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Multiple mechanisms for the regulation of haem synthesis during erythroid cell differentiation

Possible role for coproporphyrinogen oxidase

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Murine erythroleukaemia (MEL) cells are virus-transformed erythroid precursor cells that, when induced to differentiate by dimethyl sulphoxide (DMSO), will initiate haem biosynthesis by the induction and synthesis *de novo* of all of the enzymes of the haem-biosynthetic pathway. The activities of porphobilinogen (PBG) deaminase (EC 4.3.1.8), coproporphyrinogen oxidase (EC 1.3.3.3), protoporphyrinogen oxidase (EC 1.3.3.4), ferrochelatase (EC 4.99.1.1) and NADH: ferric iron reductase, as well as the synthesis of the enzyme ferrochelatase and the levels of excreted porphyrins, were monitored during DMSO-induced differentiation of MEL cells in culture. The data demonstrate that PBG deaminase and protoporphyrinogen oxidase activities rise rapidly and early, in comparison with ferrochelatase activity, which rises more slowly, and coproporphyrinogen oxidase activity, which decreases by 60% within 24 h of induction before returning to initial levels by 72 h. NADH: ferric iron reductase activity increases slightly, but is always present at levels higher than needed for haem synthesis. Total immunoprecipitable ferrochelatase also rises slowly and parallels the increase in its activity, suggesting that it is not synthesized early in a slowly processed precursor form. Examination of culture media demonstrated that, whereas excretion of protoporphyrin and coproporphyrin occurs within 24 h of induction, coproporphyrin is excreted in amounts 4–15 times greater than protoporphyrin.

INTRODUCTION

In animal cells the regulation of the haem-biosynthetic pathway differs between erythroid and non-erythroid tissues. In the liver and other non-erythroid cells the rate of haem biosynthesis is controlled by the levels of the first pathway enzyme, 5-aminolaevulinate (ALA) synthase [1,2]. Haem may regulate its own synthesis by feedback inhibition of ALA synthase and by controlling the synthesis, intracellular translocation and processing of ALA synthase [3–7]. In differentiating erythroid cells it is now generally agreed that ALA synthase is not under the same regulatory mechanism, but at present there is no clear consensus as to what and how many factors may limit haem production.

During erythropoietic differentiation one of the key events is the increase in the synthesis of haem for haemoglobin production. A variety of studies has clearly demonstrated that in this system haem plays not only the role of cofactor but also appears to serve a signal function in that haem formation is a prerequisite for continued differentiation. In murine erythroleukaemia (MEL) cells it has been shown that any mutational or chemically induced blockage of haem synthesis results in the arrest of the normal erythropoietic programme [8–13]. Addition of haem, but not protoporphyrin, to these cells allows the continuation of the normal programme. As a result of this demonstrated effect of haem on erythroid differentiation in MEL cells, the control of haem biosynthesis and the induction of the pathway enzymes in relation to differentiation has attracted considerable interest.

Using MEL cells as a model for normal haematopoiesis, a variety of researchers has shown that the first four enzymes of the haem-biosynthetic pathway are induced within hours of dimethyl sulphoxide (DMSO) addition to the medium [1,8,14]. One model for regulation of haem biosynthesis suggests that ferrochelatase activity is rate-limiting [12]. However, the dramatic and rapid rise in ferrochelatase activity reported in that study has never been reproduced by other workers. One set of data not considered in previous models is the level of activity of coproporphyrinogen oxidase and protoporphyrinogen oxidase during differentiation. Although these enzymes have been largely ignored in MEL cells, data from studies in yeast cells have suggested that coproporphyrinogen oxidase may play a controlling role in haem biosynthesis in those cells [15].

The present work adds three significant new pieces of information, namely (1) protoporphyrinogen oxidase is induced to high levels within 48 h, (2) ferrochelatase is induced more slowly than the other enzymes and (3) coproporphyrinogen oxidase activity falls by approx. 60% in the first 24 h after induction, followed by a rise to near pre-induction levels by 72 h.

MATERIALS AND METHODS

MEL cell cultures

MEL cell strains DS-19 and 270 were continuously cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% foetal bovine serum (FBS). Cells were subcultured as previously described [16,17], and only cultures that showed 90% differentiation on addition of 2% DMSO were used. All reported cell numbers refer to viable cell counts as determined by Trypan Blue exclusion.

Ferrochelatase assay

DMSO-induced MEL cells were harvested by centrifugation for 5 min at 1300 *g* and washed once with Hanks balanced salt solution (HBSS) plus 20 mM-Hepes (pH 7.4). Cell pellets were resuspended to a concentration of 10^8 cells/ml in lysis buffer

Abbreviations used: MEL cells, murine erythroleukaemia cells; DMSO, dimethyl sulphoxide; PBG, porphobilinogen; ALA synthase, 5-aminolaevulinate synthase; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; HBSS, Hanks balanced salt solution.

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containing 20 mM-Tris/HCl (pH 7.2), 150 mM-NaCl, 2 mM-EDTA, 1.0% Triton X-100, 0.05% sodium cholate, 0.5 mM-dithiothreitol and 1 μg of phenylmethanesulphonyl fluoride/ml. Incubation mixtures, each containing 0.1 ml of the lysed cell suspension, 0.5 ml of 0.1 M-Tris/acetate (pH 8.1), 0.1 ml of 50 mM-dithiothreitol, 0.1 ml of 2 mM-mesoporphyrin IX and 0.1 ml of 4 mM-[^{59}Fe]ferrous citrate (4 μCi per sample) were prepared. Control samples were also prepared with 100 μl of mouse liver mitochondria [18] or boiled MEL cells in place of the lysed-MEL-cell suspension. The samples were incubated in the dark at 37 °C for 1 h. The extraction of product was performed using a modification of the method described by Dailey *et al.* [19]. The reaction was stopped by addition of 0.5 ml of 2 M-HCl. Haemin (10 μl of a 1 mM solution) was added as carrier to each sample. Mesohaem was extracted with 1.5 ml of ethyl methyl ketone. All samples were vortex-mixed, chilled on ice, then centrifuged for 10 min at 800 ***g*** to complete the separation of phases. Equal portions of each organic phase (0.2 ml) were transferred to scintillation vials and evaporated. After the addition of 0.1 ml of 30% H_2O_2 to decolorize the mesohaem, the samples were left overnight. The following morning the vials were heated at 105 °C for 15 min. After cooling, 10 ml of Scinti-Verse I scintillation fluid was added to each vial and radioactivity was quantified with a Beckman LS 3801 scintillation counter.

Haem synthase assay

A haem synthase assay was used to measure NADH-dependent ferrochelatase activity [20]. DMSO-induced MEL cells were centrifuged and washed as described above. Cell pellets were resuspended to a final concentration of 10^8 cells/ml in 0.1 M-Tris/acetate (pH 8.1). Incubation mixtures, each containing 0.1 ml of the cell suspension, 0.5 ml of 0.1 M-Tris/acetate (pH 8.1) and 0.1 ml of 5.0 mM-NADH (to provide reduced iron [21,22]), were prepared in serum tubes. Control samples were prepared with 0.1 ml of mouse liver mitochondria or boiled MEL cells. The tubes were stoppered, evacuated and filled with nitrogen. After the addition of 4 μCi per sample of 0.1 ml of 4 mM-[^{59}Fe]ferric citrate (or 0.1 ml of 4 mM-[^{59}Fe]ferrous citrate for the mitochondria control) and 0.1 ml of 2 mM-mesoporphyrin, the tubes were incubated in the dark at 37 °C for 1 h. The reaction was stopped, mesohaem was extracted and organic phases were collected and counted for radioactivity as described above.

Coproporphyrinogen oxidase assay

Coproporphyrinogen oxidase was assayed in cell extracts and total product formed was determined by fluorescence-detection h.p.l.c. The assay buffer at 37 °C contained, in 1 ml, 0.1% (w/v) Triton X-100, 0.1 mM-EDTA, 25 mM-Hepes (pH 7.5), 0.1 mM-dithiothreitol, 50 μM-coproporphyrinogen III and cell extract from 10^7 cells. Coproporphyrinogen III was prepared from coproporphyrin III by reduction with sodium amalgam immediately before use [23]. All reactions were carried out in dim light to prevent photocatalysed autoxidation of the substrate. The assay was stopped by addition of 1 ml of acetic acid/saturated sodium acetate (4:1, v/v). Porphyrinogens were oxidized to their corresponding porphyrins by addition of 0.1 ml of 0.005% I_2 under an oxygen atmosphere in the light. After addition of mesoporphyrin as an internal standard, porphyrins were extracted into ethyl acetate (3 ml). A 2 ml portion of the ethyl acetate layer was removed to fresh tubes and the porphyrins were extracted into 0.5 ml of 1.5 M-HCl. A sample of this was subjected to h.p.l.c. to separate and quantify coproporphyrin and protoporphyrin. In these assays less than 10% of the total substrate was converted into product during the 30 min incubation. The h.p.l.c. system used a Spheri-S RP18 column (10 cm × 0.46 cm) (Pierce Chemical Co.) with a two-solvent program. Solvent A was 1 M-ammonium acetate (pH 5.16)/acetonitrile (9:1, v/v). Solvent B was acetonitrile/methanol (1:9, v/v). The column was equilibrated with 70% Solvent A/30% Solvent B. After sample loading, a 5 min linear gradient up to 95% Solvent B was run, followed by a hold for 5 min at 95% Solvent B.

For separation and determination of culture porphyrins this same column and solvents were used, but a different program was employed. For separation of porphyrins the column was equilibrated with 75% Solvent A/25% Solvent B. Solvent B was increased to 35% in a 2 min linear gradient and then held for 1.5 min. Solvent B was then increased to 70% in 0.5 min and then held at that level for 10.5 min. It was then increased to 95% in a 5 min linear gradient and held for 4.5 min. Detection was by fluorescence.

Ferric iron reductase assay

DMSO-induced MEL cells were centrifuged, washed and resuspended (10^8 cells/ml) as described for the haem synthase assay. Cells (10^7) were assayed for ferric iron reductase activity by a modification of the method published by Moody & Dailey [22]. In order to measure the activity of ferric iron reductase, the ferrous iron chelator 3-(pyrid-2-yl)-1,2,4,-triazine-5,6-bis(benzene-4-sulphonic acid) (ferrozine) was employed as a ferrous iron trap. The reaction mixture contained 0.1 ml of MEL cell suspension, 0.1 ml of 0.1 M-Tris/acetate (pH 8.1), 0.1 ml of 10 mM-ferrozine, 50 μl of 4 mM-ferric citrate and 50 μl of 0.8 mM-NADH. Absorbance was measured at 562 nm with a Cary 219 spectrophotometer.

Porphobilinogen (PBG) deaminase and protoporphyrinogen oxidase assays

PBG deaminase was assayed by a fluorimetric assay described previously [24]. Protoporphyrinogen oxidase was assayed by monitoring the increase in fluorescence due to protoporphyrin production. Cell extracts from 10^7 cells were assayed as described by Dailey & Karr [23].

Fluorimetric haem assay

Cellular haem was assayed in MEL cells (10^7) by the fluorimetric method described by Sassa *et al.* [17]. Fluorescence was determined with a Perkin–Elmer 650-40 fluorimetric spectrophotometer (405 nm excitation, 635 nm emission). Levels of endogenous free porphyrins were determined by fluorescence after lysis of cells with 1% SDS,

Protein determination

MEL cell protein concentration was determined by the BCA protein assay [25] with BSA as a standard.

L-[^{35}S]Methionine labelling

DMSO-induced MEL cells (6×10^7) were harvested by centrifugation at 1300 ***g*** for 5 min and washed once in HBSS + 20 mM-Hepes (pH 7.4). Cell pellets were resuspended in 10 ml of DMEM + 15% FBS warmed to 37 °C. [^{35}S]Methionine (0.25 mCi) was added and cells were incubated at 37 °C under 5% CO_2. After the appropriate length of time, the labelled cells were centrifuged at 1300 ***g*** for 5 min and washed once with HBSS + 20 mM-Hepes (pH 7.4). Cell pellets were resuspended to 10^8 cells/ml in buffer containing 20 mM-Tris/HCl (pH 7.6), 150 mM-NaCl, 2 mM-EDTA, 1.0% Triton X-100, 0.05% sodium cholate, 0.1% SDS, 2 mM-methionine, 0.4 mM-digitonin and 1 μg of phenylmethanesulphonyl fluoride/ml.

Immunoprecipitation and gel electrophoresis

MEL cell extracts were centrifuged at 5900 *g* for 10 min. Each supernatant was preadsorbed for 30 min at 25 °C with 0.2 ml of a suspension of fixed *Staphylococcus aureus* (Sigma), which was washed twice in immunoprecipitation buffer [50 mM-Tris/HCl (pH 7.5), 150 mM-NaCl, 1 mM-EDTA, 1.0 % Triton X-100 and 2 mM-methionine] plus 0.1 % SDS. The samples were centrifuged at 5900 *g* for 10 min and the supernatants removed. Rabbit anti-(mouse ferrochelatase) antibody [26] was added to each sample (50 μl), which was incubated overnight at 4 °C. Control samples were also prepared with 50 μl of non-immune rabbit serum or 50 μl of anti-ferrochelatase antibody which was added to 0.1 ml of purified ferrochelatase 1 h before immunoprecipitation. *S. aureus* Protein A (50 μl) (Calbiochem), washed twice in immunoprecipitation buffer plus 0.4 % SDS, was added to each sample and incubated for 30 min at 25 °C. Samples were centrifuged for 15 min at 1000 *g* and the supernatants were discarded. Precipitated pellets were washed four times in immunoprecipitation buffer plus 0.4 % SDS. Final pellets were resuspended in 50 μl of sample buffer containing 0.5 M-Tris/HCl (pH 6.8), 8 % 2-mercaptoethanol, 5 % SDS, 17 % glycerol and 17 μg of Phenol Red/ml and heated at 100 °C for 3 min. The samples were applied to a 10 %-polyacrylamide gel containing 0.1 % SDS. Autoradiography was performed by the method described by Bonner & Laskey [27].

Sources of material

DMEM was purchased from JRH Biosciences (Lenexa, KS, U.S.A.). FBS and *S. aureus* were obtained from Sigma. HBSS was supplied by Gibco (Grand Island, NY, U.S.A.). Mesoporphyrin IX, porphobilinogen, coproporphyrin III, porphyrin standards and protoporphyrin IX were purchased from Porphyrin Products (Logan, UT, U.S.A.). $^{59}FeCl_3$ and [^{35}S]methionine were obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). *S. aureus* Protein A was purchased from Calbiochem (San Diego, CA, U.S.A.). All other reagents were of the highest purity available.

RESULTS

Induction of enzyme activities and haem production in suspension cultures

DMSO-induced DS-19 MEL cells grown in suspension cultures were harvested every 24 h over a 5-day period and assayed for haem content and PBG deaminase, coproporphyrinogen oxidase, protoporphyrinogen oxidase and ferrochelatase activities. In addition to DS-19 MEL cells, 270 MEL cells were assayed for these activities. The data for both cell lines were, within experimental limits, identical. The production of haem in these cultures and the decline in cellular protein content are shown in Fig. 1. Previously it has been shown that PBG deaminase activity increases rapidly over the first 24–48 h after induction by DMSO [14]. DS-19 MEL cells, when grown as described above, showed an increase in PBG deaminase activity after the first 24 h, with the most rapid rise 48 h after induction (Fig. 2).

Protoporphyrinogen oxidase activity has not previously been measured during differentiation of MEL cells. In the present study the activity of this enzyme was found to decrease by 20 % in the first 24 h after DMSO induction before rising rapidly over 3-fold by 72 h (Fig. 2). Except for the small decrease in activity, which is not viewed as highly significant, since the S.D. at this activity is approx. 20 %, the pattern of induction is similar to that seen with PBG deaminase.

It has been reported that, in 707 MEL cells induced to differentiate with DMSO, coproporphyrinogen oxidase activity had doubled at 96 h over that in non-induced cells [12]. There was no report of the time course of the induction of this enzyme. In the present study we have assayed coproporphyrinogen oxidase at several time points after induction. The data shown in Fig. 3 clearly demonstrate a 60 % decrease in activity in the first 24 h, followed by a return to pre-induction levels by 3 days after induction.

Induction of ferrochelatase activity in MEL cells has previously been reported to occur in clone 745 cells with the same rate as PBG deaminase [9], and slowly in 707 cells (with measurable activity increasing at about 72 h) [12]. The precise time course of induction is significant, since the late induction of ferrochelatase, corresponding to the time of the appearance of haem, would suggest that ferrochelatase may be a rate-limiting step in haem biosynthesis. To examine ferrochelatase activity in this study, a sensitive ^{59}Fe assay was employed. The results shown in Fig. 4

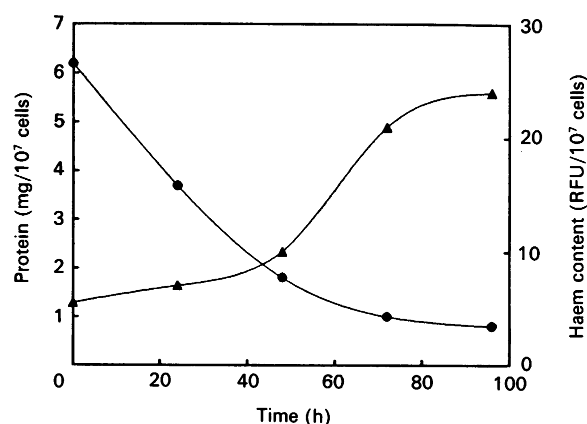

Fig. 1. Haem synthesis and protein concentration during differentiation of DS-19 MEL cells

Following DMSO induction the level of cellular haem formed and cell protein concentration was determined as described in the Materials and methods section. Haem (▲) was determined as free porphyrin after removal of iron by the oxalic acid method. Values are listed as relative fluorescence units (RFU) per 10^7 cells. Protein (●) is shown as mg per 10^7 cells. All data shown are the averages of three determinations. S.D. for any one point was less than ±10 %.

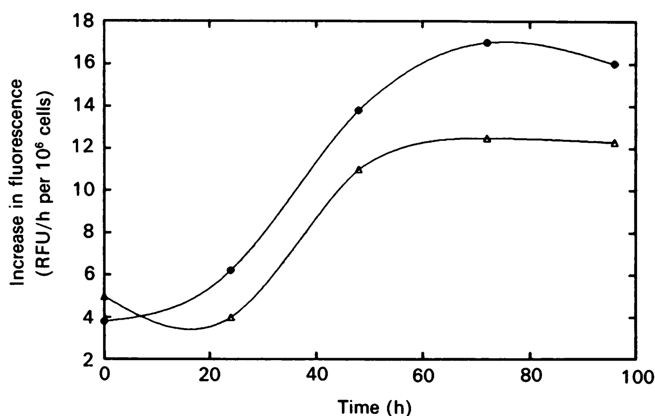

Fig. 2. PBG deaminase and protoporphyrinogen oxidase activity in differentiating DS-19 MEL cells

Activities were determined as described in the text. PBG deaminase (●) and protoporphyrinogen oxidase (△) activities are averages of three determinations. For PBG deaminase S.D. values were less than 10 %, and for protoporphyrinogen oxidase S.D. values at 0 and 24 h were 20 %, and the remaining points were less than 15 %.

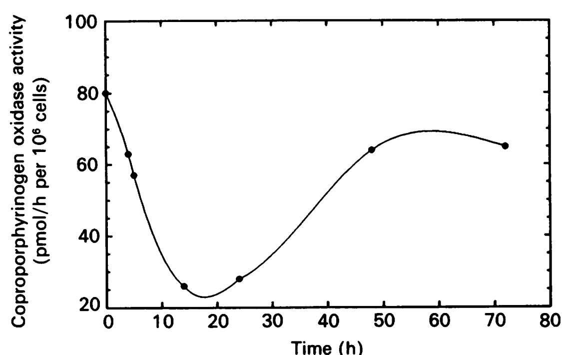

Fig. 3. Coproporphyrinogen oxidase activity in differentiating DS-19 MEL cells

Enzyme activity determinations are described in the text. The data shown are averages of five determinations for times after 12 h and three determinations at 12 h and less.

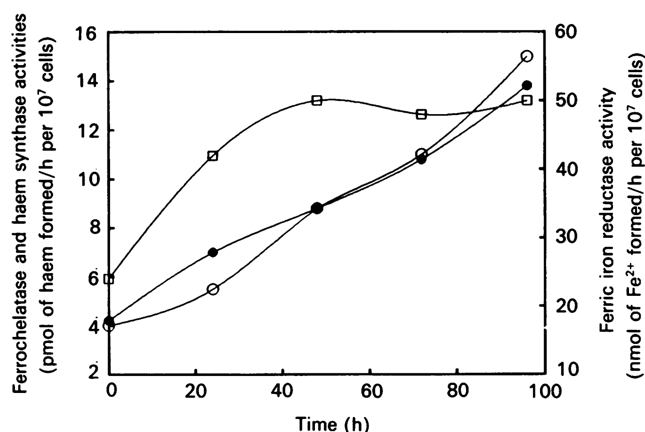

Fig. 4. Ferrochelatase and ferric iron reductase activities during differentiation of DS-19 cells

Enzyme activities were determined as described in the Materials and methods section. Ferrochelatase (●) and haem synthase (○) activities are expressed as pmol of mesohaem formed/h per 10^7 cells. Ferric iron reductase (□) is shown as nmol of Fe^{2+} formed/h per 10^7 cells. Data shown for iron reductase are averages of at least six determinations, and S.D. values are less than ±15%. Ferrochelatase data are from 20 separate determinations and haem synthase data from six determinations: S.D. values are less than ±7%.

Table 1. Rate of ferrochelatase synthesis during MEL cell differentiation

DMSO-induced MEL cells were pulsed at the indicated times for 2 h intervals with 0.25 mCi of [^{35}S]methionine as described in the text. Whole-cell extracts were prepared, immunoprecipitated and analysed by gel electrophoresis and fluorography. The ferrochelatase protein band was scanned by a densitometer and the amount of ferrochelatase was calculated for each post-induction pulse interval. For the zero time point uninduced cells were pulse-labelled for 2 h.

Post-induction time (h)	Immunoprecipitable ferrochelatase formed
0	1.0
12–14	5.3
24–26	3.4
36–38	3.3
48–50	3.3

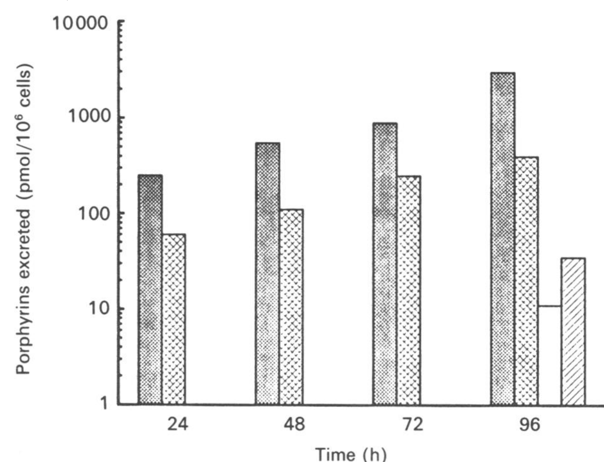

Fig. 5. Porphyrin excretion patterns for DS-19 MEL cells after induction

These data represent the rate (pmol/24 h per 10^6 cells) of porphyrin accumulation in the culture medium. Cells were transferred to fresh media every 24 h, and porphyrins present in the spent medium were extracted and analysed by h.p.l.c. with fluorescence detection as described in the text. Only those porphyrins shown on the Figure were detected, and all values are for porphyrins, since any porphyrinogens that might have been present were oxidized in the extraction procedure. However, since culture conditions are aerobic, it would be expected that any excreted porphyrinogens would be chemically oxidized in the medium. ▩, Coproporphyrin III; ▨, protoporphyrin IX; □, uroporphyrin III; ▧, uroporphyrin I.

clearly show that in DS-19 (and 270) cells ferrochelatase activity increases slowly but steadily over the entire length of the experiment, with an approx. 3.5-fold increase by 96 h.

Since ferrochelatase required ferrous iron and will not use ferric iron [19,28], and because it has been shown that fractions from other mammalian cells can catalyse an NADH-dependent ferrochelatase activity [21,29], this activity, termed haem synthase [20], was monitored to determine if iron reduction for ferrochelatase is rate-limiting. In these determinations detergents were not added to cell extracts, since disruption of the mitochondrial electron-transport chain results in decreased iron reduction [21]. These data (Fig. 4) show that the increase in haem synthase activity parallels that of ferrochelatase. As an independent check of this, the activity of NADH:ferric iron reductase was also determined in differentiating MEL cells. The data indicate that iron reductase activity, although it increases overall about 2-fold, appears always to be in excess of ferrochelatase activity at all points (Fig. 4). Elimination of NADH from the haem synthase assay abolished activity.

The data presented above are on a per-viable-cell basis (as determined by Trypan Blue exclusion). Because DMSO-induced MEL cells eventually cease division and show an actual fall in total protein content [11], the values for these same parameters will be appreciably larger when expressed per mg of protein. In the present study there was a 6-fold decrease in protein concentration over 96 h, with the largest decrease occurring in the first 48 h (Fig. 1).

Induction of ferrochelatase

The data presented above show that ferrochelatase activity increases at a different time and rate from PBG deaminase and protoporphyrinogen oxidase. These data do not demonstrate if the enzyme activity coincides with its synthesis. To determine the time course of the synthesis of ferrochelatase, MEL cells in suspension culture were induced and pulse-labelled with [^{35}S]methionine for 2 h pulses. The synthesis of the protein had

increased 5-fold above uninduced cells 12–14 h after induction. The rate then fell slightly to about 3.4-fold for the remaining 48 h (Table 1).

Porphyrin excretion during MEL cell differentiation

Previously it has been reported that an unquantified amount of protoporphyrin was found in the medium from 707 MEL cells 4 days after induction [12]. No other porphyrins were observed. In the current study we examined porphyrin excretion by both DS-19 and 270 MEL cells and found qualitatively identical excretion patterns for both of these cell lines. The data in Fig. 5 are the rates of porphyrin excretion by DS-19 cells. Porphyrin accumulation in the medium was found, within experimental error, to be additive with respect to the 24 h rates shown. Non-induced porphyrin excretion, which was approx. 10 and 3 pmol/day per 10^6 cells for protoporphyrin IX and coproporphyrin III respectively, was subtracted from these values. Coproporphyrin III is the major porphyrin excreted at all times, although some protoporphyrin IX is found and by 96 h there is a small amount of uroporphyrin I and III excreted. We were unable under any culture conditions to eliminate coproporphyrin or significantly increase the amount of protoporphyrin (results not shown).

DISCUSSION

The data presented above contribute significantly to our understanding of two questions concerning haem biosynthesis: (1) the rate of induction of the enzymes of haem biosynthesis in differentiating MEL cells, and (2) the synthesis and membrane assembly of the terminal three enzymes of the pathway. In regard to this second item, it has been proposed previously that the terminal two or three enzymes of the pathway may be present in a complex on the mitochondrial inner membrane [28,30,31]. If such a complex were a stable, rather than dynamic, one, then it might be expected that there would be some co-ordination between the synthesis and processing of these three enzymes. The current work clearly shows in differentiating MEL cells that this is not the case. Indeed, all three of these enzymes have unique rates of synthesis, as monitored by functional activity, which would not support a model of co-ordinate synthesis and processing.

The other item is a model for the induction of haem synthesis in differentiating MEL cells that is consistent with available data. Previous models have suggested everything from sequential enzyme induction to control at a single step [1,10,12,14]. It now appears most likely that a variety of control mechanisms exists at several stages.

The current data add new pieces to the puzzle of control of the induction of haem synthesis during DMSO-induced differentiation of MEL cells. The rapid rise in protoporphyrinogen oxidase (as compared with PBG deaminase), the decrease in coproporphyrinogen oxidase and the slower increase in ferrochelatase taken together with previously published data clearly demonstrate that this process is not a simple one. The interesting findings by Nordmann's laboratory [9,10] with PBG deaminase in differentiating MEL cells may now be largely explained by the finding from Jordan's group that apo-PBG deaminase requires PBG synthesis *in vivo* to allow production of the enzyme-bound dipyrrole cofactor [32]. In the absence of PBG synthesis (such as occurs when PBG synthase is inhibited) newly synthesized apo-(PBG deaminase) will remain as an inactive precursor which may be subjected to rapid intracellular degradation. Data from Ponka's group, which indicate that the iron status of the cell has a direct bearing on the level of haem produced after induction [33–35], might be explained, at least in part, by the recent discovery of a putative translational iron-regulatory element in the sequence of the erythroid-specific ALA synthase [36]. Taken as a whole, these previous reports would suggest that control of the pathway may be via a number of subtle control elements located throughout the pathway, rather than at a single major site.

Thus at the present time the fluctuations in the temporal levels of enzyme activities measured *in vitro* at all steps in the pathway is known, but what is not known is what controls the induction of these enzymes or whether the activities measured *in vitro* are yielding an accurate picture of actual catalysis rates *in vivo*.

The 20-fold increase in coproporphyrin excretion into the medium after induction probably reflects the increased activity of the early pathway enzymes along with the decreased activity of coproporphyrinogen oxidase. The physiological relevance of this excretion by MEL cells is not known, but the routine finding of coproporphyrin in faeces of healthy humans [2] may be a consequence of normal erythroid development. Interestingly, although secreted protoporphyrin levels are always lower than those of coproporphyrin, the molar ratio of these two remains almost constant. An explanation for this is difficult, since ferrochelatase activity is steadily increasing over the entire time after DMSO induction.

Thus control mechanisms may exist at at least four points: (1) ALA synthase, (2) PBG deaminase, (3) coproporphyrinogen oxidase and (4) ferrochelatase. The current data do not explain how or why coproporphyrinogen oxidase activity falls, nor do they explain why ferrochelatase activity rises so slowly. A possible regulatory role for coproporphyrinogen oxidase in MEL cells, although interesting, is not unique, since there is evidence that the enzyme may serve a similar role in yeast and some photosynthetic bacteria [21].

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REFERENCES

1. Andrew, T. L., Riley, P. G. & Dailey, H. A. (1990) in Biosynthesis of Heme and Chlorophylls (Dailey, H. A., ed.), pp. 163–200, McGraw-Hill, New York
2. Kappas, A., Sassa, S. & Anderson, K. E. (1983) in The Metabolic Basis of Inherited Disease (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L. & Brown, M.S., eds.), pp. 1301–1384, McGraw-Hill, New York
3. Hayashi, N., Terasawa, M., Yamauchi, K. & Kikuchi, G. (1980) J. Biochem. (Tokyo) **88**, 1537–1543
4. Hayashi, N., Watanabe, N. & Kikuchi, G. (1983) Biochem. Biophys. Res. Commun. **115**, 700–706
5. Srivastava, G., Borthwick, I. A., Booker, J. D., May, B. K. & Elliott, W. H. (1982) Biochem. Biophys. Res. Commun. **109**, 305–312
6. Yamamoto, M., Hayashi, N. & Kikuchi, G. (1982) Biochem. Biophys. Res. Commun. **105**, 985–990
7. Yamauchi, K., Hayashi, N. & Kikuchi, G. (1980) J. Biol. Chem. **255**, 1746–1751
8. Marks, P. & Rifkind, R. A. (1978) Annu. Rev. Biochem. **47**, 419–448
9. Beaumont, C., Deybach, J. C., Grandchamp, B., DaSilva, V., de Verneuil, H. & Nordmann, Y. (1984) Exp. Cell Res. **154**, 474–484
10. Grandchamp, B., Beaumont, C., de Verneuil, H. & Nordmann, Y. (1985) J. Biol. Chem. **260**, 9630–9635
11. Parker, D. & Housman, D. (1985) J. Biol. Chem. **260**, 604–609
12. Rutherford, T., Thompson, G. C. & Moore, M. R. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 833–836
13. Sassa, S. (1983) in Regulation of Hemoglobin Biosynthesis (Goldwasser, E., ed.), pp. 359–383, Elsevier, New York
14. Sassa, S. (1976) J. Exp. Med. **143**, 305–315
15. Labbe-Bois, R. & Labbe, P. (1990) in Biosynthesis of Heme and Chlorophylls (Dailey, H. A., ed.), pp. 235–285, McGraw-Hill, New York
16. Fadigan, A. B. & Dailey, H. A. (1987) Biochem. J. **243**, 419–424

17. Sassa, S., Granick, J. L., Eisen, H. & Ostertag, W. (1978) in *In Vitro* Aspects of Erythropoiesis (Murphy, M. J., ed.), pp. 268–270, Springer-Verlag, New York
18. Guerra, F. C. (1974) Methods Enzymol. **31**, 299–305
19. Dailey, H. A., Fleming, J. E. & Harbin, B. M. (1986) Methods Enzymol. **123**, 401–415
20. Dailey, H. A. & Lascelles, J. (1977) J. Bacteriol. **129**, 815–820
21. Barnes, R., Connelly, J. L. & Jones, O. T. G. (1972) Biochem. J. **128**, 103–105
22. Moody, M. D. & Dailey, H. A. (1983) Anal. Biochem. **134**, 234–239
23. Dailey, H. A. & Karr, S. W. (1987) Biochemistry **26**, 2697–2701
24. Anderson, P. M. & Desnick, R. J. (1982) Enzyme **28**, 146–157
25. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fukimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) Anal. Biochem. **150**, 76–85
26. Harbin, B. M. & Dailey, H. A. (1985) Biochemistry **24**, 366–370
27. Bonner, W. M. & Lasky, R. A. (1974) Eur. J. Biochem. **46**, 83–88
28. Dailey, H. A. (1990) in Biosynthesis of Heme and Chlorophylls (Dailey, H. A., ed.), pp. 123–161, McGraw-Hill, New York
29. Barnes, R. & Jones, O. T. G. (1973) Biochim. Biophys. Acta **304**, 304–308
30. Ferreira, G. C., Andrew, T. L., Karr, S. W. & Dailey, H. A. (1988) J. Biol. Chem. **263**, 3835–3839
31. Grandchamp, B., Phung, N. & Nordmann, Y. (1978) Biochem. J. **176**, 97–102
32. Jordan, P. M. (1990) in Biosynthesis of Heme and Chlorophylls (Dailey, H. A., ed.), pp. 55–120, McGraw-Hill, New York
33. Laskey, J. D., Ponka, P. & Schulman, H. M. (1986) J. Cell. Physiol. **129**, 185–192
34. Ponka, P. & Schulman, H. M. (1985) J. Biol. Chem. **260**, 14717–14721
35. Ponka, P., Schulman, H. M. & Cox, T. M. (1990) in Biosynthesis of Heme and Chlorophylls (Dailey, H. A., ed.), pp. 393–434, McGraw-Hill, New York
36. Dierks, P. (1990) in Biosynthesis of Heme and Chlorophylls (Dailey, H. A., ed.), pp. 201–233, McGraw-Hill, New York

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