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A Requirement for Dietary Lipids for Induction of Cytochrome *P*-450 by Phenobarbitone in Rat Liver Microsomal Fraction

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1. Rats fed on purified synthetic diets have a markedly lower cytochrome *P*-450 concentration and hydroxylating enzyme activity in liver microsomal fraction than rats fed on stock pellets. 2. When both groups are treated with phenobarbitone the difference is even greater, the purified diet allowing only 50% of the cytochrome *P*-450 concentrations of controls. 3. Addition of herring oil, linoleic acid or 0.1% oxidized sitosterol to the diets allows induction of cytochrome *P*-450 to take place. 4. Addition of coconut oil to the diet does not permit induction of cytochrome *P*-450. 5. The interactions between dietary protein and the lipid substances are explored. 6. The mechanism of induction of microsomal hydroxylation enzymes by drugs is discussed in the light of these requirements.

It has been found that protein-deficient diets decrease the liver's ability to metabolize drugs (Kato, Chiesara & Vassanelli, 1962; McLean & McLean, 1966; Marshall & McLean, 1969*a*). We found that protein-deficient rats could still respond to exposure to phenobarbitone or DDT† by production of increased microsomal hydroxylating activity and increased cytochrome *P*-450. However, the new concentrations attained were less than one-third of those found in rats fed on stock diet under the same stimulus.

The present experiments concern the observation that, even when protein is added to purified diets, these diets still do not support the response to phenobarbitone. After preliminary experiments with extracts of stock pellets, we found that rats require not only dietary protein but also lipid substances for normal synthesis of microsomal cytochrome *P*-450 and hydroxylating enzymes. Preliminary reports of this nature have been published by Century & Horwitt (1968) and by ourselves (Marshall & McLean, 1969*b*; McLean & Marshall, 1970). Either linoleic acid or oxidized sterols (Brown, Miller & Miller, 1954) can fulfil this function of permitting a full response to the inducing effect of phenobarbitones.

The interrelations between these two major groups of dietary requirements for induction (protein and certain lipids) are explored in this paper.

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† Abbreviation: DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane.

MATERIALS AND METHODS

Rats. Male rats of a Wistar strain were bought (from A. Tuck and Son, Rayleigh, Essex, U.K.) and housed in mesh-floored cages. Food and water were available *ad libitum*, and the rats were weighed at the beginning, middle and end of feeding experiments.

Phenobarbitone. This was given as a solution containing 1 mg of sodium phenobarbitone/ml in water as the sole source of drinking water (Marshall & McLean, 1969*a*). The amount drunk was estimated from the disappearance of water from the bottles, and the rats took the water in amounts such that phenobarbitone dosage was 80–100 mg/kg body wt. per day on all diets except protein-free diets, where intake was in the region of 70 mg/kg body wt. per day.

Diets. Purified diets were made with materials purchased as previously described (McLean & McLean, 1966) except that the fat content was varied at the expense of starch; the content of α-tocopherol acetate in all diets was increased to 450 mg/kg of diet and vitamins A and D were added at 5000 and 1000 i.u./kg of diet respectively in an aqueous preparation (Decamin fortior aquosum; Ferrosan, Copenhagen, Denmark). The stock pellets were a modified diet 41B (Bruce & Parkes, 1956) (Oxoid Ltd., London S.E.1, U.K.).

Lipid factors. Coconut oil (1-pound Pigeon Brand tins; Hwa Hong Mfg Co., Singapore) was obtained from Bombay Emporium, Grafton Way, London W.C.1, U.K., and linoleic acid (75% purity, batch no. 45B1260) from Sigma (London) Chemical Co., London S.W.6, U.K., and herring oil was a gift from British Cod Liver Oils (Hull and Grimsby) Ltd., Marfleet, Hull, U.K.

Cholesterol and sitosterol (Sigma Chemical Co.) were oxidized and purified as described by Brown *et al.* (1954).

Chemicals. $NADP^+$ was bought from Boehringer Corp. (London) Ltd., London W.5, U.K. and other chemicals were bought from British Drug Houses Ltd., Poole, Dorset, U.K.

Tissue samples. Each rat was lightly anaesthetized with ether and exsanguinated from the carotid artery, and the liver was removed and weighed. A 2g portion was cut off and put into 18ml of buffered KCl (150mM-KCl in 25mM-tris–HCl buffer, pH 7.5 at 0°C). The liver was homogenized for 6s in a blender (Ultra-Turrax, TP 18/2; Janke und Kunkel, Staufen i. Br., Germany). The heavy particles were removed from the homogenate by centrifugation at $7000g_{max.}$ for 10min at 4°C.

The supernatant from this centrifugation was used for enzyme assays. For preparation of microsomal fraction the supernatant was given a further centrifugation at $104000g_{av.}$ for 1h and the resulting pellet resuspended in 150mM-KCl.

Assays. Cytochrome *P*-450 was measured by the method of Omura & Sato (1964) by using the forward-cell position of a Unicam SP.800 spectrophotometer and an external amplifier and recorder. Microsomal preparations were diluted in 100mM-KCl buffered with 25mM-NaH_2PO_4 adjusted to pH 7.4 with NaOH so that 1ml contained microsomal fraction from 50mg of liver or less, to minimize errors due to light-scattering.

Hydroxylation of aniline was measured by the method of Kato & Gillette (1965), with 5mM-aniline in the incubation medium previously described for measurement of amidopyrine demethylation (Marshall & McLean, 1969*a*).

RESULTS

Table 1 shows that a purified diet containing 20% casein and 5% olive oil, which supports near-maximal growth and reproduction, is not adequate for the induction of the cytochrome *P*-450 synthesis forced by phenobarbitone. A fat-free diet or one containing 15% coconut oil is similarly deficient. However, the addition of 5% linoleic acid instead of coconut oil allows near-maximal concentrations of cytochrome *P*-450 to be attained.

All these diets gave good growth during the 10 days for which they were given, and the intake of phenobarbitone in water did not alter to any appreciable extent.

Table 2 shows the separate and combined effects of feeding with a diet containing 20% casein and 15% of a highly unsaturated oil, herring oil, as compared with fat-free and protein-free diet. In the absence of phenobarbitone feeding with a no-fat–no-protein diet leads to low concentrations of cytochrome *P*-450. Both protein and fat are needed to bring concentrations up to those found in animals fed on stock diets. But neither casein nor herring oil is an inducer in the same sense as phenobarbitone. The amounts needed are large, and the quantities of cytochrome *P*-450 made are relatively small when no phenobarbitone is given.

When phenobarbitone is given the cytochrome *P*-450 concentrations increase. In the absence of protein and oil only small amounts are formed. Both casein and oil allow increased concentrations.

Table 1. *Effect of dietary fat on cytochrome* P-450 *concentrations in the liver of rats treated with phenobarbitone*

Male rats (weighing 140–180g) were fed for 10 days on stock pellets or else on purified diets containing various fats, prepared as described in the Materials and Methods section. All groups except the last were given a solution of 1mg of sodium phenobarbitone/ml to drink instead of water for the whole 10-day period. The results are given as means±S.D. with the numbers of determinations in parentheses.

Diet	Microsomal cytochrome *P*-450 (nmol/g of liver)
Stock pellets	113±18 (53)
20% Casein+5% olive oil	66±13 (13)
20% Casein, no fat	55±15 (39)
20% Casein+15% coconut oil	59±15 (5)
20% Casein+10% coconut oil+5% linoleic acid	103± 8 (5)
20% Casein+15% herring oil	123±19 (4)
Stock pellets (no phenobarbitone)	31± 4 (8)

Table 2. *Interaction of dietary protein and polyunsaturated fat (herring oil) on concentrations of cytochrome* P-450 *in the liver and on change of body weight, in rats with or without phenobarbitone treatment*

Male rats (initial body weight 110–130g) were fed on the diets for 10 days with four rats in each group. The phenobarbitone-treated groups received a solution of 1mg of sodium phenobarbitone/ml instead of drinking water. The oil used was herring oil. The results are given as means (±S.D.).

	Microsomal cytochrome *P*-450 (nmol/g of liver)		Mean change of body weight (g/day)	
Treatment ... Diet	Water	Phenobarbitone	Water	Phenobarbitone
Stock pellets	30±3	124± 7	+7.2	+7.3
No protein, no fat	8±1	24± 6	−1.0	−1.4
No protein+15% oil	13±1	53±17	−1.5	−1.4
20% Casein, no fat	18±3	60± 7	+6.0	+6.0
20% Casein+15% oil	24±7	123±19	+6.0	+5.1

Table 3. *Hydroxylation of aniline to* p-*aminophenol in liver of rats fed on fat-free or protein-free diets*

Rats were fed on diets as described in Table 2. The oil was herring oil. Aniline hydroxylation was measured as described in the Materials and Methods section. The results are given as means ± S.D.

	Aniline hydroxylation (μmol/h per g of liver)	
Treatment ... Diet	Water	Phenobarbitone
Stock	1.9 ± 0.1	5.2 ± 0.5
No protein, no fat	0.42 ± 0.01	1.1 ± 0.3
No protein + 15% oil	0.83 ± 0.23	2.3 ± 0.4
20% Casein, no fat	1.3 ± 0.2	4.1 ± 0.4
20% Casein + 15% oil	1.7 ± 0.1	5.7 ± 0.8

Table 4. *Liver weight as a percentage of body weight in rats fed on fat-free or protein-free diets*

Rats were fed on diets as described in Table 2, with and without herring oil. The results are given as means ± S.D. for four determinations.

	Liver wt. (% of body wt.)	
Treatment ... Diet	Water	Phenobarbitone
Stock	5.1 ± 0.1	7.0 ± 0.4
No protein, no fat	5.4 ± 0.2	5.7 ± 0.6
No protein + 15% oil	5.4 ± 0.2	6.5 ± 0.7
20% Casein, no fat	6.0 ± 0.1	7.3 ± 1.3
20% Casein + 15% oil	5.9 ± 0.3	6.5 ± 1.8

The combination of casein and oil gives normal synthesis of cytochrome *P*-450.

Simultaneous determinations of aniline hydroxylation (Table 3) showed that enzyme activity exhibited a similar requirement for both protein and unsaturated fat.

Liver weights showed some changes in that feeding with casein and phenobarbitone in the drinking water led to an increase in relative liver weight (Table 4). The herring oil has little effect on liver weight.

In other experiments the addition of benzo[*a*]-pyrene or DDT to phenobarbitone treatment did not permit full synthesis of cytochrome *P*-450 in rats fed on fat-free diets with adequate protein (Table 5), nor did addition of saturated fatty acids, such as lauric acid, palmitic acid or stearic acid (10% of diet), permit induction of cytochrome *P*-450. The non-essential fatty acid linolenic acid (2%) did, however, permit induction.

Table 6 shows that the addition of oxidized sitosterol allowed a considerable degree of induction of cytochrome *P*-450 by phenobarbitone while having little inducing effect on its own. Pure crystalline sitosterol had no effect of its own, nor did cholesterol, but addition of oxidized cholesterol to diets did permit induction with phenobarbitone in a similar way to oxidized sitosterol.

Table 5. *Effect of benzo*[a]*pyrene, DDT, and some saturated lipids on cytochrome* P-450 *concentrations in the liver of rats treated with phenobarbitone*

Male rats were fed on the diets for 10 days. All groups were given a solution of 1 mg of sodium phenobarbitone/ml to drink. Benzopyrene was dissolved in corn oil (10 mg/ml) and given as a single intraperitoneal dose of 20 mg/kg body weight, 4 days before death. DDT was dissolved in olive oil (100 mg/ml) and given as a single subcutaneous injection of 100 mg/kg body weight, 1 week before death of the animals. The results are given as means ± S.D.

Diet	Microsomal cytochrome *P*-450 (nmol/g of liver)
Stock pellets	131 ± 11 (4)
30% Casein + 5% olive oil	58 ± 11 (4)
30% Casein + 5% olive oil + benzo[*a*]pyrene	76 ± 6 (4)
20% Casein + 15% lauric acid	55 ± 8 (4)
20% Casein + 15% tripalmitin	59 ± 6 (4)
15% Casein, no fat	48 ± 2 (4)
15% Casein, no fat + DDT	46 ± 10 (4)

Table 6. *Effect of feeding diets containing oxidized sitosterol or cholesterol on the induction of microsomal cytochrome* P-450 *in rats by phenobarbitone*

Groups of four rats each were fed on the diets for 10 days. The diets, which contained no other fat, were prepared as described in the Materials and Methods section. Cholesterol was recrystallized from ethanol; sterols were oxidized as described in the Materials and Methods section. The results are given as means ± S.D.

	Microsomal cytochrome *P*-450 (nmol/g of liver)	
Treatment ... Diet	Water	Phenobarbitone
15% Casein	14 ± 1	66 ± 5
15% Casein + 0.5% sitosterol	18 ± 1	64 ± 18
15% Casein + 0.1% oxidized sitosterol	16 ± 3	95 ± 16
15% Casein + 1% recrystallized cholesterol	12 ± 2	44 ± 17
15% Casein + 0.5% oxidized cholesterol	16 ± 1	110 ± 15

DISCUSSION

It is clear that a purified diet, adequate for life and reproduction in the rat (20% casein + 5% olive oil), does not allow maximal induction of cytochrome *P*-450 by phenobarbitone. Two types of lipids that

permit induction are indicated by the present experiments, namely linoleic acid and oxidized sterols. These substances have a little inducing power on their own (Brown *et al.* 1954), but permit massive increases in the quantity of cytochrome *P*-450 induced by phenobarbitone. In this respect they are different from other inducers, such as DDT or benzopyrene, and we have called the effect a 'permissive' effect (Marshall & McLean, 1969*b*).

The lack of effect of coconut oil, 5% olive oil and saturated fatty acids suggests that it is an aspect of the polyunsaturated fatty acids that is responsible for the permissive effect. The linoleic acid preparation used is only 75% pure, and herring oil contains a mixture of highly unsaturated fatty acids. It is possible that it is either the degree of unsaturation or the liability to lipid peroxidation that makes these latter compounds active in permitting induction of cytochrome *P*-450.

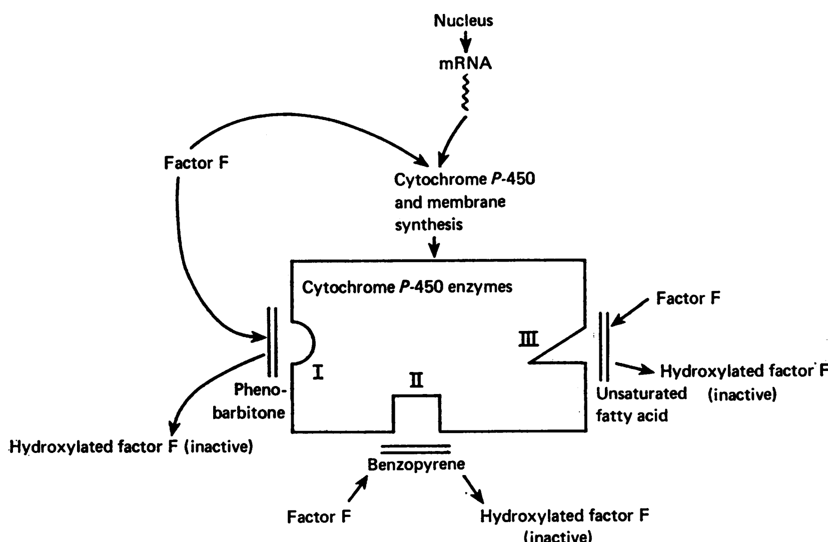
The induction of cytochrome *P*-450 by drugs involves DNA-dependent RNA synthesis and requires protein synthesis (Conney, 1967), but the intracellular site of action of the inducers is unknown. It seems improbable that all the 200 or so drugs cited by Conney (1967) should act on a DNA repressor site in the classical fashion (Bretscher, 1968; Nebert & Gelboin, 1970). It seems to us more likely that an endogenous factor is the final common pathway that causes increased synthesis of cytochrome *P*-450 (and related endoplasmic-reticulum components). The increased synthesis of the cytochrome *P*-450 system caused by inducers is mediated indirectly through such a factor (Factor F in Scheme 1). We suggest that the endogenous factor is normally inactivated at cytochrome *P*-450 sites.

Any substance capable of binding to the cytochrome *P*-450 sites (I, II or III in Scheme 1) either as a substrate or an inhibitor, could block the inactivation of endogenous factor. The increased amount of factor available would then result in increased production of cytochrome *P*-450 until a new steady state was achieved. This mechanism allows for the diversity of inducing drugs.

With such a model the unsaturated fatty acids or oxidized sterols must act as substrates at a different set of cytochrome *P*-450 sites (III) from those where phenobarbitone (I) or benzo[*a*]pyrene (II) act, and so provide a rational explanation for the observations that these unrelated lipids are not essential nutrients and do not have powerful inducing activity of their own, but permit massive accumulation of cytochrome *P*-450 in the liver when combined with phenobarbitone (Scheme 1).

The model implies that the endogenous factors were preferentially inactivated at a first 'phenobarbitone' site (I) and that blockage of a second type of site (III) by oxidized cholesterol on its own would have little effect. When the first site was occupied by phenobarbitone the endogenous factors could still be inactivated at the second site. But when both sites (I and III) were blocked, inactivation of endogenous factor would be markedly inhibited, and a powerful induction effect could then take place.

How much permissive substance is present in human and other natural diets is a matter for



Scheme 1. Model of induction of microsomal hydroxylation enzymes, describing the interaction between inducers such as phenobarbitone and permissive factors such as oxidized sterols.

conjecture, but may well have important consequences in terms of metabolism of drugs and environmental contaminants (McLean & McLean, 1969).

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