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Catabolites produced by the deacetylation of hexamethylenebisacetamide play a key role in murine erythroleukaemic-cell differentiation

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N-Acetyl-1,6-diaminohexane and 1,6-diaminohexane, formed by deacetylation of the inducer hexamethylenebisacetamide (HMBA), are shown to accumulate rapidly inside murine erythroleukaemic cells. The appearance of these molecules preceded the differentiation-associated changes in intracellular polyamines. A quantitative relationship was observed between the accumulation of these molecules and the changes in intracellular polyamines. In the absence of HMBA, exogenous *N*-acetyl-1,6-diaminohexane was able not only to cause changes in polyamine biosynthesis, but also to induce the complete differentiation process. These results imply that these catabolites of HMBA are directly responsible for the changes in polyamine biosynthesis and probably also for initiating other events regulatory for the differentiation of these cells.

INTRODUCTION

After the initial observation that HMBA could induce differentiation in MEL cells [1], this compound was shown to induce the differentiation of, or change the phenotype in, a number of other cell lines [2–5]. Therefore we thought it of interest to elucidate the molecular mechanisms by which chemical inducers act, since this will probably help us to understand normal differentiation control. Despite the widespread use of HMBA and other diacetylated diamines as inducing agents, the mechanism by which these molecules alter cellular functions has not been determined. Even whether the inducer molecules act specifically at defined site(s) or pleiotropically on cellular functions or structures such as membranes is not known.

We have recently shown [6] that changes in intracellular polyamines are an intimate part of the differentiation programme induced in MEL cells by HMBA, and they appear to be associated with a regulatory event in this process [6a]. A large body of evidence has demonstrated that the activity of the first polyamine-biosynthetic enzyme, ODC, is negatively controlled by synthetic α-ω diamines as well as by the natural polyamines (reviewed in [7]).

Since HMBA is a diacetylated diamine, its deacetylation would produce molecules that could potentially regulate polyamine biosynthesis. Incorporation of the acetyl moieties of the inducer molecule into lipid and protein has been previously reported [8]; however, diamines produced by this intracellular deacetylation were not detected in the cell extracts.

The results described in this paper show that: (1) the products of intracellular deacetylation of HMBA accumulate in the cells shortly after the addition of the inducer to the culture medium; (2) these catabolites alone are probably responsible for the changes in polyamine biosynthesis previously reported; (3) in the absence of HMBA they can induce the complete differentiation process in MEL cells.

MATERIALS AND METHODS

Cell culture

Maintenance of MEL cells (clone DS 19), measurements of cell growth, cell viability and proportion of cells committed to differentiate were performed as previously described [9].

Analytical methods

Measurement of ODC activity in cytosol extracts and preparation of acid-soluble extracts for polyamine analysis were performed as previously described [6].

Polyamines were analysed using a method developed for the Pharmacia FPLC apparatus equipped with a 100 μl injection loop and a Pharmacia Pep RPC HR 5/5 column. The column was developed at a flow rate of 1 ml/min with a gradient formed from two buffers both containing 2 mM-octane sulphonate (Pic B8; Waters Associates): Buffer A, 0.1 M-acetic acid, pH 3; Buffer B, 0.2 M-sodium acetate, pH 4.5, plus 0.3 vol. of acetonitrile. The gradient shown in Fig. 1(*a*) gave the best separation of putrescine from *N*-acetyl-1,6-diaminohexane and 1,6-diaminohexane. The break-points (time, % buffer B) of this gradient were: 0 min, 20%; 7 min, 33.5%; 12 min, 33.5%; 20 min, 58%; 28 min, 90%; 28.1 min, 100%; 32 min, 100%; 32.1 min, 20%. The next sample could be injected at 40 min. The effluent from the column was mixed with *o*-phthalaldehyde reagent (0.5 ml/min) [10], and after it had flowed through a coil of PTFE (polytetrafluorethylene) tubing [750 mm × 1 mm (int. diam.); 37 °C] the fluorescence was measured with a Waters model 420 detector equipped with the appropriate standard filters. The amounts of the individual polyamines

Abbreviations used: HMBA, hexamethylenebisacetamide (*NN*-diacetyl-1,6-diaminohexane); ODC, ornithine decarboxylase (EC 4.1.1.17); MEL, murine erythroleukaemic; f.p.l.c., fast protein liquid chromatography.

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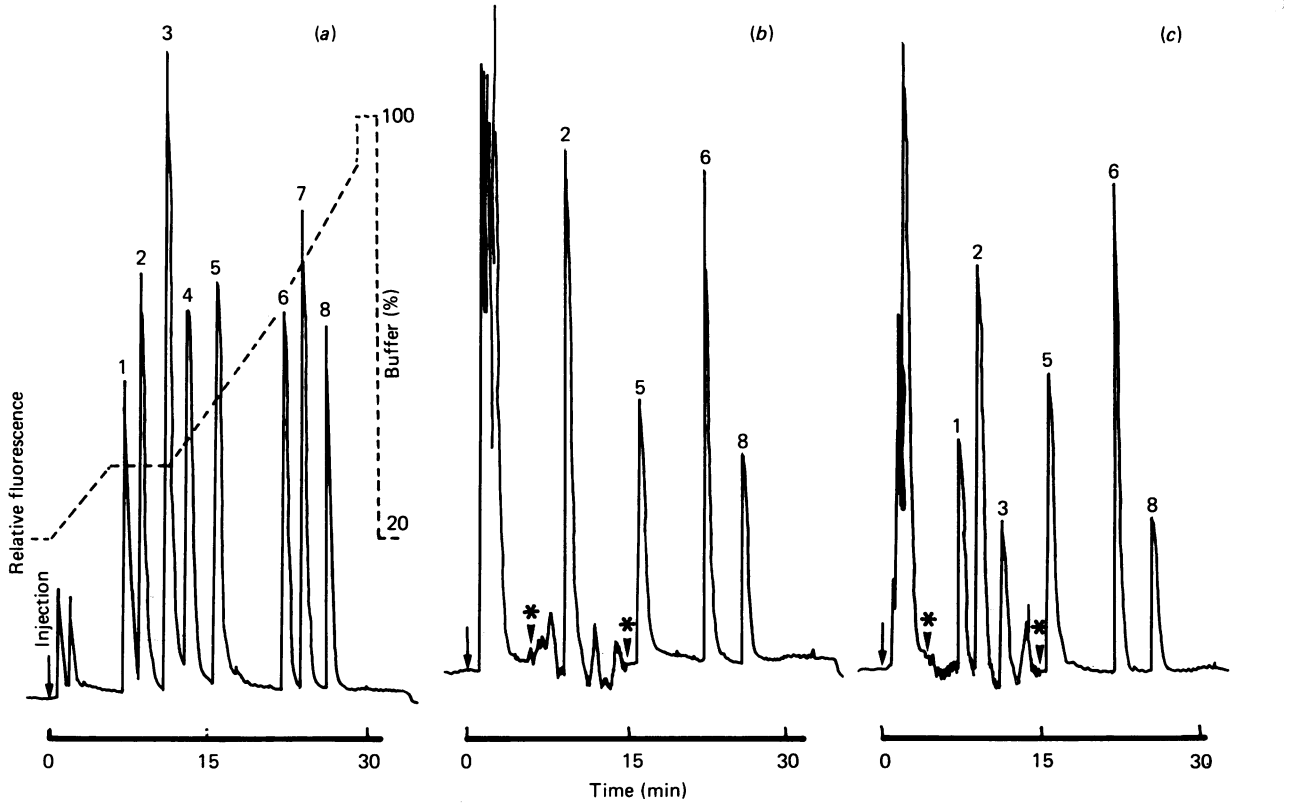

Fig. 1. Chromatograms of polyamine standards and cell extracts

Polyamine analysis was performed as described in the Materials and methods section by using a Pharmacia FPLC apparatus and *o*-phthaladehyde post-column derivatization with fluorescence detection. (*a*) Polyamine standards; (*b* and *c*) acid-soluble extracts prepared from cells cultured in the absence of HMBA (*b*) or in the presence of 4 mM-HMBA for 3 h (*c*). The numbered peaks and amounts used in polyamine standards are: 1, *N*-acetyl-1,6-diaminohexane, 0.4 nmol; 2, putrescine, 0.6 nmol; 3, 1,6-diaminohexane, 0.6 nmol; 4, N^1-acetylspermidine, 0.6 nmol; 5, 1,7-diaminoheptane, 0.4 nmol; 6, spermidine, 0.4 nmol; 7, *N*-acetylspermine, 0.6 nmol; 8, spermine, 0.4 nmol. 1,7-Diaminoheptane (2 nmol/10^6 cells) was added to the cell extracts as an internal standard. Between the asterisks the detector gain increased 8-fold. The broken line in (*a*) represents the pumped gradient. Owing to the large amounts of 1,6-diaminohexane (17 mmol) manipulated during the synthesis of *N*-acetyl-1,6-diaminohexane, cell extracts prepared with the same apparatus could become contaminated with this molecule or 1,6-diaminohexane. The small peaks in (*b*) with retention times corresponding to these molecules show the maximum degree of such a contamination which was observed.

were quantified by planimetry of chart-recorder tracings and comparison with the tracings obtained with known amounts of pure natural or synthetic polyamines. Polyamines standards were from Fluka, and all solvents were of hplc grade.

Histone H1[9] was extracted essentially as described by Johns [11] and analysed by gel electrophoresis [12].

Synthesis of *N*-acetyl-1,6-diaminohexane

N-Acetyl-1,6-diaminohexane was synthesized by allowing 1,6-diaminohexane to react with acetic anhydride. 1,6-Diaminohexane (2 g; 17 mmol) was dissolved in 10 ml of acetic acid. Acetylation was performed in two steps with an interval of 24 h. In each reaction acetic anhydride was added dropwise to the 1,6-diaminohexane with heating (50–60 °C) and stirring during a 3 h period. At the end of the second reaction equimolarity of acetic anhydride and 1,6-diaminohexane had been obtained. After evaporation to dryness *in vacuo*, the residue was chromatographed on Bio-Rex 70 and eluted with sodium acetate (0.2 M), pH 6.5. The fractions containing *N*-acetyl-1,6-diaminohexane were identified by chromatography (chloroform/methanol/acetic acid, 49:49:2, by vol.) on silica-gel thin-layer plates with a concentrating zone (E. Merck, Darmstadt, Germany) (R_F values: diaminohexane, 0.00; *N*-acetyl-1,6-diaminohexane, 0.35; HMBA, 0.60). Salts present in the solution were eliminated by chromatography on Dowex AG 50 WX8 resin, and *N*-acetyl-1,6-diaminohexane was eluted with 6 M-HCl. This solution was freeze-dried (yield: 680 mg, 25%). The purity of the final product was controlled by chromatography on thin-layer plates and by f.p.l.c. as described above. Neither diacetylated diaminohexane (HMBA) nor non-acetylated diaminohexane were detectable.

Synthesis of tritiated *N*-acetyl-1,6-diaminohexane and HMBA

[*acetyl*-^{3}H]*N*-Acetyldiaminohexane was prepared by the procedure described above, except that [^{3}H]acetic anhydride was used (500 mCi/mmol; Amersham, Les Ulis, France). The specific radioactivity of the final product was 1.34×10^9 c.p.m./mmol. In this case the yield was only 10%, owing to the smaller quantities involved.

[*acetyl*-^{3}H]HMBA was synthesized by first allowing

the 1,6-diaminohexane to react with [^{3}H]acetic anhydride. The reaction was then completed by addition of excess acetic anhydride (yield, 52%; specific radioactivity, 2.57×10^9 c.p.m./mmol).

Uptake of *N*-acetyl-1,6-diaminohexane

[*acetyl*-^{3}H]*N*-Acetyl-1,6-diaminohexane was added to the culture medium (8 mM) from a stock solution (80 mM in pH 7.4 medium; 1.34×10^9 c.p.m./mmol). At selected time intervals three aliquots of 7×10^5 cells were taken, collected by centrifugation (200 *g*, 5 min, 4 °C) and washed three times with 15 mM-Hepes (pH 7.4)/135 mM-NaCl. The radioactivity in the final pellet after solubilization with Soluene (0.2 ml, 2 h, 20 °C) was determined by liquid-scintillation spectrometry using a Triton X-100-based scintillation fluid.

RESULTS

In the present study the analysis of intracellular polyamines was performed by using a method developed for the Pharmacia FLPC apparatus. The elution gradient was designed so that an optimum separation between potential catabolites of HMBA and putrescine was achieved. Fig. 1(*a*) shows the elution profile for a mixture of natural and synthetic polyamines and some of their monoacetylated derivatives. The total cycle time was 40 min and the detection limit was 5 pmol of polyamine. The elution profiles for acid-soluble extracts

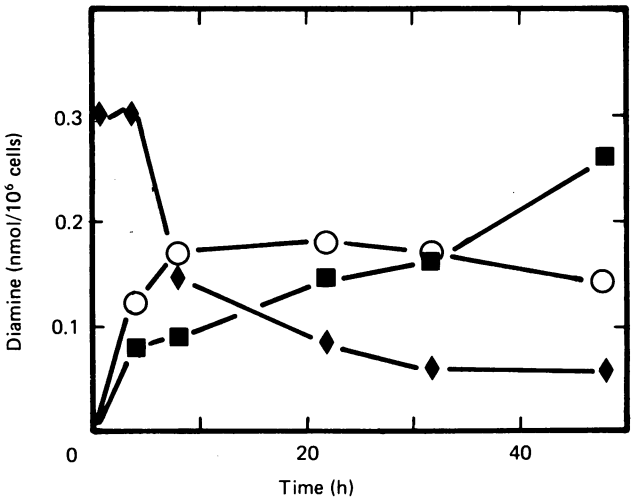

Fig. 2. Temporal relationship between the accumulation of HMBA catabolites and the decrease of putrescine

MEL cells grown to stationary phase in medium supplemented with 15% foetal-calf serum were diluted to 1×10^5 cells/ml into medium supplemented with 5% fetal-calf serum. HMBA (4 mM) was added to the culture 15 h after this dilution. Aliquots of cells (1×10^6) were taken at the indicated times and intracellular acid-soluble polyamines were analysed; quantification of the chromatogram was performed by comparing the fluorescence of a particular peak with that of a known amount of the corresponding standard molecule. ◆, Putrescine; ○, *N*-acetyl-1,6-diaminohexane; ■, 1,6-diaminohexane. The results shown are from three separate experiments and the reproducibility between experiments was normally better than ±10% of the average values.

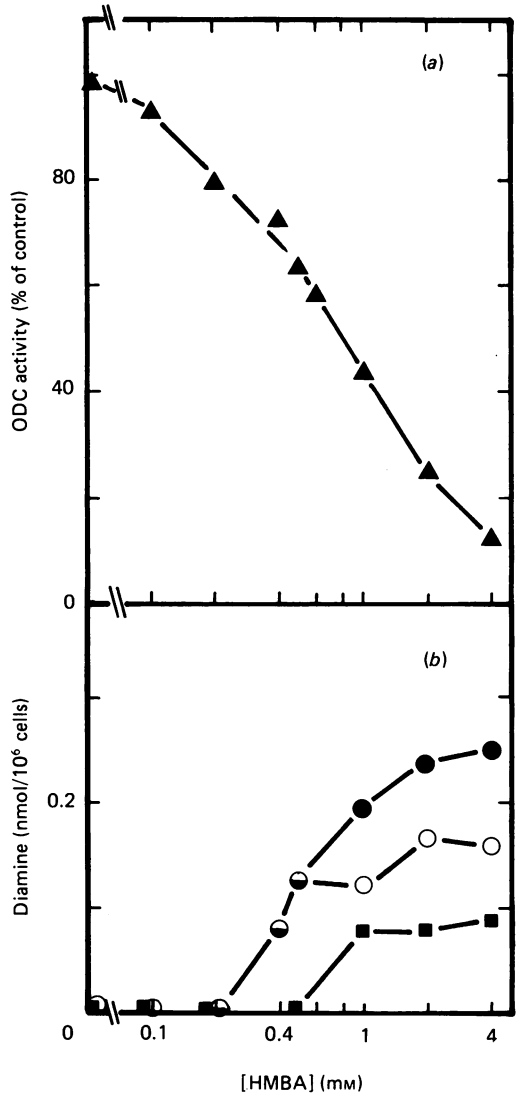

Fig. 3. Quantitative relationship between ODC activity and accumulation of HMBA catabolites

MEL cell were cultured as in Fig. 2, but with various concentrations of HMBA. At 8 h after the addition of HMBA, aliquots [7×10^5 cells (duplicate) for ODC measurements and 1×10^6 cells for polyamine analysis] were taken. At this time duplicate aliquots of cells were also taken for ODC measurements from a culture that was similar but lacked HMBA. The ODC activity (*a*) was determined in duplicate on each aliquot (total of four values) by measuring the $^{14}CO_2$ released from L-[1-^{14}C]ornithine. The activity in the extracts from cells cultured in the presence of HMBA relative to that in the cells cultured without HMBA was calculated (activity as % of control). Although the absolute value of the ODC activity varied between the separate experiments (for typical values, see Fig. 4) the relative values (activity as % of control) at each HMBA concentration varied by less than 5% between the different experiments. The accumulation of HMBA catabolites (*b*) was determined as in Fig. 2. ○, *N*-Acetyl-1,6-diaminohexane; ■, 1,6-diaminohexane; ●, sum of catabolites. Results shown are the averages from two experiments (max. range less than ±10% of average).

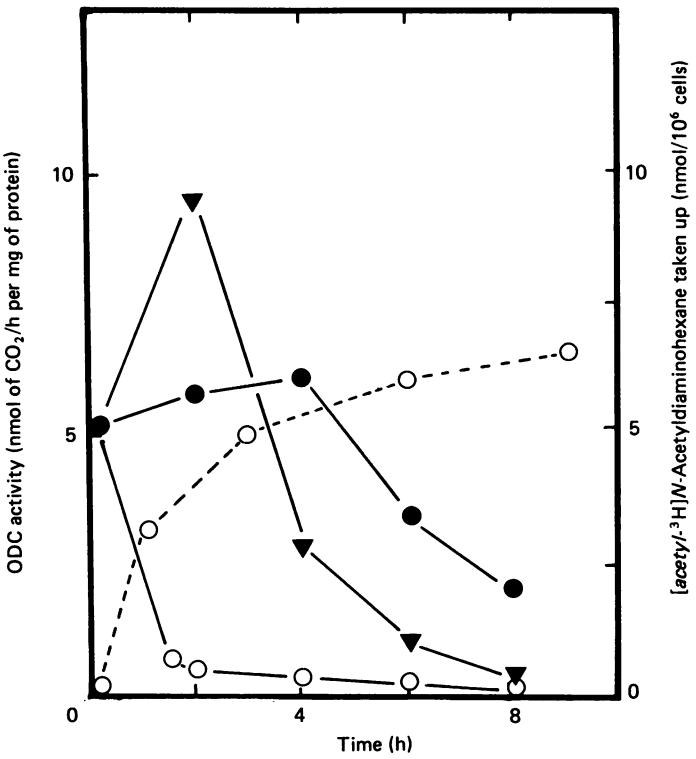

Fig. 4. Effect of exogenous *N*-acetyl-1,6-diaminohexane on ODC activity: uptake of [acetyl-^{3}H]*N*-acetyl-1,6-diaminohexane

MEL cells were cultured as in Fig. 2; 15 h after dilution of the cells no drugs (●), 4 mM-HMBA (▼) or 8 mM-*N*-acetyl-1,6-diaminohexane (stock solution: 80 mM in pH 7.4 medium) (○) was added to the cultures. At the indicated times, aliquots were taken and the ODC activity measured as in Fig. 3. Results shown are the averages from two separate experiments (max. range/average ⩽ 10%). The uptake of [*acetyl*-^{3}H]*N*-acetyl-1,6-diaminohexane (○----○) was measured as described in the Materials and methods section (max. range/average ⩽ 5%).

from cells cultured in the absence or presence of inducer are shown in Figs. 1(*b*) and 1(*c*) respectively. The ratio of putrescine to spermidine or spermine was about 1:10, in agreement with analyses of MEL-cell intracellular polyamines performed by h.p.l.c. [13,14]. Comparison of Figs. 1(*b*) and 1(*c*) shows that *N*-acetyl-1,6-diaminohexane and 1,6-diaminohexane were detected in extracts from cells cultured for only a few hours in the presence of HMBA. The appearance of these deacetylation products of HMBA was the most outstanding early change in the intracellular polyamines caused by the presence of the inducer. That the molecules forming these peaks in the elution profile were derived intracellularly from HMBA was confirmed as follows. Acid-soluble extracts were prepared from cells cultured in the presence of [*acetyl*-^{3}H]HMBA for 24 h and polyamine analysis was performed. Radioactivity was only found in the peak corresponding to the *N*-acetyl-1,6-diaminohexane. When [*acetyl*-^{3}H]HMBA was incubated in medium with 15% (v/v) foetal-calf serum (37 °C, 24 h) and without cells, no deacetylation of the HMBA could be detected. Furthermore, quantification of the *N*-acetyl-1,6-diaminohexane from the measurement of either the post-column *o*-phthalaldehyde fluorescence or the radioactivity gave identical results.

Kinetics of the accumulation of HMBA catabolites

We have previously shown [6] that the decrease in intracellular putrescine associated with the differentiation of MEL cells induced by HMBA occurs shortly after a similar change in the ODC activity.

In Fig. 2 the kinetics of the accumulation of *N*-acetyl-1,6-diaminohexane and 1,6-diaminohexane are compared with those of the decrease in intracellular putrescine. They show that 70% of the increase in *N*-acetyl-1,6-diaminohexane had occurred before any decrease in intracellular putrescine was detected. Cells committed to erythrodifferentiation first appeared in these cultures 15–24 h after the addition of HMBA to the medium [6]. The data given in Fig. 2 shows that during this time interval there is a switch from putrescine to 1,6-diaminohexane as the major intracellular diamine. However, before the appearance of committed cells in the culture, the *N*-acetyl-1,6-diaminohexane was the most abundant catabolite of HMBA.

Relationship between changes in polyamine biosynthesis and accumulation of HMBA catabolites as a function of HMBA concentration

In the culture conditions used, the decrease in ODC activity was completed between 6 and 8 h after the addition of 4 mM-HMBA to the medium [6]. Therefore cells were cultured for 8 h in the absence or the presence of various concentrations of HMBA. The ODC activity in the cells cultured with HMBA relative to that in the cells cultured in the absence of HMBA was then determined (Fig. 3*a*). A significant decrease ($19 \pm 7\%$) in the ODC activity was observed for HMBA concentrations as low as 0.2 mM. Analysis of the polyamines extracted from these cells showed that the decrease in ODC activity was associated with a decrease in intracellular putrescine (results not shown). The data in Fig. 3(*b*) show that the two catabolites of HMBA accumulate and the activity of ODC decreases within the same range of HMBA concentrations. Furthermore, a strong reciprocal relationship was found between the accumulation of the total HMBA catabolites (*N*-acetyl-1,6-diaminohexane + 1,6-diaminohexane) (Fig. 3*b*) and the decrease in ODC activity (Fig. 3*a*). At low concentrations of HMBA, a decrease in ODC activity was observed in the absence of detectable amounts of HMBA catabolites in the cell extracts. This is probably because at low HMBA concentrations the ODC activity in a cell extract is easily measured, whereas the amount of HMBA catabolite is very low and could even be below the detection limit of the method used. However, these small amounts of HMBA catabolites could be functionally active.

Effect of exogenous *N*-acetyl-1,6-diaminohexane on polyamine biosynthesis

The changes in polyamine biosynthesis observed during the HMBA-induced differentiation of MEL cells could result from a synergy between the intracellular catabolites of HMBA and the inducer molecule itself. In order to exclude this possibility, the effect of exogenous *N*-acetyl-1,6-diaminohexane (added directly to the culture medium) on the ODC activity and on the intracellular polyamine levels was investigated. In preliminary experiments we observed that 8 mM-*N*-acetyl-1,6-di-

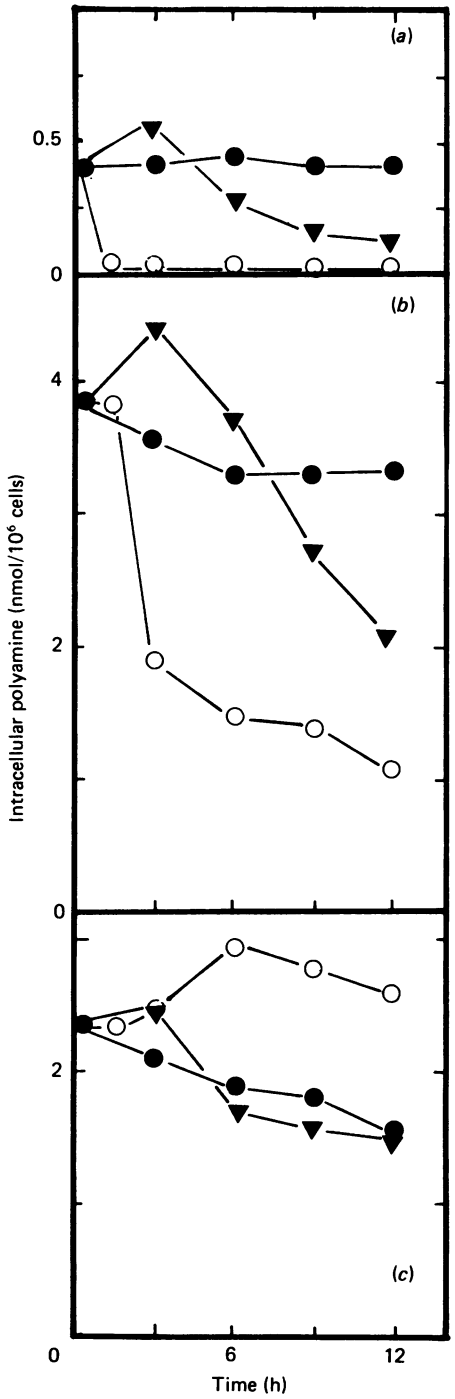

Fig. 5. Effect of exogenous *N*-acetyl-1,6-diaminohexane on intracellular polyamines

MEL cells were cultured as in Fig. 4 in the presence of 4 mM-HMBA (▼), 8 mM-*N*-acetyl-1,6-diaminohexane (○) or in the absence of drugs (●). The acid-soluble putrescine (*a*), spermidine (*b*) and spermine (*c*) were quantified as in Fig. 2. Results shown are from two separate experiments and the reproducibility between experiments was better than ±10% of the average values.

aminohexane had no effect on cell growth or viability. With 10 mM-*N*-acetyl-1,6-diaminohexane the viability of the cells after 48 h was slightly affected (less than 10% Trypan Blue-positive). Optical microscopy showed that most of these cells had a serrated appearance with 'blebs'. Therefore, in the experiments described below, 8 mM-*N*-acetyl-1,6-diaminohexane was used. It was verified, by using [*acetyl*-^{3}H]*N*-acetyl-1,6-diaminohexane, that this molecule was not acetylated by serum enzymes or intracellularly to form HMBA.

The data in Fig. 4 show that the addition of *N*-acetyl-1,6-diaminohexane to the culture medium induced a rapid fall in the ODC activity. This decrease was more rapid than that observed in the presence of HMBA, which is shown for comparison in Fig. 4. The uptake of exogenous *N*-acetyl-1,6-diaminohexane is also shown in Fig. 4. The rate of *N*-acetyl-1,6-diaminohexane uptake was greatest during the first hour, at which time the amount present in the cells was about 20-fold that accumulated by deacetylation of HMBA after 8 h (compare Fig. 2). This probably explains the faster decrease in ODC activity in cells cultured with *N*-acetyl-1,6-diaminohexane than with HMBA. In these experiments the ratio of intracellular 1,6-diaminohexane/*N*-acetyl-1,6-diaminohexane was approx. 0.1 throughout the first 24 h of culture with *N*-acetyl-1,6-diaminohexane (results not shown).

Changes in the amounts of intracellular putrescine and spermidine consistent with this rapid decrease in ODC activity were observed (Fig. 5). As observed with the ODC activity, the variations in intracellular putrescine and spermidine that occur in the presence of *N*-acetyl-1,6-diaminohexane are similar to those induced with HMBA, which are also shown in Fig. 5 for comparison, but they appeared much earlier and with a greater amplitude. However, spermine accumulated in the cells 3–6 h after the addition of *N*-acetyl-1,6-diaminohexane. Such an accumulation was not observed with HMBA. A possible explanation for this accumulation of spermine is that the *N*-acetyl-1,6-diaminohexane, owing to the similarity of its structure to an acetylated polyamine, could inhibit enzymes responsible for the conversion of spermine into spermidine. Such an inhibition combined with that of the ODC activity would also explain the very rapid decrease in intracellular spermidine.

Induction of MEL-cell differentiation by *N*-acetyl-1,6-diaminohexane

It was of interest to determine whether *N*-acetyl-1,6-diaminohexane could induce the complete differentiation process in MEL cells.

The data shown in Fig. 6(*a*) demonstrate that the differentiation of MEL cells can be induced by *N*-acetyl-1,6-diaminohexane in a dose-dependent manner. The kinetics of the induction of differentiation by *N*-acetyl-1,6-diaminohexane (8 mM) and by HMBA (4 mM) are shown in Fig. 6(*b*). The rate of appearance of committed cells with *N*-acetyl-1,6-diaminohexane was nearly half of that observed with HMBA. The differentiation induced by *N*-acetyl-1,6-diaminohexane presented characteristics similar to that induced by HMBA. The differentiation induced by both of these molecules was associated with an accumulation of histone H1^0 (results not shown) [15] and was efficiently inhibited (80%) by dexamethasone (10^{-6} M) (results not shown) [5,16,17]. The regulatory events associated with both of these characteristics are probably independent of the changes in polyamine metabolism. Firstly, the kinetics of histone H1^0 accumulation is not affected by the inhibitors of polyamine biosynthesis [methylglyoxal

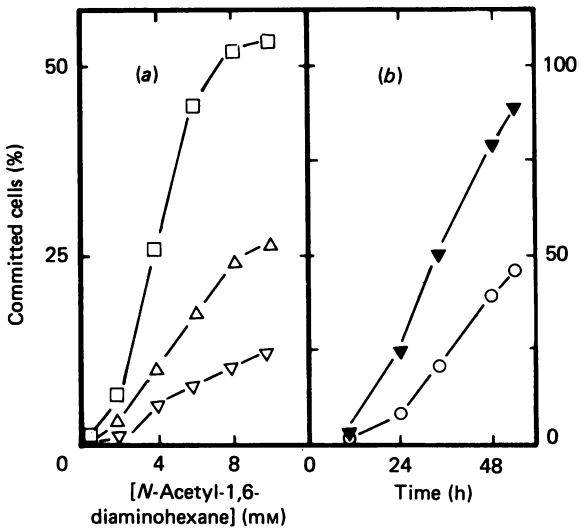

Fig. 6. Effect of exogenous *N*-acetyl-1,6-diaminohexane on MEL cell differentiation

Cells were cultured as in Fig. 4, (*a*) in the presence of various concentrations of *N*-acetyl-1,6-diaminohexane, or (*b*) in the presence of 8 mM-*N*-acetyl-1,6-diaminohexane (○) or 4 mM-HMBA (▼). The proportion of cells committed to erythrodifferentiation was determined in (*a*) after 24 h (▽), 34 h (△) and 48 h (□), and in (*b*) at the indicated times, by transferring aliquots of the cells to medium without inducer. The proportion of cells staining with benzidine 24 h afterwards is equivalent to the proportion of committed cells at the time of transfer. Note that the scale in (*a*) is twice that in (*b*). The results shown are representative for eight experiments.

bis(guanylhydrazone) and α-difluoromethylornithine] (A. Chabanas & H. B. Osborne, unpublished work) which can modulate the HMBA-induced differentiation process [6a]. Secondly, dexamethasone inhibited the HMBA-induced differentiation without affecting the changes in intracellular polyamines normally observed [6,18].

DISCUSSION

The present results show that *N*-acetyl-1,6-diaminohexane and 1,6-diaminohexane, formed by deacetylation of HMBA, can accumulate rapidly inside MEL cells cultured with this inducer (Fig. 1). The accumulation of these catabolites of HMBA preceded the changes in polyamine biosynthesis (Fig. 2) associated with this differentiation process [6]. A reciprocal relationship was observed between the quantity of HMBA catabolites accumulated and the decrease in ODC activity (Fig. 3). That HMBA was not required for these changes in polyamines biosynthesis to occur was demonstrated by culturing MEL cells with exogenous *N*-acetyl-1,6-diaminohexane. Like HMBA, this molecule is deacetylated to produce 1,6-diaminohexane intracellularly. However, no acetylation of *N*-acetyl-1,6-diaminohexane to form HMBA, intracellularly or in the medium, was detected. Under these conditions a very rapid decrease in ODC activity and in intracellular polyamines was observed.

Several mechanisms by which natural or synthetic diamines regulate ODC activity have been proposed. Synthesis of a protein inhibitor of ODC ('ODC antizyme') is stimulated by putrescine, spermidine, spermine and synthetic diamines (reviewed in [7]). However, we have detected no antizyme activity in MEL cells cultured for up to 8 h with 4 mM-HMBA (H. B. Osborne, unpublished work). Recently, Kahana & Nathans [19] have shown that the translation of ODC mRNA is regulated by putrescine in a cell line that overproduces ODC. Since ODC mRNA has a long 5′ leader containing four AUG initiator codons [20], the above authors suggested that putrescine or other polyamines could exert their effect on ODC mRNA translation either by changing the secondary structure of this leader or by modifying the binding of specific proteins to this sequence. Whether the two catabolites of HMBA whose accumulation is correlated with a decrease in ODC activity can act by a similar mechanism requires further investigation.

It should be noted that, for low HMBA concentrations (between 0.2 mM and 0.5 mM), the only catabolite of HMBA detected was *N*-acetyl-1,6-diaminohexane. However, 0.5 mM-HMBA was sufficient to cause a 50% decrease in the ODC activity. Therefore, in this cellular system, it appears that *N*-acetyl-1,6-diaminohexane alone can negatively control the ODC activity. Mamont *et al.* [21] showed that the addition of N^8-acetylspermidine to the culture medium of HTC cells caused a decrease in the ODC activity. Since N^8-acetylspermidine can be rapidly converted into spermidine, the identity of the active molecular species was not clear in this case.

In addition to the key role in the regulation of polyamine biosynthesis described above, we observed that exogenous *N*-acetyl-1,6-diaminohexane was able to induce the differentiation of MEL cells. Similarly to that induced by HMBA, the differentiation observed in the presence of *N*-acetyl-1,6-diaminohexane was associated with an accumulation of the histone $H1^0$ and could be inhibited by dexamethasone. Whether *N*-acetyl-1,6-diaminohexane or HMBA were used as inducer, 1,6-diamino hexane is formed intracellularly. Although 1,6-diaminohexane when added to the culture medium is very toxic [1] (4 mM causes 40% cell death after 24 h; E. Meilhoc unpublished work) and does not induce the differentiation of MEL cells, we cannot exclude a role for 1,6-diaminohexane in this differentiation process. Cells may respond differently to 1,6-diaminohexane formed intracellularly or applied extracellularly. It should be noted that, when the differentiation was induced with exogenous *N*-acetyl-1,6-diaminohexane, the intracellular concentration of 1,6-diaminohexane was greater than when HMBA was present. However, the rate of appearance of committed cells was slower with *N*-acetyl-1,6-diaminohexane as inducer than with HMBA. Moreover, in the presence of HMBA, *N*-acetyl-1,6-diaminohexane was the major intracellular catabolite present before the appearance of committed cells in the culture. These observations suggest that, in the differentiation process induced by HMBA, *N*-acetyl-1,6-diaminohexane is functionally more important than 1,6-diaminohexane.

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