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

EFFECT OF 3-METHYLCHOLANTHRENE INDUCTION ON ACTIVITY AND BINDING KINETICS*

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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 13 and 14 with either corn oil or 3-methylcholanthrene (3-MC) (20 mg/kg) in corn oil. Twenty-four hr after the last injection, animals were decapitated and liver microsomes were prepared. Contents of microsomal protein, phosphatidylcholine and hemoprotein (cytochrome P-450 or cytochrome P-448 or both) were measured and used as the basis of expression for spectral and enzyme kinetic constants; the activities of cytochrome c and P-450 reductases were also determined. Protein deficiency reduced microsomal protein and hemoprotein, but did not alter phosphatidylcholine (PC); 3-MC treatment produced increases in protein, hemoprotein and PC/mg of protein, but a decrease in PC/hemoprotein. In general, 3-MC produced greater percentage increases in the above parameters with protein deficiency, although the absolute amounts remained considerably below that achieved with groups 2 and 3. The 3-MC-induced groups showed the characteristic spectral shift for the P-450–CO complex to 448 nm. Ethylmorphine (EM) metabolism was decreased by both protein deficiency and 3-MC induction, whereas aniline (AN) metabolism was decreased by protein deficiency but increased by 3-MC treatment. Spectral binding (ΔA_{max}) of EM was decreased by protein deficiency and unchanged by 3-MC when expressed on a protein basis, but was reduced when expressed on a hemoprotein basis, indicating the loss of the type I site in the 3-MC-induced hemoprotein. Aniline binding, when based on protein, was decreased by protein deficiency but remained unchanged when expressed on a hemoprotein basis, whereas 3-MC treatment increased the ΔA_{max} when expressed on a protein basis, but did not change this parameter when expressed on a hemoprotein basis, indicating the retention of the type II site after 3-MC induction. Protein deficiency reduced both cytochrome c and P-450 reductase activities, whereas 3-MC treatment did not alter these parameters. Based on these and previous data, a role for the type I binding site as a "substrate effector site" was proposed.

THE ACTIVITY of the hepatic microsomal enzyme system is related in considerable degree to dietary or nutritional status, which influences the ability of an animal to respond to toxicological or pharmacological insult.[1–11] For example, in growing rats, dietary protein deficiency markedly reduces ethylmorphine and aniline metabolism, related in part to a reduction in the normal rate of liver cell proliferation and

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a concomitant reduction of microsomal protein (enzyme), and in part to an effect upon the specific microsomal enzyme activity.[7] This latter effect has been further characterized in studies designed to relate the kinetics of binding[10] and catalysis[11] for this enzyme system, and an important role for phosphatidylcholine has been suggested. The hypothesized role for phosphatidylcholine in the functionality of the terminal oxidase of the enzyme system for drug catalysis and binding, cytochrome P-450, is largely based on the differing capacities of protein-deficient and -sufficient animals to be induced with the broad spectrum inducer, phenobarbital (PB). In contrast, 3-methylcholanthrene (3-MC) is a classical narrow spectrum inducer[12] causing the appearance of a cytochrome exhibiting a reduced-CO complex peak at 448 nm for the spectrophotometric difference spectrum[13] and possessing a preferential catalysis for type II substrates[12, 14] such as aniline (AN), as well as polycyclic hydrocarbons such as benzpyrene.[15] In earlier studies of others, phosphatidylcholine was proposed to be involved in type I but not type II* substrate binding to cytochrome P-450.[16–18] Since the catalytic site of type II compounds may not be identical with the binding site(s) measured by the difference spectrum, [16] additional studies were undertaken to determine what effect dietary protein deficiency might have on microsomal enzyme inducibility with 3-MC. Studies of this sort provide fundamental information on the manner in which dietary protein insufficiency, which is prevalent in so large a segment of the world's population, modifies the biological activity of pharmacologically and toxicologically active chemicals. Almost always, toxicological data applied to nutritionally deficient human populations are derived from evaluations using nutritionally adequate experimental animals. Also, combined treatments of dietary protein insufficiency and administration of foreign compounds produce certain interactions on the microsomal mixed-function oxidase system that are quite informative of mechanistic relationships.

EXPERIMENTAL PROCEDURE

Animal treatment. Male, weanling (50–55 g) Sprague–Dawley-derived rats obtained from Flow Research Laboratories, Dublin, Va., were used. 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 for acclimatization, 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.* All groups received tap water *ad lib.* during the 15 days of feeding. The 20% casein diet previously described by Weatherholtz *et al.*[19] was used. The 5% casein diet was kept isocaloric to the 20% casein diet by replacing casein with an equivalent amount of sucrose.

After 12 days of experimental feeding, the dietary groups were divided into experimental and control subgroups containing at least seven animals each. On days 13 and 14, the experimental subgroups received intraperitoneal injections of 20 mg/kg of 3-MC in corn oil while the control subgroups received corn oil only.

* 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.

Preparation of microsomes. Twenty-four hr after the last injections, the rats were sacrificed by decapitation and the livers excised, weighed and perfused with 0·9% saline and pooled for each subgroup. Microsomes were prepared according to the method previously published.[10] The final suspension of microsomes contained the equivalent of 1 g liver/ml.

Determination of microsomal constituents. Microsomal protein was determined by the method of Lowry *et al.*[20] and cytochrome P-450 by the method of Omura and Sato.[21] An extinction coefficient of 91 $mM^{-1} \times cm^{-1}$ was used to calculate P-450 content[21] in both the control and induced subgroups, although it is recognized that the coefficient for the 3-MC-induced hemoprotein (cytochrome P-448) may be slightly different.[22] For general considerations, we will refer to the cytochrome component as the hemoprotein (HP) but will, however, denote it as either P-448 or P-450 where specificity is desired. Microsomal phosphatidylcholine was determined as choline content by the method of Ackerman and Salmon,[23] as modified by this laboratory.[10] Microsomal choline was used as an estimate of the phosphatidylcholine content, since Glaumann and Dallner [24] and Rogers [25] have independently shown that phosphatidylcholine is the major microsomal component containing the choline moiety.

Determination of binding constants. The spectral binding constants (K_s) and maximal spectral change (ΔA_{max}) were determined using spectrophotometric titrations essentially as described by Guarino *et al.*[26] Substrate concentrations ranging from 0·02 to 5·0 mM for EM and 0·15 to 1·5 mM for AN were successively added to the cuvette in minimal volumes. The change in absorbancy between 420 and 500 nm to compute the binding constants for EM and between 430 and 500 nm for the AN binding constants. The K_s and ΔA_{max} were calculated using a Fortran computer program devised by Cleland.[27]

Determination of kinetic constants. Aniline *p*-hydroxylase was determined essentially as described by Guarino *et al.*[26] and EM *N*-demethylase as reported by Davies *et al.*[28] as modified in this laboratory.[11]

Determination of cytochrome c and P-450 reductase. Cytochrome c reductase and cytochrome P-450 reductase activities were determined by a slightly modified method of Gigon *et al.*,[29] except that 15 μl of 0·1 M 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.

All statistical determinations were made using a Wang 600-14 programmable calculator employing the Wang statistical program based on Student's *t*-test for paired data.[30]

RESULTS

As noted previously,[10,11] 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.

TABLE 1. EFFECT OF DIETARY PROTEIN AND 3-MC INDUCTION ON FOOD CONSUMPTION AND GROWTH OF MALE RATS*

Rats	Dietary casein (%)	Treatment	No. of experiments	No. of animals per experiment	Food consumption (g)	Final body wt (g)	Liver wt g/100 g body wt
Group 1	5	Corn oil	7	8	61 ± 3^{x}	56 ± 2^{x}	$5{\cdot}6 \pm 0{\cdot}2^{x}$
	5	3-MC	7	8	62 ± 3	55 ± 2	$6{\cdot}6 \pm 0{\cdot}1$†
Group 2	20	Corn oil	7	8	61 ± 3^{x}	80 ± 3^{y}	$4{\cdot}7 \pm 0{\cdot}5^{y}$
	20	3-MC	7	8	61 ± 3	78 ± 2	$5{\cdot}3 \pm 0{\cdot}5$†
Group 3	20	Corn oil	7	7	122 ± 5^{y}	129 ± 6^{z}	$5{\cdot}9 \pm 0{\cdot}3^{x}$
	20	3-MC	7	7	125 ± 2	128 ± 5	$6{\cdot}4 \pm 0{\cdot}2$†

—* Animals were fed for 15 days according to the protocol presented in the text. Groups 1 and 2 were pair-fed; group 3 was fed *ad lib.* 3-MC (20 mg/kg) in corn oil was administered i.p. on days 13 and 14. Control animals were injected with corn oil on the same days. Data represent means ± S.E. Statistical significance between dietary treatments (subgroup controls) is indicated by lettered superscripts (*x*, *y*, *z*); data which are not significantly different ($P < 0{\cdot}05$) show the same symbol.

† Statistically significant difference between 3-MC-induced animals and their respective controls ($P < 0{\cdot}05$).

Shown in Table 1 are the effects of protein deficiency and 3-MC induction on food consumption and growth parameters for these rats. Both restriction of food intake (group 2 vs 3) and deficiency of protein (group 1 vs 2) significantly reduced the body weight gain, a finding which is consistent with our previous results.[10,11] In all three dietary groups, the administration of 3-MC significantly increased liver weight, in agreement with the reports of others.[31,32]

Table 2 shows the data for the microsomal contents of protein, hemoprotein (HP, P-450 and/or P-448) and phosphatidylcholine. Protein deficiency reduced microsomal protein by 39 per cent (group 1 vs 2) and HP by 75 per cent (group 1 vs 2), but resulted in a slight insignificant ($P > 0{\cdot}05$) decreased in the phosphatidylcholine content. Microsomal protein was only slightly higher (1·9 to 8·0 per cent) in the 3-MC-induced groups, whereas HP was increased by 102–200 per cent, both observations

TABLE 2. EFFECT OF DIETARY PROTEIN AND 3-MC INDUCTION ON RAT LIVER MICROSOMAL CONSTITUENTS*

Rats	Dietary casein (%)	Treatment	Microsomal protein (mg/g liver)	Cytochrome P-450 (nmoles/mg protein)	Choline (phosphatidylcholine) (μg/mg protein)	(μg/nmole P-450)
Group 1	5	Corn oil	$7{\cdot}0 \pm 0{\cdot}5^{x}$	$0{\cdot}12 \pm 0{\cdot}02^{x}$	$25{\cdot}8 \pm 1{\cdot}5^{x}$	$230{\cdot}7 \pm 45{\cdot}3^{x}$
	5	3-MC	$7{\cdot}5 \pm 0{\cdot}6$	$0{\cdot}48 \pm 0{\cdot}10$†	$45{\cdot}1 \pm 4{\cdot}9$†	$69{\cdot}2 \pm 9{\cdot}8$†
Group 2	20	Corn oil	$11{\cdot}4 \pm 0{\cdot}4^{y}$	$0{\cdot}48 \pm 0{\cdot}10^{y}$	$28{\cdot}8 \pm 3{\cdot}5^{x}$	$58{\cdot}9 \pm 19{\cdot}2^{y}$
	20	3-MC	$12{\cdot}3 \pm 0{\cdot}6$	$1{\cdot}03 \pm 0{\cdot}21$†	$54{\cdot}3 \pm 12{\cdot}4$†	$36{\cdot}9 \pm 9{\cdot}6$
Group 3	20	Corn oil	$12{\cdot}9 \pm 0{\cdot}8^{y}$	$0{\cdot}42 \pm 0{\cdot}04^{y}$	$30{\cdot}6 \pm 4{\cdot}8^{x}$	$63{\cdot}7 \pm 11{\cdot}3^{z}$
	20	3-MC	$13{\cdot}1 \pm 0{\cdot}2$	$0{\cdot}85 \pm 0{\cdot}15$†	$49{\cdot}0 \pm 11{\cdot}6$†	$47{\cdot}5 \pm 6{\cdot}6$†

* Assay methods are described in Experimental Procedure. Data represent means ± S.E. for a minimum of 4–7 experiments with 7–8 animals/experimental subgroup; pooled livers for each subgroup and 2–3 replicates/assay. Statistical significance between dietary treatments (subgroup controls) is indicated by lettered superscripts (*x*, *y*, *z*); data which are not significantly different ($P < 0{\cdot}05$) show the same symbol.

† Statistically significant difference between 3-MC-induced animals and their respective controls ($P < 0{\cdot}05$).

being consistent with previous reports.[31–33] For both constituents, treatment with 3-MC caused greater percentage increases in the protein-deficient group, but these increases did not, however, become as high as the induced levels seen in groups 2 and 3. Alvares *et al.*[34] observed a similar relationship when 130–150 g male rats fed a protein-free diet for 7 days and given 3-MC were compared to normal animals injected with 3-MC.

Dietary protein deficiency in group 1 caused a marked increase (260–290 per cent) in the ratio of phosphatidylcholine/HP when compared to either group 2 or 3; 3-MC administration, on the other hand, caused a highly significant reduction in this ratio. A similar phenomenon was also observed for PB induction.[10]

In the 3-MC-treated groups, the characteristic shift in the absorption maximum from 450 to 448 nm[13] was observed for the reduced hemoprotein–CO complex.

Metabolism kinetics are presented in Tables 3 and 4 for ethylmorphine *N*-demethylase and aniline hydroxylase activities respectively. The estimates for V_{max} and K_m are apparent values only, in that this system is membrane bound, multicomponent and multisubstrate, requiring NADPH, O_2 and an oxidizable substrate. The estimates for V_{max} are presented for several weight bases.

For EM *N*-demethylase (Table 3), the V_{max} ($\times$ mg microsomal protein^{-1}) is reduced both by protein deficiency and by 3-MC administration. The dietary protein effect is consistent with the results of our earlier studies,[11] as well as those of Alvares *et al.*;[34] in addition, the lower V_{max} obtained after 3-MC treatment agrees with similar responses noted for hexobarbital hydroxylation[31,33] and aminopyrine *N*-demethylation.[33] The decrease in the V_{max} ($\times$ mg microsomal protein^{-1}) is accounted for by the much lower V_{max} ($\times$ nmole HP^{-1}), which more than offsets the increase in the HP content seen after 3-MC administration. Although diet did not cause consistent changes in the K_m for EM, administration of 3-MC significantly increased this parameter for EM in all three groups, in contrast to the decreases of the K_m seen after 3-MC treatment for benzpyrene hydroxylation in four species[35] and hexobarbital hydroxylation in male and female rats.[33]

TABLE 3. EFFECT OF DIETARY PROTEIN AND 3-MC INDUCTION ON ETHYLMORPHINE N-DEMETHYLASE OF RAT LIVER MICROSOMES*

Rats	Dietary casein (%)	Treatment	V_{max} per† Microsomal protein (mg)	V_{max} per† nmole P-450 ($\times 10^{-3}$)	V_{max} per† 100 g final body wt ($\times 10^{3}$)	K_m (mM)
Group 1	5	Corn oil	345 ± 48^{x}	4·31 ± 1·16^{x}	12·9 ± 1·4^{x}	0·15 ± 0·02^{x}
	5	3-MC	285 ± 38‡	0·62 ± 0·11‡	13·6 ± 2·1	0·24 ± 0·03‡
Group 2	20	Corn oil	938 ± 42^{y}	3·39 ± 1·18^{x}	48·4 ± 7·6^{y}	0·16 ± 0·02^{x}
	20	3-MC	889 ± 52	1·18 ± 0·21‡	42·1 ± 7·3	0·29 ± 0·03‡
Group 3	20	Corn oil	898 ± 64^{y}	2·19 ± 0·28^{x}	65·2 ± 7·0^{y}	0·20 ± 0·03^{x}
	20	3-MC	743 ± 33	0·98 ± 0·25‡	61·5 ± 10·7	0·27 ± 0·03‡

* Assay methods are described in Experimental Procedure. Data represent means ± S.E. for a minimum of 7 experiments with 7–8 animals experimental subgroup; pooled livers for each subgroup and 2–3 replicates/assay. Statistical significance between dietary treatments (subgroup controls) is indicated by lettered superscripts (*x*, *y*, *z*); data which are not significantly different ($P < 0·05$) show the same symbol.

† Nmoles *p*-aminophenol produced/hr, expressed for each weight basis.

‡ Statistically significant difference between 3-MC-induced animals and their respective controls ($P < 0·05$).

TABLE 4. EFFECT OF DIETARY PROTEIN AND 3-MC INDUCTION ON ANILINE HYDROXYLASE OF RAT LIVER MICROSOMES*

Rats	Dietary casein (%)	Treatment	V_{max} per† Microsomal protein (mg)	V_{max} per† nmole P-450	V_{max} per† 100 g final body wt ($\times 10^3$)	K_m (mM)
Group 1	5	Corn oil	9·9 ± 1·1^x	100 ± 24^x	0·384 ± 0·044^x	0·028 ± 0·003^x
	5	3-MC	15·4 ± 1·2	32 ± 8‡	0·743 ± 0·091‡	0·027 ± 0·004
Group 2	20	Corn oil	20·0 ± 2·2^y	51 ± 13^y	1·098 ± 0·174^y	0·023 ± 0·001^x
	20	3-MC	31·9 ± 4·3‡	34 ± 6	2·030 ± 0·376‡	0·021 ± 0·003
Group 3	20	Corn oil	19·7 ± 2·7^y	46 ± 8^y	1·515 ± 0·272^z	0·028 ± 0·002^x
	20	3-MC	27·0 ± 2·7‡	37 ± 8	2·289 ± 0·263‡	0·025 ± 0·002

* Assay methods are described in Experimental Procedure. Data represent means ± S.E. for a minimum of 7 experiments with 7–8 animals/experimental subgroup; pooled livers for each subgroup and 2–3 replicates/assay. Statistical significance between dietary treatments (subgroup controls) is indicated by lettered superscripts (x, y, z); data which are not significantly different ($P < 0.05$) show the same symbol.

† Nmoles p-aminophenol produced/hr, expressed for each weight basis.

‡ Statistically significant difference between 3-MC-induced animals and their respective controls ($P < 0.05$).

The effects of diet and 3-MC on AN hydroxylation are shown in Table 4. As with EM N-demethylation, protein deficiency is associated with a marked reduction of the V_{max} ($\times$ mg microsomal protein^{-1}) and confirms our previous report[11] as well as like observations on aniline hydroxylase activities reported by Kato *et al.*[6] The lower V_{max} ($\times$ nmole HP^{-1}) found in the 3-MC treatment groups for AN hydroxylase is supported by similar findings of Gram *et al.*,[31] although Kato *et al.*[33] reported little or no change for male and female rats. The K_m for AN remained essentially unaltered by either dietary or 3-MC treatment. The absence of an effect on K_m contrasts with the finding of Kato *et al.*[33] that the K_m was increased two- to three-fold after 3-MC treatment.

TABLE 5. EFFECT OF DIETARY PROTEIN AND 3-MC INDUCTION ON ETHYLMORPHINE BINDING TO RAT LIVER MICROSOMES*

Rats	Dietary casein (%)	Treatment	K_s (mM)	ΔA_{max} per Microsomal protein ($\times 10^3$) (mg)	ΔA_{max} per nmole P-450 ($\times 10^3$)
Group 1	5	Corn oil	0·150 ± 0·016^x	2·90 ± 0·33^x	22·4 ± 4·2^x
	5	3-MC	0·147 ± 0·076	2·73 ± 0·86	3·2 ± 0·6†
Group 2	20	Corn oil	0·047 ± 0·011^y	7·09 ± 1·46^y	10·8 ± 1·2^y
	20	3-MC	0·059 ± 0·010	6·63 ± 0·69	4·9 ± 1·1†
Group 3	20	Corn oil	0·080 ± 0·009^z	7·86 ± 0·16^y	19·0 ± 3·4^{x,y}
	20	3-MC	0·091 ± 0·008	5·89 ± 0·64†	7·0 ± 1·6†

* Ethylmorphine was added directly to the microsomal suspension in the cuvette according to the procedure outlined in the Experimental Procedure. Data represent means ± S.E. for a minimum of 3 experiments; pooled livers for each subgroup and 2–3 replicates/assay. Statistical significance between dietary treatments (subgroup controls) is indicated by lettered superscripts (x, y, z); data which are not significantly different ($P < 0.05$) show the same symbol.

† Statistically significant difference between 3-MC-induced animals and their respective controls ($P < 0.05$).

TABLE 6. EFFECT OF DIETARY PROTEIN AND 3-MC INDUCTION ON ANILINE BINDING TO RAT LIVER MICROSOMES*

Rats	Dietary casein (%)	Treatment	K_s (mM)	ΔA_{max} per Microsomal protein ($\times 10^3$) (mg)	ΔA_{max} per nmole P-450 ($\times 10^3$)
Group 1	5	Corn oil	0·249 ± 0·109^x	1·64 ± 0·25^x	10·0 ± 1·9^x
	5	3-MC	0·121 ± 0·041†	9·18 ± 2·32†	11·3 ± 2·6
Group 2	20	Corn oil	0·216 ± 0·029^x	6·30 ± 0·23^y	11·4 ± 2·5^x
	20	3-MC	0·096 ± 0·014†	13·60 ± 3·7†	9·6 ± 2·8
Group 3	20	Corn oil	0·253 ± 0·020^x	6·28 ± 1·62^z	16·7 ± 7·6^x
	20	3-MC	0·107 ± 0·035†	12·60 ± 3·6†	18·7 ± 10·9

* Aniline–HCl was added directly to the microsomal suspension in the cuvette according to the procedure outlined in Experimental Procedure. Data represent means ± S.E. for a minimum of 3 experiments; pooled livers for each subgroup and 2–3 replicates/assay. Statistical significance between dietary treatments (subgroup controls) is indicated by lettered superscripts (x, y, z); data which are not significantly different ($P < 0·05$) show the same symbol.

† Statistically significant difference between 3-MC-induced animals and their respective controls ($P < 0·05$).

Shown in Tables 5 and 6 are the binding constants, ΔA_{max} and K_s, for EM and AN respectively. With reference to the ΔA_{max} ($\times$ nmole HP^{-1}) for EM, 3-MC treatment caused very large decreases, which were partially alleviated in the expression ΔA_{max} ($\times$ mg microsomal protein^{-1}). This alleviation is due to the increase in HP contents, which were quite substantial after 3-MC treatment (Table 2). Kato *et al.*[33] likewise found highly significant decreases in the ΔA_{max} ($\times$ nmole HP^{-1}) after 3-MC treatment of rats, when type I compounds such as hexobarbital and aminopyrine were used as the binding ligands. Also, Kutt *et al.*[36] found very large decreases after 3-MC treatment in the ΔA_{max} ($\times$ mg microsomal protein^{-1}) for phenobarbital (85 per cent) and benzphetamine (39 per cent). The estimates of ΔA_{max} ($\times$ nmole HP^{-1}) for AN were not significantly related either to dietary group or to 3-MC treatment. Since these values (ΔA_{max} $\times$ nmole HP^{-1}) were not different, the ΔA_{max} ($\times$ mg microsomal protein^{-1}) was essentially related to the HP content in each group, i.e. increased by 3-MC treatment and decreased by protein deficiency. Increases in ΔA_{max} ($\times$ mg microsomal protein^{-1}) for AN after 3-MC treatment have consistently been found by several other laboratories.[33,35,36]

In our experience, measurements of K_s for either substrate have been somewhat difficult to reproduce and are quite variable. However, in both this report and a previous one,[10] we found that protein deficiency increased the K_s for EM, whereas 3-MC treatment had no effect. On the other hand, protein deficiency had no significant effect on the K_s for AN binding, but 3-MC treatment reduced this parameter approximately 50 per cent in all three dietary groups. Other laboratories have reported a similar reduction of the K_s for AN after 3-MC treatment,[33,36] as well as for type I substrates such as benzphetamine,[36] hexobarbital[33] and phenobarbital.[36] In general, the decreases in the K_s for type II substrates are more consistent and more substantial than those for type I substrates.

In Table 7, reductase activities for the exogenous cytochrome c and endogenous HP are presented. As with our previous findings,[11] opposing dietary effects were

TABLE 7. EFFECT OF DIETARY PROTEIN AND 3-MC INDUCTION ON CYTOCHROME C AND CYTOCHROME P-450 REDUCTASE ACTIVITIES*

Rats	Dietary casein (%)	Treatment	Cytochrome c reductase†	Cytochrome P-450 reductase‡
Group 1	5	Corn oil	31·7 ± 1·4	7·2 ± 1·0
	5	3-MC	32·9 ± 2·6	6·9 ± 0·9
Group 2	20	Corn oil	55·3 ± 1·8	19·3 ± 1·2
	20	3-MC	56·3 ± 3·0	19·8 ± 1·5
Group 3	20	Corn oil	42·0 ± 0·7	16·5 ± 1·7
	20	3-MC	45·4 ± 3·2	17·9 ± 0·4

* Assay methods are described in Experimental Procedure.

† Nmoles cytochrome c reduced/mg microsomal protein/min ± S.E.; assays were run in duplicate.

‡ Nmoles cytochrome P-450 reduced/mg microsomal protein/min ± range; assays were run in duplicate.

observed, in that protein deficiency reduced (group 1 vs 2) but restricted food intake (quasi-starvation?) increased these enzyme activities. 3-MC treatment showed no effect. Similarly, others have not been able to show any appreciable effect of 3-MC treatment on cytochrome c or P-450 reductase activities.[37–39*]

DISCUSSION

Paramount in any consideration of the induction of the hepatic microsomal mixed-function oxidase activity by polycyclic hydrocarbons such as 3-MC is the altered activity resulting from the presence of cytochrome P-448, which is an aberrant form of cytochrome P-450 lacking the type I binding site [16] and possessing an altered substrate specificity. For example, there was a consistent decrease in the apparent V_{max} ($\times$ mg microsomal protein^{-1}) for EM *N*-demethylase after 3-MC administration in all dietary groups. This is most likely due to a partial replacement of cytochrome P-450 with cytochrome P-448. Another factor that may be partially responsible is the failure of type I substrates to cause stimulation of the rate of reduction of cytochrome P-448,[39*] as is the case for cytochrome P-450;[29,40] therefore, if, as several workers have shown,[41–43] the rate of reduction of cytochrome P-450 is rate limiting, the lack of stimulation would be expected to produce lower V_{max} values. The increased apparent K_m for EM observed after 3-MC treatment may be indicative of the altered substrate specificity of cytochrome P-448.[44–46] These apparent kinetic parameters most likely represent composite values, reflecting the K_m and V_{max} for both hemoprotein-containing systems. The net V_{max} ($\times$ nmole HP^{-1}) would be expected to be lower in the 3-MC-induced groups, due to the dual effects resulting from: (1) the partial replacement of cytochrome P-450 with the less active P-448, and (2) the lower rate of reduction of cytochrome P-448 *in vitro*.

After administration of PB or 3-MC, a decrease in the ratio of phosphatidylcholine per nmole of HP was observed, produced by a greater increase in the content of HP than in the content of phosphatidylcholine. The observed decrease in type I binding

* Reference 39 cited in reference 38.

after PB treatment may be due to a lower contribution of phosphatidylcholine to cytochrome P-450;[10] however, in the 3-MC-treated animals, the loss of type I binding may also be due to replacement of cytochrome P-450 with cytochrome P-448, which lacks the type I binding site.[16,47]

The very significant decrease in the ΔA_{max} ($\times$ nmole HP^{-1}) for EM after 3-MC treatment, combined with the observation that 3-MC administration does not produce significant alterations in the K_s, indicates that the remaining type I binding may be caused by residual cytochrome P-450. If cytochrome P-448 completely lacks the type I binding site, then only partial replacement of P-450 would be expected to depress significantly the ΔA_{max} but leave the K_s value unchanged. No attempt was made to determine the ratio of cytochrome P-448 to cytochrome P-450 in these studies, although it is assumed that this ratio would be relatively large. Jefcoate and Gaylor[48] have estimated, from electron paramagnetic resonance studies, that approximately 50 per cent of the microsomal hemoprotein from 3-MC-treated animals was cytochrome P-448 4 days after a single dose of 3-MC.

The administration of 3-MC enhanced AN metabolism when expressed as V_{max} ($\times$ mg microsomal $protein^{-1}$), but decreased metabolism when expressed as V_{max} ($\times$ nmole HP^{-1}), although these results are in apparent contrast to the partially purified system of Lu *et al.*[49] Clearly, the increase in the quantity of cytochrome P-448 more than offsets the loss of specific activity seen for P-448. As with EM, P-448 does not appear to metabolize AN as well as P-450, although the relative loss of activity due to the hemoprotein replacement is not as great for AN as for EM.

The alterations in AN binding are probably not affected by the altered phosphatidylcholine ratios, since these ratios were earlier shown not to affect appreciably P-450–type II substrate binding interactions.[10] Aniline binding was significantly increased in all dietary groups after 3-MC administration, as evidenced by the increase in the ΔA_{max} per mg of protein; however, when the ΔA_{max} was expressed per nmole of HP, no significant changes were evident. The K_s of AN binding is decreased in all dietary groups after 3-MC treatment, indicating an altered substrate specificity for binding of AN to cytochrome P-448. The binding of type II compounds to cytochrome P-448, characterized by the lower K_s, probably takes place at the iron of the heme moeity as it does with cytochrome P-450.[50]

Induction by 3-MC had no effect on the activity of the reductase for either cytochrome c or cytochrome P-450. Two alternative explanations might be considered. If the flavoprotein is rate-limiting in the control animals, then the production of additional HP would not increase its rate of reduction. Alternatively, the new cytochrome P-448 may have a reduced capacity to accept electrons from the flavoprotein, which offsets the increase in the quantity of P-448.

The effects of protein deficiency were very similar in this experiment to those found in our previous studies with PB; however, one difference found was the effect on the K_s for EM. In the PB studies, no alteration in the K_s for EM was found. On the other hand, the K_s for EM appeared to be increased by protein deficiency in the 3-MC studies. Perhaps this alteration in the 3-MC studies was due to the use of corn oil, which may contain some substance that interferes with this determination.

One relationship seen here and even more apparent in the previous studies with PB induction demonstrates that the "total quantity of binding sites" for EM, which is conventionally indicated by the ΔA_{max} ($\times$ nmole HP^{-1}), is inversely correlated

with the K_m for EM. This relationship is particularly impressive in the previous studies with PB, and reappears in these studies with 3-MC, although these latter 3-MC studies may be somewhat confounded because of the presence of P-448. This relationship suggests that the type I binding site may be better visualized as a "substrate effector site," which enhances the availability of the substrate to the catalytic site. The substrate may bind to the type I site, which enhances the approach of the substrate to the catalytic site; however, if the type I site were not available, the substrate would have to diffuse to the catalytic site through an environment that may sufficiently hinder the diffusion and decrease the overall rate of catalysis. This may, in part, explain why Liebman and Estabrook[17] found a 60 per cent reduction in catalysis after destroying the type I binding site by isooctane extraction. The substrate effector site would presumably depend on the presence and functionality of phosphatidylcholine. The saturation of this site would be indicated by the ΔA_{max} ($\times$ nmole HP^{-1}) and the occupation of these sites might enhance the rate of catalysis through a mechanism associated with a decrease in the K_m. The suggested role for this substrate effector site is in contrast to the facilitative function of the type I site as proposed by Chaplin and Mannering.[16] They suggested that conformational changes may take place upon substrate binding to the type I site, thereby facilitating the flow of electrons within the drug–HP complex and enhancing the overall reaction rate. On the other hand, Holtzman and Rumack[40] have suggested that substrate enhancement of reductase activity may result from binding of EM to HP sites completely different from the type I site.

The most significant aspect of these studies, designed to ascertain the interaction between dietary protein deprivation and induction of the microsomal mixed-function oxidase system, was that even though the microsomal enzyme system of protein-deprived animals can be induced, the induced activity never reached that of the protein-sufficient controls. As has been previously mentioned,[10] large segments of the world's population suffer from protein-calorie malnutrition and are, nevertheless, given drugs and/or exposed to foreign compounds at levels applicable to normal individuals. The biological activities and tolerance limits for such chemicals are, unfortunately, too often based on data derived from normal, well nourished experimental animals; therefore, care should be used in determining tolerance levels of drugs or toxic chemicals which are to be administered and/or exposed to populations with inadequate protein levels in their diet.

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