This is to certify that the

dissertation entitled
THE ASSESMENT OF EPIDERMAL GROWTH FACTOR BINDING AND PROTEIN KINASE ACTIVITY AS BIOMARKERS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN TOXICITY IN RAINBOW TROUT

presented by

JOHN LESLIE NEWSTED

has been accepted towards fulfillment
of the requirements for

Doctoral degree in Environmental Toxicology/ Fisheries and Wildlife

John P. Geisy
Major professor

Date 8-1-91

THE ASSESSMENT OF EPIDERMAL GROWTH FACTOR BINDING AND PROTEIN KINASE ACTIVITY AS BIOMARKERS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN TOXICITY IN RAINBOW TROUT

BY

JOHN LESLIE NEWSTED

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Fisheries and Wildlife
and
Institute for Environmental Toxicology

1991

THE A
AN
2,

Haloge
synthetic
dibenzodio
toxicity,
their rela
HAHs are
species ir
in the Gr
that are
reproduct
stages o
environme
isosterec
of anima
common m
receptor.
in devel
mediated
of the bi
marker of

ABSTRACT

THE ASSESSMENT OF EPIDERMAL GROWTH FACTOR BINDING AND PROTEIN KINASE ACTIVITY AS BIOMARKERS OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN TOXICITY IN RAINBOW TROUT

BY

JOHN LESLIE NEWSTED

Halogenated aromatic hydrocarbons (HAHs) are a group of synthetic contaminants that includes polychlorinated-dibenzodioxins (PCDDs) and biphenyls (PCBs). Due to their toxicity, tendency to bioaccumulate in aquatic food chains, their relative persistence and widespread distribution, these HAHs are of particular concern to the health of aquatic species in the Great Lakes. Several studies have shown that in the Great lakes, salmon accumulate HAHs to concentrations that are sometimes sufficient to cause adverse effects in the reproduction, development and survival of the early life stages of lake trout. Although HAHs exist as complex environmental mixtures, many HAHs are approximate isostereomers and produce similar toxic symptoms in a variety of animals. Research has shown that HAHs work through a common mode of action that is mediated through the Ah receptor. Some HAH-elicited responses are similar to changes in development and are related to alterations in hormone-mediated systems. In HAH-treated mammals, "down regulation" of the binding of epidermal growth factor (EGF) is a sensitive marker of exposure in tissues and cells.

In this

the binding

rainbow trou

stimulated t

have an EGF

and saturab

that seen i

activity th

receptor and

tyrosine kin

kinase inhi

Reduct

dependent ma

The reduct

protein ki

alteration

specific fo

with a rain

alterations

correlated

synthesis.

In this study we have examined the relationship between the binding of EGF and TCDD-induced lesions in the liver of rainbow trout. Characterization of EGF binding and the EGF-stimulated tyrosine kinase activity showed that rainbow trout have an EGF receptor-like protein that binds EGF in a specific and saturable manner and has a molecular weight similar to that seen in mammals. Upon binding, EGF elicited a kinase activity that autophosphorylated tyrosine residues on the receptor and exogenous polypeptide substrates. EGF-stimulated tyrosine kinase activity was significantly reduced by tyrosine kinase inhibitors.

Reduction in the binding of EGF was time and dose dependent manner TCDD-exposed rainbow trout and RTH-149 cells. The reduction in binding was accompanied by an increase in protein kinase C and tyrosine kinase activities. The alteration in EGF-binding and protein kinase activities were specific for HAHs that could induce cytochrome P-450. Studies with a rainbow trout hepatoma cell line, RTH-149, showed that alterations in EGF binding and protein kinase activity were correlated to increases in cell proliferation and DNA synthesis.

Dedicated to my wife, Pam.

I woul
Kevern, Mor
Giesy for
doctoral s
professor,
a scientist
and opport
also like
providing t
did.

Thank
would alsc
more thing
life times
my everlas
thank all
projects a
the years.

ACKNOWLEDGEMENTS

I would like to thank my graduate committe, Drs. Niles Kevern, Monte Mayes, Robert Roth, Steven Bursian and John Giesy for their time and guidance over the course of my doctoral studies. I would also like to thank my major professor, John Giesy, for his guidance in my development as a scientist and professional and for allowing me the freedom and opportunity to pursue my scientific rainbows. I would also like to thank Bob Hoke for his friendship and for providing the inspiration for me to finish my degree before he did.

Thanks also go to my family for their faith and love. I would also like to thank my wife, Pam. She has put up with more things than most people could ever put up with in several life times while I have completed my doctorate. To her I give my everlasting thanks and love. Additionaly, I would like to thank all the people in the laboratory for their help in my projects and for the friendship that they have given me over the years.

TABLE OF CONTENTS

Tabl

LIST OF TABLES

Figu

1.

2.

3.

4.

5.

6.

7.

8.

9.

10.

11.

12.

13.

14.

LIST OF FIGURES

Figure

15. West
stim

16. Dose
125I-

17. Scatc
and 1

18. Compa
activ
in TC

19. Chang
(PKC)
EGF-r
of t
TCDD/

20. 32-P
immun
study

21. Dose-
kinas
the h

22. Effec
Proli

23. Effec
incor

24. Conce
to RT

25. Time-
TCDD-

26. Scatc

LIST OF FIGURES (cont.)

Figure

27. Effe
tyro
(EGF
cell

28. Effe
tyro
phos

LIST OF FIGURES (cont.)

tr

Gr

pre

McL

to r

Lake

Huron

repro

reach

spawn.

survive

al. 198

is of g

sport an

economie

concerned

populatio

problems

coastal ar

GENERAL INTRODUCTION

Problem Definition

During the late 1940s and throughout the 1950s the lake trout (Salvelinus namaycush) populations in the Laurentian Great Lakes were decimated by overfishing, sea lamprey predation and degradation of spawning habitat (Wells and McLain 1973). Despite massive restocking programs, attempts to restore self-sustaining lake trout populations in the Great Lakes have been only successful in Lake Superior and Lake Huron (Swanson and Swedberg, 1980). In areas of where the reproduction of lake trout is not self-sustaining, lake trout reach sexual maturity, produce viable gametes and successfully spawn. However, only a negligible number of their young survive (Jude et al. 1981; Nester and Poe, 1984; Marsden et al. 1988). The lack of self-supporting lake trout populations is of great concern to many of the Great Lake states in that sport and commercial fisheries are an important part of their economies (Brown, 1982). Regulatory agencies are also concerned by the possibility that the failure of these fish populations to be self-sustaining may be indicative of future problems in human health for populations that live near coastal areas and who consume Great Lakes fish (Fein et al.

1984). This concern is based on the observation that field and laboratory studies have suggested a possible role of halogenated aromatic hydrocarbons (HAHs) in the increase in salmoninae early life stage mortality (Berlin et al. 1981; Mac 1988).

The problem of increased mortality of Great Lakes salmoninae became evident during the early 1980s when several hatcheries in the Great Lakes region observed an unexplained increase in rearing mortality in chinook salmon (Oncorhynchus tshawytscha) fry. An examination of this problem determined that the cause of the mortality was not due to bacterial, fungal or viral infection (Flagg, 1982). Chemical analysis of the fry showed that moribund fry had greater concentrations of several DDTs and PCBs than did live fry. In subsequent studies, it was also demonstrated that sampling location was the most significant influence on observed fry mortality. Mac et al. (1985) found that the concentration of total PCBs and organochlorine pesticides was positively correlated with early life stage mortality of lake trout eggs collected from the Great Lakes. In that study, the eggs collected from Lake Michigan contained the greatest concentrations of PCBs and organochlorine pesticides, while eggs collected from Lakes Huron and Superior contained lesser concentrations. Rates of mortality of fertilized eggs from Lakes Superior, Huron and Michigan was 4, 22, and 30% respectively, while mortality of the fry equaled 41, 52, and 96%, respectively. When fry that

develope

exhibite

lethargy

syndrome

observed

they die

collected

the labor

other stu

sample lo

parameters

establishe

(1986) dem

correlatio

and early

only about

principle c

involved i

(1988) dire

PCB congen

lake trout

relationshi

To dat

presence

implicated

contaminate

developed from eggs collected from Lake Michigan died they exhibited loss of equilibrium, erratic swimming behavior and lethargy which is characteristic of "swim-up" or "drop-out" syndrome (Walker et al. 1991). Abnormal behaviors were observed in fry that died from Lakes Superior and Huron when they died. In a similar study, coho salmon eggs were collected from Lakes Michigan, Ontario and Erie and reared in the laboratory (Morrison et al. 1985a; 1985b). As in the other studies, mortality observed in the fry was correlated to sample location but no relationships between intrinsic egg parameters and inorganic and organic contaminants could be established. Using principle component analysis, Giesy et al. (1986) demonstrated that there was a statistically significant correlation between the concentration of toxaphene and PCBs and early life stage mortalities of chinook salmon. However, only about 30% of the total variance was explained by the principle components indicating that there are other variables involved in the observed mortalities. Recently, Mac et al. (1988) directly correlated the concentration of a single toxic PCB congener 3,3',4,4'-tetrachlorobiphenyl in Lake Michigan lake trout with egg mortality but did not examine this relationship in sac-fry.

To date, laboratory and field evidence indicates that the presence of persistent environmental contaminants are implicated for the mortality that has been documented in the contaminated regions of the Great Lakes. These contaminants

include polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) which may act in a combined fashion to cause the poor survival of early life stages of some species of salmoninae and as a consequence reduce the reproductive success of these species in the Great Lakes (Walker et al. 1991).

Halogenated Aromatic Hydrocarbons

PCDDs, PCDFs and PCBs belong to a family of polyhalogenated aromatic hydrocarbons. These compounds are poorly metabolized, very persistent and lipophilic. Thus, they bioaccumulate in the food chain (Tanabe et al. 1989). There are 75, 135, and 209 possible congeners of the PCDDs, PCDFs and PCBs respectively (Poland and Knutson 1982). Of these compounds, only 21 congeners are considered extremely toxic and these compounds constitute the planar halogenated hydrocarbons (PHHs) (Safe et al. 1985; EPA 1989). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic of these compounds and is considered the prototype for the toxicity of the other PHH congeners (Poland and Knutson, 1982).

TCDD is a chlorinated, planar tricyclic halogenated carbon-oxygen compound that is extremely toxic to most animal species (Harris et al. 1973). Inputs of TCDD into the environment come from three principal sources. The first source includes chemical manufacturing where TCDD appears as synthesis intermediates and by-products. Specific examples of

these sources include the production of 2,4,5-T and Silvex herbicides where PCDDs occurred in the ppb to ppm range (Rappe et al. 1978; Esposito et al. 1980). Trace concentrations of TCDD have also been found in samples of bleached pulp, in bleached kraft pulp effluents and in some products manufactured from the bleached pulp (Anon 1987). The second source is the combustion of chlorinated hydrocarbons. PCDDs are formed by alkaline hydrolysis of chlorophenolate salts at temperatures that exceed 180°C (Blair 1983; Ballschmiter et al. 1988). Burning of coal, chlorophenols and/or solid waste can result in the formation of PCDDs (Long and Hanson 1983). Evidence for this source is that PCDDs have been found in flyash and flue gas emissions of municipal and industrial incinerators (Lustenhouwer et al. 1980). The third source of PCDDs incorporates the disposal of the wastes of the first and second source and includes chemical waste dumps, sludge from incinerators, sediments and contaminated soils. Transport from these sites can occur by aerial transport, ground water leaching, surface water runoff or through accidental release. Examples of the later type of release include the Seveso incident (Reggiani 1980) and the accidental spraying of waste oil contaminated with 2,3,7,8-TCDD on dirt roads and horse arenas at various locations in Missouri including Times Beach (Carter et al. 1975). It has been hypothesized that atmospheric deposition of particulate-bound contaminants produced by combustion is the major source of PCDDs in the

prod

Grea

TCDD

have

(198

that

the

ng T

fille

fish

of 6

1984)

O'Kee

conce

estab

each

trout

for

avera

Lake

Lake

low

infor

Lakes

Lakes

produced by combustion is the major source of PCDDs in the Great Lakes system (Czuczwa and Hites 1986).

TCDD in Great Lakes Fish

Concentrations of many PCDDs, including 2,3,7,8-TCDD, have been found in Great Lakes salmoninae. Petty et al. (1983) analyzed six species of Great Lakes salmon and found that an averaged 33.4 ng/kg TCDD per whole body weight for all the fish sampled. In Lake Ontario, lake trout averaged 30.8 ng TCDD/kg and 22 ng TCDD/kg wet tissue weight in livers and fillets, respectively (Tosine et al. 1983) while commercial fish taken during 1980 contained 2.0 to 38.5 ng TCDD/kg in 21 of 62 samples with detectable concentrations (Ryan et al. 1984). These findings were collaborated by the results of O'Keefe et al. (1983) where Lake Ontario lake trout had TCDD concentrations ranging from 51 to 107 ng/kg body weight. To establish a statistical baseline for the comparison between each of the Great Lakes, De Vault et al.(1989) analyzed lake trout taken from each of the Great Lakes and Lake St. Clair for PCDDs and PCDFs. They found total PCDD concentrations averaging 7.2 ng/kg b.w. in Lake Superior, 64.5 ng/kg b.w.in Lake Ontario, 1.0 ng/kg in Lake Superior and 48.9 ng/kg in Lake Ontario. Due to their extreme hydrophobicity and very low environmental concentrations, there is currently no information on the water concentration of PCDDs in the Great Lakes (Czczwa and Hites 1984). Whether the observed Great Lakes fish TCDD concentrations are sufficient to induce the

observed toxic symptoms in salmoninae of these lakes is still not known at this time.

TCDD Toxicity

The toxicity of PHH is determined by their structure (Safe, 1990). Results of acute and chronic toxicity studies have reported that the tetra- to hexachloro 2,3,7,8-substituted PHH analogs are more active than those congeners which contain less than four lateral chlorines substituents (Poland and Knutson, 1982). Moreover, as the degree of chlorine substitution at the nonlateral positions increase in PHHs with lateral chlorines there is a decrease in the observed toxicity. Structure activity studies have shown that the ideal PHH ring structure is planar and covers an area of 3 X 10 A. Of the PHHs, 2,3,7,8-TCDD is the most toxic isostereomer and has been used as a model PHH to examine the molecular mechanism of toxicity for this class of compounds.

The observed toxicity of TCDD is dependent on a number of factors including the dose, the age, strain, species and sex of the animals tested (Safe 1986). LD50s from several animal species range from 600 to 2,000 ng/kg b.w.in the guinea pig, 22,000 to 45,000 ng/kg b.w. in the rat, 25,000 to 50,000 ng/kg b.w.in the chicken, 70,000 ng/kg b.w. in the monkey, 115,000 ng/kg b.w. in the rabbit, 114,000 to 284,000 ng/kg b.w. in the mouse, ≥1,000,000 ng/kg b.w. in the bullfrog and 1,157,000 to 3,000,000 µg/kg b.w.in the hamster (Kociba and Schwetz 1982; Tucker et al. 1982). TCDD toxicity differences are also

observed within the same species which females being more sensitive than males of the same reproductive maturity (Schwetz et al. 1973).

Two signs observed in most animals acutely poisoned with TCDD are loss of body weight (wasting syndrome) and lymphoid depletion. Body weight loss can be as much as 50% of the critical body weight but does not appear to be strictly related to a reduction in food intake (Gasiewicz et al. 1980; McConnell 1980). The mechanism of TCDD-induced lymphoid tissue loss is believed to be the result of an alteration of thymus epithelium which results in the alteration in the maturation and the loss of T-lymphocyte precursors (Vos and Moore 1974; Greenlee et al. 1985). Other TCDD effects include liver necrosis, enlargement of the liver due to hyperplasia and hypertrophy of parenchymal cells and fatty infiltration (McConnell 1980). Commonly observed TCDD-induced effects in mammals is hyperplasia and hyperkeratosis of the epidermis resulting in an acnegenic response and the production of comedones and cysts (Kimbrough 1983). In man, chloracne is most commonly observed in individuals exposed to TCDD (May 1973).

TCDD Effects in Fish

Fresh water fish are uniquely sensitive to the effects of TCDD. Unlike mammals, fish do not show the wide range in sensitivity to TCDD. Of six species of fresh water fish from five families, only a five-fold difference in TCDD LD50s was

observed (Kleeman et al. 1988). The LD50s for these species were not affected by the exposure route in that both intraperitoneal injection and waterborne exposure yielded similar LD50 values (Spitsbergen et al. 1988b). The LD50 range for these fish was similar to that observed in the most sensitive mammal, the guinea pig (Schwetz et al. 1973). However, early developmental stages of fish are more sensitive to TCDD than the adult fish. In rainbow trout(Oncorhynchus mykiss), TCDD exposure of swim-up fry resulted in a concentration of 900 ng/kg and 45% mortality (Mehrle et al. 1988). In newly hatched rainbow trout sac fry, the LD50 was 300 ng/kg (Walker 1991) while juvenile trout had an LD50 of 16 µg/kg (Kleeman et al. 1988). Thus, newly hatched sac-fry are 3 and 30 times more sensitive to the lethal effect of TCDD than trout swim-up fry and juveniles, respectively.

TCDD Effects on Fish Early Life Stages

Early life stages fish have been shown to be very sensitive to the effects of waterborne TCDD (Helder, 1980; Helder, 1981). Rainbow trout eggs, sac fry, and juveniles exposed directly to TCDD in water (0.1 ng/l) for 96 h exhibited reduced survival. Exposure of sac fry to 10 ng/l for 96 h caused 100% mortality. For older fry, 100 ng/l was required to cause 100% mortality. In exposed eggs, these concentrations caused underdevelopment, hemorrhages, edema and malfunctions of the jaws (Helder, 1981). Northern pike eggs exposed 0.1, 1.0, and 10 ng/l for 24 h exhibited a 23%

reta

Thes

expo

nort

was c

in eg

(Mac

toxic

(199

reduc

TCDD/

occur

Effec

the p

Conce

cont

grea

stag

hatc

b.w.

TCDD

effec

and

deter

retardation in the development rate relative to control eggs. These concentrations also caused delayed mortality of fry. A exposure to 10 ng TCDD/l resulted in 100% mortality. Eggs of northern pike exhibited a tail-first hatching phenomena that was dose-dependent. This finding was similar to that observed in eggs sampled that are hatched from Lake Michigan lake trout (Mac 1988).

To relate the concentration of TCDD in eggs to the toxicity manifested during early development, Walker et al. (1991) exposed lake trout eggs to TCDD. Hatchability was reduced at egg concentrations greater than or equal to 226 ng TCDD/Kg wet weight. The greatest TCDD-related mortality occurred at the sac-fry stage. The No Observable Adverse Effect Level for cumulative mortality was 34 ng/kg b.w. while the Lowest Observable Adverse Effect Level was 55 ng/Kg b.w. Concentrations of TCDD in eggs that caused 50% mortality above control at swim-up (LD50) was 65 ng/Kg b.w. While the greatest TCDD-induced mortality occurred during the sac-fry stage, a greater percentage of the larvae (8-17%) died half-hatched when concentrations equaled or exceeded 226 ng TCDD/kg b.w.

TCDD Effects on Juvenile Fish

Juvenile fish are also very sensitive to the toxic effects of TCDD. Guppies exposed to TCDD in water for 24 h and then observed for 69 d exhibited an increase in fin deterioration (necrosis and fungus infection). Toxic

threshold for the fin deterioration was estimated to be between 0.08 and 0.8 ng TCDD per gram wet weight (Miller et al., 1979). Fathead minnows exposed to 0.0, 0.1, 1.0, 10 and 100 ng TCDD/l for 1, 2, 3, or 4 d exhibited mortality that was time of exposure and dose-dependent (Adams et al., 1986). In juvenile rainbow trout, the Maximum Acceptable Toxicant Concentration was between 1 and 38 pg TCDD/L while the No Observable Adverse Effect Level was less than 38 pg TCDD/L in rainbow trout (Mehrle et al. 1988). TCDD induced significant alterations in feeding behavior and general activity levels at 38 pg TCDD/L. While rainbow trout body TCDD concentrations were not measured in Mehrle's study, Kleeman et al (1986) determined for rainbow trout that no overt signs of toxicity were observed until body burdens reached a threshold of 150-250 ng TCDD/kg. As was observed in mammalian studies, lethality in fish was delayed 1 to 3 weeks post exposure.

Histopathologic Effects in Fish

The effects of TCDD exposure in laboratory studies using fish are very similar to the effects observed in laboratory studies with mammals. In both mammals (McConnell 1980; Poland and Knutson 1982) and fish (Kleeman et al 1986; Spitsbergen et al. 1988 a,b), the primary sites of TCDD-induced lesions are epithelial and lymphomeyloid tissues. TCDD exposure results in altered epithelial differentiation and proliferation (metaplastic/hyperplastic) with different organ systems affected depending on the species of test animal. The

generalized edematous response, thymic involution and lymphoid depletion, leukopenia, thrombocytopenia, hyperplastic lesions of bile ducts and gastric mucosa, and fibrous pericarditis observed in fish have also been previously reported in either laboratory mammals (McConnell 1980) or birds (Allen 1964; Gilbertson 1982). Slow metabolism and elimination of TCDD in rainbow trout compared to that of mammals may explain the appearance of hyperplastic lesions in rainbow trout bile ducts and gastric mucosa after single TCDD exposures (Kleeman et al. 1986).

Fin margin necrosis appears to be a common indicator of TCDD exposure in fish. TCDD is known to enhance terminal differentiation in cultured human epidermal cells (Osborne and Greenlee 1985). Terminal differentiation in fish skin exposed to TCDD could decrease regenerative capacity and result in lesser resistance to shear stress of swimming. Separation of epidermal cells as a result of immune responses is not considered a plausible explanation for fin necrosis since epidermal inflammation is typically absent (Spitsbergen et al. 1988 a).

Exposure of lake trout early life stages to TCDD results in several well-defined lesions that correspond to lesions observed in eggs collected from the Great Lakes (Spitsbergen et al. 1991). In eggs exposed to 400 ng TCDD/L, embryos develop normally until one week prior to hatch. At this time retrobulbar, meningeal and subcutaneous edema become evident.

Following hatching, sac-fry develop severe subcutaneous edema with cessation of blood circulation in the yolk sac and body. Necrosis of the retina, brain and spinal cord were also present in the embryos. Thus, the cardiovascular system appears to be the initial tissue affected by TCDD in developing trout. Other lesions in the brain, retina and liver develop as a result of circulatory derangements expressed as anemia and hypoxia (Spitsbergen et al. 1991).

As in mammals, the type and severity of lesions observed in fish do not account for the observed mortality. The "wasting syndrome" also does not play a role in fish mortality since some species exhibit no weight loss after TCDD exposure (Spitsbergen et al. 1988 b) and most necropsied fish exhibit adequate body reserves of fat. While the mechanism of TCDD-induced lesions in both mammals and fish remains unknown, it appears to be an evolutionarily conserved phenomenon because of the similarity of observed effects in a number of phylogenetically disparate groups.

Biochemical Effects OF TCDD Toxicity

TCDD and other PHHs cause diverse biochemical responses in animals that include the induction of both phase I and phase II drug-metabolizing enzymes (Poland et al. 1979; Goldstein and Safe 1989). TCDD induces both cytochrome P-4501A1 and cytochrome P-4501A2 hemoproteins and their associated microsomal monooxygenases which include ethoxyresorufin O-deethylase (EROD) and aminopyrine

demethylase (APDM). TCDD also induces glutathione S-transferase (Baars et al. 1980), glucuronyl transferase (Owens 1977) and several other enzymes including ornithine decarboxylase (Nebert et al. 1980), epidermal transglutaminase (Puhvel et al. 1984) and DT diaphorase (Beatty and Neal 1976).

Many of the effects of TCDD are similar to those associated with modulation of hormone-mediated responses. The "wasting syndrome" observed in TCDD toxicity is similar to that seen in animals that have altered hormone metabolism. TCDD-mediated reduction of thyroid hormones in rats and tadpoles has been correlated with toxic endpoints such as mortality and reduction in growth and a retardation of development (McKinney 1985). Moreover, thyroid hormones may have a role in modulating the toxicity of TCDD in that radiothyroidectomy protected rats against TCDD-mediated T-cell immunotoxicity, mortality and body weight loss (Rozman et al. 1985). TCDD-induced lesions are also similar to several glucocorticoid-induced effects such as lymphoid involution, edema,lipid mobilization (McConnell 1980; Safe 1986), elevation of steroid metabolizing enzyme activities (Poland and Knutson 1982) and teratogenesis in mice (Birnbaum et al. 1985). Other TCDD-hormone associated effects include the reduction of progesterone and estrogen (Romkes et al. 1987; Kleeman et al. 1990) and Vitamin A (Hakansson et al. 1986). In animals, hormone levels are regulated through intercellular signalling systems while their biological

activity is mediated through cellular receptors. TCDD and other PHHs have been shown to alter the membrane integrity of cells through lipid peroxidation (Alsharif et al., 1990).

TCDD can affect membrane function in target organs of exposed mammals. In rats, a single subacute TCDD dose can reduce the hepatic biliary excretion of ouabain which was correlated to an inhibition of Ca^{2+}-Mg^{2+} and Na^{+}-K^{+} ATPases of the biliary plasma membrane (Eaton and Klaussen 1979; Peterson et al. 1979 a, b). SDS-PAGE electrophoresis analysis of TCDD-treated animal membranes revealed significant alterations in the band profile of plasma membrane proteins concentrations (Brewster et al., 1982). Receptor binding assays of TCDD-treated membranes revealed a reduction in the binding of insulin, glucagon, concanavalin A and epidermal growth factor to their receptors (Matsumura et al. 1984).

Alterations of membranes have also been implicated in TCDD-induced hyperlipidemia (Swift et al. 1981). The hyperlipidemia is characterized by the accumulation of cholesterol esters and triglyceride-carrying lipoproteins in the blood. It is associated with the reduction of low density lipoprotein receptors in adipocytes (Bombick et al. 1984) and the reduction in lipoprotein lipase (LPL) activity (Brewster et al. 1984). Both of these systems are active in the control of cholesterol, free fatty acid and concentrations of triglyceride in the blood (Chapman 1980).

Of the intrinsic plasma membrane proteins studied to date, alteration of EGF binding to plasma membrane receptors has been shown to be the most sensitive to TCDD exposure (Madhukar et al. 1984). In the transformed keratinocyte cell line (SCC-12F), TCDD was found to reduce the binding of EGF in a dose-dependent manner (ED50=1 μM) (Hudson et al. 1985; Osborne and Greenlee 1985). The reduction in the binding of EGF was the result of a loss in high affinity binding sites (Hudson et al. 1985). Polycyclic aromatic hydrocarbons (PAHs) and phorbol esters have also been shown to reduce the binding of EGF in several cell lines (Ivanovic and Weinstein 1982; Karenlampi et al. 1983). The reduction in EGF binding was compound-specific and transient (< 24 hours). Only benz(a)anthracene, 3-methylcholanthrene, and benzo(a)pyrene reduced binding while p.p'-DDT, phenobarbital and testosterone had no significant effect on binding (Ivanovic and Weinstein 1982). However, PAHs and phorbol esters only caused a short term reduction in binding (<24 hours) while TCDD significantly reduced binding (40% of control) for up to 10 days after the removal of TCDD from the culture media (Hudson et al., 1985). The dose-dependent and compound specific reduction in the binding of EGF seen in various cell types has also been observed in rats, mice, guinea pigs and hamsters exposed to TCDD (Madhukar et al. 1984). The formation of cleft palate in newborn mice has also been attributed to alteration of EGF receptor homeostasis in the fusion of the shelves of the

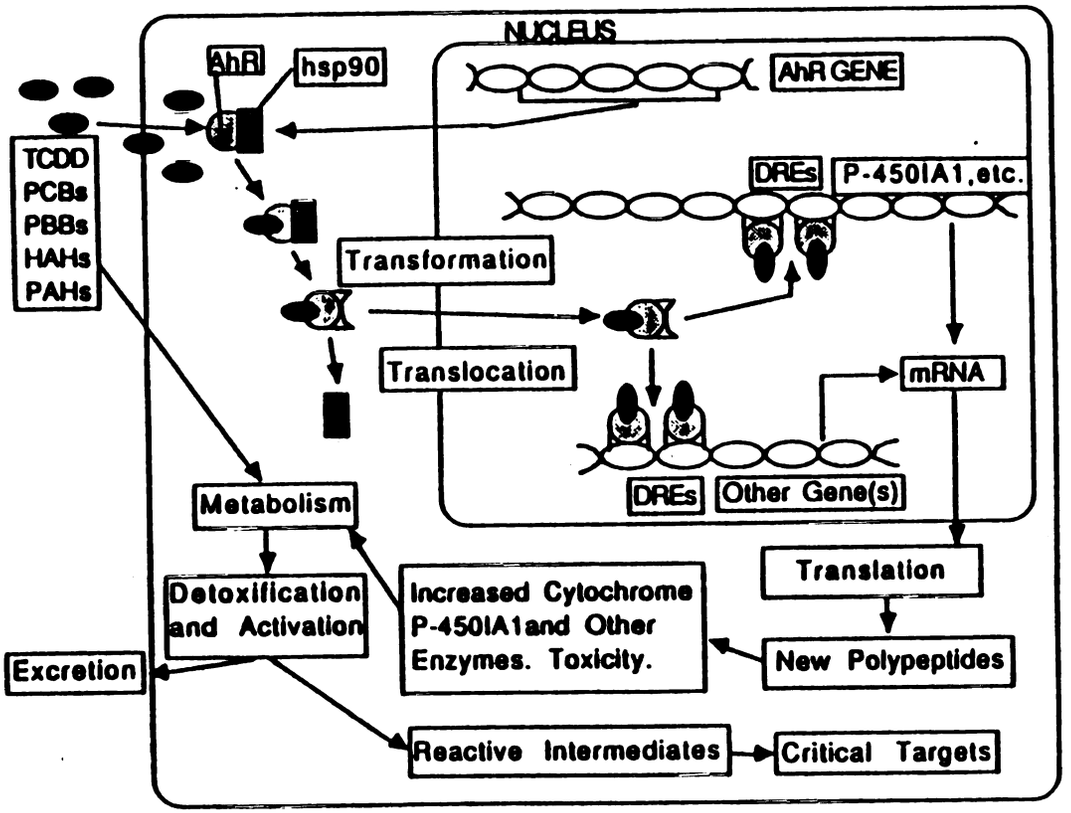
palate (Abbott and Birnbaum 1990). Alteration of EGF receptor homeostasis has also been linked to hyperkeratinization of the epidermis of TCDD-treated animals (Osborne and Greenlee 1985).

Molecular Mechanisms of TCDD Toxicity

One common effect seen in all TCDD-responsive animals is the induction of microsomal cytochrome P-450 and its associated mixed-function oxidase activities. Early studies of the induction of hepatic aryl hydrocarbon hydroxylase (h_AHH) activity by homologous series of PHHs revealed a relationship between PHH structure and their ability to induce AHH activity (Poland and Knutson 1982). Further investigations showed that in genetically inbred aryl hydrocarbon (Ah) responsive (C57BL/6) and nonresponsive (DBA/2) mice and their backcrosses there was at least one order of magnitude difference in their sensitivity to TCDD-induced AHH acitivity (Poland and Glover 1980). In comparison, 3-methylcholanthrene was inactive as an inducer in the nonresponsive mice. Genetic studies with the inbred strains and their backcrosses demonstrated that AHH induction is inherited as a simple autosomal dominant trait (Poland et al. 1974). These studies were the basis for the theory that the induction of AHH activity was receptor- mediated and that the reduced responsiveness of DBA/2 mice was due to a defect in the Ah receptor. Subsequent studies have confirmed that the differential responsiveness of the inbred mice strains was due to differences in affinity of TCDD for a cytosolic

Figure 1. Mechanism of action of halogenated aromatic hydrocarbons (M. Denison).

MECHANISM OF ACTION OF HALOGENATED AROMATIC HYDROCARBONS

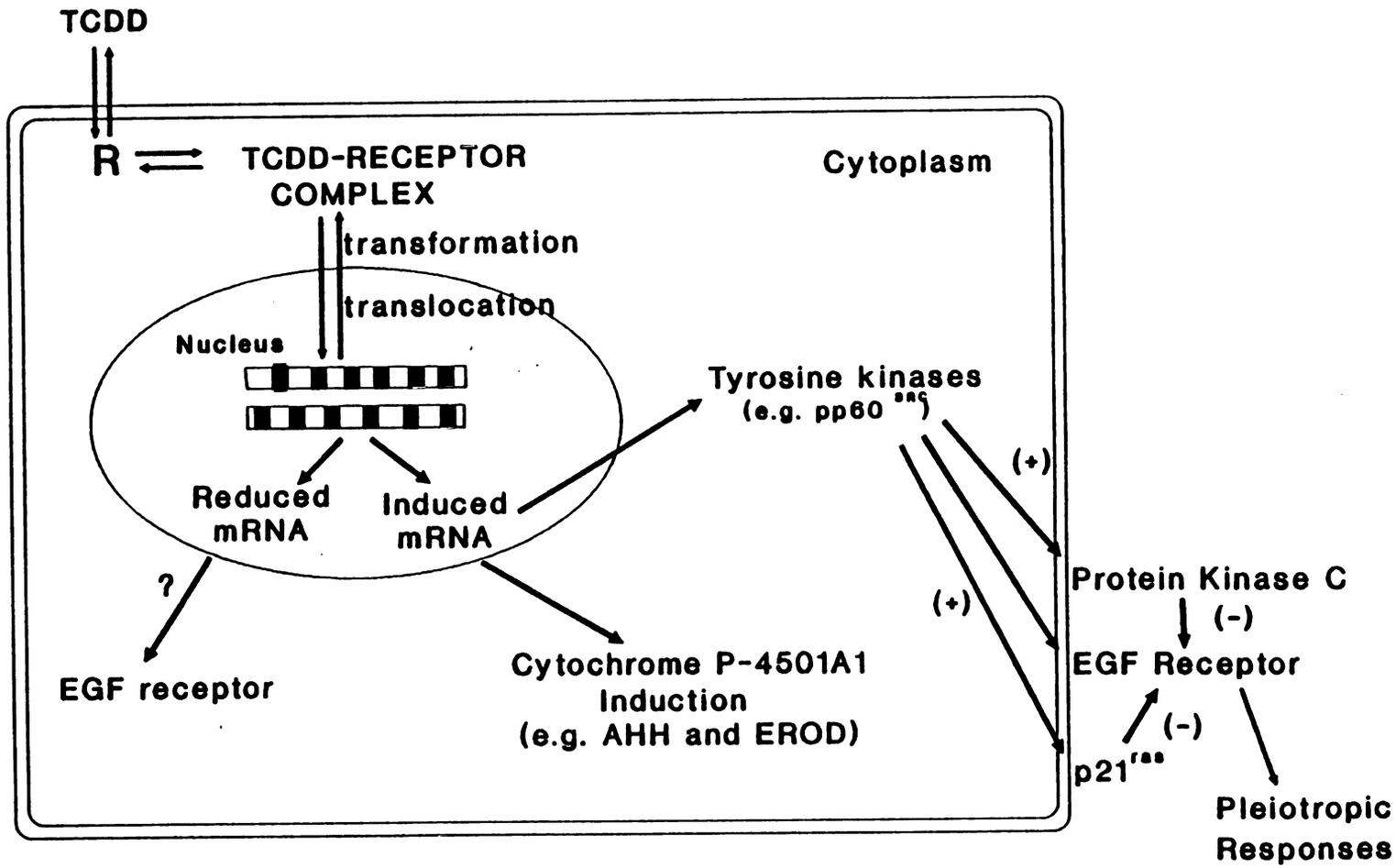


receptor, Ah or TCDD receptor (Poland and Knutson 1982; Okey et al. 1989). Subsequent research revealed that the ability of PHHs to bind to this receptor correlated well with its ability to induce AHH activity and to induce toxicity such as thymic involution, wasting syndrome and epidermal hyperkeratinization (Poland and Knutson 1982; Safe, 1990). As a result of these studies, a common mode of action has been suggested for PHH compounds that is based on a model that incorporates alteration of receptor-mediated gene expression (Figure 1) (Whitlock 1987). Initial binding of TCDD to the cytosolic Ah receptor is followed by atransformation where a heat shock protein dissociates from the TCDD:AhR complex and occupied receptor complexes accumulate in the nucleus. Binding of the nuclear complexes to specific DNA sequences or dioxin responsive elements (DREs) located in the 5'-upstream region from the CYP1A1 gene lead to the enhancement of CYP1A1 gene expression and the subsequent accumulation of cytochrome P-450 specific mRNA (Whitlock 1990).

Other TCDD-induced lesions have been hypothesized to be the result of altered proliferation and differentiation (Bombick 1986). In this model (Figure 2), alteration of EGF-binding homeostasis is the key event in the disruption of normal cell growth and differentiation. As is seen in cytochrome P-450 induction, the interaction of the TCDD:Ah-receptor complex with DREs is suspected to be the initial event of the toxic response. However, instead of cytochrome

Figure 2. Molecular model for the role of the EGF-receptor in TCDD toxicity. (Bombick, 1986).

TCDD-EGF RECEPTOR MODEL



P-450 induction being the next event, the activation of specific oncogenes is the catalyst for the observed toxic lesions. Oncogene products are protein kinases and the elevation in kinase activity has been shown to be important in the activation and inactivation of cellular proteins and enzymes that control cell growth and differentiation (Hunter 1984; Coggin 1986). Studies supporting this model have demonstrated that TCDD induces $pp60^{src}$ and $p21^{ras}$, both of which are related to disruption of growth processes. Furthermore, Abbott and Birnbaum (1990) have shown that TCDD exposure altered the regulation of growth factors and their receptors in cleft palate formation in mice.

Objectives and Significance

Fish are very sensitive to the toxic effects of TCDD. Many of the TCDD-induced lesions observed in fish are also observed in mammals that suggests that the mode of toxic is similar between these species (Spitsbergen et al. 1988 a,b). One common TCDD-induced effect is the induction of hepatic cytochrome P-450s that are functionally similar to those observed in mammals (Stegeman and Kloepper-Sams 1987). Moreover, the Ah receptor has been identified in rainbow trout (Heilmann et al. 1988) and in the rainbow trout cell line RTH-149 (Lorenzen and Okey 1990). Based on this information, it appears that the mechanism of TCDD induction in fish is mechanistically similar to that in mammals. However, there is

little information on molecular mechanisms of TCDD- induced toxicity in fish.

The objectives of this dissertation are to examine the applicability of a mammalian model of TCDD-induced toxicity to rainbow trout that is based on the alteration of EGF-receptor homeostasis (Figure 2). This model will serve as the characterization and assessment of TCDD-induced alterations in the binding of EGF and the stimulation of protein kinase activity as biomarkers of TCDD toxicity in rainbow trout.

We will test the null hypothesis that there is no effect on TCDD on the binding of EGF or on the activity of protein kinases in hepatic plasma membranes of rainbow trout. We will use two-tailed tests since we do not know what effect TCDD will have on these parameters.

TCDD was chosen as the prototype PHH because of its extreme toxicity and the large amount of toxicological information available on TCDD effects to rainbow trout. Specific objects include:

1. To quantify and characterize the binding of EGF to the hepatic plasma membranes of rainbow trout.
2. To examine the relationship between the binding of EGF and the activation of protein kinase activity in the hepatic membranes of trout.
3. To examine the relationship between TCDD dose and time on the binding of EGF and protein kinase activity in the liver of rainbow trout.

4. To correlate the relationship between other TCDD-induced responses and the alteration of EGF binding in rainbow trout.
5. Use a rainbow trout hepatoma cell line , RTH-149, as an *in vitro* model to examine the effect of TCDD on the binding of EGF and to other processes of cell proliferation and differentiation.

The studies were grouped into two sections. The first set of studies characterized the binding, physiology and biochemistry of the EGF receptor in hepatic tissues of rainbow trout. The second set of studies was designed to examine the effect of TCDD on the EGF receptor in the hepatic plasma membrane of rainbow trout and in the cell line RTH-149.

LIST OF REFERENCES

Alsharif,N.Z., Grandjean,C.J., Murray,W.J. and Stohs,S.J. (1990). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced decreases in the fluidity of rat liver membranes. Xenobiotica 20: 979-988.

Abbott,B.D., and Birnbaum,L.S. (1990). TCDD-induced altered expression of growth factors may have a role in producing cleft palate and enhancing the incidence of clefts after coadministration of retinoic acid and TCDD. Toxicol. Appl. Pharmacol. 106:418-432.

Allen, J.R. (1964). The role of "toxic fat" in the production of hydropericardium and ascites in chickens. Am.J. Vet. Res. 25:1210-1219.

Anon. (1987). Assessment of potential health risks from dermal exposure to dioxin in paper products: Technical bulletin No 534. Envirologic Data for the Natl. Council of the Paper Industry for Air and Stream Improvement. N.Y.

Baars, A.J., Jensen,M., and Breimer, D.D. (1978). The influence of phenobarbital, 3-methycholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin on glutathione S-transferase activity of rat liver cytosol. Biochem. Pharmacol. 27: 2487-2494.

Ballschmiter,K., Braunmiller,I., Niemczyk,R. and Swerev,M. (1988). Reaction pathways for the formation of polychlor-

dibenzo-p-dioxins (PCDDs) and dibenzofurans in combustion processes. II. Chlorobenzenes and chlorophenols as precursors in the formation of polychloro-dibenzo-p-dioxins-dibenzofurans in flame chemistry. Chemosphere 17: 995-1001.

Beatty, P.W. and Neal, R.A. (1976). Induction of DT-diaphorase by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Biochem. Biophys. Res. Comm. 68: 197-204.

Berlin, W.H., Hesselberg,R.J. and Mac,M.J. (1981). Growth and mortality of fry of Lake Michigan lake trout during chronic exposure to PCBS and DDE. In: Chlorinated Hydrocarbons as a Factor in the Reproduction and Survival of Lake Trout (Salvelinus namaycush) in Lake Michigan. USFW SER. Rep. Paper 105, p11-22.

Birnbaum,L.S., Weber,H., Harris,M.,Lamb,J.C. and McKinney, J.D. (1985). Toxic interaction of specific polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin: increased incidence of cleft palate in mice. Toxicol. Appl. Pharmacol. 77: 292-302.

Blair,E.H. (1983). Chlorodioxins- origin and fate. Adv. Chem. Ser. 120:141.

Bombick,D.W. (1986). Initial biochemical alterations involved in 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity. PhD dissertation, Michigan State University, East lansing, Mi.

Bombick,D.W., Madhukar,,B.V., Brewster,D.W. and Matsumura,F. (1985). TCDD causes an increase in protein kinases particularly protein kinase C in the hepatic plasma membrane of rat and guinea pig. Biochem. Res. Comm. 271: 296-302.

Bombick,D.W., Matsumura,F., and Madhukar,B.V. (1984). TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) causes reduction in the low density lipoprotein (LDL) receptor activities in the hepatic plasma membrane of the guinea pig and rat. Biochem. Biophys. Res. Comm. 118:548-554.

Brewster,D.W., Madhukar,B.V., Matsumura,F. (1982). Influence of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the protein composition of the plasma membrane of hepatic cells from rat. Biochem. Biophys. Res. Comm. 107: 68-74.

Brewster,D.W., and Matsumura,F. (1984). TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) reduces lipoprotein lipase activity in the adipose tissue of the guinea pig. Biochem. Biophys. Res. Comm. 122: 810-817.

Brown, T.L. (1982). Regional economics of Michigan salmonid program: Does a $20 million fishery really make a difference? In: Michigan Tourism: How can research help? Fridgen,J.D., Allen,D.J.(eds.). Michigan Ag. Exp. Station, E. Lansing, Mi. p156.

Carter,C.D., Kimbrough,R.D., Liddle,J.A., Cline,R.F., Zack,M.M., Barthel,W.F., Koehler,R.E. and Phillips, P.E.

(1975). Tetrachlorodibenzodioxin: an accidental poisoning episode in horse areas. Science 188: 738-740.

Chapman,M.J. (1980). Animal lipoproteins: chemistry, structure and comparative aspects. J. Lipid Res. 21: 789-853.

Coggin,J.H. Jr. (1986). The implications of embryonic gene expression in neoplasia. CRC Critical Reviews in Oncology/Hematology 5: 37-55.

Czuczwa,J.M. and Hites, R.A. (1984). Environmental fate of combustion generated polychlorinated dioxins and furans. Environ. Sci. Technol. 18: 444-450.

Czuczwa,J.M. and Hites, R.A. (1986). Airborne dioxins and dibenzofurans: Sources and fates. Environ. Sci. Technol. 20: 195-200.

DeVault,D., Dunn,W., Berqvist,P., Wiberg,K. and Rappe,C. (1989). Polychlorinated dibenzofurans and polychlorinated dibenzo-p-dioxins in Great Lake fish: A baseline and interlake comparison. Environ. Toxicol. Chem. 8:1013-1022.

Eaton, D.L. and Klaussen,C.D. (1979). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin, kepone and polybrominated biphenyls on transport systems in isolated rat hepatocytes. Toxicol. Appl. Pharmacol. 51: 138-144.

Edsall,C.C. and Mac,M.J. (1982). Comparative hatching success of lake trout eggs in Lake Michigan water and well water. Prog. fish-cult. 44:47-48.

Esposito, M.P., Tierman, T.O., Dryden, F.E. (1980). Dioxins. EPA-600/ 2-80-179. Industrial Environmental Research Laboratory, Cincinnati, OH. p351.

Fein,C.G., Jacobsen,J.L., Jacobsen,S.W., Schwartz,P.W. and Dowler, J.K. (1984). Prenatal exposure to polychlorinated biphenyls: effects on birth size and gestation age. J. Ped. 105: 315-320.

Flagg, R.M. (1982). Case history information from an epizootic in fall chinook received at Garrison Dam NFH as eggs from the Little Manistee, Lake Michigan. USFW Service Report CHN 82-87 and 82-81.

Gasiewicz,T.A., Holscher,M.A. and Neal,R.A. (1980). The effect of total parental nutrition on the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the rat. Toxicol. Applied Pharmacol. 54: 469-488.

Giesy,J.P., Newsted, J.L. and Garling, D.L. (1986). Relationship between chlorinated hydrocarbon concentrations and rearing mortality to chinook salmon eggs from Lake Michigan. J. Great Lakes Res. 12:82-89.

Gilbertson, M. (1982). Etiology of chicken edema disease in herring gulls in the lower Great Lakes. Can. Tech. Rep. Fish. Sci. 1120.

Goldstein, J.A. and Safe, S. (1989). Mechanism of action and structure-activity relationships for the chlorinated dibenzo-p-dioxins and related compounds. In: Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and

Related Products. Kimbrough, R.D. and Jensen, A.A., Eds. Elsevier/North Holland, Amsterdam, p239-263.

Greenlee, W.F., Osborne,R., Dold,K.M., Hudson,L.G., and Toscono,W.A., Jr. (1985). Toxicity of chlorinated aromatic compounds in animals and humans: in vitro approaches to toxic mechanisms and risk assessment. Environ. Health Perspect. 60: 69-76.

Hakansson, H. Ahlborg,U.G., and Gottling,L. (1986). The effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the distribution and excretion of the endogenous pool of vitamin A in rats with low liver vitamin A stores. Chemosphere 15: 1715-1723.

Harris,M.W., Moore,J.D., Vos,J.G., and Gupta,B. (1973). General biological effects of TCDD on laboratory animals. Environ. Health. Perspect. 5:101-109.

Heilmann,L.J., Sheen,Y.Y, Bigelow,S.W., and Nebert,D.W. (1988). Trout P450IAI:cDNA and deduced protein sequence, expression in liver, and evolutionary significance. DNA 7, 279-387.

Helder, T. (1980). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on early life stages of the pike (Esox lucius, L.). Sci. Total Environ. 14: 255-264.

Helder,T. (1981). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on early life stages of rainbow trout (Salmo gairdneri Richardson). Toxicology 19:101-112.

Hudson, L.G., Toscano,W.A., and Greenlee,W.F. (1985). Regulation of epidermal growth factor binding in human keratinocyte cell line by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Appl. Pharmacol. 77:251-259.

Hunter,T. (1984). The proteins of oncogenes. Sci. Ameri. 251: 60-69.

Ivanovic,V., and Weinstein,I.B. (1982). Benzo(a)pyrene and other inducers of cytochrome P_1-450 inhibit binding of epidermal growth factor to cell surface receptors. Carcinogenesis 3: 505-510.

Jude, D.J., Klinger, S.A. and Enk, M.D. (1981). Evidence of natural reproduction by planted lake trout in Lake Michigan. J. Great Lakes Res. 57-61.

Karenlampi,S.O., Eisen,H.J., Hankinson,O., and Nebert,D.W. (1983). Effects of cytochrome P1-450 inducers on the cell-surface receptors of epidermal growth factors, phorbol 12,13-dibutyrate, or insulin of cultured mouse hepatoma cells. J. Biol. Chem. 258: 10378-10383.

Kimbrough,R.D. (1983). Morphology of lesions produced by dioxins and related compounds. In: Human and Environmental Risks of Chlorinated Dioxins and Related Compounds. Tucker,R.E., Young,A.L.,and Grey,A.P., (eds). Plenum Press, New York, pp. 527-538.

Kleeman, J.M., Moore,R.W. and Peterson,R.E. (1990). Inhibition of testicular steroidgenesis in 2,3,7,8-tetrachlorodibenzo-p-dioxin(TCDD)-treated rats: evidence

that the key lesions occur prior to or during pregnenolone formation. Toxicol. Appl. Pharmacol. 106: 112-125.

Kleeman,J.M., Olson,JR., Chen,S.M., and Peterson,R.E. (1986). Metabolism and disposition of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rainbow trout. Toxicol. Appl. Pharmacol. 83, 391-401.

Kleeman,J.M., Olson,J.R., and Peterson,R.E. (1988). Species differences in 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity and biotransformation in fish. Fundam. Appl. Toxicol. 10: 206-213.

Kociba,R.J. and Schwetz,B.A. (1982). A review of the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with comparison of the toxicity of the other chlorinated dioxin isomers. Assoc. Food Drug Officials Q. Bull. 46:168-188.

Long,J.R. and Hanson,D.J. (1983). Dioxin. Chem. and Engineer. News 61: 23-36.

Lorenzen,A. and Okey,A.B. (1990). Detection and characterization of [^{3}H]2,3,7,8-tetrachlorodibenzo-p-dioxin binding to Ah receptor in a rainbow trout hepatoma cell line. Toxicol. Appl. Pharmacol. 106:53-62.

Lustenhouwer, J.W.A., Olie,K., and Hutzeinger, O. (1980). Chlorinated dibenzo-p-dioxins and related compounds in incinerator effluents. Chemosphere 9:501-522.

Mac,M.J. (1988). Toxic substances and survival of Lake Michigan salmonids: field and laboratory approaches. p 389-401. In: Evans,MS. (ed). Toxic Contaminants and Ecosystem Health: A Great Lakes Focus. John Wiley & Sons, New York, NY.

Mac, M.J., Edsall,C.C. and Seeyle,J.G. (1985). Survival of lake trout eggs and fry reared in water from the upper Great Lakes. J. Great Lakes Res. 11: 520-529.

Mac,M.J., Schwartz,T.R. and Edsall,C.C. (1988). Correlating PCB effects on fish reproduction using dioxin equivalents. Soc. Environ. Toxicol. Chem. 19 Annu. Meeting. Abstract p116.

Madhukar,B.V., Brewster,D.W., and Matsumura,F. (1984). Effects of in vivo administered 2,3,7,8-tetrachlorodibenzo-p-dioxin on receptor binding of EGF in hepatic plasma membrane of rat, guinea pig, mouse and hamster. Proc. Natl. Acad. Sci. USA. 81: 7407-7411.

Madhukar,B.V., Ebner,K., Matsumura,F., Bombick,D.W., Brewster,D.W., and Kawamoto,T. (1988). 2,3,7,8-tetrachlorodibenzo-p-dioxin causes an increase in protein kinases associated with epidermal growth factor in the hepatic plasma membrane. J. Biochem. Toxicol. 3: 261-277.

Marsden,J.E., Krueger,C.C, and Schneider,C.P. (1988). Evidence of natural reproduction by stocked lake trout in Lake Ontario. J. Great Lakes Res. 14: 3-8.

Matsumura,F., Brewster,B.W., Madhukar,B.V, and Bombick, D.W. (1984). Alteration of rat hepatic plasma membrane functions by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Arch. Environ. Contam. Toxicol. 13:509-515.

May,G. (1973). Chloracne from the accidental production of tetrachlorodibenzodioxin. Br. J. Int. Med. 30: 276-283.

McConnell,E.E. (1980). Acute and chronic toxicity, carcinogenesis, reproduction, teratogenesis and mutagenesis in animals. In: Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Compounds. Kimbrough, R.D. (ed). Elsevier, N.Y., pp 109-150.

McKinney, J.D. (1985). The molecular basis of chemical toxicity. Environ. Health Perspect. 61:5-10.

Mehrle,P.M., Buckler,D.R., Little,E.E., Smith,L.M., Petty,J.D., Peterman,P.H., Stalling,D.L., DeGraeve,G.M., Coyle,J.J. and Adams, W.J. (1988). Toxicity and bioconcentration of 2,3,7,8-tetrachlorodibenzo-p-dioxin and 2,3,7,8-tetrachlorodibenzofuran in rainbow trout. Environ. Toxicol. Chem. 7: 47-62.

Miller,R.A., Norris, L.A., and Loper,B.R. (1979). The response of coho salmon and guppies to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in water. Trans. Amer. Fish. Soc. 108: 401-407.

Morrison, P.F., Leatherland, J.F. and Sonstegard,R.A. (1985a). Comparative fecundity and egg survival of coho salmon

(Oncorhynchus kisutch) from Lakes Ontario, Erie, and Michigan. Can. J. Zool. 63:1096-1100.

Morrison, P.F., Leatherland,J.F. and Sonstegard,R.A. (1985b). Proximate composition, organochlorine and heavy metal composition of eggs from Lake Ontario, Lake Erie, and Lake Michigan coho salmon (Oncorhynchus kisutch, Walbaum) in relation to egg survival. Aquat. Toxicol. 6: 73-86.

Nebert, D.W., Jensen,N., Perry, J., and Oka,T. (1980). Associations between ornithine decarboxylase E.L.4.1.1.17 induction and the Ah locus in mice treated with polycyclic aromatic compounds. J. Biol. Chem. 255: 6836-6842.

Nester, R.T. and Poe,T.P. (1984). First evidence of successful natural reproduction of planted lake trout in Lake Huron. Am. J. Fish Manage. 4: 126-128.

O'Keefe,P., Meyer,C., Hilker,D., Aldous,K. Jelus-Tyror,B., Dillon,K., Donnelly,R., Horn,Z., and Sloan,R. (1983). Analysis of 2,3,7,8-tetrachlorodibenzo-p-dioxin in Great Lakes fish. Chemosphere 12: 325-332.

OKey,A.B., Denison,M.S., Prokipcak,R.D., Roberts,E.A. and Harper,P.A. (1989). Receptors of polycyclic aromatic hydrocarbons. In: Biologie Prospective. Galteau, M.M. and Henny,J. Eds., pp 605-610. John Libbey, Eurotext. Paris.

Osborne,R., and Greenlee,W.F. (1985). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) enhances terminal

differentiation of cultured human epidermal cells. Toxicol. Appl. Pharmacol. 77: 434-443.

Owens,I.S. (1977). Genetic regulation of UDP-glucuronsyl transferase induction by polycyclic aromatic hydrocarbons in mice. J. Biol. Chem. 252: 2827-2833.

Peterson,R.E., Hamada,N., Yang,K.H., Madhukar,B.V., and Matsumura,F. (1979a).Reversal of 2,3,7,8-tetrachlorodibenzo-p-dioxin induced depression of ouabain biliary excretion by pregnenolone-16α carbonitrile and spirolactone in isolated perfused rat livers. Toxicol. Appl. Pharmacol. 50: 407-416.

Peterson,R.E., Madhukar,B.V., Yang,K.H., and Matsumura,F. (1979b). Depression of adenosine triphosphatase activities in isolated liver surface membranes of 2,3,7,8-tetrachlorodibenzo-p-dioxin treated rats: correlation with effects on ouabain biliary excretion and bile flow. J. Pharmacol. Exper. Therapeutics 210: 275-282.

Petty,J.D., Stalling,D.L., Smith,L.M. and Johnson,J.L. (1983). Occurrence and potential impact of PCDFs and PCDDs in aquatic ecosystems. In: Trace Substances in Environmental Health XVII. p 96-102. Hemphill, D.D. (ed). University of Missouri Press, Columbia, MO.

Poland,A., and Glover,E. (1980). 2,3,7,8-tetrachlorodibenzo-p-dioxin: segregation of toxicity with the Ah locus. Mol. Pharmacol. 17:86-94.

Poland,A., Glover,E., Robinson,J., and Nebert,D.W. (1974). Genetic expression of aryl hydrocarbon hydroxylase activity. Induction of monooxygenase activities and cytochrome P-450 formation by 2,3,7,8-tetrachlorodibenzo-p-dioxin in mice generally "nonresponsive" to other aromatic hydrocarbons. J. Biol. Chem. 249: 5599-5606.

Poland,A., Greenlee,W.F. and Kende, J.C. (1979). Studies on the mechanism of action of chlorinated dibenzo-p-dioxins and related compounds. Ann. N.Y. Acad. Sci. 30: 214-230.

Poland, A., and Knutson,J. (1982). 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanisms of toxicity. Annu. Rev. Pharmacol. Toxicol. 22:516-554.

Puhvel,S.M., Ertl,D.C., and Lynberg,L. (1984). Increased epidermal transglutamase activity following 2,3,7,8-TCDD: in vivo and in vitro studies in mouse skin. Toxicol. Appl. Pharmacol. 73: 42-47.

Rappe,C., Buser,H.R., and Bosshardt, H.P. (1978). Identification and quantification of polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in 2,4,5-T ester formulations and herbicide Orange. Chemosphere 7:431-438.

Reggiani, G. (1980). Acute human exposure to TCDD in Seveso Italy. J. Tox. Environ. Health. 6:27-43.

Romkes,M. and Safe,S. (1988). Comparative activities of 2,3,7,8-tetrachlorodibenzo-p-dioxin and progesterone as

antiestrogens in the female rat uterus. Toxicol. Appl. Pharmacol. 92: 368-380.

Rozman,K., Rozman,T., Scheufler,E., Pazdernik, T., and Greim,H. (1985). Thyroid hormones modulate the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. J. Toxicol. Environ. Health 16: 481-491.

Ryan,J. Lau,J.C., Pilon,J.C.,Lewis,D., McLeod,H.A., and Gervaris,A. (1984). Incidence and levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin in Lake Ontario commercial fish. Environ. Sci. Technol. 18: 719-721.

Safe,S. (1986). Comparative toxicology and mechanisms of action of polychlorinated dibenzo-p-dioxins and dibenzofurans. Annu. Rev. Pharmacol. Toxicol. 92: 368-380.

Safe,S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). CRC Reviews in Toxicology 21: 51-88.

Safe, S. Bandiera,S., Sawyer,T., Robertson,L., Safe,L., Parkinson,A., Thomas,P.E., Ryan,D.E., Reik,L.M., Levin,W., Denomme,M.A. and Fujita,T. (1985). PCBs: Structure-function relationships and mechanism of action. Environ. Health Perspect. 60:47-66.

Schwetz,B.A., Norris,J.M., Sparschu,G.L., Rowe,V.K., Gehring,P.J., Emerson,J.L., and Gerbig,C.C. (1973).

Toxicology of chlorinated dibenzo-p-dioxins. Environ. Health Perspect. 5: 87-99.

Spitsbergen, J.M., Kleeman,J.M. and Peterson,R.E. (1988a). Morphologic lesions and acute toxicity in rainbow trout (Salmo gairdneri) treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. J. Toxicol. Environ. Health 23: 333-358.

Spitsbergen,J.M., Kleeman,J.M. and Peterson, R.E. (1988b). 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in yellow perch (Perca flavescens). J. Toxicol. Environ. Health 23: 359-383.

Spitsbergen,J.M., Walker,M.K., Olson,J.R., and Peterson,R.E. (1991). Pathologic alterations in early life stages of lake trout,Salvelinus namaycush, exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin as fertilized eggs. Aquatic Toxicol. 19:41-72.

Stegeman,J.J., and Kloepper-Sams,P.J. (1987). Cytochrome P-450 isozymes and monooxygenase activity in aquatic animals. Environ. Health Perspect. 71: 87-95.

Swanson, B.L. and Swedberg, D.V. (1980). Decline and recovery of the Lake Superior Gull Island Reef lake trout (Salvelinus namaycush) population and the role of sea lamprey (Petromyzon marinus) predation. Can. J. Aquat. Sci. 37: 2074-2080.

Swift, L.L., Gasiewicz,T.A., Dunn,G.D., Saule,A.D. and Neal, R.A. (1981). Characterization of the hyperlipidemia in

guinea pigs induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Appl. Pharmacol. 59: 489-499.

Tanabe,S., Kannan,N., Wakimoto,T., Tatsukawa,R., Okamota,T., and Masuda,Y. (1989). Isomer-specific determination and toxic evaluation of potentially hazardous coplanar PCBs, dibenzofurans and dioxins in tissues of "Yusho" PCB poisoning victims and in the causal oil. Toxicol. Environ. Chem. 24:215-226.

Tosine,H., Smillie,D., and Rees, G.A.V. (1985). Comparative monitoring and analytical methodology for 2,3,7,8-TCDD in fish. In: Human and Environmental Risks of Chlorinated Dioxins and Related Compounds. Tucker, R.E., Young,A.L. and Grey, A.P. (eds). Plenun Press, NY. pp. 127-142.

Tucker, R.E., Young, A.L. and Grey,G.A.V. (1982). Human and Environmental Risks of Chlorinated Dioxins and Related Compounds. Plenum Press, NY. pp.823.

USEPA. (1989). Interm procedures for estimating risks associated with exposures to mixtures of chlorinated dibenzo-p-dioxins, and -dibenzofurans (CDDs and CDFs) and 1989 update. EPA/625/3-89/016. Risk Assessment Forum. Washington,DC.

Vos,J.G. and Moore,J.A. (1974). Suppression of cellular immunity in rats and mice by maternal treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Int. Arch. Allergy Appl. Immunol. 47: 777-794.

Walker,M.K., Spitsbergen,J.M., Olsen,J.R. and Peterson, R.E. (1991). 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity during early life stage development of lake trout (Salvelinus namaycush). Can. J. Fish. Aquat. Sci. (in press).

Wells, L. and McLain, A.L. (1973). Lake Michigan: Man's effect on native fish stocks and other biota. Great Lakes Fish. Comm. Tech. Rep. 20, p55.

Whitlock,J., Jr. (1987). The regulation of gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Pharmacol. Rev. 39: 147-174.

Whitlock,J. Jr. (1990). Genetic and molecular aspects of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Annu. Rev. Pharmacol. Toxicol. 30:251-277.

CHAPTER 1

CHARACTERIZATION OF EPIDERMAL GROWTH FACTOR BINDING TO THE HEPATIC PLASMA MEMBRANES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

INTRODUCTION

Epidermal growth factor (EGF) is a 6,045 dalton, single chain polypeptide which was first discovered in the mouse submaxillary gland (Cohen, 1962). EGF is classified into a group of cytomodulatory factors that are hormone-like in their behavior and biological potency. EGF, when administered to mammals, produces several significant biological responses in epithelial and epidermal cells (Carpenter and Cohen, 1979). In vivo, EGF induces proliferation and keratinization of epidermal cells, stimulation of corneal and intestinal cell regeneration, hepatic hypertrophy and hyperplasia, and the inhibition of gastric and thyroid hormone secretion (Ahren, 1987; Soderquist and Carpenter, 1983). In vitro, EGF induces ion transport in cells, the turnover of phosphotidylinositol and ATP, the induction of ornithine decarboxylase, and the activation of RNA, DNA and protein synthesis (Cohen, 1983). EGF exerts its biological effects through a cell surface transmembrane receptor (Adamson and Rees, 1981).

The mouse EGF receptor is a single chain glycoprotein (M_r= 170,000) that has no subunit structure, but can be separated into three functional domains. A heavily glycosylated external domain contains the EGF recognition site

and is involved in ligand binding. The second domain consists of a transmembrane segment that functions in anchoring the receptor to the cell and in signal transmission. The cytoplasmic domain contains an intrinsic tyrosine kinase. This kinase can phosphorylate specific tyrosine residues on the receptor itself and other intracellular proteins and is believed to be the mechanism by which this receptor mediates its mitogenic effects (Das, 1983; Downward et al., 1984).

The purpose of the present study was to characterize the EGF receptor in the liver of adult rainbow trout. Binding of EGF to tissues in fish has been demonstrated in several fish species (Naftel 1989). In a phylogenetic survey of ^{125}I-EGF binding, EGF specific binding was demonstrated in three bony fishes (Fundulus heteroclitus, Stenotemus chrysoes, and Prionotus earlinus) and one cartilagenous fish (Mustelus canis) (Naftel, 1989). In the fishes which bound EGF, the greatest amount of binding was seen in the liver. Comparative binding experiments between the dogfish (Mustelus canis) and mouse liver preparations showed that the EGF association and saturation binding curves were nearly identical. Here we report the binding characteristics of the EGF-specific high affinity, limited capacity binding sites on the plasma membranes of rainbow trout liver.

MATERIALS AND METHODS

Materials

Receptor grade EGF, dexamethasone, glucagon, insulin, cortisol, FSH, LH, and prolactin were purchased from Sigma Chemical Co. (St. Louis, MO). Transforming growth factor-α (TGF-α) and EGF-fragment were purchased from Calbiochem (San Diego, CA). ^{125}I-EGF (100-150 μC/μg) was obtained from ICN Radiochemicals (Irvine, CA).

Fish

Rainbow trout (Oncorhynchus mykiss; RBT) were obtained from Baldwin Fish Farming Enterprise, Big Rapids, MI. Fish were held at 14 ± 1° in 1,000 l tanks supplied with a continuous flow of oxygenated, dechlorinated tap water. The tanks were cleaned twice a week and the fish were fed (Purina trout chow) to satiation three times weekly. The photoperiod was kept constant at 12:12 hr, light:dark.

Plasma Membrane Isolation

Hepatic plasma membrane fractions were prepared by the following procedure. Fish were killed by a sharp blow to the head and their livers were immediately removed and placed in ice-cold 0.25 M sucrose-50mM Tris-HCl (pH 7.2) buffer. The liver was dissected free of the gall bladder and connective tissue, blotted dry and weighed. The livers were then finely minced with scissors and gently homogenized in a loose fitting Dounce homogenizer (2 passes) in 0.25 M sucrose-50mM Tris-HCl, pH 7.2, (1.5 w/v) containing the following protease

inhibitors: aprotinin (100 kallikrein units/ml), leupeptin (10 μg/ml) and PMSF (2mM). The homogenate was passed through double layered gauze and the membranes were separated by differential centrifugation as described by Lutz (1973). The purified membranes were resuspended to about 4-6 mg protein per ml in 0.25 M sucrose-50mM Tris-HCl (pH 7.2) which contained protease inhibitors and aliquots were stored under liquid nitrogen. Protein analysis was by the method of Lowry et al (1951) with bovine serum albumin (BSA) as the standard.

Binding Assays

The binding of ^{125}I-EGF to rainbow trout hepatic membranes was carried out by a modification of the procedure of Carpenter (1985). Briefly, aliquots of membrane protein (50 μg) were incubated in a Krebs-Ringer bicarbonate buffer developed for salmon (Wolf, 1963), pH 7.4 with 0.1% BSA. ^{125}I-EGF was added to each tube in the presence (nonspecific bound) or absence (total bound) of a 1,000-fold excess of unlabeled EGF. The assays were typically conducted at 24° for 180 min unless otherwise stated. The binding assays were terminated by dilution with ice-cold buffer and the bound ^{125}I-EGF was separated from the free ^{125}I-EGF by rapid filtration through Whatman GF/F glass fiber filters. Each filter was washed three times with 3 ml of ice-cold buffer and counted for radioactivity in a Packard autogamma counter (70% efficiency). Specific binding was calculated as the difference between total and nonspecific binding and expressed

as fmol ^{125}I-EGF bound/mg protein.

Binding and Statistical Analysis

The binding curves were fitted and factors extracted by use of the LIGAND/KINETIC computer program package (McPhearson, 1985). The association (K_1) and dissociation (K_{-1}) constants were calculated by the KINETIC program and are expressed as $M^{-1}*min^{-1}$ and min^{-1}, respectively (Rodbard, 1984). The equilibrium dissociation constant (K_D) and the apparent maximum binding capacity (B_{max}) were calculated with the LIGAND program (Munson and Rodbard, 1980). The K_D and B_{max} were expressed as nM and fmol of ^{125}I-EGF bound per mg protein, respectively. Individual saturation binding curves were plotted according to the methods of Scatchard (1949) and Klotz (1982).

Data were expressed as the mean ± standard error of the mean (SEM). Treatment differences were examined with either Students-T test or by Duncan's New Multiple Range test (Steel and Torrie, 1980). Differences were considered significant at $p \leq 0.05$ for all statistical procedures.

RESULTS

Plasma Membrane

The hepatic plasma membrane isolation procedure used yielded the majority of the EGF binding activity in the fraction between the 10% and 32% layers of the sucrose gradient. This fraction corresponds to the hepatic plasma membrane fraction isolated and characterized by Lutz(1973)

from rainbow trout. Due to the efficiency of our procedure and the high yield of plasma membrane relative to liver weight, we used this method in all later studies. The duration of membrane storage did have a significant effect on the binding characteristics in that after one month we observed a 5% loss of specific binding activity. Thus, in all of our assays we only used plasma membranes that had been stored for less than one month.

Optimization of Binding

Preliminary studies were performed to identify optimal conditions for EGF binding assays with rainbow trout (RBT) membranes. Time course studies of the binding of EGF with receptors exhibited temperature dependence for binding kinetics. The rate of binding at 24° was more rapid than that observed at lesser temperatures (Fig. 3). A steady state for binding was reached after approximately 200 min at both 14° and 24° while at 4° steady state was not observed until after 350 min. Analysis of the association data by KINETIC showed that a two site model gave a statistically better fit than that of a single site model. At 24°, the apparent association rate constant (K_{11}) for the high affinity site was $7.2 \times 10^{8} M^{-1} * min^{-1}$ while the apparent rate constant for the low affinity site (K_{12}) was $1.01 \times 10^{7} M^{-1} * min^{-1}$. The association rate constants at 4° and 14° were also calculated but only the 4° rate constant was found to differ statistically from the value observed at 24° (Table 1).

Figure 3. Association of ^{125}I-EGF to RBT liver plasma membranes. Liver plasma membranes (50 μg) which were incubated in triplicate for designated times at 4°(o), 14°(▲), and 24°(■) in 0.2 ml Krebs-Ringer bicarbonate buffer (pH 7.2) with 0.1% BSA. Incubations were started with the addition of 8.3×10^{-10} M (100,000 cpm) ^{125}I-EGF. Non-specific binding was determined in duplicate in the presence of a 1,000-fold excess unlabeled EGF.

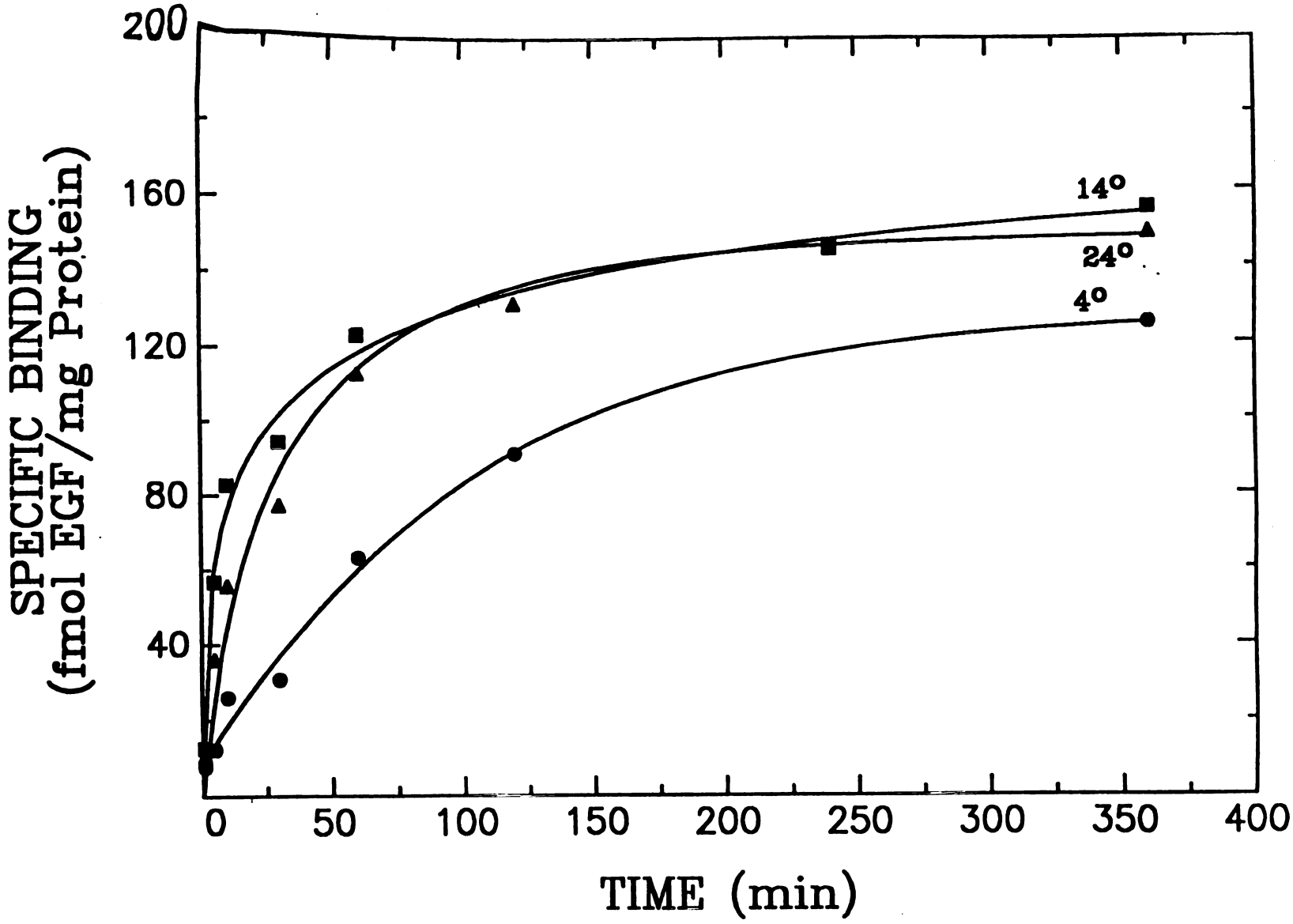

SPECIFIC BINDING
(fmol EGF/mg Protein)
200
160
120
80
40
0
50
100
150
200
250
300
350
400
TIME (min)
14°
24°
4°

Figure 4. Dissociation of bound ^{125}I-EGF from RBT hepatic plasma membranes. After a standard binding assay, ^{125}I-EGF bound to the membranes was displaced by the addition of 4 ml of buffer which contained a 1000-fold excess unlabeled EGF. The amount of bound activity was determined at various time intervals by filtration and each activity measurement was corrected for nonspecific binding. Assays were conducted at 4°(o), 14°(▲), and 24°(■).

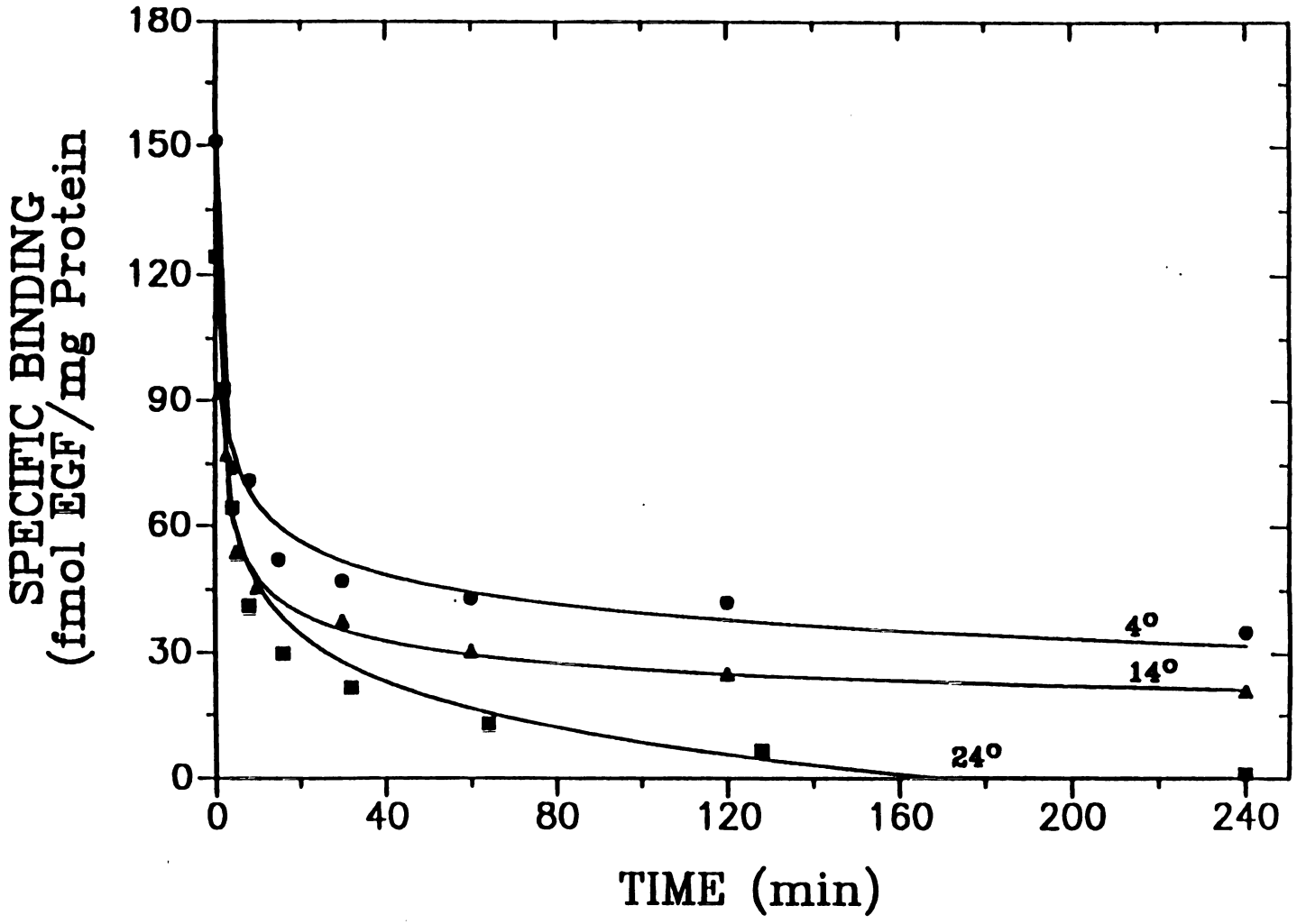

SPECIFIC BINDING
(fmol EGF/mg Protein
180
150
120
90
60
30
0
0
40
80
120
160
200
240
TIME (min)
4°
14°
24°

Table 1. Kinetic analysis of EGF binding to rainbow trout hepatic plasma membranes.

Temperature (°C)		Association ($M^{-1}*min^{-1}$)	Dissociation (min^{-1})	KD (M)
4	Site 1	$3.63x10^{8}$	$1.54x10^{-3}$	$4.2x10^{-11}$
	Site 2	$3.61x10^{6}$	$2.33x10^{-1}$	$6.4x10^{-8}$
14	Site 1	$9.38x10^{8}$	$2.03x10^{-3}$	$2.3x10^{-11}$
	Site 2	$2.28x10^{7}$	$2.07x10^{-1}$	$9.1x10^{-9}$
24	Site 1	$7.24x10^{8}$	$2.29x10^{-2}$	$3.2x10^{-11}$
	Site 2	$1.02x10^{7}$	$1.64x10^{-1}$	$1.6x10^{-9}$

To examine the reversibility of ^{125}I-EGF binding to rainbow trout hepatic plasma membranes, dissociation experiments were conducted at 4°, 14°, and 24° (Fig. 4). The dissociation of ^{125}I-EGF from the membranes was rapid with a half-life of about 22 min. KINETIC analysis of dissociation data statistically fit a two site model. At 24°, the dissociation rate constant for the high affinity site (K_{-11}) was 2.29×10^{-2} min^{-1} while the low affinity site (K_{-12}) was 1.46×10^{-1} min^{-1}. Using the dissociation and association rate constants, estimates of the equilibrium dissociation constants (K_D) were calculated using equation 1 (Table 1)

$$K_D = K_{-1}/K_1 \qquad (1)$$

Incubation of ^{125}I-EGF with greater quantities of hepatic plasma membrane protein showed that the level of total and nonspecific binding were directly related to protein concentration. This relationship was linear for both total and nonspecific binding over a concentration range of 10 to 150 μg/200 μl assay volume. At greater concentrations of protein, the relationship between nonspecific binding and protein became nonlinear with nonspecific binding being proportionally greater than that observed for total binding. This resulted in lowered protein-normalized specific binding values.

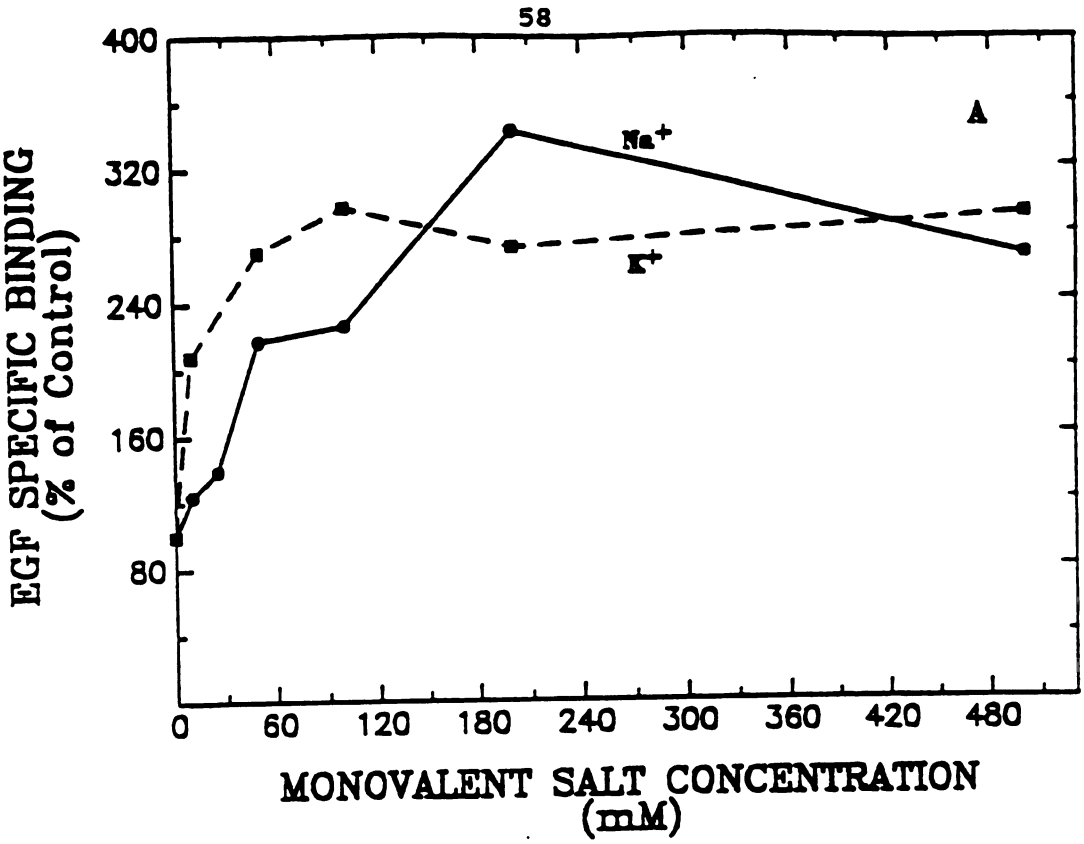
Using a wide range buffer system which contained citrate, phosphate, borate and diethylbarbiturate counterions (Hock and Hollenberg, 1980), the maximum specific binding was observed

at pH 7.2. The level of specific binding remained relatively constant between pH 6.5 to 7.5 with only a 5% loss from the optimum binding which occurred in this pH range. Above pH 7.5 or below pH 6.5 there was a dramatic, irreversible loss of specific binding.

The presence of cations can affect the binding of EGF with its receptor (Hock and Hollenberg, 1980). In rainbow trout, both monovalent (Na^+,K^+) and divalent (Ca^{2+}, Mg^{2+}, Mn^{2+}) cations resulted in a 1.5 to 2.0-fold increase in specific binding over control values (Fig. 5). Divalent cations were found to be more effective than the monovalent cations since optimal binding was observed at a lower ionic strength (0.05) in the presence of divalent cations than in the presence of monovalent cations (0.2). The stimulatory effects of the individual cations at maximal effective concentration were not additive and as a result, combinations of various cations did not increase the observed specific binding maxima above that seen with individual cations.

To confirm the specificity of the EGF ligand for the membrane receptor, a number of hormones and peptides were used in displacement assays (Table 2). Of the hormones tested, only unlabeled EGF, TGF-α and an EGF-fragment effectively displaced the ^{125}I-EGF from the receptor. These results are similar to those observed in studies of mammals in that the EGF receptor exhibits little cross-reactivity with other bioactive peptides such as insulin, glucagon or FSH.

Figure 5. Effects of cations on the binding of EGF. (A) Monovalent cations and (B) divalent cations. Protein was incubated in 20 mM HEPES buffer (pH 7.2), containing 0.1% BSA and the indicated concentrations of either NaCl, KCl, $MgCl_2$, $CaCl_2$, or $MnCl_2$. Each solution was incubated with $8x10^{-10}$ M ^{125}I-EGF in the presence or absence of 1000-fold excess unlabeled EGF.


400
320
240
160
80
EGF SPECIFIC BINDING
(% of Control)
A
Na+
K+
0
60
120
180
240
300
360
420
480
MONOVALENT SALT CONCENTRATION
(mM)

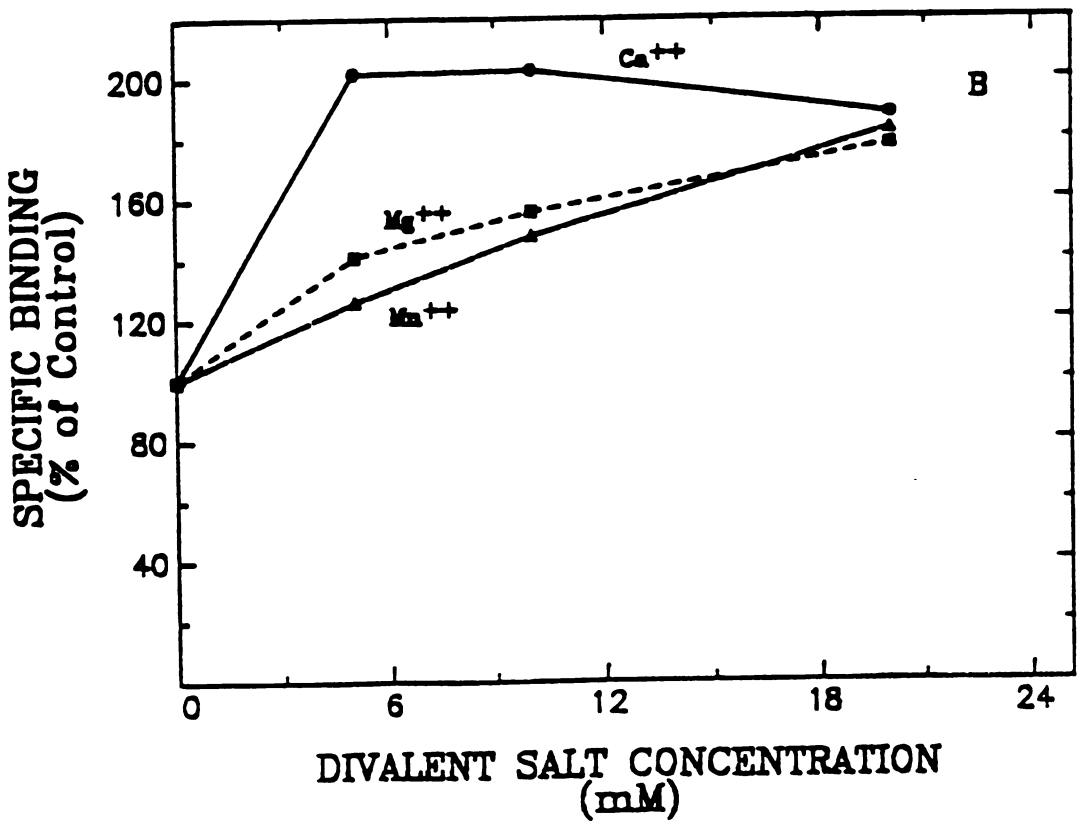

200
160
120
80
40
SPECIFIC BINDING
(% of Control)
B
Ca++
Mg++
Mn++
0
6
12
18
24
DIVALENT SALT CONCENTRATION
(mM)

Table 2. Effects of competing peptides and hormones on binding of ^{125}I-EGF to rainbow trout hepatic plasma membranes[a].

Competitors	Concentration (μg/ml)	% Displacement
None	0	0
EGF	1	70**
TGF-α	1	64**
EGF-Fragment	1	67**
Insulin	50	5
Prolactin	50	5
Glucagon	50	3.5
LH	50	2.5
FSH	50	5
Cortisol	50	8
Dexamethasone	50	5

[a] Competitors were preincubated with membranes for 15 min before ^{125}I-EGF was added to start the reaction.

** Significantly different from the control at $p \leq 0.05$.

Temperature is an important variable in the control of EGF binding kinetics in mammalian species (O'Keefe et al., 1974). This is of particular concern for fish which are poikilothermic. To examine the effect of temperature on EGF binding to rainbow trout membranes, association, dissociation and saturation studies were conducted at 4°, 14°, 24° and 37°. At 37°, for the first 10 min, the binding of EGF proceeded at a rate that was not significantly different from that observed at 24°. However, after this time , there was a dramatic loss of specific binding such that over 70% of the maximal observed binding at 10 min was lost in the following 10 min. The loss of specific binding at 37° was irreversible in that when these membranes were preincubated at 24°, no specific binding was observed. There was a small effect of temperature on EGF binding kinetics and binding capacity (Tables 1 and 3). However, these effects were not of equal magnitude as that which have been observed with mammalian receptors (Hock and Hollenberg, 1980).

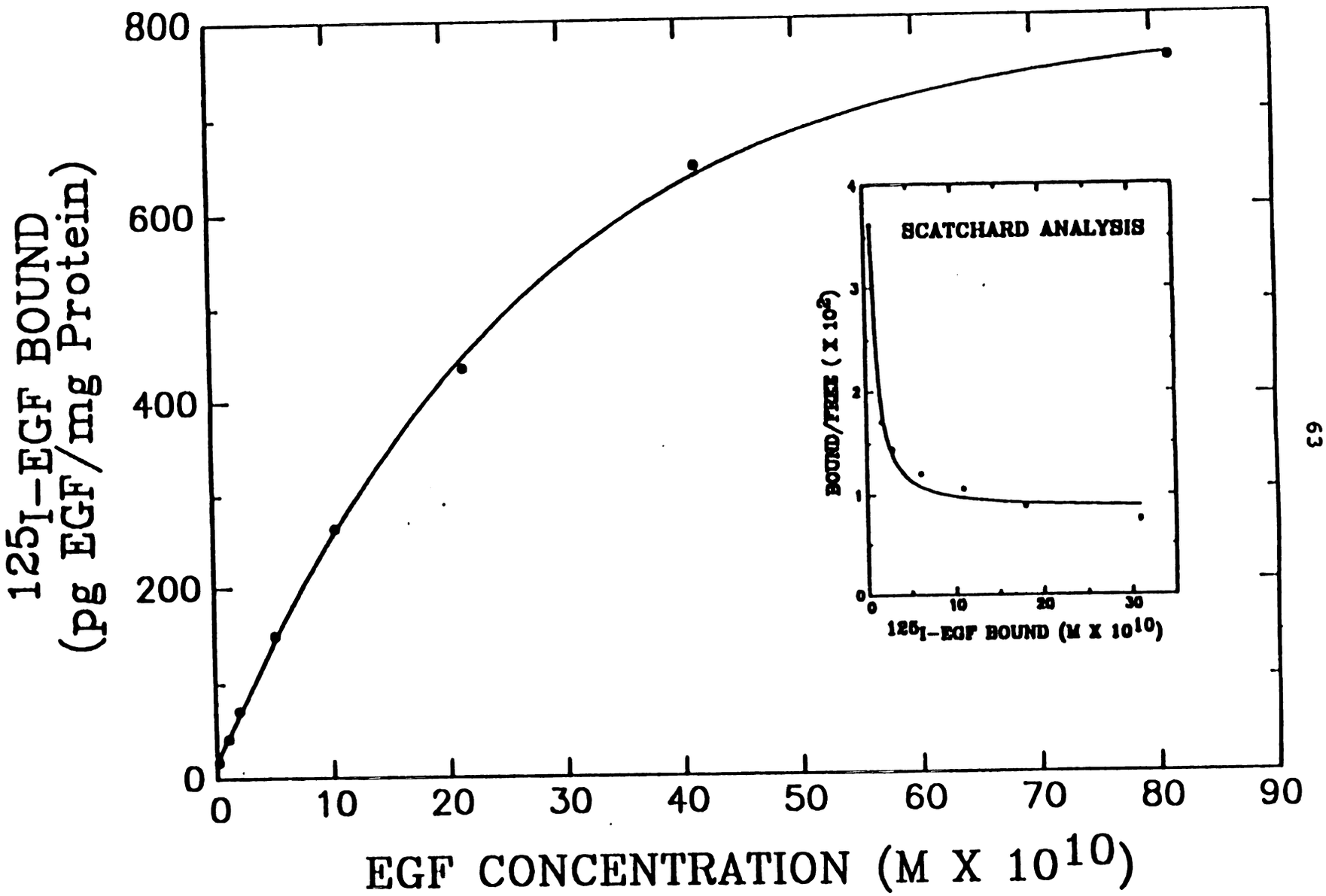
Saturation Analyses

The binding of ^{125}I-EGF to RBT hepatic membranes was saturable (Fig. 6A) with nonspecific binding in the saturation assays averaging 55% of the total binding. The half-maximal binding in these assays was 1.5×10^{-9}M ^{125}I-EGF. Klotz plots (data not shown) confirmed the saturation of the receptors (Klotz, 1982). Scatchard plots were curvilinear (Fig.6B) which indicates the presence of a heterogeneous population of

Table 3. Summary of the Dissociation constants (K_D) and maximal binding capacity (B_{max}) of ^{125}I-EGF to RBT membranes as determined by Scatchard analysis.

Temperature (°C)		K_D (M)	B_{max} (fmol/mg protein)
4	Site 1	2.81×10^{-11}	1.74
	Site 2	2.62×10^{-9}	181.57
14	Site 1	4.78×10^{-11}	11.40
	Site 2	5.22×10^{-9}	282.60
24	Site 1	6.01×10^{-11}	15.67
	Site 2	3.13×10^{-8}	259.07

Figure 6. Saturation curve of ^{125}I-EGF binding to RBT hepatic plasma membrane. Liver membranes (50 μg) were incubated with increasing concentrations of ^{125}I-EGF in 0.2 ml Krebs Ringer-bicarbonate buffer (pH 7.2) with 0.1% BSA. After 120 min at 24°, specific binding (fmol EGF bound/mg of protein) was determined as outlined in "Material and Methods". (Insert) Representative Scatchard plot of ^{125}I-EGF bound to hepatic plasma membranes of RBT.


800
600
400
200
0
0
10
20
30
40
50
60
70
80
90
125I-EGF BOUND
(pg EGF/mg Protein)
EGF CONCENTRATION (M X 10^10)
SCATCHARD ANALYSIS
BOUND/FREE (X 10^2)
125I-EGF BOUND (M X 10^10)

receptors. The equilibrium dissociation constants (K_D) and binding capacities (B_{max}) at 24° were calculated to be 6.01 x 10^{-11} M and 15.7 fmol EGF/mg protein respectively for the high affinity site and 3.13 x 10^{-8} M and 259.1 fmol EGF/mg protein respectively for the low affinity site. To confirm that the curvilinear nature of the Scatchard analysis was due to multiple receptor sites and not due to site-to-site interaction, the method of DeMeyts et al. (1976) was used. This procedure is based on the kinetics of ^{125}I-EGF dissociation from its receptor in the presence of an "infinite" dilution or dilution with the addition of an excess of unlabeled EGF. Site-to-site interactions are indicated when the dissociation rate of ^{125}I-EGF statistically differs between the two treatments. Results of this analysis indicate the curvilinear Scatchard analysis was due to a heterogeneous population of receptors (Fig. 7). This conclusion agrees with analyses conducted with LIGAND, which showed that the data were best fit to a two receptor site model. Furthermore, Hill plots of the EGF binding data resulted in slope values that were not significantly different from unity which indicates a lack of site-to-site interaction (Cuatrecasas and Hollenberg, 1976).

Displacement of labeled EGF from fish receptors with EGF, an EGF-fragment and TGF-α was conducted to confirm receptor affinities (Fig. 8). Analysis of the displacement data by LIGAND showed that a two site model best fit the data.

Figure 7. Kinetic analysis for site-to-site interaction of EGF binding to RBT hepatic membranes. Membrane protein (500 ug/ml) was incubated with ^{125}I-EGF (8.3×10^{-10}M) in the absence or presence of unlabeled EGF (9.0×10^{-7}M) for 180 min at 24° in a total volume of 2.5 ml. At the end of the incubation, specific binding was determined with a 40 μl aliquot from each tube by filtration. This value was considered as 100% at time 0. After the incubation, 40 μl aliquots were diluted in 4 ml buffer in the presence or absence of (1.6×10^{-7}M) unlabeled EGF. At indicated intervals, three tubes of each set were filtered and counted. The radioactivity on the filters, expressed as a percentage of the radioactivity at 0 min, are plotted as a function of time after dilution (o) and dilution plus unlabeled EGF (■).

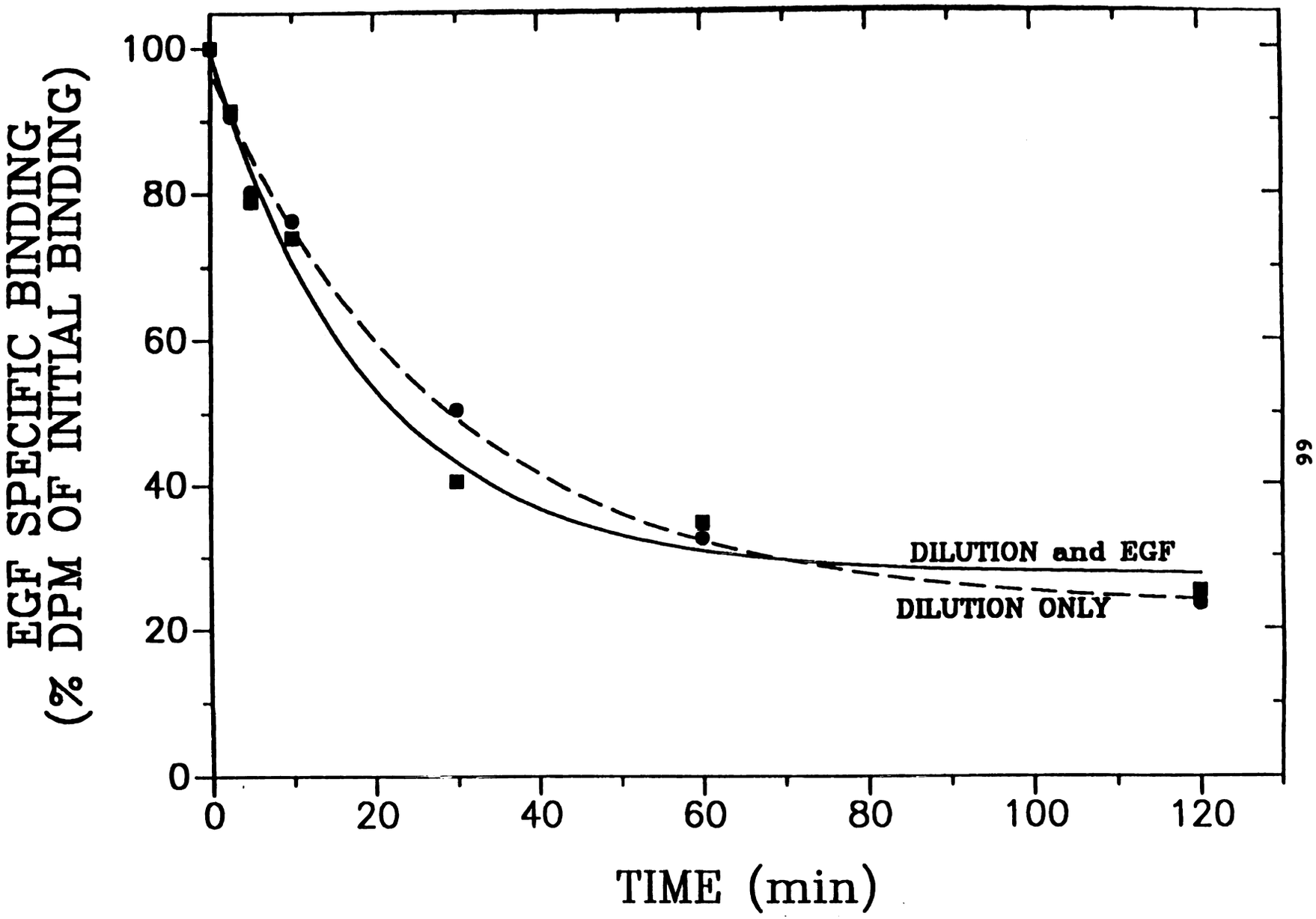
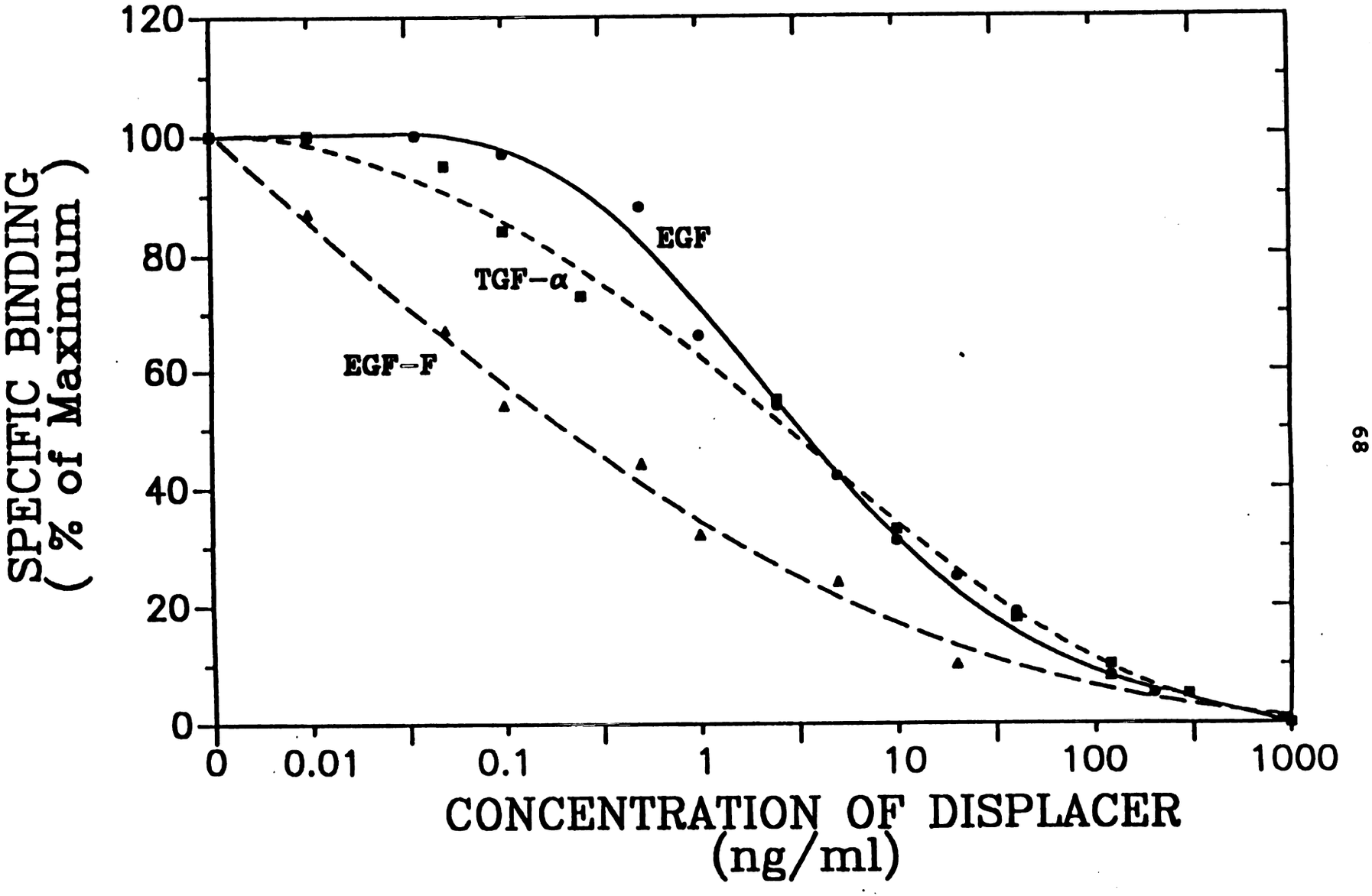

EGF SPECIFIC BINDING
(% DPM OF INITIAL BINDING)
100
80
60
40
20
0
0
20
40
60
80
100
120
TIME (min)
DILUTION and EGF
DILUTION ONLY

Figure 8. Representative displacement curves of ^{125}I-EGF from RBT hepatic membranes by EGF (●), an EGF-fragment (▲) and TGF-α (■). Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled EGF. Data are plotted as the relative decrease of saturable binding in the presence of different concentrations of unlabeled displacers.


120
100
80
60
40
20
0
SPECIFIC BINDING
(% of Maximum)
EGF
TGF–α
EGF–F
0
0.01
0.1
1
10
100
1000
CONCENTRATION OF DISPLACER
(ng/ml)

Displacement of ^{125}I-EGF by unlabeled EGF had an IC_{50} of$1.48x10^{-11}$ M for the high affinity site and an IC_{50} of $1.03x10^{-9}$ M for the low affinity site. The EGF-fragment, which contains the binding portion of the EGF ligand, displaced the ^{125}I-EGF with about equal affinity as that of the unlabeled EGF. The high affinity site had an IC_{50} of $2.01x10^{-11}$ M while the low affinity site had an IC_{50} of $1.59x10^{-9}$ M. Displacement with TGF-α was similar to that observed with unlabeled EGF. IC_{50} for the high affinity site was $1.12x10^{-11}$ M while the IC_{50} for the low affinity site was $9.11x10^{-9}$ M. The results of the displacement analyses indicate that both EGF and TGF-α compete for the same receptor sites with about equal affinity. Furthermore, the displacement assays confirm our previous finding of the existence of two EGF receptor classes in RBT liver membranes.

DISCUSSION

This is the first published report on the characterization of EGF binding sites in RBT. Of particular significance is that the binding of mouse EGF to trout receptors is very similar to that observed in mammals. In RBT, EGF-receptors consist of two classes of receptors that have different, but fixed binding affinities (King and Cuatrecasas, 1982). The apparent dissociation constant for the high and low affinity sites of EGF receptors in RBT fall within the ranges reported for mammals (Gregoriou and Rees,

1984). For human liver, the apparent dissociation constant (K_D) for the low affinity site was 1.4 x 10^{-9} M while that for the high affinity site was 1.08 x 10^{-10} M (Lev-Ran et al., 1984). The observation of two receptor classes differs from that of Naftel (1989) for dogfish liver. Reasons for this discrepancy are unknown. Based on the results of the kinetic experiments (DeMytes et al., 1976), the observed curvilinearity of the Scatchard plot was due to different receptor classes and not an artifact of our binding protocol.

The EGF kinetics of association and dissociation with receptors in RBT are similar to those observed in mammalian tissues. The rate constants for association (K_1) are 1.56 x 10^8 $M^{-1}*min^{-1}$ and 6.0 x 10^8 $M^{-1}*min^{-1}$ for human placenta and mouse liver, respectively. The rate constants for dissociation (K_{-1}) are 3.7 x 10^{-2} min^{-1} and 3.7 x 10^{-2} min^{-1} for human placenta and mouse liver, respectively (Hock and Hollenberg, 1980; O'Keefe et al., 1974). Unlike mammals, temperature caused only a small shift in RBT binding kinetics over the temperature range we studied.

EGF bound maximally to RBT liver membranes at pH 7.0 and at slightly basic conditions (pH 7.5). No other information is currently available for comparison in fish but, human placenta receptors bind with a pH optimum of pH 7.6. Like fish, the EGF binding with receptors remained stable over the same pH range, pH 6.0 to pH 7.5 (Hock and Hollenberg, 1980).

The ionic requirements of EGF-binding are similar to those reported for human placental tissue. Optimum binding was observed at an ionic strength (I) for monovalent cations of 0.1 for human placenta while optimal binding in RBT was observed at an ionic strength of 0.2. For divalent cations, optimal binding of EGF occurs at an ionic strength of 0.015 for placental membranes while in RBT it was 0.05. While the cations are not required for EGF-binding in RBT and human placental tissues, they do increase the binding maximum above that of the control values.

The binding of EGF to RBT receptors is very specific in that only unlabeled EGF, an EGF-fragment and TGF-α displaced the ^{125}I-EGF. TGF-α is a single chain polypeptide that is structurally and functionally related to EGF and may be important in fetal development (Marquardt et al., 1984; Pike et al., 1982). In displacement studies with rainbow trout hepatic membranes, TGF-α and unlabeled EGF displaced ^{125}I-EGF from the receptors with about equal efficacy in that they had comparable IC_{50}s. These results are consistent with observations of the behavior of EGF in mammalian tissues.

Before a binding site can be considered a physiologically relevant receptor, various criteria must be met. These criteria include; 1) that the ligand bind to the receptor with high affinity; 2) that the binding be saturable and reversible: 3) that there be a high degree of chemical specificity for the receptor in regards to binding to the

receptor by other ligands, and 4) the binding of the ligand results in a physiological effect (Laduron, 1984). The first three criteria have been met in this study. To study the fourth criteria we are now examining the effect of EGF binding on the activation of a tyrosine kinase that is associated with the EGF receptor in RBT hepatic membranes. However, the EGF binding properties of the RBT hepatic membranes in this study were consistent with the premise that the site of ^{125}I-EGF binding represents a form of EGF receptor that is homologous to that found in mammalian EGF-receptors.

REFERENCES

Adamson, E.D. and Rees, A.R. (1981). Epidermal growth factor receptors. Mol. Cell. Biochem. 34, 129-152.

Ahren, B. (1987). Epidermal growth factor (EGF) inhibits stimulated thyroid hormone secretion in the mouse. Peptides 8, 743-745.

Carpenter, G. (1985). Binding assays for epidermal growth factor. In "Methods in Enzymology" (L. Birnbaumer and B.W. O'Malley Eds), Vol 109, pp.101-115. Academic Press, New York.

Carpenter, G. and Cohen, S. (1979). Epidermal growth factor. Ann. Rev. Biochem. 48, 193-216.

Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal. J. Biol. Biol. 237, 1555-1562.

Cohen, S. (1983). The epidermal growth factor (EGF). Cancer 51, 1787-1791.

Cuatrecasas, P. and Hollenberg, M.D. (1976). Membrane receptors and hormone action. Adv. Protein Chem. 30, 251-289.

Das, M. (1983). Epidermal growth factor receptor and mechanisms for animal cell division. In " Current Topics in Membranes and Transport: Membrane Receptors."

(A. Kleinzeller and B.R. Martin, Eds), Vol. 18., pp. 381-405. Academic Press, Inc., New York.

DeMeyts, P., Bianco, A.R., and Roth, J. (1976). Site-site interactions among insulin receptors. J. Biol. Chem. 251, 1877-1888.

Downward, J., Parker, P., and Waterfield, M.D. (1984). Autophosphorylation sites on the epidermal growth factor receptor. Nature 311, 483-484.

Gregoriou, M. and Rees, A.R. (1984). Properties of a monoclonal antibody to epidermal growth factor receptor with implications for the mechanism of action of EGF. EMBO J. 3, 929-937.

Hock, R.A. and Hollenberg, M.D. (1980). Characterization of the receptor for epidermal growth factor-ugastrone in human placenta membranes. J. Biol. Chem. 255, 10731-10736.

King, A.C. and Cuatrecasas, P. (1982). Resolution of high and low affinity EGF receptors. J. Biol. Chem. 257, 3053-3060.

Klotz, I.M. (1982). Numbers of receptor sites from Scatchard graph: Facts and fantasies. Science 217, 1247-1249.

Laduron, P.M. (1984). Criteria for receptor sites in binding studies. Biochem. Pharmacol. 313, 833-839.

Lev-Ran, A., Huang, D., Josefsberg, Z., Barseghian, G., Kemeny, M., Meguid, M., and Beatty, D. (1984). Binding of epidermal growth factor (EGF) and insulin to human liver

Lowry

Lutz

Marq

McPh

Muns

Naft

O'Ke

Pike

microsomes and golgi fractions. Biochem. Biophys. Res. Commun. 119, 1181-1185.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-268.

Lutz, F. (1973). Isolation and some characteristics of liver plasma membranes form rainbow trout. Comp. Biochem. Physiol. 45B, 805-811.

Marquardt, H., Hunkapiller, M.W., Hood, L.E. and Todaro, G.J. (1984). Rat transforming growth factor type 1: Structure and relation to epidermal growth factor. Science 233, 1079-1082.

McPherson, G.A. (1985). Analysis of radioligand binding experiments: A collection of computer programs for the IBM PC. J. Pharmacol. Meth. 14, 213-228.

Munson, P.J. and Robard, D. (1980). LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107,220-239.

Naftel, J. (1989). Personal communication.

O'Keefe, E.J., Hollenberg, M.D., and Cuatrecasas, P. (1974). Epidermal growth factor: Characteristics of specific binding in membranes from liver, placenta and other target tissues. Arch. Biochem. Biophys. 164, 518-526.

Pike, L.J., Marquardt, H., Todaro, G.J., Gallis, B.,

Casnellie, J.E., Bornstein, P., and Krebs, E.G. (1982). Transforming growth factor and epidermal growth factor stimulate the phosphorylation of a synthetic tyrosine-containing peptide in a similar manner. J. Biol. Chem. 257,14628-14631.

Rodbard, D. (1984). Lessons from the computerization of a radioimmunoassay: An introduction to basic principles of modelling. In " Computers in Endocrinology", (D. Rodbard and G. Forti, Eds.) Raven Press, New York.

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51, 660-672.

Soderquist, A.M., and Carpenter, G. (1983). Developments in the mechanism of epidermal growth factor activation: Activation of protein kinase by epidermal growth factor. Fed. Proc. 42, 2615-2620.

Steel, R.G. and Torrie, J.H. (1980). "Principles and Procedures of Statistics". 2nd Ed. McGraw-Hill Book Co., New York.

Wolf, K. (1963). Physiological salines for fresh-water teleosts. The Prog. Fish Cult. 25, 135-140.

CHAPTER 2

EPIDERMAL GROWTH FACTOR RECEPTOR-PROTEIN KINASE INTERACTIONS IN THE LIVER MEMBRANES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

pol

in

Coh

spe

cel

eff

oua

ara

Ree

gas

198

(Sc

sur

ran

rec

aff

a l

Sch

a p

INTRODUCTION

Epidermal growth factor (EGF) is a 6,045 Dalton polypeptide that has mitogenic and growth stimulatory activity in cells of epidermal and epithelial origin (Carpenter and Cohen, 1979; Gospodarowicz, 1981). Binding of EGF to a specific receptor located on the plasma membrane of responsive cells will cause a number of biological responses. In vitro effects include increased amino acid transport, activation of ouabain-sensitive Na^+/K^+ ATPase activity , stimulation of arachidoniate metabolism and DNA synthesis (Gregoriou and Rees, 1984). In vivo responses include the inhibition of gastric secretion, altered endocrine function (Ritvos et al., 1988), and the stimulation of growth hormone synthesis (Schonbrunn et al., 1980). These responses are mediated by surface receptors whose affinities (K_D) for EGF are in the range of 10^8 M^{-1} to $5x10^9$ M^{-1} for a major class of low affinity receptors and 10^{10} to 10^{11} M^{-1} for a minor class of high affinity receptors (Gregoriou and Rees, 1984).

The EGF receptor is a transmembrane glycoprotein that has a ligand-dependent tyrosine kinase activity (Carpenter, 1987; Schlessinger, 1988). The mature receptor is synthesized from a precursor (134 KDa) which is N-glycosylated to 170 KDa.

Wh
th
do
re
bi
ac
tr
wh
be
au
10
ac
bi
si
sy
19

of
Th
sa
ki
re
co
af
re
we

While it has no subunit structure, it can be divided into three major domains based on structure and function. The domains are as follows: (1) The N-terminus extracellular region which is heavily glycosylated constitutes the ligand binding domain; (2) a unique hydrophobic transmembrane region acts in anchoring the receptor and is also involved in signal transduction; and (3) the C-terminus or intracellular region which contains a tyrosine kinase. In mammals, this kinase has been shown to phosphorylate cytosolic proteins as well as autophosphorylating four tyrosine residues located at 1068, 1086 1148 and 1173 (Margolis et al., 1989). It is the activation of the tyrosine kinase that is essential for the biological function of the EGF receptor and for its role in signal transduction, receptor trafficking, stimulation of DNA synthesis and cellular transformation (Moolenaar et al., 1988).

We have previoulsy reported that hepatic plasma membranes of rainbow trout bind mouse ^{125}I-EGF (Newsted and Giesy, 1991). The binding of ^{125}I-EGF to the membranes was peptide-specific, saturable, reversible and of high affinity. Scatchard and kinetic analysis of the binding data indicated multiple receptor sites of differing affinity. Apparent dissociation constant for the high affinity (K_D= 2.3×10^{-11} M) and low affinity (K_D=9.1×10^{-9} M) sites were in agreement with values reported for mammalian species. Maximal binding capacities were 13 fmol EGF bound/mg protein and 270 fmol EGF bound/mg

protein
respecti
EGF cova
showed a
in sampl

In
phosphor
in the h
Paramete
incubati
and pept
have als
the phos
studies,
binding
demonstr
an intr
biochemi
mammalia

Material

Rec
LH, dex
phosviti
Sigma (S

protein for the high and low affinity receptor sites, respectively. Furthermore, SDS-PAGE electrophoresis of ^{125}I-EGF covalently linked to the receptor in trout membranes showed a band that corresponded to 150 KDa that was displaced in samples incubated with 1,000 fold excess of unlabeled EGF.

In this study we characterized an EGF-stimulated phosphorylation reaction and its relationship to EGF binding in the hepatic plasma membranes of rainbow trout (RT-HPM). Parameters that we have evaluated include the effect of incubation buffer and time, ATP concentration, other hormones and peptides and EGF on the phosphorylation of RT-HPM. We have also examined the protein tyrosine kinase associated with the phosphorylation induced by the binding of EGF. These studies, in conjunction with studies that characterized the binding of EGF in RT-HPM (Newsted and Giesy, 1991) have demonstrated that rainbow trout have an EGF receptor that has an intrinsic tyrosine kinase activity that is similar in biochemical activity to that which is observed in many mammalian species and cell lines.

MATERIAL AND METHODS

Materials

Receptor grade EGF, insulin, prolactin, glucagon, FSH, LH, dexamethasone, angiotensin II, [Val^5]-angiotensin II, phosvitin, casein, and histone H1 type III were purchased from Sigma (St. Louis, MO.). Transforming growth factor-α (TGF-α)

was purcha

200 μC/μg)

(Boston,

dihydroxy

tryphosti

aminobenz

protein k

protein

Laborator

receptor

Fish

Rain

Stony Cre

in 1,000

oxygenate

twice a

satiation

(light:da

Plasma Me

RT-P

(1991).

membranes

purificat

gradient.

was purchased from Calbiochem (San Diego, CA). ^{125}I-EGF (150-200 μC/μg) and ^{32}P[γ]ATP (3000 Ci/mmol) were purchased from NEN (Boston, MA). Protein kinase inhibitors methyl 2,5-dihydroxycinnamate (MDHC), lavendustin A, genistein, tryphostin, RCMA-lysozyme, 2 hydroxy-5-(2,5-dihyroxybenzyl) aminobenzoic acid (HDAA) and PKC inhibitor (19-36) as well as protein kinase substrates RR-src and acetylated- myelin basic protein (Ac-MBP) were purchased from Gibco-BRL (Gibco Laboratories, Grand Island, NY). Phosphotyrosine and EGF-receptor antibodies were purchased from Oncogene.

Fish

Rainbow trout (Oncorhynchus mykiss) were obtained from Stony Creek Trout Farm, Grant MI. Fish were held at 12 ± 1 C in 1,000 liter tanks supplied with a continuous flow of oxygenated, dechlorinated tap water. The tanks were cleaned twice a week and fish were fed (Silvercup trout Food) to satiation twice weekly. The photoperiod was 16:8 hr (light:dark).

Plasma Membrane Isolation

RT-HPM were prepared by the method of Newsted and Giesy (1991). This method was based on the isolation of crude membranes by differential centrifugation followed by a final purification step by centrifugation through a sucrose gradient. The purified RT-HPM were resuspended to about 4-6

mg protein
containing
µg/ml) and
the RT-HPM
analysis wa
serum albu

EGF Bindir

Bind
procedure
reactions
Hepes (
Nonspeci
containe
assays
buffer
by rapi
Each f
and th
autoga
calcu
bindi

Stand

fina

mg protein in 50 mM Tris-HCl,(pH 7.2) 0.25 M sucrose buffer containing aprotinin (100 kallikrein units/ml), leupeptin (10 μg/ml) and phenylmethylsulonyl fluoride (2 mM). Aliquants of the RT-HPM were then stored under liquid nitrogen. Protein analysis was by the method of Lowry et al (1951) with bovine serum albumin (BSA) as the standard.

EGF Binding Assay

Binding assays were conducted by a modification of the procedure of Carpenter (1985). Briefly, triplicate binding reactions were carried out in 100μl volumes containing 20 mM Hepes (pH 7.4), 0.1% BSA, 50 μg RT-HPM, and ^{125}I-EGF. Nonspecific binding was measured in triplicate tubes which contained a 100-molar excess of unlabeled EGF. The binding assays were terminated by dilution with ice-cold binding buffer and the bound ^{125}I-EGF was separated from the free EGF by rapid filtration through Whatman GF/F glass fiber filters. Each filter was washed three times with 3 ml ice-cold buffer and the filters were counted for radioactivity in a Packard autogamma counter (70% efficiency). Specific binding was calculated as the difference between total and nonspecific binding and expressed as fmol ^{125}I-EGF bound/mg protein.

Standard Phosphorylation Assays

Assays of RT-HPM phosphorylation were conducted in a final volume of 60 μl in microfuge tubes. Reaction mixtures

contai

$MgSO_4$,

(2.0x1

the pr

reacti

C for

µl al

were i

(10 ml

phosph

min)

scint

cockta

quant

was s

(Laem

was r

with

stain

with

expos

incor

the

scint

containing 20 mM Hepes buffer (pH 7.2), 5 mM $MnSO_4$, 20 mM $MgSO_4$, 100 μM Na_3VO_4, 0.05% Nonidet-P40 and 10 μM ^{32}P-ATP ($2.0x10^5$ cpm) were placed on ice and incubated for 10 min in the presence or absence of 0.5 μM EGF. The phosphorylation reaction was initiated by the addition of ATP and incubated 4 C for 15 min. The reaction was terminated by pippetting a 50 μl aliquot onto Whatman P81 phosphocellulose filters which were immediately immersed in a beaker of 75 mM phosphoric acid (10 ml/filter). Filters were washed 3 times (10 min each) in phosphoric acid at room temperature, extracted with acetone (5 min) and air dried. The dried filters were placed in scintillation vials and 7 ml of Safety Solv scintillation cocktail was added to the vials. Radioactivity was quantitated by liquid scintillation counting.

In a separate set of phosphorylation assays, the reaction was stopped by the addition of 30 μl of 4x SDS sample buffer (Laemmli, 1970) and heated at 100 C for 5 min. Each mixture was run on 7.5% SDS-polyacrylamide gels. The gels were fixed with acetic acid:water:methanol (10:40:50 by volume) and stained with Coomassie Blue R-250. After extensive washing with 7% methanol/7% acetic acid (v/v), the gels were dried and exposed to X-ray film for autoradiography. To quantitate ^{32}P incorporated into specific proteins, bands were excised from the dried gel and radioactivity counted by liquid scintillation counting.

Peptide Ph

Tyros

filter pape

HPM (25 µg

buffer in

substrate.

reaction w

ATP, 1000-

min at 0°C

5% trichlo

BSA/ml. S

C. Two 70

cm filter

times for

acid, once

counted b

activity w

in the pr

pmol ^{32}P i

Phosphoam

Phos

procedure

from the

100 µl 6

hydrosyla

Peptide Phosphorylation Assays

Tyrosine kinase activity was quantitated by use of a filter paper assay (Pike, 1987). Triplicate aliquants of RT-HPM (25 μg) were incubated in standard phosphorylation assay buffer in the absence and presence of a phosphorylation substrate. The final volume of the reaction was 60 μl. The reaction was initiated by the addition of ATP (50 μM [γ-^{32}P] ATP, 1000-2000 cpm/pmol) and the tubes were incubated for 15 min at 0°C. Reactions were terminated by addition of 150 μl 5% trichloroacetic acid-1.65 Mm KH_2PO_4 and 10 μl of 10 mg BSA/ml. Samples were centrifuged at 10,000xg for 15 min at 4 C. Two 70 μl aliquants of supernatant were spotted onto 2.3 cm filter disks (Whatman P81). The filters were washed 3 times for 10 min each at room temperature in 75 Mm phosphoric acid, once with acetone and air dried. Filters were then counted by liquid scintillation counting. Protein kinase activity was calculated as the difference in ^{32}P incorporated in the presence and absence of substrates and expressed as pmol ^{32}P incorporated per min per mg protein.

Phosphoamino Acid Analysis

Phosphoamino acid analysis was based on a modified procedure of Ushiro and Cohen (1986). ^{32}P-labeled membranes from the standard phosphorylation assays were hydrolyzed in 100 μl 6 N HCl at 100 C for 2 h in sealed tubes. The hydrosylates were dried under vacuum and dissolved in 25 μl of

water cc

phospho:

chromat

system c

acid/wa

ninhydr

autorad

amino a

spots

scintil

Western

RT-

prepare

Laemmli

wells

electro

electro

perform

SDS-PAG

(100 V

contain

Tris/0.

powdere

overnig

5 min a

water containing phosphoamino acid standards (phosphotyrosine, phosphoserine and phosphothreonine). Samples were chromatographed on cellulose thin layer plates with a solvent system consisting of n-butyl alcohol/isopropyl alcohol/formic acid/water (3:1:1:1, v/v). The standards were located by ninhydrin while radioactive material was located by autoradiography using X-ray film. ^{32}P-incorporated into the amino acids was quantified by scrapping the radiolabeled spots into scintillation vials and counting by liquid scintillaton .

Western Analysis of Phosphorylated Membranes

RT-HPM were phosphorylated in a standard assay and prepared for electrophoresis by heating at 100 C for 5 min in Laemmli SDS sample buffer. RT-HPM (50 μg) was applied to wells of the 4.5% stacking gel and subjected to electrophoresis on a 7.5% resolving gel in a Mini Protean electrophoresis unit (Biorad, Melville, NY). Immunoblots were performed according to the method of Towbin et al. (1979). SDS-PAGE separated proteins were electophoretically transfered (100 V for 1 h) to nitrocellulose. The nitrocellulose sheets containing the transfered proteins were rinsed in 0.02 M Tris/0.5 M NaCl (TBS), pH 7.4 for 5 min, blocked in 5% powdered non-fat milk in TBS containing 0.5% Tween 20 overnight at 4 C. The sheets were rinsed 3 times with TBS for 5 min and then incubated with the primary antibody for 1 h at

room temperature with gentle shaking. The sheets were then rinsed three times with TBS and incubated with horseradish peroxidase-linked IgG (Immunoselect system, Gibco, Grand Island, NY). Visualization and quantification were performed by autoradiography followed by densitometry.

Statistical Analysis

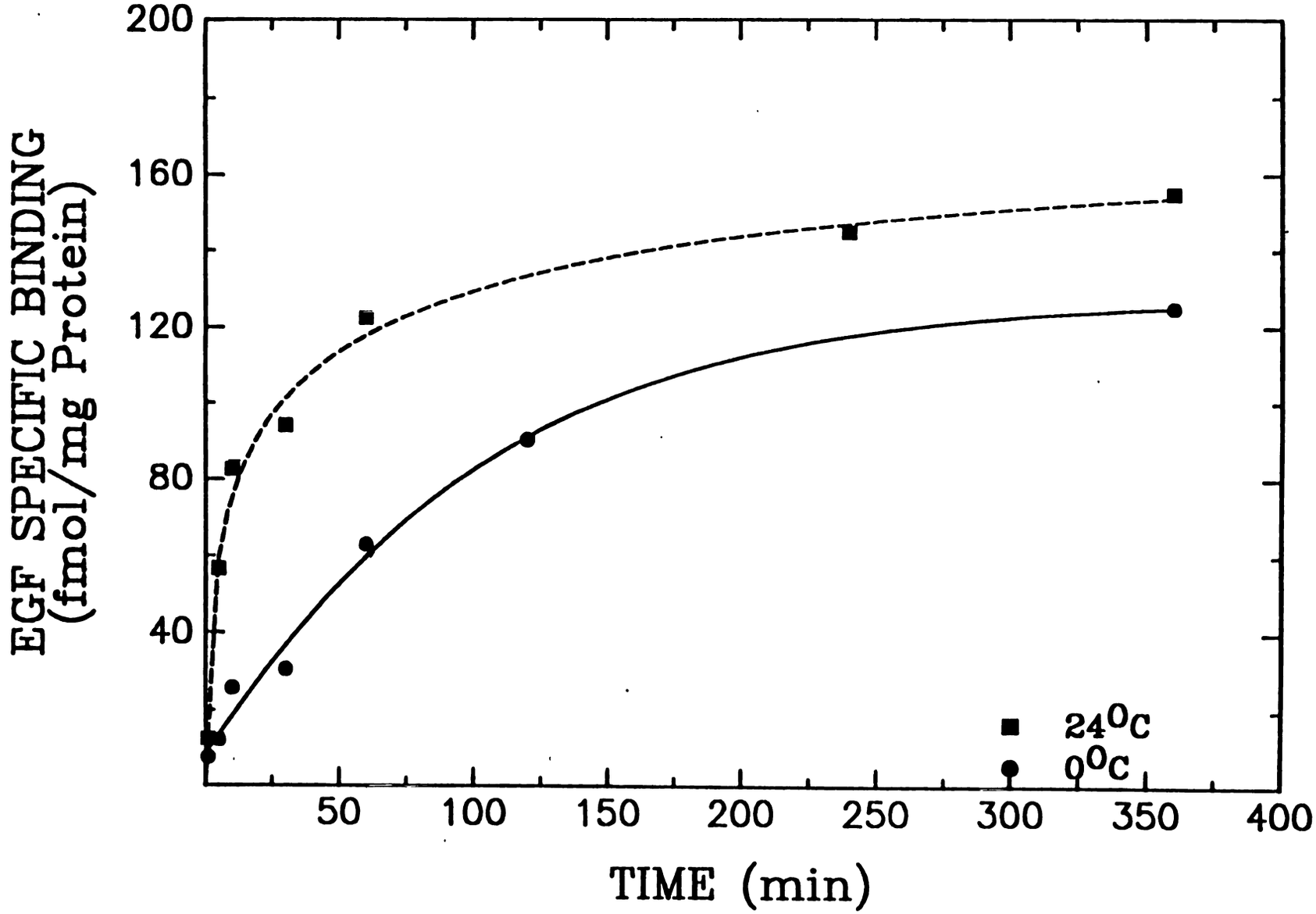
Results are expressed as means ± S.D. for each measured parameter. Statistical analyses were performed using Student's T test, analysis of varience and planned paired comparisons (Steel and Torrie, 1980). The level of significance to detect differences between treatments was $p < 0.05$.

RESULTS

Binding of ^{125}I-EGF to trout membranes

The rate of binding ^{125}I-EGF to RT-HPM was rapid and temperature dependent (Fig. 9). At 24 C, steady state binding was reached at approximately 200 min with a very rapid increase in EGF binding occurring in the first 25 min. At 4 C, the binding of EGF to RT-HPM proceeded at a much slower rate with steady state being attained by 350 min. Half maximal binding at 0 C occurred by 1 h while it was attained by 15 min at 24 C. At 0C the rate of EGF binding for the first 30 min was 1.03 fmol EGF bound/min/mg protein while the rate was 3.13 fmol EGF bound/min/mg protein at 24 C.

Figure 9. Time course of ^{125}I-EGF binding to RT-HPM. Binding reactions were initiated by the addition of ^{125}I-EGF (8.3×10^{-10}M, 100,000 cpm) to standard reaction mixtures. At the indicated times, triplicate tubes for total and nonspecific binding were quantitated.


EGF SPECIFIC BINDING
(fmol/mg Protein)
200
160
120
80
40
50
100
150
200
250
300
350
400
TIME (min)
24°C
0°C

Time

incor
mater
the r
incre
assoc
of ph
per m
min p
diffe
was s
both
in a
level

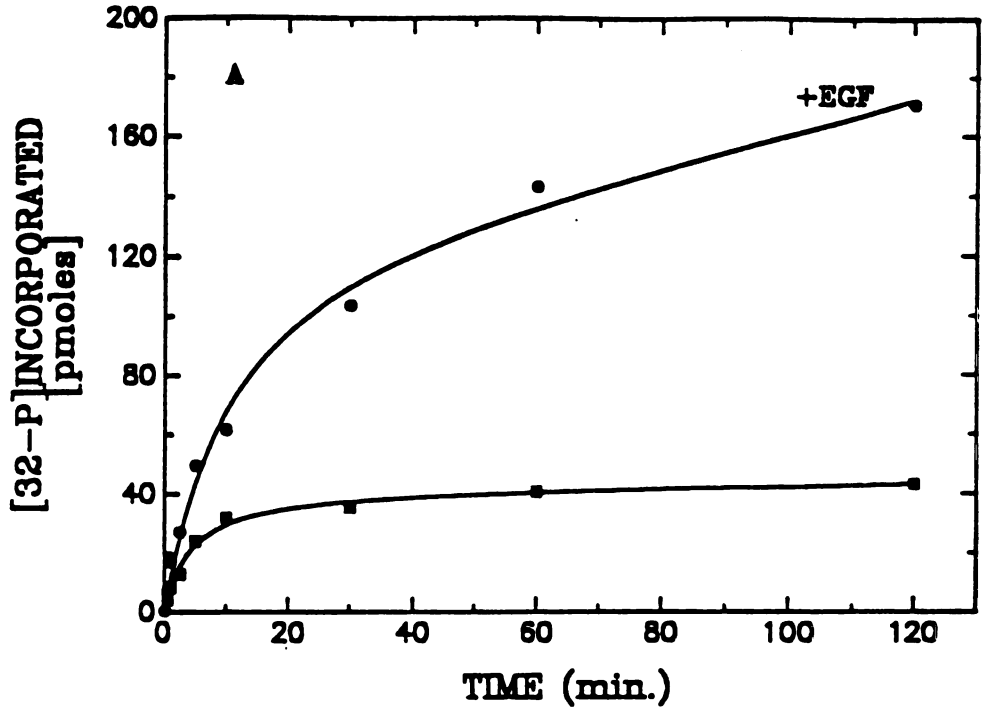
rapid
the
phosp
Appro
EGF-t
While
prese
treat
EGF-s
a sig

Time Course of Membrane Phosphorylation

The incubation of RT-HPM with [γ-^{32}P]ATP resulted in the incorporation of radioactivity into phosphoric acid insoluble material at 0 C and 24 C (Fig. 10). The addition of EGF to the reaction at both temperatures stimulated a 4- to 5-fold increase in the phosphorylation of endogenous membrane-associated proteins. In the presence of EGF, the initial rate of phosphorylation was increased from 24.2 pmole [^{32}P]phosphate per min per mg protein at 0 C to 36.1 pmole [^{32}P]phosphate per min per mg protein at 24 C (Fig. 10A and 10B). While the difference in the phosphorylation between the two temperatures was small, it was stastistically significant ($p < 0.05$). At both temperatures, the addition of EGF to the samples resulted in a stimulation of phosphorylation that reached a maxmimum level by 120 min.

The rate of dephosphorylation of $^{32}PO_4$ from RT-HPM was rapid (Fig. 11). After 40 min at 0 C, approximately 15% of the radioactivity incorporated into membranes in the basal phosphorylation reactions was displaced by the unlabeled ATP. Approximately 25% of the radioactivity was displaced in the EGF-treated membranes incubated at 0 C with unlabeled ATP. While the extent of dephosphorylation was affected by the presence of EGF, the rate of dephosphorylation between the two treatments was kinetically and statisitcally similar. In both EGF-stimulated and basal phosphorylation reactions there was a significant loss in radiolabeled phosphate from RT-HPM due

Figure 10. The incorporation of [^{32}P]-phosphate into RT-HPM from labeled ATP in the presence of EGF. A. Incubations were carried out at 0 C; B. Incubations were carried out at 24 C.


A
+EGF
[32-P]INCORPORATED
[pmoles]
200
160
120
80
40
0
0
20
40
60
80
100
120
TIME (min.)

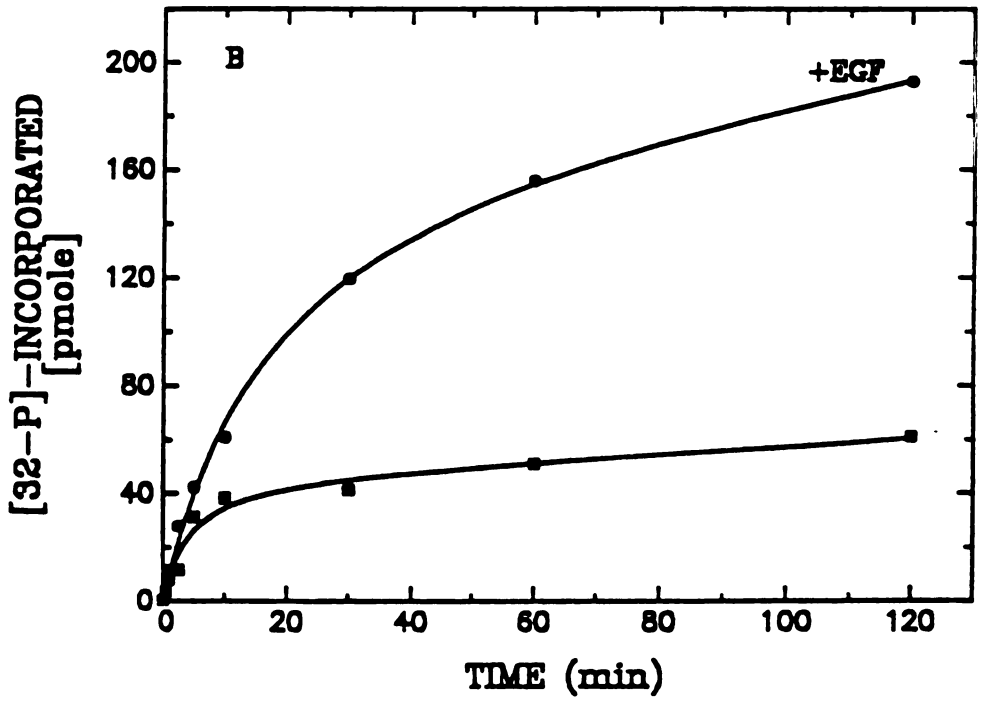
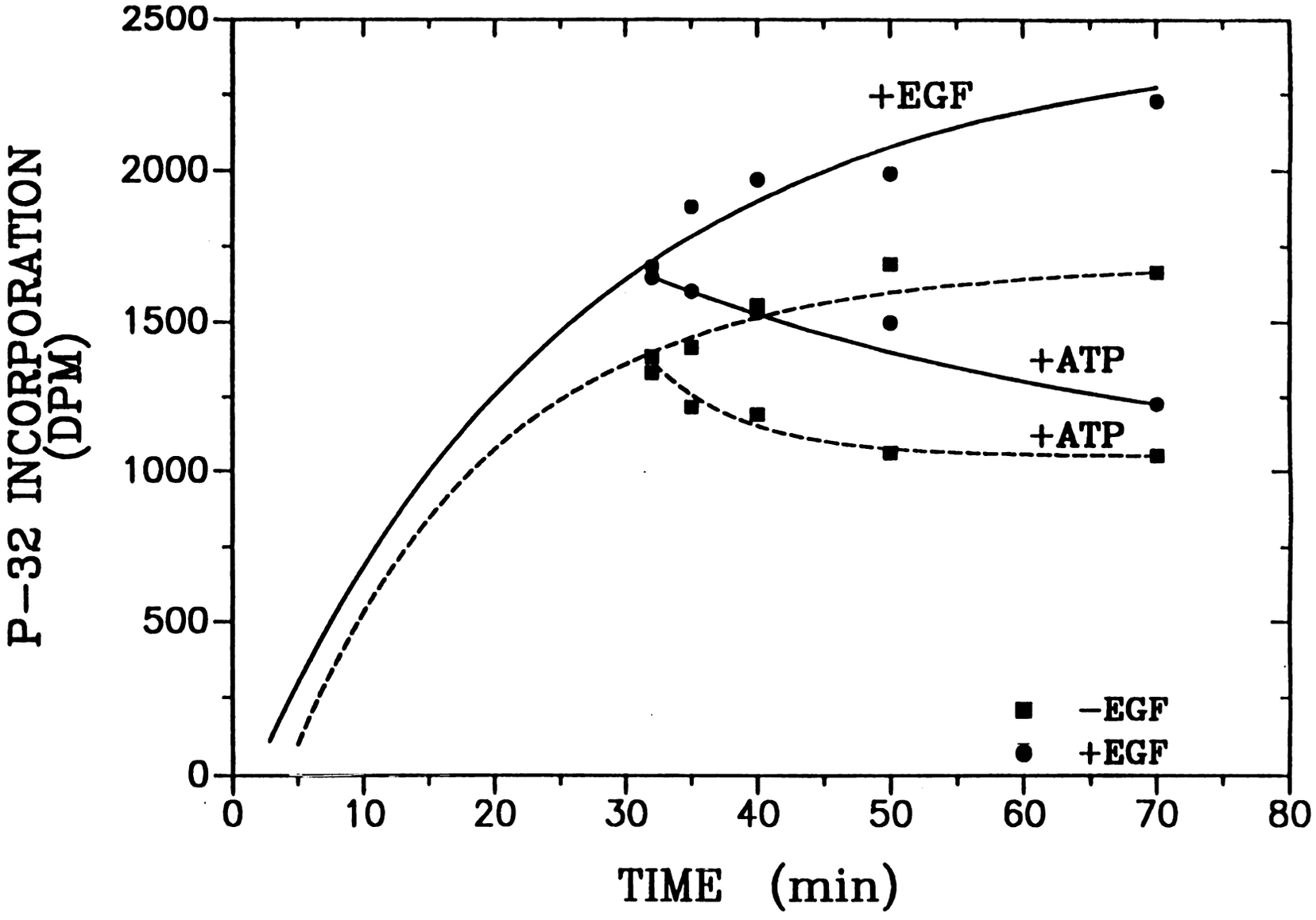

B
+EGF
[32-P]-INCORPORATED
[pmole]
200
160
120
80
40
0
0
20
40
60
80
100
120
TIME (min)

Figure 11. **Time course for membrane dephosphorylation in the presence or absence of EGF. Dephosphorylation was monitored by adding 1.5 mM ATP. At the indicated times, triplicate tubes with and without ATP were removed and the membrane associated ^{32}P was measured.**

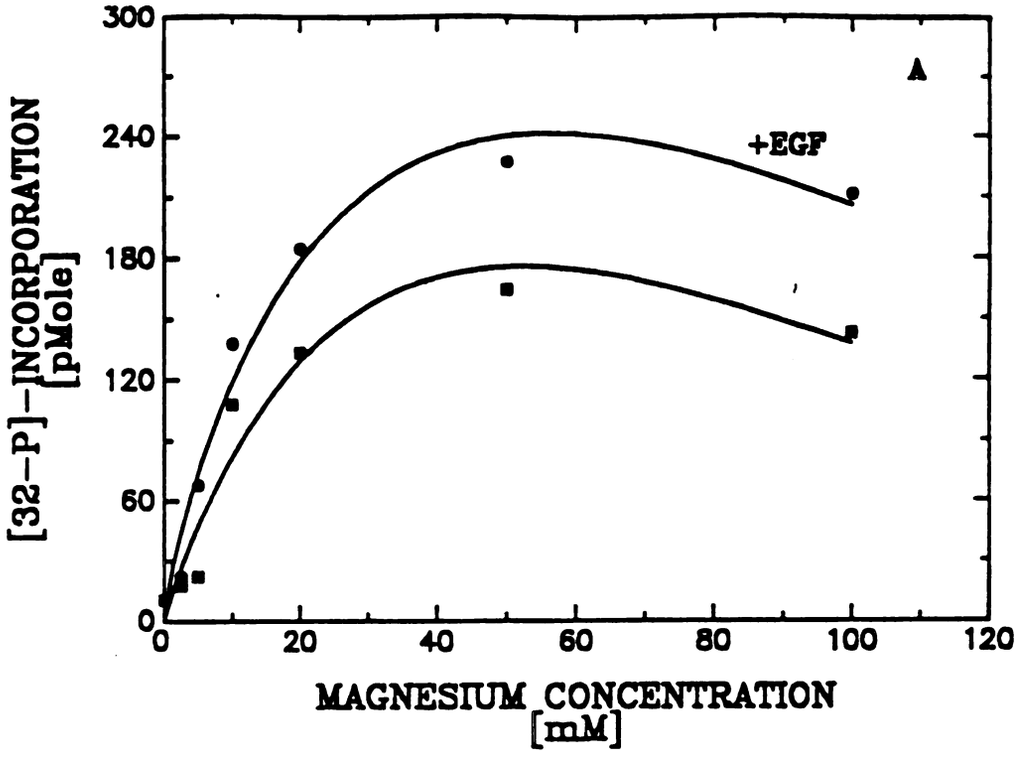

2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
70
80
P-32 INCORPORATION (DPM)
TIME (min)
+EGF
+ATP
+ATP
−EGF
+EGF

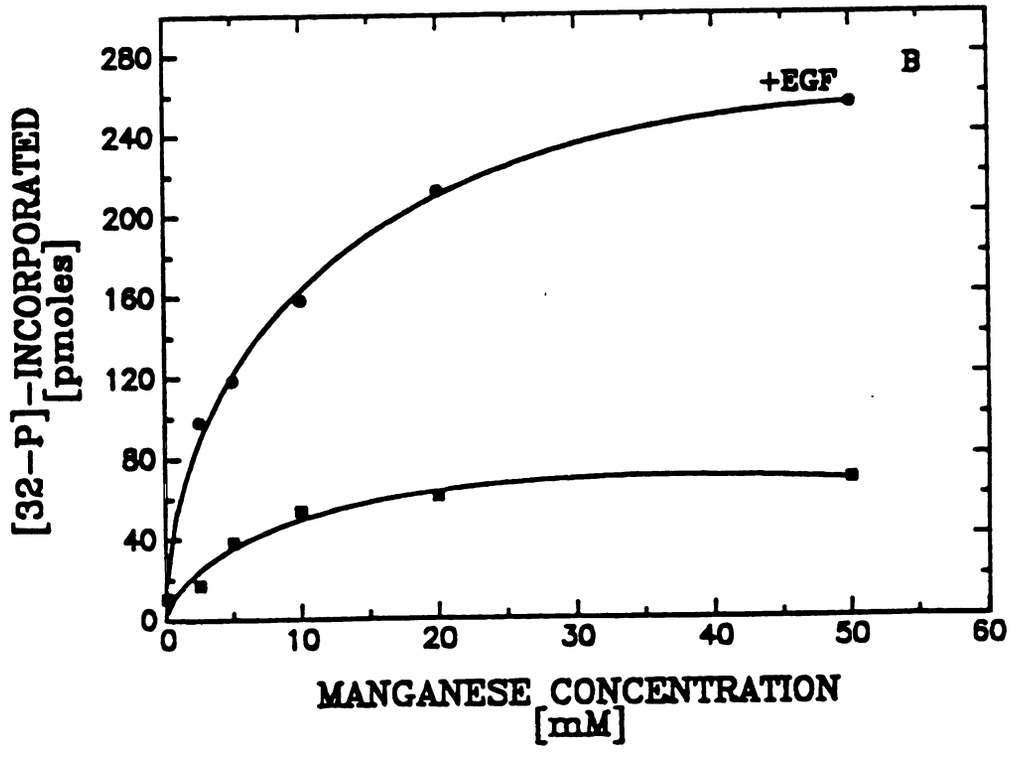
to phosphatase activity. Incubations conducted at low temperatures, 0 to 4 C did not reduce the amount of phosphatase activity. To reduce phosphatase activity in our phosphorylation reaction, we included 100 μM of orthovanadate that is a potent inhibitor of phosphatase activity (Pike, 1987).

Effect of Mg^{2+} and Mn^{2+} on Phosphorylation

EGF-stimulated phosphorylation reactions in RT-HPM require the presence of Mg^{2+} or Mn^{2+} (Fig. 12). In the absence of added metal ions, only trace phosphorylation activity wasobserved in EGF-stimulated RT-HPM. The optimum Mg^{2+} concentration for EGF-induced phosphorylation reactions was approximately 60 mM while for Mn^{2+}it was 30 mM. The stimulatory effect of Mn^{2+} and Mg^{2+} in EGF-mediated-phosphorylations are seen at all concentrations tested. In reactions conducted with Mn^{2+}, the values of basal phosphorylation remained small relative to EGF-stimulated values over the concentration range. This was not seen with Mg^{2+} where the values of basal phosphorylations increased with increasing Mg^{2+} concnetrations relative to EGF-stimulated values. In the presence of 10 mM EDTA, EGF stimulated and basal phosphorylations were completely inhibited. The combined effect of Mg^{2+} and Mn^{2+} on EGF-stimulated phosphorylation was nonadditive with nonEGF-stimulated reactions values remaining constant over the cation concentration range tested. No

Figure 12. Effects of Mg^{2+} and Mn^{2+} concentrations on EGF stimulated-phosphorylation of RT-HPM. Standard assays were carried out at 0 C for 30 min except that the assay buffer did not contain any divalent ions.


300
240
180
120
60
0
0
20
40
60
80
100
120
A
+EGF
[32-P]-INCORPORATION
[pMole]
MAGNESIUM CONCENTRATION
[mM]


280
240
200
160
120
80
40
0
0
10
20
30
40
50
60
B
+EGF
[32-P]-INCORPORATED
[pmoles]
MANGANESE CONCENTRATION
[mM]

effects on EGF-induced phosphorylations were noted with the addition of Ca^{2+} (0.1 to 20 mM), Na^{+} or K^{+} (10 to 200 mM).

Effects of ATP and EGF Concentrations on Phosphorylation

Both nonEGF- and EGF-stimulated phosphorylation reactions were increased at ATP concentrations of 10 to 120 μM (Fig. 13). Subtraction of the nonEGF- from EGF-stimulated RT-HPM reactions showed that EGF-induced a constant 1.25-fold increase in phosphorylation over the ATP concentrations used in this study. Lineweaver-Burk plots of the phosphorylation data exhibited a Km of 1.25 $x10^{-5}$M with a Vmax of 318 $x10^{-9}$M per min in EGF stimulated membranes whereas the nonEGF-treated membranes had a Km of 1.19 $x10^{-5}$M and a Vmax of 273 $x10^{-9}$M per min. This suggests that EGF did not significantly alter the Km of the phosphorylation reaction for ATP but increased the Vmax of the reaction.

The phosphorylation of hepatic plasma membranes of rainbow trout was dependent on the concentration of EGF (Fig. 14). Maximal stimulation of the phosphorylation of RT-HPM occurred at about 22 nM EGF while half maximal stimulation occurred at approximately 4 nM EGF. At 100 nM EGF, there was some inhibition of the phosphorylation of RT-HPM.

The properties of rainbow trout HPM were studied in the presence or absense of EGF by three methods: SDS-acrylamide electrophoresis, autoradiography and western blot analysis (Fig. 15). In lanes E and F, three major phosphorylated

Figure 13. **Effect of ATP concentration on phosphorylation of rainbow trout membranes. ATP was added at the indicated final concentrations with labeled ATP (1 μCi/ tube) and the assays were conducted at 0 C for 30 min.**

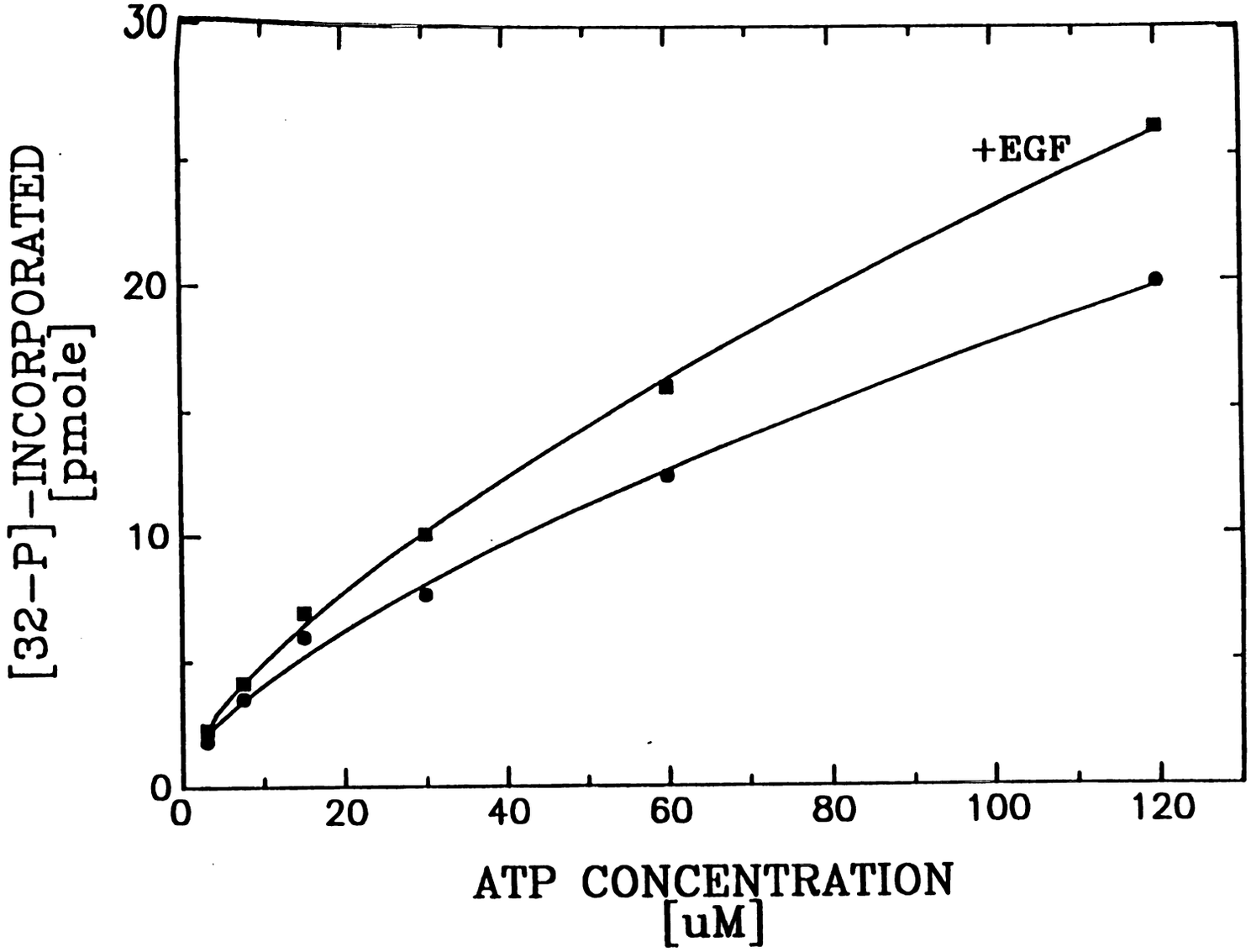

30
20
10
0
0
20
40
60
80
100
120
+EGF
[32-P]-INCORPORATED
[pmole]
ATP CONCENTRATION
[uM]

Figure 14. **Effect of EGF concentration on the phosphorylation of rainbow trout hepatic plasma membranes.**

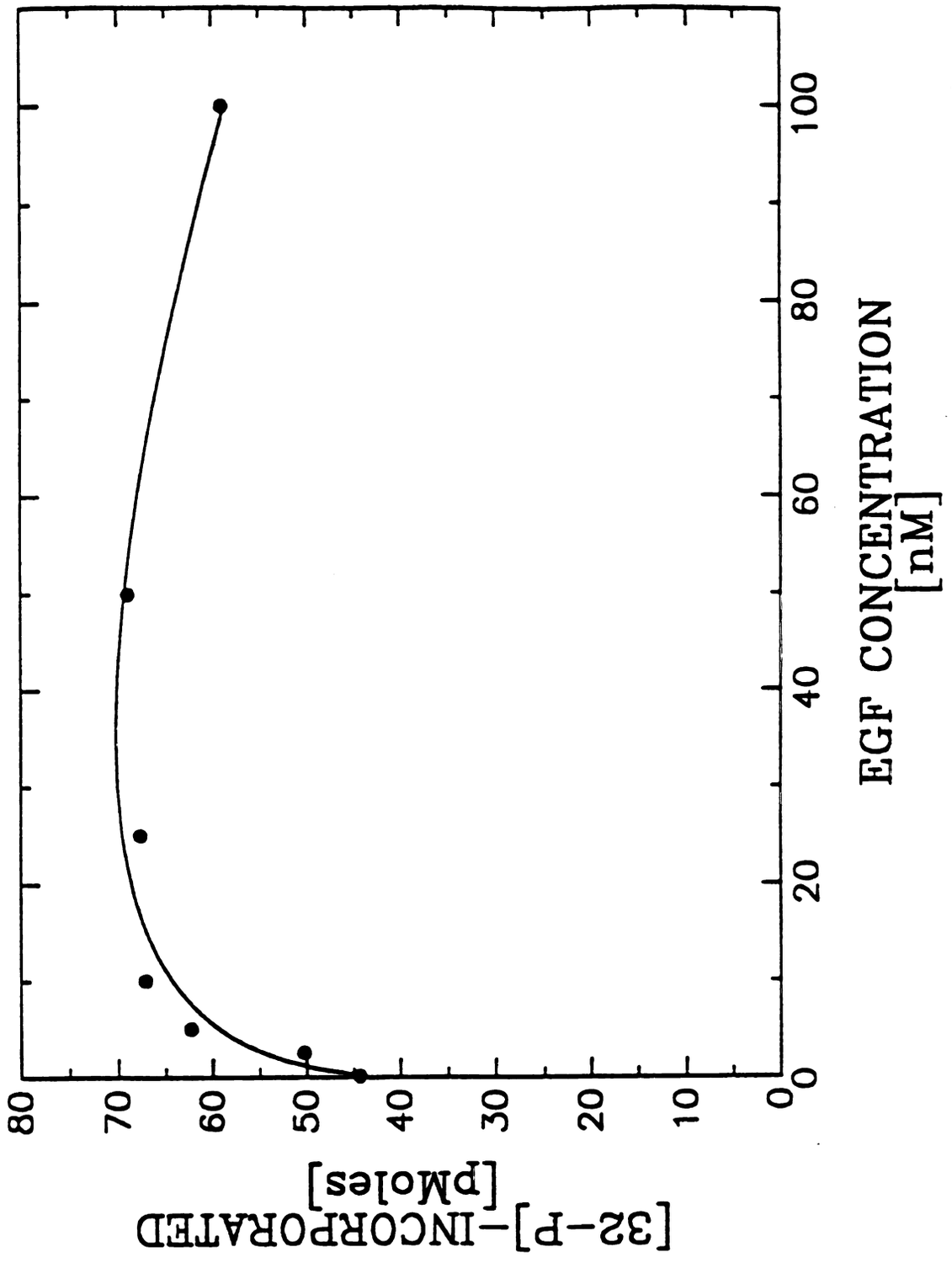
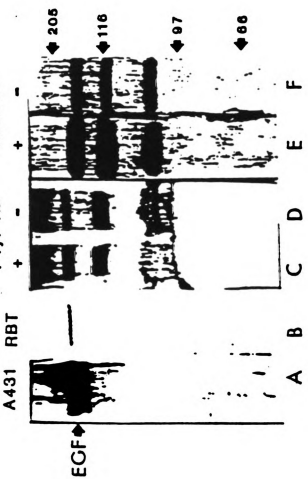

[32-P]-INCORPORATED
[pMoles]
0
10
20
30
40
50
60
70
80
0
20
40
60
80
100
EGF CONCENTRATION
[nM]

Figure 15. **Western and autoradiographic analyses of EGF-stimulated RT-HPM. Membranes were subjected to the standard phosphorylation procedures in the presence and absence of EGF for 30 min at 0 C. Lanes: A. A-431 membranes probed with AB-4 antibody; B. rainbow trout membranes probed with AB-4 antibody; C and D, phosphotyrosine antibody western of rainbow trout membranes phosphorylated with (C) and without (D) EGF; E and F, autoradiography of membrane components phosphorylated in the presence (E) and absence (F) of EGF.**


A431
RBT
EGF
PTyr-AB
Autorad
+
-
+
-
205
116
97
66
A
B
C
D
E
F

bands can be observed that have molecular weights of approximately 150,000 116,000 and 66,000 daltons. No other phosphorylated bands were observed in either lane of the autoradiograph. Westerns of RT-HPM using a phosphotyrosine monoclonal antibody showed a major band at 180,000 daltons with a minor set of doublets at approximately 130,000 and 116,000 daltons. Western analyses conducted on duplicate gels with an EGF-receptor monoclonal antibody (AB-4) exhibited protein bands at 150,000, 116,000, 105,000 and 66,000 daltons. The 180,000 band seen in lanes C and D corresponds to the EGF receptor band observed in lane A for A-431 membranes.

Phosphoamino acid analysis was conducted on the 180,000 protein band to examine further the increase in EGF receptor phosphoamino acid concentration. Results showed that in EGF-stimulated samples there was a greater than 2-fold increase in phosphotyrosine. The level of phosphothreonine and phosphoserine in this band remained relatively constant with only about a 10% increase in their labeled phosphate content.

Effect of Nucleotides on Membrane Phosphorylation

All protein kinase-mediated phosphorylation reactions are dependent upon the presence of triphosphate nucleotides (Cohen et al., 1980). In our assays, the presence of either cyclic AMP or cyclic GMP did not affect the phosphorylation of RT-HPM (Table 4). Also, in the presence or absence of EGF, the addition of cyclic nucleotides, (10^{-3} to 10^{-5} M) did not

Table 4. Competition among nucleosides in the phosphorylation of RT-HPM with labeled triphosphate nucleosides.

Labeled Nucleoside	Unlabeled Nucleoside	^{32}P Incorporated -EGF	+EGF
		DPM	
[gamma-^{32}P]ATP		853	1329
	ATP	693	689
	GTP	857	1003
	cAMP	874	1260
	cGMP	853	1210
[alpha-^{32}P]ATP		1033	1045
	ATP	1319	1300
	GTP	1165	1176
	cAMP	1097	1108
	cGMP	1053	1060

Reaction mixtures were incubated with either [α-^{32}P] or [γ-^{32}P]ATP (30 μM, 1.15 x10^{6} cpm) in the presence or absence of unlabeled nucleosides. Unlabeled nucleosides were added at a 20-fold excess (600 μM) prior to initiating the reactions.

stimulate the phosphorylation activity in RT-HPM preparations. GTP, which in many biochemical reactions can substitute for ATP, also did not alter the phosphorylation of basal or EGF- stimulated rainbow trout membranes.

In mammals, EGF-induced phosphorylation of the EGF receptor and of membrane proteins is dependent upon the presence of [γ-^{32}P]ATP. To examine the specificity of RT-HPM phosphorylation labeled and unlabeled triphosphates were used in a standard assay. Phosphorylation of membrane components by [γ-^{32}P]ATP is significantly reduced by a 20-fold excess of unlabeled ATP (Table 4) while GTP reduced the level of phosphorylation to a lesser extent. [α-^{32}P]ATP was anineffective donor of phosphate groups to RT-HPM either in the presence or absence of EGF.

Hormonal Specificity in Trout Membranes

A wide variety of peptide hormones were used to examine the hormonal specificity of the activation of RT-HPM phosphorylation (Table 5). Basal phosphorylation activity of rainbow trout membranes was not affected by insulin, prolactin, glucagon, FSH, LH and dexamethasone. EGF, and EGF-fragment (EGF_{1-48}) and α-TGF were effective in stimulating the phosphorylation of rainbow trout membranes. These compounds have previously been shown to have biological activity in the activation of phosphorylation of A-431 membranes (Carpenter et al., 1979) as well as having the ability to bind to the EGF

Table 5. Efficacy of hormones to stimulate phosphorylation of RT-HPM.[a]

Hormone	^{32}P-Incorporated (DPM)
None	354 (30)
EGF	672 (110)*
Insulin	345 (15)
Prolactin	346 (98)
Glucagon	313 (51)
FSH	321 (25)
LH	326 (41)
Dexamethasone	329 (46)
TGF-α	394 (35)*
EGF-fragment	452 (41)*

Reactions were carried out at 0°C for 30 min. EGF, EGF-fragment and TGF-α were added at a final concentration of 40 ng/tube. All other hormones were added at 100 ng/tube. Results are reported as averages of triplicate determinations.

[a] Numbers in parentheses are standard deviations

* Significantly different from control at $p < 0.05$.

receptor in RT-HPM (Newsted and Giesy 1991).

Substrate Specificity in Membrane Phosphorylation

The activation an EGF-stimulated tyrosine kinase was examined using several specific and nonspecific kinase substrates (Table 6). RT-HPM readily phosphorylated [Val^5]-angiotensin II, angiotensin II, RR-Src, phosvitin, and to a lesser extent casein and histone. Angiotensin I and the protein kinase C substrate, Ac-MBP, were not significantly phosphorylated in the presence of EGF. In mammalian EGF-receptor systems, [Val^5]-angiotensin II is a better phosphorylation substrate than angiotensin II, however, rainbow trout membrane EGF receptors show no such preference in that the overall extent of phosphorylation of eithersubstrate was similar. Using equimolar concentrations of substrates, the rates of EGF-stimulated phosphorylation of substrate were examined in RT-HPM. Of the substrates tested, RR-Src exhibited the greatest rate of phosphorylation (0.11 pmol $^{32}PO_4$ incorporated/min/mg) while Histone H1 Type III had the least (0.004 pmol $^{32}PO_4$/min/mg protein). The rates of phosphorylation were also similar for [Val^5]-angiotensin II and angiotensin II, 0.58 and 0.61 pmole/min/mg, respectively.

Effect of Kinase Inhibitors on Phosphorylation

To further characterize the phosphorylation activity of RT-HPM, we examined the effect of various kinase inhibitors on

Table 6. Phosphorylation of exogenous proteins by RT-HPM in the presence and absence of EGF[a].

Substrate	Phosphorylation (% of Control)
[Val^5]-Angiotensin II	227 (35)*
Angiotensin II	239 (41)*
Angiotensin I	95 (25)
Histone H1 Type III	105 (11)
Phosvitin	144 (23)*
Casein	122 (13)*
RR-SRC	347 (52)*
PKC Substrate (19-36)	109 (16)

[a] Phosphorylation assays were conducted at 0 C for 30 min. The exogenous protein substrates were added at a final concentration of 100 μM except for phosvitin, histone and casein which were added at a final concentration of 1 mg/ml. The results presented are averages of triplicate determinations (S.D) of percent differences from control values of tubes that did not contain EGF.

* Significance determined at $P < 0.05$.

the ability of RT-HPM to phosphorylate RR-Src in the presence of EGF (Table 7). Of the kinase inhibitors studied, methyl 2,5-dihydroxycinnamate (MDCH), genistein, lavendustin A, and quercitin significantly inhibited the EGF stimulated phosphorylation. Tryphotin, HDAA, PKC inhibitor and ouabain did not significantly inhibit the phosphorylation activity of RT-HPM.

Discussion

The results in this report describe a system where the binding of EGF is accompanied by the alteration of phosphorylation in RT-HPM. These events suggest that in rainbow trout, TCDD-induced alteration of EGF receptor phosphorylation status may have an important role in the modulation of extracellular signals that regulate cell function (Cohen, 1983). Of the other hormonally responsive systems that have been examined in mammals, only the insulin receptor and its associated biological activity have been examined in fish. Thus, this is the first report that examines the EGF receptor and its associated tyrosine kinase activity in rainbow trout.

In previous studies of the binding of EGF (Newsted and Giesy, 1991), we found that RT-HPM membranes bound approximately 250 fmol of ^{125}I-EGF bound/mg protein. This binding value of EGF to RT-HPM is equivalent to EGF binding values in human fibroblasts but is approximately 100-fold less than that observed in A-431 cells (Carpenter et al., 1979).

Table 7. Effect of protein kinase inhibitors on the EGF stimulated phosphorylation of RT-HPM.

Kinase Inhibitor	Concentration (μM)	Kinase Activity (pmol/mg)
Control		47.4 (6.53)
MDHC	50	17.4 (5.13)*
Lavendustin A	50	27.9 (5.74)*
Genistein	50	23.9 (10.4)*
Tyrphotin	50	19.5 (30.1)*
HDAA	50	39.3 (3.96)
PKC Inhibitor	50	40.8 (5.43)
Ouabain	100	64.8 (17.1)
Quercitin	100	4.5 (3.51)*
RCAM	50	10.8 (4.52)*
cAMP Inhibitor	100	49.6 (10.2)

* Significant differences from control values determined at $p < 0.05$.

Binding of ^{125}I-EGF results in the formation of EGF receptor complexes that is accompanied by an 1.5- to 3-fold stimulation in the phosphorylation of RT-HPM (Fig 10, A and B). The increase in EGF-stimulated phosphorylation of RT-HPM was observed by 2 min at 0 c and 24 C. The lack of difference between the EGF stimulated phosphorylation rates in fish membranes at both temperatures is interesting in that the lower temperature reduced the rate and extent of EGF-stimulated phosphorylation of mammal membranes (Carpenter et al., 1979). However, the phosphorylation rate in RT-HPM was slower than that observed in mammals where an increase in EGF-stimulated membrane phosphorylation can be detected in less than 1 min. These results point to a major difference between cold-temperature adapted water fish and mammals in regards to possible membrane composition and its effect on enzyme function at low temperatures.

EGF concentrations required for maximal and half-maximal EGF-stimulated phosphorylation reaction values were approximately half the concentration necessary for maximal and half-maximal binding of EGF to RT-HPM. Furthermore, at 0 C, the binding of EGF to RT-HPM will have reached a steady state under standard binding and phosphorylation conditions. From these data we can conclude that complete saturation of the EGF receptor sites is required for stimulation of phosphorylation activity. The finding in RT-HPM is similar to that observed in A-431 cells where there was a 7-fold difference between

half-maximal stimulation of phosphorylation and half-maximal EGF binding saturation concentration (Carpenter et al., 1979).

EGF-stimulated phosphorylation reactions in RT-HPM were dependent on the presence of Mg^{2+} and Mn^{2+}. This observation is consistent with data from mammalian studies that show Mg^{2+} and Mn^{2+} dependence of the EGF receptor-associated tyrosine kinase activity (Hunter and Cooper, 1985). Furthermore, induction of basal phosphorylation reactions at high Mn^{2+} concentrations was relatively low compared to the EGF-stimulated reactions. This result is consistent with data from mammalian studies that show Mn^{2+} inhibit ATPases and other phosphorylation reactions in EGF-stimulated membranes.

Binding of EGF to RT-HPM was accompanied by the incorporation of phosphotyrosine into a 180,000 dalton protein that corresponded to the same molecular weight as that of the A-431 EGF receptor (Fig. 15). This band was also observed in westerns conducted with the EGF receptor monoclonal antibody (AB-4). While a 180,000 dalton band was not evident in radioactivity incorporated into EGF-stimulated RT-HPM, a 150,000 daltons was observed in the autoradiographs. The dichotomy between these analyses may indicate that there was EGF receptor degradation occurring during our analyses. One explanation for these results is that the phosphotyrosine-antibody may have recognized other phosphotyrosine-containing proteins in our RT-HPM preparation while the AB-4 monoclonal antibody would have recognized only the glycosylated epitope

of the EGF receptor of either intact or partially degraded receptor. The existance of the 180,000 dalton protein band in the phosphotyrosine and EGF receptor antibody westerns supports the receptor degration hypothesis since both 180,000 and 150,000 dalton bands are observed in mammalian EGF receptor where degradation has occurred (Carpenter, 1987).

The activation of tyrosine kinases is important in cell proliferation, carcinogenesis and cell differentiation through mechanisms seeming to involve the control of intracellular signals and protein trafficking (Schlessinger, 1988). A major event in the binding of EGF to its receptor is the autophosphorylation of tyrosine residues and the phosphorylation of cellular proteins. These reactions are carried out by an intrinsic tyrosine kinase located on cytoplasmic domain of the EGF receptor. Examples of *in vivo* EGF receptor tyrosine kinase substrates include: p42; p34-39, the lipocotins or calpactins; $p185^{neu}$, a protein encoded by the *neu* proto-oncogene; and phospholipase C-gamma which is linked to the induction of EGF-mediated phosphatidylinositol turnover (Velu, 1990). In our study, we have reported that EGF stimulated the phosphorylation of various tyrosine kinase substrates in a manner that is similar to that observed in mammals. EGF-stimulated trout membranes readily phosphorylated angiotensin II, [Val^5]-angiotensin II and RR-Src protein while casein and histones were phosphorylated to a much lesser extent. This phosphorylation pattern has also

been described for the rat in a study that compared the phosphorylation of exogenous substrates by insulin and EGF (Klein et al., 1985). In EGF-mediated phosphorylations, the significance of substrate specificity, in vivo or in vitro, in the physiological mediation of the EGF-mediated mitogenic signal is unknown at this time in both mammalian systems and in fish.

To characterize further the EGF-mediated tyrosine kinase activity in rainbow trout membranes, we used specific inhibitors of tyrosine kinase activity as well as inhibitors of other protein kinases. The use of these specific inhibitors provides a valuable tool for the elucidation of the role of EGF-mediated events that involve the biological function of EGF receptor cell proliferation and differentiation. Results from our study show that EGF-mediated phosphorylation was inhibited by specific tyrosine kinase inhibitors. These inhibitors included 4-hydroxycinnamamide derivatives which are competitive inhibitors of substrate binding to the EGF receptor-associated protein tyrosine kinase and Lavendustin A, a derivative from Streptomyces griseolavendus, a competitive inhibitor of ATP binding to the tyrosine kinase. Of the compounds that did not inhibit EGF stimulated phosphorylation, the lack of inhibition by HDAA and tyrphotin is puzzling in that both are potent inhibitors of tyrosine phosphorylation activity. This was especially true of tryphotin which is a specific inhibitor

of EGF-mediated tyrosine kinase activity (Gazit et al., 1989). As expected, inhibitors of protein kinase C, ATPases and of cAMP-dependent kinase did not reduce the EGF-stimulated phosphorylation of RR-Src. Quercitin, a biflavone, is a nonspecific inhibitor that inhibits most protein kinases such A-kinase, C-kinase, tyrosine kinase and casein kinase I and II (Shiaishi et al., 1987). In our assays, quercitin was a potent inhibitor of EGF-stimulated tyrosine kinase activity and protein kinase C activity in RT-HPM. Ouabain, an ATPase inhibitor, stimulated the phosphorylation of RR-Src in EGF-stimulated membranes. This result indicates that ATPases may have a role in mediating the phosphorylation of membrane components such as the EGF receptor in RT-HPM through dephosphorylation of membrane protein or through competition for triphosphate nucleotides such as ATP. The results observed in these assays are similar to those observed in mammals where treatment with tyrosine kinase inhibitors reduce tyrosine kinase activity associated with the EGF-receptor. The reduction in EGF receptor tyrosine kinase activity has been shown to correlate with altered regulation of cell growth and proliferation (Klein et al., 1985).

We have observed differences between mammals and fish relative to EGF-stimulated phosphorylation and EGF-binding in RT-HPM. Since rainbow trout are poikilotherms and evolutionarily divergent from mammals, the role that the EGF receptor might play in cell growth and differentiation is a

question that needs further research. The similarity between the characteristics of binding of EGF and EGF-induced phosphorylation in mammals and rainbow trout indicates that many factors that control cell proliferation and differentiation in mammals may also be present in fish.

REFERENCES

Carpenter,G. (1985). Binding assays for epidermal growth factor. In Methods in Enzymology (L. Birnbaumer and B.W. O'Malley, Eds), Vol 109, pp. 101-115. Academic Press, New York.

Carpenter,G. (1987). Receptors for epidermal growth factor and other polypeptide mitogens. Ann. Rev. Biochem. 56, 881-914.

Carpenter, G. and Cohen, S. (1979). Epidermal growth factor. Ann. Rev. Biochem. 48, 193-216.

Carpenter,G., King,L., Jr. and Cohen,S. (1979). Rapid enhancement of protein phosphorylation in A-431 cell membrane preparations by epidermal growth factor. J. Biol. Chem. 254, 4884-4891.

Cohen,S. (1983). The epidermal growth factor (EGF). Cancer 51, 1787-1791.

Cohen,S., Carpenter,G., and King,L., Jr. (1980). Epidermal growth factor-receptor-protein kinase interactions: co-purification of receptor and epidermal growth factor-enhanced phosphorylation. J. Biol. Chem. 255, 4834-4842.

Gazit,A., Yaish,P., Gilon,C., and Levitzki, A. (1989). Tyrphostins I: Synthesis and biological activity of protein tyrosine kinase inhibitors. J. Med. Chem. 32, 2344-2352.

Gospodarowicz,D. (1981). Epidermal and nerve growth factors in

mammalian development. Ann. Rev. Physiol. 43, 251-263.

Gregoriou, M. and Rees, A.R. (1984). Properties of monoclonal antibody to epidermal growth factor receptor with implications for the mechanism of action of EGF. The EMBO J. 3, 929-937.

Hunter, T. and Cooper, I.A. (1985). Protein tyrosine kinases. Ann. Rev. Biochem. 54, 897-930.

Klein,H.H., Freidenberg,G.R., Cordera,R., and Olefsky,J.M. (1985). Substrate specificities of insulin and epidermal growth factor receptor kinases. Biochem. Biophys. Res. Comm. 127, 254-263.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Margolis,B. Lax,I., Kris,R., Dombalagian,M., Honegger,A.M., Hocuk,R., Givol,D., Ullkrich,A., and Schlessinger,J. (1989). All autophosphorylation sites of epidermal growth factor (EGF) receptor and HER2/neu are locted in their carboxy-terminal tails: identification of a novel site in EGF receptors. J. Biol. Chem. 264, 1-667-10671.

Moolenaar,W.H., Bierman,A.J., Tilly,B.C., Verlaan,I., Honegger,A.M., Ullrich,A., and Schlessinger,J. (1988). A point mutation at the ATP-binding site of the EGF-recpetor abolishes signal transduction. EMBO J. 7, 707-710.

Newsted,J. and Giesy, J. (1991). Characterization of

epidermal growth factor binding to the hepatic plasma membrane of rainbow trout (Oncorhynchus mykiss). Gen. Compara. Endocrin. (in press).

Pike,L. (1983). Assay of growth factor stimulated tyrosine kinases using synthetic peptide substrates. In Methods in Enzymol. Vol. 146, pp. 353-362, Peptide Growth Factors, (D.Barnes and D.A. Sirbasku, Eds), Academic Press, New York.

Ritvos,O., Jalkanen,J., Pekonen,F., Stenman,U.M., and Ranta,T. (1988). Epidermal growth factor but not insulin-like growth factor I potentiates adenosine 3',5'-monophosphate-mediated chorionic gonadotropin secretion by cultures human choriocarcinoma cells. Endocrin. 123, 859-865.

Schlessinger,J. (1988). The epidermal growth factor receptor as a multifunctional allosteric protein. Biochem. 27, 3119-3123.

Schonbrunn,A., Kranshoff,M., Westendorf,J.M., and Tashjian,A.H., Jr. (1980). Epidermal growth factor and thyrotropin-releasing hormone act similarly on a clonal pituitary cell strain. J. Cell. Biol. 85, 786-797.

Shiraishi,T., Domoto,T., Imai,N., Shimada,Y., and Watanabe,K. (1987). Specific inhibitors of tyrosine-specific protein kinase, synthetic 4-hydroxycinnamamide derivatives. Biochem. Biophys. Res. Comm. 147, 322-328.

Steel, R.G. and Torrie, J.H. (1980). Principles and Procedures

in Statistics. 2nd Edition, McGraw-Hill Book Co., New York.

Towbin,H., Staehelin,T., and Gordon,J. (1979). Electrophoretic transfer of proteins form polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350-4354.

Ushiro,H. and Cohen,S. (1986). Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. J. Biol. Chem. 255, 8363-8365.

Velu, T.J. (1990). Structure, function and transforming potential of the epidermal growth factor receptor. Mol. Cell. Endocrinol. 70, 205-216.

CHAPTER 3

EFFECT OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) ON THE EPIDERMAL GROWTH FACTOR RECEPTOR IN HEPATIC PLASMA MEMBRANES OF RAINBOW TROUT

INTRODUCTION

Many species of salmon are very sensitive to halogenated aromatic hydrocarbons (HAHs) such as polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated biphenyls (PCBs), polychlorinated naphthalenes (PCNs), polychlorinated azobenzenes (PCABs) and polychlorinated dibenzofurans (PCDFs) (Kleeman et al., 1988; Kleinow et al., 1987; Dauble et al., 1988). Of the HAHs, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is regarded as the most toxic. Rainbow trout are among the species most sensitive to TCDD with an 80 day LD50 of 10,000 ng/kg body weight (Spitsbergen et al., 1988). TCDD is found as a contaminant in most Great Lakes fish. Fish taken from the most highly contaminated areas average about 0.4 ng TCDD/g body weight (DeVault et al., 1989). Of the HAHs, only PCBs and PCNs have been manufactured commercially while the other HAHs are found as by-products and impurities of the manufacture of other chemical products such as chlorophenols and PCBs (Bowes et al., 1975; Rappe and Buser, 1980). Combustion of chlorinated chemicals of industrial and municipal origin also are a source of HAHs to the Great Lakes (Ballschmitter et al., 1983; Buser, 1979). As a result of their use and misuse, HAHs are found throughout the biotic and

abiotic compartments of most freshwater and marine aquatic ecosystems. Due to their lack of metabolism, long residence time in the environment, their lipophilicity and toxic potency, TCDD as well as the other HAHs pose potentially serious health effects to many fish populations in the Great Lakes.

The administration of HAHs to animals elicits a number of common biologic and toxic responses in a diverse number of species (Poland and Knutson, 1982; Spitsbergen et al., 1988). Exposure to TCDD will result in a variety of species- and tissue-specific effects that include tumor promotion, teratogenicity, immunotoxicity, hepatotoxicity, epithelial hyperplasia and enzyme induction (Safe, 1986). The mechanism of toxic action TCDD and HAH are mediated through an intracellular receptor, the Ah receptor (Poland and Knutson, 1982; Whitlock, 1987). Toxic effects elicited by TCDD and HAHs are thought to be a result of the stereospecific, reversible binding of these compounds to the Ah receptor with a subsequent alteration of gene expression initiated by the ligand-receptor complex. Events known to be mediated through this mechanism include the induction of ornithine decarboxylase, cytochrome P-450 monooxygenases and glutathione S-transferases (Safe, 1986). While induction of enzymes is a though to be a significant event in TCDD exposure, induction alone is not directly related to TCDD's lethal action since the most sensitive mammalian species, the guinea pig, does not

exhibit significant induction mixed-function monooxygenases at concentrations of TCDD which are lethal (Poland and Knutson, 1982). Furthermore, the broad spectrum of biologic and toxic responses observed in different tissues of TCDD exposed animals indicate a more complex mechanism than that of a simple direct-acting toxicity.

Many of the effects of TCDD are comparable to those associated with the modulation of several hormone-mediated responses (Safe, 1986). The fact that TCDD effects are more pronounced in young, growing animals than those observed in mature animals has led to the hypothesis that many of TCDDs toxic effects may be a result of an alteration of the developmental process. This hypothesis is supported by evidence that TCDD and its isomeric analogs cause an alteration in the proliferation and/or differentiation of epithelial cells of the skin, intestine, urinary bladder and thymus (Greenlee et al., 1987). The TCDD-induced effects noted above are consistent with effects induced by the alteration of cell surface structure and function such that there is an alteration of membrane-associated constituents such as enzymes, receptors, ion channels and surface glycoproteins (Madhukar et al., 1988).

In mammals, the alteration of plasma membrane has been shown to be one of the features of TCDD-induced toxicity (Matsumura et al., 1984). In vivo studies have shown that exposure to TCDD results in a reduction in the levels of

insulin, low density lipoprotein, and epidermal growth factor activities as well as a reduction of concanavalin A binding and ATPase activity in the hepatic membranes of the rat. Of these parameters, the reduction in the binding of EGF was the most sensitive. The reduction in the binding of EGF was dose-dependent and compound specific (Madhukar et al., 1984). Along with the reduction in EGF binding, there was an observed increase in protein kinase activities that are known to be important in the modulation of the biological potency of the EGF receptor (Cochet et al., 1984; Downward et al., 1985). Alteration of EGF binding in TCDD-treated animals is believed to be mediated by the Ah receptor since degree of TCDD-induced reduction of the binding of EGF segregated with the presence of the Ah locus.

The significance of the relationship between the alteration in EGF receptor homeostasis and TCDD-induced lesions is the close similarity of the effects induced by both TCDD and EGF in target tissues. For example, young mice treated with either EGF or TCDD induce early eye opening and early tooth eruption (Cohen and Elliot, 1962; Heinberg et al., 1965; Madhukar et al., 1988). Other similar lesions include cleft palate formation, proliferation of gastric and intestinal mucosa, and fatty infiltration of the liver. Finally, both TCDD and EGF cause an increase in the phosphorylation of the EGF receptor and the subsequent activation of intrinsic tyrosine kinase activity (Bombick

1986). Protein tyrosine kinases are involved in the control of normal and abnormal cell growth such as development of embryonic tissues, hepatocarcinogensis, and nonmalignant hyperplastic cell growth (Gentleman et al., 1984; Hunter and Cooper, 1985: Olson 1985).

Pathological lesions observed in rainbow trout which have been treated with TCDD are similar to those observed in mammals (Miller et al., 1973; McConnell, 1980; Spitsbergen et al., 1988). As in mammals, lymphomyeloid and epithelial tissues are the primary sites of TCDD toxicity in fish. Additional evidence for a similar mode of toxic action for TCDD between fish and mammals is the induction of mixed-function oxygenases which are regulated by the Ah receptor (Kleinow et al., 1987; Vodicnik and Lech,1982). The Ah receptor has been identified in rainbow trout liver (Heilmann et al., 1988) and in the rainbow trout hepatoma cell line RTH-149 (Lorezen and Okey, 1990).

This study was undertaken to examine the effect of TCDD on EGF binding in the hepatic membranes of rainbow trout. Specific objectives were to: 1) determine the effect of time and dose on EGF binding to liver plasma membranes; 2) examine the effect of TCDD on protein kinase activities and; 3) examine the relationship between cytochrome P-450 monooxygenase activity and EGF binding in TCDD-treated fish.

MATERIALS AND METHODS

Chemicals. TCDD was purchased from Accustandard Inc. (New Haven, CT). Receptor grade EGF, protein kinase substrates RR-SRC and AC-MBP, and protein kinase C inhibitor (PKC 19-36) were purchased from Gibco-BRL (Grand Island, NY). ^{125}I-EGF (150-200 μC/μg) and $^{32}[\gamma]$P-ATP (3000 Ci/mmol) were purchased from NEN (Boston, MA). All other chemicals were purchased from Sigma (St. Louis, MO). The anti-EGF-receptor mouse monoclonal antibody was purchased from Sigma (F4-clone).

Fish. Sexually immature rainbow trout (Oncorhynchus mykiss) weighing 50-100g were obtained from Stony Creek Trout Farm, Grant MI. Fish were held at 12 ± 1 C in 1,000 liter tanks supplied with a continuous flow of oxygenated, dechlorinated tap water. Fish were fed (silvercup Trout Food) three times weekly at 1% body weight. The photoperiod was 16:8 hr (light:dark).

Treatment of Fish. Fish received a single ip injection (2 ml/kg) of TCDD dissolved in corn oil at doses indicated in the figures and tables. Corn oil was used as the vehicle for all experiments. In general, fish were sampled 7 days post-injection. For time course studies, fish were sampled 2, 5, 10, 20, and 40 days after injection. At sampling, the fish were killed and the liver was immediately excised, weighed and frozen in liquid nitrogen for further in vitro studies.

Plasma Membrane Isolation. Rainbow trout hepatic plasma membranes (RT-HPM) were prepared by the method of Newsted and Giesy (1991). Purified membranes were stored in 50 mM Tris-HCl (pH 7.2) which also contained 0.25 mM sucrose and protease inhibitors (100 kallikrein units/ml aprotinin; 10 μg/ml leupeptin and 10 μM E-64). Aliquants of the membrane preparations were stored under liquid nitrogen (-196 C). Protein analysis was by the method of Lowry et al., (1951) with BSA as the standard.

EGF Binding Assay. Binding assays were carried out by the method of Newsted and Giesy (1991). Results were reported as fmol ^{125}I-EGF bound/mg protein. Binding curves were fitted and the equilibrium dissociation constants (k_D) and the apparent maximum binding capacity (B_{max}) were calculated with the LIGAND program (McPhearson, 1985). The K_D and B_{max} were expressed as nM and fmol of ^{125}I-EGF bound/mg protein, respectively.

Tyrosine Kinase Assay. Tyrosine kinase assays were conducted in microfuge tubes by a modified method of Ribot et al. (1984). Reactions were carried out in 60 μl volumes and contained 50 mM HEPES, pH 7.4, 20 mM Mg^{2+}, 5 mM Mn^{2+}, 100 μM vanadate, 5 μM okadaic acid, 0.05% nonidet NP40, 50 μM [^{32}P] ATP (1000-2000 cpm/pmol) and 20 to 100 μg RT-HPM. To half of the tubes, 120 μM RR-SRC synthetic peptide was added while the other half received water. The assay was initiated by the

addition of [γ-^{32}P]ATP. After incubation for 30 min at 4 C, the reaction was stopped by the addition of 150 μl of 5% trichloroacetic acid and 1.56 mM KH_2PO_4. Subsequently, 10 μl of bovine serum albumin (BSA) was added to aid in protein precipitation, and the samples were incubated on ice for 15 min. The precipitated protein was removed by centrifugation at 5,500xg for 15 min at 4°C. From each tube, two 50 μl aliquots of supernatant were spotted on phosphocellulose filters (Whatman P-81). Phosphocellulose filters were washed four times in 75 mM phosphoric acid (5 min) and once in acetone (2 min). The air dried filters were counted for radioactivity in 7 ml of Safety Solv. Tyrosine kinase activity was expressed as the difference between samples with and without the RR-SRC peptide and normalized for time and protein concentration.

Protein Kinase C Assay. Protein kinase C (PKC) activity was measured by a modification of the procedure of Bell and Sargent (1987). To determine the activity of "particulate" PKC, RT-HPM were extracted in the following manner: RT-HPM were homogenized in 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, and 25 μg aprotinin and leupeptin/ml. Homogenized membranes were incubated for 30 min at 0 C and the cellular debris was removed by centrifugation for 2 min in a microcentrifuge. Assays were conducted with supernatant in microfuge tubes in 50 μl total volume. The diluted enzyme

preparation (20 μl) was assayed at 10 C for 5 min. The reaction mixture contained 20 mM HEPES, pH 7.5, 10 mM MgCl, 1 mM DTT, 20 μM vanadate, 50 μM [γ-^{32}P]ATP (1000-2000 cpm/pmol), 10 μM $CaCl_2$, 120 μM Ac-myelin basic protein (Ac-MBP), 40 μg diolein and 40 μg phosphatidylserine. Before being added to the reaction tubes, equal volumes of diolein and phosphotidylserine in chloroform were combined and the solvent was removed under nitrogen. The residue was then suspended in a small volume of 20 mM HEPES, pH 7.4, by sonication for 5 min at 0 C and the resultant liposome mixture was used in the assay. Basal activity was measured in the presence of 10 mM EGTA instead of $CaCl_2$ and liposomes. The reaction was stopped by spotting 25 μl onto phosphocellulose paper. The filters were washed twice in 150 mM phosphoric acid and then twice in distilled water. The filters were dried and counted in 7 ml Safety Solv. Protein kinase activity was calculated as the difference between the sample that contained EGTA sample without EGTA and expressed as pmol of ^{32}P incorporated per min per mg membrane protein.

Immunoprecipitation. Immunoprecipitation of EGF-related proteins was carried out as outlined by Gill et al. (1984). Plasma membrane protein (100 μg) was phosphorylated *in vitro* using [γ-^{32}P]ATP as outlined in Chapter 2 except that RR-SRC was omitted from the mixture. The phosphorylated membrane protein was solubilized in 1 ml RIPA buffer (10 mM K_2HPO_4, 0.15

M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.01% sodium dodecyl sulfate and 2 mM EDTA) for 1 hr at 0°C. The solubilized material was centrifuged at 12,000xg for 10 min and the supernatant was incubated overnight at 4 C with 10 μl of mouse anti-EGF receptor monoclonal antibody. The immunocomplex was precipitated using goat antibody to mouse immunoglobulin G (IgG) conjugated to Staphylococcus aureus cells (Tachisorb-M, Calbiochem) for 1 h at room temperature with frequent mixing. Samples were centrifuged (2,000xg for 15 min) and the supernatants removed. Pellets were washed twice with 1 ml PBS at 0 C followed by centrifugation. The final pellet was heated for 5 min at 98 C in SDS-Laemmli buffer and the solution was centrifuged to remove particulates. The supernatant was precipitated with 10% TCA, dissolved by 1 M NaOH and counted by liquid scintillation.

Phosphoamino Acid Analysis. Proteins from immunoprecipitation and phosphorylation assays were precipitated by trichloroacetic acid and the precipitate was washed twice with chloroform:methanol (2:1) and once with acetone. The pellet was air dried and hydrolyzed in 6 N HCl at 110 C for 2 h in a sealed Reacti-Vial. The HCl was removed under vacuum and the phosphoamino acids analyzed by thin-layer chromatography by the method of Cooper et al. (1983). The amino acids were visualized with ninhydrin spray, and the radioactivity was measured by scrapping the spots and counting

by liquid scintillation.

MFO Activity Assay. Microsomes from individual fish livers were prepared from post-treated fish by the method of Ankely et al.(1987). 7-Ethoxy O-deethylase (EROD) activity was measured using the fluorometric procedure of Pohl and Fouts (1980).

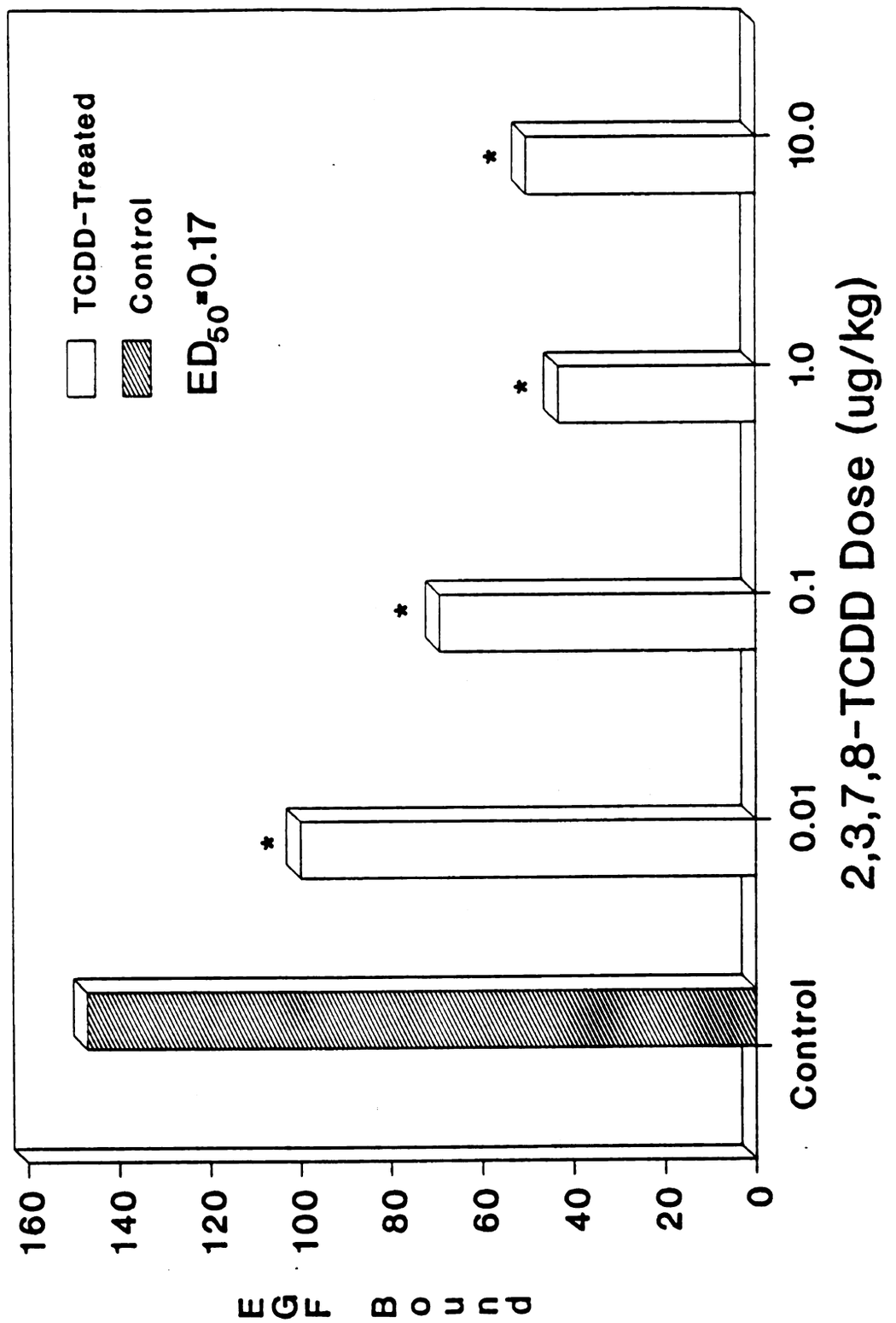
Statistical Analysis. All results are expressed as means ± S.D. for each experimental point. Statistical differences between control and treatment values analyzed by Student's t-test or by analysis of variance. Comparison among means was conducted with a posteriori test, sum of squares simultaneous test procedure (SS-STP) (Steel and Torrie, 1980). Significant differences were established at the $p < 0.05$ level.

RESULTS

Effects on EGF Binding

The effect of TCDD exposure to the EGF receptor was examined in hepatic plasma membranes of rainbow trout. The effect of a single i.p. injection of TCDD significantly decreased EGF binding at all tested concentrations by day 10 (Fig. 16). The reduction in the binding of EGF was maximal at 1.0 μg TCDD/kg body weight (b.w.) and remained unaltered at the 10 μg TCDD/kg b. w. treatment. Probit analysis of the dose-response data computed the effective TCDD dose to reduce

Figure 16. **Dose-response relationship for the binding of ^{125}I-EGF and TCDD exposure in RT-HPM. Liver plasma membrane preparations were made on day 7 post treatment. Binding of EGF to RT-HPM is reported as fmole EGF bound/ mg protein. Each sample point represents the mean of 5 fish. ED50 was calculated by probit analysis.Means that are significant different from control ($p < 0.05$) are designated by *.**


TCDD-Treated
Control
ED_{50}=0.17
EGF Bound
160
140
120
100
80
60
40
20
0
*
*
*
*
Control
0.01
0.1
1.0
10.0
2,3,7,8-TCDD Dose (ug/kg)

the binding of EGF (ED50) to be 0.17 μg TCDD/kg body weight. To study the nature of these changes, Scatchard analyses were conducted on the membrane preparations (Fig. 17) EGF binding generally exhibits a biphasic relationship which is indicative of multiple classes of receptors with differing affinities (Newsted and Giesy 1991). In the TCDD-treated animals, the high and low affinity receptors levels were reduced relative the receptor values seen in RT-HPM from untreated fish. Scatchard analysis of saturation curves showed that the reduction in EGF binding was accompanied without any apparent change in receptor affinity (K_0) of the treated trout membranes compared to control membrane values (Table 8).

To study whether the <u>in vivo</u> reduction in EGF binding wasrelated to general TCDD-induced effects, condition factors and liver somatic indices were calculated for both untreated and TCDD-treated fish. Condition factors were calculated as:

$$CF = \text{final weight}/(\text{length}^3) \times 100\% \qquad (1)$$

while the liver somatic index was calculated as:

$$LSI = (\text{liver weight}/\text{body weight}) \times 100\% \qquad (2)$$

Statistical analysis demonstrated no significant relationship between CF and the binding of EGF in TCDD-exposed rainbow trout ($R^2 = 0.15$) (Fig. 18). In addition, the correlation between the TCDD dose and CF was not statistically significant

Figure 17. **Scatchard analysis of ^{125}I-EGF binding to control (●) and TCDD treated (■) RT-HPM. Each point is the mean of 3 determinations of different membrane preparations.**

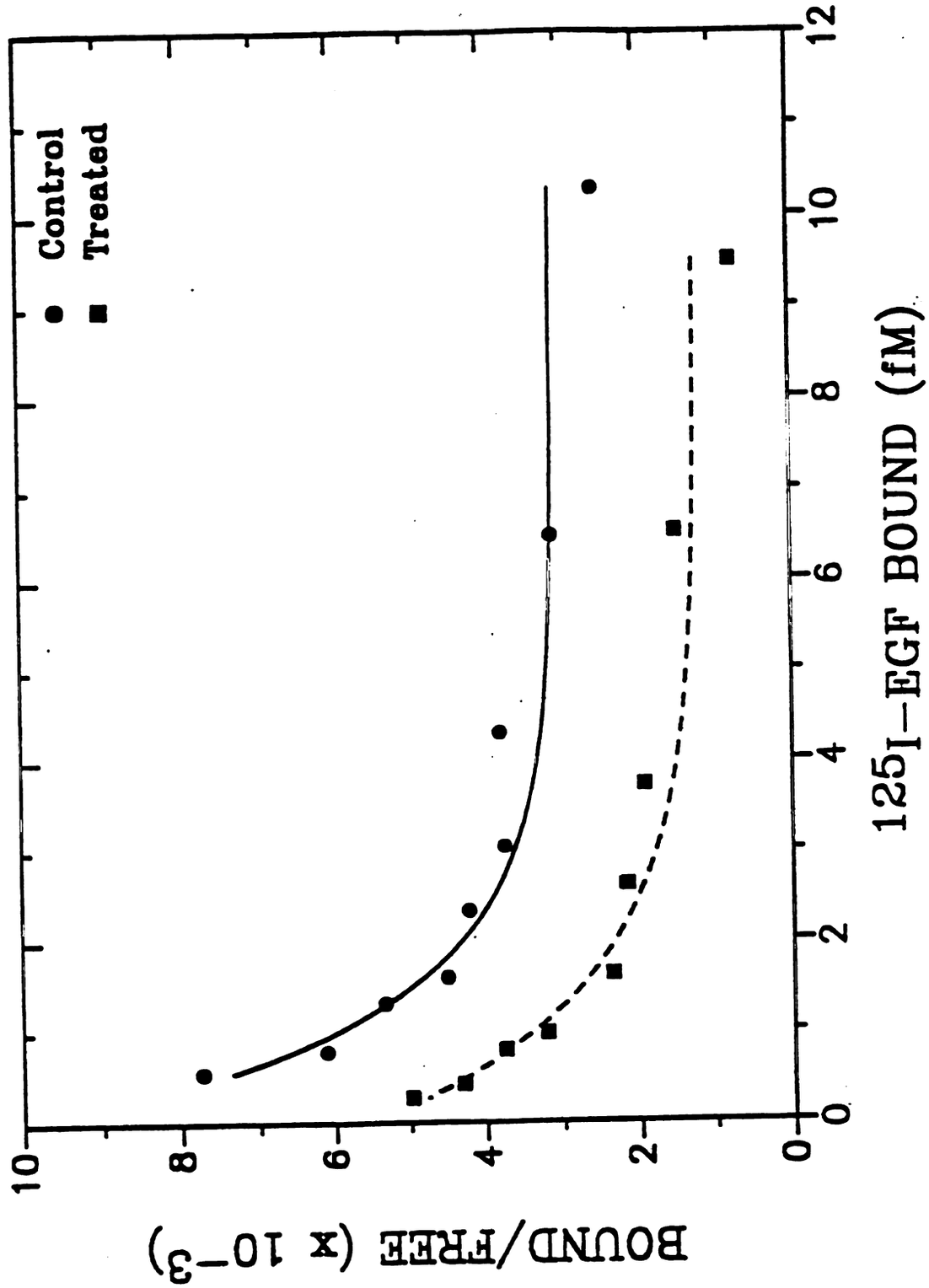

Control
Treated
BOUND/FREE (x 10⁻³)
125I-EGF BOUND (fM)
0
2
4
6
8
10
12

Table 8. Comparison of EGF binding affinities and binding capacities in plasma membranes of control and TCDD-treated rainbow trout.[a]

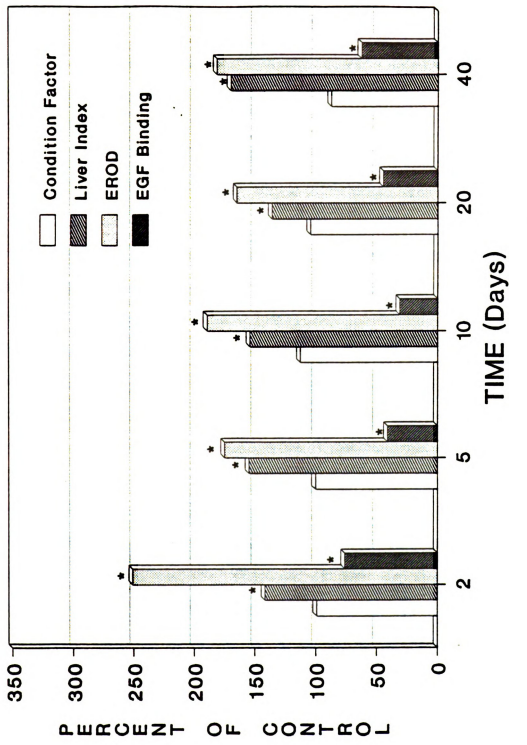
	^{125}I-EGF receptor Parameters			
Treatment[b]	KD_1 nM	KD_2 nM	B_{max1} fmol/mg	B_{max2} fmol/mg
Control	0.20	3.9	16.0	245
5.0	0.11	3.7	2.7*	244
10.0	0.12	3.6	0.9*	172*

[a] KD and Bmax of the high and low affinity EGF-receptor binding sites were analyzed by Scatchard analysis from saturation plots of the binding data.

[b] Injection concentrations were in μg TCDD/kg b.w.

* Significantly different at $p < 0.05$.

Figure 18. **Comparison (as percentages) of EGF binding, EROD activity, condition factor and liver somatic index in TCDD-treated 0(10 μg/kg) and control rainbow trout. Values are expressed as % control activities and are represented as means of 5 determinations. Means within a response that significantly differ from control ($p < 0.05$) are designated by *.**


Condition Factor
Liver Index
EROD
EGF Binding
PERCENT OF CONTROL
0
50
100
150
200
250
300
350
2
5
10
20
40
TIME (Days)

(R^2=0.03). However, the observed negative correlation between LSI vs EGF binding was highly significant (R^2= 0.937). The enlargement of the liver is consistent with previous studies that show a TCDD-induced hyperplastic response in juvenile rainbow trout (Spitsbergen et al., 1988).

The induction of cytochrome P-450 monooxygenase activity (EROD) was used in our study as a biomarker to examine the relationship between exposure to TCDD and the alteration of hepatic function in rainbow trout. EROD was chosen since it has been well established that TCDD induces EROD activity in rainbow trout at the concentrations used in this study (Kleinow et al., 1990). The seven day ED_{50} for EROD activity was 0.66 μg TCDD/kg b.w. Induction of EROD activity was negatively correlated (R^2= 0.608) to EGF binding. EROD activity was significantly elevated from conctrol levels at 0.1 μg TCDD/kg b.w. while the binding of EGF was significantly reduced from control levels at 0.01 μg TCDD/kg.

Time course experiments were conducted to examine further the effect of TCDD on the binding of EGF in rainbow trout plasma membranes (Fig. 18). Over the 40 d observation period, TCDD-treated trout exhibited no significant reduction in or growth as measured by the condition factor. While overall growth of the treated trout was unaffected, there was an increase in weight of the liver, as indexed by LSI, that was significantly greater than controls values. The increase in liver weight was first observed at day 2 and persisted

throughout the experimental period. The increase in LSI values were also associated with an increase in EROD activity. EGF binding was also significantly reduced at day 2 and remained so for the duration of the study. Binding was reduced to its lowest point by day 10 (31% below control). However, by day 20, EGF binding began to return to control values and by Day 40 was approximately 62.5% of the control EGF binding.

To test whether the phenomenon of TCDD-induced reduction in EGF binding could be associated with other toxicants that may induce general membrane alterations, we examined several other compounds that were representative of other contaminant classes (Table 9). Of the compounds tested, only TCDD and the nonortho-substituted PCBs caused a reduction in EGF binding. The doses chosen for this experiment were great relative to the TCDD dose but were based on their ability to induce MFO activity maximally in rainbow trout. No manifestations of toxicities were observed for TCDD or PCBs whereas trout treated with DDT and dieldrin exhibited some toxic symptoms but no mortality.

TCDD Effects on Kinase Activity

TCDD exposure induced alterations in protein kinase activity of RT-HPM (Fig. 19). Significant elevation of all kinase activities in treated RT-HPM was observed by day 5 and reached a maximum by day 20. Kinase activities returned to control values by day 40. This trend was also observed for

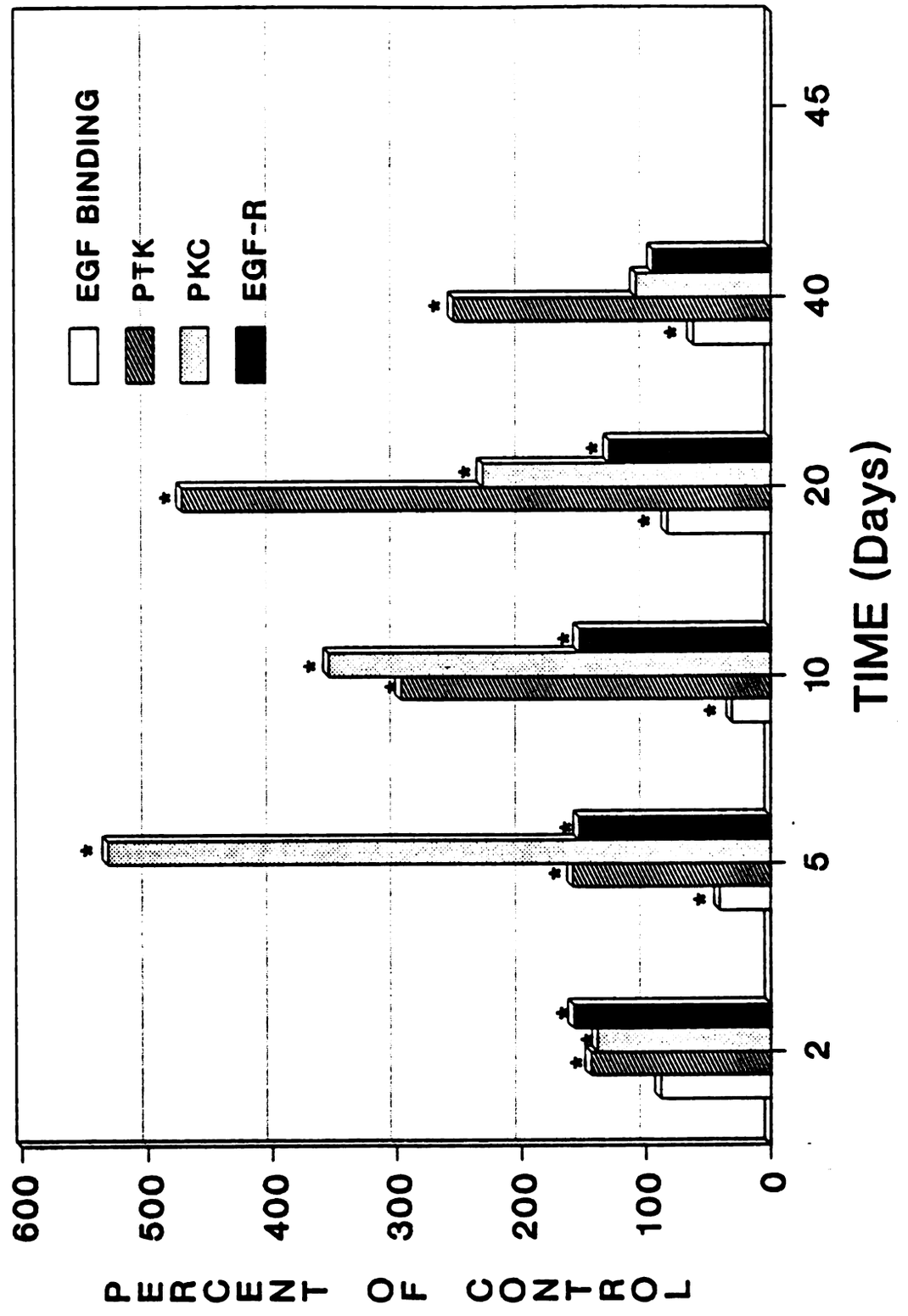
Table 9. Effects on *in vivo* administration of various toxicants on ^{125}I-EGF binding RT-HPM.[a]

Toxicant	Dose	EGF Specific Binding % of Control ± S.D
Control		100.0 ± 33.3
2,3,7,8- TCDD	10 µg/kg	29.4 ± 14.7*
3,3',4,4',5-PCB	100 µg/kg	30.8 ± 3.2*
3,3',4,4'-TCB	500 µg/kg	31.5 ± 17.5*
2,3,3',4,4'-PCB	4.5 mg/kg	68.7 ± 60.3
3,3',4,4',5,5'-HCB	500 µg/kg	31.4 ± 20.0*
DDT	10 mg/kg	95.8 ± 45.0
Dieldrin	10 mg/kg	91.2 ± 32.0

[a] Chemicals were administered in a single i.p. dose. Corn oil served as the vehicle. Fish were sampled on day 7.

* Significant differences were at $p < 0.05$.

Figure 19. **Changes in the binding of EGF, protein kinase C (PKC) and tyrosine kinase (PTK) activities, and EGF-receptor phosphorylation (EGF-R) as a function of time after a single i.p. injection (10 μg TCDD/kg). Results are reported as % control activities and are represented as means of at least 3 determinations. The data was analyzed analysis of variance and Student's T test. Differences (*) between control and treatments were determined at $p < 0.05$.**


EGF BINDING
PTK
PKC
EGF-R
PERCENT OF CONTROL
600
500
400
300
200
100
0
2
5
10
20
40
45
TIME (Days)

EGF-receptor phosphorylation where phosphorylation concentrations were elevated by day 2 and remained elevated through day 40.

Protein kinase C activity was defined as the portion of kinase activity stimulated by calcium and phosphatidylserine. Specificity of the protein kinase C assay in RT-HPM was established by the use of a specific protein kinase inhibitor (PKC 19-36) (Hardie 1988). In TCDD-treated rainbow trout, there was a significant increase in PKC activity by day 5 that reached a maximum level on day 20. However, by day 40 the protein kinase C activities had returned back to control levels. Protein kinase C activity and EGF binding were negatively related in TCDD-treated RT-HPM. As protein kinase C activity increased to a maximum by day 5 in the treated animals, there was a concurrent reduction in EGF binding such that it reached a minimum 10 d after injection with TCDD. When PKC activity returned to control values at day 20, there was an increase in the binding of EGF to control values.

Tyrosine kinase activity in TCDD-treated trout became significantly elevated by day 2, reached a maximum at day 10 and remained elevated through day 40. Examination of the increase in protein tyrosine kinase activity showed that it occurred about 2 days prior to the increase in PKC and the reduction in EGF binding. Studies have shown that ligand activated EGF-receptor has an intrinsic tyrosine kinase activity that is responsible for its biological potency

(Carpenter,1983). Thus, the elevation in tyrosine kinase activity may be linked to an overexpression of EGF receptor activity in TCDD-exposed trout.

To understand better the relationship between changes in protein kinase C and tyrosine kinase activity in the plasma membranes of TCDD-treated trout and the alteration in EGF receptor activity, we examined the phosphorylation of the EGF receptor. The results of the experiments showed that in TCDD treated trout, there was a significant increase in the phosphorylation of the EGF-receptor by day 2. The elevation in the phosphorylation of the receptor remained relatively constant throughout the duration of the study (Fig. 19). To examine further the relationship between the phosphorylation of the EGF receptor and kinase activity, we measured phosphoamino acids in immunoprecipitated EGF-receptor (Fig. 20). At all sampling times, there was an increase in phosphotyrosine and phosphothreonine values of immuno-precipitated EGF receptor protein in TCDD-treated rainbow trout. However, unlike phosphotyrosine or phosphothreonine, phosphoserine values were decreased on day 5 and returned to control by day 40. The increase in phosphothreonine corresponds to the increase in PKC activity over the study period.

TCDD dose-response studies were conducted that showed relationship between alterations in protein kinase activity and EGF binding (Fig. 21). TCDD caused a dose-dependent

Figure 20. ^{32}P incorporation into phosphoamino acids of immunoprecipitated EGF-receptor in a time course study. Trout were given a single i.p. injection (10 μg TCDD/kg) in corn oil. Data are shown as to pmol $^{32}PO_4$ incorporated into immunoprecipitated protein. Each value represents the mean of 3 determinations. Results were analyzed by analysis of variance and by Student's T test. Significant differences between control and treatments (*) were detected at $p < 0.05$. t-test.

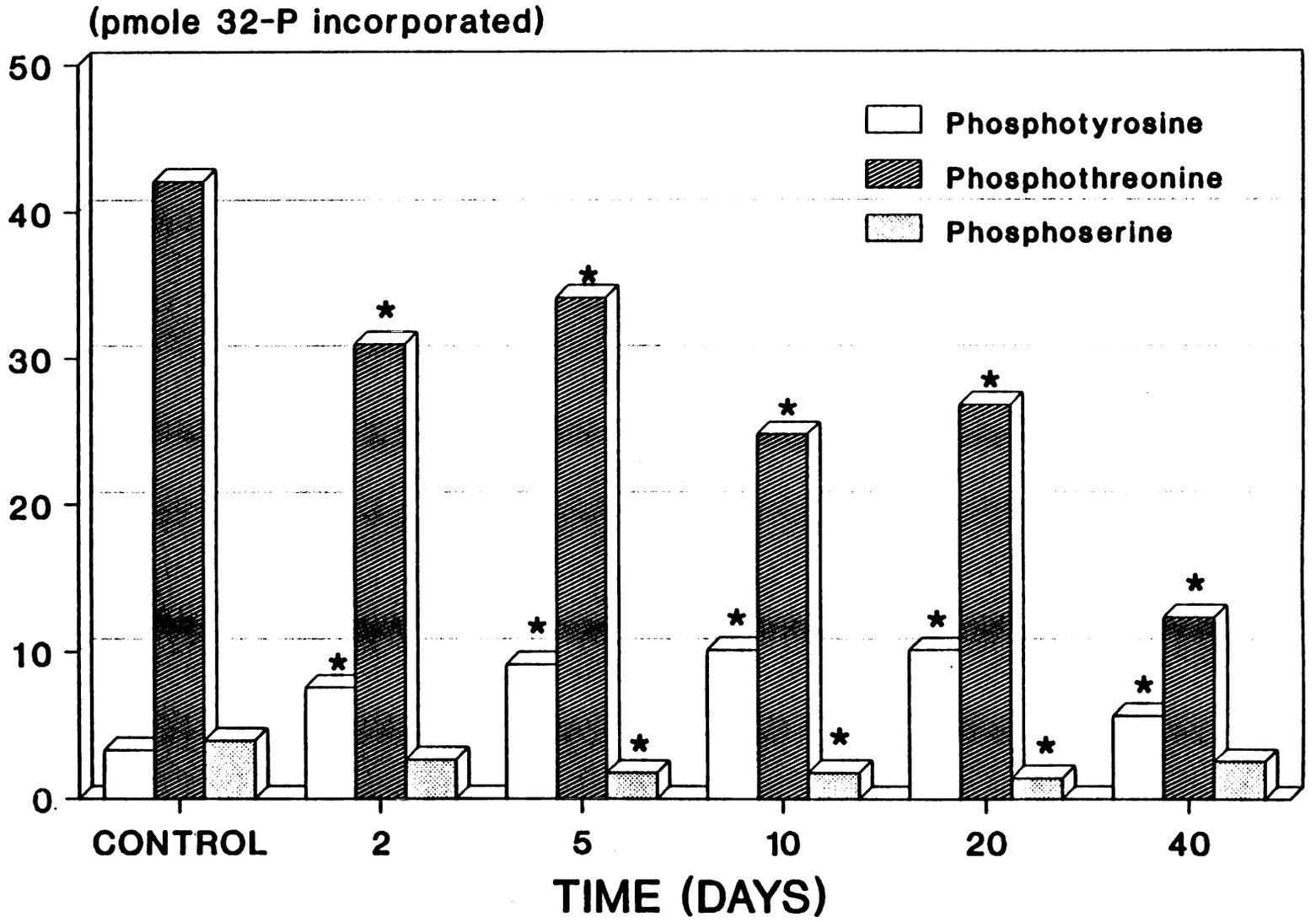
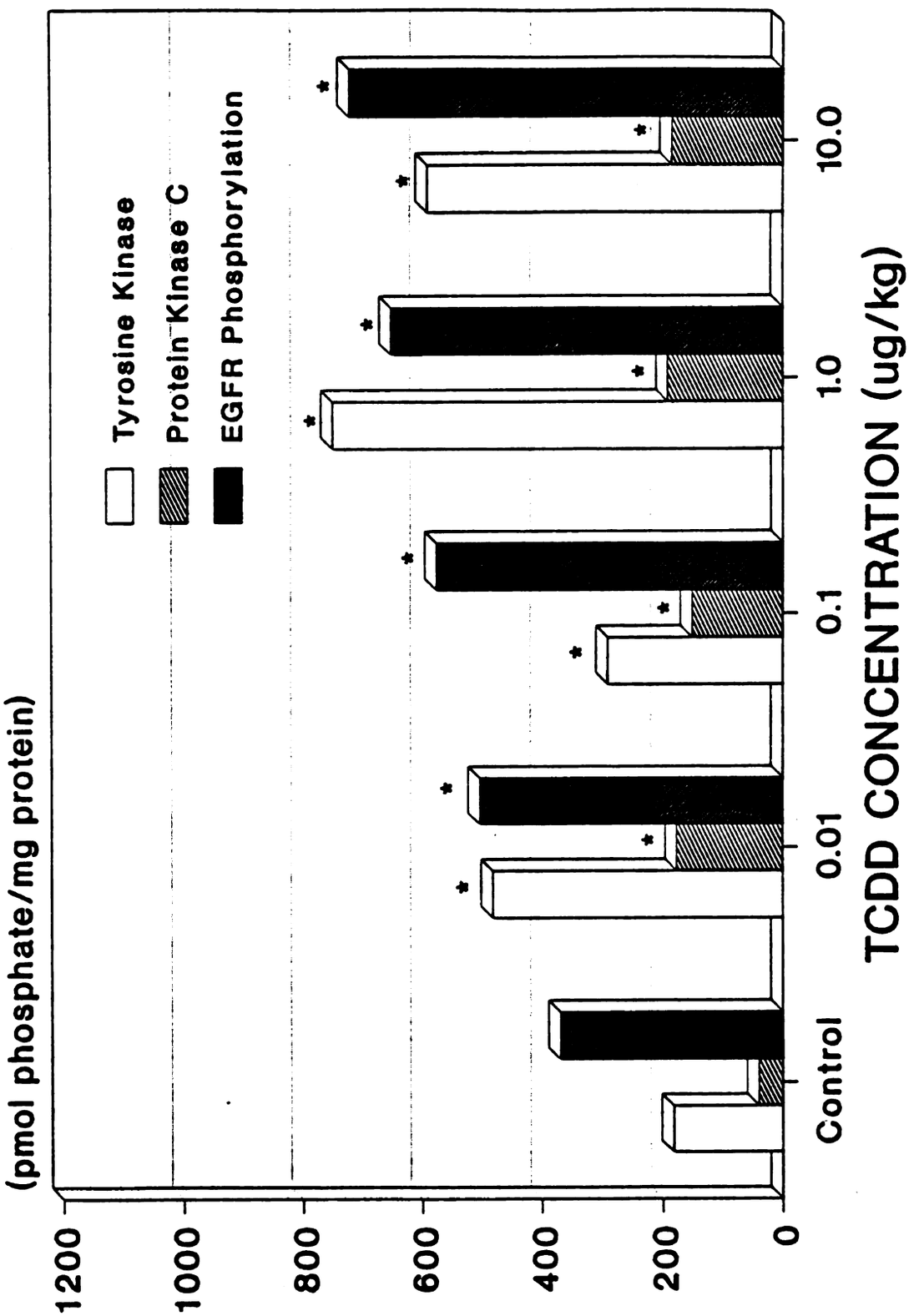

(pmole 32-P incorporated)
50
40
30
20
10
0
Phosphotyrosine
Phosphothreonine
Phosphoserine
CONTROL
2
5
10
20
40
TIME (DAYS)

Figure 21. **Dose-response relationships between TCDD dose, protein kinase activity and EGF-receptor phosphorylation in the hepatic plasma membranes of rainbow trout. Results of protein kinase C and tyrosine kinase assays are reported as pmole $^{32}PO_4$ incorporated per mg membrane protein and EGF-receptor phosphorylation was reported pmol phosphate incorporated per mg protein of original starting membrane protein. Each point represents the mean of 3 determinations. Differences between control and treatment values were determined at $p < 0.05$.**


(pmol phosphate/mg protein)
1200
1000
800
600
400
200
0
Tyrosine Kinase
Protein Kinase C
EGFR Phosphorylation
Control
0.01
0.1
1.0
10.0
TCDD CONCENTRATION (ug/kg)

increase in protein kinase C and tyrosine kinase activity that also included an increase in EGF-receptor phosphorylation over control values. These trends are opposite of that observed in the EGF binding assays where the increases in PKC (R^2=0.997), tyrosine kinase (R^2=0.789) and EGF-receptor phosphorylation (R^2= 0.963) were negatively correlated to EGF binding.

The relationship between protein kinase activity and the EGF-receptor phosphorylation state was examined further in the dose-response study. Correlation analysis of the results show that there was a positive correlation between PKC and tyrsoine kinase activity (R^2= 0.715) and PKC and EGF receptor phosphorylation (R^2=0.609). These data are consistent with the hypothesis that kinase activity regulates the phosphorylation of the EGF-receptor in TCDD toxicity. Phosphoamino acid analysis of the receptor showed that phosphotyrosine was increased approximately 2.5 times over control levels while phosphoserine and phosphothreonine increase approximately 2 and 6 times over control values, respectively, for the dosage regime. Statistical analysis showed a positive relationship for phosphotyrosine concentration and protein tyrosine kinase activity as well as between phosphoserine and phosphothreonine concentrations and PKC activity. However, no significant correlations were found between tyrosine kinase activity and EGF-receptor phospho-serine or phosphothreonine levels nor was there a significant relationship between PKC and tyrosine kinase activities.

DISCUSSION

The EGF receptor is a transmembrane glycoprotein widely distributed in many tissues (Adamson and Rees 1981). This receptor is unique among many other receptor types because it contains an intrinsic tyrosine kinase activity that is responsible for its biological activity. The EGF receptor has been shown to be involved in embryonic development and cellular repair processes as well as in neoplastic cellular growth responses (Carpenter 1983; Hunter 1984). Expression of the biological activity of the EGF receptor is believed to be under the control of tyrosine kinases which act to stimulate the activity of the receptor through the phosphorylation of tyrosine residues of the receptor. Activity of the EGF receptor can be reduced by PKC through the phosphorylation of serine and threonine residues. The phosphorylation of the EGF receptor by PKC can result in the reduction in the binding of EGF and in the tyrosine kinase activity associated with the receptor (Cochet et al., 1984; Downward et al., 1985). Alteration of gene function has been demonstrated in TCDD-treated female rats where TCDD induced both a reduction of EGF binding and a parallel decrease in steady-state levels of uterine EGF receptor mRNA (Astroff et al., 1990) Thus, the control of cell surface EGF receptor levels and of their biological potency are under biochemical and genetic control. Alteration of either of these mechanisms may result in the

alteration of cell processes.

In these studies we have examined the relationship between TCDD administered *in vivo* and alterations of EGF receptors in the plasma membranes of rainbow trout. These data agree with results observed in mammals in that TCDD caused a reduction in the binding of EGF as well as an increase in protein kinase activity in hepatic plasma membranes (Matsumura et al., 1984). As seen in mammalian studies, reduction in EGF binding in rainbow trout hepatic membranes was accompanied by an increase in tyrosine kinase activity and the phosphorylation of the EGF receptor. Although no definite cause-effect can be proven by this data, the decrease in the binding of EGF may be due in part to increased tyrosine kinase activity that phosphorylates tyrosine residues of the EGF receptor. Data to support this hypothesis is the increased phosphotyrosine concentration of the EGF receptor in RT-HPM of TCDD-exposed rainbow trout. However, while the reduction of EGF binding was correlated to the increase in tyrosine kinase activity and the increase in EGF receptor phosphorylation, there was a less significant relationship between tyrosine kinase activity and EGF receptor phosphorylation. This suggests that other kinases may be involved in the regulation of EGF binding.

Other classes of toxic agents have been shown to reduce EGF binding in a manner similar to that observed in TCDD exposures. Examples of these agents include oncogenic

retroviruses such as v-src (Erikson et la., 1981), hormones such as thyroid hormone T3 (Hayden and Severson 1983), and tumor promoters such as phorbol esters (Lee and Weinstein 1979). While the mode of action of these agents are not similar, many of their effects are believed to be mediated through protein kinase C. An example would be 12-O-tetradecanoyl-phorbol-13-acetate (TPA), where the activation of protein kinase C results in the subsequent phosphorylation of serine and threonine residues and results in the down regulation of EGF receptors (Lee and Weinstein 1978). Increased PKC activity is also related to the alteration of the phosphoinositol cycle, calcium mobilization, induction of ornithine decarboxylase, enhancement of glucose transport and the stimulation of mitogenic activity (Takai et al., 1985).

In this study, we observed a dose and time dependent increase in PKC that was associated with a reduction in the binding of EGF in RT-HPM. The increase in PKC activity was also correlated with an increase in EGF receptor phosphorylation an in EGF receptor phosphothreonine concentration. The observed increase in protein kinase C activity occcured at approximately the sampling time as that observed for tyrosine kinase activity (Fig. 19). This differs from observation of TCDD-exposed mammals where induction of tyrosine kinase activity preceeds the induction of protein kinase C (Bombick, 1986). Thus, in rainbow trout, the activation of protein kinase C and tyrosine kinase by TCDD

may be mediated through separate biochemical mechanisms that result in the phosphorylation of the EGF receptor and the subsequent reduction in the binding of EGF in RT-HPM. Our results support this hypothesis since the increase in EGF receptor phosphoamino acid concentration is related to elevations in PKC and tyrosine kinase activities in TCDD-exposed RT-HPM.

Chemical class and toxicant structure were closely associated to their ability to reduced the binding of EGF in RT-HPM and to their ability to induce cytochrome P-450 in rainbow trout (Kleinow et al., 1987). DDT, dieldrin and PCB 105 did not significantly reduce EGF binding in our study nor were they potent inducers of cytochrome P-450 monooxygenase activity in rainbow trout microsomes. This finding supports the hypothesis that TCDD toxicity is mediated through the Ah receptor and that those species with low levels of activated, nuclear Ah receptors are less sensitive to TCDD-induced toxic effects (Whitlock, 1987). The significance of the relationship between Ah responsiveness and TCDD toxicity in rainbow trout is unknown since there is a lack of information on the biochemical and physiological characteristics Ah receptor in rainbow trout. However, TCDD induces similar enzymatic effects in rainbow trout such as mixed function oxygenase induction and glutathione S-transferase induction that are known to mediated through the Ah receptor in mammals. The similarity in enzymatic response implies a common mode of

toxic action between mammals and rainbow trout exposed to TCDD.

In conclusion, we have shown that a single dose of TCDD results in a significant alteration in plasma membrane function. In particular, there is a decrease in EGF binding accompanied by increases in various protein kinase activities. While the significance of these effects are still unknown in fish, it is important to note the strong comparison between these changes and those seen in mammalian studies. However, one must be careful in relating the alterations of EGF binding and phosphorylation to *in vivo* lesions and symptoms since the role of the EGF-receptor and kinase activity still has to be established in rainbow trout.

REFERENCES

Adamson, E.D. and Rees, A.R. (1981). Epidermal growth factors. Mol. Cell. Biochem. 34, 129-152.

Ankely, G.T., Blaser,V.S., Reinert,R.E., and Agosin,M. (1987). Metabolism of alkoxyphenoxanzones by channel catfish liver microsomes: effects of phenobarbital, Aroclor 1254, and 3-methylcholanthrene. Biochem. Pharmacol. 36, 1379-1381.

Astroff,B., Rowlands,C., Dickerson,R., and Safe,S. (1990). 2,3,7,8-tetrachlorodibenzo-p-dioxin inhibition of 17B-estradiol-induced increases in rat uterine EGF-receptor binding activity and gene expression. Mol. Cell. Endocrin. 72, 247-252.

Ballschmiter,K., Braumiller,I., Niemczyk,R. and Swerev,M. (1988). Reaction pathways for the formation of polychlorinated dibenzo-p-dioxins and dibenzofurans in combustion processes. II. Chlorobenzenes and chlorophenols as precursors in the formation of polychlorodibenzo-p-dioxins-dibenzofurans in flame chemistry. Chemosphere 17, 995-1001

Bell,M.V. and Sargent,J.R. (1987). Protein kinase C activity in the spleen of trout (Salmo gaidneri) and the rectal gland of dogfish (Scyliorhinus canicula) and the effects of phosphatidylserine and diacylglycerol containing (n-3) polyunsaturated fatty acids. Comp. Biochem. Physiol. 87B,

875-880.

Bombick,D.W. (1986). Initial biochemical alterations involved in 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity. PhD Dissertation, Michigan State University, East Lansing, MI.

Bowes, G.W., Mulvihill,M.J., Simoneit, B.R.T., Burlingame, A.L., and Risenbrough, R.W. (1975). Identification of chlorinated dibenzofurans in American polychlorinated biphenyls. Nature 256, 305-307.

Buser, H.R. (1979). Formation of polychlorinated dibenzofurans and dibenzo-p-dioxins from pyrolysis of chlorobenzenes. Chemosphere 8, 415-424.

Carpenter,G. (1983). The biochemistry and physiology of the receptor kinase for epidermal growth factor. Mol. Cell. Endocrin. 31, 1-19.

Cochet,C., Gill,G.N., Meisenhelder,J., Cooper,J.A., and Hunter,T. (1984). C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor stimulated tyrosine protein kinase activity. J. Biol. Chem. 259, 2553-2558.

Cohen, S. and Elliott,G.A. (1962). The stimulation of epidermal keratinization by a protein isolated from the submaxillary gland of the mouse. J. Invest. Dermatol. 40, 1-5.

Cooper,J.A., Sefton,B.M., and Hunter,T. (1983). Detection and quantification of phosphotyrosine in proteins. In Hormone

Action: Part F Protein kinases, (J.D. Corbin and J.G. Hardman) Methods in Enzymology 99, 387-402.

Dauble,D.D., Dankovic,D.A., Abernathy,C.S. and Grey, R.H. (1988). Induction of hepatic aryl hydrocarbon hydroxylase activity in rainbow trout (Salmo gairdneri) exposed to coal liquids. Comp. Biochem. Physiol. 91C, 431-435.

DeVault,D., Dunn,E., Bergquist,P., Wiberg,K., and Rappe,C. (1989). Polychlorinated dibenzofurans, and polychlorinated dibenzo-p-dioxins in Great Lake fish: a baseline and interlake comparison. Environ. Toxicol. Chem. 8, 1013-1022.

Downward,J., Waterfield,M.D., and Parker,P.J. (1985). Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. J. Biol. Chem. 260, 14538-14546.

Erickson,E., Shealy,P., and Erikson,R.L. (1981). Evidence that viral transforming gene products and epidermal growth factor stimulate phosphorylation of the same cellular protein with the same specificity. J. Biol. Chem. 256, 716-722.

Gentleman,S., Martensson,T.M., Digiovanna,J.T., and Chader,G.J. (1984). Protein tyrosine kinase and protein phosphotyrosine phosphatase in normal and psoriatic skin. Biochem. Biophys. Acta. 798, 53-59.

Gill,G., Kawamoto,T., Le,A.D., Mendelsohn,J., Sato,G.H., and Mendelsohn,J. (1984). Monoclonal anti-epidermal growth

factor receptor antibodies which are inhibitors of epidermal factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein kinase activity. J. Biol. Chem. 259, 7755-7760.

Greenlee, W.F., Osborne,R., Dold,K.M. Hudson,L.G., Young,M.J., and Toscano,W.A. Jr. (1987). Altered regulation of epidermal cell proliferation and differentiation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In: Reviews in Biochemical Toxicology Vol. 8,(J.R. Bend, and R.M. Philpot, Eds.), pp. 1-35, Elsevier, New York.

Hardie, G. (1988). Pseudosubstrates turn off protein kinases. Nature 355, 592-593.

Hayden,L.J., and Severson, D.L. (1983). Correlation of membrane phosphorylation and epidermal growth factor binding to hepatic membranes isolated from triiodothyronine-treated rats. Biochim. Biophys. Acta 730, 226-230.

Heilmann,L.J., Sheen,Y.Y., Bigelow,S.W., and Nebert,D.W. (1988). Trout P450IAI:cDNA and deduced protein sequence, expression in liver, and evolutionary significance. DNA 7, 379-387.

Heinberg,M., Weinstein,I., LeQuire,V.S., and Cohen,S. (1965). The induction of fatty liver in neonatal animals. Life Sci. 4, 1625-1633.

Hunter,T. (1984). The proteins of oncogenes. Sci. American 251, 60-69.

Hunter,T. and Cooper,I.A. (1985). Protein tyrosine kinases. Ann. Rev. Biochem. 54, 897-930.

Kleeman,J.M., Olson,J.R., and Peterson,R.E. (1988). Species differences in 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity and biotransformation in fish. Fundam. Appl. Toxicol. 10, 206-213.

Kleinow,K.M., Melancon,M.J., and Lech,J.J. (1987). Biotransformation and implications for toxicity, bioaccumulation and monitoring of environmental xenobiotics in fish. Environ. Health Perspect. 71, 105-119.

Lee,L.S., and Weinstein, I.B. (1978). Tumor-promoting phorbol esters inhibit binding of epidermal growth factor to cellular receptors. Science 202, 313-315.

Lee, L.S., and Weinstein, I.B. (1979). Mechanism of tumor inhibition of cellular binding of epidermal growth factor. Proc. Natl. Acad. Sci. USA 76, 5168-5172.

Lorenzen,A. and Okey,A.B. (1990). Detection and characterization of [^{3}H]2,3,7,8-tetrachlorodibenzo-p-dioxin binding to Ah receptor in a rainbow trout hepatoma cell line. Toxicol. Appl. Pharmacol. 106, 53-62.

Lowry,O.H., Rosenbrough,N.D., Farr,A.L., and Randall,R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Madhukar,B.V., Brewster,D.W., and Matsumura,F. (1984). Effects of in vivo administered 2,3,7,8-tetrachlorodibenzo-p-

dioxin on receptor binding of EGF in the hepatic membrane of rat, guinea pig, mouse, and hamster. Proc. Natl. Acad. Sci. USA. 81, 7407-7411.

Madhukar,B.V., Ebner,K., Matsumura,F., Bombick,D.W., Brewster,D.W. and Kawamoto,T. (1988). 2,3,7,8-tetrachlorodibenzo-p-dioxin causes an increase in protein kinases associated with epidermal growth factor receptor in the hepatic plasma membrane. J. Biochem. Toxicol. 3, 261-277.

Matsumura,F., Brewster,D.W., Madhukar,B.V., and Bombick,D.W. (1984). Alteration of rat hepatic plasma membrane functions by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Arch. Environ. Contam. Toxicol. 13, 509-515.

McConnell,E.E. (1980). Acute and chronic toxicity, carcinogenesis, reproduction, teratogenesis and mutagenesis in animals. In: Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Compounds.(R.D. Kimbrough, Ed.), pp. 109-190, Elsevier, New York.

McPherson, G.A. (1985). Analysis of radioligand binding experiments: a collection of computer programs for the IBM PC. J. Pharmacol. Meth. 14, 213-228.

Miller,R.M., Norris, L.A., and Hawkes,C.L. (1973). Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in aquatic organisms. Environ. Health Perspect. 5, 177-186.

Newsted, J.L., and Giesy,J.P. (1991). Characterization of

epidermal growth factor binding to its hepatic plasma membrane receptor in rainbow trout (Oncorhynchus mykiss). Gen. Compar. Endocrin. (in press).

Olson, J.W. (1985). Liver tyrosine kinase activation during early stages of chemical hepatocarcinogenesis. J. Cell. Biochem. 27, 175-180.

Pohl, J.D. and Fouts, J.R. (1980). A rapid method for assessing the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. Anal. Biochem. 107, 150-158.

Poland,A., and Knutson, J.C. (1982). 2,3,7,8-tetrachloro-dibenzo-p-dioxin and related halogenated aromatic hydrocarbons: Examination of the mechanism of toxicity. Ann. Rev. Toxicol. 22, 517,554.

Rappe,C., and Buser, H.R. (1980). Chemical properties and analytical properties. In Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products, (R.D. Kimbrough, ed.), pp. 41-76, Elsevier, Amsterdam.

Ribot, H.D., Eisenman,E.A., and Kinsey,W.H. (1984). Fertilization results in increased tyrosine phosphorylation of egg proteins. J. Biol. Chem. 259, 5333-5338.

Safe,S.H. (1986). Comparative toxicology and mechanism of action of polychlorinated dibenzo-p-dioxins and dibenzofurans. Ann. Rev. Pharmacol. Toxicol. 26, 371-399.

Spitsbergen, J.M., Kleeman,J.M., and Peterson, R.E. (1988). Morphologic lesions and acute toxicity in rainbow trout (Salmo gaidneri) treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. J. Toxicol. Environ. Health 23, 333-358.

Steel, R.G. and Torrie, J.H. (1980). Principles and Procedures of Statistics, 2nd Edition, McGraw-Hill Book Co., New York.

Takai,Y., Kaibachi,K., Tsuda,T., and Hoshijima,M. (1985). Role of protein kinase C in transmebrane signaling. J. Cell. Biochem. 29, 143-155.

Vodicnik, M.J., and Lech,J.J. (1982). Characterization of the induction response of the hepatic microsomal monooxygenase (MO) system in fish. In Cytochrome P-450 Biochemistry, Biophysics and Environmental Implications (E. Hietanen, M. Laitinen, and O. Hanninen. Eds.). pp. 225-231. Elsevier Biomedical Press, Amsterdam.

Whitlock,J. Jr. (1987). The regulation of gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Pharmacol. Rev. 39, 147-174.

CHAPTER 4

THE EFFECTS OF TCDD ON EPIDERMAL GROWTH FACTOR BINDING AND PROTEIN KINASE ACTIVITY IN THE RAINBOW TROUT CELL LINE, RTH-149

INTRODUCTION

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic congener of a larger group of structurally similar compounds, the halogenated aromatic hydrocarbons (HAHs). Due to their toxicity and persistence in the environment, these compounds represent a serious concern for human health and impact aquatic species. Several fish species have been shown to be sensitive to TCDD and other HAHs such as polychlorinated biphenyls (PCBs), polychlorinatd dibenzofurans (PCDFs) and polychlorinated dibenzodioxins (PCDDs) (Kleeman et al., 1986; Kleinow et al., 1987; Kleeman et al., 1988). Of the fish species tested, rainbow trout has been shown to be among the most sensitive with a 80 day LD50 of 10 μg TCDD/kg, body weight (Spitsbergen et al., 1988). While these compounds are extremely toxic to fish and may have a significant impact on fish populations in various aquatic environments (Walker et al., 1991), little is known about the mechanisms by which fish recognize and respond to HAHs.

In mammals, TCDD-induced toxicity, as well as enzyme induction, are mediated through the binding of this compound to an intracellular protein, the Ah receptor (Poland and Knutson, 1982; Whitlock, 1987). It is through this mechanism that TCDD regulates a variety of biochemical responses in

target tissues. Many of these responses are associated with changes in cell proliferation and altered patterns of differentiation (Hudson et al., 1985). Examples of these effects seen in both fish and mammal species, include dermal hyperkeratinization (fin necrosis in fish), teratogenicity, lymphoid involution, immunotoxicity and carcinogenesis (Safe, 1986; Spitsbergen et al., 1988a, 1988b).

The proliferation of many cell types in culture are regulated by biochemical mediators which include polypeptide growth factors such as epidermal growth factor (EGF) (Schlessinger et al., 1982; Carpenter, 1987; Velu, 1990). The biological activity of EGF is mediated through a transmembrane receptor that has a ligand-dependent tyrosine kinase activity (Schlessinger, 1988). The EGF receptor activity is regulated by the down regulation of receptors and internalization of the receptors from the cell surface. The decrease in EGF binding has also been observed in cells exposed to polycyclic aromatic hydrocarbons (Lee and Weinstein, 1978), other growth factors such as platelet derived growth factor (PDGF) (Bowen-Pope et al., 1983) and phorbol esters such as TPA (Karenlampi et al., 1983). Xenobiotics such as TCDD have also been shown to reduce EGF binding to hepatic plasma membranes (Madhukar et al., 1984). This reduction was accompanied by an increase in cellular protein kinase activity which included the EGF-associated tyrosine kinase (Madhukar et al., 1988).

Previously, we have shown that rainbow trout have hepatic EGF receptors that are associated with an intrinsic tyrosine kinase activity (Newsted and Giesy, 1991). Furthermore, we have shown that *in vivo* TCDD exposure of rainbow trout results in a significant decrease in EGF binding in the liver that is accompanied by the elevation of protein kinase C and tyrosine protein kinase activities (Chapter 3). TCDD also causes an increase in the phosphothreonine and phosphotyrosine content of the EGF receptor that is associated with the increase in EGF receptor associated tyrosine kinase activity. However, we were not able to correlate the alteration in the binding of EGF to other cellular alterations that are known to be regulated by the EGF receptor (Carpenter, 1987). Thus, there is need to further examine the relationship between TCDD-induced alteration of EGF receptor ligand binding and tyrosine kinase activity with changes in cell growth and differentiation.

In this study we report on the effect of TCDD on EGF binding and protein kinase activity in a rainbow trout hepatoma cell line (RTH-149) and how alterations in these parameters relate to TCDD-induced alterations in nucleic acid synthesis and cell proliferation. Our null hypothesis was: "TCDD does not cause an alteration in the binding of EGF or protein kinase activity that is associated with changes to cell growth or proliferation". This cell line was selected as a model system because it is induced by TCDD, and the

cytosolic Ah receptor has been identified (Lorenzen and Okey, 1990). This allowed us to make certain assumptions relative to the Ah model of TCDD toxicity as it pertains to the modulation of binding of EGF in rainbow trout.

METHODS

Chemicals

Epidermal growth factor was purchased from UBI (Lake Placid, NY). [^{3}H]-thymidine, [γ-^{32}]ATP and [^{125}I]-EGF were purchased from Dupont/NEN Research Products (Bannockburn, IL). All cell culture materials including fetal bovine serum and protein kinase substrates, RR-Src and Ac-MBP, were purchased from GIBCO-BRL (Grand Island, NY). All other chemicals were from Sigma (St. Louis, MO).

Cell Culture

The RTH-149 cell line employed in this study was derived from an aflatoxin-induced hepatoma in an adult rainbow trout (Lannan et al., 1984). Cultures were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown as a monolayer in minimum essential medium (MEM) (Eagle, modified) with Earle's salts, glutamine, bicarbonate, 50 μg/ml gentamicin and supplemented with 10% fetal bovine serum. Cells were maintained in a normal atmosphere at 22 C and the media was changed every third day. At confluence, cells were harvested by trypsinization. Cells were washed in cold phosphate-buffered saline [2.5 mM KCl, 1.5 mM KH_2PO_4, 1.5

mM NaCl, 8 mM Na_2HPO_4, 1 mM EDTA] to remove trypsin from the cells prior to platting or reseeding of flasks.

Cell Membrane Isolation

All procedures were conducted at 0 C. Washed cells were allowed to swell for 15 min in a hypotonic buffer [10 mM HEPES, pH 7.4, 1 mM $MgCl_2$] containing 100 mM leupeptin, 0.3 trypsin inhibitor units of aprotinin per ml, 0.5 mM E-64 (Calbiochem) and 1 mM sodium vanadate. Cells were broken by 25 strokes in a Dounce homogenizer with a tight fitting pestle. NaCl (5 M) was then added to a final concentration of 0.15 M. The homogenate was then centrifuged at 800xg to collect a nuclear fraction (P1). The postnuclear supernatant was then centrifuged for 1 hour at 40,000xg to form a membrane fraction (P2) and cytosolic fraction (S1). The P2 fraction was washed once with homogenization buffer and stored at -80 C for analysis. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

Cell Proliferation

Cells were seeded into 12 well plates (Falcon) at an initial density of 2.2 $x10^5$ cells/well and incubated overnight. They were then washed with PBS, and the medium was replaced with culture medium supplemented with 1% fetal bovine serum (FBS). After 24 h, EGF or TCDD was added at the appropriate concentration and cells were incubated for the times indicated. Medium was changed after 48 h. Cell number

was determined in duplicate using a hemocytometer.

Measurement of DNA Synthesis

DNA synthesis was estimated by the incorporation of [^{3}H]thymidine (90 Ci/mmol) into trichloroacetic acid-insoluble material. Subconfluent cultures in 24-well plates (Falcon), were made quiescent by a 48 h incubation in a serum free medium. Cultures were then exposed to TCDD for 24 h. [^{3}H]Thymidine (1 μCi/ml) was added to each well and the cultures were incubated for 4 h room temperature. Monolayers were washed 4 times with 1 ml ice-cold PBS, 2 times with 1 ml 10% trichloroacetic acid (10 min each) and once with ethanol. The cells were solubilized with 300 μl of 0.5 M NaOH and the radioactivity was measured by liquid scintillation counting. The [^{3}H]thymidine incorporation was normalized to cell protein.

EGF Binding Studies

Binding studies were conducted as a modified procedure of Newsted and Giesy (1991). RTH-149 P2 membranes (50 μg) were incubated in binding buffer [20 mM Hepes, pH 7.2, 180 mM NaCl, 1 mM DTT, 0.1% BSA] containing 10 mM leupeptin, 0.3 trypsin inhibitory units of aprotinin /ml and 20 μM E-64 with varying concentrations of ^{125}I-EGF (1.1 μCi/nmol) ranging from 0.05 to 10 nM. After 2 hr at 18 C, the reactions were stopped by the addition of 3 ml of ice cold buffer and filtered through GF/F filters (Whatman). The filters were washed 3 times with buffer and counted in a Packard auto-gamma counter. Non-

specific binding was defined as binding in the presence of a 750-fold excess of unlabeled EGF at each [^{125}I]EGF concentration. EGF receptor numbers and affinities were estimated from plots by fitting the data with the LIGAND program as modified by McPherson (1985).

Tyrosine Kinase Assay

Tyrosine kinase assays were conducted in microfuge tubes by a modified method of Ribot et al. (1984). Reactions were carried out in 60 μl volumes and contained 50 mM HEPES, pH 7.4, 20 mM Mg^{2+}, 5 mM Mn^{2+}, 100 μM vanadate, 5 μM okadaic acid, 0.05% nonidet NP40, 50 μM [γ-^{32}P]ATP (1000-2000 cpm/pmol) and 20 to 100 μg membrane protein. To half of the tubes, 120 μM RR-SRC synthetic peptide was added while the other half received water. The assay was initiated by the addition of [γ-^{32}P]ATP. After incubation for 30 min at 4°C, the reaction was stopped by the addition of 150 μl of 5% trichloroacetic acid and 1.56 mM KH_2PO_4. Subsequently 10 μl of bovine serum albumin (BSA) was added to aid in protein precipitation and the samples were incubated on ice for 15 min. The precipitated protein was removed by centrifugation at 5,500xg for 15 min at 4°C. From each tube, two 50 μl samples of supernatant were spotted on phosphocellulose filters (Whatman P-81). Phosphocellulose filters were washed four times in 75 mM phosphoric acid (5 min) and once in acetone (2 min). Radioactivity of air dried filters was quantified for in 7 ml of Universol (ICN, Irvine, CA). Tyrosine kinase activity was

expressed as the difference between samples with and without the RR-SRC peptide and normalized for time and protein concentration.

Protein Kinase C Assay

Protein kinase C activity (PKC) was measured by a modification of the procedure of Bell and Sargent (1987). To determine the activity of "particulate" PKC, plasma membranes were extracted in the following manner: Membranes were homogenized in 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% triton X-100, and 25 μg aprotinin and leupeptin/ml. Homogenized membranes were incubated for 30 min at 0 C and the cellular debris was removed by centrifugation for 2 min in a microcentrifuge. Assays were conducted with supernatant in microfuge tubes in 50 μl total volume. The diluted enzyme preparation (20 μl) was assayed at 10 C for 5 min. Each assay contained 20 mM HEPES, pH 7.5, 10 mM MgCl, 1 mM DTT, 20 μM vanadate, 50 μM [γ-^{32}]ATP (1000-2000 cpm/pmol), 10 μM $CaCl_2$, 120 μM Ac-myelin basic protein (Ac-MBP), 40 μg diolein and 40 μg phosphatidylserine. Before being added to the reaction tubes, equal volumes of diolein and phosphotidylserine in chloroform were combined and the solvent was removed under nitrogen. The residue was suspended in a small volume of 20 mM HEPES, pH 7.4, by sonication for 5 min at 0 C and the resultant liposome mixture was used in the assay. Basal activity was measured in the presence of 10 mM EGTA instead of $CaCl_2$, and liposomes. The reaction was stopped by spotting 25

μl onto phosphocellulose paper. The filters were washed twice in 150 mM phosphoric acid and then twice in distilled water. The filters were dried and counted in 7 ml Safety Solv. Protein kinase activity was calculated as the difference between the EGTA treatment and expressed as pmol of ^{32}P incorporated per min per mg membrane protein.

Immunoprecipitation

Immunoprecipitation of EGF-related proteins was carried out as outlined by Gill et al. (1984). Plasma membrane protein (100 μg) was phosphorylated *in vitro* using [γ-^{32}P]ATP as outlined previously except that RR-SRC was omitted from the mixture. The phosphorylated membrane protein was solubilized in 1 ml RIPA buffer (10 Mm K_2HPO_4, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.01% sodium dodecyl sulfate and 2 Mm EDTA) for 1 hr at 0 C. The solubilized material was centrifuged at 12,000g for 10 min and the supernatant was incubated overnight at 4 C with 10 μl of mouse anti-EGF receptor monoclonal antibody. The immunocomplex was precipitated by using goat antibody to mouse immunoglobulin G (IgG) conjugated to *Staphylococcus aureus* cells (Tachisorb-M, Calbiochem) for one hour at room temperature with frequent mixing. Samples were centrifuged (2,000xg for 15 min) and the supernatants removed. Pellets were washed twice with 1 ml PBS at 0 C followed by centrifugation. The final pellet was heated (98 C for 5 min) in SDS Laemmli sample buffer and centrifuged. The supernatant was precipitated with 10% TCA,

dissolved by 1 M NaOH and counted by liquid scintillation.

Statistical Analysis

The data were analyzed by analysis of variance planned comparisons of the means. Relationships between measured parameters were investigated by Pearsons pairwise correlations. The level of significance to detect differences between treatments was $p < 0.05$.

RESULTS

Modulation of DNA Synthesis and Cell Growth by TCDD

Proliferation of RTH-149 cells low serum conditions was increased by exposure to TCDD at concentrations ranging from 3.0 to 150 pM (Fig. 22). At concentrations below 3 pM, cells remained viable, as judged by trypan blue exclusion, but no statistical increase in cell number was observed. The TCDD-induced increase in cell number was similar to that observed in cells grown in MEM supplemented with 10% FBS. No cytotoxicity was observed at any of the TCDD concentrations used in this study. The incorporation of [^{3}H]thymidine into TCA-precipitated material was dose-dependent with half-maximal stimulation occurring at about 3.0 pM (Fig. 23). The greatest stimulation of [^{3}H]thymidine uptake occurred at 150 pM. The dose-dependent increase in [^{3}H]thymidine incorporation corresponded to the observed increase in cell proliferation over the same concentration range. There was a positive correlation between protein normalized [^{3}H]thymidine uptake

Figure 22. **Effect of TCDD on the stimulation of cell proliferation of RTH-149 cells. Triplicate subconfluent monolayers of RTH-149 cells were incubated with the serum poor medium and varying concentrations of TCDD. Percent of control values are given, and significant differences ($p < 0.05$) between control and treated cell values are denoted with *.**

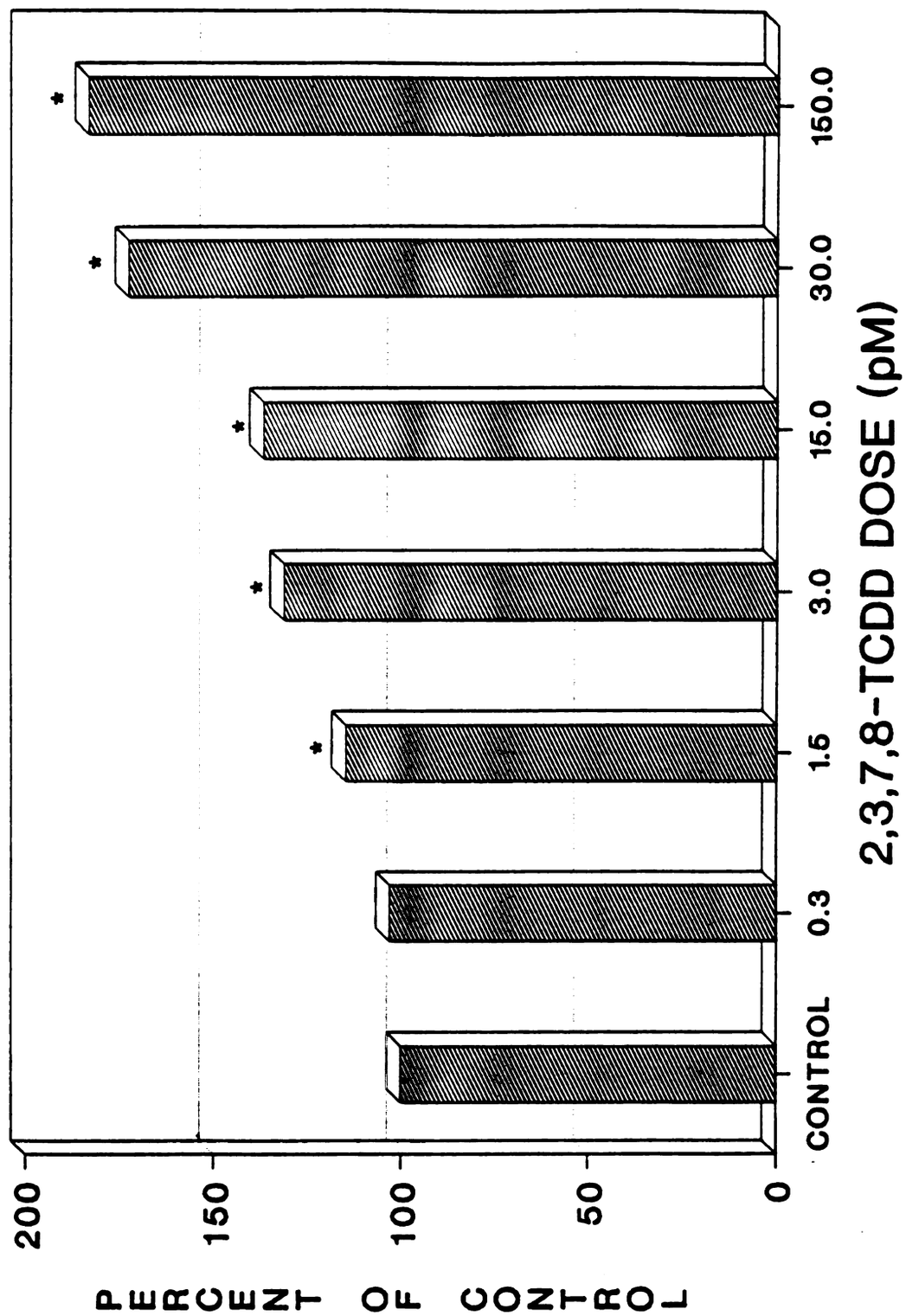
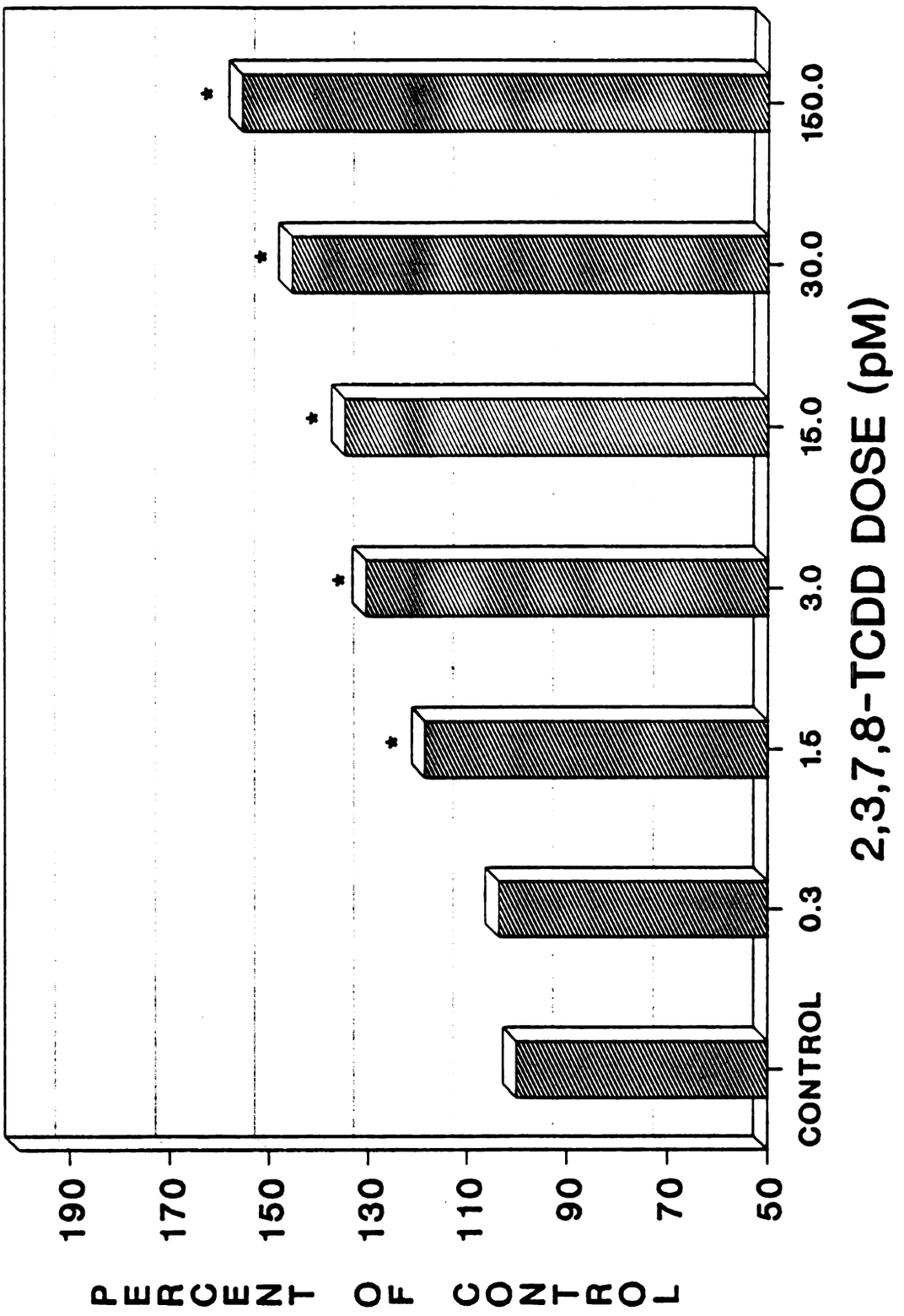

PERCENT OF CONTROL
200
150
100
50
0
CONTROL
0.3
1.5
3.0
15.0
30.0
150.0
2,3,7,8-TCDD DOSE (pM)
*
*
*
*
*
*

Figure 23. **Effect of TCDD on the stimulation of [^{3}H]thymidine incorporated in RTH-149 cells. Triplicate confluent RTH-149 monolayers were incubated with [^{3}H]thymidine in a serum-poor medium for 4 h after a 24 h incubation in the presence of TCDD. Percent of control values are given, and significant differences ($p < 0.05$) between control and TCDD-treated cells are denoted with *.**


PERCENT OF CONTROL
190
170
150
130
110
90
70
50
CONTROL
0.3
1.5
3.0
15.0
30.0
150.0
2,3,7,8-TCDD DOSE (pM)

and cell proliferation (R^2= 0.951). The increase in [^{3}H]thymidine uptake and cell proliferation exhibited the same trends for the duration of the experiments.

Effect of TCDD on EGF binding

Treatment of RTH-149 cells with TCDD resulted in a concentration-dependent decrease in specific binding of EGF (Fig. 24). The EC50 for inhibition of EGF binding was 27.5 pM. The diminished EGF binding in cultures treated with TCDD was not due to loss of cells from the dish, as was measured by total protein, or to a reduction in cell viability as measured by trypan blue exclusion. Binding of EGF was significantly inhibited within 6 h post-treatment and reached a maximal inhibition by 72 h that remained unaltered until 96 h (Fig. 25). The effect of TCDD on the magnitude of EGF binding was persistent. Binding remained reduced (53%) for at least 7 d after the removal of TCDD from the growth medium. The reduction of EGF binding was not due to a loss of TCDD from the culture medium or to a depletion of serum since the reduction in EGF binding at 72 h did not differ from 96 h even though medium and TCDD was replenished at 72 h.

The binding in the control cultures was curvilinear, whereas in the TCDD-treated cultures there was a progressive loss in linearity of the Scatchard analysis (Fig. 26). Analysis of the control cultures by LIGAND indicated the presence of two EGF binding sites with differing affinities for ^{125}I-EGF. The binding capacity of the high affinity site

Figure 24. **Concentration-dependent inhibition of EGF-binding to RTH-149 by TCDD. Confluent cultures of RTH-149 cells were treated with isooctane (0.05%) or TCDD (0.3 to 150 Pm) for 24 h prior to analysis of EGF-specific binding at 18 C for 2 h. The data shown represents means of triplicate determinations on duplicate cultures and are expressed as a percentage of the isooctane control. Significant differences ($p < 0.05$) between control and TCDD-treated cells are indicated with *.**

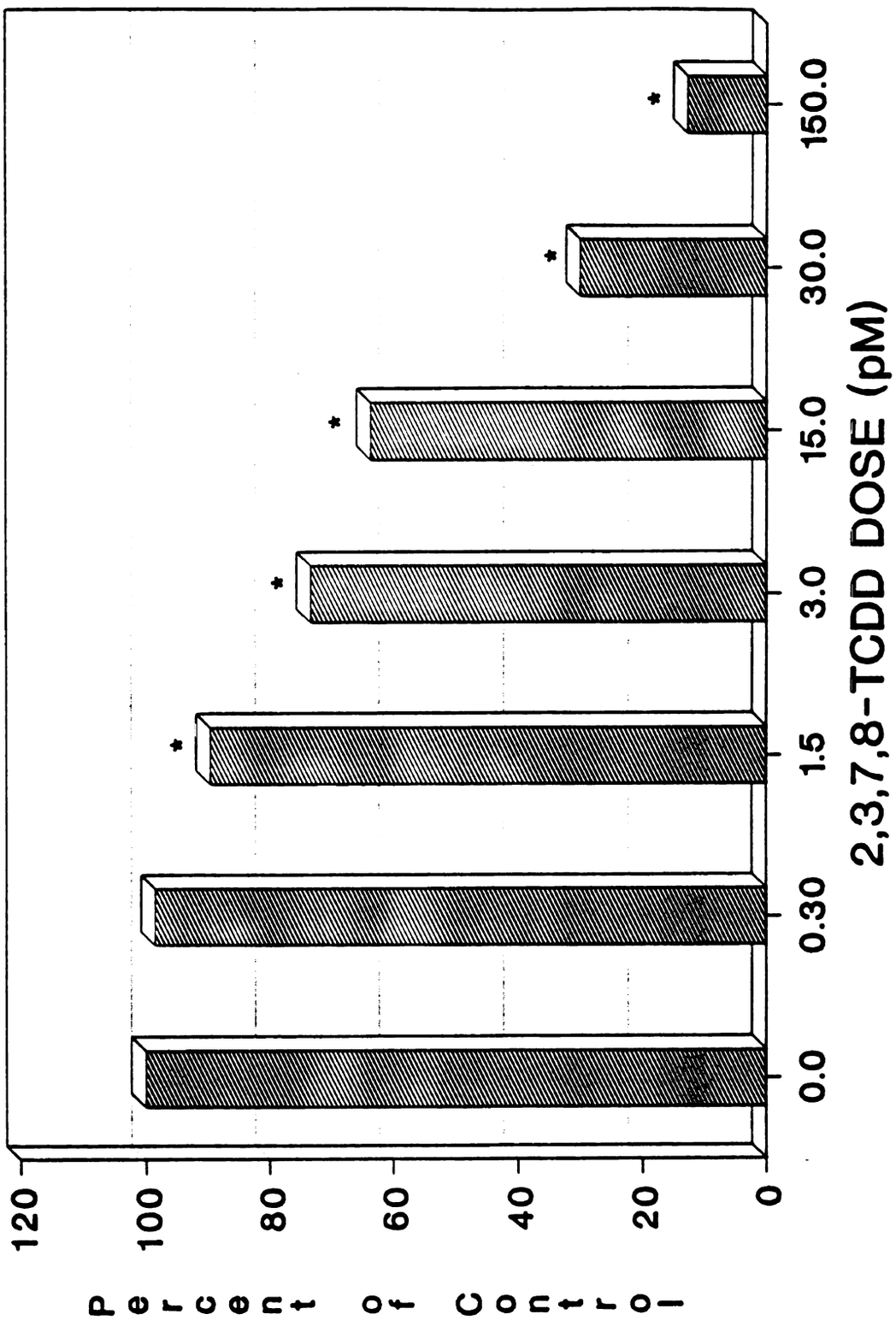

Percent of Control
120
100
80
60
40
20
0
0.0
0.30
1.5
3.0
15.0
30.0
150.0
2,3,7,8-TCDD DOSE (pM)
*

Figure 25. Time-dependent inhibition of binding of EGF in TCDD-treated RTH-149 cells. Confluent monolayers of RTH-149 cultures were treated with isooctane (0.05% v/v) or TCDD (150 pM) for the indicated times. The data shown represents means of triplicate determinationson duplicate cultures and are expressed as a percentage of the isooctane control. Significant differences ($p < 0.05$) between control and treated cells are indicated with *.

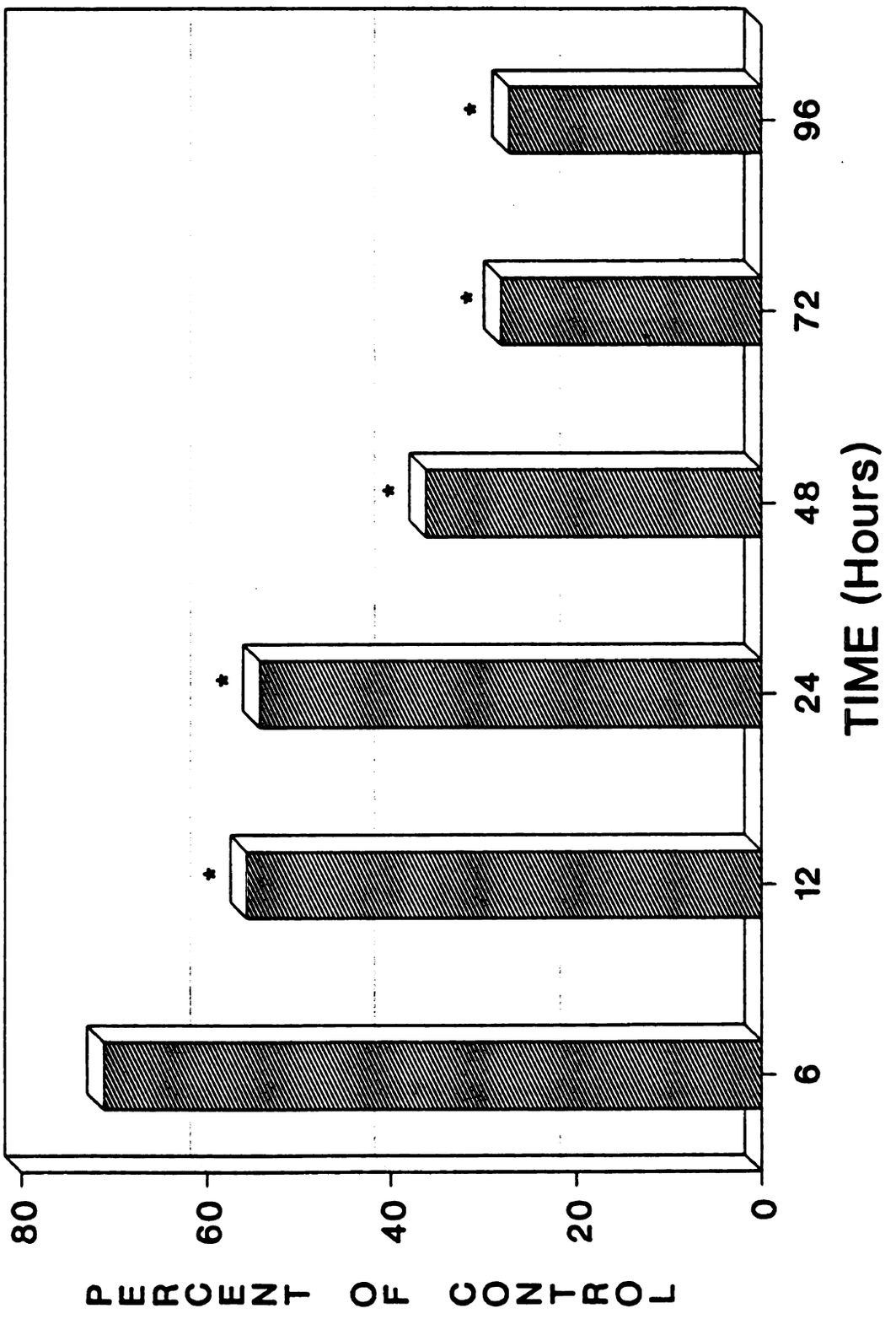
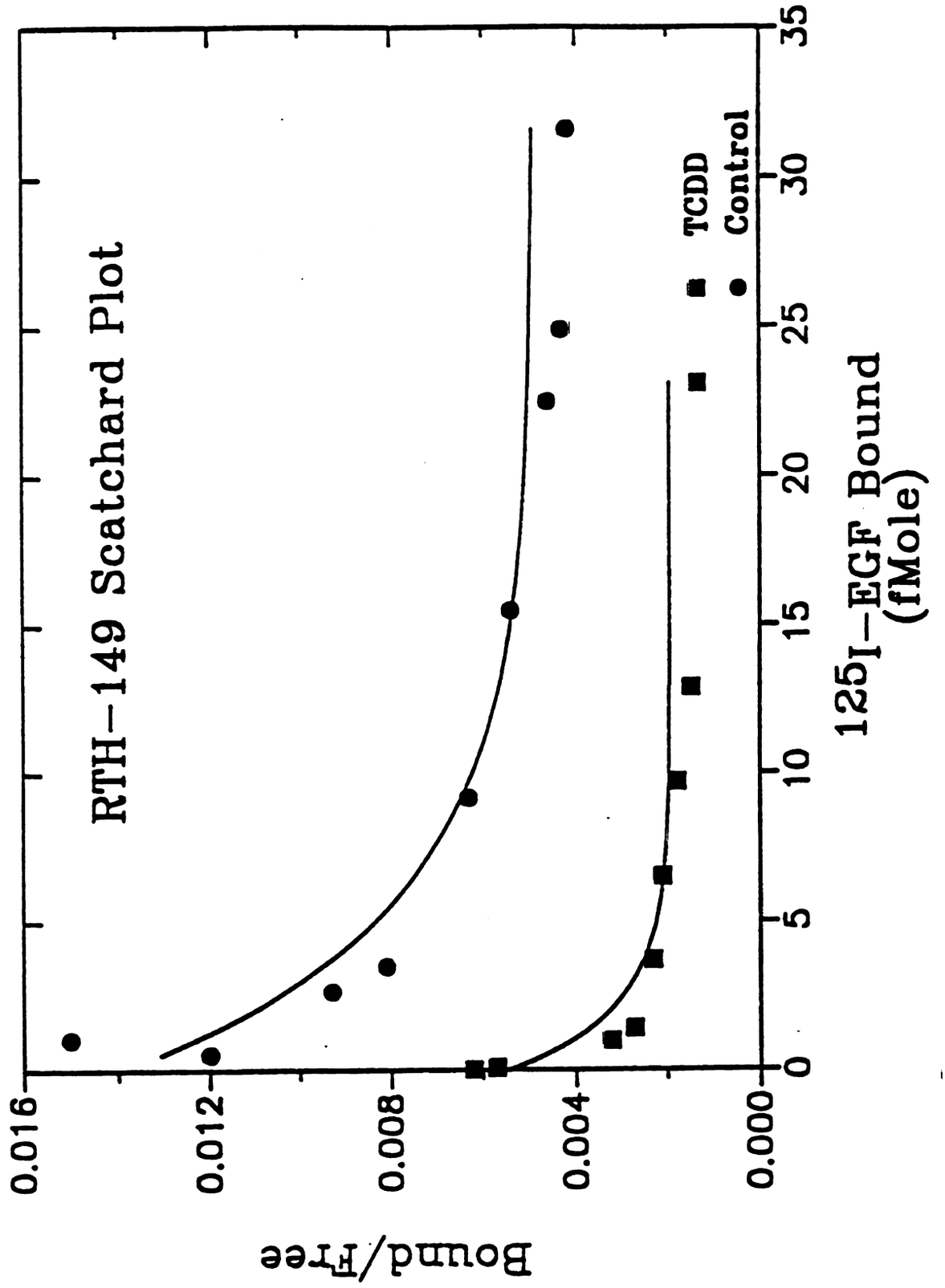

PERCENT OF CONTROL
0
20
40
60
80
6
12
24
48
72
96
TIME (Hours)
*
*
*
*
*

Figure 26. Scatchard analysis of EGF binding data. Confluent cultures of RTH-149 cells were treated with 0.05% (v/v) isooctane (○) or 150 pM TCDD (■) for 24 h prior to the isolation of the cell membranes. Membranes were incubated with increasing concentrations of ^{125}I-EGF in the presence or absence of 750-fold excess unlabeled EGF. EGF-specific binding was measured as outlined in Methods. Each point is the mean of triplicate determinations of duplicate cultures.

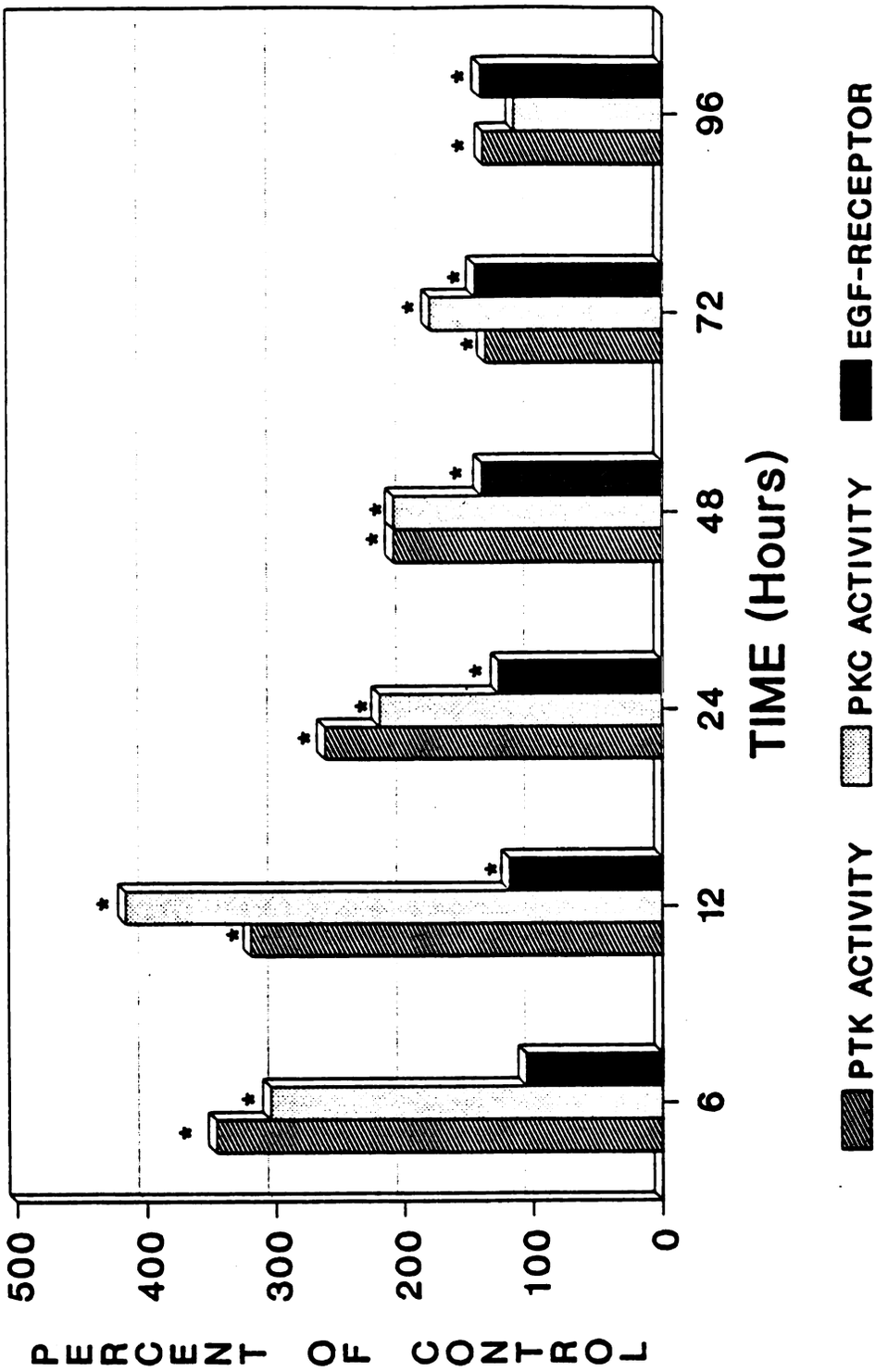

RTH-149 Scatchard Plot
Bound/Free
0.016
0.012
0.008
0.004
0.000
0
5
10
15
20
25
30
35
125I-EGF Bound
(fMole)
TCDD
Control

(K_D =1.5 x 10^{-10}M) was 10.5 fmole/mg protein while the binding capacity (B_{max}) for the low affinity site (K_D= 4.0 x 10^{-9}) was 153 fmole/mg protein. Cells treated with TCDD exhibited fewer high affinity binding sites (B_{max}= 0.5 fmole/mg) and only a small decrease in low affinity binding sites (B_{max}= 145 fmole/mg). EGF binding affinity (K_D) of low affinity and high affinity binding sites of the TCDD-treated cells were unchanged from control values. The reduction in EGF binding in TCDD-treated RTH-149 cells was negatively correlated to the increase in cell number (R^2= 0.987) and to the increase in thymidine uptake (R^2= 0.957). These results indicate that TCDD itself may be potentially mitogenic in RTH-149 cells by altering EGF receptor homeostasis.

Effect of TCDD on Protein Kinase Activity

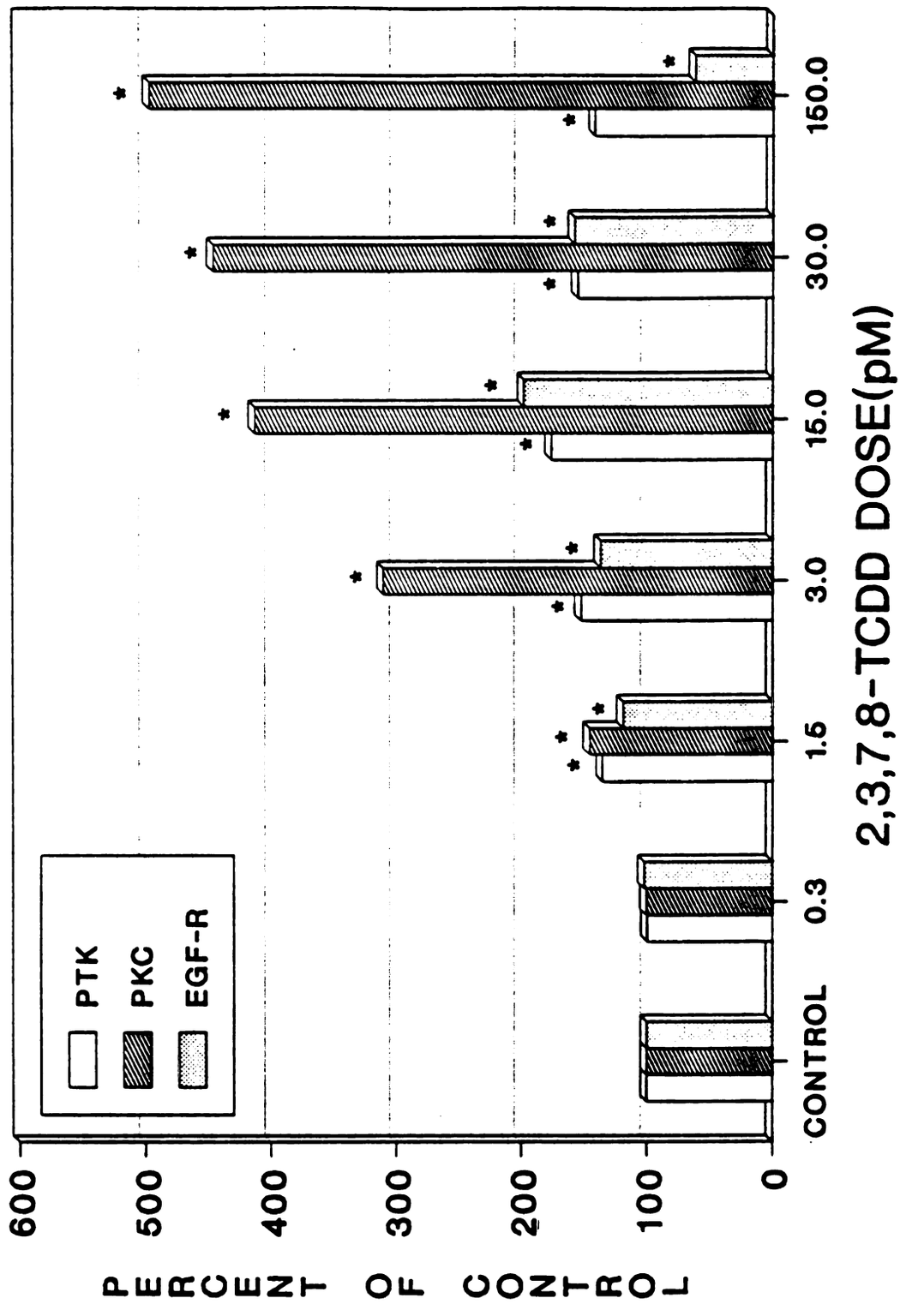
Protein kinase activities were significantly greater in RTH-149 cells treated with TCDD for 24 h (Fig. 27). TCDD exposure resulted in approximately a 4-fold increase in protein kinase C activity. The time-dependent increase corresponded to the reduction of EGF binding in the cells (Fig. 25). Protein kinase C activity was greater after 12 h and then returned to control values by 96 h (Fig. 27). Induction of protein kinase C activity was dose-dependent with an EC50 of about 23 pM (Fig. 28). This value agrees closely with the EC50 value for the reduction in EGF binding. Correlation analysis showed a significant negative correlation (R^2= 0.835) between EGF binding and protein kinase C activity.

Figure 27. **Effect of TCDD on protein kinase C (PKC) activity, tyrosine kinase (PTK) activity and EGF receptor (EGF-R) phosphorylation in confluent RTH-149 cells. Cells were treated with 0.05% (v/v) isooctane or 150 pM TCDD and incubated in serum poor medium for 24 h. Cell membranes were isolated at the indicated times and the protein kinase activity was determined as outlined in Methods. Each point represents the mean of triplicate determinations of duplicate cultures. Significant differences ($p < 0.05$) between control and TCDD-treated cell values are denoted with *.**



C (PKC)
vity and
cion in
treated
TCDD and
h. Cell
ted times
etermined
epresents
tions of
fferences
D-treated

Figure 28. **Effect of TCDD-dose on protein kinase C (PKC) activity, tyrosine kinase (PTK) activity and EGF receptor (EGF-R) phosphorylation in confluent RTH-149 cells. Cells were treated with 0.05% (v/v) isooctane or TCDD at the indicated doses incubated for 24 h. Cell membranes were isolated and protein kinase assays were conducted as outlined in Methods. Each point represents the mean of triplicate determinations of duplicate cultures and are expressed as a percent of the control. Significant differences ($p < 0.05$) between control and treated cells are denoted with *.**


PTK
PKC
EGF-R
PERCENT OF CONTROL
600
500
400
300
200
100
0
CONTROL
0.3
1.5
3.0
15.0
30.0
150.0
2,3,7,8-TCDD DOSE(pM)

The observed increase in protein kinase C activity was also correlated with [^{3}H]thymidine uptake (R^2= 0.901) and increase in cell number (R^2= 0.794).

Protein tyrosine kinase activity, in TCDD treated RTH-149 cells, was maximally elevated by 6 h (Fig. 27). By 24 h post-treatment, tyrosine kinase activity had started to return control values, but remained elevated through 96 h. As was observed for protein kinase C activity, tyrosine kinase activity was positively correlated with TCDD concentration (Fig. 28). Activity reached a maximum at 3.0 pM TCDD and remained unaltered up to 150 pM TCDD. The increase in tyrosine kinase activity was significantly correlated with the reduction of EGF binding (R^2= 0.672) but was poorly correlated with the increase in protein kinase C activity (R^2= 0.218). The lack of relationship between tyrosine kinase activity and protein kinase C is not surprising in that both protein kinases are activated through separate mechanisms. Correlations between protein tyrosine kinase and [^{3}H]thymidine (R^2= 0.726) and cell number (R^2=0.583) were also significant.

Immunoprecipitation of phosphorylated RTH-149 cell membranes showed a time-related increase in the radioactivity incorporated into EGF receptor immunoreactive protein (Fig. 27). The increase in receptor phosphorylation was initially observed at 12 h and remained elevated through the 96 h period of the study. The increase in EGF receptor phosphorylation occurred approximately 6 h after the initial increase in

protein kinase C and tyrosine kinase activities. As was seen in protein kinase activity, the phosphorylation of the EGF receptor increased in a dose-dependent manner (Fig. 28). Increased phosphorylation of the EGF receptor was first observed at 1.5 pM TCDD and reached a maximum at 15 pM. However, at 150 pM, there was a decrease in the level of phosphorylation. Some of this decrease may be attributed to the inhibition of EGF binding (77%) which occurred at this TCDD concentration (Fig. 24).

The dose-response relationship between protein kinase C activity and EGF receptor phosphorylation was not significant (R^2= 0.147) in TCDD-treated RTH-149 cells . However, EGF receptor phosphorylation values and protein tyrosine kinase activity was positively correlated (R^2= 0.752).

DISCUSSION

TCDD modulates the binding of EGF to the rainbow trout hepatoma cell line, RTH-149. A loss of high affinity EGF binding sites following treatment with TCDD was observed (Fig. 26). This loss was both time- and dose-dependent and correlated with altered biological responses such as greater synthesis of DNA (Fig. 23). In RTH-149 cells, a significant reduction in EGF binding occured at a lesser concentrations of TCDD (27.5 pM) than that required to cause an induction of aryl hydrocarbon hydroxylase (AHH) activity (500 pM) (Lorenzen and Okey, 1990). The association between TCDD concentration and the observed effects supports the hypothesis that the

reduction in binding of EGF is in part mediated by the Ah receptor (Ivanovic and Weinstein, 1982). The Ah receptor has recently been detected in this cell line. RTH-149 cells have fewer Ah sites (about 20 fmol/mg cytosolic protein) than most mammalian species but have sites of higher affinity, K_D~1 nM (Lorenzen and Okey, 1990).

The reduction in the binding of EGF binding caused by TCDD in RTH-149 cells was similar to that observed in (SCC-12F) human keratinocyte cells. Exposure of SCC-12F cells to TCDD resulted in a dose-dependent reduction of binding of EGF that was also similar in scope to the reported dissociation constant for binding of TCDD to the Ah receptor of mouse liver (Poland and Knutson, 1982). In RTH-149 cells, the EC50 for the reduction of binding of EGF was 30-fold greater than the apparent dissociation constant of the RTH-149 Ah receptor. This indicates that while the Ah receptor may have a role in the modulation of EGF binding, some of the reduction in binding may be caused by other cellular mechanisms.

The TCDD-induced reduction in binding of EGF capacity can be regulated by two different mechanisms. The first mechanism involves altered gene expression of the EGF receptor where down regulation of the receptor is mediated by the alteration in protein synthesis. The alteration of EGF receptor gene expression has been observed in rat uterus where TCDD has been shown to inhibit the increase in EGF receptor binding activity by 17β-estradiol through reduced mRNA transcription (Astroff

et al., 1990). The second mechanism by which TCDD may down regulate EGF receptor binding activity is through the activation of protein kinases that can either activate or inactivate the receptor with the subsequent internalization of the receptor from the cell surface (Carpenter, 1987). Studies of TCDD-exposed rats and guinea pigs have shown protein kinase C and protein tyrosine kinase activities were correlated with the down regulation of both high and low affinity EGF receptor sites in hepatic plasma membranes (Madhukar et al., 1988). The second mechanism is consistent with findings from our study.

In vivo studies of rainbow trout have shown that TCDD induced morphological changes are consistent with altered epithelial differentiation and proliferation of target tissues (Spitsbergen et al., 1988). Similarly, the TCDD-induced alteration in rainbow trout liver morphology is consistent with liver hyperplasia and fatty infiltration which are also induced by EGF. Rainbow trout given a single i.p. dose of TCDD exhibit a dose- and time-dependent decrease in hepatic EGF binding that was correlated with the increase in protein kinase C activity (see Chapter 3). The increase in protein kinase C was also observed in TCDD treated RTH-149 cells. As was observed in rainbow trout hepatic plasma membranes, protein kinase C activity was correlated in a time- and dose-dependent manner with the reduction in EGF binding. The increase in protein kinase C was also significantly correlated

with an elevation in [^{3}H] thymidine uptake which is indicative of altered cell proliferation. Protein kinase C is a calcium- and phospholipid-dependent protein kinase that is a key enzyme for intracellular signal induction and the control of cell proliferation and mitogenesis (Nishizuka, 1988). Protein kinase C has also been identified as a major cellular receptor for the potent skin-tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA)(Lee and Weinstein, 1978). TPA-stimulated protein kinase C is thought to phosphorylate serine and threonine residues of critical target proteins which may directly or indirectly regulate the expression of specific genes associated with tumor promotion and cell growth. In A-431 cells, the activation of protein kinase C has been shown to decrease EGF binding via phosphorylation of serine and threonine residues on the EGF receptor (Downward et al., 1985). Thus, elevation of protein kinase C activity in RTH-149 cells and the subsequent induction of cell proliferation and [^{3}H] thymidine uptake may have significant implications relative to altered plasma membrane homeostasis as it relates to TCDD-induced toxicity (Matsumura et al., 1984).

Tyrosine protein kinase activity was also elevated in TCDD treated RTH-149 cells (Fig. 28). The increase in tyrosine kinase activity was significantly correlated with the reduction of EGF binding which may indicate activation of the receptor with subsequent internalization and the loss of receptors from the cell surface (Velu, 1990). Studies have

suggested that the tyrosine kinase activity of the EGF receptor is essential for signal transduction, normal receptor "trafficking", stimulation of DNA synthesis and transformation (Moolenaar et al., 1988). Significant correlations were observed between tyrosine kinase activity and cell growth (R^2= 0.683) and [^{3}H] thymidine uptake (R^2= 0.826) over the dose range used in this study. Similar results were also seen in the *in vivo* rainbow trout study where significant correlations were observed between protein tyrosine kinase activity and the reduction in EGF binding. The significance of this finding is that there was a positive correlation between the increase in tyrosine kinase activity and receptor phosphorylation levels. The increase in EGF-receptor phosphorylation may indicate the activation of the EGF receptor in both TCDD-treated rainbow trout hepatic plasma membranes and RTH-149 cells. However, the dose-response relationship between cell number and EGF receptor phosphorylation were not statistically significant and may indicate that other regulatory mechanisms are involved in the cell growth. One such mechanism may be TCDD-induced stimulation of protein kinase C activity that has been shown to be related to increased cell proliferation and differentiation (Nishizuka, 1988).

In conclusion, our previous findings indicate that altered binding of EGF in TCDD-treated rainbow trout is related to changes in protein kinase activity. Data from the current study corroborates these findings in the rainbow trout

cell line, RTH-149. We have also shown that TCDD alters cell growth and [^{3}H]thymidine uptake in a time-and dose-dependent manner that correlates with increases in protein kinase activity. These effects are similar to those observed in TCDD-treated mammals and may indicate a similar mode of toxic action between fish and mammals. These results argue that TCDD may exert multiple effects on the EGF-induced proliferative pathway, one of which may involve an alteration of protein kinase C activity and of EGF receptor associated and other cell surface tyrosine kinases.

REFERENCES

Adamson,E.D. and Rees,A.R. (1981). Epidermal growth factors. Mol. and Cell. Biochem. 34: 129-152.

Astroff,B., Rowlands,C., Dickerson,R., and Safe,S. (1990). 2,3,7,8-tetrachlorodibenzo-p-dioxin inhibition of 17B-estradiol-induced increases in rat uterine EGF-receptor binding activity and gene expression. Mol. Cell. Endocrin. 72: 247-252.

Bell,M.V. and Sargent,J.R. (1987). Protein kinase C activity in the spleen of trout (Salmo gairdneri) and the rectal gland of dogfish (Scyliorhinus canicula) and the effects of phosphatidylserine and diacylglycerol containing (n-3) polyunsaturated fatty acids. Comp. Biochem. Physiol. 87B: 875-880.

Bowen-Pope,D.F., Dicorleto,P.E., and Ross,R. (1983). Interactions between the receptors for platelet-derived growth factor and epidermal growth factor. J. Cell Biol. 96: 679-683.

Bradford,M.M. (1976). A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Carpenter,G. (1987). Receptors for epidermal growth factor and

other polypeptide mitogens. Ann. Rev. Biochem. 56: 881-914.

Downward, J., Waterfield,M.D., and Parker, P.J. (1985). Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. J. Biol. Chem. 260: 14538-14546.

Gill,G., Kawamoto,T., Le, A.D., Mendelson, J., Sato,G.H. and Mendelson,J. (1984). Monoclonal anti-epidermal growth factor receptor antibodies which are inhibitors of epidermal factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein kinase activity. J. Biol. Chem. 259: 7755-7760.

Hudson,L.G., Toscano,W.A., and Greenlee, W.F. (1985). Regulation of epidermal growth factor binding in human keratinocyte cell line by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Appl. Pharmacol. 77: 251-259.

Ivanovic, V., and Weinstein,I.B. (1982). Benzo[a]pyrene and other inducers of cytochrome P_1-450 inhibit binding of epidermal growth factor to cell surface receptors. Carcinogenesis 3: 505-510.

Karenlampi,S.O., Eisen,H.J., Hankinson,O., and Nebert,D.W. (1983). Effects of cytochrome P-450 inducers on the cell-surface receptors for epidermal growth factor, phorbol 12,13-dibutyrate, or insulin of cultured mouse hepatoma cells. J. Biol. Chem. 258: 10378-10383.

Kleeman,J.M., Olson,J.R., Chen,S.M., and Peterson,R.E. (1986). Metabolism and disposition of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rainbow trout Toxicol. Appl. Pharmacol. 83: 391-401.

Kleeman,J.M., Olson,J.R, and Peterson,R.E. (1988). Species differences in 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity and biotransformation. Fundam. Appl. Toxicol. 10: 206-213.

Kleinow,K.M, Melancon,M.J., and Lech,J.J. (1987). Biotransformation and implications for toxicity, bioaccumulation and monitoring of environmental xenobiotics in fish. Environ. Health Perspect. 71: 105-119.

Lee,L-S., and Weinstein,I.B. (1978). Tumor promoting phorbol esters inhibit binding of epidermal growth factor to cellular receptors. Sci. 202: 313-315.

Lorenzen,A., and Okey,A.B. (1990). Detection and characterization of [^{3}H] 2,3,7,8-tetrachlorodibenzo-p-dioxin binding to Ah receptor in a rainbow trout hepatoma cell line. Toxicol. Appl. Pharmacol. 106: 53-62.

Madhukar,B.V., Brewster,D.W., and Matsumura,F. (1984). Effects of in vivo administered 2,3,7,8-tetrachlorodibenzo-p-dioxin on receptor binding of EGF in the hepatic membranes of rat, guinea pig, mouse and hamster. Proc. Natl. Acad. Sci. USA. 81: 7407-7411.

Madhukar,B.V., Ebner,K., Matsumura,F., Bombick,D.W.,

Brewster,D.W., and kawamoto,T. (1988). 2,3,7,8-tetrachlorodibenzo-p-dioxin causes an increase in protein kinases associated with epidermal growth factor receptor in the hepatic plasma membrane. J. Biochem. Toxicol. 3: 261-277.

Matsumura,F., Brewster,D.W., Madhukar,B.V., and Bombick,D.W. (1984). Alteration of rat hepatic plasma membrane functions by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Arch. Environ. Contam. Toxicol. 13: 509-515.

McPhearson,G.A. (1985). Analysis of radioligand binding experiments: a collection of computer programs for the IBM PC. J. Pharmacol. Meth. 14: 213-228.

Moolenaar,W.H., Bierman,A.J., Tilly,B.C., Verlaan,I., Honegger,A.M., Ullrich,A., and Schlessinger,J. (1988). A point mutation at the ATP-binding site of the EGF-receptor abolishes signal transduction. EMBO J. 7: 707-710.

Newsted,J.L. and Giesy, J.P. (1991). Characterization of epidermal growth factor binding to the hepatic plasma membranes of rainbow trout (Oncorhyncchus mykiss). Gen. Compar. Endocrinol. (in press).

Nishizuka,Y. (1988). The molecular heterogeneity of protein kinase C and its implication for cellular regulation. Nature 334: 661-665.

Poland,A. and Knutson,J. (1982). 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons:

examination of the mechanisms of toxicity. Ann. NY Acad. Sci 30: 214-230.

Ribot,H.D., Eisenman,E.A., and Kinsey,W.H. (1984). Fertilization results in increased tyrosine phosphorylation of egg proteins. J. Biol. Chem. 259: 5333-5338.

Safe,S. (1986). Comparative toxicology and mechanisms of action of polychlorinated dibenzo-p-dioxins and dibenzofurans. Ann. Rev. Pharmacol. Toxicol. 92: 368-380.

Schlessinger,J. (1988). The epidermal growth factor receptor as a multifunctional allosteric protein. Biochem. 27: 3119-3123.

Schlessinger,J., Schreiber,A.B., Levi,A., Lax,I., Liberman,T. and Yarden, Y. (1982). Regulation of cell proliferation by epidermal growth factor. CRC Critical Reviews in Biochemistry. 14: 93-111.

Spitsbergen,J.M., Kleeman,J.M., and Peterson, R.E. (1988). Morphologic lesions and acute toxicity in rainbow trout (Salmo gairdneri) treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. J. Toxicol. Environ. Health 23: 333-358.

Spitsbergen,J.M., Kleeman,J.M., and Peterson, R.E. (1988b). 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in yellow perch (Perca flavescens). J. Toxicol. Environ. Health 23: 359-383.

Velu,T.J. (1990). Structure, function and transforming potential of the epidermal growth factor receptor. Mol. Cell. Endocrinol. 70: 205-216.

Walker,M.K., Spitsbergen,J.M., Olsen,J.R., and Peterson,R.E. (1991) 2,3,7,9-tetrachlorodibenzo-p-dioxin toxicity during early life stage development of lake trout (Salvelinus namaycush). Can. J. Fish. Aquat. Sci. 48: 875-883.

Whitlock,J. Jr. (1987). The regulation of gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Pharmacol Rev. 39: 147-174.

SUMMARY

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a potent environmental toxicant that has been shown to affect many fish species at low environmental concentrations. Evaluation of the possible effects of TCDD on fish populations is difficult in that TCDD exists in the environment in a complex mixture of structuraly similar compounds. Thus, methods to monitor the effects of TCDD and TCDD-like compound on fish should be sensitive and specific and be based on the mode of toxic action of these compounds. While fish are extremely sensitive to the effects of TCDD, little or nothing is known about the mechanisms of toxicity of TCDD in these species. The results presented in this report are the first to examine the role of the epidermal growth factor receptor (EGF-R) in the toxicity of TCDD to fish.

Since little was known about EGF-R in fish, we conducted several studies to characterize the binding and the biochemistry of this receptor in rainbow trout. Our results show that in rainbow trout, EGF-binding was peptide specific, saturable, reversible, and of high affinity. Binding of EGF to rainbow trout membranes caused an increase in a tyrosine kinase activity that phosphorylated tyrosine residues of endogenous polypeptide substrates and of the EGF receptor like protein. These observations are similar to those seen in

mammalian tissues treated with EGF. However, care must be taken when extrapolating the data generated in these studies to that of the possible biological action of the EGF-R in other species of fish. Before these extrapolations can be made, research need to be conducted to examine the role of EGF in fish. Future research should examine how EGF alters the regulation of normal cell growth and differentiation and the development of early life stages. One of the more sensitive measures of EGF effects is the early eyelid opening in newborn mice. Analogous studies should be conducted in fish. Efforts should also be made to isolate both the EGF-receptor and its ligand in rainbow trout.

Many of the toxic effects induced by TCDD are mediated through the alteration of membrane receptors. Studies conducted on mammals to characterize changes in plasma membrane homeostasis have shown that alterations in EGF receptor binding are a sensitive measure of TCDD exposure. We have shown that a similar mechanism may be operating in fish. As was seen in mammals, TCDD reduced EGF binding in hepatic membranes in a time- and dose-dependent manner. The reduction in binding was associated with an increase in protein kinase C and protein tyrosine kinase activities. While these activities correlated with other biological measures of toxicity (cytochrome P-450 induction), actual documentation of hepatic lesions was not made during these studies. The lack of information makes it difficult to interpret the alterations

of EGF-binding and protein kinase activities with toxicological lesions known to occur at the concentrations used in this study. Studies with the RTH-149 rainbow trout hepatoma cell line demonstrated that alterations in EGF binding and protein kinase activities were correlated with changes in cell proliferation and DNA synthesis. These data provide some evidence for alterations in EGF receptor activity as it relates to TCDD toxicity. However, evidence for the *in vivo* role of the EGF-receptor in TCDD toxicity is needed before this biomarker of TCDD toxicity can be used in any monitoring program.