Inhibition by Cycloheximide of Degradation of Cytochrome *P*-450 in Primary Cultures of Adult Rat Liver Parenchymal Cells and *in vivo*

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Degradation of cytochrome *P*-450 was studied in adult rat liver parenchymal cells in primary monolayer culture. In cells incubated in standard culture medium, the amount of cytochrome *P*-450 decreased at an accelerated rate relative to either the rate of degradation of total protein in the cells or the turnover of cytochrome *P*-450 *in vivo*. This change was succeeded by a spontaneous increase in the activity of haem oxygenase, an enzyme system that converts haem into bilirubin *in vitro*, measured in extracts from the cultured cells. This finding suggests that the rate of cytochrome *P*-450 breakdown may be controlled by factor(s) other than the activity of haem oxygenase. The decline in cytochrome *P*-450 and the subsequent increase in haem oxygenase activity was prevented by incubation of hepatocytes in medium containing an inhibitor of protein synthesis such as cycloheximide, puromycin, actinomycin D, or azaserine. The effect of cycloheximide appeared to be due to decreased breakdown of microsomal ¹⁴C-labelled haem. By contrast, cycloheximide was without effect on the degradation of total protein, measured either in homogenates or in microsomal fractions prepared from the cultured cells. These results suggest that the conditions of cell culture stimulate selective degradation of cytochrome *P*-450 by a process that is inhibited by cycloheximide and hence may require protein synthesis. The findings in culture were verified in parallel studies of cytochrome *P*-450 degradation *in vivo*. After administration of bromobenzene, the degradation of the haem moiety of cytochrome *P*-450 was accelerated *in vivo* in a manner resembling that observed in cultured hepatocytes. Administration of cycloheximide to either bromobenzene-treated rats or to untreated rats decreased the degradation of the haem moiety of cytochrome *P*-450. However, the drug failed to affect degradation of haem not associated with cytochrome *P*-450, suggesting that cycloheximide is not a general inhibitor of haem oxidation in the liver. These findings confirm that the catabolism of hepatic cytochrome *P*-450 haem is controlled by similar cycloheximide-sensitive processes in the basal steady state *in vivo*, as stimulated by bromobenzene *in vivo*, or in hepatocytes under the conditions of cell culture. We conclude that the rate-limiting step in this process appears to require protein synthesis and precedes cleavage of the haem ring.

The endoplasmic reticulum of the liver contains a family of haemoproteins, known collectively as cytochrome *P*-450, which function in, and in some instances may limit, the oxidation of a wide variety of compounds, including drugs and carcinogens, as well as endogenous lipophilic substances. The amount of cytochrome *P*-450 itself is subject to modification by numerous factors, including drugs. For example, cytochrome *P*-450 increases after administration of such drugs as phenobarbital to the intact animal (Remmer & Merker, 1965), largely due to an increase in the synthesis of haemoprotein (Greim *et al.*, 1970). Decreases in cytochrome *P*-450 in the liver have been observed in a variety of pathological or abnormal physiological states (Kato, 1977), including severe hepatitis and cirrhosis (Schoene *et al.*, 1972), hepatoma (Miyake *et al.*, 1974), and deficiency of protein

(Anthony, 1973), vitamin A (Becking, 1973) or ascorbic acid (Zannoni *et al.*, 1972). It is not known whether these decreases are due to decreased synthesis or increased degradation of the haemoprotein (or both).

Turnover of cytochrome *P*-450 *in vivo* has generally been studied by measuring the disappearance of radioactive label from the haem moiety, the assumption being that metabolism of the haem prosthetic group reflects metabolism of the holocytochrome. This assumption is necessary because practical methods for measuring the individual apoprotein forms of cytochrome *P*-450 are not available. However, labelling of haem possesses several advantages over labelling of protein for this kind of study, because the labelled compound (δ -aminolaevulinic acid) is a highly specific haem precursor and because

the radioactive haem is converted into equimolar quantities of bilirubin and carbon monoxide, end-products that are not reutilized.

These studies have demonstrated that turnover of cytochrome *P*-450 in the basal steady state [approx. 50% of hepatic cytochrome *P*-450 in 24 h (Greim *et al.*, 1970; Levin & Kuntzman, 1969; Meyer & Marver, 1971)] is rapid compared with the turnover of other major haemoproteins in the liver cell [mitochondrial cytochromes and microsomal cytochrome *b*₅ (Marver & Schmid, 1972)]. This suggests that the amounts of cytochrome *P*-450 may be altered rapidly by changes in the rate of its degradation, as has been shown for many short-lived enzymes (Goldberg & Dice, 1974). However, little is known about the mechanisms of degradation of cytochrome *P*-450 or of their control, because until recently there have been few experimental models developed in which cytochrome *P*-450 is affected predominantly by changes in its rate of degradation. These models include treatment of rats with bromobenzene (Guzelian & Bissell, 1976*a,b*), cobalt (Maines & Kappas, 1975), or endotoxin (Bissell & Hammaker, 1976*a*), and dietary-induced selenium deficiency in rats (Correia & Burk, 1976).

It has been reported that adult rat hepatocytes maintained in primary monolayer culture (Bissell *et al.*, 1973) demonstrate a striking selective loss of cytochrome *P*-450 during the first 24 h of incubation (Bissell & Guzelian, 1975; Guzelian & Bissell, 1976*b*; Michalopoulos *et al.*, 1976*a*; Guzelian *et al.*, 1977). The sustained viability of the liver cells in culture, which has been documented previously (Bissell *et al.*, 1973; Bissell & Guzelian, 1975; Bissell, 1976; Guzelian & Bissell, 1976*b*; Guzelian *et al.*, 1977), provides an opportunity to study the control of cytochrome *P*-450 degradation under defined conditions *in vitro*. In the present paper we show that the loss of cytochrome *P*-450 in culture involves, as a major component, accelerated degradation. In examining the regulation of cytochrome *P*-450 degradation, we have found that the process is sensitive to inhibitors of protein synthesis. These investigations in culture have been confirmed by supplementary studies of the degradation of cytochrome *P*-450 *in vivo*.

Experimental

Primary culture of adult-rat liver parenchymal cells in monolayer

The method for preparation of monolayer cultures of hepatocytes, described previously (Bissell *et al.*, 1973), was used with minor modifications. In brief, 4 or 5 days after a partial (two-thirds) hepatectomy, the regenerated liver was perfused *in situ* with 0.03% buffered collagenase solution. Hepatocytes were separated from other cellular components of the liver

by centrifugation, and were placed in plastic tissue-culture dishes (60mm diam.) in a final volume of 3.0ml of chemically defined medium. Inoculation of plates with 5.0×10^6 parenchymal cells leads to formation of a viable monolayer in 12–18 h. In a few experiments (see Tables 2 and 3 below), culture dishes pre-coated with rat tail collagen were used as recommended by Bonney *et al.* (1974). This modification had little effect on amounts of cytochrome *P*-450 after 24h of incubation and therefore was not used routinely in these studies. The standard medium, termed 'L-16,' contains amino acids, salts and vitamins, and is free of protein, including serum and hormones (Guzelian & Bissell, 1976*b*). Substances were added to the L-16 medium directly, and the solution was sterilized by passage through a Millipore filter (0.22 μ m pore size).

Experimental approach

All experiments with cultured cells were carried out during the first 48 h after plating of hepatocytes in culture medium, because changes in cytochrome *P*-450 occur largely during this time interval. In each experiment, freshly isolated hepatocytes were prepared from a single donor liver and were seeded with plates containing control medium or, in some instances, modified culture media. Cultures were terminated at various times of incubation according to protocol, and the cells were analysed by one of the procedures below.

Materials

Male Sprague-Dawley rats weighing 220–280g were purchased from Flow Laboratories, Dublin, VA, U.S.A. and were housed in wire-bottomed cages with free access to chow and water. Collagenase type I was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; Eagle's Minimal Essential Vitamins (100 $\times$) from Grand Island Biological Company, Grand Island, NY, U.S.A.; bromobenzene from Eastman Chemical Company, Rochester, NY, U.S.A.; [¹⁴C]leucine and [¹⁴C]valine were obtained from New England Nuclear, Boston, MA, U.S.A.; δ -amino[5-¹⁴C]laevulinic acid was purchased from either New England Nuclear, or from R.P.I. Co, Elk Grove Village, IL, U.S.A., with specific radioactivity of 50–60mCi/mol. Other chemicals were of the highest purity available commercially.

Assay procedures

For cultures incubated for 12h or longer, the medium was removed and the monolayer was washed twice with iced phosphate-buffered saline (4mM-potassium phosphate in 0.14M-NaCl, pH7.4) and scraped into 0.1M-potassium phosphate buffer,

pH 7.4. The cells were disrupted by sonication (Bissell *et al.*, 1973) and centrifuged for preparation of 10000g supernatant or microsomal fractions as described previously (Bissell *et al.*, 1973). By comparison with disruption of cells by rapid decompression in a nitrogen bomb, sonication had no effect on the amount of cytochrome P-450 or haem oxygenase activity. Cells in culture for less than 12 h are incompletely attached to the plates and may be lost by washing; therefore an alternative procedure was used that consisted of scraping the cells directly into the culture medium of the dish and transferring the cells plus medium to iced centrifuge tubes. The cells were sedimented at 600g for 2 min, and the medium was removed. The cell pellet was suspended in phosphate-buffered saline and re-sedimented twice and finally resuspended in 0.1 M-potassium phosphate buffer. During the first 24 h of incubation, recovery of cells, as judged by measurement of DNA, ranged between 75 and 85% of the initial inoculum.

Protein degradation in cultured hepatocytes. Donor animals were labelled with [^{14}C]valine and the labelled livers were perfused with collagenase solution containing 10 mM-valine to impede reincorporation of the labelled amino acid into protein. The cells were placed in culture, and at various times thereafter the monolayers were washed, removed from the plate and disrupted by sonication for preparation of cell homogenates and microsomal fractions. The protein in the cell lysates or microsomal fractions was precipitated with chilled trichloroacetic acid (10%, w/v, final concn.), and was centrifuged at 1000g for 5 min. The precipitate was washed three times in 5 mM-valine in 5% trichloroacetic acid and finally dissolved in 0.6 ml of 0.2 M-NaOH with 2% (w/v) Na_2CO_3 . Portions of this solution were combined with 2.0 ml of Soluene 350 (Packard Instrument Company, Downers Grove, IL, U.S.A.) and incubated at 50°C for 16 h to digest the protein completely. Radioactivity was counted with addition of 15 ml of Dimilume (Packard). Efficiency was 82–93% by external standardization. Results were expressed as the ratio of the total d.p.m. recovered in the trichloroacetic acid precipitate from either homogenate or microsomal fractions to the total amount (μg) of DNA in the homogenate.

Analysis of hepatic [^{14}C]haem catabolism *in vivo* by ^{14}CO excretion. In the standard protocol, rats weighing 180–200 g with free access to food and water received an intraperitoneal injection of 5 μCi of δ -amino[5- ^{14}C]laevulinic acid, to label hepatic haem with negligible labelling of erythrocyte haem. After 16–20 h, when degradation of free, rapidly exchangeable haem pools was completed (Bissell & Hammaker, 1976a), each of the rats was placed in a separate metabolic cage, and the exhaled breath was collected by a slight modification of the system described previously (Guzelian & Bissell, 1976b). Expired $^{14}\text{CO}_2$ was

removed by passage through soda lime ($\text{CaO}/\text{NaOH}/\text{H}_2\text{O}$). The ^{14}CO that remained was converted by heated Hopcalite into $^{14}\text{CO}_2$ and in this form was trapped in 4 ml of ethanolamine/methoxyethanol (1:1, v/v). A portion (1 ml) of this solution was counted for radioactivity as described previously (Guzelian & Bissell, 1976b). Air flow through the system was maintained at 2.5 litres/min by using a flowmeter connected to a vacuum pump. Rats were studied in pairs in parallel CO -collecting systems to permit comparisons of ^{14}CO excretion between treated and control animals. The observed rate of ^{14}CO excretion was corrected for the efficiency of ^{14}CO recovery, which was always greater than 85%. This was determined after each experiment by releasing into each cage a known quantity of ^{14}CO formed by addition of [^{14}C]formate to sulphuric acid. Detailed studies of this protocol (Bissell & Hammaker, 1976a) have provided evidence for the assumption that labelled cytochrome P-450 haem in the liver is the major source of ^{14}CO excretion. Although renal [^{14}C]haem contributes a minor fraction of basal ^{14}CO excretion, bromobenzene and endotoxin have no effect on renal [^{14}C]haem content (P. S. Guzelian, unpublished work). Thus the observed changes in ^{14}CO excretion after bromobenzene treatment may reasonably be inferred to arise from degradation of labelled hepatic haem.

Cytochrome P-450 was measured as the CO -binding haemoprotein in the dithionite-reduced-minus-oxidized difference spectrum as described previously (Guzelian & Bissell, 1976b). Extraction and crystallization of labelled haem (Labbe & Nishida, 1957) was carried out with rat erythrocytes as haem carrier as previously described (Guzelian & Bissell, 1976b). Protein was measured by the method of Schacterle & Pollack (1973), with crystalline bovine albumin as standard. Published procedures were used for measurement of microsomal haem oxygenase activity (Tenhunen *et al.*, 1969) and DNA content (Burton, 1956).

Results

Effect of cell-culture conditions on amounts of cytochrome P-450

We have documented previously that in adult rat hepatocytes incubated in the standard culture medium for 24 h, the concentration of cytochrome P-450 falls to approx. 20% of that present in the liver *in vivo* (Guzelian & Bissell, 1976b; Guzelian *et al.*, 1977). The time course of this change occurred reproducibly. The decrease in the amount of cytochrome was detectable 4–6 h after plating of the cells, proceeded at a relatively rapid rate for a subsequent 8 h period, then at a more gradual rate after 12 h of incubation (Fig. 1). The decrease in the amount of cytochrome P-450 from

Fig. 1. Effect of cycloheximide on the loss of cytochrome in cultured hepatocytes

Isolated hepatocytes prepared from a single rat liver were plated in either control medium or in medium containing cycloheximide ($10\mu\text{M}$). At the indicated times, the cells pooled from three dishes were disrupted by sonication for preparation of 10000g supernatant. Haem oxygenase activity (a) and cytochrome *P*-450 (b) were measured as described in the Experimental section. Results for control cells (○) or for cells exposed to cycloheximide (●) are the average values from duplicate pairs of plates in two experiments and expressed on the basis of protein content in the 10000g supernatant. The data of Meyer & Marver (1971) for turnover of cytochrome *P*-450 haem in rats *in vivo* are shown for comparison [(b), △].

steady-state values was due in part to increased breakdown, as judged by a comparison of the rate of decline in the net amount of cytochrome *P*-450 in the cultured cells with published estimates of the turnover of cytochrome *P*-450 in the liver *in vivo*. The latter, shown as the △ curve in Fig. 1, would represent the concentration of cytochrome *P*-450 in the cells if one were to assume (a) that synthesis of cytochrome *P*-450 had ceased completely at the time of cell plating and (b) that the rate of breakdown of the cytochrome in culture was unchanged from that *in vivo*. It is apparent that the rate of degradation of cytochrome *P*-450 in hepatocytes in culture is markedly accelerated, because 12h after plating of the cells the amount of cytochrome *P*-450 was less than one-third of the value that would be expected even in the complete absence of synthesis. Associated with the decline in cytochrome *P*-450 in culture was a reciprocal rise in

the activity of haem oxygenase, a microsomal enzyme that converts haem into bile pigment *in vitro* (Tenhunen *et al.*, 1969). It was consistently observed, however, that this change in haem oxygenase activity occurred after, and not before, the initial fall in cytochrome *P*-450 (Fig. 1).

Effect of cycloheximide on microsomal function in cultured hepatocytes

The increased rate of cytochrome *P*-450 catabolism in culture represents a minimum estimate, since the experimental curve (Fig. 1) represents the net effect of continued synthesis of the cytochrome and accelerated degradation. Inhibitors of protein synthesis were used to estimate the contribution of synthesis in these experiments. Thus cycloheximide would be expected to decrease cytochrome *P*-450 to even lower concentrations than those seen under standard culture conditions. However, culture of hepatocytes in medium containing cycloheximide at a concentration that inhibited 90–95% of general protein synthesis markedly decreased the rate of decline in cytochrome *P*-450 as compared with cells plated in standard culture medium (Fig. 1). The effect of cycloheximide to prevent partially the fall in cytochrome *P*-450 concentration in culture was apparent after 4–8 h of incubation. After 24h, cells incubated in the presence of cycloheximide contained a 3-fold higher concentration of cytochrome *P*-450 than did cells incubated in control medium (Fig. 1; Tables 1, 2 and 3). The effect of cycloheximide was apparent regardless of whether 10000g supernatant or microsomal fractions were assayed, and whether cytochrome *P*-450 concentration was expressed per mg of DNA or per culture dish. This indicates that cells incubated in the presence of cycloheximide contain specifically a higher net content of cytochrome *P*-450 rather than a lower protein content. Cycloheximide does not appear to interfere with measurement of cytochrome *P*-450; at a concentration of $10\mu\text{M}$ the drug was without effect on the dithionite-reduced-minus-oxidized CO difference spectrum in microsomal fractions prepared from normal rat liver. Moreover, microsomal fractions prepared from cultured hepatocytes exposed to cycloheximide gave a spectral peak of CO-binding haemoprotein at 450–451 nm, as is observed in microsomal fractions obtained from control hepatocyte cultures (Guzelian & Bissell, 1976b) or from intact liver. Finally, a parallel relationship was observed between cytochrome *P*-450 concentration and aminopyrine *N*-demethylase or aniline hydroxylase activities in 10000g supernatant prepared from control or cycloheximide-treated cultures (results not shown). This suggests that the CO-binding haemoprotein in cells exposed to cycloheximide represents functional cytochrome *P*-450.

The magnitude of the difference between the con-

centration of cytochrome *P*-450 in cycloheximide-treated cells as compared with controls and the extent of inhibition of protein synthesis was dependent on the concentration of cycloheximide (Table 1). Inhibitors of protein synthesis other than cycloheximide had similar effects on the concentration of cytochrome *P*-450 (Table 1). The possibility that in some way these agents increase synthesis of cytochrome *P*-450 seems unlikely, because net formation of microsomal haem and of total protein was inhibited by 80% (Table 2) and 95% (Table 1), respectively. These results suggest that protein synthesis is obligatory for accelerated catabolism of cytochrome *P*-450 in cultured hepatocytes.

Effect of cycloheximide on degradation of microsomal haem

The studies with inhibitors of protein synthesis provide important, albeit indirect, evidence that cycloheximide affects degradation, rather than synthesis, of cytochrome *P*-450 in the cultured cells. As

a more direct approach for examining degradation of haem in culture, the heme of hepatic haemoproteins (largely cytochrome *P*-450) (Bissell & Hammaker, 1976a) was labelled *in vivo* with a pulse of δ -amino-[5-¹⁴C]laevulinic acid and, 16 h later, isolated hepatocytes were prepared from the 'labelled' animal. On incubation of the radioactively labelled cells in the standard culture medium, the amount of microsomal [¹⁴C]haem declined to 22% of its initial value after 24 h (Table 3, Exp. I and II). The presence of cycloheximide in the culture medium decreased the amount of decay of microsomal [¹⁴C] haem, consistent with decreased degradation of haem. Cycloheximide also decreased the loss of [¹⁴C]microsomal haem in cells prepared 2 h after pulse administration of δ -amino-laevulinic acid. In the above studies we measured total [¹⁴C]haem in microsomal fractions, because cultured cells from a single liver contain insufficient material to allow preparations of submicrosomal cytochrome *P*-450 particles (Comai & Gaylor, 1973) with acceptable concentrations of CO-binding haemo-

Table 1. *Effect of inhibitors of protein synthesis on cytochrome P-450 in hepatocyte monolayers*

Cells were plated in either control medium or in medium containing an inhibitor of protein synthesis. After incubation for 24 h the monolayers were scraped off and 10000 g supernatant was prepared. Cytochrome *P*-450 concentration was measured as described in the Experimental section. Results were expressed as a percentage of cytochrome *P*-450 concentrations in incubated control cells at 24 h, which averaged 0.021 ± 0.0098 (s.d., $n = 12$) nmol per mg of protein in 10000 g supernatant. In two experiments the rate of protein synthesis was also measured as the rate of [¹⁴C]leucine incorporation into trichloroacetic acid-precipitable material. The average percentage inhibition of protein synthesis relative to control cultures was as follows: cycloheximide (10 μ M), 95%; cycloheximide (1 μ M), 61%; actinomycin D, 44%; puromycin, 79%.

Additions to L-16 medium	Concn.	[Cytochrome <i>P</i> -450] (% of control at 24 h)	Range	No. of expts.
None (control)	—	100	—	—
Cycloheximide	10 μ M	272	151–459	10
Cycloheximide	1 μ M	135	125–150	3
Actinomycin D	30 μ M	184	158–205	5
Puromycin	50 μ M	201	195–203	2

Table 2. *Effect of cycloheximide on synthesis of microsomal haem in cultured hepatocytes*

Isolated hepatocytes were cultured in control medium in the presence or absence of cycloheximide, added at the time of cell plating. After 18 h of incubation, the rate of haem synthesis in the cells was assessed by addition of 10 μ Ci of [¹⁴C]glycine to the medium in each plate. Rather than using glycine-free medium, the concentration of glycine was kept at 200 mg/litre (as it is in the standard medium), to diminish any effects cycloheximide may produce on concentrations of intracellular glycine. Labelling of the cells was carried out at 37°C and was terminated after 60 min. The labelled medium was removed and the cells were washed and scraped into 0.1 M-phosphate buffer, pH 7.4. Microsomal fractions were prepared from cell homogenates for measurement of cytochrome *P*-450, [¹⁴C]haem content and protein concentration as described in the Experimental section.

Expt.	Additions to L-16 medium	Cytochrome <i>P</i> -450 (nmol/mg of protein)	[¹⁴ C]Haem (d.p.m./mg of protein)
I	None	0.080	160
	Cycloheximide (10 μ M)	0.255	46
II	None	0.102	200
	Cycloheximide (10 μ M)	0.194	41

protein and labelled haem. A lack of complete correspondence between the effects of cycloheximide on cytochrome *P*-450 and [^{14}C]microsomal haem was noted in these experiments (Table 3, Expts. I and II). It is possible that the pulse-labelled fraction of cytochrome *P*-450 is degraded at a rate different from the overall rate for degradation of all forms of the haemo-protein. This is suggested by the closer correspondence of cytochrome *P*-450 and microsomal [^{14}C]haem in the cells prepared at a shorter time after administration of δ -amino[5- ^{14}C]laevulinic acid (Table 3, Expt. III).

Effect of cycloheximide on total protein turnover

The specificity of the effect of cycloheximide on degradation of cytochrome *P*-450 was next examined because cycloheximide has been reported to decrease turnover of general hepatic protein (Khairallah & Mortimore, 1976). Breakdown of protein was studied in hepatocytes isolated from animals labelled by pulse administration of [^{14}C]valine *in vivo*. The cells were cultured in media supplemented with unlabelled valine (10mM) to decrease recycling of the radioactive amino acid. Approx. 50% of the radiolabelled protein of the cultured cells was lost during the first 24h of

Table 3. *Degradation of microsomal [^{14}C]haem in hepatocytes cultured in the presence or absence of cycloheximide for 24h* Donor animals were injected intravenously with 10 μCi of δ -amino[5- ^{14}C]laevulinic acid and operated on 16h later. The liver was perfused with collagenase solution for preparation of isolated hepatic parenchymal cells, and these were plated in complete culture medium, with or without cycloheximide. At zero time and after 24h in culture, cells were disrupted and microsomal fractions were prepared for measurement of protein and cytochrome *P*-450 and for quantification of labelled haem as described in the Experimental section. Each value represents an analysis of the cells pooled from four culture plates or the equivalent amount of freshly isolated cells. In the latter cells the concentration of [^{14}C]haem (d.p.m./mg of microsomal protein) was 3150 in Expt. I, 2360 in Expt. II, and 5144 in Expt. III. The initial concentration of cytochrome *P*-450 (nmol/mg of microsomal protein) was 0.169 in Expt. I and 0.173 in Expt. II.

Expt.	Additions to the L-16 medium	Cytochrome <i>P</i> -450 (nmol/mg of protein)	[^{14}C]Haem (d.p.m./mg of protein)
I	None	Not tested	693
	Cycloheximide		1030
II	None	0.042	441
	Cycloheximide	0.076	608
III	None	0.022	1320
	Cycloheximide	0.065	3480

Table 4. *Degradation of radiolabelled protein in cultured hepatocytes after 24h of incubation*

In each experiment (A and B), protein in the liver of the donor animal was labelled by a single intraperitoneal injection of 30 μCi of [^{14}C]valine. 4h later the liver was perfused with the standard collagenase solution supplemented with 10mM-valine. The isolated hepatocytes were seeded in L-16 culture medium containing 10mM-valine with no other supplements (control), with cycloheximide, or with dialysed foetal-calf serum. At zero time and after 24h of incubation, the medium was removed, the cells were washed twice with phosphate-buffered saline and scraped into 0.1M-potassium phosphate buffer, pH 7.4. Cell homogenate and microsomal fractions were prepared for assay of radioactivity in protein precipitated with trichloroacetic acid as outlined in the Experimental section. DNA concentration was measured in the cell homogenates. Results are presented as percentages of the value in freshly isolated cells, which were (d.p.m./ μg of DNA) 186.1 (A) and 172.5 (B) for the homogenate and 22.3 (A) and 27.5 (B) for microsomal fractions.

Additions to the medium	Expt. ...	Radiolabelled protein (% of initial value)			
		Homogenate		Microsomal fraction	
		A	B	A	B
None (control)		57.4	56.7	41.1	38.0
Dialysed foetal-calf serum (5%)		56.6	50.8	41.2	34.2
Cycloheximide (10 μM)		59.3	55.9	43.7	35.1

incubation (Table 4). Serum deprivation in some cultured-cell systems causes an increase in the rate of protein degradation, an effect that is blocked by cycloheximide (Poole & Wibbo, 1973; Epstein *et al.*, 1975). By contrast, we observed no effect of addition to the medium of dialysed foetal-calf serum or cycloheximide on the turnover of protein, regardless of whether cell homogenates or microsomal fractions were tested (Table 4). Furthermore, supplementation of the medium with 2, 5 or 10% dialysed foetal-calf serum was without effect on the concentrations of cytochrome P-450. From these experiments we conclude that, in contrast with the degradation of general or microsomal protein, catabolism of cytochrome P-450 is selectively sensitive to cycloheximide in cultured hepatocytes.

Effect of cycloheximide on the breakdown of cytochrome P-450 haem in vivo

The catabolism of hepatic cytochrome P-450 *in vivo* was examined both in untreated animals and in rats given bromobenzene. The latter chemical accelerated the rate of degradation of cytochrome P-450 haem, as has been reported previously for endotoxin (Bissell & Hammaker, 1976a) or cobalt (Maines & Kappas, 1975), thereby providing *in vivo* a perturbation of cytochrome P-450 metabolism similar to that occurring spontaneously in cultured hepatocytes. Degradation was quantified as ^{14}CO excretion from animals with hepatic cytochrome P-450 [^{14}C]haem, labelled

endogenously (see the Experimental Section). Utilizing a new technique described by Bissell & Hammaker (1976a), we gave rats a pulse injection of δ -amino[5- ^{14}C]laevulinic acid to label preferentially the haem of cytochrome P-450, and 16 h later we determined the rate of ^{14}CO excretion as a reflection of the rate of degradation of cytochrome P-450 haem (Bissell & Hammaker, 1976a). Administration of bromobenzene increased the rate of ^{14}CO excretion and also increased haem oxygenase activity (Table 5, Expt. I). Prior administration of cycloheximide to bromobenzene-treated animals abolished the stimulated breakdown of cytochrome P-450. At a dose that inhibits 90% of general hepatic protein synthesis (Ch'ih *et al.*, 1977), cycloheximide decreased the rate of ^{14}CO excretion (Table 5, Expt. II) and blocked the rise in haem oxygenase activity when compared with bromobenzene-treated rats given 0.9% NaCl instead of cycloheximide. Similar effects of cycloheximide are found in rats treated with endotoxin (Guzelian & Bissell, 1976a) or with cobalt (P. S. Guzelian unpublished work). In untreated animals, cycloheximide reproducibly decreased the rate of ^{14}CO excretion, relative to excretion by the paired control (Table 5, Expt. III). These experiments suggest that the rate-limiting step(s) for the stimulated degradation of cytochrome P-450 by hepatotoxins and for the turnover of cytochrome under basal conditions are similar in being sensitive to cycloheximide.

A final set of experiments concerned the possibili-

Table 5. *Effect of cycloheximide on bromobenzene-stimulated degradation of cytochrome P-450 in rats labelled with δ -amino[5- ^{14}C]laevulinic acid*

Under (a) (Expts. I-III), each of a pair of animals was given intravenous pulse injections of $10\mu\text{Ci}$ of δ -amino[5- ^{14}C]laevulinic acid and 16 h later were placed in tandem metabolic cages to determine that the basal rates of ^{14}CO excretion in the two rats were the same. Next, one of the animals received an 'experimental' treatment while the other served as 'control'. Bromobenzene (5 mmol/kg dissolved in warmed corn oil) or cycloheximide (4 mg/kg dissolved in saline) were given intraperitoneally, and ^{14}CO collections were made for the next 6-8 h. In Expt. IV, the treatments were given first, followed 2 h later by administration of δ -amino[5- ^{14}C]laevulinic acid and immediate collections of the 'early peak' of ^{14}CO . Under (b) data are the cumulative ^{14}CO excretion for the 'experimental' animal expressed as the percentage (mean $\pm$ s. D.) increase or decrease relative to the value for the 'control' animal. (c) Pairs of unlabelled animals were treated as indicated and were killed, and hepatic microsomal fractions were prepared as outlined in the Experimental section. Haem oxygenase activity measured in these microsomal fractions was calculated as nmol of bilirubin formed/min per mg of protein. Data are given for the experimental animals expressed as percentage of the activity in the controls.

Expt.	(a) Treatment		^{14}CO collection times (h after δ -amino[5- ^{14}C]- laevulinic acid)	(b) ^{14}CO excretion	(c) Haem oxygenase activity
	Experimental	Control			
I	Bromobenzene	Saline (0.9% NaCl)	16-24	+33.3% $\pm$ 17.0 ($n = 4$)*	+832% $\pm$ 44 ($n = 7$)*
II	Bromobenzene + cycloheximide	Bromobenzene	16-24	-46.1% $\pm$ 17.4 ($n = 4$)*	-94% $\pm$ 12 ($n = 4$)*
III	Cycloheximide	Saline	16-24	-17.9% $\pm$ 9.0 ($n = 3$)*	+120% $\pm$ 41 ($n = 7$)
IV	Cycloheximide	Saline	0-8	+27.0% $\pm$ 10.0 ($n = 4$)*	+110% $\pm$ 38 ($n = 6$)

* $P < 0.05$ by Student's *t*-test.

ties that, in the intact animal, cycloheximide either inhibited the oxidation of haem *per se* (rather than the degradation of the holo-cytochrome) or interfered with measurement of this process at a subsequent step, e.g., elimination of ^{14}CO from the rat. These possibilities were investigated by examining the effect of cycloheximide on the 'early peak' of ^{14}CO excretion that is observed during the first few hours after a pulse of δ -amino[5- ^{14}C]laevulinic acid (Guzelian & Bissell, 1976a; Bissell & Hammaker, 1976b). The source of this burst of ^{14}CO is believed to be a pool of 'free' or 'unassigned' haem in the liver unrelated to cytochrome *P*-450 or any other known hepatic haemoprotein (Schmid, 1973; Bissell & Hammaker, 1976b). When 2 h after treatment with cycloheximide (4 mg/kg) rats were given pulse injections of δ -amino[5- ^{14}C]laevulinic acid, no decrease in ^{14}CO excretion was detected during the ensuing 5 h (Table 5, Expt. IV). Indeed, cycloheximide appeared to increase the 'early peak' of ^{14}CO excretion. These experiments confirm that 16 h after administration of δ -amino[5- ^{14}C]laevulinic acid to rats, cycloheximide decreases ^{14}CO excretion by selectively inhibiting the degradation of cytochrome *P*-450 haem rather than by interfering generally with the oxidation of hepatic haem to CO or by blocking the release of hepatic ^{14}CO from the animal.

Discussion

The present studies were initiated with the observation that the loss of cytochrome *P*-450 associated with isolation and primary culture of adult rat liver parenchymal cells is preventable with cycloheximide. The selective change in cytochrome *P*-450, which has been confirmed by others in primary cultures of hepatocytes derived from either rats (Michalopoulos *et al.*, 1976a) or mice (Renton *et al.*, 1978), appears to be an adaptive response of the cells to culture conditions, rather than a reflection of declining viability of the cells *in vitro* (Bissell *et al.*, 1973; Bissell & Guzelian, 1975; Guzelian & Bissell, 1976b; Guzelian *et al.*, 1977; Bissell, 1976). The degradation process in the cultured hepatocytes resembles, in several respects, physiologic catabolism of cytochrome *P*-450 *in vivo*. Normally, the haem of cytochrome *P*-450 is degraded to bilirubin; likewise, microsomal haem in cultured hepatocytes appears to be converted into bilirubin (P. S. Guzelian, unpublished work). Accompanying this process *in vivo* is the appearance of haem oxygenase activity, in proportion to the rate of turnover of cytochrome *P*-450 (Bissell & Hammaker, 1976a), and this too was observed in the cultured cells (Fig. 1). By these criteria the changes in cytochrome *P*-450 seen in cultured hepatocytes appear to reflect, at least in part, activation of certain physiologic processes. The culture system offers a way

to characterize the control of these processes, as in the present study by their sensitivity to cycloheximide.

An obligatory role for protein synthesis in catabolism of protein has been observed previously in other systems. Cycloheximide and other inhibitors of protein synthesis block proteolysis of general cellular protein in perfused rat liver (Khairallah & Mortimore, 1976; Levitan & Webb, 1969; Seglen, 1971; Woodside, 1976), and cultured hepatoma cells (Epstein *et al.*, 1975; Knowles & Ballard, 1976), and also decrease the turnover of specific enzymes in these systems (Goldberg & Dice, 1974). However, decreased protein degradation is demonstrable only under experimental conditions that have in common some form of nutritional deprivation as, for example, starvation of rats (Millward, 1970; Garlick *et al.*, 1975), culture of hepatoma cells in the absence of serum (Epstein *et al.*, 1975) or perfusion of intact liver without adequate amino acids (Mortimore *et al.*, 1972). Common to these states of decreased nutrition is an increment in the rate of protein turnover, which is blocked by inhibitors of protein synthesis. By contrast, the basal rate of protein catabolism is unaffected by these agents. These observations have been interpreted to show that nutritional deprivation leads to the induction of an alternate mechanism for protein disposal that differs from normal proteolytic pathways by its sensitivity to cycloheximide.

By analogy with this concept, transfer of hepatocytes from the liver *in vivo* to culture could represent 'step-down' (Auricchio *et al.*, 1969) nutritional conditions, with an attendant stimulation of the catabolism of proteins, including cytochrome *P*-450. However, protein degradation in the cultured hepatocytes is unaffected by supplementation of the culture medium with serum (Table 4). Furthermore, inhibition of protein synthesis with cycloheximide has little if any effect on protein catabolism in the hepatocytes in culture (Table 4), a result similar to those reported for freshly isolated hepatocytes in suspension (Seglen & Reith, 1976; Crane & Miller, 1977). Thus accelerated breakdown of cytochrome *P*-450 haem in cultured hepatocytes is mediated by a selective catabolic process sensitive to inhibitors of protein synthesis. This characteristic of cytochrome *P*-450 degradation has practical importance for those interested in modifying the hepatocyte culture system such that cytochrome *P*-450 is maintained at concentrations observed *in vivo*. Agents that increase cytochrome *P*-450 in culture, such as drugs (Michalopoulos *et al.*, 1976b), interferon (Renton *et al.*, 1978), or complex hormonal and nutritional supplements (Decad *et al.*, 1977; Paine & Legg, 1978) should be tested for possible inhibitory effects on general protein synthesis.

It has been proposed that haem oxygenase may control the rate of degradation of cytochrome *P*-450 (Maines, 1976; Maines & Kappas, 1977). If this

postulate were correct, then the decrease in degradation of cytochrome P-450 haem observed with cycloheximide might be due to blocked synthesis of this specific catabolic enzyme. However, available evidence, including that from the present studies, does not conform with this idea. For example, we have found that cytochrome P-450 concentration in cultured hepatocytes declined before the rise in haem oxygenase activity (Fig. 1). Conversely, in hepatocytes cultured in a more complex medium, stimulation of haem oxygenase may occur without a loss of cytochrome P-450 concentration (Paine & Legg, 1978), although it should be noted that the rate of degradation of the haemoprotein was not measured in these studies. Furthermore, in studies of rats given cadmium and actinomycin D, accelerated degradation of cytochrome P-450 haem is not accompanied by stimulation of haem oxygenase activity in the liver (Krasny & Holbrook, 1978). Finally, in the present study (Table 5, Expt. III), the turnover of cytochrome P-450 haem in the basal steady state was decreased by cycloheximide, whereas the activity of haem oxygenase in hepatic microsomal fractions prepared from these animals was not decreased. Therefore although turnover of cytochrome P-450 haem is intimately associated with haem oxygenase, continued synthesis of the latter enzyme is unlikely to be rate-limiting for this process, and identification of the cycloheximide-sensitive step remains to be determined.

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References

- Anthony, L. E. (1973) *J. Nutr.* **103**, 811–820
- Auricchio, F., Martin, D., Jr. & Tompkins, G. M. (1969) *Nature (London)* **224**, 806–808
- Becking, G. C. (1973) *Can. J. Physiol. Pharmacol.* **51**, 6–11
- Bissell, D. M. (1976) in *Progress in Liver Diseases* (Popper, H. & Schaffner, F., eds.), pp. 69–82, Grune and Stratton, New York
- Bissell, D. M. & Guzelian, P. S. (1975) in *International Symposium on Gene Expression and Carcinogenesis in Cultured Liver* (Gershenson, L. E. & Thompson, E. B., eds.), pp. 119–136, Academic Press, New York
- Bissell, D. M. & Hammaker, L. E. (1976a) *Arch. Biochem. Biophys.* **176**, 91–102
- Bissell, D. M. & Hammaker, L. E. (1976b) *Arch. Biochem. Biophys.* **176**, 103–112
- Bissell, D. M., Hammaker, L. E. & Meyer, U. A. (1973) *J. Cell Biol.* **59**, 722–734
- Bonney, R. J., Becher, J. E., Walker, P. R. & Potter, V. R. (1974) *In Vitro* **9**, 399–413
- Burton, K. (1956) *Biochem. J.* **62**, 315–323
- Ch'ih, J., Procyk, R. & Devlin, T. M. (1977) *Biochem. J.* **162**, 501–507
- Comai, K. & Gaylor, J. L. (1973) *J. Biol. Chem.* **248**, 4947–4955
- Correia, M. A. & Burk, R. F. (1976) *Arch. Biochem. Biophys.* **177**, 642–644
- Crane, L. J. & Miller, D. L. (1977) *J. Cell Biol.* **72**, 11–25
- Decad, G. M., Hsieh, D. P. H. & Byard, J. L. (1977) *Biochem. Biophys. Res. Commun.* **78**, 279–287
- Epstein, D., Elias-Bishko, S. & Hershko, A. (1975) *Biochemistry* **14**, 5199–5204
- Garlick, P. J., Millward, D. J., James, W. P. T. & Waterlow, J. C. (1975) *Biochim. Biophys. Acta* **414**, 71–84
- Goldberg, A. L. & Dice, J. F. (1974) *Annu. Rev. Biochem.* **43**, 835–869
- Greim, H., Schenkman, J. B., Klotzbucher, M. & Remmer, H. (1970) *Biochim. Biophys. Acta* **201**, 20–25
- Guzelian, P. S. & Bissell, D. M. (1976a) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1393
- Guzelian, P. S. & Bissell, D. M. (1976b) *J. Biol. Chem.* **251**, 4421–4427
- Guzelian, P. S., Bissell, D. M. & Meyer, U. A. (1977) *Gastroenterology* **72**, 1232–1239
- Kato, R. (1977) *Xenobiotica* **7**, 25–92
- Khairallah, E. A. & Mortimore, G. E. (1976) *J. Biol. Chem.* **251**, 1375–1384
- Knowles, S. E. & Ballard, F. S. (1976) *Biochem. J.* **156**, 609–617
- Krasny, H. C. & Holbrook, D. J., Jr. (1978) *Biochem. Pharmacol.* **27**, 364–366
- Labbe, R. F. & Nishida, G. (1957) *Biochim. Biophys. Acta* **26**, 437
- Levin, W. & Kuntzman, R. (1969) *J. Biol. Chem.* **244**, 3671–3676
- Levitan, I. B. & Webb, T. C. (1969) *J. Biol. Chem.* **244**, 4684–4688
- Maines, M. D. (1976) *J. Exp. Med.* **144**, 1509–1519
- Maines, M. D. & Kappas, A. (1975) *J. Biol. Chem.* **250**, 4171–4177
- Maines, M. D. & Kappas, A. (1977) *Science* **198**, 1215–1221
- Marver, H. S. & Schmid, R. (1972) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B. & Frederickson, D. S., eds.), p. 1098, McGraw-Hill, New York
- Meyer, U. A. & Marver, H. S. (1971) *Science* **171**, 64–66
- Michalopoulos, G., Sattler, G. L. & Pitot, H. C. (1976a) *Life Sci.* **18**, 1139–1144
- Michalopoulos, G., Sattler, C. A., Sattler, G. L. & Pitot, H. C. (1976b) *Science* **193**, 907–909
- Millward, D. J. (1970) *Clin. Sci.* **39**, 591–603
- Miyake, Y., Gaylor, J. L. & Morris, H. P. (1974) *J. Biol. Chem.* **249**, 1980–1987
- Mortimore, G. E., Woodside, K. H. & Henry, J. E. (1972) *J. Biol. Chem.* **247**, 2776–2784
- Paine, A. J. & Legg, R. F. (1978) *Biochem. Biophys. Res. Commun.* **81**, 672–679
- Poole, B. & Wibo, M. (1973) *J. Biol. Chem.* **248**, 6221–6226
- Remmer, H. & Merker, H. J. (1965) *Ann. N. Y. Acad. Sci.* **123**, 79–97

- Renton, K. W., Deloria, L. B. & Mannering, G. J. (1978) *Mol. Pharmacol.* **14**, 672-681
- Schacterle, G. R. & Pollack, R. L. (1973) *Anal. Biochem.* **51**, 654-655
- Schmid, R. (1973) *Drug Metab. Dispos.* **1**, 256-258
- Schoene, B., Fleischman, R. A., Remmer, H. & Oldershausen, H. F. v. (1972) *Eur. J. Clin. Pharmacol.* **4**, 65-73
- Seglen, P. O. (1971) *Biochim. Biophys. Acta* **230**, 319-326
- Seglen, P. O. & Reith, A. (1976) *Exp. Cell Res.* **100**, 276-280
- Tenhunen, R., Marver, H. S. & Schmid, R. (1969) *J. Biol. Chem.* **244**, 6388-6394
- Woodside, K. H. (1976) *Biochim. Biophys. Acta* **421**, 70-79
- Zannoni, V. G., Flynn, E. G. & Lynch, M. (1972) *Biochem. Pharmacol.* **21**, 1377-1392