3-1977

Role of Cytochrome P-450 in Methemoglobin Formation by Primaquine with Hamster Liver Microsomes

Tommy L. Hicks

Abstract

ROLE OF CYTOCHROME P-450 IN
METHEMOGLOBIN FORMATION BY PRIMAQUINE
WITH HAMSTER LIVER MICROSOMES

by Tommy L. Hicks

The role of cytochrome P-450 in in vitro methemoglobin formation by primaquine was investigated through a series of experiments with golden-Syrian hamsters, treated before sacrificing with phenobarbital or 20-methylcholanthrene to increase the cytochrome P-450 content or with cobaltous chloride to cause its decrease. To determine the extent of induction in the liver microsomal cytochrome P-450-dependent mixed-function oxidases, cytochrome P-450 content and formaldehyde from the O-demethylation of p-nitroanisole were measured. A 90% increase occurred in cytochrome P-450 content with phenobarbital pretreatment and an increase of 102% was observed in p-nitroanisole O-demethylation. Formaldehyde from primaquine increased 64%. The methylcholanthrene treatment produced a 55% increase in P-448. In vitro methemoglobin-forming ability was examined by incubating washed human red blood cells with primaquine, hamster liver microsomes and a NADPH-generating cofactor mixture. In the control flasks, the cofactor mixture was omitted. After the incubations, the red blood cells were separated from the other components of the incubation flask by differential centrifugation and the methemoglobin and oxyhemoglobin contents were measured. The ability to produce methemoglobin

by primaquine was greatly enhanced by microsomes with cofactors and only traces of methemoglobin were produced in their absence. Hamster liver microsomes, in the presence of the cofactor mixture, were 2.5 times more active in producing methemoglobin than mouse liver microsomes in comparable experiments. This process is induced to a limited extent (15%) with phenobarbital, while methylcholanthrene failed to produce induction. Hamsters pretreated with cobaltous chloride demonstrated decreases in both P-450 levels and the ability to produce methemoglobin. With the combination of the phenobarbital and cobaltous chloride pretreatments, a decrease from phenobarbital-induction levels was noted. The methemoglobin-forming ability of primaquine correlated well with increasing cytochrome P-450 in the non-phenobarbital-treated hamsters. The possible primaquine metabolite, 5,6-dihydroxyprimaquine, was an active methemoglobin-former in the system without cofactors but its activity was tripled in the presence of cofactors. The metabolism of dihydroxyprimaquine was also induced with phenobarbital particularly at a low microsomal protein concentration. A negative relationship was observed between methemoglobin formation from dihydroxyprimaquine with non-phenobarbital-induced microsomes. In conclusion, cytochrome P-450 does appear to be involved in the metabolism of primaquine by hamster liver microsomes to methemoglobin-forming compounds, but its involvement appears to be in only a part of the overall process.

LOMA LINDA UNIVERSITY

Graduate School

ROLE OF CYTOCHROME P-450 IN METHEMOGLOBIN FORMATION BY PRIMAQUINE WITH HAMSTER LIVER MICROSOMES

by

Tommy L. Hicks

A Thesis in Partial Fulfillment of the
Requirements for the Degree Master of Science
in the Field of Pharmacology

March 1977

Each person whose signature appears below certifies that this thesis in his opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.

Ian M. Fraser, Chairman
Ian M. Fraser, Professor of Pharmacology

Allen Strother
Allen Strother, Professor of Pharmacology

R. Bruce Wilcox
R. Bruce Wilcox, Professor of Biochemistry

ACKNOWLEDGMENTS

I wish to thank Dr. Ian M. Fraser for his guidance in the laboratory and suggestions for the preparation of this manuscript. I would also like to thank Drs. Allen Strother and R. Bruce Wilcox for their ideas and service on my guidance committee.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Primaquine (Figure 1), an 8-aminoquinoline, has proven to be very useful in the prevention and treatment of malaria. However, it, like several other drugs, produces significant side effects, particularly in individuals whose erythrocytes are deficient in the enzyme glucose-6-phosphate dehydrogenase (G6PD) (Kellermeyer et al., 1962; Alving et al., 1962). Manifestations of the primaquine-induced effects on these erythrocytes include increases in methemoglobin and Heinz bodies, a decrease in reduced glutathione and hemolysis (Kellermeyer et al., 1962; Beutler, 1969). Since the usefulness of primaquine and other 8-aminoquinolines in malaria therapy is limited by these undesirable effects, the elucidation of the

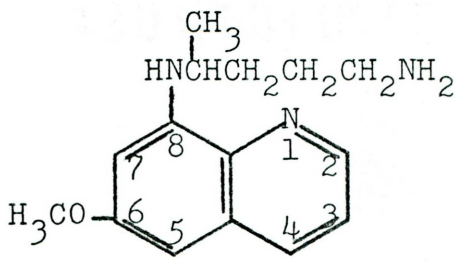


Figure 1: The structure of primaquine.

cause of these effects could prove valuable in the development of a relatively non-toxic but highly effective antimalarial drug.

Early work with the 8-aminoquinoline antimalarials suggested that the parent compounds were relatively innocuous

and that metabolites of the drug are responsible for the toxicity of these compounds. In 1950, Brodie and Udenfriend (1950) using an 8-aminoquinoline, pamaquine, demonstrated that an unstable metabolite obtained from human and dog urine samples produced methemoglobin and some hemolysis when incubated with either human or dog red blood cells; pamaquine, except at extremely high concentrations, was unable to produce these toxic effects. They suggested that the pamaquine molecule is hydroxylated in the 5 position allowing the reversible formation of a quinonimine, which then acted as an oxidant in the conversion of oxyhemoglobin to methemoglobin. In the following year, Josephson et al. (1951) reported the isolation of a compound from the droppings of pamaquine-treated chickens with characteristics very similar to 8-amino-5,6-quinolinequinone. It was 16 times more active than pamaquine in producing methemoglobin.

Using carbon^{14}-labeled drug samples in the Rhesus monkey, Elderfield and L. Smith (1953), and later C. Smith (1956), investigated the <u>in vivo</u> metabolism of pentaquine, a compound which differs from pamaquine and primaquine only in the 8-amino side chain. Elderfield and L. Smith demonstrated that the 8-amino side chain can be removed. C. Smith observed a very rapid removal of the 6-methoxy group. He postulated that the rapid removal of the 6-methoxy group is

followed by a 5-hydroxylation forming the 5,6-dihydroxy derivative that can reversibly convert to the 5,6-quinolinequinone derivative (Figure 2). Josephson et al. (1951) have shown that quinolinequinones are very active methemoglobin-forming compounds.

Fraser and Vesell (1968a), using urine from dogs given primaquine, demonstrated that when these urine samples were incubated with dog erythrocytes there was considerable methemoglobin production and almost total hemolysis. Two compounds, 8-amino-5,6-quinolinediol and 5,6-dihydroxypentaquine, serving as models for the proposed metabolites of primaquine, were shown to be very active in vitro with human erythrocytes as methemoglobin-forming compounds while primaquine was relatively inactive (Fraser and Vesell, 1968b). More methemoglobin was produced in G6PD-deficient than in normal erythrocytes.

In a report delivered to the Sixth International Congress of Pharmacology, evidence was presented for the tentative identification of 5-hydroxy- and 6-hydroxyprimaquine in the urine of dogs which had received primaquine (Strother et al., 1975). However, when the isolated metabolites were compared with samples of known 5-hydroxy- and 6-hydroxyprimaquine, they proved to be more potent methemoglobin-forming compounds than the known samples. These samples of known structure varied

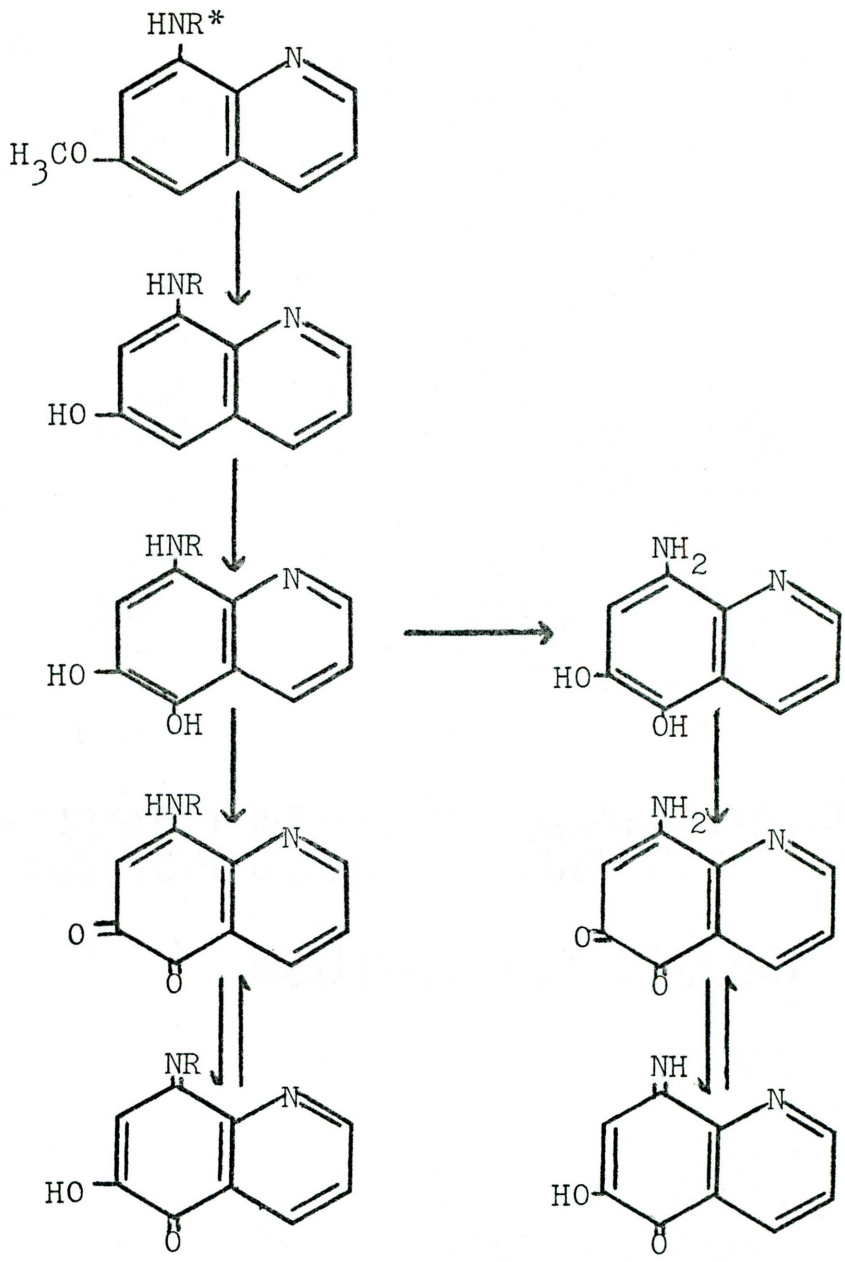


Figure 2: Proposed metabolic pathway for the 8-aminoquinoline antimalarials (from Fraser and Vesell, 1968a). * R=$CH(CH_3)(CH_2)_3NH_2$ for primaquine; $=CH(CH_3)(CH_2)_3N(C_2H_5)_2$ for pamaquine; $=(CH_2)_5NHCH(CH_3)_2$ for pentaquine.

in their abilities to produce methemoglobin when incubated with human red blood cells; 6-hydroxyprimaquine formed only 2%, while 5-hydroxyprimaquine formed 35% and primaquine produced none. It was also reported that primaquine, when incubated with a mouse liver microsomal enzyme system, human red blood cells and a NADPH-generating cofactor mixture, was capable of producing 20% methemoglobin. In a later report (Fraser et al., 1976), primaquine and its 5-hydroxy, 6-hydroxy and 5,6-dihydroxy derivatives were all shown to produce methemoglobin in human erythrocytes when incubated with cofactors and the mouse liver microsomal enzyme system. In the case of the 5-hydroxy and 5,6-dihydroxy derivatives, methemoglobin production was much greater in the presence of the cofactors and microsomal system than in their absence. These results suggest that the mechanisms proposed by Brodie and Udenfriend (1950) and C. Smith (1956) do not account for the total methemoglobin-forming ability of primaquine as metabolized by liver microsomal enzymes.

The hemoprotein, cytochrome P-450, serves as terminal oxidase for the microsomal mixed-function oxidase system responsible for the metabolism of steroids, fatty acids and many drugs (Cooper et al., 1970; Goldstein et al., 1974). Because such processes as ring-hydroxylation, O-demethylation,

N-demethylation (Goldstein et al., 1974) and some N-oxidations (Uehleke and Hellmer, 1971; Hlavica, 1972; Thorgeirsson et al., 1973; Hinson et al., 1975) are cytochrome P-450-dependent reactions, it is of interest to investigate the role of cytochrome P-450 in the metabolism of primaquine to methemoglobin-forming compounds. Of particular interest is the possiblility of an N-hydroxylation of the 8-amino nitrogen of primaquine because N-hydroxy derivatives of arylamines are potent methemoglobin-forming compounds (Uehleke, 1962; Kiese, 1965, 1966). Since hamster liver microsomes have been shown to be very effective in catalyzing the N-hydroxylation of the arlyamides, 2-acetylaminofluorene (Lotlikar et al., 1973; Thorgeirsson et al., 1973; Lotlikar and Zaleski, 1974; Lotlikar et al., 1974) and p-chloroacetanilide (Hinson et al., 1975, 1976), and at N-oxidizing the tertiary arylamine, N,N-dimethylaniline (Lotlikar et al., 1973), hamster liver microsomes were chosen for this present study of primaquine-induced methemoglobin formation.

Cytochrome P-450 involvement can be studied through induction and inhibition experiments. Known inducers of this cytochrome are phenobarbital, which causes an increase in the cytochrome with an absorption peak at 450 mμ, and 20-methyl cholanthrene, which causes an increase in the cytochrome with an absorption peak at 448 mμ (Conney, 1967; Lu et al., 1973).

Phenobarbital treatment also causes increases in the activity of NADPH-cytochrome P-450 reductase (Peters, 1973; Tsyrlov et al., 1976). Methylcholanthrene does not increase the amount of NADPH-cytochrome P-450 reductase and actually causes a moderate decrease in NADPH-cytochrome c reductase (Tsyrlov et al., 1976). When cytochrome P-450 content is increased by these inducing agents, changes occur in the ability to oxidize specific substrates so that differences are observed between control, phenobarbital and methylcholanthrene pretreated microsomes (Lu et al., 1973; Peters, 1973; Tsyrlov et al., 1976).

Cobaltous chloride ($CoCl_2 \cdot 6\ H_2O$) inhibits the synthesis of cytochrome P-450 presumably by inhibiting ferrochelatase, the enzyme responsible for the incorporation of iron into the heme molecule (Wagner and Tephly, 1975). Treatment with cobaltous chloride has been shown to bring about decreases in cytochrome P-450 content. When cobaltous chloride and phenobarbital treatments are combined, the cobalt decreases the cytochrome P-450 level compared to the level of induced-controls, while no effect is observed on the induction of NADPH-cytochrome c reductase. A good correlation has been established between cytochrome P-450 content and the N-demethylation of ethylmorphine through these induction and inhibition experiments (Tephly and Hibbeln, 1971).

The aim of this study is to determine if cytochrome P-450 is involved in the *in vitro* metabolism of primaquine by hamster liver microsomes to methemoglobin-forming compounds. Since the level of cytochrome P-450 can be varied through phenobarbital-induction and cobalt-inhibition, these techniques were chosen as the means for investigating a dependence on the presence of cytochrome P-450 by the processes responsible for the increases in primaquine methemoglobin-forming ability.

MATERIALS AND METHODS

Male golden-Syrian hamsters were obtained in groups of a few dozen over several months from Engle Laboratory Animals, Inc., Farmersburg, Indiana. The animals were 7 to 9 weeks old when sacrificed and ranged in weight from 67 to 87 grams. Their diet consisted of Purina Lab Chow and water ad lib and it was not restricted before sacrificing.

For the induction experiments phenobarbital, USP, was obtained from Millinckrodt Chemical Works, cobalt chloride from J. T. Baker Chemical Co. and 20-methylcholanthrene from Sigma Chemical Co. Phenobarbital and cobaltous chloride were dissolved in phosphate buffered-0.85% saline with the pH adjusted to allow the compounds to just dissolve in the saline solution. The pH was between 10 and 11 in the phenobarbital solution and was between 4 and 5 in the cobalt solution. Methylcholanthrene was suspended in Mazola corn oil. All injections were administered IP at 48, 41, 24 and 17 hours before sacrificing the animal. When no drug treatment was to be given at an injection time, buffered-0.85% saline was administered instead. Phenobarbital was given in 80 mg/kg doses at all four of the injection times. The cobalt treatments were 60 mg/kg doses at both 41 and 24 hours or just 24 hours before sacrificing the animals. Controls for these experiments received 4 injections of buffered saline at a volume equivalent to that received by the treated animals

and at the corresponding times. Methylcholanthrene induction was obtained by giving a single 100 mg/kg dose in 0.5 ml corn oil 24 hours before sacrificing, with control hamsters recieving corn oil alone.

The hamsters were sacrificed by cervical dislocation, the livers excised and placed in a 4 X volume of cold KCl-Tris (1.15% KCl in 25 mM Tris buffer at pH 7.35). Unless otherwise stated, two livers were combined. At all times the liver preparations were kept on ice or at near zero temperatures. The livers were homogenized in a glass homogenized with a motor-driven pestle and centrifuged in a Model RC-2 Sorvall refrigerated centrifuge at 9000 g for 20 minutes. The 9000 g supernatant was removed so as not to disturb the pellet and centrifuged in a Beckman Model L3-50 ultracentrifuge at 165,000 g (middle of tube) for 35 minutes to obtain the microsomal fraction. The supernatant was discarded, the pellet suspended in KCl-Tris, and the suspension recentrifuged for 30 minutes at 165,000 g. The final microsomal pellet was resuspended in KCl-Tris up to its original volume. The microsomal suspensions were stored on ice and were used the same day they were prepared.

In the determination of methemoglobin formation by drug metabolism, washed human erythrocytes were used. These red blood cells were obtained from G6PD-normal donors and collected

in BD-Vacutainer tubes with EDTA as anticoagulent. The blood sample was centrifuged at 2500 rpm in an IEC PR-6000 refrigerated centrifuge for 10 minutes. After the supernatant was removed, only erythrocytes remained. This erythrocyte pellet was washed twice with equal volumes of saline-dextrose (10 mM dextrose in phosphate buffered-0.85% saline solution). After the second-wash-supernatant was discarded, the cells were resuspended in an equal volume of saline-dextrose, yielding a 50% suspension of washed red blood cells.

Incubation flasks for the measurement of methemoglobin-forming ability contained 0.5 ml of the 50% suspension of washed erythrocytes, 110 mM Tris buffer, pH 7.35, 62 mM KCl, 1 to 2.5 mg microsomal protein, 0.1 mM primaquine or 0.1 mM 5,6-dihydroxyprimaquine and a NADPH-generating cofactor mixture consisting of 1.7 mM $NADP^+$ (4.2 µmoles/flask), 10.1 mM glucose-6-phosphate (25.2 µmoles/flask), 6.0 mM $MgCl_2 \cdot 6\ H_2O$ (15 µmoles/flask) and 1 unit/flask G6PD. In the control flasks, the cofactor mixture was omitted. All incubations were in 25 ml Erlenmeyer flasks containing a final volume of 2.5 ml. The flasks were placed in a 37° C shaking incubator bath for 30 minutes for the reaction to take place. After the 30 minutes, the reaction was stopped by placing the flasks in ice for 5 minutes. Each reaction mixture was poured into a 15 ml centrifuge tube and centrifuged for 10

minutes at 2500 rpm. The pellet will contain only erythrocytes since the microsomes require a much greater gravitational force than that produced under these conditions before they will become a part of the pellet. After removing the supernatant by a suction device, the erythrocyte pellet was washed with 2 ml saline-dextrose, mixed and recentrifuged. The pellet was washed a second time, resuspended in an equal volume of saline-dextrose and mixed to provide a 50% suspension of red blood cells. A 0.1 ml aliquot was removed and treated according to the method of Robin and Harley (1964) as modified by Fraser and Vesell (1968b). Duplicate 0.1 ml aliquots of nonincubated red blood cells were used as a standard for the total hemoglobin content in a 50% solution of a particular donor's erythrocytes. Measurements were made on a Gilford 300-N spectrophotometer.

Methemoglobin values per incubation flask were standardized by the following formula: $[\text{metHb/Tot Hb}]_{sam}$ X Tot Hb_{std} X 2 X 0.25 ml RBC's/flask = μmoles Hb oxidized/flask, where, $[\text{metHb/Tot Hb}]_{sam}$ is the ratio of hemoglobin oxidized to the total hemoglobin present in the measured aliquot, Tot Hb_{std} is the total hemoglobin present in an unincubated standard red blood cell sample in μmoles Hb/ml of red blood cell sample for that donor, and the dilution factor is 2, due to the 50% suspensions used. It was necessary to use

this standardization calculation to minimize the effects of differences in hemoglobin content between the various donors and because of losses of erythrocytes during the washing procedure following incubation.

Formaldehyde production from the O-demethylation of p-nitroanisole and primaquine was measured by the method of Nash (1953). Incubation flasks contained 160 mM Tris buffer, pH 7.35, 7 mM semicarbazide hydrochloride, 62 mM KCl, 1 to 2.5 mg microsomal protein, 0.1 mM primaquine or 0.2 mM p-nitroanisole and a cofactor mixture consisting of 1.7 mM $NADP^+$ (4.2 μmoles/flask), 10.1 mM glucose-6-phosphate (25.2 μmoles/flask), 6.0 mM $MgCl_2 \cdot 6\ H_2O$ (15 μmoles/flask) and 1 unit/flask G6PD. The final volume in each flask was 2.5 ml.

Cytochrome P-450 content was measured by the method of Omura and Sato (1964) with samples of the microsomal suspension diluted two-fold with 250 mM Tris buffer, pH 7.35 (microsomal protein ranged from 0.67 to 1.67 mg). The measurement was made on a Perkin-Elmer Model 202 UV-Visible spectrophotometer with the use of the extinction coefficient, 91.0 cm^{-1} mM^{-1}, in the calculation of the cytochrome P-450 valves from the difference in absorption between 450 mμ and 490 mμ.

Microsomal protein was measured at 500 mμ by the method of Lowry et al. (1951) using a bovine serum albumin standard.

The data from each experimental procedure were standardized by describing the results per mg of microsomal protein present.

Statistical determinations were made according to Weinberg and Schmaker (1969). For statistical purposes, N refers to datum from one microsomal suspension in a single experiment. The difference between two means was analyzed by a two-tailed t-test.

RESULTS

Table I summarizes the effects of the inducing agents on the total body weight, total liver weight per 100 grams of body weight and microsomal protein per gram of liver. Two animals were combined to make one experimental determination (N). Usually there was only one determination per treatment in a given experiment. The values referring to total animal weight and total liver weight are actually the average weights for the two animals combined to make one determination. Only phenobarbital significantly increased the amount of liver ($P<.01$) and microsomal protein per gram of liver ($P<.05$). However, significant ($P<.01$) increases were observed with both treatments in cytochrome P-450 (or P-448) content (see Tables II, IV and VI). Saline control cytochrome P-450 values agree with those reported by Thorgeirsson et al. (1976) for male golden-Syrian hamsters.

To establish that induction had occurred in the microsomal mixed-function oxidases, the O-demethylation of p-nitroanisole was measured by following the production of formaldehyde. Netter and Seidel (1964) have shown that this O-demethylation is inducible with phenobarbital. In Table II, it can be seen that this pathway was increased by over 100% with the phenobarbital treatment. When primaquine was used as the substrate in parallel experiments, a 64% increase was noted in formaldehyde production. Formaldehyde is most

Treatment	N	Animal Weight (grams)	Liver Weight (grams)	Liver Wt/100 gm Animal	Microsomal Protein (mg prot/ gm liv)
Control (Saline)	10	80.8 ±1.5	3.7 ±.1	4.5 ±.1	17.7 ±.8
Pheno-barbital	6	78.5 ±2.0	4.4 ±.7	5.6** ±.2	20.9* ±.7
Control (Corn oil)	4	82.3 ±5.7	3.5 ±.3	4.2 ±.1	18.0 ±.6
Methylchol-anthrene	4	84.6 ±5.1	3.6 ±.2	4.2 ±.3	19.2 ±.4

Table I: Effects of induction on some liver parameters. Values are the mean ± standard error of the mean. Two hamsters were combined to make one experimental determination (N). The data are from several experiments. *Significantly different from controls ($P<.05$). **Significantly different from controls ($P<.01$).

Treatment	Formaldehyde (nmoles/ mg protein)						Cytochrome P-450		
	N	From p-Nitro-anisole 0.2 mM	% Control	N	From Prima-quine 0.1 mM	% Control	N	Content (nmoles/ mg prot)	% Control
Control (Saline)	8	43 ±3	100	6	22 ±1	100	7	1.0 ±.1	100
Pheno-barbital	7	87** ±8	202	6	36** ±1	164	7	1.9** ±.1	190

Table II: Phenobarbital induction and the production of formaldehyde from p-nitroanisole and primaquine. Values are means ± standard error of the mean. Two hamster livers were combined to make one experimental determination (N). The data presented are from several experiments. **Significantly different from controls ($P < .01$).

	N	μmoles Hb oxidized/ mg protein
Mouse liver microsomes	4	.18 ±.01
Hamster liver microsomes	10	.46** ±.01

Table III: Comparison between methemoglobin produced by primaquine with mouse and hamster liver microsomes. Values are the mean ± standard error of mean. N refers to separate microsomal preparations. **Significantly different from mouse microsomes ($P < .01$).

likely resulting from the 0-demethylation at the 6 position of primaquine (cf. Smith, 1956; Strother et al., 1975).

Tables III to VI summarize the measurements of methemoglobin production by the hamster liver microsomal system. These values give the net formation of methemoglobin after the control (the flask without cofactors) values were subtracted. Although not shown, primaquine without cofactors produced negligible (0.02 μmoles/flask) methemoglobin while dihydroxyprimaquine produced an average of 0.25 μmoles/flask.

The hamster liver microsomes proved to be very effective at producing methemoglobin-forming metabolites from primaquine being, in fact, two and one half times more active than mouse liver microsomes (Table III). As seen in Table IV, this process is significantly ($P < .01$) inducible with phenobarbital but only to a limited extent (15%). Preliminary experiments (Figure 3) indicated that methemoglobin production from metabolized primaquine is linear with increasing protein concentrations over the range used in the experiments presented in the table, i.e., 1 to 2.5 mg/flask. However, since in these experiments the protein concentration was not adjusted before the incubation and only a small increase was observed with phenobarbital-induced microsomes, a series of experiments at a low protein concentration (0.5 mg/flask) was carried out to check if the higher protein was masking some

Treatment	N	µmoles Hb oxidized/mg protein				Cytochrome P-450 Content (nmoles/ mg prot)	% Control
		With Primaquine 0.1 mM	% Control	With Dihydroxy-Primaquine 0.1 mM	% Control		
Control (Saline)	10	.46 ±.02	100	.53 ±.03	100	1.1 ±.1	100
Cobaltous Chloride (24 hrs)	6	.39 ±.05	85	.60 ±.07	113	.8** ±.1	73
Cobaltous Chloride (41 + 24 hrs)	3	.30** ±.01	65	.62 ±.06	117	.5** ±.1	45
Phenobarbital	6	.53** ±.02	115	.64 ±.05	121	2.0** ±.1	182
Phenobarbital + Cobaltous Chloride (24 hrs)	5	.45 ±.04	98	.69 ±.06	130	1.4* ±.2	127
Phenobarbital + Cobaltous Chloride (41 + 24 hrs)	4	.36* ±.07	78	.57 ±.08	108	1.4** ±.1	127

Table IV: Comparison of phenobarbital induction and cobalt inhibition of methemoglobin production with primaquine and 5,6-dihydroxyprimaquine. Times refer to hours before sacrificing. Values are the mean ± standard error of the mean. Two hamster livers were combined to make one experimental determination (N). The data are from several experiments. *Significantly different from control ($P < .05$). **Significantly different from control ($P < .01$).

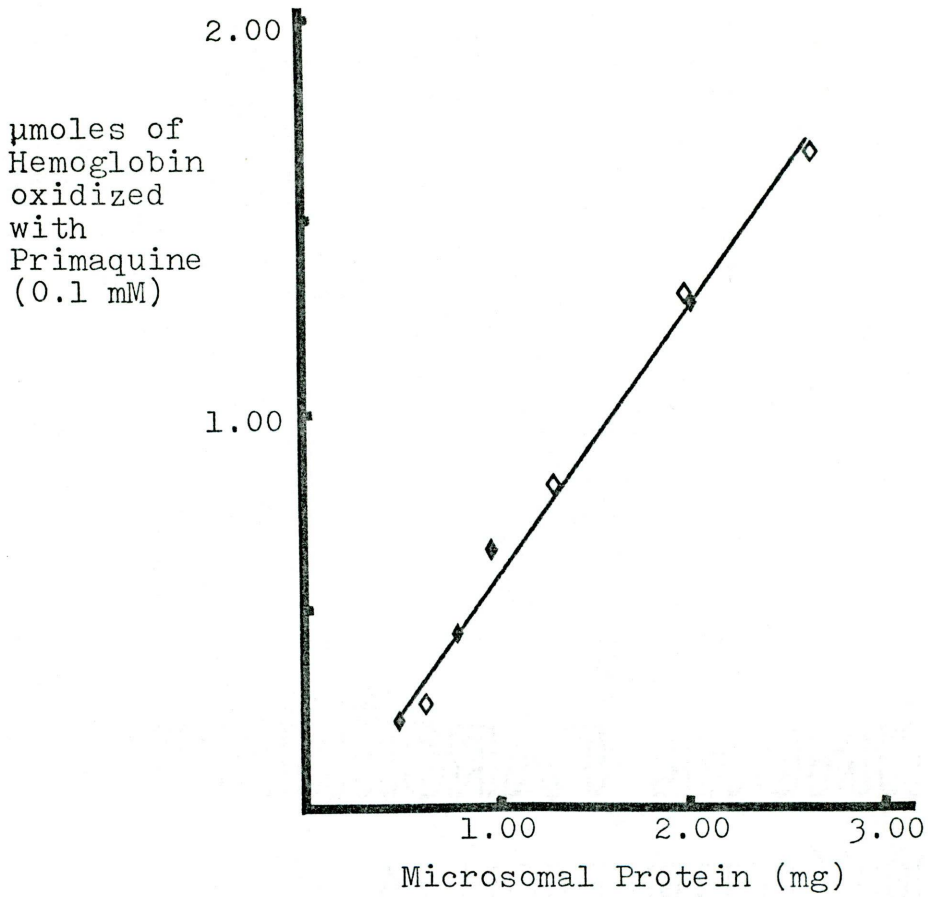


Figure 3: The relationship between microsomal protein concentration and methemoglobin formation with primaquine. Each point represents the average of duplicate determinations. The data is from two experiments. Determinations from the first experiment, represented by ◊, were made with G6PD-deficient erythrocytes. Results from the second experiment, depicted by ♦, were made with normal erythrocytes. The correlation coefficient, r, for these points is 0.99+ and the slope, m, is equal to 0.7.

of the induction effects. The results (Table V) reveal that even at the low protein concentration of 0.5 mg/flask there was still only a 15% increase in methemoglobin formation due to primaquine metabolism by phenobarbital-treated microsomes; this was not a significant induction ($P > .05$) of the process. But, at this lower protein concentration, dihydroxyprimaquine methemoglobin-forming ability was increased 46% by phenobarbital (significantly different from control with $P < .01$) where it was only increased 21% before (Table IV). It appears that high protein concentrations may have limited the measured enzyme induction effects with phenobarbital in the case of dihydroxyprimaquine but not with primaquine. This agrees with a preliminary experiment carried out recently by Fraser (personal communication) indicating that the rate of methemoglobin formation by dihydroxyprimaquine is greatest at a level less than 1 mg microsomal protein. From 1 to 2 mg microsomal protein, the rate is decreased and above 2 mg there is a drop in total methemoglobin-forming ability. Note that the specific activity for primaquine metabolism in the experiments in Table V is lower than that reported in Table IV. This is possibly because these experiments were ran in early winter and the group of hamsters used were showing a seasonal variation in metabolism. Further investigation of this possibility may be desirable.

Treatment	N	µmoles Hb oxidized/mg protein			
		With Primaquine 0.1 mM	% Control	With Dihydroxy-primaquine 0.1 mM	% Control
Control	8	.20 ±.02	100	.67 ±.05	100
Pheno-barbital	8	.23 ±.01	115	.98** ±.03	146

Table V: Methemoglobin production with primaquine and 5,6-dihydroxyprimaquine at 0.5 mg microsomal protein/ flask. Values are the mean ± standard error of the mean. N refers to determinations made with separate microsomal preparations. Only one liver was used in each preparation. The data are from two experiments. **Significantly different from control ($P < .01$).

Treatment	µmoles Hb oxidized/ mg protein				Cytochrome P-450 or P-448 Content (nmoles/ mg prot)	
	N	With Prima-quine 0.1 mM	N	With Dihydroxy-primaquine 0.1 mM	N	
Control (Corn oil)	4	.35 ±.04	2	.41 ±.10	4	1.1 ±.1
Methylchol-anthrene	4	.33 ±.03	2	.37 ±.10	4	1.7** ±.1

Table VI: Methylcholanthrene induction and methemoglobin production by primaquine and 5,6-dihydroxy-primaquine. Values are means ± standard error of the mean. Two hamsters were combined to make one experimental determination (N). The data are from four experiments. **Significantly different from control ($P < .01$).

The results of the experiments on the effects of cobalt alone or in combination with phenobarbital are summarized in Table IV. Cobalt did significantly ($P < .01$) decrease the cytochrome P-450 content in each instance that it was given and consequently, several levels of cytochrome P-450 content were established by the two treatments. Figure 4 and 5 are graphical representations of Table IV. Figure 4 attempts to correlate the methemoglobin produced by primaquine metabolites with the cytochrome P-450 content of the microsomes involved. This relationship is best described by two separate lines instead of a single all inclusive one. Line a consists of points from saline and cobalt-treated animals and shows a strong correlation, with the correlation coefficient, r, equal to 0.96. Line b has a weaker correlation ($r = 0.84$) and consists of points from phenobarbital-treated animals plus those that received both treatments. The slope of line b is slightly less than that of line a and line b is shifted to the right, indicating a decreased ability to produce methemoglobin-forming metabolites from primaquine by the induced forms of cytochrome P-450.

In Figure 5, the methemoglobin formed by dihydroxyprimaquine metabolites is examined for its dependence on cytochrome P-450 content. Here, as in Figure 4, the saline group displays a different relationship than the phenobarbi-

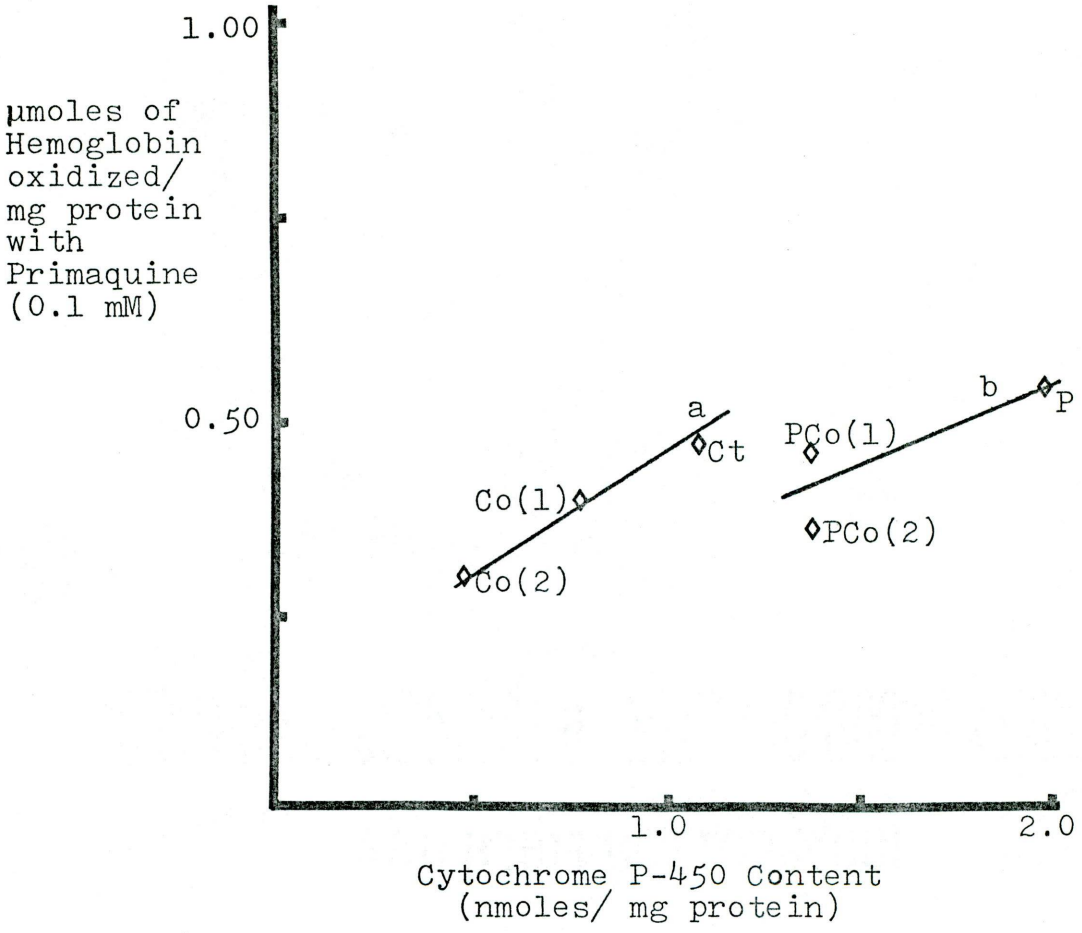


Figure 4: Relationship between cytochrome P-450 and methemoglobin production with primaquine. For line a the slope, m, is 0.3 and the correlation coefficient, r, is 0.96. For line b, m = 0.2 and r = 0.84. Symbols refer to treatments used to obtain that point. The data is listed in Table IV. Ct = control, Co(1) = one cobalt injection, Co(2) = two cobalt injections, P = phenobarbital, PCo(1) = phenobarbital + one cobalt injection, PCo(2) = phenobarbital + two cobalt injections.

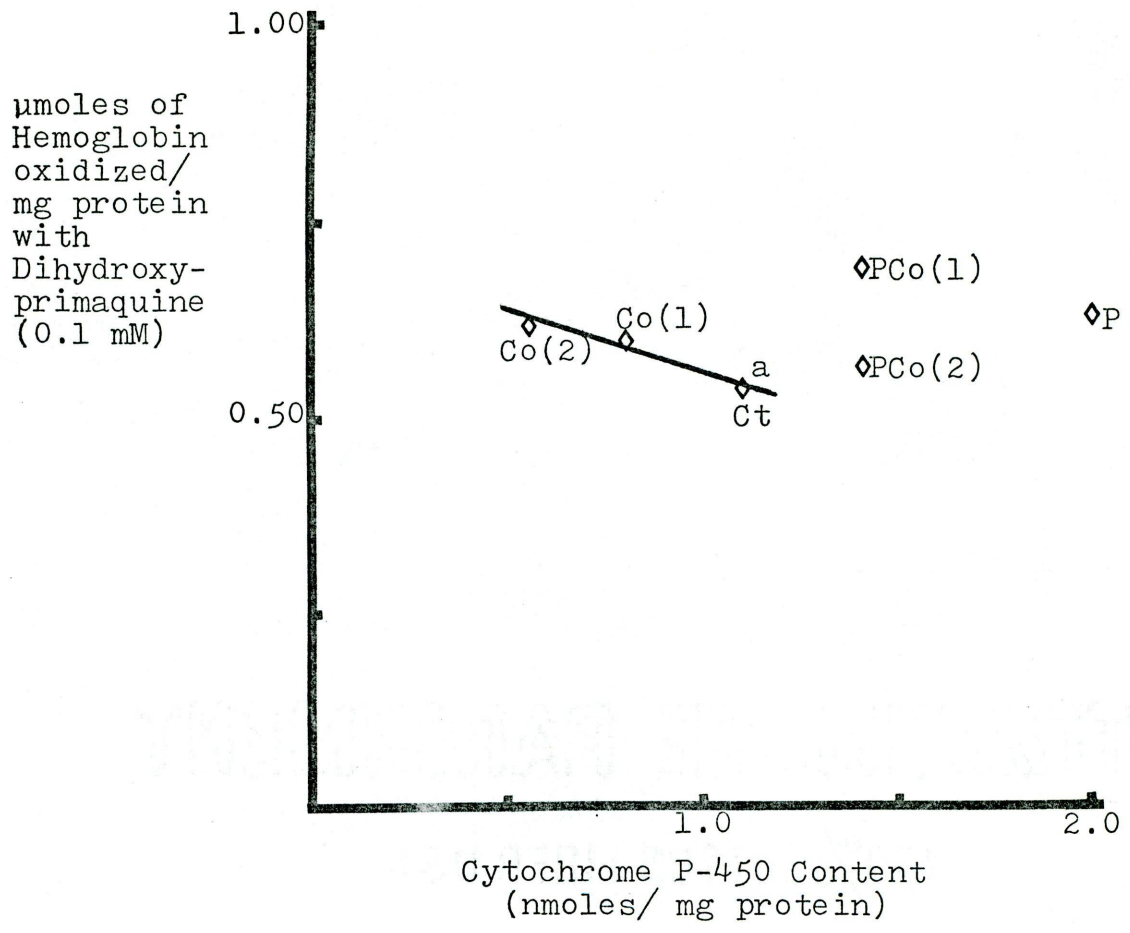


Figure 5: Relationship between cytochrome P-450 and methemoglobin production with 5,6-dihydroxyprimaquine. For line a, m = -0.2 and r = -0.99+. The data is presented in Table IV. Symbols are explained in Figure 4.

tal-treated group. The saline group has a correlation coefficient of -0.99+ as seen in line a. The phenobarbital group shows a poor correlation (r = 0.10) to cytochrome P-450 content.

When methylcholanthrene was given as an inducing agent, no induction of primaquine methemoglobin-forming ability was observed (Table VI). The data indicates a 55% increase in cytochrome P-448 with the treatment but no increase in methemoglobin formation. Also included in Table VI are the averaged results from two experiments where dihydroxyprimaquine served as substrate for methylcholanthrene-treated microsomes. These results also indicate an inability for methylcholanthrene to induce methemoglobin formation.

DISCUSSION

The data presented earlier in this report indicate that microsomal enzymes, in the presence of NADPH, metabolize primaquine through one or more steps to a substance capable of oxidizing the iron of hemoglobin from the ferrous to the ferric state. This process was shown to be inducible in the hamster by phenobarbital but not by methylcholanthrene. As seen in Figure 4, this process is related to the amount of cytochrome P-450 present in the system, but the relationship is complex since large increases in cytochrome P-450 bring only small increases in methemoglobin production.

The most likely sites of action by the cytochrome P-450 dependent mixed-function oxidase system on the primaquine molecule would be at the 5, 6 and 8 positions. Previous studies (Strother et al., 1975; Fraser et al., 1976) have shown that the 5-hydroxylated derivative of primaquine is a very active methemoglobin-forming compound. The 6-methoxy group could also be removed by these enzymes leaving 6-hydroxyprimaquine. This has been shown to occur with pentaquine in the Rhesus monkey (Smith, 1956) and appears to be happening with primaquine in the dog (Strother et al., 1975) and mouse (Strother, personal communication). In this report, formaldehyde production from primaquine was increased 64% with phenobarbital-induced microsomes as compared to controls. The most likely source of the formaldehyde is the

removal of the methoxy group at the 6 position. The O-demethylation of p-nitroanisole, as previously reported by Netter and Seidel (1964), was also induced by phenobarbital. It is likely that an O-demethylation is occurring with primaquine to form the 6-hydroxy derivative. However, without cofactors, 6-hydroxyprimaquine is not a very active methemoglobin-forming compound (Strother et al., 1975; Fraser et al., 1976), while 5,6-dihydroxyprimaquine is very active (Fraser et al., 1976). Therefore, 5,6-dihydroxyprimaquine may be formed as described for pentaquine by Smith (1956). The removal of the N-substituted side chain at position 8 is another possible P-450 mediated alteration of the primaquine molecule. Since the side chain is large and relatively complex, it is less likely that an N-dealkylation does take place (Smith and Rosazza, 1975); however, Elderfield and Smith (1953) did show that the side chain of pentaquine, which is also long, is removed in the Rhesus monkey. If any or all of these processes do occur in primaquine metabolism, particularly the 5-hydroxylation added with the 6-demethylation with the reversible formation of the quinolinequinone as suggested for pentaquine by Smith (1956), methemoglobin might be formed. Figure 2 diagrams this postulated scheme for the metabolic changes of primaquine as it goes to a quinolinequinone.

This scheme does explain how methemoglobin-forming

metabolites of primaquine can be produced, but it does not offer an explanation for several observations. As mentioned previously, methemoglobin production at best shows only a 1:3 ratio with changes in cytochrome P-450 content (Figure 4). If cytochrome P-450-dependent changes alone are responsible for methemoglobin formation a ratio closer to 1:1 would be expected. When the proposed metabolites of primaquine (5-hydroxy-, 6-hydroxy- and 5,6-dihydroxyprimaquine) were incubated with cofactors and microsomes, large increases, over the controls without cofactors, in methemoglobin-forming ability occurred (Fraser et al., 1976). When Strother et al. (1975) isolated from dog urine metabolites of primaquine which migrated on thin-layer chromatography with primaquine, 5-hydroxy- and 6-hydroxyprimaquine and incubated these with red blood cells, much larger amounts of methemoglobin were produced by the primaquine and 6-hydroxyprimaquine migrating compounds than by primaquine and 6-hydroxyprimaquine themselves. It seems that, in the migrating samples, there is possibly more than one metabolite acting to produce methemoglobin and these are related closly enough to allow a similar migration pattern. It is likely that the microsomal enzymes are changing primaquine and its "model" metabolites in some way which is increasing their methemoglobin-forming ability.

In experiments presented in this report, when 5,6-dihy-

droxyprimaquine was subjected to the same incubations as primaquine, a negative relationship was observed between methemoglobin formation and the amount of cytochrome P-450 with non-phenobarbital-induced animals (Figure 5). No correlation exists between the phenobarbital-induced increases in cytochrome P-450 and methemoglobin formation, even though the process was induced; at a low protein concentration the induction was close to 50% (Table V). However, according to the pathway of Smith (1956), this compound should be oxidized to a quinolinequinone requiring no further cytochrome P-450 involvement. The negative relationship between the amount of cytochrome P-450 and methemoglobin formation is puzzling because it seems to say that cytochrome P-450 dependent oxidases are changing dihydroxyprimaquine to a compound less able to produce methemoglobin. A possible cytochrome P-450 mediated alteration on this molecule would be the N-dealkylation step at the 8 position. However, 8-amino-5,6-quinolinediol, the compound formed by this N-dealkylation, shows high activity as a methemoglobin-former and displays little difference from dihydroxyprimaquine (Fraser et al., 1976), so that this would not be a detoxification mechanism for dihydroxyprimaquine. Other cytochrome P-450 mediated changes could be ring-hydroxylations at the 2, 3, 4 and 7 positions. Hydroxylations at these positions

have not been studied and so the effects and/or the possibility of such substitutions occurring is not known. Greenberg et al. (1951) did show a lack of antimalarial activity by 2,6-dihydroxy-8(diethylaminopropylamino) quinoline both *in vivo* and *in vitro*, while the corresponding 6-hydroxy compound was very active *in vitro* against *Plasmodium gallinaceum*. The 2-hydroxylation must be an inactivating mechanism for this compound, but its role in methemoglobin formation is not known. However, this does not fully explain the data; since ring-hydroxylations would be induced with phenobarbital, decreases in methemoglobin would, therefore, be expected if the ring-hydroxylations were inhibiting methemoglobin-forming ability. On the other hand, if some factor that is inducible with phenobarbital (eg. NADPH-cytochrome P-450 reductase) is limiting to the reaction which is increasing the methemoglobin-forming ability of dihydroxyprimaquine, then the level of methemoglobin formation would be raised if the amount of the factor is increased as a result of phenobarbital treatments. In the liver microsomes of non-induced animals this factor could limit the amount of dihydroxyprimaquine that can be metabolized to greater methemoglobin-forming compounds which possibly would allow an increase in the metabolism of dihydroxyprimaquine through other pathways, eg. the cytochrome P-450-dependent ring-hydroxylations at the 2, 3, 4 and 7

positions.

Quinone formation is not the only possible mechanism in which a methemoglobin-forming compound can be produced from primaquine. The N-substituted 8-amino group could be the site of an N-hydroxylation reaction (Figure 6) and N-hydroxy derivatives are known to be potent methemoglobin-forming compounds (Uehleke, 1962; Kiese, 1965, 1966). Uehleke (1962) states that N-hydroxylation is increased *in vitro* by a substitution on aromatic amines at the *para* position. The 5-hydroxylation of primaquine is such a p-substitution, but primaquine can not be considered a typical aromatic amine because of its quinoline structure. As stated earlier, hamster liver microsomes have been shown to be excellent in N-hydroxylating several compounds especially the arylamides, 2-acetylaminofluorene and p-chloroacetanilide (Lotlikar et al., 1973; Hinson et al., 1975). The reaction is inducible by methylcholanthrene (Lotlikar et al., 1973), but not by phenobarbital (Hinson et al, 1975), and cytochrome P-450 (P-448) is known to be involved in the process (Thorgeirsson et al., 1973; Lotlikar and Zaleski, 1974; Hinson et al., 1975). Because the 8-amino nitrogen of primaquine is a secondary amine and not an amide as in acetylaminofluorene, the ability to N-hydroxylate these two compounds is apt to be different. This was shown by Lotlikar et al. (1973) with hamster liver

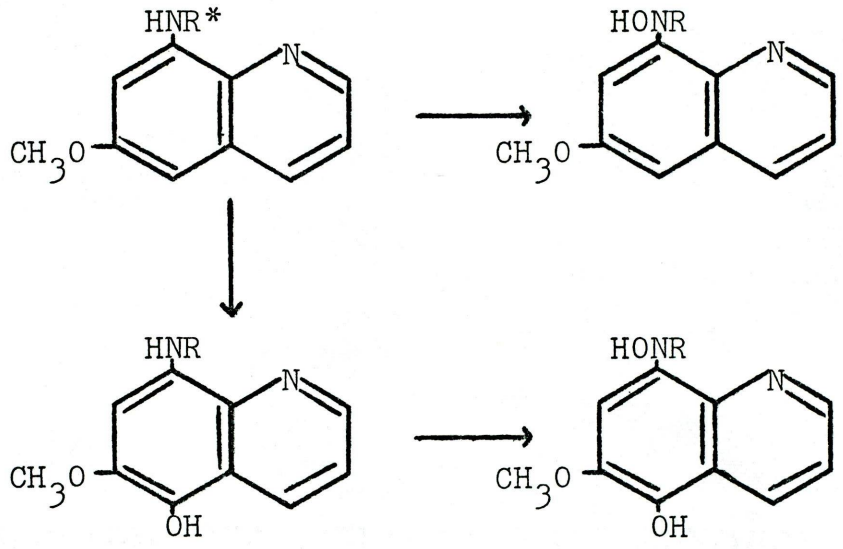


Figure 6: Possible pathway for the N-hydroxylation of primaquine at the 8-amino nitrogen. *R = $CH(CH_3)(CH_2)_3NH_2$.

microsomes; evidence was presented demonstrating that the N-oxidation of the tertiary arylamine, N,N-dimethylaniline, requires a different oxidase than the N-hydroxylation of acetylaminofluorene. Although the N-oxidations of secondary and tertiary arylamines do not behave the same in every instance (Hlavica and Kiese, 1969), it is generally considered that they proceed by the same mechanism (see Uehleke, 1971, 1973). This difference between N-substituted arylamines and arylamides in N-oxidation could explain why methylcholanthrene failed to induce methemoglobin production from the metabolites of primaquine (Table VI) while phenobarbital did induce it (Table IV). However, in work with the N-oxidation of the secondary arylamines, N-methyl- and N-ethylaniline, the role of cytochrome P-450 is nebulous and phenobarbital-treated animals failed to demonstrate any induction in N-oxidation (Lange, 1967; Uehleke, 1967).

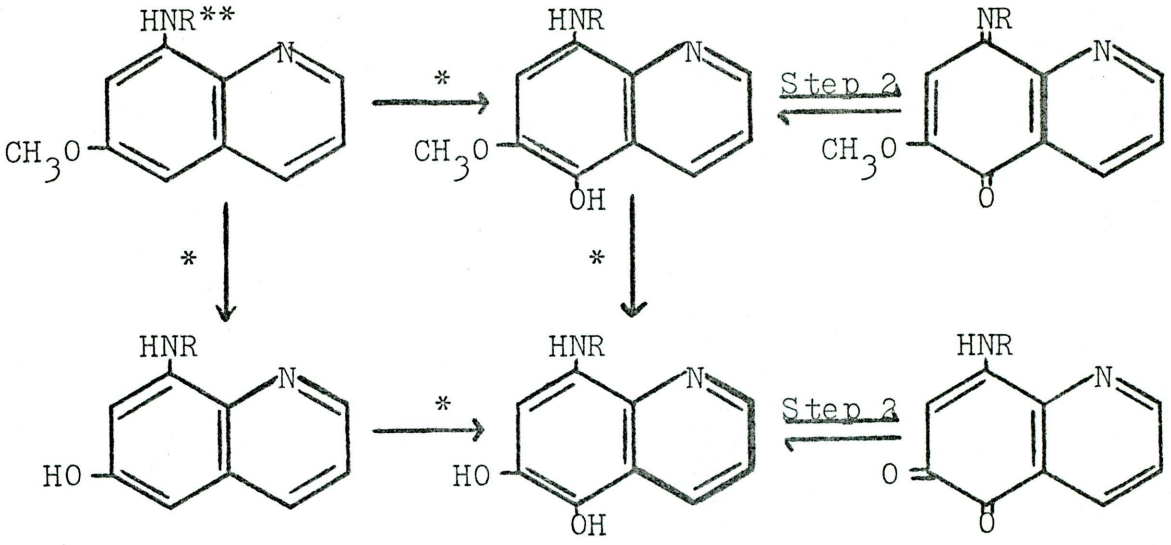
An amine oxidase that selectively acts on secondary and tertiary arylamines has been isolated from porcine liver (Zeigler and Pettit, 1966; Zeigler and Mitchell, 1972). The isolated oxidase is a flavoprotein containing no iron (and therefore no cytochrome P-450) and effectively N-oxidizes several secondary and tertiary arylamines (Zeigler and Mitchell, 1972). This enzyme was not inducible with phenobarbital (Masters and Zeigler, 1971). However, in the rat

a non-isolated liver microsomal tertiary amine oxidase was shown to be inducible with diethylbarbital (Machinist et al., 1968). Since the rat amine oxidase was not isolated nor characterized, it is not possible to state that it is identical with the porcine amine oxidase. From these studies with the rat and pig it can be concluded that there are species differences in the ability to N-oxidize secondary and tertiary arylamines. Data comparing rats and hamsters in the activity of another enzyme, aryl hydrocarbon hydroxylase, induced with methylcholanthrene and phenobarbital confirm that these two animals differ in the inducibility of enzyme systems (Nebert et al., 1972), so that the ability to induce hamsters with phenobarbital cannot be inferred from the data presented in the rat and pig, yet it does not exclude its possibility. Even if the N-oxidation of the 8-amino nitrogen of primaquine is not inducible in the hamster with phenobarbital, the 5-hydroxylation, which is inducible, may increase the N-oxidation in a similar manner to that reported for p-substituted arylamines by Uehleke (1962). This could explain why the role of cytochrome P-450 in the production of methemoglobin-forming metabolites is limited.

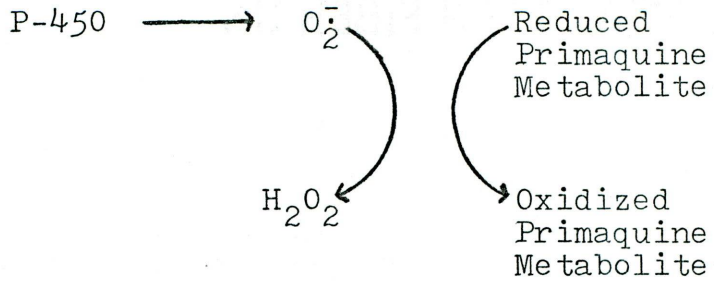
In a recent report on the hepatotoxic effects of α-methyldopa, Dybing et al. (1976) demonstrated the production by cytochrome P-450 of a superoxide anion which forms hydro-

gen peroxide as it oxidizes α-methyldopa. The process was not inducible with phenobarbital, but decreases in cytochrome P-450 after cobaltous chloride treatment paralleled decreases in irreversible binding of α-methyldopa to rat and mouse liver microsomal protein. This presents interesting possibilities for primaquine metabolism since hydrogen peroxide is a known methemoglobin-forming agent (Kiese, 1966) and it has been observed in erythrocytes after primaquine administration (Cohen and Hochstein, 1964) as well as after incubation with the primaquine metabolites, 8-amino-5,6-quinolinediol, 5-hydroxy-, 6-hydroxy- and 5,6-dihydroxyprimaquine (Fraser et al., 1975). The 5-hydroxylated derivatives of primaquine were by far the most effective in generating hydrogen peroxide.

The role of cytochrome P-450 could be two-fold in the process of methemoglobin formation (Figure 7). The first would be to convert primaquine to easily oxidizable compounds like the 5-hydroxy derivatives which can donate an electron to the superoxide thus forming hydrogen peroxide plus a quinone or quinonimine derivative of primaquine; both products could then interact with oxyhemoglobin to produce methemoglobin. The second role would be its direct involvement in the production of the superoxide anion as described by Dybing et al. (1976). However, Wallace and Caughey (1975) have de-



Step 1: Possible cytochrome P-450 dependent changes on the primaquine molecule. *Denotes a process requiring cytochrome P-450. **R = $CH(CH_3)(CH_2)_3NH_2$ or H (if N-dealkylated).



Step 2: Possible role of cytochrome P-450-produced superoxide anion in the oxidation of primaquine metabolites (adapted from Dybing et al., 1976).

Figure 7: Possible mechanism for the production of methemoglobin-forming compounds from primaquine by cytochrome P-450 dependent processes.

scribed a mechanism by which hydrogen peroxide and methemoglobin are produced through a direct reduction of dioxygen bound to hemoglobin by an electron from an external oxidant donor (Figure 8). This too suggests an explanation for the

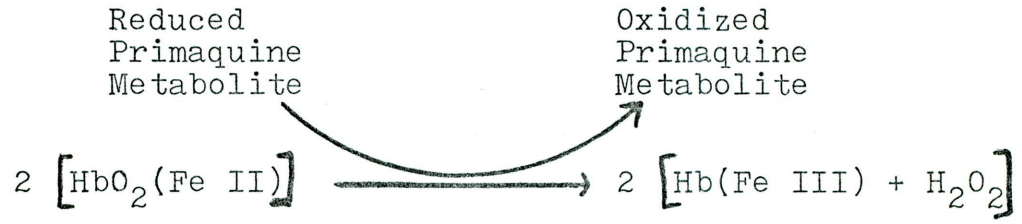


Figure 8: Proposed mechanism of direct oxidation of hemoglobin by reduction of bound dioxygen and a primaquine-metabolite-oxidant (adapted from Wallace and Caughey, 1975).

methemoglobin and hydrogen peroxide observed in erythrocytes but with this mechanism the role of cytochrome P-450 is limited to Step 1 as diagramed in Figure 7.

The results of the experiments presented in this report can be interpreted in the light of the peroxide-superoxide mechanisms. A 15% increase in methemoglobin production with phenobarbital could represent the increased formation of easily oxidizible metabolites of primaquine, the quinolinequinones and quinolinequinonimes, through increases in the cytochrome P-450 dependent and inducible processes of 5-hydroxylation, 6-O-demethylation and N-dealkylation. The limited extent of the induction of methemoglobin formation

agrees with the finding of Dybing et al. (1976) that the generation of superoxide anions by cytochrome P-450 is not inducible with phenobarbital. The cobalt treatment results also agree with the results of Dybing et al. (1976) because they show a dependence of methemoglobin formation on P-450 content only at lower than control levels.

The negative relationship between cytochrome P-450 and methemoglobin formation from dihydroxyprimaquine might be accounted for by this pathway also. At lower cytochrome P-450 levels, there would be less ring-hydroxylations at positions that possibly prevent or reduce the extent of oxidation (eg. 2, 3, 4 or 7) thus allowing the oxidation of more dihydroxyprimaquine molecules, particularly through the mechanism of Wallace and Caughey (1975). At the higher P-450 levels, the ring-hydroxylations could decrease the ability of the molecule to act as an oxidant in either of the two mechanisms discussed, and therefore, a decrease in methemoglobin production would be observed. Problems arise, however, when this explanation is applied to the induced animals. As stated earlier, a decrease would be expected in methemoglobin-forming ability if these extra ring-hydroxylations were actually involved. Also, Table V shows that at a lower protein concentration an almost 50% induction of this process occurred. Other factors must limit the metabolism of dihydroxyprimaquine

to more potent methemoglobin-forming compounds. Possibly, the increases in reductases with phenobarbital induction (Peters, 1973; Tsyrlov et al., 1976) and the increased ratio of reductases to cytochrome P-450 after cobalt treatment could be involved. If dihydroxyprimaquine is converted to the quinolinequinone by the reduction of either superoxide or bound dioxygen with the subsequent production of hydrogen peroxide, then there may be a greater demand for the reductases to supply reducing equivalents to convert the quinone back to dihydroxyprimaquine, which could then by oxidized to produce more hydrogen peroxide. Thus the final result would be larger amounts of hydrogen peroxide than that produced before, and this, added with the quinolinequinone formation, might account for the induction of methemoglobin formation with dihydroxyprimaquine. Clearly, more research is needed to determine if the reductases are limiting to this reaction or if other factors are involved.

CONCLUSIONS AND SUMMARY

It is very likely that cytochrome P-450 is involved in the metabolism of primaquine by hamster liver microsomes to methemoglobin-forming compounds. The cytochrome P-450 mediated steps appear to be only portions of the total process leading to increases in methemoglobin-forming since only a 15% increase occurred with phenobarbital-induced microsomes. The most likely involvements of cytochrome P-450 are in the mechanisms of 5-hydroxylation, 6-O-demethylation and, to a lesser extent, N-dealkylation (Figure 9). Also the possibility exists for either an N-hydroxylation at the 8-amino nitrogen or quinone formation through oxidation of the hydroxylated primaquine derivatives and these could be responsible for much of the methemoglobin produced. The oxidized primaquine derivatives can arise via cytochrome P-450-generated superoxide anions and/or a direct reduction of hemoglobin-bound dioxygen. All of these mechanisms may be true to some extent. In any case, isolation and quantification of the metabolites of primaquine produced *in vitro* and *in vivo* will be necessary to determine the major mechanism of methemoglobin production by primaquine, and ultimately, the development of a relatively non-toxic yet effective antimalarial agent.

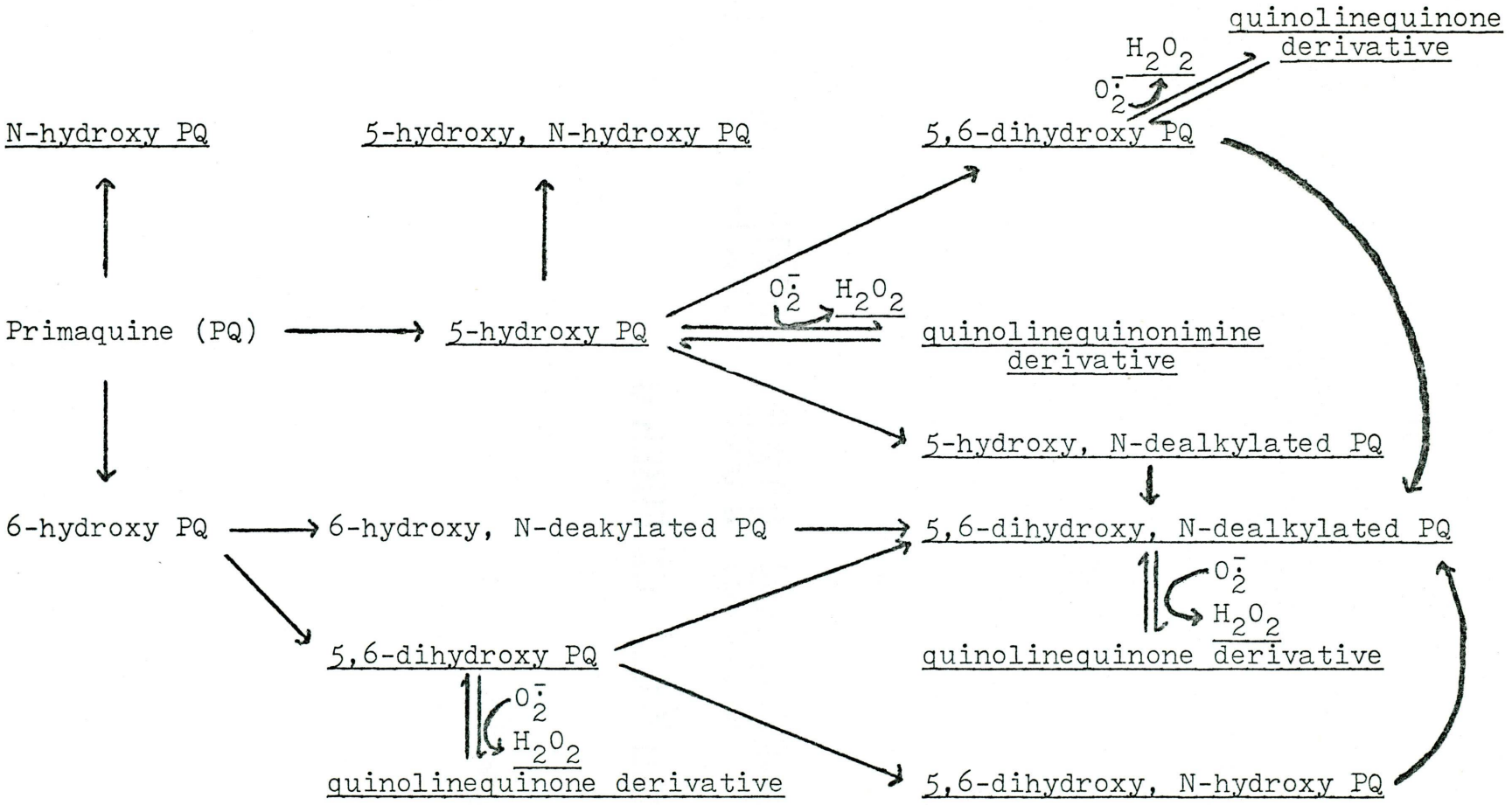


Figure 9: A summary of the postulated pathways for the metabolism of primaquine to methemoglobin-forming compounds. Underlined terms refer to either known or postulated potent methemoglobin-forming compounds. The superoxide anion, O_2^-, is assumed to be formed from cytochrome P-450.

BIBLIOGRAPHY

Alving, A. S., Powell, R. D., Brewer, G. J. and Arnold, J. D.: Malaria, 8-aminoquinolines and haemolysis. In Drugs, Parasites and Hosts, ed. by L. G. Goodwin and R. H. Nimmo-Smith, pp. 83-115, J. & A. Churchill, Ltd., London, 1962.

Beutler, E.: Drug-induced hemolytic anemia. Pharm. Rev. 21: 73-103, 1969.

Brodie, B. B. and Udenfriend, S.: Metabolites of pamaquine in urine. Proc. Soc. Exp. Biol. Med. 74: 845-848, 1950.

Cohen, G. and Hochstein, P.: Generation of hydrogen peroxide in erythrocytes by hemolytic agents. Biochemistry 3: 895-900, 1964.

Conney, A. H.: Pharmacological implications of microsomal enzyme induction. Pharm. Rev. 19: 317-366, 1967.

Cooper, D. Y., Schleyer, H. and Rosenthal, O.: Some chemical properties of cytochrome P-450 and its carbon monoxide compound (P-450-CO). N. Y. Acad. Sci. Ann. 174:205-217, 1970.

Dybing, E., Nelson, S. D., Mitchell, J. R., Sasame, H. A. and Gillette, J. R.: Oxidation of α-methyldopa and other catechols by cytochrome P-450-generated superoxide anion: possible mechanism of methyldopa hepatitis. Mol. Pharm. 12: 911-920, 1976.

Elderfield, R. C. and Smith, L. L.: On the physiological disposition of pentaquine labeled in the side chain with N^{15}. J. Amer. Chem. Soc. 75: 1022-1024, 1953.

Fraser, I. M., Tilton, B. E. and Strother, A.: The relationship of hydrogen peroxide production to glutathione depletion and methemoglobin formation by model metabolites of primaquine and phenacetin in normal and glucose-6-phosphate dehydrogenase deficient erythrocytes. Pharmacologist 17: 250, 1975.

Fraser, I. M., Tilton, B. E. and Strother, A.: Methemoglobin formation in normal and glucose-6-phosphate dehydrogenase (G6PD) deficient human erythrocytes by primaquine and a mouse liver microsomal system. Fed. Proc. 35: 244, 1976.

Fraser, I. M. and Vesell, E. S.: Effects of drugs and drug metabolites on erythrocytes from normal and glucose-6-phosphate dehydrogenase-deficient individuals. N. Y. Acad. Sci. Ann. 151: 777-794, 1968a.

Fraser, I. M. and Vesell, E. S.: Effects of metabolites of primaquine and acetanilid on normal and glucose-6-phosphate dehydrogenase-deficient erythrocytes. J. Pharm. Exp. Ther. 162: 155-165, 1968b.

Goldstein, A., Aronow, L. and Kalman, S. M.: Principles of Drug Action: The Basis of Pharmacology, 2nd ed., pp. 242-251, John Wiley & Sons, Inc., New York, 1974.

Greenberg, J., Taylor, D. J. and Josephson, E. S.: Studies on Plasomodium gallinaceum in vitro: II. The effects of some 8-aminoquinolines against the erythrocytic parasites. J. Infect. Dis. 88: 163-167, 1951.

Hinson, J. A., Mitchell, J. R. and Jollow, D. J.: Microsomal N-hydroxylation of p-chloroacetanilide. Mol. Pharm. 11: 462-469, 1975.

Hinson, J. A., Mitchell, J. R. and Jollow, D. J.: N-hydroxylation of p-chloroacetanilide in hamsters. Biochem. Pharm. 25: 599-601, 1976.

Hlavica, P.: Interaction of oxygen and aromatic amines with hepatic microsomal mixed-function oxidase. Biochim. Biophys. Acta 273: 318-327, 1972.

Hlavica, P. and Kiese, M.: N-oxygenation of N-alkyl- and N,N-dialkylanilines by rabbit liver microsomes. Biochem. Pharm. 18: 1501-1509, 1969.

Josephson, E. S., Taylor, D. J., Greenberg, J. and Ray, A. P.: A metabolic intermediate of pamaquine from chickens. Proc. Soc. Exp. Biol. Med. 76: 700-703, 1951.

Kellermeyer, R. W., Tarlov, A. R., Brewer, G. J., Carson, P. E. and Alving, A. S.: Hemolytic effect of therapeutic drugs: Clinical considerations of the primaquine-type hemolysis. J. A. M. A. 180: 388-394, 1962.

Kiese, M.: Relationship of drug metabolism to methemoglobin formation. N. Y. Acad. Sci. Ann. 123: 141-155, 1965.

Kiese, M.: The biochemical production of ferrihemoglobin-forming derivatives from aromatic amines, and mechanisms of ferrihemoglobin formation. Pharm. Rev. 18: 1091-1161, 1966.

Lange, G.: Hydroxylase-Aktivität und Hämoprotein-Gehalt von Mikrosomen aus Kaninchenleber. N. S. Arch. Pharmak. Path. 259: 221-238, 1968.

Lotlikar, P. D., Luha, L. and Zaleski, K.: Reconstituted hamster liver microsomal enzyme system for N-hydroxylation of the carcinogen 2-acetylaminofluorene. Biochem. Biophys. Res. Comm. 59: 1349-1355, 1974.

Lotlikar, P. D., Wertman, K. and Luha, L.: Role of mixed-function amine oxidase in N-hydroxylation of 2-acetamidofluorene by hamster liver microsomal preparations. Biochem. J. 136: 1137-1140, 1973.

Lotlikar, P. D. and Zaleski, K.: Inhibitory effect of carbon monoxide on the N- and ring-hydroxylation of 2-acetamidofluorene by hamster hepatic microsomal preparations. Biochem. J. 144: 427-430, 1974.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.: Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275, 1951.

Lu, A. Y. H., Levin, W., West, S. B., Jacobson, M., Ryan, D., Kuntzman, R. and Conney, A. H.: Reconstituted liver microsomal enzyme system that hydroxylates drugs, other foreign compounds, and endogenous substrates: VI. Different substrate specificities of the cytochrome P-450 fractions from control and phenobarbital-treated rats. J. Biol. Chem. 248: 456-460, 1973.

Machinist, J. M., Dehner, E. W. and Ziegler, D. M.: Microsomal oxidases: III. Comparison of species and organ distribution of dialkylarylamine N-oxide dealkylase and dialkylarylamine N-oxidase. Arch. Biochem. Biophys. 125: 858-864, 1968.

Masters, B. S. S. and Ziegler, D. M.: The distinct nature and function of NADPH-cytochrome c reductase and the NADPH-dependent mixed function amine oxidase of porcine liver microsomes. Arch. Biochem. Biophys. 145: 358-364, 1971.

Nash, T.: The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochemistry 55: 416-421, 1953.

Nebert, D. W., Gielen, J. E. and Goujon, F. M.: Genetic expression of aryl hydrocarbon hydroxylase induction: III. Changes in the binding of n-octylamine to cytochrome P-450. Mol. Pharm. 8: 651-666, 1972.

Netter, K. J. and Seidel, G.: An adaptively stimulated O-demethylating system in rat liver microsomes and its kinetic properties. J. Pharm. Exp. Ther. 146: 61-65, 1964.

Omura, T. and Sato, R.: The carbon monoxide-binding pigment of liver microsomes: II. Solubilization, purification, and properties. J. Biol. Chem. 239: 2379-2385, 1964.

Peters, M. A.: Relative selectivity of some microsomal drug metabolizing enzyme inducers. Arch. Int. Pharm. Ther. 203: 30-45, 1973.

Robin, H. and Harley, J. D.: Practical applications of the simultaneous determination of oxyhaemoglobin and methaemoglobin concentrations. Aust. Ann. Med. 13: 313-319, 1964.

Smith, C. C.: Metabolism of pentaquine in the rhesus monkey. J. Pharm. Exp. Ther. 116: 67-76, 1956.

Smith, R. V. and Rosazza, J. P.: Microbial models of mammalian metabolism. J. Pharm. Sci. 64: 1737-1759, 1975.

Strother, A., Fraser, I. M. and Tilton, B.: Studies of the metabolism of the antimalarial agent primaquine. Paper presented at Sixth International Congress of Pharmacology, Helsinki, Finland, 1975.

Tephly, T. R. and Hibbeln, P.: The effect of cobalt chloride administration on the synthesis of hepatic microsomal cytochrome P-450. Biochem. Biophys. Res. Comm. 42: 589-595, 1971.

Thorgeirsson, S. S., Jollow, D. J., Sasame, H. A., Green, I. and Mitchell, J. R.: The role of cytochrome P-450 in N-hydroxylation of 2-acetylaminofluorene. Mol. Pharm. 9: 398-404, 1973.

Thorgeirsson, S. S., Sasame, H. A., Mitchell, J. R., Jollow, D. J. and Potter, W. Z.: Biochemical changes after hepatic injury from toxic doses of acetaminophen or furosemide. Pharmacology 14: 205-217, 1976.

Tsyrlov, I. B., Zakharova, N. E., Gromova, O. A. and Lyakhovich, V. V.: Possible mechanism of induction of liver microsomal monooxygenases by phenobarbital. Biochim. Biophys. Acta 421: 44-56, 1976.

Uehleke, H.: Relations between structure, velocity of biological N-hydroxylation and toxicity of aromatic amines. In Proceedings First International Pharmacological Meeting, ed. by B. B. Brodie and E. G. Erdos, Pergamon Press, Oxford, vol. 6, pp. 31-37, 1962.

Uehleke, H.: Stimulierung einiger mikrosomaler Fremdstoff-Oxydationen durch Phenobarbital, Methylcholanthren und Chlorophenothan, einzeln und in Kombinationen. N. S. Arch. Pharmak. Path. 259: 66-90, 1967.

Uehleke, H.: N-hydroxylation. Xenobiotica 1: 327-338, 1971.

Uehleke, H.: The role of cytochrome P-450 in the N-oxidation of individual amines. Drug Metab. Dispos. 1: 299-313, 1973.

Uehleke, H. and Hellmer, K. H.: Competitive inhibition of the microsomal N-hydroxylation of 4-chloroaniline by metyrapone and cyclohexane. N. S. Arch. Pharmak. 268: 242-246, 1971.

Wagner, G. S. and Tephly, T. R.: A possible role of copper in the regulation of heme biosyntheses through ferrochelatase. In Cytochromes P-450 and b_5: Structure, Function and Interaction, ed. by D. Y. Cooper, O. Rosenthal, R. Synder and C. Witmer. Advances in Experimental Medicine and Biology, vol. 58, Plenum Press, New York, pp. 343-354, 1975.

Wallace, W. J. and Caughey, W. S.: Mechanism for the autoxidation of hemoglobin by phenols, nitrite and "oxidant" drugs: Peroxide formation by one electron donation to bound dioxygen. Biochem. Biophys. Res. Comm. 62: 561-567, 1975.

Weinberg, G. H. and Schumaker, J. A.: Statistics, an Intuitive Approach, 2nd ed. Wadsworth Publishing Co., Belmont, California, 1969.

Zeigler, D. M. and Mitchell, C. H.: Microsomal oxidase IV: Properties of a mixed-function amine oxidase isolated from pig liver microsomes. Arch. Biochem. Biophys. 150: 116-125, 1972.

Zeigler, D. M. and Pettit, F. H.: Microsomal oxidases I: The isolation and dialkylarylamine oxygenase activity of pork liver microsomes. Biochemistry 5: 2932-2938, 1966.