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THE EFFECT OF DIETARY LIPIDS ON 3, 4, BENZO[A]PYRENE METABOLISM IN THE HEPATIC ENDOPLASMIC RETICULUM

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Abstract—The effect of variations of the nature of the lipid content of the diet has been studied on the rate of formation of hydroxylated derivatives of benzo[a]pyrene in liver endoplasmic reticulum. Measurements were made by a fluorimetric determination of the hydroxylated products and by separation of radioactive products from labelled benzo[a]pyrene. Hydroxylation was minimal when a fat free diet was fed, little affected by addition of 10% lard (containing mainly saturated and monounsaturated fatty acids but only 6% linoleic acid) but increased to a significant extent by 10% corn oil (containing 50% linoleic acid). Addition of 10% herring oil (containing 15.4% polyunsaturated fatty acids but only 1.7% linoleic acid) to the diet did not increase the rate of hydroxylation. Methyl linoleate significantly increased the rate of hydroxylation, Vitamin E did not affect it and cholesterol was inhibitory by some tests.

These experiments demonstrate similarities and important differences in the responses of oxidative demethylation of drugs and hydroxylation of carcinogens to dietary lipids. Both processes are enhanced by a supply of dietary linoleic acid but cholesterol and Vitamin E which stimulate oxidative demethylation do not significantly affect hydroxylation of benzo[a]pyrene. These different requirements for dietary lipids may demonstrate that membranes of different structure are required for maximum activity of the two enzyme systems.

The quantity and composition of lipid in the diet play an important role in the regulation of the activity of the microsomal drug oxidation enzyme system and the concentration of cytochrome P450, both before and after induction with phenobarbitone. Polyunsaturated fatty acids, and expecially linoleic acid, sterols and vitamin E are essential in the diet for maximum activity of the hepatic drug metabolising enzyme systems [1–4].

The oxidative metabolism of carcinogenic polycyclic hydrocarbons, which is likely to be essential for the conversion of hydrocarbons such as benzo[a]pyrene to the active carcinogenic form [5–7], occurs in two stages. The first stage which is NADPH dependent, produces epoxides which are converted by an epoxide hydratase to diols. The formation of hydroxylated derivatives of hydrocarbons in the liver and lung but not the kidney is decreased by feeding a low protein diet. It is also influenced by dietary lipid but the actual factors in the dietary lipids responsible for the variation are uncertain [8].

Experiments were therefore designed to investigate the nature of the dietary lipid factors which influence the enzymes involved in hydrocarbon hydroxylation and establish whether these were the same or different from those which we had shown regulate the activity of oxidative drug metabolism [4].

MATERIALS AND METHODS

Rats. Male albino rats, 6–7 weeks old, weight 120–150 g were used in all experiments. Food and water were available *ad lib.*

Diets. Purified fat free diets were prepared according to Diplock *et al.* [9] and contained 25% casein (Unigate Ltd.), 30% sucrose, 20% wheat starch (Adcol Ltd.), 10% dried yeast (Bovril Ltd.), 10% lipid and 5% Cox's salt mix. The diet was supplemented with Rovimix AD_3 (Roche Ltd.), supplying 5,000 i.u. Vit. A and 1,000 i.u. Vit. D_3/kg diet.

Dietary lipid components. Corn oil (Mazola C.P.C.) or lard, free from antioxidants (Unigate Ltd.) or refined herring oil (Marfleet Refining Co., Marfleet, Hull) were added as the dietary lipid to give a final concentration of 10% lipid. The 10% lipid fraction of some diets was made up of 50% antioxidant free lard and 50% redistilled methyl linoleate (Koch Light Ltd.). Pure cholesterol (BDH, Analar) 2 g cholesterol/kg diet), purity determined by t.l.c. [10] and D-α-tocopherol (Sigma) (120 mg/kg diet) were added to some diets after mixing into the lard.

The iodine numbers of the dietary lipids were determined according to Wij's method [11], the mixed tocopherol content and the fatty acid composition of the dietary lipids by the methods described by Rowe and Wills [4] and the cholesterol concentration in the unsaponifiable matter of the dietary lipids by the method of Mann [12].

Preparation of microsomal fraction. Each rat was killed by cervical fracture and the liver blotted dry and weighed. A 5 g sample of each liver was finely chopped and homogenised for 1 min in 5 vol. of ice cold 125 mM KCl using a Potter–Elvehjem homogeniser. The cell debris, nuclei and mitochondria were removed by centrifuging for 25 min at 9,000 *g* av in a MSE 18 refrigerated centrifuge. The supernatant was removed and centrifuged at 100,000 *g* av in a

MSE 50 centrifuge. The pellet was washed and resuspended in 125 mM KCl (0.5 g liver/ml) to make a 50% microsomal fraction. Phenobarbitone induction of the microsomal aryl hydrocarbon hydroxylase activity after 21 days on special diets was achieved by giving animals 1 mg/ml sodium phenobarbitone in their drinking water [13].

Benzo[a]pyrene hydroxylation. The rate of hydroxylation benzo[a]pyrene which is dependent on epoxidation and hydration of the epoxide was assayed by the method of Nebert and Gelboin [14] and by a modified method of Waterfall and Sims [15]. The assay medium of the second method contained 0.6 mM NADPH, 1.25 mM $MgCl_2$, 0.05 M Na_2HPO_4/NaH_2PO_4, pH 7.4, 62.5 mM KCl and 20–30 mg microsomal protein or protein equivalent to 1 g liver, in a final vol. of 10 ml. The reaction was started by addition of 1 μmole benzo[a]pyrene in 0.5 ml acetone and 0.1 μmoles of radioactive benzo[a]pyrene (G-[^{3}H]benzo[a]pyrene), incubated for 30 min at 37° in a shaking water bath and was stopped by addition of 50 ml ice cold ethyl acetate. Ethyl acetate was added to the blank samples prior to incubation and all samples were shaken mechanically for 10 min. The ethyl acetate phase was removed, the aqueous phase re-extracted with a further 50 ml ethyl acetate and the combined extracts (100 ml) were dried over 15 g anhydrous sodium sulphate for 18 hr.

The ethyl acetate was evaporated under reduced pressure at 35° and the residue taken up in 0.5 ml ethyl acetate. The sample (0.3 ml) was spotted onto a 10 × 20 cm thin layer chromatographic plate spread with 0.2 mm silica gel, and developed in a solvent containing benzene–ethanol (9:1 v/v). The products were located and examined under an ultra-violet lamp, wave length 350 nm, and the fluorescent bands were marked.

Sections of silica gel were scraped off into scintillation vials and 10 ml 0.4% BBOT in toluene added, shaken well and the radioactivity measured by counting on a Packard Tricarb liquid scintillation spectrometer. All the samples were corrected with an external normalisation standard at 100% efficiency because the quenching caused by various coloured components of the benzo[a]pyrene metabolites was unknown. Specific activity of the radioactive benzo[a]pyrene was determined and the proportion of the total quantity of benzo[a]pyrene converted to each metabolite was calculated. The quantities of metabolites formed by non-enzymic oxidation were allowed for and the results expressed as pmoles benzo[a]pyrene converted to each metabolite/mg protein/30 min.

Experimental plan. Each diet was fed to a group of 16 rats for 21 days after which phenobarbitone was incorporated into the drinking water so that each animal received 10 mg/100 g body weight/day. Four animals were killed on each of the days 7, 9, 11 and 14 after commencing the phenobarbitone treatment so that the total period of feeding special diets ranged from 28 to 35 days.

RESULTS

Diet analysis. The fatty acid composition of the lard, corn oil and herring oil are shown in Table 1 and the relative quantities of vitamin E and cholesterol are shown in Table 2.

Table 1. Fatty acids analysis of dietary lipids

Fatty acids	% Total fatty acid		
	Lard	Corn oil	Herring oil
C 14:0	—	1.1	4.3
C 16:0	31.4	12.9	12.6
C 16:1	2.4	—	5.8
C 18:0	12.6	2.4	2.1
C 18:1	46.3	33.0	12.6
C 18:2	5.9	49.9	1.7
C 18:3	—	—	1.2
C 18:4	—	—	5.4
C 20:1	—	—	15.8
C 22:1	—	—	21.0
C 22:5	—	—	2.4
C 22:6	—	—	6.4
Minor constituents unidentified	1.4	0.5	8.7

Benzo[a]pyrene metabolism. Six metabolites were formed from benzo[a]pyrene after incubation with the microsomal fraction. The R_f values of the products and their fluorescent properties in u.v. light before and after exposure to ammonia are shown in Table 3. Metabolite 1 was identified as 3-hydroxybenzo[a]pyrene by use of an authentic standard and the major part of the radioactivity, 50–60%, was also found in the fraction, but the other 5 metabolites were not clearly identified because no authentic standards were available. However, by comparison of the R_f values and the fluorescent properties of the metabolites with those described by Waterfall and Sims [15] and Sims [16], we considered that metabolite 2 was likely to be 7, 8-dihydro-7, 8-dihydroxy benzo[a]pyrene, metabolite 3 was likely to be 9, 10-dihydro-9, 10-dihydroxy benzo[a]pyrene and metabolite 5 was likely to be 1,2-dihydro-1,2-dihydroxy benzo[a]pyrene.

Effects of feeding diets containing no fat, lard, corn oil or herring oil on benzo[a]pyrene hydroxylation. Changes in the lipid constitution of the diet caused significant changes in the quantity of 3 hydroxy benzo[a]pyrene whether determined fluorimetrically or measured by the formation of total radioactive metabolites or metabolites 1, 4, 5 and 6. No significant

Table 2. Vitamin E and cholesterol content of diets

Diet	Vitamin E content mg/kg diet	Cholesterol content mg/kg diet
Fat free	—	—
10% Lard	2.25	90
10% Corn oil	101.0	—
10% Herring oil	11.9	745
5% Lard + 5% methyl linoleate	1.15	45
10% Lard + 0.2% cholesterol	2.25	2090
10% Lard + vit E*	120.0	90

* 120 mg D-α-tocopherol/100 g lard or /kg diet.

Table 3. Analysis of metabolites of benzo[a]pyrene by thin layer chromatography

Compound	R_f in benzene–ethanol (9:1 v/v)	Fluorescence in u.v. light	
		Immediate	After exposure to ammonia
Benzo[a]pyrene	0.76	Violet	Violet
Metabolite (1) 3-hydroxybenzo[a]-pyrene	0.60	Violet	Green
Metabolite (2)	0.54	Blue/Violet	Violet
Metabolite (3)	0.44	Violet/Blue	Violet
Metabolite (4)	0.36	Violet	Violet
Metabolite (5)	0.29	Violet	Violet
Metabolite (6)	0.21	Blue	Blue

The solvent system was benzene–ethanol (9:1 v/v) stationary phase silica gel G.

changes were observed in metabolites 2 and 3 after feeding each of the diets used. Feeding a fat free diet caused the formation of a smaller quantity of total metabolites and of each separated metabolite, than each of the fat containing diets tested (Table 4). Metabolite 5 which was formed to a much smaller extent after feeding a fat free diet than after feeding any other diet may be of special interest (Table 4).

If 10% lard (containing 5.9% linoleic acid) was added to the diet, the formation of the total quantity of hydroxylated products of benzo[a]pyrene was increased to a small and not significant extent over that observed after feeding a fat free diet but much greater quantities of metabolites 5 and 6 were produced. Feeding a diet containing 10% corn oil caused large and significant increases in 3 hydroxy benzo[a]pyrene determined spectrofluorimetrically and in the total metabolites and metabolites 1, 4, 5 and 6, determined by the radioactive method and separated by t.l.c. (Table 4). After feeding a herring oil diet (containing 55.2% monoethenoid fatty acids but also 15.4% polyethenoid fatty acids), the formation of hydroxylated products of benzo[a]pyrene was not significantly different from that observed after feeding a fat free or lard diet (Table 4).

These experiments indicated that dietary linoleic acid was likely to be important for the metabolism of benzo[a]pyrene in the liver and this was tested by addition of 5% methyl linoleate to 5% lard diet. This additive caused the oxidation of benzo[a]pyrene as measured by the rate of formation of metabolite 1 and by total radioactive metabolites to increase to values similar to those obtained after feeding a diet containing 10% corn oil. Measurements of the rate

Table 4. The effect of dietary lipids on the formation of hydroxylated derivatives of benzo[a]pyrene

Diet	Radioactivity measurement (pmoles benzo[a]pyrene converted to each metabolite/mg protein/30 min)							Spectrofluorimetric determinations (pmoles 3-hydroxybenzo [a]pyrene formed/ mg protein/30 min)
	Total Metabs.	Metab. 1	Metab. 2	Metab. 3	Metab. 4	Metab. 5	Metab. 6	
Fat free	8397 ± 820	4359 ± 876	1875 ± 250	733 ± 137	923 ± 294	196 ± 74	311 ± 60	6486 ± 287
10% Lard	9716 ± 923 NS	4798 ± 380 NS	1500 ± 240 NS	1128 ± 286 NS	1185 ± 94 NS	1105 ± 151 P < 0.001	499 ± 58 P < 0.05	6682 ± 254 NS
10% Corn oil	12480 ± 1135 P < 0.01	7030 ± 663 P < 0.02	NS	NS	1635 ± 149 P < 0.05	665 ± 105 P < 0.001	575 ± 56 P < 0.01	9353 ± 501 P < 0.01
10% Herring oil	8782 ± 1730 NS	NS	NS	NS	NS	837 ± 125 P < 0.001	689 ± 102 P < 0.01	6992 ± 309 NS
5% Lard + 5% methyl linoleate	11950 ± 1904 NS	6955 ± 721 P < 0.02	NS	NS	NS	664 ± 138 P < 0.05	NS	8084 ± 587 P < 0.05
10% Lard + vit. E*	9403 ± 2004 NS	NS	NS	NS	NS	342 ± 93 P < 0.001	NS	7149 ± 230 NS
10% Lard + 0.2% cholesterol	6997 ± 770 P < 0.05	NS	NS	NS	NS	148 ± 62 P < 0.001	NS	7258 ± 396 NS

Results are expressed as the mean ± SEM s of 16 animals. Significance tests for 10% lard, herring oil and corn oil diets related to values obtained for fat free diet. Significance tests for lard and methyl linoleate, lard and Vit. E and lard and cholesterol related to values obtained after feeding 10% lard diets. P values >0.05 classed as NS.

of formation of 3 hydroxy benzo[a]pyrene were greater than those after feeding lard but of much smaller significance (Table 4).

Effect of dietary cholesterol. The failure of the herring oil diet to stimulate oxidative metabolism of benzo[a]pyrene may have been due to the high cholesterol content of the oil, which raised the cholesterol content of the diet to 745 mg/kg (Table 2).

The effect of dietary cholesterol was studied by supplementing the 10% lard diet with 2 g cholesterol/kg diet. This addition decreased the rate of formation of metabolite 1 to a small and not significant extent but greatly depressed the formation of metabolite 5 (Table 4). Total metabolites determined by each method were not significantly different from results obtained after feeding a lard diet.

Effect of dietary vitamin E. Feeding a 10% corn oil diet containing 100 mg vitamin E/kg diet resulted in a higher rate of hydroxylation of benzo[a]pyrene than after feeding a diet containing 10% lard containing only 2.3 mg vitamin E/kg diet (Table 4). However, supplementation of the 10% lard diet with 120 mg. D-α-Tocopherol/kg diet caused no significant changes in the rate of hydroxylation of the benzo[a]pyrene as measured by the total metabolites formed and spectrofluorimetrically (Table 4). These results indicate that Vitamin E was not responsible for the high rate of hydroxylation of benzo[a]pyrene observed after feeding a diet containing 10% corn oil and that this effect was much more likely to be caused by the supply of a high concentration of linoleic acid provided by the corn oil (Table 1).

DISCUSSION

The oxidative metabolism of benzo[a]pyrene is catalysed by two enzyme systems, firstly, an NADPH dependent system which catalyses epoxidation and secondly an epoxide hydratase which converts the epoxides to diols. In this investigation we have only studied the end products of the process, the hydroxylated derivatives of benzo[a]pyrene and the effects of the diets tested may have been mediated by changes in the activity of the enzyme system involved in epoxidation, in the epoxide hydratase or both. In view of the fact that it has been demonstrated [4] that dietary lipids affect the oxidative metabolism of drugs it would appear that it is more likely that effects observed are a result of changes in activity of the epoxidation system. There is a clear requirement for lipid in the diet for a maximum rate of hydroxylation in benzo[a]pyrene because the enzyme activity was lower after feeding a fat free diet than after feeding a diet containing any fat tested. Although the very large increases in metabolite 5 caused by feeding lipid containing diets may be very important it is difficult to be certain about the significance of the finding because, as explained in the Results section, its precise identification was not established. If it is 1, 2 dihydro, 1-2 dihydroxy benzo[a]pyrene, as suggested in the Results section, then changes in the composition of the membrane lipids, caused by dietary change may alter the specificity of the enzyme system so that benzo[a]pyrene is preferably oxidised to form this product (Table 4). Inclusion of polyunsaturated fatty acids in the diet is mainly responsible for the increased rate of benzo[a]pyrene oxidation when fats are added to the diet. This view is supported by the fact that when the concentration of linoleic acid in the diet was substantially increased by incorporating corn oil or methyl linoleate it caused a very marked increase in the rate of benzo[a]pyrene oxidation (Table 4).

The constitution of dietary lipids has a profound effect on the fatty acid composition of the phospholipids of the microsomal membrane [2, 3], and incorporation of linoleic acid into the phospholipids of the hepatic endoplasmic reticulum appears to be of vital importance in maintaining the maximum activity of the membrane dependent enzyme system which catalyses oxidative metabolism of the carcinogen benzo[a]pyrene as well as being essential for the oxidative drug metabolising enzyme system. Phospholipids containing linoleic acid in the fatty acyl chains may be essential for the formation of membranes of correct structure and configuration for support of drug and carcinogen metabolising enzymes, or alternatively, the active sites of these enzymes may be altered by the presence of linoleic acid in the phospholipids of the endoplasmic reticulum so that, as a result, the enzyme activity is increased.

In addition to supplying linoleic acid, corn oil provides a good source of natural vitamin E while lard supplies a minimal quantity of the vitamin. Vitamin E in the diet is essential for maximum activity of oxidative drug metabolism, expecially aminopyrine *N*-demethylation [4] and is believed to increase the stability of the microsomal membrane lipids and maintain the structural configuration of the membranes [17].

Supplementation of the lard diet with 120 mg D-α-tocopherol/kg diet did not, however, increase the rate of hydroxylation of benzo[a]pyrene above that found after feeding a diet containing lard. Therefore oxidation of benzo[a]pyrene may require a less stable membrane for activity than aminopyrine demethylase or the structure of the membrane required for benzo[a]pyrene hydroxylation may differ from that which is required for aminopyrine demethylation in its vitamin E content.

A diet containing 10% herring oil strongly enhances oxidative demethylation of drugs in the endoplasmic reticulum compared with a lard or fat free diet [4], but feeding this diet resulted in a rate of hydroxylation of benzo[a]pyrene which was similar to that after feeding a diet containing lard (Table 4). Herring oil contains a very low concentration (1.7%) of diene (Table 1) and although this may be responsible for its failure to enhance hydroxylation of benzo[a]pyrene the large concentrations of highly unsaturated fatty acid in the oil appear to compensate for the linoleic acid which is required for oxidative demethylation [4].

Herring oil also contains a high concentration of cholesterol (Table 2) and we have already shown* that the addition of cholesterol to a lard diet stimulates the activity of the enzyme system involved in oxidative drug metabolism. However, supplementation of the lard diet with cholesterol did not increase the rate of hydroxylation of benzo[a]pyrene and by some criteria reduced the rate (Table 4). The

* L. Lambert and E. D. Wills, unpublished observations.

low rate of hydroxylation of benzo[a]pyrene activity observed after feeding a diet containing 10% herring oil may therefore be caused by the presence of high concentrations of cholesterol in the herring oil (Table 2). This could imply that the membrane with a high concentration of cholesterol is of unsuitable configuration for the support of the oxidation of cyclic hydrocarbons, or cholesterol could act as a substrate analogue for benzo[a]pyrene, preferably undergoing oxidative metabolism at the expense of the carcinogen. The oxidative metabolism of drugs such as aminopyrine and oxidative metabolism of benzo[a]pyrene on the liver endoplasmic reticulum both require a supply of dietary linoleic acid for maximum activity but Vitamin E which enhances oxidative drug demethylation has no effect on benzo[a]pyrene metabolism and cholesterol which also enhances oxidative drug metabolism has a weak inhibitory effect on benzo[a]pyrene oxidation.

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