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Effect of polyamine depletion on macromolecular synthesis of the malarial parasite, *Plasmodium falciparum*, cultured in human erythrocytes

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DL-α-Difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase, prevented the increases in putrescine and spermidine, but not in spermine, in human erythrocytes infected with the malarial parasite *Plasmodium falciparum*. The addition of putrescine to these polyamine-depleted cultures restored the normal concentrations of spermidine, whereas that of putrescine even exceeded that of the control cultures. DFMO also inhibited the incorporation of radioactive amino acids into the proteins of parasitized erythrocytes. Electrophoresis on polyacrylamide gels revealed that the synthesis of some proteins was completely blocked by DFMO, but the synthesis of others was not affected. DFMO also caused a partial inhibition of RNA synthesis, and DNA synthesis was completely blocked in polyamine-depleted parasitized erythrocytes. It has been suggested that putrescine and/or spermidine are required for the synthesis of certain proteins in parasitized erythrocytes and that at least one of those proteins is related to the synthesis of DNA of the malarial parasite. It appears that polyamines regulate the schizogony process of *P. falciparum*.

INTRODUCTION

Parasites of the genus *Plasmodium* undergo a complex developmental cycle inside erythrocytes of the vertebrate host. The maturation of the young stages of *Plasmodium falciparum* to the multinucleated segmenter is accompanied by a massive synthesis of macromolecules (McCann *et al.*, 1981; Assaraf *et al.*, 1984; Gritzmacher & Reese, 1984; Inselburg & Banyal, 1984; De Rojas & Wasserman, 1985). The naturally occurring polyamines spermidine and spermine and the diamine putrescine play a functional role in cell differentiation and proliferation processes (Cohen, 1971; Bachrach, 1973; Tabor & Tabor, 1984). These organic polycations interact with nucleic acids (Gosule *et al.*, 1978) and may control DNA transcription (Blair, 1985) and translation (Igarashi *et al.*, 1982).

It has been reported that the amounts of polyamines increase in *P. falciparum*-infected erythrocytes (Assaraf *et al.*, 1984; Whaun & Brown, 1985), as did the activity of the biosynthetic enzymes ornithine decarboxylase (ODC, EC 4.1.1.17) and *S*-adenosyl-L-methionine decarboxylase (SAMDC, EC 4.1.1.50) (Assaraf *et al.*, 1984). DL-α-Difluoromethylornithine (DFMO) is an irreversible inhibitor of ODC, the key enzyme in polyamine biosynthesis (Metcalf *et al.*, 1978). This inhibitor interferes with the development of the parasites; schizogony is blocked and the asexual cycle is arrested at the early trophozoite stage (McCann *et al.*, 1981; Assaraf *et al.*, 1984). This inhibition is readily reversed by the addition of putrescine to polyamine-depleted cells (Assaraf *et al.*, 1986).

Using this system we investigated the effect of polyamine depletion on the dynamics of synthesis of nucleic acid and protein. Some specific effects of DFMO on the physiology of the intraerythrocytic schizogony process are presented.

EXPERIMENTAL

Reagents

[^{3}H]Hypoxanthine (sp. radioactivity 10 Ci/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A. [^{35}S]Methionine (sp. radioactivity 1160 Ci/mmol) was from The Radiochemical Centre (Amersham, Bucks., U.K.), and L-[^{3}H]leucine (sp. radioactivity 38.3 Ci/mmol) was the product of the Nuclear Research Center (Negev, Israel). Putrescine dihydrochloride and phenylmethanesulphonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Methionine-free RPMI-1640 medium was purchased from BioLab (Jerusalem, Israel). Oligo(dT)–cellulose was the product of Pharmacia (Uppsala, Sweden). DFMO was kindly provided by Dr. P. McCann, Merrell Dow Research Center, Cincinnati, OH, U.S.A.

Parasite culture

Plasmodium falciparum (FCR-3 TC) was maintained in culture by the candle-jar method (Trager & Jensen, 1976), with some modifications: the growth medium, RPMI-1640 (GIBCO), was supplemented with D-glucose (final concn. 22 mM). Erythrocytes from asynchronous cultures were washed three times with RPMI-1640 and

Abbreviations used: ODC, ornithine decarboxylase; DFMO, DL-α-difluoromethylornithine.

suspended in 5% (v/v) sorbitol for 15 min at room temperature (Lambros & Vanderberg, 1979). The resulting population, containing mainly ring forms, was washed, diluted with fresh red blood cells, adjusted to an initial parasitaemia of 0.5–3%, and returned to culture for 20–30 h. Thereafter, parasites were further synchronized as described by Assaraf *et al.* (1986). Briefly, mature schizonts were placed into medium containing 10 mM-DFMO. After incubation for 38–45 h, the drug-containing medium was removed and cultures were given growth medium containing 0.25 mM-putrescine, or fresh medium containing DFMO. Parasitaemias and differential stage distribution were determined from Giemsa-stained thin blood films, by counting 5000 cells and 500 parasitized erythrocytes respectively. Mean values of three cultures are presented.

Polyamine extraction and determination

Polyamines were extracted with 5-sulphosalicylic acid in 0.1 M-HCl from the infected erythrocytes. Polyamines in the acid-soluble fraction were separated by ion-exchange chromatography and quantified as described by Desser *et al.* (1980) or Seiler (1970).

Incorporation of labelled precursors into parasite macromolecules

Cultures of *P. falciparum* (with an initial parasitaemia of 4–15% and a 5% haematocrit) were distributed into 96 microculture trays (100 μl/well), and arrested by DFMO at the early trophozoite stage. Then DFMO-containing medium was removed and a putrescine-supplemented one was added to some cultures. [^{3}H]Hypoxanthine (1–2 μCi/well) or L-[^{3}H]leucine (2 μCi/well) was added in 10 μl quantities of growth medium and incubated for 4 h. The cells were first harvested, sedimented, washed three times, and the macromolecules were finally precipitated on glass-fibre filters with cold 5% trichloroacetic acid. Radioactivity was determined by liquid-scintillation counting.

Protein labelling and analysis

Parasitized erythrocytes from cultures treated with DFMO were concentrated by the gelatin sedimentation technique (Jensen, 1978). The resulting population of 70–90% parasitaemia (of more than 99% early trophozoites) was suspended in RPMI-1640 medium supplemented with 10% serum to 0.5% haematocrit and distributed (2 ml/plate). Parasite proteins were then labelled for 2 h in 1 ml of methionine-free RPMI-1640 medium (BioLab) containing 40 μCi of [^{35}S]methionine. In some cultures DFMO (10 mM) or putrescine (0.25 mM) was also included in the methionine-free medium. Cells were finally sedimented (2000 *g* for 5 min) washed three times and stored at −80 °C. Frozen samples were lysed at 37 °C with double-distilled water containing 5 mM-phenylmethanesulphonyl fluoride. Freezing and thawing was repeated three times, and the protein content of the homogenates was determined colorimetrically (Lowry *et al.*, 1951). The incorporation of radioactive methionine was measured by precipitation of 15 μl samples of the homogenate with ice-cold 5% trichloroacetic acid on glass-fibre filters. Preparation of samples and the electrophoretic procedures were carried out as described by Laemmli & Favre (1973). Samples containing approx. 150 μg of protein (15×10^4 c.p.m.) were applied to each slot and separated in either a 10% or a continuous 5–15% polyacrylamide gel containing SDS, for 6 h at 200 V/80 mA. Kodak X-ray films were exposed to fixed, washed and dried gels. The autoradiograms were analysed by scanning densitometry (Quick Scan R & D, Helena Labs, Beaumont, TX, U.S.A.).

Extraction and measurement of nucleic acids

Parasitized erythrocytes labelled with [^{3}H]hypoxanthine were washed twice with ice-cold RPMI-1640 medium and sedimented at 2000 *g* for 5 min. Cells were then lysed and nucleic acids extracted with trichloroacetic acid as described by Inselburg & Banyal (1984). RNA was digested with 0.3 M-NaOH for 12 h at 37 °C. Total nucleic acids and DNA were precipitated with ice-cold 10% trichloroacetic acid and washed with cold ethanol on glass-fibre filters. The filters were dried and radioactivity was measured by liquid-scintillation counting. RNA was determined by subtracting the radioactivity of DNA from that of total nucleic acids.

RNA fractionation

Gelatin-enriched suspensions of 1% haematocrit and 80% parasitaemia (98% at the trophozoite stage) of normal and DFMO-treated cultures were washed three times. Extraction of RNA and its fractionation on agarose gels were performed as described by Wallach & Kilejian (1982). Fractionation of RNA on agarose gels

Table 1. Effect of DFMO on the polyamine content of erythrocytes infected with *P. falciparum*

Cultures of *P. falciparum* were treated with 10 mM-DFMO for 40 h. Cells (99.5% at the early trophozoite stage) were then harvested before (29% parasitaemia) or after gelatin enrichment (82% parasitaemia). Untreated cultures (94.5% early trophozoites) were harvested (9.2% parasitaemia), whereas from other cultures, parasitized erythrocytes were enriched with gelatin (85% parasitaemia). Polyamine contents (means ± S.D. of three or four determinations) were expressed as pmol/10^8 normal or parasitized erythrocytes.

Erythrocytes	Treatment	Polyamine content (pmol/10^8 cells)		
		Putrescine	Spermidine	Spermine
Parasitized	None	1720 ± 17	5090 ± 585	1270 ± 70
Parasitized	DFMO	27 ± 3	352 ± 27	1210 ± 107
Gelatin-enriched parasitized	None	1125 ± 57	5500 ± 287	531 ± 123
Gelatin-enriched parasitized	DFMO	60 ± 3	645 ± 133	66 ± 81
Normal	None	1.9 ± 0.3	18 ± 2	86 ± 10

containing formaldehyde was carried out essentially as described by Lehrach *et al.* (1977).

Separation of polyadenylated RNA on oligo(dT)–cellulose

A 400 μg sample of total trophozoite RNA from both normal and DFMO-treated cultures in ice-cold buffer [10 mM-Tris/HCl (pH 7.5)/0.3 M-NaCl] were loaded on a 1 cm-long Pasteur pipette column of oligo(dT)–cellulose. The effluent was reloaded three times to ensure maximal binding. Thereafter, ice-cold elution buffer [10 mM-Tris/HCl (pH 7.5)/0.05% SDS/1 mM-EDTA] was added and 0.5 ml fractions were collected. The RNA content of the various fractions was determined spectrophotometrically.

The polyadenylated fraction was determined by using a metabolically ^{3}H-labelled RNA. To free the RNA from contaminating DNA and tRNA, samples (110 μg) of total [^{3}H]RNA from both normal and DFMO-treated parasites were treated overnight at 4 °C with 2 M-LiCl. Thereafter, the RNA was sedimented and reprecipitated with 2% (w/v) potassium acetate and dissolved in distilled water as described elsewhere (Wallach, 1982). From the resulting DNA-free RNA, triplicate 0.5 ml portions were precipitated with cold 5% trichloroacetic acid on GF/C filters and the radioactivity was determined. Samples (5×10^5–15×10^5 c.p.m.) of [^{3}H]RNA were chromatographed on oligo(dT)–cellulose columns. The polyadenylated fraction was estimated from the radioactivity retained by the column.

RESULTS

Polyamine contents in normal and DFMO-treated cultures of *P. falciparum*

When DFMO (10 mM) was added to parasite-infected erythrocytes and polyamines were measured as described by Desser *et al.* (1980), a substantial decrease in cellular putrescine was noted (Table 1). DFMO also caused a decrease in spermidine in parasitized erythrocytes. On the other hand, DFMO hardly affected the concentrations of spermine, whereas a moderate increase in spermine was observed in gelatin-enriched parasitized erythrocytes, after treatment with DFMO (Table 1).

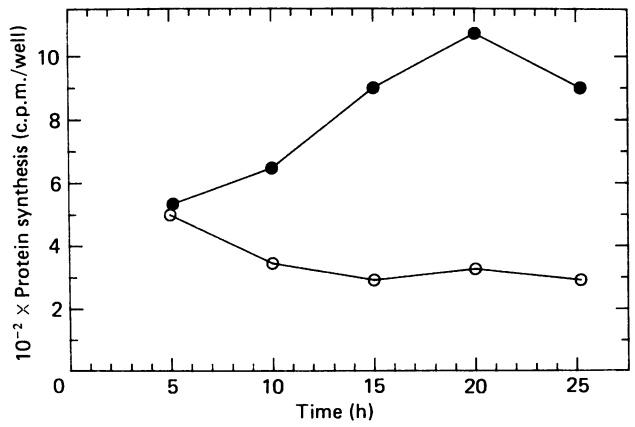

Fig. 1. Effect of putrescine on the inhibition of protein synthesis

Cultures of *P. falciparum* were treated with 10 mM-DMFO for 40 h and then grown in medium containing DFMO (○) or in the presence of 0.25 mM-putrescine (●). Triplicate micro-cultures were pulsed with [^{3}H]-leucine for 5 h periods, and the incorporation of ^{3}H into trichloroacetic acid-insoluble material was then determined.

Effect of polyamine depletion on the synthesis of parasite proteins

Treatment of *P. falciparum* cultures (15% parasitaemia) with DFMO (10 mM) for 40 h interfered with the incorporation of [^{3}H]leucine (Fig. 1). The addition of putrescine (0.25 mM) to the cultures resulted in an

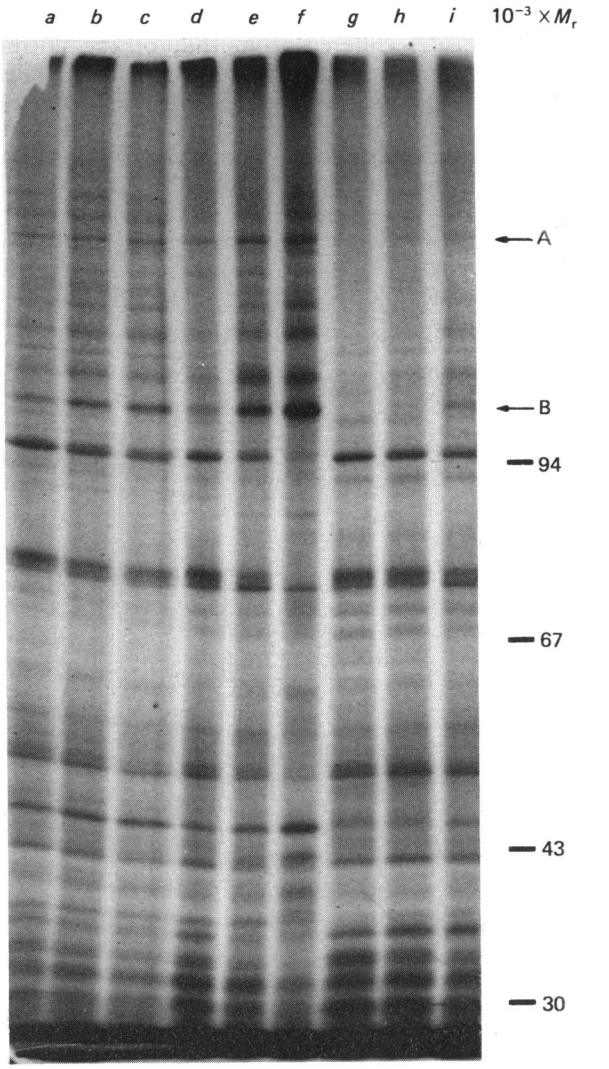

Fig. 2. Electrophoretic pattern of [^{35}S]methionine-labelled proteins of control and DFMO-treated parasites

Cultures were grown for 43 h in a medium containing 10 mM-DFMO. In the control experiment, young trophozoites were incubated in a medium without any addition. To some DFMO-treated cultures (after gelatin enrichment) putrescine (0.25 mM) was added. Cultures were then pulsed with [^{35}S]methionine for 2 h periods at the following times: 5 h (lanes *a*, *d*, *g*), 10 h (lanes *b*, *e*, *h*) and 14 h (lanes *c*, *f*, *i*). Labelled proteins were separated by electrophoresis on a 10%-polyacrylamide gel. Lanes *a*–*c*, continuous treatment with DFMO. Lanes *d*–*f*, treatment with DFMO for 43 h, followed by the addition of putrescine (for times indicated above). Lanes *g*–*i*, control, of young trophozoites, without DFMO or putrescine treatment. Positions of M_r ($\times 10^{-3}$) standards are indicated on the right.

increase in the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable proteins, and maximal synthesis was obtained after 20 h (Fig. 1). To test whether putrescine stimulated the synthesis of all parasite proteins, or only specific polypeptides in the depleted parasitized erythrocytes, cultures were labelled with [^{35}S]methionine at given times after putrescine addition. The radiolabelled proteins were separated by SDS/polyacrylamide-gel electrophoresis, followed by autoradiography. Young trophozoites not treated with DFMO served as controls. Fig. 2 shows that the electrophoretic profile of proteins derived from DFMO-treated parasites differed from that of the untreated young trophozoites. The incorporation of methionine into some proteins was enhanced, but a decrease was noted in the incorporation into others. Putrescine did not affect the incorporation of methionine into most proteins, yet the synthesis of two high-M_r fractions was stimulated by putrescine (Fig. 2, lanes *d–f*; A and B). When the M_r of these two proteins was determined by gel electrophoresis on a 5–15% polyacrylamide gradient, under denaturing conditions, values of 103000 and

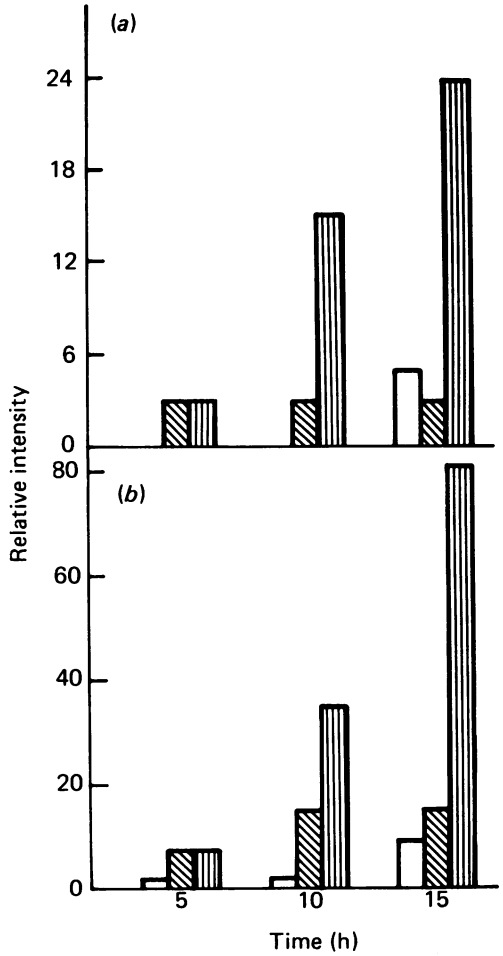

Fig. 3. Scanning densitometric analysis of the polypeptides of M_r 170000 and 103000

The labelling intensity of the M_r-170000 and -103000 proteins of the control (□), DFMO-treated (▨) and putrescine-treated (▥) groups were analysed by scanning densitometry, and the corresponding area of the bands was plotted versus time: (*a*) and (*b*) indicate the M_r-170000 and -103000 proteins respectively.

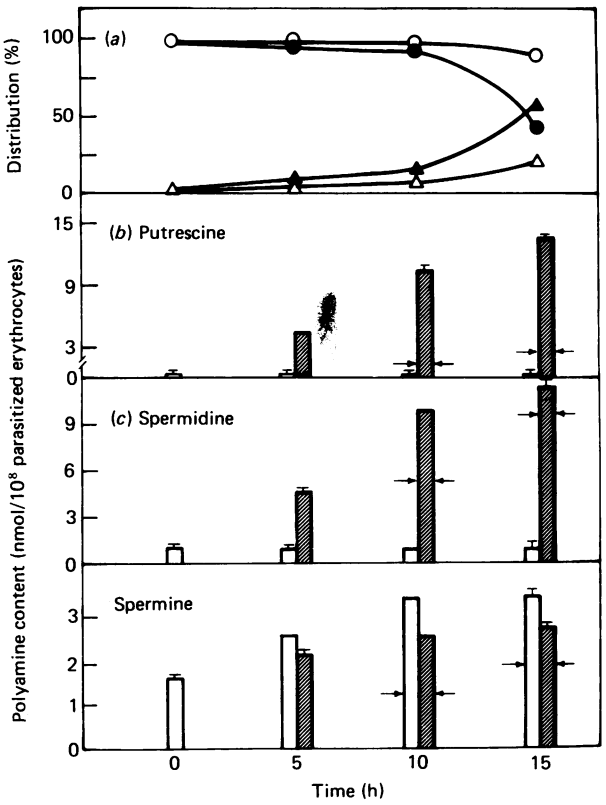

Fig. 4. Restoration of the intracellular pool of polyamines by adding putrescine to DFMO-treated cultures

Cultures of *P. falciparum* (2.8×10^8 parasitized erythrocytes/culture) were treated with 10 mM-DFMO for 43 h, followed by gelatin enrichment (78% parasitaemia; 99.8% early trophozoites). In some cultures the DFMO-containing medium was replaced by growth medium containing 0.25 mM-putrescine (●, ▲; ▨), whereas others grew continuously in DFMO alone (○, △; □). At times indicated, samples were analysed for polyamines and differential counts were also carried out. Horizontal arrows indicate the amounts of polyamines in control cultures (at the appropriate developmental stage) when they were not treated with DFMO. ○, ●, Trophozoites; △, ▲, schizonts.

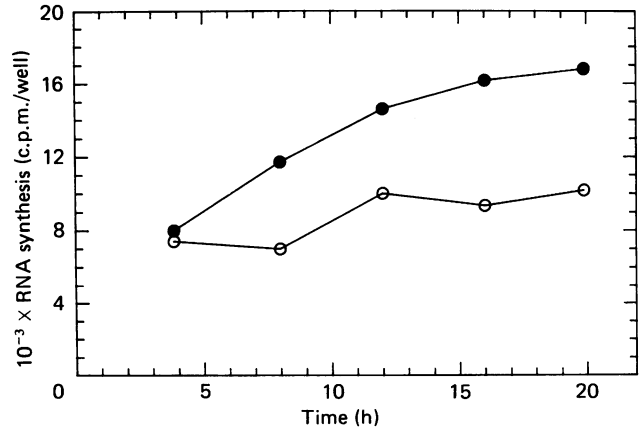

Fig. 5. Effect of putrescine on RNA synthesis in DFMO-treated cultures

Micro-cultures were pulsed with [^{3}H]hypoxanthine for 4–5 h and processed for RNA synthesis as described in the Experimental section: ○, DFMO; ●, putrescine.

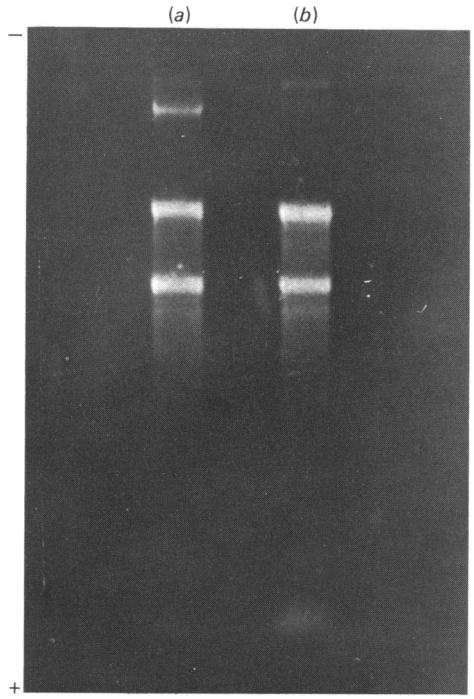

Fig. 6. Analysis of *P. falciparum* total RNA on agarose gel

Total RNA (10 μg) of non-treated young trophozoites (lane *a*) or DFMO-arrested trophozoites (lane *b*) was electrophoresed in a 1.5%-agarose gel and stained with ethidium bromide.

170000 were obtained (results not shown). The sequential increase in the labelling of these bands was confirmed by scanning densitometry. Thus, 14 h after the addition of putrescine to the DFMO-treated parasites, the peak incorporation of methionine into the M_r-103000 protein was 5.4- and 9-fold higher than that observed in DFMO-treated parasites and into young trophozoites, respectively (Fig. 3). It may therefore be concluded that putrescine preferentially stimulated the synthesis of certain, but not all, proteins in polyamine-depleted cultures of *P. falciparum*.

In the experiment described above, putrescine (0.25 mM) was added to DFMO-treated parasites. To study the effect of exogenously added putrescine on the cellular pool, diamines and polyamines were extracted from the cells and analysed as described by Seiler (1970).

Table 1 and Fig. 4 show that DFMO caused a dramatic decrease in cellular putrescine and that addition of putrescine to the depleted cells led to an accumulation of this diamine, even above the control values. Spermidine also increased 5, 10 and 15 h after the addition of putrescine to the depleted cells, whereas spermine contents were hardly affected (Fig. 4).

Effect of polyamine depletion on the synthesis of nucleic acids

We have studied the influence of polyamine depletion on the synthesis of RNA and DNA. The incorporation of a purine precursor, [^{3}H]hypoxanthine, into parasite RNA was partially inhibited by DFMO (Fig. 5). Removal of the DFMO-containing medium did not result in a significant increase in RNA synthesis. On the other hand, the addition of putrescine caused an immediate increase in RNA synthesis. The rRNA of *P. falciparum* represents 80–90% of total RNA (Vezza & Trager, 1981). Separation on agarose gel of total RNA from normal and DFMO-treated trophozoites did not reveal a dramatic change in a specific RNA species (Fig. 6). Electrophoresis on agarose gels under denaturing conditions revealed that these major species migrated as 26S and 19S rRNA (results not shown), confirming the previous study of Vezza & Trager (1981). In addition, 1.5–2.0% of the RNA isolated from both normal and DFMO-treated cultures was retained by an affinity chromatography column of oligo(dT)–cellulose. DNA synthesis was totally inhibited in DFMO-treated cultures. The addition of putrescine to this culture resulted in the synthesis of DNA after 10 h, which reached a peak about 8 h later (Fig. 7). In the presence of putrescine, the rate

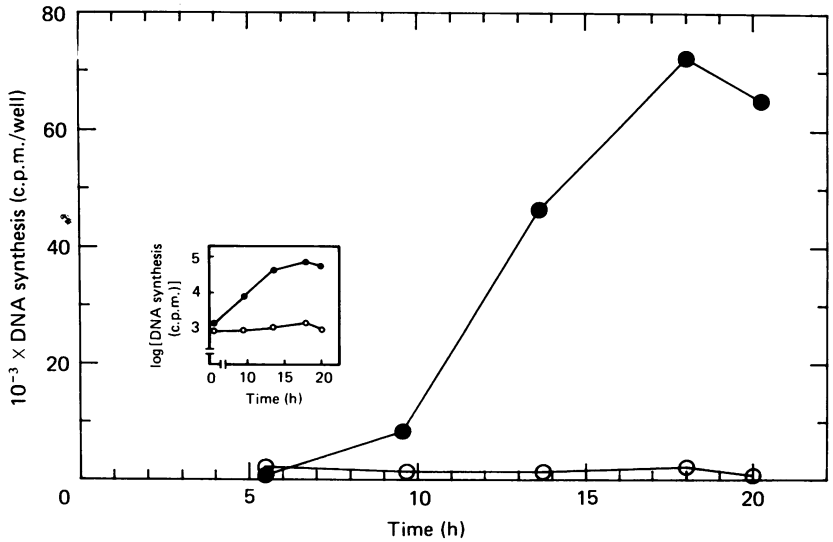

Fig. 7. Effect of putrescine on the recovery of DNA synthesis in DFMO-treated cultures

Experimental conditions are those described in the legend to Fig. 5, except that the incorporation was estimated for DNA synthesis. The DNA synthesis was also analysed by a log plot (insert). ○, DFMO; ●, putrescine.

of DNA synthesis between 10 and 18 h was exponential (Fig. 7, see the insert).

DISCUSSION

DFMO is known as an inhibitor of ODC and thus leads to the decrease in cellular putrescine. This depletion in turn causes a decrease in spermidine, but not in spermine (Jänne *et al.*, 1983). Table 1 shows that DFMO causes a significant decrease in putrescine and spermidine in parasitized erythrocytes. However, spermine was not decreased by DFMO, but instead was slightly increased.

Polyamines affect many cellular processes, including DNA replication (Knutson & Morris, 1978), transcription (Blair, 1985) and translation (Igarashi *et al.*, 1982; Chroboczek, 1985). McCann *et al.* (1981) reported that DFMO inhibited the incorporation of [^{3}H]hypoxanthine into the nucleic acids of *P. falciparum*, but incorporation into parasite proteins was only slightly affected. Our findings confirm these results and show that DFMO indeed inhibited protein synthesis; this was reversed by the addition of putrescine at physiological concentrations (Fig. 1). It was not clear from that experiment whether the synthesis of all protein species was affected similarly, or whether the synthesis of certain molecules was preferentially inhibited. Figs. 2 and 3 show that the synthesis of some proteins was more dependent on the presence of diamines and spermidine than was that of others. Igarashi & Morris (1984) have suggested that polyamines (directly or indirectly) stimulate DNA replication by regulating the synthesis (or function) of specific DNA-replicating proteins. Fig. 7 demonstrates that the synthesis of DNA in parasitized erythrocytes is strictly dependent on the presence of putrescine and spermidine. The addition of putrescine to the DFMO-treated cultures reversed the block and led to the exponential synthesis of DNA after 10 h. A similar delay in DNA synthesis after restoration of normal polyamine concentrations has been reported by Igarashi & Morris (1984). They explained it by assuming that polyamines are required for the synthesis of a DNA-replicating protein such as DNA polymerase.

Synthesis of RNA in parasitized erythrocytes is only partially inhibited by DFMO, and the synthesis of RNA species and total polyadenylated mRNA is equally affected (Figs. 5 and 6). These findings may be related to the regulation of the cell cycle of *P. falciparum*. DNA is synthesized during the early-trophozoite–late-schizont stages (Inselburg & Banyal, 1984). At this time, polyamines accumulate in the infected erythrocytes (Assaraf *et al.*, 1984). The syntheses of 'early' (in the ring-form stage) parasite proteins as well as of rRNA is not strictly dependent on elevated concentrations of polyamines (Figs. 1, 5 and 6). The 'early' proteins may thus be expressed at the initial stages of the cell cycle when cellular polyamines are relatively low. The expression of accumulate. It appears that the asexual cycle of the 'late' proteins (in the trophozoite and schizont stages) and DNA replication will occur only when polyamines human malarial parasite *P. falciparum* is regulated by polyamines.

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