Haem synthesis from exogenous 5-aminolaevulinate in cultured chick-embryo hepatocytes

Effects of inducers of cytochromes *P*-450

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The effects of inducers of cytochrome *P*-450 on haem biosynthesis from 5-aminolaevulinate were examined by using cultured chick-embryo hepatocytes. Cultures treated with either 2-propyl-2-isopropylacetamide or 3-methylcholanthrene contained increased amounts of cytochrome *P*-450 and haem. After treatment for 3 h with 5-amino[4-¹⁴C]laevulinate, the relative amounts of radioactivity accumulating as haem corresponded to the relative amounts of total cellular haem, but not to increases in the amounts of cytochrome *P*-450. Treatment with 5-aminolaevulinate did not alter cellular haem or cytochrome *P*-450 concentrations in either control or drug-treated cultures. The mechanism of the enhanced accumulation of radioactivity in haem was investigated. Although 2-propyl-2-isopropylacetamide enhanced the uptake of 5-aminolaevulinate and increased the cellular concentration of porphobilinogen 1.5-fold, these changes did not account for the increases in haem radioactivity. The inducing drugs had no effect on the rates of degradation of radioactive haem, but appeared to enhance conversion of protoporphyrin into haem. This latter effect was shown by: (1) a decreased accumulation of protoporphyrin from 5-aminolaevulinate in cells treated with inducers, and (2) complete prevention of this decrease if the iron chelator desferrioxamine was present. We conclude that inducers of cytochrome *P*-450 may increase haem synthesis not only by increasing activity of 5-aminolaevulinate synthase, but also by increasing conversion of protoporphyrin into haem.

INTRODUCTION

A substantial proportion of newly synthesized hepatic haem is incorporated into cytochrome *P*-450 (Meyer & Schmid, 1978). Many chemicals which induce cytochrome *P*-450 also induce 5-aminolaevulinate (ALA) synthase, the first and rate-controlling enzyme of the haem-biosynthetic pathway (for reviews, see De Matteis, 1978; Marks, 1978; Bonkovsky, 1982). It has been assumed that increases in this enzyme result in increased production of haem for incorporation into newly synthesized induced apoprotein.

In preliminary studies we have found that, in chick hepatocyte cultures treated with inducers of cytochrome *P*-450, accumulation of radioactivity in haem from excess [¹⁴C]ALA was increased compared with control cultures. This was an unexpected result, since the excess [¹⁴C]ALA should have bypassed the rate-controlling step in the pathway and equal amounts of haem should have been synthesized. Therefore increased accumulation of radioactivity in haem in induced hepatocytes suggested that steps in the haem-synthetic pathway beyond ALA synthase might become rate-limiting under some conditions and be altered by inducing chemicals. The use of ALA loading to detect limiting steps in the haem pathway has been reported previously (Healey *et al.*, 1981; Sinclair *et al.*, 1983, 1984, 1986a; Ferioli *et al.*, 1984; Doss *et al.*, 1985; Bonkovsky *et al.*, 1986).

In the study reported here, we investigated whether the increased accumulation of radioactivity in haem in induced hepatocyte cultures was due to increases in ALA uptake, to increases in synthetic steps of the pathway beyond ALA synthase, or to decreases in degradation of newly synthesized radioactive haem. We found that changes in ALA uptake and rates of haem breakdown could not account for our findings. Instead, we found that drug treatments increased the rates of conversion of [4-¹⁴C]ALA into haem. Measurement of PBG and porphyrin accumulation in the absence and presence of the iron chelator desferrioxamine indicated that the increases occurred at the final step of haem biosynthesis, namely the conversion of protoporphyrin into haem.

MATERIALS AND METHODS

Hepatocyte cultures and cytochromes *P*-450 induction

Primary cultures from 16-day-old White Leghorn chick embryos were prepared as previously described (Sinclair *et al.*, 1982). For the initial 18 h in culture, cells were maintained in Williams E medium (GIBCO, Grand Island, NY, U.S.A.), to which was added 1 µg of insulin (Sigma, St. Louis, MO, U.S.A.)/ml, 1 µg of tri-iodothyronine (Sigma)/ml, and 0.3 µg of dexamethasone (Organon, West Orange, NJ, U.S.A.)/ml. Cells were then maintained in Williams E medium containing tri-

Abbreviations used: ALA, 5-aminolaevulinate; 3-MC, 3-methylcholanthrene; PBG, porphobilinogen; PIA, 2-propyl-2-isopropylacetamide.

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iodothyronine and dexamethasone, but no insulin. Culture plates (6 cm diameter) were inoculated with 1.4×10^7 cells containing 1.5–1.8 mg of protein.

Culture plates (3.5 cm diameter) and 1 cm diameter wells (on multiwell plates) were inoculated with 5.2×10^6 and 1.3×10^6 cells respectively. For induction of two different isoenzymes of cytochrome *P*-450, cells were exposed for 20 h to either 140 μ M-PIA or 1 μ M-3-MC. PIA was a gift from Hoffmann-La Roche (Nutley, NJ, U.S.A.) and was dissolved in 50% ethanol. 3-MC from Sigma was dissolved in dimethyl sulphoxide. No more than 5 μ l of 50% ethanol or 2 μ l of dimethyl sulphoxide was added/ml of medium.

Analytic techniques

Total haem was measured in cells homogenized in 0.01 M-sodium phosphate 0.15 M-NaCl 1mM-EDTA, pH 7.4, by the pyridine haemochromogen method (Porra & Jones, 1963) using a $\Delta\epsilon_{557-541}$ of 20.7 $\text{mm}^{-1}\cdot\text{cm}^{-1}$. PBG was measured separately in cells and medium as follows: Medium removed from cultures was saved and the cell monolayer rapidly rinsed once with cold 0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.4. Cells were then scraped into 1 ml of 1.2 M-HClO₄, and, after mixing, centrifuged at 250 *g* for 5 min. The supernatant was then added to an equal volume of modified Ehrlich's reagent (Urata & Granick, 1963). The medium was added directly to an equal volume of Ehrlich's reagent and then centrifuged. The chromophore was measured on a Hewlett-Packard 8154A diode-array spectrophotometer, a $\Delta\epsilon_{555-610}$ value of 61 $\text{mm}^{-1}\cdot\text{cm}^{-1}$ being assumed.

Porphyrins were analysed spectrofluorometrically as uro-, copro- and proto-porphyrin by the method of Grandchamp *et al.* (1980). Cells were harvested in 2.3 M-HClO₄/methanol/water (1:1:2, by vol), mixed, and the porphyrins measured in the supernatant after centrifugation (250 *g* for 5 min). Cells and medium together or medium alone were mixed with an equal volume of 2.3 M-HClO₄/methanol (1:1, v/v), centrifuged and the porphyrins analysed. Cytochrome *P*-450 concentrations were determined in 8700 *g* supernatants of detergent-solubilized homogenates as previously described (Sinclair *et al.*, 1979). Proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Radiolabelled ALA experiments

Cells were exposed to either [4-¹⁴C]ALA (52 mCi/mmol) (New England Nuclear, Boston, MA, U.S.A.) or [2,3-³H]ALA (60 Ci/mmol) (Research Products International, Mount Prospect, IL, U.S.A.) for the times indicated in the Figure legends. Unlabelled ALA was added with the labelled ALA to achieve the final concentrations indicated. For assessment of short-term ALA uptake, cells were exposed to the label for various periods of time, after which they were quickly rinsed four times with cold 0.15 M-NaCl to remove the remaining radioactivity in the medium and the cells were scraped with a rubber policeman into 1 ml of water, which was then transferred into scintillation-counting fluid (Aqua-sol; New England Nuclear).

To determine the radioactivity in haem, haem was extracted by using a modification of the method of Healey *et al.* (1981), as follows. After removing the medium, the cell monolayer was rinsed once with 0.15 M-NaCl, and the haem extracted with acetone/conc.

HCl/water/human erythrocyte lysate containing 1.2 mM-haem (25:1.3:4:1, by vol.). A 1.5 ml portion of extracting solution was used for the 3.5 cm-diameter plates and 2 ml for the 6 cm-diameter plates. After the extracts were transferred into glass scintillation vials, the haem was taken into diethyl ether (5 ml) and washed twice with 2 ml of 2 M-HCl to remove porphyrins and ALA. The diethyl ether was evaporated, and radioactivity in haem determined. The recovery of haem from the cell monolayers was $89 \pm 7\%$ (mean $\pm$ S.D., $n = 6$), and the validity of this method of haem extraction has recently been confirmed by h.p.l.c. analysis (Bonkovsky *et al.*, 1986). Radioactivity was determined in a Packard 3255 scintillation counter and converted to d.p.m. by using internal standardization.

All experiments were carried out at least twice. When duplicate culture plates were used, only those experiments whose values differed by $< 10\%$ were accepted. When triplicate plates or more were used, significant differences (P values ≤ 0.05) were determined by using Student's *t*-test (for two groups) or analysis of variance (for three or more groups). To determine significant changes in values which represent differences between the means of two groups, the standard error of the difference of the means was calculated and an approximate *t* test applied according to Satterwhaitte's Approximation (Snedecor & Cochran, 1980).

RESULTS

Cellular haem content of hepatocytes treated with PIA and 3-MC and effects of exogenous ALA exposure

PIA and 3-MC induce different isoenzymes of cytochrome *P*-450 in cultured chick-embryo hepatocytes (Sinclair *et al.*, 1986b). As shown in Table 1, induction of cytochrome *P*-450 by PIA was associated with a 1.7-fold increase ($P < 0.001$) in total cellular haem, and induction by 3-MC was associated with a 1.2-fold increase in haem ($P = 0.028$). The increases in total cytochromes *P*-450 content of these cultures were 3.3–3.8-fold with PIA and 2.3–2.7-fold with 3-MC. In the control cultures, the amount of haem in cytochromes *P*-450 represented only 21% of total haem. But with PIA and 3-MC treatment, the haem associated with cytochromes *P*-450 increased to 41 and 43% of the total haem. As shown in the third column of Table 1, PIA treatment also increased the amount of non-cytochrome *P*-450 haem by almost 23% ($P = 0.05$ for cells not treated with ALA and $P = 0.01$ for cells treated with ALA), whereas treatment with 3-MC did not. Adding 34 μ M-ALA (or 150 μ M-ALA; results not shown) to the cultures for 3 h did not alter the haem or cytochrome *P*-450 levels in the cells.

Accumulation of radiolabelled ALA in haem in cells treated with PIA and 3-MC

As shown in Fig. 1, when the chemically induced cultured hepatocytes were exposed to radiolabelled ALA, radioactivity in haem was increased at 1, 3 and 6 h. The increases in accumulations of radiolabel in haem were similar to the increases in amounts of total cellular haem (i.e. in this experiment there were 2.3–2.4-fold increases in both haem radioactivity and total haem content in the PIA-treated cells 3 h after ALA exposure and 1.4–1.6-fold increases in these values in the 3-MC-treated cells). However, the increases in accumulation of

Table 1. Effects of inducers of cytochrome(s) *P*-450 on total cellular haem in cultured hepatocytes

Cells on 6 cm-diameter plates were left untreated (control), or treated with 140 μ M-PIA or 1 μ M-3-MC for 24 h. For the last 3 h, some cultures were also treated with 34 μ M-ALA as indicated. Total cellular haem and cytochrome *P*-450 levels were determined on separate plates as described in the Materials and methods section. Values are the means $\pm$ s.d. for five to eight plates.

Inducer	Content			
	Haem (pmol/plate)	Cytochrome <i>P</i> -450 (pmol/plate)	Non-cytochrome <i>P</i> -450 haem* (pmol/plate)	Cell protein (mg/plate)
Control	390 $\pm$ 70	80 $\pm$ 8	309 $\pm$ 25	1.34 $\pm$ 0.07
+ ALA	363 $\pm$ 40	74 $\pm$ 10	289 $\pm$ 18	1.29 $\pm$ 0.06
PIA	649 $\pm$ 62	268 $\pm$ 53	380 $\pm$ 29	1.42 $\pm$ 0.05
+ ALA	670 $\pm$ 45	285 $\pm$ 29	385 $\pm$ 22	1.35 $\pm$ 0.06
3-MC	452 $\pm$ 28	193 $\pm$ 37	259 $\pm$ 16	1.32 $\pm$ 0.03
+ ALA	453 $\pm$ 50	201 $\pm$ 37	252 $\pm$ 25	1.24 $\pm$ 0.10

* The 'non-cytochrome *P*-450 haem' is the difference between the means in columns 1 and 2, and the standard error of the difference of the means (Snedecor & Cochran, 1980) is shown.

Fig. 1. Effect of PIA or 3-MC treatment of chick-embryo hepatocytes on accumulation of [14 C]ALA in cellular haem

Cells on 6 cm-diameter plates were either untreated (○), or treated with 140 μ M-PIA (△) or 1 μ M-3-MC (□) for 20 h and then 0.25 μ Ci of [14 C]ALA (final concn. 34 μ M) was added. At various times indicated, cultures were rinsed and the cellular haem extracted as described in the Materials and methods section. Each point is the mean $\pm$ s.d. for three plates. The haem and cytochrome *P*-450 levels in parallel cultures treated identically but exposed to unlabelled ALA for 3 h were: control, 296 pmol of haem and 84 pmol of cytochrome *P*-450/plate; for PIA, 676 and 307; and for 3-MC, 422 and 229 respectively.

radioactivity in haem in the induced hepatocytes were less than the increases in cytochromes *P*-450.

Effect of exogenous ALA concentration on radiolabelled ALA conversion into haem

PIA treatment of cultured chick hepatocytes increases activity of ALA synthase (Shedlofsky *et al.*, 1983), leading to increases in pools of unlabelled ALA and haem precursors which would dilute radiolabelled-haem-precursor pools after exposure to [14 C]ALA. It was therefore important to determine whether changing the exogenous ALA concentration would affect the accumulations of the radiolabel in haem. As shown in Table 2, when concentrations of exogenous ALA of less than 18 μ M were used, no increase in radiolabel accumulation in haem in the induced cells occurred. With 0.013 μ M-ALA there was even less radiolabel found in the haem of PIA-treated cells than in that of control cells, and the specific radioactivity of the haem was less than 50% of the control value. In cells exposed to 1.4 or 2.3 μ M-ALA, the specific radioactivities of the haem in the treated cells were still less than the control values. These findings most likely resulted from dilution of the exogenous radiolabel by endogenous unlabelled haem-precursor pools, but might also be due to some other effect of maintaining less-than-maximal substrate concentrations along the pathway and converting fewer total equivalents of the [14 C]ALA into haem. By using an ALA concentration of 18 μ M or more, however, effects of dilution and low substrate concentration apparently were overcome, and the increased accumulations of radiolabel in haem in the treated cells were the same as the increases in total cellular haem. At 18 μ M- and 34 μ M-ALA, the specific radioactivities of the haem in control and induced hepatocytes became identical (Table 2).

Having demonstrated that cytochrome *P*-450 inductions are associated with increases in both total hepatocyte haem and accumulation of radiolabel in haem from [14 C]ALA, we next investigated the possible reasons for this latter effect. Since the accumulation of radiolabel in

Table 2. Effect of exogenous ALA concentration on accumulation of radiolabelled ALA in haem in hepatocytes treated with inducers of cytochrome P-450

Cells on 6 cm-diameter plates were either left untreated (control) or induced with 140 μM -PIA or 1 μM -3-MC for 24 h. For the last 3 h in culture, cells were exposed to radiolabelled ALA at the final medium concentration indicated. (Unlabelled ALA was added to the radiolabelled ALA to achieve these concentrations.) At the end of incubation, haem was extracted and the radioactivity in haem determined as described in the Materials and methods section. The [^3H]ALA values are from one experiment performed with duplicate plates, and the [^{14}C]ALA values are from another experiment performed with triplicate plates. Maximum variation was < 10%. Haem specific radioactivities were calculated from haem determinations performed in the [^{14}C]ALA experiment (see the legend to Fig. 1.)

[ALA] (μM)	Isotope	Radioactivity ($\mu\text{Ci}/\text{plate}$)	10 ⁻³ × Radioactivity in haem (d.p.m./plate)			Specific radioactivity (d.p.m./pmol of haem)		
			Control	PIA	3-MC	Control	PIA	3-MC
0.013	^3H	3.9	385.0	316.0	—	1301.0	467.0	—
1.4	^{14}C	0.25	22.5	24.0	24.3	76.0	35.5	57.6
2.3	^3H	3.9	278.0	27.0	—	939.0	410.0	—
18.0	^{14}C	0.25	4.6	11.0	7.2	15.5	16.3	17.1
34.0	^3H	3.9	36.0	83.0	—	122.0	123.0	—
34.0	^{14}C	0.25	2.6	6.1	4.1	8.8	9.0	9.7

haem is a function of several factors, including the cellular uptake of the ALA, its conversion into haem, and the stability of the labelled haem, we studied the effects of the two chemical inducers on each of these processes.

Effects of PIA and 3-MC treatment upon uptake of exogenous ALA

With a concentration of 34 μM radiolabelled ALA the effects of PIA and 3-MC on the initial uptake of the radiolabel were investigated. As shown in Fig. 2, there was linear uptake of the radiolabel for 20 min in all cultures, with some decline in the rate of uptake at 30 min, similar to the findings of Israels *et al.* (1974) in chick hepatocyte suspensions. Pretreatment with 3-MC had no effect on this initial uptake, but treatment of cells with PIA increased the rate of ALA uptake by 50% over that of the control and 3-MC-treated cells. This increased uptake was also seen at ALA concentrations of 0.04, 1.6 and 150 μM (results not shown). With 34 μM -ALA, by 30 min, the proportion of the exogenous ALA taken up by the cells was 0.54, 0.86 and 0.54% for the control, PIA-treated and 3-MC-treated cells respectively, so that exogenous ALA concentrations were not affected during the experiment.

PBG accumulations in control and induced hepatocytes

Since the exogenous ALA taken up would be converted into PBG, the next intermediate in the haem pathway, we analysed the effects of both PIA and 3-MC treatment on PBG levels to see if alterations in PBG formation and/or accumulation might explain the increase in radioactive haem accumulations. PBG was measured in cells and in the overlying medium for 3 h after exposure to 34 μM -ALA (Fig. 3). PBG could not be detected in cells or medium without added ALA, nor could PBG be detected in the medium until 2 h after ALA addition. Although cellular PBG and total PBG in the culture increased with time, the increases in cellular PBG were not linear, and

Fig. 2. Cellular uptake of [^{14}C]ALA by cultured hepatocytes

Cells on 3.5 cm-diameter plates were either untreated (○) or treated with 140 μM -PIA (△) or 1 μM -3-MC (□) for 20 h and then exposed to 0.25 μCi of [^{14}C]ALA at a final medium concentration of 34 μM . At the times indicated after adding the label, the radioactive culture medium was removed and the cells quickly rinsed four times with 2 ml of cold 0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.4. The last rinse contained < 200 d.p.m. The radioactivity in the cells was determined as described in the Materials and methods section. Each point is the mean for duplicate plates with the range indicated. Cell protein determined on parallel plates treated with unlabelled ALA was 0.53, 0.55, and 0.49 mg protein for the control, PIA- and 3-MC-treated cultures respectively.

by 2 h at least 14–22% of the PBG was found in the medium.

From 30 min to 3 h after ALA exposure, the amount of PBG in PIA-treated cultures was 30% higher than in the control and 3-MC-treated cultures, a finding which agrees with the increased [^{14}C]ALA uptake seen in the PIA-treated cells (Fig. 2). Table 3 shows that increasing the ALA concentration from 34 to 150 μM approximately doubled total PBG concentrations in control and induced cultures.

Porphyrin accumulations in control and induced hepatocytes

To assess the next intermediates in the haem pathway, porphyrins were analysed in cultures after ALA exposure. The amounts of total porphyrin which accumulated in control and treated cultures in 3 h are shown in Table 3. Increasing the exogenous ALA from 34 to 150 μM did not change the amounts of porphyrins. This finding confirms that 34 μM -ALA led to accumulation of cellular PBG to concentrations above the K_m of PBG deaminase, thereby providing for maximal rates of porphyrin and haem synthesis.

Table 3 also shows that both PIA and 3-MC treatment substantially lowered the amounts of porphyrins which accumulated after 3 h. Shown in Fig. 4 are the time courses of accumulation of each of the individual porphyrin types in control, PIA-treated and 3-MC-treated cultures. These patterns of porphyrin accumulation indicate that the decreases in total porphyrins found in the induced cultures (Table 3) are due to decreases in protoporphyrin accumulation, especially marked with PIA treatment, a phenomenon which has been noted previously in chick hepatocytes (Ferioli *et al.*, 1984; Bonkovsky *et al.*, 1986). Also shown in Fig. 4 is an increase in the amounts of uroporphyrin, which accumulated with both inducers, as reported previously with various inducers of cytochrome *P*-450 (de Verneuil *et al.*, 1983; Marks *et al.*, 1983; Ferioli *et al.*, 1984; Bonkovsky *et al.*, 1986; Sinclair *et al.*, 1986a).

To see whether the same amount of protoporphyrin would accumulate in induced cultures as in control cultures if conversion of the protoporphyrin to haem was blocked, the iron chelator desferrioxamine was added, together with the exogenous ALA. As shown in Fig. 5, when cultures were also exposed to 178 μM -desferrioxamine, the PIA- and 3-MC-treated cells accumulated the same amount of protoporphyrin as control cells. However, desferrioxamine did not affect the increased

Fig. 3. PBG in cultured hepatocytes exposed to exogenous ALA

Cells on 3.5 cm-diameter plates were left untreated ($\circ, \bullet$) or induced with 140 μM -PIA ($\triangle, \blacktriangle$) or 1 μM -3-MC ($\square, \blacksquare$) for 20 h and then exposed to 34 μM -ALA in the culture medium. At the times indicated, cellular and medium PBG levels were determined as described in the Materials and methods section. PBG was not detected in cultures not exposed to ALA. Each point is the mean for duplicate plates whose values differed by < 10%. The solid symbols and lines represent total (cells+medium) PBG, and the open symbols and lines represent cellular PBG only.

accumulation of uroporphyrin. This concentration of desferrioxamine, when added to control cultures immediately before their exposure to 34 μM -[^{14}C]ALA, decreased the 3 h accumulation of radiolabel in haem by 51%.

Stability of radiolabelled haem in control and induced hepatocytes

After radiolabelling of haem, its degradation can be monitored by removing the exogenous [^{14}C]ALA and diluting the remaining labelled haem-precursor pools with unlabelled ALA (150 μM). Preliminary experiments revealed that labelling haem with 34 μM -[^{14}C]ALA led to intracellular labelled-haem-precursor pools that could

Table 3. Comparison of PBG levels with porphyrin levels in hepatocyte cultures exposed to exogenous ALA

Cells on 3.5 cm-diameter plates were left untreated (control) or induced with 140 μM -PIA or 1 μM -3-MC for 20 h, and then either 34 μM or 150 μM exogenous ALA was added to the medium. After 3 h the cultures were harvested (cells and medium together) and analysed for either PBG or porphyrins as described in the Materials and methods section. Values are means $\pm$ S.D. for triplicate plates.

[ALA] (μM)	Total PBG (pmol/mg of protein)			Total porphyrins (pmol/mg of protein)		
	Control	PIA	3-MC	Control	PIA	3-MC
34	2770 $\pm$ 250	3820 $\pm$ 260	2640 $\pm$ 140	581 $\pm$ 20	353 $\pm$ 12	506 $\pm$ 27
150	5540 $\pm$ 50	6700 $\pm$ 270	4660 $\pm$ 40	685 $\pm$ 55	317 $\pm$ 17	540 $\pm$ 32

Fig. 4. Porphyrins in cultured hepatocytes exposed to exogenous ALA

Cells in 1 cm-diameter wells were left untreated (○) or induced with 140 μM -PIA (Δ) or 1 μM -3-MC ($\square$) for 20 h and then exposed to 34 μM -ALA in the culture medium. At the times indicated after ALA exposure, cultures were analysed for porphyrins as described in the Materials and methods section. Values are means for duplicate wells and values differed by < 10%.

not be effectively diluted by the chase with unlabelled ALA. Therefore 1.6 μM - $[^{14}\text{C}]$ ALA was used for haem labelling. As shown in Fig. 6, the rates of radiolabelled haem loss between 30 min and 3 h after stopping label incorporation into haem were first-order and virtually identical for both control and induced cells. Since only 1.6 μM exogenous $[^{14}\text{C}]$ ALA was used in this experiment, the specific radioactivities of the haem in PIA- and 3-MC-treated cells were only approx. 45 and 76% of the specific radioactivity of the haem in control cells (see Table 2; specific-radioactivity values for the 1.4 μM - $[^{14}\text{C}]$ ALA). Therefore the absolute rates of haem degradation (pmol/h) in the PIA- and 3-MC-treated cells were actually 2.6-fold and 1.6-fold that of the control respectively. Clearly haem degradation was not less in the induced cells, and decreased haem degradation could not account for the increased radiolabelled haem accumulation seen with cytochrome *P*-450 inductions.

DISCUSSION

The results of the present study show that treatment of chick-embryo hepatocyte cultures with PIA or 3-MC,

Fig. 5. Porphyrins in cultured hepatocytes exposed to exogenous ALA and desferrioxamine (DES)

Cells in 1 cm-diameter wells were treated and analysed as described in Fig. 4, except that the iron chelator desferrioxamine (178 μM) was added with the ALA.

two chemicals that induce different forms of cytochrome *P*-450 (Althaus *et al.*, 1979; Sinclair *et al.*, 1979; Sinclair *et al.*, 1986b), both increased the accumulations of radioactivity from $[^{14}\text{C}]$ ALA in haem (Fig. 1). The increased accumulations of radioactivity were in association with increases in the concentrations of total cellular haem and cytochrome *P*-450 haem (Table 1). For most of our experiments we used a sufficiently high dose of exogenous ALA to 'swamp' any endogenous haem-precursor pools that would complicate data interpretation and, by so doing, assured maximal saturation of the haem pathway with substrate (Table 2). We considered three possible explanations for the increased labelling of haem from $[^{14}\text{C}]$ ALA in the drug-treated cultures, namely that drug treatments (1) increased cellular uptake of ALA, (2) increased conversion of ALA into haem, and/or (3) decreased the rate of haem breakdown. We now consider the evidence for and against each of these possibilities.

(1) Drug treatments increase cellular uptake of ALA

As shown in Fig. 2, the initial rate of uptake of $[^{14}\text{C}]$ -ALA by cultures treated with PIA was greater than the uptake by control or 3-MC-treated cultures. Since 3-MC treatment produced increased haem labelling without increasing ALA uptake, it seems that changes in ALA uptake alone cannot account for our findings. However,

the greater increase in cellular ALA uptake produced by PIA than by 3-MC might help to account for the greater haem labelling at high concentrations of ALA in the PIA-treated than in the control or 3-MC-treated cultures (Fig. 1). To play a rate-controlling role, however, ALA uptake would need to be the rate-limiting step in the pathway from exogenous ALA to cellular haem. The data of Table 3 and Figs. 3–5 show that the rate of conversion of PBG into porphyrinogens (or porphyrins) is the rate-limiting step, since large quantities of PBG accumulate, with or without treatment with PIA or 3-MC. The conclusion that PBG deaminase activity is rate-limiting for conversion of ALA into porphyrins and haem is in agreement with our studies (Healey *et al.*, 1981) and those of others (Doss *et al.*, 1985) using rat liver homogenates and chick hepatocyte cultures. Regardless of drug treatment, there was little additional porphyrin accumulation when the ALA concentration was increased from 34 to 150 μM (Table 3), indicating that 34 μM -ALA provides sufficient PBG to saturate PBG deaminase. Thus we conclude that increased uptake of [^{14}C]ALA into hepatocytes cannot account for the increased accumulation of radioactivity in haem in the drug-treated cultures.

(2) Drug treatments increase the rate of conversion of ALA into haem

For the reasons just described, this possible explanation can be thought of as an increased rate of PBG conversion into haem. The effect of the drug treatments to decrease porphyrin accumulations (Table 3 and Fig. 4) might at first suggest that there was decreased conversion of PBG into haem, particularly with PIA treatment. However, in the presence of desferrioxamine, a chelator of Fe^{3+} that blocks conversion of protoporphyrin to haem, the rates of accumulation of protoporphyrin became the same for both control and drug-treated hepatocytes (Fig. 5). In spite of the increased accumulation of uroporphyrin in the drug-treated cells, finding nearly equal amounts of protoporphyrin in control and drug-treated cells in the presence of desferrioxamine indicates that the drug treatments did not substantially impede the conversion of ALA or PBG into protoporphyrin. The lower accumulations of protoporphyrin in the drug-treated (especially the PIA-treated) cultures in the absence of desferrioxamine must therefore be the result of increased conversion of protoporphyrin into haem.

The increase in uroporphyrin accumulation observed in PIA- and 3-MC-treated cultures (Figs. 4 and 5) agrees with previous observations (Marks *et al.*, 1983; de Verneuil *et al.*, 1983; Ferioli *et al.*, 1984; Sinclair *et al.*, 1986; Bonkovsky *et al.*, 1986). This accumulation occurs despite the increases in the amounts of cellular haem (Table 1), and therefore could not represent a substantial inhibition of the haem pathway at uroporphyrinogen decarboxylase. The increased accumulation of uroporphyrin is perhaps due to the oxidation of uroporphyrinogen, the substrate of uroporphyrinogen decarboxylase, to uroporphyrin, which is not a substrate for the decarboxylase. Other data, previously discussed (Sinclair *et al.*, 1986), suggest that uroporphyrinogen oxidation in chick hepatocyte cultures can be caused by action of some species of cytochrome *P*-450, perhaps by forming a reactive oxygen radical.

Fig. 6. Haem degradation in control hepatocytes and hepatocytes treated with PIA and 3-MC

Cells on 3.5 cm-diameter plates were left untreated (○) or induced with 140 μM -PIA (△) or 1 μM -3-MC (□) for 20 h. After removing and saving 1.5 ml of the medium from each plate, 0.125 μCi of [^{14}C]ALA was added to the remaining 1.5 ml of medium at a final concentration of 1.6 μM -ALA. After 3 h of labelling, the radioactive medium from each culture was removed, the cells quickly rinsed twice with 2 ml of 37 °C 0.01 M-sodium phosphate/0.15 M-NaCl, pH 7.4, and the original (saved) medium 'spiked' with 150 μM unlabelled ALA re-added. The cultures were then incubated at 37 °C for the times indicated, and the cellular haem extracted and counted for radioactivity as described in the Materials and methods section. Each point represents the mean $\pm$ S.D. for triplicate plates. Best-fit lines were determined by linear-regression analysis. The slopes of the lines were: control, -2740 d.p.m./h per mg; PIA, -3329 d.p.m./h per mg; and 3-MC, -3219 d.p.m./h per mg.

(3) Drug treatments decrease the rate of haem catabolism

In addition to an increased conversion of protoporphyrin to haem, drug treatments might have also decreased the rate of haem breakdown. However, as shown in Fig. 6, during the first 3 h after the labelling period, the rates of loss of radioactivity from haem were first-order and the same for control and drug-treated hepatocytes. Since drug-treated cultures contained more haem than did control cultures (Table 1), these results imply that the total amounts of haem degraded in drug-treated cultures were greater than in controls. From the data in Fig. 6 and the haem specific radioactivities for 1.4 μM -[^{14}C]ALA in Table 2, the absolute rates of haem breakdown are calculated to be 36, 94 and 56 pmol of haem/h per mg of protein for the control, PIA-treated and 3-MC-treated cultures respectively. We can therefore conclude that the increase in radioactivity in haem produced by drug treatments cannot be ascribed to a decrease in the fractional or absolute rates of haem catabolism.

Thus our results establish that, in addition to several other previously described effects of PIA and 3-MC on cultured chick hepatocytes, these drugs also increase the rate of haem formation from protoporphyrin in the intact cells. This mechanism of increased haem formation is likely to be important for increasing haem synthesis

during cytochrome *P*-450 inductions. This last point is strengthened by our previous finding that 3-MC does not increase ALA synthase activity in cultured chick hepatocytes (Shedlofsky *et al.*, 1984), even though the present study demonstrates increased cellular haem content after 3-MC treatment. Perhaps PIA and 3-MC increase the activity of ferrochelatase, as has been reported for phenobarbital-treated rats (Hasegawa *et al.*, 1970) and isolated rat hepatocytes (Canepa *et al.*, 1984), or perhaps they increase availability of Fe²⁺ at the mitochondrial site of ferrochelatase action.

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