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Received June 27, 1966. P.S.E.B.M., 1966, v123.

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Chronic Toxicity of Methyl Linoleate Hydroperoxide for the Rabbit. (31459)

M. G. KOKATNUR, J. G. BERGAN, AND H. H. DRAPER
Division of Nutritional Biochemistry, Department of Animal Science, University of Illinois, Urbana

Although the toxicity of oxidized fats for animals is well known(1-6), the explanation of their toxic effect is still unclear. There is evidence that the oral toxicity of air-oxidized oils is associated with their peroxide concentration; however, since peroxides apparently are destroyed in the intestine their toxicity presumably is exerted at this site(5,6). Whether fatty acid peroxides are formed in the tissues of animals in general, and of vitamin E deficient animals in particular, is a subject of current controversy. Clearly, if peroxides are formed *in vivo* they must be rapidly decomposed, as no appreciable accumulation occurs and their continued presence would have serious implications for the structural integrity of cell membranes and subcellular particles(7).

The muscular dystrophy which results from a deficiency of vit E may be attributable to peroxidation of lipids in the cellular and subcellular membranes which proceeds in the absence of this vitamin(8). If this explanation is correct, chronic parenteral administration of small amounts of preformed hydroperoxides might be expected to enhance the appearance of the disease by increasing the rate of vit E consumption in the tissues for decomposition of the peroxide or for destruction of additional free radicals whose formation may be catalyzed in its presence. This hypothesis was tested in the present study by determining the effects on the occurrence of vit E deficiency of administering small doses of purified methyl linoleate hydroperoxide to rabbits over protracted periods of time.

Experimental. New Zealand White weanling rabbits weighing 1100-1600 g were housed individually in metal cages and maintained on a vit E deficient diet for 10 days to deplete their tocopherol reserves. The composition of the diet was as follows(%): Labco casein 20.0, glucose (Cerelose) 20.4, starch 40.0, distilled lard 7.0, salts 4164(9) 4.0, cellulose 5.0, cod liver oil 3.0, choline chloride 0.1 and vit premix(9) 0.5. Methyl linoleate hydroperoxide (MLHP), prepared fresh according to a method described previously(10), was suspended in isotonic saline with the aid of 2% Tween 80. An emulsion of pure methyl linoleate was prepared in the same way.

In Experiment I, 2 groups of 6 vitamin E-depleted rabbits each were injected daily over a period of 10-14 days, one group with 1 ml of an emulsion containing 50 mg of MLHP and the other with the same quantity of emulsified methyl linoleate. The dose was administered intravenously through an ear vein. During the course of injections observations were made on the excretion of creatine and creatinine in the urine(11), body weight changes and development of muscular incoordination. At the end, the animals were examined for gross lesions of the internal organs and the livers were preserved in 10% formalin for histopathological examination.

Experiment II was designed to determine the influence of tocopherol or selenium administration on the chronic toxicity of MLHP. Thirty-two animals depleted of vit E reserves as described above were divided into 5 groups. Group 1 was maintained as an untreated control, Group 2 received daily intravenous injections of 50 mg of methyl linoleate in emulsion and the remaining animals were given a similar daily dose of MLHP. Group 5 received, in addition, a daily oral supplement of 100 mg of d-α-tocopheryl acetate prior to the injection, and Group 6 was given a supplement of 1 μg of selenium per g of diet in the form of sodium selenite. All the animals were killed after 10-14 days and histological examinations made made of their livers.

Experiment III was carried out to determine whether the incipient signs of vit E deficiency could be induced in rabbits depleted of their tocopherol stores by continuous intravenous infusion of MLHP. As peroxides are known to be rapidly metabolized *in vivo,* continuous administration of these compounds is probably necessary to maintain a circulating titer. After being maintained on a vit E deficient diet for 12-14 days, weanling animals were shaved in the neck region and fine polyethylene tubing was introduced 2-3 inches into the jugular vein through an 18-gauge heparinized hypodermic needle. The rabbits subsequently were kept in a restraining cage and given free access to food and water. Fresh emulsions of MLHP (peroxide value 4800-5400 meq per kg) were prepared in isotonic saline containing 100 mg% Tween 80 and 50 mg% animal lecithin. The hydroperoxide was dispersed in this medium at a concentration of 2 mg per ml by shaking gently and then bubbling nitrogen through the mass vigorously for 5 minutes. Emulsions thus prepared were stable for the duration of the infusion. As intravenous administration of fat emulsions is known to result in some physiological reactions which may be confused with those observed in vit E deficiency(12), a group of control rabbits was infused with a preparation containing methyl linoleate in place of MLHP. The emulsions were administered by means of a continuous infusion pump at a rate of 0.103 ml (206 μg MLHP) per minute for a period of 26-30 hours. The parameters chosen as criteria of incipient vit E deficiency were red blood cell hemolysis and creatinuria. Samples of blood and urine were taken at the beginning and end of the infusions (and, where possible, during the experiment) for analysis by published methods(11,13).

Results. Experiment I. The rabbits which received daily MLHP injections underwent a sharp increase in the ratio of creatine to creatinine in the urine during a 10-14 day period (Table I) and exhibited either a general loss of muscle tone or gross symptoms of incoordination which were evident in the righting reaction. In the methyl linoleate-treated group, no gross signs of dystrophy were detected and most of the creatine:creatinine ratios remained within the normal range. Two animals in the control group exhibited a mild creatinuria as might be expected of rabbits maintained on the deficient diet for 3 weeks. On the other hand, 5 of the 6 MLHP-injected rabbits experienced pronounced creatinuria as indicated by values in excess of 1.00. The range of ratios for the 12 animals at the start of the injection period was 0.03-0.33. Whereas the linoleate-treated group gained some weight during the experiment, the weights of most of the hydroperoxide-treated animals remained about constant or declined.

TABLE I. Effect of Intravenous Administration of Methyl Linoleate or MLHP on Urinary Creatine Excretion and Occurrence of Gross Symptoms of Muscular Dystrophy in Rabbits.

Rabbit No.	Methyl linoleate group* Body wt Initial	Final	Urinary creatine/creatinine Initial	Final	Righting reaction	MLHP group* Body wt Initial	Final	Urinary creatine/creatinine Initial	Final	Righting reaction
1	1100	—	.16	.04	—	1350	—	.10	.65	—
2	1570	1900	.10	.54	—	1550	1440	.03	2.92	+
3	1470	1510	.12	.73	—	1380	1080	.10	1.38	+
4	1095	1190	.33	.20	—	1090	1110	.17	1.73	—
5	1490	1570	.11	.12	—	1400	1380	.09	1.81	+
6	1320	1390	.16	.08	—	1470	1580	.10	1.08	—

* 50 mg per day injected intravenously for 10-14 days. Peroxide value of the MLHP preparation was 5000 meq/kg.

Gross examination of the livers of the MLHP-injected rabbits revealed the presence of white mottled areas on the surface of the lobes. Histopathological examination showed that these areas were marked by diffuse centrolobular fatty changes of varying degree with scattered foci of necrosis and calcification. Giant cell formation and bile duct proliferation also were observed in some livers. These lesions were not observed in any of the animals which received methyl linoleate.

Experiment II. Administration of large amounts of α-tocopherol orally prevented the occurrence of creatinuria and other gross signs of toxicity in rabbits injected with small daily doses of MLHP, but did not entirely prevent the liver lesions (Table II). Selenium had no influence on the incidence of hepatic lesions or on the increased creatine excretion. Interestingly, one case of hepatic lesions was observed in the methyl linoleate group, suggesting that peroxidation of this compound occurred in the vit E-depleted liver of this animal.

Experiment III. Table III shows that the continuous infusion of either methyl linoleate or MLHP for 26-30 hours was associated with an increase in erythrocyte hemolysis and creatine excretion. However, the hemolysis values for the MLHP-treated rabbits were consistently greater than those for the control group, and in 3 animals the creatine:creatinine ratios reached exceptionally high levels. The results suggest that the hydroperoxide induced a degeneration of red blood cell membranes and initiated additional destructive processes affecting other cells. These effects appear to be more extensive than can be attributed to the non-specific effect of infusing lipid emulsions.

Discussion. The rate of MLHP administration used in these studies (about 12 μM per 100 g body weight per day) was well below the LD_{50} intraperitoneal dose of 150-170 μM per 100 g reported for rats(6) and a comparable figure reported for mice(4). The toxicity of linoleate hydroperoxides is much greater by the intraperitoneal route than by the oral route(3,4,6), and at acute doses seems to be unaffected by tocopherol administration (6). The ameliorating effect of vit E on peroxide-induced creatinuria observed in the present study indicates that if the amounts of hydroperoxide administered are small the vitamin is capable of partially destroying them *in vivo*, probably by reduction to the corresponding hydroxy acid. Considering the large amounts of vit E employed in this experiment, however, and the incomplete protection obtained, it is not surprising that at much higher rates of hydroperoxide administration no protective effect was discernible(6).

TABLE II. Influence of Vit E or Selenium on Toxic Effects of Intravenously Administered MLHP.

Treatment*	Oral supplement†	No. of rabbits: In group	With liver lesions	With incoordination	Urinary creatine/creatinine: Avg	Range
None	none	3	0	0	.15	.10– .20
Methyl linoleate	"	8	1	2	.14	.08– .30
MLHP	"	6	5	3	.69	.13–1.81
"	vit E	7	3	1	.12	.04– .20
"	Se	8	8	1	.64	.23–1.67

* 50 mg per day injected intravenously for 10-14 days. Peroxide value of the MLHP preparation was 5800 meq/kg.
† 100 mg d-α-tocopheryl acetate per day; 1 ppm Se as sodium selenite.

TABLE III. Effect of Continuous Infusion of Methyl Linoleate or MLHP on Erythrocyte Hemolysis and Urinary Creatine Excretion in Rabbits.

Rabbit No.	Methyl linoleate group Infusion time, hr	% RBC hemolysis	Urinary creatine/creatinine	MLHP* group Infusion time, hr	% RBC hemolysis	Urinary creatine/creatinine
1	0	0	.11	0	0	.13
	29	31	.34	30	49	.59
2	0	0	.01	0	0	.32
	30	9	.73	30	100	3.12
3	0	0	.31	0	0	.08
	30	32	.37	30	49	12.90
4	0	0	.004	0	0	.20
	26	21	.38	30	100	3.80

* Peroxide values of the MLHP preparations ranged from 4800 to 5400 meq/kg. Infusion rate was 206 μg/min.

The results of these experiments indicate that the chronic administration of small doses of hydroperoxide leads to a more rapid destruction of tocopherol in the tissues and an accelerated appearance of deficiency symptoms (creatinuria, erythrocyte hemolysis, muscular dystrophy). It has been reported by several workers that the concentration of vit E in the tissues of exhaustively depleted animals declined to undetectable levels. Under these conditions the formation of hydroperoxides *in vivo* may be presumed to increase, and there is considerable evidence that the damaging effect of these compounds on subcellular particles, sulfhydryl enzymes and other proteins(14) may account for the biochemical and histological changes observed in vit E deficiency. It has been reported(15) that cerebellar disorders characteristic of tocopherol deficiency can be induced in chicks by intravenous injection of 10 mg of methyl linoleate hydroperoxide.

The fatty degeneration of the liver found in this study does not occur in vit E deficient rabbits and has not been reported in other animals following short-term hydroperoxide administration. The lesion is distinct from the dietary necrotic liver degeneration described by Schwartz(16), as indicated by the histopathology and the failure of selenium to exert a protective effect. Intravenous administration of preformed hydroperoxides may lead to rapid localized liver damage before these compounds are reduced by tissue antioxidants. Holman and Greenberg(1) have observed that a hydroxy acid with a conjugated diene system (the likely reduction product of a linoleate hydroperoxide) is much less toxic for rats than the corresponding hydroperoxide.

Summary. Small quantities of purified methyl linoleate hydroperoxide (MLHP) were administered by daily intravenous injection (50 mg/day) or by continuous intravenous infusion (206 μg/min) to rabbits depleted of their vitamin E stores. After 10-14 days the injected animals exhibited fatty degeneration and necrosis of the liver, creatinuria and an increased incidence of muscular incoordination. The creatinuria was prevented by large oral doses of vitamin E (100 mg/day) but sodium selenite had no effect on the creatinuria or the incidence of liver lesions. Infusion of MLHP for 26-30 hr led to an increased fragility of the red blood cells and a marked creatinuria. These results indicate that chronic administration of small quantities of MLHP leads to a more rapid de-

struction of vitamin E in the tissues and an accelerated appearance of deficiency symptoms.

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Received June 27, 1966. P.S.E.B.M., 1966, v123.

Thyroxine Augmentation of Growth Hormone-Induced Endochondral Osteogenesis.* (31460)

EMILIJA RIEKSTNIECE AND C. WILLET ASLING
Departments of Anatomy, University of California, Berkeley and San Francisco

The actions of thyroxine in enhancing body growth, skeletal and somatic, are among the most sensitive of this hormone's effects(1), but remain incompletely studied and understood. Thyroxine participates both in (*A*) incremental growth, and in (*B*) differentiation.

A, 1. It has long been known that thyroxine augments the action of pituitary growth hormone, in the absence of the pituitary and/or the thyroid glands, as well as in intact animals (2). Whether this augmentation is true synergism is unresolved, but the demonstration by Geschwind and Li(3) that a dose of thyroxine so minute as to have no discernible effect alone could yet increase the sensitivity of the "tibia line" assay for growth hormone, appears strong evidence for synergism.

A, 2. Alone, thyroxine induces a discernible, but slight and unsustained, elongation of bones in hypophysectomized rats, a transient effect which is thus independent of pituitary growth hormone(4).

B. In differentiation, thyroxine can induce the appearance of new epiphyseal ossification centers and subsequently their fusion to the diaphysis through resorption of the epiphyseal cartilage plate. This action occurs in the absence of the pituitary(4) or the thyroid(5,6), or both(7). It is apparently effected through continued erosion of cartilage in the absence of an equivalent rate of chondrogenesis; "chondroclasts" have appeared at the marrow-cartilage junction during thyroxine administration(8).

In most circumstances, the principles underlying these actions are not known; in one circumstance (growth in thyroxine-treated

* Aided by USPHS Research Grant AM-00664 and a grant from Univ. of California Research Committee. This study was conducted while the first author was a Post-Doctoral Research Fellow in Department of Nutritional Sciences, Training Grant 1 T1 DE 139-02, Nat. Inst. Health.

thyroidectomized rats) it has been well-documented that repair of the pituitary and restoration of endogenous growth hormone secretion is a substantial part of the action(9,10, 11). To complicate the situation, distinctions have not always been well drawn between the immediate effects of hormone administration (*i.e.*, observation after a few days or even weeks) and the effects seen following nearly a year of sustained treatment. Nor does it follow that the widening of an epiphyseal cartilage plate (such as is used as an indicator of growth in the assay of growth-promoting substances) is always associated with active bony elongation.†

The present inquiries arose, in part, from the observation that it proved impossible to stimulate skeletal growth to the point of gigantism in thyroidectomized rats by long-continued administration of high doses of growth hormone, although it was possible to achieve this effect in intact or hypophysectomized rats(12). The growth hormone stimulated osteogenesis, as such, as judged by widening and thickening of bones, but not the elongation of bones which depends on chondrogenesis at epiphyseal cartilage plates. Only when traces of thyroxine were added to the treatment was full endochondral osteogenesis resumed. As a possible explanation, it appeared that growth hormone alone might support osteogenesis, but that thyroxine might be necessary for growth hormone to stimulate sustained chondrogenesis; conceivably, the needs for thyroid hormone were so low that they might be met by endogenous activity even in hypophysectomized rats. (Taurog *et al*(13) have shown continued, albeit low-level thyroxine formation in rats 8-9 weeks after hypophysectomy.)

The present study is intended to search for the lower limit of this potential thyroxine requirement for endochondral ossification. It was decided to use, as the basic test situation, the standard tibia line assay procedure, for this test depends upon evocation of chondrogenesis in the epiphyseal cartilage plate. The conditions for performing the test have been defined carefully by Geschwind and Li(3), who also describe the earlier contribution of Marx, Simpson, and Greenspan in the test's development. In brief, female rats are hypophysectomized when 26-28 days of age, and after a 12-day post-operative interval are screened for good condition and presumptive evidence of completeness of the operation. The hormonal material under test is injected daily for a 4-day period; 24 hours after the last injection the animals are autopsied and the tibias dissected free, split, and "stained" with silver nitrate solution to mark calcified regions (von Kossa reaction). The width of the uncalcified portion of the proximal epiphyseal cartilage plate is measured in a compound microscope with a micrometer eyepiece. Typically the width in untreated controls is of the order of 150 μ. If, following treatment, the width does not achieve at least 200 μ, the effect is considered to be in the "non-specific" range(3). With a potent growth hormone a linear dose-response increase is found over a wide range of dosages. Dose is always expressed as total administered during the 4-day test.

Experiment 1. A standard dose of growth hormone was sought to form the base of comparison for all further tests. The preparation of growth hormone employed was highly purified. To give still further assurance of minimal contamination with thyrotropic hormone —TSH—(which might stimulate the thyroid, if present, or even aberrant thyroid follicles such as have been seen in the thymus(15)), the hormone was treated with periodate(16) to inactivate any residual glycoprotein moieties.‡ Table I shows the epiphyseal plate widths encountered in the various experimental groups. At 40 μg total dose this preparation gave a response in the "non-specific" range; 50 μg gave a well-defined response, almost 50% above the control level. The dose chosen for further testing was 60 μg, since this gave an increase which was invariably significantly above the non-specific range.

† Of several situations which support this remark, one may mention an experiment in which bone length was greater in a thyroxine-growth hormone-treated group than with growth hormone alone, but the epiphyseal cartilage plates were narrower in the doubly-treated group(14).

‡ We acknowledge gratefully a generous supply of such growth hormone from Dr. Lawson L. Rosenberg.

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Long-term Rat Feeding Study with Used Frying Fats

GRANVILLE A. NOLEN, J. CRAIG ALEXANDER [1] AND NEIL R. ARTMAN [2]
The Procter & Gamble Company, Miami Valley Laboratories, Cincinnati, Ohio

ABSTRACT A study was made to learn whether fats which had been exposed to the heat and aeration of actual frying differ significantly from fresh fats in their nutritional properties. Partially hydrogenated soybean oils, cottonseed oil, and lard were used for frying under practical restaurant-type frying conditions until they became unfit for further use owing to excessive foaming during frying. The used fats were fed to groups of 50 male and 50 female rats as 15% of the diet for 2 years. The used fats were slightly less absorbable than unheated control fats, and gave correspondingly slower growth rates. Other than this there were no differences in clinical, metabolic, or pathological criteria to suggest that the used fats adversely affected the rats consuming them. Mortality among the heated-fat groups was no higher than among the control groups. Distillable non-urea-adductable fractions concentrated from the used fats proved somewhat toxic when large doses of them were administered by stomach tube to weanling rats. The results indicate that, although heating of fats under actual frying conditions does cause the formation of substances which can be shown to be toxic, the level of such substances and the degree of their toxicity are so low as to have no practical dietary significance.

Fatty materials undergo chemical reactions when they are strongly heated in air. Evidence has been offered that some of the reaction products which form under certain heating conditions are toxic when fed to rats (1, 2). It seemed important to learn whether fats which had been exposed to the heat and aeration of actual frying differ significantly from fresh fats in their nutritional properties.

Of the publications reporting toxicity in heated fats, some (3–5) have dealt with fats which had been heated under extreme conditions of temperature, or aeration, or both. Others (6, 7) report the concentration of small quantities of toxic materials from large volumes of heated fat. And in still others it appears that manifestations ascribed to fat toxicity should have been ascribed to oxidative deterioration of improperly stored mixed diets (8–11). The significance of these results as they relate to human nutrition is not clear.

Work has been reported in which fats that actually had been used for restaurant and commercial frying were evaluated nutritionally by short-term feeding studies and were found to be unimpaired in value (12, 13). The long-term feeding studies which have been reported used fats that had been oxidized to high peroxide value at low temperature (14), or polymerized at high temperature in the absence of air (15), or bubbled with air during heating (16). In each case no ill effects were observed in the animals that consumed these fats, but, since the conditions used for preparing the fats differed considerably from practical frying conditions, these experimental results also are not directly applicable to practical human nutrition.

In a recently published study, Lanteaume et al. (17) fed rats with diets containing 15% of heated fats. The fats were grapeseed oil which had been used 60 times for frying potatoes without replenishment of fat lost by absorption in the potatoes, or grapeseed oil which had been heated 2 days or 4 days in an open beaker at 200°. The feeding study started with rats 7 months of age and lasted for one year. Unheated grapeseed oil was slightly more absorbable than the fat used for frying and gave slightly greater weight gains. The artificially heated fats were slightly less absorbable, and gave slightly lower weight gains. Otherwise no significant differences were observed among the groups according to the several clinical, biochemical, and histological observations made.

Received for publication May 3, 1967.
[1] Present address: Department of Nutrition, University of Guelph, Guelph, Ontario, Canada.
[2] To whom inquiries should be addressed.

We wished to conduct an experiment whose results, more nearly than any of those cited above, would demonstrate whether fats repeatedly used in actual frying practice have any meaningful effects in normal diets. For this experiment we studied 7 fats. Two of these were unheated controls. Four had been used for frying until they reached the end of their frying usefulness as evidenced by excessive foaming tendency. One was used for frying until it had attained a steady state of foaming tendency. The distillable non-urea-adducting fractions of these fats were isolated and bioassayed, and the fats themselves were fed to rats over a 2-year period.

EXPERIMENTAL

Fats. The fresh and used fats studied in this experiment are described in table 1. The table also shows the code letter assigned to each fat, which will be used to identify it in the other tables. All the fats had been prepared by normal factory or pilot plant processes. The cottonseed oil and soybean oils had been refined, bleached, and deodorized. Analyses of the fats are reported in table 2.

Frying procedure. The used fats were prepared by heating them in 60-kg (fat capacity) gas-fired kettles. The fats were heated at 182° each weekday from 8 AM to 4 PM. At 9 AM and at 2 PM in each kettle 3.95 kg each of frozen potatoes, breaded scallops, and onion rings, in that order, were fried for 5 minutes, 2.5 minutes, and 3 minutes, respectively. These foods were obtained through normal commercial food channels. At 4 PM daily 2.47 kg of fat were added to each kettle to replace the fat that had been absorbed by the fried foods. The kettles were allowed to cool overnight and on weekends. During the frying of the onion rings, the height of foam was measured above the previously measured level of still fat in the kettle. When the foam height exceeded 75 mm that fat was removed from the kettles and protected against oxidative changes which might have occurred during storage by the addition of 39 ppm butylated hydroxytoluene plus 31 ppm butylated hydroxyanisole. The fats were sealed in 453-g cans under nitrogen before storage in a frozen-food warehouse. Fat D (soybean oil, IV 108, with silicone) never attained the 75-mm foam height end point owing to the stabilizing effect of the silicone. Frying in that fat was discontinued after it became apparent that a steady state of foam height had been reached due to fat turnover.

TABLE 1
Fats studied

Code	Description
A	Soybean oil, fresh (not used for frying)
B	Soybean oil, hydrogenated to IV 108, fresh
C	Soybean oil, hydrogenated to IV 108, used for frying
D	Soybean oil, hydrogenated to IV 108, with 1.6 ppm methyl silicone added, used for frying
E	Soybean oil, hydrogenated to IV 70, used for frying
F	Cottonseed oil, used for frying
G	Lard, used for frying

Total duration of the heating ranged from just over a week to over 5 weeks. Table 2 shows the number of hours each of the fats was heated and the analytical values after heating. Samples of used fats were withdrawn from storage and analyzed at intervals up to 2 years; the zero- and 2-year analyses are also shown in the table.

Feeding study. The fats were incorporated at a level of 15% into the semipurified diet shown in table 3. The diets were prepared fresh each week and kept refrigerated until dispensed into the feeding cups. Feeding was carried out 3 times per week, and any feed remaining in the cups was discarded, so that the longest period that any of the feed was unrefrigerated after mixing was 3 days. Previous experiments (unpublished) had shown that the results obtained with such a system of diet handling were indistinguishable from results obtained with daily mixing of diets, whereas weekly feeding had led to relatively poor growth with diets containing used fats.

Four hundred each of male and female rats [3] were received as weanlings. After 3 days the extremely light and heavy animals were discarded. The remainder were sorted into 7 groups of 50 males and 50 females, balanced on the basis of weight and litter. The rats were kept individually

[3] Simonsen Standard Laboratory, Sprague-Dawley-originated, Caesarean-derived rats from Simonsen Laboratories, White Bear Lake, Minnesota 55110.

TABLE 2
Analyses of fats

Fat	Frying time	Storage time	Lovibond color [1]	Free fatty acid [1]	Iodine value [1]	Peroxide value	n_D^{60}	Polar fraction [2]	Conjugated diene [2]	Conjugated triene [2]	Fatty acid composition by gas-liquid chromatography						
											14:0	16:0	16:1	18:0	18:1	18:2	18:3
	hours	years		%				%	%	%	%	%	%	%	%	%	%
A	0	0	10/1.0	0.04	129.0	1.6			50.6	7.3	0	11	0	5	27	51	6
	0	2	12/1.2	0.03	130.0	0.5	1.4603	1.4	51.2	7.7	0	11	0	4	26.5	52	6
B	0	0	3/0.3	0.02	108.2	1.2	1.4578		33.5	3.0	tr	11	0	5	45	35	4
	0	2	4/0.4	0.02	108.5	0.1	1.4577	1.3	33.2	3.1	0	11.5	0	6	46	34.5	2
C	0	0	3/0.3	0.02	108.2	1.2	1.4578		33.5	3.0	0	12	0	6	50	31	1
	60	0	300/80	0.65	100.5	3.8	1.4597	13.6	28.1	2.3	0	13	0	6.5	50	30	0.3
	60	2	300/80	0.76	101.0	3.5	1.4597	14.6	28.1	2.3							
D	0	0	3/0.3	0.02	108.5		1.4578				tr	11	0	5	46	35	3
	216	0	3500/430	8.10	101.5	0	1.4589	30.2	33.6	3.0	0	11	0	6	50	32	1
	216	2	1800/400	8.20	101.5	0.7	1.4588	32.5	28.5	2.7	0	12	0	6.5	49.5	30.5	1.5
E	0	0	3/0.2	0.03	69.7	0	1.4533		5.1	0.5	0	15	0	12	66	7	0
	84	0	300/60	1.30	63.5	5.5	1.4552	20.1	3.2	0.08	tr	16	0	14	65	5	tr
	84	2	300/80	1.30	66.4	1.9	1.4551	19.0	2.6	0.1	0	16	0	14.5	65.5	4.0	0
F	0	0	8/0.8	0.03	109.0	0.6	1.4581		53.8	0.44	1	27	0	2	17	53	0
	49	0	200/50	0.45	102.5	4.6	1.4601	14.2	44.9	0.33	1	31	0	2	17	48	0
	49	2	200/60	0.48	102.0	4.4	1.4598	15.2	46.5	0.6	1	30	0	2.5	17	49.5	0
G	0	0	1/0.1	0.05	64.4	0	1.4527		13.1	0.98	1	24	0	13	46	10	3
	116	0	600/110	2.30	55.6	3.0	1.4550	23.4	7.5	0.42	1	30	0	15	46	6	0
	116	2	500/140	2.40	55.7	1.3	1.4550	25.5	7.5	0.40	2	31	1.5	15	44.5	5.5	0

[1] AOCS method (24)
[2] Measured by adsorption chromatography on silicic acid containing 4% methanol; eluted with methanol:benzene 1:49. Method of E. N. Frankel (unpublished).
[3] AOCS method (24) involving alkali isomerization and UV absorption, as linoleate and linolenate.

TABLE 3
Composition of diet

	%
Casein [1]	5.0
Non-fat dry milk [2]	21.0
Ground whole wheat	43.0
Dried egg white [3]	3.0
Dried liver, defatted [4]	3.0
Vitamin mixture in sucrose [5]	3.0
L-Lysine·HCl	0.5
Calcium phosphate, dibasic [6]	1.0
Salt mixture [7]	3.5
Vitamin mixture in soybean oil [8]	2.0
Experimental fat	15.0
Kilocalories/g	4.4

[1] Labco, Vitamin-Free, Whitson Products Division of the Borden Company, New York.
[2] Land O'Lakes Creameries, Minneapolis.
[3] Nutritional Biochemicals Corporation, Cleveland; biotin was added at a level of 1 mg/100 g of albumen.
[4] Wilson Laboratories, Inc., Chicago.
[5] Furnished the following in mg/kg of diet: menadione, 1.8; thiamine, 2.4; riboflavin, 3.0; niacin, 12.0; folic acid, 0.15; Ca pantothenate, 12.0; pyridoxine, 2.4; inositol, 12,000; p-aminobenzoic acid, 60.0; biotin, 0.015; cyanocobalamin, 90.0; ascorbic acid, 60.0; and choline chloride, 1800.
[6] Added to maintain calculated Ca and P at 2% and Ca:P ratio at 1.1:1.0.
[7] Phillips and Hart (25) but with the addition of 0.005% cobalt chloride; obtained from Nutritional Biochemicals Corporation, Cleveland.
[8] Soybean oil furnished essential fatty acids, and the mixture furnished the following IU per kg of diet: 12,000 vitamin A; 3,200 vitamin D_3; and 100 vitamin E as d-α-tocopheryl acetate.

in galvanized steel cages, 26 × 30 × 17 cm, at 23 ± 1° and 50 ± 5% relative humidity. They received 12 hours of light per day uniformly. The individuals within the groups were so distributed as to avoid bias caused by position within the racks or the room. Feed and water were given ad libitum. Feed consumption records were maintained for each animal. Each week the animals were weighed and carefully examined for abnormalities. As they aged, sick ones were separated from the rest, and their values were removed from the subsequent growth and feed data, but were included in the longevity and pathology data.

Metabolic studies. For the determination of fat absorbability, and coefficient of absorbability, feces were collected from 10 randomly selected rats per sex per dietary group for two weeks at 2, 12, and 21 months, by means of wire-screen collectors fastened below the regular cages. The feces were dried in vacuo at 80°, cleaned, and pulverized. Total fatty acids were determined by saponification with alcoholic KOH, acidulation, dilution with water, extraction with petroleum ether, drying and evaporating the extract, and weighing the dried residue. Fecal nitrogen was determined by the Kjeldahl method. For the calculations, endogenous fat was assumed to be 0.03 g/day for males and 0.02 g/day for females; these values were based on our experience with similar animals fed purified rations.

Urine was collected from 10 randomly selected animals per sex per group at 2, 12, and 21 months. The rats were given water but not feed during the 12-hour collection period, which followed a 12-hour fast. Rats were kept in metabolism cages for the collection. The urine was collected under toluene; each sample was diluted to 25 ml and analyzed for nitrogen (Kjeldahl), and for sugar, albumin, bilirubin, and ketone bodies by means of test tablets and strips. [4]

Clinical studies and pathology. From each group and sex there were chosen at random 5 rats at 12 months and 10 rats at 24 months. Blood samples were obtained from these animals by caudal artery puncture for blood cell counts, hematocrit, hemoglobin, and blood glucose analyses. The same animals were anesthetized with ethyl ether, and 5-ml samples of blood were drawn from the heart for plasma cholesterol (18) and plasma phospholipid determinations (19). The animals were then put to death by excess of the ether; the heart, liver, and kidneys were removed and weighed for the calculation of organ-to-body weight ratios. The heart, liver, and kidneys were assayed for sodium, potassium, and calcium by flame photometry at 12 months and by atomic absorption spectroscopy at 24 months. Liver cholesterol and phospholipid values were determined. Peritoneal fats were converted to methyl esters, and their fatty acid compositions were determined by gas-liquid chromatography. Thymus, heart, lung, liver, stomach, pancreas, spleen, adrenal, kidney, mesenteric lymph nodes, ileum, gonads, and any apparent neoplasms were removed for histological examination. Slides were prepared and studied within our laboratories, and were also examined by a consulting veterinary pathologist. Animals that died or were killed because moribund were autopsied, and if putrescence had not started the same tissues were removed for similar histology.

[4] Ames Company, Inc., Elkhart, Indiana.

Statistical treatment. Where applicable the analysis of variance was used to test for significance. The final growth and feed consumption data were tested by the use of harmonic means because of the extreme disproportionalities produced in the data by intermediate deaths (20). Minimum significant differences were computed by the method of Tukey as described by Scheffe (21).

Isolation and bioassay of distillable non-urea-adducting fractions (DNUA). The DNUA's were isolated by a modification of the method of Crampton (22). A 1-kg portion of each fat was saponified with aqueous-alcoholic KOH. The soaps were acidulated, and the free fatty acids were extracted. The acids were converted to ethyl esters by twice refluxing for 30 minutes with 2 volumes of ethanol containing 0.5% H_2SO_4. The esters were distilled under high vacuum at pot temperatures up to 240°. Each distillate was mixed with 4 times its weight of powdered urea and 4 times its volume of ethanol. The slurry was held at 55° for 30 minutes before cooling overnight to room temperature. After filtration the filter cake was washed with urea-saturated alcohol. The combined alcohol solutions were mixed with water and extracted with hexane, then ether. The extracts were concentrated, dried, and evaporated under reduced pressure, leaving behind the DNUA. Yields of DNUA are shown in table 4.

For bioassay 0.5 ml of a DNUA was administered daily for 3 consecutive days to each of 5 weanling male rats (7). Administration was by stomach tube. The rats were given a commercial pelleted ration and water ad libitum during the experiment. Survivors were killed on the tenth day, at which time body and thymus weights were determined. As controls, similar groups of rats were not given any DNUA, or else were given the DNUA from olive oil which had been heated 50 hours at 182°, the latter having previously been shown in our laboratory to be toxic under these conditions. Results of the bioassay are shown in table 4.

RESULTS

Fats. Analytical values on the fresh fats (table 2) showed them to be typical of their respective kinds. The analytically measurable changes which took place in the fats during frying were generally similar to the changes observed by many other

TABLE 4
Acute toxicity test

Sample characteristics [1]	Level of DNUA in fat	Avg body wt gain [2]		Thymus wt [3]	Diarrhea incidence	Mortality
		3 days	10 days			
	% by volume	*g*	*g*	*mg*	*%*	*%*
DNUA of Fat A	2.0	17.2	69.4	425	0	0
DNUA of Fat B	1.5	19.6	71.2	446	0	0
DNUA of Fat C	2.1	0.3	54.7	359 (81)	0	40
DNUA of Fat D	2.0	9.2	59.6	391	0	0
DNUA of Fat E	2.0	−10.4	24.0	167 (32)	100	20
DNUA of Fat F	2.3	7.8	55.6	328	0	0
DNUA of Fat G	1.7	9.8	57.3	409 (88)	0	20
Positive control [4]	2.4	−10.0	39.3	142 (84)	40	40
Negative control, pellets only	—	21.6	66.6	445	0	0

[1] There were 5 weanling male rats/group, and each rat was given 0.5 ml of lipid/day for 3 consecutive days. DNUA = distillable non-urea-adductable fraction.
[2] These results are for surviving animals only.
[3] Weights shown are averages for animals living at 10 days. Values in parentheses are for dead rats; normal rats of the same age as the dead ones have thymus weights averaging 210 mg.
[4] DNUA of olive oil heated 50 hours at 182°

TABLE 5

Cumulative growth, feed consumption, and feed efficiency[1]

Fat	Avg wt gain			Avg feed consumption			Feed efficiency [2]			Coefficient of absorbability [3]		
	2 months	12 months	21 months	2 months	12 months	21 months	2 months	12 months	21 months	2 months	12 months	21 months
	g	g	g	g	g	g	%	%	%	%	%	%
Male												
A	365 [a]	727 [a]	783 [a]	974 [a]	6619 [a,b]	11870 [a]	37.4 [a]	11.0 [a]	6.6 [a]	96.2 [a]	96.5 [a]	97.0 [a]
B	358 [a,b]	725 [a,b]	763 [a]	963 [a]	6511 [a]	11747 [a,b]	37.2 [a]	11.1 [a]	6.5 [a]	95.5 [a]	96.8 [a]	96.6 [a]
C	339 [b]	677 [b]	764 [a]	951 [a]	6435 [a]	11674 [a,b]	35.7 [a,b]	10.5 [a,b]	6.5 [a]	90.3 [a]	92.4 [a]	94.2 [a]
D	344 [a,b]	704 [a,b]	774 [a]	965 [a]	6604 [a,b]	11848 [a,b]	35.6 [a,b]	10.6 [a,b]	6.5 [a]	91.5 [a]	92.7 [a]	92.2 [a]
E	339 [b]	682 [b]	727 [a]	962 [a]	6506 [a]	11861 [a,b]	35.3 [a,b]	10.1 [b]	6.0 [b]	82.2 [c]	88.2 [c]	86.9 [c]
F	340 [b]	703 [a,b]	747 [a]	1001 [a]	6929 [a,b]	12292 [a,b]	34.0 [a,b]	10.1 [b]	6.0 [b]	87.9 [a]	90.0 [a]	89.8 [a]
G	335 [c]	679 [a,b]	737 [a]	976 [a]	6855 [a,b]	12380 [b]	34.7 [a,b]	9.9 [c]	6.0 [b]	87.8 [a]	89.4 [a,c]	89.3 [a]
Female												
A	200 [d]	405 [c]	525 [b]	732 [b]	4897 [c]	9409 [c]	27.5 [d]	8.2 [c]	5.6 [c]	96.8 [a]	97.5 [a]	97.6 [a]
B	204 [d]	392 [c]	518 [b]	726 [b]	4825 [c]	9238 [c]	28.0 [d]	8.1 [b,c]	5.6 [c]	96.4 [a]	96.8 [a]	96.4 [a]
C	196 [d]	375 [c]	466 [b]	731 [b]	4901 [c]	9164 [c]	26.9 [d,e]	7.6 [b,c]	5.0 [c,b]	92.1 [a]	92.7 [a]	92.5 [a]
D	201 [d]	377 [c]	463 [b]	744 [b]	4974 [c]	9323 [c]	27.1 [d]	7.6 [b,c]	4.9 [c,b]	92.7 [a]	94.0 [a]	93.6 [a]
E	189 [c]	362 [c]	446 [b]	746 [b]	5018 [c]	9262 [c]	25.5 [d]	7.1 [c]	4.8 [b]	87.6 [b]	89.6 [b]	89.6 [b]
F	196 [d]	374 [c]	486 [b]	741 [b]	4913 [c]	9145 [c]	26.4 [d]	7.6 [b,c]	5.3 [c,b]	91.1 [a]	91.8 [a]	92.0 [a]
G	196 [d]	372 [c]	454 [b]	749 [b]	5131 [c]	9441 [c]	26.1 [d]	7.2 [c]	4.8 [b]	89.8 [a]	91.9 [a]	90.3 [b]

[1] Any 2 values within a column which are followed by the same letter are not significantly different ($P \geq 0.05$) from each other.
[2] Feed efficiency = weight gain × 100/feed consumption.
[3] For experimental fat level only. Allowance was made for fat-soluble vitamin mixture and metabolic fat.

given for coefficient of absorbability (table 5). Two factors appear to have influenced absorbability: heating, and degree of unsaturation. The unheated fats were most absorbable, and also, on account of the experimental design, the most unsaturated. Among the heated fats, the 2 samples of lightly hydrogenated soybean oil (C and D) were more absorbable than the lard or cottonseed oil, while Fat E, the soybean oil which had been hydrogenated to IV 70 before frying, showed the lowest values for coefficient of absorbability. This same relationship of absorbabilities prevailed in both sexes at all ages. The lower absorbability of the heated fats is probably due to their content of unabsorbable (22) polymeric materials, formed during heating.

Detailed results of the many clinical analyses carried out — fecal nitrogen, urine volume, pH, nitrogen, and protein, blood counts, hemoglobin, hematocrit, blood glucose, plasma cholesterol and phospholipid, organ-to-body weight ratios, liver lipid, phospholipid and cholesterol, liver and kidney calcium, sodium, and potassium — are not shown in the tables. The values were generally within normal ranges and were not remarkable. Many of the values show differences between the sexes and at different ages, but there were few statistically significant ($P = 0.05$) differences assignable to dietary groups within the age-sex groupings. All of the statistically significant differences which were seen are summarized in table 6. Most of these differences appear to be of a random nature, showing no pattern which can be correlated with fresh vs. used fats. An exception to this is the liver cholesterol-to-phospholipid ratio at 24 months; the relatively high values observed in the male groups receiving unheated fats might be related to the relatively high incidence of obesity in those groups. The biological significance of differences in mineral content of the various organs is questionable, since these values showed wide variations, both within and among the various groups. Analyses of peritoneal fats showed their fatty acid compositions to be generally parallel to the fatty acid compositions of the respective dietary fats.

Longevity and pathology. Table 7 shows the percentage of animals surviving at

workers in the past. There were small but irregular increases in the free fatty acid levels, peroxide values, and refractive indices, and small decreases in the iodine values. There were small decreases in the relative levels of polyunsaturated fatty acids, presumably as a result of oxidation and polymerization. The content of polar material in each fat increased markedly during frying. The ultraviolet, gas chromatographic, and urea adduction results show that the majority of the fatty acid chains were unaffected by the heating procedures. The properties of the fats did not change appreciably during storage for 2 years, showing that the storage conditions were adequate for preservation of the fats in their originally prepared states.

Two-year feeding study. Table 5 shows mean growth and feed consumption data for 2, 12, and 21 months. Values for intermediate times were generally similar to the ones shown, and are omitted for brevity. The 24-month growth data are omitted since deaths among the senescent animals caused extreme variations within the groups. During the early, rapid growth stage the two fresh fats produced generally more rapid growth than the others, although Fat D (soybean oil, IV 108, with silicone) was nearly equivalent. The growth differences were more pronounced among the male than the female rats. The differences in body weights which arose during the early growth period tended to persist throughout the experiment, although they became relatively smaller with time, and statistically insignificant with increasing variance among the individuals. Part of the reason for the diminution of weight differences with time was that the heavier groups, namely those receiving Fats A (fresh soybean oil), B (fresh soybean oil, IV 108), and D (used soybean oil, IV 108, with silicone), had slightly higher mortality rates than the others, and these deaths occurred mostly among the more obese individuals. Deaths of the more obese animals in the heavier groups lowered the mean weights of those groups.

The differences in growth could not be attributed to differences in feed consumption. Instead, rapid growth appears to have been associated with high feed efficiency, and is accounted for by the values

TABLE 6

Statistically significant differences in metabolic and clinical values

Analysis	Sex	Age	Direction of difference
		months	
Fecal nitrogen	F	21	B > A, D, G
Urine volume	F	12	E > A, C, D, F
Heart wt/body wt	M	12	C > E
Liver wt/body wt	F	12	E > A, B, D
Liver wt/body wt	F	24	F > A
Liver cholesterol	M	24	A > B, C, D, E, F, G
Liver cholesterol/phospholipid	M	24	A, B > C, D, E, F, G
Kidney calcium	F	12	F > D
Liver potassium	F	24	A, B, C > D, E, F, G
Kidney potassium	F	12	G > C
Kidney potassium	F	24	A, C > E
Kidney sodium	F	24	A > B, D, F, G

TABLE 7

Percentage of animals surviving at various ages

Diet group	Males, age in months				Females, age in months				Total, both sexes
	12	18	21	24	12	18	21	24	
A	96	84	80	62	92	80	60	33	48
B	98	87	73	53	100	91	80	58	56
C	96	96	87	67	98	91	80	60	63
D	88	73	66	53	98	89	80	58	55
E	100	87	82	69	96	89	80	67	68
F	100	96	82	64	100	93	87	56	60
G	98	93	91	62	98	96	93	84	73
Avg	96.5	88	80	61	97	90	80	59	60

several ages. Sixty percent of the animals survived for 2 years. There was only a slight difference between the sexes in most groups. Survival rates were slightly lower for animals receiving the two unheated fats and Fat D (soybean oil, IV 108, with silicone, used) than for animals receiving the other heated fats. This difference parallels and is attributed to the larger mean weights, the higher incidence of obesity, and the better utilization of fat in those same groups. The table shows what appears to have been a high incidence of early mortality in the males receiving Fat D (soybean oil, IV 108, with silicone, used). The value shown resulted from 6 deaths within the first year; two were from unknown causes, one from a tumor of the prostate, two from pneumonia, one from a bladder infection. The pneumonia and bladder infection are not uncommon among such animals as these in our laboratory, and we see no reason to suppose that the distribution of mortality was not random.

Table 8 shows the incidence of tumors, respiratory disease, nephritis, and liver pathology observed grossly in all rats. The females developed more tumors than the males, owing to their susceptibility to mammary carcinoma. The higher incidence of tumors in the females contributed to their having mortality rates equal to the males. No diet-related differences in tumor incidence appeared in the males, but the females receiving the two fresh fats had a higher incidence of tumors than those receiving used fats. It has been reported (23) that a direct relationship exists between caloric intake and tumor risk in the rat, and, as pointed out above, the two fresh fats furnished more absorbable energy than the used fats.

The histological findings on the 10 rats killed from each group are summarized in table 9. In both sexes the incidence of

TABLE 8

Percentage incidence of various pathological conditions [1] (all animals, all ages)

Fat	A		B		C		D		E		F		G	
Sex	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Tumors [2]	20.0	55.6	22.2	53.3	17.8	37.8	22.2	28.9	22.2	33.3	26.7	28.9	13.3	31.1
Respiratory disease [3]	22.2	11.1	15.6	6.7	22.2	13.3	20.0	11.1	11.1	8.9	2.2	6.7	11.1	6.7
Nephritis	30.0	8.0	24.0	14.0	20.0	12.0	22.0	6.0	30.0	12.0	18.0	12.0	30.0	18.0
Liver pathology	24.0	34.0	48.0	38.0	6.0	28.0	18.0	24.0	28.0	30.0	12.0	26.0	22.0	30.0

[1] Grossly observable tumors and disease symptoms only.
[2] Male tumor incidence, 20.6%; female tumor incidence, 38.4%.
[3] Severe respiratory disease with clinical symptoms; male respiratory disease incidence, 14.9%; female respiratory disease incidence, 9.2%.

fatty infiltration of the liver was relatively high in animals receiving the unheated fats and quite low in animals receiving Fat E (soybean oil, IV 70, used) or Fat G (lard, used). Thus it appears that this condition also may be related to the intake of absorbable energy, but not specifically to the ingestion of fresh or used fats. None of the other frequently seen pathology appears to have been related to dietary differences. Various other pathological conditions were observed only infrequently, and could not be related to dietary differences.

Acute toxicity test. Crampton et al. (22) showed that the toxic substances formed by heating linolenate under carbon dioxide could be concentrated into the DNUA fraction. The distillable urea-adducting fraction contained esters of unchanged fatty acids, while the non-distillable fraction contained polymers which, although not nutritious, were non-toxic by reason of their non-absorbability. For assaying the presence of toxic substances in the DNUA fractions of heated fats, we have used a method (7) which involves forced feeding of weanling rats with large quantities (ca. 30 g/kg) of the DNUA over a 48-hour period. Our experiences with this procedure show that its results parallel those observed by Crampton, but it is quicker and uses much less material. Table 4 shows that, as expected, DNUA fractions from the fresh fats did not harm the rats receiving them, while those from the heated fats were all harmful in one or more respects, causing diminished weight gains, premature involution of the thymus, diarrhea, or death. The number of animals used was too small to permit quantitative comparisons among the various used fats; it is clear that all of them contained low levels of substances toxic to the weanling rat.

DISCUSSION

The aim of this experiment was to feed high levels of fats which had been heated under conditions as severe as any ever likely to be encountered as constituents of fried foods in the normal American diet. Therefore we chose what we believed to be the worst possible conditions for preparing the fats, short of conditions which

TABLE 9

Percentage incidence of pathology in two-year-old rats (histological examination of ten rats/group, per sex)

Fat	A		B		C		D		E		F		G	
Sex	M	F	M	F	M[1]	F	M	F	M	F	M[1]	F	M	F
Fatty liver	40	50	30	20	22	10	20	20	0	—	11	10	10	—
Chronic pyelonephritis	50	50	80	50	77	20	80	30	60	10	55	50	40	50
Tubule mineralization in kidney	—	40	—	20	—	40	—	60	—	50	—	30	—	30
Adrenal telangiectasis	—	30	30	70	22	70	—	30	10	20	—	40	20	40
Alveolar foam cells	20	10	20	—	—	—	20	10	20	30	66	20	10	20
Tumors:														
Mammary	—	60	—	70	—	40	—	20	—	30	—	30	—	20
Others	20	—	10	20	33	0	20	20	10	—	22	—	30	10

[1] Only 9 slides/group were examined by the consulting pathologist.

would have been wholly unrealistic in relation to culinary practice.

Of the ways in which fats are used in cooking, three involve relatively severe heat treatment. We may distinguish these as 1) pan frying, 2) continuous frying, and 3) intermittent deep-fat frying. In pan-frying or sautéing, fats are heated strongly, but only for short periods of time. Such fats are not ordinarily saved and re-used, and hence there is little opportunity for any transformation products which might form to build up to substantial levels.

In many commercial frying operations, such as doughnut or potato chip manufacturing, where frying is continuous, fat is constantly absorbed by the food and carried out, to be replaced by fresh fat. The turnover time is short enough so that there is little opportunity for significant changes in fat composition to occur; the levels of color, flavor, foaming tendency, and free fatty acids are not objectionable, so it is seldom or never necessary to discard the fat and replace it with new.[3] It seems clear that if one is to look for nutritionally undesirable changes in used frying fat, they should be sought not here, but rather in the low fat-turnover operations of small volume, batch-frying establishments.

Some restaurants frequently keep their fat hot for many hours a day, fry in it occasionally, allow it to cool overnight, and reheat it next day. While this pattern of use continues, the polymer content and viscosity of the fat increase. Eventually it either foams over the sides of the kettle or becomes unacceptably dark in color; then it is discarded and replaced. (In many restaurants, of course, the frying volume is great enough so that there is a rapid turnover of fat, and here the situation is not unlike that which exists in the continuous frying operations discussed above.) In our opinion, those fats used for frying until they threaten to foam out of the kettle are the most severely heated fats likely to be consumed in the normal human diet, and it was such fats that we chose for our feeding study. Along with them we fed a fat (D) whose frying life had been greatly extended through the use of silicone, and which had been used for frying until its content of transformation products (as indicated by foam height) appeared to have reached a steady state through fat turnover, even though the quantity of food being fried in it was relatively small.

Most previous workers have studied fats which had been heated at higher temperatures or with more vigorous aeration than would be found in actual frying practice. Attempts to apply conclusions based on such work to human nutrition seem unwarranted, owing to the likelihood that such artificially abused fats may differ from practically used fats in the kind, as well as the levels, of transformation products which they contain.

[3] Robertson, C. J. 1966 The principles of deep fat frying for the bakery. Bakers Dig., 40 (5): 54.

Some factor of exaggeration is necessary and appropriate in feeding studies for detecting mild toxicity or low levels of toxic substances. Such exaggeration was achieved in the acute bioassay stage of our work by concentrating the altered fatty acids from used fats through urea adduction. The DNUA fractions of the used fats elicited manifestations of acute toxicity, and even caused death in some cases, when 1.5-ml doses of them were given to weanling rats over a 48-hour period. It is clear from this that toxic substances did form in our fats during the heating program, although at quite low levels. To administer in whole fat the same quantity of these toxic substances as was administered in the DNUA concentrate would have required dosing each rat with more than his own body weight of used fat during a 48-hour period.

Chronic toxicity of the DNUA's and of other used fat components were evaluated in the long-term stage of our experiment. In the 2-year study, a considerable degree of exaggeration compared with human diets was maintained (a) by using the fats up to the practical end-point of their usefulness for frying, rather than to some intermediate point corresponding to the average of the fats being used in actual food preparation, (b) by feeding them at the high level of 15% of the diet, and (c) by feeding them steadily throughout the lifetime of the animals. Even with these exaggerations, the experiment produced no evidence that used frying fats adversely affected the health or longevity of the animals consuming them.

The literature reports that artifically abused fats contained substances which are toxic to laboratory animals. From such reports it has been inferred that it may be harmful for humans to consume used frying fats. We have found that actual used frying fats contain only very small quantities of substances which are toxic when administered in large doses to weanling rats, and that the fats themselves produce no appreciable ill effects on animals consuming them.

ACKNOWLEDGMENT

Doyle Johnson furnished valuable information about commercial frying conditions and supervised the preparation of the fats used in this study.

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Protein Quality of a Soybean Protein Textured Food in Experimental Animals and Children [1,2]

RICARDO BRESSANI, FERNANDO VITERI, LUIZ G. ELIAS, SILVIA DE ZAGHI, JORGE ALVARADO AND A. D. ODELL
Institute of Nutrition of Central America and Panama (INCAP), Guatemala, Central America

ABSTRACT Studies were carried out in experimental animals and children to evaluate the protein quality of a textured food simulating ground beef, and made from isolated soybean protein with added egg albumin and wheat gluten. On the basis of a PER value of 2.50 for casein, the PER of the textured food and of natural dehydrated beef was 2.30 and 2.34, respectively. Highest weight gain was obtained with diets containing 16.7 and 16.3% protein, from the soybean protein textured food and casein, respectively. NPU values were 62.6 for casein and 59.1% for the soybean protein textured food. Heating of the soybean protein textured food increased weight gain but not the PER. Heating of the protein isolate and of the fiber made from it improved both. Apparently this treatment caused the elimination of adverse physiological factors inherent in soybean, or the removal of substances in the product derived from the preparation process. At the 10% protein level, supplementation with lysine and methionine added together, but not alone, improved protein quality. Growth and nitrogen balance studies with dogs indicated that the soybean protein textured food had essentially the same protein quality as that of dehydrated beef. True protein digestibility and biological value were 92.3 and 65.3%, respectively, for the soybean protein textured food, and 87.0 and 67.4% for the dehydrated beef. The results in children show that, at a protein intake level of 2 g/kg/day, no difference in quality was evident between skim milk and the soybean protein textured food. Nitrogen equilibrium was obtained when the children received approximately 138 mg of nitrogen from the soybean protein textured food, as compared with 97 mg from milk. The true protein digestibility and biological value was 92.3 and 65.3%, respectively. It was concluded that the protein quality of the soybean protein textured food was about 80% of that from milk. It was readily accepted by the children and free of adverse physiological effects.

Highly purified proteins are being isolated now from oil-free, food-grade protein concentrates such as soybean, cottonseed and sesame. Among these, protein isolates prepared from oil-free soybean flakes have received the greatest attention. The protein content of the isolate runs as high as 95%, and the products are bland in taste and have none of the flavors normally associated with the flours and other similar products (1–3).

The protein isolates are available in monofilament, granular or powder forms, which make them suitable for a wide range of functional uses, such as whipping, emulsifying, gelling, stabilizing, thickening and moisture-binding. Thus, the number of food products which can be made from them is practically unlimited.[3-6]

The essential amino acid pattern present in such isolates is, in the majority of cases, essentially the same as that in the material from which it was prepared. However, the process of isolation, eliminating certain protein fractions, as well as the use of variable temperatures, treatment with chemicals and pH changes, may alter the nutritive value of such products. Information on the nutritive value of the protein of these isolates is not very extensive, and

Received for publication January 30, 1967.
[1] Supported by U. S. Public Health Service Research Grant no. AM-3811 from the National Institute of Arthritis and Metabolic Diseases, and a grant from the General Mills, Inc., Minneapolis.
[2] INCAP Publication I-428.
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Purification of Cyclic Fatty Acid Esters: a GC-MS Study[1]

EDWARD G. PERKINS[2] and WAYNE T. IWAOKA,[3] Department of Food Science,
The Burnsides Research Laboratory, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Gas chromatographic analysis of cyclic monomeric concentrates and fractions from argentation chromatography on packed columns containing SE-30, OV-25 and Apiezon L stationary phases yielded incompletely separated peaks representing the various isomers present in the mixture. Somewhat better separation was achieved using a 6 ft x 1/8 in. column packed with 15% EGS on Chromosorb W. This column, when coupled to a mass spectrometer, yielded information concerning the composition of each of the isomeric components. Comparable results were obtained using a 50 ft x 0.02 in. S.C.O.T. column with DEGS stationary phase and a 150 ft x 0.01 in. capillary column coated with Apiezon L. While argentation thin layer chromatography proved useful, an argentation column method using silicic acid coated with 10% $AgNO_3$ proved more efficient for larger scale preparations. Elution of the column with 2% diethyl ether in petroleum ether yielded material essentially free of conjugated linolenate. A comparison of the behavior upon argentation thin layer chromatography of conjugated methyl linolenate, methyl linoleate and cyclic monomer esters indicated that these esters migrated to the same relative position as methyl oleate.

INTRODUCTION

The cyclization of linolenic and linoleic acids has been studied extensively. Schofield and Cowan demonstrated that linolenic acid could be converted to a cyclized structure by heating in the presence of solvent and alkali (1). Subsequent papers have concerned themselves with methods for improving the preparation and yields of saturated cyclized product (2), its separation from straight chain fatty acids (3), determination by gas liquid chromatography (GLC) (4), and a structural study of these products as mixtures of the corresponding saturated and aromatic isomers (5). Extensive earlier structural work has been reviewed by Friedrich (15), including work on cyclic products from eleostearate.

Much of this interest in cyclic fatty acids stems from their potential as industrial chemicals (6). However of equal if not greater importance is the fact that cyclic monomers of fatty acids (primarily linoleic acid) have been found in fats heated under relatively mild conditions. For instance, cyclic monomers have been found as a component of heated cottonseed oil (7) and heated linoleic acid (8,9). They have been isolated from soybean oil (10) and corn oil (11) heated in deep fryers. In addition, they are formed during the thermal oxidation in the laboratory of pure triglycerides containing oleic (12) and linoleic (13) acids. Furthermore cyclic monomers have been isolated as by-products from the hydrogenation of fats and oils (14,15). The field of cyclic compounds in fats and oils has recently been reviewed comprehensively by Artman (16).

As part of our program to determine the metabolic properties of heated fat components, a mixture of cyclic fatty acids was prepared from pure linolenic acid. These experiments were to precede our objective of preparing a purified ^{14}C-labeled cyclic monomer for use in metabolic studies. It was therefore important to remove as much starting material as possible from the product, since the presence of such material, especially in the labeled conjugated form, would yield ambiguous results. The present paper is concerned with the preparation and partial characterization of a purified monomer fraction.

[1] Presented at the AOCS Meeting, Los Angeles, April 1972.

[2] Author to whom inquiries should be addressed.

[3] Abstracted in part from the dissertation to be submitted to the University of Illinois Graduate College in partial fulfillment of the requirements for the Ph.D. degree.

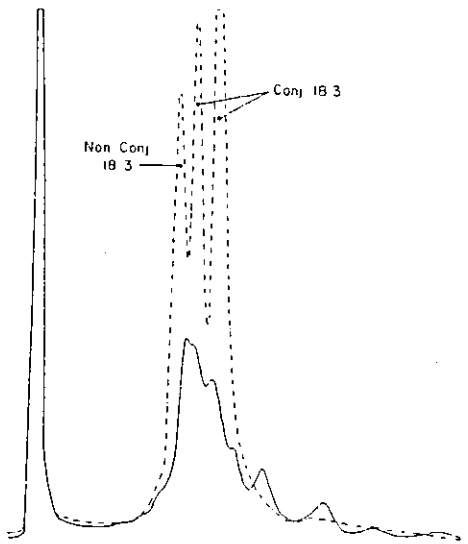


FIG. 1. Gas liquid chromatogram of cyclic monomer reaction product methyl esters with both conjugated and nonconjugated methyl linolenates (18:3) (6 ft x 1/8 in. S.S., with 15% EGS, Chromosorb W [AW] 60-80 mesh).

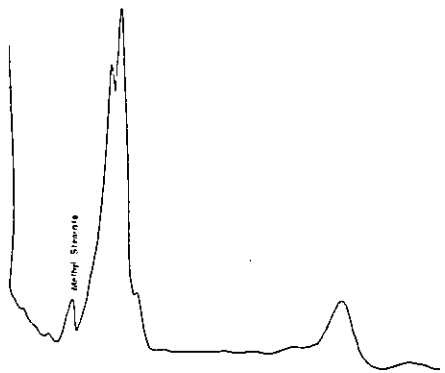


FIG. 2. Gas liquid chromatogram of hydrogenated cyclic monomer reaction product methyl esters (6 ft x 1/8 in. S.S., with 15% EGS, Chromosorb W [AW] 60-80 mesh).

EXPERIMENTAL PROCEDURES

Pure linolenic acid was obtained from the Nu Chek Prep Co. and was analyzed as >99% by both GLC and thin layer chromatography (TLC). Pure aromatized cyclic monomer was obtained through J.C. Cowan, Northern Regional Research Lab., Peoria, Ill.

Methyl esters were prepared by reaction with diazomethane in diethyl ether followed by evaporation of solvent (17) and by reaction with methanol containing 2% H_2SO_4 (17) followed by the usual workup (17).

Samples were hydrogenated at atmospheric pressure with stirring by dissolving the sample (~5 mg) in ethyl acetate (10 ml) with 1-2 mg PtO_2 in an all glass system (18).

The cyclic monomers of pure linolenic acid were prepared according to a modification of the method of Schofield and Cowan (1) as follows: A typical preparation of the cyclic monomer of linolenic acid utilized 10 g pure linenic acid dissolved in 100 g technical grade diethylene glycol and placed in a 250 ml three-necked pyrex flask. To this mixture was added 80-100% M excess of sodium hydroxide pellets (Reagent) and the flask and contents heated under an atmosphere of nitrogen gas for 1 hr at 240-245 C. During this time the level of solvent was kept constant by addition of diethylene glycol. The mixture was cooled, adjusted to pH 2 with 10% aqueous H_2SO_4 and the fatty acids extracted with petroleum ether. The organic extract was washed, dried over anhydrous sodium sulfate and evaporated to dryness. The crude product was stored at 0 under nitrogen.

TLC was carried out according to Stahl (19). Glass plates (20 x 20 cm) were coated with a slurry of Silica Gel G (EM) containing 12% $AgNO_3$ in NH_4OH in layers 1 mm thick. Samples (80-100 mg) were applied as a narrow streak at 2 cm from the bottom of a plate. The plate was placed in a developing tank and developed twice with a solvent composed of petroleum ether-diethyl ether-glacial acetic acid 95:5:1. After drying, the plates were sprayed lightly with 1% of 2,7'-dichlorofluorescein in ethanol and viewed under UV light. The separated bands were removed by scraping into petroleum ether. The slurry was filtered and the fraction recovered by evaporation under N_2 to remove solvent. Analytical TLC was carried out under identical conditions as for preparative chromatography but employed a thinner silica gel layer (750 μ).

Purification of the crude cyclic monomer methyl ester was also carried out using column chromatography as follows: Mallinckrodt silicic acid (100-200 mesh) was washed with 50% aqueous HCl followed by distilled water rinses until succeeding rinses were Cl^- ion free. The washed silicic acid was heated in an oven at 150 C until dry. Silver nitrate (12.5% wt) was dissolved in distilled water and 100 g of the washed silicic acid added to form a slurry. This slurry was redried at 150 C and packed as a slurry in petroleum ether into a 2 cm diameter column. The average size column employed was 40 cm x 2 cm and contained ca. 80 g of packing. Samples of crude cyclic monomer methyl ester (2 g) were applied to the column and eluted with varying percentages of diethyl ether in petroleum ether. The elution was monitored by TLC and hydrogenation followed by GLC of the products.

Gas chromatography was accomplished using several columns. For ordinary analysis a 6 ft x 1/8 in. S.S. column packed with 15% EGS coated on 60-80 mesh Chromosorb W (Supelco, Inc., Bellefonte, Pa.) was employed. Other

FIG. 3. Argentation TLC of cyclic, conjugated and nonconjugated fatty acid methyl esters: 1. methyl stearate, 2. methyl oleate, 3. methyl linolenate, 4. methyl linolenate, 5. conjugated methyl linoleate isomer mixture, 6. conjugated methyl linolenate isomer mixture, 7. methyl esters of aromatized cyclic monomer from linolenate, 8. methyl esters of cyclic monomer reaction product (dimeric and polymeric materials removed).

SE-30 on 60-80 mesh Chromosorb W (AW-DCMS) (Supelco, Inc.), a 6 ft x 1/8 in. S.S. column packed with 3% OV-25 on 60-80 mesh Chromosorb W (AW-DCMS) (Supelco, Inc.), a 50 ft x 0.02 in. S.C.O.T. column with DEGS stationary phase (Perkin-Elmer Corp., Norwalk, Conn.), and a 150 ft x 0.01 in. capillary column coated with Apiezon L (Perkin-Elmer Corp.). These columns were utilized at a column temperature of 170-185 C, at optimum flow rates for each column, and coupled with a flame ionization detector. The instruments employed were a Beckman GC-5 and a Varian Aerograph A-60A.

Mass spectra were determined with a Perkin-Elmer Hitachi RMU6E double focusing mass spectrometer adjusted to a resolution of 1000 and coupled with a gas chromatographic inlet system. The helium separator was maintained at 250 C, as was the ion source and short heated transfer line from the GLC. The ionizing current was 55 μA and the voltage set at 70 volts. Spectra were recorded every 3 sec to m/e = 600 during the elution of a GLC peak (1-10 μg material) as determined by both the FID detector of the gas chromatograph (10% split) and the continuous record produced by the total ion monitor. The mass spectra so obtained were stored on magnetic tape for further processing. High resolution element maps were obtained on selected samples at a resolution of 10,000 with a Varian Mat SM-1B instrument. The Varian Aerograph series 1200 Gas Chromatograph coupled to the mass spectrometer employed a 6 ft x 1/8 in. S.S. column packed with 15% EGS coated on 60-80 mesh acid washed Chromosorb W. A helium flow rate of 20-22 ml/min was employed. Approximately 90% of the column effluent was diverted to the mass spectrometer. Elution of individual components was accomplished by isothermal operation of the column at 175 C. The mass spectra collected (30-40 per sample) were processed using an on-line data system. The background contribution of the EGS column and that due to peak overlap were subtracted to yield spectra representative of the eluted components. The spectra were also averaged for changing ion current to obtain a representative spectrum for any one component.

RESULTS AND DISCUSSION

The formation of cyclic monomers of fatty acids in oils, which have been heated under both simulated and actual deep fat frying conditions, represents a potential public health problem. Artman and Smith (7) have shown that

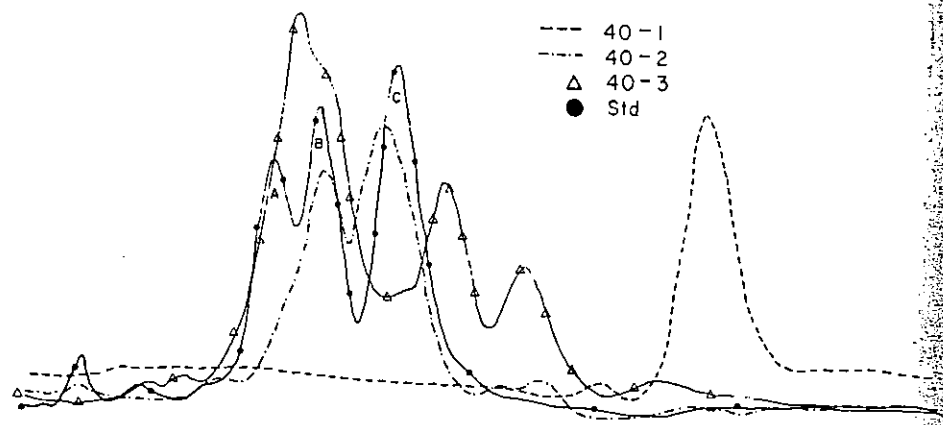


FIG. 4. Gas liquid chromatogram of fractions obtained by argentation thin layer chromatography of cyclic fatty acid methyl esters. Composition of standard: A. nonconjugated methyl linolenate, B. and C. conjugated isomers of linolenate (6 ft x 1/8 in. S.S., with 15% EGS on 60-80 mesh Chromosorb W [AW]).

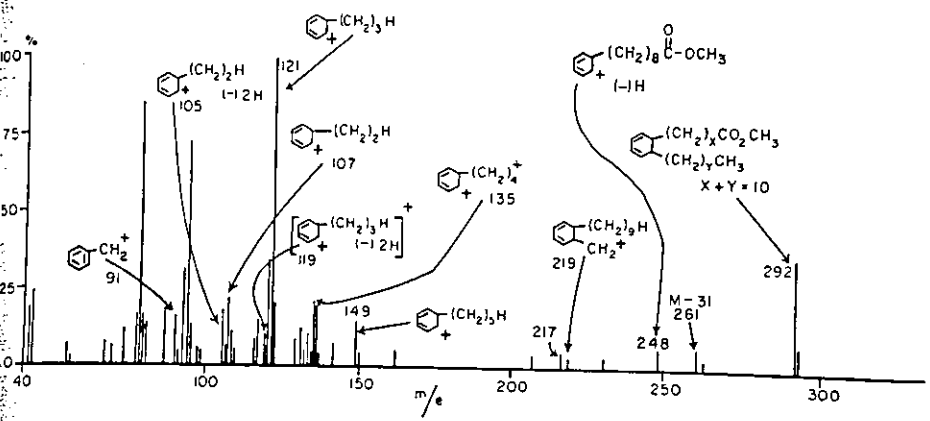


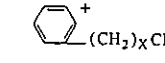
FIG. 6. Mass spectrum (70 eV) of isomeric cyclic monomer methyl esters (fraction 40-2).

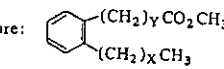
cyclic monomer occurs in heated cottonseed oil as well as soybean oil heated in a deep fat fryer (10). Furthermore it appears that cyclic monomer may be the one component of heated oils with the most toxic potential that occurs in the largest quantities (7). For this reason it was essential to study the purification of cyclic monomer to remove as many other side reaction products as possible, with a view toward synthesis of approximately labeled compounds for future metabolic and toxicological studies.

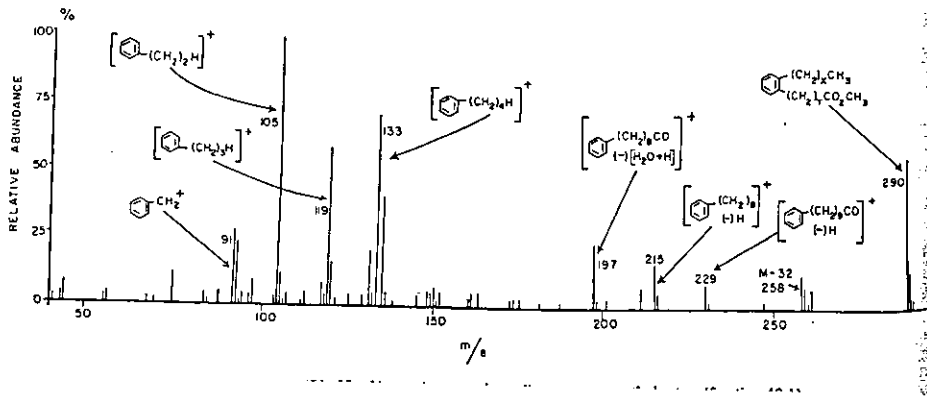
The method of producing the cyclic monomer mixture used in the present study was a somewhat modified version of that reported previously (1). The formation of some small amounts of dimeric products appears to be a byproduct of the reaction at the high temperature and is probably due to a Diels Alder condensation between two molecules of linolenic acid. When the mechanism of cyclic acid formation (5) is considered, the side product of the reaction is, in addition to a small amount of dimeric material, primarily conjugated linolenic acid which is not readily separable from the reaction product.

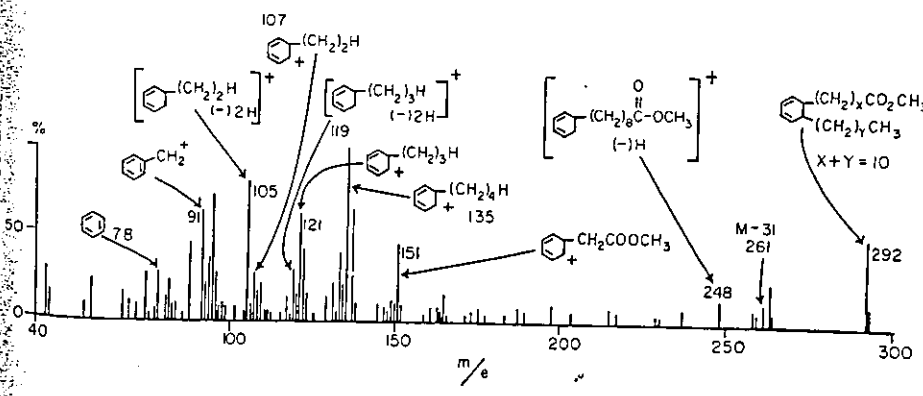
Examination of the reaction products from a typical cyclization reaction using an ethylene glycol succinate (EGS) column (Fig. 1) indicated an ill-defined mixture of isomers similar to those reported for the cyclic monomer from linseed oil. There is considerable overlap indicated with the conjugated isomer mixture from methyl linolenate. The amount of conjugated linolenate isomers in the reaction mixture may be determined by hydrogenation and subsequent rechromatography of the hydrogenated crude reaction product (Fig. 2). The chromatogram of this sample indicated the presence of a small amount of methyl stearate (7-9%) and the presence of a component which did not change in retention time upon hydrogenation, probably due to an aromatic isomer.

Although the effect of conjugation on the Rf values of compounds separated via argentation TLC has not been fully investigated, it has been shown that the effect of silver complexing increased as the distance between double bonds increased (20). A comparison of the effects of argentation on the separation of conjugated and nonconjugated fatty acid methyl esters and cyclic monomer which demonstrates this effect is shown in Figure 3. The influence of increasing the number of double bonds in decreasing the Rf value in the series Rf 18:0>18:1>18:2>18:3, where the double bonds are all of the *cis* configuration, is readily apparent. However it is interesting to note that the complex mixture of geometrical and positional isomers representing conjugated 18:2 and 18:3 methyl esters exhibits the same Rf value as methyl oleate. The two lower spots in the conjugated 18:3 lane may be due to mixtures containing lower percentages of *trans* double bonds; all three components have identical mass spectra. The aromatized cyclic monomer and the present reaction product behave in the same fashion. The reaction product, methyl ester, separated into three poorly resolved bands upon preparative argentation TLC. Each of the fractions were collected and the organic material extracted and analyzed by GLC on an EGS column coupled directly to the mass spectrometer. The mass spectra of each peak was recorded. The gas chromatogram of each fraction is outlined in Figure 4, and the origin of the fractions is indicated on the photograph of the TLC plate (Fig. 3, lane 8). Fraction 1, the topmost spot, was composed of one major component, which did not change retention time upon hydrogenation and represented ca. 10% of the total cyclic material. The average mass spectrum of this peak indicated that it was a mixture of isomeric aromatic cyclic monomers with a molecular weight of 290 as the methyl ester (Fig. 5).

An examination of the mass spectrum indicates the presence of an intense molecular ion as well as ions at m/e 258 and 259, representing loss of methanol from the methyl ester. Cleavage of the alkylcarbonyl side chain leads to the formation of a series of ions at m/e 105 (C_8H_9, 105.0693, calculated 105.0704), 119 (C_9H_{11}, 119.0857, calculated 119.0861), and 133 ($C_{10}H_{13}$, 133.1015, calculated 133.1017) with the general structure  . The ions at m/e 119 and 133 contained a small percentage of ions (<2%) with the structure C_8H_7O (m/e 119.0490, calculated 119.0497) and C_9H_9O (m/e 133.0650, calculated 133.0653) due to a ketene ion. In addition, an intense ion at m/e 197 corresponding to $C_{15}H_7$ (m/e 197.1325, calculated 197.1330) may have originated from a corresponding ketene ion by loss of water and a proton. Less intense ions at m/e 215, $C_{15}H_{19}O$ (m/e 215.1436, calculated 215.1436) and m/e 229, $C_{16}H_{21}O$, (m/e 229.1592, calculated 229.1593) seem to be ketene type ions that have lost one proton. Ion structures that have been written in monosubstituted forms may also be written in the disubstituted form, as may be obtained by partial cleavage of one substituent. A fairly intense ion is present at m/e = 91 representing the tropilium ion.

The mass spectra of this fraction are quite similar to those published by Zeman et al. (21) for pure synthetic aromatic cyclic monomer, and by Scharmann et al. (8) for aromatic acids produced from linolenate and linoleate with the general structure:  X + Y = 10





The second fraction isolated was composed of two major components that were poorly resolved (Fig. 4). Peak number two coincided with the retention time of a conjugated 18:3 isomer mixture as shown by hydrogenation of this fraction. A small percentage (2.7%) of methyl stearate was observed, indicating overlap between conjugated 18:3 isomers and a major cyclic component. This overlap caused serious problems when evaluating the mass spectra of these components, since the mass spectra of conjugated methyl linolenate and its isomers were identical to those of the component cyclic esters. Several mass spectra were taken during elution of each of the major peaks. The mass spectra were all identical, varying only in intensity, indicating a series of closely related isomers. A typical spectrum, after spectrum averaging and background subtraction, is shown in Figure 6. The mass spectrum shown indicates a base peak at m/e = 121 (C_9H_{13}, 121.1008, calculated 121.1017) for an alkyl ion with a propyl substituent; other ions were present, representing alkyl substituents of up to 10 carbon atoms. Other ions representing a tropilium ion, formed by dehydrogenation, disubstituted ions, as well as a peak at m/e = 261 indicating loss of methanol are present. An ion at m/e = 248, which represented cleavage of the alkyl side chain from the molecular ion of one isomer, was also produced ($C_{16}H_{24}O_2$, 248.1764, calculated 248.1776). This spectrum and other obtained closely resembled those obtained by other workers (8,9,22) for cyclohexadienoid structured and aromatized monomers. This particular spectrum represented a mixture of isomers in which a short alkyl substituent group predominated. In the other major peak of the chromatogram (Fig. 4), the mass spectrum indicated predominance of compounds with other combinations of substituent groups varying in the number of carbon atoms in both the alkyl and carboxyl containing side chains.

Fraction 3, taken from the bottom portion of the band corresponding to the cyclic monomer reaction product as indicated in Figure 3, was subjected to GLC (Fig. 4). Another more complex series of poorly separated isomers was obtained consisting of a large doublet and two other fairly well resolved components. In this case also, there was considerable overlap with both linolenate and its conjugated isomers, and, upon hydrogenation of this fraction, 3.7% stearate was present. The mass spectra of each of the peaks were determined periodically during the elution of the peak, resulting in over 40 spectra. All spectra were identical with only intensity differences, as observed previously. This would be expected, since the gas chromatographic peaks represent a concentration of various isomers in terms of both substituent chain length differences as well as geometrical configuration. The average mass spectrum of the major component of the fraction is reproduced in Figure 7. The mass spectrum indicates the molecular weight as 292 with a peak at 261, representing loss of methoxy (methanol). Peaks are also present for alkyl-substituted cyclohexadiene and aromatic ions at m/e 105, 107, 119, 121, 135, 137, 151 and 165. Ions are present at m/e 78 and 91, formed by dehydrogenation and cleavage of the corresponding ion at m/e 93.

The intense ion at m/e 151 was resolved into three ions as follows. A major ion corresponding to $C_{10}H_{15}O$ (151.1111, calculated 151.1123) and minor ions corresponding to $C_{11}H_{19}$ (151.1463, calculated 151.1486) and $C_9H_{11}O_2$ (151.0748, calculated 151.0759). The second ion represents the alkyl substituted cyclohexadiene ion and the $C_9H_{11}O_2$ ion that fragment from which the alkyl group has been removed. The ion corresponding to $C_{10}H_{15}O$ probably represents a ketene ion. Other higher molecular weight ions consisted primarily of a homologous series of ketene ions.

This spectrum is also in accord with those reported earlier and is similar in fragmentation to that obtained for

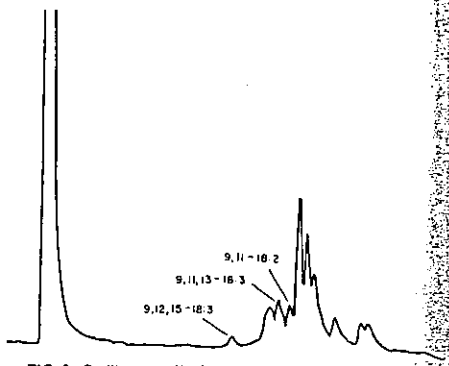
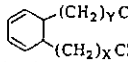


FIG. 8. Capillary gas liquid chromatogram of cyclic monomer ethyl ester reaction product with added 9,11-methyl linolenate, 9,11,13 and 9,12,15-methyl linolenate (150 ft x 0.01 in. capillary coated with Apiezon L).

aromatic isomers (8,9,21). The mass spectral studies have confirmed the structures of fractions 2 and 3 as mixtures of isomeric ω-(2-alkylcyclohexadienyl) carboxylic acids as shown:



X + Y = 10

Additional gas chromatographic studies using columns with OV-25 and SE-30 packings yielded inferior separations compared to that obtained with EGS. Results obtained with a 50 ft x 0.02 in. S.C.O.T. column coated with DEGS were not superior to those obtained from the EGS column. However the separation of cyclic monomer was improved considerably using a 150 ft x 0.01 in. capillary column coated with Apiezon L (Fig. 8). As indicated in the gas chromatogram, the separation of the cyclic monomer isomer, as well as octadecadienoate and trienoate samples was enhanced compared to that obtained on the 6 ft EGS column. However this separation is not of practical value for preparative use.

Argentation TLC has therefore allowed the partial removal of the undesirable side reaction products, such as conjugated linolenate and polymeric material, from crude cyclic fatty acid preparations. This method allowed partial fractionation of the isomeric monomers and produced one fraction very low in conjugated linolenate. The amount of material that may be fractionated by this method is low although in the case of a ^{14}C-labeled substrate this would not be a major factor. Using a microgram scale (10-20 μg), separations are quite good and yielded complex chromatograms which appeared to be composed of separated positional and geometrical isomers of the cyclic monomer. However attempts to increase the plate loadings to preparative scale (20 mg) decreased the separation of the cyclic monomer and conjugated linolenate.

In order to facilitate the preparation of larger quantities of isomeric cyclic monomer mixtures free of conjugated linolenate isomers, argentation column chromatography was investigated. A column packed with acid-washed silicic acid coated with 10% $AgNO_3$, when eluted with 10% diethyl ether in petroleum ether, eluted large amounts of conjugated linolenate and cyclic material. When the ether content of the eluting solvent was decreased to 5% (v/v) less conjugated methyl linolenate isomers eluted. A fraction of pure cyclized methyl linolenate was eluted with 2% v/v ether in petroleum ether. With this solvent, yields of over 70% cyclized material were obtained by direct application of reaction mixture methyl esters to the column. Other reaction products, such as dimeric and higher polymeric colored products were retained on the column. With this solvent system, the conjugated methyl linolenate was successfully removed from sample sizes of up to 10 g. The eluted material had on the average less than 1% conjugated linolenate isomers as determined by hydrogenation.

Although argentation TLC allowed partial separation of isomeric cyclic monomers and the aromatic isomer, column chromatography yielded a batch type separation of conjugated linolenate from the reaction product. The final purified cyclization product contained ca. 10% of the aromatic isomer, as well as those isomeric cyclohexadienyl products described previously.

ACKNOWLEDGMENTS

G. Rao, E. Mayhood, M. Sullivan and T. Fielder rendered technical assistance. Partial support was obtained from the U.S. Public Health Services Grant FD 00049, the Illinois Agriculture Experiment Station and the Biomedical Support Grant at the University of Illinois at Urbana-Champaign.

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[Received June 16, 1972]

Studies on Peroxidative Hemolysis and Erythrocyte Fatty Acids in the Rabbit: Effect of Dietary PUFA and Vitamin E

LYNDA R. HORN, MYRA O. BARKER, GWENDOLYN REED AND MYRON BRIN
Department of Biochemical Nutrition, Hoffmann-La Roche Inc., Nutley, New Jersey 07110

ABSTRACT Clinical signs of vitamin E deficiency were not manifested in the rabbit prior to the onset of dietary PUFA supplementation. PUFA consumption by vitamin E-deficient rabbits tended to promote peroxidative hemolysis by dialuric acid, as well as osmotic fragility when determined in the presence of either hydrogen peroxide or the peroxide-generating system of glucose oxidase and glucose in a continuous salt gradient. Analysis of erythrocyte fatty acids by GLC demonstrated incorporation of the administered PUFA. Peroxidation of RBC PUFA was observed in vitro but not in vivo. Fatty acids of RBC phospholipids were also analyzed for alterations under the experimental conditions. Possible mechanisms of the participation of vitamin E in lipid metabolism and membrane stabilization are discussed. J. Nutr. *104:* 192–201, 1974.

INDEXING KEY WORDS vitamin E · PUFA · rabbits · RBC

In 1947 Hove and Harris (1) observed that vitamin E-deficient animals developed symptoms of muscular dystrophy when dietary polyunsaturated fatty acids (PUFA) were increased. Borgman (2) noted no increase in dystrophic symptomatology when he fed oleic acid to vitamin E-deficient rabbits. However, increased dietary linoleate did cause nutritional muscular dystrophy in E-deficient rabbits, confirming the earlier observation by Hove and Harris (1).

Many investigators have proposed a relationship between the amount of dietary PUFA and the requirement for the antioxidant, vitamin E. Tappel (3) and Pritchard and Singh (4) have postulated a destruction of rat tissue PUFA in the absence of vitamin E which they attribute to in vivo lipid peroxidation. However, Hayes et al. (5), having shown that the canine requirement for vitamin E is directly related to PUFA consumption, associate this phenomenon with lipid metabolism, not solely connected to an antioxidant effect. In their investigation of fatty acid turnover in rat erythrocyte membranes, Oliveira and Nason (6) found no correlation between vitamin E status of the rats and the ability of their erythrocytes to acylate glycerophospholipids. Thus, the role of vitamin E in lipid metabolism is still in question. Although Horwitt (7) has demonstrated an increased requirement for tocopherol in the chick in the presence of an elevated intake of linoleic acid, other workers (8, 9) have been unable to extend these results in studies with rats fed PUFA. Also, El Khatib et al. (10), in their studies of rats and rabbits, found no difference in the amounts of lipid peroxidation in tissues of E-deficient and control animals. The increased requirement for vitamin E in the presence of increased dietary PUFA is therefore still controversial.

The actual mechanism of peroxidative hemolysis of RBC from vitamin E-deficient animals is still in dispute. Studies by Younkin et al. (11) indicate that lipid peroxidation precedes erythrocyte hemolysis. According to Jacob and Lux (12) membrane-bound phosphatidylethanolamine (PE) is selectively attacked by peroxides generated by glucose oxidase and glucose. This observation was contradicted

Received for publication June 4, 1973.

by studies performed by Heikkila et al. (13) who found that during peroxidative hemolysis all phospholipids of the erythrocyte membrane were destroyed in approximately equal ratios.

The present study was undertaken to further elucidate the synergistic effect of vitamin E deprivation and increased dietary PUFA upon rabbits. We have noted an effect of increasing consumption of PUFA on the fatty acid composition of RBC membranes and the peroxidative hemolysis of these cells.

MATERIALS AND METHODS

Animals and Diets. White male New Zealand rabbits [1] were raised from weaning on a pelleted vitamin E-deficient or supplemented diet (table 1). The tocopherol-supplemented diet was identical to the deficient diet except for the addition of 225 mg DL-α-tocopherol per kilogram diet. In addition, designated groups of animals were given daily oral supplements of 1 ml cod-liver oil (CLO), 3 ml stripped safflower oil containing 78% linoleic acid [2] or 1 ml 50% ethyl arachidonate.[3]

Procedures. All solvents were reagent grade and were redistilled prior to use. Plasma tocopherol was assayed by the micro method of Hashim and Schuttringer (14).[4] Hemolysis with dialuric acid was performed according to Friedman et al. (15); glucose oxidase–glucose (GO-G) hemolysis was carried out by a modification of the procedure of Jacob and Jandl (16) using a final concentration of glucose of 27.8 mM and 3.0 U/ml glucose oxidase. Direct hydrogen peroxide hemolysis was assayed according to Lubin et al. (17). Erythrocyte density distribution was determined by the method of Danon and Marikovsky (18). Creatine phosphokinase activity was assayed by a modification of the procedure of Kjekshus and Sobel (19). These values have been reported elsewhere by this laboratory.[5]

Osmotic fragility measurements [6] were performed as described by Barker et al. (20). This procedure measures the change in osmotic fragility of RBC in a continuous salt gradient. The fragility curve that is generated as a function of light transmitted through the cell suspension is continuously recorded on a chart. The derivative of the fragility curve is also recorded. A shift to the left is indicative of increased susceptibility to osmotic lysis, while a rightward shift suggests a slight resistance to hemolysis conferred upon the cells by the increased tonicity of the solution (21).

Blood was drawn from the ear vein of the animals or obtained by heart puncture from rabbits anesthetized with Penthrane using EDTA as an anticoagulant. The plasma and buffy coat were removed by centrifugation [7] at 1,000 × *g* for 10 minutes followed by aspiration. RBC were washed three times and hemolyzed according to the procedures of Dodge et al. (22). Membrane ghosts were extracted with chloroform–methanol (2:1). The aqueous phase was removed by aspiration and the remaining chloroform layer was dried with anhydrous sodium sulfate. After evaporation of the sample under nitrogen to dryness, fatty acid methyl esters (FAME) and dimethylacetals (DMA) were prepared by heating the lipid residue with 2 ml methanol–sulfuric acid (9:1) in sealed tubes at

TABLE 1

Synthetic rabbit stock colony diet

Ingredients	g/kg
Casein, GBI Vitamin Free Test	200.00
Cornstarch	260.00
Cane sugar	260.00
Agar, U.S.P.	20.00
Nonnutritive fiber	120.00
Stripped lard	80.00
Salt mix, U.S.P. XIV	60.00
Vitamin mix[2,3]	

[1] General Biochemicals, Chagrin Falls, Ohio. [2] Vitamin mix contributed the following vitamins per kg diet: (in mg) calcium pantothenate, 45; choline chloride, 6000; i-inositol, 300; menadione, 2.25; niacin, 600; pyridoxine·HCl, 21; riboflavin, 21; thiamin·HCl, 21; DL-α-tocopherol, 225; as well as vitamin A, 4750.2 U/kg and vitamin D, 264.31 U/kg. [3] DL-α-tocopherol omitted from vitamin E-deficient diet.

[1] Edgely Research Farm, Columbus, N. J.
[2] Donated by Dr. Winifred Cort of Hoffmann-LaRoche Inc., Nutley, N. J. The stripping procedure was effected by heating in a molecular still at 270° in a V. L. Watt 10-stage rotary glass evaporator manufactured by Vitamins Ltd., England, at 1 μ vacuum.
[3] Nu-Chek Prep, Elysian, Minn.
[4] DL-α-Tocopherol for standard curves was donated by Dr. Ray Bunnell of Hoffmann-La Roche Inc., Nutley, N. J.
[5] Brin, M., Barker, M. O., Horn, L. & Reed, G. (1973) Analysis of skeletal muscle from vitamin E-deficient and supplemented rabbits fed polyunsaturated fatty acids. Federation Proc. *32:* 943 (abstr.).
[6] Frigiligraph Model D-2, Elron Electronic Instruments, Ltd., Haifa, Israel.
[7] Sorvall RC2-B, HS-4 rotor, Ivan Sorvall, Inc., Norwalk, Conn.

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TABLE 2

Hemolysis of rabbit erythrocytes with glucose oxidase–glucose or hydrogen peroxide

Tocopherol status	Oil	No.	Percentage hemolysis with glucose oxidase: Mean	Percentage hemolysis with glucose oxidase: Range	Percentage hemolysis with hydrogen peroxide: Mean	Percentage hemolysis with hydrogen peroxide: Range
Control	None	(7)	2.1	0–13	0.8	0–1.5
Deficient	None	(13)	10.3	0–27	23.0	0–47
Control	CLO	(11)	0.5	0–6.4	6.3	
Deficient	CLO	(5)	1.1	0–3.1	7.6	
Control	Safflower	(4)	1.7	0–2.7	6.4	4.2–6.6
Deficient	Safflower	(2)	0		53.2	7–99
Control	Arachidonate	(3)	0		5.0	0–7.9
Deficient	Arachidonate	(2)	24.8		97.4	96.8–98

100° for 90 minutes. Following extraction into hexane, FAME and DMA were chromatographed on a 183-cm stainless steel column of 10% EGSS-X on 100/120 mesh Gas Chrom P at 185° to 195° [8] employing helium as carrier gas. Plasma lipids were extracted into 20 volumes of ethanol–isopropyl ether (2:1), filtered, evaporated to dryness, and transmethylated as described above.

Two-dimensional thin-layer chromatography was performed in a glass tank in a Plexiglass box under nitrogen. Lipid extracts were dissolved in chloroform and applied to 20 by 20 cm Silica Gel G plates.[9] Phospholipids were first resolved in a solvent system of chloroform–methanol–50% ammonia (65:27:6) and then in chloroform–methanol–acetone–glacial acetic acid–water (50:20:10:8:4). Following detection with a solution of 0.01% 4′,5′-dichlorofluorescein in redistilled absolute ethanol under ultraviolet light, phospholipid spots were scraped off and extracted into 20 volumes chloroform–methanol–50% acetic acid (65:25:14). Extracts were washed, the chloroform layer was dried with sodium sulfate, and FAME and DMA were prepared and analyzed as described above.

TABLE 3

Percentage hemolysis of rabbit erythrocytes after 4 to 6 weeks feeding of oil diets

Tocopherol status	Oil	Percentage hemolysis[1]: Glucose oxidase–glucose	Dialuric acid	Hydrogen peroxide
Control	None	0	1.5	0.4
Deficient	None	1	0.1	99
Control	Arachidonate	0	1.4	3
Deficient	Arachidonate	85	13	98
Control	Safflower	4	0	6
Deficient	Safflower	35	0	76

[1] All percentage hemolysis values reading horizontally were obtained from the same rabbit blood sample.

RESULTS

The rabbits were considered to be vitamin E deficient when their plasma tocopherol levels fell below 300 μg/100 ml compared to the control level of at least 1,000 μg/ml. Even after 6 months at this deficient level, the rabbits displayed no signs of muscular weakness when given no oral oil supplement.

In our earlier studies with rats we used susceptibility of RBC to peroxidative hemolysis as an index of vitamin E deficiency. The results shown in table 2 demonstrate that only the blood from the arachidonate-supplemented E-deficient rabbits displayed appreciable hemolysis when treated with the peroxide-generating system of glucose oxidase and glucose. The values presented in table 3 are representative of a typical hemolysis experiment with a second group of rabbits. These data indicate that blood from all E-deficient rabbits, excluding those receiving no oil supplements, exhibited considerable hemolysis following treatment with glucose oxidase and glucose. Some hemolysis with dialuric acid was noted for the blood from arachidonate-supplemented deficients, and in subsequent studies, as much as 59% hemolysis was observed following dialuric acid treatment of blood from safflower oil-fed E-deficient rabbits.

[8] Hewlett-Packard gas chromatograph Model 7620A, Hewlett-Packard Company, Avondale, Pa.

[9] Quanta Gram Q1, Quantum Industries, Fairfield, N. J.

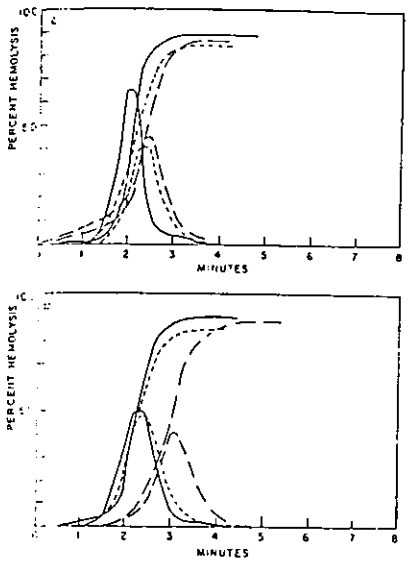

Fig. 1 Fragility curves of blood from control and vitamin E-deficient rabbits given no oil supplements: 1A (top), deficient rabbit; 1B (bottom), control rabbit. Key: ———, fragility curve of blood in phosphate–saline only; — — —, fragility curve of blood in phosphate–saline plus glucose oxidase and glucose; – – – –, fragility curve of blood in phosphate–saline plus 3% hydrogen peroxide; cells washed, resuspended in phosphate–saline.

Peroxidative hemolysis of the rabbit blood in an osmotic gradient was studied. Although blood from vitamin E-deficient rats has been shown to be extremely sensitive to this procedure (20), rabbit blood is not. Figure 1 depicts fragility curves from vitamin E-deficient and control rabbits fed no oil supplements. There is no leftward shift of the curve in the presence of either peroxidizing agent, indicating no increased hemolysis under the experimental conditions. Nor did the blood from any of the vitamin E-sufficient rabbits display a leftward shift of the fragility curve of their blood. Figure 2A shows the hemolysis of blood from a vitamin E-deficient safflower oil-supplemented rabbit. Although no leftward shift of the fragility curve was initially noted in the presence of glucose oxidase and glucose, a shift was apparent after a 15-minute incubation of blood with hydrogen peroxide. Blood from a CLO-fed E-deficient rabbit also hemolyzed in the presence of hydrogen peroxide even though it did not with glucose oxidase and glucose (fig. 2B).

Hemolysis profiles of blood from E-deficient rabbits fed ethyl arachidonate exhibit biphasic hemolysis curves (fig. 3), suggesting that there were two populations of erythrocytes, one of which lysed before the other. Density distribution studies further confirmed the presence of two populations as the mean RBC density shifted from 1.110 to 1.102 g/ml within 3 weeks after the onset of ethyl arachidonate supplementation. Reticulocyte staining also demonstrated increased reticulocytosis in the blood of arachidonate-fed control and deficient rabbits, thereby again explaining the biphasic osmotic fragility and the density shift.

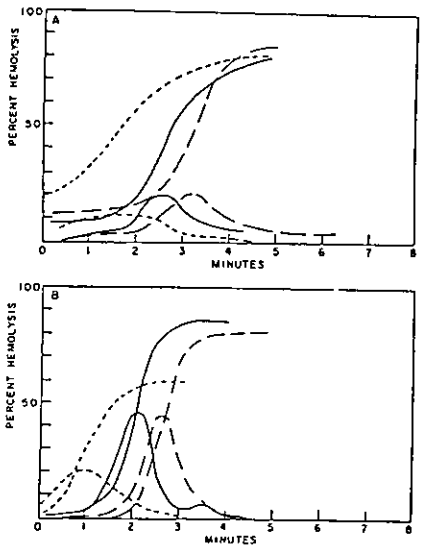

Fig. 2 Fragility curves of blood from vitamin E-deficient rabbits given either safflower oil (15D) or CLO (8D). 2A, rabbit 15D; 2B, rabbit 8D. Key: same as for figure 1.

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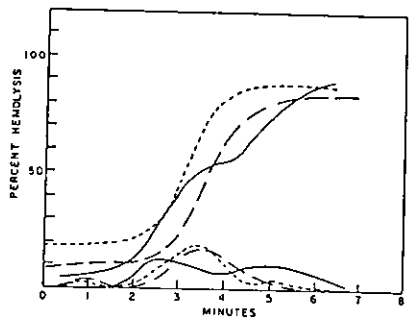

Fig. 3 Fragility curves of blood from a vitamin E-deficient, ethyl arachidonate-supplemented rabbit. Key: same as for figure 1.

Initial investigation of the RBC fatty acid distribution of arachidonate-fed rabbits showed that relatively large amounts of dietary arachidonic acid were incorporated (table 4). RBC from safflower oil-fed rabbits exhibited an increased linoleate content with no concomitant rise in the percentage of arachidonate, presumably due to the rabbits' inefficient conversion of dietary linoleate to arachidonate. The results of a second study, depicted in table 5, also indicate that there was a large increase in the amount of arachidonic acid incorporated into the RBC of ethyl arachidonate-fed rabbits, but no increase for the unsupplemented or safflower oil-fed animals.

On the other hand, the linoleate content of the RBC remained constant in both untreated and safflower oil-fed animals, while decreasing substantially in the arachidonate-supplemented rabbits. The level of eicosadienoic acid, an intermediate in the biosynthesis of arachidonic acid from linoleic acid, was observed to rise in RBC of all animals examined except the E-deficient rabbits fed arachidonate or CLO. No such increases were noted in eicosadienoic acid content of the plasma (table 6), although plasma arachidonic acid content of arachidonate-fed rabbits did increase. Not only did the plasma level of this fatty acid not increase in animals unsupplemented with ethyl arachidonate, but the deficient animals had a consistently lower level of arachidonic acid than the controls.

In vitro peroxidation of blood from unsupplemented rabbits by glucose oxidase and glucose (GO–G) is depicted in table 7. Blood drawn at the same time from the same rabbit was incubated with phosphate-saline buffer (P–S). Peroxidation caused a lowering of the arachidonate content in RBC of the deficients but not in those of the controls. Reduced levels of arachidonic acid are also evident in peroxidized RBC of all the PUFA-fed E-deficient rabbits. Peroxidation also caused a decrease in the relative levels of linolenic and docosahexaenoic acids in RBC from E-deficient rabbits fed CLO.

Table 8 illustrates the fatty acid composition of erythrocyte membrane PE over the time course of the study. There were increased levels of the 16 carbon aldehyde in PE of PUFA-fed deficient rabbits, but not in controls or untreated rabbits. Linoleate content increased significantly in the PE of safflower oil-fed rabbits, but not in that of arachidonate-fed rabbits. Arachidonate content rose substantially in the

TABLE 4

Fatty acid distribution in rabbit erythrocytes

Animal no.	Tocopherol status	Oil	Area percentage of major fatty acids[1]					
			16	18	18:1	18:2	18:3	20:4
16C	Control	None	28.5	11.2	25.7	20.0		8.7
8D	Deficient	None	27.2	9.9	27.0	24.9		11.8
18C	Control	Arachidonate	22.3	10.4	30.2	19.6		14.8
2D	Deficient	Arachidonate	20.4	8.9	24.6	18.4		21.7
17C	Control	Safflower	23.5	10.4	30.9	28.0		5.0
7D	Deficient	Safflower	19.6	7.9	33.6	30.6		6.7
18C	Control	CLO	33.5	13.6	33.3	11.0	2.3	2.3
8D	Deficient	CLO	28.8	13.1	31.9	19.0	1.2	5.5

[1] In this and subsequent tables, all fatty acids from 14:0 to 22:6 were analyzed, but, for the sake of simplicity, only the major ones are shown.

TABLE 5

Fatty acid distribution in rabbit erythrocytes

Tocopherol status	Oil	No.	Area percentage of selected fatty acids[1]					
			18:2		20:2		20:4	
			i	f	i	f	i	f
Control	None	(4)	18.5	22.1	6.3	10.6	6.7	8.0
Deficient	None	(5)	18.0	23.5	4.1	8.6	5.1	5.2
Control	Arachidonate	(3)	18.5	11.2	6.3	14.3	6.7	14.9
Deficient	Arachidonate	(3)	18.0	11.8	4.1	1.2	5.1	13.3
Control	Safflower	(4)	18.5	17.2	6.3	12.1	6.7	7.7
Deficient	Safflower	(4)	18.0	18.2	4.1	7.7	5.1	7.5
Control	CLO	(8)	18.5	9.3	6.3	11.2	6.7	8.4
Deficient	CLO	(9)	18.0	16.4	4.1	2.4	5.1	7.3

[1] Initial values were obtained from blood drawn prior to onset of PUFA feeding. Final values were obtained from blood drawn at sacrifice of animals 3 to 7 weeks later.

PUFA-supplemented controls, but to a lesser extent in those given safflower oil than in the arachidonate-fed control rabbits. The level of arachidonic acid in PE from safflower oil-supplemented deficients remained constant.

Analysis of the distribution of fatty acids and fatty aldehydes in erythrocyte phosphatidylcholine (PC) indicates an increased level of the 16 carbon aldehyde in the arachidonate-fed deficient animals (table 9). The relatively high initial level of linoleic acid in the arachidonate-fed deficients returned to the same final value as the controls at the time of sacrifice. Linoleate content increased significantly ($P < 0.005$) in the safflower oil-fed controls, but not in the corresponding deficients. The levels of arachidonic acid decreased in erythrocyte PC of the untreated deficient, but increased in RBC PC derived from both types of PUFA-fed deficient rabbits.

DISCUSSION

After 6 months on a lard-based tocopherol-deficient diet, rabbits did not exhibit characteristic symptoms of muscular dystrophy. These rabbits were judged to be deficient on the basis of RBC hemolysis data, a reduction of muscle creatine phosphokinase activity,[8] as well as the low plasma tocopherol levels. It was only after oil supplementation that the rabbits became fatally dystrophic.

Although blood from the vitamin E-deficient rat is susceptible to dialuric acid hemolysis, it has been reported (23) that blood from the vitamin E-deficient rabbit is not. Other species whose blood is largely refractory to dialuric acid hemolysis include the calf, pig, monkey and human. However, we have demonstrated that, subsequent to PUFA feeding, rabbit blood becomes susceptible to hemolysis by dialuric acid. After ethyl arachidonate ingestion, for

TABLE 6

Fatty acid distribution in rabbit plasma

Tocopherol status	Oil	No.	Area percentage of fatty acids[1]					
			18:2		20:2		20:4	
			i	f	i	f	i	f
Control	None	1	16.8	13.4	2.4	3.0	2.0	2.0
Deficient	None	1	14.2	10.7	2.7	0.9	1.1	0.4
Control	Arachidonate	3	16.8	11.1	2.7	5.2	2.0	5.8
Deficient	Arachidonate	3	14.2	11.0	1.9	1.5	1.1	7.3
Control [1]	Safflower	4	16.8	17.4	2.2	2.9	2.0	2.4
Deficient	Safflower	2	14.2	18.8	2.9	2.3	1.1	1.4

[1] See footnote 1, table 5.

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TABLE 7

Fatty acid distribution in rabbit erythrocytes following peroxidation

Animal no.	Tocopherol status	Oil	Incubation condition	Area percentage of fatty acids						
				16	18	18:1	18:2	18:3	20:4	22:6
16C	Control	None	P-S	28.5	11.2	25.7	20.0		8.7	
			GO-G	32.0	9.8	23.9	20.7		11.8	
8D	Deficient	None	P-S	27.2	9.9	27.0	24.9		10.7	
			GO-G	27.5	14.1	30.9	23.2		4.4	
18C	Control	Arachidonate	P-S	22.3	10.4	30.2	19.6		14.8	
			GO-G	26.7	10.5	22.2	21.8		14.1	
2D	Deficient	Arachidonate	P-S	24.0	8.9	24.6	18.4		21.7	
			GO-G	34.5	8.0	32.2	21.4		3.9	
17C	Control	Safflower	P-S	23.5	10.4	30.9	28.0		5.0	
			GO-G	23.5	9.2	25.1	33.8		8.4	
7D	Deficient	Safflower	P-S	19.6	7.9	33.6	30.6		6.7	
			GO-G	29.6	9.6	23.8	33.8		4.0	
15BC	Control	CLO	P-S	32.4	13.8	30.2	15.2		2.2	
			GO-G	26.2	13.3	28.5	14.5		4.7	
13D	Deficient	CLO	P-S	25.2	13.4	34.8	13.2	2.0	3.8	7.6
			GO-G	26.5	12.0	47.4	11.4	0.4	1.7	0.4

instance, the rabbit RBC becomes rich in that fatty acid, thereby more closely resembling the rat erythrocyte. The change in rabbit blood susceptibility may therefore be a reflection of the altered composition of rabbit erythrocyte phospholipids. This might also be responsible for the increased osmotic fragility under peroxidizing conditions of RBC from PUFA-fed deficient rabbits as demonstrated by their hemolysis profiles.

Our findings confirm Dinning's observation (24) that there is no difference in hematocrit values between deficient and control animals. In addition, we noted the lack of effect of PUFA feeding on hematocrit values, a finding which is in disagreement with that reported by Hayes and coworkers (5) who noted that, in dogs, hematocrits were significantly depressed by PUFA consumption.

That dietary PUFA were incorporated into erythrocyte membranes was clearly shown by analysis of erythrocyte fatty acids. Other indications of PUFA incorporation included the reticulocytosis exhibited by blood of ethyl arachidonate-fed rabbits. The fatty acid composition of the erythrocyte phospholipids also reflected the fatty acid composition of the diets, especially in the case of ethyl arachidonate supplementation, where a high degree of arachidonic acid was detected in both PC and PE. Despite the apparent lack of increased in-

TABLE 8

Fatty acid distribution in rabbit erythrocyte phosphatidylethanolamine

Tocopherol status	Oil	No.	Area percentage of fatty acids[1]											
			16DMA		16		18		18:1		18:2		20:4	
			i	f	i	f	i	f	i	f	i	f	i	f
Control	None	(1)	1.7	1.4	20.7	25.4	8.4	8.1	31.3	27.1	21.1	20.6	10.2	12.2
Deficient	None	(1)	2.3	1.8	27.2	28.0	3.0	8.2	20.5	27.2	15.4	19.5	8.5	9.9
Control	Arachidonate	(3)	1.2	2.5[a]	23.0	22.2	11.6	10.5	35.0	28.2	12.7	13.0	9.7	18.8
Deficient	Arachidonate	(3)	1.1[a]	4.8[b,c]	25.7	19.2	8.0	7.9	26.5	27.0	18.6	14.3	11.7	14.3
Control	Safflower	(4)	5.5	1.4	25.6	20.4	6.5	8.2	34.1	27.0	12.8[a]	28.3[c]	4.5[a]	9.9[a,b]
Deficient	Safflower	(4)	1.7	4.0	24.3	18.7	0.3	0.5	33.3	37.3	13.5	19.1	4.1	5.4[a]

[1] i = initial values, from blood drawn 2 weeks after the onset of PUFA-feeding; f = final values, from blood drawn at sacrifice of the animals 3 to 7 weeks later. Numbers with matched superscripts were significantly different from each other at the level of $P < 0.01$.

TABLE 9

Fatty acid distribution in rabbit erythrocyte phosphatidylcholine

Tocopherol status	Oil	No.	Area percentage of fatty acids[1]											
			16DMA		16		18		18:1		18:2		20:4	
			i	f	i	f	i	f	i	f	i	f	i	f
Control	None	(1)	3.0	2.4	35.4	34.3	5.0	8.6	20.4	27.5	20.8	20.4	1.0	2.8
Deficient	None	(1)	4.2	2.1	32.8	35.9	7.0	11.0	26.5	27.5	14.6	21.0	5.2	1.7
Control	Arachidonate	(3)	1.0	2.4[b]	35.2	34.0	9.4	10.0	27.3	29.4	11.9	13.3	6.4	6.6
Deficient	Arachidonate	(3)	0.7[a]	5.6[b,c]	28.8	38.2	9.6	7.7	15.3	25.0	35.5	12.8	3.0	9.0
Control	Safflower	(4)	4.0	1.7	30.7	31.1	5.6	9.3	29.2	28.2	10.9[a]	24.9[c]	1.9	2.5
Deficient	Safflower	(4)	2.4	3.3	33.3	30.5	8.2	8.2	28.1	30.4	17.7	21.1	0.7	2.6

[1] See footnote 1, table 8. [a] $P < 0.005$; [b] $P < 0.02$; [c] $P < 0.05$.

corporation of linoleate into the erythrocyte in the rabbits fed safflower oil in the second study, the phospholipid fraction of the RBC membrane had significantly elevated levels of linoleic acid. It is well known that the β position of both PE and PC is highly specific for PUFA. Thus, it may be postulated that the linoleic acid derived from safflower oil is concentrated in the erythrocyte at the β position of the phospholipids.

The displacement of endogenous fatty acids by dietary fatty acids is generally accepted. Analyses by Century and Horwitt (23) of various tissues of the chick demonstrated the substitution of essential fatty acids, especially arachidonic acid, by the fatty acids native to the administered cod-liver oil. We too have noted the incorporation of fatty acids of administered cod-liver oil into the rabbit erythrocyte.

In vitro peroxidation by the glucose oxidase and glucose system caused a decrease in the arachidonate content in the erythrocytes of all vitamin E-deficient animals. Despite Tappel's postulation of in vivo lipid peroxidation in the absence of vitamin E (3), it is apparent from our analyses of fatty acid distribution in the erythrocyte that there was no in vivo destruction of arachidonic acid in erythrocytes of deficient rabbits. In fact, RBC from deficient animals often contained a higher percentage of arachidonic acid than did control RBC.

There are two major methods for incorporation of dietary fatty acids into RBC: 1. synthesis of erythrocytes in bone marrow, and 2. transacylation of RBC phospholipids by plasma fatty acids. Our data show that plasma from vitamin E-deficient rabbits had a lower level of PUFA than did control plasma, suggesting that circulating RBC deficient in PUFA utilized this source to incorporate the necessary fatty acids into PC. Experiments by Van Deenen et al. (26) have demonstrated that lecithin is the only phospholipid in rabbit RBC that undergoes transacylation by plasma fatty acids. We have observed that PUFA content of RBC PC did not differ between control and vitamin E-deficient rabbits. However, the PUFA content of PE of RBC derived from deficient animals was considerably lower than in controls. (Note especially the values for safflower oil-supplemented rabbits.) Thus, it appears likely that the observed difference reflects alterations occurring during RBC synthesis in the bone marrow, as PUFA deficits of the PC fraction can be corrected by exchange with plasma fatty acids whereas those of the PE fraction cannot.

Further support for this hypothesis may be obtained from the work of Winterbourn and Batt (27) who studied the incorporation of plasma fatty acids into human erythrocytes which had been separated on the basis of age. They found that younger RBC incorporated plasma fatty acids into PE much less efficiently than did older RBC. The considerable amount of reticulocytosis exhibited by the rabbits suggests, therefore, that most of the fatty acids incorporated from plasma into phospholipids would be found in the PC fraction. This effect was apparently great enough to overcome the phenomenon described by Jacob and Lux (12) of greater incorporation of fatty acids into erythrocyte PE by vitamin E-deficient rats than by controls.

The fact that both RBC PE and plasma phospholipids derived from blood of ethyl arachidonate-fed vitamin E-deficient rab-

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bits have no PUFA deficit is most likely due to the large excess of PUFA available from the diet. Suggestions that observed differences in PUFA content of PE may reflect in vitro decomposition in the absence of antioxidant can be discounted, as initial values showed no difference in PUFA levels between PE of control and vitamin E-deficient rabbits.

The differences seen most likely reflect changes in lipid biosynthesis in the absence of vitamin E. An interesting observation made by Century and Horwitt (25) in the tocopherol-deficient chick was that fatty acids of the linolenic series found in cod-liver oil inhibit the in vivo conversion of linoleic acid to arachidonic acid in erythrocytes and phospholipids of skeletal muscle and brain mitochondria. We have found that erythrocyte PE from deficient rabbits fed safflower oil contains significantly less arachidonic acid than does that from control animals. Perhaps vitamin E is necessary for the biosynthesis of arachidonic acid from linoleic acid via eicosadienoic acid. From the data presented in table 5 it is apparent that, in all groups, the final values for eicosadienoic acid content are higher in RBC of control rabbits than in RBC of the corresponding deficients. This effect might be due to an inhibition of the conversion of linoleate to eicosadienoate in the absence of vitamin E. Assuming that vitamin E does not itself participate in the reaction, some intermediate produced in the absence of the vitamin might interfere with the biosynthesis of eicosadienoic acid such that an alternate pathway of arachidonic acid synthesis might now be favored. The extent of such inhibition presumably would be enhanced by end product inhibition in the case of ethyl arachidonate feeding.

The inhibition may be a reflection of any of three theoretical functions of vitamin E. The first is the antioxidant effect postulated by Tappel (3), in which some product of lipid peroxidation, such as a free radical, might act as the inhibitor. Another is the lipotropic effect ascribed to tocopherol by Hayes et al. (5), in which tocopherol, in a role not necessarily related to antioxidation, would accelerate the removal of abnormal lipids, such as polymerized PUFA. Any of these polymers might be implicated in the regulatory process. The third role is the physicochemical stabilization of erythrocyte membranes by vitamin E. In a structural model proposed by Lucy (28) the methyl groups of the phytyl side-chain of α-tocopherol interact with the *cis* double bonds of esterified arachidonic acid. In the presence of excess PUFA, membranes are more vulnerable to enzymic degradation by phospholipases (28), leading to a higher metabolic "pool" of free fatty acids, whose accumulation might result in the end product inhibition mentioned above. The elucidation of the complex interactions of vitamin E and PUFA and their role in nutrition indeed merits further study.

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[Received November 17, 1975]

A Guide for Authors is Located in Lipids 11(January):85(1976)

Nutritional Effects of the Cyclic Monomers of Methyl Linolenate in the Rat

WAYNE T. IWAOKA[1] and E.G. PERKINS,[2] Department of Food Science, Burnsides Research Laboratory, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Low levels (0.0075, 0.0225, and 0.15%) of cyclic fatty acid methyl esters (>98% pure) were incorporated into diets of weanling rats fed different levels of protein. Animals on low protein diets (8-10% casein) exhibited decreased wt gains and feed consumption with increasing levels of cyclic esters in their diets after 6 weeks. Liver enlargements due to a significant ($P<0.01$) accumulation of liver lipid were noted in animals receiving 0.15% cyclic fatty acid esters in their diets.

INTRODUCTION

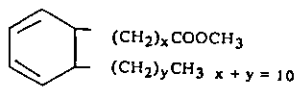
Many studies have been conducted in which a heat-abused fat or fractions from such fats were fed (1-12). Such heated oils produced adverse nutritional and physiological conditions when fed to laboratory animals as parts of normal diets (8-12). Moreover, the non-urea adduct forming fatty acids, a concentrate of the cyclic fatty acids and other polar materials present in such oils, caused a high number of deaths among rats fed this material (11). Repeated studies of the non-urea adduct forming fraction from heated oils have identified disubstituted aromatic and alicyclic fatty acids as major and minor components of these fats (13-16).

Although studies of others (8-12) have shown that cyclic fatty acids in diets cause adverse physiological effects when fed to rats, these diets contained only uncharacterized concentrated fractions fed at relatively high levels rather than the pure cyclic fatty acids themselves. In the present work, the nutritional effects of low levels of highly purified isomeric ω(2-alkyl cyclohexadienyl) carboxylic acids in nutritionally adequate diets containing different levels of protein (casein) were determined.

MATERIALS AND METHODS

Synthesis of Cyclic Fatty Acids

Cyclic fatty acids were synthesized by a modification of the method used by Scholfield and Cowan (17). The crude cyclic fatty acids were converted to the corresponding methyl esters which were further purified by argentation chromatography on silicic acid, with 2% diethyl ether in hexane as eluant. This procedure eliminated all of the dimeric or polymeric material formed and most of the isomers of conjugated linolenic acid as determined by thin layer chromatography. Subsequent gas chromatography after hydrogenation indicated that the isomeric ω(*o* alkyl cyclohexadiene) carboxylic acid methyl ester mixture made up ca. 90% of the sample; the remainder of the sample was the corresponding aromatic isomer mixture, the general structure of which is:



Experimental Animals and Diets

Male weanling SPF Albino rats (40-50 g) of Sprague Dawley descent, obtained from Murphy Breeding Laboratories (Plainfield, IN), were used for all experiments and housed in galvanized iron wire cages with mesh bottoms. The composition of the diets used (in g/100 g diet) was as follows: salt mixture w (a modification of the Osborne Mendel salt mix containing only inorganic constituents [18]), 3.5; vitamin premix,[3] 1.0; choline chloride, 0.15; corn oil containing either 0, 0.05, 0.15, or 1.0% cyclic fatty acid methyl ester, 15; casein, 8, 10, or 15; dextrose, to 100. All diets were made up in 1 kg batches and were isocaloric containing 4.72 calories/g. Thus, each diet contained 0.0075, 0.0225, and 0.15% cyclic fatty acid methyl ester. The diets were stored under nitrogen in the cold between feedings. Each group of animals was fed the diets for 6 weeks.

[1]Present address: Institute for Food Science and Technology, College of Fisheries, Department of Nutrition and Food Science, University of Washington, Seattle, WA 98195.

[2]Author to whom correspondence should be addressed.

[3]Vitamin mix (mg vitamin/kg diet): retinyl acetate, 20,000 IU; calciferol, 2,000 IU; α tocopherol, 100 IU; ascorbic acid, 22; inositol, 112; menadione bisulfite, 48; para-amino benzoic acid, 22; niacin, 100; Ca Panthothenate, 67; riboflavin, 22; pyridoxine HCl, 22; thiamine HCl, 100; biotin, 0.45; folic acid, 4; cyanocobalamin, 0.03.

Lipid extractions were accomplished according to the method of Folch et al. (19).

Statistical Treatment of Data

Data mean values are expressed as standard error of the mean (SEM). The F test for significance was employed (20).

RESULTS

Average wt gain, total and average feed consumed, and feed and energy efficiency data for animals in the dietary study are presented in Table I. In diets containing 8 and 10% protein, the average wt gained and feed consumed exhibited a progressively decreasing trend for animals in groups fed with increasing amounts of cyclic fatty acid methyl esters. There is one exception to the trend (group 6) where average wt gains and feed consumption tended to be less than that of a group with a higher content of cyclic esters in the diet. Group 8 gained significantly less wt ($P>0.05$) and consumed less food than did other groups fed the same protein level. Wt gains and feed consumption of animals fed 15% protein diets showed no signficant influence of cyclic monomer level.

The action of the cyclic monomer in the diet can also be observed in the values obtained for energy efficiency. These values measured the efficiency of utilization of food for growth; an increase indicated that the food was not utilized as well for growth. The modified protein efficiency ratios (PER) obtained (Table I) indicated that the lowest ratios were obtained for groups 4 and 8 compared with other groups consuming the same amount of protein. There was not a significant trend for animals fed 15% protein. Values reported for these modified PERs seem to lie on a curve similar to those obtained by Bunce and King (21) in which a maximum PER was obtained between 10-14% protein (casein). Lower PER values were obtained for lower and higher levels of protein.

Liver and Liver Lipid Wts

The percentages of liver:body and lipid:liver wt ratios of rats fed different levels of protein and cyclic fatty acids for different lengths of time are indicated in Table II. Certain experiments took longer to complete than others, and thus the liver:body wt ratios represent average ratios obtained during the period the experiments were conducted. Animals fed the 8% protein diets were fed the diets longer because of their small size upon completion of the 6-week dietary study. Therefore no tests of significance were applied. There were, however, small differences among liver:body wt ratios of animals fed different levels of cyclic acids and protein.

The lipid:liver wt ratios showed a large (probably significant) difference for rats that consumed 0.15% cyclic fatty acids in their diets in groups 1, 2, and 3. The amount of lipid in the liver of animals consuming 0.15% cyclic fatty acids in their diets was slightly higher than the others. The livers removed from rats that consumed 0.15% cyclic fatty acids (groups 1, 2, and 3) were spongy to the touch and white-yellow in color. It was clearly seen that certain lobes exhibited more pronounced fatty infiltration than others.

DISCUSSION

The feed consumption and wt gain data presented in Table I, especially with respect to animals fed 8 and 10% protein, showed trends which were observed by others who reported wt gains among animals fed various heated unfractionated fats (1-3,11,12). Altered organ wts, modified enzyme activities, and an acceleration in the formation of certain types of abnormal tissue have been reported in animals fed heated fats (22). Gottenbos and Thomasson (23) fed rats fish oils which contained low levels of aromatic cyclic fatty acids. Lower wt gains and feed consumption trends were observed in those animals fed increasing levels of cyclized fish oils. In a study comparing body wt changes in toxicity tests, Frazer (24) showed that wt gain and food intake tended to parallel each other. Animals fed small amounts of known toxic compounds also displayed differences in behavior and scattered significantly more food. Animals in the present dietary study also exhibited differences in behavior, and those fed 8% protein were clearly more tense and excitable than rats fed higher levels of protein. Animals fed different levels of protein and increasing amounts of cyclic fatty acids in their diets did not scatter any more food than the control animals. Lower wt gains achieved by these animals were not due to palatability, since preliminary studies in our laboratory indicated that diets containing cyclic compounds seemed to be preferred to diets without them.

A number of investigators have reported wts of and lipid content of livers from experimental animals fed heated fats (1,2,4-6,25,26). The results of liver wts reported varied from highly significant wt differences (5) to no wt changes for rats on heated and fresh oil diets (2,25,26). The protein content in the experimental diets ranged from 18% (5,25) to 30% (4), while others (1,2) provided diets in which the exact

TABLE I

Wt Gained and Feed Consumed by Rats Fed Different Levels of Cyclic Fatty Acids and Protein for 6 Weeks

Group	Protein fed (% by wt)	Number of animals	Cyclic fatty acids in diet (%)	Average wt gain[a] (g)	Average feed consumed (g)	Energy[b] efficiency	Wt gain[c]/wt protein consumed
1	8	8	0.0000	78.6 ± 4.5	448.9 ± 15.4	25.5 ± 1.9	2.06 ± 0.11
2	8	8	0.0075	79.0 ± 4.0	433.6 ± 19.2	25.2 ± 0.9	2.23 ± 0.07
3	8	8	0.0225	70.1 ± 3.2	415.5 ± 13.6	26.5 ± 0.8	2.06 ± 0.08
4	8	8	0.1500	62.0 ± 6.0	382.2 ± 21.6	27.2 ± 0.6	1.96 ± 0.13
5	10	10	0.0000	123.9 ± 10.4	488.0 ± 30.5	19.0 ± 0.7	2.49 ± 0.08
6	10	10	0.0075	102.7 ± 5.8	437.8 ± 11.4	20.0 ± 1.1	2.34 ± 0.11
7	10	10	0.0225	110.9 ± 5.5	451.6 ± 27.9	19.3 ± 0.4	2.44 ± 0.06
8	10	10	0.1500	89.0 ± 7.1[d]	400.9 ± 19.3	21.8 ± 1.3	2.21 ± 0.11
9	15	9	0.0000	213.4 ± 5.2	616.5 ± 8.4	13.5 ± 0.4	2.30 ± 0.06
10	15	10	0.0075	218.1 ± 5.5	640.4 ± 9.0	13.8 ± 0.3	2.26 ± 0.04
11	15	10	0.0225	219.6 ± 7.0	621.7 ± 14.8	13.4 ± 0.3	2.35 ± 0.05
12	15	10	0.1500	212.5 ± 6.6	602.5 ± 10.9	14.9 ± 0.8	2.34 ± 0.07

[a]Mean values ± SEM.

[b] $= \frac{\text{Caloric intake}}{\text{wt gain}}$

[c]A modified protein efficiency ratio.

[d]Significant at $P<0.05$ from groups fed the same protein level.