ACUTE REGULATION OF ACETYL-CoA CARBOXYLASE BY COVALENT MODIFICATION

A Thesis

Presented to the

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The University of Manitoba

In Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

Patrick M. Gillevet

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BY

PATRICK M. GILLEVET

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To all the free spirits and their will to power.

REALITY: nothing but patterns in the mind-
but what of the patterns that describe the
patterns that describe the patterns....

ACKNOWLEGEMENTS

I would like to thank Dr. K. Dakshinamurti for the privilege of working with him in his laboratory. It was excellent learning experience that will always be remembered.

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ABSTRACT

The acute regulation of lipogenesis through the covalent modification of Acetyl-CoA carboxylase was investigated to determine if the phosphorylation of the enzyme resulted in decreased enzyme activity. Initial experiments determined that an insoluble form of the enzyme could be phosphorylated by a cAMP-independent mechanism while a soluble form could not. In neither case was phosphorylation correlated with the inactivation of the enzyme. Phosphorylation of an extract of both of the above forms resulted in the formation of small molecular weight phosphopeptides that migrated at the dye front on 5% PAGE. It was subsequently demonstrated that the enzyme was proteolytic nicked during isolation and that the nicked enzyme remained as an intact protomer. The enzyme was phosphorylated at sites that migrated as phosphopeptides upon denaturation. Both a cAMP-independent and cAMP-dependent process phosphorylated the enzyme at these sites as well as phosphorylating the intact uncleaved protomer. Neither of the above mechanisms significantly inactivated the enzyme. A Fatty acid Synthesizing complex was isolated on sucrose gradients that contained Acetyl-CoA carboxylase, Fatty acid synthetase and ATP-citrate lyase. The interaction of the enzymes of this complex was decreased by altering the dietary status of the animal and by incubating the complex with ATP and cAMP. It is hypothesized that glucagon initiates the phosphorylation of the lipogenic enzymes which decreases their interaction and

this subsequently results in a decrease in lipogenesis.

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ABREVIATIONS

ACC:	Acetyl-CoA carboxylase
FAS:	Fatty acid Synthetase
CL:	ATP-Citrate lyase
ATP:	Adenosine 5' triphosphate
-CoA:	Coenzyme A
K:	kilo
ul:	microlitre
mM:	millimolar
mg:	milligram
ml:	millilitre
uCi:	microcurie
umole:	micromole
N:	normal
TRIS:	Tris(hydroxymethyl)aminomethane
SDS:	sodium dodecyl sulphate
xg:	times gravity
gm:	gram
mamp:	milliampere
EDTA:	[Ethylenebis(oxyethlenenitrilo)]-tetraacetic acid
cm:	centimeter
Kg:	kilogram
DEAE:	N,N-Diethylaminoethyl
nm:	nanometer
^{o}C:	degree Celcius
M_r:	molecular weight
mUnits:	milliunits
PAGE:	polyacrylamide gel electrophoresis
B-:	beta-

SECTION I

INTRODUCTION

Hormones have diverse effects on all tissues of the animal body ranging from short term (acute) effects on enzyme activities, metabolite levels and substrate transport to long term (chronic) effects on enzyme levels through alterations in protein synthesis and degradation.

This thesis deals with the acute hormonal regulation of long chain fatty acid synthesis and is based on the hypothesis that acetyl-CoA carboxylase plays a pivotal role in the regulation of this metabolic pathway. The hormonal regulation of this enzyme is considered to be mediated through a phosphorylation-dephosphorylation mechanism. We hypothesize that this covalent modification does not alter the intrinsic activity of the enzyme but alters the interaction between the lipogenic enzymes, acetyl-CoA carboxylase, fatty acid synthetase and ATP-citrate lyase.

The thesis is divided into six parts. The first part, a general review of the literature, will be restricted to animal systems with specific attention being paid to mammalian systems. The main emphasis will be placed on acetyl-CoA carboxylase while the discussion on fatty acid synthetase and ATP citrate lyase will be limited to aspects that directly involve similarities with acetyl-CoA carboxylase. The interaction of various enzymes with subcellular structure and with other enzymes in metabolic

pathways will be discussed briefly.

The experimental results are divided into four sections with a specific literature review and discussion for each section. The first section deals with the purification of avian liver ACC and the production of antibodies to the enzyme. The second section describes our initial phosphorylation experiments and the identification of a cytosolic form and a microsomal form of ACC. The proteolytic cleavage of the phosphorylated enzyme is also described. The third section investigates the latter proteolytic phenomena and describes both cAMP-dependent and independent phosphorylation of the enzyme and the corresponding effects on enzyme activity. The last experimental section identifies a "Fatty acid synthesizing complex" that is regulated through covalent modification.

A general discussion will attempt to coorrelate the results of the individual experimental section and arrive at a general hypothesis.

SECTION II

LITERATURE REVIEW

FATTY ACID SYNTHESIS

1. Overview of Liver Metabolism:

A major function of lipogenesis is to store the excess energy ingested by an organism in a readily available form as triacyl glycerols. Thus, the lipogenic status of an organism must be amenable to short term regulation to accomodate fluctuations in dietary intake. This acute regulation is accomplished by the modulation of enzyme activities through various mechanisms such as limiting substrate supply, through allosteric effectors or through covalent modification. As the major site of the synthesis of endogenous fatty acids is the liver and the major site of storage is peripheral adipose tissue,the likely candidate for co-ordinating these two tissues are the hormones glucagon and insulin acting on the liver and insulin and epinephrine acting on the adipose tissue.

It is hypothesized that glucagon alters the metabolic activity of the liver, through short-term control mechanisms, with the result that under its influence the organ is primarily active in glycogenolysis, gluconeogenesis and ketogenesis the net result of which is to mobilize stored energy sources. Insulin, on the other

hand, helps to stores excess energy by inhibiting the above catabolic pathways and stimulating glycogenesis, glycolysis, lipogenesis and fatty acid esterification. It has been hypothesized that these two hormones elicit their opposing effects through covalent modification of enzymes at key points in these metabolic pathways. The exact molecular mechanism for the action of insulin has yet to be elucidated but it has been suggested that the hormone may elicit its effects by directly antagonizing the formation of cAMP by stimulating phosphodiesterase, acting through an alternative second messenger such as cGMP or by phosphorylating enzymes at sites different than those involved with cAMP phosphorylation.

These pathways also seem to be regulated acutely by various allosteric effectors such as malonyl-CoA, long-chain acyl CoA esters, acetyl CoA, fructose 1,6-bisphosphate and citrate. Hormones alter the level of these effectors by changing the flux through the pathways, via changes of the enzyme Vmax, and by altering substrate availability, via changes in membrane permeability and transport. Furthermore, the interdependence of these two regulatory mechanisms (ie. covalent modification and allosteric regulation) is exemplified by the observation that phosphorylation affects the Km for various allosteric metabolites.

The key enzymes that are hypothesized to be co-ordinately regulated by phosphorylation-

dephosphorylation mechanisms are pictured in Figure 1. Glycogen synthase, phosphofructokinase,, pyruvate kinase, pyruvate dehydrogenase, acetyl-CoA carboxylase and B-hydroxy-B-methylglutaryl-CoA reductase are thought to be active in a dephosphorylated state which is favoured by low cAMP levels (i.e. high insulin levels). The exact effects of phosphorylation on ACC, phosphofructokinase and ATP-citrate lyase have yet to be clarified. The allosteric effectors in this situation are fructose 1,6-bisphosphate which activates pyruvate kinase, pyruvate which inhibits pyruvate dehydrogenase kinase and citrate which inhibits phosphofructokinase and activates acetyl-CoA carboxylase. Thus glycolysis, lipogenesis and cholesterolgenesis would be co-ordinately regulated.

Under the influence of high glucagon levels the above enzymes are found in a phosphorylated and inactive form while glycogen phosphorylase, phosphorylase kinase and fructose 1,6-bisphosphatase are in a phosphorylated and active form. This results in a decreased flux through the anabolic pathways and results in the formation of glucose and ketone bodies.

1 : Phosphorylase

2 : Glycogen synthase

3 : Phosphofructokinase

4 : Pyruvate kinase

5 : Pyruvate dehydrogenase

6 : ATP-citrate lyase

7 : Acetyl-CoA carboxylase

8 : Glycerol phosphate acyl transferase

9 : Hormone sensitive lipase

10: Hydroxymethylglutary-CoA reductase

G-6-P: glucose 6-phosphate

F-6-P: fructose 6-phosphate

G-1-P: glucose 1-phosphate

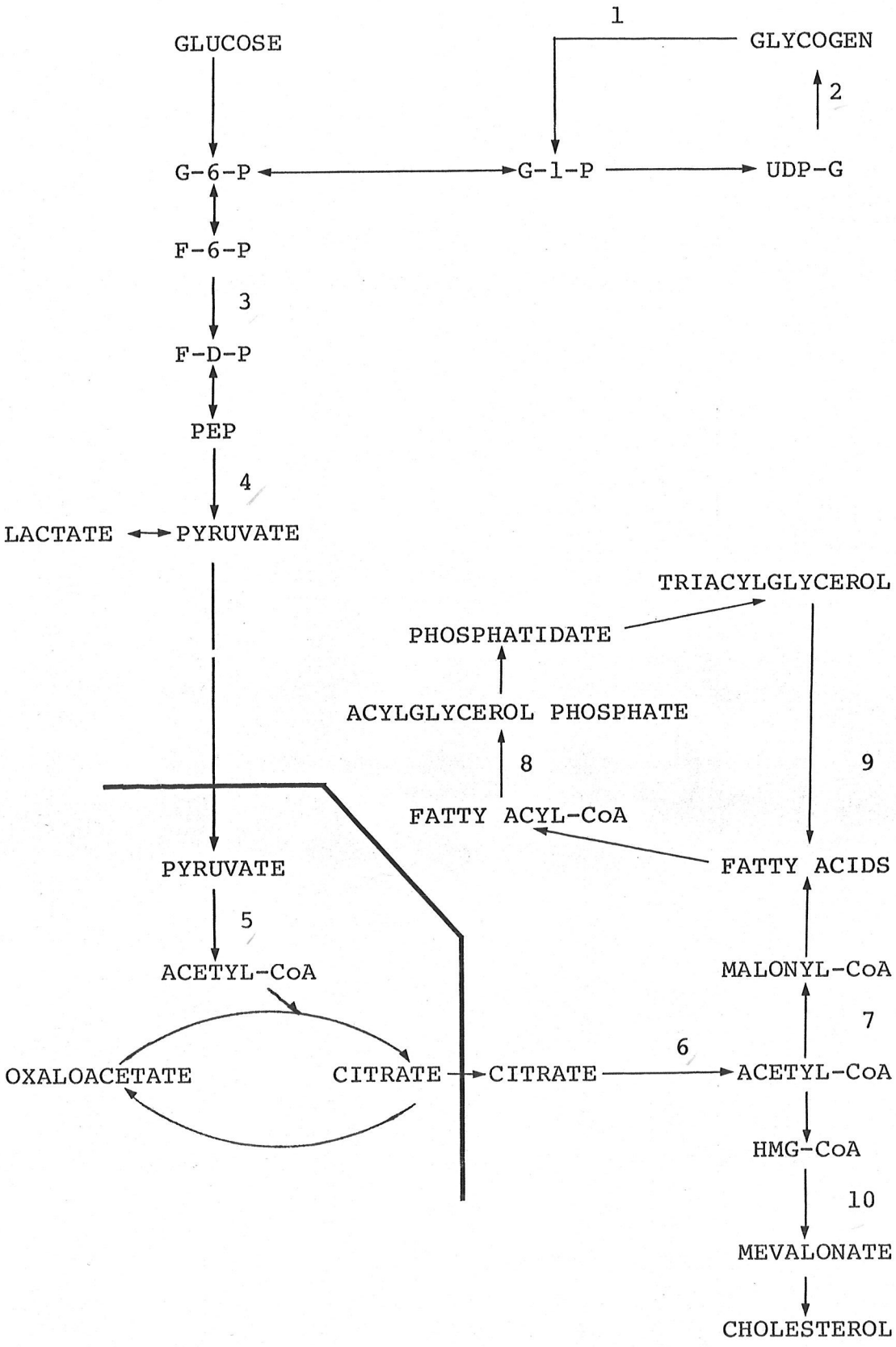
UDP-G: uridine diphosphate glucose

F-D-P: fructose 1,6-diphosphate

PEP : phosphoenolpyruvate

HMG-CoA: hydroxymethylglutaryl coenzyme A

FIGURE 1


GLUCOSE
1
GLYCOGEN
2
G-6-P
G-1-P
UDP-G
F-6-P
3
F-D-P
PEP
4
LACTATE
PYRUVATE
TRIACYLGLYCEROL
PHOSPHATIDATE
ACYLGLYCEROL PHOSPHATE
8
9
FATTY ACYL-CoA
FATTY ACIDS
PYRUVATE
5
ACETYL-CoA
MALONYL-CoA
7
6
OXALOACETATE
CITRATE
CITRATE
ACETYL-CoA
HMG-CoA
10
MEVALONATE
CHOLESTEROL

2. De Novo Synthesis of Saturated Fatty Acids:

The stoichiometry for the synthesis of palmitate, the major fatty acid produced in most organisms, is:

$$8 \text{ acetyl CoA} + 7 \text{ ATP} + 14 \text{ NADPH} + 14 \text{ H}^+ \longrightarrow$$

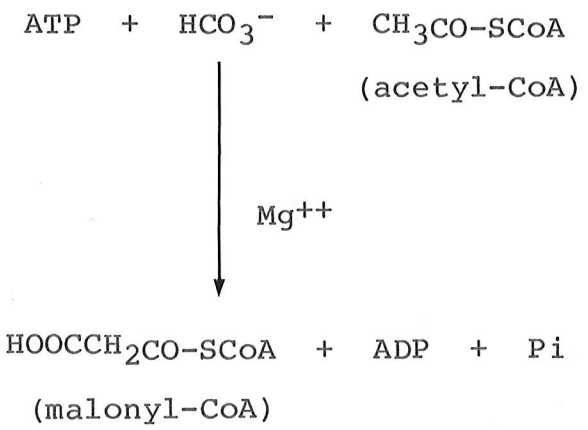
$$\text{Palmitic acid} + 8 \text{ CoA-SH} + 7 \text{ ADP} + 7 \text{ Pi} + 14 \text{ NADP}^+ + 6 \text{ H}_2\text{O}$$

The two enzymes involved in the above reaction are acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS). ACC catalyzes the first committed step in the synthesis of fatty acids from acetyl-CoA and hence is considered to be the major site of short-term regulation of the pathway (Volpe and Vagelos, 1976; Block and Vance, 1977). The carbon source for fatty acid synthesis is generally considered to be mitochondrial citrate which is translocated into the cytosol by the citrate-malate transport system where it is then cleaved into acetyl-CoA and oxaloacetate by ATP-citrate lyase (Geelen et al, 1980). Recently, reports have indicated that cytosolic acetyl-CoA can be derived from acetoacetate via the cytosolic enzymes acetoacetyl-CoA synthetase and acetoacetyl-CoA thiolase (Bergstrom et al, 1982) suggesting mitochondrial acetoacetate as an alternative source of the acetyl-CoA for lipogenesis. This pathway may be utilized in the liver

under certain conditions such as a high fat diet. FAS, on the other hand, is considered to play a major role in the long term adaption of the pathway to different dietary or developmental conditions (Guynn et al, 1972).

3. Acetyl-CoA Carboxylase:

Acetyl-CoA carboxylase catalyzes the carboxylation of acetyl CoA forming malonyl-CoA:



Homogeneous ACC has been purified from avian liver (Gregolin et al, 1968a), bovine adipose tissue (Moss et al, 1972), rat liver (Inoue and Lowenstein, 1972), rat mammary gland (Ahmad et al, 1978), rabbit mammary gland (Hardie and Cohen, 1978a) and pig adipose tissue (Bhuller and Dakshinamurti, 1982). All sources yield an enzyme with a protomeric molecular weight between 400 K and 500 K daltons containing two subunits of a molecular weight between 220 K and 260 K daltons. The enzyme contains 1

biotin prothetic group per subunit. The pure enzyme exists in an equilibrium between the protomeric and high moleculor weight polymeric (4 to 10 million daltons) form of the enzyme. The polymeric form is favoured by the presence of citrate, acetyl-CoA, high protein concentrations and a pH of 6.5 - 7.0 while the protomeric form is favoured by the presence of Cl^-, palmityl-CoA, low protein concentration, a pH greater than 7.5 and by the carboxylation of the enzyme (Volpe and Vagelos, 1976). The enzyme is subject to allosteric activation by citrate which is accompanied by polymerization of the inactive or less active protomers along with conformational changes in the enzyme (Gregolin et al, 1966b). The maximal velocity of the enzyme is increased in the presence of citrate with little effect on the Km values for substrates (Numa et al, 1964; Ryder et al, 1967). The significance of citrate activation of the enzyme in vivo has been highly debated as over 70% of the citrate in the mitochondria (Capuzzi et al, 1974) and estimates of the cytoplasmic concentration are between 0.1 and 0.2 mM (Greenbaum et al, 1971) while the Km for citrate is between 2 and 6 mM, an order of magnitude higher than the in vivo concentrations (Volpe et al, 1976).

Long-chain fatty acyl-CoA thioesters, in uM concentrations, allosterically inhibit and depolymerize mammalian ACC (Bortz and Lynen, 1963a). The inhibition is competitive with respect to citrate and noncompetitive with

respect to acetyl-CoA, bicarbonate and ATP. The above inhibition is reversed by (+)-palmitylcarnitine which acts as a structural analogue and displaces palmityl-CoA but does not alter the activity of the enzyme (Volpe and Vagelos, 1976). The concentration of long-chain fatty acyl-CoA derivatives ranges between 15 and 140 uM in mammalian liver (Bortz and Lynen, 1963b) and as the apparent Ki of palmityl-CoA is around 0.8 to 1.1 uM, inhibition by these derivatives could be significant *in vivo* (Nishikori *et al*, 1973). Recently it has been shown that rat liver ACC binds reversibly to palmityl-CoA in a 1:1 molar ratio with a Ki as low as 5.5 nM (Ogiwara *et al*, 1978) further emphasizing the significance of feedback inhibition of the enzyme *in vivo*.

ACC activity undergoes long term adaptive changes attributed to the alteration of enzyme levels in the cell. The most striking alterations occur when fasted animals are refed a fat-free diet (Allred and Roehrig, 1973). During fasting both a decreased rate of enzyme synthesis and an increased rate of enzyme degradation contribute to the lowered levels of ACC, while an increased rate of synthesis contribute to the rise in ACC levels in a refed state (Majerus and Kilburn, 1969; Nakanishi and Numa, 1970b).

5. Fatty Acid Synthetase:

Fatty acid synthetase is a multienzyme complex that catalyzes the synthesis of saturated fatty acids from malonyl-CoA and acetyl-CoA.

$$\underset{\text{(acetyl-CoA)}}{CH_3CO\text{-}SCoA} + 7\ \underset{\text{(malonyl-CoA)}}{HOOCCH_2CO\text{-}S\text{-}CoA} + 14\ NADPH + 14\ H^+ \downarrow$$

$$\underset{\text{(Palmitic acid)}}{CH_3CH_2(CH_2CH_2)_6CH_2COOH} + 7\ CO_2 + 14\ NADP^+ + 8\ CoASH + 6\ H_2O$$

FAS has been purified to homogeneity from pigeon liver (Hsu et al, 1965), avian liver (Hsu and Yun, 1970), rat liver (Burton et al, 1968), human liver (Roncari, 1974) and rabbit mammary gland (Hardie and Cohen, 1978b). The molecular weight of the enzyme ranges between 450 K to 550 K daltons with one mole of 4'-phosphopontetheine per mole of complex (Burton et al, 1968). An exception is the rabbit mammary gland enzyme which has a molecular weight of 910 K (Carey and Dils, 1970). The enzyme readily dissociates into subunits of approximately 250 K daltons.

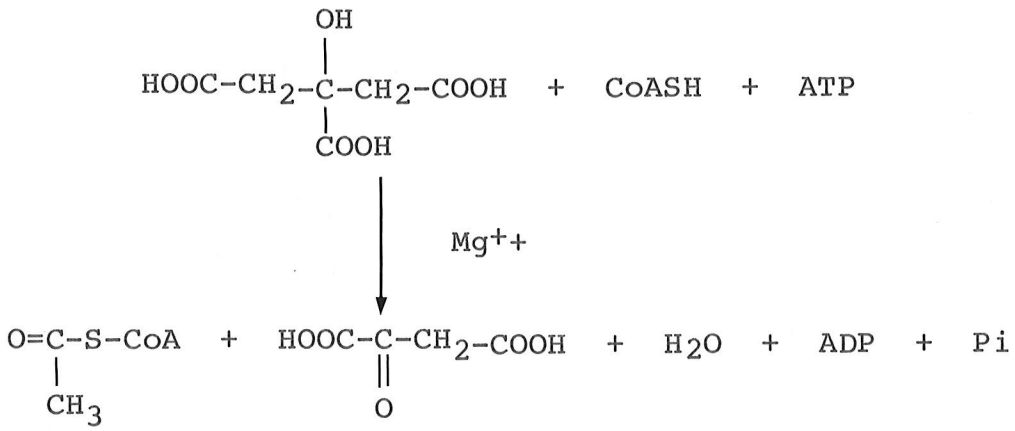
Phosphorylated sugars, especially fructose 1,6-diphosphate, allosterically activate FAS by decreasing the Km for NADPH, thus co-ordinating glucose metabolism and lipogenesis (Wakil et al, 1966). Palmityl-CoA inhibits FAS

activity *in vitro* in rat liver (Tubbs and Garland, 1963) but the *in vivo* significance of this phenomenon has been questioned as it seems to be irreversible and thus may be the result of the detergent action of the thioester (Taketa and Pogel 1966).

FAS undergoes long term alterations especially in rats that are fasted for 48 hours and refed a high-carbohydrate, low fat diet (Allred and Roehrig, 1973). During starvation the rate of degradation of the enzyme was fourfold greater than in the refed state and the rate of synthesis of the enzyme was sixfold less than in the refed state thus resulting in a 20 fold difference in enzyme content (Volpe *et al*, 1973). As malonyl-CoA utilization is limiting under the above conditions it is generally agreed that FAS is the lipogenic enzyme regulated during chronic manipulation of the dietary state (Guynn *et al*, 1972).

6. ATP Citrate Lyase:

ATP citrate lyase (CL) or the citrate cleavage enzyme cleaves citrate into acetyl-CoA and oxaloacetate making the carbons of the citric acid cycle available for lipogenesis:



Citrate lyase (CL) has been purified from rat liver (Linn and Srere, 1979), rat hepatocytes (Ramakrishna and Benjamin, 1979) and rabbit mammary gland (Guy et al, 1981) and the molecular weight of the enzyme ranges between 440 K and 470 K daltons. The enzyme is a tetramer with subunits ranging from 116 K to 123 K daltons (Alexander et al, 1979). Like ACC and FAS, the activity of CL increases under conditions favouring the biosynthesis of fatty acids in liver, adipose tissue and mammary gland, and is decreased under conditions that lower lipogenesis (Ballard and Hanson, 1967; Angielski and Szutowicz, 1967; Kornacker and Lowenstein, 1965; and Smith and Abraham, 1970).

7. Hormonal Regulation of Lipogenesis:

It has been reported that glucagon inhibits the incorporation of tritiated water into long chain fatty acids in liver slices (Harris and Yount, 1975), hepatocytes (Witters et al, 1979; Geelen et al, 1978) and in intact animals (Cook et al, 1977). It has also been shown that upon incubating hepatocytes with insulin there is a rapid stimulation of fatty acid synthesis (Geelen et al, 1978) and the inhibitory effect of glucagon on lipogenesis is antagonized by insulin (Beynen et al, 1979). As lactate or pyruvate only partially relieves the glucagon induced inhibition of fatty acid synthesis, it has been suggested that the effect is on the lipogenic pathway per se and not on glycolysis (Watkins et al, 1977).

It has been observed that the level of malonyl-CoA is rapidly decreased in response to glucagon (Cook et al, 1977) while insulin elevates it (Beynen et al, 1979). This suggest that acetyl-CoA carboxylase catalyzes the step of lipogenesis that is regulated by the above hormones. Furthermore, the activity of ACC is stimulated or depressed after incubation of rat liver cells with insulin and glucagon respectively (Muller et al, 1976; Geelen et al, 1978; Witters et al, 1979).

It was initially observed that purified ACC contains covalently bound phosphate (Inoue and Lowenstein, 1972). It has been suggested that in adipose tissue, insulin and epinephrine respectively activate and inactivate ACC. This

is accompanied by a decrease or increase in the phosphate content of the enzyme (Brownsey et al, 1979; Kim, 1979). It has been shown that crude preparations of ACC can be phosphorylated by both cAMP-dependent and independent kinases and that phosphorylation is accompanied by inactivation of the enzyme (Carlson and Kim, 1973; Lee et al, 1973; Lee and Kim, 1977). These phosphorylated preparations have been reported to be dephosphorylated with protein phosphatase resulting in the activation of the enzyme (Hardie and Cohen, 1979; Krakower and Kim, 1980).

The above hypothesized phosphorylation-dephosphorylation mechanism has been disputed by several laboratories, who report cAMP-independent phosphorylation of ACC without affecting enzyme activity (Brownsey et al, 1977; Pekala et al, 1978; Desjardins and Dakshinamurti, 1978).

Several complications arise when trying to interpret the hormonal affects on enzyme activity. One is the cold sensitivity of ACC. Lowering the temperature to 4°C inactivates the enzyme by depolymerizing it into inactive protomers (Numa, 1965). Another is that proteolysis can result in the activation of the enzyme (Swanson et al, 1968) and in the loss of phosphate (Guy et al, 1981). Finally the activity of the enzyme is usually assayed in the presence of citrate which activates the enzyme and masks short-term dietary and hormonal effects (Allred and Roehrig, 1973). It is possible that

covalent modification and allosteric modulation are complimentary and the activity of the enzyme may be dependent on the presence of allosteric effectors and the interaction of the allosteric effectors with the enzyme may be regulated by the phosphorylation state of the enzyme.

INTERACTIONS BETWEEN SOLUBLE ENZYMES AND SUBCELLULAR STRUCTURE

The term cytosolic fraction, containing the so called "soluble" components of the cell, should not be taken as an indication that these components are freely dissolved *in vivo*. It is an operational definition applied to the subcellular fractionation process and does not establish the *in vivo* localization of proteins or metabolites associated with this fraction. Recent data suggests that many enzymes present in the cytosolic fraction may be interacting with subcellular structures forming multienzyme complexes (Masters, 1981). These include the binding of hexokinase to mitochondria (Wilson, 1972) and the binding of lactate dehydrogenase (Ehmann and Hultin, 1973), aldolase, pyruvate kinase and phosphofructokinase to particulate elements of tissues (Masters, 1978). The possibility has also been suggested that all cytosolic

enzymes may be associated to some degree with the microtrabecullar lattice or the network of microtubles that can be visualized under the electron microscope and that this association may play a regulatory role in metabolic pathways (Wolosewick and Porter, 1979). The possibility then arises that the kinetic parameters associated with individual enzymes may be altered upon association as a multienzyme complex exemplified by the so called glycosome of muscle (Clarke and Masters, 1976). The term ambiquitous has been coined for these enzymes to describe their tendency to be localized in either a soluble or membrane bound form (Wilson, 1978).

Some reports suggest that Acetyl-CoA carboxylase may be one of these ambiquitous enzymes. Acetyl-CoA carboxylase has been found to be associated with particulate frations in yeast (Abraham et al, 1961), fungus (Vorisek and Lojda, 1979) and in barley seedlings (Reitzel and Nielsen, 1976).

PURIFICATION OF AVIAN ACETYL-CoA CARBOXYLASE

INTRODUCTION

To investigate the covalent modification of an enzyme in impure preparations, it is necessary to have a simple and rapid method to isolate the modified protein in question. This is usually accomplished with the use of a specific antibody to precipitate and isolate the enzyme. We, therefore, set out to purify ACC from a readily available source and prepare the antibody to the protein.

Gregolin et al (1968a) purified ACC from avian liver and found the subunit composition to be 117 K, 129 K, and 139 K daltons in a ratio of 2:1:1 (Guchhait et al, 1974). It has been subsequently shown by Mackall and Lane (1977) that the above are actually proteolytic fragments of the enzyme and the intact subunit has a molecular weight of 230 K daltons. Similarly, the rat liver enzyme has been shown to consist of a native 230 K dalton subunit that is cleaved into 124 K and 118 K fragments (Tanabe et al,1975). Recently, using avidin affinity chromatography to quickly isolate the enzyme, it has been suggested that the native subunit of rat liver ACC has an even higher molecular weight of 260 K daltons (Song and Kim, 1981).

Majerus and Kilburn (1969) prepared antibody against isolated avian liver ACC and demonstrated that it cross-reacted with rat liver enzyme. They then used the antibody to study the synthesis and degradation of the enzyme under various lipogenic states which were altered by dietary manipulation.

The purification proceedure of Gregolin et al (1966a) was adapted to isolated ACC from laying hen liver (fowl liver) as this tissue was easily obtained and contained high levels of ACC. Antibody to the pure enzyme was produced by inoculating rabbits with pure protein and collecting the antisera produced.

EXPERIMENTAL

(a) MATERIALS:

Electrophoretic reagents were obtained from Sigma Chemical Company; goat anti-rabbit sera from Miles Laboratory and [^{14}C] sodium bicabonate from New England Nuclear. Gel filtration standards and electrophoretic standards were supplied by Pharmacia Fine Chemicals. NCS tissue solubilizer and OCS scintillant were obtained from Amersham Corporation.

(b) ACETYL-CoA CARBOXYLASE ASSAY:

Acetyl-CoA carboxylase activity was determined by the [^{14}C]-bicarbonate fixation assay of Dakshinamurti and Desjardins (1969). The enzyme (25 - 50 ul) was incubated in the presence of 60 mM TRIS HCl; 3.0 mM reduced glutathione; 8 mM MgCl ;20 mM potassium citrate; 0.6 mg/ml bovine serum albumen, 2.0 mM ATP; 0.2 mM acetyl-CoA and 10 mM [^{14}C]-sodium bicarbonate (0.6 uCi/umole), in a total reaction volume of 0.5 ml. The reaction was stopped with 100 ul of 6N HCL and a 200 ul aliquot was dried and counted in an aqueous scintillation fluid. One unit of enzyyme was defined as the incorporation of one umole of bicarbonate into acid stable malonyl-CoA per minute.

(c) PROTEIN ASSAYS:

Protein was determined by the dye binding method using

the reagent supplied by Bio-Rad (Bradford 1976; Biorad, 1977). Alternatively, the concentration of pure protein was estimated using the relationship of A_{280nm} x 0.86 = mg/ml as outlined by Gregolin et al, (1968a).

(d) SDS GEL ELECTROPHORESIS:

Polyacrylamide tube gels were run using the discontinuous system of Laemmli and Favre (1973) employing 5% acrylamide running gels and 3% acrylamide stacking gels. The gels were run for approximately 4 hours (at 3 mamps per tube). The gels were stained overnight in a solution of 0.25% coomassie blue G, 25% isopropanol and 10% acetic acid. The gels were then destained in 5% methanol and 7.5% acetic acid. The molecular weight of proteins on the SDS gels were determined using standards supplied by Pharmacia.

(e) PURIFICATION OF AVIAN LIVER ACC:

(i) HOMOGENIZATION AND AMMONIUM SULFATE FRACTIONATION:

Batches (1.0 kg. to 1.5 kg.) of frozen fowl livers were homogenized with a waring blender in 1.5 volumes of 85 mM K_2HPO_4, 9 mM KH_2PO_4 and 0.1 mM EDTA. The homogenate was centrifuged for 90 minutes at 10,000 xg in a GS-3 rotor. The supernatant was filtered through cheesecloth and brought to 25% saturation with solid ammonium sulfate while maintaining the temperature at 4°C and the pH at 7.0.

After allowing the protein to precipitate for 30 minutes the suspension was centrifuged for 60 minutes as above and the pellet was suspended in 10 mM potassium phosphate buffer pH 7.0 containing 0.1 mM EDTA and 5mM B-mercaptoethanol. The resuspended enzyme was then dialyzed against the above buffer overnight at 4°C.

(ii) CALCIUM PHOSPHATE GEL ADSORPTION AND ELUTION:

The dialyzed enzyme was then adsorbed onto Calcium phosphate gel (1 gm. protein/ 1.5 gm. dry weight of gel) and stirred gently for 15 minutes at 4°C. The gel suspension was centrifuged at 1600 rpm for 10 minutes in an I.E.C. PR6 centrifuge and the supernatant was then discarded. The enzyme was extracted from the gel with 3 litres of 120 mM potassium phosphate buffer pH 7.0 containing 0.1 mM EDTA and 5 mM B-mercaptoethanol.

The extraction was repeated three times and the pooled extract was brought to 50% saturation with ammonium sulfate and centrifuged. The pellet was resuspended in 2 litres of 100 mM KPO_4 pH 7.0 containing 25% $(NH_4)_2SO_4$ and recentrifuged at 10,000 xg for 30 minutes. The pellet was frozen until later use.

(iii) DEAE CELLULOSE CHROMATOGRAPHY:

The dialysed pellets of three batches of liver (4 Kg) from the calcium phosphate gel step were pooled and applied to a DEAE cellulose column (5 x 20 cm) equilibrated by

washing it with 0.5 M potassium phosphate pH 7.0 followed by washing with 0.01 M potassium phosphate containing 0.1 mM EDTA and 5 mM B-mercaptoethanol. Elution was completed with a 6 liter linear gradient (0.01 M to 0.3 M potassium phosphate containing 0.1 mM EDTA and 5 mM B-mercaptoethanol) at a flow rate of 5 ml/minute. The effluent was collected in 10 ml fractions and assayed for absorbance at 280 nm, ACC activity and conductance. The peak of carboxylase activity appeared in the eluant when the phosphate concentration reached 150 mM. The active fractions were pooled and the enzyme was precipitated with the addition of 0.66 volumes of 100 mM potassium phosphate saturated with ammonium sulfate containing 0.1 mM EDTA and 5 mM B-mercaptoethanol. After centrifugation the pellet was dissolved in 30 ml. of 10 mM potassium phosphate buffer pH 7.0 containing 0.1 mM EDTA, 5 mM B-mercaptoethanol and 20 mM potassium citrate and dialysed overnight under an atmosphere of nitrogen at room temperature.

(iv) SEPHAROSE 2B CHROMATOGRAPHY OF POLYMERIZED ACC:

The enzyme fraction from the above was concentrated with sucrose (to approximately 20 ml), clarified by centrifugation and applied to a 2.5 x 90 cm. column of sepharose 2B equilibrated with the above dialysis buffer (Numa et al, 1964). The column was eluted with the equilibration buffer at a flow rate of 2 ml / minute and

fractions (5ml) were collected and assayed for absorbance at 280 nm and ACC activity. Fractions with the approximately the same specific activity were pooled and the enzyme was precipitated by the addition of an equal volume of 100 mM potassium phosphate buffer pH 7.0 saturated with ammonium sulfate containing 0.1 mM EDTA and 5mM B-mercaptoethanol. The precipitated enzyme was cetrifuged in aliquots at 10,000 xg for 15 minutes and the pellets were frozen and stored.

(v) SEPHAROSE 2B CHROMATOGRAPHY OF DEPOLYMERIZED ACC:

Pellets from the above step were resuspended and dialyzed against 50 mM TRIS buffer pH 7.0 containing 0.1 mM EDTA, 5 mM B-mercaptoethanol and 0.5 M NaCl overnight at 4°C under an atmosphere of nitrogen (Numa et al, 1964). The depolymerized enzyme was then applied to a 2.5 x 40 cm sepharose 2B column equilibrated with the above buffer. Fractions were collected as in the preceeding section and the ACC activity and absorbance at 280 nm was determined. Active fractions were pooled, concentrated with sucrose and dialysed against 10 mM phosphate buffer pH 7.0 containing 20 mM citrate 0.25 sucrose, 0.1 mM EDTA and 5 mM B-mercaptoethanol under nitrogen at 4°C. Aliquots were stored at -20°C until use.

(f) PREPARATION OF ANTIBODY TO ACC:

Anti acetyl-CoA carboxylase serum was prepared

essentially by the procedure of Marshall and Cohen (1961) as modified by Nakanishi and Numa (1970a). Rabbits were injected intradermally along their sides with 1 mg of pure protein emulsified in an equal volume of Freund's complete adjuvant. After a three week interval another 1 mg of the pure enzyme was injected intravenously . Two weeks later a final injection of 1 mg of the enzyme was given intravenously and the serum was collected 8 days later by heart puncture. The immunoglobulin G fraction of the antiserum was prepared by ammonium sulfate frationation as described by Marshall and Lane, (1977).

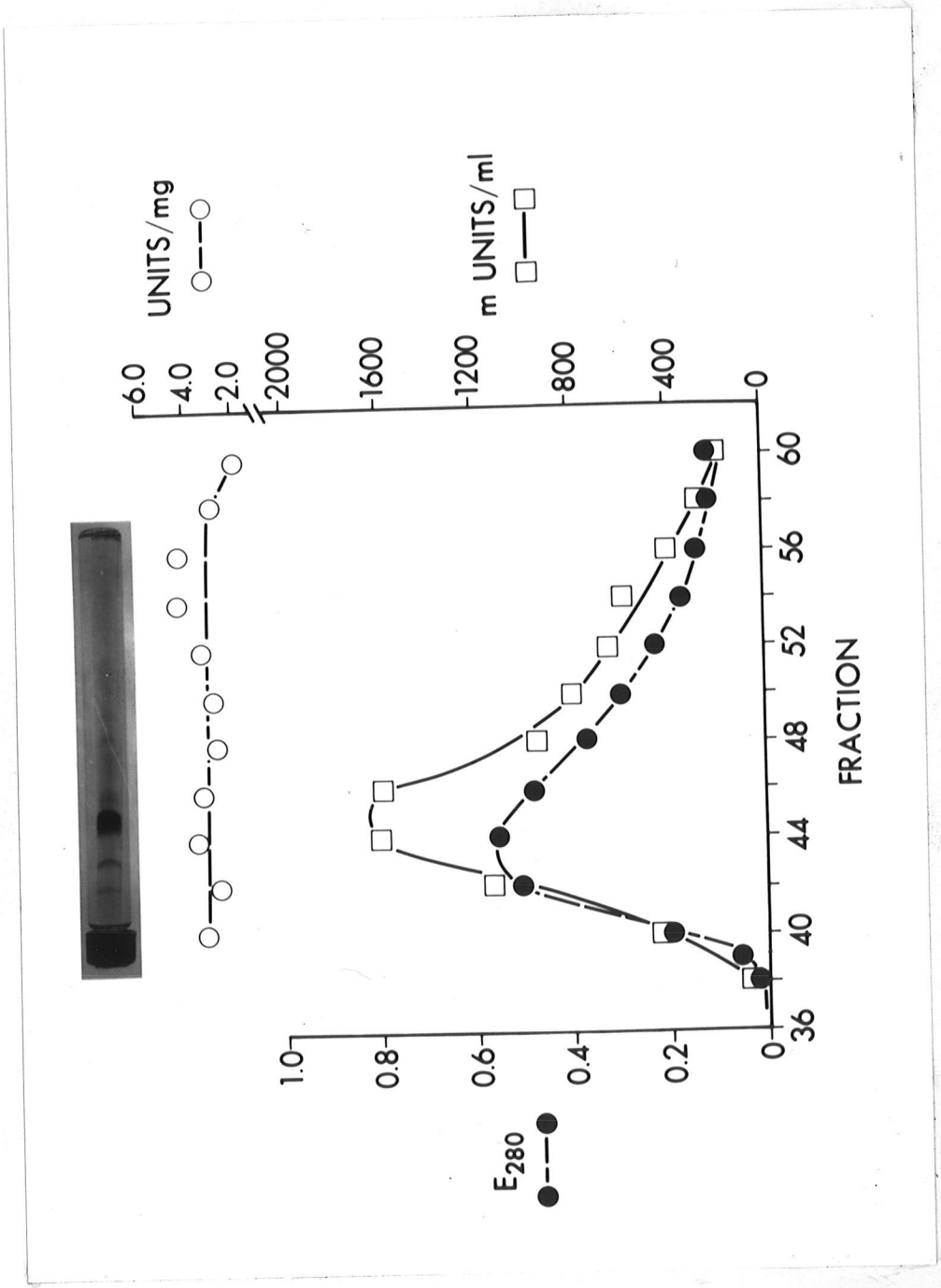
The antibody titre was monitored as outlined by Majerus and Kilburn (1969). Aliquots of freshly prepared 30% $(NH_4)_2SO_4$ fraction of rat liver cytosol were preincubated with appropriate volumes of anti-ACC or control sera for 30 minutes at 37°C in the presence of 20 mM potassium citrate. Subsequently an appropriate volume of the above was assayed by the standard ACC assay.

Acetyl-CoA carboxylase was immunoprecipitated by the method of Harrington et al (1971). The enzyme was incubated for 30 minutes at 37°C with the antibody in the presence of 0.85% NaCl. Goat anti-rabbit gamma globulin was added to precipitate the antibody enzyme complex and this mixture was incubated at 37°C for 15 minutes in the presence of 1% polyethylene glycol (M_r 6,000) at which time precipitation of the immune complex was complete.

RESULTS

The separation of polymerized ACC on sepharose 2B has been described by Nakanishi and Numa (1970) for the rat liver enzyme. A single trailing peak of protein was observed that eluted near the void volume of the column that corresponded to enzyme activity (figure 2) with constant specific activity throughout the peak. Upon subjecting the pooled fractions to SDS gel electrophoresis three major bands were observed corresponding to the 230 K native subunit and proteolytic cleavage products of 139 K and 129 K to 117 K (Mackall and Lane, 1977). To confirm that these other bands were indeed proteolytic products the enzyme was depolymerized in the presence of 0.5 M NaCl (Gregolin et al, 1966a). Two peaks of protein and correspondingly two peaks of enzyme activity were observed, the second peak corresponding to the protomeric form of the enzyme (figure 3). Only the broad band corresponding to the proteolytic fragments of 129 K to 118 K were observed when the second peak was subjected to SDS gel electrophoresis. This suggests that the enzyme that has not been proteolytically cleaved has a different susceptability to depolymerization by NaCl and migrated in the first peak. It would seem that this peak of enzyme has been denatured as there is a very low specific activity associated with it.

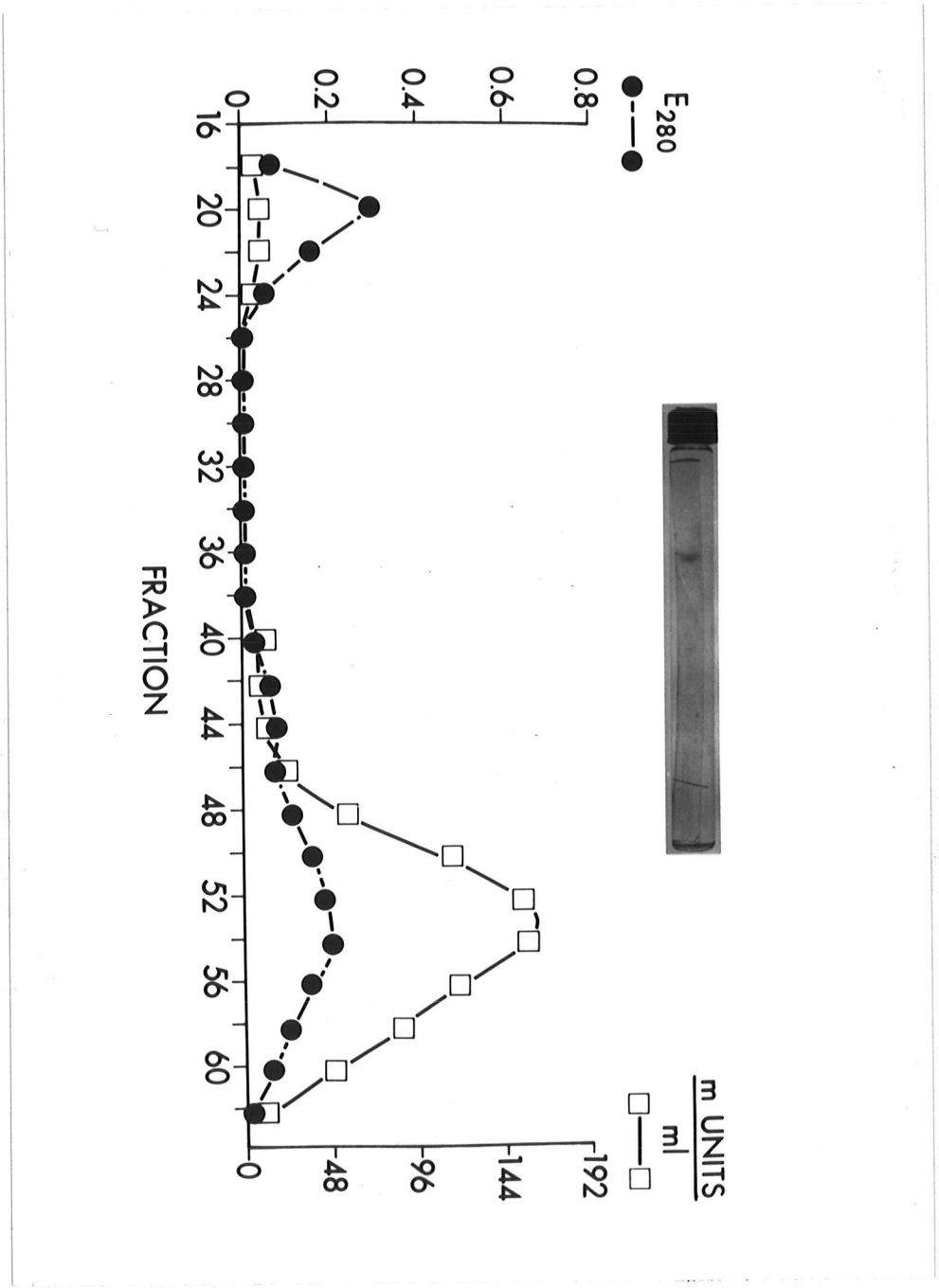
FIGURE 2


UNITS/mg
m UNITS/ml
E_{280}
FRACTION
6.0
4.0
2.0
2000
1600
1200
800
400
0
1.0
0.8
0.6
0.4
0.2
0
36
40
44
48
52
56
60

SEPHAROSE 2B CHROMATOGRAPHY OF POLYMERIZED ACC

The polymerized enzyme was subjected to chromatography on sepharose 2B and fractions were collected and assayed for absorbance at 280 nm. (● ——— ●) and ACC activity (□ ——— □). The specific activity of the enzyme was also plotted (o ——— o) and an aliquot of the pooled peak was subjected to SDS gel electrophoresis (inset).

FIGURE 3


E280
0
0.2
0.4
0.6
0.8
16
20
24
28
32
36
40
44
48
52
56
60
FRACTION
m UNITS
ml
0
48
96
144
192

SEPHAROSE 2B CHROMATOGRAPHY OF DEPOLYMERIZED ACC

The depolymerized enzyme was subjected to chromatography on sepharose 2B in the presence of 0.5 M NaCl and fractions were collected and assayed for absorbance at 280 nm. (● ——— ●) and ACC activity (□ ——— □). An aliquot of the pooled second peak was subjected to SDS gel electrophoresis (inset).

This has been confirmed recently by Beaty and Lane (1982) who observed that the unmodified enzyme displayed different kinetic properties from the proteolytically modified enzyme.

The yield of pure protein from the final sepharose 2B column was approximately 4 mg/kg tissue (table 1) with a specific activity of 8 to 10 Units/ mg, corresponding well with published values.

The final titre of the isolated gamma globulin fraction of the anti ACC serum is shown in figure 4 and by extrapolating the initial slope it was found that 3.5 munits of rat liver ACC were inactivated by one ul of the gamma globulin fraction. This also indicated that the antibody to the avian enzyme cross reacts with the rat liver enzyme. When SDS gel electrophoresis was performed on the immunoprecipitate using rat liver enzyme only one major band of 250 K was precipitated by the antibody (figure 5). It was difficult to determine if minor components are precipitated by the antibody due to the presence of large bands corresponding to the heavy and light chains of the immunoglobulin molecule.

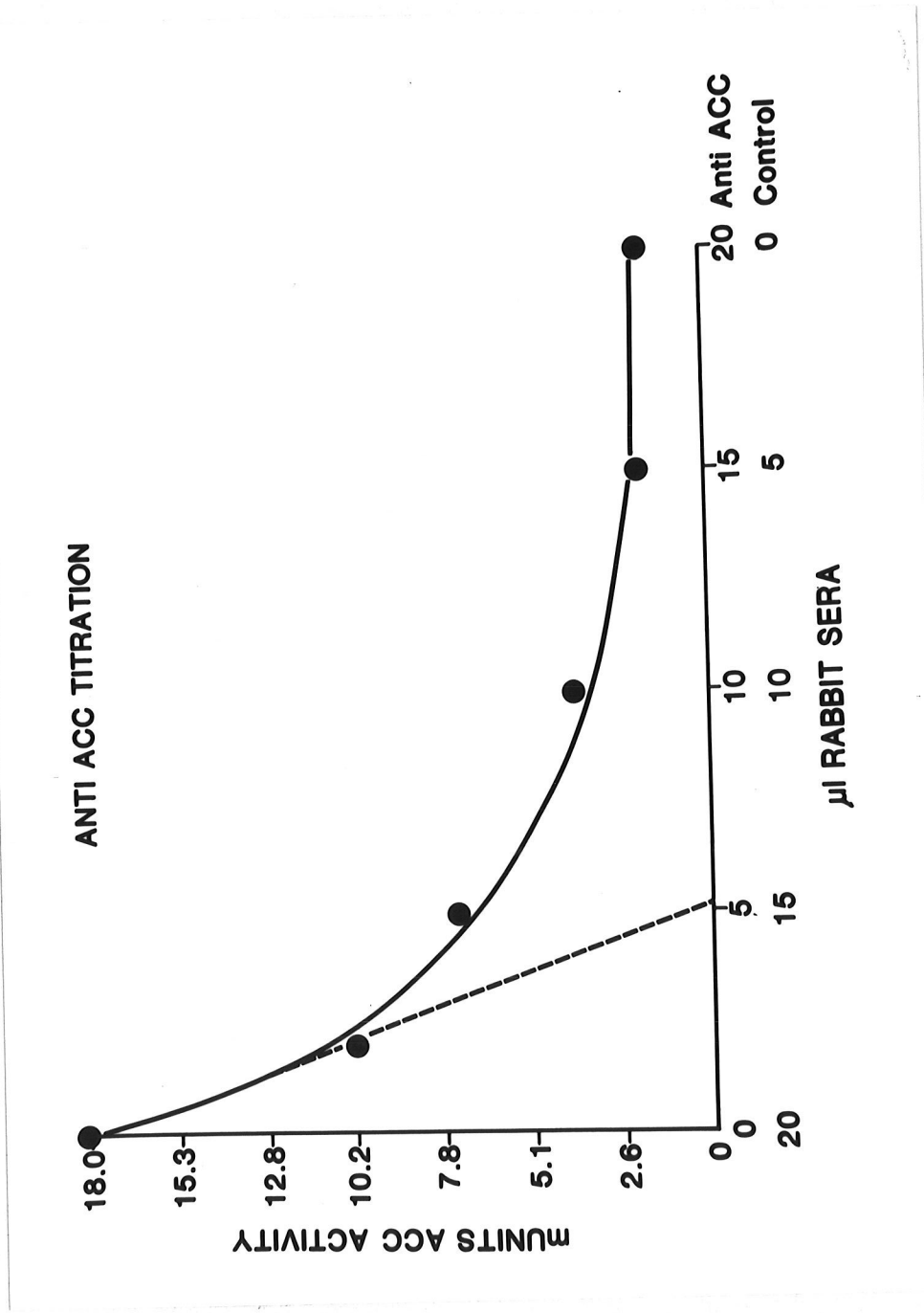
TABLE 1

PURIFICATION OF ACETYL-CoA CARBOXYLASE

Step	Total Units	Protein (mg)	S.A. (units/mg)
100K xg supernatant	145	61,600	0.0024
Ammonium sulfate fraction	437	55,120	0.008
Calcium phosphate gel extract	195	5,242	0.037
DEAE cellulose chromatography*	195	198	0.98
Polymerized Sepharose 2B*	146	23	6.2
Depolymerized Sepharose 2B*	111	13	8.5

* Three batches of liver were processed through to the Calcium phoshate gel step and then pooled for the subsequent steps

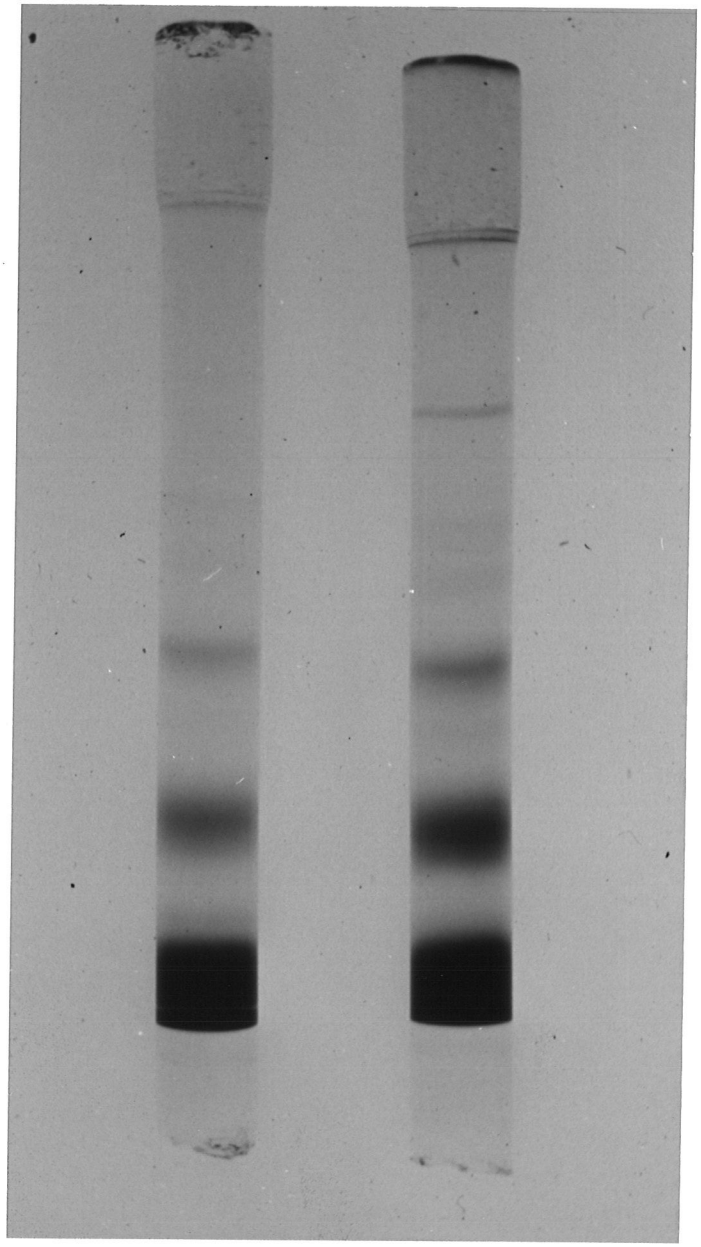
FIGURE 4


ANTI ACC TITRATION
mUNITS ACC ACTIVITY
18.0
15.3
12.8
10.2
7.8
5.1
2.6
0
0
5
15
20
µl RABBIT SERA
0 Control
20 Anti ACC
5
10
15
10
20

IMMUNOTITRATION OF RAT LIVER ACC

Increasing amounts of rabbit anti-sera was incubated with a constant amount of rat liver ACC for 30 minutes at 37°C and then assayed for ACC activity (● ——— ●). Control sera was added to standardize the total amount of sera in each tube.

FIGURE 5



SDS GEL ELECTROPHORESIS OF THE IMMUNOPRECIPITATE FROM RAT LIVER

Immunoprecipitates obtained by incubating the rat liver ACC with either control (gel A) or anti-ACC (gel B) sera were denatured at 100°C for 2 minutes in the presence of 1% SDS and 5% B-mercaptoethanol and subjected to SDS gel electrophoresis.

SECTION III

DISCUSSION

Antibody was produced against avian liver ACC that cross-reacted with rat liver ACC inactivating and precipitating the enzyme. The antibody was actually prepared against the 117 K and 129 K proteolytic fragments of the avian liver enzyme. This is similar to the procedure employed by Lee and Kim (1977) who prepared antibody to the proteolytic fragment (125 K) of rat liver ACC that specifically precipitated the native enzyme. Polyethylene glycol was used to speed up immunoprecipitation as the conventional procedure, involving incubation overnight at 4°C results in non-specific precipitation of proteins in the crude 100,000 x g supernatants (Majerus and Kilburn, 1969).

The antibody prepared was used to investigate the covalent modification of ACC by analyzing the incorporation of label from [γ-^{32}P]-ATP into the enzme protein.

SECTION IV

ISOLATION OF CYTOSOLIC AND MICROSOMAL ACC

INTRODUCTION

Evidence that acetyl-CoA carboxylase (ACC) undergoes covalent modification by a phosphorylation-dephosphorylation mechanism is steadily accumulating (Krebs and Beavo, 1979). Kim and coworkers (Carlson and Kim, 1973; Carlson and Kim, 1974a; Carlson and Kim, 1974b; Lee and Kim, 1977) have reported on a phosphorylation-dephosphorylation mechanism in which the phosphorylated enzyme (ACC-b) has a decreased response to citrate and is more sensitive to palmityl CoA inhibition. Recently, they have demonstrated that the inactivation and phosphorylation of the enzyme under low citrate levels is stimulated by cAMP and that phosphorylation is accompanied by the depolymerization of the active polymer into a species that is intermediate in size between the protomer and polymer (Lent et al, 1978). Furthermore treatment of epididymal fat tissue in culture with epinephrine resulted in the formation of a catalytically less active enzyme that sedimented as an intermediate-sized form (Lee and Kim, 1978).

Although the exact mechanism of ATP inactivation is still under investigation recent reports indicate that ACC

is phosphorylated (Lane et al, 1974). Hardie and Cohen (1978a) reported the phosphorylation of rabbit mammary gland ACC by a cAMP-dependent as well as a cAMP-independent process but failed to report any direct change in enzyme activity following phosphorylation. Pekala et al (1978) phosphorylated ACC in cultured chick liver cells but reported that the phosphorylated protein (ACC-b) was enzymatically as active as the nonphosphorylated protein (ACC-a). Previous work in our laboratory (Desjardins and Dakshinamurti, 1978) provided evidence suggesting that the effect of ATP on the enzyme was due to disaggregation of the polymeric form.

Several criteria have been outlined for identifying the physiological significance of protein phosphorylation-dephosphorylation cycles (Krebs and Beavo, 1979). These are:

(1). Demonstration of stoichiometric phosphorylation and dephosphorylation in vitro at significant rates with appropriate kinases and phosphatases.

(2). Demonstration that functional properties of the enzyme undergo changes that correlate with the degree of in vitro phosphorylation.

(3). Demonstration of phosphorylation and dephosphorylation *in vivo* or *in situ* with accompanying functional changes.

(4). Demonstrate a relationship between the degree of phosphorylation and cellular levels of kinase and phosphatase effectors.

With the above in mind we have investigated the *in vitro* phosphorylation of ACC to assess the role of phosphorylation in the regulation of lipogenesis.

EXPERIMENTAL

Preparation of partially purified Rat Liver ACC for Inactivation and Phosphorylation Studies

(i) Procedure A:Preparation of Phosphate Extracted ACC.

Fresh rat livers were homogenized with a teflon pestle in 3 volumes of 100 mM potassium phosphate buffer pH 7.5 containing 0.25 M sucrose, 5 mM B-mercaptoethanol and 1.0 mM EDTA and the high-speed supernatant (100,000 x 60 min) was prepared. This was brought to 30% saturation with $(NH_4)_2SO_4$ (pH 7.2 to 7.5) and the enzyme allowed to precipitate for 30 minutes after which it was collected at 10,000 x g for 20 minutes. The pellet was then resuspended and dialysed against 100 mM potassium phosphate buffer pH 7.5 containing 5 mM B-mercaptoethanol and 0.1 mM EDTA. All these operations were carried out at 4°C.

(ii) Procedure B:Preparation of TRIS Extracted ACC.

Fresh rat livers were homogenized in 3 volumes of 50 mM TRIS buffer pH 7.5 containing 0.25 M sucrose, 1.0 mM EDTA and 5 mM B-mercaptoethanol (TRIS-sucrose buffer). A 30% ammonium sulfate precipitation was performed on the high speed supernatant as above. It was then dialysed against a 50 mM TRIS buffer pH 7.5 containing 0.1 mM EDTA

and 5mM B-mercaptoethanol (TRIS buffer) for 3 hours. The dialysed enzyme was then clarified by centrifugation and passed through a sephadex G-25 column, equilibrated with the dialysis buffer. The protein fraction was then collected.

(iii) Procedure C:Preparation of Microsomal and Cytosolic Acc.

Fresh rat livers were homogenized in 1.5 volumes of 10 mM potasium phosphate buffer, pH 7.5 containing 0.25M sucrose, 5 mM B-mercaptoethanol and 0.1 mM EDTA (PO_4-sucrose buffer). Subcellular fractionation was done according to Easter and Dils (1968). This consisted of centrifuging the homogenate at 10,000 rpm in a Sorval centrifuge for 20 minutes followed by centrifuging the resultant low speed supernatant at 100,000xg for 60 minutes. The high speed supernatant (100,000xg) was brought to 35% ammonium sulphate saturation. The precipitated protein was collected and dialysed against TRIS buffer then mixed with 0.6 volumes of glycerol and frozen until use. This preparation contained the cytosolic species of ACC.

The microsomal pellet obtained during subcellular fractionation was rehomogenized in two volumes of TRIS buffer. After centrifugation at 105,000 x g for 60 minutes the supernatant was dialysed against TRIS buffer. This procedure yielded a microsomal species of ACC.

In some experiments the cytosolic and microsomal species of ACC was subjected to 35% ammonium sulphate fractionation and dialysed against TRIS buffer.

(iv) Procedure D:Preparation of Calcium Phosphate Extracted Microsomal and Cytosolic ACC.

The 35% ammonium sulfate fractionated cytosolic and microsomal enzyme preparations (see procedure C), were adsorbed on calcium phosphate gel and eluted with 1 volume of 150 mM phosphate buffer (pH 7.5) containing 0.1 mM EDTA and 5 mM B-mercaptoethanol. The eluant was frozen in aliquots until use.

ATP Inactivation-Activation Assay:

The enzyme (3-5 mg/ml) was inactivted at 37°C in the presence of 50 mM TRIS pH 7.5, 2 mM $MgCl_2$ and 1 mM ATP. At appropriate times, 50 ul aliquots were removed and assayed by the [^{14}C]-bicarbonate fixation assay (Dakshinamurti and Desjardins, 1969) except that the final $MgCl_2$ concentration in the assay medium was 9.3 mM and the final ATP concentration was 2.1 mM.

The enzyme was activated as above except that the concentration of $MgCl_2$ was increased to 13 mM and ATP was omitted. Controls with ATP alone being omitted were included along with the inactivation assay.

Labelling of ACC with [γ-^{32}P]-ATP:

The enzyme was incubated as described for inactivation of the enzyme but with the exception that cold ATP was replaced with 1 mM [γ-^{32}P]-ATP. After incubation for 30 minutes the labelled enzyme was passed through a column of sephadex G-25 (1.5 x 30 cm) equilibrated with 50 mM TRIS plus 0.15 M KCl. The first peak of radioactivity was collected and was incubated for 2 hours with Sepharose-avidin (Landman and Dakshinamurti, 1973). The above solution was then filtered on a millipore filter, washed with 50 mM TRIS pH 7.5 plus 0.3 M KCl then with 3 M guanidine HCl. The filter was then counted using a toluene-ethanol scintillant (Dakshinamurti and Desjardins, 1969).

Alternatively the phosphorylated enzyme fraction from the G-25 column (equilibrated in TRIS buffer) was incubated for 30 minutes at 37°C with rabbit anti-ACC antibody in the presence of 0.85% NaCl. Goat anti-rabbit -globulin was added to precipitate the rabbit anti-ACC complex and the above mixture was incubated at 37°C for 15 minutes in the presence of 0.85% NaCl and 1% polyethylene glycol (M_r 6000) at which time precipitation of the immune complex was complete (Harrington et al, 1971).

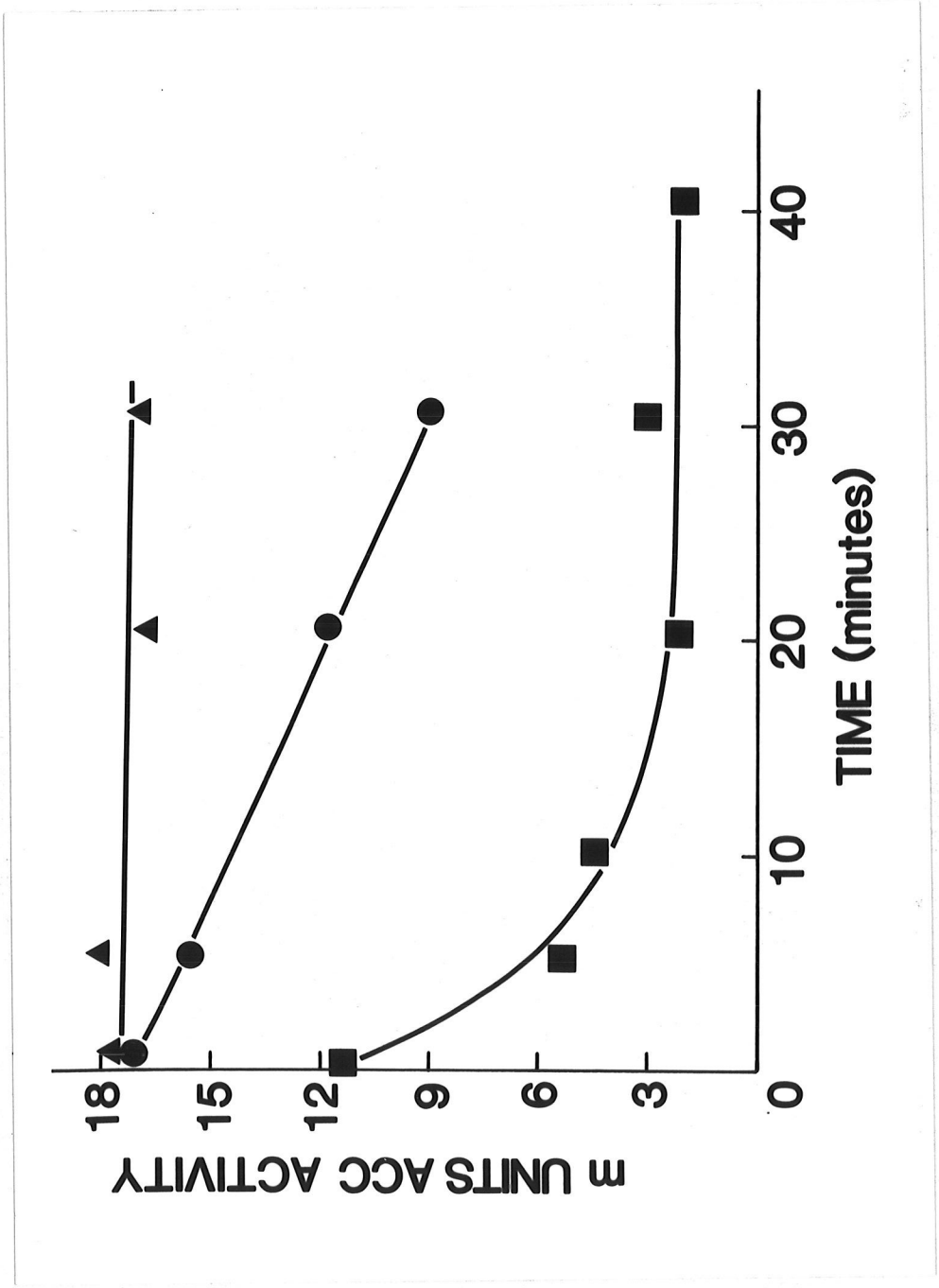
RESULTS

Inactivation and Phosphorylation of ACC

We initially proceeded to investigate the inactivation, by ATP, of the 30% Ammonium sulphate fraction from rat liver homogenized in a phosphate sucrose buffer. This fraction contains both ACC and a protein kinase (Carlson and Kim, 1974a). The ACC specific activity of this preparation was 20 munits/mg. We observed inactivation of ACC in the presence of 1 mM ATP and 2 mM $MgCl_2$ (Fig. 6) while 2 mM $MgCl_2$ alone and 13 mM $MgCl_2$ stabilized or prevented this inactivation. When [$-^{32}P$]-ATP was used in this incubation no label was bound to Sepharose-avidin.

The results confirm earlier observation in our laboratory (Desjardins and Dakshinamurti, 1978) that the inactivation of ACC by ATP can occur without concommitant phosphorylation. We reinvestigated the phosphorylation reaction by an alternate procedure involving the extraction of liver in a TRIS-sucrose buffer. The inactivation curve of the TRIS extracted enzyme is presented in Figure 7. A four fold increase in enzyme (ACC) specific activity was observed over the previous Phosphate extracted preparation. The inactivation of ACC in this preparatiion was accompanied by the incorportion of about 1 pmole of ^{32}P per 50 mUnits ACC activity after a 30 minute incubation

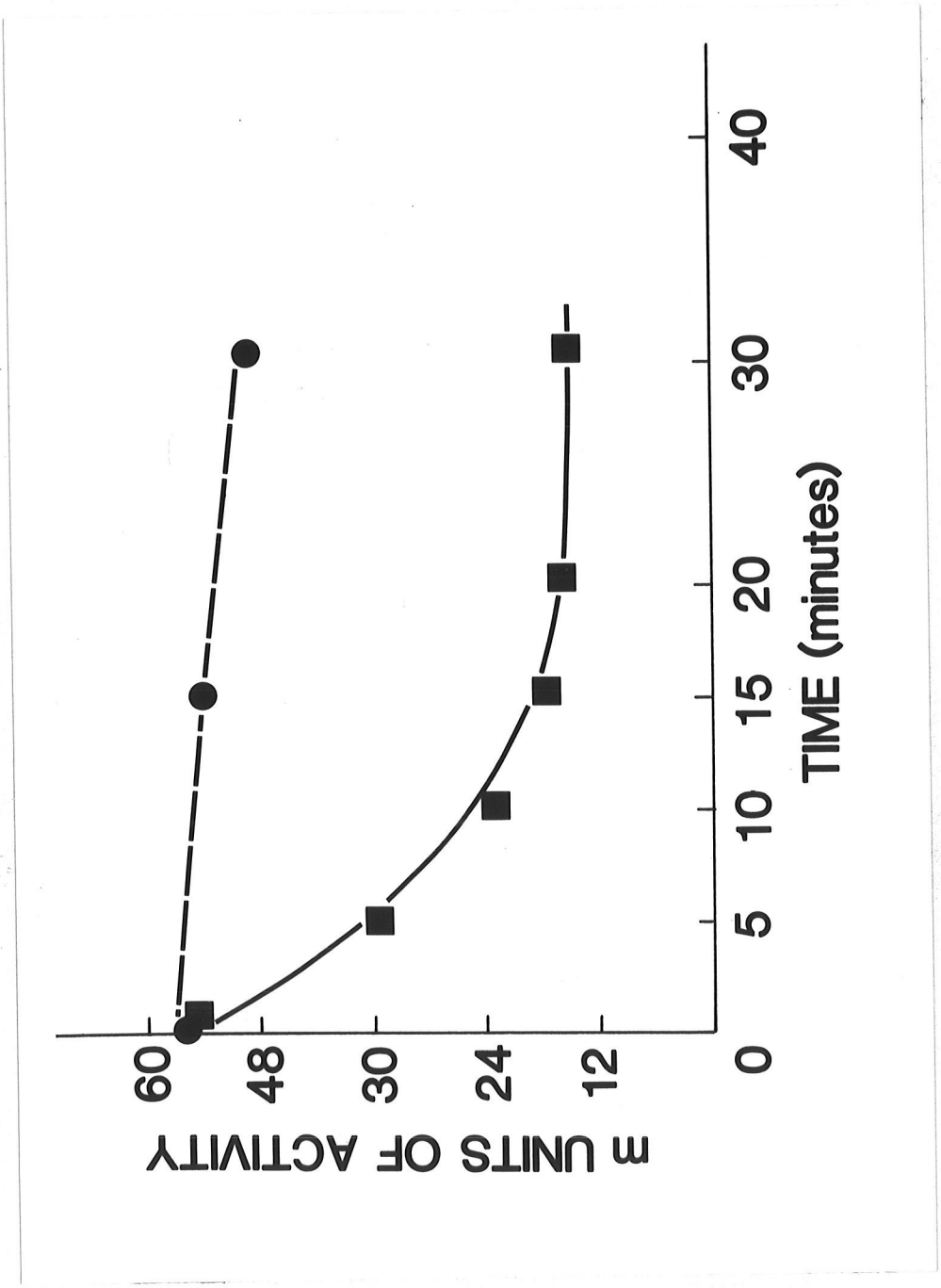
FIGURE 6



INACTIVATION PHOSPHATE EXTRACTED ACC BY ATP AND Mg^{++}

Rat liver was extracted with a phosphate sucrose buffer (procedure A) and dialysed against a phosphate buffer (enzyme SA 20 mUnits/mg). The enzyme was then incubated at 37°C in the presence of 13 mM $MgCl_2$ (triangles), 2 mM $MgCl_2$ (circles), or 2 mM $MgCl_2$ and 1 mM ATP (squares) and at approriate times aliquots were withdrawn and assayed for ACC activity. Alternatively enzyme was incubated with 2 mM $MgCl_2$ and 1 mM [γ-^{32}P]-ATP for 30' x 37°C and Sepharose avidin bindable label was determined as described in methods.

FIGURE 7



INACTIVATION TRIS EXTRACTED ACC BY ATP AND Mg^{++}

Rat liver was extracted in a TRIS sucrose buffer (procedure B) and dialysed against a TRIS buffer (enzyme SA 76 mUnits/mg). The enzyme was incubated at 37°C in the presence of 2 mM $MgCl_2$ (● — ●) or 2 mM $MgCl_2$ and 1 mM ATP (■ — ■) and assayed as in Fig. 6. Alternatively the enzyme was incubated with 2 mM $MgCl_2$ and 1 mM [γ-^{32}P]-ATP for 30 minutes and label incorporated into ACC was determined as in the previous experiment.

determined by the Sepharose-avidin method. Additional experiments indicated tha t dialysis of ACC in a TRIS buffer did not increase the activity of ACC over a phosphate (PO_4) buffer dialysate.

To eliminate any differential buffer effects on the enzyme activity, pooled rat livers were extracted in either a 50 mM KPO_4 - 0.25 M sucrose buffer or a 50 mM TRIS-0.25 M sucrose buffer and after ammonium sulfate precipitation both fractions were dialysed against TRIS buffer. A two fold higher specific activity in the TRIS extracted enzyme was observed with a three fold increase in the total yield as compared to the phosphate extracted enzyme (Table II). The inactivation of the TRIS extracted enzyme by ATP with the concommitant incorporation of label from [^{32}P] ATP into Sepharose-avidin bindable material is shown in Figure 8. It was also seen that the phosphate extracted enzyme, on the other hand, was not affected by ATP and showed no incorporation of the ^{32}P label from ATP.

In conclusion these results suggest that the PO_4 buffer extracted a species of ACC that underwent inactivation by ATP without phosphorylation while the TRIS buffer extracted an additional species of the enzyme and the latter preparation underwent inactivation and phosphorylation.

ACC is generally considered to be a cytosolic enzyme. However, Easter and Dils (1968) have demonstrated a species

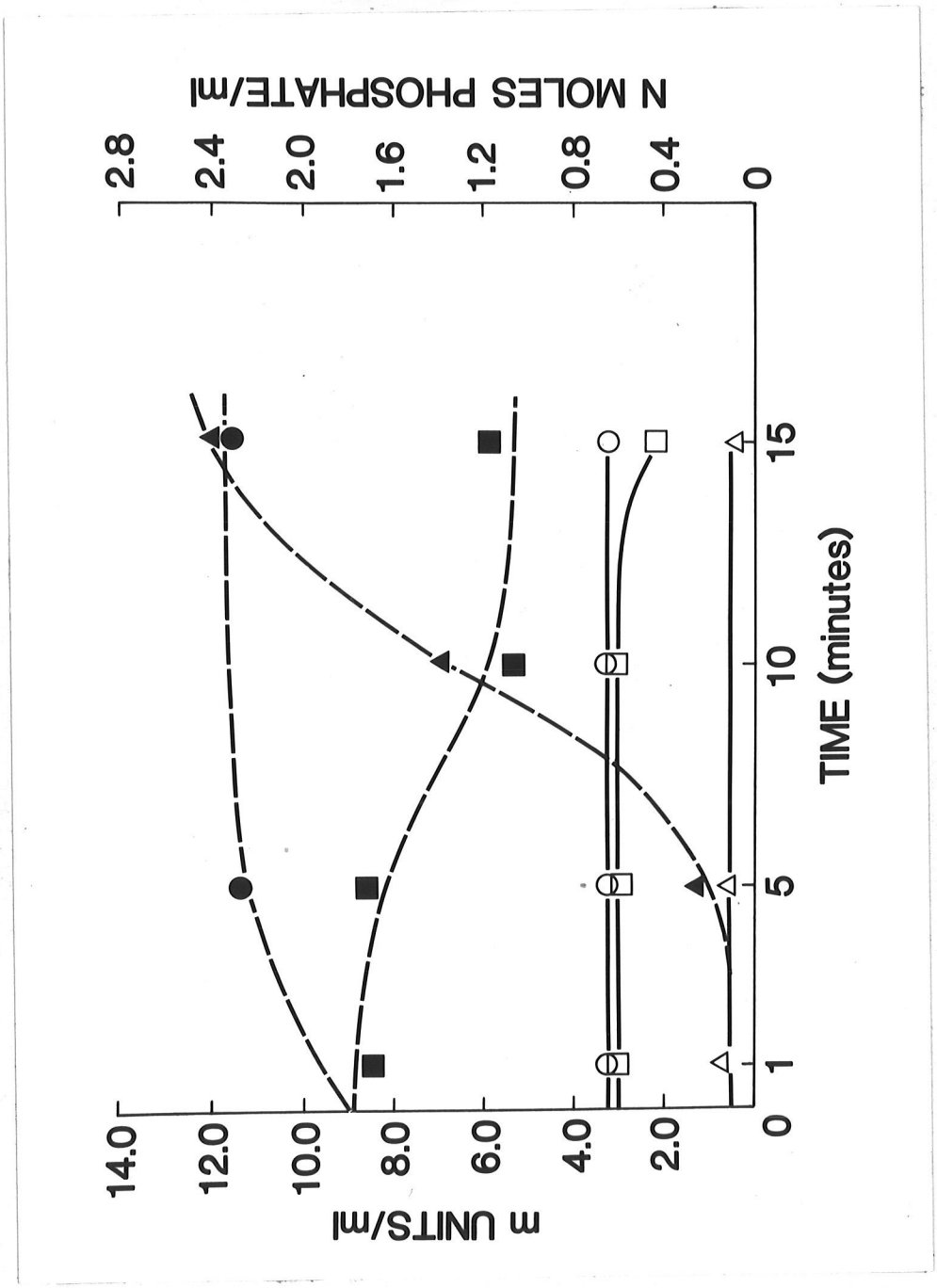
TABLE II

Yield and specific activity of rat liver ACC extracted in TRIS or phosphate buffer.

	TRIS extract	PO_4 extract
mUnits/ml	5.6	0.96
mg/ml	1.1	0.44
mUnits/mg	5.0	2.20
mUnits/gm wet weight	13.3	4.20

Pooled rat livers were divided into two batches and homogenized in either a TRIS-sucrose buffer or a PO_4-sucrose buffer (procedures A or B). After $(NH_4)_2SO_4$ precipitation the fractions were dialysed against TRIS buffer and passed through a sephadex G-25 column equilibrated with the same buffer. The extracts were then assayed for ACC activity and protein content.

FIGURE 8


N MOLES PHOSPHATE/ml
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
m UNITS/ml
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
1
5
10
15
TIME (minutes)

INACTIVATION AND PHOSPHORYLATION OF TRIS AND PHOSPHATE EXTRACTED ACC

The TRIS extracted enzyme (solid figures) or PO_4 extracted enzyme (open figures) was prepared as outlined in Table II. The extracts were incubated with 2 mM $MgCl_2$ (circles) or 2 mM $MgCl_2$ and 1 mM ATP (squares) at 37°C and at the indicated times aliquots were withdrawn and assayed for ACC activity. Alternatively the enzyme extracts were incubated with 2 mM $MgCl_2$ and 1 mM [γ^{32}P]-ATP and at the indicated times aliquots were mixed with 10 mM EDTA and incorporated label was determined using Sepharose-avidin binding (triangles).

of ACC that is bound to the microsomes in the lactating rabbit mammary gland. Margolis and Baum (1966) reported a cytosolic form of ACC that was extracted in Phosphate buffer while TRIS-NaCl buffers extract both a cytosolic and a microsomal form of the enzyme. With this in mind a cytosolic extract and a microsomal wash from rat liver was prepared as outlined in Figure 9 (procedure D).

It is known that phosphate buffers (Gregolin et al, 1966a) tend to keep ACC in the active polymeric form while TRIS and NaCl promote depolymerization of ACC (Gregolin et al, 1966b). The relationship between the cytosolic and microsomal species of ACC to the polymerizing conditions employed during homogenization was further investigated. The 10,000 x g supernatant from livers homogenized in 0.25 M sucrose was extracted separately in a TRIS buffer, a PO_4 - 1 mM citrate buffer or a PO_4 - 25 mM citrate buffer respectively. Subsequently subcellular fractionation was performed and the microsomes were washed in TRIS buffer and resedimented. The results, given in Table III,indicate that extraction under increasing polymerizing conditions (ie. increasing citrate concentration) leaves a greater percentage of the enzyme with the microsomes.

FIGURE 9

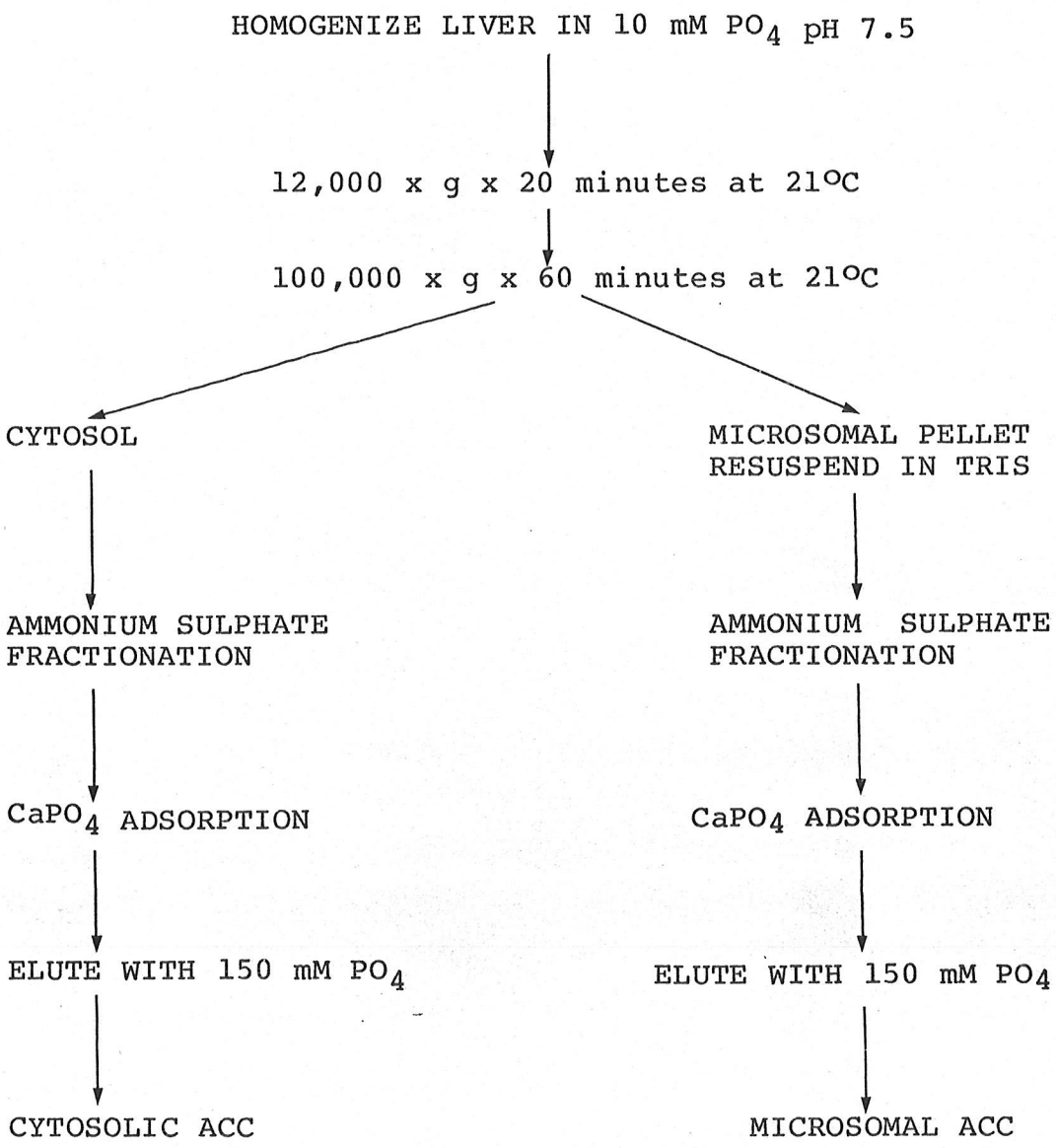

HOMOGENIZE LIVER IN 10 mM PO_4 pH 7.5
12,000 x g x 20 minutes at 21°C
100,000 x g x 60 minutes at 21°C
CYTOSOL
MICROSOMAL PELLET RESUSPEND IN TRIS
AMMONIUM SULPHATE FRACTIONATION
AMMONIUM SULPHATE FRACTIONATION
$CaPO_4$ ADSORPTION
$CaPO_4$ ADSORPTION
ELUTE WITH 150 mM PO_4
ELUTE WITH 150 mM PO_4
CYTOSOLIC ACC
MICROSOMAL ACC

TABLE III

Extraction of ACC from rat liver under conditions of increasing polymerization.

Total mUnits	TRIS	1mM citrate	25mM citrate
Cytosol	1,432	1,683	1,158
Microsomal Wash	178	344	363
% Bound to Microsomes	12	20	37

Normal rat livers were homogenized in 0.25 M sucrose and spun at 10,000 x g for 30 minutes. Aliquots of this low speed supernatant were made up to either 50 mM TRIS, 10 mM PO_4 + 1 mM potassium citrate or 10 mM PO_4 + 25 potassium citrate. Subcellular fractionation was performed (see procedure C) and the microsomes were washed in TRIS buffer. All fractions were dialysed against PO_4 buffer and assayed.

Inactivation of cytosolic and microsomal ACC:

The rates of inactivation of the ammonium sulfate fractionated cytosolic and microsomal enzyme preparations are depicted in Figure 10. After incubation for 12 minutes in the presence of ATP, a 50% decrease in the activity of the cytosolic enzyme was observed with respect to the control without ATP. The microsomal enzyme on the other hand was inactivated by only 15% during the same time interval. When the extent of phosphorylation was monitored (Table IV) it was found that the microsomal enzyme was phosphorylated about four times more than the cytosolic enzyme.

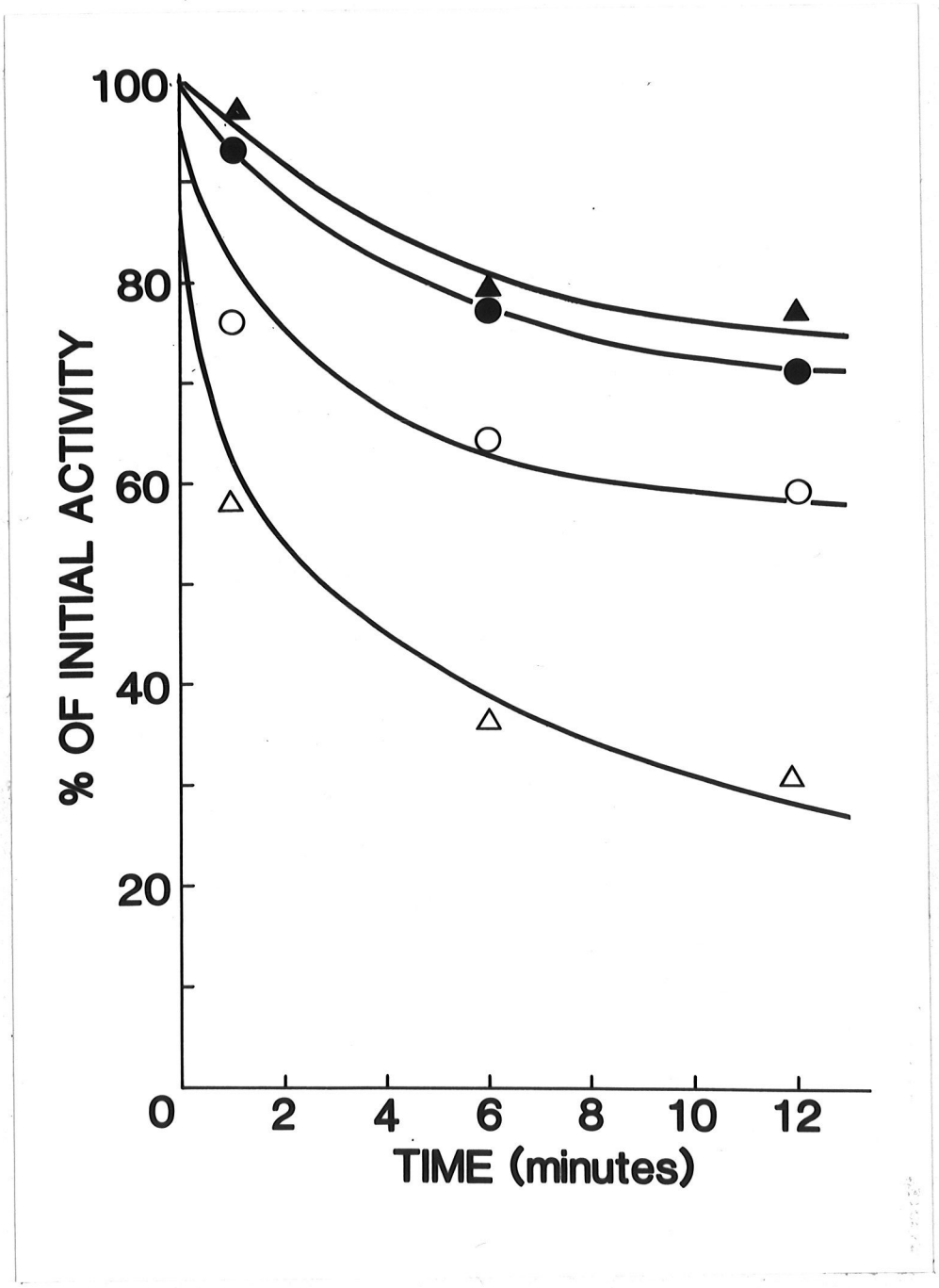
To determine that these differences in the extent of phosphorylation were not due to the lack of a fully active kinase in the cytosolic preparation we mixed equal amounts of the cytosolic and microsomal preparations and proceeded to incubate this mixture in the presence of ATP as described above. There was no increase in phosphorylation when the two preparations were mixed indicating that the microsmal preparation did not contain a kinase that was absent in the cytosolic preparation, but a 40% decrease in the expected counts was observed. This decrease could be due to competition for ATP by other enzymes or by the presence of a NaF insensitive phosphatase. The titration of enzyme activity by Ab

indicated that the microsomal enzyme was 3 times as active as the cytosolic species.

In contrast to the above, the rate of inactivation of the calcium phosphate extracted cytosolic and microsomal enzyme was the same (Table V). The microsomal enzyme was phosphorylated to a greater extent (4 times) than the cytosolic enzyme and again, mixing the two preparations did not increase the rate of phosphorylation but decreased it by about 60%.

To eliminate the possibility that the cytosolic preparation lacked a kinase, the incorporation of γ-^{32}P label from ATP into total protein was investigated (Figure 11). The microsomal preparation was phosphorylated quickly reaching a plateau after 12 minutes. The cytosolic preparation exhibited an initial rapid burst of phosphorylation which leveled off after 1 minute. The microsomal preparation was then phosphorylated first with cold ATP and then incubated with γ-^{32}P labelled ATP and cytosolic protein. The extent of phosphorylation of the above mixture was the same as for the cytosol preparation alone. Thus, by first phosphorylating the microsomal protein with cold ATP, the rate and extent of phosphorylation of the cytosolic protein was determined to be the same in the presence or absence of the microsomal kinase. Furthermore the same kinetic pattern was observed in the presence or absence of NaF suggesting that the lack of phosphorylation in the cytosolic preparation was not due

FIGURE 10



INACTIVATION OF AMMONIUM SULPHATE FRACTIONATED CYTOSOLIC AND MICROSOMAL ACC

The ammonium sulphate fractionatd cytoslic (Triangles) and microsomal (Circles) enzymes were incubated with 2 mM Mg^{++} and 50 mM NaF in the presence (open symbols) or absence (closed symbols) of 2 mM ATP. Aliquots were then withdrawn and assayed for ACC activity at appropriate times.

TABLE IV

PHOSPHORYLATION OF AMMONIUM SULPHATE FRACTIONATED CYTOSOLIC AND MICROSOMAL ACC.

	pmoles ACC	pmoles ^{32}P	pmole* ratio
Microsome	3.8	5.2	1.4
Cytosol	16.7	4.9	0.3
Microsome + Cytosol	1.9 + 8.6	3.3 (5.1)	

( ) Expected

The ammonium sulfate fractionated cytosolic and microsomal enzymes were incubated as in Figure 7 except that 2 mM [γ-^{32}P]-ATP was used (1,000 dpm/pmole). Immunoprecipitation was then performed and the Ab pellet was solubilized and counted.

* pmoles phosphate / pmole 400K protomer

TABLE V

PHOSPHORYLATION AND INACTIVATION OF CALCIUM PHOSPHATE EXTRACTED CYTOSOLIC AND MICROSOMAL ACC

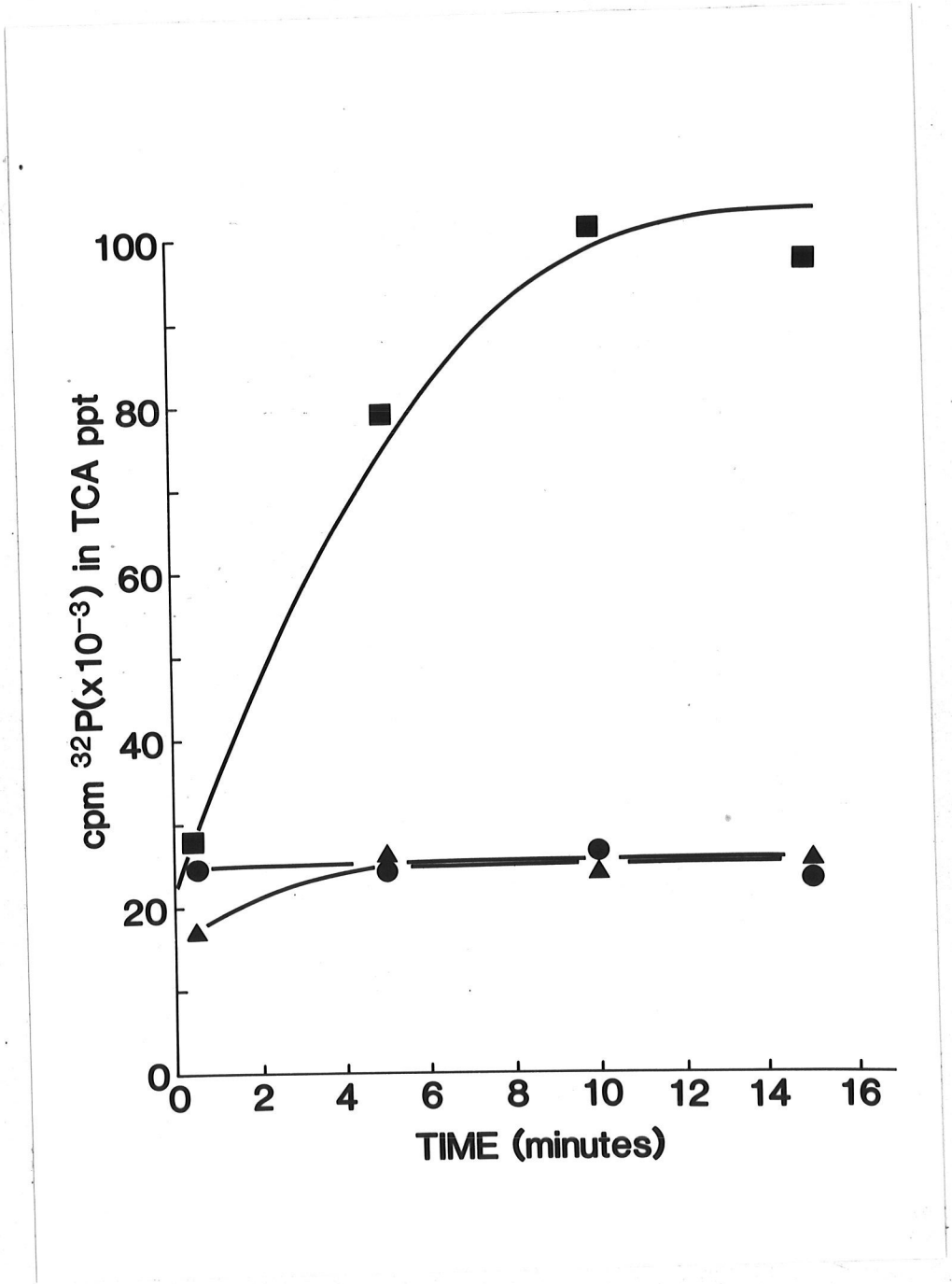
	mUnits ACC	pmoles ^{32}P incorporated	% inactivation	pmole* ratio
Cytosol	9.3	3.2	37	0.34
Microsomes	10.7	14.5	36	1.36
Cytosol + Microsomes	4.7 + 5.4	3.6 (8.9)		

( )Expected

The Calcium Phosphate extracted cytosolic and microsomal enzyme was incubated as in Figures 7 & 8 and the percent inactivation and the extent of phosphorylation was determined.

* pmoles phosphate / pmole 400K protomer

FIGURE 11


100
80
60
40
20
0
cpm ^{32}P(x10^{-3}) in TCA ppt
0
2
4
6
8
10
12
14
16
TIME (minutes)

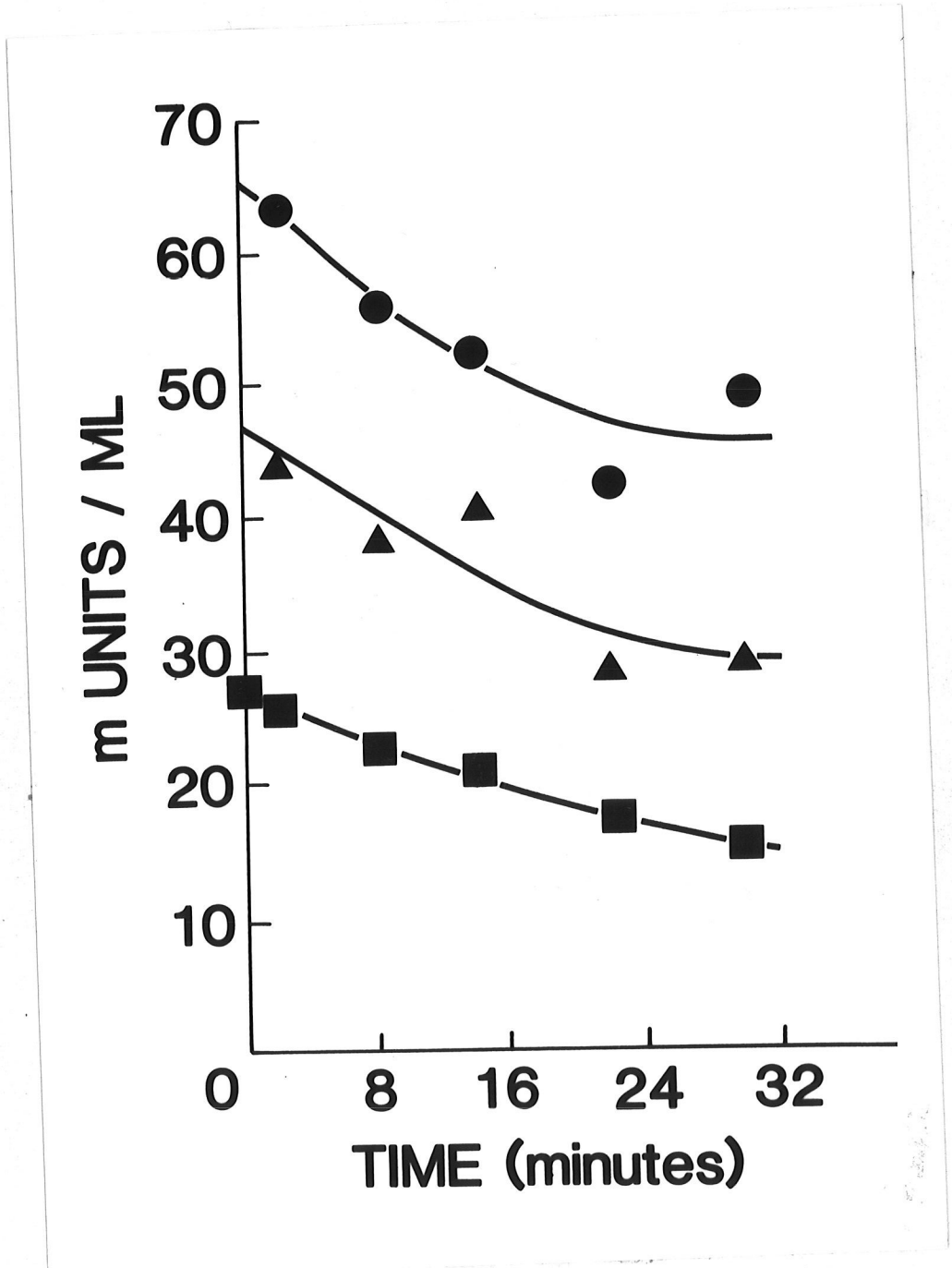
TOTAL PHOSPHORYLATION OF CALCIUM PHOSPHATE EXTRACTED CYTOSOLIC AND MICROSOMAL PREPARATION

The Calcium phosphate gel extracted cytosolic (circles) and microsomal (squares) enzyme preparations were incubated in the presence of 0.5 mM [γ-^{32}P] ATP (1000 dpm/pmole), 1.0 mM Mg^{++} and 50 mM NaF and the incorporation of ^{32}P into total TCA precipitable material was determined. The microsomal preparation was then phosphorylated with cold ATP and then hot ATP was added along with an aliquot of the cytosolic enzyme and total incorporation was determined as above (triangles).

to the presence of a phosphatase.

It has been suggested that (+) palmitylcarnitine reverses the inhibition of ACC by long chain fatty acyl CoA's in rat liver (Fritz and Hsu, 1967), in chick liver (Goodridge, 1972) and bovine adipose tissue (Moss et al, 1972). We have i nvestigated the effects of (+) palmitylcarnitine on the inactivation of ACC by ATP to determine the possible relationship of fatty acyl-CoA to the depolymerization phenomena. Preliminary experiments showed that (+) palmitylcarnitine, at concentrations between 10^{-4} M and 10^{-3} M, maximally stimulated the ACC activity in the partially purified preparations used in these experiments. The inactivation of ACC by ATP in the presence of respectively 0, 10^{-4} and 10^{-3} M (+) palmitylcarnitine is represented in Figure 12. It is seen that 100 uM and 1,000 uM (+) palmitylcarnitine increases the initial activity by 1.6- and 2.4-fold respectively (Table VI). Although there was substantial inactivation by ATP in each case it can be seen that upon increasing the concentration of (+) palmitylcarnitine the extent of inactivation decreased. The presence of (+) palmitylcarnitine did not prevent the incorporation of label from [^{32}P] ATP showing that phosphorylation itself was not affected by (+) palmitylcarnitine. This suggests that there is a population of ACC that is inhibited by palmityl CoA and the addition of (+) palmitylcarnitine relieves that inhibition (the initial rise in activity).

FIGURE 12



INACTIVATION OF TRIS EXTRACTED ACC IN THE PRESENCE OF PALMITYLCARNITINE

Rat livers were extracted with a TRIS buffer as in Fig. 2. Aliquots were incubated at 37°C in the presence of 2 mM ATP, 2 mM $MgCl_2$ and no palmitylcarnitine (squares); 100 uM palmitylcarnitine (triangles) and 1,000 uM palmitylcarnitine (circles). At appropriate times aliquots were removed and assayed for ACC activity.

TABLE VI:

Inactivation and phosphorylation of the TRIS extracted ACC in the presence of ATP and (+) palmitylcarnitine.

Palmitylcarnitine	Initial Increase	% Inhibition	cpm ^{32}P ml enzyme
0	0	41	23K
10^{-4}	1.6	34	55K
10^{-3}	2.4	30	N.D.

N.D. not done

The enzyme was inactivated and assayed as in Figure 8. Aliquots were incubated with 2 mM [γ-^{32}P]-ATP and 2 mM $MgCl_2$ +/- 100 uM palmitylcarnitine at 37°C x 30 minutes. The reaction mixture was subjected to Ab precipitation as described in Figure 2 and the washed pellets were counted after solubilization in NCS.

It was seen that the subsequent inactivation by ATP was slightly reversed by (+) palmitylcarnitine.

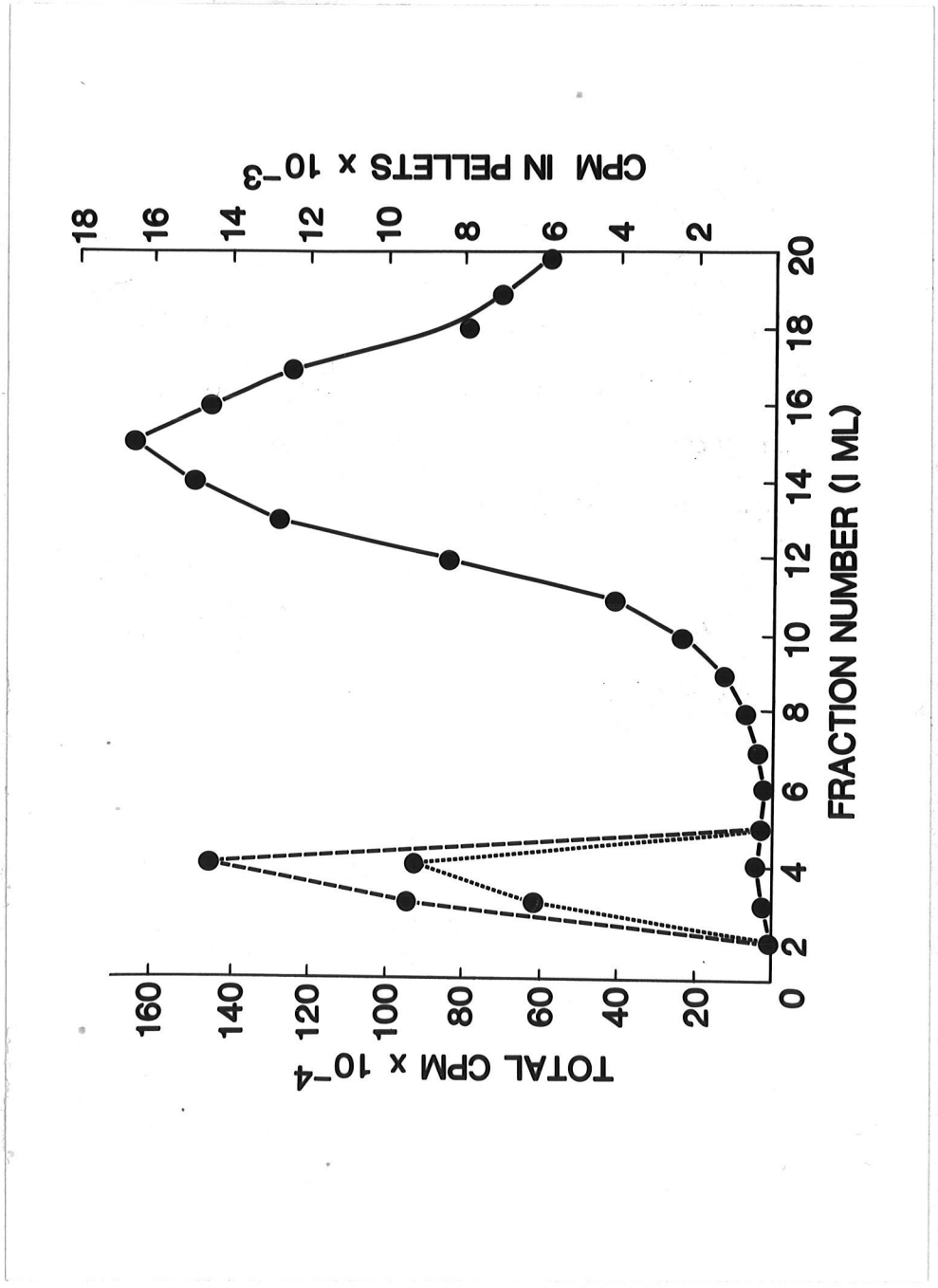
Identification of labelled ACC

When the TRIS extracted enzyme was phosphorylated and run on a Sephadex G-25 column a peak of labelled protein eluted ahead of the labelled ATP peak (Fig. 13). About 50% of the label associated with the initial peak was TCA precipitable and about 64% of this was precipitated by the double antibody techinque. This double antibody procedure (Harrington et al, 1971) can be completed in less than one hour and avoids the time consuming partial purification step employed by other investigators (Nakanishi and Numa, 1970, Majerus and Kilburn, 1969).

To determine if the ^{32}P label was actually incorporated into ACC, SDS-gel electrophoresis was performed on the denatured immunoprecipitates. In initial experiments SDS gel electrophoresis was done in tube gels by the method of Laemmli (1970) before and after partial purification on mini DEAE cellulose columns (Lent et al, 1978). Figure 14A indicates that 30% of the ^{32}P label incorporated into the immunoprecipitate migrated at the dye front. Evidence will be presented in section V that suggests that proteolytic nicking of the protomer had occurred with subsequent release of phosphopeptides upon denaturation. When the enzyme was first partially purified on DEAE cellulose prior to SDS PAGE (Figure 14B) the

phosphopeptides migrating at the dye front were lost along with about 50% of the total label indicating that the proteolytically nicked protomer does not bind to the column.

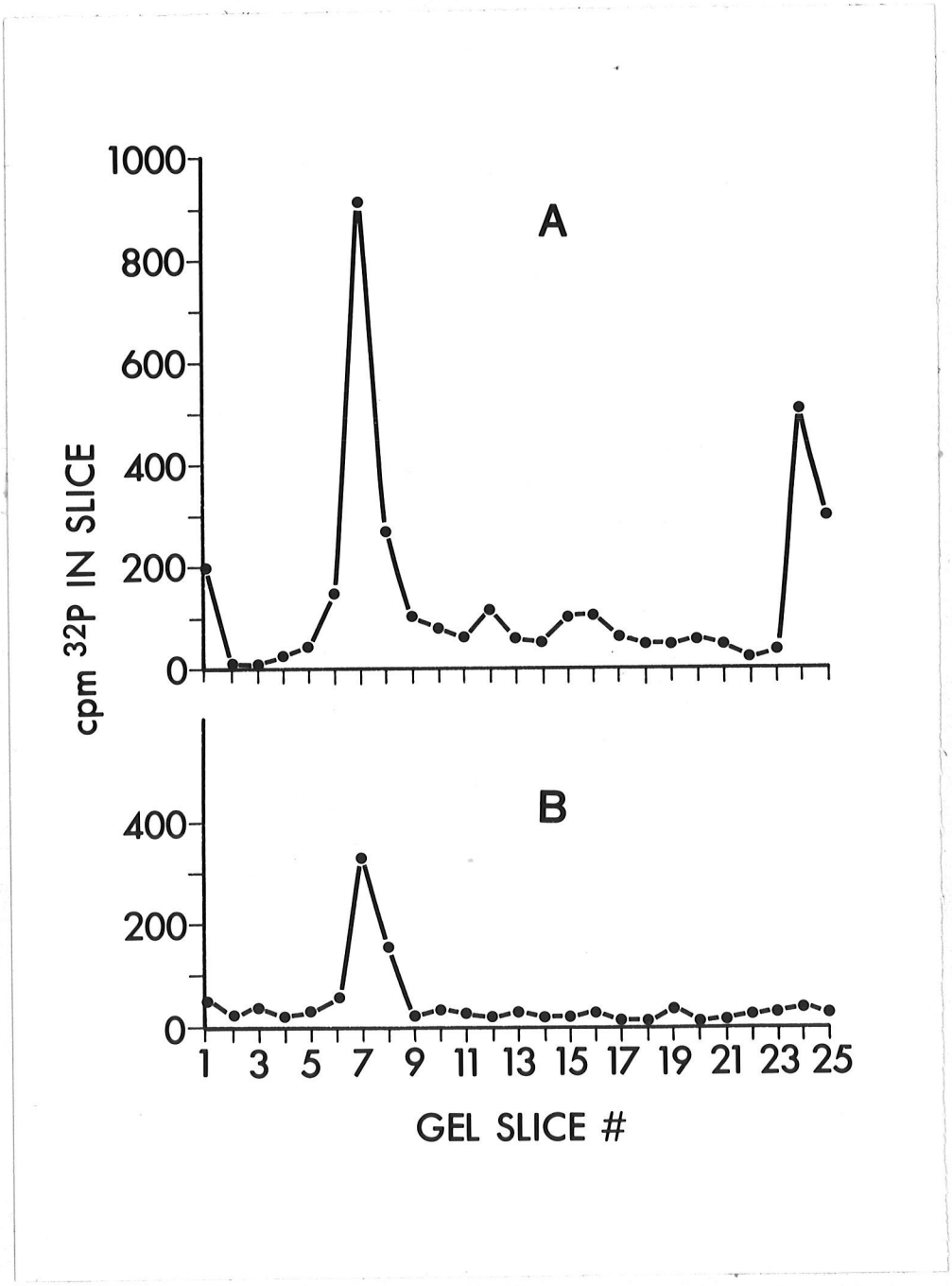
FIGURE 13


CPM IN PELLETS x 10^-3
18
16
14
12
10
8
6
4
2
FRACTION NUMBER (1 ML)
0
2
4
6
8
10
12
14
16
18
20
TOTAL CPM x 10^-4
160
140
120
100
80
60
40
20

ELUTION PROFILE OF PHOSPHORYLATED TRIS EXTRACTED ACC ON SEPHAROSE G-25

The 35% $(NH_4)_2SO_4$ dialysate (100 mUnits) from rat liver extracted in TRIS sucrose buffer was incubated with 2 mM [γ-^{32}P]-ATP, 2 mM $MgCl_2$, 50 mM NaF and 50 mM TRIS buffer pH 7.5 for 30' x 37°C in a total of 1 ml. Subsequently the above mixture was applied to a 1.5 x 30 cm Sephadex G-25 column equilibrated and eluted with TRIS buffer. Aliquots from the eluted fractions were either counted for total radioactivity (——) indicated on the left ordinate, or TCA precipitable material (- - - -), and Ab precipitable material (·····),indicated on the right ordinate.

FIGURE 14


1000
800
600
400
200
0
A
cpm 32P IN SLICE
400
200
0
B
1 3 5 7 9 11 13 15 17 19 21 23 25
GEL SLICE #

SDS GEL ELECTROPHORESIS OF PHOSPHORYLATED ACC

Immunoprecipitation was performed on the phosphorylated ammonium sulfate fraction of the TRIS extracted enzyme (procedure B) before (panel A) or after (panel B) chromatography on DEAE cellulose. After subjecting the precipitate to electophoresis on 5% tube gels the gels were sliced and counted.

Discussion

The data presented here suggests that two forms of ACC are found *in vivo*; a cytosolic form which is isolated upon extraction of liver with a phosphate-sucrose buffer and a microsomal form isolated from the microsomal fraction in TRIS buffer. As the cytosolic form cannot be phosphorylated it would appear that it is already in the phosphorylated state (ACC-b) while the microsomal species which can be phosphorylated is in the nonphosphorylated state (ACC-a).

Under conditions favouring polymerization of ACC a shift occured in the equilibrium between the cytosolic and the microsomal content of ACC towards the microsomal fraction. Carlson *et al* (1974) have suggested that ACC-b is less sensitive to polymerization by citrate and more sensitive to inactivation by palmityl-CoA. As ACC-b would be depolymerized and inactive *in vivo* and ACC-a would be polymerized and active; ACC-a may tend to sediment with the microsomes possibly the result of the trapping of the large linear ACC aggregate in the sedementing matrix. On the other hand Kleinschmidt *et al* (1969) has suggested that the organization exhibited by the ACC polymer might have a structural role in addition to the known catalytic and regulatory function and ACC may be physically associated with the microsomes.

The cytosolic form of ACC (ACC-b) seems to undergo inactivation independent of phosphorylation especially when the enzyme is in mildy polymerizing conditions suggesting that this inactivation might be due to depolymerization by fatty acyl CoA's. The initial two to three fold increase in activity observed upon the addition of (+) palmitylcarnitine may be due to the alleviation of allosteric inhibition of ACC-b by acyl CoA. It is not clear why the ATP in the incubation permits inactivation while no inactivation occurs in the control which lacks ATP. Perhaps this could be due to carboxylation of the enzyme which promotes depolymerization or an allosteric effect of ATP itself (Pekala et al, 1978).

In latter the experiments of this section the concentration of ATP in the phosphorylation reaction mixture was decreased from 2 mM to 0.5 mM to increase the specific activity of the [$-^{32}P$] ATP employed. We observed that this change had no significant affect on the rates of phosphorylation as reported by Kim (1979).

There still seems to be inactivation and phosphorylation in the presence of (+) palmitylcarnitine and ATP, although increasing concentrations of (+) palmitylcarnitine decrease the extent of this inhibition. Possibly this is the result of the phosphorylation of ACC-a which increases its sensitivity to acyl CoA as suggested by Carlson and Kim (1974). Ogiwara et al (1978) has recently shown that inhibition of rat

liver ACC by palmityl CoA results in the formation of a molecular aggregate that is intermediate in size (20-25 S) between the 40-60 S polymer and the 13-16 S protomer and a similar observation has been made by Lent et al (1978) during the phosphorylation of ACC. Alternatively, the inactivation observed may be the result of the allosteric effects of ATP (Desjardins and Dakshinamurti, 1978).

In summary two major obervations have been made in our investigation of the phosphorylation of ACC. The first is the existance of a soluble or cytosolic form of the enzyme that was not phosphorylated by a cAMP-independent mechanism. In contrast an insoluble or microsomal form of the enzyme was phosphorylated by a cAMP-independent mechanism. The second observation is that the phosphorylated enzyme, isolated by antibody precipitation seems to be cleaved into phosphopeptides. These two observations will be addressed in the two subsequent sections.

PROTEOLYSIS OF ACETYL CoA CARBOXYLASE

INTRODUCTION

The first aspect to be investigated was the proteolytic cleavage of the ACC that occured during initial phosphorylation experiments. The main purpose in this study was to determine if there was any causal relationship between phosphorylation and proteolysis of the enzyme (Dakshinamurti and Gillevet, 1982).

Kim and co-workers have hypothesized that rat liver acetyl-CoA carboxylase (ACC) is inactivated and activated by a phosphorylation-dephosphorylation mechanism (Carlson and Kim, 1973; Lee and Kim, 1977; Lent et al, 1978). In these studies, phosphorylation was shown to occur concomitantly with enzyme inactivation in a crude ammonium sulfate fraction of rat liver cytosol in the absence of added cAMP. Subsequently, Hardie and Cohen (1978a) showed that pure rabbit mammary gland ACC was phosphorylated and inactivated by endogenous cAMP-dependent and cAMP-independent (ACC-PK2) protein kinases. Their ACC preparation contained so called "structural" phosphates (Hardie and Guy, 1980) and further phosphorylation by the above kinases occurred whether the enzyme was first

dephosphorylated or not. ACC isolated from any source contains endogenous structural phosphates in a ratio of 2 to 6 moles of phosphate per mole of ACC subunit. These data suggest the existence of a third, as yet uncharacterized protein kinase, that phosphorylates ACC at these structural phosphate sites. Rat liver and rat mammary gland ACC have been reported to undergo phosphorylation and inativation by cAMP-dependent protein kinase (Hardie and Guy, 1980; Tipper and Witters, 1982). It has been suggested that proteolysis occurs at the phosphorylated sites and can mimic the increase in activity accompanying dephosphorylation of the enzyme (Guy and Hardie, 1981).

With the above in mind we set out to investigate the causal role between phosphorylation at these "structural sites" and it's relation to proteolytic cleavage of the enzyme. In this section, we present evidence to suggest that rat liver ACC in crude preparation is phosphorylated by a cAMP-independent protein kinase at sites on the enzyme that have already undergone proteolytic cleavage or nicking and that this phosphorylation has little effect on enzyme activity. Furthermore, the enzyme is also phosphorylated by the catalytic subunit of cAMP-dependent protein kinase and the rate of this phosphorylation does not correlate with inactivation of the enzyme.

EXPERIMENTAL

Materials.

D[carbonyl-^{14}C] Biotin (51 mCi/mmol) was obtained from Amershan Corporation . Electrophoretic reagents were obtained from Sigma Chemical Company. Gel filtration standards and electrophoretic standards were supplied by Pharmacia Fine Chemicals. NCS tissue solubilizer and OCS scintillant were obtained from Amersham Corporation.

Preparation of ACC.

Fresh rat livers were homogenized in 2.0 volumes of 50 mM TRIS buffer (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA and 5 mM B-mercaptoethanol and the 100,000 Xg supernatant was prepared. This was brought to 30% saturation with ammonium sulfate. The enzyme was allowed to precipitate for 30 minutes at 4^{o}C and was then collected by centrifugation. The pellet was resuspended and dialysed in 50 mM TRIS buffer (pH 7.5) containing 0.1 mM EDTA and 5 mM B-mercaptoethanol. The dialysate was clarified by centrifugation. Half of the enzyme was mixed with 0.6 volumes of glycerol and frozen in aliquots until use. The other half was adsorbed on calcium phosphate gel and eluted with one volume of 150 mM phosphate buffer (pH 7.5) containing 0.1 mM EDTA and 5 mM B-mercaptoethanol and

frozen in aliquots until use. In some experiments male Long-Evans rats (150 gm) were starved for 48 hours and then refed a high carbohydrate diet for another 48 hours. During refeeding the rats were injected with (carbonyl)-[^{14}C] Biotin (10 uCi/day). The enzyme was prepared as above except that a further 30% ammonium sulfate fractionation was performed followed by dialysis against TRIS buffer.

ACC Inactivation and Phosphorylation.

ACC preparations were incubated at 37°C in the presence of $MgCl_2$ and ATP as described in the legend to the figures. At appropriate times aliquots were removed and assayed for activity by the [^{14}C] bicarbonate fixation assay (Dakshinamurti and Desjardins, 1969) without preincubation with citrate. Control incubations were identical except that ATP was omitted. In experiments comparing the two preparations of ACC, the ammonium sulfate fractionated enzyme was diluted in 150 mM phosphate buffer to the same enzyme concentration as the calcium phosphate gel extracted enzyme, before additions were made to the reaction mixture. Both ACC preparations were labelled under identical conditions. In these experiments cold ATP was replaced with [γ-^{32}P] ATP (100-500 dpm/pmole). The reaction was terminated by addition of 10 mM EDTA and the phosphorylated enzyme was incubated for 30 minutes at 37°C, in the presence of antibody prepared in rabbits against the

purified avian liver enzyme. The above mixture was then further incubated in the presence of goat anti rabbit IgG and 1% polyethylene glycol (Mr 6,000) for 15 minutes at which time precipitation of the immune complex was complete (Harrington et al, 1971). Controls employing IgG from non-immunized rabbits were used to determine any non-specific precipitation.

The immunoprecipitates were washed three times with 0.85% NaCl and dissolved in 0.1 N NaOH. This latter procedure denatures the pellet and removes any non-covalent label that may be trapped there (Nimmo et al,1976). After reprecipitation with an equal volume of 10% TCA the pellets were solubilized in NCS and counted in a liquid scintillation counter. Immunoprecipitation of the enzyme was independent of its monomeric or polymeric state (Majerus and Kilburn, 1969). For SDS gel electrophoresis, the immunoprecipitate was centrifuged through a discontinuous sucrose gradient consisting of 0.5 M sucrose and 1 M sucrose containing 1% SDS and 1% TRITON X-100. This procedure removed any non-specific material adsorbing to the immune complex and is a standard procedure for the isolation of specific polysomes by immunoprecipitation (Taylor and Schimke, 1974).

SDS Polyacrylamide Gel Electrophoresis.

The immunopreciptate was denatured in 1% SDS and 5% B-mercaptoethanol by heating at 100°C for 5 minutes.

Aliquots were subjected to slab gel electrophoresis by the method of Kwoland (1974) employing 3% acrylamide stacking gels and 5% acrylamide running gels were employed. These highly crosslinked gels resolve a broader molecular weight range allowing the resolution of the previously mentioned band at the dye front (section IV). The slab gels were dried and autoradiographed using Kodak Xomat film. Samples of the denatured immunoprecipitate were also spotted on filter disc and washed in TCA (Butcher, 1971) to determine the total counts in the immunoprecipitate.

RESULTS

Inactivation and phosphorylation of the ammonium sulfate fraction and the calcium phosphate eluant.

It has been suggested that preincubation with citrate artificially polymerizes ACC masking the effects of phosphorylation (Allred and Roehrig, 1973). In order to study the direct effect of phosphorylation on ACC activity, the enzyme assays in our experiments were done in the absence of preincubation with citrate. The inactivation of the ammonium sulfate fractionated enzyme by ATP and Mg^{++} is shown in Figure 15. The control, lacking ATP, decreased in activitiy by 40% in 18 minutes while the addition of ATP increased the inactivation to 75%. These results indicate that the ACC preparation was in a partially active state in the absence of preincubation with citrate. When the enzyme that was further purified on calcium phosphate gel was used, there was no increase in the rate of inactivation produced by ATP (Figure 15). Although the extent of inactivation at 18 minutes was 20% lower than the ammonium sulfate fractioned enzyme, the calcium phosphate gel extracted enzyme was maximally phosphorylated to a greater extent (Table VII).

From immunotitration data, it was calculated that the ACC antibody bound equivalent amounts of enzyme activity units in both preparations (48.4 munits /10ul Ab and

TABLE VII

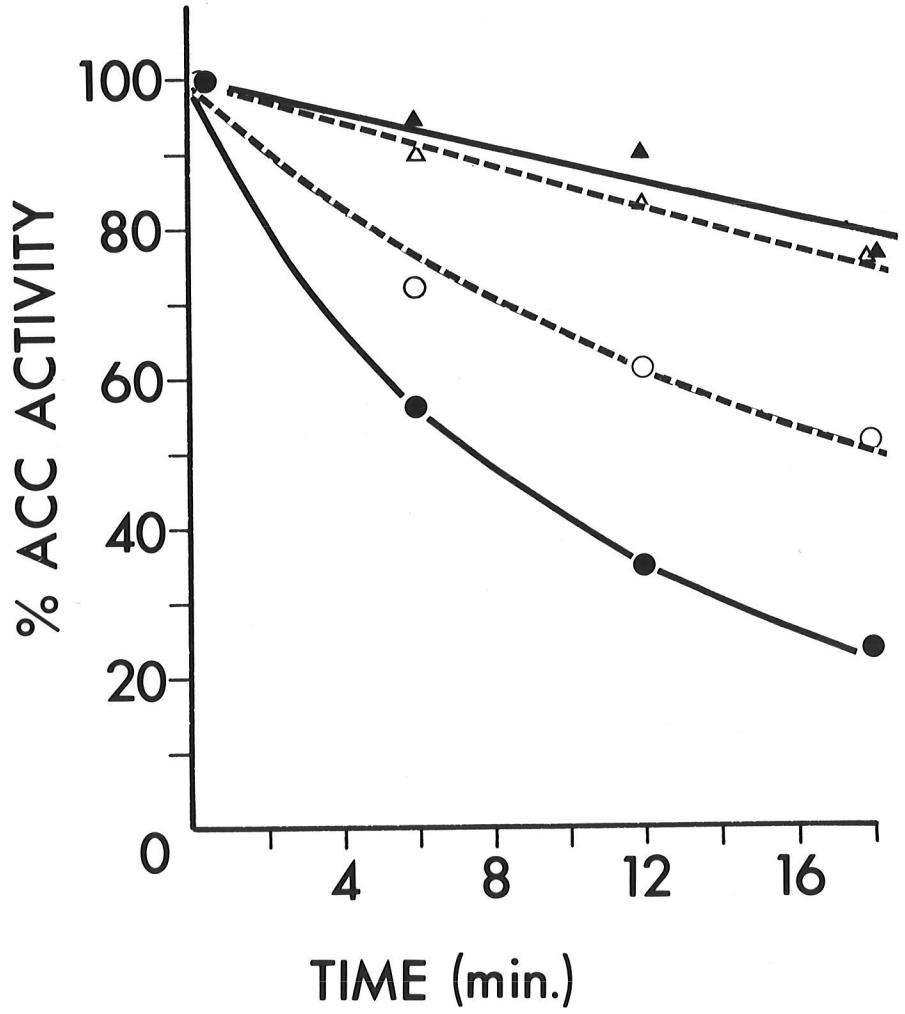
INACTIVATION AND PHOSPHORYLATION OF AMMONIUM SULFATE FRACTION AND CALCIUM PHOSPHATE ELUENT

	Ammonium Sulfate Fraction	Calcium Phosphate Gel Eluent
m units inactivated	48.4	49.7
calculated pmoles ACC*	8.1	8.3
pmoles ^{32}P in Ab ppt	42.6	51.7
pmoles ^{32}P per pmole ACC	5.3:1	6.2:1

Experimental conditions were the same as in Fig. 1 except that [γ-^{32}P] ATP was substituted for cold ATP. After incubation for 30 min the phosphorylated enzyme was isolated using antibody to avian ACC as described under methods.

* assuming enzyme S.A. of 15 units/mg and a molecular weight of 400,000 dalton.

FIGURE 15



THE INACTIVATION OF ACC IN THE PRESENCE OF ATP AND Mg^{2+}.

The 30% ammonium sulfate fraction (solid symbols) or the calcium phosphate gel eluant (open symbols) was incubated in the presence of 0.8 mM Mg^{2+} (triangles)or with 0.8 mM Mg^{2+} and 0.4 mM ATP (circles). At appropriate times aliquots were assayed for ACC activity as described.

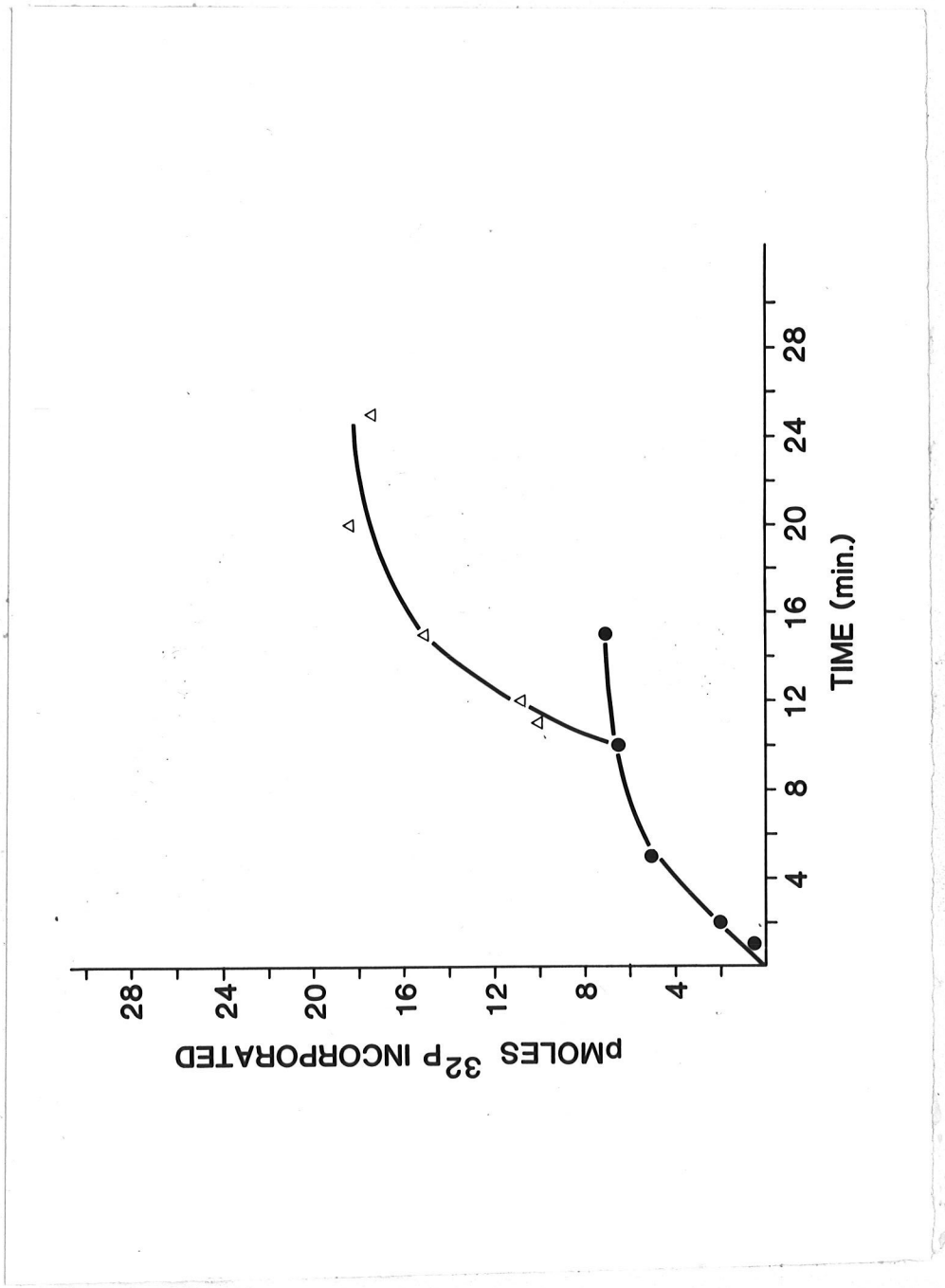
49.7 munits / 10ul Ab for the ammonium sulfate fractionated and calcium phosphate gel fractionated enzymes respectively) indicating that both ACC preparations had the same intrinsic specific activity. The calcium phosphate gel eluent was phosphorylated to 125% of the extent seen in the ammonium sulfate preparation. The number of phosphates incorporated into the ammonium sulphate fraction, i.e. 5.3 pmoles phosphate per pmole ACC dimer, is in close agreement with previously published values (Hardie and Cohen, 1978a).

The lack of correlation between enzyme activity and phosphorylation could be the result of a direct effect of ATP on the polymeric state of the enzyme (Desjardins and Dakshinamurti, 1978). Calcium phosphate gel extraction removes endogenous inhibitors like long chain fatty acyl CoA esters (Gregolin et al, 1968a) which may have a role in enhancing the depolymerization of ACC by ATP (Desjardins and Dakshinamurti, 1978). This purification step also separates ACC from fatty acid synthetase (Gregolin et al, 1968b). It has been suggested that ACC and fatty acid synthetase are coupled in vivo (Hansen et al, 1971) and this may sterically hinder phosphorylation of ACC.

Effects of phosphorylation on ACC labelled with [^{14}C] biotin

We investigated the kinetics of phosphorylation using enzyme that had been labelled with [^{14}C] Biotin and further purified by a second ammonium sulfate

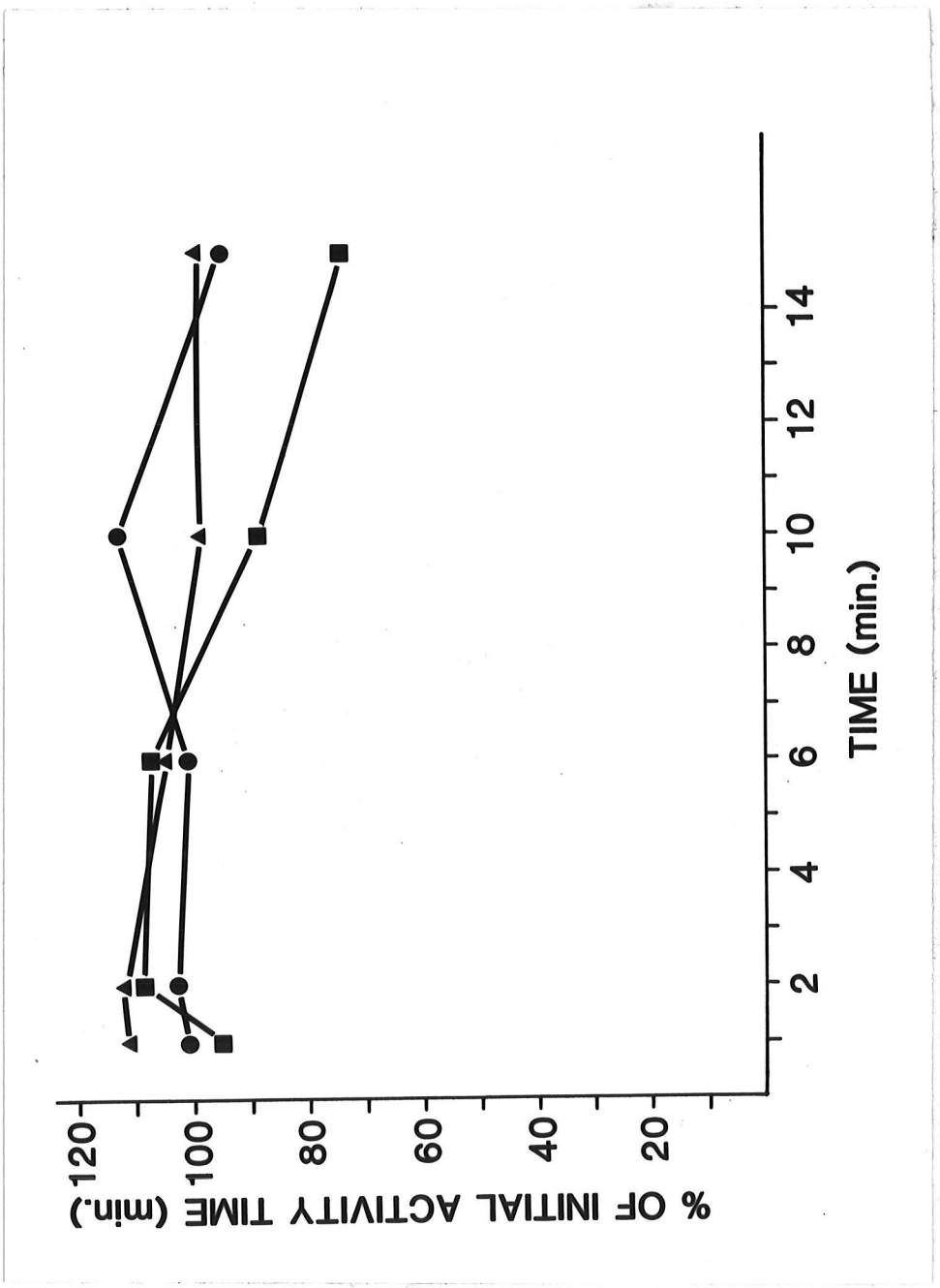
FIGURE 16


pMOLES ^{32}P INCORPORATED
4
8
12
16
20
24
28
TIME (min.)
4
8
12
16
20
24
28

THE RATE OF PHOSPHORYLATION OF ACC.

The second ammonium sulfate fraction of the ^{14}C labelled enzyme was incubated with 2 mM Mg^{2+}, 0.5 mM [γ-^{32}P] ATP (● ——— ●) and the label incorporated into the immunoprecipitate was determined. After phosphorylating the enzyme as above for 10 minutes, 20 Units/ml of the catalytic subunit of cAMP dependent protein kinase was added and further incorporation of label (△ —— △)was determined.

FIGURE 17


% OF INITIAL ACTIVITY TIME (min.)
120
100
80
60
40
20
2
4
6
8
10
12
14
TIME (min.)

THE EFFECT OF PHOSPHORYLATION ON ACC ACTIVITY.

The second ammonium sulfate fraction of the ^{14}C labelled enzyme was incubated with 2 mM Mg^{2+} (●—●), 2 mM Mg^{2+} and 0.5 mM ATP (▲——▲) or 2 mM Mg^{2+}, 0.5 mM ATP and 20 Units/ml of the catalytic subunit (■——■). Aliquots were withdrawn and assayed for enzyme activity at the appropriate times.

fractionation. The enzyme was labelled with [^{14}C] Biotin to monitor the extent of proteolysis and a second ammonium sulfate fractionation was performed to concentrate the labelled enzyme. The rate of incorporation of label from [γ-^{32}P] ATP into the immunoprecipitate by a cAMP-independent and cAMP-dependent kinases is shown in Figure 16. It is observed that phosphorylation by the independent mechanism is complete by 10 minutes and the enzyme can be further phosphorylated by the catalytic subunit of cAMP-dependent protein kinase and this further phosphorylation event is complete by another 10 minutes.

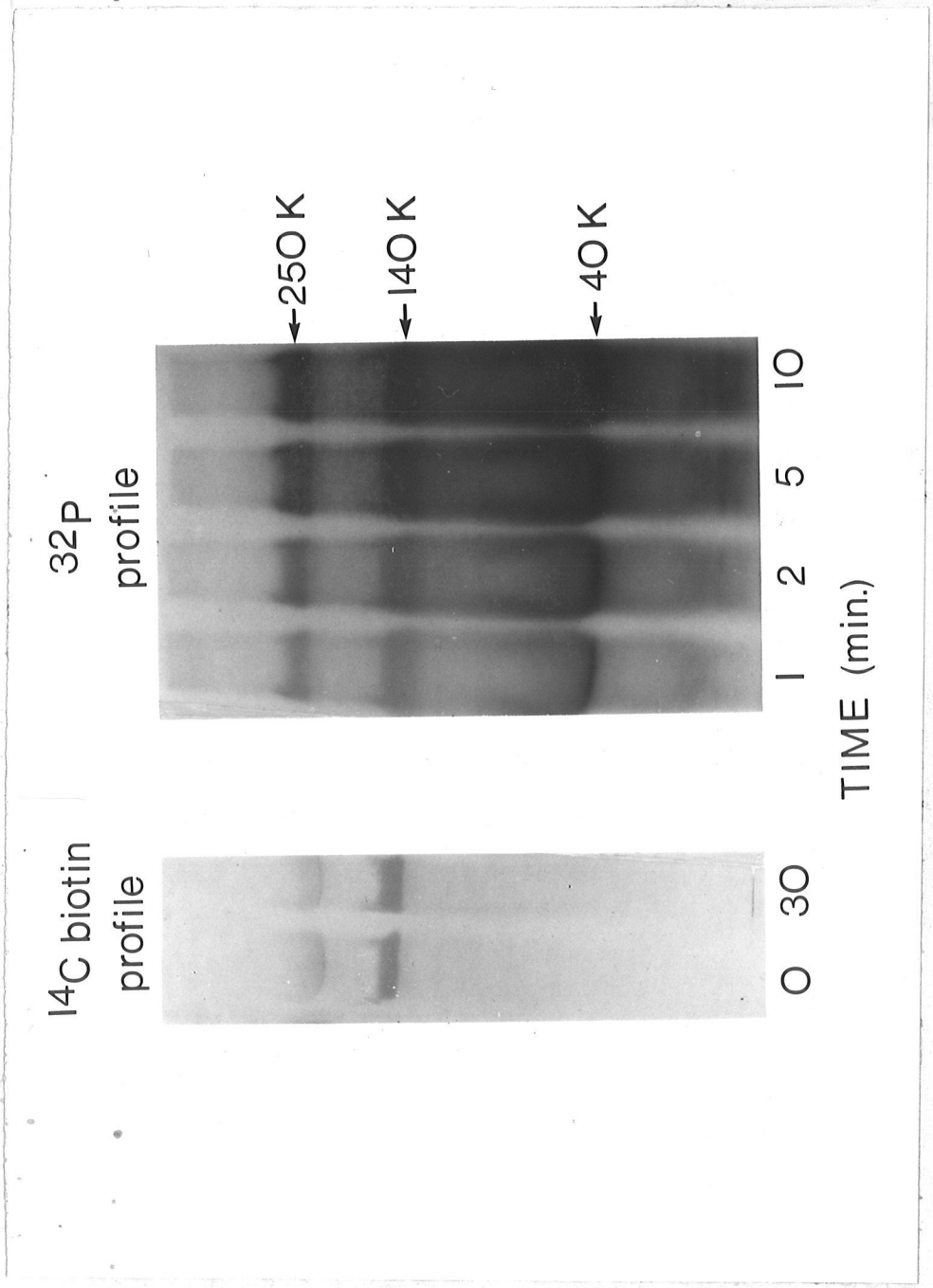
When the effect of these phosphorylation reactions on enzyme activity was investigated it was found that neither the cAMP-dependent nor the cAMP independent phosphorylation had any large effect on the direct activity of the enzyme purified further by a second ammonium sulphate fractionation (Figure 17). The lack of any effect of phosphorylation could be the result of the removal of endogenous inhibitors. Phosphorylation by the catalytic subunit seemed to decrease the activity of the enzyme by about 20% in 15 minutes although phosphorylation was complete in ten minutes.

To clarify that the peak of radioactivity at the dye front (Figure 14) consisted of phosphopeptides we subjected the immunoprecipitates, obtained after phosphorylating the enzyme preparation, to SDS gel electrophoresis on high cross linked polyacrylamide gels (Kwoland, 1974). The

autoradiographs of the immunoprecipitate, obtained from the enzyme that was phosphorylated by the cAMP-independent kinase, is shown in Figure 18. It can be seen that the 250K native subunit of ACC along with a 140K and a 40K phosphopeptide are phosphorylated under these conditions. The [^{14}C] biotin profile which contains a labelled 250K subunit and a 140K proteolytic product does not change after phosphorylation even after 30 minutes. This indicates that the enzyme has been proteolytically nicked during isolation and it is on these cleaved fragments that phosphorylation occurs. The autoradiographic profile of cAMP-dependent phosphorylation of the enzyme is shown in Figure 19. It can be seen that after 10 minutes of cAMP-independent phosphorylation the catalytic subunit further phosphorylates the 250 K and 140 K phosphopeptides and produces an additional 120 K phosphopeptide.

Finally to determine if the phosphopeptides are actually part of the 400 K protomer of ACC and not free immunoreactive peptides we first subjected the phosphorylated enzyme (cAMP-independent) to gel filtration on sepharose 6B. It can be seen in Figure 20 that a peak of labelled protein eluted off the column in the 400 K position. When material from this peak was subjected to immunoprecipitation and SDS electrophoresis the previously defined phosphopeptides of 250 K, 140 K and 40 K were again seen on the autoradiograph.

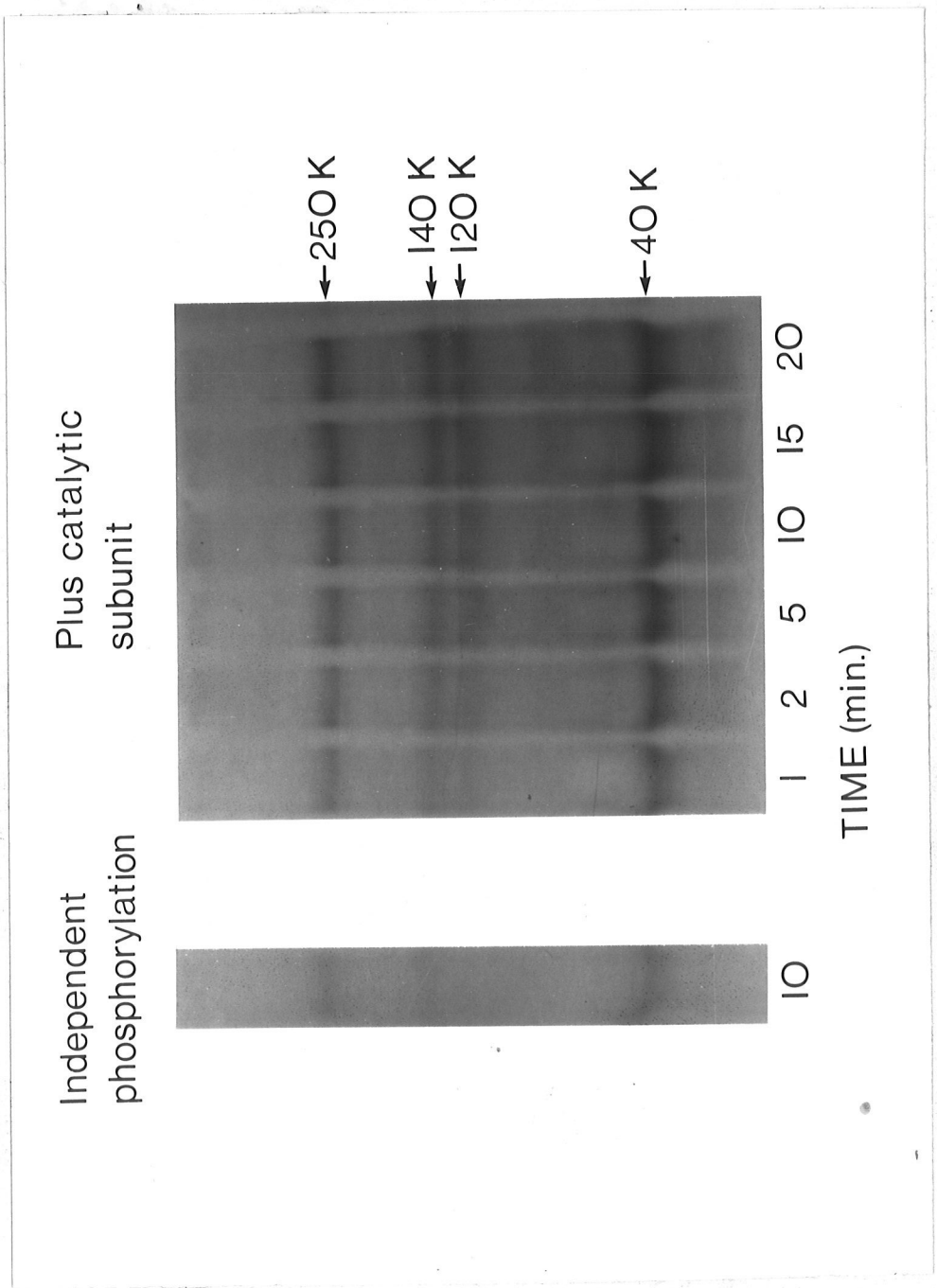
FIGURE 18


250 K
140 K
40 K
32P profile
14C biotin profile
0 30
1 2 5 10
TIME (min.)

AUTORADIOGRAPHS OF THE ^{14}C AND ^{32}P PROFILES OF ACC PHOSPHORYLATED BY A cAMP-INDEPENDENT MECHANISM.

The ^{14}C labelled enzyme was phosphorylated in the absence of the catalytic subunit, as in Figure 3, in the presence of either [γ-^{32}P] ATP or cold ATP to visualize the ^{32}P and ^{14}C profiles respectively. The immunoprecipitates, obtained at appropriate times, were subjected to SDS slab gel electrophoresis and the gels were dryed and autoradiographed.

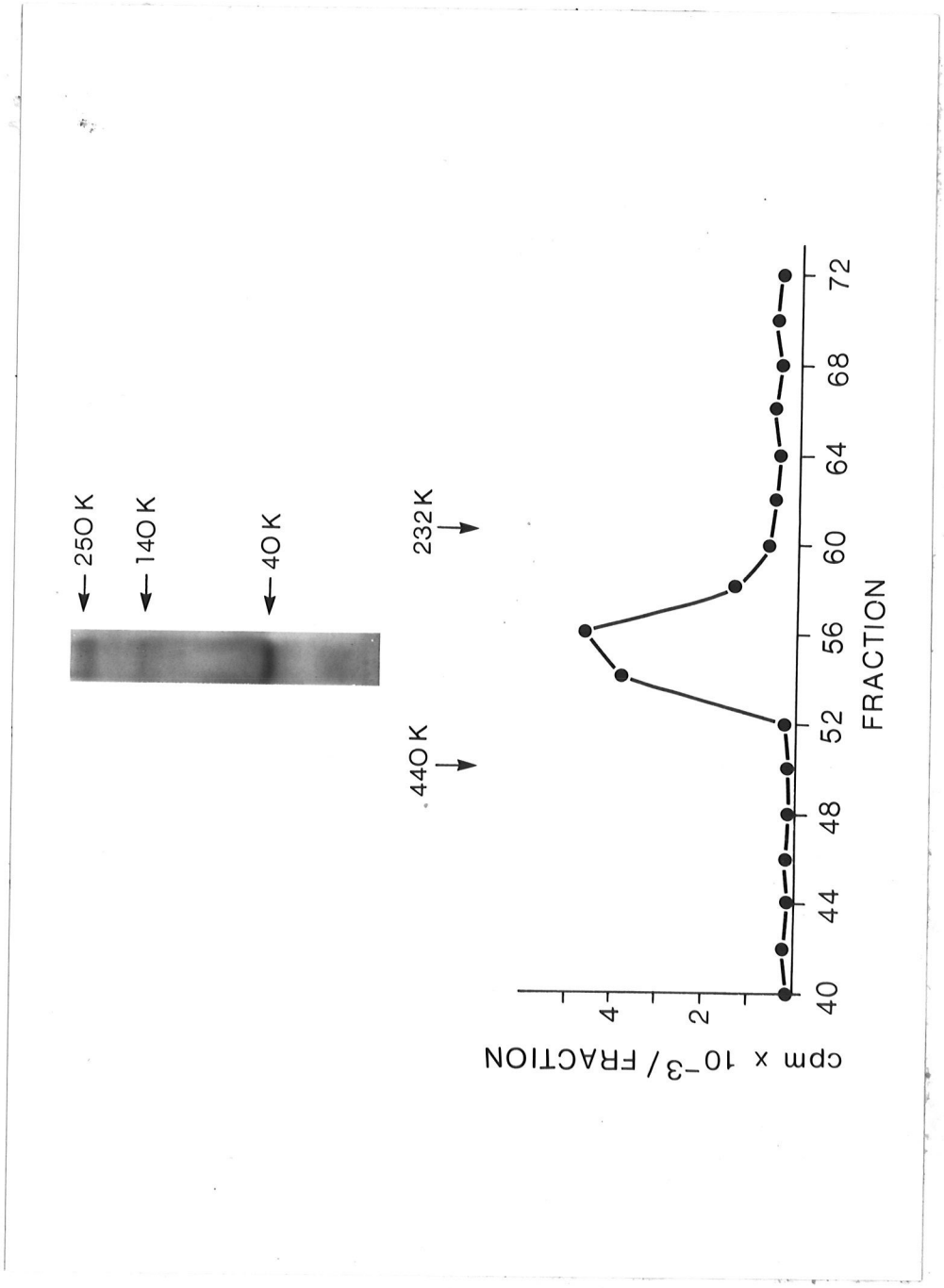
FIGURE 19


Independent phosphorylation
Plus catalytic subunit
250 K
140 K
120 K
40 K
10
1
2
5
10
15
20
TIME (min.)

AUTORADIOGRAPHS OF THE ^{32}P PROFILES OF ACC PHOSPHORYLATED BY THE cAMP-INDEPENDENT AND cAMP-DEPENDENT MECHANISMS.

The immunoprecipitates obtained in Figure 3 were subjected to slab gel electrophoresis and autoradiographed as in Figure 5.

FIGURE 20


250 K
140 K
40 K
232 K
440 K
cpm x 10⁻³/ FRACTION
4
2
40
44
48
52
56
60
64
68
72
FRACTION

SEPHAROSE 6B CHROMATOGRAPHY OF THE ^{14}C LABELLED ENZYME AFTER cAMP-INDEPENDENT PHOSPHORYLATION.

The second ammonium sulfate fraction was phosphorylated by the cAMP independent mechanism, as in Figure 3, and was subjected to chromatography on a 2.5x 80 cm. Sepharose 6B column. The peak of ^{32}P label that eluted at about 400,000 daltons was pooled and after immunoprecipitation was subjected to slab gel electrophoresis and autoradiography. The protein markers were Ferritin (M_r 440 K) and Catalase (M_r 232 K).

DISCUSSION

A crude preparation of rat liver ACC seemed to undergo inactivation upon incubation under phosphorylating conditions. However, when the enzyme was purified further this inactivation was abolished but the phosphorylation of the enzyme by a cAMP-independent mechanism was not. This suggests that cAMP-independent phosphorylation has no direct effect on the enzyme activity. The enzyme is phosphorylated on both the intact and nicked protomers as the 250 K native subunit and smaller peptides are labelled with [γ-^{32}P] ATP.

Witters and Vogt (1981) have purified the enzyme from rat hepatocytes and reported that the sites phosphorylated in vivo are sensitive to tryptic cleavage which produced a 140 K phosphopeptide and smaller peptides that ran at the dye front in SDS PAGE. As we see phosphorylation of previously nicked protomers it would seem that the sites susceptible to proteolytic cleavage are on the outside of the molecule and are sensitive to cleavage regardless of their phosphorylation state.

The lack of any effect of this phosphorylation on enzyme activity is similar to that reported by Song and Kim (1981) who has purified rat liver enzyme with a high phosphate content (6 phosphates / subunit). Removal of 3 of these phosphates did not increase the activity of the

enzyme and they concluded that these "structural" phosphates have no relationship to enzyme regulation. Similarily Bhullar and Dakshinamurti (1982) have purified ACC from porcine adipose tissue that could be phosphorylated by a cAMP-independent mechanism, incorporating 0.25 PO_4 / dimer, without affecting enzyme activity. Tipper and Witters (1982) observed a 26% decrease in activity of pure rat liver ACC following the incorporation of 0.26 moles of phosphate per 480K protomer by a cAMP-independent protein kinase. Thus it would seem that cAMP-independent phosphorylation has no significant effect on ACC activity.

Alternatively McNeillie et al (1981) have reported that rat mammary gland extracts contained a cAMP-independent kinase and a Ca^{++} ,Mg^{++} sensitive phosphatase. They observed about a 90% decrease in activity upon phosphorylation and about a 2 fold increase in the activity of the enzyme upon dephosphorylation. Lent and Kim (1982) purified a cAMP-independent kinase from rat liver that was associated with the enzyme. Incorporation of 0.9 PO_4 / subunit by the kinase resulted in 80% inactivation of the enzyme. Using exogenous phosphorylase phosphatase about 2-fold increase in activity and decrease from 0.7 to 0.2 PO_4 / subunit was observed. These reports suggest that cAMP-independent phosphorylation does have an effect on enzyme activity but in all cases dephosphorylation by both endogenous and exogenous

phosphatases does not completely reactivate the enzyme.

Guy and Hardie (1981) have shown that the rabbit mammary enzyme prepared in the presence of NaF, and containing 6 phosphates per 250 K subunit can be activated by tryptic cleavage of the enzyme which resulted in the removal of about one mole of phosphate. It is possible that the enzyme used in our studies is fully activated by proteolytic nicking and is no longer susceptible to inactivation by phosphorylation. The results in the present report indicate that the site phosphorylated by the catalytic subunit of cAMP-dependent protein kinase is different from the so called "structural phosphate" sites reported by Hardie and Guy (1980). In our experiments, if one considers the inactivation at 15 minutes significant, the rate of phosphorylation was faster than the rate of inactivation and it is difficult to assign any causal role to phosphorylation. It must also be considered that the 250 K native subunit is phosphorylated and if cAMP-dependent phosphorylation caused inactivation there should have been a more significant decrease in the enzyme activity. Bhullar and Dakshinamurti (1982) has reported that pure porcine adipose ACC can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase,incorporating 0.65 PO_4 / dimer, which resulted in 25% inactivation of the enzyme. Tipper and Witters (1982) using pure rat liver observed a 57% decrease in ACC activity that was accompanied by the incorporation of 1

mole of phosphate per 480 K subunit after a 60 minute incubation in the presence of the catalytic subunit of cAMP-dependent protein kinase. The significance of the inactivation observed is questionable as they dilute the enzyme 10 fold in cold buffer before assaying it. It is well known that the rat liver enzyme is cold sensitive and the active polymer is in equilibrium with the protomeric form of the enzyme (Numa, 1965). Thus, assaying the enzyme by their method one would tend to depolymerize and inactivate the enzyme until the new equilibrium is reached. The presence of ATP may just speed up this process and the intrinsic activity of the enzyme may not be affected.

Witters (1981) observed that insulin stimulated the phosphorylation of ACC in rat hepatocytes and insulin and glucagon phosphorylated the enzyme additively. Brownsey et al (1981) using ammonium sulfate fractionated rat adipose ACC reported a Mg^{2+}-dependent membrane bound kinase that phosphorylated and increased the direct activity of the enzyme whereas cAMP-dependent phosphorylation inactivated both the direct and preincubated activity. This brings up the possibility that the "structural phosphates" may be the result of insulin dependent phosphorylation which activates the enzyme masking the inhibitory effects of cAMP-dependent phosphorylation.

In summary, we have demonstrated that the rat liver ACC can be phosphorylated by a cAMP-independent mechanism and that the enzyme is already proteolytically nicked.

Thus, this phosphorylation does not sensitize the enzyme to proteolysis. It is not known whether the rapid phosphorylation of the rat liver enzyme causes a slow depolymerization of the enzyme or whether it is the presence of ATP that affects the depolymerization of the enzyme. In light of these results it would be advisable to investigate the cAMPdependent phosphorylation-dephosphorylation phenomena after first completely dephosphorylating the enzyme to remove the competitive effects of other phosphorylations.

SECTION VI

FATTY ACID SYTHESIZING COMPLEX

INTRODUCTION

Acetyl-CoA carboxylase (ACC) is generally considered to be a cytosolic enzyme. However, Easter and Dils (1968) have demonstrated that a species of ACC is sedimented with the microsomes in lactating rabbbit mammary gland. We reported earlier on the *in vivo* distribution of ACC from rat liver between a cytosolic species and one that sedimented with the microsomal pellet (Dakshinamurti and Gillevet, 1980; Gillevet and Dakshinamurti, 1982a; Gillevet and Dakshinamurti, 1982b). Similar observations were subsequently reported by Witters *et al* (1981).

In this section evidence is presented which suggests that the high molecular weight polymer of ACC observed *in vitro* does not exist *in vivo* under conditions favouring lipogenesis. In addition the form of the enzyme referred to as the microsomal form in section IV is actually associated in a complex that sediments at a heavier density than the protomeric form of the enzyme. Fatty acid synthetase and ATP-citrate lyase (EC 4.1.3.8) are also found to sediment in the same region of the sucrose gradient as ACC. It is hypothesized that the enzymes of fatty acid synthesis are associated in an aggregate with either themselves or some subcellular structure such as

microtubles and that the interaction between the components of this complex or aggregate seems to be regulated by a cAMP-dependent phosphorylation mechanism.

EXPERIMENTAL

Materials: D[1-^{14}C] Pantothenic acid (57 mCi/mmol) was obtained from New England Nuclear Corporation and D[carbonyl-^{14}C] Biotin (51 mCi/mmol) from Amershan Corporation . Electrophoretic reagents were obtained from Sigma Chemical Company. Gel filtration standards and electrophoretic standards were supplied by Pharmacia Fine Chemicals. NCS tissue solubilizer and OCS scintillant were obtained from Amersham Corporation.

Preparation of animals: Male Long-Evans rats (150 gm) were starved for 48 hours and then refed a high carbohydrate diet for another 48 hours. During refeeding the rats were injected with either [^{14}C] Biotin or [^{14}C] Pantothenate (10 uCi/day) to label ACC and FAS respectively. They were then sacrificed by decapitation. The livers were homogenized with a teflon pestle in 0.25 M sucrose, 50 mM phosphate buffer pH 7.5, 1 mM EDTA and 5 mM B-mercaptoethanol at room temperature. The homogenate was centrifuged at 10,000 xg for 20 min. at 21^{o}C. The low speed supernatant was then filtered through cheesecloth and applied on the sucrose gradients.

To monitor changes in the distribution of ACC and FAS during different lipogenic states the two enzymes were

labelled as above. The rats were then divided into two sets, one of which was fasted overnight, the other being kept on the high carbohydrate diet. The following morning the low speed supernatant was prepared as described.

Sucrose Gradient: Aliquots of the low speed supernatant were centrifuged through a 20% to 40% sucrose gradient (30 ml) prepared on a Beckman Sucrose Density Gradient Former. The gradient contained 50 mM phosphate buffer pH 7.5, 1 mM EDTA and 5 mM B-mercaptoethanol. One ml aliquots of the low speed supernatant were layered on top of the gradientand were centifuged for either 2.0 or 2.5 hours(as indicated in the lengends) at 60,000 rpm at 21°C in a Beckman 70Ti fixed angle rotor. Fractionation (1.2 ml) was accomplished through the use of a peristaltic pump to remove the gradient from the bottom of the tube. Thyroglobulin (M_r 669K) was run as a molecular weight marker and in some instances pure Avian ACC was also run.

SDS Gel Electrophoresis: A 50 ul aliquot of each of the above fractions was denatured in 1% SDS and 5% B-mercaptoethanol by heating at 100°C for five minutes and was then subjected to SDS-gel electrophoresis on slab gel with a 3% acrylamide stacking gel and a 5% acrylamide running gel according to the method of Laemmli (1970). Standards were run and molecular weights were determined from plots of the relative mobility verses the log of the

molecular weight.

Enzyme Assays: Acetyl-CoA carboxylase activity was determined by the ^{14}C-bicarbonate fixation assay of Dakshinamurti and Desjardins (1969), after preincubating the enzyme at 37°C for 30 minutes in the presence of 20 mM potassium citrate. Fatty acid synthetase (FAS) activity was assayed by following the incorporation of [^{14}C] malonyl-CoA into long chain fatty acids (Gibson. 1958). The ATP-citrate lyase activity was monitored by following the incorporation of label from [1,4-^{14}C] citrate into fatty acid through a coupled assay with fatty acid synthetase (Gibson, 1958; Dakshinamurti and Desjardins, 1969).

Determination of label incorporated into ACC and FAS: Initially 10% TCA precipitation at 4°C was done to determine protein bound label. The TCA precipitate was washed with 5% TCA and solubilized in NCS tissue solubilizer and counted using OCS scintillant. Alternatively, to speed up the analysis of the gradients, aliquots from the gradient fractions were solubilized and counted directly without TCA precipitation.

Concentration of labelled ACC and FAS: After locating the position of the biotin and pantothenate labels, by counting fractions directly, the two label peaks

were concentrated separately in an Amicon Ultrafiltration Cell under a stream of nitrogen using a YM10 filter at room temperature. The concentrate was diluted with 50 mM phosphate buffer pH 7.5, 1 mM EDTA and 5 mM B-mercaptoethanol and reconcentrated at room temperature to remove sucrose. This concentrate was then reapplied to new sucrose gradients.

RESULTS

Distribution of Enzyme Activities on Sucrose Gradients

To investigate the nature of the microsomal form of ACC, previously descibed in section IV, we subjected the low speed supernatant prepared from rats fed a high carbohydrate diet to sedimentation on 20%-40% sucrose density gradients. The distribution of acetyl-CoA carboxylase (ACC), ATP-citrate lyase (CL) and fatty acid synthetase (FAS) activities is shown in Figure 21. ACC activity sedimented as a broad peak between the major peaks of cytosolic and microsomal proteins. This form of ACC formed under high lipogenic conditions and isolated by the method described did not sediment with the purified, polymerized avian enzyme which sedimented with the microsomes. The activity of FAS and CL sedimented as peaks partly overlapping the ACC peak. Where the ACC peak and the FAS peak overlapped there seemed to be a decrease or trailing out of the ACC activity. The majority of the FAS peak and part of the CL peak ran ahead of the Thyroglobulin marker.

SDS Gel Profile of Sucrose Gradient Fractions

The fractions from the above gradient were analysed by SDS gel electrophoresis. The molecular weight of the subunits of ACC and FAS is 250K and a band can be seen migrating at this molecular weight in fractions 8 to 14

that contained fatty acid synthetase activity (Figure 22). The subunit of citrate lyase has a molecular weight of 120K and another band can be seen in this region in fractions 8 to 14.

Distribution of TCA Precipitable [^{14}C] Biotin and [^{14}C] Pantothenate

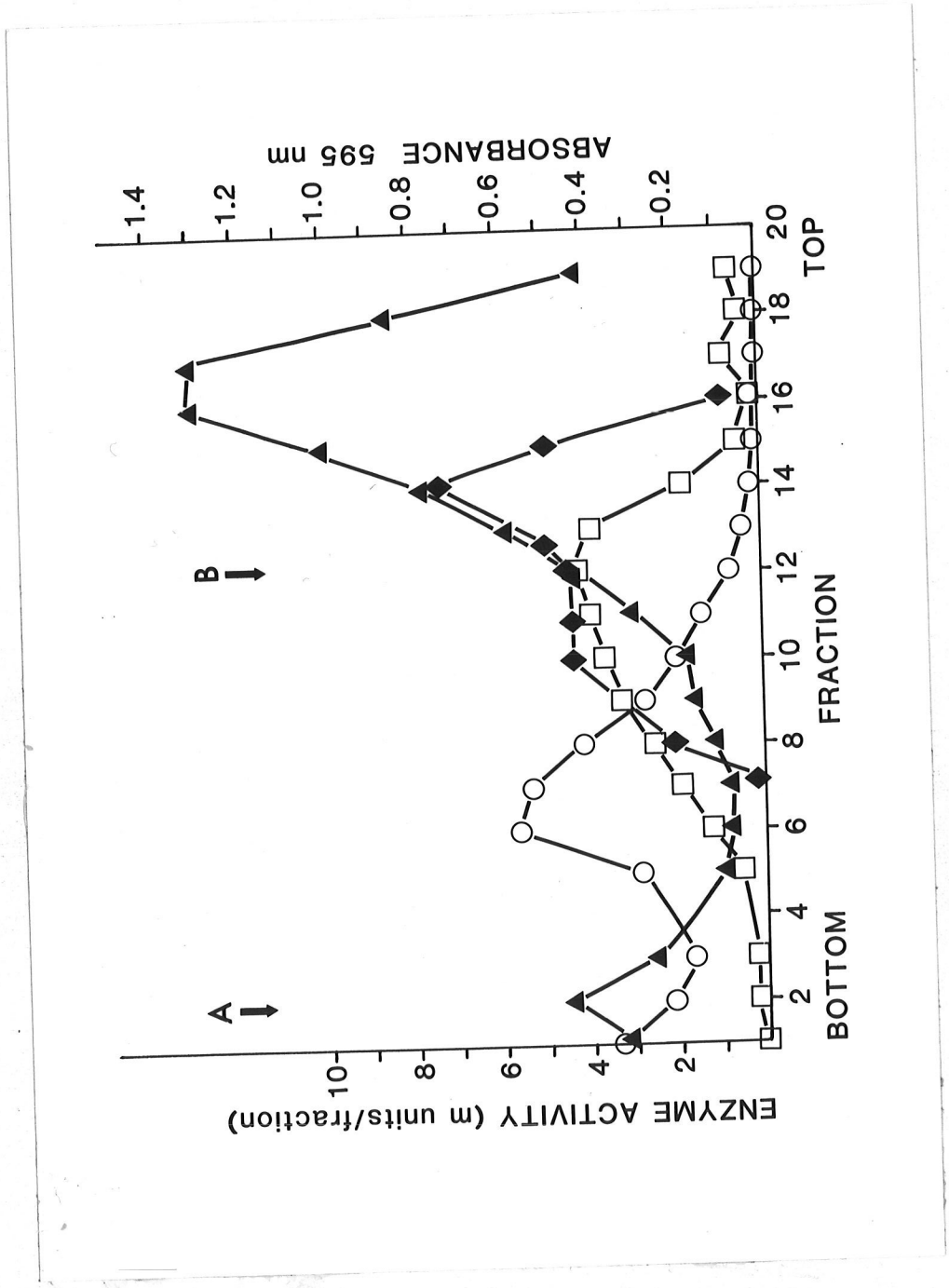
ACC and FAS were labelled with [^{14}C] biotin and [^{14}C] pantothenate respectively in separate animals to further define the distribution of these two enzymes as FAS interferes with the assay for ACC. Labelled low speed supernatant was prepared and analyzed on separate sucrose gradients under identical conditions. Two peaks of [^{14}C] biotin label and two peaks of [^{14}C] pantothenate label that were TCA precipitable were observed (Figure 23). One peak of biotin migrating near the top of the gradient just under the Thyroglobulin marker (fractions 11 and 12) which corresponds to the same position in the gradient that the peak of FAS activity sedimented at in Figure 21. A peak of pantothenate label was also observed to also sediment at the above position (fractions 11 and 12) It would seem then that the trailing peak of ACC activity observed in Figure 21 was do to FAS intefering with the ACC assay as there is biotin label sedimenting at the same density (Figure 23) indicating the presence of ACC in this area of the gradient. Another peak of pantothenate was observed to sediment in the lower molecular weight region of the

gradient (fractions 14 to 16) and a second peak of biotin label sedimented at a density (fractions 8 to 10) lower then the first peak of biotin label. When the homogenate was incubated in the presence of 1 mM citrate a dramatic shift in the profile of [^{14}C] biotin label was seen towards a heavier density. This was the result of either the formation of the artificial polymer of ACC or of the formation of some aggregate that sedimented further into the gradient.

Distribution of Total [^{14}C]-Biotin and [^{14}C]- Pantothenate Under Different Lipogenic Conditions

To determine if the association of the two enzymes was altered under low lipogenic conditions, rats were labelled in separate experiments with [^{14}C] biotin or [^{14}C] pantothenate as before and one set was fasted overnight and another set was maintained on the high carbohydrate diet. The profiles of the distribution of [^{14}C] biotin and [^{14}C] pantothenate in the sucrose gradient fractions were determined in both the refed and starved rats (Figure 24). Preliminary experiments indicated the aggregated forms of the two enzymes (ACC and FAS) were not very stable (data not shown). To facilitate the isolation of these aggregates, TCA precipitation of the fractions was omitted and the gradients were only centrifuged for 2.0 hours. This allowed the isolation and concentation of the aggregates to be performed on the same day.

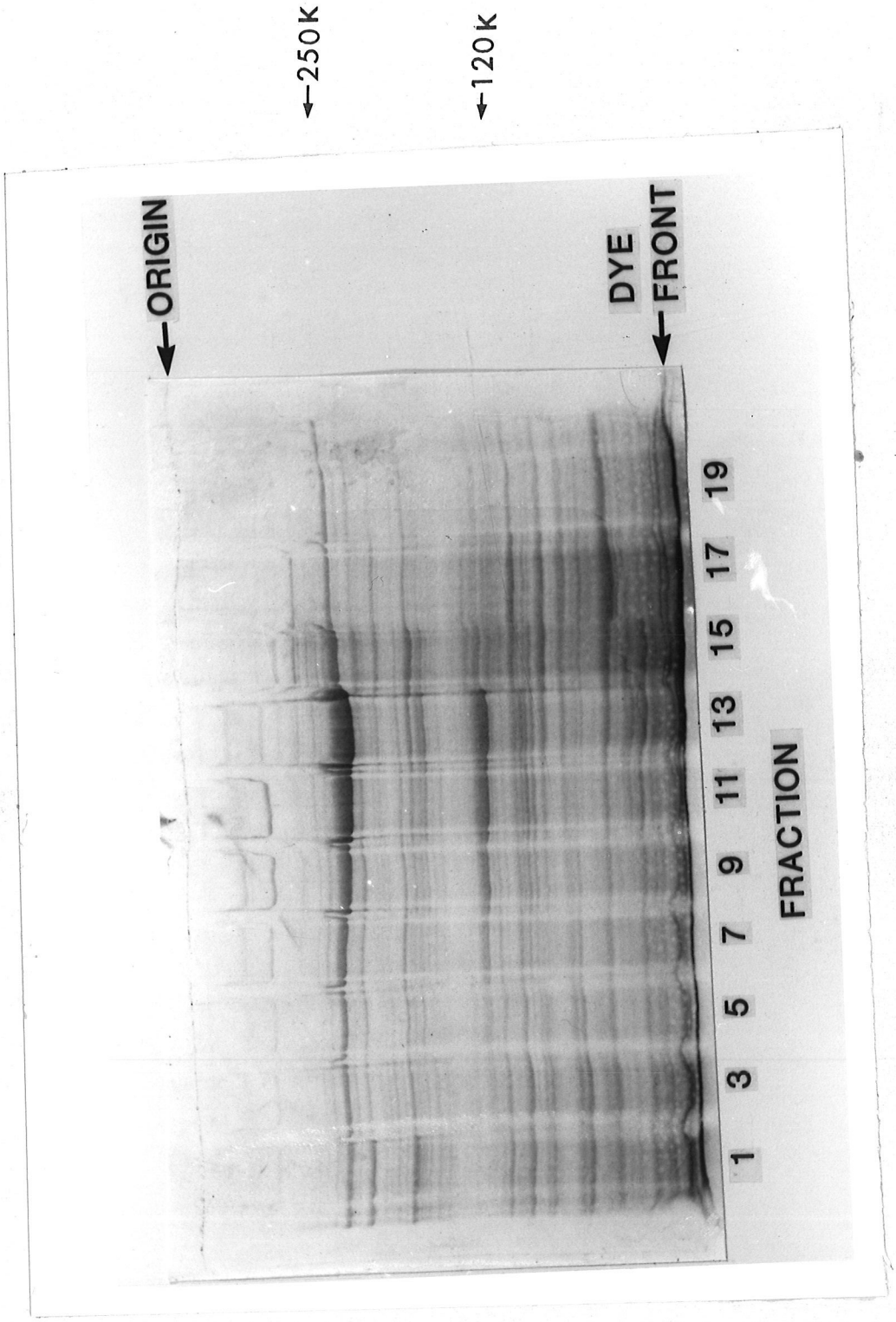
FIGURE 21


ENZYME ACTIVITY (m units/fraction)
ABSORBANCE 595 nm
A
B
BOTTOM
TOP
FRACTION
2
4
6
8
10
12
14
16
18
20
0.2
0.4
0.6
0.8
1.0
1.2
1.4

DISTRIBUTION OF ACETYL-CoA CARBOXYLASE AND FATTY ACID SYNTHETASE ACTIVITIES ON SUCROSE DENSITY GRADIENTS.

Rats were fed a high carbohydrate diet and the low speed supernatant was prepared from the liver. This was then centrifuged through a 20% to 40% sucrose gradient for 2.5 hours as outlined in the methods. The gradient was fractionated and assayed for protein (▲—▲), Acetyl-CoA carboxylase activity (○—○), fatty acid synthetase activity (□—□) and ATP-citrate lyase activity (◆—◆). Marker A is the pure polymerized avian acetyl CoA carboxylase and marker B is thyroglobulin (molecular weight 669,000).

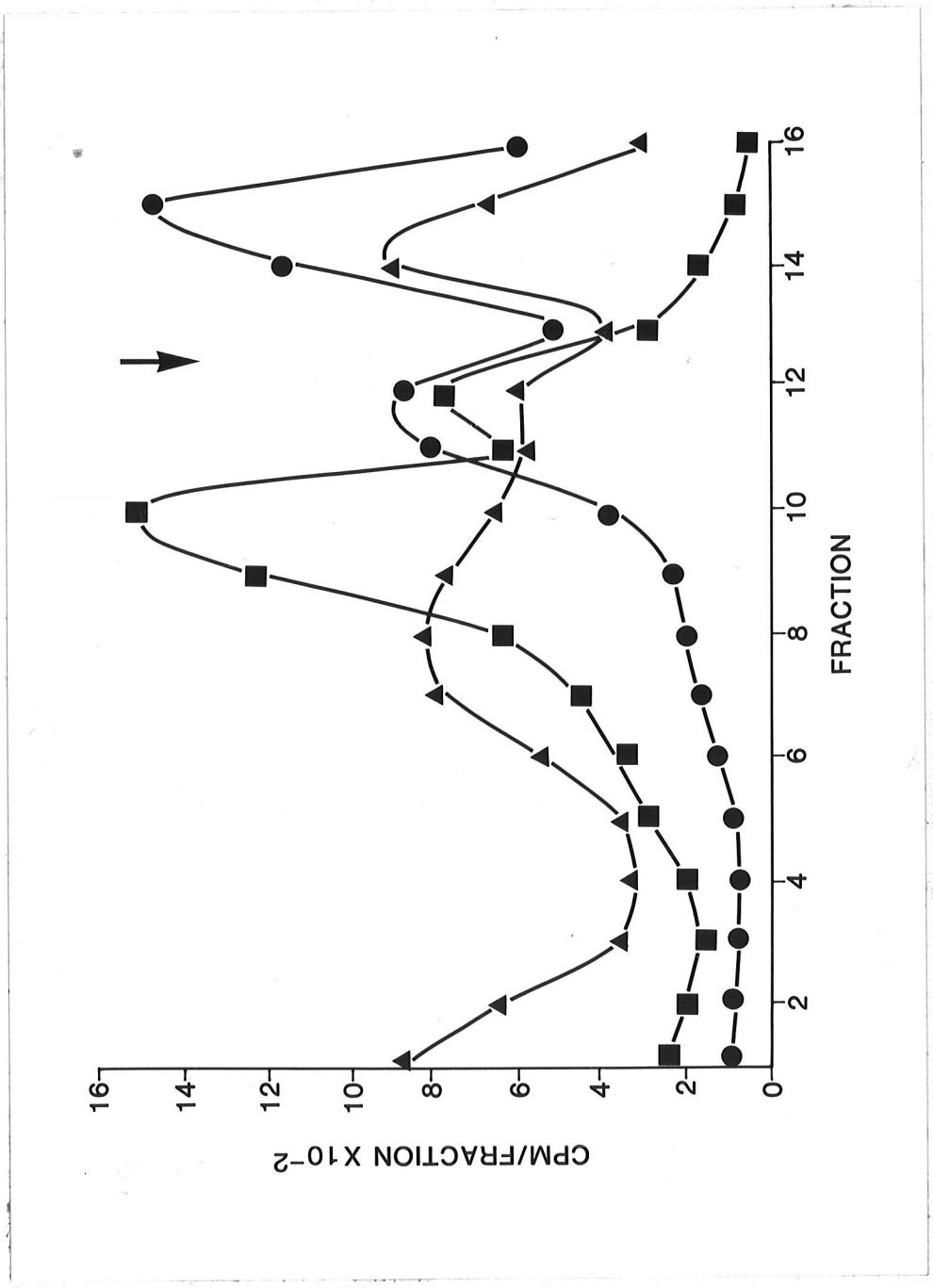
FIGURE 22



SDS GEL PROFILE OF SUCROSE GRADIENTS.

Aliquots from the gradient fractions of Fig. 1 (25 ul) were denatured and analyzed on a 5% SDS polyacrylamide gel. The positions of 250,000 daltons and 140,000 daltons is indicated as determined from molecular weight standards.

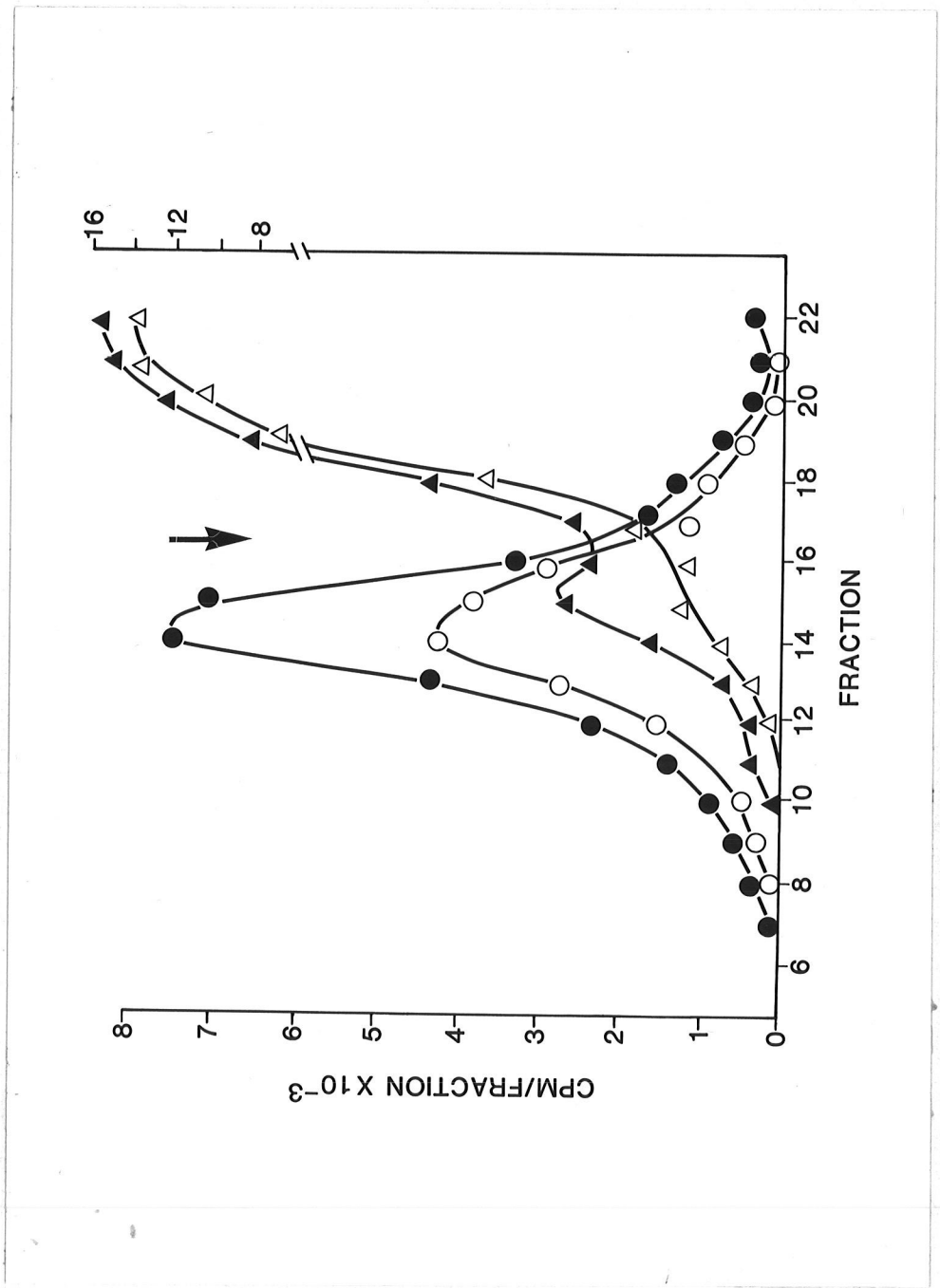
FIGURE 23



DISTRIBUTION OF TCA PRECIPITABLE [^{14}C] BIOTIN AND [^{14}C] PANTOTHENATE ON SUCROSE GRADIENTS.

Separate groups of animals were labelled with either ^{14}C-Biotin or ^{14}C-Pantothenate and the low speed supernatants were prepared as described in the methods. The low speed supernatants were run on separate sucrose gradients for 2.5 hours and TCA precipitation was performed on the fractions. The arrow indicates the position of the Thyroglobulin marker. The distribution of biotin label (■— ■) and pantothenate label (●—●) in the absence of citrate in the homogenization media and the distribution of biotin label (▲—▲) in the presence of citrate in the homogenization media is depicted.

FIGURE 24


CPM/FRACTION X10⁻³
FRACTION
0
1
2
3
4
5
6
7
8
6
8
10
12
14
16
18
20
22
8
12
16

DISTRIBUTION OF TOTAL [^{14}C]- BIOTIN AND [^{14}C]-PANTOTHENATE ON SUCROSE GRADIENTS UNDER HIGH AND LOW LIPOGENIC CONDITIONS.

Rats were labelled *in vivo* as outlined in the methods and then divided into sets of two groups each. One set (consisting of a biotin labelled group and pantothenate labelled group) was fasted overnight while the other set was maintained on the high carbohydrate diet. The low speed supernatant was prepared from all four groups and sedimented on sucrose gradients for 2.0 hours and 1.2 ml fractions were counted directly with TCA precipitation. The distribution of biotin label (circles) and pantothenate label (triangles) from rats fed a high carbohydrate diet (closed symbols) or fasted overnight (open symbols) is shown.

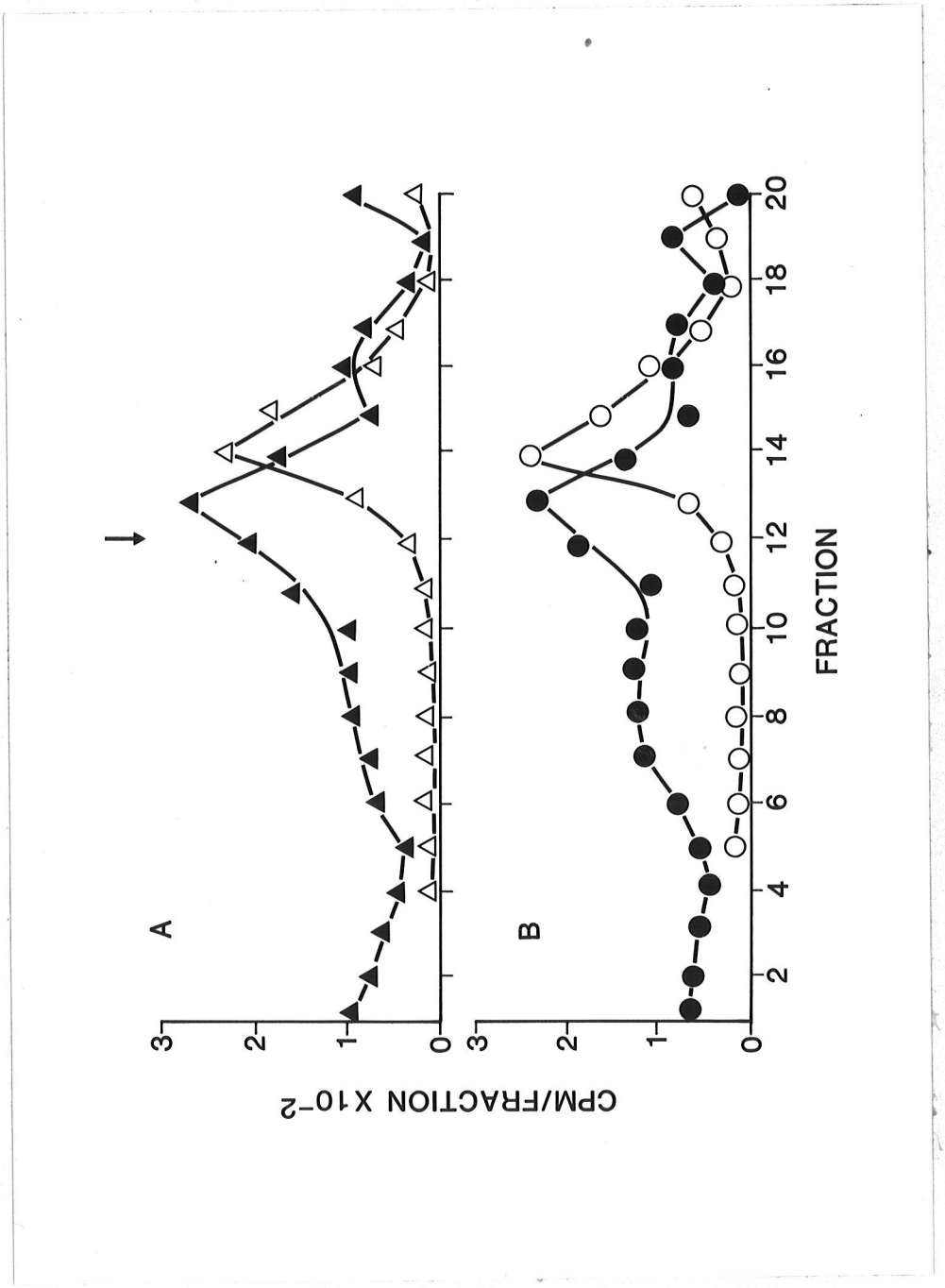
Although there was not the clear separation of biotin and pantothenate peaks as observed in Figure 23 a biotin peak and a pantothenate peak were observed that sedimented ahead of the Thyroglobulin marker (fractions 8 to 16). The biotin label and the pantothenate label did not quite overlap in rats fed a high carbohydrate diet and probably consisted of two species of ACC and one of FAS (as in Figure 23) that had not been separated do to the shorter sedimentation time. The majority of the pantothenate label sedimenting in the lower molecular region (fractions 16 to 22; Figure 24) was free pantothenate or fatty acyl-CoAs as they could be removed by TCA precipitation (as in Figure 23). After an overnight fast a 50% reduction in the height of both the biotin and pantothenate peaks that sedimented ahead of the Thyroglobulin marker was observed (Figure 24).

Alteration in the Distribution of [^{14}C] Biotin and [^{14}C] Pantothenate Upon Incubation Under Phosphorylating Conditions

An attempt was made to try to elucidate the *in vitro* effect of cAMP dependent phosphorylation on the aggregation of ACC and FAS. Fractions 8-15 from the refed gradients of Figure 24 were pooled and concentrated separately as described in the methods. These aggregates were then resedimented in the presence or absence of 0.1 mM cAMP, 1 mM ATP and 2 mM $MgCl_2$ (Figure 25) for 2.5 hours. Both the biotin and the pantothenate labels resedimented as a broad

distribution throughout the gradient in the absence of ATP, Mg^{++} and cAMP. The broad distribution can be explained in part by the increased sedimentation time but it would seem that the concentration procedure is altering the proprties of the aggregates. Upon incubation under phosphorylating conditions (in the presence of ATP, Mg^{++},and cAMP) a concomittant shift in both labels was observed toward the top of the gradient behind the Thyroglobulin marker.

FIGURE 25



REDISTRIBUTION OF THE ISOLATED FATTY ACID SYNTHESIZING COMPLEX UPON INCUBATION WITH ATP, Mg^{++} and cAMP.

Fraction 8 to 15 from the gradient obtained from the refed rats (Figure 24) which contained the peaks of biotin or pantothenate label sedimenting ahead of the Thyroglobulin marker were pooled, concentrated and run on the gradients in the presence (open symbols) or absence (closed symbols) of 1 mM ATP, 0.1 mM cAMP and 2 mM $MgCl_2$ for 2.5 hours. Panel A is the pantothenate label and panel B is the biotin label. The arrow is the position of the Thyroglobulin marker.

DISCUSION

We have suggested that the low activity of ACC in fractions 8 to 14 (Figure 21) might be an artifact of the bicarbonate fixation assay as the labelled malonyl-CoA formed by ACC is decarboxylated by FAS. As there is a peak of TCA precipitable [^{14}C] biotin co-sedimenting with the pantothenate label (Figure 23) it would seem that the low ACC activity in this area of the gradient is due either to the above mentioned artifact of the assay reaction or to the existance of an inactive form of ACC. The latter possibility is unlikely as the fractions were preincubated with citrate to maximize ACC activity and a completely inactive form of the enzyme has never been reported. The molecular weight of ACC, CL and FAS protomers is 400 K and as they all sediment ahead of the thyroglobulin marker (molecular weight 669 K) it would seem that the enzymes are associated in some sort of high molecular weight complex but they may not be associated with each in a stoichiometric fashion. It would seem that the enzymes themselves may be bound to the microtubular lattice and it is this complex that is observed on the gradients. Under the centrifugation condition employed in these experiments, the microtubular lattice would sediment between the low molecular weight region at the top of the gradient and the band of microsomes near the bottom of the gradient. The observation that FAS strongly inhibits the ACC assay

suggest, on the other hand, that these two enzymes may be associated with each other or are interacting with each other.

The exact size of the complex found on the gradient is difficult to determine as the sedimentation velocity is dependent on the stokes radius of the complex. Thus large branched complexes cannot be directly compared to globular protein markers. An attempt was made to use dextran blue (M_r 2,000K) to further calibrate the gradient but it did not enter the gradient, probably due to its branched structure and no other large molecular weight markers were available.

The exact identity of the 250K and 140K bands on the SDS gel (Figure 22) cannot be unequivically assigned to ACC, FAS and CL unless specific antibody to these proteins is employed to specifically remove these bands by immunoprecipitation. It is well documented, on the other hand, that the synthesis of these enzymes are increased by refeeding a high carbohydrate diet and under these conditions constitute a major fraction of the cellular protein (Majerus and Kilburn, 1969; Volpe et al, 1973; Smith and Abraham, 1970). Thus, the prominance of these bands and their molecular weight suggests that they are the lipogenic enzymes. It should also be noted that there is a distribution of many proteins or subunits of proteins of the same molecular weight throughout the complete gradient range. This suggests that this aggregation phenomena may

not be specific to the lipogenic enzymes but may of a common phenomena of many proteins as suggested by Masters (1981). Thus, we may be observing proteins binding to the so called lattice or network of microtubles (Masters,1981) and as this lattice is sedimented the shear forces break it up leaving behind a trail of protein.

The preparation of the low speed supernatant was done at 21°C in order to maintain the *in vivo* form of ACC as it has been suggested that cooling the rat liver enzyme to 4°C inactivates the enzyme and thus abolishes the *in vivo* stimulation of the enzyme activity by insulin (Witters *et al*, 1979). When the low speed supernatant was incubated with citrate, the [^{14}C] biotin label was distributed toward the bottom of the gradient along with the appearance of a peak sedimenting at the top of the gradient suggesting that the form of the enzyme produced by incubation with citrate is an *in vitro* form and does not exist *in vivo* under lipogenic conditions.

The *in vivo* regulation of fatty acid synthesis was investigated (Figure 24). Lipogenesis in rat liver is inhibited by a 48 hour fast and upon refeeding a high carbohydrate diet there is a prompt rise in fatty acid synthesis within 48 hours (Boxer and Stetten, 1944; Allman *et al*, 1965) as the result of enzyme synthesis (Majerus and Kilburn, 1969; Nakanishi and Numa, 1970). The dietary manipulation employed in these experiments would involve the long term stimulation of the lipogenic capacity of the

liver, through increased enzyme content, during refeeding (Majerus and Kilburn, 1969) and the short term hormonal regulation of that capacity during the overnight fast (Katz and Ick, 1981). Acetyl-CoA carboxylase from rats refed a high carbohydrate diet has a half life of about 55 hours and this is decreased to about 31 hours after a 48 hour fast (Nakanishi and Numa, 1970). Thus the 50% decrease in the biotin and pantothenate peaks (fraction 8 to 16, Figure 24) can not be solely contributed by enzyme degradation. It is possible that redistribution of the label is occuring presumably by the disassociation of the aggregated forms of the enzymes.

The in vitro affects of phosphorylation on the aggregated forms of ACC and FAS were investigated (Figure 25). The shift in the biotin and pantothenate profiles following the incubation with ATP would seem to indicate that cAMP dependent phosphorylation alters the aggregation properties of ACC and FAS altough further experiments are required to confirm this. Preliminary experiments have indicated that ACC is phosphorylated under these conditions. It is not known whether it is the phosphorylation of the enzymes themselves or other components of the aggregate or aggregates that alters this property. The results also indicate that the aggregates of ACC and FAS contain a kinase associated with them. Lent and Kim (1982) have also reported that a protein kinase is found associated with ACC suggesting that a complex may

exist that contains endogenous kinases and phosphatases similar to the pyruvate dehydrogenase complex. Both fatty acid synthetase (Hardie and Cohen, 1978) and ATP-citrate lyase (Alexander et al, 1982) undergo cAMP dependent phosphorylation and as yet no function for these phosphorylations has been elucidated.

In conclusion, we hypothesize that the enzymes of lipogenesis are associated together either directly as a "Fatty acid Synthesizing Complex" or indirectly in some subcellular structure such as the microtrabecular lattice where the enzymes can interact in the synthesis of fatty acids. It is also hypothesized that cAMP dependent phosphorylation of these lipogenic enzymes contributes to a decrease in the interaction of these enzymes either through the dissociation of the aggregate or by decreasing coupled activity of these enzymes and thus is the mechanism for the hormonal regulation of lipogenesis.

GENERAL DISCUSSION

It is generally hypothesized that fatty acid synthesis is regulated through the combined effects of allosteric regulation, covalent modification and enzyme synthesis and degradation. The key enzyme that seems to be regulated during the acute regulation of the above metabolic pathway is acetyl-CoA carboxylase. These mechanisms are by no means exclusive of one another as it has been observed that covalent modification alters the Km and Ki of various allosteric effectors of ACC and the degree of allosteric activation (ie the degree of polymerization) seems to decrease the susceptability of the enzyme to proteolytic degradation. The acute regulation of ACC, in the liver, is thought to be mediated by the hormone glucagon through a phosphorylation mechanism and the present investigation has been limited to this latter aspect, that is the effect of phosphorylation on ACC activity.

To be certain that a enzyme is regulated by a phosphorylation-dephosphorylation mechanism, the following criteria must be fullfilled:

(1). Demonstration of stoichiometric phosphorylation and dephosphorylation <u>in vitro</u> at significant rates with appropriate kinases and phosphatases.

(2). Demonstration that functional properties of the enzyme undergo changes that correlate with the degree of *in vitro* phosphorylation.

(3). Demonstration of phosphorylation and dephosphorylation *in vivo* or *in situ* with accompanying functional changes.

(4). Demonstrate a relationship between the degree of phosphorylation and cellular levels of kinase and phosphatase effectors.

There is substantial evidence suggesting that glucagon acting through cAMP decreases lipogenesis *in vivo*, although it as been difficult to demonstrate functional changes in in the lipogenic enzymes. It has been suggested that ACC catalyzes the step that is acutely regulated by the hormone yet it has been very difficult to show distinct changes in the activity of the enzyme upon *in vivo* manipulation of the hormonal status of the tissue. This in itself would suggest that some other process is involved in the regulation of fatty acid synthesis besides direct changes in enzyme activity. Several laboratories have demonstrated that crude preparations as well as pure acetyl-CoA carboxylase can be phosphorylated at significant rates *in vitro* and in some instances cAMP-dependent phosphorylation has been shown to inactivate the enzyme. In these experiments the pure enzyme was assayed in the presence of high citrate concentrations which artificially polymerize the enzyme and thus, any change in activity

observed may not be indicative of the in vivo situation.

Several phosphatases have been reported to dephosphorylate and activates ACC but no correlation has been demonstrated between the induction or activation of these phosphatases by insulin. In summary the hypothesis that ACC is regulated directly by a phosphorylation-dephosphorylation mechanism mediated by cAMP has not satisfied all the criteria defined above.

In any experiment with ACC it is difficult to distinguish between allosteric activation of the enzyme through polymerization and activation of the intrinsic activity of the enzyme. All observations to date indicate that phosphorylation of the enzyme does not affect intrinsic activity as the Vmax is not changed while the Km for allosteric effectors is altered. The Km and Vmax of many ambiquitous enzymes are altered upon binding to subcellular structures. Thus, it is quite possible that the primary effect exerted by insulin is a conformational change that induces the enzyme to bind to other structures or enzymes and that in the absence of these ligands (ie in a pure enzyme preparation) the enzyme binds to itself. It is hypothesized that ambiquitous enzymes exist in a equilibrium between the bound and soluble form. Thus glucagon may antagonise this association resulting in a shift in the equilibrium favouring the soluble or unbound form. It is interesting to note, in this context, that both fatty acid sythetase and citrate lyase have protomeric

molecular weights of around 400K, the same moleccular weight as that of the ACC protomer suggesting that the components of the complex have a common unit size and that ACC could quite feasably bind to itself in the absence of other ligands. Thus, the polymerization and activation observed in vitro may be secondary effects, the more important effect being the interaction with other enzymes or subcellular structures. The importance of the allosteric effects cannot be completely disregarded as both mechanisms are probably complementary to each other.

In the present investigation it was initially demonstrated that ACC was associated with the microsomal fraction and that this species of ACC could be phosphorylated while a cytosolic species could not. The cytosolic species underwent inactivation during incubation with ATP as the result of a depolymerization phenomenon and not due to phosphorylation and the extent of this inactivation was decreased upon further purification of the enzyme due the removal of endogenous inhibitors. The microsomal form of the enzyme isolated under these conditions is not necesarily associated with the endoplasmic reticulum per se but in some sort of complex large enough to sediment with the microsomal fraction. Finally, it was obseved that the phosphate that was incorporated into the enzyme protein was situated on portions of the enzyme that were proteolytically nicked and thus ran at the dye front on SDS gel electrophoresis.

We have presented further evidence, using an enzyme extract containing both ACCa and ACCb, demonstrating that cAMP-dependent and independent protein kinases phosphorylate ACC *in vitro* at significant rates. Yet the effect of such phosphorylation on enzyme activity is ambiguous. It was also demonstrated that the inactivation obseved in the presence of ATP can be abolished by further purification of the enzyme. It was seen that proteolysis occured during isolation of the enzyme and the cleaved enzyme was preferentially phosphorylated by both cAMP-dependent and independent mechanisms possibly due to local unfolding and the exposure of these sites after proteolytic nicking occured. To determine the significance of phosphorylation in further investigations on ACC *in vitro* it is imperative that a preparation of the enzyme that has a low phosphate content and little or no proteolytic cleavage be used.

We have presented evidence in this investigation showing that ACC is associated in a high molecular weight complex with Fatty acid synthetase and ATP-citrate lyase and that the association between these enzymes is altered upon alteration of the lipogenic status of the animal. Furthermore, incubation of the complex under phosphorylating conditions also altered this association. The high molecular weight filamentous form of the enzyme polymer does not seem to exist *in vivo* and is an artifact of preincubating the enzyme in the presence high

concentrations of citrate. Our observation may explain why numerous investigators have reported conflicting results over observations involving the acute regulation of ACC in vivo. Various investigators have observed decreases in vivo of the activity of ACC upon exposing liver to glucagon and this decrease was observed whether the enzyme was assayed with or witout preincubation with citrate. In contrast activation of the enzyme, after exposure of the tissue to insulin, could only be observed when the enzyme was assayed directly without preincubation as the activity of ACC in the presence or absence of insulin is the same upon preincubation with citrate. This suggests that phosphorylation may alter the allosteric sensitivity of the enzyme in a low lipogenic state but the activation of lipogenesis involves another mechanism that does not alter the total intrinsic activity of the enzyme but decreases the activity of the enzyme if monitored without exogenously added citrate.

We therefore hypothesize that lipogenesis is regulated by glucagon through a covalent modification mechanism that alters the allosteric sensitivity of ACC and modulates the interaction of the various lipogenic enzymes. The exact mechanism through which insulin acts is not known and could be through the dephosphorylation of the sites phosphorylated by glucagon, through the phosphorylation of alternate sites or through both of the above mechanisms. In view of the observation that glucagon affects both the

preincubated and direct activity of ACC while insulin only affects the direct activity, we would hypothesize that insulin seems to exert its effects on ACC by an alternate mechanism than glucagon such as second site phosphorylation.

To confirm this hypothesis it is necessary that the actual phosphorylation of ACC, FAS and CL be monitored to correlate the degree of phosphorylation with the degree of dissociation of the complex using the catalytic subunit of cAMP-dependent protein kinase as it is assumed that the efffects of glucagon are mediated by the classical cAMP cascade. The correlation of the enzymatic activity of the complex (ie the coupled activities of the enzymes) with the degree of physical association of the individual enzymes must also be demonstrated. In these experiments a pure preparations of the enzymes are required that are minimally phosphorylated and have undergone little proteolysis. With respect to ACC, a preparation of pure enzyme has been isolated in this laboratory from porcine adipose tissue that has a very low phosphate content and that has an intact 250K subunit.

The association of the enzymes in this complex must be shown to vary <u>in vivo</u> during hormonal manipulation of the animals or tissue and corespondingly the coupled activity of the complex must be shown to reflect the lipogenic status of the animal. The dissociation induced by phosphorylation must be shown to be reversible, possibly

by employing phosphatases to reverse glucagon (cAMP) mediated phosphorylation or by cAMP-independent phosphorylation. Investigation of the latter mechanism may be more productive and in this context it is interesting to note recent reports that indicate that a membrane bound cAMP-independent kinase, that is activated by insulin, phosphorylates and activates ACC (Brownsey et al, 1981).

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