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EFFECT OF PROTEIN DEFICIENCY ON THE INDUCIBILITY OF THE HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYME SYSTEM—I

EFFECT ON SUBSTRATE INTERACTION WITH CYTOCHROME P-450*

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Abstract—Male, weanling rats divided into three groups were maintained for 15 days on a semipurified diet containing either 5% casein fed *ad lib.* (group 1), 20% casein pair-fed to group 1 (group 2), or 20% casein fed *ad lib.* (group 3). After each group was further subdivided, animals were injected i.p. on days 11, 12, 13 and 14 with either 0·9% saline or phenobarbital (80 mg/kg) in 0·9% saline. Twenty-four hr after the last injection, animals were decapitated and liver microsomes were prepared. Contents of microsomal protein, phosphatidylcholine and cytochrome P-450 were measured and used as bases of expression for spectral dissociation constants (K_s) and maximal spectral changes (ΔA_{max}) associated with the binding of ethylmorphine and aniline to the cytochrome P-450 hemoprotein of microsomes. Phenobarbital administration increased microsomal protein, cytochrome P-450, and phosphatidylcholine in all three dietary groups; however, in all groups, the increase in P-450 was relatively greater than that for phosphatidylcholine. Protein deficiency (group 1 vs 2) decreased P-450 and microsomal protein, but had no effect on phosphatidylcholine contents. The effect of total food restriction (group 2 vs 3) on each of these parameters was not significant. These data suggest that a portion of the induced cytochrome P-450 binding sites may be dependent on an association with phosphatidylcholine. The fraction of such phosphatidylcholine-associated sites relative to the total sites was greater during protein deficiency and was in agreement with a greater ΔA_{max} per nanomole P-450 for ethylmorphine. Phenobarbital induction decreases the proposed fraction of phosphatidylcholine-associated P-450 sites relative to the total P-450 sites and results in a decrease in the ΔA_{max} per nanomole P-450 for ethylmorphine. Phenobarbital increased the ΔA_{max} per milligram of microsomal protein for aniline, which paralleled the increase in total P-450, thus indicating that the type II site may be independent of any association of cytochrome P-450 with phosphatidylcholine. These results indicate that phosphatidylcholine may play an important role in distinguishing the effects of dietary deficiency on type I substrate binding and the corresponding capacity for induction of the rat liver microsomal enzyme system.

IT IS well known that the injection of many foreign compounds induces the activity of liver microsomal enzymes.[1,2] On the other hand, low protein intake reduces microsomal enzyme activity.[3–6] Previous work from this laboratory[6] has shown that liver microsomes isolated from weanling rats fed 5% casein diets for 15 days compared to animals pair-fed a diet containing 20% casein were characterized by lower V_{max}-values for both ethylmorphine (EM) and aniline (AN).

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Protein deficiency may also affect induction of microsomal enzyme activity. Marshall and McLean[7] found that when rats fed a low protein diet are given phenobarbitone, the level of cytochrome P-450 rises, but is still only a quarter of that found in phenobarbitone-treated rats given a normal protein diet. Recently, these same investigators[8] showed that not only is additional dietary protein required for restoration of the inductive process but also needed is a dietary source of polyunsaturated fat or oxidized sterol as indicated by cytochrome P-450 content or aniline hydroxylation.

Induction of enzyme activity may be associated with modified spectral changes produced by the addition *in vitro* of certain substrates to microsomes.[9–12] Studies by Remmer *et al.*[9] and Schenkman *et al.*[13] have established two types of spectral changes which occur upon substrate binding to the microsomal cytochrome P-450 hemoprotein, termed type I and type II.* An alteration of the difference spectrum as a function of substrate concentration is used to calculate both the binding constant (K_s) and the maximal spectral change (ΔA_{max}).

The present studies were undertaken to elucidate the effect of dietary protein deprivation on the substrate binding characteristics of microsomes in order to further characterize both the deficiency and its effect on the inductive process. These experiments have also enabled us to obtain additional information on the possible role of phosphatidylcholine in mediating the binding of substrate to microsomal hemoprotein in protein-deficient animals. Several laboratories[14–17] have presented evidence in support of a role for phosphatidylcholine in the maintenance of the functional integrity of cytochrome P-450.

MATERIALS AND METHODS

Animal treatment. Male weanling Sprague–Dawley-derived rats, obtained from Flow Research Laboratories, Dublin, Va., were used in all experiments. Upon procurement, they were housed individually in stainless steel, wire-bottom cages with no bedding. The animals were housed under controlled humidity and exposed to a 12-hr light–dark schedule. They were fed a semipurified 20% casein diet for 2 days for acclimatization. The animals were then divided into three groups of 8–14 animals each. Group 1 animals were fed a 5% casein diet *ad lib.*; group 2, a 20% casein diet pair-fed to group 1; and group 3, a 20% casein diet fed *ad lib.* All groups received tap water *ad lib.* during the 15 days of feeding. The basal diet (20% casein) previously described by Weatherholtz *et al.*[18] consisted of the following (in grams): sucrose, 69·6; vitamin-free casein, 20·0; Mazola corn oil, 4·0; Jones–Foster salt mixture (Nutritional Biochemicals Corp.), 4·0; vitamin diet fortication mixture (Nutritional Biochemicals Corp.), 2·2; and D,L-methionine, 0·2. The 5% casein diet was kept isocaloric by replacing the casein with an equivalent amount of sucrose.

Each dietary group was divided into a control and experimental subgroup. The experimental subgroup received i.p. injections of 80 mg/kg of phenobarbital (PB) in 0·9% saline and the control subgroup received 0·9% saline only. Injections were given on days 11–14. The animals were sacrificed by decapitation 24 hr after the last injection.

Tissue preparation. Livers were immediately removed, weighed and perfused with 0·9% saline and placed in an ice-bath. Individual livers were pooled for the various

* Type I ligands produce a difference spectrum characterized by an absorption peak at 385–390 nm and a trough at 419–425 nm, whereas type II substrates produce a difference spectrum characterized by an absorption peak at 426–435 nm and a trough at 390–405 nm.

subgroups. All subsequent manipulations were carried out at 0–5°. The livers were finely minced and homogenized in 2 vol. of ice-cold 1·15% KCl containing 20 mM Tris–HCl (pH 7·4) in a motor-driven Potter–Elvehjem Teflon–glass homogenizer at 600 rev/min using six complete strokes. The same homogenizer was used in each experiment.

The homogenate was centrifuged at 9000 *g* for 20 min in a refrigerated centrifuge to remove unbroken cells, nuclei and mitochondria. The supernatant was carefully decanted through glass wool and centrifuged at 105,000 *g* in a Spinco model L2-65B centrifuge to sediment the microsomes. The microsomal pellet was floated off the glycogen pellet and resuspended in the KCl–Tris buffer by gentle homogenization to give a suspension equivalent to 1 *g* liver/ml.

Analytical procedures. Microsomal protein was determined by the method of Lowry *et al.*[19] The method of Omura and Sato[20] was used for the determination of cytochrome P-450. Microsomal choline was determined by a modification of the method of Ackerman and Salmon.[21] One ml of microsomal suspension was added to 15 ml of 25% HNO_3 and digested for 3 hr at 80–90°. The digest was filtered to remove any residue and the volume made up to 25 ml. NaOH pellets were added to obtain a pH above 12. After the solution was chilled, 3 ml of freshly prepared 5% ammonium reineckate in methanol was added. The solution was then placed in the cold in the absence of light for 3 hr and subsequently filtered through Celite (Johns–Manville Hyflo SuperCel) previously washed with acetone and 1-propanol. The reineckate–choline complex retained on the Celite was washed to remove excess ammonium reineckate four times with 2 ml of 1-propanol; the reineckate–choline complex was recovered with 10 ml acetone; and the absorbancy of the acetone eluate was measured at 526 nm.

The spectral binding constant (K_s) and maximal spectral change (ΔA_{max}) were determined using spectrophotometric titrations essentially as described by Guarino *et al.*[11] Substrate concentrations ranging from 0·02–50 mM for EM and 0·15–1·5 mM for AN were successively added to the sample cuvette in minimal volumes of buffer. The change in absorbancy was determined with a Unicam SP-800 spectrophotometer, using the change in absorbancy between 420 and 500 nm to compute the binding constants for EM and between 430 and 500 nm for AN. The K_s and ΔA_{max} were calculated by a Fortran computer program devised by Cleland.[22]

Statistical analyses were determined by the Student's *t*-test.[23]

RESULTS

Throughout the presentation of these results, the assumption will be made that protein deficiency, *per se*, distinguishes the differences shown between groups 1 and 2, whereas restricted food intake accounts for the differences between groups 2 and 3. It is, however, recognized that the restricted food intake of group 2, relative to group 3, represented a reduced intake imposed by pairing of group 2 with group 1, which in turn consumed less, presumably because of protein deficiency.

Table 1 shows the effects of protein deficiency* and PB treatment on growth and food consumption. Protein deficiency was associated with a decreased food consumption (indicated by a comparison of pair-fed groups 1 and 2 vs group 3) and a

* Previous work in our laboratory has shown that saline injections do not significantly alter the effects of protein deficiency on the various parameters under study.

TABLE 1. EFFECT OF DIETARY PROTEIN AND PHENOBARBITAL INDUCTION ON FOOD CONSUMPTION AND GROWTH OF MALE RATS*

	Group 1		Group 2		Group 3	
Dietary casein (%)	5	5	20	20	20	20
Treatment	Saline	PB	Saline	PB	Saline	PB
No. of exp.	6	6	6	6	6	6
Animals/exp.	7	7	6	6	6	6
Food consumption (g)	76 ± 4[a]	77 ± 3	77 ± 3[a]	76 ± 4	159 ± 10[b]	152 ± 5
Final body wt (g)	57 ± 2[a]	60 ± 2	86 ± 2[b]	88 ± 2	141 ± 3[c]	144 ± 3
Liver wt (g/100 g body wt)	5·8 ± 0·2[a]	7·9 ± 0·1†	4·7 ± 0·3[b]	6·0 ± 0·2†	5·2 ± 0·1[b]	6·7 ± 0·1†

* Animals fed for 15 days according to protocol presented in text. Groups 1 and 2 were pair-fed; group 3 was fed *ad lib.* Phenobarbital (PB, 80 mg/kg), administered i.p. on days 11–14 in 0·9% saline. Control animals injected on the same days with 0·9% saline. Standard errors are shown where appropriate. Statistical significance between dietary treatments (subgroup controls) is indicated by lettered superscripts; data which are not significantly different at the 5 per cent level of probability show the same letter.

† Statistically significant difference between PB-induced animals and their respective controls ($P < 0{\cdot}05$).

60 per cent lower final body weight (group 1 vs group 2). Restricted food intake reduced the final body weight by 39 per cent (group 2 vs group 3). Liver weight as a per cent of body weight was 24 per cent higher in the protein-deprived animals (group 1 vs group 2).

Although not detailed in Table 1, PB treatment caused slight increases in food intake and body weights during the 4 days of administration. These increases did not markedly influence the total 15-day changes. Liver weights as a per cent of body weight were increased by 26–35 per cent by PB treatment, which is consistent with earlier reports.[1,8]

Table 2 represents the effects of the experimental treatments on liver microsomal constituents. Protein deficiency decreased the quantity of microsomal protein per g of liver by 41 per cent and cytochrome P-450 by 61 per cent (group 1 vs group 2). Microsomal choline was used as an estimate of the phosphatidylcholine content, since Glaumann and Dallner[24] and Rogers[25] showed that phosphatidylcholine is the major microsomal component containing the choline moiety. Protein deficiency did not cause a significant change in phosphatidylcholine per milligram of microsomal protein; even though we have observed that there was lipid infiltration in the livers from the protein-deprived rats.* Since phosphatidylcholine has been implicated in the binding of substrates to cytochrome P-450, the ratio of phosphatidylcholine to P-450 may be important. Therefore, if phosphatidylcholine is expressed per nanomole of P-450, protein deficiency elicited a 161 per cent increase (group 1 vs group 2).

PB treatment produced a 40–44 per cent increase in microsomal protein in groups 1 and 2, both of which were under dietary stress, and only a 17 per cent increase in group 3. Cytochrome P-450 was increased after PB administration by 240–350 per cent in groups 1 and 2, but by only 160 per cent in group 3. Dietary stress encountered with protein or caloric insufficiency, or both, appears to be associated with an increase

* M. U. K. Mgbodile and T. C. Campbell, unpublished observations.

TABLE 2. EFFECT OF DIETARY PROTEIN AND PHENOBARBITAL INDUCTION ON RAT LIVER MICROSOMAL CONSTITUENTS*

	Group 1		Group 2		Group 3	
Dietary casein (%)	5	5	20	20	20	20
Treatment	Saline	PB	Saline	PB	Saline	PB
Protein (mg/g liver)	6·9 ± 0·4[a]	9·9 ± 0·6†	11·7 ± 0·6[b]	16·4 ± 1·0†	12·9 ± 1·1[b]	15·1 ± 0·7†
Cytochrome P-450 (nmole/mg protein)	0·16 ± 0·03[a]	0·54 ± 0·07†	0·41 ± 0·07[b]	1·84 ± 0·12†	0·47 ± 0·11[b]	1·24 ± 0·18†
Choline (phosphatidylcholine)						
(μg/mg protein)	26·5 ± 0·6[a]	35·5 ± 3·2†	26·0 ± 1·5[a]	45·8 ± 2·2†	23·8 ± 1·4[b]	45·2 ± 2·3†
(μg/nmole P-450)	165·6 ± 34·3[a]	65·7 ± 9·4†	63·4 ± 9·2[b]	24·9 ± 2·0†	50·6 ± 9·5[b]	36·5 ± 5·9†

* Same as footnote to Table 1. Data represent means ± S.E. for a minimum of six experiments with four to seven animals per experimental subgroup (24–42 animals/experiment); pooled livers for each group, and two to three replications per assay. Statistical treatment same as in Table 1.

† Statistically significant difference between PB-induced animals and their respective controls ($P < 0·05$).

TABLE 3. EFFECT OF DIETARY PROTEIN AND PHENOBARBITAL INDUCTION ON ETHYLMORPHINE BINDING TO RAT LIVER MICROSOMES*

	Group 1		Group 2		Group 3	
Dietary casein (%)	5	5	20	20	20	20
Treatment	Saline	PB	Saline	PB	Saline	PB
K_s (mM)	0·056 ± 0·014[a]	0·025 ± 0·010†	0·051 ± 0·009[a]	0·050 ± 0·015	0·048 ± 0·015[a]	0·044 ± 0·027
ΔA_{max}/mg protein × 10^3	4·84 ± 0·71[a]	3·92 ± 0·37	7·41 ± 0·75[b]	7·92 ± 1·41	7·08 ± 1·26[a]	4·75 ± 0·75†
ΔA_{max}/nmole P-450 × 10^3	21·26 ± 4·55[a]	6·05 ± 1·19†	14·90 ± 2·56[a]	5·17 ± 1·12†	14·00 ± 2·12[a]	4·46 ± 0·94†

* Ethylmorphine added directly to microsomal suspension in cuvette according to procedure outlined in Materials and Methods. Data represent means ± S.E. for a minimum of six experiments with four to seven animals per experimental subgroup (24–42 animals/experiment); pooled livers for each group, and two to three replications per assay. Statistical treatment same as in Table 1.

† Statistically significant different between PB-induced animals and their respective controls ($P < 0·05$).

TABLE 4. EFFECT OF DIETARY PROTEIN AND PHENOBARBITAL INDUCTION ON ANILINE BINDING TO RAT LIVER MICROSOMES*

	Group 1		Group 2		Group 3	
Dietary casein (%)	5	5	20	20	20	20
Treatment	Saline	PB	Saline	PB	Saline	PB
K_s (mM)	0.14 ± 0.04^a	0.35 ± 0.09†	0.35 ± 0.09^b	0.71 ± 0.04†	0.55 ± 0.17^b	0.48 ± 0.04
ΔA_{max}/mg protein × 10^3	2.5 ± 0.3^a	6.8 ± 0.7*	$6.5 \pm 2.6^{a,b}$	26.0 ± 2.1†	6.1 ± 1.0^b	14.1 ± 1.0†
ΔA_{max}/nmole P-450 × 10^3	15 ± 4^a	12 ± 1	14 ± 3^a	13 ± 1	16 ± 5^a	12 ± 1

* Aniline added directly to microsomal suspension in cuvette according to procedure outlined in Materials and Methods. Data represent means ± S.E. for a minimum of six experiments with four to seven animals per experimental subgroup (24–42 animals/experiment); pooled livers for each group, and two to three replications per assay. Statistical treatment same as in Table 1.

† Statistically significant difference between PB-induced animals and their respective controls ($P < 0.05$).

in the relative inducibility of microsomal protein and cytochrome P-450, although the final levels for the protein-deficient group were still considerably lower than those for either group 2 or group 3.

Phosphatidylcholine per milligram of microsomal protein was increased by PB treatment in all dietary groups. The protein-deficient group (group 1) showed a 34 per cent increase in phosphatidylcholine after PB treatment; the group on restricted food intake (group 2), a 76 per cent increase; and the *ad lib.* group (group 3), a 90 per cent increase. Protein deficiency significantly impaired the ability of PB to increase to equivalent levels the phosphatidylcholine content ($P < 0{\cdot}05$). On the other hand, PB treatment significantly decreased the ratio of phosphatidylcholine per nanomole of P-450 in all dietary groups.

The relationship between the effects of protein deficiency and PB induction on the contents of phosphatidylcholine and cytochrome P-450 suggested to us that we determine the binding constants for substrate interactions with cytochrome P-450. Table 3 represents the effects of protein deficiency and induction on the binding constants for EM, which is a type I substrate. The diets under study had no significant effect on the K_s for EM. However, the ΔA_{max} per milligram of protein was decreased 35 per cent (group 1 vs group 2) by protein deficiency. In contrast, protein deficiency increased by 36 per cent ($P = 0{\cdot}07$) the ΔA_{max} per nanomole of P-450 (group 1 vs group 2). The effect of PB treatment on the alterations of the ΔA_{max} per milligram of microsomal protein was associated with decreases in groups 1 ($P = 0{\cdot}14$) and 3 ($P = 0{\cdot}04$) and a slight increase in group 2 ($P = 0{\cdot}14$). The variability observed in these latter data was apparently due, in part, to the variable increases in cytochrome P-450 content after PB treatment. When ΔA_{max} is expressed per nanomole of P-450, however, PB treatment produced very marked and consistent decreases (65–72 per cent) in all dietary groups.

Table 4 shows the effect of dietary protein and PB treatment on the binding constants for AN, a type II substrate. In contrast to the effects of protein deficiency on the K_s for EM, the K_s for AN was decreased both by protein deficiency (60 per cent, group 1 vs group 2) and by restricted food intake (36 per cent, group 2 vs group 3). Although the ΔA_{max} per milligram of protein was decreased 62 per cent by protein deficiency, the ΔA_{max} per nanomole of P-450 was not altered. PB treatment increased the K_s in the two groups (1 and 2) under dietary stress and slightly reduced the K_s in group 3. PB treatment greatly increased the ΔA_{max} per milligram of protein in all groups, but did not significantly affect the ΔA_{max} per nanomole of P-450.

DISCUSSION

Two of the components of the mixed-function oxidase system that have been shown to be affected by phenobarbital induction are the contents of cytochrome P-450[26] and phospholipid.[27] Several studies have implicated a function for phosphatidylcholine in substrate binding to cytochrome P-450. Chaplin and Mannering[14] eliminated type I binding, but enhanced type II binding with phospholipase *c*-treatment of microsomes. They postulated that the type I binding site was closely associated with membrane phospholipids, which may be essential for the specific conformation of the binding sites. Moreover, they proposed that aniline could bind to both type I and type II sites,

causing the two spectra produced to cancel each other, thereby producing a diminished type II measurement. They found that removal of phosphatidylcholine and the proposed loss of type I binding increased the measurement of type II spectra.[14] As an alternative explanation, Chaplin and Mannering[14] suggested that the ligand state of the hemoprotein may be affected by its association in the membrane with phospholipid. Leibman and Estabrook[15] showed that extraction of microsomal phospholipids with isooctane caused the loss of type I binding and an enhancement of type II binding. They suggested that the form of cytochrome P-450 which binds type I substrates is dependent on the presence of phospholipid. Eling and DiAugustine[16] demonstrated a decrease in the benzphetamine (type I ligand)-induced spectral change following treatment *in vitro* of hepatic microsomes with phospholipase *c*. They attributed the decrease to a smaller pool size of substrate binding sites, and suggested that the type I spectral change was phospholipid dependent as opposed to the type II site, which appears not to require phosphatidylcholine. And finally, Strobel *et al.*[17] have shown that phosphatidylcholine is required for fatty acid, hydrocarbon, and drug hydroxylation in a reconstituted liver microsomal enzyme system.

In the present experiments, an examination of the effects of protein deficiency and phenobarbital induction on the phosphatidylcholine contents, while seemingly complex, nevertheless demonstrates a consistent and dependent relationship with substrate binding to the hemoprotein. Microsomal phosphatidylcholine (per milligram of microsomal protein) was increased in all dietary groups after PB administration. However, if phosphatidylcholine contents are expressed per nanomole of cytochrome P-450, very much reduced ratios were shown after phenobarbital treatment for all three groups. The decreased ratios associated with phenobarbital induction are due to greater levels of induction for cytochrome P-450 than for phosphatidylcholine. One means of viewing this relationship is to assume that type I substrate binding (and catalysis[13]) may occur either to a greater or lesser degree, depending on whether there is or is not the appropriate phosphatidylcholine association respectively. Chaplin and Mannering[14] showed, for example, that when type I binding of microsomes was eliminated with phospholipase *c* treatment, subsequent metabolism of ethylmorphine or aminopyrine was not completely eliminated but was retained—albeit with a higher K_m. These same investigators[14] proposed that the conformational changes of the cytochrome P-450 which occur in the presence of phosphatidylcholine may facilitate the flow of electrons within the drug–hemoprotein complex so as to enhance the reaction rate. This facilitated reaction rate (with the lower K_m) would presumably be the preferential rate *in vivo* where the phosphatidylcholine association remained intact; however, the alternate reaction rate was proposed to occur after disruption of this association. In other words, the extent of binding, which Schenkman *et al.*[13] suggested as indicative of the formation of the enzyme–substrate complex preceding catalysis, should depend upon the fraction of the total P-450 which is functionally associated with the phosphatidylcholine. Therefore, of the total cytochrome P-450 measured in our preparations (Tables 2 and 3), a smaller fraction may possess the requisite phosphatidylcholine association in the phenobarbital-induced groups. According to this viewpoint, this smaller fraction of phosphatidylcholine-associated P-450 should reduce the capacity of the total P-450 to bind at the type I site. This was evidenced in each group by the highly significant 65–71 per cent decreases in the ΔA_{max} per nanomole of cytochrome P-450 after phenobarbital induction. According

to this concept, comparisons of the values for ΔA_{max} per milligram of microsomal protein are not as informative of the mechanism associated with the capacity for substrate binding, since one must know not only the cytochrome P-450 content but also the proposed degree of association with phosphatidylcholine.

Protein deficiency did not significantly alter the phosphatidylcholine per milligram of microsomal protein, but did increase the ratio of phosphatidylcholine to cytochrome P-450, primarily as a result of a slight increase in phosphatidylcholine and a marked decrease in P-450. Similar to the above argument, this ratio increase was associated with a 43 per cent increase in the ΔA_{max} per nanomole of P-450 for ethylmorphine.

With a type II substrate such as aniline, a large increase in the ΔA_{max} per milligram of protein was observed after phenobarbital treatment in each dietary group. Guarino *et al.*[28] similarly found a 4- to 7-fold increase in the type II ΔA_{max} after PB administration. Both their data[28] and ours exhibited parallel increases of the ΔA_{max} and cytochrome P-450 concentrations. Since aniline is presumed to bind primarily to the iron at the CO-binding site of the heme,[13] and since phospholipid most likely is not significantly involved with this type II site, then all of the phenobarbital-induced P-450 molecules would appear to remain functional insofar as AN binding is concerned. In accordance with this suggestion, Guarino *et al.*[28] pointed out that phenobarbital may induce quantities of hemoprotein which are active in the binding of aniline but not in its metabolism.

Protein deficiency did not change the type II ΔA_{max} per nanomole of P-450 since, as noted above, such a spectral change should parallel P-450 content for type II compounds.

These studies suggest that the effects of dietary protein deficiency on microsomal enzyme substrate binding characteristics, *per se*, as well as the inducibility of that binding, are related to the relative contents of both cytochrome P-450 and phosphatidylcholine and are consistent with the proposed role of the latter[10–12] in modifying substrate binding.

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