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13. ABSTRACT *(Maximum 200 words)*

This report outlines our research progress regarding toxicological investigations of perfluoro-*n*-octanoic acid (PFOA) and perfluoro-*n*-decanoic acid (PFDA). These compounds and related fluorocarbon compounds are useful in Air Force applications as solvents, lubricants, and surfactants. Unfortunately, many of these compounds display hepatotoxic effects. Recent studies in our laboratory have shown that PFDA displays unique effects upon liver metabolism which are not observed with PFOA treatment. Specific effects on phospholipid metabolism include an induction in phosphatidylcholine-specific phospholipase C activity and inhibition in CTP:phosphocholine cytidylyltransferase activity. These enzymes regulate phosphatidylcholine degradation and biosynthesis, respectively. These data suggest that diacylglycerol may be elevated which can affect various cellular processes through a second-messenger signaling mechanism. Other studies in the laboratory have shown that PFDA inhibits hepatic glycogen synthesis by decreasing glucose transport activity. PFDA-treated rats show rates of glucose transport which are 1.8-fold less than that for corresponding controls ($p = 0.02$). PFDA-treatment also impacts the relative activity of pyruvate carboxylase and pyruvate dehydrogenase—enzymes involved in the regulation of both carbohydrate and lipid metabolism. These research activities further our understanding of the mechanisms involved in the hepatotoxicity associated with these important Air Force chemicals.

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ANNUAL TECHNICAL REPORT

Title: Hepatic Metabolism of Perfluorinated Carboxylic Acids and Polychlorotrifluoroethylene: A Nuclear Magnetic Resonance Investigation *in Vivo*

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Report Period: December 15, 1992 to December 14, 1993

Date Submitted: January 5, 1994

I. Introduction/Research Objectives

The primary objective of this research program is to gain a better understanding of the toxicological mechanisms associated with perfluorocarboxylic acids. These studies involve the application of nuclear magnetic resonance (NMR) spectroscopy *in vivo* and strive to further our understanding of the metabolism of these compounds, their effects on endogenous liver metabolism and, in general, to expand the applicability of the NMR technique in the field of toxicology.

The hepatotoxicity associated with perfluoro-*n*-octanoic acid (PFOA) and perfluoro-*n*-decanoic acid (PFDA) in rats has been the primary focus of our research efforts. Our investigations during the past year have led to a new realm of study involving biochemical assays for specific enzyme activities and metabolite concentrations. The laboratory is rapidly gaining experience and learning new techniques in radioisotope assays. This expansion in capabilities has enabled our research program to obtain more detailed information regarding the metabolic consequences accompanying hepatotoxicity. In a beautifully complementary fashion, the NMR studies provide information to identify the specific metabolic pathways which are affected by the perfluorocarboxylic acids, and standard biochemical analyses then confirm enzymatic involvement and alterations in the concentration of various important intermediary metabolites. NMR data obtained from tissue *in vivo* and complementary information from biochemical assays provide a more complete picture of the metabolic processes under investigation. The cumulative information obtained from these experiments provides new insight toward our understanding of the mechanisms involved in the hepatotoxicity associated with this class of compounds.

II. Publications

Full Journal Publications

C.M. Goecke, B.M. Jarnot, and N.V. Reo. "Effects of the Peroxisome Proliferator, Perfluoro-n-decanoic Acid, on Hepatic Gluconeogenesis and Glycogenesis: A ^{13}C NMR Investigation." *Chem. Research Toxicol.* (in press; will appear in Jan/Feb 1994 issue).

N. V. Reo, C. M. Goecke, L. Narayanan, and B. M. Jarnot. "Effects of Perfluoro-*n*-octanoic Acid, Perfluoro-*n*-decanoic Acid, and Clofibrate on Hepatic Phosphorus Metabolism in Rats and Guinea Pigs *in Vivo*." *Toxicol. Appl. Pharmacol.* **124** (in press).

Published Abstracts/Presentations

C.M. Goecke, N.V. Reo, J. Wyman and B. M. Jarnot: "Effects of Perfluoro-*n*-Decanoic Acid on Hepatic Glucose Transport." *The Toxicologist*. Society of Toxicology, Annual Meeting, Dallas, TX, March 1994 (Accepted).

N. V. Reo, L. Narayanan and C. M. Goecke: "Induction of Liver Phospholipase C Activity by the Peroxisome Proliferator, Perfluorodecanoic Acid." International Society for the Study of Xenobiotics, *ISSX Proceeding*, **4**, 103 (1993). Presented at the Fifth North American ISSX Meeting, Tucson, AZ, October 1993.

C. M. Goecke, L. Narayanan, B. M. Jarnot and N. V. Reo: "Effects of the Peroxisome Proliferator Perfluorodecanoic Acid on Hepatic Glucose and Alanine Metabolism." International Society for the Study of Xenobiotics, *ISSX Proceeding*, **4**, 166 (1993). Presented at the Fifth North American ISSX Meeting, Tucson, AZ, October 1993. Received student award for *Best Scientific Abstract and Presentation*.

Manuscript in Preparation

N. V. Reo, C. M. Goecke, and L. Narayanan. "Induction of Liver Phospholipase C Activity by the Peroxisome Proliferator, Perfluoro-n-decanoic Acid." Planned submission to either *Toxicol. Lett.* or *J. Biol. Chem.*

III. Personnel

Laboratory Technician

The AFOSR grant provides support for a full-time research laboratory technician. Latha Narayanan held this position from July 1992 until September 7, 1993 when she resigned to take another job at Wright-Patterson AFB. Latha is a very skilled biochemist and helped to foster our research progress in the area of biochemical assays. Her expertise in the laboratory is greatly missed; three months were spent in a job search to fill this vacancy.

Recently, on December 13, 1993 Katrina Kling was hired as a research technician. Katrina has a M.S. degree in Zoology and she is currently becoming familiar with our laboratory and research program.

Students

Currently, the laboratory has two Ph.D. students, Carol Goecke and Mehdi Adinehzadeh, who are working on their dissertation research in the AFOSR-funded program.

Carol Goecke, who is supported by an ASSERT grant, has recently completed all experimentation and is currently writing her dissertation. She expects to complete the degree program and graduate by March 1994. Carol has expressed a desire to continue working in the laboratory, and I am hopeful that money will be available in the AFOSR grant to support her in a postdoctoral capacity.

Mehdi is a second-year Ph.D. student who joined the laboratory in June 1993 and is supported by a graduate stipend from the AFOSR grant.

Marjorie Artz was a Ph.D. graduate student who worked in this laboratory briefly in 1992 and then left in January 1993. She was supported for only one month on the AFOSR program before leaving to join another laboratory at WSU.

IV. Interactions with Air Force and Other DoD Laboratories

Our laboratory participates in an active ongoing collaboration with Capt. Bruce Jarnot, Ph.D. of the Toxicology Division, Armstrong Laboratory, Wright-Patterson AFB. We meet on a regular basis to discuss data and plan experiments. Bruce is also a co-author on the two manuscripts which were accepted for publication this past year.

Another collaboration was also initiated this year with Lt. Commander John Wyman of the Naval Medical Research Institute (NMRI), Toxicology Detachment, Wright-Patterson AFB. Dr. Wyman is an expert in rat liver perfusion and has assisted our laboratory in a series of experiments to investigate the effects of PFDA on hepatic glucose transport (*vide infra*). These experiments involved personnel from my laboratory and Dr. Wyman's laboratory and used equipment at the NMRI laboratory at Wright-Patterson AFB. These experiments were completed in December 1993. We continue to interact with Dr. Wyman and anticipate manuscript preparation during the early part of 1994.

V. Additional Information

Our scientific achievements received recognition this year at the Fifth Annual North American Conference of the International Society for the Study of Xenobiotics which was held in Tucson, Arizona on October 17-21, 1993. Carol Goecke, a Ph.D. student in my laboratory, was the recipient of an award for *best student abstract and presentation* for our work involving the effects of PFDA on hepatic carbohydrate metabolism (copy of award letter attached; Appendix I). All student-submitted abstracts (39 total) were evaluated by a distinguished panel of judges and four winners were chosen. This award signifies the excellent work that Carol has done in the laboratory and, additionally, it acknowledges the recognition and significance of our research within the toxicology scientific community.

VI. RESEARCH ACCOMPLISHMENTS

A. Effects of PFDA on Hepatic Carbohydrate Metabolism

This research program has provided the first demonstration that PFDA has dramatic effects on liver carbohydrate mechanism. This conclusion is predominantly supported from results of ^{13}C NMR studies of rat liver *in vivo*. A detailed description of this work including significance, results, and discussion was presented in last year's Annual Technical Report (submitted January 14, 1993).

This work culminated into a manuscript which was recently accepted for publication in *Chemical Research in Toxicology* (preprint attached, Appendix II). The following discussion pertains to new data in this area of study, and a more detailed presentation of data which existed only in preliminary form last year.

The main objective of this particular series of experiments was to examine the effects of PFDA on the processes of hepatic glycogenesis from glucose and alanine—a glucogenic amino acid. These studies employed ^{13}C labeled compounds in conjunction with ^{13}C NMR spectroscopy to monitor specific metabolic pathways. Gluconeogenesis and glycogenesis from [3-^{13}C] alanine are active in both PFDA-treated and corresponding pair-fed control rats. Interestingly, however, differences between these two groups appear in the ^{13}C labeling pattern observed in glutamate. The analyses of these data were not complete at this time last year and, thus, it was presented as preliminary data in last year's report. The final data analysis and interpretation are given below.

Glutamate Labeling - Entry of Pyruvate into TCA Cycle

The metabolism of alanine involves its conversion to pyruvate and the entry of pyruvate into the TCA cycle either as oxaloacetate via pyruvate carboxylase (PC), or as acetyl-CoA via pyruvate dehydrogenase (PDH). Ultimately, glutamate attains ^{13}C labeling from the TCA cycle intermediate, α-ketoglutarate. The ^{13}C labeling of glutamate reflects the percentage of pyruvate which enters as oxaloacetate and that which enters as acetyl-CoA. Pyruvate which enters the TCA cycle as oxaloacetate yields C2 and C3 labeled glutamate, whereas the C4 carbon of glutamate attains a ^{13}C label only from pyruvate which is converted to acetyl-CoA. Thus the (C2 + C3)/C4 ratio of glutamate carbon labeling is related to the relative activity of PC versus PDH. This ratio is directly obtained from the peak intensities in the ^{13}C NMR spectra of liver. Figure 1 clearly shows well resolved resonances for the individual carbons of the glutamate molecule.

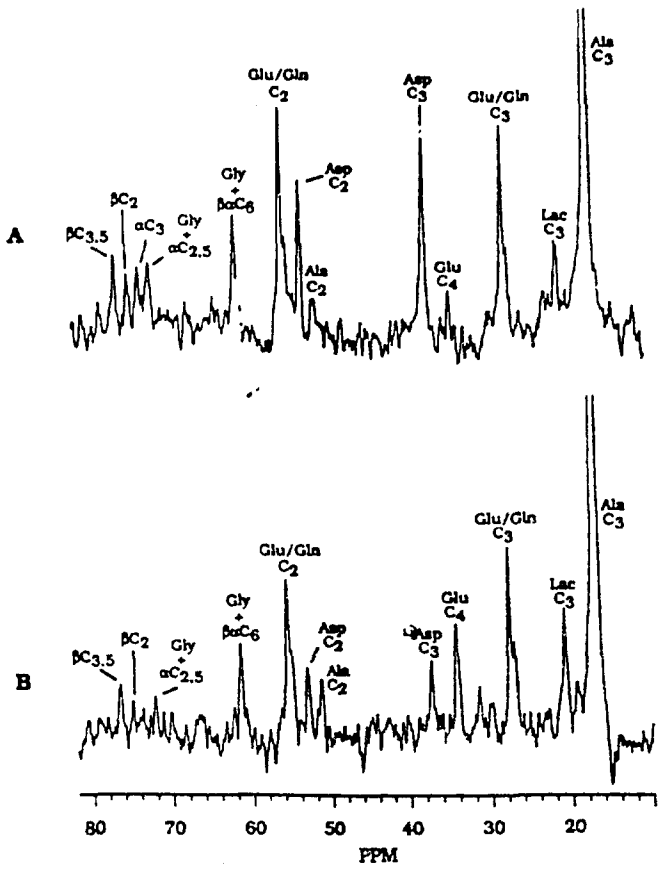

Figure 1. Expanded region of proton-decoupled ^{13}C NMR liver difference spectra, obtained 15 min post iv [3-^{13}C]alanine bolus, representing 10 min of signal averaging. Data acquisition and processing parameters are as given in Experimental Procedures except that an exponential filter producing 20-Hz line broadening was used. (A) Pair-fed control rat receiving only [3-^{13}C]alanine (Group 4C); (B) PFDA-treated rat receiving only [3-^{13}C]alanine (Group 4T). The labeled ^{13}C NMR peaks include those due to the α- and β-anomers for D-glucose: βC-2, βC-3, βC-5, βC-6, αC-2, αC-3, and αC-6. Abbreviations: Gly, glycogen; Glu, glutamate; Gln, glutamine; Ala, alanine; Asp, aspartate; Lac, lactate. Carbon-13 chemical shifts are given relative to TMS at 0 ppm by setting the C_3 of alanine at 17.2 ppm.

The $(C_2 + C_3)/C_4$ glutamate intensity ratios for PFDA and control rats are shown in Table 1. The experiment involved the administration of [3-^{13}C] alanine with (Group 5) or without (Group 4) an intravenous infusion of unlabeled glucose. The C and T suffixes on the group names identify control and treated (PFDA) animals, respectively.

Table 1. Ratios of $(C_2 + C_3)/C_4$ Glutamate Intensities (Mean ± SE) for PFDA and Pair-Fed Control Rats Measured in ^{13}C NMR Liver Spectra

time (min) post-alanine[a]	Group 4C: control, (−) glucose	Group 4T: PFDA, (−)-glucose	Group 5C: control (+)-glucose	Group 5T: PFDA, (+)-glucose
15	6.6 ± 1.0[b,c]	3.9 ± 0.5[b,d]	2.9 ± 0.5[c]	2.1 ± 0.5[d]
25	7.9 ± 1.1[b,c]	3.6 ± 0.1[b,d]	2.5 ± 0.3[c]	2.9 ± 1.0[d]
35	7.1 ± 1.3[b,c]	3.7 ± 0.4[b,d]	3.4 ± 0.8[b,c]	2.1 ± 0.4[b,d]

[a] Time points represent the midpoint in time of each 10-min difference spectrum acquired post-alanine administration. [b] Denotes a significant difference ($p \leq 0.05$) between PFDA rats and their corresponding pair-fed control. [c] Denotes a significant difference ($p \leq 0.05$) between control rats receiving only alanine (Group 4C) and control rats receiving alanine and unlabeled glucose (Group 5C). [d] Denotes a significant difference ($p \leq 0.05$) between PFDA rats receiving only alanine (Group 4T) and PFDA rats receiving alanine and unlabeled glucose (Group 5T).

Within each group, the $(C_2 + C_3)/C_4$ glutamate ratio remains relatively constant through time. Comparison of the glutamate ratios between PFDA and control animals, however, reveals a significantly lower ratio in PFDA rats receiving only alanine (Group 4T) relative to control (Group 4C) from 15 to 35 min post-alanine. In addition, PFDA rats receiving alanine and glucose (Groups 5T) show a significantly lower ratio compared to control (Group 5C) at 35 min post-alanine.

These data suggest that PFDA rats have a greater relative activity of PDH versus PC as compared to controls. Differences in PDH activity may arise from differences in lipolytic activity. Our data suggest a dysfunction in fatty acid metabolism in PFDA rats and corroborate other studies which show that PFDA inhibits fatty acid oxidation. This greater PDH activity also suggests that pyruvate serves as the predominant source of acetyl-CoA in treated rats.

At all time points examined, PFDA rats receiving alanine and unlabeled glucose (Group 5T) show a significantly lower ratio than PFDA rats receiving only alanine (Group 4T). In addition, control rats receiving alanine and unlabeled glucose (Group 5C) show a significantly lower ratio than control rats receiving only alanine (Group 4C). The magnitude of the decrease in this ratio, however, is greater between control groups than between treated groups. These results suggest that the addition of supplementary glucose yields a greater relative activity of PDH versus PC. The reason for this is not known.

Mechanism for Inhibition of Hepatic Glycogenesis

Results from the ^{13}C NMR studies demonstrate that PFDA inhibits glycogen synthesis from glucose but not from alanine. Note that the glycogenic pathways from glucose and alanine converge at glucose-6-phosphate (G6P) and then proceed via a common pathway to glycogen. This suggests that the inhibition in this metabolic process must lie in the transport of glucose into the hepatocyte and/or the phosphorylation of glucose by glucokinase. This hypothesis was further tested by administering 2-deoxyglucose (2-DG) to PFDA and control rats and measuring hepatic 2-deoxyglucose-6-phosphate (2-DG6P) concentrations. Control rats yield a *ca.* 2.6-fold greater concentration of 2-DG6P (mean ± SE) than PFDA rats at 5 days post-dose, 1.90 ± 0.11 *versus* 0.74 ± 0.07 mmol/kg of protein, respectively.

Like glucose, 2-DG is transported into hepatocytes via the glucose transporter and is phosphorylated by glucokinase to form 2-DG6P; however, 2-DG6P is not a substrate for glycolysis nor glycogenesis. Since PFDA rats show significantly less 2-DG6P than corresponding controls, this data is consistent with the hypothesis that PFDA inhibits either the transport of 2-DG into the hepatocyte and/or its phosphorylation by glucokinase.

Test of Hepatic Glucose Transporter Activity

To accomplish our objectives in an expeditious manner with regard to the effects of PFDA on the transport of glucose into liver, it was helpful to establish a collaboration with LCDR, John Wyman, Ph.D., of the Naval Medical Research Institute, Toxicology Detachment, Wright-Patterson AFB. Dr. Wyman's laboratory is equipped with a rat liver perfusion apparatus and he is exceptionally skilled in this technique.

Generally, this experiment involved perfusion of liver with a radioisotope-labeled glucose analog in order to determine glucose transport activity in both PFDA-treated and control rats. This experiment proved to be quite difficult and the experimental procedures required considerable refinement in order to obtain reliable data. Our efforts were successful, however, and the results show that PFDA severely impacts carbohydrate metabolism by inhibiting glucose transporter activity at the cell membrane. Details of this experiment are outlined below.

Experimental Technique/Methods

The goal of this study was to determine the effect of PFDA on hepatic glucose transport. This was accomplished using a paired tracer first pass extraction technique in perfused rat livers. In brief, livers were excised from PFDA and control rats on day 5 posttreatment and were perfused with tracer quantities of a radiolabeled glucose analog, 3-O-[^{14}C-methyl]-D-glucose ([^{14}C]3-O-MG). Similar to D-glucose, [^{14}C]3-O-MG equilibrates rapidly between the intracellular and extracellular spaces, but unlike glucose, this analog is not metabolized. Others have shown that the affinity for glucose transport is approximately equal for D-glucose and 3-O-MG. Radiolabeled sucrose, [fructose-1-^{3}H(N)] ([^{3}H]sucrose), was also administered as a marker of extracellular volume. In the isolated perfused liver, [^{3}H]sucrose does not penetrate the cell and, therefore, diffuses only into the extracellular space. Sucrose thus provides a marker of the extracellular volume and hepatic glucose transport was calculated from the change in the ratio, [^{14}C]3-O-MG/[^{3}H]sucrose, during passage through the liver.

Animal Treatments. Male Fischer-344 rats (225-280 g) were treated with a single ip injection of 50 mg PFDA/kg dissolved in vehicle, 1:1 v/v propylene glycol/water. PFDA animals (n=7) received *ad libitum* access to food and water. Weight-paired control animals (n=8) received a single ip injection of vehicle and were given *ad libitum* access to water but were fed the same amount of food as their PFDA-treated partners had consumed during the previous 24-h period (pair-feeding).

Perfusion apparatus. The perfusion apparatus employed two vessels (hot and cold), each containing 100 ml of Krebs's Ringer solution. The "hot" vessel, however, also contained [^{14}C]3-O-MG (0.25 µCi) and [^{3}H]sucrose (0.25 µCi). Perfusion from the cold vessel used a recirculating system and flowed through a membrane oxygenator (12 feet of sialastic tubing through which 95% O_2 and 5% CO_2 was bubbled), temperature probe, filter, bubble trap, pH probe, influent O_2 probe, and influent pressure gauge before reaching the liver. The effluent passed through an O_2 probe, effluent pressure gauge, and collected back into the original vessel. The hot vessel was used in a non-recirculating manner with effluent collected directly into scintillation vials as described below.

Liver perfusion. At 5 days posttreatment, rats were anesthetized with halothane (5% for induction; 1% maintenance) and livers were surgically exposed by a xiphoid-pubis midline incision. Intestines were gently placed to the animals left in moist gauze and the bile duct was cannulated (PE10). Caudate lobes were freed by tying two ligatures around the esophagus and incising between the ligatures. The portal vein was cannulated (Bard-A-Cath., 16 gauge) and livers were initially perfused with 0.8 ml of a heparin/saline solution (1:1 v/v) followed by perfusion with warm, oxygenated Krebs-Ringer solution (10 ml/min). The thorax was opened to expose the heart and the inferior vena cava was cannulated (PE 240-1.7 mm ID) via an incision in the right atrium. Livers were excised, washed with warm saline, transferred to the perfusion cabinet (maintained at 37 °C) and connected into the perfusion circuit. Livers were placed into a petri dish lined with a silk cloth, cannulae positions were optimized, and perfusion flow rate was increased to 25 ml/min. Livers were perfused with Krebs improved Ringers I solution (hemoglobin-free) in an anterograde direction using a recirculating system for a stabilization period of 30 min. During this time, hepatic pressure, effluent perfusion rate, and bile flow were monitored. In addition, perfusate samples (1.0 ml) were obtained at 0, 10, 20, and 30 min for lactate dehydrogenase (LDH) and sorbitol dehydrogenase (SDH) determinations. These samples were stored on ice until analyzed (no longer than 6 hrs).

Perfusion of the tissue with the radiolabeled isotopes was initiated following the 30 min stabilization period. The "hot" perfusate was contained in a separate vessel from which flow to the liver was controlled with a stopcock. Perfusion of the tissue from this vessel was non-recirculating. A sample of the perfusate was withdrawn from the hot vessel before perfusion of the liver. When flow to the liver was begun, tissue effluent was collected manually into scintillation vials at 2 sec intervals for 2 min. Following collection of the last effluent sample, the influent tubing was disconnected from the portal vein catheter and an influent sample was collected. At the end of the experiment, livers were blotted dry and weighed.

Analytical methods. Influent and effluent samples were prepared for liquid scintillation spectrometry by adding 10 ml of scintillant (Scintevest) to each sample and allowing time for temperature equilibration in the scintillation counter prior to data acquisition. The ^{14}C and ^{3}H activities in each sample were counted for 10 min in a Hewett Packard Liquid Scintillation Spectrometer with external standardization. Each sample was counted twice and averaged. The percent hepatic extraction (%E) of [^{14}C]3-O-MG relative to the extracellular reference [^{3}H]sucrose was derived from the ratios of the isotopes in the influent ($R_{in} = {}^{14}C_{in}/{}^{3}H_{in}$) and effluent samples ($R_{out} = {}^{14}C_{out}/{}^{3}H_{out}$) as follows:

$$\%E = 100 \bullet [1-(R_{out}/R_{in})]$$

Hepatic extraction of [^{14}C]3-O-MG measured in the first 3 samples (6 sec after appearance of measurable amounts of [^{3}H]sucrose) was calculated and averaged for each rat. These early samples give the closest estimation of maximal glucose uptake due to unidirectional influx of glucose into hepatocytes. Mean values of %E were then calculated for both PFDA and control groups.

Statistical analyses. Data were analyzed using the Student t-test for unpaired data, and are considered to be statistically significant at a value of $p \leq 0.05$.

Results and Discussion

In this study, the effect of PFDA on hepatic glucose transport was assessed in perfused rat liver from PFDA and control rats on day 5 posttreatment. Control rats yield a *ca.* 1.8-fold greater percent hepatic glucose extraction (mean ± SE) compared to PFDA rats, 27.1 ± 3.6 versus 15.5 ± 2.2, respectively.

LDH is a cytoplasmic enzyme which serves as an indicator of irreversible cell death. LDH values for PFDA and control rats show no significant difference at any time ($p < 0.05$), suggesting that liver viability is similar in both experimental groups. Therefore, the difference in percent glucose extraction (%E) between PFDA and control groups is specifically due to treatment which is not attributable to differences in liver viability between groups.

These studies clearly demonstrate that the inhibition in hepatic glycogen synthesis, which was observed in earlier ^{13}C NMR experiments following PFDA treatment, is predominately due to a severe dysfunction in glucose transport. A cursory comparison of the percent inhibition in glucose transport and the percent decrease in 2-DG6P concentrations (*vide infra*) in PFDA-treated rats, suggests that PFDA inhibits both glucose transport and glucokinase activity. A more thorough analysis of the data is currently in progress.

Glucose is transported into the liver via the glucose transporter, GLUT 2, which is localized primarily in the plasma membrane. Others have shown that glucose transport activity is influenced by the nature of the plasma membrane; it is altered by bilayer physical state, lipid acyl chain length and degree of unsaturation, and bilayer cholesterol content. PFDA may indirectly alter hepatocellular glucose transport by influencing the nature of the plasma membrane. This interpretation is consistent with other data from our laboratory which show significant effects of PFDA on phospholipid metabolism (*vide infra*) and suggests a specific membrane interaction. M. George and coworkers at Wright-Patterson AFB have also suggested (in past publications) that PFDA may act through changes in membrane composition and fluidity. Additionally, ^{13}C NMR data from our laboratory have shown that PFDA rats possess improved spectral resolution in the ^{13}C liver spectra along with an increase in the intensity of the methylene resonance (at 30 ppm) relative to control rats. This is believed to reflect either an increase in membrane fluidity and/or an increase in liver triglycerides and/or free fatty acids. Thus data from various experiments seem to indicate an interaction of PFDA with membranes and phospholipids which, in turn, impacts a variety of important cellular functions.

VI.

B. Studies of the Effects of Perfluorocarboxylic Acids on Phospholipid Metabolism

Phosphorus-31 NMR studies of liver *in vivo* have revealed an interesting observation with regard to the effects of PFDA treatment. Liver phosphocholine (PCho) concentration is significantly increased following PFDA exposure. This effect is unique to PFDA and is not seen with PFOA nor clofibrate (a hypolipidemic drug and classic peroxisome proliferator). The quinea pig, a nonresponsive species with regard to proliferation of liver peroxisomes, is also not affected in this way by PFDA treatment. These data where discussed in last year's Annual Technical Report, and a manuscript has recently been accepted for publication in *Toxicology and Applied Pharmacology* (preprint enclosed; Appendix III).

The results of these studies suggest that PFDA significantly impacts hepatic phospholipid metabolism. Further investigations in this area during the past year have focused on the measurement of specific enzyme activities involved in phosphatidylcholine metabolism. Some very interesting results have emerged. The experimental procedures, data analyses, and discussion are presented below.

Methods

These studies involved the measurement of liver phospholipase C and CTP:phosphocholine cytidylyltransferase activities in PFOA-, PFDA-treated and corresponding pair-fed control rats. PFOA and PFDA were administered via a single ip injection (150 mg/kg PFOA; 50 mg/kg PFDA). The dosing volume was less than 0.4 mL. Control animals were dosed according to the same regiments with an equal volume of the vehicle solution. Liver experiments were initiated at 3 days and 5 days post treatment with PFOA or PFDA, respectively.

The following separate experiment was designed to examine differences between control groups with regard to cytidylyltransferase activity. Rats were dosed with vehicle solution and then separated into three groups differing only in the quantity of food they were given to eat and the day post-dose that the liver was assayed for enzyme activity. These groups included: rats fed *ad libitum* and examined at 5 days post-dose, and two groups which were fasted in a similar manner to which PFOA and PFDA rats typically respond with regard to food consumption. These groups will be referred to as PFOA-like and PFDA-like controls, and enzyme assay experiments were conducted at 3 and 5 days post-dose, respectively. Essentially, the PFOA-like controls received little food (2 - 6 g/day) prior to the experiment, and the PFDA-like controls received little food for the first two days post-dose and then were completely fasted for the remaining three days prior to the experiment. The *ad libitum* fed animals showed reduced food consumption on the day of the vehicle dosing, but then consumed approximately 19 g per day thereafter.

Sample Preparation. Rats were anesthetized with halothane (5% induction; 1% maintenance). Livers were surgically exposed and then removed by freeze-clamping the tissue between aluminum plates chilled in liquid N_2. The livers were homogenized in 50 mM ice-cold Tris-HCL buffer (~3 mL/g tissue; pH 7.4). A cytosolic fraction was prepared by sequential centrifugation at 10,000 g for 20 min and 50,400 g for 60 min. Protein concentrations were estimated using the procedure described by Lowry with bovine serum albumin as standard. Enzyme activities are reported relative to total protein content.

Enzyme Assays. Liver cytosolic fractions were prepared from PFOA-, PFDA-treated, and corresponding pair-fed control rats for each enzyme assay. Separate groups of animals were used for each assay. Each treatment group and its corresponding control group were analyzed on consecutive days using the same reagents and radioisotope stock solutions. This procedure helps to maintain consistency in the assay sensitivity and ensures reliable comparisons between groups. The control studies involving *ad libitum*-fed, PFOA-like, and PFDA-like groups involved an analogous procedure such that all three groups were analyzed using

identical assay media. These three groups were only analyzed for cytidylyltransferase activity.

Phospholipase C. Phospholipase C (phosphatidylcholine cholinephosphohydrolase) enzyme activity was measured at 37 °C using a phospholipase C– alkaline phosphatase coupled assay. Briefly, this procedure utilizes a phosphatase to liberate inorganic phosphate from the phosphobase product of the phospholipase C reaction. Inorganic phosphate is then determined spectrophotometrically by measuring absorbance at 820 nm. Each liver preparation was run in triplicate using an assay volume of 1 mL. Triplicate assays deviated by less than 5%.

Cytidylyltransferase. In a separate study, CTP:phosphocholine cytidylyltransferase activity was measured at 37 °C in liver cytosolic fractions. Enzyme activity was determined by measuring the formation of radioactive CDP-choline from phospho[methyl-^{14}C]choline. The CDP-choline was recovered by absorption to charcoal. This radiolabeled product was eluted from the charcoal and collected directly into scintillation vials under reduced pressure using a multiple sample filtration manifold (#FH225V, Hoefer Scientific Inst., San Francisco, CA). The assay was run in duplicate and the enzyme activity is reported per mg protein.

Data Analyses. Statistical analyses employed the appropriate Student t-test for paired and unpaired data.

Results

PLC and cytidylyltransferase activities for PFOA-, PFDA-treated and corresponding control groups are shown in Table 2. PFOA-treated rats show no change in PLC activity, while PFDA causes a 1.8-fold elevation in this enzyme activity as compared to corresponding pair-fed controls ($p = 0.0003$). With regard to liver cytidylyltransferase activity, PFDA-treated rats have 36% less activity than corresponding controls, while PFOA rats are not different from their pair-fed controls ($p < 0.01$).

The effects of nutritional status can be seen in the comparison between the *ad libitum*-fed group and the PFOA-like and PFDA-like controls (Table 3). The PFDA-like control rats were completely fasted for 72 hours prior to the experiment and show cytidylyltransferase activity which is 2.5-fold greater than that observed for fed animals ($p = 0.001$). The PFOA-like controls are not different from the *ad libitum*-fed group. A thorough analysis and interpretation of this data is not yet complete, and it will not be discussed further in this report.

TABLE 2

Enzyme Activity (nmol/mg protein/min) at 37 °C in Rat Liver Cytosolic Fractions

Groups ‡	Phospholipase C	Cytidylyltransferase
PFOA-treated	9.09 ± 0.92	1.36 ± 0.12
Pair-fed Control to PFOA group	8.75 ± 0.76	1.47 ± 0.16
PFDA-treated	14.71 ± 0.87*	2.26 ± 0.20*
Pair-fed Control to PFDA group	7.94 ± 0.48	3.51 ± 0.34

‡ Values are Mean ± SD (n = 3) for each group. PFOA-treated (150 mg/kg) and corresponding controls were examined on day 3 post-dose. PFDA-treated (50 mg/kg) and corresponding controls were examined on day 5 post-dose.

* Denotes a significant difference from corresponding control group ($p \leq 0.005$).

TABLE 3

Cytidylyltransferase Activity at 37 °C in Rat Liver Cytosolic Fractions. Values are Mean ± SD (n = 2).

Groups	**Activity** (nmol/mg protein/min)
Ad libitum-fed	1.26 ± 0.09
PFOA-like Control	1.29 ± 0.01
PFDA-like Control	3.13 ± 0.004

Discussion

This study clearly demonstrates that PFDA affects important enzymes associated with phospholipid metabolism in liver. A significant induction in PLC activity and decrease in cytidylyltransferase activity are associated with PFDA treatment, while PFOA does not affect these enzyme activities. Previous ^{31}P NMR studies from our laboratory have shown that PFDA causes a significant increase in the concentration of liver PCho (Appendix III). As shown in Figure 2, PCho is a key metabolite in phosphatidylycholine (PtCho) metabolism. It is a by-product of PtCho degradation via PLC and it is a precursor for the biosynthesis of PtCho through the CDP-choline pathway. The rate-determining step in this biosynthetic pathway to PtCho is catalyzed by cytidylyltransferase. The inhibition of cytidylyltransferase and induction of PLC, which results from PFDA treatment, suggests an overall breakdown of phospholipids. Specifically, these data indicate that PFDA induces membrane degradation through a phosphatidylycholine-specific phospholipase C activated mechanism (Fig. 2). This is substantiated by the significant increase in liver PCho following PFDA treatment. Indeed, PFDA-treated rats show an increase in liver PCho concomitant with increased activity of PLC and decreased activity of cytidylyltransferase. PFOA treatment, however, has no effect on PCho levels nor the activity of these enzymes. Thus, PFDA causes an increase in liver PCho by stimulating the breakdown of PtCho via PLC and inhibiting the utilization of PCho in the biosynthesis of phospholipids.

A rather intriguing and important mechanistic implication of these finding relates to the effect that these processes may have on the concentration of diacylglycerol (DAG). The hydrolysis of PtCho by PLC generates DAG in addition to PCho. Thus our data suggest that DAG levels may also be elevated in PFDA-treated rats. Current studies in our laboratory are focused on the measurement of liver DAG concentrations in response to PFDA and PFOA treatments.

DAG is a key metabolite since it is a precursor for both phospholipid and triglyceride synthesis. PFDA-treated rats are known to have elevated liver triglycerides; our studies are providing a clearer understanding of the mechanisms involved in this process. More importantly, however, is the role of DAG as a second messenger in signal transduction and the involvement of PtCho as a source for this metabolite. Elevated DAG concentrations can activate protein kinase C and ultimately affect many cellular processes. The effects of PFDA on phospholipid metabolism may provide a mechanism by which this compound influences a variety of cellular responses including cell proliferation, hepatomegaly, and induction of cytochrome P450 enzymes.

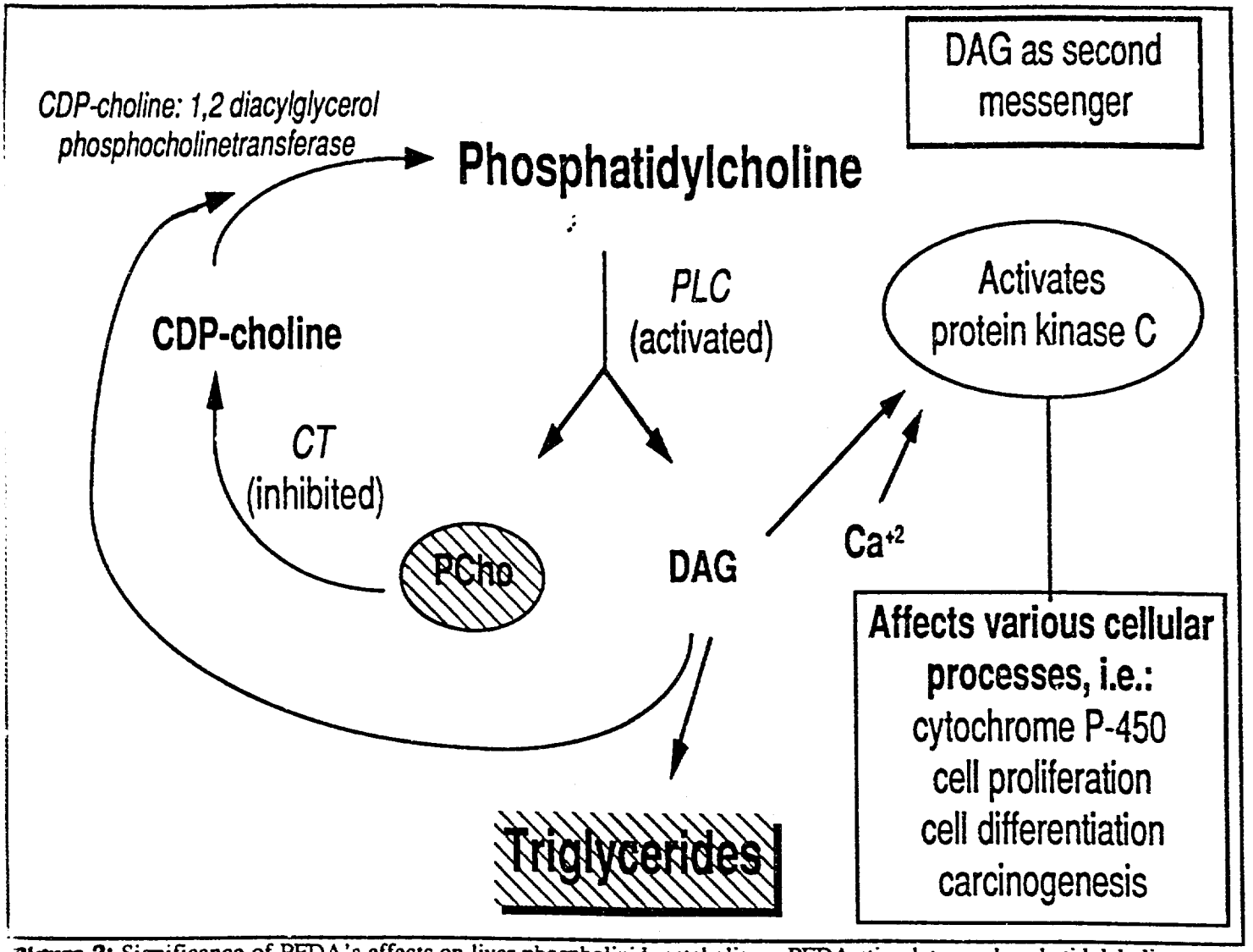

Figure 2: Significance of PFDA's effects on liver phospholipid metabolism. PFDA stimulates a phosphatidylcholine-specific PLC and inhibits CT activity causing an increase in PCholine. The importance of diacylglycerol in the biosynthesis of phosphatidylcholine and triglycerides, and its role as a second messenger are outlined. Our data suggest that the toxicological mechanism associated with PFDA treatment may involve activation of protein kinase C. Key: **PCholine** - phosphocholine, **DAG** - diacylglycerol, **PLC** - phospholipase C, **CT** - CTP:phosphocholine cytidylyltransferase.

Interestingly, R. Thurman and coworkers have recently shown that certain peroxisome proliferators, including PFOA, can stimulate protein kinase C activity in microsomes. They further suggest that this activation of protein kinase C may play a central role in the mechanism of peroxisome proliferator-induced hepatocarcinoma. Future studies in our laboratory will involve the measurement of protein kinase C activity. We hypothesize that PFDA may be an activator of protein kinase C mediated through a DAG-second-messenger signal. Such cellular events can have profound effects on metabolic processes and may constitute a probable mechanism for the hepatotoxicity associated with PFDA.

VI.

C. Preliminary Studies to Investigate Whether PFDA Penetrates the Cell Membrane.

The effects of PFDA on liver phospholipid metabolism lead to an interesting question: "Does PFDA cross the plasma membrane and become internalized in the cell?" It is quite likely that PFDA may not enter the cell but only interact with the membrane, possibly imbedding itself in the membrane and disrupting membrane structure and function of membrane proteins. Such an interpretation may explain many of the hepatotoxic differences observed between PFDA and PFOA.

During this past year, we attempted to address this question through the use of NMR spectroscopy in conjunction with chemical shift reagents. These studies were designed to quickly evaluate if NMR techniques could be used *in vivo* to distinguish intracellular versus extracellular PFDA. We probed various paramagnetic transition metal and lanthanide ions for the capacity to shift the ^{19}F NMR resonances of PFDA. These included Co^{+2}, Y^{+3}, and Eu^{+3} in varying mole ratios relative to PFDA. For Co^{+2} we also investigated the effects on the ^{13}C NMR signals of PFDA. Additionally, Gd(DTPA) — a nuclear relaxation agent — was used as a means to selectively affect the ^{19}F relaxation rate of the extracellular PFDA. If this was effective, then specific NMR experiments could be used to selectively observe only the intracellular PFDA signal.

Although early results with some of these shift and relaxation agents showed promise, further studies in biological-type systems proved to add a great deal more complexity to the experiments. Initial studies were done in simple aqueous solutions, but later experiments involving rat serum showed that the PFDA interacts very strongly with albumin. This interaction makes PFDA much less accessible to the shift or relaxation agent and significantly weakens the effect. These experiments were temporarily discontinued in order to concentrate our focus on the metabolic impact of the perfluorocarboxylic acids.

VII. Overall Conclusions

During the past year, this research program has focus in two main areas relating to the effects of PFOA and PFDA on hepatic metabolism, namely phospholipid and carbohydrate metabolism. Through the use of NMR spectroscopy and standard biochemical assays, these studies have probed specific metabolic pathways and examined the impact of perfluorocarboxylic acid exposure. This investigative strategy will delineate the metabolic effects exerted by these compounds and aid in developing a clearer understanding of the hepatotoxic mechanisms at play.

In summary, these studies have demonstrated that PFDA treatment exhibits unique metabolic effects which are not observed with PFOA. PFDA depresses glucose transport into hepatocytes and inhibits glycogen synthesis. It also shows dramatic effects upon hepatic phospholipid metabolism. PFDA activates a phosphatidylcholine-specific phospholipase C which has important implications with regard to various cellular processes. It is likely that this effect of PFDA may trigger a cellular signaling mechanism through diacylglycerol and subsequent activation of protein kinase C. Current investigations in our laboratory are addressing these cellular metabolites, enzymes, and pathways. These research endeavors will provide new information regarding the mechanisms of toxicity associated with a class of compounds which are important in various Air Force applications.

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