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Three studies were conducted to determine the effects of cyclopropenoid fatty acids (CPFA) on the membrane components of livers of rainbow trout (Salmo gairdneri). In the first study, ^{14}C-sterculic acid was administered by intraperitoneal injection into rainbow trout and the trout maintained for 72 hours. The labelled sterculic acid was found in choline phospholipids (CP) and ethanolamine phospholipids (EP). Smaller amounts of label were found in other microsomal membrane lipid components. No label was found associated with the proteins of the microsomal membrane. Phospholipase A_2 treatment of isolated CP and EP showed ^{14}C-sterculic acid to be preferentially esterified to the 1-position of the glycerol backbone.

In the second study, the cleavable bifunctional protein crosslinking reagent dimethyl 3,3'-dithiobispropionimidate·2HCl (DTBP) was used in an attempt to study alterations in the spatial arrangement of proteins in liver microsomal and plasma membranes that might be induced by dietary CPFA. The use of this reagent failed to yield a clear picture of protein-protein interactions in the microsomal membrane due to the formation of high molecular weight aggregates that were not resolvable on polyacrylamide gels. On the other hand, the use of DTBP failed to crosslink the proteins of the plasma membrane.

In the third study, two-dimensional polyacrylamide gel electrophoresis was used to assess the effects of dietary CPFA on protein composition of trout liver microsomal and plasma membranes. Proteins were separated in the first dimension on the basis of their isoelectric points and in the second dimension on the basis of their molecular weights. No major alterations in the compositon of liver microsomal or plasma membranes were found to be induced by dietary CPFA.

Effect of Cyclopropenoid Fatty Acids on Membrane Components of Liver of Rainbow Trout (*Salmo gairdneri*)

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	2
Structure and Occurrence of CPFA	2
Physiological Effects of CPFA	3
Cocarcinogenicity of CPFA	4
Membrane Related Effects of CPFA	5
Cocarcinogenicity and Promotion	7
Phorbol Esters	8
The Two-Stage Model of Carcinogenesis	9
Mechanism of Action of Tumor Promoters	10
Membrane Effects of Phorbol Esters	11
Mechanism of CPFA in Tumor Promotion	12
MATERIALS AND METHODS	14
Animals	14
Chemicals and Equipment	14
Isolation of Microsomal Membranes	15
Isolation of Plasma Membranes	15
Purification of Plasma Membranes	16
Administration of ^{14}C-Sterculic Acid	17
Lipid Analysis of Radiolabelled Microsomes	17
Two-Dimensional TLC	18
Autoradiography	18
Isolation of CP and EP From Fish Microsomes	19
Phospholipase A_2 Treatment of Purified CP and EP	19
Protein Analysis of Radiolabelled Microsomes	20
Cholesterol and Phosphate Determinations on Microsomal Membranes	21
Crosslinking of Isolated Microsomal Membranes	21
Crosslinking of Isolated Plasma Membranes	22

Page

MATERIALS AND METHODS (Continued)
Two-Dimensional Gel Electrophoresis..... 23
Staining of Isoelectric Focusing Gels..... 26
pH Gradient of Isoelectric Focusing Gels..... 26
Silver Staining of Gels..... 26
Photography of Gels..... 27
Comparison of Gels..... 28

RESULTS..... 31
Incorporation of ^{14}C-Sterculic Acid into Microsomal Lipids.... 31
Protein Analysis of Labelled Microsomes..... 38
Cholesterol and Phospholipid Determinations..... 38
Crosslinking Studies..... 44
Isoelectric Focusing of Membrane Proteins..... 54
Two-Dimensional Gel Electrophoresis of Microsomal Membranes... 54
Two-Dimensional Gel Electrophoresis of Plasma Membranes..... 61

DISCUSSION..... 66
Incorporation of ^{14}C-Sterculic Acid into Microsomes..... 69
Protein Analysis of Labelled Microsomes..... 69
Effect of CPFA on Cholesterol Levels of Microsomal Membranes.. 69
Crosslinking Studies..... 70
Two-Dimensional Gel Electrophoresis..... 74
CPFA and the Promotion of Carcinogenesis..... 79

SUMMARY AND CONCLUSIONS..... 82

REFERENCES..... 83

LIST OF FIGURES

Figure		Page
1.	Template used for comparison of gels.	30
2.	TLC and distribution of label in lipids from microsomes isolated from trout liver after administration of ^{14}C-sterculic acid.	33
3.	Distribution of label in TLC fractions of lipids from microsomes isolated from trout liver after administration of ^{14}C-sterculic acid.	35
4.	Two-dimensional TLC of lipids from microsomes isolated from trout liver after administration of ^{14}C-sterculic acid.	37
5.	TLC of phospholipase A_2 treated CP and EP.	40
6.	TLC and distribution of label in phospholipase A_2 treated CP and EP.	42
7.	Gel electrophoretic pattern of trout liver microsomal proteins treated with DTBP.	46
8.	Gel electrophoretic pattern of trout liver microsomal proteins treated with DTBP.	48
9.	Two-dimensional gel electrophoretic pattern of trout liver microsomal proteins treated with DTBP.	51
10.	Gel electrophoretic pattern of trout liver plasma membrane proteins treated with DTBP.	53
11.	Two-dimensional gel electrophoretic pattern of trout liver plasma membrane proteins treated with DTBP.	56
12.	First dimensional isoelectric focusing gel of trout liver microsomal membrane proteins and the associated pH gradient.	58
13.	Two-dimensional separation of microsomal membrane proteins isolated from trout liver.	60
14.	Two-dimensional separation of plasma membrane proteins isolated from trout liver.	63

Figure		Page
15.	Two-dimensional separation of purified plasma membrane proteins isolated from trout liver.	65
16.	Crosslinking of proteins with DTBP.	72
17.	One-dimensional SDS-PAGE of trout liver microsomal proteins.	77

EFFECT OF CYCLOPROPENOID FATTY ACIDS ON MEMBRANE COMPONENTS OF LIVER OF RAINBOW TROUT (*Salmo gairdneri*)

INTRODUCTION

Cyclopropenoid fatty acids (CPFA) are a group of structurally unusual fatty acids that have been shown to cause various physiological effects in a number of species. One of the effects that has been receiving increased attention is the cocarcinogenic activity of CPFA. CPFA have been shown to markedly enhance the carcinogenic effect of aflatoxin B_1 and its metabolites in the liver of rainbow trout.

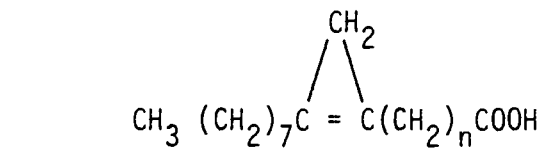
The mechanism by which CPFA enhance the carcinogenic activity of a carcinogen is not known. Work on other cocarcinogenic agents has suggested that these compounds may exert their effect through their action on cell membranes. This work, together with the known effects of CPFA on biological membranes suggest that CPFA may also exert their cocarcinogenic effect through a membrane mediated process.

The purpose of this study was to examine the effects of CPFA on the plasma membranes and microsomal membranes of rainbow trout liver using several techniques. An attempt was then made to relate these research findings to current theories on the mechanism of action of cocarcinogenic agents.

LITERATURE REVIEW

Structure and Occurrence of Cyclopropenoid Fatty Acids

Cyclopropenoid fatty acids (CPFA) are a group of structurally unusual fatty acids found naturally in plants of the order Malvalis. Their unusual structure is due to the presence of a highly strained cyclopropene ring in the center of the long chain fatty acid. The two principal fatty acids identified in these plants are 7-(2-octyl 1 cyclopropenyl) heptanoic acid and 8-(2-octyl 1 cyclopropenyl) octanoic acid, commonly referred to as malvalic and sterculic acid, respectively (Nunn, 1952; MacFarlane et al., 1957; Phelps et al., 1965). The structure of these compounds is as follows:



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

The highest concentration of CPFA is found in the oil of Sterculia foetida, which has been shown to contain up to 70% CPFA (Shenstone et al., 1965). Although not considered an edible oil, Sterculia foetida oil has been extensively used as a source of CPFA for research purposes.

CPFA are found in a number of species from which oils for human and animal consumption are derived. The most important of these are Gossypium hirsutum, from which cottonseed oil is obtained, and Eriodendron anfractuosum, from which kapok oil is derived. Levels of CPFA found in these crude oils have been reported at between 1.5 and 2.4% for cottonseed oil (Christie, 1970) and between 12 and 14% in kapok oil (Kawase et al., 1968).

CPFA are very reactive compounds due to the high degree of steric strain present in the cyclopropene ring (Turner et al., 1968). For this reason they readily undergo a variety of reactions such as

oxidation, reduction, addition, and polymerization (Carter and Frampton, 1964; Christie, 1970). In fact, a widely used method for the detection of CPFA, the Halphen test, is based upon the addition of sulfur compounds across the cyclopropene ring (Christie, 1970). Because of their reactivity, modern methods of oil processing usually destroy most of the cyclopropene components of the oil (Phelps et al., 1965). However, small amounts of CPFA have been shown to survive commercial processing (Mattson, 1973; Kawase et al., 1968). In addition, significant amounts of CPFA can be found in oils consumed in underdeveloped countries, where modern methods of commercial oil processing are less prevalent (Berry, 1980; Berry, 1982).

Physiological Effects of CPFA

CPFA have been shown to be responsible for a number of adverse physiological effects in a variety of animals. The first known effect of CPFA is the classic pink white discoloration defect of eggs. This problem caused severe egg losses in the United States in the early part of this century and is manifested by the appearance of a pink white and a large, watery, salmon colored yolk in eggs during storage. The cause of this defect was initially attributed to a halphen positive substance present in the cottonseed meal fed as a part of the diet of the hens (Lorenz et al., 1933). A number of investigators subsequently confirmed CPFA as the causative agent of pink white discoloration in eggs (Masson et al., 1957; Shenstone and Vickery, 1959; Nordby et al., 1962; Abou-Ashour and Edwards, 1970). Other effects on poultry which have been attributed to CPFA include a delay of sexual maturity (Schneider et al., 1962), reduced egg production and hatchability (Kemmerer et al., 1965; Kemmerer et al., 1967), increased embryo mortality (Schneider et al., 1961; Donaldson and Fites, 1970) decreased weight gain (Swanson, 1982), and increased plasma cholesterol levels (Tennet et al., 1959; Goodnight and Kemmerer, 1967).

Similar effects have been found in rats fed CPFA and include increased prenatal and postnatal mortality (Miller et al., 1969),

retarded growth rates (Schneider et al., 1968; Nixon et al., 1974), impaired reproduction (Nixon et al., 1977a), as well as acute toxic effects (Schneider et al., 1968).

Rainbow trout are also susceptible to the effects of CPFA. Among the reported effects are reduced growth rates (Roehm et al., 1970) bile duct hyperplasia (Malevski et al., 1974), increased liver size, unusual glycogen deposition in the liver, and necrosis of hepatocytes (Struthers et al., 1975).

Recently, CPFA have been shown to cause increases in liver cholesterol, plasma cholesterol, and plasma triglycerides of New Zealand rabbits, as well as a higher incidence of aortic atherosclerosis in these animals (Ferguson et al., 1976).

Cocarcinogenicity of CPFA

Interest in CPFA has increased since they were shown to potentiate the carcinogenic effect of aflatoxin B_1 in the liver of rainbow trout (Sinnhuber et al., 1966; Sinnhuber et al., 1968a). In these studies it was found that trout receiving basal diets containing 4 ppb aflatoxin B_1 + 220 ppm CPFA showed a 90% incidence of liver tumors after six months, while trout receiving only 4 ppb aflatoxin B_1 in their diet developed only a 20% incidence of liver tumors after nine months. In addition, the volume of tumor tissue in CPFA fed fish was approximately 100 times that of tumors found in fish fed aflatoxin B_1 alone. Subsequent studies have confirmed these results (Lee et al., 1967; Lee et al., 1968) and have shown that CPFA are cocarcinogenic with metabolites of aflatoxin B_1 including aflatoxin Q_1 (Hendricks et al., 1978; Hendricks et al., 1980), aflatoxin M_1 (Sinnhuber et al., 1974), and aflatoxicol (Schoenhard et al., 1981). CPFA have also been shown to enhance the carcinogenic effect of 2-acetylaminofluorine (Lee et al., 1968). Studies with pure methyl esters of CPFA have indicated that methyl sterculate is a stronger cocarcinogen than methyl malvalate (Lee et al., 1971). The powerful cocarcinogenic property of CPFA was believed to cause the high

incidence of liver tumors found in trout fed aflatoxin contaminated cottonseed meal (Sinnhuber et al., 1968b; Jackson et al., 1968).

More recently, CPFA were shown to strongly promote the formation of liver tumors in embryos given a single exposure to aflatoxin B_1 (Hendricks et al., 1981). In this study, trout embryos were given a single exposure to aflatoxin B_1 for 15 to 60 minutes. The subsequently hatched trout were fed either a control diet or a diet containing 50 ppm CPFA for periods of up to 12 months. Trout receiving the CPFA diet showed as much as a 53% increase in tumor incidence over trout receiving control diets. This study is significant in that it demonstrates that CPFA act as a tumor promoter in the classical sense, whereas previous studies demonstrated only the cocarcinogenic activity of CPFA.

In a recent study, Swanson (1982) demonstrated that high dietary levels of CPFA stimulated the synthesis and release of avian myeloblastosis virus particles in White Leghorn chickens. Thus, it appears that CPFA have the capacity to influence viral induced as well as chemically induced carcinogenesis.

Membrane Related Effects of CPFA

CPFA have been shown to have pronounced effects on biological membranes. In fact, a number of the physiological effects attributed to CPFA are believed to be mediated through CPFA induced alterations in membrane systems. For example, the classic pink white discoloration of stored eggs was shown to be caused by an alteration in the permeability of the vitelline membrane surrounding the egg yolk. This allowed iron to diffuse through the membrane from the yolk into the white where it complexed with the conalbumin of the white to give the characteristic pink color (Schaible and Bandemer, 1946). Miller et al., (1969) suggested that CPFA were responsible for rat infant mortality by altering membrane permeability and increasing capillary fragility. In support of this, Nixon et al. (1974) found CPFA to affect two membrane systems in the young rat. In this study CPFA were shown to increase erythrocyte hemolysis rates and reduce the

glutathione induction of mitochondrial swelling in young rats. Scarpelli et al. (1974) found CPFA to alter the rough surfaced endoplasmic reticulum of trout hepatocytes. These alterations, when analyzed by electron microscopy, appeared as dilations of the membrane cisternae by sheaf-like aggregates of membrane material.

The activity of membrane bound enzyme systems is also affected by CPFA. The most striking of these is the effect of CPFA on the fatty acyl desaturase system, the membrane bound enzyme complex involved in the desaturation of fatty acids. A number of investigators have demonstrated that CPFA strongly inhibit the activity of this enzyme in a wide variety of biological systems including hen liver (Johnson et al., 1967; Johnson et al., 1969; Allen et al., 1967; Fogerty et al., 1972), White Leghorn hens (Pearson et al., 1972), green algae (James et al., 1968), rat liver (Raju and Reiser, 1967), rats (Reiser and Raju, 1967), and mice (Raju and Reiser, 1973).

It is believed the inhibition of this enzyme system is responsible for the subsequent increase in the ratio of saturated to unsaturated fatty acids found in tissue lipids (Raju and Reiser, 1967; Evans et al., 1962; Allen et al., 1967; Johnson et al., 1967; Coleman and Friedman, 1971; Roehm et al., 1970; Nixon et al., 1974) and membrane lipids (Selivonchick and Williams, 1982; Swanson, 1982) of animals fed CPFA.

CPFA have also been shown to affect the drug metabolizing enzymes of rainbow trout. Eisele et al. (1978) found CPFA to decrease cytochrome P-450 levels and inhibit NADPH cytochrome c reductase, the two membrane bound enzymes which together are responsible for the metabolism of many xenobiotic compounds as well as some endogenous substances such as steroids, bile components, and fatty acids. These results were confirmed by Loveland et al. (1979) and Bailey et al. (1979). Other membrane associated enzymes shown to be affected by CPFA include codeine demethylase (Nixon et al., 1974), benzo(a)pyrene hydroxylase (Eisele et al., 1978), and aldrin epoxidase (Loveland et al., 1979).

How CPFA cause these various membrane related effects is not known. It has been proposed independently by two research groups

(Kircher, 1964; Ory and Altschul, 1964) that CPFA exert their biological activity by reaction of the cyclopropene ring with sulfhydryl groups of enzymes, thus inactivating them. Raju and Reiser (1967) subsequently postulated this to be the mechanism by which CPFA inhibit fatty acyl desaturase. Other investigators (Allen et al., 1967; Johnson et al., 1969; Fogerty et al., 1972) have produced results which support this theory. However, Jeffcoat and Pollard (1977) in studies with [^{3}H]-sterculic acid found that CPFA do not covalently bind with fatty acyl desaturase. Thus, the mechanism of action of CPFA on this enzyme is not clear.

It has also been suggested that CPFA exert their effect by altering membrane lipid composition (Nixon et al., 1974). For example, it is well known that the membrane bound cytochrome P-450 drug metabolizing enzyme system has a specific requirement for lipid (Marshall and McLean, 1971), and that lipids are essential for the proper functioning of these enzymes (Wade and Norred, 1976). Thus, by alteration of membrane lipid composition, CPFA may affect the activity of these membrane bound enzymes.

Several investigators have also shown that CPFA are readily incorporated into tissue (Roehm et al., 1971) and membrane lipids (Nixon et al., 1977b; Eisele et al., 1979). Furthermore, it was shown in these studies that the methylene carbon of the cyclopropene ring was not oxidized, suggesting that the cyclopropene ring remained intact. The presence of this ring may itself be disruptive to the normal functioning of biological membranes.

Carcinogenicity and Promotion

Much of the research done in recent years on mechanisms of carcinogenesis has lended support to the concept that cancer is not a phenomenon that occurs as a result of a single cellular event, but in all probability is a multistep process (Farber, 1980). However, the specific cellular and biochemical events involved in this process have remained largely undiscovered (Sivak, 1979).

It is well known that cancer can be produced by sufficient exposure to any number of chemical carcinogenic agents, generally known as tumor initiators (Miller, 1970). However, the whole concept of carcinogenicity has become more complex with the discovery of substances that can modify this carcinogenic response. Substances that can modify carcinogenesis to give either an increased or decreased tumor yield have come to be known as cocarcinogens (Boutwell, 1974). Within this category is a group of compounds called tumor promoters. A promoter is capable of eliciting tumors when applied together with or after an effective tumor initiator, but by itself is not capable of producing a carcinogenic response, nor will it potentiate the carcinogenic response when applied a sufficient time before the administration of an initiator. Thus, the essential characteristic function of a tumor promoter is that it completes a process begun by initiators. Because of these properties it is thought that initiators and promoters have different roles in the process of tumor formation (Boutwell, 1974).

Phorbol Esters

Most of what is known about tumor promotion has come from the study of a group of compounds known as phorbol esters. The promoting properties of these compounds was first discovered by Berenblum in 1941 in his classic experiments on carcinogenesis in mouse skin. In these experiments Berenblum found that application of a single large dose of the known carcinogenic agent 3,4-benzpyrene to the shaved backs of mice induced tumor formation. As this dose was reduced progressively, an amount of benzpyrene could be identified which caused no detectable incidence of skin tumors over the life span of the mouse. Berenblum called this the subthreshold dose. However, if croton oil was subsequently applied to the backs of mice treated with a subthreshold dose of benzpyrene, multiple tumors appeared within six to ten weeks. No tumors appeared if the croton oil was applied without prior treatment with a carcinogen, nor did they occur if the order of the treatments was reversed. The active components in croton

oil were subsequently identified as esters of the tetracyclic diterpene phorbol (Hecker, 1971). Eleven active components were identified, the most potent being phorbol 12-myristate 13-acetate. The group as a whole has come to be known generally as phorbol esters. Since then, a number of other compounds have been discovered that have tumor promoting capabilities such as anthralin, iodoacetic acid, various phenols, as well as CPFA.

The Two-Stage Model of Carcinogenesis

The work on phorbol esters has led to the concept of a two-stage model of mouse skin carcinogenesis (Boutwell, 1974). The essential features of this model are described in the following discussion. The carcinogenic process is divided into two steps. The first step, initiation, is accomplished by administration of a subcarcinogenic quantity of a known carcinogenic agent (the initiator). Although this process is considered irreversible, no tumors are produced. The second step, promotion, is accomplished by administration of a suitable promoting agent, after which many tumors develop. Thus, tumor promoters complete a process begun by initiators. No tumors are produced if the order of treatments is reversed. Initiation is considered irreversible because of the length of time that can occur between application of the initiator and promoter with subsequent tumor development. In contrast, the effect of promoters is reversible and therefore not permanent.

The extent of the mouse skin promotion phenomenon and whether or not it is involved in the development of other types of carcinogenesis is not known. There have been reservations about its significance because a phenomenon found only in mouse skin might not be representative of carcinogenesis in general (Marx, 1978). However, experiments in recent years have suggested that promotion plays a significant role in the development of several cancers, including those of the lung, colon, breast, bladder, and liver of man and other species (Wynder *et al.*, 1978; Sivak, 1979). Cigarette smoke, for example, contains a number of known carcinogens, but scientists

believe their concentration is not sufficient to account for the full incidence of cancer associated with smoking (Marx, 1978). However, analysis of cigarette smoke has shown it to contain a number of promoting agents, which may explain the high incidence of cancer among smokers (Wynder et al., 1978; Van Duuren and Goldschmidt, 1976). In addition, it is believed that many carcinogens may have the ability to both initiate and promote tumor formation, which could make promotion a very widespread occurrence. In fact, it has been suggested that in terms of control of overall human carcinogenesis, promotion may be a more important factor than initiation (Marx, 1978; Wynder et al., 1978; Sivak, 1979).

Mechanism of Action of Tumor Promoters

The precise biochemical mechanisms underlying the two-stage initiation-promotion model of carcinogenicity are not known. Although there is not agreement on this subject, a number of leading investigators in this field believe that initiation results in the formation of permanent and heritable but unexpressed changes in the genome (DNA) of a cell. This position is supported by the fact that many initiating agents are known to react with, or are metabolized to compounds that react with DNA. In fact, a number of these DNA-initiator complexes can be found in cells of organisms treated with initiating agents (Miller, 1970; Miller and Miller, 1977). Treatment with a promoter then causes this altered DNA to be expressed, resulting in altered metabolism and morphology of the cell and ultimately, tumor formation (Sabine, 1980; Berenblum and Armuth, 1981).

How tumor promoters cause altered DNA to express itself is again, not known. There are a large number of theories on the mechanism of promoter action, probably because of the large number of diverse biochemical effects produced by these compounds (Blumberg, 1980; Blumberg, 1981). However, a number of investigations have suggested that the critical site of action of tumor promoters may be at the level of cellular membranes. This evidence, along with the knowledge

of the critical role that cell membranes play in regulatory processes of the cell (Sivak, 1979) make this an attractive theory. Wallach (1975) in fact, has gone so far as to state that "cancer is a membrane disease."

Most of the work done on the mechanism of action of promoters has been done with phorbol esters. The tremendous volume of literature on this subject has been throughly reviewed by Blumberg (1980; 1981). As can be seen from this review, the action of phorbol esters on biological systems is widespread and varied. Nevertheless, there is a substantial body of information suggesting that phorbol esters may exert their promoting effect through their action on cell membranes.

Membrane Effects of Phorbol Esters

Sivak and Van Duuren (1967) first suggested that phorbol esters interact with cell membranes. Their suggestion was based on their observation of the structure of phorbol esters, which contains both hydrophobic and hydrophilic groups, making interaction with biological membranes a clear possibility. In support of this theory, they subsequently demonstrated that phorbol esters disrupted membranes and caused the release of certain membrane bound enzymes, both in artificial and biological membrane systems (Weissman et al., 1968; Van Duuren and Sivak, 1968). Sivak et al. (1969) subsequently demonstrated that phorbol esters altered the permeability and structural properties of cell membranes of mouse embryo fibroblasts. Further evidence of a phorbol ester-membrane interaction was obtained when isotopically labelled phorbol esters were shown to bind to cell membrane fractions of mouse embryo fibroblasts (Sivak and Van Duuren, 1971), rat liver (Kubinski et al., 1973), and murine spleen cells (Wenner et al., 1978). Phorbol esters have also been shown to alter the activity of membrane bound enzymes including Na^+-K^+ATPase and 5'-nucleotidase (Sivak et al., 1972; Bos and Emmelot, 1974).

In a test system using platelets, phorbol esters caused cell aggregation, alterations in morphology, and release of nucleotides from the cells (Blumberg, 1980). The rapidity of these effects

together with the absence of a nucleus in these cells suggest a membrane site of action.

Other investigators (Rohrschneider et al., 1972; Balmain and Hecker, 1974) found that phorbol esters rapidly induced the synthesis of phospholipids in mouse skin, suggesting that a phorbol ester-membrane interaction could be the initial event in their mechanism of action. Rohrschneider and Boutwell (1973) confirmed this response and found that stimulation of phospholipid metabolism was specific for tumor promotion.

Surface active properties of phorbol esters were examined by Jacobson et al. (1975). These investigators found phorbol esters to be strongly surface active and to penetrate phospholipid monolayers. Subsequent fluorescent studies on the interaction of phorbol esters with biological membranes suggested that they caused alterations in membrane structure and fluidity (Van Duuren et al., 1976; Fisher et al., 1979; Fisher et al., 1981). Wenner et al. (1974) found phorbol esters to alter electrophoretic mobility of Ehrlich ascites tumor cells, further suggesting a membrane interaction. Phorbol esters have also been shown to decrease the level of a major cell surface protein in chick embryo fibroblasts (Blumberg et al., 1976).

Recently, a number of studies have suggested that phorbol esters may react with specific receptors present on the cell membrane surface (Shoyab et al., 1979; Shoyab and Todaro, 1980; Driedger and Blumberg, 1980; Delclos et al., 1980; Dunphy et al., 1980; Horowitz et al., 1981; Cooper et al., 1982). This position is supported by the fact that extremely small amounts of phorbol esters are required to exert their effects. In addition, two research groups (Yamasaki et al., 1979; Braun et al., 1981) have shown phorbol esters to alter surface properties of cells, and that these alterations were specific to tumor promoters, thus implicating the plasma membrane as the site of action.

Mechanism of CPFA in Tumor Promotion

In contrast to the situation with phorbol esters, very little work has been directed toward the study of the mechanism of the

promoting activity of CPFA. Studies on the effect of CPFA on the drug metabolizing enzymes of trout liver (Eisele et al., 1978) and the effect on metabolic activation of aflatoxin B_1 (Loveland et al., 1979) have not provided a plausible mechanism for the powerful promoting activity of CPFA.

The evidence indicating that phorbol esters interact with cell membranes, together with the known membrane effects of CPFA suggest that CPFA also exert their promoting activity through a membrane mediated process. In fact Scarpelli, in investigating the cocarcinogenic activity of CPFA, found them to be mitogenic in both rainbow trout hepatocytes (Scarpelli, 1974) and rat pancreatic acinar cells (Scarpelli, 1975). This author suggested that the mitogenic effect of CPFA may explain their cocarcinogenic activity. He went on to suggest that the mechanism by which CPFA augment cell division is through the alteration of the lipid composition of the plasma membrane, which has a central role in the regulation of mitosis.

In a recent study, Selivonchick and Williams (1982) found CPFA to induce changes in both the plasma membrane and microsomal membrane lipids of trout liver. The most notable change was an increase in the level of saturation of these lipids. Preliminary work has also indicated changes in the protein composition of trout liver microsomal membranes (Selivonchick et al., 1981).

MATERIALS AND METHODS

Animals

All 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 50 and 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.

Chemicals and Equipment

All solvents and chemicals were of reagent grade quality. β-Mercaptoethanol and phospholipase A_2 from Crotalus adamanteus were obtained from Sigma Chemical Company, St. Louis, MO. The crosslinking reagent dimethyl 3,3'-dithiobispropionimidate·2HCl (DTBP) was obtained from Pierce Chemical Company, Rockford, IL. Methyl sterculate, labelled in the 9,10 methylene bridge of the cyclopropene ring (600 uCi/mmol), was synthesized in this laboratory by the method of Pawlowski et al. (1974). Sodium dodecyl sulfate (SDS) and lithium dodecyl sulfate (LDS) manufactured by BDH Chemicals were 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 SDS 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), β-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 and isoelectric focusing.

Isolation of Microsomal Membranes

Fish were killed by a cranial blow, the liver rapidly removed and perfused with ice-cold 0.9% NaCl. Approximately 10 grams of liver representing livers from three to five fish were combined, diced, and chopped with a razor blade to a fine mince in a petri dish on ice. Four volumes of ice-cold buffer (0.25 M sucrose in 0.01 M Tris HCl, pH 7.4) were added and the livers homogenized with 6 passes in a motor driven Teflon pestle glass homogenizer. The homogenate was centrifuged at 2000 x g for 30 minutes in a Sorvall SS-34 rotor. The supernatant was transferred to another centrifuge tube and centrifuged at 12,000 x g for 30 minutes. The supernatant was then centrifuged at 105,000 x g for 90 minutes in a Sorvall 627 rotor. The resulting microsomal pellet was washed by rehomogenization in 30 ml of 0.05 M phosphate buffer, pH 7.6 with 10^{-3}M EDTA, using 3 passes in the above homogenizer. The homogenate was centrifuged at 105,000 x g for 60 minutes, the supernatant poured off, and the resulting microsomal pellet frozen in dry ice-methanol, blanketed with nitrogen, and stored at -70°C until subsequent analysis.

Isolation of Plasma Membranes

Liver cell plasma membranes were isolated by a method developed in this laboratory (Selivonchick and Williams, 1982). Fish were killed by a cranial blow, the liver rapidly removed and placed on ice. Nine grams of liver representing livers from between three to five fish were used for each isolation. Livers were trimmed of excess connective tissue, cut into small pieces, and chopped with a razor blade to a fine mince in a petri dish on ice. The macerated tissue was washed with 35 ml of ice-cold buffer (10% sucrose (w/w) in 5 mM Tris HCl, pH 7.4), transferred to a Kontes Dounce homogenizer, and homogenized twice with two strokes of a loose pestle. The homogenate

was diluted to 150 ml with the above buffer, transferred to four centrifuge tubes and centrifuged for 6 minutes at 120 x g in a Sorvall SS-34 rotor. The supernatant was aspirated, the resulting pellets were transferred back into the Dounce homogenizer, the volume brought to 35 ml with ice-cold sucrose-Tris buffer, and homogenized twice as before. The homogenate was filtered through 1 mm nylon mesh (Tetko, Inc., Elmsford, NY), diluted to 150 ml with ice-cold buffer, and centrifuged as before. The supernatant was aspirated, the resulting pellets transferred back to the Dounce homogenizer, brought to 35 ml with buffer, and homogenized with 14 vigorous strokes of the loose pestle. The total volume was brought to 40 ml with buffer and 20 ml layered onto each of two discontinuous sucrose gradients consisting of 5 ml 38% sucrose (w/w) in 5 mM Tris HCl, pH 7.4 and 8 ml 33% sucrose (w/w) in the same buffer. The gradients were centrifuged at 105,000 x g for 150 minutes in a Sorvall 627 rotor. The resulting membranes were collected at the 10% and 33% sucrose interface using a bent pasteur pipette. The membranes were transferred to a 40 ml centrifuge tube, filled to volume with ice-cold 5 mM Tris HCl buffer, pH 7.4, inverted several times, and centrifuged at 27,000 x g for 30 minutes in a Sorvall SS-34 rotor. The supernatant was aspirated and the resulting milky white pellet containing plasma membranes was frozen in dry ice-methanol, blanketed with nitrogen, and stored at -70°C until analyzed.

Purification of Plasma Membranes

For certain analyses the plasma membranes obtained by the above procedure were further purified as follows. The milky white pellet obtained from the last centrifugation was suspended in 2 ml 1 mM ice-cold $NaHCO_3$ and layered on top of 26 ml of a 1 to 24% continuous sucrose gradient (w/w in 1 mM $NaHCO_3$) on top of 5 ml of a 50% sucrose cushion (w/w in 1 mM $NaHCO_3$). The gradients were centrifuged at 105,000 x g for 30 minutes and the purified membranes collected at the interface between the continuous gradient and the cushion. The

membranes were transferred to a 40 ml centrifuge tube and washed, centrifuged, and stored as above.

Administration of ^{14}C-Sterculic Acid

Trout which had been fed 300 ppm CPFA for 20 weeks were used for the radiolabel study. The spinal column was transected 1 cm caudal to the gills one day prior to administration of the label. The ^{14}C-methyl sterculate was converted to the free acid by saponification with 0.5 N 95% ethanolic KOH at 45-50°C for 30 minutes just before injection. The ^{14}C-sterculic acid (in 0.7 ml corn oil, 4.8 uCi/ml) was injected intraperitoneally into the transected trout. A total of four trout were injected. The fish were maintained for 72 hours in specially built plexiglass metabolic chambers. Aerated 12°C well water was continually passed over the gills. At the end of this time period the trout were killed by a cranial blow and liver microsomes isolated as previously described.

Lipid Analysis of Radiolabelled Microsomes

Total lipid was extracted from the microsomes by the method of Waechter et al. (1973). The microsomes were suspended in 1 ml 0.05 M phosphate buffer, pH 7.6. Aliquots of 100 µl were extracted once with 2 ml $CHCl_3/CH_3OH$ (2:1, v/v) and then twice more with 1 ml aliquots of the same solvent. The combined extracts were washed once with 0.8 ml of 0.9% NaCl and once with 0.8 ml of 0.9% $NaCl/CH_3OH$ (1:1, v/v). The washed extracts were evaporated to dryness under nitrogen and taken up in 100 µl $CHCl_3/CH_3OH$ (2:1, v/v) prior to separation of the lipids by thin layer chromatography (TLC).

Phospholipids were separated on 20 cm long glass plates coated with 0.5 mm silica gel H. Plates were washed in a solvent system consisting of $CHCl_3/CH_3OH/NH_4OH$ (65:35:4, v/v/v) and then activated at 100°C for 45 minutes. Lipids were applied in a 1 cm band 2 cm from the bottom of the plate. Plates were run in $CHCl_3/CH_3OH/H_2O$ (70:30:4, v/v/v) until the solvent front migrated 15 cm (about 45 minutes).

Bands were visualized by exposure to I_2 vapor. Following visualization, 0.5 cm bands widths were scraped into a scintillation vial, 0.5 ml H_2O and 4.5 ml Aquasol (New England Nuclear, Boston, MA) added, the vials shaken, and then counted in a Beckman LS 7500 scintillation counter.

Neutral lipids were separated and counted in the same manner as that described for phospholipids only hexane/diethyl ether/acetic acid (80:20:1, v/v/v) was used as the solvent system. Pure samples of cholesterol oleate, triglyceride, free cholesterol, and diglyceride were run as standards. Bands were visualized by spraying with 50% H_2SO_4 followed by charring in a 200°C oven.

Two-Dimensional Thin Layer Chromatography

Total lipid was extracted from the isolated microsomes which were suspended in 2 ml 0.05 M phosphate buffer, pH 7.60, by the method of Bligh and Dyer (1959). The extracted lipid was taken up in 0.5 ml benzene prior to TLC. Two-dimensional TLC was done on a 20 cm x 20 cm glass plate coated with 0.5 mm silica gel H. The plate was washed and activated as previously described. A fraction of lipid extract (10,000 dpm) was spotted in a 1 cm band in the corner of the plate. The plate was run in the first dimension in $CHCl_3/CH_3OH/NH_4OH$ (65:35:5, v/v/v) and in the second dimension in $CHCl_3$/acetone/CH_3OH/acetic acid/H_2O (10:4:2:2:1, v/v/v/v/v). Spots were visualized by spraying with 50% H_2SO_4 and charring in a 200°C oven.

Autoradiography

The two-dimensional TLC plate was exposed to Kodak XAR-5 x-ray film for two weeks. The film was processed on a Kodak RP X-OMAT automatic processor, using a 95°F development temperature and a 90 second cycle.

Isolation of Choline and Ethanolamine Phospholipids From Fish Microsomes

Choline (CP) and ethanolamine (EP) phospholipids were extracted and purified from isolated microsomes of trout following administration of ^{14}C-sterculic acid. The isolated microsomes were suspended in 2 ml 0.05 M phosphate buffer, pH 7.60 and lipid extracted by the method of Bligh and Dyer (1959). The extract was evaporated to dryness under nitrogen and taken up in 0.5 ml benzene. A fraction of the phospholipids was separated by TLC (70:30:4, $CHCl_3/CH_3OH/H_2O$, v/v/v) and visualized by spraying with dichlorofluoroscein and viewing under UV light. The bands corresponding to CP and EP were scraped into 50 ml $CHCl_3/CH_3OH/H_2O$ (30:50:20, v/v/v) and stored at -20°C overnight. The extract was then passed through a 2 cm x 20 cm glass column with a plug of glass wool at the bottom to filter out the silica gel, rinsed with 35 ml $CHCl_3$ and then with 10 ml H_2O. The extract was then transferred to a 250 ml separatory funnel, the bottom $CHCl_3$ layer collected, dried in a rotary evaporator, taken up in 1 ml benzene, and stored at -70°C. CP and EP standards were prepared in the same manner from control fish, only whole liver was used as the starting material and total lipid extracted by the method of Folch *et al*. (1957).

Phospholipase A_2 Treatment of Purified Choline and Ethanolamine Phospholipids

The location of ^{14}C-sterculic acid in the glycerol backbone of microsomal CP and EP was determined using phospholipase A_2. A total phosphorous determination was done on the isolated lipids by the method of Bartlett (1959). Fractions of CP and EP corresponding to 1500 dpm of lipid (0.79 umol of CP and 0.49 umol of EP) were each added to 1.5 cm x 12 cm screw cap test tubes. Purified CP and EP standards were treated in the same manner. The lipid was dried under nitrogen and 2 ml diethyl ether added to each tube. Phospholipase A_2 was freshly prepared by dissolving 1000 units (2.1 mg) of enzyme in 1

ml buffer (10 mM HEPES, 5 mM $CaCl_2$). The enzyme was then added to the tubes containing the purified phospholipids. A 200 ul aliquot (200 units) of enzyme preparation was added to each tube containing EP, and a 300 ul aliquot (300 units) to each tube containing CP. The total volume of buffer was made to 0.4 ml, a small stirring bar added to each tube, and the reaction allowed to proceed with moderate mixing for 12 hours. Following completion of the reaction, the ether was evaporated, 1.6 ml H_2O added, and the lipid extracted with 8 ml $CHCl_3/CH_3OH$ (2:1, v/v). The aqueous fraction was extracted once more with an equal volume of $CHCl_3/CH_3OH/H_2O$ (86:14:1, v/v/v), the two extracts combined, dried under nitrogen, taken up in 100 ul benzene, and separated by TLC (70:30:4, $CHCl_3/CH_3OH/H_2O$, v/v/v). The radiolabelled phospholipids were visualized by exposure to I_2 vapor, scraped into scintillation vials, and counted as previously described. The phospholipase A_2 treated CP and EP standards were separated in the same manner and the bands visualized by spraying with 50% H_2SO_4 followed by charring in a 200°C oven.

Protein Analysis of Radiolabelled Microsomes

Protein levels in suspended microsomes were determined by the method of Lowry et al. (1951). The protein residue remaining after lipid extraction was boiled for 10 minutes in sample buffer (0.0625 M Tris HCl, pH 6.80, 5% LDS, 2% β-mercaptoethanol, 10% glycerol). The solubilized proteins were separated by gel electrophoresis using the method of Laemmli (1970), only LDS was used instead of SDS. Protein (1.0 mg) was loaded onto 10% acrylamide tube gels (10 cm x 0.5 cm, i.d.) with a 1 cm stacking gel of 4.5% acrylamide. Gels were electrophoresed at 1.5 mA per gel constant amperage for 1.5 hours followed by 3 mA per gel for an additional 3.5 hours. Gels were stained in 0.25% Coomassie blue R-250 in 50% methanol, 10% acetic acid overnight followed by diffusion destaining in the same solvent. Gels were then cut into 2 mm slices with a razor blade and the corresponding slices from different gels pooled and placed in a scintillation vial. Protein was solubilized from the gel by adding

0.6 ml of NCS tissue solubilizer (NCS:H_2O, 9:1, v/v) purchased from Amersham, Arlington Heights, IL and incubating the tubes at 50°C in a water bath with gentle shaking for 2 hours. Following solubilization, 240 µl glacial acetic acid were added along with 5 ml Aquasol, the tubes shaken, and counted as previously described.

Cholesterol and Phosphate Determinations on Microsomal Membranes

The microsomal pellet was suspended in 2 ml H_2O and the protein assayed by the method of Lowry et al. (1951). Total lipid was extracted from a 1 ml aliquot by the method of Bligh and Dyer (1959). The lipid extract was taken up in 100 ul benzene and the total cholesterol determined by the method of Zlatkis et al. (1953). Total phosphate was assayed by the method of Bartlett (1959).

Crosslinking of Isolated Microsomal Membranes

Microsomal membranes were crosslinked with DTBP using a modification of the procedure of Markwell and Fox (1980). Microsomal membranes were suspended in 0.2 M triethanolamine hydrochloride buffer, pH 8.5 (TEA buffer) and adjusted to a protein concentration of 4 µg/µl. Protein was assayed by the method of Lowry et al. (1951). Aliquots of microsomal membrane suspension containing 800 µg (200 ul) protein were used for crosslinking. A stock solution of DTBP at a concentration of 20 µg/µl in TEA buffer was freshly prepared and kept on ice. Aliquots of this stock solution were added to the membrane suspensions to provide concentrations of DTBP ranging from 0.01 to 28 mM. Following addition of the crosslinking reagent the reaction mixture was incubated for 30 minutes at 0°C. The reaction was terminated by addition of a stock solution of ammonium acetate (1 M in TEA buffer) to a final concentration of 50 mM. N-Ethylmaleimide was then added from a freshly prepared stock solution (1 M in ethanol) to a final concentration of 50 mM to prevent sulfhydryl interchange. The mixture was then incubated for 30 minutes at room temperature. Samples were made 5% in LDS, 10% in glycerol, adjusted to pH 6.80 with

3N HCl, and boiled for 2 minutes in preparation for gel electrophoresis. When appropriate, the crosslinking was reversed by addition of β-mercaptoethanol to 2%, followed by boiling for 2 minutes.

Crosslinked samples (150 μg protein) were electrophoresed on 30 cm x 14 cm x 0.15 cm linear acrylamide gradient slab gels. Samples were run in one case on a 7.5 to 15% acrylamide gradient stabilized with from 5 to 17.5% sucrose, with a 2 cm stacking gel consisting of 5% acrylamide. In another instance samples were run on a 4 to 15% acrylamide gradient, using a 2% acrylamide stacking gel stabilized with 0.7% agarose. Buffers and conditions of electrophoresis were the same as reported in the section on two-dimensional electrophoresis. Gels were stained with Coomassie blue R-250 as previously described.

Crosslinked samples were analyzed in two dimensions as follows. The crosslinked proteins were electrophoresed in the first dimension by the method of Laemmli (1970). Samples containing 150 ug of protein were applied to the top of a 10% acrylamide tube gel (10 cm x 0.3 cm i.d.) with a 1 cm stacking gel of 4.5% acrylamide. Following electrophoresis the crosslinking was reversed by soaking the tubes in reducing buffer (0.125 M Tris HCl, pH 6.8, 3% B-mercaptoethanol, 0.1% SDS) for 10 hours. The tube gels were then laid on top of a 14 cm x 16 cm x 0.15 cm slab gel consisting of 10% acrylamide with a 1 cm stacking gel of 4.5% acrylamide. The tube gel was affixed to the slab gel with a solution of 1% agarose in reducing buffer. A sample well for the inclusion of molecular weight standards was made by placing a small plastic spacer in the stacking gel prior to polymerization. Electrophoresis was carried out at 10 mA per gel until the bromophenol blue tracking dye was 1 cm from the bottom of the plate. Gels were stained with Coomassie blue R-250 as previously described.

Crosslinking of Isolated Plasma Membranes

Isolated plasma membranes were crosslinked using the same method as for microsomal membranes except that 25 ul (100 ug protein) aliquots were crosslinked. Crosslinked samples were electrophoresed on a 14 cm x 16 cm x 0.15 cm slab gel utilizing a 10% separating gel

and a 4.5% stacking gel by the method of Laemmli (1970). The gel was stained as previously described.

Analysis in two dimensions was done as described for crosslinked microsomal membranes, only a 7% acrylamide tube gel was used in the first dimension and a 7.5 to 15% linear acrylamide gradient gel in the second dimension. Gels were silver stained as described in the section on silver staining.

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis of isolated microsomal and plasma membranes was performed using a modification of the method of Vlasuk and Walz (1980) which is a modification of the original technique developed by O'Farrell (1975). Microsomal or plasma membranes were prepared for electrophoresis as follows. The frozen membranes were suspended in dispersing buffer (0.1 M phosphate, pH 6.80, 20% glycerol w/v) to a concentration of about 10 mg/ml. Protein levels were determined by the method of Lowry et al. (1951). Two volumes of lysis buffer (3% Triton X-100, 1.5% pH 3.5-10 ampholytes, 0.5% pH 5-7 ampholytes, 5% β-mercaptoethanol, 9.5 M urea) were added to the suspended membranes, the mixture saturated with urea, and the pH adjusted to 4.3 with 3N HCl.

Isoelectric focusing was done in 120 mm long acrylamide gels that were formed in 140 mm x 3 mm i.d. glass tubes. The glass tubes were precoated with Sigmacote (Sigma Chemical Company, St. Louis, MO) to facilitate removal of gels from the tubes. A 10 mm long parafilm plug was inserted in the bottom of each tube to provide a well for the inclusion of lysis buffer. The isoelectric focusing gels consisted of 55% urea, 3.8% acrylamide, 0.2% bisacrylamide, 2% Triton X-100, 1.5% pH 3.5-10 ampholytes, 0.5% pH 5-7 ampholytes, 0.07% TEMED, and 0.01% ammonium persulfate. The gel solution was loaded into the tubes with a six inch 20 gauge needle and syringe, overlayed with 8 M urea, and allowed to polymerize for 1 to 2 hours. This solution was then removed and replaced with lysis buffer, overlayed with H_2O, and allowed to sit overnight. The parafilm plug was then removed from the

bottom of the tube, the well filled with lysis buffer, and the end of the tube covered with dialysis tubing and secured with a small rubber band, taking care to exclude air bubbles. The gels were placed in a tube gel electrophoresis chamber, the lysis buffer and water removed from the top of the gels, and 20 ul fresh lysis buffer added. The upper buffer chamber was filled with 0.01 M H_3PO_4 and the bottom chamber with degassed 0.02 M NaOH. The gels were prerun for (a) 200 volts for 15 minutes, (b) 300 volts for 30 minutes, and then (c) 400 volts for 30 minutes, with the upper buffer reservoir serving as the anode and the lower buffer reservoir the cathode.

The upper reservoir buffer was then emptied and the lysis buffer and H_3PO_4 removed from the surface of the tubes. Microsomal or plasma membrane proteins (100 to 150 ug) were loaded on top of the gels and overlayed with 15 ul of sample overlay solution (0.25% pH 5-7 ampholytes, 0.75% pH 3.5-10 ampholytes, 5% β-mercaptoethanol, 8 M urea, pH 4.3). The upper buffer chamber was refilled and the gels electrophoresed at 400 volts for 12 hours followed by 800 volts for one hour, constant voltage.

At the end of the run, gels were carefully removed from the tubes by rimming with a 25 gauge needle. The gels were placed in 5 ml sample buffer (0.0625 M Tris HCl, pH 6.80, 2.3% LDS, 5% β-mercaptoethanol, 10% glycerol) in a 1 cm x 15 cm test tube and shaken at room temperature on a mechanical shaker for 2 hours. At this point gels were either run directly in the second dimension or frozen in dry ice-methanol and stored at -70°C until electrophoresis in the second dimension.

The tube gels were electrophoresed in the second dimension on a 30 cm x 14 cm x 0.15 cm linear acrylamide gradient slab gel consisting of from 7.5 to 15% acrylamide stabilized with from 5 to 17.5% sucrose. The gradients were poured using a standard gradient former and peristaltic pump. Light acrylamide solution (5% sucrose, 0.375 M Tris HCl, pH 8.8, 7.5% acrylamide, 0.2% bisacrylamide, 0.1% ammonium persulfate, 0.04% TEMED; 35 ml) and 35 ml heavy acrylamide solution (17.5% sucrose, 0.375 M Tris HCl, pH 8.8, 14.6% acrylamide, 0.4% bisacrylamide, 0.1% ammonium persulfate, 0.04% TEMED) were poured into

each chamber of the gradient former. The gradients were poured at a flow rate of 8 ml per minute. All gradients were overlayed with water saturated butanol and allowed to polymerize for 1 to 2 hours. At the end of this time period, the top of the gel was rinsed with H_2O and a 2 cm stacking gel poured that consisted of 4.9% acrylamide, 0.14% bisacrylamide, 0.125 M Tris, HCl, pH 6.80, 0.1% ammonium persulfate, and 0.1% TEMED. A small plastic spacer was inserted in the stacking gel prior to polymerization to form a well for the application of molecular weight standards.

Following polymerization of the stacking gel, the first dimension tube gel was laid on top of the slab gel using a solution of 1% agarose in sample buffer to hold the gel in place. 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 filled with running buffer that contained 0.1% LDS and 1.2 mM EDTA. Bio-Rad low molecular weight standards (15 ug in 15 ul sample buffer containing 0.001% bromophenol blue) were applied to the sample well. Gels were electrophoresed at 20 mA per gel constant amperage at 4°C until the bromophenol blue tracking dye was 4 cm from the bottom of the gel. Electrophoresis was usually complete in 15 to 18 hours.

A total of 20 two-dimensional separations from three separate plasma membrane isolations was performed. These consisted of 10 separations of plasma membrane proteins from control fish, 5 from trout fed 50 ppm CPFA, and 5 from trout fed 300 ppm CPFA. The CPFA fed trout used in this study had been on a CPFA diet for 10, 12, and 20 weeks at the time of isolation of the plasma membrane fractions.

A total of 17 two-dimensional separations from three separate microsomal membrane isolations was performed. These consisted of 8 separations of microsomal proteins from control fish, 5 from trout fed 50 ppm CPFA, and 4 from trout fed 300 ppm CPFA. The CPFA fed trout used in this study had been on a CPFA diet for 9, 20, and 21 weeks at the time of isolation of the microsomal membrane.

Staining of Isoelectric Focusing Gels

Isoelectric focusing gels were stained by the method of Reisner et al. (1975). The gels were placed in a 1 cm x 15 cm test tube and 5 ml staining solution added. Staining solution consisted of 0.04% (w/v) Coomassie blue G-250 in 3.5% (w/v) perchloric acid. This solution had been filtered through Whatman No. 1 filter paper and then through a 0.45 µm Millipore filter. Best staining occurred when the tubes were placed in a 37°C water bath overnight. Gels were stored in test tubes containing 0.04% (w/v) Coomassie blue G-250 in 2.5% (w/v) perchloric acid that had been filtered as above.

pH Gradient of Isoelectric Focusing Gels

The pH gradient established in isoelectric focusing gels was measured by two methods. In one method, the gels were cut into 5 mm segments, 2 ml of deionized, degassed water added, and the pH measured 12 hr later. In the other method, the pH of the gel was measured directly with a MI-410 Micro-Combination Glass pH Probe obtained from Microelectrodes, Inc., Londonberry, NH.

Silver Staining of Gels

Slab gels were silver stained using a modification of the method of Wray et al. (1981). Gels were stained in 9 in x 12 in glass baking dishes that had been coated with Sigmacote to minimize adherence of the gels to the vessels. Distilled deionized water was used in all solutions used in this procedure. Best results were obtained using the following procedure. Gels were fixed in 10% trichloroacetic acid (TCA) for 30 minutes, followed by soaking in H_2O for 30 minutes. Gels were then placed in 50% methanol, 0.05% formaldehyde overnight (approximately 12 hours). The gels were then allowed to swell in water for 30 minutes, and returned to the methanol-formaldehyde solution for a minimum of 2 hours. Gels could

be stored at this point for as long as 2 weeks without affecting the staining pattern.

Just prior to staining, gels were swelled in H_2O for 5 minutes. Freshly prepared staining solution was then added to the gel. Staining solution was prepared by dissolving 4 g silver nitrate in 20 ml H_2O, and this solution added dropwise with rapid mixing to a solution of 105 ml 0.36% NaOH and 7 ml concentrated ammonium hydroxide. The final volume was made to 500 ml with H_2O. Gels were stained for 20 minutes, with constant gentle agitation provided by a mechanical shaker. The staining solution was then removed, the gel rinsed once with H_2O, and then agitated for 5 minutes in H_2O. The H_2O was removed and the gel developed by addition of freshly prepared developer solution that consisted of 2.5 ml of 1% citric acid and 0.25 ml formaldehyde in a total volume of 500 ml. Development was usually complete in 10 to 20 minutes, after which the gel was returned to 50% methanol.

Gels were destained to remove unwanted background by immersion in Kodak Rapid Fix (film strength). When the desired amount of background had been removed, the Rapid Fix was removed with a 5 minute water wash, a 20 minute wash in Kodak Hypo Clearing Agent (film strength), another 5 minute water wash, and finally the gel returned to 50% methanol. Gels were photographed within two days, as the stain gradually faded upon prolonged storage.

Photography of Gels

Gels were photographed by a professional photographer using a Linhof Technika 4 x 5 camera with a 13.5 cm f3.5 Zeiss Planar lens. All exposures were at $f32^+$ (maximum aperature). The gels were placed on a fluorescent copy table illuminated from below by five 48 in cool white tubes and diffused by opal glass. The camera was mounted on a vertical copystand over the gels, with an average gel to film plane distance of 67 cm. Silver stained gels were photographed using either a No. 58 green filter with an exposure time of three seconds, or no filter and an exposure time of one-half second. Coomassie blue

stained gels were photographed using a No. 47B yellow filter with an exposure time of three seconds. Exposures were developed in DK50:H_2O (1:1) for 2 minutes.

Comparison of Gels

Because of the complexity in the staining pattern of the two-dimensional gels, comparisons among the many gels run were very difficult. For this reason, the following procedure was adopted. The stained gels were photographed and an 11 in x 14 in print of each gel obtained. In comparing the staining pattern of two gels (one control and one test) run on a given day, any differences noted were marked on two corresponding pieces of paper. These paper "templates" had been divided into seven regions based on the position of the molecular weight standards on the gel. An example of one of the templates is shown in Figure 1. If a protein spot was noted in a control gel that was not seen in a test gel, a mark was made on one of the paper templates in the region corresponding to the position of the spot in the gel. The same was done on the other paper template if a protein spot was seen in a test gel that was not seen in the control. This analysis was carried out for each pair of gels run on a given day. Thus, one template was obtained for each gel. The paper templates from all the gels were then compared. Any consistant differences between control and test gels should appear as a reproducible pattern of marks on the paper templates.

Figure 1. Template used for comparison of two-dimensional gels. A. Phosphorylase B (MW 92,500). B. Bovine serum albumin (MW 66,200). C. Ovalbumin (MW 45,000). D. Carbonic anhydrase (MW 31,000). E. Soybean trypsin inhibitor (MW 21,500). F. Lysosyme (MW 14,400).

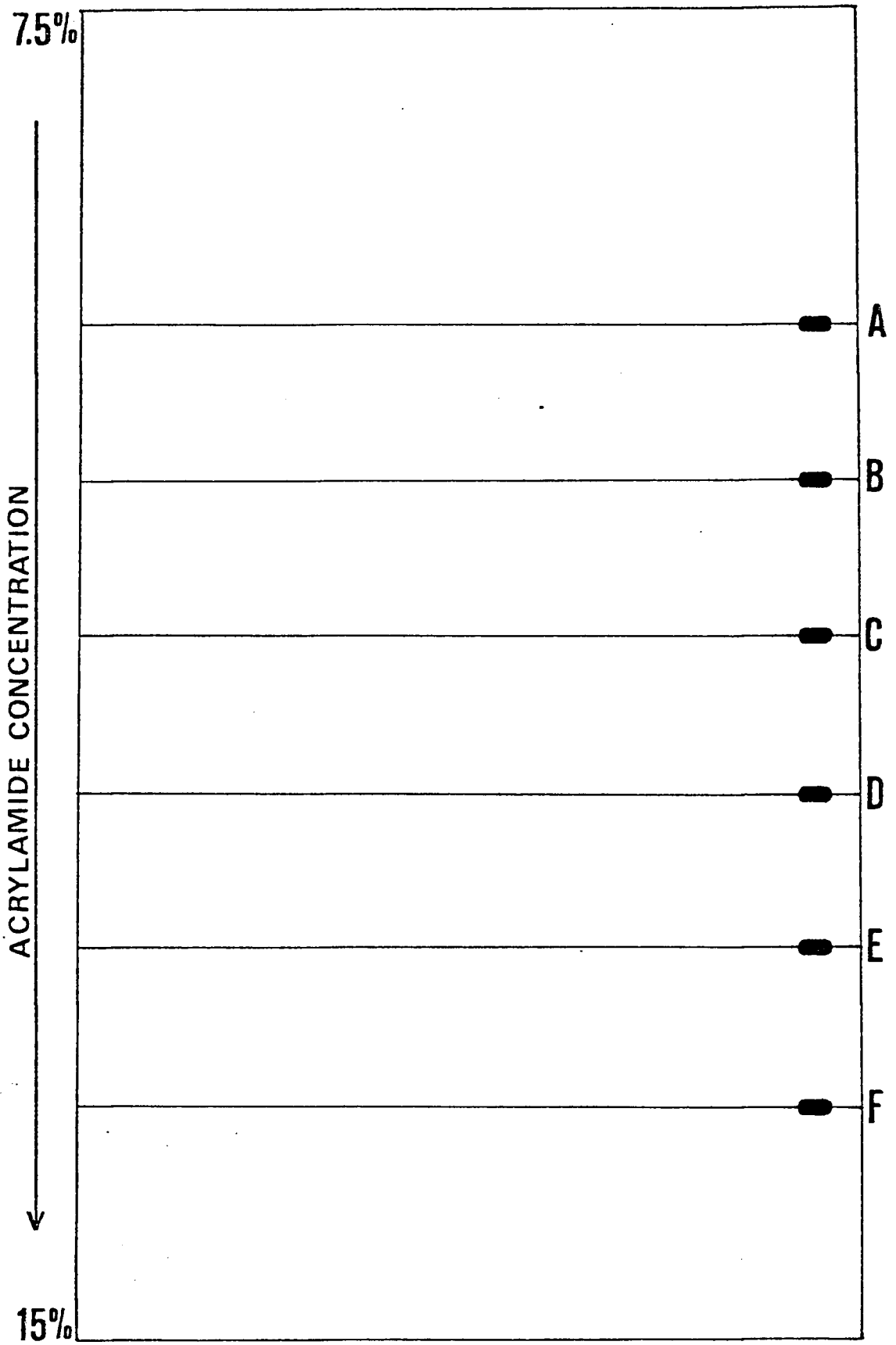


FIGURE 1

RESULTS

Incorporation of ^{14}C-Sterculic Acid into Microsomal Lipids

Radioactivity from intraperitoneally administered [9,10-methylene-^{14}C] sterculic acid was found to be incorporated into neutral and polar lipids of the liver. After 72 hours an average of 8.0% of the administered radioactivity was recovered in the total lipids of liver (3.3, 5.0, 5.1, and 8.4 x 10^5 dpm per liver, respectively).

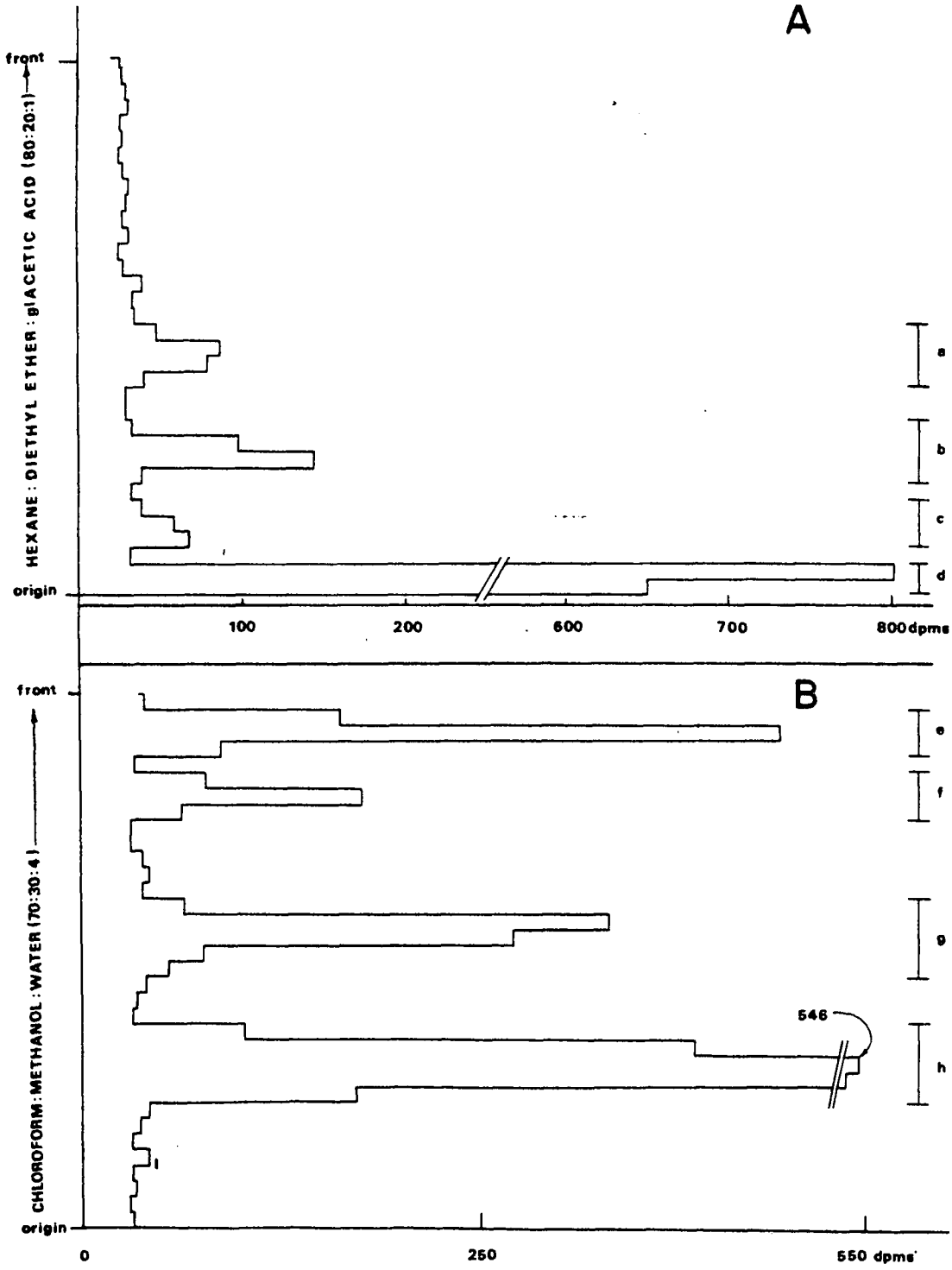
Analysis of neutral lipids by TLC (Figure 2A) showed that the polar lipid at the origin contained most of the radioactivity. Of the neutral lipids that were separated, the free fatty acids contained the highest amount of radioactivity. A lower amount of radioactivity was found in triacylglycerols, with a minor amount in diacyglycerols. Separation of polar lipids and the amount of radioactivity incorporated into the various components is shown in Figure 2B. Most of the label was found in CP and EP.

A summary of the distribution of label into specific lipid components is shown in Figure 3. The highest percentage (78%) of the label was found in the phospholipid fraction, with most of that being in CP (54%) and EP (20%). A significant amount (10.2%) of the labelled sterculic acid appeared to be present as the free fatty acid and 7.3% was found in the triacylglycerol fraction. A small amount of label (2.7%) was also incorporated into diacylglycerols.

Two-dimensional TLC of the extracted lipids (Figure 4) and autoradiography of the plate confirmed these results. Most of the radioactivity was present in CP, with a lesser amount present in EP. Small amounts of radioactivity were also present in sphingomyelin, inositol phospholipids, serine phospholipids, neutral lipid, and free fatty acids.

Phospholipase A_2 was used to determine the location of ^{14}C-sterculic acid in the glycerol backbone of the isolated CP and EP. This enzyme specifically cleaves the fatty acid from the 2-position of phospholipids. The results of the treatment of CP and EP standards

Figure 2. Radioactivity in TLC fractions of liver microsomal lipids, 72 hours post-injection. (A) Neutral lipid solvent system: Hexane-diethyl ether-glacial acetic acid, 80:20:1. (B) Polar solvent system: Chloroform-methanol-water, 70:30:4. Aquasol scintillator. Migration rates of standards: (a) triacyglycerols, (b) fatty acids, (c) diacylglycerols, (d) polar lipids, (e) neutral lipids, (f) fatty acids, (g) ethanolamine phospholipids, (h) choline phospholipids. Results are typical of 4 separate experiments.


A
front
HEXANE : DIETHYL ETHER : glACETIC ACID (80:20:1)
origin
a
b
c
d
100
200
600
700
800dpms
B
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CHLOROFORM : METHANOL : WATER (70:30:4)
origin
e
f
g
546
h
0
250
550 dpms

Figure 2

Figure 3. Distribution of label in TLC fractions of lipids from microsomes isolated from trout after administration of ^{14}C-sterculic acid. A. $CHCl_3/CH_3OH/H_2O$ (70:30:4). B. Hexane/diethyl ether/acetic acid (80:20:1).

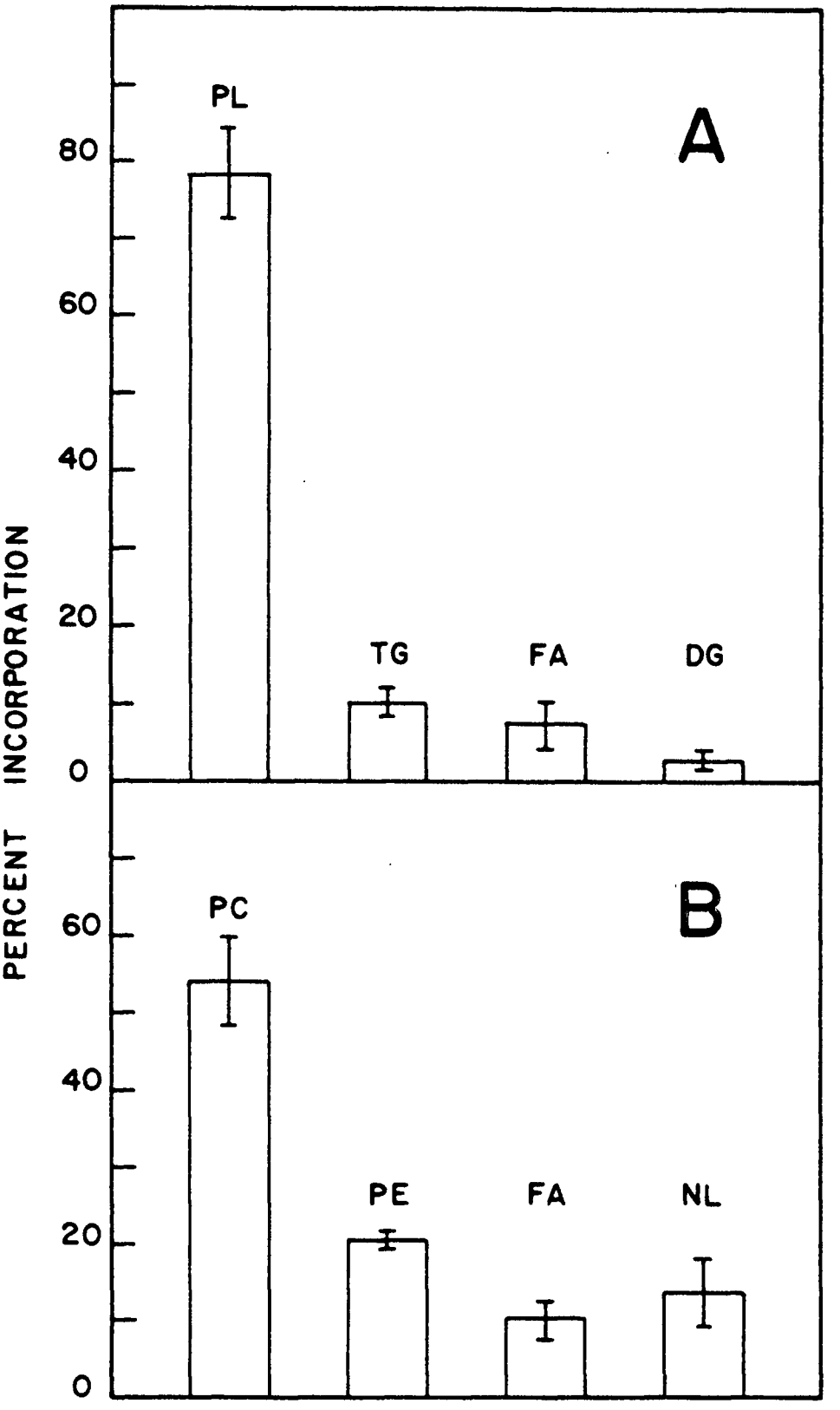
PERCENT INCORPORATION
A
PL
TG
FA
DG
0
20
40
60
80
B
PC
PE
FA
NL
0
20
40
60

Figure 3

Figure 4. Two-dimensional TLC of lipids from microsomes isolated from trout liver after administration of ^{14}C-sterculic acid. (a) inositol phospholipids + sphingomyelin, (b) serine phospholipids, (c) choline phospholipids, (d) ethanolamine phospholipids, (e) free fatty acids, (f) neutral lipids.

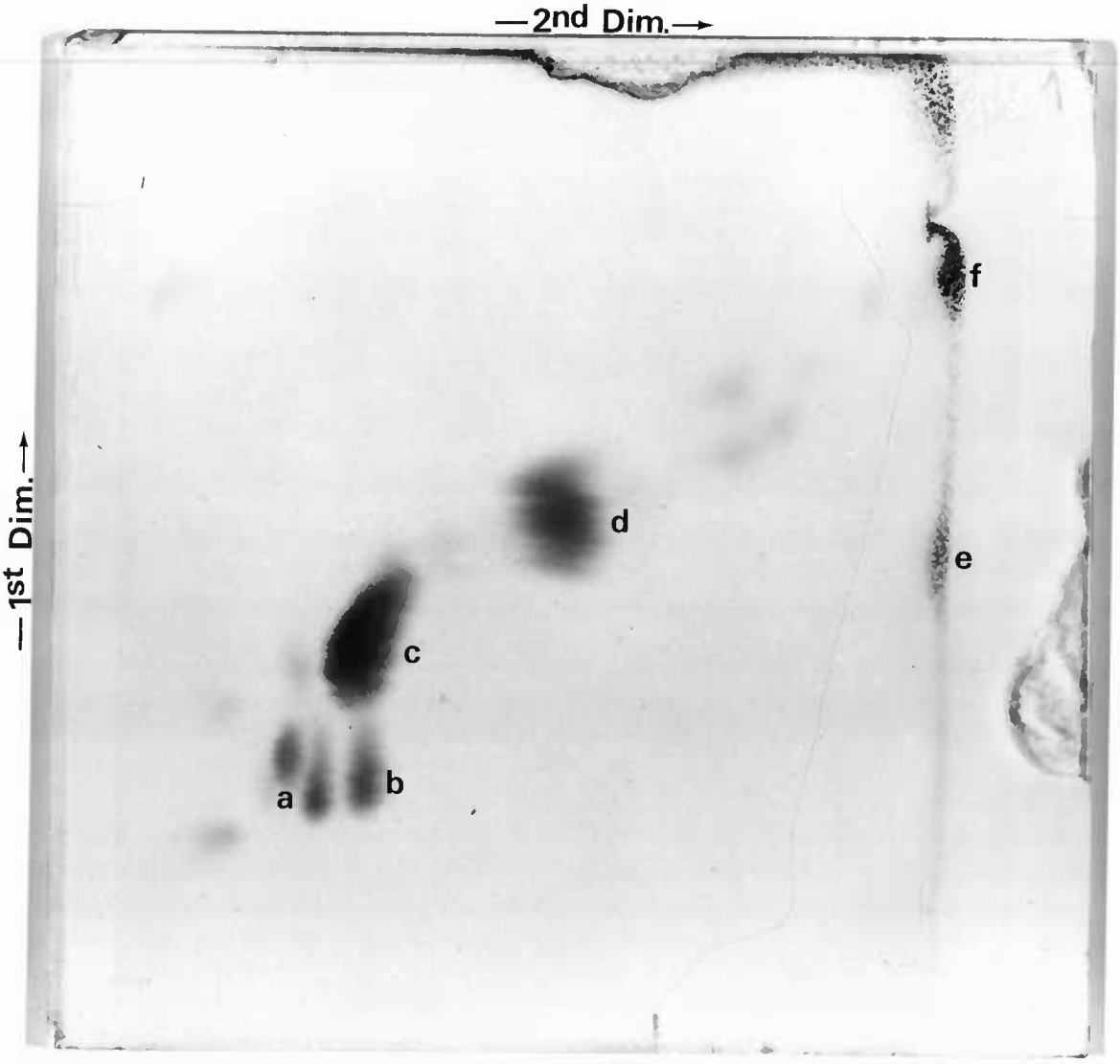

Figure 4

with phospholipase A_2 are shown in Figure 5. Phospholipase A_2 appeared to cleave all of the CP into component free fatty acids and lysoCP. EP were also cleaved into the component free fatty acids and lyso compounds. However, a small amount of EP were not affected by phospholipase A_2 treatment, as revealed by a light spot in the region of the TLC plate corresponding to EP.

Separation of phospholipase A_2 treated CP and EP which were isolated from liver microsomes of trout administered ^{14}C-sterculic acid is shown in Figure 6. The percent distribution of radioactivity in the various components is also shown in Figure 6. In phospholipase A_2 treated CP, 61% of the label was found in lysoCP and 39% in the free fatty acid. No label was found in the area of the plate corresponding to CP. In phospholipase A_2 treated EP, 80.7% of the label was found in lysoEP and 6.6% in the free fatty acid. A small amount of label (11.1%) was found in intact EP which were not cleaved by the action of phospholipase A_2. These results indicate a 2:1 preference of ^{14}C-sterculic acid for the 1-position of CP. EP on the other hand, were almost exclusively labelled in the 1-position.

Protein Analysis of Labelled Microsomes

A small amount of radioactivity was consistently found in the protein precipitate which remained following extraction of lipids from microsomal membranes. The level of radioactivity found in the protein was typically about 2% of that found in the lipid extract. In an attempt to find out if this radioactivity was associated with a specific protein component, the proteins were separated in polyacrylamide tube gels, the gels sliced into sections, and each section measured for radioactive content. No counts above background were found in any of the sections.

Cholesterol and Phospholipid Determinations

Results of the effect of CPFA on cholesterol and phospholipid levels of liver microsomal membranes are shown in Table 1. A one-way

Figure 5. TLC of CP and EP standards. I. CP II. CP treated with phospholipase A_2; (a) lysoCP, (b) free fatty acids. III. EP IV. EP treated with phospholipase A_2; (c) lysoEP (d) free fatty acids.

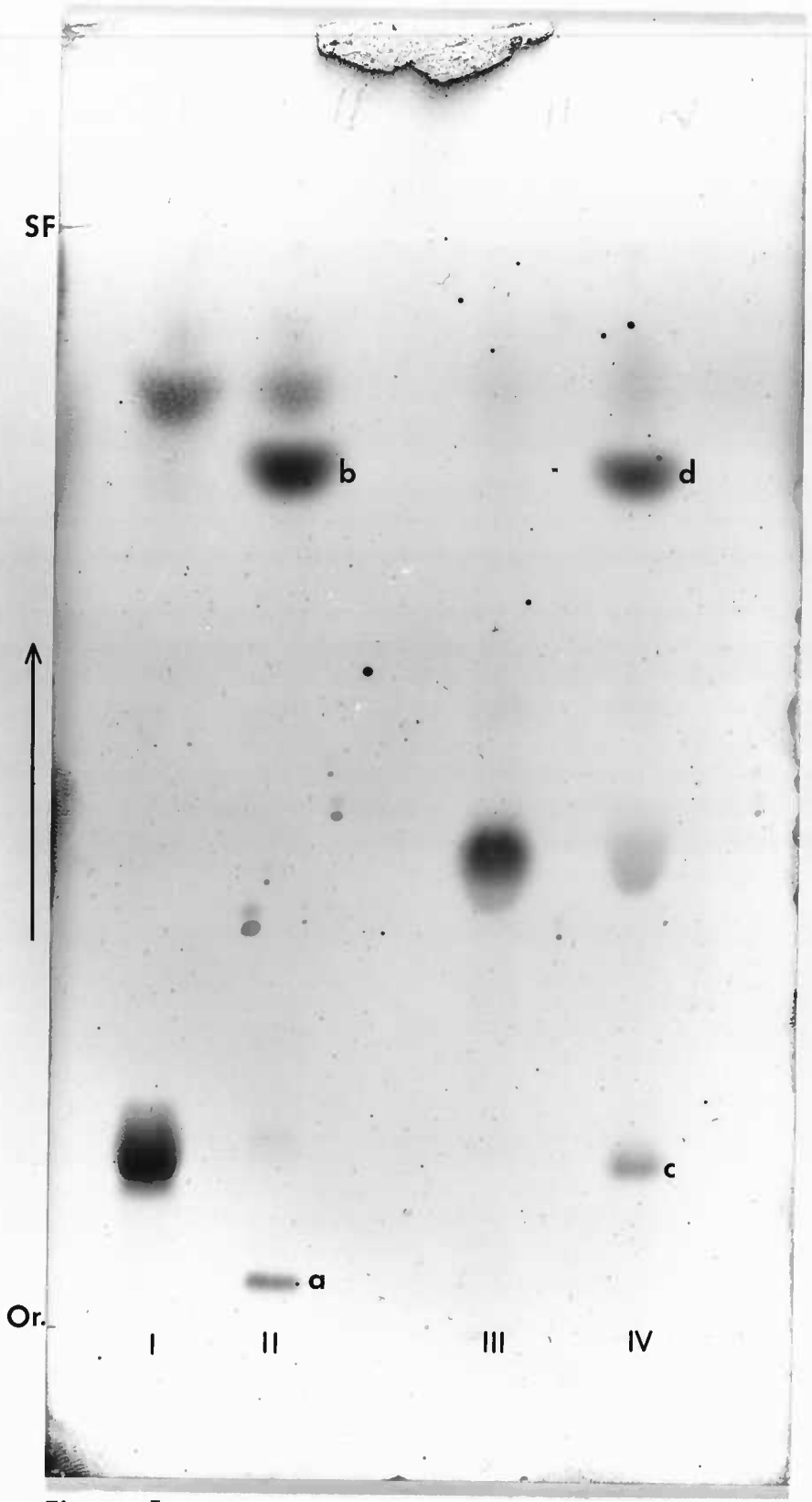

Figure 5

Figure 6. TLC and percent distribution of label in phospholipase A_2 treated CP and EP purified from isolated microsomes of trout following administration of ^{14}C-sterculic acid.

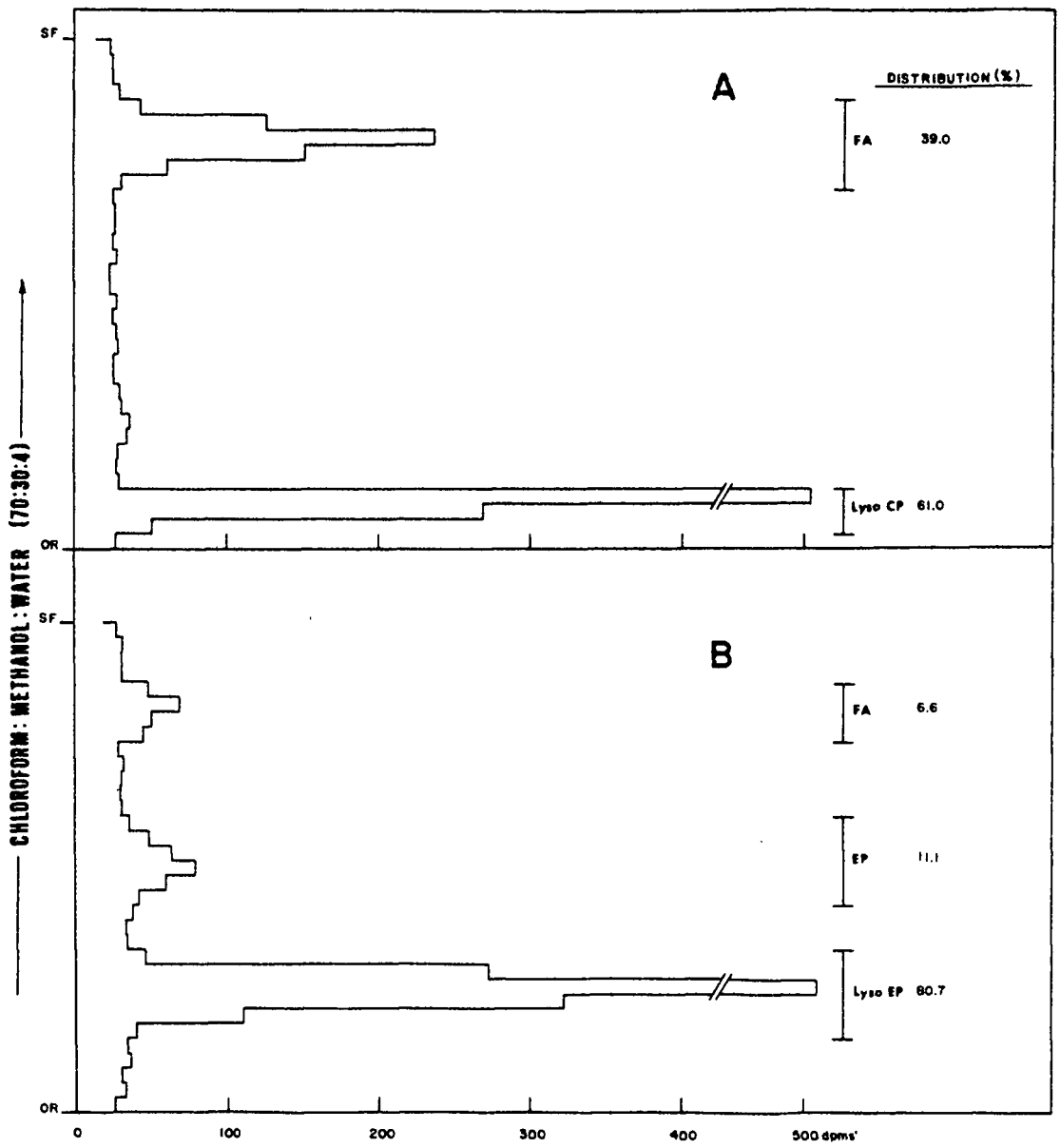
SF
OR
A
DISTRIBUTION (%)
FA 39.0
Lyso CP 61.0
SF
OR
B
FA 6.6
EP 11.1
Lyso EP 80.7
CHLOROFORM: METHANOL: WATER (70:30:4)
0
100
200
300
400
500 dpms'

Figure 6

Table 1. Cholesterol and phospholipid levels of microsomal membranes isolated from trout on control, 50 ppm, and 300 ppm CPFA diets.

Treatment	Control $n^1=7$	50 ppm n=5	300 ppm n=6	F Value[2]
Cholesterol (umoles/mg protein)	0.276	0.277	0.264	0.265
Phospholipid (umoles/mg protein)	0.771	0.712	0.693	0.565
Cholesterol/phospholipid ratio	0.361	0.398	0.393	0.687

[1] n represents a separate microsomal isolation.

[2] $F_{.05(2,15)} = 3.68$

analysis of variance showed no significant differences in cholesterol levels, phospholipid levels, or cholesterol/phospholipid ratios among control, 50, and 300 ppm CPFA fed fish.

Crosslinking Studies

Results of the crosslinking of microsomal membrane proteins of trout liver with DTBP are shown in Figures 7, 8, and 9. Figure 7 shows a linear acrylamide gradient gel ranging from 4 to 15% acrylamide. The stacking gel is a 2% acrylamide gel stabilized with 0.7% agarose. The level of crosslinker used to crosslink the proteins increases from left to right (lanes 3 through 14) from a level of 0.14 mM to 14.0 mM. Low molecular weight standards are in lane 1, high molecular weight standards in lane 15, and control microsomes in lane 2. As seen in this figure, crosslinking of microsomal proteins resulted in the formation of very high molecular weight complexes that were retained at the top of either the separating gel or the stacking gel. As the concentration of crosslinking reagent was reduced, a smaller amount of high molecular weight material was formed, but no intermediate molecular weight material that was resolvable on the gels appeared. The high molecular weight complexes were not resolvable on composite agarose-acrylamide gels with an acrylamide content as low as 1.75% (data not shown). The addition of urea to a concentration of 8 M also had no effect on the solubility of these complexes.

The fact that these high molecular weight complexes were due to the formation of crosslinked protein aggregates was confirmed by reversal of the crosslinked proteins through cleavage of the disulfide bonds with B-mercaptoethanol. These results are shown in Figure 8. Figure 8 shows a linear acrylamide gradient gel ranging from 7.5 to 15.0% acrylamide with a 5% stacking gel. The level of crosslinker used ranges from 0 (lane 1) to 28 mM (lane 13). Lane 14 contains low molecular weight standards. Lane 10 contains microsomal proteins crosslinked with 7 mM DTBP, then reversed by addition of 2% B-mercaptoethanol followed by boiling for 2 minutes. As can be seen from this figure, treatment with B-mercaptoethanol regenerated the

Figure 7. Gel electrophoretic pattern of trout liver microsomal proteins treated with various concentrations of DTBP. Levels of DTBP used and the corresponding lanes are as follows: (1) Low MW standards (2) Control (3) 0.14 mM (4) 0.71 mM (5) 1.07 mM (6) 1.42 mM (7) 2.85 mM (8) 4.27 mM (9) 5.69 mM (10) 7.11 mM (11) 8.53 mM (12) 9.96 mM (13) 11.38 mM (14) 14.2 mM (15) High MW standards.

Figure 7

Figure 8. Gel electrophoretic pattern of trout liver microsomal proteins treated with various concentrations of DTBP. Levels of DTBP used and the corresponding lanes are as follows: (1) Control (2) Blank (3) 0.014 mM (4) 0.071 mM (5) 0.14 mM (6) 0.71 mM (7) 1.42 mM (8) 4.27 mM (9) 7.11 mM (10) 7.11 mM, then treated with 2% B-mercaptoethanol (11) 11.38 mM (12) 14.2 mM (13) 28.5 mM (14) Low MW standards.

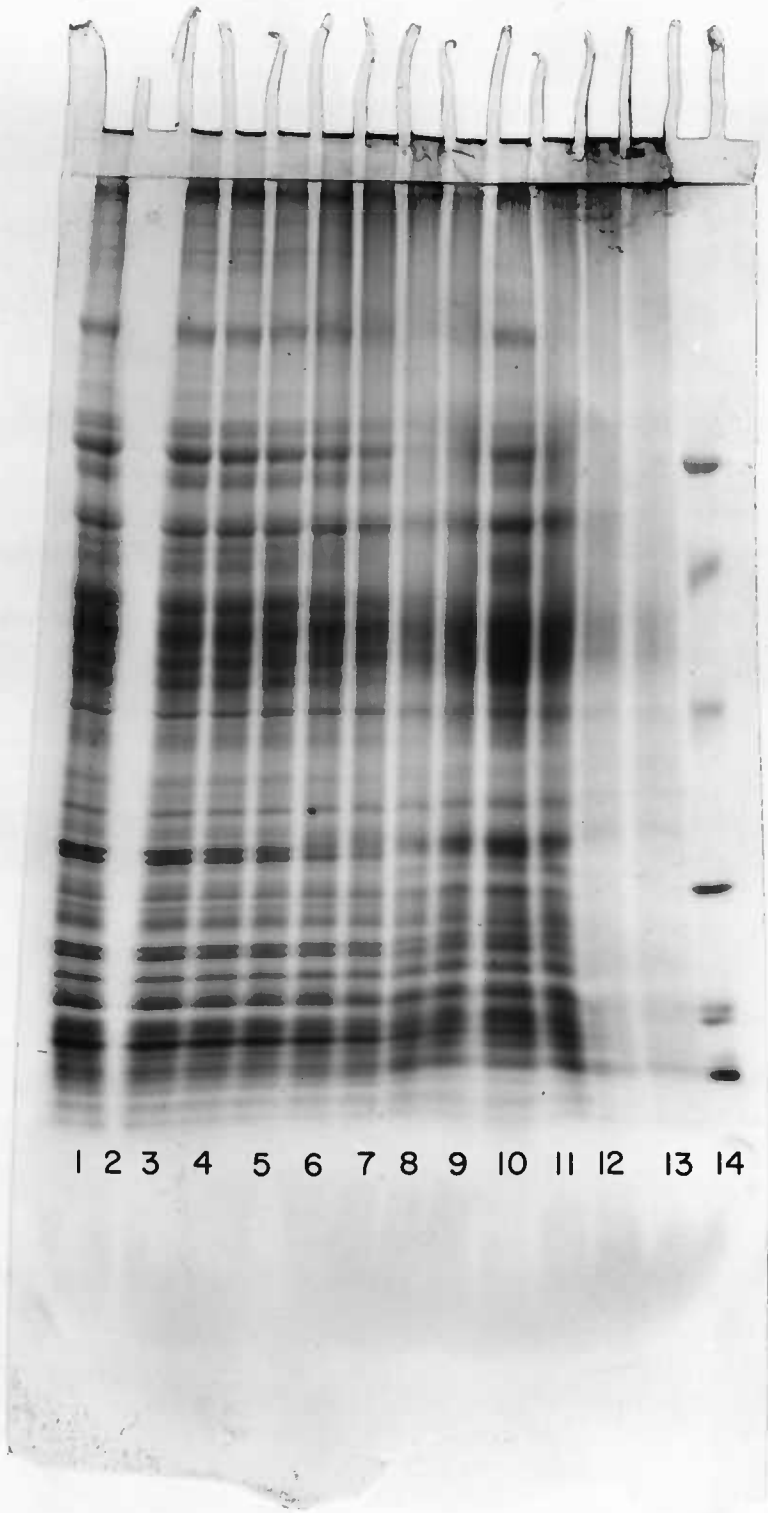

Figure 8

normal pattern of proteins seen in non-crosslinked microsomes. Lane 9 contains microsomes crosslinked with the same level of crosslinker (7 mM), but not treated with B-mercaptoethanol. However, it should be noted that the crosslinks between proteins in lanes 9 and 11 have been partially reversed due to diffusion of B-mercaptoethanol through the gel from lane 10. This is evident from looking at lane 8, which contains microsomes crosslinked with a lower level of DTBP (4.3 mM) than those in lane 9 (7.0 mM). The effect of the reversal of the crosslinks between proteins would have been clearer had an empty lane been left between reversed and non-reversed crosslinked samples. Microsomes crosslinked with the same level of DTBP (7 mM) as in lane 10 but not reversed can also be seen in lane 10 of Figure 7. The effect of reversing the crosslinking is more dramatic if one compares these two lanes.

Figure 9 shows the separation of control and crosslinked microsomes in 10% acrylamide tube gels, followed by reversal of the crosslinks and separation in the second dimension on a 10% acrylamide slab gel. As seen in Figure 9A, crosslinking again resulted in the formation of high molecular weight complexes which stacked at the top of the separating and stacking gels. Reversal of these crosslinked proteins regenerated the smaller molecular weight components which separated as a line in the second dimension (Figure 9C and D).

Figure 10 shows the results of the crosslinking of trout liver plasma membrane proteins. Figure 10 shows the separation of crosslinked plasma membrane proteins on a 10% acrylamide slab gel with a 4.5% acrylamide stacking gel. In lanes 1, 6, and 10 are non crosslinked membranes, in lanes 3, 7 and 11 are membranes crosslinked with 2.9 mM DTBP, and in lanes 4, 8, and 12 are membranes crosslinked with 7.1 mM DTBP. Lanes 14 and 15 contain low molecular weight standards. In contrast to the results obtained with microsomal membranes, the use of DTBP failed to crosslink the proteins of isolated plasma membranes of trout liver. As can be seen in Figure 10, the use of a concentration of DTBP which resulted in the formation of a substantial amount of high molecular weight crosslinked material in microsomal

Figure 9. Gel electrophoretic patterns of trout liver microsomal proteins treated with two levels of DTBP. A. Levels of DBTP used and the corresponding tubes are as follows: (1) Low MW standards (2) Control (3) 4.27 mM (4) 11.38 mM. B. Separation of control microsomal proteins (tube 2 of figure 9A) in the second dimension. C. Separation of microsomal proteins treated with 4.27 mM DTBP in the second dimension after treatment of the first dimensional tube gel (tube 3 of figure 9A) with 3% B-mercaptoethanol. D. Separation of microsomal proteins treated with 11.38 mM DTBP in the second dimension after treatment of the first dimensional tube gel (tube 4 of figure 9A) with 3% B-mercaptoethanol.

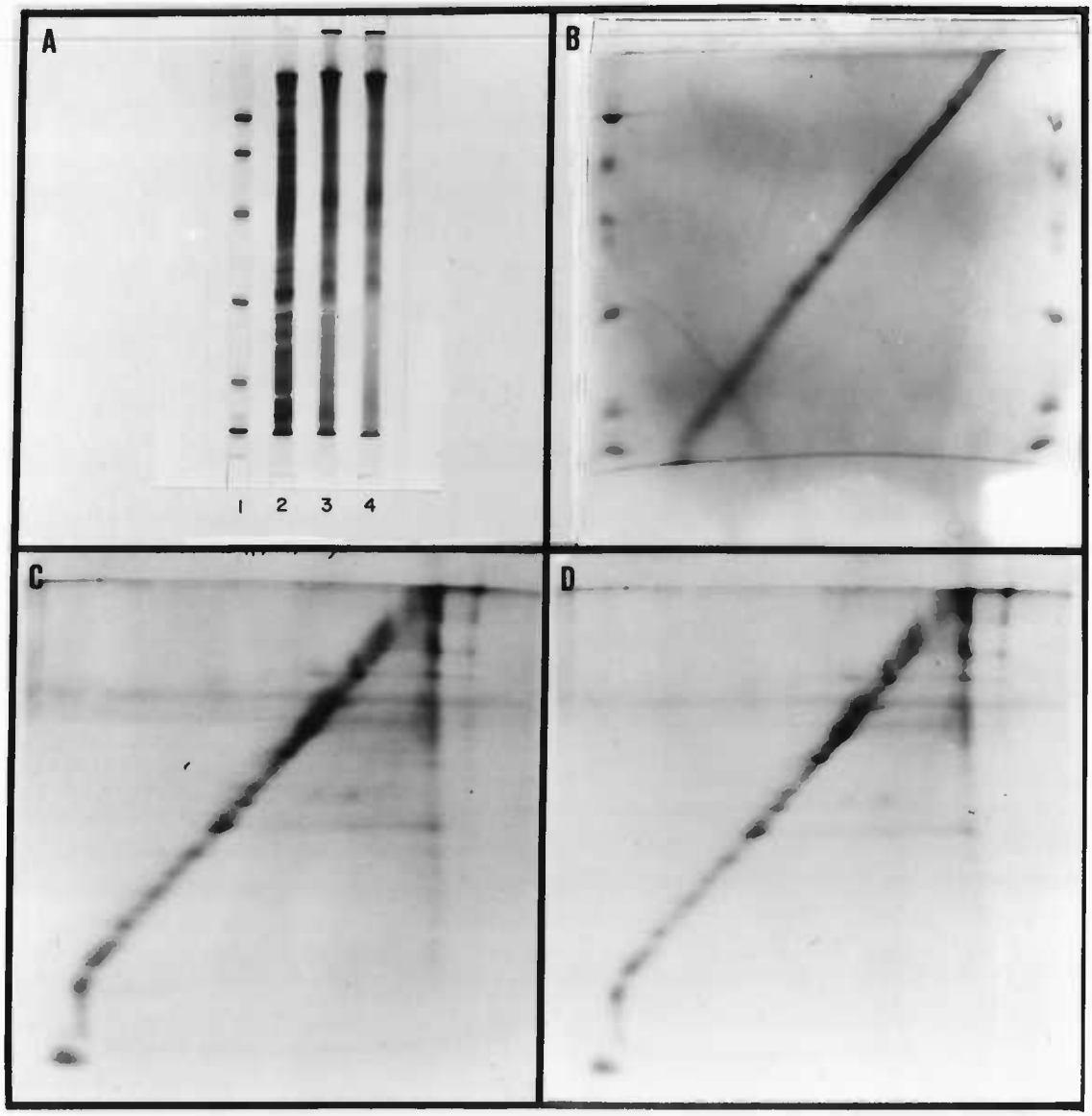

Figure 9

Figure 10. Gel electrophoretic pattern of trout liver plasma membrane proteins treated with two levels of DTBP. Levels of DTBP used and corresponding lanes are as follows: (1) Control (2) Blank (3) 2.9 mM (4) 7.1 mM (5) Blank (6) Control (7) 2.9 mM (8) 7.1 mM (9) Blank (10) Control (11) 2.9 mM (12) 7.1 mM (13) Blank (14) Low MW Standards (15) Low MW standards.

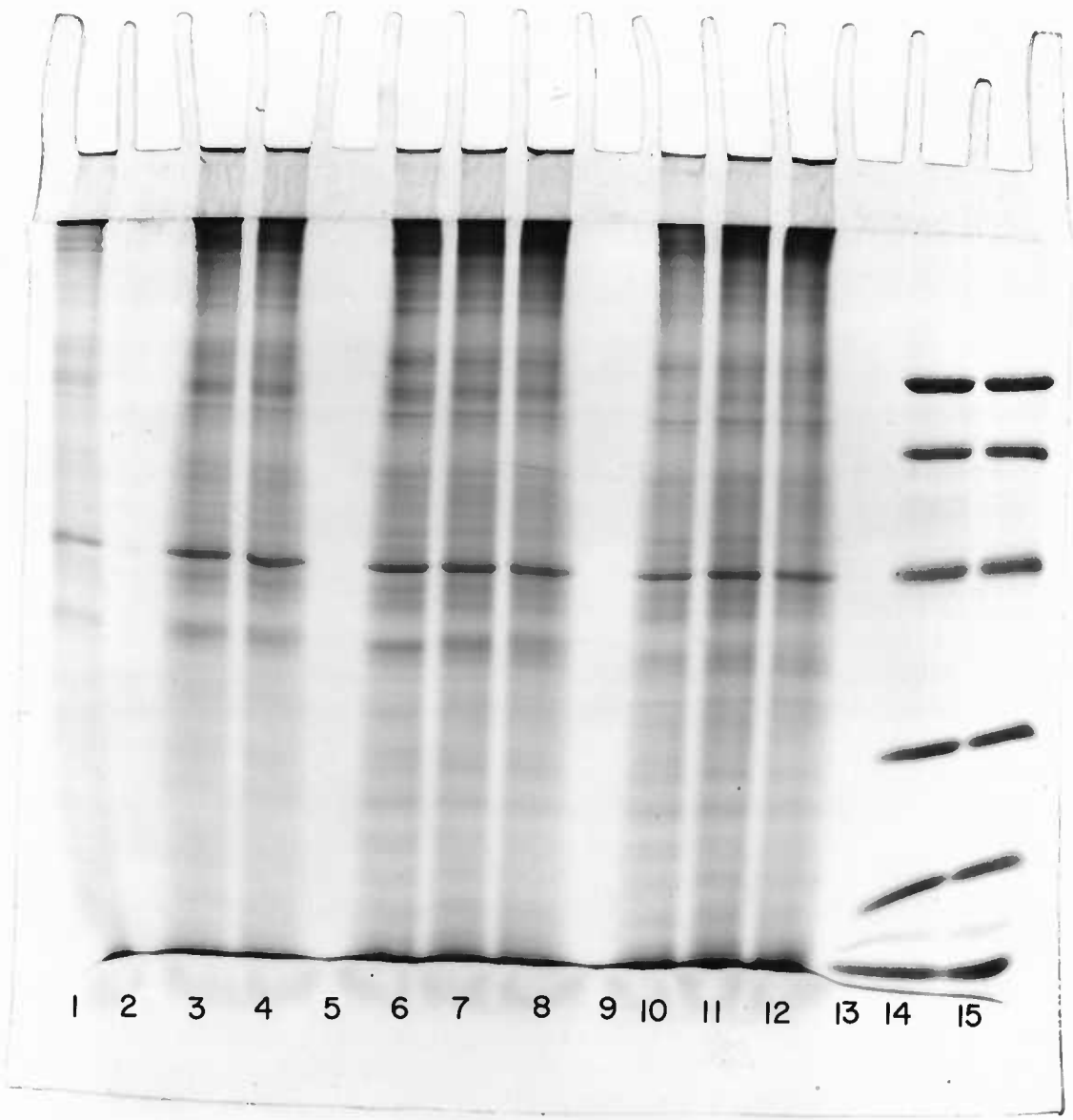

Figure 10

membranes (Figure 7) failed to yield crosslinked aggregates in plasma membranes.

These results were confirmed by two-dimensional electrophoresis of plasma membrane proteins that had been treated with DTBP. In this experiment, plasma membranes were treated with 14.2 mM DTBP and separated in the first dimension on a 7% acrylamide tube gel. The tube gel was then treated with B-mercaptoethanol and the proteins separated in the second dimension on a 7.5 to 15% acrylamide gradient gel (Figure 11B). Crosslinked proteins should appear as spots below the diagonal in the second dimension. The lack of such spots (Figure 11B) confirms that no crosslinking took place.

Isoelectric Focusing of Membrane Proteins

Figure 12 shows a typical isoelectric focusing gel of trout liver microsomal proteins and the associated pH gradient. Similar results were obtained for plasma membrane proteins. No differences were noted in the focusing pattern of membrane proteins between control and CPFA fed trout.

Two-Dimensional Gel Electrophoresis of Microsomal Membranes

Figure 13 shows a typical two-dimensional separation of liver microsomal membrane proteins from control, 50 ppm, and 300 ppm CPFA fed trout. The reproducibility of these separations was very good from one gel to the next. However, analysis of these gels proved to be very difficult due to the complexity of the staining patterns.

The procedure used to compare these gels revealed no major differences among liver microsomal proteins from control, 50 ppm and 300 ppm CPFA fed trout. However, there was one small difference that appeared to be relatively consistent. This difference was in a group of proteins in the 37,000 dalton range (see arrows in Figure 13). These proteins consistently appeared as a group of from four to six proteins in the CPFA gels, while only two to four proteins were

Figure 11. Separation of plasma membrane proteins in the second dimension. A. Control membranes, after separation in a first dimensional tube gel. B. Membranes treated with 14.2 mM DTBP, followed by separation in a first dimensional tube gel and then treated with 3% B-mercaptoethanol.

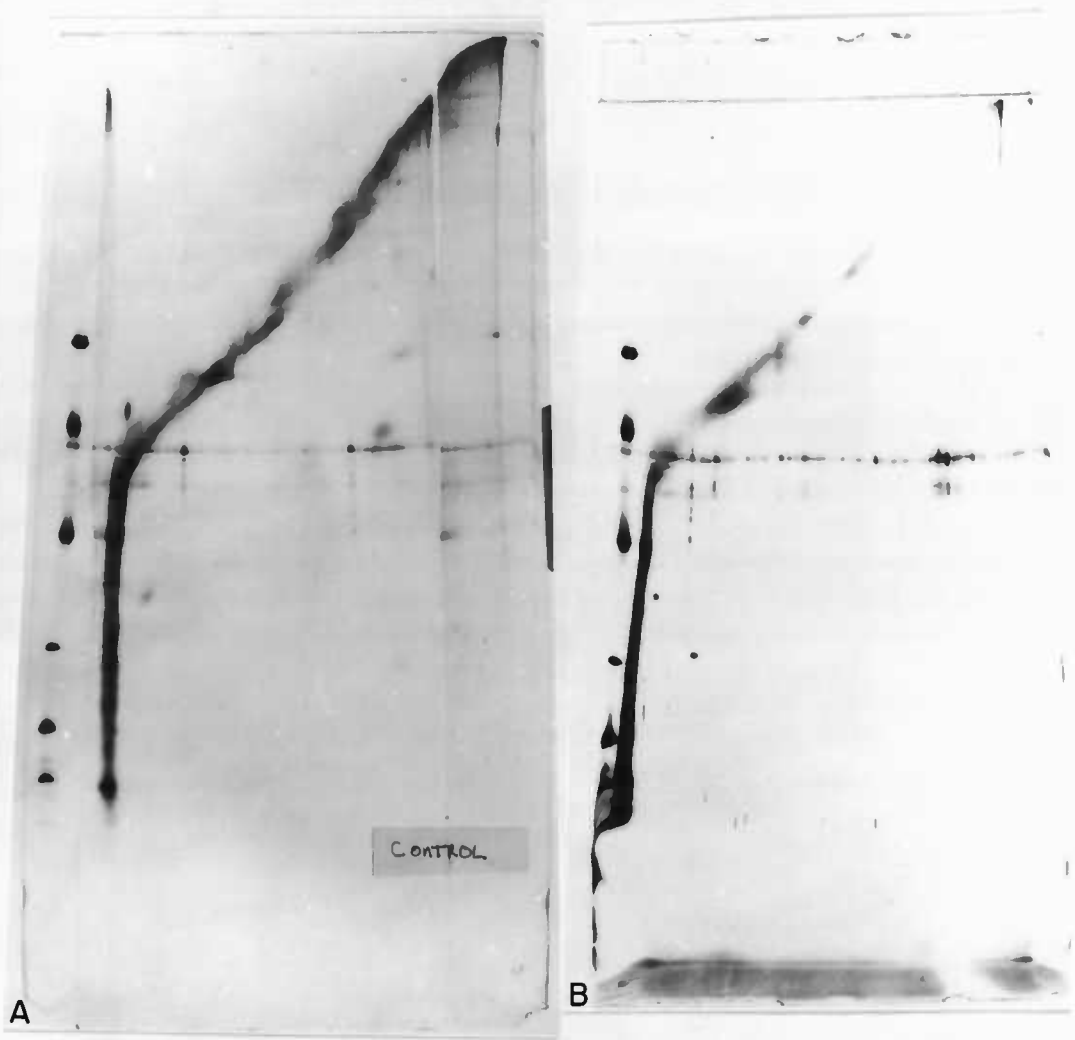

Figure 11

Figure 12. First dimensional isoelectric focusing gel of trout liver microsomal membrane proteins and the associated pH gradient.

Figure 12

Figure 13. Two-dimensional separation of microsomal membrane proteins isolated from trout liver. A. Control. B. 50 ppm CPFA. C. 300 ppm CPFA.

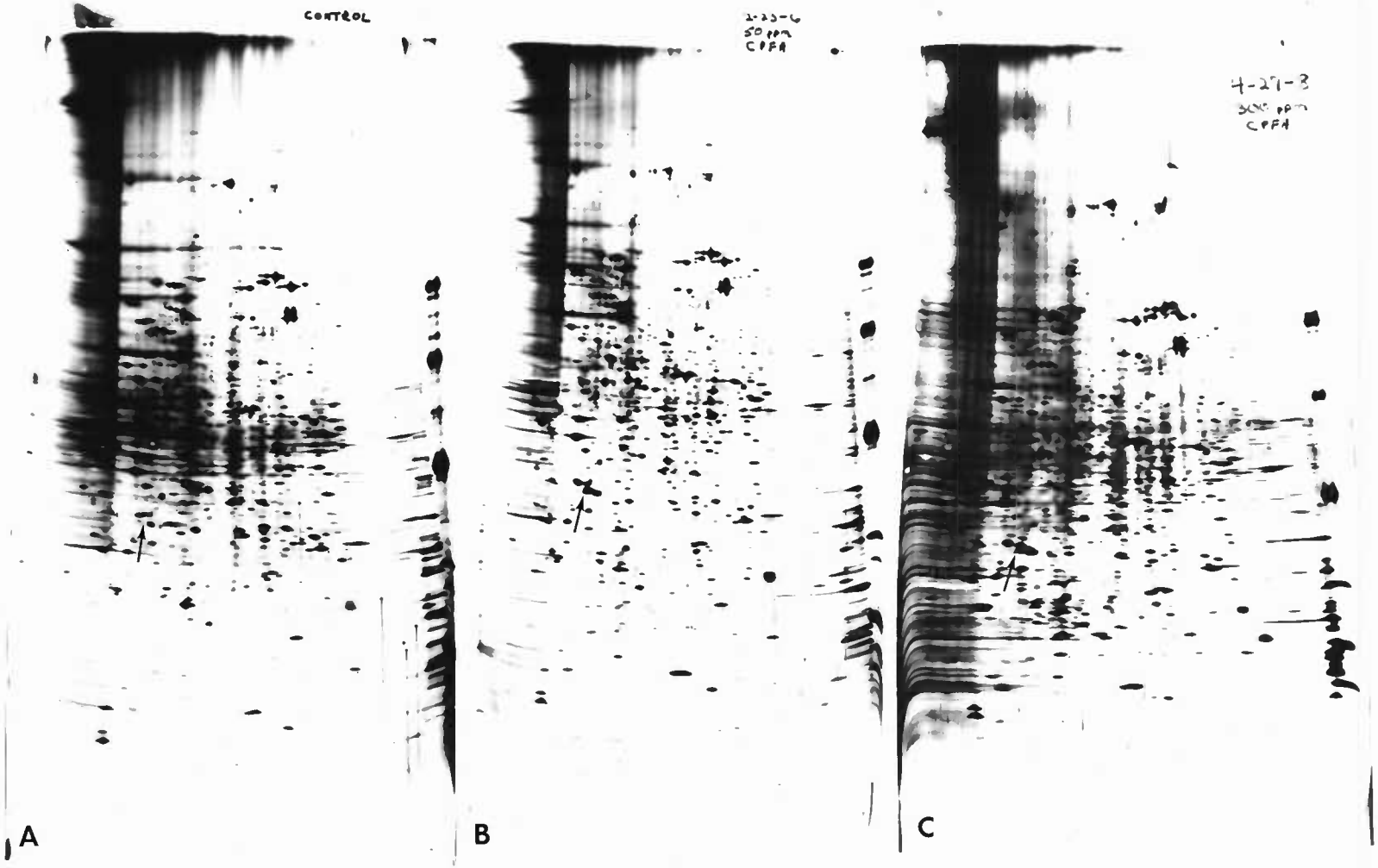

Figure 13

present in control gels. This difference was noted in all but one set of gels.

Two-Dimensional Gel Electrophoresis of Plasma Membranes

Figure 14 shows a typical two-dimensional separation of liver plasma membrane proteins from control, 50 ppm, and 300 ppm CPFA fed trout. Because of the dark background seen in these gels, it was in some cases difficult to compare the staining pattern between gels. For this reason, a further purification of plasma membranes was carried out in an attempt to obtain a purer membrane fraction and to reduce background staining. Figure 15 shows a two-dimensional separation of the purified plasma membranes. As can be seen from these gels the background was significantly reduced, facilitating comparison of the staining pattern of the gels.

The procedure used to compare the gels did not reveal a completely reproducible pattern of differences between control and CPFA fed trout liver plasma membrane proteins. Although some differences were noted between gels run on the same day, these differences were apparently not reproducible from one run to the next, with one possible exception. There was consistently a greater amount of high molecular weight material in the gels of 50 ppm and 300 ppm CPFA fed trout liver plasma membranes (See arrows in Figures 14 and 15). This material, which ranged from a molecular weight of 93,000 to 125,000 was observable in 9 of the 10 gels from CPFA fed fish and seemed to be present in higher concentration than in control gels. In fact, this material was observed in only two of the ten control gels that were run.

Figure 14. Two-dimensional separation of plasma membrane proteins isolated from trout liver. A. Control. B. 50 ppm CPFA. C. 300 ppm CPFA.

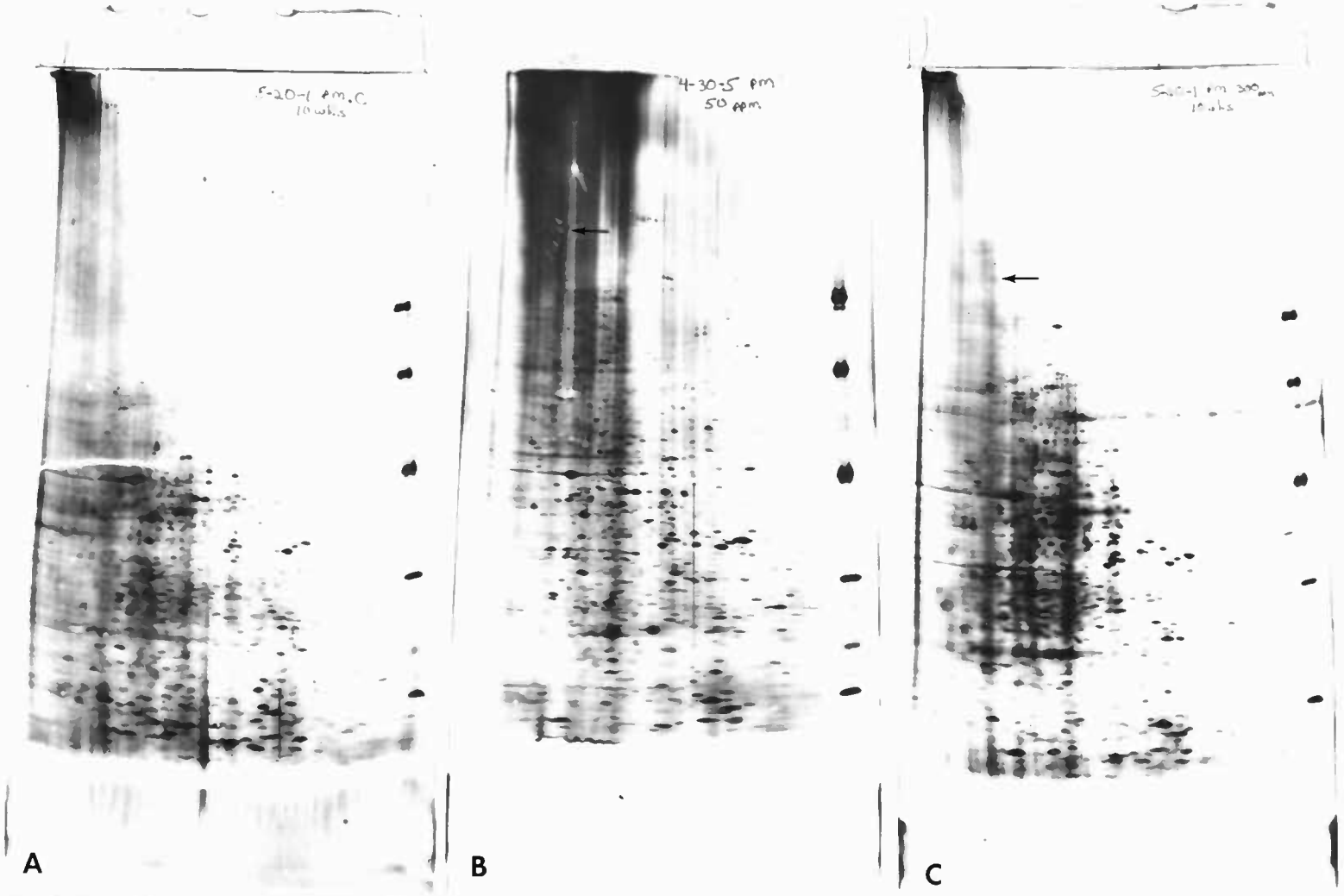

Figure 14

Figure 15. Two-dimensional separation of purified plasma membrane proteins isolated from trout liver. A. Control. B. 50 ppm C. 300 ppm.

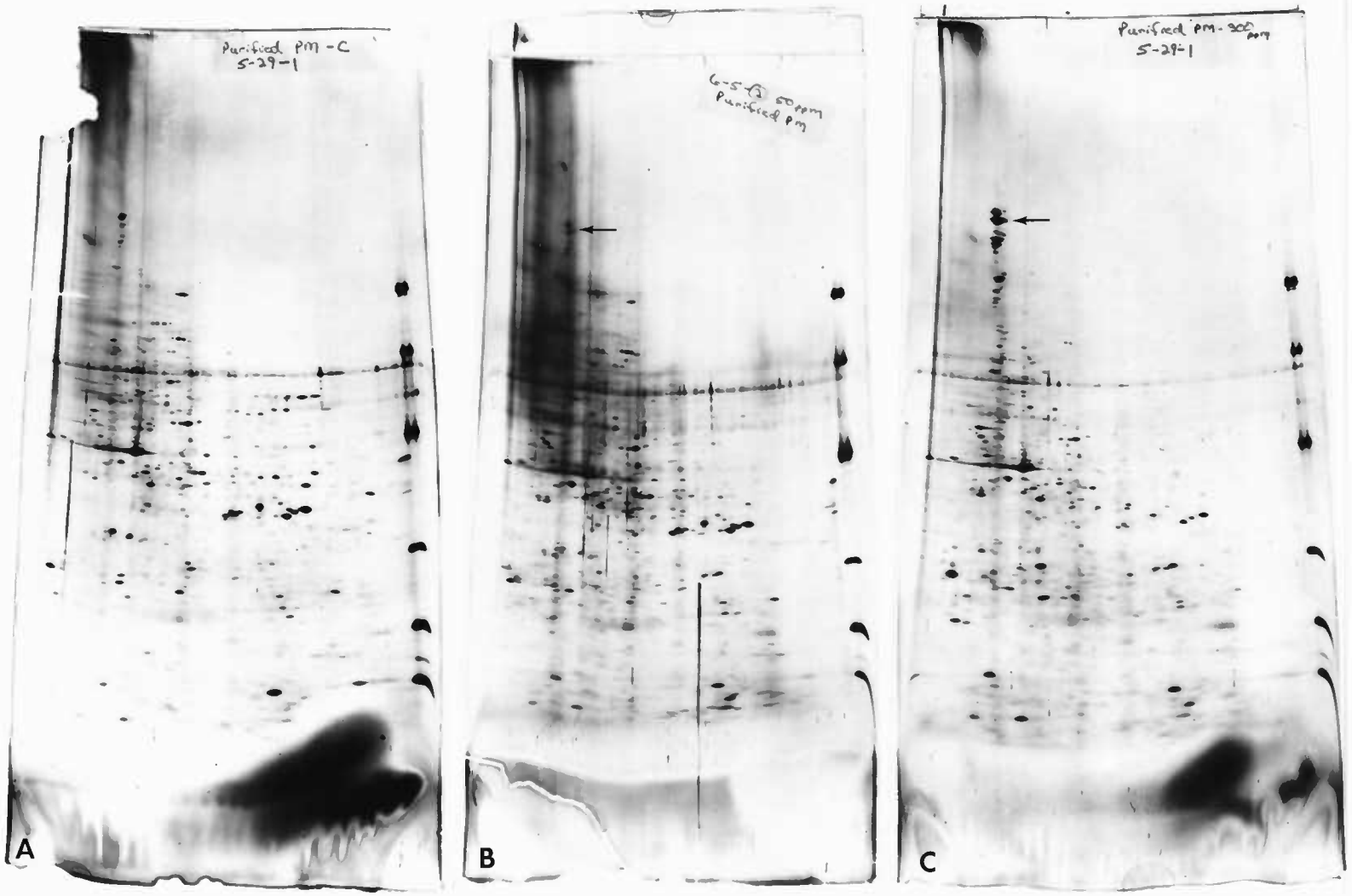
Purified PM - C
5-29-1
A
6-5-A 50rem
Purified PM
B
Purified PM - 300 rem
5-29-1
C

Figure 15

DISCUSSION

Incorporation of ^{14}C-Sterculic Acid into Microsomal Lipids

The incorporation of ^{14}C-sterculic acid into trout liver microsomal membrane lipid components suggest that CPFA readily enter into the normal pathways of phospholipid biosynthesis. In addition to this, the distribution of ^{14}C-sterculic acid into the various lipid components seems to reflect the composition of the microsomal membrane. For example, CP are known to be the main component of trout liver microsomal membranes (Selivonchick and Williams, 1982) as well as most other biological membranes (Thompson, 1980). EP are the second most predominant phospholipid, followed by lower levels of inositol phospholipids, serine phospholipids, and sphingomyelin (Selivonchick and Williams, 1982; Thompson, 1980). Thus, it appears that CPFA are readily used in the synthesis of all phospholipids and are probably incorporated into other cellular membranes such as the plasma membrane.

These results are not entirely consistent with the results obtained by Eisele *et al.* (1979). These investigators studied the incorporation of ^{14}C-sterculic acid into various organs of the trout, including the liver. They found 76% of the radioactivity incorporated into the triglyceride fraction, and only 18.5% incorporated into phospholipids. However, they analyzed a total extract from whole liver, not a subcellular membrane such as was done in this study. Since membranes are greatly enriched in phospholipids and contain virtually no triglyceride, the discrepency in radiolabel incorporation between these studies is easily explained.

The treatment of isolated lipid components with phospholipase A_2 shows that sterculic acid is preferentially esterified to the 1-position of CP and EP. Since the fatty acids found esterified to the 1-position of naturally occurring phospholipids are almost exclusively saturated species (Lands and Crawford, 1976), it appears that the acyltransferases involved in the biosynthesis of phospholipids perceive sterculic acid as a saturated fatty acid. The

biosynthetic activity of these enzymes is thought to be sensitive to the configuration of the acyl chain in a way that they normally do not catalyze the esterification of fatty acids containing cis double bonds to the 1-position of the glycerol backbone (Okuyama et al., 1972). However, the altered bond angles present in a cyclopropene fatty acid such as sterculic acid evidently results in a molecular configuration which more closely resembles that of a saturated fatty acid than one containing cis double bonds.

The presence of CPFA in a biological membrane could have implications in terms of the structure and function of the membrane. In order to discuss these possible effects, it is necessary to briefly review the role of lipids in membranes.

It is thought that a major role of membrane lipids is to provide an environment of proper fluidity for optimal enzyme function (Thompson, 1980). The fluidity of a biological membrane is, to a large extent, determined by interactions between the fatty acyl chains of the constituent phospholipid molecules (Cossins and Prosser, 1978). Saturated fatty acids tend to render the membrane less fluid, while unsaturated fatty acids have the opposite effect (Farias et al., 1975).

However, a portion of the lipids present in the membrane are not associated with the fluid matrix of the lipid bilayer. These lipids, which consist of about 20% of the lipid content of the membrane, are believed to be associated with the proteins of the membrane and are referred to as boundary lipid or lipid annulus (Lenaz, 1978). These lipids are in direct contact with the intrinsic membrane protein molecules and are strongly immobilized in contrast to the lipids of the fluid bilayer. They do not show transition from the liquid crystalline phase to a more rigid state, called the gel phase, when the temperature is lowered to a characteristic point as do lipids of the fluid bilayer (Thompson, 1980). It is thought that boundary lipid plays an important role in the activity of such membrane bound enzymes as Ca^{+2}-activated ATPase of mammalian sarcoplasmic reticulum (Dean and Tanford, 1979; Warren et al., 1975), mitochondrial cytochrome oxidase (Awasthi et al., 1970), and the microsomal drug metabolizing enzymes

(Wade and Norred, 1976; Stier et al., 1979). Boundary lipid may function by providing the environment necessary for an enzyme to assume its proper tertiary structure, thus allowing the active site to assume the conformation necessary for optimal activity (Lenaz, 1978).

The presence of CPFA could have an effect upon both boundary lipid and the fluidity of a membrane. However, the evidence suggests that CPFA do not significantly alter the fluidity of the membranes of trout liver. The acyl group in the 2-position of animal phospholipids is believed to be principally responsible for the degree of fluidity of the membrane (Lands and Crawford, 1976). Since sterculic acid is preferentially esterified to the 1-position, it is likely to have a minimal effect upon membrane fluidity. In addition, a study done in this laboratory showed no effect of CPFA upon membrane fluidity (Selivonchick, unpublished results). In this study the fluidity of liposomes prepared from liver phospholipids isolated from control and CPFA fed trout were measured by fluorescence polarization. The results showed no difference in fluidity between the two samples. The high degree of unsaturation of trout liver phospholipids apparently overrides any effect which might be introduced by the presence of a cyclopropene ring. It is more likely that CPFA have an effect on boundary lipid. The close association of cyclopropene rings with the proteins of the membrane may alter the boundary lipid environment. This could result from the interference by cyclopropene rings with the normal packing of the hydrocarbon chains of phospholipids around proteins. This could in turn affect the function of these proteins.

The presence of CPFA in membranes could have far reaching consequences in terms of overall metabolic regulation of the cell. For example, membranes are involved in such processes as transport of materials into and out of the cell, energy production, and protein synthesis. They also serve as antigenic sites, anchoring sites for enzyme systems, and as hormone receptor sites. The plasma membrane is believed to be an important controlling factor in the regulation of cell division (Pardee, 1971). Interference in any of these functions through alterations in membranes could lead to metabolic derangement of the cell.

Protein Analysis of Labelled Microsomes

No ^{14}C-sterculic acid was found associated with a specific protein component of the microsomal membrane. Similar results were obtained by Jeffcoat and Pollard (1977). These investigators incubated rat liver microsomes with ^{3}H-sterculic acid and looked for the association of the label with protein components of the membrane. They did not find ^{3}H-sterculic acid to be covalently attached to proteins of the microsomal membrane. Thus, it does not appear that CPFA react with sulfhydryl groups of enzymes as suggested by Kircher (1964) and Ory and Altschul (1964). The presence of a small amount of radioactivity in the protein precipitate was probably due to residual lipids which were not extracted.

Effect of CPFA on Cholesterol Levels of Microsomal Membranes

CPFA have been shown to cause increases in liver cholesterol and plasma cholesterol of New Zealand rabbits (Ferguson et al., 1976). For this reason it was of interest to determine the effect of CPFA on the membrane cholesterol level of trout liver.

Cholesterol is known to be an important constituent of cell membranes. It is thought to act as a stabilizing factor in membranes and to modulate the fluidity of the membrane. These effects have been attributed to the ability of cholesterol to influence the packing of hydrocarbon chains of phospholipids, possibly by way of an equimolar cholesterol-phospholipid complex (Harrison and Lunt, 1980). Changes in cholesterol levels of biological membranes have been associated with increased osmotic fragility and altered permeability characteristics of these membranes (Papahadjopoulos, 1973).

Since no significant difference in microsomal membrane cholesterol levels was found between control and CPFA fed fish, it appears that CPFA do not alter the characteristics of trout liver microsomal membranes through an effect on cholesterol level. Scarpelli et al. (1974), in investigating changes in the endoplasmic reticulum of trout hepatocytes induced by dietary CPFA, suggested

these changes may be related to the cholesterol content of the membrane. The results of the present study indicate this not to be the case.

Crosslinking Studies

The alteration in the lipid portion of membranes of trout fed CPFA is twofold. First of all, the cyclopropene ring is readily incorporated into the lipid bilayer of the membrane. Secondly, the saturation of the membrane lipids of trout fed CPFA is markedly increased in both microsomes and plasma membranes (Selivonchick and Williams, 1982). These alterations may have secondary effects upon membrane structure and function. One possible consequence is that the structural organization and protein-protein interactions of the membrane are altered.

A technique that has been used in recent years to assess spatial relationships between proteins has involved the use of bifunctional protein crosslinking reagents. These reagents are, in general, small organic molecules containing two chemical groups which are reactive toward the functional groups on the side chains of proteins. Most of the reagents that have been used react with the nucleophilic -SH and $-NH_2$ groups of proteins. Under appropriate conditions, such reagents will provide a covalent link between reactive groups in proteins which are a certain distance apart. Crosslinked products are then analyzed by either one or two-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE). These reagents have provided useful information about the spatial arrangement of proteins in such systems as the 50S ribosomal subunit of Escherichia coli (Traut and Kenny, 1976), human erythrocyte membranes (Wang and Richards, 1975), sarcoplasmic reticulum of rabbit skeletal muscle (Louis and Shooter, 1972), and paramyxoviruses (Markwell and Fox, 1980). This technique has also been used to study the subunit arrangement of such membrane bound enzyme complexes as the Ca^{2+} and Mg^{2+}-activated ATPase of the inner membrane of E. coli (Bragg and Hou, 1975).

The crosslinking reagent used in the present study was dimethyl 3,3'-dithiobispropionimidate·2HCl (DTBP), a water soluble imidoester that forms a cleavable disulfide linkage between amino groups 11 °A apart (Figure 16). This reagent has been used to study the spatial arrangement of proteins in animal viruses (Markwell and Fox, 1980), rabbit muscle sarcoplasmic reticulum (Louis et al., 1977), and human erythrocyte membranes (Wang and Richards, 1975).

The use of DTBP in the present study failed to provide a clear picture of protein-protein interactions in the microsomal membrane. This was due to the formation of very high molecular weight aggregates which were not resolvable on polyacrylamide gels. DTBP therefore could not be used to assess differences between membranes from control and CPFA fed trout. For this technique to be used successfully, it is necessary that the higher molecular weight complexes which are formed upon crosslinking be resolved so that it is possible to determine both the molecular weight of the complex and the molecular weights of the subunits formed upon reversal of the crosslinking. Sometimes it is possible to form a higher, but resolvable, molecular weight complex by using the proper concentration of crosslinking reagent. However, in this study the use of lower concentrations of DTBP only resulted in the formation of a lesser amount of the very high molecular weight material. It did not result in the formation of intermediate molecular weight complexes which might have been resolvable on polyacrylamide gels.

The failure of this technique to provide useful information on protein interactions in a complex membrane system such as the microsomal membrane is consistent with the information found in the crosslinking literature. A number of investigators have reported the formation of high molecular weight complexes upon crosslinking which were not resolvable by SDS-PAGE in such systems as sarcoplasmic reticulum (Ji, 1979) and camel erythrocytes (Eitan et al., 1976). In fact, most crosslinking studies done on membranes have shown an intense band of crosslinked products retained at the top of the gels (Kiehm and Ji, 1977; Ji, 1979; Das and Fox, 1979). The use of this technique in complex membranes is further hindered by extreme

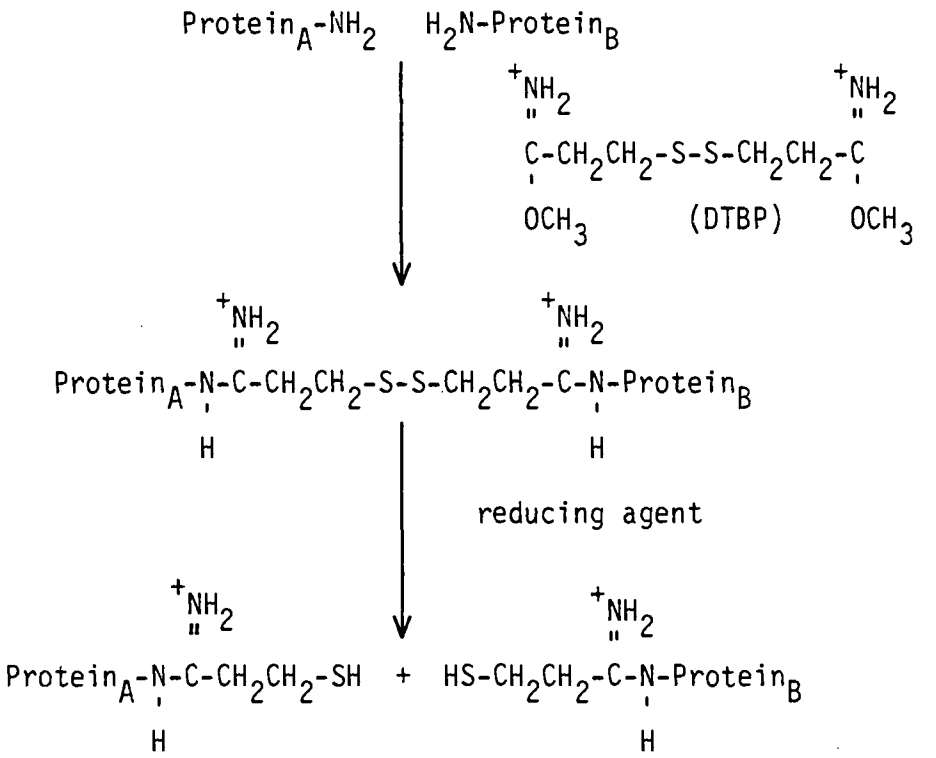

Figure 16. Crosslinking of proteins with DTBP.

difficulty in the identification of crosslinked products (Heymann and Mentlein, 1980; Jaffe et al., 1980). For these reasons the use of crosslinking reagents has been restricted to enzyme complexes solubilized from membranes rather than on intact membranes themselves (Freedman, 1979; M.A. Markwell, personal communication). An example of this approach has been in the study of beef heart mitochondrial ATPase (Satre et al., 1976). Work on intact membranes has been concentrated on systems containing only a few protein components such as the erythrocyte membrane and animal viruses, where the problems involved with the use of this technique are minimized.

In contrast to the results obtained with microsomal membranes, the use of DTBP failed to crosslink the proteins of isolated plasma membranes of trout liver. It is difficult to explain why similar conditions and levels of DTBP would extensively crosslink microsomal membrane proteins yet fail to introduce crosslinks between proteins of the plasma membrane, since both membranes are rich in protein components. Perhaps the spatial arrangement of proteins in the plasma membrane is such that they are not readily crosslinked with DTBP. The use of crosslinking reagents which crosslink over different distances than DTBP may have produced different results.

Another explanation may lie in the fact that the plasma membrane is rich in glycoproteins. The carbohydrate moiety of these proteins is usually joined to the protein through an amino group, usually of asparagine (Harrison and Lunt, 1980). Thus, the availability of free amino groups with which DTBP reacts may be greatly reduced in proteins of the plasma membrane. If this is the case, the use of crosslinking reagents which react with groups other than amino groups might provide more fruitful results. Finally, the carbohydrate moiety of the glycoproteins of the plasma membrane may restrict the accessibility of DTBP to free amino groups or may impart a structural organization to these proteins which render them unavailable to crosslinking with this particular reagent.

Two-Dimensional Gel Electrophoresis

SDS-PAGE has been an extremely useful technique in the separation and quantification of proteins from complex biological systems. This method separates proteins primarily on the basis of molecular weight (Laemmli, 1970). However, it suffers from the drawback that in complex biological mixtures there are likely to be a number of proteins with similar molecular weights. These proteins will comigrate in polyacrylamide gels and thus cannot be resolved from one another. In order to achieve a more effective separation of proteins from complex biological sources, two-dimensional separation techniques have been developed in which proteins are separated on the basis of one parameter in the first dimension and a different parameter in the second dimension. The most widely used two-dimensional separation technique of this nature is based upon the method developed by O'Farrell (1975) for analysis of bacterial cell proteins. In this technique, proteins are separated on the basis of their isoelectric point in the first dimension, followed by separation on the basis of molecular weight in the second dimension. Since these two parameters are unrelated, a much more powerful separation of complex mixtures of proteins can be achieved than with conventional SDS-PAGE.

However, application of this technique to analysis of eukaryotic membranes has not been widespread. This has probably been due to problems in the solubilization of membrane proteins (Ames and Nikaido, 1976; Sato et al., 1977; Vlasuk and Walz, 1980) as well as the general difficulty of the technique. The large expense involved in the detection of separated proteins with the use of radiolabels may also have restricted the use of this technique. Conventional staining methods are not sensitive enough for the detection of the very small amounts of proteins which are typically resolved on two-dimensional gels. However, the use of recently developed silver staining techniques have reduced the need for expensive and time consuming autoradiographic techniques. Silver staining has been reported to be 100 times more sensitive than the widely used conventional Coomassie blue stain (Merril et al., 1981).

Figure 17 shows a typical one-dimensional SDS-PAGE separation of trout liver microsomal proteins. The tremendous improvement in the resolution of these proteins by two-dimensional electrophoresis can be seen by comparing this figure to Figure 13.

It was not possible to conclusively demonstrate an alteration in the protein composition of trout liver microsomal or plasma membranes induced by dietary CPFA. Although small alterations in the protein composition of these membranes are suggested by the data, confirmation through additional experimentation is necessary.

A major problem in the comparison of the various two-dimensional gels involved the use of the silver staining technique. A number of compounds that are routinely used in two-dimensional separations such as ampholytes, urea, Triton X-100, SDS, glycine, and glycerol all interfere with the silver stain (Wray et al., 1981). Thus, many washing steps are necessary to remove these interfering compounds. However, when the silver staining procedure was used as published in the literature (Wray et al., 1981), one of the molecular weight standards, soybean trypsin inhibitor (MW 21,500), either did not appear on the gel or was greatly reduced in intensity. This did not occur when the gels were fixed with 10% trichloroacetic acid (TCA) for one-half hour prior to the washing steps. Thus, it appears there may have been losses of proteins from some of the earlier gels during the washing steps. These losses were apparently prevented by TCA, which is a better fixative than the 50% methanol solution that was used as the fixative in the original procedure. However, fixation in 10% TCA for two hours resulted in an excessively high background stain, so careful control in the fixation step was necessary.

Another problem encountered with the use of the silver stain was that different gels seemed to stain with different intensities for no apparent reason. To minimize this variable, it was found best to overstain all the gels, then destain them to approximate equal intensity. This was not easily done. Different solutions will swell polyacrylamide gels to different degrees depending upon the degree of dehydration of the gel. This alters both the intensity of the protein spots and the background. For example, the destaining solution

Figure 17. One-dimensional SDS-PAGE of trout liver microsomal membrane proteins on a 7.5 to 15% linear polacrylamide gradient slab gel. Lane 1 contains high molecular weight standards and lane 10 contains low molecular weight standards. All other lanes contain samples of microsomal membrane protein.

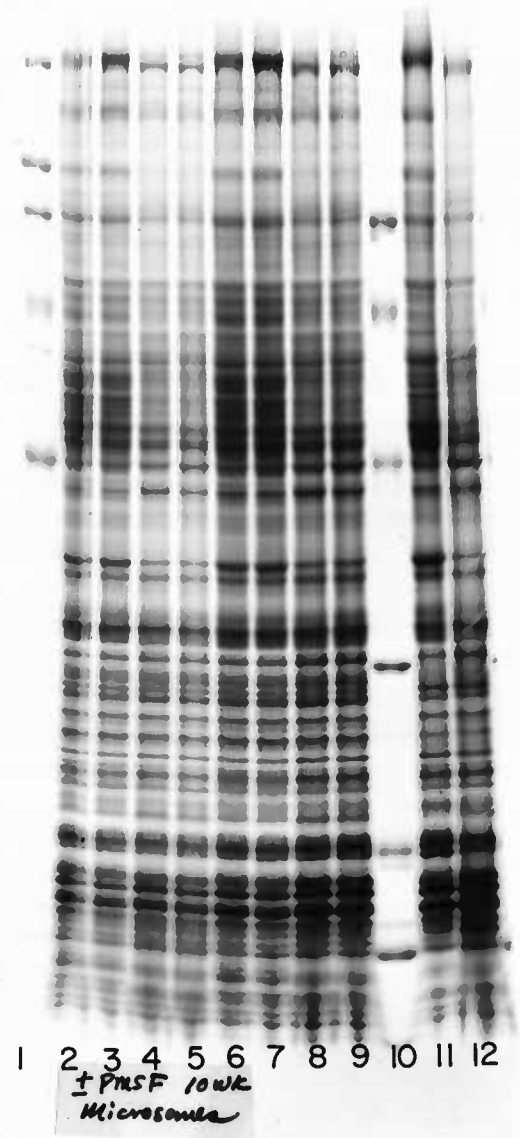

Figure 17

swelled the gel to a considerable degree. Therefore, it was necessary while destaining to anticipate the intensity of the protein spots and the degree of background stain in a more shrunken state (in 50% methanol), the condition in which the gels were photographed. This was more of an art than a science.

The variations in the use of the silver staining procedure undoubtedly contributed to some of the variability seen among the many gels run in this study. Nevertheless, the extreme sensitivity of this stain was verified. The use of Coomassie blue R-250 to stain a two-dimensional gel resulted in the appearance of only a few very faintly stained spots.

Photography of the gels also represented a source of variability, especially in trying to compare the intensity of spots between two gels. Through experience it was found that photographing a gel under different conditions could alter the appearance of the gel to a considerable extent. Variations in the development of the photographs also could change the appearance of the gel. Since the photography was done by a professional photographer, it was not possible to control this source of variability other than to ask that the gels be photographed as uniformly as possible.

Despite these problems, the two-dimensional electrophoretic technique used here should be able to detect any major alterations in the protein composition of a biological membrane. The increased resolution of this technique over that of conventional one-dimensional methods has already been discussed. The lack of any major changes in membrane protein composition upon feeding of CPFA is not entirely unexpected. Due to the importance of membranes in the structure and function of a cell, any major changes in the protein composition of membranes could easily prove toxic to the cell and the organism, and would therefore not be seen in a study such as this one.

The detection of more subtle changes in the composition of biological membranes requires a refinement of the two-dimensional technique used here. The use of autoradiography for the detection of proteins would probably improve the quantitative capabilities of this technique by eliminating the variabilities associated with the use of

the silver staining procedure. The enormous problem of quantitation and comparison of data from two-dimensional gels has been noted by other investigators (Garrels, 1979; Lemkin and Lipkin, 1981b). For this reason, methods for computer analysis of two-dimensional gels have been developed (Garrels, 1979; Lemkin and Lipkin, 1981a; Lemkin and Lipkin, 1981b). However, the knowledge of computer programming and the sophisticated instrumentation required for this analysis may preclude its use by many investigators.

CPFA and the Promotion of Carcinogenesis

No unified concept related to the biochemical mechanism of promoter action has been accepted or even proposed. The diverse biochemical effects produced by these agents has hindered the search for effects which are specific to promoting activity. Nevertheless, a number of leading investigators feel that promoters act by way of an epigenetic mechanism, i.e. a mechanism not having a direct effect upon DNA. This line of thought is consistent with what is known about the control of cell growth and division.

It is believed that every nucleated cell of a multicellular organism contains the same complement of DNA (Sabine, 1980). However, the organism can survive only if each individual cell plays an appropriate role in the functioning of the organism as a whole. This role depends upon the proper expression of the DNA of a cell, which must be regulated and coordinated with the function of all the other cells in the organism. This implies that a high degree of intercellular communication is necessary to allow coordination and control of the proliferation, differentiation, and other activities of the component cells (Pitts, 1980).

Mechanisms of intercellular communication are poorly understood but are believed to involve membrane associated structures called gap junctions. These junctions are regions of close contact between the membranes of adjacent cells and are believed to consist of specialized membrane channels through which regulatory substances that control cell growth and differentiation can pass (Revel *et al.*, 1980;

Flagg-Newton, 1980). Gap junctions are found in most animal tissues and are thought to be responsible for the high degree of metabolic cooperation found among cells of animal tissues (Loewenstein, 1979; Pitts, 1980).

It has been proposed that promoting agents exert their activity by inhibiting intercellular communication, possibly through their effect on the cell membrane and the associated gap junctions (Yotti et al., 1979; Williams, 1981). This proposal is supported by work showing that a variety of promoting agents inhibit intercellular communication. These include phorbol esters (Yotti et al., 1979; Murray and Fitzgerald, 1981; Fitzgerald and Murray, 1980; Enomoto et al., 1981), phenobarbitol (Williams et al., 1980), DDT (Williams et al., 1981), and saccharin (Trosko et al., 1980).

The role of these promoting agents in the two-stage model of carcinogenesis is as follows. An initiator acts by damaging or changing some component of cellular DNA. This change in the genome of the cell renders it capable of the unrestrained growth and multiplication that is characteristic of neoplasia. However, this initiated cell is held in control by regulatory factors transferred to it by surrounding cells. If communication between this cell and surrounding cells is disrupted (by a promoting agent, for example), the initiated cell is no longer subject to the restraining influence of neighboring cells and becomes free to undergo unrestrained growth and division. Once a sufficient mass of neoplastic cells has been achieved, the majority of the cells are further isolated from interaction with surrounding normal cells and are free to develop into an overt tumor. At this point the neoplastic cells are no longer dependent upon the activity of a promoting agent for unrestrained growth.

There are several lines of evidence that support the concept that normal cells have a growth controlling influence on initiated cells. The long interval that can occur between initiation and promotion with subsequent production of tumors (Van Duuren et al., 1975) suggest that during this interval initiated cells are kept under control by normal cells. Nettesheim et al. (1981) have demonstrated that the number of

initiated cells produced by exposure of the respiratory tract to a carcinogen far exceeds the number that will eventually become tumors. Finally, tumor cells from a variety of sources have been found to exhibit reduced levels of intercellular communication (Loewenstein, 1979).

The inhibition of intercellular communication could be the mechanism by which CPFA exert their promoting activity. This could occur as a result of the effect of CPFA on structure and function of biological membranes. Such effects might include changes in boundary lipid, protein composition, enzymatic activity, gap junction structure and function, or other properties of the membrane which may be involved in intercellular communication. Only further research in this area can answer these questions.

SUMMARY AND CONCLUSIONS

Several studies were conducted to determine the effects of CPFA on membranes of liver of rainbow trout. ^{14}C-sterculic acid was shown to be readily incorporated into the lipids of the microsomal membrane. Most of the labelled sterculic acid was found in CP and EP, with smaller amounts found in other microsomal phospholipids. Phospholipase A_2 treatment of isolated CP and EP showed sterculic acid to be preferentially esterified to the 1-position of the glycerol backbone.

Cholesterol determinations on microsomal membranes showed no statistical difference in cholesterol levels between control trout and trout fed a diet containing CPFA.

The use of DTBP failed to yield a clear picture of protein-protein interactions in the microsomal membrane. This was due to the formation of high molecular weight aggregates that were not resolvable on polyacrylamide gels. On the other hand, DTBP failed to crosslink the proteins of the plasma membrane. Thus, this technique could not be used to assess alterations in protein-protein interactions in membranes that may be induced by dietary CPFA.

Two-dimensional gel electrophoresis was used for analysis of the protein composition of liver microsomal and plasma membranes. No major differences in the protein composition of these membranes were found between control trout and trout fed diets containing CPFA. Minor alterations in protein composition were suggested by the data. However, a refinement of the two-dimensional technique used here is required to confirm the subtle alterations in the plasma and microsomal membrane that may be induced by dietary CPFA.

The relevance of these research findings to current theories on the mechanism of action of promoting agents was discussed. It was suggested that CPFA exert their promoting activity through their effect on biological membranes. Such an effect might include an inhibition of intercellular communication, which may be an important factor in the development of cancer.

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