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Title: _The Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin on_

Acetyl-CoA Carboxylase and Other Lipogenic Enzymes.

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Acetyl-CoA carboxylase (ACC) is the pivotal enzyme in the de

novo synthesis of fatty acids and is the only carboxylase with a

biotin-containing subunit greater than 200,000 daltons. The biotin

moiety is covalently linked to the active site and has a high

affinity (K_d - 10^{-15} M) for the protein avidin. This relationship

has been used in previous studies to identify ACC isolated from

mammalian species. However, ACC has not been isolated and

characterized in a poikilothermic species such as the rainbow trout.

The present study describes the isolation and identification of ACC

in the cytosol of rainbow trout (_Salmo gairdneri_) liver. The enzyme

was isolated using two distinct procedures; polyethylene glycol

precipitation (PEG) and avidin-Sepharose affinity chromatography.

The subunit molecular weight of the major protein was 230,000 daltons

± 3.3%. The PEG precipitation yielded 200 μg protein (4.4 μg/g

liver), with a specific activity of 5 nmoles malonyl-CoA/min/mg

protein, whereas avidin affinity chromatography yielded 1.75 ± 1.1 mg

protein (9.0 μg/g liver), with a specific activity of 1.37 ±

0.18 μmoles malonyl-CoA/min/mg protein.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is an extremely toxic contaminant found in chlorophenoxy herbicides. The compound is preferentially concentrated in liver tissues and has been shown to alter lipid metabolism. It therefore seemed likely that this compound could also affect the enzymes involved with lipogenesis. Due to the extreme toxicity of TCDD and the difficulties involved with handling this compound, it was not possible to study its affects on hepatic ACC in the rainbow trout. Therefore, the experimental model was changed to the rat.

Rats given a single i.p. dose of TCDD and sacrificed nine days later showed a 67% reduction in hepatic ACC activity. ACC mRNA levels were also reduced 40%, but enzyme levels were not appreciably changed. This implies that the pronounced decrease in ACC activity was due to a post-translational modification, while the reduced mRNA levels most likely suggest a long term response which would eventually result in a reduction of hepatic ACC. Indirect evidence indicates that TCDD decreased the amount of fatty acid synthetase. Pyruvate carboxylase levels were also diminished.

The Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin
on Acetyl-CoA Carboxylase and Other Lipogenic Enzymes

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Dr. Daniel P. Selivonchick served in an advisory role throughout the course of this work, as well as assisting with various laboratory procedures in the second manuscript. Dr. Henry W. Schaup served in an advisory role throughout every aspect of this study. In addition, Dr. Schaup provided technical assistance and instruction on the use of various analytical equipment. Dr. Koenraad Marien also served in an advisory role as well as assisting with various laboratory procedures in both manuscripts.

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I. LITERATURE REVIEW

Introduction

The de novo synthesis of fatty acids in animals is carried out by acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS), two complex enzyme systems that function in sequence (1). ACC and FAS are considered to be the primary lipogenic enzymes and are directly involved with the biosynthesis of palmitic acid. The reaction occurs in two distinct steps (2,3). The initial step is the carboxylation of acetyl-CoA yielding malonyl-CoA, by ACC. This is followed by a series of reactions all catalyzed by FAS, in which acetyl-CoA is attached to malonyl-CoA to form the end product (palmitic acid).

The stoichiometry of this reaction is shown below.

$$8 \text{ acetyl-CoA} + 7 \text{ ATP} + 14 \text{ NADPH} + 14 \text{ H}^{\pm} \longrightarrow \text{palmitic acid} \quad (4)$$
$$+ 8 \text{ CoA-SH} + 7 \text{ ADP} + 7 \text{ Pi} + 14 \text{ NADP}^{+} + 6 \text{ H}_2\text{O}$$
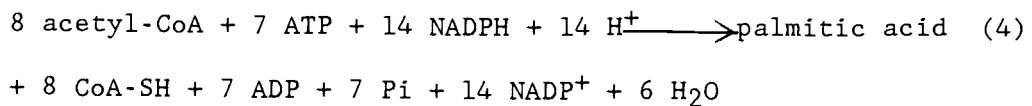
The sequence above is made up of several partial reactions catalyzed by specific enzymes.

The first studies elucidating the mechanism of ACC carboxylations were done in plants and bacteria (4,5). These studies revealed that three enzymes were required to complete the carboxylation reaction. The first was the biotin carboxy carrier protein (BCCP). This peptide possesses a biotin moiety and is responsible for moving the biotin to the second peptide, biotin

carboxylase (BC). BC uses bicarbonate to carboxylate the biotin group. The third enzyme, carboxy transferase (CT), transfers the carbonate from biotin to the substrate acetyl-CoA, yielding malonyl-CoA. The identification of these activities was possible because they can be separated and isolated individually from plants and bacteria. This early work suggested that three separate, but closely associated enzymes were responsible for ACC activity. However, attempts to isolate these components from animal tissue were unsuccessful (6). Additional studies in animals have shown conclusively that the enzyme activities are tightly linked to one another and cannot be isolated (1,5,6).

FAS has seven specific activities associated with its function. This complex enzyme system is responsible for the final steps in fatty acid synthesis resulting in palmitic acid (1). The activities of this enzyme were also first described in plants and bacteria (3,4). However, once again the polypeptides associated with these activities could not be separated in animal preparations. Thus, ACC and FAS are representative of a new class of enzymes - multifunctional enzymes (6-8). Such enzymes are characterized by two distinct properties. First, they consist of a single type of polypeptide chain and second, they exhibit more than one enzyme activity (6,8). The ability of these enzymes to function is dependent upon folding of the enzyme resulting in globular structures or domains, each with its own activity (6).

It is clear that both ACC and FAS are complex and important enzyme systems. These enzymes control lipogenesis, a metabolic pathway that is closely associated with energy usage/storage and

with the synthesis of biological membranes. The structural and biochemical properties as well as the regulation of both enzymes are discussed below with emphasis given to ACC. In addition, some consideration is given to pyruvate carboxylase (PC). The primary role of this enzyme is in gluconeogenesis; however, it is also an important indirect component of lipogenesis (9,10).

A discussion is provided on the toxic properties of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and its known affects on lipogenic enzymes and lipid disposition. Chapter 2 discusses the first identification and purification of ACC from a poikilotherm species and compares its biochemical properties to ACC isolated from homoiotherms. Finally, Chapter 3 examines the effects of TCDD on ACC, FAS, and PC synthesis and activity, in rat liver.

Acetyl-CoA Carboxylase

ACC was first discovered by Wakil et al. (1958) (11) and was shown to be dependent on biotin, ATP, acetyl-CoA, Mn^{2+} and bicarbonate for activity (11). Since then ACC has been shown to be a unique and complex multifunctional enzyme responsible for catalyzing the first committed step in the de novo synthesis of fatty acids; the conversion of acetyl-CoA to malonyl-CoA (1,12). The carboxylation process involves two distinct steps requiring three enzymes; BCCP, BC, and CT (1,4,5,6,13). The first step is the carboxylation of the biotin group of the BCCP domain by BC. This is followed by transfer of the carboxyl group by CT to the substrate acetyl-CoA to form malonyl-CoA,

$$BCCP + HCO_3^- + ATP \xrightleftharpoons{BC,\ Mn^{2+}} BCCP - COO^- + ADP + Pi \qquad (1)$$

$$BCCP + COO^- + CH_3COSCoA \xrightleftharpoons{CT} BCCP + \overset{\displaystyle COO^-}{\underset{|}{CH_2COSCoA}}$$
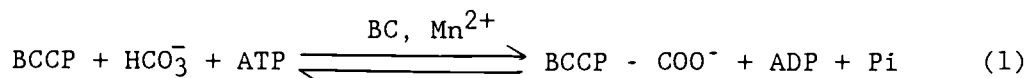
The overall reaction requires several cofactors that include ATP, HCO_3, and Mn^{2+} (1,11). Biotin is essential for ACC activity and is covalently linked to a lysine ϵ-amino group. The molecular arrangement of ACC is extremely complex. The active form of the enzyme is a polymer consisting of more than 40 units ($M_r = 4$-8 million) in close association (6,14). Each protomer ($M_r = 400,000$) is comprised of two identical subunits ($M_r = 230,000$-$260,000$) possessing one mole of biotin and the functions of BCCP, BC, and CT. The active form of the enzyme is the polymer with fully intact subunits (6,14).

Early studies aimed at determining the subunit structure of ACC produced conflicting results (15,16). The subunit was initially described as having two nonidentical polypeptides; one equal to 117,000 daltons and the other equal to 129,000 daltons (15). The biotin moiety was only associated with the 117,000 dalton polypeptide. Subsequent work by Tanabe et al. (1975) (17) showed that ACC was highly susceptible to proteolysis during the purification procedure and that the smaller polypeptides reported earlier were derived from a larger 230,000-260,000 dalton polypeptide. This study showed that the active subunit of ACC is the 230,000-260,000 band and that it is this polypeptide that comprises the active ACC protomer.

ACC has been purified to homogeneity and characterized from several animal tissues including rat liver (17-21), rabbit mammary gland (22,23), chicken liver (24,25) and goose uropygial gland (26). In all cases the enzyme showed essentially the same subunit structure (M_r = 230,000-260,000) and catalytic properties.

ACC and FAS catalyze the synthesis of palmitic acid in the cytosol of cells (4). However, Allred et al. (1985) (27) has recently provided evidence suggesting the existence of a mitochondrial form of the enzyme. The mitochondrial ACC is inactive and has a slightly larger subunit molecular weight. Additionally, it was shown that the amount of mitochondrial ACC versus cytosolic ACC is determined by diet (27). Further, rats made diabetic with injections of alloxan (28) showed a decrease in ACC activity. This decrease in cytosolic ACC activity is accompanied by a decrease in the amount of cytosolic enzyme with a concomitant increase in mitochondrial enzyme. These data suggest that the mitochondria may provide a storage depot for carboxylase and that movement to and from this subcellular storage compartment is mediated by diet and hormones. ACC has also been shown to be closely associated with, but not bound to, endoplasmic reticulum (29,30). The enzyme associated with this fraction is catalytically active.

ACC is an extremely large enzyme with a subunit molecular weight of 230,000-260,000 daltons. Correspondingly, it has a large mRNA (~10 kilobases) (20,24), present in extremely small amounts in cells (31). These properties have made molecular characterization of the enzyme difficult experimentally. Recently, the entire primary structure of ACC has been sequenced in both rat (20) and chicken

liver (32). In rat liver cDNA clones isolated from a λgt11 library were shown to direct the synthesis of recombinant proteins. These proteins were subsequently shown to react with anti-ACC serum. By isolating and then sequencing cDNA clones that correspond to the entire ACC coding region, a cDNA derived sequence of the primary structure of ACC was obtained. The protein consists of 2346 amino acids with the biotin containing BCCP region located roughly 800 bases from the 5'-end. The sequence of the biotin binding region is shown below.

$$\begin{array}{ccccccc} \text{GTAATGAAGATGGTAATGACT} & & & & & & \text{(20)} \\ \text{V} & \text{M} & \text{K} & \text{M} & \text{V} & \text{M} & \text{T} \end{array}$$

Although this region is highly conserved among all the carboxylases at the amino acid level, differences in the third base of several codons is apparent. Also, while valine (V) precedes the "M K M" sequence in ACC, alanine precedes this sequence in all other known biotin proteins. For comparison the cDNA and amino acid sequence for the biotin binding region of rat and chicken liver ACC are shown.

| Rat (ACC) | GTAATGAAGATGGTAATGACT | (20) |
| Chicken (ACC) | GTGATGAAAATGGTGATGACA | (32) |

These studies have provided information essential to studying the synthesis and regulation of ACC at a molecular level.

Fatty Acid Synthetase

Fatty acid synthetase (FAS) like ACC is a large and complex multi-enzyme system (33,34), located in the cytosol and responsible

for the synthesis of long chain fatty acids from acetyl-CoA and
malonyl-CoA precursors (1). The primary end product of this enzyme
is palmitic acid and its synthesis requires seven sequential
reactions. In prokaryotic systems all seven enzymes are loosely
associated and hence could be separated and isolated individually
(3,4). In animals the enzymes are covalently combined forming a
single polypeptide chain (M_r = 250,000) (1). The active synthetase
unit in animals consists of two identical subunits arranged in a head
to tail manor (1). Each subunit possesses a prosthetic group
(4'-phosphopantetheine) that is attached to a peptide responsible for
binding all acyl-intermediates, thus, it is called the acyl-carrier
protein (ACP) (1). Analyses have shown that the entire FAS subunit
is synthesized from a single mRNA, 16 kilobases, in length (35,36).

The complex multifunctional nature of FAS has led to studies
aimed at determining its native arrangement. It is now believed that
FAS as well as other multifunctional enzymes form globular domains
that are connected by polypeptide bridges. These bridges are more
sensitive to proteolytic cleavage than the globular regions
(1,37,38). This characteristic structure has made it possible to
isolate active regions using various proteolytic enzymes (1). To
this end chymotrypsin exhibited the most controlled cleavage by
hydrolyzing the subunits into only two pieces. One fragment of
M_r = 230,000 and the other of M_r = 33,000 (1,39). These studies
enabled investigators to construct a two-dimensional diagram of the
synthetase polypeptide. The polypeptide is divided into three
domains with each domain possessing a set of enzyme activities. The

diagram below depicts the head to tail arrangement of the subunits as well as their functional division.

FUNCTIONAL
DIVISON

Substrate
Entry

AcCoA

MalCoA

AT

KS

cys

MT

Reduction

DH

KR ACP

ER

TE Palmitate
Release

Chain HS Elongation

HS

Chain SH Elongation

SUBUNIT
DIVISON

(1)

Palmitate
Release

TE

ACP KR

ER

DH

Reduction

MT

cys

SH

SH

KS

AT

MalCoA

Substrate
Entry

AcCoA
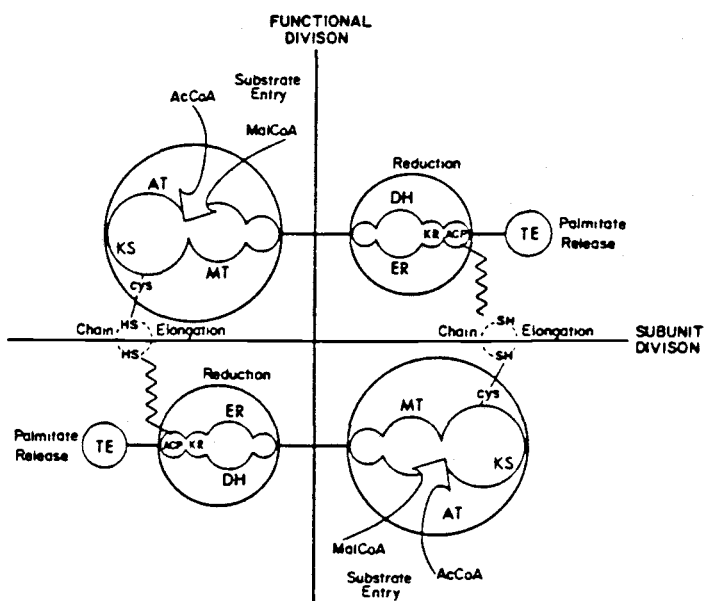
Substrate entry and chain elongation occurs at Domain I which contains acetyl and malonyl transacylases (AT, MT) as well as the condensing enzyme site. The region responsible for the reduction of the carbonyl carbon to a methylene group, by β-ketoacyl reductase (KR), dehydratase (DH), and enoyl reductase (ER) activities is Domain II. Domain III, possesses the acyl-carrier protein (ACP) and the 4'-phosphopantetheine prosthetic group, and is responsible for chain termination and palmitate release.

Pyruvate Carboxylase

Pyruvate carboxylase (PC) is a biotin-containing enzyme predominantly located in the mitochondria of cells (40). The native

enzyme exists as a tetramer of identical subunits (M_r = 130,000) (40). The enzyme is found in large quantities in liver, kidney, lactating mammary gland, and adipose tissue. PC catalyzes the first committed step in gluconeogenesis; the energy-dependent fixation of carbon dioxide to pyruvate resulting in the formation of oxaloacetate (41). PC activity is regulated by availability of substrate (pyruvate) (9,42,43), allosterically (9,42,44) by ADP (negative) and acetyl-CoA (positive), and by covalent modification (9,42,45).

In addition to its role in the formation of glucose, PC is also considered an important lipogenic enzyme (9). PC functions in this capacity by transporting acetyl groups, as citrate, and reducing equivalents as malate from the mitochondria to the cytosol (9). In addition, PC activity is high in both adipose and liver (9,46) tissue; the primary sites of fatty acid synthesis. It has also been shown that the mouse preadipocyte cell line 3T3-L1, accumulates lipid upon differentiation. This increase in lipid has been correlated to increases in the activities of lipogenic enzymes (9). ACC (47), FAS (48,49), and PC (50) levels all increase in parallel during the development of these cells, suggesting a close association between ACC, FAS, and PC.

Regulation of Acetyl-CoA Carboxylase

A. Allosteric Mechanisms

ACC is an allosteric enzyme that is controlled positively by citrate and negatively by long chain fatty acids. Citrate is the primary source of cytosolic acetyl-CoA. When a citrate molecule

binds to ACC the enzyme undergoes a conformational change, followed by polymerization into a filamentous form (51,52). Dissociation of the polymerized form back to the protomeric form causes inactivation. This conversion process is controlled by citrate and long chain fatty acids. Thus, early studies suggested that the primary mechanism of ACC regulation was by allosteric processes, via changes in the balance between active polymers and inactive protomers (4,5,6,12).

The citrate activation constant K_a for ACC is between 3 mM and 8 mM (53,54). In most species examined thus far, 10 mM is used to achieve maximal activity in vitro assays. There is little doubt that citrate causes a positive stimulation of ACC in vitro; however, its significance in vivo is still unclear (12). Total citrate levels in the liver of fed rats is estimated between 0.3 mM and 1 mM; these values are well below the levels reported for maximal activity in vitro (12). Several studies have attempted to correlate changes in cellular citrate levels with changes in the rate of fatty acid synthesis (23,55,56,57). Establishing this relationship has been difficult because a satisfactory method for measuring metabolically active compounds, such as citrate, has not been established (12). It is clear that citrate acts as a stimulator of ACC in vitro, but its significance in vivo has yet to be shown.

Long chain fatty acids inhibit ACC by causing depolymerization (12,58). A mole of palmityl-CoA per mole ACC inhibits the enzyme. The inhibition constant K_i for palmityl-CoA is 5 nM (12). There is some evidence suggesting that these levels are significant in vivo. However, recent studies by Mikkelsen and Knudsen (1987) (59) report that while the total concentration of fatty acyl-CoAs in the rat

hepatocyte is 1-10 μM, the amount of unbound or free fatty acyl-CoA actually present may be significantly less due to an abundance of cytosolic fatty acid/acyl-CoA binding proteins. Thus, the significance of the allosteric mechanism of ACC regulation, in vivo is still under consideration.

B. Phosphorylation

Covalent modification such as phosphorylation-dephosphorylation has been extensively investigated over the past few years. Early studies by Inoue and Lowenstein (1972) (15) reported that ACC was a phosphoprotein and proposed that its activity may be determined by its phosphorylation state. In 1977, Lee and Kim showed that the phosphorylation-dephosphorylation of ACC and the associated decrease or increase in activity occurs in the enzyme itself (60). In these experiments ACC was purified from rat liver. The final preparation contained the enzyme and a kinase which catalyzes the phosphorylation of ACC. Radiolabeled phosphate was added to the purified enzyme and incorporated activity was determined. These experiments showed that the incorporation of phosphate into the enzyme was inversely proportional to activity. Subsequent studies (22) revealed that the purified ACC preparation was contaminated with a cyclic-AMP-dependent kinase activity. This was evidenced by the fact that when cAMP was added to the preparation, phosphorylation proceeded at a much faster rate. In order to elucidate the mechanism of phosphorylation which occurred in the absence of cAMP, a potent inhibiter of the cAMP-dependent kinase was employed.

Four preparations were prepared as follows:

A) Contained enzyme, cAMP and phosphate

B) Contained enzyme, cAMP, phosphate, and kinase inhibitor

C) Contained enzyme, phosphate

D) Contained enzyme, phosphate and kinase inhibitor

Reaction (A) showed a high rate of phosphate incorporation. When the kinase inhibitor was added (B) the rate of incorporation was dramatically decreased. However, the suppression was equal to the rates observed in reaction (C) which contained no cAMP, only enzyme and phosphate. When the kinase inhibitor was included in this preparation (preparation D) there was no significant affect on the phosphate incorporation rates. These data indicate that the phosphorylation of ACC in the absence of cAMP is catalyzed by a different kinase. These authors suggest that this kinase should be called acetyl-CoA carboxylase kinase-2. They further conclude that purified ACC is contaminated with both a cAMP dependent kinase and an acetyl-CoA carboxylase kinase-2.

In contrast Pekela et al. (1978) (61) and Tipper et al. (1983) (62) showed that phosphorylation of ACC from rat liver and from cultured chicken liver cells had no affect on ACC activity. Moreover, several studies have shown that in fat cells exposed to insulin, ACC activity increases. This increase is due to phosphorylation not dephosphorylation of the enzyme (63,64). Thus, there was some disagreement as to the role of phosphate groups in the regulation of ACC activity. More recently, it has been shown that ACC purified by a avidin affinity chromatography possesses little

activity in the absence of citrate (18). The enzyme was shown to possess 8.3 mol phosphate/mol subunit. Addition of citrate (10 mM) resulted in a 10-fold increase in activity. This study also showed that a protein (M_r = 90,000) isolated from rat liver can activate the purified ACC enzyme 10-fold in the absence of citrate (18). The protein was identified as a manganese (Mn^{2+}) dependent phosphoprotein phosphatase. Analysis of the dephosphorylated enzyme following incubation with the purified phosphatase showed 5.0 mol phosphate/mol subunit. Although this value is less than the 8.3 mol phosphate/mol subunit reported for the phosphorylated enzyme, it is clear that phosphate groups are still associated with the enzyme. This suggests that the number of phosphate groups may not be as significant as the site of phosphorylation.

This study is significant because it is the first example of high levels of ACC activity in the absence of citrate. More evidence for the regulatory effects of phosphorylation-dephosphorylation was presented by Thampy and Wakil (1988) (14). In this study the effects of a fast/refeed diet regime on the phosphorylation state and activity of purified rat liver ACC were investigated. This feeding protocol resulted in an increase of ACC specific activity with a subsequent decrease in phosphate content. Moreover, when ACC from 48 hr fasted rats was incubated with acetyl-CoA carboxylase-phosphatase-2, the enzyme was activated in the absence of citrate. This indicated that the low activity observed initially was due to an increased phosphate content. These investigators also established the existence of two polymeric forms of the enzyme. One is a highly active polymer consisting of more than 40 subunits. The other a less

active polymer consisting of only 8 subunits. The large polymer is
most abundant in the livers of fed rats while the octamer
predominates in fasted rats. In addition, the octamer can be
converted to the highly active polymer by dephosphorylation (14,65).
This study has again shown the importance of the phosphate in the
regulation of ACC activity and has provided a mechanism for the
observed increase in the activity of ACC in rats that are fasted then
refed.

It is now clear that several kinases, including cAMP and
Ca^{2+}/calmodulin-dependent protein kinases, protein kinase C, casein
kinase-2 and AMP-phosphokinase are capable of phosphorylating ACC
(66). There are seven major sites of phosphorylation within the
carboxylase subunit (66,67,68,69). The locations of these sites in a
rat and chicken subunit are shown below.

(66)
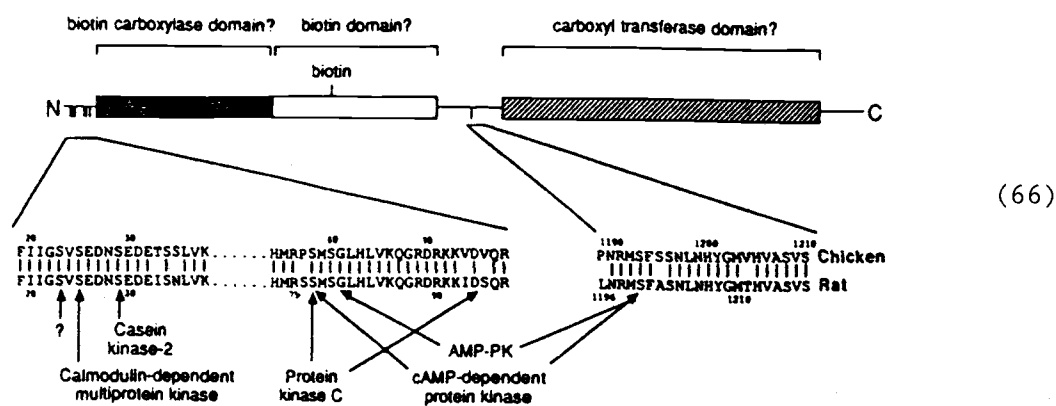
These sites are highly conserved between chicken and rat, except for
Ser95 which is not seen in the chicken sequence. Most of the sites
are located in the N-terminal half of the protein in a protease
sensitive region (66). The cAMP dependent kinases that phosphorylate

sites Ser77 and Ser1200 cause a slight inactivation of the carboxylase. In contrast, the sites phosphorylated by AMP-phosphokinase (Ser79 and Ser1200) cause a marked reduction in ACC activity (67). Studies by Sim and Hardie (1988) (70) showed that Ser79 and Ser1200 are phosphorylated in both hepatocytes and adipocytes and that phosphorylation increases with addition of cAMP elevating agents. However, Ser77 is not phosphorylated under any of the conditions examined. This information suggested to the authors that AMP-phosphokinase is the important regulator of ACC, and that ACC is not a physiologic substrate for cAMP dependent kinase.

Thus, new information concerning the role of phosphorylation in the regulation of ACC activity, suggests that this mechanism may be more important in vivo than allosteric regulators.

Hormonal Regulation of Acetyl-CoA Carboxylase, Fatty Acid Synthetase, and Pyruvate Carboxylase

Several studies have shown that fatty acid synthesis rates and ACC activity increase with exposure to insulin or high carbohydrate diets (14,63,64,71,72,73). There is, however, some disagreement as to the mechanism of the observed increase in ACC activity (14,63,64). The de novo synthesis of long chain fatty acids is significantly influenced by many hormones, including insulin (72,73,74,75), thyroid (74,76,77,78), glucagon (79), glucocorticoids (75), and adrenaline (80). Only insulin and thyroid hormones will be dealt with in the following discussion because serum levels of these hormones are affected by TCDD, and because the objective of the present study is to determine the effects of TCDD on lipogenesis.

It has been well established that rats fasted then refed a high carbohydrate diet show an increase in the synthesis and activity of lipogenic enzymes. It is believed that this inductive response is due to a rapid increase in serum insulin levels during this fast-refeed regime. This in turn results in insulin-induced changes in the phosphorylation state of ACC.

ACC isolated from rat epididymal fat pads exposed to insulin revealed a 2-fold increase in ACC activity (71). Subsequent studies by the same group (64) showed that insulin increased cAMP-dependent kinase activity, and that increased phosphorylation of ACC was the cause of enhanced activity. These data contradict what has been generally accepted as the role of phosphorylation on the activity of ACC, that phosphorylation causes inactivation while dephosphorylation causes activation (14). It should be mentioned that the studies producing these data were done with ACC from adipocytes and it has been proposed that regulation of ACC in adipocytes may be different. More conclusive studies are needed to determine the mechanism of regulation in adipocytes.

Other investigators have also shown that insulin increases ACC activity. This enhanced activity is due to dephosphorylation of the enzyme (14,18,65). Thus, increased ACC activity by insulin can be attributed to activation of a phosphatase enzyme and subsequent dephosphorylation of ACC. In addition to ACC, several lipogenic enzymes are positively affected by insulin; these include FAS and malic enzyme (ME) (81,82,83,84). Kinetic studies examining the rates of synthesis and degradation of ACC, FAS, and ME indicate that regulation in the long term by insulin stimulation is due to

increased enzyme synthesis. When insulin levels are normal or below normal, enzyme degradation exceeds synthesis. However, the rate of degradation is not altered (85). It is important to note that with insulin treatment the initial increase in ACC activity was observed prior to changes in mRNA levels (75). This implies that enzymes, such as ACC, that are highly regulated by phosphorylation and allosteric mechanisms, may be controlled initially by post-translational mechanisms, while long term regulation is mediated at the level of transcription/translation.

The thyroid hormone triiodothyronine (T_3) has also been shown to alter lipogenic rates (82,86). ACC hepatic activities are increased with increasing levels of T_3 (77,78). Diamant et al. (1972) (78) reported that ACC and FAS activities are dramatically increased upon administration of T_3 to rats and suggested that this increase is due to increased rates of protein synthesis. It is evident that insulin and thyroid T_3 are lipogenic hormones capable of stimulating fatty acid synthesis by increasing the activities of the primary lipogenic enzymes. Further, this stimulation can be caused by increased synthesis of the enzyme and/or regulation of the enzyme itself; covalent modification and/or allosteric mechanisms.

Since it is difficult to affect the synthesis and activity of ACC without affecting FAS, a brief discussion on the hormonal regulation of FAS follows. FAS has not been reported to be an allosteric enzyme, or a phosphoprotein as in the case of ACC. The enzyme is, however, highly regulated by dietary and hormonal influences (77,78,81). It appears that the same hormones affecting ACC also affect FAS (77). The difference is in how the influence is

mediated. As mentioned above, insulin and thyroid hormones cause a marked increase in lipogenesis. Both ACC as well as FAS are stimulated by insulin or T_3 exposure. The initial response of ACC to insulin or T_3 is a rapid increase in activity. This initial increase in activity occurs prior to changes in its mRNA or enzyme quantities (75). In contrast FAS is regulated rapidly and efficiently at the level of transcription and translation (86). For example, administration of T_3 to rats causes a significant increase in hepatic FAS mRNA and enzyme activity (85,86). In order to determine if active protein synthesis is necessary in order to see T_3-induced synthesis of FAS mRNA an inhibitor (puromycin) of total protein synthesis was included in the experiment. Under these conditions FAS mRNA levels did not increase. Therefore, ongoing protein synthesis is required in order for T_3 to cause its stimulatory affect on mRNA synthesis. These data were interpreted to mean that T_3 regulates FAS activity by controlling the synthesis of its mRNA. The same study showed that while T_3 controlled FAS synthesis and activity at the level of transcription, insulin which has also been shown to stimulate FAS activity had no affect on mRNA synthesis, but did increase enzyme activity 3-fold (86). When T_3 was present in the incubation medium with insulin, mRNA levels increased 2-fold with a corresponding 14-fold increase in enzyme synthesis, as compared to hepatocytes incubated with T_3 only. Thus, insulin exerts its affects on the translational efficiency of pre-existing mRNA, while T_3 controls mRNA synthesis (83,86).

Additional evidence for regulation of FAS at the level of transcription is reported by Paulauskis and Sul (1988) (83). Mouse

liver mRNA, enriched for high molecular weight mRNA by sucrose density gradient centrifugation, was used to prepare cDNA. This was subsequently cloned and the clones containing the FAS cDNA sequences were identified. The cDNA segment was used as a probe to monitor the levels of FAS mRNA in mouse liver and in 3T3-L1 adipocyte cells. Mice that were fasted then refed showed a significant increase in FAS mRNA levels. Treatment of the 3T3-L1 cells with insulin elicited a 3-fold increase in both FAS mRNA and enzyme quantities. These results suggest that control of FAS enzyme levels and activity are at the level of transcription and translation. From the discussion above it is clear that FAS is stimulated by insulin, T_3, and fast/refeed diets (81,82,83,84,86,87). This stimulation is at the level of transcription, as evidenced by changes in mRNA levels (82,83,86) or at the level of translation as judged by alterations in translation efficiency (83,86,88). These properties of regulation are considerably different from those described for ACC, which is initially controlled post-translationally.

PC activity is also altered in the presence of lipogenic hormones. In 1979, Weinberg and Utter (42) compared the effects of thyroid status on the rate of synthesis and concentrations of PC in rat liver. PC activity in the livers of hyperthyroid rats was twice the values of normal rats. Hypothyroid rats showed a significant decrease in activity. By utilizing immunochemical techniques it was shown that the observed changes in PC activity were controlled by enzyme quantity.

In another study (89), PC activity was shown to increase 2-fold in the livers of diabetic (i.e. low insulin) rats. The observed

change in activity was associated with an increase (2.0 to 2.5-fold) in the rate of PC synthesis. Thus, under the influence of either thyroid or insulin, PC activity like FAS activity is determined by enzyme levels.

2,3,7,8-Tetrachlorodibenzo-p-dioxin and Lipids

One of the most toxic compounds known today is the dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This compound is not readily metabolized and is persistent both in the environment and in organisms. TCDD is a contaminate formed during the production of chlorophenoxy herbicides, an example of which is 2,4,5-trichlorophenoxy acetic acid, (2,4,5-T). The first step in the manufacturing process of 2,4,5-T occurs when 1,2,4,5-trichlorobenzene is reacted with methanol and sodium hydroxide to form 2,4,5-trichlorophenol (2,4,5-TCP). If the temperatures are not tightly regulated during this phase, highly toxic dioxin compounds such as TCDD can be formed (90).
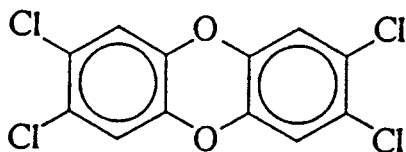
2,3,7,8-Tetrachloro-dibenzodioxin

TCDD is extremely toxic with LD_{50} values that vary significantly depending on the species, strain, and substrain of animal used (91). In a recent study TCDD toxicity was examined in two strains of rats; the Han/Wistar and Long-Evans. This study showed that the Han/Wistar

rat was more resistant to TCDD (LD_{50} of 3000 μg/kg), while the Long-Evans strain was more sensitive (LD_{50} of 10 μg/kg) to TCDD (92,93). In another study TCDD's lethal toxicity was compared in four substrains of Fischer rats. LD_{50} values from this study ranged from 164 μg/kg to 340 μg/kg (94). It is apparent that the toxicity of this compound is not only dependent upon dose and time of exposure, but also to species, strain, substrain, and sex (91-95).

Due to its hydrophobic nature, TCDD distributes itself throughout the body using chylomycrons and other lipoproteins. The primary site of accumulation is the liver, followed by the adipose tissue (96). Thus, the liver is considered the primary target organ. Fatty liver is a common toxic response to low and high doses of TCDD. These studies have provided the impetus for several investigations into TCDD's affect on hepatic lipids.

Albro et al. (1978) (95) examined the effects of TCDD on various serum and liver lipid classes. In this study female Fisher rats were dosed orally with 10, 50, and 100 μg/kg TCDD in corn oil. The animals were sacrificed 1, 3, 10, 14, or 21 days later. Fatty liver developed at all doses, as evidenced by an increase in free fatty acids, phospholipids, triacylglycerol, and cholesterylesters. When the fatty acid composition of the various hepatic lipids was examined, it was found that liver triacylglycerols had a significant (p < .05) reduction in palmitic (16:0) acid, while stearic (18:1) and linoleic (18:2) acids were increased.

Alterations in hepatic and serum lipid composition were also observed by Swift et al. (1981) (97). Using guinea pigs, Swift showed that the animals became hyperlipidemic as judged by a 19-fold

increase in serum very low density lipoproteins (VLDL) and a 4-fold increase in serum low density lipoproteins (LDL). The size of VLDL lipoproteins in control and experimental preparations were similar, however, VLDL from TCDD animals contained less cholesterylester and showed variations in C apoproteins when analyzed on sodium dodecyl sulfate-polyacrylamide gels. LDL from TCDD animals contained more phospholipid and apoprotein C, but less protein than LDL from control animals. Serum levels of linoleic (18:2) acid were also increased in exposed animals. This is the principle fatty acid found in adipose tissue, suggesting an increased mobilization of adipose tissue fatty acids. Indeed, it has been shown that adipose tissue hormone sensitive lipase activity is stimulated by TCDD exposure (97).

Two of the most obvious and reoccurring morphological signs of TCDD toxicity are: 1) the "wasting syndrome," characterized by decreased food intake, loss of weight and death; and 2) liver enlargement (hepatomegaly) due to increased fat content (95,97,98,99,100). It is clear from the preceding discussion that TCDD causes substantial alterations in hepatic and serum lipid profiles. Recent studies by Lakshman et al. (1988) (100) using rats treated with a single sublethal dose of TCDD showed that hepatic fatty acid synthesis was decreased relative to pair-fed controls, in a dose dependent manor, after 7 days. This reduction in synthesis was quite substantial (62%) in animals receiving 20 μg/kg TCDD. Subsequent investigations by the same group (101) examined the effects of TCDD on the activities of the primary lipogenic enzymes, ACC and FAS. The report revealed that both ACC and FAS activities were inhibited in a progressive and dose dependent manor. After a

two week exposure to 10 μg/kg TCDD, ACC activity was inhibited 46% (p < .01), while FAS activity was inhibited 78% (p < .001). This study, however, did not describe a mechanism for the observed inhibition nor were the actual quantities of the enzymes measured.

Recent studies using plants sprayed with chlorophenoxy herbicides have shown that ACC is specifically inhibited and that this results in plant mortality (102,103,104). It was proposed that death caused by ACC inhibition is due to a decrease in acyl-lipid and membrane synthesis.

In addition to disruption of hepatic lipid metabolism, TCDD also affects hormone levels. Thyroxine (T_4) and insulin, are two important hormones responsible for controlling the physiologic pathways involving hepatic carbohydrate and fat metabolism (105). Potter et al. (1983) (106) reported that TCDD (45 μg/kg) administered to rats caused a decrease in T_4, one week after dosing. Pancreatic and serum insulin levels were also reduced by 25% and 76%, respectively. Rozman et al. (1984) (107) reported that thyroidectomized rats were protected from TCDD toxicity, when given high doses (100 μg/kg). In this study, rats with intact thyroid showed a 70-80% mortality compared to thyroidectomized animals. Feed intake in thyroid competent animals decreased while no change in feed intake was observed in thyroid incompetent animals. These data suggest that thyroid hormones may play an important role in mediating TCDD toxicity.

In a more recent study (108) serum levels of T_4, T_3, and thyroid stimulating hormone (TSH), were evaluated in TCDD-exposed animals. The data revealed that T_4 levels were greatly decreased, T_3 levels

were increased, while TSH decreased with increasing dose. These data suggest that at high doses TSH is decreased, this would inhibit the animals ability to release more T_4. The T_4 already present would be converted to T_3, which accounts for the decrease in T_4 levels while T_3 increases. Thus, over a longer exposure period one would expect T_3 levels to decrease rapidly. Gorski and Rozman (1987) (109) examined the effects of dose and time on thyroid and insulin serum levels in rats exposed to TCDD. This work showed a decrease in T_4 levels by day 8. T_3 was maximally suppressed by day 8, but returned to normal levels by day 16, then decreased steadily by day 32. Suggesting again that T_4 is converted to T_3 thus reducing its concentration. Once TSH is sufficiently inhibited T_3 levels begin to drop. Insulin levels were also decreased by day 16 then showed rapid recovery, even beyond normal levels. This hyperinsulin effect was attributed to an increased sensitivity or up regulation of the insulin receptor.

It is evident from the preceding discussion that TCDD has a profound affect on lipid distribution, composition, and synthesis. Additionally, TCDD decreased the serum levels of thyroxine and insulin; two important hormones that control key metabolic pathways. The primary objective of the present study was to examine the effects of TCDD on the activity and synthesis of ACC, and other lipogenic enzymes, and to propose a mechanism for its toxicity.

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II. Isolation and Identification of Acetyl-CoA Carboxylase
     From Rainbow Trout (<u>Salmo gairdneri</u>) Liver

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ABSTRACT

Acetyl-CoA carboxylase (ACC) is the pivotal enzyme in the de novo synthesis of fatty acids and is the only carboxylase with a biotin containing subunit greater than 200,000 daltons. The biotin moiety is covalently linked to the active site and has a high affinity ($K_d = 10^{-15}$ M) for the protein avidin. This relationship has been used in previous studies to identify ACC isolated from mammalian species. However, ACC has not been isolated and characterized in a poikilothermic species such as the rainbow trout. The present study describes the isolation and identification of ACC in the cytosol of rainbow trout (Salmo gairdneri) liver. The enzyme was isolated using two distinct procedures; polyethylene glycol precipitation (PEG) and avidin-Sepharose affinity chromatography. Identification of the isolated protein as ACC was made by the following: 1) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); 2) avidin binding; 3) in vivo labeling with [14C]biotin; and 4) ACC specific activity. The subunit molecular weight of the major protein was 230,000 daltons $\pm$ 3.3%. This protein was shown to bind avidin ($M_r = 16,600$) prior to SDS-PAGE, indicating the presence of biotin. In addition, protein isolated from fish that had previously received intraperitoneal injections of [14C]biotin, showed the majority of radioactivity associated with the 230,000 dalton protein. The PEG precipitation yielded 200 μg protein (4.4 μg/g liver), with a specific activity of 5 nmoles malonyl-CoA/min/mg protein, whereas avidin affinity chromatography yielded 1.75 $\pm$ 1.1 mg protein (9.0 μg/g liver), with a

specific activity of 1.37 ± 0.18 μmoles malonyl-CoA/min/mg protein.
The enzyme was citrate dependent showing maximum activity between
10 mM and 20 mM. ACC specific activity decreased by 50% in the
presence of 0.2 M NaCl. These findings suggest that the major
protein (M_r = 230,000) purified from rainbow trout liver is ACC with
enzyme characteristics comparable to mammalian ACC.

INTRODUCTION

Acetyl CoA-carboxylase (ACC) [EC 6.4.1.2] is a biotin-containing enzyme responsible for the first committed step in the de novo synthesis of fatty acids (1-3). The enzyme has been isolated and characterized from several mammalian tissues, including rat liver (4-7), rat mammary gland (8) and rabbit mammary gland (9,10), as well as from chicken liver (11) and goose uropygial gland (12). These studies reported a subunit (M_r) for ACC that ranged from 225,000 to 260,000 daltons. However, this enzyme has not been characterized or purified to homogeneity in a poikilothermic species.

The rainbow trout (Salmo gairdneri) is currently being used as an alternative vertebrate model (13-15) for testing the carcinogenic effects of environmental and dietary toxicants, such as nitrosamines (16,17), aflatoxin B_1, and cyclopropenoid fatty acids (CPFA) (18-20). These studies have shown that the liver is the primary site of toxicity for these compounds. Because the liver is also the principle organ for lipogenesis in fish (21) it is possible that these compounds as well as others may adversely affect the primary lipogenic enzymes ACC and/or fatty acid synthetase.

In this laboratory, preliminary studies using rainbow trout identified ACC (M_r = 220,000) from the microsomal fraction of liver homogenates and revealed that the enzyme was closely associated with, but not bound to liver endoplasmic reticulum. It was also observed that fish exposed to dietary CPFA showed a significant reduction in liver microsomal ACC (22). The presence of this enzyme in the microsomal fraction is of interest because it is well known that the

biochemical pathway involved with lipogenesis occurs in the cytosol of cells. Previous studies (4-12) have, therefore, concentrated on isolating the enzyme from the soluble fraction of tissue preparations. Recently, other workers have reported mammalian ACC to be associated with the mitochondria (23) and endoplasmic reticulum (24). These investigations not only support our earlier findings in trout, but also suggest that fatty acid synthesis and, hence, ACC are not confined to the cytosol.

Before toxicity data obtained from a poikilotherm model can be used to predict similar effects in a homoiotherm model it is necessary to fully understand the similarities and differences of the enzyme affected in both systems. We report here the first purification of ACC from the cytosol of rainbow trout liver.

MATERIALS AND METHODS

Chemicals

[^{14}C]Biotin and [^{14}C]sodium bicarbonate were purchased from Amersham, Inc. (Arlington Heights, IL). Polyacrylamide (99%), protein standards and all other electrophoresis supplies were obtained from Bio Rad, (Richmond, CA). All other reagents were purchased from Sigma Chemical, Co. (St. Louis, MO); and were of the purest grade available.

Animals and Diets

Mt. Shasta strain rainbow trout (Salmo gairdneri) were used for all analyses. These were spawned and reared at the Oregon State University Food Toxicology and Nutrition Laboratory. The control diet (diet A) consisted of a dextrose and casein-gelatin mixture containing 10% lipid. The test diet (diet B) contained 20% carbohydrate with no lipid or biotin. All animals were approximately 1 yr of age and weighed 300 to 700 g.

Isolation of Acetyl-CoA Carboxylase

Fish were killed by a sharp blow to the head and the livers were excised immediately and placed into ice cold homogenization buffer (Tris-HCl, 100 mM, pH 7.4; sucrose, 0.25 M; EDTA 2 mM; β-mercaptoethanol, 15 mM; and phenylmethylsulfonyl fluoride, 0.4 mM) as a rinse. After rinsing, the livers were placed into an ice cold glass blender pitcher, that contained a small volume of the homogenization buffer. When all livers had been removed and placed in the pitcher, three volumes of ice cold homogenization buffer were

added and the livers homogenized by pulsating (5 sec intervals) at high speed for 20 sec. The homogenate was allowed to settle and the foam was removed by aspiration. The homogenate was filtered through cheese cloth to remove fibrous materials and then centrifuged at 100,000 x g for 1 hr at 4°C. The supernatant was subjected to ammonium sulfate precipitation (35% of saturation) and two 3% polyethylene glycol (PEG) precipitations as described previously (9). Protein concentrations were determined either by absorbance at 280 nm or the Bradford assay (25).

Acetyl-CoA Carboxylase Activity

ACC activity was assayed using the $[^{14}C]$bicarbonate fixation procedure (26,27). The reaction buffer, (Tris-Acetate, 70 mM, pH 7.5; potassium acetate, 120 mM; reduced glutathione, 4 mM; potassium citrate, 10 mM; ATP, 1 mM; bovine serum albumin, 0.6 mg/ml; acetyl-CoA, 0.35 mM; and magnesium acetate, 10 mM) was essentially as described by Allred and Roehrig (26) with the following modifications: nonlabeled bicarbonate was omitted to increase sensitivity, and samples were incubated with 7 mM (0.25 μCi) of sodium $[^{14}C]$bicarbonate (0.1 μCi/μmole) at 37°C for 2-3 min. Final reaction volumes were 0.5 ml and reactions were started by addition of enzyme. Controls were performed in the absence of enzyme or acetyl-CoA.

Binding Avidin to PEG Isolated Acetyl-CoA Carboxylase Prior to SDS-PAGE

Ten fish (45 g liver tissue) were killed and prepared according to the method previously described. Avidin was bound to ACC

essentially as described by Goodson et al. (1984) (5). ACC (3 μg)

was added to 0.15 ml of sample buffer (Tris-HCl, 0.0625 M, pH 6.8;

4% sodium dodecyl sulfate (SDS) w/v; 2% dithiothreitol; 5% glycerol

and 0.003% bromophenol blue) and the mixture was heated 2-3 min at

95°C. After cooling, 50 μg of N-acetylated monomeric avidin was

added and the mixture was allowed to stand for 5 min. The sample was

loaded onto an acrylamide gel (18.0 cm x 16.5 cm x 0.2 cm) consisting

of a 3.5% stacking gel and a 5% resolving gel (28). The sample was

electrophoresed at 21 mA constant current for 7 hr, stained overnight

with Coomassie Blue and destained with a 50% methanol, 10% acetic

acid solution for several hours.

In Vivo [^{14}C]Biotin Incorporation Into Acetyl-CoA Carboxylase

Ten fish (450-600 g) were fasted for 1 month, then fed diet B

for 5 days. During this feeding period, each fish received daily

i.p. injections of 2 μCi [^{14}C]biotin in 0.2 ml distilled water.

After sacrifice, ACC was isolated by the PEG procedure run on

SDS-PAGE and stained Coomassie Blue, as previously described. The

gel was sliced into 2 mm segments, each of which was placed into a

1.5 ml Eppendorf micro centrifuge tube and dried in an oven at 60°C.

Following the addition of 0.1 ml 30% hydrogen peroxide, each segment

was incubated again at 60°C to solubilize the gel. Once solubilized,

each tube received 0.4 ml of water and 5.0 ml ACS scintillation fluid

and the radioactivity in each segment was determined using a Beckman

model LS 3801 scintillation counter as previously described (7).

Avidin Affinity Chromatography

Avidin was cross-linked to the Sepharose according to Thampy and Wakil (4) as modified from Beaty and Lane (11). The biotin binding capacity of the column was 13 nmoles/ml of packed column. Approximately 200 g of liver tissue was removed and prepared as described above, except that the PEG precipitations were omitted. The 100,000 x g supernatant was subjected to ammonium sulfate precipitation (35% or 26% of saturation) and the pellet collected by centrifugation at 20,000 x g for 20 min. The $(NH_4)_2SO_4$ pellet was resuspended in a minimum volume of column buffer (Tris-HCl, 100 mM, pH 7.5; EDTA 1.0 mM; dithiothreitol 1.0 mM; NaCl 0.5 M; glycerol 5.0% (v/v) and phenylmethylsulfonyl fluoride 0.4 mM) and added to the avidin-Sepharose gel forming a slurry. To prevent settling and ensure uniform mixing the preparation was gently shaken at 4°C for 4-6 hr. The suspension was then gently poured into a 150 ml glass fritted funnel mounted on a vacuum flask and washed with 4-6 liters of column buffer (precooled to 4°C) to remove unbound protein. The cleaned avidin-Sepharose gel, now containing bound ACC, was transferred to a Bio Rad econo column, 10 cm in length by 2.5 cm in diameter with a 50 ml capacity. ACC was eluted with 0.2 mM biotin in column buffer without the 0.4 mM phenylmethylsulfonyl fluoride. The column was attached to a peristaltic pump and the flow rate set at 3 to 5 ml/hr. Fractions were collected in 1.0 ml aliquots and analyzed for protein content on a Beckman spectrophotometer at 280 nm. The fractions containing protein were pooled and dialyzed 4-5 hr at 4°C against column buffer without NaCl. This dialysis is important because NaCl will greatly decrease ACC activity. ACC

specific activity and protein content were then determined. The final preparation was aliquoted, frozen rapidly in liquid nitrogen, and stored at -70°C.

RESULTS

Acetyl-CoA carboxylase (ACC) was initially isolated and purified using the polyethylene glycol (PEG) procedure first described by Hardie and Cohen (9). The protein concentration in the final preparation was 200 μg (45 g liver tissue, 4.4 μg ACC/g liver). A portion of the final preparation was subjected to SDS-PAGE and the protein visualized by Coomassie Blue staining. As shown in Figure 1, lanes 2 and 3, the major band detected had a (M_r) of 230,000 daltons ± 3.3% as determined by its electrophoretic mobility (Figure 2). Two minor protein components (Figure 1, lanes 2 and 3, (a,b)) were closely associated with the putative ACC.

Biotin is covalently bound to all known carboxylases. Therefore, to show the presence of biotin in the 230,000 dalton protein, another portion of the PEG isolated protein was incubated with N-acetylated monomeric avidin. Because the binding affinity between avidin and biotin is high, ($K_d = 10^{-13}$, for monomeric avidin), the avidin ($M_r = 16,600$) bound to the putative ACC forms a stable ACC-avidin complex. The molecular weight of this complex was found to be approximately equal to the sum of the two subunits; 248,000 daltons (Figure 1, lane 1 and Figure 2). In Figure 1, lane 1, both the major 230,000 dalton band and minor component (b) bind to avidin. This results in an increased molecular weight and a decreased electrophoretic mobility. Subsequently, both bands appear above their counterparts in lanes 2 and 3. This evidence suggests that protein (b) also possesses biotin and is most likely a proteolytic fragment of the major 230,000 dalton band. Protein (a)

on the other hand did not bind avidin and did not change its position relative to lanes 2 and 3. Therefore, component (a) is not visible in lane 1 (the ACC-AV complex is co-eluting with protein (a)) suggesting that it did not react with avidin and, therefore, could not be an active subunit of ACC.

Additional evidence showing the presence of biotin in the 230,000 dalton protein is shown in Figure 3. These data represent the in vivo incorporation of [^{14}C]biotin into the 230,000 dalton protein. The protein was isolated by the PEG procedure and analyzed by SDS-PAGE. The graph depicts the distribution of radioactivity in the gel, showing the majority of activity present in the 230,000 dalton region.

The PEG isolation was adequate for making a preliminary identification of the 230,000 dalton band as ACC. However, the final preparation was not homogeneous and proteolysis was apparent. Several attempts to improve the quality of the isolation were unsuccessful. In addition the specific activity of citrate activated (10 mM) ACC (5.0 nmoles/min/mg protein) was extremely low in this procedure.

Therefore, to improve the purification procedure and verify the data obtained from the PEG isolation, fresh liver tissue was extracted, homogenized, and centrifuged at 100,000 x g and subjected to 26% (NH$_4$)$_2$SO$_4$ precipitation, as described previously for the PEG procedure. However, instead of continuing with the PEG purification the (NH$_4$)$_2$SO$_4$ fraction was further purified using avidin-affinity chromatography. Figure 4 shows the elution of ACC from the column as determined by its absorbance at 280 nm. Total ACC activity

(μmoles malonyl-CoA/min/ml) in selected fractions showed a single peak that coincided with the protein peak. The amount of protein recovered was 1.75 mg $\pm$ 1.1 mg (9.0 μg ACC/g liver) and the activity in the combined fractions was 1.37 $\pm$ 0.18 μmoles malonyl-CoA/min/mg ACC. A sample of the column purified ACC was analyzed by SDS-PAGE (Figure 5). The Coomassie Blue stained gel revealed a single protein band with a subunit (M_r) of 230,000 daltons. The two minor components observed in the PEG isolation procedure (Figure 1) were not present.

A typical purification using avidin affinity chromatography is shown in Table 1. Data obtained from mammalian and avian species using essentially the same protocol are included for comparison. It is difficult to accurately access ACC activity in crude cell fractions due to interference by malonyl-CoA decarboxylase and contamination by mitochondrial pyruvate carboxylase (29). However, the values are useful as a relative comparison between species and to monitor the success of the purification procedure. Data presented in Table 1 can be summarized as follows: 1) Throughout the purification ACC total activity in trout was significantly lower than in the other species; 2) each step in the procedure resulted in a several fold increase in purity for all species; 3) the specific activity of trout ACC in the final fraction, although lower, was comparable with values obtained in rat, chicken and goose; and 4) the overall recovery of trout ACC per gram of liver was less than the other species.

Activity assays performed at various citrate concentrations (0, 2, 5, 10, 20, and 40 mM) revealed the enzyme was citrate dependent with optimal citrate levels between 10 and 20 mM. At 40 mM

and at higher citrate concentrations, ACC specific activity decreased. In addition, sodium chloride (0.2 M) in the reaction mixture decreased ACC specific activity by 50%. These findings are all characteristic of ACC isolated from homoiotherms and are consistent with data reported in previous studies (12).

DISCUSSION

Acetyl-CoA carboxylase (ACC) has been identified and characterized from several mammalian species. The activity of ACC in various fish species has been studied only in crude cell fractions (30-32). The present investigation provides the first description and characterization of ACC purified from rainbow trout liver cytosol. To accomplish this, advantage was taken of the fact that ACC is predominantly a cytosolic enzyme with a subunit (M_r) greater than 200,000 daltons. The remaining carboxylases are confined to the mitochondria and possess a subunit (M_r) less than 200,000 daltons (3,5). In addition, ACC (as well as the other carboxylases) contains a covalently linked biotin moiety associated with each subunit. Thus, a protein isolated from the soluble fraction that possesses biotin and has a subunit (M_r) greater than 200,000 daltons is consistent with the characteristic properties of ACC.

The subunit (M_r) of the major protein isolated from rainbow trout using two different techniques was determined to be 230,000 daltons. This is in agreement with the (M_r) range of 225,000-260,000 daltons reported for mammals. To establish that this protein also possessed a biotin moiety essential for carboxylase activity, it was incubated with monomeric avidin. The resulting protein-avidin complex showed an increase in the (M_r) as judged by a reduction in its electrophoretic mobility. These data suggest that the entire 230,000 dalton band in Figure 1, lane 1, was composed of the biotinyl protein. The fact that protein (b) also bound avidin, strongly suggests that it is a proteolytic fragment of the larger protein.

This proteolytic fragment was also observed by Hardie and Cohen (10). Protein (a) (Figure 1, lane 1) did not move up relative to its position in lanes 2 and 3. This suggests that (a) did not possess biotin and therefore must be a contaminant. The electrophoretic properties and close association of band (a) with ACC suggests that protein (a) is fatty acid synthetase, which has been shown to electrophorese slightly above ACC (10). To be certain that the observed ACC-avidin complex (Figure 1, lane 1) was the result of a specific biotin-avidin interaction and not random association of avidin with the 230,000 dalton protein, [^{14}C]biotin was incorporated into the carboxylase in vivo. The results of this experiment confirmed the presence of biotin in the 230,000 dalton band and showed that the observed interaction between avidin and the suspected ACC (M$_r$ = 230,000) was associated with the presence of this moiety.

The PEG isolation procedure was useful in establishing the presence of biotin in the 230,000 dalton protein. However, ACC specific activity, as well as the amount of protein obtained (4.4 μg/g liver), was extremely low. Additionally, small amounts of contaminant proteins were apparent. Therefore, avidin-Sepharose affinity chromatography was used to purify trout ACC to homogeneity.

This procedure yielded a homogeneous ACC preparation without the proteolysis observed using the PEG procedure. In addition, ACC specific activity in the final fraction was substantially higher than the PEG isolation and comparable with ACC activity reported in homoiothermic species. The average amount of ACC recovered per gram of liver increased from 4.4 μg to 9.0 μg.

The data presented here provides good evidence that the 230,000 dalton protein isolated from rainbow trout liver cytosol is ACC, with a subunit (M_r) and enzyme characteristics comparable with mammalian ACC. These similarities are important because the rainbow trout is being used to study the effects of various toxicants on lipogenesis. The fact that the fish ACC possesses many of the same biochemical properties as rat ACC, suggests that toxicants directly affecting this important lipogenic enzyme in trout may also cause the same effects in mammals. Finally, these data provide an important impetus to studies currently underway in our laboratory and aimed at understanding the mechanisms by which xenobiotics affect ACC and the processes involved with lipogenesis in the rainbow trout model.
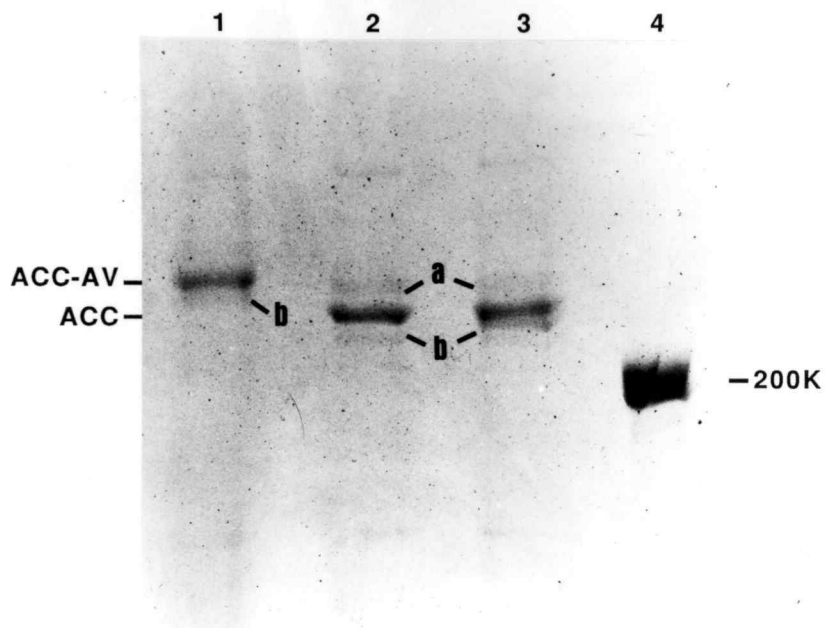
FIGURE II.1. SDS-PAGE analysis (5% polyacrylamide) and subsequent staining with Coomassie Blue of the putative acetyl-CoA carboxylase (ACC) at 230,000 daltons obtained from the PEG precipitation procedure. Lane 1 shows the formation of a carboxylase-avidin complex (ACC-AV) and the subsequent increase in molecular weight from 230,000 to 248,000. Lane 2 represents 3 µg ACC after 4 hr dialysis. Lane 3 is 3 µg ACC without dialysis. Lane 4 is a myosin standard marker. The bands identified as (a) and (b) are minor components (see Results).
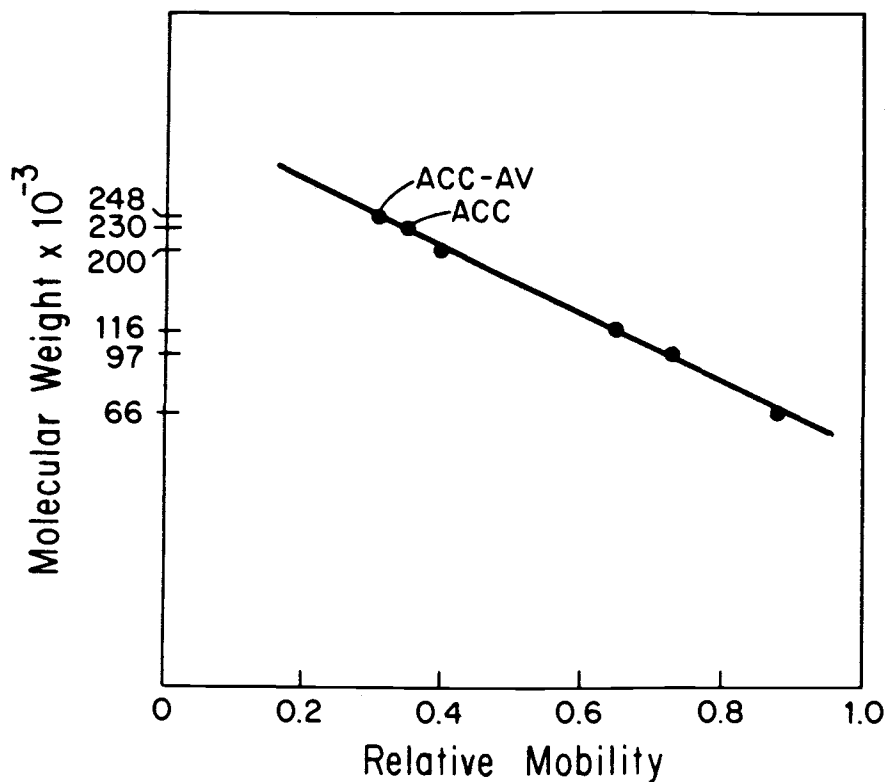
FIGURE II.2. Molecular weight determination for rainbow trout acetyl-CoA carboxylase (ACC). Subunit molecular weights (MW) of the Bio Rad protein standards, (x 10^{-3}) were as follows: Myosin (200), β-galactosidase (116), phosphorylase b (97) and bovine serum albumin (66). The putative ACC was (230), and the acetyl-CoA carboxylase-avidin complex (ACC-AV) was (248). The graph was derived by plotting the $\log_{10}$ values of the subunit (MW) against their relative mobility. Actual MWs (x 10^{-3}) are shown for easier evaluation.
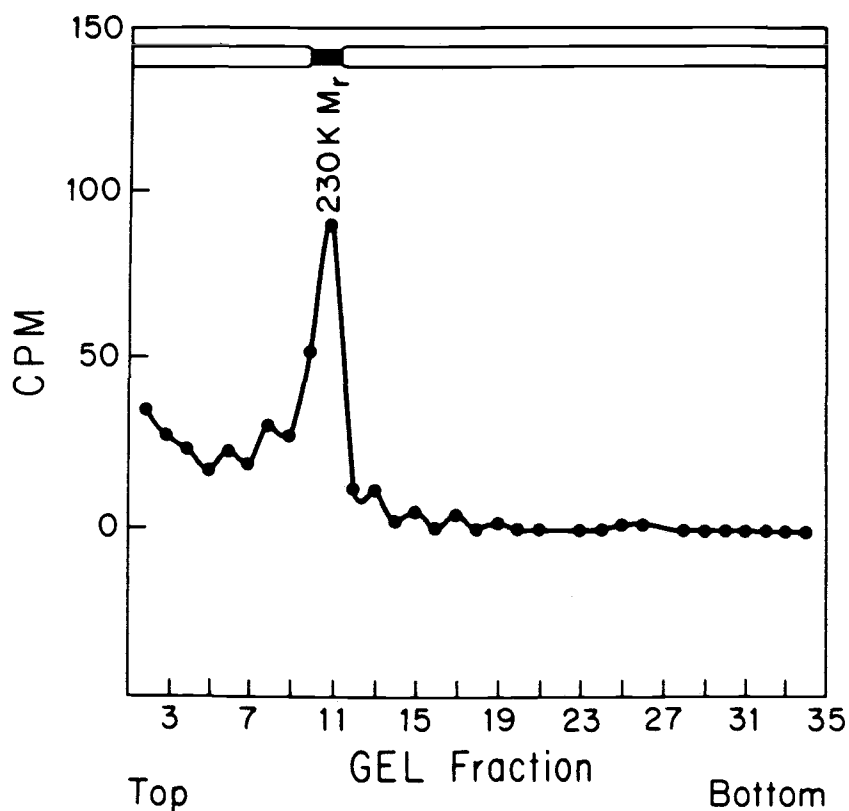
FIGURE II.3. SDS-PAGE analysis (5% polyacrylamide) of [^{14}C]biotin labeled acetyl-CoA carboxylase (ACC), isolated by the PEG procedure. The amount of protein used was 25 μg, (12.0 CPM/μg protein). After electrophoresis at 21 mA constant current for 7 hr, the gel was stained Coomassie Blue (gel not shown) and sliced into 2 mm segments. The radioactivity content of each slice was determined as described in Materials and Methods and the distribution of [^{14}C]biotin plotted. The majority of the radioactivity was observed in the 230,000 dalton region of the gel.
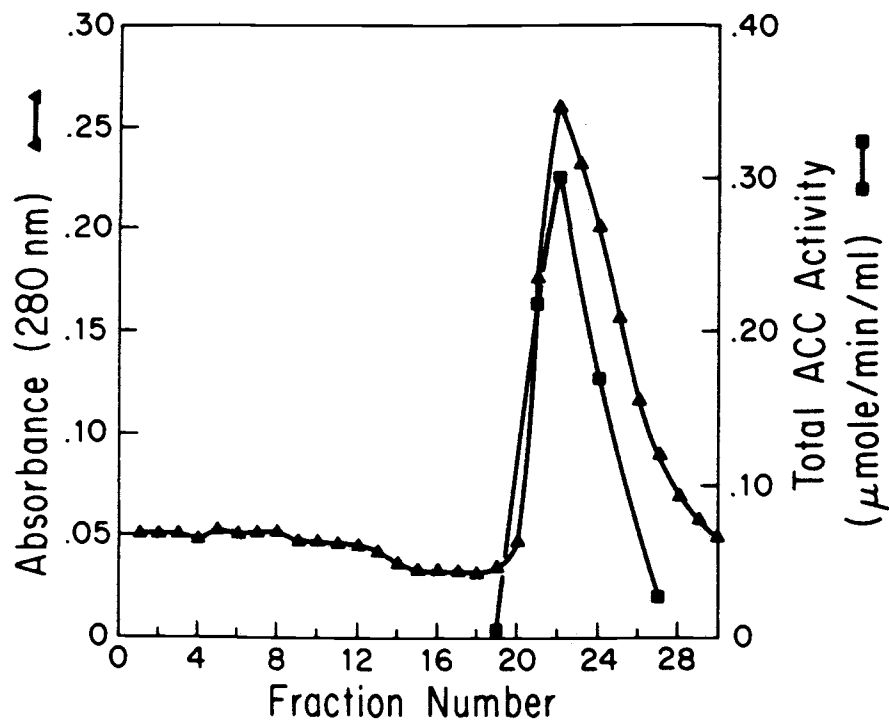
FIGURE II.4. Elution of acetyl-CoA carboxylase (ACC) from the avidin-Sepharose column using 0.2 mM biotin in column buffer. Fractions were collected in 1.0 ml aliquots at a flow rate of 3-5 ml/hr. ACC total activity coincided with the protein peak. The biotin binding capacity was 13 nmoles/ml packed gel.
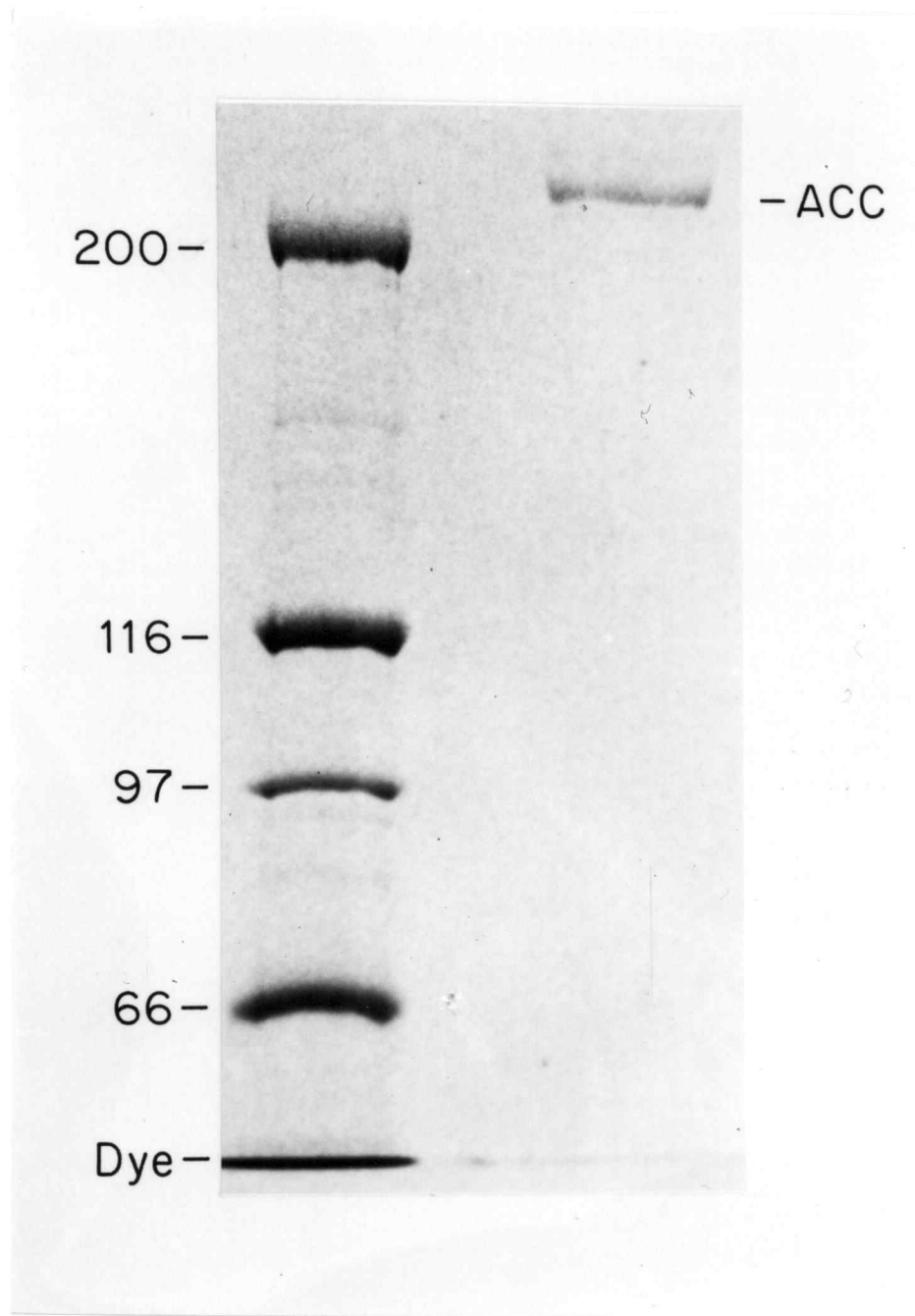
FIGURE II.5. SDS-PAGE analysis (5% polyacrylamide) and subsequent staining with Coomassie Blue of protein recovered from the avidin-Sepharose column. Lane 1 contains Bio Rad high molecular weight standards (x 10^{-3}). Lane 2 received 5 μg protein and shows a single protein (M_r = 230,000) component.

TABLE II.1. Purification of Acetyl–CoA Carboxylase (ACC) From Rat, Chicken, Goose, and Rainbow Trout Using Avidin–Sepharose Affinity Chromatography.

Fraction	Species	Tissue	Total Protein (mg)	Total[a] Act.(U)	Specific Act.(U/mg)	Purification (-fold)
100,000 x g						
	Rat[b]	Liver	11,169	40.1	.004	1.0
	Chicken[c]	Liver	4,000	400.0	.010	1.0
	Goose[d]	Uropy	2,240	44.0	.020	1.0
	Trout[e]	Liver	11,800	21.0	.002	1.0
(NH4)2SO4						
35%	Rat	Liver	1,248	31.2	.025	6.25
25%	Chicken	Liver	360	162.0	.450	45.00
30%	Goose	Uropy	60	35.0	.590	30.00
26%	Trout	Liver	172	3.0	.020	10.00
Avidin–Sepharose Column						
	Rat	Liver	20.0	37.10	1.830	458.00
	Chicken	Liver	29.4	153.00	5.200	520.00
	Goose	Uropy	3.0	15.00	5.000	250.00
	Trout	Liver	1.0	1.24	1.240	620.00

[a] Activity in all studies was determined using the [^{14}C]bicarbonate fixation assay at 37°C. One unit (U) equals μmoles malonyl–CoA produced per min at 37°C.

[b] From 200 g liver; ACC production was induced by diet, [Ref (6)].

[c] From 500 g liver; citrate was included throughout the isolation, [Ref (11)].

[d] From five uropygial glands; gel filtration preceded the 30% (NH4)2SO4, [Ref (12)].

[e] From 200 g liver; fish received standard diet.

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III. The Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin on
Acetyl-CoA Carboxylase and Other Lipogenic Enzymes

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ABSTRACT

Rats given a single i.p. dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and sacrificed nine days later showed a 67% reduction in hepatic acetyl-CoA carboxylase (ACC) activity. ACC mRNA levels were also reduced 40%, but enzyme levels were not appreciably changed. This implies that the pronounced decrease in ACC activity may be due to a post-translational modification of the enzyme, while the reduced mRNA levels suggest a long term response which would eventually result in a reduction of the enzyme. Indirect evidence indicates that TCDD also decreased the amount of fatty acid synthetase. Pyruvate carboxylase levels were also diminished. These lipogenic enzymes regulate the de novo synthesis of fatty acids. Suppression of the activity and/or synthesis of these key enzymes could alter membrane function and enhance TCDD toxicity.

INTRODUCTION

The chlorinated dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a contaminate formed during the production of chlorophenoxy herbicides, is a toxic environmental and occupational pollutant (1-3). TCDD toxicity coupled with its persistence in organisms and the environment have made this compound the focus of extensive investigations aimed at identifying the mechanism(s) of its toxicity (4-14). Such studies have produced valuable information about the physiological and biochemical affects of TCDD, but have not revealed adequate molecular mechanism(s) for its toxicity.

The most apparent and reoccurring physiological and toxicological change observed in animals exposed to TCDD is the "wasting syndrome," characterized by decreased food consumption, weight loss, and death. Weight loss in TCDD-treated animals is greater than in pair-fed controls which implies that decreased food consumption alone is insufficient to account for the accelerated weight loss and death. Therefore, the mechanism of TCDD-induced mortality may be indirectly linked to the wasting syndrome via alterations in energy metabolism (9,11,12). Further, animals exposed to TCDD show alterations in thyroid and insulin levels. These hormones control a wide range of metabolic responses including lipid and carbohydrate metabolism (9-13,15).

The liver accumulates the highest levels of TCDD after o.p. or i.p. administration (16). Therefore, this organ provides a logical choice for studying the biochemical alterations caused by this compound. Effects on lipid metabolism resulting in fatty liver are

characterized by increased serum fatty acids, hepatic triglycerides, phospholipids, and lipoproteins (17-19). A decrease in the de novo synthesis of fatty acids in the liver of TCDD-treated Wistar (1-20 μg/kg) rats has been reported (20). However, in another study Sprague-Dawley rats receiving high doses (125 μg/kg) of TCDD revealed an increase in hepatic fatty acid synthesis (21).

De novo synthesis of fatty acids in all organisms examined requires the cooperative activity of acetyl-CoA carboxylase (ACC) [EC 6.4.1.2], fatty acid synthetase (FAS) [EC 2.3.1.85], and other enzymes such as pyruvate carboxylase (PC) [EC 6.4.1.1] and malic enzyme [EC 6.4.1.1] (22-24). A recent study by Lakshman et al. (1989) (25) showed that TCDD decreases the activity of ACC and FAS in a dose dependent manner in rat liver. However, these authors did not provide evidence for the mechanism of this observed reduction. Recent studies with plants have shown that ACC is the target of several chlorophenoxy herbicides (26-28). Plant death was linked to inhibition of ACC and the subsequent decrease in acyl-lipid and membrane synthesis.

In this study we investigated the effects of TCDD on the function and synthesis of hepatic ACC and other key lipogenic enzymes in rats. This was accomplished by comparing ACC enzyme activity, quantity, and mRNA levels between control and experimental animals. To our knowledge this study provides the first such comparison. These data should provide new insight into the complex mechanism(s) of TCDD-induced toxicity, and its affects on lipogenesis.

MATERIALS AND METHODS

Chemicals

2,3,7,8-Tetrachlorodibenzo-p-dioxin was purchased from Cambridge Isotope Laboratories (Woburn, MA) presolubilized in nonane at 50 μg/ml. [^{14}C]-NaHCO$_3$ and γ[^{32}P]-ATP were obtained from Amersham, Inc. (Arlington Heights, IL). Polyacrylamide (99%), protein molecular weight standards, 1-Methyl-2-Pyrrolidinone (MPO), Tween-20, avidin-HRP, HRP-developer and Zeta Probe membrane were purchased from Bio Rad (Richmond, CA). Poly (A)$^+$ mRNA was isolated using a kit obtained from Invitrogen (San Diego, CA). The RNase inhibitor RNasin was purchased from Promega (Madison, WI). Agarose, EDTA, formaldehyde, and diethylpyrocarbonate were of the highest grade obtainable from Sigma Chemical, Co. (St. Louis, MO). Formamide (ultra pure) was from International Biotechnologies, Inc. (New Haven, CT). 5'-End-labeling of oligonucleotide probes were done with T4 polynucleotide kinase obtained from Pharmacia (Piscataway, NJ). Sephadex-25 quick spin columns were from Boehringer Mannheim Biochemicals (Indianapolis, IN). RNA molecular weight markers were from Bethesda Research Labs (Gaithersburg, MD).

Preparation of TCDD for Injection

All procedures were performed in a contained area under a fume hood. Double layer gloves were worn when handling TCDD and all contaminated disposable materials were placed in biohazard bags and incinerated. Non disposable glass wear was rinsed with tolulene and then washed with soap and water. 2.0 ml of the nonane TCDD solution were added to 10 ml of corn oil in a 50 ml glass beaker. The

mixture was stirred under a fume hood for 48 hr in order to insure uniform mixing and to allow complete evaporation of the nonane carrier. The corn oil/TCDD solution was then analyzed by gas chromatography to ascertain the TCDD concentration.

Animals

Male Wistar rats (140-160 g) were used in these investigations. The animals were housed individually in wire bottom cages, paired according to weight, separated into two groups of eight and fed standard rat chow ad libitum for five days. Eight animals from each group were i.p. injected with 53 μg/kg TCDD in 1 ml of corn oil. Pair-fed controls were injected with corn oil only. Two additional animals were injected with corn oil and were used as free fed controls. The diet received by controls was determined by the amount consumed by experimental animals.

Histopathologic Preparation

Tissue samples for pathologic analysis were placed in bouins fixative (23.3% Formaldehyde, 4.8% Acetic acid, 0.7% Picric acid) for 2 days. The samples were stained with osmium tetroxide prior to being embedded in paraffin. This stain is specific for fat which appears as black spots (29).

Tissue Preparation

Five days after exposure to TCDD, experimental and pair-fed controls were fasted for 48 hr and then fed a standard rat chow diet (< 4% fat) for 48 hr. This was done to be sure that conditions were optimal for lipogenic enzymes. Animals in group I were weighed and

then killed by decapitation. Livers were removed immediately,
weighed and portions removed for histopathologic analysis and mRNA
isolation. The remaining tissue was placed into ice cold
homogenization buffer, (Tris-HCL, 20 mM, pH 7.4; Mannitol, 0.3 M;
EDTA, 2 mM; dithiothreitol, 1 mM; and phenylmethylsulfonyl fluoride,
0.2 mM). Two control and two experimental livers were combined and
homogenized in 3 volumes of homogenization buffer in a blender by
pulsating (5-10 sec) at high speed for ⁻30 sec. The homogenate was
filtered through cheese cloth to remove fibrous materials. The
homogenates were centrifuged for 10 min at 20,000 x g at 4°C. The
supernatant was removed and centrifuged again at 100,000 x g for 1 hr
at 4°C. Portions of the supernatant were used to conduct enzyme
activity assays. The remaining supernatant was aliquoted and stored
in 10% Glycerol at -70°C.

Activity Assays

Cytosolic acetyl-CoA carboxylase was assayed as described
previously (30) as modified from Allred and Roehrig (31). The final
reaction mixture (0.5 ml) contained, (Tris-Acetate, 70 mM, pH 7.5;
Potassium acetate, 120 mM; reduced glutathione, 4 mM; potassium
citrate, 10 mM; ATP, 1 mM; bovine serum albumin, 0.6 mg/ml; acetyl-
CoA, 0.35 mM; magnesium acetate, 10 mM; and 7 mM (0.25 μCi) [^{14}C]-
NaHCO$_3$). Blanks were performed without protein and without
substrate. Pyruvate carboxylase activity was assayed essentially as
described by Ballard and Hanson (32). The reactions in both assays
were stopped with 0.05 ml of concentrated HCL. Unincorporated label
was removed by drying an aliquot (200 μl) of the reaction mixture at

95°C for 30 min. This was followed by resolubilization with 0.2 ml distilled water and 5.0 ml Aqueous Counting Scintillant fluid. The reaction was started with addition of protein and incubated 2-3 min for ACC and 10 min for PC at 37°C.

Polyacrylamide Gel Electrophoresis of Cytosolic Protein

Cytosolic proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (33). The sample was cooled and loaded onto a SDS-PAGE (18 cm x 16.5 cm x 0.2 cm) consisting of 3% stacking gel and a 5% resolving gel. The gel was electrophoresed at 21 mA constant current for 4 hr, packed in ice. The gel was stained overnight with Coomassie Blue and destained with 50% methanol, and 10% acetic acid, for several hours.

Western Blotting and Avidin-Horse Radish Peroxidase Stain for Biotin

Protein (200 μg) was separated according to size by SDS-PAGE as described above. Immediately after electrophoresis the gel was cut in half. One section was stained with Coomassie Blue. The other was equilibrated in transfer buffer for 30 min at room temperature in order to swell the gel and to remove excess sodium dodecyl sulfate (SDS). The gel was then placed in the transfer cassette on 2 pieces of 3 mM filter paper and a scotch pad. Zeta Probe membrane, presoaked in transfer buffer (Tris-HCl 25 mM; and Glycine 192 mM, pH 8.3), was then placed on the anode (+) side of the gel. Care was taken to insure that no air bubbles were trapped between the gel and membrane. Two pieces of 3 MM presoaked in buffer were placed on top of the membrane, followed by another scotch pad. The cassette was closed and placed in the Bio Rad Trans-Blot[R] apparatus. Ice cold

transfer buffer was added (3.5 l) and the proteins were transferred on ice with stirring at 50 V (~0.560 Amps) for 6.5 hr. The membrane was then placed in a plastic dish (10 cm x 10 cm) and incubated with 100 ml of Borate Tween blocking buffer (Na_2B_4O · 10 H_2O; 0.05 M; NaCl 0.5 M; 5% MPO; 0.2% Tween-20, pH 9.3) on a rotating shaker for 10 min; the procedure was repeated 2Xs. The membrane was then washed 3Xs with 100 ml Tris-Tween buffer (0.02 M Tris; 0.5 M NaCl, pH 7.5; 0.2% Tween-20; and 5.0% MPO), for 5 min. Avidin-HRP was prepared by adding 150 μl of Bio Rad's premixed avidin-HRP to 100 ml of the Tris-Tween buffer. The avidin-HRP was allowed to bind for 1.5 hr at room temperature on a rotating shaker. Excess avidin-HRP was removed by washing 2Xs with 100 ml a Tris-Tween buffer (0.02 M Tris; 0.5 NaCl; 0.2% Tween-20; 5.0% MPO), and then 2Xs with 100 ml Tris-buffered saline (0.02 M Tris; 0.5 M NaCl, pH 7.5). The bound avidin-HRP was visualized using HRP-developer, (60 mg developer in 20 ml ice cold methanol and 0.015% H_2O_2 in 100 ml distilled water. The membrane was developed for 30 min with no background problems. Development was stopped by soaking the membrane in distilled water.

Slot Blot Analysis of mRNA

Purified Poly (A+) mRNA (20 μg) was applied to the Zeta Probe nylon membrane in 0.050 ml of sample buffer, (13 mM, NaPO4, pH 7.0; 1.3 mM EDTA; 6.5 mM sodium acetate; 2 M Formaldehyde, 50% Formamide; 0.025% bromophenol blue) in 25 μl portions. The membrane was then dried at 80°C for 2 hr. Prehybridization was (6X SSC; 50 mM $NaPO_4$, pH 7.5; 1% SDS; 0.5% milk powder (w/v); 250 μg/ml RNA yeast; 100 μg/ml chicken DNA; 50% Formamide), for 8-12 hr at 42°C. The

oligonucleotide complementary to rat ACC mRNA was synthesized on an Applied Biosystems DNA Synthesizer at the Center for Gene Research, Oregon State University. The DNA was 5´-end-labeled with $\gamma[^{32}P]$-ATP using T4 polynucleotide kinase. Unincorporated label was removed by Sephadex-25 quick spin columns. The labeled DNA was then added to the prehybridization solution and hybridization continued at 42°C for 18-20 hr. The membrane was washed for 30 min in 2X SSC at 30°C and then with 1X SSC at 30°C for 30 min. The membrane was dried at 45°C between 3 MM paper for 30 min and was then exposed to film (Kodak X-OMAT) using an intensifying screen for 5-7 days. The autoradiograph was analyzed using a laser scanning densitometer.

RESULTS

Rats given a single i.p. injection of (53 μg/kg) TCDD showed a significant weight loss at the end of the nine day period. Liver enlargement was apparent both visually and by liver weight to body weight ratios which increased from 4 to 6 percent (Table 1). The observed increase in liver size was attributed to a dramatic increase in the lipid content of TCDD-treated versus control livers (Figure 1, A and B). In addition, ACC specific activity was suppressed 67% in TCDD-exposed animals (Table 1).

To determine whether the observed reduction in ACC activity was pre or post-translational, a synthetic oligonucleotide probe [5'-GTAATGAAGATGGTAATGACT] 100% homologous to the biotin-binding region of ACC mRNA was prepared from known ACC cDNA sequence data (34). Poly (A)+ mRNA from control and TCDD liver was isolated and analyzed by slot blot analysis. Comparisons were made by analyzing the autoradiographs with a laser scanning densitometer (Figure 2). These analyses showed a 40% decrease in the probes ability to hybridize Poly (A)+ mRNA isolated from TCDD-treated animals, relative to controls.

In order to assess the effects of TCDD on ACC protein levels, equal amounts of cytosolic protein from control and experimental livers were analyzed by SDS-PAGE (Figure 3). Localization of ACC on the gel was determined using purified rainbow trout hepatic ACC as a standard (M_r = 230,000) (lane 3), which has been shown previously by McKim et al. (1989) (30) to have the same biochemical and electrophoretic properties as rat hepatic ACC. It was apparent from

Figure 3 that the 230,000 dalton protein believed to be ACC has dramatically decreased in experimental (lane 1) versus control animals (lane 2).

A unique property of carboxylases is the presence of a covalently linked biotin moiety. If the observed decrease in protein levels was due to reduced ACC, then the biotin content of the 230,000 dalton band should have been reduced proportionately. To determine if this had occurred, a western transfer was done and the membrane was incubated with avidin-HRP which has a high binding affinity for biotin. This enabled us to visualize all of the biotin-containing proteins present in the soluble fraction. Figure 4 showed that the amount of ACC protein (M_r = 230,000) was not decreased in TCDD (lane 4) versus control preparations (lane 3). The higher molecular weight form of ACC visualized with the avidin-HRP probe (a doublet) was present in all of the control and experimental crude cell preparations. This doublet was also present in the purified rainbow trout ACC preparation. Although this higher molecular weight band has been reported by several others (19,28), its exact source has not been clearly defined. It is important to note, however, that in all control and TCDD samples analyzed in this study, there was no visible difference between the high and low molecular weight forms. In our laboratory, as well as others (19,28), it was the smaller subunit (M_r = 230,000-260,000) that comprised the major portion of purified preparations. The reduced staining intensity observed in Figure 3, lane 1, as compared to lane 2, implies that ACC co-migrated with one or more protein(s).

A smaller biotin-containing protein ($Mr = 125,000$) was also observed (Figure 4) and was clearly suppressed in TCDD (lane 4) versus control animals (lane 3). The mitochondrial protein pyruvate carboxylase (PC), has a reported molecular weight of 130,000 daltons, and the presence of this enzyme in cytosolic preparations has been previously established (32). To verify the presence of PC in our preparations, activity assays were performed. These assays revealed the presence of this enzyme in the cytosolic preparations, and showed that PC specific activity in TCDD-treated animals was reduced by 58%. This decrease in activity was attributed to decreased protein levels as shown by SDS-PAGE analysis (Figure 3, lanes 1 and 2) and by western blots analyzed with a biotin specific probe (Figure 4, lanes 3 and 4).

DISCUSSION

Animals given TCDD showed a reduction in body weight after nine days compared to pair-fed controls. Hepatomegaly was also observed with liver weight to body weight ratios increasing significantly over controls. In addition, TCDD had a suppressive affect on ACC, a key enzyme in the de novo synthesis of fatty acids. These data indicate that although ACC specific activity was suppressed by 67% (Table 1), actual amounts of the enzyme were not different when analyzed with biotin specific staining techniques. However, a reduction in ACC mRNA levels occurred in TCDD-treated animals. This suggests that post-translational regulation, characterized by decreased ACC activity without loss of protein, was the initial response to TCDD. This initial suppression was accompanied by a reduction in the overall levels of ACC mRNA synthesis. Over a longer period of time, it is likely that the amount of ACC protein would also decrease.

Fatty acid synthetase (FAS) is the only protein that is known to co-electrophorese with ACC on low percentage acrylamide gels (23,35). Both ACC and FAS activities were greatly increased following a fast/refeed diet protocol as described here. The increased activity of FAS was due to increased translational efficiency (36,37), resulting in higher quantities of this enzyme. The increase in ACC activity, however, is due at least initially to regulation of the enzyme itself (38,39). Additionally, Lakshman et al. (1989) (25) has shown that FAS activity is greatly reduced by TCDD. Our data suggest that this decrease in activity was due to decreased enzyme levels.

From these characteristics, we believe that the primary component of the 230,000 dalton band in Figure 3 was FAS and that TCDD-treatment resulted in decreased quantities of this protein (Figure 3, lane 1).

A second biotin-containing protein was identified in the western transfer/avidin-HRP procedure. This protein had a molecular weight of 125,000 daltons which was essentially the same as that reported for pyruvate carboxylase (PC). The presence of PC in the soluble fraction was verified using a PC activity assay. The results showed that PC was present in our preparations and that PC activity was at least one order of magnitude greater than ACC activity. PC activity, as well as protein levels, were greatly decreased in TCDD-treated animals. Based on molecular weight, biotin content, and enzyme activity, we conclude that the 125,000 dalton band in Figures 3 and 4 was PC. These data are in good agreement with data recently reported by Lebofsky et al. (1989) (40). They showed a decrease in two other gluconeogenic enzymes; phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in rats exposed to TCDD.

The present study has shown that two important metabolic enzymes, ACC and PC, were suppressed in the presence of TCDD. There was also good evidence of TCDD suppressive affects on FAS. The observed inhibitory affects on ACC and FAS are significant because a reduction in fatty acid biosynthesis could alter membrane synthesis, structure, and function which could lead to alterations in ion flux and eventual cell death. This possibility was supported by a recent report showing a substantial increase in the influx of exterior Ca^{2+} into TCDD treated thymocytes (8).

The data presented here complements previous studies that examined the in vivo regulation of ACC as well as the effects of TCDD on hormones, fat disposition, and enzyme activities. Figure 5 shows diagrammatically how the information presented here as well as data reported by others might be linked together. We suggest that post-translational regulation of ACC is the initial response to TCDD exposure while long term exposure may lead to a reduction in enzyme quantity. Because in vivo levels of citrate (0.5 mM) are considerably lower than the levels required for maximal ACC activity (10 mM) (23), and because our assays were performed under conditions of optimal activity, it is unlikely that the observed reduction in ACC activity was due to citrate stimulated changes in enzyme structure (25). It has, however, been shown that ACC is regulated rapidly and specifically by phosphorylation of specific sites within the enzyme and that the kinase responsible for this phosphorylation (5'-AMP PK) can be activated by nanomolar amounts of fatty acyl-CoAs via a kinase-kinase cascade (38,39,41) (Figure 5). Under normal circumstances the negative allosteric affects of fatty acyl-CoAs on ACC are negligible due to the presence of acyl-binding proteins and the subsequent low levels of free fatty acyl-CoAs (41). However, the dramatic increase in hepatic lipids caused by TCDD exposure may saturate these binding proteins, and in combination with the 5'-AMP kinase allow the fatty acyl-CoAs to exert an inhibitory affect on ACC. These suppressive affects would be a result of allosteric regulation and increased phosphorylation. Therefore, we propose that the reduction in ACC activity without loss of protein was due initially to an increase in phosphorylation and/or allosteric

inhibition of ACC by fatty acyl-CoAs. This inhibition was followed by a decrease in mRNA that could lead to reduced enzyme quantities given sufficient time. Current investigations in our laboratories are aimed at identifying the link between TCDD-induced hormonal changes, phosphorylation, and ACC regulation.
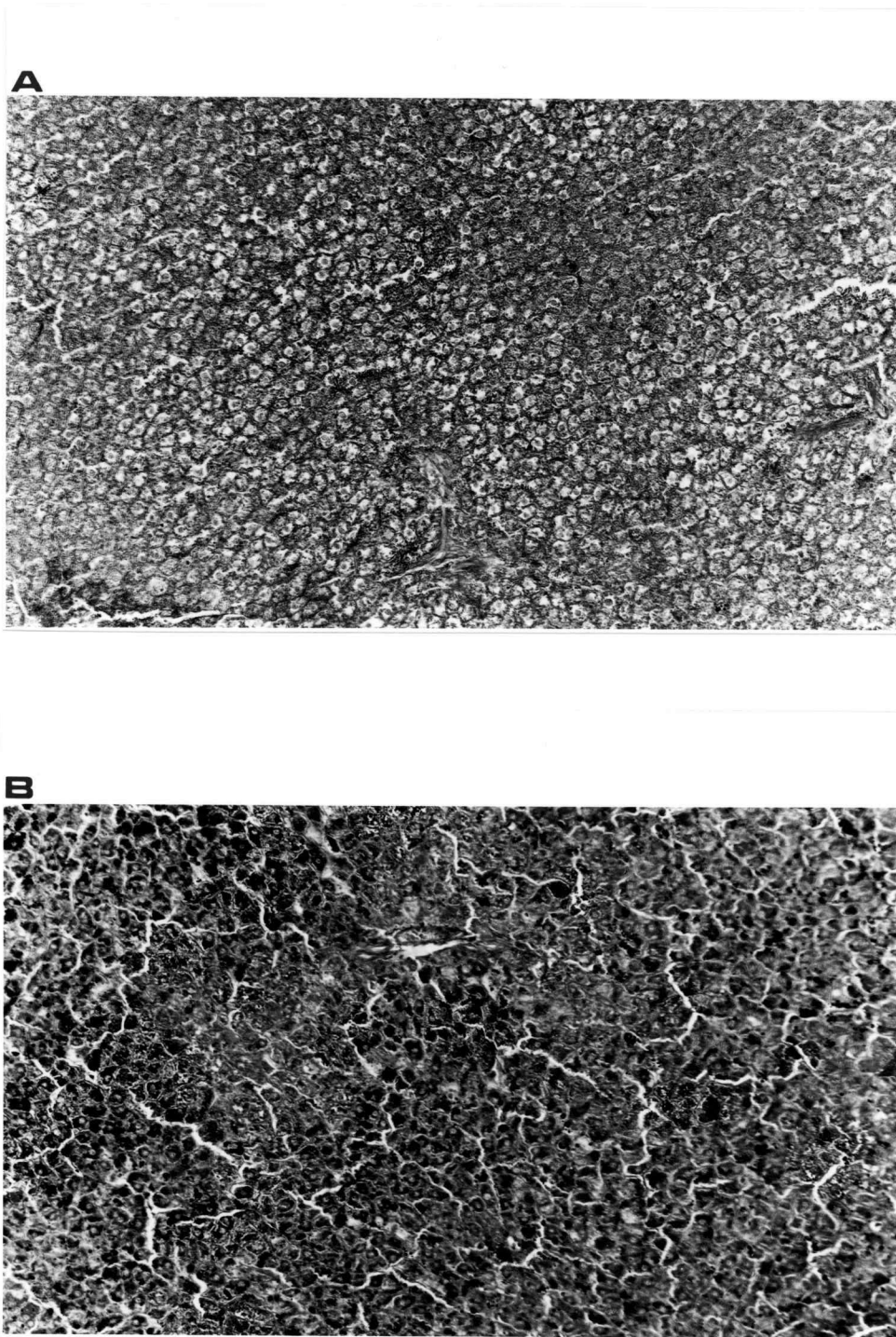
FIGURE III.1. Photomicrographs (250X) of control (A) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) treated (B) liver stained with osmium tetroxide in order to visualize fat. The large black spots apparent in (B) are the fat deposits.

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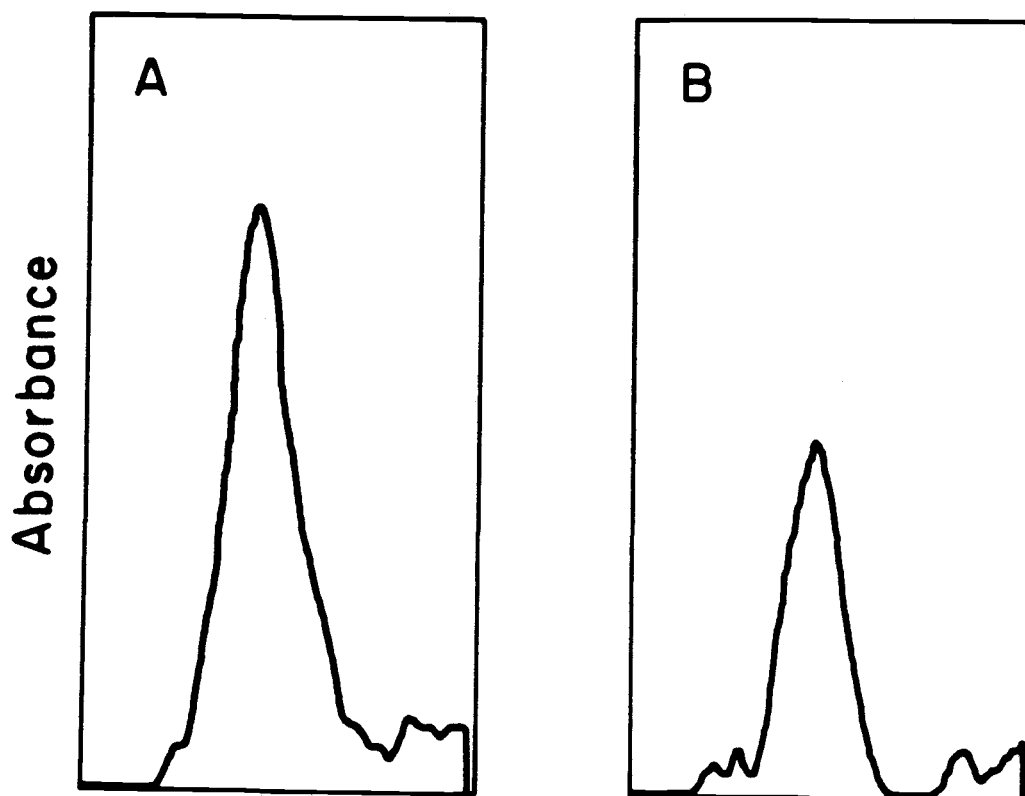
FIGURE III.2. Laser scanning densitometry of mRNA slot blots probed with an acetyl-CoA carboxylase (ACC) specific oligonucleotide (A) control scan (B) experimental scan. Poly (A)$^+$ mRNA was purified using a kit manufactured by Invitrogen (San Diego, CA). Poly (A)$^+$ mRNA (10 or 20 μg) was applied to the Zeta Probe nylon membrane using a slot blot apparatus.
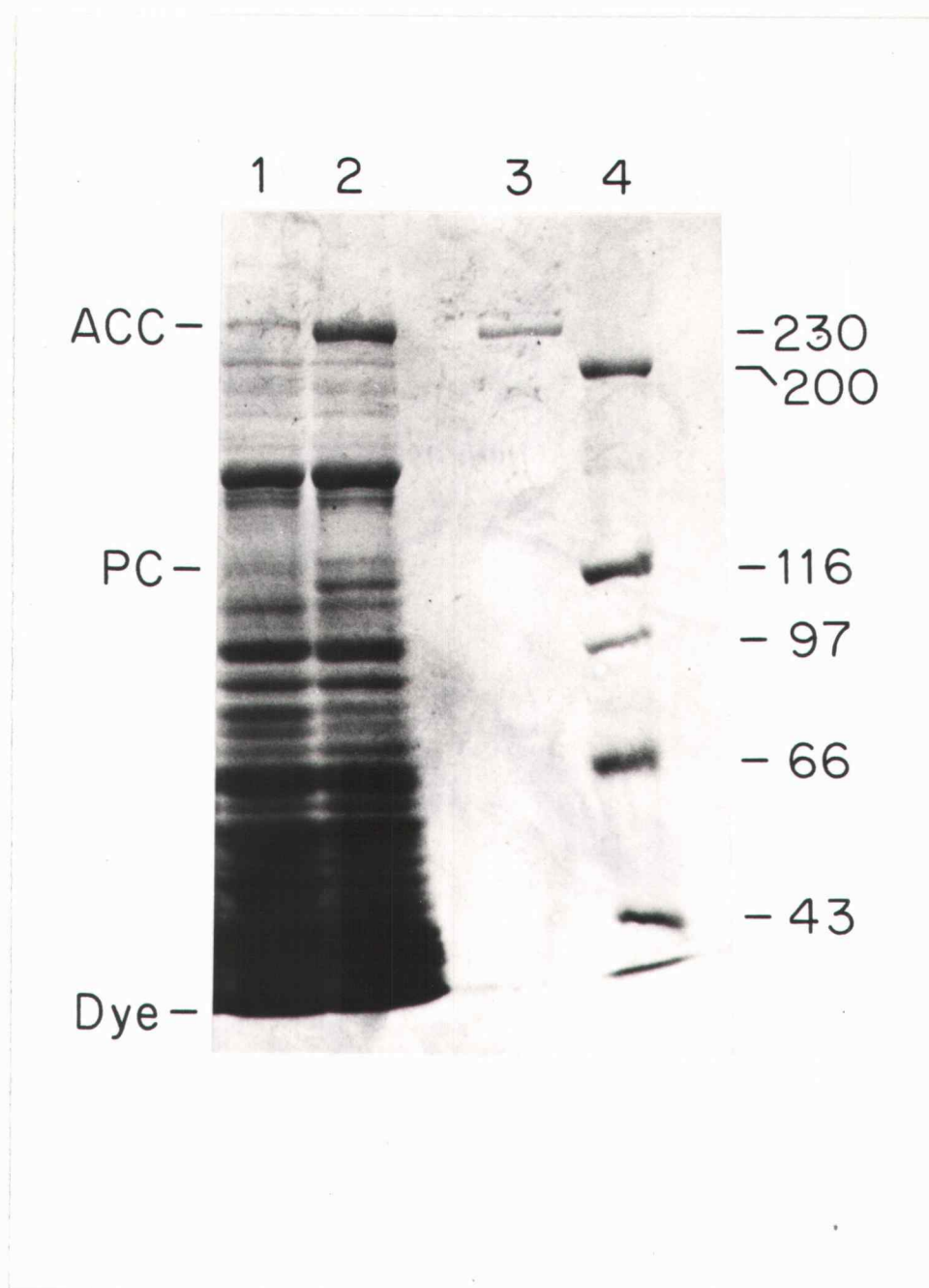
FIGURE III.3. SDS-PAGE analysis (5% polyacrylamide) of cytosolic proteins. Lane 1, 200 μg TCDD treated liver cytosolic protein. Lane 2, 200 μg of control liver cytosolic protein. After electrophoresis for 4 hr at 21 mA the gel was stained with Coomassie Blue. Acetyl-CoA carboxylase (ACC) and pyruvate carboxylase (PC) bands are identified. Lane 3 is an avidin-Sepharose purified rainbow trout liver ACC, which has been shown to have essentially the same biochemical and electrophoretic properties as rat liver ACC (30). Lane 4 contains Bio Rad high molecular weight standards (x 10^{-3}).
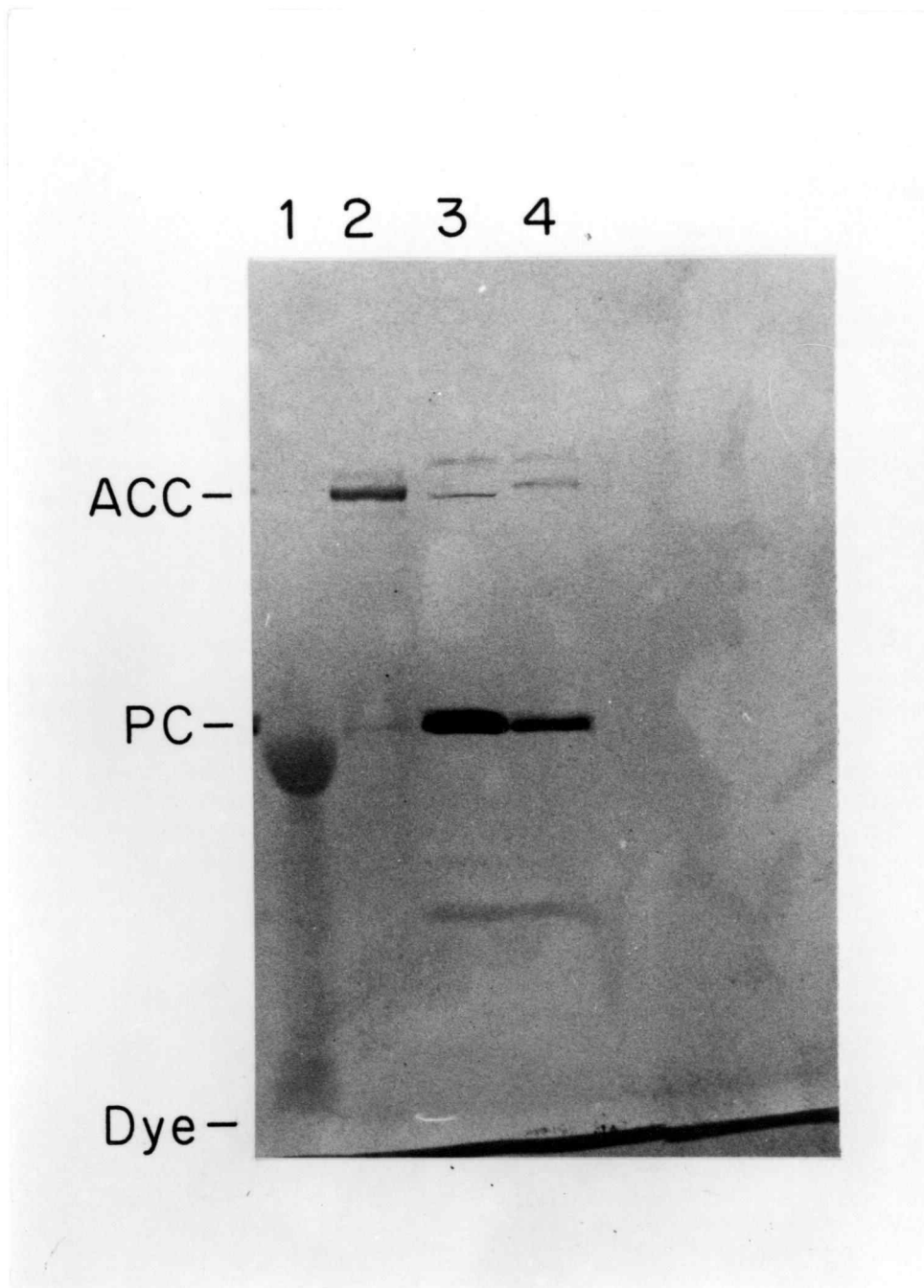
FIGURE III.4. Western transfer and biotin specific staining of protein with avidin-HRP. Lane 1 is a prestained β-galactosidase (M_r = 116,000) standard obtained from Diversified Biotech (Newton Centre, MA). Lane 2 is 3 μg purified rainbow trout liver ACC. Lane 3, 200 μg cytosolic protein from control liver. Lane 4, 200 μg cytosolic protein from TCDD liver.

Blood   **Cell Membrane**   **Cytosol**

Lipogenesis

($\downarrow$)Thyroid[9-11]

($\downarrow$)Insulin[11]   Glucose   **Glycolysis**   ($\uparrow$)Exogenous[17]
                                                                                    Fatty Acids
($\downarrow$)Glucose[40]

($\uparrow$)FFA[17]   Acetyl-CoA ⟶ Malonyl-CoA ⟶ ($\downarrow$)FAS[*,25] ⟶ ⟶ Fatty Acids($\downarrow$)[20]
                                         Pyruvate
                                                                        ($\downarrow$)ACC[*,25]
                                                                              Active
                                                                                              Kinase-Kinase ◀— Fatty Acyl-CoAs
                                         Pyruvate ⟶ Acetyl-CoA
                                                                                                Inactive
                                            ($\downarrow$)PC *   AMP-Activated
                                                                              ACC   AMP-Activated   Kinase
Phosphoenol ($\downarrow$)PEPCK[40]   Oxaloacetate   Inactive   Kinase
Pyruvate   TG($\uparrow$)[17]
   Citrate ⟶ Citrate

                                                                              PL   ($\uparrow$)VLDL[19]
    ($\downarrow$)G-6-P[40]

                                                                           Membranes
    Glucose   **Mitochondria**   (alterations)

                                                                           ($\uparrow$)Ca^{2+} influx[8]
Gluconeogenesis   **Citric Acid Cycle**
                                                                           Alts PLs[17]
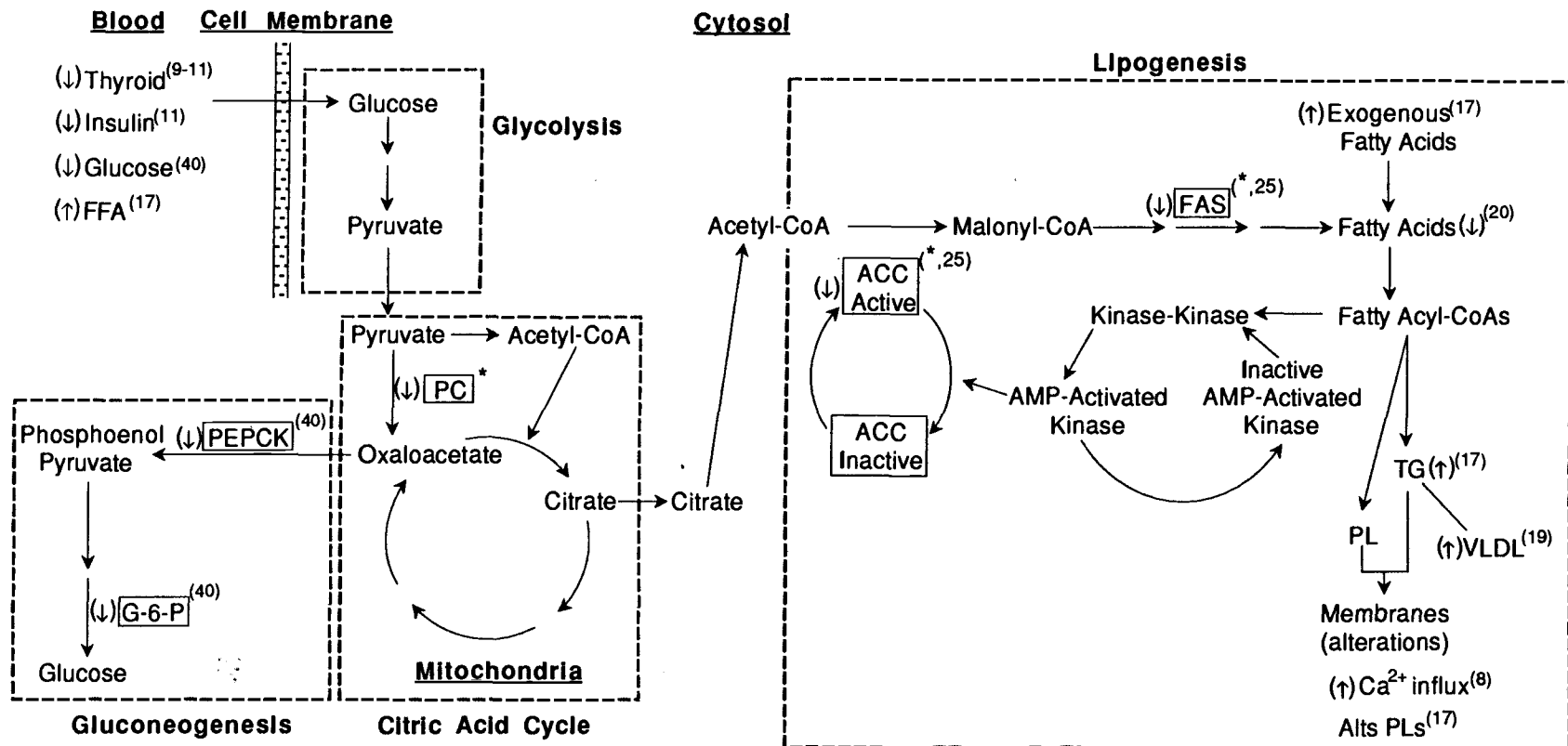
FIGURE III.5. Diagrammatic representation of three key physiologic pathways known to be affected by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The figure links information reported in earlier studies to the findings presented in this study (*). The numbers in parentheses correspond to appropriate references. Parts of the lipogenesis pathway were taken from Hardie et al. (1989) (41).

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TABLE III.1. Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin on Animal Body Weight, Liver Weight, and Acetyl-CoA Carboxylase (ACC) Specific Activity.

Group	(N)	Body Wt.	Liver Wt.	$\dfrac{\text{Liver Wt.}}{\text{Body Wt.}} \times 100$	*ACC Activity (U/mg)	% Inhibition
Control (corn oil only)	(8)	228.1 ± 17.5	9.30 ± 1.64	4.0	1.00	0.0
Experimental (53 μg/kg TCDD)	(8)	202.0 ± 13.7^a	12.02 ± 1.71^a	6.0	0.33^b	67.0

*(U) equals nmoles of malonyl-CoA produced per minute at 37°C.
a = $p < .01$; b = $p < .001$

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