VEGETABLE OILS, OLEIC AND
LINOLEIC ACIDS #105 Vol I

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MONOGRAPH ON VEGETABLE OILS OLEIC AND LINOLEIC ACIDS

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Table of Contents

Summary	1
Chemical Information	
Vegetable Oils	7
Oleic Acid	19
Linoleic Acid	27
Biological Data	
Vegetable Oil	23
Oleic Acid	34
Linoleic Acid	40
Biochemical Aspects	42
Bibliography	53
Documents (A through E)	205
Documents (F through Anon.)	Vol. II

VEGETABLE OILS

Summary

As a result of their widespread use in the preparation of foods and as food additives, vegetable oils, oleic acid, and linoleic acid have been the subjects of a considerable amount of research.

The digestion and metabolism of the title substances have been studied in detail. Through tests with 225-250 g rats, Basu and Nath (129) found that mustard, coconut, olive, peanut, and sesame oils are readily absorbed when fed via stomach tube. The digestibility of 34 different vegetable oils was determined by Langworthy (1147) through 3-day oral tests with human volunteers. The coefficient of digestibility was found to be greater than 87% for all cases and greater than 97% for 20 of the 34 oils (1147). Bergstrom et al. (166) found that rats absorb 66-92% of orally administered oleic acid. Renner and Hill (1615) determined the metabolizable energy value and percent utilization of oleic acid to be 3770 cal/lb and 88.4, respectively, for the chick. In feeding studies with rats, Carroll and Richards (306) found the coefficients of digestibility for oleic and linoleic acids to be 73 and 84%, respectively.

According to Kayden et al. (1034) analysis of intestinal lumen contents of man after fat feeding has shown a separation of glycerides into two phases: an oil phase which contains mainly diglycerides and triglycerides and some fatty acid, and a micellar phase which is a combination of bile salts, free fatty acids, and monoglycerides. From the micellar solutions monoglycerides and free fatty acids are absorbed through the intestinal wall, leaving the bile salts within the lumen to form additional micelles. The fate of the free fatty acids within the mucosal cell is determined by their chain length and saturation; those of less than 12 carbons are preferentially absorbed by rats directly into the portal blood, whereas longer chain fatty acids are incorporated mainly into triglycerides, and appear in lymph chylomicrons. Monoglycerides within epithelial cells may be esterified to di- and triglycerides or cleaved to glycerol and free fatty acids. The 2-monoglycerides are esterified especially rapidly to triglycerides, but esterification of 1-monoglycerides lags and these are more likely to undergo hydrolysis particularly if the fatty acids are of short chain length.

Fatty acids play an important role as an energy source. Their oxidation is prominent in higher animals which can store large amounts of neutral fat as a fuel reserve. It is estimated that fatty acid oxidation provides at least half of the oxidative energy in the liver, kidneys, heart muscle and resting skeletal muscle. Fatty acids under-

going oxidation in tissues of higher animals come either from extracellular fluid or from endogenous intracellular lipids. Vertebrate blood contains considerable amounts of triacylglycerols but very small amounts of free fatty acids. The major endogenous source of fatty acids for fuel is storage fat, in the form of fat droplets in the cytoplasm, which consists largely of triacylglycerol. Before the fatty acids can undergo activation and oxidation, the triacylglycerols first must undergo hydrolysis by intracellular lipases to yield free fatty acids and glycerols. Free fatty acids are therefore derived either from the blood stream, in which they are transported bound to serum albumin, or prior hydrolysis of intracellular lipids. They are first activated by esterification with CoA to form acyl CoA esters of the outer mitochondrial membrane. Then the fatty acyl group from CoA is carried across the inner membrane by the carrier molecule carnitine. Finally the fatty acyl group is transferred from carnitine to intramitochondrial CoA: through several enzymatic reactions. A two-carbon fragment of the fatty acid is removed as acetyl CoA and two pairs of hydrogen atoms are given to specific acceptors. A long chain fatty acid will go through several spirals. Thus the 16-carbon palmitic acid undergoes a total of 7 such cycles, to yield 8 molecules of acetyl CoA and 14 pairs of hydrogen atoms. The acetyl CoA formed as a product of the fatty acid oxidation will then enter the tricarboxylic acid cycle, which is the final common pathway of oxidation catabolism of all fuel molecules in aerobic cells (1172).

In tests with 3-month-old male Wistar rats, Cenedalla and Allen (323) found that approximately 25% of an orally administered dose of linoleic acid was excreted in the form of CO_2 within a week. Bergstrom et al. (166) observed that rats fed ^{14}C-labelled oleic acid or methyl oleate expired about 16% of the absorbed (63% absorbed) activity as CO_2 within 24 hours.

Oleic and linoleic acids have been found to produce, in isolated mitochondria, manifold changes which may be summarized as follows: activation of latent ATPase (1566, 238); inhibition of the ATP-Pi exchange reaction (908, 2060, 1964); uncoupling of oxidative phosphorylation (908, 1964); and induction of mitochondrial swelling (1173, 88, 1964, 2095). An investigation by Basu and Nath (130) on calcium and phosphorus metabolism in four human subjects led to the conclusion that the addition of mustard, sesame, or peanut oil to the diet greatly favored the absorption of Ca and P, while with the addition of coconut oil the elimination of Ca through the urine and feces was greatly increased, the fecal Ca being in 3 cases greater than the amount of dietary Ca. Calvery and Kennedy (288) studied the effect of peanut oil, coconut oil and hydrogenated cottonseed oil on Ca and P metabolism in the rat and concluded that the addition of 5% of each of the oils to a normal diet increased the fecal excretion of Ca, paralleled by a decrease in the retention of Ca and P. Rao and De (1598) reported that the inclusion of 10% fat (coconut oil, saturated and unsaturated fatty acid fractions) in a normal diet increased the utilization of Ca in young growing rats, while Sodasivan (1666) reported that adult rats showed a positive Ca balance with coconut oil as diet fat. Male albino

rats of the Holtzman strain were observed by Janssen (964) to exhibit a rise in plasma cholesterol when fed a diet containing 15% free linoleic acid. Renaud and his coworkers (1614, 1612, 1613) reported that oleic and linoleic acids appear to be atherogenic to rats when included in a hyperlipemic diet. Schneider (1708) reported that gastric secretion in the rat can be inhibited by the intraduodenal administration of olive oil, oleic acid, or sodium oleate.

Schneider (1707) has shown that a supplementation of 2 g/day of oleic acid to pullets partially overcomes the adverse effects of S. foetida oil (inhibition of ovary and oviduct development and consequently egg production). Studies by Tove (1916) have shown that the addition to the diet of 4% of fats rich in either oleate or linoleate prevents the poor growth and high mortality that results when diets containing high levels (20-40%) of either palmitate or stearate are fed to mice. The daily administration of 2000-2500 i.v. of tetracycline to mice was found by Gomazkov et al. (689) to increase the resistance of the animals to lethal doses of oleic acid.

A considerable amount of research into the possible carcinogenicity of vegetable oils has been carried out. The administration of peanut oil to a total of 97 XVII X C57 (Black) hybrid mice by four different routes - cutaneous application, s.c. injection, i.p. injection, and feeding by stomach tube - was found by Gothoskar and Ranadive (701) to produce no evidence of malignancy at the site of treatment or in the remote organs of the test animals even though the animals were observed from the age of 10-12 weeks till death. During an observation period of 2 years, Belman et al. (154) observed no tumors in 12 rats which had received i.p. injections of 0.5 ml of peanut oil twice weekly for 3 months. In a study by Roe and Carter (1634), 16 male CB stock rats given 20 weekly i.m. injections of 0.2 ml of peanut oil developed no tumors within the 63-week observation period. Observation by Dickens et al. (489) for over 2 years revealed the formation of no tumors in 12 male rats injected s.c. with doses of 0.5 ml of peanut oil twice weekly for 57 weeks. Fitzgerald et al. (595) reported that daily topical application of peanut oil to 12 albino mice resulted in retardation of hair growth and slight to moderate exfoliation of the epidermis by day 14. In 4 rabbits, Fitzgerald et al. (595) observed that twice-daily topical application of peanut oil induced a slight erythema after 5-9 days of treatment. The administration of mustard oil to a total of 86 hybrid mice (XVII x C57 black) by cutaneous application, s.c. injection, i.p. injection, and feeding by stomach tube was observed by Ranadive and Gothoskar (1591) to produce no evidence of malignancy due to the mustard oil at the site of treatment or in the remote organs even though the animals were observed for their complete life span of over two years. Following repeated i.p. injections of olive oil (unspecified dosage and number) in 40 rats and 60 mice, Burrows et al. (281) observed that peritoneal tumors developed in one rat after 27 weeks and in two mice after 46 and 62 weeks, respectively, while eight rats given weekly s.c. injections of 1.0 ml of olive oil developed no tumors. Observation by Weisburger et al. (2033), for 9 months, of 20 Sprague-Dawley rats given 1 ml of sesame oil by gastric intubation daily for 45 days revealed the formation of only one tumor.

Although O'Gara et al. (1453) found that no tumors were produced in rats by the s.c., i.m. or oral administration of fresh whole corn oil, the i.m. injection of the non-urea-adduct fraction of the corn oil resulted in a significant increase in the incidence of sarcomas of the injection site, indicating the presence of a carcinogen in the corn oil. Studies by Szepsenwol (1855, 1856) in which mice were maintained for several generations on diets supplemented with corn oil (100-150 mg/day) with or without the addition of cholesterol (4-5 mg/day) led to the conclusion that corn oil increases the incidence of tumor formation. A semisynthetic high-corn oil (20%) diet was found by Gammal et al. (648) to enhance the development of mammary cancer induced by DMBA in female Sprague-Dawley rats as compared to 2 other groups of similarly treated rats fed a high-coconut oil (20%) and a low-fat (0.5%) semisynthetic diet.

The carcinogenicity of oleic acid has also been studied extensively. Following treatment of 16 mice with a total of 33 injections of 0.5 mg of oleic acid per injection administered at a rate of 2 injections per week and of 15 mice with a total of 10 injections of 0.1 mg each administered 3 times per week, Swern et al. (1851) concluded that oleic acid exhibits no carcinogenic activity when administered in this manner. The intradermal injection by Berlin and Wyman (168) of solutions (25-100%) of oleic acid, in 0.1 ml doses, into the backs of two guinea pigs produced local inflammation and necrosis. The application of undiluted oleic acid to the backs of male white mice 6 times per week for 1 month was found by Setala et al. (1739) to produce hyperplasia in the interfollicular epidermis. Holsti (884) found that topical application of oleic acid to mice once daily 6 days per week for 24 weeks resulted in tumor formation only when the animals were previously treated with DMBA, in which case oleic acid exhibited considerable tumor-promoting activity (884, 885, 886). Bottomley and Twort (245) reported the observation of no more than about a dozen benign tumors among 1,000 mice painted with oleic acid, however, they found that the presence of oleic acid enhances the carcinogenic activity of chrysene and 1:2:5:6-dibenzanthracene. The administration by Shubik (1752) of undiluted oleic acid to the skin of 10 mice twice weekly for 20 weeks starting 3 weeks after initiation with DMBA resulted only in slight epidermal hyperplasia without the promotion of any tumors.

Based on the data obtained through a series of experiments, Carroll and Noble (305) concluded that oleic acid, at a dietary level of 15%, does not effect the growth or general health of rats, but greatly impairs their reproduction, through interference with parturition and repression of mammary gland development and lactation.

Through tests on rabbits, guinea pigs, and mice, Flesch (606) found oleic and linoleic acids to be potent local depilatory agents, producing a marked, although reversible, irritating effect on the animal skin, characterized by crusting, ulceration and thickening.

The acute toxicity of the vegetable oils has not received a great

deal of consideration. Oro and Wretlind (1467) determined the LD_{50} of cottonseed oil, when administered i.v., to be greater than 15 g/kg for mice. No adverse effects were observed by Schwartze (1719) in mice, rats, guinea pigs, rabbits or cats following i.p. injections of 1-10 ml of cold-pressed peanut oil.

A little more data is available on the acute toxicity of oleic acid, however, only one report deals with linoleic acid. The LD_{50} of oleic acid for mice was determined by Oro and Wretlind (1467) to be 230 mg/kg when injected intravenously. Ungar et al. (1953) reported that single intrasplenic injections of 0.15-0.5 ml of a 25-50% emulsion of oleic or linoleic acid into rats caused a variable and transient parenchymal injury and, in a few cases, death. Kinosita et al. (1067) reported that single i.p. injections of 10 mg Na oleate into rats resulted in reversible changes in the liver. Intravenous doses of 0.25 ml of oleic acid were found by Jefferson and Necheles (966) to have little effect on dogs, while doses of 0.5-5 ml were highly toxic, causing reduced blood pressure, urination, defecation, rapid forced respiration, coughing, and massive pulmonary edema, followed, with the higher doses, by death. Baker et al. (100) reported that the i.v. injection of 0.07 ml of oleic acid/kg in dogs resulted in an immediate drop in arterial O_2 partial pressure, compliance and surfactant activity.

The short-term toxicity of vegetable oils has been tested by several workers, all of whom used rats as test animals. Through a series of 6-week feeding tests with rats, Thomasson (1894) investigated the biological action of 20 oils and fats and found that while the various oils and fats exhibited some differences in growth-promoting effect when fed at various levels up to 73 cal %, only rapeseed and kapokseed oils were harmful to the animals, producing increased mortality of the higher dietary levels. Cameron et al. (291) found that the daily i.p. injection of 0.1 ml of peanut oil into rats for 17-18 days caused testicular atrophy and decreased growth rate. Gothoskar and Ranadive (701) observed no adverse effects when rats were fed 0.1 ml of peanut oil by stomach tube daily for 6 months. Bruce and Tobin (266) reported that daily i.p. injections of 0.25-1.0 ml of sesame oil for several weeks produced toxic effects in normal male rats, manifested in decreased growth rate, decreased testicular weight and increased adrenal weight. After feeding rats for 90 days on a diet containing 25% refined coconut oil, Harris and Mosher (793) concluded that coconut oil is harmless to rats when fed at this level.

Chicks, rats, and rabbits have been used in testing the short-term toxicity of oleic and linoleic acids. Groups of day-old chicks maintained for 4 weeks by Sunde (1839) on a chick starting ration supplemented with 5% oleic or linoleic acid exhibited no deleterious effects consequent of their diet. Gyorgy et al. (765) reported that a diet containing 16% linoleic acid with or without 0.06% butter yellow produced toxic effects in rats, manifested in loss of weight, progressive anemia of the secondary type, leucopenia and, eventually, death. Scaling and reversible hair loss on the ears was observed by Flesch (605) in rabbits following oral administration of 40 ml of

oleic acid in 4 equal doses every other day.

In the only long-term study reported, mice were given weekly or biweekly s.c. injections of oleic acid (0.25-0.5 ml, 5% emulsion) by Robertson et al. (1629) from 5 weeks to 400 days of age. The oleic acid had no appreciable effect on the growth of the mice but reduced the mean life duration from 772 to 661 days for the males and from 780 to 702 days for the females (1629).

The effect of heating on the nutritive value of vegetable oils has received extensive study. Roy (1651) reported that the absorbability of vegetable oils is decreased by thermal treatment. In a series of tests by Peacock et al. (1514), cottonseed oil heated under various conditions was fed to mice at levels of 1-5% of the diet and was found to significantly increase the incidence of tumors of the forestomach and hepatomata. Beck and Peacock (148) reported that rats maintained on an adequate basal diet supplemented with previously heated cotton-seed oil developed signs of avitaminosis-A within a year, accompanied by ulceration and papillomatosis of the fore-stomach. Dietary additions of 10% previously heated linseed, rapeseed, corn, and peanut oil and 20% previously heated soybean oil have been found by Crampton et al. (415) to significantly depress the growth of rats and the efficiency of feed utilization. Significant adverse effects on weight gain were observed by Raju and Rajagopalan (1584) when previously heated peanut, sesame, and coconut oils were incorporated into the diets of rats to give 15% fat. When the same oils were fed at a level of 30%, all of the rats died within one week (1584). Steiner et al. (1818) and Beck (147) reported that previously heated sesame and cotton-seed oils showed carcinogenic activity when administered to mice subcutaneously. However, Chalmers (340) and Arffman (62) found no evidence of carcinogenicity in animals treated with previously heated cottonseed or soybean oil.

VEGETABLE OILS

Chemical Information

The following information on vegetable oils was obtained from the Merck Index (1296).

Coconut Oil. Copra oil. Expressed oil from kernels of *Cocos nucifera* L., *Palmae*. *Constit.* Trimyristin, trilaurin, tripalmitin, tristearin; also various other glycerides.

White, semisolid, lard-like fat; stable to air. Remains bland and edible for several years under ordinary storage conditions. d_{4}^{40} 0.903. mp 21–25°. n_{D}^{40} 1.4485–1.4495. Sapon. no. 255–258. Iodine no. 8–9.5. Acid no. not over 6. Surface tension at 20° = 33.4 dyn/cm, at 80° = 28.4 dyn/cm. Practically insol in water and 95% alcohol, more sol in absolute alcohol; very sol in chloroform, ether, carbon disulfide. Solubility data: Rao, Arnold, *J. Am. Oil Chemists' Soc.* 33, 389 (1956).

USE: Manuf soap, edible fats, chocolate, and candies; in baking instead of lard; in candles and night lights; in dyeing cotton; as an ointment base; in hair dressing and in massage.

Corn Oil. Maize oil; Mazola oil; Maydol. Obtained as a byproduct by wet milling the grain of *Zea mays* L., *Gramineae* for the manuf of corn starch, corn syrup, glucose, dextrins, etc.: E. W. Eckey, *Vegetable Fats and Oils* (Reinhold, New York, 1954). *Constit.* Glycerides of the following fatty acids: Myristic 0.1–1.7%, palmitic 8–12%, stearic 2.5–4.5%, hexadecenoic 0.2–1.6%, oleic 19–49%, linoleic 34–62%. Unsaponifiable fraction: 1–3% (γ-tocopherol 0.1%, the rest is mostly isomeric sitosterols and wax such as myricyl and ceryl alcohols). The crude oil may contain up to 2% phospholipids (vegetable lecithin, inositol esters). The following constants are for the refined product.

Yellow oil. Faint characteristic odor and taste. d_{25}^{25} 0.916–0.921. mp −18 to −10°. Titer 14–20°. Flash pt 610°F (321°C). Ignition pt 740°F (393°C). n_{D}^{25} 1.470–1.474; n_{D}^{40} 1.464–1.468. Acid value 2–6. Saponification value 187–196. Iodine value 109–133. Thiocyanogen value 71–77. Hydroxyl value 5–12. Reichert-Meissl value <0.5. Polenske value <0.5. Hehner value 92–96. Classed as a semidrying oil. On prolonged exposure to air it thickens and becomes rancid. Miscible with chloroform, ether, benzene, petr ether. Slightly sol in alc.

USE: Mainly as salad and cooking oil. In pharmacy as vehicle for i.m. injections. Some use in the preparation of non-yellowing enamel paint.

MED USE: Promoted as a source of polyunsaturated fatty acids in special diets.

Cottonseed Oil. Fixed oil from seeds of cultivated varieties of *Gossypium herbaceum* L. or of other species of *Gossypium*.

Pale yellow, oily, practically odorless liq. d_{25}^{25} 0.915–0.921. Solidif 0° to −5°. n_{D}^{40} 1.4645–1.4655. Sapon. no. 190–198. Iodine no. 105–114. Surface tension at 20° = 35.4 dyn/cm, at 80° = 31.3 dyn/cm. Slightly sol in alcohol; miscible with chloroform, ether, carbon disulfide, petr ether.

USE: Manuf soaps, oleomargarine, hydrogenated fats, lard substitute, glycerol, leather dressings, lubricants, cosmetics; also as salad and cooking oil; packing fish.

MED USE: Has been used as emollient laxative; promoted as a source of polyunsaturated fatty acids in special diets.

VET USE: Delousing agent. Combined with 2 parts of pine tar for ear ticks of horses. Mild laxative and emollient in small animals. *Dose:* dogs: 15–60 ml; cats: 4–30 ml.

Linseed Oil. The oil obtained by expression of linseed. *Constit.* Glycerides of linolenic, linoleic, oleic, stearic, palmitic and myristic acids. *Ref:* T. P. Hilditch, *The Chemical Constitution of Natural Fats*, 3rd ed (London, 1956, p 175 sqq. E. W. Eckey, *Vegetable Fats and Oils* (New York, 1954), pp 535–547.

Yellowish liq, peculiar odor, bland taste. Exposed to air it gradually thickens, becomes darker, and acquires a more pronounced odor and taste. d 0.925–0.935. n_{D}^{40} 1.4725–1.4750. Does not congeal above −20°. Sapon no. 187–195. Iodine no. not below 170. Unsaponifiable matter not over 1.5%. Slightly sol in alcohol, miscible with chloroform, ether, petr ether, carbon disulfide, oil turpentine.

USE: In varnishes, paints, putty, oilcloths, linoleum, printing inks, artificial rubber, tracing cloth, tanning and enameling leather; applied to paper and fabrics to render them waterproof and tough.

MED USE: Emollient in liniments, pastes, medicinal soaps.

VET USE: Laxative for large animals. *Dose:* horses: 500–750 ml; sheep and swine: 120–180 ml. *Note:* Not suitable for small animals.

Olive Oil. A fixed oil obtained from ripe olives, the fruit of the cultivated olive tree *Olea europaea* L., *Oleaceae*. Produced almost exclusively in the countries adjoining the Mediterranean Sea, Spain being the largest producer. Whole olives are crushed in edge-runner mills and the oil is expressed in open hydraulic presses. *Constit.* Mixed glycerides of oleic acid 83.5%, of palmitic acid 9.4%, of linoleic acid 4.0%, of stearic acid 2.0%, of arachidic acid 0.9%. Minor constituents are squalene, up to 0.7%, phytosterol and tocopherols about 0.2%. *Reviews and bibliographies:* José M. de Soroa y Pineda, *El aceite de oliva* (1944, Dossat, Madrid); R. F. Simari, G. B. Martinenghi, *Olivicoltura e Oleificio* (1950, Hoepli, Milano); P. G. Garoglio, *Tecnologia de los Aceites Vegetales* (1951, Mendoza, Argentina); E. W. Eckey, *Vegetable Fats and Oils* (1954, Reinhold, New York).

Pale yellow or light greenish-yellow oil with a pleasing, delicate flavor. Becomes rancid on exposure to air. Begins to get turbid at +5 to +10°C, below 0°C it forms a whitish, granular mass. Flash pt 437°F (225°C). Ignition temp 650°F (343°C). d_{15}^{15} 0.914–0.919; d_{25}^{25} 0.909–0.915. n_{D}^{25} 1.466–1.468; n_{D}^{40} 1.460–1.464. Titer 17–26°. Acid value 0.2–2.8. Saponification value 187–196. Iodine value 79–90. Thiocyanogen value 75–83. Hydroxyl value 4–12. Reichert-Meissl value 0.2–1.0. Unsaponifiable 0.5–1.3%. Slightly sol in alcohol. Miscible with ether, chloroform, carbon disulfide.

USE: As food in salads, with sardines, for cooking and baking. In the manuf of soaps, textile lubricants, sulfonated oils, cosmetics and pharmaceutical preparations.

MED USE: Laxative, cholagogue, emollient.

VET USE: Laxative for dogs, cats. *Dose:* dogs: 15–60 ml; cats: 5–15 ml.

Palm Oil, From Fruit. Palm butter. Obtained from the fruit *Elaeis guineensis* Jacq., *Palmae*. *Constit.* Chiefly palmitin; some stearin, linolein.

Reddish-yellow to dark dirty red, fatty mass; faint odor of violet. d 0.920–0.927. mp 27–42.5°. n_{D}^{40} 1.453–1.459. Sapon no. 200–205. Iodine no. 53–57.

USE: In manuf of soap, candles; lubricant.

Palm Oil, From Seed. An oil obtained from seed of *Elaeis guineensis* Jacq., *Palmae*.

White to yellowish. d 0.952. mp 26–30°. Sapon. no. about 247. Iodine no. about 15.

USE: Manuf soap; with coconut oil in manuf plant butter; also has been used in liniments and ointment.

Peanut Oil. Arachis oil; groundnut oil; earthnut oil; katchung oil. Prepd by pressing the shelled and skinned seeds of *Arachis hypogaea* L., *Leguminosae:* N. J. Morris, F. G. Dollear, *Abstract Bibliography of the Chemistry and Technology of Peanuts*, Southern Regional Res. Lab. (New Orleans, 1949); E. W. Eckey, *Vegetable Fats and Oils* (Reinhold, New York, 1954). *Constits.* of cold pressed oil: Glycerides of the following fatty acids: Palmitic 8.3%, stearic 3.1%, arachidic 2.4%, behenic 3.1%, lignoceric, 1.1%, oleic 56.0%, linoleic 26.0%. Traces of capric and lauric acids have been reported. Unsaponifiable matter 0.8%. The unsaponifiable fraction includes tocopherols (0.022 to 0.059%), sterols (0.19 to 0.25%), squalene (0.027%) and other hydrocarbons.

Greenish-yellow or almost colorless oil. Mild, pleasant odor. Bland taste. d_{15}^{15} 0.917–0.921. d_{25}^{25} 0.910–0.915. Clouds at low room temp. Solidifies at about −5°. A.S.T.M. cloud point +4.5°; A.S.T.M. pour point +1°. Titer 26 to 32°. Flash pt 540°F. (283°C.); ignition temp 833°F. (443°C.). n_{D}^{25} 1.466–1.470; n_{D}^{40} 1.4605–1.4645. Acid value 0.08–6; saponification value 188–195; iodine value 84–102; thiocyanogen value 67–73; hydroxyl value 2.5–9.5; Reichert-Meissl value 0.2–1.0; Polenske value 0.2–0.7. On prolonged exposure to air, very slowly thickens and becomes rancid. Miscible with ether, petr ether, chloroform, carbon disulfide. Soluble in benzene, carbon tetrachloride, oils. Very slightly sol in alcohol.

USE: Edible oil: For salad oil as is; in hydrogenated state as shortening, in mayonnaise, in confections. For the manuf of margarine, soaps, paints. In pharmacy as vehicle for i.m. medication, in the laboratory as heat transfer medium in melting point apparatus.

MED USE: Solvent. In ointments and liniments.

VET USE: *See* Olive Oil.

Poppy Oil. Poppy-seed oil. Expressed from poppy seeds. Contains no morphine or other opium alkaloids.

Pale yellow, drying oil; pleasant odor and taste. d 0.924–0.927. Solidif about −18°. n_{D}^{20} 1.4766–1.4774. Sapon. no. 189–197. Iodine no. 133–158.

USE: Manuf paints, varnishes, and soaps. Edible grades are marketed in Europe and Asia.

See also Ethiodol; Lipiodol.

Rapeseed Oil. Colza oil. Oil expressed from seeds of *Brassica campestris* L., *Cruciferae.*

Pale yellow, rather viscid liquid. d 0.913–0.917; n_{D}^{20} 1.4720–1.4752. Solidif −2° to −10°. Sapon no. 170–177. Iodine no. 97–105. Soluble in chloroform, ether, carbon disulfide.

USE: Lubricant; manuf rubber substitutes, margarine, soft soaps, blown oils; oiling woolens.

Safflower Oil. The oil from the seed of *Carthamus tinctorius* L., *Compositae.* Milling and extraction procedures: Winter, *J. Am. Oil Chemists' Soc.* 27, 82 (1950). Purification and stabilization: Freedman, Shapiro, U.S. pat. 2,978,381 (1961). Monograph: R. E. Woodward, G. M. Severson, *Industrial Survey of Safflower*, Chemurgy Dept. Report no. S-3, Agricult. Expt. Sta., Lincoln, Nebraska, 1951. Fatty acids present as glycerides: Palmitic 6.4%, stearic 3.1%, arachidic 0.2%, oleic 13.4%, linoleic 76.6–79.0%, linolenic 0.04–0.13%.

Edible drying oil, intermediate between soybean and linseed oil. d_{25}^{25} 0.9211–0.9215. Titer: 15–18°. n_{D}^{25} 1.472–1.475. n_{D}^{40} 1.4690–1.4692. Acid value 1.0–9.7. Saponification value 188–194. Iodine value 140–150. Thiocyanogen value 82.5–86.0. Reichert-Meissl value below 0.5. Hydroxyl value 2.9–6.0. Unsaponifiable below 1.5%. Thickens and becomes rancid on prolonged exposure to air. Soluble in the usual oil and fat solvents.

USE: As linseed oil in paints. For salad oil blends, in hydrogenated state as shortening.

MED USE: Dietary supplement in hypercholesteremia (and possible prophylaxis and treatment of atherosclerosis).

Sesame Oil. Benne, teel or gingilli oil. From the seeds of cultivated varieties of *Sesamum indicum* L., *Pedaliaceae. Constit.* Olein, stearin, palmitin, myristin, linolein, sesamin, sesamolin. *Review:* Budowski, Markley, *Chem. Revs.* 48, 125 (1951).

Pale yellow oil; almost odorless; bland taste. d 0.916–0.920. Solidif about −5°. $[\alpha]_{D}^{25}$ +1° to +9°. n_{D}^{40} 1.4650–1.4665. Sapon no. 188–193. Iodine no. 103–122. Insoluble in water; sol in chloroform, ether, petr ether, carbon disulfide; slightly sol in alcohol.

USE: Manuf oleomargarine, cosmetics, iodized oil, etc.

MED USE: Has been used as laxative, extern. as skin softener. Vehicle for i.m. medication.

VET USE: Contains components active against lice of farm animals.

Soybean Oil. Obtained from soybeans by solvent extraction using petroleum hydrocarbons or, to a lesser extent, by expression using continuous screw press operations. *Constit.* Triglycerides of oleic acid 26%, of linoleic acid 49%, of linolenic acid 11%, of saturated acids 14%. Free fatty acids are usually less than 1%. Phospholipids (lecithin) 1.5–4%. Another 0.8% consists of stigmasterol sitosterols, and tocopherols. The phospholipids and sterols are removed by refining with alkali. *Reviews and bibliographies:* K. S. Markley, *Soybeans and Soybean Products*, 2 vols. (1951, Interscience, New York); E. W. Eckey, *Vegetable Fats and Oils* (1954, Reinhold, New York); J. C. Cowan in Kirk-Othmer, *Encyclopedia of Chemical Technology* vol. 12 (1954, Interscience, New York), pp 689–701.

Pale yellow to brownish-yellow oil. Slight characteristic odor and taste. The flavor of crude soybean oil has been described as paint-like and grass-like, and is somewhat distasteful to the palate of occidentals. d_{25}^{25} 0.916–0.922. Flash pt 540°F (282°C). Ignition temp 833°F (445°C). n_{D}^{25} 1.471–1.475. Titer 22–27°. Solidifies at −10 to −16°. Viscosity in centipoises: 172.9 at 0°; 99.7 at 10°; 50.09 at 25°; 28.86 at 40°. Acid value 0.3–3.0. Saponification value 189–195. Iodine value 127–138. Thiocyanogen value 77–85. Diene no. 0.7. Hydroxyl value 4–8. Reichert-Meissl value 0.2–0.7. Polenske value 0.2–1.0. Miscible with abs alcohol, ether, petr ether, chloroform, carbon disulfide.

USE: Directly as food in the Orient, also in blends with olive oil. In the manuf of margarine, shortenings, candy, soap. In drying oil industries (paints, varnishes, linoleum, printing ink). The foots or residue from refining has been used to obtain soybean fatty acids which can be used in the manuf of alkyd resins.

Sunflower Seed Oil. The oil obtained by milling the seeds of *Helianthus annuus* L., *Compositae.* Produced on a large scale in U.S.S.R., India, Egypt, Argentina. *Reviews:* E. W. Eckey, *Vegetable Fats and Oils* (Reinhold, New York, 1954), pp 772–777; L. H. Bailey, *Standard Cyclopedia of Horticulture* (Macmillan, New York, 1953) vol. II, pp 1445–1449.

Pale yellow oil. Bland, agreeable taste. d_{15}^{15} 0.922–0.926. d_{25}^{25} 0.915–0.919. mp −18°. Titer: 16–20°. Acid value: 0.6. Saponification value: 188–194. Iodine value: 125–136. Thiocyanogen value: 78. Hydroxyl value: 14–16. Reichert-Meissl value below 0.5. Polenske value below 0.5. Unsaponifiable below 1.5%. n_{D}^{25} 1.472–1.474. n_{D}^{40} 1.466–1.468. Palmitic acid 6.4%, stearic acid 1.3%, arachidic acid 4.0%, behenic acid 0.8%, oleic acid 21.3%, linoleic acid 66.2% (the glycerides in sunflower oil consist mainly of mixed triglycerides, each contg one or two linoleic acid radicals), linolenic acid <0.1%. Vitamin E (mixed tocopherols) 75 mg/100 g. This approaches the tocopherol content of wheat germ oil which is 103 mg/100 g. Slightly sol in alc. Miscible with benzene, chloroform, carbon tetrachloride. Classified as a semidrying oil. Forms a "skin" after exposure to air for 2–3 weeks.

USE: Food and salad oil, in candy manuf, in oleomargarine. Like wheat germ oil in dietary supplements. Industrially in oil-modified alkyd resins and soap manuf.

Grieco and Piepoli analyzed the oils extracted from 36 different species of seeds and fruits for fatty acid composition. The results they obtained are presented in Tables 1 and 2 (723).

A study similar to that of Grieco and Piepoli was performed by Amelotti et al. (40). The fatty acid compositions of 76 oils extracted from the seeds of plants belonging to 21 families are shown in Table 3.

A more complete analysis of vegetable oils was carried out by Fedeli and Jocini. In addition to the fatty acid composition (see Table 4). They also analyzed the unsaponifiable fraction of the oils (generally less than 2%), and found it to contain chiefly tocopherols, sterols, squalene, terpenic alcohols, and hydrocarbons (see attached article for details) (573).

Table 4
COMPOSITION OF SOME COMMON SEED OILS

Acids	Corn	Safflower	Rape	Linseed	Soybean	Cotton	Peanut	Olive	Palm	Sunflower	Sesame
14:0	–	Tr[a]	–	–	Tr	1	Tr	Tr	1	–	–
16:0	13	8	4	6	11	29	6	14	48	11	10
18:0	4	3	2	4	4	4	5	2	4	6	5
20:0	Tr	Tr	–	Tr	Tr	Tr	2	Tr	–	–	–
22:0	Tr	–	–	–	Tr	–	3	–	–	–	–
24:0	–	–	–	–	–	–	1	–	–	–	–
14:1	–	–	–	–	–	Tr	–	–	–	–	–
16:1	–	–	–	–	–	2	Tr	2	–	–	–
18:1	29	13	19	22	25	24	61	64	38	29	40
20:1	–	–	13	–	–	–	–	–	–	–	–
22:1	–	–	40	–	–	–	–	–	–	–	–
18:2	54	75	14	16	51	40	22	16	9	52	45
18:3	–	1	8	52	9	–	–	–	–	–	–

[a]Tr = trace.

Table 1

Semi e frutti oleosi	Nome botanico	Famiglia	Provenienza	Contenuto in olio (etere di petrolio) %	Acidità dell'olio (in acido oleico) %	Laurico 12:0	Miristico 14:0	Palmitico 16:0	Palmitoleico 16:1	Eptadecanoico 17:0	Eptadecenoico 17:1	Stearico 18:0	Oleico 18:1	Linoleico 18:2	Linolenico 18:3	Arachico 20:0	Eicosenoico 20:1	Eicosadienoico 20:2	Beenico 22:0	Erucico 22:1	Docosadienoico 22:2	Lignocerico 24:0	Tetracosenoico 24:1
Anacardio o Cajù	Anacardium	Anacardiaceae	Mozambico	49,9	2,5	—	tracce	10,8	0,5	0,1	tracce	8,2	60,8	18,9	0,4	0,3	tracce	—	—	—	—	—	—
Arachide	Arachis Hypogeae	Leguminosae	Gambia	45,1	1,6	—	tracce	9,1	0,3	tracce	—	3,7	63,4	17,0	—	1,4	1,2	—	2,5	—	—	1,3	—
Arachide	» »	»	Nigeria	48,1	1,2	—	tracce	10,3	0,3	tracce	—	3,8	60,2	19,4	—	1,3	1,2	—	2,3	—	—	1,2	—
Arachide	» »	»	Rhodesia	47,5	0,7	tracce	tracce	12,5	0,2	tracce	—	4,5	44,2	31,9	tracce	1,7	1,0	—	3,2	—	—	0,7	—
Arachide	» »	»	Senegal	48,3	1,1	—	tracce	11,0	0,3	tracce	—	3,7	57,6	21,0	—	1,4	1,2	—	2,6	—	—	1,2	—
Arachide	» »	»	Sudan	45,2	1,2	—	tracce	12,2	0,3	tracce	—	4,9	46,1	29,3	—	1,7	1,1	—	3,3	—	—	1,0	—
Arachide	» »	»	Sud Africa	46,0	0,6	—	tracce	11,7	0,1	tracce	—	4,0	40,4	35,7	—	1,9	1,1	—	3,6	—	—	1,5	—
Arachide	» »	»	Sud Africa	45,5	0,4	—	tracce	12,5	0,2	tracce	—	4,6	44,5	31,2	—	1,8	0,9	—	3,2	—	—	1,1	—
Canapa	Cannabis Sativa	Moraceae	Italia	31,7	3,5	—	tracce	5,6	0,3	0,1	tracce	2,6	10,6	59,4	19,4	1,9	tracce	—	tracce	—	—	—	—
Cartamo	Carthamus Tinctorius	Compositae	Stati Uniti	30,9	2,8	—	0,1	6,8	0,6	—	—	2,9	13,3	75,4	—	0,5	0,3	—	—	—	—	—	—
Cipolla	Allium Cepa Linn	Liliaceae	Italia	21,7	11,6	—	0,1	9,8	0,4	—	—	2,2	24,1	62,3	0,1	0,1	0,2	—	tracce	0,7	—	—	—
Colza	Brassica Species	Cruciferae	Canadà	40,1	1,2	tracce	tracce	2,6	0,3	tracce	tracce	1,3	31,0	17,5	8,5	0,5	11,1	0,5	0,3	24,9	0,3	tracce	1,2
Colza	» »	»	Cina	41,1	1,8	—	tracce	3,4	0,2	—	—	1,2	18,9	13,4	7,5	0,7	11,0	0,3	0,6	42,1	tracce	—	0,7
Colza	» »	»	Eritrea	46,3	1,2	—	tracce	3,3	0,1	—	—	1,2	13,0	16,2	13,8	0,7	10,1	0,8	tracce	40,7	0,6	0,2	1,7
Colza	» »	»	Francia	40,9	1,0	tracce	tracce	3,2	0,3	0,1	—	1,3	13,1	13,8	8,5	tracce	9,5	0,5	tracce	49,0	0,5	—	1,1
Colza	» »	»	It. (Emilia)	40,8	0,7	tracce	tracce	3,2	0,2	tracce	—	1,1	16,1	12,0	6,7	0,7	9,2	0,3	tracce	48,9	0,5	—	1,0
Colza	» »	»	It. (Lombardia)	37,8	0,7	tracce	tracce	2,8	0,2	—	—	1,1	11,5	14,2	10,8	0,4	10,1	0,5	tracce	46,9	0,7	—	0,7
Colza	» »	»	It. (Veneto)	41,2	0,8	—	tracce	3,8	0,3	0,1	—	1,0	13,7	12,5	6,3	0,6	10,4	0,3	tracce	49,8	0,5	—	0,6
Colza «Dadu»	» »	»	Pakistan	37,6	0,9	—	tracce	2,6	0,2	—	—	1,2	13,2	15,0	10,1	0,5	8,2	0,6	1,5	45,1	0,9	—	0,8
Colza «Naw»	» »	»	Pakistan	40,3	0,8	—	tracce	2,5	0,2	—	—	1,2	13,7	15,6	9,1	0,6	8,4	0,5	0,9	45,6	0,6	—	1,0
Colza	» »	»	Svezia	41,9	1,3	tracce	tracce	3,2	0,2	tracce	—	1,0	11,0	13,9	9,3	0,9	7,6	0,5	tracce	51,0	0,7	—	0,7
Cotone	Gossypium Species	Malvaceae	Italia	24,8	1,1	tracce	0,9	25,2	0,3	0,2	0,1	2,7	18,6	51,1	0,1	0,3	—	—	tracce	—	—	—	—
Girasole	Helianthus Annuus	Compositae	Bulgaria	46,1	1,1	—	tracce	7,5	0,1	tracce	—	3,8	29,6	57,3	0,2	0,8	0,1	—	0,6	—	—	—	—
Girasole	» »	»	It. (Veneto)	43,1	2,7	—	tracce	6,3	0,1	tracce	—	3,9	32,7	55,8	tracce	0,3	0,1	—	0,7	—	—	—	—
Girasole	» »	»	It. (Veneto)	37,5	1,2	—	tracce	5,5	0,1	tracce	—	3,7	30,6	58,2	tracce	0,2	0,1	—	0,6	—	—	—	—
Girasole	» »	»	It. (Veneto)	24,7	1,1	—	tracce	5,6	0,2	tracce	tracce	3,7	37,4	51,9	0,1	0,2	0,1	—	0,7	—	—	—	—
Girasole	» »	»	Egitto	29,1	0,5	—	tracce	5,3	0,4	tracce	tracce	3,0	49,5	40,5	0,1	0,3	0,2	—	0,7	—	—	—	—
Girasole	» »	»	Egitto	27,0	0,7	—	tracce	5,9	0,4	tracce	tracce	3,4	52,0	37,0	0,1	0,2	0,2	—	0,8	—	—	—	—
Grano duro (Germe)	Triticum Durum	Gramineae	Italia	15,1	4,4	tracce	0,1	17,4	0,2	tracce	—	0,6	17,1	53,6	7,4	tracce	1,5	—	tracce	—	—	—	—
Grano tenero (Germe)	Triticum Vulgare	»	Italia	12,5	4,1	—	tracce	17,8	0,5	tracce	—	0,7	14,7	58,3	6,7	tracce	1,3	—	tracce	—	—	—	—
Granone (Germe di amideria)	Zea Mais	»	Argentina (Plata)	52,5	1,8	—	tracce	14,0	0,3	tracce	—	2,5	36,7	45,0	0,7	0,3	0,2	—	—	—	—	—	—
Granone bianco (Ger. di mulino)	» »	»	Italia	16,2	4,7	—	tracce	14,2	0,2	tracce	—	2,7	39,3	42,4	0,6	0,4	0,2	—	tracce	—	—	—	—
Granone giallo (Ger. di mulino)	» »	»	Italia	23,0	3,8	—	tracce	13,0	0,2	tracce	—	2,3	30,6	52,5	0,9	0,3	0,2	—	tracce	—	—	—	—
Kenaph	Hibiscus Cannabinus	Malvaceae	Egitto	non determinato		tracce	0,2	20,0	0,7	tracce	0,5	2,2	25,9	49,0	0,7	0,5	0,3	—	—	—	—	—	—
Lino	Linum Usitatissimum	Linaceae	It. (Catania)	37,0	0,3	—	tracce	6,2	0,2	0,1	tracce	4,5	18,0	14,4	56,4	—	tracce	—	0,2	—	—	—	—
Lino	» »	»	It. (Puglie)	38,1	0,5	—	tracce	6,5	0,2	tracce	tracce	4,8	24,0	13,5	51,0	—	tracce	—	tracce	—	—	—	—
Lino	» »	»	It. (Puglie)	40,3	0,4	—	tracce	6,6	0,2	tracce	tracce	6,3	26,0	15,6	45,2	—	tracce	—	tracce	—	—	—	—
Lino	» »	»	It. (Trapani)	36,1	0,5	—	tracce	6,2	0,2	tracce	—	4,3	17,5	13,5	58,3	—	tracce	—	tracce	—	—	—	—
Mandorle amare	Prunus Amygdalus	Rosaceae	It. (Puglie)	52,3	0,6	—	tracce	5,8	0,4	0,1	0,1	2,6	70,6	20,3	tracce	tracce	—	—	—	—	—	—	—
Mandorle dolci	Prunus Amygdalus	»	It. (Puglie)	58,7	0,7	—	tracce	6,5	0,6	0,1	0,1	2,3	72,0	18,4	tracce	tracce	—	—	—	—	—	—	—
Neuk	Guizotia Abyssinica	Compositae	Eritrea	42,8	0,9	—	0,1	9,7	0,4	tracce	—	7,9	7,7	72,6	0,4	0,5	—	—	0,7	—	—	—	—
Nocciole	Corylus Avellana	Betulaceae	Italia	69,4	0,2	—	tracce	6,2	0,4	tracce	tracce	2,9	83,5	6,8	0,2	tracce	—	—	—	—	—	—	—
Noci (Gheriglio)	Juglans Regia	Juglandaceae	Italia	66,0	0,1	—	tracce	7,5	0,2	tracce	—	2,4	16,2	61,5	12,1	tracce	tracce	—	tracce	—	—	—	—
Peperone	Capsicum Annuum	Solanaceae	Italia	non determinato		—	0,2	11,7	0,5	tracce	tracce	2,7	9,9	75,0	tracce	tracce	tracce	—	tracce	—	—	—	—
Pomodoro	Solanum Lycopersicum	»	It. (Emilia)	28,2	1,6	—	0,1	15,0	0,5	0,1	0,4	5,0	21,3	55,4	2,0	0,2	—	—	tracce	—	—	—	—
Ravizzone	Brassica Napus	Cruciferae	It. (Veneto)	36,9	1,5	—	tracce	3,1	0,3	—	—	1,3	12,3	14,7	10,4	0,8	10,0	0,5	tracce	45,0	0,9	—	0,6
Ricino	Ricinus Communis	Euphorbiaceae	Etiopia	50,2	0,3	—	—	1,8	tracce	—	—	1,5	5,7	6,7	0,3	0,2	0,5		RICINOLEICO 83,3			—	—

Table 1 (cont.)

Semi e frutti oleosi	Nome botanico	Famiglia	Provenienza	Contenuto in olio (etere di petrolio) %	Acidità dell'olio (in acido oleico) %	Laurico 12:0	Miristico 14:0	Palmitico 16:0	Palmitoleico 16:1	Eptadecanoico 17:0	Eptadecenoico 17:1	Stearico 18:0	Oleico 18:1	Linoleico 18:2	Linolenico 18:3	Arachico 20:0	Eicosenoico 20:1	Eicosadienoico 20:2	Beenico 22:0	Erucico 22:1	Docosadienoico 22:2	Lignocerico 24:0	Tetracosenoico 24:1
Riso (Gemma)	Oryza Sativa	Gramineae	Italia	26,5	12,1	tracce	0,2	19,4	0,4	tracce	—	2,0	33,6	42,0	1,1	0,9	0,3	—	tracce	—	—	—	—
Riso (Pula)	Oryza Sativa	»	Italia	20,0	13,3	tracce	0,3	17,1	0,5	tracce	—	1,3	40,6	37,2	1,3	0,9	0,5	—	0,3	—	—	—	—
Senape bianca	Sinapis Alba	Cruciferae	Italia	[illegible]	0,9	tracce	tracce	3,0	0,2	tracce	tracce	1,0	20,2	9,7	10,6	0,8	10,0	0,2	0,3	41,6	0,1	—	[illegible]
Senape nera	Sinapis Nigra	»	It. (Puglie)	[illegible]	1,0	—	tracce	3,4	0,2	—	—	2,0	13,3	15,2	12,6	1,1	10,2	0,7	0,7	39,0	tracce	—	[illegible]
Sesamo	Sesamum Indicum	Pedaliaceae	Egitto	60,5	0,7	—	—	9,7	0,2	tracce	—	5,5	39,7	[illegible]	0,2	0,6	0,1	—	—	—	—	—	—
Sesamo	» »	»	Messico	52,5	0,8	—	tracce	9,5	0,2	tracce	—	5,2	39,5	44,5	0,2	0,6	0,2	—	—	—	—	—	—
Sesamo	» »	»	Nigeria	52,0	1,0	—	—	9,2	0,6	tracce	—	5,1	36,5	41,4	0,2	0,7	0,2	—	tracce	—	—	—	—
Sesamo	» »	»	Sudan	52,1	0,9	—	tracce	10,1	0,2	tracce	—	6,4	42,2	[illegible]	0,3	0,6	0,1	—	tracce	—	—	—	—
Sesamo	» »	»	Sudan	45,5	0,7	—	tracce	10,1	0,1	tracce	—	6,4	41,9	[illegible]	0,3	0,6	0,1	—	tracce	—	—	—	—
Soia	Glycine Hispida	Leguminosae	Brasile	18,0	0,8	—	0,1	11,7	0,2	tracce	—	3,9	22,1	[illegible]	6,9	0,2	0,1	—	tracce	—	—	—	—
Soia	» »	»	Cina	18,5	1,2	—	tracce	11,5	0,3	0,1	tracce	4,2	19,1	[illegible]	10,0	0,2	0,1	—	0,4	—	—	—	—
Soia	» »	»	Egitto	22,2	1,0	—	0,1	12,9	0,4	tracce	—	3,5	21,4	53,1	7,9	0,3	0,1	—	0,4	—	—	—	—
Soia	» »	»	Stati Uniti (Illinois)	19,4	0,8	—	tracce	10,9	0,3	0,1	tracce	4,3	21,5	54,2	7,8	0,2	0,2	—	0,4	—	—	—	—
Soia	» »	»	Stati Uniti	19,9	0,9	—	tracce	12,0	0,2	tracce	—	3,8	23,0	[illegible]	6,7	0,2	0,1	—	tracce	—	—	—	—
Soia	» »	»	Stati Uniti	17,3	0,9	—	tracce	11,5	0,2	tracce	—	4,4	27,5	48,2	5,7	0,2	0,1	—	tracce	—	—	—	—
The	Thea Sasanqua	Theaceae	Sud Africa	21,3	2,9	—	0,1	19,7	0,2	0,2	—	2,9	54,3	[illegible]	0,7	0,1	1,1	—	—	—	—	—	—
Vinaccioli	Vitis Vinifera	Vitaceae	Italia	15,5	1,2	—	tracce	7,3	0,2	—	—	4,5	18,0	[illegible]	0,4	0,2	tracce	—	—	—	—	—	—
Vinaccioli	» »	»	Italia	[illegible]	1,5	tracce	tracce	7,7	0,2	tracce	—	3,8	14,6	[illegible]	0,4	0,2	tracce	—	—	—	—	—	—
Vinaccioli	» »	»	Italia	16,4	1,1	—	tracce	7,3	tracce	—	—	3,5	14,5	74,3	0,4	tracce	tracce	—	—	—	—	—	—
Zucca	Cucurbita Pepo	Cucurbitaceae	Italia	30,2	5,3	0,1	0,2	14,2	0,3	0,3	0,1	7,3	34,1	42,3	0,6	0,4	—	—	—	—	—	—	—

Table 2

Semi e frutti oleosi	Nome botanico	Famiglia	Provenienza	Contenuto in grasso (etere di petrolio) %	Acidità del grasso (in acido oleico) %	Capronico 6:0	Caprilico 8:0	Caprinico 10:0	Laurico 12:0	Miristico 14:0	Palmitico 16:0	Palmitoleico 16:1	Eptadecanoico 17:0	Stearico 18:0	Oleico 18:1	Linoleico 18:2	Linolenico 18:3	Arachico 20:0	Eicosenoico 20:1
Babassù	Attalea funifera	Palmae	Brasile	—	5,8	0,5	7,1	6,3	44,5	16,0	8,7	tracce	—	3,0	11,9	1,9	—	—	—
Cocco	Cocos nucifera	»	Ceylon	—	7,6	0,5	6,8	5,9	48,9	18,8	8,6	—	—	2,5	6,2	1,8	—	—	—
Cocco	» »	»	Filippine	62,5	1,8	0,4	7,3	5,5	49,1	17,2	8,9	—	—	3,1	7,9	1,6	—	—	—
Illipè	Bassia longifolia	Sapotaceae	India	55,6	5,8	—	—	—	—	tracce	17,2	tracce	tracce	48,3	32,0	0,8	—	1,2	—
Illipè	» »	»	India	57,3	6,5	—	—	—	—	tracce	16,2	0,2	0,1	47,7	32,9	0,8	—	2,3	—
Mowrah	» latifolia	»	India	59,5	7,2	—	0,2	0,1	0,1	0,3	23,7	0,3	0,1	24,7	35,2	14,5	0,2	0,6	—
Mowrah	» »	»	India	54,6	5,1	—	0,2	0,1	0,4	0,3	24,5	0,2	0,1	23,9	34,8	14,3	0,3	0,7	0,1
Murumuru	Astrocaryum murumuru	Palmae	Brasile	—	7,9	tracce	6,9	5,9	49,7	14,5	8,1	tracce	—	2,8	10,8	1,3	—	—	—
Palma (*)	Elaeis guineensis (polpa del frutto)	»	Congo	—	6,9	—	tracce	tracce	0,1	[illegible]	43,9	0,3	tracce	5,6	37,7	10,7	0,3	0,4	tracce
Palma (*)	» »	»	[illegible]	—	4,5	—	tracce	tracce	0,1	[illegible]	44,6	0,4	tracce	5,6	38,0	9,8	0,2	0,3	tracce
Palmisto	» » (mandorla)	»	[illegible]	51,1	10,5	0,2	4,0	[illegible]	49,3	[illegible]	9,5	tracce	—	2,5	13,3	2,4	—	—	—
Palmisto	» »	»	Africa Occ.	45,3	12,7	0,4	5,0	4,3	50,2	15,4	7,9	—	—	2,2	12,8	1,8	—	—	—
Shea e Karità	Butyrospermum Parkii	Sapotaceae	[illegible]	52,1	7,3	—	—	—	—	tracce	3,7	—	1,1	45,5	43,5	4,8	—	1,4	tracce

Table 1. Composition of the oils extracted from oil seeds and fruits.

Key:

1. Oil Seeds and fruits

    a. pistachio or cashew
    b. peanut
    c. hemp
    d. safflower
    e. onion
    f. colza
    g. cotton
    h. sunflower
    i. (1) hard corn (seed)
       (2) soft corn (seed)
    j. Maize (bitter kernel)
       (1) white maize (mill seed)
       (2) yellow maize (mill seed)
    k. kenaph
    l. linseed
    m. (1) bitter almonds
       (2) sweet almonds
    n. neuk
    o. walnut
    p. nut (kernel)
    q. chili
    r. tomato
    s. rapeseed
    t. ricinus
    u. rice (bud and chaff)
    v. mustard
       (1) white
       (2) black
    x. sesame
    y. soybean
    z. tea
    aa. grapenuts
    bb. pumpkin

2. Botanical Name

3. Family

4. Origin

    a. Mozambique
    b. South Africa
    c. Italy
    d. United States

e. China
f. Erythrea
g. France
h. Italy (Lombardy)
i. Italy (Venetia)
j. Sweden
k. Egypt
l. Ethiopia
m. Mexico
m. Brazil

5. Oil content (petroleum ether) %

6. Acidity of the oil (in oleic acid) %

7. Fatty acids %

a. Lauric
b. Myristic
c. Palmitic
d. Palmitoleic
e. Heptadecanoic
f. Heptadecenoic
g. Stearic
h. Oleic
i. Linoleic
j. Linolenic
k. Arachic
l. Eicosenoic
m. Eicosadienoic
n. Beenic
o. Erucic
p. Docosadienoic
q. Lignoceric
r. Tetracosenoic

tracce = traces non determinato = undetermined

Table 2. Composition of fats extracted from oil seeds and fruits.

Key:

1. oil seeds and fruits

    a. babassu
    b. coco palm
    c. illipe
    d. mowrah
    e. murumuru
    f. palm *traces of pentadecanoic acid
    g. palmisto
    h. shea or karite

2. botanical name

3. family

4. origin

    a. Brazil
    b. Philippines
    c. West Africa

5. Fat content (petroleum ether) %

6. Acidity of the fat (in oleic acid) %

7. Fatty acids %

    a. Caproic
    b. Caprilic
    c. Capric
    d. Lauric
    e. Myristic
    f. Palmitic
    g. Palmitoleic
    h. Heptadecanoic
    i. Stearic
    j. Oleic
    k. Linoleic
    l. Linolenic
    m. Arachic
    n. Eicosenoic

Table 3

1. Nome Comune	2. Famiglia e denominazione botanica	3. Lipidi totali % s.s.	4. Acidità % s.s.	5. ACIDI GRASSI %																									6. insaturi saturi	7. oleico linoleico
				$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0m}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:1}$	$C_{20:2}$	$C_{20:3}$	$C_{20:4}$	$C_{22:0}$	$C_{22:1}$	$C_{22:2}$	$C_{24:0}$	$C_{24:1}$		
	Anacardiaceae																													
Lentisco a.	Pistacia lentiscus	n.d.	n.d.	—	—	0,1	0,2	0,4	tr.	21,6	1,6	tr.	tr.	1,5	57,0	17,0	0,4	0,1	tr.	—	—	—	—	—	—	—	—	—	3,2	3,3
	Betulaceae																													
Nocciolo b.	Corylus avellana																													
semi lung. (1)	» »	65,3	0,4	—	—	—	tr.	tr.	tr.	5,6	0,2	tr.	0,1	3,2	76,8	13,7	0,2		0,1	—	—	—	—	—	—	—	—	—	10,3	5,6
» ovali (2)	» »	67,5	0,5	—	—	—	tr.	tr.	—	5,3	0,2	0,1	0,1	3,7	82,6	7,5	0,2		0,1	—	—	—	—	—	—	—	—	—	9,9	11,0
» tondi (3)	» »	69,7	0,5	—	—	—	tr.	—	—	5,7	0,3	tr.	tr.	3,2	81,4	9,0	0,3		0,1	—	—	—	—	—	—	—	—	—	10,2	9,0
	Cannabaceae																													
Canapa c.	Cannabis sativa	28,2	1,0	—	—	—	0,1	—	—	8,2	0,2	tr.	—	2,8	18,2	52,2	17,0	1,0	tr.	—	—	—	0,3	—	—	—	—	—	7,0	0,3
	Compositae																													
Carciofo d.	Cynara scolymus	24,5	0,3	—	—	—	0,1	tr.	—	12,5	0,3	tr.	—	2,2	16,7	67,9	tr.	0,3	tr.	—	—	—	—	—	—	—	—	—	5,6	0,2
Cardo e.	Cynara cardunculus	24,7	0,4	—	—	—	tr.	tr.	—	12,7	0,1	—	—	3,1	25,6	58,0	tr.	0,2	0,1	—	—	—	—	—	—	—	—	—	5,2	0,4
Girasole f.	Helianthus annuus	49,3	1,1	—	—	—	0,1	tr.	tr.	6,0	0,1	tr.	tr.	4,3	38,3	50,9	0,3		0,1	—	—	—	tr.	—	—	—	—	—	8,6	0,8
Neuk g.	Guizotia abyssinica	43,6	1,0	—	—	—	0,1	tr.	—	11,8	0,4	tr.	—	9,8	7,2	69,8	0,4	0,5	tr.	—	—	—	tr.	—	—	—	—	—	3,5	0,1
	Cruciferae																													
Canolina h.	Canolina sp.	n.d.	n.d.	—	—	—	0,1	tr.	tr.	5,9	0,4	tr.	—	1,9	20,9	14,2	9,0	0,7	9,7	0,6	tr.	—	1,1	33,1	0,3	0,6	1,5	8,7	1,5
Cavolfiore i	Brassica oler. Botrytis	40,0	1,3	—	—	—	tr.	—	—	2,5	0,1	—	—	1,1	14,5	16,7	10,9	0,7	11,3	0,6	0,4	—	—	39,7	0,6	tr.	0,8	22,2	0,3
Cav. verza j.	Brassica oler. sab.	43,2	1,9	—	—	—	tr.	—	—	3,6	0,2	—	—	0,9	15,9	11,9	9,1	0,6	8,9	0,5	0,3	—	—	47,2	tr.	tr.	0,8	18,6	1,3
Rapa k.	Brassica rapa	42,3	1,1	—	—	—	tr.	—	—	3,8	0,3	tr.	—	1,0	14,5	13,4	9,4	0,9	9,9	0,5	tr.	—	—	44,7	0,2	tr.	1,1	16,5	1,1
Ravanello l	Raphanus raphanistrum	43,4	2,8	—	—	—	tr.	—	—	5,9	0,2	—	—	1,6	18,4	16,8	10,8	0,8	9,4	0,7	0,6	—	—	32,2	tr.	0,5	2,0	10,4	1,1
Senapa m.	Sinapis nigra	32,9	1,2	—	—	—	0,1	—	—	3,3	0,3	—	—	1,1	10,2	13,6	12,5	0,9	13,1	1,3	tr.	—	0,9	39,6	0,9	0,7	1,4	13,2	0,7
	Cucurbitaceae																													
Anguria a (1) semi b.chi grossi	Citrullus maxima	24,3	1,0	—	—	—	0,1	tr.	—	11,0	0,1	0,1	—	5,5	13,6	69,1	0,4		0,1	—	—	—	—	—	—	—	—	—	4,9	0,2
semi neri (2) piccoli	»	26,0	0,8	—	—	—	0,1	tr.	—	11,0	0,1	tr.	—	5,7	10,5	72,1	0,4		0,1	—	—	—	—	tr.	—	—	—	—	4,9	0,1
semi neri (3) grossi	» »	25,3	0,9	—	—	—	tr.	tr.	—	10,5	0,1	0,1	—	7,1	14,9	66,8	0,4		0,1	—	—	—	—	tr.	—	—	—	—	4,6	0,2
Cetriolini o.	Cucumis sativus	34,2	1,2	—	—	—	tr.	tr.	tr.	15,4	0,4	—	—	7,0	10,6	66,3	0,3		—	—	—	—	—	—	—	—	—	—	3,4	0,2
Cetriolo	» »	29,1	1,1	—	—	—	tr.	—	—	13,7	0,1	tr.	tr.	6,2	10,5	68,6	0,7		0,1	—	—	—	—	—	—	—	—	—	4,0	0,2
Melone p.	Cucumis melo																													
» liscio (1)	» »	28,9	1,7	—	—	—	tr.	tr.	—	9,0	tr.	tr.	tr.	5,7	13,1	71,9	0,3		tr.	—	—	—	—	—	—	—	—	—	5,8	0,2
» rugoso (2)	» »	28,7	1,6	—	—	tr.	tr.	tr.	tr.	9,6	0,1	tr.	tr.	5,6	14,5	69,9	0,2		0,1	—	—	—	—	—	—	—	—	—	5,6	0,2
Zucca q.	Cucurbita maxima																													
semi gialli (1)	» »	31,1	3,2	—	—	—	0,1	—	—	12,5	0,2	—	—	5,8	28,1	52,7	0,2	0,3	tr.	—	—	—	—	—	—	—	—	—	4,3	0,2
semi b.chi (2)	» »	30,8	3,5	—	—	tr.	0,1	—	—	14,4	0,2	tr.	tr.	6,8	19,3	58,6	0,1	0,3	tr.	—	—	—	tr.	—	—	—	—	—	3,6	0,2
Zucchetta	Cucurbita pepo	43,2	0,7	—	—	—	0,1	—	—	12,3	0,1	—	—	6,9	37,1	43,1	tr.	0,3	tr.	—	—	—	—	—	—	—	—	—	4,1	0,2
	Juglandaceae																													
Noce r.	Juglans regia	70,3	0,2	—	—	—	tr.	—	—	8,0	0,2	tr.	tr.	2,4	16,0	61,0	11,8	tr.	0,4	tr.	—	—	—	—	—	—	—	—	8,6	0,2
Noce selv.	Juglans nigra	63,2	0,2	—	—	—	tr.	tr.	—	3,9	0,2	tr.	tr.	1,3	15,7	71,3	7,4	tr.	0,2	—	—	—	—	—	—	—	—	—	18,2	0,2
	Lauraceae																													
Alloro s.	Laurus nobilis	21,1	0,2	0,8	1,9	47,7	1,5	—	tr.	9,2	0,3	—	—	0,4	15,9	21,2	0,5	tr.	0,6	—	—	—	—	—	—	—	—	—	0,6	0,2
	Leguminosae																													
Soja t.	Soja hyspida	23,0	1,0	—	—	—	0,1	—	—	11,3	0,1	0,1	0,1	4,5	24,0	51,0	7,7	0,5	0,3	—	—	—	0,3	—	—	—	—	—	4,9	0,5
Carruba u.	Ceratonia siliqua	2,1	0,1	—	—	tr.	0,1	tr.	0,2	13,3	0,7	0,2	tr.	4,1	32,4	46,6	1,3	0,7	—	—	tr.	—	—	tr.	—	—	—	—	4,3	0,7
Lino comune v.	Linum usitatissimum	4,3	n.d.	—	—	0,1	0,2	0,4	tr.	15,5	1,6	1,7	0,3	8,0	30,8	29,6	4,4		1,9	—	—	—	5,0	—	—	8,0	—	n.d.	1,1	1,1

Table 3 (cont.)

Nome Comune	Famiglia e denominazione botanica	Lipidi totali % s.s.	Acidità % s.g.	ACIDI GRASSI %																								Insaturi saturi	oleico linolenico
				$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$ $C_{20:0}$	$C_{20:1}$	$C_{20:2}$	$C_{20:3}$	$C_{20:4}$	$C_{22:0}$	$C_{22:1}$	$C_{22:2}$	$C_{24:0}$	$C_{24:1}$		
	Liliaceae																												
Cipolla	*Allium cepa*	24,9	1,8	—	—	—	tr.	—	—	8,6	0,2	—	—	2,2	29,6	59,4	tr.	tr.	—	—	—	—	—	—	—	—	8,3	0,5
»	» »	24,1	5,0	—	—	—	0,2	—	—	7,6	0,5	tr.	tr.	3,1	29,6	58,7	0,3	0,2	—	—	—	—	—	—	—	—	8,2	0,5
	Oleaceae																												
Olivo semi	*Olea europaea*	45,6	1,8	—	—	—	tr.	—	—	9,6	0,3	0,1	tr.	2,8	68,0	17,0	0,6	0,6	0,6	—	—	—	0,4	—	—	—	6,4	4,0
» frutto	» »	n.d.	n.d.	—	—	—	—	—	—	9,9	0,6	tr.	tr.	2,6	79,0	6,2	0,7	0,4	0,6	—	—	—	—	—	—	—	6,7	12,7
	Ombrelliferae																												
Sedano	*Apium graveolens*	22,1	0,4	—	—	—	tr.	tr.	—	4,9	1,6	tr.	tr.	1,0	68,5	20,4	0,7	1,1	0,5	1,3	—	—	—	—	—	—	15,8	3,3
	Pedaliaceae																												
Sesamo Eg.	*Sesamum indicum*	61,5	0,7	—	—	—	tr.	—	—	9,4	0,2	tr.	—	6,0	40,6	42,4	0,4	0,8	0,1	—	—	—	tr.	—	—	—	5,2	1,0
» Sudan	» »	53,2	0,8	—	—	—	tr.	—	—	9,8	0,3	tr.	—	6,2	41,0	41,0	0,5	0,7	0,2	—	—	—	tr.	—	—	—	5,0	1,0
» Messico	» »	54,4	0,8	—	—	—	tr.	—	—	9,8	0,4	tr.	—	5,5	37,8	44,3	0,8	0,9	0,3	—	—	—	—	—	—	—	5,2	0,9
	Pinaceae																												
Pino (semi)	*Pinus pinea*	54,8	0,7	—	—	—	tr.	—	—	7,3	0,3	tr.	—	3,1	38,8	46,8	0,6	0,4	0,8	tr.	0,4	1,5	—	—	—	—	8,2	0,8
»	» »	54,1	n.d.	—	—	—	0,1	tr.	—	6,6	0,3	tr.	—	3,7	40,8	44,2	0,6	0,4	0,8	tr.	0,4	1,7	tr.	—	—	—	8,2	0,9
»	» »	55,0	n.d.	—	—	—	0,1	—	—	6,3	0,2	tr.	—	3,8	39,6	45,0	0,7	0,5	0,9	tr.	0,5	2,1	tr.	—	—	—	8,3	0,9
»	» »	53,8	n.d.	—	—	—	tr.	tr.	—	7,1	0,2	tr.	tr.	3,5	38,0	47,6	0,6	0,5	0,6	tr.	0,4	1,4	—	—	—	—	8,0	0,8
	Rosaceae																												
Albicocca	*Prunus armeniaca*	13,2	0,5	—	—	—	tr.	—	—	6,3	0,8	tr.	0,1	1,7	60,6	30,1	0,3	tr.	tr.	—	—	—	—	—	—	—	11,5	2,0
Mandorle CC	*Prunus dulcis communis*	57,6	0,2	—	tr.	0,1	0,1	tr.	tr.	6,4	0,6	0,2	0,1	2,4	69,4	18,4	0,8	0,4	0,6	—	—	—	—	—	—	—	9,4	3,8
» dolci	» »	55,5	0,3	—	—	—	tr.	—	—	6,1	0,4	tr.	tr.	1,8	72,8	18,8	tr.	tr.	—	—	—	—	—	—	—	—	11,6	3,9
» amare	» »	53,1	1,1	—	—	—	tr.	—	—	5,9	1,0	tr.	tr.	1,2	63,8	28,1	tr.	tr.	tr.	—	—	—	—	—	—	—	13,1	2,3
Pesca dd.	*Prunus persica*	48,8	0,3	—	—	—	tr.	tr.	tr.	6,1	0,4	tr.	0,1	1,8	69,0	22,2	0,1	0,1	0,1	—	—	—	tr.	—	—	—	11,5	3,1
»	» »	25,5	2,0	—	—	—	tr.	—	—	3,0	0,2	tr.	tr.	1,8	32,7	55,2	0,8	0,5	0,4	—	—	—	0,3	—	—	—	8,4	0,6
Mela	*Pyrus malus*	24,7	1,2	—	—	—	tr.	—	—	8,6	0,4	tr.	tr.	1,6	30,9	52,0	6,0		0,4	tr.	—	—	tr.	—	—	—	n.d.	0,6
Pera	*Pyrus communis*																												
Rosa can.		n.d.	n.d.	—	—	0,3	0,1	tr.	0,2	4,8	0,3	tr.	tr.	3,6	17,7	53,6	19,4	—	tr.	—	—	—	—	—	—	—	10,1	0,3
frutt. int.		6,8	1,7	tr.	tr.	0,3	0,1	—	0,2	4,9	0,4	tr.	tr.	3,7	17,6	53,2	19,6	—	tr.	—	—	—	—	—	—	—	9,9	0,3
semi																													
	Rubiaceae																												
Caffè (cr.)	*Coffea arabica*	15,6	2,8	—	—	—	tr.	—	—	35,6	tr.	0,2	—	9,3	9,2	41,1	4,3		0,2	—	—	—	0,1	—	—	—	n.d.	0,2
» (lost)	» »	n.d.	n.d.	—	—	—	tr.	tr.	—	36,7	tr.	tr.	—	7,3	8,1	43,6	3,6		0,1	—	—	—	0,6	—	—	—	n.d.	0,2
	Rutaceae																												
Arancio	*Citrus sinensis*	n.d.	n.d.	—	—	—	0,1	—	tr.	26,1	1,3	0,4	0,2	5,0	23,9	38,5	4,4	tr.	tr.	—	—	—	—	—	—	—	2,2	0,6
Limone	» limonia	n.d.	n.d.	—	—	—	tr.	—	tr.	22,2	0,8	tr.	0,2	5,8	30,7	32,9	9,1	tr.	0,2	—	—	—	—	—	—	—	2,8	0,9
Mandarino	» nobilis	n.d.	n.d.	—	—	—	tr.	tr.	tr.	22,2	0,7	tr.	0,2	5,7	28,2	38,9	3,7	tr.	0,2	—	—	—	—	—	—	—	2,6	0,7
	Solanaceae																												
Melanzana	*Solanum melongena*	19,6	6,0	—	—	tr.	0,1	tr.	—	8,8	0,2	tr.	—	3,4	18,1	67,7	1,4	tr.	0,2	—	—	—	tr.	—	—	—	7,1	0,3
Peperone ros.	*Capsicum annuum*	24,3	0,7	—	—	tr.	0,1	tr.	tr.	11,2	0,5	0,4	0,1	2,7	10,3	71,9	0,8		1,1	—	—	—	0,3	—	—	—	5,7	0,2
»	» »	27,3	1,8	—	—	tr.	0,1	tr.	tr.	12,4	0,4	tr.	tr.	3,0	11,1	72,1	0,2	0,2	tr.	—	—	—	0,2	—	—	—	5,3	0,2
»	» »	25,4	0,7	—	—	tr.	0,1	tr.	tr.	12,5	0,4	tr.	0,2	3,2	11,6	71,3	tr.	0,3	tr.	—	—	—	0,4	—	—	—	5,1	0,2
»	» »	n.d.	n.d.	—	—	—	0,1	tr.	—	11,3	0,5	tr.	0,1	2,8	11,0	73,6	0,2	0,2	0,1	—	—	—	0,2	—	—	—	5,8	0,2
Pomodoro ros.	*Solanum lycopersicum*	n.d.	n.d.	—	—	—	0,3	—	tr.	16,2	0,8	tr.	0,4	5,8	21,0	52,0	2,8	0,6	tr.	—	—	—	tr.	—	—	—	3,4	0,4
semi (s)		n.d.	n.d.	—	—	—	0,1	—	—	14,8	0,9	tr.	0,3	4,7	19,9	54,3	2,9	2,0	tr.	—	—	—	tr.	—	—	—	3,6	0,4
» bucce	» »																												

(segue)

Table 3 (cont.)

Nome comune	Famiglia e denominazione botanica	Lipidi totali % s.s.	Acidità % s.g.	ACIDI GRASSI %																								insaturi / saturi	oleico / linoleico
				$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0R}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{22:1}$	$C_{20:2}$	$C_{20:3}$	$C_{20:4}$	$C_{22:0}$	$C_{22:1}$	$C_{22:2}$	$C_{24:0}$	$C_{24:1}$		
	Sterculiaceae																												
Burro di cacao	*Theobroma cacao*	n.d.	n.d.	—	—	tr.	0,2	tr.	tr.	28,3	0,5	0,3	—	34,9	30,5	3,0	tr.	2,3	—	—	—	—	tr.	—	—	—	—	0,5	10,2
Cacao [illegible]	» »	—	—	—	—	—	0,1	tr.	tr.	25,0	0,5	0,3	tr.	34,5	34,8	3,7	tr.	1,1	—	—	—	—	tr.	—	—	—	—	0,6	9,4
»	» »	—	—	—	—	—	0,1	—	—	26,3	0,3	0,2	—	35,7	34,6	1,9	tr.	0,8	—	—	—	—	tr.	—	—	—	—	0,6	18,2
»	» »	—	—	—	—	[illegible]	0,2	—	—	25,9	0,3	0,2	—	34,2	34,7	3,5	tr.	1,0	—	—	—	—	tr.	—	—	—	—	0,6	9,9
»	» »	—	—	—	—	tr.	0,2	—	—	25,9	0,3	0,2	—	34,8	34,2	3,5	tr.	0,9	—	—	—	—	tr.	—	—	—	—	0,6	9,8
»	» »	—	—	—	—	0,1	0,3	tr.	—	26,0	0,7	0,4	—	33,8	34,0	3,5	tr.	1,1	—	—	—	—	tr.	—	—	—	—	0,6	9,7
»	» »	—	—	—	—	—	0,2	—	—	27,0	0,4	0,2	—	34,7	33,3	3,0	tr.	1,1	—	—	—	—	tr.	—	—	—	—	0,6	11,1
»	» »	—	—	—	—	tr.	0,2	tr.	—	25,4	0,6	0,3	—	36,9	32,7	3,0	0,1	0,7	—	—	—	—	tr.	—	—	—	—	0,6	10,9
»	» »	—	—	—	—	—	0,1	tr.	—	25,4	0,4	0,2	—	36,9	33,3	2,8	—	0,8	—	—	—	—	tr.	—	—	—	—	0,6	11,9
»	» »	—	—	—	—	—	0,1	tr.	—	26,6	0,3	0,2	—	36,2	32,8	2,8	—	0,9	—	—	—	—	tr.	—	—	—	—	0,6	11,7
»	» »	—	—	—	—	—	0,1	tr.	—	25,8	0,4	0,3	—	34,5	34,3	3,5	tr.	1,0	tr.	—	—	—	—	—	—	—	—	0,6	9,8
	Theaceae																												
The [illegible]	*Thea sasanqua*	23,8	2,7	—	tr.	0,1	0,2	—	—	18,5	0,4	0,3	tr.	3,6	53,3	21,8	0,8		0,9	—	—	—	—	—	—	—	—	3,4	2,4
	Vitaceae																												
Vinaccioli	*Vitis vinifera*	19,3	0,3	—	—	—	0,2	—	—	8,2	0,9	0,6	tr.	5,9	19,0	64,5	0,6	tr.	—	—	—	—	—	—	—	—	—	5,7	0,3

KEY TO TABLE

1. Common Name

a. lentisk
b. hazelnut
   (1) long seeds
   (2) oval seeds
   (3) round seeds.
c. hemp
d. artichoke
e. thistle
f. sunflower
g. neuk
h. camellia
i. caulifiower
j. cabbage
k. turnip
l. radish
m. mustard
n. (1) watermelon with large white seeds
   (2) with small black seeds
   (3) with large black seeds
o. cucumbers
p. melon
   (1) smooth
   (2) wrinkled
q. pumpkin
   (1) yellow seeds
   (2) white seeds
r. walnut
s. laurel
t. soybean
u. carruba
v. tamarind
w. onion
x. olive (1) seeds
   (2) fruit
y. celery
z. sesame (1) Egyptian
   (2) Sudan
   (3) Mexico
aa. pine (seeds)
bb. apricot
cc. almonds
   (1) sweet
   (2) bitter
dd. peach
ee. apple
ff. pear
gg. sweet-briar
hh. coffee cr. - crude tos t. - refined

ii. orange
jj. lemon
kk. mandarin orange
ll. eggplant
mm. chilibean
nn. tomato (1) seeds
(2) skin
oo. cocoa butter
pp. cocoa
qq. tea
rr. grapenuts

2. Family and botanical name

3. total lipides

4. Acidity

5. Fatty acids %

6. unsaturated/saturated

7. oleic/linoleic

tr -- traces n.d. -- undetermined

OLEIC ACID

Chemical Information

I. Nomenclature

A. Common Name

1. Oleic Acid

B. Chemical Name

1. cis-9-Octadecenoic acid

C. Trade Names

None

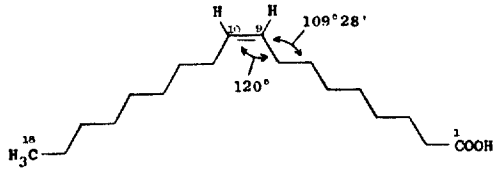
D. Chemical Abstracts Number

000112-80-1

II. Empirical Formula

$C_{18}H_{34}O_2$

III. Structural Formula



IV. Molecular Weight

282.45

V. Specifications

Food Chemicals Codex

Acid value	Between 196 and 204
Iodine value	Between 83 and 103
Saponification value	Between 196 and 206
Titer (Solidification point)	Not above 10°

Limits of Impurities

Arsenic (as As)	Not more than 3 ppm
Heavy metals (as Pb)	Not more than 10 ppm
Residue on ignition	Not more than 0.01%
Unsaponifiable matter	Not more than 2%
Water	Not more than 0.4%

VI. Description

A. General Characteristics

Pure oleic acid is a colorless to pale yellow, oily liquid with a characteristic lard-like odor and taste.

B. Physical Properties

The specific gravity of oleic acid is 0.895. It solidifies to a crystalline mass at 4 degrees and, with a pressure of 100 mm Hg, boils at 286 degrees. Its index of refraction for sodium light at 18 degrees is 1.463 and at 26 degrees is 1.4585. Oleic acid is practically insoluble in water, but is miscible with alcohol, benzene, chloroform, ether, and fixed and volatile oils.

C. Stability

Upon exposure to air oleic acid gradually absorbs oxygen and acquires a yellow to brown color and a rancid odor. When strongly heated (80-100 degrees) in air, it is decomposed with the production of acrid vapors.

VII. Analytical Methods

An excellent review of the methods of analysis of fatty acids was compiled by Fedeli and Jacini in 1971. They reported that the most versatile and most widely used method of analysis is gas-liquid chromatography, and that numerous other methods are also in current use (573).

LINOLEIC ACID

Chemical Information

I. Nomenclature

A. Common Names

1. Linoleic Acid
2. Linolic Acid

B. Chemical Names

1. cis-9, cis-12-Octadecadienoic Acid
2. 9,12-Linoleic Acid

C. Trade Names

None

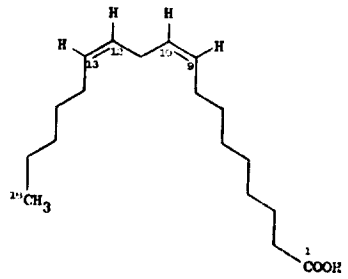
D. Chemical Abstracts Number

000060-33-3

II. Empirical Formula

$C_{18}H_{32}O_2$

III. Structural Formula



IV. Molecular Weight

280.44

V. Specifications

No Information Available

VI. Description

A. General Characteristics

Pure linoleic acid is a colorless oil.

B. Physical Properties

Linoleic acid exhibits the following characteristics:

d_4^{18}	0.9038
d_4^{22}	0.9007
mp	-12°
$bp_{1.4}$	202°
bp_{16}	230°
$n_D^{11.5}$	1.4715
n_D^{20}	1.4699
$n_D^{21.5}$	1.4683
n_D^{50}	1.4588
Iodine value	181.1
Thiocyanogen value	96.7

Linoleic acid is freely soluble in ether and is soluble in absolute alcohol. One ml of the acid dissolves in 10 ml of petroleum ether. It is also miscible with dimethylformamide, fat solvents, and oils.

C. Stability

Linoleic acid is easily oxidized by air and cannot be distilled without decomposition. Storage in ester form is recommended.

VII. Analytical Methods

See Oleic Acid

Biological Data

I. Acute Toxicity

Mice

In an attempt to determine the LD_{50} for a carrier emulsion containing cottonseed oil, Oro and Wretlind found on intravenous injection of a maximal volume - i.e., 60 ml/kg of the 25% cottonseed oil emulsion - into mice only a transient depression of the respiration rate. Therefore, they concluded that the LD_{50} of cottonseed oil for mice is higher than 15 g/kg (1467).

In a study by Schwartze, from 1 to 10 ml of cold-pressed peanut oil was injected into the peritoneal cavity of rats, mice, guinea-pigs, rabbits and cats. A few of the animals received oil containing 1% lecithin, and a few more received oil containing Sudan III. Observation of the animals revealed no untoward physiological effects resulting from the oil treatment. Post mortem examination of the animals several months after the injection disclosed that a considerable amount of the oil which had been injected remained, in most cases, within the peritoneal cavity. In the rats and mice the occurrence of single or flattened conglomerations of small cyst-like spheres containing oil was noted. The cyst-like bodies became very evident by the end of the first month and by the 24th week contained much semi-solid matter, suggesting a calcification process. In the guinea-pigs and rabbits a light yellow fibrin-like shaggy and cheesy material formed within 24 to 48 hours after injection and was still present at the beginning of the second month. The cats, and to a lesser degree the rabbits and guinea pigs, showed a tendency for a little serous exudation into the peritoneal cavity (1719).

II. Short-Term Studies

Rats

Through a series of 6-week feeding experiments with rats, Thomasson investigated the biological action of 20 oils and fats. Each of the oils was administered to groups of 8-12, 21-day-old Wistar rats in 7 different dosages from 10 to 73 Cal.%. Results of the tests are presented in Table 1. As shown in the table, the various oils and fats exhibited some differences in growth-promoting effect. Nevertheless, the food-efficiency of the test substances, except for shea butter and palm oil, seemed to have a constant value. Increased mortality was observed among some of the groups receiving rapeseed oil and kapokseed oil (1894).

Cameron et al. found that the daily intraperitoneal injection of 0.1 ml of peanut oil into rats for 17 or 18 days caused testicular atrophy and a decrease in the rate of growth. Upon post mortem examination the organ surfaces in the peritoneal cavity presented a peculiar glistening appearance due to a coating of minute oil droplets; this

Table 1

Mean increase in weight after 1, 3 and 6 weeks (gm)

Cal. %	LARD				SUMMER BUTTERFAT I				SUMMER BUTTERFAT II			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	12	24	99	195	12	27	107	206	12	25	100	196
20	10	22	100	199	..	..	...	...	..	..	...	..
30	12	25	109	216	12	26	109	218	12	25	104	211
40	12	25	107	211	12	27	108	211	12	25	105	206
50	12	25	105	218	12	25	111	220	12	24	105	218
60	12	24	104	220	12	26	110	211	12	24	103	217
73	12	23	105	220	12	21	103	216	12	21	99	208
Standard [1]	12	22	97	194	12	28	111	212	12	25	104	205

Cal. %	OLIVE OIL				COTTONSEED OIL				WINTER BUTTERFAT			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	..	..	...	...	12	23	101	202	12	20	79	153
20	12	24	103	201	11	21	98	202	12	19	81	171
30	..	..	...	...	12	24	103	209	12	19	82	171
40	12	22	96	195	12	24	107	217	12	15	80	176
50	..	..	..	.	12	22	97	204	12	18	78	170
60	12	19	99	203	11	21	99	213	12	14	78	177
73	12	17	90	185	12	17	87	196	12	13	70	181
Standard [1]	12	24	102	195	12	24	101	209	12	18	80	166

Cal. %	BEEF FAT				SHEA BUTTER				MAIZE OIL			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	12	28	108	208	12	25	95	179	9	21	93	188
20	10	26	105	196	12	24	95	178	9	23	98	199
30	12	28	108	210	12	22	89	169	9	22	94	192
40	12	26	105	202	12	21	90	171	9	23	99	202
50	12	27	106	210	12	20	87	169	9	25	104	216
60	12	27	106	209	12	20	89	169	9	21	92	195
73	12	18	93	201	12	19	88	174	8	16	74	181
Standard [1]	9	28	111	213	12	22	93	181	10	22	94	189

Cal. %	SOYABEAN OIL				GROUNDNUT OIL			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	11	26	102	190	10	25	107	214
20	11	26	100	184	10	24	104	215
30	11	26	103	198	10	25	109	219
40	11	24	92	184	9	22	102	210
50	11	24	95	188	10	22	105	215
60	11	23	97	200	10	21	99	210
73	11	17	82	184	10	14	84	193
Standard [1]	10	25	102	192	10	24	106	219

[1] Twenty Cal. % summer butterfat.

Table 1 (cont.)

Mean increase in weight after 1, 3 and 6 weeks (gm)

Cal. %	Palm fat				Sunflowerseed oil				Poppyseed oil			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	9	27	106	204	11	26	102	204	11	24	98	197
20	9	28	104	203	12	26	103	208	11	24	98	199
30	9	30	110	207	11	25	101	208	12	24	101	207
40	9	29	110	212	12	24	103	207	11	24	99	202
50	8	27	107	214	12	24	101	214	11	24	101	207
60	8	23	100	197	10	21	88	191	11	20	89	200
73	9	16	91	193	12	17	75	177	11	13	69	177
Standard [1]	9	29	111	218	12	26	105	204	11	25	104	208

Cal. %	Coconut fat				Sesame oil				Owalanut oil			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	12	25	98	196	11	22	89	179	12	23	96	...
20	12	23	95	195	10	24	97	192	12	21	89	...
30	12	24	98	197	11	21	90	186	12	22	92	...
40	12	22	92	191	10	20	86	183	12	18	85	...
50	12	21	92	198	11	19	81	182	12	18	81	...
60	11	18	83	182	11	19	76	173	12	15	75	...
73	11	13	65	156	9	10	49	139	12	6	31	...
Standard [1]	12	26	102	203	11	22	91	190	12	22	94	...

Cal. %	Whale oil I				Whale oil II				Herring oil			
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	n	1 w	3 w	6 w
10	11	25	99	187	10	24	97	201	12	29	107	209
20	12	23	85	169	10	20	84	181	12	23	96	189
30	11	22	85	168	10	20	85	178	12	22	90	180
40	11	20	82	163	10	20	80	177	12	19	82	164
50	11	19	74	141	10	18	76	175	12	14	70	151
60	12	14	61	149	11	13	67	168	12	12	60	127
73	11	6	47	135	8	6	48	158	12	5	44	104
Standard [1]	11	23	97	186	10	24	100	214	12	29	108	212

Cal. %	Rapeseed oil				Kapokseed oil				
	n	1 w	3 w	6 w	n	1 w	3 w	6 w	
10	8	21	93	183	12	16	88	187	
20	10	22	81	154	12	15	73	169	
30	9	19	75	149	11	12	57	142	
40	9	13	56	131	12	7 animals died (mean 18 days)			
50	9	12	52	125	12	9 animals died (mean 15 days)			
60	9	7	35	96	12	all animals died (mean 14 days)			
73	9 (all animals died, mean 17 days)				11	all animals died (mean 13 days)			
Standard [1]	9	24	94	177	11	24	104	212	

[1] Twenty Cal. % summer butterfat.

was especially noticeable on the surfaces of the liver, kidneys, and spleen (291).

To study the effect of peanut oil consumption on the liver, Gothoskar and Ranadive carried out a short-term feeding experiment on 24 Wistar rats. Twelve of the rats were fed 0.1 ml of the oil by stomach tube daily for six months, while the rest were maintained as controls. The animals were sacrificed one month after termination of treatment and the body weights and liver weights were determined. The relative liver weight of the treated animals did not vary significantly from the controls and no gross abnormalities were observed (701).

In an effort to ascertain the possible adverse effects of sesame oil, Bruce and Tobin injected 119 normal, castrated, and adrenalectomized rats with various doses of sesame oil. The dosage levels and results of sesame oil administration are presented in Table 2. It was observed that daily injections of 0.25 to 1.0 ml of sesame oil for several weeks produced toxic effects in normal male rats, including inhibition of growth, decreased weight of testes, and increased renal weight (266).

TABLE 2. THE EFFECTS OF SESAME OIL ON NORMAL, CASTRATED, AND ADRENALECTOMIZED RATS

No. of rats	Initial body weight		Operative treatment	Sesame oil per animal		Effects
	Mean	(Range)		Daily[1]	Total	
	gm.			cc.	cc.	
10	131	(119–148)	none	—	—	
4	141	(134–148)	none	0.05 S	3.25	Survived until killed on 63rd day
5	130	(127–134)	none	0.25 I	15.75	Inhibited growth, body weight only 85% of controls, increased adrenal weight, decreased testis weight, slightly decreased seminal vesicle weight, after 63 days of injections
5	150	(144–157)	none	1.0 I	10 to 55	1 died on 55th, 57th, and 58th days, 2 survived 42 days until killed, visible effects on body weight
5	52	(46–60)	none	—	—	
5	47	(42–54)	none	0.50 I	3.5	No significant effects on body weight or viscera
5	49	(42–56)	castrated	—	—	Typical castration effects
5	50	(46–50)	castrated	0.50 I	3.5	Same as controls, no effects on viscera
5	44	(40–49)[2]	spayed	—	—	Typical castration effects
5	47	(44–52)[2]	spayed	0.50 I	14.35	Same as controls, no effects on viscera
36	57	(35–90)	adrenal-ectomized	—	—	Mean survival time 5.6 (3–13) days, gained 12 gm., then lost 8 gm.
23	65	(45–88)	adrenal-ectomized	0.05 S		Mean 9.2 (4–24) days, gained 4 gm. then lost 8 gm.
6	60	(54–68)	adrenal-ectomized	0.50 I		Mean 8.3 (7–10) days, practically no gain in body weight

[1] "S" refers to subcutaneous injections; "I" refers to intraperitoneal injections. Injections of adrenal-ectomized animals were started on the 1st postoperative day, whereas those for castrated animals were started on day of operation.

[2] Females (all others were males).

To evaluate the possible toxicity of refined coconut oil, Harris and Mosher conducted a feeding study in which groups of 25 (12 male and 13 female) albino Wistar rats, 120 days old, were fed a diet consisting of 72% extracted skim milk powder, 3% extracted brewer's yeast and 25%

refined coconut oil or butterfat, with supplements of vitamins A and D and iron. For controls, 4 male and 4 female rats were fed an acceptable stock diet. After feeding of these diets for 15, 30, 60, and 90 days, animals from each test group were sacrificed and sections of the liver, heart, lungs, kidneys, skin, subcutaneous fat, adrenals and femur of each were taken for microscopic examination. The coconut oil-fed rats showed greater growth rate than the butterfat group, but not as great as the controls. There was no evidence of pathological tissue changes in any of the rats. However, the animals on both test diets developed a slight fatty infiltration of the body and liver cytoplasm (793).

III. Long-Term Studies

None

IV. Special Studies

Cancer

Certain vegetable oils are commonly used as vehicles in the administration of compounds in tests for carcinogenicity. In none of the numerous studies of this sort which were encountered in the preparation of this monograph were any vegetable oils implicated as causative in tumor formation. In most of these studies the oils were administered only in very small quantities and as a result the studies provide no information on the safety or toxicity of the oils. Therefore, only those studies felt to provide information relevant to the safety or toxicity of the vegetable oils were included in this monograph.

Gothoskar and Ranadive tested peanut oil for carcinogenic effects through administration of the oil to a total of 97 XVII X C57 (Black) hybrid mice by four different routes: (1) cutaneous application, (2) subcutaneous injection, (3) intraperitoneal injection, and (4) feeding by stomach tube. The treatment was started between the age of 10 and 12 weeks and the animals were observed till death or were sacrificed when they appeared weak and emaciated. No evidence of malignancy was observed at the site of treatment or in the remote organs of the test animals, and although some minor abnormalities were observed in the tissues the authors concluded that they could not be positively attributed to the oil treatment (701).

As controls in a study by Belman et al., 12 male Wistar albino rats received i.p. injections of 0.5 ml of peanut oil twice weekly for 3 months. Observation of the animals for two years, and post mortem examination disclosed the formation of no tumors (154).

In a study by Roe and Carter, 16 male CB stock rats, 5 to 6 weeks old at the beginning of the experiment, were given 20 once-weekly intramuscular injections of 0.2 ml of peanut oil in the right flank.

Observation of the animals for 63 weeks disclosed the development of no tumors nor other adverse effects (1634).

Dickens et al. injected 12 male rats subcutaneously with doses of 0.5 ml of peanut oil twice weekly for 57 weeks. Observation of the animals for over 2 years revealed the formation of no tumors. Nine of the rats survived more than a year, 7 for 1.5 years, 5 for 95 weeks, and 3 for over 2 years (489).

In a study by Fitzgerald et al., peanut oil was tested for topical irritation by daily application of a film of the oil onto the backs of 12 male albino Spartan source mice for periods up to 28 days. Two animals were killed after 1, 2, 3 or 4 weeks of exposure and at 1 or 2 weeks after the last exposure. The application of peanut oil resulted in retardation of hair growth and slight to moderate exfoliation of the epidermis by day 14. Histological examination of the application site revealed that the treated mice developed mild to moderate hyperkeratosis, acanthosis, and parakeratosis with neutrophil and mononuclear cell infiltration of the dermis after 14 days of treatment. These changes regressed with continued treatment and essentially returned to normal (595).

Fitzgerald et al. also tested peanut oil for topical irritation by twice daily application of a film of the oil to the backs of 4 New Zealand white rabbits for 1-2 weeks. Single animals were killed after 1 and 2 weeks of exposure and at 1 and 2 weeks after cessation of exposure. Gross observation showed that the peanut oil induced a slight erythema after 5.9 days of treatment. Upon microscopic examination it was disclosed that the oil induced mild acanthosis, hyperkeratosis with some hair follicle plugging, and a focal dermal inflammatory response. After the applications were discontinued, the skin changes regressed; however, epidermal thickening with increased exfoliation or keratosis, some follicle dilatation and plugging, and patchy lymphoid cell infiltration were still present 14 days after treatment (595).

To test for possible carcinogenic effects, Ranadive and Gothoskar administered mustard oil to a total of 86 hybrid mice (XVII X C57 black) by cutaneous application, subcutaneous injection, intraperitoneal injection, and feeding by stomach tube. Gross observations and histopathological studies disclosed no evidence of malignancy due to the mustard oil at the site of treatment or in the remote organs even though the animals were observed for their complete life span of over two years (1591).

Following repeated intraperitoneal injections of olive oil (unspecified dosage and number) in 40 rats and 60 mice, Burrows et al. observed that peritoneal tumors developed in one rat after 27 weeks and in two mice after 46 and 62 weeks, respectively. In all three the tumors were multiple and consisted of cells with round, deeply staining nuclei and relatively little cytoplasm. The cells were supported by a delicate reticulum. There was widespread enlargement of the lymph glands with abundant perivascular collections of round cells in the lungs, liver, kidneys and other organs. In the mice large masses of growth were present in the mediastinum and were invading the pericardium and other

structures. Leucemia or advanced leucocytosis was also present in one of the mice: no blood-counts were made, but abnormal numbers of white cells were visible in the blood in the microscopic sections of the tissues. Eight rats given weekly injections of 1.0 ml of olive oil subcutaneously developed no tumors (281).

As controls in a study by Weisburger et al., 20 Sprague-Dawley rats, 40-45 days old, were given 1 ml of sesame oil by gastric intubation daily for 45 days. The animals were weighed weekly and thoroughly examined for the appearance of mammary tumors or other grossly apparent lesions. Animals which appeared ill for any reason were observed twice daily and killed when death seemed imminent. After 9 months, all animals were carefully necropsied, gross lesions were noted, and select tissues were examined microscopically after fixation in 10% formalin and conventional histologic processing. The only tumor observed was a large fibroadenoma in an animal killed after 237 days. In a separate group of 132 controls which were part of a simultaneous series of related experiments, there were 4 rats with fibroadenomas or fibroadenomatous hyperplasia, while 1 rat had multiple tumors, namely 3 mammary carcinomas and 1 fibroadenomatous hyperplasia (2033).

The analysis, by Fabian, of coconut oil which had been produced without activated carbon revealed the presence of carcinogenic aromatic hydrocarbons, primarily 3,4-benzopyrene, in concentrations of 19 to 32 mu-g/kg. Olive oil was also found to contain carcinogens, although in lower concentrations (559).

The results of studies by O'Gara et al. on the carcinogenicity of heated fats and fat fractions are presented in Tables 3 and 4. Although no tumors were produced in the test rats by the subcutaneous, intramuscular, or oral administration of fresh whole corn oil, the intramuscular injection of the non-urea-adduct fraction of the corn oil resulted in a significant increase in the incidence of sarcomas at the injection site, indicating the presence of a carcinogen in the corn oil (1453).

In a study by Szepsenwol, mice of the T. M. strain were placed at the age of 4 weeks on Purina mice chow (Group 1, control) supplemented with refined corn oil (Group 2) and with corn oil and cholesterol (Group 3). The mice were bred and their offspring maintained on the same diet, so as to have mice of several generations on the above diets from the day they were born. Each mouse received daily 100 to 150 mg of corn oil (Group 2) and 4 to 5 mg of cholesterol (Group 3). The results were as follows: The mice of groups 2 and 3 developed a relatively high incidence of lung tumors, while the incidence of mammary cancer was 5% in Group 2 and 10.7% in Group 3. Lymphosarcoma, which was completely absent from Group 2, ocurred in 3 out of 130 mice of Group 3. The lymphoid organs in most of the experimental mice were atrophic and depleted of lymphocytes. The mammary tumors occurring in these mice were atypical, resembling the human ductal type of neoplasm (1856).

To test carcinogenicity, Szepsenwol placed mice of the T. M. strain

TABLE 3.—Tumors obtained in NIH Black rats fed cooking fats and fat fractions

Material	Dose % of diet	Number of rats*	Sex	Median age at death (months)	Range (months)	Tumors of alimentary tract (age in months)†	Other tumors (age in months)†
Fresh whole hydrogenated fat	20%	21	F	23	6–23	0	Reticular cell sarcoma (17) Adenocarcinoma of uterus (23) Fibroadenoma of breast (19)
		19	M	12	10–12	0	—
Fresh whole hydrogenated fat + benzo[*a*]pyrene	20% 0.5 mg/wk	21	F	23	7–23	0	Fibromyxosarcoma of pelvis (20) Adenocarcinoma of endometrium (23) Fibroadenoma of breast (23)
		17	M	23	6–23	Squamous cyst of F.S.	Fibromyxosarcoma of neck (23) Lymphosarcoma of abdomen (14) Adenocarcinoma of ureter (23)
Heated whole hydrogenated fat	20%	27	F	22	11–23	0	Rhabdomyosarcoma of abdominal cavity (15) Reticulum cell sarcoma (17)
		21	M	21	7–23	2 papillomas of F.S. (21)	Squamous carcinoma of nasal cavity (19)
Heated whole hydrogenated fat + benzo[*a*]pyrene	20% 0.5 mg/wk	29	F	22	10–23	2 papillomas of F.S. (22–23) 2 squamous cysts of F.S.	Lymphosarcoma (11)
		20	M	22	11–23	Papillomas of F.S. (21)	Subcutaneous fibrosarcoma (23)
Fresh whole corn oil	10%	28	F	23	11–23	0	Adenocarcinoma of endometrium (23)
		19	M	12	6–12	2 papillomas of F.S. (12) Squamous cyst of F.S.	—
Fresh whole corn oil + benzo[*a*]pyrene	10%	26	F	22	12–23	Papilloma of F.S. (22)	Two fibroadenomas of breast (12 and 19)
	0.5 mg/wk	18	M	22	11–23	Squamous carcinoma of F.S. (21)	Mesothelioma of heart (20) Lymphosarcoma (21)
Urea adduct of heated corn oil and fresh whole corn oil	5% 5%	22	F	20	10–24	2 squamous carcinomas of F.S. (24)	Fibroadenoma of breast (24) Adenocarcinoma of endometrium (22) Granulosa cell carcinoma of ovary (24) Adenoma of adrenal cortex (24)
		18	M	22	9–24	Squamous cyst of F.S.	Lymphosarcoma of abdominal nodes (22)

Table 3 (cont.)

Urea adduct of heated corn oil and fresh whole corn oil + benzo[a]pyrene	5% 5% 0.5 mg/wk	27	F	21	9–23	0	Two fibroadenomas of breast (23 and 23) Adenocarcinoma of pancreas with metastases (23) Lymphosarcoma of abdominal nodes (13)
		17	M	23	11–23	Squamous carcinoma of esophagus (11) Papillomas of F.S. (21) Adenocarcinoma of cecum (23) Squamous cyst of F.S.	Fibrosarcoma of salivary gland (21)
Nonurea adduct of heated corn oil and fresh whole corn oil	5% 5%	23 15	F M	10 10	9–10 6–24	0 Papilloma of F.S. (9)	— —
Nonurea adduct of heated corn oil and fresh whole corn oil + benzo[a]pyrene	5% 5% 0.5 mg/wk	28 13	F M	22 23	6–24 20–24	0 2 papillomas of F.S. (20 & 21) Squamous cyst of F.S.	Adenocarcinoma of endometrium with metastases (22) —
Dregs of heated hydrogenated fat	20%	5 8	F M	12 10	12 8–12	0 0	— —
TOTAL.....		442					

*Only rats 6 months of age or older are included.

†The first number indicates the number of rats (if more than one) with a specific tumor. Papillomas of the forestomach (F.S.) are solitary unless designated as multiple by an "s."

TABLE 4.—Tumors obtained in NIH Black rats given biweekly intramuscular injections of cooking fats and fat fractions

Material	Dose (ml)	Number of rats*	Sex	Median age at death (months)	Range (months)	Sarcomas at injection site	Ages at time of first and last tumors	Other tumors and related lesions
Fresh whole corn oil	0.1	17	F	22	11–22	0	—	Hyperplastic nodule of liver
	.1	14	M	22	20–22	0	—	Hyperplastic nodule of liver Papilloma of forestomach Lymphosarcoma of abdominal lymph nodes
Fresh whole hydrogenated fat†	.1	16	F	12	12–23	0	—	Adenocarcinoma of endometrium
	.1	7	M	14	14	0	—	Squamous cyst of forestomach
Heated whole hydrogenated fat†	.1	23	F	14	14–23	0	—	Lymphosarcoma of kidney and ovary; Squamous cyst of forestomach Fibroadenoma of breast
	.1	14	M	14	12–14	0	—	Papilloma of forestomach
Nonurea adduct of fresh hydrogenated fat	.1	17	F	22	13–22	2	22	Cystadenoma of bile duct Squamous cyst of forestomach
	.1	17	M	13	6–22	0	—	Lymphosarcoma, abdominal and thoracic lymph nodes
Nonurea adduct of heated hydrogenated fat	.1	20	F	16	9–23	6‡	11–21	Two fibrosarcomas of abdominal cavity Adenocarcinoma of colon Squamous carcinoma of ovary
	.1	18	M	18	7–22	2	16–20	Two lymphosarcomas of ilcocecal region
Nonurea adduct of fresh corn oil	.1	19	F	22	7–22	4	21–22	Lymphosarcoma, generalized
	.1	18	M	22	8–22	1	22	—
Nonurea adduct of heated corn oil	.1	13	F	19	11–22	9‡	15–20	—
	.1	14	M	16	12–22	7	15–22	Lymphosarcoma of abdomen Papillomatosis of forestomach and ulcers
Volatiles of corn oil heated at 200° C	.04	8	F	23	6–23	0	—	Squamous carcinoma of cervix uteri Adenocarcinoma of endometrium
	.01–.02	8	M	23	13–23	0	—	—
TOTAL		243				31		

*Only rats 6 months of age or older are included.

†Originally given a single subcutaneous injection of nonurea adduct of fresh and heated hydrogenated fat. Intramuscular injections begun at the age of 8 months.

‡Three tumors in each of these groups had metastasized.

at the age of 4 weeks on Purina mice chow (control) supplemented with refined corn oil (100-150 mg/mouse/day), cholesterol (4-5 mg/mouse/day), or both. The mice of each group were bred and their offspring maintained on the same diets. The mice were kept until they died or became moribund and were killed. The incidence of brain nerve cell tumors is presented in Table 5 (1855).

Table 5

Diet	Control	Cholesterol	Corn oil	Corn oil and Cholesterol
Total number of mice	360	212	159	106
Number of mice brain examined	188	80	11	22
Number of mice with brain tumors	0	20 (25%)	1 (9.1%)	7 (31.8%)

A semisynthetic high-corn oil (20%) diet was found by Gammal et al. to enhance the development of mammary cancer induced by 7,12-dimethylbenzanthracene in intact female Sprague-Dawley rats as compared to 2 other groups of similarly treated rats fed a high-coconut oil (20%) and a low-fat (0.5%) semisynthetic diet, respectively (648).

OLEIC ACID

Biological Data

I. Acute Toxicity

Substance	Animal	Sex & No.	Route	Dosage mg/kg Body Weight	Measurement	Ref.
Oleic Acid	Mice	60	i.v.	230	LD_{50}	1467

Mice

To determine the acute toxicity of oleic acid, Oro and Wretlind injected mice (6 groups of 10; 13-34 g) intravenously with various doses of a 2% emulsion of the acid. An LD_{50} of 230 ± 18 mg/kg was determined. When doses near this level were injected the animals immediately had convulsions and collapsed on their sides. Respiration ceased within 1-2 minutes, sometimes after hyperpnoea (1467).

Rats

In a study by Ungar et al., 18 white male rats were subjected to a single intrasplenic injection of 0.15-0.5 ml of a 50% oleic acid emulsion (in gum acacia). Three of the rats died within 24 hours after injection and the remaining animals were killed at various intervals up to 21 days after injection. Post mortem examination of the animals which died within 24 hours after injection revealed extensive necrosis of the liver, chiefly in peripheral areas but occasionally extended toward the central vein. Sinusoidal dilation and congestion were conspicuous, and hemorrhages were numerous, particularly in the necrotic regions. At 48 hours the same extensive necrosis and focal hemorrhage were still visible. In addition, there were numerous mitoses throughout the remaining uninvolved parenchyma. Most of the viable hepatic cells contained small sudanophilic droplets, and in several areas larger fat droplets were lying free in sinusoids. After 4 days the liver parenchyma appeared wholly intact, and the only vestige of injury was the presence of moderately increased numbers of lymphocytes in Glisson's spaces. Occasionally a variable number of lymphoid cells were seen within sinusoids without a particular pattern of distribution. In all rats that survived a week or more, all the lobes of the liver were fused together, and the capsule was slightly opaque grossly but still glistening. Microscopically, the capsule was markedly thickened, at first by loose connective tissue and young fibroblasts, later by dense, less cellular fibrous tissue (1953).

Kinosita et al. reported that a single intraperitoneal injection of 10 mg sodium oleate in 1 ml water resulted in changes in the liver of the rat. Within a few days the liver became plump with rounded edges. The cytoplasm of the liver cells contained unstained patches of irregular shape, electron microscopy of which demonstrated the lack of ribosomes and alteration of the endoplasmic reticulum, which, instead of cristae, formed a network of narrow canals or became fuzzy and filamentous.

These changes were reversible, except that the liver remained plump in form and hyperplastic (1067).

Dogs

In a study of acute toxicity by Jefferson and Necheles, 36 normal, starved, unanesthetized, mongrel dogs were subjected to intravenous doses of oleic acid (95% oleic acid and 5% other long chain fatty acids), ethyl oleate and sodium oleate. In addition, a few tests were performed on dogs anesthetized with pentobarbitol sodium. Doses of 0.25 ml of oleic acid were found to have little effect (6 dogs), while doses of 0.5 to 5 ml were toxic (19 dogs). The outstanding symptoms were: drop in blood pressure, urination and defecation, and rapid, forced, deep respiration, coughing, and development of massive pulmonary edema with large amounts of bloody, frothy exudate from the mouth and nose, followed by collapse and death. Death occurred an average of 40 minutes after the injection of 5 ml. Further details and the results of post mortem examination are reported in the attached article. Ethyl oleate injected in doses of 0.5 to 5.0 ml (3 dogs) produced toxic effects similar to those of oleic acid, but no edema or hemorrhage of the lungs and no hemolysis was observed and the ethyl oleate was never lethal. The injection of 2 g of sodium oleate (3 dogs) resulted in the death of the animals within a few minutes, the blood showing nearly complete hemolysis (966).

Baker et al. reported that the intravenous injection of 0.07 ml of oleic acid per kg body weight in 8 normotensive, normovolemic dogs resulted in an immediate drop in arterial O_2 partial pressure, compliance and surfactant activity. Pathological examination of the test animals revealed grossly hemorrhagic lungs that were 2-3 times as heavy as the control lungs. Occasional proteinaceous exudates in the alveoli and decreased cellularity of the alveolar septa were noted. Evaluation of the blood coagulation mechanism disclosed a sharp thrombocytopenia (100).

II. Short-Term Studies

Chicks

Groups of 25 day-old chicks were maintained for 4 weeks by Sunde on a chick starting ration supplemented with 5% oleic acid. No deleterious effects resulted from the consumption of this diet. Feed utilization was improved by the supplement (1839).

Rabbits

In a study by Flesch, 2 groups of 4 albino rabbits (3-4 kg) each were given oleic acid by stomach tube, the first group receiving a total of 40 ml of oleic acid administered in 4 equal doses every other day, while of the second group, 2 rabbits received 2 doses of 2.5 ml of oleic acid and two received the same amount of ethyl oleate at 5 days' intervals. None of the animals of the second group showed any effects from this treatment. However, of the group receiving the higher

doses, one animal died and the remaining animals developed, on the 5th to 7th day of treatment, scaling "seborrheic" lesions on the outer sides of both ears. Shortly after the onset of scaling reversible hair loss was observed (605).

III. Long-Term Studies

Mice

A group of 36 male and a group of 36 female 5-week-old albino mice were given subcutaneous injections of an oleic acid emulsion (approx. 5%) in a set of experiments by Robertson et al. Each of the mice was given a weekly injection of 0.25 ml of the emulsion until they reached 20 g in weight, whereupon the amount of injection was increased to 0.5 ml. After the animals reached 210 days of age the injections were given fortnightly and after 400 days were discontinued. The authors found that the oleic acid emulsion had no appreciable effect on the growth curve of either the male or female mice but the mean life duration of the males was reduced from a previously determined value of 772 days down to 661 days, of the females from 780 days to 702 days (1629).

IV. Special Studies

Carcinogenicity

Oleic acid was tested by Swern et al. for carcinogenic activity by repeated subcutaneous injections in young female Swiss-Webster mice. The treatment of 16 mice with a total of 33 injections of 0.5 mg of oleic acid (dissolved in tricaprylin) per injection administered at a rate of 2 injections per week and of 15 mice with a total of 10 injections of 0.1 mg per injection administered at a rate of 3 injections per week resulted in the formation of no tumors at the site of injection. One carcinoma of the breast was observed among the treated animals, however, the authors concluded that oleic acid exhibits no carcinogenic activity when administered in the aforementioned manner (1851).

Solutions of oleic acid, in 0.1 ml doses, were injected by Berlin and Wyman intradermally into the skin on the back of two guinea pigs in concentrations of 0, 25, 50, 75 and 100% dissolved in peanut oil. All of the sites at which oleic acid was injected showed local inflammation and necrosis. The peanut oil alone produced no changes (168).

The application of undiluted oleic acid to the backs of male white mice 6 times per week for 1 month was found by Setala et al. to produce hyperplasia in the interfollicular epidermis. The development and nature of the hyperplasia is described by the authors in detail (refer to attached article) (1739).

A series of experiments were conducted by Twort and Fulton to investigate the possible carcinogenicity of oleic acid. By bi-weekly application (unspecified method) of oleic acid to mice, tests were

performed (a) on 100 animals in an experiment lasting 40 weeks which gave no tumors, (b) on 200 animals in an experiment lasting 40 weeks which gave one benign tumor on the 35th week, and (c) on 100 animals in an experiment lasting 23 weeks which gave one benign tumor on the 15th week. In another experiment which was not completed at the time of writing, application of oleic acid 5 times per week induced the formation of a tumor by the 7th week, but as the experiment progressed only an occasional tumor arose, and at the time of writing there was no evidence of any change to malignancy in the tumors under observation (1939).

The tumor promoting action of 4 fatty acids was investigated by Holsti using groups of 22 male and 22 female mice. Following initiation by a single treatment with DMBA, the acids were applied to the skin of the mice once daily 6 days per week for 24 weeks. The maximum tumor incidence observed during the test period is presented in Table 6. In the group treated with undiluted oleic acid, the first tumor appeared 10 weeks after the beginning of treatment. A sex difference was observed among the tumor-bearing animals, the males exhibiting a significantly greater tumor incidence than the females (884).

Table 6

Primary treatment (initiation)	Secondary treatment (tumor promotion)	Max. tumor incidence (in %)
0.3% DMBA	none	0
none	undiluted oleic acid	0
0.3% DMBA	undiluted oleic acid	56
0.3% DMBA	20% oleic acid	32
0.3% DMBA	20% lauric acid	31
0.3% DMBA	20% stearic acid	0
0.3% DMBA	20% palmitic acid	0
0.3% DMBA	chloroform	0

Using groups of 22 male and 22 female mice, 4 to 6 months old, Holsti tested the tumor-promoting effects of oleic acid, sorbitan mono-oleate (Span 80), and polyoxyethylene sorbitan mono-oleate (Tween 80) in mouse skin. Initiation was accomplished by a single local application of an 0.3% solution of 9,10-dimethyl-1,2-benzanthracene in colorless, non-fluorescent liquid paraffin. Treatment with the test compounds was started 24 hours after the initiation and the compounds were applied with a water color brush once daily (six times per week) to the previously initiated area of skin on the animals back. The tumors were recorded once weekly and the incidence of tumors was calculated from the number of animals surviving at the time of appearance of the first

tumor in the series concerned. The results presented in Figure 1 and Table 7 indicate that oleic acid exhibits considerable tumor-promoting activity (885).

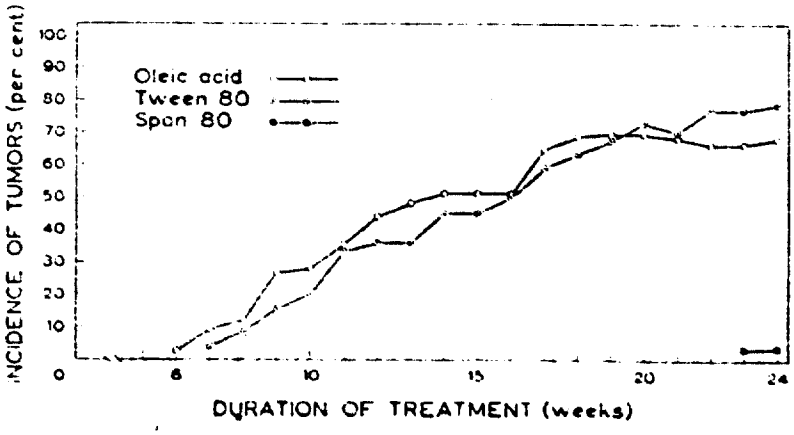


Fig. 1. — Development of local skin tumors with daily (6 times a week) treatment with oleic acid, Tween 80 and Span 80 after initiation with a single local application of 0.3 per cent DMBA in liquid paraffin.

TABLE 7

Test Compound	First Tumor		Maximum Incidence of Tumors		No. of Mice Alive at 21 Weeks
	Time (Weeks)	No. of Mice Alive	Time (Weeks)	Tumors per Tumor-Bearing Mouse	
Oleic acid	6	43	19	131/30	36
Tween 80	7	44	24	143/35	43
Span 80	23	38	23	11	38

Later studies by Holsti, performed under similar conditions, further validated his observation that oleic acid exhibits significant tumor-promoting activity when applied to the skin of mice once daily (6 times per week). In addition, the promoting action of oleic acid was shown to be linked to the frequency of application. That is, the more frequently oleic acid was applied, the earlier the tumors developed and the more steep was the increase in the incidence of tumors. Also, in these studies, a greater number of tumors was exhibited by the male mice than the females. Histological examination of all induced tumors revealed typical papillomas (886).

Bottomley and Twort reported the observation of no more than about a dozen benign tumors among 1,000 mice painted with oleic acid. However, they found that the presence of oleic acid enhances the carcinogenic activity of chrysene and 1:2:5:6-dibenzanthracene (245).

The carcinogenicity of oleic acid was tested by Shubik by administration of undiluted oleic acid to the skin of 10 mice twice weekly for 20 weeks starting 3 weeks after initiation with a single application of 9,10-dimethyl-1,2-benzanthracene in liquid paraffin. This treatment resulted only in slight epidermal hyperplasia without the promotion of tumors (1752).

Reproduction

Carroll and Nobel conducted a series of experiments to determine the influence of a dietary supplement of oleic acid on the fertility of the rat. They found that the maintenance of weanling rats (Sprague-Dawley and Wistar) on a diet supplemented with 15% oleic acid for 16 weeks produced no impairment of the fertility of the males. Of 4 female weanling rats similarly treated all 4 were able to become pregnant - however, 2 of the females died at parturition, 1 liter was eaten at birth, and the remaining liter died within 3 days of birth. In addition, the mating of 7 adult female rats after maintainence on a diet containing 15% oleic acid for 10 weeks resulted in the production of 52 young - of which 44 survived 1 week, 16 survived 2 weeks, and 11 survived 3 weeks. Throughout the tests all of the animals appeared normal except for their reproductive capacity. Post-mortem examination of the treated rats revealed that the degree of mammary development was much less than expected and that a few rats had cysts of the ovary. However, no lesions were found in organs other than those of reproduction. The results of a feeding test in which four immature male Sprague-Dawley rats were maintained on a diet containing 15% oleic acid for 5 months indicate that the rats grew normally on this regimen. The authors concluded that oleic acid, at a dietary level of 15%, does not effect the growth or general health of rats, but impairs reproduction through interference with parturition and repression of mammary gland development and lactation (305).

Depilation

The depilatory action of oleic acid was tested by Flesch on 5 rabbits, 2 guinea pigs and 12 mice by a single application of doses of 1-2, 1, and 0.3 ml, respectively. Oleic acid was found to be a potent depilatory agent in all cases, producing a marked irritating effect on the animal skin, characterized by crusting, ulceration and thickening. Histological examination of the application sites disclosed hyperkeratosis, pronounced acanthosis, keratotic plugs in the follicles, hyperplasia of the sebaceous glands, and disappearance of the hair shafts and of many of the hair follicles. All of these changes were observed to be reversible (606).

Biological Data

I. Acute Toxicity

Rats

Single doses of 0.15 to 0.5 ml of linoleic acid, in a 25 or 50% emulsion (in gum acacia), were intrasplenally injected by Ungar et al. into 10 and 7 white male rats, respectively. Four of the rats receiving 50% linoleic acid died within 24 hours after injection, and the remaining animals from both groups were killed at various intervals up to 28 days after injection. Post mortem examination of the animals which died within 24 hours after injection revealed extensive necrosis of the liver, chiefly in peripheral areas but occasionally extended toward the central vein. Sinusoidal dilation and congestion were conspicuous, and hemorrhages were numerous, particularly in the necrotic regions. There was a striking polymorphonuclear neutrophile infiltration in and around the necrotic foci as well as a scattering of those cells in the sinusoids. At 48 hours the same extensive necrosis and focal hemorrhage were still visible. In addition, there were numerous mitoses throughout the remaining uninvolved parenchyma. Most of the viable hepatic cells contained small sudanophilic droplets, and in several areas larger fat droplets were lying free in sinusoids. The polymorphonuclear neutrophile response was still abundant. After 4 days the liver parenchyma appeared wholly intact, and the only vestige of injury was the presence of moderately increased numbers of lymphocytes in Glisson's spaces. Occasionally a variable number of lymphoid cells were seen within sinusoids without a particular pattern of distribution. In all rats that survived a week or more, all the lobes of the liver were fused together, and the capsule was slightly opaque grossly but still glistening. Microscopically, the capsule was markedly thickened, at first by loose connective tissue and young fibroblasts, later by dense, less cellular fibrous tissue (1953).

II. Short-Term Studies

Chicks

Groups of 13-15 day-old chicks were maintained for 4 weeks by Sunde on a chick starting ration supplemented with 5% linoleic acid. No deleterious effects resulted from the consumption of this diet. Feed utilization was improved by the linoleic acid supplement (1839).

Rats

An experimental diet containing 16% linoleic acid and 0.06% butter yellow was found by Gyorgy et al. to be toxic for rats. Sixty-seven rats fed this diet *ad libitum* lost weight rapidly in spite of satisfactory intake of food. They exhibited marked, progressive anemia, secondary in type, which was accompanied, as a rule, by leucopenia. They became

infested with pediculi, often before the development of anemia when the animals were still very active and not nearly moribund. The toxic effect was even more pronounced when the butter yellow was omitted from the diet. In this group comprising 23 rats all the animals died before the 80th experimental day, with an average survival time of 55 days. Four rats showed leucopenia but no anemia before death. The toxic effect of the rations containing linoleic acid with or without butter yellow could, as a rule, be neutralized preventively and therapeutically by the daily addition of 0.5 to 1 gm of yeast (765).

III. Long-Term Studies

None

IV. Special Studies

Depilation

The depilatory action of linoleic acid was tested by Flesch on 5 rabbits and 10 mice by a single application of doses of 1-2 and 0.3 ml, respectively. Linoleic acid was found to be a potent depilatory agent in all cases, producing a marked irritating effect on the animal skin, characterized by crusting, ulceration and thickening. Histological examination of the application sites disclosed hyperkeratosis, pronounced acanthosis, keratotic plugs in the follicles, hyperplasia of the sebaceous glands, and disappearance of the hair shafts and of many of the hair follicles. All of these changes were observed to be reversible (606).

Biochemical Aspects

I. Breakdown

In the processing and cooking of food products, vegetable oils are often subjected to heating, the conditions of heating varying widely. A number of studies have been conducted to determine the effect of heating on the nutritive value of vegetable oils.

As a result of the data he obtained (see Table 1) Roy concluded that the absorption of vegetable oils decreases when they are subjected to thermal treatment (1651).

TABLE I.

Oil.	Temp. °C	Saponification value.	Iodine value.	Acid value.	Percentage of absorption.
Groundnut	Normal	184	83.5	9.6	99.7
	200	184	81.5	7.2	99.7
	250	186.5	83.0	5.3	99.7
	275	190	84.2	2.8	99.7
	300	192.5	76.0	2.5	99.5
Cocoanut	Normal	250	10.0	3.6	98.4
	200	246	10.0	3.4	98.3
	250	246	10.0	1.1	98.3
	275	246	7.4	0.5€	96.4
	300	240	6.6	...	94.9
Mustard	Normal	174	100.0	5.4	95.5
	200	172	99.5	3.8	95.2
	250	172	92.6	3.3	95.2
	275	170	87.0	1.8	91.7
	300	166.5	78.0	...	88.7
Sesame	Normal	189	109	13.4	96
	200	189	107	11.2	96
	250	187	96	4.4	95
	275	189	88	1.3	94
	300	190	83	...	92.5
[illegible]	Normal	192	187	2.6	97
	200	191	174	2.4	93.9
	250	189	144	2.2	94.0
	275	187	136	...	90.8
	300	187	98	...	78.3

In a study by Peacock et al., cottonseed oil heated under various conditions was fed to mice at levels of 1-5% of the diet and was found to significantly increase the incidence of tumors of the forestomach once hepatomata. Data obtained from the treated animals from the controls are shown in Tables 2 and 3 (1514).

TABLE 2 —*Tumour incidence in mice fed heated cottonseed oil (and possible co-carcinogens) surviving over 400 days*

Cottonseed oil heated at:	Percent	Solvent	Plus	No. of mice	Papilloma	Carcinoma	Adenosis	Hepatoma
350° C. × 4 hr.*	5		Histamine (0.5 mg. subcutaneously 3 × weekly).	12	0	0	1	2
" "*	5			31	3	0	0	0
320° C. × 2½ hr. + Fe*	5			32	3	2	1	2
350° C. × 4 hr	1	10% soap solution		56	3	1	0	4
" "	1	"	0.1% croton oil	21	1	1	2	0
" "	1	"	0.05% croton oil	35	4	0	2	2
" "	1	"	2% "lorol" sulphate†	18	2	1	0	2
" "	1	"	0.5% pepper	37	2	1	0	2
" "	1	10% C_2H_5OH		9	0	0	0	0
" "	1	"	0.05% croton oil	9	0	0	1	1
" "	1	40% C_2H_5OH		8	0	0	0	0
Total				268	18	6	7	15

*Heated in an atmosphere of nitrogen.
†"Lorol" sulphate is a mixture of sodium dodecyl and tetradecyl sulphates.

TABLE 3 —*Tumour incidence in control mice fed unheated cottonseed oil, or no cottonseed oil, and surviving over 400 days*

Percent unheated cottonseed oil	Solvent	Plus	Number of mice	Papilloma	Carcinoma	Adenosis	Hepatoma
1	10% soap solution		22			2	
1	"	0.05% croton oil	10				
0	"	"	15	1			
0	"		24	2			1
1	10% C_2H_5OH		8				
0	40% "		8				
0		Histamine (0.5 mg. subcutaneously 3 × weekly).	12			1	
0			86				1
	Control total		185	3	0	3	2

Beck and Peacock reported that rats maintained on an adequate basal diet supplemented with cottonseed oil which had repeatedly been heated at 200-220 degrees C developed within a year signs of avitaminosis-A. Cases of ulceration and papillomatosis of the fore-stomach were observed among those that died. Control animals fed with unheated fats and the same basal diet showed no gross pathological lesions (148).

Through feeding studies using white rats, Crampton et al. evaluated the nutritive value of linseed, rape seed, corn, peanut, and soybean oils which had been heated at 275 degrees C for up to 30 hours. -Feeding of those oils at 10 and 20% of the diet produced the results presented in Tables 4 and 5. The heated oils were shown to depress the growth of

the rats and the efficiency of feed utilization, the severity of these effects increasing as the proportion of heated oil in the diet was increased and as the time of heating the oil was prolonged. Growth was depressed even when the oil was fed separately from the rest of the diet, indicating that the deleterious effects exerted by heated oils are not incurred through the destruction of some essential non-lipid diet component but through some oil constituent that was directly toxic to the rats (415).

TABLE 4.

Summary of data showing the effects of heat treatment of oils as measured by daily gains of rats, feed intakes, and gains per 1,000 Calories eaten

TEST AND OIL USED	DIET NO.	OIL IN DIET	DURATION OF HEAT TREATMENT AT 275°C.	NO. OF ANIMALS	AVE. DAILY GAIN	AVE. DAILY FEED INTAKE	GAIN/ 1,000 CALORIES
		%	*hrs.*		*gm*	*gm*	*gm*
Linseed	III	10	nil	10	3.9	9.6	91
		10	2	10	3.8	9.6	91
		10	4	10	3.4[1]	9.5	80
		10	8	10	2.5[2]	8.1	69
Rapeseed	III	10	nil	10	3.4	10.0	76
		10	15	10	2.9	9.5	69
		10	30	10	2.3[1]	7.8	64
Corn	III	10	nil	12	3.6	10.4	78
		10	15	12	2.8	8.7	71
		10	30	12	2.3[1]	8.0	64
Peanut	III	10	nil	12	3.6	9.3	87
		10	15	12	3.6	10.0	80
		10	30	12	0.8[1]	5.2	30
Soybean	II	20	nil	10	4.6	10.2	90
		20	3	10	4.0	9.4	85
		20	6	10	3.3[1]	8.3	80
		20	9	10	2.9[1]	7.7	76
Herring	I	20	nil	16	2.6	8.1	64
		20	10	16	0.89[1]	6.3	28

[1] Weight gains significantly lower than those of control animals fed the unheated oil.

[2] Weight gains significantly lower than those of animals fed linseed oil heated only 4 hours.

TABLE 5

Effect of 10% vs. 20% of heated oil in the diets as measured by daily gains and feed intake ratios of rats

TEST AND OIL FED	OIL IN DIET	DURATION OF HEAT TREATMENT	NO. OF ANIMALS	AVE. DAILY GAIN	AVE. DAILY FEED INTAKE	GAIN/1,000 CALORIES
	%	*hrs.*		*gm*	*gm*	*gm*
Linseed	20	nil	10	3.7	10.1	80
	10	12	10	2.3[1]	8.2	72
	20	12	10	−0.1[2]	6.0	4
Soybean	10	nil	10	3.7	10.3	92
	10	6	10	3.5	10.4	87
Soybean	20	nil	10	4.6	10.2	90
	20	6	10	3.3[1]	8.3	76

[1] Weight gain significantly lower than that of group fed unheated oil.

[2] Weight gain significantly inferior to that of group fed heated oil as 10% of the diet.

Further studies by Crampton and his coworkers have shown that peroxidation of thermally polymerized linseed oil is not concerned in the development of any toxic factor, and also suggest that a lack of vitamin E is not involved in the growth inhibition resulting from the ingestion of the heated oil (416). On the contrary, they suggested that the primary cause of the lowered nutritive value of diets containing thermally polymerized linseed oil is the presence of one or more mono- or dimeric acyl radicals which are in some way inimical to the well-being of the animals, and that their deleterious effects could well be aggravated since they are produced at the expense of unsaturated fatty acids, some of which may be those essential to the animal for growth (417, 418).

The nutritive value of peanut, sesame, and coconut oils which were heated in open pans at 270 degrees for 8 hours, to simulate conditions which are common in the human use of the oils in India, was investigated by Raju and Rajagopalan. The heated oils were incorporated into synthetic diets to give 15% fat, and fed to albino rats. With all 3 types of oil, there was a significant adverse effect on weight gain, as compared to controls fed unheated oils (see Table 6). When the heated oils were fed at a level of 30%, all of the rats died within one week, whereas the controls were quite healthy (1584).

Table 6. Influence of Heating the Oil on its Nutritive Value and Fat Deposition in the Liver

	Average gain per rat per week (gm.)	Feed efficiency	Liver weight as percentage of body weight	Percentage fat in liver
Groundnut oil:				
Unheated	13·0 ± 0·5	1·1	3·8 ± 0·3	3·9 ± 0·3
Heated	5·0 ± 0·9	0·07	5·7 ± 0·6	7·1 ± 0·4
Sesame oil:				
Unheated	10·0 ± 0·8	1·0	4·2 ± 0·7	4·8 ± 0·3
Heated	4·3 ± 0·7	0·7	6·0 ± 0·5	7·8 ± 0·3
Coconut oil:				
Unheated	11·0 ± 1·1	1·1	4·3 ± 0·3	4·5 ± 0·3
Heated	4·5 ± 0·7	0·7	5·8 ± 0·4	7·0 ± 0·3

Beck reported that subcutaneous injection of 0.5 ml of cotton-seed oil which had previously been heated at 340-360 degrees C resulted in the formation of sarcomata in 2 of 12 mice, after 414 and 538 days. Similar treatment with unheated cotton-seed oil and cotton-seed oil which had repeatedly been heated at 200-220 degrees C produced no tumors (147).

Steiner et al. reported that sesame oil that had been heated to 350 degrees C was carcinogenic, inducing 3 sarcomas in 31 mice that had received 3 injections of 0.5 ml of the oil at intervals of 4 weeks (1818).

Chalmers reported that subcutaneous injections into mice of cotton-seed oil heated at 350 degrees for 4 hours or at 320 degrees for 2.5 hours in the presence of iron did not induce the formation of any tumors at the site of injections, although observed for more than a year (340).

No evidence of cancer was found by Arffman in newts treated with soybean oil which had been heated at 350 degrees C for 0.5 or 1 hour (62).

Kieckebusch et al. reported that the blowing of soybean oil at high temperatures (180 degrees C) greatly increases its toxicity to rats, probably due to the formation of oxypolymers. Through feeding tests with diets containing up to 20% blown soybean oil, the following effects of blown oils were established: growth inhibition connected with a considerable deterioration of the feed- and albumen efficiency, occasionally high mortality rate, disturbances of the liver function, increased central excitability, intensified spontaneous mobility of the animals, inhibition of the motor system in the gastrointestinal tract, modifications of the kidney function, increased capillary permeability, modifications of the motor performance and secretion output in the gastrointestinal tract, histologically determinable changes of the thyroid gland as well as incorporations (deposits) in the kidney (1058).

II. Absorption - Distribution

The rate of absorption of several vegetable oils was determined by Basu and Nath through tests with 225-250 g rats. The rats were given 2 ml of the oil by stomach tube and then sacrificed 2, 4, or 6 hours later and the amount of unabsorbed oil determined. Results of the tests are shown in Table 7 (129).

Table 7

Percentage of different oils absorbed after 2, 4 and 6 hours.

Type of oils.	Percentage of different oils absorbed after		
	2 hours.	4 hours.	6 hours.
Mustard oil ...	27·6 ±0·871	56·3 ±0·752	64·0 ±0·867
Coconut oil ...	36·0 ±1·063	42·7 ±0·740	49·8 ±1·171
Olive oil	41·1 ±0·478	48·9 ±0·719	59·8 ±0·858
Ground-nut oil ...	35·13 ±0·534	52·0 ±0·428	59·9 ±0·480
Sesame oil ...	36·2 ±0·335	46·1 ±0·273	57·3 ±0·191

Langworthy determined the digestibility of a number of vegetable oils through 3-day oral tests with human volunteers. Results of the tests are shown in Table 8 (1147).

TABLE 8 -DIGESTIBILITY OF SOME VEGETABLE FATS

Fat Used in Test	Melting Point °C.	No. of Tests	Digestibility Highest Per cent	Lowest Per cent	Av. Per cent	Corrected for Metabolic Products and Fat of Basal Ration Per cent
Olive oil		18	95.6	91.5	94.7	97.8
Cottonseed oil		12	96.9	91.6	94.9	97.6
Corn oil		7	94.9	88.6	93.5	96.9
Sesame oil		5	95.3	92.1	93.8	98.0
Soy-bean oil		7	95.8	92.4	93.8	97.5
Sunflower-seed oil	Room temp.	4	95.2	91.9	93.8	96.5
Japanese mustard-seed oil		3	96.6	92.6	94.9	95.8
Rapeseed oil		4	96.6	93.7	95.7	98.8
Charlock (wild mustard) seed oil		4	96.3	93.7	94.6	98.9
Cocoa butter	30	11	95.5	79.1	89.6	94.9
Peanut oil		5	97.6	93.7	96.0	98.3
Coconut oil		12	96.3	88.1	93.5	97.9
Almond oil		4	96.2	86.3	93.1	97.1
Black-walnut oil		4	96.7	91.9	94.8	97.5
Brazil-nut oil		3	95.8	92.3	94.5	96.3
Butternut oil		3	94.7	85.3	88.7	95.4
English-walnut oil		3	95.6	92.5	94.4	97.6
Hickory-nut oil		4	98.4	96.5	97.2	99.3
Pecan oil		4	96.9	90.1	93.9	96.8
Apricot-kernel oil	Room temp.	4	95.4	92.3	94.4	98.4
Cherry-kernel oil		4	95.5	93.0	95.2	98.0
Peach-kernel oil		3	96.5	87.0	91.6	96.6
Watermelon-seed oil		3	94.2	88.5	90.7	94.8
Melon- (cantaloupe) seed oil		3	97.7	92.1	94.8	98.2
Pumpkin-seed oil		2	95.0	94.9	95.0	98.2
Tomato-seed oil		3	91.3	88.1	90.3	95.8
Palm-kernel oil		4	97.5	93.9	95.3	98.0
Hempseed oil		3	94.7	94.0	94.4	98.5
Poppy-seed oil		7	93.4	87.4	91.3	96.3
Cohune oil	Near 28	4	94.7	94.0	94.6	99.1
Cupuassu fat	..	4	91.0	86.3	88.8	94.1
Avocado fat	..	3	90.8	88.7	90.0	87.9
Java-almond oil	Room temp.	2	95.5	89.0	92.2	97.0
Tea-seed oil	Room temp.	1	88.2	....	....	91.2

The available studies on the digestibility of the various vegetable oils are too numerous to allow detailed review in this monograph. Furthermore, none of the digestibility studies encountered were observed to differ significantly from the data obtained by Langworthy (1147).

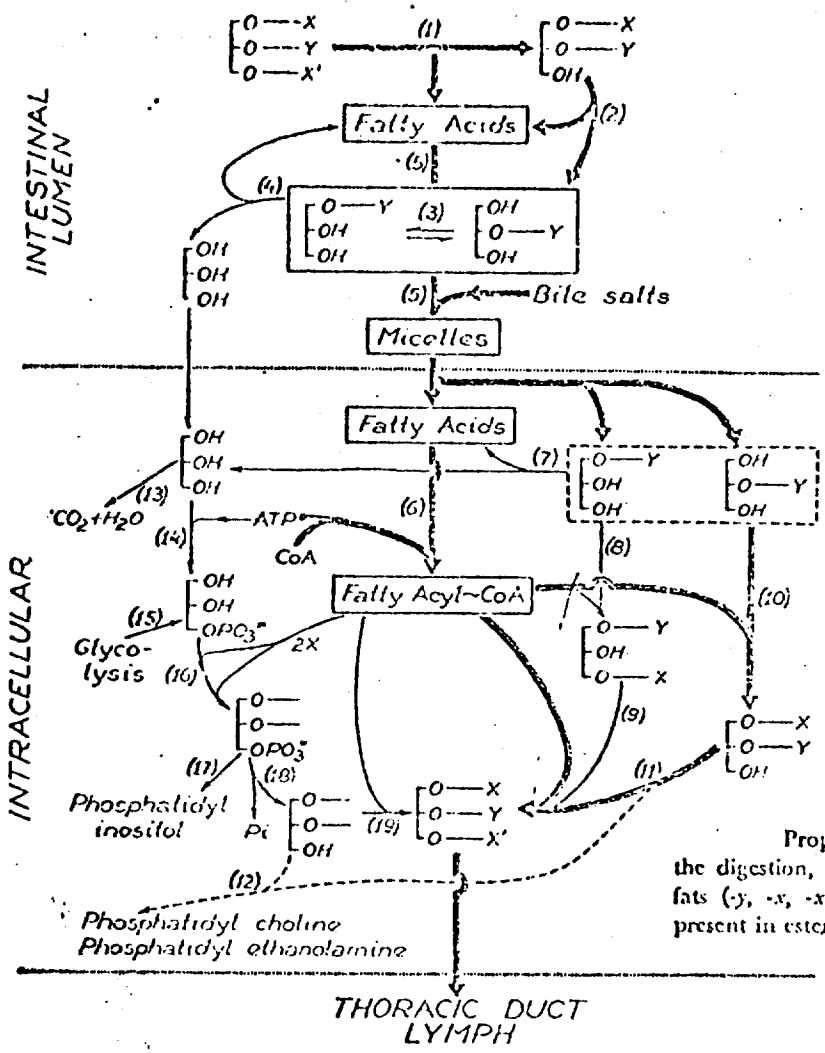
Bergstrom et al. found that when ^{14}C-labelled oleic acid was fed to rats, 66-92% of the fed activity was absorbed in 24 hours. Of the absorbed activity, 38-96% (mean 63%) was found in the lymph fat from the thoracic duct. About 2% of the activity found in the lymph lipids was in the form of phospholipids, the remainder in the form of neutral fats (166).

Renner and Hill determined the metabolizable energy value and percent utilization of oleic acid to be 3770 cal/lb and 88.4, respectively, for the chick (1615).

In feeding studies with rats, Carroll and Richards found the coefficients of digestibility for oleic and linoleic acids to be 73 and 84%, respectively (306).

According to Kayden et al. analysis of intestinal lumen contents of man after fat feeding has shown a separation of glycerides into two phases: an oil phase which contains mainly diglycerides and triglycerides and some fatty acid, and a micellar phase which is a combination of bile salts, free fatty acids, and monoglycerides. From the micellar solutions monoglycerides and free fatty acids are absorbed through the intestinal wall, leaving the bile salts within the lumen to form additional micelles. The fate of the free fatty acids within the mucosal cell is determined by their chain length and saturation; those of less than 12 carbons are preferentially absorbed by rats directly into the portal blood, whereas longer chain fatty acids are incorporated mainly into triglycerides, and appear in lymph chylomicrons. Monoglycerides within epithelial cells may be esterified to di- and triglycerides or cleaved to glycerol and free fatty acids. The 2-monoglycerides are esterified especially rapidly to triglycerides, but esterification of 1-monoglycerides lags and these are more likely to undergo hydrolysis particularly if the fatty acids are of short chain length (1034).

In a review of the literature of fat absorption, Johnston stated that the mechanism of fat absorption can be summarized as shown in the following figure.



Proposed mechanism involved in the digestion, absorption, and biosynthesis of fats (-y, -x, -x', and —— denote fatty acids present in ester linkages).

The heavy arrows denote the major pathway. The emulsified triglycerides are hydrolyzed by pancreatic lipase, at the 1 and 3 positions, forming monoglycerides and fatty acids which in combination with bile salts form a micellar dispersion (reaction 5). The monoglycerides and fatty acids form micelles which penetrate the lumen membrane by an energy-independent mechanism, presumably by diffusion (reaction 5). The monoglycerides and fatty acids migrate to the endoplasmic reticulum, where the fatty acids are activated to their CoA derivatives (reaction 6) and are resynthesized into triglycerides by the acylation of monoglycerides in a multienzyme complex (reactions 10 and 11) (983).

III. Metabolism and Excretion

Fatty acids play an important role as an energy source. Their oxidation is prominent in higher animals which can store large amounts of neutral fat as a fuel reserve. It is estimated that fatty acid oxidation provides at least half of the oxidative energy in the liver,

kidneys, heart muscle and resting skeletal muscle. Fatty acids undergoing oxidation in tissues of higher animals come either from extracellular fluid or from endogenous intracellular lipids. Vertebrate blood contains considerable amounts of triacylglycerols but very small amounts of free fatty acids. The major endogenous source of fatty acids for fuel is storage fat, in the form of fat droplets in the cytoplasm, which consists largely of triacylglycerol. Before the fatty acids can undergo activation and oxidation, the triacylglycerols first must undergo hydrolysis by intracellular lipases to yield free fatty acids and glycerols. Free fatty acids are therefore derived either from the blood stream, in which they are transported bound to serum albumin, or prior hydrolysis of intracellular lipids. They are first activated by esterification with CoA to form acyl CoA esters of the outer mitochondrial membrane. Then the fatty acyl group from CoA is carried across the inner membrane by the carrier molecule carnitine. Finally the fatty acyl group is transferred from carnitine to intramitochondrial CoA: through several enzymatic reactions. A two-carbon fragment of the fatty acid is removed as acetyl CoA and two pairs of hydrogen atoms are given to specific acceptors. A long chain fatty acid will go through several spirals. Thus the 16-carbon palmitic acid undergoes a total of 7 such cycles, to yield 8 molecules to acetyl CoA and 14 pairs of hydrogen atoms. The acetyl CoA formed as a product of the fatty acid oxidation will then enter the tricarboxylic acid cycle, which is the final common pathway of oxidation catabolism of all fuel molecules in aerobic cells (1172).

Cenedella and Allen determined the amount of ^{14}C-linoleate oxidized to $^{14}CO_2$ by 3-month-old male Wistar rats during various time intervals after feeding. They found that a total of approximately one fourth of the administered dose was excreted through this route within a week (see Table 9). In addition they measured the degree of incorporation of the linoleate into cholesterol (see Table 10) (323).

TABLE 9

Per Cent of the Total Endogenous ^{14}C-Labeled Fatty Acid Carbon Oxidized to Respiratory $^{14}CO_2$ Per Hour by the Intact Rat During Various Time Intervals After Feeding 1-^{14}C-Linoleate

Hours after feeding	Number of rats	$^{14}CO_2$ (^{14}C-Linoleate-fed rats)
5--6	4	20.94 ± 7.16
24--29	5	2.43 ± 0.59
48--53	3	0.79 ± 0.22
72--77	4	0.80 ± 0.20
168--173	1	0.67

TABLE 10

Per Cent of the Total Absorbed 1-^{14}C-Linoleate Carbon Found Incorporated Into the Total Cholesterol of the Entire Rat, 6, 29 and 77 hr After Feeding 1-^{14}C-Linoleate

Hours Killed	Number of rats	^{14}C-Cholesterol (^{14}C-Linoleate-fed rats)
6	3	1.252 ± 0.342
29	4	0.804 ± 0.222
77	4	0.800 ± 0.122

Bergstrom et al. observed that rats fed ^{14}C-labelled oleic acid or methyl oleate expired about 16% of the absorbed (63% absorbed) activity as CO_2 within 24 hours (166).

IV. Effects on Enzymes and other Biochemical Parameters

Oleic and linoleic acids have been found to produce, in isolated mitochondria, manifold changes which may be summarized as follows: activation of latent ATPase (1566, 238); inhibition of the ATP-Pi exchange reaction (908, 2060, 1964); uncoupling of oxidative phosphorylation (908, 1964); and induction of mitochondrial swelling (1173, 88, 1964, 2095).

The influence of dietary fat on calcium and phosphorus metabolism was investigated by Basu and Nath in four human subjects. The inclusion of mustard, sesame or peanut oil in the diet greatly favored the absorption of Ca and P and the utilization of the minerals was better than in the absence of added fat. With coconut oil, however, the elimination of Ca through the feces was greatly increased, the fecal Ca being in 3 cases greater than the amount of dietary calcium. The urinary excretion of Ca on coconut oil diet was also generally higher than with other fats (130).

Calvery and Kennedy studied the effect of peanut, coconut oil and hydrogenated cottonseed oil on calcium and phosphorus metabolism in the rat and concluded that the inclusion of 5% of each of the oils in a normal diet increased the fecal excretion of Ca, paralleled by a decrease in the retentions of Ca and P (288).

Rao and De reported that the inclusion of 10% fat (coconut oil, saturated and unsaturated fatty acid fractions) in a normal diet increased the utilization of calcium in young growing rats (1598).

In contrast to the findings of Basu and Nath (130), Sadasivan reported that adult rats showed a positive Ca balance with coconut oil as diet fat (1666).

Male albino rats of the Holtzman strain were observed by Jansson to exhibit a rise in plasma cholesterol when fed a diet containing 15%

free linoleic acid (964).

Renaud and his coworkers reported that linoleic acid appears to be slightly atherogenic when included in the hyperlipemic diet of rats. Under these circumstances linoleic acid was found to be non- or even anti-thrombogenic. Oleic acid appeared to exhibit a greater atherogenic activity than linoleic acid (1614, 1612, 1613).

Schneider reported that spontaneous gastric secretion in the rat can be inhibited by the intraduodenal administration of olive oil; however, large amounts (180 mg/20 min) have to be used. Previous hydrolysis of the oil markedly enhances the inhibitory effect. In addition, oleic acid and sodium oleate are potent inhibitors of both spontaneous and insulin-stimulated gastric secretion (1708).

V. Drug Interaction

The administration of 200 mg of S. foetida oil per day to sexually developing S. C. White Leghorn pullets inhibits ovary and oviduct development and consequently egg production. Schneider has shown that a supplementation of 2000 mg/day of oleic acid partially overcomes the effect of the S. foetida oil and allows egg production to rise to 68% of that of the non-supplemented controls (1707).

Studies by Tove have shown that the addition to the diet of 4% of fats rich in either oleate or linoleate prevents the poor growth and high mortality that results when diets containing high levels (20-40%) of either palmitate or stearate are fed to mice (1916).

The daily administration of 2000-2500 I.U. of tetracycline to mice was found by Gomazkov et al. to increase the resistance of the animals to lethal doses of oleic acid (689).

VI. Consumer Exposure

The vegetable oils are used in the manufacture of a variety of food products. One of the best known uses is in the production of oleomargarine. Many salad oils, cooking oils, and shortenings consist of or contain vegetable oils. Mayonnaise also has a high vegetable oil content. Vegetable oils are often used in baking and in the production of confections.

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THE ACID COMPOSITION OF SOME VEGETABLE OILS

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In the course of investigatons concerning the lipidic metabolism of vegetables that are under way in the Institute of Agrarian Chemistry of the University of Milan, the acid composition of 76 oils extracted from plant seeds belonging to 21 families was determined by means of gas chromatography.

We believed it to be of some use to publish the results obtained, insofar as for some kinds of oils the acid composition has not yet been noted (obtained by gas chromatography), and for the remaining oils we made a comparison with the experimental results obtained by other authors.

EXPERIMENTAL PORTION

Materials and methods

Extraction of the total lipides from seeds ground in toto in Soxhlet with ethyl ether; determination of the degree of acidity according to the official methods of analysis of the M.A.F. Preparation of the methyl esters by means of transesterification with methyl alcohol and sodium methylate, for re-precipitation, according to Anselmo, Boniforti and Monacelli (1).

Gas chromatography with Fractovap Mod B/F with a cold filament. Transport gas: helium. Temperature of the column: 210°C. Evaporation temperature: 320°C. Length of the column: 2 m. Diameter of the column: 6 mm. Repartition liquid: CEAS (polyesters of succinic acid and ethyleneglycol at 20% on celite C 22 bk 60-80 mesh).

Results

The data relating to the total lipide content and to the acid composition are reported in the attached table. Examination of them allows for the drawing of the following conclusions:

Anacardiaceae:

The gas chromatographic examination of the oil of seeds of Pistacia lenticus revealed a large prevalence of oleic acid (57.0%). Worthy of note is the marked presence (21.6%) of palmitic acid, which, according also to Vodret (2), represents one of the principal acid components.

Betulaceae:

The acid composition of three cultivars of Corylus avellana was studied in this family. The results obtained reveal a high content of total lipides, which agrees with the value found by other authors (3-6).

Oleic acid is present in great preponderance (from 76.8 to 82.6%), while linoleic acid varies from 7.5 to 13.7%. Traces of $C_{14:0}$, $C_{15:0}$, $C_{16:0}$ ramified were found.

Cannabaceae:

The total lipide content of seeds of Cannabis sativa (28.2%) was found to be slightly inferior to that reported by Eckey (7), who considers values moving between 30 and 35% to be normal contents.

As concerns the gas chromatographic findings, which confirm the data of the literature concerning the high unsaturation of this oil, they reveal a great preponderance of linoleic acid (52.2%), a high linolenic acid content (17.0%), present in nearly the same proportion as oleic acid (18.2%).

Compositae:

On the basis of the fat level of the four genera examined, a separation of them into two groups is suggested: the first including Cynara scolimus and Cynara cardunculus, the seeds of which have a fat content of about 24%, and the second including Hebantus anuus and Guizotia abyssinica, characterized by a lipide content that is notably higher (over 40%). However, this is only an apparent distinction in that, if Ekstein (8)found a 25.2% content in the seeds of C. cardunculus, other authors (2) registered a value of 32.5%. Besides this, it has been noted (7) that the composition of sunflower seeds is markedly variable and largely influenced by atmospheric and variety factors. This was confirmed by the recent data of Grieco and Piepoli (9), who, in three cultivars of Heliantus, found contents varying from a minimum of 24.7 to a maximum of 43.1%. Besides this, the same authors found that the sunflower seeds cultivated in Bulgaria have an oil content that is consistently greater than that of the same seeds cultivated in Venetia.

For our part, in the course of examinations intended to characterize the glyceride structure of the oils extracted from sunflower seeds of various varieties and origins, we found total lipide levels equal to 46.5% (Bulgarian sunflower), 50.0% (Rumanian sunflower), 42.7% (Italian sunflower), and 28.6% (Italian sunflower).

As concerns their acid composition, the acid with the

greatest representation in all cases is linoleic acid (from a minimum of 50.9% to a maximum of 69.8%); the content of oleic acid varies more (from 7.2% in the neuk to 38.3% in the sunflower).

Beyond this, the presence of a relatively high quantity of stearic acid in the neuk (which perhaps differs considerably from the other Compositae oils considered here) should be mentioned.

Cruciferae

In agreement with that reported by Echey (7), we registered a markedly high level of total lipides (greater than 40%), with the exception of seeds of Sinapis nigra, which contains 32.9%.

As regards the acid composition, the presence of a conspicuous quantity of erucic acid (from 32 to 47%) was confirmed; however, this acid is not to be considered a component peculiar to the Cruciferae oils, in that it is present in the majority, but not in all, the genera of this family (10, 11).

The presence of a relatively large quantity of eicosenoic acid which we confirmed agrees with the data of other authors (14-16); this acid is characterized, according to Hopkins (12) and Baliga and Hilditch (13) by the presence of the double bond corresonding to C_{11}. From among the authors who have confirmed its presence, Miller et al. (11) found that it is largely diffused and represents one of the major components (32.5%) in 4 of the 102 species of Crucifere analyzed.

However, eicosenoic acid is not specific for the crucifers, in that it has been found (10-11%) in the fats of seeds of Delphinium (Ranunculaceae), Pongamia and Erytrina (Papilionatae) (10), as well as in the seeds of a Composita, Marshallia cioespitosa (17), for which, uniquely, it is the major component.

Another characteristic of the crucifer oils is the presence, sometimes in a measurable quantity, sometimes in traces, of the acids $C_{20:2}$, $C_{20:3}$, $C_{22:2}$, $C_{24:0}$, $C_{24:1}$.

As has been said, our data are in general in agreement with those cited in the literature. An exception are the analytical reports regarding the seeds of Camelina sp. While in fact we have registered in this species, analyzed impromptu, a level of 33.1% of erucic acid, Miller et al. (18) found 3.8%. It is a matter of considerable differences, presumably attributable to differences in the variety and atmospheric conditions, and which need further confirmation.

Cucurbitaceae:

The fat content in the genera we analyzed varies, in accord with the variations registered in the literature (7), from a minimum of 24.3% (Citrullus maxima) to a maximum of 43.2% (Cucurbita pepo).

The predominant acid is linoleic (around 70% in Citrullus maxima, in Cucmis sativus, in Cucumis melo; from 43 to 58% in the Cucurbita). It is followed, according to the case, by palmitic acid and by oleic acid.

Juglandaceae:

The oil of seeds of Juglans regia and Juglans nigra, present in the respective proportions of 70.3 and 63.2%, notably greater than those found by other authors (3, 7), is composed 95% by unsaturated acids, among which linoleic acid predominates, followed by oleic and linolenic. This composition coincides with that ascertained by Jamieson and Mckinney (19).

Lauraceae:

The value of the total lipide content of the drupe in toto agrees with that registered by Lotti and Faloppini (3) and is close to the minimum limit given by Eckey (7); on the other hand, it deviates from the value found by Chiarlo (20) who, however, used a different method of extraction.

As relates to the gas chromatographic reports, lauric acid was found to be obviously predominant (47.7%). Linoleic acid and oleic acid follow. Worthy of note is the presence of acids of lower molecular weight, such as $C_{8:0}$ (0.8%) and $C_{10:0}$ (1.9%), which the literature (7, 10) does not list among the acids commonly present in the drupes of Laurus nobilis. On the other hand, we did not detect the presence of valeric and caproic acid, which were found by Chiarlo (20).

Leguminosae:

Of the numerous genera belonging to this family, we examined the seeds of Soya hyspida, Ceratonia siliqua, Tamarindus indica. As concerns the fat of the soybean, the content registered by us is included within the extreme limits (13-26%) that are valid according to Eckey (7) when a large gamut of varieties and atmospheric conditions is considered. These limits move to 18-22% if only the most common varieties are taken into consideration.

The gas chromatographic data, which agree with the values reported by other authors (9, 21, 22, 23), denote a great predominance of linoleic acid, followed by oleic and palmitic. Also confirmed is the relatively high level of linolenic acid and the presence in small quantity of beenic acid.

The lipide content we found in the seeds of Ceratonia siliqua coincides with the values reported in the literature (24).

The acid composition, which, at least as we found, has not yet been studied using gas chromatography, is characterized by the prevalence of linoleic acid, followed by oleic , and by the presence of traces or small amounts of the saturated acids ramified to C_{12}, C_{14}, and C_{16}.

For the oil of seeds of Tamarindus indica we likewise found no gas chromatographic reports in the literature. Hilditch and Williams (10) report data on acid composition obtained by Grindley in 1954 and Tawakley et al in 1953 for two varieties, from the Sudan and India, considerably divergent from the values we obtained. In fact, in contrast to a linoleic acid content of 40% found by the above-named authors, we found a content of 20.6%. It is presumable that this discordance may be due not so much to the different analytical methods

used (in the case of soybeans there were considerable agreements in spite of the disparity in methods) as to the difference between varieties. In the oil of tamarind seeds there were also evident traces or small quantities of saturated acids from C_{12} to C_{15}. Particularly worthy of note is the relatively high content of lignoceric acid.

Liliaceae:

The level of total lipides of the seeds of Allium coepa (24.9%) was slightly higher than that found by Loew (19.4%) and Phadnis (20%) (25).

The gas chromatographic analysis revealed the same picture: great predominance of linoleic acid followed by oleic acid and palmitic acid.

Oleraceae:

Worthy of note is the presence of measurable amounts of beenic acid (0.4%) in the oil of seeds of Olea europaea characterized otherwise by a composition that is qualitatively and quantitatively very close to that of the oil extracted from the fruit.

Ombrelliferae:

The total lipide content of the seeds of Apium graveolens (22.1%) was slightly higher than that registered in the literature (16-17%) (26).

As concerns the gas chromatographic reports, it should be noted above all that the datum relating to $C_{18:1}$ includes both oleic acid and petroselinic acid (octadece-6-noic). Worthy of note otherwise is the presence of $C_{20:3}$ in a measurable quantity (1.3%).

Pedaliaceae:

The fat level found in the three varieties of Sesamum indicum examined is equal to, and sometimes exceeds (in the sample of Egyptian origin) the maximum limit (54%) given by Eckey (7).

In the gas chromatographic analysis, the oil in question was found to contain practically the same propotions of oleic and linoleic acid. In sum, the data obtained are in agreement with the results of Grieco and Piepoli (9), of Jamieson and Bangman (27), of Rudakov (28), of Hilditch et al. (29) and of Hilditch and Riley (30).

Pinaceae:

Four different samples of pinoles, all characterized by a fat level of 54%, greater than the value of 47.7% given by Eckey (7) as an average value, were examined.

The gas chromatographic analysis revealed almost equal quantities of linoleic acid (about 45%) and oleic acid (39%). As concerns the acids with a higher number of carbon atoms, the traces we obtained contained portions relating to the

acids C_{20}:1 and C_{20}:3, and after these, a portion whose retention time corresponds, in our opinion, to arachidonic acid.

Rosaceae:

In accord with the literature, both in regard to the fat level and the acid composition, two groups are differentiated: the first including the seeds of the peach, almonds and the apricot, with a high lipide content and with a clear predominance of oleic acid over linoleic acid, and the second including seeds of the pear, the apple and the sweet-briar, with a lower level of lipides and with a predominance of linoleic acid over oleic. In the sweet-briar we found the presence, sometimes in traces, sometimes in measurable amounts, of saturated acids with a low molecular weight (C_8, C_{10}, C_{12}, C_{14}) and acid ramified to C_{16}.

Rubiaceae:

As has been noted, the fat level of the seeds of Coffea arabica is very variable; according to Eckey (7), the most typical values for the ether extract move from a minimum of 10 to a maximum of 15%.

The gas chromatographic determinations made on the oil extracted from the seeds of coffee, both crude and refined, revealed an acid picture considerably different from that usually characterizing vegetable oils. In accord with the data of Schuette et al. (31), palmitic acid was in fact found in the proportion of about 35%; also, the stearic acid level was found to be higher than in the other vegetable fats. Linoleic acid constitutes a little less than 40%. The presence of beenic acid was ascertained; our gas chromatograms did not, on the other hand, reveal the presence of unsaturated hydroxy acids and of tetracosenoic acid, evidence by other authors (32).

Rutaceae:

At least as far as we found, the literature does not register gas chromatographic data relating to the oils of the seeds of Citrus sinensis, Citrus limonia and Citrus nobilis. For these species as well, palmitic acid was found in a rather high quantity (22-26%); otherwise there is a prevalence of linoleic acid opposed to oleic acid. In contrast to that observed by other authors, we did not find measurable quantities of arachic acid in the seeds of Citrus sinensis and Citrus nobilis (33, 34).

Solanaceae:

In the case of the seeds of Solanum melongena and Capsicum anuum, the principal component is linolenic acid; palmitic and oleic acids are present in proportions that are nearly equal in the second, while in the first there is a clear predominance of oleic over palmitic. Measurable quantities of

beenic acid were found.

The lipides of tomato seeds differ slightly from, the oils mentioned above due to a lesser linoleic acid content and a higher proportion of palmitic and oleic acid. The data relating to Solanum lycopersicum agree with the values reported by Cescon and Giovetti (35).

Sterculiaceae:

The gas chromatographic data that we report here refer to cocoabutter, sold as such or extracted from cocoa or commerical chocolate. Notwithstanding the disparity in the samples examined, a notable consistency in composition, characterized by the presence of nearly equal amounts of linoleic and oleic acid, is evident. Worthy of note is the presence of $C_{17:0}$ in a clearly measurable quantity.

Theaceae:

The oil content of the seeds of Thea sasanqua is extremely variable and can reach 58-60% (referring to the pure almond) (7). The value we found (23.8%) is in accord with that evidenced by Grieco and Piepoli (21.3%) (9) and by Martos et al. (26.3%) (36). In agreement with that found by other authors, the acid most represented is oleic, followed by linoleic and then by palmitic.

Vitaceae:

In the samples of grapenuts we used (Hamburg muscat variety) we found a fat content and an acid composition not very different from that found by Grieco and Piepoli (9) and by other authors cited by Eckey (7). The major component is linoleic acid, followed by oleic.

CONLUSIONS

The results we obtained, taken in sum, confirm that which has hitherto been noted concerning the high degree of unsaturation of vegetable oils. With the exception of cocoa butter and the oil extracted from the drupe of Laurus nobilis, all the vegetable oils analyzed are in fact characterized by a unsaturated acid/saturated acid ratio greater than 1 and varying from a minimum of 2.2 to a maximum of 22.2.

The two acids most represented are usually oleic and linoleic, with a clear predominance of the latter in the oils of seeds of plants belonging to the following families: Cannabaceae, Compositae, Leguminosae, Liliaceae, Pinaceae, Rubiaceae, Rutaceae, Solanaceae and Vitaceae. In the case of the Rosaceae, only a part of the oils analyzed (Pirus malus, Pirus communis,, Rosa canina) showed a prevalence of oleic acid. In the oils of seeds of plants belonging to the remaining families there was evidenced, on the other hand, an oleic acid level clearly superior to the linoleic acid content.

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1. Nome Comune	2. Famiglia e denominazione botanica	3. Lipidi totali % s.s.	4. Acidità % s.g.	5. ACIDI GRASSI %																								6. insaturi / saturi	7. oleico / linoleico
				$C_{8:0}$	$C_{10:0}$	$C_{11:0}$	$C_{12:0}$	$C_{13:0}$	$C_{14:0}$	$C_{14:1}$	$C_{15:0}$	$C_{15:1}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:1}$	$C_{20:2}$	$C_{20:3}$	$C_{20:4}$	$C_{22:0}$	$C_{22:1}$	$C_{23:0}$	$C_{24:0}$	$C_{24:1}$		
	Anacardiaceae																												
Lentisco a.	Pistacia lentiscus	n.d.	n.d.	—	—	0,1	0,2	0,4	tr.	21,6	1,6	tr.	tr.	1,5	57,0	17,0	0,4	0,1	tr.	—	—	—	—	—	—	—	—	3,2	3,3
	Betulaceae																												
Nocciolo b.	Corylus avellana																												
semi lung. (a)	» »	65,3	0,4	—	—	—	tr.	tr.	tr.	5,6	0,2	tr.	0,1	3,2	76,8	13,7	0,2		0,1	—	—	—	—	—	—	—	—	10,3	5,6
» ovali (a)	» »	67,5	0,5	—	—	—	tr.	tr.	—	5,3	0,2	0,1	0,1	3,7	82,6	7,5	0,2		0,1	—	—	—	—	—	—	—	—	9,9	11,0
» tondi (a)	» »	69,7	0,5	—	—	—	tr.	—	—	5,7	0,3	tr.	tr.	3,2	81,4	9,0	0,3		0,1	—	—	—	—	—	—	—	—	10,2	9,0
	Cannabaceae																												
Canapa d.	Cannabis sativa	28,2	1,0	—	—	—	0,1	—	—	8,2	0,2	tr.	—	2,8	18,2	52,2	17,0	1,0	tr.	—	—	—	0,3	—	—	—	—	7,0	0,3
	Compositae																												
Carciofo d.	Cynara scolymus	24,5	0,3	—	—	—	0,1	tr.	—	12,5	0,3	tr.	—	2,2	16,7	67,9	tr.	0,3	tr.	—	—	—	—	—	—	—	—	5,6	0,2
Cardo e.	Cynara cardunculus	24,7	0,4	—	—	—	tr.	tr.	—	12,7	0,1	—	—	3,1	25,6	58,0	tr.	0,2	0,1	—	—	—	tr.	—	—	—	—	5,2	0,4
Girasole f.	Helianthus annuus	49,3	1,1	—	—	—	0,1	tr.	tr.	6,0	0,1	tr.	tr.	4,3	38,3	50,9	0,3		0,1	—	—	—	tr.	—	—	—	—	8,6	0,8
Neuk g.	Guizotia abyssinica	43,6	1,0	—	—	—	0,1	tr.	—	11,8	0,4	tr.	—	9,8	7,2	69,8	0,4	0,5	tr.	—	—	—	—	—	—	—	—	3,5	0,1
	Cruciferae																												
Camelina h.	Camelina sp.	n.d.	n.d.	—	—	—	0,1	tr.	tr.	5,9	0,4	tr.	—	1,9	20,9	14,2	9,0	0,7	9,7	0,6	tr.	—	1,1	33,1	0,3	0,6	1,5	8,7	1,5
Cavolfiore i	Brassica oler. Botrytis	40,0	1,3	—	—	—	tr.	—	—	2,5	0,1	—	—	1,1	14,5	16,7	10,9	0,7	11,3	0,6	0,4	—	—	39,7	0,6	tr.	0,8	22,2	0,3
Cav. verza j	Brassica oler. sab.	43,2	1,9	—	—	—	tr.	—	—	3,6	0,2	—	—	0,9	15,9	11,9	9,1	0,6	8,9	0,5	0,3	—	—	47,2	tr.	tr.	0,8	18,6	1,3
Rapa k.	Brassica rapa	42,3	1,1	—	—	—	tr.	—	—	3,8	0,3	tr.	—	1,0	14,5	13,4	9,4	0,9	9,9	0,5	tr.	—	—	44,7	0,2	tr.	1,1	16,5	1,1
Ravanello l	Raphanus raphanistrum	43,4	2,8	—	—	—	tr.	—	—	5,9	0,2	—	—	1,6	18,4	16,8	10,8	0,8	9,4	0,7	0,6	—	—	32,2	tr.	0,5	2,0	10,4	1,1
Senape m.	Sinapis nigra	32,9	1,2	—	—	—	0,1	—	—	3,3	0,3	—	—	1,1	10,2	13,6	12,5	0,9	13,1	1,3	tr.	—	0,9	39,6	0,9	0,7	1,4	13,2	0,7
	Cucurbitaceae																												
Anguria n.																													
semi bchi grossi (a)	Citrullus maxima	24,3	1,0	—	—	—	0,1	tr.	—	11,0	0,1	0,1	—	5,5	13,6	69,1	0,4		0,1	—	—	—	—	—	—	—	—	4,9	0,2
semi neri piccoli (a)	» »	26,0	0,8	—	—	—	0,1	tr.	—	11,0	0,1	tr.	—	5,7	10,5	72,1	0,4		0,1	—	—	—	tr.	—	—	—	—	4,9	0,1
semi neri grossi (a)	» »	25,3	0,9	—	—	—	tr.	tr.	—	10,5	0,1	0,1	—	7,1	14,9	66,8	0,4		0,1	—	—	—	tr.	—	—	—	—	4,6	0,2
Cetriolini o	Cucumis sativus	34,2	1,2	—	—	—	tr.	tr.	tr.	15,4	0,4	—	—	7,0	10,6	66,3	0,3		—	—	—	—	—	—	—	—	—	3,4	0,2
Cetriolo p.	» »	29,1	1,1	—	—	—	tr.	—	—	13,7	0,1	tr.	tr.	6,2	10,5	68,6	0,7		0,1	—	—	—	—	—	—	—	—	4,0	0,2
Melone q.	Cucumis melo																												
» bianco (a)	» »	28,9	1,7	—	—	—	tr.	tr.	—	9,0	tr.	tr.	tr.	5,7	13,1	71,9	0,3		tr.	—	—	—	—	—	—	—	—	5,8	0,2
» rugoso (a)	» »	28,7	1,6	—	—	tr.	tr.	tr.	tr.	9,6	0,1	tr.	tr.	5,6	14,5	69,9	0,2		0,1	—	—	—	—	—	—	—	—	5,6	0,2
Zucca r.	Cucurbita maxima																												
semi gialli (a)	» »	31,1	3,2	—	—	—	0,1	—	—	12,5	0,2	—	—	5,8	28,1	52,7	0,2	0,3	tr.	—	—	—	—	—	—	—	—	4,3	0,5
semi bchi (a)	» »	30,8	3,5	—	—	tr.	0,1	—	—	14,4	0,2	tr.	tr.	6,8	19,3	58,6	0,1	0,3	tr.	—	—	—	tr.	—	—	—	—	3,6	0,3
Zucchetta	Cucurbita pepo	43,2	0,7	—	—	—	0,1	—	—	12,3	0,1	—	—	6,9	37,1	43,1	tr.	0,3	tr.	—	—	—	—	—	—	—	—	4,1	0,9
	Juglandaceae																												
Noce r.	Juglans regia	70,5	0,2	—	—	—	tr.	—	—	8,0	0,2	tr.	tr.	2,4	16,0	61,0	11,8	tr.	0,4	tr.	—	—	—	—	—	—	—	8,6	0,3
Noce selv.	Juglans nigra	63,2	0,2	—	—	—	tr.	tr.	—	3,9	0,2	tr.	tr.	1,3	15,7	71,3	7,4	tr.	0,2	—	—	—	—	—	—	—	—	18,2	0,2
	Lauraceae																												
Alloro s.	Laurus nobilis	21,1	0,2	0,8	1,9	47,7	1,5	—	tr.	9,2	0,3	—	—	0,4	15,9	21,2	0,5	tr.	0,6	—	—	—	—	—	—	—	—	0,6	0,8
	Leguminosae																												
Soja t.	Soja hyspida	23,0	1,0	—	—	—	0,1	—	—	11,3	0,1	0,1	0,1	4,5	24,0	51,0	7,7	0,5	0,3	—	—	—	0,3	—	—	—	—	4,9	0,5
Carruba u.	Ceratonia siliqua	2,1	0,1	—	—	tr.	0,1	tr.	0,2	13,3	0,7	0,2	tr.	4,1	32,4	46,6	1,3	0,7	—	—	tr.	—	tr.	—	—	—	—	4,3	0,7
Lupino v.	Lupinus mutica	4,3	n.d.	—	—	0,1	0,2	0,4	tr.	15,5	1,6	1,7	0,3	8,0	30,4	29,6	4,4		1,9	—	—	—	5,9	—	—	8,6	—	n.d.	1,5

Nome Comune	Famiglia e denominazione botanica	Lipidi totali % s.s.	Acidità % s.s.	ACIDI GRASSI %																						Insaturi saturi	oleico linoleico
				$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:1}$	$C_{20:2}$	$C_{20:3}$	$C_{20:4}$	$C_{22:0}$	$C_{22:1}$	$C_{22:2}$	$C_{24:0}$	$C_{24:1}$		
	Liliaceae																										
Cipolla	Allium cepa	24,9	4,8	—	—	—	tr.	—	—	8,6	0,2	—	—	2,2	29,6	59,4	tr.		tr.	—	—	—	—	—	—	—	8,3	0,5
»	»	24,1	5,0	—	—	—	0,2	—	—	7,6	0,5	tr.	tr.	3,1	29,6	58,7	0,3		0,2	—	—	—	—	—	—	—	8,2	0,5
	Oleraceae																										
Olivo semi (a)	Olea europaea	45,6	1,8	—	—	—	tr.	—	—	9,6	0,3	0,1	tr.	2,8	68,0	17,0	0,6	0,6	0,6	—	—	—	0,4	—	—	—	6,4	4,0
» frutto (a)	»	n.d.	n.d.	—	—	—	—	—	—	9,9	0,6	tr.	tr.	2,6	79,0	6,2	0,7	0,4	0,6	—	—	—	—	—	—	—	6,7	12,7
	Ombrelliferae																										
Sedano	Apium graveolens	22,1	0,4	—	—	—	tr.	tr.	—	4,9	1,6	tr.	tr.	1,0	68,5	20,4	0,7		1,1	0,5	1,3	—	—	—	—	—	15,8	3,3
	Pedaliaceae																										
Sesamo Eg (a)	Sesamum indicum	61,5	0,7	—	—	—	tr.	—	—	9,4	0,2	tr.	—	6,0	40,6	42,4	0,4	0,8	0,1	—	—	—	tr.	—	—	—	5,2	1,0
» Sudan (a)	»	53,2	0,8	—	—	—	tr.	—	—	9,8	0,3	tr.	—	6,2	41,0	41,0	0,5	0,7	0,2	—	—	—	tr.	—	—	—	5,0	1,0
» Messico (a)	»	54,4	0,8	—	—	—	tr.	—	—	9,8	0,4	tr.	—	5,5	37,8	44,3	0,8	0,9	0,3	—	—	—	—	—	—	—	5,2	0,9
	Pinaceae																										
Pino (semi)	Pinus pinea	54,8	0,7	—	—	—	tr.	—	—	7,3	0,3	tr.	—	3,1	38,8	46,8	0,6	0,4	0,8	tr.	0,4	1,5	—	—	—	—	8,2	0,8
»	»	54,1	n.d.	—	—	—	0,1	tr.	—	6,6	0,3	tr.	—	3,7	40,8	44,2	0,6	0,4	0,8	tr.	0,4	1,7	tr.	—	—	—	8,2	0,9
»	»	55,0	n.d.	—	—	—	0,1	—	—	6,3	0,2	tr.	—	3,8	39,6	45,0	0,7	0,5	0,9	tr.	0,5	2,1	tr.	—	—	—	8,3	0,9
»	»	53,8	n.d.	—	—	—	tr.	tr.	—	7,1	0,2	tr.	tr.	3,5	38,0	47,6	0,6	0,5	0,6	tr.	0,4	1,4	—	—	—	—	8,0	0,8
	Rosaceae																										
Albicocca	Prunus armeniaca	43,2	0,5	—	—	—	tr.	—	—	6,3	0,8	tr.	0,1	1,7	60,6	30,1	0,3	tr.	tr.	—	—	—	—	—	—	—	11,5	2,0
Mandorle dolci (a)	Amygdalus communis	57,6	0,2	—	tr.	0,1	0,1	tr.	tr.	6,4	0,6	0,2	0,1	2,4	69,4	18,4	0,8	0,4	0,6	—	—	—	—	—	—	—	9,4	3,8
» amare (a)	»	55,5	0,3	—	—	—	tr.	—	—	6,1	0,4	tr.	tr.	1,8	72,8	19,8	tr.	tr.	—	—	—	—	—	—	—	—	11,6	3,9
Pesca	Prunus persica	43,1	1,1	—	—	—	tr.	—	—	5,9	1,0	tr.	tr.	1,2	63,8	28,1	tr.	tr.	tr.	—	—	—	—	—	—	—	13,1	2,3
»	»	48,8	0,3	—	—	—	tr.	tr.	tr.	6,1	0,4	tr.	0,1	1,8	69,0	22,2	0,1	0,1	0,1	—	—	—	tr.	—	—	—	11,5	3,1
Susina	Pirus radus	24,5	2,0	—	—	—	tr.	—	—	8,0	0,2	tr.	tr.	1,8	32,7	55,2	0,8	0,5	0,4	—	—	—	0,3	—	—	—	8,4	0,6
Pera	Pirus communis	24,7	1,2	—	—	—	tr.	—	—	8,6	0,4	tr.	tr.	1,6	30,9	52,0	6,0		0,4	tr.	—	—	tr.	—	—	—	n.d.	0,6
Rosa canina frut. int.		n.d.	n.d.	—	—	0,3	0,1	tr.	0,2	4,8	0,3	tr.	tr.	3,6	17,7	53,6	19,4	—	tr.	—	—	—	—	—	—	—	10,1	0,3
semi		6,8	1,7	tr.	tr.	0,3	0,1	—	0,2	4,9	0,4	tr.	tr.	3,7	17,6	53,2	19,6	—	tr.	—	—	—	—	—	—	—	9,9	0,3
	Rubiaceae																										
Caffè (cr.)	Coffea arabica	15,6	2,8	—	—	—	tr.	—	—	35,6	tr.	0,2	—	9,3	9,2	41,1	4,3		0,2	—	—	—	0,1	—	—	—	n.d.	0,2
» (tost)	»	n.d.	n.d.	—	—	—	tr.	tr.	—	36,7	tr.	tr.	—	7,3	8,1	43,6	3,6		0,1	—	—	—	0,6	—	—	—	n.d.	0,2
	Rutaceae																										
Arancio (a)	Citrus sinensis	n.d.	n.d.	—	—	—	0,1	—	tr.	26,1	1,3	0,4	0,2	5,0	23,9	38,5	4,4	tr.	tr.	—	—	—	—	—	—	—	2,2	0,6
Limone (a)	» limonia	n.d.	n.d.	—	—	—	tr.	—	tr.	22,2	0,8	tr.	0,2	3,8	30,7	32,9	9,1	tr.	0,2	—	—	—	—	—	—	—	2,8	0,9
Mandarino (a)	» nobilis	n.d.	n.d.	—	—	—	tr.	tr.	tr.	22,2	0,7	tr.	0,2	5,7	28,2	38,9	3,7	tr.	0,2	—	—	—	—	—	—	—	2,6	0,7
	Solanaceae																										
Melanzana	Solanum melongena	19,6	6,0	—	—	tr.	0,1	tr.	—	8,8	0,2	tr.	—	3,4	18,1	67,7	1,4	tr.	0,2	—	—	—	tr.	—	—	—	7,1	0,3
Peperone (cr.)	Capsicum annuum	24,3	0,7	—	—	tr.	0,1	tr.	tr.	11,2	0,5	0,4	0,1	2,7	10,8	71,9	0,8		1,1	—	—	—	0,3	—	—	—	5,7	0,2
»	»	27,3	1,8	—	—	tr.	0,1	tr.	tr.	12,4	0,4	tr.	tr.	3,0	11,1	72,1	0,2	0,2	tr.	—	—	—	0,2	—	—	—	5,3	0,2
»	»	25,4	0,7	—	—	tr.	0,1	tr.	tr.	12,5	0,4	tr.	0,2	3,2	11,6	71,3	tr.	0,3	tr.	—	—	—	0,4	—	—	—	5,1	0,2
»	»	n.d.	n.d.	—	—	—	0,1	tr.	—	11,3	0,5	tr.	0,1	2,8	11,0	73,6	0,2	0,2	0,1	—	—	—	0,2	—	—	—	5,8	0,2
Pomodoro semi (a)	Solanum lycopersicum	n.d.	n.d.	—	—	—	0,3	—	tr.	16,2	0,8	tr.	0,4	5,8	21,0	52,0	2,8	0,6	tr.	—	—	—	tr.	—	—	—	3,4	0,4
» buccia (a)	»	n.d.	n.d.	—	—	—	0,1	—	—	14,8	0,9	tr.	0,3	4,7	19,9	54,3	2,9	2,0	tr.	—	—	—	tr.	—	—	—	3,6	0,4

(segue)

Nome comune	Famiglia e denominazione botanica	Lipidi totali % s.s.	Acidità % s.g.	ACIDI GRASSI %																												
				$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{15:0}$	$C_{16:0R}$	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{23:1}$	$C_{23:2}$	$C_{20:3}$	$C_{20:4}$	$C_{22:0}$	$C_{22:1}$	$C_{22:2}$	$C_{24:0}$	$C_{24:1}$	insaturi / saturi	oleico / linoleico
	Sterculiaceae																															
Burro di cacao	Theobroma cacao	n.d.	n.d.	—	—	tr.	0,2	tr.	tr.	28,3	0,5	0,3	—	34,9	30,5	3,0	tr.	2,3	—	—	—	—	tr.	—	—	—	—	0,5	10,2
Cacao	»	»	—	—	—	—	0,1	tr.	tr.	25,0	0,5	0,3	tr.	34,5	34,8	3,7	tr.	1,1	—	—	—	—	tr.	—	—	—	—	0,6	9,4
»	»	»	—	—	—	—	0,1	—	—	26,3	0,3	0,2	—	35,7	34,6	1,9	tr.	0,8	—	—	—	—	tr.	—	—	—	—	0,6	18,2
»	»	»	—	—	—	—	0,2	—	—	25,9	0,3	0,2	—	34,2	34,7	3,5	tr.	1,0	—	—	—	—	tr.	—	—	—	—	0,6	9,9
»	»	»	—	—	—	tr.	0,2	—	—	25,9	0,3	0,2	—	34,8	34,2	3,5	tr.	0,9	—	—	—	—	tr.	—	—	—	—	0,6	9,8
»	»	»	—	—	—	0,1	0,3	tr.	—	26,0	0,7	0,4	—	33,8	34,0	3,5	tr.	1,1	—	—	—	—	tr.	—	—	—	—	0,6	9,7
»	»	»	—	—	—	—	0,2	—	—	27,0	0,4	0,2	—	34,7	33,3	3,0	tr.	1,1	—	—	—	—	tr.	—	—	—	—	0,6	11,1
»	»	»	—	—	—	tr.	0,2	tr.	—	25,4	0,6	0,3	—	36,9	32,7	3,0	0,1	0,7	—	—	—	—	tr.	—	—	—	—	0,6	10,9
»	»	»	—	—	—	—	0,1	tr.	—	25,4	0,4	0,2	—	36,9	33,3	2,8	—	0,8	—	—	—	—	tr.	—	—	—	—	0,6	11,9
»	»	»	—	—	—	—	0,1	tr.	—	26,6	0,3	0,2	—	36,2	32,8	2,8	—	0,9	—	—	—	—	tr.	—	—	—	—	0,6	11,7
»	»	»	—	—	—	—	0,1	tr.	—	25,8	0,4	0,3	—	34,5	34,3	3,5	tr.	1,0	tr.	—	—	—	—	—	—	—	—	0,6	9,8
	Theaceae																															
The	Thea sasanqua	23,8	2,7	—	tr.	0,1	0,2	—	—	18,5	0,4	0,3	tr.	3,6	53,3	21,8	0,8		0,9	—	—	—	—	—	—	—	—	3,4	2,4
	Vitaceae																															
Vinaccioli	Vitis vinifera	19,3	0,3	—	—	—	0,2	—	—	8,2	0,9	0,6	tr.	5,9	19,0	64,5	0,6	tr.	—	—	—	—	—	—	—	—	—	5,7	0,3

KEY TO TABLE

1. Common name
   a. lentisk
   b. hazelnut
      (1) long seeds
      (2) oval seeds
      (3) round seeds
   c. hemp
   d. artichoke
   e. thistle
   f. sunflower
   g. neuk
   h. camellia
   i. cauliflower
   j. cabbage
   k. turnip
   l. radish
   m. mustard
   n. (1) watermelon with large white seeds
      (2) with small black seeds
      (3) with large black seeds
   o. cucumbers
   p. melon
      (1) smooth
      (2) wrinkled
   q. pumpkin
      (1) yellow seeds
      (2) white seeds
   r. walnut
   s. laurel
   t. soybean
   u. carruba
   v. tamarind
   w. onion
   x. olive (1) seeds
      (2) fruit
   y. celery
   z. sesame (1) Egyptian
      (2) Sudan
      (3) Mexico
   aa. pine (seeds)
   bb. apricot
   cc. almonds
      (1) sweet
      (2) bitter
   dd. peach
   ee. apple
   ff. pear
   gg. sweet-briar
   hh. coffee cr. - crude tos t.- refined
   ii. orange
   jj. lemon
   kk. mandarin orange
   ll. eggplant
   mm. chilibean
   nn. tomato (1) seeds (2) skin

oo. cocoa butter
pp. cocoa
qq. tea
rr. grapenuts

2. Family and botanical name
3. total lipides
4. Acidity
5. Fatty acids %
6. unsaturated/saturated
7. oleic/linoleic

tr -- traces n.d. -- undetermined

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HEATED FATS AND ALLIED COMPOUNDS AS CARCINOGENS

Studied by the Newt Test

By

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Received 4.x.63

Extensive studies through decades have failed to clarify whether heated and oxidated fats have carcinogenic properties. This appeared from a review of accumulated experimental results (*Arffmann* 1960) pointing to the incapability of the conventional methods of bioassay to yield conclusive evidence. It seems therefore highly desirable to develop new methods for the demonstration of carcinogenicity which at the same time may be expected to reveal unknown effects of carcinogens. A series of experiments on the newt test (*Arffmann & Christensen* 1961, *Arffmann* 1962, 1963 and 1964) has shown a specificity sufficient for a screening method, if the chemical compounds are given in oil, which makes the method convenient for the study of heated fats and allied compounds.

Heating and Oxidation of Fats.

The chemical changes induced by heating of lipids depend on the presence or absence of oxygen, the active components being primarily the unsaturated fatty acids (*Axt* 1959). Most important is the oxidative transformation, corresponding to autoxidation which has found excellent description in works by *Paquot* (1958), *Axt* (1959) and *Glavind* (1963). The first step is the formation of hydroperoxides (lipoperoxides) formed by a free-radical mechanism and proceeding as a chain reaction. This is terminated by polymerization. Decomposition of the peroxides leads amongst others to a formation of hydroxyl-, aldehyde- and ketone groups as well as acids, and the products include a series of low-mole-

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cular-weight unsaturated carbonyl compounds. Also epoxides are formed late in the process.

If oxygen is absent during heating oxygen-free polymers, often of a cyclic structure, are formed. Decomposition products are paraffins, aldehydes, acids and ketones.

The most simple analytical methods to follow the oxidation are determinations of the peroxide value and of the carbonyl (aldehyde) value. The peroxide value expresses the actual quantity of hydroperoxide groups and is therefore not a measure of the total oxidation (*Deutsch et al.* 1941). The carbonyl value measures the secondary oxidation products, which are determined as aldehydes (*Holm et al.* 1957).

The present knowledge of the chemical changes in heating and oxidation of fats is, however, only elementary. In summary, complicated processes of oxidation, polymerization and decomposition lead to a vast mixture of reaction products, including polymers, peroxides, aldehydes, ketones, epoxides, lactones and free fatty acids (*Wurziger & Ostertag* 1960).

Heating of cholesterol probably leads to oxidation, and the formation of ketonic steroids is suggested by *Haven & Bloor* (1956). The products may possess hydroperoxide- or epoxide groups (*Fieser* 1954).

MATERIAL AND METHODS

Substances. Soyabean oil was chosen as test object for the study of heated fats. This oil is used for the preparation of edible emulsifiers, especially for the manufacture of margarine and baking compounds, and besides its content of the more unsaturated fatty acids is rather high.

Simple heating was performed on an infrared radiation bath (BB Infrabath, Bie & Berntsen, Copenhagen) furnished with a relay and a contact thermometer. The fresh soyabean oil (supplied by Dansk Sojakagefabrik) was contained in a Pyrex beaker or retort and the contact thermometer put into the oil. A temperature of 350° C was reached after 45 to 60 minutes and maintained for exactly ½ or 1 hour. During this time the temperature was not below 350° C and not above 355° C. After the heating period the oil was immediately removed from the warm plate. Air had free admittance, but was not bubbled through. The end-product was a dark, brownish and distinctly viscous material.

Some of the soyabean oil was heated in nitrogen atmosphere. The fresh oil was bubbled through with nitrogen for 6 hrs. 20 min. before being poured into a retort, which was sealed from the surrounding air. The warm-up time was 105 minutes and a strong current of nitrogen through the retort was maintained until 300°-320° C was reached after about 70 minutes. The sealed system was heated at 350° 355° C for exactly 1 hour. The nitrogen current was reestablished during the first hour of cooling, and then the retort was closed. The end-product was of a lighter colour and less viscous than that after heating in the presence of air.

Chemical data of the polymerized soyabean oils are given in Table 1.

The viscosity of the heated oils made it necessary to dilute the products with 5 parts of fresh soyabean oil (v/v) before injection.

Commercially polymerized soyabean oil was kindly supplied by Grindstedvaerket, Viby J., Denmark. It is used in an edible emulsifier and produced by heating at 300° C in vacuum (about 20 mm Hg) for about 48 hours until the desired viscosity has been reached. Chromatographic analysis (performed by Professor Jacini, Milan) after saponification of the oil showed the following approximate percentages: monomeric acids: 43-50, dimeric acids: 32-38, polymeric acids: 15-16.5. Molecular weight

of the monomeric glyceryl fraction was about 920, while total oil molecular weight was 2470–3120. Because of the viscosity the product was administered as a 25 per cent in one experiment 50 per cent solution in fresh soyabean oil (w/w).

TABLE 1

Chemical Analyses of Heat-Polymerized Soyabean Oil.

Substance	Peroxide value m.e./kg	Carbonyl value mg%	Iodine value	% Urea filtrate	% Non-distillable methyl esters
				Acids	
Soyabean oil	1.4	6.2	131	6	5
Soyabean oil heated in N-atm. at 350° C for 1 hr.	4.7	217	75	58	53
Soyabean oil heated in air at 350° C for 1 hr.	5.1	206	73	66	56
Soyabean oil heated in air at 350° C for ½ hr.	...	...	...	...	51

The peroxidized soyabean oils were prepared by the Laboratory at Grindstedvaerket. Moderate heating, vigorous stirring of the oil and a constant current of air through the retort were maintained until the peroxide content had risen to the highest obtainable values. Before that, specimens had been taken out at lower levels with proper intervals. In experiments Nos. 11 and 12 (Table 3) the P.V.[1] 4.5 oil was oxidated at 37° C for 131 hours, while the P.V. 858 specimen was made at 100° C for 21 hours. The same temperature was applied to the P.V. 293 oil through 28 hours, but after the oxidation this specimen was mixed with equal part of fresh soyabean oil (the peroxide value is determined on the mixture). In spite of several trials it proved impossible once more to reach the high peroxide value of 8–900 m.e./kg. The applied temperatures were 70° C and down to 40° C in order to avoid heat-induced reversion of the process which ran for up to 95 days. The highest peroxide value obtained in these latter experiments was 409 m.e. kg.

For parallel control, specimens of the peroxidized oils were quickly heated at 200° C for 10 minutes at 10 mm Hg in order to destroy the peroxides. These samples are designated B in the tables.

The peroxidized soyabean oils were—with a few exceptions—injected without any dilution.

Ethyl linoleate hydroperoxides were kindly prepared by Johs. Glavind, D.Sc., Polytechnic Institute, Copenhagen, who also supplied the ethyl linoleate. The technique of preparation is described by *Glavind, Sondergaard & Dam* (1961). The peroxide values (*Glavind & Hartmann* 1955) of the three specimens were 2000, 1500 and 750 m.e./kg. Part of the P.V. 1500 sample was reduced following the method of *Privett & Nickell* (1956), and the peroxide value thereby lowered to 70 m.e./kg. The reduction lasted 1 hour. In most experiments the ethyl linoleate preparations were solved in peanut oil to reduce toxicity, concentrations being v/v.

Epoxidized soyabean oil was obtained from General Mills, U.S.A., and is designated Epoxygen (type 90 premium grade). The following analytical values are specified: Epoxy content, per cent of theoretical: 90 per cent min. Oxirane content: 6.8 per cent min. Iodine value (Wijs): 2.0 max. Acid value: 0.2 max. Saponification value: 180–184. Molecular weight: 1000 appr. Because of viscosity the epoxygen had to be diluted with fresh soyabean oil before injection to make a 25 per cent solution (v/v).

The various isomers of dicpoxybutane (*Feit* 1960) were kindly supplied by Dr. P. W. Feit, the Leo Pharmaceutical Products, Copenhagen. The pure d-, l-, meso- and dl- compounds were all easily solved in peanut oil without the use of heat. The concentrations are weight/volume except in experiment No. 65 (v/v).

16 α, 17 α-epoxyoestratrien-3-ol, synthetisized and kindly provided by Professor H. Breuer, Bonn, was tested in the newts. This monoepoxide, which is a naturally

[1] P.V. = peroxide value, determined a.m. Wheeler (DGF Einheitsmethoden C-VI 6a (57)).

occurring product in estrogen metabolism, is closely related to oestradiol, which was chosen for parallel control and kindly supplied by the Leo Pharmaceutical Products. Both substances were found difficult to solve in peanut oil requiring vigorous stirring and heating on waterbath (<100° C) for 5 to 11 minutes to obtain 0.5 per cent solutions. In most instances slight secondary precipitation after cooling was eliminated before injection by heating over open fire for a few seconds.

Cholesterol was obtained from The British Drug Houses Ltd. (Laboratory Reagent. Melting point: 145° to 149° C. Specific rotation (α) $\frac{20}{D}$ (in chloroform): −38°.5 to −40°.5. Moisture: Not more than 0.5 per cent. Sulphated ash: Not more than 0.1 per cent). Cholesterol was heated on the BB Infrabath the compound being placed in a Pyrex glass, which together with the contact thermometer was put into a beaker with silicon oil. One sample was heated at 350° C, another at 200° C, both for exactly 1 hour. The warm-up time was 30 and 14 minutes, respectively. After the 350° C heating cholesterol appeared darkbrown to black and after cooling was paste-like. The lower temperature changed the white colour to light yellow-brown. Both specimens were, like untreated cholesterol, easily solved in oils by the use of stirring and heating on waterbath (<100° C) for 1 1½ minutes.

Technical procedures have been described before (*Arffmann* & *Christensen* 1961, *Arffmann* 1962, 1963). In a few experiments Triton marinoratus was used instead of Triton cristatus, the latter being at that time not available.

RESULTS

The results are shown in Tables 2–10. Negative and positive controls are included. The various heat-polymerized soyabean oils gave a negative outcome in all experiments (Table 2).

Strongly peroxidized soyabean oil was submitted to two tests (Table 3). The results are slightly varying, probably because of a less reliable technique in these early experiments, but indicate clearly a weak or moderate, positive reaction to the two oils with a peroxide value of 858 and 293 m.e./kg respectively. In contrast, the P.V. 4.5 oil gave a result not different from that seen after fresh oil, while heavy dilution (2 per

TABLE

Experiments with Heat-Polymerized Soyabean Oil.

Experiment No.	35-36			40						41			
Substance	Soyabean oil	Soyabean oil heated in air at 350°C for ½ hr. 25% in soyabean oil*	Dibenz-[a,h]anthracene 0.1% in soyabean oil	Soyabean oil		Soyabean oil heated in N-atm. at 350°C for 1 hr. 25% in soyabean oil		Soyabean oil heated in air at 350°C for 1 hr. 25% in soyabean oil		Soyabean oil		Soyabean oil heated in N-atm. at 350°C for 1 hr. 25% in soyabean oil	
Site of application	Tail		Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back
Number of animals (Triton cristatus)	4	8	8	4	4	4	4	4	4	4	4	4	4
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on													
3.- 4. day													
6.- 7. "													
8.-10. "													
11.-12. "													
15. "													
18. "													
Animals died during experiment	0	1 (included)	3 (2 included)	1	0	3 (1 included)	1	2	0	0	0	2 (included)	0

Experiment No.	42							50			51			52	
Substance	Soyabean oil heated in air at 350°C for 1 hr. 25% in soyabean oil		Soyabean oil	Soyabean oil heated in N-atm at 350°C for 1 hr. 25% in soyabean oil	Soyabean oil heated in air at 350°C for 1 hr. 25% in soyabean oil	Benzo-a pyrene 0.5% in soyabean oil	Soyabean oil	Commercially polymerized soyabean oil 25% in soyabean oil	Benzo-a pyrene 0.1% in soyabean oil	Soyabean oil	Commercially polymerized soyabean oil 25% in soyabean oil	Benzo-a pyrene 0.1% in soyabean oil	Soyabean oil	Commercially polymerized soyabean oil 50% in soyabean oil	Chrysene 0.5% in peanut oil
Site of application	Tail	Back	Tail					Tail			Tail			Tail	
Number of animals (Triton cristatus)	4	4	8	8	8	8	8	8	8	8	8	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on															
3.- 4. day															
6.- 7. "															
8.-10. "															
11.-12. "															
15. "															
18. "															
Animals died during experiment	1	1	3	2 (included)	3 (1 included)	2 (1 included)	0	2	1 (included)	1	1	1 (included)	0	0	0

* Unsuited because of epidermolysis.

TABLE 2

Experiments with Strongly peroxidized Soyabean Oil.

TABLE 3

Experiment No.	11								12								
Substance	Peanut oil	Perox. soya-bean oil (P.V.* 4.5)	Perox. soya-bean oil (P.V. 293)	Perox. soya-bean oil (P.V. 858)	Perox. soya-bean oil (P.V. 4.5) 2% in peanut oil	Perox. soya-bean oil (P.V. 293) 2% in peanut oil	Perox. soya-bean oil (P.V. 858) 2% in peanut oil	3-Methyl-cholan-threne 0.1% in peanut oil	Soya-bean oil	Perox. soya-bean oil (P.V. 4.5)	Perox. soya-bean oil (P.V. 293)	Perox. soya-bean oil (P.V. 858)	Perox. soya-bean oil (P.V. 4.5) 2% in soya-bean oil	Perox. soya-bean oil (P.V. 293) 2% in soya-bean oil	Perox. soya-bean oil (P.V. 858) 2% in soya-bean oil	3-Methyl-cholan-threne 0.1% in soya-bean oil	
Site of application	Tail								Tail								
Number of animals (Triton cristatus)	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																	
3. day	—			—			—	—	+	(+)	—	+	—	—	+		
6. -	—		(+)	—		+		(+)			—	+	—	—	+	—	
9. -	—		—		—		(+)			+	—	+	—	—	—	+	
12. -	—			+	+			—	—	—	—	+	—	—	—	(+)	
15. -	—	—	+		—		—	+	—	—	—	—	—	—	(+)	+	
18. -	—	—	+	+	—		—	+		—	+	—	—	—	+	—	
21. -	—	—			—	—	—	—	—	—	—	+	—	—	—	—	
Animals died during experiment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

* P.V. = = peroxide value (m.e./kg).
† No signs of the injected oil.
‡ Sections technically bad.

TABLE 4

Experiments with Peroxidized Soyabean Oil before (A) and after (B) Heat-Reduction.

Experiment No.			45	46	47	49	50	51	52	54	69	70
Substance	P.V.*	C.V.§	Positive reactions Total number of animals									
Oil 0	1.9	12.5	1/5	0/5	0/6	...	...	...	...	...	...	...
Oil 0	2.2	3.7	...	...	...	0/8	0/8	0/7	0/8	...	...	...
Oil 0	1.4	10.2	...	...	...	...	...	...	...	1/6	...	...
Oil 0	3.0	2.5	...	...	...	...	...	...	...	...	1/8	0/8
Oil 1A	33.0	9.5	0/7	0/6	...	...	...	...	...	...	...	...
Oil 2A	55.0	...	...	...	...	0/8	...	...	...	0/6	...	...
Oil 3A	70.0	9.1	0/6	0/6	...	...	...	...	...	...	...	...
Oil 4A	104.0	...	...	...	...	0/8	...	...	...	0/6	...	...
Oil 5A	161.0	13.1	2/7	1/6	...	...	...	...	...	...	...	...
Oil 6A	191.0	...	...	...	...	0/8	...	1/8	...	0/6	...	...
Oil 7A	296.0	22.0	0/2	0/5	2/4	...	...	...	...	...	...	...
Oil 8A	362.0	...	...	...	...	0/8	0/8	0/8	0/8	0/6	...	...
Oil 9A	409.0	57.2	...	...	...	...	...	...	...	...	2/8	3/16
Oil 1B	1.5	47.0	0/3	0/5	...	...	...	...	...	...	...	...
Oil 2B	0.6	67.0	...	...	...	0/8	...	...	...	0/5	...	...
Oil 3B	1.8	90.0	0/6	2/5	...	...	...	...	...	...	...	...
Oil 4B	0.7	137.0	...	...	...	0/8	...	...	...	0/5	...	...
Oil 5B	2.6	162.0	0/6	0/6	...	...	...	...	...	...	...	...
Oil 6B	1.3	310.0	...	...	...	0/8	...	0/7	...	0/6	...	...
Oil 7B	4.1	165.0	0/3	0/6	0/4	...	...	...*	...	...	...	...
Oil 8B	3.8	640.0	...	...	...	1/8	0/7	0/8	0/8	0/6	...	...
Oil 9B	3.1	336.0	...	...	...	...	...	...	...	...	2/7	3/16
Dibenz(a.h)-anthracene 0.1%			2/2	...	...	...	...	...	...	...	...	...
Chrysene 0.5%			...	2/5	...	...	...	...	3/8	...	...	...
Benzo(a)-pyrene 0.1%			...	...	3/6	...	1/8	4/8	...	...	5/7	...
— 0.5%			...	...	...	...	...	...	...	...	...	8/8
Benz(a)-anthracene 0.1%			...	...	...	0/6	...	...	...	...	...	...
— 0.5%			...	...	...	...	...	...	...	2/5	...	...

* P.V. = peroxide value (m.e. kg).
§ C.V. = carbonyl value (mg%).

cent) of the P.V. 858 and 293 oils weakened the positive response distinctly.

The following extensive experiments on the reactivity of the newt epidermis to peroxidized soyabean oil are summarized in Table 4. The oil numbering is arbitrary and in series A refers to the increasing peroxide value of the samples, while series B represents the same specimens after heat destruction of the peroxides. O oils are untreated, fresh soyabean oils. Positive reactions cover + and (+) responses, while doubtful reactions (±) are not included. Total number of animals represents effective total. Eleven positive reactions to soyabean oils with a peroxide value of 161 or more were seen among 108 newts. With one exception all of these responses fall in experiments 45–47, being made in the autumn, and in experiments 69–70, using hibernating animals from the preceding year's batch, which in both cases means that

Table [illegible]. *Experiments on a Possible [illegible] of Oxidated Soyabean Oil.*

Experiment No.																													
Substance	Soya-bean oil 0* (P.V. 1.0 C.V. 22.5)†	Soya-bean oil 7B (P.V. 4.1 C.V. 165)	Soya-bean oil 7A (P.V. 286 C.V. 22)	Benzo[a]pyrene 0.05 % in soya-bean oil 0	Benzo[a]pyrene 0.05 % in soya-bean oil 7B	Benzo[a]pyrene 0.05 % in soya-bean oil 7A	Benzo[a]pyrene 0.1 % in soya-bean oil 0	Soya-bean oil 0 (P.V. 3.0 C.V. 2.5)	Soya-bean oil 9B (P.V. 3.1 C.V. 336)	Soya-bean oil 9A (P.V. 400 C.V. 57.2)	Benzo[a]pyrene 0.025 % in soya-bean oil 0	Benzo[a]pyrene 0.025 % in soya-bean oil 9B	[illegible]	Benzo[a]pyrene 0.1 % in soya-bean oil 0	Soyabean oil 0 (P.V. 3.0 C.V. 2.5)	Soyabean oil 9B (P.V. 3.1 C.V. 336)	Soyabean oil 9A (P.V. 400 C.V. 57.2)	Benzo[a]pyrene 0.025 % in soyabean oil 0	Benzo[a]pyrene 0.025 % in soyabean oil 9B	Benzo[a]pyrene 0.025 % in soyabean oil 9A	Benzo[a]pyrene 0.1 % in soyabean oil 0
Site of application	Tail							Tail							Tail	Pr. T.‡	Tail	Pr. T.	Tail	Pr. T.	Tail	Pr. T.	Tail	Pr. T.	Tail	Pr. T.	Tail	Pr. T.	Tail	Pr. T.
Number of animals (Triton cristatus)	6	6	6	6	6	6	6	8	8	8	8	8			8	3	3	8	8	8	8	3	3	3	3	3	3	3	3	3
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀			♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																													
3.- 4. day								(+)																					
6. -																													
9. -																													
12. -						(+)	(+)	+	(+)																				
14.-15. -			+ (+)		(+)			+	(-)																				
18. -									(+)																				
Animals died during experiment	1 (included)	3 (1 included)	2	0	0	0	0	2 (included)	2 (1 included)	0	1 (included)	1	2	0	0	0	0	0	1 (included)	1 (included)	1	0	0	1	0	0	0		

* Numbering of the oils is the same as in Table 4.
P.V. = peroxide value (m.e./kg), C.V. = Carbonyl value (mg%).
† Triton marmoratus in exper. no. 47.
‡ Omitted because of heavy skin-infection.
§ Part of tail proximal to secondary tail.

the animals have been in a rather sensitive state (cfr. *Arffmann* 1964). Experiments 49–54, holding only one positive response, were made in the spring on newly received and therefore more resistant animals. This is reflected by the positive controls in experiments 49–50. In the B series the result of experiments 69–70 equals that in the A series. Besides, the positive reactions are few, but it is difficult to explain why 2 out of 5 animals in experiment No. 46 react to an oil with carbonyl value 90 considering the many negative responses at higher carbonyl values.

A possible cocarcinogenic effect of oxidated soyabean oil was examined in three experiments shown in Table 5. The first experiment (No. 47) was indicative of such an effect, but could not be confirmed. Unfortunately an unexpected high reactivity to benzpyrene was seen at a concentration of 0.025 per cent, and signs of promotion could not be demonstrated, when oxidated soyabean oil was used as vehicle instead of the fresh oil.

The first experiment with ethyl linoleate hydroperoxide is not tabulated. Since the preparation of the compound several months earlier the peroxide value had fallen from 3000 to 2000 m.e./kg. The oil was injected undiluted into 11 Triton marmoratus and 1 Triton cristatus. From the 4th day severe toxic lesions with extensive necrosis and deep ulceration were seen in the tails, so that the experiment had to be terminated within 9 days. The lesions were grossly like those seen after tributyrin (*Arffmann* 1962), but microscopically the epithelial reaction was of quite different degree with extensive and strong hyperplasia. Less striking was the slight or moderate infiltrative downgrowth at the edges or in the bottom of the ulceration. The severe inflammatory lesions made it impossible to estimate the epithelial reaction exactly in terms of degree, but the changes strongly indicated further experimentation.

Tables 6 and 7 show the results with ethyl linoleate hydroperoxide of peroxide value 750 and 1500 m.e./kg and in various concentrations, compared to the effect of reduced ethyl linoleate hydroperoxide and pure ethyl linoleate. The degree of toxic symptoms was practically equal

TABLE 6

Experiments with Ethyl Linoleate Hydroperoxide (High Concentrations).

Experiment No.	54				57					55					58					61				
Substance	Soyabean oil	Ethyl linoleate hydroperoxide 750 m.e. kg	Ethyl linoleate hydroperoxide 1500 m.e. kg	Benzo-a-anthracene 0.5% in soyabean oil	Soyabean oil	Ethyl linoleate	Dibenzo-a,h-anthracene 0.025% in soyabean oil	Peanut oil	Ethyl linoleate hydroperoxide 750 m.e. kg 50% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 50% in peanut oil	[illegible]	Peanut oil	Ethyl linoleate 50% in peanut oil	Reduced ethyl linoleate hydroperoxide 1500→70 m.e. kg 50% in peanut oil	Ethyl linoleate hydroperoxide 750 m.e. kg 50% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 50% in peanut oil	Dibenzo-a,h-anthracene 0.025% in peanut oil	Peanut oil	Ethyl linoleate 25% in peanut oil	Reduced ethyl linoleate hydroperoxide 1500→70 m.e. kg 25% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 25% in peanut oil	Benzo-a-pyrene 0.5% in peanut oil
Site of application	Tail				Tail			Tail						Tail					Tail				Back
Number of animals (Triton cristatus)	6	6	6	6	8	8	8	8	8	8	8	8	8	8	8	8	8	4	8	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																							
5.-7. day	+	-*	-*																				
8.-9. -	+	-*	-*		—	—	(+)	—	+	—	—	—	—	(+)	—	—	—	—	—	—	—	+	(+)
12.-13. -	—	+	+	—	—	—	—	—	+	+	—	—	—	—	—	—	+	+	—	—	+	—	—
14.-15. -	—	(+)	-*	—	—	—	—	—	+	—	—	—	—	—	—	—	+	+	—	—	—	—	+
18.-19. -	—			+	—	—	—	—	+	—	—	—	—	—	—	—	+	+	—	—	—	—	+
Animals died during experiment	0	1 (included)	0	0	0	1 (included)	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	1	0

* Severe necrosis. † Sections technically bad.

for the two peroxides (Fig. 1) and the reduced form, but decreased with concentration, being absent at 10 per cent. Undiluted ethyl linoleate had negligible toxicity. A few cases (in experiments 54 and 55) could not be classified because of severe necrosis. Ethyl linoleate hydroperoxide P.V. 1500 induced a strong, positive reaction in the newt when undiluted and as a 50 per cent solution in peanut oil (Fig. 2). The response decreased with the concentration and was totally negative at 1 per cent. The reaction to the reduced compound was a little less, but also positive. Ethyl linoleate hydroperoxide P.V. 750 induced a clearly weaker positive response than the P.V. 1500 compound, and pure ethyl linoleate can be estimated as negative.

The toxic lesions being similar to those observed after the injection of tributyrin made it interesting to compare the latter compound with ethyl linoleate hydroperoxide in a few experiments. Toxicity showed to be of the same order. In one experiment (No. [illegible]) a 50 per cent solution (v/v) of tributyrin in peanut oil had the same carcinogen-like effect as ethyl linoleate hydroperoxide P.V. 750, and even at 10 and 1 per cent single positive reactions were seen. This result seems to indicate that tributyrin is not a totally indifferent compound (cfr. Hoffmann 1962). Peroxide value of the tributyrin was 0 and carbonyl value 1.4.

A completely negative result followed the injection of epoxidized soyabean oil in three experiments (Table 8). The same applies to 16 α, 17 α-epoxyoestratrien-3-ol which was submitted to three separate tests comprising [illegible] animals with intact tails. Also estradiol appeared newt-negative, while 0.5 per cent benzo(a)pyrene in the positive control experiments provoked epithelial proliferation in 20 out of 24 animals.

In contrast, the testing of a low-molecular diepoxide, diepoxybutane, showed positive reaction to all isomers (Table 9). A preliminary experiment ([illegible]) with di-diepoxybutane revealed a moderate irritative

TABLE

Experiments with Ethyl Linoleate Hydroperoxide (Low Concentrations).

Experiment No.	50							56	60										
Substance	Peanut oil	Ethyl linoleate 10% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in peanut oil	Reduc. ethyl linoleate hydroperoxide 1500→70 m.e. kg 10% in peanut oil	Dibenz-[a,h]-anthracene 0.00005 % in peanut oil	Peanut oil	Tributyrin 1% in peanut oil	Tributyrin 10% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 1% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 1% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 10% in peanut oil	Benzo-a-pyrene 0.5% in peanut oil	Peanut oil	Ethyl linoleate 1% in peanut oil	Reduc. ethyl linoleate hydroperoxide 1500→70 m.e. kg 1% in peanut oil	Ethyl linoleate hydroperoxide 1500 m.e. kg 1% in peanut oil	Benzo-a-pyrene 1% in peanut oil
Site of application	Tail								Tail							Tail			
Number of animals (Triton cristatus)	8	8	8	8	8	8 (Tr. crist. & vulg.)	8	[illegible]	[illegible]	8	8	8	8 (Tr. crist. & vulg.)	4	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																		
5.-7. day											–							
8.-9.	±	– –	– +	– –	– –	±	– –	–	– –	– –	– –	–	– –	–	– –	– ±	+ +	
12.-13.	–	– –	– +	– –	+ +	±	– –	–	– –	– –	±	–	– –	–	– –	– –	– +	
14.-15.	–	– –	– –	– –	+ +	– –	+ –	–	– –	– –	±	–	– –	–	– –	– ±	+ +	
18.-19.	±	– –	– –	±	+ –	–	– –	–	– –	– –	±	–	– –	–	– –	– –	+ +	
Animals died during experiment	0	0	0	0	0	0	0	[illegible]	[illegible]	1	1 (included)	0	0	0	0	0	1 (included)	0

effect at a concentration of 10 per cent, but at 1 and 0.5 per cent such lesions were not seen. In two out of three experiments the positive reaction to the meso-compound was slight, but else there was no significant difference between the various isomers (Fig. 3).

In seven different experiments 0.1 or 0.5 per cent solutions of cholesterol induced no epithelial reaction in newts. After heating of the cholesterol at 200° C for 1 hour, one single positive response occurred among 16 animals, injected with 0.5 per cent solutions. Preheating at 350° C, however, formed a product which in the same concentration provoked epithelial reaction in about half the animals (Fig. 4). Experiment No. 66 confirmed this finding, being made 7 months later. A 0.1 per cent solution of the heated cholesterol had no effect. (Table 10).

DISCUSSION

The present results with peroxides suggest a weak carcinogenicity of such compounds. Most impressing was the reaction to ethyl linoleate hydroperoxide at high peroxide value and in high concentrations, while peroxidized soyabean oil had a weak and inconstant effect. This difference parallels the variation in peroxide value, indicating a causality which is further supported by experiments Nos. 11 and 12, and by the quantitatively different response to the two ethyl linoleate hydroperoxides (P.V. 1500 and 750). The results may, however, have been influenced by the fact that the linoleate peroxide is a low-molecular, more diffusible and therefore immediately active compound, while the peroxy groups in the soyabean oil are connected to larger and less mobile molecules. The severe toxic reaction to ethyl linoleate hydroperoxide is another expression of this circumstance and may have added to the

epithelial response. The carcinogenicity of peroxides is considered weak, because positive reaction demands a very high peroxide value of the oil or the use of sensitive animals.

The positive results obtained with reduced peroxides indicate that the activity is connected with other oxidation products as well, even if peroxy groups seem to be the most active. The carbonyl value is a summarized expression of the secondary oxidation products in the thermo-reduced soyabean oils (B series), while reduction of ethyl linoleate hydroperoxide probably leads to the formation of alcohol groups (*Glavind* 1963). The exact nature and quantities of these various reaction products is not known.

That the observed effect is connected with oxidation of the oils is supported by the other results of the present experiments. Heating of cholesterol is supposed to yield oxidative transformation products (*Fieser* 1954, *Haven & Bloor* 1956), and cholesterol heated at 350° C provoked a positive response in newts. Diepoxybutane likewise showed a distinct activity, while the negative result with epoxidized soyabean oil may be explained by the high molecular weight and low mobility of the active groups. The results with 16 α, 17 α-epoxyoestratrien-3-ol may express inactivity of monoepoxides.

Commercially polymerized soyabean oil is heated in vacuum, and the soyabean oil heated at 350° C in the presence of air showed only a mild degree of oxidation, the carbonyl value not exceeding that seen after heating in nitrogen atmosphere. Also *Wurziger & Ostertag* (1960) found a moderate rise in carbonyl value on heating of oils in a current of nitrogen. Table 4 shows that with a few exceptions oils in series B with a carbonyl value below 300 gave no positive reactions. Furthermore, the high-molecular-weight of polymerized oils will restrain the effectivity of any oxidized groups. So the negative effect of heat-polymerized soyabean oil is in accordance with the hypothesis that oxidation is the major factor responsible for the carcinogen-like activity of some heated lipids in newts.

Figs. 1–4.

Fig. 1. Large ulcerative defect with necrotic tissue remnants on 12th day after injection of ethyl linoleate hydroperoxide (P.V. 1500), 50 per cent in peanut oil. Strong epithelial hyperplasia is seen at the edge of the ulceration and on the other side of the tail. (10 ×).

Fig. 2. Epithelial hyperplasia and infiltrative downgrowth on 19th day after injection of ethyl linoleate hydroperoxide (P.V. 1500), 50 per cent in peanut oil. (40 ×).

Fig. 3. Strong epithelial hyperplasia and infiltrative downgrowth on 15th day after injection of 1-diepoxybutane, 1 per cent in peanut oil. (40 ×).

Fig. 4. Epithelial hyperplasia and downgrowth on 18th day after injection of heated cholesterol (350° C for 1 hour), 0.5 per cent in peanut oil. (40 ×).

Fig. 1–4.

TABLE 8

Experiments with Epoxidized Soyabean Oil.

Experiment No.	39						40				41						
Substance	Soyabean oil		Epoxy soyabean oil 25 % in soyabean oil		Benz(a)-anthracene 0.1 % in soyabean oil		Soyabean oil		Epoxy soyabean oil 25 % in soyabean oil		Soyabean oil		Epoxy soyabean oil 25 % in soyabean oil				
Site of application	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back	Tail	Back			
Number of animals (Triton cristatus)	4	4	4	4	4	4	4	4	4	4	4	4	4	4			
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																	
5. day		−				±											
9. -	−		−	−	−			−	−	−	−	−		−			
11.-12. -		±		−	−			−	−	−	−	−		−			
15. -	−	−	−	−	+	−	−	−	−	−	−	−	−	−			
18. -	±		−	−	+	−	−	−	−			−		−			
Animals died during experiment	0	0	0	0	0	1 (included)	1	0	0	2 (included)	0	0	3	0			

TABLE 9

Experiments with Diepoxybutane.

Experiment No.	65		66					67					68					
Substance	dl-Diepoxybutane 10% in peanut oil	dl-Diepoxybutane 1% in peanut oil	Peanut oil	d-Diepoxybutane 0.5% in peanut oil	l-Diepoxybutane 0.5% in peanut oil	meso-Diepoxybutane 0.5% in peanut oil	Benzo[a]pyrene 0.5% in peanut oil	Peanut oil	d-Diepoxybutane 1% in peanut oil	l-Diepoxybutane 1% in peanut oil	meso-Diepoxybutane 1% in peanut oil	Benzo[a]pyrene 1% in peanut oil	Peanut oil	d-Diepoxybutane 1% in peanut oil	l-Diepoxybutane 1% in peanut oil	dl-Diepoxybutane 1% in peanut oil	meso-Diepoxybutane 1% in peanut oil	Benzo[a]pyrene 1% in peanut oil
Site of application	Part of tail proximal to secondary tail		Part of tail prox. to secondary tail					Part of tail prox. to secondary tail					Part of tail proximal to secondary tail					
Number of animals (Triton cristatus)	6	6	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on																		
6. day																		
9. -	(+)	–	–	–	–	–	+	–	–	–	–	+	–	±	–	–	+	–
12. -	+	(+)	–	(+)	–	(+)	+	(+)	(+)	+	–	+	–	–	–	(+)	–	+
15. -	(+)	–	(+)	–	+	(+)	–	–	(+)	–	±	(+)	(+)	+	–	(+)	±	+
18.-19. -	–	+	–	(+)	–	+	+	+	+	(+)	+	–	–	–	(+)	(+)	–	–
Animals died during experiment	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3 (1 included)	1 (included)

Experiments with Cholesterol and Heated Cholesterol.

Experiment No.	38		11											
Substance	Peanut oil	Cholesterol 0.5% in peanut oil	Soyabean oil	Cholesterol 0.5% in soyabean oil	Dibenz-a,h-anthracene 0.1% in soyabean oil	Soyabean oil	Cholesterol 0.1% in soyabean oil	Cholesterol heated at 350° C for 1 hr 0.1% in soyabean oil	Dibenz-a,h-anthracene 0.025% in soyabean oil	Peanut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 350° C for 1 hr 0.25% in peanut oil	Dibenz-a,h-anthracene 0.05% in peanut oil
Site of application	Tail		Part of tail proximal to secondary tail			Tail				Tail			
Number of animals (Triton crista)	6	6	6	6	6	8	8	8	8	8	8	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on													
9 day	–	–	–	–	–	–	–	–	–	–	–	–	–
12.	–	–	–	+	–	–	–	–	–	–	–	–	–
15.	–	–	–	–	–	–	–	–	+	–	–	–	–
18.	–	–	–	–	–	–	–	–	–	–	–	–	–
Animals died during experiment	0	0	0	0	0	0	0	0	0	1	0	0	0

Experiment No.	50				46				60				61			
Substance	Peanut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 350° C for 1 hr 0.5% in peanut oil	Dibenz-a,h-anthracene 0.025% in peanut oil	Peanut oil	Cholesterol heated at 350° C for 1 hr 0.5% in peanut oil	Benzo-a-pyrene 0.5% in peanut oil	Peanut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 300° C for 1 hr 0.5% in peanut oil	Benzo-a-pyrene 1% in peanut oil	Peanut oil	Cholesterol 0.5% in peanut oil	Cholesterol heated at 300° C for 1 hr 0.5% in peanut oil	Benzo-a-pyrene 0.5% in peanut oil
Site of application	Tail				Part of tail proximal to secondary tail			Tail				Tail			Back
Number of animals (Triton crista)	8	8	8	8	8	8	8	4	4	8	8	4	4	8	8
Sex	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀	♂♀
Epidermal reaction on															
9 day	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
15.	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–
18.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Animals died during experiment	0	0	0	0	0	1	0	0	0	0	0	0	0	0*	0

* Minimal signs of the injected oil.

SUMMARY

Some heated and oxidated fats and a few allied compounds were tested in newts. Positive results of varying degree were obtained with peroxidized soyabean oil and ethyl linoleate hydroperoxide, but the carcinogen-like activity of the peroxides is considered rather weak, when the response is compared with the magnitude of peroxide values and the sensitivity of the animals. Reduction of the peroxides through heating or by chemical means weakened the activity only slightly. Epoxidized soyabean oil and 16 α, 17 α-epoxyoestratrien-3-ol induced no epithelial reaction, while the testing of dieposybutane proved positive for all isomers. Heating of cholesterol at 350° C for 1 hour gave a newt-positive product, while untreated cholesterol and moderately heated cholesterol (200° C for 1 hour) were negative. Commercially polymerized soyabean oil and soyabean oil heated at 350° C for ½ or 1 hour had no effect on the skin of newts. It is concluded that oxidation may be considered the most important process in the transformation of lipids leading to newt-positive and therefore carcinogen-suspected products.

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A SPECTROPHOTOMETRIC STUDY OF THE SPONTANEOUS AND MICROSOME-INDUCED SWELLING OF RAT-LIVER MITOCHONDRIA

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SUMMARY

The effect of microsomes on the swelling of rat liver mitochondria has been studied. A kinetic study of the changes in the optical density of the mitochondrial suspension during swelling revealed that the swelling is preceded by a lag. Addition of microsomes shortens this lag period, but otherwise the microsome-induced swelling resembles closely spontaneous swelling. The duration of the lag depends on the concentration of mitochondria, pH, temperature and osmotic pressure. The active component of the microsomes has the properties of a fatty acid. Indeed, fatty acids have been shown to produce the same effect. Some aspects of the spontaneous and the microsome-induced swelling are discussed.

INTRODUCTION

Mitochondria isolated from an isotonic sucrose homogenate swell in a hypotonic sucrose medium in accordance with the osmotic law[1]. However, there exists some doubt whether the whole mitochondrion responds to the osmotic pressure or only an inner compartment which is relatively impermeable to sucrose[2,3,4]. The almost instantaneous adjustment of mitochondria to the prevailing osmotic pressure is followed by a slower phase of swelling due to the penetration of sucrose into an internal compartment[5,6]. This so-called spontaneous swelling takes place even in an isotonic sucrose solution and is accompanied by an activation of the latent mitochondrial ATPase[7], release of mitochondrial ATP[8] and other autolytic changes. There is also a strong indication that the permeability of the inner membrane to sucrose increases during the slow phase of swelling[9].

A variety of compounds is known to accelerate the structural deterioration of mitochondria[10]. Some of them increase the rate of the spontaneous swelling[9] while others seem to act by a different mechanism[11]. Of special interest is the study of the effect on the swelling of normal cell constituents likely to contaminate the mitochondrial cell fraction. Such constituents are the microsomes which adhere tenaciously to mitochondria[12] and affect various mitochondrial activities[13]. It is shown in the present investigation that addition of microsomes accelerates markedly the swelling in isotonic sucrose. This microsome-induced swelling, like the effect of thyroxine[9], is essentially a stimulation of the spontaneous swelling.

EXPERIMENTAL

Particle preparations

Mitochondria. Rat liver and kidney mitochondria were prepared by the method of SCHNEIDER[14], as modified by SWANSON[15], rat heart sarcosomes as described previously[16], and rat brain mitochondria by the procedure of BRODY AND BAIN[17].

Microsomes were separated as described previously[16] and washed once with distilled water.

Alcohol–ether extract of microsomes

Freeze-dried microsomes (1 g) were refluxed for 2 h with 200 ml of an ethanol (3 vol.)–diethyl ether (1 vol.) mixture and filtered. The volume of the filtrate was reduced to 2 ml *in vacuo*. Ice-cold acetone (20 ml) was added and the mixture left overnight at 0°. The precipitate formed was removed by centrifuging (acetone-insoluble fraction) and the supernatant was evaporated to dryness in a stream of nitrogen. An aliquot of the dry residue was hydrolyzed with a 20 % alcoholic potassium hydroxide solution for six hours. The free fatty acids and the nonsaponifiable material were separated by the usual procedure. To assay the activity of the various fractions of the microsomal extract or of glycerides and fatty acids, a 1 % solution of the lipid was prepared in ethanol. This stock solution was diluted 100 fold with water and sonicated in a Raytheon sonic oscillator of 10 kc for 15 min on the day of the experiment.

Methods of assay

The standard assay medium for the spectrophotometric experiments contained 0.02 *M* Tris buffer of pH 7.4, 0.25 *M* sucrose, and mitochondria in an amount to give an optical density of 0.500 at 600 mμ in the Beckman spectrophotometer (Corex or Pyrex cells of 1 cm light path). For liver mitochondria, this amount is equivalent to 30 μg N/ml. The turbidity was measured against a blank containing all the components with the exception of mitochondria. As a rule, experiments were conducted at room temperature (16–18°). When the effect of temperature was investigated, the cells were kept in a water bath at the required temperature, except during the time of measurement (approx. 20 sec). Viscosity was measured in an Ostwald viscosimeter at 18°.

Chemicals

Lecithin was prepared according to HANAHAN[18]. Other chemicals were obtained from the following commercial sources: glycerides and fatty acids, BDH; crystalline ATP, Sigma; *d,l*-thyroxine, Mann Research Laboratories; crystalline bovine serum albumin, serum globulin and ovalbumin, Armour Lab., dialysed for 48 h against 0.002 *M* Tris buffer of pH 7.4; cellulose methyl-ether (methocel), Dow Company.

RESULTS

Comparison of the kinetics of the spontaneous and of the microsome-induced swelling

The kinetics of optical density changes which occurred in rat liver mitochondria, suspended in buffered 0.25 *M* sucrose, are presented in Fig. 1. It is apparent that a lag preceded the spontaneous swelling which was shortened on the addition of

microsomes. After the completion of the swelling, the final level of optical density was the same, irrespective of whether microsomes were added or not. By increasing the amount of microsomes added, the lag could be suppressed almost completely. The kinetics of swelling in isotonic KCl or NaCl were essentially the same as in sucrose, but the duration of the lag was shorter.

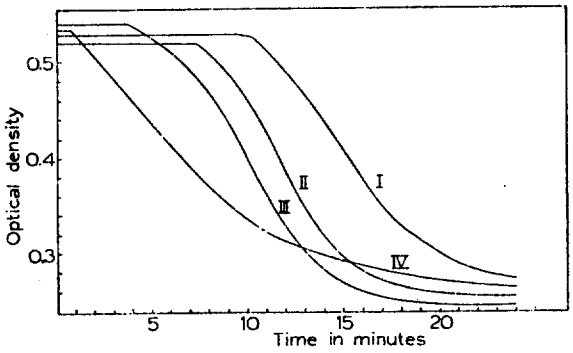

Fig. 1. Kinetics of spontaneous and microsome-induced swelling. Standard assay medium as described in EXPERIMENTAL. Temp. 18°. Curve I: spontaneous swelling; Curves II, III and IV: 10 μg, 20 μg, 200 μg (dry weight) of microsomes added/ml of medium.

The relative sensitivity of mitochondria obtained from various organs to the microsomal effect decreased in the following order: liver > kidney ≫ heart > brain. This order of sensitivities was observed also in the case of the spontaneous swelling and is similar to that reported by TAPLEY AND COOPER for thyroxine-induced swelling[20]. Microsomes prepared from kidney had about half the effect of liver microsomes when compared on the basis of dry weights.

The influence of the reaction conditions on the kinetics of the spontaneous and microsome-induced swelling

(a) *Osmotic pressure.* Under the standard conditions of the experiment, 0.25 *M* sucrose was used for the maintenance of the osmotic pressure. When the concentration of the sucrose was varied between 0.03 *M* and 0.25 *M*, only the initial optical density changed but the duration of the lag remained almost constant (Table I). In a sucrose concentration higher than 0.25 *M*, the lag became longer. The latter increase in stability is not connected with the higher viscosity of the medium, since the viscosity

TABLE I

EFFECT OF SUCROSE CONCENTRATION ON THE DURATION OF THE LAG

0.02 *M* Tris buffer, pH 7.4 and sucrose in various concentrations. 20 μg/ml (dry weight) of microsomes has been added when indicated. Temperature 18°.

Concn. of sucrose (M)	*Initial optical density*	*Duration of lag (in minutes)*	
		Spontaneous	*Microsomes added*
0.50	0.406	24.0	18.0
0.25	0.420	11.0	5.5
0.10	0.350	11.5	6.5
0.03	0.250	12.0	6.5

of the isotonic sucrose could be increased tenfold by the addition of 0.05 % methocel solution without affecting the duration of the lag phase.

(b) *Concentration of mitochondria.* The duration of the lag phase decreased markedly with dilution of the mitochondria, while the effect of the microsomes depended on the ratio of the two cell fractions (Table II). The considerable influence of the dilution suggested the loss of a protective factor. It could, indeed, be shown that when mitochondria were diluted at 0° to the same extent as under the conditions of the optical assay, centrifuged immediately in the cold, and brought to the original volume, the particles obtained showed an impaired stability. Furthermore, the lag of such "exhaustively" washed mitochondria became almost independent of concentration (Table II). Replacement of sucrose in the assay medium by the supernatant of the washed mitochondria increased by 20–40 % the lag in a standard preparation but proved to be ineffective in the case of the exhaustively washed particles. The latter results recall the observation of WERKHEISER AND BARTLEY[3] on the preserving effect of their "sucrose supernatant" on the morphological and physiological integrity of mitochondria.

TABLE II

EFFECT OF THE CONCENTRATION OF MITOCHONDRIA ON THE DURATION OF THE LAG

Standard assay medium. Temp. 18°. "Exhaustively" washed mitochondria were prepared as described in the text. 20 μg/ml (dry weight) of microsomes or 1 mM ATP or 1 mM KCN was added as indicated.

Concn. of mitochondria (μg/ml)	*Duration of lag (in minutes)*				
	Standard mitochondria		*"Exhaustively" washed mitochondria*		
	Spontaneous	*Microsomes added*	*Spontaneous*	*ATP added*	*KCN added*
10	3.5	1.0	—	—	—
20	5.0	2.0	—	—	—
40	7.5	4.5	1.5	8.0	10.0
80	14.0	12.0	—	—	—
160	20.0	—	2.0	10.0	12.0
200	29.0	—	—	—	—

pH. In the range between pH 6 to pH 8 the lag period of the spontaneous swelling as well as that of the microsome-induced swelling decreased with increasing pH. The shortening of the lag caused by the addition of microsomes became more pronounced the higher the pH was. According to HARMAN AND KITIYAKARA[21], mitochondria increase their size with increasing pH, and concomitantly their structure is loosened up. Such opening up of the structure could increase the number of sensitive sites accessible for the action of the microsomes.

(c) *Temperature.* LEHNINGER *et al.*[9] investigated the effect of temperature on the time required to reach one fourth of the maximum extent of swelling. The Q_{10} was found to be very high and showed a maximum at 20°. Since during the present investigation it became apparent that the duration of lag is a highly characteristic parameter of mitochondrial swelling, its temperature dependence was studied. The reciprocal value of the duration of the lag was taken as the overall rate. The value of 100 was assigned arbitrarily to the "rate" at 32° in the absence of microsomes

and the logarithm of the relative velocities was plotted against $1/T$ (Fig. 2). From the slope of this Arrhenius plot the apparent energy of activation was calculated by the usual method. The values obtained ranged between 19,000 and 21,000 cal/"mole" for five different batches of mitochondria. Addition of microsomes which shortened the lag period, and of ATP which stabilized the mitochondria (see below), were found to affect merely the position, but not the slope of the plot.

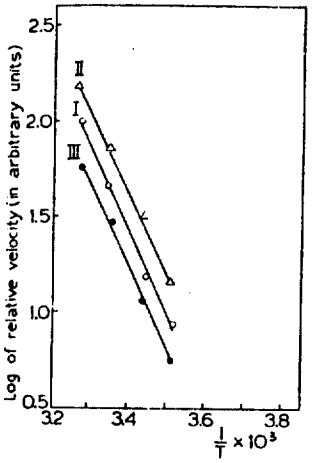

Fig. 2. Arrhenius plot for the lag phase. Curve I: spontaneous swelling; Curve II: 20 μg/ml microsomes added; Curve III: 0.33 m*M* ATP added. Standard reaction medium.

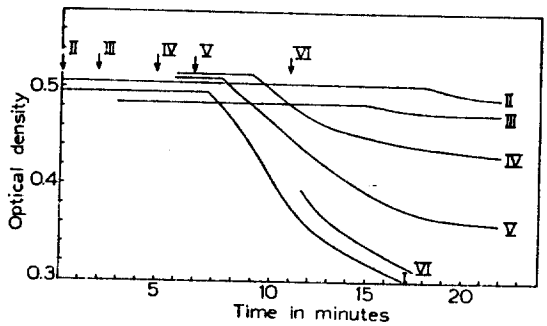

Fig. 3. Effect of the time of addition of KCN. Standard medium. Temp. 18°. Experimental points before the addition of KCN not indicated. Curve I: No KCN added; Curves II, III, IV, V, VI: 1 m*M* KCN added at the time indicated by an arrow and the number of the respective curve.

Effect of KCN and ATP on spontaneous and microsome-induced swelling

A great variety of compounds is known to confer increased stability upon the mitochondria[9,10]. Of special interest among them is KCN, which presumably affects the state of oxidation of the respiratory carriers and of ATP, which is able not only to stabilize intact mitochondria but also to reverse the swelling of mitochondria under certain experimental conditions[9,22,23]. In order to localize the phase in which the damage to the osmotic barrier occurs, the effect of the time of addition of the stabilizing agents in relation to the protection attained has been investigated. It can be seen from Fig. 3 that when the addition of KCN was made during the lag phase its effect manifested itself both in an elongation of the lag of spontaneous swelling and in a higher final level of optical density. Addition at the end of the lag or during the decline in optical density affected only the final level, but not the lag. The sooner the addition was carried out, the greater was its effect. Quite similar results were obtained when ATP was added at various phases of the swelling process. The pattern of action of the two stabilizers on the microsome-induced swelling was qualitatively the same as on the spontaneous swelling and for the sake of brevity will not be described here. The exhaustively washed mitochondria still responded to the action of KCN and ATP. 1 m*M* KCN or 1 m*M* ATP was able to increase the lag from 2 to approx. 10 min.

Effect of microsomes on "reconstituted" mitochondria

The effect of microsomes was also investigated on mitochondria which were once swollen but then contracted under the influence of added ATP. These experiments

were conducted in a 0.15 *M* KCl suspension of mitochondria since according to LEHNINGER *et al.* no reversal takes place in isotonic sucrose[9]. In accordance with the observation of LEHNINGER *et al.*[9], a partial reversal of the decline in light absorption took place when ATP was added to the test system, after the swelling had proceeded already to a considerable extent (Fig. 4). The duration of the newly established lag phase was unaffected, however, by the addition of microsomes.

In contrast to the lack of response of the reconstituted particles, the unswollen mitochondria remained sensitive to the microsomal effect even in the presence of ATP (lag in KCl medium in the presence of 1 m*M* ATP: 12 min; of ATP in conjunction with 20 μg of microsomes: 7 min). When in the experiment for the study of ATP reversal, 25 m*M* sucrose was added simultaneously with ATP, the reversal occurred to the same extent as before, but it was followed by an immediate decline in the optical density (Fig. 4).

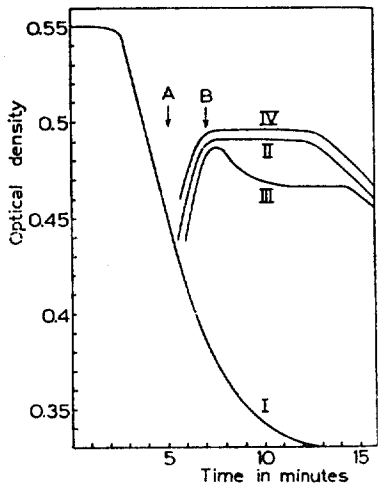

Fig. 4. Reversal of swelling by ATP (1 m*M*) and the effect of microsomes (20 μg dry weight/ml) and of sucrose (25 m*M*) upon it. Assay medium: 0.02 *M* Tris, pH 7.4, KCl, 0.15 *M*. Curve I: No addition made; Curve II: ATP added at the time indicated by arrow "A"; Curve III: ATP + sucrose added simultaneously at time "A"; Curve IV: ATP added at time "A" and microsomes at time "B".

Effect of microsomal and other lipids on swelling

The swelling-inducing activity of microsomes was found to be insensitive to heating at 100° for 15 min. Sonication increased their activity. After refluxing the microsomes with a mixture (3:1) of ethanol and ether, all the activity was found in the extract. The activity of the acetone-soluble material was greater than that of the acetone-insoluble substance. Removal of the non-saponifiable material from the acetone-soluble fraction left the largest part of the active principle behind (Table III). Since isolated microsomes are known to contain large amounts of free fatty acids[13] and since, as shown in Table III, a number of fatty acids are able to stimulate swelling to a similar extent as does the microsomal extract, it was unlikely that the microsomal activity could be connected with a single specific substance. No further fractionation was attempted therefore. It can be seen from Table III that free fatty acids are more active than glycerides. Most active are unsaturated acids, and among the saturated ones those of intermediate chain length. These results compare well with those of PRESSMAN AND LARDY on the activation of the latent mitochondrial ATPase by an acetone extract of microsomes and by free fatty acids[13]. The effects described by these authors are probably manifestations of microsome-induced deterioration of the mitochondrial structure.

Since serum albumin is known to bind free fatty acids with great affinity[21], its effect on the stimulation of swelling caused by microsomes, by the acetone–ether extract and by palmitic acid was compared. The concentration of the stimulants was chosen in such a manner that they shortened the lag by approx. 50 % (20 μg/ml of microsomes, 6 μg/ml of alcohol–ether extract, and 0.005 mM of palmitic acid). When 0.01 % of bovine serum albumin was added to the system, it increased the lag in the control experiment by 15–20 % while it suppressed the accelerating action of the three agents completely. On the other hand, 1 % of serum globulin or ovalbumin had no effect either on spontaneous or on microsome-induced swelling.

TABLE III

COMPARISON OF THE EFFECTIVENESS OF VARIOUS SUBFRACTIONS OF THE CRUDE ETHANOL–ETHER EXTRACT OF MICROSOMES (see METHODS), OF GLYCERIDES AND OF FREE FATTY ACIDS AS STIMULATORS OF SPONTANEOUS SWELLING

Standard conditions of assay. Temp. 18°.

Fraction or substance added	*Concentration causing a shortening of the lag phase by 50 %*	
	µg/ml	*mM*
Microsomes	20	
Sonicate of microsomes	10	
Crude extract	6.6	
Acetone-soluble fraction	1.8	
Acetone-insoluble fraction	6.3	
Non-saponifiable material	6.0	
Free "fatty acid" fraction	0.8	
Tristearate		0.2
Trioleate		0.1
Lecithin		0.1
Caprylic acid		0.01
Caproic acid		0.01
Lauric acid		0.003
Myristic acid		0.002
Palmitic acid		0.005
Stearic acid		0.007
Oleic acid		0.0015
Linoleic acid		0.0015

DISCUSSION

For an interpretation of the spectrophotometric data obtained in the present study in terms of an osmotic process some assumptions have to be made in accordance with recent views on spontaneous swelling. These assumptions are:

(a) the existence of an inner mitochondrial compartment, impermeable to sucrose, in the freshly isolated particles (*cf.* ref. [3–6,9]); (b) a connection between the state of oxidation of some constituents of the membrane and permeability (*cf.* ref. [9]).

There are a number of observations, such as the effect of dilution or exhaustive washing on the duration of the lag, which indicate that during the process of isolation protective substances are removed from the mitochondria. Since KCN or ATP were found to have no effect on the lag, once the swelling started, but were still effective

in the washed preparation, one must assume that the latter is only sensitized but nevertheless still possesses an intact osmotic barrier.

The magnitude of the energy of activation (19,000–21,000 cal./"mole") calculated for the hypothetical reaction which takes place during the lag phase, is compatible with a biological oxidation process dependent on consecutive enzyme reactions (*e.g.* 16,000 cal./"mole" for succinoxidase[25]). The elongation of the lag by KCN could thus be due to the inhibition of the oxidative process. ATP presumably effects the lag by a different mechanism since it is able under certain conditions *to reverse* the swelling. Most probably it exerts a direct stabilizing effect on the structure of the membrane, as suggested by RAABLAUF and co-workers[8,26]. There exists, however, an intimate relationship between the factor controlled by ATP and the one regulated by the oxidation process since the same energy of activation was found for spontaneous or microsome-induced swelling and for the swelling in the presence of ATP. Such interrelation could be visualized by assuming the existence of a *contractile principle* in the mitochondrial membrane, as it has been pointed out already by RAABLAUF[26]. The contractile unit is kept in a contracted state when sufficient ATP is present, but unfolds when this stabilizer is lost (*e.g.* by the action of surface-active agents like free fatty acids originating from microsomes). The unfolding exposes sensitive groups, which regulate the permeability, to oxidation. The change in permeability and—as a corollary—swelling can thus be avoided either by keeping the membrane in its contracted state (ATP) or by inhibiting the oxidation of the sensitive groups (anaerobiosis[27], KCN, other inhibitors of respiration[9]).

The lag is terminated by a transitory phase in which addition of KCN or ATP is unable to stop the decline in the optical density, but still has an effect on the final level of the turbidity reached. WERKHEISER AND BARTLEY[3] considered the possibility that the mitochondrial population is not homogenous osmotically. Differences might exist between one mitochondrion and another also with regard to their state of oxidation. Therefore, a part of the mitochondria might have reached the critical state of oxidation before the others and will no longer respond to the stabilizers. The non-responding fraction will be larger the later the stabilizers are added and the final optical density will be correspondingly lower.

The elucidation of the partial reversal of the decrease in the optical density in KCl suspensions of mitochondria[9] by ATP requires further experiments. The results shown in Fig. 4 indicate that the once-swollen mitochondria did not regain their former relative impermeability to sucrose. They differ also from the unswollen particles in their lack of response to microsomes.

In a hypertonic sucrose solution, the lag is considerably extended. Probably extensive shrinkage of the mitochondria reduces the number of sites which are exposed to the influence of deleterious factors. Since, according to HARMAN AND KITIYAKARA[21], mitochondria contract also in an acid medium, the increased stability at a lower pH might have a similar explanation. It seems, however, that already in 0.25 *M* sucrose all the sensitive sites are bared and therefore additional stretching of the membrane under hypotonic conditions will not reduce further the duration of lag phase.

Since no qualitative differences were found between microsome-induced and spontaneous swelling, it is not possible yet to evaluate the part played by contaminating microsomes in the so-called spontaneous swelling. The microsomal fraction

originates mainly from the cytoplasmic system of membranes and granules described as endoplasmic reticulum[28]. In view of the intimate contact which exists between the reticulum and mitochondria[28], the possibility has to be considered that the reticulum influences the permeability of the mitochondrial membrane also in the cell. The main vehicles for the microsomal effect seem to be free fatty acids which were liberated from the reticulum as a result of the action of esterases. Owing to their surface activity, fatty acids are likely to stimulate the unfolding of the contractile unit in the membrane. Fatty acids and especially those of intermediary chain-length were shown to denature ovalbumin[29]. While the largest part of the fatty acids found in the microsomes is liberated only during the procedure of isolation[13], even the much smaller amount which might occur in the intact cell could modify the mitochondrial structure.

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EXPERIMENTAL FAT EMBOLISM IN DOGS

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Multiple fractures and soft tissue trauma may be followed by mild or severe respiratory insufficiency (6, 23, 27). Fat embolism is associated with a large percentage of such cases coming to postmortem examination (8, 9, 22). Embolization of neutral fat and bone marrow from injury sites is well documented, but the exact role of embolic neutral fat in the etiology of respiratory insufficiency is not clear (6, 7, 9, 22, 24). Both a mechanical-chemical (17) and a physical-chemical (2) theory have been postulated in the pathogenesis of this condition. Other investigators have implicated a disturbed lipoprotein state with hypercoaguability and intravascular coagulation as the mechanism (13). This study was designed to observe the effects of the intravenous injections of neutral fat and free fatty acids in otherwise normal dogs.

METHODS

Thirteen healthy mongrel dogs, weighing 10 to 15 kilograms, were divided into 4 groups (Table 1). They were anesthetized with light pentobarbital anesthesia with an endotracheal tube in place. A small incision was made to isolate the femoral vein and artery for blood collection, and a central venous pressure catheter was passed via the jugular vein into the right atrium. An indwelling catheter was placed in the urinary bladder and Ringers solution was given (75-100 ml per hour) to maintain urinary flow. Triolein (1 ml/kg) or oleic acid (0.07 ml/kg) was injected intravenously. Fat embolism was the only major trauma the animals sustained.

The efficiency of ventilation was measured by: 1) lung compliance; 2) arterial pO_2, pCO_2, pH, and % saturation; 3) chest X-rays; and 4) surfactant activity. A Harvard respirator delivering 250 to 300 ml of room air per stroke, Statham pressure transducers, and a Gilson recorder were used to measure compliance. Arterial gases were measured on an Instrumentation Laboratories blood gas analysis system. Surfactant activity was determined by the previously described technique utilizing a Wilhelmy balance (12).

The blood clotting mechanism was evaluated by: 1) platelet counts; 2) plasma prothrombin times; 3) activated partial thromboplastin times; and 4) recalcified clotting times. The platelet counts were performed on the Coulter counter, uti-

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TABLE I

	NO. ANIMALS	MATERIAL INJECTED	RESPIRATOR
GROUP I	3	SALINE (CONTROL)	YES
GROUP II	2	TRIOLEIN	YES
GROUP III	5	OLEIC ACID	YES
GROUP IV	3	OLEIC ACID	NO

lizing edetic acid as the anticoagulant (4). The other studies were done on the Fibrometer for automatic timing with Simplastin* and Platelin** as reagents.

Serum lipase was measured by the modified Cherry and Crandall method (7). Plasma lactate levels were done by the modified method of Lowry (16).

Baseline studies were obtained after induction of anesthesia and fat embolism produced by the intravenous injection over a 5-minute period. All studies were repeated at 30, 60, 120, 180, and 240 minutes, and after 24 hours. The animals were given repeated small doses of pentobarbital to maintain anesthesia for 4 hours and for final induction at 24 hours. The animals were sacrificed at this time and the lungs immediately removed. One-gm samples were excised for surfactant determinations, and the remainder of the lung inflated with 10% formalin at atmospheric pressure and the bronchus ligated. The lung was then placed in 10% formalin for 7 days. Haemotoxylin and eosin and Oil Red O stains were made.

RESULTS

The control animals (Group I) showed no alteration in any of the parameters studied. The results in one animal are illustrated in Table II.

The triolein injected animals (Group II) showed no change in compliance, arterial gases, platelet counts, prothrombin times, activated partial thromboplastin times, serum lipase, or plasma lactate levels (Table II). There was mild tachypnea after 4 hours but none after 24 hours. Hypotension was not noted. Focal areas of hemorrhage were found on the gross and microscopic examination, but they were not extensive. In the hemorrhagic areas surfactant activity was reduced (Fig. 1), but it was unchanged in the normal appearing areas of the lung. Oil Red O staining showed severe alveolar capillary fat embolism present (Fig. 2).

Animals injected with oleic acid and maintained on room air with a volume respirator (Group III) developed no change in arterial pH, but there was a mild ventilator induced hyperventilation with a fall in the pCO_2 (Table II). A prompt fall in pO_2, compliance, and diminished surfactant activity was noted

* Simplastin, Warner-Chilcott.

** Platelin, Warner-Chilcott.

TABLE II

Representative Values of a Single Animal in Each Group

	pH	pO_2 (mm Hg)	% Sat.	pCO_2 (mm Hg)	Platelets (no/cu ml)	Pro-time (Seconds)	P.T.T. (Seconds)	Recalcified Clotting Time (Seconds)	Lipase (Units)	Lactate mM	Compliance L/cm	Hct.
Group I												
Baseline	7.39	87	95	35		8.4	13.5		0.14	1.71		39
30 min	7.41	86	95	32	191,000	8.4	14.2					39
60 min	7.40	96	97	33	226,000	8.0	14.0					36
120 min	7.41	103	97	32	231,000	7.9	14.7					37
180 min	7.41	105	97	33	226,000	8.4	14.7					38
240 min	7.44	112	97	29	230,000	9.4	16.0		0.22	1.12	0.030	39
24 hours	7.42	90	98	31		9.0	14.6			1.60		34
Group II												
Baseline	7.43	85	95	34	288,000	8.4	15.0		0.45	1.29	0.025	39
30 min	7.41	90	95	29	290,000	8.9	15.9				0.021	39
60 min	7.46	93	96	28	272,000	7.9	13.4				0.023	37
120 min	7.53	97	97	25	278,000	7.9	15.4				0.022	40
180 min	7.51	92	96	27	252,000	8.4	15.9				0.022	40
240 min	7.51	85	96	28	202,000	8.4	16.4				0.022	47
24 hours	7.54	75	94	25	201,000	8.6	16.4		0.17	1.46	0.020	43
Group III												
Baseline	7.50	82	97	27	283,000	8.9	21.0		0.09	1.36	0.025	30
30 min	7.40	80	95	28	191,000	8.2	21.0				0.023	27
60 min	7.45	72	93	29	234,000	8.5	19.4				0.019	25
120 min	7.44	53	86	30	234,000	8.2	19.4				0.016	31
180 min	7.46	51	85	28	197,000	8.2	21.0				0.019	34
240 min	7.42	56	88	27	176,000	8.4	21.0		0.26	2.50	0.016	37
24 hours												
Group IV												
Baseline	7.34	82	97	41	233,000	9.0	14.9	56	0.10	1.22	0.017	42
30 min	7.24	50	81	51	135,000	8.4	13.7	56				44
60 min	7.24	55	87	43	109,000	8.6	14.4	74			0.012	44
120 min	7.35	66	95	38	142,000	8.7	15.6	74				46
180 min	7.35	57	91	37	144,000	8.4	13.0	55				46
240 min	7.40	69	97	26	142,000	8.4	13.0	55		0.98	0.010	48
24 hours	7.36	58	92	33	144,000	8.0	15.4	50		1.13	0.012	41

(Fig. 1). Chest X-rays after injection showed the development of patchy infiltrates in all lung fields, especially in the lower lobes (Fig. 3). The platelet counts fell sharply, but spontaneous bleeding did not occur. Serum lipase did not change, but a rise in plasma lactate was noted in some animals. Hypotension did not occur.

The animals injected with oleic acid and not placed on a respirator (Group IV) immediately developed tachypnea of 50 to 100 respirations per minute. The other findings were similar to Group III, except that acidosis was more prominent (Table II).

The pathological examination of all oleic acid injected animals revealed grossly hemorrhagic lungs that were two to three times as heavy as the control lungs.

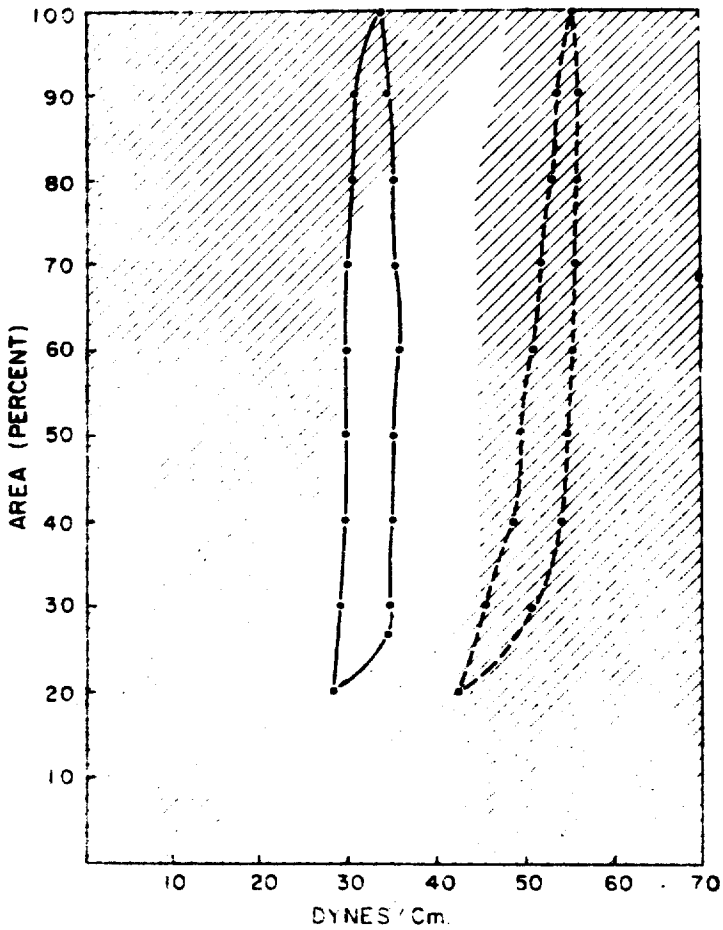

FIG. 1. Surfactant determinations. Hysteresis loops typical of various groups. Relative area of the surface (ordinate) is plotted against the surface tension (abcissa). Control loop -----shaded background; Triolein loop ·····; Oleic acid loop ·-·-·-·-·

Occasional proteinaceous exudates in the alveoli and decreased cellularity of the alveolar septa were noted (Fig. 4). No neutral fat was found by Oil Red O staining.

DISCUSSION

Our animals were not severely affected by the triolein embolization. This was most probably due to the fact that the animals were all well hydrated and not in shock. Under such circumstances the severe mechanical obstruction of the pulmonary bed is well tolerated.

Recent work has confirmed the hypothesis that such emboli of neutral fat are broken down by hydrolysis to release free fatty acids (1, 10, 11, 19). The effects of the intravenous injection of oleic acid were much more dramatic, and

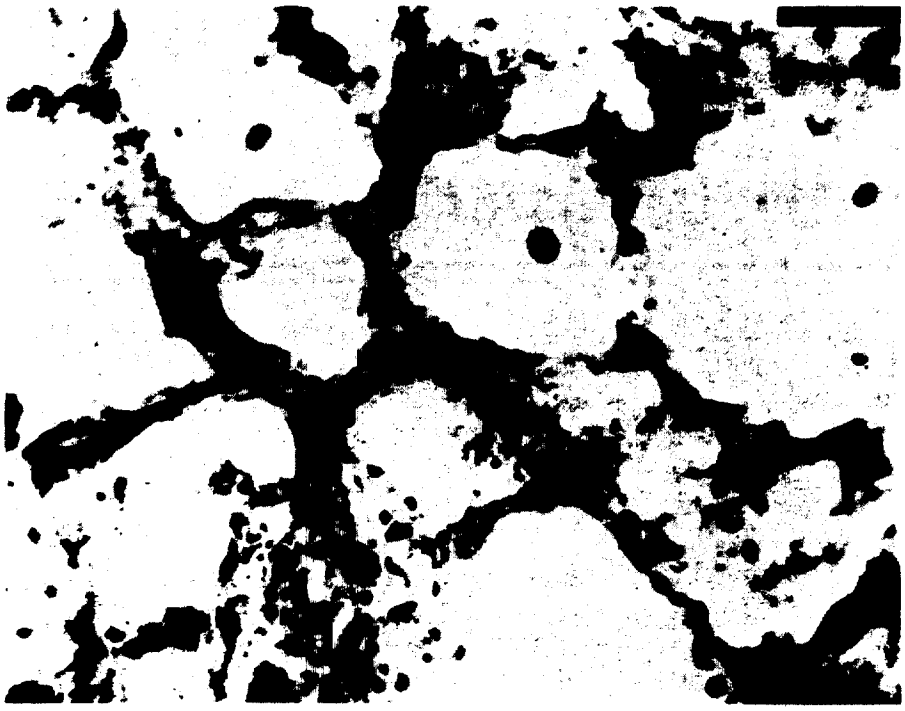

FIG. 2. Triolein embolism. Dark globular areas are droplets of embolic triolein in the alveoli of the lung. Note severe congestion of alveoli. ×200.

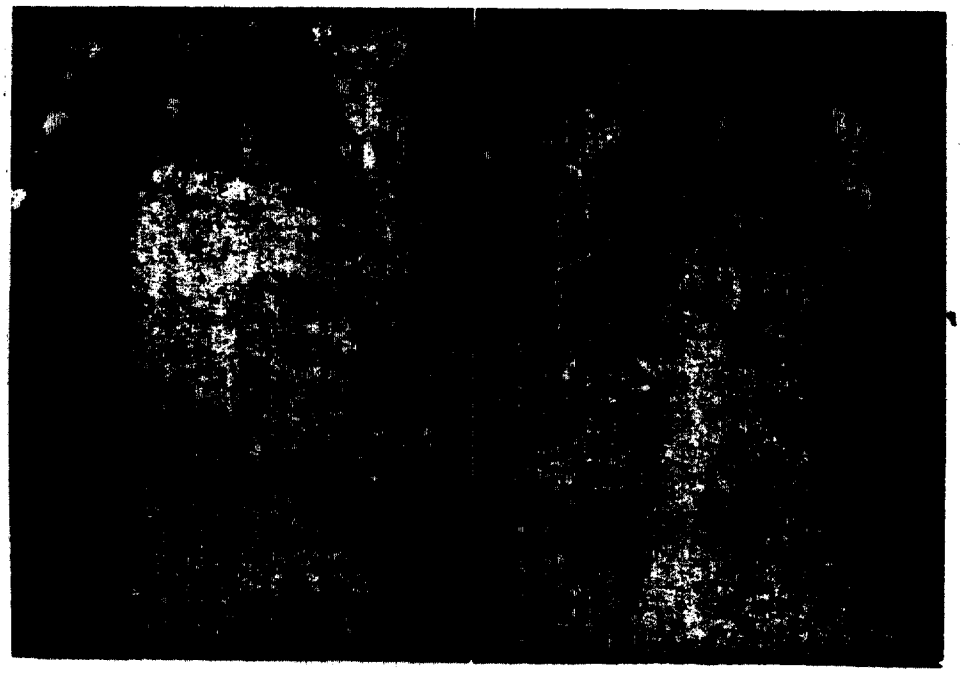

FIG. 3. Oleic acid injection.
Left - Before oleic acid injection. Right - Twenty-four hours after oleic acid injection.

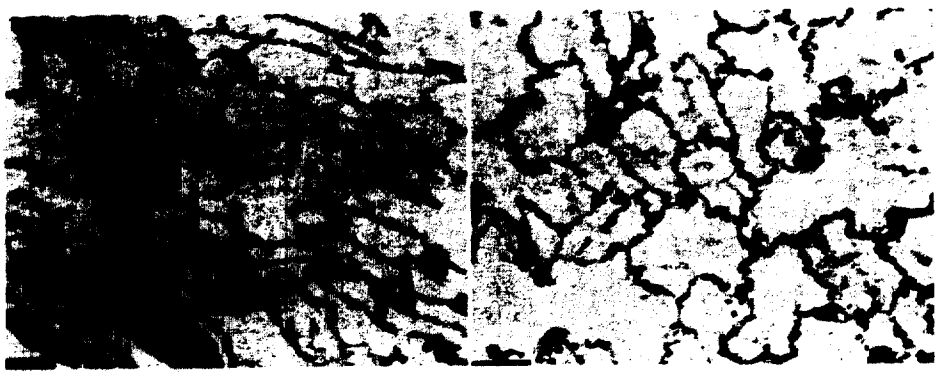

FIG. 4. Oleic acid injection. Left—Relatively normal appearing microscopic examination with severe clinical involvement. Right—Other areas of lung showing alveolar exudates. ×220.

more closely resembled the classical picture of fat embolism as it is observed in man. Oleic acid embolism produces severe respiratory distress accompanied by a decreased compliance and a falling pO_2. Lung surfactant activity is promptly decreased as a result of the local effect of the fatty acids on the surfactant, rather than due to the effect of anoxia on the cells producing surfactant (12, 15). Plasma lactate levels varied considerably in our studies, and did not correlate well with arterial oxygen levels, in contrast to the published work in endotoxic and hemorrhagic shock, where there is close correlation between oxygen deficit and blood lactate levels (21).

The pathological examination of the triolein injected animals revealed extensive capillary occlusion without severe hemorrhage, consolidation, or congestion. The severe hemorrhage, consolidation, and congestion found in the oleic acid injected animals is similar to that found in the "shock lung" (14). Pulmonary hypoperfusion reported by others (5) could explain the fall in arterial pO_2, surfactant, and compliance. The continuation of this process produces exudates in the alveoli similar to those described in hyaline membrane disease (17).

Of particular interest were the clotting studies, which failed to demonstrate disseminated intravascular coagulation. Indeed, only the platelet counts were affected. We have demonstrated a striking increase in platelet adhesiveness to glass beads coated with a thin layer of triolein or oleic acid (20). Thrombocytopenia is regularly observed in patients with clinical fat embolism (27). The collection of large numbers of platelets adherent to fat droplets in the lung, with subsequent breakdown to release platelet amines, could account for the immediate increase in lung compliance on the basis of bronchospasm (26). This is accompanied by perivascular edema following disruption of the capillary endothelium from the local effect of the acids themselves also decreasing compliance (25).

The toxicity of embolic oleic acid in the lungs appears to depend upon its own

chemical properties. The presence of abnormal amounts of free fatty acids in a congested, poorly perfused pulmonary capillary bed results in endothelial damage, platelet aggregation, damage to lung surfactant, and increased capillary permeability with resulting decreased lung compliance and arterial oxygenation. Such a configuration, i.e., varying degrees of hypoperfusion and varying degrees of elevated free fatty acids, may represent the basic pathogenesis in "shock lung," postperfusion lung, and the respiratory distress syndrome, as well as in fat embolism.

SUMMARY

1. The effect of intravenous triolein in normotensive and normovolemic animals is minimal.

2. In contrast, oleic acid injection in such animals results in an immediate drop in arterial pO_2, compliance, and surfactant activity.

3. The only defect in the coagulation mechanism was a thrombocytopenia.

4. The significance of these findings is discussed.

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DISCUSSION

DR. DAVID S. BRADFORD (New York, New York): Dr. Baker and co-workers have provided an interesting model for the production of fat embolism and have shown nicely a definite thrombocytopenia with pulmonary insufficiency. I do not feel, however, that the coagulation studies used rule out intravascular clotting.

Saturated fatty acids are known to activate Factors XI and XII, and unsaturated fatty acids such as oleic may exhibit a thromboplastic effect and cause platelet and red blood cell aggregation, the degree of protein binding being an important consideration.

Yet one may not assume that this model is necessarily applicable to the pathogenesis of fat embolism in man. Certainly from the work of Bergentz, Adkins, and others, a similar picture occurs after a variety of injections, particularly thromboplastin and thrombin. The lipid we see in the lungs at autopsy may come in part from platelet degeneration, cell breakdown, and intravascular clotting. Thus, I am not sure that the production of this syndrome by oleic acid implies more than the ability of the lung to respond in a similar fashion to a variety of insults.

In the fat embolism syndrome, we have felt that intravascular clotting does occur, and are therefore studying the coagulation parameters in these patients with the aid of Dr. Davidson and Dr. Nossel.

(Slide). This is one such patient admitted to our service with multiple fractures and major soft tissue trauma. Shortly after admission you will notice that her arterial oxygen saturation was normal; yet fibrinogen was decreased to 150 mgm%, Factor V to 40%, platelets to 150,000 cm³. Cryofibrinogen was present in the plasma. Over the next 48 hours she became agitated and confused. Her

platelet count had fallen to 33,000, oxygen saturation to 50%, and her chest X-ray showed scattered hazy infiltrates. The next day, with the appearance of petechiae, fibrinogen-split products were found to be elevated to 20 μg/ml, and the fibrinogen was showing a rebound phenomenon to over 700 mgm%. These findings—a decrease in Factor V, thrombocytopenia, the presence of cryofibrinogen, and elevation of split products—suggest to us that intravascular clotting was occurring concomitant with respiratory insufficiency. You will notice that the PT and PTT were normal in the early stages. Two weeks later the patient's course deteriorated with the development of gram-negative sepsis. Split products again became elevated and platelets depressed, as one might expect.

We are seeing a similar pattern emerging in patients with this syndrome who have not been in shock or received massive transfusions. If lipid is truly the culprit, we wonder if its excessive mobilization after injury serves in part along with tissue thromboplastin to activate intrinsic clotting as well as promote RE blockade and overload the alveolar wall cell.

In closing, I would like to ask the authors: did they identify intravascular fibrin deposits, and do they feel that their results would have been altered by using fatty acid bound to protein?

Dr. Tamotoco Shinozaki (Burlington, Vermont): I would like to say that we are doing the same types of experiments in connection with various animals. We are interested in the physiological progress. We subjected the animal to a very large dose of olive oil, and found that the animal usually died in 8 hours.

We also tried oxygen to avoid the pulmonary complications, but this did not bring success.

Our results seem to indicate that pulmonary fat embolism is due to pulmonary complications as in the other cases.

Dr. Samuel R. Powers (Albany, New York): This is a beautiful study, which seems to have demonstrated in a convincing manner that circulating free fatty acids produce lesions in the lung which appear to be similar to those found in patients with fat emboli.

My first question is to wonder if comparable levels of free fatty acid have been found in patients with fat embolization. Methods for the accurate determination of free fatty acids are now available in most laboratories, and it would be most interesting to know if the levels necessary to produce the lesion shown were also found in clinical cases.

My second question is concerned with the abnormalities of pulmonary function which were demonstrated. The presence of arterial oxygen unsaturation which was not increased with 100% oxygen is compatible with the so-called "shock lung." Since this finding is, however, quite non-specific, I wonder if any other studies of pulmonary function, such as compliance, were carried out.

Dr. Antonio A. Garzon (Brooklyn, New York): I want to congratulate the authors on a very interesting study. I would like to know the lung compliance values. I don't believe they were mentioned during the presentation. I am also interested in the method followed for compliance determination, and what

precautions were taken to avoid the alveolar collapse commonly seen in dogs under general anaesthesia.

DR. PHILLIP L. BAKER (closing): With regard to the comments of Dr. Bradford, let me say that we have recently done the same type of work in patients. This work is described in Dr. Peltier's article in the September 1968 issue of The Journal of Trauma.

As you know, free fatty acids are bound to proteins, and their relationship in all of this is not well understood from our point of view. Therefore, this is something that we will have to continue working on.

We also feel that these are primarily pulmonary and not cardiac abnormalities.

As to methods of compliance and methods of controlling the animal, we placed the control animal and maintained it indefinitely, at least in connection with our study, on a respirator. I used the method developed at the University of Minnesota, where they increase their levels of volume throughout a period of time.

Thank you very much.

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THE RATE OF ABSORPTION OF DIFFERENT FATS AND OILS.

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In evaluating the importance of natural oils and fats for edible purposes, two points of fundamental importance are their digestibility coefficients and rates of absorption: the first indicates the extent to which they are absorbed in the system and the second determines the amount of a particular fat or oil that is absorbed in a definite time. The latter point should be determined specially because a fat or oil which is absorbed to a maximum amount in the least time should always be preferred. The first problem has been dealt with by some investigators (Langworthy, 1923; Langworthy and Holmes, 1915; Deuel and Holmes, 1921) and with few exceptions, fats having a low melting point have been found to be almost completely absorbed from the gastro-intestinal tract of normal men. This has also been confirmed by the present authors (Basu and Nath, 1946), the fats and oils used in the experiments being those which are commonly used in different parts of India for edible purposes.

On the contrary, very few studies have been carried out on the rate of absorption of the different fats and oils especially of those which are commonly used in India. Steenbock, Irwin and Weber (1936) employing a technique described by Irwin, Steenbock and Templin (1936), which is merely a modification of the technique used by Cori (1925) for determining the rate of absorption of sugars, found the extent of absorption of a number of fats over a 6-hour period. Deuel, Hallman and Leonard (1940) determined the rates of absorption of another batch of fats and oils differing in chemical composition, which are commonly used in America.

The present paper deals with the study of the comparative rates of absorption of several fats and oils that are used in different parts of India, namely, mustard, coconut, sesame and ground-nut oils, and cow butter-fat.

Experimental.

The technique was practically the same as that of previous workers mentioned above, with some modifications at various points. The fats were fed by stomach-tubes to rats, weighing from 225 g. to 250 g. and fasted previously for 48 hours. During this period of fasting, water was given *ad libitum*, but the rats lost 8 to 10 per cent of their body-weight. The stomach-tube was inserted by holding the rat by the neck and placing a forceps between the teeth. In earlier experiments light anæsthesia was employed. A glass receptacle was fitted to the end of the stomach-tube. The melted fat was poured into it from a pipette and was then introduced into the stomach by means of a gentle push with a hand-pump fitted at the end of the glass receptacle.

The small amount of oil remaining on the receptacle and stomach-tube was recovered in each case and duly allowed for in calculating the exact quantity introduced into the stomach.

Amounts of fat absorbed after periods of 2, 4 and 6 hours were determined. At the end of the definite interval the rats were killed, the intact gastro-intestinal tract was removed and the fats remaining unabsorbed were determined by water and petroleum-ether extraction according to the procedure of Irwin, Steenbock and Templin (*loc. cit.*).

A set of preliminary experiments was also performed to determine the extent of recovery of fat immediately after it has been introduced by the stomach-tube. The results are

indicated in Table I. It has been found that the mean percentage of recovery lies between 96 and 99 per cent and often it is 100 per cent. No correction is, therefore, necessary for any amount of fat which might escape extraction from the gastro-intestinal tract.

It has been found by Irwin, Steenbock and Templin (*loc. cit.*) that sex plays no important rôle in the rate of absorption and nearly identical results were obtained by using either male or female rats. For this reason rats of both sexes have been used. It has also been found by the same workers that rats of different sizes exhibit different rates of absorption with the same oil and for the same time interval, i.e. the rate of absorption depends on the surface area of the stomach, intestine, etc., and so in all the experiments rats were chosen which were nearly of the same weight. The amount of fat remaining in the gastro-intestinal tract after a fast of 48 hours was also found to be insignificant.

Tables II to VII give the rate of absorption of the various oils and fats. Table VIII summarizes the rates of absorption of all fats investigated. Table IX shows the effect of the amount of fat administered on the rate of absorption of coconut oil for a period of 2 hours.

TABLE I.

Percentage of recovery of fat immediately after administration.

Weight of coconut oil administered, g.	Amount in the tubes, etc., g.	Total amount administered, g.	Amount recovered, g.	Percentage of recovery.	Mean percentage of recovery.
0·7868	0·0953	0·6915	0·6645	96·1	97·46
	0·0753	0·7115	0·6987	98·2	
	0·0819	0·7049	0·6873	97·5	
	0·1059	0·6809	0·6734	98·9	
	0·0982	0·6885	0·6651	96·6	

TABLE II.

Rate of absorption of mustard oil.

Time (hours).	Weight of oil taken (2 c.c.), g.	Amount remaining in the tube, g.	Exact amount administered, g.	Amount recovered, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1·6836	0·1534	1·5302	1·1264	0·4038	26·4	27·6±0·871
		0·1253	1·5583	1·1593	0·3990	25·6	
		0·1628	1·5208	1·0539	0·4669	30·7	
4	1·6836	0·1593	1·5243	0·6280	0·8963	58·8	56·3±0·752
		0·1432	1·5404	0·7070	0·8334	54·1	
		0·1523	1·5313	0·6738	0·8575	56	
6	1·6836	0·1234	1·5602	0·5570	1·0032	64·3	64 ±0·887
		0·1038	1·5798	0·6130	0·9668	61·2	
		0.1534	1·5302	0·5126	1·0176	66.5	

Table III.

Rate of absorption of coconut oil.

Time (hours).	Weight of oil taken (2 c.c.), g.	Amount remaining in the tube, g.	Exact amount administered, g.	Amount recovered, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1·7754	0·0987	1·4767	0·9997	0·4770	32·3	36·[illegible] ± 1·06[illegible]
		0·1076	1·4678	0·9262	0·5416	36·9	
		0·1030	1·4718	0·9007	0·5711	38·8	
4	1·5754	0·0943	1·4811	0·8156	0·6655	44·8	42·7 ± 0·74
		0·1237	1·4517	0·8681	0·5836	40·2	
		0·1053	1·4701	0·8365	0·6336	43·1	
6	1·5754	0·0878	1·4881	0·6920	0·7961	53·5	49·8 ± 1·17[illegible]
		0·0763	1·4991	0·7690	0·7301	48·7	
		0·0857	1·4897	0·8000	0·6897	46·3	

Table IV.

Rate of absorption of olive oil.

Time (hours).	Weight of oil taken (2 c.c.), g.	Amount recovered from stomach-tube, etc., g.	Exact amount administered, g.	Amount extracted from stomach and intestine, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1·6152	0·0856	1·5296	1·0285	0·5011	39·3	41·1 ± 0·478
		0·0793	1·5359	0·8724	0·6635	43·2	
		0·0823	1·5329	0·9075	0·6254	40·8	
4	1·6152	0·0921	1·5231	0·8164	0·7067	46·4	48·9 ± 0·719
		0·0758	1·5394	0·7605	0·7789	50·6	
		0·0843	1·5309	0·7700	0·7609	49·7	
6	1·6152	0·1053	1·5099	0·5874	0·9225	61·1	59·8 ± 0·857
		0·1042	1·5110	0·5802	0·9308	61·6	
		0·0970	1·5182	0·6566	0·8616	56·7	

Table V.

Rate of absorption of ground-nut oil.

Time (hours).	Weight of oil taken (2 c.c.), g.	Amount recovered from stomach-tube, etc., g.	Exact amount administered, g.	Amount extracted from stomach and intestine, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1·6992	0·1123	1·5869	1·0236	0·5633	35·5	35·13±0·531
		0·0913	1·6079	1·0725	0·5354	33·3	
		0·1023	1·5969	1·0124	0·5845	36·6	
4	1·6992	0·0856	1·6136	0·7987	0·8149	50·5	52 ±0·428
		0·0972	1·6020	0·7626	0·8394	52·4	
		0·1123	1·5869	0·7443	0·8426	53·1	
6	1·6992	0·0957	1·6035	0·6398	0·9637	60·1	59·9 ±0·480
		0·0823	1·6169	0·6257	0·9912	61·3	
		0·0834	1·6158	0·6636	0·9522	58·3	

Table VI.

Rate of absorption of sesame oil.

Time (hours).	Weight of oil taken (2 c.c.), g.	Amount recovered from stomach-tube, etc., g.	Exact amount administered, g.	Amount extracted from stomach and intestine, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1·7039	0·0750	1·6289	1·0412	0·5877	36·1	36·2±0·335
		0·0829	1·6210	1·0164	0·6046	37·3	
		0·0836	1·6203	1·0500	0·5703	35·2	
4	1·7039	0·0953	1·6086	0·8815	0·7271	45·2	46·1±0·273
		0·0875	1·6164	0·8583	0·7581	46·9	
		0·0913	1·6126	0·8340	0·7786	46·3	
6	1·7039	0·0859	1·6180	0·6909	0·9271	57·3	57·3±0·[illegible]
		0·1031	1·6008	0·6931	0·9077	56·7	
		0·0989	1·6050	0·6757	0·9293	57·9	

TABLE VII.

Rate of absorption of cow butter-fat.

Time (hours).	Amount administered (2 c.c.), g.	Amount recovered from gastro-intestinal tract, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1·3084	0·7988	0·5096	39·0	40·8 ±0·510
		0·7538	0·5546	42·0	
		0·7654	0·5430	41·5	
4	1·3084	0·7362	0·5722	43·4	47·97±1·390
		0·6288	0·6796	52·1	
		0·6742	0·6342	48·4	
6	1·3084	0·5306	0·7778	59·4	59·13±0·735
		0·5098	0·7986	61·3	
		0·5688	0·7396	56·7	

TABLE VIII.

Percentage of different oils absorbed after 2, 4 and 6 hours.

Type of oils.	PERCENTAGE OF DIFFERENT OILS ABSORBED AFTER		
	2 hours.	4 hours.	6 hours.
Mustard oil ...	27·6 ±0·871	56·3 ±0·752	64·0 ±0·867
Coconut oil ...	36·0 ±1·063	42·7 ±0·740	49·8 ±1·171
Olive oil	41·1 ±0·478	48·9 ±0·719	59·8 ±0·858
Ground-nut oil ...	35·13±0·534	52·0 ±0·428	59·9 ±0·480
Sesame oil ...	36·2 ±0·335	46·1 ±0·273	57·3 ±0·191
Cow butter-fat ...	40·8 ±0·511	47·97±1·390	59·13±0·735

TABLE IX.

Effect of amount of oil administered on the rate of absorption.

(Oil taken—coconut.)

Amount administered, g.	Amount recovered from stomach-tube, g.	Exact amount administered, g.	Amount extracted from stomach and intestine, etc., g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
0·7877	0·0856	0·7021	0·4739	0·2282	32·5	32·3±0·355
	0·1050	0·6827	0·4704	0·2123	31·1	
	0·0987	0·6890	0·4593	0·2297	33·3	
1·5754	0·0987	1·4767	0·9997	0·4770	32·3	36 ±0·106
	0·1076	1·4678	0·9262	0·5416	36·9	
	0·1036	1·4718	0·9007	0·5711	38·8	
2·3631	0·0973	2·2658	1·3480	0·9178	38·3	38·8±0·159
	0·0759	2·2872	1·3823	0·9049	39·3	
	0·1021	2·2610	1·3737	0·8873	38·8	

ANALYSIS OF THE DATA.

The probable error of the mean results is indicated in the tables. Analysis of the mean values obtained 2 hours after administration reveals that the difference of $13{\cdot}2 \pm 1{\cdot}01$ between the absorption of cow butter-fat and mustard oil is clearly significant. The difference of $8{\cdot}4 \pm 1{\cdot}371$ between the absorption of coconut and mustard oils as also the difference of $4{\cdot}8 \pm 1{\cdot}175$ between cow butter-fat and coconut oil are significant.

Four hours after administration the difference of $4{\cdot}3 \pm 0{\cdot}86$ between the absorption of mustard and ground-nut oils is significant. The difference of $9{\cdot}2 \pm 1{\cdot}27$ between coconut and ground-nut oils 6 hours after administration is significant being 7·24 times its probable error.

RESULTS AND DISCUSSION.

From the results it is clear that there are appreciable differences between the rates of absorption of the various fats and oils, especially when we consider the results of absorption in the first few hours. It is seen that 2 hours after administration, olive oil and cow butter-fat are the most rapidly absorbed, while mustard oil is the least absorbed. This is to be expected since the former varieties contain a larger percentage of short-chain fatty acids and as these are somewhat water-soluble, they are likely to be absorbed more rapidly than the long-chain fatty acids. The results, however, become striking 4 hours after the fat is administered when it is seen that mustard oil is absorbed to the greatest extent. The difference between the rates of absorption of olive, sesame and ground-nut oils and cow butter-fat is not marked at this time and at the 6-hour period they are practically identical except in the case of coconut oil which shows less absorption than the others. The results of our previous investigation (Basu and Nath, *loc. cit.*) have shown that, during the 24-hour period, the absorption of all the natural fats and oils is almost complete and it is probably from the results at the 6-hour period that the percentage of absorption of all the fats and oils except coconut oil tends to become equal.

In contrast to the findings of Deuel, Hallman and Leonard (*loc. cit.*) and also of Steenbock, Irwin and Weber (*loc. cit.*) it has been found that the rate of absorption of oil (coconut oil in our case) increases slightly with an increase in the amount of oil administered (*see* Table IX). With increased amounts of the oil fed the absolute amount absorbed, however, increased in all their experiments. The comparisons of the above-mentioned authors were made 3 hours after the administration of the oil, i.e. when the absorption was well on its way, while in our experiments comparisons were made 2 hours after administration. The effect of concentration may possibly change after this interval.

Summary.

The rates of absorption of mustard, coconut, olive, ground-nut and sesame oils and of butter-fat have been compared using rats (225 g. to 250 g. weight) as test animals. The fats were fed by the stomach-tube and at the end of definite intervals (2, 4 or 6 hours) the rats were killed, the intact gastro-intestinal tract was removed and the fats remaining unabsorbed were determined.

Two hours after administration olive oil and butter-fat were found to be the most rapidly absorbed fats. After 4 hours mustard oil is absorbed to the greatest extent and the others show almost equal rates of absorption. After 6 hours the fats are all almost equally absorbed, the absorption of coconut oil being slightly less than that of the others.

With an increase in the amount of coconut oil administered the rate of absorption of the oil was found to be slightly increased.

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THE EFFECT OF DIFFERENT FATS ON CALCIUM UTILIZATION IN HUMAN BEINGS.

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THE problem of the influence of dietary fat on calcium utilization has received considerable attention with somewhat conflicting results. Nearly all investigators have, however, worked with rats and have shown that the addition of fat to a fat-free diet has often a favourable influence on calcium utilization. Variations in experimental conditions and dietary régime have, however, led to diverse conclusions. Knudson and Floody (1940) and Jones (1940) furnished clear-cut evidence that moderate levels of fat in the diet favoured the utilization of calcium. Earlier investigators in this field, viz. Cronheim and Müller (1908), Bahrdt (1910), Rothberg (1907) and Birk (1907), found that as the amount of fat in the diet increased above a moderate level, calcium soaps in the stool also increased. Bosworth, Bowditch and Giblin (1918, 1918*a*) concluded that the presence of soluble ionized calcium in the intestine determined the extent of soap formation, while loss in the faeces depended not only upon the amount of calcium soaps formed but also upon their nature, calcium oleate being much more soluble in the normal intestinal fluids than was calcium palmitate or calcium stearate. It was shown by Givens (1917) that in the case of low fatty acid utilization, e.g. with ethyl palmitate and palmitic acid, the loss of calcium was increased.

Some recent publications also point to the same results. Jones (1942) and French (1942) have independently proved that in rats dietary fat increases the acidity of the intestinal contents, in particular in the jejunum and lower ileum, and that this change in acidity is responsible for increased absorption of calcium and phosphorus. Booth, Henry and Kon (1942) have found that on a high calcium, low phosphorus diet calcification of bones is definitely aided by the presence of fat in the diet. The effect is not evident with a low calcium, high phosphorus diet.

Telfer (1923, 1930) has outlined the mechanism as follows: Free fatty acids dissolve calcium phosphate with the formation of calcium soaps which are then absorbed. He has also pointed out that if fats and phosphates are both low, calcium carbonate may be excreted in an alkaline stool. The form in which calcium is eliminated from the intestinal tract is therefore, associated with the pH of the intestinal contents as a consequence of the effect of acidity upon solubility.

It was, however, indicated by the work of Boyd, Crum and Lyman (1932) that all the calcium soaps are not absorbed equally. The three soaps investigated by them were calcium oleate, calcium palmitate and calcium stearate. The first showed the highest percentage of absorption and the last-named the least.

When fats and oils are taken, they are mostly resolved, by the action of fat-splitting enzymes, into a mixture of fatty acids in the intestine. Each fat has a definite composition peculiar to itself so far as its fatty acid make-up is concerned. As already pointed out some of these fatty acids then form salts with calcium. The work of Boyd (*loc. cit.*) has definitely shown that the percentage of utilization of different calcium soaps is not equal. This being the case, it may be possible that a difference in the nature of the calcium soaps formed out of the fatty acids of different fats and oils may result in a difference in the amount of absorption of calcium.

The purpose of the present investigation was to find out whether there was any relation between the nature of the oil fed and the total amount of calcium absorbed. An attempt was also made to find whether any relation existed between the nature of the oil fed and phosphorus metabolism.

Experimental.

The experimental procedure was the same as that reported in previous communications by Basu and his co-workers (Basu and Basak, 1939; Basu, Basak and De. 1941). An experimental diet of the composition rice 600 g., pulses 70 g., fish 60 g. and vegetables 200 g. together with 60 g. of the oils was consumed by healthy male subjects for 7 days. The first 3 days were observed as a preliminary period and the urine and fæces of each subsequent period of 4 days were collected and analysed and the mean of daily analytical figure is represented. The analytical methods for the estimation of calcium and phosphorus were the same as employed in the previous investigations of Basu *et al.* (1939, 1941).

Tables I to VI show the metabolism data of calcium and phosphorus with different oils as dietary fat with different subjects :—

Table I.

Calcium metabolism with different fats.

Subject : *G. C. D., 24 years, weight 60 kg.*

Diet.	Food Ca (mg.).	Urinary Ca (mg).	Faecal Ca (mg.).	Total Ca excretion (mg.).	Balance (mg.).
Basal and fat-free	253	52·6	193·7	246·3	6·7
Basal and mustard oil ...	262	43·1	123·5	163·6	98·4
Basal and coconut oil ...	247·6	47·2	208·3	255·5	—7·9
Basal and ground-nut oil ...	259·7	35·6	182·1	217·7	42
Basal and sesame oil ...	243·2	32·4	182·7	215·1	28·1
Basal and cow butter-fat ...	241·7	36·9	176·6	212·5	39·2

Table II.

Calcium metabolism with different fats.

Subject : *A. C. D., 18 years, weight 50 kg.*

Diet.	Food Ca (mg.).	Urinary Ca (mg.).	Faecal Ca (mg.).	Total Ca excretion (mg.).	Balance (mg.).
Basal and fat-free	205·5	38·7	180·6	219·3	—13·8
Basal and mustard oil ...	209·5	34·5	132	163·5	46
Basal and coconut oil ...	217·2	78·1	253·8	328·9	—111·7
Basal and ground-nut oil ...	206	33·7	169·8	203·5	2·5
Basal and sesame oil ...	221	26·5	161·2	187·7	33·3
Basal and cow butter-fat ...	213·6	34	182·7	216·7	—3·1

TABLE III.

Calcium metabolism with different fats.

SUBJECT: *I. B. G.*, 19 *years, weight* 53 *kg.*

Diet.	Food Ca (mg.).	Urinary Ca (mg.).	Faecal Ca (mg.).	Total Ca excretion (mg.).	Balance (mg.).
Basal and fat-free	177	42·9	133·9	176·8	0·2
Basal and mustard oil ...	187	34·7	65·2	99·9	87·1
Basal and coconut oil ...	180·8	75	228	303	—122·2
Basal and sesame oil ...	180·8	36·6	115·7	152·3	28·5
Basal and ground-nut oil ...	186	25·3	149·6	174·9	11·1
Basal and cow butter-fat ...	193·6	31·3	123·7	155	38·6

TABLE IV.

Calcium metabolism with different fats.

SUBJECT: *S. C. D.*, 19 *years, weight* 52 *kg.*

Diet.	Dietary Ca* (mg.).	Urinary Ca (mg.).	Faecal Ca (mg.).	Total Ca excretion (mg.).	Balance (mg.).
Basal and fat-free	485	165·8	351·3	517·1	—32·1
Basal and mustard oil ...	487·3	62·5	330·6	393·1	94·2
Basal and coconut oil ...	482·7	88·4	508·2	596·6	—113·9
Basal and ground-nut oil ...	503	79	312	391	112
Basal and sesame oil ...	492·5	127·9	328·6	456·5	36
Basal and cow butter-fat ...	512	45·3	189	234·3	277·7
Basal and buffalo butter-fat ...	497	53·4	261·3	314·7	182·3

* The comparatively larger intake of calcium in this experiment is due to inclusion of leaves of *Cucurbita pepo* (pumpkin) in the vegetables taken. These leaves contain 240 mg. to 390 mg. of calcium per 100 g. of the moist leaf. A paper on the availability of calcium in *Cucurbita pepo* leaves is being communicated for publication.

TABLE V.

Phosphorus metabolism with different fats.

SUBJECT : *I. B. G.*, 19 *years*, *weight* 53 *kg.*

Diet.	Dietary P (mg).	Urinary P (mg.).	Faecal P (mg.).	Total P excretion (mg.).	Balance (mg.).
Basal and fat-free	387·5	195·5	145	340·5	47
Basal and mustard oil ...	375·6	154·2	139·3	293·5	82·1
Basal and coconut oil ...	392·8	163·7	161·2	324·9	67·9
Basal and sesame oil ...	385·7	162·5	146·1	308·6	77·1
Basal and ground-nut oil ...	373·5	150·2	151·6	301·8	71·7
Basal and cow butter-fat ...	395·7	171·2	152·3	323·5	72·2

TABLE VI.

Phosphorus metabolism with different fats.

SUBJECT : *S. C. D.*, 19 *years*, *weight* 52 *kg.*

Diet.	Dietary P (mg.).	Urinary P (mg.).	Faecal P (mg.).	Total P excretion (mg.).	Balance (mg.).
Basal and fat-free	721	276	215	491	230
Basal and mustard oil ...	746	189	174	363	383
Basal and coconut oil ...	715·1	221	189	410	305·1
Basal and ground-nut oil ...	732	207	232	439	293
Basal and sesame oil ...	726·3	239	235	454	272·3
Basal and cow butter-oil ...	718	186	162	348·2	369·8
Basal and buffalo butter-fat ...	705·5	193·8	227	420·8	284·7

Discussion.

The data prove definitely two things: (1) firstly, the presence of fat in the diet greatly favours the absorption of calcium and phosphorus in the body. Addition of fat in the diet lowers the excretion of the calcium and phosphorus by the urinary tract to a great extent. The excretion of phosphorus in the fæces is also decreased to some extent and the elimination of calcium through the fæces is reduced to a much greater extent in all cases except with coconut oil. Probably the fatty acids form absorbable calcium soaps with calcium which would otherwise have been excreted as insoluble calcium carbonate. (2) all fats are not equally efficient in bringing about favourable calcium absorption and indeed on basal diet and coconut oil there occurs a drainage of calcium from the body. The intake of coconut oil as the main dietary fat cannot be recommended in view of the results obtained in this investigation. When coconut oil was used as the sole dietary fat, the experimental subjects were invariably in negative calcium balance. The faecal excretion of calcium was greatly increased and in three cases out of four exceeded the intake of calcium. With this oil in the diet the urinary elimination of calcium was generally higher than with other oils and butter-fat. The reason for this peculiar behaviour of coconut oil is not clear. Probably some particular calcium soaps that are being produced in the intestine as a result of digestion of the coconut oil are not absorbed at all. Further work is in progress in order to elucidate this point.

Summary.

The influence of the dietary fat on the calcium and phosphorus metabolism in four human subjects has been investigated. Inclusion of mustard, sesame or ground-nut oil or of butter-fat in the diet greatly favoured the absorption of calcium and phosphorus and the utilization of the minerals was better than in the absence of added fat. With coconut oil, however, the elimination of calcium through the fæces was greatly increased, the faecal calcium being in three cases greater than the amount of dietary calcium. The urinary excretion of calcium on coconut oil diet was also generally higher than with other fats. The use of coconut oil as the main dietary fat cannot be recommended in view of these findings.

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SARCOMA PRODUCED BY SUBCUTANEOUS INJECTIONS OF OVERHEATED COTTON-SEED OIL INTO MICE.

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BURROWS, Hieger and Kennaway (1932) announced that fats commonly used as vehicles for subcutaneous injection were under investigation as far as the production of tumours of the connective tissue is concerned. Three tumours occurred in a group of ten control mice injected with unheated lard in Burrows' (1932) experiments, but the author was suspicious that some confusion of animals or substances injected had occurred. Tumours did not arise in any other group of the injected mice, and the positive result could not be reproduced after complete precautions had been taken.

Peacock (1933) injected fowls with a solution of dibenzanthracene in lard into the right breast, and with lard only into the left breast. Tumours were observed at both sites.

Andervont (1934) found no new growth in 190 control mice injected with lard alone after 189 days.

Barry and Cook (1934) described tumour-like tissue in 2 of 10 rats following subcutaneous injection of lard after 385 and 462 days. The mass consisted of necrotic matter, granulation tissue, and some spindle-celled areas which were not considered to be malignant.

The experiments of Burrows, Hieger and Kennaway (1936) yielded not a single tumour in 216 mice after the injection of fats, fatty acids, unsaponifiable materials and liquid paraffin. In rats, however, the injection of lard alone resulted in 5 spindle-celled tumours out of 107 rats. Three more tumours were observed, one after the injection of lard with oleic acid, another after the injection of lard with theelin, and another after the injection of olive oil with arsenious acid. More than a year elapsed before the tumours appeared. Injections of lard, heated at 340–360° C. for two hours in a stream of nitrogen gave rise to two spindle-celled tumours in rats after 509 and 608 days. No tumours were obtained in mice. Three peritoneal tumours have been observed after the intraperitoneal injection of olive oil, but the authors consider it unsafe to assume that the tumours were the result of the injection.

Roffo (1938, 1939) described papillomata, adenocarcinomata and sarcomata

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of the stomach, and mesenteric sarcomata, obtained by the feeding of vegetable and animal fats heated at 350° C. for half an hour.

Domagk (1939) attempted to elicit gastro-intestinal tumours in mice by adding various apparently unheated fats to the diet. Only a few mice responded with tumours or glandular hyperplasia. These lesions were not found in the controls.

Beck and Peacock (1941) found that fats, heated under conditions that might occur in domestic cooking (repeatedly heated at 200–220° C. for a total of 12 hours), did not produce carcinoma or sarcoma of the stomach, but interfered with the metabolism of vitamin A and produced papillomata, bordering on malignancy, as a result of vitamin A deficiency, in rats fed on a diet which was adequate for controls, but not rich in vitamin A. The effect of overheated fats, as in the experiments of Roffo, is now under investigation.

Mice have been chosen for the injection of overheated cotton-seed oil, because they do not respond with the production of sarcoma as easily as rats do.

EXPERIMENTS.

Three groups, each containing 12 mice, were subcutaneously injected with 0·5 c.c. cotton-seed oil into the right flank.

In Group 1 the cotton-seed oil was heated at 340–360° C. for an hour, in the presence of air in the fume cupboard.

In Group 2 the cotton-seed oil was repeatedly heated at 200–220° C. for a total of 12 hours.

In Group 3 unheated cotton-seed oil was injected.

A new syringe and needle were used for the injections, to be sure that the cotton-seed oil was not contaminated with carcinogenic substances.

RESULTS.

Only those mice which survived the latent period of the earliest tumour (414 days) have been taken into account.

In Group 1 six mice survived. In every mouse examined post-mortem, a cyst was found containing a brown liquid similar to the overheated cotton-seed oil injected. The cyst was palpable in vivo. The first tumour, (Mouse 1866) appeared at the site of injection 414 days after the start of the experiment, and seemed to arise at the cyst wall. The mouse was killed 43 days later, by which time the tumour had penetrated into the peritoneum. Histological examination by my colleague, Dr. L. Woodhouse Price, revealed a spindle-celled sarcoma with an intercellular reticulum. The second tumour appeared at the site of injection 538 days after the start of the experiment (Mouse 1869). The tumour was very close to a similar cyst containing brown fluid, and proved, histologically, to be a spindle-celled sarcoma with a fine reticulum. No secondaries were observed in either mouse, and grafts have not been undertaken.

In Group 2 three mice only survived. A cyst was usually found containing the injected cotton-seed oil, except in the mice which died after twelve months. No tumours occurred. The small number of surviving mice makes it necessary to repeat the experiment.

In Group 3 ten mice survived, and six lived for more than 560 days. Neither cysts nor tumours were observed in this group.

DISCUSSION.

Before injection a sample of the overheated cotton-seed oil (340–360° C.) and of the repeatedly heated cotton-seed oil (200–220° C.) was spectroscopically examined. The spectrum showed only a general fluorescence and no bands, proving that none of the known carcinogens was present, and proving also that no contamination with carcinogens could have occurred. The heated cotton-seed oils, after having been purified chromatographically, contained a substance which gave a red colour when added to antimony trichloride. The colour was different from the red of the cholestadiene reaction.

Besides the production of sarcomata by overheated cotton-seed oil, three facts are of interest:

First, the great persistence of the depot of overheated cotton-seed oil. Second, the nearness of the tumour to the depot of overheated cotton-seed oil. Third, the long latent period.

These three facts can be correlated to each other.

Peacock (1933) described the topical conditions of tumour and tar depot in fowls. The tumour never arose in immediate contact with the main tar depot, but at some distance from the smaller depots in the direction of the lymph stream.

Peacock and Beck (1938) observed the same fact for benzpyrene tumours, and suggested that the tumour occurred at a point at some distance from the depot where the optimum rate of absorption was maintained for an optimum time. It seems obvious that the sarcoma-producing substance acts while it is being absorbed. In the case of overheated cotton-seed oil, the tumour starts very near the depot; this suggests that, either the rate of absorption is very slow, coinciding with the persistence of the depot, or that the concentration of the tumour-producing substance in overheated cotton-seed oil is very low.

The long latent period can also be explained by the foregoing facts. Assuming that the tumour-producing substance acts on its way to absorption, the process of inducing sarcomata appears similar to carcinogenesis as a result of painting. In the latter case, the carcinogen is applied every few days. In the case of sarcomagenesis, we have a more continuous stream of tumour-producing substances being absorbed through the tissues. We know that a dilution of the carcinogen in painting experiments results in a prolongation of the latent period. It seems justifiable to conclude, as far as sarcomata are concerned, that a slow rate of absorption or a low concentration of the tumour-producing substance brings about the conditions for a long latent period.

SUMMARY.

(1) Subcutaneous injection of overheated cotton-seed oil (340–360° C.) produced sarcomata in two of twelve mice, at the site of injection, after 414 and 538 days.

(2) Repeatedly heated cotton-seed oil (200–220° C.) and unheated cotton-seed oil did not produce tumours.

(3) Secondary findings : (*a*) A long persistent depot of overheated cotton-seed oil, (*b*) nearness of the tumour to this depot, and (*c*) a very long latent period, are correlated to each other.

I am indebted to Dr. Peacock for his interest in the work, to Dr. L. Woodhouse Price for the histological examination of the tumours, and to Mr. A. H. M. Kirby for the spectroscopic examination and the chromatogram.

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GASTRO-PAPILLOMATOSIS DUE TO VITAMIN A DEFICIENCY INDUCED BY HEATED FATS*

BY

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Since Fibiger (1913) first described experimentally induced tumours of the fore-stomach of the rat many other workers have investigated this problem without, however, reaching a satisfactory explanation of his original results. Fibiger described three types of lesions: (*a*) multiple papillomata of the fore-stomach ; (*b*) squamous carcinoma of the fore-stomach ; and (*c*) carcinoma of the tongue. He also obtained a carcinoma in a mouse that was transplantable. These lesions he attributed to the presence of a nematode, *Gongylonema neoplasticum*, derived from cockroaches which were present in the animal-house and which were consumed by the rats in addition to their diet of white bread and water. Passey (1934) drew attention to the fact that such diet must have been deficient in vitamins, and that vitamin A deficiency in the absence of the gongylonema can lead to papillomatosis of the fore-stomach of the rat. Cramer (1937) reviewed experimental work, including his own investigations bearing on Fibiger's results, and concluded that " diet and parasite are in themselves not sufficient to account for the lesions observed by Fibiger, and that there must be yet another factor involved which has not yet been identified." More recently Roffo (1938, 1939) claimed to have obtained a wide range of pathological lesions, including ulcers, papilloma, and carcinoma of the fore-stomach, adenocarcinoma of the glandular stomach, and sarcoma of the stomach and liver, in rats fed with heated or ultra-violet-irradiated cholesterol, and with overheated animal and vegetable fats. It is clear from the copious illustrations to his papers that Roffo has induced marked hyperplasia of the epithelium of both parts of the rat's stomach, amounting in some cases to histological evidence of malignancy, though the carcinomata do not seem to have metastasized to the liver, as might have been expected. The sarcomata, however, metastasized freely. These experiments are therefore of outstanding interest and demand independent investigation. Roffo believes that carcinogenic hydrocarbons are formed from sterols by the action of heat or ultra-violet rays, but, according to Cook and Kennaway (1940), his views on the nature of the chemical changes involved are inadequately supported by his experimental evidence. The presence of carcinogenic hydrocarbons in the food has been shown by Waterman (1939) to be capable of inducing tumours in the fore-stomach of mice.

In this laboratory an examination of a variety of fats has been made, after different conditions of heating, for the possible presence of known carcinogenic hydrocarbons, but nothing having the characteristic fluorescent spectrum of these substances has yet been demonstrated. It seems possible, therefore, that other types of carcinogen may be partly responsible for Roffo's experimental results. The suggestion that ordinary cooking fats may develop carcinogenic properties under the action of heat raises a practical problem which might have a bearing on the [illegible] gastric cancer in man. The fats used in Roffo's experiments were apparently heated to 300-400° C., a higher

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range of temperature than is ordinarily reached in domestic cooking, but it seemed possible that similar changes might occur progressively in fats repeatedly heated at lower temperatures. Even in times of peace economic conditions habitually forced a large part of the community to use fats repeatedly for cooking, and our experiments were therefore designed to imitate the worst conditions of frying likely to be in daily use, and to test the fats so treated by feeding them to rats and mice.

Experimental

1. A mixed cooking fat (equal parts of cotton-seed oil and whale oil) was heated to 200-220° C. in a closed iron saucepan for twenty to thirty minutes on thirty consecutive weekdays. The total time of heating amounted to about twelve hours. White bread was fried each day in this fat, allowed to cool, and then fed to 50 Norwegian hooded rats, maintained on a basal diet of whole oats, green vegetables, and water, with the addition of about 2 c.cm. of milk per rat three times a week and 2 grammes of carrot once a week after the first two months. When this experiment had been in progress for twelve months 40 rats had died, of which 8 showed multiple papillomata—6 with central ulceration—1 had a single unulcerated papilloma, and 2 showed irregular hyperplasia of the epithelium, all the lesions occurring in the fore-stomach. One rat developed a carcinoma of the lower lip. As in the foregoing experiment the bread was heated as well as the fat, this variable factor was eliminated in subsequent work by heating the fats alone and, after cooling, spreading them on white bread for feeding to the animals, using unheated fat spread in similar amounts as a control. The survivors of this first group of rats were fed in this manner for a further period of seventeen months, with the addition of about 2 grammes of carrot per rat four times a week, for reasons given below.

2. Twenty-four inbred Wistar albino rats were fed on the basal diet, with the addition of pure lard heated on thirty consecutive weekdays to 200-220° C. for twenty to thirty minutes.

3. Twenty-four Wistar rats were fed on the same basal diet, with the addition of cotton-seed oil, heated in the same way.

4. Twelve Wistar rats were fed on the basal diet, with the addition of unheated lard.

5. Twelve Wistar rats were fed on the basal diet, with the addition of unheated cotton-seed oil. (In Groups 2 to 5, after two months, 2 grammes of carrot once a week, and after a further five months four times a week, was added to the diet.)

6. Fifty stock mice were fed on the basal diet, with the addition of repeatedly heated mixed cooking fat spread on white bread, as described in the second paragraph under Group 1. (After two months 0.5 gramme of carrot once a week, and after a further ten months six times a week, was added to the diet.)

Litters of rats were divided between Groups 2 to 5 so that the groups were genetically comparable. During the first year of these experiments symptoms suggestive of avitaminosis-A were observed in young rats, particularly in Groups 2 and 3. The difference between these groups and the controls became more marked as time passed. The animals were smaller and less robust than the controls, had dry fur and slight xerophthalmia, and avoided exercise except in pursuit of food. As the control animals showed no such symptoms it seemed that the addition of heated fat had in some way neutralized or interfered with the metabolism of the dietary vitamin A. This possibility was tested in the following manner:

7. Twenty-four Norwegian hooded rats were fed on the basal diet, with the addition of about 3 c.cm. of milk and about 2 grammes of carrot once a week. This diet cannot be con-

[illegible] of lard heated at 327° C. for one hour six days a week; (c) 6 rats were similarly fed with lard heated at 220° C. for twelve hours; and (*d*) 6 rats were similarly fed with lard heated at 220° C. for one hour. After two months 2 rats from each group were killed and their livers rapidly removed, rinsed in physiological saline, minced, and desiccated. One gramme of desiccated liver was shaken with 5 c.cm. of chloroform for five minutes, the extract was then decanted, and dilutions of 1/1, 1/2, 1/4, up to 1/128, with chloroform, were made in a series of test-tubes. To each tube, containing 0.5 c.cm. of extract, 2 c.cm. of 30% solution of antimony trichloride in chloroform was added, and the highest dilution in which the blue vitamin A colour reaction could be detected was noted. The results of these estimations are shown in Table I.

TABLE I.—*Relative Vitamin A Contents of Rat Livers*

Rat No.	Lard	Vitamin A Content of Liver (Control = 1)
375B	Unheated (control)	1
376B		1
372B	Heated, 220° C. 1 hr.	1
373B		1
363B	Heated, 220° C. 12 hrs.	1/2
366B		1/2
361B	Heated, 327° C. 1 hr.	1/4
362B		1/8

It will be seen that the vitamin A content of the liver was reduced in proportion to the degree and duration of heating to which the lard was subjected. The remaining rats in this experiment were then fed for one month on a diet rich in vitamin A (basal diet, plus 2 grammes of carrot daily); one from each group was sacrificed, and the liver vitamin A estimated in the above manner. No differences were found between them, showing that the interference with vitamin A metabolism due to the heated lard was not permanent. The remainder of the animals are still under observation.

When animals died their stomachs were washed out and then filled with 10% formol-saline and left to fix for twenty-four hours before being opened. Fixation in this way gave very satisfactory preparations, free from distortion. All the stomachs were subjected to independent histological examination by Dr. L. Woodhouse Price, who reported his findings without knowing the details of the experiment. This is the routine procedure with all histological material in this laboratory. All but three lesions classed as papilloma were histologically confirmed. The results are shown in Tables II to V.

TABLE II.—*Lesions in* 14 *out of* 44 *Norwegian Hooded Rats, surviving for More than* 4 *Months, fed on Basal Diet and repeatedly Heated Mixed Cooking Fats* (30 *Died without Gastric Lesions*)

Rat No.	Duration of Experiment at Death	Lesions
108B	139 days	9 papillomata (5 ulcerated) in fore-stomach
136B	182 ,,	15 ,, (ulcerated) ,,
112B	221 ,,	4 ,, ,, ,,
113B	225 ,,	4 ,, ,, ,,
122B	226 ,,	1 papilloma ,, ,,
141B	236 ,,	1 ,, ,,
127B	268 ,,	21 small papillomata ,,
103B	275 ,,	1 ulcerated papilloma ,,
141B	301 ,,	Marked hyperplasia of fore-stomach
148B	312 ,,	Hyperplasia of fore-stomach
149B	318 ,,	Haemorrhage in fore-stomach
150B	343 ,,	Keratinizing carcinoma of lower lip
128B	354 ,,	2 haemorrhagic ulcers in glandular stomach
105B	436* ,,	Haemorrhage in glandular stomach

* On vitamin-A-rich diet after first year.

TABLE III.—*Gastric Lesions in* 6 *out of* 19 *Wistar Rats, surviving for More than* 4 *Months, fed on Basal Diet plus repeatedly Heated Lard* (13 *Died without Lesions*)

873	192 ,,	3 ,, ,, ,,
877	209 ,,	2 papillomata (1 ulcerated) ,,
694	304 ,,	4 ulcerated papillomata ,,
713	304 ,,	3 ,, ,, ,,
714	315 ,,	1 papilloma ,,

TABLE IV.—*Gastric Lesions in* 3 *out of* 14 *Wistar Rats, surviving for More than* 4 *Months, fed on Basal Diet plus repeatedly Heated Cotton-seed Oil* (11 *Died without Lesions*)

Rat No.	Duration of Experiment at Death	Lesions
878	117 days	1 ulcerated papilloma of fore-stomach
969	196 ,,	1 ,, ,, ,,
715	318 ,,	1 ,, ,, ,,

TABLE V.—*Gastric Lesions in* 3 *out of* 45 *Stock Mice surviving for more than* 4 *Months, fed on Basal Diet plus repeatedly Heated Cooking Fat* (41 *Died without Lesions; 1 Survives*)

Mouse No.	Duration of Experiment at Death	Lesions
1104	271 days	1 papilloma of fore-stomach
1106	279 ,,	1 ,, ,,
1134	385 ,,	Diffuse hyperkeratosis of fore-stomach

In the three groups of rats receiving repeatedly heated fats in addition to the original basal diet 77 survived for more than four months, and 17 of these died within the first year of the experiment and had papillomata in the fore-stomach. Control rats (receiving unheated fats) dying within the same period showed no papillomata, but 2 had minor degrees of hyperplasia. Among 25 old rats (over 2 years) from other experiments 3 showed hyperkeratosis of the fore-stomach, 1 diffuse hyperplasia, and 1 a simple papilloma of the fore-stomach. In our experience similar lesions have not been observed in control rats under 2 years old.

The benzidine test was carried out on stomach contents in cases showing brownish discoloration of the glandular stomach, and was found to be positive, confirming the presence of blood in the stomach cavity, probably derived from small ulcers. Papillomata, ulceration, and consequent haemorrhage appear to be definitely related to the feeding of repeatedly heated fat and associated with symptoms of varying degrees of avitaminosis-A. To test the possible interrelation between these pathological states 10 rats that had been under experiments for a year and 31 for seven months were given a diet rich in vitamin A, consisting of rat-cake nuts (see accompanying formula) daily, carrot 2 grammes four times a week, and about 2 c.cm. of milk once a week, while continuing to receive the differently treated fats as before. Six of these rats died after being on the vitamin-A-rich diet for more than a year, and none of these showed any gross lesion of the stomach. Two survivors are still under observation. The others are accounted for in the tables.

Rat-cake Formula (Supplied by the North-Eastern Agricultural Co-operative Society, Ltd., Bannermill Place, Aberdeen)

Wheat offal (fine middlings No. 2)	19·2%
Ground wheat	19·2%
Sussex ground oats	19·2%
Ground barley	9·5%
Ground maize	9·5%
Meat and bone meal (45% protein)	9·5%
Dried skim-milk	7·0%
White fish meal (60% protein)	4·7%
Dried yeast (40% protein)	1·2%
Sodium chloride	0·5%
Cod-liver oil	0·5%

In more recent experiments 92 rats have been maintained for ten months on the vitamin-A-rich diet throughout, with the addition of heated lard (350° C. for one hour or 220° for about twelve hours). No pathological lesions of the stomach have been observed among 10 that have died. *In vitro* tests for vitamin A in mixtures of cod-liver or halibut-liver oil and heated fats dissolved in chloroform were carried out to see whether any direct neutralization occurred, but the usual antimony trichloride blue reaction could not be observed, as the heated fats alone gave a brilliant red colour reaction with this substance.

[illegible] whether the vitamin reaction is merely masked by the intensity of the red colour reaction. Further work is being done to clear up these points and to determine the nature of the substance in the heated fat responsible for the red colour reaction with antimony trichloride.

Discussion

The foregoing evidence suggests that there is present in repeatedly heated fats a factor which interferes in some way with the absorption or metabolism of vitamin A. Our results confirm Roffo's claim that heated fats cause papillomatosis in the fore-stomach of rats, but no evidence of glandular tumours or of sarcoma has been found. One rat developed a carcinoma of the lower lip, but the significance of a single case cannot be assessed.

Although no recognizable carcinogenic hydrocarbons were formed in heated fats under the conditions of our experiments, a factor which may provisionally be termed "anti-vitamin A" was produced, and the subsequent pathological lesions developed by rats and mice receiving this factor with an otherwise adequate diet were apparently due to an artificially induced avitaminosis-A. Thus our results correlate those of Cramer and of Passey with some of the results observed by Fibiger and by Roffo. The malignant tumours originally described by Fibiger and the more extensive series of Roffo may have been due to the action of some hitherto unrecognized factors in the presence of avitaminosis-A. Mice were less susceptible to induced avitaminosis-A and papillomatosis than rats.

So far as any application of these experiments to human pathology is concerned there is no evidence of the development in fats, under ordinary cooking conditions, of any known carcinogen, but the anti-vitamin-A factor may possibly be detrimental to health, particularly when the dietary intake of vitamin A is low. If there is any analogy between rats and humans in this respect the addition of vitamin A to the diet in the form of raw carrot seems to be sufficient to counteract the effects of the anti-vitamin. The habit of eating raw carrot seems to be prevalent at the present time, and it is possible that future statistics will show whether this has any influence on the incidence of human gastric diseases.

Summary

Groups of rats and mice were fed with a variety of repeatedly heated fats in addition to an adequate basal diet. Within a year signs of avitaminosis-A appeared among the rats, and cases of ulceration and papillomatosis of the fore-stomach were observed among those that died. Control animals fed with unheated fats and the same basal diet showed no gross pathological lesions. Extracts of the livers of rats fed with lard heated under various conditions in addition to the basal diet contained reduced amounts of vitamin A as compared with controls.

The heated fats did not contain recognizable amounts of any known carcinogen, but they included some substance which interferes with the antimony trichloride reaction for vitamin A, giving a bright red coloration.

Rats fed with repeatedly heated fats in addition to a diet rich in vitamin A (carrot) showed no gross pathological lesions in fore-stomach or reduction in liver vitamin A.

The mechanism of papillomatosis in these experiments seems to be related to an induced avitaminosis-A, due to the presence in repeatedly heated fats of an "anti-vitamin A" of undetermined nature.

We wish to acknowledge the valued help given by Dr. L. Woodhouse Price in dealing with a large number of histological preparations, and by Mr. J. C. Chalmers in examining fluorescence spectra of extracts of heated fats.

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The Carcinogenic and Mutagenic Properties of N-Hydroxy-aminonaphthalenes[1,2]

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SUMMARY

N-Hydroxy-1-aminonaphthalene (*N*-hydroxy-1-AN), *N*-hydroxy-2-aminonaphthalene (*N*-hydroxy-2-AN), 1-aminonaphthalene (1-AN), 2-aminonaphthalene (2-AN), and 2-amino-1,4-naphthoquinone were repeatedly injected i.p. into rats at a dose of 5 mg/100 gm. Lower doses of 2-amino-1-naphthol (0.15 mg/100 gm), and of *N*-hydroxy-aniline (1.3 mg/100 gm) were also given i.p. *N*-Hydroxy-1-AN was a more potent carcinogen than *N*-hydroxy-2-AN; the other compounds were not notably carcinogenic. The mutagenicity of *N*-hydroxy-1-AN and *N*-hydroxy-2-AN was demonstrated in several mutants of *Escherichia coli*. The greater carcinogenicity of *N*-hydroxy-1-AN, a metabolite of 1-AN, as compared with *N*-hydroxy-2-AN suggests that *N*-hydroxylation is by itself an insufficient metabolic step in the formation of a proximate carcinogen.

INTRODUCTION

An important characteristic of carcinogenic aromatic amines is that they produce tumors at sites distant from points of application; therefore, their carcinogenicity is attributed to their metabolites. Metabolic studies in conjunction with mouse bladder implantation tests for carcinogenicity led Clayson (15) to propose that orthohydroxyamines are the proximate carcinogens. This hypothesis seemed to explain why amines with a blocked para position, such as 2-AN,[3] was carcinogenic whereas amines, such as 1-AN, with a free para position was weakly carcinogenic. Although both amines are orthohydroxylated, 1-AN is substituted to a greater extent in the para position which is unavailable in 2-AN.

The discovery by Cramer *et al.* (17) of *N*-hydroxylation of 2-AAF led to subsequent studies which revealed that this is a general metabolic process occurring in all aromatic amines examined (2, 3, 27, 28, 30, 34, 42). The *N*-hydroxy metabolites of carcinogenic aromatic amines are as active or more carcinogenic than the parent amines; this makes this type of metabolite an additional candidate for the proximal carcinogen. The metabolism of noncarcinogenic aromatic amines, such as acetanilide and 1-AN (41), also involved *N*-hydroxylation. Comparative carcinogenicity studies are required to pinpoint the role of *N*-hydroxylation in aromatic amine carcinogenesis. *N*-Hydroxy-2-AN is carcinogenic for the rat peritoneum (10) and the mouse bladder (9). *N*-Hydroxy-1-AN has only been tested in the mouse bladder, where it is carcinogenic (9).

The present paper, in an attempt to extend these observations, demonstrates that *N*-hydroxy-1-AN is a stronger carcinogen than *N*-hydroxy-2-AN for the rat peritoneum and that both compounds are mutagenic for *E. coli*. The latter observation confirms an earlier report (32). These results indicate that the role of *N*-hydroxylation in the carcinogenic process needs to be examined further.

MATERIALS AND METHODS

N-Hydroxy-1-AN, *N*-hydroxy-2-AN, and phenylhydroxylamine were prepared by reduction of the nitro compounds according to the method of Willstätter and Kubli (45). 2-Amino-1,4-naphthoquinone was prepared according to Fieser (18). 2-Amino-1-naphthol was prepared as previously described (40). Other chemicals were commercial products.

Groups of 12–14 male Wistar albino rats (Blue Spruce Farms) of about 130 gm were used. They were maintained on Purina laboratory chow for rats (J. Dombrowski). Intraperitoneal injections were performed at the dose and frequency used by Boyland *et al.* (10). Most of the compounds were injected at a dose of 50 mg/kg in 0.5 ml peanut oil twice weekly for 3 months. The compounds were homogenized in the oil with a Virtis 45 homogenizer.

Phenylhydroxylamine and 2-amino-1-naphthol were toxic at this dose; these compounds were therefore injected at 13 mg/kg and 1.5 mg/kg respectively. The animals were examined at death or when tumors were palpable.

The method we used for the determination of *N*-hydroxy-*N*-acetyl metabolites in urine depends on the ability of these metabolites to extract copper into chloroform; the copper is back extracted into water and analyzed as its diethyldithiocarbamate complex. Control experiments demonstrated that

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[2] A preliminary report of this work was presented at the meeting of the American Association for Cancer Research in April, 1966 (5).

[3] The following abbreviations are used: AN, aminonaphthalene; AAN, acetyl-AN; AAF, acetylaminofluorene; MAB, *N*-methyl-4-aminoazobenzene; TPF, trisodium pentacyanoamine ferroate.

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one mole of copper is extracted by 2 moles of *N*-hydroxy-2-AAF and of *N*-hydroxy-2-AAN, as expected for a 2:1 complex (28).

Urine samples were collected for 24 hours after feeding small doses of aromatic amines or amides. The pooled urines were extracted with 20% v/v chloroform, which was shaken with 0.3 M cupric acetate and filtered to remove traces of the aqueous layer. The copper was extracted with 33% v/v of water to which was added 10% v/v of 0.1% diethyl-dithio carbamate. The copper concentration was determined by absorbancy at 440 mμ.

The glucuronide was measured by first incubating the urine for 18 hours at 37°C with bacterial glucuronidase (Sigma).

The method of Boyland and Nery (12) was used for the determination of *N*-hydroxy-1-AN and *N*-hydroxy-2-AN. The reagent TPF was a Fisher certified product. A 0.05% w/v solution in 0.1 M phosphate, pH 7, slowly decomposed and was freshly prepared. Standard curves were obtained by the following procedure, which includes the peanut oil used in the assay. The *N*-hydroxy compounds were dissolved in 10 ml nitrogenated ethanol containing 0.1 ml peanut oil. One-ml aliquots were added to 5 ml of the TPF solution and extracted with 3 ml $CHCl_3$ after sitting for 5 min. The optical density of the aqueous phase was read against a reagent-control in a Beckman DK-2A spectrophotometer. Absorption maxima of the TPF complexes were 560 mμ for *N*-hydroxy-2-AN, as reported by Boyland and Nery (12), and 490 mμ for *N*-hydroxy-1-AN, which they had not determined.

For stability assays the *N*-hydroxy compounds were homogenized in peanut oil; 0.1 ml aliquots were added to 10 ml ethanol when homogenization was complete (0 time) and after 30 min. One-ml aliquots of the diluted solutions were added to 5 ml TPF and analyzed as before. The 30-min interval spans the time required for the rat injections.

Mutagenicity of Aromatic Amines. Mutagenicity tests were initially conducted by measuring the frequency of reversion to amino acid independence of a number of nutritionally dependent mutants of *E. coli* by the spot test method of Iyer and Szybalski (20). The bacteria were exposed to the compounds on Petri plates prepared by spreading on the surface approximately 2×10^7 bacteria from a freshly grown overnight culture in nutrient broth. The plates contained M-9 agar medium (1) enriched with 1.25% nutrient broth. Dry crystals of the compounds were placed in the center of the plates. Revertant colonies were scored after two to six days incubation at 37°C.

Since all of the compounds on test were, at best, only moderately soluble in aqueous media, significance could be placed only upon the positive mutagenic activities of the spot test method. More recently (Mukai, in preparation), a glycerol-acetone solvent system, suggested by the studies of Martin (24), has been devised which gives quantitative studies on the mutagenicity of these aromatic amine compounds. This liquid medium testing system consists of adding aliquots of a stock solution of the compound in acetone to glycerol, then thoroughly mixing equal volumes of this solution with washed bacterial cultures in saline. Immediately after mixing, half of the culture is chilled in an ice-bath for two minutes, centrifuged once, and resuspended in saline; then appropriate aliquots are spread on the surface of Petri plates as described above. This is designated as 0 minutes incubation. The remaining half of the culture is incubated at 37°C without aeration for 45 minutes and the centrifuging and plating procedure repeated. Titers of both cultures were made on nutrient agar plates. Control samples consisted of glycerol-acetone solutions without the compounds in question treated in a similar manner. Revertant colonies were scored after two days incubation at 37°C.

RESULTS

The tumor incidence is given in Table 1. The compound 2-amino-1,4-naphthoquinone was identified as an *in vitro* oxidation product of 2-amino-1-naphthol (4), and was included in these experiments because the oxidation products of aromatic amines and their metabolites might be responsible for their carcinogenicity. Bonser *et al.* (8) obtained subcutaneous sarcomas in mice with aged solutions of 2-AN which were recently found to contain 2-naphthoquinone-N^4-2-naphthylimine (13). We had previously observed that the oxidation of 2-amino-1-naphthol was required for alteration of DNA properties (38). King and Kriek (21) could not confirm these results for reasons which are unknown.

The *N*-hydroxy-aminonaphthalenes were the only compounds which gave rise to several peritoneal sarcomas (Table 1). The pathologic findings were similar to those observed by Boyland *et al.* (10) with *N*-hydroxy-2-AN. The multiple sarcomas were scattered throughout the peritoneum usually accompanied by hemorrhagic ascites. The nodules were on the mesentery and adhering to the internal organs such as liver and kidney. The microscopic appearance of these tumors is given in Figs. 1 and 2. *N*-Hydroxy-1-AN was more carcinogenic than *N*-hydroxy-2-AN in two experiments, as shown in Table 1 and Chart 1.

The comparative potencies of these metabolites is clearly demonstrated in Chart 1, which includes the data of Boyland *et al.* (10) for comparison. The data are plotted according to Altshuler's cumulative incidence function (personal communication). The ordinate is a cumulative tumor score by which each tumor is weighted according to the reciprocal of the number of animals at risk at the time that tumor is observed.

A gratifying observation is that both experiments with *N*-hydroxy-1-AN showed parallel incidences and that our data for *N*-hydroxy-2-AN parallel those of Boyland *et al.* (10).

It is important to note that phenylhydroxylamine and 2-amino-1-naphthol could only be tested at doses of 1.5 and 13 mg/kg body weight. Higher doses caused rapid deaths in several hours to 3 days. The primary cause of death appeared to be methemoglobinemia. Similar effects of phenylhydroxylamine were reported by Miller *et al.* (27) who also observed no tumors when 10 mg/kg was injected i.p. into rats 3 times weekly for 12 weeks. A more adequate testing procedure might be to continue the injections for the lifetime of the animals.

The Stability of *N*-Hydroxy-1-AN and *N*-Hydroxy-2-AN during the Injection Procedure. *N*-Hydroxy aromatic amines are highly unstable compounds which form colored decomposition products in the presence of light, air, and heat. It is,

Table 1

Compound	Dose	Mortality (years)	Neoplasms
N-Hydroxy-1-AN (Exp. #1)	5 mg/100 gm	12/12 (1)	5 Peritoneal sarcomas
N-Hydroxy-1-AN (Exp. #2)	5 mg/100 gm	14/14 (1)	11 Peritoneal sarcomas
N-Hydroxy-2-AN (Exp. #1)	5 mg/100 gm	11/12 (2)	1 Bladder carcinosarcoma and 1 Peritoneal sarcoma
N-Hydroxy-2-AN (Exp. #2)	5 mg/100 gm	7/14 (2)	4 Peritoneal sarcomas
1-AN	5 mg/100 gm	11/12 (2)	1 Leukemia
2-AN	5 mg/100 gm	10/12 (2)	1 Leg lymphosarcoma 1 Peritoneal sarcoma
2-Amino-1,4-naphthoquinone	5 mg/100 gm	11/12 (2)	None
2-Amino-1-naphthol	0.15 mg/100 gm	12/12 (1)	1 Leukemia
N-Hydroxy-aniline	1.3 mg/100 gm	8/14 (2)	None
Peanut oil	0.5 ml	11/12 (2)	None

The carcinogenicity of aromatic amines and their metabolites by intraperitoneal injection in the rat. Each compound was injected in 0.5 ml peanut oil twice weekly for 3 months. AN, aminonaphthalene.

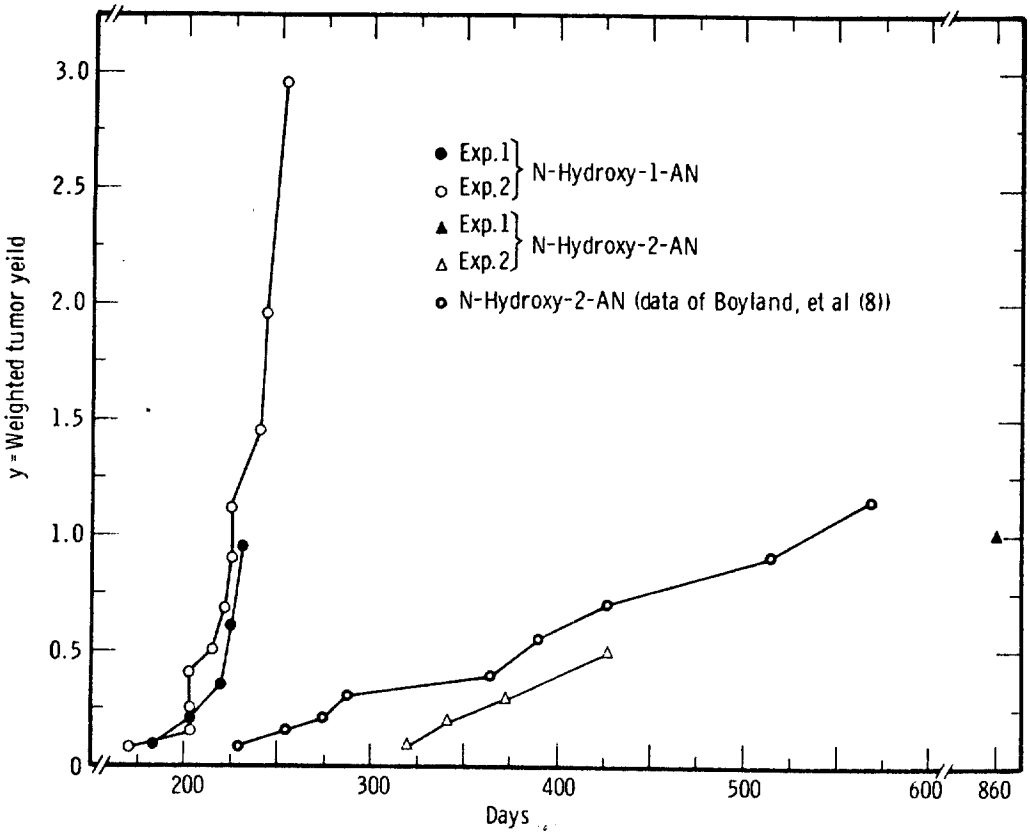

Chart 1. Tumor incidence expressed as weighted tumor yield. Each point represents an animal with tumor at designated time. The weighted tumor yield (*Y*) is computed as follows: Li = number of animals on test at time (t) of tumor scoring, each tumor given weight of $1/Li$, Y at time $t = 1/L_1 + 1/L_2 \ldots + 1/L_n$.

therefore, possible that our procedure may produce a disparate decomposition of *N*-hydroxy-1-AN and *N*-hydroxy-2-AN which might consequently be responsible for their differences in carcinogenicity. The results in Table 2 demonstrate that both compounds are relatively stable to homogenization in peanut oil for more than the 30 min required for injection. The slight loss of *N*-hydroxy-1-AN at 0 time may be due to experimental error or to the homogenization. The greater carcinogenicity of *N*-hydroxy-1-AN cannot therefore be attributed to its greater stability during the homogenization and injection procedure.

N-Hydroxy Metabolites of Carcinogenic and Noncarcinogenic Aromatic Amines and Amides.[4] The results of feeding

[4] A preliminary report of this work was presented at the meeting of the American Association for Cancer Research in April, 1965 (41).

Table 2

Time (min)	*N*-Hydroxy-1-AN (μg/ml)	*N*-Hydroxy-2-AN (μg/ml)
0	17	21
30	16	18

Stability of *N*-hydroxy-1-AN and *N*-hydroxy-2-AN to peanut oil homogenization. The concentration of each compound in the final assay solution is calculated to be 21 μg/ml. AN, aminonaphthalene.

single low doses of various amines and amides to 26 individuals are given in Table 3. *N*-Phenyl-acetamide was used instead of aniline to avoid methemoglobinemia. The results with 2-AN and 2-AAN, which form approximately similar amounts of *N*-hydroxy metabolites, indicate that man's ability to acetylate aromatic amines provides common metabolic routes for free and acetylated amines. The data indicate no major differences in the amount of *N*-hydroxy metabolites formed over a 24-hour period, between carcinogenic and noncarcinogenic amines in man.

Mutagenicity. The results of the mutagenicity test by the spot test method are given in Table 4. *N*-Hydroxy-1-AN was mutagenic for 5 mutants whereas *N*-hydroxy-2-AN was mutagenic for only 2 of these 5; in both mutants the latter appeared to be a weaker mutagen than the 1-isomer by this test. The other compounds tested in this manner were not mutagenic for the 7 strains listed. (The *N*-hydroxy-aminonaphthalenes were also tested in 30 additional strains but were not mutagenic in these.)

The inadequacy of the spot test method in detecting reliably the mutagenic activity of these compounds, which are only moderately soluble under the conditions of this method of testing, is revealed by the results of the glycerol-acetone solvent test system shown in Table 5.

This improved method of testing for mutagenicity reveals that both *N*-hydroxy-1-AN and *N*-hydroxy-2-AN are mutagenic for all of the mutants. Mutants A-53 and B-6, which did not show any increase in the number of revertants by the spot test, show approximately a two-fold increase in the numbers

Table 3

Compound fed	Dose	Patient	% *N*-Hydroxyacetyl compound excreted free in 24 hours	% *N*-Hydroxyacetyl compound excreted as a glucuronide in 24 hours	Total %
2-Naphthylamine	600 mg	A	0.53	0.33	0.86
		B	0.085	0.075	0.16
		C	0.446	0.014	0.46
		D	0.09	0.11	0.20
					0.42
N-2-Naphthyl-acetamide	400 mg	E	0.16	negligible	0.16
		F	1.23	1.23	2.46
		G	0.18	0.27	0.45
		H	negligible	0.44	0.44
					0.88
1-Naphthylamine	250 mg	I	0.41	0.52	0.93
		J	0.41	0.16	0.57
		K	2.00	0.76	2.76
		L	0.19	0.19	0.38
					1.16
Benzidine	200 mg	M	0.23	0.56	0.79
		N	2.66	3.41	6.07
		O	1.05	negligible	1.05
		P	0.192	0.048	0.24
					2.04
Dichlorobenzidine	250 mg	Q	1.54	0.45	1.99
		R	1.55	0.27	1.82
		S	0.32	0.11	0.43
		T	0.87	0.11	0.98
					1.31
N-phenyl-acetamide	400 mg	U	0.26	0.21	0.47
		V	0.02	0.41	0.43
		W	0.14	0.30	0.44
					0.45
Phenacetin	900 mg	X	0.22	0.11	0.33
		Y	0.03	0.08	0.11
		Z	0.39	negligible	0.39
					0.28

The urinary excretion of *N*-hydroxy metabolites of aromatic amines and amides in man. Averages are in italics.

Table 4

Mutant	Mutability[a]					
	N-Hydroxy-1-AN	*N*-Hydroxy-2-AN	2-Amino-1-naphthol	2-AN	1-AN	*N*-Hydroxy-aniline
A11	++	+	—	—	—	—
B36	++	+	—	—	—	—
A12	+	—	—	—	—	—
A23	+	—	—	—	—	—
B14	+	—	—	—	—	—
A53	—	—	—	—	—	—
B6	—	—	—	—	—	—

Induction of reverse mutation in *E. coli* mutants by *N*-hydroxy-aminonaphthalenes. Dry crystals of each compound were placed in the center of plates previously spread with 2×10^7 cells. The mutant colonies were counted after incubation at 37°C for 2 to 6 days. —, no increase in reversion frequency; +, less than five-fold increase; ++, five- to ten-fold increase. AN, aminonaphthalene.

[a] *N*-Hydroxy-1-AN and *N*-hydroxy-2-AN were not mutagenic in 30 additional strains.

Table 5

Mutant	Time in minutes at 37°C	Frequency of revertants/10^8 viable cells		
		Control (glycerol-acetone)	*N*-Hydroxy-1-AN	*N*-Hydroxy-2-AN
A11	0	10.0	19.8	19.3
	45	14.6	76.2	92.6
B36	0	8.8	10.0	36.5
	45	14.0	64.2	130.0
A12	0	0.6	7.2	1.8
	45	1.8	59.6	11.2
A23	0	4.3	6.1	6.2
	45	4.5	10.0	10.9
B14	0	6.5	16.7	13.4
	45	7.5	47.3	21.5
A53	0	19.4	23.8	29.0
	45	22.8	40.5	199.9
B6	0	3.0	5.1	6.9
	45	3.0	6.7	8.5

Mutagenesis in liquid medium. The compounds 1-AN, 2-AN, *N*-hydroxyaniline, and 2-amino-1-naphthol showed no significant increase in the number of revertants over that of control cultures. AN, aminonaphthalene.

of revertants. On the other hand, when the parent compounds 1-AN and 2-AN as well as *N*-hydroxy-aniline and 2-amino-1-naphthol, were tested by this improved method, these compounds gave revertant frequencies similar to that of the control cultures, and thus are not included in the table.

DISCUSSION

We have demonstrated that *N*-hydroxy-1-AN is a powerful carcinogen for the rat peritoneum and is more potent than *N*-hydroxy-2-AN at this site. These compounds are also carcinogenic for the mouse bladder (9).

The carcinogenicity of *N*-hydroxy-2-AN for these tissues is in accord with the results of others (2, 9, 27, 28, 30) who have shown that *N*-hydroxy metabolites of aromatic amines are carcinogenic *in situ* and may, therefore, represent the proximal carcinogen.

The carcinogenicity of *N*-hydroxy-1-AN at these sites is perplexing, however, because the parent amine is at most a weak carcinogen. 1-AN is a weaker bladder carcinogen than 2-AN in man, as manifested by a lower incidence and longer latent period (14). In dogs, 2-AN produces 100% incidence of bladder tumors in 1–2 years whereas 1-AN induced one bladder papilloma in one of three dogs after 8.75 years (6). In both these cases the activity of 1-AN may be attributed to the 4–10% 2-AN present. In mice, 2-AN is a liver carcinogen (7) whereas the purified 1-AN, freed of the 2-isomer, is not (16).

According to Miller and Miller (29), the criteria of relative carcinogenicity and carcinogenicity at local sites are used to define proximate carcinogenic metabolites. Ultimate carcinogenic metabolites are those which finally react with cellular constituents to initiate cancer. Both *N*-hydroxy-2-AN and *N*-hydroxy-1-AN would be defined as proximate carcinogens by these criteria. We would expect, however, the proximate carcinogen to be a metabolite of 2- but not 1-AN, or the former to be a more potent carcinogen. The results in Table 3 indicate that in man both *N*-hydroxy-1-AAN and *N*-hydroxy-2-AAN are formed at similar rates, at least over a 24-hour period. This applies only to the free and glucuronide forms which we measured.

The ultimate carcinogens in man and dog may be further reactive metabolites of *N*-hydroxy-2-AN which are either not formed from *N*-hydroxy-1-AN or formed at faster rates. Such forms may be similar to the *N*-hydroxy-esters like *N*-benzyloxy-MAB and *N*-acetoxy-AAF, which react with methionine and protein *in vitro* to form 3-CH_3-S- derivatives which were also isolated from protein of liver from rats fed MAB (23).

The reaction of *N*-acetoxy-AAF with guanine in nucleic acids (26) and the carcinogenicity of *N*-benzyloxy-MAB and *N*-acetoxy-AAF at local sites (25, 33) would support this.

The only metabolite of this nature, thus far identified in dog (11) and man (this paper), is the *N*-hydroxy-2-AAN glucuronide, whose carcinogenicity and reactivity with macromolecules have not been examined.

N-Acetoxy-2-AAN and *N*-acetoxy-1-AAN, however, did not alter the melting profile or buoyant density of DNA but did have some affect on its RNA priming activity. These compounds were also not mutagenic in the *E. coli* test system.

N-Acetoxy-2-AAF, on the other hand, had a marked effect on all 3 properties of DNA and was mutagenic as well (manuscript in preparation). This agrees with Scribner's results on the rates of hydrolysis of these compounds (35).

Other esters, such as the *N*-*O*-sulfate (11) recently synthesized, may be more reactive and are currently being investigated. Another metabolite, bis(2-amino-1-naphthyl) phosphate, which we have observed in urine of man and dog fed 2-AN (39, 43) may also be a proximate carcinogen. The corresponding phosphate was not found in urines of dogs fed 1-AN. The bis phosphate, which is a potent carcinogen for the mouse bladder (9), might be *N*-hydroxylated by the bladder mucosa (44) to form a metabolite containing both *o*-hydroxy and *N*-hydroxy functions in one molecule. We have observed a metabolite whose properties are suggestive of such a compound (37). It contained 2 amino groups per phosphate but differed from the bis phosphate in the following respects: it was ether soluble, unstable in alkali, and readily reacted with Folin-Ciocalteau phenol reagent.

Although *N*-hydroxylation has conferred strong carcinogenic properties on some noncarcinogenic aromatic amines (19) in addition to 1-AN, it is an insufficient carcinogenic process with aminoazobenzene. This was demonstrated by Sato *et al.* (34), who observed that the *N*-hydroxy metabolite of this compound was also noncarcinogenic when administered to rats by feeding, i.p. injections, or by s.c. injections as the cupric chelate of *N*-hydroxy-acetyl aminoazobenzene.[5] The authors point out that the structure of the *N*-hydroxy metabolite must govern its carcinogenicity. Here the presence of an *N*-methyl group is of critical importance.

The mutagenicity results in Table 4 agree with those of Perez and Radomski (30) who observed that *N*-hydroxy-1-AN was more mutagenic for one mutant of *E. coli* than *N*-hydroxy-2-AN. Their tests, as well as those in Table 4, were conducted with the spot test method, which now appears to be a less sensitive indicator of mutagenicity than the solution method which gave the results in Table 5. By this improved method, the mutagenicity of *N*-hydroxy-1-AN and *N*-hydroxy-2-AN appear to be equal. The differences observed seem to be related to the different sensitivities of the various mutants to these mutagenic agents. As with the spot test, 1-AN, 2-AN, 2-amino-1-naphthol, and phenyl-hydroxylamine were also nonmutagenic for the mutants listed.

Studies on the mutagenicity of aromatic amine metabolites bear directly on the hypothetical relationship between carcinogenicity and mutagenicity. These studies should help elucidate the nature of the proximal metabolite and its mechanism of action. This is indicated by the observation that three of the proximate carcinogens are also mutagenic. Nucleic acid alterations, such as the GC $\longrightarrow$ AT transitions induced by the *N*-hydroxy-aminonaphthalenes (31) and *N*-acetoxy-AAF (manuscript in preparation), can be correlated with the *in vitro* guanine reactions of *N*-hydroxyl aromatic amines (22).

Our results and those of Boyland *et al.* (10) indicate that the *N*-hydroxy metabolites of 1-AN and 2-AN are carcinogenic for the rat peritoneum and mouse bladder. Since these urinary metabolites are formed in similar amounts, it is unlikely that *N*-hydroxy-2-AN is the major proximate carcinogen of 2-AN, Other possible candidates are (*a*) the phosphate esters which are metabolites of 2-AN but not 1-AN, (*b*) further metabolites of *N*-hydroxy-2-AN, and (*c*) additional metabolites which remain to be discovered. These may be explored by further studies on the comparative metabolism of 2-AN and 1-AN and the carcinogenicities of their metabolites.

[5] The criterion of testing the copper chelates of *N*-hydroxy-acetyl-aromatic amines by s.c. injection in rats may not be generally useful. We have found that this procedure gave no tumors with the copper chelates of *N*-hydroxy-acetyl-2-AN, -1-AN, -4-aminobiphenyl, and -3-aminobiphenyl. The copper chelate of *N*-hydroxy-2-AAF, however, was carcinogenic, and produced a sarcoma incidence similar to that reported by Miller *et al.* (26).

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Route of Absorption and Distribution of Oleic Acid and Triolein in the Rat

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During the last few years a better understanding of the process of intestinal fat absorption has been gained, largely with the aid of isotopically labelled material, in combination with the technique of Bollman, Cain & Grindlay (1948) for collecting intestinal or thoracic lymph from unanaesthetized rats. The results of these investigations have shown that after absorption long-chain saturated fatty acids are mainly transported via the intestinal lymph as triglycerides both in rats and cats (Bloom, Chaikoff, Reinhardt, Entenman & Dauben, 1950; Bergström, Borgström, Carlsten & Rottenberg, 1950; Bloom, Chaikoff, Reinhardt & Dauben, 1951; Chaikoff, Bloom, Stevens, Reinhardt & Dauben, 1951; Bloom, Chaikoff & Reinhardt, 1951; Borgström, 1951, 1952*a*, *b*, *c*; Bergström, Borgström & Carlsten, 1954). Fatty acids with 10 or less carbon atoms are, however, mainly transported from the intestine via the portal blood vessels (Bloom, Chaikoff & Reinhardt, 1951; Kiyasu, Bloom & Chaikoff, 1952) largely in free form (Borgström, to be published). For summaries see Bergström & Borgström (1953, 1954).

The higher saturated fatty acids are thus not absorbed via different routes according to the state in which they enter the intestinal mucosa, i.e. as free acids or esterified with glycerol, as postulated in the original fat–fatty acid partition theory of Frazer (Frazer, 1938, 1943, 1946, 1948*a*, *b*, 1950, 1951, 1952).

There is also much experimental evidence that the unsaturated long-chain fatty acids are mainly absorbed via the lymphatics irrespective of whether they are fed as free acids or glycerides.

Munk (1880) fed oleic acid to dogs with a cannulated thoracic duct and found that there was a considerable increase in the neutral fat of the lymph. Munk & Rosenstein (1891) fed olive oil, oleic and erucic acid to a patient having a lymph fistula and obtained a high recovery of the ingested fat in the lymph. Freeman & Ivy (1935) obtained the same results after feeding olive oil to dogs. Bollman, Flock, Cain & Grindlay (1950) observed a marked increase of glycerides in the lymph after feeding oleic acid to dogs. Reiser & Bryson (1951) and Reiser, Bryson, Carr & Kuiken (1952) fed linoleic acid with conjugated double bonds to rats having a thoracic duct fistula and recovered part of the labelled acid in the lymph fat both after free acid and glyceride ingestion.

On the other hand, Frazer (1943), after feeding olive oil and oleic acid to rats and assessing the results with a technique mainly based on chylomicron counts and histological observations, concluded that 'fatty acid passes by the portal vein to the liver while neutral fat goes by the lymphatic route...' and this opinion has been reiterated in many subsequent publications (see above). Tidwell (1950), however, obtained a similar chylomicrographic response after feeding, respectively, olive oil and oleic acid to rats.

We now wish to report the results of some experiments in which [1-^{14}C]oleic acid has been fed either in free form or as triolein to rats with a thoracic-duct fistula. Some observations on the distribution of the labelled acid in these two forms to rats without a fistula have also been made.

EXPERIMENTAL

Methyl [1-^{14}C]oleate and [1-^{14}C]oleic acid were prepared according to Bergström, Pääbo & Rottenberg (1952), and triolein was prepared from oleic acid via the acid chloride prepared with oxalyl chloride.

The labelled substances were fed to rats with a thoracic-duct fistula (*A*) and also to animals without a fistula (*B*). The specific activity of the [1-^{14}C]oleic acid was 2300 counts/min./mg. (10 $\mu\mu$c/mg.).

A. *Animals with a thoracic-duct fistula*

Male rats weighing about 250 g. and starved for 12 hr. before operation were used. The procedure used for cannulating the thoracic duct was, with a slight modification, that described by Bollman *et al.* (1948). Instead of a straight cannula, a heated polythene cannula was bent at right angles about 0·5 cm. from the tip and was inserted through the left lateral body wall into the thoracic duct. This bend permits a cannula of larger calibre to be used, as the tension on the tube after insertion is reduced. After operation the rats were placed in restraining cages and had free access to 1% (w/v) NaCl solution until an adequate lymph flow was obtained. Thereafter, 0·64% (w/v) NaCl solution with 0·04% (w/v) KCl was fed *ad lib.* during the whole experiment. Three hours before the labelled substance was administered the rat had free access to white, fat-free bread. These rats were given either 0·5 ml. of labelled oleic acid or triolein by stomach tube when under light ether anaesthesia.

The lymph was collected for 24 hr., after which the animals were killed. In most of these experiments the rat in its cage was placed in a metabolic chamber and the expired CO_2 was recovered in NaOH. The lymph fat was extracted with ethanol–ether (3:1, v/v) and after the evaporation of this extract *in vacuo* the residue was extracted with light petroleum (b.p. 40–60°)-$CHCl_3$ (2:1, v/v). The total fat from the lymph samples was separated into neutral fat and phospholipids on columns of silicic acid and these fractions were saponified as described by Borgström (1952*c*).

The whole gastrointestinal tract was removed in one piece and digested, together with the faeces collected during the experiment, with a solution of 30% (w/v) KOH at 100° for 12 hr. The mixture was then acidified and the fatty acids were extracted with light petroleum (b.p. 40–60°). The amount of fat absorbed in these experiments was calculated by taking the difference between the amount of isotope administered and the amount recovered from the intestinal tract and faeces.

The small activity present in the intestinal wall has thus been included in the unabsorbed amount in these cases, but this amount is negligible in fistula animals 24 hr. after feeding.

B. *Non-cannulated rats*

The labelled material was administered by gastric intubation in amounts corresponding to 0·1 ml./sq.dm. body surface (Lee, 1929). For comparison with the cannulated animals the activity of the expired CO_2 was measured on some rats which had received 0·5 ml. labelled material through a stomach tube. At different intervals after the feeding the rats were bled to death under ether anaesthesia. The gastrointestinal tract was removed and the contents of the small intestine were washed out with saline.

The small intestine was finely dispersed in ethanol in a Waring Blendor and the total fat extracted with ethanol–ether (3:1, v/v), and after the evaporation of this extract *in vacuo* the residue was extracted with light petroleum (b.p. 40–60°)–$CHCl_3$ (2:1, v/v). The total fat was then fractionated into neutral fat and phospholipids on silicic acid columns and saponified (Borgström, 1952*c*).

Measurement of radioactivity

The determination of ^{14}C in fatty acids was done with a thin end-window Geiger-Müller tube on samples of 'infinite thinness'. Samples of an ethanolic solution were evaporated on aluminium planchets to leave less than 0·2 mg./cm^2.

In order to relate the activity of the fatty acids and the activity of the expired CO_2 that was counted as $BaCO_3$ at 'infinite thickness', suitably diluted samples were plated as described above and similar samples were oxidized to CO_2 and counted as $BaCO_3$ at 'infinite thickness' (Bergström, Borgström & Rottenberg, 1951).

RESULTS

The results of the lymph experiments (*A*) are given in Table 1. When labelled oleic acid was fed as the free acid, 66–92% (mean 78·2%) of the fed activity was absorbed in 24 hr. Rats receiving labelled triolein absorbed 57–92% (mean 76·2%) of the activity. It is seen that the fed free oleic acid was absorbed just as well as triolein in 24 hr. After feeding free [1-^{14}C]oleic acid, 38–96% of the absorbed activity was recovered in the lymph fat from the thoracic duct (mean 63·0%). The corresponding figures obtained when labelled triolein was fed were 51–83% (mean 65·5%). Feeding the labelled oleic acid as the free acid or as the glyceride made no significant difference to the recovery of the activity in the lymph lipids.

About 2% of the activity found in the lymph lipids was in the form of phospholipids, the remainder in the form of neutral fat in both groups. It was found that the labelled fatty acid in the lymph lipids followed the mono-unsaturated fatty acid within the limits of the fractionation method of

Table 1. *Recovery of [^{14}C]oleic acid and [^{14}C]triolein in thoracic lymph after oral administration to rats*

Rat no.	Substance fed	Amount of administered activity absorbed (%)	Amount of absorbed activity recovered in lymph as fatty acids (%)	Amount of lymph fat activity found in: Glyceride fatty acids (%)	Amount of lymph fat activity found in: Phospholipid fatty acids (%)	Amount of absorbed activity in expired CO_2 (%)
1	Oleic acid	91·5	64·0	98·2	1·8	4·8
2	Oleic acid	65·5	38·1	98·8	1·2	3·7
3	Oleic acid	73·0	96·4	97·4	2·6	3·5
4	Oleic acid	91·8	62·5	97·9	2·1	—
5	Oleic acid	69·0	53·8	97·5	2·5	4·5
Mean		78·2	63·0	—	—	—
6	Triolein	92·1	59·3	97·5	2·5	4·4
7	Triolein	56·5	50·5	97·7	2·3	5·0
8	Triolein	85·0	69·2	97·4	2·6	4·0
9	Triolein	71·0	82·9	98·8	1·2	—
Mean		76·2	65·5	—	—	—

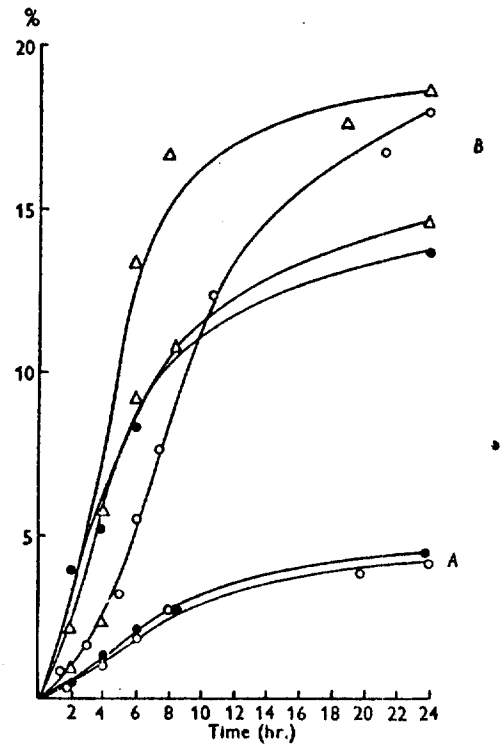

Fig. 1. Percentage of absorbed isotope in expired CO_2 after feeding [^{14}C]oleic acid as triolein (●), methyl oleate (△) or free oleic acid (○). *A*, rats with lymph fistula; every point represents the mean of three animals. *B*, rats without fistula; each point represents a single animal.

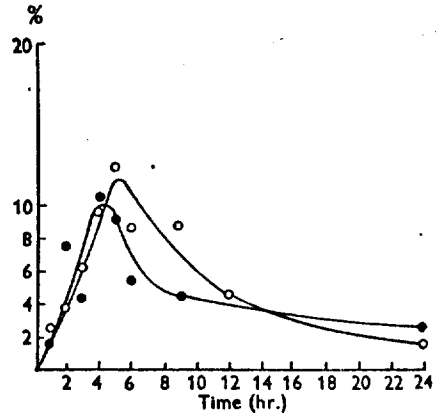

Fig. 2. Relative specific activity of phospholipid fatty acids of small intestine after feeding [^{14}C]oleic acid (○) or [^{14}C]triolein (●).

Bergström & Pääbo (1954). This method is based on chromatographic separation of the products of hydroxylation of the mixture of saturated and unsaturated fatty acid with performic acid.

While non-cannulated rats fed [1-^{14}C]oleic acid or methyl [1-^{14}C]oleate expired about 16% of the absorbed activity as carbon dioxide within 24 hr., rats with the thoracic duct cannulated expired at the same time about 4% irrespective of whether the oleic acid had been administered as glyceride or as free acid (Fig. 1).

In Fig. 2 are given the curves for the specific activities of the fatty acids of the phospholipids of the intestinal wall as percentage of the specific activity of the fed fatty acids. The specific activities of the phospholipid fatty acids increased to a maximum of about 10% of that of the fed fatty acids about 4 hr. after administration. However only a part of these phospholipids represents material that had been present in the epithelial cells of the mucosa.

DISCUSSION

After feeding labelled free oleic acid or triolein to rats with the thoracic duct cannulated, means of 63·0 and 65·5% of the absorbed activity were

recovered in the lymph lipids during the following 24 hr.

These figures give for the first time quantitative information about the route of transport of oleic acid after absorption from the small intestine.

In corresponding experiments with [^{14}C]palmitic acid dissolved in olive oil (Blomstrand, 1954), in which the same procedures were used as in the experiments reported here, a mean of 67·4 % of the absorbed activity was recovered in the thoracic duct lymph as [^{14}C]fatty acid (range 58·5–75·0 in five experiments). If these figures obtained for labelled palmitic acid are compared with those obtained for oleic acid in this investigation no significant differences are found.

The figures obtained for palmitic acid recovered in the lymph in these experiments are not as high as those given by Bloom *et al.* (1950). These authors found a mean of 75·9 % of the absorbed activity in the thoracic duct lymph as [^{14}C]fatty acid (range 58·5–91·8 in ten experiments). The differences of the means in these two series of experiments may be due to differences in the analytical methods used. Bloom *et al.* (1950) determined the radioactivity directly from the total fatty acids of the lymph obtained after saponification, while in experiments from this laboratory the neutral and phospholipid fatty acids were isolated by a chromatographic procedure before the activity determinations. In this way small losses of activity probably occurred.

Probably there are several causes for the large variation between different animals. There are certainly considerable anatomical differences between different animals in the arrangement of lymph vessels, some of which might by-pass the fistula. Possibly anastomoses exist between lymph and blood vessels which make possible an increased by-passing of the thoracic duct if the lymph flow is partly blocked by, for instance, clots that form in the lymph vessels of some cannulated animals. Also the lymph flow is probably not entirely normal with an open fistula. It is not unlikely that the rats with the highest recoveries are those most approaching normal.

It is seen in Fig. 1, however, that the amount of isotope expired during 24 hr. by a cannulated rat is roughly one-fourth to one-third of the amount expired by the non-cannulated rat. This corresponds roughly to the loss of labelled material via the fistula and shows that the absorbed labelled compounds are metabolized at a normal rate. It must be emphasized that the extent of lymphatic transport of oleic acid was found to be the same as palmitic acid when studied with the same technique (Blomstrand, 1954). No difference was observed in the recovery of labelled oleic acid in the thoracic-duct lymph lipids during the 24 hr. following the administration of labelled oleic acid as free acid or as triolein. The capacity of the intestinal cells to synthesize triglycerides is thus apparently very great. These findings are in general in agreement with the results earlier obtained on the absorption of long-chain unsaturated fatty acids in humans, dogs and rats by Munk (1880), Munk & Rosenstein (1891), Freeman & Ivy (1935), Bollman *et al.* (1950) and Fernandes (1953).

Frazer (1943) followed the absorption of oleic acid and olive oil in the rat with chylomicron counting and reached other conclusions. In the case of olive oil he observed a quicker and stronger response, which led him to the interpretation that the free oleic acid fed was absorbed directly via the portal route and rapidly metabolized in the liver, whereas the olive oil was mainly absorbed via the lymphatics and deposited in the fat depot. These interpretations formed part of the basis for the original 'fat–fatty acid partition theory'. Chylomicron counting—a method of doubtful quantitative significance—seems unsuitable for studies of this kind and has led to erroneous interpretations of this phase of the absorption process (for further discussion cf. Bergström & Borgström, 1954).

That fatty acids of different structure are incorporated into the phospholipids of the small intestinal wall to different extents has been found in earlier work with elaidic acid (Sinclair & Smith, 1937), conjugated linoleic acid (Barnes, Miller & Burr, 1941), and with different saturated fatty acids (Bergström *et al.* 1952; Borgström, 1952*d*).

The values obtained for the incorporation of labelled oleic acid in this work correspond to those found with double-bond conjugated linoleic and myristic acids.

In conclusion it must be emphasized that a very great variability in the rate of the absorption of fat and fatty acids is to be expected. Not only is the general condition of the animal of importance but the presence and nature of other food constituents greatly affect the absorption rate. The influence of these factors on the route of absorption and distribution in the body seems to be of less importance.

SUMMARY

1. The intestinal absorption of [1-^{14}C]oleic acid has been studied in rats with cannulated thoracic ducts.

2. It was found that oleic acid was transported via the thoracic-duct lymph and incorporated into triglycerides and phospholipids to the same extent as found for palmitic acid in earlier work. The same results were obtained whether the oleic acid was fed as free acid or as triolein.

3. The incorporation of the labelled acid into the lipids of the intestinal wall and the appearance of the isotope in the expired carbon dioxide has been followed.

4. The results indicate clearly that there is no detectable difference between the routes followed during absorption of fed free oleic or palmitic acids, or of glycerides containing these acids.

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Fractionation of Arlacel A[1] (35493)

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Arlacel A[2] is a synthetic oil-soluble emulsifier for the preparation of water-in-mineral oil emulsions, the type initially described by Freund as immunologic adjuvants (1). Specially-Treated Arlacel A is a partially purified form of Arlacel A that has been processed to remove toxic materials which might be injurious to tissues. The special grade of the emulsifier has been widely employed as a component of water-in-mineral oil emulsions which are used for prevention of respiratory diseases by vaccination and for repository treatment of allergy and only those batches that satisfy stringent biological tests for safety have been used in adjuvant emulsions intended for use in man (2).

Arlacel A is also called mannide monooleate by its manufacturer as an eponym for 1,3:4,6 dianhydromannitol monooleate which is presumably the principal active ingredient of this emulsifier (3). Dianhydromannitol, an anhydrosugar with two hydroxyl groups available for possible esterfication, is apparently formed during the manufacturing process and combines with oleic acid to yield mannide monooleate and mannide dioleate which are thought to be major constituents of Arlacel A. However the composition of Arlacel A is not well known and it is probable that the emulsifier also contains esters of other fatty acids (4, 5). The objectives of the present study were to ascertain the chemical and biologic properties of more highly purified mannide monooleate and mannide dioleate fractions of Arlacel.

A. Methods. 1. Analytical methods. Thin-layer chromatography (TLC) on silica gel[3] matrix was used for qualitative identification of various fractions recovered during the purification process. Chromatograms were developed with a mixture of acetone and chloroform (1:2; v/v), unless otherwise stated, and were stained by exposure to I_2 vapor. Fractions were identified by their characteristic rate of migration on TLC plates.

2. Fractionation methods. a. Distillation. Distillation was done in a closed system at 0.001 mm Hg pressure using an apparatus[4] which spread the materials to be distilled as a thin layer over the inner walls of a heated glass cylinder by means of rotating vanes. The temperature of distillation was controlled by an external heating jacket which was wrapped around the body of the still. Distillate was condensed on the outer walls of a smaller water-cooled glass column which was concentric to the heated cylinder and the condensate was collected in a side-arm flask at the reduced pressure. It was necessary to open the system to ambient pressure to recover either distillate or the undistilled residue.

b. Column chromatography. Appropriate fractions obtained by distillation were subjected to column chromatography as an additional fractionation procedure. Initially, the columns which measured about 20 mm in diameter were made with 40 to 50 g of mag-

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[2] Atlas Chemical Industries, Wilmington, Delaware.

[3] Silica Gel G, Brinkmann Instruments, Inc., Des Plaines, Illinois.

[4] Molecular Still, A. O. Smith and Company, Kankakee, Illinois.

nesium silicate,[5] which was additionally ground by mortar and pestle so as to obtain a tightly packed column which ran at less than 1 ml/min. Various fractions were removed from the columns by successive elution with suitable nonaqueous solvents. Samples of column eluates were collected in 25-ml samples, assayed by TLC; and those containing like fractions were combined and concentrated by evaporating the solvent.

B. Findings. 1. Analysis. Reference samples of mannide dioleate and mannide monooleate were prepared under special laboratory conditions[6] from higher quality mannitol and oleic acid than those customarily employed in the manufacture of Arlacel A. TLC patterns showed that the major constituent of the dioleate reference was a fraction with a R_f value of 0.8 while the major constituent of the monooleate reference was a fraction with a R_f value of 0.5. Each reference sample also contained a distinct secondary fraction: The secondary fraction in the monooleate reference had a R_f value of 0.8 while that for the dioleate reference had a R_f value of 0.5. In addition, the monoleate reference contained a minor fraction with a R_f value of 0.1. In subsequent chromatograms, the R_f-0.5 material is referred to as the monooleate fraction while the R_f-0.8 material is referred to as the dioleate fraction.

The TLC pattern of Specially-Treated Arlacel A showed at least four distinct chromatographic fractions: one with an R_f of 0.4, another with an R_f of 0.5 which presumably was a mannide monooleate fraction, a third fraction with an R_f 0.7 and, lastly, a fraction with an R_f 0.8, presumably containing isomannide dioleate. Several other batches of Arlacel A examined by TLC also contained similar fractions presenting clear evidence that the emulsifying agent was more heterogeneous a material than the mannide monooleate reference supplied by the manufacturer.

2. Fractionation. a. Vacuum distillation. Fractional distillation of Arlacel A was employed as a preliminary step, especially in recovery of mannide monooleate fractions. Because of the mode of operating the still, it was necessary to use a batch-type procedure. A large volume, usually 250 ml, of Arlacel was processed with a jacket temperature of 50 to 60° until the entire sample had passed through the still. Then the apparatus was opened to ambient pressure, the vessel for collecting the distillate was replaced, and the undistilled residue was reprocessed at a slightly higher temperature, usually 20 to 30° higher than the preceding distillation.

Distillates recovered with jacket temperatures from 50 to 100° contained appreciable amounts of a material with an R_f of 0.6 which behaved in the same manner as oleic acid on TLC. Similar material appeared in varying concentrations in distillates obtained over the entire range of temperatures employed and was even obtained from batches of Arlacel A that appeared to be without nonesterified or "free" oleic acid. It is assumed that the R_f-0.6 fraction contained oleic acid which may have been released by thermal decomposition of esters in Arlacel A. Distillates obtained at jacket temperatures between 130 and 170° consisted chiefly of the mannide monooleate fraction but also contained smaller amounts of the mannide dioleate fraction. Finally, the major part of distillates recovered with jacket temperatures above 180° consisted of the mannide dioleate fraction, but even these distillates also contained small amounts of the lower boiling fractions.

Attempts to collect materials using jacket temperatures above 300° resulted in carbonization of the undistilled residue. Redistillation of various distillates at the temperatures at which they were initially produced did not result in further purification.

b. Column chromatography. Final recovery of the fractions desired was obtained by subjecting appropriate distillates from vacuum distillation to several cycles of column chromatography. The choice of solvents used in developing the magnesium silicate columns to which the distillate fractions were applied and the order of application was based on evidence from TLC that the mannide dioleate fraction had a higher R_f value in sever-

[5] Florisil, The Floridin Company, Pittsburgh, Pennsylvania.

[6] Reference samples kindly supplied by the Atlas Chemical Industries, Wilmington, Delaware.

TABLE I. Comparative Mobilities of Isomannide Monooleate and Isomannide Dioleate on Silica Gel.

Solvent	*Rf*[a]	
	Monooleate fraction	Dioleate fraction
1.) $CHCl_3$ alone	0.6	0.9
2.) $CHCl_3$ + acetone (2:1)[b]	0.55	0.8
3.) Hexane–chloroform–acetone (18:1:1)	0.35	0.6
4.) Hexane + methanol (98:2)	0.3	0.55
5.) 5% $CHCl_3$ in hexane [hexane + $CHCl_3$ (19:1)]	0.1	0.1
6.) Skellysolve	0.1	0.1

[a] *Rf* = (distance substance migrated)/(distance solvent front migrated).
[b] Ratio by volume.

al organic solvents than did the mannide monooleate fraction. Moreover both fractions migrated faster in polar solvents than they did in nonpolar solvents (Table I). Accordingly, the columns were developed by successive application of chloroform, acetone and chloroform mixtures, acetone and methanol. The course of the chromatographic procedure was monitored by analysis of the eluates by TLC at appropriate intervals and those eluates exhibiting identical thin-layer chromatograms were combined, and then concentrated by flash evaporation of the organic solvents at partially reduced pressures.

Vacuum distillates recovered at 140 to 150° were used as the source of material for recovery of the mannide monooleate fraction by two cycles of column chromatography. Eluates recovered with 10 and 20% solutions of acetone in chloroform (v/v) were combined and subjected to a second run. The mannide monooleate fraction was eluted from the second column with 10% acetone in chloroform and the chromatogram of this product showed a single spot with an R_f of 0.5. Some dioleate material was recovered in the chloroform eluates of columns which were loaded with 140 to 150° distillates, but this procedure did not result in a high yield. The mannide dioleate fraction was obtained principally by column chromatography of vacuum distillates obtained above 180° and was eluted from the magnesium silicate columns with chloroform. In addition, after the monooleate and dioleate fractions had been removed from the columns, elution with methanol yielded a third, and as yet unidentified, fraction which had a R_f of 0.2.

C. Chemical properties. The mannide monooleate fraction was a pale straw-colored oily liquid which had a refractive index of 1.477 at 23° and sp gr, 1.05 at 23°. The monooleate fraction promoted formation of water-in-oil emulsions when added in a 10% concentration to Drakeol 6VR, a medicinal form of mineral oil. In contrast, the mannide dioleate fraction purified by magnesium silicate was a colorless oily liquid with an index of refraction of 1.473 and sp gr, 0.94. The dioleate fraction was less viscous than the monooleate fraction and, when added to medicinal mineral oil in a 10% suspension, led to production of oil-in-water emulsions which are the opposite type of those desired as adjuvants.

In addition, the monooleate fraction was more easily hydrolyzed than the dioleate fraction. When initially recovered from the magnesium silicate column, the monooleate fraction contained little free unbound acid as shown by an acid number[7] of 1.0, but after 3-years storage at 4 to 10°, the acid number increased to 16. In contrast, no free acid was found in the dioleate fraction either when freshly isolated or after 3-years storage. The monooleate fraction was also readily hydrolyzed when refluxed with sodium hydroxide. An ether extract of acidified hydrolysate yielded an oily material which accounted for 64% of the weight of the starting fraction and also had an acid number of 208. Pancreatic

[7] Number of milligrams of potassium hydroxide to neutralize an alcoholic solution equivalent to 1 g of lipid (6).

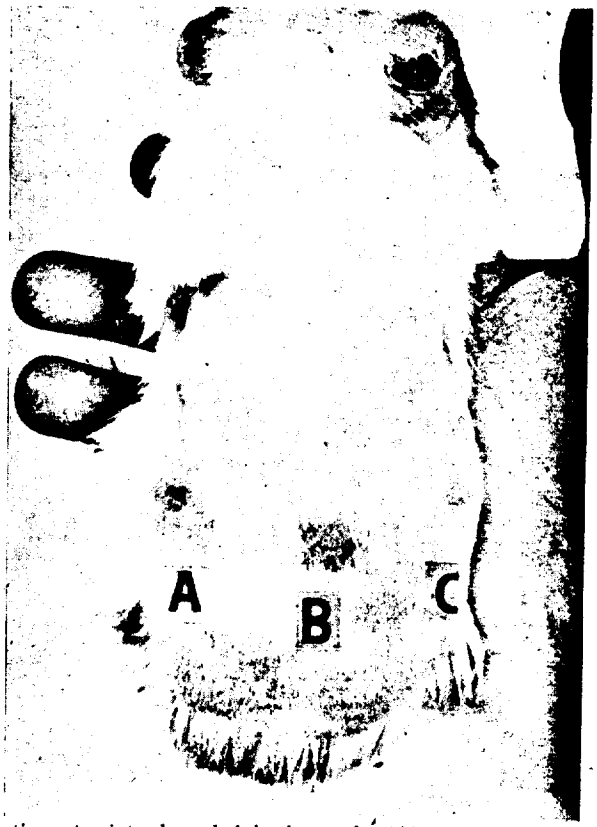

FIG. 1. Skin reactions to intradermal injections of: (A) component R_f approx. 0.2; (B) component R_f approx 0.5 (mannide monooleate fraction); and (C) component R_f approx 0.8 (mannide dioleate fraction).

lipase had greater lipolytic activity with the monooleate fraction than with the dioleate fraction, as shown by incubating each fraction at 37° overnight with the equivalent of 2 g of crude lipase[8]/g of lipid and measuring the amount of potassium hydroxide to neutralize the products of this hydrolysis. The equivalent of 150 mg of potassium hydroxide/g of monooleate fraction contrasted sharply with the value of 29 mg of potassium hydroxide/g of dioleate fraction.

D. Biologic properties. The mannide monooleate and mannide dioleate fractions were tested for intradermal toxicity in two guinea pigs using procedures based on previously described tests for biologic safety (2). Figure 1 is a photograph of the back of one guinea pig 24 hr after intradermal injection of 0.1-ml amounts of freshly obtained monooleate and dioleate fractions. The same volume of the unidentified fraction with a R_f of 0.2 was also tested in these guinea pigs. The monooleate and R_f-0.2 fractions caused severe inflammatory reactions and local tissue necrosis comparable to that seen in guinea pigs injected intradermally with toxic lots of Arlacel A or with oleic acid (2). In contrast, mannide dioleate had no greater effect than that observed in other tests following injection of acceptable batches of Specially-Treated Arlacel A. Figure 1, illustrates the greater toxicity of the R_f 0.2 and monooleate fractions. As expected, lesions caused by the toxic materials healed leaving noticeable scars.

In addition, a simple biological assay was employed to compare the tissue toxicity of the monooleate fraction with that of oleic

[8] Pancreatic Lipase, Sigma Chemicals, St. Louis, Missouri.

TABLE II. Biologic Assay of Isomannide Monooleate.

Solute/solvent ratio[a] (v/v)	Intradermal reaction; solute: Monooleate fraction a	Monooleate fraction b	Oleic acid a	Oleic acid b
Undiluted	18(N)[b]	25(N)	18(N)	20(N)
3:1	20	ND	18(N)	20(N)
1:1	20	20	20(N)	20(N)
1:3	10	15	18(N)	20(N)
Peanut oil alone	8	8	—	—

[a] Ratio of volume of isomannide monooleate fraction or oleic acid to volume of peanut oil: 0.1 ml intracutaneous.

[b] Recordings in two guinea pigs (a) and (b) denotes the diameter (ml) of the area of inflammation surrounding the site of injection at 3 days; N, the occurrence of local necrosis; ND, not done.

acid which might be a major irritant in undesirable batches of Arlacel A. Oily mixtures made with differing proportions of the monooleate fraction and peanut oil or mixtures made with similar proportions of oleic acid and peanut oil were injected in 0.1-ml amounts intradermally in the skin on the back of two guinea pigs and the local reactions were observed. Sites injected with undiluted monooleate fraction or with oleic acid developed the expected local inflammation and necrosis. The findings at 3 days (Table II), which were representative of the relative toxicity of the various materials injected, indicate that the toxicity of the monooleate fraction was rapidly diluted out by peanut oil, whereas that of oleic acid was not appreciably changed even when diluted fourfold with peanut oil. The interpretation given to these findings was that the monooleate fraction was less toxic than oleic acid by at least fourfold factor.

Discussion. These findings demonstrated that three fractions, at least, were obtained from Specially-Treated Arlacel A by fractional distillation and column chromatography. The principal products, the mannide monooleate and dioleate fractions, had disparate chemical and biologic properties. The monooleate fraction was more viscous, more dense, more readily hydrolyzed, and more toxic than the dioleate fraction. The finding suggests a possible direct relationship between the relative instability of the monooleate fraction and its toxicity in the skin of the guinea pig.

The product obtained by alkaline hydrolysis of the monooleate fraction is consistent with the assumption that this fraction consists largely of mannide monooleate. First, the acid number of the lipid recovered from the monooleate fraction approximated that for oleic acid (7). Second, the proportion by weight (64%) of the ether-soluble material recovered from the monooleate fraction approximated the proportion of oleic acid (mol wt, 282) in mannide monooleate (mol wt, 426).

A possible explanation for the tissue toxicity is that some or all of the components of the monooleate fraction are as readily hydrolyzed by tissue lipases *in vivo* as by pancreatic lipases *in vitro*. Presumably, the enzymic hydrolysis releases free fatty acids which then have direct toxic effects similar to those described by Freinkel (8). Thus it appears reasonable to expect that tissue toxicity of Arlacel A would be reduced if means could be found to prevent release of fatty acids. Evidently, those lots of Arlacel A which pass biologic tests for safety in animals do not exhibit significant toxicity in man despite their content of mannide monooleate. One explanation for the apparent lack of toxicity might be that mannide dioleate and other constituents of Arlacel A reduce the concentration of mannide monooleate in Arlacel A below the toxic level by simple dilution. In addition, these other components might also limit any enzymic hydrolysis as well as the inherent tendency of mannide monooleate to hydrolyze spontaneously.

Hardegree and Pittman (9) reported a correlation between the presence of free fatty acids in tetanus toxoid vaccines and other vaccines with the induction of abscess formation. They found that certain antigens like cholera and tetanus vaccines promoted the release of free fatty acids *in vitro* and surmised that the effect may have been due to a bacterial lipase. The susceptibility of purified mannide monooleate fraction observed in

the present studies and the ease with which it was hydrolyzed by a number of methods is consistent with the observation by Hardegree and Pittman and also suggests that it is the mannide monooleate in those vaccines which is acted upon by esterases and lipases *in vivo*.

These present studies also support an earlier concept for the production of stabilized emulsified systems. Water-in-oil emulsions may be stabilized if, in addition to an emulsifying agent which promotes that type of emulsion, a small amount of an emulsifying agent which promotes the opposite kind of emulsion is included (10). The mannide monooleate fraction served as a water-in-oil emulsifier, although it is toxic, whereas the dioleate fraction which minimized the toxicity served to produce oil-in-water emulsions, albeit an unstable one. Thus the mixture of the monooleate and dioleate fractions in Arlacel A works toward an optimal emulsifier.

Summary. Mannide monooleate and mannide dioleate fractions were obtained by vacuum distillation and column chromatography of Arlacel A. Different densities, stabilities, and different toxicities were found. The monooleate fraction was readily hydrolyzed by pancreatic lipase with consequent release of free oleic acid and it exhibited also severe tissue toxicity when injected intradermally into guinea pigs. In contrast, the dioleate fraction was not so susceptible to hydrolysis and showed minimal effects when injected intradermally. The findings support the hypothesis that the toxic effect of certain batches of Arlacel A is due to release of free oleic acid *in vivo*.

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UNCOUPLING ACTIVITY OF LONG-CHAIN FATTY ACIDS

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SUMMARY

1. At all pH values between 6.0 and 9.5 unsaturated long-chain fatty acids are more effective than the saturated acids in stimulating the latent ATPase activity of fresh rat-liver mitochondria, provided Mg^{2+} is added. Oleic, linoleic, linolenic, arachidonic and elaidic acids are about equally effective.

2. Whereas the saturated fatty acids scarcely stimulate above pH 8 the unsaturated acids are maximally active at pH 9.

3. Mg^{2+} was required for maximal stimulation by oleic acid. In the absence of added Mg^{2+} oleic acid completely inhibited the ATPase stimulated by 2,4-dinitrophenol.

4. Oleic acid concentrations that gave complete loss of ADP respiratory control or phosphate respiratory control lowered the P:O ratio with glutamate as substrate below 1. With palmitic acid loss of respiratory control was only partial.

5. Uncoupling by oleic acid could be reversed by adding serum albumin.

INTRODUCTION

The interest in the uncoupling activity of long-chain fatty acids stems from investigations of naturally occurring uncoupling agents. In 1952, PRESSMAN AND LARDY[1] isolated from microsomes a heat-stable, acetone-soluble fraction which stimulated the respiration of mitochondria in a medium deficient in phosphate acceptor, and stimulated the ATPase. In 1955, the active substance was identified as a mixture of long-chain fatty acids[2,3].

In 1956, POLIS AND SHMUKLER[4] and PULLMAN AND RACKER[5] described the formation in disintegrated, aged liver mitochondria of an uncoupler, whose action on fresh mitochondria could be counteracted by serum albumin. POLIS AND SHMUKLER[4] isolated the uncoupling agent in the form of a haemoprotein to which they gave the name mitochrome. Investigations[6-12] in this laboratory in 1958 led to the separation of mitochrome into two fractions: a haemoprotein almost free from uncoupling activity, and a lipid fraction containing a mixture of long-chain fatty acids which were found to be responsible for the uncoupling activity of mitochrome. It could be shown that the protein fraction, for which the name mitochrome has been retained, is derived from cytochrome.

Following his experiments on mitochrome HÜLSMANN[9] demonstrated that

uncoupling activity could be directly extracted with isooctane from aged rat-liver mitochondria. The formation of the uncoupler was shown to be an enzymic process stimulated by Ca^{2+} and inhibited by EDTA, ATP or diisopropyl phosphofluoridate. In similar experiments LEHNINGER AND REMMERT[13] found that an enzymically formed uncoupling factor (called U factor) could also be extracted with isooctane from aged mitochondrial fragments and they suggested that this factor might be a fatty acid. Further work on U factor, which in a preliminary analysis has been identified as a mixture of long-chain fatty acids, has recently been reported by WOJTCZAK AND LEHNINGER[14].

It was shown by HÜLSMANN[9] in 1958 (see also refs. 11 and 12) that the mixture of fatty acids extracted from a mitochrome preparation by isooctane consisted of 38.5 % saturated (C_{16}, 24 %; C_{18}, 10 %) and 61.5 % unsaturated (C_{18}, one double bond, 23 %; C_{18}, two double bonds, 10.5 %; C_{20} and C_{22}, highly unsaturated, 21 %). To determine which of these acids were responsible for the uncoupling action a series of pure long-chain fatty acids was tested for ability to stimulate the latent ATPase and uncouple oxidative phosphorylation of fresh rat-liver mitochondria. It was found that the unsaturated fatty acids as a group are far more potent as uncouplers than the saturated acids and the conclusion was drawn that the uncoupling action of the mitochrome preparation could be completely explained by the, predominantly, unsaturated long-chain fatty acids which it contained[11,12].

In this paper the experiments with pure fatty acids are reported in detail.

METHODS

Rat-liver mitochondria were prepared according to HOGEBOOM[15] as described by MYERS AND SLATER[16].

ATPase activities were measured as described by MYERS AND SLATER[16] at room temperature (22° ± 2°), in a reaction mixture containing 75 m*M* KCl, 50 m*M* Tris-acetate buffer, 0.5 m*M* EDTA, 2 m*M* ATP, 50 m*M* sucrose, 1.5 m*M* $MgCl_2$ and, when added, 0.1 m*M* 2,4-dinitrophenol. Fatty acids were added in 0.03 ml 96 % ethanol (final ethanol concentration, 2 %). Unless indicated otherwise all values were corrected for ATP breakdown in the presence of ethanol alone. Similarly the ATPase activity in the presence of dinitrophenol has been corrected for ATPase activity in its absence (no ethanol was present in most experiments with dinitrophenol).

P:O ratios were determined by the procedure of SLATER AND HOLTON[17]. The reaction mixture contained 14 m*M* KCl, 6 m*M* $MgCl_2$, 2 m*M* EDTA, 22 m*M* Tris-acetate buffer[16] (pH 7.5), 30 m*M* potassium phosphate buffer (pH 7.5), 0.3 m*M* ATP, 20 m*M* glucose, 100–200 Cori units of yeast hexokinase (prepared according to DARROW AND COLOWICK[18], omitting the final crystallization step*), 20 or 40 m*M* substrate, 50 m*M* sucrose and 0.03 ml 96 % ethanol containing varying amounts of fatty acids. The reaction was started by the addition of 0.1 ml mitochondrial suspension (2–4 mg protein). The final volume was 1 ml, the reaction temperature 25° and the reaction time varied between 14 and 24 min. Q_{O_2} values are expressed as μl/mg protein/h.

ADP respiratory control was determined by comparing the respiratory rate in this medium with that obtained in a medium from which the glucose and hexokinase

* We are grateful to Dr. R. A. DARROW and Dr. S. P. COLOWICK for giving us the details of their procedure prior to publication.

were omitted. The respiratory-control index is defined as the ratio of the respiratory rates in the presence and in the absence of a phosphate-acceptor system or an uncoupler. Phosphate respiratory control was determined similarly, as previously described[19].

The chemicals used were obtained from the same sources as described in previous publications of this series[11]. Oleic acid was a product of the British Drug Houses, Ltd. The other fatty acids were obtained from Fluka A.G. Except arachidonic acid they were all "puriss". According to the manufacturer the linoleic acid was 98–99 % pure and the linolenic acid 97–99 % pure; both acids were practically free of peroxides and they contained only traces of trans isomers. The arachidonic acid was only 65 % pure, the main contaminants being C_{20} and C_{22} unsaturated fatty acids. With oleic, linoleic, palmitic and myristic acid the concentrations of stock solutions were checked titrimetrically[20] and found to be 100 % pure within the limits of error of the procedure.

RESULTS

Adenosinetriphosphatase

Comparison of ATPase-stimulating capacity of different fatty acids at pH 6.5

In preliminary experiments with oleic acid widely different results were obtained with different enzyme preparations. Two reasons for this were found: (a) The stimulation of the ATPase by fatty acids is strongly dependent on the amount of enzyme protein used (*cf.* ref. 3). Better reproducibility was obtained when the concentration of oleic acid was expressed as μmoles/g enzyme protein, at least in the range of protein concentrations (0.2–0.4 mg protein/tube) used in the ATPase experiments. (b) The maximal ATPase activity per milligram protein that can be obtained with oleic acid varies with different mitochondrial preparations. Since stimulation by dinitrophenol varied correspondingly, we have expressed the ATPase activity obtained with fatty acids as percentage of that obtained with 0.1 m*M* dinitrophenol.

Fig. 1 shows the concentration curves obtained with oleic and stearic acid. Curves similar to that of oleic acid were obtained for elaidic (contrast ref. 3), linoleic and linolenic acids*; a curve, qualitatively similar to that of stearic acid, was obtained for myristic, palmitic and octanoic acids and for Tween 21. There were, however, quantitative differences: at low fatty acid concentrations myristic acid stimulated the ATPase more than palmitic or stearic acid (*cf.* ref. 3). From these curves two sets of values have been calculated: the amount of fatty acid necessary to stimulate the ATPase to 50 % of that obtained with 0.1 m*M* dinitrophenol, and the stimulation given by 500 μmoles fatty acid/g protein, also expressed as a percentage of the stimulation by dinitrophenol. It is clear from the results presented in Table I that the unsaturated long-chain fatty acids stimulate the ATPase far more than the saturated acids. The degree of unsaturation and the position of the double bond appears to have little effect. Deoxycholic acid is a little more active than the saturated fatty acids; the activity shows a maximum (equal to 60–90 % of that obtained with 0.1 m*M* dinitrophenol) at 1000–1500 μmoles deoxycholate/g protein whereas higher concentrations inhibit. With fatty acids inhibition is never observed at pH 6.5 even when concentrations up to 5000 μmoles/g protein are added.

* In most experiments with the unsaturated fatty acids a more pronounced S-shaped concentration curve was obtained.

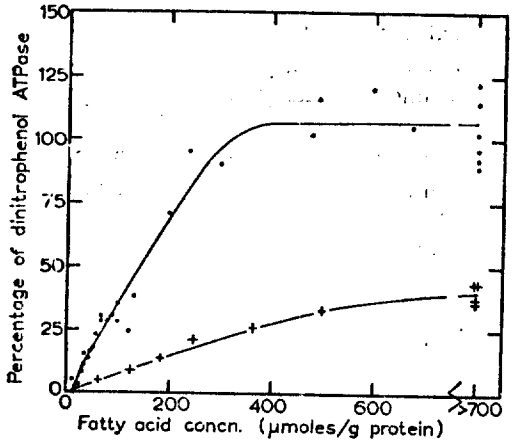

Fig. 1. Stimulation of ATPase by oleic and stearic acid at pH 6.5. 1.5 mM $MgCl_2$ present. ●—●, oleic acid (points obtained in experiments with 5 mitochondrial preparations); +—+, stearic acid (1 experiment). The ATPase activity is expressed as a percentage of the value obtained in the presence of 0.1 mM 2,4-dinitrophenol.

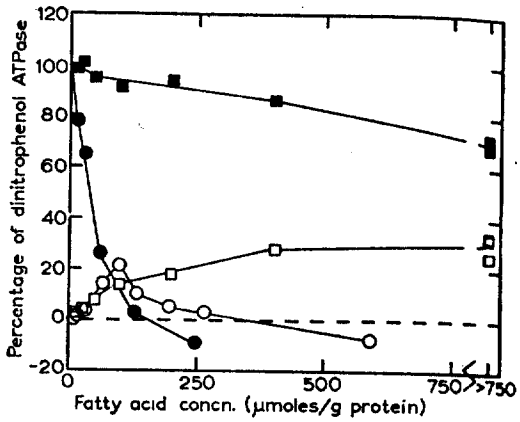

Fig. 2. Stimulation of ATPase and inhibition of dinitrophenol-stimulated ATPase in the absence of Mg^{2+} by oleic and palmitic acid. No $MgCl_2$ present; pH 6.5. ○—○, oleic acid; □—□, palmitic acid; ●—●, oleic acid and 0.1 mM dinitrophenol; ■—■, palmitic acid and 0.1 mM dinitrophenol. The values were obtained in 4 different experiments. The two negative values for high oleic acid concentrations are due to inhibition of ATPase in the presence of ethanol by oleic acid. If the values are only corrected for P_i present in the zero-time controls these two values are zero. The ATPase activity is expressed as in Fig. 1.

TABLE I

STIMULATION OF ATPase BY DIFFERENT FATTY ACIDS AND DEOXYCHOLIC ACID

ATPase was measured at pH 6.5 in the presence of 1.5 mM $MgCl_2$. The values given are calculated from concentration curves similar to those of Fig. 1.

Compound	*μmoles/g protein giving 50% stimulation**	*Percent stimulation* obtained with 500 μmoles/g protein*
Unsaturated		
Oleic acid	140	106
Linoleic acid	120	103
Linolenic acid	160	108
Arachidonic acid		109
Saturated		
Octanoic acid	—	1
Palmitic acid	950	25
Stearic acid	—	33
Deoxycholic acid	590	38

* 100% = ATPase with 0.1 mM dinitrophenol.

Effect of Mg^{2+}

In the experiments summarized in Fig. 2, $MgCl_2$ was omitted from the reaction medium. With palmitic acid the maximal stimulation of the ATPase was the same in the presence or absence of $MgCl_2$ (33 %). With oleic acid, however, complete inhibition occurred at higher concentrations of the acid in the absence of Mg^{2+} and the maximal ATPase activity was only 20 % of that obtained with dinitrophenol. A corresponding difference between oleic and palmitic acids was found when increasing amounts of fatty acid were added in the presence of dinitrophenol and absence of Mg^{2+}. Low concentrations of oleic acid inhibited the dinitrophenol-induced ATPase more than 100 % while palmitic acid was less effective as inhibitor. In the presence of $MgCl_2$ neither fatty acid had any effect on the dinitrophenol-induced ATPase. This suggests that the same enzyme system is responsible for ATPase activity induced by fatty acids and by dinitrophenol.

The dependence of the ATPase activity induced by oleic acid on added $MgCl_2$ for maximal activity was found at all pH values between 6 and 9 and similar results have been obtained with linoleic and linolenic acid. The ATPase activity induced by palmitic acid is independent of $MgCl_2$ only in the acid range. At pH values above 7, $MgCl_2$ is required for maximal activity. Similar results were obtained for myristic and stearic acid.

It should be noted that in these experiments only one $MgCl_2$ concentration (1.5 m*M*) was used. We have found that higher $MgCl_2$ concentrations have little effect on the ATPase activity induced by oleic acid at pH 6.5 but strongly inhibit at pH 9.0. Further work is required to establish the optimal Mg^{2+} concentration for different concentrations of fatty acids at all pH values.

Influence of pH

When a concentration of oleic acid sufficient for maximal stimulation of the ATPase activity at pH 6.5 was tested at other pH values, a typical and reproducible curve was obtained with maxima at 6.5 and 9* and a minimum at pH 7.5–8 (Fig. 3). Similar curves were obtained for linoleic, linolenic and arachidonic acids, and the fatty acids from mitochrome (*cf.* Fig. 2 of ref. 11). The saturated fatty acids, myristic, palmitic and stearic acid, however, induced very little activity in the alkaline region.

The form of the pH-activity curve obtained with oleic acid markedly depends upon the concentration of the oleic acid. Fig. 4 shows that at higher oleic acid concentrations the trough at pH 8.0 shown in Fig. 3 is filled up, and that at concentrations over 2000 μmoles/g protein inhibition sets in above pH 7.5, becoming more marked with increasing pH.

In Fig. 5 the maximal ATPase activity induced by oleic acid is plotted as a function of pH and the curve is compared with the pH-activity curve of the ATPase activity of aged mitochondria. The resemblance between the two curves is striking.

Oxidative phosphorylation

P:O ratio and respiratory control

In 1959 REMMERT AND LEHNINGER[21,22] described a factor, isolated from sonicated rat-liver mitochondria, which promoted loss of respiratory control in digitonin par-

* The maximum in the alkaline region may be somewhat underestimated since in most experiments more than 25 % of the ATP was used up during measurements of the ATPase activity.

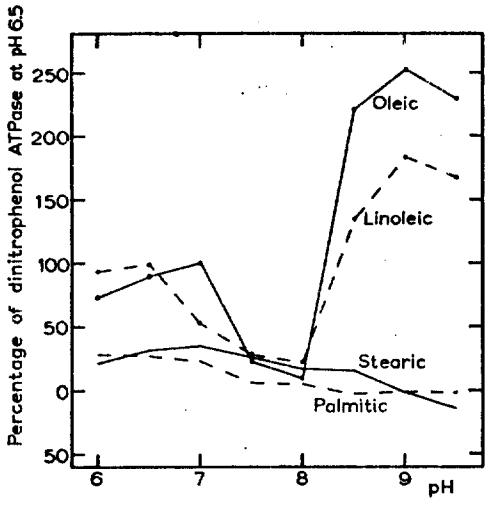

Fig. 3. pH-activity curves of ATPase stimulated by intermediate concentrations of fatty acids. 1.5 mM $MgCl_2$ present. The ATPase in the presence of 0.1 mM dinitrophenol at pH 6.5 has been set at 100. The values were obtained in 4 different experiments. ●—●, oleic acid, 430 μmoles/g protein; ●---●, linoleic acid, 340 μmoles/g protein; ---, palmitic acid, 890 μmoles/g protein; —stearic acid, 2040 μmoles/g protein.

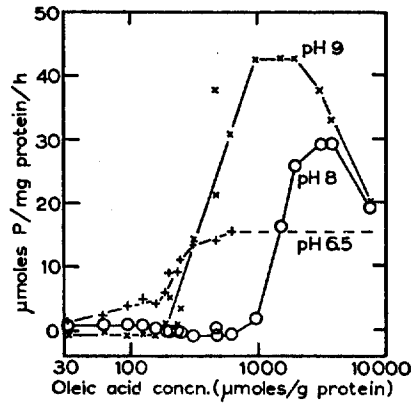

Fig. 4. Effect of oleic acid concentration on ATPase at pH's 6.5, 8 and 9. 1.5 mM $MgCl_2$ present. The values were obtained in two different experiments, the figures of which overlap between 100 and 1000 μmoles oleic acid/g protein. Note logarithmic plot of oleic acid concentration.

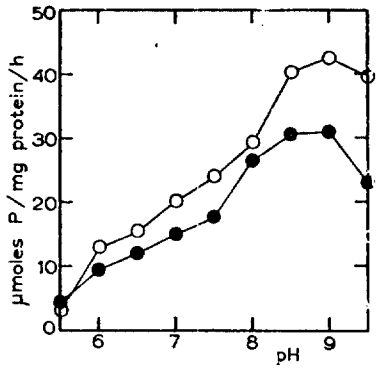

Fig. 5. Comparison of the pH-activity curves of the ATPase of aged mitochondria and the oleic acid-induced ATPase of fresh mitochondria. ●—●, liver mitochondria that had been aged and then subjected 5 times to freezing and thawing (taken from Fig. 5 of ref. 16); ○—○, maximal ATPase of fresh rat-liver mitochondria that can be obtained in the presence of oleic acid (Mg^{2+} present). The values are taken from the curves of Fig. 4 and similar curves.

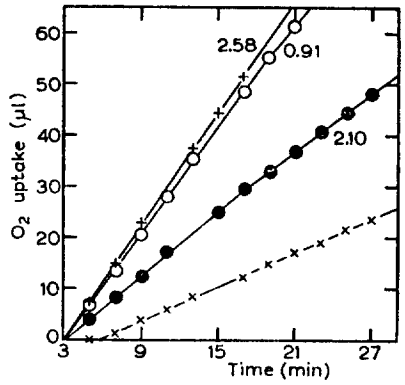

Fig. 6. Effect of oleic acid on ADP respiratory control with glutamate as substrate. Respiration measured in ADP-deficient reaction mixture with 20 mM L-glutamate as substrate, and the following special additions: ×—×, 3% ethanol; ●—●, 32 μg oleic acid + 3% ethanol; ○—○, 64 μg oleic acid + 3% ethanol; +—+, 20 mM glucose + 150 Cori units yeast hexokinase + 3% ethanol. In parallel flasks P:O ratio's were determined in the presence of glucose and hexokinase, and corresponding amounts of oleic acid; the results are indicated in the figure.

ticles without lowering the P:O ratio. As the procedure used for preparing this R factor was rather similar to procedures used in the isolation of mitochrome, we have investigated whether the properties of the R factor could be duplicated by fatty acids. Since β-hydroxybutyrate, the substrate used by REMMERT AND LEHNINGER, gave low Q_{O_2} values and poor respiratory control in our hands, glutamate was used as the substrate (*cf.* refs. 19 and 23). With this substrate ADP-respiratory-control ratio's varying between 3 and 6 were obtained. These values are a little below normal owing to the 3 % ethanol and the relatively high concentration of adenine nucleotide present, which tend to increase the rate of O_2 uptake measured in the absence of ADP. All experiments were performed at pH 7.5. An example of these experiments is given in Fig. 6. In this and similar experiments it was found that some stimulation of respiration* (measured in the absence of phosphate acceptor) could be obtained with oleic acid without a marked decrease in the P:O ratio (measured in the presence of phosphate acceptor) but that complete or near-complete loss of respiratory control was always associated with a decrease of the P:O ratio to below 1.0, unlike the results reported for the R factor[22]. Oleic acid added in the presence of 1 % serum albumin gave results similar to those obtained with oleic acid alone, the only difference being that 3–6 times more oleic acid had to be added for complete loss of respiratory control. Even high concentrations of palmitic acid gave only partial loss of respiratory control. The results of a number of experiments, in which the P:O ratio and respiratory control were measured simultaneously, are summarized in Fig. 7.

With L-proline as substrate oleic acid in concentrations sufficient to abolish ADP respiratory control also abolished P_i respiratory control (*cf.* ref. 19). Thus, as is also the case with dinitrophenol as uncoupling agent[19], respiration uncoupled by oleic acid does not require P_i.

In an attempt to see whether the different phosphorylating steps in the respiratory chain differ in their susceptibility to uncoupling by fatty acids we have compared the effect of oleic acid on the phosphorylation associated with succinate and β-hydroxybutyrate oxidation. The differences found were small and we do not attach much significance to them.

Reversibility of uncoupling by oleic acid

Partial reversibility of uncoupling by mitochrome[4,5] and the dried acetone extract from rat-liver microsomes[3] has been demonstrated. Table II shows that this

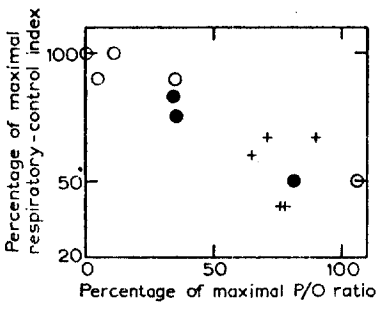

Fig. 7. Correlation between loss of respiratory control and loss of oxidative phosphorylation in the presence of oleic and palmitic acid. The effect of fatty acids on respiratory control and P:O ratio was determined in parallel flasks as shown in Fig. 6. The maximal respiratory-control index is the Q_{O_2} in the presence of glucose + hexokinase + ethanol divided by the Q_{O_2} in the presence of ethanol alone. Since this index was around 5 in most experiments 20 % is taken as the base line. The maximal P:O ratio is the P:O ratio in the presence of ethanol. ●, different oleic acid concentrations; ○, different oleic acid concentrations with 1 % serum albumin added; +, different palmitic acid concentrations. The values were obtained in experiments with 7 different mitochondrial preparations.

* For the purpose of this article it is of no importance whether the electrons were derived from the oxidation of glutamate or of the fatty acids added. In the absence of glutamate the average Q_{O_2} with oleic acid was 13, with palmitic acid 34.

is also true for uncoupling by oleic acid. Even after the mitochondria had been in contact with the oleic acid for 10 min at 25°, almost complete reversal of the uncoupling was obtained by adding serum albumin, which is known strongly to bind long-chain fatty acids[24,25]. Egg albumin was ineffective. Comparable results have recently been obtained by HELINSKI AND COOPER[26] in an extensive investigation of the effect of albumin on aged rat-liver mitochondria. The stimulatory effect of oleic acid on the ATPase was also reversed by the subsequent addition of albumin, even at pH 9.0. However, complete reversal was not obtained.

TABLE II

REVERSIBILITY OF UNCOUPLING BY OLEIC ACID

After 10 min the reaction was stopped in vessel 1, while vessels 2, 3, 4 and 7 were tipped as indicated. After 20 min vessels 2–7 were stopped. The ΔP and ΔO of vessels 2, 3, 4 and 7 have been calculated for the period after the tip by subtracting the ΔP of vessel 1 in the case of vessels 2, 3 and 4 and half of the ΔP of vessel 6 in the case of vessel 7. The latter correction was negligible.

Vessel	*Substrate*	*Oleic acid (μg)*	*Tip*	*ΔO (μatoms)*	*ΔP (μmoles)*	*P:O*
1	L-Glutamate (20 m*M*)	64	—	2.94	2.31	0.79
2	L-Glutamate (20 m*M*)	64	Water	2.78	2.11	0.76
3	L-Glutamate (20 m*M*)	64	1% Serum albumin	2.87	5.99	2.09
4	L-Glutamate (20 m*M*)	64	1% Egg albumin	2.53	2.06	0.81
5	Succinate (40 m*M*)	0	—	2.40	4.15	1.73
6	Succinate (40 m*M*)	32	—	0.93	0.01	0.01
7	Succinate (40 m*M*)	32	1% Serum albumin	1.68	2.40	1.43

DISCUSSION

The results reported in this paper have revealed four differences between oleic and palmitic acid with respect to their uncoupling activity: (a) In the presence of Mg^{2+} oleic acid induces a much greater ATPase activity at all pH values between 6 and 9.5. (b) In the same pH range maximal ATPase stimulation by oleic acid is completely dependent on the addition of Mg^{2+} to the medium. For palmitic acid this is only necessary at pH values above pH 7. (c) The ATPase induced by dinitrophenol in the absence of Mg^{2+} is completely inhibited by low concentrations of oleic acid; palmitic acid inhibits only partially even at high concentrations. (d) While oleic acid can bring about complete loss of ADP respiratory control and phosphorylation, palmitic acid can not.

These conclusions differ in two respects from those of PRESSMAN AND LARDY[3], who studied activation of ATPase by fatty acids at pH 7.5. In the first place, these authors did not find any marked differences between the effects of saturated and unsaturated fatty acids. Secondly, they did not find any effect of Mg^{2+} on the ATPase activity induced by oleic acid. Inspection of their Table II suggests, however, that this was only tested at low oleic acid concentrations. As Fig. 2 of the present paper shows, at low oleic acid concentrations no Mg^{2+} requirement can be detected. Apart from these differences our results and those of PRESSMAN AND LARDY[3] supplement one other quite satisfactorily. Table I and Fig. 2 of their paper give the relative activities of the long-chain fatty acids at low concentrations, whereas our Table I shows the relative activities at high concentrations in the presence of Mg^{2+}.

In view of the reversibility of the uncoupling by oleic acid, the inhibition of the dinitrophenol-induced ATPase by oleic acid in the absence of added Mg^{2+} can be most simply explained by assuming that oleic acid binds the intramitochondrial Mg^{2+} necessary for the dinitrophenol-induced ATPase (*cf.* ref. 27). The concentration of oleic acid required for complete inhibition is of the same order of magnitude as the Mg^{2+} concentration in fresh rat-liver mitochondria[28] (70 μmoles/g protein).

In recent years several uncouplers (or respiratory inhibitors) isolated from natural materials have been shown to contain long-chain fatty acids as the active material, *e.g.* the extract from rat-liver microsomes[3], mitochrome[11], inhibitors of electron transport isolated from rat-intestinal mucosa[29] and *Tetrahymena pyriformis*[30], and uncoupling agents present in insect sarcosome preparations[31,32]. Furthermore, the beneficial effect of serum albumin on the oxidative phosphorylation of freshly isolated tumour mitochondria[33-35] and skeletal-muscle sarcosomes[36,37] indicates that free fatty acids are possibly present in these preparations. The lipid uncoupling material present in hepatomas is under investigation by EMMELOT and coworkers[38§].

Serious consideration should be given to the possibility that other naturally occurring uncoupling agents (or respiratory inhibitors) which have been described in the literature contain fatty acids, perhaps bound to protein as in the mitochrome preparation of POLIS AND SHMUKLER[4]. The following characteristic properties of the long-chain fatty acids should make it easy to test this possibility: (a) the uncoupling activity is extracted into isooctane from acid or neutral aqueous solutions and is reextracted into the aqueous phase by alkali; (b) the activity is counteracted by serum albumin; (c) unsaturated fatty acids inhibit the dinitrophenol-induced ATPase activity of rat-liver mitochondria in the absence of Mg^{2+}; (d) they stimulate the ATPase in the presence of Mg^{2+}, giving the characteristic pH-activity relationship shown in Figs. 3 and 4. In addition long-chain fatty acids bring about loss of respiratory control, lower the P:O ratio, inhibit the ATP–$^{32}P_i$ exchange reaction[6,13,39], induce swelling of fresh rat-liver mitochondria[13,40] and inhibit the respiratory chain when added in higher concentrations[41*,43].

Uncoupling by fatty acids may be distinguished from that brought about by agents breaking up mitochondrial structure in that it is readily reversible by the subsequent addition of serum albumin. The ready reversibility of this uncoupling draws attention to the possible role of unsaturated fatty acids as regulators of oxidative metabolism. This possibility has been discussed by PRESSMAN AND LARDY[3], HÜLSMANN and co-workers[7,9,11] and LEHNINGER and co-workers[13,14]. It is further supported by the evidence of SCHOLEFIELD and co-workers[44-46] that fatty acids (only saturated acids were studied) may exert their uncoupling effects in intact ascites tumour cells.

Whether or not the uncoupling activity of unsaturated fatty acids has any physiological role, it is clear that it can be of importance in biochemical experiments *in vitro*.

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§ *Note added in proof*: In a paper[17] which appeared in January, 1962, and which reached the authors since submitting their paper, EMMELOT confirms that oleic acid inhibits the dinitrophenol-induced ATPase in the absence of Mg^{2+} and demonstrates that the low phosphorylative activity of mitochondria isolated from certain hepatomas is due to the presence in the mitochondrial preparation of unsaturated fatty acids.

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* LASER's haemolytic substance studied in this paper was later found to be *cis*-vaccenic acid[12].

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THE CARCINOGENICITY OF CHRYSENE AND OLEIC ACID

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In several of our previous publications we have referred to experiments with chrysene which indicated that this substance had a low degree of carcinogenicity for mice, and that in combination with oleic acid, itself apparently capable only of inducing benign tumour formation, we had a mixture which had a definitely greater activity than either of its constituents applied separately. Prior to our experiments Kennaway (1) had tested chrysene on mice, and came to the conclusion that it was inactive. Quite recently Barry and Cook (2) report experiments which lead them to doubt whether our results were not really due to impurities in our chrysene, and to question whether this particular hydrocarbon is *per se* carcinogenic.

Subsequent to our investigations on chrysene and oleic acid, Kennaway and his collaborators (3, 4, 5, 6), in a brilliant series of experiments, reported the isolation and synthesis of several pure hydrocarbons which have a carcinogenic activity for mice immeasurably greater than that of chrysene. The importance of these observations was obvious to all workers on cancer research, and they were of particular interest to us as they supported our experiments with chrysene, and our ideas with reference to the possible significance of the phenanthrene nucleus in the induction of cancer (7).

The earliest experiments on chrysene in these laboratories were carried out with a commercial sample which was definitely yellow (8, 9). The main impurity in this case would probably be naphthacene (2, 3 benzanthracene), which, it is now thought, is the "chrysogene" of Fritzsche (10), the contaminating substance giving the yellow colour to coal-tar chrysene. Naphthacene appears to be non-carcinogenic (9).

In another series of experiments we utilised chrysene isolated from one of the less carcinogenic fractions of our pinene tar (11). Fraction 11, one of the fractions rejected in the concentration of carcinogenic substances, consisted mainly of anthracene, but contained a fair amount of chrysene (over 5 per cent). Extraction with carbon disulphide removed the anthracene and the small quantity of tarry matter, leaving crude yellow chrysene of m.p. 240–244°. Crystallisation from toluene raised the m.p. to 245–246°. Refluxing with alcohol containing 3 to 4 per cent nitric acid removed most of the colour, giving a cream-coloured product, and a further crystallisation from toluene or pyridine raised the m.p. to 247–249°.

Recently we have found that the crude chrysene from our tar can be almost completely decolourised by refluxing six hours with maleic

anhydride in xylene, though boiling for one hour had relatively little effect (*cf.* Cook et al., 5). The acidic compounds formed have been decomposed by heat to regenerate the hydrocarbons. So far naphthacene has not been found, but an unidentified hydrocarbon of m.p. 286–289° has been isolated. This has only a pale yellow colour, dissolves readily in hot benzene, the solution having a strong blue fluorescence, and does not form a stable picrate in benzene.

In practically all the later experiments we used a sample of chrysene obtained from Kahlbaum, designated "Puriss". This was perfectly white, of m.p. 248–250° (or 251–253°, corrected for stem exposure). Its 2.7 dinitroanthraquinone compound had m.p. 295–296° (uncorr.).

In order to obtain a still purer product we proceeded as follows. "Pure" chrysene (Kahlbaum, 20 gm.) was ground to a paste with concentrated sulphuric acid (25 c.c.), and allowed to stand three hours at room temperature. After dilution with water the unchanged material was filtered off, washed, and dried. The acid filtrate yielded 1.8 gm. of barium salt which was not investigated.

The unchanged chrysene (18.7 gm.) was sulphonated by warming five hours on the steam bath with concentrated sulphuric acid (30 c.c.). Some oxidation occurred (evolution of sulphur dioxide). The solution was diluted, filtered from a trace of unchanged chrysene, and neutralised by addition of barium carbonate. Evaporation of the filtered solution gave 43.8 gm. of mixed barium sulphonates. Crystallisation from water gave a sparingly soluble salt B (9.2 gm.), and fractionation of the remainder finally yielded 16 gm. of a moderately soluble salt C, together with mixtures which were subsequently combined with corresponding fractions from another sulphonation and further fractionated into B and C.

B, dried at 100°, gave Ba = 24.35%. Loss in weight at 135° = 4.4%. $C_{18}H_{10}(SO_3)_2Ba \cdot 2H_2O$ requires Ba = 24.54%, H_2O = 6.44%. The salt is probably the dihydrate, the second molecule of water being difficult to remove.

C, dried at 100°, appeared to have lost water and to be anhydrous.

Found, Ba = 26.36%. $C_{18}H_{10}(SO_3)_2Ba$ requires Ba = 26.23%. B and C are, therefore, the barium salts of isomeric disulphonic acids of chrysene.

C was converted to the ammonium salt by treating with the equivalent amount of ammonium sulphate. The ammonium salt was very readily soluble in water, and the aqueous solution appeared to undergo some oxidation, becoming red on evaporation in an open basin. Addition of ether to an aqueous-alcoholic solution precipitated coloured salt first, the later fractions being perfectly white. The white product was redissolved and again fractionally precipitated.

Found, N = 6.61 (analysis by Mr. F. Consterdine). Calc. for anhydrous salt N = 6.64. The salt was hygroscopic.

The sodium salt was prepared by treating the above purified ammonium salt with the theoretical quantity of sodium hydroxide solution, and purified by crystallising twice from dilute alcohol. Chrysene was

regenerated from the purified sodium disulphonate by the action of superheated steam in the presence of syrupy phosphoric acid. It distilled over at 180–200° (thermometer in liquid), forming a bulky mass of white crystals of m.p. 249–250°. Crystallisation from pyridine, then from toluene, raised the m.p. to 251–251½° (254½–255° corr).

This regenerated chrysene was applied to the mice in liquid paraffin (2 per cent suspension; solubility = about 0.1 per cent), since we regarded the test as crucial, and therefore requiring a definitely negative diluent. The result, given in Table I, shows that this sample is defi-

TABLE I

Agent and Diluent	Week of 1st Tumour		Total Number of Tumours		Duration of Experiment in Weeks	Carcinogenic Potency
	B	M	B	M		
Biweekly applications						
7.5% Chrysene, etc., impure, in L.P.	45	45	1	2	60	+
" " " " " " "	44	—	1	0	60	+
" " " " " " "	—	—	0	0	30	—
" " residue " " " "	33	—	2	0	50	+
" " " " " O.A.	9	50	5	2	85	7
" " pure in L.P.	19	65	1	1	70	+
Five applications per week						
7.5% Chrysene pure in L.P.	38	48	6	1	78	4
" " " " O.A.	23	40	18	9	50	9
0.15% " " " " " "	21	—	10	0	60	7
" Tar B/19 in O.A.	22	38	10	3	60	6
0.4% Chrysene pure in benzene	36	—	1	0	60	+
" " and 0.4% O.A. in benzene	46	56	2	1	60	+
2.0% " , regenerated in L.P.	57	77	4	1	92	+
Ten applications per week						
0.15% Chrysene pure in O.A.	11	—	8	0	60	10
0.15% Tar B/19 in O.A.	11	—	5	0	55	23
2.0% Chrysene pure in O.A.	12	47	19	1	55	20
Breast applications						
0.15% Chrysene pure in O.A., Ma	—	—	0	0	60	—
" " " " " " Fe	49	—	2	0	70	+
" Tar B/19 in O.A., Ma	39	43	1	2	60	+
" " " " " " Fe	37	46	6	3	60	+

B = Benign tumour
M = Malignant tumour
L.P. = Medicinal liquid paraffin
O.A. = Oleic acid
Ma = Males
Fe = Females

nitely, if very slightly, carcinogenic. Owing to the tedious nature of the operations and the small yield over the whole cycle, the amount of regenerated chrysene available at any time was small, and it was therefore applied to the mice more sparingly than was the case with most of the other samples tested. The indicated potency is therefore probably lower than it should be, and there is probably little, if any, difference between the activity of this highly purified chrysene and that of the less pure samples employed in the other experiments.

We are fully aware of the difficulty of establishing the purity of complex hydrocarbons. Melting points can be deceptive, since some

mixtures show no appreciable depression, but such cases are rare and it seems unlikely that a mixture could be taken practically unchanged through the cycle of operations described above. The activity of the regenerated chrysene confirms our previous opinion that chrysene is genuinely carcinogenic. We think we are also justified in assuming that the chrysene used in the bulk of our experiments was of a degree of purity comparable with that of the material used by Barry and Cook (loc. cit.), and that the failure of these workers to obtain malignant tumours in mice can be attributed to more or less fortuitous circumstances. Data relating to some of our experiments are given in Table I.

As we have remarked on more than one occasion, the fact that the activity of chrysene is enhanced by the presence of oleic acid is of the first importance in view of the wide distribution of this acid in living organisms. That we have observed no more than about a dozen benign tumours among 1,000 animals painted with oleic acid prohibits our designating the acid itself as a cancer producer, although we shall not be surprised to meet, sooner or later, with a tumour showing frank malignancy. Meanwhile, then, we prefer to consider chrysene as carcinogenic and oleic acid as a sensitizer, and we cannot see how our experimental results can be interpreted in any more rational manner.

Although chrysene is so insoluble in our diluents, it nevertheless appears that a thick paste is more active than a saturated solution. It may be argued that this indicates the presence of highly potent contaminating materials, but if it were so one may ask why a higher percentage of the agent in benzene shows itself to be so little active. Let us consider for a moment some experiments with a synthetic tar and dibenzanthracene when applied in a solution of benzene and oleic acid.

It is interesting to note that fraction 19 of our pinene synthetic tar, which as a 1 per cent solution in chloroform will give rise to from 75 to 100 per cent of malignant tumours in mice, in twenty-five weeks, does not show itself to be very much more active than chrysene when applied as a 0.15 per cent dilution in oleic acid, but we know that it is infinitely more powerful when applied as a low dilution in a volatile solvent such as benzene. We have ourselves synthesised 1:2:5:6-dibenzanthracene for these experiments, and although they are not yet completed we have had some interesting results. A 0.15 per cent solution of dibenzanthracene in benzene appears to have a carcinogenic potency a little greater than that of fraction 19 of our pinene synthetic tar, but a similar dilution of the two agents in oleic acid shows a remarkable difference in activity. The dibenzanthracene in this instance is apparently much more powerful than the tar as a cancer producer, a fact quite in keeping with our contention of the probable importance of oleic acid in the induction of spontaneous cancers in the animal kingdom. We wish to emphasize this point because it is obvious that, under natural conditions, the carcinogenic agents would presumably not be acting in an environment largely composed of volatile hydrocarbons but in a medium which, in our opinion, is possibly relatively rich in oleic and allied acids. It is probable that the frequency of cancer

among animals is not only governed by the intrinsic cancer susceptibility and the concentration of particular carcinogenic extrinsic substances containing the phenanthrene nucleus, but also by the relative concentration of the unsaturated and saturated fatty acids and fats. We have shown that fats are inhibitors of skin cancers, and it is probable that they are also inhibitors of internal cancers, but, of course, not so if the contained fatty acids are set free. We may remark that, while under certain circumstances unsaturated fatty acids hasten cancer of the skin, saturated fatty acids, such as stearic acid, may inhibit. In one of our experiments addition of a 1 per cent stearic acid to shale oil resulted in a relative potency of only 48.

TABLE II

Material	Site of Injection	Last Animal Died Week	No. of Animals
0.2 c.c. Mineral oils	Subcutaneous	80	200
" " "	Intraperitoneal	90	230
" "	Intracerebral	44	14
0.15 c.c. Tar B/10, 5% in olive oil	Subcutaneous	35	50
" " " " " " "	Intraperitoneal	45	50
0.5 c.c. Chrysene, 0.4% in lard	Subcutaneous	45	50
" " " " "	Intraperitoneal	50	50
0.2% Shale oil	Subcutaneous	45	100
" " "	Intraperitoneal	45	100

The fact that Barry and Cook succeeded in getting four sarcomas in rats injected subcutaneously with chrysene in lard in itself rather suggests that chrysene might be carcinogenic for the skin of mice, although, as these authors point out, the sarcoma- and carcinoma-producing activity of some of their agents do not run parallel. As a matter of fact, this probably depends chiefly on the difference in susceptibility of the rat and the mouse, and we should not expect two agents to act relatively in a similar degree in producing sarcomas and carcinomas unless we were using a single variety of animal. We ourselves have found that two agents having a similar carcinogenicity for the skin of mice may act quite differently as regards the amount of epithelial hyperplasia produced when applied to the skin of rats.

In these laboratories efforts to obtain sarcomas in mice by injection of carcinogenic agents during the last five years have failed consistently. Some of the data relating to these experiments are given in Table II, the animals receiving one injection only.

SUMMARY

1. Chrysene is a very lowly carcinogenic agent for the skin of mice.

(*a*) No tumours were obtained on subcutaneous or intraperitoneal injection in lard.

(*b*) No malignant skin tumours were obtained with a solution in benzene except on the addition of a small quantity of oleic acid.
(*c*) A few late skin tumours were obtained with a solution in liquid paraffin.
(*d*) A definitely increased number of tumours was obtained with a solution in oleic acid.
(*e*) The thicker the paste used the greater the yield of tumours.

2. 1:2:5:6-dibenzanthracene so far as tested by us has qualitative action similar to chrysene, while quantitatively immeasurably greater.

3. The action of our pinene synthetic tar not only varies quantitatively according to the concentration of the active substances, but, compared with chrysene and dibenzanthracene, also in some respects qualitatively. The latter is especially noticeable when oleic acid is used as solvent.

4. Pure oleic acid (less than 6 per cent saturated fatty acids) induced occasional benign tumours only.

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THE EFFECTS OF SESAME OIL AND FRACTIONS OF SESAME OIL ON ADRENALECTOMIZED AND OTHER EXPERIMENTAL RATS

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SESAME OIL has been reported to be inactive when used in numerous experiments as a control oil or as a vehicle for fat-soluble substances. There are indications, however, that it may not be inactive under all conditions. Pollia (1) reported that when rats bearing sarcomas were injected intraperitoneally with sesame oil, there was marked damage to the viscera. Spurr and Kochakian (2) found that subcutaneous injections of sesame oil twice daily shortened the mean survival period of immature adrenalectomized male rats by 1.6 days as compared with untreated controls. They attributed this effect to the handling and treatment, noting that "handling and trauma are deleterious factors known to shorten the survival of adrenalectomized animals." Sandholzer (3) found that sesame oil was effective in prolonging the survival time of mice that were given a fatal injection of Friedländer's bacillus. Tobin (4) found that sesame oil definitely prolonged the survival time of adrenalectomized pregnant rats that had successful gestation, and slightly enhanced their lactation.

To determine whether sesame oil was satisfactory as a control oil or as a vehicle for fat-soluble substances, its possible effects on normal and experimental rats were ascertained first. Later the effects of fractions of sesame oil were studied on adrenalectomized rats. For comparative purposes the effects of wheat germ oil, synthetic α-tocopherol,[1] synthetic tricaproin,[1] and pure linoleic acid[1] were studied on adrenalectomized rats.

All the animals used in these experiments were albino rats from our colony, originally of the Wistar strain. They were kept in uncrowded cages containing wood shavings, in a thermostatically regulated room at a temperature of 80° F. Purina Fox Chow, a commercial food, and tap water were supplied ad libitum. Most of the operations were performed under sodium amytal anesthesia. When needed this anesthesia was supplemented by drop ether. Only those animals which had recovered completely from the operation by the 2nd day are included in these data. Injections for the castrated animals were started on the day of operation, whereas injections for the adrenalectomized animals were started on the first postoperative day, unless otherwise indicated.

The Assay of Whole Sesame Oil on Normal and Experimental Rats

The effects of whole sesame oil on 119 normal, castrated, and adrenalectomized rats are summarized in table 1.

A. Mature normal males. Twenty-four males, were divided into 10 untreated controls, and 14 that were injected. The controls were observed for a period of 63 days.

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[1] The authors wish to thank Dr. J. B. Brown of Ohio State University and Dr. D. F. Robertson of Merck & Company, Inc., for generously supplying them with samples of pure linoleic acid and synthetic α-tocopherol, respectively. The tricaproin was obtained from the Eastman Kodak Company.

Four rats were injected subcutaneously with 0.05 cc. of sesame oil daily for 63 days. Subcutaneous oil cysts were found in these animals at autopsy. No definite toxic effects were noted, probably because of the small quantity of oil injected and the slow rate of absorption via the subcutaneous route.

Five rats of the group of 24 normal males were injected intraperitoneally with 0.25 cc. of sesame oil daily for 63 days. In these animals the body growth was slowly but progressively retarded until by the 63rd day they weighed only 80% as much as the controls (fig. 1) indicating that this quantity of oil was toxic to normal males. Further evidence of toxic effects was a 38% increase in adrenal weight as compared with the controls. The fact that the seminal vesicle weight decreased slightly indicates that the injected oil produced no androgenic stimulation.

TABLE 1. THE EFFECTS OF SESAME OIL ON NORMAL, CASTRATED, AND ADRENALECTOMIZED RATS

No. of rats	Initial body weight		Operative treatment	Sesame oil per animal		Effects
	Mean	(Range)		Daily[1]	Total	
	gm.			cc.	cc.	
10	131	(119–148)	none	—	—	
4	141	(134–148)	none	0.05 S	3.15	Survived until killed on 63rd day
5	130	(127–134)	none	0.25 I	15.75	Inhibited growth, body weight only 80% of controls, increased adrenal weight, decreased testis weight, slightly decreased seminal vesicle weight, after 63 days of injections
5	150	(144–157)	none	1.0 I	10 to 35	1 died on 10th, 17th, and 20th days, 2 survived 42 days until killed, variable effects on body weight
5	52	(46–60)	none	—	—	
5	47	(42–54)	none	0.50 I	3.5	No significant effects on body weight or viscera
5	49	(42–56)	castrated	—	—	Typical castration effects
5	50	(46–50)	castrated	0.50 I	3.5	Same as controls, no effects on viscera
5	44	(40–49)[2]	spayed	—	—	Typical castration effects
5	47	(44–52)[2]	spayed	0.50 I	14.25	Same as controls, no effects on viscera
36	57	(35–90)	adrenal-ectomized	—	—	Mean survival time 8.8 (3–13) days, gained 12 gm., then lost 8 gm.
23	65	(43–88)	adrenal-ectomized	0.05 S		M.s.t. 9.2 (4–14) days, gained 4 gm. then lost 8 gm.
6	60	(54–68)	adrenal-ectomized	0.50 I		M.s.t. 8.3 (7–10) days, practically no gain in body weight

[1] "S" refers to subcutaneous injections; "I" refers to intraperitoneal injections. Injections of adrenalectomized animals were started on the 1st postoperative day, whereas those for castrated animals were started on day of operation.

[2] Females (all others were males).

Three of the five animals that were injected intraperitoneally with 1.0 cc. of sesame oil for 10 to 35 days died on the 10th, 17th and 20th days, whereas the remaining 2 survived until killed on the 42nd day. These last two rats gained 82 and 115 gm. during the experiment, while those 3 that died had first gained from 16 to 55 gm., and then had lost from 34 to 48 gm. before dying. In all instances oil was found in the peritoneal cavities, and in one rat (which died on the 20th day) the oil may have been responsible for peritonitis. Thus large doses of sesame oil over a period of several days when administered via the intraperitoneal route may be toxic to normal male rats.

B. Immature normal males. Ten immature males were divided into one group of 5 untreated controls, and another group of 5 which were injected intraperitoneally with 0.5 cc. of sesame oil daily for 7 days. No significant differences in daily body weight were noted. On the 7th day all animals were killed and autopsied. There was

accumulated oil in the peritoneal cavity of the injected animals. The viscera in all instances appeared normal.

C. Immature castrated males. Ten immature males were castrated, and 5 were injected intraperitoneally with 0.5 cc. of sesame oil daily for 7 days. There were no differences in daily body weight between the 2 groups. On the 7th day all animals were killed and autopsied. There was accumulated oil in the peritoneal cavity of each animal that had been injected. The viscera appeared normal. The average weight of the seminal vesicles in these animals dropped to one-half of the normal value, which indicated that the oil possessed no androgenic properties.

D. Immature spayed females. Ten immature female rats were spayed, and 5 of these were injected intraperitoneally with 0.25 cc. of sesame oil on the 1st day, and with 0.5 cc. of oil on 28 successive days. The animals were killed and autopsied on the 30th day. During this period the average body weight of each group showed a gain of more than 80 gm. There was very little mammary tissue in any of the animals. The vaginae had opened in a few of the rats, and all uteri were infantile. The oil possessed no estrogenic properties, according to these results.[2]

E. Immature adrenalectomized males. Sixty-five immature male rats were adrenalectomized. Thirty-six untreated controls had a mean survival of 8.8 (3 to 13) days. Twenty-three rats which were injected subcutaneously with 0.05 cc. of sesame oil daily had a mean survival of 9.2 (4 to 14) days. Sesame oil hardly altered the average survival time of these adrenalectomized rats. Six animals that were injected intraperitoneally with 0.5 cc. of sesame oil daily had a mean survival of 8.3 (7 to 10) days. This tenfold increase in dosage shortened the average survival period slightly.

The Composition of Sesame Oil and its Fractionation

Sesame oil is a semi-drying oil obtained from the seeds of *Sesamum indicum*, usually by cold expression. The physical and chemical constants for this oil have been published in the "Handbook of Chemistry and Physics" (5). The saponifiable matter of the oil consists of neutral fats or glycerides of saturated and unsaturated fatty acids. Jamieson and Baughman (6) found that the oil contained 36% linoleic acid as glycerides. The unsaponifiable matter varied from 0.9 to 1.7% of the oil, and sitosterol, sesamin and sesamolin have been isolated from the unsaponifiable matter. Adriani (7) obtained sesamin by crystallization from an ethyl alcohol solution of the unsaponifiable matter. Bertram, van der Steur, and Waterman (8) established the formula ($C_{18}H_{16}O_5$) for sesamin. Villavechia and Fabris (9) obtained a higher alcohol ($C_{21}H_{44}O_7$) by extracting the barium soap of sesame oil with ethyl alcohol. Klamroth (10) identified this compound as sitosterol. Villavechia and Fabris described the remaining unsaponifiable matter as a thick, non-crystallizable oil, free from nitrogen, and containing a substance giving the Baudouin reaction. Adriani recovered sesamolin, a glucosidic compound ($C_{20}H_{18}O_7$) which gave a positive Baudouin reaction. Boeseken, Coden and Kip (11) synthesized sesamol ($C_7H_6O_3$) and its β-glucoside from piponeral; the β-glucoside gave a positive Baudouin reaction, thus showing the mechanism of the reaction. Adriani obtained from hydrolysis of sesamolin, sesamol and another compound, samin ($C_{13}H_{14}O_5$), which failed to give the Baudouin reaction.

A. Extraction. Refluxing with either methyl alcohol or ethyl alcohol was found to be satisfactory for the extraction of some of the alcohol-soluble constituents of sesame oil. Some of the alcohol-soluble material could be extracted with 90% methyl alcohol at a temperature of 56° C. for 6 hours. A quantitative study of the differences in the extracted materials was not made.

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[2] Mr. Arthur L. Haskins assayed the sesame oil in immature rabbits by direct intrauterine application, and found that the oil possessed no progestational proliferative properties. This assay was repeated by Dr. Inés de Allende, who confirmed the negative finding of Haskins.

About 7 to 8 cc. of the alcohol-soluble material was obtained by refluxing a liter of 95% ethyl alcohol with a liter of sesame oil (table 2). After separating the oil

TABLE 2. ALCOHOLIC EXTRACTION OF SESAME OIL

Sesame oil, 1 liter	
refluxed with 1 liter 95% ethyl alcohol for 1 hour, cooled, and separated	
alcohol fractions	oil fraction
concentrated to equal volume of alcohol and residue, cooled to 0° C. for several hours, filtered through Büchner funnel	refluxed again with equal volume of alcohol, cooled, and separated; alcohol combined with 1st fraction
crystalline matter / oily filtrate	oil fraction
washed with cold alcohol, and recrystallized	alcohol evaporated by heating to 100° C.
"Sesamin," m.p. 118° C.	"remaining alcohol-soluble fraction"
	alcohol evaporated by heating to 100° C.
	"Depleted sesame oil"

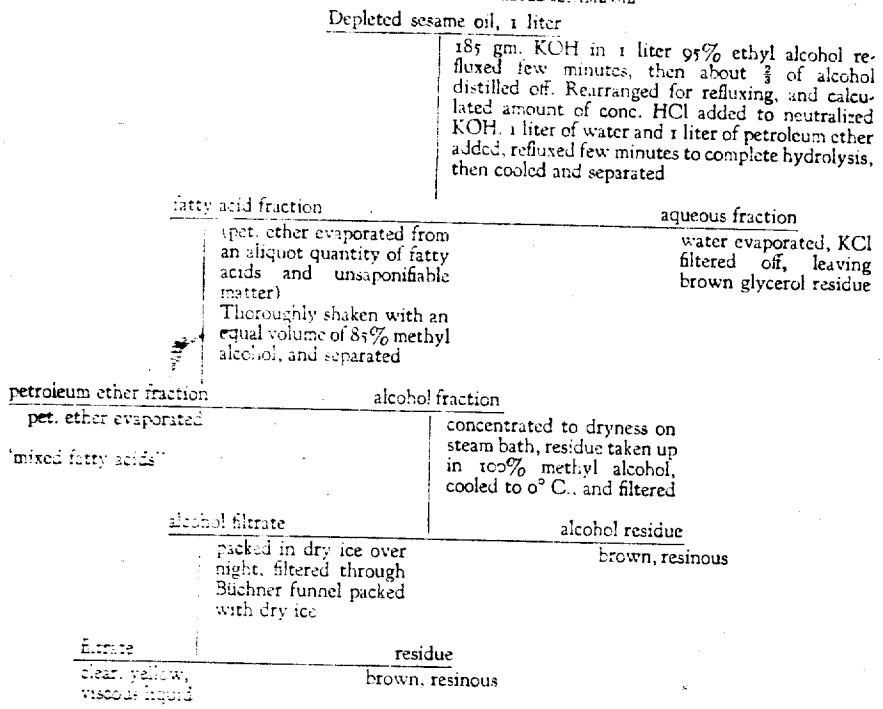
from the alcohol solution, the alcohol was distilled off at atmospheric pressure, leaving a brown liquid residue. By dissolving this residue in about 10 cc. of hot 95% ethyl alcohol and then cooling to 0° C. for at least 3 hours, about 200 mg. of crystals were obtained. The crystalline material had a melting point of 118° C., and was soluble in acetone, chloroform, pyridine, hot ethyl alcohol and acetic acid. It was insoluble in water, and it decomposed in cold concentrated sulfuric acid. These crystals, when dissolved in chloroform, produced a reddish color when 1 drop of concentrated sulfuric acid was added (Adriani's reaction); similarly in chloroform solution, a reddish blue color reaction was obtained when equal parts of concentrated sulfuric acid and acetic anhydride were added (Bömer and Winter's reaction). These color tests indicated the presence of sesamin. The result of the Baudouin reaction, which is specific for sesamolin, was negative. It was impossible to make either an acetate, benzoate, semi-carbizide, or phenylhydrazone derivative. Tocher (10), who extracted sesamin from sesame oil by means of glacial acetic acid, obtained a melting point of 118° C., and found the molecular weight to be 311 in benzene and 312 in acetic acid, making the empirical formula $C_{18}H_{18}O_5$. Although Villavechia and Fabris, Tocher, and Bömer and Winter (10) each assigned a different formula to this compound according to their own determinations of the composition and molecular weight, Bertram, van der Steur, and Waterman (8) established the formula $C_{18}H_{16}O_5$ for sesamin, with a molecular weight of 312, and a melting point of 122.7° C. Microscopic examination of the crystals obtained by crystallization from alcohol revealed two forms: colorless plates and needles. Repeated recrystallization yielded a few milligrams of plate-like crystals which had a melting point of 123° C. From these data the crystalline material was tentatively identified as sesamin.

The alcohol-soluble residue remaining after sesamin had been crystallized from the original alcohol-soluble mixture had the following characteristics. In the presence of 10 cc. of concentrated HCl and 0.1 cc. of recently distilled furfural dissolved in 10 cc. of 95% ethyl alcohol, about 1.0 cc. of this fraction developed a red color (Baudouin reaction). The alcohol-soluble residue when dissolved in chloroform in the presence of acetic anhydride and concentrated sulfuric acid developed a dark green color (Liebermann-Burchard test) indicating the presence of a steroid. Repeated attempts to isolate sitosterol in crystalline form from this residue failed. Any free fatty acids present in the sesame oil would be found in this fraction.

The oil that remained after alcoholic extraction was heated to 100° C. to evaporate a small amount of alcohol that was dissolved in it. This fraction was designated as depleted sesame oil.

B. Hydrolysis. Depleted sesame oil was first saponified and then the fatty acids were liberated by acidic hydrolysis, as indicated in table 3. Although the free fatty

TABLE 3. HYDROLYSIS OF DEPLETED SESAME OIL



acids are soluble in 100% methyl alcohol, they are not soluble in 85% methyl alcohol. The petroleum ether fraction, which contained both free fatty acids and any remaining unsaponifiable matter, was extracted in 250 cc. quantities with an equal volume of 85% methyl alcohol, and then separated. Evaporation of the petroleum ether fraction left a mixture of fatty acids, which at 20° C. separated into liquid and solid fractions. The methyl alcohol fraction which contained unsaponifiable matter was concentrated to dryness, and then dissolved in hot absolute methyl alcohol and cooled to 0° C., according to the method of Mackenzie, Mackenzie, and McCollum (12) for concentrating vitamin E in the unsaponifiable matter of wheat germ oil.

C. Comparison with effects of other lipid substances. With the exception of sesamin, found in the alcohol-soluble fraction of sesame oil, no fractionations were carried far enough to isolate and identify pure crystalline compounds. It was considered desirable, therefore, to compare the effects on adrenalectomized rats of other and related lipid substances with those obtained with sesame oil and its crude fractions. Wheat germ oil was chosen because of its high content of vitamin E. Since some beneficial effects were obtained with wheat germ oil, synthetic α-tocopherol was assayed in order to determine whether the vitamin E present in wheat germ oil was responsible for those effects. Sesame oil contains a large amount of linoleic acid as glycerides. Because linoleic acid is considered to be one of the essential fatty acids,

a pure sample of this acid was assayed. The iodine number, as determined by Dr. J. B. Brown, was 181, the same as the theoretical value. In order to ascertain whether neutral fats were responsible for the beneficial effects obtained in treated adrenalectomized lactating rats, synthetic tricaproin was chosen. We assume that this neutral fat, which is saturated and liquid at 20° C., is free from any contaminants with physiological activity.

The Assay of Fractions of Sesame Oil and Other Lipid Substances on Immature Adrenalectomized Rats

The resistance of an adrenalectomized animal to toxic substances is not as great as that of normal animals, due to the loss of adrenal cortical hormones. The survival time of an adrenalectomized rat, therefore, is a non-specific criterion for testing the toxicity of injected substances. Since the survival time of individual animals in a group varies, only the average survival time of the group may be used for purposes of comparison. When the average survival time of adrenalectomized immature rats is definitely shortened by the administration of the substance being assayed, that substance is considered to be toxic. Two hundred and sixteen immature male rats were adrenalectomized and used to assay fractions of sesame oil and other lipids (table 4). The average weight of these animals was 56 gm. A good correlation between the initial body weight and the survival time was not found. In assaying the fractions of sesame oil, a uniform daily dose of 0.05 cc., administered subcutaneously, was employed. For the crude alcohol-soluble fractions, this represented approximately 100-fold concentration, as compared with the original concentration in the oil. For the depleted sesame oil, the dose was practically equivalent to the original oil, there being only a very slight concentration.

Thirty-six uninjected adrenalectomized controls had a mean survival time of 8.8 (3 to 13) days.

The alcohol-soluble fractions of sesame oil, including crystalline sesamin dissolved in depleted sesame oil, shortened the survival period. When compared with the controls, the mean difference divided by the probable error of the mean difference for 25 animals so treated was 10.0. Thus there are present in sesame oil alcohol-soluble compounds, one of which is sesamin, that are toxic to adrenalectomized rats. It is suggested that the toxic effects of whole sesame oil upon normal male rats over a period of weeks may be attributed to the presence of these alcohol-soluble compounds.

Depleted sesame oil prolonged the average survival period of 29 rats. Since the individual survival periods ranged from 5 to 27 days, the significance of this enhanced survival is doubtful, although when compared with the controls, the ratio of the mean difference divided by its probable error was 4.0. The depleted sesame oil, however, was considered to contain some beneficial substances, as well as a slight amount of alcohol-soluble substances. A 10-fold increase in the daily dose of depleted sesame oil when administered intraperitoneally to 8 animals reduced the survival period to 7.2 days. Fractionation of the depleted sesame oil by filtration at 0° C. yielded 2 fractions one of which was solid at that temperature, and, when assayed, was almost as beneficial as the original mixture. When the depleted sesame oil, which was oxidized on the steam bath by bubbling oxygen through it for 25 minutes, was assayed on 17 animals, the survival time was 6.4 days. Hydrolysis of depleted sesame oil yielded crude aqueous, mixed fatty acid, and mixed unsaponifiable fractions. All these fractions shortened the average survival period. The inactivation of the beneficial substances in depleted sesame oil, consequently, may be the result of either oxidation or hydrolysis or a combination of both processes. The fact that an alcohol-soluble fraction was obtained after hydrolysis of the depleted sesame oil indicates

that alcoholic extraction does not remove all the alcohol-soluble constituents from sesame oil.

Wheat germ oil, synthetic α-tocopherol dissolved in depleted sesame oil, pure linoleic acid and synthetic tricaproin when assayed on immature adrenalectomized rats also shortened the average survival period (table 4).

TABLE 4. SURVIVAL TIME OF IMMATURE ADRENALECTOMIZED MALE RATS

Fraction assayed	No. of animals	Daily dose	Survival time	
			Average	Range
		cc.[1]	days	days
Controls	36	—	8.8	3–13
Alcohol-soluble				
Crude mixture	11	0.05 S	5.1	3–7
Sesamin[2]	9	0.05 S	5.1	3–10
Crude mixture minus sesamin	5	0.05 S	5.8	3–10
Depleted sesame oil	29	0.05 S	11.5	5–27
Depleted sesame oil	8	0.50 I	7.2	5–11
Depleted sesame oil, liquid at 0° C.	6	0.05 S	5.8	5–11
Depleted sesame oil, solid at 0° C.	10	0.05 S	10.1	6–14
Depleted sesame oil, oxidized	17	0.05 S	6.4	3–11
Fatty acids and unsaponifiable matter	6	0.05 S	3.8	2–7
Mixed fatty acids	7	0.05 S	5.7	5–7
Unsaponifiable matter	10	0.05 S	5.9	3–9
Glycerol and water-soluble matter	4	0.05 S	7.5	7–8
Wheat germ oil	11	0.05 S	5.9	3–9
α-tocopherol	8	0.50 mg.	7.3	5–11
α-tocopherol	7	0.05 mg.	7.1	5–9
α-tocopherol	8	1.0 mg.	6.2	4–8
Linoleic acid	5	0.03 I	less than 24 hours	
Linoleic acid	4	0.03 S	7.5	6–9
Tricaproin	15	0.05 S	5.7	4–9

[1] Subcutaneous injections are designated by "S"; intraperitoneal by "I." All injections were started on first postoperative day.

[2] 1 mg. sesamin/0.05 cc. of depleted sesame oil.

Although the adrenalectomized rat is more susceptible to histamine poisoning than the normal animal, the resistance to histamine can be raised by the administration of the adrenal cortical hormone (13). Immature male rats which weighed from 39 to 69 gm. were adrenalectomized, and were injected with histamine on the fourth postoperative day. The minimum lethal dose of histamine diphosphate for operated rats was 20 mg./100 gm. of body weight. Nine rats were injected subcutaneously with 0.05 cc. of sesame oil for 4 days, and all died from histamine poisoning within 58 to 232 minutes, at a dosage level of 10 mg./100 gm. Eight rats were injected subcutaneously with 0.05 cc. of depleted sesame oil for 4 days. They died within 59 to 377 minutes, at a dosage level of 20 mg./100 gm. Seven animals which were injected intraperitoneally with 0.15 cc. of depleted sesame oil died within 40 to 331 minutes when given the same dose as above. Five normal animals of the same weight range survived after a similar injection of histamine. Sesame oil and depleted sesame oil did not possess any cortin-like protective action against histamine poisoning.

The Effects of Depleted Sesame Oil and Other Lipid Substances on Adrenalectomized Lactating Rats

A. *Depleted sesame oil.* Rats adrenalectomized a few days before parturition will deliver their young, but will not lactate sufficiently to raise the young (14). Twelve primiparous females were adrenalectomized on the 16th to 19th day of gestation. They were then injected intraperitoneally with 0.25 cc. of the depleted sesame oil daily, beginning with the first postoperative day, and continuing for 8 to 30 days. Three females failed to wean any of their young, and 9 weaned from 14 to 100% of their suckling young. Three females survived for 81, 82 and 83 days and were killed.

TABLE 5. PRIMIPAROUS RATS ADRENALECTOMIZED 15TH TO 19TH DAY OF GESTATION

Group	No.	Daily dose	Average postoperative survival time	Young weaned
		cc.	days	%
Normal (unoperated)	8	0–0.5	—	92
Control[1] (operated)	10	—	9.6	0
Sesame oil[1]	15	0.15–0.55	21.0	0
Depleted sesame oil	19	0.1–0.5	33.0[2]	47
Wheat germ oil	9	0.1	26.6	34
α-tocopherol	9	2.5–10.0 (mg.)	26.0	—
Tricaproin[3]	13	0.05–0.10	32.4	31

[1] C. E. Tobin, in press, 1940.
[2] Not including 4 animals which had an indefinite survival time.
[3] Adrenalectomized just before, or just after parturition.

One of these 3 weaned 7 (58%) of her litter, another weaned 1 (14%) of her litter, and the third failed to wean any. At autopsy only the second female showed gross evidence of an adrenal rest. Lactation was not complete in the other 2 females, and there was no gross evidence of regenerated adrenal tissue. Consequently the cause of the prolonged postoperative survival could not be ascertained. There were 3 females, however, which weaned 100% of their young, exhibited incomplete lactation, and died of adrenal insufficiency on the 35th, 38th and 61st days. The young

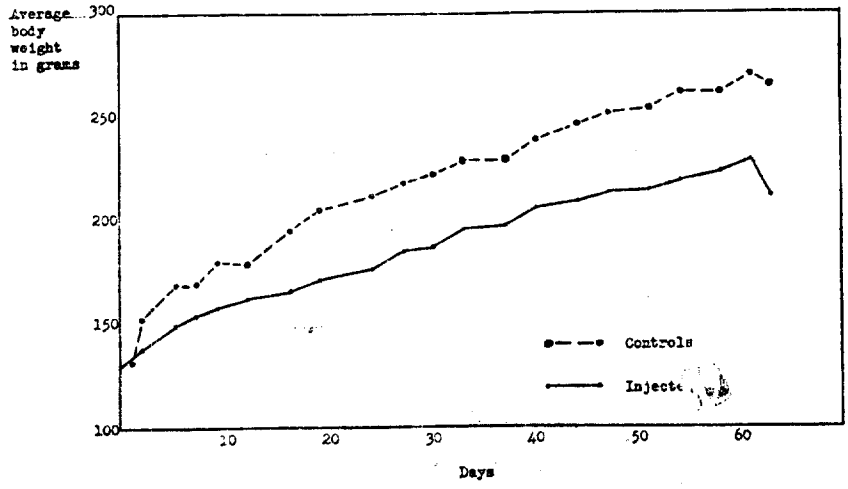


FIG. 1. THE EFFECT OF DAILY INTRAPERITONEAL INJECTIONS OF 0.25 CC. OF SESAME OIL ON THE AVERAGE BODY WEIGHT OF NORMAL MATURE MALE RATS.

were stunted during the period of suckling, but they gained rapidly as soon as they had been weaned (fig. 2).

For controls, 8 normal primiparous rats were selected, and 4 were injected intraperitoneally with 0.5 cc. of depleted sesame oil daily for 9 to 16 days during lactation. The oil was saturated with Sudan III in order to facilitate tracing its absorption. At autopsy stained fat was found in the peritoneal cavity, omentum, and hepatic lymphatics. No toxic effects were noted, and the suckling young gained weight at a normal rate. Examination of the milk in the stomachs of the suckling young showed unstained droplets of fat. The mesenteric fat of the suckling young was not colored.

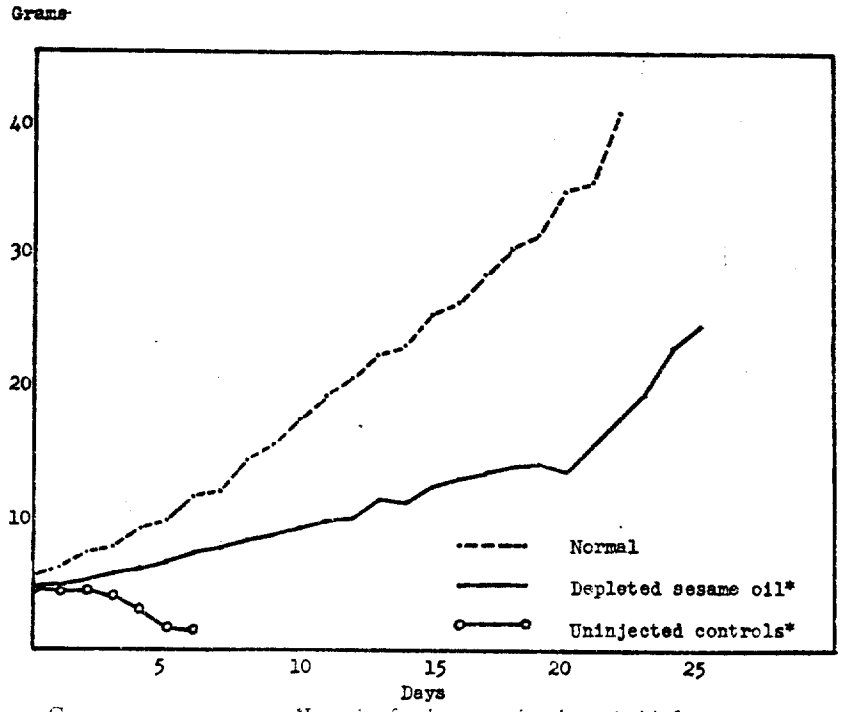


Fig. 2. Growth of suckling rats. *Lactating females were adrenalectomized before parturition, and were injected intraperitoneally, daily. All the young from the uninjected controls died within 1 week.

Seven primiparous rats were adrenalectomized on the 16th to 19th day of gestation. Beginning with the day of operation, they were injected intraperitoneally with 0.5 cc. of Sudan stained depleted sesame oil, daily for 5 to 22 days. All 7 delivered normal litters of from 7 to 13 each. Two failed to lactate adequately and lost all of their young, and the remaining 5 weaned from 60 to 100% of their young. One litter of these 5, however, survived for 21 days and the young were weaned, but they died within 2 days. All the young of the other 4 litters which had been stunted and weaned, survived and gained rapidly after being weaned. The adult females had a postoperative survival time ranging from 23 to 60 days, and all died of adrenal insufficiency. At autopsy there was stained fat in the peritoneal cavities, omenta, and hepatic lymphatics. Microscopic examination of the mammary glands and the renal fat deposits showed unstained fat in the cells. Smears of the peritoneal cavity, stained with methylene blue showed droplets of Sudan stained oil and many white cells. Droplets of fat within the cells were finely divided and gave no positive evidence of Sudan staining.

Seventy-six young rats (47%) were weaned successfully from 19 primiparous rats that were adrenalectomized before parturition and were treated with depleted sesame oil during lactation. The possibility of functional adrenal cortical tissue being responsible was eliminated by the fact that 15 of the females died of adrenal insufficiency. Furthermore, comparison of 2 rats, each 22 days old, that were from different litters, one nursed by a lactating female that had been subtotally adrenalectomized several weeks previously, the other nursed by a totally adrenalectomized lactating female maintained on depleted sesame oil showed a striking difference. The former weighed 45 gm. and appeared normal, whereas the latter weighed 20 gm. and was

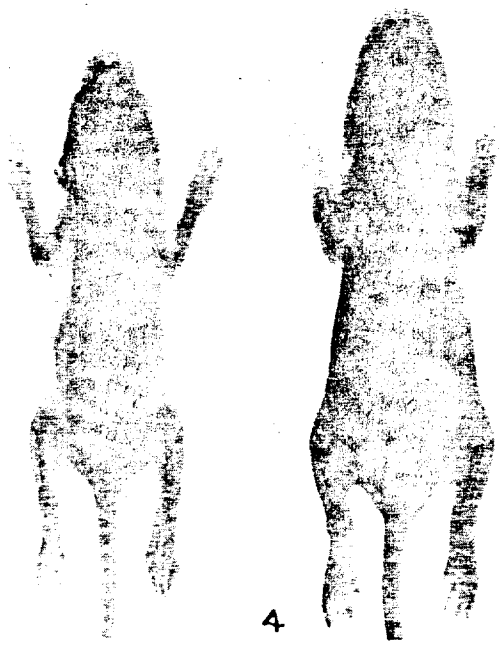


Fig. 3. STUNTED SUCKLING RAT, 16 DAYS OLD, BORN OF A RAT ADRENALECTOMIZED BEFORE PARTURITION AND TREATED WITH 0.5 CC. SUDAN-STAINED DEPLETED SESAME OIL DAILY. Weight: 12 gm.; length: 67 mm.; tibial diaphysis: 9 mm. Notice extent of ossification of tarsal and metatarsal bones. About natural size. Fig. 4. NORMAL SUCKLING RAT, 10 DAYS OLD, BORN OF A NORMAL RAT THAT WAS INJECTED WITH 0.5 CC. SUDAN-STAINED DEPLETED SESAME OIL DAILY DURING THE PERIOD OF LACTATION. Weight: 17 gm.; length: 76 mm.; tibial diaphysis: 8.5 mm. Notice extent of ossification of tarsal and metatarsal bones. About natural size.

stunted. Several of the stunted rats from 4 to 28 days old were anesthetized and photographed with Roentgen rays. The length of the tibial diaphysis, and the number and extent of ossification of the carpal and tarsal bones as well as the metacarpal and metatarsal epiphyses were taken as indices of skeletal development. Characteristic of the stunted groups was a rat 16 days old that weighed 12 gm. and exhibited skeletal development that was approximately equivalent to a normal young rat 10 days old that weighed 17 gm. (fig. 3, 4). For further comparison, a normal suckling rat 16 days old weighed 28 gm., was 37% longer, and had a tibial diaphysis 33% longer than the stunted animal of the same age chronologically. After the young rats had been weaned, however, the skeletal development was accelerated so that by the 28th day it was comparable to that of a normal animal of that age. When approximately 10 weeks old, 10 females and 7 males were mated. All females became

pregnant within the next month and delivered normal litters of 6 to 8 young. Eight males and 10 females, with an average weight of 120 gm. and about 10 weeks old, were killed and autopsied. All phases of the estrous cycle were found in the vaginal smears at autopsy. The weights of the following organs were within the range for normal animals of a similar age: testes, seminal vesicles, adrenals, kidneys, livers, abdominal viscera, hearts and lungs.

The death of some of the suckling young, from females adrenalectomized before parturition, might be attributed either to some prenatal effect of adrenalectomy, or to some dietary deficiency. The following two protocols suggest that a dietary deficiency, manifested by incomplete lactation, was responsible.

Rat 13 was a normal primiparous animal that was adrenalectomized 5 days before parturition. She delivered a litter of 10 living young, weighing 45 gm. On the 1st, 20th, and 22nd days postpartum, 3 of her litter died, and 2 died on the 23rd day. On the 24th day one more was killed for histological examination of some of the tissues. The female was injected daily for 30 days, beginning with the 1st postoperative day, with 0.25 cc. of depleted sesame oil. On the 22nd postpartum day the adrenalectomized female was exchanged with a normal female which had delivered 2 days previously a litter of 9 young, weighing 51 gm. On the day of exchange, the 9 young weighed 59 gm. but all had died by the 6th day. Thus the foster mother which had been adrenalectomized was unable to lactate sufficiently to maintain a normal litter. Meanwhile the normal female was able to feed and maintain the 4 remaining young of the litter from the adrenalectomized female. During those 4 days, there was a gain of 5.9 gm. per animal, which was equivalent to the average gain per animal in the 17 days prior to the exchange of lactating females. When the 4 young were 28 days old, and together weighed 84 gm., they were weaned, and by the 40th day they weighed 112 gm. The adrenalectomized female died on the 40th postoperative day of adrenal insufficiency. Histological sections of skeletal and cardiac muscle and the kidney of one of the young rats appeared more like fetal than normal tissues.

Rat 81 was a normal primiparous animal that delivered a normal litter, and was adrenalectomized on the first postpartum day. She was then given another litter of 8 young rats of the same age as her own litter, and was injected intraperitoneally with 0.5 cc. of depleted sesame oil daily during the period of lactation. One of the young died on the 3rd day, but the remaining 7 survived and exhibited stunted development, up to the time that they were weaned. On the 20th postpartum day the average weight of the young was 14.7 gm. as compared with an average weight of 35 gm. for normal young of the same age. The lactating female showed a slight initial loss in weight, but gained 21 gm. by the 20th day.

The average gain in body weight during the period of lactation for 8 normal, 19 uninjected adrenalectomized and 19 adrenalectomized rats treated with depleted sesame oil, are shown in figure 5. It will be noted that the sesame oil injections not only prevented the loss of body weight shown by untreated adrenalectomized animals but permitted a more prolonged gain in weight than shown by the normal stock animals.

The following protocol shows that oxidized depleted sesame oil does not produce the same results as reported above, but is toxic.

Rat 71 was a primiparous animal that was adrenalectomized the day before parturition, and was given intraperitoneally 0.5 cc. of oil daily for 8 days. She delivered a litter of 10 young, which survived for 7 days, and then lost 22 gm. before dying on the 15th day. At autopsy there was oil in the peritoneal cavity, and there was considerable congestion of the lungs, liver, spleen and kidneys.

B. Wheat germ oil. Nine primparous females were adrenalectomized on the 17th to 19th day of gestation (table 5). They were injected intraperitoneally with 0.1 cc. of wheat germ oil daily, beginning with the first postoperative day and continuing for 3 to 30 successive days. Seven females delivered living young, with from 2 to 11 in a litter; 1 delivered 7 dead fetuses, and 1 suddenly lost considerable weight at term and probably delivered, although no young were found. Five of the 7 females weaned successfully from 1 to 7 (25 to 87.5%) young of their litters. All 9 females died of adrenal insufficiency in from 12 to 57 days after the operation. Wheat germ oil under these conditions was responsible for maintaining 27 young rats until they were weaned. This represented 34% of the young in all the litters. In all instances the young were stunted, but within a few days after weaning they gained rapidly.

C. Synthetic α-tocopherol. Eleven primiparous rats were adrenalectomized on the 15th to 19th day of gestation (table 5). Two controls received 0.1 cc. of depleted sesame oil injected subcutaneously on the first postoperative day. The remaining 9 were injected subcutaneously with 2.5 to 10.0 mg. of synthetic α-tocopherol that was dissolved in 0.10 to 0.45 cc. of depleted sesame oil. All females gave birth to living young, except one which delivered 12 dead fetuses with intact membranes.

Of the 9 females that were given α-tocopherol, only 2 weaned any of their young. These 2 weaned 80% and 89% of their litters, a total of 12 young rats. In each instance the young were stunted while suckling, but gained rapidly after being weaned.

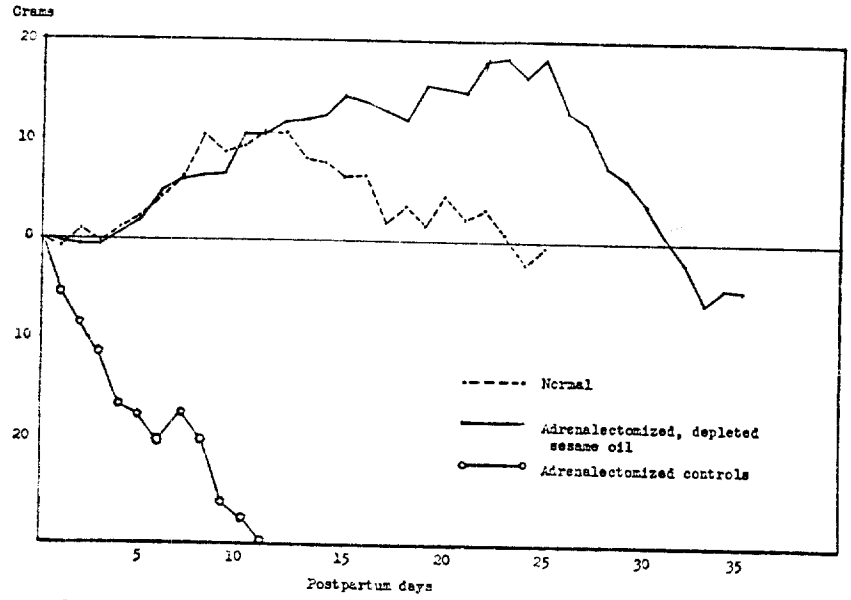


Fig. 5. THE EFFECTS OF ADRENALECTOMY AND DEPLETED SESAME OIL ON THE AVERAGE BODY WEIGHT OF LACTATING RATS.

Since one of the control animals, which was adrenalectomized before parturition, and was administered a total dose of 0.1 cc. of depleted sesame oil, weaned 75% of her litter of 8, and then died of adrenal insufficiency on the 46th day, it is considered that α-tocopherol did not benefit the 2 females which were treated with it. In view of the fact that 7 of the 9 females lost weight during lactation and failed to wean any of their litters, it is suggested that α-tocopherol is toxic to adrenalectomized rats during lactation. The α-tocopherol was also toxic to immature adrenalectomized male rats.

D. Tricaproin. Thirteen primiparous females were adrenalectomized either 2 to 4 days before or 1 day after parturition. They delivered normal litters of from 4 to 12 young each. They were injected intraperitoneally with 0.05 to 0.10 cc. daily of synthetic tricaproin, until the total dose ranged from 0.20 to 2.15 cc. Seven females lost all of their suckling young, and 6 weaned successfully 33 to 78% of their young. All the young were stunted, and those that were weaned successfully weighed on the average 14.5 gm. on the 20th day, as compared with 35 gm. for normal animals of the same age.

E. Linoleic acid. Six primiparous rats were adrenalectomized on the first or second day postpartum. They were injected subcutaneously with 0.05 to 0.10 cc. of linoleic acid daily, until the total dose ranged from 0.25 to 0.55 cc. In all, 33 young rats were

born, and only 1 survived the period of suckling and was weaned. Three others survived for 21 days. All 4 young rats were stunted. The acid formed palpable subcutaneous cysts, and was absorbed rather slowly.

The Effects of Administering Depleted Sesame Oil to the Suckling Young of Lactating Rats That Were Adrenalectomized before Parturition

Two normal primiparous rats were adrenalectomized on the 17th to 19th day of gestation. Each delivered a normal litter of 8 young rats that had an average weight of 5.1 gm. Beginning with the first day, each young rat received a total of 7 to 8 subcutaneous injections of 0.05 cc. of Sudan stained depleted sesame oil. The injections were administered about every 2nd or 3rd day, and the total quantity that any one rat received did not exceed 0.4 cc. The adult females received no injections. Thirteen of the original 16 (81%) exhibited markedly retarded gains in body weight while suckling, but survived after being weaned. On the 20th day the young rats had an average weight of 12 gm., as compared with 35 gm. for normal controls of the same age. By the 27th day, after they had been weaned, they had an average weight of 21 gm. At this time 8 were killed, and at autopsy an average of 0.1 cc. (0.05–0.20) of Sudan stained depleted sesame oil was aspirated from subcutaneous cysts. Thus the average net volume of injected and absorbed oil was about 0.25 to 0.30 cc. The adults during lactation lost 13 gm. on the 3rd day, slowly gained 19 gm., and then lost 31 and 64 gm., when they died of adrenal insufficiency on the 35th and 40th postoperative days.

DISCUSSION

Sesame oil, when injected into the rat, may be either inactive or toxic, according to the experimental conditions. Over a period of 9 weeks, daily intraperitoneal injections of 0.25 cc. of sesame oil produced toxic manifestations in normal mature males, and larger doses were sufficiently toxic to induce the death of the animal. Over a period of a few days, however, sesame oil, when assayed on immature rats, possessed neither androgenic, estrogenic, nor cortin-like properties.

The assays on the immature adrenalectomized male rats demonstrated the toxicity of the alcohol-soluble fractions of sesame oil and the beneficial effects of the depleted sesame oil (alcohol-insoluble fraction). In the alcohol-soluble fraction, both crystalline sesamin and remaining liquid residue were toxic.

Wheat germ oil (which is an excellent source of vitamin E), synthetic α-tocopherol, synthetic tricaproin, and pure linoleic acid, an essential fatty acid, failed to benefit immature adrenalectomized males. Neither oxidized depleted sesame oil nor the concentrated aqueous, the fatty acid, and the unsaponifiable fractions obtained from saponification of depleted sesame oil were beneficial when assayed with immature adrenalectomized male rats.

The depleted sesame oil consisted largely of glycerides of saturated and unsaturated fatty acids. This fraction not only prolonged the survival time of immature adrenalectomized rats as compared with untreated controls, but enabled female rats adrenalectomized before parturition to gain weight during lactation and to exhibit markedly improved lactation. In 4 litters 100% of the young survived beyond weaning, and in all litters of the group 47% of the young survived beyond weaning. Similar adrenalectomized females treated with wheat germ oil or with synthetic tricaproin lactated sufficiently to enable some of the young to survive beyond weaning. In each instance the suckling young were stunted, but they gained weight rapidly after being weaned, indicating, possibly, that there was a deficiency in the milk. The successful weaning of the litters could not be attributed to the presence of functional

adrenal cortical tissue, for most of the animals considered died of adrenal insufficiency after their young had died or had been weaned. The possibility of vitamin E was considered, for Karrer and Keller (15) reported finding 0.63% α-tocopherol in the unsaponifiable matter of sesame oil. Mason (16) found that depleted sesame oil was devoid of any vitamin E by bioassay with rats. Thus any tocopherol present in the oil was probably extracted by the alcohol.

Wang (17) found that the food intake in the rat increased enormously after parturition, and declined to the normal level after weaning. Sinclair (18) demonstrated that there was a change in the composition but not the amount of phospholipid fatty acids in the intestinal mucosa of rats. From this he suggested that phospholipids were an intermediate step in the resynthesis of neutral fat. At term the plasma neutral fat is about twice the non-pregnant level, and this lipemia does not disappear until late in lactation. Since the composition of milk fat changes relatively little, the quantity of milk is related to the amount of plasma lipids (19). Verzar and Laszt (20) found that adrenalectomized rats showed an impaired absorption of fatty acids by the intestinal mucosa, that was related to disturbances in the phosphorylating mechanism. Administration of the adrenal cortical hormone was sufficient to restore phosphorylation to normal. Barnes, Wick, Miller and MacKay (21) reported that adrenalectomized rats maintained on a 1% NaCl regimen absorbed neutral fats and methyl esters of fatty acids as readily as normal controls. They noted that the salt prevented the more severe symptoms of adrenal insufficiency, and thus the addition of salt evidently permitted fat absorption. Gaunt and Tobin (22), studying the effects of adrenalectomy during pregnancy and lactation, found they could control the percentage of young surviving until weaning by the amount of cortical extract administered. At least twice the life-maintaining dose was necessary for adequate lactation, and although lactation was improved, the young often exhibited sub-normal growth. Tobin (4) reported that sesame oil enhanced lactation of adrenalectomized rats, but none of the young survived until weaning.

Adrenalectomized animals are less resistant to toxins and infections than are normal animals, because of the lack of adrenal cortical hormone. Depleted sesame oil, from which a considerable amount of toxic substances were removed by alcoholic extraction, proved to be more beneficial to adrenalectomized rats than whole sesame oil. It is suggested that the high content of neutral fats of depleted sesame oil is responsible for the prolonged survival of immature adrenalectomized males, and for the improved lactation of females adrenalectomized before parturition. Intraperitoneal injection of neutral fat overcomes the inability of the intestinal mucosa to absorb sufficient fatty acids for the resynthesis of neutral fats to meet the requirements of lactation. Progesterone will substitute for the adrenal cortex in maintaining life in the adrenalectomized rat (23). Although immature male rats have no known source of progesterone, lactating rats are supplied with progesterone by the corpora lutea of lactation. Since injection of neutral fat into adrenalectomized and lactating rats keeps the young alive and nursing, involution of the mammary gland is prevented. After the young are weaned and the mammary gland atrophies, the mammary-anterior pituitary-ovary relationships are altered. With an apparent cessation of secretion of progesterone, the symptoms of adrenal insufficiency appear in a few days, culminating in the death of the animal.

SUMMARY

Sesame oil, when administered intraperitoneally in daily doses of 0.25 to 1.0 cc. for a period of several weeks, produces toxic effects in normal male rats. Extraction of sesame oil with ethyl alcohol yielded alcohol-soluble fractions, and an alcohol-

insoluble fraction designated as depleted sesame oil. The alcohol-soluble fractions were toxic to immature adrenalectomized male rats, as determined by shortened mean survival periods. The depleted sesame oil prolonged the survival time of immature adrenalectomized males. Oxidized depleted sesame oil, all fractions resulting from hydrolysis of sesame oil, as well as synthetic tricaproin, synthetic α-tocopherol, wheat germ oil, and pure linoleic acid shortened the average survival period of immature adrenalectomized rats.

The survival time of lactating rats that were adrenalectomized before parturition was prolonged by depleted sesame oil, and lactation was improved markedly to enable 47% of the young to survive and be weaned successfully. All the young were stunted while suckling, they gained weight rapidly after being weaned, and when mature were mated and found to be fertile. Since similar results were obtained with wheat germ oil and synthetic tricaproin, but not with synthetic α-tocopherol dissolved in depleted sesame oil, the beneficial effects are attributed to the neutral fats. It is suggested that the injected neutral fats benefit the suckling young of adrenalectomized lactating rats, and since the young are maintained and suckle, mammary involution is prevented. This in turn by relationships with the anterior pituitary and ovary possibly influences corpora lutea of lactation to secrete enough progesterone to maintain temporarily the life of the adult female rat.

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EXPERIMENTS IN CARCINOGENESIS: THE EFFECT OF THE SUBCUTANEOUS AND INTRA-PERITONEAL INJECTION OF LARD, OLIVE OIL AND OTHER FATTY MATERIALS IN RATS AND MICE.

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(Plate LXXII.)

During the last few years many experiments have been undertaken at this Institute to test the carcinogenic potency of 1 : 2 : 5 : 6-dibenzanthracene and other compounds when introduced into the subcutaneous tissue or peritoneum of rats and mice. In most of the earlier experiments of this kind the material to be tested was dissolved in lard or olive oil. For purposes of control, lard, olive oil and other fatty materials free from added carcinogenic compounds were injected into rats and mice in the same way; the results of these control experiments are described below.

I. Injections of fats, fatty acids, unsaponifiable materials and liquid paraffin sub cutem *in mice.*

Table I records the completion of control experiments on mice which were mentioned in an earlier paper (Burrows, Hieger and Kennaway, 1932). All the substances named were injected in amounts of 0·2 to 0·5 c.c. *sub cutem* in the groin with the exception of the solutions of oleic and myristic acids (5 per cent. in olive oil) which were injected in the pectoral region. The table includes figures referring to 216 mice from the material dealt with in the present paper and to these we have added the data obtained by Barry and Cook (1934) in this Institute for mice receiving injections of lard, making a total of 236 animals. In no single instance was a tumour produced at the site of injection.

Andervont (1934) injected lard (0·25 c.c. twice and 0·5 c.c. once in 90 days) *sub cutem* in 190 mice and obtained no tumours in 189 days, when the experiment ended, while tumours appeared at the site of injection in 153 out of 368 mice which received during the same period similar injections of lard containing 1 : 2 : 5 : 6-dibenzanthracene. Schabad (1935) injected sunflower oil (0·25 to 0·5 c.c. on three occasions) *sub cutem* in 16 mice and

obtained no tumours in 6½ months, while in mice receiving 1 : 2 : 5 : 6-dibenzanthracene in the same oil sarcomas appeared in 5 out of 15 between the 4th and 8th months.

TABLE I.

Injections of fats, fatty acids, unsaponifiable materials and liquid paraffin sub cutem *in mice.*

Material injected.	Number of mice.		Day of death of last mouse, or of conclusion of experiment.
	Initial.	Survived four months.	
Pig fat	50	21	613
Lard	30	16	410
Unsaponifiable fraction of lard	18	8	577
Oleic acid (technical)	8	3	259
Unsaponifiable fraction of oleic acid (technical)	20	8	337
Olive oil	10	7	540
Linseed oil	10	2	196
Cod-liver oil	10	7	534
Wheat germ oil	10	2 (7 months)	333
Triolein	10	7	540
Oleic acid 5 per cent. in olive oil	10	6	574
Myristic acid 5 per cent. in olive oil	10	3	179
Liquid paraffin	20	9	533
	216	99	...
Lard (Barry and Cook, 1934)	20	10	581

No tumours resulted.

II. Injections of lard and olive oil sub cutem *in rats.*

Care was taken to avoid accidental contamination with any recognised carcinogenic compound, though in most instances the animals were housed in the main animal room. In expts. 3, 6, 15, 16, 19, 20 and 21 (tables II and V) more rigid precautions were exercised; the rats, freshly obtained from a dealer, were placed in new cages and housed in a room in which no animals had previously been kept. All apparatus and utensils used were new and, together with the animals, were retained throughout in the same compartment. The injections were made early in the day, before the operator had engaged in any other work in the laboratories. In addition to these control experiments four have been included in which the injected fat contained theelin (Parke Davis and Co.) (expt. 7), œstrone (expts. 12 and 13) or arsenious acid (expt. 14). The method was to give weekly injections of 1 c.c. until a bulky collection of oil was present under the skin. After this stage had been reached the intervals between the injections were increased, being regulated by the size of the subcutaneous collection of oil. The rats used were from mixed stocks of no special strain.

Among 193 rats given lard or olive oil subcutaneously, eight spindle-celled tumours arose at the site of injection (table II)

TABLE II.

Subcutaneous injections of lard and olive oil in rats.

No. of expt.	Duration in weeks.	Number of rats.	Survived four months.	Material injected.	Site of injection.	Spindle-celled tumour at site of injection.	Period in weeks from first injection to death of tumour-bearing animals.	Other treatment.
1	146	20	17	Lard	Groin	2	79, 146	None
2	99	7	6	Lard	Groin	0	...	None
3	121	10	8	Lard	Groin	1	121	None
4	81	10	6	X-radiated lard	Groin	0	...	None
5	69	10	5	Lard	Groin	1	98	None
6	89	10	7	Lard+5 per cent. oleic acid	Groin	1	79	None
7	72	7	7	Lard+theelin (Parke Davis and Company)	Groin	1	56	None
8	53	10	7	Lard	Nape	1	53	None
9*	105	20	16	Lard	Nape	0	...	Emulsified olive oil into peritoneum
10	94	30	23	Lard	Nape	0	...	1 : 2 : 5 : 6-dibenzanthracene into peritoneum
11	80	8	6	Olive oil	Groin	0	...	None
12	71	8	6	Olive oil+œstrone (0·03 per cent.)	Groin	0	...	None
13	48	3	2	Olive oil+œstrone (0·03 per cent.)	Groin	0	...	None
14	106	40	28	Olive oil+arsonious acid †	Groin	1	97	None
Totals	...	193	144	...	...	8	...	...

* See also table V.

† Contained 0·0173 per cent. $NaAsO_2$, which gives a concentration of As (0·01 per cent.) of the order of that found in the hyperkeratotic skin of persons liable to arsenic cancer.

Unsuccessful attempts were made to graft the tumours of expts. 3, 6, 7, 8 and 14 into young rats, ten of which were used for each attempt.

A polypus of the uterus was present in one of the tumour-bearing rats of expt. 1. In expt. 12, in which the injected olive oil contained œstrone, mammary tumours appeared in two of the rats (31st and 64th weeks) and a sarcoma of the liver adjacent to a *cysticercus* cyst in another.

A *cysticercus* sarcoma was present also in one of the rats of expt. 14. In expt. 13, in which œstrone was present in the injected oil, lymphosarcomatosis appeared in one of the rats. In expt. 10, in which 1 : 2 : 5 : 6-dibenzanthracene was injected into the peritoneum, 23 of the animals survived for four months or longer and peritoneal tumours appeared in 12 (52 per cent.) of these.

Two of the tumours occurred among the animals treated under the rigid and isolated conditions mentioned above (expts. 3 and 6). In one of the eight instances the tumour formation followed the injection of lard which contained theelin and in another the injection of olive oil containing arsenious acid. These two cases have been included in the tables although it is possible that the theelin or the arsenious acid might have had some part in causing the tumours. Of the 193 animals, 144 survived for periods of more than four months. Tumours are not commonly induced by 1 : 2 : 5 : 6-dibenzanthracene in less than four months, and therefore this period has been arbitrarily chosen when arranging the experimental results in a numerical form, all animals dying at an earlier stage being omitted from further consideration. On this basis the incidence of tumours at the site of injection of lard or olive oil is 5·7 per cent. as compared with 46·3 per cent. after similar injections of lard or olive oil containing 1 : 2 : 5 : 6-dibenzanthracene (table III). It will be observed (eighth column of table II) that the tumours which followed the injections of fat required a long period for their appearance, none developing during the first year. The average time up to the death of the rat in these cases was 93 weeks. Of the total number of rats treated 99 lived for a year or more, and it was in 8 of these that the spindle-celled tumours occurred.

TABLE III.

Subcutaneous or intraperitoneal injections of 1 : 2 : 5 : 6-*dibenzanthracene dissolved in lard or olive oil, without other treatment.*

Animal.	Number.	Surviving four months.	Tumour at site of injection.	Percentage.	Tumours elsewhere.
Rat . .	50	41	19	46·3	0
Mouse .	10	8	2	25·0	0
Totals .	60	49	21	42·8	0

No spindle-celled tumours arose in any other part of the body in these animals, nor do such tumours commonly develop spontaneously in the groin of rats; it is not easy therefore to escape the conclusion that the tumours were a consequence of the treatment. This would not necessarily mean that lard and olive oil have neoplastic properties themselves. Most of the known carcinogenic compounds are fat-soluble and perhaps it may have been that the artificially established subcutaneous depots of lard or olive oil served to concentrate some neoplastic agent from the body fluids. Another possibility is that the tumours were caused

Plate LXXII

Fig. 1.—Spindle-celled tumour from rat injected with lard (expt. 1). ×430.

Fig. 2.—Spindle-celled tumour from rat injected with lard containing 5 per cent. oleic acid (expt. 6). ×430.

Fig. 3.—Same as fig. 2, showing mammary duct included in tumour. ×155.

Fig. 4.—Spindle-celled tumour from rat injected with lard containing theelin (expt. 7). ×430.

Fig. 5.—Abdominal tumour in mouse following intraperitoneal injections of olive oil (expt. 20). ×430.

Fig. 6.—Mediastinal tumour from same mouse. ×430.

EXPERIMENTAL CARCINOGENESIS

FIG. 1.

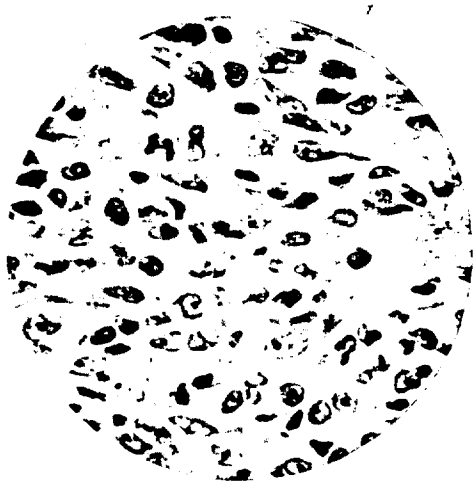

FIG. 2.

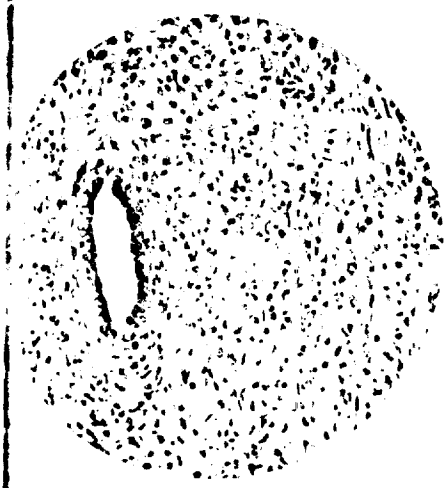

FIG. 3.

FIG. 4.

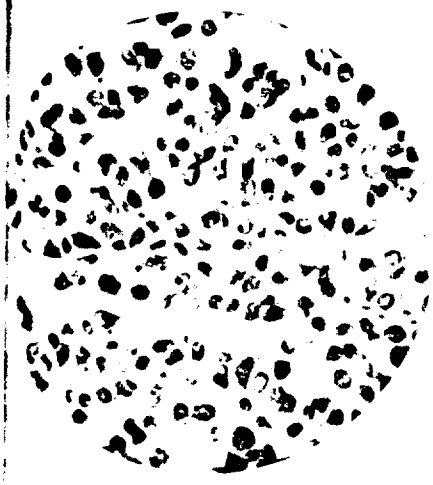

FIG. 5.

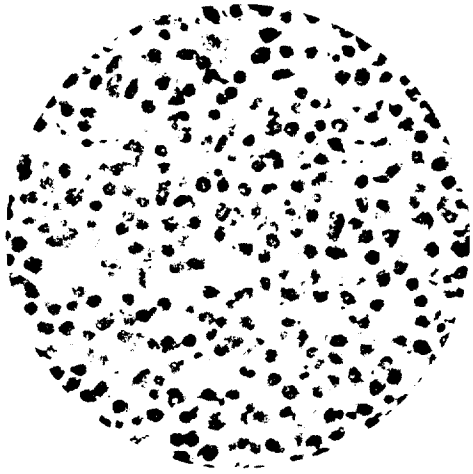

FIG. 6.

by " chronic irritation " produced by the lard or olive oil, for the opinion is still held in some quarters that cancer may result from chronic, non-specific irritation.

Nature of the tumours :—When comparing the effects of injecting 1 : 2 : 5 : 6-dibenzanthracene dissolved in lard or olive oil with those produced by injecting the same fatty solvents without the 1 : 2 : 5 : 6-dibenzanthracene, the relatively long periods required for the appearance of neoplasia in the latter series should be recognised.

The tumours which followed the injections of lard or olive oil consisted of spindle cells (figs. 1, 2, 3 and 4), were firm to the touch, grew to a large size, eventually invaded surrounding structures, underwent central necrosis, ulcerated through the skin and killed the hosts. On the other hand no metastases were observed, and the tumours when grafted into other rats failed to grow in every case. Such attempts were made with the tumours occurring in expts. 3, 6, 7, 8 and 14, 10 young rats being grafted on each occasion without any of the grafts becoming established. Considered together, the characters of these tumours suggest that they may represent a low grade of malignant neoplasia. In certain details they differ from the frankly malignant tumours induced by 1 : 2 : 5 : 6-dibenzanthracene and other carcinogenic compounds produced in these laboratories. Tumours induced by the latter, in contrast with those which have followed injections of lard or olive oil only, show more rapid invasion of neighbouring structures, produce metastases, and can generally be transmitted by grafting.

Spindle-celled tumours arising at the site of local injections of lard have already been reported from this Institute. Barry and Cook have described two examples occurring in a batch of 10 control rats given subcutaneous injections of lard alone. The rats died in the 55th and 66th week of the experiment. The tumours consisted largely of necrotic matter and granulation tissue, with some spindle-celled areas. No metastases were found and grafts made from these tumours did not become established.

Special reference should be made, perhaps, to expt. 7, in which a spindle-celled tumour developed in the 56th week at the site of injection of lard containing theelin, for Cori (1927) has recorded an example in which a sarcoma arose in a mouse at the site of injections of ovarian hormone dissolved in " oil " (see addendum).

III. *Injections of heated lard* sub cutem *in rats and mice.*

Some experiments were carried out with heated fat (table IV) in view of the results obtained in this and in other laboratories upon the production of carcinogenic substances by heat. Lard was heated in a metal bath for two hours at 340-360° C. in a stream of nitrogen. The product was shaken with petroleum ether and

watery Na_2CO_3 solution: (a) the petroleum ether extract was washed with water, the solvent distilled off in CO_2 and the residue injected (series 6); (b) the alkaline extract was acidified and the precipitated acids washed with water and mixed with lard (1 : 3 of lard) for injection (series 6A).

TABLE IV.

Injections of heated lard sub cutem *in rats and mice.*

	Series 6.	Series 6A.
Initial number (rats)	9	5
,, ,, (mice)	7	5
Alive after 1 year (rats) . . .	6	5
,, ,, 1 ,, (mice) . . .	4	5
Spindle-celled tumour in one rat . .	Day 608	Day 509
Last rat dead	Day 713	Experiment stopped, day 588
Last mouse dead	Day 497	Experiment stopped, day 609

On the 608th day a large tumour at the site of injection in a rat of series 6 was autografted and grafted. On the 616th day the rat (L.R. 175) died; the tumour showed spindle-celled areas but was mostly of a more fibromatous character than are the great majority of the tumours produced in connective tissue by carcinogenic hydrocarbons. On the 509th day a spindle-celled tumour of a more cellular character was found at the site of injection in a rat (L.R. 162) of series 6A, and was grafted. No metastases were found accompanying either of these tumours, and the grafts proved negative. Another rat (L.R. 261) of series 6A produced a densely fibrotic tumour containing hardly any cells at all. The mice in these experiments yielded no tumours.

IV. *Intraperitoneal injections of lard or olive oil in rats and mice.*

Repeated intraperitoneal injections of lard or olive oil were made in 60 rats and 80 mice (table V). At first these injections were given once a week, and later at gradually lengthening intervals. No spindle-celled tumours appeared in this series, but peritoneal tumours developed after injections of olive oil in one rat (27th week) and two mice (46th and 62nd weeks). In all three the tumours were multiple and consisted of cells with round, deeply staining nuclei and relatively little cytoplasm (figs. 5 and 6). The cells were supported by a delicate reticulum. There was widespread enlargement of the lymph glands with abundant perivascular collections of round cells in the lungs, liver, kidneys and other organs. In the mice large masses of growth were present in the mediastinum and were invading the pericardium and other structures. Leucæmia or advanced

TABLE V.

Repeated intraperitoneal injections of lard and olive oil.

No. of expt.	Duration in weeks.	Animal.	Number of animals.	Material injected.	Peritoneal tumours.	Period in weeks from first injection to death of tumour-bearing animals.	Other treatment.	Remarks.
9*	105	Rat	20	Olive oil	1†	27	Injections of lard into nape	Multiple tumours
15	89	Rat	10	Lard+5 per cent. oleic acid	0	...	None	...
16	124	Rat	10	Lard	0	...	None	1 mammary tumour, 124th week
17	114	Rat	20	Olive oil	0	...	None	...
18	60	Mouse	20	Emulsified olive oil	1†	46	None	Multiple tumours, leucæmia
19	43	Mouse	20	Olive oil	0	...	None	...
20	64	Mouse	20	Olive oil	1†	62	None	Multiple tumours, including large mediastinal tumour invading œsophagus. 10 mice grafted: no takes
21	40	Mouse	20	Lard+5 per cent. oleic acid	0	...	None	

* This experiment is also scheduled in table II (no. 9).

† Round-celled tumours.

leucocytosis was also present in one of the mice: no blood-counts were made, but abnormal numbers of white cells were visible in the blood in the microscopic sections of the tissues.

Although in these instances new growths were present in the abdomen it seems unsafe to assume that the disease began there or that it was a result of the injections. To establish such a connection a more extended experience would be required. A similar condition of lymphosarcomatosis without obvious leucæmia was seen in one of the rats (expt. 8, see table II) subjected to no other treatment than repeated subcutaneous injections of lard into the nape of the neck.

Summary.

Experiments carried out in this Institute, including those of Barry and Cook, upon the injection *sub cutem* of fats and allied materials have yielded 12 spindle-celled tumours in 217 rats and no tumours in 248 mice.

Repeated intraperitoneal injections of lard or olive oil into 60 rats and 80 mice in no instance caused spindle-celled tumours, but in one of the rats and two of the mice lymphosarcomatosis occurred.

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Addendum.

Since this paper was written, Gardner, Smith, Strong and Allen (1936) have reported the development of spindle-cell sarcomas in all of five mice belonging to a pure cancerous strain (Strong C3H) and treated by large daily doses of theelin followed by weekly injections of œstrone benzoate dissolved in oil.

GARDNER, W. U., SMITH, G. M., STRONG, L. C., AND ALLEN, E. Development of sarcoma in male mice receiving estrogenic hormones, *Arch. Path.*, 1936, xxi. 504.

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THE EFFECT OF FAT ON CALCIUM AND PHOSPHORUS METABOLISM IN NORMAL GROWING RATS UNDER A NORMAL DIETARY REGIME [1]

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Much work has been done on the effect of fat on calcium and phosphorus metabolism. In most instances either the diet or the animals employed, or both, have been abnormal. The criteria used for assessing results also have varied. Thus Westerlund ('34a, '34b), investigating the influence of tripalmitin, triolein and tributyrin upon calcium metabolism in the adult rat, found that the feeding of tripalmitin caused negative calcium balances while neither triolein nor tributyrin produced such an effect. It is to be noted, however, that the level of calcium fed was kept extremely low to promote the highest degree of calcium utilization. French ('42) and French and Elliott ('43) fed diets containing oleo oil at levels ranging from 0.28% to 45.46% to adult rats. The diets contained the suboptimum level of 0.3% of calcium and a calcium-to-phosphorus ratio of 1:1. They concluded that oleo oil interferes to a slight degree with calcium retention, though the evidence was of a low order of significance.

Knudson and Floody ('40) fed rats a low-fat rachitogenic diet containing a known amount of vitamin D, 1.27% calcium

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and a calcium-to-phosphorus ratio of 5:1. They found that the addition of 5% hardened cottonseed oil promoted better healing in rachitic rats. Larger amounts of fat were not so efficacious. Similarly, Jones ('40) found that the addition to rachitogenic diets of lard to comprise up to 10% increased the bone ash of rachitic rats over a wide range of calcium levels (0.38%–1.0%) and calcium-to-phosphorus ratios (4:1–50:1). Higher levels of lard exerted less influence.

As a result of many studies involving the addition of peanut oil to a high calcium-low phosphorus rachitogenic diet, Booth, Henry and Kon ('42) concluded that fats exert a definite antirachitic effect. They do not exert such an effect in high phosphorus-low calcium diets. Bunkfeldt and Steenbock ('43) fed cottonseed oil as a supplement to various diets and concluded that calcification was depressed in a low-phosphorus rachitogenic ration (phosphorus 0.075%). When phosphorus was optimum (0.25%), calcification was increased. Further increases in phosphorus content indicated a much smaller beneficial effect of fat, which was quite independent of the calcium-to-phosphorus ratio.

While fat most certainly affects the utilization of calcium and phosphorus, it appears that the absolute level of calcium and of phosphorus, their ratio to each other, the fat used and the condition of the animal are important factors. It seemed to us desirable to determine the influence of fat on calcium and phosphorus utilization when a normal diet is fed to normal, growing rats.

EXPERIMENTAL

The percentage composition of the ration used was egg albumen (cooked), 18.0; dextrinized starch, 73.4; and Jones and Foster ('45) salt mixture, 3.6. To this was added 5% of the fat to be used–completely hydrogenated cottonseed oil (HCSO), peanut oil (PNO) or coconut oil (CNO). One group was given an additional 5.2% of dextrinized starch in lieu of fat, the salt mixture being dropped to 3.4% so as to preserve

a constant ratio of calories to minerals. Daily vitamin supplements of 80 μg thiamine hydrochloride, 160 μg riboflavin, 120 μg pyridoxine hydrochloride and 100 μg of calcium pantothenate were given. Vitamins A and D were supplied by one drop of cod liver oil twice each week. The rations provided approximately 0.6% of calcium and 0.4% of phosphorus.[3]

Twelve 60-gm male rats were placed on each diet. In the case of the hydrogenated cottonseed oil, however, the feces and urine collections were from 10 rats, as the urine from two of the animals was inadvertently discarded after the completion of the feeding. The rats were kept in individual mesh-bottomed metabolism cages, each of which was set in a funnel in which had been placed a copper screen for the separation of feces and urine. Collections were started following a two-day preliminary feeding period. Feces were removed from the screen each day and the cage bottom, screen, and funnel washed with hot water acidified with sulfuric acid to a pH of 4.6 to 4.8. Twice each week, the urine, plus washings, was collected from beakers placed under the funnels and stored in glass jars at 7 to 8°C. until analyzed.

Food wastes and contamination with excreta were held to negligible quantities by the use of a specially prepared feeding device. A Franke-type cup was placed in the center of a can having a diameter of about 5 inches, the can being cut off at an angle of approximately 30° and the whole assembly covered with quarter-inch mesh and suspended on the side of the cage. By this arrangement, sleeping on the feeding cup was prevented and scattered food fell through the mesh into the large can, permitting subsequent recovery and weighing.

At the conclusion of a 28-day feeding period, the rats were killed with chloroform and the femurs removed for bone ash determinations. Calcium and phosphorus determinations were made of the diets and samples of the feces and urine.

[3] The actual percentage of calcium and phosphorus was as follows: HCSO diet — Ca 0.68, P 0.42; CNO diet — Ca 0.63, P 0.40; PNO diet — Ca 0.65, P 0.40; no-fat diet — Ca 0.61, P 0.44.

The methods of Morris, Nelson and Palmer ('31) were used in these determinations.

RESULTS

Calcium and phosphorus balances

Data on food intakes, weight gains and calcium and phosphorus determinations are given in table 1. Initially an attempt was made to control rigidly and equalize the amount of food eaten. This did not prove feasible, possibly because of a difference in appetite for the various fats. Sufficient

TABLE 1

Results of chemical determinations and feeding trials

CATEGORY OF INTEREST	DIET			
	HCSO	CNO	PNO	No-fat
Food intake (gm)	297.3	307.7	295.5	311.0
Weight gain (gm)	121.4	115.5	116.5	117.7
Calcium				
In food (gm)	2.0 ± 0.08 [1]	1.9 ± 0.04	1.9 ± 0.06	1.9 ± 0.12
In feces (gm)	0.8 ± 0.07	0.7 ± 0.05	0.6 ± 0.08	0.4 ± 0.23
In urine (gm)	0.1 ± 0.07	0.1 ± 0.02	0.2 ± 0.05	0.2 ± 0.12
Phosphorus				
In food (gm)	1.3 ± 0.05	1.2 ± 0.02	1.2 ± 0.08	1.4 ± 0.10
In feces (gm)	0.2 ± 0.02	0.3 ± 0.03	0.3 ± 0.04	0.3 ± 0.08
In urine (gm)	0.2 ± 0.06	0.1 ± 0.04	0.1 ± 0.04	0.1 ± 0.08
Femur ash (%)	61.9 + 1.90	62.8 + 0.70	62.0 + 1.80	62.0 + 1.00

[1] Standard deviation.

control was maintained, however, so that the mean variation of the amounts eaten was not large. All animals gained well.

Calculated values of the proportion of calcium and phosphorus in the feces and urine and that which was retained are given in table 2. The significance of the differences between the means of calcium and phosphorus in the feces and in the urine for each fat and the comparable mean for the no-fat diet were tested, using Fisher's ('30) modification of "Student's" t test.

Fecal calcium increased with increase in the melting point of the fat used. The animals on the no-fat diet excreted the least calcium in the feces. The differences between the means of the HCSO and CNO diets, respectively, and that of the no-fat diet were highly significant; between the mean of the PNO diet and the no-fat diet, the differences were below the significant level.

In general, urinary calcium varied inversely to fecal calcium, though the variations were of smaller magnitude. Hence, retentions of calcium varied nearly as greatly as the fecal

TABLE 2

Partition of dietary calcium and phosphorus

CATEGORY OF INTEREST	DIET			
	HCSO	CNO	PNO	No-fat
Calcium				
Fecal (%)	42 ± 2.5 [1]	35 ± 3.2 [1]	30 ± 4.5	27 ± 4.4
Urinary (%)	3 ± 0.1 [1]	7 ± 1.9	7 ± 3.1	8 ± 1.8
Retention (%)	55 ± 2.6 [1]	59 ± 2.7 [1]	63 ± 1.7	65 ± 4.3
Phosphorus				
Fecal (%)	18 ± 2.9	27 ± 3.4 [1]	26 ± 4.8 [1]	20 ± 1.8
Urinary (%)	19 ± 2.1 [1]	6 ± 1.7	5 ± 1.8	6 ± 2.1
Retention (%)	63 ± 4.4 [1]	67 ± 4.1 [1]	69 ± 4.9 [2]	74 ± 3.6

[1] The difference between this value and that of the fat-free diet is highly significant ("t" value exceeds that tabulated for P = 0.01).

[2] The difference between this value and that of the fat-free diet is significant ("t" value exceeds that tabulated for P = 0.05).

calcium, but in inverse order. Again the difference between the means was highly significant in the case of the HCSO and CNO diets, but not significant in the case of the PNO diet.

The excretion of fecal phosphorus revealed no constant relationship either to the characteristics of the fat used or to the presence or absence of fat in the diet. Animals on the CNO and PNO diets excreted more phosphorus than those on the no-fat diet. There was little difference between results on the HCSO and no-fat diets.

The very high excretion of phosphorus in the urine of rats on the HCSO diet was the only noteworthy feature of urinary phosphorus excretion. It lowered the retention of phosphorus to a value less than that obtained on any other diet; consequently retentions of phosphorus varied in the same manner as retentions of calcium. The difference between the means of the HCSO and CNO diets and that of the no-fat diet were highly significant; that between the means of the PNO diet and the no-fat diet was significant.

Excretion of calcium soaps

Holt, Courtney and Fales ('20) suggested that certain instances of poor calcium retention were due to the formation of insoluble calcium soaps. In order to discover the extent

TABLE 3

Neutral fat and fatty acids of feces

CATEGORY OF INTEREST	DIET			
	HCSO	CNO	PNO	No-fat
Dry weight of feces (gm)	25.36	18.22	16.19	17.94
Neutral fat in feces (gm)	2.28	0.58	0.82	0.60
Free fatty acids in feces (gm)	10.64	1.13	1.96	1.44
Total (as triglycerides) (gm)	13.41	1.76	2.86	2.10

to which this reaction might be responsible for the higher fecal calcium noted in rats fed the fat-containing diets, we attempted to determine the quantity of calcium soaps excreted in the feces. Several methods were tried and that of Augur, Rollman and Deuel ('47) was finally used, although it did not prove entirely satisfactory. The results are given in table 3.

Rats on the no-fat diet excreted considerable quantities of neutral fat and soaps. These, as Augur et al. have pointed out, must be regarded as of "metabolic" origin.

The animals on the CNO diet excreted even less neutral fat and soaps; hence it would appear that all of the fat in

their diet was absorbed, since the quantities found in the feces were of such small magnitude that they could all be accounted for as metabolic. This agrees with the findings of Hoagland and Snider ('43a) that coconut oil is 98.9% digestible at the 5% level. The relatively high fecal excretion of calcium noted in rats on this diet cannot be attributed to the formation of calcium soaps.

Rats on the PNO diet excreted slightly larger quantities of neutral fat and soaps than did rats on the no-fat diet.

Particularly noteworthy is the much greater amount of feces passed by animals on the HCSO diet than that by animals on the other diets. All of this increase was accounted for by increases in the amounts of free fat and soap excreted, which was expected in view of the higher fecal excretion of calcium observed.

Bone formation

Data on the analysis of the femurs for ash is given in table 1. It can readily be seen that, at least in the 28 days of feeding, the differences in mineral metabolism obtained on the diets did not effect the formation of the bones.

DISCUSSION

The inclusion of 5% fat in the diet increased fecal calcium. From the work of Holt, Courtney and Fales ('20) one might conclude that the rather large increase in fecal calcium in rats on the HCSO diet was probably due to poor absorption of calcium stearate formed in the digestive tract. Hoagland and Snider ('43b) have also reported on the poor absorption of tristearin and stearic acid. The HCSO was completely hydrogenated, hence it would have contained a large percentage of stearate. The large amounts of free fatty acids (soaps) noted in the feces of these animals would tend to substantiate such a theory.

On the other hand, the excretion of free fatty acids (soaps) by rats on the CNO and PNO diets was much smaller, so that it seems improbable that such a reaction was a factor in the

increases of fecal calcium noted in these two groups of animals. Calcium stearate is utilized by the rat, to a limited extent at least, as Boyd, Crum and Lyman ('32) have shown. It would seem likely that the much smaller amounts of calcium stearate which might have been formed on the CNO and PNO diets were largely absorbed.

Bergeim ('26) demonstrated that calcium is excreted into the intestine. Hence, the presence of calcium in the feces may be due to lack of absorption or to excretion. The extent of excretion varies over a considerable range, depending on the diet. Following a period of feeding calcium citrate, Steggerda and Mitchell ('46) found fecal calcium to be as much as twice the calcium intake. Greenberg ('45) found that about 18.0% of an injected dose of radioactive calcium appeared in the feces, whereas 32.5% appeared when the same amount was administered orally. It was not possible in the present experiments to determine whether the increased calcium in the feces of those rats fed the CNO and PNO diets was due to decreased absorption, to increased excretion, or to both.

It does seem that the increased excretion of fecal calcium imposed no strain on the economy of calcium in the body except, possibly, in those rats on the HCSO diet. Urinary calcium remained nearly constant except for the latter group. Bone ash apparently was not affected in any group, which is interesting in view of the marked variation in retention from group to group.

Phosphorus, too, is excreted into the intestine, as Bergeim ('26) has shown, and its presence in the feces may be due to lack of absorption or to excretion. Though the addition of CNO and PNO to the diet increased fecal phosphorus, HCSO did not have such an effect. This may have been due to the removal of interfering calcium from the intestine, as calcium stearate, when HCSO was fed. With the CNO and PNO diets there was little, if any, increase in soap formation but there was an increase in calcium excretion, possibly as a phosphate, thus accounting for the observed increase in

phosphorus excretion. Certainly there was no indication that the inclusion of fat, per se, increased fecal phosphorus.

Urinary phosphorus, like urinary calcium, was not much affected except in the group on the HCSO diet. Though fecal phosphorus was the lowest in this group, urinary phosphorus was over three times as great as in the other groups. Such an effect might have been due to a higher plasma concentration after absorption, giving rise to an excessive excretion before the phosphorus could be used, or to a lack of calcium ions which would aid in fixing phosphorus in the tissues.

The intimate relationship existing between calcium and phosphorus metabolism was evident in the values obtained for phosphorus retention. In spite of the irregular and divergent effect of the different fats on phosphorus excretion in the feces and urine, its retention varied in a manner similar to that of calcium.

When calcium is in excess, as in many rachitogenic diets, and the fat is partly composed of triglycerides of long-chain, saturated fatty acids, the formation of calcium soaps, with consequent lowering of phosphorus lost in the feces, may bring about some improvement in phosphorus utilization. This may possibly explain the results of Knudson and Floody ('40) and of Jones ('40). The observation of Booth, Henry and Kon ('42) that the equicaloric inclusion of peanut oil in the diet lowered both the fecal excretion of phosphorus and the urinary excretion of calcium is in direct contrast to our results. It was possibly due to the higher calcium and lower phosphorus content of their rachitogenic diet (calcium 0.9%, phosphorus 0.18%) and the formation of calcium soaps when fat was included.

CONCLUSIONS

1. The inclusion in a normal diet of 5% fat (completely hydrogenated cottonseed oil, coconut oil, or peanut oil) increased the fecal excretion of calcium.

2. When fats containing many of the poorly absorbed glycerides of the long-chain, saturated fatty acids were fed,

the increase in fecal excretion was probably due to the formation of relatively insoluble calcium soaps. When this was the case, the excretion of phosphorus in the feces was not increased.

3. When fats containing mostly the more rapidly absorbed glycerides of long-chain, unsaturated or short-chain, saturated fatty acids were fed, the increase in fecal excretion of calcium was of lesser magnitude. It did not seem to be due to the formation of calcium soaps and it apparently carried phosphorus with it, since fecal phosphorus was also increased.

4. The excretion of calcium and phosphorus in the urine was affected only indirectly by the presence of fat in the diet. When the fecal excretion of calcium was considerably increased there was a decrease in the urinary excretion of calcium but a marked increase in the urinary excretion of phosphorus.

5. The cumulative result of the inclusion of 5% fat in the diet was a decrease in the retentions of calcium and of phosphorus which roughly paralleled the increase in the fecal excretion of calcium.

6. The inclusion of 5% fat in the normal diet, well supplied with calcium and phosphorus and having an optimum calcium-to-phosphorus ratio, did not apparently affect the formation of bone in healthy, growing rats.

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THE EFFECTS OF OESTROGENS AND OF MILD CHRONIC STARVATION ON THE WHITE RAT[1]

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Abstract

Daily injections of peanut oil for 17 or 18 days cause decrease in rate of growth of the rat, and testicular atrophy. Hence results in experiments in which peanut oil (and probably similar oils) are used as solvent vehicles for administration of material by injection may be misinterpreted. Oral administration of oestradiol (5 ngm. daily) to young mature rats for three weeks or more causes decrease in growth rate, relative decrease in size of kidneys, heart, spleen, muscle, and ovaries, little effect on the liver, marked decrease in size of testes, even to actual atrophy, and frequent enlargement of the adrenals in males, with occasional enlargement in females. The adrenals are discoloured to a maroon shade, whether enlarged or not, and evidence is advanced that they are undergoing a pathological change; any enlargement is not in the nature of hypertrophy. Oral administration of stilboestrol gives similar results. The general effects of oestrogens are more marked in male than in female animals. Loss of appetite and diminished food intake are among the general effects, but the mild chronic starvation so produced can only contribute in very minor degree to the other oestrogenic effects.

In mild chronic starvation from food restriction the liver is invariably affected, while the adrenals are never enlarged nor discoloured.

The effects of combined oestrogenic and thyroid administration seem to be neither additive nor truly antagonistic.

Introduction

Effects of Oestrogens

Published data show some lack of agreement, probably owing to such causes as method of dosage, size of dose, vehicle used for injection, variation in length of administration, variation in potency of the oestrogen used, and in age and strain of the test animal (cf. 15). The discrepancies in the published results are best revealed by considering separately the effects on growth rate, and on the different body organs. The rat is referred to, unless it is otherwise stated.

Growth rate is said to be slightly decreased by injected oestrone (8, 32), markedly decreased in young animals by oestradiol (25), and initially increased in mature females by oestradiol (23). It is decreased by stilboestrol given orally (21, 24), or by pellet implantation (22), or injected dissolved in sesame oil (20) or in corn oil (15). Stilboestrol administered orally to parent females

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depresses the growth rate of their suckling young, although growth rate is relatively less retarded in young than in mature animals (21). It is decreased to actual loss of weight by benzestrol, whether injected, given orally, or implanted in pellets (2).

The heart, kidneys, and liver are said to show a relative decrease in size in normal animals (25), and a relative or actual increase in size in gonadectomized animals (14) following treatment with oestradiol or other natural oestrogens. The spleen shows a relative decrease (25). Benzestrol is said to produce no appreciable effect on kidneys or liver (2).

The thyroid, following injections of oestradiol, is variously reported to be but little affected (25), decreased in size (7), and initially hypertrophied, then atrophied (23). Both natural oestrogens and stilboestrol are said to produce destruction of acinar walls (17). Benzestrol is said to produce no appreciable effect (2).

The ovaries are said to hypertrophy if sufficient oestrogen is injected (12) but most investigators claim that they atrophy (7, 20, 21, 22, 25, 23). Oestradiol and stilboestrol both produce atrophy of the testes (2, 25, 20, 21, 22).

(The islets of Langerhans in mice are said to enlarge following administration of oestrogen (9).)

The natural oestrogens produce enlargement of the pituitary (12, 15, 7, 23, 14, 26) except in immature rats; in these, such treatment tends to produce a decrease in size (26). Stilboestrol also produces an enlargement (20, 21, 22).

(Oestrogens are reported to cause reappearance of the *X* zone in the adrenals of mice (5), and, oppositely, to produce degenerative changes (9, 10).)

In rats, enlargement of the adrenals, usually considered to be a hypertrophy, is stated to be produced by the natural oestrogens (27, 12, 7, 25, 14, 3, 26), although some investigators report little or no effect (2, 15), and even decrease in weight (8, 26), final atrophy (23), and degeneration (11). Stilboestrol is said to cause enlargement of the rat adrenal (20, 21, 22, 13) and to produce haemorrhage into the cortex (24, 28, 16), and, on the other hand, to produce little or no effect (17); it causes enlargement of guinea-pig adrenals (1). Following administration of benzestrol there is a slight tendency to enlargement with occasional slight haemorrhage (2).

Some of the effects produced by stilboestrol have been shown to be proportional to dosage. By feeding it in drinking water in gradually increasing concentrations it has been shown that growth is affected first, then the gonads atrophy, while definitely higher dosage is needed to produce enlargement of the adrenals and pituitary (21).

In so far as it is possible to draw conclusions from majority verdicts in these discrepant reports, it seems that, for the normal rat, natural oestrogens cause decrease in growth rate, relative decrease in size of heart, kidneys, liver, and spleen, atrophy of ovaries and testes, and enlargement of the pituitary and adrenals, provided dosage be adequate. A relatively greater effect is produced on male than on female animals. Stilboestrol and benzestrol

produce effects comparable to those of natural oestrogens on growth rate, the gonads, the adrenals, and the pituitary.

It is generally agreed that many of the effects of the oestrogens are mediated through the pituitary. Adrenal enlargement (28, 27, 12, 30), depression of growth rate (24), and atrophy of the ovaries (12) are usually considered to be secondary effects of this kind, though there is evidence that body growth may be directly affected (21).

Effect of Vehicle Used to Dissolve Oestrogen for Purposes of Injection

Selye (25) injected cholesterol (presumably an inert compound) dissolved in peanut oil into young rats. His results suggest a slight decrease in weights of liver and ovaries in females, and of testes in males. These effects may well be due to the peanut oil.

Richards and Kueter (24) administered stilboestrol in oil, with resulting pronounced cloudy swelling in liver and kidneys; this effect was shown to be produced by prolonged administration of the oil alone.

Heskett and Huffman (13) injected stilboestrol in corn oil, and noted that, regardless of dosage, the animals became lethargic, their coats rough and coarse, their conjunctivae hyperaemic, and they developed a nasal discharge, diarrhoea, skin eruptions, etc., and lost appetite. The results were attributed to the stilboestrol, but not improbably were partly due to the vehicle.

Bruce and Tobin (4) record that daily injections of 0.25 to 1.0 cc. of sesame oil for several weeks produce toxic effects on normal male rats, including inhibition of growth, decreased weight of testes, and increased adrenal weight.

Combined Oestrogen and Thyroid Administration

This has some practical significance, in view of the present minor vogue of treating hyperthyroid patients with oestrogens. It is well established that administration of thyroid to rats causes decreased growth rate, and hypertrophy of kidneys, heart, and adrenals, with lessened thyroid weight. Korenschevsky and Hall (14) consider that when both thyroid and oestrogen are administered they produce co-operative activity on adrenals, liver, kidneys, and heart, and a greater loss of body weight than is produced by either alone.

Claims have been made that administration of oestrogen to the experimentally hyperthyroid animal lowers oxygen consumption and decreases the basal metabolic rate (29).

Chronic Starvation

We are only concerned here with effects in the rat following chronic starvation for a period of some weeks. Mulinos and Pomerantz (18, 19) state that such chronic inanition produces atrophy of the adrenal cortex, never marked, and chiefly affecting the cytoplasm. The adrenal medulla is virtually unaffected. Loss of weight of the thyroid, spleen, and liver is relatively greater than that of the whole body. The weight and size of the ovaries are not markedly altered; anoestrus is usual.

Present Investigation

The experiments were begun in 1941 to ascertain whether the combined effects of oestradiol and thyroid administration were or were not additive. At first the oestradiol was injected dissolved in peanut oil, but since injections of peanut oil alone were found to produce definite effects, oestradiol was thereafter given orally. It seemed to produce lessened appetite, and the food intake was found to be measurably smaller. Hence the effect of mild chronic starvation was studied for comparison. Finally the effects of oestradiol and stilboestrol were compared, special attention being given to the adrenal glands.

Experimental Part

General

Each of many separate experiments were carried out with rats of the same sex and litter, each rat isolated in a separate cage. Individual variations of growth rates and of weights of organs of litter mates kept under precisely the same conditions have necessitated grouping the animals and averaging the results of groups. The muscle weighed for comparison in the experiments has been the right anterior tibialis.

Effect of Peanut Oil

When 0.1 cc. was injected intraperitoneally daily for 17 or 18 days, either alone or as vehicle for oestradiol, and then the rats were killed and their body cavities were opened up, the organ surfaces presented a peculiar glistening appearance due to a coating of minute oil droplets; this was especially noticeable on the surfaces of the liver, kidneys, and spleen.

In a few experiments the same dosage of peanut oil was injected alone and for the same periods as when used as solvent for oestradiol. The animals so treated showed a decrease in rate of growth and in weight of testes of the same order as that produced by the oestradiol solution. For example, the initial weight of a control male rat was 54.5 gm. It gained 49 gm. in 18 days and was then killed. Its testes weighed 1.33 gm. A litter mate initially weighed 56.5 gm. After 18 days' injection of 0.1 cc. peanut oil daily, it had only gained 31.5 gm., and its testes weighed only 0.38 gm.

It therefore seemed that results from experiments with oestradiol dissolved in peanut oil might be open to misinterpretation, and that conclusions as to the effect of oestradiol could be drawn more safely if it were given orally.

Experiments with Oestradiol, Given Orally

Table I gives the average results for groups of animals of the same sex and of similar initial body weights. In each single experiment the figures for the control, or the mean figures for controls, were taken for comparison. The effect on growth was calculated as the percentage ratio of the relative increases of body weight of the experimental and control animals during the experimental period. The effects on organ weights were calculated from the figures for percentage organ weights (i) based on body weights at the beginning

TABLE I

EFFECT OF OESTRADIOL ON THE GROWTH RATE AND BODY ORGANS OF THE WHITE RAT

	Males				Females			
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
No. of treated rats	2	6	3	2	3	6	7	2
Length of treatment, days	15	21 (av.)	21 (av.)	31	15	23 (av.)	22 (av.)	31
Average initial body wt., gm.	72	60	102	106	69	64	80	105
Average gain in wt., %	55	53	37	42	80	61	38	64
Organ wt. cpd. with normal, %								
Liver	96 (112)	106 (128)	90 (116)	87 (123)	110 (119)	100 (118)	103 (124)	99 (115)
Kidneys	91 (105)	97 (118)	88 (110)	78 (111)	101 (108)	96 (113)	87 (102)	80 (93)
Heart	87 (97)	96 (117)	82 (100)	76 (108)	97 (105)	92 (107)	91 (110)	86 (100)
Spleen	82 (96)	71 (85)	69 (85)	74 (103)	84 (91)	81 (95)	85 (105)	94 (111)
Muscle	76 (88)	91 (103)	78 (96)	69 (98)	87 (97)	90 (109)	—	91 (106)
Gonads	60 (69)	23 (27)	58 (72)	25 (35)	85 (91)	65 (76)	79 (93)	74 (86)
Adrenals	98 (112)	128 (155)	130 (165)	121 (173)	107 (116)	102 (118)	106 (132)	101 (117)
Thyroid	62 (74)	119 (144)	96 (121)	71 (100)	107 (115)	103 (119)	93 (113)	80 (88)

of the experimental period, and (ii) based on the final body weights. In Table I and subsequent tables the latter values are given in parentheses.

Throughout each experiment a daily dose of 3 mgm. crystalline oestradiol was fed each experimental animal. Attempts to give it with barley meal failed, but it was fairly well taken when mixed with carrot juice (controls got the carrot juice alone).

The effect on body organs can be analysed by applying the following criteria (remembering that values in parentheses are calculated from the percentage organ weights in terms of the final body weight):—

(*A*) When percentage organ weights, referred to initial body weight (i) are greater than those of controls (and thus exceed 100), actual enlargement is indicated; (ii) are equal to those of controls (giving a value approximately 100), there is no effect.

(*B*) When percentage organ weights are less than those of controls, when referred to initial body weight, but greater, when referred to final body weight, the effect on the organ is less than that of the whole body.

(*C*) When percentage organ weights, referred to final body weight, (i) are equal to those of controls (giving a figure in parentheses approximating to 100), the effect on the organ is proportional to that on the whole body; (ii) are less than those of controls, the effect is relatively greater than that on the whole body and may indicate an actual atrophy.

Examining Table I in light of these desiderata the following conclusions seem permissible. The figures show considerable variation (the variations for individual animals in a group were still greater). There is a greater effect

on males than on females. As might be expected there seems to be a more marked effect with longer treatment, though the number of animals in individual groups is too small to stress this.

Growth rate is invariably diminished.

Liver: there is no effect in females, a slight but negligible relative loss of weight in males.

Kidneys and heart: there is a relative decrease in size, but the effect is less than that on the whole body.

Thyroid: very variable results are shown; no definite conclusion can be drawn from them.

Spleen: in females it tends to remain proportional to the whole body, in males to be more markedly affected.

Muscle: in females it is less affected than the whole body; there is a somewhat greater effect in males.

Testes: marked relative decrease in weight, and sometimes actual atrophy.

Ovaries: the effect is greater than that on the whole body, but less than on the testes.

Adrenals: usually, but not invariably, there is an actual enlargement in male animals; there is usually but little effect in females, but sometimes some degree of enlargement.

Examination of individual records shows that when the body weight of male rats at the beginning of treatment was between 50 and 60 gm., oestradiol always produced an actual atrophy of the testes so that at the end of a 21-day experiment their combined weight varied from 0.16 to 0.25 gm.; with initial body weights between 60 and 80 gm., such atrophy was only occasionally produced, although there was always an arrest of growth. With still greater initial body weights, only some arrest of growth was obtained.

The colour of the adrenals in normal rats, in our experiments, has varied from bright yellow (rare) to dull and grey yellow, sometimes with a slightly reddish tinge. In practically all the animals fed (or injected) with oestradiol the adrenals showed a definite colour change, variously recorded as grey, brown, dark brown, and most frequently as brown red or maroon. Such change of colour was noted in enlarged glands, but also in others in which there was no definite enlargement, or even slight relative loss of weight.

Appetite

In association with the striking diminution in growth rate, it was observed that the rats fed oestradiol appeared to eat less food. Hence measurement of food intake was carried out in two experiments. Each day, weighed quantities of food were given in known excess of requirements, and the amount uneaten the following morning was removed, and weighed. Correction was made for partial drying of residues by several determinations of the water content of fresh material and of mixed residues. The correction is only

approximate, so that the measurements of food consumption also are only approximate. Nevertheless they should be comparable.

In Experiment 1, during the experimental period, each of a litter of four males and five females was fed daily 20 gm. bread and 25 gm. milk.

In 13 days the two control males ate respectively 446 and 465 gm. of the mixture (average 455.5 gm.). In the same period two males receiving 3 mgm. oestradiol daily ate 361 and 360 gm. (average 360.5); their intake was thus 21% less.

The corresponding figures for two control females were 369 and 431 gm. (average 400 gm.), and for the three females fed the same dose of oestradiol 429, 382, and 343 gm. (average 385 gm.); there was only a 4% diminished intake.

In Experiment 2 a litter of nine females was similarly treated. Four controls ate in 22 days 727, 733, 741, and 824 gm. (average 756 gm.). Five animals fed oestradiol ate 577, 625, 670, 726, 730 gm. (average 666 gm.), a diminished intake of 12%.

The apparently lesser effect on appetite of females than of males agrees with the other effects recorded.

The diminished food intake, although not great, suggests that some part of the effect produced by oestradiol might be traceable to a chronic mild starvation. Hence the effects of such a mild starvation were determined.

Effects of Chronic Mild Starvation

Five experiments were carried out in each of which animals on an insufficient diet were compared with controls of the same sex and litter. The controls were fed daily 20 gm. bread and 25 gm. milk; the experimental animals 8 gm. bread and 10 gm. milk. All residual food was removed each morning: the experimental animals left little. In a sixth experiment the daily allowance of the experimental animals was lowered to 4 gm. bread and 5 cc. milk and they actually lost weight, while becoming somewhat hyperactive. The results are given in Table II.

Analysis of the figures in Table II in accordance with the principles already stated permits the following conclusions. Liver, spleen, ovaries, and thyroid show a relative lessened gain of weight greater than that of the whole body. Kidneys, heart, testes, and muscle show a relative lessened gain of weight less than, or proportional to that of the whole body. Adrenals show a lessened gain of weight relatively less than that of the whole body in males, a somewhat greater effect in females.

In chronic starvation of the duration and degree of severity tested, enlargement of adrenals never occurred while the glands always retained their normal colour.

It is to be noted that the effect on growth rate was greater than that produced by oestradiol, in our experiments, so that the effects on body organs are as great or greater than can be attributed to the potential mild starvation

TABLE II

SUMMATION OF EFFECTS OF CHRONIC STARVATION ON GROWTH AND BODY ORGANS

	Males		Females	
	Group 1	Group 2	Group 1	Group 2
Type of starvation	Mild	Mild	Mild	Severe
No. of treated rats	3	7	5	2
Duration of expt., days	21	20-21	21	20
Average initial body wt., gm.	71	84	70	76.5
Relative increase in body wt. as cpd. with controls, %	26	19	35	−80
Organ weights cpd. with normal, %				
Liver	69 (97)	58 (88)	69 (89)	34 (97)
Kidneys	69 (107)	68 (104)	72 (95)	51 (143)
Heart	64 (106)	67 (104)	76 (100)	47 (134)
Spleen	55 (89)	58 (89)	72 (96)	31 (83)
Muscle	73 (122)	81 (124)	88 (113)	29 (79)
Gonads	82 (133)	74 (116)	61 (80)	23 (64)
Adrenals	81 (127)	84 (127)	67 (90)	41 (114)
Thyroid	52 (83)	58 (89)	59 (77)	47 (130)

of the oestradiol-fed rat. A comparison of the two sets of results indicates several definite differences. The liver is practially unaffected by oestradiol, but is very susceptible to inadequacy of food intake. Oestradiol has a marked effect on the testes, mild starvation but a slight effect. Chronic starvation never produces adrenal enlargement or colour change; oestradiol frequently produces enlargement, and usually produces colour change. Hence it seemed reasonable to conclude that the general effects produced by oestradiol are primary effects and are not appreciably affected by the loss of appetite it induces.

Effects of Stilboestrol

Pure crystalline diethylstilboestrol was used, and fed in varying doses in different experiments. Some difficulty was experienced in feeding it. It was not well taken in carrot juice, but was eaten moderately well in carrot pulp, or by alternately mixing it with carrot pulp and with bread and milk (controls being of course fed the medium alone). The results are summed up in Table III.

Comparison of Tables I and III suggests general similarity in the effects of oestradiol and stilboestrol. As with oestradiol, male animals appear to be more markedly affected than females. Markedly increased dosage does not appear to produce marked difference in results, but the number of animals in each group is too few to stress this, especially since there was not complete control of the actual amounts eaten in these oral experiments.

Stilboestrol rarely produced enlargement of the adrenals, though the same mahoon discoloration was produced as with oestradiol. The apparent enlargement of the livers of female rats was probably fortuitous. The uteri of the stilboestrol animals were markedly distended (with a clear fluid); those of the oestradiol-fed animals were less affected.

TABLE III

EFFECTS OF STILBOESTROL ON GROWTH, ETC.

	Males		Females		
	Group 1	Group 2	Group 3	Group 4	Group 5
No. of test animals	4	3	4	4	3
Daily dosage, mgm.	3	6	2	3	6
Duration of expt., days	30–35	31	22	30–35	31
Average initial body weight, gm.	111.5	109	114	101	99.5
Relative increase in body wt. as cpd. with controls, %	27	36	29	49	54
Organ wt. cpd. with normal, %					
Liver	92 (140)	93 (130)	104 (122)	120 (140)	108 (128)
Kidneys	82 (118)	77 (110)	84 (99)	96 (113)	92 (109)
Heart	84 (119)	81 (113)	98 (118)	96 (113)	90 (110)
Spleen	73 (105)	66 (92)	82 (97)	77 (90)	79 (96)
Muscle	71 (102)	72 (102)	84 (103)	89 (95)	85 (100)
Gonads	17 (24)	18 (25)	114 (137)	89 (100)	94 (111)
Adrenals	108 (154)	95 (133)	77 (91)	102 (119)	83 (98)
Thyroid	66 (94)	50 (72)	76 (90)	101 (119)	66 (76)

Effect of Oestrogens on the Adrenals

An attempt was made in some of the later experiments to ascertain the nature of the adrenal enlargement and change in colour by determination of water content. No difference in colour as produced by either of the oestrogens could be definitely asserted, though the adrenals of the oestradiol-fed animals were (perhaps imagined to be) a little greyer.

The adrenals were transferred to closed weighing bottles as rapidly as possible after dissection, weighed, and then dried at 105° C. for 24 hr. The results are shown in Table IV.

The results suggest that there is a tendency to increased water content, whether or not there is an actual enlargement of the glands.

Since it has previously been shown that, in acute starvation, enlargement of the adrenals is in the nature of a hydropic degeneration, with increased water content, and decreased number of cells in corresponding areas of the adrenal cortex (6), an attempt was made to estimate the cell numbers in corresponding areas of the adrenal cortexes of animals fed oestrogens. The technique has already been fully described (6). The cell counts were made by counting nuclei in five 1 × 10 cm. strips running inward from the periphery of the cortex in a series of photomicrographs (magnification × 200), of which three or four were made for each adrenal, selecting central sections. The figures refer to results per square cm. of the measured areas. Data are given in Table V and are for a single litter of rats.

The results in Table V indicate that the slight enlargement of the adrenals produced in a male rat by stilboestrol was accompanied by a decreased number of cells in the outer part of the cortex. Examination of the photomicrographs also indicated a definite narrowing of the glomerular zone. The relative

TABLE IV

EFFECT OF OESTROGENS ON WATER CONTENT OF ADRENALS

Sex	Controls			Stilboestrol-fed					Oestradiol-fed				
	No. of animals	Adrenal dry wt.		Daily dose	No. of animals	Adrenal size	Adrenal dry wt.		Daily dose	No. of animals	Adrenal size	Adrenal dry wt.	
		Extremes	Mean				Extremes	Mean				Extremes	Mean
		%	%	Mgm.			%	%	Mgm.			%	%
Male	5	28–32	30	3	2	Enlarged	27–28	27.5	—	—		—	—
				3	1	?		26	—			—	—
				6	2	Relatively small	29–30	29.5	3	2	Enlarged	25–25	25
Female	7	27–32	30	2	4	Relatively smaller	26–29	27	—			—	—
				3	3	Relatively smaller	25–28	26	—			—	—
				6	1	Relatively smaller		21	—			—	—

TABLE V

CELL COUNTS IN COMPARABLE AREAS OF ADRENAL CORTEXES

	Rat 1, male	Rat 2, male	Rat 3, female	Rat 4, female	Rat 5, female	Rat 6, female	Rat 7, female
	Control	Stilboestrol	Control	Oestradiol	Oestradiol	Stilboestrol	Stilboestrol
Daily dose, mgm.	—	6	—	3	3	6	6
Initial body wt., gm.	109	123	86	110.5	105.5	105.5	99.5
Gain in body wt., gm.	54	18	53	20.5	21.5	19	18
Left adrenal							
Actual wt., mgm.	13.5	19.4	24.7	30.6	27.4	19.8	23.6
Relative wt., mgm. %*	12.4	15.8	28.7	27.8	26.0	18.8	23.7
Cell count	63.2	42.9	26.1	27.3	26.0	29.9	28.0
Right adrenal							
Actual wt., mgm.	13.4	12.3	18.4	26.0	25.2	21.2	15.6
Relative wt., mgm. %*	12.3	10.0	21.4	23.5	23.9	20.1	15.7
Cell count	56.6	44.9	26.5	31.9	27.4	29.6	27.6
Both adrenals							
Actual wt., mgm.	26.9	31.7	43.1	56.6	52.6	41.0	39.2
Relative wt., mgm. %*	24.7	25.8	50.1	51.2	49.9	38.9	39.4
Cell count	59.6	43.7	26.3	29.1	26.7	29.7	27.8
Mean values				27.9		28.7	

* *To initial body weight.*

decrease in weight of the adrenals of the stilboestrol-fed female rats was not accompanied by a change in cell count. There was an occasional thinning of the glomerular zone. In female animals fed oestradiol it produced no change in the adrenal weights. There was no change in cell count, but there was a marked decrease in the width of the glomerular layer.

Effect of Combined Oral Administration of Oestradiol and Desiccated Thyroid

A few experiments were carried out to ascertain this effect. In each, animals of the same sex and litter were fed daily either (i) desiccated sheep thyroid (0.32% iodine) in daily dosage of 1 : 5000 of the (initial) body weight, or (ii) 3 mgm. oestradiol, or (iii) a combined dose of the two. One or more animals (also of this litter and sex) were used as controls.

The results were not clear cut. They were certainly not additive. Thus, since both oestradiol and thyroid feeding depress growth rate, jointly they should give a greater depression, but this was not the case. Since (at least in male animals) both tend to produce adrenal enlargement, jointly they should produce greater enlargement, but they did not do so. They both depress the relative weight of the muscle tested, and jointly should do so to a greater extent, but did not do so. They both depress the development of the testes, but jointly produced no greater effect than oestradiol alone.

Nor did the results indicate a definite antagonism between the actions of the two, except perhaps on the heart; in both sexes, instead of the enlargement produced by thyroid feeding, following the combined treatment the weight of the heart remained practically normal.

The data obtained were so indefinite that it did not seem worthwhile to carry out further experiments.

Discussion of Results

General

In determining the effects of any particular treatment on growth or on one or several of the body organs of the rat it is usually considered adequate to take a number of animals of the same sex and of approximately the same initial body weight, and to divide them into two groups, one to be treated, the other kept as controls. In recorded experiments the size of such groups varies considerably. Careful examination of our records of control animals made during the past 25 years shows very marked variations both for rate of growth and for the organ weights at any particular body weight, so that large groups of randomly selected animals are essential to permit accurate conclusions concerning qualitative differences in results, unless the observed differences are constant and large. This is still more true where attempts are made to draw conclusions as to quantitative differences.

It is generally assumed that the degree of normal variation in litter mates of the same sex is much smaller than in a random selection. Our records show that this is usually the case, and that in plotted results, lines connecting values of litter mates for a particular organ (heart, kidneys, adrenals, etc.), tend to

parallel the average curve for such an organ, so that the percentage values tend to be in good agreement. Yet there are by no means infrequent exceptions, in which the reverse of such parallelism is seen, so that even with comparisons with litter mates, the average of a number of comparisons is desirable.

In some of the experiments now recorded it is recognized that the number of animals used has not been adequate; the results in such cases have not been stressed.

Peanut Oil

The definite effects on rate of growth and on the testes following injection of peanut oil indicate that there may be danger in drawing conclusions from experiments in which this oil is used as solvent vehicle for test material. It has been pointed out that Bruce and Tobin (4) obtained very similar results with sesame oil, while some of the pathological effects reported by Heskett and Huffman (13) may well be due to injected corn oil. It seems likely that the inertness of such oils has been unwarrantedly assumed. While the amounts employed therapeutically are almost certainly without significance, those used in experiments with small animals are relatively much greater and their potential effect should not be ignored.

The Effects of Administered Oestrogens

Oestradiol, in the dosage used for the periods of time stated, produced in our experiments results almost identical with the majority verdicts set out in the introduction. Effect on liver, however, seems negligible, and actual atrophy of ovaries seems doubtful, while enlargement of the adrenals was rare in female animals. There was definitely a greater effect on males than on females. Stilboestrol produced similar effects, our results in general agreeing with those of Noble (21), who administered it in drinking water.

The Effect of Oestrogens on the Adrenals

The enlargement has usually been considered a hypertrophy. We have shown that the so-called adrenal hypertrophy produced by acute starvation is in reality a pathological process in the nature of a hydropic degeneration, and we have adduced evidence from the literature that the similar enlargement that results from thiamin deficiency is also not a hypertrophy. We have stressed that whenever experimental treatment results in marked discoloration of the adrenals, this indicates presence of a pathological process (6).

We have now shown that such discoloration follows treatment with oestrogen (oestradiol or stilboestrol), the typical colour being a maroon or grey-maroon, and differing from the dark grey or dirty grey colour typical of the adrenal of the moribund acutely starved rat. This maroon discoloration occurs whether or not the adrenal is enlarged, or is even relatively somewhat smaller than normal.

We have also adduced some evidence suggesting a tendency to increased water content, not definitely associated with enlargement. Examination of histological sections of such glands suggested presence of no marked abnor-

mality such as haemorrhage, but there was a tendency to thinning of the glomerular zone, not definitely associated with enlargement. Cell counts in comparable areas of the cortex were normal in absence of enlargement, and decreased when enlargement had occurred.

Too few determinations and examinations were made to stress these findings, but they all fit together to permit the suggestion that oestrogens produce no hypertrophy of the adrenals, but rather, in the dosage used, something in the nature of a pathological process.

Mild Chronic Starvation

Our results are in moderate agreement with those of Mulinos and Pomerantz (18, 19), which were of longer duration. The absence of enlargement of the adrenals is to be stressed. The effects produced by oestrogens include loss of appetite and diminished food intake but the mild chronic starvation resulting can only influence the oestrogen results in very minor degree.

Combined Oestrogen and Thyroid Administration

Results in the few experiments performed were not in good agreement with those of Korenschevsky and Hall (14) and indicated neither an additive effect, nor a true antagonism.

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INFLUENCE OF A DIETARY SUPPLEMENT OF ERUCIC ACID AND OTHER FATTY ACIDS ON FERTILITY IN THE RAT

STERILITY CAUSED BY ERUCIC ACID[1]

K. K. CARROLL AND R. L. NOBLE

Abstract

Male rats were raised on a diet of powdered Masters Meal with a supplement of 10% or more by weight of erucic acid. The animals suffered a progressive reduction in spermatogenesis and became completely sterile after about 5 months, despite their normal rate of growth and their apparently excellent state of health. Subsequent experiments have suggested that similar effects can be produced, though less readily, in adult rats on the same diet. Recovery of spermatogenesis apparently can take place provided testicular degeneration has not advanced too far. The impairment of spermatogenesis appears to be a specific effect of the erucic acid in the diet. The estrous cycles of female rats on the diet usually remained undisturbed but there was some interference with parturition. Few of the offspring survived for long because of deficient mammary development and lactation in the mother. The same impairment occurred also when the animals were fed the diet with 15% by weight of oleic acid. The defects resemble those which occur in animals when fed a diet deficient in essential fatty acids. The suggestion is offered, therefore, that erucic acid may interfere with reproduction by interfering with the metabolism of the essential fatty acids. The administration of erucic acid in the diet may afford a new lead for the control of fertility.

Introduction

During the past few years we have been interested in the nutritional influence of certain fatty acids on metabolism and function. There is evidence that some of the fatty acids may have a special metabolic function. We have endeavored to elucidate these functions by means of nutritional experiments with diets containing various proportions of individual fatty acids. Erucic acid, a C_{22}-monoethylenic fatty acid, and a prominent constituent of rapeseed and related seed oils, is known to cause a marked accumulation of cholesterol in the adrenal cortex of the rat (4), and to induce an increased excretion of cholesterol in the feces of rats maintained on a sterol-free diet (6). It has been suggested that nervonic acid, the C_{24}-homologue, or related compounds present in the lipids of the brain and spinal cord may be beneficial in the treatment of demyelination in humans (18).

In some of our early experiments it was observed that the cholesterol content of the ovaries was increased in the rats that had been fed rapeseed oil, but no obvious dysfunction of the ovaries had been noted (5). Although we could not be sure that the increase in the cholesterol content of the ovaries was attributable to the action of erucic acid in the rapeseed oil supplement, we decided to examine the effect of the continuous feeding of erucic acid, to successive generations of rats, on the fertility of the animals. This communication describes the results of feeding tests that have been in progress during the past $1\frac{1}{2}$ years.

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Contribution from the Collip Medical Research Laboratory, University of Western Ontario, London, Canada.

The animals used in the experiments to be described were of both sexes, mostly of the Sprague-Dawley strain, but included some Wistars. Except in the earliest experiments vaginal smears were taken daily from all female animals. A complete and detailed chart of the estrous cycles of each animal was obtained. The presence of a vaginal plug or sperm in the vaginal smear provided evidence that mating had occurred. Implantation was assumed to have occurred if the following signs appeared: cessation of the estrous cycle, appearance of blood in the vaginal smear after the 10th day, a rapid increase in body weight. If parturition did not occur and the body weight decreased, resorption of the fetus was assumed to have taken place. Careful observation was made of the number and state of health of the newborn, but in some cases the mothers destroyed the offspring at birth.

The supplement of fatty acid or rapeseed oil was added in the proportion by weight indicated below, and thoroughly mixed with the stock powdered diet (Masters Meal), which is fed to the breeding colony of this laboratory. The erucic acid was obtained commercially (Archer–Daniels-Midland Co., Midland, Michigan,) and was melted to facilitate its incorporation in the diet. In more recent experiments purified erucic acid prepared in our laboratory was used. The acid was purified by twice recrystallizing the commercial erucic acid from three volumes of methanol at 3° C. The purified material was a white solid, m.p. 32° C. with an iodine value (Hanus) of 71 and a neutralization equivalent of 333 (theoretical: I.V.—74.8, neutral equivalent 338.5). The oleic acid* was obtained from Nutritional Biochemicals, Cleveland, and the rapeseed oil was kindly provided by Dr. B. M. Craig of the Prairie Regional Laboratory of the National Research Council, Saskatoon.

The animals were killed after varied periods on the diet. At autopsy, the testes, seminal vesicles, and prostate were removed, and fixed in formalin or Bouin's solution before weighing. Other organs were weighed in the natural state.

Results

Young Male Rats Maintained on the Supplemental Diet after Weaning

The data in Table I indicate the effect of the feeding of a diet containing erucic acid, oleic acid, or rapeseed oil for various periods of time. Of the rats that were fed the diet with erucic acid, three groups were the offspring of mothers which had also been fed the diet containing 15% by weight of erucic acid. Groups C and D represent the first and second generations respectively in one experiment, while group B represents the first generation in another experiment. In these cases the young were considered to have started to eat the diet at 3 weeks of age. The animals in groups A and E were from mothers that had been fed the standard control stock diet and were given the erucic acid supplement after they were weaned at 21 days of age.

* Analysis of a sample of this oleic acid through the courtesy of Dr. O. S. Privett of the Hormel Institute, Austin, Minnesota, gave oleic 88.1%, linoleic 3.0%, linolenic 0.6%, saturated acids 7.4%, and preformed diene 0.8%.

In initial tests males and females on the diet were mated, but the males that had received the erucic acid supplement were later mated with females fed the stock diet (usually one male with two females).

TABLE I

INFLUENCE OF DIETARY SUPPLEMENT OF FATTY ACID ON FERTILITY IN THE GROWING MALE RAT

Group	Dietary supplement	Period on special diet before mating (weeks)	Procedure	Results
Normal controls	Stock diet (without fatty acid)		1 M mated with 4 F 2 M mated with 6 F	10 litters, pregnancy occurring on estrous cycles after mating — 3 on 1st, 3 on 2nd, 3 on 3rd, and 1 on 5th
		Mated with treated* females		
	25% rapeseed oil	16	3 M mated with 3 F	3 litters, pregnancy occurring on 1st cycle after mating
	15% oleic acid	16	4 M " " 4 F	4 litters, pregnancy occurring in 3 cases on 1st, and 1 on 2nd cycle
A	15% erucic acid	11–15	2 M " " 10 F	7 litters
A	15% " "	20–28	4 M " " 10 F	0 litters, no matings
B	15% " "	9–13	6 M " " 5 F	1 litter, pregnancy occurring on 1st cycle after mating — 1 resorption, 5 sterile matings with pseudo-pregnancy
C	15% " "	9–13	4 M " " 9 F	0 litters
D	15% " "	10–16	2 M " " 3 F	0 litters
E	10% recrystallized erucic acid	16–20	5 M " " 5 F	2 litters, pregnancy occurring on 1st cycle after mating — 3 sterile matings with pseudopregnancy
E	10% " "	20–24	4 M " " 4 F	0 litters,7 sterile matings with pseudo-pregnancy
		Mated with normal females		
A	15% erucic acid	29–45	4 M mated with 8 F	0 litters, no matings
B	15% " "	17–21	6 M " " 12 F	0 litters, 1 sterile mating with pseudo-pregnancy
C	15% " "	17–25	4 M " " 8 F	0 litters, no matings

* Female animals on the same diet and for the same duration as the males.

It is evident that the fertility of the males was markedly affected after they had been maintained on the erucic-supplemented diet for a period of approximately 3 months. In many cases sterile mating occurred in animals that had subsisted for 3 to 5 months on the diet, but after 5 months mating rarely occurred. In contrast to the results with the diet with erucic acid, the feeding of the diet supplemented with 15% oleic acid or 25% of rapeseed oil has so far produced no impairment of the fertility of the male rats.

Mature Male Rats Maintained on the Supplemented Diet

The study on the effects of erucic acid supplemented diet on adult male rats has not yet been completed. The data so far suggest that the adult may be more refractory than the actively growing animal to the effects of such a diet. In the various fertility experiments, 10 adult males have been examined, after having been fed the diet for 2 to 3 months. Most of these animals showed no evidence of sterility. In one case, however, a male was housed with one pair of females for 2 weeks, and another pair for the following 2 weeks. Although a total of 14 estrous cycles were detected during the month, the male did not mate. In another experiment, a male mated seven times during the first 6 weeks on the diet. During the subsequent 6 weeks, when

it was housed with three females which exhibited a total of 14 estrous cycles, it made no attempt to mate. A third male on the diet sired three litters within 2 months. At the end of the 4th month one of the testes was removed from the last-mentioned male. The gland weighed 1725 mg. and was found to be normal. During the interval between $4\frac{1}{2}$ and 6 months on the diet the male has made four sterile matings and the remaining testis has become small and soft on palpation. In an experiment started more recently 25 male animals on the diet with a 15% erucic acid supplement have shown little palpable change in the testes after 3 months. However, in two of five animals on a 30% erucic acid supplement the testes have become small and soft.

Weight of Endocrine Organs

Some observations have been made on the weights of various endocrine organs from male rats which had been maintained for different periods of time on the diet supplemented with erucic acid. In most cases unilateral castration was performed in order that the continued effects of the diet could be observed. The total weight of the testes was estimated as being double the weight of the testis removed. The weights are shown in Table II.

TABLE II

WEIGHT OF ORGANS FROM RATS ON DIET SUPPLEMENTED WITH ERUCIC ACID

Period on diet (weeks)	Testes (mg.)	Seminal vesicles (mg.)	Prostate (mg.)
	Rats started on diet when adult		
Control animal on stock diet but used for breeding experiments	3335(1)*	740	940
4	3286(2)	877(2)	983(2)
8	3350(2)		
12	3575(2)	927(2)	1414(2)
16	3450(1)		
	Rats started on diet when immature		
17	1882(6) 760–3618		
23–25	1245(7) 900–1774	1225(1)	1330(1)
29–37	1296(5) 813–1646	512(3)	977(3)

* Numeral in parenthesis indicates the number of animals.

The weight of the testes was markedly affected in the growing animals that were fed the erucic acid supplemented diet, and the deleterious influence of the supplement became clearly evident after the animals had been 4 months on the diet. In contrast, the weight of the seminal vesicles and prostate appeared to be relatively normal. Little change has been noted in the weight of the testes of adult animals maintained on the supplemented diet, but so far the animals have been under observation for periods only up to 4 months.

Morphological Changes in the Male Rats

The most prominent histological evidence of change was observed in the spermatogenic cells of the testes. A gradually increasing degenerative change takes place after the animals have been approximately 3 months on the erucic supplemented diets. Although in most cases advanced degenerative changes have been observed, an irregular patchy type of degeneration occurred during earlier stages. Degeneration was evident only in some of the tubules, and in those the presence of giant cells was a prominent feature. Many of the tubules, however, appeared to be normal. All of the basal cells showed degenerative change, and in the advanced condition only Sertoli cells remained within the shrunken tubule. Fluid accumulation around the tubules was pronounced. Mature sperm were absent in the tubules and in the epididymis. The interstitial cells appeared to be little affected. The typical lesions are evident in the microphotographs shown in Figs. 1 and 2. In contradistinction to these changes in the tubules the condition of the testes of adult rats fed the supplemented diet for 4 months showed no difference from normal.* In the male animals which did not mate spermatogenesis appeared to be normal, and sections of the epididymis showed a large number of sperm, although possibly the number may have been fewer than in controls. The "normal" condition of the testis of an adult rat after 1 month on the supplemented diet is indicated in Fig. 3. In some animals the thyroid gland was enlarged and weighed up to 25 mg. A weight of 13 mg. is about the normal for rats of comparable body weight. The thyroid appeared brownish in color, and on section, showed a slightly increased colloid content but no evidence of hyperactivity. The parathyroid glands in some cases appeared more deeply stained than occurs with the normal tissue. The liver, kidneys, and pituitary were of normal appearance.

Recovery of Spermatogenesis

Only a few experiments have been completed to this date on the recovery from testicular impairment in immature animals maintained on the erucic acid supplemented ration. In some of the animals one testis was removed and the rats were then transferred to the stock (unsupplemented) diet. The data has been summarized in Table III.

It may be noted that in only one group of rats was there any indication that recovery of the damage to the testes caused by the erucic acid supplement may take place. These animals had been on a 10% erucic acid supplement, and degeneration of the testes had not advanced to a marked degree before they were transferred to the stock diet.

Young Female Rats Maintained on the Supplemented Diet after Weaning

As in the experiments previously described, female animals on the erucic acid supplemented ration were mated with male animals also on the same supplemented diet. In most cases the same females were subsequently mated with normal males, and the fertility assessed. Since the females were

* Results recently obtained, however, have shown that the typical picture of degeneration was present after 5 months on the supplemented diet.

allowed to survive after mating, it has not been possible to determine exactly what happened when litters were not born. However, there has been evidence of failure of pregnancy with resorptions, and of sterile matings. In some cases, when newborn animals died, normal pups were placed with the mother to see if she could rear them. The observations pertaining to the rats which became pregnant are presented in Table IV. This table refers to the same groups of animals as referred to in Table I. From the two tables the number of females which did not produce litters may be ascertained.

TABLE III

RECOVERY OF DEGENERATION OF THE TESTES AFTER REMOVAL OF ERUCIC ACID FROM THE DIET

No. of rats	Period on diet with erucic acid supplement (weeks)	Notes	Period on stock diet (weeks)	Notes
1	9½	Right testis removed = 700 mg.	8	Left testis 350 mg., no recovery
1	7½	Sterile, testes very soft and small	4	No recovery apparent on histological examination
1	6½	Testes very soft and small	16	Testes unchanged, no matings
3	5½	Right testis removed, averaged 576 mg. (450–740)	8	Palpation indicates no improvement in condition of testes
5*	5	Right testis removed, averaged 1053 mg. (650–1824)	8	Palpation indicates slight improvement in condition of testes†

* Fed 10% erucic acid supplement.
† In a comparable group of four rats not changed to stock diet the testes, over the same period of time, have continued to decrease in size.

TABLE IV

RESULTS OF THE FEEDING OF FATTY ACID SUPPLEMENT TO GROWING FEMALE RATS

Group	Dietary supplement	Period on diet before mating (weeks)	No. of rats	No. of litters	Fate of young, etc.
Normal controls	Stock diet		10	10	89 young, of which 3 died at birth; 69 survived 3 weeks (26 young of 7 litters averaged 37 g. body weight at 3 weeks)
		Mated with treated* males			
	25% rapeseed oil	16	3	3	1 litter of 8 died at birth; 2 litters of 16 young all died within 6 days
	15% oleic acid	16	4	4	2 females died at parturition; 1 litter eaten at birth; 1 litter died within 3 days
A	15% erucic acid	11–15	10	7	32 of 51 young survived 3 weeks, average weight = 25 g. (12 of 51 young survived 4 weeks)
B	15% " "	9–13	5	1	1 litter of 6 eaten — 1 resorption, 5 sterile matings with pseudopregnancy
E	10% recrystallized erucic acid	16–20	4	2	1 litter of 4 dead within 4 days; 1 litter of 8, of which 5 survived 3 weeks, average weight = 30 g. — 3 sterile matings with pseudopregnancy
		Mated with normal males			
A	15% erucic acid	29–34	10	4	1 female resorbed; 15 sterile matings with 3 pseudopregnancies; 2 litters, 10 died within 3 days, 2 normal pups died by 3 days; 1 litter eaten but 2 normal pups reared — average weight 3 weeks = 20 g., 1 female died at parturition
B	15% " "	17–21	5	0	3 rats showed resorptions; 1 rat showed 6 sterile matings with no pseudopregnancies
B	15% " "	21–34	2	1	Litter eaten at birth; 1 rat — 5 sterile matings with pseudopregnancies
C	15% " "	19	5	5	45 young of which 5 survived 3 weeks, average weight = 30 g.
D	15% " "	21	3	3	17 young eaten within 24 hours

* Male animals on the same diet and for the same duration as the females.

PLATE I

FIG. 1. Moderately severe changes in testis after maintenance for 5 months on a diet supplement with 10% erucic acid. (Magnification × 100)

FIG. 2. Severe changes in testis after maintenance for 5 months on a diet supplement with 10% erucic acid. (Magnification × 100)

FIG. 3. "Normal" testis of adult rat maintained for 1 month on a diet supplement with 15% erucic acid. (Magnification × 100) FIG. 4. Lactating breast of normal rat. (Magnification × 100) FIG. 5. Breast of rat which failed to lactate after erucic acid diet. (Magnification × 100)

The results obtained indicate that a high intake of certain free fatty acids in the diet of rats from the time of weaning markedly influences their fertility when they approach the adult stage. Although some females showed a reduced fertility, as indicated by the frequency of sterile matings, the majority of animals eventually became pregnant. Sterile matings in 16 cases were followed by pseudopregnancy. In 18 cases pseudopregnancy failed to occur, but the number of animals involved was small. In four cases fetal resorption probably took place, and in three cases the females died during parturition. In such cases the fetuses appeared large, and in some cases labor did not occur until 1 to 2 days after the expected date. Although the litters were of approximately normal size, in many cases death occurred shortly after birth, and the young were eaten. A greatly increased mortality was observed among the surviving animals. Those which survived 3 weeks were much underweight. The mother in some instances failed also to rear normal pups which had been placed with her after the loss of her own litter. In one instance normal pups (2) were reared but they were much underweight. This failure is believed to be attributable to a failure of lactation. It is of interest that only the erucic acid supplement in the diet caused physiological impairment in the male animals and that in the form of the failure of spermatogenesis. In the females all three of the supplementary fatty acids produced dysfunction, manifested by impairment of parturition and lactation.

Mature Female Rats Maintained on the Supplemented Diet

Adult female rats were fed the diet supplemented with erucic acid and after various intervals they were placed with fertile males. The assessment of the fertility of the females is indicated in Table V.

It is evident that the feeding of the erucic acid supplement to the mother for more than 8 weeks resulted in a poor survival of the young. The supplement containing oleic acid also showed the same effect in a single experiment.

TABLE V

RESULTS OF FEEDING FATTY ACID SUPPLEMENT TO ADULT FEMALE RATS

Dietary supplement	Period on diet (weeks)	No. of rats with litters	Fate of young
15% oleic acid	10	7	52 young of which 44 survived 1 week, 16 survived 2 weeks, and 11 survived 3 weeks
15% erucic acid	5	3	24 young, of which 19 survived 3 weeks
15% " "	6–7	2	1 litter of 8, 4 survived 3 weeks, average weight = 26 g.; 1 litter of 8, all died within 8 days
15% " "	7–8	5	44 young, of which 40 survived 3 weeks
15% " "	8–9	3	3 litters, all dead by 3 days; 2 of the females each given 2 normal pups; these all died in 6 days
15% " "	10–11	2	2 litters all dead within 6 days
15% " "	12–13	4	3 litters all dead within 24 hours; 1 litter of 9, 6 dead within 4 days

In a few cases not shown in the table, females on the erucic acid supplemented diet have not become pregnant. They have, however, shown a series of sterile matings followed by pseudopregnancy.

Morphological Changes in the Female Rats

The animals which died during parturition, or were killed, showed only occasional changes in endocrine organs. A few rats had cysts of the ovary filled with clear fluid. The non-pregnant uterus was frequently of a brownish color. The pituitary and adrenal glands showed no unexpected changes. The thyroid was increased in weight up to 20 mg. in some cases. Striking differences, however, were seen in the mammary gland development. In animals on the dietary supplement the amount of mammary tissue was much less than expected, and when the tissue was cut, no evidence of milk secretion was found. Comparable sections of the breast of a control and an experimental rat were prepared. Fig. 4 indicates the breast development of a normal mother 3 days after parturition. The animal had been nursing four young. Fig. 5 shows the relatively small amount of breast tissue in the mammary gland of an animal which had been fed the erucic acid supplement. It was killed 3 days after parturition. The young had survived only 2 days. Apparently, not only was the milk secretion reduced in the animal, but the mammary development was also different.

Recovery of Normal Fertility

As it did in the male rat, the prolonged feeding of the erucic acid supplement was found to cause impairment of the reproductive process in the female. When the animals which had failed to produce any offspring were again fed the stock diet, a variable degree of recovery occurred, as indicated in Table VI.

TABLE VI

RECOVERY OF FERTILITY IN FEMALE RATS AFTER REMOVAL OF ERUCIC ACID FROM THE DIET

Period on diet with erucic acid supplement (weeks)	Period on stock diet (weeks)	Results (on individual rats)
2	4	6 sterile matings, 3 pseudopregnancies
2	4	Litter of 9, 1 alive at 3 weeks (growth stunted)
5	8	1 sterile mating
5	8	Litter of 8, all dead at birth
18	2–4	1 sterile mating, no pseudopregnancy
30	4	1 sterile mating, no pseudopregnancy
		Litter and mother died at parturition
30	1–5	6 sterile matings, no pseudopregnancies
30	2	1 sterile mating and pseudopregnancy
	8	Litter of 8, 5 alive at 3 weeks
30	4–16	7 sterile matings and pseudopregnancies
36	2	1 resorption
	8–16	6 sterile matings, no pseudopregnancies

It may be noted that recovery of function did not occur in the animals which had been fed the erucic acid supplemented diets for longer than $4\frac{1}{2}$ months. Only one animal which had been fed the stock diet for 8 weeks had a litter of eight, of which five survived for 3 weeks. From these preliminary results it would appear that even after short intervals of feeding the erucic acid supplemented diet to female rats, recovery of fertility proceeds slowly and may be limited.

Estrous Cycle

Only in a few animals has the estrous cycle been found to be influenced by the supplemented experimental diets. In no case has the animal become acyclic, but the duration of the cycle became longer. In most cases, however, no alteration in the estrous cycle has been observed. In one experiment two animals which had been placed on the erucic acid supplemented diet for 9 months were injected daily with 2 μg. of stilboestrol for 1 month. The vaginal smears indicated a continuous state of estrus during this period.

Influence of the Erucic Acid Supplemented Diet on the Body Weight

All of the animals which have been described have appeared normal except for their reproductive capacity. Even after a year on the experimental diet the animals have remained healthy and vigorous. Post-mortem examination has shown no lesions in other organs than those of reproduction. The weights of four groups of the animals are given in Fig. 6. Two groups of

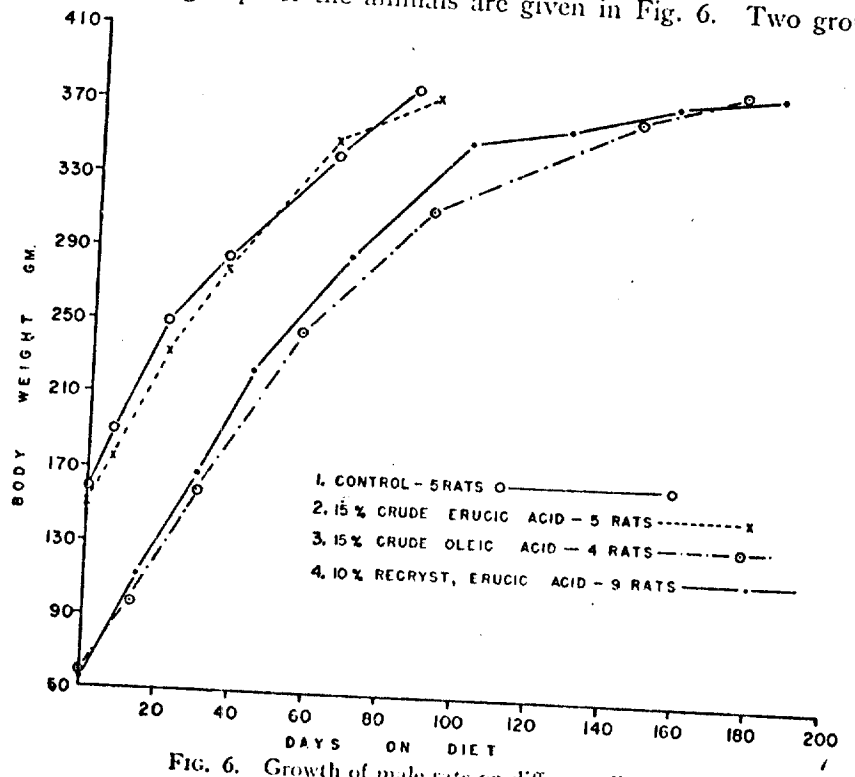

FIG. 6. Growth of male rats on different diets.

immature male Sprague-Dawley rats, which were fed the diet supplemented with 10% recrystallized erucic acid and with the 15% level of oleic acid respectively, showed a normal rate of gain in body weight. The group of adult male Wistar rats on the 15% level erucic acid supplement increased in body weight as rapidly as the group on the stock diet. Even animals fed a diet containing a supplement of 30% crude erucic acid suffered only a temporary (10 days) retardation in growth rate, and thereafter continued to grow at a normal rate and remained in excellent health after 5 months on the diet.

Discussion

The findings which have been reported demonstrate that the feeding of 10% or more, by weight, of erucic acid to an otherwise complete diet profoundly impairs spermatogenesis in the rat. If animals were placed on this diet when they were weaned, degeneration of tubules of the testes began after approximately 3 months, and sterility was total in about 5 months. Only the tubules were affected; little change was observed in the androgen-controlled secondary sex organs. Preliminary observations indicate that sterility also develops in the adult animal maintained on the erucic acid supplemented diet, although the impairment possibly develops at a slower rate. Efforts to restore normal function to the testes by placing the animals again on the stock diet produced a degree of improvement which suggests that recovery may occur if the degeneration has not reached an advanced degree. Male rats which are being fed the diet containing 15% by weight of oleic acid or 25% by weight of rapeseed oil have not shown these deleterious changes. The rapeseed oil used in these experiments contained approximately 50% by weight of erucic acid in the triglycerides, hence the net proportion of the fatty acid in the experimental ration was at least 10%. However, the presence of other fatty acids in the rapeseed oil, including the essential fatty acids, may have moderated the effect of the erucic acid. Von Euler *et al.* (10, 11) observed no change in the reproductive capacity of rats in their study in which the diet was supplemented with Swedish margarine, which contained as much as 47% of hydrogenated rapeseed oil. It may be added that the impure oleic acid used in our experiments contained about 3% of linoleic acid.

The histological picture of the testicular lesions observed in the rats on the erucic acid supplemented diet is closely similar to that described by Evans, Lepkovsky, and Murphy (14) in rats maintained on a diet deficient in the essential fatty acids. Similar lesions were observed in the testes of rats subjected to prolonged vitamin E deficiency (16), but the changes observed in our female rats on the erucic acid supplements were more typical of an essential fatty acid deficiency than of vitamin E deficiency. There is little evidence in our findings that erucic acid acts primarily by inhibiting the function of any of the sex hormones. Similarly, inhibition of the function of some of the anterior pituitary hormones is not compatible with the experimental observations described.

It appears plausible to suppose that erucic acid may act directly through an antagonism or competition with fatty acids essential for normal testicular function, particularly since the testis is rich in highly unsaturated fatty acids (17). Such an antagonism also might account for the previously observed effect of erucic acid in increasing the cholesterol content of the adrenal and liver, since this occurs also in these tissues in essential fatty acid deficiency (1,7). Furthermore, the adrenal, like the testis, contains a relatively large proportion of the highly unsaturated fatty acid -- arachidonic acid (19). Other workers (2,7,15) have observed that the development of symptoms of essential fatty acid deficiency may be accelerated and accentuated by the addition of hydrogenated fatty acids, *trans*-isomers of unsaturated fatty acids, mineral oil, or cholesterol, to fat-free diets.* To our knowledge, such symptoms (i.e. of essential fatty acid deficiency) have not been produced by adding these substances to diets containing essential fatty acids.

It is apparent from our studies that the feeding of erucic acid in the diet did not produce all of the effects of essential fatty acid deficiency (3,9). Lesions of the skin and kidney, and an increased water requirement, for example, have not been observed in our study. Rats fed the diet containing added erucic acid appeared remarkably healthy and their normal growth rate was unaffected. The normal growth rate observed in the present study is, however, in contrast to the earlier results obtained with synthetic diets (6), and to the experiments of Thomassen and Boldingh (20), in which erucic acid added to a semisynthetic diet caused a definite retardation of the rate of increase in body weight.

It may be argued that the symptoms observed in rats maintained on a fat-free diet for a long period are indicative of a multiple fatty acid deficiency, even though complete recovery may be obtained by the administration of linoleic or arachidonic acids. If this were so, erucic acid may possibly produce some of the symptoms of essential fatty acid deficiency without giving rise to the complete syndrome. If erucic acid does act by antagonizing or competing with certain highly unsaturated fatty acids, the change produced must be to a large extent irreversible. It is known that an increase in the level of adrenal cholesterol can be produced by feeding rapeseed oil which contains a large proportion of esterified linoleic acid in addition to erucic acid. Testicular lesions were produced also by adding erucic acid to a complete diet, although there is evidence that these lesions may not be induced by the feeding of rapeseed oil in the diet.

The effect of erucic acid on the female rat cannot be considered as specific since oleic acid caused a similar change. The estrous cycles were prolonged in some animals but in most cases were normal. Most of the animals became pregnant. Fetal resorption occurred in four of the animals which had been fed the erucic acid supplemented diet for more than 3 months. In most

* H. Dam and his associates in a series of papers (see Brit. J. Nutrit. **10**, 292 (1956) have described testicular degeneration and interference with reproduction in the female as a result of feeding supplements of various partially hydrogenated fats. Our results with erucic acid bear a close resemblance to their description and it seems possible that their results may be partially due to the formation of erucic acid or closely related fatty acids during hydrogenation.

cases, however, the young were born alive. In three instances there was interference with parturition and the mothers died in labor. The females which did not become pregnant gave evidence of frequent sterile matings. The failure of conception could not always be attributed to sterility of the male. Most of the animals showed a typical pseudopregnancy after sterile mating, but some of them mated at 5-day intervals without any cessation of the estrous cycle. All these deviations from the normal behavior cannot be attributed to the length of time the animals were maintained on the special diet. Animals might exhibit sterile matings and then become pregnant, only later to lose the litter, or conversely, pregnancy might be followed by a series of sterile matings. Relatively prolonged periods of feeding the maintenance stock diet were necessary to cause even a partial recovery of fertility. In contrast to the behavior of the male rats, some of the females showed signs of disturbed fertility after relatively short periods of maintenance on the erucic acid supplement.

The high mortality among the offspring of animals maintained on the special diet was clearly evident. Many of the litter died shortly after birth. The animals which survived exhibited a poor growth rate and many more died before the time of weaning. A few were stunted in growth but showed no other abnormality. When newborn animals from normal mothers were placed with the experimental mother, they either died or grew at a reduced rate. At autopsy, the offspring which died appeared normal but emaciated, and their stomachs were empty. It became apparent that the failure to survive was attributable to deficient mammary gland development and lactation in the mother. In all cases mammary development which normally occurs during pregnancy was markedly reduced, and lactation failed to occur.

The findings in the female rats on the special diet resembled more closely the type of change seen in essential fatty acid deficiency than those in vitamin A or vitamin E deficiency. The vaginal smears showed no continuous presence of cornified cells indicative of vitamin A deficiency (12), and the small incidence of fetal resorption compared to the frequency of live births would tend to eliminate vitamin E deficiency as a cause (12). The effects observed in the females after mating, and in parturition and lactation, however, closely resemble those of essential fatty acid deficiency in the rat (8, 13). The effects noted on parturition of feeding an oleic acid supplement are not readily explained, since the addition of fat to the diet is generally considered to have a favorable effect on reproduction (8).

The reduction in fertility as observed in the male rat, if reproducible in other species, may have significance for the control of fertility in the human. Erucic acid is readily available and palatable in the diet; it is metabolizable and produces no harmful side effects. When ingested in adequate amounts it may reduce fertility by partial suppression of spermatogenesis. Our findings suggest furthermore that recovery of function may follow the withdrawal of the acid from the diet. An extension of the study has been undertaken to test the influence of erucic acid in the diet on fertility in species other than the rat.

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FACTORS AFFECTING DIGESTIBILITY OF FATTY ACIDS IN THE RAT

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In the experiments described in the preceding paper (Carroll, '58), a number of saturated and mono-unsaturated fatty acids were fed to rats in synthetic diets and their coefficients of digestibility were measured. In most of those experiments, the fatty acids were fed in the non-esterified form, whereas normally they are ingested in the form of triglycerides. The present study has therefore been concerned with differences between the digestibility of non-esterified fatty acids and those fed as triglycerides. In addition, the effect of a number of other variables on digestibility has been investigated, such as the level of protein in the diet, the type of salt mixture used, and the age of the animals.

METHODS

The general procedure employed in these experiments and the diets used were the same as those described in the preceding paper. Some of the experiments were carried out before our investigation of the methods for extraction of fecal lipid, and in these the method of Augur, Rollman and Deuel ('47) for measuring digestibility was used, with the modifications previously indicated. Otherwise the fecal lipids were extracted by the ether-acetic acid method (Carroll, '58). The amount of free fatty acid in the neutral ether extract was

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determined by titrating an aliquot with N/50 potassium hydroxide in ethanol, using phenolphthalein as indicator. The neutral equivalent was taken to be that of the dietary fatty acid. The total glycerol content was determined on another aliquot following hydrolysis with approximately 10 volumes of 15% potassium hydroxide in 60% ethanol on a steam bath for 40 minutes. The hydrolysis mixture was diluted with water, acidified and filtered. The filtrate was made to volume and an aliquot used for determination of glycerol by the method of Lambert and Neish ('50) as modified by Korn ('55). A further aliquot of the original ether extract was taken to dryness and the residue was oxidized with 60% perchloric acid. It was then taken up in water and aliquots were analyzed for phosphorus (King, '32) and calcium (Wang, '35).

RESULTS

Digestibility of triglycerides versus non-esterified fatty acids

The results of these experiments are presented in table 1. It can be seen that triolein and trilinolein were almost completely digested in contrast to the rather low digestibilities of oleic acid and linoleic acid. Trierucin was also better digested than erucic acid, but the triglycerides of the saturated fatty acids appeared to be digested to a lesser extent than the free fatty acids. The improved digestibility of triolein and trilinolein over that of the corresponding free acids was due mainly to a reduction in the amount of neutral lipid excreted in the feces whereas the improved digestibility of trierucin resulted from a decrease in the fatty acids excreted as soaps. When stearic acid and palmitic acid were fed as triglycerides, most of the fecal lipid was in the neutral fraction but when they were fed as free acids, most of the fecal lipid was in the soap fraction.

Further examination of the neutral fecal lipid fraction from the various experiments helped to explain the observed differences in digestibility (table 2). There appeared to be little or

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TABLE 1

Digestibility of triglycerides versus non-esterified fatty acids [1]

(Results are expressed as mg/rat/day)

FAT OR FATTY ACID FED [2]	BODY WEIGHT [3]		FAT INGESTED	WEIGHT OF DRY FECES	FAT EXCRETED			FAT ABSORBED	COEFFICIENT OF DIGESTIBILITY
	Initial	Final			Neutral fraction	Soap fraction	Total (corrected for metabolic fat)		
	gm	gm							
Triolein	86	163	1230	850	25	10	10	1220	99
Oleic acid	85	162	1240	1240	290	80	340	900	73
Trilinolein	73	135	990	810	40	20	30	960	97
Linoleic acid	78	156	1160	1090	140	75	190	970	84
Trierucin	85	123	970	990	230	160	360	610	63
Erucic acid	83	139	1410	1660	290	470	740	670	48
Tristearin	85	149	1370	2200	1110	100	1180	190	14
Stearic acid	83	132	1410	2180	330	780	1080	330	24
Tripalmitin	84	159	1420	2230	920	220	1110	310	22
Palmitic acid	87	143	1340	1760	240	610	820	520	40

[1] Purified casein (Nutritional Biochemicals, Cleveland, Ohio) was used in these diets, and the salt mixture was formulated according to Steenbock and Nelson ('23) except that double the amount of calcium phosphate was used.

[2] Triolein, trilinolein, tristearin and tripalmitin were obtained from the Hormel Institute, Austin, Minnesota. Trierucin, oleic acid, linoleic acid and erucic acid were provided through the courtesy of Dr. B. M. Craig, Prairie Regional Laboratory, National Research Council, Saskatoon, Sask. Stearic acid and palmitic acid were obtained from Nutritional Biochemicals, Cleveland, Ohio. All fats were fed as 10% by weight of the diet.

[3] Diets were fed for 16 days.

no glyceride in this fraction except when tripalmitin or tristearin was fed. These two triglycerides seemed to be less readily hydrolyzed during their passage through the gastrointestinal tract and this accounts for the large amount of neutral lipid in the feces when they are fed.

TABLE 2

Composition of neutral fecal extracts

(Results expressed as mg/rat/day)

FAT OR FATTY ACID	WEIGHT OF NEUTRAL FRACTION	FREE FATTY ACID	GLYCERIDE [1]	CALCIUM [2] PHOSPHORUS COMPLEX	CALCIUM [3] SOAP
Triolein	25	8	3	2	0
Oleic acid	290	70	3	105	105
Trilinolein	40	7	3	3	0
Linoleic acid	140	75	5	45	30
Trierucin	230	40	5	60	45
Erucic acid	260	100	3	75	30
Tristearin	1110	230	930	10	5
Stearic acid	440	225	0	0 [4]	0
Tripalmitin	920	215	735	0 [4]	0
Palmitic acid	340	155	2	0 [4]	0

[1] Calculated as triglyceride from the amount of glycerol in the hydrolyzed extract, but no distinction was made between mono-, di- and triglyceride.

[2] Calculated from the amount of phosphorus in the extract according to the formula of Swell et al. ('56b), substituting the dietary fatty acid for oleic acid where necessary.

[3] Calculated from the excess of calcium over that required for complex formation.

[4] A small amount of phosphorus was present in these extracts but there was no detectable calcium.

The neutral lipid fraction from the feces of rats fed oleic acid, linoleic acid, erucic acid or trierucin contained relatively large amounts of calcium and phosphorus. This appeared to be present in the form of a complex similar to that described by Swell, Trout, Field and Treadwell ('56 a, b) as occurring in the feces of rats fed oleic acid. In agreement with their results and those of Pihl ('55) and Kim and Ivy ('52), much less lipid-soluble phosphorus was found in the feces of rats

fed saturated fatty acids or triglycerides of either saturated or unsaturated fatty acids, with the exception of trierucin. Our experiments have also included feeding tests with the monounsaturated fatty acids eicosenoic and nervonic and the results have shown that eicosenoic acid promotes an excretion of calcium and phosphorus comparable with that of oleic acid while nervonic acid is no more effective than the saturated fatty acids.

In table 2 the weight of the calcium and phosphorus complex has been calculated from the weight of phosphorus excreted, on the basis of the formula suggested by Swell et al. ('56b), and assuming that the fatty acid portion of the molecule was derived from the dietary fat. The crude fecal extracts in the experiments with oleic acid, linoleic acid, erucic acid and trierucin contained an excess of calcium over that required for complex formation and this has been shown in the table as calcium soap of the dietary fatty acid. These figures for complex and soap excretion are intended only to give a rough measure of the amounts that could be present and should not be taken to imply that all of the phosphorus and calcium in the extracts is combined in this manner. However, further experiments, particularly with the fecal extract of rats fed erucic acid, have shown the presence of a substance containing calcium, phosphorus and erucic acid which was soluble in ether and petroleum ether, could be precipitated by acetone or methanol and which after several precipitations gave analytical figures similar to those of Swell et al. ('56b). It has also been demonstrated that the calcium salts of oleic, linoleic and erucic acid can be partially extracted by neutral ether in a Sohxlet so it is possible for these soaps to be present in the neutral ether extract.

Effect of salt mixtures on digestibility

The salt mixture used in our earlier synthetic diets was formulated according to Steenbock and Nelson ('23) except that double the recommended amount of calcium phosphate

was used. In later experiments this was replaced by the Phillips and Hart ('35) salt mixture and it appeared that the change was associated in some cases with a reduction in the digestibility of individual fatty acids. It was suspected that the reduced digestibility might have resulted from the increased amount of calcium in the Phillips and Hart mixture since Cheng, Morehouse and Deuel ('49) showed clearly that increasing the amount of calcium and magnesium in the diet lowered the digestibility of higher melting simple triglycerides and hydrogenated fats.

A survey of a number of salt mixtures recommended for use in synthetic diets revealed wide variations in calcium content and in the calcium-phosphorus ratio. The magnesium varied less and was present in much smaller quantities than the calcium in all mixtures. The salt mixtures could be classified into three main groups on the basis of their calcium and phosphorus content. Those formulated by McCollum and Simmonds ('18) and by Steenbock and Nelson ('23) were low in calcium (7% by weight) and relatively high in phosphorus (Ca: P ratio 0.60 to 0.64). The Wesson ('32) modification of the Osborne-Mendel mixture, the U.S.P. XIV, the Jones-Foster ('42) and the Phillips-Hart ('35) mixtures contained about twice as much calcium and somewhat less phosphorus (Ca: P ratio 1.39 to 1.94). Finally, the Hubbell-Mendel-Wakeman ('37) mixture contained three times as much calcium and had the lowest phosphorus content (Ca: P ratio 4.25). This latter mixture was designed to be added to diets at the 2% level rather than the more usual 4 to 5% but it has frequently been used at the 4 to 5% level (Norcia and Lundberg, '54; Scribante and Favarger, '54, Swell, Flick, Field and Treadwell, '55; Okey and Lyman, '56).

Because most of these salt mixtures are being used in various laboratories for the preparation of synthetic diets it seemed desirable to carry out a few experiments in order to assess the magnitude of the effects that they might have on the digestibility of fatty acids. When oleic and erucic acid were fed in a diet containing the Phillips and Hart salt mix-

ture at the 5% level or the Hubbell-Mendel-Wakeman mixture at the 2% level, their digestibilities were the same or slightly lower than those observed previously with the modified Steenbock salts (table 3). When the Hubbell-Mendel-Wakeman mixture was raised to the 5% level, much lower coefficients of digestibility were obtained. On the other hand, both fatty acids were more than 90% digested when a salt mixture containing no calcium was used.

In these experiments, decreased digestibility of oleic acid was associated mainly with an increase in weight of the neutral fecal lipid fraction, while decreased digestibility of erucic acid was associated with a greater increase in the soap fraction. This difference may be due partly to the method of extraction since calcium oleate was found to have a greater solubility in neutral ether than calcium erucate. On the oleic acid diet, the calcium content of the neutral ether extract of feces increased from 0.24 mg/rat/day with the calcium-free salt mixture to 64 mg/rat/day with the 5% Hubbell-Mendel-Wakeman mixture. Corresponding figures for the erucic acid diet were 0.60 and 7.0 mg/rat/day respectively. The phosphorus content of the extract also was increased more on the oleic acid diet (0.11 to 6.8 mg/rat/day) than on the erucic acid diet (0.22 to 1.6 mg/rat/day), but the calcium phosphorus complex formed with either fatty acid appeared to readily soluble in ether.

It seems possible that the effects of different salt mixtures on digestibility may be greater for non-esterified fatty acids than for triglycerides. In one experiment, however, a coefficient of digestibility of 35 was obtained for trierucin fed in a diet containing 5% of the Hubbell-Mendel-Wakeman salt mixture. This is considerably lower than that obtained for trierucin fed with the modified Steenbock salt mixture (table 1), but triglycerides like triolein and trilinolein would perhaps be almost completely digested regardless of the salt mixture.

TABLE 3

Effect of different salt mixtures on digestibility
(Results are expressed as mg/rat/day)

SALT[1] MIXTURE	PERCENT IN DIET	FATTY ACID FED	BODY WEIGHT[2]		FAT INGESTED	WEIGHT OF DRY FECES	FAT EXCRETED			FAT ABSORBED	COEFFICIENT OF DIGESTIBILITY
			Initial	Final			Neutral fraction	Soap fraction	Total (corrected for metabolic fat)		
			gm	gm							
Calcium free	5	Oleic	86	120	880	570	25	5	5	875	99
Phillips-Hart	5	Oleic	84	169	1570	1570	220	150	340	1230	78
Hubbell-Mendel-Wakeman	2	Oleic	84	150	1160	930	220	45	240	920	79
Hubbell-Mendel-Wakeman	5	Oleic	88	165	1400	1930	740	100	810	590	42
Calcium free	5	Erucic	85	120	940	540	90	20	80	860	92
Phillips-Hart	5	Erucic	82	158	1630	2160	370	750	1090	540	33
Hubbell-Mendel-Wakeman	2	Erucic	86	148	1260	1430	175	505	650	610	48
Hubbell-Mendel-Wakeman	5	Erucic	84	165	1590	2600	220	1070	1260	330	21

[1] The salt mixtures and casein used in these diets were obtained from Nutritional Biochemicals, Cleveland, Ohio. Analysis of the casein showed 0.77% phosphorus and little or no calcium. The calcium-free mixture consisted of magnesium chloride 47.5%, potassium citrate 20.7%, potassium chloride 19.7%, sodium chloride 8.2%, potassium sulfate 2.1%, ferric citrate 1.6%, potassium iodide 0.04%, sodium fluoride 0.04%, manganous sulfate 0.02% and potassium alum 0.008%. It contained 12.1% of magnesium.

[2] Diets were fed for 16 days.

Effect of other factors on digestibility

The effect of other dietary alterations and of age of the animals on the digestibility of erucic acid have also been examined and the results are presented in table 4. Digestibility appeared to be increased by a high-protein and decreased by a low-protein diet. It should be noted, however, that the animals on the low-protein diet lost weight during the experiment. The addition of desiccated thyroid or of thiouracil had relatively little effect on digestibility although the amount of fatty acid ingested was much greater on the diet containing thyroid. Experiments with rats of different ages suggested that the digestibility of erucic acid was somewhat decreased in older rats.

DISCUSSION

The results obtained in these experiments indicate that the triglycerides of unsaturated fatty acids are better digested than the corresponding non-esterified fatty acids. In fact, one would expect to find that triolein and trilinolein are almost completely digested since most naturally-occurring fats and oils have coefficients of digestibility of 95 or above (Deuel, '55).

Conversely, the triglycerides of palmitic and stearic acid appeared to be digested to a lesser extent than the free fatty acids. Low digestibility values for saturated triglycerides have also been obtained by Cheng et al. ('49), Mattson, Alexander, Baur and Reller ('56) and Karvinen, Lin and Ivy ('57), but considerably higher values were reported by Norcia and Lundberg ('54) and Scribante and Favarger ('54). The results of Norcia and Lundberg were influenced by the type of conditioning to which the animals were subjected, but those of Scribante and Favarger are not readily explained, particularly since Buensod and Favarger ('56) reported values for palmitic acid and stearic acid which are in reasonable agreement with ours. It should be noted, however, that rather wide variations in digestibility have been observed for any individual fatty acid in our experiments (Carroll, '58).

TABLE 4

Digestibility of erucic acid under various conditions[1]

	NO. OF DETERMINATIONS	FAT INGESTED	FAT EXCRETED			FAT ABSORBED	COEFFICIENT[2] OF DIGESTIBILITY
			Neutral fraction	Soap fraction	Total[3] (corrected for metabolic fat)		
High protein	2	850	200	200	310	540	63 (54–72)
Low protein	2	650	140	400	440	210	33 (29–37)
0.1% thyroid	2	1310	390	410	640	670	51 (42–60)
0.3% thiouracil	2	900	250	300	450	450	50 (49–51)
Young rats (84 gm)	6	1140	280	360	540	600	53 (37–61)
Adult rats (221 gm)	4	1760	360	740	970	790	45 (35–56)
Old rats (386 gm)	4	1700	390	830	1040	660	39 (35–43)

[1] The modified method of Augur et al. ('47) was used in these experiments. The standard diet was the same as that used in earlier experiments. (Carroll, '58.) Additions of thyroid (desiccated thyroid — Burroughs Wellcome) and of thiouracil were made to this diet. For the high- and low-protein diets, the casein was increased to 65% and decreased to 5.5% by weight respectively at the expense of the glucose.

[2] The metabolic fat was determined in each experiment by feeding a fat-free diet having the appropriate additions or alterations.

[3] The figures within parentheses give the range of values.

The presence of large amounts of glyceride in the feces of rats fed tristearin and tripalmitin indicates that these triglycerides are not readily hydrolyzed during their passage through the gastrointestinal tract. Ivy, Karvinen and Lin ('57) obtained similar results with tripalmitin but found that trielaidin was readily hydrolyzed.

The poor digestibility of oleic acid, linoleic acid and erucic acid relative to their corresponding triglycerides is associated with increased fecal excretion of calcium and phosphorus in the lipid fraction extractable with neutral ether. It seems probable that the fatty acids are poorly digested because they form calcium soaps and calcium-phosphorus containing complexes which are not readily absorbed, particularly since the digestibility of oleic acid and erucic acid was decreased still further by increasing the calcium content of the diet (table 3).

Relatively large amounts of free fatty acid are also liberated in the intestine during the digestion of triglycerides (Borgstrom, '52; Mattson, Benedict and Beck '54) but much larger amounts would be present when the fatty acids are administered in the non-esterified form. There is good evidence that a large proportion of triglyceride fat is absorbed without being completely hydrolyzed (Reiser, '55) and it has been reported by Buensod and Favarger ('56) that monoglycerides are better digested than either triglycerides or free fatty acids.

It is interesting that calcium and phosphorus were present in quantity in the fecal extracts of rats fed erucic acid either as the triglyceride or as the free fatty acid while this was not true for oleic or linoleic acids. The difference may be related to the slow rate of absorption which we have observed for erucic acid and which has also been reported for rapeseed oil (Thomasson, '56).

Our experiments with different salt mixtures confirm the findings of Cheng et al. ('49) that the amount of calcium in the diet may play an important role in the digestibility of fatty acids and this should be borne in mind when choosing a

salt mixture for synthetic diets. It would appear that calcium is more important than magnesium in this respect.

Our studies on the effect of a low protein diet in decreasing digestibility also confirm the results of other workers (Deuel, '55). We are not aware of previous studies on the relation of age or thyroid activity of rats to their ability to digest fat.

SUMMARY

Triolein, trilinolein and trierucin were more completely digested than the corresponding non-esterified fatty acids. Palmitic and stearic acids were very poorly digested either as triglycerides or as free acids. When diets containing oleic, linoleic, eicosenoic or erucic acids were fed, the neutral ether extract of the feces contained large amounts of calcium and phosphorus. This also occurred with trierucin but not with other triglycerides, saturated fatty acids or nervonic acid.

The calcium content of the diet was found to play an important role in determining the digestibility of fatty acids. This should be considered in preparing synthetic diets because commonly-used salt mixtures cover a wide range of calcium concentrations.

The digestibility of erucic acid appeared to improve as the protein level in the diet was increased. The addition of desiccated thyroid or of thiouracil did not affect the digestibility although the total amount of fatty acid ingested was much greater in the former case. The digestibility of erucic acid seemed to be lower in old than in young rats.

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Differences Between the Metabolism of Linoleic and Palmitic Acid: Utilization for Cholesterol Synthesis and Oxidation to Respiratory CO_2[1]

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ABSTRACT

Measurements were made of the incorporation of intragastrically administered 1-C^{14}-labeled linoleic and palmitic acid carbon into the total body cholesterol of the intact rat and of the rat's ability to oxidize these two labeled acids to respiratory CO_2. As compared with palmitic acid, the intact rat and isolated rat tissues exhibit a greater ability to metabolize linoleic acid. This is evidenced by a greater utilization of linoleic acid carbon for synthesis of total body cholesterol and also by a preference for the oxidation of linoleic acid. The greater incorporation of linoleic acid carbon into cholesterol appears to reflect the preferential oxidation of linoleic acid by the liver, a main site of fatty acid oxidation and cholesterol biosynthesis. This preference of the rat to utilize linoleic acid carbon for the synthesis of cholesterol may help to explain the well documented observation that the plasma cholesterol of the rat increases as the linoleic acid content of the diet increases.

INTRODUCTION

In recent years there has been much interest in differences between the metabolism of essential and nonessential fatty acids and particularly in relationships between the fatty acids and the metabolism of cholesterol. Considerable attention has been devoted to relationships between the composition of the dietary fatty acids and the cholesterol content of the plasma (1) and also the composition of the plasma cholesterol esters (1,2). Investigations of this area have revealed that in the rat plasma cholesterol levels increase as the linoleic acid content of the diet increases (1); whereas, in man the circulatory levels of cholesterol decrease in response to an increase in the polyunsaturated fatty acid intake (3). The reasons for these changes are largely unexplained. Since large amounts of acetyl CoA are formed during catabolism of fatty acids, it is conceivable that these substances contribute a significant portion of the total carbon required for the cholesterol biosynthesis and that differences between the degree to which fatty acids furnish carbon for cholesterol synthesis may partially explain the relationships between intake of a particular type of fatty acid and changes in the levels of body cholesterol. In an effort to investigate this possibility and to further study differences between the metabolism of essential and nonessential fatty acids, the present study measures the incorporation of labeled carbon from intragastrically administered 1-^{14}C-linoleic acid, an essential fatty acid, and 1-^{14}C-palmitic acid, a nonessential fatty acid, into the total body cholesterol of the intact rat. In addition, simultaneous measurements were also made on the extent of oxidation of these two labeled fatty acids to respiratory CO_2 in order to provide an index of the rates at which these two fatty acids were generating acetyl-CoA. A preliminary report on these experiments has been presented (4).

EXPERIMENTAL PROCEDURES

Three month old male rats of the Wistar strain, maintained on Purina rat chow, were fed by stomach tube, 1 ml of USP olive oil to which was added 12 μc of either 1-^{14}C-palmitic or 1-^{14}C-linoleic acid, having a specific activity of 1 μc/mg. Since the palmitic acid and linoleic acid content of USP olive oil is essentially identical (5), the specific activity of the 1-^{14}C-palmitate and 1-^{14}C-linoleate absorbed from the intestines should also be essentially identical. At various time intervals after administration of the labeled fatty acids, respiratory CO_2 was collected for a few hours (6) and the animals then killed. Rats were given access to their regular diet during the time period between administration of the labeled fatty acids and collection of respiratory CO_2.

[1]This work is taken from a thesis by R. J. Cenedella in partial fulfillment of the requirements for the Ph.D. degree in biochemistry.

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TABLE I

Per Cent of the Total Absorbed 1-^{14}C-Palmitate and Linoleate Carbon Found Incorporated Into the Total Cholesterol of the Entire Rat, 6, 29 and 77 hr After Feeding 1-^{14}C-Palmitate or Linoleate[a]

Hours Killed	Number of rats	^{14}C-Cholesterol (^{14}C-Palmitate-fed rats)	Number of rats	^{14}C-Cholesterol (^{14}C-Linoleate-fed rats)
6	3	0.266 ± 0.024	3	1.252 ± 0.342
29	4	0.231 ± 0.030	4	0.804 ± 0.222
77	4	0.249 ± 0.067	4	0.800 ± 0.122

[a] Each value is expressed as the arithmetic mean ± 1 SD. The statistical significance of the differences between the incorporation of 1-^{14}C-palmitate and linoleate at each time studied is less than 0.01 (as measured by the Student's "t" test).

Unabsorbed lipid was collected by washing the entire gastrointestinal tract with acidic-aqueous-ethanol followed by petroleum ether and combining these washings with the excreted fecal matter. The unabsorbed lipid fraction and the entire rat were than saponified separately in alcoholic KOH and the fatty acids and colesterol extracted, essentially as described by Van Bruggen et al. (7). The isolated fatty acids were oxidized to CO_2 by wet combustion (8) and converted to baruim carbonate; the cholesterol converted to the digitonide (9), and the respiratory CO_2 precipitated as baruim carbonate. All ^{14}C-containing samples were plated to infinite thickness and counted with the Nuclear Model C110B detector. The total activity of all radioactive samples were calculated by multiplying the specific activity as baruim carbonate multiplied by the amount of carbon in the sample. The amounts of the ^{14}C fatty acids absorbed from the intestines were calculated by subtracting the total ^{14}C recovered in the fecal plus intestinal contents from the total ^{14}C fed. About 60% of both the fed 1-^{14}C-palmitate and linoleate was absorbed by the sixth hour after feeding; about 80% of each was absorbed by the twenty ninth and seventy-seventh hours. The percentage of absorbed ^{14}C fatty acid carbon incorporated into cholesterol in the rat was calculated directly by dividing the total activity of the cholesterol isolated from the whole rat by the total activity of the absorbed ^{14}C-labeled fatty acids multiplied by 100. The percentage of endogenous ^{14}C-labeled fatty acid carbon undergoing conversion to respiratory CO_2 per hour during various time intervals after feeding the labeled fatty acids was calculated as follows: the average-hourly total activity of the respiratory $^{14}CO_2$ collected during the 1 or 5 hr time intervals was divided by the total ^{14}C fatty acid activity recovered from the entire rat, minus intestinal contents immediately after completion of the respiratory CO_2 collection. This value was then multiplied by 100.

Inasmuch as conversion of ^{14}C-labeled fatty acid carbon to cholesterol and respiratory CO_2 in some instances was measured several days after feeding of the labeled fatty acids, the question arose as to whether the endogenous ^{14}C-labeled fatty acids at such time were still essentially the same as the fed ^{14}C-labeled fatty acids. By chromatographic separation of the endogenous fatty acids into major classes (1), followed by ^{14}C assay (11), it was shown that the ^{14}C-fatty acids derived from the administered labeled palmitate and linoleate were present in the rat predominantly as saturated and diunsaturated fatty acid respectively even 7 days after the fatty acids were administered.

TABLE II

Per Cent of the Total Absorbed 1-^{14}C-Palmitate and Linoleate Carbon Found Incorporated Into the Total Cholesterol of Various Tissue Fractions 6 hr After Feeding These ^{14}C-Labeled Fatty Acids[a]

Fatty acid fed	Number of rats	Carcass[b]	Liver	Gut[c]	Skin[d]	Organs[e]
1-^{14}C-Pal.	3	0.058±0.015	0.085±0.020	0.055±0.025	0.050±0.027	0.017±0.004
1-^{14}C-Lin.	3	0.325±0.147	0.472±0.161	0.208±0.050	0.132±0.071	0.108±0.046

[a] Each value is expressed as the arithmetic mean ± 1 SD.
[b] Carcass refers to the tissue fraction remaining after removal of the liver, gut, skin and organs.
[c] Gut refers to the gastrointestinal tract from the stomach to the anus inclusive.
[d] Skin refers to the entire hide, ie., subcutaneous fat, skin and hair.
[e] Organs refers to the heart, spleen, pancreas, kidneys, adrenals and reproductive system.

TABLE III

Per Cent of the Total Endogenous ^{14}C-Labeled Fatty Acid Carbon Oxidized to Respiratory $^{14}CO_2$ Per Hour by the Intact Rat During Various Time Intervals After Feeding 1-^{14}C-Palmitate or Linoleate[a]

Hours after feeding	Number of rats	$^{14}CO_2$ (^{14}C-Palmitate-fed rats)	Number of rats	$^{14}CO_2$ (^{14}C-Linoleate-fed rats)
5--6	4	10.88 ± 2.99	4	20.94 ± 7.16
24--29	5	2.11 ± 0.51	5	2.43 ± 0.59
48--53	3	0.94 ± 0.10	3	0.79 ± 0.22
72--77	4	0.75 ± 0.49	4	0.80 ± 0.20
168--173	1	0.59	1	0.67

[a] Each value is expressed as the arithmetic mean ± 1 SD. The difference between the oxidation of 1-^{14}C-palmitate and linoleate during the 5 to 6 hr time interval is significant to the 0.05 level (as measured by the Student's "t" test). The differences at the other time intervals are not statistically significant.

RESULTS

The results shown in Table I indicate that at 6, 29 and 77 hr after feeding of the labeled fatty acids, a significantly greater fraction of the absorbed ^{14}C-linoleate than ^{14}C-palmitate carbon was recovered as ^{14}C-cholesterol in the entire rat. For example, after one or three days, about 0.8% of the absorbed ^{14}C-linoleate carbon was present as cholesterol compared to about 0.2% of the absorbed ^{14}C-palmitate. In several additional experiments it was demonstrated that this preferential incorporation of administered linoleate carbon into cholesterol in the entire rat was occurring in all of a large variety of tissues examined following the feeding of the labeled fatty acids (Table II).

The observed differences between the fraction of absorbed linoleate and palmitate carbon converted to cholesterol appears to be the result of events that occurred soon after the labeled fatty acids were absorbed, since as shown in Table I, the concentration of ^{14}C in the cholesterol of the rats fed either labeled palmitate or linoleate remained essentially constant between one and three days after administration of the fatty acids.

The explanation for the preferential incorporation of absorbed linoleate carbon into cholesterol appears related to the observation that there was an accompanying preferential oxidation of ^{14}C-labeled linoleate compared to ^{14}C-labeled palmitate shortly after administration of these fatty acids. This is shown in both Tables III and IV. Between the fifth and sixth hours after feeding the labeled fatty acids, approximately twice as much endogenous ^{14}C-fatty acid carbon was oxidized to CO_2 per hour by the intact, 1-^{14}C-linoleate-fed rats than by the intact, 1-^{14}C-palmitate-fed rats (Table III). In addition, when removed from the rats 6 hr after feeding and incubated in vitro, both the liver and skeletal muscle of these animals showed a preference for oxidation of endogenous linoleate (Table IV). As further equilibration of the labeled fatty acids with the body

TABLE IV

Per Cent of the Endogenous Tissue ^{14}C-Labeled Fatty Acid Carbon Oxidized to Respiratory $^{14}CO_2$ Per Gram of Tissue (Wet Weight)[a,b]

Hours after feeding	Number of rats	$^{14}CO_2$ (^{14}C-Palmitate-fed rats)		Number of rats	$^{14}CO_2$ (^{14}C-Linoleate-fed rats)	
		Liver	Muscle		Liver	Muscle
6	4	1.43 ± 0.41	8.67 ± 2.50	4	2.82 ± 0.23	16.61 ± 3.63
29	2	1.07 ± 0.07	2.29 ± 0.10	2	1.13 ± 0.01	2.83 ± 0.03
77	4	1.24 ± 0.08	1.62 ± 0.56	4	0.98 ± 0.06	2.22 ± 0.83

[a] At 6, 29 and 77 hr after feeding the ^{14}C-labeled fatty acid 1 g samples of liver and skeletal muscle were removed, sliced (Stadie tissue slicer) and incubated in 20 ml of Krebs phosphate buffer (pH = 7.4) for 2 hr at 37 C.

[b] Each value is the mean ± 1 SD. Significantly more endogenous ^{14}C from 1-^{14}C-linoleate than 1-^{14}C-palmitate was oxidized to $^{14}CO_2$ by both liver and muscle at the sixth hour, $P(t) < 0.02$ (as measured by the Student's "t" test).

lipid occurred over a period of a day or more, the fraction of endogenous ^{14}C-fatty acid carbon oxidized to CO_2 per hour in the linoleate-fed rat was similar to that observed in the palmitate-fed rat (Tables III and IV).

DISCUSSION

Since the liver is a primary site of oxidation of fatty acids recently absorbed from the intestines and also a main site of cholesterol biosynthesis, the observed preferential utilization of dietary 1-^{14}C-linoleate carbon for cholesterol biosynthesis by the intact rat could largely represent a preferential catabolism of 1-^{14}C-linoleate by the liver. Indeed, the data indicate that soon after ingestion of 1-^{14}C-linoleic and palmitic acids the liver was oxidizing significantly more 1-^{14}C-linoleic acid carbon than 1-^{14}C-palmitic acid carbon. The greater oxidation of the fed 1-^{14}C-linoleate than 1-^{14}C palmitate by the liver perhaps could reflect a smaller pool of hepatic linoleic acid available to dilute the newly absorbed ^{14}C-linoleate than hepatic palmitic acid to dilute the ^{14}C-palmitate. It is also possible that the enzymes of the β oxidation pathway exhibit a preference for linoleic over palmitic acid as substrate for oxidation.

Irrespective of the mechanism involved, as judged by ability to oxidize linoleic and palmitic acids and to utilize the carbon of these acids as substrate for cholesterol synthesis, the rat exhibits a greater ability to catabolize linoleic than palmitic acid. The greater incorporation of linoleic acid carbon into cholesterol most probably reflects the preferential oxidation of linoleic acid by the intact rat and by the rat liver in particular. Although utilization for cholesterol biosynthesis is a quantitatively minor pathway of fatty acid metabolism, the fact that animals ingest considerable amounts of fatty acids and that cholesterol represents a small fraction of the total body carbon makes it likely that a large fraction of the carbon required for total cholesterol synthesis is derived from fatty acid carbon. Thus, the observed preference of the intact rat to utilize linoleic acid carbon for the synthesis of cholesterol may help to explain the observation of Klein (1) and others (12) that the plasma cholesterol of the rat increases as the linoleic acid content of the diet increases. By comparison, the decrease in plasma cholesterol of humans in response to increased intake of linoleic acid may relate to the observation of Fredrickson and Gordon (13) that the human does not catabolize linoleic acid as readily as palmitic and oleic acids.

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Heat Transformation Products of Cottonseed Oil

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The high incidence of cancer of the stomach has indicated the possibility that heated foodstuffs contain a carcinogen. However, in this laboratory examination of fats heated to relatively high temperatures in cooking has not revealed the presence of agents of the aromatic hydrocarbon type, which show characteristic fluorescence. Biological tests of the carcinogenicity of heated cottonseed-oil products by feeding experiments on mice have been described by Peacock, Buck & Chalmers (1953) and additional tests made by subcutaneous injection into mice will be described later in this communication.

In the feeding experiments to which reference has been made, the following cottonseed-oil products have been used: (1) Cottonseed oil heated at the b.p. (350°) for 4 hr. (2) Cottonseed oil heated at 320° for 2·5 hr. in the presence of iron, which favours ketonization of the oil.

Considering all tests carried out in this biological investigation, in 268 animals surviving 400 days and over, there were eighteen papillomata and six carcinomata of the forestomach. Although this is a smaller percentage than that which might be expected to follow the administration of a carcinogenic hydrocarbon, it is similar to the percentage of deaths in the human species due to gastric cancer. The present work was undertaken in an attempt to determine the nature of the heated products used in these biological tests. For a preliminary communication of this work see Chalmers (1952).

EXPERIMENTAL AND RESULTS

Apparatus. In the earlier biological experiments (Peacock *et al.* 1953), the oil was heated in a stream of N_2 in a flask fitted with an electrically heated column as described for use in the distillation of fatty esters (Hilditch, 1947). In the present experiments, owing to difficulties due to frothing, the heated column was replaced by a vacuum-jacketed column (3 ft. × 2 in. diam.). A small receiver was inserted immediately above the vacuum jacket, and water formed during the reaction together with a small quantity of liquor smelling strongly of acrolein were run off. The still-head carried a jet for the admission of N_2 in case of frothing: the main receiver was attached directly to the still-head. The path to the receiver and exhaust was about 1 in. wide.

Injection experiments. In addition to the feeding experiments to which reference has been made, several of the cottonseed-oil products were tested by subcutaneous injection, either alone or in solution in tricaprylin, into mice which were 3 months old at the start of the experiment (Table 1). Some of these experiments are still in progress. A number of the animals died within the first year of the experiment, owing to an intercurrent infection. In Expts. 3 and 4 few of the mice lived much longer than 12 months and the results of the tests are therefore inconclusive. There has been no obvious reaction at the site of injection in any of these experiments, although in some of the older animals remote tumours were observed.

Table 1. *Biological tests with heated cottonseed-oil products*

(For experimental details see text.)

Expt. no.	Temperature and duration of heating	Product tested	Mice surviving at (months) 12	15	18	24	Remote tumours observed
1	350° for 4–6 hr.	Crude product and unsaponifiable fraction	14	14	14	7	Mammary adenoma after 18 months. Hepatoma after 24 months. Lung adenocarcinoma after 24 months
2	320° for 2–3 hr. (+ iron)	Crude product	13	10	10	4	Lung adenoma after 23 months. Lung adenoma and hepatoma after 27 months. Squamous carcinoma of skin and generalized lymphomatosis of lymph nodes and lungs after 25 months
3	300° for 22–25 hr.	Crude product	8	2	2	0	Lung adenoma after 18 months
4	Peroxidized at room temperature	Crude product	8	4	2	0	Lung adenoma after 22 months

Decarboxylation of cottonseed oil. When testing for the presence of CO_2, the volatile products of pyrolysis carried over in the stream of N_2 were led from the still-head through a wash bottle containing aqueous $Ca(OH)_2$.

Piper *et al.* (1931) described the preparation of certain aliphatic ketones by heating the corresponding fatty acids under reflux at temperatures in the region of 300° in the presence of an iron ring, and it was anticipated that heating cottonseed oil in the presence of iron would result in the formation of ketonic products. In the present experiments, it was found that in the presence of iron filings a vigorous reaction was reached after 0·5 hr. at 340°, after 1·25 hr. at 320° or after 4 hr. at 300°. Iron strips catalysed the decarboxylation reaction, although not quite so effectively as iron filings. The surface area of the iron strips used was equal to that of an iron vessel capable of holding the volume of oil heated. It was considered that the results of this experiment would be comparable with those of Grün, Ulbrich & Krezil (1926) who prepared aliphatic ketones by heating the corresponding fatty acids in an iron vessel and also with ordinary domestic cooking in iron utensils. On heating cottonseed oil alone under the conditions described above, CO_2 was first detected at 345°; in the presence of iron strips it was detected at 305° and in the presence of iron filings at 250°.

Polymerization of cottonseed oil. In the present experiments cottonseed oil was heated at 300° in an atmosphere of N_2. There was a steady increase in viscosity on heating up to 20 hr., between 20 and 25 hr. there was a sharp rise and from 25 to 35 hr. the steady rate of increase was resumed. Fig. 1 shows changes in iodine and free fatty acid values during polymerization.

The polymerization of a number of highly unsaturated oils, including cottonseed oil, by heating at 280° for periods up to 20 hr. has been described by Lips, Promislow & Grace (1953). Under the conditions used by these workers, free fatty acid values remained low and the changes in viscosity and iodine value were lower than those found in the present experiments.

Peroxidation of cottonseed oil. This was done by passing air through a sample of cottonseed oil at room temperature until a high peroxide value was obtained. The sample used for biological test had a peroxide value of 165 and a free fatty acid value of 4·3%.

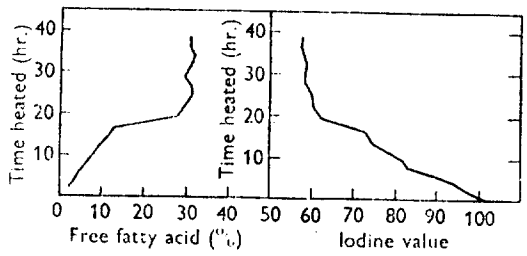

Fig. 1. Iodine and free fatty acid values during polymerization of cottonseed oil.

Fractionation of heated cottonseed oil. The heated product (100 g.) was well stirred with hot acetone (1 l.) and allowed to stand overnight at room temperature. 2 *N*-NaOH was used to separate the free fatty acid (*FFA*) from an ether solution of the acetone-soluble material. Saponification of the remainder of the acetone-soluble material gave acid (*FA2*) and unsaponifiable (*U1*) fractions. The acetone-insoluble fraction of the heated product was also saponified giving acid (*FA3*) and unsaponifiable (*U2*) fractions. An examination has been made in this way of the composition of the heated cottonseed-oil products used in the biological tests and a comparison made with cottonseed oil polymerized by heating at 300° for 22 hr. The results shown in Table 2 are typical, but there were variations in the results for samples heated on different occasions.

Polymerized cottonseed oil. An examination of the polymeric material in the acidic products has been made, using the method of Cowan, Falkenburg & Teeter (1944). The methyl ester of the acid was distilled in an alembic-type still under reduced pressure and the distillate collected in small fractions. The refractive index of the fractions was determined and plotted against the total percentage distilled up to the mid-point of the fraction. The acetone-insoluble fraction (*FA3*) of the oil heated at 350° for 4 hr., the free fatty acids (*FFA*) and the fatty acids of the neutral oil (*FA2*) were examined and found to contain polymeric

Table 2. *Comparison of heated cottonseed-oil products*

(There was a significant production of free fatty acid on polymerizing cottonseed oil at 300°. At 350°, less polymer (acetone-insoluble fraction) was produced and there was a corresponding increase in free fatty acid and unsaponifiable material (acetone-soluble fraction). Heating in the presence of iron filings resulted in the production of acetone-soluble and acetone-insoluble unsaponifiable material. Some of these fractions were ketonic but there was also a large amount of chemically inert material of a tarry nature.)

	Acetone-soluble fraction			Acetone-insoluble fraction	
	Free acids			Free acids,	
Temperature and duration of heating	*FFA* (%)	*FA*2 (%)	Unsaponifiable (%)	*FA*2 (%)	Unsaponifiable (%)
300° for 22 hr.	18	10	1·5	60	2
350° for 4 hr.	48	11	11	18	4
320° for 2·5 hr. (+iron)	2	0	35	0	62

Table 3. *Analysis of methyl esters of heated cottonseed oil*

(The figures in parentheses are percentages of the total heated product. For other details see text.)

		Monomer	Intermediate fraction	Dimer	Non-volatile residue
Fraction of heated product (350° for 4 hr.)					
Free fatty acid, *FFA* (48%)	Yield (%)	40	10	15	30
	Mol.wt.	308		531	1205
	n_D^{50}	1·4260–1·4435		1·4775	1·4925
Neutral oil, *FA*2 (11%)	Yield (%)	50	15	10	25
	Mol.wt.	329		466	1263
	n_D^{30}	1·4325–1·4445		1·4805	1·5020
Acetone-insoluble polymer, *FA*3 (18%)	Yield (%)	15	10	7	67
	Mol.wt.	309		544	934
	n_D^{30}	1·4330–1·4470		1·4780	1·4970
Fraction of heated product (300° for 22 hr.)					
Acetone-insoluble polymer, *FA*3 (60%)	Yield (%)	35	15	15	32
	Mol.wt.			440	932
	n_D^{50}	1·4355–1·4450		1·4750	1·4900

material. The results of these distillations and, for comparative purposes, of the distillation of the *FA*3 fraction of cottonseed oil polymerized by heating at 300° for 22 hr., are shown in Table 3. The acetone-insoluble polymeric fraction of cottonseed oil heated at 350° contained less monomer, less dimer, and more non-volatile polymeric material than the product heated at 300°. The free fatty acid fraction of the oil heated at 350° contained a significant amount of polymeric material and the importance of this is increased by the fact that almost half of this heated product was free fatty acid. The polymerization of the free fatty acid from soy-bean oil has been described by Croston, Tubb, Cowan & Teeter (1952) and presumably in the present case the fatty acid liberated on heating was subsequently polymerized. The neutral-oil fraction was also shown to contain polymeric material.

Cooking tests. Chang, Teben & Watts (1952) examined the effect of deep-fat frying on the component fatty acids of lard and corn oil. They found that the iodine value of the corn oil dropped from 126·1 to 122·1 after 4 hr. at 180° and that the percentage of conjugated diene showed a slight increase. Changes in fatty acid values were slight, the most marked being shown by linoleic acid, which dropped from 55·0 to 52·2%.

In this laboratory a sample of cottonseed oil which had been used in a local factory in frying potato crisps has been examined by the methods which have been described. In the factory the fat was heated in a deep iron pan to a temperature of about 250°. The sample did not contain any acetone-insoluble polymeric material and the methyl ester of the fatty acids from the neutral oil behaved similarly to the unheated cottonseed oil on distillation in the alembic still, consisting almost entirely of monomer.

In a further experiment potatoes were fried in cottonseed oil in the laboratory. In view of the catalytic effect of iron referred to earlier in this paper, an iron and an aluminium pan were used in parallel experiments. One litre of cottonseed oil was used for each experiment and when the temperature reached 190° the potatoes were added. The temperature dropped to 120° and remained low for about 15 min. Subsequently the temperature rose steadily to 140° at which point the chip potatoes were cooked brown. The time of heating was approx. 30 min. The operation was repeated using the same oil until the total time of heating was 7 hr. An analysis of the oil remaining and of the oil used commercially gave the results shown in Table 4. In the laboratory experiments, the reduction in the iodine value of the used oil was greater than the corresponding increase in free

Table 4. *Effect of potato-frying temperatures on cottonseed oil*

(For details see text.)

Test	Unused cottonseed oil	Cottonseed oil used in frying potatoes		
		Commercially (iron pan)	Laboratory (iron pan)	Laboratory (aluminium pan)
Solubility in acetone	Soluble	Soluble	Soluble	Soluble
Iodine value	104·4	100·1	91·4	89·2
Free fatty acid (%)	None	0·85	0·38	0·33
Peroxide value	1·2	4·7	13·3	9·9
Refractive index (n_D^{30})	1·4680	1·4685	1·4710	1·4720

fatty acid content when compared with oil heated at 300°. In this respect the commercially heated oil occupied an intermediate position. The laboratory heated oil was also oxidized to a greater extent than the commercially heated oil but this was possibly due to the exposure of a relatively greater surface to air. It was noted that the refractive index values varied with the iodine value. Distillation of the fatty acid methyl esters from the neutral fraction of cottonseed oil which had been used commercially in frying potatoes gave no evidence of polymer formation.

DISCUSSION

There is evidence that distillation of the glycerides of saturated fatty acids in the presence of iron converts the fatty acids into the corresponding ketones. Holleman & Koolhaas (1939) distilled coconut oil in the presence of iron and isolated an aliphatic ketone from the distillate. They concluded that small quantities of higher aliphatic ketones are generally formed when food is fried.

There is also evidence that on heating cottonseed oil at 320–340° in the presence of iron the palmitic acid fraction of the oil is converted into palmitone (Chalmers, 1951). In previous experiments, cottonseed oil was distilled in the presence of iron and the solid distillate fractionated by treatment with ethanol. In the higher melting fraction palmitic acid was identified. It was presumed that the fatty acid was first split from the fat and under suitable conditions, e.g. in the presence of iron at about 320°, converted into the corresponding ketone.

Subsequently it was suggested by Hendry, Homer, Rose & Walpole (1951) that ketonization of heated fat might take place with formation of glycidyl esters. Although heated fat products have been tested in this laboratory for the presence of glycidyl esters, with negative results, it is possible that the glycidyl esters are unstable intermediates.

In addition to palmitic acid, cottonseed oil contains oleic and linoleic acids as major components. Isomerization of these acids has been described by Bauer & Krallis (1931) who heated oleic acid in nitrogen at 250° and were able to identify an isomeric acid in the heated product. In the course of a study of the synthesis of triolein, Desnuelle & Naudet (1945) found that '*iso*oleic acid' was formed in amounts varying from 5·1 to 11·8% when oleic acid or triolein was heated at 135–180°.

In a recent paper, Barker, Crawford & Hilditch (1951*a*) have described the interchange of acyl groups in triglycerides containing saturated and unsaturated acids. They heated oleodistearin at 300° in glass or silica vessels and after 10 hr. at this temperature the composition of the mixture was tristearin 25, oleodistearin 54·2, stearodiolein 20·2 and triolein 0·6%. The production of free fatty acid was the only side reaction detected. Acyl migration in the presence of catalysts has been demonstrated by other workers in the case of the glycerides of soy-bean oil or cottonseed oil and similar results were obtained with mixtures of tripalmitin and triolein in the presence of stannous hydroxide at 225° (Norris & Mattil, 1946, 1947) or of tristearin and triolein at 205° in the presence of sodium methoxide (Naudet & Desnuelle, 1947).

The primary change which takes place on heating an oil containing polyethenoid acids is a rearrangement of unconjugated pentadienes to the corresponding conjugated dienes. This change, which thermally takes place at about 300°, can take place at a lower temperature in the presence of catalysts. Following the conjugation stage, polymerization of the oil takes place. The mechanism of polymerization is not as yet completely understood but is a subject that has received considerable study. Thus the kinetics of thermal polymerization of isomers of methyl linoleate have been investigated by Paschke, Jackson & Wheeler (1952) and chemical changes taking place on thermal polymerization of sunflower-seed and linseed oils have been described by Barker, Crawford & Hilditch (1951*b*). Sunflower-seed oil, like cottonseed oil, contains oleic and linoleic acids as major component unsaturated acids, and Hilditch and his co-workers consider that dimerization is effected by a single cross-linking between two carbon atoms in different linoleic acid radicals, intramolecularly in the earlier and intermolecularly in the later stages.

Polymerization increases the flavour stability of oils containing unsaturated acids but has been found to have a deleterious effect on the nutritional value. Lassen, Bacon & Dunn (1949) investigated

the digestibility of polymerized sardine oil incorporated in the diet of rats and found that the polymerized portion of the oil was not absorbed by the gastro-intestinal tract. Further work from a nutritional standpoint has been carried out by Crampton, Common, Farmer, Berryhill & Wiseblatt (1951) and Crampton, Common, Farmer, Wells & Crawford (1953), who fractionated polymerized linseed oil by distillation at low pressure and then by the capacity of the distillate to form urea adducts. When fed to rats, the non-distillable polymeric fraction was largely unabsorbed and the portion of the distillate which did not form urea adducts was found to be toxic. Chemical aspects of this thermal effect on the nutritive value of the polymerized oil have been examined by Wiseblatt, Wells & Common (1953) and by Wells & Common (1953). In the first paper the authors give analytical data, including changes in the fatty acid composition, for linseed oil polymerized by heating at 275° in an atmosphere of CO_2 for various times up to 17 hr. Analytical data are also presented for soy-bean oil heated at various time intervals up to 30 hr. It is suggested that the relationship between the values for polymeric acyl radicals and mean molecular weights of the heated oils indicates the presence of intrapolymers. In passing, it may be mentioned that, unlike in the present experiments with cottonseed oil, little free fatty acid was formed (0·69 % after 30 hr. heating of soy-bean oil). The second paper describes the fractionation of linseed oil polymerized as before by heating in CO_2 at 275° for 12 hr. Distillation of the ethyl esters at low pressure gave a monomeric distillate of which one-fifth was incapable of forming urea adducts. It is suggested, on the basis of fatty acid composition, that this fraction contains cyclic acids. Estimations of acetyl values and of the proportion of terminal groups excluded the presence of hydroxy acids and branched fatty acids.

The possible carcinogenicity of a mixed fat (equal parts of cottonseed oil and whale oil) which had been repeatedly heated at 200–220° was examined by Beck & Peacock (1941). The heated fat was added to an adequate basal diet containing 0·5 % cod liver oil and fed to rats and mice. Within a year signs of avitaminosis-A appeared among the rats and this was followed by gastropapillomatosis in the forestomach. The papillomatosis, however, could be avoided or cured by the addition of raw carrot to the heated fat diet. Beck & Peacock also showed by experiments in which rats were fed a diet supplemented by milk, carrot and heated lard that 'the vitamin-A content of the liver was reduced in proportion to the degree and duration of heating to which the lard was subjected'. They concluded that 'there is present in repeatedly heated fats a factor which interferes in some way with the absorption or metabolism of vitamin A'. It is therefore possible that this condition was caused by the inefficient absorption of vitamin A from the diet due to its retention in the polymeric material of the heated lard which was, to a greater or less extent, unabsorbed.

In the present work consideration was given to the nature of the agent inducing tumours of the forestomach of mice fed a diet containing heated cottonseed oil (Peacock *et al.* 1953). The oil tested was heated (*a*) at 350° for 4 hr. and (*b*) at 320° in the presence of iron for 2·5 hr. These products were fractionated by treatment with acetone and by distillation of the fatty acid after methanolysis in an alembic still. For comparative purposes a sample of cottonseed oil polymerized by heating at 300° for 22 hr. was also examined. In the case of the oil heated at 350°, a high percentage of free fatty acid was formed and this fraction, as well as the acetone-soluble (monomeric acid) and acetone-insoluble (polymeric acid) fractions, contained appreciable amounts of undistillable polymeric material. While the figures given for the oil heated at 320° in the presence of iron indicate that this product is mainly unsaponifiable, some of the samples used in the biological tests contained polymers. Moreover, the unsaponifiable fractions contained, in addition to ketonic material, chemically inert substances of a tarry nature which presumably were decomposition products of polymeric material. In a preliminary account of this work (Chalmers, 1952) the presence of polymers in heated cottonseed-oil products was stressed.

No tumours occurred at the site of injection in the present experiments with cottonseed oil polymerized at 300° or oil heated at 350° for 4 hr. and at 320° for 2·5 hr. in the presence of iron. The experiments with oil polymerized at 300° were inconclusive since many of the animals under test died at a comparatively early age due to an infection. However, in the test with oil heated at 350°, which also contained polymeric material, more animals were used and there was a good survival rate. In this experiment, also, no tumours were found at the site of injection. In the feeding tests, where the route of administration is more satisfactory (Peacock *et al.* 1953), cottonseed oil heated at 350° gave fewer tumours of the forestomach of mice than the product heated at 320° in the presence of iron. It therefore appears that the substance responsible for the induction of tumours of the forestomach of mice fed a heated fat diet is a weak carcinogenic agent. Although there is at present no conclusive evidence concerning the nature of this carcinogen, it is possible that it may be a polymer.

In considering the abnormal products in heated cottonseed oil from the point of view of possible carcinogenicity, reference may be made to the sterol fraction of the oil which, although small in

amount, may be important in view of the carcinogenic activity of cholesterol-rich extracts from biological sources (Hieger, 1947). It is also possible that small quantities of polymeric acyl radicals are formed during potato frying as carried out in the laboratory, although the heated fat did not contain demonstrable amounts of polymeric material. Another possibility would be the formation in heated fats of compounds of a 'cyclic' type, the toxicity of which was described by Crampton *et al.* (1953) in the case of heated linseed oil.

Although there is no evidence associating stearoylethyleneimine with heated fat products, it is interesting that it has been suggested that polymerization plays a part in the action of this synthetic carcinogen in the cell. It has been postulated by Hendry *et al.* (1951) that cytotoxic agents condense intermolecularly within the cell with the formation of polymer units with chemically reactive side chains, which then combine with the protein or nucleoprotein components of the cell. These authors suggested that certain carcinogens acted in the same way and prepared stearoylethyleneimine, which was regarded as a micelle-forming substance capable of reacting with cell components, and showed it to be carcinogenic.

SUMMARY

1. An analysis has been made of cottonseed oil heated (*a*) at 350° for 4 hr. and (*b*) at 320° for 2·5 hr. in the presence of iron. These products, which have been reported to induce tumours of the forestomach of mice, were fractionated by treatment with acetone and by distillation in an alembic-type still. Product (*a*) contained polymeric material in both the acetone-soluble and acetone-insoluble fractions. The major component of (*b*) was unsaponifiable material.

2. Subcutaneous injections into mice of these products and also of polymerized and peroxidized cottonseed oil have not so far, after more than a year, induced any tumours at the site of injection.

3. The changes observed in cottonseed oil used in frying potatoes were comparatively slight and polymerized products were not detected.

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STUDIES TO DETERMINE THE NATURE OF THE DAMAGE TO THE NUTRITIVE VALUE OF SOME VEGETABLE OILS FROM HEAT POLYMERIZATION [1, 2]

I. THE RELATION OF AUTOXIDATION TO DECREASE IN THE NUTRITIONAL VALUE OF HEATED LINSEED OIL

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Ingestion of heat polymerized linseed oil was followed by lowered growth rates in rats, this growth depression becoming more severe with increased lengths of heating time of the oils and with increased amounts of oil in the diet (Crampton et al., '51). Our earlier experiments indicated that the low nutritive value of heated linseed oil diets is attributable to the oil itself and not to the effects of the heated oil upon non-lipid diet constituents.

As thermal treatment causes oil to be less stable to oxidation, it has been proposed that biologically deleterious products may accumulate through progressive autoxidation during storage of the oil-containing diets at room temperature. Oven temperatures used during the baking of the diets may also promote oxidative changes.

The extent of autoxidation can be controlled by the addition of an anti-oxidant. A mixture of nordihydroguararetic acid (NDGA) with citric acid has been shown to be an effective

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[2] Issued as paper No. 260 of the Canadian Committee on Food Preservation.

anti-oxidant for edible fats, increasing stability even in baked products (Mattil et al., '45; Higgins and Black, '45).

The question of vitamin E deficiency in heated oils also seemed worthy of consideration at this time, since polymerization at 275°C. would destroy any of this vitamin inherent in the original oil. The absence of vitamin E, a natural antioxidant, presumably permits a more rapid onset of autoxidation. In addition, a relation of vitamin E to growth, over and above its stabilization of linoleic acid, has been shown in insects by Fraenkel and Blewett ('46). In the feeding of heated lard to rats, Morris and his co-workers ('43) noted a depression of growth and also a paralysis simulating a vitamin E deficiency. Therefore it was possible that in our tests, thermal destruction of vitamin E was responsible in part for the adverse effects we had observed to result from feeding heat-polymerized linseed oil.

EXPERIMENTAL

Feeding trials were conducted to examine the effects of baking, of controlled autoxidation, and of vitamin E supplementation of diets containing heat-polymerized linseed oil.

Oil preparation

Polymerization and acetone segregation were performed according to the method of Privett, McFarlane and Gass ('47). Preliminary trials in our laboratories showed no nutritional difference between whole heated oil and its acetone soluble fraction, beyond a decrease in intake of the diets containing the latter. This effect was thought to be due to residual traces of acetone (Langerman, '49). Therefore in the present test, in which acetone segregation of heated oil was involved, the control (unheated) oil was subjected to like treatment with acetone.

Where oils were artificially stabilized (test II), an antioxidant mixture of NDGA with citric acid was added as 0.05% of the oil. Peroxide values on these oils when freshly prepared

and after 28 days of storage at room temperature were estimated by the method of Skellon and Wills ('48). Peroxide determinations on chloroform extracts of the oil-containing diets were made at intervals throughout the 28-day feeding trial by a modification of the Skellon and Wills method (see appendix).

Biological procedure

The animals used in these trials were albino rats ranging in age from 21 to 28 days at the outset of the test period. They received, ad libitum, the diets described in table 1.

TABLE 1

Composition of diets

INGREDIENTS	DIET A (test II)	DIET B (tests I and III)
White flour (%)	47	54
Milk powder (%)	19	19
Casein (%)	11.5	11.5
Oil (%)	20	10
Bone meal (%)	2	2
Salt (%)	0.5	0.5
Yeast (%)	..	3
Vitamins		
Thiamine	10 p.p.m.	..
Niacin	230 p.p.m.	..
Riboflavin	5 p.p.m.	..

Diets minus vitamins or yeast were baked (except where specified in test I) for 20 minutes at 375°F. and the resulting biscuit granulated and air-dried for 24 hours. Vitamin supplements in diet A, or yeast in the case of diet B, were then added. Weekly doses of cod liver oil were administered orally to supply 175 I.U. of vitamin A and 35 I.U. of vitamin D.

In addition to these vitamins, half of the animals in test III each week received orally alpha-tocopherol dissolved in corn oil. The doses, administered two days following the A and D supplementation, were of such amount as to supply 7 mg of vitamin E to each animal.

RESULTS

When the oils were stabilized, there was no appreciable accumulation of easily reducible peroxides. However, definite signs of progressive peroxidation were shown in the unstabilized oil over the 28-day trial. These facts are evident from the peroxide values given in table 2 and calculated at the beginning and the end of the test period on oil extracted from the feed and also on oil that was not combined with the feed.

It is true that peroxide values are not a full indication of rates of total oxygen absorption and that other diet components may have been attacked by peroxides as rapidly as

TABLE 2

Peroxide values

(Calculated as milligrams of peroxide oxygen per kilogram)

	OIL EXTRACTED FROM FEED		STORED OIL	
	Fresh	After 28 days	Fresh	After 28 days
Unheated oil	99	276	47	1,334
Unheated stabilized oil	26	55	49	172
Heated oil	116	333	0	270
Heated stabilized oil	25	38	0	51

these were formed. In such an event, no accumulation of easily reducible peroxides would have been noted. However, a steady increase in peroxide values in unstabilized oils throughout the test points to the likelihood that extensive peroxide decomposition had not yet begun in the stabilized samples.

The growth rates and feed intakes of the animals in test II, given in table 3, show that the stabilized oil was no better nutritionally than the unstabilized control oil. The equality of the growth responses of animals fed heated oils regardless of anti-oxidant treatment makes it unlikely that autoxidation is responsible for the low nutritive value of polymerized linseed oil.

This is further supported by the data from test I, where the growth responses of the animals show that baking did not influence the growth inhibition exerted by the heated oil. Exposure to oven temperatures would, if anything, encourage

TABLE 3

Effect of baking, of anti-oxidant stabilization, and of vitamin E supplementation on the nutritive value of unheated or heated whole linseed oil or on the acetone soluble fraction of the heated oil

TEST NO.	OIL USED	TREATMENT	OIL IN DIET	NO. ANIMALS	AVE. DAILY GAIN	AVE. DAILY FEED INTAKE	GAINS PER 1,000 CAL.[1]
			%		*gm*	*gm*	*gm*
I	Unheated	Diet baked	10	8	4.3	12.1	72
	Unheated	Diet unbaked	10	8	4.2	12.4	69
	Heated	Diet baked	10	8	3.1	10.6	60
	Heated	Diet unbaked	10	8	2.7	9.7	57
II	Unheated	nil	20	10	3.7	10.1	68
	Unheated	Oil stabilized[2]	20	10	3.8	9.6	73
	Heated (acetone soluble fraction)	nil	20	10	—0.1	6.0	
	Heated (acetone soluble fraction)	Oil stabilized[2]	20	10	—0.1	5.8	
III	Heated	nil	10	24	2.4	10.0	49
	Heated	Vitamin E supplementation to diet	10	24	2.3	9.8	48

[1] Gross calories (calculated from heats of combustion): 10% fat diet contained 4.9 Cal./gm; 20% fat diet contained 5.4 Cal./gm.

[2] Protected against autoxidation with NDGA and citric acid.

autoxidation. These results imply that the damaging factor in heated oil is not produced oxidatively.

Supplementation with vitamin E failed to alter the nutritive value of polymerized linseed oil (test III).

CONCLUSIONS

The results of these experiments show that peroxidation of polymerized linseed oil is not concerned in the development of any toxic factor, and also suggest that a lack of vitamin E is not involved in the growth inhibition resulting from the ingestion of heated oil.

ACKNOWLEDGMENTS

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Thanks are also due to Miss Barbara Clark for her aid in conducting these feeding tests.

APPENDIX

Modification of the Skellon and Wills procedure for peroxide determinations on oil extracted from diets

A weighed sample of biscuit containing about 1 gm of the oil was shaken up with a 25-ml portion of chloroform and the filtrates were combined. The extract was evaporated to 10 ml by passing over it oxygen-free nitrogen *without heating*, and transferred to a 250-ml flask containing 2 gm sodium bicarbonate. A stopper was fitted, carrying a short piece of glass tubing capped by a rubber Bunsen valve. This served as a one-way pressure relief valve. Fifteen milliliters of glacial acetic acid were used to rinse the last traces of chloroform extract into the reaction flask, and the acid-bicarbonate reaction released CO_2 which swept air out of the flask through the relief valve. When foaming subsided, 1 ml of saturated fresh potassium iodide solution was added, and the tightly stoppered flask was left in the dark for one hour. Fifty milliliters of boiled, cooled water were then added, and the iodine titrated with 0.002 N thiosulfate.

Peroxide values were calculated as milligrams of peroxide oxygen per kilogram of oil, thus:

$$PV = \frac{V \times N \times 16 \times 10^3}{W \times P}$$

where,

V = Volume of thiosulfate used (ml)
N = Normality of thiosulfate
W = Sample weight (gm)
P = Per cent oil in sample.

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STUDIES TO DETERMINE THE NATURE OF THE DAMAGE TO THE NUTRITIVE VALUE OF SOME VEGETABLE OILS FROM HEAT TREATMENT [1, 2]

II. INVESTIGATION OF THE NUTRITIOUSNESS OF THE PRODUCTS OF THERMAL POLYMERIZATION OF LINSEED OIL

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FOUR FIGURES

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INTRODUCTION

Heating unsaturated oils to polymerization temperatures has been advocated as a practical method of reducing or eliminating the flavor reversion of shortening produced from such oils (Privett et al., '45). However, if oils treated in this manner are included in the diets of rats, then the rate of gain, the feed intake and the feed efficiency are decreased. These effects may be due to the action of some toxic material (Crampton et al., '51).

Of the oils which have been tested in this laboratory, linseed oil has proved to be the one most susceptible to nutritive damage by heating. Accordingly, linseed oil was selected for further studies on the nature of the material responsible for the toxic effects. The results of these experiments are reported in the present paper.

[1] Contributon from the Faculty of Agriculture, McGill University, Macdonald College, Quebec, Canada. Journal Series No. 280.

[2] Issued as paper 263, Canadian Committee on Food Preservation.

Unsaturated fats yield dimeric, trimeric and higher polymeric glycerides when heated at 275°C. in the absence of oxygen. It is rather generally accepted that polymerization does not proceed to any appreciable extent beyond dimerization so far as the fatty acid radicals themselves are concerned (Privett et al., '47). The extent to which such dimerization of the fatty acid radicals may result in the presence of intrapolymeric glycerides remains controversial. Indeed, Bernstein ('48) has dismissed the possibility on theoretical grounds. Adams and Powers ('44), on the other hand, produced considerable experimental evidence in support of the view that such intrapolymers are formed in the early stages of polymerization, and that they may in turn give rise to dimeric and higher polymeric glycerides by transesterification. Experimental evidence obtained in our laboratories supports the contention that thermally polymerized linseed oil may contain considerable amounts of intrapolymeric material (see appendix, table 1). Kass ('47) studied the thermal polymerization of eleostearates, and, as well as observing normal polymerization products, described side reactions leading to production of free fatty acids, aldehdyes, hydrocarbons and acids and esters of reduced molecular weight. He found that some of the latter fragments also polymerize, and produced evidence of cyclization leading to the production of cyclic aliphatic monomers and mono- and polycyclic aromatic compounds. We have essayed to indicate in plate 1 the nature of the substances which are thought to be the main products of thermal polymerization.

At the outset we suspected that polymerized triglycerides might be mainly responsible for the lower nutritive value of heated linseed oil. Consequently we sought to obtain for feeding tests a fraction which would consist essentially of non-polymeric glycerides. Privett et al. ('47) have described the preparation from heated linseed oil of an acetone-soluble segregate which, they reported, was free from "dibasic acids and was, therefore, non-polymeric in nature." Bernstein ('48) has described the use of propanol for the segre-

gation of monomeric from higher polymeric glycerides, such monomeric fractions being regarded by him as free from dimeric acids. In view of these reports, segregates were prepared by the use of acetone and also by the use of propanol. Our chemical data (see appendix, table 2) indicate that propanol was more effective than acetone for recovery of a non-polymeric glyceride fraction, thus supporting the findings of Bernstein in this respect. We have shown, however, that the acetone-soluble segregate of polymerized linseed oil appears to contain appreciable amounts of intrapolymeric material (see appendix, table 1). The propanol segregate does not contain dimeric or higher polymeric glycerides, but we have not examined this fraction for the presence of intrapolymers. Presumably the principal polymerized material, if any, in the propanol segregate is intrapolymeric. The acetone-soluble fraction is probably composed mainly of the molecular types indicated in plate 1, figure 2, while the propanol-soluble fraction is probably composed of those indicated in plate 1, figure 3.

It was also thought possible that the dimeric fatty acid radicals themselves might be implicated in the nutritional defects of polymerized linseed oil. Since solvent segregations offered little promise for the separation of true monomeric glycerides from intrapolymeric glycerides, it was decided to esterify the heated oil and then to separate monomeric esters from dimeric esters by distillation under vacuum. The esterification yielded the fractions indicated in plate 1, figure 4, and the succeeding distillation under vacuum achieved an essentially complete separation of polymeric material (see appendix, table 3). The distillate consisted of monomeric esters, but it may possibly have included some cyclic monomeric material. The undistillable residue consisted essentially of esters of dimeric acids, together with such sterols as were present and presumably some non-volatile products of thermal decomposition which were soluble in acetone.

EXPERIMENTAL PROCEDURE

The materials prepared for feeding comprised fractions of heated linseed oil composed largely of (a) monomeric esters, and (b) dimeric esters, as well as several fractions which contained various mixtures of monomeric and dimeric glycerides and of intrapolymers. In addition to examining biologically the fractions of the oil segregated by solvents (acetone and propanol) and by distillation of the esters of the fatty acids, crude and refined heated oils were compared to discover some indication as to whether or not the non-glyceride constituents of the oil were concerned in growth inhibition.

Polymerization and acetone segregation were carried out by the technique of Privett, McFarlane and Gass ('47). The polymerizations were based on a treatment of 12 hours at 275°C. in an all-glass apparatus while passing through it a current of carbon dioxide. A sufficient flow of carbon dioxide was maintained to keep the oil surging vigorously; attention to this detail resulted in products of excellent color and blandness and with remarkably low acid values (of the order of 0.6 to 1.0% F.F.A.). Propanol segregations were performed according to the directions of Bernstein ('48). Esterifications were carried out by interesterification in the presence of the appropriate anhydrous alcohol, using sodium hydroxide as catalyst; the oil or fraction was alkali-refined to remove F.F.A. prior to esterification. Esters were distilled at about 0.5 mm Hg absolute pressure (see appendix).

The several fractions of linseed oil, indicated in table 1, were fed to albino rats as the source of dietary fat for 28-day periods. The animals ranged from 21 to 28 days of age at the outset of the tests.

The basal diets had the composition described in table 2. Diets were baked for 20 minutes at 375°F. and the resulting biscuit granulated and air-dried for 24 hours. Vitamin B supplements, in the case of diet II; or yeast, in the case of diet III, were then added. Weekly doses of cod liver oil were

administered orally to supply 175 I.U. of vitamin A and 35 I.U. of vitamin D.

FEEDING TRIAL RESULTS

Table 1 is a summary of the weight gains and feed intakes of animals fed the various fractions of linseed oil. It is recognized that negative gains are unsatisfactory as quantitative measures of the nutritional values of rations, nor are

TABLE 1

Summary of average daily gains and average feed intakes of animals fed diets containing 20%[1] of various fractions of linseed oil

TEST NO.	OIL FRACTION	TREATMENT AT 275°C.	PRINCIPAL COMPONENTS OF FRACTION	NO. ANIMALS	AVE. DAILY GAINS	AVE. DAILY FEED INTAKE
		hrs.			*gm*	*gm*
1. A	Whole	none	Monomeric glycerides	5	3.0	6.8
B	Whole	12	Monomeric glycerides Intrapolymers Dimeric and polymeric glycerides	5	—3.0	4.6
C	Acetone-soluble	12	Monomeric glycerides Intrapolymers Dimeric glycerides	5	—3.0	5.3
2. A	Acetone-soluble, alkali-refined	12	Monomeric glycerides Intrapolymers Dimeric glycerides	6	—2.1	4.5
B	Acetone-soluble, crude	12	Monomeric glycerides Intrapolymers Dimeric glycerides Non-glyceride constituents	6	—0.7	3.5
3. A	Whole	none	Monomeric glycerides	10	3.8	10.0
B	Acetone-soluble	12	Monomeric glycerides Intrapolymers Dimeric glycerides	10	—1.4	6.0
C	Propanol-soluble	12	Monomeric glycerides Intrapolymers	10	0.2	5.5
4. A	Whole	none	Monomeric glycerides	8	3.4	9.7
B	Whole ethyl esters	none	Monomeric esters	8	2.3	7.5
C	Acetone-soluble ethyl esters	12	Monomeric and dimeric	8	0.8	5.8
D	Distillate of 4C	12	Monomeric esters	8	0.7	4.5
E	Residue of 4C	12	Dimeric esters	8	[2]	3.5

[1] Excepting test 2, where 36% fat was used.

[2] No gain could be calculated, as 6 out of 8 animals died within the first week. The remaining two survived three and 4 weeks, respectively.

statistical analyses of weight losses justified. In these trials such data are nevertheless indicative of the differences in nutritive quality of the diets studied. Where weight losses were encountered (tests 1, 2, and 3), the diets may be judged unwholesome without the benefit of statistics. However, it was possible to make an analysis of the variance of both gain and feed intake in test 4. Differences significant at the 5% point were apparent between A and B, B and C, and B and D. C and D were not different.

TABLE 2

Composition of diets

INGREDIENTS	DIET I (test 2)	DIET II (test 1)	DIET III (tests 3 and 4)
White flour (%)	26.5	47.0	44.0
Casein (%)	15.0	11.5	11.5
Milk powder (%)	20.0	19.0	19.0
Linseed oil (%)	36.0	20.0	20.0
Bone meal (%)	2.0	2.0	2.0
Salt (%)	0.5	0.5	0.5
Yeast (%)	..	..	3.0
Thiamine	[1]	10 p.p.m.	..
Niacin	[1]	230 p.p.m.	..
Riboflavin	[1]	5 p.p.m.	..

[1] Where diet I was fed (test 2), thiamine, niacin and riboflavin were administered orally to provide daily intakes of 0.1 mg, 2.3 mg, and 0.5 mg, respectively, per rat.

It is realized that purified esters are of uncertain feeding value because of their susceptibility to oxidative changes (Quackenbush et al., '42). For this reason, the effects on rat growth of esters vs. the triglycerides were compared. Animals fed esterified unheated oil gained significantly less weight than animals fed the unheated triglyceride; the feed intake of ester-containing diets decreased progressively during storage at room temperatures, indicating decreasing palatability of those diets. However, the utilization of feed for producing weight gain was not significantly different between the ester- and triglyceride-containing diets, nor could any clinical in-

dication of the toxicity of the esters be found. Esters of heated linseed oil showed the same adverse clinical effects as did the heated triglycerides, as will be seen in table 1.

DISCUSSION

The data shown in table 1 confirm that 12 hours' heating at 275°C. impairs the nutritive value of linseed oil.

A comparison of heated, whole oil with the acetone-soluble fraction (test 1) shows both of them to be equally incapable of supporting normal growth in rats. One may conclude, therefore, that acetone does not segregate toxic from nontoxic material.

There was no difference between the crude and refined oils (test 2), suggesting that the deleterious material in polymerized oil is derived from the glyceride component.

The propanol-soluble fraction was not significantly better nutritionally than the acetone-soluble fraction (test 3). It thus follows that the glyceride polymerization is not responsible for growth inhibition by heated linseed oil. Since neither acetone nor propanol is effective in segregating out the toxic material in heated linseed oil, one naturally turns to a consideration of the intrapolymers as the offending material. An interpolymer is a triglyceride within which two fatty acid chains are dimerized, and one might suspect the dimeric fatty acid structure of being the deleterious factor in heated oil. It is the only quantitatively important component of the propanol-soluble fraction of heated oil that is not present in unheated oil. How far this surmise is correct is evident from test 4, in which the "dimeric" fraction separated from the ethyl esters of the acetone segregate of polymerized oil was fed.

This undistillable "dimeric" fraction, which may also contain minor amounts of non-fatty material, proved lethal to rats in approximately 5 days.

The animals fed the distillable fraction (i.e., monomeric esters), although they did not grow, were thrifty in appear-

ance and excreted well-formed feces. Those fed the undistillable residue had oily, matted coats and excreted dark, sticky feces. This condition was also noted, but to a lesser degree, with the rats fed the mixed monomeric plus dimeric esters, and had also been recorded in previous tests in which the acetone-soluble fraction of heated oil had been fed. It appears possible, therefore, that the explanation of the failure to gain in weight on a diet containing the monomeric esters may be the absence of an essential unsaturated fatty acid, presumably because these more reactive acids had become polymerized or isomerized during heating. Thus dimerization may not only remove essential fatty acids, but produce toxic radicals as well. The strong adverse reaction of the rats to the dimeric esters may be the consequence of the absence of a needed growth factor plus the presence of a toxic agent.

CONCLUSIONS

On the basis of these results one might tentatively conclude that the primary cause of the lowered nutritive value of diets containing thermally polymerized linseed oil is the presence of one or more dimeric fatty acid radicals which are in some way inimical to the well-being of the animals. Their deleterious effects could well be aggravated since they are produced at the expense of unsaturated fatty acids, some of which may be those essential to the animal for growth.

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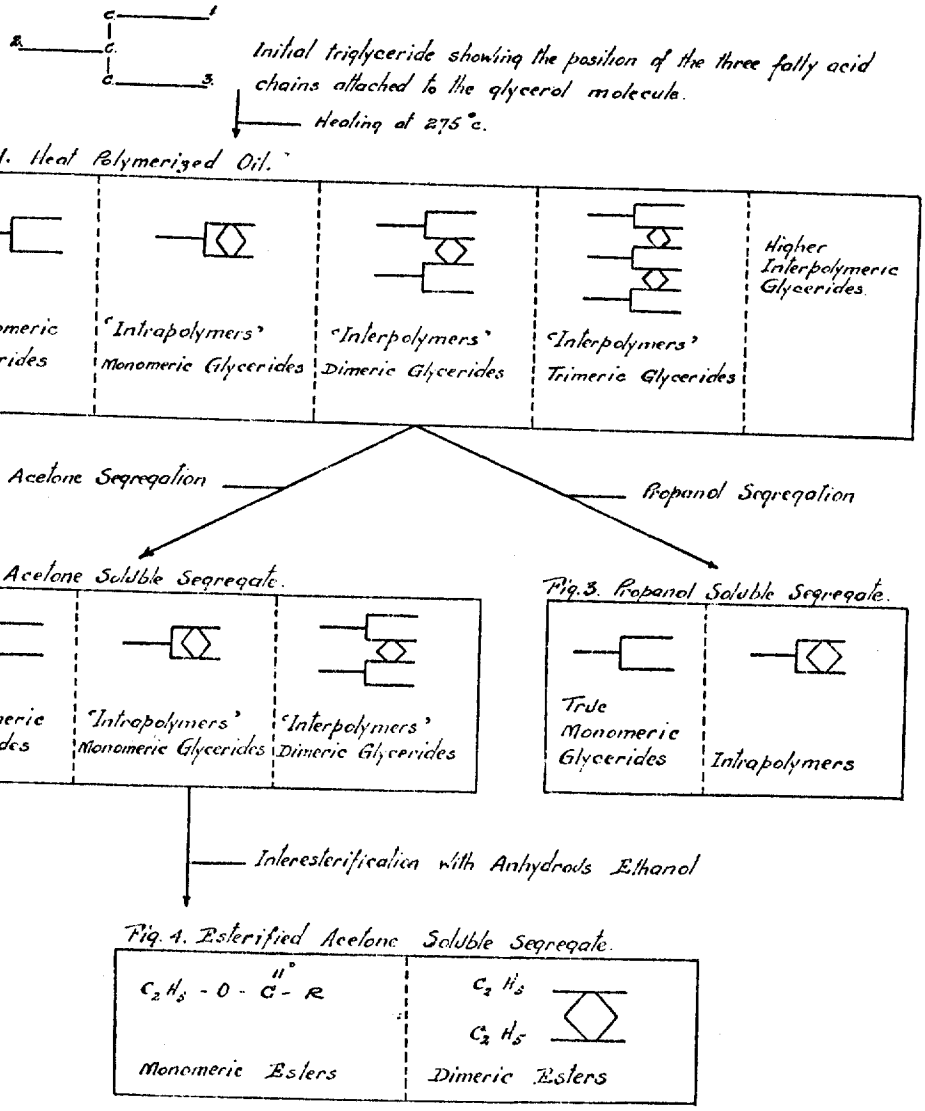
DIAGRAMMATIC REPRESENTATION OF THE MAIN CONSTITUENTS OF LINSEED OIL FOLLOWING THERMAL POLYMERIZATION, SOLVENT SEGREGATION AND ESTERIFICATION.



APPENDIX

Distillation of ethyl esters of the acetone-soluble fraction from polymerized linseed oil

The distillation of the esters was carried out using simple distilling bulbs and receivers and a glycerol bath. The distilling bulb was filled with clean glass wool to reduce bumping and frothing. Distillation temperature limits were established on esters of alkali-refined whole linseed oil, and these were observed closely in all distillations of esters from polymerized oils. The distillation pressures were of the order of 0.5 mm Hg. The distillable esters usually distilled completely between 155° and 175°C. When the vapor temperature had passed its maximum, the bath temperature was taken steadily up to 240°C. If no further rise in vapor temperature occurred, the bath was removed and the residue allowed to cool rapidly in the bulb without breaking the vacuum.

Demonstration of the presence of intrapolymers in the acetone-soluble fraction of polymerized linseed oil

A series of linseed oils were polymerized in a current of CO_2 for varying periods at 275°C. The methyl esters of their acetone-soluble segregates were prepared by interesterification. Cryoscopic determinations of mean molecular weight were carried out on the acetone-soluble fractions themselves and on the methyl ester fractions. The cyclohexane (British Drug Houses) used as cryoscopic solvent was carefully purified by successive treatment with fuming sulfuric acid and barium hydroxide, followed by distillation in a Stedman column. The purified solvent had M.P. 6.4°C., refractive index (25°C.) = 1.4237, values which are close to those recorded for very pure cyclohexane (Glasgow et al., '46). The observed mean molecular weights were corrected for the presence of free fatty acids by the method of Bernstein ('48), which assigns to the free fatty acids a dimeric average mean molecular weight of 558; i.e., assumes that association of the free fatty acids is practically complete. From the cryoscopic mean molecular weights of the acetone-soluble fraction, and assuming the presence of monomeric and dimeric glycerides of theoretical mean molecular weights of 878 and 1756, it was possible to calculate the percentages of monomeric and dimeric glycerides. From these figures, and assuming that polymerization of fatty acid radicals had not proceeded beyond the dimeric stage (Privett et al., '47), a theoretical yield of monomeric and dimeric esters could be calculated. The difference between these values and the observed

values for monomeric and dimeric esters obtained by distillation may be reasonably ascribed to the presence of intrapolymers, which would behave as monomeric glycerides in the mean molecular weight determinations. The percentage differences can be converted to a triglyceride basis by multiplying by 3/2. The results are set out in appendix table 1, and provide support for the belief that the acetone-soluble fractions of heated linseed oil contain considerable amounts of dimeric fatty acid in the form of intrapolymers.

Even after 13 hours of heating, the acetone-soluble fraction contained approximately 30% of its total fatty acids in the form of dimeric acids, but only approximately 12% could be accounted for as dimeric glyceride and some 19% of dimeric acid was probably present as intrapolymers. The amount of intrapolymers (soluble in acetone) would be expected to decline as polymerization progresses. It seems quite clear from these results that solvent segregation methods are unlikely to be effective in differentiating between intrapolymers and true monomeric glycerides.

Superiority of propanol over acetone in segregating a non-polymeric glyceride fraction from thermally polymerized linseed oil

The analytical data in table 2 show that both the viscosity and the refractive index of the propanol-soluble fraction were lower than those of the acetone-soluble fraction. The data confirm that propanol is the more effective solvent in segregating a non-polymeric fraction from thermally polymerized linseed oil.

Effectiveness of vacuum distillation in separating monomeric from dimeric ethyl esters of the acetone-soluble segregate of polymerized linseed oil

The analytical data presented in appendix table 3 relate to two samples of methyl esters prepared from acetone-soluble fractions of heated linseed oil. The data show that the "distillable" fraction consisted essentially of methyl esters of monomeric fatty acids, the mean molecular weights being 296 and 295. The "non-distillable" fraction consisted essentially of methyl esters of dimeric fatty acids, the mean molecular weights being 587 and 596.

The fact that refractive indices and iodine values for the recombined samples were practically the same as the values for the charge before distillation is evidence that the heating during distillation had little, if any, effect on the constitution of the esters.

APPENDIX TABLE 1

Calculation of the intrapolymer content of the acetone-soluble fractions of thermally polymerized linseed oil

HOURS HEATED	MEAN MOLECULAR WEIGHT OF GLYCERIDES OF HEATED OIL BY CRYOSCOPY	CALC. % DIMERIC GLYCERIDE	CALC. % DIMERIC METHYL ESTERS	OBSERVED % DIMERIC METHYL ESTER BY DISTILLATION	DIFFERENCE TO BE ASCRIBED TO INTRA-POLYMERS
5	1,010	36.1	8.7	21.7	13.0
9	1,030	29.6	9.7	23.1	13.2
13	1,060	34.4	11.5	30.7	19.2
17	970	19.0	6.3	14.8	8.5

APPENDIX TABLE 2

Analyses of acetone and propanol fractions from heated alkali-refined linseed oil

FRACTION	YIELD	REF. IND. 25°C.	VISCOSITY AT 250°C. CP.	IODINE VALUE
	%			
1. Unheated, recovered from acetone	. .	1.4789	50	183
2. Heated, acetone-soluble	45	1.4811	180	134
3. Heated, propanol-soluble	28	1.4791	127	135
4. Heated, propanol-insoluble	72	1.4851	890	121

APPENDIX TABLE 3

Properties of methyl esters and fractions obtained in distillation

SAMPLE	FRACTION	PER CENT YIELD	MEAN MOLECULAR WEIGHT	REF. IND. AT 250°C.	IODINE VALUE
1	Charge (acetone-soluble)	. .	334	1.4618	128.0
	Distillate	82.0	296	1.4600	137.2
	Residue	17.5	587	1.4896	105.5
	Recombined sample [1]	. . .	. . .	1.4647	127.6
2	Charge (acetone-soluble)	. . .	336	1.4647	129.0
	Distillate	81.9	295	1.4600	135.6
	Residue	17.6	598	1.4904	102.8
	Recombined sample [1]	. . .	. . .	1.4647	128.4

[1] Distillate and residue blended in the exact residue of their yields, to approximate original sample.

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STUDIES TO DETERMINE THE NATURE OF THE DAMAGE TO THE NUTRITIVE VALUE OF SOME VEGETABLE OILS FROM HEAT TREATMENT [1, 2]

III. THE SEGREGATION OF TOXIC AND NON-TOXIC MATERIAL FROM THE ESTERS OF HEAT-POLYMERIZED LINSEED OIL BY DISTILLATION AND BY UREA ADDUCT FORMATION

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ONE FIGURE

(Received for publication July 28, 1952)

INTRODUCTION

The deleterious effects of heating certain vegetable oils at polymerization temperatures on their nutritional value have been described in a previous paper (Crampton et al., '51c). There is some evidence (Crampton et al., '51a) that these deleterious effects are not a consequence of peroxidation. It has also been shown (Crampton et al., '51b) that the deleterious effects may be demonstrated by feeding the ethyl esters of heated linseed oil.

When the whole esters of heated linseed oil were separated by distillation at pressures of the order of 0.5 mm into a distillable fraction and a non-distillable fraction, then the non-distillable fraction was deleterious to experimental ani-

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[2] Issued as paper 277, Canadian Committee on Food Preservation.

mals. Rats receiving this fraction at the level of 20% of the diet failed to grow; had oily, matted coats; excreted dark, sticky feces; and displayed a heavy mortality. Rats receiving the distillable fraction survived the experiment in good condition and excreted well-formed feces. However, they did not grow as well as rats receiving the whole esters of unheated linseed oil. In fact, their growth was no better than that of rats receiving the whole esters of heated oil. At this stage it seemed possible that the major defect of the heated oil arose from the presence of dimeric acid radicals. However, the distillable monomeric ester fraction was also nutritionally inferior to the esters of whole unheated linseed oil. This fact could not be related to differences in susceptibility to peroxidation, for the esters of the whole unheated oil were considerably more susceptible to peroxidation than the distillable esters of the heated oil (unpublished results).

It seemed possible, therefore, that the monomeric ester fraction might contain material other than straight chain esters, and that such material might play a part in determining the deleterious nature of the heated oils.

The formation of such materials by the exposure of the oils to polymerization temperatures carries implications of some importance so far as oils intended for food are concerned. Accordingly, the preliminary work was repeated and extended, and this paper deals with these later experiments.

EXPERIMENTAL

The nutritional test was performed in two replicates, each comprising observations on 60 animals. Six diets were tested. These diets differed only in (a) the nature of the oil component and (b) the proportion of white flour as adjusted to compensate for the use of 10 or 20% levels of oil.

The formula for the diets, in per cent, was as follows: white flour 53.65 or 43.65; skim milk powder 19; casein 11.5; oil preparation 10 or 20; brewers' dried yeast 3; feeding bone-

meal 2; iodized salt 0.5; chromium sesquioxide[3] 0.25; and ferric citrate 0.1.

The oil preparations were certain fractions of the ethyl esters of heat-polymerized linseed oil (12 hours at 275°C. in a strong current of carbon dioxide). The preparation of these fractions is described in detail in the Appendix. They will be designated in this paper for convenience as follows:

1. *Straight chain monomers from whole oil:* that portion of the ethyl esters of heated linseed oil which formed urea adducts with ease.
2. *"Cyclized" monomers and dimers from whole oil:* that portion of the esters of heated oil which did *not* form urea adducts.
3. *Straight chain plus "cyclized" monomers from distillate:* that portion of the esters of heated linseed oil which was distillable at pressures around 0.5 mm.
4. *Straight chain monomers from distillate:* that portion of the distillable esters which formed urea adducts.
5. *"Cyclized" monomers from distillate:* that portion of the distillable esters which did *not* form urea adducts.
6. *Dimers by distillation:* the residue after removal of the monomeric esters by distillation.

The probable general chemical relationships of these 6 fractions are conveniently indicated by a flow sheet (fig. 1). It is to be understood that the designations of the fractions and the symbols used in the flow sheet do not imply that the nature of the fractions is established. While it is reasonably certain that fraction 6 consists mainly of esters of dimeric acids (see table 5) it cannot be considered as finally established whether these are linked by single carbon-to-carbon bonds or by a cyclohexene system. Again, whether the non-adduct-forming monomeric material of fractions 2, 3 and 5 is cyclized or branched is not established: the term "cyclized" must be read as denoting a mode of preparation and not as asserting the presence of a cyclic structure. The chemical characteristics of the fractions are reported in table 5: the

[3] For determination of digestibility.

mean molecular weights confirm the validity of the designations so far as the terms monomeric and dimeric are concerned, but do not provide evidence as to the presence or otherwise of cyclic structures. It may be recalled, however, that Paschke and Wheeler ('49) suggested the presence of cyclic monomers in heat-polymerized methyl linoleate.

The hydroxyl contents of the fractions were determined by the method of Ogg, Porter and Willits ('45). The data

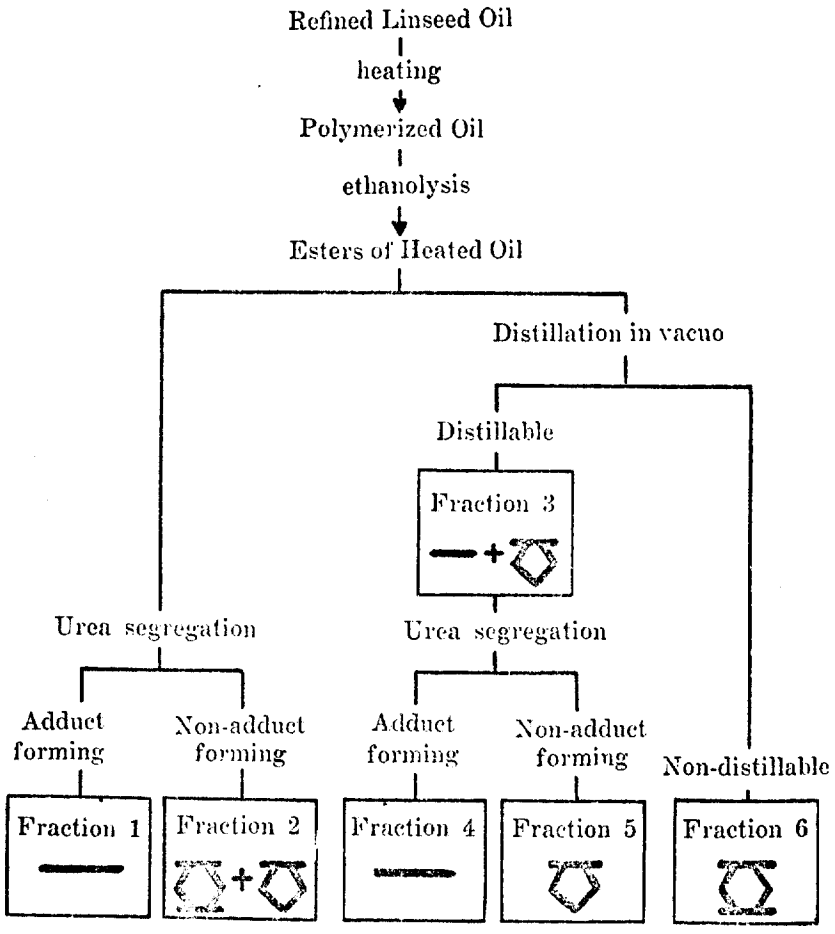

Fig. 1 Flow sheet illustrating preparation of fractions of esters of heated linseed oil used in feeding trials. (For details of preparation, see Appendix.)

are reported in table 5. So far as the monomeric fractions 1, 4 and 5 are concerned, the values correspond to not more than 8, 5 and 15%, respectively, of monohydroxy C18 acids. The available evidence therefore supports the view that hydroxy acids did not constitute a major proportion of the non-adduct-forming monomeric material.

The feeding trials lasted 28 days. Live weight changes and feed consumption were recorded. The apparent digestibilities were determined by the Cr_2O_3 index technique (Schürch et al., '50). Digestible calories were calculated from the gross calorific values of the diets, the feed intake and the simultaneously determined digestibility coefficients. Live

TABLE 1

Comparison of the effect of 10% versus 20% of fractions of the esters of heat-polymerized vegetable oil in the diet of rats (60 rats at each level)

OIL IN DIET	RATS SURVIVING 28 DAYS OF TEST	AVERAGE GAIN IN 28 DAYS	AVERAGE FEED IN 28 DAYS	DIGESTIBILITY OF FEED	GAIN ADJUSTED TO EQUAL INTAKE OF DIGEST. CALORIES
%	%	gm	gm	%	gm
10	83	73	262	91	67
20	63	27	177	83	52
Least sig. diff. (P = 0.05)	..	11	17	..	8

weight gains were adjusted by simple regression to equal intake of digestible calories to provide a measure of the relative efficiency of the diets. Least significant differences between means, where shown, were calculated from the expression

$$\text{L.S.D.} = \frac{\sigma}{\sqrt{n}} \times \sqrt{2} \times t_{0.05}.$$

In the preliminary tests, the use of diets containing 20% of certain ester fractions similar to those used in the present experiments had resulted in heavy mortality. Consequently, half of the rats in each of the lots in the tests now reported received a diet containing 10% oil. The differences between the responses of the rats receiving these two levels of oil are summarized in table 1.

Efficacy of protection of the fractions and diets against autoxidation

Since peroxidized fats and fatty acid esters may be toxic to rats, it is necessary to ensure that the results of feeding such materials are not vitiated by the effects of peroxidation. It has been shown that the deleterious effects of heated linseed oil are not related to the degree of susceptibility of the heated oils or of the diets containing the oils to peroxidation (Crampton et al., '51a). In the present experiments the fractions used in the diets were prepared immediately

TABLE 2

Peroxide values of oils extracted from the mixed feeds at two weeks and 4 weeks after preparation. Feeds stored in refrigerator

FRACTION CONTAINED IN THE DIET	PEROXIDE VALUE, MG PEROXIDE OXYGEN PER KG OIL	
	After 2 weeks	After 4 weeks
Fraction 1	44	98
Fraction 2	38	88
Fraction 3	42	90
Fraction 4	46	102
Fraction 5	38	92
Fraction 6	33	75

before feeding, and were protected by addition of an antioxidant and storage in the refrigerator in the dark. The stability of the oils in the diets was tested at the end of the second week of the second replicate and again at the end of the 4th week. Peroxide values of the lipid components of the feed were determined by the method of Skellon and Wills ('48) as described by Crampton et al. ('51a). Peroxidation was relatively slight in all fractions (table 2). From unpublished observations it is believed that peroxidation would not have begun to interfere with the nutritional well-being of the rats until the peroxide value of the oil extracted from the feed attained values of the order of 1,000 mg per kilogram of oil.

It may be concluded, therefore, that the results of the present feeding experiments were due to the effects of constituents other than products of peroxidation.

RESULTS

With diets containing 20% oil

The results of feeding these 6 oil fractions in diets in which they constituted 20% by weight are summarized in table 3. The first salient feature of the results relates to the *mortality*. It is clear that the presence of the "cyclized" monomers in the diet was associated with toxicity, though the mode of action of this material remains obscure. Rats consuming fraction 5, which contained the highest concentration of this material, survived only 5 to 10 days in spite of a greatly reduced feed intake (and hence low intake of toxic material).

A comparison of the results for diet 2, containing a mixture of approximately three parts dimeric esters to one part "cyclized" monomer, with the results for diet 6, shows clearly the much more harmful nature of the monomeric material. The deaths in group 6 were associated with partial starvation and excessive diarrhoea, whereas the animals of group 2 displayed neither diarrhoea, digestive disturbances, nor overt clinical symptoms suggestive of the cause of death.

The animals receiving fractions consisting mainly of straight chain monomers — i.e., groups 1 and 4 — all survived the test and remained in good health. Their gains were comparable to those secured in previous tests with esters of whole unheated linseed oil.

The *gains* of the rats receiving the various fractions also reflected the toxic effects of the "cyclized" monomeric material and the adverse effects of the dimeric esters. The dimeric ester fraction was associated with low digestibility, and this was doubtless the cause of the diarrhetic condition of the rats receiving this material.

The *relative efficiencies* of the diets *per unit of digestible calories eaten* showed that the straight chain monomeric

TABLE 3

The effect of feeding fractions of polymerized linseed oil at 20% level in rat diets (10 rats per lot)

OIL FRACTION FED	PRINCIPAL OIL CONSTITUENTS	NO. OF ANIMALS SURVIVING TEST	AVERAGE GAIN	AVERAGE FEED INTAKE (dry matter)	DIG. OF DRY MATTER	DIG. OF FAT FRACTION	MEAN GAINS ADJUSTED TO EQUAL INTAKE OF DIG. CAL.
			gm	*gm*	*%*	*%*	*gm*
1	Straight chain monomers of whole oil	10	101	266	93	94	72
2	"Cyclized" monomers plus dimers of whole oil	2	—32	141	81	77	4
3	Straight chain plus "cyclized" monomers of distillate	9	4	118	94	97	52
4	Straight chain monomers of distillate	10	57	193	94	95	63
5	"Cyclized" monomers of distillate	0	..	..	..	..	..
6	Dimers	7	13	168	55	29	71

esters and the dimeric esters were equally well "metabolized," whereas the presence of "cyclized" monomeric material resulted in a sharp reduction in the utilization of digestible calories.

The *low feed intake* by group 5 made it possible that this might have caused the death of some of the rats receiving the "cyclized" monomers. This point was examined by feeding a supplementary group of 5 rats the basal oil-free diet ad libitum. They were dosed daily by dropper with a quantity of "cyclized" monomer equal to 10% by weight of their measured voluntary intake of the fat-free mixture. The intake of the basal diet under these conditions was low, but it was adequate for maintenance and in one case permitted some gain. Nonetheless, all of these rats died within 16 days, thus confirming that the mortality in group 5 could not be explained on the grounds of low food intake.

It will be noted from the schematic outline (fig. 1) of the preparation of the fractions that fractions 1 and 4 ought to consist mainly of the straight chain adduct-forming material. However, fraction 1 had a lower iodine number and a lower refractive index than fraction 4 (table 5). Moreover, the yield of fraction 1 represented 46%, whereas that of fraction 4 was 49%, of the total esters. These differences in yield and in the characteristics of presumptive straight chain monomeric material according to the route by which it has been prepared, suggest that in the case of fraction 4 the adduct-forming fraction had probably carried with it by entrainment a small proportion of material incapable of itself of forming an adduct; i.e., of "cyclized" monomer.

Fraction 1 was also superior to fraction 4 in nutritional value. This circumstance reinforces the view that fraction 4 contained a small proportion of the non-adduct-forming material ("cyclized" monomer), and that the "cyclized" monomeric ester is either of itself the deleterious component or that the deleterious material accompanies this ester fraction.

TABLE 4

The effect of feeding fractions of polymerized linseed oil at 10% level in rat diets (10 rats per lot)

OIL FRACTION FED	PRINCIPAL OIL CONSTITUENTS	NO. OF ANIMALS SURVIVING TEST	AVERAGE GAIN	AVERAGE FEED INTAKE (dry matter)	DIG. OF DRY MATTER	DIG. OF FAT FRACTION	MEAN GAINS ADJUSTED TO EQUAL INTAKE OF DIG. CAL.
			gm.	*gm.*	%	%	*gm.*
1	Straight chain monomers of whole oil	10	107	300	95	94	72
2	"Cyclized" monomers plus dimers of whole oil	10	39	205	87	73	58
3	Straight chain plus "cyclized" monomers of distillate	10	66	228	94	96	66
4	Straight chain monomers of distillate	10	108	299	94	95	75
5	"Cyclized" monomers of distillate	0	..	..	91	93	..
6	Dimers	10	77	276	87	69	64
Least significant difference (P = 0.05)		..	23	36	..	..	16

With diets containing 10% fat

The results of feeding the diets in which the fat levels were reduced to 10% are shown in table 4. They were in all respects what would have been predicted from the results of feeding the 20% fat levels. The toxic effect of the "cyclized" monomers had been lessened, though it was still clearly in evidence. Gains, feed intake, digestibility and efficiency of utilization from the straight chain monomers were practically identical as between rats in lot 1 with 20% fat and those of lots 1 and 5 with 10% fat. Even at this lower fat level none of the rats receiving the "cyclized" monomeric material (lot 5) survived 28 days; where this "cyclized" monomeric material constituted only 2.5% of the diet intake (lots 2 and 3), the rats were able to complete the test, but with significantly smaller gains.

CONCLUSIONS

1. Heat polymerization of alkali-refined linseed oil results in the formation of monomeric acyl radicals whose esters can be distilled at low pressures but which do not form urea adducts. These esters are highly digestible when eaten but are not utilized for growth. The esters contain a small proportion of hydroxyl groups, but are apparently mainly esters of "cyclized" or branched structure.

2. When a fraction consisting essentially of these esters of "cyclized" monomeric acids comprises as much as 10% of the diet, it renders the diet "toxic." The harmful effect is measurable when this fraction comprises 2.5% of the diet. The effect is ascribed tentatively to the "cyclized" monomeric esters as such, rather than to the small proportion of hydroxy acid present in this and other fractions.

3. Dimeric acyl radicals are also formed on heating linseed oil at polymerization temperatures. A fraction consisting mainly of the esters of the dimeric acids is non-toxic, but this material is largely unabsorbed from the gastrointestinal tract. Its presence results in excreta of the nature and consistency of a mixture of soft feces and fresh varnish.

4. Esters of straight chain monomeric acids (i.e., capable of forming urea adducts) are apparently as nutritious as are esters prepared directly from normal unheated triglyceride oils which, in turn, are but slightly inferior to the triglycerides of normal food.

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APPENDIX

Preparation of ethyl esters of polymerized linseed oil

Commercial alkali-refined linseed oil (Canada Linseed Oil Mills, Montreal, P.Q.) was heated in a stream of CO_2 for 12 hours at 275°C. The oil was heated in batches of about 1,200 gm at a time, and the CO_2 was passed at a rate sufficient to keep the oil surging fairly freely. The resultant polymerized oil was of a clear light straw color.

The polymerized oil was allowed to cool while maintaining the current of CO_2. The polymerized oil was then shaken up with one-third of its volume of absolute ethanol to remove any fatty acids present. The washings were discarded.

The washed polymerized oil was used for the preparation of the ethyl esters by alcoholysis, using caustic soda at the rate of 0.5% by weight of the oil as catalyst (Feuge and Gros, '49). The esters were washed with large volumes of hot distilled water in a large separatory funnel. The total yield of mixed esters reckoned on the weight of oil taken was approximately 90% (varying from 88 to 92 with different batches).

Fractionation of the ethyl esters for the feeding trials

A portion of the total ethyl esters was subjected to vacuum distillation as described by Crampton et al. ('51b). The non-distillable fraction, comprising the ethyl esters of dimeric fatty acids, constituted fraction 6. The distillable portion, comprising the ethyl esters of monomeric acids, constituted fraction 3.

Immediately after distillation Tenox II * (Tennessee Eastman Corp.) was added to the portions of fractions 3 and 6 reserved for feeding at the rate of 0.1%. The protected ester fractions were at once removed to the cold room (5°C.), and held there until incorporated in the diets.

To another portion of fraction 3, Tenox II was added at the rate of 0.025%. This portion was subjected to fractionation by urea adduct formation (Schlenk, '49). The following technique was used.

Five hundred grams of the esters were dissolved in 2 l absolute commercial ethanol in a 4-l beaker. Solid urea was added slowly with stirring, while maintaining the mixture at 50°C. In all 2 kg urea were added, since approximately 3 gm urea are required for each gram of C18 ester for adduct formation. The mixture was maintained at 50°C. for about 30 minutes and then allowed to stand at room temperature overnight. Next morning the adduct and excess urea were filtered off and washed on the Buchner funnel with absolute ethanol saturated with urea. The adduct was decomposed by being thrown into warm water. Immediately the oily layer separated, Tenox II was added at the rate of approximately 0.25% of the adduct-forming material. The oily material was washed with water and dried under high vacuum with shaking. This material constituted fraction 4, and presumably consisted mainly of straight chain monomeric esters (see table 5). The non-adduct-forming material in the supernatant and ethanolic washings was separated by the addition of copious amounts of water. No Tenox

* Tenox II is a preparation containing 20% butylated hydroxyanisole, 4% citric acid, 6% propyl gallate and 70% propylene glycol.

II was added, since presumably that present before adduct formation would tend to be concentrated in this fraction, which constituted fraction 5. The addition of small quantities of sodium chloride facilitated the separation of both fractions 4 and 5 from the aqueous phase. Fraction 5 was dried in the same fashion as was fraction 4. Both fractions were now further protected against autoxidation by the further addition of 0.1% Tenox II and stored under the same conditions as fractions 3 and 6.

Another portion of the total esters was separated into adduct-forming (fraction 1) and non-adduct-forming fractions (fraction 2) by the technique described above, except that the total weights of the esters and ethanol were correspondingly reduced, since the total esters of heated oil contained a much smaller proportion of straight chain monomeric material than did the distillable esters (fraction 3). The same precautions were taken against peroxidation as when preparing fractions 4 and 5.

The analytical characteristics of the fractions are presented in table 5.

TABLE 5

Yields and characteristics of fractions of ethyl esters used in the feeding trials

FRACTION NUMBER	YIELD AS % TOTAL ESTERS OF HEATED LINSEED OIL	IODINE NO.[1]	REFRACTIVE INDEX n_D^{25}	MEAN MOLECULAR WEIGHT	HYDROXYL[2] VALUE AS PER CENT (— OH)
1 Adduct-forming fraction of total esters	46	118.2	1.45345	293	0.23
2 Non-adduct-forming fraction of total esters	54	162.7	1.47561	472	0.46
3 Distillable esters	60	130.1	1.45684	294	0.22
4 Adduct-forming fraction of distillable esters	49	124.8	1.45494	293	0.14
5 Non-adduct-forming fraction of distillable esters	11	170.7	1.46986	300	0.39
6 Non-distillable esters	40	159.9	1.48017	550	0.64

[1] Iodine numbers by the method of Benham and Klee ('50), using one-hour reaction time (Klee and Benham, '50).

[2] Hydroxyl values by the method of Ogg, Porter and Willits ('45).

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THE EFFECT OF HEAT TREATMENT ON THE NUTRITIONAL VALUE OF SOME VEGETABLE OILS

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INTRODUCTION

Oil-seed crops are becoming increasingly important to Canadian economy. While the meal plays a significant role in livestock feeding, the oils pressed from seeds have been adapted to many uses. Of current concern is the manufacture of vegetable oils into edible products such as margarine and salad oils.

Because certain of the more highly unsaturated vegetable oils develop undesirable odors and flavors on exposure to heat or light, their use as edible products was hindered. In 1945 Privett and his co-workers showed thermal polymerization to be a simple and yet satisfactory method for rendering such vegetable oils less susceptible to this "flavor reversion." Polymerization has reportedly been used in the processing of herring oil for human consumption in the United States (Lassen et al., '49), and in Norway (Schwitzer, '48) to replace olive, cotton seed and groundnut oils with herring oil for canning, cooking and to some extent as a table and salad oil.

However, when linseed oil prepared by the method of Privett et al. ('45) was fed in 28-day tests to rats in this laboratory (Crampton and Millar, '46) a high incidence of death occurred.

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Because unheated linseed oil is known to be wholesome and nutritious (Molotkow, '32), it was suspected that heat polymerization had somehow deleteriously affected the nutritional value of that oil.

Reports in the literature of adverse affects on fats of severe heat are scanty. Roffo ('44) found that rats fed sunflower seed and olive oils heated to 350°C. for 30 minutes developed stomach tumors which became cancerous over a period of 12 to 30 months. Although no other published reports confirm these observations, Morris et al. ('43) noted growth failure and weight loss in rats fed diets containing 50% lard which had been heated at 300°C. for 120 minutes. Accompanying these effects on growth, Morris observed in some animals a paralysis similar to that produced in rats fed a vitamin E-deficient diet by Mackenzie et al. ('40); also some cirrhosis of the liver and chronic gastric ulcers. However, both these groups of workers used fat heated in air, where oxidation may have been an important factor, whereas Privett's polymerization is conducted in an atmosphere of inert gas.

In 1947, Harris fed rats fish oils heated at 280°C. under a vacuum of 10 mm of mercury for 8 to 12 hours and observed symptoms of retarded growth and general ill-health.

Subsequent to our original findings, a series of experiments was conducted, the most pertinent of which are reported herein, to investigate further the influence of heat on the nutritiousness of herring oil, linseed oil and other vegetable oils which are potentially important to the food industry.

EXPERIMENTAL PROCEDURE

The general plan of each of the tests in this study involved feeding to young growing rats basal diets in which one or the other of the several fats, subjected to heats of varying duration, was incorporated at levels of 10% or 20% by weight. Fats examined in the tests reported include linseed, soybean, rapeseed, corn, peanut, and herring oils.

The vegetable oils were heated in a salt bath at 275°C. for the desired time in all-glass apparatus. Carbon dioxide was blown through the oil continuously to exclude air and to remove volatile decomposition products thoroughly. The herring oil was a caustic soda-refined, bleached, deodorized product of a process reportedly used in Norway (Schwitzer, '48), involving heating while passing through steam at 280°C. under a vacuum of 10 mm of mercury for 10 hours.

The effect of the heat treatment on the nutritive value of the oils was measured by the growth rates of the rats, their feed consumption, and their general conditions of health and thrift as compared to those of animals fed identical basal diets but containing the unheated oil. Post mortem examination of the viscera was performed on all rats failing to survive the planned feeding period and on representatives of the groups at the close of the test.

Equal numbers of male and female white rats of approximately 28 days of age were allotted at random within sexes to the test groups, which ranged in size from 6 to 16 animals per lot. Thus each lot contained equal numbers of male and female animals. They were housed in individual wire-bottom cages, and were allowed feed and water ad libitum throughout each 28-day test period. Weekly weight gain and feed consumption for each rat were recorded.

Diets

The percentage compositions of the three basal diets used are shown in table 1.

It will be noted that diets I and III differed only in the level of fat, which varied by weight at the expense of the flour. Diet II was a modification of diet I through the addition of yeast in place of the thiamine, riboflavin and niacin. The level of protein in the basal diets was such that it supplied between 20% and 24% of the Calories.

In preparation for feeding, the oils were added to the thoroughly mixed dry ingredients and the whole baked at 375°F.

for approximately 20 minutes. The resulting biscuit was then granulated and air-dried for 24 hours, after which the supplements were added. Diets were refrigerator-stored until the beginning of the test period, during which time they were held at room temperature.

In addition to the diet, weekly doses of cod liver oil were administered orally to supply 175 I.U. of vitamin A and 35 I.U. of vitamin D.

TABLE 1

Percentage compostion of the basal diets

INGREDIENTS	I	II	III
White flour	47.0	44.0	57.0
Casein	11.5	11.5	11.5
Milk powder	19.0	19.0	19.0
Oil	20.0	20.0	10.0
Bone meal	2.0	2.0	2.0
Salt	0.5	0.5	0.5
Yeast [1]	..	3.0	..
Thiamine [1]	10 p.p.m.	..	10 p.p.m.
Niacin [1]	230 p.p.m.	..	230 p.p.m.
Riboflavin [1]	5 p.p.m.	..	5 p.p.m.
Protein in the diet (%)	20.4	20.4	24.3

[1] Added after baking.

RESULTS OF FEEDING TRIALS

Clinical observations

Symptoms of defective diet were most pronounced in those animals fed polymerized linseed oil, and the severity of the symptoms varied with the amount of oil incorporated in the diet. However, animals fed any of the polymerized oils, and whose weight gains were significantly lower than those of their controls, excreted dark, sticky feces. Lassen et al. ('49) noted abnormal feces from adult rats fed polymerized sardine oil as 5% of the diet. In our tests, in addition to the sticky fecal residue, animals fed polymerized oil as 20% of the diet showed oily, matted coats.

Growth and feed consumption

In examination of the results which follow it must be understood that the data presented were collected from a series of individual tests. Control groups of animals fed the appropriate unheated oils were carried in each experiment, and quantitative comparisons of the data shown in these tables are strictly valid only within tests.

TABLE 2

Summary of data showing the effects of heat treatment of oils as measured by daily gains of rats, feed intakes, and gains per 1,000 Calories eaten

TEST AND OIL USED	DIET NO.	OIL IN DIET	DURATION OF HEAT TREATMENT AT 275°C.	NO. OF ANIMALS	AVE. DAILY GAIN	AVE. DAILY FEED INTAKE	GAIN/ 1,000 CALORIES
		%	*hrs.*		*gm*	*gm*	*gm*
Linseed	III	10	nil	10	3.9	9.6	91
		10	2	10	3.8	9.6	91
		10	4	10	3.4[1]	9.5	80
		10	8	10	2.5[2]	8.1	69
Rapeseed	III	10	nil	10	3.4	10.0	76
		10	15	10	2.9	9.5	69
		10	30	10	2.3[1]	7.8	64
Corn	III	10	nil	12	3.6	10.4	78
		10	15	12	2.8	8.7	71
		10	30	12	2.3[1]	8.0	64
Peanut	III	10	nil	12	3.6	9.3	87
		10	15	12	3.6	10.0	80
		10	30	12	0.8[1]	5.2	30
Soybean	II	20	nil	10	4.6	10.2	90
		20	3	10	4.0	9.4	85
		20	6	10	3.3[1]	8.3	80
		20	9	10	2.9[1]	7.7	76
Herring	I	20	nil	16	2.6	8.1	64
		20	10	16	0.89[1]	6.3	28

[1] Weight gains significantly lower than those of control animals fed the unheated oil.

[2] Weight gains significantly lower than those of animals fed linseed oil heated only 4 hours.

In table 2 is presented a summary of the average daily gains and feed intakes of animals fed in 6 different tests covering a series of potentially edible oils.

It should be noted that the duration of heating stated is not necessarily the earliest point at which the nutritional value of the oil is damaged. Our experiments, for example, have shown linseed oil heated two hours at polymerizing temperatures, and soybean oil heated three hours, to be as nutritious as the unheated control oils. The intermediate periods between 15 and 30 hours for rapeseed, corn, and peanut oils have not been tested, nor the periods up to 10 hours with herring oil.

TABLE 3

Effect of 10% vs. 20% of heated oil in the diets as measured by daily gains and feed intake ratios of rats

TEST AND OIL FED	OIL IN DIET	DURATION OF HEAT TREATMENT	NO. OF ANIMALS	AVE. DAILY GAIN	AVE. DAILY FEED INTAKE	GAIN/1,000 CALORIES
	%	*hrs.*		*gm*	*gm*	*gm*
Linseed	20	nil	10	3.7	10.1	80
	10	12	10	2.3 [1]	8.2	72
	20	12	10	—0.1 [2]	6.0	4
Soybean	10	nil	10	3.7	10.3	92
	10	6	10	3.5	10.4	87
Soybean	20	nil	10	4.6	10.2	90
	20	6	10	3.3 [1]	8.3	76

[1] Weight gain significantly lower than that of group fed unheated oil.

[2] Weight gain significantly inferior to that of group fed heated oil as 10% of the diet.

That the duration of heating is a factor in the extent of the damage in linseed and soybean oils is clearly indicated by the progressive decrease in weight gains as the heating period was prolonged.

The data presented in table 3 indicate that the diet becomes progressively poorer as the proportion of heated oil in it increases.

When linseed oil, heated 12 hours, was fed as 10% of the diet, the growth resulting over a 4-week period was significantly lower than that of animals fed unheated oil. When the heated oil was included in the diet as 20% by weight, the final

weight of the group was even lower than the starting weight, showing extreme growth inhibition.

When soybean oil was fed as 10% of the diet, oil heated 6 hours appeared as nutritious as the unheated oil. However, when oil treated in the same manner was fed as 20% of the diet, significantly lower gains resulted.

While the results from this series of trials leave no doubt that heat polymerization does adversely affect the nutritive value of the oils examined, the mechanism of this damage was not elucidated. Digestion studies rule out incomplete absorption as a causal factor. It can be postulated that growth inhibition was either the result of some factor in the heated oil toxic to the animals, or that it was a consequence of the destruction by abnormal products of fat polymerization of some necessary dietary component other than fat. To differentiate between these two possibilities as a guide to further investigations, the effect of feeding polymerized linseed oil by dropper was compared to that of feeding the oil mixed into the diet.

Animals fed the oil by dropper were given free access for 16 hours daily to diet III minus the fat. Subsequently, and following three hours of fasting, an amount of oil was administered equal to 10% of the fat-free diet consumed during the preceding 16-hour feeding period. Five hours then elapsed before access to the diet was again permitted.

The results of a 28-day feeding trial conducted in this manner are summarized in table 4.

The method of oil feeding had no effect on the growth of animals receiving unheated oil. However, the growth of animals fed the heated oil by dropper appeared superior to that of animals receiving the heated oil as part of the diet mixture, perhaps because the oil-free diet was more acceptable than the mixture containing the heated fat. However, comparison of gains made in relation to equal caloric intake shows that feed utilization was equally poor in the diets in which heated oil was included, regardless of the method of oil feeding.

We are not prepared at this point to explain why appetite was depressed only when the heated oil was a diet component. However, the observation that growth was depressed even when the oil was fed apart from the rest of the diet indicates that the deleterious effects exerted by polymerized oils were not incurred through the destruction of some essential non-lipid diet component, but through some oil constituent that acted in a toxic manner directly upon the animals' metabolic pools. This is in accord with the previous observations that the growth depression was intensified as the level of polymerized oil in the diet was increased, and also as the time of heating was lengthened.

TABLE 4

Summary of daily gains, feed intake and gains per 1,000 Calories eaten of animals fed polymerized linseed oil by dropper and as a dietary component

TREATMENT OF OIL	METHOD OF OIL FEEDING	NO. OF ANIMALS	AVE. DAILY GAIN	AVE. DAILY FEED INTAKE	GAIN/1,000 CALORIES
			gm	*gm*	*gm*
Unheated	As a diet component	8	4.2	12.4	87
Unheated	by dropper	8	4.1	12.5	85
Heated 12 hrs.	As a diet component	8	2.7	9.7	71
Heated 12 hrs.	by dropper	8	3.4	12.4	71

It appears logical at this time, therefore, to conclude that the toxic material is some product of polymerization and that its development is related to the unsaturation of the oil. Linseed is the most highly unsaturated of all the oils tested and was the most rapidly susceptible to nutritive damage by heating.

A series of nutritional studies of various fractions of polymerized oils designed to identify the toxic substance or substances will be reported in a subsequent paper.

CONCLUSIONS

From the data reported herein, the following conclusions are warranted:

1. Ingestion of certain oils heat-polymerized at 275°C. has been shown to depress rat growth and the efficiency of feed utilization. This reflects a lowered nutritive value of those oils which varies in severity with the degree of unsaturation of the oil, the length of time of heating each oil, and the level at which the heated oil is incorporated in the diet.

2. The factor or factors responsible for these adverse nutritional effects appear to be present in the oil itself, and act in a manner directly toxic to the animal.

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FURTHER TESTS ON THE CARCINOGENICITY OF SORBIC ACID IN THE RAT

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IT was earlier reported (Dickens and Jones, 1963) that parasorbic acid, the naturally occurring lactone of 5-hydroxy-2-hexenoic acid, induced local sarcomas in 8 of 11 rats after its repeated subcutaneous injection in doses of 0·2–2·0 mg. This finding, which was quite consistent with our similar series of observations on the carcinogenicity of other $\alpha\beta$-unsaturated lactones, led us (Dickens, Jones and Waynforth, 1966) to investigate the effect of multiple injections into the rat of sorbic acid—an antifungal material very widely used as a preservative in the food industry and therefore one which could reasonably be required to be free of any suspicion of carcinogenic activity. Sorbic acid (trans-trans-2,4-hexadienoic acid) is chemically simply derived from parasorbic acid by acid or alkali treatment, which causes opening of the 6-membered lactone ring of parasorbic acid accompanied by the loss of the elements of water from the intermediately formed 5-hydroxy-2-hexenoic acid. Since the use for commercial production of sorbic acid of the catalytic condensation of crotonaldehyde and ketene (Hagemeyer, 1949), this material has been available cheaply and in quantity. Both the free acid and its salts, particularly the potassium and calcium salts which have also found wide use as food preservatives, are the subject of British Standards Institution specifications of purity (British Standard, 1967, No. 4234 and 4233 respectively). They are now used as food additives in some 14 countries and are variously added to cheese, fishpastes, "delicatessen", margarine, preserved egg, fruit pulp and jams, non-alcoholic drinks, wine, dried fruits, pickles, confectionery and bread (Lück, 1968). In the United Kingdom their use seems to be mainly restricted to cheese and confectionery. The F.A.O./W.H.O. Expert Committee (W.H.O., 1965; F.A.O., 1965) laid down an "unconditional zone of acceptability" of 0–12·5 mg. sorbic acid/kg. body wt. and a "conditional zone" of 12·5–25 mg./kg. body wt., which for a 70 kg. man might reach a daily dietary intake of 1·75 g. of sorbic acid.

In view of these facts, our finding of a tumorigenic effect of subcutaneous injection of sorbic acid into rats needed confirmation, particularly as it was based on a group of only 6 rats each of which received twice weekly for 65 weeks a subcutaneous injection of 2 mg. sorbic acid in 0·5 ml. arachis oil. Of the 5 survivors, local sarcomata developed in 4 at the injection site after 82 weeks and in the fifth rat after 102 weeks. These tumours were all histologically malignant, although one tumour which was judged non-malignant on histological grounds arose in this series in 1 of 6 control rats injected with the same oil solvent alone (Dickens *et al.*, 1966).

Our earlier tests also included a group of 6 rats which received 10 mg. sorbic acid/100 ml. neutralized with sodium bicarbonate in their drinking water for

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periods up to 64 weeks, but at the end of that time only one rat (which had ingested a total of 2 g. sorbic acid) survived and no tumours were observed in any of these rats (Dickens *et al.*, 1966). This survival was insufficient for a clear-cut result.

In the present paper we report the results of further series of similar tests using samples of sorbic acid or its potassium salt obtained from two different commercial sources.

EXPERIMENTAL

Materials

Sorbic acid: (*a*) From L. Light & Co. (now Koch-Light Laboratories Ltd.), Colnbrook, Bucks., England. This was the original sample used by Dickens *et al.* (1966) which had been kept in a closed container in our laboratory at room temperature since it was purchased in 1963.

(*b*) A freshly obtained preparation from Farbwerke Hoechst A.G., Frankfurt/Main, W. Germany. Both materials *a* and *b* were white crystalline preparations which satisfactorily passed the usual physical and chemical tests of identity and purity (see below).

Potassium sorbate: Again 2 samples were tested, specimen (*c*) from L. Light & Co. and specimen (*d*) from Hoechst A.G.

The potassium salt is freely soluble in cold water and therefore could be dissolved directly in water either for injection, or in the drinking water in those experiments in which it was given in this way. The free acid is only sparingly soluble in cold water and fine crystals slowly appeared in the aqueous solution used for injection purposes. Where arachis oil (B.P.) was used as solvent, the free acid was dissolved by warming for a few minutes under the hot-water tap (50° C.).

Animal experiments

Twice-weekly subcutaneous injections were made into the right flank of groups of either 6 or 12 male rats for a total period of 56 to 60 weeks. The solvent was water for both samples (*c* and *d*) of potassium sorbate and also for sorbic acid sample *a*; in each instance 2 mg. substance/0·5 ml. water at each injection. Sorbic acid (*b*) was injected at the same dosage but in arachis oil. In addition, 12 rats received 0·5 ml. arachis oil twice weekly as controls on the solvent.

For oral administration only potassium sorbate, sample *c*, was tested, 6 rats receiving 0·1 % added to all their food (ground-up cubes of standard rat cake diet no. 86, North Eastern Agricultural Co-operative Society, Aberdeen) while a further 6 rats had 0·3 % dissolved in their drinking water throughout the experiment. These animals underwent laparotomy under ether anaesthesia at the 65th week of experiment, when no liver tumours were detected, and continued on the same regime for up to 100 weeks or when all the animals had died.

At death or at the termination of all the experiments all animals were carefully examined post mortem, with histological examination where appropriate as described by Dickens and Jones (1961).

RESULTS

Injection experiments

Of the 12 control rats injected twice weekly for 57 weeks with 0·5 ml. oil alone, 9 survived more than a year, 7 for 1½ years, 5 for 95 weeks, and 3 for over 2 years (Table I). In none of these animals was any tumour detected, either locally or

TABLE I.—*Effects of Prolonged Administration of Sorbic Acid and Potassium Sorbate to Rats*

(All injections were of 2 mg. substance/0·5 ml. twice weekly for 56–60 weeks).

	Initial No. of rats	Total No. of tumours	Tumour rats/Survivors after (weeks):					
S.C. Injections			54	66	78	95	109	198
Sorbic acid (*a*) in water	6	2*	1/4	1/2	0/0	—	—	—
K Sorbate (*c*) in water	6	0	0/4	0/4	0/4	0/4	0/3†	—
Sorbic acid (*b*) in oil	12	0	0/10	0/7	0/4	0/4	—	0/2
K Sorbate (*d*) in water	12	0	0/9	0/8	0/6	0/3	—	0/2
Oil only (controls)	12	0	0/9	0/7	0/7	0/5	—	0/3
Oral Administration (for 60 weeks)								
K Sorbate (*c*) (0·1% in food)	6	0	0/6	0/3 (L)	0/1	0/0 (all dead)		
K Sorbate (*c*) (0·3% in drink)	6	0	0/5	0/5 (L)	0/4	0/3	0/3†	—

* Local fibrosarcomata at injection site in 2 rats of the 6.
† Some rats had small inflammatory liver lesions (see text).
(L. Position at exploratory laparotomy at 65 weeks. No liver tumours seen in any of these.

distant. The incidence of spontaneous tumours in our closed colony of Wistar rats has consistently been extremely low over many years, and in previously reported prolonged control injections with arachis oil we have also found only a very low incidence of local tumours (Dickens *et al.*, 1966, p. 149).

Subcutaneous injection of sorbic acid (sample *a*) in water into 6 rats resulted in local tumours in 2 rats at 54 and 66 weeks respectively, all 6 rats being dead by the 78th week. Histological evaluation of these tumours showed them to be typical fibrosarcomata having interlacing bundles of fusiform cells lying in a collagen matrix, though this was of greatly varying density between the 2 tumours. In the tumour appearing at 54 weeks very few mitoses were apparent while these were abundant in the second tumour at 66 weeks (Fig. 1), associated also with the occasional presence of large binucleate cells. This same sample of sorbic acid dissolved in arachis oil had given local malignant tumours in 5 of 6 rats at 82–102 weeks (Dickens *et al.*, 1966). Sorbic acid (sample *b*) in oil solution similarly injected into 12 rats gave no local tumours at all in spite of satisfactory periods of survival in this series (Table I).

Potassium sorbate from both manufacturers was injected (Table I) in aqueous solution into either 6 rats (sample *c*) or 12 rats (sample *d*). One rat injected with sample *d* showed an abnormal bilateral macroscopic appearance of the testes which, on histological examination were both found to have an interstitial cell tumour. The connection between the tumour and the injection of potassium sorbate (sample *d*) was thought not to be close. A few rats injected with potassium sorbate (*c*) showed the presence at post mortem of small inflammatory liver lesions, as described below, but these were non-neoplastic.

Oral administration

Two further groups of 6 rats each were maintained for 60 weeks with constant oral addition of potassium sorbate, sample *c*, either to their food or in their drinking water (see *Materials*, above). Both food and drink were readily consumed by the rats and their survival and general condition (Table I) was also satisfactory,

especially those given the material in their drink (5% solution) over $1\frac{1}{2}$ years).

Neither at an exploratory laparotomy at 65 weeks, nor at the end of the experiment was any special pathological feature detected in the animals fed potassium sorbate and in those given this substance in their drinking water the only abnormal

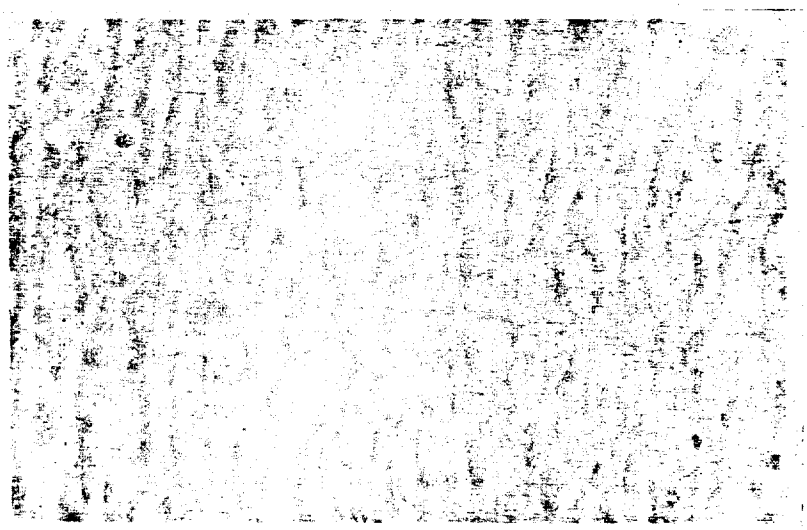

FIG. 1.—Actively proliferating fibrosarcoma from the injection site of a male rat treated subcutaneously with 2 mg. Sorbic acid in water twice a week for 56 weeks. ×400.

finding was the presence of scattered small whitish nodules on the surface of the liver in several of these rats. Histological examination confirmed that in no instance were these lesions neoplastic and they were evidently small inflammatory lesions only. No tumours were detected post mortem in any of the animals in either group.

DISCUSSION

The twice-weekly subcutaneous injection for more than a year of 0·5 ml. quantities of arachis oil into 12 rats gave no tumours in this series, in agreement with our earlier solvent control experiments (Dickens and Jones, 1965; Dickens *et al.*, 1966).

One rat with a neoplastic condition of the testes, possibly of spontaneous origin, was seen in the group injected with potassium sorbate (sample *d*) dissolved in 0·5 ml. water. No other tumours, either local or distant were observed in this group or in rats injected with another sample (*c*) of the same compound.

In our previous experiments (Dickens *et al.*, 1966) we reported the production of local sarcomata in 5 of 6 surviving rats which had been injected subcutaneously with repeated 2 mg. doses of a particular commercial specimen of free sorbic acid (sample *a*) dissolved in 0·5 ml. arachis oil. Sufficient of this particular batch of sorbic acid remained for the repetition of this experiment under similar conditions

except that [illegible] (0·5 ml.) was used instead of arachis oil as the injection medium. The result, reported above, showed that of 4 survivors after 54 weeks 1 rat had developed a local subcutaneous tumour and a second tumour appeared in 1 of the 2 remaining survivors at 66 weeks, both tumours being histologically typical fibrosarcomata.

Since this material (*a*) had been stored in the laboratory for some 3 years, it was possible that some decomposition might have occurred during this period. Consequently for the present work a fresh batch (sample *b*) of pure sorbic acid of the quality supplied for food preservation was obtained from a second commercial source. On injection of 2 mg. samples of this material twice weekly for a prolonged period as a solution in 0·5 ml. arachis oil, no tumours appeared in any of 12 rats so treated.

Sorbic acid is chemically not a particularly stable compound, since it possesses 2 conjugated double bonds which very slowly undergo atmospheric oxidation, especially in solution at elevated temperatures (Marx and Sabalitscka, 1963*a*, *b*) at first forming peroxides or hydroperoxides which subsequently may decompose yielding carbonyl compounds and organic acids (acrolein, oxyacrolein, crotonaldehyde, malondialdehyde, malonic acid and formic acid according to the above authors and Petropavlovskii and Ustinova, 1967). The Merck Index (1960), however, merely states that in the solid state sorbic acid should be kept at temperatures below 40° C., whereas the maximum room temperature in our laboratory probably never reached 30° C., so that the occurrence of extensive decomposition was judged most unlikely. Our own stock of the original sorbic acid was unfortunately no longer available, but through the kindness of Dr. E. Lück, Farbwerke Hoechst A.G., Frankfurt, Germany, to whom we had previously sent a small sample of sorbic acid, specimen *a*, we are permitted to report his own comparative chemical and physical tests of this material, in comparison with similar tests made on freshly prepared sorbic acid, sample *b*. The latter was a highly purified material supplied for use as a food additive, and of course conformed to the British Standards Specifications for this purpose.

Dr. Lück reports as follows on sample (*a*): "The assay showed that the sorbic acid content is slightly under the norm of 99·6–100 %. Beyond that the sample conformed to the British Standard No. 4234 (1967). The purity tests by gas chromatography, by spectrophotometry and fluorescence measurements did not show any difference from normal and highly purified sorbic acid as we produce it."

Therefore, apart from a slightly lower titration value to phenolphthalein in ethanol ("Determination of Sorbic Acid Content", British Standard 4234), no difference between samples *a* and *b* of sorbic acid was detectable by the British Standards methods specified for the testing of this substance for use in foodstuffs.

On the other hand, sample *a* has produced tumours in rats after its subcutaneous injection either in oil (Dickens *et al.*, 1966) or in water (the present work). Sample *b* on the contrary has now been shown by us to give no tumours when injected in oil into 12 rats under closely similar conditions of experiment. A recent report on Hoechst A.G. sorbic acid has also shown no tumours after its repeated injection into mice as the solution in arachis oil (Gericke, 1968).

We are unable to explain the reasons underlying this difference in biological behaviour of sorbic acid samples *a* and *b*, and it appears to us rather unlikely that the differences in chemical purity could be sufficient to account for the marked difference in tumorigenicity observed by us. However, in view of the considerable

disquiet produced by our earlier experience with this material, which is so widely used in food preservation, we thought it desirable to put the present results on record at once.

A consideration favourable to the use of certain of these products is the fact that we have observed only one, possibly unrelated, tumour following the injection of potassium sorbate (2 different commercial samples, *c* and *d*) and no tumours after the addition of the former material (sample *d* was not tested in this way) to either the food or drink of the rats. Whether or not this fact is related to the much higher solubility in water (58·2 % at 20° C.) of the potassium salt compared with that of sorbic acid itself (0·25 % at 30° C.), we are unable to say.

SUMMARY

1. Two different commercial samples of sorbic acid, and also 2 of potassium sorbate, have been tested for carcinogenic activity by their repeated subcutaneous injection as 2 mg. doses into rats.

2. One sample of sorbic acid (*a*) which had been previously shown by us (Dickens *et al.*, 1966) to induce subcutaneous tumours after its injection in oil was again found to induce fibrosarcomata, in 2 of 6 rats, when it was injected in water.

3. A further sample of sorbic acid (*b*), supplied by Farbwerke Hoechst, A.G. as used in the food industry as a preservative, gave no tumours in any of 12 rats after its repeated subcutaneous injection in oil solution.

4. Two samples of commercial potassium sorbate failed to induce local tumours after their repeated subcutaneous injection in aqueous solution into rats. One distant testicular tumour however was observed, but the significance of this was felt to be questionable.

5. One of these samples of potassium sorbate did not induce any tumours either when fed continuously to rats (1 % in their food) or when dissolved in their drinking water (0·3 %) throughout the experiment. The other sample was not tested in this way.

6. As only trifling chemical or physical differences were detected between samples *a* and *b* of sorbic acid, no explanation is available of the marked difference in their biological behaviour in these tests.

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