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Turnover of messenger RNA, apoprotein and haem of cytochrome *P*-450b+e induced by phenobarbitone in rat liver

Honnavar RAVISHANKAR* and Govindarajan PADMANABAN
Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

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A single injection of phenobarbitone elicits asynchronous behaviour in the pattern of induction of mRNA, apoprotein and haem of cytochrome *P*-450b+e. The mRNA content reaches a maximum around 16h, the apoprotein content reaches a maximum around 30–36h, and the holoprotein content shows biphasic behaviour, with maxima around 16h and 30–36h. With the use of $CoCl_2$ to block fresh transcription of cytochrome *P*-450b+e mRNA, the half-life of the preinduced mRNA was found to be 3h. The apoprotein and haem moieties of cytochrome *P*-450b+e turn over with half-lives of 16h and 8h respectively. The pattern of induction of δ-aminolaevulinate synthase shows biphasic behaviour, with maxima around 16h and 30–36h. The biphasic behaviour of the holoprotein content is thus due to differences in the extent of saturation of the apoprotein with haem, the process being regulated by the activity of the rate-limiting δ-aminolaevulinate synthase and the independent faster turnover rate of haem with respect to the apoprotein. Massive degradation of the haem moiety of preformed cytochrome *P*-450b+e results in the subsequent degradation of the apoprotein.

It is now well established that prototype drugs such as phenobarbitone and 3-methylcholanthrene induce specific species of cytochrome *P*-450 in liver (Lu & West, 1980; Ryan *et al.*, 1982; Yuan *et al.*, 1983). This induction is associated with an increase in specific mRNA (Bhat & Padmanaban, 1979; Kumar & Padmanaban, 1980; Adesnik *et al.*, 1981; Morville *et al.*, 1983) and the prototype drugs have been shown to act at the level of transcription (Hardwick *et al.*, 1983; Atchison & Adesnik, 1983).

Dubois & Waterman (1979) reported that, whereas the translatable cytochrome *P*-450 mRNA activity in response to a single injection of phenobarbitone to rats reaches a maximum around 16h, the holoprotein content measured spectrally reaches a maximum around 45h. In a preliminary study, we had reported that the cytochrome *P*-450b+e species (inducible by phenobarbitone) in the normal uninduced animal turns over with half-lives around 12h and 5h for the apoprotein and haem moieties respectively (Kumar *et al.*, 1980). It appears feasible that the cytochrome *P*-450 mRNA may turn over much faster than the apoprotein, and this may explain the temporal correlation between the induction of the two parameters. Spectrally measured total holoprotein (Dubois & Waterman, 1979) would include all the haem-saturated species of cytochrome *P*-450, and therefore temporal correlation would also need the measurement of the specific protein species induced.

In the present study, the half-lives of the mRNA, apoprotein and the haem prosthetic group of cytochrome *P*-450b+e, induced by phenobarbitone in rat liver, have been examined in an attempt to explain the temporal relationship between the induction of these parameters. In addition, the possible role of haem in influencing the stability of the apoprotein has been examined. We have previously shown that haem regulates cytochrome *P*-450b+e gene transcription (Ravishankar & Padmanaban, 1985).

Experimental

Treatment of animals

Male rats of the Institute strain were injected with phenobarbitone (8mg/100g, intraperitoneally), $CoCl_2,6H_2O$ (6mg/100g, subcutaneously), cycloheximide (1mg/100g intraperi-

Abbreviation used: SDS, sodium dodecyl sulphate.
* To whom reprint requests should be addressed.

toneally) or 2-allylisopropylacetamide (30mg/100g, subcutaneously), where indicated. Pooled livers were used for the isolation of microsomal fraction of polyribosomes. For the isolation of microsomal fractions, the livers were homogenized in 1.15% (w/v) KCl, and the pellet (after centrifugation at 100000g for 1 h) was used for the spectral measurement of total cytochrome *P*-450, and of cytochrome *P*-450b+e content by radial immunodiffusion and half-life measurements. Liver polyribosomes were isolated by the Mg^{2+} precipitation method of Palmiter (1974) and served as the source for the isolation of poly(A) containing RNA by oligo(dT)-cellulose chromatography (Krystosek *et al.*, 1975).

Measurement of total cytochrome P-450 and cytochrome P-450b+e contents

Total cytochrome *P*-450 was measured spectrally by recording the difference in absorbance between dithionite-reduced CO-treated microsomal suspension and dithionite-reduced microsomal suspension in the range 500−400nm. Total cytochrome *P*-450 content was computed from the ε_{mM} value of 91 for A_{450}–A_{490} (Omura & Sato, 1964).

Cytochrome *P*-450b+e protein content was measured by the immunodiffusion method of Thomas *et al.* (1979), with anti-(cytochrome *P*-450b+e) antibody as described previously (Ravishankar & Padmanaban, 1983; Sathyabhama & Padmanaban, 1984). The specific protein contents were calculated from a standard curve relating known concentrations of purified cytochrome *P*-450b and the square of the ring diameter. The protocols used for cytochrome *P*-450b purification from phenobarbitone-treated rats and for isolation and purification of antibodies have been described previously (West *et al.*, 1979; Bhat & Padmanaban, 1979).

Quantification of cytochrome P-450b+e RNA

Assay of messenger activity. The translatable activity of cytochrome *P*-450b+e mRNA was quantified by translating fixed concentrations of poly(A)-containing RNA, isolated from the liver polyribosomes of treated animals, in the reticulocyte-lysate cell-free system (Pelham & Jackson, 1976) as described previously (Ravishankar & Padmanaban, 1983; Sathyabhama & Padmanaban, 1984). Poly(A)-containing RNA was added at a fixed concentration of 2μg/25μl reaction mixture. It was found that the amount of total protein (trichloroacetic acid-precipitable) and cytochrome *P*-450b+e (antibody-precipitable) synthesized was directly proportional to the amount of RNA used in these experiments. The incubation mixture was processed for the measurement of total protein synthesized and of cytochrome *P*-450b+e synthesized by using purified antibody and *Staphylococcus aureus* cells to adsorb the antigen–antibody complex. The cells were washed, dissociated with buffer containing 2% (w/v) SDS, 50mM-Tris/HCl (pH6.8) and 5% (v/v) mercaptoethanol in a boiling-water bath for 2min and then analysed on SDS/8%-polyacrylamide gels as described by Laemmli (1970). The gels were stained with Coomassie Blue and subjected to fluorography, or the cytochrome *P*-450b+e band was cut out, digested with H_2O_2 and its radioactivity was measured (Ravishankar & Padmanaban, 1983; Sathyabhama & Padmanaban, 1984).

Assay of mRNA content with a cloned probe

We have described the construction of a cDNA clone (clone 91) for cytochrome *P*-450e mRNA in *Escherichia coli* by using pBR 322 vector. The clone consists of a 1100-base-pair insert and represents two-thirds of the mRNA for cytochrome *P*-450e (Ravishankar & Padmanaban, 1985). Cytochrome *P*450b and *P*-450e have extensive homologies at the protein and mRNA levels (Yuan *et al.*, 1983; Atchison & Adesnik, 1983; Mizukami *et al.*, 1983). The insert was labelled by nick translation with [α-^{32}P]dCTP (3×10^7 c.p.m./μg) and used to quantify cytochrome *P*-450b+e mRNA in the different preparations. Total poly(A)-containing RNA (500ng) was loaded on nitrocellulose filters in the presence of 20×SSC (3M-NaCl/0.3M-sodium citrate) and hybridization was carried out with 100ng of [^{32}P]DNA (10^6c.p.m./ml), and filters were then washed as described by Thomas (1980). The radioactivity in the filters was evaluated and taken as a measure of the cytochrome *P*-450b+e mRNA content.

Measurement of the half-life of the apoprotein and haem moieties of cytochrome P-450b+e

Rats were injected with phenobarbitone, and 12h after the drug injection each animal received 500μCi of $NaH^{14}CO_3$. The animals were killed at various time intervals starting from 12h (zero-time point) after the injection of $NaH^{14}CO_3$. The microsomal fractions were isolated, solubilized with 1% (w/v) sodium cholate and immunoprecipitated as described by Bhat & Padmanaban (1979). Under these conditions the holoprotein could be recovered quantitatively in the immunoprecipitate, as measured by spectral analysis. The precipitate was dissolved in alkali, and radioactivity was measured with Triton/toluene (1:2, v/v) containing 0.5% (w/v) 2,5-diphenyloxazole as scintillant. Non-specific-precipitation controls were performed by carrying out a second immunoprecipitation on the supernatant after removal of the first immunoprecipitate by adding fresh antigen and excess antibody. Non-specific precipitation ac-

counted for about 10–15% of radioactivity in the specific immunoprecipitate, and the values obtained were corrected accordingly.

To measure the half-life of the prosthetic group, a similar analysis was carried out after injection of 20 μCi of δ-amino[4-^{14}C]laevulinic acid hydrochloride to each animal, 18h after phenobarbitone administration. The animals were killed at various time points starting 6h (zero-time point) after administration of the labelled precursor.

Assay of δ-aminolaevulinate synthase

Enzyme activity was assayed in liver homogenates as described by Narisawa & Kikuchi (1966). δ-Aminolaevulinate formed in the reaction was measured after condensation with acetylacetone and fractionation on Dowex-1 acetate columns, by using modified Ehrlich's reagent.

Results

The effect of a single injection of phenobarbitone on translatable cytochrome b+e mRNA, total holo-cytochrome *P*-450 and cytochrome *P*-450b+e protein contents as a function of time is given in Fig. 1. Total holo-cytochrome *P*-450 was measured spectrally, whereas cytochrome *P*-450b+e content was measured by the radial-immunodiffusion assay. The translatable cytochrome *P*-450b+e mRNA activity reaches a maximum around 16h and then shows a rapid decline. The cytochrome *P*-450b+e protein content reaches a maximum around 30–36h and then shows a decreasing trend. The total holo-cytochrome *P*-450 content shows two peaks, one at 16h and another around 30–36h, the former peak being smaller than the latter. The transient decrease in the holoprotein content after the first peak is consistently

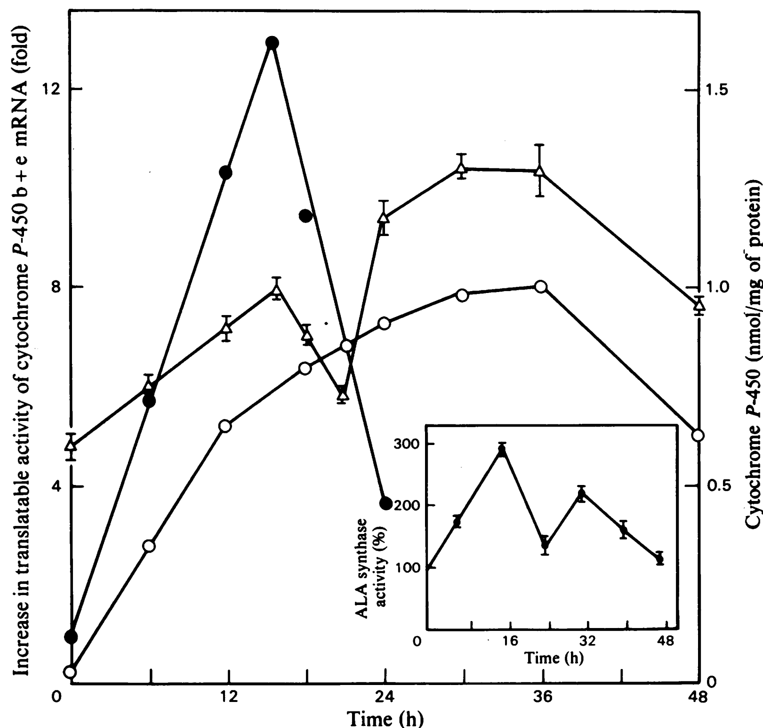

Fig. 1. *Effect of a single injection of phenobarbitone on the time course of induction of total cytochrome P-450, cytochrome P-450b+e protein species and their mRNAs, and δ-aminolaevulinate synthase activity*

Rats were given a single injection of phenobarbitone and killed at different times afterwards. The liver microsomal fraction was used for the measurement of holo-cytochrome *P*-450 spectrally and of cytochrome *P*-450b+e protein content by the radial-immunodiffusion method. Polyribosomal poly(A)-containing RNA was used for the quantification of cytochrome *P*-450b+e mRNA by translation assay. δ-Aminolaevulinate synthase was assayed in liver homogenates. The data are average values from two separate animals, except the values of holo-cytochrome *P*-450, which are the means ± S.D. obtained from four rats. ●, Cytochrome *P*-450b+e mRNA; △, holo-cytochrome *P*-450; ○, cytochrome *P*-450b+e. In the inset the δ-aminolaevulinate (ALA) synthase activity of control animals is taken as 100% and corresponds to 20±1.5 nmol of δ-aminolaevulinate formed/g of liver. The values are the means ± S.D. for four different experiments.

observed in all the experiments, although the first peak is observed around 12–18h in the different experiments.

A detailed analysis of the measurement of half-lives of the mRNA, apoprotein and haem moieties of cytochrome *P*-450b+e was then undertaken. In Fig. 1, the declining part of the curve depicting mRNA activity indicates a half-life of 5h for the cytochrome *P*-450b+e mRNA. We have shown that $CoCl_2$ is an effective inhibitor of cytochrome *P*-450 gene transcription (Ravishankar & Padmanaban, 1985), and therefore it can be used to measure mRNA half-life by eliminating any contribution caused by continued transcription of mRNA. $CoCl_2$ was administered 12h after phenobarbitone administration, and the cytochrome *P*-450b+e mRNA activity was measured by translation, and its content by hybridization with the cloned probe, every hour afterwards. Fig. 2 depicts a fluorogram showing that the mRNA activity falls appreciably 4 and 6h after $CoCl_2$ administration. A quantititative assessment is given in Fig. 3. After the administration of $CoCl_2$, the mRNA activity declines, with a half-life around 3h. Estimation of mRNA content by hybridization with the cloned probe gives essentially a similar result.

We have examined the half-lives of apo-cytochrome *P*-450b+e and the haem prosthetic group, after a single injection of phenobarbitone. The results indicate a single component with a half-life of 16h for the protein moiety and 8h for the haem prosthetic group.

The results indicate an independent turnover for the apoprotein and the haem prosthetic groups, and this can perhaps explain the pattern of induction of the holo-protein, which shows two peaks after a single injection of phenobarbitone. δ-Aminolaevulinate synthase activity was measured at different time intervals after phenobarbitone administration, and the induction pattern shows a two-peak behaviour (Fig. 1, inset). The two peaks of δ-aminolaevulinate synthase activity and of holo-cytochrome *P*-450 content occur around the same time, namely at 16h for the first and at 30–36h for the second.

The independent faster turnover rate for the haem prosthetic group prompted us to examine the fate of the protein, under conditions when the haem pool is decreased. We have used three treatments, namely $CoCl_2$, cycloheximide and 2-allyl-isopropylacetamide, to examine changes in the pre-induced amounts of total cytochrome *P*-450 measured spectrally and cytochrome *P*-450b+e measured by radial immunodiffusion. Since the

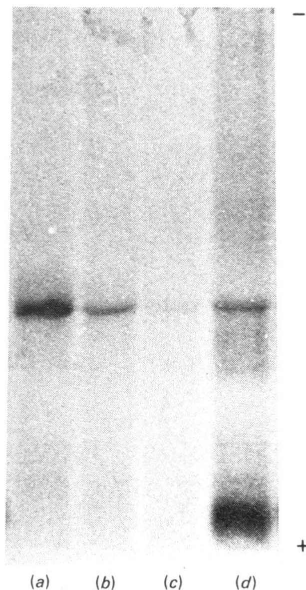

Fig. 2. *Effect of $CoCl_2$ on the preinduced contents of cytochrome P-450b+e mRNA*
$CoCl_2$ was given to rats 12h (zero time point) after phenobarbitone administration. The animals were killed 4h or 6h after $CoCl_2$ administration and cytochrome *P*-450b+e mRNA was quantified by translation assay. Treatments: *a*, phenobarbitone, 12+4h; *b*, $CoCl_2$, 4h; *c*, $CoCl_2$, 6h; *d*, phenobarbitone, 12+6h.

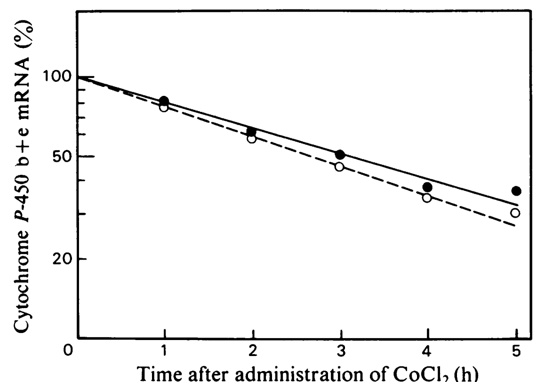

Fig. 3. *Determination of the half-life of cytochrome P-450b+e mRNA in phenobarbitone treated rats*
$CoCl_2$ was given to rats 12h after phenobarbitone administration. The animals were killed at different time intervals after $CoCl_2$ administration, and cytochrome *P*-450b+e mRNA was quantified by translation (●) and hybridization to the cloned probe (○). The zero-time value is taken as 100%: 100% activity corresponds to 200c.p.m. in cytochrome *P*-450b+e/μg of poly(A)-containing RNA, and 100% content corresponds to 800c.p.m. hybridized/μg of poly(A)-containing RNA.

inhibition of protein synthesis by cycloheximide lasts for only 6–8 h, we have confined our measurements within this time period. It is noteworthy

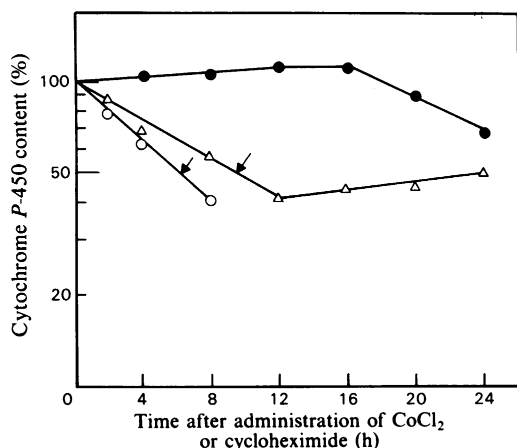

Fig. 4. *Effect of $CoCl_2$ and cycloheximide on the half-life of cytochrome P-450 holoprotein*

Rats received $CoCl_2$ or cycloheximide 24 h after the administration of phenobarbitone (zero time point) and were killed every 2 h afterwards. Total holo-cytochrome *P*-450 in the microsomal fractions was measured spectrally. Treatments: ●, phenobarbitone; △, phenobarbitone + $CoCl_2$; ○, phenobarbitone + cycloheximide. The values represent averages from two experiments: 100% corresponds to 1.25 nmol/mg of microsomal protein.

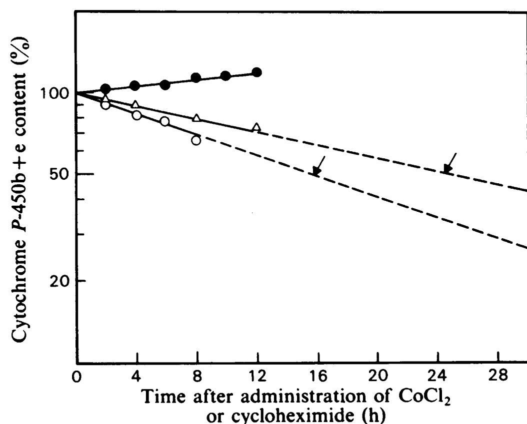

Fig. 5. *Effect of $CoCl_2$ and cycloheximide on the half-life of cytochrome P-450b + e protein moiety*

The experimental conditions are as described in Fig. 4. Cytochrome *P*-450b + e protein was measured by the radial-immunodiffusion method. Treatments: ●, phenobarbitone; △, phenobarbitone + $CoCl_2$; ○, phenobarbitone + cycloheximide. The values represent averages of two experiments: 100% corresponds to 1.05 nmol/mg of microsomal protein.

Table 1. *Effect of a single injection of 2-allylisopropylacetamide on amounts of preinduced total holoprotein and cytochrome P-450b + e*

Rats were given two injections of phenobarbitone at an interval of 24 h. 2-Allylisopropylacetamide was given 24 h after the second phenobarbitone injection (zero time point) and the animals were killed at different time intervals afterwards. Total cytochrome *P*-450 was measured spectrally and cytochrome *P*-450b + e was measured by the radial-immunodiffusion method. The values represent the average from two experiments.

Treatment	Total cytochrome *P*-450 (nmol/mg of protein)	Cytochrome *P*-450b + e (nmol/mg of protein)
Control (saline)	0.75	0.07
Phenobarbitone: 24 + 24 h (A) (zero time)	1.45	1.68
A + 2-allylisopropylacetamide (B)		
3 h	0.33	1.74
5 h	0.38	0.87
7 h	0.38	0.90
10 h	0.38	0.93
Phenobarbitone: 34 h	1.50	1.70

that, soon after cycloheximide administration, there is a rapid decline in holo-cytochrome *P*-450 content measured spectrally, indicating a half-life around 6 h (Fig. 4). The measurement of cytochrome *P*-450b + e protein content by radial immunodiffusion indicates a half-life around 16 h (Fig. 5). With $CoCl_2$ administration, the half-life values for both the total holo-cytochrome *P*-450 and cytochrome *P*-450b + e protein increase to 9.5 h (Fig. 4) and 24 h respectively (Fig. 5). We have also examined the effects of a massive degradation of haem on the stability of the apoprotein after administration of 2-allylisopropylacetamide to phenobarbitone-pretreated rats (De Matteis, 1970). The results presented in Table 1 indicate that 3 h after 2-allylisopropylacetamide administration there is nearly an 80% decrease in the spectrally measurable total cytochrome *P*-450 content. However, at this time point the cytochrome *P*-450b + e protein content does not decrease. However, at 6 and 12 h after 2-allylisopropylacetamide administration, there is nearly a 50% decrease in the cytochrome *P*-450b + e protein content.

Discussion

In agreement with previous observations (Dubois & Waterman, 1979), the cytochrome *P*-450b + e mRNA activity reaches a maximum around 16 h after a single injection of phenobarbi-

tone. It then shows a rapid decline, although by 24h the mRNA activity is still 3-fold higher than in the controls. Estimation of mRNA activity beyond this time point indicates that it is still 1.5–2-fold more than in the controls, at least up to 30h, although the sensitivity of the translation assay does not permit an exact estimate. The natural decay of the mRNA activity in the declining portion of the curve indicates a half-life of 5h. When $CoCl_2$ is used to block transcription, the mRNA decays with a half-life of 3h, measured in terms of both translatable activity and content by hybridization to a cloned probe. It is not clear at this stage whether $CoCl_2$ may also destabilize cytochrome *P*-450b+e mRNA in addition to blocking transcription. It is clear that there is a small amount of synthesis of cytochrome *P*-450b+e mRNA and therefore of the protein even at 24–30h after phenobarbitone administration.

Although cytochrome *P*450b+e mRNA activity reaches a peak around 16h, the cytochrome *P*-450b+e content reaches a maximum around 30–36h, in agreement with the reported observation by Dubois & Waterman (1979), although the exact time intervals corresponding to the peak values are different in the two studies. Since cytochrome *P*-450b+e protein acounts for nearly 70–80% of the total cytochrome *P*-450 after phenobarbitone administration (Ravishankar & Padmanaban, 1983), the changes in the content of the other species of cytochrome *P*-450 cannot account for these results. The small amount of synthesis of cytochrome *P*-450b+e taking place even during 24–30h after a single injection of phenobarbitone, coupled with its longer half-life (16h) compared with that of the mRNA (3h), can explain the temporal relationship between the induction of the mRNA and its protein (Fig. 1).

The total cytochrome *P*-450 content measured spectrally, after a single injection of phenobarbitone, shows two peaks, one around 16h and another around 30–36h. The decrease in the cytochrome *P*-450 content after the first peak is consistently observed, although the time co-ordinates vary by a few hours in different experiments. Measurement of δ-aminolaevulinate synthase activity also shows two peaks, their timings more or less coinciding with those of the holo-cytochrome *P*-450. Measurement of δ-aminolaevulinate synthase in control animals at various intervals indicates values differing by 10–15%, but not revealing any distinct diurnal variation. It is, however, possible that, since the enzyme activity is low and rate-limiting in liver, regulation by the end-product, haem, may give rise to an oscillatory behaviour, which is easily discernible only under induced conditions. The oscillatory behaviour of induced δ-aminolaevulinate synthase has been reported (Schachter *et al.*, 1976; Waxman *et al.*, 1966). This possibly determines the extent of saturation of the protein with the prosthetic group, thus explaining the anomalous behaviour of the holoprotein, whereas the apoprotein content continually increases. The feasibility of this proposition is strengthened by the observation that the haem moiety turns over faster than the apoprotein.

In a preliminary study we had reported that, in normal animals injected with saline, apo-cytochrome *P*-450b+e and the prosthetic group turn over with half-lives of 12h and 5h respectively (Kumar *et al.*, 1980). The half-life values of 16h and 8h obtained for the apoprotein and the haem moiety after a single injection of phenobarbitone in the present study agree with the values reported by Gasser *et al.* (1982) and Sadano & Omura (1983) within the limits of experimental manipulation and conditions. These values are, however, different from those reported by Parkinson *et al.* (1983), who have estimated the half-lives 11 days after Aroclor 1254 (mixture of polychlorinated biphenyl) to be 37h and 28h for the apoprotein and haem moieties respectively. The reason for the differences in the values obtained is not clear, although all the reports agree with our original observation that the haem prosthetic group turns over faster than the apoprotein.

The changes in the concentration of the spectrally measurable total cytochrome *P*-450 and cytochrome *P*-450b+e protein contents, preinduced with phenobarbitone, after cycloheximide, $CoCl_2$ or 2-allylisopropylacetamide administration permit some interesting conclusions. Changes in preinduced enzyme activity or content after blocking protein synthesis with cycloheximide have been taken to reflect the degradation rate of the enzyme in question (Schimke & Doyle, 1970), provided that the inhibitor does not by itself cause stabilization or superinduction. The present studies reveal that, after cycloheximide administration, the preinduced total holo-cytochrome *P*-450 content measured spectrally decreases with a half-life of around 6h. This obviously should reflect the half-life of the haem moiety, since the cytochrome *P*-450b+e protein measured by radial immunodiffusion decreases with a half-life of 16h, which is in agreement with the data obtained from radioactivity-decay experiments. In addition, it is known that δ-aminolaevulinate synthase is a fast-turning-over protein, with a half-life of 1h (Hayashi *et al.*, 1970), and cycloheximide treatment would block its synthesis and therefore that of the haem prosthetic group. It is noteworthy that $CoCl_2$ treatment, which depletes the haem pool and blocks transcription of cytochrome *P*450b+e mRNA (Ravishankar & Padmanaban, 1985), appears to stabilize the protein. This is observed from

measurement of both holo- and apo-protein contents in relation to the values obtained after cycloheximide treatment. It is known that $CoCl_2$ administration results in cobalt-protoporphyrin formation and replacement of the haem moiety of the preinduced cytochrome *P*-450 with cobalt-protoporphyrin, which is a poor substrate for haem oxygenase (Sinclair *et al.*, 1982; Yoshida & Kikuchi, 1978), and might stabilize the protein against degradation.

Finally, massive degradation of haem from preinduced cytochrome *P*-450 caused by 2-allylisopropylacetamide administration has an interesting effect. The degradation of the haem moiety as indicated by the holoprotein measurement is very fast, of the order of 80% within 3h. At this time there is no change in cytochrome *P*-450b+e protein content, which is the dominant species induced by phenobarbitone. Subsequently, however, the apoprotein content decreases to 50% of the maximal value. Thus, even though haem and the apoprotein turn over at independent rates, the prosthetic group seems to protect the bulk of the protein against degradation. Although we would like to suggest that the loss of haem results in the degradation of the apoprotein, the possibility that some of the metabolites of allylisopropylacetamide and the non-enzymic degradation products of haem directly damage the apoprotein cannot be ruled out. The reason for only a partial decrease in the apoprotein even at later time points is not clear. It is possible that part of the degraded protein is still bound to the membrane in a form recognized by the antibody that is used to quantify the protein.

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