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Some Properties of Hepatic Reduced Nicotinamide Adenine Dinucleotide Phosphate–Cytochrome *c* Reductase†

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ABSTRACT: A purified NADPH–cytochrome *c* reductase prepared by trypsin solubilization and chromatography from rabbit liver microsomes had the following properties: (1) it contained two flavines per single polypeptide of mol wt 6.8×10^4 (sodium dodecyl sulfate gel electrophoresis) or 7.9×10^4 (sedimentation equilibrium); (2) one flavine was FAD, the other, FMN, approximately equimolar; (3) upon reduction by NADPH in the presence of O_2, an O_2-stable neutral semiquinone containing one flavine free radical per two flavines formed; the other flavine appeared to be fully oxidized; (4) excess NADPH in the absence of O_2 only partially reduced the flavine semiquinone, and the NADPH-residual free radical had properties similar to those of the O_2-stable semiquinone; and (5) the NADPH-reduced enzyme autoxidized by both one-electron equivalent and two-electron equivalent mechanisms. A free radical similar to the O_2-stable flavoprotein semiquinone appeared in whole microsomes when reduced by NADPH in the presence of O_2.

Liver NADPH–cytochrome *c* reductase (NADPH: cytochrome *c* oxidoreductase, EC 1.6.99.2) was first isolated from beef liver acetone powder by Horecker (1950) and was subsequently purified from hog and beef liver microsomes by Williams and Kamin (1962), Phillips and Langdon (1962) and Baggot and Langdon (1970), from rabbit liver microsomes by Nishibayashi *et al.* (1963), and from rat liver microsomes by Omura and Takesue (1970). These various investigators used either trypsin or a lipase to solubilize the enzyme, and reported that it contained one to two molecules of FAD per molecule of enzyme and was free of heavy metal. The enzyme catalyzed one-electron transfer such as a typical one-electron reduction of quinones (Iyanagi and Yamazaki, 1969), cytochrome *c*, and ferricyanide (Masters *et al.*, 1965a,b). The reduction mechanism was clarified by Masters *et al.* (1965a,b), who concluded from spectrophotometric and

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kinetic data that the reduction of cytochrome *c* and ferricyanide involved cycling of the flavine in a shuttle between its fully and half-reduced forms. The physiological role of this enzyme, however, is the transfer of two electrons from NADPH to cytochrome P-450 during the catalysis of mixed-function oxidations in liver endoplasmic reticulum, and the relationship between structure, mechanism, and physiological function remains unknown. In the present paper we report a purification from rabbit and pig microsomes which produced a high activity, homogeneous form of the enzyme containing one molecule each of FAD and FMN. This enzyme formed an O_2-stable semiquinone at one reducing equivalent above the fully oxidized state, and remained partially semiquinonoid after complete reduction with NADPH. Its autoxidation appeared to involve both one- and two-equivalent redox reactions. A free radical appeared in aerobic microsomes treated with NADPH which had properties similar to the O_2-stable semiquinone of NADPH–cytochrome *c* reductase.

Experimental Section

Methods. Optical spectra were measured with a Cary Model 14 spectrophotometer, in a sample compartment thermostatted at 25°. Cytochrome P-450 was determined from the difference spectrum, reduced-CO complex minus reduced, $\Delta\epsilon_{450-500nm}$ 91 mM^{-1} cm^{-1}; and cytochrome b_5 was determined from the aerobic difference spectrum, reduced minus oxidized, $\Delta\epsilon_{423-409nm}$ 185 mM^{-1} cm^{-1} (Omura and Sato, 1964a). Electron paramagnetic resonance (epr) derivative absorption spectra were observed with a Varian V-4500 spectrometer at 100-kHz field modulation and 9 kMHz, using a Varian variable-temperature accessory. A flat cell was used at room temperature.

Velocity and equilibrium sedimentations were carried out at 20° with a Spinco Model E analytical ultracentrifuge. Fluorescence measurements for flavine determinations (Bessey *et al.*, 1949) were made with an Aminco Bowman spectrophotofluorimeter. Gel electrophoresis was carried out as described by Weber and Osborn (1969) using 8% acrylamide gel at pH 8.0 (0.3 M Tris-acetate buffer) in the presence of 0.1% sodium dodecyl sulfate, and an Ortec apparatus. Protein was then stained with Coomassie Blue (Fairbanks *et al.*, 1971). Prior to electrophoresis, protein samples were incubated in 2% sodium dodecyl sulfate and 2% mercaptoethanol at room temperature overnight, then at 60° for 60 min. Homogeneous γ-globulin (mol wt 160,000), bovine serum albumin (mol wt 67,000), ovalbumin (mol wt 44,000), bovine heart cytochrome *c* (mol wt 12,400), lactoglobulin (mol wt 18,500), and chymotrypsinogen (mol wt 26,000) were used as molecular weight standards for gel electrophoresis and gel filtration. Protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin standard (Labtrol).

Materials. NADPH, NADH, FAD, FMN, Tos-PheCH$_2$Cl,[1] cytochrome *c* (horse heart, Sigma type III), Triton N-101, and trypsin (bovine pancreas, type III, twice recrystallized) were purchased from Sigma, Sephadex G-100 and DEAE-Sephadex A-50 from Pharmacia, DEAE-cellulose from Schleicher and Schuell, and desoxycholate from Mann Research Laboratories. Snake venom (*Naja naja*) was obtained from the Ross Allen Reptile Institute. Acrylamide, *N,N'*-methylenebisacrylamide and sodium dodecyl sulfate were purchased from Matheson, Coleman & Bell. FAD and FMN were purified from commercial samples by the method of Massey and Swoboda (1963), but the purified FMN sample from this source still contained fluorescing contaminants (Table III). FAD and FMN were also prepared from D-amino acid and *Peptostreptococcus elsdenii* flavodoxin by the method of Mayhew and Massey (1969). FAD and FMN concentrations were determined spectrometrically using ϵ_{450} 11.3 × 10^3 M^{-1} cm^{-1} (pH 7.0) and ϵ_{445} 12.5 × 10^3 M^{-1} cm^{-1} (pH 7.0), respectively (Koziol, 1971). Flavodoxin concentration was determined spectrometrically using ϵ_{455} 10,200 M^{-1} cm^{-1} (Mayhew and Massey, 1969). The extinction coefficient for NADPH–cytochrome *c* reductase was found to be 10.7 × 10^3 M^{-1} cm^{-1} at 455 nm by the method of Mayhew and Massey (1969). Apoflavodoxin was prepared by the method of Mayhew (1971). D-Amino acid oxidase was purified by the method of Kubo *et al* (1960) from pig kidney. Tos-PheCH$_2$Cl-treated trypsin was prepared by the method of Schoellmann and Shaw (1963). All other reagents were of the best grade commercially available.

Preparation of Microsomes. Male rabbits (2–2.5 kg) were given intraperitoneal injections of 60 mg of sodium phenobarbital/kg, each day for 5 days. The animals were then sacrificed by air embolism, and the livers were removed immediately and perfused with cold isotonic saline, then homogenized intermittently for a total of 2 min in four volumes of ice-cold 0.15 M KCl, using a cold Waring blender. The homogenate was centrifuged at 10,000*g* for 20 min; the supernatant was then centrifuged at 35,000 rpm (Beckman Ti-15 rotor) for 90 min. The sediment was resuspended in 0.15 M KCl and recentrifuged at the same speed, to give the *microsomal fraction.* All subsequent operations were carried out near 4°. Microsomes were prepared from pig liver by the same method.

Preparation of NADPH–Cytochrome c Reductase from Trypsin-Treated Microsomes. KCl-washed microsomes were suspended in cold 0.1 M potassium phosphate buffer (pH 7.7), containing 1 mM EDTA. The NADPH–cytochrome *c* reductase was released and purified from the membranes by the general method of Omura and Takesue (1970) except that Tos-PheCH$_2$Cl-treated trypsin was employed. Chymotrypsin activity is inhibited by this treatment (Singer, 1967).

Tos-PheCh$_2$Cl-trypsin (80 mg) was added to microsomes (about 20 g of protein in 1.7 l. of 0.1 M potassium phosphate buffer (pH 7.7) containing 1 mM EDTA). The suspension was stirred for 12 hr at 0°, under anaerobic conditions. The trypsin-digested suspension was then centrifuged at 35,000 rpm (Beckman Ti-15 zonal rotor) for 120 min to sediment the microsomal residue. The supernatant fraction, containing about 80% of the original NADPH–cytochrome *c* reductase activity and less than 10% of the original cytochrome b_5, gave a precipitate with ammonium sulfate at pH 7.7 between 45 and 75% saturation. The precipitate was dissolved in a small volume of 0.1 M potassium phosphate buffer (pH 7.7) and applied to a Sephadex G-100 column (4.5 × 50 cm) previously equilibrated then eluted with 0.02 M potassium phosphate buffer (pH 7.7), containing 0.2 mM EDTA. The reductase fractions from the column were combined, and applied to DEAE-cellulose (2.0 × 30 cm) equilibrated with 0.02 M potassium phosphate buffer (pH 7.7), containing 0.2 mM EDTA (buffer A). The reductase was eluted by a linear gradient (0–0.5 M KCl) in buffer A. High purity enzyme was obtained by repeating the gel filtration

[1] Abbreviations used are: Tos-PheCH$_2$Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Triton N-101, nonylphenoxy-poly(ethoxyethanol).

and DEAE-cellulose chromatography steps. Enzyme activity was assayed by measuring the rate of cytochrome *c* reduction (Omura and Takesue, 1970). The activity of these preparations was about 38–41 μmol/min per mg of protein at pH 7.7, about the same as obtained by Omura and Takesue in (1970) and Masters and Zeigler (1971). The purified preparations of other investigators had activities ranging from 8.1 to 21 μmoles per min per mg of protein (Horecker, 1950; Phillips and Langdon, 1962; Masters *et al.*, 1965a; Williams and Kamin, 1962; Hernandez *et al.*, 1967).

Preparation of Detergent-Solubilized NADPH–Cytochrome c Reductase and Cytochrome b_5. Desoxycholate and Triton N-101 solubilized NADPH–cytochrome *c* reductases were prepared by a modification of the method of Fujita *et al.* (1970). A suspension of microsomes, about 10 g of protein in 500 ml of 0.1 M Tris-acetate buffer (pH 7.6), was stirred for 30 min at 0° with 2.5 g of deoxycholate, 10 ml of Triton N-101, 25 ml of glycerol, and 186 mg of EDTA; the precipitate recovered by centrifugation for 60 min at 105,000*g* was discarded. The supernatant solution was applied to a DEAE-cellulose column (6.0 × 75 cm) previously equilibrated with 0.1 M Tris-acetate (pH 7.6), containing 2.5% glycerol, 0.1% Triton N-101 and 1 mM EDTA (buffer B). The column was washed with 4 l. of buffer B to elute P-450 and P-420 particles. Cytochrome b_5 adsorbed at the middle of the column. The top 17 cm of the column was removed, and repacked as another column. The reductase was subsequently eluted from this column with buffer B containing 0.5 M KCl. Active fractions were combined and dialyzed against 2 l. of buffer B overnight, then applied to a DEAE-cellulose column (2.5 × 45 cm), equilibrated with buffer B. The column was washed with buffer B containing 0.1 M KCl; the reductase was then eluted with buffer B containing 0.5 M KCl. The active yellow fraction was applied to a Sephadex G-100 column (4.5 × 50 cm) equilibrated with buffer B. The active fractions were combined and applied to DEAE-Sephadex A-50 (2.0 × 30 cm) equilibrated with buffer B. The column was washed with the buffer containing 0.2 M KCl. The active yellow fraction was dialyzed against 2 l. of buffer B overnight, applied to a DEAE-Sephadex A-50 (2.0 × 30 cm), and equilibrated with buffer B and the reductase was eluted with a linear gradient, 0–0.5 M KCl in the buffer B. The active fraction had a specific activity of 12.4 μmol/min per mg (cytochrome *c* reduction) and 1.06 μmol/min per mg (detergent–cytochrome b_5 reduction). The enzyme contained flavine, 15.5 nmol/mg by the method of Bessey *et al.* (1949), and the ratio of FMN to FAD was 0.86. The samples contained contaminating proteins, including P-420 and cytochrome b_5, as shown in Figure 3. Detergent–cytochrome b_5 was purified to homogeneous form with DEAE-cellulose and Sephadex G-100 columns; it had mol wt 16,000 (gel electrophoresis in the presence of sodium dodecyl sulfate). Its epr signal at 22°K was identical with that of trypsin-solubilized cytochrome b_5 (Ichikawa and Yamano, 1970).

Results

The Stable Free Radical in Microsomes. A narrow g = 2.00 signal behaving like a free-radical signal has been reported in resting hepatic microsomes (Murakami and Mason, 1967; Miyake *et al.*, 1967). A g = 2.00 signal, observed at room temperature, also arose from microsomes upon addition of NADPH in the presence of O_2 (Figure 1). A similar signal was observed upon aerobic addition of NADH. The NADPH-dependent signal diminished upon anerobic addition of NADPH or ferricyanide. The signal, with g = 2.00, about 80-G wide was similar to the signal arising from purified NADPH–cytochrome *c* reductase (Figure 1E), as we shall describe. Both signals were stable in the presence of O_2. Similar results were obtained with low-temperature epr spectroscopy.

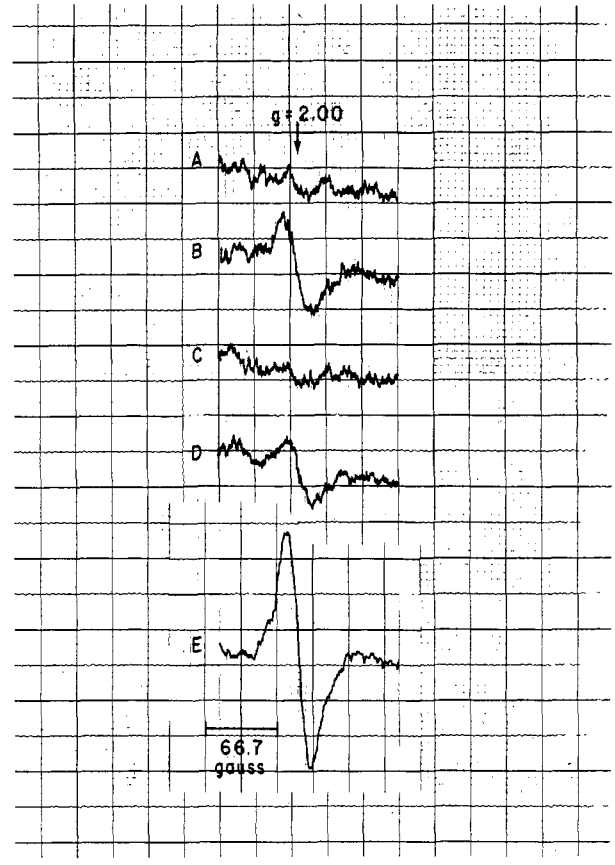

FIGURE 1: Room temperature epr spectra of phenobarbital-induced, oxidized, microsomes in the g = 2.00 region. Curve A, resting microsomes (53 mg/ml) suspended in 0.1 M potassium phosphate buffer (pH 7.7), containing 1 mM EDTA; curve B, microsomes, and curve E, purified NADPH–cytochrome *c* reductase (17.7 μM) after 330-sec incubation with 300 μM NADPH in the presence of air, 23°; curve C, sample B, after oxidation with 2 mM ferricyanide: curve D, excess NADPH (2 mM) added to sample B. The cytochrome P-450 and flavine contents given in Table I. Modulation amplitude, 6.5 G; microwave power, 25 mW; scanning rate, 200 G/min; magnetic field increased from left to right.

Relationship between NADPH–Cytochrome c Reductase and the g = 2.00 Signal from Microsomes. The relationship between flavine content, NADPH–cytochrome *c* reductase activity, and the signal height of the stable microsomal free radical is summarized in Table I. Forty per cent of the 10% trichloroacetic acid extractable microsomal flavine was recovered in the supernatant after treatment of microsomes with Tos-PheCH$_2$Cl-trypsin. The extent of solubilization of the stable free radical corresponded to that of the flavines and of NADPH–cytochrome *c* reductase activity. The signal height of the solubilized stable free radical in the trypsin-treated supernatant also followed the NADPH–cytochrome *c* reductase activity during purifications of the enzyme (Table II). The capacity for development of the O_2-stable g = 2.00 signal thus paralleled purification of NADPH–cytochrome *c* reductase. During gel filtration of the ammonium sulfate fraction (40–75%) in Sephadex G-100, the elution peak of

TABLE I: Flavine Content, Activity, and Signal Height at $g = 2.00$ in Microsomes, Trypsin-Treated Microsomes, Microsomal Supernatant, and Purified Enzyme.[a]

System	Signal Height at $g = 2.00$ (Arbitrary Units)	nmol/ml (nmol/mg of Protein)		Protein (mg/ml)	NADPH–Cytochrome *c* Reductase Act.	
		FAD	FMN		Total Act./ml	Sp Act.[b]
1. Oxidized (resting) microsomes, as prepared	12	19.6 (0.37)	5.3 (0.10)	53	10.8	0.204
2. (1) +NADPH	85					
3. (2) +Ferricyanide	24					
4. Trypsin-treated microsomes	16	14.2 (0.33)	1.7 (0.04)	43	1.76	0.041
5. (4) +NADPH	29					
6. Trypsin-treated supernatant	10	5.1 (0.73)	4.6 (0.66)	7	11.7	1.67
7. (6) +NADPH	80					
8. Purified NADPH–cytochrome *c* reductase	100	4.0 (12.5)	3.4 (10.6)	0.32		37.4

[a] Tos-PheCH$_2$Cl-trypsin (10 mg) was added to microsomes, 30 ml (53 mg/ml in 0.1 M potassium phosphate buffer (pH 7.7), containing 1 mM EDTA), and the mixture was stirred at 0° for 10 hr under anaerobic conditions. The trypsin-digested mixture was diluted to 60 ml in the same buffer and the mixture was centrifuged at 105,000*g* for 120 min, to obtain the supernatant and trypsin-treated microsomes. Flavine content was measured by the method of Bessey *et al.* (1949) after 15-min incubation with 10% trichloroacetic acid at 0°. Signal height was measured at −170° after 60-sec incubation with 300 μM NADPH in the presence of air at 23°. Signal height was also measured at −170° after 60-sec incubation with 2 mM ferricyanide at 23°. Induced microsomes contained 3.2 nmol/mg of P-450. [b] μmol/min per mg of protein.

TABLE II: Correlation between NADPH–Cytochrome *c* Reductase Activity and the $g = 2.00$ Signal Height During Purification of NADPH–Cytochrome *c* Reductase from Phenobarbital-Induced Rabbit Liver Microsomes.

	Total Protein (mg)	NADPH–Cytochrome *c* Reductase Act.		Signal Height at $g = 2.00$	
		Total	%	Total	%
1. Microsomes	5930	4.00	100	170	100
2. Trypsin-treated microsomes	4860	0.96	24	40	24.1
3. Supernatant after trypsin treatment	1120	4.13	103.3	125	70.3
4. Ammonium sulfate precipitate					
0–40% saturation	560	0.24	6	13.8	8.1
40–75% saturation	265	2.90	72.4	58.3	34.3
75–100% saturation	130	0.23	5.7	16.5	9.7
5. DEAE-cellulose	13.3	1.90	47.8	110.7	65.1

enzymic activity was the same as that of the capacity to produce the $g = 2.00$ signal with aerobic NADPH.

The response of the signal arising from the O_2-stable free radical in microsomes and that from purified NADPH–cytochrome *c* reducatse to changes in incident microwave power at both −40 and −170° are shown in Figure 2. The spectra are broadened inhomogeneously at both temperatures with increase of microwave power. A similar result was reported by Beinert and Orme-Johnson (1967) for a preparation of the enzyme ascribed to Masters and coworkers (*cf.* Masters *et al.*, 1965a), and was interpreted by them as arising from a flavine semiquinone free of interaction with metal. The inhomogeneity of the broadening could be due to the presence in the system of a mixture of signals, and to unresolved hyperfine structure. Since the microsomal signal is similar to the (lipid-free) enzyme signal, since the capacity to produce it follows the enzymic activity during purification from the microsomes, and since the signal is ferricyanide and NADPH reversible in both systems, it appears likely that the microsomal signal is related to that of flavoprotein and not to sulfhydryl or peroxyl free radicals. Cytochrome b_5 reductase, which is a flavoprotein component of hepatic microsomes, does not form an O_2-stable semiquinone.

Characterization of NADPH–Cytochrome c Reductase. The minimal molecular weight of our purified rabbit NADPH–cytochrome *c* reductase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The extent of migration of the major component did not depend on the presence or absence of mercaptoethanol, and a molecular weight of 6.8×10^4 was observed. Trace amounts of contaminating protein or degraded enzyme were always present (Figure 3A, 1 and 2). The major band was also observed by isoelectric focusing at pH 5.6, and a minor band at about pH 5.4. On the other hand, desoxycholate–Triton-

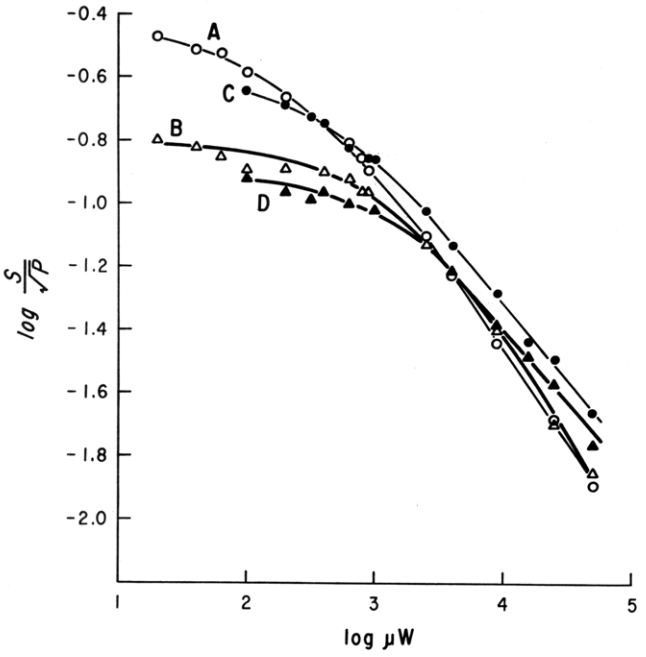

FIGURE 2: Microwave power saturation characteristics of $g = 2.00$ signal and from purified NADPH–cytochrome *c* reductase (curves A and B) and from microsomes (curves C and D) reduced by NADPH in the presence of air. Curves A and B, NADPH–cytochrome *c* reductase: A, −170°; B, −40°. Curves C and D, microsomes: C, −170°; D, −40°. Protein concentration: microsomes, 53 mg/ml in 0.1 M potassium phosphate (pH 7.7), containing 1 mM EDTA; NADPH–cytochrome *c* reductase, 7.4 μM in the same buffer. Modulation amplitude, 12.5 G; scanning rate, 250 G/min. Cytochrome P-450 and flavine contents of the two systems are given in Table I.

N-101-solubilized reductase showed a molecular weight of about 7.5×10^4 (sodium dodecyl sulfate gel electrophoresis) although it was not wholly pure (Figure 3B). Using the gel filtration method with Sephadex G-100, a molecular weight of 6.6×10^4 was obtained with the same sample. These results show that trypsin- and detergent-solubilized NADPH–cytochrome *c* reductases are single polypeptide chains. The apparent variations in molecular weight may have been due to charge effects, reported by Tung and Knight (1971).

The sedimentation patterns of the purified enzyme showed a single symmetrical peak with a sedimentation coefficient $s_{20,w} = 4.9$ S in 0.1·M NaCl, protein concentration of 0.56%. The molecular weight of the enzyme was also determined by sedimentation equilibrium at 8219 rpm for 120 hr, $T = 20°$. If the values of $\bar{v}$ and ρ were assumed to be 0.738 and 0.999639 g per cm^3, respectively, a molecular weight of 7.9×10^4 was obtained (Yphantis, 1964). The log *c* *vs.* r^2 plot was a straight line.

Flavine Components of NADPH–Cytochrome c Reductase. The flavine of purified NADPH–cytochrome *c* reductase was analyzed by three methods: (*1*) *The fluorimetric method of Bessey et al. (1949), after flavine was liberated with 10% trichloracetic acid* (5 min at 0°), and protein removed by centrifugation. The enzyme contained both FAD and FMN. The ratio of FAD to FMN in purified preparations of the enzyme showed fairly constant values near unity (FAD 49.8 ± 2.0%, FMN 50.2 ± 2.0%, five preparations). (*2*) *Chromatography:* an aliquot of flavine solution prepared from the enzyme by the method of Mayhew and Massey (1969) was submitted to paper chromatography with butanol–acetic acid–water, developed in the dark (Yagi, 1951). The chromatogram showed two spots under ultraviolet light, one corresponding to an FAD standard obtained from D-amino acid oxidase, and the other to an FMN standard prepared from flavodoxin (Figure 4). (*3*) *Apoflavodoxin reconstitution:* FAD and FMN in the reductase were determined with snake venom phosphodiesterase (*Naja naja*) and apoflavodoxin, which binds FMN specifically (Mayhew, 1971). The addition of snake venom phosphodiesterase to the total flavine released from NADPH–cytochrome *c* reductase resulted in an increase of 1.82-fold in fluorescence at 520 nm, that is, in FMN, due to hydrolysis of FAD (Table III). Furthermore, addition of about twofold excess of apoflavodoxin to the phosphodiesterase-treated flavines resulted in decrease of 50-fold in fluorescence at 520 nm. The same result was obtained with purified trypsin-solubilized pig NADPH–cytochrome *c* reductase. These results provide strong evidence that the hepatic NADPH–cytochrome *c* reductase obtained in this study contained FAD and FMN in approximately equimolar quantities and that there were about 2 mol of total flavine/mol of protein. The minimal molecular weight of the enzyme calculated from flavine content was about 4.2×10^4–4.5×10^4/mol of total flavine. The holoenzyme was not further activated by incubation with FAD, FMN, or mixture of these flavines.

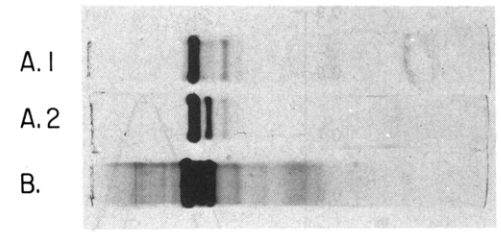

FIGURE 3: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of NADPH–cytochrome *c* reductase. Part A (1 and 2): two preparations of NADPH–cytochrome *c* reductase (trypsin-solubilized); part B: detergent-solubilized NADPH–cytochrome *c* reductase.

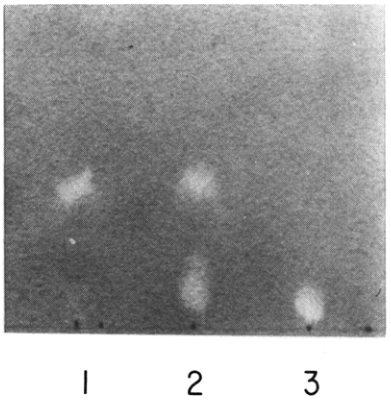

FIGURE 4: Identification of the prosthetic groups of NADPH–cytochrome *c* reductase: paper chromatography developed by the upper phase of 1-butanol–acetic acid–water (4:1:5). Flavine solutions (0.5 μmol/ml) were submitted to paper chromatography. Flavines were detected by fluorescence in ultraviolet light: (1) FMN (flavodoxin standard) (5 μl); (2) flavines from NADPH–cytochrome *c* reductase (10 μl); and (3) FAD (D-amino acid standard) (5 μl).

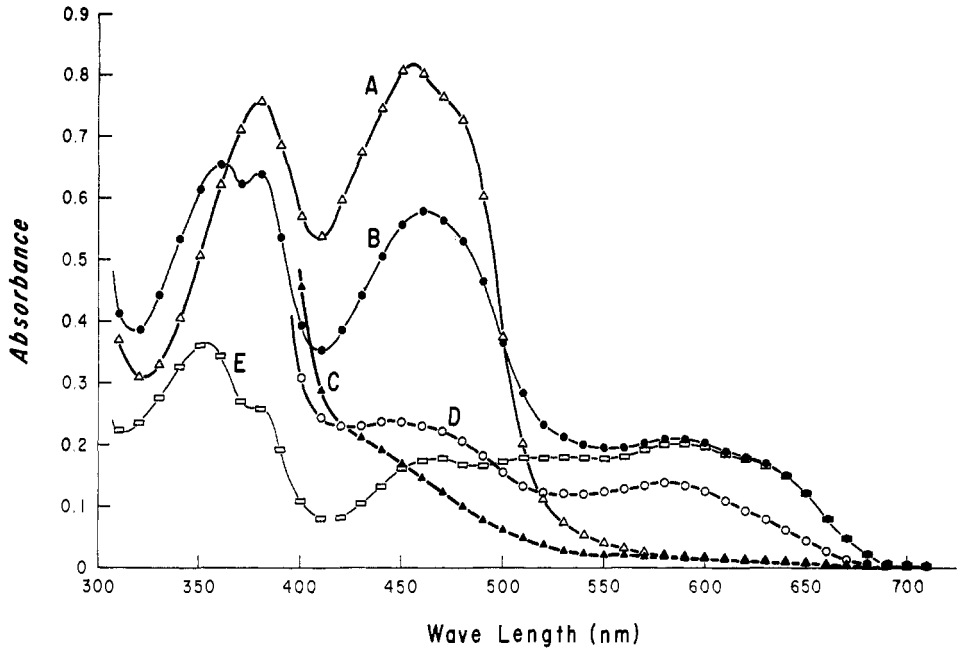

FIGURE 5: Absorption spectra of purified NADPH–cytochrome *c* reductase (trypsin solubilized). The stable semiquinone was prepared by adding NADPH (50 μM, final concentration) in the presence of air to purified enzyme (76.6 μM) in 0.1 M potassium phosphate buffer (pH 7.7), containing 1 mM EDTA, and the system was allowed to stand for about 10 min: curve A, oxidized; curve B, stable semiquinone form; curve D, after additional NADPH (2 mM, final concentration) under anaerobic conditions; curve C, hydrosulfite reduced; and curve E, curve B minus one-half curve A.

Our results therefore differ from those of Horecker (1950), Williams and Kamin (1962), Masters *et al.* (1965), and Nishibayashi and Sato (1970) in respect to the nature of the flavines in NADPH–cytochrome *c* reductase, but are in approximate agreement with recent estimations of molecular weight (*e.g.*, Baggot and Langdon, 1970).

The Oxidation Level of the O_2-Stable Semiquinone. The visible absorption spectrum of our purified NADPH–cytochrome *c* reductase at several oxidation levels is depicted in Figure 5. The oxidized enzyme (*i.e.*, as obtained) has absorption peaks at 380 and 455 nm (ϵ_{445} 10,700 M^{-1} cm^{-1}), typical of a flavoprotein. The O_2-stable semiquinone was obtained by reduction with NADPH in the presence of oxygen. The spectrum is typical of neutral flavoprotein semiquinone (*cf.* Müller *et al.*, 1972) but with ϵ_{585} 2700 M^{-1} cm^{-1}. *This extinction coefficient is much smaller than the values reported for neutral flavoprotein semiquinones*, which lie in the range 4000–5500 M^{-1} cm^{-1} (Müller *et al.*, 1972; Hinkson and Bulen, 1967; Mayhew *et al.*, 1969) (Table IV). At the same time, the extinction at 455 nm, a measure of the oxidized for, is high compared to other neutral flavoprotein semiquinones (Table IV) and the ratios of absorbance at 455 and 585 nm indicate that *the O_2-stable semiquinone of this enzyme contains fully oxidized flavine.* The ratio A_{455}/A_{585nm} is about 2.8 for this enzyme; an even larger value (3.12) is apparent from the spectrum of the O_2-stable semiquinone of NADPH–cytochrome *c* reductase characterized by Masters *et al.* (1965, Figure 5), whereas Müller *et al.* (1972) report values of less than one for several neutral flavoprotein semiquinones (Table IV). The question was investigated further by comparing the properties of the semiquinones of flavodoxin and NADPH–cytochrome *c* reductase directly, and by titrating the reducing equivalents present.

TABLE III: FMN and FAD of NADPH–Cytochrome *c* Reductase, Estimated with Apoflavodoxin.[a]

Sample	Initial Fluorimetric Reading at 520 nm	Reading after Phosphodiesterase Addition (0.8 mg/ml)	Reading after Apoflavodoxin Addition (1.6×10^{-6} M)
FMN (0.83×10^{-6} M)	45	45	8.5
FAD (0.84×10^{-6} M)	4.5	45	1
D-Amino acid oxidase (0.97×10^{-6} M)	4.5	47.5	1
NADPH–cytochrome *c* reductase (0.88×10^{-6} M)	25	45.5	1
	25	Not treated	2.5

[a] Method of Mayhew (1971).

The optical spectra of the oxidized and semiquinonoid forms of NADPH–cytochrome *c* reductase and of flavodoxin are compared in greater detail in Figure 6. The electron transfer flavoprotein, flavodoxin, contains one molecule of FMN per molecule of protein. Its (half-reduced) semiquinonoid form is typically neutral (Müller *et al.*, 1972; Mayhew and Massey, 1969).

The spectrum of the O_2-stable semiquinone of NADPH–cytochrome *c* reductase is similar to that of half-reduced flavodoxin in the 500- to 700-nm region, but the extinction coefficient of the reductase at 585 nm per flavine is about half. The semiquinone concentration in flavodoxin semiquinone is an almost quantitative measure of the total flavine (Mayhew and Massey, 1969). In Table IV the molar extinction coefficient of the oxidized and semiquinonoid states of flavodoxin semiquinone and the O_2-stable NADPH–cytochrome *c* reductase semiquinones are compared. *The flavine radical/total flavine*

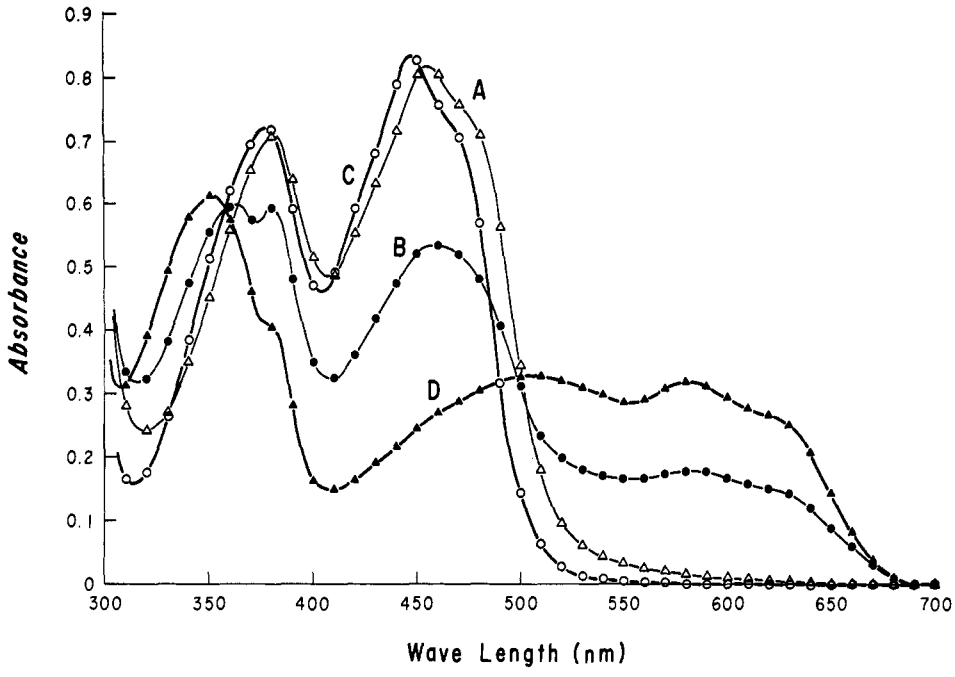

FIGURE 6: Absorption spectra of NADPH–cytochrome *c* reductase and flavodoxin compared: (curve A) NADPH–cytochrome *c* reductase, 76.6 μM in 0.1 M potassium phosphate buffer (pH 7.7) and 1 mM EDTA; (curve B) the stable semiquinone prepared as described in Figure 5; (curve C) flavodoxin, 81.6 μM in 0.1 M potassium phosphate buffer (pH 7.7)–0.06 M EDTA; (curve D) its semiquinone, obtained by light irradiation (Mayhew *et al.*, 1969a); semiquinone concentration, 71 μM.

TABLE IV: Molar Absorptivities of Some Neutral Flavoprotein Semiquinones.

Flavoprotein	Wavelength (nm)	Molar Absorptivity (M^{-1} cm^{-1}) Oxidized	Semiquinone	Absorbancy Ratios
Flavodoxin (Mayhew and Massey, 1969)	445	10,200	2100	0.47[a]
	580		4500	
Azotobacter flavoprotein (Hinkson *et al.*, 1967)	452	10,600	2570	0.47[b]
	580		5440	
NADPH–cytochrome *c* reductase (Masters *et al.*, 1965a)	455	11,300	8100[e]	3.12[c,e]
	580		2600[e]	
NADPH–cytochrome *c* reductase (present work)	455	10,700	7500 (1) 7000 (2) 7100 (3)	2.78 (1)[d] 2.92 (2)[d] 2.73 (3)[d]
	585		2700 (1) 2400 (2) 2600 (3)	

[a] Ratio A_{445}/A_{580} in the semiquinone form. [b] Ratio A_{452}/A_{580} in the semiquinone form. [c] Ratio A_{455}/A_{580} in the stable semiquinone form. [d] Ratio A_{455}/A_{585} in the stable semiquinone form; sample 1, 2, 3. [e] Calculated from Figure 5, in Masters *et al.* (1965a).

ratio of the O_2-stable reductase semiquinone is clearly about half of that in half-reduced flavodoxin. The observed spectrum of the O_2-stable semiquinone minus one-half the observed spectrum of the oxidized enzyme gives a difference spectrum (Figure 5, curve E) very similar to the spectrum of a half-reduced flavodoxin (Figure 6, curve D). The point is of importance because the O_2-stable semiquinonoid state was implicated by Masters *et al.* (1965a,b) in the "shuttle" mechanism which they proposed for this enzyme ($2FADH_2 \rightleftharpoons 2FADH\cdot + 2H\cdot$), and this oxidation level now appears to contain a substantial proportion of fully oxidized flavine.

Comparison of the Epr Signals and Spin Concentrations of the O_2-Stable Semiquinone of NADPH–Cytochrome c Reductase and the Semiquinones of Flavodoxin and Other Flavine Semiquinones. A quantitative estimate of the spin concentration in the O_2-stable semiquinone of NADPH–cytochrome *c* reductase was made by a comparison of the double integral of its epr signal observed at different temperatures and at different incident microwave powers, with the corresponding double integral of the epr signals from *p*-benzosemiquinone (Narni *et al.*, 1966), Cu-EDTA, and half-reduced (anaerobic) flavodoxin semiquinone (Table V). Neither the signal of the flavodoxin semiquinone nor that of the O_2-stable NADPH–cytochrome *c* reductase saturated at microwave powers up to 2 mW; at 9 mW both signals lost about 10% of their presumptive nonsaturated height at $g = 2.00$. The signals of the flavine semiquinones are quantitative measures at powers less than 2 mW, and that of flavodoxin semiquinone is also

TABLE V: Spin Concentrations of the O_2-Stable Semiquinone of NADPH–Cytochrome *c* Reductase, Estimated by Epr Spectrometry against Three Standards.

Standard	Enzyme Concn (μM)	Estimated Spin Concn (μM)	Ratio of Spin Concn/Flavine (%)	Microwave Power (mW)
p-Benzosemiquinone (23°)	76.6	33.3	44	9
Cu-EDTA (−40°)	76.6	38.7	51	0.1
Flavodoxin, half-reduced (23°)	76.6	29.1	38	0.25–2

a measure of total flavine (Mayhew and Massey, 1969). Using three standards of measurement, the epr estimations of spin concentration in the O_2-stable reductase semiquinone ranged from 38 to 51% of the total flavine concentration, thus confirming the estimates based on optical measurements within the wider limits of precision of epr estimations.

Oxidation Level of the O_2-Stable Semiquinone of NADPH–Cytochrome c Reductase. The effective oxidation level of the O_2-stable semiquinone of NADPH–cytochrome *c* reductase was found to be 1 equiv more reduced than its fully oxidized state, by titration of the O_2-stable semiquinone with $K_3Fe(CN)_6$ (Figure 7). One oxidizing equivalent of ferricyanide was consumed per two flavines judged by decrease of absorbancy at 585 nm, and the enzyme simultaneously became fully oxidized, as judged by the increase in absorbancy at 455 nm. This result is in good general agreement with the optical and epr estimates of the flavine semiquinone content of the enzyme in the O_2-stable semiquinonoid state. Thus, within their limits of accuracy, the various estimates of semiquinonoid and fully oxidized flavine in our O_2-stable semiquinone of NADPH–cytochrome *c* reductase show that it has an oxidation level corresponding to the structure

$$\begin{bmatrix} F \\ FH\cdot \end{bmatrix}$$

but the results do not show whether or not the two flavines in the molecule have distinct oxidation levels, or stand in overall equilibrium with one another, or whether each flavine site contains FMN or FAD solely, or in mixture.

The NADPH–Residual Semiquinone. The O_2-stable reductase semiquinone was not completely reduced by excess NADPH even under anaerobic conditions: 58% (room temperature epr) or 51% (−40°, epr) of the stable semiquinone concentration remained unaffected (Figure 8). Masters *et al.* (1965) report the same phenomenon. The extent of decrease of optical absorption at 585 nm in the presence of excess NADPH under the same conditions was in good agreement with the decrease of spin concentration as judged by epr spectrometry. The spectral maximum at 585 nm was identical in both the O_2-stable and NADPH–residual semiquinones. Redox titrations are being performed and will be described elsewhere. The microwave power dependence of the O_2-stable semiquinone signal and of the NADPH–residual signal in the presence of excess NADPH is shown in Figure 8. The NADPH–residual signal, which represents about 25% of the total flavine in the O_2-stable semiquinone, has the same shape as, and saturation characteristics similar to, that of the O_2-stable semiquinone. The NADPH–residual signal saturated inhomogeneously.

Autoxidation of NADPH–Cytochrome c Reductase. Strobel and Coon (1971) and Aust and his coworkers (1972) have reported that the autoxidation of NADPH–cytochrome *c* reductase gives rise to superoxide anion, O_2^-. In the present study we utilized the relatively slow autoxidation of NADPH-reduced enzyme in order to determine whether both flavines acted in a coordinated way in this reaction, cycling through fully reduced and half-reduced forms, and whether differences in the reactivities of the flavines toward O_2 could be observed. Molecular oxygen is a very poor substrate, and therefore convenient for detecting intermediate states of the NADPH-reduced enzyme during oxidation–reduction. We investigated changes in epr signal intensity, changes in absorbance at 580 nm (a measure of free radical), changes at 455 nm (a measure of oxidized flavine), at 340 nm (a measure of oxidation of NADPH), simultaneously (Figure 9). The rate of appearance of the semiquinone signal during the autoxidation of NADPH-reduced enzyme by O_2 (Figure 9, curve A) paralleled the increase of semiquinone absorbance at 580 nm (Figure 9, curve B), as expected. However, the initial rate of increase at 580 nm was similar to the initial rate of decrease at 455 nm, indicating that both free-radical production (one-equivalent oxidation–reduction) and fully oxidized flavine reduction took place together under these conditions. After the initial reduction, free-radical production continued without further loss of oxidized flavine. The reason for this must lie in the complex redox relationships between the two flavine moieties, as we indicate in the following discussion.

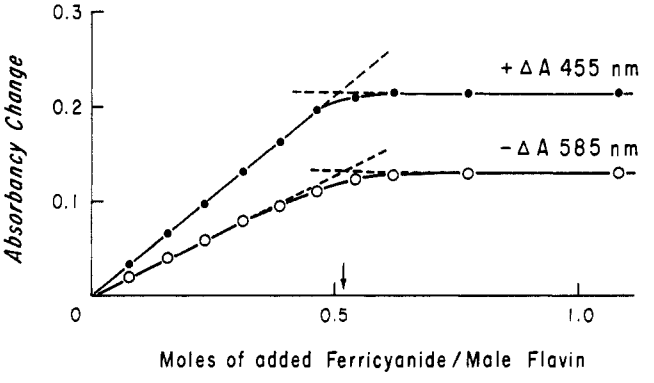

FIGURE 7: Ferricyanide titration of the O_2-stable semiquinone of NADPH–cytochrome *c* reductase. Absorbance changes at 455 nm (positive, increase of oxidized flavine) and at 585 nm (negative, decrease of O_2-stable semiquinone (are plotted against ferricyanide added per flavine present. NADPH–cytochrome *c* reductase, 64.3 μM in 0.1 M potassium phosphate, pH 7.7 (1-ml total volume), was titrated with 1 mM ferricyanide in 0.1 M potassium phosphate (pH 7.7) at 25°. The volume change during titration was less than 0.5% of the original volume.

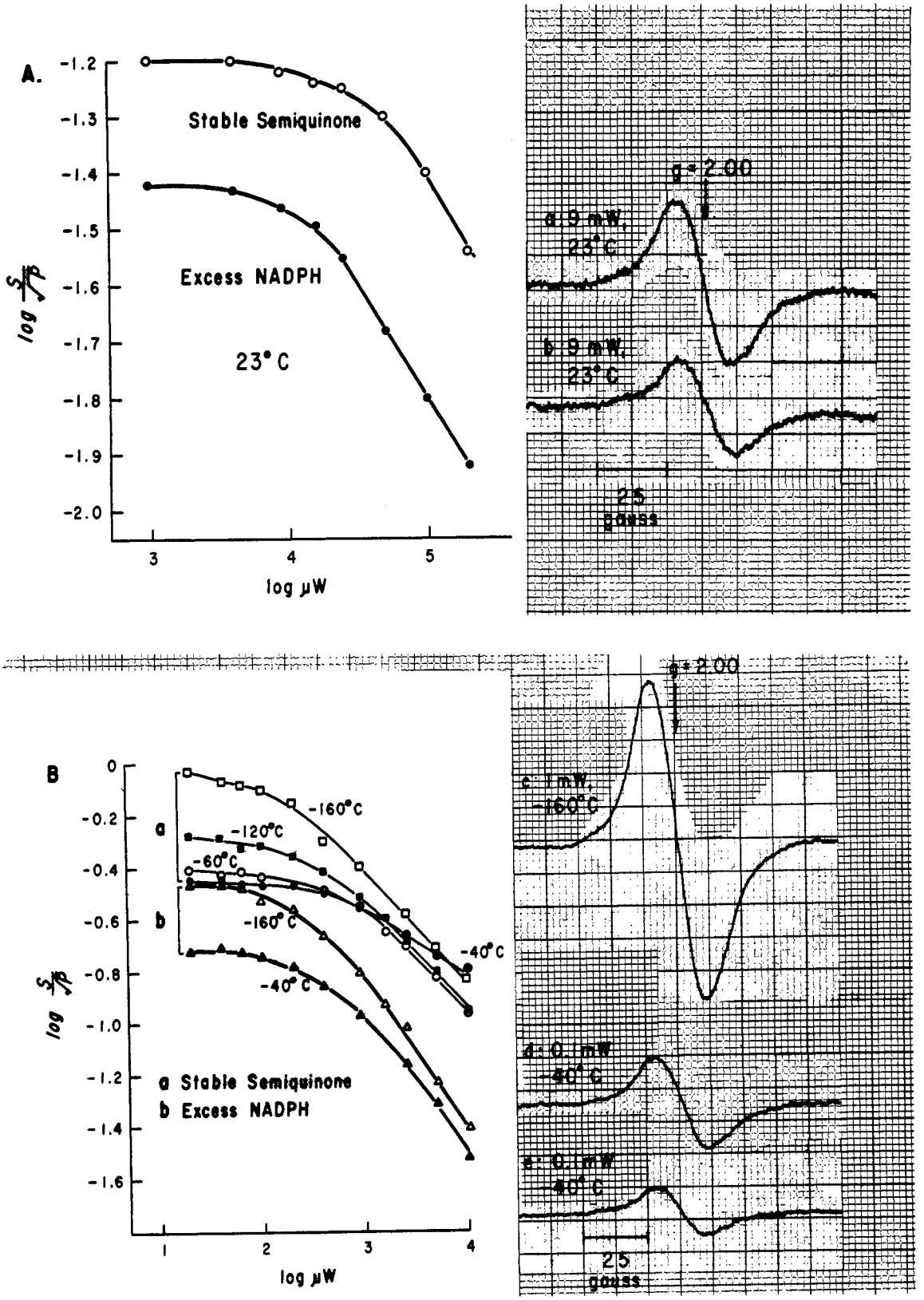

FIGURE 8: Microwave saturation curves and representative epr spectra of the O_2-stable and the NADPH-residual semiquinones of NADPH–cytochrome *c* reductase. Part A: saturation curves and epr spectra at 23°. The samples described in Figure 6 were used in these experiments. Epr spectrum, curve a, of the O_2-stable flavoprotein semiquinone, was observed at 9-mW, modulation amplitude, 6.5 G, scanning rate, 25 G/min, the magnetic field increasing from right to left epr spectrum; curve b was observed by adding NADPH anaerobically to a final concentration of 2 mM to sample a, under the same epr conditions. The spin concentrations for curves a and b were 33.3 and 25.5 μM, respectively. Part B, the same saturation curves and epr spectra observed at −40, −60, −120, and −160°, and at 1 mW (spectrum c) and at 0.1 mW (spectra d and e). The spin concentrations were similar to the concentrations used in part A.

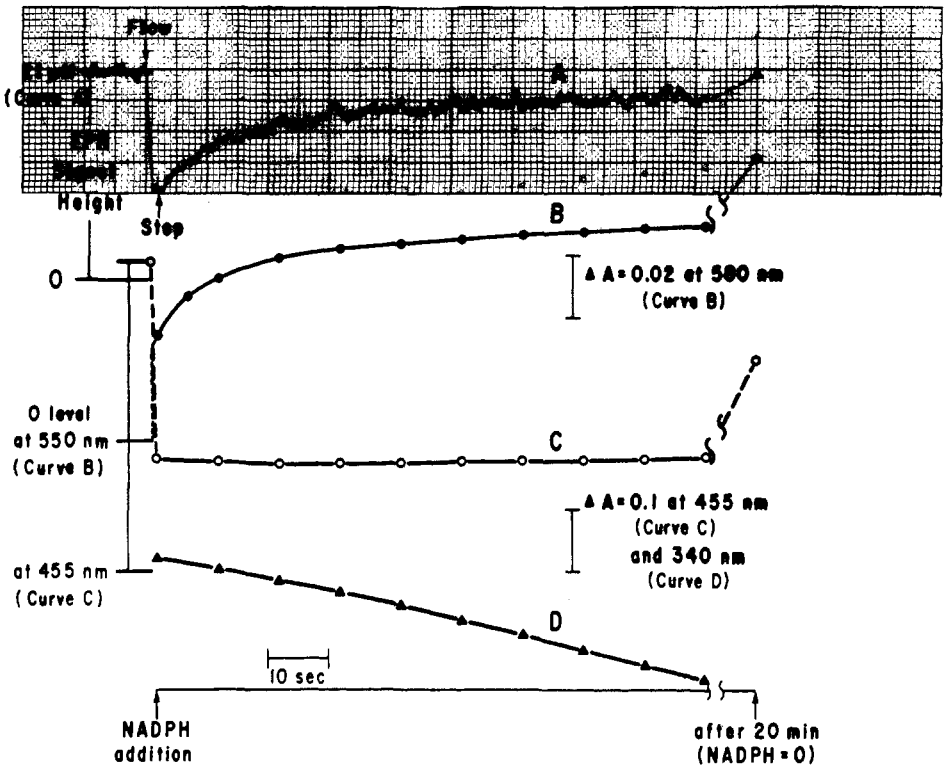

FIGURE 9: Time course of flavine free-radical formation during autoxidation of NADPH–cytochrome *c* reductase, observed optically and by epr. At "Flow" (curve A), enzyme and NADPH were rapidly mixed (dead time, about 30 msec) aerobically in an epr flat cell with a four-jet mixer, so as to produce a final concentration of 47.2 μM NADPH–cytochrome *c* reductase and 160 μM NADPH in 0.1 M potassium phosphate buffer (pH 7.7) at 23°. The magnetic field was set at the signal maximum. Curves B and C show flavine oxidation reduction at 580 and 455 nm, respectively, when the same system was observed spectrophotometrically. Curve D shows NADPH oxidation at 340 nm.

Discussion

In the present study hepatic NADPH–cytochrome *c* reductase was obtained with mol wt 6.8×10^4 or 7.9×10^4 (depending upon method), consisting of a single polypeptide, $s_{20,w} = 4.9$ S, containing two flavine molecules, and having an activity in the range 37.8–40.8 μmol of cytochrome *c* reduced sec^{-1} mg^{-1} of protein at pH 7.7. In these respects it resembled preparations already described by Omura and Takesue (1970), and by Masters and Zeigler (1971). Other preparations already described had about 50% or less of its activity, and lower flavine content. However, our preparation differed from all others in two important respects: the flavine consisted of approximately one molecule each of FMN and of FAD per polypeptide, and it gave an O_2-stable semiquinone at an oxidation level one reducing equivalent above the fully oxidized state.

There are several possible explanations for the substantial amount of FMN observed in our preparation but not observed in those of other investigators. The apoenzyme has a higher affinity for FMN than for FAD (Nishibayashi and Sato, 1970; Baggot and Langdon, 1970), and it is possible that an equilibration of flavines among FAD, FMN, and the enzyme occurred during preparation. FMN has been reported to comprise about one-third of the total flavine in hepatic microsomes (Mason *et al.*, 1965). It is also possible that one-half of the FAD content of the enzyme was hydrolyzed to FMN during preparation, or that the FMN content of the native enzyme was inexplicably missed by other investigators. In our work, two procedures (Tos-PheCH$_2$Cl-trypsin solubilization and detergent soubilization) produced enzyme having equimolar quantities of FMN and FAD and we obtained identical results with both rabbit and pig liver preparations. Our trypsin solubilization procedure differed from that of Omura and Takesue (1970) only in minor respects: the use of rabbit or pig liver rather than rat liver, the use of Tos-PheCH$_2$Cl-treated trypsin under N_2 rather than trypsin in air, the appearance of less than 10% of the microsomal cytochrome b_5 with NADPH–cytochrome *c* reductase in the trypsin supernatant, instead of 70–80%, and the use of an ammonium sulfate fractionation between 45 and 75% of the supernatant, rather than freeze-drying it for recovery.

Our enzyme preparations gave an O_2-stable flavoprotein semiquinone, with between 38 and 51% of the flavine in an epr-detectable semiquinonoid state, the remainder of the flavine fully oxidized, as judged by spectrophotometry and by titration with ferricyanide. The (less active) preparation described by Masters *et al.* (1965a) also produced an O_2-stable semiquinone under conditions similar to those used in the present work, but their O_2-stable semiquinone had a much higher ratio, fully oxidized flavine to semiquinonoid flavine, than ours (Table IV) and a much lower epr-detectable content of semiquinone, for structural reasons not now understood. The optical spectrum of our O_2-stable semiquinone resembled that of neutral flavine semiquinone except for its content of oxidized flavine (Müller *et al.*, 1972). It was reduced anaerobically by NADPH to a state containing 25% or less of the total flavine in an epr-detectable form, not demonstrably different with respect to its microwave saturation characteristics from the O_2-stable semiquinone. The remainder of the flavine was fully reduced.

In principle, NADPH–cytochrome *c* reductase can exist in nine redox states:

$FADH_2$	$FADH_2$	FADH·
$FMNH_2$	FMNH·	$FMNH_2$
I	II	III
FADH·	FADH·	FAD
FMNH·	FMN	FMNH·
IV	V	VI
FAD	$FADH_2$	FAD
FMN	FMN	$FMNH_2$
VII	VIII	IX

Whether or not these states are all observably distinct from one another will depend upon several factors, including the midpotential of each one-electron redox couple, the degree of overlap of the potentials of the $FADH_2$–FADH·–FAD and $FMNH_2$–FMNH·–FMN systems, and the degree of spin coupling between the two flavine molecules. In the present study we have observed two states: state I (dithionite reduced), and state VII (ferricyanide oxidized). The O_2-stable flavoprotein semiquinone lies one reducing equivalent above fully oxidized enzyme, state VII, *i.e.*, either state V and/or VI, or a mixture of them. The NADPH–residual semiquinonoid state appears to lie about 3.5 reducing equiv above state VII. Presumably it is a mixture of states I and II which forms because the redox potential of one of the flavine couples lies, like that of the $FMNH_2$–FMNH· couple of *P. elsdenii* flavodoxin at −0.373 V, pH 7 (Mayhew *et al.*, 1969), above the midpotential of NADPH–$NADP^+$.

It has been suggested that the catalytic cycle of mixed function oxidations involving cytochrome P-450 require two separate one-equivalent reductions (Huang and Kimura, 1971; Tyson *et al.*, 1972). In two systems, adrenal mitochondria and *Pseudomonas putida*, these equivalents are supplied by the iron–sulfur proteins, adrenodoxin and putidaredoxin, respectively. In the case of hepatic microsomal mixed function oxidations involving cytochrome P-450, no iron–sulfur protein nor non-heme iron protein of the rubredoxin type has been detected (Miyake *et al.*, 1967; Ichikawa and Yamano, 1970; Peisach and Blumberg, 1970; Ichikawa and Mason, 1973)[2] and the active system has been reconstituted from rat microsomal fractions containing cytochrome P-450, NADPH–cytochrome *c* reductase, and phosphatidylcholine (Lu and Coon, 1968; Lu *et al.*, 1969, 1971) although admittedly crude. The "factor x" which has been proposed as a requirement for electron transport in the microsomal system (Omura and Sato, 1962, 1964b; Hildebrandt and Estabrook, 1971) may be the phospholipid structural factor. Cytochrome b_5 has also been postulated as an electron transfer factor in the system (Estabrook *et al.*, 1971) but is not established as such (Ichikawa and Loehr, 1972; Gillette *et al.*, 1972). In any case, NADPH–cytochrome *c* reductase appears to supply at least one, and probably two, reducing equivalents to this mixed-function oxidation. The steric relationship between the reductase and cytochrome P-450 must be relatively restricted in the membrane, and we suggest that each flavine may have an individual role in the overall process, *e.g.*, to accept two reducing equivalents from NADPH, on the one hand, and to donate one reducing equivalent at two stages of the catalytic cycle, on the other. The overall process will require a two-equivalent gap between the enzyme charged with, and the enzyme after transfer of, the two reducing equivalents. The work of Masters *et al.* (1965a,b) with artificial electron acceptors strongly suggests that enzyme redox states in which one fully reduced flavine and one semiquinone, respectively are present participate in the catalytic cycle. We suggest that these cannot be states I and IV, as they propose, because those redox states have in fact not been observed in the presence of NADPH, O_2, or substrate, although Vanneste *et al.* (1972) may have observed forms of state IV during rapid freeze experiments. The reduced states II or III, or equilibrium mixtures of these with state I, could equally well be involved as donor states; and the corresponding states IV, V, or VI, 2 equiv more oxidized, may represent NADPH–acceptors. If NADPH–cytochrome *c* reductase is directly involved in the hepatic microsomal reduction of cytochrome P-450 during the mixed function oxidase cycle as here suggested, the mechanism of its action may therefore be less well defined than Masters and her colleagues (1965a,b) have concluded.

We examined the epr spectra of the flavoprotein reductase semiquinones at room temperature and at temperatures below 0° because Beinert and Orme-Johnson have reported and discussed "wings" which appear in the epr spectra of NADPH–cytochrome *c* reductase at temperatures in the region of 0° and above (Beinert and Orme-Johnson, 1967), and a study of the temperature effects upon the saturation curves was performed (Figure 8). While temperature did have some effect upon the shape of the saturation curves, the "wings" reported by Beinert and Orme-Johnson were not markedly visible in our own spectra even at 200 mW, room temperature, and modulation amplitude 6.5 G. Our results throw no light on the origin of this phenomenon.

Acknowledgments

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