315 $\pm$ 10	405 $\pm$ 5

Fig. 6. Evolution of the polyribosome profile in putrescine- and spermidine-depleted HTC cells exposed to spermidine Experimental conditions were as described in the legend of Table 3. At the indicated time after addition of spermidine, samples of the cell suspension were taken for polyribosome analysis as described in the Materials and methods section., + 5 mM-DFMeOrn; —, + 5 mM-DFMeOrn + 1 μ M-spermidine.

between control and 5 mM-DFMeOrn-treated cells was observed during the first 24h after dilution, and no significant difference in total apparent ribosome content was observed. The effects on ribosome distribution could therefore result from a decrease in mRNA content and/or a defect in initiation, elongation or termination. Exposure of cells to low concentrations of cycloheximide is known to slow elongation and termination to an extent that they become rate-limiting. These and

related conditions of decreased elongation rate permit the accumulation of ribosomes on available mRNA (Stanners, 1966; Fan & Penman, 1970; Lodish, 1971; Oleinick, 1977). As shown in Fig. 7, this was indeed observed. The polyribosome profiles showed that monoribosomes and subunits had been recruited into polyribosomes, giving distribution patterns essentially identical with those of control cells after similar treatment. Identical results were obtained with emetine. Thus

Fig. 7. Effect of low concentrations of cycloheximide on ribosome distribution of putrescine- and spermidine-depleted HTC cells. High-density cultures were diluted in the absence or presence of 5 mM-DFMeOrn as described in the legend of Fig. 1. Then 24 h later, portions of each cell culture were exposed to 1 μ g of cycloheximide/ml for 30 min. Samples were then taken for analysis of polyribosomes as described in the Materials and methods section. (a) DFMeOrn-treated cells, and (b) control cells, in the absence (····) and presence (—) of cycloheximide.

there appeared to be no gross differences in total available mRNA or ribosome content between DFMeOrn-treated and control cells at 24 h.

Discussion

When high-density cultures of HTC cells are diluted into fresh medium, the amount of ribosomes in polyribosomes and the rate of leucine incorporation into protein rapidly increase.

The results presented here clearly indicate that protein-synthetic activity of HTC cells, evaluated from polyribosome profiles and from leucine-incorporation data, was significantly decreased during treatment with DFMeOrn. This decrease appears to have been initiated during the first 3–6 h of exposure and reached a minimum between 12 and 24 h. It correlated with the decrease in spermidine content, since putrescine was diminished by 6 h and since spermine contents remained essentially unchanged.

Brief exposure of DFMeOrn-treated HTC cells to a low concentration of cycloheximide or emetine produced a polyribosome profile comparable with that of similarly treated control cells (Fig. 7). This result indicated that there was no gross difference in either mRNA or ribosome content after 24 h in the presence of 5 mM-DFMeOrn.

The perturbation of polyribosome formation appears to be related to the ODC-inhibitory properties of DFMeOrn and not to a direct effect of this ornithine analogue. This was evidenced by the dose-dependence of the effect on polyamine contents (Table 1) and on ribosome distribution (Fig. 4).

Our replenishment studies showed that putrescine, spermidine and spermine can reverse the effects of DFMeOrn treatment on the polyribosomes. Since putrescine was not accumulating in the reversal experiments, it can be excluded as being directly involved in the re-establishment of a normal polyribosome profile apart from its conversion into spermidine and spermine. Spermine accumulated to supranormal concentrations and gave results comparable with those with spermidine. It appears that the cells were able to use the excess endogenous spermine to replace the missing spermidine. This is not surprising, since numerous studies *in vitro* have shown that spermidine and spermine have qualitatively similar effects on enzyme activities that are modified by binding of polycations to anionic sites. Spermine is usually effective at lower concentrations than spermidine (for references see Maurel & Douzou, 1978). One reason for this is most probably the higher binding energy of spermine (Bloomfield & Wilson, 1981).

Whether spermidine can replace spermine in a comparable situation *in vivo* cannot be studied at present, because it is not possible to deplete cellular spermine specifically. On the other hand, it is highly unlikely that a lack of spermine would be responsible for the perturbation of the polyribosome profile in DFMeOrn-treated HTC cells, since its cellular contents were essentially normal during the time when the decrease in polyribosomes was observed. Under conditions where putrescine and spermidine are depleted, a significant fraction of the endogenous spermine is most probably sequestered from sites within the protein-synthetic machinery which, when occupied by a polyamine, permit optimum protein synthesis. Thus the evidence strongly suggests that the decrease in the amount of ribosomes in polyribosomes in DFMeOrn-treated cells is a consequence of the depletion of spermidine.

The effect of cycloheximide and emetine on ribosome distribution in putrescine- and spermidine-depleted cells is characteristic of systems where the rate of initiation is insufficient with respect to that of elongation (Stanners, 1966; Fan & Penman, 1970; Lodish, 1971). Furthermore, the shift in ribosome distribution during depletion of putrescine and spermidine is similar to that observed when initiation of protein synthesis is impaired (Reichman & Penman, 1973; Lodish, 1971). These results suggest that the rate of initiation of translation may be decreased in putrescine- and spermidine-depleted cells. This possibility is supported by several studies *in vitro* in eukaryotic cell-free systems which suggest that spermidine plays a specific role in initiation (Raymondjean *et al.*, 1977; Kramer *et al.*, 1979; Igarashi *et al.*, 1980). Our experiments do not distinguish, however, between a direct or an indirect effect of spermidine depletion on initiation. Indeed, our observations could also be explained by an increased rate of premature termination (Abraham & Pihl, 1980). More refined studies in intact cells will be required to determine to what extent and for what reasons the individual steps may be affected.

Several investigators have shown that the growth rate of cultured cells is closely linked to the status of protein-synthetic machinery. In the presence of very low concentrations of protein-synthesis inhibitors, doubling times are increased proportionately to the extent of inhibition of protein synthesis. This is mainly due to prolongation of the G1 phase of the cell cycle, thereby delaying initiation of DNA synthesis (Pardee & James, 1975; Brooks, 1977; Rossow *et al.*, 1979; Liskay *et al.*, 1980). Our results show that comparable correlations can be made between spermidine-limited protein synthesis and cell growth in

DFMeOrn-treated HTC cells. Furthermore, several investigators have observed that, during polyamine depletion, cells accumulate in the phase of the cell cycle in which they would normally accumulate under conditions of nutrient deprivation (see Heby, 1981; Morris & Harada, 1980). These observations are consistent, therefore, with an effect of polyamine depletion on protein synthesis. Our results also lend support to the idea that the prevention of virus synthesis after pre-treatment of a host cell with DFMeOrn may be due in part to altered protein-synthetic activity of the host cell (Tuomi *et al.*, 1982).

Impairment of DNA synthesis has been considered for many cell lines, including HTC cells, as the most prominent early cellular response to polyamine depletion (Heby & Jänne, 1981). Our data show that a decrease in protein-synthetic activity occurred concomitantly with the depletion of spermidine in HTC cells stimulated to proliferate in the presence of DFMeOrn. The major portion of the decrease in the amount of ribosomes in polyribosomes had occurred by 12h. A decrease in DNA synthesis was only detectable at later times, and did not parallel the decrease in putrescine or spermidine. It follows therefore that, in HTC cells, protein synthesis is affected before DNA synthesis during depletion of spermidine. Hölttä *et al.* (1979) came to a similar conclusion during polyamine depletion in phytohaemagglutinin-stimulated human lymphocytes. It is well established that unperturbed protein synthesis is a prerequisite for normal DNA-synthetic activity in proliferating cells (for references see Baserga, 1976; Prescott, 1976). On the basis of these observations, the results discussed herein indicate that the initial perturbation of DNA synthesis observed in cultured cells during spermidine depletion by DFMeOrn and the cytostatic effect of this compound could be a consequence of decreased protein-synthetic activity.

We thank M. Siat for routine maintenance of cell cultures, Dr. J. Wagner for some of the polyamine analyses, J.-J. Schwartz for assistance with the leucine analyses, Dr. W. Higgins for discussion and helpful comments on the manuscript, and I. Boutillon for skilful secretarial assistance. This work was performed by B. B. R. in partial fulfilment of the requirements for a Ph.D. degree from the University of Pennsylvania, Philadelphia, PA, U.S.A.

References

- Abraham, A. K. & Pihl, A. (1980) *Eur. J. Biochem.* **106**, 257–262
- Algranati, I. D. & Goldemberg, S. H. (1977) *Trends Biochem. Sci.* **2**, 272–274

- Baserga, R. (1976) *Multiplication and Division in Mammalian Cells*, Marcel Dekker, New York
- Bey, P. (1978) in *Enzyme-Activated Irreversible Inhibitors* (Seiler, N., Jung, M. J. & Koch-Weser, J., eds.), pp. 27–41, Elsevier/North-Holland Biomedical Press, Amsterdam
- Bloomfield, V. A. & Wilson, R. W. (1981) in *Polyamines in Biology and Medicine* (Morris, D. R. & Marton, L. J., eds.), pp. 183–206, Marcel Dekker, New York
- Brooks, R. F. (1977) *Cell* **12**, 311–317
- Erwin, B. G. & Pegg, A. E. (1982) *Biochem. Pharmacol.* **31**, 2820–2823
- Fan, H. & Penman, S. (1970) *J. Mol. Biol.* **50**, 655–670
- Gelehrter, T. D. & Tomkins, G. D. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 723–730
- Grove, J., Schechter, P. J., Hanke, N. F. J., de Smet, Y., Agid, Y., Tell, G. & Koch-Weser, J. (1982) *J. Neurochem.* **39**, 1618–1622
- Heby, O. (1981) *Differentiation* **19**, 1–20
- Heby, O. & Jänne, J. (1981) in *Polyamines in Biology and Medicine* (Morris, D. R. & Marton, L. J., eds.), pp. 243–310, Marcel Dekker, New York
- Hershko, A., Mamont, P., Shields, R. & Tomkins, G. M. (1971) *Nature (London) New Biol.* **232**, 206–211
- Hogan, B. L. M. & Korner, A. (1968) *Biochim. Biophys. Acta* **169**, 129–138
- Hölttä, E., Jänne, J. & Hovi, T. (1979) *Biochem. J.* **178**, 109–117
- Igarashi, K., Kojima, M., Watanabe, Y., Maeda, K. & Hirose, S. (1980) *Biochem. Biophys. Res. Commun.* **97**, 480–486
- Kramer, G., Odom, O. W. & Hardesty, B. (1979) *Methods Enzymol.* **60**, 555–567
- Liskay, R. M., Kornfield, B., Fullerton, P. & Evans, R. (1980) *J. Cell. Physiol.* **104**, 461–467
- Lodish, H. (1971) *J. Biol. Chem.* **246**, 7131–7138
- Mamont, P. S., Böhlen, P., McCann, P. P., Bey, P., Schuber, F. & Tardif, C. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1626–1630
- Mamont, P. S., Duchesne, M.-C., Joder-Ohlenbusch, A.-M. & Grove, J. (1978) in *Enzyme-Activated Irreversible Inhibitors* (Seiler, N., Jung, M. J. & Koch-Weser, J., eds.), pp. 43–54, Elsevier/North-Holland Biomedical Press, Amsterdam
- Mamont, P. S., Bey, P. & Koch-Weser, J. (1980) in *Polyamines in Biomedical Research* (Gaugas, J. M., ed.), pp. 147–165, John Wiley, Chichester
- Mamont, P. S., Joder-Ohlenbusch, A.-M., Nussli, M. & Grove, J. (1981a) *Biochem. J.* **196**, 411–422
- Mamont, P. S., Seiler, N., Siat, M., Joder-Ohlenbusch, A.-M. & Knödgen, B. (1981b) *Med. Biol.* **59**, 347–353
- Martin, T. E., Wool, I. G. & Castles, J. J. (1971) *Methods Enzymol.* **20**, 417–429
- Maurel, P. & Douzou, P. (1978) in *Frontiers in Physicochemical Biology* (Pullman, B., ed.), pp. 421–457, Academic Press, New York
- Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P. & Vevert, J. P. (1978) *J. Am. Chem. Soc.* **100**, 2551–2553
- Morris, D. R. & Harada, J. J. (1980) in *Polyamines in Biomedical Research* (Gaugas, J. M., ed.), pp. 1–16, John Wiley, Chichester
- Oleinick, N. (1977) *Arch. Biochem. Biophys.* **182**, 171–180
- Pardee, A. B. & James, L. J. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4994–4998
- Pegg, A. E. & McCann, P. P. (1982) *Am. J. Physiol.* **243**, C212–C221
- Perry, R. P. & Kelley, D. E. (1968) *J. Mol. Biol.* **35**, 37–59
- Pösö, H., Seely, J. E., Zagon, I. S. & Pegg, A. E. (1983) *Adv. Polyamine Res.* **4**, 603–613
- Prescott, D. M. (1976) *Reproduction of Eukaryotic Cells*, Academic Press, New York
- Raymondjean, M., Bogdanovsky, D., Bachner, L., Kneip, B. & Schapira, G. (1977) *FEBS Lett.* **76**, 311–315
- Reichman, M. & Penman, S. (1973) *Biochim. Biophys. Acta* **324**, 282–289
- Rosow, P. W., Riddle, V. G. H. & Pardee, A. B. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4446–4450
- Roth, M. & Hampai, A. (1973) *J. Chromatogr.* **83**, 353–356
- Scott, W. A., Shields, R. & Tomkins, G. M. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2937–2941
- Seiler, N. & Knödgen, B. (1980) *J. Chromatogr.* **221**, 227–235
- Seiler, N. & Sarhan, S. (1983) *Neurochem. Int.* **5**, 625–633
- Seiler, N. & Schmidt-Glenewinkel, T. (1975) *J. Neurochem.* **24**, 791–795
- Stanners, C. P. (1966) *Biochem. Biophys. Res. Commun.* **24**, 758–764
- Stanners, C. P. & Becker, H. (1971) *J. Cell. Physiol.* **77**, 31–42
- Tabor, C. W. & Tabor, H. (1976) *Annu. Rev. Biochem.* **45**, 285–306
- Tuomi, K., Raina, A. & Mäntyjärvi, R. (1982) *Biochem. J.* **206**, 113–119
- Van Venrooij, W. J. W., Henshaw, E. C. & Hirsch, C. A. (1972) *Biochim. Biophys. Acta* **259**, 127–137
- Wagner, J., Danzin, C. & Mamont, P. S. (1982) *J. Chromatogr.* **227**, 349–368
- Zylber, E. A. & Penman, S. (1970) *Biochim. Biophys. Acta* **204**, 221–229