Redox Properties of the Reduced Nicotinamide Adenine Dinucleotide Phosphate–Cytochrome P-450 and Reduced Nicotinamide Adenine Dinucleotide–Cytochrome b_5 Reductases†

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ABSTRACT: Hepatic microsomal NADPH–cytochrome P-450 reductase, an enzyme containing one molecule each of FMN and FAD per protein molecule (Iyanagi and Mason (1973), *Biochemistry 12*, 2297), was completely reduced by 1 equivalent of dithionite/mol of flavine (2 electron equivalents/mol); accordingly there were no redox active components in the enzyme other than flavine. Experimentally, the reduction appeared to take place in three steps. The first step (system 1) consumed 1.0 electron equivalent/2 flavines, $E_0' = -109$ mV, $N = 1.1$; the second step (system 2) consumed 2.0 electron equivalents/2 flavines, $E_0' = -279$ mV, $N = 0.9$ (NADPH titration) and -274 mV, $N = 0.9$ (dithionite titration); the last step (system 3) consumed 1.0 electron equivalent/2 flavines, $E_0' = -371$ mV, $N = 0.9$ (dithionite titration) at pH 7.0 and 25°. The overall potentiometric and spectrophotometric titration curves with dithionite as a reductant were analyzed by computer, and a reasonably close fit was obtained with a computed curve based upon the assumption of four one-equivalent redox couples. Three mechanisms for the NADPH-dependent catalytic reaction, based upon assumptions of two-equivalent transfers from NADPH and intramolecular transfers of reducing equivalents from acceptor flavine to donor flavine, are discussed. The midpoint potential of NADH–cytochrome b_5 reductase, a microsomal flavoprotein which may be functionally related to NADPH–cytochrome P-450 reductase, was $E_0' = -280$ mV, $N = 2$ (pH 7.5).

Reduced nicotinamide adenine dinucleotide phosphate–cytochrome P-450 reductase of hepatic microsomes is a flavoprotein, mol wt about 69,000, containing one molecule each of FAD and of FMN (Iyanagi and Mason, 1973). The enzyme is specific for NADPH, which reduces cytochrome P-450 in the microsomal membrane (Omura and Sato, 1964). Since NADPH supports mixed-function oxidations dependent upon cytochrome P-450, and since antibodies to NADPH–cytochrome P-450 reductase inhibit these oxidations (Omura, 1969; Masters *et al.*, 1971a,b; Nelson *et al.*, 1971; Schacter *et al.*, 1972), the enzyme is believed to be the electron-transfer protein in the hepatic microsomal mixed-function oxidase system. The active mixed-function oxidase system can be reconstituted from microsomal fractions containing the reductase, cytochrome P-450, and phosphatidylcholine (Lu and Coon, 1968; Lu *et al.*, 1969, 1971). No other electron-transferring protein, *e.g.*, analogous to adrenodoxin of adrenal mitochondria or putidaredoxin of *Pseudomonas putida* (each a part of a mixed-function oxidase system dependent upon cytochrome P-450), has been detected in liver microsomes (Miyake *et al.*, 1967; Ichikawa and Yamano, 1970; Peisach and Blumberg, 1970; Ichikawa and Mason, 1973[1]). It is therefore important to determine the oxidation–reduction characteristics of NADPH–cytochrome P-450 reductase and their relationship to the oxidation–reduction reactions of the enzyme which participate in cytochrome P-450 catalyzed mixed-function oxidations in hepatic microsomes. We have examined the oxidation–reduction properties of purified NADPH–cytochrome P-450 reductase and NADH–cytochrome b_5 reductase in this study, both spectroscopically and potentiometrically.[2]

Experimental Section

Enzyme Preparation. Rabbit liver NADPH–cytochrome P-450 reductase was prepared by a method described elsewhere (Iyanagi and Mason, 1973). Flavine content was determined by the method of Iyanagi and Mason (1973), using phosphodiesterase. The ratio of FAD to FMN was 0.94 ± 0.02. Enzyme concentration expressed as flavine concentration was determined spectrophotometrically, using $\epsilon_{455} = 10.7$ mM^{-1} cm^{-1} (Iyanagi and Mason, 1973). Pig liver NADH–cytochrome b_5 reductase was prepared by the method of Takesue and Omura (1970) with some modification (Iyanagi and

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[1] Y. Ichikawa and H. S. Mason, unpublished epr observations at 15°K.

[2] Abbreviations used are: epr, electron paramagnetic resonance; N, the number of electrochemical equivalents involved in any given oxidation–reduction process (Clark, 1960); $E_{0,1}'$, the midpoint potential at pH 7.0 with respect to the standard hydrogen electron potential taken as zero, of the first (most positive) redox couple (system 1) encountered during the reductive titration of NADPH–cytochrome P-450 reductase; $E_{0,2}'$, the midpoint potential of the second redox reaction (system 2) encountered during the reductive titration of NADPH–cytochrome P-450 reductase; $E_{0,3}'$, the midpoint potential of the third redox couple (system 2) encountered during the reductive titration of NADPH–cytochrome P-450 reductase; and $E_{0,4}'$, the midpoint potential of the fourth (most negative) redox couple; E_h, measured potential with reference to a standard hydrogen electrode at zero volts. For purposes of discussion, FAD, FADH·, and $FADH_2$, and FMN, FMNH·, and $FMNH_2$ are used to symbolize the flavine species, F1H, oxidized flavine; $F1H_2$, neutral flavoprotein semiquinone, and $F1H_3$, neutral fully reduced flavoprotein (Hemmerich *et al.*, 1965); where the individual species of flavines are not known but are relevant to the discussion, they are referred to as F_1, F_1H·, and F_1H_2, and F_2, F_2H· and F_2H_2, respectively, where F_1 is the oxidized form of the redox couple with the most positive E_0'.

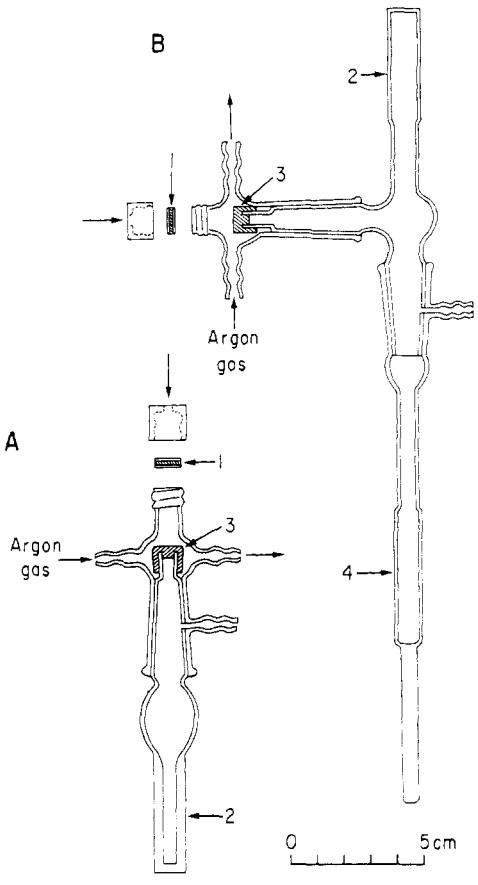


FIGURE 1: Apparatus for anaerobic titrations: (A) spectrophotometer cuvette, and (B) combined spectrophotometer–epr cell; 1, gas chromatography inlet septum; 2, 1.0-cm optical path-length cuvette; 3, rubber serum tube cap; 4, epr flat cell. The apparatus is derived from the design of Burleigh *et al.* (1969) with the addition of a gas lock in the injection port.

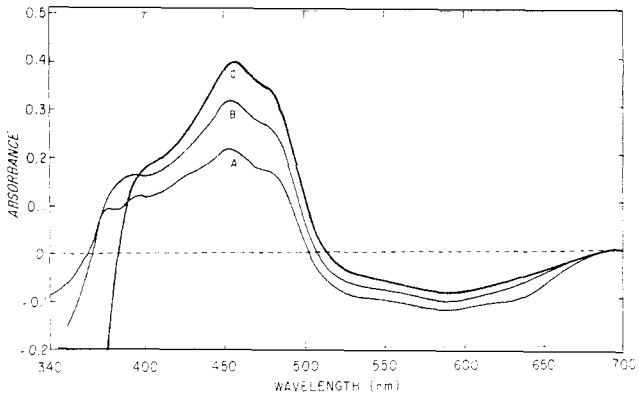


FIGURE 2: Difference spectra: (A) oxidized NADPH–cytochrome P-450 reductase (56.5 μM) minus stable flavoprotein semiquinone; (B) oxidized NADPH–cytochrome P-450 reductase (56.5 μM) minus stable flavoprotein semiquinone reduced anaerobically with NADPH (14 μM); (C) curve B after addition of NADPH to 200 μM. The stable semiquinone was prepared by adding NADPH (56 μM, final concentration) in the presence of air, to purified enzyme (56.5 μM) dissolved in 0.1 M potassium phosphate buffer (pH 7.7).

Yamazaki, 1969). Enzyme concentration was determined spectrophotometrically, $\epsilon_{460} = 10.2$ mM^{-1} cm^{-1} (Strittmatter and Velick, 1956).

Materials. NADPH, NADH, and FAD were purchased from Sigma. FAD was purified from commercial samples by the method of Massey and Swoboda (1963). Indigodisulfonic acid ($E_{m,7} = -116$ mV at 25° and $\epsilon_{613} = 2.22 \times 10^4$ M^{-1} cm^{-1} (pH = 7.0)) (Preisler *et al.*, 1959) was purchased from the National Aniline Corp. Safranine T ($E_{m,7} = -289$ mV at 30° (Stieliler *et al.*, 1933) $\epsilon_{520} = 48.0$ mM^{-1} cm^{-1} (pH 7.0) (Huang and Kimura, 1973)), and methylviologen ($E_{m,7} = -440$ mV at 30° (Michaelis and Hill, 1933) and $\epsilon_{602} = 14$ mM^{-1} cm^{-1} (pH 7.0) (Huang and Kimura, 1973)) were purchased from the Aldrich Chemical Co., Inc. Sodium dithionite solutions were prepared, and standardized spectrophotometrically with FAD, under anaerobic conditions. FAD concentration was determined with the extinction coefficient, $\epsilon_{455} = 11.3$ mM^{-1} cm^{-1} (pH 7.0). Argon (Matheson, minimum purity, 99.999%) was used in the titration systems, and nitrogen (prepurified, passed through two bubble towers containing vanadyl sulfate solution (Meites and Meites, 1948) to remove traces of O_2) was used for making solutions anaerobic.

Methods. Optical spectra were measured with a Cary Model 14 spectrophotometer, in a sample compartment thermostatted at 25 ± 1°. Epr derivative absorption spectra were observed with a Varian V-4500 spectrometer at 100-kHz field modulation, and other conditions as noted. Spin concentration was determined by the method of Iyanagi and Mason (1973) using flavodoxin semiquinone as standard.

The optical titration and combined optical epr titration cells are shown in Figure 1A,B. These systems were made anaerobic by evacuation and flushing with nitrogen. The evacuation and flushing process was repeated at least five times. A small argon flow was maintained through the gas lock protecting the cell units and reagents were added with a Hamilton gas-tight microsyringe, 10 or 50 μl, through a compound gas chromatograph entry port septum.

The potentiometric titration techniques were essentially the same as those employed by Dutton (1971). The anaerobic titration vessel contained NADPH–cytochrome P-450 reductase, an appropriate mediator or mixture of mediators, and aliquots of reductant added anaerobically. The conditions are described in the legends to Figures 7 and 8. The resultant potentials were measured with a system comprising a Type P 101 platinum electrode (Radiometer, Copenhagen), a saturated calomel electrode (Type K 401, Radiometer), and an Orion Model 801 digital pH/mV meter. When potentials were determined electrometrically, the apparatus was checked against the ferrocyanide–ferricyanide system (O'Reilly, 1973), using the value $E_{m,7} = +0.244$ V at 25° for the saturated calomel electrode (Ives and Janz, 1961). We also measured the potentials of the mediators, indigodisulfonate and safranine T, and FAD, using this apparatus. Neither the absorption spectra nor the potentials drifted during the measurements.

Computer Calculations. A Digital Equipment Corp. PDP-11 computer was used with FOCAL-11 language.

Results

Absorption Spectra. Fully oxidized NADPH–cytochrome P-450 reductase had absorption maxima at 277, 380, and 455 nm, and a pronounced shoulder at 485 nm; $\epsilon_{455} = 10.7$ mM^{-1} cm^{-1} (Iyanagi and Mason, 1973). The difference spectrum, fully oxidized enzyme minus O_2-stable flavoprotein semiquinone, is shown in Figure 2 (curve A). Peaks appeared at 395, 455, and 590 nm, with shoulders at 485 (positive) and 635 nm (negative). When NADPH was added to the reference cuvette under anaerobic conditions, the positive maxima intensified and the shoulder at 635 nm disappeared. This may be ascribed to the reduction of the flavoprotein semiquinone

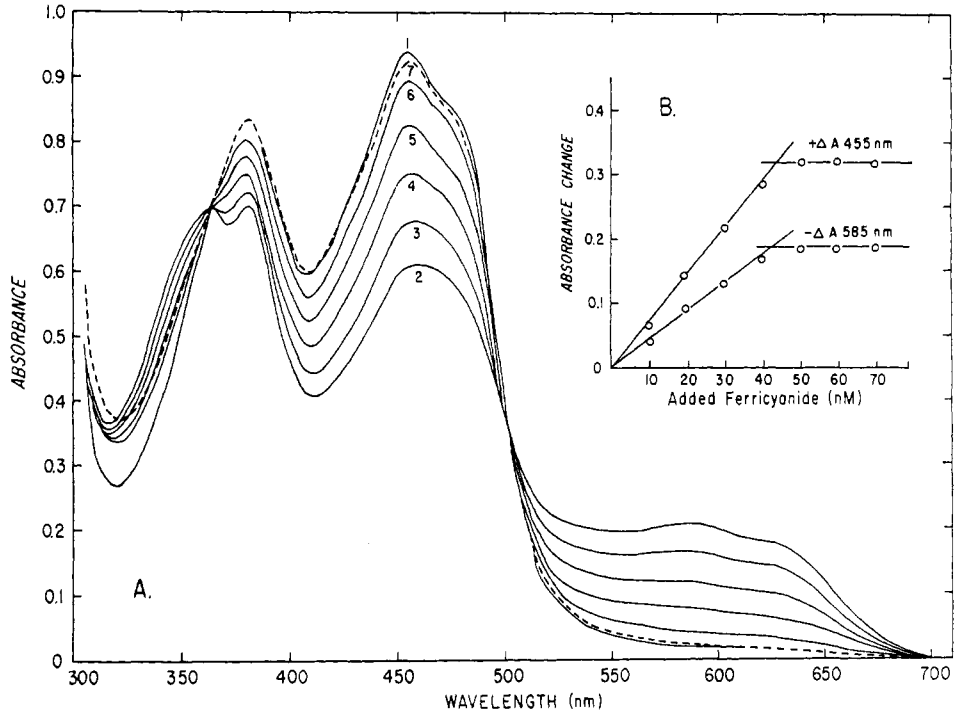


FIGURE 3: Stepwise oxidation of the O_2-stable semiquinone of NADPH–cytochrome P-450 reductase by ferricyanide. The semiquinone reductase (87.4 μM) in 0.1 M potassium phosphate buffer (pH 7.7) was titrated with 1.0 mM potassium ferricyanide in 0.1 M potassium phosphate buffer (pH 7.7) at 25°. The O_2-stable semiquinone was prepared as described by Iyanagi and Mason (1973). Curve 1, fully oxidized enzyme; curves 2–7, equilibrium absorption spectra after the addition of 10, 20, 30, 40, 50, and 70 μM ferricyanide, respectively. The dashed line is that of curve 7.

arising from one of the two distinct flavines. The process is brought to completion by addition of a greater excess of NADPH (curve C). No absorbance was detected between 700 and 1000 nm, presumably due to lack of interaction between adjacent flavine semiquinone molecules, and it appears possible that these spectra reflect some chemical discrimination between the two flavines in the reductase molecule.

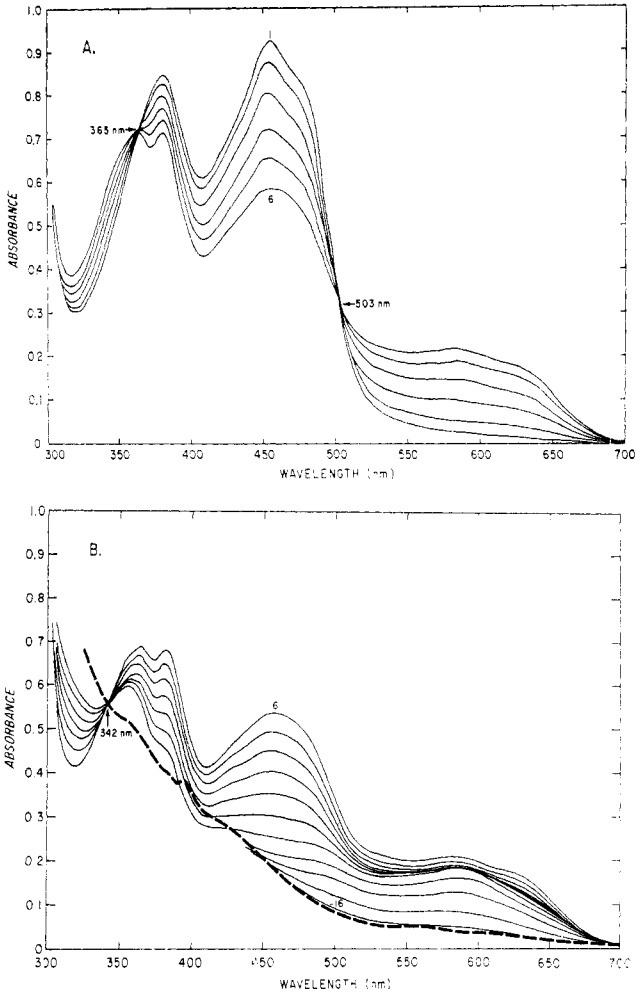
Oxidative Titration of the O_2-Stable Flavoprotein Semiquinone of NADPH–Cytochrome P-450 Reductase with Potassium Ferricyanide. The O_2-stable flavoprotein semiquinone of NADPH–cytochrome P-450 reductase was prepared according to the method described by Iyanagi and Mason (1973), then titrated with aliquots of potassium ferricyanide. The successive absorption spectra obtained, and the relationship between absorbance changes at 455 and 585 nm, to ferricyanide added, are depicted in Figure 3. Maximum increase at 455 nm and maximum decrease at 585 nm took place when 1 mol of ferricyanide had been added per 2.0 mol of flavine, that is, 1 oxidizing equivalent/2 flavines, confirming a previous report that the O_2-stable flavoprotein semiquinone is 1 equivalent more reduced than the fully oxidized enzyme (Iyanagi and Mason, 1973). As ferricyanide was added, the absorption bands at 380 and 455 nm became more intense while the absorption above 505 nm and below 380 nm became weaker; the whole spectrum gradually approached that of fully oxidized flavoprotein (*cf.* curves 1 and 7), and isosbestic points were discernible at 364 and 503 nm. During the oxidation, the absorption at 585 and 635 nm disappeared together, that is, coherently, in contrast to their behavior upon reduction with NADPH. Accordingly, there is only a single flavine involved in the semiquinonoid structure of the flavoprotein semiquinone, which we call F_1. Presumably, an additional flavine, F_2, gives rise to the flavine semiquinone in the NADPH-reduced state; that is, in the one state, either FADH· or FMNH· is involved, and in the other, the other flavine semiquinone.

Anaerobic Reduction of NADPH–Cytochrome P-450 Reductase with Sodium Dithionite. The anaerobic titration of NADPH–cytochrome P-450 reductase with sodium dithionite was carried out in the presence of a small amount of methylviologen because the rate of reduction of the flavoprotein was slow in its absence. The spectroscopic results are depicted in Figure 4. The reduction by dithionite took place in three steps. In the first, 0.25 mol of dithionite (0.5 electron equivalent) was consumed per mol of flavine (1.0 electron equivalent/2 flavines) (Figure 4A,C). The absorption maximum of oxidized flavine at 455 nm decreased and a new maximum at 585 nm appeared. During this process, isosbestic points appeared at 365 and 503 nm, indicating a reductive transformation of one species of the enzyme into another species, presumably a flavine semiquinone.

$$\begin{matrix} F_1 \\ F_2 \end{matrix} + H\cdot \rightleftharpoons \begin{matrix} F_1H\cdot \\ F_2 \end{matrix}$$

A further addition of 0.5 mol of dithionite/mol of enzyme flavine (1 electron equivalent/flavine, or 2 electron equivalents/2 flavines) caused a decrease of absorbance at 455 nm with an accompanying small change at 585 nm (Figure 4B,C) and an isosbestic point appeared at 342 nm, indicating the reductive transformation of one species of enzyme into one other species, corresponding finally to the fully reduced enzyme. An additional 0.25 mol of dithionite/mol of flavine (1 electron equivalent/2 flavines) caused a rapid change of absorbance at 585 nm to the fully reduced state (Figure 4B,C); the same isosbestic point at 342 nm was involved.

The three phases of the titration are clearly indicated by the plot of absorbancy changes at 455 and 585 nm as functions of



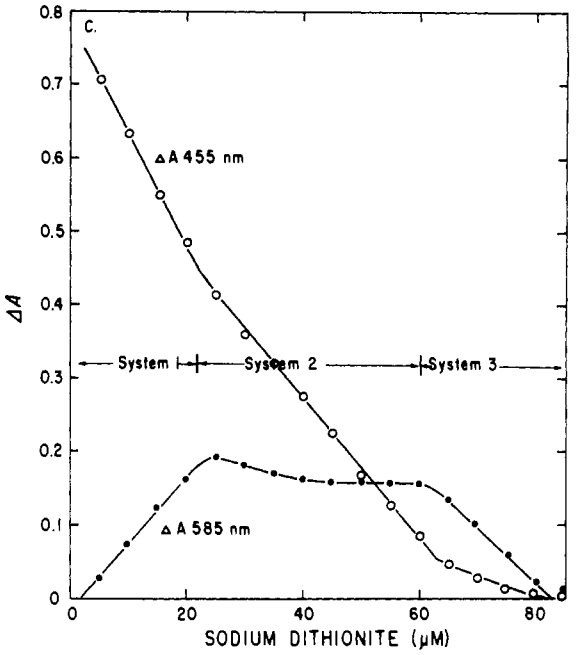


FIGURE 4: Anaerobic titration of NADPH–cytochrome P-450 with dithionite: spectrophotometric results. NADPH–cytochrome P-450 reductase, 86 μM in 0.1 M potassium phosphate, (pH 7.7), was titrated at 25° in the presence of methylviologen (2 μM) with sodium dithionite (7 mM) dissolved in 0.01 M potassium phosphate buffer (pH 8.35). The experimental curves were not corrected for dilution. Parts A and B represent a single continuous titration but sets of curves are separated in the two graphs to show two individual sets of isosbestic points occurring during the titration. Part C is a plot of the decrease of absorbancy at 455 nm and the changes at 585 nm which occurred as dithionite was added; the data were taken from parts A and B. The dashed line (Figure 4B) represents fully reduced reductase.

amounts of added dithionite (Figure 4C). We call the first phase, redox "system 1," the second, redox "system 2," and the third, redox "system 3." The isosbestic point shown in Figure 4B, at 342 nm, arises presumably because flavine semiquinone is being converted to fully reduced flavine, although the net concentration of flavine semiquinone is changing only slightly (system 2); finally, flavine semiquinone is converted to fully reduced flavine (system 3) giving rise to the same isosbestic point. A total of 2 electron equivalents was consumed per flavine during the overall titration.

Anaerobic Reduction of NADPH–Cytochrome P-450 Reductase with NADPH. The anaerobic titration of NADPH–cytochrome P-450 reductase with NADPH was also carried out in the presence of a small amount of the mediator, methylviologen, because the rate of intramolecular equilibration was otherwise slow. The spectroscopic results are depicted in Figure 5A,B. The addition of 0.25 mol of NADPH/mol of flavine produced a spectroscopic effect similar to that of the addition of 0.25 mol of dithionite/mol of flavine (Figure 4A). Upon further addition of 0.50 mol of NADPH/mol of flavine, spectroscopic changes took place at 455 and 585 nm similar to those observed with the same number of reducing equivalents of dithionite (Figure 4B,C), but still further additions of NADPH up to 1.0 mol/mol of flavine caused only small changes at 455 and 585 nm, while an increase in absorbance around 340 nm indicated that unreacted NADPH was accumulating. Accordingly, the oxidation–reduction potential of system 3 (final consumption of 1 electron equivalent to the complete reduction of the 2 flavine molecules) must be such that the oxidation–reduction potential or the $NADP^+$–NADPH couple is not sufficiently low to bring about perceptible reduction of the residual flavine semiquinone concentration at the concentration ratios, $[NADP^+]/[NADPH]$, which developed during this titration.

The flavine semiquinone formed during the anaerobic titration of the reductase with NADPH was also followed by simultaneous observation of absorbance at 585 nm and the intensity of epr signal on the same sample (arbitrary units) using the anaerobic optical epr titration cell depicted in Figure 1B. The results are shown in Figure 6. During the initial addition of NADPH (system 1), the decrease in absorbancy at 455 nm, and the increase in absorbancy at 585 nm, paralleled the increase in spin concentration. During the second phase of the titration (system 2, consumption of 2 electron equivalents per 2 flavine molecules), the spin concentration continued to parallel the absorbance at 585 nm, and there was little change in flavine semiquinone concentration by these criteria, but the concentration of oxidized flavine diminished, as shown by a decrease in absorbance at 455 nm (Figure 6). In addition, the spin concentration of the enzyme measured when the 585-nm band was at a maximum height was 34% of the total flavine concentration when flavodoxin semiquinone was used as a standard of comparison (*cf.* Iyanagi and Mason, 1973). The result is consistent with our interpretation that in system 2, fully reduced flavine accumulates, oxidized flavine diminishes, while the flavine semiquinone concentration remains relatively constant.

Oxidation–Reduction Potentials of NADPH–Cytochrome P-450 Reductase. The presence of mediators was required during the potentiometric titration of NADPH–cytochrome

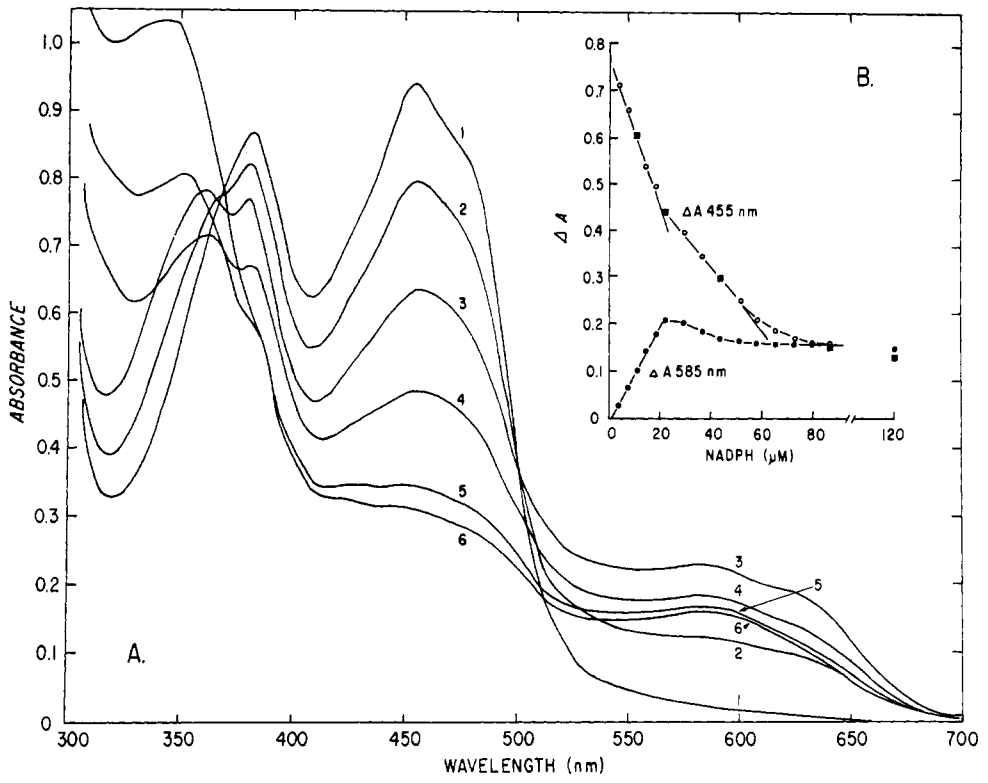


FIGURE 5: Anaerobic titration of NADPH–cytochrome P-450 reductase with NADPH. NADPH–cytochrome P-450 reductase, 86 μM in 0.1 M phosphate buffer (pH 7.7) was titrated with NADPH, 5.04 mM in the same buffer. The experimental curves were not corrected for dilution. Curve 1, fully oxidized enzyme; curves 2–6, absorption spectra observed after the addition of 14, 21, 42, 84, and 120 μM NADPH. The inset, Figure 5B, shows the changes occurring at 455 and 858 nm during the titration with the absorbance of fully reduced enzyme (Figure 4B, C) taken as zero.

P-450 reductase because the potentials developed in their absence were unsteady, particularly in system 1, indicating that equilibrium was difficult to reach. Consequently, system 1 potentials were determined in the presence of high concentrations of a mediator, indigodisulfonate (*e.g.*, reductase, 74.5 μM; indigodisulfonate, 43.2 μM). This mixture was titrated with NADPH and the changes in the concentrations of oxidized and reduced (semiquinonoid) reductase, and oxidized and reduced dye stuff, were observed spectrophotometrically. The percentage reduction of the fully oxidized reductase, compared to maximum reduction in this system taken as 100% (1 reducing equivalent per 2 flavines, as discussed above), was calculated from the decrease in absorbance of the enzyme at 462 nm, an isosbestic point between oxidized and reduced indigodisulfonate at pH 7.0, when small increments of NADPH were added and the titration system was allowed to come to equilibrium. The contribution by the enzyme semiquinone to absorbance at 610 nm was determined from the percentage reduction of the reductase (*cf.* Figure 4A) and the percentage reduction of the indigodisulfonate was then calculated from the decrease in absorbance at 610 nm after the correction has been made (Preisler *et al.*, 1959). The potentials for system 1 were measured by direct potentiometry. The potentials for NADPH–cytochrome P-450 reductase and for indigodisulfonate at each step of reduction are shown in Figure 7A. The N values were determined from the corresponding plots of log [ox.]/[red.]. The $E_{0,1}'$ for NADPH–cytochrome P-450 reductase obtained from these plots, for system 1, was −109 mV and N = 1.1 at pH 7.0 and 25°.

The values of E_0' for systems 2 and 3, as defined by Figure 4C, were measured by direct potentiometry using low concentrations of mediators in the titration systems. The equilibrium potentials at each titration step with dithionite or NADPH were measured and the values, E_h, were plotted against the percent reduction of the enzyme system compared to the maximum reduction observed in systems 2 and 3 by following the decrease in absorbance at 455 nm (Figures 4C and 5). The results of the dithionite titrations are depicted in Figures 7B (system 2) and 7D (system 3), and those of the NADPH titration in Figure 7C. The calculated midpoint potentials were: system 2, −274 mV, N = 0.9 (dithionite) (Figure 7B), and −279 mV, N = 0.9 (NADPH) (Figure 7C); system 3, −371 mV, N = 0.9, at pH 7.0 and 25°.

Oxidation–Reduction Potential of NADH–Cytochrome b_5 Reductase. The oxidation–reduction potential of NADH–cytochrome b_5 reductase was measured by direct potentiometry in the apparatus already described, titrating with dithionite as a reductant and ferricyanide as an oxidant, in the presence of small amounts of indigodisulfonate and Safranine T as mediators. The midpoint potential, $E_{0,7.5}'$, was calculated from the titration data depicted in Figure 8 to be −280 mV, N = 2, at pH 7.5 and 25°.

Discussion

Computer Analysis of the Potentiometric Titration Curve of NADPH–Cytochrome P-450 Reductase. Although direct graphical analysis of the potentiometric titration curves for NADPH–cytochrome P-450 reductase readily yielded the three midpoint potentials just described, there were two unusual features of the results: during system 2 reduction, the concentration of flavine semiquinone remained approximately constant (Figure 4C), and although the system required 2 reducing equivalents, its N value was 1. We attempted to resolve

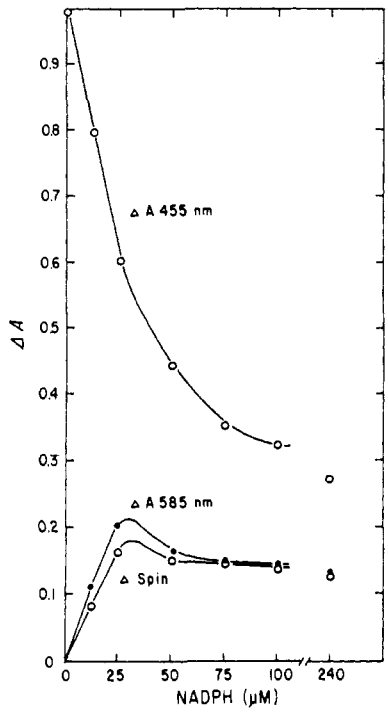


FIGURE 6: Correlation of free-radical concentration changes with optical changes during the anaerobic titration of NADPH–cytochrome P-450 reductase with NADPH. NADPH–cytochrome P-450 reductase, 93 μM in 2.5 ml of 0.1 M potassium phosphate buffer (pH 7.7), was titrated with NADPH, 10 mM in the same buffer. The free-radical concentration (arbitrary scale) was determined with epr (Iyanagi and Mason, 1973). The ordinate shows ΔA. Spin concentrations are given in the text.

the redox mechanism by assuming that two different flavine semiquinones with different optical and redox properties formed during the titration. The experimental optical and potentiometric titration curves could then precisely be fitted by computer-calculated curves.

The following reactions were assumed to occur, where F_1 and F_2 are the two flavines in the enzyme:

$F_1 + H\cdot = F_1H\cdot$	midpoint potential, $E_{0,1}'$	$N = 1$
$F_1H\cdot + H\cdot = F_1H_2$	midpoint potential, $E_{0,2}'$	$N = 1$
$F_2 + H\cdot = F_2H\cdot$	midpoint potential, $E_{0,3}'$	$N = 1$
$F_2H\cdot + H\cdot = F_2H_2$	midpoint potential, $E_{0,4}'$	$N = 1$

Then the measured potential of the system at any stage in the titration was

$$\begin{aligned} E_h &= E_{0,1}' + \frac{RT}{F}\ln\frac{[F_1]}{[F_1H\cdot]} \\ E_h &= E_{0,2}' + \frac{RT}{F}\ln\frac{[F_1H\cdot]}{[F_1H_2]} \\ E_h &= E_{0,3}' + \frac{RT}{F}\ln\frac{[F_2]}{[F_2H\cdot]} \\ E_h &= E_{0,4}' + \frac{RT}{F}\ln\frac{[F_2H\cdot]}{[F_2H_2]} \end{aligned} \tag{1}$$

The total concentrations of the two kinds of flavine were constant and equal

$$\begin{aligned} [F_1] + [F_1H\cdot] + [F_1H_2] &= f_1 \\ [F_2] + [F_2H\cdot] + [F_2H_2] &= f_2 \end{aligned} \tag{2}$$

where f_t = total flavine and the ratios of concentrations, from the Nernst equations, were

$$\begin{aligned} \frac{[F_1]}{[F_1H\cdot]} &= e^{(F/RT)(E_h - E_{0,1}')} = x_1 \\ \frac{[F_1H\cdot]}{[F_1H_2]} &= e^{(F/RT)(E_h - E_{0,2}')} = x_2 \\ \frac{[F_2]}{[F_2H\cdot]} &= e^{(F/RT)(E_h - E_{0,3}')} = x_3 \\ \frac{[F_2H\cdot]}{[F_2H_2]} &= e^{(F/RT)(E_h - E_{0,4}')} = x_4 \end{aligned} \tag{3}$$

Solving the simultaneous equations, (2) and (3)

$$\begin{aligned} [F_1] &= \frac{x_1x_2}{1 + x_2 + x_1x_2}f_1 \\ [F_1H\cdot] &= \frac{x_2}{1 + x_2 + x_1x_2}f_1 \\ [F_1H_2] &= \frac{1}{1 + x_2 + x_1x_2}f_1 \end{aligned} \tag{4}$$

and corresponding equations for $[F_2]$, $[F_2H\cdot]$, and $[F_2H_2]$, where x_1, x_2, x_3, and x_4 are defined by eq 3, were obtained.

In the actual calculation, f_t was usually set to be 86 μM, the concentration present, but in some cases it was set to be 1, in order to determine the percentage of each component. In the computer program for numerical calculation, E_h was gradually decreased from 0 to −0.5 V with decrements of 0.002 V, and the concentration of each flavine species was calculated for each E_h value assuming a set of four E_0' values, which were varied systematically to obtain a high degree of fit of the calculated relationships to the observed relationship. When the abscissas of the computed curves were degree of reduction, R, the values were expressed on a scale of 0–1, where 1 equalled four reducing equivalents added per two flavine molecules per protein molecule. This value was calculated by the relationship

$$R = \frac{[F_1H\cdot] + 2x[F_1H_2] + [F_2H\cdot] + 2x[F_2H_2]}{f_t}$$

Six components derived from the two flavines were considered to be in equilibrium during dithionite titration of the enzyme: F_1, $F_1H\cdot$, and F_1H_2, and F_2, $F_2H\cdot$, and F_2H_2. Relative E_0' values of the redox couples were then assigned according to three sets of assumptions, as follows (becoming more negative from left to right).

Mechanism 1

$$F_1 \overset{E_{0,1}'}{\rightleftarrows} F_1H\cdot \qquad F_1H\cdot \overset{E_{0,2}'}{\rightleftarrows} F_1H_2$$

$$F_2 \overset{E_{0,3-4}'}{\rightleftarrows} F_2H_2$$

Mechanism 2

$$F_1 \overset{E_{0,1}'}{\rightleftarrows} F_2H\cdot \qquad F_1H\cdot \overset{E_{0,2}'}{\rightleftarrows} F_1H_2$$

$$F_2 \overset{E_{0,3}'}{\rightleftarrows} F_2H\cdot \overset{E_{0,4}'}{\rightleftarrows} F_2H_2$$

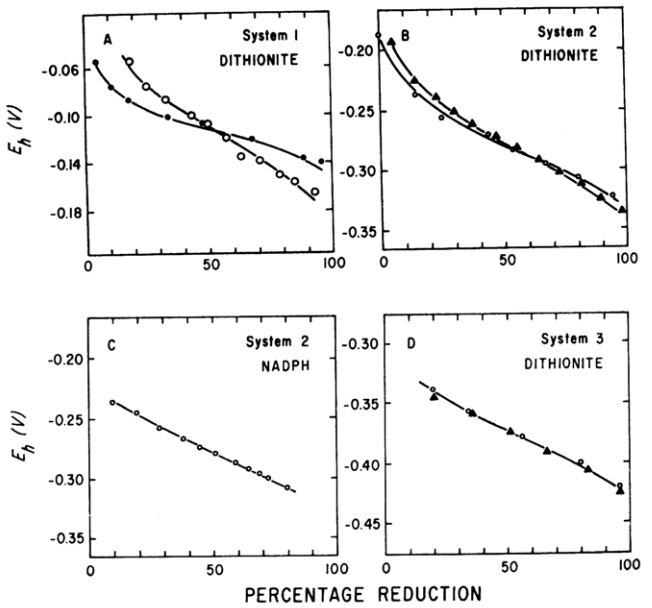


FIGURE 7: Potentiometric titration of NADPH–cytochrome P-450 reductase. (A) System 1 titration. Values for E_h are plotted against the percent reduction of NADPH–cytochrome P-450 reductase compared to the maximum reduction in system 1 (Figure 4A) taken as 100%. NADPH–cytochrome P-450 reductase, 74.5 μM, and indigo disulfonate, 43.2 μM, in a total volume of 5 ml, 0.1 M potassium phosphate buffer (pH 7.0). This mixture was titrated with NADPH, 10 mM in the same buffer (pH 7.0). The circles represent the titration of reductase, and the dots, the reduction of indigo disulfonate, calculated as described in the test. Parts B and C represent the potentiometric titration of NADPH–cytochrome P-450 reductase, E_h plotted against the percent reduction of the enzyme compared to complete reduction in system 2 (Figure 4B) taken as 100%. NADPH–cytochrome P-450 reductase, 100 μM, and mediator, a mixture of indigo disulfonate, 2 μM, Safranine T, 2 μM, methyl viologen, 2 μM in a total volume of 5 ml of 0.1 M potassium phosphate buffer (pH 7.0). This mixture was titrated with either sodium dithionite, 7 mM in 0.01 M potasium phosphate buffer (pH 8.35) (B), or NADPH, 10 mM in 0.01 M potassium phosphate buffer (pH 7.0) (C). The triangles represent back-titration with ferricyanide, 10 mM in 0.1 M potassium phosphate (pH 7.0). Part D represents the potentiometric titration of NADPH–cytochrome P-450 reductase with dithionite in system 3. The measured potential values, E_h, are plotted against the percent reduction of the enzyme compared to fully reduced enzyme (Figure 4B) taken as 100%. The experimental conditions are the same as used for Figure 7B. The triangles represent back-titration with ferricyanide, 10.0 mM in 0.1 M potassium phosphate buffer (pH 7.0) at 25°.

Mechanism 3

$$F_1 \underset{}{\overset{E_{0,1}'}{\rightleftharpoons}} F_1H\cdot \overset{E_{0,2}'}{\rightleftharpoons} F_1H_2$$

$$F_2 \overset{E_{0,3}'}{\rightleftharpoons} F_2H\cdot \overset{E_{0,4}'}{\rightleftharpoons} F_2H_2$$

Mechanism 1 does not readily fit the experimental results with dithionite titration because the N value for the second redox step (system 2) was approximately 1 and because computed titration curves based upon this hypothesis fitted neither the potential titration curve (Figure 11) nor absorbancy changes at 585 nm (Figure 9). However, this conclusion applies only to the dithionite titration curve; the mechanism must be kept in mind for the functional reduction by NADPH, and for complex interactions and transfers of reducing equivalents between the flavine moieties, as in the mechanism for NADPH–sulfite reductase proposed by Siegel *et al.* (1971, 1972).

Mechanisms 2 and 3 were discriminated from one another

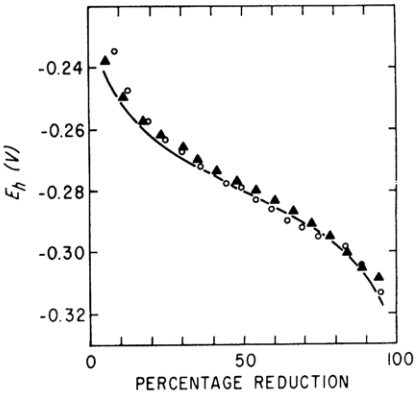


FIGURE 8: Potentiometric titration of NADH–cytochrome b_5 reductase. NADH–cytochrome b_5 reductase, 87.6 μM; mediator, indigo disulfonate, 2 μM, plus safranine T, 2 μM, and in 5 ml of 0.1 M potassium phosphate buffer (pH 7.5), titrated with dithionite under anaerobic conditions (circles), and back-titrated with ferricyanide (triangles). The line without symbols in the calculated curve for an $N = 2$ titration.

by assuming sets of different E_0' values for the four redox couples

Mechanism 2

$$E_{0,1}' > E_{0,3}' \approx E_{0,4}' > E_{0,2}'$$

Mechanism 3

$$E_{0,1}' > E_{0,2}' \approx E_{0,3}' > E_{0,4}'$$

The absorbance change at $A_{585\text{ nm}}$ during the titration, calculated on the basis of mechanism 2, was found to be almost symmetrical with a maximum absorbance value at 585 nm at the middle of the titration curve. It was impossible to obtain a good fit with the observed titration curve. On the other hand, computed spectrophotometric titration curves based upon mechanism 3 had a relatively flat shape in the middle of the system 2 titration. By assuming an extinction coefficient for $F_1H\cdot$, $\epsilon = 4900\ M^{-1}\ cm^{-1}$, and for $F_2H\cdot$, $\epsilon = 4500\ M^{-1}\ cm^{-1}$,

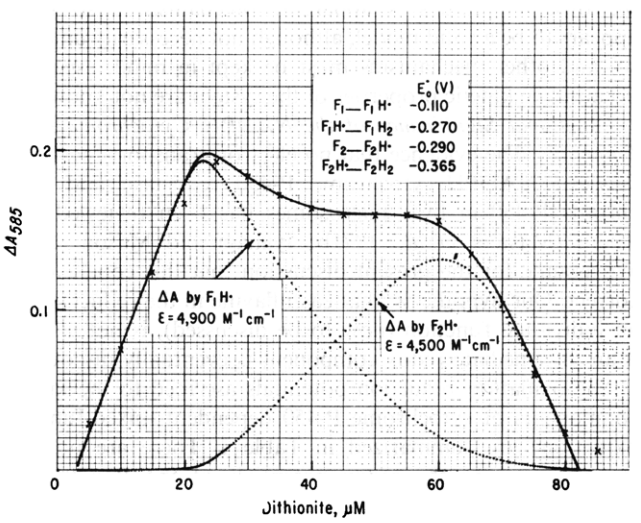


FIGURE 9: Computer simulation for the spectrophotometric titration of cytochrome P-450 reductase. Crosses show the observed values (*cf.* Figure 4C). The solid line was computed according to mechanism 3, and the assumptions that $E_{0,1}' = -0.110$ V, $E_{0,2} = -0.270$ V, $E_{0,3}', = -0.290$ V, $E_{0,4}' = -0.365$ V, and (total F_1) = (total F_2) = (1/2) (total flavine) = 43 μM. It was also assumed that the system contained 1.5 μM O_2 at the initial stage and that the end point was at 81.5 μM dithionite, in order to obtain the best fit to experimental values.

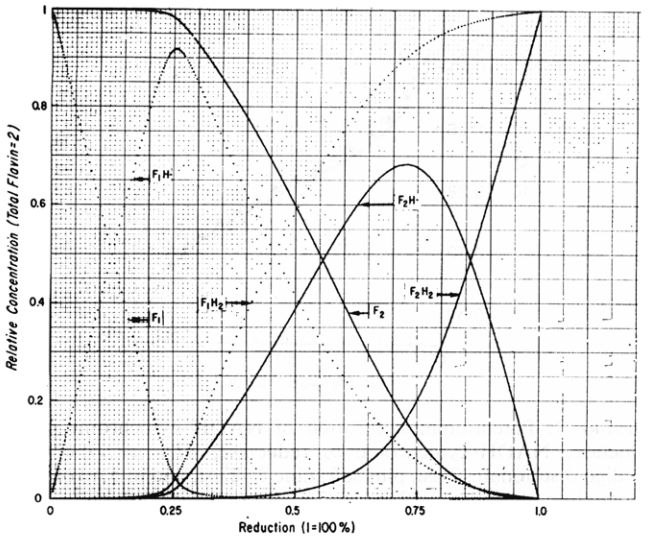


FIGURE 10: Computed curves for the change in concentration of each flavine species during the reduction of liver NADPH–cytochrome P-450 reductase. Values of E_0' were the same as assumed in Figure 9. The ordinate was taken as (total F_1) + (total F_2) = 2.

and with $E_{0,1}' = -0.110$ V, $E_{0,4}' = -0.365$ V, $E_{0,2}' = -0.270$ V, $E_{0,3}' = -0.290$ V, a very precise fit of the calculated to the observed titration curves was obtained (Figure 9). The fit was very sensitive to the assumed values of E_0' and the extinction coefficients of the flavine semiquinones, and it seems probable that the E_0' values so obtained were accurate to within ± 0.005 V if there is no error in the experimentally determined values. About 3 μM dithionite was apparently consumed by an adventitious oxidant because absorbancy changes at 585 nm did not commence at the origin. The calculated *concentration* changes, F_1 and F_2, $F_1H\cdot$ and $F_2H\cdot$, and F_1H_2 and F_2H_2, are shown in Figure 10. A clear distinction between the redox properties of $F_1H\cdot$ and $F_2H\cdot$ are apparent. The computed curve, relative reduction *vs.* measured potential, is shown in Figure 11. The closeness of the computed values to the observed values is apparent. Mechanism 3 so tested appears to be a valid model, although not necessarily uniquely so, and other mechanisms must be kept in mind, especially because the kinetic properties of the system are not known.

Functional Significance of the NADPH–Cytochrome P-450 Potentials. The evidence that no electron-transport factor lies between NADPH–cytochrome P-450 reductase and cytochrome P-450 in liver microsomes has been reviewed elsewhere (Masters *et al.*, 1971b; Iyanagi and Mason, 1973). We assume for the present discussion that this conclusion is correct. Our hypothesis, that each flavine in the reductase has an individual function (now to be discussed), is not affected if an intermediate redox component is eventually discovered.

The oxidation–reduction potentials ascribed to the known components of cytochrome P-450 dependent mixed-function oxidations are summarized in Table I. There are no consistencies among the redox potentials of the *Pseudomonas putida*, adrenal mitochondrial, and liver microsomal systems, other than that all component potentials are negative, that in the systems in which iron sulfur protein intervenes, the flavoprotein reductases have more negative potentials than the iron sulfur proteins but not more negative than the cytochrome P-450s which are thereby reduced, and have $N = 2$ values in the (equilibrium) titrations whereas their substrates are one-equivalent acceptors. Substrate causes the redox potential of *P. putida*$_{cam}$ P-450 to become about 100 mV more positive; the corresponding effects have not been measured with the other cytochromes P-450, but are probably of a similar magnitude.

In any case it has been demonstrated that in the *Pseudomonas* system, the two reducing equivalents required are supplied by putidaredoxin in two separate one-equivalent reductions to be acceptor system probably having two different redox potentials (Huang and Kimura, 1971; Tyson *et al.*, 1972). If the mechanisms of the *Pseudomonas* and hepatic microsomal mixed-function oxidations are similar in this respect, the liver system will also require the injection of two separate reducing equivalents each with $N = 1$. We suggest that the reductase supplies these equivalents through the low potential flavine, F_2, using one or both of the couples, $E_{0,4}' = -0.365$ V and $E_{0,3}' = -0.290$ V. F_2 may thus be the *donor flavine* of the reductase; similarly, NADPH/NADP$^+$ ($E_{0,7}' = -0.32$ V) may supply the reducing equivalents required by the mixed function oxidation through F_1, the *acceptor flavine*, at $E_{0,1}' = -0.110$ V and $E_{0,2}' = -0.270$ V. The overlap of $E_{0,2}'$ (F_1H/F_1H_2) and $E_{0,3}'$ (F_2/F_2H) provides, in any case, a mechanism for transfer of single reducing eauivalents from the acceptor flavine to the donor flavine and thus to cytochrome P-450 during its catalytic cycle (mechanism 3A or 3B).

Mechanism 3A: interaction of NADPH–cytochrome P-450 reductase with P-450 during mixed-function oxidation: 2 reducing equivalents from F_2H_2

1 $\quad \mathrm{NADPH + H^+ + F_1 \rightleftharpoons NADP^+ + F_1H_2}$

2 $\quad \mathrm{F_1H_2 + F_2 \rightleftharpoons F_1 + F_2H_2}$

3 $\quad \mathrm{F_2H_2 + P\text{-}450^{3+}\text{-}SH \rightleftharpoons F_2H\cdot + P\text{-}450^{2+}\text{-}SH}$

4 $\quad \mathrm{(P\text{-}450^{2+}\text{-}SH + O_2 \rightleftharpoons P\text{-}450^{2+}\text{-}SH\text{-}O_2)}$

5 $\quad \mathrm{F_2H\cdot + P\text{-}450^{2+}\text{-}SH\text{-}O_2 \rightleftharpoons F_2 + P\text{-}450^{3+} + SOH + OH^-}$

Mechanism 3B: interaction of NADPH–cytochrome P-450 reductase with cytochrome P-450 during mixed-function oxidation: 2 reducing equivalents from the reductase at the oxidation level, $F_1H_2/F_2H\cdot$

2′ $\quad \mathrm{F_1H_2 + F_2 \rightleftharpoons F_1H\cdot + F_2H\cdot}$

3′ $\quad \mathrm{F_2H\cdot + P\text{-}450^{3+}\text{-}SH \rightleftharpoons F_2 + P\text{-}450^{2+}\text{-}SH}$

4′ $\quad \mathrm{P\text{-}450^{2+}\text{-}SH + O_2 \rightleftharpoons P\text{-}450^{2+}\text{-}SH\text{-}O_2}$

5′ $\quad \mathrm{F_1H_2 + P\text{-}450^{2+}\text{-}SH\text{-}O_2 \rightleftharpoons F_1H\cdot + P\text{-}450^{3+} + SOH + OH}$

6′ $\quad \mathrm{NADPH + F_1H\cdot/F_2 \rightleftharpoons NADP^+ + F_1H_2/F_2H\cdot}$

However, there are no kinetic data available on the reduction of highly purified NADPH–cytochrome P-450 reductase by NADPH, and it is quite possible that this reduction may commence with a 2-equiv transfer from NADPH to F_2, which would then become the acceptor flavine (mechanism 4).

Mechanism 4: interaction of NADPH–cytochrome P-450 reductase with cytochrome P-450 during mixed function oxidation: 2 reducing equiv from F_1H_2

1 $\quad \mathrm{F_2 + NADPH + H^+ \rightleftharpoons F_2H_2 + NADP^+}$

2 $\quad \mathrm{F_2H_2 + F_1 \rightleftharpoons F_2H\cdot + F_1H\cdot \rightleftharpoons F_2 + F_1H_2}$

3 $\quad \mathrm{F_1H_2 + SH\text{–}F^{3+}\ (P\text{-}450) \rightleftharpoons F_1H\cdot + SH\text{–}Fe^{2+}\ (P\text{-}450) + H^+}$
($\mathrm{F_2}$–$\mathrm{F_1H\cdot}$ is the O_2-stable flavoprotein)

4 $\quad \mathrm{F_2\text{–}F_1H\cdot + NADPH + H^+ \rightleftharpoons F_2H_2\text{–}F_1H\cdot + NADP^+}$

5 $\quad \mathrm{F_2H_2\text{–}F_1H\cdot + SH\text{–}Fe^{2+}O_2(P\text{-}450) \rightleftharpoons F_2H\cdot\text{–}F_1H\cdot = F_2\text{–}F_1H_2 + SOH + Fe^{3+}(P\text{-}450) + OH^-}$

A similar proposal has been made by Siegel *et al.* (1971, 1972) to account for intramolecular transfer of reducing equivalents in NADPH–sulfite reductase. This enzyme, like NADPH–cytochrome P-450 reductase, contains both FMN

TABLE I: Oxidation–Reduction Potentials of Components of Cytochrome P-450 Dependent Mixed-Function Oxidase Systems from Various Sources.

Component	$E_{0,7}'$ (V)	N	Tissue or Cell
Flavoprotein reductases			
NADH–putidaredoxin reductase (Marbach, 1972)	−0.283	2	*Pseudomonas putida*$_{cam}$
NADPH–adrenodoxin reductase (Schleyer *et al.*, 1971)	−0.345		Adrenal mitochondria
(Chu and Kimura, 1973)	−0.274		
NADPH–cytochrome P-450 reductase (present work)	−0.365 ($E_{0,4}'$)	1	Liver microsomes
	−0.290 ($E_{0,3}'$)	1	
	−0.270 ($E_{0,2}'$)	1	
	−0.110 ($E_{0,1}'$)	1	
NADPH–cytochrome P-450 reductase (Ichikawa and Yamano, 1972)[a]	−0.310	2	
Iron–sulfur proteins			
Putidaredoxin (Wilson, 1969; Wilson *et al.*, 1973)	−0.235	1	*Pseudomonas putida*$_{cam}$
Adrenodoxin (Schleyer *et al.*, 1971, 1973; Cooper *et al.*, 1970)	−0.305	1	Adrenal mitochondria
(Huang and Kimura, 1973)	−0.270	1	
Cytochrome P-450			
Pseudomonas putida$_{cam}$ (Gunsalus and Lipscomb, 1972)	−0.270	1	
Adrenal mitochondria (Schleyer *et al.*, 1971, 1973; Cooper *et al.*, 1970)	−0.170 (with substrate)	1	
	−0.400		
Liver microsomes (Waterman and Mason, 1972)	−0.34 to −0.40	1	(Absence of substrate)
(Cohen and Wilson)[b]	−0.335	1	

[a] E_0' based upon $A_{445\ nm}$ only during dithionite titration in the presence of mediator. [b] B. S. Cohen and D. F. Wilson, personal communication. Their work indicated that microsomal cytochrome P-450 may be heterogeneous.

and FAD. Siegel and his coworkers proposed a mechanism for catalysis in which FAD cycles between fully oxidized and fully reduced forms (probably *via* a semiquinone), and FMN cycles between fully reduced and semiquinone forms. This mechanism, which includes a disproportionation step, permits a "step-down" from the two-electron donor, NADPH, to a succession of equipotential one-electron transfer steps.

Figure 12 depicts a computer analysis of the relative concentrations of each redox state of each flavine, F_1 and F_2, as functions of E_h and of the per cent of NADPH in a mixture of NADPH and $NADP^+$. It is apparent that even at the lowest levels of NADPH concentration there is virtually no F_1 (fully oxidized form of F_1) associated with the reductase; conversely, there is little fully reduced F_2H_2 associated with the reductase except at very high NADPH/$NADP^+$ ratios. Sies and Kandel (1970) have reported that the ratio of NADPH/$NADP^+$ varies between 2.2 and 4 in liver cells, depending upon the presence or absence of the (substrate) drug, hexobarbital. Under these conditions, the concentration of F_2H_2 would vary between 18 and 25% of the sum $[F_2] + [F_2H] + [F_2H_2]$. On the other hand, the concentrations of F_1H_2 and $F_2H\cdot$, the postulated source of 2 electrons for mixed-function oxidation according to mechanism 3B, remain high and relatively constant throughout the physiological range of NADPH/$NADP^+$ ratios which supports this mechanism, but kinetic,

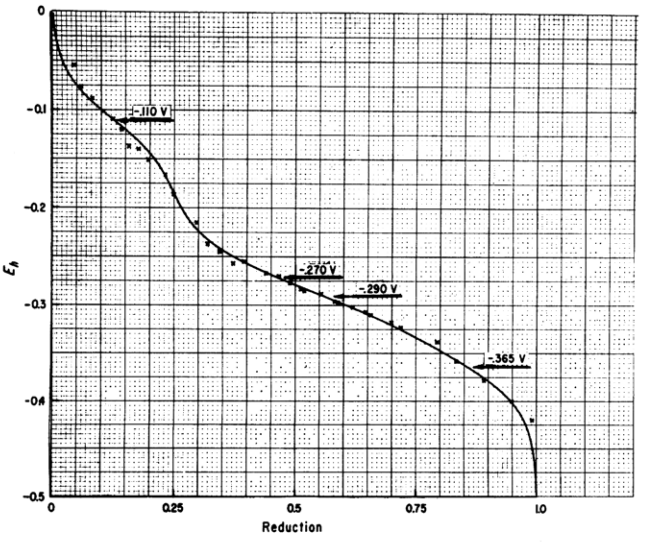


FIGURE 11: Computer simulation for the potentiometric titration of cytochrome P-450 reductase. Crosses show the observed values. Solid line was computed according to mechanism 3 (see text), making the same assumptions as in Figure 9.

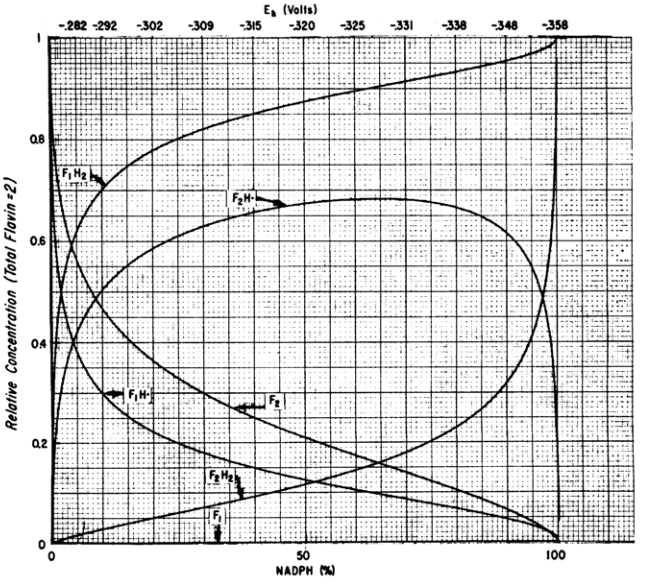


FIGURE 12: Computed curves for the change in concentration of flavine species as functions of E_h (top abscissa) and % NADPH in a mixture of NADPH and $NADP^+$, using the same assumptions as for Figure 9. The ordinate was taken as (total F_1) + (total F_2) = 2.

rather than equilibrium, information about the whole system will be required to assess these or other hypotheses. In any case the simple shuttle mechanism for this enzyme proposed by Masters and her coworkers (Masters *et al.*, 1965) is improbable if only because the two flavines are structurally different and have quite different redox properties. It is interesting that the potentials of the couples $F_1H_2/F_1H\cdot$ and $F_2H\cdot/F_2$ are similar to that of adrenodoxin (Table I). We have sought direct evidence of electronic coupling of $F_1H\cdot$ and $F_2H\cdot$ by epr spectroscopy, in accordance with the suggestion of Van Voorst (1966) that a $\Delta m = 2$ transition giving an epr signal at about 1500 G would be definite proof for the existence of two flavine radicals coupled only by dipolar spin–spin interaction. However, as Figure 10 shows, at half-reduction of the enzyme, the concentration of each flavine semiquinone is 40% of the total of each flavine; hence the number of protein molecules containing one each of the flavine semiquinones would be 40% × 40% = 16% of the total flavine. Even under favorable conditions ([E] = 1.0 mM, 20°K, 200 mW), the signal to noise ratio in the region of 1500 G from this system was too low to give a definitive result. We cannot say that orbital overlap between the two flavines, F_1 and F_2, occurs in any of their states of oxidation–reduction; but direct electronic overlap would not be required for facilitated electron transfer between the $E_{0,2}'$ and $E_{0,3}'$ couples in system 2. Additional structural and kinetic studies of the enzyme, particularly in the assembled mixed-function oxidase system, are obviously required before an exact description of the mechanism of hepatic mixed-function oxidation dependent on cytochrome P-450 and its reductase can be given.

Acknowledgments

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References

Burleigh, B. D., Jr., Foust, G. P., and Williams, C. H., Jr. (1969), *Anal. Biochem. 27*, 536.

Chu, J. W., and Kimura, T. (1973), *J. Biol. Chem. 248*, 2089.

Clark, W. M. (1960), Oxidation-reduction Potentials of Organic Systems, Baltimore, Md., Waverly Press.

Cooper, D. Y., Schleyer, H., and Rosenthal, O. (1970), *Ann. N. Y. Acad. Sci. 174*, 205.

Dutton, P. L. (1971), *Biochim. Biophys. Acta 226*, 63.

Gunsalus, I. C., and Lipscomb, J. D. (1972), *in* Molecular Basis of Electron Transport, Schulz, J., Ed., New York, N. Y., Academic Press, p 179.

Hemmerich, P., Veeger, C., and Wood, H. C. S. (1965), *Angew. Chem., Int. Ed. Engl. 4*, 671.

Huang, J. J., and Kimura, T. (1971), *Biochem. Biophys. Res. Commun. 44*, 1065.

Huang, J. J., and Kimura, T. (1973), *Biochemistry 12*, 406.

Ichikawa, Y., and Yamano, T. (1970), *Biochim. Biophys. Acta 200*, 220.

Ichikawa, Y., and Yamano, T. (1972), *J. Biochem. (Tokyo) 71*, 1053.

Ives, D., and Janz, G. (1961), Reference Electrodes, New York, N. Y., Academic Press, p 161.

Iyanagi, T., and Mason, H. S. (1973), *Biochemistry 12*, 2297.

Iyanagi, T., and Yamazaki, I. (1969), *Biochim. Biophys. Acta 172*, 370.

Lu, A. Y. H., and Coon, M. J. (1968), *J. Biol. Chem. 243*, 1331.

Lu, A. Y. H., Junk, K. W., and Coon, M. J. (1969), *J. Biol. Chem. 244*, 3714.

Lu, A. Y. H., Kuntzman, R., West, S., and Conney, A. H. (1971), *Biochem. Biophys. Res. Commun. 42*, 1200.

Marbach, W. J. (1972), Thesis, Univ. Illinois.

Massey, V., and Swoboda, B. E. P. (1963), *Biochem. Z. 338*, 474.

Masters, B. S. S., Baron, J., Taylor, W. E., Isacson, E. L., and LoSpalluto, J. (1971a), *J. Biol. Chem. 246*, 4143.

Masters, B. S. S., Kamin, H., Gibson, Q. H., and Williams, C. H., Jr. (1965), *J. Biol. Chem. 240*, 921.

Masters, B. S. S., Nelson, E. B., Ziegler, D. M., Baron, J., Raj, P. P. and Isacson, E. L. (1971b), *Chem.-Biol. Interactions 3*, 296.

Meites, L., and Meites, T. (1948), *Anal. Chem. 20*, 948.

Michaelis, L., and Hill, E. S. (1933), *J. Gen. Physiol. 16*, 859.

Miyake, Y., Mason, H. S., and Landgraf, W. (1967), *J. Biol. Chem. 242*, 393.

Nelson, E. B., Raj, P. P., Belfi, K. J., and Masters, B. S. S. (1971), *J. Pharmacol. Exp. Ther. 178*, 580.

Omura, T. (1969), *in* Microsomes and Drug Oxidations, Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R., Mannering, G. J., Ed., New York, N. Y., Academic Press, pp 160–161.

Omura, T., and Sato, R. (1964), *J. Biol. Chem. 239*, 2370.

O'Reilly, J. E. (1973), *Biochim. Biophys. Acta 292*, 509.

Peisach, J., and Blumberg, W. E. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 172.

Preisler, P. W., Hill, E. S., Loeffel, R. G., and Shaffer, P. A. (1959), *J. Amer. Chem. Soc. 81*, 1991.

Schacter, B. A., Nelson, E. B., Marver, H. S., and Masters, B. S. S. (1972), *J. Biol. Chem. 247*, 3601.

Schleyer, H., Cooper, D. Y., and Rosenthal, O. (1971), *in* Magnetic Resonances in Biological Research, Franconi, C., Ed., New York, N. Y., Gordon and Breach, Inc., p 247.

Schleyer, H., Cooper, D. Y., and Rosenthal, O. (1973), Oxidases and Related Redox Systems, Second Symposium, Baltimore, Md., University Press p 469.

Sies, H., and Kandel, M. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 9*, 205.

Stieliler, R. D., Chen, Tung-Ton, and Clark, W. M. (1933), *J. Amer. Chem. Soc. 55*, 891.

Strittmatter, P., and Velick, S. F. (1956), *J. Biol. Chem. 221*, 277.

Siegel, L. M., Faeder, E. J., and Kamin, H. (1972) *Z. Naturforsch. 27*, 1087.

Siegel, L. M., Kamin, H., Rueger, D. C., Presswood, R. P., and Gibson, Q. H. (1971), *in* Flavins and Flavoproteins, Proceedings of the Third International Symposium, H. Kamin, Ed., Baltimore, Md., University Press, p 523.

Takesue, S., and Omura, T. (1970), *J. Biochem. (Tokyo) 67*, 267.

Tyson, C. A., Lipscomb, J. D., and Gunsalus, I. C. (1972), *J. Biol. Chem. 247*, 5777.

Van Voorst, J. D. W. (1966), *in* Flavines and Flavoproteins, Slater, E. C., Ed., Elsevier, Amsterdam, p 155.

Waterman, M. R., and Mason, H. S. (1972), *Arch. Biochem. Biophys. 150*, 57.

Wilson, G. S. (1969), *in* Mossbauer Spectroscopy in Biological Systems, Debrunner, P., Tsibris, J. C. M., and Munck, E., Ed., Urbana, Ill., University of Illinois.

Wilson, G. S., Tsibris, J. C. M., and Gunsalus, I. C. (1973), *J. Biol. Chem.* (in press).