Post-translational modification of the protein-synthesis initiation factor eIF-4D by spermidine in rat hepatoma cells

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The rates of synthesis and turnover of the rare amino acid hypusine [*N*⁶-(4-amino-2-hydroxybutyl)-2,6-diaminohexanoic acid] in protein were studied in relationship to polyamine metabolism and growth rates in rat hepatoma tissue-culture (HTC) cells. Hypusine is selectively formed in the eukaryotic translation initiation factor eIF-4D, by a post-translational mechanism involving spermidine [Cooper, Park, Folk, Safer & Braverman (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1854–1857]. The half-life of the hypusine-containing protein was longer than 24 h. In cells whose intracellular spermidine pools had been initially depleted, by using DL- α -difluoromethylornithine (DFMO), maximum synthesis rates of hypusine in protein were 5–10 times higher, on restoration of endogenous spermidine contents by exogenous addition, than those observed in untreated exponential-phase cultures. In cells pretreated with DFMO, the rate of hypusine synthesis was constant for up to 1 h after the addition of 5 μ M-spermidine, whereas endogenous spermidine contents varied from less than 1 to more than 10 nmol/mg of protein. However, the overall amount of hypusine formed, during the first 1 h after the addition of various concentrations of spermidine (0.05–10 μ M) to the culture medium, was markedly dependent on the final endogenous spermidine content achieved at the end of the 1 h measurement interval. Early in exponential-phase growth, protein-bound hypusine was synthesized at a rate of 1–2 pmol/h per mg of protein. This rate decreased to less than 0.5 pmol/h per mg of protein when cell growth rates decreased as cultures reached high cell densities. Analysis of the polyamine substrate specificity for hypusine formation showed that *N*¹-acetylspermidine did not compete with spermidine in the reaction, nor did *N*¹-(buta-2,3-dienyl)-*N*²-methylbutane-1,4-diamine, an irreversible inhibitor of polyamine oxidase, block the reaction. On the basis of comparative radiolabelling experiments, spermine was either a poor substrate, or not a substrate, for hypusine formation. These results confirm that spermidine is the likely precursor of the aminohydroxybutyl moiety of hypusine, and show that overall hypusine formation, but not necessarily the synthesis rate, is dependent on the endogenous spermidine concentration, especially under conditions where spermidine concentrations are initially low, as is the case after DFMO treatment, and then increase.

INTRODUCTION

Hypusine [*N*⁶-(4-amino-2-hydroxybutyl)-2,6-diaminohexanoic acid], an unusual amino acid originally discovered by Shiba *et al.* (1971), is present as its free or as its protein-bound form in various mammalian tissues (Nakajima *et al.*, 1971; Imaoka & Nakajima, 1973; Sano *et al.*, 1984). As demonstrated by Folk and his group (Park *et al.*, 1981, 1982; Cooper *et al.*, 1982), hypusine synthesis proceeds by a multi-step mechanism and occurs as a post-translational modification of a peptidyl-lysine residue of a single protein, the translation initiation factor eIF-4D (Cooper *et al.*, 1983). Although the biochemical mechanism of this protein modification has not been entirely clarified from the enzymic point of view, it appears that the polyamine spermidine is the precursor of the aminobutyl group of hypusine (Park *et al.*, 1981, 1984).

Polyamines play essential regulatory functions in cell replication processes (reviewed by Pegg & McCann, 1982; Tabor & Tabor, 1985). Interestingly, the post-translational modification of eIF-4D occurs after

initiation of cell proliferation (Cooper *et al.*, 1982; Chen, 1983; Torrelío *et al.*, 1984). These facts led us to study more precisely the relationship between polyamine metabolism, growth rate and the modification of this protein, using growing rat hepatoma tissue-culture (HTC) cells or cells depleted of their spermidine content by the use of DL- α -difluoromethylornithine (DFMO), an irreversible inhibitor of L-ornithine decarboxylase (Metcalf *et al.*, 1978).

EXPERIMENTAL

Materials

DFMO (Metcalf *et al.*, 1978), *N*¹-(buta-2,3-dienyl)-*N*²-methylbutane-1,4-diamine (Bey *et al.*, 1985) and *N*¹-acetylspermidine (Tabor *et al.*, 1971; Bolkenius & Seiler, 1981) were synthesized at our Institute. [*terminal methylenes*-³H]Spermidine trihydrochloride and [*tetra-methylene*-1,4-¹⁴C]spermine tetrahydrochloride were purchased from New England Nuclear. Cycloheximide was from Sigma (St. Louis, MO, U.S.A.) and putrescine,

Abbreviations used: eIF-4D, eukaryotic initiation factor 4D; HTC cells, hepatoma tissue-culture cells; DFMO, DL- α -difluoromethylornithine.

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spermidine and spermine hydrochloride were obtained from Fluka (Buchs, Switzerland). Authentic hypusine was generously given by Dr. J. E. Folk (N.I.H., U.S.A.).

Methods

HTC cells were grown as a suspension as described previously (Mamont *et al.*, 1981a). Horse serum (Gibco) replaced newborn-calf serum in experiments with polyamines and their acetyl derivatives. To deplete the putrescine and spermidine contents of the cells, cultures were incubated in the presence of 5 mM-DFMO for either 24 or 42 h (Mamont *et al.*, 1978). For the determination of polyamines, HClO_4 cell extracts were prepared (Mamont *et al.*, 1978) and analysed by h.p.l.c. as described by Seiler & Knodgen (1980). HClO_4 -insoluble fractions were washed twice with cold 0.1 M- HClO_4 , and hydrolysed in 6 M-HCl at 120 °C for 18 h. After drying in an evacuated desiccator, residues were dissolved in 0.1 M-HCl and analysed by h.p.l.c. Radioactivities were determined by scintillation counting of 1 min fractions collected from the post-column effluent supplemented with 10 ml of Aquasol-2 (NEN Chemicals, Dreieich, Germany). For gel electrophoresis, cells (2×10^6) were solubilized in 0.5 ml of 62.5 mM-Tris/HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 2% mercaptoethanol and 0.001% Bromophenol Blue. Single-dimension electrophoresis was performed as described by Laemmli (1970), except that *NN'*-diallyltartardiamide was substituted for bisacrylamide. Thereafter, gels were fractionated into 1 mm slices, dissolved by addition of periodic acid, and radioactivity was determined after addition of 10 ml of Econofluor (NEN Chemicals). Proteins were measured by the method of Lowry *et al.* (1951).

RESULTS

Identification of newly formed protein-bound hypusine in HTC cells

In preliminary studies, we demonstrated that, as in other cultured cells (Cooper *et al.*, 1982; Chen, 1983; Torrelío *et al.*, 1984), radiolabelled spermidine added to the culture medium was essentially incorporated into a single protein of M_r 18000, most probably eIF-4D (Fig. 1a). Analysis by h.p.l.c. of the HClO_4 -insoluble cell extracts, after acid hydrolysis, revealed the presence of four radioactive areas (Fig. 1b). The major peak, which accounted for 60% of the total radioactivity and was eluted slightly before putrescine, was identified as hypusine by chromatographic comparison with an authentic standard. Spermidine and spermine were also present in the acid hydrolysate, as were unidentified radioactive materials which were eluted at the beginning of the gradient.

Degradation of the hypusine-containing protein

By taking advantage of the selective labelling of the 18 kDa protein by radioactive spermidine, its degradation was first determined by the measurement of radiolabelled hypusine covalently bound to protein after a chase with unlabelled spermidine. It has been suggested that the post-translational formation of hypusine may occur soon or after protein translation (Park *et al.*, 1982). Consequently, cycloheximide was used to prevent ribosome translocation and synthesis *de novo* of the eIF-4D protein during the chase. As illustrated in Fig. 2, the content of radiolabelled protein-bound hypusine did

Fig. 1. Identification of newly formed protein-bound hypusine

At 2 days after subculture, cells were incubated in the presence of [*terminal methylenes*- ^3H]spermidine (2 $\mu\text{Ci/ml}$, 44.3 Ci/mmol) for 1 day. At that time, HClO_4 -soluble and insoluble fractions of the cells were prepared as described in the Experimental section. SDS/polyacrylamide-gel electrophoresis (a) was performed with 50 μg of whole-cell proteins; 21 K and 14 K indicate the migration of M_r marker proteins. To identify hypusine, 2 mg of proteins were acid-hydrolysed and analysed by h.p.l.c. (b). Abbreviations: HY, hypusine; PUT, putrescine; CAD, cadaverine; HIS, histamine; SPD, spermidine, SPM, spermine; DAHp, diaminoheptane.

Fig. 2. Turnover of the hypusine-bound protein

HTC cells were incubated in the presence (●) or in the absence (○) of 5 mM-DFMO for 42 h. Cells were then pulse-labelled with radioactive spermidine (2 μ Ci/ml, 44.3 Ci/mmol) for 2 h. At that time, cells were washed and further incubated in fresh medium supplemented with 5 μ M unlabelled spermidine and 200 μ M-cycloheximide. At the indicated times, radioactivities in protein-bound hypusine were determined by h.p.l.c. as described in the Experimental section.

not decrease in either DFMO-treated or -untreated cells after the 6 h period of chase.

Since it can be argued that the 18 kDa protein might be partially degraded into peptidic fragments large enough to be acid-precipitable, the 18 kDa-protein-associated radioactivity was also measured in a second series of experiments (Table 1). Moreover, cycloheximide was omitted to prevent any deleterious effects on the proteolytic degradative processes. No decrease of either protein-bound hypusine radioactivity (Expt. 1) or 18 kDa protein-associated radioactivity (Expt. 2) was noticed up to 6 h after the beginning of the chase period. These radioactivities declined, however, by 60 and 30% relative to the initial values at 24 and 48 h respectively. These decreases inversely correlated with the increase in cell numbers.

Protein-bound hypusine synthesis

The time-dependence of protein-bound hypusine formation was studied in control and DFMO-pretreated cells as an attempt to determine rates of hypusine synthesis. After addition of radioactive spermidine (0.09 μ M) to the culture medium of control cells (Fig. 3, upper panel), the intracellular spermidine specific radioactivity continuously increased during the first 1 h period of labelling to reach maximum values thereafter. The calculated amount of total protein-bound hypusine synthesized *de novo* was about 3 pmol/mg of protein. Formation of hypusine as a function of time was not linear at the beginning of the labelling, most probably because of rapid changes of the specific radioactivity of

Table 1. Degradation of the hypusine-containing protein

HTC cells were pretreated with DFMO for 42 h as described in the Experimental section. Cultures were then pulse-labelled for 2 h with either 2 (Expt. 1) or 20 μ Ci (Expt. 2) of [*terminal methylenes*-³H]spermidine (44.3 Ci/mmol)/ml. Cells were washed and further incubated in fresh medium supplemented with 5 μ M unlabelled spermidine. At the indicated times, radioactivities in protein-bound hypusine (Expt. 1) or associated with the 18 kDa protein (Expt. 2) were determined by h.p.l.c. or gel-electrophoresis analyses, respectively. Gel electrophoresis was performed with 80 μ g of whole cell proteins.

	Time (h)	10 ⁻⁵ × Cell no. (/ml)	10 ⁻³ × Radioactivity
Expt. 1			In protein-bound hypusine (d.p.m./mg of protein)
	0	1.00	6.86
	3	1.10	6.86
	6	1.20	7.02
	24	1.84	4.41
	48	3.47	1.98
Expt. 2			In 18 kDa protein (d.p.m./sample)
	0	1.00	2.85
	6	1.20	2.71
	24	1.95	1.58

spermidine. Taking into account the linear part of the curve, hypusine synthesis rate could be estimated to be 1.7 pmol/h per mg of protein.

Pretreatment of the cells with DFMO for 42 h depleted the intracellular putrescine and spermidine to undetectable values (Fig. 3, lower panel). Radioactive spermidine (5 μ M) added to the culture medium resulted in a linear increase in the intracellular spermidine concentration to normal control values within 1 h. By 2 h after addition, endogenous spermidine concentrations were nearly twice control values. Under these conditions, the intracellular specific radioactivity of spermidine remained constant during the 2 h labelling interval. Hypusine formation increased linearly during the first 1 h at a calculated rate of 10.6 pmol/h per mg of protein. Thereafter, hypusine synthesis continued at a slower rate, as suggested by previous longer-term labelling studies (Gerner & Mamont, 1985).

Relationship between hypusine formation, growth rates and spermidine concentration in HTC cells

Within 6 h after induction of cell proliferation, formation of peptidyl hypusine increased almost 2-fold to reach a value of 2 pmol/h per mg of protein (Fig. 4). This rate was maintained during the exponential phase of growth and declined to a value of 0.4 pmol/h per mg of protein when cultures reached high cell density and cell growth rates decreased. During this same time, the spermidine content of the cells displayed qualitatively similar changes to those found for hypusine formation, although spermidine increased between 6 and 48 h from 7.5 to nearly 10 nmol/mg of protein, whereas hypusine synthesis rates remained essentially constant. Although the data shown in Fig. 4 are from a single representative

Fig. 3. Protein-bound hypusine synthesis in control and DFMO-pretreated cells

HTC cells were preincubated for 42 h in the absence (upper panel) or in the presence (lower panel) of 5 mM-DFMO. At that time, media were changed and cells were then labelled with radioactive spermidine (2 $\mu\text{Ci}/\text{ml}$, 22.5 Ci/mmol); 5 μM unlabelled spermidine was concomitantly added with radioactive spermidine to the culture medium of DFMO-treated cells. At the indicated times, intracellular polyamine contents (●, ■) and polyamine specific radioactivities (○, □) were measured. Newly formed protein-bound hypusine of DFMO-untreated (upper panel) and -treated cells (lower panel) were calculated after determination of protein-bound hypusine radioactivity as follows:

$$\text{hypusine (pmol/mg of protein)} = \frac{\text{d.p.m. of hypusine}}{\frac{1}{2} \times \text{SRA} \times P}$$

where SRA = spermidine specific radioactivity (d.p.m./pmol) and P = protein content of the sample (mg). The factor $\frac{1}{2}$ was used because one of the two labelled methylene groups from spermidine is incorporated into hypusine.

experiment, qualitatively similar results were found in two replicate experiments.

Concentration-dependent effects of spermidine on hypusine formation in DFMO-treated cells

To gain better information on the relationship between the spermidine contents of the cells and hypusine formation, DFMO-induced spermidine-depleted cells were used. Intracellular concentration of spermidine was differentially restored by addition of increasing amounts of radioactive spermidine to the culture medium, maximum intracellular spermidine content being reached at exogenous concentration of spermidine $\geq 2.5 \mu\text{M}$ (Fig. 5). Hypusine synthesis increased as a function of the intracellular spermidine concentration in a non-linear fashion. As low as $0.05 \mu\text{M}$ exogenously added radiolabelled spermidine, a concentration that did not apparently elevate the residual spermidine content of DFMO-treated cells, led to incorporation into hypusine. Maximum hypusine formation occurred at intracellular spermidine contents $\geq 12 \text{ nmol}/\text{mg}$ of protein. These spermidine concentrations were of the same order of magnitude as those found during the exponential phase of growth (Fig. 4).

Amine substrate specificity of the post-translational modification of eIF-4D

Spermidine, but not its precursor putrescine, is a substrate of the enzymic reactions which lead to the formation of protein-bound hypusine (Park *et al.*, 1981). It has not been, however, considered that *N*¹-acetylspermidine, a natural metabolite of spermidine, may in fact be the amine precursor of hypusine. This hypothesis is strengthened by the demonstration that only the butylamino group of spermidine is incorporated into hypusine (Park *et al.*, 1981, 1982) and that *N*¹-acetylspermidine is oxidatively cleaved into putrescine and 3-acetamidopropanal by the polyamine oxidase enzyme (Bolkenius & Seiler, 1981). To verify this hypothesis, competition experiments for hypusine formation, between radioactive spermidine and unlabelled *N*¹-acetylspermidine, were performed in spermidine-deficient cells. *N*¹-(Buta-2,3-dienyl)-*N*²-methylbutane-1,4-diamine (MDL 72.521), an irreversible inhibitor of polyamine oxidase (Bey *et al.*, 1985), was also added at 25 μM concentration to the culture medium, 2 h before the addition of the amines, to ensure complete inhibition of *N*¹-acetylspermidine catabolism *in situ* (Seiler *et al.*, 1985).

In the absence of MDL 72.521, exogenously added

Fig. 4. Relationship between hypusine formation, growth rates and spermidine concentration

High-density cell cultures (8×10^5 cells/ml) were diluted into fresh medium at a cell density of 1.5×10^5 cells/ml. At the indicated times, samples were pulse-labelled for 1 h with $2 \mu\text{Ci}$ of radioactive spermidine (44.3 Ci/mmol)/ml. At the end of the pulse-labelling, cell number (●), spermidine content (▲) and protein-bound hypusine formation (○) were determined as described in the Experimental section.

N^1 -acetylspermidine accumulated intracellularly and elevated 5-fold the residual spermidine content of DFMO-treated cells (Table 2). As previously demonstrated (Mamont *et al.*, 1981b), increase in spermidine content resulted from the rapid consumption of the putrescine, produced from N^1 -acetylspermidine, by the spermidine synthase reaction. This reaction was greatly facilitated by the high concentration of decarboxylated

S -adenosylmethionine present in DFMO-treated cells (Mamont *et al.*, 1982). In cells whose polyamine oxidase activity was inhibited by MDL 72,521, N^1 -acetylspermidine accumulated as such, with no elevation of the residual spermidine content. As shown in Table 2, incorporation of radioactive spermidine into hypusine, which resulted from the concomitant labelling of the cells with tracer amount of spermidine ($0.045 \mu\text{M}$), was neither decreased by the accumulation of N^1 -acetylspermidine nor dependent on the catabolism of this acetyl derivative of polyamines into putrescine and subsequent re-accumulation of spermidine. Of most interest, inhibition of polyamine oxidase activity did not affect hypusine formation in any of the experimental conditions used.

Contribution of spermine to hypusine formation in spermidine-deficient cells

Park *et al.* (1981) have provided evidence for the failure of [*aminopropyl*- $3\text{-}^3\text{H}$]spermine to enter hypusine. We further investigated to what extent spermine might contribute to hypusine formation in condition of spermidine deficiency, by pulse-labelling with [*tetramethylene*- ^{14}C]spermine. As shown in Table 3, barely detectable amounts of radioactivity were incorporated into hypusine from radiolabelled spermine, despite a 2.8-fold increase in the intracellular spermine content of the cells. This incorporation most probably occurred through the formation of spermidine from spermine via the retroconversion pathway (Bolkenius & Seiler, 1981). These data show that spermine either is not a substrate, or is a poor substrate, for hypusine formation.

DISCUSSION

In HTC cells, it is confirmed that, like other cultured cells (Cooper *et al.*, 1982; Chen, 1983; Torrelío *et al.*, 1984), radioactive spermidine is incorporated predominantly into a single protein of M_r 18000, most probably eIF-4D (Cooper *et al.*, 1983). On acid hydrolysis, the

Fig. 5. Concentration-dependent effects of spermidine on hypusine formation on DFMO-treated cells

Cells were preincubated for 42 h in the presence of DFMO. At that time medium was changed, and cells were re-incubated in fresh medium for 1 h in the presence of DFMO and increasing concentrations of radioactive spermidine. Intracellular spermidine contents, spermidine specific radioactivities and hypusine formation were determined as described in the Experimental section. Results of two experiments are represented (○, ●).

Table 2. Effects of *N*¹-acetylspermidine in the presence or in the absence of MDL 72.521 on hypusine formation

HTC cells were incubated in the presence of DFMO for 24 h to deplete the intracellular spermidine. Culture samples were then incubated for 2 h in the presence of 25 μ M-MDL 72.521 to inhibit polyamine oxidase activity. Radiolabelled spermidine (2 μ Ci/ml; 44.3 Ci/mmol) and unlabelled amines were then added for 2 h. Abbreviation: n.d., none detected.

Addition	MDL 72.521 present	Protein-bound hypusine (d.p.m./mg of protein)	Intracellular polyamine (nmol/mg of protein)	
			Spermine	<i>N</i> ¹ -Acetylspermidine
None	+	13 348	0.20	n.d.
<i>N</i> ¹ -Acetylspermidine (10 μ M)	+	13 221	0.21	1.59
	—	15 007	1.11	0.75
Spermidine (5 μ M)	+	5 671	7.60	n.d.
	—	6 465	6.12	n.d.

protein liberated radioactive hypusine, whose quantitative analysis has been used to study the relationship between hypusine formation, polyamine metabolism and cell growth rates.

In agreement with previous findings (Park *et al.*, 1981), spermidine is the likely amine precursor of hypusine synthesis. *N*¹-Acetylspermidine does not compete with spermidine *in situ* in the enzymic reactions leading to hypusine formation. It was expected that intracellular spermidine produced from *N*¹-acetylspermidine, via the transient formation of putrescine, would decrease the incorporation of radioactivity into hypusine because of the dilution of the exogenously added radiolabelled spermidine. This decrease does not occur, most probably as a result of the increased amount of hypusine formation caused by the elevation of the intracellular spermidine content. The rate of hypusine synthesis does not correlate directly with the spermidine content of cells (Fig. 3, lower panel), as a constant rate of hypusine formation was observed during the first 1 h after addition of 5 μ M exogenous spermidine, whereas endogenous spermidine concentrations increased by more than 1 order of magnitude. The overall amount of hypusine formation was dependent on the endogenous spermidine content in cells whose spermidine pools had been previously depleted with DFMO (Fig. 5). These data show that the overall amount, but not necessarily the rate, of hypusine formation is dependent on the endogenous spermidine content, especially in cells treated with DFMO.

Hypusine synthesis proceeds by a multi-step mechanism: covalent binding of spermidine to the ϵ -NH₂ group of protein-bound lysine leads to bound deoxyhypusine, which is subsequently hydroxylated (Park *et al.*, 1982). More recently, Park *et al.* (1984) provided more precise evidence in favour of this scheme. The imino group of hypusine derives from lysine and not from spermidine, and C-5 of 1,8-diamino-4-azaoctane (spermidine) is linked to lysine. These findings may explain the ineffectiveness of the polyamine oxidase inhibitor to inhibit hypusine synthesis, since polyamine oxidase enzyme generates putrescine and not 4-aminobutanol (Bolkenius & Seiler, 1981).

Our attempts to determine precisely hypusine synthesis rates in various experimental conditions are not entirely satisfactory. In growing cells, labelling by exogenous radioactive spermidine leads to a time-dependent change

of the intracellular specific radioactivity, whereas a time-dependent change of the intracellular concentration of spermidine follows the exogenous addition of the amine to the culture medium of spermidine-deficient cells. Despite the limitations of the methods used, kinetic studies of incorporation of spermidine into hypusine suggest that the rate of hypusine synthesis in growing cells has a value of 1–2 pmol/h per mg of protein. This value is of the same order of magnitude as those calculated from reported amounts of radiolabelled hypusine formed in other cultured cell systems after a 24 h period of labelling (Torrelío *et al.*, 1984). This calculation is also based on the assumption that the hypusine-modified protein is poorly degraded in these other cells, as demonstrated here for HTC cells. As shown in cultured CHO cells (Torrelío *et al.*, 1984), the rate of hypusine formation in HTC cells is a function of the growth conditions, maximum rates being observed when cells are in the exponential phase of growth. It seems unlikely, however, that in HTC cells spermidine, as the co-substrate of the post-translational modification, plays an essential role in the regulation of hypusine formation, since spermidine concentrations only vary by a factor of 2, after induction of cell proliferation, and are in the range (5–10 nmol/mg of protein) where hypusine formation is least dependent on endogenous spermidine content. It is possible, however, that in other cell systems, such as in stimulated lymphocytes, the striking elevation of spermidine concentrations, from values less than 1 nmol/mg of protein to concentrations similar to those seen in the HTC cells, does contribute to increased rates of eIF-4D modification.

On restoration of the spermidine content, the initial rate of hypusine formation in DFMO-treated cells is apparently 10 times higher than in untreated control cultures. Further studies are needed to determine whether or not this higher rate is due to an increased accumulation of the unmodified protein caused by DFMO treatment and/or to an increased activity of the processing enzymes.

Finally, depletion of spermidine leads to a marked decrease in hypusine synthesis in HTC cells and total cell protein synthesis both in HTC cells (Rudkin *et al.*, 1984) and in mitogen-activated lymphocytes (Holttä & Hovi, 1985). In hepatoma cells, the contribution of residual spermine to hypusine formation via the formation of spermidine is minimal. It is, however, premature to

Table 3. Hypusine formation with either spermidine or spermine as substrate

HTC cells were treated with DFMO for 42 h. Radiolabelled spermidine ($2 \mu\text{Ci}/\text{ml}$; $1.9 \text{ Ci}/\text{mmol}$) or [*tetramethylene- ^{14}C*]spermine ($0.75 \mu\text{Ci}/\text{ml}$; $74 \text{ mCi}/\text{mmol}$) were then added for 4 h.

Addition	Spermidine		Spermine		Hypusine (pmol/mg of protein)
	Hypusine (d.p.m./mg of protein)	(d.p.m./mg of protein)	(nmol/mg of protein)	(d.p.m./mg of protein)	
Spermidine	31 701	2.13×10^7	5.75	9.08×10^6	17.12*
Spermine	62	150×10^4	0.08	2.95×10^6	0.33†

*pmol of hypusine/mg of protein = (d.p.m./mg of protein)/(d.p.m./pmol of spermidine).

†pmol of hypusine/mg of protein = (d.p.m./mg of protein)/(d.p.m./pmol of spermine).

attribute the decrease in cell protein synthesis to the decrease in hypusine formation, since the function of eIF-4D has not been clarified and its role as a protein-synthesis initiation factor has been questioned (Thomas *et al.*, 1982). Germane to this point, it still remains to be demonstrated that the post-translational modification of eIF-4D contributes to activate or impair its biological function. Whatever the answer, it is striking that, on restoration of the spermidine content of DFMO-treated cells, spermidine is immediately incorporated into eIF-4D before the residual spermidine content of the cells is elevated. This finding emphasizes again the biochemical importance of this post-translational modification.

E. W. G. was supported in part by U.S. Public Health Service grants from the National Cancer Institute CA-17343, CA-18273 and CA-30052. The excellent technical assistance of Margaret Tome is appreciated.

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Received 31 January 1986/2 June 1986; accepted 27 June 1986