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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1 Nov 90	FINAL 01 Jun 85 to 31 May 90

4. TITLE AND SUBTITLE
Toxicology of Perfluorodecanoic Acid

5. FUNDING NUMBERS
G - AFOSR-85-0207
PE - 61102F
PR - 2312
TA - A5

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8. PERFORMING ORGANIZATION REPORT NUMBER
AFOSR-TR- 90 1183

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
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AFOSR/NL
Building 410
Bolling AFB, DC 20332

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Despite hypothyroxinemia, PFDA-treated rats are not functionally hypothyroid. Furthermore, any alteration in functional thyroid status can be dissociated from the overt toxicity (i.e., severe hypophagia and body weight loss). PFDA exerts effects on neutral lipid metabolism in both liver and carcass of the rat. At 7 days following a single administration of PFDA, hepatic esterification of free fatty acid into TG and CE was increased yet the expected augmentation in the export of these neutral lipids from liver into plasma was absent. An efficient chemical method was developed for the purification of commercially available PFDA that contains contaminants of mono- and diprotio-substituted materials. PFDA of high specific activity has been synthesized with ^{14}C-labeling in the C-1 position. In vivo experiments indicate that perfluorinated acid derived radioactivity found in rat tissues behaves similarly to PFDA or PFOA added directly to frozen rat tissue. Hepatic oxidation of long-chain medium-chain and short-chain fatty acids was unaffected by pretreatment with PFDA or PFOA. Similarly, esterification was not affected.

14. SUBJECT TERMS	15. NUMBER OF PAGES
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
(U)	(U)	(U)	(U)

U.S AIR FORCE OFFICE OF SCIENTIFIC RESEARCH

FINAL TECHNICAL REPORT

TOXICOLOGY OF PERFLUORODECANOIC ACID

AFOSR-85-0207

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Period Covered 6/1/1985 to 5/31/1990

I. Perfluorodecanoic Acid (PFDA) and Thyroid Status.

A. Statement of Problem:

1. Toxic doses of PFDA result in reduction of feed intake, body weight, serum concentrations of thyroxine (T_4) and triiodothyronine (T_3), resting heart rate and body temperature (Langley and Pilcher, 1985). Some of these effects resemble changes characteristic of hypothyroidism.

B. Objective:

1. To define the role of thyroid dysfunction in PFDA toxicity.

C. Experimental Findings:

1. Reduction in basal metabolic rate of PFDA-treated rats was probably not due to hypothyroidism, but rather was secondary to PFDA-induced hypophagia and weight loss (Van Rafelghem et al., 1986; 1987a).

2. Thermogenesis, as determined by oxygen consumption and body core temperatures, was not greatly affected by the administration of PFDA and these changes were paralleled in vehicle-treated rats pair-fed to those receiving the perfluorinated acid (Van Rafelghem et al., 1986; 1987a).

3. Energy expenditure, evaluated indirectly by measuring oxygen consumption and carbon dioxide production, decreased in a dose-dependent manner, but was similar in PFDA-treated rats and their pair-fed counterparts at a given dose (Van Rafelghem et al., 1987b).

4. Following the administration of PFDA, L-glycerol-3-phosphate dehydrogenase, a liver mitochondrial enzyme sensitive to thyroid status, exhibited a modest augmentation in activity (Kelling et al., 1987). Yet, the activity of cytosolic malic enzyme, also a thyroid-responsive hepatic enzyme, was increased dramatically in PFDA-treated rats (Kelling et al., 1987). The administration of PFDA to adult male rats in both the fed and fasted nutritional states has been found to regulate hepatic malic enzyme by not only increasing enzyme quantity but also by augmenting the specific activity, (i.e., catalytic state) of the enzyme (Kelling et al.; 1986). Thus, the pattern of activity for thyroid-responsive liver enzymes after administration of PFDA to

the adult rat is not compatible with a functional shift toward a lessened thyroid status.

D. Conclusion:

1. Despite the hypothyroxinemia, PFDA-treated rats are not functionally hypothyroid. Furthermore, any alteration in functional thyroid status can be dissociated from the overt toxicity (i.e., severe hypophagia and body weight loss).

II. Perfluorodecanoic Acid (PFDA) and Lipid Metabolism in the Rat.

A. Statement of Problem:

1. PFDA in a dose-dependent manner decreased both feed intake and body weight of adult male rats. Body weight loss was greater in PFDA-treated rats than their pair-fed partners. Yet, PFDA-treated rats were found to have a higher carcass fat content than their pair-fed partners. Thus with the same caloric intake, rats receiving PFDA lost more weight yet maintained a greater body fat content than their pair-fed counterparts (Van Rafelghem et al., 1987b).

B. Objective:

1. To define what effects, if any, PFDA might have on lipid metabolism in the adult male rat.

C. Experimental Findings (Van Rafelghem et al., 1987c):

1. At 7 days after PFDA treatment, a dose-dependent decrease in carcass triacylglycerols (TG) due to hypophagia was found. Yet at every dose examined, rats receiving PFDA had a greater carcass TG content than their pair-fed partners.
2. Hepatic content of TG was increased in a dose-dependent manner in PFDA-treated rats, yet it decreased in their pair-fed counterparts.
3. Despite the elevated amount of liver TG in PFDA-treated animals, no concomitant increase in the concentration of TG in plasma was found.
4. A dose-related increase in hepatic content of cholesteryl esters (CE) also characterized PFDA-treated rats.

D. Conclusions:

1. PFDA exerts effects on neutral lipid metabolism in both liver and carcass of the rat.
2. At 7 days following a single administration of PFDA, hepatic esterification of free fatty acid into TG and CE was increased yet the expected augmentation in the export of these neutral lipids from liver into plasma was absent.

III. Synthesis of ^{14}C-Labeled Perfluorodecanoic Acid.

A. Statement of Problem:

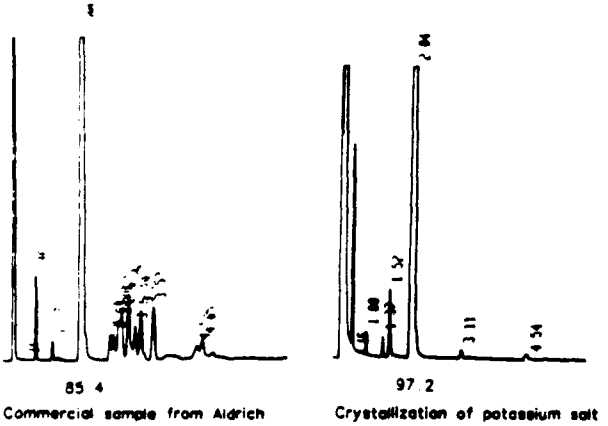
Investigation of PFDA metabolism is thought to be crucial in understanding the effects of the perfluorinated acid on energy metabolism, especially that of lipids (Van Rafelghem et al., 1987b;c). The availability of ^{14}C-labeled PFDA would provide a sensitive tracer for its metabolism. But ^{14}C-labeled perfluorinated acids are not available commercially.

B. Objectives:

1. To synthesize perfluoro-n-decanoic acid (PFDA) with ^{14}C-labeling in the C-1 position.
2. To devise a chemical purification for the commercially available PFDA as it contains mono- and diprotio substituted impurities (George and Andersen, 1986).

C. Experimental Findings (Reich et al., 1987):

1. Refluxing the potassium salt of commercial PFDA resulted in nearly complete removal of the impurities after crystallization of the salt.



Purification of $CF_3(CF_2)_8COOH$

2. Perfluorononyl iodide was synthesized from the silver salt of the purified PFDA using Hunsdiecker reaction.
3. The ^{14}C-carboxyl labeled PFDA was prepared by carbonation of perfluorononyllithium at -100°C.

D. Conclusions:

1. An efficient chemical method was developed for the purification of commercially available PFDA that contains contaminants of mono- and diprotio-substituted materials.
2. PFDA of high specific activity has been synthesized with ^{14}C-labeling in the C-1 position.

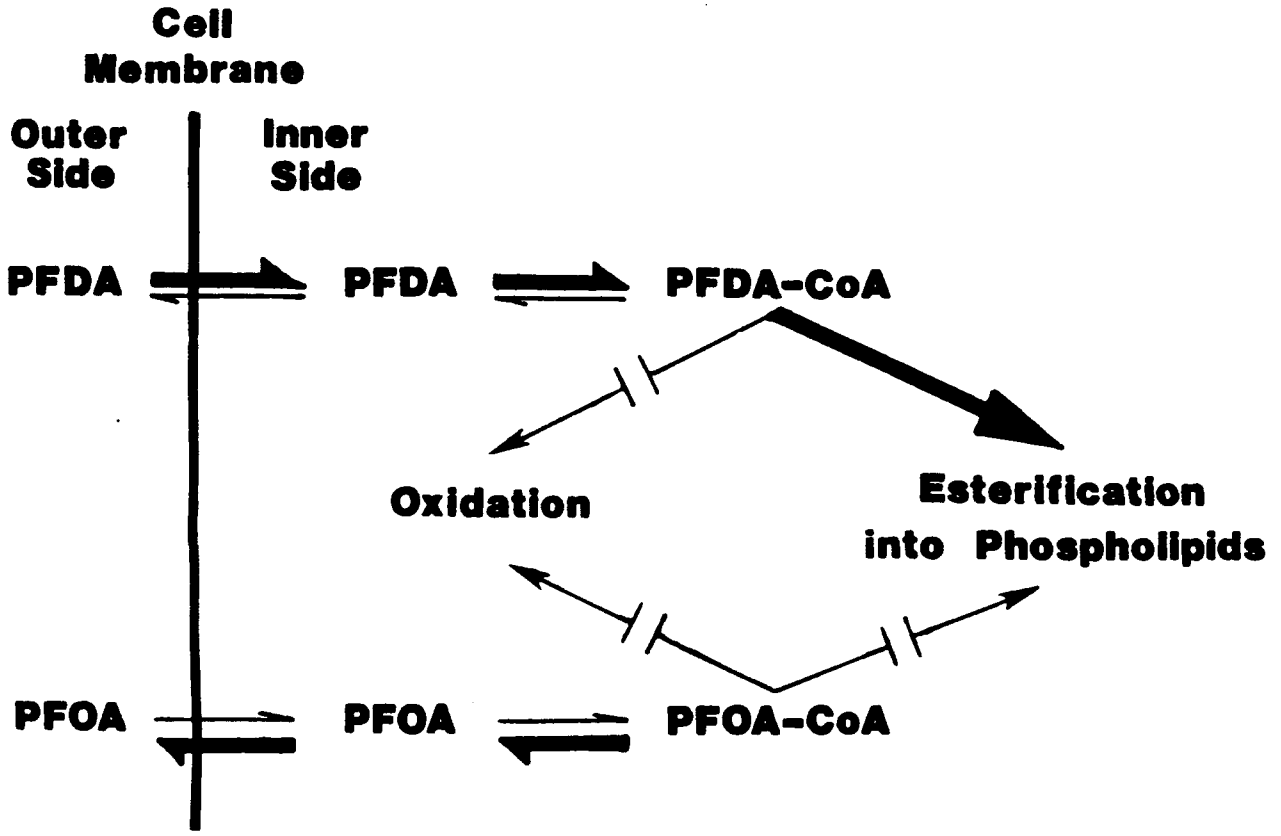
IV. Metabolism of Perfuorodecanoic Acid (PFDA) in the Rat

A. Statement of the Problem:

1. The pattern of acute toxicity of PFDA and perfluorooctanoic acid (PFOA) including lethality, is identical when extremely high doses of either perfluorinated acid are given (Olsen and Andersen, 1983). When lower doses of PFDA are administered, a starvation-like syndrome with delayed lethalities and disruption of hepatic architecture in rats is observed (Van Rafelghem et al., 1982). With smaller doses of PFOA, toxic effects are minimal and transient and all rats survive (Olsen and Andersen, 1983).
2. Even though administration of PFDA results in changes in certain of the neutral lipid classes in both liver and carcass of the rat (Van Rafelghem et al., 1987c), separation of liver lipids by thin-layer chromatography has indicated that PFDA was found to the greatest degree in the most polar fraction, e.g. phospholipids, (George and Andersen, 1986). George and Andersen (1986) have suggested that at least part of the hepatic PFDA is in a much more polar compound than the parent carboxylic acid but this is not a phospholipid.

B. Hypothesis:

CELLULAR METABOLISM OF PFDA AND PFOA



Thus, it is hypothesized that esterification of PFDA into phospholipids occurs in all cells, including hepatocytes. Yet, it is postulated that PFOA can be activated into its coenzyme A ester but cannot be esterified into phospholipids. Thus, PFDA esterified into phospholipids, most likely incorporated into cellular membranes, serves as an intracellular

store that is not available for PFOA. The presence of such a storage capability for PFDA (but not PFOA) might explain the differences in toxic potency of these two compounds.

C. Development of procedure to isolate PFDA-containing lipids:

1. Objective:

a. Develop extraction procedures for the recovery of [1-^{14}C]PFDA and [9,10-^{3}H]palmitic added to rat tissues.

b. Develop column chromatographic procedures to separate simple (eg. fatty acids, triacylglycerols, cholesterol and cholesteryl esters) and complex lipids (phospholipids) such that PFDA partitions with the neutral lipids.

c. Develop a thin layer chromatographic procedure to separate the neutral lipids such that incorporation of PFDA, as well as palmitic acid, into neutral lipids such as triacylglycerols can be examined.

d. Examine if the developed procedures are applicable to <u>in vivo</u> studies.

2. Experimental Findings (Vanden Heuvel *et al.*, 1989)

a. Due to the unique physical structure of the perfluorinated acids, conventional lipid extraction procedures were unable to sufficiently recover [1-^{14}C]PFDA or [1-^{14}C]PFOA added to tissues. The inclusion of dilute sulfuric acid in the aqueous solvent systems greatly increased the partitioning of PFDA or PFOA

organic phases. The final recovery of ^{14}C from the extraction procedures was approximately 80% for the perfluorinated acids added to liver, heart, gastrocnemius, testis, epididymal fat pad, kidney and plasma. Essentially 100% of [^{3}H]palmitic acid, used to label the endogenous fatty acids, was extracted from tissues.

b. Silica gel chromatography has been used extensively to separate phospholipids from simple lipids such as triacylglycerols, fatty acids and cholesteryl esters. Due to the electron withdrawing ability of the fluorine atoms within PFDA and PFOA, these compounds were not recovered with the endogenous fatty acids as desired. The use of the strong organic acid, trifluoroacetic acid, in the eluting solvent mixture vastly improved the recovery of the perfluorinated fatty acids from the column. The mixture of diethyl ether/trifluoroacetic acid (100:1, v/v) eluted 100% of the palmitic acid, PFDA or PFOA added to the column, while recovering less than 3% of the phospholipids.

c. The determination of possible PFDA or PFOA hybrid-lipid formation, such as a perfluorinated acid containing triacylglycerol or cholesteryl ester, requires the separation of the simple lipids into various classes. Thin layer chromatography is often used for this purpose. The use of conventional lipid

isolation procedures caused PFDA or PFOA to remain at the origin, making detection of a metabolite difficult. The inclusion of trifluoroacetic acid to the developing solvent caused PFDA and PFOA to migrate similarly to the endogenous fatty acids decanoic and octanoic acids.

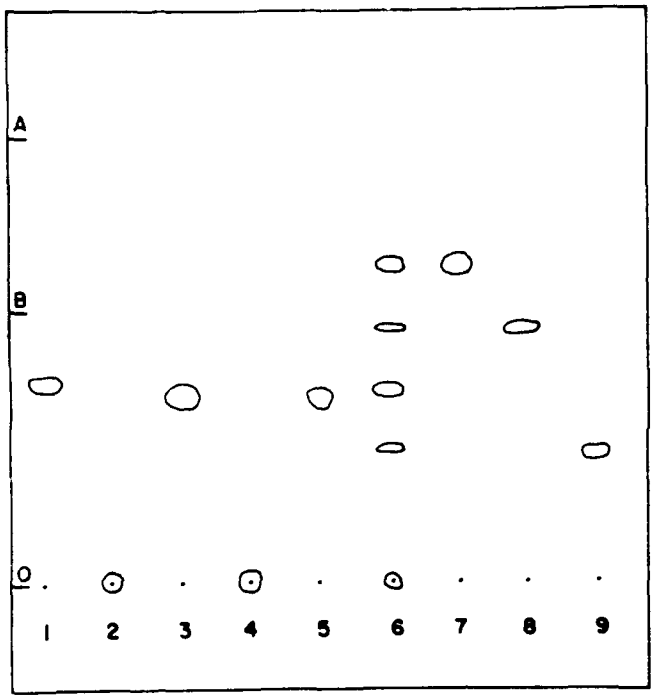


FIG. 1. TLC separation of fatty acid and simple lipid standards, using glacial acetic acid in the developing solvents. Lipids were spotted 3 cm (O) from the bottom of the TLC plate and developed to a height of 16 cm (A) in a solvent system of petroleum ether/diethyl ether/glacial acetic acid (70:30:1, v/v). The plate was air-dried and developed in the same direction in diethyl ether/petroleum ether/glacial acetie acid (70:30:1, v/v) to a height of 11 cm (B). Standards were dissolved in chloroform/methanol (2:1, v/v) and spotted as follows: 1, palmitic acid (20 μg); 2, PFDA (50 μg); 3, decanoic acid (50 μg); 4, PFOA (50 μg); 5, octanoic acid (50 μg); 6, mixture of palmitic acid, PFDA, cholesteryl palmitate, tripalmitin, and cholesterol; 7, cholesteryl palmitate (20 μg); 8, tripalmitin (20 μg); 9, cholesterol (20 μg).

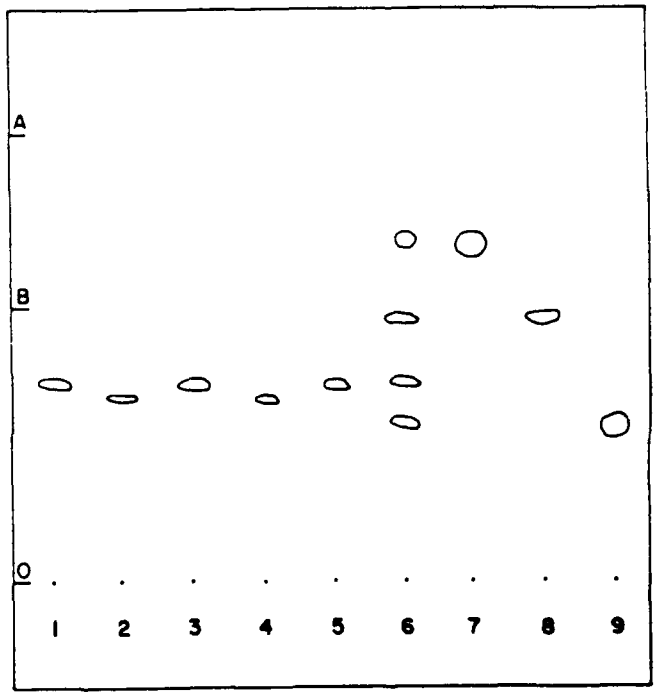


FIG. 2. TLC separation of fatty acid and simple lipid standards, using trifluoroacetic acid in the developing solvents. All conditions were the same as described in the legend to Fig. 1, except that glacial acetic acid was replaced by trifluoroacetic acid in the TLC developing system.

d. In order to validate our methodology following <u>in vivo</u> administration, male rats were given a single ip dose of 9.4 μmol/kg of either [1-^{14}C]PFDA or [1-^{14}C]PFOA and tissues removed 4 days post-treatment. The extent of extraction

following *in vivo* treatment was similar to that seen for the *in vitro* labelling experiments. In addition, the separation of phospholipids and simple lipids using silica gel columns yielded similar results, with essentially no radioactivity being eluted with the phospholipids.

3. Conclusions:

a. The use of the modified extraction, separation and isolation procedures enabled the metabolism of PFDA and PFOA to be examined with endogenous fatty acids such as palmitic acid.

b. The procedures developed have been validated for *in vivo* study of PFDA and PFOA metabolism. The *in vivo* experiments indicate that the perfluorinated acid derived radioactivity found in these rat tissues behaves similarly to PFDA or PFOA added directly to frozen rat tissue.

IV. *In Situ* Perfused Rat Liver

1. Objectives:

a. To develop, implement, and characterize an *in situ* rat liver perfusion system.

b. To examine the effects of PFDA or PFOA treatment on the oxidation and esterification of various fatty acids. A representative long-chain (oleic acid), medium chain (octanoic acid) and short-chain (pyruvic acid) will be examined.

c. To determine if fed or fasted rats are more sensitive to the effects of PFDA and PFOA on fatty acid utilization.

2. Experimental Design:

a. Treatment groups.

Male Sprague-Dawley rats, either fasted (48 hr) or fed, were given a single ip injection of PFDA or PFOA (9.4mol/kg) or vehicle (propylene glycol/water 1:1, v/v).

b. Liver perfusion technique

The liver from the rats treated as stated above 2 hr previously were perfused in situ (Hems *et al.*, 1966). With this technique the liver remains in the animal (in situ) but its circulation is isolated. The success of liver perfusion was assessed by hepatic glycogen, ATP content, wet to dry weight ratio of the liver and perfusion flow rate.

c. Fatty acid infusion

[1-^{14}C]Fatty acids in a 10% albumin complex were infused directly into the inflow line at a constant rate such that the final concentration of fatty acid was 0.2 mM. The infusion began following a 20 min equilibration period and continued for 30 min.

d. Fatty acid oxidation

The effects of PFDA or PFOA treatment on the oxidation of oleic, octanoic and pyruvic acids was examined in the collected perfusate. $^{14}CO_2$, acid soluble radioactivity and ketone body concentrations were examined to determine if pretreatment with PFDA or PFOA was affecting fatty acid

oxidation.

e. Fatty acid esterification

Oleic and octanoic acid incorporation into lipids such as triacylglycerols and cholesteryl esters was examined using procedures similar to those described above for PFDA and PFOA metabolism. The specific activity of the lipid classes in the liver following fatty acid infusion was determined by a lipid charring, liquid scintillation spectroscopic technique (Noland and Shand, 1980).

3. Experimental Findings.

a. There was no effect due to fatty acid infusion nor due to pre-treatment with PFDA or PFOA on the viability of the in situ rat liver perfusion preparation. The levels of glycogen and ATP, the wet to dry weight ratio and the perfusion flow rates were not affected.

b. Effects on fatty acid oxidation

The release of $^{14}CO_2$ and acid soluble products derived from [1-^{14}C] fatty acids into the perfusate was examined. PFDA or PFOA pre-treatment had no effect on the oxidation of oleic, octanoic or pyruvic acids under these experimental conditions. Fasted rats generally had a higher rate of fatty acid oxidation, but there was no difference among the fasted treatment groups. The amount of ß-hydroxybutyrate and acetoacetate in the media, also indices of fatty acid oxidation, were examined. Once again PFDA and PFOA had no

effect, with fasted rats having a higher content of ketone bodies but no significant effects between treatment groups.

c. Effects on fatty acid esterification

The specific activity (μCi/mass) of triacylglycerols, cholesteryl esters, cholesterol and free fatty acids was examined in the liver following the 30 min infusion period. Neither PFDA nor PFOA had an effect on the mass, μCi of fatty acid incorporated or on the specific activity of the lipid classes. The total extraction of radioactivity from the tissue was also equivalent among the treatment groups. The fed rats exhibited higher rates of fatty acid esterification, but no treatment effect was observed.

4. Conclusions:

a. The hepatic oxidation of long-chain (oleic acid), medium-chain (octanoic acid) and short-chain (pyruvic acid) fatty acids was unaffected by pre-treatment with 9.4 μmol/kg PFDA or PFOA.

b. Similarly, the esterification of oleic and octanoic acid into various lipid classes was not affected by these low doses of PFDA or PFOA.

c. Fasted rats and fed rats are equally sensitive to these doses of PFDA and PFOA

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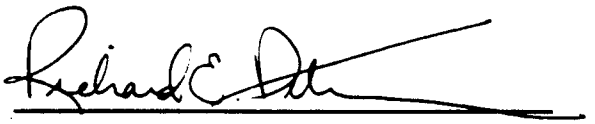

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