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

EFFECT ON ENZYME KINETICS AND ELECTRON TRANSPORT SYSTEM*

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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 11–14 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. Apparent V_{max} and apparent K_m kinetic constants were determined for ethylmorphine and aniline. The V_{max} per milligram of microsomal protein was 64–66 per cent lower in the protein-deficient group. Equivalent reductions of the content of cytochrome P-450 and activities of cytochrome P-450 and *c* reductases were also observed. Phenobarbital induction increased specific enzyme activities (V_{max} per milligram of microsomal protein) in all groups with slightly greater percentage increases seen in the protein-deficient animals. Increases were also noted for the cytochrome P-450 content and cytochromes P-450 and *c* reductase activities. It was suggested that phosphatidylcholine and cytochrome P-450 both play important roles in the kinetics of metabolism determined after protein deficiency or phenobarbital induction, or both.

DIETARY variables that have been shown to affect the mixed-function oxidase system are: starvation;[1,2] ascorbic acid depletion;[3] dietary carbohydrate;[4,5] dietary lipid;[6,7] iron, riboflavin and iodide deficiencies;[8] and dietary protein.[9–12] Employing an enzyme kinetic approach, we have found that protein deficiency produces marked decreases in the apparent V_{max} values for ethylmorphine (EM) *N*-demethylation and aniline (AN) *p*-hydroxylation.[11] Also, the administration of drugs and exposure to foreign compounds are related to alterations of the microsomal mixed-function oxidase system; in turn, large segments of the world's population suffer from protein malnutrition. Significant interactions between dietary protein insufficiency and toxicological or pharmacological response are therefore readily apparent and prompted us to investigate the relationship between induction of the drug oxidase system and dietary protein deprivation.

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Phenobarbital, a broad spectrum inducer, was chosen as a representative inducer of the mixed-function oxidase system for these studies, since its inductive properties have been well documented.[13] Previous studies have indicated that induction by phenobarbital may be affected by dietary protein deficiency,[14,15] although this relationship has not been elucidated in terms of a mode of action at the enzyme level. Earlier studies from our laboratory have shown the effects of protein deficiency and phenobarbital induction on type I and type II substrate binding spectra produced by EM and AN respectively.[12] To further elucidate this relationship, we now report the effect on certain kinetic and electron transport properties characteristic of EM and AN microsomal metabolism.

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 in groups of four to five, in wire-bottomed, stainless-steel cages with no bedding, in a humidity controlled rat room with a 12-hr day–night lighting schedule. After feeding a 20% semipurified casein diet for 2 days, animals were randomized and subdivided into three groups. Group 1 was 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.* The 20% casein diet previously described by Weatherholtz *et al.*[16] was used. The 5% casein diet was kept isocaloric to the 20% casein diet by replacing casein with an equivalent amount of sucrose. Water was provided *ad lib.* throughout.

After 10 days of experimental feeding, the dietary groups were divided into experimental and control subgroups containing at least four animals each. On days 11–14, the experimental subgroup received i.p. injections of 80 mg/kg of sodium phenobarbital (PB) in 0·9% saline, while the control subgroup received 0·9% saline only.

Preparation of microsomes. Twenty-four hr after the last injections, the rats were sacrificed by decapitation, and the livers were excised, weighed and perfused with 0·9% saline and pooled for each subgroup. After weighing, all subsequent tissue manipulations were carried out at 0–5°. Livers were then finely minced and homogenized with six complete strokes in 2 vol. of 1·15% KCl containing 0·2 M Tris–HCl (pH 7·4) using a motor-driven Potter–Elvehjem Teflon–glass homogenizer at 600 rev/min. The same homogenizer was used throughout all experiments. The homogenate was centrifuged at 9000 *g* for 20 min in a Sorval refrigerated centrifuge. The supernatant was carefully decanted through glass wool and centrifuged at 105,000 *g* in a type 30 rotor for 1 hr using a Beckman L265B preparative ultracentrifuge. The microsomes were then floated off the glycogen pellet and resuspended in the same buffer to give a suspension containing the equivalent of 1 g liver/ml.

Determination of microsomal constituents. Microsomal protein was determined by the method of Lowry *et al.*[17] and cytochrome P-450 by the method of Omura and Sato.[18]

Enzyme assays. Aniline *p*-hydroxylase was determined essentially as described by Guarino *et al.*[19] and EM *N*-demethylase essentially as reported by Davies *et al.*[20] Incubations were carried out in open 25-ml Erlenmeyer flasks at 37° in a Dubnoff metabolic shaking incubator at a shaking rate of 120 oscillations/min. Each reaction mixture was comprised of 0·5 ml of microsomal suspension containing: 3·5 to 8·0 mg

protein; 1·5 ml of 400 mM Tris–HCl buffer (pH 7·4); 0·5 ml of the appropriate substrate solution; 1·0 ml of the NADPH-generating system;* and 1·5 ml of 1·15% KCl containing 5·0 mM $MgCl_2.6H_2O$. The concentrations of substrates in the final reaction mixtures were 0·01 to 1·5 mM for AN and 0·2 to 2·0 mM for EM. Within these ranges, five substrate concentrations, each run in duplicate, were used for the determinations of kinetic values. The amounts of product produced were determined spectrophotometrically as described previously.[19,20] Reaction rates for all three groups were linear with time and protein concentration. NADPH concentrations were provided in excess of requirements. The kinetic parameters were determined using a FORTRAN computer program devised by Cleland.[21]

Cytochrome *c* reductase and cytochrome P-450 reductase activities were determined essentially by the method of Gigon *et al.*,[22] except that 15 μl of 0·1M NADPH was added to the cuvettes in lieu of an NADPH-generating system. All spectrophotometric determinations were made with a Unicam SP-800 UV-Vis spectrophotometer using a Honeywell Electronik 19 external recorder.

Statistical analyses. Statistical significance was determined with Student's *t*-test.[23]

RESULTS

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

Shown in Table 1 are the effects of diet and PB induction on liver weights and liver contents of microsomal protein and cytochrome P-450.† The reductions of liver microsomal protein and cytochrome P-450 associated with protein deficiency are in agreement with the findings of others.[9,11,14] The same animals used to generate the data on the binding studies presented in the first paper[12] were also used to determine most of the enzyme kinetic parameters presented here and thereby permit more appropriate comparisons of catalysis with binding. These animals initially weighed 50–55 g and remained in the growth phase throughout the 14-day feeding period.

Table 2 shows the effect of diet and PB induction on the kinetic parameters‡ for EM *N*-demethylation. Any discussion of the interpretation of maximal velocities must take into consideration the unit of weight employed in the calculation. Most drugs and foreign compounds are metabolized by the liver microsomal enzyme system and these metabolic rates have often been correlated with activity *in vivo*.[13] Therefore, the V_{max} per 100 g of body weight should be the most appropriate basis insofar as relating

* The NADPH-generating system consisted of $NADP^+$ (2 mM), glucose 6-phosphate (50 mM), and Torula yeast glucose 6-phosphate dehydrogenase (2 E.U./ml).

† These data are portions of Tables 1 and 2 of the first paper in this series[12] and are presented again as a convenient reference for results shown here.

‡ Throughout these studies, we recognize that the V_{max} and K_m kinetic parameters are apparent values only, since the microsomal preparations used are multicomponent, multisubstrate, membrane bound, and poorly defined mechanistically.

TABLE 1. EFFECT OF DIETARY PROTEIN AND PHENOBARBITAL INDUCTION ON RAT LIVER WEIGHTS AND MICROSOMAL CONSTITUENTS*

	Group 1		Group 2		Group 3	
Dietary casein (%)	5	5	20	20	20	20
Treatment	Saline	PB	Saline	PB	Saline	PB
No. of experiments	6	6	6	6	6	6
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†
Microsomal 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†

* Animals fed for 15 days according to protocol presented in text. Phenobarbital (80 mg/kg) administered i.p. on days 11–14 in 0·9% saline. Control animals injected with 0·9% saline. Data represent four to seven animals per experimental subgroup (24–42 animals/experiment); pooled livers for each group, and two to three replications per assay. 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% level of probability show the same letter.

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

TABLE 2. EFFECT OF DIETARY PROTEIN AND PHENOBARBITAL INDUCTION ON ETHYLMORPHINE METABOLISM BY RAT LIVER MICROSOMES*

	Group 1		Group 2		Group 3	
Dietary casein (%)	5	5	20	20	20	20
Treatment	Saline	PB	Saline	PB	Saline	PB
V_{max} per†						
mg microsomal protein	404 ± 48[a]	1670 ± 320‡	1200 ± 70[b]	4730 ± 590‡	1110 ± 220[b]	2720 ± 310‡
nmole P-450 ($\times 10^{-3}$)§	2·52	3·09	2·93	2·57	2·36	2·19
100 g final body wt ($\times 10^{-3}$)	16·7 ± 3·2[a]	138 ± 31‡	67·0 ± 6·1[b]	432 ± 75‡	67·7 ± 9·4[b]	284 ± 56‡
K_m (mM)	0·12 ± 0·03[a]	0·19 ± 0·04‡	0·12 ± 0·02[a]	0·40 ± 0·07‡	0·11 ± 0·02[a]	0·29 ± 0·04‡

* Same as first footnote to Table 1.

† Nanomoles of formaldehyde produced per hr, expressed for each weight basis.

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

§ Calculated from V_{max}/milligram of microsomal protein divided by cytochrome P-450/milligram of microsomal protein.

liver microsomal metabolism to biological activity *in vivo* is concerned. Protein deficiency resulted in a 4-fold lower activity, in agreement with our previous findings.[11] About one-third of this reduction was caused by a decreased liver microsomal protein content (per 100 g of body weight) and was apparently associated with the attendant reduction in rate of liver cell proliferation.[24] The remaining two-thirds of the reduction was associated with a decrease of the specific enzyme activity (V_{max} per milligram of microsomal protein). This latter decrease may be associated with the 61 per cent loss of cytochrome P-450 content or the 64 per cent loss of cytochrome P-450 reductase activity, or with both, which are shown later (see Table 4). The V_{max} activities per nanomole of P-450 for both substrates were not appreciably different between groups 1 and 2, and support the impression that either P-450 activity (content?) or its rate of reduction is important in limiting the V_{max} per milligram of protein in the protein-deficient animals. Food restriction (group 2 vs group 3) reduced the V_{max} per 100 g of body weight only to a minimal extent.

PB induction resulted in a 4·2- to 8·3-fold increase in V_{max} per 100 g of body weight for EM (Table 2), with the greatest increase in the protein-deficient group. Approximately one-third of this increase could be accounted for by larger contents of liver microsomal protein per 100 g of body weight, with the remainder being due to increases in specific enzyme activities. In turn, the 2·5- to 4·1-fold increase in specific enzyme activity (V_{max} per milligram of microsomal protein) was related to a 2·6- to 4·5-fold increase in P-450 content and a 6·0- to 7·9-fold increase in P-450 reductase activity shown later (see Table 4). Clearly, P-450 content, as opposed to P-450 reductase activity, was better correlated with specific enzyme activity in these experiments.

Similar kinetic parameters for AN are shown in Table 3. Protein deficiency reduced V_{max} per 100 g of body weight by 3·8-fold, with approximately one-third of this reduction being due to a decrease in microsomal protein per 100 g of body weight and the remaining two-thirds to a decrease in specific enzyme activity. This latter 2·8-fold decrease was related to a 2·6-fold decrease in P-450 content and a 6·2-fold decrease in P-450 reductase activity. Restriction of food intake again showed only a very minor effect on V_{max} per 100 g of body weight. When V_{max} values for all six subgroups are expressed on a basis of P-450 content, it is interesting to note that there is little or no difference, leaving the impression that cytochrome P-450 is limiting insofar as enzyme activity per milligram of microsomal protein is concerned.

The most significant observation noted for the K_m parameters is that, for every group, these values were increased after administration of the inducer, 1·6- to 3-fold for EM and 2·3- to 8·7-fold for AN. On the other hand, diet treatment appeared to have little or no effect and appears to contrast with the earlier work of Mgbodile and Campbell.[11] However, the K_m concentration determined here for EM cannot be compared with that of the earlier study, since K_m is a function of substrate concentration* and different substrate concentrations were used for each study.

Presented in Table 4 are the results of the reductase activities. Protein deficiency decreased P-450 reductase by 64 per cent and *c* reductase by 37 per cent, whereas restriction of food intake increased activities by 47 and 72 per cent respectively. That is, the increases in reductase activities caused by food intake, consistent with the report

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

TABLE 3. EFFECT OF DIETARY PROTEIN AND PHENOBARBITAL INDUCTION ON ANILINE METABOLISM BY RAT LIVER MICROSOMES*

	Group 1		Group 2		Group 3	
Dietary casein (%)	5	5	20	20	20	20
Treatment	Saline	PB	Saline	PB	Saline	PB
V_{max} per†						
mg microsomal protein	8·59 ± 1·24[a]	30·3 ± 13·6	23·8 ± 1·8[b]	75·8 ± 8·9‡	21·1 ± 2·0[b]	52·4 ± 4·4‡
nmole P-450 × 10^{-3})§	53·7	56·1	58·0	41·2	44·9	42·3
100 g final body wt (× 10^{-3})	0·325 ± 0·035[a]	2·45 ± 1·10	1·24 ± 0·16[b]	6·91 ± 0·84‡	1·65 ± 0·24[b]	4·24 ± 0·75‡
K_m (mM)	0·03 ± 0·004[a]	0·26 ± 0·08‡	0·03 ± 0·002[a,b]	0·14 ± 0·01‡	0·06 ± 0·01[b]	0·14 ± 0·02‡

* Same as first footnote to Table 1.
† Nanomoles of *p*-aminophenol produced per hr, expressed for each weight basis.
‡ Statistically significant difference between PB-induced animals and their respective controls ($P < 0·05$).
§ Calculated from V_{max}/milligram of microsomal protein divided by cytochrome P-450/milligram of microsomal protein.

TABLE 4. EFFECT OF DIETARY PROTEIN AND PHENOBARBITAL INDUCTION ON CYTOCHROME *c* AND CYTOCHROME P-450 REDUCTASE ACTIVITIES*

	Group 1		Group 2		Group 3	
Dietary casein (%)	5	5	20	20	20	20
Treatment	Saline	PB	Saline	PB	Saline	PB
Cytochrome P-450 reductase†	6·1 ± 1·0[a]	37·9 ± 0·8‡	17·1 ± 3·3[b]	135 ± 39‡	11·6 ± 0·7[b]	69·9 ± 1·3‡
Cytochrome *c* reductase§	28·0 ± 1·1[a]	59·6 ± 2·3	44·5 ± 3·3[b]	93·4 ± 7·2‡	25·9 ± 1·2[a]	68·3 ± 2·4

* Same as first footnote to Table 1.
† Nanomoles of cytochrome P-450/milligram of microsomal protein/min ± S.E.M. for saline subgroups and ± range for PB subgroups.
‡ Statistically significant difference between PB-induced animals and their respective controls ($P < 0·05$).
§ Nanomoles of cytochrome *c* reduced/milligram of microsomal protein/min ± S.E.M.

of Kato,[25] who showed an increase of NADPH cytochrome *c* reductase by starvation for 72 hr, may be completely offset by protein deficiency. However, separate mechanisms could be involved.

Administration of PB increased the activities of P-450 reductase by 6·0- to 7·9-fold and *c* reductase by 2·1- to 2·6-fold, apparently independent of diet.

DISCUSSION

The interpretations most consistent with these data must take into account our previous results on the kinetics of binding of EM and AN to cytochrome P-450.[12] Using a similar experimental protocol of simultaneous protein deficiency and PB induction, we suggested that phosphatidylcholine played a significant role in the effects of these treatments on the EM binding to cytochrome P-450, whereas binding of AN remained solely a function of cytochrome P-450 content. After PB administration, cytochrome P-450 content was increased to a greater degree than was phosphatidylcholine, such that the proposed "phosphatidylcholine-associated P-450 sites" ($P\text{-}450_{PC}$) were diluted relative to the total P-450 sites ($P\text{-}450_T$). On the other hand, protein deficiency appeared to concentrate such $P\text{-}450_{PC}$ sites because of an enhancement in the ratio of phosphatidylcholine relative to the hemoprotein. This hypothesis was supported by the observation that the fraction of the proposed $P\text{-}450\text{-}_{PC}$ sites (relative to $P\text{-}450_T$) varied directly with the ΔA_{max} values for EM, which has been shown to depend on phosphatidylcholine functionality in microsomes.[26,27] For a type II compound such as AN, the measured ΔA_{max} was strictly a function of $P\text{-}450_T$ and appeared to be independent of phosphatidylcholine.

Chaplin and Mannering[27] found that treatment of microsomes with phospholipase *c* increased both the apparent K_m for EM *N*-demethylase and the type II spectral change by AN. They also showed that, although phospholipase *c* treatment essentially eliminated type I binding, a considerable percentage of the original catalytic activity was nevertheless retained. As one of their alternative interpretations of these findings, they suggested that two cytochromes P-450 may exist, only one of which is associated with phospholipid to form a type I site. In the absence of the phospholipid association, the resulting catalysis is characterized by the higher K_m. Also, these workers proposed that the increased type II spectral change apparently resulted from the loss of a certain amount of type I binding by AN, thereby reducing the cancellation of the true type II spectral changes caused by simultaneous type I binding.

Our data appear to be analogous to those of Chaplin and Mannering[27] in that PB induction increased both the K_m for EM reported here and the type II spectral change per milligram of microsomal protein shown previously.[12] That is, the analogy lies in the fact that PB induction resulted in a decrease in the concentration of $P\text{-}450_{PC}$ sites, resulting in a very marked decrease in the ΔA_{max} per nanomole of $P\text{-}450_T$ for EM reported in our earlier work; an increase in the ΔA_{max} for AN (per milligram of microsomal protein); and an increase in the K_m for EM *N*-demethylase. We previously suggested that the increase in the ΔA_{max} for AN was due to an increase in the concentration of $P\text{-}450_T$ and remains independent of phosphatidylcholine, since it is believed that AN binds to the iron at the CO-binding site of the heme.[28] Our data also showed an increase in the K_m for EM *N*-demethylase and are in agreement with a 19 per cent increase observed by Rubin *et al.*,[29] although they did not report significance. Our increases in the K_m for EM *N*-demethylase after PB induction are also in

agreement with Chaplin and Mannering,[27] when phosphatidylcholine involvement is assumed.

The most appropriate unit of weight for the expression of V_{max} activities should be considered with care and depends on the primary objectives of the study. Activity per 100 g of body weight is the best representative insofar as hepatic metabolism related to biological activity *in vivo* is concerned. Activities per gram of liver, per milligram of microsomal protein and per nanomole of cytochrome P-450 are useful when considered collectively toward interests in understanding mechanistic relationships.

V_{max} activities per milligram of microsomal protein, as related either to diet or to PB induction, were fairly well correlated with either cytochrome P-450 content, cytochrome *c* reductase activity and/or cytochrome P-450 reductase activity. However, considering all data collectively, none of these assays independently was advantageous in explaining alterations of specific enzyme activities.

The pharmacological and toxicological implications of understanding the simultaneous effects of protein deficiency and drug induction are clearly evident. Large segments of the world's population suffer from protein-calorie malnutrition and are nevertheless given drugs or exposed to foreign compounds at levels applicable to normal individuals. The resulting biological activities and tolerance limits for such chemicals are, unfortunately, too often based on data derived from normal, well nourished experimental animals.

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