

3M Specialty Materials

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AR 226_0644

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September 19, 2000

FYI-00-001378
450000000 SQS

Ms. Vanessa Williams
EPA Document Control Office
US Environmental Protection Agency
401 M Street, S.W.
Washington, DC 20460-001

FAX 202-260-9555

Dear Ms. Williams:

Regarding an August 31, 2000 submission to EPA by 3M entitled "Information on Perfluorooctane Sulfonate and Related Compounds", one specific environmental study included in the submission was claimed as CBI. All other studies and information included in that submission are considered to be non-CBI by 3M.

Best regards,

William A. Wagoner

William A. Wagoner, Ph.D.
Director
Environmental, Health, Safety & Regulatory Affairs
Specialty Material Markets Group
3M Center, Bldg. 236-1B-10
St. Paul, MN 55144

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St. Paul, MN 55144-1000
651 733 1110

AR 226_0644

FYI - 0900 - 1378

MR FHSJ
AR 39320



August 31, 2000

VIA OVERNIGHT DELIVERY

CONFIDENTIAL

Dr. Charles Auer
Director
Chemical Control Division
Office Of Pollution Prevention And Toxics
United States Environmental Protection Agency
401 M Street, Southwest
Room 403 East Tower (Mail Code 7405)
Washington, D. C. 20460

2000 SEP - 1 PM 12:29

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2000 SEP 11 AM 10:15

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Re: Information On Perfluorooctane Sulfonates And Related Compounds

Dear Charlie:

Pursuant to our recent communications, 3M is enclosing additional information on perfluorooctane sulfonates and related compounds. The enclosed information supplements information submitted to you previously, including our letters dated April 21, May 4, May 18, May 25, and June 28, 2000. Again, we are providing this information on a voluntary basis as part of our continuing discussions with EPA regarding fluorochemistry.

We are enclosing information on perfluorooctane sulfonates and related compounds that either post-dates our most recent submission or has been located as part of our continuing file search. The enclosed information includes:

- > Copies of post-1976 studies and certain other information relating to the following health effects areas: (i) acute toxicity; (ii) genotoxicity; (iii) repeated-dose toxicity; (iv) pharmacokinetics; (v) teratology; and (vi) medical surveillance and epidemiology. We have organized this information in labeled file folders for each compound, and provided a detailed index, to aid EPA's review. Some acute studies are enclosed, while less significant acute studies appear only as bibliographic entries on the index. The index also notes a few minor corrections to earlier indices.
- > Copies of post-1976 studies and certain other information relating to the following environmental science areas: (i) environmental fate and transport and (ii) environmental monitoring. These materials are contained in a separate file folder with an index in table form.



000001a

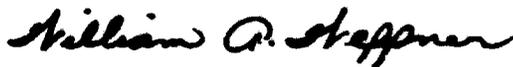
8009001233E

One enclosed environmental document contains information that qualifies as confidential business information (CBI); we have enclosed a sanitized version of that document, and are submitting the unredacted version in a sealed envelope.

As in our prior submissions, many of the studies refer to the test substance only by a 3M product identification number with a "T", "L" or "FC" prefix. Although analysis of the test substance has been included where available, it is often not possible to determine from the study report itself the identity of the test substance. Thus, we have 3M's historical records keyed to the 3M product number to determine the composition of the test substance. Where available, the index provides composition information such as percent composition, solvent context, salt form and purity grade for compounds not so described in prior submissions. Please note that the term "narrow range" refers to a higher purity grade of a compound. Finally, it should be recognized that product formulations have evolved over the years; composition information may not be contemporaneous with the toxicology studies, and some of the test substances do not constitute current products.

3M is continuing its file review and will supplement the enclosed information as appropriate. 3M looks forward to discussing the enclosed information with you and other EPA staff. In the meantime, please do not hesitate to contact me with any questions.

Very truly yours



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Safety And Regulatory Affairs
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Enclosures

000002

**ATTACHMENT TO LETTER TO C. AUER DATED AUGUST 31, 2000
SUPPLEMENTAL SUBMISSION**

TOXICOLOGY AND MEDICAL SURVEILLANCE

PFOS	Perfluorooctane sulfonate
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Medical Surveillance and Epidemiologic

1. Interim Report #1, Determination of Serum Half-Lives of Several Fluorochemicals, June 8, 2000 (previously submitted to TSCA 8(e) docket, included here for convenience)
2. Data regarding general population blood samples from several countries analyzed by 3M's Environmental Laboratory in 1999 (no report was prepared).

Acute Toxicity

The following pre-1976 study was listed in the bibliography of acute studies with a prior submission, but a copy is now being submitted for EPA's convenience:

1. Report by WARF Institute, Inc. and accompanying documentation, Primary Skin Irritation and Primary Eye Irritation in Albino Rabbits, Sample T-1117 (FC-95), November 7, 1974

Acute Toxicity (Bibliography Only - Copy Not Submitted)

1. Final Report, Acute Ocular Irritation Test with T-2997CoC in Albino Rabbits, Safety Evaluation Laboratory, Riker Laboratories, Inc., Study No. 0882EB0009, 3M Reference No. T-2997 (perfluoroethylcyclohexylsulfonate DEA salt), January 25, 1982.

Genotoxicity

1. Final Report, revised, In Vitro Microbiological Mutagenicity Assays of 3M Company Compound T-2816CoC, SRI International, SRI Study No. LSC-8958, 3M Reference No. T-2816CoC (PFOS DEA salt), March 1980.
2. Final Report, In Vitro Microbiological Mutagenicity Assays of 3M Company's Compound T-2997 CoC (perfluoroethylcyclohexylsulfonate DEA salt), SRI International, SRI Study No. LSC-8958, January 1981.

Teratology

Correction to May 25 Index: Materials provided were incorrectly indexed as Letter from E. Marshall Johnson to William C. McCormick. Index should read Letter from E. Marshall Johnson to Dr. Franklin D. Griffith, regarding Riker teratology studies on PFOS and ethyl FOSE, November 12, 1982.

000003

**ATTACHMENT TO LETTER TO C. AUER DATED AUGUST 31, 2000
SUPPLEMENTAL SUBMISSION**

Repeat Dose

1. **Additional Data on 26-Week Capsule Toxicity Study with Perfluorooctane Sulfonic Acid Potassium Salt (PFOS, 3M Reference T-6295):**
 - a) **Draft Pathology Report (Ancillary Study), Electron Microscopic Evaluation of Liver in Cynomolgus Monkeys (Recovery Animals), Study No. 6329-223, Pathology Associates International, PAI Study Number EM 99.76, May 30, 2000.**
 - b) **Data on TSH and Lipids in Cynomolgus Monkeys, Anilytics Incorporated, reported May 2, 2000, with accompanying correspondence including letter from Andrew Seacat, Ph.D. to Peter J. Thomford, Ph.D. dated March 6, 2000, and letter from Saroj R. Das, Ph.D. to Andrew Seacat, Ph.D. dated April 5, 2000 regarding methodology**

Pharmacokinetic

1. **The following study was previously identified in the list of published literature; a copy now being submitted for convenience:**

An Investigation of the Effects of Fluorocarbons on Liver Fatty Acid-Binding Protein, thesis submitted to the Graduate School of the University of Minnesota, by Deanna J. Nabbefeld, April 1998.

**Legend: FC1 = perfluorooctane sulfonic acid
FC2 = ammonium perfluorooctanoate
FC3 = N-Ethyl FOSE
FC4 = N-ethylperfluorooctane sulfonamide**

2. **Final Report, 28 Day Percutaneous Absorption Study with FC-98 in Albino Rabbits, Safety Evaluation Laboratory, Riker Laboratories, Inc., 3M Reference No. T-4980, (perfluoroethylcyclohexylsulfonate), Experiment No. 0979AB0630, March 15, 1981.**
3. **Final Report, Single-Dose Intravenous Pharmacokinetic Study of T-6684 in Rabbits, Corning Hazleton, Study No. CHW 6329-199, 3M Reference No. T-6684 (didecyldimethyl ammonium salt of PFOS), December 31, 1997.**
4. **Final Report, 5-Daily Dose Dermal Absorption/Toxicity Pharmacokinetic Study of T-6684 in Rabbits, Covance Laboratory, Study No. 6329-200, 3M Reference No. T-6684 (didecyldimethyl ammonium salt of PFOS), December 31, 1997. [Draft of this report was submitted to EPA with letter dated May 18, 2000.]**

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**ATTACHMENT TO LETTER TO C. AUER DATED AUGUST 31, 2000
SUPPLEMENTAL SUBMISSION**

PFOS Mixtures	
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Acute Toxicity (Bibliography Only)

1. Final Report, Primary Eye Irritation Study in Rabbits, Hazelton Laboratories America, Inc., 3M Reference No. T-4016, January 28, 1987.
2. Final Report, Acute Oral Toxicity Study in Rats, Hazelton Laboratories America, Inc., 3M Reference No. T-4016, February 9, 1987.
3. Final Report, Primary Dermal Irritation Study in Rabbits, Hazelton Laboratories America, Inc., 3M Reference No. T-4016, February 27, 1987.

POSF	Perfluorooctanesulfonyl fluoride
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Ongoing Studies

1. Study Outline dated June 22, 2000 and Protocol dated December 30, 1998 for Pharmacokinetic Study of POSF in Rats, 3M Reference No. T-7098.1.

N-EtFOSE alcohol	N-ethyl perfluorooctane sulfonamidoethanol
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Acute Toxicity

1. Final Report, Acute Oral Toxicity (LD₅₀) Study in Rats, FM-3422, International Research and Development Corporation, 3M Reference No. T-2253, Study No. 137-084, June 7, 1978

Pharmacokinetic

1. Biotransformation of ¹⁴C-N-Ethyl FOSE in Rats After Administration in Feed for One Week, Riker Laboratories, Inc., January 28, 1983.

The copy of the following item is submitted under PFOS:

An Investigation of the Effects of Fluorocarbons on Liver Fatty Acid-Binding Protein, thesis submitted to the Graduate School of the University of Minnesota, by Deanna J. Nabbefeld, April 1998.

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**ATTACHMENT TO LETTER TO C. AUER DATED AUGUST 31, 2000
SUPPLEMENTAL SUBMISSION**

Teratology

- 1. Teratology Studies on FM 3422, 3M Reference T-3352:
 - a) Final Report, Pilot Rat Teratology Study, T-3352, Hazleton Laboratories America, Inc., Project No. 154-159, May 11, 1983.
 - b) Final Report, Rat Teratology Study, T-3352, Hazleton Laboratories America, Inc., Project No. 154-161, March 21, 1984.
 - c) Protocol, Rat Teratology Study (Segment II), T-3352, Hazleton Laboratories America, Inc., June 2, 1983.

Correction To May 25 Index: Materials provided were incorrectly indexed as Letter from E. Marshall Johnson to William C. McCormick. Index should read Letter from E. Marshall Johnson to Dr. Franklin D. Griffith, regarding Riker teratology studies on PFOS and ethyl FOSE, November 12, 1982

Ongoing

Note: Mechanistic research on N-ethyl FOSE, 3M Reference Nos. T-3616.10, FM 3923, is ongoing at the University of Minnesota under the direction of Kendall Wallace. Documents regarding this research were submitted with the May 4, 2000 submission on PFOS (see Mechanistic 1)(a)-(f)) but were not indexed under N-ethyl FOSE.

N-EtFOSA	N-ethyl perfluorooctanesulfonamide
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Acute Toxicity

- 1. Final Report, Acute Oral Toxicity Screen with T-3067CoC in Albino Rats, Safety Evaluation Laboratory, Riker Laboratories, Inc., Experiment No. 0981AR0147, 3M Reference No. T-3067 (narrow range ethyl FOSA), May 15, 1987.

Correction to May 18 Submission: Three studies conducted by Corning Hazelton (CHW 61101149, CHW 61101150, and CHW 61101151) were erroneously listed on the May 18, 2000 bibliography under N-EtFOSA (Acute Toxicity bibliography only items 1 - 3). These studies should not have been listed there. They did correctly appear on the May 18 bibliography under PFOS and were provided under PFOS Acute Toxicity 1(a)-(c).

**ATTACHMENT TO LETTER TO C. AUER DATED AUGUST 31, 2000
SUPPLEMENTAL SUBMISSION**

Pharmacokinetic

1. Final Report, Single-Dose Intravenous Pharmacokinetic Study of T-5904 in Rabbits, Hazleton Wisconsin, 3M Reference No. T-5904 (95-99%), Study No. HWT 6329-117, October 14, 1994.

Submitted above under PFOS:

An Investigation of the Effects of Fluorocarbons on Liver Fatty Acid-Binding Protein, thesis submitted to the Graduate School of the University of Minnesota, by Deanna J. Nabbefeld, April 1998.

Ongoing

Mechanistic research at the University of Minnesota under the direction of Kendall Wallace includes N-Et FOSA, 3M Reference No. T-6868.1. Documents regarding this research have been previously submitted with the May 4, 2000 submission on PFOS (see Mechanistic 1)(a)-(f)).

PFHS	Perfluorohexane sulfonate
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Medical Surveillance and Epidemiologic

See item 1 under PFOS: Interim Report #1, Determination of Serum Half-Lives of Several Fluorochemicals, June 8, 2000.

FOSA or PFOSA	Perfluorooctanesulfonamide
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Medical Surveillance and Epidemiologic

See item 1 under PFOS: Interim Report #1, Determination of Serum Half-Lives of Several Fluorochemicals, June 8, 2000.

Ongoing - Pharmacokinetic

1. Protocol for Study No. T-7132.2; ST-39, Pharmacokinetic Study of Perfluorooctane Sulfonamide in Rats, 3M Medical Department, Corporate Toxicology Laboratory, In-Life End Date November 2, 1999, report on in life study pending. We are providing a copy of the Laboratory Report, Analytical Report of Data for PFOSA Pharmacokinetic Study in Rats (Sera and Liver), 3M Environmental Laboratory, Laboratory Report No. FACT-TOX-145 (W2814), 3M Reference No. T-7132.2, December 6, 1999.

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**ATTACHMENT TO LETTER TO C. AUER DATED AUGUST 31, 2000
SUPPLEMENTAL SUBMISSION**

PFOSAA	Perfluorooctane sulfonylamido (ethyl)acetate
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Medical Surveillance and Epidemiologic

See item 1 under PFOS: Interim Report #1, Determination of Serum Half-Lives of Several Fluorochemicals, June 8, 2000.

Acute Toxicity (Bibliography Only)

1. Final Report, Acute Oral Toxicity Study in Rats, Hazleton Laboratories America, Inc., Study No. 70905392, 3M Reference No. T-4101, FC-109 (19-23% PFOSAA Potassium salt in water), January 21, 1988.
2. Final Report, Acute Dermal Toxicity Study in Rabbits (OECD Guidelines), Hazleton Laboratories America, Inc., Study No. 70905393, 3M Reference No. T-4101, FC-109 (19-23% PFOSAA Potassium salt in water), January 18, 1988.
3. Final Report, Primary Dermal Irritation/Corrosion Study in Rabbits, Hazleton Laboratories America, Inc., Study No. 70905394, 3M Reference No. T-4101, FC-109 (19-23% PFOSAA Potassium salt in water), January 18, 1988.
4. Final Report, Primary Eye Irritation/Corrosion: Study in Rabbits, Hazleton Laboratories America, Inc., Study No. 70905395, 3M Reference No. T-4101, FC-109 (19-23% PFOSAA Potassium salt in water), January 18, 1988.

Ongoing

Mechanistic research at the University of Minnesota under the direction of Kendall Wallace includes PFOSAA, 3M Reference No. T-6896.1. Documents regarding this research have been previously submitted with the May 4, 2000 submission on PFOS (see Mechanistic 1)(a)-(f)).

PFOA	Perfluorooctanoic acid
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Medical Surveillance and Epidemiologic

See item 1 under PFOS: Interim Report #1, Determination of Serum Half-Lives of Several Fluorochemicals, June 8, 2000.

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**ATTACHMENT TO LETTER TO C. AUER DATED AUGUST 31, 2000
SUPPLEMENTAL SUBMISSION**

Acute Toxicity

1. Final Report, Acute Oral Toxicity Study in Rats, Hazleton Laboratories America, Study No. HLA 91201739, 3M Reference No. T-4416 (approximately 45% FC-143 solids - $C_7H_{15}COO-NH_4^+$, 2.2% homologs, 50% water, 2.5% inerts), April 2, 1990.
2. Final Report, Primary Dermal Irritation/Corrosion Study in Rabbits (OECD Guidelines), Hazleton Laboratories America, Study No. HLA 91201740, 3M Reference No. T-4416, February 14, 1990.
3. Final Report, Primary Eye Irritation/Corrosion Study in Rabbits (OECD Guidelines), Hazleton Laboratories America, Study No. HLA 91201740, 3M Reference No. T-4416, February 28, 1990.

Genotoxicity

1. Final Report, Mutagenicity Test on T-6358 Measuring Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells, Corning Hazleton Inc., Study No. 17388-0-437, 3M Reference No. T-6358, FC-143, Lot 332, April 25, 1996, with protocol.
2. Final Report, Mutagenicity Test on T-6358 in an *In Vivo* Mouse Micronucleus Assay, Corning Hazleton Inc., Study No. 17388-0-455, 3M Reference No. T-6358, FC-143, Lot 332, May 14, 1996, with protocol and protocol amendments.

Pharmacokinetics

Submitted above under PFOS:

An Investigation of the Effects of Fluorocarbons on Liver Fatty Acid-Binding Protein, thesis submitted to the Graduate School of the University of Minnesota, by Deanna J. Nabbefeld, April 1998

Ongoing

1. Mechanistic research at the University of Minnesota under the direction of Kendall Wallace includes PFOA, 3M Reference No. T-6889.1. Documents regarding this research have been previously submitted with the May 4, 2000 submission on PFOS (see Mechanistic 1)(a)-(f)).

000009

**ATTACHMENT TO LETTER TO C. AUER DATED AUGUST 31, 2000
SUPPLEMENTAL SUBMISSION**

ENVIRONMENTAL

Title	Laboratory or Author	Completion Date	Type
Fluorochemical Characterization of Surface Water Samples Columbus, GA (W2336)	Centre Analytical Laboratories, Inc.	5/24/00	Final Report
Fluorochemical Characterization of Surface Water Samples Cleveland, TN (W1973)	Centre Analytical Laboratories, Inc.	5/24/00	Final Report
Fluorochemical Characterization of Surface Water Samples Decatur, AL (W1979)	Centre Analytical Laboratories, Inc.	5/24/00	Final Report
Fluorochemical Characterization of Surface Water Samples Mobile, AL (W2151)	Centre Analytical Laboratories, Inc.	5/24/00	Final Report
Fluorochemical Characterization of Surface Water Samples Pensacola, FL (W2176)	Centre Analytical Laboratories, Inc.	5/24/00	Final Report
Fluorochemical Characterization of Surface Water Samples Port St. Lucie, FL (W2363)	Centre Analytical Laboratories, Inc.	5/24/00	Report
Fluorochemical Characterization of Surface Water Samples Port St. Lucie, FL (W2363)	Centre Analytical Laboratories, Inc.	August, 2000	Final Report
Final Multi-City Study. Field Report for Mobile Alabama and Pensacola Florida - Battelle Duxbury Activities	Battelle Memorial Institute	9/29/99 (Addendum signed on 10/13/99)	Final Report
Final Multi-City Study. Field Report for Cleveland Tennessee and Decatur Alabama - Battelle Duxbury Activities	Battelle Memorial Institute	7/9/99 (Addendum signed on 12/6/99)	Final Report
Final Multi-City Study. Field Report for Columbus Georgia and Port St. Lucie Florida - Battelle Duxbury Activities	Battelle Memorial Institute	10/26/99 (Addendum signed on 12/6/99)	Final Report
Port St. Lucie Florida Water and Sediment Resample for the Empirical Human Exposure Assessment Multi-City Study	Pace Analytical Services, Inc.	8/15/00	Report
Sulfonated Perfluorochemicals: U.S. Release Estimation - 1997 Part 1: Life-Cycle Waste Stream Estimates - Executive Summary	Battelle Memorial Institute	4/21/2000	Final Report

000010

AR 226-0645

PFOS

Medical Surveillance/Epi - !

**Interim Report #1
Epidemiology, 220-3W-05
Medical Department
3M Company
St. Paul, MN 55144**

Date: June 8, 2000

Title: Determination of Serum Half-Lives of Several Fluorochemicals

**Study
Start Date:**

Protocol Number # 0007
IRB Approval # 98095
Exempt XX Expedited

**Estimated Date of
Final Report:**

June, 2004

IRB Approval Date:

14 October 1998

Principal Investigator:

Jean M. Burris, RN, MPH¹

Co-investigators:

Geary Olsen, DVM, PhD
Cathy Simpson, RN²
Jeffrey Mandel, MD, MPH¹

Study Director:

Jeffrey Mandel, MD, MPH¹

Study Sponsor:

Corporate Occupational Medicine Department
3M Company
220-3W-05
Saint Paul, MN 55144

1. Occupational Medicine, Medical Department, 3M Company, 220-3W-05, St. Paul, MN 55114
2. 3M Decatur Specialty Adhesives and Chemicals Plant, P.O. Box 2206, Decatur, AL 35609-2206

ABSTRACT

Previous estimates regarding the human serum half-life of perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) ranged between 1000-1500 days and 365-530 days respectively [Olsen et al., 1999; Ubel et al., 1980]. These estimates were based on very few subjects. We are currently in the process of collecting data from 27 retired, fluorochemical production employees in an effort to more completely estimate the half-life of PFOS and PFOA along with five other fluorochemical analytes. To date, preliminary analyses from three serum collection periods utilizing a one compartment model suggests that the serum half-life of PFOS in these retirees is likely to be four-fold lower than the range of 1000-1500 days that was previously suggested. The serum half-life of PFOA in these retirees appears to be approximately one year, which is comparable to the estimate suggested by Ubel et al [1980]. Because of the lower limit of quantitation assayed for the other fluorochemical analytes, we believe additional collection periods are necessary before serum half-life calculations can be determined. There are several limitations to the current report, the most important being the paucity of data available to date and the range of the serum levels measured (PFOS range 0.2-2.0ppm; PFOA 0.1-3.1 ppm). We anticipate completing two additional serum collection cycles before December 2000. Additional data should allow a better assessment of fluorochemical half-lives of all participants in this study.

INTRODUCTION

Although it has been reported that the serum half-life of perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) in humans may range between 1000-1500 days and 365-530 days respectively [Olsen et al., 1999; Ubel et al., 1980], such estimations have been based on very few subjects. The purpose of this study is to determine the serum half-life from a group of Decatur and Cottage Grove retirees for the following fluorochemicals: PFOS, PFOA, perfluorohexanesulfonate (PFHS), N-ethyl perfluorooctanesulfonamidoacetate (PFOSAA), N-methyl perfluorooctanesulfonamidoacetate (M570), perfluorooctanesulfonamide (PFOSA), and perfluorooctanesulfonamidoacetate (M556).

METHODS

Twenty-four Decatur retirees and three Cottage Grove retirees have voluntarily participated in multiple serum sample collection cycles for fluorochemical analysis. The majority of the twenty-seven participants are male, only two are female. All participants are long-term 3M employees. They have worked an average of 28 years in either the Decatur or Cottage Grove chemical division. The average age of the participants at the time of the first collection is 60 years (range: 55-74). The mean number of months from retirement to the start of the study is 30 months (range: 5-130 months).

To date, there have been three Decatur collection periods: November 1998, June 1999 and November 1999. Cottage Grove retirees have participated in two collection periods: June 1999 and November/December 1999. The next serum sampling is scheduled for May 2000. Participants have received letters informing them of their

individual fluorochemical results as data have become available from the laboratory. Each participant completed a brief medical history questionnaire containing information about current medications, and disease diagnosis. Responses have been keyed into a SAS dataset and analyzed by JMP software.

High-performance liquid chromatography mass spectrometry/mass spectrometry has been utilized to analyze all serum samples (NWB,1999). All samples were analyzed by Northwest Bioanalytical Laboratory, Salt Lake City, Utah with the exception of the M556 and M570 from November 1998. The 3M Environmental Technology and Services Laboratory analyzed these samples.

Half-lives were calculated assuming a one compartment model. A log-linear relationship was used to estimate the serum fluorochemical elimination half-life in participating retirees. In this log linear relationship, the slope of the line is related to the elimination constant ($-k_{el}$) via the equation: Slope = $-k_{el}(2.303)$. Once the elimination constant is calculated, the half-life is determined using the relationship: $t_{1/2} = 0.693/k_{el}$ (Medinsky & Klassen, 1996).

RESULTS

A total of 24 participants provided serum samples for each collection period. A half-life was calculated for each of these individuals for PFOS and PFOA. Except for PFHS, half-lives for the other fluorochemicals were not calculated as the majority was below the limit of quantitation by the third collection. Because the assay measurement of PFHS was inconsistent (e.g., many subsequently collected samples were at higher levels

than initial samples), the calculation of the PFHS half-life was deferred until further blood collections occur.

Table 1 presents the mean, median, range and 95% confidence interval of the half-lives calculated for those retirees (n=18 for PFOS, n=20 for PFOA) who were judged to have a good fit ($r^2 \geq 0.6$) for a linear one compartmental model for PFOS and PFOA. At this time we have not included the other participants in these analyses for two reasons: 1) lack of three data points for some subjects; and 2) lack of fit of model (i.e., $r^2 < 0.6$). The latter was often due to the fact that the second measurement was higher than the first. At this time we are uncertain whether this may be due to subsequent exposure, biological variation or assay error and/or variation. [Note: We are scheduled to collect two more data points by December 2000. By that time we anticipate including all participants in the analysis.]

The range of serum PFOS measured was 0.2 - 2.0 ppm (Table 1). The range of serum PFOA measured was 0.1 - 3.1 ppm. All PFOS serum half-life calculations were of male retirees. Two of the 20 PFOA serum half-life calculations were of female retirees.

As can be seen from Table 1, the median serum half life of PFOS was 270 days (range 139-640) for the 18 male retirees whose log linear regression model had an $r^2 \geq 0.6$. The median serum half life of PFOA was 344 days for the 20 male and female retirees whose log linear regression model had an $r^2 \geq 0.6$. Restricting the models to $r^2 \geq 0.8$ did not substantially change the half-life calculations for PFOS or PFOA. It should be noted that the highest PFOA half-life calculations were from the two female retirees 654 and 1308 days. Neither age nor the number of months retired was associated with the serum half-life calculations for PFOS or PFOA.

DISCUSSION

The results from this first interim analysis suggest that the serum half-life of PFOS in humans is likely to be four-fold lower than the range of 1000-1500 days that was initially suggested [Olsen et al., 1999]. The serum half-life of PFOA in humans appears to be approximately one year, which is comparable to the estimate suggested by Ubel et al [1980]. Additional collection cycles will further refine these half-life estimates and take into account the retirees who were excluded in this interim report due to lack of fit of the one compartment model. This, of course, assumes a one-compartment model is appropriate at the low ppm serum levels measured in this group of retirees.

Our results from this preliminary interim analysis should be interpreted cautiously due to the paucity of data available to date. The fourth data point is scheduled to be collected and analyzed by mid-summer. There are several additional limitations to these interim data. To date, Cottage Grove retirees have participated in only two serum collection cycles. Three of the twenty-four Decatur retirees have indicated at each serum collection cycle that they have worked in a consulting capacity in the 3M Decatur plant at some point during the intervening six months. However, their log linear regression models were considered to be of reasonably good fit (i.e., r^2 for PFOS $\geq .80$; r^2 for PFOA $\geq .57$). Four of the seven fluorochemical analytes (PFOSAA, PFOSA, M556 and M570) measured below the lower limit of quantitation for more than 80% of the samples prohibiting a reasonable half-life calculation. At this time we are uncertain why many subsequent PFHS serum levels were higher than the first measurement. With additional data we hope to define half-lives in these compounds although if levels remain below the LLOQ this may not be feasible.

The next collection period will occur in May 2000 with anticipated results due back from Northwest Bioanalytical by mid-summer. Additional data should allow a better assessment of the half-lives of all retirees. The next interim report should be available by August 2000.

REFERENCES

Olsen GW, Burris JM, Mandel JH, Zobel LR. Serum perfluorooctane sulfonate and hepatic and lipid clinical chemistry tests in fluorochemical production employees. *J Occup Env Med* 41:1999:799-806.

Ubel F, Sorenson S, Roach D. Health status of plant workers exposed to fluorochemicals: a preliminary report. *Am Ind Hyg Assoc J* 1980;41:584-589.

NWB. Quantitative Determination of PFOS, PFOSA, PFOSAA, POAA, and PFHS in Human Serum by LC/MS/MS. 1999. Salt Lake City, Northwest Bioanalytical (NWB) A Division of NWT Inc.

Medinsky MA, Klaasen CD. Toxicokinetics (In) Klaasen CD (ed) Casarett & Doull's Toxicology Fifth Edition. 1996. New York: McGraw-Hill, pages 187-198.

Table 1

Half-Life Summary Data In Days For Those Retirees With Three Collection Periods
Whose Log Linear Models Had $R^2 > 0.6$

	<u>N</u>	<u>Mean</u>	<u>Median</u>	<u>Range</u>	<u>95% CI of Mean</u>
<u>PFOS</u>					
$R^2 \geq 0.6$	18	303	270	139-640	228-378
$R^2 \geq 0.8$	16	303	259	139-640	217-388
<u>PFQA</u>					
$R^2 \geq 0.6$	20	389	344	109-1308	269-509
$R^2 \geq 0.8$	13	395	324	109-1308	204-585

AR 226-0646

PFOS

Medical Surveillance/Epi - 2

Internal Correspondence



August 2000

Memo to File re: OUS pooled samples

Pursuant to a review of my file, there are raw data for several other pooled human sera sample results for PFOS dating from 1999. This data were assembled in our own 3M laboratory from European blood banks subsequent to the preparation of the January 21, 1999 3M document entitled "Perfluorooctane Sulfonate: Current Summary of Human Sera, Health and Toxicology Data." The data are very consistent with that presented in that paper. No formal report was made relative to these data. It is my understanding that each pool is comprised of 15-20 donors in each of three European countries. The results which have been measured in ppb are:

1. Belgium – six pooled samples analyzed; range = (4.9 to 22.2); average = 17 ppb
2. Netherlands – five pooled samples analyzed; range = (39-61); average = 53 ppb
3. Germany – six pooled samples analyzed; range = (32-45.6); average = 37 ppb

Jeff Mandel

000024

AR 226 - 0647

PFOS

Acute Toxicity - I

Interoffice Correspondence

300

Subject:

November 14, 1974

irc: H. A. Birnbaum - 220-2E
E. J. Deviny - 236-2B
C. G. Klaus - 236-3A
J. D. Lazerte - 236-1
J. A. Pendergrass - 220-2E
F. A. Ubel - 220-2E

TO: D. R. RICKER - COMMERCIAL CHEMICALS - 236-2B

FM: J. E. LONG - MEDICAL DEPT. - TOXICOLOGY SERVICES - 220-2E

Attached please find copies of the reports on the primary skin irritation and eye irritation assessments conducted with your FC-95 (T-1117) by WARF Institute.

FC-95 was found to be non-irritating to the skin of albino rabbits under the study conditions utilized. No signs of dermal irritation were observed in any of the test animals at any time during the study period.

This product was found to be minimally to mildly irritating to the eyes of albino rabbits under the study conditions utilized. The ocular irritation was limited to that of the conjunctivae in the six test animals at one, twenty-four, and forty-eight hours post-instillation. No conjunctival irritation was observed at the 72 hour examination. No signs of corneal opacity or iridal irritation were observed in any of the test animals at any time during the study period.

Flushing the eyes with plenty of water immediately upon contact should arrest/reverse the irritation process.

Precautionary label text should indicate that this product may be irritating to eyes.

J. E. Long
JEL:mn

000026

bcc: H. A. Birnbaum - 220-2E NOTE FOR 3M COPIES
E. J. Deviny - 236-2B
C. G. Klaus - 236-3A T-1117 = FC-95, CC#7410-16
J. D. LaZerte - 236-1
J. A. Pendergrass - 220-2E
F. A. Ubel - 220-2E

October 24, 1974

Dr. Donald L. Harris
WARF Institute
Biological Department
P.O. Box 2599
Madison, WI 53701

Dear Dr. Harris:

Under separate cover, I am forwarding one 3M sample labeled T-1117 to your laboratory. Please consider this to be a request/authorization for WARF Institute to conduct eye irritation and primary skin irritation assessments with this sample.

In order to assess the eye irritation potential, please instill one-tenth of one gram of test sample into the conjunctival sac of six albino rabbits. Hold the eyelid shut for one second and then examine the test eyes at one, 24, 48, and 72 hours post-instillation and score for ocular irritation according to the procedure of Draize.

For the purpose of assessing the primary skin irritation potential, apply five-tenths of one gram of test sample to intact (dry and wet) and abraded (dry and wet) skin test sites. At 24 and 72 hours post-application, examine the skin test sites and score for dermal irritation according to the procedure of Draize.

Our purchase order for \$200 will be forwarded to you in the nearffuture.

We thank you for your assistance and cooperation in this matter.

Sincerely yours,

J. E. Long, Sc.D.
Medical Department
Manager, Toxicology
Services

JEL:mun

000027

Interoffice Correspondence **300**

T-1117

c. c. E. J. Deviny 236-2A
C. G. Klaus 236-3A
J. D. LaZerte 236-1

Subject: Toxicity Testing of
FC-95, CC 7410-16

October 16, 1974

TO: J. E. Long - Toxicology - 220-2E

FROM: D. Ricker - Comm. Chems. - 236-2B

As per your suggestion, please arrange to have the following studies conducted on FC-95:

- 1) Primary Skin Irritation Assessment
- 2) Eye Irritation Assessment.

I will send you a sample of FC-95 to be used for this testing. Please let me know under what T No. this testing will be done.

Thank you

D. Ricker

DR:dl



000028

WARF INSTITUTE, INC.

MADISON, WISCONSIN

Reports are submitted to clients on a confidential basis. No reference to the work, the results or to the Institute in any form of advertising, news release or other public announcement may be made without written authorization from the Institute.

REPORT

Sample: T-1117

WARF No. 4102871

Date Received: 10-31-74

Submitted by:

J. E. Long
3M Company
St. Paul, Minnesota 55101

Testing requested:

Skin Irritation
Eye Irritation

Conclusion:

Under the conditions specified the product has a primary skin irritation index of zero and zero for both dry and wet applications and eye irritation scores of 8.00, 9.33, 3.33 and zero at 1, 24, 48 and 72 hours respectively.

The product is not irritating to the skin and is irritating to the eye.

Signed



By and for WARF Institute, Inc.

Report approved by:

Date: November 7, 1974


Donald L. Harris, D.V.M.
Chief Pathologist/Toxicologist

000029

WARF INSTITUTE, INC.
MADISON, WISCONSIN

Sample: T-1117

WARF No. 4102871

Primary Skin Irritation:

Method: Albino rabbits were used for this work. They were housed in individual screen bottom cages and fed water and rabbit chow ad libitum. The hair was clipped from the back and flanks of the animal. Five tenths of one gram of test material was placed in intimate contact with the skin on prepared test sites (intact-wet; abraded-wet), and covered with gauze patch and tape to maintain contact with the skin. After a 24 hour contact period, the coverings were removed and the degree of erythema and edema was recorded according to the scale below. Readings were taken again at 72 hours and the 24 and 72 hour readings used to determine the primary skin irritation score.

Results:

<u>Animal Number</u>	<u>24 Hours</u>		<u>72 Hours</u>		<u>Average</u>
	<u>Abraded</u>	<u>Unabraded</u>	<u>Abraded</u>	<u>Unabraded</u>	
1					
2					
3					
4					
5					
6					

no noted irritation
all sites read negative

Primary skin irritation score: 0

Scale for Evaluation of Skin Reaction

<u>Erythema and Eschar Formation</u>	<u>Score</u>	<u>Edema Formation</u>	<u>Score</u>
Slight erythema	1	Slight edema (barely perceptible)	1
Defined erythema	2	Defined edema (edges definite rising)	2
Moderate to severe erythema	3	Moderate edema (area raised 1 mm)	3
Severe erythema to slight eschar formation	4	Severe edema (raised more than 1 mm)	4

Score equals sum of erythema and edema readings.
Skin irritation index equals average of 24 and 72 hour scores.

000650

WARF INSTITUTE, INC.
MADISON, WISCONSIN

Sample: T-1117

WARF No. 4102871

Primary Skin Irritation:

Method: Albino rabbits were used for this work. They were housed in individual screen bottom cages and fed water and rabbit chow ad libitum. The hair was clipped from the back and flanks of the animal. Five tenths of one gram of test material was placed in intimate contact with the skin on prepared test sites (intact-dry; abraded-dry), and covered with gauze patch and tape to maintain contact with the skin. After a 24 hour contact period, the coverings were removed and the degree of erythema and edema was recorded according to the scale below. Readings were taken again at 72 hours and the 24 and 72 hour readings used to determine the primary skin irritation score.

Results:

Animal Number	24 Hours		72 Hours		Average
	Abraded	Unabraded	Abraded	Unabraded	
1					
2					
3					
4					
5					
6					

no noted irritation
all sites read negative

Primary skin irritation score: 0

Scale for Evaluation of Skin Reaction

<u>Erythema and Eschar Formation</u>	<u>Score</u>	<u>Edema Formation</u>	<u>Score</u>
Slight erythema	1	Slight edema (barely perceptible)	1
Defined erythema	2	Defined edema (edges definite rising)	2
Moderate to severe erythema	3	Moderate edema (area raised 1 mm)	3
Severe erythema to slight eschar formation	4	Severe edema (raised more than 1 mm)	4

Score equals sum of erythema and edema readings.
Skin irritation index equals average of 24 and 72 hour scores.

00631

WARF INSTITUTE, INC.
MADISON, WISCONSIN

Sample: T-1117

WARF No. 4102871

Eye Irritation:

Method: Adult albino rabbits of the New Zealand White variety were placed in a collar such that the animals could not rub their eyes. One-tenth of a milliliter (0.1 gm for solids) of test substance was instilled in one eye, the other untreated eye served as a control. A series of six albino rabbits was used for each substance. The reaction to the test material was read according to the scale of scoring for damage to the cornea, iris and the bulbar and palpebral conjunctivae at 1, 24, 48 and 72 hours after eye instillation. Any residue of the test material and accumulated discharge are flushed from the eye each time they are scored.

Administration:

Concentration of test sample: as submitted

Diluent or solvent: none

Special washing: none

Results:

	Rabbit Number	Cornea		Iris	Conjunctivae		
		Opacity	Area		Redness	Chemosis	Discharge
1 hour	1	0	0	0	2	1	1
	2	0	0	0	2	1	1
	3	0	0	0	2	1	1
	4	0	0	0	2	1	1
	5	0	0	0	2	1	1
	6	0	0	0	2	1	1
1 hour eye irritation score: 6.00							
24 hours	1	0	0	0	2	1	1
	2	0	0	0	2	1	1
	3	0	0	0	2	2	1
	4	0	0	0	2	1	1
	5	0	0	0	2	2	1
	6	0	0	0	2	2	2
24 hour eye irritation score: 9.33							
48 hours	1	0	0	0	1	0	0
	2	0	0	0	1	0	0
	3	0	0	0	1	1	0
	4	0	0	0	1	0	0
	5	0	0	0	1	1	0
	6	0	0	0	1	1	1
48 hour eye irritation score: 3.33							

72 hour eye irritation score was zero. All readings were negative.

000032

C 06

AR 226 - 0648

PFOS

Genotoxicity - /

000033

SRI International



**IN VITRO MICROBIOLOGICAL MUTAGENICITY ASSAYS
OF 3M COMPANY COMPOUND T-2816CoC**

Final Report, Revised

March 1980

**By: Kristien E. Mortelmans, Ph.D.
Director, Microbial Genetics Department
and
Anne Pomeroy, Microbiological Technician**

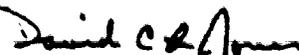
Prepared for:

**3M COMPANY
Medical Department
General Offices, 3M Center
St. Paul, Minnesota 55101**

**Attention: W. C. McCormick
Toxicology Services**

SRI Project LSC-8958

Approved:


**David C. L. Jones, Director
Toxicology Laboratory**


**W. A. Skinner, Executive Director
Life Sciences Division**

333 Ravenswood Ave. • Menlo Park, California 94025
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000034

SUMMARY

SRI International examined 3M Company's Compound T-2816CoC for mutagenic activity with strains TA1535, TA1537, TA1538, TA98, and TA100 of Salmonella typhimurium in the standard Ames Salmonella/microsome assay and with the yeast Saccharomyces cerevisiae D3. Each assay was performed in the presence and in the absence of a rat liver metabolic activation system. Compound T-2816CoC was not mutagenic or recombinogenic in any assay performed.

INTRODUCTION

SRI International examined 3M Company's Compound T-2816CoC for mutagenicity by in vitro microbiological assays with strains TA1535, TA1537, TA1538, TA98, and TA100 of the bacterium Salmonella typhimurium in the standard Ames Salmonella/microsome assay and with the yeast Saccharomyces cerevisiae D3. An Aroclor 1254-stimulated, rat liver homogenate metabolic activation system was included in the assay procedures to provide metabolic steps that the bacteria either are incapable of conducting or do not carry out under the assay conditions.

The assay procedure with S. typhimurium has proven to be 80 to 90% reliable in detecting carcinogens as mutagens, and it has about the same reliability in identifying chemicals that are not carcinogenic. The assay procedure with S. cerevisiae is about 60% reliable in detecting carcinogens as agents that increase mitotic recombination. However, because the assay systems do not always provide 100% correlation with carcinogenicity investigations in animals, neither a positive nor a negative response conclusively proves that a chemical is hazardous or nonhazardous to man.

METHODS

Salmonella typhimurium Strains TA1535, TA1537, TA1538, TA98, and TA100

The Salmonella typhimurium strains used at SRI are all histidine auxotrophs by virtue of mutations in the histidine operon. When these histidine-dependent cells are grown on minimal medium agar plates containing a trace of histidine, only those cells that revert to histidine independence (his⁺) are able to form colonies. The small amount of histidine allows all the plated bacteria to undergo a few divisions; in many cases, this growth is essential for mutagenesis to occur. The his⁺ revertants are easily visible as colonies against the slight background growth. The spontaneous mutation frequency of each strain is relatively constant, but when a mutagen is added to the agar, the mutation frequency is increased, usually in a dose-related manner.

We obtained our S. typhimurium strains from Dr. Bruce Ames of the University of California at Berkeley. In addition to having mutations in the histidine operon, all the indicator strains have a mutation (rfa) that leads to a defective lipopolysaccharide coat; they also have a deletion that covers genes involved in the synthesis of the vitamin biotin (bio) and in the repair of ultraviolet (uv)-induced DNA damage (uvrB). The rfa mutation makes the strains more permeable to many large aromatic molecules, thereby increasing the mutagenic effect of these molecules. The uvrB mutation causes decreased repair of some types of chemically or physically damaged DNA and thereby enhances the strains' sensitivity to some mutagenic agents. Strain TA1535 is reverted to his⁺ by many mutagens that cause base-pair substitutions. TA100 is derived from TA1535 by the introduction of the resistance transfer factor, plasmid pKM101. This plasmid is believed to cause an increase in error-prone DNA repair that leads to many more mutations for a given dose of most mutagens. In addition, plasmid pKM101 confers resistance to the

antibiotic ampicillin, which is a convenient marker to detect the presence of the plasmid in the cell. The presence of this plasmid also makes strain TA100 sensitive to some frameshift mutagens [e.g., ICR-191, benzo(a)pyrene, aflatoxin B₁, and 7,12-dimethylbenz(a)anthracene]. Strains TA1537 and TA1538 are reverted by many frameshift mutagens. Strain TA98 is derived from TA1538 by the addition of the plasmid pKM101, which makes it more sensitive to some mutagenic agents.

All indicator strains are kept at 4°C on minimal agar plates supplemented with an excess of biotin and histidine. The plates with the plasmid-carrying strains also contain ampicillin (25 µg/ml) to ensure stable maintenance of the plasmid pKM101. New stock culture plates are made every 4 to 6 weeks from single colony isolates that have been checked for their genotypic characteristics (his, rfa, uvrB, bio) and for the presence of the plasmid. For each experiment, an inoculum from the stock culture plates is grown overnight at 37°C in nutrient broth (Oxoid, CM57).

Aroclor 1254-Stimulated Metabolic Activation System

Some carcinogenic chemicals (e.g., of the aromatic amino type or the polycyclic hydrocarbon type) are inactive unless they are metabolized to active forms. In animals and man, an enzyme system in the liver or other organs (e.g., lung or kidney) is capable of metabolizing a large number of these chemicals to carcinogens. Some of these intermediate metabolites are very potent mutagens in the S. typhimurium test. Ames has described the liver metabolic activation system that we use. In brief, adult male rats (250 to 300 g) are given a single 500-mg/kg intraperitoneal injection of Aroclor 1254 (a mixture of polychlorinated biphenyls). This treatment enhances the synthesis of enzymes involved in the metabolic conversion of chemicals. Four days after the injection, the animals' food is removed but drinking water is provided ad libitum. On the fifth day, the rats are killed and the liver homogenate is prepared as follows.

The livers are removed aseptically and placed in a preweighed sterile glass beaker. The organ weight is determined, and all subsequent operations are conducted in an ice bath. The livers are washed with an equal volume of cold, sterile 0.15 M KCl (1 ml/g of wet organ), minced with sterile surgical scissors in three volumes of 0.15 M KCl, and homogenized with a Potter-Elvehjem apparatus. The homogenate is centrifuged for 10 minutes at 9000 x g, and the supernatant, referred to as the S-9 fraction, is quickly frozen in dry ice and stored at -80°C.

The metabolic activation mixture for each experiment consists of, for 10 ml:

- 1.00 ml of S-9 fraction
- 0.20 ml of MgCl₂ (0.4 M) and KCl (1.65 M)
- 0.05 ml of glucose-6-phosphate (1 M)
- 0.40 ml of NADP (0.1 M)
- 5.00 ml of sodium phosphate buffer (0.2 M, pH 7.4)
- 3.35 ml of H₂O.

Assays in Agar

To a sterile 13 x 100 mm test tube placed in a 43°C heating block, we add in the following order:

- (1) 2.00 ml of 0.6% agar*
- (2) 0.05 ml of indicator organisms
- (3) 0.50 ml of metabolic activation mixture (if appropriate)
- (4) 0.05 ml of a solution of the test chemical.

This mixture is stirred gently and then poured onto minimal agar plates.[†] After the top agar has set, the plates are incubated at 37°C for 3 days. The number of his⁺ revertant colonies is counted and recorded.

*The 0.6% agar contains 0.05 mM histidine, 0.05 mM biotin, and 0.6% NaCl.

[†]Minimal agar plates consist of, per liter, 15 g of agar, 10 g of glucose, 0.2 g of MgSO₄·7H₂O, 2 g of citric acid monohydrate, 10 g of K₂PO₄, and 3.5 g of Na₂H₂PO₄·4H₂O.

For negative controls, we use steps (1), (2), and (3) and 0.05 ml of the solvent used for the test chemical. For positive controls, we test each culture by specific mutagens known to revert each strain, using steps (1), (2), (3) and (4).

Saccharomyces cerevisiae D3

The yeast S. cerevisiae D3 is a diploid microorganism heterozygous for a mutation leading to a defective enzyme in the adenine-metabolizing pathway. When grown on medium containing adenine, cells homozygous for this mutation produce a red pigment. These homozygous mutants can be generated from the heterozygotes by mitotic recombination. The frequency of this recombinational event may be increased by incubating the organisms with various carcinogenic or recombinogenic agents. The recombinogenic activity of a compound or its metabolite is determined from the number of red-pigmented colonies appearing on test plates.

A stock culture of S. cerevisiae is stored at 4°C. For each experiment, broth containing 0.05% MgSO₄, 0.15% KH₂PO₄, 0.45% (NH₄)₂SO₄, 0.35% peptone, 0.5% yeast extract, and 2% dextrose is inoculated with a loopful of the stock culture and incubated overnight at 30°C with shaking.

The in vitro yeast mitotic recombination assay in suspension is conducted as follows. The overnight culture is centrifuged and the cells are resuspended at a concentration of 10⁸ cells/ml in 67 mM phosphate buffer (pH 7.4). To a sterile test tube are added:

- 1.00 ml of the resuspended culture
- 0.50 ml of either the metabolic activation mixture or buffer
- 0.20 ml of the test chemical
- 0.30 ml of buffer.

Several doses of the test chemical are tested in each experiment, and appropriate controls are included.

The suspension mixture is incubated at 30°C for 4 hours on a roller drum. The sample is then diluted serially in sterile physiologic

saline, and 0.2 ml of the 10^{-5} and 10^{-6} dilutions is spread on plates containing the same ingredients as the broth plus 2.0% agar; five plates are spread with the 10^{-5} dilution and three plates are spread with the 10^{-6} dilution. The plates are incubated for 2 days at 30°C , followed by 2 days at 4°C to enhance the development of the red pigment indicative of adenine-deficient homozygosity. Plates containing the 10^{-5} dilution are scanned with a dissecting microscope at 10 X magnification, and the number of mitotic recombinants (red colonies or red sectors) is recorded. The surviving fraction of organisms is determined from the total number of colonies appearing on the plates of the 10^{-5} dilution.

The number of mitotic recombinants is calculated per 10^5 survivors. A positive response in this assay is indicated by a dose-related increase of more than 3-fold in the absolute number of mitotic recombinants per milliliter as well as in the relative number of mitotic recombinants per 10^5 survivors.

RESULTS AND DISCUSSION

Compound T-2816CoC was tested for mutagenicity in the Ames Salmonella/microsome assay and with the yeast Saccharomyces cerevisiae D3 in the presence and in the absence of a metabolic activation system. The compound was tested at least twice on separate days in both assays. The results are presented in Tables 1 through 5.

In the Ames Salmonella/microsome assay, T-2816CoC was initially tested in a preliminary assay with strain TA100 over a wide range of concentrations, from 10 to 5,000 µg/plate. Toxicity was observed at a dose of 5,000 µg/plate (Table 1). Ethanol was used as the solvent in all assays.

The results of our tests of T-2816CoC with five strains of S. typhimurium in the Ames Salmonella/microsome assay are presented in Tables 2 and 3. No toxicity or dose-related increase in the number of revertants was observed in these assays.

The results of the microbiological assays with S. cerevisiae D3 on T-2816CoC are presented in Tables 4 and 5. The compound was tested at concentrations from 0.05 to 5.0%. No toxicity or significant dose-related increase in the number of mitotic recombinants above background was observed.

We therefore conclude that Compound T-2816CoC was not mutagenic with S. typhimurium or recombinogenic with S. cerevisiae D3.

Table 1

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM
COMPOUND T-2816Coc
 Experiment Date: 13 February 1980

<u>Compound</u>	<u>Metabolic Activation</u>	<u>Amount of Compound Added per Plate</u>	<u>Hisidine Revertants per Plate TA100</u>
Negative Control			
Ethanol	-	50 µl	114
	+	50	111
Positive Controls			
2-Anthramine	-	1.0 µg	102
	+	1.0	427
Sodium Azide	-	0.5 µg	367
Compound T-2816Coc			
	-	10.0 µg	127
	-	50.0	123
	-	100.0	93
	-	500.0	102
	-	1,000.0	97
	-	5,000.0	toxic
	+	10.0 µg	146
	+	50.0	116
	+	100.0	110
	+	500.0	108
	+	1,000.0	108
	+	5,000.0	toxic

Table 2

IN VITRO ASSAYS WITH *SALMONELLA TYPHIMURUM*
COMPOUND T-2816CoC
 Experiment Date: 20 February 1980

Compound	Metabolic Activation	Amount of Compound Added Per Plate	Histidine Revertants per Plate				
			TA1535	TA1537	TA1538	TA98	
Negative Control							
Ethanol	-	50.0 µl	19 14	15 7	18 18	30 29	100 117
	-	50.0	26 21	5 14	9 8	31 27	120 91
	+	50.0	31 27	20 13	24 24	44 55	92 108
	+	50.0	25 32	21 20	55 26	67 56	140 114
Positive Controls							
2-Anthracene	-	1.0 µg			12 13	38 31	125 115
	+	1.0			320 330	299 319	542 550
	-	2.5	21 26	8 15			
	+	2.5	201 197	110 109			
Sodium Azide	-	0.5 µg					260 245
	-	1.0	293 370				
9-Aminoacridine	-	50.0 µg		180 193			
2-Nitrofluorene	-	5.0 µg			530 490	402 399	
Compound T-2816CoC							
	-	5.0 µg	17 18	9 11	8 18	39 37	111 114
	-	10.0	32 20	7 9	8 15	38 28	93 89
	-	50.0	28 31	15 16	17 15	36 38	105 111
	-	100.0	28 18	16 9	14 15	33 34	110 99
	-	500.0	26 19	6 9	15 16	27 40	104 108
	-	1,000.0	29 28	6 8	18 12	37 32	97 103
	+	5.0	18 30	17 22	27 37	42 99	98 114
	+	10.0	26 24	12 13	36 27	61 55	122 120
	+	50.0	19 30	8 12	30 27	39 44	140 99
	+	100.0	26 38	16 12	19 28	49 53	126 101
	+	500.0	39 31	18 23	35 37	42 61	113 117
	+	1,000.0	36 30	6 9	19 42	51 63	113 115

Table 3

IN VITRO ASSAYS WITH SALMONELLA TYPHIMURIUM
COMPOUND T-2816Coc
 Experiment Date: 25 February 1980

Compound	Metabolic Activation	Amount of Compound Added per Plate	Hlatidine Revertants per Plate									
			TA1535	TA1537 ^a	TA1538	TA98						
Negative Control												
Ethanol	-	50.0 µl	20	16	7	9	16	13	45	48	90	96
	+	50.0	36	31	19	3	24	19	77	69	137	139
Positive Control												
2-Anthramine	-	1.0 µg					13	19	43	40	126	96
	-	2.5	21	26	9	9						
	+	1.0					163	134	250	230	662	706
	+	2.5	219	176	137	118						
Sodium Azide	-	0.5 µg									403	369
	-	1.0	304	331								
9-Aminoacridine	-	50.0 µg										
2-Nitrofluorene	-	5.0 µg										
Compound T-2816Coc												
	-	5.0 µg					934	813	517	461		
	-	10.0	25	24	8	7	17	15	33	44	111	87
	-	50.0	19	26	7	8	9	7	35	54	137	114
	-	100.0	38	34	4	3	21	13	54	53	111	97
	-	500.0	32	35	6	9	10	11	56	41	122	87
	-	1,000.0	33	33	12	6	7	10	46	51	98	104
	+	5.0 µg	22	26	6	7	14	18	41	48	97	123
	+	10.0	38	37	17	9	36	33	70	62	126	116
	+	50.0	23	35	8	20	21	31	46	68	124	117
	+	100.0	30	39	9	15	20	21	53	53	140	124
	+	500.0	27	34	21	13	27	34	47	53	93	89
	+	1,000.0	34	28	16	14	28	23	50	57	110	97
	+		25	33	9	13	22	28	59	52	116	110

^a Retested on 6 March; control values of 25 February were invalid.

000045

Table 4

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE
COMPOUND T-2816Coc
 Experiment Date: 13 February 1980

Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Survivors		Mitotic Recombinants	
			Cells per ml ($\times 10^{-7}$)	Percent	Per ml ($\times 10^{-3}$)	Per 10^5 Survivors
Negative Control						
Ethanol	-		6.3	100	6.0	9.0
	+		5.6	100	7.0	12
Positive Control						
1,2,3,4-Diepoxybutane	-	0.025	6.4	102	980	1,500
	+	0.025	7.3	116	1,000	1,400
Compound T-2816Coc						
	-	0.05	3.3	50	7.0	21
	-	0.1	6.6	105	5.0	7.6
	-	0.5	5.7	90	15	26
	-	1.0	5.0	79	7.0	14
	-	5.0	2.9	46	5.0	17
	+	0.05	3.4	61	3.0	8.8
	+	0.1	6.4	114	7.0	11
	+	0.5	5.3	95	11	21
	+	1.0	5.7	102	4.0	7.0
	+	5.0	5.2	93	11	21

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Table 5

IN VITRO ASSAYS WITH SACCHAROMYCES CEREVISIAE
COMPOUND T-2816Coc
 Experiment Date: 21 February 1980

Compound	Metabolic Activation	Percent Concentration (w/v or v/v)	Survivors		Mitotic Recombinants	
			Cells per ml ($\times 10^{-7}$)	Percent	Per ml ($\times 10^{-3}$)	Per 10^5 Survivors
Negative Control						
Ethanol	-		5.4	100	4.5	8.5
	+		5.5	100	6.0	11
Positive Control						
1,2,3,4-Diepoxybutane	-	0.025	5.8	107	1,100	1,900
	+	0.025	5.7	104	1,100	1,900
Compound T-2816Coc						
	-	0.05	9.4	174	10	11
	-	0.1	9.4	174	16	17
	-	0.5	8.0	148	12	15
	-	1.0	8.1	150	9.0	11
	-	5.0	9.7	180	8.0	8.2
	+	0.05	8.0	160	9.0	11
	+	0.1	8.1	147	9.0	11
	+	0.5	8.6	156	5.0	5.8
	+	1.0	8.4	153	10	12
	+	5.0	8.4	153	4.0	4.8

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PFOS

Pharmacokinetic - /

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**AN INVESTIGATION OF THE EFFECTS OF FLUOROCARBONS ON LIVER
FATTY ACID-BINDING PROTEIN.**

DEANNA J. NABBefeld
Masters Thesis Key

FC1: Perfluorooctane Sulfonic Acid (PFOS)

FC2: Ammonium Perfluorooctonate (APFO)

FC3: N-ethylperfluorooctane Sulfonamide Ethanol (N-EtFOSE)

FC4: N-ethylperfluorooctane Sulfonamide (N-Et FOSE Amide; FX-12)

**AN INVESTIGATION OF THE EFFECTS OF FLUOROCARBONS ON
LIVER FATTY ACID-BINDING PROTEIN.**

**A THESIS
SUBMITTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY
DEANNA J. NABBefeld**

**IN FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE/ENVIRONMENTAL HEALTH**

APRIL, 1998

000115

AN INVESTIGATION OF THE EFFECTS OF FLUOROCARBONS ON LIVER FATTY ACID-BINDING PROTEIN.

ABSTRACT

The objective of this study was to investigate the hypothesis that certain Fluorocarbons (FCs) bind liver fatty acid-binding protein (L-FABP) and displace endogenous fatty acids (FAs) as an initial event leading to peroxisome proliferation. The goals of the study were to assess the effect of FCs on L-FABP function as evaluated by the ability of the fluorescent FA analogue 11 - (5-dimethylaminonaphthalenesulphonyl) - undecanoic acid (DAUDA) to bind to L-FABP isolated from rats and guinea pigs treated and not treated with FC1 *in vivo*; and to assess the potency of FC1, FC2, FC3 and FC4 for binding to L-FABP.

Results show a decreased maximum binding capacity of L-FABP from FC1 treated rats without an increase in K_d . The most potent L-FABP binder was FC1, followed by FC4 and (with equal IC_{50} s) FC3 and FC2. Results for guinea pig L-FABP samples were inconclusive. This may be because guinea pig samples were only partially purified; thus resulting in a high degree of interference from remaining cellular debris and a lower concentration of L-FABP, as a proportion of total protein, as compared to rat samples.

INTRODUCTION

STUDY OBJECTIVES

This study was designed to investigate the hypothesis that certain fluorocarbons (FCs) bind to liver fatty acid-binding protein (L-FABP) and displace endogenous fatty acids (FAs) as an initial event leading to peroxisome proliferation. To examine this hypothesis, the kinetics of FA and FC binding to L-FABP were investigated with an *in vitro* binding assay using the fluorescent FA analogue 11 - (5-dimethylaminonaphthalenesulphonyl) - undecanoic acid (DAUDA). FC1 and FC2, known peroxisome proliferators, and FC3 and FC4, suspect peroxisome proliferators, were examined. Wyeth-14,643 (WY), a well known peroxisome proliferator, was used as the positive control and methanol as the negative control. Oleic acid, a FA known to bind to L-FABP with a very high affinity, was used to measure the maximum L-FABP binding. (Figure 1 - structures). L-FABP from

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male rats (considered to be strong responders to peroxisome proliferators) and male guinea pigs (considered to be weak or non-responders to peroxisome proliferators) (Svoboda, Grady and Azarnoff, 1967; Orton *et al.*, 1984; Lake and Gray, 1985; Elcombe and Mitchell, 1986), treated or not treated with FC1 *in vivo*, were examined.

The goals of the study were as follows:

- 1) to assess the effect of FCs on L-FABP function, as evaluated by the ability of DAUDA to bind to L-FABP isolated from rats and guinea pigs; and
- 2) to assess the potency of the various FCs for binding to L-FABP.

The first goal was accomplished as follows:

- a. L-FABP from rats and guinea pigs, treated and not treated with FC1, was isolated;
- b. the maximum binding capacity or receptor number (B_{max}) of each L-FABP sample and the dissociation constant or affinity (K_d) of DAUDA for each L-FABP sample were calculated; and
- c. the concentration of oleic acid which inhibited 50% of specific DAUDA binding to isolated L-FABP samples, the oleic acid IC_{50} for each sample, was measured.

The second goal was achieved by calculating the IC_{50} of each FC for the binding of DAUDA to the isolated control rat L-FABP sample.

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FIGURE I - STRUCTURES.

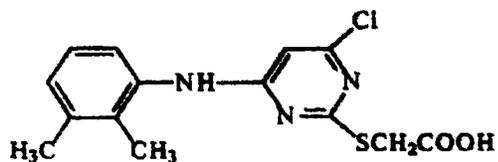
EC1

EC2

EC3

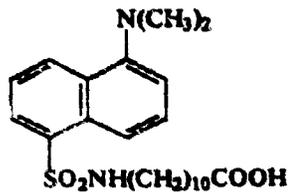
EC4

Wyeth-14,643



Methanol
CH₃-OH

DAUDA



Oleic Acid
CH₃-(CH₂)₇-CH=CH-(CH₂)₇-COOH

BACKGROUND

LIVER FATTY ACID-BINDING PROTEIN

The exact role of L-FABP, a member of the intracellular lipid-binding protein (iLBP) family, is unclear (Bass, Kaikaus and Ockner, 1993). It is found predominately in the liver, although it is also present in the small intestinal and colonic enterocytes, gastric brush border, and enteroendocrine cells (Bass, 1985; Bass, 1988; Sweetser, Heuckeroth and Gordon, 1987; Vincent and Muller-Eberhard, 1985; Chan *et al.*, 1985; Gordon *et al.*, 1982; Sorof and Custer, 1987). Accepted functions of L-FABP include binding and transporting FAs within the cell, regulating lipid metabolism, and protecting the cell by maintaining the concentration of free fatty acids (FFAs) below toxic levels (Bass *et al.*, 1993). L-FABP is unique to the iLBP family in that it has a larger binding cavity (Thompson *et al.*, 1997); broader ligand specificity (binds multiple hydrophobic compounds such as heme, certain eicosanoids, bilirubin, thyroxine, steroids, specific carcinogens and peroxisome proliferators as well as FAs) (Kaikaus, Bass and Ockner, 1990; Ockner *et al.*, 1972; Rolf *et al.*, 1995; Thumser, Voysey and Wilton, 1994; Khan and Sorof, 1990; Levi, Gatmaitan and Area, 1969); and the ability to bind two molecules per protein while other iLBPs bind only one (Thompson *et al.*, 1997).

The crystal structure of rat L-FABP (Thompson *et al.*, 1997) reveals two short antiparallel α -helices positioned over one end of an 11-stranded antiparallel β -barrel. This differs from other iLBPs, which are 10-stranded, but does not significantly alter the conformation of the protein. A cavity is formed within the β -barrel that serves as an internalized ligand

binding site with polar and nonpolar residues and bound water. In addition to a normal gap between the two β -strands, L-FABP has a second gap formed by missing hydrogen bonds. The function of this gap is unknown, but the missing hydrogen bonds increase the range of motion in L-FABP compared to other iLBPs. This localized conformational flexibility may contribute to the broad ligand specificity exhibited by L-FABP.

Two L-FABP binding sites exist, and interact allosterically. Crystal structures of the protein prepared with oleic acid have characterized the primary binding site by an internalized carboxylate and a U-shaped hydrocarbon chain. Fatty acids bound in the primary binding site interact with Arg¹²², a conserved residue in all iLBPs, and are surrounded by protein atoms, structural water and nearby atoms of the second bound FA. The oleic acid in the primary binding site is involved in hydrogen bond interactions at the carboxyl group with Ser³⁹, Arg¹²² and Ser¹²⁴. The secondary binding site is characterized by having the carboxylate of the second oleic acid near the surface, and the hydrocarbon tail inserted toward the center of the molecule and between the U-shaped hydrocarbon chain in the primary binding site. The carboxylate at this site is solvent-accessible, but still involved in a network of hydrogen bonds with residues forming the entrance to the primary binding cavity. The two ligands are in physical contact and it is believed that they influence each others relative affinities. Structural data suggest the second site may not exist until the primary site is filled, or that the prior presence of a FA in the primary site may be required for anything larger than a C₁₄ FA to bind the secondary site (Thompson *et al.*, 1997).

FLUOROCARBONS

Fluorocarbons (FCs) are compounds structurally analogous to hydrocarbons with the hydrogens replaced with fluorines. The FCs under investigation resemble long chain FAs, having a hydrophobic tail and a polar head group. The tails of FCs are more rigid in structure than the tails of FAs, however, and thus the conformational flexibility of FCs is more restricted than that of FAs (Zisman, 1964). FCs have unique chemical and physical properties such as being very heat stable, inert and chemically and electrically nonreactive (Bryce, 1964; Bankes, 1970; George and Anderson, 1986; Gilliland, 1992; Clark *et al.*, 1973). Such characteristics make them ideal for use in many consumer products and industrial procedures (Bryce, 1964). FCs are components of products including household cleaners, leather treatments, insecticides and fire-extinguishing foams; used as surfactants in the aqueous polymerization of fluorinated monomers; and used in industrial processes such as insulating, cooling, wetting, and corrosion inhibition (Bryce, 1964; Bankes, 1970; George and Anderson, 1986; Gilliland, 1992; Clark *et al.*, 1973). Despite the usefulness of these chemicals, some are known to cause mitochondrial inhibition, cholestasis, peroxisome proliferation and tumor formation in rodents (Gilliland and Mandel, 1996; Langely, 1990; Ikeda *et al.*, 1985; Pastoor *et al.*, 1987; Harrison *et al.*, 1988; Abdellatif *et al.*, 1991).

Permadi *et al.* (1993) suggest chain length of FCs influences the severity of effect, finding the greatest significance exhibited by C₈ compounds followed closely by C₁₀ compounds, and increasingly less severe consequences exhibited with shorter chained molecules. The work of Feller and Intrasukri (1993) agreed with that of Permadi *et al.* (1993), and added

that a carboxylic function was important for the stimulation of peroxisome proliferation. Similar results were reported by Kennedy *et al.* (1998), who analyzed FCs ranging in length from 4-9 carbons for the effect of structure on toxicity. They found C₈ FCs to produce effects at a 10-fold lower dose than C₄ FCs, and short chained FCs to be the least toxic. Although the effects seen in rodents have not been seen in humans, the potential for cumulative and long-term human toxicity resulting from continuous exposure to low concentrations of FCs is of concern (Gilliland and Mandel, 1996; Gilliland, 1992).

PEROXISOMES & PEROXISOME PROLIFERATION

According to Small (1993), peroxisomes (also called microbodies or, in plants, glyoxysomes) are single membrane-limited cytoplasmic organelles present in most eukaryotic cells. The major function of peroxisomes is the β -oxidation of FAs and FA derivatives (Mannaerts and Van Veldhoven, 1993). Peroxisomes contain no DNA; rather, their proteins are synthesized on free polyribosomes in the cell cytosol and imported into pre-existing peroxisomes post-translationally. It is believed that new peroxisomes form by fission from existing peroxisomes.

Peroxisomes have been shown to proliferate following exposure to a diverse class of chemicals referred to as peroxisome proliferators (Green, Issemann and Tugwood, 1993). The mechanism by which this occurs is unclear. Peroxisome proliferation is thought to be mediated by peroxisome proliferator activated receptors (PPARs), nuclear hormone receptors which, upon binding ligand, recognize specific DNA sequence motifs located upstream of the peroxisome proliferator target genes (peroxisome proliferator response

elements (PPREs)), and activate specific gene transcription (Isseman and Green, 1990; Dryer *et al.*, 1993). Due to the diversity of peroxisome proliferators shown to activate PPAR (Green *et al.*, 1993), speculation exists over a direct modulation of PPAR by peroxisome proliferators, and an indirect mechanism is suggested. In addition to chemical and xenobiotic peroxisome proliferators, natural factors such as a high fat diet, starvation and diabetes (Flatmark *et al.*, 1988; Ishii *et al.*, 1980; Ishii, Horie and Suga, 1980; Horie, Fukumori and Suga, 1991; Gottlicher, Widmark and Gustafsson, 1992) have been shown to cause peroxisome proliferation. This correlation between peroxisome proliferation and FA metabolism suggests that PPAR serves an important role in lipid homeostasis (Vanden Heuvel, 1996). It is probable, thus, that PPAR activation represents a physiological response to a biological stimulus, likely a factor involved in FA metabolism (Green *et al.*, 1993). Possible stimuli/PPAR ligands include steroids, FAs and derivatives of FA metabolism, and cholesterol metabolites (Green *et al.*, 1993). Target genes include those for acyl-CoA oxidase (Tugwood *et al.*, 1992; Feller and Intrasuksri, 1993), L-FABP (Isseman *et al.*, 1992) and P450_{IVA1} genes (Green *et al.*, 1993).

Significant interest surrounds the issue of peroxisome proliferation because some peroxisome proliferators have been shown to cause hepatocellular carcinomas in laboratory rodents (ivfoody *et al.*, 1991; Vanden Heuvel, 1996). The mechanism by which peroxisome proliferators cause cancer in rodents is unknown. They are classified as a novel class of epigenic chemical carcinogen (Vanden Heuvel, 1996), are nonmutagenic in the Ames assay and do not appear to bind DNA (Conway *et al.*, 1989; Cohen and Grasso, 1981; Reddy and Lalwani, 1983; Stott, 1988; Reddy and Rao, 1989; Lake *et al.*, 1990).

Multiple mechanisms have been proposed to explain peroxisome proliferator-induced liver tumor formation, including oxidative stress (Reddy and Rao, 1989), enhanced cell replication (Marsman *et al.*, 1988) and promotion of spontaneously formed lesions (Schulte-Hermann *et al.*, 1989). Green *et al.* (1993) propose peroxisome proliferators are "complete carcinogens" which exhibit a combination of initiation (oxidative radical production) and promotion (liver mitogenesis), possibly leading to sustained DNA replication depending on the compound and dose. Doubt about a causal relationship between peroxisome proliferation and carcinogenesis in rodents exists, however, and the relevance to human health is unclear (Tucker and Orton, 1993).

Mammalian species differ in their response to peroxisome proliferators (Lake and Gray, 1985; Rodricks and Turnbull, 1987). Rats are considered strong responders, and guinea pigs and nonhuman primates low to non-responders (Svoboda *et al.*, 1967; Orton *et al.*, 1984; Lake and Gray, 1985; Elcombe and Mitchell, 1986). Slight to no increase in peroxisomes were found in human patients treated with colfibrate (Hanefeld, Kemmer and Kadner, 1983) and fenofibrate (Blumcke *et al.*, 1983), drugs used in the treatment of hypercholesterolemia and known peroxisome proliferators in rodents. Many hypothesize that if a causal relationship does exist between peroxisome proliferation and hepatocarcinogenesis, it is specific to rodents and not a risk to man (Tucker and Orton, 1993).

Other well documented effects of peroxisome proliferators in rodents include inhibition of mitochondria β -oxidation (Elcombe and Mitchell, 1986; Eacho and Foxworthy, 1988;

Foxworthy and Eacho, 1988; Lock, Mitchell and Elcombe, 1989; Wallace, 1998), induction of peroxisomal β -oxidation and ω -oxidation in the ER (Reddy and Lalwani, 1983; Hawkins *et al.*, 1987), induction of L-FABP expression (Bass, Manning and Oelzner, 1985; Das, Gourisankar and Mukherjee, 1989; Fleischner *et al.*, 1975), cholestasis (Elcombe and Mitchell, 1986; Foxworthy and Eacho, 1988; Lock *et al.*, 1989; Van Raefighem *et al.*, 1988) and hepatomegaly (Moody *et al.*, 1991).

FATTY ACID CATABOLISM IN THE HEPATOCYTE

Free fatty acids (FFAs), formed by the breakdown of triacylglycerols stored in adipocytes, are carried in the blood by serum albumin and transported into hepatocytes by what is thought to be a plasma membrane bound fatty acid-binding protein (FABPpm) (Stremmel, Strohmayer and Berk, 1986; Stremmel *et al.*, 1985). Once in the cell, FFAs are picked up by L-FABP and, under routine conditions, the majority are transported to the mitochondria for β -oxidation, a process by which FAs are degraded to acetyl-CoA by the sequential removal of two carbon segments (Moran and Scrimgeour, 1994). Mitochondrial β -oxidation is coupled to the generation of high energy phosphate bonds via oxidative phosphorylation, and results in the synthesis of ATP and ketone bodies. In order to gain entry into the mitochondria, FA must first be converted to acyl-CoA esters by acyl-CoA synthetases, FA specific enzymes located in the mitochondrial outer membrane (Singh, Derwas and Poulos, 1987). The rate of FA entry into the mitochondria is regulated by carnitine acyl-transferase I, a second enzyme located in the outer membrane of the mitochondria, which converts acyl-CoA esters to acylcarnitines (Murthy and Pande, 1987). Once inside the mitochondria, acylcarnitines are converted back to acyl-CoA

esters by carnitine acyltransferase II, and degraded by mitochondrial β -oxidation to acetyl-CoA (McGarry and Foster, 1980; Bieber, 1988). Acetyl-CoA is shuttled into the cytosol by the citrate transport system for cholesterol and lipid synthesis (Stryer, 1994). The key factor regulating the rate of mitochondrial β -oxidation is the amount of FA entering the mitochondria which, as stated above, is controlled by carnitine acyl-transferase I. The activity of carnitine acyl-transferase I is controlled by the abundance of malonyl-CoA, the first committed intermediate in FA synthesis (McGarry and Foster, 1980). According to Bass *et al.* (1993), under conditions of increased FA biosynthesis, malonyl-CoA production is increased. Malonyl-CoA is produced from acetyl-CoA in a reaction catalyzed by acetyl-CoA carboxylase, an enzyme controlled by reversible phosphorylation responding to hormone signals and the presence of fatty acyl-CoA (Moran and Scrimgeour, 1994). When fatty acyl-CoA levels are low, acetyl-CoA carboxylase activity is high. This enhances the conversion of acetyl-CoA to malonyl-CoA. When malonyl-CoA is plentiful, the activity of carnitine-acyltransferase I is limited. This causes the rate of mitochondrial β -oxidation to decrease; FFAs to accumulate; acyl-CoA production and hence cholesterol synthesis to slow; and the rates of alternative routes of FA catabolism, peroxisomal β -oxidation and ω -oxidation in the endoplasmic reticulum (ER), to increase (Lock *et al.*, 1989).

Peroxisomal β -oxidation is normally responsible for catabolizing most, if not all, of the very long chain fatty acids brought into the hepatocyte (Singh *et al.*, 1981; Singh *et al.*, 1984; Lazo *et al.*, 1990; Jakobs and Wanders, 1991). This system is also capable of oxidizing medium and long chain FAs and previously activated CoA esters of medium and

long chain dicarboxylic acids. Under normal conditions, however, mitochondrial β -oxidation is the dominant route of catabolism for such substrates (Singh *et al.*, 1987). Peroxisomal β -oxidation proceeds through similar steps as does mitochondrial β -oxidation, however, important differences exist. First, the enzymes used in each process are different proteins (Hashimoto, 1987). Secondly, peroxisomal β -oxidation does not degrade FAs to their two carbon fragments as does mitochondrial β -oxidation; rather, peroxisomal β -oxidation stops after a few cycles, only shortening the carbon chain (Lazarow, 1978; Thomas *et al.*, 1980). Thirdly, peroxisomal β -oxidation is not coupled to an electron transport chain and oxidative phosphorylation as is mitochondrial β -oxidation (Lazarow and de Duve, 1976; Mannaerts *et al.*, 1979). Thus, while mitochondrial β -oxidation produces ATP and ketone bodies, peroxisomal β -oxidation produces hydrogen peroxide and heat. The rate of peroxisomal β -oxidation is thought to be controlled by substrate supply, specifically the activity of acyl-CoA oxidase, which reduces molecular oxygen to hydrogen peroxide in the first step of peroxisomal β -oxidation (Mannaerts *et al.*, 1979; Miyazawa *et al.*, 1983). Like substrates for mitochondrial β -oxidation, substrates for peroxisomal β -oxidation must be esterified to their acyl-CoA derivatives; however, peroxisomal β -oxidation is not dependent on carnitine acyl transferase I, as is mitochondrial β -oxidation (Mannaerts and Van Veldhoven, 1993).

ω -Oxidation in the ER, a P450_{IVAI} mediated process, is responsible for converting monocarboxylic acids to dicarboxylic acids. Dicarboxylic acids are activated in the ER by

dicarboxyl-CoA synthetase, an enzyme absent in mitochondria and peroxisomes. CoA esters of dicarboxylic acids are almost entirely dependent on mitochondrial β -oxidation for catabolism (Suzuki *et al.*, 1989). The ER also oxidizes bile acid intermediates and is able to esterify very long chain fatty acids (Singh and Poulos, 1988; Lazo *et al.*, 1990). A prerequisite of esterification is activation of FAs to their CoA derivatives (Mannaerts and Van Veldhoven, 1993). ω -Oxidation in the ER is enhanced in cases of FA overload (eg uncontrolled diabetes) or inhibition of mitochondrial β -oxidation (Mortensen and Gregersen, 1981; Golden and Kean, 1984; Mortensen, 1986; Vianey-Liaud *et al.*, 1987); and like peroxisomal β -oxidation, ω -oxidation is not dependent on carnitine acyl transferase I (Mannaerts and Van Veldhoven, 1993).

HYPOTHESIS

As stated above, exposure to FCs leads to mitochondrial inhibition, cholestasis, peroxisome proliferation, and tumor formation in rodents. Three of these endpoints - mitochondrial inhibition, cholestasis and peroxisome proliferation - are directly linked to FA metabolism. This study was designed to test the hypothesis that an initial step in FC-induced peroxisome proliferation is displacement of FAs from L-FABP by FCs. This hypothesis is supported by the fact that L-FABP has been shown to bind nongenotoxic peroxisome proliferators, including certain FCs, *in vitro* (Vanden Heuvel, 1996; Issemann *et al.*, 1992), with relative strengths of binding that parallel their ability to elicit peroxisome proliferation (Brandes *et al.*, 1990; Kanda *et al.*, 1990; Cannon and Eacho, 1991). According to the theory under question, upon displacement of FAs from L-FABP, the intracellular levels of fatty acyl-CoA would decrease. This would increase the activity

of acetyl-CoA carboxylase and enhance the conversion of acetyl-CoA to malonyl-CoA. An increase in the level of malonyl-CoA would repress the activity of carnitine acyltransferase I, and inhibit mitochondrial β -oxidation. ω -Oxidation in the ER would be enhanced, increasing the production of dicarboxylic acids. PPARs would be activated, by the binding of FAs or metabolic intermediates such as dicarboxylic acids, and specific gene transcription of acyl-CoA oxidase, L-FABP and P450_{IVAI} would be induced. A positive relationship between the amount of L-FABP and the rate of peroxisomal β -oxidation has been found (Appelkvist and Dallner, 1980), and the level of acyl-CoA oxidase is thought to determine the rate of peroxisomal β -oxidation (Mannaerts *et al.*, 1979; Miyazawa *et al.*, 1983). Thus, increased transcription of acyl-CoA oxidase and L-FABP would increase rates of peroxisomal β -oxidation and elicit peroxisome proliferation. Induction of P450_{IVAI} genes would further increase the rate of ω -oxidation in the ER. Cholesterol synthesis would eventually cease in response to mitochondrial inhibition and lack of acetyl-CoA production, and decreased esterification by the ER due to decreased acyl-CoA. This would lead to cholestasis. The mechanisms by which carcinogenesis could be induced or promoted will not be discussed.

MATERIALS AND METHODS

MATERIALS

Wyeth-14,643 (WY) was obtained from ChemSyn Science Laboratories, Lenexa, KS; FCs were provided by 3M Speciality Chemicals Division, St. Paul, MN; Optifluor LSC-cocktail was obtained from the Packard Instrument Company, Meriden, CT; AMICON YM-5 membrane was purchased from Amicon Corporation, Lexington, MA; BCA Protein Assay was obtained from Pierce Chemical Company, Rockford, IL; and 11-(5-Dimethylaminonaphthalenesulphonyl)-undecanoic acid (DAUDA) was purchased from Molecular Probes, Eugene, OR. All other chemicals were obtained from VWR Scientific, West Chester, PA.

ANIMALS AND TREATMENT

Male rats and guinea pigs, 6-8 weeks of age, weighing between 150 and 250 grams were purchased from Charles River Labs, Wilmington, MA. Following an adaptation period of one week after arrival at 3M, animals were weighed, ear-tagged and exposed. The treatment groups consisted of the following:

1. Guinea Pig Vehicle Control - Tween 80, 2% (n = 4);
2. Rat Vehicle Control - Tween 80, 2% (n = 4);
3. Guinea Pig FC1 - FC1 in Tween 80, 2% (n = 4); and,
4. Rat FC1 - FC1 in Tween 80, 2% (n = 4).

All treatments were administered by intraperitoneal (ip) injection. The vehicle control groups were dosed at 5ml / kg body weight 2% Tween 80. The FC1 groups were dosed at

5 ml / kg body weight with a suspension of 32 mM FC1 in Tween-80, 2% (86 mg FC1 / kg body weight). All animals were housed individually in controlled environments and observed for mortality and clinical signs of toxicity during the first four hours after dosing, at 24 hours, and daily thereafter for the duration of the study. Animals were sacrificed with CO₂ 12 days after dosing. Body weights and selected organ weights (liver, kidneys, testes) were recorded at necropsy. Organ tissues and body fluids were stored frozen at -70°C for biochemical analysis or in 10% buffered formaldehyde for subsequent histological analysis. Selected livers were perfused with and stored in gluteraldehyde for future histological analysis by light microscopy.

PURIFICATION OF L-FABP

Protein purification was performed at the University of San Francisco, CA (UCSF) Liver Research Center. Three frozen livers (rat FC1, guinea pig vehicle control, and guinea pig FC1) were shipped in dry ice from 3M to UCSF. These livers and one fresh liver, from a non-treated rat (control) sacrificed at UCSF, were purified.

Frozen livers (approximately 10g each) were thawed and weighed. The fresh liver (approximately 10g) was isolated and perfused with isotonic saline. Each liver was homogenized 30% (w/v) in ice-cold 10mM potassium phosphate buffer, pH 7.4, using a Teflon-glass Potter-Elvehjem tissue homogenizer. The homogenates were centrifuged for 20 minutes at 10,000g in a Sorvall superspeed RC2-B centrifuge maintained at 4°C. The supernatants were removed and subsequently centrifuged for one hour at 38-40,000 rpm (4°C) in a Beckman L7 Ultracentrifuge. The resulting supernatant (cytosol) was labeled with 0.5µCi of 1-¹⁴C oleate to trace the L-FABP during purification.

Purification steps were performed in a cold room at 4°C. The cytosol was loaded on a Sephadex G50 M column (5 x 60 cm) equilibrated with 10mM potassium phosphate buffer, pH 7.4. Protein was eluted from the column at a flow rate of approximately 1.4 ml/minute. One hundred fractions were collected (approximately 14.5 ml/fraction). Optifluor LSC-cocktail (5 ml) was added to a 20µl aliquot of each fraction and a Packard Tri-carb 4530 scintillation counter was used to assess L-FABP activity. Fractions with L-FABP activity were pooled and concentrated to approximately 5ml using a vacuum filter apparatus fitted with an AMICON YM-5 membrane. Guinea pig samples (control and FC1 treated) were concentrated and frozen at this point. Rat samples (control and FC1 treated) were further purified as follows.

The concentrated solution was loaded on a Sephadex G50 (fine) gel filtration column (2.5 x 45 cm) equilibrated with 10mM potassium phosphate buffer, pH 7.4. The flow rate was approximately 0.9 ml/minute. Sixty fractions were collected at a volume of 3.48ml/fraction. The fractions containing L-FABP activity were pooled and concentrated to approximately 5ml. The concentrated cytosol was dialyzed overnight at 4°C against 30mM Tris-HCl, pH 9, using a Spectrapore Membrane MWCO 3,500. The sample was then applied to a DEAE-cellulose column (Whatman DE-52, 1.25 x 15cm or 2.5 x 15cm) previously equilibrated with 30mM Tris-HCl, pH 9, (degassed). The column was eluted with 30mM Tris-HCl, pH 9, (degassed) followed by a linear gradient of NaCl (0-0.2M) in 30mM Tris-HCl, pH 9. The flow rate was approximately 1ml/minute. Twenty fractions, 7.8 ml each, were collected.

Homogeneity of the final rat control and rat FC1 fractions was assessed using sodium dodecyl sulfate / polyacrylamide-gel electrophoresis (SDS PAGE) analysis, and confirmed by the appearance of a dominant protein band at molecular weight (MW) 14,000 Daltons (Da.) (Figure 4). Protein concentration of all samples was determined using the bicinchonic acid (BCA) protein assay by Pierce with BSA as the standard (Table 1). None of the samples were delipidated.

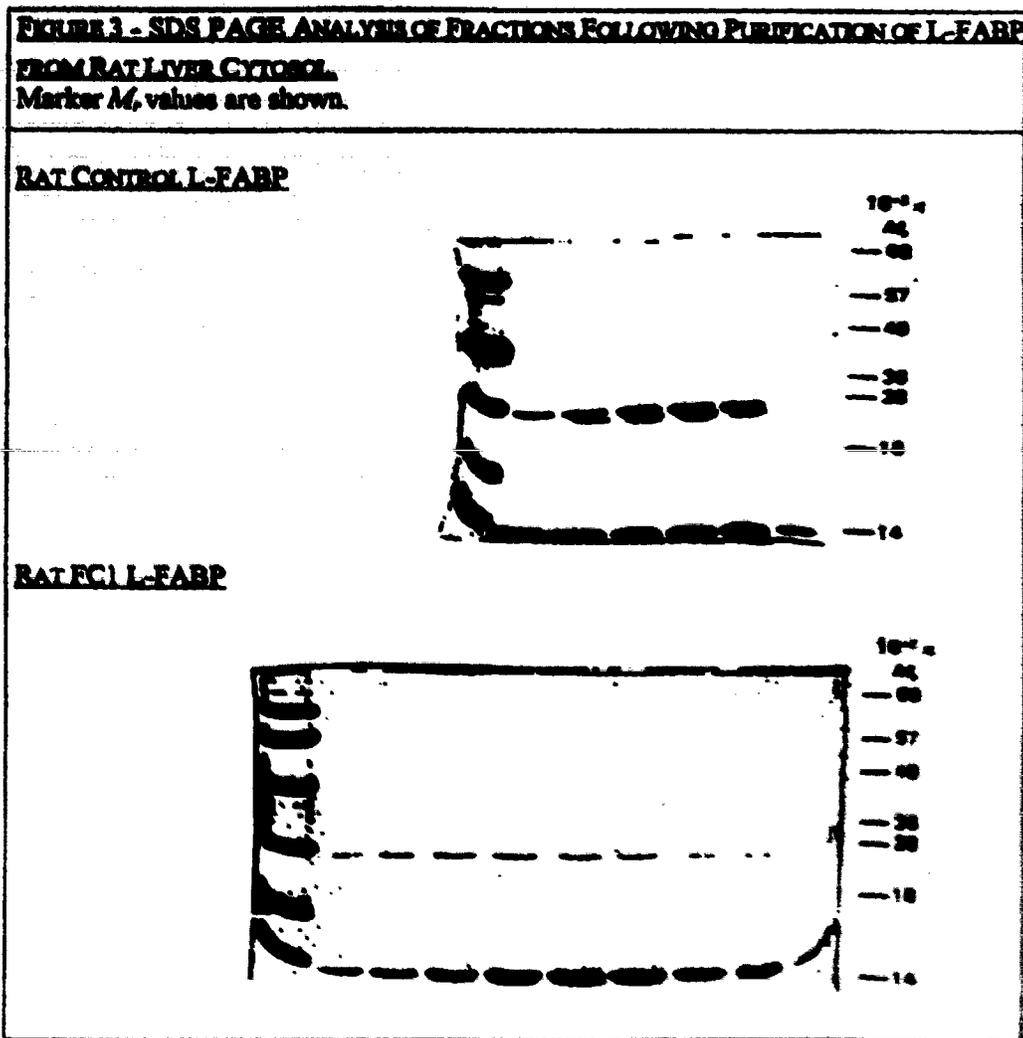


TABLE I - PROTEIN CONCENTRATION	
Protein concentration was determined using the BCA protein assay with BSA as the standard. Values are a mean \pm standard deviation of 2 trials.	
FRACTIONS	TOTAL PROTEIN CONCENTRATION ($\mu\text{g/ml}$)
<i>PURIFIED</i>	
Rat Control	123.1 \pm 13.7
Rat FCi	308.8 \pm 1.4
<i>PARTIALLY PURIFIED</i>	
Guinea Pig Vehicle Control	1755.7 \pm 10.1
Guinea Pig FC1	1021.0 \pm 129.7

FLUORESCENCE MEASUREMENTS

Fluorescence measurements were based on the work of Wilkinson and Wilton (1986). All assays were carried out at room temperature using a slit width of 5nm in a SPEX 1681 0.22m spectrometer, SPEX Industries, Incorporated. A stock solution of DAUDA, 0.1mM, was prepared by slowly adding 50mM potassium phosphate (KH_2PO_4) buffer, pH 7.2, to 1mM DAUDA in methanol. All further dilutions of DAUDA were in 50mM KH_2PO_4 , pH 7.4. All dilutions of L-FABP samples were in 50mM KH_2PO_4 , pH 7.4. All FCs, WY and oleic acid were dissolved in methanol. All measurements were made after binding had reached equilibrium.

FLUORESCENCE CHARACTERIZATION

Emission and Excitation Maxima and Average Maximum Fluorescence Intensity

The maximum emission and excitation wavelengths (nm) and average maximum fluorescence intensity (FI) (cpm) were determined for 1 μM DAUDA binding to each L-

FABP sample. L-FABP, from original undiluted stock, was added to 2ml 1 μ M DAUDA in aliquots of 1.6 - 114 μ l (depending on the concentration of protein) until no further change in emission or excitation wavelength or FI was detected. The range of protein concentrations analyzed for each L-FABP sample was 0.1 μ M-3 μ M. Excitation scans, from 250-400nm using an emission wavelength of 500nm, and emission scans, from 350-600nm upon excitation at 350nm, were performed with each addition. FI (Em. 500nm, Ex., 350nm) was measured following each addition of L-FABP. Curves of FI versus concentration of L-FABP, representing an average \pm standard deviation of 3 trials, were constructed for each L-FABP sample. The three highest FI values for each curve were averaged to determine the average maximum FI for DAUDA binding to each sample.

Specific DAUDA Binding

Total binding of DAUDA (0-8 μ M) to each L-FABP sample was determined by adding 2-20 μ l aliquots of 0.1mM DAUDA to 2ml 1 μ M L-FABP. Increased FI (Em. 500nm, Ex., 350nm) due to the binding of DAUDA to protein was measured. Nonspecific binding was assessed by saturating L-FABP binding sites with oleic acid and performing the same titration. Aliquots, 2-20 μ l, of 0.1mM DAUDA were added to a 2ml solution of 1 μ M L-FABP and 100 μ l oleic acid. Specific binding of DAUDA to each L-FABP sample was determined by subtracting nonspecific binding from total binding.

ANALYSIS OF THE EFFECT OF FCS OF L-FABP

Calculation of DAUDA Binding Constants (K_d , B_{max})

K_d and B_{max} values were determined for each protein. Specific binding was transformed to units of bound DAUDA (μ M) by dividing the specific FI (cpm) by the maximum FI per

1 μ M DAUDA (cpm) for each L-FABP sample. Computer assisted nonlinear regression (GraFit Version 3, Erithacus Software Limited) was used to construct curves of specific bound DAUDA versus free DAUDA representing an average of 3-6 trials. The following equation was used, $\text{Bound} = ([L] \times B_{\text{max}}) / (K_d + [L])$.

Calculation of Oleic Acid IC₅₀'s

The concentration of oleic acid which inhibits 50% of specific DAUDA binding, the IC₅₀, was calculated for each combination of oleic acid and L-FABP sample. Cuvettes contained 2ml 1 μ M L-FABP and 1 μ M DAUDA. Oleic acid, 1mM in 10% methanol, was added in 0.4-20 μ l aliquots. FI (cpm) (Em. 500nm, Ex. 350nm) due to the binding of DAUDA to protein following each addition was measured. Curves of percent inhibition of specific DAUDA binding versus oleic acid concentration, representing an average \pm standard deviation of 3-6 trials corrected for the effect of methanol, were constructed.

ANALYSIS OF THE POTENCY OF VARIOUS FCs FOR BINDING TO L-FABP

Competitive Binding Experiments - Calculation of IC₅₀'s

Cuvettes contained 2 ml 1 μ M rat control L-FABP and 1 μ M DAUDA. Ligands, 1mM (FCs and WY in 100% methanol, and methanol in 50mM KH₂PO₄, pH 7.2), were added in 0.4-20 μ l aliquots. FI (cpm) (Em. 500nm, Ex. 350nm) due the binding of DAUDA to protein following each addition was measured. Curves of percent inhibition of specific DAUDA binding versus competitor concentration, representing an average \pm standard deviation of 3-6 trials corrected for the effect of methanol were constructed. The concentration of each competitor which inhibited 50% of specific DAUDA binding, the IC₅₀, was calculated.

RESULTS & DISCUSSION

FLUORESCENCE MEASUREMENTS

FLUORESCENCE CHARACTERIZATION

Emission and Excitation Maximum

The emission and excitation spectra of each protein binding DAUDA was analyzed to determine the optimal conditions for the study. The maximum emission and excitation wavelengths for all proteins were approximately 500 and 350nm respectively (Table 2). Values for guinea pig L-FABP samples were slightly higher than those for rat samples. This difference is likely because guinea pig samples were only partially purified; thus, more cellular debris remained to potentially interfere with DAUDA binding, and less L-FABP as a proportion of total protein was present. A "blue shift" in both emission and excitation wavelength occurred upon the addition of each L-FABP sample to DAUDA. Excitation wavelength shifted from approximately 330 to 340nm (Figure 4), and emission wavelength shifted from approximately 550 to 500nm (Figure 5). This shifting of wavelength is characteristic of DAUDA binding to a nonpolar site on L-FABP (Wilkinson and Wilton, 1986). According to Thumser *et al.* (1994a), DAUDA only binds one of the two oleate-binding sites of L-FABP. Due to the stringent conformational requirements of primary site binding (internalized carboxylate/polar group and a U-shaped hydrocarbon/hydrophobic chain), DAUDA most likely binds the secondary site. Similar shifting of fluorescence wavelength and emission and excitation maxima were found by Wilkinson and Wilton (1986) and Thumser, Vosey and Wilton (1996).

TABLE 2 - EMISSION AND EXCITATION MAXIMA.
 Fluorescence emission maxima (nm) were measured upon excitation at 350nm. Excitation maxima (nm) were measured using an emission wavelength of 500nm. L-FABP was added to 1µM DAUDA until no further change in emission or excitation wavelength was detected. Each value is an average ± standard deviation of 3 trials.

SAMPLE	EMISSION MAXIMUM (nm)	EXCITATION MAXIMUM (nm)
DAUDA only	551.33 ± 1.53	331.00 ± 1.00
Rat Control L-FABP	502.67 ± 4.62	344.33 ± 2.08
Rat FCI L-FABP	502.33 ± 4.04	339.67 ± 6.66
Guinea Pig Vehicle Control L-FABP	511.00 ± 7.81	340.67 ± 5.13
Guinea Pig FCI L-FABP	511.00 ± 7.81	333.67 ± 1.15

FIGURE 4 - EXCITATION MAXIMA.
 Fluorescence excitation maxima (nm) were measured using an emission wavelength of 500nm. L-FABP was added to 2ml 1µM DAUDA until no further change in excitation wavelength was detected. Each curve is representative of 3 trials (1µM DAUDA, 3µM L-FABP). KEY: a - Guinea Pig FCI L-FABP; b - Guinea Pig Vehicle Control L-FABP; c - Rat FCI L-FABP; d - Rat Control L-FABP.

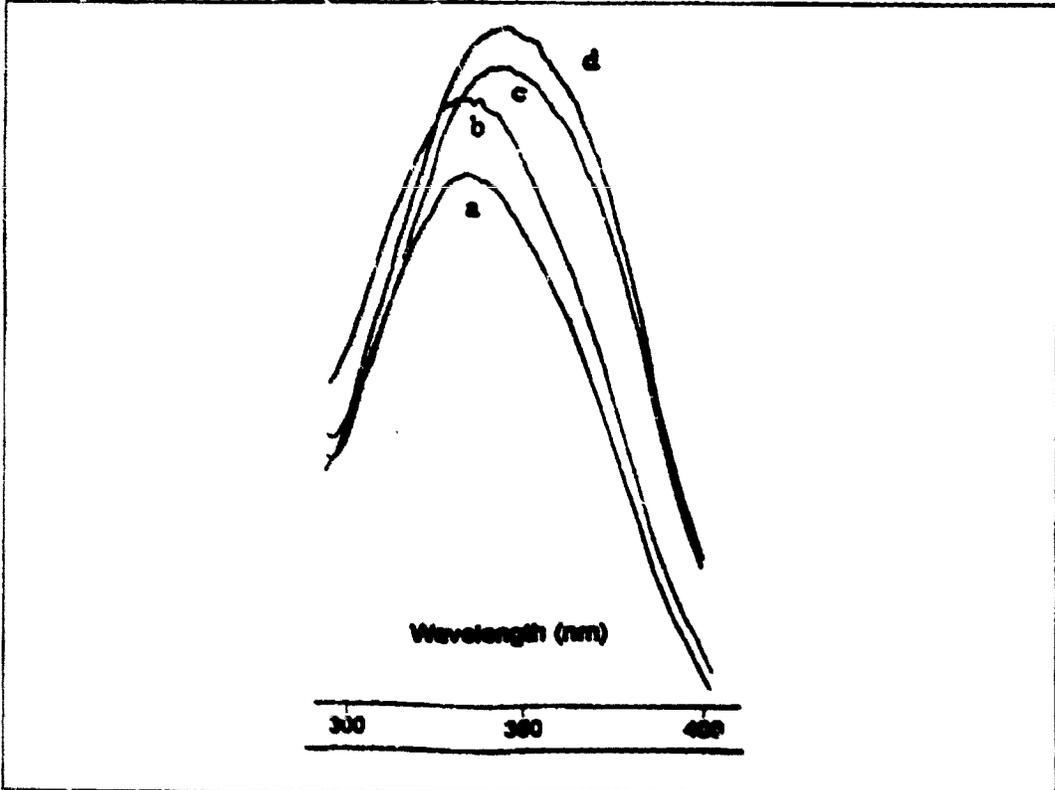
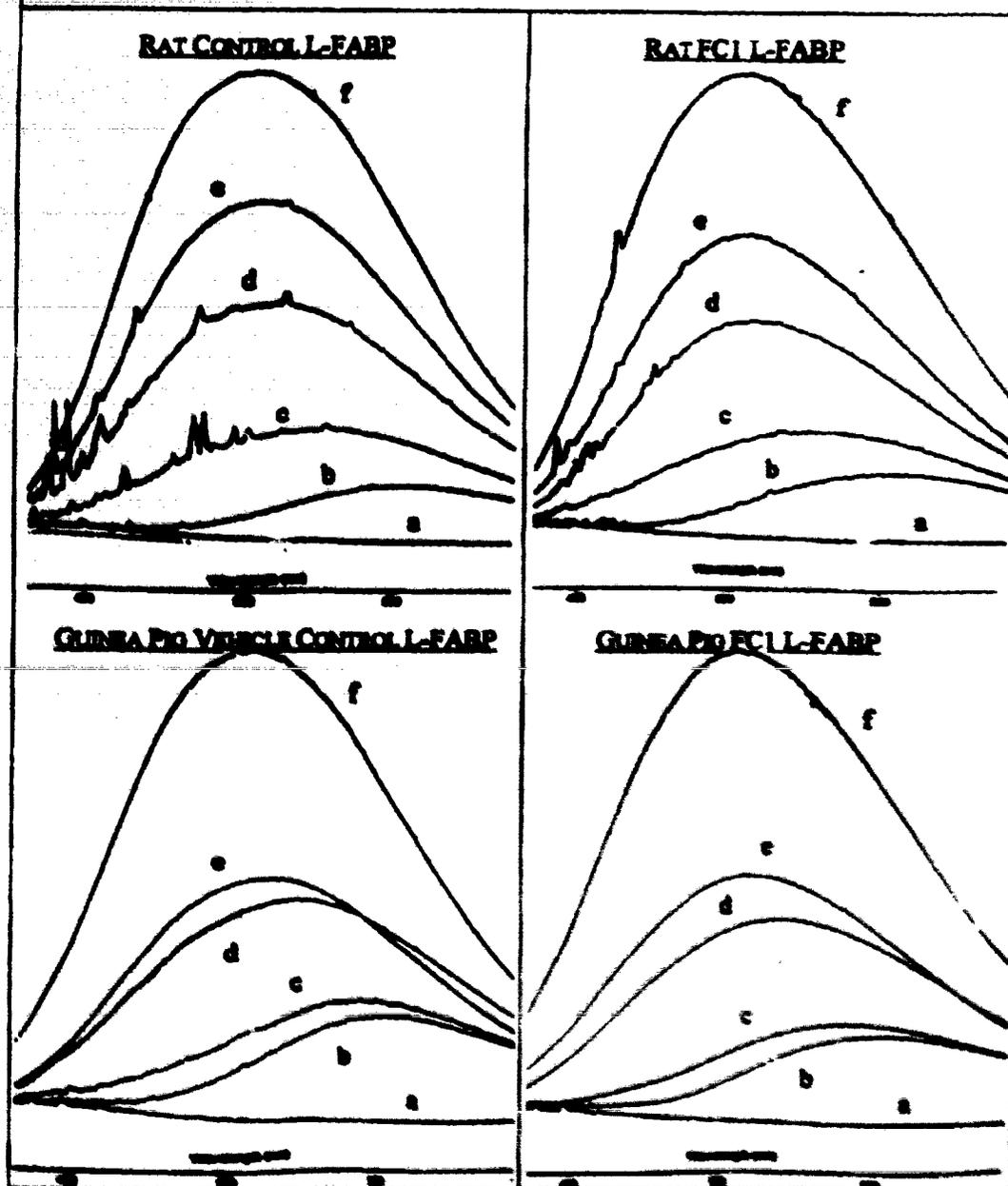


FIGURE 5 - EMISSION MAXIMA.

Fluorescence emission maxima (nm) were measured upon excitation at 350nm. L-FABP was added to 2ml 1 μ M DAUDA until no further change in emission wavelength was detected. Each curve is representative of 3 trials.

KEY: a = 0 DAUDA, 0 L-FABP, 50mM KH₂PO₄; b = 1 μ M DAUDA, 0 L-FABP; c = 1 μ M DAUDA, 0.1 μ M L-FABP; d = 1 μ M DAUDA, 0.5 μ M L-FABP; e = 1 μ M DAUDA, 1 μ M L-FABP; f = 1 μ M DAUDA, 3 μ M L-FABP.



Average Maximum Fluorescence Intensity

The average maximum FI (cpm) of $1\mu\text{M}$ DAUDA binding to L-FABP from FCI treated and non-treated animals did not substantially differ. The average maximum FI was approximately 850,000 cpm for rat L-FABP samples, and 660,000 cpm for guinea pig L-FABP samples (Table 3). Maximum FI was reached following the addition of $1\mu\text{M}$ rat control L-FABP, $2.5\mu\text{M}$ rat FCI L-FABP, $2.5\mu\text{M}$ guinea pig vehicle control L-FABP and $2\mu\text{M}$ guinea pig FCI L-FABP (Figure 6). The lower the affinity of receptor for probe, the higher the concentration of receptor needed for binding, and vice versa (Matthews, 1993). Thus, these data suggest that the rat FCI L-FABP sample had a decreased affinity for DAUDA as compared to the rat control L-FABP sample, and that the guinea pig FCI sample had an increased affinity for DAUDA as compared to the guinea pig vehicle control L-FABP sample. The average maximum FI for guinea pig samples was 77.6% lower than that for rat samples. This may be due to the impurity of the guinea pig samples resulting in a high degree of interference in DAUDA binding and a lower concentration of L-FABP as a proportion of total protein.

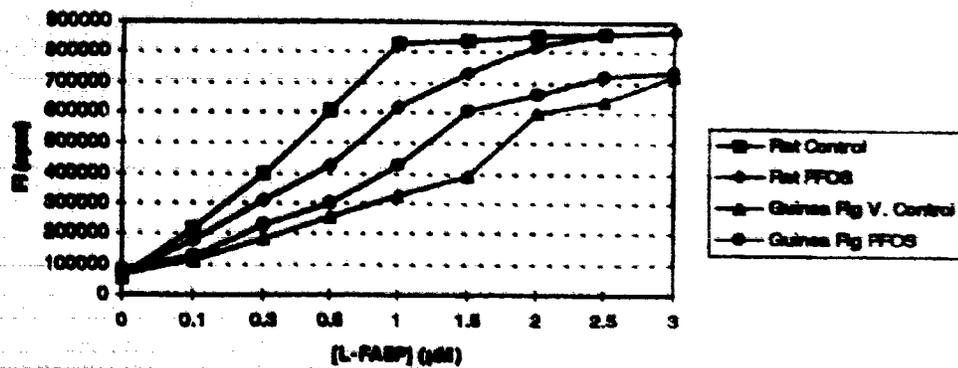
TABLE 3 - MAXIMUM FI OF $1\mu\text{M}$ DAUDA

Cuvettes containing 2 ml $1\mu\text{M}$ DAUDA were titrated with rat and guinea pig L-FABP samples (0.1mM) to determine the maximum FI of $1\mu\text{M}$ DAUDA binding to each sample. Three trials per protein were performed. Values are the average \pm standard deviation of the 3 highest average FI values per protein.

L-FABP SAMPLE	MAXIMUM FI (cpm)
Rat Control	848,093 \pm 13,639
Rat FCI	852,438 \pm 24,776
Guinea Pig Vehicle Control	655,348 \pm 63,812
Guinea Pig FCI	669,632 \pm 33,619

FIGURE 6 - AVERAGE MAXIMUM FI OF 1 μ M DAUDA FI vs [L-FABP]

Cuvettes containing 2 ml 1 μ M DAUDA were titrated with rat and guinea pig L-FABP samples (0.1 mM) to determine the maximum FI of 1 μ M DAUDA binding to each sample. FI, due to the binding of DAUDA to protein, was measured after each addition. Each curve is an average of 3 trials.



Specific DAUDA Binding

Because of its well documented high affinity for L-FABP (Thumser *et al.*, 1994 a,b; Thumser and Wilton, 1994; Thumser *et al.*, 1996; Thumser and Wilton, 1995), oleic acid was chosen as the displacing ligand to determine nonspecific binding of DAUDA to L-FABP. Excess oleic acid was added to occupy all L-FABP binding sites and prevent the specific binding of DAUDA to L-FABP. DAUDA was titrated into the assay and FI, due to nonspecific binding, was observed. Total binding was determined by performing the assay in the absence of oleic acid; specific binding was calculated by subtracting nonspecific binding from total binding. Absolute FI values are given in Table 4. The breakdown of total binding into percent specific and nonspecific binding is shown in Figure 7.

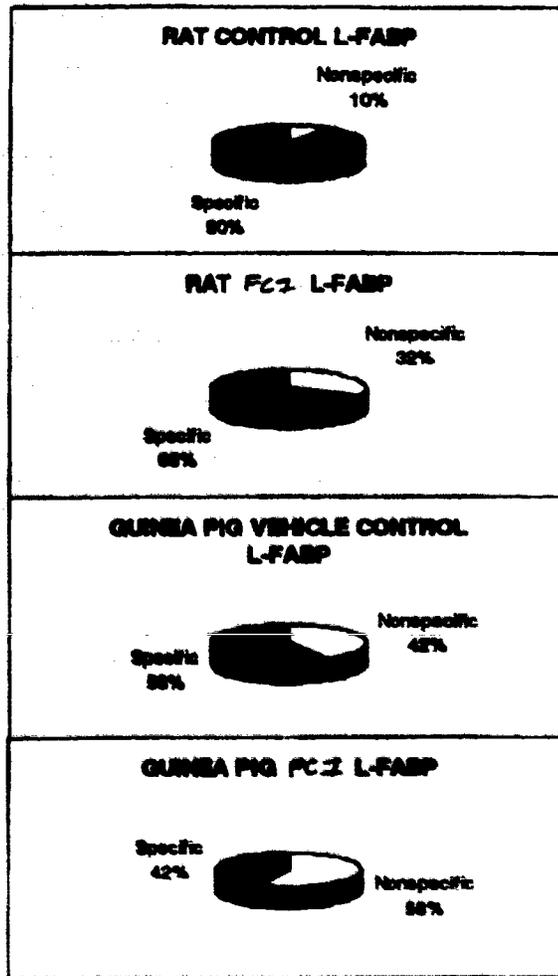
TABLE 4 - 1 μ M DAUDA BINDING TO 1 μ M L-FABP.

Total binding of DAUDA to each L-FABP sample was determined by adding aliquots of 0.1mM DAUDA to 2ml 1 μ M L-FABP. Increased FI due to the binding of DAUDA to protein was measured. Nonspecific binding was measured in the presence of 100 μ M oleic acid. Specific binding of DAUDA to each L-FABP sample was determined by subtracting nonspecific binding from total binding. Values are an average \pm standard deviation of 3-6 trials (cpm).

L-FABP SAMPLE	TOTAL	NONSPECIFIC	SPECIFIC
Rat Control	818,000 \pm 124,000	78,000 \pm 26,000	740,000 \pm 124,000
Rat FCI	302,000 \pm 81,000	96,000 \pm 21,000	206,000 \pm 81,000
Guinea Pig V. Control	219,000 \pm 138,000	92,000 \pm 23,000	127,000 \pm 138,000
Guinea Pig FCI	162,000 \pm 15,000	94,000 \pm 65,000	68,000 \pm 15,000

FIGURE 7 - DAUDA BINDING

Total binding of DAUDA to each L-FABP sample was determined by adding aliquots of 0.1mM DAUDA to 2ml 1 μ M L-FABP. Increased FI due to the binding of DAUDA to protein was measured. Nonspecific binding was measured in the presence of 100 μ M oleic acid. Specific binding of DAUDA to each L-FABP sample was determined by subtracting nonspecific binding from total binding. Values are an average of 3-6 trials.



Specific binding of DAUDA to rat control L-FABP represented approximately 90% of total binding, while specific binding of DAUDA to rat FC1 L-FABP accounted for only 68% of total binding. When a ligand has lower affinity for a receptor, a larger proportion of total binding is nonspecific (Matthews, 1993); thus, these data suggest the rat FC1 L-FABP sample had a three fold lower affinity for DAUDA than the rat control L-FABP sample. This is consistent with the results of the average maximum FI analysis of the rat L-FABP samples, which also suggests the affinity of L-FABP for DAUDA was decreased in the FC1 sample. Less of a difference was seen between the two guinea pig L-FABP samples. Specific binding of $1\mu\text{M}$ DAUDA to $1\mu\text{M}$ L-FABP represented 58% of total binding in the guinea pig vehicle control L-FABP sample, and 42% of total binding in the guinea pig FC1 sample. The data suggest the guinea pig FC1 L-FABP sample had lower affinity for DAUDA than the guinea pig vehicle control L-FABP sample. This is not consistent with the results of the average maximum FI analysis, which suggest the relative affinity of the guinea pig FC1 L-FABP sample was higher than that for the guinea pig vehicle control L-FABP sample. This inconsistency may be due to the crudeness of the guinea pig samples giving rise to a high degree of interference in the binding of DAUDA to L-FABP.

The overall crudeness of the guinea pig L-FABP samples, as compared to the rat L-FABP samples, is reflected in a lower absolute total DAUDA binding (cpm), a higher percent nonspecific binding and a lower percent specific binding.

ANALYSIS OF THE EFFECT OF FC1 ON L-FABP

Calculation of DAUDA Binding Constants

DAUDA binding constants were calculated for each L-FABP sample. The purpose of this analysis was to assess the effect of FC1 on the functionality of L-FABP; and to confirm the results shown thus far, which suggest the affinity of L-FABP for DAUDA was decreased in FC1 treated rat L-FABP samples, and that guinea pig L-FABP samples were too impure to accurately analyze.

The maximum binding capacity or receptor number (B_{max}) of each L-FABP sample and the dissociation constant or affinity (K_d) of each L-FABP sample for DAUDA were determined using computer assisted nonlinear regression (Table 5 and Figure 9). Specific binding of DAUDA to each L-FABP sample was converted to μM by dividing the FI (cpm) due to specific DAUDA binding (Table 4) by the average maximum FI (cpm) per $1\mu M$ DAUDA for each L-FABP sample (Table 3).

The B_{max} of rat control L-FABP was nearly $1\mu M$. This agrees with the work of Thumser *et al.* (1994 a,b), Thumser and Wilton (1994) and Thumser *et al.* (1996) who all found DAUDA to bind to one of the oleic acid binding sites on L-FABP. The B_{max} for the rat FC1 L-FABP sample was approximately $0.35\mu M$, suggesting the capacity of rat FC1 L-FABP to bind DAUDA was nearly one third that of rat control L-FABP. One possible explanation for this decreased capacity is that FC1 is bound to L-FABP in the FC1 sample, rendering fewer available binding sites and allowing less DAUDA to bind. The rigid tail of

FC1 (Zisman, 1964) and strict conformational requirements for primary site binding to L-FABP (Thompson *et al.*, 1997) suggest FC1 binding would occur in the secondary binding site. Another possible explanation is that the vehicle (Tween 80, 2%) somehow affected the binding of DAUDA to L-FABP in the FC1 treated sample. This is unlikely, however, since Tween 80 is a mild, non-ionic detergent, designed to allow solubilized proteins to retain their native structure (Sigma, 1998); and because a low concentration of Tween 80 used (see methods). This does point out, however, the importance of including a vehicle control in the experiment.

The Kds for the rat control L-FABP and rat FC1 L-FABP samples were not notably different and were comparable to the Kd of $0.38 \pm 0.02\mu\text{M}$ found by Thumser *et al.* (1996). Thus, although the data for average maximum FI and percent specific binding suggest a decreased affinity of rat FC1 L-FABP for DAUDA as compared to rat control L-FABP, the Kd and Bmax values suggest the maximum binding capacity/number of binding sites rather than the binding affinity was affected.

The Bmax of the guinea pig vehicle control L-FABP sample was much lower than expected, $0.281\mu\text{M}$ as compared to approximately $1\mu\text{M}$ for the rat control L-FABP sample. The Kd for the guinea pig vehicle control L-FABP sample was higher than expected, $0.48\mu\text{M}$ as compared to approximately $0.3\mu\text{M}$ for the rat control L-FABP. One possible explanation for these differences in results is that rat L-FABP and guinea pig L-FABP are different. Although the crystal structure of guinea pig L-FABP has not been deduced, sequence identity for FABPs of the same tissue type from different species is

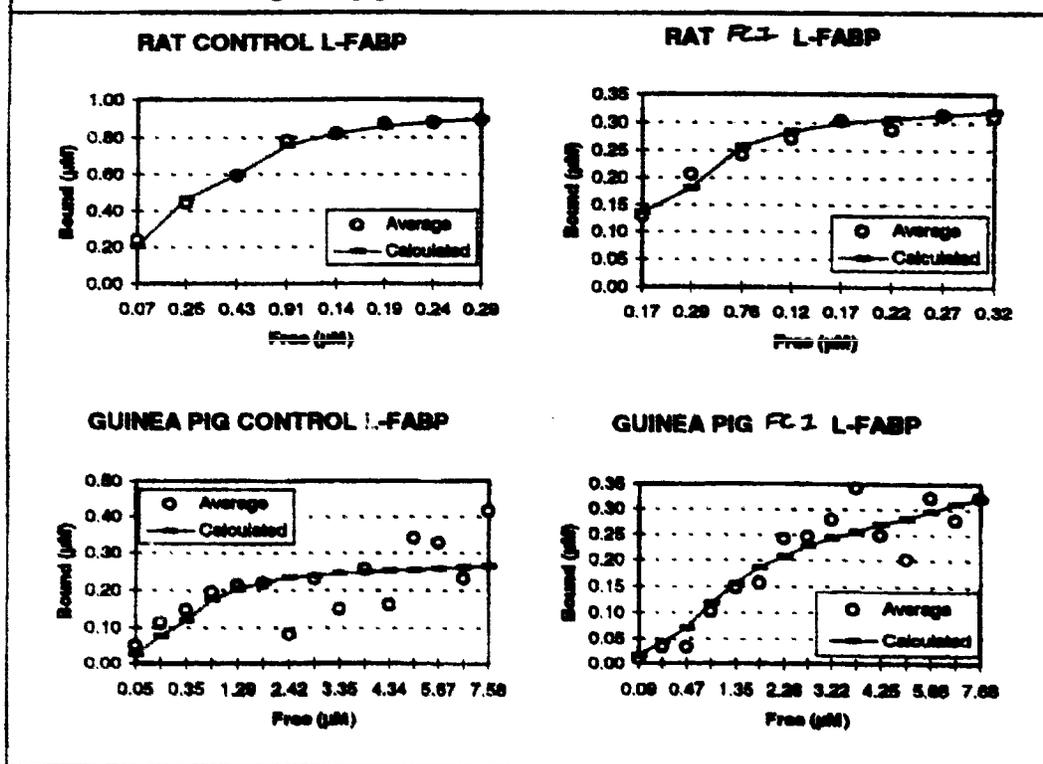
approximately 82-92% (Richieri, Ogata and Kleinfeld, 1994). One can, therefore, expect the binding constants of control guinea pig and rat L-FABP to be comparable. Other possible explanations for this difference are that the vehicle (Tween 80, 2%) had some effect on the L-FABP, or that the crudeness of the guinea pig sample caused a great deal of interference with the binding of DAUDA to L-FABP. As mentioned above, the effect of Tween 80 is presumably minimal; thus, the crudeness of the sample is likely to be to blame for the unexpected binding constants.

As discussed previously, the results for specific binding of DAUDA to guinea pig L-FABP suggest the guinea pig FC1 L-FABP sample had lower affinity for DAUDA than the guinea pig vehicle control L-FABP sample. This suggestion was counter to the results for average maximum FI, which indicate the guinea pig FC1 sample had an increased affinity for DAUDA as compared to the guinea pig vehicle control L-FABP sample. These contradictory data, when combined to calculate a K_d and B_{max} for each guinea pig L-FABP sample, resulted in a K_d for the guinea pig FC1 L-FABP sample that was about 4.5 times that of the vehicle control sample, and a capacity of the guinea pig FC1 L-FABP sample that was about twice that of the guinea pig vehicle control L-FABP sample. These results are inconsistent and inconclusive, likely due to the impurity of the guinea pig samples, as discussed above.

TABLE 5 - DAUDA BINDING CONSTANTS.
 Values are an average \pm standard deviation of 3 trials as calculated by computer assisted nonlinear regression. Bound = $([L] \times B_{max}) / (K_d + [L])$.

L-FABP SAMPLE	B _{max} (μ M)	K _d (μ M)
Rat Control	0.987 \pm 0.016	0.278 \pm 0.020
Rat FC1	0.345 \pm 0.012	0.260 \pm 0.063
Guinea Pig Vehicle Control	0.281 \pm 0.043	0.480 \pm 0.391
Guinea Pig FC1	0.413 \pm 0.060	2.240 \pm 0.452

FIGURE 8 - SPECIFIC BOUND VS FREE DAUDA.
 Computer assisted nonlinear regression was used to construct curves of specific bound DAUDA versus free DAUDA (Bound = $([L] \times B_{max}) / (K_d + [L])$). Each curve is an average of 3-6 trials. The reduced chi square value for each regression was as follows: rat control L-FABP, 3.83e-16; rat FC1 L-FABP, 7.83e-16; guinea pig vehicle control L-FABP, 6.12e-15; and guinea pig FC1 L-FABP, 1.67e-15.



Calculation of Oleic Acid IC₅₀'s

An IC₅₀ was calculated for each combination of oleic acid and L-FABP sample to assess the functionality of L-FABP from FC1-treated and non-treated rats and guinea pigs. Oleic acid, 1mM in 10% methanol, was titrated into 2 ml 1μM L-FABP and 1μM DAUDA. FI (cpm), due the binding of DAUDA to protein, was measured following each addition. Curves of percent inhibition of specific DAUDA binding versus oleic acid concentration were constructed (Figure 9). Table 6 shows the percent inhibition of specific DAUDA binding by 2μM oleic acid and the IC₅₀ values for each L-FABP sample. As discussed in the introduction, oleic acid is capable of binding both primary and secondary L-FABP binding sites; and once a ligand has bound the primary site, binding to the secondary site is facilitated (Thompson *et al.*, 1997). This relatively unconstrained binding of oleic acid to L-FABP is reflected in a sharp decrease in FI (cpm) and an increase in DAUDA-specific binding inhibition upon the addition of micro-molar quantities of oleic acid to solutions of L-FABP and DAUDA.

Ninety one percent of specific DAUDA binding to rat control L-FABP was inhibited by 2μM oleic acid; and the IC₅₀ of oleic acid for the rat control L-FABP sample was 0.01μM. This percent inhibition was higher than that found by Thumser *et al.* (1994b), Thumser *et al.* (1996) and Thumser and Wilton (1995), of 80.5%, 76% and 80% respectively, under similar conditions. The percent inhibition was much lower (52%) and IC₅₀ much higher (0.5μM) for the rat FC1 L-FABP sample as compared to the rat control L-FABP sample. This suggests the ability of L-FABP from the FC1-treated rat to bind oleic acid was decreased. One explanation for this decrease in functionality, is that FC1 was bound to L-

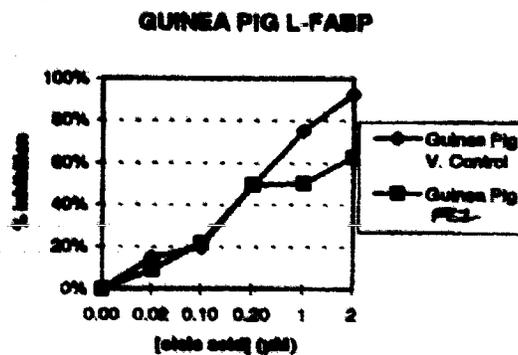
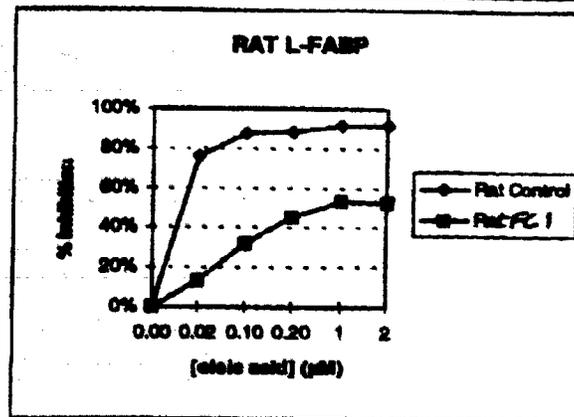
FABP in the FCI treated sample, rendering fewer sites available to bind oleic acid and requiring more oleic acid to achieve 50% inhibition. This agrees with the results presented for the analysis of average maximum FI, specific DAUDA binding and DAUDA binding constants in rat L-FABP samples.

The percent inhibition of specific DAUDA binding to the guinea pig vehicle control L-FABP sample upon addition of $2\mu\text{M}$ oleic acid was 93%; that for the guinea pig FCI sample was 63%. This is similar to the results for the rat samples; however, the curves of percent inhibition versus oleic acid concentration for rat and guinea pig samples were very different (Figure 9). The curves for rat samples were hyperbolic while the curves for guinea pig samples were more linear and scattered. The resulting IC_{50} values for both guinea pig L-FABP samples were the same, $0.2\mu\text{M}$ oleic acid. The data are ambiguous, and are likely due to the crudeness of the guinea pig samples.

TABLE 6 - OLEIC ACID IC_{50}'S & PERCENT INHIBITION OF SPECIFIC DAUDA BINDING.		
The IC_{50} of oleic acid for each L-FABP sample was calculated by adding oleic acid, 1mM in 10% methanol, to a solution of $1\mu\text{M}$ L-FABP and $1\mu\text{M}$ DAUDA. Values are IC_{50} 's of oleic acid for each L-FABP sample and percent inhibition of specific DAUDA binding by $2\mu\text{M}$ oleic acid.		
L-FABP Sample	Oleic Acid IC_{50} (μM)	% Inhibition by $2\mu\text{M}$ oleic acid
Rat Control	0.01	91
Rat FCI	0.5	52
Guinea Pig Vehicle Control	0.2	93
Guinea Pig FCI	0.2	63

FIGURE 9- % DAUDA INHIBITION VS [OLEIC ACID].

Oleic acid, 1mM in 10% methanol, was added to a solution of 1 μ M L-FABP and 1 μ M DAUDA. Curves represent an average of 3-6 trials corrected for the effect of methanol.

**ANALYSIS OF THE POTENCY OF FCs FOR BINDING TO L-FABP****Competitive Binding Experiments - Calculation of IC_{50} 's**

An IC_{50} of each FC, WY and methanol was calculated for DAUDA binding to the rat control L-FABP sample. Each ligand (1mM, in 100% methanol or 50mM KH_2PO_4 , pH 7.2) was titrated into 2ml 1 μ M rat control L-FABP and 1 μ M DAUDA. The addition of

competitor to L-FABP and DAUDA induced a shift of emission wavelength back towards the curve of DAUDA with no L-FABP (Figure 10). This is indicative of DAUDA being displaced from a nonpolar binding site on L-FABP, such as the secondary binding site. To varying degrees, similar shifting and decreases in FI occurred upon addition of each competitor. FI (cpm), due the binding of DAUDA to protein, was measured following each addition of competitor, and curves of percent inhibition of specific DAUDA binding versus ligand concentration constructed (Figure 11). Table 8 gives the percent inhibition of specific DAUDA binding by 10 μ M competitor and the IC₅₀ values for each competitor.

FC1 was the strongest inhibitor of specific DAUDA binding, with 69% inhibition upon the addition of 10 μ M and an IC₅₀ of 4.9 μ M. FC4 was the next strongest, with 51% inhibition upon the addition of 10 μ M and an IC₅₀ of 9.7 μ M. Wyeth followed FC4, with 50% inhibition upon the addition of 10 μ M, and an IC₅₀ of 10 μ M. FC3 and FC2 both inhibited 43% of specific DAUDA binding upon the addition of 10 μ M and had IC₅₀'s greater than 10 μ M.

Thumser and Wilton (1996) suggest that ligands for L-FABP need to have both a hydrophobic and a hydrophilic domain. Each FC and WY fits this description to varying degrees. Due to the rigid tail of the FCs and the bulky structure of WY, it is likely that binding of each of these competitors to L-FABP occurs in the secondary binding site on L-FABP. It is conceivable that the FCs bind with their rigid hydrophobic CF tails, analogous to the flexible CH tail of oleic acid, in the secondary binding site and that the polar head groups of FCs are solvent exposed as is the carboxylate group on oleic acid.

To reason by structure, the varying potencies of each FC to binding L-FABP is difficult. Thompson *et al.* (1997) suggest that anything larger than a C₁₄ molecule may require prior binding in the primary site. As was mentioned in the introduction, C₈ and C₁₀ FCs are the most potent peroxisome proliferators. It may be the case that C₈ and C₁₀ molecules bind most readily to the secondary binding site on L-FABP, not requiring a molecule to be bound in the primary binding site. All of the FCs examined in the present study were C₈ molecules; thus, an analysis of chain length cannot be undertaken at this time. The disparate potencies of binding L-FABP between FCs do not appear to be due to differences in polarity and hydrogen bonding ability. For example, FC2, having a very polar carboxylate head group, and capable of extensive hydrogen bonding, has a higher IC₅₀ than FC4, which has a less polar head group and less ability to hydrogen bond (see Figure 1).

The binding of WY to the secondary binding site on L-FABP is difficult to reason. In place of the hydrophobic tail, present on FCs and oleic acid, WY has two aromatic rings. The conformational flexibility of L-FABP may accommodate this bulky structure internally, and position the carboxylate externally, in a solvent exposed manner able to participate in hydrogen bonding. Thompson *et al.* (1997) also speculate that a third binding site, located completely on the exterior of L-FABP, may exist. Only the first few carbons of a molecule binding to such a site would be bound in an organized fashion, the remaining portion of the ligand would be disordered. This, although highly speculative, may be the site of WY binding to L-FABP.

Methanol was chosen as the negative control because a non-peroxisome proliferator, similar in structure to the FCs being researched, has not been identified. Upon the addition of 10 μ M methanol, 21% of specific DAUDA binding was inhibited. The IC₅₀ of methanol was much greater than 10 μ M. It is reasonable to suggest methanol is capable of weakly binding the secondary L-FABP binding site, with the CH₃ group internally located and the OH group exposed to the exterior of the protein. The structure of methanol renders it an unlikely candidate for looping into a U-shape and participating in primary site binding.

FIGURE 10 - CHROMATOGRAM.

The addition of competitor to L-FABP and DAUDA induced a shift of emission wavelength back towards the curve of DAUDA with no L-FABP. This is indicative of DAUDA being displaced from a nonpolar binding site on L-FABP. To varying degrees, similar shifting and decreases in FI occurred upon addition of each competitor. The example shown is for FC2 binding rat control L-FABP.

KEY : a = 1 μ M DAUDA, 1 μ M L-FABP; b = 1 μ M DAUDA, 1 L-FABP, 10 μ M FC2; c = 1 μ M DAUDA, 1 μ M L-FABP, 15 μ M FC2; d = 1 μ M DAUDA, 1 μ M L-FABP, 20 μ M FC2; e = 1 μ M DAUDA; f = 0 DAUDA, 0 L-FABP, 30mM K₂HPO₄.

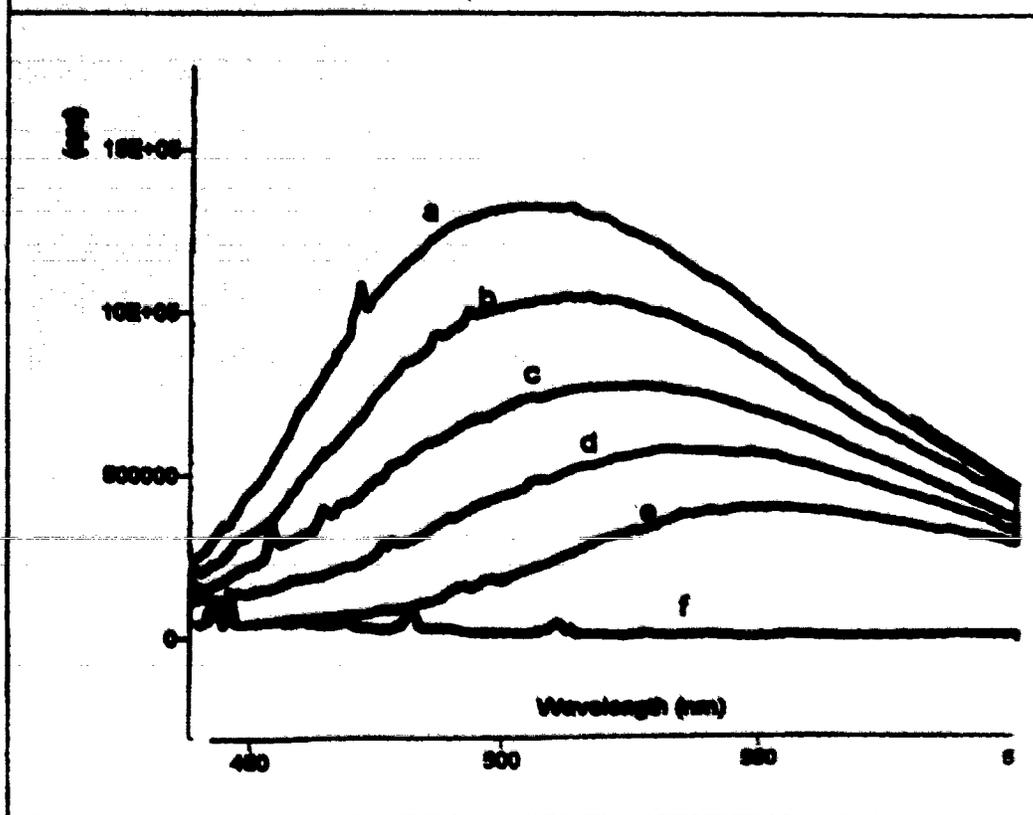
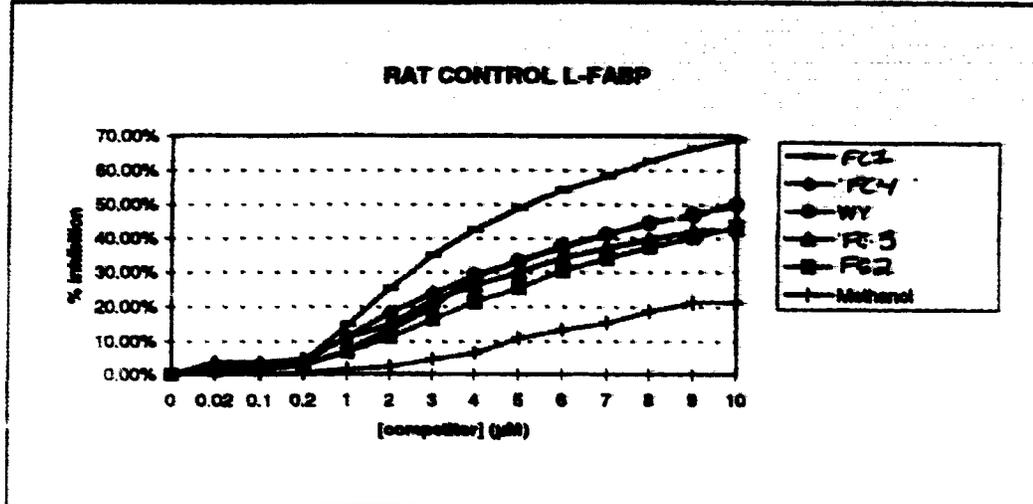


FIGURE 11 - % INHIBITION OF SPECIFIC DAUDA BINDING VS [COMPETITOR].

Each competitor, 1mM, was added to 1 μ M L-FABP and 1 μ M DAUDA. Measurements of FI were made upon each addition. Curves represent an average of 3-6 trials corrected for the effect of methanol.

**TABLE 8 - IC₅₀'S OF FC'S AND WY & PERCENT INHIBITION OF SPECIFIC DAUDA BINDING.**

The IC₅₀ of each competitor for the rat L-FABP sample was calculated by adding competitor (1mM) to 1 μ M L-FABP and 1 μ M DAUDA. Values are the IC₅₀'s of each competitor and % inhibition of specific DAUDA binding by 10 μ M of each competitor.

COMPETITOR	IC ₅₀ (µM)	% INHIBITION
FC1	4.9	69
FC4	9.7	51
WY	10	50
FC3	>10	43
FC2	>10	43
METHANOL	>10	21

CONCLUSIONS & FUTURE DIRECTIONS

This study was designed to investigate the hypothesis that certain Fluorocarbons (FCs) bind to liver fatty acid-binding protein (L-FABP) and displace endogenous fatty acids (FAs) as an initial event in peroxisome proliferation. To examine this hypothesis, the kinetics of FA and FC binding to L-FABP were investigated using DAUDA, a fluorescently labeled octanoic acid analogue. L-FABP from male rats (considered to be strong responders to peroxisome proliferators) and male guinea pigs (considered to be weak or non-responders to peroxisome proliferators) (Svoboda *et al.*, 1967; Orton *et al.*, 1984; Lake and Gray, 1985; Elcombe and Mitchell, 1986) were examined.

The first goal was to assess the effect of FCs on L-FABP function as evaluated by the ability of DAUDA to bind to L-FABP isolated from rats and guinea pigs treated and not treated with FC1 *in vivo*. Results show a decreased maximum binding capacity of L-FABP from FC1-treated rats without an increase in K_d . This was demonstrated by a lower B_{max} , higher oleic acid IC_{50} and unchanged K_d in L-FABP from rats treated with FC1 as compared to samples from control rats. These results are likely due to FC1 binding to the secondary binding site on L-FABP; thus preventing the binding of DAUDA to L-FABP. Results for guinea pig L-FABP samples were inconclusive. This is presumably because guinea pig samples were only partially purified, resulting in a high degree of interference from remaining cellular debris and a lower concentration of L-FABP, as a proportion of total protein, as compared to the more highly purified rat samples. A second attempt will be made to analyze the difference in response to FCs seen in rodent and guinea pig L-FABP. This analysis will be performed on fully purified L-FABP samples.

The second goal of the study was to assess the potency of the various FCs for binding to L-FABP, as suggested by IC_{50} values. Results indicate the most potent L-FABP binder is FC1, followed by FC4, WY and (with equal IC_{50} s) FC3 and FC2. Binding of FCs to L-FABP likely occurs in the secondary binding site; and the variance in potency is likely due to structural differences.

Future work will focus on correlating new data with the results of this study, and relating the effects seen in rodents and guinea pigs to the relevance they pose to human health. The possibility of completing a new set of kinetics assays using a probe capable of binding both L-FABP binding sites will be looked into. This would help characterize the interaction of DAUDA with L-FABP and create a new set of results for analysis. Human L-FABP will be examined, and the relevance to human health of FC induced peroxisome proliferation in rodents will be investigated. FCs of different chain length will be investigated, and the effect of chain length on ability to bind L-FABP analyzed. The ability of each FC to induce peroxisome proliferation in rodent L-FABP will be assessed using the method of Lazarow and de Duve (1976). An attempt will be made to correlate this capacity with the relative potency of each FC to bind L-FABP, as determined in the current study. Electro-Spray Mass Spectrometry analysis on the same L-FABP samples as analyzed in this study is currently underway. The objective is to quantify FC1 bound to L-FABP from FC1 treated animals, correlate this with the decrease in L-FABP capacity observed in this study and with the ability of FC1 to induce peroxisome proliferation in rodents. The long range goal is to refine the correlation between the amount of FC bound to L-FABP with peroxisome

proliferation, and develop a biomarker for peroxisome proliferation based on bound FC levels. This biomarker would be used as a method of screening FCs for their ability to induce peroxisome proliferation.

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Mar 17 1981
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TOXICOLOGY

28 Day Percutaneous Absorption Study
with FC-98
in Albino Rabbits

Experiment No.:

0979AB0630

Conducted At:

Safety Evaluation Laboratory
Riker Laboratories, Inc.
St. Paul, Minnesota

Dates Conducted:

October 25, 1979 to December 17, 1979

Conducted By:

K. D. O'Malley 3/11/81
K. D. O'Malley, BS Date
Advanced Toxicologist
Study Director

Reviewed By:

K. L. Ebbens 7/15/81
K. L. Ebbens, BS Date
Supervisor, Acute Toxicology

- dc: M. T. Case
- K. L. Ebbens
- F. D. Griffith
- W. C. McCormick

Summary

A 28 day percutaneous absorption study with FC-98 was conducted from October 25, 1979 to December 17, 1979 at Riker Laboratories, Inc., St. Paul, Minnesota using male and female albino rabbits ranging in body weights from 1.88 to 2.30 kg. The test article was administered by dermal application to ten male and ten female rabbits each a dosage level of 5,000 mg/kg body weight for a 24 hour exposure period.^a No mortalities, untoward behavioral reactions or body weight losses were noted during the 28 day study. Necropsies were performed on all animals upon termination of the study with no visible lesions noted. Preliminary serum analysis (see Appendix V) indicates dermal absorption of FC-98 in albino rabbits, however, due to the limited number of samples analyzed, no concrete conclusion may be drawn.

Introduction

The objective of this study^b was to determine the percutaneous absorption potential of FC-98 in male and female albino rabbits. The study, which was initiated at Riker Laboratories, Inc., St. Paul, Minnesota on October 25, 1979 and completed on December 17, 1979, was not conducted to support a government submission or marketing permit and is therefore not regulated by the Good Laboratory Practice Regulation of 1978. The raw data generated by the Study Director and the final report are stored in the conducting laboratory's archives.

^a A preliminary rangefinder study was conducted to determine the appropriate dosage level to be used in this study.
^b Riker Toxicity Experiment No.: 0979AB0630, Test Method 699

Method

Young adult albino rabbits of the New Zealand breed^a were used in this test. All animals were held under quarantine for several days prior to testing with only animals which appeared to be in good health and suitable as test animals at the initiation of the study used. The rabbits were housed individually in stainless steel, wire-bottomed cages and maintained on a standard laboratory ration^b with food and water available ad libitum.

An initial rangefinding study was conducted using two male and two female rabbits for each dosage level. The trunk of each animal was clipped free of hair and the test article placed on the surface of the intact skin which covered approximately 40% total body surface area. After administration of the test article, a flexible plastic collar was fitted on each animal and the trunk wrapped with impervious plastic sheeting which will occlude the test article. The animals were returned to their cages for a 24 hour period after which time the test article was removed from the dermal surface of the animals. The animals were observed for pharmacotoxic reactions both during the exposure period (immediately post dose administration, one and two hours) and after removal of the test article (daily for 14 days following dose administration) with all reactions recorded (Table 3). Initial and final body weights were also recorded (Table 1).

The information derived from the initial rangefinder was used in determining the dosage level for the 28 day percutaneous study. Preparation of 10 male and 10 female animals for dosing and application of the test article were conducted in the same manner as the rangefinder study with the exception of the collection of blood samples from the orbital sinus plexus prior to application and again on days 1, 7, 14 and 28 after initiation of the study for serum which was frozen for sponsor analysis. After the 24 hour exposure

^a Pel Freez, Inc., Rogers, AR

^b Purina Rabbit Chow, Kalston Purina, St. Louis, MO

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period the test article was removed from the dermal surface of the animals and the animals returned to their cages for the following 28 days. Initial, 7, 14 and 28 day body weights were recorded (Table 2) as were any pharmacotoxic signs noted during the 28 day observation period (Table 4). A gross necropsy was conducted on all animals sacrificed on day 28 and all findings recorded (Table 2). The protocol, principal personnel involved in the study, composition characteristics, and Quality Assurance statement are contained in Appendices I - IV.

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TABLE I

4.

ACUTE DERMAL RANGEFINDER TOXICITY STUDY - ALBINO RABBITS
with FC-98
Mortality and Body Weight Data

Dose ^a (mg/kg)	Sex	Animal Number	Individual Body Weights (kg)		Number Dead Number Tested	Percent Dead
			Test Day Number 0	14		
5000	M	9B2599	2.37	2.53	0/4	0
	M	9B2602	2.41	2.62		
	F	9B2617	2.42	2.31		
	F	9B2614	2.15	2.52		
1000	M	9B2605	2.22	2.51	0/4	0
	M	9B2608	2.47	2.55		
	F	9B2618	2.25	2.16		
	F	9B2621	2.35	2.45		

^a Test article was dosed as a suspension in water

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TABLE 2

5.

ACUTE PERCUTANEOUS ABSORPTION TOXICITY STUDY - ALBINO RABBITS

with PC-98

Mortality and Body Weight Data

Dose ^a (mg/kg)	Sex	Animal Number	Individual Body Weights (kg)				Number Dead Number Tested	Percent Dead
			Test Day Number					
			0	7	14	28		
5000	M	9B3041	2.16	2.58	2.47	2.63	0/10	10
	M	9B3047	2.30	2.03	2.27	2.68		
	M	9B3053	2.16	2.13	2.45	2.79		
	M	9B3059	2.15	2.10	2.33	2.54		
	M	9B3049	2.30	2.15	2.47	2.79		
	M	9B3049	2.11	1.96	2.11	2.37		
	M	9B3055	2.19	2.22	2.47	2.75		
	M	9B3061	2.18	2.22	2.31	2.46		
	M	9B3045	2.30	2.33	2.52	2.79		
	M	9B3051	2.22	2.15	2.38	2.70		
5000	F	9B2921	2.25	2.03	2.25	2.66	1/10	10
	F	9B2927	2.12	2.00	2.29	2.78		
	F	9B2933	2.18	1.87	2.01	2.37		
	F	9B2939	1.99	1.72	2.09	2.42		
	F	9B2923	2.08	1.99	2.30	2.65		
	F	9B2929	1.88	1.72	1.90	2.21		
	F	9B2935	2.11	2.14	2.33	2.85		
	F	9B2941	2.02	2.13	2.25	2.48		
	F	9B2925	2.12	1.98	1.70	<u>b</u>		
	F	9B2931	1.94	1.94	2.14	2.37		

^a Test article was dosed as a suspension in water

^b Animal sacrificed on day 14 due to accidental back injury inflicted during the day 7 bleeding period

Necropsy

Necropsies performed on all animals upon termination of the study revealed no visible lesions. Animal 9B2925 which was sacrificed on day 14 due to posterior paralysis, also revealed no visible lesions.

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TABLE 3
 ACUTE DERMAL RANGEFINDER TOXICITY STUDY - ALBINO RABBITS
 with PC-98

Summary of Reactions

Dose (mg/kg)	Sex	Reaction	Number Affected Number Dosed	Time of Onset Following Dose Administration	Cessation of Reaction Following Dose Administration	Time of Death Following Dose
5000	M	No significant reactions noted		----	----	----
	F	No significant reactions noted		----	----	----
1000	M	No significant reactions noted		----	----	----
	F	No significant reactions noted		----	----	----

TABLE 4
 ACUTE PERCUTANEOUS TOXICITY STUDY - ALBINO BABBITS
 with PC-98

Summary of Reactions

Dose (mg/kg)	Sex	Reaction	Number Affected Number Dosed	Time of Onset Following Dose Administration	Cessation of Reaction Following Dose Administration	Time of Death Following Dose
5000	M	No significant reactions noted		----	----	----
	F	No significant reactions noted		----	----	----

Riker Experiment No.: 04-14H30630APPENDIX I
METHOD:

8.

TEST: Single Dose 28 Day Percutaneous Absorption Study
 SPONSOR: 3M Commercial Chemicals Division
 CONDUCTED BY: Safety Evaluation Laboratory, Riker Laboratories, Inc., St. Paul, Minnesota
 TEST ARTICLE: FC-98 Lot 315
 CONTROL ARTICLE: N/A
 PROPOSED STARTING/COMPLETION DATE OF STUDY: 11-79 / 1-80
 TEST SYSTEM AND SOURCE: New Zealand White Albino Rabbits Sex: M+F
Pei Freuz, Inc., Rogers, Arkansas Number: 10-10
 Weight Range: 2-5 kg

OBJECTIVE: The objective of this study will be to determine the percutaneous absorption potential of the test article in albino rabbits. Rabbits were selected as the test system for their historical use in dermal absorption studies, ease of handling and general availability.

METHOD: The animals, selected from a larger colony by health and body weight, will be randomly housed in standard wire-mesh cages in temperature and humidity controlled rooms with food^a and water offered ad libitum. Each animal will be assigned a numbered ear tag, which will correspond to a card affixed to the outside of the cage. The trunk of each animal will be clipped free of hair and the test article applied as a single dosage of 5,000 mg/kg to intact skin covering approximately 10% total body surface area. A flexible plastic collar^b will be fitted on each animal and the trunk wrapped with impervious plastic sheeting, which will occlude the test article. The animals will then be returned to their cages for a 24 hour exposure period after which the test article will be removed. Prior to the application, blood samples will be collected from the orbital sinus plexus and again on days 1, 7, 14, and 28 after initiation of the study for serum which will be frozen for sponsor analysis. A gross necropsy will be conducted on all animals which may die during the conduct of the study as well as all animals sacrificed on day 28. All gross findings will be recorded and tissue samples of liver, spleen, brain, kidney and bone marrow (sternum) will be fixed in 10% buffered formalin for possible future microscopic examination. Initial, 7, 14, and 28 day body weights will be recorded as well as any pharmacotoxic signs noted during the conduct of the study. All raw data, other than the blood analysis data which will be the responsibility of the sponsor, and the final report will be stored in the Riker Laboratory's Archives, St. Paul, Minnesota.

^a Purina Rabbit Chow, Ralston Purina, St. Louis, Missouri

^b The collar will be worn for the duration of the study to reduce oral ingestion of residual test article.

W. C. McCormick, Inc. 11/79
Sponsor Date

W. O. Malley 11/79
Study Director Date

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TEST: Acute Dermal Toxicity Manufacturing Study
SPONSOR: JM Commercial Chemical
CONDUCTED BY: Safety Evaluation Laboratory, Riker Laboratories, Inc., St. Paul, Minne Divi
TEST ARTICLE: FC-98
CONTROL ARTICLE: N/A
PROPOSED STARTING/COMPLETION DATE OF STUDY: 11/19 - 11/20
TEST SYSTEM AND SOURCE: New Zealand White Albino Rabbits Sex: ♀
Pul-Fruuz, Inc., Rogers, Arkansas Number: 212
Body Weight Range: 2.5-3.0

OBJECTIVE: The objective of this study will be to approximate the acute dermal toxicity of the test article in albino rabbits. Rabbits were selected as the test system for their sensitivity of response, historical data, ease of handling and general availability.

METHOD: The animals, selected from a larger colony by health and weight, will be randomly housed in standard wire-mesh cages in temperature and humidity controlled rooms with food^a and water offered ad libitum. Each animal will be assigned a numbered ear tag, which will correspond to a card affixed to the outside of the cage. The trunk of each animal will be clipped free of hair and the test article placed on the surface of the intact skin at single dosages of 500, 2000, 1000 mg/kg, however, if these dosage levels do not adequately characterize the toxicity of the test article, additional animals will be administered the test article at supplemental dosage levels. Any additional dosage levels will be documented and filed with this protocol. The test article will be administered to the animals in the form received from the sponsor. After administration of the test article, a flexible plastic collar^b will be fitted on each animal and the trunk wrapped with impervious plastic sheeting which will occlude the test article. The animals will be returned to their cages for a 24 hour exposure period after which time the test article will be removed from the dermal surface of the animal. The animals will be observed for pharmacotoxic reactions both during the exposure period (immediately post dose administration, one and two hours) and after removal of the test article (daily for 14 days following dose administration) with all reactions being recorded. Initial and final body weights will also be recorded. The acute median lethal dose (LD50) of the test article will be approximated. All raw data and the final report will be stored in the Riker Laboratories Archives, St. Paul, Minnesota.

^a Purina Rabbit Chow, Ralston Purina, St. Louis, Missouri
^b The collar will be worn for the duration of the study to reduce oral ingestion of residual test article.

N/A
Sponsor _____ Date _____

KO [Signature]
Study Director _____

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1. Due to the unavailability of rabbits, the 2,000 mg/kg
change group will be omitted on the remaining studies

K.D. Malley 10/24/79
Study Director Date

2. The 1-minute, 15 and 30-minute observations were deleted
on the remaining studies due to the type of study

K.D. Malley 10/20/79
Study Director Date

3. The studies identified ~~with~~ corresponding numbers for the body
in this study will be changed to K.D. Malley (00)
(001) KI studies

K.D. Malley 11/12/79
Study Director Date

4. The weight range was expanded to 1.8-30 kg in order
to initiate the study

K.D. Malley 11/20/79
Study Director Date

5. Due to a delay in report processing and sponsor
analysis the completion date is extended to
3/78

K.D. Malley 7/16/79
Study Director Date

6.

Study Director Date

7.

Study Director Date

8.

Study Director Date

APPENDIX II

Principal Participating Personnel Involved in the Study

<u>Name</u>	<u>Function</u>
K. I. Ebbens, BS	Supervisor, Acute Toxicology
K. D. O'Malley, BS	Advanced Toxicologist Study Director
Dr. V. Pothapragada	Commercial Chemicals Chemist
G. C. Pecore	Supervisor Animal Laboratory

APPENDIX III

Composition Characteristics

This study is not regulated by the Good Laboratory Practice Regulation of 1978 and therefore information pertaining to composition characteristics is not applicable for inclusion in this study.

APPENDIX IVQuality Assurance Statement

This study is not regulated by the Good Laboratory Practice Regulation of 1978 and therefore a statement signed and prepared by the Quality Assurance group is not applicable. This study was, however, audited by the Quality Assurance group.

In addition to the data audit, different significant phases for studies underway in the Toxicology Laboratory are inspected weekly on a recurring cycle, and the facilities are examined by Laboratory Quality Assurance on a three month schedule.

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D. R. Ricker

F. D. Griffith - 220-2E

F. A. Ubel - 220-2E

APPENDIX 5

K. L. EBBENS - RIKER SAFETY EVALUATION LAB - 203-1

W. C. McCORMICK - MEDICAL DEPT. - TOXICOLOGY SERVICES - 220-2E

SKIN ABSORPTION STUDIES ON FC-143, FC-95, FC-90, FC-134, FC-135, FC-128, FC-129 and FC-98
JUNE 27, 1980



Please consider this an authorization for your laboratory to release the dermal toxicity/skin absorption studies conducted on the above mentioned compounds.

It is understood that the studies are being issued in an incomplete form insofar as the fluorochemical analysis of the serum samples have not been completed and will not be included in the report. Preliminary serum sample analysis indicates absorption of the compounds. The serum data analysis are not sufficient enough to draw any concrete conclusions concerning comparative toxicity. However, the animal data you have generated addresses this matter in a broader context. It is not certain when the remaining samples will be analyzed and their completion should not hold up your report any longer.

Thank you for your patience in this matter.

W. C. McCormick

WCM:kjh

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~~CONFIDENTIAL~~
 K. A. Prokop - 236-28
 L. D. Winter - 236-28

APPENDIX 3 (Concluded)

COMMERCIAL CHEMICALS DIVISION
 ANALYTICAL LAB REPORT #146

To: W. C. MCCORMICK - 220-2E-02
 From: V. POTHAPRAGADA AND V. BUNNELLE - 236-3A
 Subject: NIKEA SKIN ABSORPTION STUDY
 Date: June 9, 1980



Reference: Commercial Chemicals Division Analytical Request #13669

For lack of time, only a selected set of serum samples was analysed.

Compound	TOTAL F. ppm					
	Females			Males		
	Day 1	Day 28	Day 1	Day 28	Day 1	Day 28
PC-129	26.1	69.6	11.4	23.3		
PC-134	0.2	18.1	18.8	23.9		
PC-138	4.4	16.5	1.6	10.5		
PC-98	226.4	93.1	271.9	94.3		
PC-135	6.9	20.8	2.3	7.6		
PC-95	0.9	128.0	10.3	130.2		
PC-99	42.5	111.5	129.1	73.5		
	22.2	129.6	72.7	66.6		

Compound	Females						Males		
	Females			Males			Males		
	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28	Day 7	Day 14	Day 28
PC-143	10.1	12.1	3.5	5.4	6.8	6.0			

Method of Analysis: Oxygen Bomb/CC: Technique (See Be-Hite and D. F. Nagel, Anal. Biochem; 87, 543, 1978).

V. A. Bunnelle
 V. A. Bunnelle
 VAB/hc

V. Pothapragada
 V. Pothapragada

Read and Reviewed
 by L. D. Winter
L. D. Winter

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