Role of Haem in the Synthesis and Assembly of Cytochrome *P*-450

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By using 3-amino-1,2,4-triazole, an inhibitor of haem synthesis, and 2-allyl-2-isopropylacetamide, a drug that degrades the haem moiety of cytochrome *P*-450, the involvement of haem in cytochrome *P*-450 synthesis and assembly was investigated. Phenobarbital was used to stimulate apo-(cytochrome *P*-450) synthesis. Degradation of preformed cytochrome *P*-450 haem does not result in a concomitant release of the apoprotein from the endoplasmic reticulum. The availability of haem for cytochrome *P*-450 synthesis in the normal animal is not rate-limiting. Prolonged inhibition of haem synthesis *in vivo* decreases the rate of apo-(cytochrome *P*-450) synthesis, although this effect is not discernible under conditions of short-term inhibition of haem synthesis. Under the former conditions exogenous haemin is able to counteract the decrease in the rate of apoprotein synthesis. In animals receiving successive injections of phenobarbital plus 3-amino-1,2,4-triazole, compared with those receiving phenobarbital only, the holo-(cytochrome *P*-450) content measured spectrally shows a greater decrease than could be accounted for by the decrease in the content of the total apoprotein. In addition to less haem being available under these conditions, the free apoprotein appears to have undergone some modification, such that its haem-binding capacity is considerably decreased. This particular effect could be due to a direct interaction of 3-amino-1,2,4-triazole or its metabolites with cytochrome *P*-450 rather than a consequence of haem deficiency. Apo-(cytochrome *P*-450) is capable of binding to the endoplasmic reticulum in a form and at a site, which can be reconstituted with haemin to yield the functional protein.

Haemoproteins are ideal candidates for the study of the role of the prosthetic group in the synthesis and assembly of the holoprotein. Haemoglobin has been a favourite choice in this field, and it is now known that haem, in addition to its involvement in the structural and functional aspects of the protein molecule, also regulates the initiation step in the synthesis of this protein (Maxwell & Rabinovitz, 1969; Adamson *et al.*, 1972; Legon *et al.*, 1973). There is evidence to indicate that haem may serve as a regulator of the initiation step in general protein synthesis also (Lodish & Desalu, 1973; Beuzard *et al.*, 1973). The role of haem in the synthesis and assembly of liver haemoproteins is not clear. Beattie & Stuchell (1970) and Beattie (1971) reported that the drug 2-allyl-2-isopropylacetamide, a potent inducer of δ -aminolaevulinic synthase, enhances the content of mitochondrial cytochromes in rat liver, and also reported that the drug enhances primarily the rate of haem synthesis, followed by an increase in the rate of cytochrome oxidase synthesis. Sardana *et al.* (1976) also found that 2-allyl-2-isopropylacetamide and δ -aminolaevulinic, the rate-limiting precursor of the haem-biosynthetic pathway, increase the contents of mitochondrial cytochromes, which have components made on mitochondrial ribosomes. However, Barnes *et al.* (1971) and Druyan & Kelly

(1972) could not detect any change in the contents of mitochondrial cytochromes after the administration of 2-allyl-2-isopropylacetamide or δ -aminolaevulinic. In addition, even though in most systems studied haem has been found to regulate mitochondrial haemoprotein synthesis, there are reports to the contrary in certain other systems and the reason for this variability is not clear (Gonzalez-Cadavid, 1974).

As regards cytochrome *P*-450, the key haemoprotein of the liver endoplasmic reticulum, there has been a view that haem availability could be rate-limiting for the synthesis of this protein (Granick, 1966; Marver, 1969; Baron & Tephly, 1969). At the same time there have also been reports of an increase in cytochrome *P*-450 content under conditions when δ -aminolaevulinic synthase, the rate-limiting enzyme of the haem-biosynthetic pathway, fails to show a significant increase in activity (Song *et al.*, 1971; Bock *et al.*, 1971; Rajamanickam *et al.*, 1972). In addition, Rajamanickam *et al.* (1975) have shown that a drug such as phenobarbital primarily increases apo-(cytochrome *P*-450) synthesis, which is later followed by an increase in the rate of haem synthesis. The present study describes an analysis of the possible involvement of haem in cytochrome *P*-450 synthesis and assembly.

Materials and Methods

Male rats (110–120 g) of the Indian Institute of Science strain were used in these experiments. 2-Allyl-2-isopropylacetamide was generously supplied by Hoffman–La Roche, Basle, Switzerland. [^3H]Leucine and [^3H]valine were obtained from The Radiochemical Centre, Amersham, Bucks, U.K. All other biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

The animals were injected with different drugs at the following concentrations: phenobarbital dissolved in water containing Na_2CO_3 followed by sodium phosphate buffer (pH 8.0), 80 mg/kg (intraperitoneal); 3-amino-1,2,4-triazole in 0.9% NaCl, 3 g/kg (intraperitoneal); 2-allyl-2-isopropylacetamide in 0.9% NaCl, 400 mg/kg (subcutaneous). The schedule of injections is described in the appropriate Tables and Figures.

Processing of liver

Usually livers from two or more animals were pooled after perfusion and homogenized in 6 vol. of 1.15% (w/v) KCl. The homogenates were centrifuged at 15000g for 15 min and the supernatant was spun at 100000g for 60 min to isolate the microsomal fraction. This pellet was used for the determination and isolation of cytochrome *P*-450.

Isolation and purification of cytochrome *P*-450

The microsomal fraction isolated from three or more livers was solubilized with cholate and a partial purification was effected with two $(\text{NH}_4)_2\text{SO}_4$ fractionation steps (Lu & Levin, 1972). This preparation was subjected to SDS*/polyacrylamide-gel electrophoresis and the cytochrome *P*-450 species were detected with Coomassie Blue stain. Gels were also run under conditions where the cytochrome *P*-450 sample was dissociated with SDS in the absence of mercaptoethanol, such that some residual haem would still be associated with the protein, thus allowing identification of the protein by using benzidine staining (Welton & Aust, 1974). Benzidine is a carcinogen, but a more recent method uses 3,3',5,5'-tetramethylbenzidine for the staining procedure and this derivative is non-carcinogenic (Thomas *et al.*, 1976). Gel slices containing the cytochrome *P*-450 species identified in the marker gels on the basis of the above criteria were pooled and stacked in a glass tube over a short length (about 1 cm) of polyacrylamide gel. The protein was eluted electrophoretically into a dialysis tubing tied to the other end of the tube. The preparation was subjected to a second SDS/polyacrylamide-gel electrophoresis and the cyto-

chrome *P*-450 band was then used for the determination of specific radioactivity in labelling experiments *in vivo*. Plate 1 depicts the gel-electrophoretic pattern of the final preparation from normal animals. The preparation from normal animals consists of bands, although not distinctly separated in this gel system, ranging in molecular weight from 46000 to 54000. Microheterogeneity in cytochrome *P*-450 species is now well documented, and the molecular-weight range obtained here is in keeping with the SDS/polyacrylamide-gel pattern of highly purified cytochrome *P*-450 preparations reported previously (Welton & Aust, 1974; Haugen *et al.*, 1975; Ryan *et al.*, 1975).

Quantification of holo- and apo-(cytochrome *P*-450)

Holo-(cytochrome *P*-450) was determined in microsomal suspensions by measuring the difference spectrum between dithionite-reduced and dithionite-reduced/CO-treated portions in the range 500–400 nm in a Cary-14 spectrophotometer. The cytochrome *P*-450 content was computed from the spectrum by using an absorption coefficient of $91 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ for $A_{450}-A_{490}$ (Omura & Sato, 1964).

Apo-(cytochrome *P*-450) was estimated by two methods. In the first method, based on the report of Correia & Meyer (1975), perfused liver homogenates were divided into two equal portions and incubated in the presence of phosphatidylcholine and phosphatidylethanolamine with and without haemin. After the incubation, microsomal fractions were isolated and the holo-(cytochrome *P*-450) content was measured by the spectral method. The extent of reconstitution obtained with haemin is taken as a measure of free apo-(cytochrome *P*-450) content, that is the portion not combined with haem *in vivo*.

In the second method total apo-(cytochrome *P*-450) content was estimated. In this method, based on the report of Siekevitz (1973), microsomal preparations were solubilized and the preparation was subjected to SDS/polyacrylamide-gel electrophoresis. The apo-(cytochrome *P*-450) bands identified on the gels by the criteria mentioned above were stained with Coomassie Blue. After the gel had been destained with 50% (v/v) methanol containing 7% (v/v) acetic acid, the stained cytochrome *P*-450 bands were eluted from the gel slices by using 25% (v/v) pyridine, and the A_{605} was measured. In some experiments the gel slices without the Coomassie Blue stain were pooled and eluted electrophoretically. The eluted protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. There is a reasonable correlation between the increase in the intensity of the apoprotein band stained with Coomassie Blue and the increase in the content measured spectrally, after phenobarbital treatment (Table 2).

* Abbreviation: SDS, sodium dodecyl sulphate,

EXPLANATION OF PLATE I

SDS/polyacrylamide-gel-electrophoretic pattern of the purified cytochrome P-450 preparation from normal animals
The experimental details are given in text. Gel D carried marker proteins (1) bovine serum albumin, (2) ovalbumin and (3) cytochrome c. Gels A, B and C depict the mobilities of the purified cytochrome P-450 species when 40 μ g, 20 μ g and 10 μ g of protein were loaded on the gels respectively.

EXPLANATION OF PLATE 2

Effect of 2-allyl-2-isopropylacetamide injection on apo-(cytochrome P-450) content in phenobarbital-pretreated rats
 The animals received three injections of phenobarbital at 24h intervals. 2-Allyl-2-isopropylacetamide was given 24h after the last phenobarbital injection and the animals were killed at different time intervals after the drug injection. Some 250 μ g of protein of the solubilized microsomal preparations was placed on each gel. (1) Protein standards (bovine serum albumin, ovalbumin and cytochrome c); (2) and (3), 24h and 36h after the last phenobarbital injection; (4), (5) and (6), 2, 6 and 12h after 2-allyl-2-isopropylacetamide injection.

Studies on the binding of holo- and apo-(cytochrome P-450) to isolated microsomal preparations

These experiments are based on the observations and experimental approaches described by Yang & Strickhart (1975) and Brown & Kupfer (1975). Yang & Strickhart (1975) have shown that solubilized cytochrome P-450 preparations are capable of specific binding to isolated microsomal preparations, as shown by an increase in benzopyrene hydroxylase activity. Brown & Kupfer (1975) have shown that incubation of microsomal preparations isolated from phenobarbital-treated animals with haemin results in enhanced activity of benzopyrene hydroxylase as a result of saturation of apo-(cytochrome P-450) with haemin. By using a combination of the two experimental approaches described, microsomal preparations isolated from normal animals were incubated with solubilized holo- and apo-(cytochrome P-450) preparations and the microsomal preparations were reisolated by centrifugation. The benzopyrene hydroxylase activity of these preparations was assayed in the presence and absence of haemin. In addition, the reisolated microsomal fractions were passed through a Sepharose 4B column to separate them from aggregated unchanged cytochrome P-450 that could have co-sedimented with the microsomal fraction (Yang & Strickhart, 1975). The holo- and apo-(cytochrome P-450) contents in such preparations were measured spectrally and after SDS/polyacrylamide-gel electrophoresis respectively.

Microsomal fractions (1 mg of protein/ml) were incubated with different concentrations of solubilized holo- or apo-(cytochrome P-450) preparations at 37°C for 30 min. The incubation mixture also contained 0.06 M-potassium phosphate, pH 7.4, 2.5 mM-MgCl₂, 0.1 mM-EDTA and 10% (v/v) glycerol. At the end of the incubation, the microsomal fractions were reisolated by centrifugation at 120000g for 90 min. Sepharose 4B chromatography was carried out as described by Yang & Strickhart (1975).

Benzopyrene hydroxylase activity was assayed in microsomal suspensions as described by Yang (1974). The reaction mixture (total volume of 1 ml) contained 100 µmol of potassium phosphate, pH 7.4, 5 µmol of MgCl₂, 0.4 µmol of NADPH, 80 nmol of benzopyrene (in 0.05 ml of acetone) and 100–150 µg of microsomal protein. To the microsomal suspension, buffer was added followed by haemin (2 µM) in some cases, NADPH and finally benzopyrene. The incubation was carried out at 37°C for 10 min. The reaction was stopped by the addition of 1 ml of acetone and 3 ml of hexane, and 2 ml of the organic phase was extracted with 4 ml of 1 M-NaOH; the fluorescence of the alkali phase was measured in a spectrofluorimeter at 522 nm with excitation at 396 nm. The assay and extraction were carried out in dim light. The product of the reaction was characterized

as 3-hydroxybenzopyrene on the basis of the fluorescence spectra. The results are expressed as percentage emission actually observed per 100 µg of microsomal protein.

Preparation of apo-(cytochrome P-450)

Preliminary attempts to remove haem from cytochrome P-450 preparations by using HCl/ethyl methyl ketone (Teale, 1959) were not satisfactory. Therefore attempts were made to generate apo-(cytochrome P-450) *in vivo*. For this purpose, animals were given four injections of phenobarbital at 24 h intervals followed by a single injection of allylisopropylacetamide. It is known that the latter drug brings about a massive degradation of haem, especially that of cytochrome P-450 (De Matteis, 1970; Satyanarayana Rao & Padmanaban, 1973). The animals were killed 2 h after the administration of allylisopropylacetamide. The results reported below reveal that under these conditions, the apo-(cytochrome P-450) content of microsomal fractions is not altered (Plate 2; Tables 1 and 2). Thus microsomal fractions from animals treated with phenobarbital or phenobarbital plus 2-allyl-2-isopropylacetamide were solubilized by using cholate and the preparations were partially purified by (NH₄)₂SO₄ fractionation and calcium phosphate-gel steps (Lu & Levin, 1972). The apoprotein contents of the holo- and apo-(cytochrome P-450) preparations as estimated by SDS/polyacrylamide-gel electrophoresis were identical. Spectral measurements revealed that the apo-(cytochrome P-450) preparation in fact contained 20% holo-(cytochrome P-450).

Measurement of the rate of microsomal haem synthesis

Animals treated with the required drugs received either [2-¹⁴C]glycine (5 µCi) or [⁵⁹Fe]ferric citrate (5 µCi) intraperitoneally. The animals were killed after 1 h and the microsomal fractions were isolated from the perfused liver homogenate by the usual procedure. The pellet was suspended in 1.15% (w/v) KCl and the microsomal fractions were reisolated by centrifugation. The pellet was suspended in 0.05 M-phosphate buffer (pH 7.0) and haem was extracted by using ethyl acetate/acetic acid as described by Levin *et al.* (1972). The ethyl acetate layer freed of porphyrins by extraction with 2 M-HCl was directly used for radioactivity measurements (Satyanarayana Rao & Padmanaban, 1973). A portion of the microsomal suspension was also used for protein determination by the method of Lowry *et al.* (1951).

Binding of ¹⁴C-labelled haem to apo-(cytochrome P-450) on gels

Cytochrome P-450 preparations were subjected to SDS/polyacrylamide-gel electrophoresis and the gels

were incubated with ^{14}C -labelled haemin under conditions described by Siekevitz (1973). The gels were then thoroughly washed to remove the unchanged haemin and cut into 1 mm-thick slices which were digested with H_2O_2 (30% solution), and the radioactivity was measured (Rajamanickam *et al.*, 1975). ^{14}C -labelled haemin was prepared by growing *Neurospora crassa* in a medium containing $[2\text{-}^{14}\text{C}]\text{-glycine}$. Haemin was isolated and crystallized from the mycelial homogenates after the addition of carrier blood (Labbe & Nishida, 1957).

SDS/polyacrylamide-gel electrophoresis was performed on 8% gels as described by Laemmli (1970). Radioactivity measurements were made in a Beckman LS-100 scintillation counter. ^{59}Fe was measured in a well-type scintillation counter with NaI crystal attached to a decade scaler (Nuclear-Chicago, Des Plaines, IL, U.S.A.).

Results and Discussion

Most studies reported on the amounts of cytochrome *P*-450 in liver under a wide variety of conditions have used the spectral method (Omura & Sato, 1964) for quantifying the amount of this protein. As the presence of haem moiety is essential for the manifestation of the difference spectrum, the presence or formation of only the apoprotein would not be detected by this method. In the present study, in order to assess the role of haem in cytochrome *P*-450 formation, an attempt was made to quantify the apo-(cytochrome *P*-450) content in the microsomal fraction under conditions of treatment with the drugs that interfere with the haem concentration.

2-Allyl-2-isopropylacetamide and 3-amino-1,2,4-triazole were the drugs used for this purpose. As stated above 2-allyl-2-isopropylacetamide is a powerful inducer of δ -aminolaevulinic synthase and at early time intervals brings about a massive

degradation of the haem moiety of cytochrome *P*-450 especially after phenobarbital pretreatment (De Matteis, 1970; Satyanarayana Rao & Padmanaban, 1973). 3-Amino-1,2,4-triazole is an inhibitor of haem synthesis and specifically decreases the activity of δ -aminolaevulinic dehydratase (Baron & Tephly, 1969; and Table 3). In one case, the animals were pretreated with two injections of phenobarbital and then given a single injection of 2-allyl-2-isopropylacetamide. The question to be answered in this case is whether under these conditions of rapid haem breakdown the apoprotein still remains bound to the microsomal membranes. In the second set of experiments, 3-amino-1,2,4-triazole was given along with phenobarbital, so that haem synthesis would remain decreased, although phenobarbital would tend to boost apoprotein synthesis. It has been shown that phenobarbital primarily enhances apo-(cytochrome *P*-450) synthesis (Rajamanickam *et al.*, 1975). Here, the question whether concomitant and continued haem synthesis is necessary for the formation of the apoprotein can be answered.

The results presented in Table 1 indicate that in phenobarbital-pretreated animals receiving a single injection of 2-allyl-2-isopropylacetamide, some reconstitution is possible. In animals receiving simultaneous or successive phenobarbital and 3-amino-1,2,4-triazole treatments, no such reconstitution could be demonstrated in liver homogenates. However, the reconstitution obtained with the homogenates is very variable and the method does not appear to be sensitive. The results obtained by the second method used for quantifying the total apo-(cytochrome *P*-450) contents are presented in Table 2. It is once again clear that 2-allyl-2-isopropylacetamide injection after phenobarbital treatment does not result at least in an immediate change in the apoprotein content. This is also borne out by the pattern seen in Plate 2. A decrease in the apoprotein

Table 1. *Reconstitution of cytochrome P-450 in liver homogenates with haemin*

Male rats (100–110g) received either of two treatments. In the first set, the animals received two injections of phenobarbital, followed by a single injection of 2-allyl-2-isopropylacetamide, at 24 h intervals, and the animals were killed 2 h after the injection of the latter drug. In the second set, the animals received two injections of phenobarbital or phenobarbital plus 3-amino-1,2,4-triazole at 24 h intervals. The animals were killed 16 h after the second injection. The liver homogenates were incubated with and without haemin as described in the Materials and Methods section. Cytochrome *P*-450 content was measured in the microsomal fraction isolated from the incubated homogenates. The results obtained in three independent experiments (I–III) are presented.

Treatment	Cytochrome <i>P</i> -450 content (nmol/mg of microsomal protein)						Reconstitution (%)		
	+Haemin			–Haemin					
	I	II	III	I	II	III	I	II	III
	Phenobarbital	1.85	1.90	1.72	1.75	1.90	1.70	5.4	0.0
Phenobarbital + 3-amino-1,2,4-triazole	1.15	0.95	1.20	1.12	1.05	1.20	2.0	–5.2	0.0
Phenobarbital + 2-allyl-2-isopropylacetamide	1.20	1.10	1.01	0.82	0.90	0.90	25.0	18.2	10.9

Table 2. *Apo-(cytochrome P-450) content in the drug-treated animals*

The treatment schedule is as described in Table 1. Microsomal preparations were made from the liver homogenates, and the solubilized preparation (250 µg of protein) was subjected to SDS/polyacrylamide-gel electrophoresis. The gels were stained with Coomassie Blue. The apo-(cytochrome P-450) band was eluted with 2 ml of 25% (v/v) pyridine and the A_{605} was measured. The results obtained in two different experiments are presented.

Treatment	Cytochrome P-450 content			
	Holoprotein (nmol/mg of protein)		Apoprotein (A_{605} /mg of protein)	
	I	II	I	II
Nil	0.86	0.75	0.36	0.30
Phenobarbital	1.78	1.63	0.70	0.64
Phenobarbital+3-amino-1,2,4-triazole	1.12	1.01	0.60	0.52
Phenobarbital+2-allyl-2-isopropylacetamide	0.92	0.89	0.72	0.62

Table 3. *Effect of phenobarbital, δ -aminolaevulinate and 3-amino-1,2,4-triazole on microsomal haem synthesis*

The animals were starved overnight and received single injections of phenobarbital (8 mg/100 g) or δ -aminolaevulinate (neutralized, 20 mg/100 g) or 3-amino-1,2,4-triazole (300 mg/kg) or phenobarbital plus 3-amino-1,2,4-triazole. Some animals in the last two groups received an additional injection of 3-amino-1,2,4-triazole 12 h before the final drug injections. At 2 h after the final drug administration, the animals received 5 µCi of [14 C]glycine. Animals treated with δ -aminolaevulinate received 5 µCi of [59 Fe]ferric citrate. The animals were killed 1 h later and radioactivity in microsomal haem was measured as described in the text. The values given are the averages from two experiments.

Treatment	Microsomal haem-specific radioactivity (c.p.m./mg of protein)	
	[14 C]Glycine	59 Fe
Control	626	283
Phenobarbital	686	—
3-Amino-1,2,4-triazole (1 injection)	395	—
3-Amino-1,2,4-triazole (2 injections)	335	—
Phenobarbital+3-amino-1,2,4-triazole (1 injection)	419	—
Phenobarbital+3-amino-1,2,4-triazole (2 injections)	346	—
δ -Aminolaevulinate	—	1232

content is evident only 12 h after 2-allyl-2-isopropylacetamide treatment. The results obtained by Siekevitz (1973) in this regard are not conclusive. For animals receiving simultaneous or successive phenobarbital and 3-amino-1,2,4-triazole treatments, there is a decrease in total apo-(cytochrome P-450) content as estimated by gel electrophoresis (Table 2).

Thus quantification of apo-(cytochrome P-450) content, free and total, by the two methods indicates

that loss of haem from preformed cytochrome P-450 on microsomal membranes does not result in an immediate release or breakdown of the apoprotein. Concomitant inhibition of haem synthesis during the formation and assembly of cytochrome P-450 does result in decreased total apo-(cytochrome P-450) being present on the microsomal membranes. However, the decrease in the total apo-(cytochrome P-450) content is not proportional to the decrease in holo-(cytochrome P-450) content (Table 2). These results could be influenced by the following considerations: (1) requirement of haem for apo-(cytochrome P-450) synthesis; (2) requirement of haem for the integration of the protein to its ultimate site on the microsomal membrane; (3) the stability and the possible modification which the protein can undergo before or after integration to the endoplasmic reticulum, in the absence of adequate haem supply.

To measure the effect of haem on apo-(cytochrome P-450) synthesis, it was necessary to purify the cytochrome P-450 species. In the present study, the rate of cytochrome P-450 synthesis was measured at early times after phenobarbital treatment and as such no detectable change in the content and distribution of the different species was expected. The procedure described in the literature for the purification of any one species of cytochrome P-450 after prolonged treatment with a particular drug probably selects out the dominant species responding to the particular drug treatment (Welton & Aust, 1974; Ryan *et al.*, 1975; Haugen *et al.*, 1975). However, the interpretation of the results of the present study is based on the assumption that the band recovered from the gels contains only apo-(cytochrome P-450), but this has not been proved conclusively.

The results presented in Table 3 indicate that 3-amino-1,2,4-triazole does inhibit haem synthesis under conditions of both short-term and long-term treatment. This effect is discernible in both the presence and the absence of phenobarbital. As

Table 4. *Effect of phenobarbital, δ -aminolaevulinate and 3-amino-1,2,4-triazole on cytochrome P-450 synthesis*

The animals were starved overnight and received a single injection of phenobarbital (intraperitoneal; 8mg/100g) or δ -aminolaevulinate (intraperitoneal; neutralized; 20mg/100g) or 3-amino-1,2,4-triazole (intraperitoneal; 300mg/100g). At 2h after the drug administration, the animals received 100 μ Ci of [3 H]leucine. After 1h labelling the animals were killed and cytochrome P-450 was isolated for the determination of specific radioactivity as described in the Materials and Methods section. As a routine, gel slices from four gels were pooled and the apo-(cytochrome P-450) was eluted electrophoretically in about 0.2ml volume, and portions were used for radioactivity and protein measurements. The values represent the mean $\pm$ S.D. obtained from four independent experiments.

Treatment	Specific radioactivity (c.p.m./mg of protein)	
	Microsomal fraction	Cytochrome P-450
Control	1022 $\pm$ 59	1369 $\pm$ 122
Phenobarbital	1224 $\pm$ 96	2033 $\pm$ 166 ($P < 0.02$)*
3-Amino-1,2,4-triazole	1059 $\pm$ 42	1447 $\pm$ 122
Phenobarbital + 3-amino- 1,2,4-triazole	1136 $\pm$ 71	2074 $\pm$ 193 ($P < 0.02$)*
δ -Aminolaevulinate	1109 $\pm$ 53	1395 $\pm$ 82

* P value (Student's t test) calculated with respect to control.

expected, δ -aminolaevulinate enhances microsomal haem synthesis considerably. Phenobarbital does not have a significant effect on the rate of haem synthesis during the time interval examined. The results presented in Table 4 indicate that phenobarbital administration enhances the rate of [3 H]leucine incorporation into the cytochrome P-450 species within 2h, confirming previous results obtained by a different purification procedure (Rajamanickam *et al.*, 1975) and also the results reported by Haugen *et al.* (1976) using the SDS/polyacrylamide-gel procedure. δ -Aminolaevulinate does not enhance the rate of cytochrome P-450 synthesis. Simultaneous administration of 3-amino-1,2,4-triazole along with phenobarbital does not counteract the effect of phenobarbital. However, if the animals have received two successive injections of 3-amino-1,2,4-triazole, the rate of cytochrome P-450 synthesis is decreased (Table 5). This is observed irrespective of whether the animals are subsequently treated with phenobarbital or not. That the effect of 3-amino-1,2,4-triazole is related to its inhibitory effect on haem synthesis is indicated by the fact that administration of haemin 30min before the injection of the label is able to counteract the effects of drug in this regard.

Table 5. *Effect of prolonged inhibition of haem synthesis on cytochrome P-450 synthesis*

The animals received one injection of phenobarbital or two injections of 3-amino-1,2,4-triazole. The second injection of 3-amino-1,2,4-triazole was given 12h after the first. Some animals received phenobarbital along with the second 3-amino-1,2,4-triazole injection. At 2h after the final drug injection, the animals received 100 μ Ci of [3 H]valine and the animals were killed 1h after the tracer administration. Haemin when administered (intraperitoneally) was given 30min before the administration of the label. The results represent means $\pm$ S.D. obtained from four independent experiments.

Treatment	Specific radioactivity (c.p.m./mg of protein)	
	Microsomal fraction	Cytochrome P-450
Control	393 $\pm$ 36	451 $\pm$ 45
Phenobarbital	482 $\pm$ 25	739 $\pm$ 73
3-Amino-1,2,4-triazole	420 $\pm$ 39	359 $\pm$ 38
Phenobarbital + 3-amino- 1,2,4-triazole	446 $\pm$ 52	478 $\pm$ 53
Phenobarbital + 3-amino- 1,2,4-triazole + haemin	450 $\pm$ 9	641 $\pm$ 77 ($P < 0.05$)*
Haemin	383 $\pm$ 36	434 $\pm$ 42

* P value calculated with respect to phenobarbital + 3-amino-1,2,4-triazole treatment.

In addition, the treatments with δ -aminolaevulinate, haemin and 3-amino-1,2,4-triazole do not affect total microsomal protein synthesis. Haemin when given alone does not significantly affect the rate of cytochrome P-450 synthesis. Thus it appears likely that the observed effects of 3-amino-1,2,4-triazole on cytochrome P-450 syntheses are due to its interference with the haem status. The results presented in Tables 4 and 5 show that the availability of haem is not rate-limiting for cytochrome P-450 synthesis in normal animals, and under conditions of short-term inhibition of haem synthesis the rate of apo-protein synthesis is not affected. However, when there is prolonged inhibition of haem synthesis, the synthesis of apo-(cytochrome P-450) is inhibited.

The next question concerns the requirement of haem for the integration of cytochrome P-450 to its ultimate site on the endoplasmic reticulum. It is not known whether the site of nascent protein formation in relation to a proximal site on the membrane is the same as that of the ultimate site of location of the mature functional protein. However, one possibility considered by several workers (Dehlinger & Schimke, 1972; Ichikawa & Mason, 1974) is that the protein could be present in the cytosol (soluble phase) and membrane proteins could be assembled from and degraded as cytosolic components. Thus in the

Table 6. *Binding of holo- and apo-(cytochrome P-450) to microsomal preparations*

Microsomal preparations (1 mg of protein/ml) from normal animals were incubated with different concentrations of holo- or apo-(cytochrome *P*-450) preparations as described in the Materials and Methods section. The two preparations had the same amount of cytochrome *P*-450 in terms of apoprotein concentration. The reisolated microsomal preparation was used for the assay of benzopyrene hydroxylase activity in the presence and absence of haemin, the estimation of holo-(cytochrome *P*-450) content spectrally, and the measurement of apo-(cytochrome *P*-450) content by SDS/polyacrylamide-gel analysis, after Sepharose 4B chromatography. The cytochrome *P*-450 preparations did not have detectable benzopyrene hydroxylase activity. The values are expressed per mg of microsomal protein except where indicated.

Preparation	Amount of holo-(cytochrome <i>P</i> -450) in the added preparations (nmol)	Holo-(cytochrome <i>P</i> -450) content in reisolated microsomal fraction (nmol)	Apo-(cytochrome <i>P</i> -450) content in reisolated microsomal fraction (A_{605})	Benzopyrene hydroxylase activity in reisolated microsomal fraction (% of emission at 522 nm/100 μ g of protein)	
				-Haemin	+Haemin
Microsomal fraction	Nil	0.80	0.36	10	8.3
Microsomal fraction + holo-(cytochrome <i>P</i> -450)	4.80	1.85	0.86	66	56
	7.20	2.32	1.04	85	79
	9.60	2.95	1.36	103	100
Microsomal fraction + apo-(cytochrome <i>P</i> -450)	0.90	0.82	0.87	22	57
	1.35	0.85	1.01	27	103
	1.80	0.90	1.26	35	104

present study, an attempt was made to examine the binding of solubilized holo- and apo-(cytochrome *P*-450) preparations to microsomal membranes. The binding was measured on the basis of (1) spectral measurement of holo-protein content, (2) measurement of apo-(cytochrome *P*-450) content after SDS/polyacrylamide-gel electrophoresis, (3) assay of benzopyrene hydroxylase activity in the presence and absence of haemin. The basis of these experiments and the procedure are described in the Materials and Methods section. The results presented in Table 6 indicate that both holo- and apo-(cytochrome *P*-450) preparations are capable of binding to the microsomal membrane as evidenced by an increase in apo-(cytochrome *P*-450) contents in the reisolated microsomal preparations. The binding of holo-(cytochrome *P*-450) is associated with a corresponding increase in benzopyrene hydroxylase activity. The smaller increase in enzyme activity observed for the apo-(cytochrome *P*-450) binding is due to the fact that the preparation contained 20% holo-protein. This is further confirmed by the observation that when the holo- and apo-(cytochrome *P*-450) bound microsomal preparations were assayed for benzopyrene hydroxylase activity in the presence and absence of haemin, the apo-(cytochrome *P*-450)-bound microsomal preparations showed a striking response to the addition of haemin. It must be mentioned, however, that the results obtained with haemin are somewhat erratic, as, for example, the activation obtained with apo-(cytochrome *P*-450)-bound microsomal preparations is variable and with holo-(cytochrome *P*-450)-bound microsomal preparations there is actually a slight inhibition. This has been attributed

to the quenching effect of haemin in the fluorimetric estimation of the hydroxylated product and has been circumvented by the use of radioactive substrate (Brown & Kupfer, 1975). In the present study only the fluorimetric assay procedure could be adopted, and despite the variations the evidence is overwhelmingly in favour of apo-(cytochrome *P*-450) binding to the endoplasmic reticulum in a specific manner, as the holo-protein would do. These results do not, however, prove conclusively that binding takes place to sites specific for the haemoprotein.

In animals receiving simultaneous and successive doses of phenobarbital and 3-amino-1,2,4-triazole, initially the rate of enhanced cytochrome *P*-450 synthesis would thus not be affected. Subsequently, however, the supply of haem would become rate-limiting and the rate of apoprotein synthesis would be decreased. The net result would be a decreased cytochrome *P*-450 content in animals treated with phenobarbital plus 3-amino-1,2,4-triazole compared with animals receiving phenobarbital only. However, under these conditions the total apo-(cytochrome *P*-450) content has decreased by only 20%, whereas the holoprotein has decreased by 50% (Table 2). That apo-(cytochrome *P*-450) is capable of specific binding to the endoplasmic reticulum in the absence of haem would indicate that this step is also probably not affected *in vivo*. Since the decrease in total apo-(cytochrome *P*-450) content is only 20%, perhaps degradation of the apoprotein in the absence of adequate haem supply either before or after binding to the microsomal membrane is also not a major factor under these conditions. The decrease in the apoprotein content can thus be accounted for by a decrease in

Fig. 1. Binding of [^{14}C]haemin to apo-(cytochrome *P*-450) on SDS/polyacrylamide gels

Solubilized microsomal preparation (250 μg of protein) from animals treated with phenobarbital or 3-amino-1,2,4-triazole plus phenobarbital were subjected to SDS/polyacrylamide-gel electrophoresis. The animals received two successive injections of the drugs at 24h intervals. The gels were thoroughly washed to remove SDS and then incubated with [^{14}C]haemin as described by Siekevitz (1973). Each gel was incubated in 10ml of the incubation mixture containing 3mg of haemin (20000 c.p.m./mg of haemin). The gels were once again thoroughly washed and slices were counted for radioactivity. ●, Microsomal preparation from phenobarbital-treated animals; ▲, microsomal preparation from animals treated with phenobarbital plus 3-amino-1,2,4-triazole. The apo-(cytochrome *P*-450) contents of the two preparations estimated on SDS/polyacrylamide gels by Coomassie Blue staining and elution were 0.18 and 0.14 (A_{605} unit) respectively. The experiment has been repeated three times and the results presented are those obtained in a typical experiment.

its synthesis. But the greater decrease in holoprotein content measured spectrally raises certain possibilities. It is known that the cytochrome *P*-450 content of young animals is lower than that of adults. Siekevitz (1973) has shown that in young animals the apo-(cytochrome *P*-450) content is higher than the holoprotein content. In this case he considered the possibilities that (1) availability of haem could be limiting and (2) the membrane conformation in young animals may be such that the apoprotein may not be accessible to haem. Although it is difficult to examine whether the latter situation can account for the results obtained with animals treated with phenobarbital plus 3-amino-1,2,4-triazole, results presented in Table 2 show that at the homogenate level haemin is not able to bring about the reconstitution

of cytochrome *P*-450 in such animals. Again, the results presented in Fig. 1 indicate that the binding of exogenous ^{14}C -labelled haemin to the cytochrome *P*-450 region on SDS/polyacrylamide gels is considerably decreased in the solubilized microsomal preparation from animals treated with phenobarbital plus 3-amino-1,2,4-triazole, compared with that from animals receiving phenobarbital only. Siekevitz (1973) has shown that the binding of exogenous haemin to the cytochrome *P*-450 region of the gel is fairly specific under conditions of incubation of the gel with haemin. In the present study some non-specific binding is noticed, but the binding to the cytochrome *P*-450 region of the gel in the preparation from phenobarbital-treated animals is significantly above this background. The results of these binding studies would then indicate that under conditions of prolonged 3-amino-1,2,4-triazole treatment, apart from the decreased availability of haem to bind to the apoprotein, the cytochrome *P*-450 could also have undergone some subtle change so as to result in a decreased binding affinity for haem. This particular result could be a consequence of a direct interaction of 3-amino-1,2,4-triazole or its metabolites with the cytochrome *P*-450 species rather than being the result of prolonged haem deficiency. This could also explain why no reconstitution with haemin was possible with liver homogenates from rats treated with phenobarbital plus 3-amino-1,2,4-triazole (Table 1). Thus in the present study although δ -aminolaevulinic acid, haemin and 3-amino-1,2,4-triazole have been used as agents interfering specifically with the haem content, results due to certain effects outside the haem-biosynthetic machinery cannot be entirely ruled out. This is particularly applicable to prolonged treatment with 3-amino-1,2,4-triazole.

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