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Polyamines and HeLa-cell DNA replication

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HeLa cells were synchronized for S-phase DNA synthesis by the double thymidine-block procedure. A comparison was made of the polyamine content and S-phase DNA synthesis in cells from control cultures and cultures to which an inhibitor of polyamine biosynthesis, α-difluoromethylornithine, was added to the synchronization medium. Control cells showed a peak of synchronous DNA synthesis at 3 h and a maximum concentration of polyamines at 6–9 h after release of the second thymidine block. Cells from cultures containing the inhibitor were severely inhibited in the synthesis of DNA and contained no putrescine and only traces of spermidine while the spermine content was lowered by as much as 80%. Supplementation of cultures containing α-difluoromethylornithine with a polyamine, at the time of release of the second thymidine block, replenished the intracellular pool of the administered polyamine and partially restored S-phase DNA synthesis, with a lag of 3–6 h. Almost complete restoration of DNA synthesis in cells depleted of polyamines was achieved by the addition of a polyamine to cultures at least 10 h before release of the second thymidine block. The lag in initiation of synchronous S-phase DNA synthesis was eliminated in these cells. It is concluded that reversal by polyamines of the deficiency in S-phase DNA synthesis, in polyamine-depleted HeLa cells, is a time-dependent process indicative of the necessity for the replenishment of replication factors or their organization into an active replication complex.

INTRODUCTION

The essential requirement for polyamines in the proliferation of cells was first demonstrated in bacteria (Herbst & Snell, 1948, 1949). Later, stimulation by polyamines of the proliferation of animal and plant cells in culture was also observed (Ham, 1964; Bertossi *et al.*, 1965). More recently, the essential role of polyamines in cell proliferation has been confirmed in polyamine auxotrophs of *Escherichia coli* (Hafner *et al.*, 1979) and *Saccharomyces cerevisiae* (Cohn *et al.*, 1978) and in animal cells in which ornithine decarboxylase (EC 4.1.1.17), the first enzyme in the pathway of polyamine biosynthesis, was irreversibly inhibited by α-difluoromethylornithine (DFMO) (Mamont *et al.*, 1978). An inhibitor of the enzymic conversion of putrescine into spermidine and spermine, methylglyoxal bis(guanylhydrazone), was utilized by Fillingame *et al.* (1975) to demonstrate a requirement for polyamines in synthesis of DNA by primary cultures of lymphocytes. Subsequently Knutson & Morris (1978) showed that DNA synthesis in nuclei, isolated from bovine lymphocytes in which polyamines were depleted, was inhibited by more than 50%. The addition of polyamines to the assays did not increase DNA synthesis by the isolated lymphocyte nuclei. Krokan & Eriksen (1977) similarly demonstrated the inhibition of DNA synthesis by nuclei isolated from HeLa cells in which the biosynthesis of spermidine and spermine was inhibited by methylglyoxal bis(guanylhydrazone). Like Knutsen & Morris (1978), they were unable to re-activate DNA synthesis in isolated nuclei by the addition of polyamines. Seyfried & Morris (1979) showed that DFMO decreased DNA synthesis in bovine lymphocytes comparably with the degree of depletion of cellular polyamines. Branca & Herbst (1980) blocked the proliferation of HeLa cells by the induction of the antizyme of ornithine decarboxylase and demonstrated that S-phase DNA synthesis was inhibited by 50–75% in HeLa cells in which polyamines were depleted by the combined action of antizyme and DFMO (Herbst & Branca, 1981). Herbst & Elliott (1981) showed that DNA synthesis *in vitro* was blocked in nuclei isolated from HeLa cells in which polyamine biosynthesis was inhibited by DFMO during synchronization of the cells for S-phase. In that study, in agreement with previous investigations (Knutson & Morris, 1978; Krokan & Ericksen, 1977), DNA synthesis was not increased by the addition of polyamines to assays *in vitro* of nuclei from polyamine-depleted cells. Partial reversal of the effects of depletion of polyamines on DNA synthesis in HeLa cells and isolated nuclei was achieved by the addition of polyamines to the cell cultures before the assay of DNA synthesis (Herbst & Branca, 1981; Herbst & Elliott, 1981).

In the study reported here, the time course of the replenishment of polyamines in HeLa cells, after supplementation of individual polyamines to cultures grown in the presence of DFMO, is characterized. A relationship between intracellular polyamines and S-phase DNA synthesis of HeLa cells has been established.

EXPERIMENTAL

Chemicals

Putrescine, spermidine and spermine were obtained from Calbiochem–Behring Corp. (La Jolla, CA, U.S.A.) as hydrochlorides. Thymidine was purchased from Calbiochem or Sigma Chemical Co. (St. Louis, MO,

Abbreviations used: DFMO, α-difluoromethylornithine; BME medium, Basal Modified Eagle's medium.

U.S.A.). DFMO was generously given by Dr. Peter P. McCann, Merrell Dow Research Laboratories, Cincinnati, OH, U.S.A. Solutions of these compounds were sterilized by filtration through 0.22 μm-pore-size Nalgene membrane filters (VWR Scientific Co., Boston, MA, U.S.A.).

Cell cultures

HeLa-cell stocks were obtained from Dr. G. C. Mueller, McArdle Laboratory for Cancer Research (Madison, WI, U.S.A.). The culture media and conditions for maintenance of cell stocks and growth and synchronization of HeLa cells in suspension cultures for S-phase DNA synthesis were based on procedures of that laboratory (Mueller & Kajiwara, 1969). Stock cultures were grown in silicone-coated spinner flasks at 37 °C in Ca^{2+}- and Mg^{2+}-free Basal Modified Eagle's (BME) medium (GIBCO, Grand Island, NY, U.S.A.). The BME medium was supplemented with 10% (v/v) horse serum (GIBCO), 0.1 mM-glycine, 0.1 mM-serine, 0.01 mM-*myo*-inositol (Calbiochem–Behring Corp.) and 0.1% (w/v) of the surfactant Pluronic F-68 (Wyandotte Chemical Co., Parsippany, NJ, U.S.A.). Experimental suspension cultures were incubated in a rotary shaker–incubator (Psychrotherm; New Brunswick Scientific Co., New Brunswick, NJ, U.S.A.) at 37 °C in silicone-treated flat-bottom boiling flasks. All cultures were gassed with air/CO_2 (19:1). Cell counts were determined with a Royco Cell Counter (Royco Instruments, Menlo Park, CA, U.S.A.). Silicone coating of spinner flasks, boiling flasks and culture tubes was accomplished by rinsing the glassware with a 1% (v/v) solution of dichlorodimethylsilane (Aldrich Chemicals, Milwaukee, WI, U.S.A.) in benzene.

Synchronization and polyamine depletion of cells

HeLa cells were synchronized for S-phase DNA synthesis by the double thymidine-block procedure of Mueller & Kajiwara (1969). To obtain cells that were deficient in polyamines, the synchronization medium was supplemented with 1 mM-DFMO (Herbst & Elliott, 1981).

Control cultures were synchronized in complete BME medium at an initial density of 1×10^5 cells/ml. Polyamine-depleted cultures were synchronized in complete BME medium, containing 1 mM-DFMO, at an initial density of 1.25×10^5 cells/ml. After 32 h of incubation, 2 mM-thymidine was added to both cultures. Incubation was continued for 16 h, at which time the first thymidine block was reversed (at 48 h) by centrifuging the cells (500 *g*, 10 min at 25 °C), washing the cell pellets by two cycles of resuspension in serum-free BME medium, and resuspending the washed cell pellets in either complete BME medium (control culture) or complete BME medium containing 1 mM-DFMO (polyamine-depleted culture). After incubation for 8 h to permit recovery of the cells from the first thymidine block, 2 mM-thymidine was added to both cultures (at 56 h) and the second thymidine block was applied by incubation for an additional 16 h. Thus the total period of the protocol for synchronization and polyamine depletion of the cells was 72 h.

The control and the polyamine-depleted synchronized cells were harvested by centrifugation, and the cell pellets were washed in serum-free BME medium to remove residual thymidine as described above. The cells were resuspended in complete BME medium (control culture) or in complete BME medium containing 1 mM-DFMO (polyamine-depleted culture) at 5×10^5 cells/ml and incubated to initiate S-phase DNA synthesis.

[^{3}H]Thymidine incorporation into DNA

Control and polyamine-depleted synchronized cells were incubated as suspension cultures in a rotary shaker–incubator. Commencing with the initiation of incubation of the synchronized cultures and at intervals thereafter, four replicate 2.5 ml samples were aseptically transferred to 12 ml sterile silicone-treated centrifuge tubes fitted with gum-rubber stoppers. To each sample, 1 μCi of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) in 0.1 ml of sterile water was added and, after gassing with air/CO_2 (19:1), the tubes were stoppered and three replicates were incubated on an 8° slant at 37 °C for 30 min. Vigorous rotary shaking of the tubes was applied in some experiments as indicated in certain protocols. The incubation was terminated by adding 5 ml of cold saline to each tube and, after centrifugation (1000 *g*, 4 °C, 10 min), the cells were resuspended in 5 ml of cold saline and re-centrifuged. The cell pellets were resuspended in 3 ml of cold saline and filtered on 24 mm GFC glass-fibre filters on a manifold (Millipore Corp., Bedford, MA, U.S.A.) under low vacuum. The cells on the filters were washed with 3 ml of cold saline and, after filtration of the saline wash, 15–20 ml of cold 4% (w/v) $HClO_4$ was added to each filter well and allowed to pass slowly through each filter without the application of a vacuum. The precipitates on the filters were washed with 15–20 ml of cold 80% (v/v) ethanol, followed by 15–20 ml of cold ethanol under vacuum filtration. The filters were transferred to scintillation vials, dried under a heat lamp, and the [^{3}H]thymidine incorporated into the precipitated DNA was measured by radioactivity counting in Omnifluor/toluene (New England Nuclear Corp., Boston, MA, U.S.A.) in a Beckman model LS 7000 liquid-scintillation spectrometer.

Analysis of cells for polyamines

At each interval at which S-phase DNA synthesis was assayed, samples of the synchronized cultures containing 12.5×10^6–25×10^6 cells were removed and centrifuged (1000 *g*, 5 min, 4 °C), and cell pellets were washed twice by resuspension in 0.5 vol. of cold saline, followed by centrifugation after each wash. The washed cell pellets were resuspended in 0.5 ml of cold 0.2 M-$HClO_4$ and kept overnight at 4 °C. After centrifugation (1000 *g*, 4 °C, 5 min), the $HClO_4$ supernatant was removed. The analysis of polyamines was performed on the $HClO_4$ extracts of the cells by the modification of Seiler's dansyl (5-dimethylaminonaphthalene-1-sulphonyl) procedure previously described (Herbst & Dion, 1970).

RESULTS AND DISCUSSION

Synchronization of HeLa cells for S phase

A variety of procedures, including the selective harvesting of metaphase cells and the use of metabolic inhibitors, have been employed to accumulate HeLa cells behind a blockade of DNA synthesis or mitosis (Mueller & Kajiwara, 1969). The sequential thymidine-block procedure for the synchronization of HeLa cells for

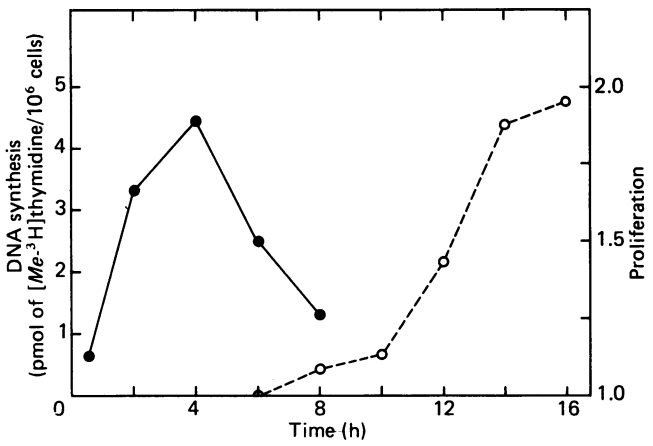

Fig. 1. Synchrony of S-phase DNA synthesis and cell proliferation in HeLa-cell cultures after release from double thymidine block

Samples (2.5 ml) of HeLa-cell suspension culture, synchronized by the double thymidine-block procedure, were removed at intervals after release of the second thymidine block. The incorporation of [^{3}H]thymidine into DNA was assayed, and each point (●) is the mean of the values obtained for triplicate samples. Incubation of the synchronized culture was continued for 16 h after release of the second thymidine block, and cell counts (○) were determined.

S-phase DNA synthesis was selected for several reasons. Firstly, a high degree of synchrony has been reported in HeLa-cell suspension cultures by the application of this metabolic-inhibitor protocol (Mueller & Kajiwara, 1969). Most importantly, the time of incubation utilized to accomplish synchronization by this method is identical with the period (72 h) required to deplete HeLa cells of polyamines when cultured in media containing DFMO (Herbst & Elliott, 1981).

A high degree of synchrony of S-phase DNA synthesis and cell proliferation in control cultures was achieved by the double thymidine-block protocol (Fig. 1). The incorporation of [^{3}H]thymidine into control HeLa cells was minimal after the second thymidine block terminated at 72 h by the removal of the thymidine-containing culture medium (0 h in the time course of Fig. 1). During the subsequent incubation of the culture in fresh BME medium, synchronous DNA synthesis occurred, as indicated by the incorporation of [^{3}H]thymidine into cells assayed at 2–4 h. An additional indication of the synchrony of DNA replication in these cells is the relatively short interval (4 h) in which essentially complete proliferation of the cells occurred (10–14 h in the time course of Fig. 1).

Relationship between S-phase DNA synthesis and polyamines

A possible relationship between S-phase DNA synthesis and the intracellular polyamines of HeLa cells was

Table 1. DNA synthesis and polyamine content of HeLa cells synchronized for S phase in the absence or presence of DFMO: effect of a supplement of spermine

HeLa cells were synchronized for S phase in the absence or presence of 1 mM-DFMO. After S phase was initiated by the release of the cells from the second thymidine block, samples of each culture were removed at intervals, as indicated, for the assay of [^{3}H]thymidine incorporation into DNA and the analysis of polyamines. The effect of 20 μM-spermine, added to a culture synchronized in media containing DFMO (DFMO/spermine) at the time that S phase was initiated, on DNA synthesis and polyamine content was determined. The values are the means of assay results on triplicate samples removed from the cultures at each interval. Variability of assay results between individual samples was less than $\pm 10\%$. Abbreviation: N.D., not detectable.

Culture	Time after initiation of S phase (h) ...	[^{3}H]Thymidine incorporation into DNA (pmol/10^6 cells)				
		0.5	3	6	9	12
Control		3.42	9.52	2.53	0.16	0.16
DFMO		0.64	1.78	2.59	2.56	1.86
DFMO/spermine		0.73	3.23	5.18	7.83	3.52
	Time after initiation of S phase (h) ...	Polyamines (nmol/10^6 cells)				
		0.5	3	6	9	12
Control						
Spermine		2.68	2.68	2.63	5.11	3.57
Spermidine		1.93	2.04	1.76	3.48	2.29
Putrescine		0.92	0.85	0.41	0.80	0.44
DFMO						
Spermine		1.59	0.88	0.55	1.30	1.20
Spermidine		0.18	0.09	N.D.	0.27	0.08
Putrescine		N.D.	N.D.	N.D.	N.D.	N.D.
DFMO/spermine						
Spermine		1.96	2.33	1.44	3.95	1.80
Spermidine		0.09	0.09	N.D.	0.15	0.09
Putrescine		N.D.	N.D.	N.D.	N.D.	N.D.

Table 2. DNA synthesis and polyamine content of HeLa cells synchronized for S phase in the absence or presence of DFMO: effect of a supplement of spermidine

Conditions for the initiation of S phase and for the assay of culture samples for [^{3}H]thymidine incorporation into DNA and polyamines are indicated in the legend of Table 1. The effect of 20 μM-spermidine, added to a culture synchronized in media containing DFMO (DFMO/spermidine) at the time that S phase was initiated, on DNA synthesis and polyamine content was determined.

Culture	Time after initiation of S phase (h) ...	[^{3}H]Thymidine incorporation into DNA (pmol/10^6 cells)				
		0.5	3	6	9	12
Control		2.13	6.05	1.04	0.20	0.15
DFMO		0.87	1.04	0.58	1.37	1.07
DFMO/spermidine		1.24	2.08	1.49	4.53	2.08
	Time after initiation of S phase (h) ...	Polyamines (nmol/10^6 cells)				
		0.5	3	6	9	12
Control						
Spermine		3.97	3.86	4.40	3.62	1.30
Spermidine		2.72	2.67	3.19	2.14	0.92
Putrescine		1.40	1.18	1.65	0.67	0.10
DFMO						
Spermine		1.70	1.79	1.43	0.65	0.85
Spermidine		0.20	N.D.	N.D.	N.D.	N.D.
Putrescine		N.D.	N.D.	N.D.	N.D.	N.D.
DFMO/spermidine						
Spermine		1.87	0.95	2.38	1.70	0.77
Spermidine		1.67	2.30	2.98	2.89	1.30
Putrescine		N.D.	N.D.	0.87	N.D.	N.D.

investigated in the following synchronized cultures: (*a*) cultures in which polyamine biosynthesis was not inhibited (control); (*b*) cultures in which polyamine biosynthesis was inhibited (DFMO); (*c*) cultures in which polyamine synthesis was inhibited by DFMO and to which a polyamine was added at the beginning of S phase (DFMO/spermine, DFMO/spermidine, or DFMO/putrescine).

In Table 1, the time course of changes in DNA synthesis and polyamine content of synchronized cells from control, DFMO and DFMO/spermine experimental cultures is summarized. The synchronous pattern of DNA synthesis in the control cells, analogous to the S-phase curve illustrated in Fig. 1, is evident in the [^{3}H]thymidine incorporation by these cells from samples of the culture assayed at intervals between 0.5 and 12 h. In contrast, cultures synchronized in the presence of DFMO yield cells in which DNA synthesis is depressed and asynchronous. Cultures synchronized in the presence of DFMO and to which 20 μM-spermine was added when the second thymidine block was released at 72 h (zero time of initiation of S phase) yielded cells in which synchronous DNA synthesis was restored. The peak incorporation of [^{3}H]thymidine was lower than in control cells, and there was a delay of 6 h in the attainment of the highest S-phase synthesis of DNA.

The concentrations of individual polyamines in the control cells were somewhat variable at the intervals during S-phase DNA synthesis at which the cells were assayed. Generally, at each interval the concentration of spermine was highest, that of spermidine was intermediate, and that of putrescine was lowest. There was no evidence of an elevated concentration of either polyamine at the interval corresponding to the peak of synchronous DNA synthesis. There was a rather sharp elevation of the concentration of spermine and spermidine at the 9 h interval in the G2 phase of the cell cycle of these synchronized cultures (Fig. 1). A lower concentration of the polyamines, expressed in nmol/10^6 cells, at 12 h is indicative of the division of the cellular polyamine pools that occurs as a result of the synchronized cell proliferation at this time interval (Fig. 1).

Results comparable with those obtained when spermine was added to synchronized and polyamine-depleted cultures were obtained with supplements of spermidine. In Table 2, cells from a synchronized and polyamine-depleted culture to which spermidine was added at 72 h (DFMO/spermidine) are compared with cells from control and DFMO cultures. The high degree of S-phase synchrony of DNA synthesis in the control cells and the polyamine content of these cells is similar to the data of Table 1. Only traces of spermidine and no putrescine occur in the DFMO cells, and at the 9 h interval only 18% of the spermine is conserved. Cells from the DFMO culture to which spermidine was added at the time of initiation of S phase at 72 h, designated DFMO/spermidine, accumulate spermidine rapidly to achieve intracellular concentrations comparable with those in control cells during S phase. Spermine concentrations are increased, but not to control values, and putrescine was detected at only one time point. As in the previous experiment in which spermine was added to reverse the

Table 3. DNA synthesis and polyamine content of HeLa cells synchronized for S phase in the absence or presence of DFMO: effect of a supplement of putrescine

Conditions for the initiation of S phase and for the assay of culture samples for [^{3}H]thymidine incorporation into DNA and polyamines are indicated in the legend of Table 1. The effect of 50 μM-putrescine, added to a culture synchronized in media containing DFMO (DFMO/putrescine) at the time that S phase was initiated, on DNA synthesis and polyamine content was determined.

Culture	Time after initiation of S phase (h) ...	[^{3}H]Thymidine incorporation into DNA (pmol/10^6 cells)				
		0.5	3	6	9	12
Control		1.94	4.84	2.84	0.29	0.29
DFMO		0.65	1.04	0.63	1.72	1.78
DFMO/putrescine		1.26	2.15	4.02	3.62	4.06
	Time after initiation of S phase (h) ...	Polyamines (nmol/10^6 cells)				
		0.5	3	6	9	12
Control						
Spermine		3.13	2.85	2.64	4.51	3.24
Spermidine		2.07	2.12	1.70	3.31	2.08
Putrescine		0.77	0.77	0.66	1.08	0.28
DFMO						
Spermine		1.22	1.33	0.97	0.97	0.95
Spermidine		N.D.	N.D.	N.D.	N.D.	N.D.
Putrescine		N.D.	N.D.	N.D.	N.D.	N.D.
DFMO/putrescine						
Spermine		0.59	1.00	0.79	1.42	1.68
Spermidine		0.10	0.39	0.74	1.53	1.79
Putrescine		0.56	5.40	5.06	5.40	2.57

polyamine deficiency, peak S-phase DNA synthesis was delayed by 6 h.

When a supplement of putrescine was utilized to reverse the polyamine deficiency (Table 3), high concentrations of putrescine, 7-fold greater than the putrescine content of control cells in this experiment, were attained within 3 h. Surprisingly, spermine pools were not increased substantially, and spermidine concentrations reached only about 50% of the control value 9 h after polyamine supplementation. DNA synthesis was quite asynchronous in these putrescine-loaded cells, and there was a 3 h lag in the attainment of peak S-phase DNA synthesis, which occurred at 6 h.

Thus none of the three polyamines, provided as supplements to the culture medium utilized to initiate S phase, completely overcomes the deficiency of intracellular polyamines and the deficiency in S-phase DNA synthesis that is induced by DFMO. Partial and unbalanced polyamine pools result from supplements of individual polyamines to HeLa-cell cultures containing DFMO.

Reversal of the polyamine deficiency before the initiation of S phase

The lag in S-phase DNA synthesis in polyamine-deficient cells supplemented with polyamines suggested that the synthesis or utilization of DNA-replication factors, dependent on polyamines, was essential before the initiation of S phase. To characterize the time-frame of polyamine reversal of the deficiency in S-phase DNA synthesis, supplements of spermidine (20 μM) to HeLa cell cultures synchronized in the presence of DFMO were made at intervals preceding the release of the second thymidine block. Elimination of the lag in S phase and nearly complete reversal of the inhibition of DNA synthesis was achieved by the addition of 20 μM-spermidine to these cultures at least 10 h before release of the second thymidine block (e.g. 62 h; Fig. 2). Supplements of spermidine (or other polyamines) provided at earlier time points were equally effective (results not shown), but supplements administered later than 10 h before initiation of S phase (e.g. 64 and 66 h; Fig. 2) did not overcome the deficiency.

The results of this experiment suggest that the inhibitory effect of DFMO on DNA replication by HeLa cells is completely reversible by the provision of spermidine at least 10 h before the initiation of S phase of the cell cycle. The failure of supplements of polyamine, added later in the synchronization schedule, to initiate DNA synthesis on the same time course as control cultures suggests that the polyamines function in the 'staging' of the cell cycle. A deficiency of polyamines induced by DFMO delays the traverse of G1 cells through S phase. The delay is avoided if exogenous polyamine is provided at least 10 h before the initiation of S phase. The results indicate that the maintenance of 'cycling' HeLa cells, culminating in the proliferation of the cells in each 22–24 h traverse of the cycle, is absolutely dependent on polyamines. Furthermore, the provision of exogenous polyamine, e.g. spermidine, before the 'staging' of the cell cycle for S-phase DNA

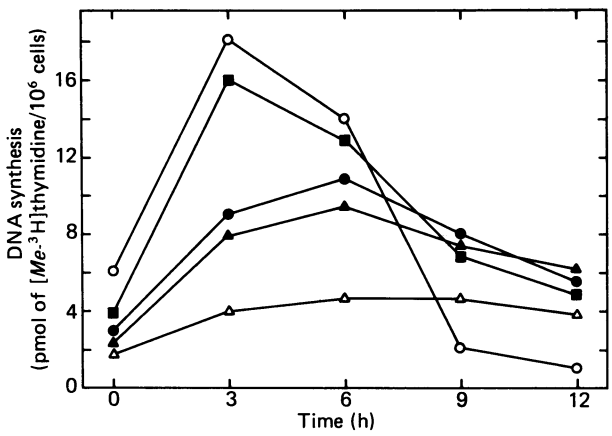

Fig. 2. Reversal of DFMO-induced inhibition of DNA synthesis in HeLa cells by spermidine

HeLa-cell cultures were synchronized for S-phase DNA synthesis in the absence or presence of DFMO (1 mM). Spermidine (20 μM) was added to cultures containing DFMO at 62, 64 or 66 h of the synchronization procedure, which entails removal of the second thymidine block and initiation of S-phase DNA synthesis at 72 h. DNA synthesis was assayed on 2.5 ml samples of culture taken at the indicated intervals after removal of the second thymidine block. [*Me*-^{3}H]Thymidine (1 μCi; 0.67 Ci/mmol) was added to each sample in silicone-treated assay tubes, which were incubated with vigorous rotary agitation for 30 min at 37 °C. Filtration of the cells, precipitation of the DNA on glass-fibre filters and liquid-scintillation counting of the filters was performed as described in the Experimental section. Each experimental point is the mean of assay results on triplicate samples, corrected for an unincubated blank. Variability of assay results between replicates was less than $\pm 10\%$. ○, Control; △, DFMO; ▲, DFMO/spermidine at 66 h; ●, DFMO/spermidine at 64 h; ■, DFMO/spermidine at 62 h.

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synthesis, completely reverses the deficiency induced by DFMO. Thus the cytostatic effect of DFMO on HeLA cells appears to be confined to the irreversible inactivation of ornithine decarboxylase, culminating in a deficiency of intracellular polyamines.

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