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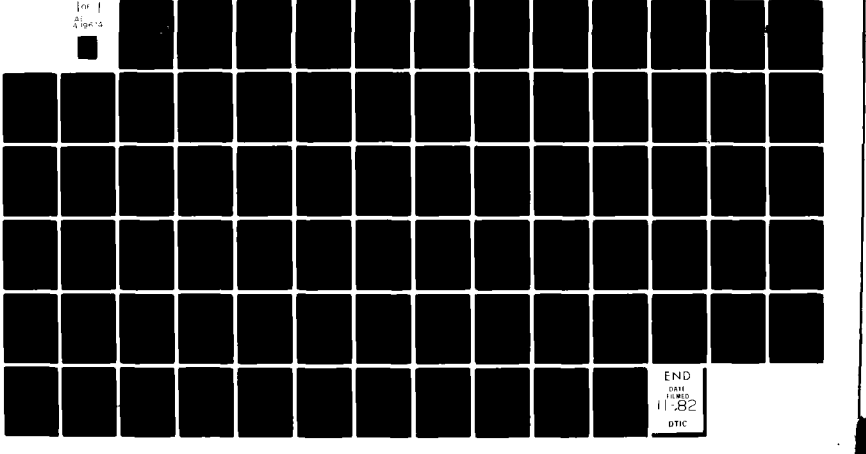

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EFFECTS OF NONADECAFLUORODECANOIC ACID ON TISSUE FATTY ACIDS OF THE RAT

By

Carl Thomas Olson, Ph.D.

The Ohio State University, 1982

Professor Roger A. Yeary, Adviser

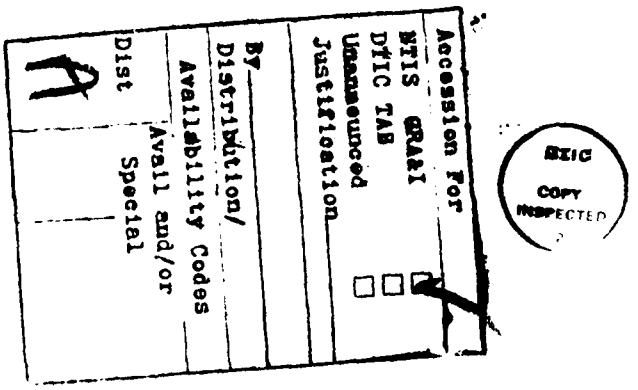
The effects of intraperitoneal injection of nonadecafluoro-n-decanoic acid (NDFDA) on liver, testes, and blood fatty acids were investigated. The hypothesis that NDFDA affects the relative amounts of the six major tissue fatty acids was tested. Effects and fatty acid ratios observed after NDFDA exposure were compared with those seen after injection of propylene glycol, injection of propylene glycol with pair-feeding, and injection of perfluorooctanoic acid (PFOA).

The lethal response of rats injected with NDFDA, when plotted on a log dose versus time basis, does not appear similar to that of rats injected with PFOA. At higher doses, there is a similarity in response, but, as the dosage is lowered, a second mechanism of toxicity seems to occur in NDFDA-treated rats. The $LD_{50/14}$ in 200 g rats for a single ip injection of NDFDA is 64 mg/kg and the $LD_{50/30}$ is 41 mg/kg. The $LD_{50/14}$ of PFOA injection in the same strain of rats is 189 mg/kg, which is also the $LD_{50/30}$.

At doses of 100 mg/kg PFOA and 50 mg/kg NDFDA, the liver appears enlarged and heart and body weights are significantly decreased (0.05 level) from both propylene glycol and propylene glycol/pair-fed controls, but mean testes and adrenals weights are significantly less than controls only for NDFDA-treated rats.

Compared with control ratios, the greatest changes in relative amounts of the six major fatty acids after treatment with NDFDA occur in the liver. There is a relative increase in palmitate and oleate and a relative decrease in stearate and arachidonate that cannot be attributed to reduced food intake. There is a similar trend after exposure to PFOA but this appears to be of a lesser magnitude and of a more transient nature. This shift in fatty acid ratio appears to be unrelated to microsomal lipid peroxidation. Levels of conjugated dienes measured in liver microsomes of NDFDA-treated rats 4 and 12 days post exposure are lower than in propylene glycol-treated control rats.

The mechanisms of toxicity of NDFDA remain in doubt. Oxidation of fatty acids leading to production of prostaglandins or other biologically-active derivatives which adversely affect physiological activities may be occurring. The decrease in relative fraction of arachidonic acid may give credence to this theory. A direct effect on membrane function can also be advocated since recent experimentation has demonstrated a decreased erythrocyte osmotic fragility in rats injected with NDFDA.



EFFECTS OF NONADECAFLUORODECANOIC ACID ON TISSUE FATTY ACIDS OF THE RAT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Carl Thomas Olson, D.V.M., M.S.

* * * * *

The Ohio State University

1982

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INTRODUCTION

Nonadecafluoro-n-decanoic acid (NDFDA) is a straight chain, perfluorinated, ten-carbon acid (CF_3 $(CF_2)_8$ CO_2H) with formula weight of 514.08. It has a melting point of 77-79°C, a boiling point of 218°C at 740 mm Hg pressure, and is readily obtainable in crystalline form.[1] Perfluorocarboxylic and perfluorosulfonic acids are used commercially in plating systems and as wetting agents and corrosion inhibitors. Seven to nine carbon perfluorinated acids are also used to impart water and oil resistance to paper, fabrics, and leather. Aqueous film-forming foams, used as fire extinguishants, contain commercial mixtures of derivatized perfluorinated fatty acids. Perfluorinated compounds have also been proposed for use as vascular replacement fluids and contrast media in computer assisted tomography (CAT).

Perfluorinated compounds are generally considered to be metabolically inert, but perfluorohexane causes reduction of microsomal cytochrome P-450 *in vitro* and perfluoro-n-octanoic acid (PFOA) causes lipid peroxidation *in vitro*. While investigating the toxicity of aqueous film-forming foams, Naval personnel compared the effects of some perfluorinated fatty acids, including PFOA and NDFDA. The only observable effect following intraperitoneal injection of PFOA at a dose of 100 mg/kg in young adult male Fischer 344 rats was a transient decrease in

[1] Aldrich Chemical Co., Milwaukee WI.

weight. Recovery appeared to be complete within one week. At intraperitoneal or oral doses of NDFDA of 100 mg/kg, rats became anorectic, lost weight, and died within two to three weeks of treatment. Pathologic findings associated with administration of this compound included: cachexia, thymic hemorrhage, and atrophy; bone marrow depletion; gastritis; bile duct proliferation and hepatic necrosis; and tubular degeneration, atrophy and necrosis of the testes.

It was hypothesized that NDFDA affects the relative amounts of the six major tissue fatty acids. This research was performed to test that hypothesis.

LITERATURE REVIEW

Perfluorinated compounds have wide application. Perfluorocarboxylic and perfluorosulfonic acids have found commercial utilization in plating systems and as wetting agents and corrosion inhibitors. Seven to nine carbon perfluorinated fatty acyl compounds are used to impart water and oil resistance to paper, fabrics, and leather (Rozner and Taves, 1980). Many perfluorinated organic liquids are synthesized for industrial use as heat exchangers, leak detectors, and hydraulic fluids because they are electrically nonconductive, chemically nonreactive, and heat stable (Clark et al., 1973). Aqueous film-forming foams, used as fire extinguishants, contain commercial mixtures of derivatized perfluorinated fatty acids (Andersen, 1980). The use of perfluorinated compounds for vascular fluid replacement has been advocated due to "the virtual lack of toxicity of these materials coupled with their high gas solubility capacities" (Sargent and Seffl, 1970). Perfluorinated organic liquids have also been proposed for use as computer-assisted tomography (CAT) scan media (Enzmann and Young, 1979).

Perfluorinated compounds have generally been considered biologically inert (Clark et al., 1973). Holaday (1970) wrote that due to the high carbon-fluorine bond strength, fluorocarbon liquids were chemically inert and no metabolism in vivo had been observed. Ullrich and Diehl (1971) reported that perfluoro-n-hexane, in contrast to n-hexane, is not hydroxylated by the microsomal monooxygenation system

in liver microsomes of phenobarbital-treated rats. These authors also stated that the fluorocarbon forms an enzyme-substrate complex, stimulating NADPH oxidation, and acting as a dead-end inhibitor of the microsomal monooxygenase system. Triner et al. (1970) reported that during liver perfusion with fluorocarbon suspensions, an extremely high rate of gluconeogenesis and a doubling of oxygen consumption was observed compared to controls perfused with blood.

Spitzer et al. (1970) found that mice injected with $^{32}PO_4$ and immersed in a fluorocarbon bath equilibrated with 100% oxygen showed a marked reduction of $^{32}PO_4$ incorporation into phospholipids when compared with mice swimming in fluorocarbon. Danis et al. (1981) stated that another halocarbon, 2-bromooctanoate, was an inhibitor of the β-oxidation of acyl CoA compounds.

The avid binding of perfluorooctanoic acid (PFOA) with serum albumin was reported in the 1950's (Nordby and Luck, 1955; Ellenbogen and Maurer, 1956). Evidence for the existence of organic compounds of fluorine in human plasma has been provided (Guy et al., 1976; Guy, 1979) and higher than normal levels of organic fluorine have been found in the blood of workers exposed to fluorochemicals in an industrial environment (Ubel et al., 1981). Organic fluorine content of blood appears to be independent of the fluoride concentration of the water supply (Guy et al., 1976; Guy, 1979), and the finding of little or no organic fluorine in the blood of animals other than humans suggests an environmental origin of this organofluorine.

Guy et al. (1976) reported that "little has been published about the metabolic handling and toxicology of perfluorinated fatty acid

derivatives." These authors stated that computer-assisted literature searches using Medline, Toxline, and Chemcon developed no information on these subjects and wrote that "this was surprising with respect to the widespread commercial use of such compounds." Since that time, several papers have been written on methods for determining total fluorine or PFOA content in various biological samples (Guy, 1979; Belisle and Hagen, 1980) and papers have also been written on metabolism and toxicity of PFOA (Ophaug and Singer, 1980; Rozner, 1980) and ammonium perfluorooctanoate (Griffith and Long, 1980).

Griffith and Long (1980) reported an acute oral LD_{50} for ammonium perfluorooctanoate, a commercial surfactant, in 180-221 g ChR-CD rats of 540 mg/kg with a 95% confidence interval of 389-759 mg/kg. In repeated dose rodent studies the liver was the target organ. Rozner and Taves (1980) hypothesized, based on the work of Ullrich and Diehl (1971), that perfluorinated compounds stimulated microsomal membrane peroxidation. Lipid peroxidation has been proposed as a mechanism of cellular injury in some disease processes and in some chemically-induced toxicities (Recknagel and Goshal, 1966; DiLuzio and Hartman, 1967).

Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids. The peroxidative process leads to the formation of free radical intermediates which can lead to autocatalysis (Plaa and Witschi, 1976). The proposed scheme for radical formation, generalizing for site of formation, is given in Figure 1 (Dahle et al., 1962).

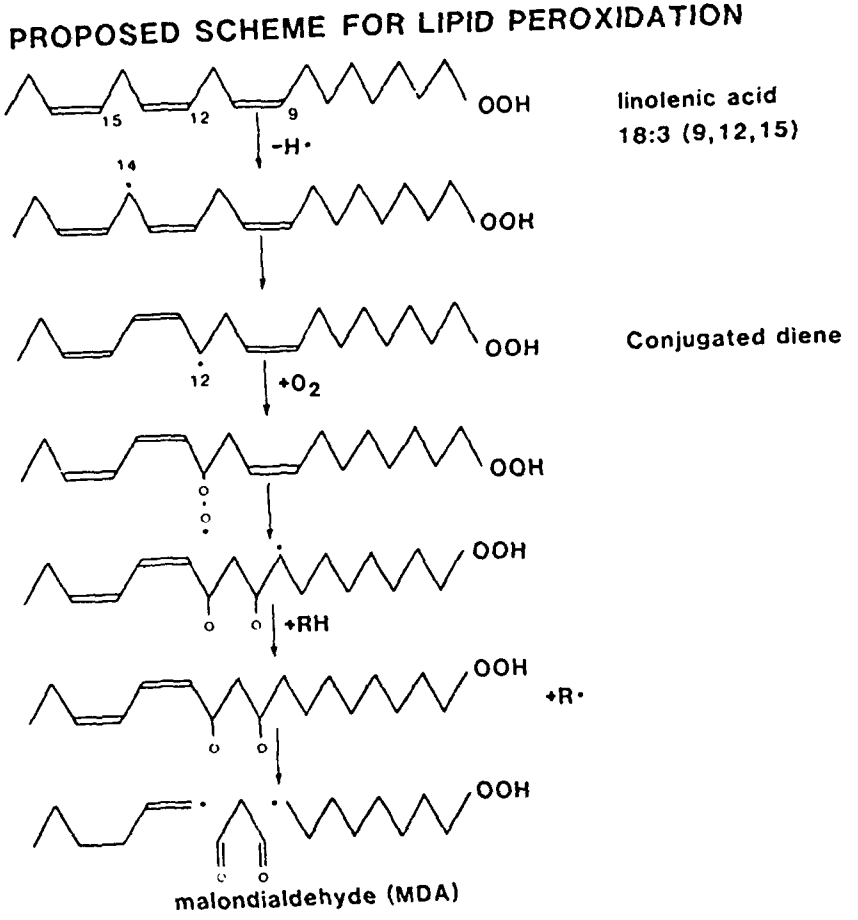


Figure 1. Proposed scheme for radical formation by lipid peroxidation. From Dahle et al., 1962.

There are various methods available for measuring lipid peroxidation (Dahle et al., 1962; Sawicki et al., 1963; Placer et al., 1966; Csallany and Ayaz, 1976; Bernheim et al., 1948; Waller and Recknagel, 1977; Frank et al., 1980). The most commonly used method is the spectrophotometric determination of a thiobarbituric acid (TBA)-reacting substance, generally thought to be malondialdehyde (MDA), formed in the autoxidation of polyunsaturated lipids. The TBA assay appears to be a reliable method for estimating extent of lipid peroxidation in vitro but despite its wide application in in vivo experimentation, this assay may not be suitable. MDA is rapidly metabolized in vivo and also reacts with tissue components (Bus and Gibson, 1979). Another useful method for measurement of lipid peroxidation is examination of tissue extracts for the presence of conjugated dienes which are also formed in the peroxidation of unsaturated fatty acids and exhibit spectra characterized by intense absorption at 233 nm (Waller and Recknagel, 1977).

Rozner (1980) examined the effects of PFOA on microsomal membrane peroxidation in vitro using MDA analysis. He found that MDA was produced to an extent similar to that seen after carbon tetrachloride treatment. He concluded, however, that PFOA and carbon tetrachloride stimulate peroxidation by different mechanisms. He based this conclusion on several findings:

1. Concentrations of PFOA that produced maximum peroxidation significantly reduced microsomal electron transport, unlike carbon tetrachloride.

2. Addition of EDTA reduced PFOA peroxidation to a level indistinguishable from controls but reduced carbon tetrachloride peroxidation by only 70%.

3. PFOA or ferrous iron, but not carbon tetrachloride, stimulated peroxidation in the presence of ascorbic acid, a non-enzymatic reducing agent.

Rozner postulated that PFOA stimulated peroxidation by partially solubilizing microsomal membranes. He found that Tween 20, a surface active agent, exhibited effects upon non-enzymatic peroxidation of microsomal membranes that were qualitatively similar to the effects of PFOA.

Though lipid peroxidation is often observed together with liver injury, the data for a causative role are not conclusive (Plaa and Witschi, 1976). Stacey and Klassen (1981) have shown that sodium vanadate induced high levels of lipid peroxides in isolated hepatocytes but without associated cell injury. These authors were also able to inhibit lipid peroxidation without preventing cellular injury in isolated rat hepatocytes.

Fox (1981) wrote of work being done by Porter and coworkers (Porter et al., 1981) at Duke University and Pryor and colleagues at Louisiana State University on autoxidation of polyunsaturated fatty acids. These researchers found that physiologically-active products such as leukotrienes and prostaglandins may be found as a result of fatty acid oxidation.

In a review of the literature, toxicity studies on NDFDA were limited to reports from the Air Force Aerospace Medical Research Laboratory (Bacon et al., 1981; Andersen et al., 1981a; Andersen et

al., 1981b; Rogers et al., 1982). In a teratologic evaluation in rats (Bacon et al., 1981), intraperitoneal injection of NDFDA produced no terata but decreased dam and fetal weights and, at day nine of pregnancy, single doses of 15 mg/kg or higher did cause some maternal and/or fetal deaths. Andersen et al. (1981a) reported steep dose response curves for NDFDA. In male rats given 90 mg/kg intraperitoneally, the time to death was 18 ± 2 days and rats lost more than half their body weight before dying (225 ± 4 to 106 ± 2 g). Pathologic evaluation after NDFDA exposure revealed hypocellularity of bone marrow, bile duct proliferation and hepatocellular alterations in both nucleus and cytoplasm, testicular atrophy and necrosis, mild gastritis with hyperkeratosis of the squamous portion of the stomach, and severe thymic atrophy. Concentrations of 500 ug/ml or greater caused lysis of cultures of L5178Y cells, and cells treated with 0.5 ug/ml were unable to grow in soft agar. Survival time at the LD_{50}, toxic signs, and histopathology found with NDFDA intoxication were strikingly similar to the effects noted after 2,3,7,8-tetrachlorodibenzodioxin (TCDD) exposure (Andersen et al., 1981a; ibid, 1981b).

Cronan (1974) wrote:

"In virtually all natural phospholipids, unsaturated fatty acids are preferentially esterified at position 2 and saturated fatty acids at position 1 of the glycerol molecule. This asymmetric fatty acid distribution is thought to be of major importance in the functional and structural roles of phospholipids in membrane processes. Perturbations in the fatty acid content of membrane phospholipids can produce drastic disturbances in cellular physiology." Neal et al. (1979) stated that

TCDD had a marked effect on lipid metabolism in all animal species studied. However, when they compared fatty acid composition of liver, plasma, and adipose tissue of TCDD-treated guinea pigs with pair-fed controls, they were unable to detect any major differences. Wood and Harlow (1969a) analyzed Ehrlich ascites carcinoma cell lipids and found that "randomly biosynthesized diglycerides are used at random for the biosynthesis of triglycerides and diacyl phosphatidylcholine; these results are in contrast with the selectivity observed in rat liver."

Lipids are water-insoluble organic biomolecules that can be extracted from cells and tissues by non-polar solvents (Lehninger, 1977). Fatty acids are incorporated in complex lipids such as acylglycerols, phosphoglycerides, and sphingolipids and as such serve as structural components of membranes, as storage and transport forms of metabolic fuel and as cell-surface components concerned in cell recognition, species specificity, and tissue immunity. It was earlier thought that lipids were metabolically relatively inactive and served primarily for storage of energy, remaining in situ until energy requirements exceeded that available from the diet. However, even in animals that are not growing and are in a steady state in terms of body mass, it was determined that there is a continual turnover of tissue glycerides, requiring a rate of glyceride biosynthesis equivalent to the rate of glyceride degradation (Masoro, 1968). The turnover of adipose tissue triacylglycerol (triglyceride) is vigorous, with a high rate of biosynthesis occurring in this tissue. Liver also has a high rate of triacylglycerol biosynthesis and turnover. Phosphoglycerides turn over in

mammals in the steady state but it is difficult to assess quantitatively the extent of this process (Masoro, 1968). Some of the phosphoglyceride molecules are apparently quite stable and turn over very slowly; others appear to turn over at rapid rates. It is clear, however, that even in the steady state, there is a significant biosynthesis and turnover of phosphoglycerides in the tissue of mammals.

Fatty acids are obtained from the diet and from de novo synthesis. Palmitic acid and stearic acid, to some extent, are the products of de novo synthesis. From these, chain elongation and/or desaturation reactions can occur, forming many other fatty acids. Linoleic, linolenic, and arachidonic acids are considered essential fatty acids since they cannot be synthesized de novo and thus are required in the diet.

Each tissue has a unique biochemical nature that regulates the fatty acid composition of specific lipid subclasses (Masoro, 1968; Cronan, 1974). Species also influence the fatty acid composition of lipid subclasses, this difference being maintained even when the diet is markedly modified. Many papers have been written about fatty acid composition of various tissues, of different species, and of intracellular organelles, in normal and diseased states (Skipski et al., 1965; Dod and Gray, 1968; Pfleger et al., 1968; Ray et al., 1969; Wood and Harlow, 1969a; Wood and Harlow, 1969b; Wood, 1970; Keenan et al., 1970; Van Hoeven and Emmelot, 1972; Ruggieri and Fallani, 1978; Milner and Perkins, 1978; Ruggieri et al., 1979; Baker, 1979). Since fatty acids are a major component of most lipids, and since fatty acid composition is regulated, it is often the properties of these fatty acids

that endow lipids with specific characteristics that are significant in the structure and function of living cells.

Lipids are generally isolated from tissues or membranes by the method developed by Folch (Folch et al., 1957). To determine fatty acid content, it is common practice to esterify fatty acids (Luddy et al., 1960; Metcalfe and Schmitz, 1961; Mason and Waller, 1964; Metcalfe et al., 1966; Supelco Bulletin 721C, 1975) to increase volatility and then analyze the fatty acid esters by gas chromatography and/or mass spectrometry (Stoffel et al., 1959; Murata, 1978; Schwarzenbach and Fisher, 1978).

MATERIALS AND METHODS

Male, Fischer 344 rats[2] were obtained approximately two weeks before they were to be used for experimentation. Two rats from each shipment were sacrificed and examined by veterinary pathologists to assure the health of experimental animals. Animals arrived weighing 125-150 g and were 200 g at the time of injection. Rats were maintained at the Air Force Aerospace Medical Research Laboratory's vivarium until two days before use, when they were transported to the Toxic Hazards Division laboratory and caged individually, in random order, in a portable containment system.[3] Rat chow[4] and tap water were given ad libitum. Food consumption and animal weights to the nearest gram were recorded daily.[5]

Perfluorinated acid solutions were prepared one to two days prior to injection with propylene glycol-water (1:1) as diluent. Concentrations were adjusted to maintain a dosing volume of 2 ml/kg body weight. To estimate the dose lethal to 50% of a treated population (LD_{50}) and to gather information on survival times, doses of 25, 40, 50, 60, 80,

1 Aldrich Chemical Co., Milwaukee WI.

2 Charles River Breeding Laboratories, Inc., Wilmington MA.

3 PCS-80, Hazleton Systems, Inc., Aberdeen MD.

4 Purina Rat Chow, Ralston Purina Co., St. Louis MO.

5 Ohaus Dial-O-Gram Model 1600 Balance, Ohaus Scale Corp., Florham Park NJ.

100, 150, 200, 300 and 500 mg/kg of NDFDA and doses of 50, 100, 200, 500 and 1000 mg/kg of PFOA were administered intraperitoneally to ten rats per dose group. Three additional PFOA dose groups were included at a later date to more accurately assess the LD_{50} of this compound. Ten rats were given intraperitoneal doses of 2 ml propylene glycol-water (1:1)/kg and served as controls.

For fatty acid composition studies, doses were 50 mg NDFDA/kg and 100 mg PFOA/kg. Six groups of rats were treated. In each group, four rats received 50 mg NDFDA/kg intraperitoneally; four received 100 mg PFOA/kg; and another four received propylene glycol-water (1:1). The following day, four additional rats were given propylene glycol-water (1:1) and each rat fed the amount of food consumed by an individual NDFDA-treated rat (pair-fed controls). Considering the day of injection as day 0, rats were killed at 2, 4, 8, and 16 days after the single ip injections.

At the time of sacrifice, animals were anesthetized with halothane in a bell jar, the heart exposed and blood samples obtained by cardiac puncture. Brain, liver, testes, heart, kidneys, and adrenals were removed and weighed. Liver and testes were weighed to the nearest 0.1 g[6] and other tissues weighed to the nearest 0.001 g[7]. Two-gram samples of liver and testes and two-milliliter samples of blood were homogenized[8] for 45 seconds in ten milliliters of chloroform-methanol (2:1). Liver

[6] Mettler P1200 Balance, Mettler Instrument Corp., Heightstown NJ.

[7] Roller-Smith Precision Balance, Roller-Smith, Bethlehem PA.

[8] Tekmar SDT Series Tissumizer, Tekmar Co., Cincinnati OH.

homogenates were immediately filtered through a sintered glass filter with vacuum. Homogenates of testes and blood were centrifuged at 1500 rpm for ten minutes and then filtered through sintered glass filters with vacuum. Filtrates were added to ten milliliters of physiological saline in a separatory funnel and allowed to layer. The bottom chloroform layer was separated into a pre-weighed[9] scintillation vial and the vial placed in an evaporator at 60°C in a hood. When the samples were evaporated to dryness, the vials were reweighed (samples were weighed for approximately half of the experimental animals).

Two milliliters of benzene was added to each vial and then poured into a 50-ml 14/20 standard taper reflux flask. One milliliter of 3N methanolic-HCl[10] was used to rinse the vial and added to the reflux flask. Refluxing was accomplished with stirring in a hood for a minimum of 30 minutes to obtain esterification of fatty acids. Two samples were refluxed at a time. After refluxing, samples were poured into labeled test tubes and 1.0 milliliter of distilled water added. Tubes were gently agitated and samples allowed to separate for ten minutes. The top benzene layer was aspirated and placed in labeled 2.7 ml vials. Solvents were evaporated in the hood overnight. One milliliter of methylene chloride was added to liver samples and 0.2 milliliter to testes and blood samples.

Fatty acid esters were analyzed by gas chromatography. One microliter samples were injected on a 10 meter SE30, methyl silicone capillary

[9] Beta N-IV (Type SCN) Balance, Ainsworth, Denver CO.

[10] Supelco, Inc., Bellefonte PA.

column[10] and peak areas were determined by integration.[11] The chromatograph was programmed with an initial oven temperature of 50°C which was maintained for two minutes and then raised 8°C/ minute until 230°C was reached and maintained for five minutes. Nitrogen was used as a carrier gas with a flow rate of 1.0 ml/min and a split ratio of 10:1. Air flow at the flame ionization detector was 450 ml/min and hydrogen 30 ml/min. Detector and injector temperatures were maintained at 200°C.

To assure proper identification of peaks, RM-3,[10] an oil reference mixture, was injected on this column as was a methyl docosahexaenoate standard.[10] The purpose of the experiment was to compare the relative amounts of the six major fatty acid components - palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), arachidonic (20:4), and docosahexaenoic (22:6) acids - before and after administration of NDFDA. Methyl esters of these acids, except arachidonic, are present in the standards used. Methyl arachidate, the saturated 20-carbon ester, is present in the RM-3 standard. Samples were also injected on a column of a gas chromatograph/mass spectrometer system[12] and peaks compared with those in the National Bureau of Standards and Wiley computer libraries. Results were compatible with those fatty acids anticipated.

Data on relative amounts of fatty acids in the liver, testes, and blood of NDFDA-treated, PFOA-treated, and control animals, along with

[11] Hewlett-Packard 5880A Gas Chromatograph System, Hewlett-Packard, Palo Alto CA.

[12] Hewlett-Packard 5985 GC-MS System, Hewlett-Packard, Palo Alto CA.

data on body and organ weights, and weight of chloroform-extractable material were analyzed at The Ohio State University and at Wright-Patterson AFB using a computerized multivariate analysis of variance program.[13]

13 Statistical Analysis System, SAS Institute, Cary, NC.

RESULTS

To determine the intraperitoneal LD_{50} of NDFDA in 200 g male Fischer rats, ten dose groups of ten rats each were used: 25, 40, 50, 60, 80, 100, 150, 200, 300, and 500 mg/kg. At doses of 80 mg/kg or greater all ten animals in each dose group died. At 60 mg/kg, nine animals died; at 50 mg/kg, eight animals died; at 40 mg/kg, 5 animals died, and at 25 mg/kg, all animals survived. Means and standard errors of the mean for survival time for these groups are given in Table 1. When plotted on a log-normal scale (Figure 2), a biphasic response is evident. A probit analysis yields a 14 day LD_{50} ($LD_{50/14}$) of 63.6 mg/kg with 95% probability limits of 52.0 and 74.9 mg/kg. Using the same analysis system the 30 day LD_{50} ($LD_{50/30}$) is 41.4 mg/kg with 95% probability limits of 34.1 and 46.7 mg/kg.

Initially, five doses of PFOA were injected intraperitoneally into 200 g male Fisher rats: 50, 100, 200, 500, and 1000 mg/kg. Each dose group consisted of ten animals. There were no mortalities in the 50 and 100 mg/kg dose groups within 30 days postexposure. Seven animals died at a mean time of 2.7 days in the 200 mg/kg dose group. All animals in the 500 and 1000 mg/kg dose groups died at mean times of 0.09 and 0.06 days, respectively. Based on these results, three additional groups of animals were injected at doses of 140, 170 and 270 mg/kg. At 140 mg/kg, no deaths were observed within 30 days. Two of the ten rats given 170

Table 1

Mean Survival Time of Animals
Injected Intraperitoneally with NDFDA

Dose (mg/kg)	Mean Time (days)	SEM[a]
40	16.12	0.25
50	15.26	0.55
60	15.62	0.39
80	14.11	0.96
100	12.31	0.55
150	7.23	0.89
200	1.84	0.60
300	0.76	0.08
500	0.27	0.01

[a] Standard error of the mean.

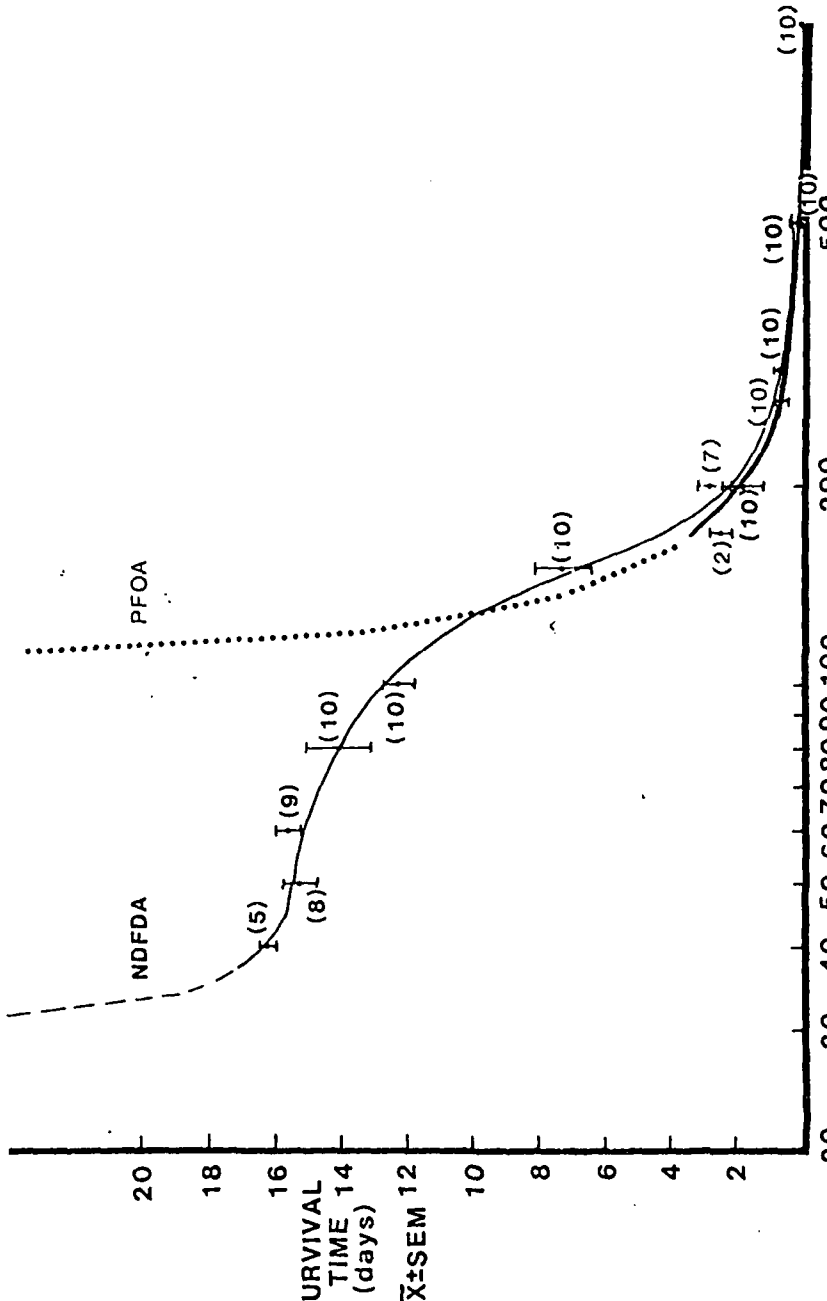


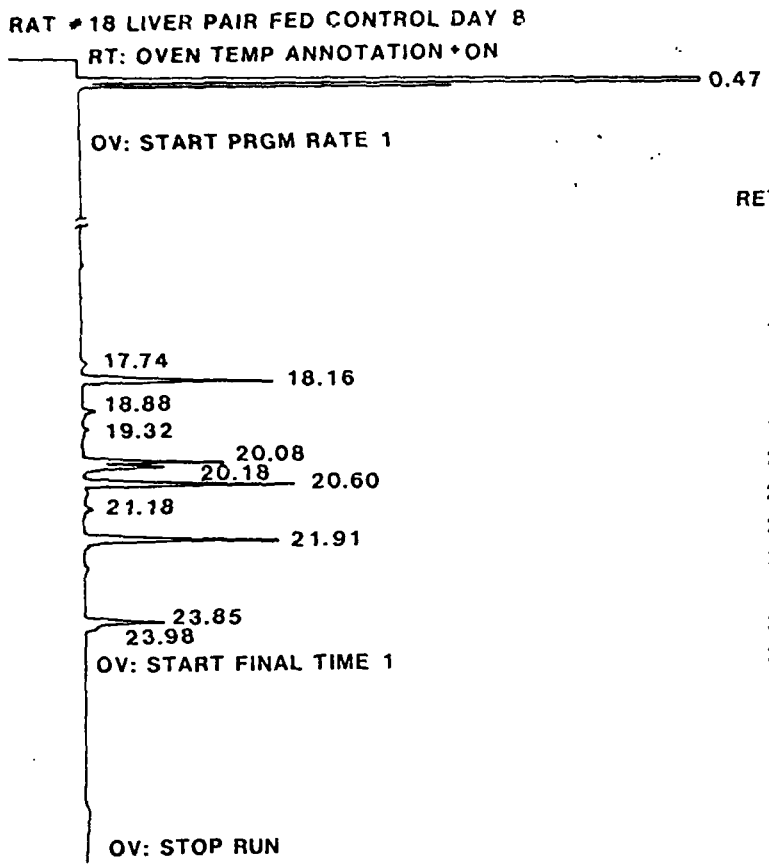
Figure 2. Mean survival times after IP injection of PFOA and NDFDA. In Parentheses after plotted points are the number of animals responding.

mg/kg PFOA died at 2.0 and 3.0 days. All ten rats injected with 270 mg/kg died with a mean survival time of 0.66 days with a range of 0.28 to 1.95 days. Probit analysis yielded an estimated $LD_{50/14}$ (or $LD_{50/30}$) of 188.7 mg/kg with 95% probability limits of 175.4 and 209.4 mg/kg.

When plotted, mean survival time versus dose of PFOA on a log-normal scale (Figure 2), the curve at higher doses appears very similar to the curve for NDFDA. At doses of 100 mg/kg or less, however, these curves differ significantly.

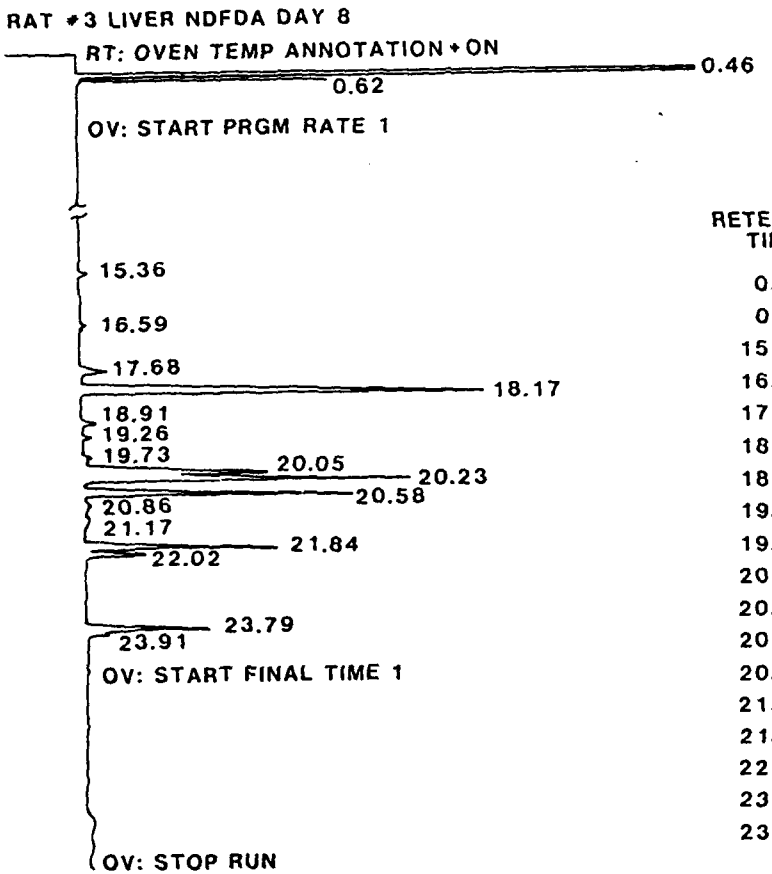
Doses selected for analyzing effects on fatty acids were 100 mg/kg of PFOA and 50 mg/kg of NDFDA. Relative fatty acid contents were calculated by adding the areas under the peaks (obtained from integrator of gas chromatograph)[14] for the six major fatty acids and dividing the area under each peak by the total area under the six peaks (Figures 3 and 4). The mean values of six animals for these relative distributions for liver, testes, and whole blood at days 2, 4, 8, and 16 are given in Tables 2 through 13. Those values significantly different at the 0.05 level from both propylene glycol and pair-fed controls are annotated. Liver fatty acid composition was more markedly altered than that in any other tissue (Figures 5 through 8). By the second day after NDFDA injection, stearic, arachidonic and docosahexaenoic acid fractions are reduced, and oleic and linoleic fractions are increased. Two days after PFOA exposure, palmitic and oleic acid fractions are increased. By day 4, palmitic and oleic acid fractions are significantly greater in NDFDA-treated than controls and

[14] Hewlett-Packard 5880A System, Hewlett-Packard, Palo Alto CA.



RETENTION TIME	FATTY ACID ESTERS	AREA %
0.47		96.768
0.64		0.381
17.74		0.010
18.16	PALMITATE	0.528
18.88		0.040
19.32		0.012
20.08	LINOLEATE	0.373
20.18	OLEATE	0.243
20.60	STEARATE	0.653
21.18		0.030
21.91	ARACHIDONATE	0.667
23.85	DOCOSAHEXAENOATE	0.257
23.98		0.038

Figure 3. Gas chromatograph of liver sample from pair-fed control animal.



RETENTION TIME	FATTY ACID ESTERS	AREA %
0.46		89.560
0.62		0.231
15.36		10.032
16.59		0.027
17.68		0.160
18.17	PALMITATE	2.659
18.91		0.097
19.26		0.039
19.73		0.054
20.05	LINOLEATE	1.412
20.23	OLEATE	2.118
20.58	STEARATE	1.445
20.86		0.070
21.17		0.043
21.84	ARACHIDONATE	1.045
22.02		0.242
23.79	DOCOSAHEXAENOATE	0.640
23.91		0.127

Figure 4. Gas chromatograph of liver sample from NDFDA-treated animal.

Table 2

Mean Relative Distribution of the 6 Major Fatty Acid Components of Liver at Day 2[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.243	0.273[b]	0.248	0.211
Stearic	0.257	0.206[c]	0.206[c]	0.296
Oleic	0.076	0.146[b]	0.153[b]	0.048
Linoleic	0.158	0.134	0.171[b]	0.132
Arachidonic	0.187	0.183	0.164[c]	0.234
Docosahexaenoic	0.080	0.058[c]	0.058[c]	0.079

[a] Mean values for six animals in each treatment group.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

Table 3

Mean Relative Distribution of the 6 Major Fatty Acid Components of Liver at Day 4[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.245	0.251	0.275[b]	0.208
Stearic	0.269	0.235[c]	0.199[c]	0.277
Oleic	0.060	0.125[b]	0.168[b]	0.057
Linoleic	0.150	0.199	0.160	0.132
Arachidonic	0.195	0.212	0.143[c]	0.243
Docosahexaenoic	0.083	0.059[c]	0.055[c]	0.084

[a] Mean values for six animals in each treatment group.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

Table 4

Mean Relative Distribution of the 6 Major Fatty Acid Components of Liver at Day 8[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.241	0.238	0.292[b]	0.205
Stearic	0.261	0.251	0.159[c]	0.263
Oleic	0.062	0.099[b]	0.216[b]	0.069
Linoleic	0.152	0.127	0.150	0.131
Arachidonic	0.200	0.209	0.103[c]	0.238
Docosahexaenoic	0.085	0.076	0.081	0.093

[a] Mean values for six animals in each treatment group.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

Table 5

Mean Relative Distribution of the 6 Major Fatty Acid Components of Liver at Day 16[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.242	0.242	0.271[b]	0.220
Stearic	0.263	0.261	0.189[c]	0.248
Oleic	0.064	0.070	0.196[b]	0.078
Linoleic	0.146	0.140	0.131	0.146
Arachidonic	0.201	0.204	0.112[c]	0.201
Docosahexaenoic	0.085	0.084	0.103	0.107

[a] Mean values for six animals in each treatment group.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

Table 6

Mean Relative Distribution of the 6 Major Fatty Acid Components of Testes at Day 2[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.360	0.370	0.360	0.357
Stearic	0.085	0.077	0.077	0.081
Oleic	0.148	0.154	0.158	0.156
Linoleic	0.068	0.066	0.067	0.070
Arachidonic	0.163	0.148	0.159	0.161
Docosahexaenoic	0.176	0.184	0.180	0.177

[a] Mean values for six animals in each treatment group.

Table 7

Mean Relative Distribution of the 6 Major Fatty Acid Components of Testes at Day 4[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.367	0.382	0.334	0.337
Stearic	0.084	0.089	0.069	0.082
Oleic	0.151	0.156	0.163	0.158
Linoleic	0.068	0.061	0.069	0.072
Arachidonic	0.157	0.149	0.165	0.164
Docosahexaenoic	0.174	0.164	0.200	0.187

[a] Mean values for six animals in each treatment group.

Table 8

Mean Relative Distribution of the 6 Major Fatty Acid Components of Testes at Day 8[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.359	0.352	0.380[b]	0.360
Stearic	0.084	0.081	0.071	0.083
Oleic	0.148	0.159	0.151	0.152
Linoleic	0.064	0.059	0.052[c]	0.060
Arachidonic	0.161	0.165	0.140[c]	0.153
Docosahexaenoic	0.184	0.185	0.206	0.193

[a] Mean values for six animals in each treatment group.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

Table 9

Mean Relative Distribution of the 6 Major Fatty Acid Components of Testes at Day 16[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.350	0.359	0.359	0.359
Stearic	0.082	0.086	0.077	0.070
Oleic	0.150	0.154	0.178[b]	0.143
Linoleic	0.068	0.065	0.040	0.042
Arachidonic	0.161	0.160	0.134[c]	0.153
Docosahexaenoic	0.191	0.176	0.212	0.234

[a] Mean values for six animals in each treatment group.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

Table 10

Mean Relative Distribution of the 6 Major Fatty Acid Components of Blood at Day 2[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.317	0.308	0.315	0.296
Stearic	0.143	0.135	0.138	0.170
Oleic	0.125	0.150	0.138	0.118
Linoleic	0.141	0.132	0.130	0.127
Arachidonic	0.183	0.198	0.205	0.209
Docosahexaenoic	0.090	0.077	0.075	0.081

[a] Mean values for six animals in each treatment group.

Table 11

Mean Relative Distribution of the 6 Major Fatty Acid Components of Blood at Day 4[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.288	0.301	0.278	0.290
Stearic	0.161	0.156	0.114[b]	0.154
Oleic	0.132	0.124	0.156	0.136
Linoleic	0.142	0.134	0.136	0.122
Arachidonic	0.193	0.212	0.223	0.211
Docosahexaenoic	0.083	0.075	0.093	0.086

[a] Mean values for six animals in each treatment group.

[b] Significantly less than control values at 0.05 level.

Table 12

Mean Relative Distribution of the 6 Major Fatty Acid Components of Blood at Day 8[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.297	0.284	0.343[b]	0.300
Stearic	0.165	0.161	0.128[c]	0.167
Oleic	0.114	0.142	0.146	0.122
Linoleic	0.153	0.129	0.120	0.110
Arachidonic	0.196	0.206	0.178	0.214
Docosahexaenoic	0.075	0.079	0.086	0.089

[a] Mean values for six animals in each treatment group.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

Table 13

Mean Relative Distribution of the 6 Major Fatty Acid Components of Blood at Day 16[a]

Fatty Acid	Propylene Glycol Control	PFOA	NDFDA	Pair-Fed Control
Palmitic	0.285	0.306	0.328	0.282
Stearic	0.148	0.152	0.133	0.139
Oleic	0.129	0.141	0.157[b]	0.126
Linoleic	0.160	0.142[d]	0.128[d]	0.114
Arachidonic	0.199	0.191	0.159[c]	0.233
Docosahexaenoic	0.079	0.069	0.094	0.107

[a] Mean values for six animals in each treatment group.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

[d] Significantly different from but between control values.

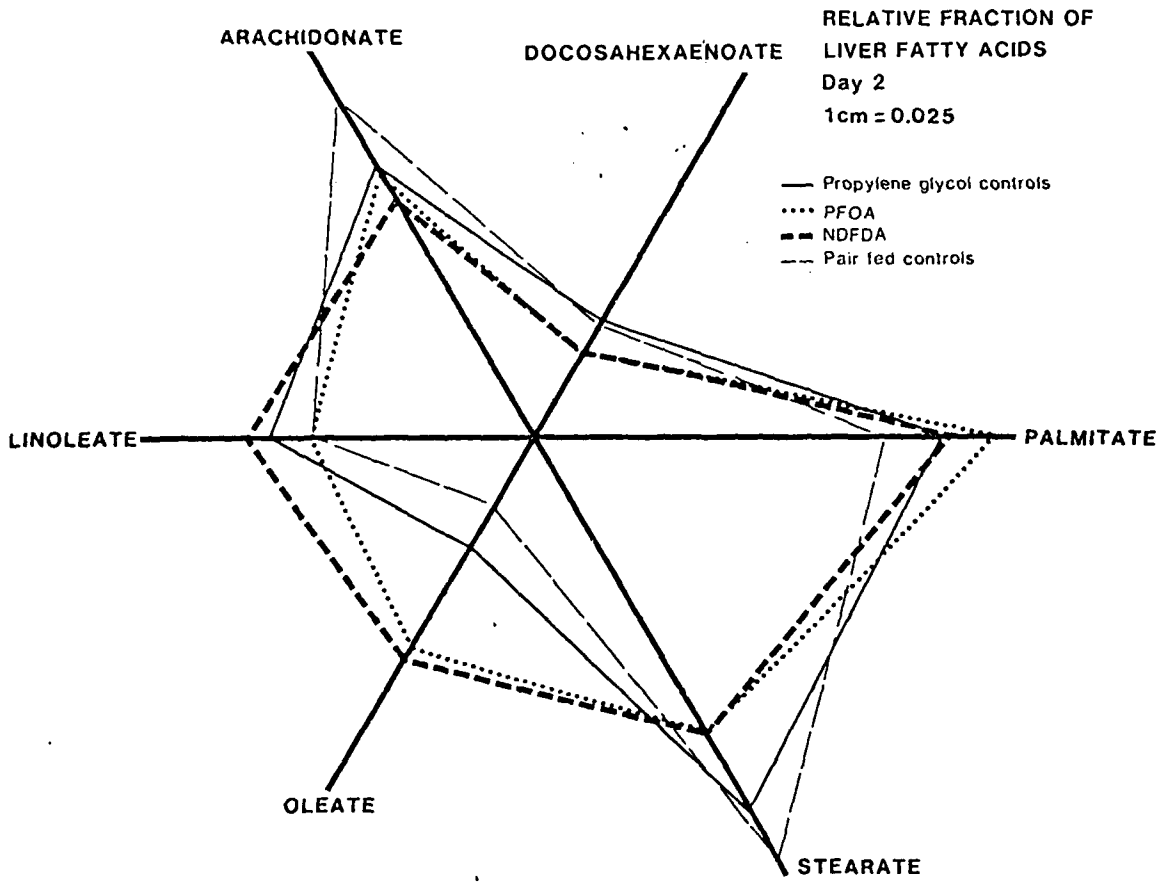


Figure 5. The relative fractions of the six major fatty acids in the livers of rats two days after treatment with NDFDA, PFOA, propylene glycol or propylene glycol with pair-feeding. Each equally separated "spoke" represents one fatty acid with the center representing 0.0 and each centimeter from the center equaling a relative fraction of 0.025. The relative fractions for each treatment are connected to illustrate differences and similarities in response.

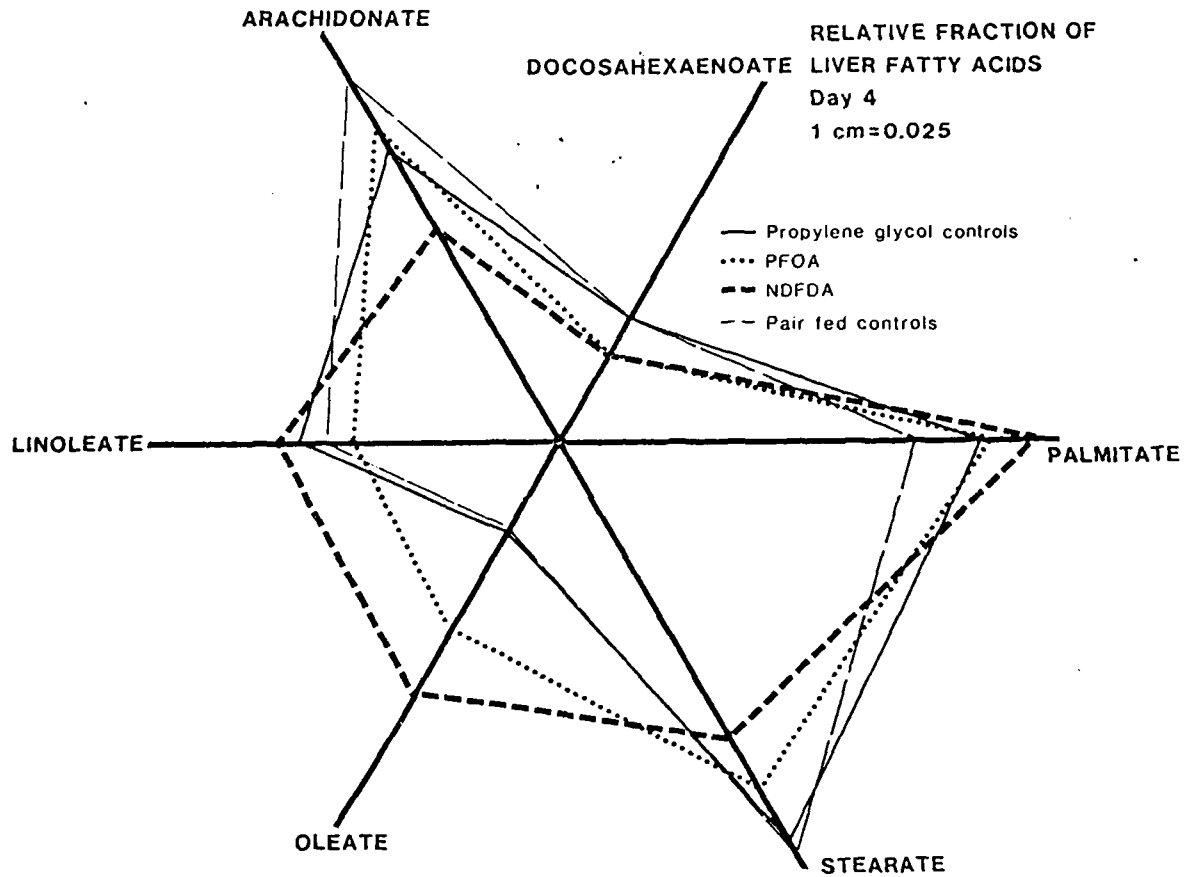


Figure 6 The relative fractions of the six major fatty acids in the livers of rats four days after treatment with NDFDA, PFOA, propylene glycol or propylene glycol with pair-feeding. Each equally separated "spoke" represents one fatty acid with the center representing 0.0 and each centimeter from the center equaling a relative fraction of 0.025. The relative fractions for each treatment are connected to illustrate differences and similarities in response.

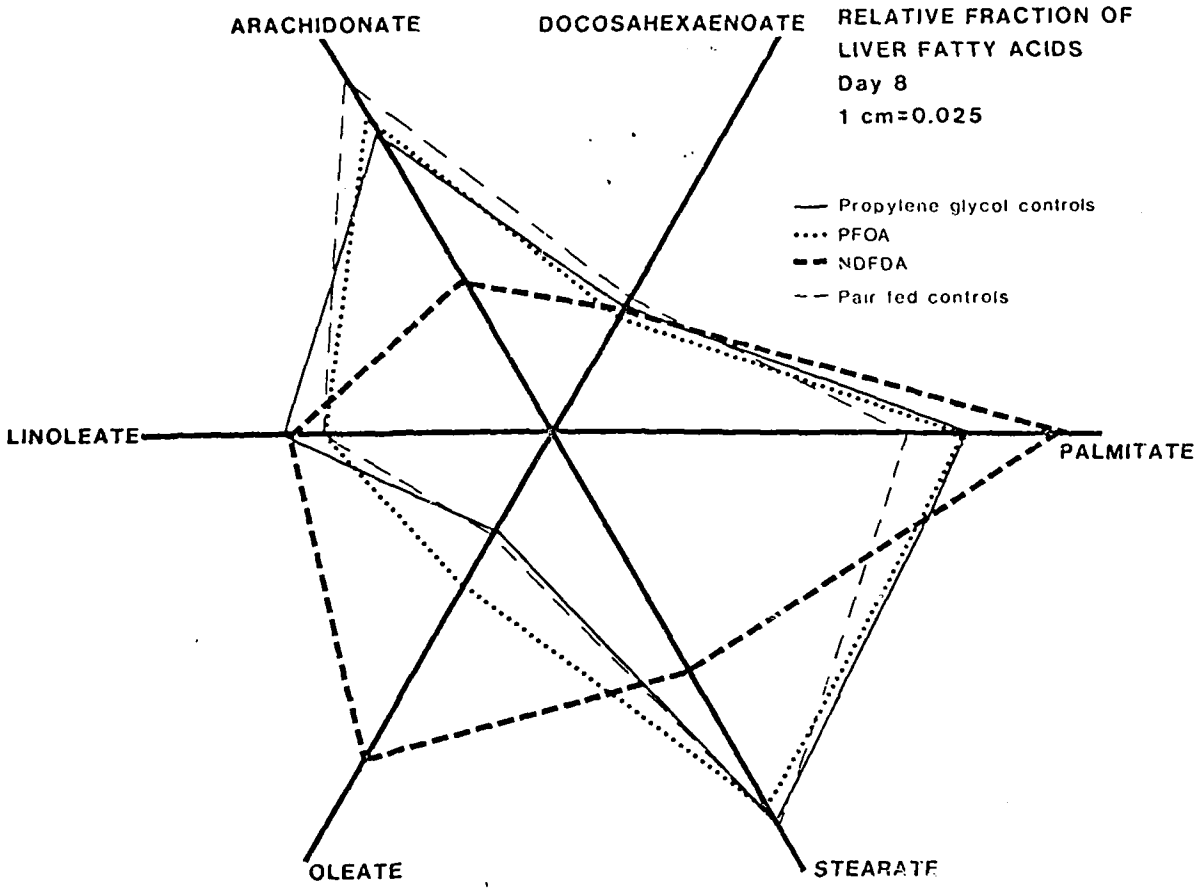


Figure 7. The relative fractions of the six major fatty acids in the livers of rats eight days after treatment with NDFDA, PFOA, propylene glycol or propylene glycol with pair-feeding. Each equally separated "spoke" represents one fatty acid with the center representing 0.0 and each centimeter from the center equaling a relative fraction of 0.025. The relative fractions for each treatment are connected to illustrate differences and similarities in response.

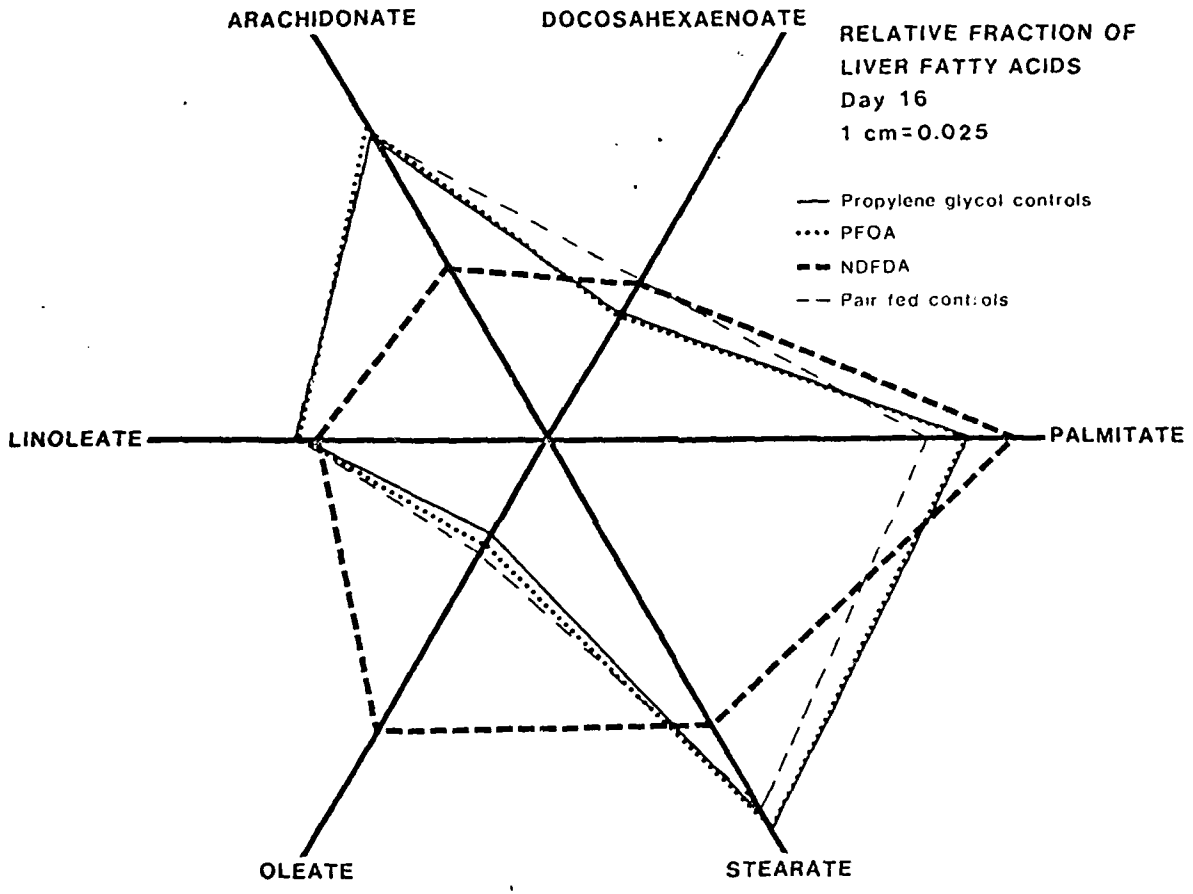


Figure 8. The relative fractions of the six major fatty acids in the livers of rats 16 days after treatment with NDFDA, PFOA, propylene glycol or propylene glycol with pair-feeding. Each equally separated "spoke" represents one fatty acid with the center representing 0.0 and each centimeter from the center equaling a relative fraction of 0.025. The relative fractions for each treatment are connected to illustrate differences and similarities in response.

stearic, arachidonic and docosahexaenoic acid fractions are reduced. PFOA-injected animals still show reduced stearic and docosahexaenoic acid fractions and an increased oleic acid fraction. On the eighth day, PFOA-injected animals exhibit a higher than normal fraction of oleic acid but this is decreasing and is not significantly different from controls by day 16. On both days 8 and 16, NDFDA-exposed animals exhibited increased fractions of palmitic and oleic acids and decreased fractions of stearic and arachidonic acids.

Another method of analyzing the data is to compare ratios of fatty acids which have an established relationship. Stearate is primarily formed from palmitate by a chain elongation reaction. Oleic acid is formed from stearic acid by a desaturase reaction. Arachidonic acid is believed to be formed from linoleic acid by elongation and desaturation. Therefore, one could evaluate a stearate to palmitate ratio (SPR), oleate to stearate ratio (OSR), and arachidonate to linoleate ratio (ALR). This was done using computerized statistical analysis. Only those sample means demonstrating a difference at the 0.05 significance level from both propylene glycol and pair-fed controls were deemed significant. There were no ratios from blood samples that met these criteria at any day tested. For testes, the SPR was significantly lower in NDFDA-treated animals only at day eight. Again, the greatest changes were in the liver. At day two, the SPR and ALR were decreased and the OSR increased for NDFDA-treated animals. The SPR was decreased, OSR increased, and ALR was between control values in PFOA-treated animals. At day four, the same conditions existed for SPR and OSR but the ALR was significantly decreased for only the NDFDA-treated rats. At day eight,

the SPR was decreased only for the NDFDA-treated animals while the OSR was increased and the ALR decreased for both PFOA and NDFDA treatments. At day 16, the SPR and ALR were decreased and the OSR increased only for the NDFDA-treated groups. This information is tabulated in Table 14.

Five rats given 50 mg/kg NDFDA that survived to day 30 were sacrificed and livers analyzed for relative distribution of the six major fatty acids. The average of the relative fractions were: palmitate - 0.261; stearate - 0.257; oleate - 0.160; linoleate - 0.062; arachidonate - 0.197; and docosahexaenoate - 0.063. Comparing these values with those from day 16 propylene glycol and pair-fed control animals, stearate and arachidonate fractions appear relatively normal, while palmitate and oleate levels appear elevated and linoleate and docosahexaenoate fractions appear depressed.

Mean organ weights at days 2, 4, 8, and 16 after treatment are presented in Tables 15 through 18. At day two, the mean liver weight of PFOA-treated animals is significantly (0.05 level) greater than both control groups and the heart weight is significantly less than controls for both PFOA and NDFDA-treated rats.

At day four, liver weights in PFOA-treated animals are still significantly greater than in controls and the mean heart weight of NDFDA-treated animals is significantly less. At day eight, the mean liver weight of PFOA-treated rats is still significantly greater than controls and that for NDFDA-treated animals is significantly different from controls but between the mean values for propylene glycol and pair-fed controls. Adrenal weights for NDFDA-treated animals are significantly less than controls as are body weights. Body weight mean for

Table 14

Significant Changes in Fatty Acid Ratios
with Treatment and Time

LIVER

Time	Ratio	Treatment NDFDA	PFOA
Day 2	SPR[a]	↓	↓
	OSR[b]	↑	↑
	ALR[c]	↓	*
Day 4	SPR	↓	↓
	OSR	↑	↑
	ALR	↓	–
Day 8	SPR	↓	–
	OSR	↑	↑
	ALR	↓	↓
Day 16	SPR	↓	–
	OSR	↑	–
	ALR	↓	–

---

[a] Stearate to palmitate ratio.

[b] Oleate to stearate ratio.

[c] Arachidonate to linoleate ratio.

↑ Significantly greater than control values at 0.05 level.

↓ Significantly less than control values at 0.05 level.

* Between control values.

– Not significantly different from both control group values.

Table 15

Mean Organ Weights (Grams) - Day 2

	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Organ:				
Brain	1.753	1.733	1.690	1.745
Liver	8.4[a]	10.4[b]	8.4	6.0
Testes	2.5[a]	2.6	2.6	2.5
Kidneys	1.607	1.579	1.553	1.522
Adrenals	0.037	0.039	0.035	0.039
Heart	0.596	0.553[c]	0.523[c]	0.594
Body Weight	207.3	197.7	185.2	193.8

[a] Liver and testes weighed to the nearest 0.1 gram.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

Table 16

Mean Organ Weights (Grams) - Day 4

	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Organ:				
Brain	1.750	1.710	1.700	1.763
Liver	8.9[a]	10.5[b]	7.9	5.4
Testes	2.7[a]	2.6	2.4	2.6
Kidneys	1.717	1.633	1.503	1.466
Adrenals	0.039	0.038	0.035	0.044
Heart	0.625	0.546	0.483[c]	0.560
Body Weight	221.2	201.8	173.5	186.7

[a] Liver and testes weighed to the nearest 0.1 gram.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

Table 17

Mean Organ Weights (Grams) - Day 8

	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Organ:				
Brain	1.776	1.754	1.691	1.686
Liver	9.0[a]	10.8[b]	7.6[d]	4.1
Testes	2.7[a]	2.6	2.3	2.5
Kidneys	1.766	1.794	1.343	1.271
Adrenals	0.036	0.038	0.032[c]	0.039
Heart	0.628	0.646	0.396	0.477
Body Weight	229.5	217.5[d]	139.8[c]	158.5

[a] Liver and testes weighed to the nearest 0.1 gram.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

[d] Significantly different from but between control values.

Table 18

Mean Organ Weights (Grams) - Day 16

	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Organ:				
Brain	1.776	1.759	1.645	1.684
Liver	9.9[a]	10.3	7.6[c]	3.0
Testes	2.8[a]	2.7	1.7[b]	2.2
Kidneys	1.870	1.892	1.214	1.110
Adrenals	0.040	0.041	0.027[b]	0.040
Heart	0.673	0.613[c]	0.304[b]	0.368
Body Weight	252.3	241.7[c]	109.2[b]	130.8

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[a] Liver and testes weighed to the nearest 0.1 gram.

[b] Significantly less than control values at 0.05 level.

[c] Significantly different from but between control values.

PFOA animals is significantly different from but between values for propylene glycol and pair-fed controls.

At day 16, mean testes, adrenals, heart and body weight values are less than both control groups for NDFDA-treated rats. Liver weights are also significantly different but between control group weights. Heart and body weight mean values for PFOA-treated animals are different from but between control group means.

Organ weights were also analyzed by dividing by body weight or brain weight. These data are presented in Tables 19 through 26.

The weights of chloroform extractable material from 2 g of liver, 2 g of testes, and 2 ml of blood were obtained from a limited number of animals. At day 2 postexposure, with 2 observations per treatment group, there were no significant differences (0.05 level) in weight of chloroform-extractable material in PFOA or NDFDA-treated animals from both propylene glycol injected and pair-fed controls. At day 4 post-exposure, with 3 observations per treatment group, no significant differences existed. At day 8, with 2 observations per treatment group, the weight of chloroform extractable material from 2 g of liver from NDFDA-treated rats was significantly greater than that from propylene glycol or pair-fed controls and from PFOA-treated rats.

By day 16, with 3 rats in the NDFDA and propylene glycol-treated groups and 4 in the other two treatment groups, the only significant difference was in the testes of the NDFDA-treated group which showed a greater amount of chloroform extractable material than any of the others.

Mean body weights and food consumption for the four treatment groups are presented in Table 27. The PFOA group shows a transient

Table 19

Organ Weights Divided by Body Weight[a] - Day 2

Organ:	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Brain	0.008	0.009	0.009	0.009
Liver	0.040	0.052[b]	0.045[b]	0.031
Testes	0.012	0.013	0.014[b]	0.013
Kidneys	0.0078	0.0080	0.0084[b]	0.0079
Adrenals	0.00018	0.00020	0.00019	0.00020
Heart	0.0029	0.0028	0.0028	0.0031

[a] Individual organ weights divided by body weight and then averaged for the six groups.

[b] Significantly greater than control values at 0.05 level.

Table 20

Organ Weights Divided by Brain Weight[a] - Day 2

	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Organ:				
Liver	4.77	5.99[b]	4.99	3.46
Testes	1.43	1.49	1.52	1.43
Kidneys	0.918	0.912	0.918	0.872
Adrenals	0.021	0.023	0.021	0.022
Heart	0.340	0.319[c]	0.309[c]	0.341
Body Weight	118.3	114.1	109.6	111.0

[a] Individual organ weights divided by brain weight and then averaged for the six groups.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

Table 21

Organ Weights Divided by Body Weight[a] - Day 4

	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Organ:				
Brain	0.008	0.009	0.010	0.009
Liver	0.040	0.052[b]	0.046[b]	0.029
Testes	0.012	0.013	0.014	0.014
Kidneys	0.0078	0.0081	0.0087[b]	0.0079
Adrenals	0.00017	0.00019	0.00020[d]	0.00023
Heart	0.0028	0.0027[c]	0.0028	0.0030

[a] Individual organ weights divided by body weight and then averaged for the six groups.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

[d] Significantly different from but between control values.

Table 22

Organ Weights Divided by Brain Weight[a] - Day 4

	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Organ:				
Liver	5.11	6.16[b]	4.66	3.03
Testes	1.54	1.50	1.43	1.49
Kidneys	0.981	0.953	0.884	0.832
Adrenals	0.022	0.022	0.021	0.025
Heart	0.357	0.319	0.284[c]	0.318
Body Weight	126.5	118.0[d]	102.0	106.0

[a] Individual organ weights divided by brain weight and then averaged for the six groups.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

[d] Significantly different from but between control values.

Table 23

Organ Weights Divided by Body Weight[a] - Day 8

	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Organ:				
Brain	0.008	0.008	0.012[b]	0.011
Liver	0.039	0.050[b]	0.054[b]	0.026
Testes	0.012	0.012	0.016	0.015
Kidneys	0.0077	0.0082	0.0096[b]	0.0080
Adrenals	0.00016	0.00017	0.00023	0.00024
Heart	0.0027	0.0030	0.0028	0.0030

[a] Individual organ weights divided by body weight and then averaged for the six groups.

[b] Significantly greater than control values at 0.05 level.

Table 24

Organ Weights Divided by Brain Weight[a] - Day 8

	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Organ:				
Liver	5.09	6.18[b]	4.47[d]	2.41
Testes	1.53	1.47	1.36	1.45
Kidneys	0.997	1.023	0.794	0.755
Adrenals	0.020	0.021	0.019	0.023
Heart	0.354	0.367	0.234	0.283
Body Weight	129.3	124.0[d]	82.8[c]	94.0

[a] Individual organ weights divided by brain weight and then averaged for the six groups.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

[d] Significantly different from but between control values.

Table 25

Organ Weights Divided by Body Weight[a] - Day 16

	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Organ:				
Brain	0.007	0.007	0.015[b]	0.013
Liver	0.039	0.042[b]	0.069[b]	0.023
Testes	0.011	0.011	0.016	0.017
Kidneys	0.0074	0.0078	0.011[b]	0.0085
Adrenals	0.00016	0.00017	0.00025[d]	0.00030
Heart	0.0027	0.0025[c]	0.0028	0.0028

[a] Individual organ weights divided by body weight and then averaged for the six groups.

[b] Significantly greater than control values at 0.05 level.

[c] Significantly less than control values at 0.05 level.

[d] Significantly different from but between control values.

Table 26

Organ Weights Divided by Brain Weight[a] - Day 16

	Propylene Glycol Controls	PFOA	NDFDA	Pair-Fed Controls
Organ:				
Liver	5.57	5.84	4.61[c]	1.76
Testes	1.58	1.55	1.02[b]	1.29
Kidneys	1.052	1.075	0.740[c]	0.659
Adrenals	0.022	0.023	0.017[b]	0.023
Heart	0.379	0.348[c]	0.185[b]	0.218
Body Weight	142.0	137.4	66.5[b]	77.7

[a] Individual organ weights divided by brain weight and then averaged for the six groups.

[b] Significantly less than control values at 0.05 level.

[c] Significantly different from but between control values.

Table 27

Mean Body Weight and Food Consumption

Day	Number of Animals	Propylene Glycol Controls		PFOA		NDFDA		Pair-Fed Controls	
		Body Weight (g)	Food (g)	Body Weight (g)	Food (g)	Body Weight (g)	Food (g)	Body Weight (g)	Food (g)
-1	24	199	15	203	14	202	16	206	17
0	24	204	16	208	15	207	16	210	16
1	24	206	15	193	2	197	8	204	7
2	24	210	16	196	10	189	7	199	7
3	18	214	15	199	11	182	4	195	5
4	18	217	16	202	12	175	4	191	4
5	12	219	15	205	13	167	2	186	2
6	12	220	16	207	14	158	2	181	3
7	12	226	15	213	15	150	1	174	2
8	12	230	16	217	15	143	1	169	1
9	6	232	14	217	14	140	0	172	1
10	6	235	16	221	15	134	0	165	0
11	6	237	14	225	14	127	1	159	1
12	6	241	16	228	14	122	0	150	0
13	6	242	16	230	16	116	1	145	1
14	6	247	16	233	15	112	1	137	1
15	6	251	16	238	16	110	2	134	2
16	6	252	15	242	16	109	3	131	3

decrease in food consumption and a rapid weight loss with return to control-like values in approximately one week. The NDFDA-treated group shows a continual weight loss and decreased food consumption for the 16-day period of observation. Pair-fed controls, animals fed the same quantity of food eaten by NDFDA-treated rats, showed substantial but less weight loss than the NDFDA-treated rats.

If lipid peroxidation is occurring as a result of NDFDA injection, one would expect a decrease in unsaturated fatty acids. The relative arachidonic acid content is definitely decreased but a marked decrease in linoleic and docosahexaenoic acids is not evident (Figures 5 through 8). To determine if lipid peroxidation similar to that caused by carbon tetrachloride is a significant mechanism of injury following NDFDA exposure, two rats were injected with 50 mg/kg NDFDA at 2 ml/kg intraperitoneally and two were injected with 2 ml/kg of the 1:1 propylene glycol/water solvent to serve as controls. These animals were sacrificed at days 4 and 12 post injection and liver microsomal lipids from two 2 g samples for each animal were isolated following the techniques of Waller and Recknagel (1977). On both days of sacrifice, an additional rat was given 250 ul carbon tetrachloride per 100 g body weight, killed 90 minutes later and liver microsomal lipids isolated. The liver from one untreated rat was processed similarly. Samples were dried, weighed, and dissolved in 10 ml of spectrophotometric grade cyclohexane. Optical densities (OD) at 233 nm were determined spectrophotometrically[15] to measure conjugated diene content. A standardized OD was calculated

15 Beckman, ACTA III Spectrophotometer, Beckman Instruments, Inc., Irvine CA.

by multiplying observed density by the reciprocal of the weight (in mg) of the microsomal lipids per ml of cyclohexane for each sample.

To compare optical densities, the average of the standardized densities for the two samples from each rat was calculated and this density from NDFDA and propylene glycol-treated and untreated animals was divided by that for carbon tetrachloride-treated rats. Results are tabulated in Table 28. Although the optical densities vary, the ratios are quite consistent. Based on optical densities, the conjugated diene content of the liver was greater in propylene glycol control rats than in rats treated with NDFDA dissolved in propylene glycol. Days 4 and 12 were chosen because if NDFDA-treated animals respond similarly to PFOA-treated animals, the response at day 4 may be similar to that expected in PFOA-treated animals and by day 12 a second mechanism, if such exists, might be in effect. Results obtained here appear to show that NDFDA does not cause extensive microsomal lipid peroxidation.

Table 28

Conjugated Diene Measurements in Liver Microsomes

Sample	Time	Standardized OD at 233 nm	$\frac{\text{Standardized OD Sample}}{\text{Standardized OD Carbon Tetrachloride}}$
Propylene Glycol S#1	Day 4	1.27	$\frac{1.27}{1.52} = 0.84$
Propylene Glycol S#2	Day 4	1.26	
NDFDA S#1	Day 4	1.11	$\frac{1.03}{1.52} = 0.68$
NDFDA S#2	Day 4	0.94	
Carbon Tetrachloride S#1	90 Min	1.52	
Carbon Tetrachloride S#2	90 Min	1.52	
Untreated Control S#1		0.64	$\frac{0.61}{1.52} = 0.40$
Untreated Control S#2		0.57	
Propylene Glycol S#1	Day 12	1.13	$\frac{1.05}{1.20} = 0.88$
Propylene Glycol S#2	Day 12	0.97	
NDFDA S#1	Day 12	0.78	$\frac{0.78}{1.20} = 0.65$
NDFDA S#2	Day 12	0.77	
Carbon Tetrachloride S#1	90 Min	1.24	
Carbon Tetrachloride S#2	90 Min	1.16	

DISCUSSION

Although perfluorinated compounds have generally been considered biologically inert (Sargent and Seffl, 1970; Clark et al., 1973), the $LD_{50/30}$ for intraperitoneal injection of NDFDA in 200 g, male F-344 rats was determined in this study to be approximately 41 mg/kg. The $LD_{50/30}$ for PFOA, a compound of little structural difference, was approximately 189 mg/kg. After injection with NDFDA, the lethal response of rats, when plotted on a log dose versus time basis, does not appear similar to that of rats injected with PFOA. At higher doses, there is a similarity in response, but as the dosage is lowered a second mechanism of toxicity seems to occur in NDFDA-treated rats.

Liver w. ights appear to increase and heart and body weights are significantly ($p < 0.05$) lower than in propylene glycol and pair-fed controls for both NDFDA and PFOA-treated rats. Mean testes and adrenals weights are significantly ($p < 0.05$) lower than controls only in NDFDA-treated rats and these were first seen at the 8th day. Organ weight changes in PFOA-treated rats appear earlier and last a shorter duration than those in rats treated with NDFDA.

There is a definite change in fatty acid content in livers of NDFDA-treated rats with a relative increase in palmitate and oleate and a relative decrease in stearate and arachidonate that cannot be attributed to reduced food intake. Testes and blood fatty acids show

similar changes but to a lesser degree. A similar trend is seen after exposure to PFOA but this is of lesser magnitude and more transient in nature. This shift in fatty acid content appears unrelated to microsomal lipid peroxidation at day 4 or day 12 post-exposure. Levels of conjugated dienes measured in microsomes isolated from livers of NDFDA-treated rats at these times were lower than in propylene glycol-treated control rats, although only one treated rat was examined at each day.

Mechanisms of toxicity of NDFDA remain in doubt. It is possible that, as Fox (1981) reported, oxidation of fatty acids leads to the production of prostaglandins and other biologically-active derivatives such as leukotrienes which may adversely affect physiological activities. The decrease in arachidonic acid, a prostaglandin and leukotriene precursor, may give credence to this hypothesis.

Rozner (1980) examined the effects of PFOA on microsomal membrane peroxidation in vitro using malondialdehyde (MDA) formation for analysis. He found that MDA was produced to an extent similar to that seen after carbon tetrachloride treatment. He concluded, however, that PFOA and carbon tetrachloride stimulate peroxidation by different mechanisms. He based this conclusion on several findings:

1. Concentrations of PFOA that produced maximum peroxidation significantly reduced microsomal electron transport, unlike carbon tetrachloride. He also found, but could not explain, no reduction in NADPH levels.

2. Addition of EDTA reduced PFOA peroxidation to a level indistinguishable from controls but reduced carbon tetrachloride peroxidation by only 70%.

3. PFOA or ferrous iron, but not carbon tetrachloride, stimulated peroxidation in the presence of ascorbic acid, a non-enzymatic reducing agent.

Thiobarbituric acid (TBA) is used to detect MDA by means of a color reaction. This test is also used to detect endoperoxides of fatty acids. TBA tests are being conducted on blood samples in Japan where some scientists are attributing a variable TBA response to fluctuations in prostaglandin precursors (Fox, 1981). The TBA reaction is also being used to measure lipoxygenase activity (Bailey and Chakrin, 1981). Platelet lipoxygenase requires ferric iron for leukotriene formation. By contrast, platelet cyclooxygenase is dependent on ferrous iron for prostaglandin formation (Bailey and Chakrin, 1981). It is therefore possible that Rosner was measuring prostaglandin precursors rather than lipid free radical formation.

A direct effect of NDFDA on membrane function can also be advocated. Recent experimentation in this laboratory has demonstrated a decreased osmotic fragility and increased cell membrane fluidity of erythrocytes from rats injected with NDFDA (George, 1982). Changes in saturation of fatty acids in cell membrane phospholipids affects fluidity within and transport across the membrane. Prostaglandin synthesis has been shown to alter cell membrane fluidity (Das, 1981; Knazek et al., 1981).

The effects of NDFDA on treated rats and the similarity to effects of dioxin injection create much interest in this compound. Additional research on cell membrane fluidity, fatty acid composition, and prostaglandin and various enzyme levels will be carried out.

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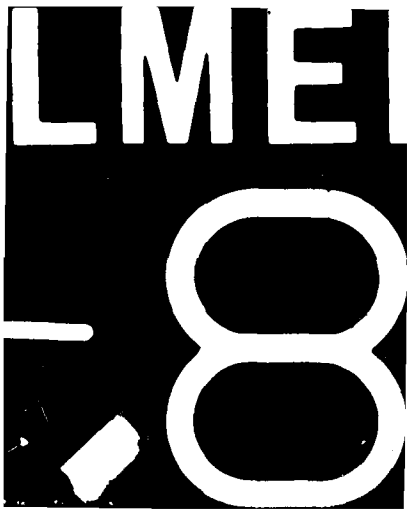
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