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# CORRELATIONS BETWEEN SPIN EQUILIBRIUM SHIFT, REDUCTION RATE, AND *N*-DEMETHYLATION ACTIVITY IN LIVER MICROSOMAL CYTOCHROME P-450 AND A SERIES OF BENZPHETAMINE ANALOGUES AS SUBSTRATES

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**Abstract**—Cytochrome P-450 forms a thermal ferric spin equilibrium which is significantly shifted by substrate binding. Within a series of benzphetamine analogues the liver microsomal enzyme system exhibits a close correlation of the substrate induced spin equilibrium shift towards the high spin state and both the rate of P-450 reduction, and of substrate turnover, as well. The spin equilibrium regulates the first electron transfer by favoured high spin state reduction and rapid pre-equilibration with respect to the low spin fraction.

Cytochrome P-450 (P-450, EC 1.14.14.1) catalysed reactions are characterized to proceed in a distinct sequential reaction cycle, as proposed by several authors [1–4]. Starting with the substrate binding process, followed by the reduction of the heme iron and binding of molecular oxygen, the reaction is finished by the introduction of a second electron, oxygen cleavage, and transfer of one oxygen atom to the substrate concomitant with the formation of water from the second one. Whereas the first reaction steps have been studied extensively, the oxygen activation and the substrate conversion process on the other hand, are rather less understood. Respective investigations are restricted to appropriate model systems, thus limiting the derived hypotheses therefrom [5, 6].

Substrate (type I) binding shifts the spin equilibrium of ferric P-450 towards the high spin state [7]. The functional importance of this spin conversion is obvious because of the concomitant increase of the apparent redox potential of the heme iron [8], which causes a favoured P-450 reduction in the respective substrate complex [3, 9–12]. The amount of the substrate induced high spin shift and the rate of the P-450 reduction could be shown to be quantitatively correlated [13, 14]. The substrates selected for that investigation, however, differed in chemical structure and, thus, were converted via different catalytic processes, e.g. cyclohexane, hexobarbital by hydroxylation and benzphetamine by *N*-demethylation, respectively. Therefore, the observed correlation is impaired, especially with respect to a correlation of both quantities with the substrate conversion process. Consequently, in the present paper the effects of a series of benzphetamine analogues on spin equilibrium, NADPH supported P-450 reduction and *N*-demethylation, respectively, were analysed. Evidence is provided that a threefold correlation of the parameters investigated exists, the causal relationships of which are discussed especially with respect to the spin equilibrium control of the reduction and the conversion reaction.

## MATERIALS AND METHODS

*Preparation of microsomes.* Male wistar rats (120–150 g) were pretreated for 3 days with sodium phenobarbital (75 mg/kg of body weight i.p. or by 1.0 g/l contained in the drinking water). 24 hr before use the animals were deprived of food, while drinking *ad lib.* was maintained. After decapitation and bleeding, the livers were removed and stored in ice-cold 0.1 M phosphate buffer, pH 7.4, containing 2 mM EDTA. The further preparational steps were performed according to ref. [15].

The microsomal suspension (40 mg protein/ml) was stored in small aliquots for, at the most, 4 weeks at −20° after freezing in liquid nitrogen. During storage neither a loss in demethylase activity nor an increase in the P-420 content was observed. The thawed samples were used within 1 day. The P-420 content in all samples did not exceed 10%. The use of only one frozen stock preparation provided reliable conditions for comparative studies.

*Chemicals.* D,L-Benzphetamine and the related substrates were prepared according to prescriptions summarized in ref. [16]. In most cases the amines were alkylated with the appropriate alkyl halides. The basic products of the reactions were separated by extraction with ether and the free tertiary amines then isolated by fractional distillation under reduced pressure. The amine hydrochlorides were prepared

by addition of hydrochloric acid in ether to the solution of the free amines in absolute ethyl alcohol, and were purified by repeated cyrstallization from ethyl alcohol/ether. D-Benzphetamine was prepared by fractional crystallization of the diastereomeric (+)-tartrates formed by reaction of racemic benzphetamine with (+)-tartaric acid. *p*-Chlorobenzyl chloride and *o*-chlorobenzyl chloride were obtained from Fluka AG, Buchs. All other chemicals for the syntheses of substrates were purchased from VEB Laborchemie, Apolda.

The chemicals used in the expts were purchased as follows: NADPH, glucose oxidase, AMP (VEB Arzneimittelwerk, Dresden); glucose-6-phosphate (Reanal, Budapest); glucose-6-phosphate dehydrogenase, catalase, (Boehringer GmbH, Mannheim); semicarbazide (Riedel-de Haen AG, Hannover).

*Analytical methods.* The concentrations of cytochromes P-450 and P-420 were determined from the CO-difference spectra using the absorption coefficients $\Delta\varepsilon_{450/490\,nm} = 91$ ($mM^{-1}\cdot cm^{-1}$) and $\Delta\varepsilon_{420/490\,nm} = 111$ ($mM^{-1}\cdot cm^{-1}$) after correction for the negative contribution of P-450 with $\Delta\varepsilon_{420/490\,nm} = -41$ ($mM^{-1}\cdot cm^{-1}$).

The substrate induced $\Delta A_{max}$ values were derived from the difference spectra of the P-450 substrate complex vs the substrate free enzyme [$\Delta A_{max\ 387/417\,nm}$ ($mM^{-1}\cdot cm^{-1}$)]. The magnitude of the spin shift was calculated from $\Delta A_{max}$ by use of an absorption coefficient $\Delta\varepsilon_{387/417\,nm} = 110$ ($mM^{-1}\cdot cm^{-1}$) for the total spin transition [17].

The spectrophotometric measurements were performed at room temperature using a Shimadzu UV 300 (Japan) spectrophotometer or a Unicam SP-800 (U.K.) device.

*Kinetic measurements.* The kinetics of the cytochrome P-450 reduction by NADPH was followed via the formation of the respective CO-complex. Since the CO binding reaction is not rate limiting [$k_{on} \simeq 10^5$ ($M^{-1}\cdot sec^{-1}$)] this way the reduction process is directly accessible. The expts were performed by use of a dual-detector stopped-flow spectrophotometer Durrum D-110, D-137 (U.S.A.). By means of filter technique the reaction was monitored in the 450/500 nm difference mode.

Anaerobicity was obtained by gassing the reaction solutions with highly purified nitrogen and CO, respectively, for about 30 min. An auxiliary enzyme system—glucose oxidase 4.6 U/ml, glucose 60 mM, catalase 4680 U/ml—was additionally added to remove traces of oxygen in the solutions. The stopped-flow apparatus was nitrogen flushed for about 60 min before introduction of the reaction solutions.

The reactions were initiated by rapidly mixing solution A (containing the microsomal P-450, substrate (0.1–1.0 mM), auxiliary enzyme system) with solution B [NADPH (1 mM), CO (saturated), auxiliary enzyme system]. The expts were run at pH 7.4 and 20° (reaction chamber and drive syringes were thermostated).

Data converting and processing (1000 data points per run) were performed by use of a transient recorder Datalab DL 905 (U.K.) and a microprocessor ZFK Rossendorf MPS 4944 (G.D.R.). For further analysis a BESM 6 (U.S.S.R.) computer was used.

The analysis of the reaction curves was performed by means of a multiexponential nonlinear estimation procedure, based on Provencher [18]. The $n$ independent rate constants $k_i$ of the amplitude/time function

$$y = b_0 + \sum_{i=1}^{n} a_i e^{-k_i t}$$

were calculated.

*Determination of the enzymatic activity.* The catalytic activity of P-450 was determined in 0.1 M phosphate buffer, pH 7.4 at 37°. The reaction mixture (1.2 ml) contained the following constituents (final concentrations in parentheses): microsomal protein (0.6 mg/ml), determined by the Biuret method, NADPH (0.5 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (0.5 U/ml), AMP (2 mM), $MgCl_2$ (7 mM), semicarbazide (6 mM) and the respective substrate (33–1000 $\mu$M).

The reaction mixtures were incubated for 8 min in a Warburg shaker at 37° with air in the gaseous phase. The reactions were stopped by addition of 0.3 ml 33.3% trichloroacetic acid, resulting in a final concentration of 6.67%.

The formaldehyde formation was determined by the method of Nash [19] after addition of double concentrated NASH-reagent to an aliquot of 1 ml and heating at 60° for 10 min. The reaction rates were expressed as nmoles formaldehyde/nmole P-450·min. The determination was performed with some modifications according to ref. [20]. $V_{max}$ and $K_M$ values were derived from Lineweaver–Burk plots.*

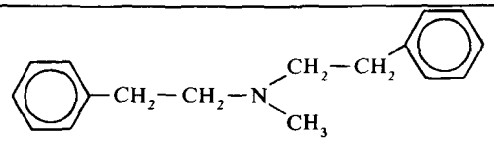
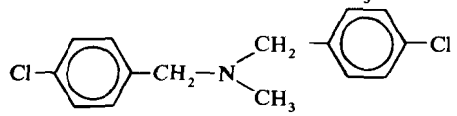
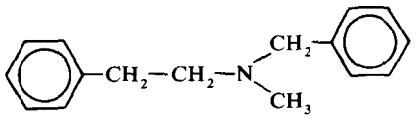
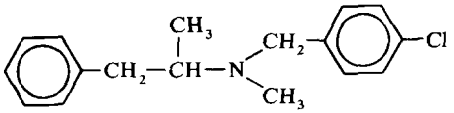
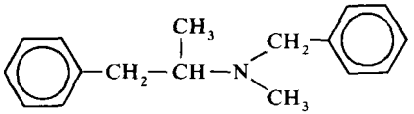
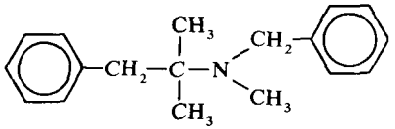
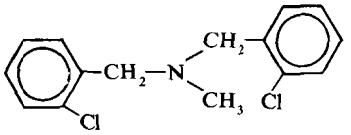
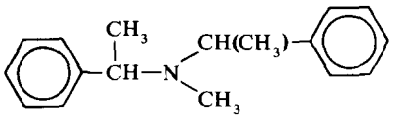
## RESULTS

A series of tertiary amines related to benzphetamine has been investigated with respect to: (1) the maximal absorption amplitudes of the enzyme substrate complex in the difference spectrum (against the free enzyme) $\Delta A_{max\ 387/417\,nm}$ and the apparent substrate binding constants $K_S$ as well; (2) the rate constant and phase distribution data of the NADPH dependent P-450 reduction, $k_n$, $\phi_n$; and (3) the kinetic parameters of the *N*-demethylation, $K_M$, $V_{max}$. The experimental data obtained are summarized in Table 1.

The compounds investigated so far induce like benzphetamine type I difference spectra with significantly different $\Delta A_{max}$ values (column 1). As previously shown [13, 14] $\Delta A_{max}$ represents the extent of the spin equilibrium shift towards the high spin state of P-450. The spin shift data are drawn in column 2. The resulting total high spin contents (column 3) were calculated on the basis of a 42% value intrinsic to the microsomal P-450 in the absence of substrate according to ref. [21] from the 645 nm absorption. The substrates are listed in the sequence of increasing $\Delta A_{max}$, i.e. of increasing high spin shift.

* The calculation of the $V_{max}$ and $K_M$ values by Dr. H. Hoppe, Institute of Physiological Chemistry, Friedrich-Schiller University, Jena, by means of a HP-20 calculator are gratefully acknowledged.

Table 1. Spin equilibrium and kinetic parameters of P-450 complexes with benzphetamine analogues

| | Substrates | $\Delta A_{max\ 387/417\ nm}$ ($mM^{-1} \cdot cm^{-1}$) | High spin shift $\Delta\alpha$ (%) | High spin content $\alpha^*$ (%) | $K_s$ ($\mu M$) | $k_1 \cdot 10^1$† ($sec^{-1}$) | $K_M$ ($\mu M$) | $V_{max}$ (nmoles HCHO/nmole P-450 · min) |
|---|---|---|---|---|---|---|---|---|
| 1 |  | 19.0 ± 0.9 | 17.3 | 59.3 | 35.4 ± 5.7 | 5.9 ± 1.0 | 101.1 ± 3.0 | 3.24 ± 0.1 |
| 2 |  | 20.0 ± 0.5 | 18.2 | 60.2 | 16.7 ± 1.6 | 4.4 ± 0.3 | 88.3 ± 5.2 | 3.76 ± 0.4 |
| 3 |  | 26.2 ± 0.6 | 23.8 | 65.8 | 44.0 ± 3.5 | 5.8 ± 0.4 | 163.9 ± 10.6 | 6.04 ± 0.5 |
| 4 |  | 32.0 ± 0.8 | 29.1 | 71.1 | 33.9 ± 2.6 | 5.7 ± 0.7 | 140.2 ± 4.9 | 6.64 ± 0.2 |
| 5 |  | 32.5 ± 0.6 | 29.5 | 71.5 | 34.2 ± 2.1 | 6.0 ± 0.6 | 172.4 ± 10.1 | 7.04 ± 0.4 |
| 6 |  | 37.6 ± 0.7 | 34.4 | 76.4 | 22.9 ± 1.5 | 6.0 ± 0.6 | 139.9 ± 4.1 | 7.16 ± 0.2 |
| 7 |  | 39.6 ± 0.3 | 36.0 | 78.0 | 7.2 ± 2.5 | 7.1 ± 0.7 | 214.4 ± 16.0 | 10.2 ± 1.0 |
| 8 |  | 45.0 ± 0.6 | 40.9 | 82.9 | 11.2 ± 1.6 | 7.4 ± 0.6 | 210.6 ± 12.0 | 13.5 ± 1.1 |

* Intrinsic high spin content in absence of substrate 42% (20°).
† Reduction rate constant, $k_1$, in absence of substrate $3.4 \pm 0.7 \cdot 10^{-1}$ ($sec^{-1}$).

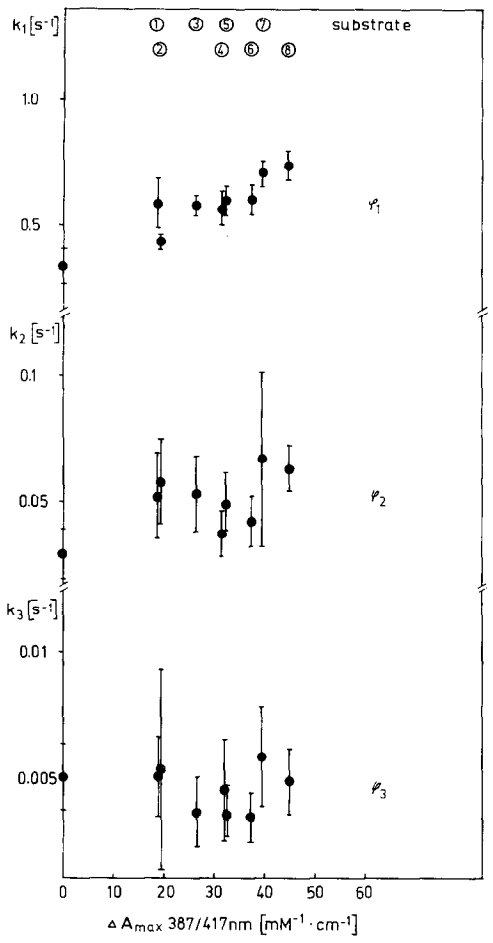



Fig. 1. Three-exponential graph of the NADPH-dependent cytochrome P-450 reduction in liver microsomes 0.1 M phosphate buffer, pH 7.4, 20°. 1–8 denote the substrates (cf. Table 1). The initial abscissa values ($\Delta A_{max} = 0$) refer to the substrate-free P-450.

Obviously *p*-chlorination (compounds **2** and **4**) is less effective as compared to *o*-chlorination (compound **7**), an increase with the introduction of $CH_3$ groups (compounds **4–6, 8**) is evident. The maximal spin shift amounts to 40.9%, i.e. a corresponding total high spin content of 82.9% was obtained. The observed spin shift does not correlate with the $K_S$ values of the P-450 substrate complexes.

The anaerobic NADPH reduction of the microsomal P-450 proceeds inhomogeneously and exhibits a three-exponential time function. The phase distribution amounts to about $\phi_1/\phi_2/\phi_3 = 60/20/20\%$ irrespective of the substrate. The first order rate constants $k_1$ ($\phi_1$), $k_2$ ($\phi_2$), $k_3$ ($\phi_3$) differ by about one order of magnitude each. (Fig. 1).

The rate constant of the catalytically most relevant fast phase in the absence of substrate $k_1 = 0.34 \pm 0.07$ sec$^{-1}$ increases up to $0.74 \pm 0.06$ sec$^{-1}$ with the most effective substrate (column 5). $k_1$ correlates well with the substrate induced $\Delta A_{max}$ and, thus, with the spin shift $\Delta\alpha$ (Fig. 2).

The correlation function was determined with $k_1$ (sec$^{-1}$) $= 0.35 \pm (0.0088 \pm 0.0026)\ \Delta\alpha$, the regression coefficient amounts to $r = 0.81$. The second

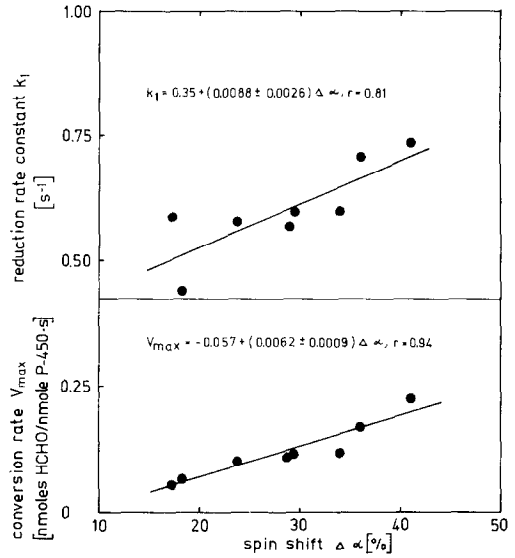



Fig. 2. Correlation of spin equilibrium shift and reduction rate constant, $k_1$ (sec$^{-1}$) and substrate turnover (sec$^{-1}$), respectively. *r*, Correlation coefficients.

phase rate constants, $k_2$, in the order 0.05 sec$^{-1}$ indicate a respective correlation. The third phase, on the other hand, seems not to be influenced by substrates. The order of this partial reaction is 0.005 sec$^{-1}$.

The compounds studied so far provide the common presupposition to be catalytically converted by *N*-demethylation. The respective $K_M$ and $V_{max}$ values, as derived from Lineweaver–Burk plots, are drawn in columns 6 and 7. The sequence of $K_M$ does not follow the selected sequence of $\Delta A_{max}$. The $V_{max}$ values, however, significantly follow that sequence, meaning that they correlate well with the observed spin shift. The conversion parameter, $V_{max}$, depends on the spin shift with $V_{max}$ (nmoles formaldehyde/nmole P-450·sec) $= -0.057 + (0.0062 \pm 0.0009)\ \Delta\alpha$. The regression coefficient was calculated with $r = 0.94$.

## DISCUSSION

Within a series of closely related substrates a significant correlation of the substrate induced spin equilibrium shift towards the high spin state ($\Delta\alpha$) and both the rate constant of the P-450 reduction in the rapid phase ($k_1$) and of the catalytic turnover ($V_{max}$) has been observed. The evidenced correlations arise the question for their causal connections in the reaction mechanism.

The spin state specific P-450 reduction ($v_r$) can be formulated by use of the high spin and low spin rate constants ($k_{hs}$, $k_{ls}$) as follows

$$v_r = \text{P-450}_{hs} \cdot k_{hs} + \text{P-450}_{ls} \cdot k_{ls}.$$

Introducing $\alpha$ and $1-\alpha$ for the respective P-450 high spin and low spin fraction results in

$$v_r = \text{P-450}\,(\alpha \cdot k_{hs} + (1-\alpha) \cdot k_{ls}).$$

The high spin rate constant, $k_{hs}$, must exceed the low

spin one ($k_{ls}$) because of the higher redox potential of the high spin P-450 [8]. Therefore, with respect to differentiated rate constants ($k_{hs}$, $k_{ls}$) a homogeneous reduction can only occur if the spin equilibrium is capable of rapid spin transition kinetics, i.e. $\alpha$ = constant. It could be evidenced that the spin state transition in P-450 CAM* [22] and in liver microsomal P-450 [23] is rapid. Recently, by means of laser temperature jump expts [24] the spin conversion in P-450 LM2† has been found to proceed in the *ns* time scale. Therefore, any rate limiting spin state equilibration with respect to the reduction, as supposed by Backes *et al.* [25, 26], can be excluded. With constant $\alpha$, P-450 thus must be reduced with the apparent rate constant $k_{app}$

$$k_{app} = \alpha \cdot k_{hs} + (1-\alpha) \cdot k_{ls}.$$

This general treatment has been applied to the catalytically most relevant first phase 'cluster' reduction [27–29]. Since no significant phase shift has been observed after substrate binding, substrate dependent cluster formation specificities may be excluded. The determined rate constants, $k_1$, correlate well with $\Delta A_{max}$ and, thus, with the substrate induced spin shift $\Delta\alpha$ (Figs. 1 and 2). Moreover, the substrate free P-450 is reduced with an apparent rate constant ($k_1 = 0.34 \pm 0.07\,sec^{-1}$) which corresponds to the intrinsic high spin content ($\alpha = 42\%$). Obviously, the observed $k_1/\Delta\alpha$ correlation is only part of a general $k_1/\alpha$ correlation. The validity of this extrapolation is supported by the respective behaviour of the aniline complex. The substrate induced decrease in high spin content is paralleled by a decrease of the reduction rate constant in comparison to the substrate free P-450 [13, 14]. That implies that the P-450 reduction is controlled by the overall high spin content of the P-450 population instead of only the substrate induced fraction of the substrate binding species.

The observed correlations, $k_1/\Delta\alpha$ and $k_1/\alpha$, respectively, may be perturbed further by the isozyme pattern of the microsomal P-450. Recent data from Guengerich *et al.* [30] evidence an amount of about 70% phenobarbital induced form (PB-$B_2$). Considering the multiplicity of isozymes and their substrate specificities, the investigations refer to the main fraction of the rat microsomal P-450. Moreover, isozyme specificities are not resolved. The correlation, therefore, excludes significant differences in reactivity of major isozyme fractions.

The extrapolation of the $k_1/\alpha$ correlation to $\alpha = 0$ indicates that the low spin state reduction rate constant $k_{ls}$ approaches zero. The above equation then reduces to

$$k_{app} = k_1 = \alpha \cdot k_{hs}.$$

This way the P-450 population can be reduced only in the high spin state either directly or by rapid pre-equilibration with respect to the low spin fraction.

The indicated $k_2/\alpha$ correlation (Fig. 1) points to some further spin state regulation of the 'noncluster' reduction ($\phi_2$). Both phases contribute by about 60% ($\phi_1$) and 20% ($\phi_2$), respectively, to the reduction process, i.e. the spin state regulation thus refers to at least 80% of the microsomal P-450.

The third phase ($\phi_3$), comprehending the remaining 20%, obviously exhibits no $k_3/\alpha$ correlation. The molecular origin of this partial reaction is rather unknown.

A resolution of reaction phases, in addition to the two main contributions ($\phi_1$, $\phi_2$), could be proved also by other authors. Oprian *et al.* [29], using a reconstituted P-450 LM4‡ system, observed three reaction phases at a low reductase/P-450 ratio, which they ascribed to biphasic reduction in a reductase–P-450 complex and to that of uncomplexed cytochrome. Recently, Ruf [31] with fresh microsomes from phenobarbital induced rats evidenced even four reaction phases with a more rapid first phase ($k_1 \sim 1.5\,sec^{-1}$) and a significant amplitude increase by benzphetamine (45% → 60%) instead of a $k_1$ increase by that substrate [12]. Thus, species differences and preparational conditions may influence the reaction.

The observed $V_{max}/\Delta\alpha$ correlation and the $k/\Delta\alpha$ and $k/\alpha$ correlation of the P-450 reduction, as well, suggest that the substrate turnover may be controlled via the spin state regulated P-450 reduction. That implies the existence of a general $\alpha$ correlation of $V_{max}$ too. The microsomal system, however, fails to exhibit a $V_{max}/\alpha$ correlation. That could be due, at least in part, to catalytically inactive isozymes which impair a reasonable interpretation of the molecular basis of the turnover regulation. At least, however, the substrate induced spin shift, $\Delta\alpha$, indicates the catalytic activity of P-450 towards the respective substrate, possibly due to the capability of the substrate to specifically modify the conformation of the ternary complex. That could effect the second electron transfer (redox potential) or other steps behind, included a shift in the ratio of oxygenase and oxidase function.

The apparent rate divergence between electron transfer and turnover, especially with respect to temperature correction (the 20° $k_1$ values increase about 7–8-fold at 37° [27]), may be closed by considering the isozyme pattern of the microsomal P-450, the uncoupling contribution, the phase distribution of the reduction, reoxidation under aerobic conditions, endogenous substrate interference, etc. In order to improve the experimental support with respect to the outlined problems further investigations with reconstituted LM2 systems were initiated.

* P-450 CAM, Bacterial camphor hydroxylating cytochrome P-450 (*Pseudomonas putide*).

† P-450 LM2, Phenobarbital induced pure isozyme of rabbit liver microsomal cytochrome P-450.

‡ P-450 LM4, 5,6-Naphthoflavone induced pure isozyme of rabbit liver microsomal cytochrome P-450.

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