Nutritional and Metabolic Studies of Noncyclic Dimeric Fatty Acid Methyl Esters in the Rat

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ABSTRACT

Methyl esters of noncyclic dimeric fatty acids were prepared by fractionation of the crude product obtained by heating a mixture of methyl oleate and di-*tert*-butyl peroxide for 48 hr at 130 C in a high pressure reactor. Rats fed diets containing less than 1% of the noncyclic dimer methyl ester showed no significant difference in growth, feed efficiency, and liver size, from those fed nutritionally adequate diets. The ^{14}C-labeled dimer was also synthesized and administered to rats via gastric intubation. The results indicated that an average of 85% of the radioactivity was recoverable from the gastrointestinal tract and feces within 48 hr of administration and that such an amount was not absorbed by the rat. Within a 48 hr period ca. 5% of the ^{14}C was recovered in the expired CO_2 and about 1% in the urine. Prefeeding rats diets containing dimer did not appear to influence the metabolism of either dimer or methyl oleate.

INTRODUCTION

Investigations into the effects of heated fats have been numerous, and reviews on the subject continue to appear in the reference literature. Most of the dietary work prior to the 1970s was concerned with heated fat as a single entity. Investigations have assessed the nutritional effects of less complex mixtures resulting from fractionating polymeric components from heated fats (1,2). Only recently have investigations for nutritional evaluation (3-5,7,8) been carried out with purified compounds found in heated fats.

The use of polymeric fractions from heated fats in nutritional studies has been popular because of their relative ease of isolation and because these compounds represent the fat in a state of modification rather than decomposition. While the volatile oxidation products of heated fats are known to be toxic (6), the effects of high molecular weight fractions appear to be more subtle. The limited absorption of heated fat polymers appears to be largely responsible for the lack of dramatic symptoms in animals after ingestion of such materials.

Among many of the nutritional studies that have been carried out with heated fat polymers, an almost universal criticism (7-9) has been the use of abnormally high levels of polymers in the animals' diets. It has been previously demonstrated that in many used frying fats, the levels of high molecular weight materials did not exceed 5% after 20 hr of use (10). In an animal diet containing 15% fat, this polymer level represents only 0.75% of the total diet. In the present study, therefore, we employed up to 0.75% dimeric fatty acid in rat diets, paralleling a study by Hsieh (5). As objectives of the present study, both the nonlabeled and ^{14}C-labeled noncyclic dimer of methyl oleate (11) was prepared; its absorption and nutritional effects were studied, and the results compared with those previously obtained with similar experiments involving the cyclic dimer of methyl oleate (5).

EXPERIMENTAL PROCEDURES

Synthesis of Dimeric Fatty Acids

Methyl oleate of 99% or greater purity was purchased from a commercial source (Nu-Check-Prep Inc., Elysian, MN). The purity was determined by gas liquid chromatography on 10% SP-2340 coated on 100-120 mesh Supelcoport (Supelco, Inc., Bellefonte, PA).

The gas chromatographic conditions were: column 195 C, injector 225 C, detector 224 C, (flame ionization), and carrier gas flow 20 ml/min of nitrogen. Quantitation was accomplished with the electronic integrator of the HP3380 instrument employed, and the aid of quantitative standard mixtures of methyl esters.

Isomeric dimeric fatty acid methyl esters were prepared by the method of Paschke et al. (11). Methyl oleate and di-*tert*-butyl peroxide were mixed in a molar ratio of 8.2 to 1 (peroxide 12 mole % of the oleate) and heated to 130 C under nitrogen for 48 hr in a Parr Pressure Reaction Apparatus (Parr Instrument Co., Moline, IL) equipped with stainless steel reaction vessel, stirring motor, and thermostat.

After reaction and cooling, the product was first distilled at 35 C under reduced pressure to remove the *tert*-butyl alcohol formed and then at 170 C and 0.25 mm Hg to remove the unreacted monomer.

The residue, consisting of dimer and polymer, was purified by gel permeation chromatography. A glass column 120 x 4.8 cm fitted at the bottom with a fritted glass insert equipped with a stopcock was packed to a bed volume of 1400 cm^3 with a slurry of Bio-Beads S-X1 (Bio-Rad) swollen overnight in chloroform. A layer of clean sand 1/2 in. high was poured on top of the gel to prevent the gel from floating. Samples of 3 g each of impure dimer mixed with an equal volume of chloroform were injected onto the column. The effluent was monitored with a Waters Model 401 Differential Refractometer (Waters Associates, Framingham, MA) connected to a chart recorder. The dimer fraction, identified by its K_{av} value (10), was collected. Solvent was removed in vacuo with a rotary vacuum evaporator.

Thin layer chromatography of the dimer for purity verification was carried out with Silica Gel G (Merck & Co., Darmstadt, Germany) coated on 5 x 20 cm glass plates to a thickness of 0.5 mm using a stainless steel applicator. The plates were activated at 120 C prior to use and were developed in a solvent system consisting of isooctane-ethyl acetate (90:10, v/v). Spots were visualized by spraying with sulfuric acid-dichromate spray (12) and then charring in an oven at 110 C.

The isolated, purified material was characterized as follows: infrared spectroscopy indicated 65% *trans* double bond. Proton nuclear magnetic resonance spectrometry (pNMR) verified the structural elements of dehydro-dimer methyl ester as did the ^{13}C-NMR spectrum. As with pNMR, the ^{13}C-NMR data provide only supportive data. The mass spectrum of the material was determined and found to be consistent with that proposed by Paschke et al. (11) for purified noncyclic dimer isomer mixtures.

Experimental Animals and Diets

Male weanling SPF Albino rats (Murphy Breeding Lab-

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TABLE I

Weight Gain and Food Consumption of Male Rats Fed for 6 Weeks on Diets Containing Different Levels of Dimers

Group	% Dimer in oil	Average weight gained (g)	Average food consumed (g)	Feed efficiency[a]
I	0.0	187 ± 15[b]	605 ± 29[b]	0.308
II	0.1	196 ± 15	621 ± 21	0.316
III	1.0	211 ± 18	638 ± 20	0.330
IV	5.0	193 ± 1.0	610 ± 29	0.316

[a]Feed efficiency = $\frac{\text{weight gain}}{\text{feed consumed.}}$

[b]Mean value ± SEM, for eight animals in each group.

oratory, Plainfield, IN) (40-50 g) of Sprague-Dawley descent were housed in galvanized iron wire cages with mesh bottoms. The composition of the diets used was as follows: (in g/kg diet) salt mixture (No. 4164 General Biochemicals, Chagrin Falls, NY), 40; vitamin premix (10 g of vitamin premix in 1 kg of diet contained vitamin A Acetate, 20,000 IU; vitamin D, 2,000 IU; vitamin E, 100 IU; and the following in mg/kg of diet; inositol, 112; menadione bisulfite, 48; para-amino benzoic acid, 110; nicotinic acid, 100; calcium pantothenate, 67; riboflavin, 22; pyridoxine HCl, 22; thiamine HCl, 22; biotin, 0.45; folic acid, 2; and cyanocobalamine, 0.03), 10; choline chloride, 1.65; casein, 150; dextrose, 648.4; and corn oil containing either 0.0, 0.1, 1.0, or 5.0% methyl esters of dimeric fatty acids, 150. All diets were prepared weekly or as needed. The animals were provided with food ad libitum, and the stock diets were stored in a 5 C cold room. The animals were fed the different diets for 6-8 weeks.

Procedures for In Vivo Metabolic Studies

Methyl esters of ^{14}C-labeled dimeric fatty acids were prepared from uniformly labeled methyl oleate (Dhom Products, Hollywood, CA) with the same procedure described previously for synthesis of unlabeled dimers, and fed in corn oil via gastric intubation to individual rats. These rats had been conditioned by receiving the special diets for 8 weeks and then were immediately placed in all-glass metabolic cages for 48 hr after injection of the labeled sample. The expired CO_2, urine, and feces were recovered, and the radioactivity was determined by liquid scintillation counting according to the procedures previously described (13). The rats were sacrificed after the 48 hr period and their livers, stomach, small and large intestines, epididymal fat, and perirenal fat were quickly excised, blotted, weighed, and stored at 0 C.

Extraction and Scintillation Counting Procedures for Tissues

Weighed samples of the liver, epididymal fat, and perirenal fat were solubilized in 2.0 ml of Protosol (New England Nuclear, Boston, MA). To each sample were then added 10 ml of Aquasol (New England Nuclear). The samples were then counted in a liquid scintillation counter. Efficiency of the counting was measured by the addition of an internal standard.

The feces, stomach contents, and intestinal contents were extracted with chloroform-methanol (2/1, v/v). An aliquot of the extract was taken and evaporated to near dryness. To each sample were added 10 ml of Aquasol. Samples were counted as described above.

Statistical analyses of the data were performed by t-test or by completely randomized design and the variance analyzed by F-test (14).

RESULTS AND DISCUSSION

The purity of the dimer used in the present study was estimated by thin layer chromatography (TLC) and gel permeation chromatography to be greater than 99%; however, the percentage of noncyclic dehydro-dimer in the preparation was greater than 90%. Repeated attempts at further separation on preparative columns did not achieve further purification. Dimeric fatty esters produced by free-radical reaction will always result in mixtures of chemically nearly identical compounds. All impurities were dimer-like in behavior on TLC plates. One spot had an R_f value greater than the dimeric components. Thin layer chromatographic comparison of the dimer with methyl oleate and cyclic monomer of methyl oleate as well as gas chromatography verified that the spot was neither of these. Thus, while "dimer-like impurities" do exist in the preparation, they will not interfere with later dietary absorption studies but will, we believe, be treated as dimeric materials.

The effects from feeding rats low levels of the dehydro-dimer of methyl oleate on weight gain are shown in Table I. The diets of these animals were nutritionally adequate. The recommended level of fat in the diet of the rat is about 5% (15). In the present study, 15% fat was used since most heated fat studies have employed this level of fat, thus enabling comparisons among studies. The levels of non-cyclic dimers added to the diets were based on results from previous work of Paulose and Chang (16) which indicated the presence of up to 5% dimers in fats used under actual frying conditions. These dimer levels were also employed by Hsieh (5) in his work with the cyclic dimer of methyl oleate.

As the data in Table I show, there were only minor differences in weight gains among the groups. There was no statistical significance ($P < 0.05$) among the groups. Furthermore, no differences in food consumption were observed which indicates that food palatability was not a problem. Earlier investigations (1,17,18) employing 12-20% of crude dimers in the diets of rats demonstrated increased mortality rates in the animals. Recent work (19) has shown these levels to be unrealistically high, and since malabsorption and diarrhea may complicate the picture, the actual effects of dimeric fatty acids may have been exaggerated. Enlarged livers, due to the accumulation of lipid, have been commonly reported as a result of feeding heated or oxidized fats (7) since these components, such as the cyclic monomer (4), can be readily absorbed.

In the present work there were no significant differences in liver weight/body weight ratios (Table II). Therefore, while some heated fat isolates do lead to enlarged livers when incorporated into rat diets, both the present study and Hsieh's study with cyclic dimers (4) indicate that dimeric fatty acids do not lead to enlarged or fatty livers. Such enlargement may be a function of the absorption of such materials; if they are absorbed to a very limited extent, no gross effect on liver lipid accumulations may be observed.

TABLE II

Liver Body:Weight Ratio of Rats Fed Different Levels of Dimers for 6 Weeks

Group	% Dimer in oil	Liver weight / Body weight
I	0.0	3.13 ± 0.16[a]
II	0.1	3.34 ± 0.24[a]
III	1.0	3.19 ± 0.13[a]
IV	5.0	3.35 ± 0.09[a]

[a]Mean ± SEM, for four animals in each group.

TABLE III

Percentage of Recovered Radioactivity in Selected Tissues, Urine, and Exhaled CO_2, after 48 Hr in Rats Prefed Dehydro-Dimer[a] for 8 Weeks and Then Force Fed the Labeled Dehydro-Dimer of Methyl Oleate

	Group			
	I	II	III	IV
Tissue	Percentage of recovered radioactivity[b]			
Gastrointestinal tract and feces	86.43	88.72	86.38	79.34
Liver	1.98	4.14	4.69	11.12
Epididymal fat	1.41	0.6 2	1.99	1.70
Perirenal fat	4.37	0.86	0.41	1.10
Urine	1.25	0.97	1.10	1.01
CO_2	4.55	4.62	5.42	5.72
	99.99	99.99	99.99	99.99

[a]% Dimer fed in diet: group I, 0.000; group II, 0.015; group III, 0.150; group IV, 0.750.

[b]Mean value from two animals.

It is important that absorption of dimeric fatty acids occur if biological effects are to be observed. The data from the metabolic studies (Table III) indicate that an average of 85% of the recovered radioactivity was found in the gastrointestinal tract and feces. This compares favorably with an average of 80% reported by Hsieh (5). These findings suggest that absorption is limited. A previous report by Bottino (2) had found 30-70% absorption of heated fat dimers. However, the dimer fraction which he isolated by molecular distillation was not characterized and probably contained components more readily absorbed than dimers.

The radioactivity recovered as $^{14}CO_2$ was less than 6% of the total when labeled noncyclic dimers were force fed, whereas the $^{14}CO_2$ recovered when uniformly labeled methyl oleate was force fed, was 60 to 80% of the total recovered radioactivity. A minimal amount of tissue incorporation was observed in liver and in the adipose tissues analyzed. Based on these data, it appears that the absorption of noncyclic dimers is limited to ca. 10%, which is then metabolized.

The urinary excretion of radioactive label from noncyclic dimers (average 1.1%) was comparable to that excreted when cyclic dimers were fed by Hsieh (1.2%). This finding demonstrates that the major structural difference between the dimers, the presence of a ring in the cyclic dimers, seems to exert little effect upon urinary excretion of radioactivity. Furthermore, a comparison of the CO_2 excretion in the two studies indicated an average of 5% $^{14}CO_2$ excreted in rats fed noncyclic dimers vs. 1.7% in those fed cyclic dimer (5). This divergence may be explained by a difference in the manner of metabolism of the cyclic product. The noncyclic dimer, although it is an isomer mixture, probably presents more carbon atoms to the enzyme systems for degradation to carbon dioxide.

The present study indicates that, in a short term experiment, the presence of noncyclic in the diet did not appear to exhibit any profound effects either on the growth or metabolism of the rat when moderate levels of the compound were present in the diet.

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