Form Approved
OMB No 0704-0188

2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
14JAN93	Annual Technical Report 15DEC91-14JAN9

4. TITLE AND SUBTITLE
Hepatic Metabolism of Perfluorinated Carboxylic Acids and Polychlorotrifluoroethylene: A Nuclear Magnetic Resonance Investigation in Vivo

5. FUNDING NUMBERS
G-AFOSR-90-0148
PE 61102F
PR 2312
TA AS

6. AUTHOR(S)
Nicholas V. Reo, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wright State University
3640 Colonel Glenn Highway
Dayton, OH 45435

Wright State University
School of Medicine
Cox Institute
3525 Southern Blvd.
Dayton, OH 45429

8. PERFORMING ORGANIZATION REPORT NUMBER
AFOSR-TR- 93 0 3

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Air Force Office of Scientific Research
AFOSR/NL
Building 410
Bolling AFB, DC 20332-6448

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)*

This report describes our studies of the effects of perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA) on hepatic carbohydrate and phospholipid metabolism. Previously we have shown that PFDA inhibits hepatic glycogenesis from glucose in rats. In recent studies using carbon-13 nuclear magnetic resonance (NMR) spectroscopy, PFDA-treated rats show active gluconeogenesis from [3-^{13}C]alanine and the incorporation of the ^{13}C label into hepatic glycogen. The rate of alanine utilization is 40% greater in controls than PFDA-treated rats, and liver glucose-6-phosphate levels are about 40% lower in PFDA rats as compared to controls ($p \leq 0.02$). These results suggest that the PFDA-induced inhibition in glycogenesis from glucose is due to a dysfunction in the glucose transporter and/or glucokinase activity. In separate studies involving liver phosphorus metabolism, ^{31}P NMR was used to examine the effects of PFDA, PFOA, and clofibrate (CLOF) in both rats and guinea pigs. A unique effect is revealed in PFDA-treated rats in which a significant increase is observed in liver phosphocholine from 2.31 ± 0.23 μmol/g tissue on day 1 post dose to 4.56 ± 0.21 μmol/g on day 5. These level are 2 to 4-fold greater than those measured in controls. The results indicate an enhanced turnover of liver phosphatidylcholine. Ongoing research efforts focus on the effects of PFDA on diacylglycerol levels, phospholipase C activity, and will examine the physical interaction of PFDA with phospholipid membranes.

14. SUBJECT TERMS
Toxicology

15. NUMBER OF PAGES
23 (inclusive)

16. PRICE CODE

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

ANNUAL TECHNICAL REPORT

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

Principal Investigator: Nicholas V. Reo, Ph.D.
Assistant Professor
Department of Biochemistry
Magnetic Resonance Lab
Wright State University
Dayton, OH 45435

Report Period: December 15, 1991 to January 14, 1993

Date Submitted: January 14, 1993

Annual Technical Report **Submitted January 14, 1993**

Introduction

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 perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA) in rats has been the primary focus of our research efforts. During the past year our laboratory has investigated the effects of these compounds on hepatic carbohydrate, high-energy phosphate, and phospholipid metabolism *in vivo*. This report will discuss the results of these studies and outline our future objectives.

Research Accomplishments/Productivity

Publication

Studies designed to investigate the metabolism of PFOA and PFDA in rats using ^{19}F NMR culminated in the publication cited below. A reprint was forwarded to Dr. Walter Kozumbo in November 1992.

C.M. Goecke, B.M. Jarnot, and N.V. Reo. "A Comparative Toxicological Investigation of Perfluorocarboxylic Acids in Rats by Fluorine-19 NMR Spectroscopy." *Chem. Research Toxicol.*, **5** (4), 512 - 519 (1992).

Published Abstracts/Presentations

N.V. Reo, C.M. Goecke, M. M. Artz, and B.M. Jarnot: "Liver Phosphorous Metabolic Response to Perfluorocarboxylic Acids and Clofibrate in Rats and Guinea Pigs: A ^{31}P NMR Study." International Society for the Study of Xenobiotics, *ISSX Proceeding*, **2**, 54 (1992). Presented at the Fourth North American ISSX Meeting, Bal Harbour, FL, November 1992.

C.M. Goecke, N.V. Reo, and B.M. Jarnot: "Effects of the Peroxisome Proliferator, Perfluoro-n-decanoic Acid, on Hepatic Gluconeogenesis and Glycogenesis: A ^{13}C NMR Study." International Society for the Study of Xenobiotics, *ISSX Proceeding*, **2**, 56 (1992). Presented at the Fourth North American ISSX Meeting, Bal Harbour, FL, November 1992.

B.M. Jarnot, C. A. Taylor, M. M. Artz, C.M. Goecke, and N.V. Reo: "Hepatic Peroxisome Induction by Perfluoro-n-decanoic Acid and Clofibrate in the Rat: Proliferation Versus Activity." International Society for the Study of Xenobiotics, *ISSX Proceeding*, **2**, 143 (1992). Presented at the Fourth North American ISSX Meeting, Bal Harbour, FL, November 1992.

B.M. Jarnot, C.M. Goecke, and N.V. Reo: "31P NMR of Altered Hepatic Phospholipids Following Exposure to the Peroxisome Proliferator-Perfluoro-n-decanoic Acid." *The Toxicologist* **12** (1), 39 (1992). Presented at the Society of Toxicology, Annual Meeting, Seattle, WA, February, 1992.

Manuscripts in Preparation

N. V. Reo, C. M. Goecke, L. Narayanan, and B. M. Jarnot. "Perfluoro-n-decanoic Acid is Unique Among Peroxisome Proliferators in its Effects on Hepatic Phospholipid Metabolism." Will be submitted to: *Chem. Research in Toxicol.*

C.M. Goecke, B.M. Jarnot, and N.V. Reo. "Inhibition of Hepatic Glucose and Glycogen Metabolism by the Peroxisome Proliferator--Perfluoro-n-decanoic Acid: A Nuclear Magnetic Resonance Investigation *in Vivo*." Will be submitted to: *Toxicol. Appl. Pharm.*

Personnel

Laboratory Technician

In July 1992 a research technician (Latha Narayanan) was added to the laboratory with the support provided by the AFOSR grant. Latha has a M.S. degree in mathematics and 10 years experience as a technician in a research laboratory in the Department of Pharmacology and Toxicology at the Medical College of Georgia. She brings experience in biochemical techniques and assays which has begun to expand our capabilities and our approach toward research objectives. NMR data obtained from tissue *in vivo* and complementary information from various biochemical assays provide a more complete picture of the metabolic processes under investigation.

Students

The AFOSR grant provided a graduate student stipend for Carol Goecke, a fifth-year Ph.D. student in my laboratory, from December 1991 until June 1992. In June this stipend was moved to a recently awarded AASERT grant.

In September 1992 Marjorie Artz, a second-year Ph.D. student joined the laboratory and her graduate stipend was assumed by the AFOSR grant. Only one month of the stipend was paid, however, since this student decided to pursue her graduate degree on a part-time basis while working full-time. Currently, I am actively seeking new graduate students to join the laboratory.

Interactions with Air Force Laboratories

On May 22, 1992 myself and two students from my laboratory attended the *Fourth Basic Research Round Table Colloquium* at the Toxicology Division, US Air Force Armstrong

Laboratory, Wright-Patterson AFB, Ohio. This meeting offers an informal forum in which colleagues present data and exchange ideas relating to the toxicology of perfluorocarboxylic acids and other classes of peroxisomes proliferators. The participants included investigators from various institutions and are listed below.

G. Gordon Gibson	Molecular Toxicology Group Department of Biochemistry University of Surrey Guilford, Surrey, U.K.
Frank Witzmann	Department of Biology Indiana University-Purdue University at Indianapolis, Columbus Campus Columbus, IN
Paul Serve	Department of Chemistry Wright State University Dayton, OH
Nicholas Reo Carol Goecke Marjorie Artz	Department of Biochemistry Kettering-Scott Magnetic Resonance Lab Wright State University and Kettering Medical Center Kettering, OH
Larry Mathes Mark Pollman Debbie Nelson	Department of Veterinary Pathobiology The Ohio State University Columbus, OH
Marilyn George Bruce Jarnot Nick DelRaso Greg Harvey Steve Channel	Toxicology Division Armstrong Laboratory Wright-Patterson AFB, OH

RESEARCH PROGRESS

I. EFFECTS OF PERFLUOROCARBOXYLIC ACIDS ON CARBOHYDRATE METABOLISM

We have been actively investigating the hepatic metabolic consequences of PFOA and PFDA exposure. In the Annual Technical Report submitted January 24, 1992 experiments were described in which the effects of PFDA on hepatic glucose and glycogen metabolism were studied by carbon-13 NMR spectroscopy. The results of these studies showed that PFDA inhibits hepatic glycogenesis from [1-^{13}C]glucose. Previous reports in the literature have described pronounced effects of perfluorocarboxylic acids on lipid metabolism, but this was the first demonstration of the impact of PFDA on carbohydrate metabolism. Delineating the mechanism by which PFDA affects glycogenesis has become an immediate goal of our research efforts. This information will provide greater insight into understanding the mechanisms associated with the hepatotoxicity of this class of fluorocarbon compounds.

The goal of recent studies from our laboratory as to determine if PFDA similarly inhibits hepatic glycogenesis from [3-^{13}C]-alanine. Alanine is a major glucogenic amino acid and studies employing ^{13}C NMR can provide information about gluconeogenesis in the intact rat liver. The details of the experimental protocol and the results of these studies are described below.

A. Methods

Male F-344 rats (230-310 g) were paired according to similar body weights. Treated rats received a single ip. injection of PFDA (50 mg/kg) dissolved in vehicle (1:1 v/v propylene glycol/water), while their pair-fed controls received an equal volume of vehicle. Rats were subjected to one of two experimental protocols: *(i)* PFDA (n=5) and control (n=5) rats received an intravenous (iv) bolus of [3-^{13}C]-alanine (600 mg/kg); *(ii)* PFDA (n=5) and control rats (n=7) received an iv bolus of [3-^{13}C]-alanine (600 mg/kg) and a continuous iv infusion of glucose (6.39 mg/kg·min) throughout the entire experiment. Supplementary glucose was given in the latter protocol to promote the incorporation of a greater percentage of ^{13}C-glucose produced from [3-^{13}C]-alanine into hepatic ^{13}C-glycogen. On 5 days post-treatment, the liver was surgically exposed and proton-decoupled ^{13}C-NMR liver spectra were acquired *in vivo* using a surface coil probe at 8.5 T. Following baseline data accumulation, an iv bolus injection of [3-^{13}C]-alanine was given and subsequent NMR spectra were collected with 5 min time resolution.

B. Results

Glucogenesis from Alanine

Figure 1A is a natural abundance ^{13}C-liver spectrum from a control rat on day 5 post-treatment. The low-frequency region of overlapping signals between 15 and 50 ppm is due to the methyl and methylene carbons primarily from phospholipids, free fatty acids, and triacylglycerols. The peak at 54.6 ppm is attributed to the methyl carbons of the choline head groups and serves as an internal chemical shift reference. The two regions appearing at higher frequency constitute the single- and double-bonded allylic carbons associated with unsaturated fatty acids at ca. 130 ppm and the carbonyl carbons at ca. 172 ppm. The peak at ca. 196 ppm is from an external CS_2 reference. Figure 1B is a ^{13}C-liver spectrum obtained from the same control rat 42.5 min post-alanine. In addition to the C_3-alanine signal, newly formed resonances attributable to glucose and to the C_1-glycogen are present. The incorporation of alanine into glucose and glycogen was observed in all control rats (n=12). Figure 2A is the natural abundance ^{13}C-liver spectrum from a PFDA treated rat on day 5 post-treatment. Note the improvement in spectral resolution and the increase in intensity of the methylene resonance at 30 ppm as compared to control (Fig. 1A). This is believed to reflect an increase in

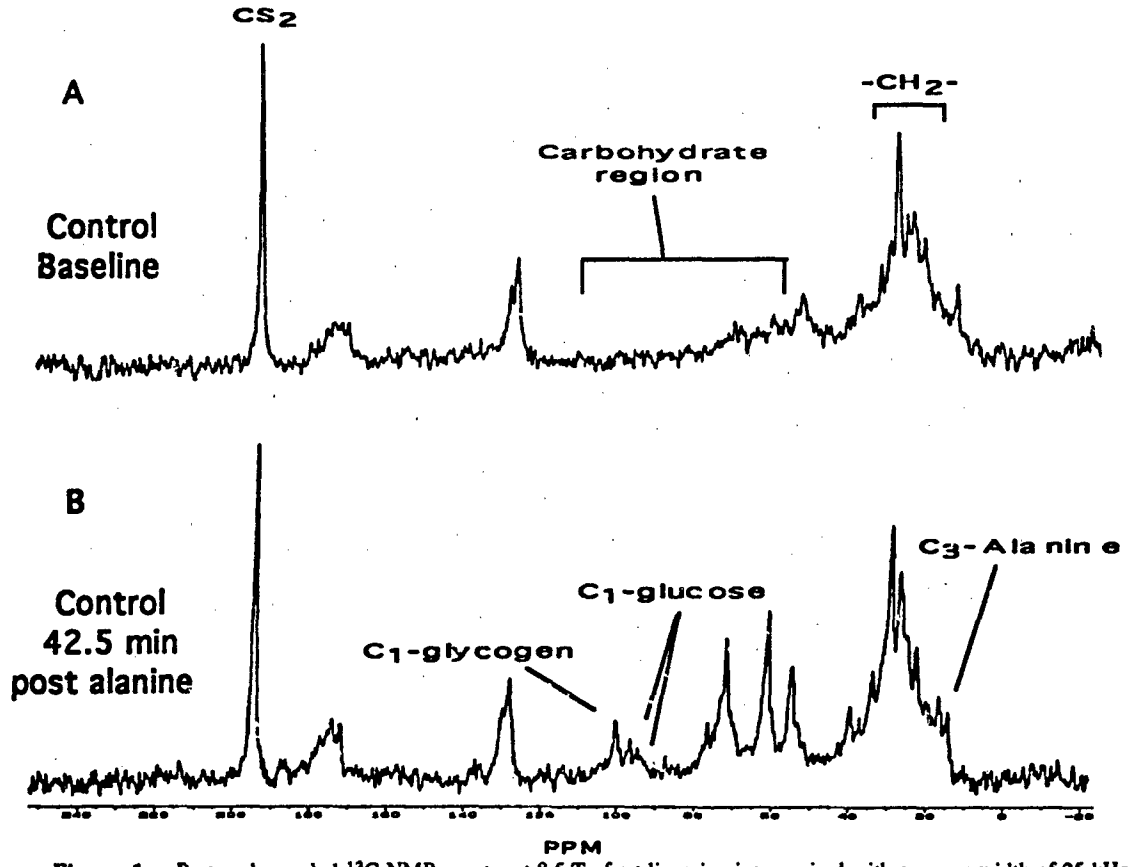

Figure 1. Proton-decoupled ^{13}C-NMR spectra at 8.5 T of rat liver *in vivo* acquired with a sweep width of 25 kHz and 5 min of signal averaging. Spectra were processed using 8K total data points and a 30 Hz exponential filter. Chemical shifts are reported relative to TMS via the methyl-carbon choline signal which is assigned a value of 54.60 ppm. (A) Baseline natural abundance ^{13}C-liver spectrum acquired from a control rat on day 5 post treatment with vehicle solution. (B) Spectrum acquired from the same control rat at 42.5 min post alanine dose. The C_1 carbon atom of glycogen is clearly observed at 100.5 ppm.

liver triglycerides, free fatty acids and/or an increase in membrane fluidity. Figure 2B is a ^{13}C-liver spectrum obtained from the same PFDA rat, 42.5 min post alanine. This spectrum also shows resonances attributable to glucose and to the C_1-glycogen. The incorporation of alanine into glucose was observed in all PFDA rats (n=10) while its incorporation into glycogen was observed in five of these animals.

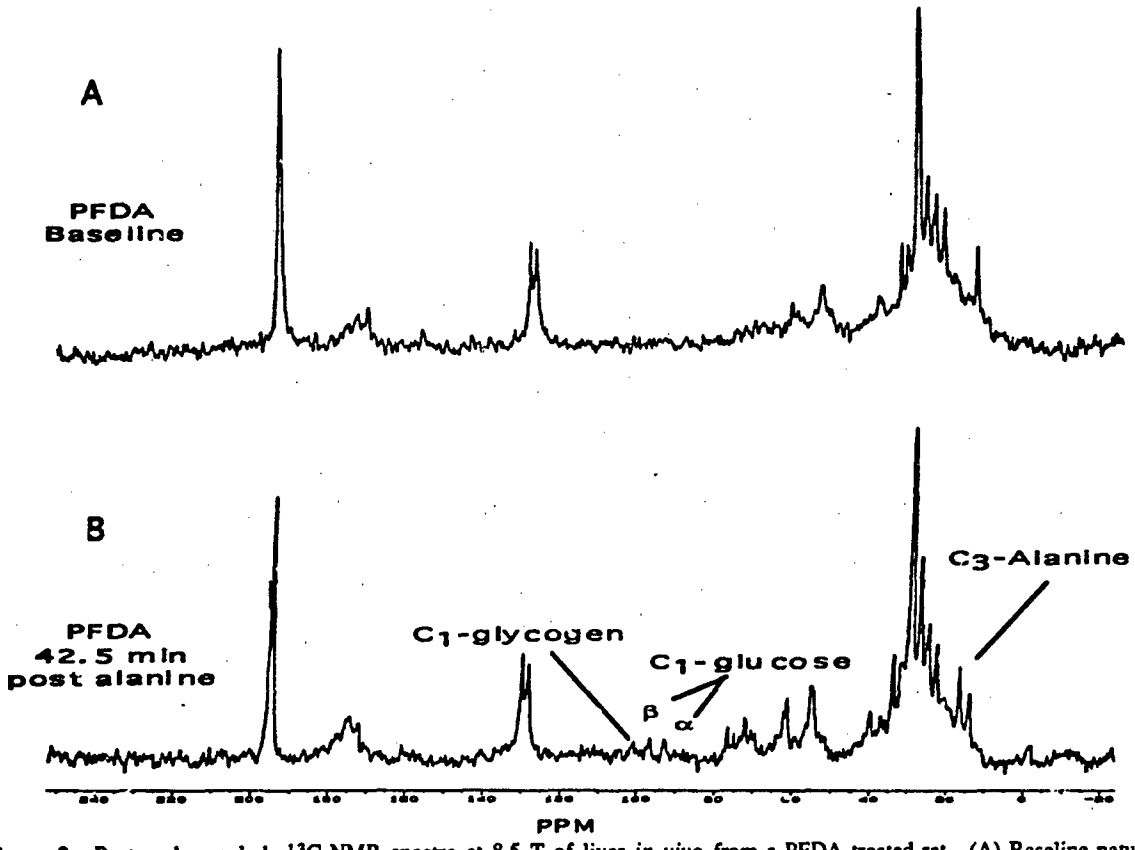

Figure 2. Proton-decoupled ^{13}C-NMR spectra at 8.5 T of liver *in vivo* from a PFDA treated rat. (A) Baseline natural abundance spectrum acquired on day 5 post-treatment. (B) Spectrum acquired from the same PFDA rat at 42.5 min post alanine dose. Acquisition and processing parameters are identical to those described for Figure 1.

Alanine Utilization

Following iv. bolus of [3-^{13}C]-alanine, subsequent ^{13}C-liver spectra reveal a large intensity attributable to the C_3-alanine. A plot of the mean percent change in alanine intensity over time in PFDA and control rats receiving only [3-^{13}C]-alanine is shown in Figure 3. PFDA rats have a significantly higher mean liver alanine intensity from 32.5 to 47.5 min post alanine as compared to controls ($p < 0.05$). Figure 4 is a plot of the mean percent change in hepatic alanine intensity for PFDA and control rats receiving both glucose and [3-^{13}C]-alanine. Again PFDA rats display a significantly higher mean liver alanine level from 22.5 to 52.5 min post alanine, as compared to control rats ($p < 0.05$). The curves (alanine intensity vs time) for individual animals were fit to a third order polynomial and the slopes were calculated at

specific time points from the first derivative of the polynomial expression. These values represent the relative rates of alanine utilization and are shown in Table 1 for all groups at various times post-alanine (mean ± SEM). The rates of alanine utilization are significantly greater for controls versus PFDA treatment from 12.5 to 22.5 min post alanine for rats receiving only alanine, and at 17.5 min for rats receiving both alanine and glucose ($p \leq 0.05$). Controls show rates which are about 40% greater than PFDA-treated rats wihtout added glucose (from 12.5 to 22.5 min), and about 60% greater with glucose (from 12.5 to 27.5 min).

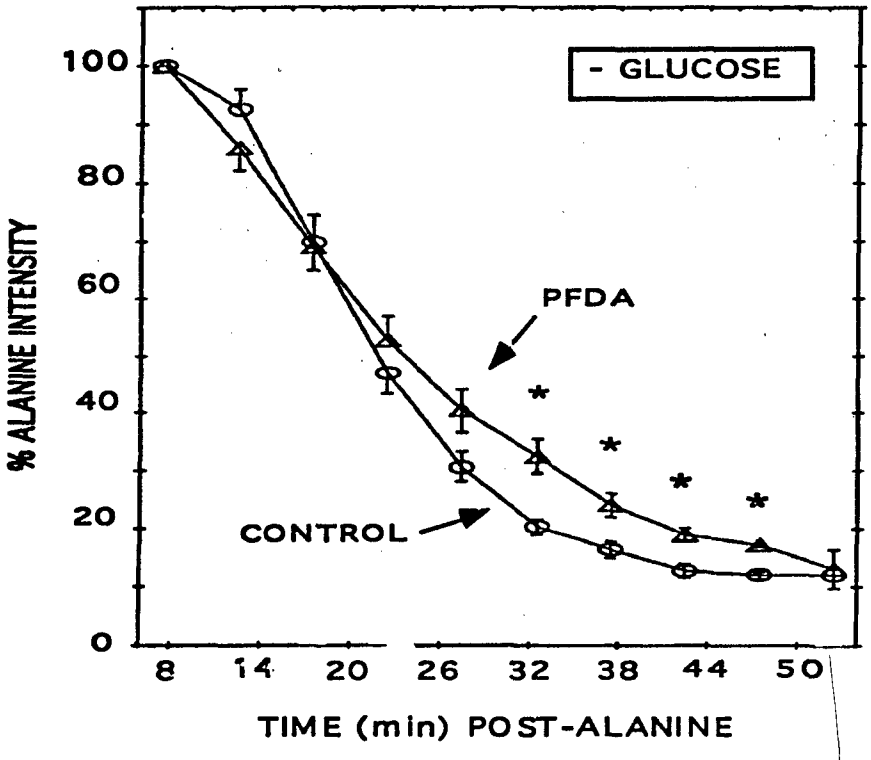

Figure 3: Percent change in the NMR intensity (mean±SEM) of the C_3-alanine signal for control (circles, n=5) and PFDA rats (triangles, n=5) following an iv. injection of [3-^{13}C]-alanine. The C_3-alanine signal was normalized to 100% in the second spectrum acquired post alanine. The asterisk (*) indicates a significant difference from control.

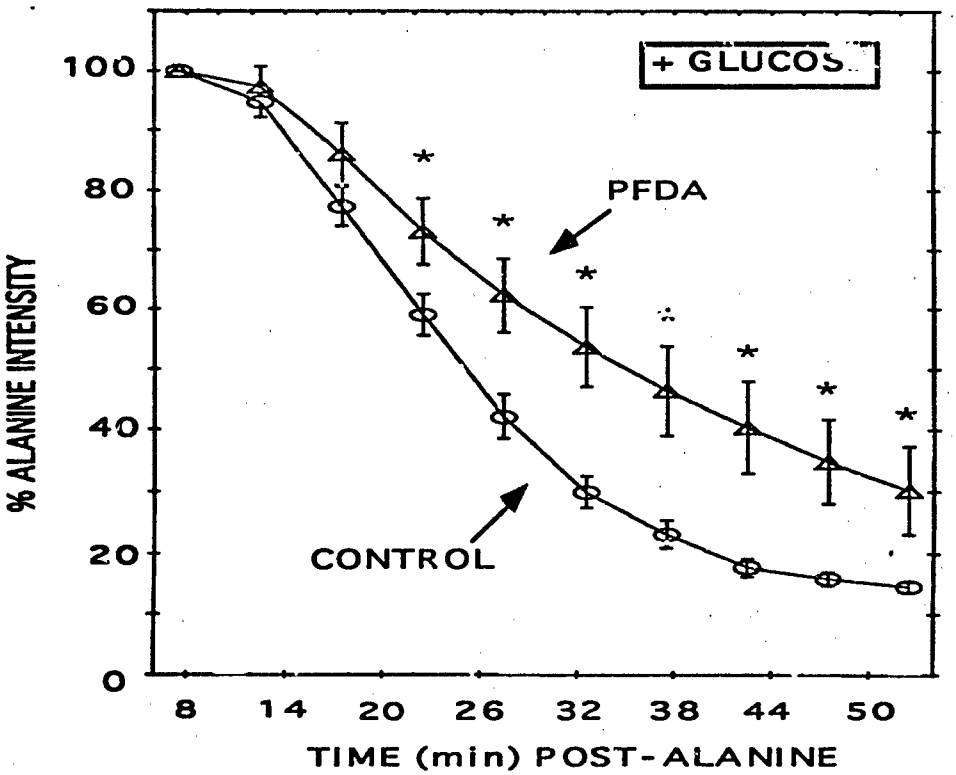

Figure 4: Percent change in the NMR intensity (mean±SEM) of the C_3-alanine signal for control (circles, n=7) and PFDA rats (triangles, n=5) receiving [3-^{13}C]-alanine and glucose. The C_3-alanine signal was normalized to 100% in the second spectrum acquired post alanine. The asterisk (*) indicates a significant difference from control.

TABLE 1

Relative rates (mean ± SEM) of alanine utilization at specific times post-alanine

Time (min) Post-Alanine	Treatment (- glucose)		(+ glucose)	
	PFDA	Control	PFDA	Control
12.5	4.0 ± 0.4[a]	5.5 ± 0.3[a]	2.4 ± 0.5	3.8 ± 0.4
17.5	3.3 ± 0.2[a]	4.6 ± 0.1[a]	2.4 ± 0.3[b]	3.8 ± 0.3[b]
22.5	2.6 ± 0.2[a]	3.6 ± 0.2[a]	2.2 ± 0.2	3.7 ± 0.4
27.5	2.1 ± 0.2	2.6 ± 0.3	2.0 ± 0.3	3.3 ± 0.5
32.5	1.6 ± 0.2	1.7 ± 0.3	1.8 ± 0.3	2.7 ± 0.6

[a] Indicates a significant difference in PFDA vs. control rats which received only [3-13C]-alanine ($p<0.5$).

[b] Indicates a significant difference in PFDA vs. control rats which received [3-13C]-alanine and glucose ($p<0.5$).

C-13 Labeling Pattern of Glutamate

The liver glutamate level increases following alanine infusion and the glutamate acquires ^{13}C enrichment. The ^{13}C labeling pattern of glutamate was analyzed in the NMR spectra and provides information about the entry of pyruvate into the Krebs cycle as explained in the *Discussion Section*. The fraction of labeling at the C2 and C3 carbons as compared with the C4 carbon was calculated from the NMR signal intensities and expressed as an intensity ratio, (C2+C3)/C4. Analysis of the data shown in Table 2 indicates that the signal intensity ratio is significantly lower in PFDA-treated rats as compared with corresponding controls at both 15 and 25 min post alanine ($p \leq 0.05$).

TABLE 2

^{13}C Labeling of Glutamate: C2 + C3/C4 Intensity Ratio (Mean ± SEM)

Time (min) post-alanine	PFDA	Control
15	4.0 ± 0.5	6.8 ± 1.1
25	3.6 ± 0.06	8.3 ± 1.4

C. Discussion

The results of this study demonstrate that PFDA-treated rats show reduced rates of hepatic alanine utilization, however, gluconeogenesis from alanine remains functional in all PFDA rats. More importantly, glycogenesis from alanine remains functional following PFDA treatment, although this was not evident in all rats. We suspect that the PFDA animals have a greater need for glucose than controls and may be utilizing it rather than storing it as glycogen. These results are quite interesting since previous studies from our laboratory show complete inhibition in hepatic glycogenesis from glucose at 5 days post PFDA treatment. Thus the synthesis of glycogen from glucose is blocked, yet glycogen can be synthesized through gluconeogenic pathways.

The possible path[illegible] of glycogen synthesis from glucose and alanine are depicted in Figure 5. Glycogen[illegible]is from[illegible]se can occur either via a direct pathway or an indirect pathway in which glucose is first catabolized to gluconeogenic three carbon precursors (ie. triosephosphates, pyruvate, or lactate) and then re-synthesized to glycogen. Gluconeogenesis provides a viable route for glycogen synthesis from alanine and other gluconeogenic 3-carbon precursors. Note that all glycogenic pathways merge at glucose 6-phosphate (G6P) and proceed via a common pathway to glycogen. The fact that PFDA rats show functional glycogenesis from alanine but not from glucose suggests that the glycogenic pathway from G6P to glycogen remains functional in PFDA rats. Thus, the site/sites of inhibition in glycogenesis from glucose in PFDA rats is hypothesized to lie either in the hepatocellular transport of glucose and/or in its phosphorylation by glucokinase. In fact, we have measured G6P levels by a spectrophotometric technique (O. H. Lowry, *et al.*, *J. Biol. Chem.* 1961: **236**, 2746) in liver extracts prepared from control and PFDA-treated rats. Preliminary results (Table 3) yield significantly lower levels of G6P for PFDA-treated rats as compared with control animals at 3 days post dose ($p \leq 0.02$). This data is in accordance with the hypothesis that PFDA causes some inhibition in the transport and/or phosphorylation of glucose in liver. We are currently investigating the specific site(s) of this inhibition.

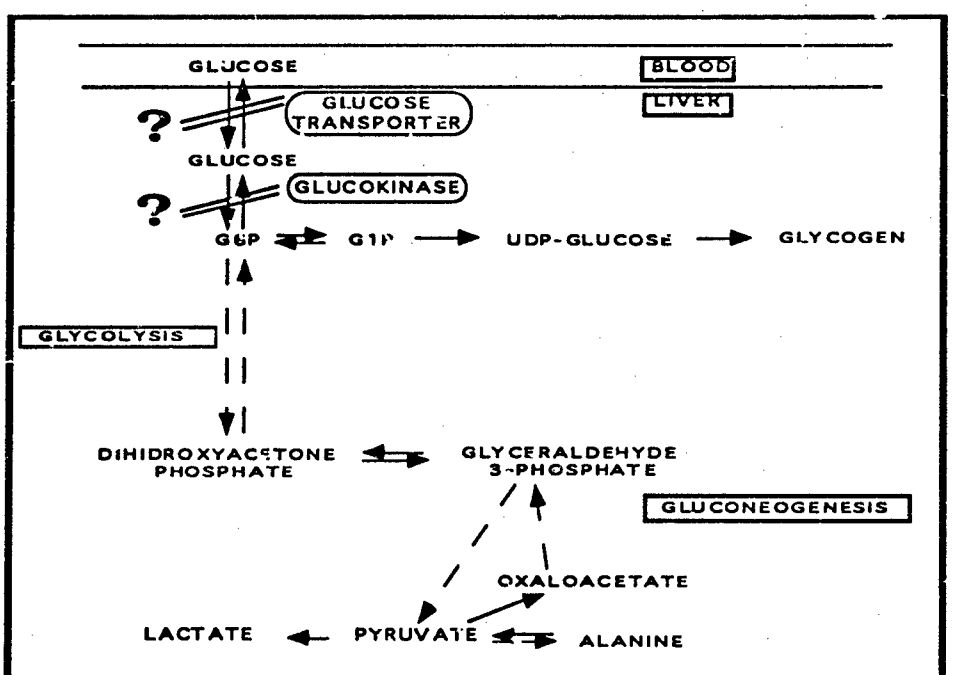

Figure 5. Metabolic pathways of glycogenesis from glucose and three carbon precursors such as alanine. Both pathways merge at glucose 6-phosphate (G6P) and proceed via a common pathway to glycogen. Question marks denote the hypothesized site/sites of glycogenic inhibition in PFDA-treated rats.

Table 3

Liver glucose-6-phosphate concentrations (mmol/kg tissue)

PFDA-Treated	Mean ± SD	Control	Mean± SD
Day 1 (n=2)	0.30 ± 0.046	Day 1 (n=2)	0.33 ± 0.033
Day 3 (n=3)	0.25 ± 0.060	Day 3 (n=5)	0.42 ± 0.073

Entry of Pyruvate into the Krebs Cycle

The use of ^{13}C enriched substrates in conjunction with NMR provides a means to follow specific metabolic pathways. One such pathway relating to the use of alanine as substrate concerns the fate of pyruvate in the mitochondria. Pyruvate enters the citric acid cycle as oxaloacetate (OAA) via the pyruvate carboxylase pathway, or as acetyl-CoA through the pyruvate dehydrogenase pathway. These pathways can be distinguished in a ^{13}C NMR experiment by observing the ^{13}C labeling in glutamate which accumulates as a result of its involvement in the transamination of alanine into pyruvate. In short, pyruvate which is converted to OAA yields glutamate labeled at the C2 and C3 carbons, while conversion of pyruvate to acetyl-CoA gives C4 labeled glutamate. These carbon resonances are well resolved in the ^{13}C NMR spectrum of liver. The ratio of intensity of the C2 and C3 carbons to that of the C4 carbon of glutamate provides quantitative information about the relative flux of pyruvate through the pyruvate dehydrogenase and pyruvate carboxylase pathways.

Analysis of the ^{13}C NMR data (Table 2) show a significantly lower signal intensity ratio ([C2+C3]/C4) in PFDA-treated rats as compared to their corresponding controls, and thus indicate that more pyruvate is converted to acetyl-CoA in PFDA rats. These results are consistent with the hypothesis that the supply of acetyl-CoA for Krebs cycle activity (which is necessary to sustain active gluconeogenesis) may be derived exclusively from pyruvate in PFDA-treated rats. The inhibition of fatty acid oxidation by PFDA (as observed by others) limits the supply of acetyl-CoA from this source, and thus pyruvate serves as the sole source of both OAA and acetyl-CoA.

D. Conclusions

These experiments yield new information regarding the effects exhibited by perfluorocarboxylic acids on cellular metabolism, and further our understanding of the toxicological mechanisms associated with this important class of Air Force compounds. Future studies will focus on the effects of these compounds on the hepatic glucose transporter and glucokinase. A manuscript is presently being prepared for publication describing our studies relating to the effects of PFDA on hepatic glycogenesis.

II. EFFECTS OF PERFLUOROCARBOXYLIC ACIDS ON HEPATIC PHOSPHORUS METABOLISM

Previous studies from our laboratory have revealed changes in the phosphorus metabolites of liver due to PFDA treatment (preliminary data were presented in last years Annual Technical Report). P-31 NMR spectra of liver *in vivo* yield resonances from various phosphorus metabolites including adenosine triphosphate (ATP), phosphomonoesters (PME) and phosphodiesters (PDE). PFDA-treated rats showed an enhanced signal intensity in the PME region of the ^{31}P liver spectrum as compared to controls. These observations have prompted a comparative study of PFDA, PFOA, and clofibrate (CLOF) in both rats and guinea pigs. All three compounds are known liver peroxisome proliferators in rodents. The guinea pig, however, represents a non-proliferative species with regard to liver peroxisomes. The purpose of this study was to identify the source of the enhanced PME resonance observed in PFDA-treated rats, its metabolic significance, and to determine if this effect is common among various peroxisome proliferators. Additionally, the inclusion of a non-proliferative species such as the guinea pig enables one to determine if these effects are related to the presence of liver peroxisomes.

A. Methods

Male F-344 rats (200-250 g) and male Hartley guinea pigs (450-550 g) were paired according to similar body weights for treatment and control groups. PFOA and PFDA were prepared in propylene glycol/H_2O (1:1 v/v), and CLOF was mixed in 0.9% saline. Treatments were given via intraperitoneal (ip) injection; control animals were dosed with an equal volume of vehicle (propylene glycol/H_2O or saline). Body weight and food consumption were monitored daily, and control animals were pair-fed with dosed partners. All animals were fasted 16-24 h prior to NMR experiments. Details relating to doses and time of NMR experiments post-dose are outlined in Table 4.

TABLE 4

Experimental protocol outlining doses for each treatment and the day post-dose that NMR experiments were conducted

Species		PFOA (single injection)	PFDA (single injection)	CLOF (multiple injection)
Rats	Dose	150 mg/kg	50 mg/kg	250 mg/kg/day for 3 days
	Day of Expt. Post-Dose	Days 1, 3, 5	Days 1, 3, 5	Day 5 post initial dose
Guinea Pigs	Dose	150 mg/kg	100 mg/kg	250 mg/kg/day for 3 days
	Day of Expt. Post-Dose	Day 3	Day 5	Day 5 post initial dose

NMR Experiments

Animals were anesthetized with halothane, the liver was surgically exposed, and data acquired using a 1 cm diameter surface coil NMR probe. Experiments involving rats utilized a Bruker AM 360 NMR spectrometer (8.5 T) operating at 145.8 MHz for ^{31}P. Data were acquired using a pulse width (PW) of 20 µsec (@ 100 W), sweep width (SW) of 10 kHz, interpulse delay of 7.05 sec., and 120 transients. Data were processed using 2K total points, exponential multiplication yielding 10 Hz linebroadening, and Fourier transformation. The guinea pig ^{31}P NMR studies employed a Bruker Biospec 2.35/400 spectrometer (2.35 T) operating at 40.6 MHz. Data acquisition used a PW of 12 µsec, SW of 2994 Hz, interpulse delay of 5.08 sec., and 420 transients. Data were processed using 1K total points, exponential multiplication yielding 5 Hz linebroadening, and Fourier transformation. Spectral peak intensities were obtained by integration and normalized relative to the total integrated intensity for the entire phosphorus spectrum.

Upon completion of the NMR experiment, livers were freeze-clamped under liquid N_2 and perchloric acid extracts prepared. High-resolution ^{31}P NMR spectra of liver extracts were obtained at 8.5 T.

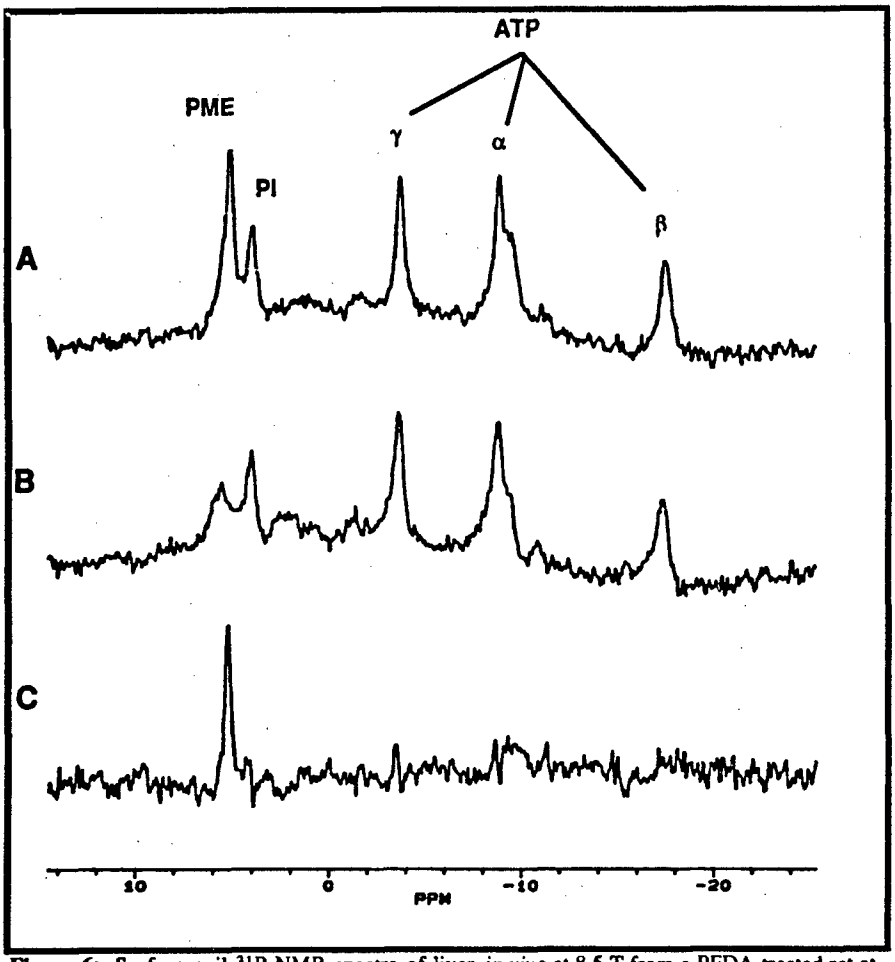

Figure 6: Surface coil ^{31}P NMR spectra of liver *in vivo* at 8.5 T from a PFDA-treated rat at 5 days post dose (A) and its pair-fed control (B). Data were acquired using a 20 μsec pulse (@ 100W), sweep width of 10 kHz, an interpulse delay of 7 sec, and 120 signal averages. The FID's were processed with 2 K total data points and an exponential filter producing a 10 Hz linebroadening. The difference spectrum (C) resulting from A-B depicts the changes which are induced by PFDA. Chemical shifts are referenced to PCr by setting the β-ATP peak to -16.0 ppm.

B. Results

Figure 6 compares the ^{31}P liver spectra for a PFDA-treated vs. a control rat at day 5 post dose. Note the enhanced signal intensity in the PME region for the PFDA rat. The β-ATP resonance serves as a monitor of ATP concentration and an indicator of overall tissue viability. The β–ATP and PME signal intensities are plotted in Figures 7 and 8 for rats and guinea pigs, respectively. For both species, the mean ATP levels among all treatment groups (within species) are <u>not</u> significantly different at the 95% confidence interval as determined by a

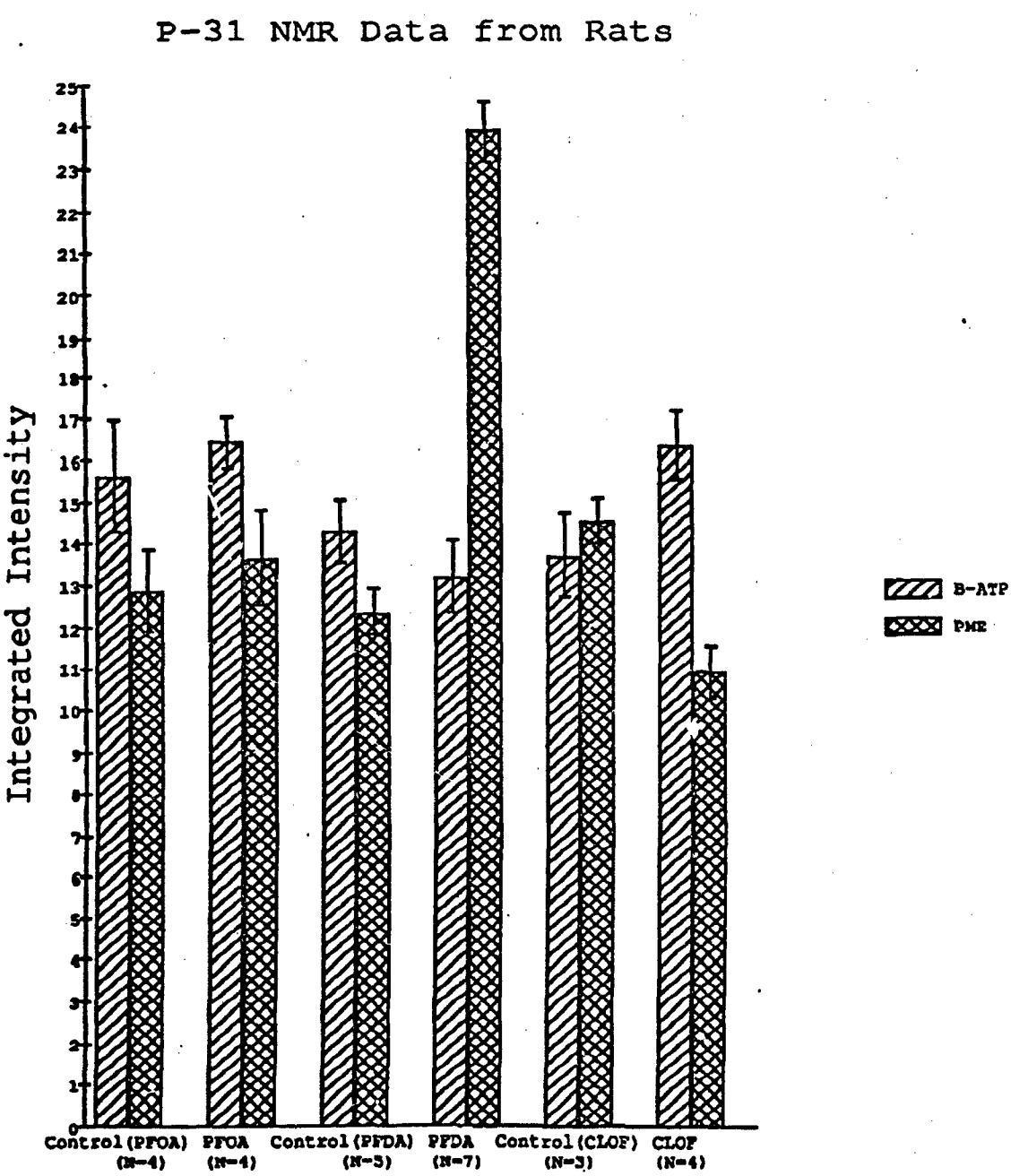

Figure 7: ^{31}P NMR spectral intensities (Mean ± SEM) for ß-ATP and PME peaks from rat livers *in vivo*. Data are shown for each treatment group and its corresponding pair-fed control group. The integrated signal intensities for both peaks in each animal spectrum were expressed relative to the integrated area for the entire ^{31}P spectrum set equal to 100.

oneway ANOVA and Bonferroni statistics. As seen in Figure 7, however, a marked increase (2-fold) in the PME signal intensity is observed in PFDA-treated rats, and a slight decrease (25%) is observed for CLOF treatment relative to corresponding controls ($p \leq 0.01$). In guinea pigs (Fig. 8), the mean PME intensity for the PFOA-treated group is one-half of the corresponding control value, while a comparison of PME levels for all other groups are not significantly different ($p \leq 0.01$).

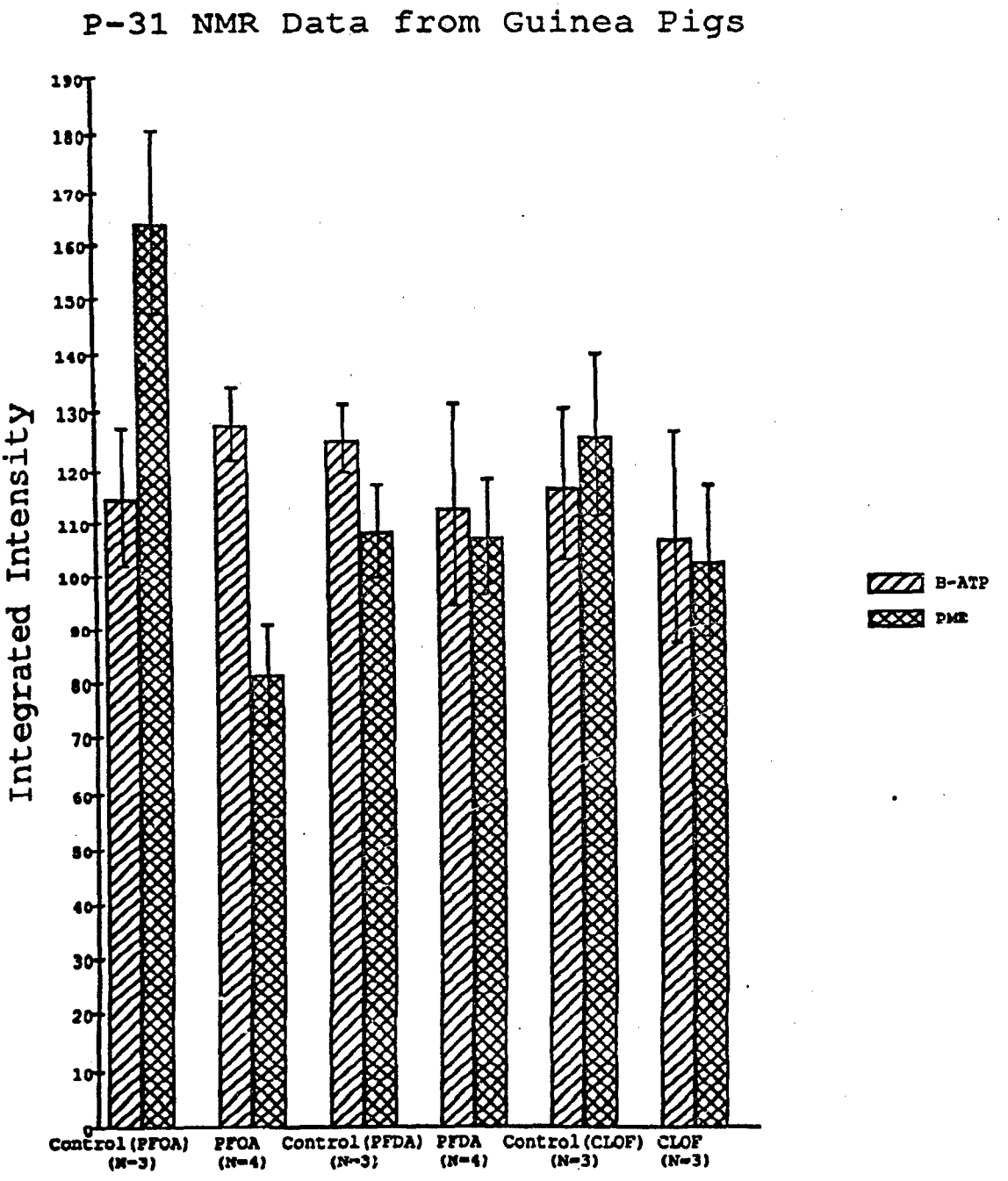

Figure 8: ^{31}P NMR spectral intensities (Mean ± SEM) for ß-ATP and PME peaks from guinea pig livers *in vivo*. Data is shown for each treatment group and its corresponding pair-fed control group. The integrated signal intensities for both peaks in each animal spectrum were expressed relative to the integrated area for the entire ^{31}P spectrum set equal to 1000.

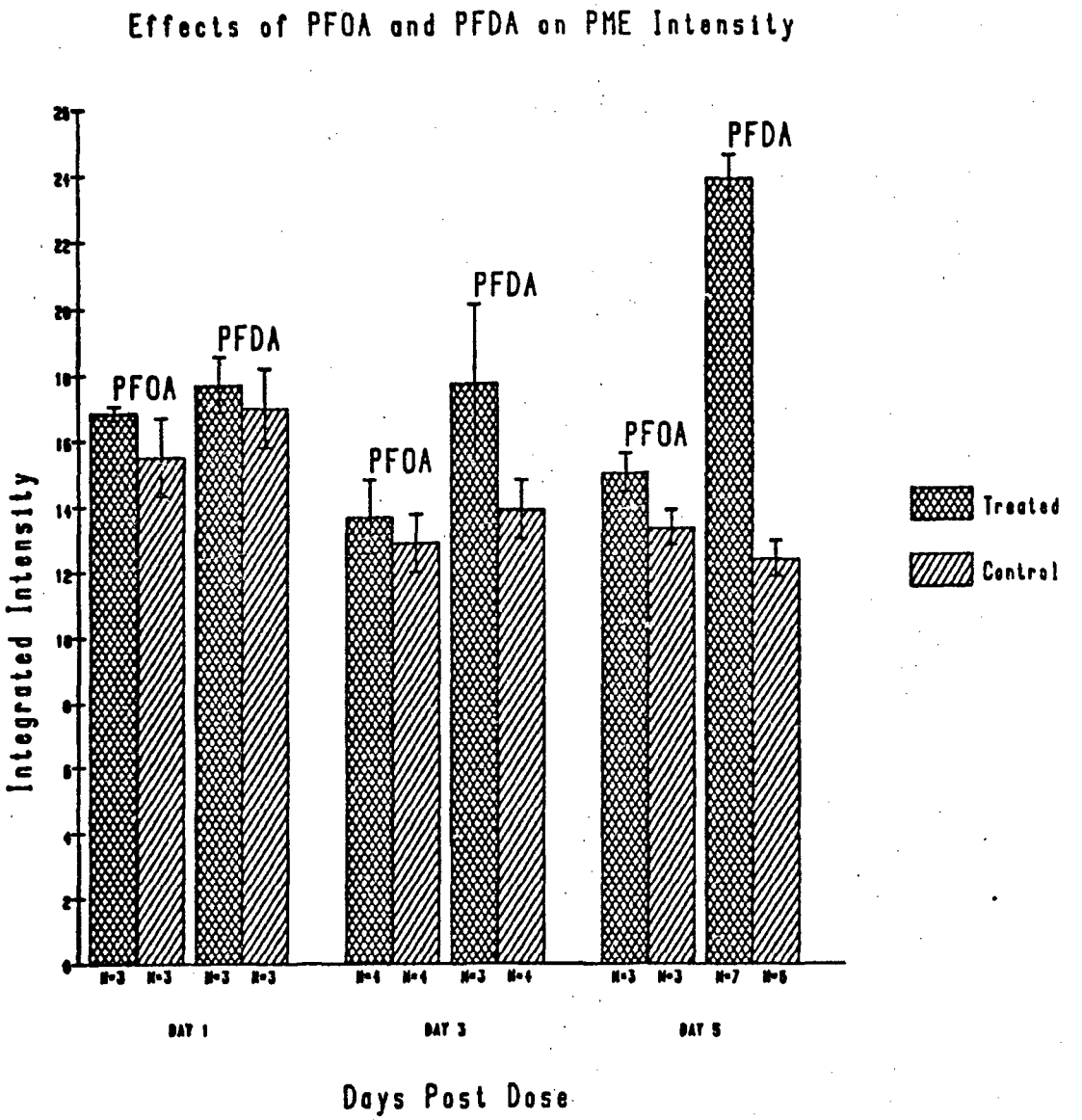

Figure 9: ^{31}P NMR spectral intensities (Mean ± SEM) for the PME peak from rat liver spectra *in vivo* for PFOA, PFDA, and corresponding control groups. Data are shown for each group at days 1, 3, and 5 post dose. Integrated signal intensities are relative to total phosphorus as described in Fig. 6 above.

Figure 9 shows the PME signal intensities for PFOA- and PFDA-treated rats (and controls) on days 1, 3, and 5 post dose. Only the PFDA group shows a deviation from control values, and is significantly higher than its control on day 5 ($p = 0.0001$). ATP levels are not different from control for all treatments at all days ($p \leq 0.01$).

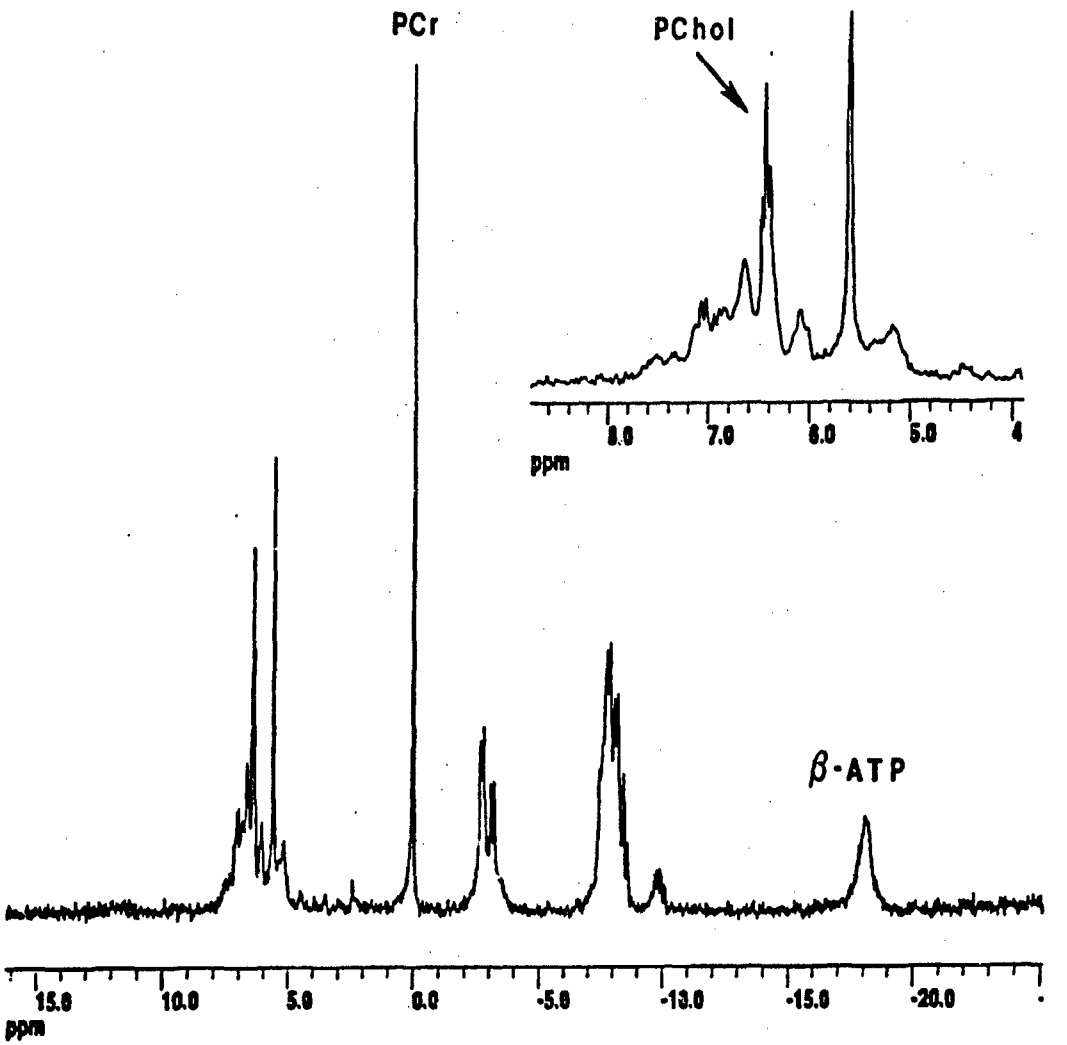

Figure 10: High-resolution ^{31}P NMR spectrum (8.5 T) of a perchloric acid liver extract from a rat at 5 days post-dose with PFDA. Phosphocreatine (PCr) was added (1 mM) as a chemical shift reference and concentration standard. The insert is an expanded plot of the phosphomonoester (PME) region. The triplet resonance at 6.4 ppm has been assigned as phosphorylcholine (PCho) and is seen exclusively in the liver spectra of PFDA-treated rats. This metabolite is responsible for the enhanced intensity observed in the PME region of the liver spectra from PFDA rats *in vivo* (see Fig. 6).

The spectrum of a liver extract prepared from a PFDA-treated rat (day 5 post dose) is shown in Figure 10. As compared to control, this sample shows an enhanced signal intensity in the PME region of the spectrum which is a single resonance (triplet) at 6.4 ppm. This resonance was identified as phosphocholine (PCho), and its assignment confirmed by addition of PCho to the extract solution.

The PCho concentration was calculated from liver extract spectra by adding a known amount of phosphocreatine (PCr) to the sample and normalizing the integrated signal intensities to the PCr peak. The results of these measurements are given in Table 5 and clearly show that the level of PCho in PFDA-treated rats increases with days post-dose and ranges from 2 to 4-fold higher as compared to control animals.

Table 5

Liver phosphorylcholine levels (Mean ± SD) in μmol/gm tissue

Days Post Dose	PFDA-Treated	Control
Day 1	2.31 ± 0.23 (n=2)	1.12 ± 0.30 (n=2)
Day 3	3.45 ± 0.63 (n=2)	1.60 (n=1)
Day 5	4.56 ± 0.21 (n=2)	1.15 (n=1)

C. Discussion

The increased concentration of liver PCho is a specific response of PFDA-treatment in rats. An increase in this metabolite may be indicative of enhanced phospholipid turnover, specifically phosphatidylcholine (PC). This response does not seem to be related to the presence of peroxisomes since neither PFOA nor CLOF produce an increase in PME. Interestingly, the observed differences between rats versus guinea pigs cannot be accounted for based solely upon species differences in the proliferous capacity for liver peroxisomes.

PCho is an integral metabolite in the processes of PC turnover (Figure 11). The level of PCho is affected by PC breakdown via phospholipases and PC synthesis via the choline/CDP-choline pathway. Further studies are necessary to discern which specific pathways are responsible for the increase in PCho observed in PFDA rats. We favor an interpretation which assumes enhanced PC synthesis involving successive acylation of G3P to yield phosphatidic acid–a precursor for both triglyceride and phospholipid synthesis. Diacylglycerol (DAG) levels may also be elevated and thereby promote PC turnover via phospholipase C through a protein kinase C activated mechanism. This interpretation is in accordance with other known metabolic effects caused by PFDA including hyperplastic hepatomegaly, disruption of mitochondrial fatty acid metabolism, decreased peroxisomal fatty acid β-oxidation, and

elevated liver triglycerides. Peterson and coworkers (M. J. Van Rafelghem,*et al. Lipids* 1988: **23**, 671) have suggested that PFDA causes a reduction in fatty acid oxidation which redirects excess liver fatty acids toward esterification and triglyceride synthesis. Our results may further imply that fatty acids are also being directed into pathways of phospholipid metabolism.

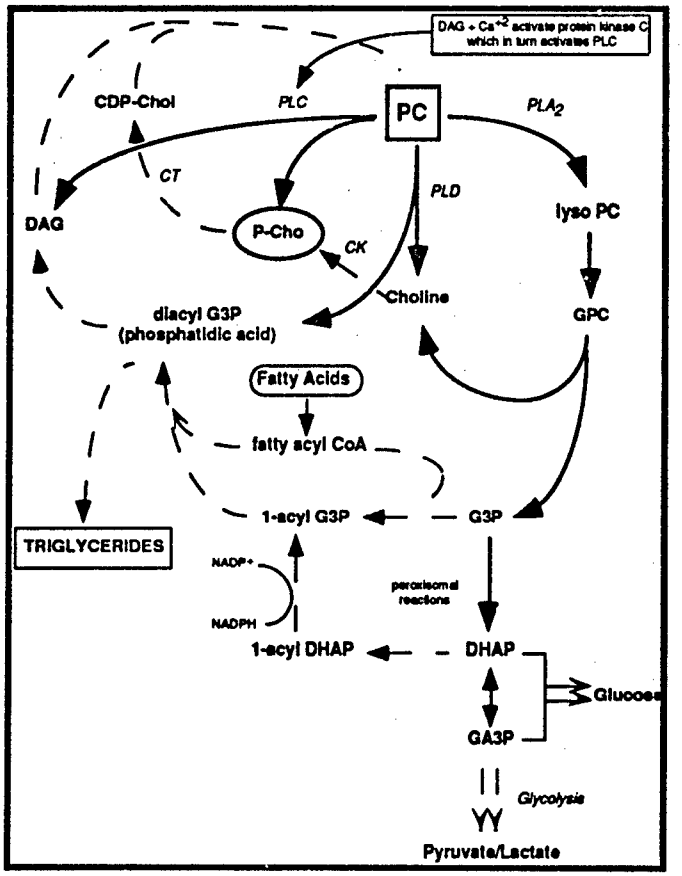

Figure 11: Pathways of phosphatidylcholine (PC) metabolism. Key: Glycerophosphocholine (GPC), glycerol-3-phosphate (G3P), dihydroxyacetonephosphate (DHAP), diacylglycerol (DAG), phosphocholine (P-Cho), phospholipases A_2, C, D (PLA_2, PLC, PLD), choline kinase (CK), CTP:phosphocholine cytidylyltransferase (CT).

D. Conclusions

The results of these studies demonstrate a unique effect of PFDA on liver phospholipid metabolism. Further studies are currently in progress to measure the levels of liver DAG and the activity of phospholipase C in both control and PFDA-treated rats. This information is necessary to gain a clear interpretation for the elevation in liver PCho levels. Additionally, the results of the present studies pose an interesting question: "Does PFDA cross the plasma membrane and become internalized in the cell?" It is quite likely the 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. We hope to address this question by using NMR spectroscopy in conjunction with chemical shift reagents. With the use of a suitable shift reagent and a membrane vesicle system, it may be possible to distinguish "intracellular" from "extracellular" PFDA from the ^{19}F or ^{13}C NMR signal. Preliminary studies are in progress to evaluate the effects of various shift reagents on the PFDA NMR signals. These investigations will provide important information concerning the partitioning of PFDA between the membrane and intracellular environment. Such knowledge may shed new light on the cellular mechanisms responsible for the toxicity of these perfluorocarbon compounds.