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INCORPORATION OF CYTOCHROME b_5 INTO RAT LIVER MICROSOMAL MEMBRANES

IMPAIRMENT OF CYTOCHROME P-450-DEPENDENT MIXED FUNCTION OXIDASE ACTIVITY

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Abstract—Cytochrome b_5 was purified to electrophoretic homogeneity from the liver microsomes of untreated rats and reincorporated into liver microsomes from phenobarbital-treated rats, resulting in an approximate three-fold enrichment of the cytochrome b_5 specific content (1.5 nmol haemoprotein · mg^{-1} protein). Our results have shown that the *N*-demethylation of benzphetamine was progressively inhibited in cytochrome b_5-fortified microsomal preparations. Using stopped flow, visible difference spectrophotometry, the NADPH-driven reduction kinetics of cytochrome P-450 were examined in the modified microsomes over the first few seconds of reaction. Increasing the amount of incorporated cytochrome b_5 resulted in a progressive inhibition of the initial, fast phase reduction rate constant of microsomal cytochrome P-450, both in the absence and presence of the type I substrate benzphetamine. Although the initial rate of NADPH-driven cytochrome b_5 reduction was the same for both native and cytochrome b_5-fortified microsomes, the extent of cytochrome b_5 reduction was greater in the fortified microsomes. If cytochrome b_5 has a positive role to play in cytochrome P-450-dependent mixed function oxidase activity either as an effector or in electron transfer or both, the former haemoprotein must be already present in sufficient concentrations in the native microsomes.

The cytochrome P-450 enzyme system of liver microsomes catalyses the oxidation of numerous xenobiotics and has been resolved into three main components, cytochrome P-450, NADPH-cytochrome P-450 reductase and phosphatidylcholine. Although NADPH is the preferred source of necessary reducing equivalents for the mixed function oxidase reaction, NADH can weakly support drug oxidation reactions [1] and additionally exerts a synergist effect in the presence of NADPH [2]. These and other findings have led to the hypothesis that cytochrome b_5 may have a role to play in cytochrome P-450-dependent drug oxidation reactions, although the precise mechanism still remains a subject of controversy [2–8]. The role of cytochrome b_5 in drug oxidation reactions is confused by the observations that cytochrome b_5 may be inhibitory [3], stimulatory [6], obligatory [9] or have no effect [3] on hydroxylation reactions. However, this confusion has been partially rationalised by the observation that the nature of the substrate and the particular isoenzyme of cytochrome P-450 under consideration is an important determinant of reactivity with cytochrome b_5 [10].

Regardless of the mechanism whereby cytochrome b_5 modulates mixed function oxidase activity, a functional interaction between cytochrome b_5 and cytochrome P-450 would appear to be necessary as it has recently been shown that cytochrome b_5 interacts with cytochrome P-450 to give a tight 1:1 molar complex [11] resulting in a type I spectral change [12], indicative of a low to high spin transition of the haem iron of cytochrome P-450 [13]. It has been proposed that the above haemoprotein interaction is dictated by highly organised binding of the hydrophobic domains present in each cytochrome [14], and a recent report [15] has shown that highly purified multiple forms of cytochrome P-450 significantly differ in their interaction with cytochrome b_5, and that the carboxyl groups of cytochrome b_5 are important in binding to cytochrome P-450. This latter study corroborated earlier findings that cytochrome b_5 preferentially undergoes complex formation with a purified, phenobarbital-induced multiple form of cytochrome P-450, resulting in a cytochrome b_5-dependent perturbation of the cytochrome P-450 spin equilibrium towards the high spin form [16]. Furthermore, the prior complexation of cytochrome b_5 to cytochrome P-450 resulted in a three-fold increase in the binding of the substrate benzphetamine (one of the preferred substrates of phenobarbital-inducible cytochrome P-450) to cytochrome P-450 and provided preliminary evidence that the cytochrome b_5-induced spin state change may have a regulatory role to play in drug oxidation reactions [16].

Accordingly, the present study was carried out to determine if cytochrome b_5-mediated modulation of substrate binding to cytochrome P-450 resulted in any change in substrate turnover rates in intact rat liver microsomal preparations.

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MATERIALS AND METHODS

Experimental animals and preparation of liver microsomes. Male Wistar albino rats (150 g ± 10.9) were supplied by the University of Surrey Breeders. Liver microsomes were prepared from phenobarbital-pretreated rats (80 mg · kg^{-1} given by intraperitoneal injection once a day for three days) by differential ultracentrifugation [17] and suspended in 50 mM potassium phosphate buffer, pH 7.4 containing 20% (v/v) glycerol. Microsomes were stored at −70° and thawed once, immediately prior to use.

Purification of cytochrome b_5. Cytochrome b_5 was purified from uninduced rat liver microsomes by a modification of the procedure described by Chiang [12]. The final cytochrome b_5 preparation was extensively dialysed against 50 mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, concentrated on an Amicon PM10 membrane and stored in aliquots at −70° until required. The final preparation had specific content of 39 nmol cytochrome b_5 · mg^{-1} protein and was spectrally similar to the original preparation of Spatz and Strittmatter [18]. The protein was electrophoretically homogeneous as determined by the sodium dodecyl sulphate/polyacrylamide gel electrophoresis system of Laemmli [19] and exhibited an apparent monomeric molecular weight of approximately 14,000 daltons. This preparation was devoid of cytochrome P-450 as determined both spectrally and electrophoretically.

Spectrophotometry. Spectrophotometric determinations were performed on an Aminco DW-2 recording spectrophotometer operating in either the split beam or dual wavelength mode. Simple colorimetric assays were carried out using a Cecil CE-292 spectrophotometer. The stopped flow reduction kinetics of cytochrome P-450 were determined on the Aminco DW-2 spectrophotometer interfaced with an Aminco-Morrow stopped flow apparatus. Absorbance data were recorded on a data storage module (Thornton, Model 492), simultaneously visualised on an oscilloscope (Telequipment, Model D1011) and hard copy created on a Servogar 120 recorder. The 100% reduced cytochrome P-450 was determined on the Aminco DW-2's own chart recorder.

The stopped flow reactions were initiated by rapidly mixing an equal volume of an anaerobic solution containing microsomes in one driving syringe and NADPH from the other. In addition, each syringe contained 100 mM potassium phosphate buffer, pH 7.25, 20% (v/v) glycerol, glucose (6.7 mM), catalase (590 Units · ml^{-1}) and glucose oxidase (13 Units/ml). NADPH concentration in one syringe was 0.6 mM (0.3 mM final concentration) and the microsomal protein concentration in the other syringe was 0.25 to 0.50 mg/ml. The above solutions were gassed with carbon monoxide (deoxygenated) for 1.5 min prior to the addition of catalase and glucose oxidase, and subsequently continued for 2.5 min. After initiation of the reaction, spectral changes were monitored through a 1 cm light path with a spectral band width of 3.0 nm. The time course of reduction of cytochrome P-450 was conveniently monitored by the increase in absorbance at 450 nm of the ferrous-carbon monoxide adduct of the haemoprotein. Under these experimental conditions the rate of binding of carbon monoxide to the ferrous haemoprotein was not rate-limiting [20] and the mixing solutions were anaerobic [21].

Reincorporation of cytochrome b_5. Liver microsomes from phenobarbital-pretreated rats were used for the reincorporation studies since these offered the lowest cytochrome b_5: cytochrome P-450 ratio. These microsomes were also used because it has previously been shown [16] that cytochrome b_5 forms a tight complex with a purified isoenzyme of phenobarbital-induced cytochrome P-450 (K_d = 275 nM). Purified cytochrome b_5 was then incorporated into the microsomes by a modification of the method of Muna and Smuckler [22] as follows. Increasing amounts of cytochrome b_5 (as indicated in the Results) were added to microsomal suspensions at a protein concentration of 15 mg · ml^{-1}. Incubation was carried out at 4° for 60 min in 100 mM potassium phosphate buffer, pH 7.25 containing 20% (v/v) glycerol. Following incubation, the contents of each tube were diluted with five times the incubation volume of the last buffer and centrifuged at 105,000 *g* av. for 60 min. The fortified microsomal pellet was resuspended in fresh buffer by vortex agitation, recentrifuged and finally resuspended in the above phosphate buffer.

Other assay procedures. Protein was determined by a modification of the Lowry method [23], after correction for interference by glycerol. Cytochrome P-450 was determined as the reduced carbon monoxide adduct in difference spectrum using a difference extinction coefficient of 91 mM^{-1} cm^{-1} for the wavelength couple 450–490 nm [24]. Cytochrome b_5 was determined from the difference spectrum of ferrous and ferric haemoproteins between 424 and 490 nm, using the difference extinction coefficient of 112 mM^{-1} cm^{-1} for this wavelength couple [25]. Reduction of cytochrome b_5 was achieved either with a few grains of solid sodium dithionite or by the addition of 25 μl of 2% (w/v) NADH. Benzphetamine *N*-demethylase activities were determined by assaying formaldehyde product formation by the colorimetric procedure of Nash [26], with reference to a formaldehyde standard curve.

RESULTS

Reincorporation of cytochrome b_5 *into hepatic microsomes*

At a fixed concentration of microsomal protein, increasing amounts of cytochrome b_5 could be incorporated into microsomal preparations, saturation being achieved at a cytochrome b_5 concentration of approximately 1.4 nmol · mg^{-1} protein, as assayed by NADH reduction of the haemoprotein (Fig. 1), resulting in an increase of the cytochrome b_5 to cytochrome P-450 molar ratio of 0.27 (native microsomes) to 0.73 at the highest added concentration of cytochrome b_5 (50 μM). Also shown in Fig. 1 is the reincorporation of cytochrome b_5 as assayed by dithionite reduction. This latter reduction method resulted in apparently increased reincorporation of cytochrome b_5 and was not as readily saturatable. The dithionite-reduced cytochrome b_5 probably includes an absorbance component of other micro-

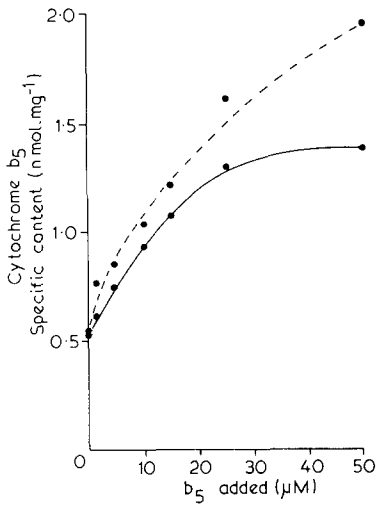

Fig. 1. Reincorporation of cytochrome b_5 into hepatic microsomal membranes. The amount of cytochrome b_5 added refers to the original amount of haemoprotein prior to resedimentaiton of the fortified microsomes, the latter at a fixed concentration of 15 mg protein · ml^{-1}, as described in Materials and Methods. The solid and dashed lines indicate reduction in the presence of NADH and dithionite respectively.

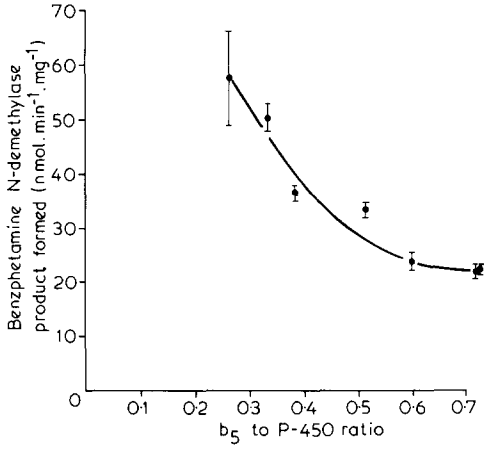

Fig. 2. Influence of reincorporated cytochrome b_5 on the *N*-demethylation of benzphetamine in hepatic microsomal membranes.

somal chromophores (including cytochrome P-450), and for this reason, the NADH-enzymatically reducible levels were subsequently used as an index of functionally reducable cytochrome b_5.

The reincorporation of cytochrome b_5 reported in Fig. 1 is higher than reported by Muna and Smuckler [22] possibly because reincorporation was carried out for a longer period in our studies (60 min as compared to 30 min). Our reincorporation of cytochrome b_5 resulted in an approximate three-fold increase in the haemoprotein specific content, as compared to a four-fold reincorporation as reported by Jansson and Schenkman [27], probably because the latter authors assayed cytochrome b_5 by using dithionite as a reductant, and may therefore not represent the true, functional, NADH-reducible amounts of the haemoprotein.

Benzphetamine N-*demethylase activity and cytochrome* P-450 *reduction kinetics in reincorporated microsomes*

The ability of the cytochrome b_5-fortified microsomal preparations to support the *N*-demethylation of benzphetamine is shown in Fig. 2. As the amount of reincorporated cytochrome b_5 is increased, there is a progressive inhibition of benzphetamine *N*-demethylase activity, resulting in an approximate halving of the activity at the highest cytochrome b_5 concentration tested.

Clearly there are several possible explanations as to why cytochrome b_5 inhibits the above microsomal enzyme activity, the most likely of which would be a cytochrome b_5-dependent blockade of the transfer of reducing equivalents from NADPH to cytochrome P-450 (i.e. haemoprotein reduction), via the flavoprotein NADPH-cytochrome P-450 reductase. Accordingly, the NADPH-driven reduction kinetics of cytochrome P-450 were investigated in the cytochrome b_5-fortified microsomes in the absence and presence of benzphetamine, as shown in Fig. 3. Firstly it should be noted that the substrate benzphetamine increases the apparent initial rate of cytochrome P-450 reduction (compare traces numbered 1 in Figs 3a and 3b) as described in other cytochrome P-450-containing systems, as a consequence of substrate modulation of the haemoprotein spin state [21]. Secondly, when the reduction of cytochrome P-450 is considered as a function of reincorporated cytochrome b_5, it is clear that increasing concentrations of the latter haemoprotein significantly inhibit cytochrome P-450 reduction in a concentration-dependent manner (compare curves 1, 2 and 3 in both Figs 3a and 3b).

The raw absorbance data in Figs 3a and 3b may then be re-expressed in semilogarithmic form (Figs 3c and 3d) and from the slope of the early reduction data, the observed, apparent rate constants for the initial, rapid rate of cytochrome P-450 reduction can then be calculated. As shown in Table 1, for the complete reincorporated cytochrome b_5 range, it is clear that increasing amounts of cytochrome b_5 inhibit the apparent initial rate constant of cytochrome P-450 reduction in a concentration-dependent manner, in an analogous manner to inhibition of benzphetamine *N*-demethylase activity as noted in Fig. 2 above.

It would then appear that cytochrome b_5 inhibits benzphetamine *N*-demethylation by inhibiting electron transfer from NADPH to cytochrome P-450 and therefore the possibility exists that cytochrome b_5 itself is reduced by NADPH, thus competing with cytochrome P-450 for the reducing equivalents derived from NADPH. As shown in Fig. 4, NADPH reduces native microsomal cytochrome b_5 and furthermore that the reincorporated cytochrome b_5 is also reducible by NADPH. It is also apparent from Fig. 4 that even in the presence of excess NADPH, cytochrome b_5 is reoxidised at later time points. Where the reducing equivalents from the reoxidation of reduced cytochrome b_5 actually are transferred to is not clear and were not addressed by our studies.

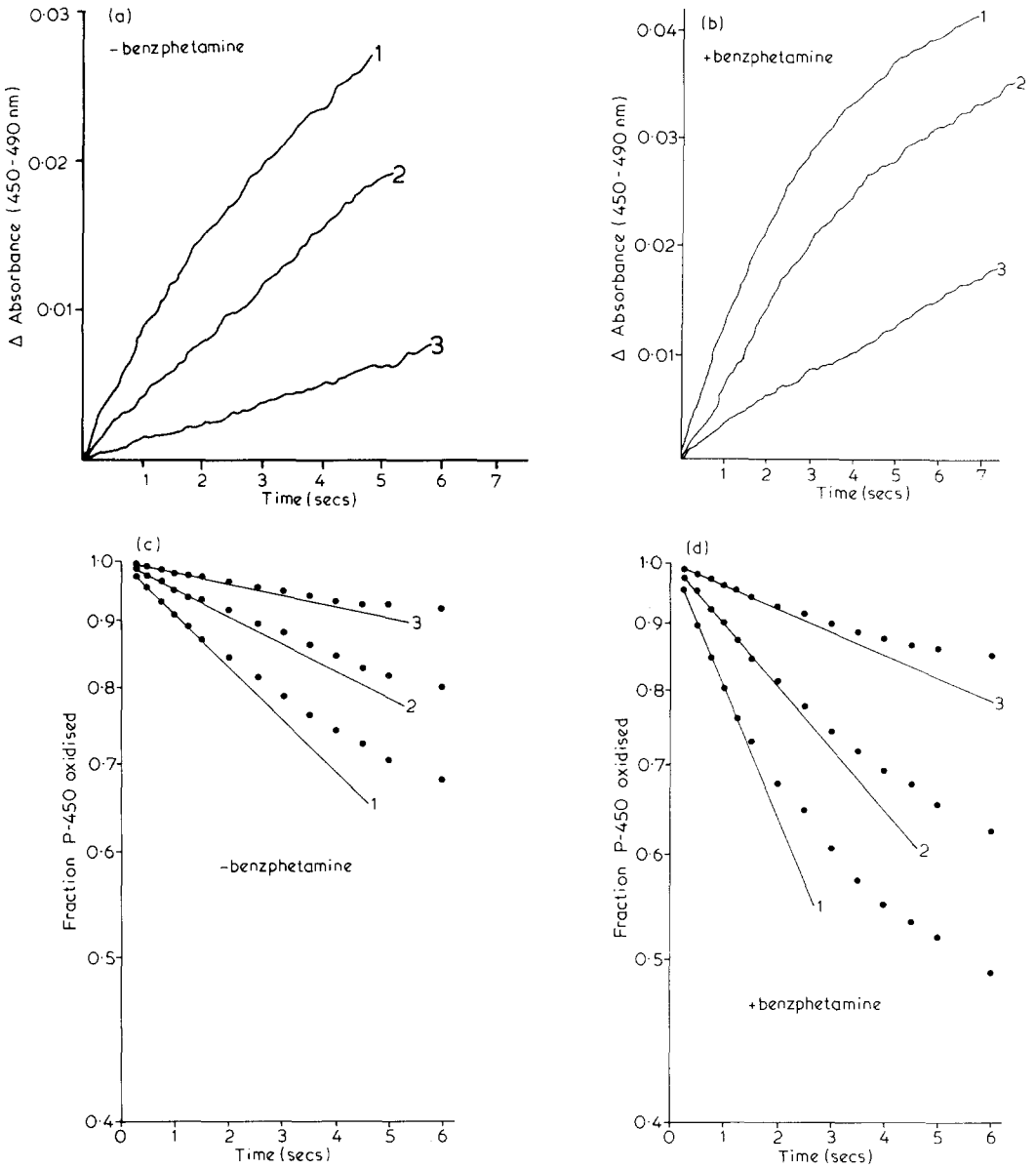

Fig. 3. Influence of reincorporated cytochrome b_5 on the reduction kinetics of hepatic microsomal cytochrome P-450. The reduction kinetics (as monitored by $\Delta A_{450-490}$) were carried out as described in Materials and Methods and is a representative set of data derived from eight separate determinations. (a) and (c) are the reduction kinetics in the absence of benzphetamine and (b) and (d) were determined in the presence of 1 mM benzphetamine. To clarify the data representation, only three separate cytochrome b_5: cytochrome P-450 molar ratios are shown corresponding to 0.27 (curve 1, native microsomes), 0.38 (curve 2) and 0.60 (curve 3). (a) and (b) were derived from continuous spectrophotometric traces and (c) and (d) show selected points derived from the original data in (a) and (b).

However, it is likely from our studies that these reducing equivalents are not utilised in the benzphetamine *N*-demethylase reaction, but rather are passed on to cytochrome P-450 and are used to activate molecular oxygen resulting in oxygen products such as superoxide or H_2O_2 as discussed by Bonfils *et al.* [7].

DISCUSSION

This study was prompted by the observation that cytochrome b_5 binds tightly to cytochrome P-450 in a 1:1 molar complex [11] resulting in a type I spectral change [12], indicative of a low to high spin transition of the cytochrome P-450 haem iron [13]. As the cytochrome b_5-induced high spin state of cytochrome P-450 binds the substrate benzphetamine much more tightly and the *substrate*-induced reduction of cytochrome P-450 is faster than in the absence of substrate, it was originally anticipated that cytochrome b_5-fortified microsomes would substantially increase benzphetamine *N*-demethylase activity

Table 1. Influence of reincorporated cytochrome b_5 on the apparent, initial reduction rate constant (k_{obs}) of cytochrome P-450 in the absence and presence of substrate

Cytochrome b_5: cytochrome P-450 molar ratio	k_{obs}* (minus benzphetamine)	k_{obs} (plus 1 mM benzphetamine)
0.27†	0.083 ± 0.009	0.211 ± 0.010
0.33	0.064 ± 0.011	0.149 ± 0.005
0.38	0.045 ± 0.006	0.122 ± 0.012
0.60	0.031 ± 0.003	0.058 ± 0.002
0.72	0.019 ± 0.001	0.025 ± 0.003
0.73	0.019 ± 0.003	0.021 ± 0.004

* k_{obs} are given in units of sec^{-1} and were calculated by least squares regression from the data in Figs 3c and 3d. Values are represented as the mean ± the standard deviation derived from eight determinations.

† Native microsomes.

based on spin state/reduction kinetic arguments. From the data presented in this communication, it is clear that the opposite influence of cytochrome b_5 on both substrate metabolism and cytochrome P-450 reduction kinetics as envisaged above is observed, and therefore the system is more complex than was originally thought. Accordingly, in the presently described system, cytochrome b_5 exerts two opposing effects on the cytochrome P-450 mediated *N*-demethylation of benzphetamine: firstly, to increase the high spin component of cytochrome P-450 and then facilitate substrate binding and reduction kinetics; secondly, and obviously more important in our system, cytochrome b_5 competes with cytochrome P-450 for NADPH-derived reducing equivalents and diverts reducing power away from cytochrome P-450, thus providing a possible explanation for the inhibition of substrate turnover.

It would appear unlikely that the cytochrome b_5-dependent changes noted are a result of non-specific effects related to protein incorporation. The fact that the re-incorporation of cytochrome b_5 is a saturable process (Fig. 1) and that both benzphetamine *N*-demethylase and the cytochrome P-450 reduction kinetics are inhibited proportional to the amount of re-incorporated cytochrome b_5 would argue against a non-specific effect. Furthermore, from the experiments described herein, it is not absolutely certain if the re-incorporated cytochrome b_5 is incorporated into the microsomal membrane at the same locus as the endogenous haemoprotein. However, the re-incorporated haemoprotein quite clearly exhibits a functional role as demonstrated by our studies.

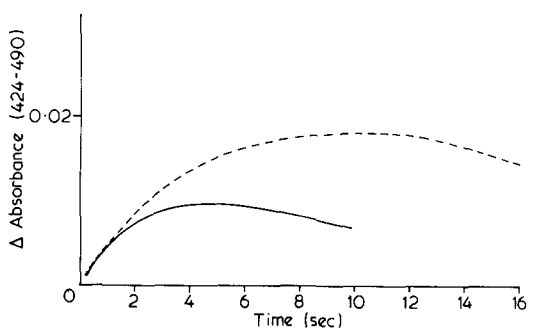

Fig. 4. NADPH-dependent reduction and re-oxidation of native and cytochrome b_5-fortified hepatic microsomes. NADPH-driven cytochrome b_5 reduction was monitored by the Δ absorbance between 424 and 490 nm by stopped flow spectrophotometry as described in Materials and Methods. The specific contents of cytochrome b_5 were 0.53 (solid line, native microsomes) and 1.40 (dashed line, fortified microsomes).

The importance of the molar ratio of cytochrome b_5 to cytochrome P-450 in hepatic microsomal membranes is thus clearly an important concept in the regulation of the rate of drug metabolism in the liver, particularly when cytochrome P-450 induction occurs after xenobiotic pretreatment. For example, most of the known inducers of cytochrome P-450 do not increase the hepatic microsomal concentration of cytochrome b_5, thus decreasing the membrane ratio of cytochrome b_5 to cytochrome P-450, and as discussed herein, causing an increased rate of drug oxidations upon induction. Therefore cytochrome b_5 has the potential to exert another level of control in drug oxidations, in addition to the well-known inducer-dependent modulation of cytochrome P-450 multiple forms that exhibit different substrate specificities. It must be re-emphasised that an additional fine-tuning of the influence of cytochrome b_5 on drug metabolism critically depends on the prevailing complement of cytochrome P-450 isoenzymes in the microsomal membrane. This statement is readily appreciated when one considers that recent reports have shown that cytochrome b_5 preferentially undergoes complex formation with only selected isoenzymes of both constitutive and induced forms of cytochrome P-450 [16, 28]. In addition, the specific chemical nature of the substrate itself is also a factor that must be taken into account when considering the role of cytochrome b_5 in drug oxidations [28]. The above factors have also been clearly highlighted by a recent report which, in addition, emphasises the importance of reconstitution conditions in influencing the effect of cytochrome b_5 on cytochrome P-450 driven reactions [29].

In conclusion, we have demonstrated that in our specified experimental system, the reincorporation of cytochrome b_5 inhibits benzphetamine *N*-demethylation. Much of the past and current controversy regarding the role of cytochrome b_5 can be best rationalised by individually considering the relative important contributions made by the system under investigation, the complement and nature of the cytochrome P-450 isoenzymes, the reconstitution conditions, the specific substrate and haemoprotein–haemoprotein interactions. It would thus appear that the concept of a universal role for cytochrome b_5 modulation of drug oxidations is untenable.

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