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SOLUBILISATION, PURIFICATION AND RECONSTITUTION OF HEPATIC MICROSOMAL AZOREDUCTASE ACTIVITY

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Abstract—Microsomal NADPH-cytochrome *c* (P-450) reductase and cytochrome P-450 were purified from the livers of phenobarbitone-treated rats. Purified NADPH-cytochrome *c* (P-450) reductase effected the NADPH-dependent reduction of FMN and FAD under anaerobic conditions in a non-enzymic manner, but was unable to reduce directly the azo dye, amaranth. In the presence of FMN, the purified reductase effected reduction of amaranth through the production of reduced FMN. Incorporation of NADPH-cytochrome *c* (P-450) reductase into the microsomal fraction increased the azoreductase activity of liver preparations from phenobarbitone-treated rats, but had no effect on azoreductase activity in preparations from control animals. Azoreductase activity was reconstituted into dilauroyl phosphatidylcholine vesicles containing purified cytochrome P-450 and purified NADPH-cytochrome *c* (P-450) reductase. In the absence of supplementary FMN, amaranth reduction was completely dependent upon all three components, but in the presence of FMN, the omission of any one component failed to abolish completely azoreductase activity.

Studies of the response of microsomal azoreductase to a variety of inhibitors have led to the postulate that two major routes of azo reduction exist in the liver. These comprise a carbon monoxide-sensitive pathway, dependent upon cytochrome P-450 haemoproteins [1–4], and a carbon monoxide-insensitive pathway attributed to the flavoprotein, NADPH-cytochrome *c* (P-450) reductase [1, 2, 5]. Treatment of rats with a number of compounds known selectively to induce or to destroy components of the microsomal electron transport chain have been reported to alter both azoreductase activity and cytochrome P-450 haemoprotein content [3, 6]. Antisera raised against cytochrome P-450 or cytochrome P-448 inhibited hepatic microsomal azoreductase activity from rats treated with phenobarbitone or 3-methylcholanthrene, respectively [7].

The azoreductase activity of the microsomal fraction towards a number of substrates may be increased by the addition of soluble flavins to the system [4–7]. These redox components have been proposed as electron carriers from certain microsomal enzymes to the azo substrates [4, 6, 8]. Analysis of the kinetics of this flavin-stimulated azo reduction showed a biphasic response to changes in flavin concentration, suggesting a complex interaction between flavin and the microsomal fraction [6].

Much attention has been directed towards the purification of hepatic microsomal enzymes with the aim of reconstituting a variety of cytochrome P-450-dependent functions [9, 10]. Purification of NADPH-cytochrome *c* (P-450) reductase has also yielded information regarding the interaction of this flavoprotein with several electron acceptors [11, 12].

The reconstitution of azoreductase activity from the purified components of this NADPH-dependent system may indicate the role of haemoprotein and flavoprotein enzymes in azo reduction in the intact microsomal membrane. Throughout this paper, amaranth has been used to exemplify azo reduction.

MATERIALS AND METHODS

Chemicals. Glycerol AR and sodium dodecyl sulphate were purchased from BDH Ltd., Poole, U.K.; dilauroyl L-α-phosphatidylcholine and Tris-base from Sigma Chemical Co. (Poole, U.K.); dithiothreitol from Aldrich Chemical Company (Gillingham, U.K.); the molecular weight markers (aldolase, bovine serum albumin, catalase, chymotrypsinogen A and hen egg albumin) from Boehringer Corp. (Lewes, U.K.); DEAE-cellulose (DE 52) from Whatman Ltd., (Maidstone, U.K.) and 2′,5′-ADP-Sepharose 4B from Pharmacia Ltd. (Hounslow, U.K.). All other chemicals and reagents were obtained as described previously [6].

Animals. Male Wistar albino rats (University of Surrey, Rodent Breeding Unit), 180–220 g body weight, were maintained as described previously [6]. Where necessary, rats were injected intraperitoneally with phenobarbitone sodium in 0.154 M saline (30 mg/ml) at 80 mg/kg body weight for three consecutive days. Animals were killed 24 hr after the final dose.

Preparation of hepatic microsomal fraction. The hepatic microsomal fraction was isolated as described by Mallett *et al.* [6] after homogenisation in 0.1 M Tris–HCl buffer, pH 7.7, containing 0.15 M KCl and 1 mM EDTA at 8°. For purification of NADPH-cytochrome *c* (P-450) reductase, the washed microsomal fraction was resuspended at 8° in 0.1 M Tris–HCl buffer, pH 7.7, containing 20% (v/v) glycerol, 0.5 mM EDTA and 0.2 mM dithiothreitol, at a pro-

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tein concentration of 30 mg/ml, and was stored under nitrogen at −40°. In preparations for the purification of cytochrome P-450, the washed microsomal fraction was stored at −40° under nitrogen in 0.025 M sucrose at a protein concentration of 30 mg/ml. All samples were used within 7 days of preparation.

Purification and characterisation of NADPH-cytochrome c *(P-450) reductase.* NADPH-cytochrome *c* (P-450) reductase was purified on two separate occasions using ion-exchange chromatography on DEAE-cellulose followed by affinity chromatography on 2′,5′-ADP Sepharose 4B [13]. These products are subsequently referred to as Fp-1 and Fp-2. All operations were performed at 8° and were protected from light. The activities of the preparations (Fp-1, 34.3 and Fp-2, 44.1 μmole cytochrome *c* reduced/min/mg protein) were in close agreement with reported values [13]. On SDS-polyacrylamide gel electrophoresis, both preparations ran as one major band, corresponding to a molecular weight of 78,000 daltons, and specific assays revealed no contamination with cytochromes P-450 and b_5, nor with cytochrome b_5 reductase. The absorption spectra of the preparations were typical of NADPH-cytochrome *c* (P-450) reductase [13, 14].

Purification and characterisation of cytochrome P-450. Cytochrome P-450 was purified by DEAE-cellulose ion-exchange chromatography [15] and all operations were performed at 8° and were protected from light. The specific cytochrome P-450 content of the preparation (14.4 nmole/mg protein) was similar to that reported by West *et al.* [15]. SDS-polyacrylamide gel electrophoresis revealed the presence of only one band, corresponding to a molecular weight of 53,000 daltons, in close agreement with reported values [15]. The carbon monoxide difference spectrum of the dithionite-reduced haemoprotein showed a single peak at 450 nm.

Polyacrylamide gel electrophoresis of purified microsomal proteins. Polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulphate was performed as described by Laemmli [16]. Chymotrypsinogen A, aldolase, hen egg albumin, catalase and bovine serum albumin were run in parallel for comparative molecular weight estimations.

Determination of NADPH-dependent azo reduction and flavin reduction by purified NADPH-cytochrome c *(P-450) reductase.* The anaerobic reduction of amaranth (50–500 μM), in the absence or presence of 300 μM FMN or FAD, was determined at a flavoprotein concentration of 60 μg/ml in stoppered, 2 mm pathlength cuvettes in 50 mM sodium phosphate buffer, pH 7.4 (0.7 ml) at 37°. A NADPH-generating system and oxygen-scavenging system were utilised as described previously [6].

The flavoprotein-dependent reduction of flavin was determined under the same conditions by substituting FAD or FMN (25–500 μM) for amaranth. An extinction coefficient of 11.30 1 $mmole^{-1}$ cm^{-1} at 450 nm was used for FAD, while FMN reduction was monitored at 445 nm using an extinction coefficient of 12.50 1 $mmole^{-1}$ cm^{-1} [17].

Incorporation of purified NADPH-cytochrome c *(P-450) reductase into microsomal fractions.* Purified NADPH-cytochrome *c* (P-450) reductase was incorporated into the microsomal fraction from control or phenobarbitone-pretreated rats by modifications to the method of Gum and Strobel [18]. The microsomal fraction was resuspended in 50 mM sodium phosphate buffer, pH 7.4, containing 0.25 M sucrose and 0.5 mM EDTA, to a protein concentration of approximately 5 mg/ml. A sample of this stock suspension (6 ml) was incubated for 20 min at 37° with various amounts of purified flavoprotein (0–200 μg/incubation) in a final volume of 8 ml, in a shaking water bath (60 cycles/min). After incubation, 17 ml of ice-cold phosphate/sucrose/EDTA buffer was added to each flask, and the microsomal fraction sedimented at 178,000 g_{av} for 35 min at 4°. The resulting pellet was resuspended in 25 ml ice-cold buffer, resedimented as above and resuspended in 6 ml buffer. Cytochrome P-450 content, NADPH-cytochrome *c* (P-450) reductase activity and azoreductase activity were determined as described below.

Reconstitution of azoreductase activity. Dilauroyl phosphatidylcholine vesicles were prepared by dissolving 3 mg of dilauroyl phosphatidylcholine in 0.2 ml chloroform, allowing the solvent to evaporate, adding 3 ml distilled water and sonicating the mixture for 10 min at 15° in a Kerry sonic bath. Aliquots (20 μg) of the resulting suspension of vesicles were mixed with 1.2 nmole of purified cytochrome P-450 and 0.06 nmole of flavoprotein in a stoppered 2 mm pathlength quartz cuvette and incubated at 37° for 1 min.

Azoreductase activity was determined at 37° with 75 μM amaranth as substrate in a final volume of 0.7 ml 50 mM sodium phosphate buffer, pH 7.4, using essentially the method described previously [6] except that the reaction was initiated by the addition of 0.5 mM $NADP^+$ to the sample cuvette. Azoreductase activity was calculated from the decrease in amaranth absorbance at 520 nm and corrected for reduction mediated by the NADPH-generating system alone. Azoreductase activity was determined under similar conditions, after the addition of 300 μM FMN to the system.

The effect of omitting a particular component of the reconstituted microsomal system on amaranth reduction was also investigated.

Specific assays. Cytochrome P-450 was determined by the method of Omura and Sato [19] and NADPH-cytochrome *c* (P-450) reductase activity by the method of Yasukochi and Masters [13]. Protein determinations were performed by the method of Lowry *et al.* [20], modified where necessary for solutions containing interfering detergents [21].

RESULTS

Flavin reduction and azo reduction mediated by purified NADPH-cytochrome c *(P-450) reductase*

The purified flavoprotein was capable of donating electrons to FMN and FAD under anaerobic conditions, and a linear relationship was found between flavin concentration (25–500 μM) and the rate of flavin reduction. FMN was reduced at a faster rate than equimolar FAD at all flavin concentrations studied. Plotting this data according to the method of Lineweaver and Burk gave lines that passed through the origin, assigning infinite values to K_m

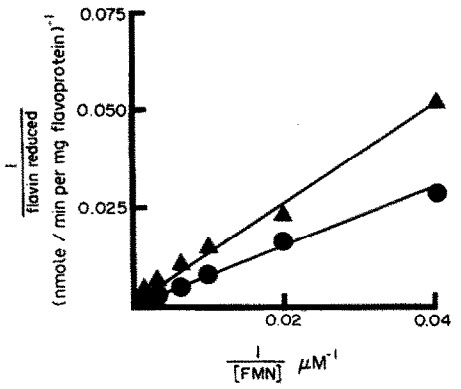


Fig. 1. Lineweaver–Burk plot of flavin reduction by purified NADPH-cytochrome *c* (P-450) reductase (Fp-1). Anaerobic incubations at 37° in stoppered cuvettes contained NADPH-cytochrome *c* (P-450) reductase (Fp-1, 60 μg/ml), a NADPH-generating system, an oxygen-scavenging system and 25–500 μM FMN, ●, or FAD, ▲, in 50 mM sodium phosphate buffer, pH 7.4 (0.7 ml). Flavin reduction was determined from the linear decrease in absorbance at 445 nm (FMN) or 450 nm (FAD) against a reference from which $NADP^+$ was omitted.

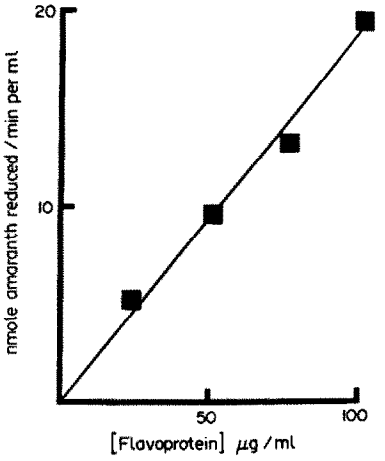


Fig. 2. Variation of FMN-supplemented azoreductase activity with the concentration of purified NADPH-cytochrome *c* (P-450) reductase (Fp-1). Azoreductase activity was determined as described in the legend to Table 2.

and V_{max} (Fig. 1). The purified flavoprotein was unable to donate electrons directly to amaranth (50–500 μM) since the rate of azo reduction in the presence of flavoprotein did not differ significantly from that mediated by the NADPH-generating system alone (Table 1). Nevertheless, inclusion of both amaranth and flavin in this anaerobic system resulted in an appreciable rate of azo reduction, which varied in direct proportion to changes in flavoprotein concentration (Fig. 2). At a flavoprotein concentration of 60 μg/ml, the rate of amaranth reduction in the presence of added flavin was dependent upon flavin concentration. A Lineweaver–Burk plot of this data (Fig. 3) again passed through the origin.

Incorporation of purified NADPH-cytochrome c *(P-450) reductase into the microsomal membrane*

Incubation of increasing concentrations of purified NADPH-cytochrome *c* (P-450) reductase with the microsomal fraction from control rats resulted in only a low incorporation of the flavoprotein into the membraneous fraction as assessed by the increases in the NADPH-dependent reduction of cytochrome *c* (Table 2a). This low incorporation produced no

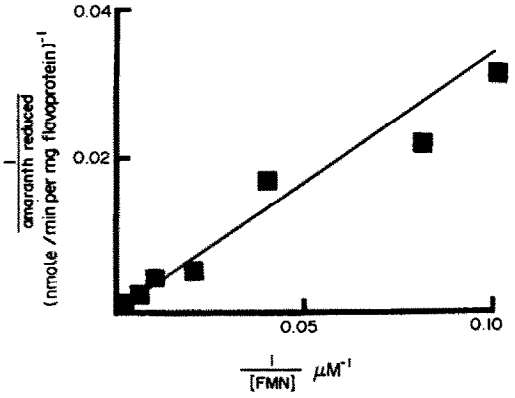


Fig. 3. Lineweaver–Burk plot of azo reduction produced by purified NADPH-cytochrome *c* (P-450) reductase (Fp-1) in the presence of soluble FMN. Azo reduction was determined as described in the legend to Table 2.

Table 1. Lack of azo reduction by NADPH-cytochrome *c* (P-450) reductase

Armaranth concentration (μM)	Azo reduction (nmole amaranth reduced/min/ml)	
	With reductase	Without reductase
50	1.1 ± 0.1	0.9 ± 0.1
100	1.9 ± 0.1	1.3 ± 0.1
150	2.4 ± 0.3	2.6 ± 0.1
300	4.5 ± 0.4	4.5 ± 0.4
500	5.6 ± 0.3	5.1 ± 0.3

Values are the means ± S.D. of triplicate determinations.

The rate of amaranth reduction at 37° was followed by the decrease in absorbance at 520 nm of anaerobic incubations in 50 mM sodium phosphate buffer, pH 7.4 (0.7 ml) containing NADPH-cytochrome *c* (P-450) reductase (Fp-1, 60 μg/ml), a NADPH-generating system, an oxygen-scavenging system and various concentrations of amaranth. The rate of reduction of amaranth by the NADPH-generating system alone was determined by omitting the reductase. The NADPH-generating system was omitted from the reference samples.

Table 2. Effect of incorporation of NADPH-cytochrome *c* (P-450) reductase into the microsomal fractions from untreated or phenobarbitone-treated rats on azoreductase activity

Additional flavoprotein (ml)	Cytochrome P-450 content (nmole/mg protein)	NADPH-cytochrome *c* reductase activity (nmole/min/mg protein)	Azoreductase activity (nmole amaranth reduced/min/mg protein)	
			−FMN	+FMN
(a) *Untreated rats*				
0.00	0.72	117.9 ± 3.2	2.5 ± 0.2	11.5 ± 0.2
0.25	0.71	124.7 ± 9.4	2.6 ± 0.1	12.3 ± 0.4
0.50	0.71	128.7 ± 7.6	2.5 ± 0.3	11.7 ± 0.4
1.00	0.70	137.3 ± 4.1*	2.3 ± 0.2	11.4 ± 0.2
(b) *Phenobarbitone-treated rats*				
0.00	1.85	113.8 ± 2.5	4.7 ± 0.1	14.4 ± 0.8
0.25	1.78	130.1 ± 6.8	4.4 ± 0.1	14.6 ± 0.6
0.50	1.87	150.3 ± 8.0	5.2 ± 0.1†	17.9 ± 1.2†
1.00	1.94	168.5 ± 6.0‡	5.2 ± 0.2†	18.5 ± 1.0*

Enzyme activities are given as mean ± S.D. of triplicate determinations.
† $P < 0.05$; * $P < 0.01$; ‡ $P < 0.001$.
The microsomal fractions from untreated or phenobarbitone-treated rats were incubated with purified NADPH-cytochrome *c* reductase (Fp-2, 200 μg protein per ml) as described in Methods. Anaerobic incubations at 37° in stoppered cuvettes contained microsomal protein (approx. 1 mg/ml), a NADPH-generating system, an oxygen-scavenging system and amaranth (75 μM) in 50 mM sodium phosphate buffer, pH 7.4 (0.7 ml) with or without the addition of FMN (300 μM). Azoreductase activity was determined from the linear decrease in absorbance at 520 nm against a reference from which $NADP^+$ was omitted.

detectable increases in azoreductase activity, either in the presence or the absence of supplementary FMN.

In contrast, the incorporation of additional flavoprotein into the microsomal fraction from phenobarbitone-treated rats was greater and enhanced azoreductase activity both in the absence and presence of supplementary flavin (Table 2b).

Reconstitution of azoreductase activity

Azoreductase activity was reconstituted into dilauroyl phosphatidylcholine vesicles containing purified cytochrome P-450 and purified NADPH-cytochrome *c* (P-450) reductase (Table 3). In the absence of supplementary FMN, amaranth reduction was completely dependent upon these three components, and the omission of any one component completely abolished the azoreductase activity. Supplementation with 300 μM FMN increased the azoreductase activity of the complete system about 7-fold. In contrast to the assay in the absence of supplementary flavin, the omission of any one component decreased but failed to abolish amaranth reduction. Thus, vesicles containing either purified flavoprotein or cytochrome P-450 showed 30% and 14%, respectively, of the azoreductase activity of the complete system, while the omission of phospholipid gave an activity that was about 60% of that expressed by the complete system.

DISCUSSION

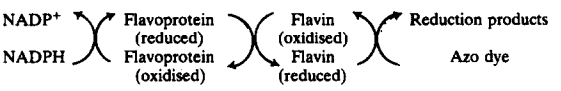
Although the purified NADPH-cytochrome *c* (P-450) reductase preparations had a high activity with respect to cytochrome *c* reduction, they were unable to support the reduction of amaranth directly, indicating that the azo dye is not a substrate for this enzyme. In the presence of added flavin, the enzyme supported reduction of the azo substrate, and since it also reduced flavin in the absence of azo dye, the mechanism proposed for azo reduction is as in Scheme 1, with the flavin acting as an electron shuttle.

Table 3. Reconstitution of azoreductase activity from purified NADPH-cytochrome *c* (P-450) reductase, purified cytochrome P-450 and dilauroyl phosphatidylcholine

Conditions	Azoreductase activity (nmole amaranth reduced/min/ml)	
	−FMN	+FMN
Complete system	1.2 ± 0.1	8.0 ± 0.6
Minus NADPH-cytochrome *c* (P-450) reductase	0.0	1.1 ± 0.1
Minus cytochrome P-450	0.0	2.4 ± 0.2
Minus dilauroyl phosphatidylcholine	0.0	4.7 ± 0.8

Values are given as mean ± S.D. for triplicate determinations.
Azoreductase activity was reconstituted from NADPH-cytochrome *c* (P-450) reductase (Fp-2, 0.06 nmole), cytochrome P-450 (1.2 nmole) and dilauroyl phosphatidylcholine (20 μg) as described in Methods. The enzymic activity was determined in the absence and presence of FMN (300 μM) as described in the legend to Table 2.

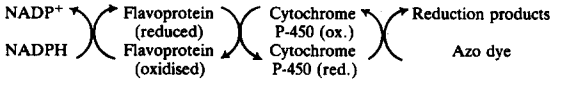
Scheme 1



Since it was not possible to saturate the enzyme with flavin, there was no evidence for the formation of a typical enzyme–substrate complex (Fig. 1). Similarly, the kinetics for the flavin-supplemented azo reduction of purified NADPH-cytochrome *c* (P-450) reductase (Fig. 3) showed no evidence for the formation of an enzyme–substrate complex. Thus amaranth reduction is solely dependent upon collisions between soluble, reduced flavin and amaranth. However, the overall rate of reduction was dependent upon the flavoprotein-mediated reduction of flavin, similar to the microbial system reported by Gingell and Walker [22].

Reconstitution of purified NADPH-cytochrome *c* (P-450) reductase and purified cytochrome P-450 into dilauroyl phosphatidylcholine vesicles demonstrated an absolute dependence of azoreductase activity upon all three of these components in the absence of added flavin (Table 3). This observation supports the view that in the intact microsomal membrane, as well as in the reconstituted system, cytochrome P-450 is the terminal electron donor to the azo substrate, as proposed in earlier studies [3, 4, 6]. The mechanism is therefore analogous to the oxidative function of this haemoprotein. Under these conditions, azo reduction would proceed as shown in Scheme 2.

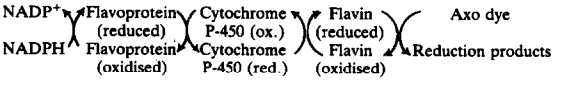
Scheme 2



In contrast, in the presence of soluble flavin, either of the enzymes in the reconstituted system could support amaranth reduction, but all three components were required for maximum activity. This suggests that, in the absence of cytochrome P-450 the mechanism of azo reduction attributed to the flavoprotein alone (Scheme 1) is also operative in the reconstituted system. In the reconstituted system, the electrons are probably passed to the soluble flavin from the FMN donor site of the flavoprotein, whereas in the intact microsomal membrane, this site is inaccessible to the soluble flavin and is normally closely coupled to the cytochrome P-450.

In the fully reconstituted system supplemented with flavin, it is apparent that cytochrome P-450 has a major role and it is proposed that the soluble flavin may act as an electron shuttle between the haemoprotein and the azo dye, as shown in Scheme 3.

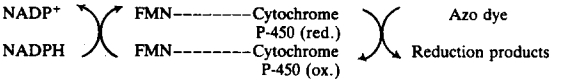
Scheme 3



Scheme 3 is also compatible with the flavin-supplemented activity seen in the isolated microsomal fraction and is supported by previous studies [4, 6] and by preliminary investigations on inhibition of the reaction by CO (unpublished observations).

The ability of the reconstituted system to support some FMN-supplemented azo reduction in the absence of NADPH-cytochrome *c* (P-450) reductase was a surprise finding. Although the rate of azo reduction was low compared with the complete system, it was double that of the non-enzymic reaction obtained with FMN and the NADPH-generating system alone (1.0 nmol/min/ml). Since cytochrome P-450 can reduce the dye directly, it is possible that the isolated cytochrome P-450 can facilitate the transfer of electrons from NADPH to the azo dye through an interaction of reduced FMN at the cytochrome's electron acceptor site, normally occupied by the reductase (Scheme 4). In view of the inaccessibility of this site in the microsomal membrane, it is unlikely that this mechanism operates in that system.

Scheme 4



The incorporation of additional NADPH-cytochrome *c* (P-450) reductase into the microsomal fraction from phenobarbitone-treated rats increased azoreductase in the absence of soluble flavin (Table 2b). This demonstrated that the increased microsomal cytochrome P-450 content after phenobarbitone induction was not matched by sufficient flavoprotein for maximum rate of reduction of the cytochrome, since the flavoprotein alone can not support azo reduction. Thus the enhancement of azo reduction produced by the additional incorporation of reductase in this system favours the mechanism in Scheme 2 for microsomal azo reductase in the absence of FMN.

The sub-optimum content of the reductase in the microsomal fraction from phenobarbitone-treated rats implies that some cytochrome P-450 is present in which the electron acceptor sites are not satisfied by the reductase. Therefore when FMN is present, the possibility that some azo reduction occurs by a mechanism analogous to Scheme 4 cannot be excluded, but the increased azoreductase activity seen on increasing the microsomal content of NADPH-cytochrome *c* (P-450) reductase favours the mechanism in Scheme 3. A contribution to the azoreductase activity by the mechanism in Scheme 1 seems very unlikely as a 16% increase in the flavoprotein content of the microsomal fraction from control animals failed to show any evidence of increased azo reduction in the presence of FMN.

Although purified NADPH-cytochrome *c* (P-450) reductase, either in solution or incorporated into phospholipid vesicles, can support azo reduction in the presence of soluble flavin, this study suggests that, in the microsomal fraction, hepatic azoreductase is totally dependent upon cytochrome P-450 both in the absence and presence of added FMN. These results do not support the suggestion by Her-

nandez *et al.* [1, 2] of the existence in the intact microsomal membranes of a flavoprotein-mediated azoreductase activity in addition to the cytochrome P-450-mediated reaction.

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