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Title: THE EFFECTS OF CYCLOPROPENOID FATTY ACIDS ON STRUCTURAL COMPONENTS OF MICROSOMAL MEMBRANES.

Abstract approved: Daniel P. Selivonchick

Studies were conducted to determine the effects of cyclopropenoid fatty acids (CPFA) on the microsomal membrane of livers of rainbow trout (Salmo gairdneri). Slab and tube gel electrophoresis of microsomes from trout fed a CPFA diet (CPFA-microsomes) for varying time periods showed a decrease in the number of protein bands resolved in the high molecular weight region. This disappearance of high molecular weight proteins was not due to increased proteolysis in the CPFA-microsomes.

Antibodies against whole microsomal protein from livers of trout fed 300 ppm CPFA were produced in rabbits. Microsomal proteins were first separated by polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose sheets (NC) and analyzed by the peroxidase-antiperoxidase (PAP) immunochemical staining procedure. Immunoabsorption of antisera directed against CPFA-microsomes by control-microsomes did not reveal any new proteins induced by the CPFA diets. However, the intensity of PAP staining was much greater in CPFA microsomes after immunoabsorption.

Hydrolysis of phospholipids in the microsomal membrane by

phospholipase A_2 failed to reveal any differences between control and CPFA fed trout. Proteolysis of microsomal membrane proteins had similar effects on NADPH cytochrome reductase and cytochrome P-450 activity on fish fed the different diets. PAGE analysis of these digests did show some differences in digested proteins between the control and CPFA group. These results may reflect a possible change in orientation of microsomal membrane proteins brought about by CPFA in the diet. Additional evidence for altered orientation of proteins was found with PAGE analysis of trypsin-digested microsomes. Moreover incubation of trypsin-digested microsomes with antisera and stained with PAP showed that proteolytic attack was different between control and CPFA microsomes. A final study with incubation of transferred proteins from control and CPFA-microsomes with antisera directed against purified cytochrome P-450 (P-450) and cytochrome P-448 (P-448) showed that CPFA had an effect on the concentration of P-448 but not P-450.

The Effects of Cyclopropenoid Fatty Acids on Structural Components of Microsomal Membranes

by

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DEDICATION

All ignorance toboggans into know

and trudges up to ignorance again:

but winter's not forever, even snow melts;

and if spring should spoil the same, what then?

. . .

e.e. cummings

This thesis is dedicated to Daniel P. Selivonchick, my advisor, who taught me that science is done for the enjoyment of science and without whose enthusiasm, cajolery, beer influenced philosophies and ability to see some order out of a heterogenous conglomeration of stuffs, I might still be lying on that beach in Mexico drinking rum and coke and thinking that I was enjoying myself.

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Table of Contents

LIST OF FIGURES

LIST OF TABLES

THE EFFECTS OF CYCLOPROPENOID FATTY ACIDS ON STRUCTURAL COMPONENTS OF MICROSOMAL MEMBRANES

I

INTRODUCTION

Cyclopropenoid fatty acids (CPFA) have biological properties that have generated a significant degree of interest in the cancer field. They are naturally occurring fatty acids that have been shown to enhance the carcinogenic effect of aflatoxin B_1 (AFB_1) in the liver of rainbow trout. In addition to being a co-carcinogen for trout, CPFA in the diet has been shown to have adverse biological effects on several species of animals.

Recently, CPFA has been found to function as a promoter for tumor enhancement in trout exposed to AFB_1 at the embryo state (Hendricks, 1981). It is probable that the physiological role for CPFA as a promoter/co-carcinogen is a membrane mediated one. CPFA has been found to incorporate into the phospholipids of cellular membranes and to alter the activity of several membrane bound enzymes (Einerson, 1982; Eisle et al., 1978). It has also been reported that CPFA induces the synthesis of a microsomal protein (Selivonchick et al., 1981).

The objectives of this study was to examine the possibility that the promotional/co-carcinogenic properties of CPFA are related to structural modifications of cellular membranes, in particular, membrane protein constituents. In addition, a second objective was to develop immunological techniques as a useful probe in the study of membrane proteins.

LITERATURE REVIEW

Protein Orientation in the Membrane

There are several theories of how proteins orientate themselves into membranes. One of the current models is the signal hypothesis (Blobel and Dobberstein, 1975), in which the protein is orientated in the endoplasmic reticulum membrane as it is being translated by the ribosomes. The N-terminal "signal sequence" is recognized by membrane receptors and facilitates its passage through the lipid bilayer. The membrane trigger hypothesis of Wickner (1979) allows the previously folded protein to be recognized by various membrane components for localization. Orientation occurs after the binding to the appropriate membrane, as the protein interacts with lipid components to fold into a conformation that exposes the hydrophobic regions to the non-polar components of the lipid bilayer.

For the majority of proteins, the hydrophobic and hydrophyllic domains of their polypeptide regions are clearly the predominant force in protein orientation (Saundermann, 1977). Primary structure determinations of numerous purified proteins indicate these polar and non-polar domains and thus their amphiphatic nature. Electron diffraction analyses of membrane proteins have allowed structural studies to predict the protein orientation. A combination of sequence and structure analyses for bacteriorhodopsin has revealed it to be an integral membrane protein composed of several

transmembrane helices of hydrophobic residues (Michel et al., 1980).

However, the lipid material itself can exert direct and indirect effects on protein alignment within the membrane. Cholesterol-modified membranes have shown that alteration of this key membrane component may alter protein position and functionality as well (Shinitzky, 1979). Phospholipids, the major lipid component of membranes, are amphipathic compounds that form bilayers with their polar heads toward the outsides of the membrane. The use of Escherichia coli mutants that are defective in total fatty acid synthesis and whose predominant membrane phospholipid fatty acids can be altered by the fatty acid supplement in the growth diet have shown several aspects of protein-lipid interaction (Baldassare et al., 1977). Specifically, when the fraction of phospholipids acylated with cis-Δ^{11}-18:1 on the 1 and 2 positions exceeded that found in normal cells grown under similar conditions, there was a progressive inactivation of membrane bound NADPH, L-α-glycerol-3-phosphate, succinate and D-lactate oxidases. This activity was restored to normal levels when membranes were incubated and fused with lysosomes containing a normal mixture of lipid molecules. Earlier work on E. coli (Hsu et al., 1970) with a defective fatty acid desaturase activity showed that there was a clear relationship between phospholipid saturation and enzymatic activity. Recently, similar results have been demonstrated with membranes of eukaryotes (Dickens and Thompson, 1980). Saundermann (1977) has reviewed numerous membrane bound enzymes that have obligatory lipid cofactors and/or modulators for enzymatic activity. Some of these include (Na^+-K^+)-ATP ase , the

cytochrome P-450 oxygenase system, isoprenoid alcohol kinase, cytochrome b_5 reductase, glucose -6- phosphatase, UDP-glucuronyl transferase and several others. Recent techniques used in the study of various lipid-protein parameters have shown further examples of this lipid-protein interaction.

Probes of Membrane Structure

With the advent of electron spin resonance (ESR) studies, it became possible to study lipid-protein dynamics without separating the two. ESR introduces stable free radical molecules into membranes and when the sample is placed in a magnetic field the absorption spectrum for radiation is measured (Anderson, 1978). The spectra allow the investigator to measure probe mobility, to calculate concentration of probe in different environments and to correlate data from membrane models to in situ membrane dynamics. Jost and coworkers (1973) used this method to demonstrate the presence of a restricted component in the ESR spectra of lipid-protein particles which was not present in the spectra of pure lipid particles. The term "boundary lipids" is now used to describe those lipid molecules in the membrane which are in direct contact with and restricted by membrane proteins. In nuclear magnetic resonance (NMR) the membrane sample is placed in a magnetic field and proton gyration is measured. The use of this technique has contributed to valuable information for testing models of polar group conformation of phospholipids in membranes (Seelig and Gally, 1976). Freeze fracture electron microscopy allows us to visualize the interior surface

of a membrane, which is split down the two halves of the bilayer. The physical features of the membrane and the protein positioning within the membrane can thus be readily observed. Wallace and Engleman (1978) have used this method to show that changes in the physical state of membrane lipids can cause changes in the relative location of surface and intramembrane components of the cell.

Use of Enzymes in Membrane Structural Analysis

In the past decade the commercial availability of phospholipases that are specific for classes of phospholipids and/or hydrolysis of specific acyl chains on the phospholipid has greatly increased the uses of this methodology. Phospholipase A_2, C, D, and sphingomyelinase have been used successfully as probes to reveal the asymmetry of phospholipids in the membrane bilayer (Op den Kamp, 1979). It is noted, however, that the hydrolysis products such as lysophospholipid, free fatty acids and diglycerides may perturb the membrane structure themselves and cause indirect alterations. Nonetheless, specific phospholipases are a useful tool for investigating various roles of the phospholipid classes in their relationship with membrane proteins (Lenaz, 1978). Loss of phosphatidyl inositol (PI) produced an inhibition of glucose-6-phosphate phosphorylase whereas changes in membrane compostion of phosphatidyl choline (PC) and phosphatidy ethanolamine (PE) produced little if any change (Crain and Zilversmit, 1981). Hydrolysis of phospholipids with phospholipase A_2 revealed a correlation of loss of tetrodotoxin to the Na^+-channel protein in the membrane to hydrolysis of PE and phosphatidyl serine (PS) (Reed,

1982). Research by several investigators have shown that PC is necessary for proper function of the cytochrome P-450 system (Coon, 1981). The stoichometry of binding appears to be one molecule of reductase, one of substrate and approximately 20 of PC per molecule of cytochrome P-450.

The asymmetrical distribution of phospholipids has been substantiated for the erythrocyte membrane (Bergelson and Barsukov, 1977) and for the membranes of several viruses (Leonard, 1978). The quantitative distribution of phospholipids across subcellular membranes is less clear. It is well established, in the endoplasmic reticulum that the biosynthesis of most of the phospholipids occurs primarily on the cytosolic side of the membrane (Coleman and Bell, 1978) (Ballas and Bell, 1981). This unidirectional synthesis may result in asymmetry of phospholipid location in the bilayer but analysis has yielded contradictory data. Phospholipase hydrolysis and several other probes have produced results that show preferential positioning on both sides of the membrane depending on the enzymes used and incubation conditions (Nillson and Dallner, 1977; Higgins and Dawson, 1977; Sundler et al., 1977). A partial explanation for this may be the fact that the hydrolysis of the phospholipids of the microsome appears to increase the translocation (from inside to outside) of the hydrolyzed lipids (Nillson and Dallner, 1975).

Researchers have been more successful in demonstrating that the protein components of biological membranes are asymmetrically located. The most classical method for examining such asymmetry in the case of closed vessicles is by treatment with proteases. Microsomes

are membrane bound vesicles primarily that are heterogenous with regard to size and density and have a proper "sidedness" of the membrane proteins (Vanderkooi, 1978). The external side of the microsomal vesicles corresponds to the cytoplasmic surface of the endoplasmic reticulum whereas the lumenal proteins remain on the internal region, imitating the situation in vivo. Proteases, like phospholipases are large macromolecules that do not normally penetrate biological membranes (Ito and Sato, 1969). Only enzymes and membrane proteins on the outside of microsomal vesicles are susceptible to proteolysis unless membrane integrity is disrupted by detergents (DePierre and Ernster, 1977). The topology of numerous enzymes of the endoplasmic reticulum have been elucidated. Most enzymes have been found to be localized asymmetrically (Nilsson et al. 1978); (Coleman and Bell, 1980). Cytochrome P-450 is one of the few enzymes studied that has been found to span the membrane. Orrenius and coworkers (1969) have done an extensive study on the effects of trypsin on the mixed function oxidase (MFO) system of rat liver microsomes. NADPH-cytochrome P-450 reductase, which donates an electron to cytochrome P-450 is located on the cytosolic surface and is thought not to interact with the phospholipid bilayer of the membrane (DePierre and Dallner, 1975). The active site of cytochrome P-450 appears, in part, to be also at the cytosolic surface in position with the reductase. Nonetheless, the majority of the cytochrome lies within the bilayer and is more likely to be hydrophobic in character. This would reflect the hydrophobic nature of the binding site for its lipid soluble substrates (Nilsson et al., 1978).

New Concepts of Membrane Structure

Data have accumulated in numerous laboratories to lend credence to several new model concepts of lipid-protein interaction and membrane architecture. As mentioned above, ESR spectral differences in measuring model membranes with cytochrome c oxidase and phospholipids showed two distinct regions differing markedly in fluidity. The boundary lipids may be looked upon as the perturbed lipid phase of the membrane that interacts with proteins and other compounds. The earliest evidence for the boundary lipid concept was noted when ether extracts of membranes removed all of the cholesterol but only one quarter of the phospholipids (Roelofsen et al., 1966). This implied that a significant portion of phospholipid was interacting with proteins in some manner. More specifically, Warren et al., (1975) have shown that 25-30 phospholipid molecules per protein molecule must be present to allow enzymatic activity of sarcoplasmic reticulum ATPase. These obligatory lipids form a boundary ring or halo around the protein. Moreover, the work of Papahadjopoulous et al., (1975) has demonstrated that boundary lipid does not take part in lipid phase transitions at different temperatures. The effect of proteins on lipid bilayers is profound. Besides the immobilization of boundary lipids, Conrad and Singer (1981) have done experiments with amphipatic molecules that demonstrate the concept that many biological membranes are characterized by alterations of their lipids by proteins which do not exist in synthetic bilayers.

A recent publication by Valtersson and Dallner (1982) has contributed to experimental evidence for lipid domains in membranes.

They have used a cross-linking agent, 1,5,-difluoro-2,4-dinitrobenzene, to cross-link microsomal membrane phospholipids of the amino lipid class PE and PS. A large amount of dimers of PE were obtained indicating clustering of these phospholipids in the bilayers. At concentrations that allowed cross-linking to go to completion, a large portion of the lipid occurred in a protein-bound form. These results demonstrated a high degree of compartmentalization of specific phospholipids in the microsomal membrane. Other investigators using various techniques, have found compartmentalization of PI in microsomes (Brophy et al., 1978), of PE in the inner mitochondrial membrane (Crain and Marinetti, 1979) and of PC in erythrocytes (Renooij et al., 1976). Although the significance of lipid compartmentalization and the existence of lipid domains awaits further study, this concept has several implications for protein-lipid interaction and changes in lipid membrane composition.

Free Fatty Acid Perturbations in Membranes

Recent work with free fatty acids (FFA) insertion into membranes also suggest the idea that different lipid domains are an important structural feature of biological membranes. FFA have been shown to alter a variety of membrane mediated cellular functions from enzymatic activity (Anderson and Jaworski, 1977) to interference with membrane surface receptors (Klausner et al., 1980). Klausner and co-workers have also found that FFA can perturb membrane lipid structure by preferentially partitioning into specific lipid domains to

produce significant changes in the packing of lipid molecules. The structure of the FFA plays a major role in its intercalation into membrane domains. FFA with cis double bonds preferentially insert themselves into fluid-phase lipid whereas trans-unsaturated or saturated FFA partition into the gel-phase. Several previous studies have also suggested this structural heterogeniety in the lipid components of membranes (Marsh and Barrantes, 1978). Klausner's laboratory has also schematically pictured the membrane as being composed of different lipid domains (see Figure 1).

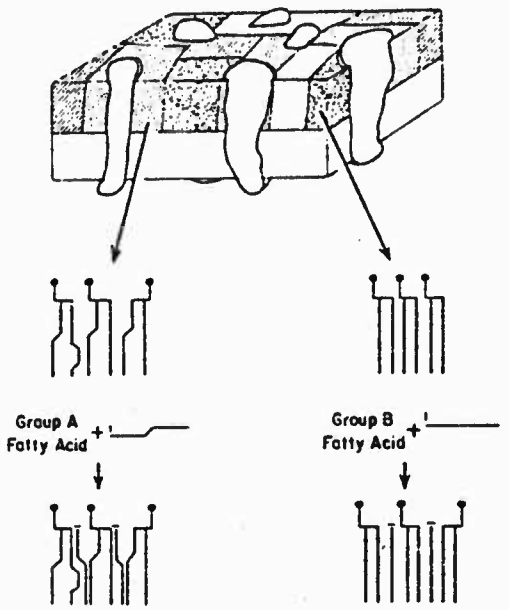
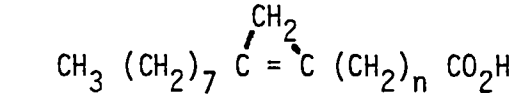


Figure 1. A highly schematized version of the plasma membrane showing the lipid organized into structurally distinct domains. Group A are the cis-unsaturated fatty acids, while Group B fatty acids represent the trans-unsaturated and saturated fatty acids (Klausner et al., 1980).

Some areas are more gel like while others are more fluid. Because saturated and unsaturated classes of FFA perturb different domains, alterations of protein orientation in these domains are variable. Tb^{3+} fluorescence, which can be used to monitor membrane protein orientation changes, since it substitutes for Ca^{2+} in many calcium binding proteins, has yielded interesting results (Pjura et al., 1982). Palmitic and stearic FFA rather than the unsaturated FFA caused the largest changes in fluorescence. Intercalation of these saturated FFA into the plasma membrane of mouse lymphoma resulted in conformational alteration of Ca^{2+} binding membrane proteins. However, a direct effect of binding FFA to the membrane protein, could not be ruled out. In either case, FFA were shown to readily insert themselves into the plasma membranes of cells and over short periods of time remain there as unesterified fatty acids. This interaction resulted in a significant perturbation of the membrane, was sensitive to the structure of FFA used and produced changes in membrane protein orientation.

Physiological Effects of Cyclopropenoid Fatty Acids

Cyclopropenoid fatty acids (CPFA) are naturally occurring fatty acids found in the plant order Malvales. Two plants have important dietary roles for man: Gossypium hirsuturm, from which cottonseed is obtained, and Eriodendron aufractuosum, from which kapok oil is derived (Christie, 1970). Both of these plants contain malvalic and sterculic acid.



(Malvalic acid, n=6; sterculic acid, n=7)

The cyclopropene ring, positioned in the center of the molecule, creates a strained structure that is very reactive and has numerous adverse biological effects (Greenberg and Harris, 1982). Studies with rats have demonstrated that CPFA in the diet leads to retarded growth, delayed sexual development and impaired reproduction in rats (Mattson, 1973).

CPFA also has shown a wide variety of effects on biological membranes. CPFA causes changes in permeability of the membrane surrounding the egg yolk, thus allowing iron to diffuse through the membrane from the yolk to the egg white. Here the iron complexes with the conalbumin of the white to give the pink white discoloration of stored eggs (Schaible and Bandemer, 1946). Nixon et al., (1974) has found that CPFA increased erythrocyte hemolysis rates in the rat and Scarpelli et al., (1974) has shown by electron microscopy that CPFA alters the rough surfaced endoplasmic reticulum of trout hepatocytes. Rainbow trout (Salmo gairdneri) have proven to be particularly sensitive to the effects of CPFA. There have been several reports showing changes in trout liver including fatty acid infiltration, the formation of fibers in liver parenchymal cells, bile duct proliferation and the presence of glycogen deposits (Lee et al., 1968; Malevski et al., 1974; Struthers et al., 1975). At very low levels, CPFA potentiates the carcinogenic effect of aflotoxin B_1 (AFB_1) in

the liver of rainbow trout (Sinnhuber et al., 1966; Sinnhuber et al., 1968). Studies with pure methyl esters of sterculic acid have shown that levels as low as 20 ppm in conjunction with AFB_1 will greatly increase the growth and the incidence of hepatoma in trout (Lee et al., 1971). High density levels of CPFA in cottonseed kernels with no detectable AFB_1 have led to increased numbers of hepatomas in trout (Hendricks et al., 1980), although it is not clear in this study whether CPFA can be considered a carcinogen by itself. Recently, dietary CPFA was found to promote the incidence of liver tumors in trout that were given a single exposure to AFB_1 at the embryo state (Hendricks, 1981). This study showed that CPFA can act as a promoter in the two stage model of carcinogenesis. The test animal was given a single dose of an initiator (AFB_1) and then continually administered a promoting agent (CPFA - by diet) which led to increased tumor levels in the host.

How CPFA works to enhance the tumor incidence of AFB_1-induced hepatomas in rainbow trout is not known. However, there are several well established biochemical changes that demonstrate the effect of CPFA on membrane proteins. There is an inhibition of Δ^9 fatty acyl desaturase which may increase the levels of saturated fatty acids in the membrane (Johnson et al., 1967). There is a significant reduction in total liver protein and an alteration in enzymatic activity of several enzymes, such as glucose-6-phosphate dehydrogenase, lactate and malate dehydrogenase (Taylor et al., 1973) as well as reduction of activity of several enzymes involved in fat and protein metabolism (Malevski et al., 1974). Eisele et al., (1978) has shown that methyl

sterculate can reduce the enzyme activity of the mixed function oxidase system, such as benzopyrene hydroxylase, cytochrome P-450 and NADPH cytochrome P-450 reductase. Loveland and co-workers (1979) have also shown that a diet containing methyl sterculate can alter the metabolic products of AFB_1. Recently, there has been reported an increase in the synthesis of a 41.5 K microsomal protein (Selivonchick et al., 1981). Its origin is being investigated. In all probability, the effects of CPFA are epigenic in nature. Although epigenic molecules do not exert a direct action on the genome (DNA), they can alter the levels of proteins and cellular metabolism by alternate means. These include interactions with hormone receptors or cellular RNA, complexing with DNA binding proteins, stimulation of mitosis, or alteration of important drug metabolizing enzymes in the endoplasmic reticulum (Weisburger and Williams, 1980). The classical tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) was shown by Balmain (1978) to stimulate the synthesis of two epidermal proteins. Hiwasa et al. (1982) has recently shown that TPA increased the synthesis of a 32K protein two-fold as early as two hours after exposure. Whether this increased synthesis is a result of altered gene expression or another cellular mechanism is not known. The possible role of this protein in cell transformation is presently being studied.

Tumor-Associated Neoantigens

Transformation of cells by chemical carcinogens often results in synthesis of *de novo* proteins that are specific for the transformed

cell line. These newly synthesized proteins are called neoantigens. Although the expression of neoantigens is not an obligatory step in carcinogensis, there is increasing evidence of cellular changes in respect to protein induction at various stages of neoplastic transformation. Embleton and Baldwin (1980) have proposed several pathways in which tumor-associated antigens may appear. Neoantigens may develop early as a consequence of carcinogen interaction with the target cell at initiation. In a different mode of action, neoantigen expression might not occur until the initiated cell is converted to the preneoplastic cell or later when transformation has completely occurred and malignant cell types develop. In all of the above pathways, neoantigen expression should persist through all stages of carcinogenesis after its initial appearance in the cell.

The classical method of demonstrating neoantigens is by transplantation experiments (Baldwin, 1955). In this method, a similar genetic strain of test animal is immunized by allowing temporary growth of a transplanted tumor, which is then surgically removed. After allowing the host's antibody titer against the transplanted tumor to develop, the animal receives a challenge with a viable tumor. Rejection of the challenge tumor by the tumor-immunized host is a positive response for the presence of tumor associated antigens. It is noted that this method of detection is primarily for neoantigens that develop on the outer surface of tumor cells. Although it is now recognized that there are several reasons for the tumor rejection, the interest in tumor-associated antigens has increased. Clinical scientists study the identification of

neoantigens for the potential they have as tumor markers for early diagnosis or in the monitoring of treatment progress (Pusztaszeri et al., 1976). Scientists interested in basic research study neoantigens to determine their identity and research their role in the process of cellular transformation.

Induction of Neoantigens by Chemical Carcinogens

With the improvement of immunochemical techniques during the past severalyears, scientists have found a variety of chemical induced neoantigens throughout the cell. Expression of several distinct tumor-associated surface antigens by 4-dimethylaminoazobenzene (DAB) has been shown with immuno-fluorescent staining of membrane surface antigens (Baldwin et al., 1971). Tumor associated antigens have been described for the polycyclic aromatic hydrocarbon 3-methylcholanthrene in several tissues including the bladder (Taranger, et al., 1972), mammary glands, respirator tract as well as 3T3 cell lines transformed in vitro (Baldwin, 1973). Several nitroso compounds have proven to be potent inducers of neoantigenic proteins including diethylnitrosamine-induced hepatomas (Zbar et al., 1969) and N-nitrosourea-induced rat brain gliomas (Tracey et al., 1978). Neoantigen expression and detection on cell lines that are transformed in vitro by chemical carcinogens may prove to be an important experimental procedure in identifying the role of neoantigens. Early antigenic changes following transformation of rat liver cells have been found with in vitro treatment of N-methyl-N-nitrosourea, 3'-methyl-dimethylaminoazobenzene, and aflatoxin B_1

(Iype et al., 1973). Furthermore, when chemically induced transformed rat liver cell lines were used for raising antibodies in rabbits, the antisera reacted with the tumor associated antigens (Yokota et al., 1978). However, correlation between these cell-line neoantigens and in vivo induced neoantigens by chemical carcinogens is difficut to prove. Moreover, not all chemically induced tumors have demonstrable neoantigens by classical transplanted tumor techniques. One notable exception is 2-acetylaminofluorene induced hepatomas (Baldwin, 1973).

The existence of tumor associated proteins has been easier to prove than their identity. Solubilization of membrane surface antigens necessitates degradative procedures that may irreversibly denature the native protein and cause loss of its immunogenicity as well as functionality. Protease digestion, KCl extraction, ion-exchange chromatography, gel filtration and immunoabsorbent columns all have been used with varying degrees of success (Price et al., 1979). Most attempts to develop cell-free immunological assays for these antigens have not proven fruitful in the past.

Western Blotting and Immunochemical Staining

With the recent development of Western blotting and indirect antibody staining techniques, subcellular localization of tumor associated neoantigens is now possible. Although most methods of Western blotting now follow modified versions of Towbin et al. (1979), the first publication describing a method of transferring

proteins from polyacrylamide gels to sheets of paper that immobilize the proteins is reported by Renart and his coworkers (1979). Simian virus 40 proteins are first separated in a cleavable crosslinked polyacrylamide gel. The crosslinker is then cleaved and the proteins are transferred onto diazobenoyloxy methyl (DBM) paper by the method of Southern (1975). The transferred proteins are detected by incubation of antisera against the proteins, followed by incubation of 125-I labeled protein A, which has a special affinity for IgG molecules.

Towbin and his coworkers (1979) have simplified the above procedure with a method for the electrophoretic transfer of polyacrylamide gels to nitrocellulose (NC) sheets. A layered sandwich is constructed of 'Scotch-brite pads', filter paper, gel and a NC sheet and placed into an electrophoretic blotting chamber. The proteins, which are dissolved in SDS and negatively charged, migrate out of the gel onto the NC sheet which is on the cathode side of the sandwich. The separation of the proteins by molecular weight remains intact throughout the transfer. In their experiments, the transfer of ribosomal proteins was found to be quantitative only in gels containing urea and not in sodium dodecyl sulfate (SDS) gels. Once transferred onto NC sheets, the proteins are immobile and can be detected by regular staining with amido black or by immunological identification.

Immunochemical methods necessitates the blocking of all additional protein binding areas of the NC with excess protein (bovine serum albumine and nonimmune animal serum). Then the NC sheets are incubated with specific antibodies. After binding of the specific

antibody to its antigen immobilized on NC, a second antibody, either radioactively labeled or conjugated to fluoresecein or to peroxidase, is directed against the first antibody. This antigen-antibody is then detected by either autoradiography, UV light or by the peroxidase reaction respectively. The method proved sensitive enough to detect μg quantities of protein and is useful for serums of low titer (Towbin et al., 1979). Furthermore, with the proper buffers and arrangement of the sandwich construction of the gel, NC, filter papers and firm support materials, proteins will transfer out of the gel onto NC sheets by diffusion in a 24-36 hour period. Although the time involved is longer, the proteins diffuse in both directions out of the gel and in essence two NC sheets become blotted at the same time with transferred protein.

The most commonly used paper to receive transferred proteins is NC. Although the binding forces are noncovalent, it is easy to handle, store and costs considerably less than others. The use of DBM paper was first described by Stellway and Dahlberg (1980). DBM requires activation but has the advantages of covalently binding proteins transferred to it. Moreover, because the unfilled protein binding sites can be inactivated chemically, this eliminates a need to saturate the paper with inert ligands as with NC sheets. Because of the cost, and its storage and handling qualities, DBM paper is less convenient to use than NC.

Proteins, transferred to NC sheets can be stained with Amido black. Immunological reactions normally utilize three different methods for visualization: flourescence, a radiolabeled technique,

or a peroxidase reaction. Fluorescence has not found great use with immunotransfer techniques since special equipment is needed to observe the reactants and to keep records. Radiolabeled methods usually are done with 125-I labeled protein A. As mentioned above this is highly specific for IgG even after the IgG is conjugated with its antigen. Although autoradiography is a necessary step, the procedure is relatively fast, inexpensive, and permanent records of the developed plates are easy to maintain. Peroxidase staining is very versatile. Peroxidase-labeled second antibodies, however, are variable in sensitivity. In this method a second antibody (e.g. goat antisera against rabbit IgG) is chemically conjugated to the peroxidase enzyme. The conjugation method and location of bond contributes to the variability. The peroxidase-anti-peroxidase (PAP) procedure developed by Sternberger (1979) is reported to be 100 times more sensitive than the peroxidase labeled second antibodies.

Initially, the procedure calls for the transfer of the antigens to the nitrocellulose sheets. After non-specific binding areas are blocked, the antigens are complexed with their primary antibodies. This is followed by a complexing of an unlabeled second antibody. The second antibody serves as a bridge for the PAP complex. The PAP complex is antibody directed against the peroxidase enzymes. This antibody is developed in the same species of animal that the primary antibody is developed in. The PAP complex is made by complexing the anti-peroxidase IgG with the enzyme. The active site of the peroxidase enzyme is not blocked and the enzyme remains highly active. The PAP complex is then incubated with the antigen-primary

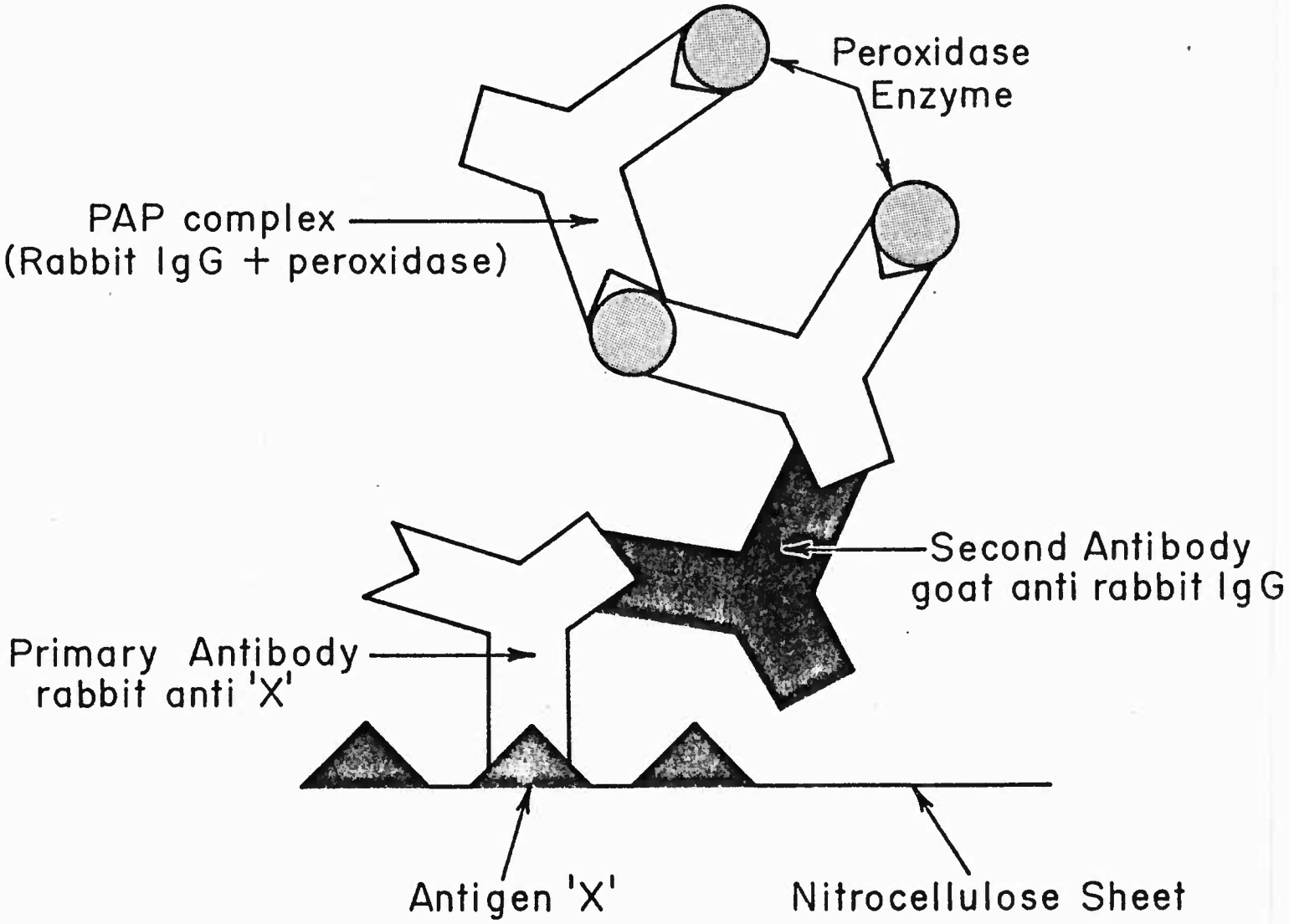

Peroxidase
Enzyme
PAP complex
(Rabbit IgG + peroxidase)
Second Antibody
goat anti rabbit IgG
Primary Antibody
rabbit anti 'X'
Antigen 'X'
Nitrocellulose Sheet

Figure 2

antibody-secondary antibody network and attaches to free ends of the secondary antibody which is specific for an antigenic site that does not interfer with the peroxidase enzyme. The PAP complexes can then form networks with each other which further increases its sensitivity. This is diagrammed in Figure 2.

Current Uses of Immunotransfer

Several investigators have employed immunotransfer methods to study nuclear proteins using monoclonal antibody or antisera against purified proteins. Research has been completed to identify non histone proteins of Drosophila melanogaster (Howard et al., 1981) associated with the active regions of polytene chromosomes. Also, the method has been used to detect various chromosomal proteins in chromatin subunits in attempts to elucidate certain areas of chromatin structure (Tahourdin, 1981). Recently, Tseng et al., (1982) have prepared monoclonal antibodies against human epidermal keratins and has used immunotransfer techniques to localize the tissue distribution of epidermis related keratins in various mammalian epithelia.

There also has been an interest in the use of this technique in neurochemistry. Greenfield et al., (1982) used immunotransfer methodology to study low molecular weight proteins in peripheral and central nervous system myelin plus homogenates from other nerve tissue. Antisera raised against the P_2 protein has been useful in identifying tissue location and P_2 biosynthesis in the nerve tissue of the developing rat.

Newcombe et al. (1982a) has used this immunological technique to identify brain proteins in the established white matter lesions characteristic of multiple sclerosis (MS). Until this method was developed, several changes were noted in polyacrylamide gel separation of MS white matter, but the identity of the proteins was not established. In their present study over 18 proteins and polypeptides showing changes have been specifically identified with highly specific antisera. As stated in their discussion, "the technique of polyacrylamide gel electrophoresis followed by immunoperoxidase staining of proteins has enabled minor changes in brain proteins to be visualized, and thus offers a possibility of identifying early biochemical events in demyelinating disease." Follow-up work, by Newcombe et al. (1982b), showed no MS-specific antibody activity directed against central nervous system (CNS) proteins. Initially, it was felt that in view of the widespread damage that occurs in both gray and white matter in MS, antibodies against CNS proteins would arise as a secondary phenomenon. Although this method failed as a fairly fast serological test for MS-related proteins, the potential for using immunotransfer techniques for clinical analysis remains a possibility. Other researchers (Braun et al., 1982) have used this procedure to identify a protein that is often associated with peripheral neuropathies of unknown etiology. Several patients experiencing this progressive neuropathy have high levels of antibody in their serum. The use of monoclonal antibodies against this protein helped establish its orgin in myelin. Immunotransfer has enabled scientists to further characterize this protein as a

glycoprotein of 90K-100K molecular weight. Isolation of the protein in the future will help to determine if this myelin component is involved in the immuno-pathogenicity of the disease.

A recent paper by Tolleshaug et al. (1982) has described an interesting study of the low density lipoprotein (LDL) receptor in familial hypercholesterolemia (FH). Altered fibroblasts from FH heterozygotes express half the normal functional LDL receptors on their cell surface. Monoclonal antibodies were directed against purified LDL receptors and were used to characterize its biochemical synthesis in cells from normal patients and those with FH. Immunotransfer techniques were used throughout the experiment and the authors discovered that the normal LDL receptor underwent a novel post translational processing in which its molecular weight increased from 120K to 160K. The antigenic segment is on the lower molecular weight component and is retained as the LDL increases in weight. The immunotransfer technique played an integral role in visualizing this biosynthesis. The researchers also found that subjects with FH apparently do not process all of their receptor proteins to the functional higher molecular weight form.

Several laboratories have used these newly developed methods in attempting to better characterize neoantigens. Glass and his coworkers (1981) were among the first to use the Western blot transfer and immunochemical staining with PAP to visualize specific antigens for transformed cells. Initially they were able to identify nonhistone protein antigens from Novikoff hepatoma cells transplanted into rats. They discovered numerous protein antigens specific to

the chromation of Novikoff hepatoma in comparison to that of normal rat liver. More recently his laboratory (Schmidt et al., 1981) has used antisera directed against their Novikoff hepatoma antigenic proteins, p 39, p 49, and p 56 purified from chromatin. The strong immuno reactivity of this antisera has allowed them to investigate other subfractions of the cell to determine location and specificity. Their results showed that although the antisera were elicited against chromatin proteins, these proteins are located also in the cell cytoplasm. The intensity of the PAP stain supports a positive location in the cytoplasm rather than chance location or cross-contamination. The absence of two hepatoma proteins in the electrophoretic transfer of total rat liver and other rat tissue chromatins indicates that these antigens may be specific for Novikoff hepatoma.

It is worthwhile to take time here to discuss the versatility of protein-directed antisera. In the papers mentioned above antisera were raised against two different types of protein. The first was a protein mixture of 300-500 different proteins representing all of the nonhistone protein of Novikoff hepatoma cells. Naturally, the proteins that are highest in quality and most antigenic in nature would develop higher antibody titers. Although less than 20% of the proteins appeared to be antigenic in antisera production, this is a significant amount and should be profitable in the search of prominent neoantigens. Moreover, immunoabsorption techniques designated neoantigens that were not present in the normal cell. The production of antisera with purified protein gives the researcher a valuable probe for subcellular localization of neoantigens.

Furthermore, the recently developed techniques of monoclonal antibody production would assure researchers of limitless supply of these probes. It is also interesting to note that the vast majority of neoantigens, studied previously to these techniques, were plasma membrane surface antigens. Immunotransfer methods allow one to search for tumor-associated antigens in all compartments of the cell.

Research that demonstrates the versatility of these described methods is reported by Duhl et al. (1982). After developing antisera to the dehistonized chromatin from human colon adenocarcinoma cell lines, they proceeded to determine the neoantigens. Immunoabsorption of the antisera with nonneoplastic colon revealed two neoantigens with molecular weights of 67K and 92K. These bands did not appear when the immunoabsorbed antisera were incubated with normal colon cells. Interestingly, antisera specific for these neoantigens did show some common neoantigens in another human colon adenocarcinoma cell line, suggesting that these tumor associated proteins are identical, or have common antigenic sites. Furthermore they have reported that this same immunoabsorbed antisera also reacts with dehistonized chromatin from liver tumor cells that have their origin in colon adenocarcinoma metastasis.

MATERIALS AND METHODS

Animals

Fish used in this study were Mount Shasta strain rainbow trout (Salmo gairdneri) spawned and raised at the Oregon State University Food Toxicology laboratory. Control fish were fed a basal semipurified diet as described by Sinnhuber et al. (1977). Test fish were fed either 50 ppm or 300 ppm CPFA in the form of methyl sterculate added to the basal diet. All fish were maintained in circular 90 cm. glass-fiber tanks receiving 12°C well water at a flow rate of 7.6 liters per minute.

Rabbits used in this study were New Zealand White, housed and fed at the Oregon State University Laboratory Animal Resource Center.

Chemicals and Equipment

All solvents and chemicals were of reagent grade quality. Lithium dodecyl sulfate (LDS) manufactured by BDH Chemicals was obtained from Boehringer Mannheim, Indianapolis, IN. All other chemicals used in electrophoresis were obtained from Bio-Rad Laboratories, Richmond, CA. Bio-Rad low molecular weight standards and Bio-Rad high molecular weight standards were used as molecular weight markers in LDS gel electrophoresis. Low molecular weight standards consisted of phosphorylase B (MW 92,500), bovine serum albumin (MW 66,200), ovalbumin (MW 45,000), carbonic anhydrase (MW 31,000), soybean trypsin

inhibitor (MW 21,500) and lysozyme (MW 14,400). High molecular weight standards consisted of myosin (MW 200,000), B-galactosidase (MW 116,250), phosphorylase B (MW 92,500), bovine serum albumin (MW 66,200), and ovalbumin (MW 45,000). Bio-Rad slab and tube gel apparatuses along with a Buchler Model 3-1500 constant power supply were used for all electrophoresis. Peroxidase anti-peroxidase soluble complex (PAP) was purchased from Sigma Chemical Company, St. Louis, Missouri. The following chemicals were also purchased from Sigma: 3,3-diaminobenzidine, trypsin, soybean trypsin inhibitor, pronase from Streptomyces griseus, phenylmethylsulfonylfluoride (PMSF) and phospholipase A_2 from Crotalus adamanteus. Antisera to trout P-450 was a gift of Dave Williams from the Agricultural Chemistry Department and horse serum was obtained under the auspices of Dr. Erwin Pearson from Oregon State University Veterinary School.

Isolation of Microsomes

Fish were sacrificed by a blow to the head and livers immediately excised, perfused with 0.9% NaCl and placed on ice. Livers were pooled and chopped with a razor blade into a fine mince in a petri dish on ice. Four volumes of ice-cold solution (0.25 M sucrose) were added and the livers homogenized with 6 passes in a motor driven Teflon pestle glass homogenizer. The homogenate was centrifuged at 800 x g for 10 minutes in a Sorvall SS-34 rotor. The postnuclear supernatant was fractionated into post-nuclear, large particle and microsomal pellets by the method of Kurup (Kurup et al., 1970). The supernatant was transferred to multiple centrifuge tubes for differential

centrifugation. The majority of the supernatant was centrifuged at 8300 x g for 10 minutes to obtain a large particle pellet (consisting mostly of mitochondria, peroxisomes and lysosomes). The supernatant collected after sedimentation of the large-particle pellet was centrifuged at 105,000 x g for 60 minutes in a Beckman 50 rotor to obtain a microsomal pellet. The other portion of the post nuclear supernatant was centrifuged at 105,000 xg for 60 minutes to obtain the post nuclear pellet (consisteing of all post nuclear membranes). All pellets were washed by resuspension in 0.25 M sucrose containing 0.03 M KCL and resedimented at the appropriate centrifugal force. A flow diagram of this isolation procedure is presented in Figure 3. All procedures were done at 4°C.

Microsomal pellets for experiments with proteases and phospholipase A_2 were prepared the same way as shown in Figure 3. However, different buffers were used. For the protease experiment the initial isolation buffer was 0.25 M sucrose, 10 mM tris (hydroxy methyl) amino methane (Tris-HCl) 1 mM ethylenediamine tetraacetic acid (EDTA), pH 7.4. The wash buffer was the same minus the EDTA.

For the phospholipase A_2 experiment the initial isolation buffers and the wash buffers were the same (0.25 M sucrose, 10 mM TRIS-HCl, pH 7.4).

Preparation of Microsomes with Protease Inhibitors and Dithiothreitol

Various protease inhibitors and dithiothreitol (DTT) were included in the sub-cellular fractionation scheme independently to

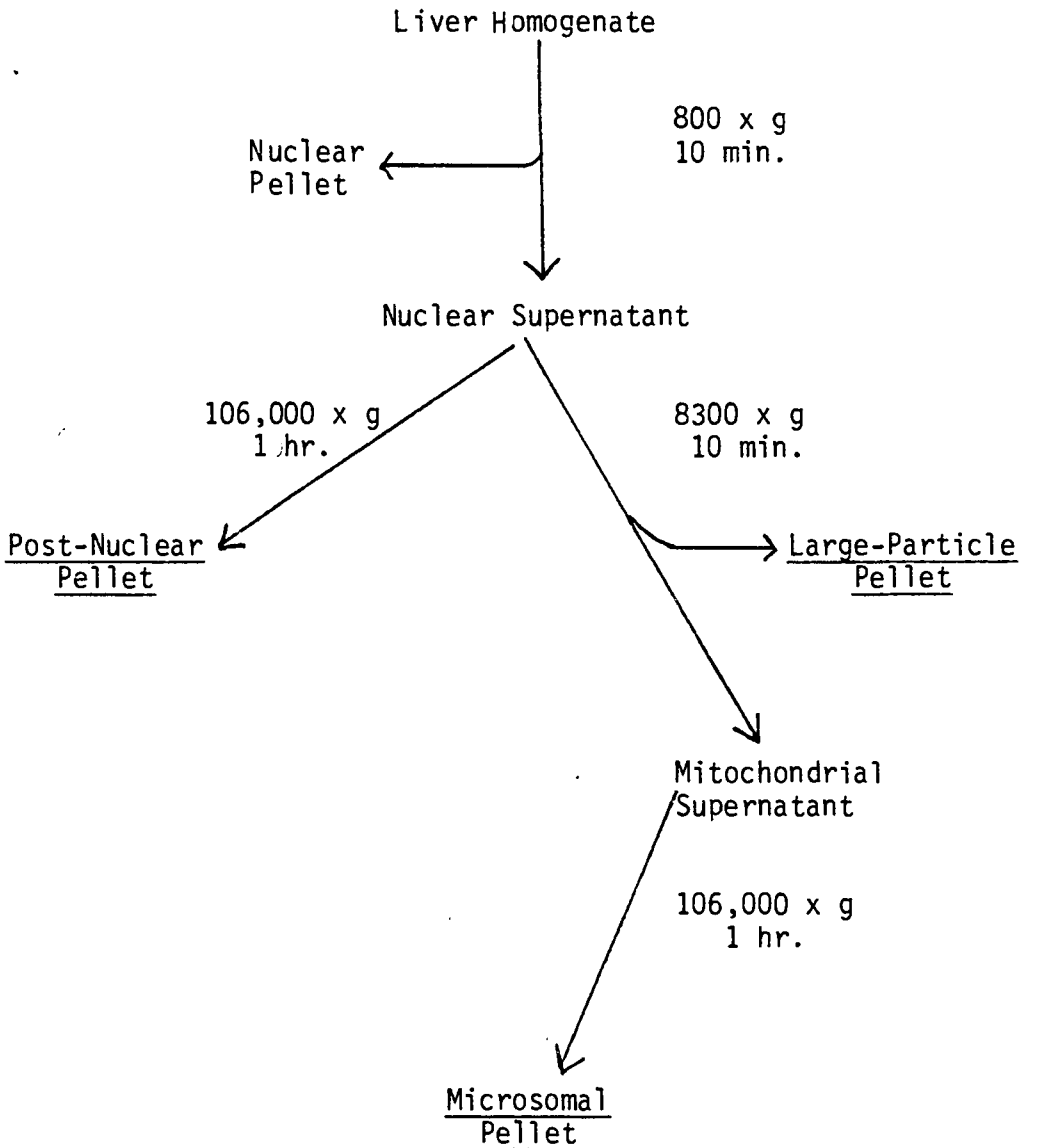


Figure 3. Flow diagram of isolation of subcellular fractions

determine their effect on microsomal protein changes. The protease inhibitors tested were iodoacetamide, EDTA, PMSF. These protease inhibitors were added to the isolation buffer in concentrations of 5mM, 1mM, 1mM respectively. DTT was added to the isolation buffer at concentrations of 5mM. Consequently, the inhibitors were present from the time the liver was minced with razor blades before homogenization until the final wash. Pellets were solubilized in 2% LDS and electrophoresed on acrylamide gels as previously described.

Polyacrylamide Gel Electrophoresis (PAGE)

Lithium dodecylsufate/polyzcrylamide sucrose gradient electrophoretic gels were performed by method of Delepelaire and Chua (1979).

Centrifuged pellets were suspended in 2% LDS buffer (2% lithium dodecyl sulfate, 12% sucrose, 0.02 M dithiotreitol (DTT), 50 mM Tris-HCL and 2 mM (EDTA), pH 6.8) by means of a microteflon homogenizer and stored at -80°C. Concentrations are approximately 5 mg/ml. Acrylamide gradient slab gels of either 15 xm x 14 cm x 0.15 cm or 30 cm x 14 cm x 0.75 cm consisting of 7.5 to 15% acrylamide stabilized with 5 to 17.5% sucrose were utilized. The gradients were poured using a standard gradient former and peristolic pump. Light acrylamide solution (5% sucrose, 0.375 M Tris-HCl, pH 8.8, 7.5% acrylamide, 0.2% N-N-methylene-bis-acrylamide (BIS), 0.1% ammonium persulfate, 0.04% N,N,N^1,N^1-Tetra methylethylenediamine (TEMED) and heavy acrylamide solution (17.5% sucrose,

0.375 M Tris-HCl, pH 8.8, 14.6% acrylamide, 0.4% BIS, 0.1% ammonium persulfate, 0.04% TEMED) were poured into each chamber of the gradient former. Solutions were either 16 ml for the short gel or 35 ml for the long gel respectively. The gradients were poured at a flow rate of 6 ml per minute at 4°C. All gradients were overlayed with water saturated butanol and allowed to polymerize for one to two hours at room temperature. At the end of this time period, the top of the gel was rinsed with distilled H_2O and a 2 cm stacking gel that consisted of 0.14% bisacrylamide, 0.125 M Tris-HCl, pH 6.80, 0.1% ammonium persulfate and 0.1% TEMED was added.

Following polymerization of the stacking gel, approximately 150 µg centrifuged protein dissolved in LDS buffer was applied to the wells of the thick or thin gel. The lower buffer reservoir was filled with running buffer (0.021 M Tris-HCl, pH 8.2, 0.16 M glycine) and the upper buffer reservoir was filled with running buffer that contained 0.1% LDS and 1.2 mM EDTA. Bio-Rad molecular weight standards (8 µg in 100 λ sample buffer containing 0.001% bromophenol blue) were applied to separate wells. Gels were electrophoresed at 10 mA or 20 mA per gel constant amperage at 4°C until the bromophenol blue tracking dye was 1 cm from the bottom of the gel. Electrophoresis was usually complete in 12-14 hours.

Tube gel electrophoresis was performed by the method of Laemmli (1970), except that LDS was substituted for SDS. All tube gels were non gradient gels that consisted of 25% buffer C (0.5 M Tris-HCl, pH 6.8, 8 mM EDTA, and 0.4% LDS), 9.7% acrylamide, 0.3% bisacrylamide,

0.1% ammonium persulfate and 0.04% TEMED and poured into glass tubes (10 cm x 0.5 cm). After polymerization of the tube gel a 4.5% acrylamide (buffer pH 6.8) stacking gel of 1 cm was poured on top. Approximately 100 µg of protein was applied to the top of the stacking gel. Tubes were positioned in their running buffer (0.5 M Tris HCl, 0.26 M Glycine, 0.1% LDS and 2 mM EDTA) and electrophoresed at 2 mA per gel constant amperage for 1 hour followed by 4 mA per gel until the tracking dye was 2 cm from the bottom of the gel. Standards (5 µg/tube) were dissolved in sample buffer without DTT and with the tracking dye.

Scanning Tube Gels

Relative migration of proteins and relative intensity of staining were determined with a Beckman Acta C III spectrophotometer equipped with a gel scanner-2 accessory. Gels were scanned at 570 nm, 0.2 mm slit width with a scan speed of 1.5 cm/min.

Staining of Polyacrylamide Gels

Tube and slab gels were stained by method of Laemmli (1970). The tube gels were placed in a 1.5 cm x 15 cm test tube and 15 ml of staining solution added. Slab gels and sufficient staining solution were placed in pyrex dishes. Both tube and slab gel were usually stained overnight. The staining solution consisted of 0.25% (w/v) Coomassie blueR-250 in 50% methanol (v/v), 40% H_2O) (v/v) and 10% glacial acetic acid (v/v). Gels were destained in the same solution without the Coomassie blue. Tube gels were placed in a Bio-Rad

destaining chamber that allowed constant agitation of the destaining solution by a magnetic stirrer. Slab gels were destained in their pyrex dishes with several washings of destaining solution.

Polyacrylamide Gels for Western Blots

Microsomal pellets were suspended in 2% LDS buffer as previously described. Acrylamide gradient slab gels of either 15 cm x 14 cm x 0.15 cm or 15 cm x 14 cm x 0.75 cm (these will be referred to as a thick or a thin gel) consisting of 7.5 to 10% acrylamide stabilized with sucrose were utilized. The components of the light acrylamide solution was the same as described above. Heavy acrylamide was essentially the same except that 10% instead of 15% acrylamide was used. Solutions were either 16 ml for the thick gel or 8 ml for the thin gel respectively. Essentially all other conditions were similar to those of regular PAGE runs described with the exception that less microsomal protein (50-100 μg) dissolved in 2% LDS buffer was applied to the wells.

Electrophoretic Transfer of Separated Proteins to Nitrocellulose Sheets

Immediately after electrophoretic separation of the proteins, the proteins were transferred to nitrocellulose (NC) sheets in the following manner. The acrylamide gel was removed from the electrophoresis unit and care was taken not to tear the gel at this point. To allow for proper orientation of the gel in the subsequent manipulation, the acrylamide gel is first marked by a small corner

cut and then the stacking gel was removed with a razor blade. The gel was then placed in 500 ml of transfer buffer (192 mM glycine, 25 mM Tris-HCl, 20% MeOH v/v) to rinse off residual LDS and equilibrate the gel to its new buffer environment. An E-C electroblot apparatus was used for the transfer (E-C Apparatus Corporation, St. Petersburg, Florida).

The gel was placed on the electrophoretic grid in a sandwich type arrangement in the following manner. The electrophoretic grid contained the electrodes, palladium wire anode on one side, and stainless steel cathode on the other side. The grid was hinged on the bottom and opened for easy placement of the gel and nitrocellulose sheets. All materials were first pre-cut for rapid construction of the gel sandwich. First a Scotch-Brite pad was soaked in the transfer buffer and then placed on the cathode side of the grid. Immediately following were two wetted Whatman No. 3 chromotography sheets cut to size. The acrylamide gel was carefully placed flat on these sheets and smoothed out with a glass rod with ample rinses of transfer buffer. The pre-cut NC sheet was laid flat in a dish containing transfer buffer and thoroughly wetted by allowing the sheet to sink to the bottom of the dish which was gently rocked. It was important to handle the NC sheets with gloves to prevent incomplete wetting. The NC sheet was then placed on top of the gel and also pressed gently but firmly to the gel surface with a glass rod and several washings of buffer. During all stages of assembly of these layers great care was taken to insure that no air bubbles were introduced since they disrupt the electrical

field and alter the patterns of protein migration from the gel to the NC sheets. The sandwich was made complete with the placement of 2 more wetted Watman No. 3 filter papers and a soaked Scotch-Brite pad. The grid is closed and held together with a plastic clasp, placed in the transfer blot chamber to which transfer buffer had been poured (approximately 6 liters). Transfers were accomplished at 200 mA for two to four hours depending on the thickness of the gel. All transfers were done at 4°C which minimizes air bubbles which may interfere with the protein transfer.

Staining of the Western Blot

NC paper to which proteins had been transblotted were cut into strips for either regular visual staining with Amido black or immunological incubation assays. Total transferred proteins were visualized by staining the nonincubated NC sheets for 10 minutes with 0.1% Amido black in 45% methanol :10% acetic acid. The NC strips were destained with three washings of a 90% methanol: 2% acetic acid solution over a period of one hour.

Immunization and Antisera Preparation

Microsomes from the livers of rainbow trout fed 300 ppm CPFA were isolated as previously described and mixed with Freund's complete adjuvant (1:1). This emulsion was administered subcutaneously to multiple back sites or intramuscularly to the hind leg quarter of female New Zealand Rabbits. Three rabbits per immunization

scheme were used. Booster shots of the same microsomal prep were prepared with Fruend's incomplete adjuvant. Table 1 shows the schedule followed in immunizing rabbits. Blood was collected by ear vein bleeding one week before the first injection and at several time intervals after each boost. Sera were separated from cells by allowing coagulation to proceed at room temperature for 2-3 hours followed by centrifugation at 10,000 xg for 10 minutes at 4°C. Sera were divided into 1 ml aliquots and stored at -80°C.

Immunoelectrophoresis

Rocket immunoelectrophoresis was performed using 1% agarose containing 0.5% Triton X-100 dissolved in sodium barbital buffer, pH 8.8. The agarose was dissolved by heating to 90°C in the buffer containing Triton X-100 and then cooled to 50°C. Antiserum to whole microsomes of trout liver was added to the warm agarose at concentrations from 5-10%. The agarose-antiserum solution was poured onto the hydrophilic side of an agarose gel support plastic plate and allowed to gel for 15 minutes. Sample wells were punched into the solidified agar with a #2 cork borer. Twenty microliters of microsome, dissolved in isolation buffer at concentrations of 5 µg/ul, were placed in the sample wells and stabilized. Gels were electrophoresed for 8 hours at 30 volts at 4°C. After electrophoresis, the gels were washed overnight at 4°C in 100 ml of TES-saline buffer (60 mm TES, 1.5 M NaCl, pH 7.3), rinsed with distilled water and stained with 0.25% Coomassie brilliant blue in ethanol/water/acetic acid (5:4:1) and destained in the same solution minus the Coomassie

Table 1. Immunization Schedules

WK	Freund's Adjuvant	Mode	Total Dose mg microsomal protein	Bleeding
Group I Rabbits 1,2,3				Pre-immune
0	Complete	IM, TP	3.2	
1 1/2	Complete	IM, TP	1.75	
2				First immune
3	Incomplete	IM	2.5	
4				Second immune
5				Third immune
6	Incomplete	IM	2.5	
7				Fourth immune
8	Incomplete	IM	2.5	
9	Incomplete	IM		Fifth immune
Group II Rabbits 4,5,6				Pre-immune
0	Complete	ID(15 sites)	4.7	
1				First immune
2	Incomplete	IM	4.7	
3				Second immune
4	Incomplete	IM	4.7	
5				Third immune
6	Incomplete	IM	4.7	
7				Fourth immune
9	Incomplete	IM	4.7	
10				Fifth immune
11				Sixth immune

Immunizations were administered according to the above schedule. Intradermal (ID) were delivered as small doses at multiple sites. Intramuscular (IM) injections were given either to the hind leg quarter or shoulder-neck area in larger doses. Toe pad (TP) injections were administered to each of the toe pads.

brilliant blue.

Immunoabsorption

Antisera were absorbed with whole microsomes of trout liver from either control or 300 ppm CPFA diets. Pooled antisera (0.2 ml) were incubated for 15 hours at 4°C with microsomal protein (1 mg/ml in phosphate buffered saline (PBS)(10 mM phosphate, pH 7.4, 0.9% NaCL) in a total volume of 1 ml. Precipitating antigen-antibody complexes were separated from nonreacting antibodies by centrifugation at 100,000 x g for 1 hour. The antisera were absorbed three to six more times by incubating the resulting supernatants with equal volumes of chromatin and separating the antigen - antibody complexes by centrifugation each time.

Immunochemical Staining of Antigenic Proteins

NC sheets containing transferred antigens were saturated with proteins by incubation in phosphate buffered saline (PBS), containing 3% bovine serum albumin (BSA) and 10% heat inactivated horse serum (HS), overnight at 4°C. This prevented non-specific binding of immunoglobulins in subsequent incubations. The protein saturated sheets were shaken gently for either 2-3 hours at room temperature or overnight at 4°C in antisera diluted into the BSA-HS buffer. Antisera dilutions ranged from 1:100 to 1:10,000. For immunoabsorption, isolated microsomes were used as an absorbent in the ratio of 1.0 mg of microsomal protein to 1.0 ml of 1: 5 diluted antisera in PBS, pH 7.2 The mixture was gently vortexed and allowed to -

incubate overnight at 4°C. Microsomes with the bound antibodies were then removed by centrifugation at 100,000 x g for 1 hr and the supernatant was collected and absorbed several more times with fresh microsomes. The absorbed antisera were used to localize immunoreactive proteins transferred to NC sheets. After exposure to either unabsorbed antisera or absorbed antisera the PAP staining proceeded as follows. All incubations were carried out at room temperature and with gentle rocking. After incubation with antisera the NC sheets were washed 5 times with 100 ml of PBS for 30 minutes. The NC sheets were next incubated for 30 minutes with goat antisera to rabbit immuno globulin G diluted 1:200 in BSA-HS buffer. The NC sheets were again washed in PBS as before, the final incubations were for 30 minutes with rabbit peroxidase antiperoxidase complex (PAP) diluted 1:300. After the sheets were again washed as above, antigenic bands were stained in 50 mM Tris-HCl pH 7.5, 3,3-diaminobenzidine (0.3 mg/ml), and 0.005% hydrogen peroxide.

Photography

Gels were photographed by a professional photographer using a Linhof Technika 4/5 camera mounted on a vertical copy stand with a 13.5 cm f 3.5 Feiss Planar lens. All exposures were f 32+ (maximum aperature). Acrylamide gels were placed on a fluorescent copy table illuminated from below. Coomassie blue stained gels were photographed using a 4713 yellow filter with an exposure time of 3 seconds. The NC sheets were placed on opaque plexiglass; illuminated

from above and photographed by the same procedure. Color photographs were done with a Nikon 35 mm camera with close-up attachment.

Phospholipase A_2 Treatment

Hydrolysis of microsomal membrane by phospholipase A_2 was carried out as follows. Phospholipase A_2 was diluted in incubation buffer (0.25 M sucrose, 10 mM Tris and 5 mM $CaCl_2$, pH 7.4) so that appropriate additions of the enzyme to the microsomal solution would be between 5-25 µl. Microsomal membrane, washed and resuspended in the incubation buffer, was diluted to a concentration of 2 mg/ml determined by protein (Lowry et al., 1951). Membranes were incubated for 30 minutes at 30°C in a metallic shaker bath (Research Specialists Co.) in the presence of phospholipase A_2 at concentrations specified. Microsomes to be used in enzyme analysis were transferred to ice, decanted immediately into appropriate tubes and centrifuged at 180,000 x g at 4°C for 75 min. No significant phospholipase A_2 activity occurs at 4°C. Pelleted membranes were resuspended in buffer (50 mM KPO_4, 1 mM EDTA, pH 7.6) put on ice and analyzed that same day. Microsomes to be used for phospholipid analyses were extracted immediately after the incubation period by the method of Bligh and Dyer (1959). The lipid extracts were dried under nitrogen and stored in benzene at -15°C until analyzed.

Analysis of Microsomal Enzymes

NADPH-cytochrome-P-450 reductase was performed as described

by Mazel (1971). Rate of reduction of cytochrome c per minute was measured at 550 nm at 25°C using approximately 0.3-0.5 mg microsomal protein per tube. Cytochrome P-450 was measured by a modification of the method described by Mazel (1971). A difference spectra of 2 mg microsomal protein per tube was recorded. The sample cell had sodium dithionite added to the microsomes while the reference cell did not. Both tubes were bubbled with carbon monoxide and absorption maxima measured at 450 minus 500 nm.

Analysis of Microsomal Phospholipids

Phospholipids were separated on 20 cm long glass plates coated with 0.5 mm silica gel H. Plates were prewashed in a solvent system of chloroform/methanol/aqueous ammonia (65:35:4, v/v/v) and activated at 100°C for 1 hour. Lipids were applied in 1 cm bands and the plates run in chloroform/methanol/water (70:30:4, v/v/v) until the solvent front migrated 15 cm from the origin. Bands were visualized by exposure to I_2 vapor and marked on silica gel. Following visualization, phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) were scraped from the plate and lipid phosphorous analyzed by the precedure of Bartlett (1959).

For fatty acid analysis of PC and PE, the phospholipids were scraped into a screw top test tube. Three ml of 4% H_2SO_4-MeOH were added, then the tube was flushed with N_2 and heated at 80°C for 90 min and then stored in a freezer. The transesterified fatty acids were extracted with hexane and analyzed by method of Hofstetter et al.,

(1964) at 180°C on a Varian aerograph series 1200-gas chromtograph using a six food GP 10% SP 2330 on 100/200 chromosorb WAW-(cyanosilicone) (Supelco) column.

For a two-dimensional separation of phospholipids, the following procedure was used. Glass plates were spread .25 mm thick with silica gel H containing 7.5% magnesium acetate. They were spotted with approximately 1 mg of lipid extracted from microsomal homogenate in lower left hand corner of plate. Plates were kept under N_2 during spotting. They were developed to 15 cm in chloroform/methanol/ammonia (65:35:5) for the first dimension. Next, the plates were dried under N_2 for 30 min and developed in the second dimension solvent system consisting of chloroform/acetone/methanol/aceticacid/H_2O HOAc/H_2O (30:40:10:7:5). Phospholipids were identified by I_2 vapor and individual spots were scraped into test tubes for Pi analysis. Total Pi was determined as previously mentioned.

Protease Treatment

Proteolysis of microsomal membranes was carried out using pronase from Streptomyces griseus and trypsin from bovine pancreas. Solutions were made the same day of the experiment by dilution of the proteases in microsomal wash buffer (0.25 M sucrose, 10 mM Tris, pH 7.4) so that the final concentrations were 0.5 mg/ml for pronase and 2 mg/ml for trypsin. Membranes (4-6 mg protein/ml) suspended in the wash buffer, were incubated for 15 minutes at 23°C for pronase and 30°C for trypsin at concentrations specified. The reaction was terminated in the following manner. Incubation tubes

were first transferred to ice. Trypsin inhibitor from egg hen white (Sigma Chemical Co.) was added to all trypsin incubations in a 5-fold excess (w/w) of inhibitor to trypsin and vortexed. Phenyl methylsufonyl fluoride (PMSF), a serine protease inactivator, was added to all pronase incubations at concentrations of 1 ul of 1 mM PMSF per 2 ug of pronase and vortexed. Immediately following this inactivation 5 ml aliquots were placed into ultracentrifuge tubes and spun down at 200,000 x g for 1 hr. Post incubation proteolytic activity was minimized under these conditions (Orrenhius et al., 1969). Pelleted membranes that were to be analyzed for biochemical assays were resuspended in buffer (50 mM KPO_4, 1 mM EDTA, pH 7.6) and analyzed that same day. Membranes that were to be used for polyacrylamide gel electrophoresis were resuspended in a LDS buffer (2.0% LDS, 12.0% sucrose, 20 mM dithiothreitol, 50 mM Tris-HCl, and 2 mM EDTA, pH 6.8) and stored at -80°C until analyzed.

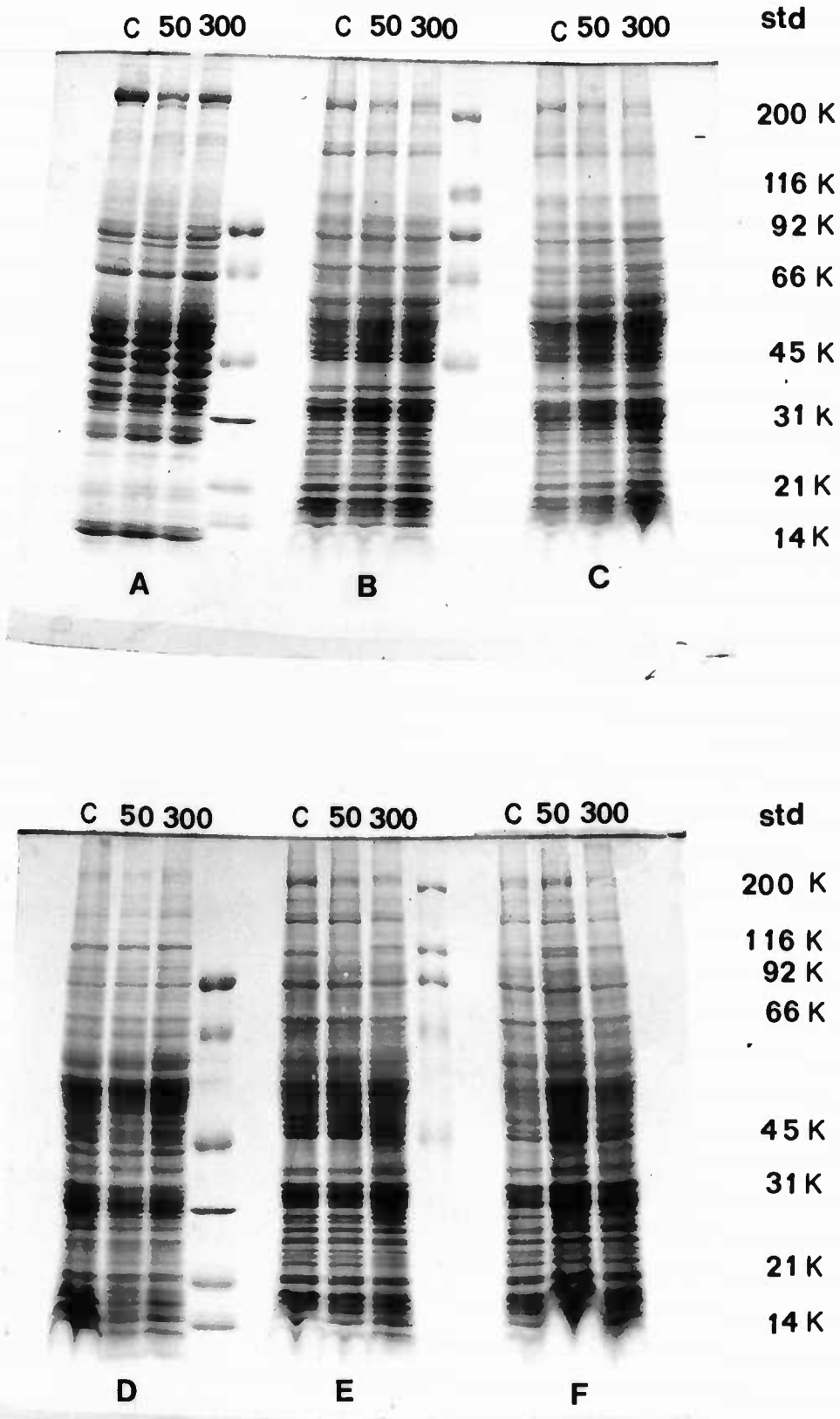
RESULTS

Polyacrylamide Gel Electrophoresis of Subcellular Fractions of Trout Liver

Subcellular fractionation of livers of rainbow trout were performed on two generations of fish that varied in age from 8-18 months and had been on CPFA diets from 5-20 weeks. It had been previously reported (Selivonchick et al., 1981) in our laboratories that CPFA induced a 41.5 kilodaltons (K) molecular weight protein after several weeks of feeding. In the two generations of fish that we studied, a 41.5 K molecular weight protein was not found in higher concentrations of CPFA-microsomes by visualization of the acrylamide gels. Although no new proteins were discovered, there were several interesting trends. Microsomes from rainbow trout fed CPFA in their diet for a minimum of five weeks had fewer higher molecular weight proteins (180K-220K) than their control counterparts. This is shown in Figure 4 in sections B, C, D and E. The two other subcellular fractions represented in sections A and D are the supernatant and the mitochondrial fraction respectively. For the most part, changes in the protein profile for these other subcellular components are less than those found in the microsomal fractions.

Since the observations of slab gels were visual, subcellular fractions were run in tube gels and scanned with a densitometer to better assess differences (Figure 5). Two areas for comparison

Figure 4. PAGE of subcellular fractions isolated from trout liver. (A) microsomal supernatant, (B) microsomes, (C) post nuclear, (D) large particles, (E) microsome (F) post nuclear


C 50 300
C 50 300
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std
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C 50 300
C 50 300
C 50 300
std
200 K
116 K
92 K
66 K
45 K
31 K
21 K
14 K
D
E
F

Figure 4

Figure 5. Densitometry scans of microsomes from trout liver electrophoresed on tube gels.

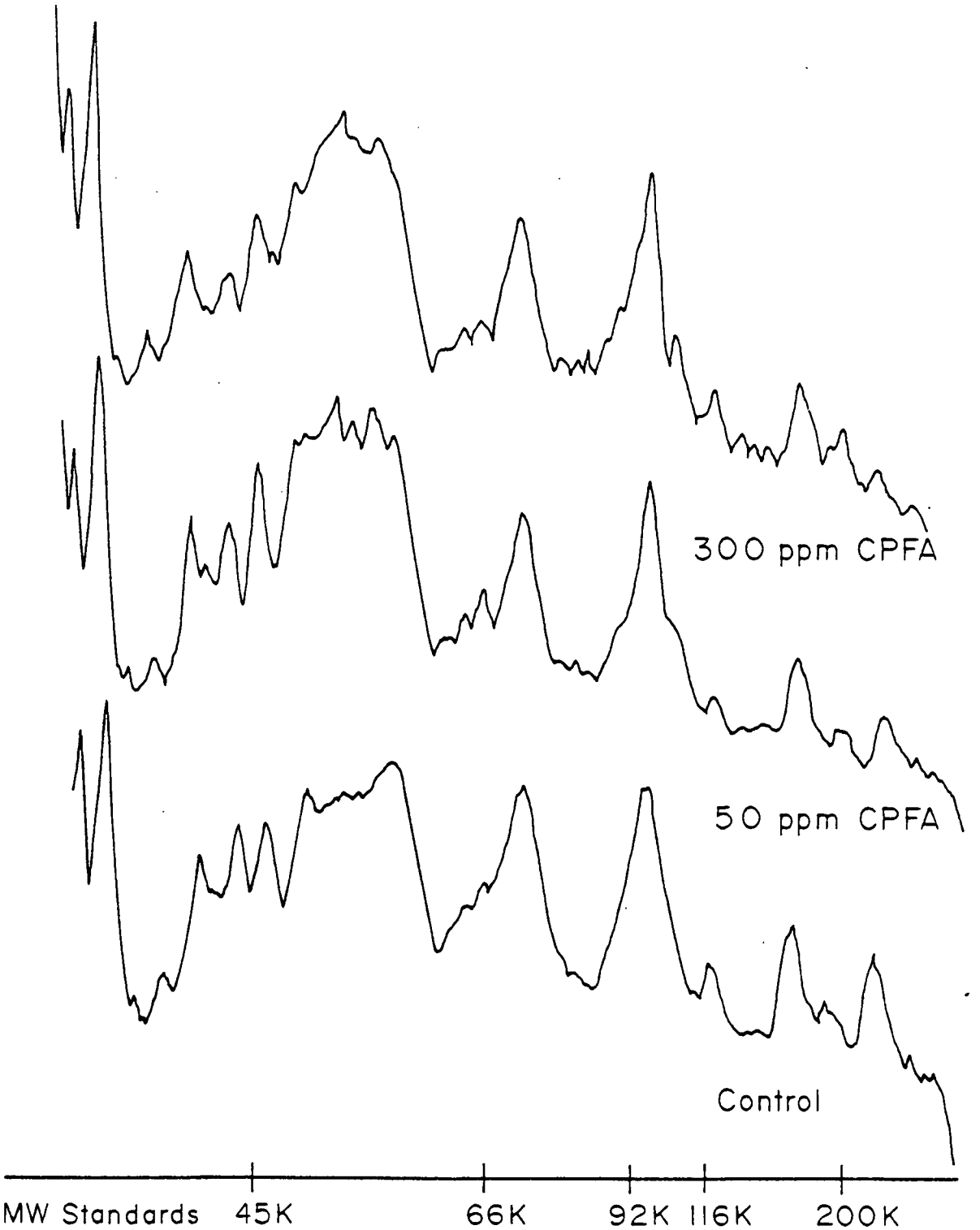

300 ppm CPFA
50 ppm CPFA
Control
MW Standards
45K
66K
92K
116K
200K

Figure 5

should be noted. A middle molecular weight region showed some variations between the diets. However, this area was so heavily populated with proteins that real differences were difficult to determine. In the high molecular weight region (180K-220K) there is a distinct reduction of peak heights of the liver subcellular proteins from CPFA treated fish. This represents a diminished protein concentration of the higher molecular weight proteins that was observed in the slab gels.

To assure ourselves that these changes were not due to endogenous protease activity, microsomal preparations with several protease inhibitors (PMSF, EDTA, iodoacetamide) were done. Isolation of microsomes with these inhibitors showed no appreciable differences from those microsomes isolated in buffer alone when resolved on polyacrylamide gels (Figure 6). These results concur with previous research in our lab with the protease inhibitors. There remained the possibility that the diminished molecular weight components or the appearance of bands with slightly less molecular weight might be the result of preferential reduction or aggregation of protein subunits in the CPFA subcellular fractions. Isolation of subcellular fractions with DTT, which would reduce all molecular subunits and inhibit aggregation was done and is shown in Figure 7. Again there is no essential difference in those subcellular fractions isolated with DTT and those without.

Figure 6. PAGE of microsomes treated with protease inhibitors (1) control-untreated, (2) control-PMSF treated, (3) control-iodacetamide treated, (4) standard, (5) 300-untreated, (6) 300-PMSF treated (7) 300-iodacetamide treated (8) standard, (9) 300-untreated, (10) 300-PMSF treated, (11) 300-iodoacetamide treated.

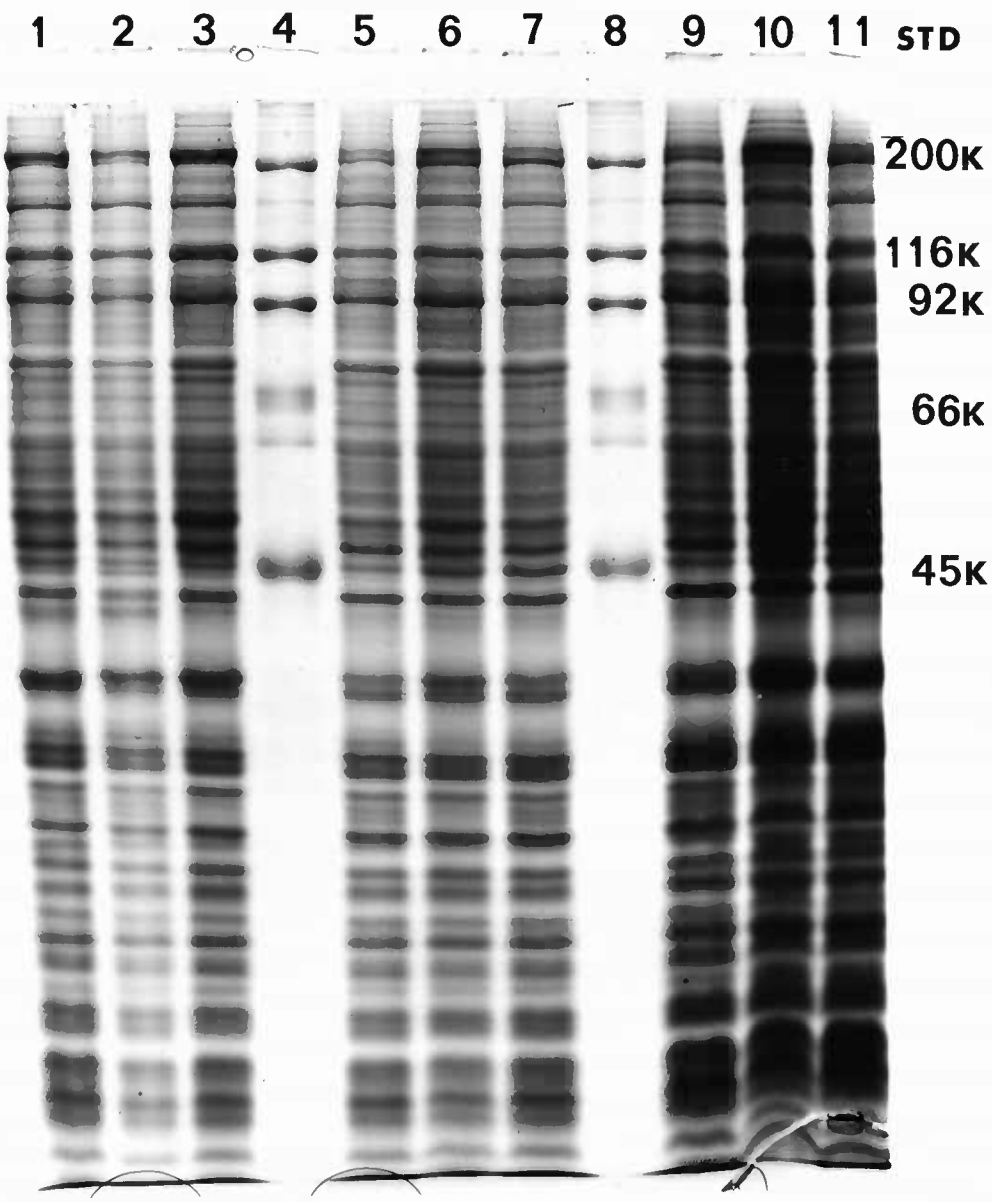
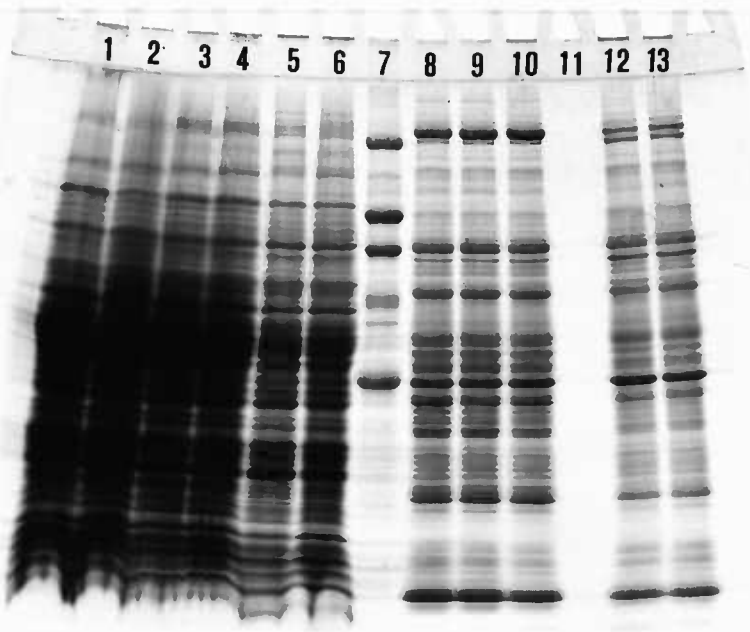
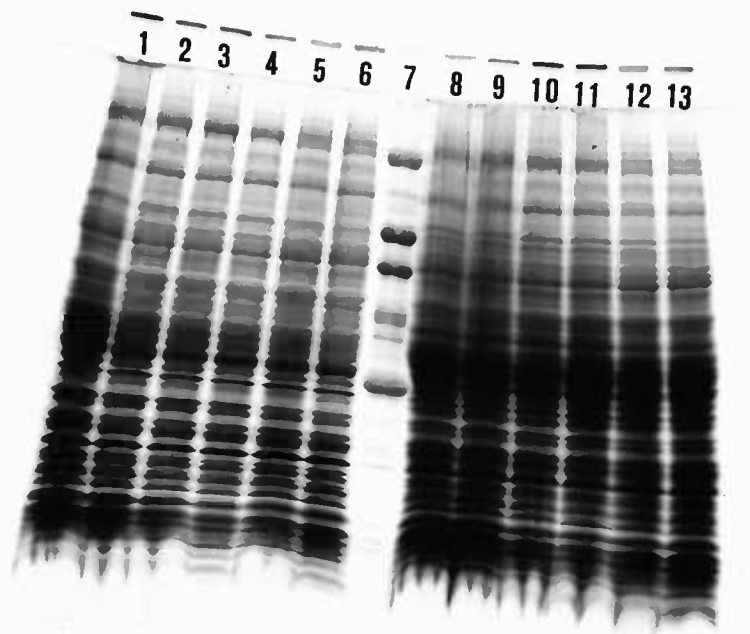


Figure 6

Figure 7. PAGE of subcellular fractions treated with DTT. (I) (1-6) large particle, (8-13) microsomal supernatant; (II) (1-6) microsome, (8-13) Post-nuclear, (7) standards. (1,8) control-no treatment, (2,9) control - DTT, (3,10) 50-no treatment, (4,11) 50-DDT, (5,12) 300-no treatment, (6,13) 300-DDT.



I



II

Figure 7

Immunological Assays

Western Blots

Transfer of proteins from an acrylamide gel to NC is shown in Figure 8. On the far left (A) side is transferred protein on a NC sheet which was cut in thirds after the transblot was made. The section shown here is stained with Amido black as described in the methods. Section B is the polyacrylamide gel from which the proteins were transblotted. Section C represents an unblotted polyacrylamide gel of the same composition that was run concurrently and stained with Coomassie blue. Although the transfer is not linear for all the proteins separated on the acrylamide gel, a substantial number of protein bands can be observed. The most active region of transfer are proteins in the molecular weight region of 40K-120K. The transferred molecular weight standards readily demonstrate this phenomenon. All but the highest molecular standards (200K) have disappeared from the blotted gel (B) and appear on the NC sheet (A). The NC sheet is larger than its parent polyacrylamide gel due to shrinking of the gels in staining solution.

Production of Antisera Against Whole Microsomes

Immunization of rabbits with microsomal proteins from the livers of rainbow trout was successful in producing antibodies to these proteins. Antisera from several of the bleedings were tested by rocket immunoelectrophoresis. Although serum proteins interfered with the staining procedure by creating a dark background and

Figure 8. PAGE of microsomes and Western blot. (A) Amido black stain of total proteins transferred to nitrocellulose sheets, (B) PAGE of various proteins and then transblotted to nitrocellulose sheet, (C) non-blotted polyacrylamide "companion" gel.

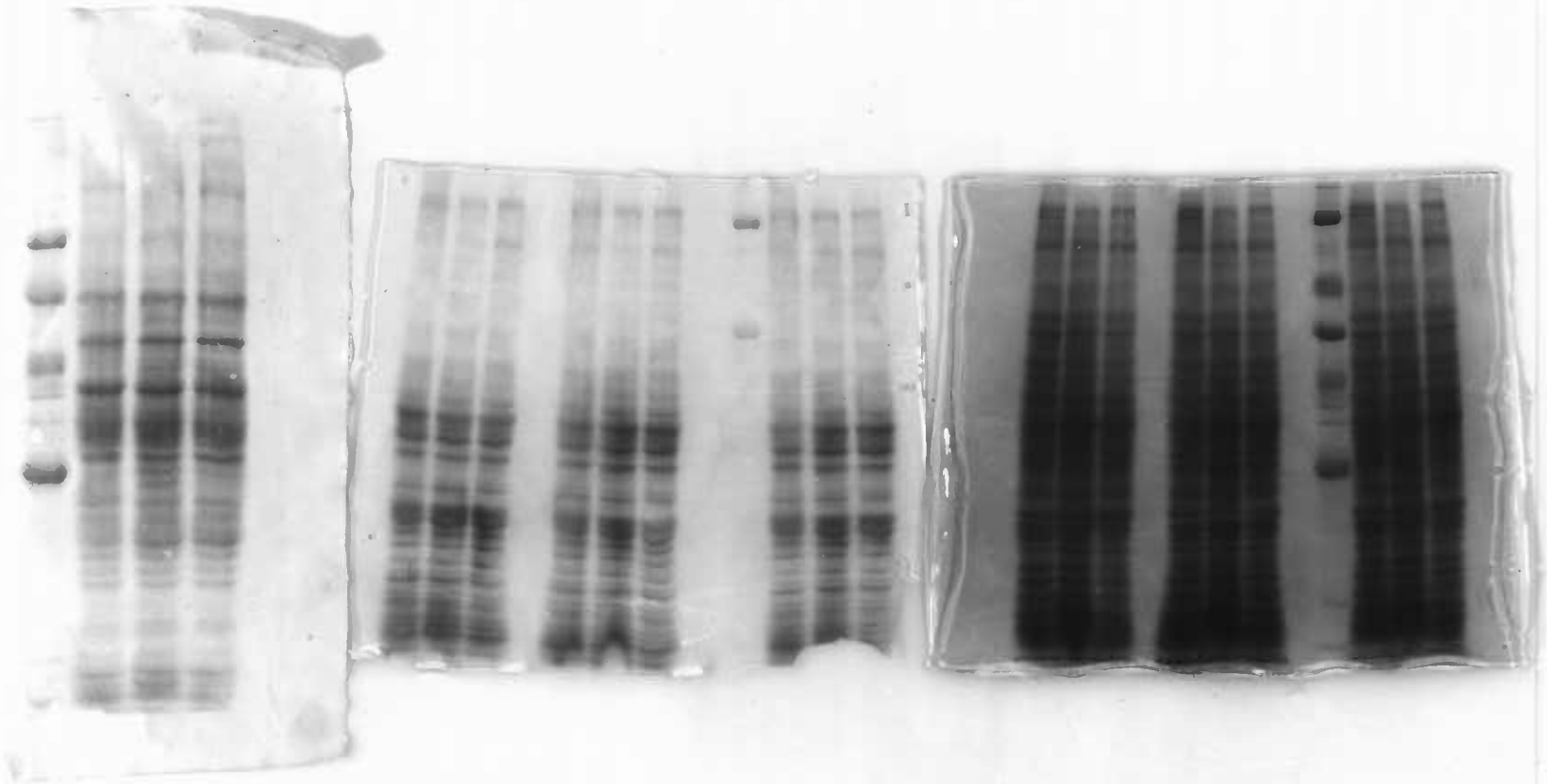


Figure 8

precluded photographs, rocket immunoelectrophoresis allowed us to choose the antisera that appears to have the greatest antigen-antibody interaction. Pooled sera from Group II rabbits-fourth bleeding (Table 1) was used for the majority of immunochemical analysis of microsmal proteins.

The electrophoretic identification of antigenic microsomal proteins by their molecular weight is shown in Figure 9-I Section A. There are numerous distinct bands throughout all the molecular weight regions. Although some minor quantitative differences existed in some bands between control and CPFA treated microsomes no definite qualitative differences could be objectively detected by incubation with unabsorbed antisera. It is of interest to note the intensity of staining in the molecular weight region above 116 K. As observed with the Western blot (B), the fewest number of proteins transfer to the NC from this region. Nonetheless, it invariably stained the darkest. Although the intensity of staining often masks the antigenic bands themselves, the proteins that appear strongest in antigenicity have approximate molecular weight values of 96K, 60K and 30K.

To insure against non specific binding of the PAP complex to the NC sheets, several control experiments were run. Figure 9-II represents a single NC sheet with transferred microsomal protein, subsequently cut into sections, and tested independently with incubations from Group II rabbits- fourth bleeding and pre-immune sera from the same rabbits. No bands appeared in the NC sheet incubated with pre-immune sera (E). Furthermore, NC sheets that had recently

Figure 9. Identification of antigenic proteins from various microsomal proteins transferred to nitrocellulose sheets. (I-A) immunochemical staining of antigens, (I-B) Amido black stain of total proteins transferred to nitrocellulose sheets, (II-C) Amido black stain of total proteins transferred to nitrocellulose sheets, (II-D) immunochemical staining of antigens, (II-E) immunochemical staining of antigens incubated with pre-immune sera.

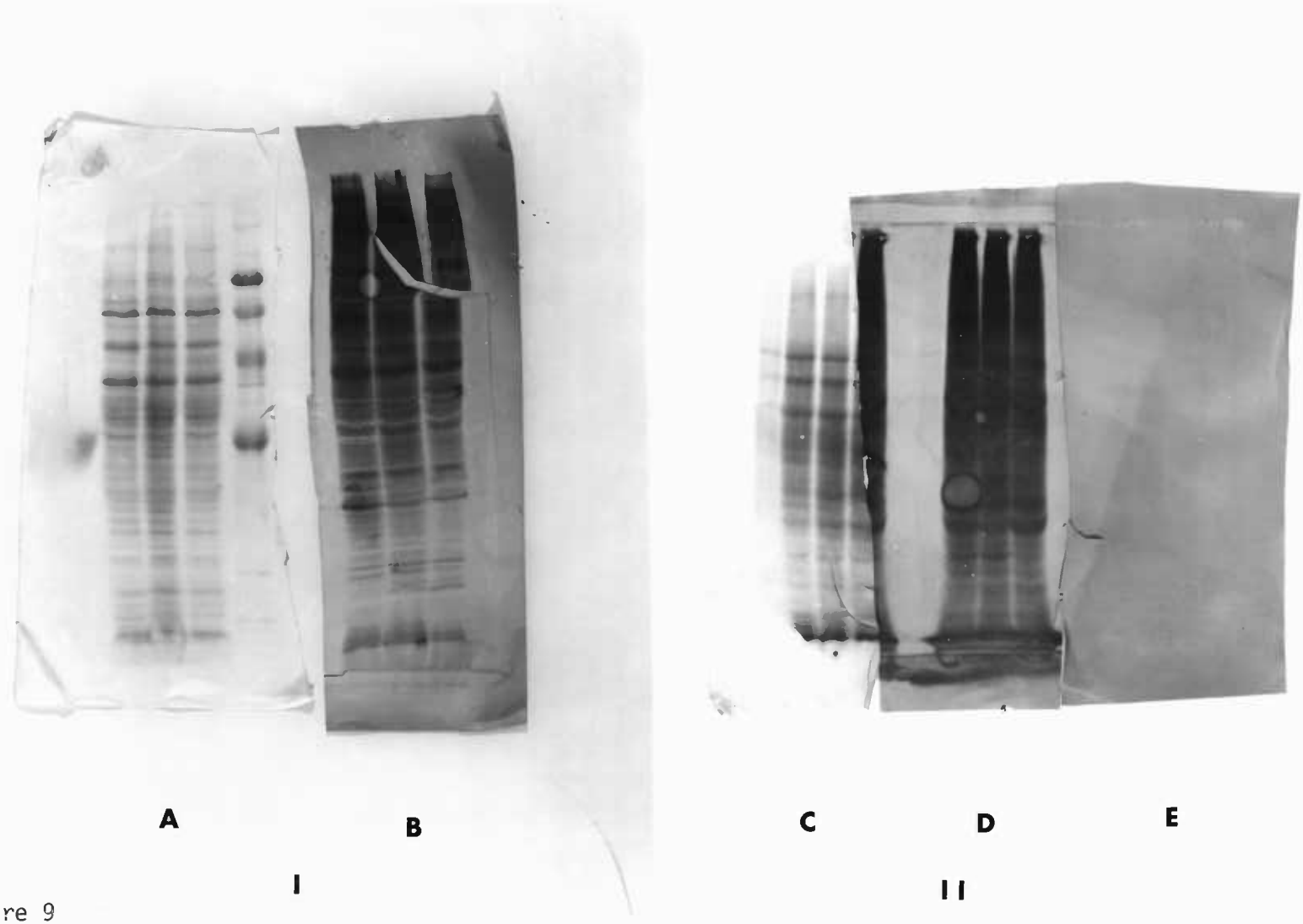


Figure 9

received transferred microsomal proteins were washed in PBS and allowed to incubate with the peroxidase substrate for an extended period of time to test for endogenous microsomal peroxidases. No bands were visualized after incubation.

Immunoabsorption

Figure 10 represents two separate experiments (I and II). Section A and D are Western blots for each of the experiments. As noted previously, proteins in the mid-molecular weight regions are more efficiently transblotted. Section B is an immunotransfer incubated with 40 µl of straight antisera and stained by PAP. In an attempt to determine if any new proteins are induced by CPFA diets, antisera was immunoabsorbed with control-microsomes as described in the methods. This has become a common procedure to eliminate antibodies that are cross-reactive between normal tissue and tissue that has undergone some profound biological changes. Section C represents an incubation with antisera that has been absorbed three times. Differences between the 300, 50 and control lanes are quantitative rather than qualitative. Rather than a disappearance of all but a few of the bands representing CPFA-induced protein, there is a gradual lessening of intensity in staining in the control and 50 ppm CPFA lanes. This is also demonstrated in experiment sections E and F. In this experiment, a sequential immunoabsorption of the same antisera with control microsomes was performed. A portion of antisera was immunoabsorbed twice then divided equally, one half was immunoabsorbed once more for a total of three times. This is shown

Figure 10. Immunochemical staining of antigens with immunoabsorbed antisera. (A) Amido black stain of total proteins transferred to nitrocellulose sheets, (B) immunochemical staining of antigens, (C) immunochemical staining of antigens with antisera absorbed 3 times, (D) Amido black stain of total proteins transferred to nitrocellulose sheets, (E) immunochemical staining of antigens with antisera absorbed 5 times, (F) immunochemical staining of antigens with antisera absorbed 3 times.

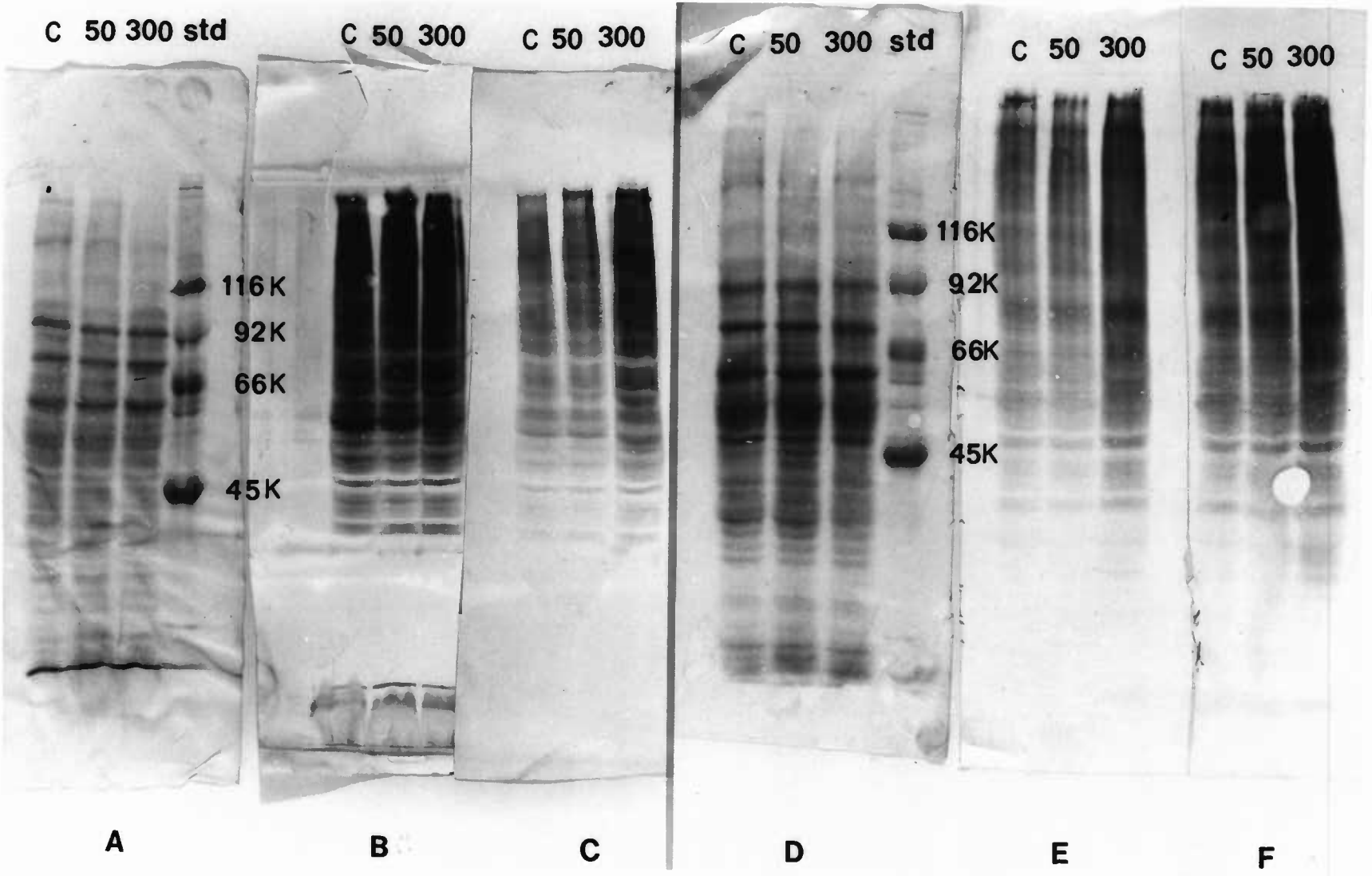

C 50 300 std
C 50 300
C 50 300
C 50 300 std
C 50 300
C 50 300
116K
92K
66K
45K
116K
92K
66K
45K
A
B
C
D
E
F
I
II

Figure 10

in section F. The other half of the twice absorbed antisera was further immunoabsorbed three more times for a total of five absorptions. This is represented in section E. Section F shows a slight loss of color in the control lanes which is further enhanced, along with the 50 lane in section E. Exhaustive immunoabsorption (seven times or greater) leads to an equal diminishing of PAP staining in all sample lanes. Although sections C and F both represent an immunoabsorption of three times, there are greater changes in section C. This is probably caused by the fact that there appears to be less initial transfer of protein to the NC sheets in experiment 1. Immunoabsorption of antisera with 300 ppm-CPFA-microsomes showed equal diminishing of PAP stain in all sample lanes.

Immunotransfer With Antisera to Cytochrome P-450 and Cytochrome P-448

Microsomes from control, 50 and 300 ppm CPFA were separated on acrylamide gels, transblotted onto NC sheets, cut into sections and incubated independently with antisera raised against trout liver cytochrome P-450 and cytochrome P-448. This is shown in Figure 11. Section A represents incubation with cytochrome P-448 and section B represents incubation with cytochrome P-450. Cytochrome P-450 is the predominant form of two mixed function oxidase systems in liver of rainbow trout. Visual observation of cytochrome P-450 by PAP staining does not show any differences in intensity of stain between the control and the CPFA diets. However, the intensity of the stain for cytochrome P-448 is substantially diminished for microsomes from fish fed a 300 ppm CPFA diet.

Figure 11. Identification of cytochrome P-450 and cytochrome P-448 with antisera against purified cytochrome P-450 (MW 54K) and purified cytochrome P-448 (MW 59K) (A) immunochemical staining of antigens incubated with cytochrome P-448 antisera, (B) immunochemical staining of antigen incubated with cytochrome P-450 antisera.

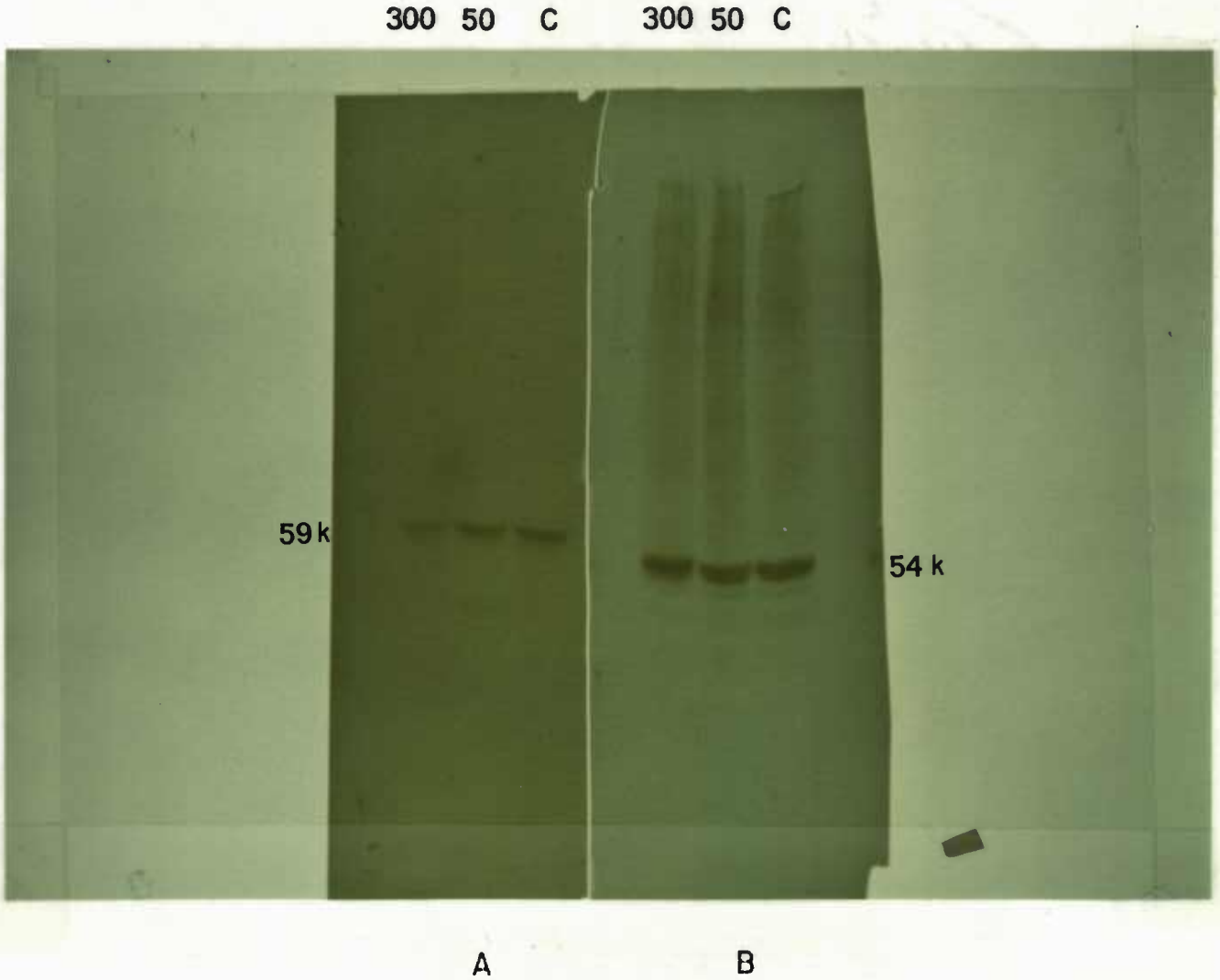


Figure 11

Phospholipid Analysis of Microsomes

The phospholipid analysis of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in microsomes from the livers of rainbow trout fed different diets has several interesting features as presented in Table 2. The increase in the saturated 18:0 to unsaturated 18:1 ratios has been used by this laboratory as a useful index of CPFA mediated effects on membrane systems. This change toward saturation has been seen even after a few weeks of dietary addition of CPFA. Since the rainbow trout exists in cold environment there is a very high level of polyunsaturated fatty acids that are found in membranes. There is an overall increase in the number of saturated fatty acids in fish fed the CPFA diet while the total polyunsaturated levels decline. This is not an across the board decline as there is a slight increase in several of the highly polyunsaturated fatty acids (20:4, 20:5). The monounsaturates, however, all decreased in concentration with CPFA. As can be seen from Table 3, the phospholipid composition of liver microsomes from fish on control and CPFA diets are similar.

Preliminary experiments of microsomal incubations with concentrations greater than 1.0 μg of phospholipase A_2 for 30 minutes showed that a maximum hydrolysis of phospholipids occurred. Hydrolysis beyond 60% of individual phospholipids indicate a reaction too exhaustive throughout the microsomal membrane to prove useful in detecting subtle changes in the membranes brought about by dietary changes. Moreover, cytochrome P-450 dependence on phospholipids for activity could not be demonstratible. With concentrations

Table 2. Composition of the Constitutent Fatty Acids (wt%) of Phosphotidyl-ethanolamine and Phosphotidylcholine from Liver Microsomes of Rainbow Trout Fed Different Levels of Cyclo-propenoid Fatty Acids for 18 weeks.

Table 2

Acyl Groups[a]	Ethanolamine Phospholipids			Choline Phospholipids		
	Control	50 ppm	300 ppm	Control	50 ppm	300 ppm
14:0	0.58 ± 0.14	0.64 ± 0.15	0.83 ± 0.07	1.91 ± 0.17	1.85 ± 0.18	1.44 ± 0.13
16:0	11.46 ± 1.18	10.99 ± 1.02	13.94 ± 0.45	28.64 ± 0.50	29.43 ± 1.51	29.90 ± 0.79
16:1	2.74 ± 0.11	1.23 ± 0.23	1.45 ± 0.36	5.84 ± 0.50	3.65 ± 0.03	3.37 ± 0.25
18:0	6.54 ± 0.32	13.96 ± 0.52	15.02 ± 0.46	6.32 ± 0.63	10.89 ± 0.68	11.67 ± 0.51
18:1	28.19 ± 1.96	19.56 ± 1.09	17.62 ± 0.72	18.00 ± 1.67	12.70 ± 1.05	13.04 ± 0.25
18:2	0.48 ± 0.07	0.27 ± 0.23	0.33 ± 0.23	0.76 ± 0.08	0.49 ± 0.03	0.54 ± 0.04
20:1	5.14 ± 0.41	4.47 ± 0.46	2.32 ± 0.08	2.19 ± 0.32	1.23 ± 0.15	0.69 ± 0.05
20:2	1.74 ± 0.27	0.16 ± 0.08	0.57 ± 0.73	1.30 ± 0.16	0.74 ± 0.50	0.44 ± 0.13
20:3	0.16 ± 0.03	0.16 ± 0.16	0.23 ± 0.07	0.46 ± 0.05	0.44 ± 0.03	0.45 ± 0.10
20:4	1.56 ± 0.27	2.27 ± 0.90	2.47 ± 1.17	1.39 ± 0.07	2.36 ± 0.36	2.49 ± 0.22
20:5	2.98 ± 0.51	3.85 ± 0.56	3.71 ± 1.01	1.97 ± 0.22	2.81 ± 0.31	4.28 ± 0.46
22:4	1.56 ± 1.99	0.76 ± 0.99	1.11 ± 1.08	0.39 ± 0.21	0.67 ± 0.59	1.86 ± 1.77
22:5	3.02 ± 0.98	2.70 ± 0.38	2.66 ± 0.67	2.92 ± 0.46	2.63 ± 0.24	3.12 ± 0.31
22:6	32.97 ± 3.35	35.72 ± 3.41	33.38 ± 0.30	26.65 ± 3.84	27.92 ± 3.39	25.53 ± 1.48
Total Saturated	18.58	25.59	29.79	34.87	42.17	43.01
Total Polyunsaturated	80.54	71.15	65.85	61.87	56.61	55.81
UFA/SFA	4.33	2.78	2.21	1.77	1.32	1.30
18:0/18:1	0.23	0.71	0.85	0.35	0.86	0.89
U.I.[b]	281	286	270	223	228	228

[a] Acyl group values represent the mean (±SD) of duplicate analysis from at least three experiments. Plasma membranes were prepared from at least six livers pooled.

[b] U.I. = unsaturated index defined as Σ (number of double bonds in each fatty acid) X (mol % of each fatty acid).

TABLE 3. Phospholipid Composition of Microsomes Isolated From the Liver of Rainbow Trout

Phospholipid	% Composition[a,b]	
	Control	CPFA
phosphatidylserine	2.9 ± 0.25	2.9 ± 0.72
phosphatidylethanolamine	17.81 ± 0.89	18.94 ± 2.27
physphatidylcholine	67.38 ± 4.38	64.47 ± 2.36
phosphatidylinositol	5.40 ± 0.26	6.47 ± 0.08
phosphatidic acid	trace	trace
sphingomyelin	3.81 ± 0.08	3.60 ± 0.06
cardiolipid	1.27 ± 0.11	2.30 ± 1.80

[a]Phospholipid content is expressed in percentage of total lypid phosphorous.

[b]Phospholipid values represent the mean (±SD) of duplicate analysis of two experiments.

from 0.1 µg - 1.0 µg of phospholipase A_2 per 2 mg microsomal protein, there is a selective hydrolysis of membrane phsopholipids. As presented in Table 4, cytochrome P-450 activity gradually decreased with increasing levels of phospholipase A_2. Interestingly, NADPH-cytochrome-C-reductase showed a stable increase at all concentrations of phospholipase A_2.

Figure 12 shows composite data for hydrolysis of the two major phospholipids for four concentrations of the enzyme. The data represents results obtained from three separate membrane preparations. There is a progressive loss of cytochrome P-450 activity with increasing phospholipid hydrolysis. However, there did not appear to be a direct relationship between initial hydrolysis and reduction of P-450 activity. At the low phospholipase A_2 concentrations the percent of hydrolysis was significantly greater than the percent loss of activity. It appears that the P-450 enzyme can accomodate a 50% loss of key phospholipids and still retain approximately 80% of its activity.

Treated microsomes from rainbow trout fed a CPFA diet for 10 weeks were compared to those on the control diet. For the most part NADPH-cytochrome c reductase and cytochrome P-450 acitvity is reduced 25-50% from their corresponding controls. However, when each group is studied independently, there are striking similarities of the effects of phospholipase A_2 treatment. One can assume that alterations in membranes due to CPFA did not affect the phospholipid content nor the ability of the phospholipase A_2 enzyme to hydrolyze the number 2 acyl position. Correspondingly, there is agreement in

TABLE 4. Cytochrome P-450 and NADPH Cytochrome C Reductase Levels of Microsomal Membrane (2 mg Protein/Sample) Isolated From Trout on Control and 300 ppm CPFA Diets and Treated With Varying Concentrations of Phospholipase A_2[a]

Sample	Cytochrome P-450 (nmol/mg microsomal protein) Phospholipase A_2 Levels (μg) 0.0	0.1	0.5	0.75
Control	0.264 ± 0.039	0.245 ± 0.090	0.079 ± 0.08	0.054 ± 0.028
CPFA	0.164 ± 0.024	6.121 ± 0.020	0.028 ± 0.025	0.032 ± 0.023
Molar Ratio CPFA/Control	0.61	0.49	0.34	0.59
	NADPH-Cytochrome c Reductase (nmol/mg microsome protein/min)			
Control	10.26 ± 1.57	9.27 ± 2.52	13.20 ± 1.37	12.91 ± 0.72
CPFA	5.24 ± 1.20	7.07 ± 1.22	6.28 ± 1.58	6.28 ± 1.00
Molar Ratio CPFA/Control	0.51	0.76	0.48	0.49

[a]Values represent the mean ± standard deviation of triplicate samples.

Figure 12. The effects of phospholipase A_2 (PLA_2) on the hydrolysis of choline and ethanolamine phospholipids of microsomes isolated from liver of rainbow trout. Control-microsomes are presented in graph form. CPFA-microsomes are presented in Table inserts.

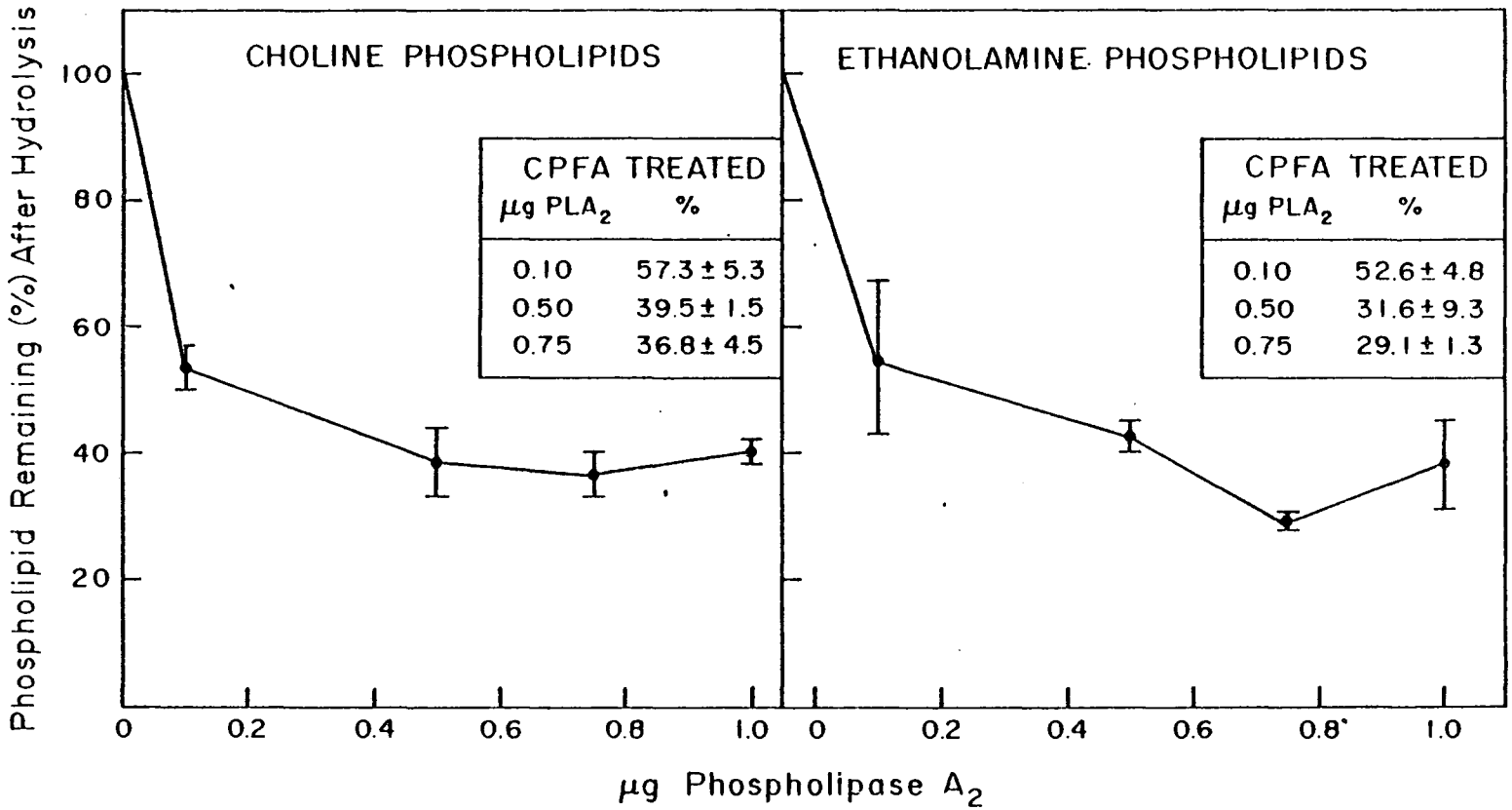

CHOLINE PHOSPHOLIPIDS
ETHANOLAMINE PHOSPHOLIPIDS
CPFA TREATED
µg PLA2 %
0.10 57.3±5.3
0.50 39.5±1.5
0.75 36.8±4.5
CPFA TREATED
µg PLA2 %
0.10 52.6±4.8
0.50 31.6±9.3
0.75 29.1±1.3
Phospholipid Remaining (%) After Hydrolysis
100
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
µg Phospholipase A2

Figure 12

enzymatic activity at different phospholipase A_2 concentrations when reported as a percentage of the respective untreated controls. Essentially there are no significant differences in treated microsomes from rainbow trout fed on a control diet and one fed on a diet of CPFA.

Protease Analysis Microsomes

Enzyme Assays and PAGE

The data presented in Table 5 demonstrate that trypsin proteolysis of NADPH cytochrome c reductase, a cytosolic-sided enzyme, was essentially complete at all levels studied. Proteolysis of cytochrome P-450, a transmembrane protein, does not reach maximum levels until an excess of 25 ug of trypsin/mg microsomal protein was used. Similar enzyme inactivation levels were found for pronase at levels of 10 ug/mg microsomal protein (data not shown). Although the levels (25 ug of trypsin and 10 ug of pronase) irreversible destroyed slightly more than 50% of the cytochrome P-450 activity, proteolytic fragments and the disappearance of high molecular weight proteins were more readily observed at these levels in polyacrylamide gel electrophoresis. Consequently, these enzyme levels were chosen for studies comparing the microsomal membrane of rainbow trout fed on a control diet and a CPFA diet.

Figure 13 represents the results of proteolysis on the activity of cytochrome P-450 and NADPH cytochrome c reductase at the protease levels used. In the untreated samples, cytochrome P-450

TABLE 5. Cytochrome P-450 and NADPH Cytochrome C Reductase Levels of Microsomal Membranes Isolated From Rainbow Trout Fed a Control Diet. The Membranes Were Incubated With Increasing Concentrations of Trypsin

Treatment (µg trypsin/mg microsomal protein)	Cytochrome P-450 (nmoles/mg microsomal protein)	NADPH cytochrome c reductase (nmoles/mg microsomal protein)
0.0	0.290	25.45
10.0	0.149	0.85
25.0	0.094	0.73
50.0	0.068	0.85
100.0	0.051	1.22

Figure 13. Digestion of microsomes by trypsin and pronase and its effect on the enzyme activity of cytochrome P-450 and NADPH-cytochrome c reductase.

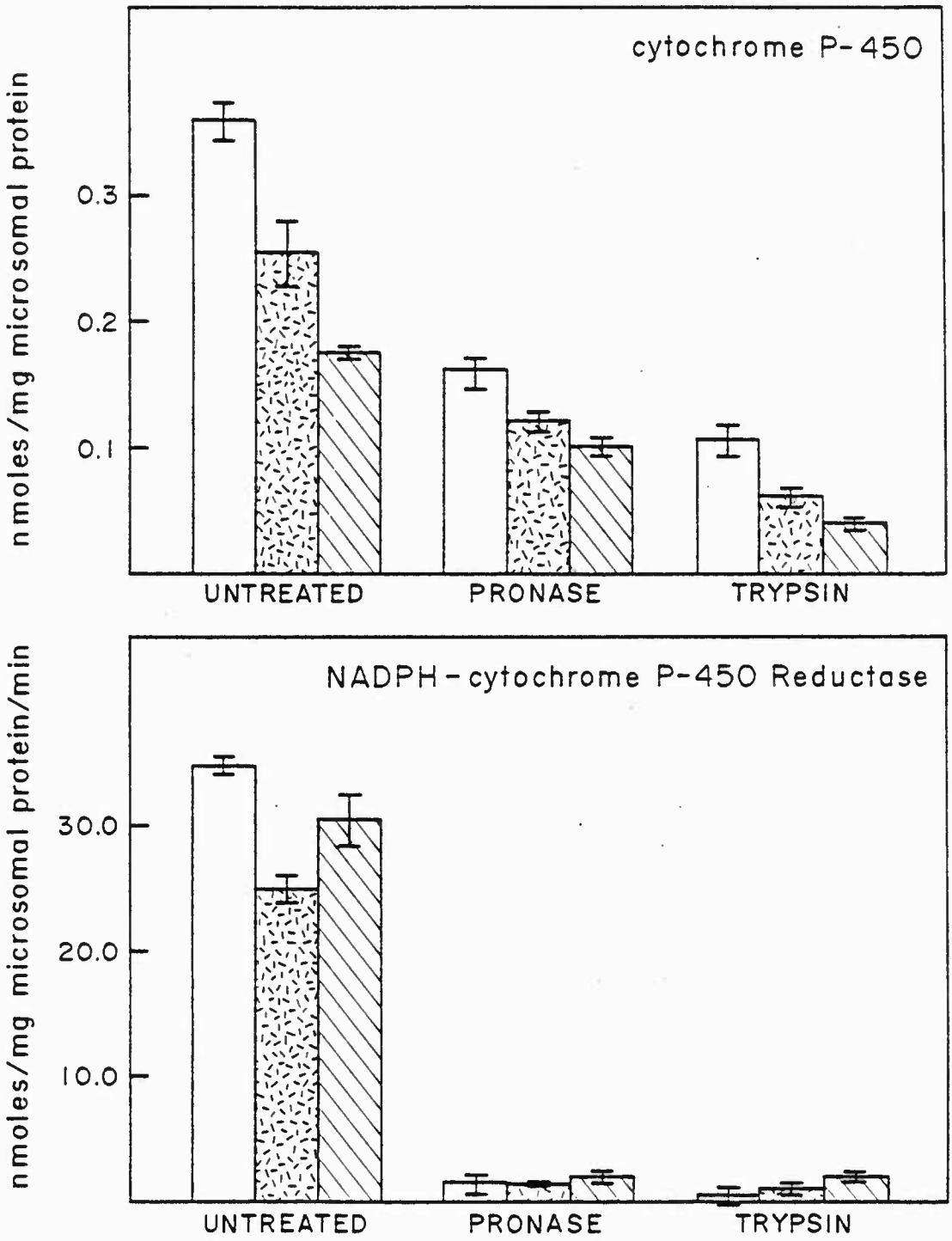

cytochrome P-450
nmoles/mg microsomal protein
0.3
0.2
0.1
UNTREATED
PRONASE
TRYPSIN
NADPH-cytochrome P-450 Reductase
nmoles/mg microsomal protein/min
30.0
20.0
10.0
UNTREATED
PRONASE
TRYPSIN

Figure 13

activity is subsequently reduced in the fish fed the 50 ppm and 300 ppm CPFA diets from those fed the control diet. The NADPH cytochrome c reductase activity also decreased in the groups fed a CPFA diet. The diet in the high CPFA range appeared to have less of an effect than a more intermediate CPFA range. Both proteases essentially destroyed all activity of NADPH-cytochrome c reductase. At the levels used, pronase inactivated 43-55% of the cytochrome P-450 activity whereas trypsin inactivated 60-75%.

Observation of the polyacrylamide gel electrophoretic patterns for the digest products demonstrate differences in digestion between control and CPFA-microsomes. Figure 14 shows a digest from rainbow trout that are a year and a half in age and have been on CPFA diet for 22 weeks. Figure 15 is a proteolytic digest profile of two different groups of ten-month old rainbow trout that have been on CPFA diet for nine weeks. In both figures, Section A represents the undigested microsomes, section B the trypsin digestion and section C on the pronase digestion. Pronase action on membrane proteins is non-specific. The membrane proteins are broken down into polypeptides of low molecular weight that are difficult to resolve in a gradient gel. The trypsin digest is more specific in that it attacks the peptide bonds on the carboxyl side of lysine and arginine. In Figure 14, section B which represents older fish and a longer exposure to CPFA, there are several easily recognizable differences. Several of the proteins around the 92K region have been removed by proteolysis in the control microsomes, while the CPFA-microsomes have retained more of their original

Figure 14. PAGE of protease digestion of microsomes from liver of 18 month old trout fed a CPFA diet for 22 weeks. (A) untreated, (B) trypsin-treated, (C) pronase treated.

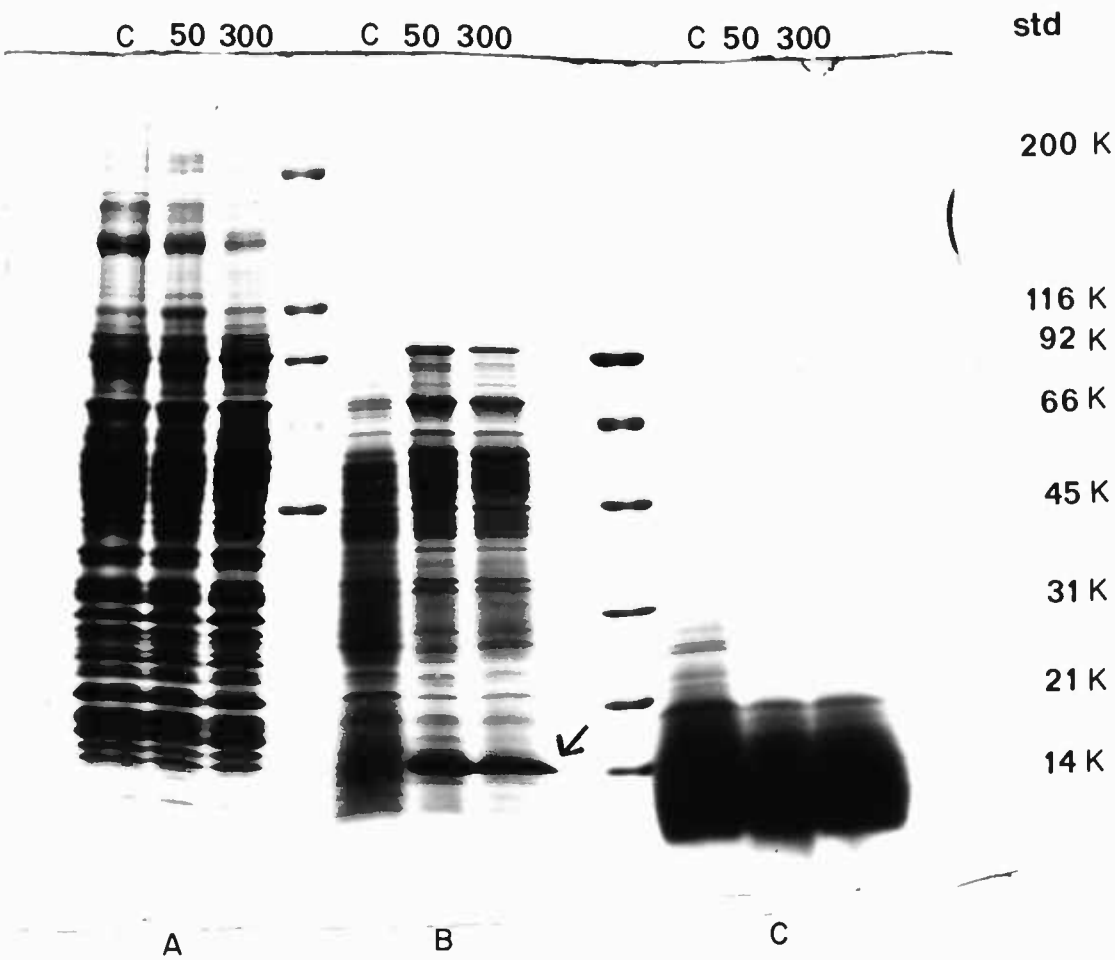

C 50 300
C 50 300
C 50 300
std
200 K
116 K
92 K
66 K
45 K
31 K
21 K
14 K
A
B
C

Figure 14

Figure 15. PAGE of protease digestion of microsomes from liver of 10 month old trout fed a CPFA diet for 9 weeks. A presentation of two separate isolations and incubations. (A) untreated, (B) trypsin-treated, (C) pronase-treated, (D) untreated, (E) trypsin-treated, (F) pronase-treated.

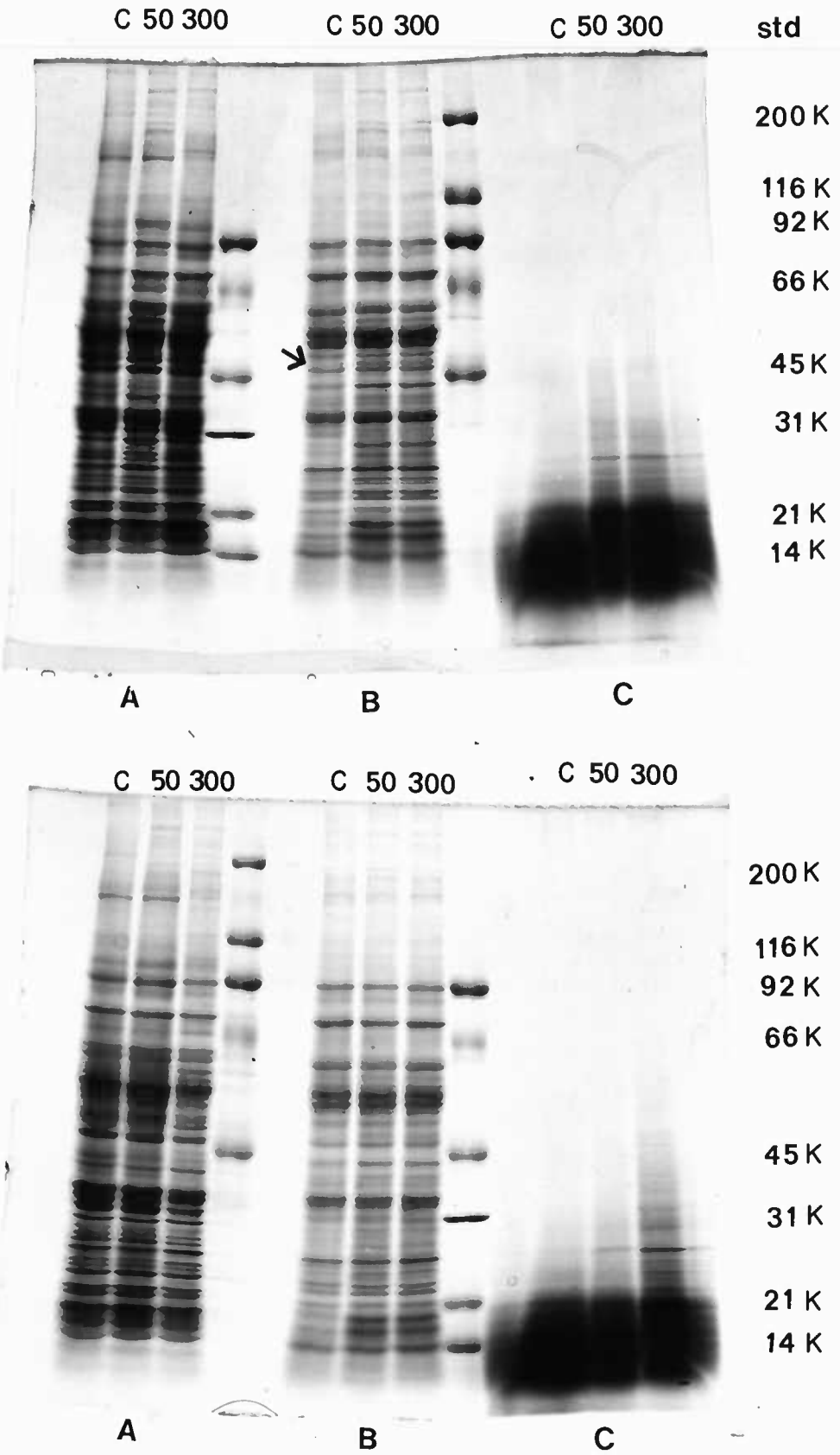

C 50 300
C 50 300
C 50 300
std
200 K
116 K
92 K
66 K
45 K
31 K
21 K
14 K
A
B
C
C 50 300
C 50 300
C 50 300
200 K
116 K
92 K
66 K
45 K
31 K
21 K
14 K
A
B
C

Figure 15

characteristics. Furthermore, there is a prominent band in 14K region (arrow) that only appears in the CPFA-microsomes. Because this band is not in section A (undigested microsomes) it must reflect a proteolytic product of the trypsin digest. Figure 15, which represents younger fish with less time fed a CPFA diet. shows some differences although not as great as in the previous figure. There is a conspicuous absence of a protein band at 46K (arrow) in the control microsomes digested with trypsin (Section C). There are also several bands in the 14-31 K region in section B that are less intense in the control microsomes when compared to the CPFA-microsomes. Overall it is possible to distinguish differences in proteolytic digest between microsomes from liver of fish fed a control died and ones fed CPFA diet.

Immunotransfer with Trypsin-Treated Microsomes

We have used antisera prepared against microsomal protein to determined if there are differences in the digestion. Figure 16 shows an incubation experiment with microsomes that have been treated with trypsin, separated on an acrylamide gel, transblotted to a NC sheet, incubated with antisera and stained by the PAP method. Section A represents the western blot of trypsin-digest microsomes. Section B is an immunotransfer of untreated microsomes. Section C and D are immunotransfers of trypsin-digested microsomes. The difference in the intensity of staining between C and D reflects the antisera concentrations used in the experiments. In both A, B and C 80 ul of antisera was used while section D was incubated with 40 ul to decrease the intensity of the stain.

Figure 16. Identification of antigenic proteins from various microsomes treated with trypsin. (A) amido black stain of trypsin-treated proteins transferred to nitrocellulose sheets, (B) immunochemical staining untreated proteins transferred to nitrocellulsoe sheets (C) immunochemical staining of trypsin-treated antigens transferred to nitrocellulose sheets and incubated with 80 µl of antisera, (D) immuno-chemical staining of trypsin-treated antigens transferred to nitrocellulose sheets and incubated with 40 µl.

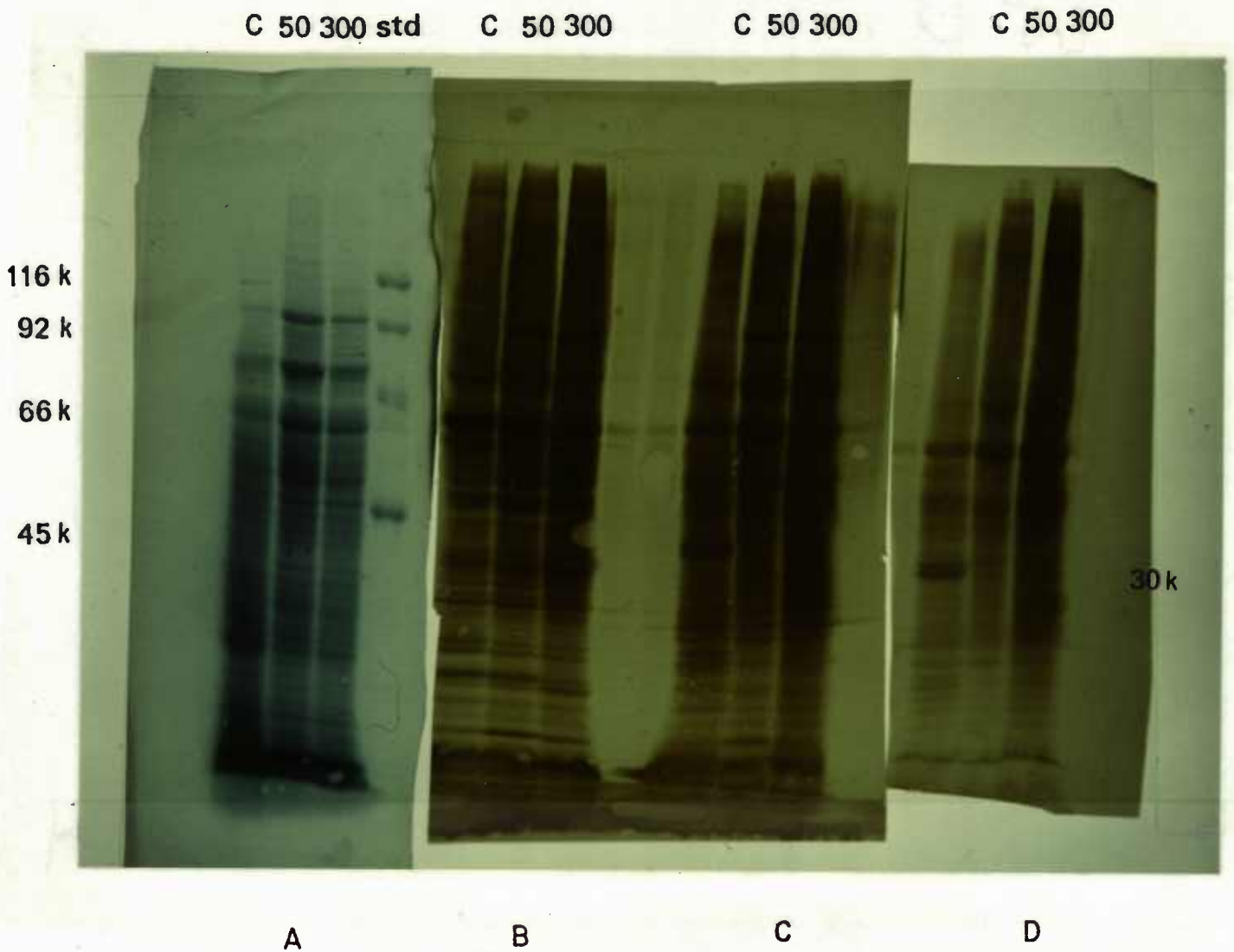


Figure 16

Several bands appear to have remained intact throughout the digestion. Microsomal proteins that are lumenal sided should retain their integrity and their antigenic properties when challenged with trypsin. Several protein bands did remain intact through the digestion. Some cytosolic proteins that are partially cleaved by trypsin may also retain their antigenic nature if the polypeptide determinants are not significantly disrupted. These would appear as new bands with lower placement in the gels, signifying a decrease in molecular weight. Both of these phenomena appear to exist in the gels. Several of the original bands are retained and some new prominant bands appear in the low molecular weight regions. In general, the transblotted lanes of microsomes that have been treated with proteases show major differences when stained by the PAP method. The most obvious is the new antigenic band at 30K that appears in control microsomes. The origin of this band cannot be determined from the undigested microsomes.

DISCUSSION

Polyacrylamide Gel Electrophoresis of Subcellular Fractions of Trout Liver

Observation of protein changes of subcellular fractions of rainbow trout liver separated by PAGE was unsuccessful in isolating distinct bands representing protein induced by CPFA. That is not to say that there were not changes in the large particle, post-nuclear and microsomal pellets. The more readily observable changes are those in the high molecular weight regions. A frequent observation was a lessening (possibly disappearing) of proteins in the CPFA-microsomes. Densitometry readings of microsomal protein separated on tube gels verify these changes. Failure to resolve gross differences in microsomal protein is consistent with the information found in this laboratory with two-dimensional gel electrophoresis (Einerson, 1982). Two dimensional separation techniques achieve a more effective separation of proteins from complex biological sources. Proteins are separated on the basis of their molecular weight in the second dimension. Although small alterations in the trout liver microsomal membranes proteins induced by CPFA were suggested by this data, it was not possible to conclusively demonstrate a constant and significant change.

Several small alterations in the high molecular weight region of the subcellular fractions are also observed with one-dimensional slab acrylamide gels and tube gels. The microsomes are

more evident by this technique. There can be several possible explanations for this. One is a decrease in protein synthesis or a decreasing translocation of the proteins to the membranes caused by CPFA. Another is the possibility that CPFA affects the fragility of membranes and lysosomes are more readily ruptured and endogenous proteases released. Isolation of subcellular fractions with the protease inhibitors in the homogenization buffer should inactivate most proteolytic activity. Comparisons of gels of subcellular fractions isolated with and without inhibitors yielded no significant differences between the two. It was also noted, predominantly in the microsome fraction, that a high molecular weight protein in the 300 ppm CPFA-microsomes appeared to have a different migratory property than the control microsomes. Another study with the strong reducing agent dithiothreitol (DTT) showed that this was not caused by an aggregation of lower molecular weight compounds during homogenization and fractionation. The proposal that CPFA increases or decreases the synthesis of proteins was an appealing one but necessitates more sensitive methods to detect this phenomenon. Recently described methods combining gel electrophoresis and immunochemical staining were subsequently utilized for these purposes.

Immunological Assays

The microsomal pellet is composed of a diverse grouping of membranes, vesicles and proteins that represent several compartments of the cell. The heterogenic make-up of the microsome is clearly

demonstrated in the gels shown in Figure 8. Although it is difficult to detect distinct changes in protein bands there are several subtle changes as noted previously. Recent work in our laboratory in separating microsomal proteins with two-dimensional gel electrophoresis has shown that there are in excess of 400 proteins that can be separated and observed with silver staining techniques (Einerson, 1982). However, it was determined with this procedure that there were no major differences in the protein composition of these membranes. Immunochemical staining with the PAP complex of mixed protein populations that are first separated on acrylamide gels and then transferred to NC sheets has proven to be a very powerful technique (Dühl, 1982). It combines the resolution of gel electrophoresis with the sensitivity of immunochemistry. Our results suggest that this technique is also useful in the study of membrane proteins.

Western Blots

The transfer of proteins from acrylamide gels to NC sheets, eventually proved to be efficient and rapid, however, several alterations of procedure and recommendations should be noted. Electrophoretic transfer is found more reproducible than transfer by passive diffusion. Furthermore, it is much less time consuming (2-4 hours compared to 24-36 hours) and does not necessitate the several washings required in the passive diffusion technique.

Its drawbacks include cost ($500-800) and the high amounts of methanol required in the transfer buffer (approx. 1 liter of the

MeOH/transfer). Whatman No. 3 filter paper is used over Whatman No. 1 because of the increase thickness and firmness of the paper that would hold the gel in a firmer gel-NC-filter paper sandwich. No measurable increase in resistance across the sandwich could be measured on a voltmeter. This increases the speed with which the gel sandwich could be assembled and would lessen the drying of the different layers. Sucrose-gradient gels are used for better resolution of a complex mixture of microsomal proteins. Any attempt to resolve an excess of 400 proteins on an acrylamide gel 15 cm in length necessitates the highest resolving power possible. Regular gels of 10% were inadequate for this task. Moreover, a straight 10% acrylamide gel would readily pass the low molecular proteins on to the NC sheet while more of the high molecular proteins would be retained in the gel. This nonquantitative transfer could possibly be avoided in a gradient gel as the low MW molecular weight proteins would need to pass through a tighter acrylamide network than proteins of higher molecular weight. Originally, we had hoped to transfer proteins from the same type of gradient gels (7.5 → 15%) that we had used in earlier experiments. Unfortunately areas of smearing would frequently appear on these NC sheets. The high degree of difference between the acrylamide concentrations from the top and the bottom of the gel would cause a gradation in the resistance to the current as well. This variation in resistance may alter the direct passage of current from the cathode to the anode and result in uneven migration of the proteins from the gel to the NC sheets and thus create a smearing effect. Various acrylamide sucrose gradient gels

were subsequently utilized to determine the most suitable combination. A sucrose gradient gel of 7 → 10% was determined to have adequate resolving power of microsomal proteins and good transfer capabilities. The smearing effect is minimized and proteins of all molecular weights are transferred. It should be noted that not all proteins are transferred equally. We find that protein in the range of 30K-120K are transferred more efficiently. It should also be noted that a change in gel thickness from 0.15 cm to 0.075 cm diminishes the smearing effect. A thin gel imparts less resistance to current flow than a thick gel would. This decrease in resistance allows less deviation of current flow especially at the edges of the gel where the smearing effects often appear.

Production of Antisera Against Whole Microsomes

The development of antibodies from microsomal proteins has been reported by several researchers (Lundkvist and Perlman, 1967; Raftell and Perlmann, 1967; Blomberg and Raftell, 1974). The detection of the number of antigenic microsomal proteins was somewhat limited by staining techniques. Although two dimensional immunoelectrophoresis revealed multiple antigenic peaks, they were seldom in excess of 15-20 precipitant bands. This is due in part to the low resolving power of a 1% agarose gel and the relative insensitivity of the stains that can be used. The PAP method of immunochemical identification has proven highly sensitive to recognizing antigen-antibody complexes (Sternberger, 1979). In our experiments as little as 20 ul of pooled antisera (representing a 1:5000 dilution in the

incubation bath) could be used to detect antigenic determinants in the microsomal protein mixture. Moreover, over 40 distinct bands can be recognized as antigenic. The intensity of staining appears to be greater in the higher molecular weight region. However, a certain degree of this is probably due to some non-specific staining. What the nature of this non-specific staining is cannot be determined by our experiments. The higher molecular weight proteins are usually more antigenic than low molecular weight proteins. Even when proteins in the high molecular weight region could not be observed on NC sheets by Amido black staining, transferred proteins incubated with antisera would appear with the immunochemical stain.

There are some quantitative differences that are recognizable in the low molecular weight range. There could be several reasons for this, the most obvious being an unequal loading of the gel lanes with microsomal protein. This was avoided by prior determination of microsomal protein per sample. Moreover all gels, blotted or companion unblotted gels are stained and checked for observable differences. Another explanation may be an unequal transfer between lanes. One-third of the NC sheet with representative lanes was usually cut and removed for Amido black staining. If there was an unequal transfer it should appear also in this staining. For the most part all vertical lanes transferred relatively evenly. A final explanation would be the actual differences in antigenic site or concentration of antigenic proteins themselves. This will be discussed in relationship to immunoabsorption experiments.

Immunoabsorption

Immunoabsorption has recently been used by several investigators to differentiate proteins from cell lines in an abnormal state from those in normal tissue. For the most part immunoabsoption is done with a semipurified protein mixture or non membrane protein. Ideally, after the immunoabsorption, the researcher is left with one or a few antibodies that represent newly synthesized proteins from cells in the abnormal state. Microsomal proteins for the most part are a complex mixture of proteins from several different membrane components and compartments in the cell. Immunoabsorption with membrane proteins has proven more difficult. In the production of antibodies, it is not clear how the host recognizes the membrane proteins. Whether the vessicles remain intact or are disrupted in a post injection immune response is a critical point in interpretation of results. Unfortunately, literature concerning this host recognition of foreign proteins is very sparse. If indeed the vessels remain intact, then antibodies should be made only to surface proteins with enough antigenic determinants exposed for recognition by antibody producing cells. Conversely, if the membrane lipid components are dissolved, then antigenic sites should be plentiful and dependent in part on conformational structure with lipid present. Immunoabsorption with intact control microsomes presented some interesting results. Invariably after a few immunoabsorptions there is a decrease in the intensity of staining in the control and 50 ppm CPFA lanes. If the immunoabsorption are carried out extensively, essentially most of the stain in all the lanes

is removed. There does not appear to be any antibodies that would be specific for CPFA induced proteins present. However, the reason for the differences in the intensity of staining raises some complex questions. Cross-reacting antibodies from the control microsomes are sequentially removed with increasing immunoabsorption runs. Exhaustive immunoabsorption (over six immunoabsorptions) would also eliminate staining of the 300 ppm CPFA lanes.
No clear pattern emerged except in immunoabsorption that is performed five times or less. There is a decrease in the intensity of immunochemical staining between the control and 50 lanes and the 300 lanes. Although there is a slight decrease in staining in the 300-lane, it is much less than the other lanes (Figure 9).

An explanation may be in the role that CPFA plays in membrane disruption. Electron micrographs show a definite disruption of plasma membranes and endoplasmic reticulum membranes (Scarpelli, et al., 1974). If proteins within these membranes are changed in their orientation in the membrane, this may be reflected in the results shown. Free fatty acids have been shown to alter protein orientation in plasma membranes as measured by Terbium flourescence (Pjura et al., 1982) and have been shown to intercalate into specific lipid domains (Klausner et al., 1980). Changes in protein orientation may affect their enzymatic activity, functionality and their antigenic nature. If enough proteins in the CPFA-microsomal-membrane are orientated differently, one could expect a different antibody response. It is very possible that not all of the membrane proteins are affected in protein orientaion, or if affected still

have exposed normal antigenic determinants. These proteins would represent regions on the NC sheets that are cross-reactive between control and CPFA-microsomes.

With immunoabsorption, one would first see the removal of all cross reactive antibodies. If all the antigenic protein bands have some cross reactivity there would be a lessening in the intensity of staining in the lanes of the control microsomes (the immunoabsorbent). The 300 ppm CPFA-microsomes would lose some intensity but for the most part remain strongly responsive to antibody-antigen reactions. The 50 ppm CPFA-microsomes would have a staining intensity inbetween depending on the overall effect of CPFA on protein orientation in the membrane. There is an eventual removal of the strong antigenic bands with continued immunoabsorption. This could be the result of nonspecific binding and entrapment of antibodies with membrane components during incubation and centrifugation. However, it should be noted there are differences based on these immunoabsorption experiments between the control microsomes and those from livers of CPFA fed fish. What exactly these differences are in relationship to antigen-antibody interaction remain unknown and should be explored in future studies.

Immunotransfer with Antisera Against Cytochrome P-448 and Cytochrome P-450

Results with the antisera made against cytochrome P-448 and cytochrome P-450 are interesting in several aspects. Recent work by Dave Williams (1982) has shown that cytochrome P-448 from the trout liver microsomes is highly responsive to the metabolism of

benzopyrene. The aflotaxin B_1 (AFB_1) that it does metabolize is directed toward less harmful by-products such as aflatoxicol M_1 which has not been shown to be carcinogenic. Cytochrome P-450 however, in reconstituted enzyme systems, metabolizes a significant amount of AFB_1 to the 2,3-diol, which probably has the 2,3-epoxide as a precursor. The 2,3 epoxide is highly reactive, difficult to isolate and considered the ultimate carcinogen of AFB_1. Correspondingly, Williams has found a several fold increase in DNA-AFB_1 binding with the reconstituted cytochrome P-450 metabolite studies in comparison to cytochrome P-448. Of the two isolated mixed function oxidase systems from the microsomes of trout liver it appears the cytochrome P-450 is more important than P-448 in hepatic carcinogenesis. Preliminary results show a dramatic increase in immunochemical staining of liver microsomal cytochrome P-448 in fish fed β-napthaflavone (BNF). Cytochrome P-450 levels appear the same in control and BNF fed fish. BNF has been shown to be a potent inhibitor of carcinogenesis in trout (Witham , 1981), and reduces AFB-DNA binding *in vivo* (Bailey, 1982). The selective induction of cytochrome P-448 by BNF may shunt the metabolism of AFB_1 to less harmful compounds and lessen the carcinogenic action. Our results, shown in Figure 11, present cytochrome P-450 as the dominant constituent of the mixed function oxidase system of rainbow trout. This may explain why the rainbow trout is very sensitive to AFB_1-induced carcinogenesis.

There did not appear to be any differences in the cytochrome P-450 between the control and CPFA microsomes. However, with

cytochrome P-448 there are apparent differences in the immunochemical staining of the microsomes from the different diets. Because the microsomal lanes are all on the same NC sheet, the differences must be in relationship to the concentration of protein. Apparently, CPFA inhibits the synthesis of cytochrome P-448. If this is indeed the case, several future studies should be done to clarify this point. This may explain in part some of CPFA effects as a potent co-carcinogen.

It should be pointed out that there are several discrepancies with this theory. The level of CPFA in the diet at 50 ppm has been shown to be a potent inducer of liver cancer in trout with AFB_1 (Lee et al., 1968). However, there are not any marked changes in cytochrome P-448 levels in our 50 ppm CPFA-microsomes. Furthermore, recent research has shown that there was not an increase in DNA-AFB_1 in the liver of CPFA fed fish when compared to controls (Bailey et al., 1982). However, it has been demonstrated that different levels of CPFA in the diet will reduce the activity of several MFO enzymes (Eisele et al., 1978) in a region where the MFO system is first induced with BNF. Concurrent feedings of CPFA and BNF in BNF-induced fish have been shown to dramatically reduce the cytochrome P-450 activity. These measurements are done on a spectrophotometer which is not able to separate the cytochrome P-448 spectra from that of cytochrome P-450. With immunotransfer techniques, and incubations with antisera made against the purified proteins, the relative quantities of these two important components of the MFO system can now be separated and analyzed.

Phopholipid Analysis of Microsomes

The phospholipid bilayer has changed from our initial understanding of it as a structural suppoty system to one that also contains obligatory components for various proteins in the membrane.

Phospholipase A_2 (PLA_2) is a specific enzyme that cleaves the acyl group in the number two position of phospholipids. It has proven to be a useful tool in studying the role of phospholipids in biological membranes such as asymmetry of structure or interaction of phospholipids with membrane proteins (3,4). In our laboratories, this enzyme has been used effectively to study the preferential acyl position of labeled cyclopropenoid fatty acids (CPFA) in phospholipids of the microsome (5). In this study we report the effects of phospholipase A_2 on microsomal membranes of rainbow trout fed a control diet and a diet containing CPFA, a potent co-carcinogen for the host species.

A decrease in NADPH-cytochrome c reductase and cytochrome P-450 activity in trout liver microsomes is a well established phenomenon in our experiments. In our experiments, these two enzyme activities were usually decreased in the fish fed 300 ppm CPFA from that found in the controls. As expected, the data presented here show that membrane phospholipids have an important role in cytochrome P-450 activity. Although we were not able to separate the effects of PC and PE hydrolysis from each other in these studies, several researchers have implicated only PC as the necessary lipid component. As our results show, even at 50% hydrolysis of the phospholipids there were sufficient molecules present to maintain high levels of cytochrome

P-450 activity. Phospholipids also play an important part in maintaining the integrity of the membrane. The dismantling of the membrane phospholipids by phospholipase A_2 could provide an effect on other membrane enzymes that are not dependent on phospholipids per se for their functionality. In the case of NADPH-cytocthrome c reductase, the hydrolysis of 50% of the phospholipids or more appeared to actually increase its activity 20-30%. Other researchers have shown that 80-90% of NADPH cytochrome c reductase activity remained in microsomes following extensive removal of neutral fat, phsopholipid and cholesterol by organic solvents (Saundermann, 1977).

Cyclopropenoid fatty acids (CPFA) have been shown to incorporate themselves into the phospholipid of trout liver microsomes. In a 72 hour incubation study with [9,10-methylene-^{14}C]-sterculic acid, the highest percentage (78%) of the label was found in the phospholipid fraction with most of that being distributed between PC (54%) and PE (20%) (Einerson, 1982). It is not known at this time whether the cyclopropene ring remains intact after CPFA is metabolized and in what form it is incorporated into membrane phospholipids.

As previously mentioned, the asymmetry of the phospholipids of the microsomal membrane is unclear. Studies with various phospholipases have shown PE, PC and PS to be preferentially in the cytoplasmic side, on the lumenal side, or evenly distributed on both sides (Op den Kemp, 1979). Because of their size, phospholipases do not cross the membrane and should hydrolyze the lipids on the outer bilayer. In the lowest levels of μg PLA_2 used there was no significant difference in the % hydrolysis of PC and PE. Whether

this would indicate an equal sidedness of PC and PE with respect to each other is not clear in these experiments, as several analyses at lower levels of PLA_2 would be necessary here. Although there does not appear to be a preference for either phospholipid at the enzyme levels studied, it should be noted that PC was four times the concentration of PE in all microsomes studied. This is in variance with the rat liver microsome which has a PC/PE ratio of approximately 2:1. There was a strong similarity in the amount of hydrolysis of phospholipids from control and CPFA fed fish. This is not surprising as the ratios of the phospholipids of the microsomes from fish fed the two diets do not differ significantly either. CPFA does not appear to effect the synthesis of the phospholipids themselves.

Several studies have been published regarding the effect of dietary lipid on drug-metabolizing enzymes (Wade and Norred, 1976). It is widely agreed that the addition of polyunsaturated fatty acids in the diet maximize the cytochrome P-450 activity. Possibly the unsaturated fatty acids may be necessary for the synthesis of phospholipids capable of facilitating electron transfer and for maintaining a membrane structure that insures proper conformation of cytochrome P-450 for optimal activity. It has been noted previously that there is a shift toward more saturation of acyl groups in the phospholipids of CPFA fed fish. This could be attributed to the findings that CPFA has been shown to inhibit the activity of the fatty acid acyl desaturase enzymes in several species. Furthermore, it appears that phospholipid synthesis enzymes appear to

recognize CPFA primarily as a saturated fatty acid since it is preferentially incorporated into the number one acyl position (Einerson, 1982). These may all contribute to the decrease in cytochrome P-450 activity in the microsomal membrane with CPFA diets.

There remains the possibility that CPFA may exert an adverse effect on microsomal membranes due to the reactiveness of the CPFA molecule itself rather than the more generalized changes described above. CPFA are reactive compounds due to the cyclopropenoid ring contained within the molecule. If the cyclopropene ring remained intact even after incorporation of CPFA into membrane lipids there would be a degree of probability of adverse reactions with nearest neighbor membrane proteins and lipids. Membrane enzymes and proteins are greatly effected by boundary lipids - those lipids whose properties are greatly effected by the presence of proteins. The optimal activity of membrane bound enzymes is dependent on the presence and composition of boundary lipids. PC and PE are important membrane lipids and can be considered boundary lipids for most membrane enzyme systems. It was felt that phospholipase A_2 hydrolysis might elucidate some characteristics of the phospholipid-CPFA species within the microsomal membrane. However, the effect of phospholipase A_2 on microsomal enzyme activity showed little difference between the control and the CPFA diet. Although it is not known in what form CPFA is incorporated into the phospholipids of the microsome, there appears to be little if any effect on the parameters we have chosen to study here.

Proteolysis of Microsomes

Enzyme Assays and PAGE

The treatemnt of trout liver microsomes with protease resulted in a greater than 90% inactivation of NADPH-cytochrome c reductase and a 40-75% inactivation of cytochrome P-450. NADPH-related enzymatic activities are more susceptible to proteolysis because of their location on the cytoplasmic side of the membrane.

Inactivation of cytochrome P-450 in isolated rat microsomes appears to necessitate higher concentrations of protease than that needed for trout, however, incubation periods were 10 minutes rather than 15 minutes as in our experiments. Changes in membrane lipids may have an effect on the protein architecture within the lipid bilayer. There have been several reports that demonstrate that changes in lipid constituents (i.e. cholesterol, changes in alteration) can result in small changes in vertical placement of membrane particles. These changes can relate to biochemical alterations in receptor mechanisms, enzymic activity and on transport systems that may have measurable physiological effects.

Our proteolysis study of two important microsomal enzymes show few differences in microsomes from trout fed a control diet and those fed a CPFA diet. Activity levels of the enzymes remained relatively constant to each other for the protease concentrations used.

Our cytochrome P-450 analysis was not sensitive enough to register significant differences in microsomes isolated from fish fed on different diets. There are inherent problems with this

method. The initial P-450 activity in CPFA-microsomes is substantially lower than the controls. With trypsin digestion there appears to be a greater percentage of P-450 decrease in the CPFA-microsome relative to the controls. However, when taken as a percentage from their initial readings there does not appear to be any significant trend.

PAGE of trypsin-digested proteins shows several areas of differences between the CPFA and the control diet. In the older trout which had been on a CPFA diet for an extended period of time (22 weeks), there are marked differences in the proteolysis of the microsomes (Figure 14). One of the more striking differences is greater disappearance of the higher molecular weight proteins in the control-microsomes treated with trypsin. Another difference is the appearance of the 14K protein in trypsin digests of the CPFA fed fish (arrow). With the younger fish (Figure 15) the small alterations that are present are less dramatic. This group had fewer weeks on the CPFA diet and membrane damage might not be as severe.

In both groups studied, the difference in the disappearance of certain bands between the control and the CPFA diet may reflect differences in the protein orientation of the membrane. CPFA's main effect may be a general disruption of membrane architecture. Gross alterations of the endoplasmic reticulum have been observed with electron microscopy (Scarpelli et al., 1974). Changes in the activity of membrane enzymes may be the result of disrupted membranes rather than alterations in protein synthesis. Although it is not possible to determine from these experiments which specific proteins may be

orientated differently and have different susceptibilities to proteolysis, PAGE of digested microsomal proteins has shown differences due to CPFA in the diet.

Pronase was much more effective in digesting of membrane proteins than trypsin as observed by PAGE and resultant gel profiles are much too diffuse to analyze.

Immunotranster of Trypsin-Digested Microsomes

Immunochemical staining has proven to be a useful tool in the study of cellular proteins. Use of flourescent antibodies or second antibodies tagged with isotopes or enzymes that catalyze colorimetric reactions have been used by numerous investigators to visualize membrane localization of specific proteins. Newly developed techniques combining the resolving power of acrylamide gel electrophoresis and immunochemical staining have proven extremely sensitive and versatile in the study of cellular proteins. We have used the PAP procedure to develop information on membrane protein architecutre with experimental rainbow trout fed different diets. As noted previously, these techniques have led us to suggest that CPFA in the diet appears to effect the protein orientation in the microsomal membrane. Our use of protease, enzyme assays and acryalmide gel resolution also lends weight to this interpretation. The use of antibodies developed against microsomal membranes has provided us with an alternative approach to study protein orientation. Microsomes for the protease study were prepared from rainbow trout that had been

on the CPFA diet three weeks longer than the trout utilized for antibody production. In the trypsin-treated microsomes there are distinct qualitative differences as well as differences in the intensity of the stain. The new double band at 30K is very prominant in the control microsomes Figure 16. This band does not appear in the 50 ppm CPFA microsomal band and only to a small degree in the 300 ppm CPFA microsomes. This could only reflect proteins in the microsome that have been digested differently from the microsomal proteins of CPFA fed fish. The origin of this doublet and other protein cleavage products that still give an antigenic response is untraceable in our experiments. However, it is likely that these proteins come from membrane systems. The majority of microsomal proteins are membrane proteins from the endoplasmic reticulum. Moreover the tryptic digest is performed in an intact microsomal system. Membrane bound proteins, anchored in a lipid bilayer with specific orientation to the outside may be digested differently depending on their orientation. Proteins that are not membrane bound and thus lack specific orientation should undergo equal digestion with trypsin. Furthermore, they would be more susceptible to protease attack and cleaved into polypeptides that would be less likely to give antigenic response. Consequently, this doublet is most likely membrane bound and orientated differently in the control-microsome than in the CPFA-microsome.

As shown in Figure 16, section D, the 300-lane still retains an intense stain compared to the other lanes as in the immunoabsorption experiments. Obviously there are some qualities of the antigenic compounds in CPFA-microsomes that are different than in

the controls. As mentioned above, some of these differences are probably alterations in protein orientation. Immunological assays have proven useful in demonstrating this phenomenon.

SUMMARY AND CONCLUSIONS

Studies were conducted to determine the effects of CPFA on microsomal membranes of liver of rainbow trout. PAGE showed that the most consistent change was a decrease in concentration of proteins in the high molecular weight region (>160K) of fish on CPFA diet. This disappearance of proteins was not due to a release of endogenous proteases in CPFA-microsomes.

Production of antisera in rabbits directed against whole microsomes of trout liver fed 300 ppm CPFA was successful. The transfer of proteins from polyacrylamide gels to nitrocellulose sheets for immunological assays was best accomplished with gradient gels of 7-15%. Immunotransfer experiments with microsomes showed several strong antigenic bands. Incubation of transferred protein with pre-immune sera gave no reaction. Immunoabsorption of antisera with control-microsomes showed differences in the type of antigenicity of membrane proteins between the two diets. These differences were in the intensity of staining of the microsomes with absorbed antisera. Microsomes from 300 ppm CPFA diets retained most of their antigenic nature after 3-5 absorptions whereas control microsomes provided much less staining intensity. These results did not show the appearance of de novo synthesis of proteins in CPFA diet. The difference in the intensity of staining may reflect changes in protein orientation in the membrane brought about by CPFA.

Phospholipid analysis did not reveal any major differences in quantification of the classes of phospholipids in the microsomal membrane. Fatty acid analysis of PC and PE showed a shift toward saturation for the majority of the monounsaturates. Although the overall saturated/unsaturated ratio increases, several of the highly polyunsaturates show no difference or a decline in the ratio. Phospholipase A_2 hydrolysis of incubated microsomes showed few differences in activity of cytochrome P-450 and cytochrome-c-reductase between the two diets.

CPFA in the diet resulted in a decrease of 25-50% of the cytochrome P-450 activity and a slight decrease in NADPH cytochrome-c-reductase activity in microsomes. Phospholipase A_2 hydrolysis showed the dependence of cytochrome P-450 on phospholipids for activity. However, there were few relative differences in the post-hydrolysis activities of these two enzymes between the two diets.

Proteolysis of microsomes with trypsin and pronase also showed few differences in cytochrome P-450 and cytochrome-c-reductase between fish fed a control diet and those fed a CPFA diet. PAGE of the trypsin and pronase digests did show several areas where the proteolytic attack was not the same between the two diets. Additional evidence for a dissimilar proteolysis was found with immunotransfer incubations of antisera and microsomes. Immunochemical staining showed some antigenic bands that migrated differently in the control microsomes.

A final study was performed with incubation of transferred

phospholipase A_2 failed to reveal any differences between control and CPFA fed trout. Proteolysis of microsomal membrane proteins had similar effects on NADPH cytochrome reductase and cytochrome P-450 activity on fish fed the different diets. PAGE analysis of these digests did show some differences in digested proteins between the control and CPFA group. These results may reflect a possible change in orientation of microsomal membrane proteins brought about by CPFA in the diet. Additional evidence for altered orientation of proteins was found with PAGE analysis of trypsin-digested microsomes. Moreover incubation of trypsin-digested microsomes with antisera and stained with PAP showed that proteolytic attack was different between control and CPFA microsomes. A final study with incubation of transferred proteins from control and CPFA-microsomes with antisera directed against purified cytochrome P-450 (P-450) and cytochrome P-448 (P-448) showed that CPFA had an effect on the concentration of P-448 but not P-450.

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