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Inhibition of ferrochelatase during differentiation of murine erythroleukaemia cells

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During dimethyl sulphoxide-induced differentiation of DS-19 murine erythroleukaemia (MEL) cells, the activity of the terminal enzyme of the haem-biosynthetic pathway, ferrochelatase (protohaem ferrolyase, EC 4.99.1.1), is thought to be the rate-limiting step for haem production. Differentiation of induced MEL cells in the presence of exogeneously supplied protoporphyrin IX showed that total haem production was affected by added porphyrin only after 48 h. These data suggest that iron insertion, the terminal step, is rate-limiting during the first 48 h of differentiation. Addition of low levels of diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine to differentiating cultures resulted in decreased haem production and decreased ferrochelatase activity. *N*-Methylprotoporphyrin at nanomolar concentrations also strongly inhibited ferrochelatase activity, but had no inhibitory effect on cellular haem production. The bivalent cations Co^{2+}, Cd^{2+} and Mn^{2+} were tested for their effect on haem production and ferrochelatase activity. All three metals were found to inhibit both haem formation and ferrochelatase activity, with Mn^{2+} being the strongest effector. These data, together with those previously published, suggest that the terminal step in haem biosynthesis is rate-limiting during the early stages of differentiation in MEL cells.

INTRODUCTION

Regulation of the haem-biosynthetic pathway in non-erythropoietic cells appears to be controlled in a manner different from that in erythropoietic cells (Kappas *et al.*, 1983). In hepatocytes the regulation is at the initial step, namely ALA synthase (Disler & Moore, 1985; Kappas *et al.*, 1983). The end product, haem, exerts negative-feedback control at the transcriptional and translational levels as well as in the translocation of ALA synthase into the mitochondrial matrix, and repression of ALA synthase activity occurs when the cellular concentration of free haem reaches approx. 10^{-7} M.

The rate of haem biosynthesis in differentiating mammalian erythroid cells appears to be controlled differently, and may not involve ALA synthase. Studies on MEL cells have suggested that the rate-limiting step is at ferrochelatase, the terminal enzyme in the pathway (Granick & Sassa, 1978; Rutherford *et al.*, 1979; Sassa, 1976). It is found that, after the treatment of MEL cells with DMSO, haemoglobin does not appear until ferrochelatase induction has occurred, even though the other enzymes of the pathway are induced much earlier. Additionally, MEL cells can be induced to differentiate by addition of haemin to cultures, although a similar response does not occur when these cells are treated with intermediates in the pathway, such as ALA or porphobilinogen (Dabney & Beaudet, 1977; Granick & Sassa, 1978; Ross & Sautner, 1976). The cell line DR-1, a mutant which does not induce ferrochelatase with DMSO, does not differentiate and synthesize haemoglobin unless it is supplemented with both DMSO and haemin (Sassa *et al.*, 1978).

The regulatory mechanisms of this pathway in different cell types can be better understood by evaluating the effects of controlling levels of products, substrates and related compounds. The present investigation examines the effects of protoporphyrin IX, DDC, *N*-methylprotoporphyin and a variety of heavy-metal ions on ferrochelatase activity and porphyrin levels in MEL cells.

MATERIALS AND METHODS

MEL cultures

Friend-virus-transformed MEL cells were maintained in culture at 37 °C, under CO_2/air (1:19), in Dulbecco's Modified Eagle Medium supplemented with 15% (w/v) heat-inactivated fetal bovine serum. The cell line used in our investigation, DS19, was kindly donated by Dr. S. Sassa (The Rockefeller University, New York, NY, U.S.A.). Cell lines were maintained in culture as described previously (Sassa *et al.*, 1978). At 24 h after transfer, cultures (10^5–10^6 cells/ml) were induced to differentiate by the addition of DMSO to a final concentration of 2%. Upon DMSO induction the following additions were made to cell cultures: protoporphyrin IX, *N*-methylprotoporphyrin, haemin, DDC, $CdCl_2$, $CoCl_2$, $MnCl_2$ or lead acetate. The porphyrins (1 mM stock solutions) were dissolved in DMSO unless otherwise stated. A stock solution of 2 mM-DDC was also prepared in DMSO. Metal stock solutions (1 mM) were freshly prepared in cell culture media for each experiment.

Determination of haem content

The fluorimetric assay for the determination of haem content has been described by Sassa *et al.* (1978). Endogenous porphyrins were subtracted from all total porphyrin determinations. Endogenous porphyrins

Abbreviations used: MEL, murine erythroleukaemia; ALA, 5-aminolaevulinic acid; DMSO, dimethyl sulphoxide; DDC, diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine; PMSF, phenylmethanesulphonyl fluoride.

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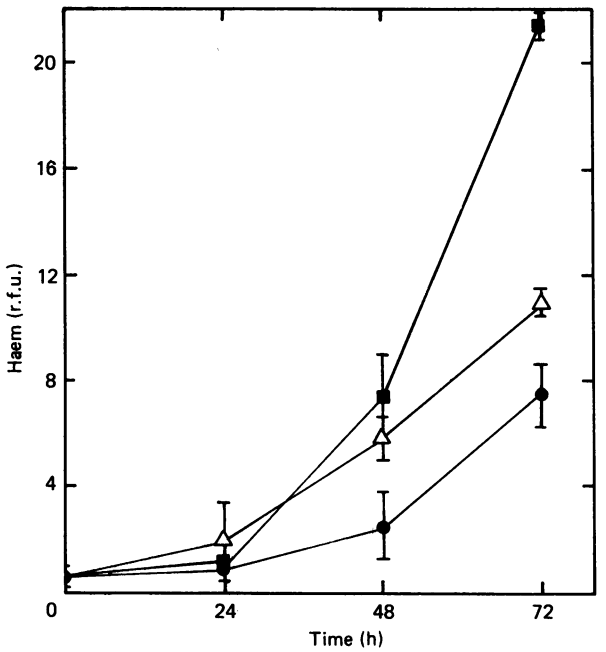

Fig. 1. Effects of 1 μM- and 2 μM-protoporphyrin IX on haem production by MEL cells induced with 2% DMSO

Haem concentration was determined by fluorimetry. The points plotted on the graph represent the means of duplicates. ●, Control; △, 1 μM-protoporphyrin IX; ■, 2 μM-protoporphyrin IX. r.f.u. are relative fluorescence units; in this experiment, 1 r.f.u. is equivalent to approx. 2 pmol of haem/10^6 cells.

in the cells were measured by preparing a tissue blank which contained the same number of cells that had been boiled for 30 s in the presence of 1.0% SDS. Fluorescence was determined with a Perkin–Elmer 650–40 fluorescence spectrophotometer (405 nm excitation, 635 nm emission).

Determination of ferrochelatase activity

The procedure for measurement of ferrochelatase activity involves the incubation of cell extracts in the presence of ^{59}Fe and either protoporphyrin IX or mesoporphyrin IX (Dailey *et al.*, 1986). MEL cells (10^7) were centrifuged at 2000 *g* for 10 min and washed once in Hanks balanced salt solution + 20 mM-Hepes. The pellets were resuspended in 100 μl of buffer containing 0.5 mM-dithiothreitol, 10 mM-Tris/HCl, pH 7.2, 150 mM-NaCl, 2 mM-EDTA, 0.5% Triton X-100, 0.05% sodium cholate, 0.4 mM-digitonin and 1 μg of PMSF/ml. An incubation mixture consisting of 0.5 ml of 0.1 M-Tris acetate, pH 8.1, 0.1 ml of 50 mM-dithiothreitol, 0.1 ml of 4 mM-ferrous citrate (4 μCi/assay) and 0.1 ml of 1 mM-protoporphyrin (or mesoporphyrin) IX was added to each sample (Dailey *et al.*, 1986). Controls contained incubation mixture without added cells, and either 100 μl of buffer or 100 μl of purified ferrochelatase. Samples were incubated in the dark for 1 h at 37 °C. The reaction was stopped by the addition of 0.5 ml of 0.2 M-HCl. Product was extracted out of the acidified aqueous phase by the addition of 1.5 ml of butan-2-one. After vortex-mixing, the layers were allowed to separate and a portion of the organic phase was removed and transferred into scintillation vials. After evaporation,

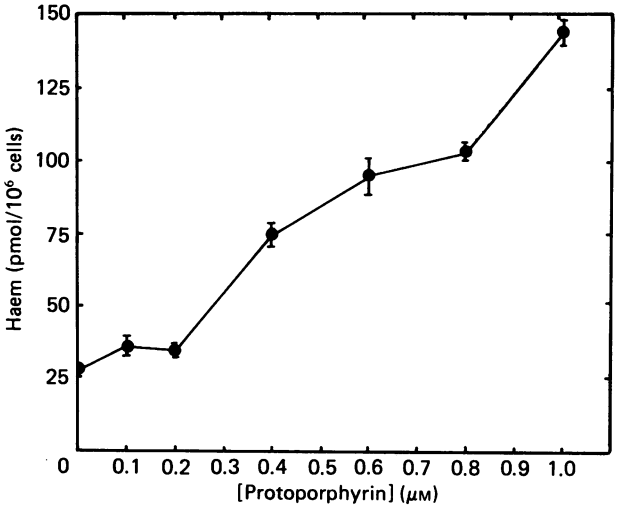

Fig. 2. Haem produced by 72 h in induced MEL cells in the presence of exogeneously supplied protoporphyrin IX

Experimental details are the same as for Fig. 1.

Table 1. Effect of protoporphyrin IX on ferrochelatase activity

Cells of MEL clone DS19 were incubated with protoporphyrin IX and 2% DMSO, and an aliquot from each was analysed 72 h post-induction for ferrochelatase activity as described in the Materials and methods section

Concentration of protoporphyrin IX added to culture (μM)	Relative ferrochelatase activity
0	100
0.25	100
0.50	124
1.0	90
2.0	118

0.1 ml of 30% H_2O_2 was added to each vial to decolorize the haem and the mixture left overnight before the addition of 10 ml of scintillation fluid (Scinti-Verse I). Radioactivity was determined by liquid-scintillation counting.

Source of compounds

Dulbecco's Modified Eagle Medium was purchased from Gibco Grand Island, NY, U.S.A. Protoporphyrin IX, mesoporphyrin IX and haemin were obtained from Porphyrin Products, Logan, UT, U.S.A. *N*-Methylprotoporphyrin was synthesized according to a previously published procedure (Kunze & Ortiz de Montellano, 1981). DDC was from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. All other reagents were of the highest purity available.

RESULTS

Effects of protoporphyrin

In an effort to determine if haem biosynthesis was limited during differentiation by the production of

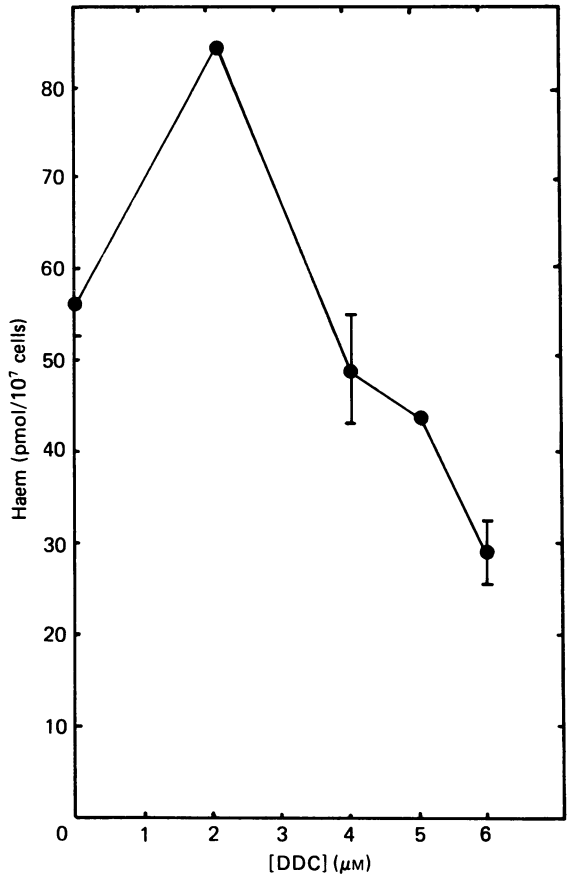

Fig. 3. Effect of DDC on haem production by MEL cells induced with 2% DMSO

Haem concentration was determined 72 h after induction. The points plotted on the graph represent the means of duplicates.

Table 2. Effect of *N*-methylprotoporphyrin and DDC on ferrochelatase

Cells of MEL clone DS19 were incubated with *N*-methylprotoporphyrin and 2% DMSO or DDC and 2% DMSO. An aliquot from each was analysed 72 h post-induction for ferrochelatase activity as described in the Materials and methods section.

Addition to culture	Relative ferrochelatase activity
None	100
DDC, 3 μM	84
DDC, 6 μM	18
N-Methylprotoporphyrin, 50 nM	12

protoporphyrin or if the terminal iron insertion step was limiting, protoporphyrin was added to differentiating cultures of MEL cells and the total amount of cellular haem produced was measured. The addition of protoporphyrin IX at concentrations of 2 μM or greater to DMSO-induced MEL cells in culture resulted in an increase in the production of haem over control levels only after 24 h. The amount of haem produced by all cultures at 24 h were identical within experimental limits. Fig. 1 displays the results when 1 μM- and 2 μM-protoporphyrin IX were introduced into the cell-culture system. Protoporphyrin IX alone dissolved in ethanol did not result in differentiation of uninduced cells. The growth of the cells in culture was not affected by the addition of protoporphyrin IX, and the level of cellular porphyrins also remained relatively constant.

Protoporphyrin was added to MEL cultures at concentrations ranging from 0 to 1 μM, and these cultures were allowed to differentiate in the presence of DMSO. At 72 h the cells were harvested and the haem produced was measured. The results of this experiment are shown in Fig. 2. The effect of exogenously supplied protoporphyrin IX on ferrochelatase activity was measured by the incorporation of ^{59}Fe *in vitro*. A 72 h period was chosen because it is at this time that the activities of the enzymes of the haem-biosynthetic pathway reach a maximum after the induction of MEL-cell differentiation. When cultured in the presence of protoporphyrin IX, MEL cells demonstrated ferrochelatase activity on the order of that seen in the control (Table 1).

Effects of DDC and *N*-methylprotoporphyrin

DMSO-induced MEL cells supplemented with 1–6 μM-DDC were assayed for the presence of haem 72 h post-induction. The concentration of haem present in 10^7 cells increased in the presence of up to 2 μM-DDC. At greater concentrations of DDC, however, the haem concentration decreased (Fig. 3). The addition of DDC, at the concentrtions used in the present study, had no deleterious effects on the growth rate or viability of MEL cells. The concentration of cellular free porphyrin remained constant, regardless of the concentration of DDC. The effects of 3 μM-DDC and 6 μM-DDC on the

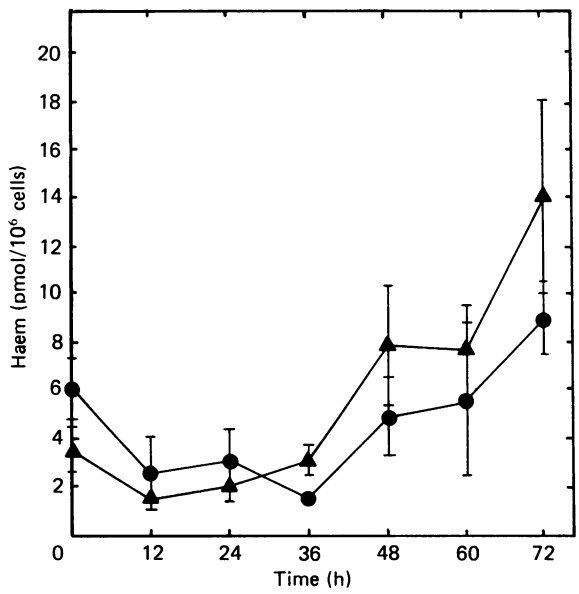

Fig. 4. Effect of 50 nM-*N*-methylprotoporphyrin on haem production by MEL cells induced with 2% DMSO

Haem concentration was determined by fluorimetry. The points plotted on the graph represent the means of duplicates. ●, Control; ▲, *N*-methylprotoporphyrin.

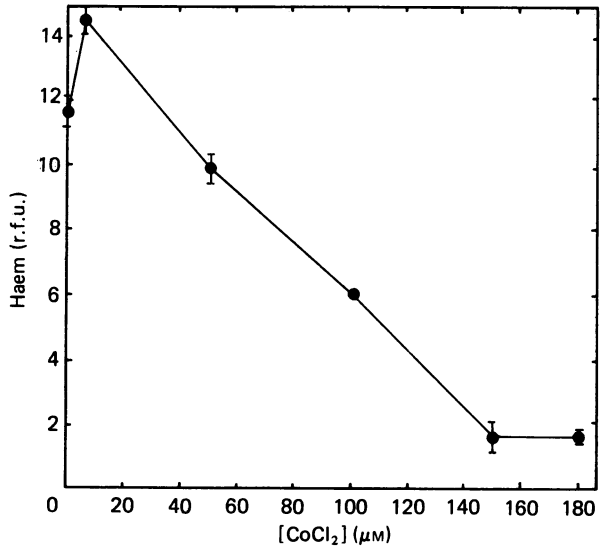

Fig. 5. Effects of $CoCl_2$ on haem production in MEL cells induced with 2% DMSO

The points that are plotted on the graph represent the means of duplicates. r.f.u. are relative fluorescence units.

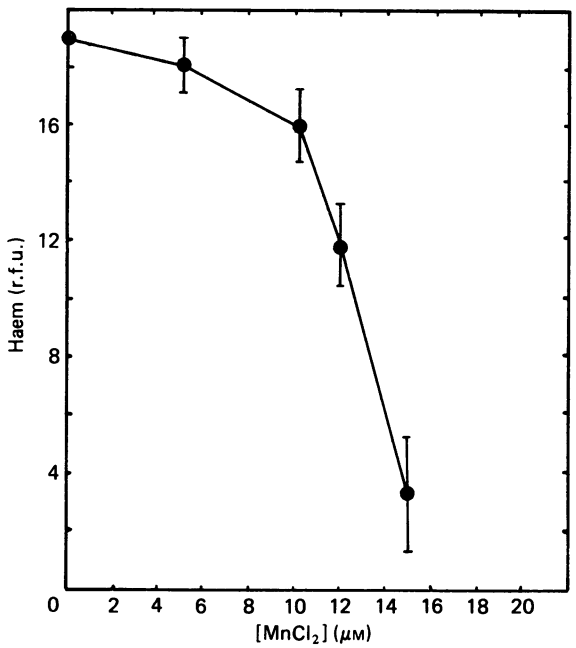

Fig. 6. Effect of $MnCl_2$ on haem production in MEL cells 72 h post-induction with 2% DMSO

The points plotted on the graph represent the means of duplicates.

Table 3. Effect of $CoCl_2$, $CdCl_2$ and $MnCl_2$ on ferrochelatase activity

Cells of clone DS19 were induced with 2% DMSO, and either $CoCl_2$, $CdCl_2$ or $MnCl_2$ was added at the concentration indicated. An aliquot from each was analysed 72 h post-induction for ferrochelatase activity as described in the Materials and methods section.

Addition to culture	Relative ferrochelatase activity
None	100
$CoCl_2$, 10 μM	100
$CoCl_2$, 50 μM	48
$MnCl_2$, 15 μM	56
$CdCl_2$, 8.6 μM	64

activity of ferrochelatase are shown in Table 2. The cultures exposed to 3 μM-DDC had a ferrochelatase activity similar to that seen in control cultures, whereas MEL cells cultured in media supplemented with 6 μM-DDC had significantly lower activity.

The addition of 50 nM-*N*-methylprotoporphyrin to induced MEL cells resulted in a slight increase in haem concentration, as measured over a 72 h period post-induction (Fig. 4). The viability of these cells, as determined by Trypan Blue exclusion and growth-rate measurements, was unaffected at this concentration. The level of cellular porphyrins was the same as control cultures throughout all samples containing *N*-methylprotoporphyrin, although culture protoporphyrin levels were not determined. To see if *N*-methylprotoporphyrin had an effect on ferrochelatase activity in cultured MEL cells, induced MEL cells cultured in media supplemented with 50 nM-*N*-methylprotoporphyrin were assayed for ferrochelatase activity. It was found that enzyme activity was significantly lower than in controls (Table 2).

Effects of $CoCl_2$, $MnCl_2$, $CdCl_2$ and lead acetate

Induced MEL cells grown in the presence of 15–180 μM-$CoCl_2$ showed decreased haem production (Fig. 5), although the haem concentrations in cells grown with less than 15 μM-$CoCl_2$ were found to be slightly higher than controls. Cell growth was not affected by the addition of $CoCl_2$ at concentrations below 200 μM, although it was noted that concentrations of 200 μM and above were cytotoxic. The concentration of cellular free porphyrins remained constant.

The effect of $CoCl_2$ added to MEL cultures on ferrochelatase activity of these cells as measured *in vitro* is shown in Table 3. In cultures supplemented with 10 μM-$CoCl_2$, ferrochelatase activity was on the order of that seen in the control. However, in cultures supplemented with 50 μM-$CoCl_2$, ferrochelatase activity was decreased more than 50%.

When MEL cells were supplemented with 5–15 μM-$MnCl_2$, a decrease in the concentration of haem resulted (Fig. 6). Cell growth was not affected measurably by the addition of 15 μM-$MnCl_2$; however, concentrations greater than 15 μM proved to be cytotoxic. The effect of $MnCl_2$ on cellular ferrochelatase activity is displayed in Table 3. In cultures supplemented with 15 μM-$MnCl_2$, ferrochelatase activity was decreased by more than 40%.

The effects of Cd^{2+} on haem biosynthesis in MEL cells are shown in Fig. 7. Increasing concentrations of $CdCl_2$ (0–50 μM) resulted in decreasing concentrations of haem. Cell growth was not affected by Cd^{2+} at the concentrations used here; however, $CdCl_2$ did prove to be cytotoxic at concentrations greater than 100 μM. No increase in cellular free porphyrins was found. The effect of $CdCl_2$ on ferrochelatase activity was shown to be inhibitory, with 8.6 μM-$CdCl_2$ decreasing the activity by 36% (Table 3).

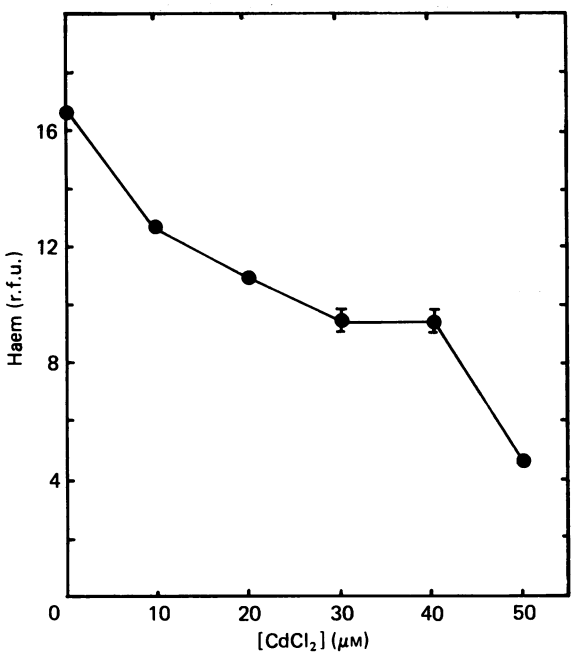

Fig. 7. Effect of $CdCl_2$ on haem production in MEL cells 72 h post-induction with 2% DMSO

r.f.u. are relative fluorescence units. The points plotted on the graph represent the means of duplicates.

MEL cells treated with low concentrations of lead (up to 25 μM-lead acetate) possessed normal levels of haem. However, concentrations greater than 25 μM proved to be cytotoxic. No increase in endogenous porphyrins was evident in cultures supplemented with low concentrations of lead acetate, and ferrochelatase activity was comparable with controls (results not shown).

DISCUSSION

An ongoing controversy in the field of haem biosynthesis concerns defining the differences in the regulatory mechanisms for haem biosynthesis in erythropoietic as opposed to non-erythropoietic cells. Hepatic and apparently other non-erythropoietic cell types have end-product-feedback regulation at the level of ALA synthase. However, studies on erythropoietic cell types suggest that ALA synthase is not negatively regulated by haem and that the terminal step of the pathway is rate-limiting during erythropoietic differentiation. It has been proposed previously that ferrochelatase may be rate-limiting in differentiating MEL cells (Rutherford *et al.*, 1979), but others have proposed that it may actually be the supply of iron which is critical (Ponka & Schulman, 1985).

To approach the question of whether ferrochelatase is rate-limiting, we have examined the effect of exogeneously supplied protoporphyrin on the total amount of cellular haem produced. The assumption was made that, if the early porphyrin-biosynthetic-pathway enzymes were limiting, the addition of protoporphyrin would bypass these steps and supply sufficient porphyrin for haem biosynthesis. Under such conditions the terminal iron-insertion step would then become limiting, although one would expect to see increased levels of haem produced. The data obtained from these experiments demonstrated three features. First, exogenously supplied protoporphyrin alone did not induce MEL-cell differentiation and did not effect the levels of ferrochelatase activity. Secondly, supplementation with protoporphyrin did not alter cellular haem biosynthesis until 48 h post-induction, suggesting that iron insertion was limiting up to that time. Thirdly, it was noted that 72 h post-induction cultures, which had been supplemented with several different amounts of protoporphyrin, had cellular haem levels that were directly proportional to the amount of added porphyrin.

These observations support a model of erythropoiesis in MEL cells where ferrochelatase or iron supply is rate-limiting for haem biosynthesis up to 48 h, at which time exogenously supplied protoporphyrin has little effect on the amount of haem synthesized, but that by 72 h post-induction they are present in excess, and it is then that the early porphyrin-producing enzymes are limiting. The fact that exogenously supplied protoporphyrin alone did not induce differentiation suggests that endogenously synthesized haem may not function as a positive-feedback stimulator of erythropoiesis, as has been previously proposed (Sassa, 1980).

A somewhat surprising finding was that DDC has an effect on haem biosynthesis in MEL cells. It is well known that DDC affects hepatocyte haem biosynthesis by inhibition of ferrochelatase via production of *N*-methylprotoporphyrin in a 'suicide' reaction with cytochrome *P*-450 (De Matteis *et al.*, 1980; Ortiz de Montellano *et al.*, 1981). One explanation for the observation that DDC administration results in inhibition of ferrochelatase in induced MEL cells is that active DDC-metabolizing cytochrome *P*-450 is present in these cells. Equally interesting, however, was the observation that exogenously supplied *N*-methylprotoporphyrin, which is an inhibitor of ferrochelatase (Dailey & Fleming, 1983), did not have a deleterious effect of haem production by these cells, even though ferrochelatase activity was drastically lower in '*in vitro*' assays. A possible explanation for the observations with DDC and *N*-methylprotoporphyrin may be that DDC causes relatively high intracellular *N*-methylprotoporphyrin concentrations, and these are not approached experimentally by the exogenously supplied *N*-alkyl compound. It is obvious that *N*-methylprotoporphyrin is getting into the cells, since ferrochelatase activity assayed *in vitro* is decreased. What is seen with DDC is that, at low (2 μM) concentrations, the cellular haem content increases, whereas at higher concentration it causes a decrease in haem content. It may be that the effect reported above with 50 nM-*N*-methylprotoporphyrin reflects the situation *in vivo* seen with low concentrations of DDC, although the finding that ferrochelatase, assayed *in vitro*, is strongly inhibited when cells are grown in 50 nM-*N*-alkyl-porphyrin, whereas it is only weakly inhibited in cells grown in 2 μM-DDC, suggests that some other factor may be involved. With the data presently available it is not possible to define all of the parameters and arrive at a completely satisfactory answer. To approach this question it will be necessary to determine accurately the intracellular concentration of *N*-methylprotoporphyrin in MEL cells, which is not presently experimentally practical.

A variety of cations that have been reported to affect ferrochelatase activity were also examined for their effect upon haem biosynthesis in differentiating MEL cells. It

was found that Mn^{2+}, Cd^{2+} and Co^{2+}, at micromolar concentrations, strongly affected haem biosynthesis. The fact that these bivalent cations were exerting their effect on haem biosynthesis is shown by their effect on ferrochelatase activity. It is interesting to note the strong inhibition by Mn^{2+}. Previous studies have shown that the K_i of bovine ferrochelatase for Mn^{2+} is 15 μM (Dailey & Fleming, 1983), which is approximately the level at which it has its strongest effect on haem production in differentiating MEL cells.

Although it might be argued that these bivalent cations have their effect at iron uptake or on some other system, with inhibition of ferrochelatase activity being of only secondary importance, the fact that similar concentrations of the metals affect both cellular haem production and ferrochelatase activity suggests that these cations express their effect by inhibition of ferrochelatase. Under no conditions did we find significant cellular accumulation of protoporphyrin. Although this might have been expected from inhibition of ferrochelatase, it is possible that any excess protoporphyrin produced was excreted into the medium. Because of the large amount of serum in our medium (15%) and the relatively low levels of porphyrin expected to be produced, we did not attempt to quantify media protoporphyrin.

In conclusion, the data presented above provide evidence for a model of erythropoiesis in MEL cells where the terminal step in haem biosynthesis is the rate-limiting one up to 48 h post-induction. Also, DDC is metabolized by differentiating MEL cells, and this metabolism produces some product, probably *N*-methylprotoporphyrin, that strongly inhibits haem formation and inhibits ferrochelatase activity. Inhibition of ferrochelatase activity by certain bivalent cations suggests that levels of ferrochelatase activity are important in the overall control of haem biosynthesis during differentation of MEL cells.

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