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STUDIES ON THE MECHANISM OF ACTION OF MAMMALIAN HEPATIC AZOREDUCTASE—I

AZOREDUCTASE ACTIVITY OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE–CYTOCHROME c REDUCTASE*†

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Abstract—Microsomal azoreductase was solubilized by steapsin, fractionated with ammonium sulfate, and purified by chromatography on DEAE-cellulose. The DEAE-cellulose eluate fraction was subjected to electrophoretic and ultracentrifugation analysis. The absorption spectrum of the purified enzyme was typical of a flavoprotein. From flavin determinations, a molecular weight between 33,000 and 45,000 was calculated. At no stage of purification was it possible to separate azoreductase and NADPH–cytochrome c reductase activities.

Kinetic studies with purified enzyme demonstrated that the K_m(NADPH) was of the same order of magnitude for the reduction of cytochrome c and of neoprontosil. The of the same order of magnitude for the reduction of cytochrome c and of neoprontosil. The enzyme could not be saturated with neoprontosil and K_m (neoprontosil) was extremely high. Azoreductase and NADPH–cytochrome c reductase activities displayed similar patterns with respect to inhibition of sulfhydryl group inhibitors, and both activities showed similar heat lability. It was concluded that purified azoreductase is probably identical with purified NADPH–cytochrome c reductase.

Microsomal azoreductase activity decreased upon solubilization, whereas NADPH–cytochrome c reductase activity increased, indicating the existence of a secondary azoreductase pathway or removal of a cofactor. In intact microsomes the reduction of the azo-linkage may not be entirely attributed to NADPH–cytochrome c reductase.

REDUCTIVE cleavage of azo compounds as an important pathway of drug metabolism was established by the classic discovery of Trefouël *et al.*[1] that prontosil is reduced to sulfanilamide in animals. Since that time, a variety of azo compounds have been shown to be reduced both *in vivo* and *in vitro* by enzymes localized mainly in the microsomal fraction of liver.[2–5]

Studies *in vitro* revealed that the microsomal enzyme was probably a flavoprotein that required NADPH as the hydrogen donor. These findings led Mueller and Miller[2–4] to suggest that the enzyme might be NADPH–cytochrome c reductase. Accordingly,

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Kamm[6] found that purified NADPH–cytochrome c reductase from porcine liver catalyzed the reduction of neoprontosil to primary amines.

The present communication compares the activities of NADPH–cytochrome c reductase and azoreductase during the process of solubilization and purification. The data confirm the observation of Kamm[6] that azo compounds are reduced by NADPH–cytochrome c reductase, but indicate that this enzyme does not account for all of the azoreductase in liver microsomes.

MATERIALS AND METHODS

Animals. The livers from adult Sprague–Dawley rats (150–300 g) of both sexes were used as the source of NADPH–cytochrome c reductase and azoreductase.

Chemicals. Most reagents and solvents (A.C.S. reagent grade) were purchased from J. T. Baker Chemical Co., including Dowex 50-X8 and *N*-ethylmaleimide (NEM), and were used without further purification. Other reagents were obtained from the following sources: ammonium sulfate (special enzyme grade) was obtained from Mann Reasearch Laboratories, Inc.; nicotinamide and *p*-chloromercuribenzoate (pCMB) from Nutritional Biochemicals Corp.; sulfanilamide and diethyl-dithiocarbamate sodium from Eastman Organic Chemicals; neotetrazolium 2, 2′, 5, 5′-tetraphenyl-3, 3′-(*p*-diphenylene)-ditetrazolium chloride, penicillamine, α-αdipyridyl, 8-hydroxyquinoline, NADP, NADPH, NAD, NADH, glucose 6-phosphate, cytochrome c (horse heart, type III), and crude porcine pancreatic lipase (steapsin) from Sigma Chemical Co.; 2-anthroquinone-sodium sulfonate from Matheson Co.; Ultra High Purity nitrogen from Southern Oxygen, Division of Air Products; crystalline bovine serum albumin from Armour Pharmaceuticals Co.; phenobarbital sodium from Merck and Co.; DEAE-cellulose from Bio-Rad Laboratories; glucose 6-phosphate dehydrogenase and riboflavin from Calbiochem; Polyacrylamide, bromphenol blue, and Amido Schwartz (naphthol blue-black) from Canal Industrial Corp. Prontosil and neoprontosil were generously supplied by Sterling–Winthrop Research Institute, Rensselaer, N.Y.

Preparation of rat liver microsomes

The animals were killed by decapitation and their livers were removed immediately and washed with ice-cold isotonic KCl (10^{-3} M EDTA). From this point on, all operations were done at 0°–4°. The washed livers were blotted, weighed, minced with scissors, and homogenized with 3 vol. isotonic KCl(10^{-3} M EDTA) in a Waring–Blendor at top speed for 90 sec. The cell debris, nuclei, and mitochondria were removed by centrifuging the liver homogenate at 12,000 *g* for 20 min in a Servall refrigerated centrifuge. The microsomal fraction was prepared by centrifuging the 12,000 *g* supernatant at 105,000 *g* for 60 min in a no. 40 rotor, or for 30 min at 198,000 *g* in a no. 50 rotor, in a Spinco centrifuge, model L or L2, respectively. The microsomal pellets were resuspended in isotonic KCl(10^{-3} M EDTA) to remove adventitious hemoglobin, the mixture was recentrifuged at either 105,000 *g* or 198,000 *g* as indicated above, and the supernatant discarded. The microsomes were resuspended in a buffer, pH 7·6, consisting of 0·1 M NaH_2PO_4–K_2HPO_4 and 10^{-3} M EDTA; each milliliter of the suspension contained the microsomes from 0·5 *g* liver (approximately 10 mg protein/ml).

Enzymatic assays employed

Azoreductase assay. The following components in a final vol. of 5 ml were added to serological flasks placed in an ice bath: 0·5 μmole NADP; 100 μmole nicotinamide; 50 μmole glucose 6-phosphate; 15 units of glucose 6-phosphate dehydrogenase,* or soluble fraction from rat liver (equivalent to 250 mg wet weight); 10 μmole neoprontosil; 5 μmole EDTA; 0·05 M NaH_2PO_4–K_2HPO_4 buffer, pH 7·6; enzyme (equivalent to microsomes from 250 mg liver). While in the ice bath, the flasks were stoppered with serological stoppers and flushed with oxygen-free nitrogen for 5 min. The flasks were then incubated at 37° with shaking for 30 min in an atmosphere of pure nitrogen that was first passed through a deoxygenizer mixture (0·5% sodium dithionite, 0·05% 2-anthroquinone sodium sulfonate in 0·1 N sodium hydroxide). The reaction was terminated by addition of 15 ml of 6·67% trichloroacetic acid (TCA). The sample was then centrifuged at 600 *g* for 10 min, and an aliquot of the supernatant was assayed for sulfanilamide.

Separation of sulfanilamide from neoprontosil. Neoprontosil was separated from sulfanilamide in the TCA supernantant by column chromatography. The column was prepared by placing an aliquot of a slurry of the acidic form of Dowex-50 in a small glass column (1·0 × 13 cm). The 1·0 × 2·0 cm resin bed was washed with distilled water. A 5-ml aliquot of the TCA supernatant was placed on the resin bed and allowed to flow through. The column was then washed with 5 ml distilled water or until the eluant was colorless. The sulfanilamide was eluted off the column with two successive portions of 10 ml 3 N hydrochloric acid. The hydrochloric acid eluates were combined and a 5-ml aliquot was adjusted to a pH value between 1·5 and 2·0 with an equal volume of a solution containing 417 g K_2HPO_4 and 33·5 ml 10N NaOH/liter or 3·0 ml of 2 M K_3PO_4. Sulfanilamide was then measured by the method of Bratton and Marshall.[7] When sulfanilamide alone was added to a complete incubation mixture, or with varying concentrations of neoprontosil, and carried through the described isolation procedure, recovery was 96–100 per cent.

Initial experiments with NADH or NADPH indicated that neoprontosil can be reduced nonenzymatically to a small extent. All experimental values were corrected for nonenzymatic formation of sulfanilamide.

NADPH–*cytochrome c reductase assay.* In most experiments NADPH–cytochrome c reductase was assayed by a method similar to that of Phillips and Langdon.[8] The mixture, in a total volume of 3·0 ml 0·05 M NaH_2HPO_4 buffer, pH 7·6, contained: 126 mμmole NADPH, 150 mμmole cytochrome c, 3 μmole KCN, 3 μmole EDTA, and enzyme equivalent to microsomes from 10 mg liver. When enzyme of high purity was assayed, KCN was omitted. Control cuvettes contained all the components, except NADPH.

The reagents were preincubated for 10 min at 25°, and the reaction was initiated by the addition of cytochrome c. The activity was followed as the change in absorbancy at 550 mμ in 1-cm light-path cuvettes by means of a Beckman DB double-beam spectrophotometer attached to a Sargeant Recorder, model SRL. The cuvette compartment temperature was maintained at 25° by a circulating water bath. Cytochrome c reduction was measured for 3–4 min at 550 mμ while the reaction rate was linear. One unit of NADPH–cytochrome c reductase is defined as the amount of enzyme

* One unit of glucose 6-phosphate dehydrogenase is the amount of enzyme that reduces 1 μmole NADP/min at pH 7·4 and 25°.

which produces a change in O.D. of 1·0 at 550 mμ in 1 min in the above system.

Neotetrazolium diaphorase assay. The method used is that described by Williams and Kamin.[9] The reaction mixture, in a total volume of 0·6 ml, contained 0·3 μmole NADP; 1·2 μmole nicotinamide; 1·8 units of glucose 6-phosphate dehydrogenase; 1·5 μmole glucose 6-phosphate; 0·15 μmole neotrazolium; 0·6 μmole EDTA; 0·05 M NaH_2PO_4–K_2HPO_4 buffer, pH 7·6; and enzyme (equivalent to microsomes from 2 mg liver). The mixture was incubated for 10 min at 37° in air and the reaction was stopped by addition of 3·0 ml of a mixture containing 40 ml water, 3·6 ml of 10% Triton X, 5·0 ml of 40% formalin, and 10 ml of 1 M formate buffer, pH 3·5. The optical densities were read in a Bausch & Lomb colorimeter at 500 mμ and the amount of formazan formed by the reduction of the tetrazolium salt was calculated from the molar extinction coefficient of 14×10^6 $cm^2 \times mole^{-1}$ determined by Lester and Smith.[10]

Protein determination. Protein was determined by the method of Lowry *et al.*[11] When samples contained more than 50 μg protein/ml, a modification of Lowry's method was used.[12] Crystalline bovine serum albumin was used as the protein standard.

Determination of flavin. Total flavins and FAD in the purified enzyme were determined by fluorometric assay.[13]

RESULTS

Intracellular distribution of azoreductase

The data shown in Table 1 indicate that azoreductase is localized in the microsomes and requires NADPH. However, microsomes with an NADPH-generating system

TABLE 1. INTRACELLULAR DISTRIBUTION OF AZOREDUCTASE

Fraction*	Relative activity†
Whole homogenate	100
Mitochondria	0
Microsomes	0
Soluble fraction (105,000 *g*)	0
Microsomes (boiled) + soluble fraction (105,000 *g*)	0
Microsomes + glucose 6-phosphate dehydrogenase (1 unit)	51
Microsomes + glucose 6-phosphate (1 unit) + boiled soluble fraction (105,000 *g*)	100
Microsomes + glucose 6-phosphate dehydrogenase (1 unit) + EDTA (10^{-3} M)	100

* The various fractions and/or the soluble fraction from 250 mg rat liver were incubated for 30 min at 37° under nitrogen in the presence of 50 μmole glucose 6-phosphate, 100 μmole nicotinamide, 0·5 μmole NADP, and 10 μmole neoprontosil, in a final volume of 5·0 ml in 0·05 M NaH_2PO_4, pH 7·6.

† Formation of sulfanilamide was determined as described under Materials and Methods. The results presented are averages of separate analyses of 2–4 flasks. The experiments have been repeated a number of times with essentially the same results.

(glucose 6-phosphate dehydrogenase) were less active than when soluble fraction was added. However, addition of EDTA (10^{-3} M) or boiled soluble fraction to the generating system restored activity. The findings thus show that the soluble fraction contains another factor in additon to glucose 6-phosphate dehydrogenase.

Conditions and requirements of the azoreductase system

Preliminary experiments revealed that the amounts of nicotinamide, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase are not rate limiting. The rate of reduction remained constant during the 30-min incubation period. Moreover, the activity was proportional to enzyme concentration when microsomes from as much as 1 g liver were assayed. The conditions and cofactor requirements of the assay were virtually identical for intact microsomes and solubilized enzyme.

Incubation of varying concentrations of NADP with the NADPH-generating system and neoprontosil in the presence and absence of microsomes revealed that neoprontosil is reduced both enzymatically and nonenzymatically. At a concentration 1×10^{-4} M NADP, there was a 4 to 5-fold difference between the enzymatic and nonenzymatic formation of sulfanilamide, which minimizes the error in the correction for nonenzymatic reduction (Fig. 1). Attempts were made to diminish the nonenzymatic reaction by changing the substrate or the conditions. However, NADPH was

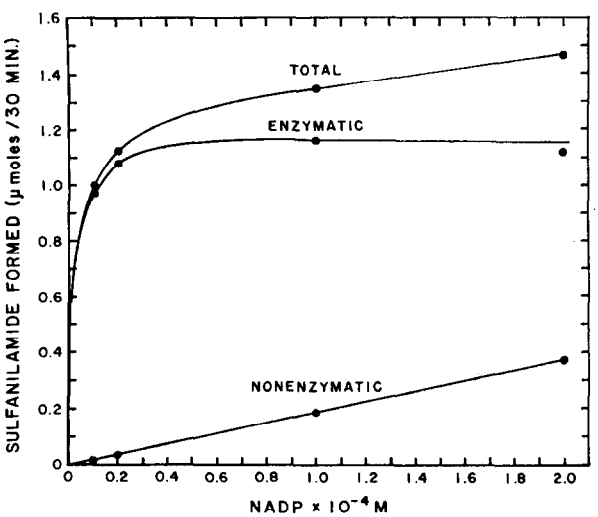

FIG. 1. Effect of varying concentrations of NADP on the reduction of neoprontosil by rat liver microsomes. Neoprontosil was reduced at 37° under anaerobic conditions for 30 min. The incubation mixture contained 10 μmole neoprontosil, 100 μmole nicotinamide, 50 μmole glucose 6-phosphate, 15 units glucose 6-phosphate dehydrogenase, 5 μmole EDTA, microsomes from from 250 mg liver, and NADP, in a final volume of 5 ml of 0·05 M NaH_2PO_4–K_2HPO_4 buffer, pH 7·6.

observed to reduce nonenzymatically a variety of azocompounds, including prontosil, azobenzene, and phenylazodiaminopyridine. Addition of NADPH to recrystallized neoprontosil still resulted in the formation of sulfaniliamide. Several chelating agents, including diethyl-dithiocarbamate, 8-hydroxyquinoline, α-α dipyridyl, EDTA, and penicillamine in concentrations of 10^{-4} M, were used in an attempt to eliminate the non-enzymatic reduction, in the absence of microsomal enzymes. However, the amount of sulfanilamide formed was not decreased in the presence of the chelating agent. Albumin (1–20 mg/ml), which may bind a metal or catalyst, if present also failed to inhibit the nonenzymatic reduction of neoprontosil.

Effect of oxygen on azoreductase activity. Incubation under 100% oxygen or under air completely inhibited the formation of sulfanilamide from neoprontosil by microsomes or purified enzyme.

Effect of neoprontosil concentration. With concentrations of neoprontosil as high as the solubility of the compound permits, the sulfanilamide formed was roughly proportional to the amount of substrate. Thus the affinity of the enzyme for either prontosil or neoprontosil is extremely low.

For the standard reaction mixture, a concentration of 2×10^{-3} M (10 μmole in 5 ml incubation mixture) was chosen. At this concentration, the nonenzymatic component is low and the enzymatic rate of sulfanilamide formation can be measured accurately.

Solubilization and purification of NADPH–*cytochrome c reductase and azoreductase*

Liver microsomes in pH 7·6 phosphate buffer were incubated with steapsin (0·07%) under nitrogen at 0° for 13 h.[14] The digest was centrifuged at 105,000 *g* for 60 min. Enough crystalline ammonium sulfate was added to the supernatant containing the solubilized protein to make a 40% saturated solution. The mixture was allowed to stand for 30 min at 4°. When ammonium sulfate was used, the pH was always adjusted to 7·6 by addition of KOH. Masters *et al.*[15] observed that the enzyme loses flavin below pH 6·0 in the presence of high concentrations of ammonium sulfate. The ammonium sulfate-precipitated protein was separated by centrifugation for 20 min at 37,000 *g* in a Servall refrigerated centrifuge. The precipitate was discarded and the supernatant made 80 per cent saturated with solid ammonium sulfate. The fraction that precipitates between 40 and 80 per cent of ammonium sulfate contained the NADPH–cytochrome c reductase and azoreductase activities. After centrifugation, the precipitates was dissolved in a small volume of 0·001 M NaH_2PO_4–K_2HPO_4, pH 7·6 (10^{-3} M EDTA) buffer and dialyzed against 200 to 300 vol. of the buffer until sulfate ions could no longer be detected by addition of $BaCl_2$.

As shown in Table 2, the ratio of the activities of azoreductase and NADPH–cytochrome c reductase decreased on treatment of liver microsomes with steapsin, but remained relatively constant thereafter.

Chromatography of NADPH–*cytochrome c reductase and azoreductase on* DEAE–*cellulose*

The 40–80 per cent ammonium sulfate fraction was placed on a DEAE-cellulose column (Fig. 2) previously equilibrated with 0·001 M NaH_2PO_4–K_2HPO_4 buffer, ph 7·6 (10^{-3} M EDTA). Nitrogen pressure was applied to obtain an elution rate of 120 ml/hr. This was followed by stepwise elution with 100 20-ml portions of buffers of increasing molarity (0·001–0·15 M), followed by 650 ml of 0·3 M buffer. Each of the 110 fractions (10 ml each) was analyzed for protein, NADPH–cytochrome c reductase, and azoreductase activities. Eluates from the 0·3 M buffer fraction containing more than 0·3 unit of NADPH–cytochrome c reductase per ml were made 80 per cent saturated with respect to ammonium sulfate and centrifuged at 27,000 *g* for 20 min The precipitated enzyme was collected and stored at −40°. Under these conditions both NADPH–cytochrome c reductase and azoreductase activities were stable for several months. A 40 per cent loss in activity was observed after 6 months. Other batches of purified enzyme had specific activities as high as 240 units per mg of

TABLE 2. PURIFICATION OF NADPH–CYTOCHROME C REDUCTASE AND AZOREDUCTASE

Fraction	Azoreductase				NADPH–cytochrome c Reductase				Activity ratio
	Units* × 10^3	% Recovery	Sp. act.†	Purification	Units‡ × 10^4	% Recovery	Sp. act.†	Purification	Azoreductase/ NADPH–cyt. c reductase
Microsomes	3322	100	208	1·0	1·43	100	0·9	1·0	231
Steapsin supernatant	1612	49	267	1·3	1·72	120	2·84	3·2	94
Ammonium sulf 40–80%	883	27	917	4·4	0·79	55	8·10	9·0	113
DEAE-cellulose eluate	428	13	15,400	74·0	0·42	29	152·0	170·0	101

* Azoreductase unit = mμmole sulfanilamide formed/30min/mg protein.
† Sp. act. = units/mg protein.
‡ NADPH–cytochrome c reductase unit = ΔO.D. 550 mμ/mg protein/min.

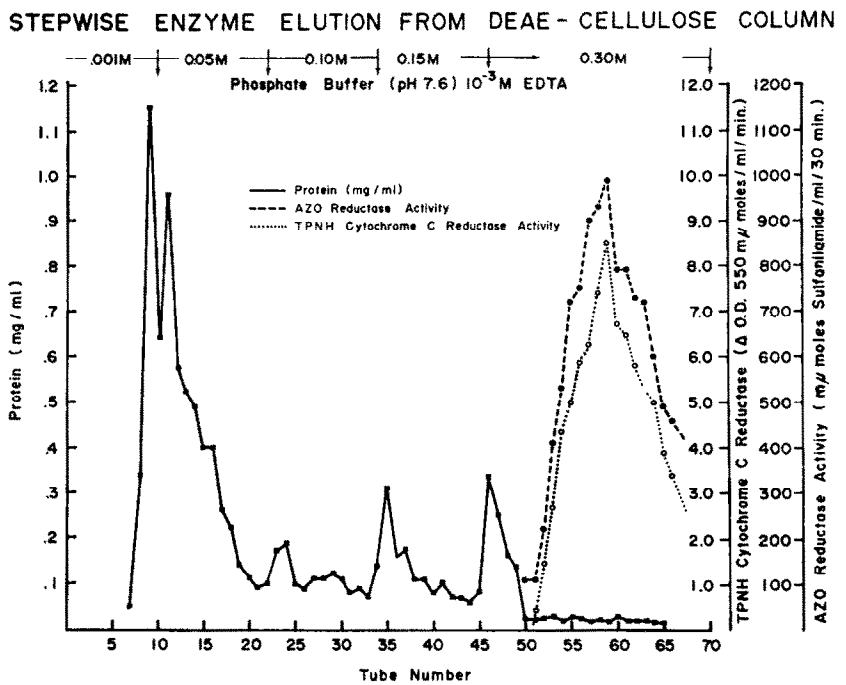

FIG. 2. Column chromatography of NADPH–cytochrome c reductase and azoreductase on DEAE-cellulose. Protein, 80% ammonium-sulfate fraction (157 mg, sp. act. 7·3 units NADPH–cytochrome c reductase/mg protein) in 9·5 ml of 0·001 M phosphate buffer, pH 7·6, was placed on a 2·5 × 35 cm column (26 g of dry adsorbent with exchange capacity of 0·87 m-Equiv g). Details of the elution are described under results.

protein in terms of NADPH–cytochrome c reductase, which is greater than the sp. act. shown in Table 2.

Characterization of homogeneity of azoreductase–NADPH–*cytochrome c reductase*

Electrophoresis. As shown in Fig. 3, preparations of the enzyme migrate as a single band during electrophoresis in acrylamide gel. When the gel (Fig. 3, tube 3) was sprayed with a solution of neotetrazolium and NADPH, deposition of colored formazan was formed at a position corresponding to the bulk of the protein. When segments of unstained gel were leeched with buffer and the buffer extract was tested for enzymatic activity, NADPH–cytochrome c reductase and azoreductase were found only in the area that displayed neotetrazolium activity (Fig. 3). These findings thus support the view that a single protein catalyzes the reduction of cytochrome c, neotetrazolium, and azo compounds.

Ultracentrifugal sedimentation. Fig. 4 shows a typical schlieren pattern for the purified enzyme as obtained from a concentrate of DEAE-cellulose eluate. Assuming that the enzyme behaves like an average protein in positive sp. vol. and diffusion characteristics, an S_{20w} of 4·56 corresponds to a molecular weight of approximately 70,000.[16, 17]

Flavin content of the purified enzyme. The flavin content of the purified enzyme was analyzed before and after hydrolysis in trichloracetic acid. On the assumption that 1

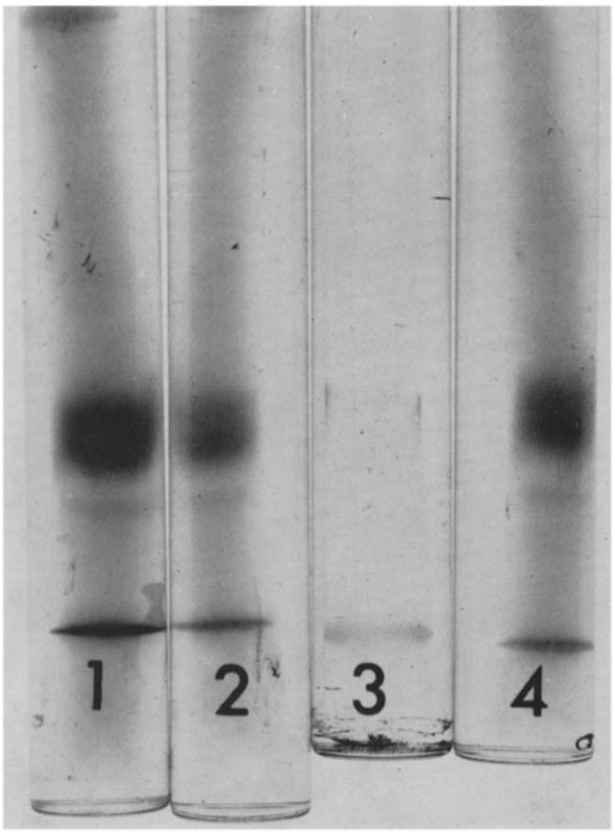

FIG. 3. Acrylamide gel electrophoresis of purified azoreductase–NADPH–cytochrome c reductase. Samples of purified enzyme (sp. act, 240 units of NADPH–cytochrome c reductase, 50–100 μg of protein) were subjected to electrophoresis in 0·1 M Tris-glycine buffer, pH 8·0, at 3 mA/cm^2 for 2 hr at 4°. All tubes were stained with amido Schwartz, except for tube 3, which was treated with neotetrazolium and NADPH. The thin lines seen in tube 3 represent the area in the gel where neotetrazolium was reduced. This same area contained azoreductase and NADPH–cytochrome c reductase activity.

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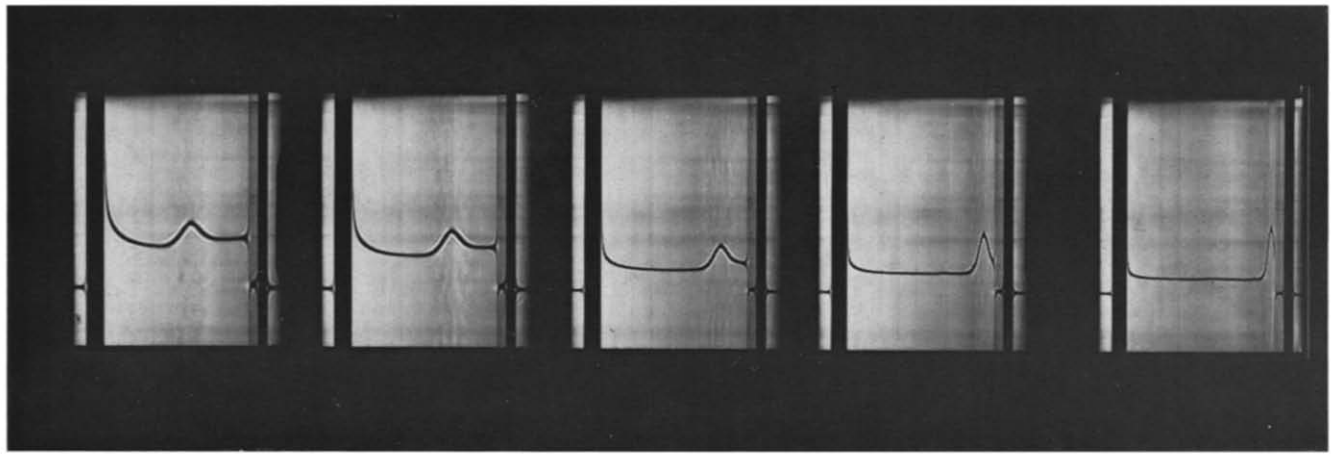


FIG. 4. Ultracentrifugation pattern of the purified enzyme. Sedimentation behaviour was determined in a Spinco model E analytical ultracentrifuge at 20°. Photographs (from right to left) were taken at 5, 16, 41, 70, and 90 min after the maximum speed (59, 780 rpm) was reached. The sedimentation coefficient, $S_{20,w}$ calculated from the sedimentation pattern was 4·56. The protein concentration was 10 mg/ml and the sp. act. of the preparation in terms of NADPH–cytochrome c reductase was 240 units/mg protein. The enzyme was dissolved in 0·05 M NaH_2PO_4–K_2HPO_4 buffer, pH 7·6 (10^{-3} M EDTA) and adjusted to ionic strength of 0·15 with sodium chloride.

mole of enzyme contains 2 mole of flavin,[15] an approximate molecular weight of 40,000 was calculated for the enzyme. Measurements done by this method in several preparations yielded values between 33,000 and 45,000 molecular weight (2 μmole of flavin per 33–45 μg of enzyme). The molecular weight of the enzyme, as calculated from the flavin content, is in agreement with the values reported by Masters *et al.*[15] for a preparation obtained from pork liver microsomes. According to Dr. E. Steers (personal communication), the higher value calculated from the sedimentation coefficient could be caused by the formation of dimers of the enzyme.

Absorption spectra of the purified enzyme. The spectrum of the oxidized enzyme was determined under anaerobic conditions[18] after bubbling oxygen-free nitrogen through the solution for approximately 20 sec. The oxidized form shows two absorption peaks, at 455 and 380 mμ. Similar spectra were obtained by Horecker[19] for pig liver reductase, and by Williams and Kamin,[9] Kamin *et al.*[20] and Phillips and Langdon[8] for purified NADPH–cytochrome c reductase prepared from liver microsomes.

Heat inactivation. The effect of heat inactivation on the purified enzyme was tested for NADPH–cytochrome c reductase, neotetrazolium diaphorase, and azoreductase. The data in Table 3 indicate that NADPH–cytochrome c reductase and azoreductse activities display a similar heat lability.

TABLE 3. EFFECT OF HEAT ON PURIFIED NADPH–CYTOCHROME REDUCTASE, AZOREDUCTASE, AND NEOTETRAZOLIUM DIAPHORASE

Temp. (°C)	NADPH–cyt. c reductase*	Azoreductase*	Neotetrazolium diaphorase*
40	82	71	51
45	55	55	36
50	19	18	18
55	0	0	0

* Expressed as % activity of the unheated controls. Purified enzyme (184 μg protein/ml) was heated at the indicated temperatures ($\pm$ 0·5°) for 10 min in a water bath. A similar preparation was kept on ice and used as control. All assays were performed as described under Materials and Methods. Each figure is the average of separate analyses of results from duplicate flask.

Effect of inhibitors on purified azoreductase, NADPH–*cytochrome c reductase and neotetrazolium diaphorase*

Williams and Kamin,[9] Phillips and Langdon,[8] and Masters *et al.*[15] reported that NADPH–cytochrome c reductase activity is influenced by sulfhydryl group inhibitors The effect of two sulfhydryl group inhibitors was tested on the ability of the purified enzyme to reduce cytochrome c, neotetrazolium, and neoprontosil in the presence of NADPH.

Concentrations of pCMB below 10^{-8} M (Table 4) were found to stimulate cytochrome c reduction, which is in agreement with the works of Masters *et al.*[15] and of Phillips and Langdon.[8] pCMB failed to stimulate the reduction of the other two substrates.

Masters *et al.*[15] were unable to inhibit purified NADPH–cytochrome c reductase with NEM, although we found that NEM inhibited NADPH–cytochrome c reductase

and azoreductase (Table 5). This discrepancy may stem from the fact that these authors preincubated their enzyme with the inhibitor for 5 min at 0°, whereas in the present studies the enzyme was preincubated with the inhibitor for 15 min at room temperature. In concentrations as high as 10^{-4} M, NEM failed to inhibit neotetrazolium diaporase.

TABLE 4. EFFECT OF *p*-CHLOROMERCURIBENZOATE (*p*-CMB) ON PURIFIED NADPH–CYTOCHROME C REDUCTASE

*p*CMB concentration (M)	Cytochrome c	Neoprontosil	Neotetrazolium
	Relative activities		
0	1·00	1·00	1·00
5×10^{-9}	1·33	1·00	1·00
1×10^{-8}	1·06	1·00	1·00
5×10^{-8}	0·86	1·00	1·00
1×10^{-7}	0·81	0·69	1·00
5×10^{-7}	0·50	0·31	0·98
1×10^{-6}	0·22	0·00	0·92
5×10^{-6}	0·00	0·00	0·16
1×10^{-5}	0·00	0·00	0·00

* The enzyme was preincubated with the inhibitor for 15 min at room temperature prior to addition of the complete incubation mixture. Enzymatic assays are described under Materials and Methods. Each figure is the average of separate analyses of results from duplicate flask.

TABLE 5. EFFECT OF *N*-ETHYLMALEIMIDE ON PURIFIED NADPH–CYTOCHROME C REDUCTASE*

N-ethylmaleimide (M)	Relative activities		
	Cytochrome c	Neoprontosil	Neotetrazolium
0	1·00	1·00	1·00
1×10^{-6}	1·00	1·00	1·00
5×10^{-6}	0·92	1·00	1·00
1×10^{-5}	0·90	0·87	1·00
5×10^{-5}	0·80	0·65	1·00
1×10^{-4}	0·65	0·51	1·00

* The enzyme was preincubated with the inhibitor for 15 min at room temperature prior to addition of the complete incubation mixture. Enzymatic assays are described underMaterials and Methods. Each figure is the average of separate analyses of results from duplicate flask.

Kinetic studies

The Michaelis constants for NADPH and various electron acceptors were determined, with the enzyme at the ammonium sulfate stage of purification. The data were plotted according to Lineweaver and Burk.[21] The ordinate represents the reciprocal of rate of product formation, which was followed at the initial rates of the reaction. The abscissa represents the reciprocal of the substrate concentration (either NADPH or one of three electron acceptors studied— cytochrome c, neoprontosil, or neotetra zolium).

Fig. 5 is a plot of data obtained when the concentration of NADPH was varied in azoreductase incubation mixtures while keeping the neoprontosil concentration constant. A Michaelis–Menten constant (K_m) for NADPH of $6{\cdot}9 \times 10^{-7}$ M was obtained.

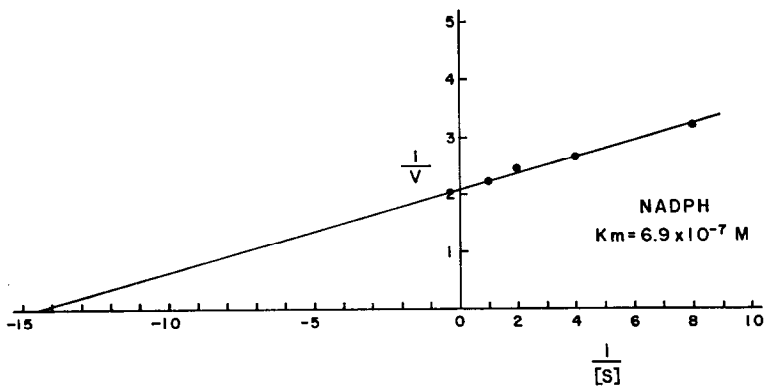


FIG. 5. Lineweaver–Burk plot for azoreductase with varying amounts of NADPH. The incubation mixtures contained 10 μmole neoprontosil, 50 μmol glucose 6-phosphate, 15 units glucose 6-phosphate dehydrogenase, 100 μmole nicotinamide, 5 μmole EDTA, enzyme (2 mg protein from fraction that precipitates between 40 and 80% saturation ammonium sulfate), in a final volume of 5 ml 0·05 M NaH_2PO_4–K_2HPO_4 buffer pH 7·6. NADP concentrations are shown as reciprocal molarities ($1/[S] \times 10^5$ M). Incubation was performed anaerobically for 30 min. The millimicromoles of sulfanilamide formed are shown as the reciprocal value ($1/V \times 10^{-3}$).

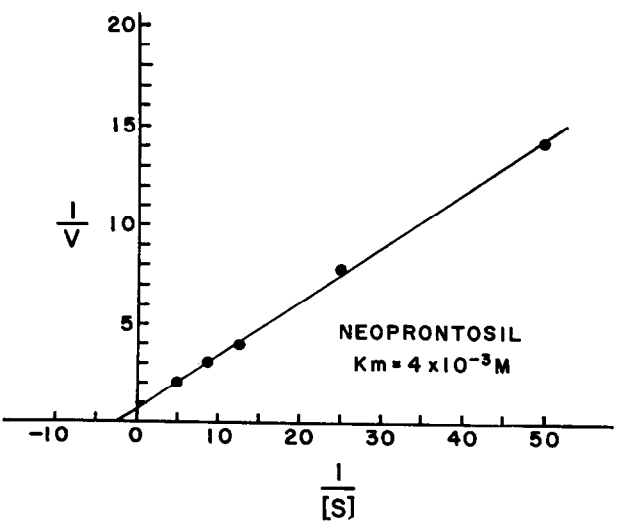


FIG. 6. Lineweaver–Burk plot for azoreductase with varying amounts of neoprontosil. The incubation mixtures contained 0·5 μmole NADP, 50 μmole glucose 6-phosphate, 15 units glucose 6-phosphate dehydrogenase, 100 μmole nicotinamide, 5 μmole EDTA, enzyme (2 mg protein from the fraction that precipitates between 40 and 80% saturation ammonium sulfate), in a final volume of 5 ml 0·05 M NaH_2PO_4–K_2HPO_4 buffer, pH 7·6. Neoprontosil concentrations are shown as reciprocal molarities ($1/[S] \times 10^2$ M). Incubation was performed anaerobically for 30 min. The millimicromoles of sulfanilamide formed are shown as the reciprocal value ($1/V \times 10^{-3}$).

A Lineweaver–Burk plot is shown (Fig. 6) for azoreductase when the concentration of neoprontosil was varied at a saturating concentration of NADPH. The K_m value for neoprontosil was 4×10^{-3} M. This value agrees with the finding that azoreductase could not be saturated within the solubility limits of neoprontosil.

Lineweaver–Burk plots of the data obtained when concentrations of NADPH and cytochrome c were varied are shown in Fig. 7 and Fig. 8. The K_m values for NADPH cytochrome c for NADPH–cytochrome c reductase are $3{\cdot}2 \times 10^{-6}$ M and $6{\cdot}2 \times 10^{-6}$ M, respectively, which agree with the values reported by Williams and Kamin[9] and by Phillips and Langdon.[8]

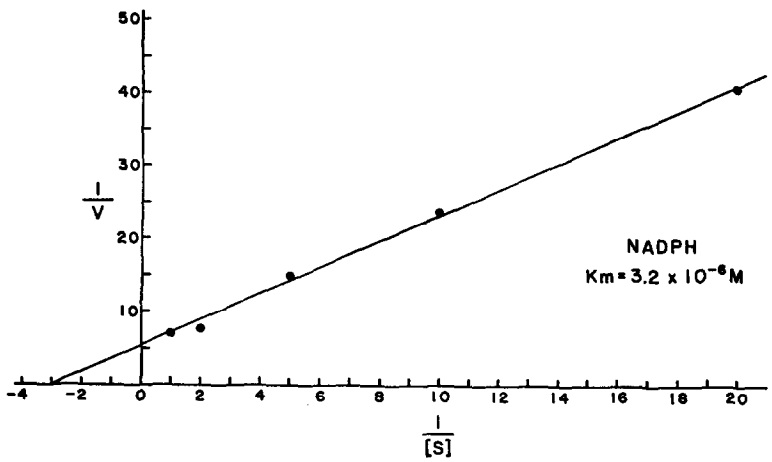


FIG. 7. Lineweaver–Burk plot for NADPH–cytochrome c reductase with varying amounts NADPH. The changes in optical density at 550 mμ were recorded using a Beckman DB spectrophotometer with a Sargeant Recorder, model SRL attached. One-cm light-path cuvettes were used. The reactions were carried out at 37° in the presence of an NADPH-generating system. The cuvettes contained 10 μmole glucose 6-phosphate, 40 μmole nicotinamide, 9 units glucose 6-phosphate dehydrogenase, 0·15 μmole cytochrome c, 3 μmole KCN, 3 μmole EDTA, enzyme (33 μg protein from the fraction that precipitates between 40 and 80% saturation ammonium sulfate), in a final volume of 3 ml 0·05 M NaH_2PO_4–K_2HPO_4 buffer, pH 7·6. NADP concentrations are shown as reciprocal molarities ($1/[S] \times 10^5$). Reference cuvettes contained all components but NADP. The millimicromoles of cytochrome c reduced/minute/ml are shown as the reciprocal value ($1/V \times 10^{-2}$) calculated from the molar extinction coefficient of $19{\cdot}1 \times 10^6$ $cm^2 \times mole^{-1}$ for the difference between reduced and oxidized cytochrome c.

The Lineweaver–Burk plots for neotetrazolium diaphorase are shown in Figs. 9 and 10. The K_m values for NADPH and neotetrazolium could not be determined, since the lines intercepted the abscissa at zero, which indicated that the K_m for the substrates approaches infinity.

DISCUSSION

These studies confirm the observation of Mueller and Miller[1–4] that azoreductase in rat liver is localized mainly to the microsomal fraction and requires NADPH and anaerobic conditions for optimum activity. The requirements for the reduction of azo-dyes were the same for both microsomes and purified enzyme.

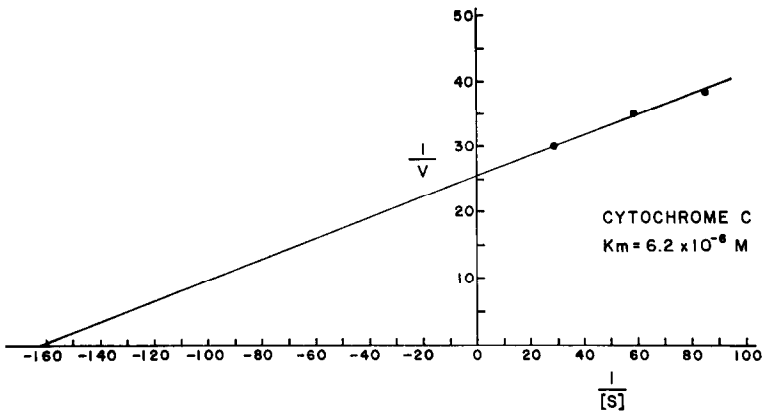


FIG. 8. Lineweaver–Burk plot for NADPH–cytochrome c reductase with varying amounts of cytochrome c. Reaction rates were determined as described under Materials and methods. The incubation mixtures contained 126 mμmole NADPH, 3 μmole KCN, 3 μmole EDTA, enzyme (33 μg protein from the fraction that precipitates between 40 and 80% saturation ammonium sulfate), in a final volume of 3 ml 0·05 M NaH_2PO_4–K_2HPO_4 buffer pH 7·6. Cytochrome c concentrations are shown as reciprocal molarities ($1/[S] \times 10^3$ M). Reference cuvettes contained all the components but NADPH. The millimicromoles of cytochrome c reduced per minute are shown as the reciprocal value ($1/V \times 10^{-2}$) calculated from the molar extinction coefficient of $19{\cdot}1 \times 10^6 cm^2 \times mole^{-1}$ for the difference between reduced and oxidized cytochrome c. The cytochrome c used was corrected for 14·9 per cent present in the reduced form.

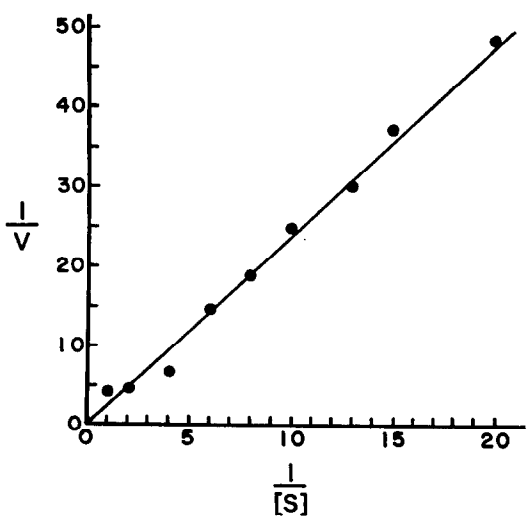


FIG. 9. Lineweaver–Burk plot for neotetrazolium diaphorase with varying amounts of NADPH. The conditions of the assay are described under Materials and methods. The enzyme used, was the fraction that precipitates between 40 and 80% saturation ammonium sulfate (150 μg protein/flask). The concentrations of NADPH are shown as the reciprocal molarities ($1/[S] \times 10$) and the millimicro-moles of formazan formed, as the reciprocal value ($1/V \times 10^{-3}$).

When purified glucose 6-phosphate dehydrogenase was used to generate NADPH, the amount of glucose 6-phosphate dehydrogenase used was far in excess of the calculated theoretical value necessary to maintain the NADPH in its reduced form. Failure to do so may lead to incorrect interpretation of the data, since the generation of NADPH may become rate limiting. For example, Gillette and Sesame[22] removed the

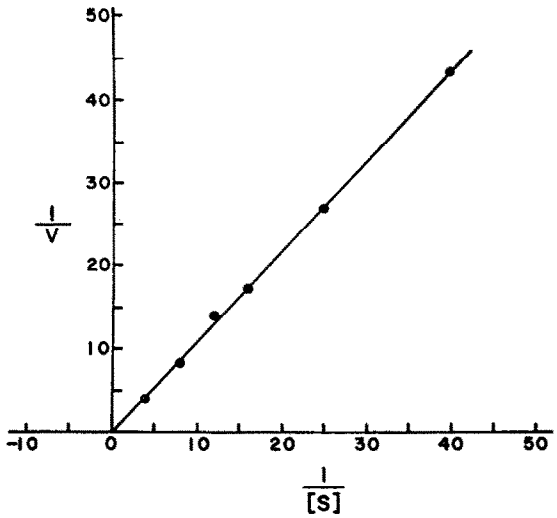


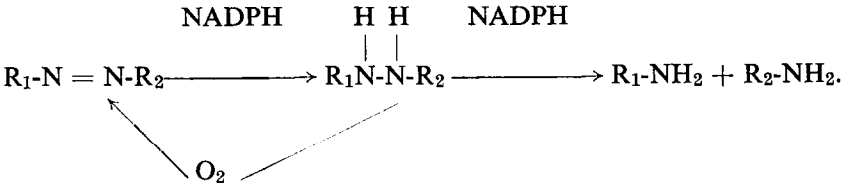
FIG. 10. Lineweaver–Burke plot for neotetrazolium diaphorase with varying amounts of neotetrazolium. The conditions of the assay are described under Materials and methods. The enzyme used was the fraction that precipitates between 40 and 80% saturation ammonium sulfate (150 μg protein/flask) and the incubation time was 5 min. The concentrations of neotetrazolium are shown as the reciprocal molarities ($1/[S] \times 10^3$ M) and the millimicromoles of formazan formed, as the reciprocal value ($1/V \times 10^{-3}$).

objection that NADPH–cytochrome c reductase could not be a component of the NADPH-dependent oxidizing system in hepatic microsomes because the K_m (NADPH) for the two systems did not agree. By using concentrations of glucose 6-phosphate dehydrogenase that were 10 times the theoretical amount, they were able to equate the K_m (NADPH) for the drug-metabolizing enzymes and for NADPH–cytochrome c reductase in mouse liver microsomes.

The findings further show that the soluble fraction, in addition to serving as a source of glucose 6-phosphate dehydrogenase, contains an unidentified factor which is required for optimum activity, since microsomes with an NADPH-generating system (glucose 6-phosphate dehydrogenase) were less active than when soluble fraction was added. However, addition of EDTA (10^{-3} M) or boiled soluble fraction restored the activity. These findings suggest that minute amounts of metals present in the reagents may affect the reaction, possibly by reacting with sulfhydryl groups of the enzyme. A partially purified azoreductase preparation was found to be highly sensitive to sulfhydryl group inhibitors, such as pCMB and NEM.

Fouts *et al.*[5] showed that incubation under oxygen inhibited azoreductase, the degree of inhibition depending on the substrate used. With neoprontosil, inhibition by air or oxygen appears to be complete. It seems possible that this inhibition might be caused by the reoxidation of hydrazo intermediates, which presumably are formed

in the reduction of azo compounds (Elson andWarren,[23] Mueller and Miller,[4] Bray *et al.*[24]). In accord with this view, hydrazo compounds are known to be sensitive to air oxidation (Fieser and Fieser[25]). The oxidation of the hydrazo intermediate may also cause a depletion of NADPH and thus may result in poor yield of the primary amine according to the following scheme:



Inhibition of azoreductase by oxygen may thus be similar to the pseudo-inhibition demonstrated for nitro-reductase by Kamm and Gillette,[26] where reoxidation of the phenylhydroxylamine intermediate is responsible for the depletion of NADPH, resulting in cessation of nitro-reduction.

The question whether NADPH–cytochrome c reductase has azoreductase activity was raised by the observations of Kamm that azoreductase could be solubilized and that a highly purified NADPH–cytochrome c reductase preparation had azoreductase activity. This was confirmed by solubilizing the microsomal enzyme, purifying it by ammonium sulfate fractionation, and chromatography on DEAE-cellulose. The NADPH–cytochrome c reductase recovered after solubilization is 20 per cent greater than the original activity present in the microsomal fraction (Table 2.) It is possible that steapsin caused the removal of an inhibitor or a change in molecular configuration of the enzyme, or both, which may account for the increased activity. In contrast, the azoreductase activity decreased upon solubilization, which resulted in a sharp drop in ratio of azoreductase activity over NADPH–cytochrome c reductase activity (Table 2) indicating that the solubilization has destroyed some of the original azoreductase activity observed in the microsomes by either inactivating a secondary pathway or by removing a cofactor. After solubilzation, however, the ratios of activities remain remarkably constant throughout the ensuing purification process. Moreover, azoreductase and NADPH–cytochrome c reductase displayed identical elution patterns. The highly purified preparation containing both activities was homogeneous in the analytical ultracentrifuge, and displayed the same mobility in polyacrylamide gel electrophoresis and stability with respect to heat inactivation. Thus, NADPH–cytochrome c reductase accounts for virtually all the azoreductase present after solubilization.

The absorption spectra of purified azoreductase, typical of a flavoprotein, are identical with those reported by Williams and Kamin,[9] Phillips and Langdon,[8] and Kamin *et al.*[20] for purified NADPH–cytochrome c reductase. On the basis of flavin content, as calculated by fluorometric assay, a molecular weight of 33,000–45,000 was found, which agrees with Masters *et al.*[15] for their purified NADPH–cytochrome c reductase.

Although the reduction of azo compounds, neotetrazolium, and cytochrome c may by catalyzed by the same protein, there is evidence that they are reduced at different sites of the enzyme. In accord with the findings of Master *et al.*[15] and of Phillips and Langdon,[8] low concentrations of pCMB stimulated the reduction of cytochrome c,

but not the reduction of neoprontosil or neotetrazolium. At high concentrations, pCMB inhibited the reduction of all three substrates, but the reduction of neotetrazolium was less sensitive to this inhibitor than was the reduction of cytochrome c or neoprontosil. Moreover, NEM inhibited NADPH–cytochrome c reduction and azoreduction to a similar extent, but did not inhibit neotetrazolium reduction.

The kinetic data further support the view that the same enzyme catalyzed the reduction of cytochrome c and that of neoprontosil with NADPH. The K_m (NADPH) when cytochrome c was used as electron acceptor, was of the same order of magnitude as that obtained when the electron acceptor used was neoprontosil. This is in agreement with the K_m (NADPH) obtained by Williams and Kamin[9] and by Phillips and Langdon[8] for their purified NADPH–cytochrome c reductase. However, the K_m (NADPH) and K_m (substrate) were too high to measure for the reduction of neotetrazolium.

None of the observations discussed above can be taken alone as evidence that NADPH–cytochrome c reductase is azoreductase. However, when analyzed as a whole, the results supply correlative evidence to conclude that it is highly probable that both activities can be attributed to the same enzyme. Thus, NADPH–cytochrome c reductase appears to be the microsomal enzyme responsible for the reduction of a number of electron acceptors. The mechanism of electron transfer from NADPH to the electron acceptor is probably mediated by a 'shuttle' between a fully reduced ($FADH_2$) and a semireduced (FADH) form of the FAD molecules of the enzyme (Kamin *et al.*[20]). This mechanism, suggested by Kamin on the basis of spectral changes of the enzyme, applies to the reduction of one-electron acceptors (cytochrome c, ferricyanide) as well as two-electron acceptors (DCl, menadione). It is possible, however, that the reduction of four-electron acceptors (NT, azo compounds) may occur by different mechanisms.

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