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A continuous spectrophotometric determination of hepatic microsomal azo reductase activity and its dependence on cytochrome *P*-450

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1. A continuous spectrophotometric determination of rat hepatic microsomal anaerobic azo reductase activity has been developed. 2. The addition of soluble flavins (riboflavin, FMN or FAD) greatly increased this NADPH-dependent activity towards a number of azo substrates. 3. Investigations with amaranth as substrate gave an apparent K_m of 34 μM and $V_{max.}$ of 4 nmol/min per mg of microsomal protein. The inclusion of a fixed concentration of FMN increased $V_{max.}$ and greatly decreased K_m, the magnitude of these changes reflecting the concentration of flavin present. 4. Investigations using a fixed amaranth concentration over a range of flavin concentrations gave biphasic double-reciprocal plots with two apparent K_m and $V_{max.}$ values. 5. Pretreatment of animals with cobaltous chloride, 2-allyl-2-isopropylacetamide, carbon tetrachloride, phenobarbitone and 3-methylcholanthrene altered azo reductase activity in parallel with changes in cytochrome *P*-450 content. 6. The significance of these results is discussed in terms of the electron-transfer components present in the hepatic microsomal fraction.

Several lipid-soluble and water-soluble aromatic azo compounds are reductively cleaved into their parent primary amines by NADPH-dependent enzymes localized within the mammalian hepatic microsomal fraction (Walker, 1970; Gillette, 1971). The identity of the enzyme(s) responsible for this activity remains controversial. Early studies with butter yellow suggested that the flavoprotein NADPH-cytochrome *c* (*P*-450) reductase was responsible for the azo reductase activity (Mueller & Miller, 1950; Fouts *et al.*, 1957). Subsequent studies with neoprontosil indicated two microsomal pathways corresponding to reduction by NADPH-cytochrome *c* (*P*-450) reductase and cytochrome *P*-450 (Hernandez *et al.*, 1967*a*,*b*). More recently, amaranth reduction was shown to be completely dependent on cytochrome *P*-450 (Fujita & Peisach, 1977, 1978*a*). The azo reductase activity of the microsomal fraction towards several substrates was increased when riboflavin, FMN or FAD were added to the incubation medium (Mueller & Miller, 1950; Williams *et al.*, 1970; Mallett *et al.*, 1977). It has been proposed that these soluble flavins act as electron carriers from the azo reductase enzyme(s) to the azo substrate (Fouts *et al.*, 1957; Gingell & Walker, 1971).

Anaerobic conditions are essential for maximum expression of azo reductase activity due to inhibition of the reaction by O_2 (Hernandez *et al.*, 1967*a*; Mason *et al.*, 1978). The commonly employed method of displacing dissolved oxygen from the reaction media by saturation with argon or N_2 (Fouts *et al.*, 1957; Hernandez *et al.*, 1967*a*,*b*; Fujita & Peisach, 1978*a*) still results in a variable lag-period before the initiation of enzymic reduction (Walker *et al.*, 1971). This effect may be overlooked in the commonly used fixed-time assays and may result in underestimation of azo reductase activity.

In this paper we report the development of a continuous spectrophotometric method for the determination of hepatic microsomal azo reductase by using the polar food-colouring agent, amaranth {the trisodium salt of 3-hydroxy-4[(4-sulpho-1-naphthyl)azo]naphthalene-2,7-disulphonic acid}, as substrate. The procedure is also applicable to other azo dyes. Anaerobiosis is maintained using glucose, glucose oxidase and catalase, which substantially decreases the lag period before dye reduction. The characteristics of the enzyme system responsible for azo reduction are also discussed.

Materials and methods

Bovine serum albumin, catalase, FAD, FMN, glucose oxidase, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, 3-methylcholanthrene and riboflavin were purchased from The Sigma

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Chemical Co., Poole, Dorset, U.K. $NADP^+$ was bought from the Boehringer Corporation, London, U.K. O_2-free N_2 was supplied by BOC, Crawley, Sussex, U.K. Phenobarbitone sodium was kindly given by May and Baker, Dagenham, Essex, U.K. 2-Allyl-2-isopropylacetamide was a gift from Dr. A. H. Gibbs, MRC Toxicology Unit, Carshalton, Surrey, U.K. AnalaR-quality $CoCl_2$, Na_2HPO_4, EDTA, glucose, KCl, NaH_2PO_4 and sucrose, and Aristar-quality carbon tetrachloride were purchased from BDH, Poole, Dorset, U.K.; all other chemicals were of reagent grade and were obtained from BDH. The azo compounds were kindly given by Williams, Hounslow, Middlesex, U.K.

Animals

Male Wistar albino rats (University of Surrey Rodent Breeding Unit), 180–220g body weight, were housed in polypropylene cages with sawdust bedding and were fed Spillers no. 1 diet and tap water *ad libitum*. The temperature and relative humidity of the experimental rooms were maintained at 22°C and 50% respectively. The lighting cycle was 06:30-18:30h.

Pretreatment of animals

In some experiments, rats received the following treatments.

(*a*) $CoCl_2$ in 0.154M-saline (60mg/ml) was injected subcutaneously at 60mg/kg body wt. on 2 consecutive days, allowing 24h between treatments. (*b*) 2-Allyl-2-isopropylacetamide in 0.154M-saline (40mg/ml) was given as a single subcutaneous injection (400mg/kg body wt.) at 5h or 20h before killing the animals. (*c*) Carbon tetrachloride (1.75ml/kg body wt.) was administered by a single intraperitoneal injection of a 1:1 (v/v) solution in corn oil. (*d*) 3-Methylcholanthrene in corn oil (20mg/ml) was administered by three consecutive daily intraperitoneal injections at 25mg/kg body wt. (*e*) Phenobarbitone sodium in 0.154M-saline (30mg/ml) was given as three consecutive daily intraperitoneal injections at 80mg/kg body wt. Control animals received equivalent volumes of saline or corn oil, as appropriate. Animals were killed 24h after receiving their final treatment, except where otherwise noted.

Preparation of microsomal fraction from rat liver

All animals were starved for 18h to decrease liver glycogen and killed by cervical dislocation. The liver was immediately excised and placed in approx. 30ml of ice-cold 50mM-sodium phosphate buffer (40.5mM-Na_2HPO_4/9.5mM-NaH_2PO_4), pH7.4, containing 0.25M-sucrose and 1mM-EDTA. A homogenate of the tissue was prepared in ice-cold sucrose/EDTA/phosphate buffer (1g of tissue to 4ml of medium) by using four return strokes of a glass Potter–Elvehjem homogenizer (size c) fitted with a Teflon pestle, power driven at 2950rev./min. Cooling of the system was maintained by a jacket of ice/water. The homogenate was centrifuged at $14000\,g_{av.}$ for 20min at 4°C (MSE HS 18; 8 × 50ml rotor) to precipitate nuclei and mitochondria. The pellet was discarded and the supernatant fraction centrifuged at $177700\,g_{av.}$ for 35min at 4°C (Beckman L5-65; 8 × 25ml rotor) to sediment the microsomal fraction. The microsomal pellet was resuspended in ice-cold 50mM-sodium phosphate buffer, pH7.4, containing 0.15M-KCl and 1mM-EDTA, and resedimented at $177700\,g_{av.}$ for 35min. The washed pellet was resuspended in ice-cold 50mM-sodium phosphate buffer, pH7.4, containing 1mM-EDTA, to a concentration of approx. 4mg of microsomal protein/ml. This stock suspension was used within 4h of preparation, and stored on ice until required.

Determination of microsomal protein

The protein content of the stock microsomal fraction was determined by the method of Lowry *et al.* (1951).

Measurement of hepatic microsomal NADPH-dependent electron-transport components

Cytochrome *P*-450 content was determined by the method of Omura & Sato (1964) and NADPH-cytochrome *c* (*P*-450) reductase activity by the method of Williams & Kamin (1962).

Determination of azo reductase activity

The reaction was performed in Teflon-stoppered 10mm-pathlength glass cuvettes, containing 3.5ml of 50mM-sodium phosphate buffer, pH7.4, at 37°C. The sample cuvette contained microsomal protein (0.5–1.0mg/ml), $NADP^+$ (0.5mM), glucose 6-phosphate (6mM), glucose 6-phosphate dehydrogenase (1unit/ml), an O_2 scavenging system (60mM-glucose, 10 units of glucose oxidase/ml and 3000 units of catalase/ml) and amaranth (75μM). The NADPH-generating system was omitted from the reference cuvette.

The microsomal fraction, NADPH-generating system and glucose oxidase system were incubated together in the cuvette at 37°C for 5min before the addition of the amaranth solution, which had been previously gassed with O_2-free N_2 for 40min at 37°C. The headspace of the cuvette was flushed briefly with O_2-free N_2, stoppered and dye reduction was measured. The azo reductase activity of the microsomal fraction was determined at 37°C in a Pye–Unicam SP.1800 spectrophotometer by recording the initial linear decrease in absorbance at 520nm over 2–3min, using an absorption coefficient of 27.32 litre·mmol^{-1}·cm^{-1}.

The kinetic constants K_m and $V_{max.}$ for this reaction were determined over a range of amaranth concentrations (25–500 μM). Stoppered cuvettes of 2 mm pathlength were utilized to compensate for the large absorbance encountered when substrate concentrations in excess of 75 μM were used.

Influence of flavin on hepatic microsomal azo reductase activity

The effect of soluble flavin on hepatic microsomal azo reductase activity was investigated by including riboflavin, FAD or FMN (0–500 μM) in the assay system. The flavin solution, previously gassed wtih O_2-free N_2 for 40 min at 37°C, was introduced into the system immediately after the addition of amaranth (see above). The head space of the cuvette was then flushed with O_2-free N_2 and azo reductase activity was measured.

Comparison of azo substrates as electron acceptors during hepatic microsomal azo reduction

A comparison was made of the azo reductase activity of the microsomal fraction towards amaranth, red 2G, red 10B, carmoisine and ponceau 4R (all at 75 μM). Assay conditions were as detailed above in the absence or presence of 300 μM-FMN. The absorption maxima and absorption coefficients for these azo substrates are given in Table 1.

Statistical analysis

Comparison between results were evaluated on an Olivetti P652 desk-top computer, using *t*-test programme ST0706. Double-reciprocal plots, constructed from three determinations at each substrate concentration, were analysed with linear regression programme 9200120 to estimate the line of best fit. In all cases the correlation coefficient was 0.97 or better.

Results

Azo reductase assay

The inclusion of the O_2-scavenging system of glucose, glucose oxidase and catalase in the assay system considerably decreased the initial lag period such that azo reduction commenced approx. 20 s after mixing the assay components. At 75 μM-amaranth, the measured rate of azo reduction was 3 nmol/min per mg of microsomal protein and this was found to be a combination of two separate processes. One of these was ascribable to the microsomal fraction, whereas the other minor activity (0.3 nmol/min per ml) represented non-microsomal azo reduction mediated by the NADPH-generating system, which was determined by omitting the microsomal fraction from the assay. All values reported in the present paper are corrected for this non-microsomal reaction. Variation of the amount of either glucose oxidase (0–20 units/ml) or catalase (0–3000 units/ml) in the reaction mixture did not affect the rate of azo reduction, only the length of the initial lag period. Under the reported conditions for the assay, the initial rate of azo reduction was linear over the range 0–2.5 mg of microsomal protein/ml. The results of five experiments, as exemplified in Fig. 1, gave an apparent K_m for amaranth of 34 ± 8 μM and a $V_{max.}$ of 4 ± 1 nmol/min per mg (means ± S.D.).

Influence of flavin on azo reductase activity

Supplementation of the assay system with FMN significantly increased the activity of the microsomal azo reductase with only minor increases in the

Table 1. *Absorbance maxima and absorption coefficients of some azo compounds*

Azo substrate	$\lambda_{max.}$ (nm)	ε (litre·mmol^{-1}·cm^{-1})
Amaranth	520	27.32
Carmoisine	514	28.69
Ponceau 4R	506	25.17
Red 2G	532	31.56
Red 10B	530	31.64

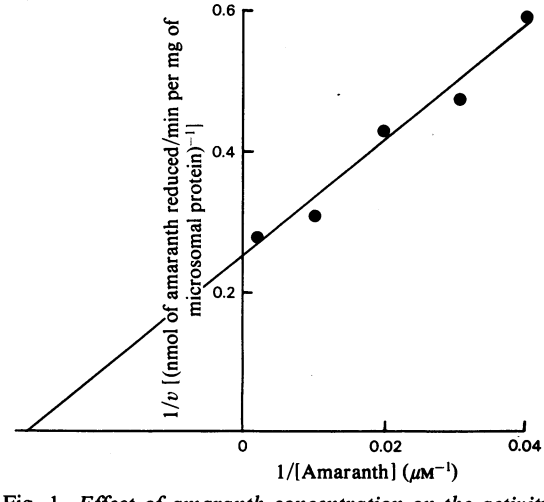

Fig. 1. *Effect of amaranth concentration on the activity of hepatic microsomal azo reductase*
Anaerobic incubations in stoppered cuvettes contained microsomal protein (approx. 1 mg/ml), $NADP^+$ (0.5 mM), glucose 6-phosphate (6 mM), glucose 6-phosphate dehydrogenase (1 unit/ml), an O_2-scavenging system and various concentrations of amaranth in 50 mM-sodium phosphate buffer, pH 7.4, at 37°C. Azo reductase activity was determined from the linear decrease in absorbance at 520 nm against a reference from which the NADPH-generating system was omitted.

reaction mediated by the NADPH-generating system alone (Fig. 2). Riboflavin, FMN and FAD all produced similar increases over a range of concentrations (Table 2), but riboflavin was incompletely dissolved beyond 100 μM and no results were obtainable. Double reciprocal plots of azo reductase activity versus amaranth concentration, when determined in the presence of 50 μM- or 500 μM-FMN, gave $V_{max.}$ values of 11 or 19 nmol of amaranth reduced/min per mg of microsomal protein, and apparent K_m values of 17 or 7 μM-amaranth respectively (Fig. 3). In contrast, double-reciprocal plots of FMN-supplemented azo reductase activity versus FMN concentration showed biphasic kinetics at the concentrations of amaranth used (50, 150 and 500 μM) as exemplified in Fig. 4. These plots showed a 'slow reaction' at concentrations of FMN less than 50 μM,

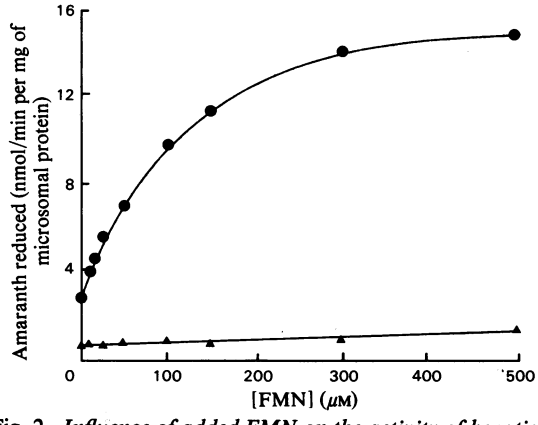

Fig. 2. *Influence of added FMN on the activity of hepatic microsomal azo reductase*
Anaerobic incubations in stoppered cuvettes contained microsomal protein (approx. 1 mg/ml), $NADP^+$ (0.5 mM), glucose 6-phosphate (6 mM), glucose 6-phosphate dehydrogenase (1 unit/ml), an O_2-scavenging system, amaranth (75 μM) and various concentrations of FMN in 50 mM-sodium phosphate buffer, pH 7.4, at 37°C. Azo reductase activity (●) was determined from the linear decrease in absorbance at 520 nm against a reference from which the NADPH-generating system was omitted. Non-microsomal azo reduction (▲) was determined in a similar manner in reactions without the microsomal fraction.

Table 2. *Comparison of riboflavin, FMN and FAD stimulation of hepatic microsomal azo reductase activity*
Results are means ± S.D. for triplicate determinations corrected for non-microsomal reduction mediated by the NADPH-generating system.

[Flavin] (μM)	Flavin-supplemented azo reductase activity (nmol of amaranth reduced/min per mg of microsomal protein)		
	Riboflavin	FMN	FAD
0	3.0 ± 0.2	3.0 ± 0.2	3.0 ± 0.2
10	4.6 ± 0.2	4.8 ± 0.4	5.7 ± 0.2
100	9.4 ± 0.4	10.0 ± 0.4	9.5 ± 0.4
300	—	18.5 ± 0.4	19.1 ± 0.4
500	—	21.2 ± 1.4	21.8 ± 0.6

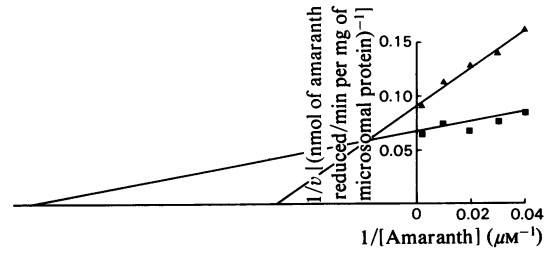

Fig. 3. *Effect of amaranth concentration on the activity of hepatic microsomal azo reductase in the presence of added FMN*
Anaerobic incubations in stoppered cuvettes contained microsomal protein (approx. 1 mg/ml), $NADP^+$ (0.5 mM), glucose 6-phosphae (6 mM), glucose 6-phosphate dehydrogenase (1 unit/ml), an O_2-scavenging system, FMN (50 or 500 μM) and various concentrations of amaranth in 50 mM-sodium phosphate buffer, pH 7.4, at 37°C. Azo reductase activity was determined from the linear decrease in absorbance at 520 nm against a reference from which the NADPH-generating system was omitted. ▲, 50 μM-FMN; ■, 500 μM-FMN.

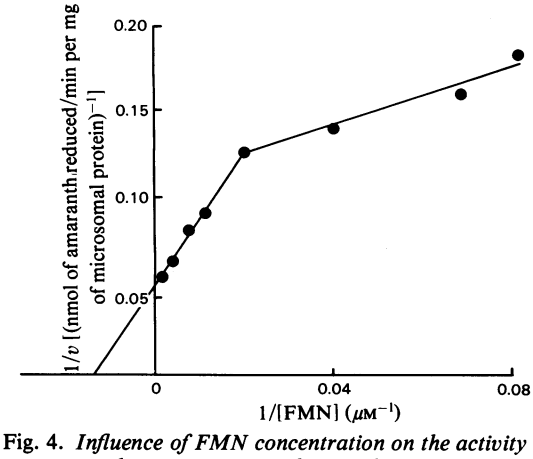

Fig. 4. *Influence of FMN concentration on the activity of hepatic microsomal azo reductase*
Incubation conditions were as described for Fig. 2, except that the concentration of amaranth used was 150 μM.

Table 3. *Influence of amaranth and flavin concentration on the kinetics of hepatic microsomal azo reductase activity*

These values were obtained from biphasic double-reciprocal plots as exemplified in Fig. 4 and are means ± S.D. as determined by linear regression analysis. Units for K_m are μM-FMN and for $V_{max.}$ are nmol of amaranth reduced/min per mg of protein.

[Amaranth]	'Slow reaction'		'Fast reaction'	
(μM)	K_m	$V_{max.}$	K_m	$V_{max.}$
50	10.8 ± 0.8	9.2 ± 0.7	93 ± 7	22 ± 2
150	8.0 ± 0.8	9.4 ± 1.0	78 ± 5	20 ± 1
500	2.9 ± 0.2	10.5 ± 0.5	48 ± 4	18 ± 2

Table 4. *Comparison of azo substrates as electron acceptors during hepatic microsomal azo dye reduction*

Azo reductase activity was determined as described in the Materials and methods section, in the absence or presence of 300 μM-FMN. Values are means ± S.D. of triplicate determinations. Azo substrate concentration was 75 μM.

Azo substrate	Azo reductase activity (nmol/min per mg of protein)	
	Non-FMN-supplemented	FMN-supplemented
Amaranth	3.0 ± 0.2	14.1 ± 0.6
Red 2G	2.5 ± 0.1	16.7 ± 1.4
Red 10B	0.5 ± 0.1	4.5 ± 0.5
Carmoisine	2.7 ± 0.3	13.8 ± 0.6
Ponceau 4R	3.0 ± 0.2	14.5 ± 1.2

having a low apparent K_m and a low $V_{max.}$ and a 'fast reaction' at concentrations of FMN above 50 μM with a high apparent K_m and high $V_{max.}$ (Table 3). These two reactions exhibited similar responses to alterations in the concentration of the azo substrate in that the $V_{max.}$ values remained essentially constant, whereas the apparent K_m values for FMN decreased with increasing azo dye concentration.

Comparison of azo substrates

Experiments with four other water-soluble azo compounds (Table 4) demonstrated that they were reduced at rates similar to amaranth, apart from red 10B, which was reduced much more slowly.

Effects of various treatments in vivo on hepatic microsomal azo reductase activity in vitro

Pretreatment of rats with $CoCl_2$, carbon tetrachloride or 2-allyl-2-isopropylacetamide resulted in decreased microsomal cytochrome *P*-450, except for 20h after treatment with 2-allyl-2-isopropylacetamide (Table 5). In contrast, NADPH-cytochrome *c* (*P*-450) reductase activity was unchanged by $CoCl_2$, decreased by carbon tetrachloride and elevated by 2-allyl-2-isopropylacetamide.

Studies on these microsomal fractions of the effects of various amaranth or FMN concentrations gave results analogous to those of control microsomal fractions in that linear and biphasic plots respectively were observed (cf. Figs. 1 and 4). For

Table 5. *Effect of various treatments in vivo on hepatic microsomal electron transfer components and azo reductase activities in vitro*

All determinations were performed as described in the Materials and methods section. Azo reductase activity was determined in the absence or presence of 300 μM-FMN. Values are means ± S.D. of group results, with four animals per group. Probabilities of a difference between control and test groups are indicated thus: $*P<0.05$; $**P<0.01$; $***P<0.001$.

	Cytochrome *P*-450 content (nmol of haemoprotein/mg of microsomal protein)	NADPH-cytochrome *c* (*P*-450) reductase activity (nmol of cytochrome *c* reduced/min per mg of microsomal protein)	Azo reductase activities (nmol of amaranth reduced/min per mg of microsomal protein)	
			−FMN	+FMN
$CoCl_2$				
Control	0.78 ± 0.09	70.5 ± 7.0	3.8 ± 0.8	16.0 ± 1.8
Test	0.55 ± 0.04**	71.9 ± 4.0	2.9 ± 0.4**	13.7 ± 1.5
Carbon tetrachloride				
Control	0.82 ± 0.03	69.8 ± 4.9	3.5 ± 0.4	16.6 ± 1.5
Test	0.19 ± 0.07***	56.0 ± 4.8**	1.4 ± 0.4***	5.9 ± 1.3***
2-Allyl-2-isopropylacetamide				
(i) After 5h				
Control	0.86 ± 0.06	82.9 ± 4.4	3.5 ± 0.3	17.9 ± 1.7
Test	0.64 ± 0.07**	99.4 ± 7.6**	2.5 ± 0.2**	14.7 ± 1.0*
(ii) After 20h				
Control	0.74 ± 0.04	73.5 ± 6.2	3.9 ± 0.4	16.5 ± 1.5
Test	0.70 ± 0.07	103.9 ± 2.8***	3.6 ± 0.6	16.1 ± 1.7

Table 6. *Effect of various treatments in vivo on hepatic microsomal electron-transfer components and azo reductase activities in vitro*

All determinations were performed as described in the Materials and methods section. Azo reductase activity was determined in the absence or presence of 300 μM-FMN. Values are means ± S.D. of group results, with four animals per group. Probabilities of a difference between control and test groups are indicated thus: $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

	Cytochrome *P*-450/*P*-448 content (nmol of haemoprotein/mg of microsomal protein)	NADPH-cytochrome *c* (*P*-450) reductase activity (nmol of cytochrome *c* reduced/min per mg of microsomal protein)	Azo reductase activities (nmol of amaranth reduced/min per mg of microsomal protein)	
			−FMN	+FMN
Phenobarbitone				
Control group	0.90 ± 0.06	76.5 ± 6.1	3.7 ± 0.4	14.9 ± 1.2
Test group	1.70 ± 0.26***	103.9 ± 3.4***	4.9 ± 0.1**	17.2 ± 1.8
3-Methylcholanthrene				
Control group	0.76 ± 0.03	95.1 ± 5.8	2.5 ± 0.5	15.6 ± 2.6
Test group	1.26 ± 0.08***	84.8 ± 5.5*	3.7 ± 0.5**	17.5 ± 3.2

carbon tetrachloride, in Fig. 1 the apparent K_m was not significantly altered but $V_{max.}$ was decreased to approx. 50% and the same results were observed for both the slow and fast reactions in Fig. 4. For all pretreatments it was demonstrated that significant changes in azo reductase activity in experiments of this type in comparison with controls could be assessed by using a determination of the enzymic activity at 75 μM-amaranth in the absence or presence of 300 μM-FMN. The results in Table 5 show that the decreased activities of azo reductase in the absence or presence of FMN generally paralleled the cytochrome *P*-450 content and not the NADPH-cytochrome *c* (*P*-450) reductase activity.

Similar experiments were performed on microsomal fractions containing enhanced amounts of cytochrome *P*-450 after pretreatment of the animals with phenobarbitone or 3-methylcholanthrene (Table 6). In the absence of added FMN, the azo reductase activities were significantly increased compared with controls, whereas the FMN-supplemented reactions were slightly but not significantly increased. The changes in azo reductase activities again paralleled the increases in cytochrome *P*-450 content and not the NADPH-cytochrome *c* (*P*-450) reductase activity and were associated with increased $V_{max.}$ values in comparison with the control value from Fig. 1.

Discussion

The method described permits the rapid determination of rat hepatic microsomal azo reductase activity compared with previous procedures (Mueller & Miller, 1950; Hernandez *et al.*, 1967*a*,*b*; Williams *et al.*, 1970). The use of glucose, glucose oxidase and catalase facilitated the rapid removal of O_2 from the assay system and thereby minimized the lag period before dye reduction commenced without altering the reduction rate.

The increase in azo reductase activity seen in the presence of added flavins is in accord with previous reports (Mueller & Miller, 1950; Shargel & Mazel, 1968; Fujita & Peisach, 1978*b*). Increasing the concentration of FMN increases the $V_{max.}$ of amaranth reduction and decreases that apparent K_m for the dye, whereas increasing the concentration of dye decreases the apparent K_m for FMN in both the slow and fast reactions. This apparent co-operativity may be due to the ability of reduced FMN to reduce the dye in a rapid non-enzymic reaction. However, the mechanism of this overall process is unclear as double-reciprocal plots of azo reductase activity versus FMN concentration are biphasic, suggesting complex interactions between substrates and enzyme(s) (Alvares & Mannering, 1970). The microsomal fraction contains two NADPH-dependent electron-transfer proteins, namely cytochrome *P*-450 and NADPH-cytochrome *c* (*P*-450) reductase, and this biphasic behaviour may represent interactions of FMN and amaranth with either or both of these.

Preliminary information on this was obtained by administering a number of chemical agents to rats, which altered the hepatic concentration of cytochrome *P*-450 and NADPH-cytochrome *c* (*P*-450) reductase. The decreases seen in azo reductase activity in the absence of FMN and in the presence of FMN, including both the slow and fast reactions where studied, were all consistent with the decreased cytochrome *P*-450 contents of the microsomal fractions (Table 5). The azo reductase activity

did not appear to be directly dependent on the NADPH-cytochrome *c* (*P*-450) reductase activity, as exemplified by 2-allyl-2-isopropylacetamide. It is possible, however, that in microsomes damaged by carbon tetrachloride or $CoCl_2$, uncoupling of the electron transfer between the flavoprotein and cytochrome *P*-450 could occur, allowing the flavoprotein to donate electrons directly to FMN and the azo dye. This could account for the poorer correlation between FMN-supplemented azo reductase activity and cytochrome *P*-450 content after these treatments.

Treatments that raised the microsomal content of different forms of cytochrome *P*-450 haemoproteins also increased the azo reductase activity, but not exactly in proportion to the cytochrome content, either in the absence or presence of supplementary FMN (Table 6). Under these conditions electron transfer from flavoprotein to haemoprotein may become rate-limiting for the reduction of amaranth and FMN. Alternatively, the different forms of cytochrome *P*-450 in control animals and those pretreated with phenobarbitone or 3-methylcholanthrene (Welton & Aust, 1974) may differ in their effectiveness in catalysing the reduction of azo compounds.

Reports in the literature suggest that hepatic microsomal azo reductase activity is partially (Hernandez *et al.*, 1967*a*,*b*) or totally (Fujita & Peisach, 1978*a*) dependent on cytochrome *P*-450 for activity, whereas the increased rate of azo reduction observed on supplementation with flavins has been assumed to reflect the reaction at NADPH-cytochrome *c* (*P*-450) reductase (Fouts *et al.*, 1957; Shargel & Mazel, 1972). The data from the present study indicate that cytochrome *P*-450 is the major terminal electron donor responsible for the reduction of azo compounds either in the absence or presence of added flavins.

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