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Dietary Induction of Hepatic Microsomal Enzymes by Thermally Oxidized Fats

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Functional changes associated with the hepatomegaly commonly observed upon feeding thermally oxidized (TO) fats were investigated. Rats were fed purified diets in which the fat consisted of fresh corn oil, TO oil, or the proportional amount of non-urea-adduct-forming material (NUAF) from TO oil plus fresh oil. Increases in relative liver weights and the concentrations of microsomal protein and endogenous malondialdehyde were observed when TO oil or NUAF plus fresh oil were fed rather than pure fresh oil with two types of dietary protein, casein and soy. Both the basal and DDT-induced mixed function oxidase activities were higher in animals fed TO oil and NUAF than in those given fresh oil. The TO oil also increased cytochrome P-450 and the activity of *S*-adenosylmethionine:phosphatidylethanolamine methyltransferase whereas the NUAF did not. Oxidized fat thus appears to stimulate SER proliferation and induce a complex of microsomal enzymes.

Certain natural food constituents such as fats are subject to changes during storage, processing, or cooking. Most cooking oils marketed today are rich in polyunsaturated fats which are highly susceptible to peroxidation. Thus, the heating of such cooking oils at high temperatures and in the presence of oxygen, as in deep fat frying, results in their oxidative deterioration. For example, Steibert and Koj (1973) found that, in an industrial scale deep fat frying of meat using rapeseed oil, the resulting fried meat products contained 0.63–1.1% of the nonvolatile oxidation products of the fatty acids taken up from the heated cooking oil. Similarly, Kurkela and Karjalainen (1973) have observed that French fried potatoes absorb some of the cooking fat including its high molecular weight secondary oxidation products. Peroxidation reactions *in vivo*, as well as the accumulation of peroxidation products, like polymerized fatty acids, produce varying degrees of cellular damage (Tappel, 1973).

The prolonged laboratory heating of fats in the presence of air, to simulate the conditions of deep fat frying, has been reported to cause appetite and growth depression, diarrhea, histological changes in various tissues, kidney and liver enlargement, and even death in some cases when fed to experimental animals (Binet and Wellers, 1966; Nolen *et al.*, 1967; Ohfuji and Kaneda, 1973; Kajimoto and Yoshida, 1973). Liver enlargement is frequently accompanied by an increased activity of microsomal enzymes resulting from proliferation of the smooth endoplasmic reticulum (SER). Most toxicological investigations on the effects of thermally oxidized fats have been quite general with little consideration of events at the molecular level. In this present study, the functional changes associated with the hepatomegaly commonly observed upon feeding a thermally oxidized fat and the non-urea-adduct-forming fatty acids contained therein were investigated. The investigation specifically considered the possi-

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Table I. Effects of Protein Type and Thermally Oxidized Fat in the Diet on Indices of Hepatic Microsomal Monooxygenase and Methyltransferase Activities[a]

Treatment	Total feed consumed, g	Body wt, g	Rel liver wt, g/100 g body wt	EPN[c] oxidn, μg/ 50 mg of liver per hr	PNA[c] O-demeth, μg/ 50 mg of liver per hr	Microsomal protein, mg/g of liver	Cyto. P-450,[b] ΔOD × 10^2 per mg of protein	Methyltransferase,[d] cpm × 10^{-4}/ mg of micr. protein/hr
Casein diets								
Corn oil	186	161	4.27	0.55	1.24	27.27	0.912	2.816
TO oil	198	171	4.65	0.73	1.43	31.21	1.060	4.052
NUAF in corn oil	191	171	4.58	0.85	1.75	32.12	1.230	3.692
Corn oil + DDT	197	174	4.41	1.00	2.03	28.03	1.912	3.120
TO oil + DDT	202	177	5.12	1.58	2.41	30.75	2.138	3.548
NUAF in corn oil + DDT	203	176	4.79	1.80	2.65	38.84	1.700	3.212
Soy protein								
Corn oil	193	162	3.93	0.83	1.82	28.07	1.100	3.956
TO oil	187	158	4.80	1.02	1.84	40.66	1.276	4.448
NUAF in corn oil	199	169	4.49	0.88	2.02	38.06	1.348	3.748
Corn oil + DDT	185	162	4.40	1.92	2.78	29.88	2.406	4.936
TO oil + DDT	185	166	4.92	2.53	3.46	34.02	2.892	4.860
NUAF in corn oil + DDT	194	168	5.00	2.46	3.43	36.85	2.310	4.528

[a] The experiment compared the capacity of casein to soy protein at levels of 20% in a purified isocaloric diet to sustain normal and induced hepatic microsomal enzyme activities. Fresh corn oil, thermally oxidized corn oil, and the non-urea-adducting fatty acid fraction of thermally oxidized corn oil were critical covariables. *p,p'*-DDT was used as an enzyme inducer (50 ppm in the diet). The feeding period was 15 days. All tests were conducted on the day of killing except microsomal measurements made the following day. [b] Measured as the 490–450 nm absorbancy change for the difference between reduced microsomes in the reference cell and reduced microsomes plus CO in the sample cell. [c] Activity expressed as μg of *p*-nitrophenol liberated per 50 mg of liver (equivalent) per hour using 10,000*g* supernatant of liver homogenate. [d] *S*-Adenosylmethionine:phosphatidylethanolamine (PE) methyltransferase assayed as conversion of [methyl-^{3}H]SAM to the butanol-soluble form (methylated PE).

bility of altered xenobiotic metabolism by rats fed such materials.

EXPERIMENTAL SECTION

Reagents. Diet materials purchased from Nutritional Biochemicals Co., Inc., included casein, soy α protein, salt mixture W, and Vitamin Diet Fortification Mixture (without choline chloride).

***S*-Adenosylmethionine (SAM).** [*methyl*-^{3}H]SAM was obtained from New England Nuclear (specific activity, 4.55 Ci/mmol) and diluted 1:10 with cold SAM (2.2 mmol/ml), Sigma Chemical Co., and with a tenfold excess of 0.1 *M* Tris buffer (pH 8.5).

NADP, glucose 6-phosphate, and uridine diphosphate glucuronic acid were purchased from Sigma Chemical Co.

Procedures. *Preparation of Thermally Oxidized Oil and the Non-Urea-Adduct-Forming Fatty Acid Fraction.* Corn oil (1200 g) was heated with slow constant stirring at 180° for 24 hr. The resultant thermally oxidized (TO) oil (600 g) was then used to obtain non-urea-adduct-forming fatty acids (NUAF) according to the method of Firestone *et al.* (1961). First, the TO oil was saponified by refluxing with ethanol and 50% v/v aqueous KOH for 1.5 hr. The saponified mixture was purified of nonsaponifiables by extraction with ether. The aqueous phase obtained after this extraction was then acidified with HCl and extracted four times with ether. The ether was subsequently removed and the fatty acids so obtained redissolved in 90% ethanol at 50°. The ethanol solution was brought to saturation with urea and cooled overnight at room temperature. The fatty acid–urea adduct formed was filtered off and that precipitate washed three times with urea-saturated ethanol. The combined filtrate and washings were then diluted with an equal volume of water, acidified, and extracted with ether. The yield of NUAF was 26% of the TO oil.

Dietary Treatment. Isocaloric diets were prepared containing casein or soy protein, 20%; cornstarch, 53%; α-cellulose, 5%; and a 2:1 mineral–vitamin mixture, 10%. Fat, 12%, was included as either fresh corn oil, the TO oil, or fresh corn oil to which the NUAF material was added in an amount proportional to its yield from heated corn oil. These diets were fed to 12 groups of five female Sprague-Dawley rats for 15 days using a balanced factorial design. Groups had equal mean body weights ± 2 g at the start of the feeding period.

In addition to the variables of protein type and fat, the insecticide 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p'*-DDT, Aldrich Chemical Co.) was included as a variable to induce the microsomal mixed function oxidase enzyme system. "Pair-feeding" techniques were used to equalize the food intake among fat groups within a given protein by inducer diet.

Liver Preparation. At the end of the 15-day feeding period, the rats were weighed and killed, and their livers removed and weighed. One-gram portions of the livers, taken for use in the different *in vitro* assays, were then homogenized in 4 ml of a solution of 1.15% (w/v) KCl and 0.25% (w/v) nicotinamide. The microsomal KCl fraction was isolated by differential centrifugation at 85,000*g* for 1 hr.

Enzyme and Other Assays. Total microsomal protein concentration was measured by the Lowry *et al.* (1951) method. The *in vitro* oxidation of EPN (*O*-ethyl *O*-(4-nitrophenyl)benzene thiophosphonate) and O-demethylation of PNA (*p*-nitroanisole) were determined as by Kinoshita *et al.* (1966). Cytochrome P-450 concentration in microsomes was measured after Omura and Sato (1964). The *S*-adenosylmethionine:phosphatidylethanolamine methyltransferase activity assay was adapted from Bremer (1969). The methyltransferase activity was found to be very sensitive to pH changes and rigid controls on the assay medium at pH 7.8 had to be maintained. Glucuronyltransferase was assayed with *p*-nitrophenol as substrate (Temple *et al.*, 1971). The thiobarbituric acid assay for malondialdehyde was taken from Kitabachi (1967).

Table II. Analysis of Variance for Data in Table I

Variable	df	Mean squares for response analyzed[a] Total feed consumed	Body wt.	Rel liver wt.	EPN oxidn	PNA O-demeth	Microsomal protein	Cyto. P-450	Methyltransferase
Replication	4	91.92	121.98	0.303**	0.599	0.586	71.95**	0.178×10^{-4}	0.371*
Protein type	1	484.50**	836.27**	0.033	4.045*	6.131*	155.62**	0.236×10^{-3}**	3.795**
Fat type	2	211.59	211.26	2.067**	1.094*	1.262	353.67**	0.363×10^{-4}	0.387*
Inducer (DDT)	1	57.04	385.06*	1.562**	17.238**	18.526**	0.39	0.172×10^{-2}**	0.232
Prot. × fat	2	279.25*	70.86	0.072	0.120	0.052	37.54	0.030×10^{-4}	0.218
Prot. × inducer	1	710.70**	96.27	0.030	1.530	0.739	77.22	0.744×10^{-4}	0.847**
Fat × inducer	2	7.74	36.07	0.005	0.354	0.230	54.38	0.522×10^{-4}	0.157
Prot. × fat × inducer	2	56.90	76.07	0.180	0.001	0.077	28.33	0.019×10^{-4}	0.027
Error	44	66.09	93.77	0.063	0.227	0.432	17.49	0.215×10^{-4}	0.210

[a] Significant effects due to each variable as interpreted by the F-test are indicated at two probability levels: single asterisk, $P < 0.05$, and double asterisk, $P < 0.01$.

Table III. Effects of Fresh and Thermally Oxidized Oil in the Diet of Male Rats on Hepatic Microsomal Function[a]

Treatment	EPN oxidation, μg of PNP/50 mg of liver per hr		Glucuronyltransferase, μg of PNP/mg of protein per hr		Liver malondialdehyde, ΔOD/40 mg of liver	
	Fresh	TO	Fresh	TO	Fresh	TO
Soy protein	2.2 ± 0.03	2.4 ± 0.30	5.4 ± 1.8	8.9 ± 7.6	0.07 ± 0.00	0.14 ± 0.00
Soy protein + DDT	6.2 ± 0.30	10.0 ± 0.70	8.8 ± 2.9	8.6 ± 6.5	0.22 ± 0.01	0.19 ± 0.00
Casein	2.0 ± 0.10	3.6 ± 0.20	4.0 ± 3.3	4.9 ± 0.54	0.04 ± 0.00	0.20 ± 0.01
Casein + DDT	7.3 ± 0.60	8.2 ± 0.60	4.7 ± 1.9	4.4 ± 0.86	0.09 ± 0.00	0.23 ± 0.00

Factorial anal.	df	Mean square	Mean square	Mean square × 10^2
Replication	4	1.35	3.98	1.61[b]
Fat type (F)	1	28[b]	7.63	9.03[b]
Protein type (P)	1	0.20	126.56[b]	0.28[b]
Inducer (I)	1	315[b]	9.03	5.09[b]
F × P	1	0.50	3.73	4.40[b]
F × I	1	2.63	16.40	0.73[b]
P × I	1	0.17	7.66	1.49[b]
F × P × I	1	12.18[b]	4.43	0.08[b]
Error	26	1.11	7.49	0.01

[a] This is a summary of preliminary experimental results in which male rats were fed purified diets containing 12% fresh or oxidized corn oil and the indicated protein at 20% for a period of 10 days. All other conditions were the same as those in the major study. [b] Significant, $P < 0.01$.

RESULTS AND DISCUSSION

Table I lists data obtained for food consumption, final body weights, and relative liver weights at the end of the experiment as well as several indices of microsomal enzyme activity. EPN oxidation, PNA demethylation, and cytochrome P-450 concentration are presented as parameters of mixed function oxidase response. Also listed are microsomal protein concentration and methyltransferase activity data. Table II is a summary of the statistical analysis of variance conducted on all the data obtained in the experiment. Cytochrome P-450 acts as an oxygen-activating enzyme as well as the site of substrate interaction for oxidative transformations of different foreign lipophilic compounds (Sato *et al.*, 1969). Phosphatidylcholine, the specific phospholipid required for drug hydroxylations in reconstituted microsomes (Coon *et al.*, 1971), is known to be synthesized by methylation of phosphatidylethanolamine *in situ* through the action of *S*-adenosylmethionine-dependent methyltransferase in the SER (Natori, 1963). In the hepatic endoplasmic reticulum this path predominates over the Kennedy pathway which involves direct incorporation of choline (Nabazawa *et al.*, 1972).

As had been observed in preliminary experiments conducted in this laboratory, in which male and female rats were employed (see Tables III and IV), thermally oxidized fat had a pronounced effect on liver weight as well as most indices of microsomal enzyme activity. In the present study, the levels of microsomal protein, EPN oxidation, and methyltransferase were significantly influenced by oxidized fat. In most cases, the NUAF fraction appeared to be as effective as the TO oil itself. Both forms of oxidized oil products were relatively more potent in effect on liver weight, microsomal protein, and methyltransferase activity (at least in the case of the casein diets) than was the specific inducer DDT. On the other hand, DDT generally affected the microsomal enzyme responses to a greater level than did the oxidized oil products.

Since the protein and fat variables each affected the activities of both methyltransferase and the mixed function oxidase system, the former may be a key regulatory enzyme for the monooxygenase system. Although the pathway from CDP-choline (Kennedy and Weiss, 1956) is also a major route for lecithin formation, the results of various other investigations strongly indicate that stepwise meth-

Table IV. Effects of Fresh and Thermally Oxidized Oil in the Diet of Female Rats on Hepatic Microsomal Function[a]

Treatment	EPN oxidation, μg of PNP/ 50 mg of liver per hr		Glucuronyl-transferase, μg of PNP/mg of protein per hr		Liver malondialdehyde, ΔOD/40 mg of liver	
	Fresh	TO	Fresh	TO	Fresh	TO
Soy protein	1.2 ± 0.2	1.8 ± 0.2	7.9 ± 0.7	17.1 ± 3.4	0.03 ± 0.01	0.07 ± 0.03
Soy protein + DDT	5.3 ± 0.2	8.0 ± 0.7	12.3 ± 1.9	16.3 ± 2.9	0.03 ± 0.00	0.06 ± 0.01
Casein	0.5 ± 0.2	1.3 ± 0.3	4.2 ± 0.7	5.9 ± 0.3	0.01 ± 0.00	0.05 ± 0.02
Casein + DDT	2.4 ± 0.4	4.4 ± 0.4	6.4 ± 2.2	5.3 ± 2.2	0.02 ± 0.00	0.07 ± 0.03

Factorial anal.	df	Mean square	Mean square	Mean square × 10^3
Replication	4	0.68	9.33	3.94[c]
Fat type (F)	1	16.24[b]	177.6[b]	18.20[b]
Protein type (P)	1	12.25[b]	765.1[b]	0.59
Inducer (I)	1	111.0[b]	17.1	0.24
F × P	1	0.21	99.0[c]	0.12
F × I	1	6.84	16.0	0.02
P × I	1	15.96	15.3	0.77
F × P × I	1	0.21	3.5	0.45
Error	26	11.75	13.4	1.30

[a] This is a summary of the results of preliminary experiments on female rats treated in the same manner as the male rats (Table III). [b] Significant, $P < 0.01$. [c] Significant, $P < 0.05$.

ylation of phosphatidylethanolamine influences hepatic drug hydroxylation. Direct correlation of drug metabolizing *vs.* methyltransferase activities in hepatic microsomes conducted in Feuer's laboratory (Cooper and Feuer, 1973; Feuer *et al.*, 1973; Acheampong-Mensah and Feuer, 1974) provides the most definitive evidence for this regulatory function. Feuer and his coworkers found that the effect of dietary treatments on the induction of the monooxygenase system by drugs parallels the changes in the synthesis of membrane-bound phospholipids, particularly the formation of phosphatidylcholine through the action of methyltransferase.

Choline was omitted in our diets in order to establish a dependency on methionine as the methyl source. While this unnatural condition might appear to confound the results and their interpretations, it is unlikely that the choline insufficiency had an appreciable effect. Experiments by Beare-Rogers (1971) indicate that, even in advanced choline deficiency, the phospholipid changes observed reflect a need for methyl groups rather than for choline itself. Our own unpublished data show that choline supplementation has an effect on drug metabolizing activity which is, at best, intermediate between that seen in rats totally deficient in methyl group donors and rats fed methionine-supplemented diets.

Street *et al.* (1971) observed that basal mixed function oxidase activities and their increases upon induction by insecticides were minimal in female rats fed purified diets containing high quality proteins such as casein and egg protein. With soy protein, which is deficient in methionine, the basal and induced enzyme levels were markedly higher. On the other hand, methionine supplementation of the soy protein resulted in depression of enzyme activities to values typical of those obtained with high quality proteins. There is, however, a sex difference in the effect on mixed function oxidase activity of soy protein substitution for casein in the diet (as is evident in the data of Tables III and IV). Nevertheless, oxidized fat in the diet affected mixed function oxidase activities similarly in both sexes. It appears important that, in this present work with females, DDT did not affect the activity of methyltransferase.

These observations suggest that the diet variables of protein type and the condition of dietary fat may establish the basal status of the hepatic microsomal monooxygenase system which is then potentiated upon specific induction by compounds such as DDT.

Normal home cooking usage is unlikely to cause the marked changes in the character of the oil and its effects on the liver as herein described. However, a survey conducted in institutional kitchens and restaurants showed that while some maintain frying fat in good condition during use, others do allow severe deterioration to accumulate through heating, quite similar to the thermal abuse of oil as used by us for experimental purposes (Thompson *et al.*, 1967). Toxicological investigation of such abused shortening from commercial fryers may therefore reveal enzymic effects comparable to those observed with experimentally oxidized oils.

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Cytochrome P-450 Optical Difference Spectra of Insecticides. A Comparative Study

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The cytochrome P-450 optical difference spectra of insecticides were examined using hepatic microsomes from sheep, rabbit, rat, and mouse as well as abdominal microsomes from insecticide-resistant (Fc) and insecticide-susceptible (CSMA) house flies. The results generally conform to the hypothesis that type II binding spectra are elicited by compounds containing a nitrogen atom with a sterically accessible pair of nonbonded electrons and, in addition, certain compounds with nucleophilic oxygen atoms. Other compounds generally exhibit type I spectral binding or do not form detectable spectra. Exceptions include certain pyrethroids which, while giving typical type I difference spectra with mammalian microsomes, gave rise to an unusual spectrum in insects, one with a peak at 415–418 nm and trough at 445–447 nm. Rotenone also gave rise to unusual difference spectra with hepatic microsomes of sheep and rabbit. Differences in the ratios of spectral size between species indicate that qualitative differences in the cytochrome P-450 of different species are common.

The microsomal hemoprotein, cytochrome P-450, has been implicated as the terminal oxidase in the microsomal mixed-function oxidase system responsible for the metabolism of xenobiotics (including insecticides) in mammals (Hodgson, 1968; Gillette *et al.*, 1972; Parke, 1968) and insects (Hodgson, 1968; Hodgson and Plapp, 1970; Wilkinson and Brattsten, 1972). The first step involved in the oxidation of a xenobiotic by the mixed-function oxidase system is its binding to oxidized cytochrome P-450 (Gillette *et al.*, 1972), a step which can be studied by optical difference spectroscopy. Two principal types of difference spectra are recognized with oxidized cytochrome P-450: type I, with a peak at 385–390 nm and a trough at 416–420 nm (Cooper *et al.*, 1965; Remmer *et al.*, 1966), and type II, with a peak at 424–430 nm and a trough(s) between 390 and 410 nm (Cooper *et al.*, 1965; Remmer *et al.*, 1966; Schenkman *et al.*, 1967; Sasame and Gillette, 1969). Type I spectra are known to be caused by many compounds including drugs and pesticides (Schenkman *et al.*, 1967; Sasame and Gillette, 1969; Kuwatsuka, 1970; Mailman and Hodgson, 1972; Baker *et al.*, 1972) while type II is characteristic of nitrogen-containing compounds with a sterically accessible pair of nonbonded electrons as well as certain alcohols (Cooper *et al.*, 1965; Remmer *et al.*, 1966; Schenkman *et al.*, 1967; Jefcoate *et al.*, 1969; Jefcoate and Gaylor, 1969; Diehl *et al.*, 1970; Mailman *et al.*, 1974; Kulkarni *et al.*, 1974).

Compounds which give rise to type I spectra are usually substrates for the microsomal mixed-function oxidase system. Previous investigators, with few exceptions (Schenkman *et al.*, 1967; Imai and Sato, 1967; Remmer *et al.*, 1969), have failed to demonstrate a correlation between spectrum formation by different compounds and the rates of their oxidative metabolism (Temple, 1971). This is to say K_s, the spectral binding constant, is not correlated in any systematic way with K_m, the Michaelis constant for the enzymatic reaction. Insecticides are not only metabolized by the mixed-function oxidase system but may also function as inhibitors or inducers (Hodgson, 1968; Hodgson and Plapp, 1970; Wilkinson and Brattsten, 1972; Baker *et al.*, 1972; Stevens *et al.*, 1973; Stevens and Greene, 1973; Greene, 1972; Greene and Stevens, 1973). Little is known concerning either the binding of insecticides to cytochrome P-450 or the variation between

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