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AD-A224 766

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: UNCLASSIFIED	1b. RESTRICTIVE MARKINGS: N/A
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S): AFOSR-TR- 90 0665
6a. NAME OF PERFORMING ORGANIZATION: Indiana University	6b. OFFICE SYMBOL (If applicable):
6c. ADDRESS (City, State, and ZIP Code): 4601 Central Avenue, Columbus IN 47203	7a. NAME OF MONITORING ORGANIZATION: AFOSR
	7b. ADDRESS (City, State, and ZIP Code): Building 410, Bolling AFB DC 20332-6448
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: AFOSR	8b. OFFICE SYMBOL (If applicable): NL
8c. ADDRESS (City, State, and ZIP Code): Building 410, Bolling AFB DC 20332-6448	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: AFOSR 89-0077
	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 61102F; PROJECT NO. 2312; TASK NO A5; WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification): Two-dimensional electrophoretic analysis of subcellular liver fractions and isolated hepatocytes from normal and PFDA treated rats

12. PERSONAL AUTHOR(S): Frank A. Witzmann, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 12/15/88 TO 12/14/89	May 28, 1990	19

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	perfluoro-n-decanoic acid; two-dimensional electrophoresis; hepatotoxicity; cell fractions; liver

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Perfluoro-n-decanoic acid (PFDA) effects on protein expression in the rat liver was studied following in vivo exposure to PFDA's LD50 and a lesser dose. Two-dimensional protein patterns were resolved on polyacrylamide gels for whole liver homogenate, mitochondrial, microsomal, cytosolic, and isolated hepatocyte protein fractions. PFDA exposure caused the deletion and induction of numerous proteins often in a dose related manner. Several proteins have been associated with those involved in lipid metabolism alterations characteristically induced by PFDA toxicity. Their actual identification awaits further study. The results of this project indicate that PFDA has significant effect on the quality and quantity of specific hepatocellular protein expression but whether these alterations are primary or secondary PFDA manifestations remains to be determined.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Lt. Col. T.J. Cerveny	22b. TELEPHONE (Include Area Code): 202-767-5021 / 22c. OFFICE SYMBOL: NL

DD Form 1473, JUN 86 — Previous editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

UNCLASSIFIED

9 MAY 1990

Report AFOSR-89-0077

AFOSR-TR- 90 0665

TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS OF SUBCELLULAR LIVER FRACTIONS AND ISOLATED HEPATOCYTES FROM NORMAL AND PFDA TREATED RATS

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28 May 1990

Final Report for Period 15 December 1988 - 14 December 1989
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INTRODUCTION

Perfluorocarboxylic acids and other structurally related compounds have been used commercially for their surfactant properties. One such compound in particular, perfluoro-n-decanoic acid (PFDA), has been extensively studied after it was found to elicit acute hepatotoxic effects after a single dose in rodents (Anderson, 1981). In its principle target, the liver, PFDA has been shown to induce hepatomegaly (George and Andersen, 1986) associated with lipid accumulation, hypophagia associated with body weight loss (Olson and Andersen, 1983), peroxisome proliferation (Harrison, 1988; Ikeda, 1985; Van Rafelghem, 1987), and various enzymic alterations (Harrison, 1988; Ikeda 1985; Kelling, 1987; Van Rafelghem, 1985; Van Rafelgehm and Andersen, 1988). Those enzymes involved with peroxisomal, microsomal, and cytosolic lipid metabolism appear to be most affected by PFDA.

For example, Van Rafelghem (1988) observed a significantly elevated accumulation of esterified compounds such as triacylglycerols in the carcasses of PFDA-treated rats due to a shift from lipid oxidation to esterification in the liver. In similary exposed rats, the hepatic ratio of saturated to unsaturated fatty acids increased significantly as did absolute levels of total fatty acids (George and Andersen, 1986). Despite the significant hepatomegaly observed in that study, liver protein decreased as a consequence of PFDA intoxication. This decline in protein concentration was not associated with a quantitative change in microsomal protein implying that qualitative changes occur. It appears that PFDA intoxication impairs microsomal electron transport perhaps as a result of decreased cytochrome b5 activity (Van Rafelghem and Andersen, 1988). Oddly, cytochrome P-450 concentration per mg microsomal protein has not been shown to be affected by PFDA despite a twenty- to

fortyfold increase in the activity of peroxisomal fatty acyl-CoA oxidase (Harrison, 1988).

Whether these changes in enzyme activity are due to primary effects of PFDA on quantitative protein expression, qualitative protein expression, or composition of the membranes in which these enzymes are embedded remains to be determined. Perhaps these effects are merely secondary to some singular PFDA effect on lipid metabolism. A better understanding of the mechanisms whereby PFDA and similar chemicals exert their hepatotoxicity can be gained by studying the proteins expressed by the hepatocytes under varying conditions through examination of protein patterns generated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) . Recently, similar projects were successfully carried out that examined the effect of several chemical toxicants on hepatic protein expression in whole homogenates and cell fractions (Anderson, 1985) as well as PFDA toxicity in a lymphoma cell-line (Witzmann, 1990).

The present study was proposed and undertaken to extend recent studies of PFDA's hepatotoxicity conducted at AFAMRL/THB by examining the effect of PFDA on qualitative and quantitative protein expression and/or post-translational activities in the whole homogenates of liver cells and in specific hepatic subcellular fractions. At the outset, no specific protein species was singled out for study although it was anticipated that those enzymes in both microsomal and mitochondrial fractions figuring prominently in lipid metabolism, the peroxisomal enzymes, the cytochrome p-450's, and other P-450 dependent enzymes would be likely candidates for PFDA-induced alteration.

The actual primary and secondary effects of PFDA in the liver have not been well clarified nor conclusively determined. Furthermore, doses well below the LD50 of 50mg/kg in Fischer 344 rats have poorly defined hepatotoxic effects. A sensitive yet broadly based tool for detection and identification of those cellular components responsible for the entry, binding, or metabolism or all

three, i.e. the cellular protein population, was warranted. The proposed method, 2D-PAGE, involves the separation of thousands of proteins contained in a complex cellular mixture in a polyacrylamide gel matrix. First described by O'Farrell (1975) and refined by the Andersons (Anderson and Anderson, 1978), 2D-PAGE first separates proteins on the basis of their individual charge (isoelectric focusing, IEF) and then on the basis of molecular weight (sodium dodecyl sulfate PAGE). The proteins separated in this way assume reproducible and predictable x,y coordinate positions on a slab gel and can be identified by any of a number of visualization techniques. It is both natural and valid to assume that alterations in such a protein pattern induced by toxic exposure can be viewed as important initial features of the chemical toxicity as well as secondary consequences of cell injury and death.

OBJECTIVES

The primary aim of this one-year project was the generation of high resolution 2D gels on which hepatocyte proteins of various fractions have been separated and appropriately stained. These gels were then to be visually inspected and the PFDA-induced alterations noted. It was also our aim to compare patterns from subcellular fractions with those derived from whole homogenates and isolated hepatocytes in both treated and control samples.

MATERIALS AND METHODS

PFDA Treatment and Tissue Preparation. PFDA exposure and tissue preparation were carried out in the Biochemistry Branch Laboratories, Toxic Hazards Division of AFAMRL at Wright-Patterson AFB. Male Fischer-344 rats (200-250g) were obtained from Charles River Breeding Labs, housed individually in metabolic containment units and maintained on rat chow and water ad libitum until the experimental groups were injected intraperitoneally with either 20 or

50mg PFDA/kg. Twenty-four hours later, control rats were injected with the propylene glycol vehicle. One control rat was matched with each PFDA-treated rat for pair feeding. The food consumption of each PFDA-treated rat was monitored daily and the same amount given to the matched control on the following day. Rats were euthanized after 8 days.

Whole homogenates of rat liver samples were prepared for electrophoresis by excising the entire liver from the PFDA-treated and control rats and selecting the same specific region or lobe for sampling from each animal. A suitable piece was then homogenized in 8 volumes of a lysis buffer containing 9M urea, 2% NP-40 detergent, 2% mecaptoethanol, and 2% ampholytes (pH 9-11) at a final pH of 9.5 (Anderson, 1988). After solubilization at room temperature the samples were centrifuged at 15,000g for 15 min and the supernates stored at -70 °C. A second piece of liver was homogenized and subjected to differential centrifugation to obtain mitochondrial (Chappall and Hansford, 1972), microsomal (Tata, 1972), and cytosolic (Chappall and Hansford, 1972) subcellular fractions. These fractions were then solubilized in an appropriate volume of lysis buffer and stored at -70 °C until electrophoresis.

Two-dimensional Electrophoresis. Protein separation was accomplished using the ISO-DALT System (Hoefer Sci. Instr.) and the large scale approach described by Anderson (1988). First dimension ISO gels (23cm x 1.5mm dia.) containing 4% acrylamide, 2% NP-40, 9M urea, and 2% ampholyte (1.2% pH2-11; 0.8% pH5-7) were loaded with protein sample and focused for 30,000 V-hr. After equilibration with a sodium dodecyl sulfate-containing buffer, the ISO gels were run in the second dimension on DALT slab gels (20cm x 25cm x 1.5mm) containing a linear 8-18% acrylamide along with molecular weight standards. The DALT gels were electrophoresed for 12-16hr at 10 °C and later stained with Coomassie brilliant blue. Stained gels were photographed with Ilford Pan-F film on a fluorescent light box and visually inspected.

RESULTS

Whole homogenates and isolated hepatocytes. Two-dimensional gels on which whole liver homogenate proteins were separated contained approximately 600-800 protein or polypeptide spots. While these were distributed fairly evenly in terms of molecular weight, the bulk of the proteins were in the 30-80 kilodalton (kD) range and had isoelectric points between pH 5 and 7. Figures 1 through 4 illustrate the 2D patterns of rat liver whole homogenates under the conditions indicated. PFDA exposure altered 28 proteins (or polypetide groups) to varying degrees in both the 20 and 50 mg/kg concentrations. Of these 28, 13 proteins were consistently altered on each gel from each sample. The remaining 15 were altered in some samples but to a lesser degree in others and have been indicated by circles. Six of the protein spots have been tentatively identified based on their x,y coordinate position with respect to molecular weight and carbamylated charge standardization of the gels. These are mitochondrial (M1, M2, and M3) and cellular actin, alpha tubulin, and beta tubulin (Anderson, 1985).

Upon visual inspection, a magnitude of change was assigned to the altered proteins relative to corresponding control spot intensities. These data are listed in Table 1a. It is interesting to note the absence of any noticeable difference in the ad lib pattern versus that of the pair-fed rats. The most notable changes are those which appear to be related to PFDA dose. The protein pair (probably charge variants of the same protein) labeled 35 (approx. 35kD) undergoes a dose related decrease in intensity with increasing PFDA levels. This is also true of proteins 29, 36.3, 38.7, and albumin. It is important to note that isolated hepatocyte gels (not shown) were nearly identical to whole homogenate gels with the exception that in the cultured hepatocytes, albumin spot intensity remained the same despite PFDA intoxication. Other protein spots underwent an increase in spot intensity and therefore an assumed increase

in concentration. These include the proteins labeled 30.3, 40.8, 52.5, 69, 80, and M1-M3; several with clear dose relationships. Non-muscle actin and the tubulins were not affected by PFDA.

Microsomal proteins. Microsomal protein patterns are illustrated on Figures 5 and 6 and altered proteins are listed in Table 1b. Eleven proteins underwent reproducible alterations in the 50mg PFDA/kg treated group, the only treatment group analyzed. Only two protein spots decreased in intensity while the rest underwent visible increases. Several known microsomal marker proteins were conspicuously absent from the patterns. None of the altered proteins were readily identifiable by either MW or pI.

Mitochondrial proteins. Mitochondrial fraction proteins are illustrated on Figures 7 and 8 while those spots altered by PFDA treatment are listed in Table 1c. The expression of the effected proteins was enhanced as suggested by their increased spot intensity. Additionally, proteins M2 and M3 were augmented as well. When microsomal and mitochondrial fraction 2D patterns are visually compared, a number of protein spots are seen to be common to both fractions. Several responded to PFDA treatment similarly.

Cytosolic proteins. Due to technical difficulties, this cell fraction was provided to us early in the study but not in later experiments. Therefore, Coomassie blue stained gel photos are not included in this report. However, polychromatically silver stained gels were visually analyzed and the results of those analyses are included in this report in Table 1d. All 8 cytosolic proteins altered by PFDA treatment underwent varying degrees of decrease in intensity.

DISCUSSION

Much previous effort has been dedicated to the investigation of PFDA's hepatotoxic effects. This study represents an initial attempt to examine the influence of toxic levels of PFDA on rat liver cells from a rather new and unique perspective. Given the evidence that PFDA alters the hepatic architecture, its lipid metabolic machinary, and perhaps its myriad membranes' composition, we felt it was very likely that significant alterations would also be found in hepatic protein expression. From our experience with lymphocytes and from the experiences of others with liver cells, it is clear that two-dimensional electrophoresis is a powerful albeit somewhat ancillary tool in toxicologic investigation.

The results of this preliminary investigation are encouraging while at the same time their interpretation points out several technical limitations in our approach. As seen in Figures 1-4, hundreds of whole homogenate proteins were separated with high resolution and sensitivity and are very comparable to those appearing in the literature. In fact, based on internal MW and pI standardization, several well known marker proteins can be visually identified with a high degree of certainty. The identification of others is barely speculative. Due to differences in running conditions, peculiarities of denaturing agents, and interlaboratory variations, it is difficult to comment with any certainty whether a given spot with an estimated pI or MW is indeed congruent with similar protein spots present in the scientific literature.

To address this uncertainty, we turned our attention to those proteins previously reported to be induced or reduced (activity or content) by PFDA. Enoyl-CoA hydratase (crotonase) is an 80kD protein whose activity and PAGE spot intensity reportedly increase in liver cells in response to PFDA intoxication (Ikeda, 1985) thus acting as a peroxisome proliferation marker. While

positive identification of this protein was beyond the scope of this project, we did observe the induction of an 80kD protein at both 20 and 50mg PFDA/kg doses. The fact that NADPH cytochrome P450 reductase is also an 80kD liver enzyme (Hodgson 1987) further confounds the results. Another enzyme associated with peroxisomes, fatty acyl-CoA oxidase, undergoes at least a tenfold amplification in response to PFDA treatment (VanRafelghem 1985; Harrison 1988). Judging by the augmented protein spots observed in the present study, any one of them is a likely candidate. The induction of mitochondrial M2 and M3 proteins (Figs. 1-4; 7 & 8) contrasts decreases observed with Aroclor 1254 intoxication (Anderson 1986). Conversely, hepatic stearoyl-CoA desaturase activity is abolished by PFDA (Van Rafelghem, 1988) and accounts for the shift in stearic and oleic acids in the liver (Olson 1983). Several whole homogenate proteins disappear with PFDA treatment, most notably spot 35. Since the molecular weight of stearoyl CoA desaturase is 33kD it is a likely candidate.

Albumin (MW 66.7kD), as identified by MW and pI standardization as well as literature comparisons, declined in intensity in the PFDA-treated rats. However, in isolated and cultured hepatocytes whose protein pattern was nearly identical to that of homogenates, albumin spot intensity was unaltered. This may be the result of PFDA binding to albumin, an effect which may alter albumin's solubility during sample preparation or its cellular concentration in vivo (i.e., decreased synthesis/increased degradation). It is also possible that albumin, present in tissue culture media remains as an in vitro artifact masking the PFDA effect observed in vivo. The exact significance of albumin's decline and its primary or secondary nature remains to be determined.

While significant and reproducible protein pattern alterations in liver whole homogenates are readily apparent in Figs. 1-4, their compartmental origin is not clear. To address this issue, liver cell fractions were isolated and their protein complement resolved by 2D-PAGE. Cytosolic fractions were

initially analyzed by silver stain. Later experiments were fraught with technical difficulties and cytosol was eliminated from consideration for the moment. However, in the initial gels that were obtained, several proteins were observed to decrease in intensity (Table 3). Due to the problems associated with running cytosol, these alterations are of questionable reliability and match rather poorly with proteins resolved in the whole homogenate gels. The primary difficulty with cytosol is related to the severe dilution caused by the isolation procedures. As will be discussed later, future experiments will incorporate steps to concentrate the cytosolic proteins into a usable range. This fraction must be better analyzed since others have observed protein pattern alterations in response to other xenobiotics (Anderson et al., 1987).

Perhaps the cellular compartment most often and most extensively altered by xenobiotic treatment is the endoplasmic reticulum or microsomal fraction (Hodgson and Levi, 1987). It is in this fraction that we observed the most numerous and most extensive protein pattern alterations. While none of the altered proteins in this fraction were identified, one in particular labelled 52.5b may very well be cytochrome P452. This protein has been shown to be readily induced by xenobiotics such as clofibrate (Sharma et al., 1988). The 52.5b kD protein appears to be induced by PFDA. Work is already underway to address this probability. Speculation on the identity of other proteins is more difficult. This is due to the similarity between the microsomal and mitochondrial patterns. According to Anderson (personal communication), there is very little qualitative overlap between the major constituents of these fractions. On this score our data are clearly in error. Recently the laboratory where these fractions were prepared noted a significant loss of calibration in their preparative centrifuges. Cross-contamination is therefore the likely explanation for the similarity between the sample gels. Despite the error, it is clear that 2D-PAGE is a powerful tool for quality-control

determinations. Furthermore, we did observe a significant number of protein pattern alterations. Some of these have candidates for potential identification while most do not. It is clear that PFDA has cellular targets not easily explained by the metabolic and membrane studies conducted previously.

Future directions. With recently awarded multi-year funding to continue these studies it will be possible to systematically identify the many proteins altered by PFDA. Significant changes in the cell fractionation protocol, acquisition of protein blotting equipment and suitable antibodies, procurement of purified forms of the enzymes previously shown to be PFDA targets, and application of computerized imaging for statistical analysis of altered patterns will all be part of an improved approach and a more intense effort to explain PFDA's toxic mechanism in the liver.

CONCLUSION

The primary goal of this project was achieved. High-resolution two-dimensional polyacrylamide gels containing separated whole homogenate and cell fraction proteins were obtained. A significant number of individual proteins were altered, many according to PFDA dose. The results encourage further study; in particular systematic protein identification and subcellular localization of specific PFDA effects.

Manuscript in Preparation:

Witzmann, F.A. Hepatic protein pattern alterations following perfluorodecanoic acid exposure. Toxicology Letters (in preparation).

Papers Presented:

Witzmann, F., N. DelRaso, and M. George. Two-dimensional electrophoretic analysis of PFDA hepatotoxicity. The Toxicologist 10(1): 250, 1990. Presented at the 29th annual meeting of the Society of Toxicology.

Witzmann, F.A. Two-dimensional protein electrophoresis: Hepatotoxic applications. To be presented at the 11th annual meeting of the Society of Environmental Toxicology and Chemistry, 11/11-15/90.

Witzmann, F.A. Induction of cytochrome P452 in liver cell fractions by perfluoro-n-decanoic acid: an electrophoretic analysis. To be presented at the Third North American Meeting of the International Society for the study of Xenobiotics, 10/21-25/90.

TABLE 1. The effects of PFDA treatment and dose on relative 2D protein spot intensity as compered to that of the untreated control.

A. Whole liver homogenate proteins

Molecular Weight	20mg/kg	50mg/kg
29,000	-	↓
30,300	↑	↑↑
35,000	↓	↓↓↓
36,300	↓	↓↓
38,700	↓	↓↓
40,800	↑↑	↑↑↑
52,500	↑	↑
69,000	↑	↑↑
80,000	↑↑	↑↑
Albumin (68kD)	↓	↓↓
M1	-	↑
M2	↑	↑↑
M3	↑	↑↑
Actin (42kD)	-	-
Tubulins	-	-

B. Microsomal Proteins

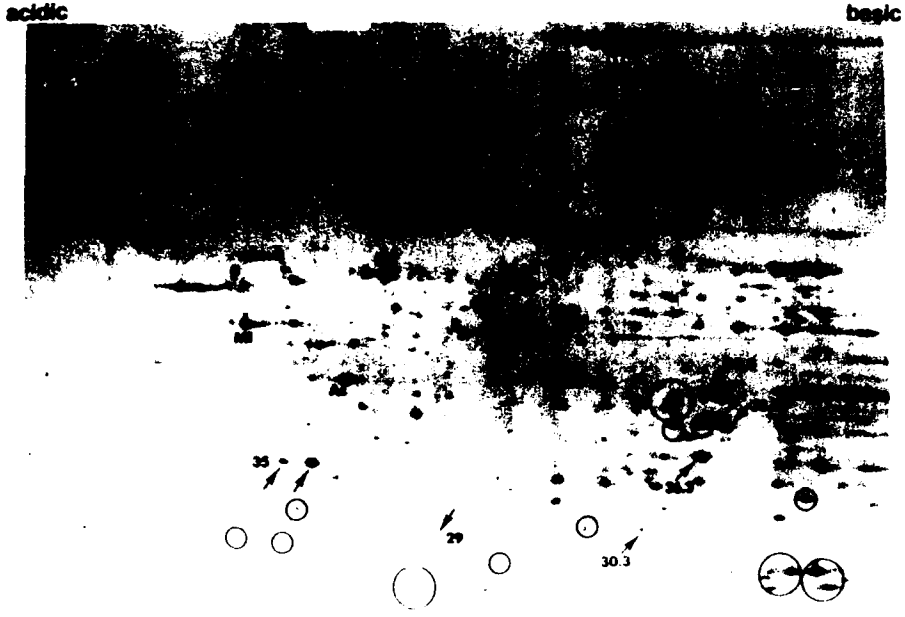
Molecular Weight	50mg/kg
31,000	↓
32,300	↑↑
36,300	↑
38,500	↓
40,700	↑
42,000	↑↑
45,000	↑↑
51,500	↑↑
52,500a	↑↑
52,500b	↑
69,000	↑

c. Mitochondrial Proteins

Molecular Weight	50mg/kg
30,300	↑
39,800	↑
40,000	↑
41,000	↑↑
69,000	↑
80,000	↑

d. Cytosol Proteins

Molecular Weight	50mg/kg
18,800	↑↑
26,000	↑↑
26,500	↑↑
31,000	↑
35,600	↑
36,500	↑
37,200	↑↑↑
37,400	↑↑



LIVER HOMOGENATE Fig. 1 Ad Lib

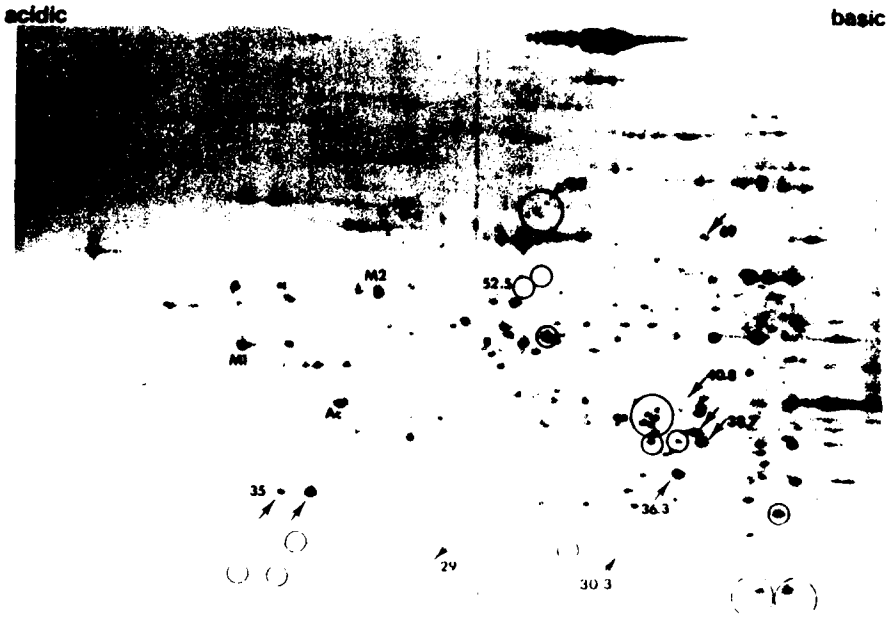


Fig 2

Figures 1 and 2. Two-dimensional electrophoretic patterns of liver whole homogenate proteins from (1) untreated rats fed ad libitum and (2) vehicle treated rats pair fed with respect to 50mg/kg PFDA treated rats. In these figures as in those to follow, protein spots with specific numbers are altered consistently in each sample examined and have been given a number based on their sodium dodecyl sulfate molecular weight estimates. Circled protein spots are those whose alterations were inconsistently observed. Alpha and beta denote the tubulins, M1, M2 and M3 are well characterized mitochondrial proteins, Ac is most likekly actin, and Al is albumin.

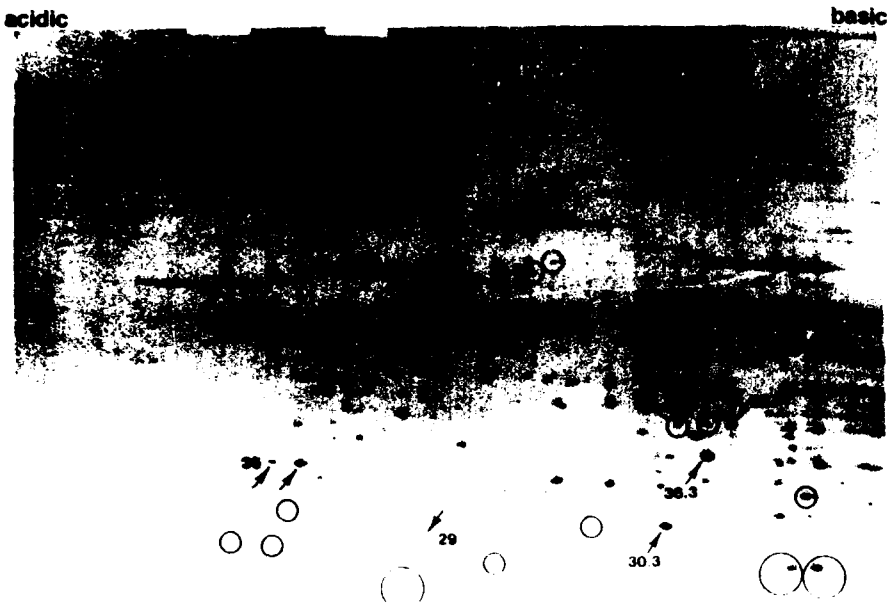


Fig. 3 20 mg/kg

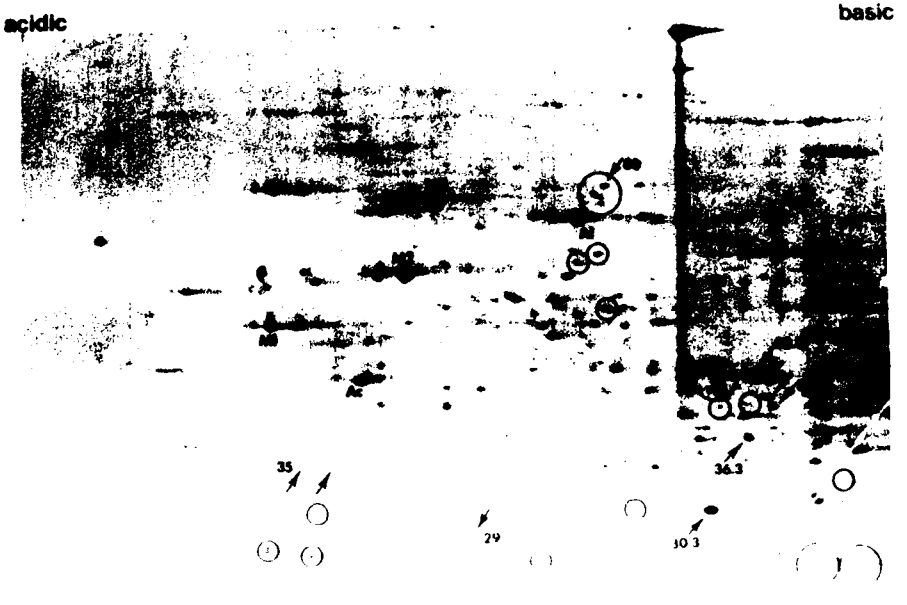
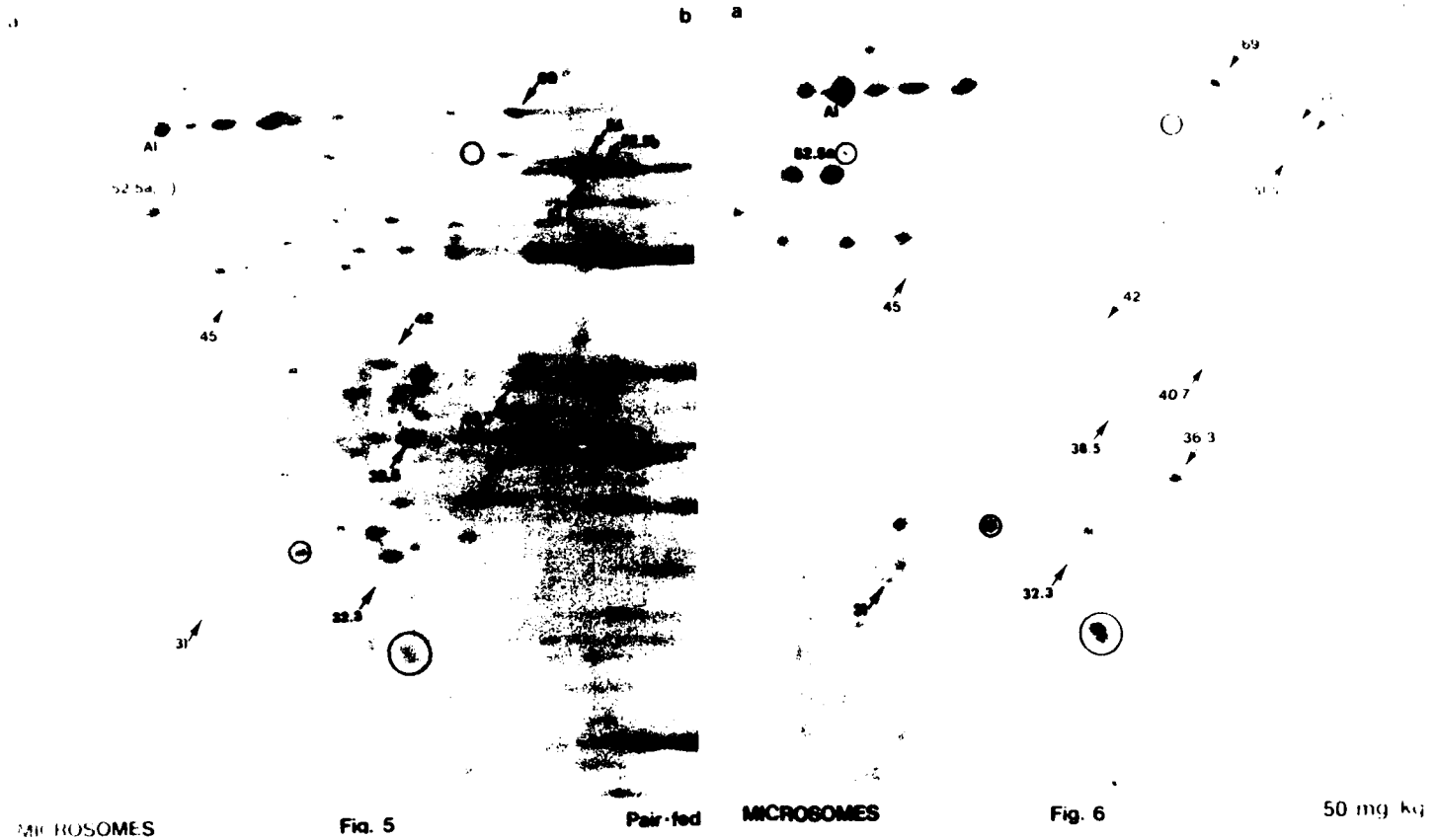


Fig. 4 50 mg/kg

Figures 3 and 4. Two-dimensional electrophoretic pattern of liver whole homogenate proteins from (3) rats treated with 20mg/kg PFDA and (4) rats treated with 50mg/kg PFDA. (see Figures 1 and 2 for details).



Figures 5 and 6. Two-dimensional electrophoretic patterns of hepatic microsomal proteins from (5) the pair-fed vehicle treated control and (6) the 50mg/kg PFDA treated rats.

MITOCHONDRIA

Figures 7 and 8. Two-dimensional electrophoretic patterns of hepatic mitochondrial proteins from (7) the pair-fed vehicle treated control and (8) the 50mg/kg PFDA treated rats.