REGULATION OF ACETYL-CoA CARBOXYLASE

AND

ROLE OF BIOTIN IN CELLULAR FUNCTIONS

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Doctor of Philosophy

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REGULATION OF ACETYL-CoA CARBOXYLASE AND ROLE OF BIOTIN IN CELLULAR FUNCTIONS

BY

RAJINDER P. BHULLAR

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

To my parents

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ABSTRACT

Acetyl-CoA carboxylase (ACC) was isolated from chicken liver to prepare antibodies against ACC. Using these antibodies, the relative synthesis of acetyl-CoA carboxylase was investigated in HeLa cells in culture. Culturing of HeLa cells in lipid-free medium or Waymouth's serum free medium resulted in an increase in the activity and relative synthesis of ACC as compared to cells cultured in medium containig fetal bovine serum. Addition of glucagon to the culture medium caused a decrease in the activity as well as in the relative synthesis of acetyl-CoA carboxylase in HeLa cells cultured in Waymouth's medium. This was reflected in a matching decrease in the rate of fatty acid synthesis.

Acetyl-CoA carboxylase was purified from porcine adipose tissue. Inclusion or ommission of sodium fluoride during the purification scheme had no effect either on the specific activity or the alkali-labile phosphate content of the purified porcine adipose tissue acetyl-CoA carboxylase. Purified catalytic subunit of cAMP-dependent protein kinase was able to phosphorylate and only partially inactivate (20%) porcine adipose tissue acetyl-CoA carboxylase. It is concluded that (i) long-term regulation of acetyl-CoA carboxylase involves changes in the relative synthesis of enzyme protein and (ii) that phosphorylation-dephosphorylation does not play a significant role in the regulation of activity of porcine adipose tissue acetyl-CoA carboxylase.

The non-prosthetic group functions of biotin in the cell were investigated. The effects of biotin-deficiency on protein synthesis and cellular growth were investigated in HeLa cells in culture. Biotin-deficiency caused a decrease in the rate of protein synthesis and cell growth. Addition

of biotin to the culture medium of biotin-deficient cells restored the rate of protein synthesis to the level observed in HeLa cells cultured continuously in the presence of biotin. Appearance of newly synthesized RNA in the cytoplasm was responsible for the observed increase in rate of protein synthesis. Biotin-supplementation of biotin-deficient cells caused an increase in DNA synthesis and cell growth. Continuous protein synthesis was found to be necessary for the increased rate of DNA synthesis. The rate of cell growth in the biotin-supplemented cells matched the rate of growth observed in cells cultured continuously in the presence of biotin. It is concluded that biotin stimulates the synthesis of certain proteins which are necessary for the continuous growth of HeLa cells in culture.

The sub-cellular distribution of biotin in rat liver was studied after an injection of [^{3}H] biotin. 8% of the total radioactivity present in the liver homogenate was associated with the purified nuclei. The biotin in the nuclei was noncovalently bound and could be displaced by unlabelled biotin. The purified nuclei did not contain any biotin-dependent carboxylases like, propionyl-CoA carboxylase or acetyl-CoA carboxylase. A biotin-binding protein was isolated from purified rat liver nuclei. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate provided an apparent molecular weight of 60K dalton. Sucrose density gradient analysis provided an apparent molecular weight of 240K dalton for the biotin-binding protein. The protein binds biotin <u>in vitro</u> in a reversible manner with a maximum binding of 3.54 pmoles of biotin per µg protein and has a dissociation constant for biotin of 2.2×10^{-7}M. It is suggested that the biotin-binding protein isolated from the nucleus might play a role in the expression of effects of biotin in the nucleus.

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ABBREVIATIONS

ACC: Acetyl-CoA carboxylase

ATP: Adenosine-5'-triphosphate

BDFBS: Biotin-deficient fetal bovine serum

cm: Centimetre

-CoA: Coenzyme A

Ci: Curie

^{o}C: Degree Celsius

DNA: Deoxyribonucleic acid

DEAE: N,N-Diethylaminoethyl

DPM: Disintegrations per minute

DTT: Dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

FBS: Fetal bovine serum

xg: Times gravity

gm: Gram

hr: Hour

µl: Microlitre

M: Molar

mM: Millimolar

µM: Micromolar

mg: Milligram

mCi: Millicurie

µCi: Microcurie

µmol: Micromole

min: Minute

M_r: Molecular weight

PBS: Phosphate-buffered saline

PAGE: Polyacrylamide gel electrophoresis

RNA: Ribonucleic acid

hnRNA: Heterogenous RNA

mRNA: Messenger RNA

rpm: Revolutions per min

SDS: Sodium dodecyl sulfate

Tris: Tris(hydroxymethyl)aminomethane

SECTION I

INTRODUCTION

INTRODUCTION

The best understood role of biotin is as the prosthetic group of various carboxylases and hence its obligatory role in lipid metabolism, gluconeogenesis, and certain amino acid metabolism. Acetyl-CoA carboxylase (ACC), the rate limiting enzyme of fatty acid synthesis, is under dietary and hormonal regulation. Short term regulation is believed to occur through phosphorylation-dephosphorylation of the enzyme while long term regulation occurs through changes in the relative synthesis of enzyme. Apart from its role as the prosthetic group of various carboxylases, biotin has been implicated in protein and RNA synthesis. It is present in the cell nucleus where no known biotin-containing enzymes exist. The objective of this thesis was to study the regulation of acetyl-CoA carboxylase through changes in enzyme synthesis and phosphorylation and to investigate the role of biotin in cellular processes where it does not function as the prosthetic group of various carboxylases.

The thesis is divided into eight parts. The first part provides a brief review of literature dealing with the discovery of biotin and its role in metabolism. The second part provides a review of literature on the regulation of lipogenesis with the main emphasis being placed on acetyl-CoA carboxylase.

The experimental results are divided into four sections with a specific 'literature review' and 'discussion' for each section. The first section (part A) deals with the purification of chicken liver acetyl-CoA carboxylase, the production and characterization of antibodies against ACC. Part B of first section describes the regulation of activity and relative synthesis of ACC in HeLa cells by the hormone, glucagon. The second section deals with the purification and physicochemical properties of porcine adipose tissue ACC. Phosphorylation of the enzyme by the catalytic subunit of cAMP-dependent

protein kinase and the corresponding effect on enzyme activity is also described. The third section deals with the effect of biotin-deficiency in HeLa cells on protein synthesis and cell growth. The last experimental section identifies a "Biotin-Binding Protein" from rat liver nuclei that binds biotin *in vitro*.

A general discussion is presented in the last section.

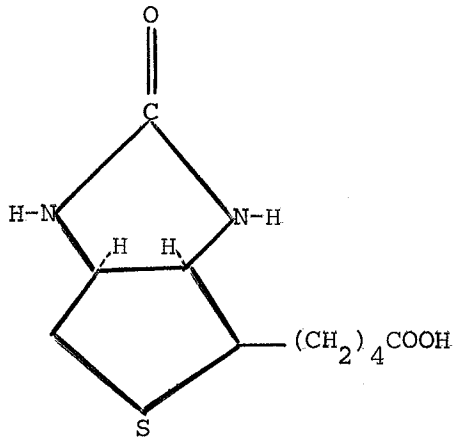
SECTION II

BIOTIN: AN OVERVIEW

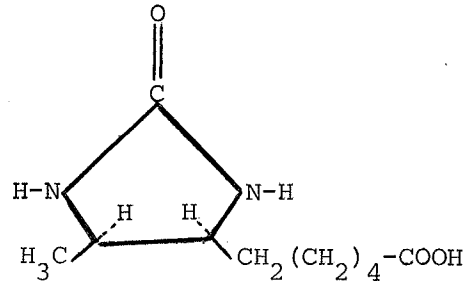
A. Biotin: Discovery, Structure and Related Compounds

The discovery of biotin (vitamin H) and the eventual elucidation of its structure and role in metabolism involved diverse investigations spanning many decades. Kögl and Tönnis (1936) made a major contribution by isolating crystalline biotin from egg yolk. Subsequently Du Vigneaud et al (1942a) determined the chemical structure and Harris et al (1943) chemically synthesized biotin from cysteine and chloroacetic acid. Biotin (Fig. 1a) was shown to be a (+)-cis-hexahydro-2-keto-1,H-thieno-(3,4)-imidazole-4-valeric acid. Only the (+)-stereoisomer of biotin exhibits significant biological activity. The molecular weight of biotin is 244.3 g/mole and it is soluble in water (0.02% w/v), ethanol (0.08% w/v) and dilute alkali.

Various biotin derivatives, analogs and antagonists are also known. Dethiobiotin (Fig. 1b) is a sulphur-free analog of biotin and is believed to be the direct precursor of biotin during biotin biosynthesis in microorganisms (Guillerm et al, 1977). In biocytin (Fig. 1c), the biotin molecule is covalently attached to the ε-amino group of lysine. Biocytin is released upon the enzymatic digestion of biotin containing proteins (Lynen, 1967). The released biocytin is cleaved by the enzyme biotinidase into equimolar amounts of biotin and lysine, thus making the released biotin available for reutilization. Biotinidase has been found in hog liver and kidney, chick pancreas (Thoma and Peterson, 1954;Knappe et al, 1963) and the serum of the human, rat, guinea pig and rabbit (Koivusalo and Pispa, 1963).



(a)



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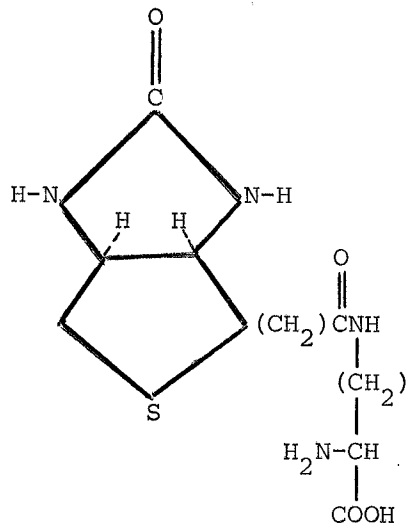
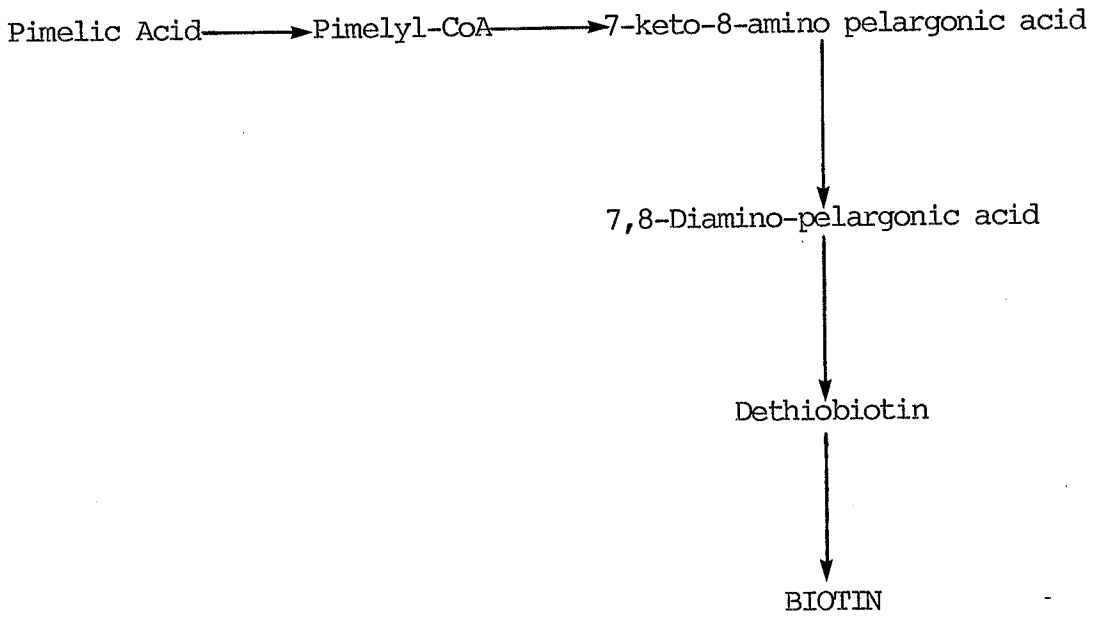


(c)

Fig. 1. Structure of (a) biotin, (b) dethiobiotin and (c) biocytin.

B. Biotin: Biosynthesis and Metabolism

Biotin is produced by many microorganisms including the intestinal bacteria of rats, cattle and man (McElroy and Jukes, 1940;Wegner et al, 1940). The earlier studies in biotin biosynthesis involved the precursor-product relationship between pimelic acid and biotin and between dethiobiotin and biotin (Du Vigneaud et al, 1942b;Eakin and Eakin, 1942;Tatum, 1945). The availability of radioactively labelled pimelic acid, cysteine, CO_2 and biotin mutants of E.Coli K-12 helped investigators in the identification of intermediates in the biosynthetic pathway of biotin (Eisenberg, 1962;Elford and Wright, 1962;Rolfe and Eisenberg, 1968). The sequence of reactions in the biosynthetic pathway of biotin based on the aforementioned studies could be formulated as follows:



The enzymes involved in the above pathway have all been isolated and partially characterized (Eisenberg and Star, 1968;Izumi et al 1972a, 1972b, 1973, 1975;Parry and Kunitani, 1976).

Biotin is degraded by microorganisms (Christner et al, 1964). The degradation of biotin by broken cell preparations requires Mg^{2+}, ATP, CoA and NAD^+ (Brady et al, 1966) to yield urea, NH^{4+}, HCO_3^- and L-cysteine (McCormick and Wright, 1971). Such complete degradation of the biotin molecule does not take place in higher organisms (Mistry and Dakshinamurti, 1964), only the side chain appears to be degraded (Baxter and Quastel, 1953).

C. Biotin: Physiological Role

The best known and understood role of biotin is as the prosthetic group of several biotin containing enzymes. The four major biotin containing enzymes in higher organisms are: propionyl-CoA carboxylase (PCC), pyruvate carboxylase (PC), 3-methylcrotonyl-CoA carboxylase (MCC) and acetyl-CoA carboxylase (ACC). PCC (EC 6.4.1.3) is a mitochondrial enzyme and is involved in the catabolic pathway for isoleucine, threonine, methionine, valine, branched chain and odd carbon fatty acids, cholesterol and thymine (Moss and Lane, 1971). PC (EC 6.4.1.2) is another biotin dependent enzyme localized in the mitochondria. PC occupies a key position in gluconeogenesis as part of the "dicarboxylic acid shuttle" and also influences lipogenesis as acetyl-CoA must combine with oxaloacetate in the mitochondria to form citrate before it can pass into the cytoplasm to enter the lipogenic pathway. MCC (EC 6.4.1.2) is also located in the mitochondria and is involved in the catabolism of leucine. ACC (EC 6.4.1.2) plays an essential role in the biosynthesis of fatty acids and was the first to be recognized as a biotin containing enzyme (Wakil and Gibson, 1960). A more detailed presentation on the role of ACC in fatty acid synthesis and its regulation will be presented in section III of this thesis.

Apart from its role as the prosthetic group of above mentioned enzymes,

biotin appears to be involved in several other areas of metabolism. Numerous reports detail decreased protein synthesis in biotin-deficiency (Ravel et al, 1961;Dakshinamurti and Mistry, 1963b;Dakshinamurti and Litvak, 1970). Studies with biotin-deficient rats have indicated a stimulatory role for biotin in RNA synthesis (Boeckx and Dakshinamurti, 1975), protein synthesis (Boeckx and Dakshinamurti, 1974) and guanylate cyclase activity (Vesley, 1982). A more detailed discussion on the role of biotin in areas other than fatty acid synthesis will be presented in section VI of this thesis.

SECTION III

REGULATION OF ACETYL-CoA CARBOXYLASE

1. Lipogenesis: An Overview

Lipids are an essential component of all living cells, functioning as an energy store and playing an important role in all biologic membranes. The major function of lipogenesis is to store the chemical energy of foodstuffs ingested above the immediate requirements of an organism in a readily available form as triacylglycerols. However, the lipogenic status of an organism must be amenable to regulation to accomodate fluctuations in dietary intake and energy requirements. The lipogenic enzymes are subject to reversible control at the level of protein synthesis. However, since these enzymes generally have half-lives over 24 hr or more, there is clearly a need for acute regulation of enzyme activity as well as the long term regulation of total enzyme protein.

The acute regulation is accomplished by the modification of enzyme activities through various mechanisms such as limiting substrate supply, through allosteric factors, hormones or covalent modification. Liver is the major site of *de novo* fatty acid synthesis although adipose tissue is also very active in triacylglycerol synthesis. The likely candidate for co-ordinating these two tissues are the hormone pair glucagon and insulin acting on the liver and insulin and epinephrine acting on the adipose tissue. The synthesis of fatty acids is stimulated within 15 min by insulin, and inhibited by glucagon or epinephrine. It is hypothesized that glucagon alters the metabolic activity of liver through short-term control mechanisms, with the result that under its influence the organ is essentially active in glycogenolysis, gluconeogenesis and ketogenesis. The net result of this

action of glucagon is to mobilize stored energy sources. Insulin, on the other hand, helps to store excess energy by inhibiting the catabolic pathways mentioned above and stimulating glycogenesis, glycolysis, lipogenesis and triacylglycerol synthesis. These controls ensure that lipids are synthesized in times of plenty (when insulin levels are high) but not during times of stress or starvation (when catelcholamines and/or glucagon are released).

It has been suggested that these two hormones elicit their opposing effects through covalent modification (phosphorylation-dephosphorylation) of enzymes at key points in the metabolic pathways. The key enzymes that are hypothsized to be regulated by phosphorylation-dephosphorylation mechanism are listed in figure 2. Under high levels of insulin, glycogen synthase, phosphofructokinase, pyruvate kinase, pyruvate dehydrogenase, acetyl-CoA carboxylase and β-hydroxy β-methylglutaryl-CoA reductase are thought to be in a dephosphorylated and active state. The exact effects of phosphorylation on acetyl-CoA carboxylase, phosphofructokinase and ATP-citrate lyase have yet to be clarified. The allosteric effectors in this situation are fructose 1,6-biphosphate which activates pyruvate kinase, pyruvate which inhibits pyruvate dehydrogenase kinase, citrate which inhibits phosphofructokinase and activates acetyl-CoA carboxylase and fatty acyl-CoA which inhibits acetyl-CoA carboxylase.

Under the influence of high glucagon the aforementioned enzymes are found in a phosphorylated and inactive form while glycogen phosphorylase, phosphorylase kinase and fructose 1,6-biphosphatase are in a phosphorylated and active form. This results in a decreased flux through anabolic pathways and results in the formation of glucose and ketone bodies. Through their effects, these two hormones thus co-ordinately regulate glycolysis, glycogenesis, lipogenesis and cholesterolgenesis.

LEGEND TO FIGURE 2

ENZYMES:

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: Hydroxymethylglutaryl-CoA reductase

INTERMEDIATES:

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

OA : oxaloacetate

SYMBOLS:

*: enzyme inactivated by phosphorylation

**: enzyme activated by phosphorylation

*?: enzyme phosphorylated but function unclear

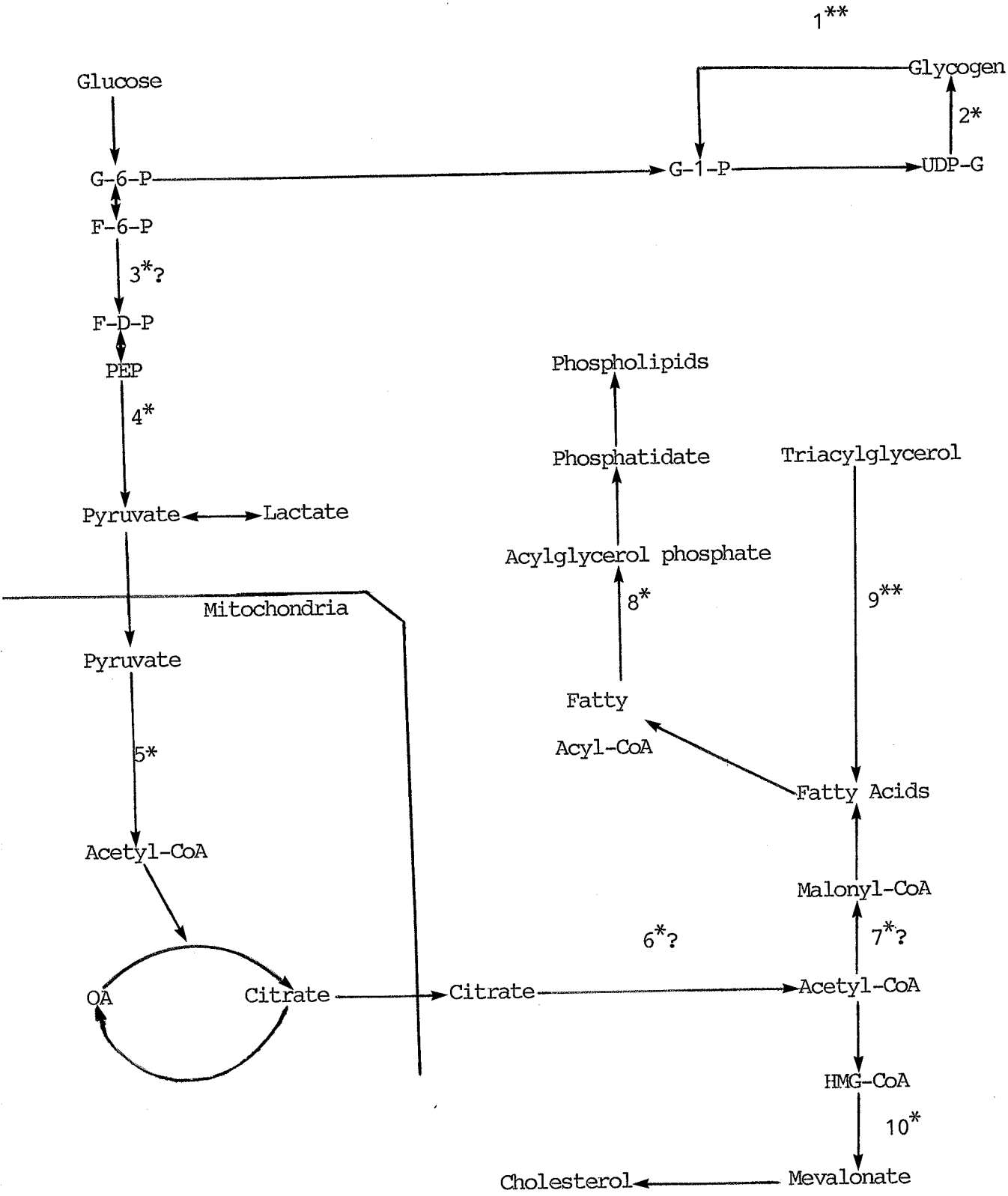


Fig. 2. Summary of pathways in the conversion of carbohydrate into lipid.

Glucagon is believed to bring about its effects through a second messenger in the form of cyclic AMP (cAMP). 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 directly by antagonizing the formation of cAMP, by stimulating phosphodiesterase, acting through an alternative second messenger such as cyclic GMP (cGMP) or by phosphorylating enzymes at sites other than those associated with cAMP mediated phosphorylation. Macaulay et al (1982) and Larner et al (1982) have suggested the presence of a as yet to be characterized second messenger that is formed from cell membranes under the influence of insulin. This mediator specifically inhibits the cAMP-dependent protein kinase, activates cAMP-phosphodiesterase and protein phosphotases (Kono, 1983). Saltiel et al (1983) have presented evidence which indicates that insulin causes the release of small molecular weight substances from rat liver plasma membrane and these substances stimulate acetyl-CoA carboxylase activity.

Long-term regulation of fatty acid synthesis is achieved through changes in the quantity of fatty acid synthesizing enzymes. The rate of fatty acid synthesis fluctuates under a variety of metabolic conditions. These conditions include dietary, hormonal, developmental and genetic alteration. Generally the enzymes in liver and adipose tissue are subject to these adaptive changes. For instance, animals fed a normal diet have a high rate of fatty acid synthesis but it is decreased in animals that have been fasted. Fatty acid synthesis is decreased in the liver of alloxan diabetic rats, while it is increased in the liver of genetically obese mice. These effects on fatty acid synthesis have been correlated with the activity and relative rate of synthesis of lipogenic enzymes.

2. De Novo Synthesis of Saturated Fatty Acids

De novo synthesis of palmitate, the major fatty acid produced in most organisms involves the following steps of reactions:

$$\text{Acetyl-CoA} + \text{HCO}_3^- \xrightarrow{Mg^{2+}} \text{Malonyl-CoA} + \text{ADP} + P_i \qquad (1)$$

$$1\ \text{Acetyl-CoA} + 7\ \text{Malonyl-CoA} + 14\ \text{NADPH} + 14\ H^+ + H_2O \xrightarrow{\text{FAS}} \text{Palmitic Acid} + 7\ CO_2 + 8\ \text{CoASH} + 14\ \text{NADP}^+ \qquad (2)$$

The two enzymes involved in the above reactions are acetyl-CoA carboxylase (ACC;rxn 1) and fatty acid synthetase (FAS;rxn 2). ACC catalyzes the first committed step and is considered to be the rate-limiting step in the biogenesis of long chain fatty acids from acetyl-CoA (Volpe and Vagelos, 1976;Bloch and Vance, 1977). The carbon source for fatty acid synthesis is generally considered to be the mitochondrial citrate which is translocated into the cytoplasm by the citrate-malate shuttle system where it is then cleaved into acetyl-CoA and oxaloacetate by ATP-citrate lyase (Geelen et al, 1980). However, recent 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 that mitochondrial acetoacetate may serve as an alternative source of acetyl-CoA for lipogenesis.

3. Acetyl-CoA Carboxylase:

Acetyl-CoA carboxylase (ACC) catalyzes the ATP-dependent carboxylation of acetyl-CoA in the formation of malonyl-CoA:

$$CH_3CO\text{-}SCoA + HCO_3^- + ATP \xleftrightarrow{Mg^{2+}} HOOCH_2CO\text{-}SCoA + ADP + P_i$$

(Acetyl-COA) (Malonyl-CoA)

Homogenous ACC has been purified from 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, 1978b), chicken liver (Beaty and Lane, 1982) and rat adipose tissue (Ramakrishna and Benjamin, 1983). All sources yield an enzyme preparation with a protomeric molecular weight between 400K and 500K daltons, containing two identical subunits of a molecular weight between 220K and 260K daltons. The enzyme contains 1 mole of biotin per mole of subunit as the prosthetic group. The pure enzyme has been shown to exist in an equilibrium between the inactive protomeric form and a high molecular weight (4 to 10 million daltons) active polymeric form (Moss and Lane, 1972). The protomer-polymeric transition of animal ACC is under the influence of various conditions (Volpe and Vagelos, 1976):

PROTOMER (Inactive) ⟷ POLYMER (Active)

Favored by:	Favored by:
$ATP.Mg^{2+}+HCO_3^-$	Citrate, Isocitrate
Malonyl-CoA	Phosphate
Fatty acyl-CoA	Albumin
Alkaline pH	pH 6.5-7.0
NaCl (>0.2 M)	High enzyme concn.
Low enzyme concn.	

Fig. 3. Protomer-polymer transition of mammalian ACC.

The enzyme is subject to allosteric activation by citrate which is accompanied by polymerization of the inactive protomer into the active polymeric form along with conformational changes in the enzyme (Gregolin et al, 1966). The maximal velocity of the enzyme catalyzed reaction is increased in the presence of citrate with little effect on the K_m value for the substrates (Numa et al, 1964;Ryder et al, 1967). The physiological significance of citrate activation of the enzyme has been highly debated as over 70% of citrate in vivo is present in the mitochondria (Capuzzi et al, 1974) and estimates of the cytoplasmic concentration are between 0.1 mM and 0.2 mM (Greenbaum et al, 1971) while the K_a for citrate is between 2 mM and 6 mM, an order of magnitude higher than the in vivo concentrations (Volpe and Vagelos, 1976). In spite of some of these uncertainties, the properties of citrate activation of ACC as studied by Lane et al (1974) leave little doubt as to citrate being a positive allosteric effector of ACC activity (Kim, 1983).

Long-chain fatty acyl-CoA thioesters, in μM concentrations allosterically inhibit and depolymerize mammalian ACC (Bortz and Lynen, 1963a). This inhibition is competitive with respect to citrate and noncompetitive with respect to acetyl-CoA, HCO_3^- and ATP. The inhibition is reversed by (+)-palmitylcarnitine which acts as a structural analogue and displaces palmityl-CoA (Volpe and Vagelos, 1976). The concentration of long-chain fatty acyl-CoA derivatives ranges between 15 μM and 140 μM in mammalian liver (Bortz and Lynen, 1963b) and as the apparent K_i of palmityl-CoA for ACC is around 0.8 μM to 1.1 μM, inhibition by these derivatives could be significant in vivo (Nishikori et al, 1973). Furthermore, rat liver ACC has been shown to bind reversibly palmityl-CoA in a 1:1 molar ratio with a K_i of as low as 5.5 nM (Ogiwara et al, 1978) thus emphasizing the significance of feedback inhibition of ACC in vivo by the long-chain fatty acyl-CoA derivatives.

4. Fatty Acid Synthetase:

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

$$CH_3CO\text{-}SCoA + 7\ HOOCCH_2CO\text{-}SCoA + 14\ NADPH + 14\ H^+ \longrightarrow CH_3CH_2(CH_2CH_2)_6CH_2COOH + 7\ CO_2 + 14\ NADP^+ + 8\ CoASH + 6\ H_2O$$

FAS has been purified to homogeneity from pigeon liver (Hsu et al, 1965), rat liver (Burton et al, 1968), avian liver (Hsu and Yan, 1970), human liver (Roncari, 1974), rabbit mammary gland (Hardie and Cohen, 1978b) and rat adipose tissue (Ramakrishna and Benjamin, 1983). The molecular weight of the enzyme ranges from 450K to 550K daltons with one mole of 4'-phosphopantetheine per mole of enzyme (Burton et al, 1968). The enzyme readily dissociates into subunits of approximately 250K daltons which exhibit no enzyme activity (Stoops et al, 1979). Phosphorylated sugars, especially fructose 1,6-diphosphate activate FAS 3- to 5-fold by decreasing the K_m for NADPH, thus co-ordinating glucose metabolism and lipogenesis (Wakil et al, 1966). Palmityl-CoA inhibits rat liver FAS activity in vitro (Tubbs and Garland, 1963). As this inhibition in vitro is irreversible, the in vivo significance of this phenomenon has been questioned as it may be due to the detergent action of the thioester (Taketa and Pogell, 1966).

FAS also undergoes long-term alterations as the rate of degradation of the enzyme in starved rats was 4-fold greater and the rate of synthesis 6-fold less than in the refed state (Volpe et al, 1973). Glucagon also causes a decrease in the rate of synthesis of FAS in rat liver (Volpe and Marasa, 1975).

5. Hormonal Regulation of Acetyl-CoA Carboxylase:

The suggestion that ACC catalyzes the rate limiting step in fatty acid synthesis comes from several studies with the hormones glucagon and insulin. It has been reported that glucagon inhibits the incorporation of ^3H_2O 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 fatty acid synthesis is antagonized by insulin (Beynen et al, 1979). In response to glucagon the level of malonyl-CoA is rapidly decreased (Cook et al, 1977) while insulin elevates it (Beynen et al, 1979). 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). As lactate or pyruvate only partially relieve the glucagon induced inhibition of fatty acid synthesis, it has been suggested that the effect of glucagon is on the lipogenic pathway per se and not on glycolysis (Watkins et al, 1977).

It was initially observed that pure ACC contains covalently bound phosphate (Inoue and Lowenstein, 1972). It has been suggested that in adipose tissue, insulin and epinephrine activate and inactivate ACC respectively. This is accompanied by decrease or increase in the phosphate content of the enzyme (Brownsey et al, 1979;Kim, 1979). It has been shown that crude preparationss of ACC can be phosphorylated by both cAMP-dependent and -independent protein 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 phosphatases resulting in activation of the

enzyme (Hardie and Guy, 1980;Shiao et al, 1981;Wada and Tanabe, 1983). However, other reports dispute the suggestion of phosphorylation-dephosphorylation as the primary regulatory mechanism for ACC (Halestrap and Denton, 1974;Desjardins and Dakshinamurti, 1978;Pekala et al, 1978;Gillevet and Dakshinamurti, 1983).

Besides playing a role in the acute regulation of ACC activity, hormones also influence the long-term regulation of ACC by affecting the amount of total enzyme protein. For instance, insulin causes a 2-fold increase in ACC activity and this increase is abolished in the presence of cycloheximide suggesting that the observed change in activity is due to the increased rate of ACC synthesis (Geelen and Gibson, 1975). L-thyroxine causes an increase in ACC activity in the liver and a decrease in adipose tissue of rats (Roncari and Murthy, 1975). This increase or decrase in ACC activty in the presence of hormone(s) has been associated with similar changes in total enzyme protein.

SECTION IV

A. PURIFICATION OF CHICKEN LIVER ACC

AND

PRODUCTION OF ANTIBODY AGAINST CHICKEN LIVER ACC

PURIFICATION OF CHICKEN LIVER ACETYL-CoA CARBOXYLASE

INTRODUCTION

To investigate the relative synthesis of a protein, it is necessary to have a simple and rapid method for quantitating the protein in question. This is usually accomplished with the use of a specific antibody that will precipitate the protein. We, therefore, set out to purify ACC from a readily available source and prepare the antibody to the pure protein.

Various procedures have been employed to purify ACC from different tissue sources. Most of these earlier procedures involved ammonium sulfate precipitation and ion-exchange chromatography. Gregolin et al (1968) purified ACC from chicken liver and suggested that it consisted of three nonidentical subunits with molecular weights of 139K, 129K and 117K daltons in a ratio of 1:1:2 respectively (Guchhait et al, 1974). In recent years, ACC has been purified in the presence of protease inhibitors and these studies have shown that ACC is susceptible to proteolytic degradation during purification. It has now been established that the intact subunit of chicken liver ACC has a molecular weight of 230K daltons with proteolytic degradation producing the smaller molecular weight fragments (Mackall and Lane, 1977). Similarily, the rat liver enzyme has been shown to consist of a native 230K dalton subunit that is cleaved into 124K and 118K dalton fragments (Tanabe et al, 1975). Recently, using monomeric 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 260K daltons (Song and Kim, 1981).

We used chicken liver as a source for purifying ACC as this tissue was

easily obtained and contained high levels of ACC. Antibodies to the pure enzyme was produced by inoculating rabbits with pure protein and collecting the antisera produced. The antibodies produced were used to study the effect of glucagon on acetyl-CoA carboxylase activity and its relative synthesis.

EXPERIMENTAL

(a) Materials

Polyethylene glycol (M_r 6000) was purchased from Fisher Scientific Co. (Fairlawn, N.J.). Bio-Rad protein dye reagent, DEAE-cellulose and electrophoresis reagents were obtained from Bio-Rad Laboratories (Mississauga, Ontario). $NaH^{14}CO_3$ (S.A. 40-60 mCi/mmol) and Aquasol-2 scintillation cocktail were purchased from New England Corp. (Montreal, Canada). Acetyl-CoA, ATP and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, Mo.). High molecular weight protein marker kit was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). NCS tissue solubilizer and OCS scintillation cocktail were purchased from Amersham-Searle Corp. (Oakville, Ontario). Freund's complete adjuvant was purchased from Calbiochem-Behring Corp. (La Jolla, Ca.). All other chemicals used were reagent grade.

(b) Acetyl-CoA Carboxylase Assay

Enzyme activity was determined by the [^{14}C]-bicarbonate fixation assay of Dakshinamurti and Desjardins (1969). The enzyme activity was determined either immediately or after preincubation at 37°C for 30 min to fully activate ACC. The preincubation medium contained: Tris-HCl, 40 mM; glutathione reduced, 3 mM; bovine serum albumin, 0.6 mg/ml; potassium citrate, 10 mM; $MgCl_2$, 10 mM. The assay mixture in a final volume of 500 µl contained: Tris-HCl (pH 7.5), 40 mM; glutathione reduced, 3 mM; magnesium chloride 10 mM; potassium citrate, 10 mM; bovine serum albumin, 0.6 mg; ATP, 4 mM; acetyl-CoA, 0.1 mM; $KH^{14}CO_3$, 10 mM (S.A. 1000 cpm/µmole). The reaction was started in a fumehood by the addition of enzyme and carried out at 37°C for 3 min. The

reaction was terminated by the addition of 100 µl 6N HCl. After centrifugation, 200 µl of supernatant was transferred to a scintillation vial and dried under a fumehood. 0.5 ml of H_2O followed by 10 ml of Aquasol-2 scintillant was added and radioactivity determined in a Beckman LS 280 liquid scintillation spectrometer.

One unit of enzyme activity is defined as that amount which catalyzes the carboxylation of 1 µmole of acetyl-CoA per min at 37°C into acid stable product.

(c) Protein Determination

Protein was determined by the dye binding method using the reagent supplied by Bio-Rad (Bradford, 1976; Biorad Technical Bulletin, 1977). Bovine serum albumin was used to construct the standard curve.

(d) Polyacrylamide Gel Electrophoresis in the presence of SDS (SDS-PAGE)

Polyacrylamide slab gels were run using the discontinous system of Laemmli (1970) employing 5% running and 3% stacking acrylamide gels. The gels were run for approximately 5 hrs at a current of 25 mA per slab gel. The gels were stained overnight in a solution of 0.25% Coomassie blue G, 50% ethanol and 10% acetic acid. The gels were destained in a solution containing 50% methanol and 7.5% acetic acid.

The molecular weight of the proteins on SDS-PAGE was determined by comparing the mobility of unknown protein with that of proteins with known molecular weight.

(e) PURIFICATION OF CHICKEN LIVER ACC

(i) Homogenization and Ammonium Sulfate precipitation:

200gm batches of fresh chicken liver were homogenized using a Waring blender in 300 ml of 10 mM phosphate-buffer (pH 7.5) containing 0.1 mM EDTA, 5 mM β-mercaptoethanol and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 10,000 x g for 90 min. The supernatant was collected by filtering through two layers of cheesecloth. The filtrate was made 25% in $(NH_2)SO_4$ by the slow addition of solid ammonium sulfate. The pH of the solution was kept between 7.3-7.4 by the addition of 5M KOH. The solution was allowed to stir gently for 30 min at 4°C and the precipitated protein collected by centrifuging at 7,000 rpm for 30 min. The collected precipitate was resolubilized in homogenization buffer and dialyzed overnight against the same buffer. Insoluble material from the dialyzed sample was removed by centrifugation and the supernatant collected.

(ii) Polyethylene glycol precipitation:

The supernatant obtained in step (i) was made 5% in polyethylene glycol (PEG) by the slow addition of a stock 50% PEG solution and allowed to stand at room temperature for 30 min. The precipitated protein was collected by centrifugation and resolubilized in the homogenization buffer. The supernatant was brought to 5% in PEG by the slow addition of a stock 50% PEG solution and allowed to stand at room temperature for 30 min. The precipitated protein was collected by centrifugation and the precipitate resolubilized in a minimal volume of homogenization buffer which now also contained 30% glycerol.

(iii) DEAE-cellulose chromatography:

After removing insoluble material by centrifugation the supernatant obtained from step (ii) was loaded onto a DEAE-cellulose column (5 cm x 20 cm) which had been previously equilibrated with homogenization buffer containing 30% glycerol. The column was washed with the buffer until A_{280} reached zero. The enzyme was then eluted off the column using a linear gradient of 10 mM-300 mM phosphate-buffer (pH 7.5) containing 0.1 mM EDTA, 5 mM β-mercaptoethanol, 0.2 mM PMSF and 30% glycerol. The effluent was collected in 10 ml fractions. ACC activity, conductance and A_{280} was determined in each fraction. Fractions containing enzyme activity were pooled.

(iv) Final PEG precipitation:

Pooled material from the DEAE-cellulose column was made 5% in PEG as previously mentioned. This was allowed to stand at room temperature for 1 hr and the precipitated protein collected by centrifugation. The precipitate was resolubilized in a minimal volume of 10 mM phosphate-buffer (pH 7.5) containing 0.1 mM EDTA, 5 mM β-mercaptoethanol, 25 mM potassium citrate and 30% glycerol and stored at -20°C as the final pure enzyme preparation.

(f) Preparation of antibody to chicken liver ACC:

Anti chicken liver acetyl-CoA carboxylase serum was prepared essentially according to the procedure of Marshall and Cohen (1961) as modified by Nakanishi and Numa (1970). 1 mg of pure ACC protein was emulsified in an equal volume of Freund's complete adjuvant and rabbits were injected intradermally along their sides with this emulsion. After a three week

interval another 1 mg of pure enzyme was injected intravenously. Two weeks later a final injection of 1 mg of pure enzyme was given intravenously and blood collected 8 days later by incision of the ear vein. The immunoglobulin G fraction of the antiserum was prepared from collected serum by ammonium sulfate fractionation as described by Marshall and Lane (1977).

The antibody titre was monitered as outlined by Majerus and Kilburn (1969). Aliquotes of purified chicken liver ACC were preincubated with appropriate volumes of anti-ACC or control sera for 30 min at 37°C in the presence of 20 mM citrate. At the end of this incubation period an aliquot of the above was assayed for ACC activity.

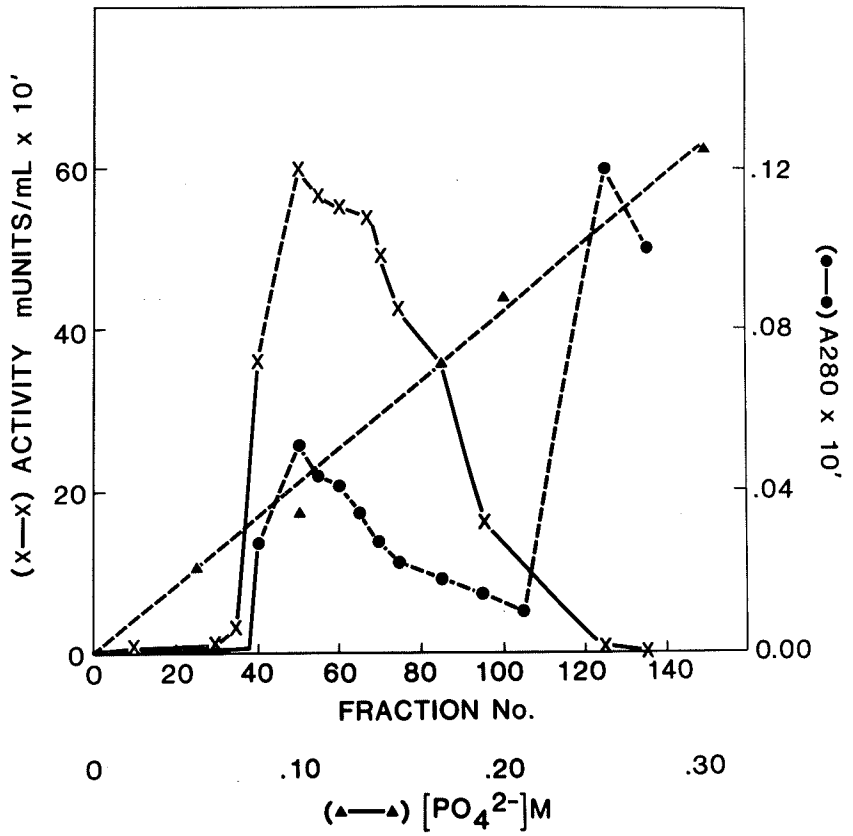
To investigate the relative synthesis of ACC, one must be able to specifically precipitate the enzyme from the cytosol. Acetyl-CoA carboxylase was immunoprecipitated by the method of Harrington et al (1971). The sample containing enzyme was incubated for 30 min at 37°C in the presence of 0.15 M NaCl with anti-ACC gamma globulin or control gamma globulin. Goat anti-rabbit gamma globulin was then added to precipitate the antibody-enzyme complex by incubating at 37°C for 15 min in the presence of 1% PEG (M_r 6,000) followed by 15 min at 4°C at which time the precipitation of the immune complex was complete. This immune complex was then centrifuged through a sucrose gradient (0.5 M-1.0 M, containing 1.0% Triton X-100 and 1% sodium deoxycholate) according to the procedure of Taylor and Schimke (1974). The precipitate was washed three times with ice cold 0.15 M NaCl containing 1% PEG. The final precipitate was then analyzed by SDS-PAGE. When radioactively labelled proteins were used as a source for immunoprecipitation, radioactivity associated with the immune complex was determined by solubilizing the final pellet in 0.9 ml of NCS at 56°C. To the solubilized pellet 40 µl of glacial acetic acid followed by 10 ml of OCS scintillant was added and radioactivity determined in a Beckman LS 2800 liquid scintillation spectrometer.

RESULTS

Acetyl-CoA carboxylase from chicken liver was purified using a combination of ammonium sulfate-polyethylene glycol precipitation and ion-exchange chromatography. During DEAE-cellulose chromatography the carboxylase appeared in the eluate when the phosphate-buffer concentration reached approximately 0.075 M (Fig. 4). The yield of pure ACC protein from the method used here was approximately 10 mg/kg of tissue with a final specific activity of 9.4 units/mg enzyme protein (Table 1). This represented a 470-fold purification of ACC from the starting material. When the final preparation of ACC was analyzed by SDS-PAGE, three major protein staining bands were observed corresponding to the 230K dalton native subunit of the enzyme and proteolytic cleavage products of 129K and 117K dalton (Fig. 5).

ACC purified above, when injected into rabbits produced sera that were able to inactivate chicken liver ACC activity. The final titre of the isolated gamma globulin fraction of anti-ACC serum as determined by extrapolating the intial slope was found to be 2.6 munits of chicken liver ACC inactivated by one µl of the isolated gamma globulin fraction (Fig. 6). The antibody prepared against chicken liver ACC was also able to inactivate rat liver ACC, porcine adipose tissue ACC and HeLa cell ACC. This indicates that the antibody prepared against chicken liver ACC has the ability to cross react with ACC from other sources. When SDS gel electrophoresis was performed on the immunoprecipitate prepared using rat liver cytosol, protein band of 250K dalton corresponding to native rat liver ACC was observed in addition to the proteins present in the antiserum (Fig. 7). This indicated that the antibodies prepared were able to specifically precipitate ACC from the cytosol. When ^{14}C-pantethenic acid labelled rat liver cytosol was used for

immunoprecipitation, no radioactivity was recovered in the pellet indicating that the antobodies raised against chicken liver ACC did not cross-react with the fatty acid synthetase complex.



DEAE-CELLULOSE CHROMATOGRAPHY

Fig. 4. The resolubilized protein after the 2nd PEG precipitation was loaded onto a DEAE-cellulose column. Fractions were collected and assayed for absorbance at 280 nm (●——●) and ACC activity (X——X).

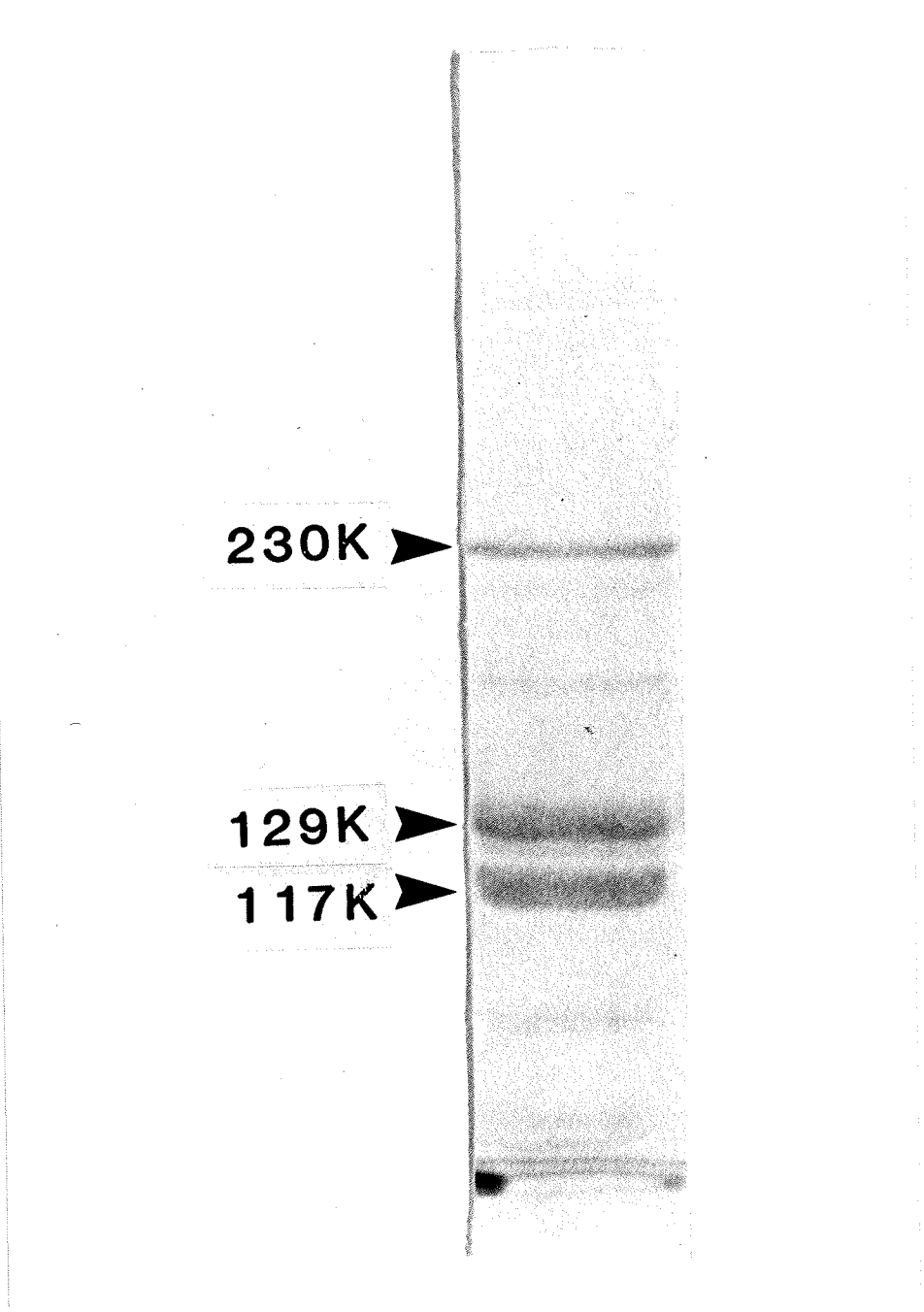
TABLE 1

PURIFICATION OF CHICKEN LIVER ACETYL-CoA CARBOXYLASE

STEP	TOTAL ACTIVITY (UNITS)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (Units/mg)
Homogenate[a]	2,334	105,800	0.02
25% $(NH_4)_2SO_4$ fraction	867	14,950	0.06
5% PEG pptn.	639	1580	0.41
5% PEG pptn.	548	220	2.52
DEAE-cellulose pool[b]	350	74	4.72
5% PEG pptn.	170	18	9.43

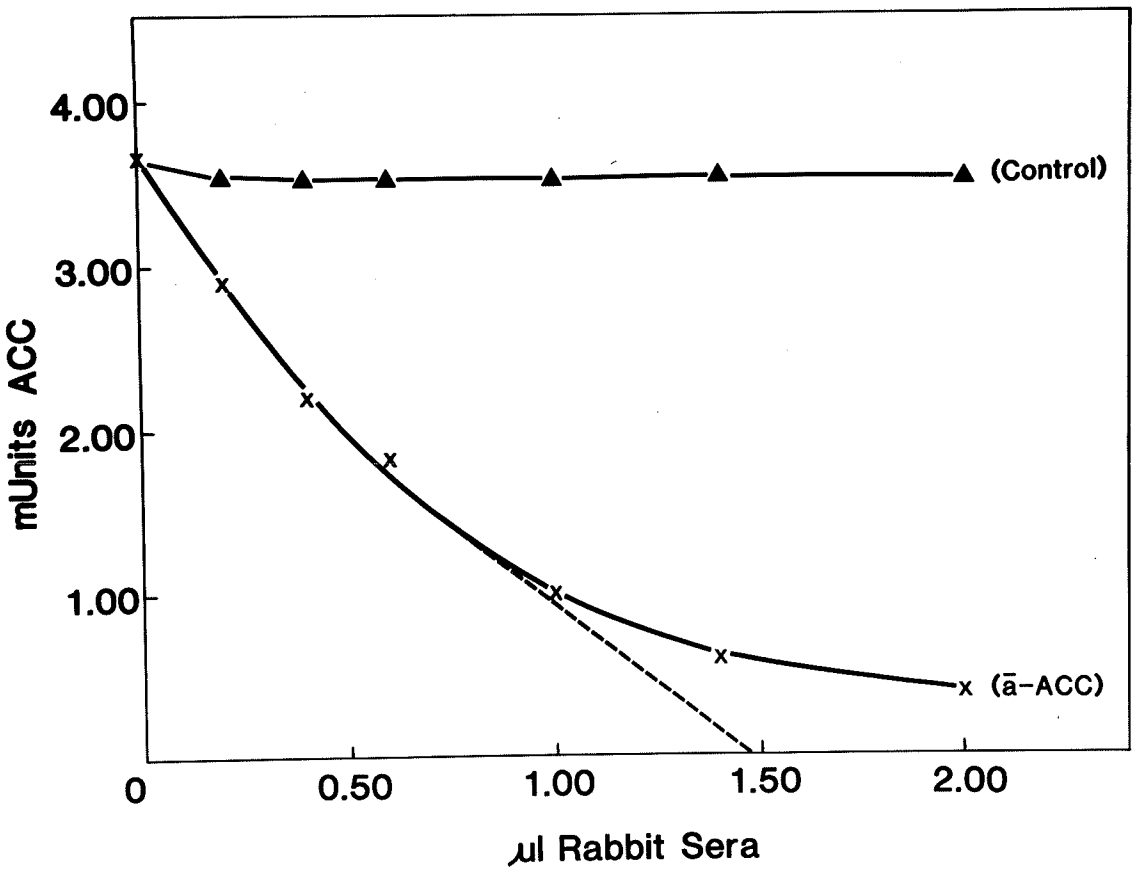
a: from 1,700gm of chicken liver.

b: fractions 35-95 pooled.



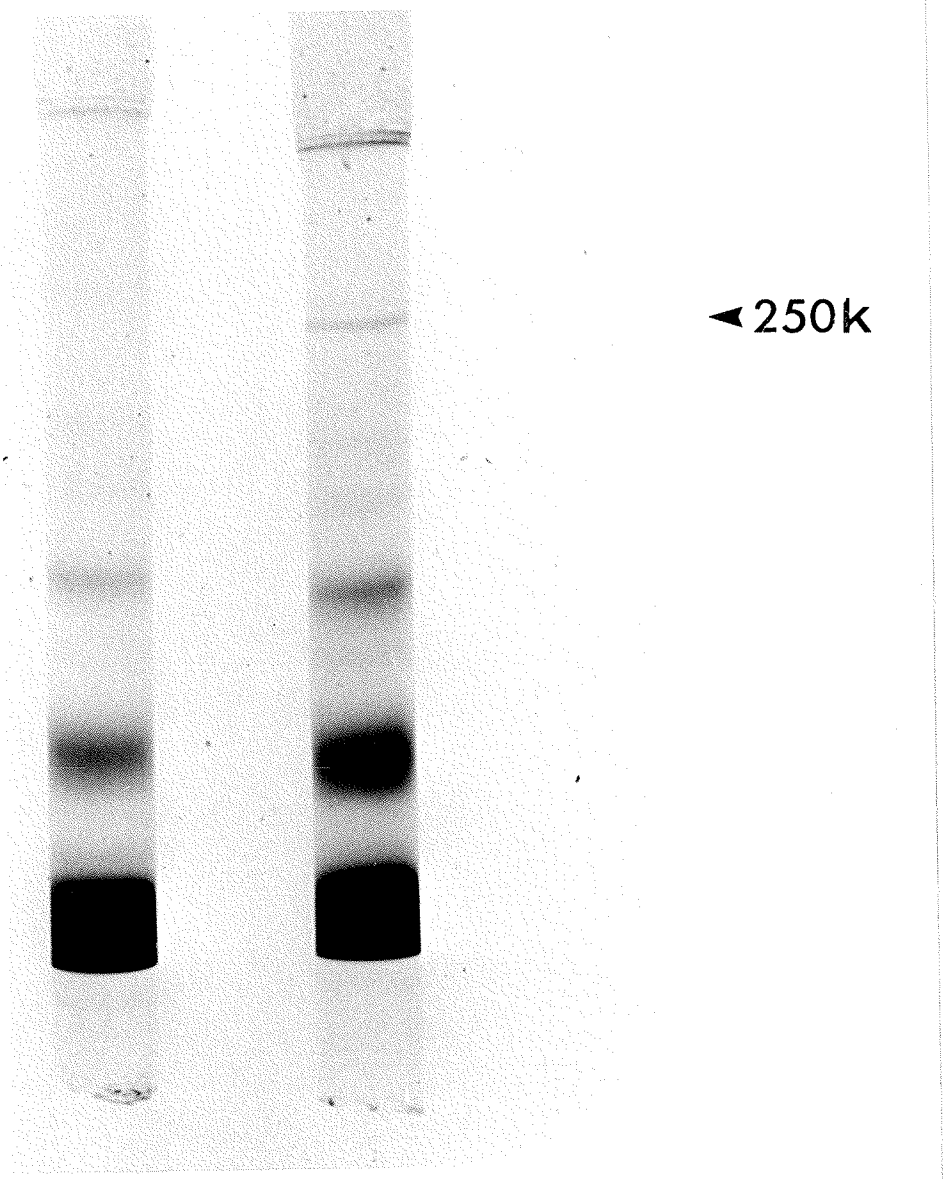
SDS-PAGE OF PURIFIED CHICKEN LIVER ACC

Fig. 5. Purified chicken liver ACC (7 μg) was denatured by heating at 100°C for 2 min in the presence of 1% SDS and 5% β-mercaptoethanol and analyzed on SDS-PAGE as described in procedures section.



IMMUNOTITRATION OF CHICKEN LIVER ACC

Fig. 6. Increasing amounts of anti-ACC serum (X—X) or control serum (▲—▲) was incubated with constant amount of chicken liver ACC for 30 min at 37°C and then assayed for ACC activity.



SDS-PAGE OF THE IMMUNOPRECIPITATE FROM RAT LIVER CYTOSOL

Fig. 7. Immunoprecipitates obtained by incubating the rat liver cytosol with either control (gel A) or anti-ACC (gel B) serum were denatured at 100°C for 2 min in the presence of 1% SDS and 5% β-mercaptoethanol and subjected to SDS gel electrophoresis.

DISCUSSION

ACC from chicken liver was prepared using polyethylene glycol as it permits rapid purification and thus avoids long delays in obtaining the pure enzyme. Although, chicken liver ACC was purified in the presence of the protease inhibitor, PMSF, proteolytic degradation of the enzyme had occured as indicated by SDS-PAGE analysis resulting in a preparation that had a relatively high specific activity. However, the results are in accordance with other reports in literature in terms of proteolytic degradation and high specific activity (Gregolin et al, 1968;Nakanishi and Numa, 1970;Inoue and Lowenstein, 1972;Mackall and Lane, 1977). The higher specific activity of these preparations is due partially to activation of ACC by proteolytic degradation (Guy and Hardie, 1980). In recent years monomeric avidin-Sepharose affinity chromatography has been employed to purify ACC quickly but even these preparations show slight amount of proteolytic degradation (Witters and Vogt, 1981). Therefore, the antibody was produced against chicken liver ACC that had undergone partial proteolysis during preparation. However, the antibody produced was able to inactivate ACC from various sources and could precipitate specifically the native subunit of ACC from rat liver cytosol. Lee and Kim (1977) prepared antibody to the 125K dalton fragment of rat liver ACC and used this antibody to specifically precipitate the native subunit of ACC. Thus, the use of proteolytically degraded enzyme was satisfactory in preparing antiserum.

The antibody prepared was used to investigate changes in the relative of synthesis of ACC under the influence of the hormone, glucagon.

SECTION IV

B. REGULATION OF THE RELATIVE SYNTHESIS OF ACC

INTRODUCTION

Long-term regulation of a protein involves changes in the relative rate of synthesis and/or degradation. The need for long-term regulation of the content of a particular protein may be due to changes in the nutritional status of an animal, due to genetic alterations and/or due to alterations in the hormonal status of the animal. One of the proteins that undergoes such changes in its total content is the enzyme acetyl-CoA carboxylase. Majerus and Kilburn (1969) showed that the rate of synthesis of rat liver acetyl-CoA carboxylase was stimulated 5- to 10-fold when previously fasted rats were fed a fat-free diet. Nakanishi and Numa (1970) provided evidence that the relative rate of synthesis of acetyl-CoA carboxylase in rat liver was decreased 1.9-fold and 1.7-fold by fasting and diabetes respectively, while the rate of degradation was found to be essentially the same in normal and diabetic rats but was accelerated in fasted rats. Analogous studies revealed that the elevated content of ACC in genetically obese mice can be attributed principally to changes in the rate of synthesis of the enzyme (Nakanishi and Numa, 1971). Studies carried out with cultured hepatocytes (JTC-25 P3) indicated that the addition of fatty acids to the culture medium caused a decrease in the rate of synthesis of ACC and had no effect on its rate of degradation (Kitajima et al, 1975). Acetyl-CoA carboxylase was stimulated 2-fold when rat hepatocyte cultures were exposed to insulin (0.1 μM) and this insulin-dependent induction of ACC activity was prevented by the addition of α-aminitin or cordycepin suggesting a transcriptional regulation of the enzyme level (Katz and Ick, 1981;Giffhorn and Katz, 1984). Studies carried out with rat liver pyruvate dehydrogenase and pyruvate carboxylase suggest that the changes in enzyme content under the influence of thyroid hormone reflected

alterations in the rate of synthesis of the enzymes with no effect on the degradation rates of the enzymes (Weinberg and Utter, 1979). The regulation of malic enzyme of chick liver cells in culture under the influence of insulin, triiodothyronine and glucagon was also correlated to alterations in the relative rate of synthesis of the enzyme while the rate of degradation remined unaffected (Goodridge and Adelman, 1976). Thus, it would appear that hormones tend to affect the relative rate of synthesis of an enzyme rather than its rate of degradation.

Although extensive work has been done on the long-term regulation of ACC in vivo under different dietary and genetic manipulations, little work has been carried out to investigate the effect of hormones on the long-term regulation of ACC. It is difficult to unravel the precise in vivo mechanism which might bring about a change in the content of ACC under the influence of a particular hormone. The effects observed could be secondary to further changes in metabolism produced by the hormone being studied. Therefore, attempts have been made to set up model systems to study the long-term regulation of ACC by hormones in cell culture. Studies performed using human fibroblasts in culture suggested that insulin causes an approximately 2-fold increase in ACC activity by 2 days and that this observed increase in activity was abolished in the presence of cycloheximide, suggesting that new ACC synthesis was responsible for the observed increase in ACC activity in the presence of insulin (Shafrir and Bierman, 1981).

We, using HeLa cells, have investigated the long-term effect of glucagon on the activity and relative synthesis of ACC as well as the rate of fatty acid synthesis.

(a) Materials

Eagle's Minimal Essential Medium (EMEM), fetal bovine serum (FBS), penicillin G, streptomycin, fungizone, trypsin solution (2.5%), Earle's Balanced salt solution and Waymouth's Medium (MD 705/1) were purchased from Grand Island Biological Co. (St. Louis, Mo.). L-[4,5-^{3}H] leucine (S.A. 62 Ci/mmol) was obtained from Amersham-Searle Corp. (Oakville, Ontario). [^{3}H] H_2O (S.A. 19 μCi/mmol) was purchased from New England Nuclear Corp. (Montreal, Quebec). Millipore filters (0.22 μm) were obtained from Millipore Ltd. (Mississauga, Ontario). The source of all other reagents used has been previously mentioned (section IV A).

(b) Preparation of Lipid-free Fetal Bovine Serum (LF)

Lipid-free fetal bovine serum was prepared essentially according to the procedure of Horwitz et al (1974). 55 ml of FBS was added dropwise with stirring to a chilled (-20°C) mixture of ethanol, diethyl ether and water (900:300:7, v/v). This was allowed to extract with stirring for 2 hr at -20°C. The suspension was centrifuged at -20°C (600 x g, 10 min) and the pellet reextracted with stirring for 4 hr at -20°C in a mixture of ethanol, ether and water (900:300:62, v/v). The suspension was then centrifuged at -20°C (600 x g, 10 min) and the pellets washed twice with chilled ether (-20°C). After these washings the pellet was resuspended in 1.0 litre of chilled ether and extracted overnight with stirring at -20°C. The suspension was centrifuged (600 x g, 10 min) and the pellets washed twice in chilled

ether. The final pellet was dried under vacuum and weighed. The dried material was dissolved by gentle stirring in Earle's balanced salt solution at a final concentration of 53.5 mg/ml. The final solution was sterilized by passing it through a 0.22 μm Millipore filter.

(c) Culturing of HeLa Cells

HeLa 229 cells (Nelson-Rees and Flandmeyer, 1977) were cultured as a monolayer in fetal bovine serum (FBS), lipid-free FBS (LF) and Waymouth's medium (WD) as follows:

(i) Culturing in FBS

HeLa cells were routinely cultured as a monolayer in EMEM supplemented with 5% FBS, 100 units/ml penicillin G, 100 μg/ml streptomycin and 2.5 μg/ml fungizone. To subculture, the cells were removed from culture dish by the addition of 2.0 ml of trypsin (0.25%) solution and split into three culture plates which contained fresh culture medium.

(ii) Culturing in LF and WD

HeLa 229 cells cultured in the presence of 5% FBS were subcultured as described above. The subcultured cells were allowed to remain in EMEM containing 5% FBS for 6 hr so that they will attach to the plates. At the end of this attachment period the medium was poured off and the plates rinsed gently with warm EMEM. The cells were then supplemented with EMEM containing 5% LF serum or with Waymouth's medium and allowed to grow.

(d) Protein Measurement

Protein in HeLa cell extracts was measured according to the procedure of Lowry et al (1951) using bovine serum albumin to construct the standard curve.

(e) Assay for ACC in HeLa Cells

HeLa cells were removed from culture dishes by the addition of trypsin and washed three times in phosphate-buffered saline (PBS, pH 7.5). The cell pellet was suspended in 0.3 ml of 10 mM Tris-HCl (pH 7.8) containing 1.0 mM EDTA and 1 mM dithiothreitol and sonicated in a Bronwill sonicator. The sonicate was centrifuged for 10 min at 16,000 rpm to obtain the cytosolic fraction. The cytosolic fraction was used as the source of ACC. The ACC activity was measured either immediately or after preincubation at 37°C for 30 min to fully activate the enzyme. The preincubation medium contained: Tris-HCl (pH 7.8), 40 mM; glutathione reduced, 3 mM; bovine serum albumin, 0.6 mg/ml; potassium citrate, 10 mM and $MgCl_2$, 10 mM. The assay mixture in a final volume of 500 µl contained: Tris-HCl (pH 7.8), 40 mM; glutathione reduced, 3 mM; magnesium chloride, 10 mM; potassium citrate, 10 mM; bovine serum albumin, 0.6 mg; ATP, 4 mM; Acetyl-CoA, 0.1 mM; $KH^{14}CO_3$, 20 mM (S.A. 3000 dpm/µmol). The reaction was started by the addition of enzyme and carried out at 37°C for 5 min in the fumehood. The reaction was terminated by the addition of 100 µl 6N HCl. After centrifugation, 200 µl of the supernatant was transferred to a scintillation vial and dried under a fumehood. 0.5 ml of water was added to dissolve the dried material followed by 10 ml of Aquasol-2 scintillant for the determination of radioactivity in a Beckman LS 2800 scintillation spectrometer. One unit of enzyme activity is defined as that amount which catalyzes the carboxylation of 1 µmole of

acetyl-CoA into acid-stable product per min at 37°C.

(f) Measurement of the Relative Synthesis of ACC

HeLa cells were removed from culture dishes by trypsinization, washed with PBS and resuspended in 1.0 ml of EMEM lacking leucine. The cells were then labelled for 20 min at 37°C with L-[U-^{14}C] leucine (10 µCi/ml). At the end of this labelling period, cells were washed three times with cold PBS and suspended in 0.35 ml of 10 mM Tris-HCl (pH 7.2) containing 0.15 M NaCl. This suspension was sonicated using a Bronwill sonicator and centrifuged for 10 min at 10,000 x g to obtain the cytosolic fraction. A portion of the cytosol was used to determine the radioactivity associated with total cellular proteins. Another equal portion of the cytosol was used to determine radioactivity associated with ACC.

To determine the radioactivity associated with total cellular proteins, a portion of the cytosol was placed on a 3 MM filter paper, dried, washed successively with cold 10% Trichloroacetic acid (TCA), 5% TCA (2x) and ethanol. The filter paper was dried, transferred to a scintillation vial containing 10 ml of Aquasol-2 and radioactivity determined in a Beckman LS 2800 liquid scintillation spectrometer.

To determine the ACC associated radioactivity a portion (same volume as used in total protein associated radioactivity) of the sonicate was incubated with an excess of antibodies raised to chicken liver ACC for 30 min at 37°C. Then goat anti-rabbit gamma globulin was added to precipitate the antibody-enzyme complex by incubating this mixture in the presence of 1% PEG at 37°C for 15 min followed by 15 min at 4°C. The precipitated immune complex was then centrifuged through sucrose gradient (section IV A) according to the procedure of Taylor and Schimke (1974). The immune complex precipitate was

then washed three times with ice-cold 0.15 M NaCl containing 1% PEG. The final washed precipitate was dissolved in 0.9 ml of NCS by incubating at 56°C overnight. 50 µl of glacial acetic acid was added to the redissolved precipitate followed by 10 ml of OCS scintillant and radioactivity determined in Beckman LS 2800 liquid scintillation spectrometer. Non-specific radioactivity associated with the immune complex was determined by substituting control antiserum for the immunoprecipitation step.

The relative rate of synthesis of ACC is expressed as dpm associated with ACC/dpm associated with total protein.

(g) Fatty Acid Synthesis in HeLa Cells

HeLa cells were removed from culture dish and suspended in 2.0 ml of EMEM containing [^{3}H] H_2O (1 mCi/ml) and incubated at 37°C for 60 min. At the end of this time period cells were washed with cold PBS and sonicated in 0.3 ml of Tris-HCl (pH 7.2) containing 0.15 M NaCl. A portion of the sonicate was used for protein determination while another portion was used for fatty acid extraction. Fatty acid extraction was performed according to the method of Geelen et al (1978) using methanol:chloroform (2:1, v/v). Fatty acids were extracted in petroleum ether, the solvent evaporated under a stream of nitrogen, the residue dissolved in 10 ml of Aquasol-2 scintillant and radioactivity determined in a Beckman LS 2800 scintillation spectrometer.

(h) Activity and Relative Synthesis of ACC under Different Culture Conditions

HeLa cells were cultured in EMEM containing either 5% FBS or 5% LF serum or in Waymouth's medium for 2 days as previously described. The cells were then removed from culture dishes, washed three times with PBS and divided into

two portions. One portion was used for measuring ACC activity while the second portion was used for measuring the relative rate of synthesis of ACC as previously described.

(i) Effect of Glucagon on ACC Activity and Synthesis and Fatty Acid Synthesis

The effect of glucagon on ACC activity and its relative rate of synthesis was investigated in cells cultured in Waymouth's medium. HeLa cells were cultured in Waymouth's medium as previously described except that varying concentrations of glucagon were included in the medium. After two days the cells were removed from culture dishes, washed in PBS and used for measuring ACC actvity as previously mentioned. Glucagon was freshly prepared each time and dissolved in 10 mM HCl containing 1% bovine serum albumin. The stock glucagon solution was sterilized by passing it through a 0.22 μm Millipore filter (yellow type) and appropriate dilutions made using Waymouth's medium.

The effect of glucagon (1.5 μg/ml) on the relative rate of ACC synthesis and fatty acid synthesis was also investigated. The cells cultured in Waymouth's medium plus 1.5 μg/ml of glucagon for two days were removed from culture dishes, washed with PBS and divided into three parts. ACC activity, the relative rate of ACC synthesis and fatty acid synthesis was measured in part I, II and III respectively according to procedures described previously.

RESULTS

HeLa cells were used to investigate the role of glucagon in the long-term regulation of acetyl-CoA carboxylase. When HeLa cells are cultured in EMEM supplemented with 5% FBS, the activity of ACC in HeLa cells is very low and the relative rate of synthesis of ACC could not be detected using the immunochemical procedures (Table 2). Therefore, the first step in this investigation was to set up conditions under which the activity of ACC would be elevated. One way of elevating ACC activity in cell culture is to limit the supply of exogenous lipids in the culture media. As most cells grown in culture obtain majority of their lipid requirement from serum added to culture media, the use of a lipid-depleted serum that can still support growth of cells can enhance the ACC activity. Fetal bovine serum was made lipid-free by the use of ethanol-diethyl ether extraction at -20°C. The yield of the protein residue after lipid extraction was 2.13 ± 0.30 g (mean ± S.D. of 5 preparations) from 55 ml of FBS. When lipid-free serum was substituted for FBS, activity of ACC in HeLa cells was stimulated 8-fold and the relative rate of synthesis of ACC could also be measured using immunochemical procedures (Table 2). However, the procedure for the preparation of lipid-free serum is cumbersome and one cannot be certain about the composition of lipid-free serum from one preparation to the next. These differences may affect the reproducibility of results.

Therefore, we choose to use a chemically defined serum-free medium. One such medium that has been used extensively is Waymouth's medium (MD 705/1). When HeLa cells were cultured in Waymouth's medium the activity of ACC was stimulated 4-fold as compared to cells cultured in FBS and the relative rate of synthesis of ACC could also be measured (Table 2). It is also apparent

TABLE 2

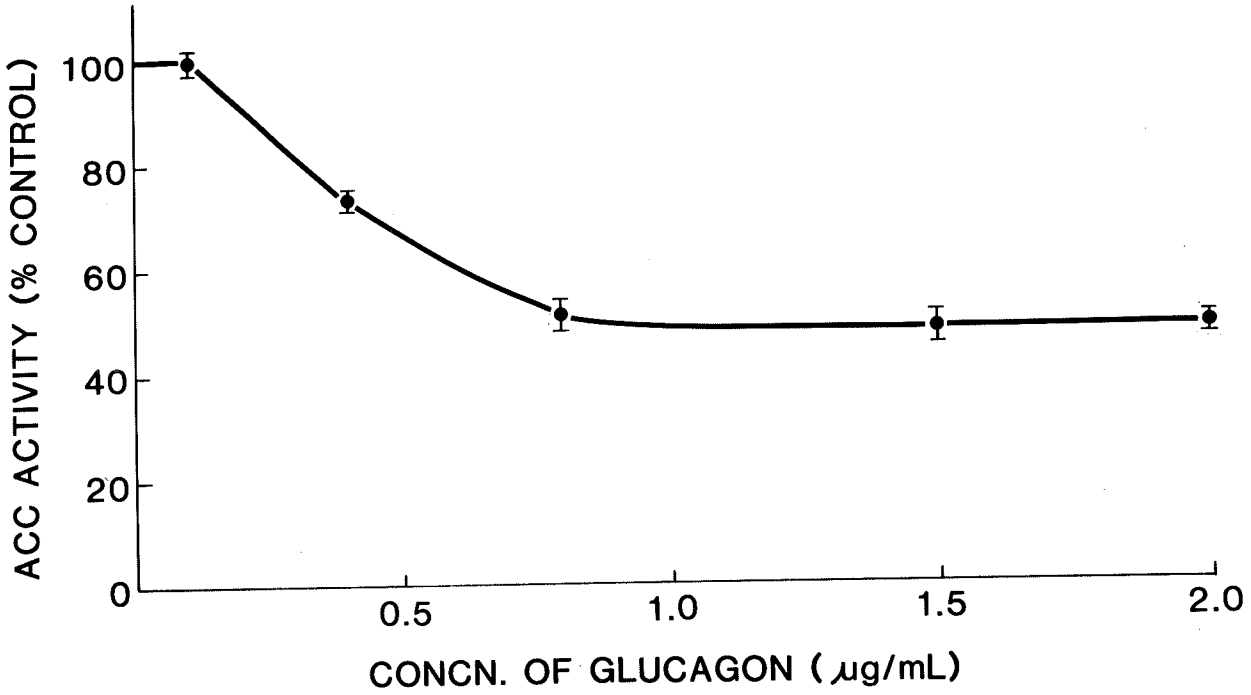
ACTIVITY AND RELATIVE SYNTHESIS OF ACC IN DIFFERENT MEDIUM

CELL CULTURE MEDIUM	ACC SPECIFIC ACTIVITY (mUnits/mg)	RELATIVE SYNTHESIS OF ACC**
5% FBS	0.25 ± 0.06 (6)	N.D.*
Waymouth's Medium	1.02 ± 0.24 (8)	0.057 ± 0.006 (3)
5% LF	1.97 ± 0.11 (8)	0.108 ± 0.008 (3)

*: Not detectable

: Results represent mean ± S.D. for the number of experiments given in ().

**: Defined as dpm associated with ACC/dpm associated with total protein.



EFFECT OF GLUCAGON ON ACC ACTIVITY

Fig. 8. HeLa cells cultured in Waymouth's medium were exposed to varying concentrations of glucagon for 2 days. At the end of this incubation period cells were removed and assayed for ACC after preincubation at 37°C for 30 min.

TABLE 3

EFFECT OF GLUCAGON ON ACTIVITY AND RELATIVE SYNTHESIS OF ACC

ADDITIONS TO MEDIUM	SPECIFIC ACTIVITY (mUnits/mg)	RELATIVE SYNTHESIS OF ACC
None	1.31 ± 0.14	0.059 ± 0.006
Glucagon	0.58 ± 0.08	0.024 ± 0.004

HeLa cells were cultured in Waymouth's medium in the presence (1.5 µg/ml) and absence of glucagon for 2 days. At the end of this time period cells were removed, assayed for ACC activity after preincubation and the relative rate of ACC synthesis was also determined as described in experimental procedures. The results are expressed as mean ± S.D. of four experiments in each set.

from the results in Table 2 that the specific activity of ACC is closely related to its rate of synthesis. Thus, one is justified in associating changes in the specific activity of ACC with changes in the relative rate of synthesis of ACC. HeLa cells cultured in Waymouth's medium when exposed to varying concentrations of glucagon, showed a decrease in the specific activity of ACC (Fig. 8). Maximum decrease observed in the specific activity was approximately 50% and was achieved at a glucagon concentration of 1.5 µg/ml in the culture medium. A similar degree of inhibition (50%) of ACC activity was also observed when cells cultured in lipid-free serum were exposed to 1.5 µg/ml of glucagon. The rate of fatty acid synthesis in the glucagon treated HeLa cells as determined by incorporation of radioactivity from $^{3}H_2O$ was also found to be decreased (50 $\pm$ 8 % of the control value, mean $\pm$ S.D. of 3 determinations). To correlate this decrease in ACC specific activity with the total enzyme protein, relative rate of synthesis of ACC in glucagon treated HeLa cells was studied. The relative rate of synthesis as determined by immunochemical techniques was found to be decreased in glucagon treated HeLa cells (Table 3).

DISCUSSION

Studies dealing with the *in vivo* long-term regulation of ACC have dealt mainly with the effect of dietary and genetic alterations (Majerus and Kilburn, 1969;Nakanishi and Numa, 1970;Nakanishi and Numa, 1971). The role of hormones in the long-term regulation of ACC is less well understood. This has been partially due to the lack of model cell culture systems that are available. However, few attempts have been made to investigate the role of hormones in the long-term regulation of ACC using cell culture systems (Shafrir and Bierman, 1981;Fischer and Goodridge, 1978).

Results of our investigation with HeLa cell culture system indicate that the specific activity of ACC is increased 8-fold and 4-fold when cells were cultured respectively in lipid-free and Waymouth's medium. This change in the specific activity of ACC was due to an increase in the relative rate of ACC synthesis. When HeLa cells were cultured in Waymouth's medium in the presence of glucagon there was a decrease (50%) in the specific activity of ACC as well as in the rate of fatty acid synthesis. Witters *et al* (1979), using rat hepatocytes have shown that during short-term incubations (60 min), insulin causes an increase while glucagon inhibits ACC activity and that this is due to changes in the dephosphorylation-phosphorylation state respectively of ACC. That the effect of glucagon on HeLa cell ACC was due to an adaptive decrease in enzyme protein is indicated by co-ordinate decrease in the specific activity and relative rate of synthesis. Shafrir and Bierman (1981), working with human skin fibroblasts, presented evidence which showed that insulin causes a 2-fold increase in ACC activity and that this increase is due to an increase in enzyme protein as the addition of cycloheximide abolished the increase observed. Fischer and Goodridge (1978) using chick liver cells in

culture have shown that insulin causes a 2-fold increase in the relative rate of synthesis of ACC and fatty acid synthetase and the addition of insulin plus glucagon to the culture abolishes the observed increase in the relative rate of synthesis of ACC but has no effect on the increased relative rate of synthesis of fatty acid synthetase (0.12 ± 0.04 vs 0.10 ± 0.02 in the presence of insulin and in the presence of insulin plus glucagon respectively) suggesting that ACC is more sensitive to the adaptive changes observed in the presence of glucagon. Results presented here along with those in the literature would suggest that the gene for ACC might be under seperate control from the fatty acid synthetase gene (Fischer and Goodridge, 1978). Whether glucagon is the primary messenger for its long-term effect on ACC or a second messenger is responsible for the effect is not clear at the present time.

SECTION V

PURIFICATION, PHYSICOCHEMICAL PROPERTIES AND REGULATION OF PORCINE ADIPOSE TISSUE ACETYL-CoA CARBOXYLASE

Acetyl-CoA carboxylase (EC 6.4.1.2) catalyzes the ATP-dependent carboxylation of acetyl-CoA to give malonyl-CoA. This is the first committed step in the de novo synthesis of long-chain fatty acids and it is generally accepted that ACC catalyzes the rate-limiting step in fatty acid synthesis (Volpe and Vagelos, 1976;Bloch and Vance, 1977). The activity of ACC is under acute and long-term regulation. Acute intracellular regulation of ACC activity may be a cosequence of allosteric modulation by citrate and fatty acyl-CoA (Bortz and Lynen, 1963a;Gregolin et al, 1966) as well as by changes in the phosphorylation of the enzyme. In this section, I will deal essentially with acute regulation of acetyl-CoA carboxylase activity by phosphorylation-dephosphorylation.

It was initially observed by Inoue and Lowenstein (1972) that pure chicken liver ACC contains covalently bound phosphate. Later, Carlson and Kim (1973) suggested that partially purified rat liver ACC could be phosphorylated using Mg^{2+}-ATP resulting in its inactivation and that rat epididymal fat tissue is activated by insulin and inactivated by epinephrine and dibutryl cAMP (Lee and Kim, 1978). Since these earlier observations, extensive effort has been made to elucidate the role of phosphorylation in the acute regulation of ACC. At the present time three distinct views exist in regards to the role of phosphorylation in the acute regulation of ACC activity. Kim and coworkers working with rat liver ACC have suggested that the phosphorylation and subsequent inactivation of acetyl-CoA carboxylase is controlled by the adenylate energy charge, so that modest increases in AMP concentration shuts off fatty acid synthesis by stimulating phosphorylation and inactivation of acetyl-CoA carboxylase (Yeh et al, 1980). Hardie's group working with

purified rabbit mammary gland and rat mammary gland ACC have provided evidence which suggests that phosphorylation by the cAMP-dependent protein kinase is responsible for the inactivation of ACC (Hardie and Cohen, 1978a;Hardie and Guy, 1980) and that protein phosphatases can cleave phosphate groups from ACC leading to an increase in enzyme specific activity (Hardie and Guy, 1980;Shiao et al, 1981;Wada and Tanabe, 1983). Others, although not refuting the suggestion of phosphorylation of ACC, have presented evidence which indicates that there is a lack of correlation between phosphorylation and inactivation of ACC (Halestrap and Denton, 1974;Desjardins and Dakshinamurti, 1978;Pekala et al, 1978;Gillevet and Dakshinamurti, 1983).

To suggest physiological significance for protein phosphorylation, one of the criteria that must be met is the demonstration that functional properties of enzyme (e.g. activity) undergo changes that correlate with the degree of in vitro phosphorylation (Krebs and Beavo, 1979). Attempts to satisfy this criteria in the regulation of ACC activity by phosphorylation have been carried out with the purified rat mammary gland and rabbit mammary gland ACC. Work done with the purified rat liver ACC appears to be contradictory (Tipper and Witters, 1982;Lent and Kim, 1983). Tipper and Witters (1982) presented evidence indicating that purified rat liver ACC could be phosphorylated by the cAMP-dependent protein kinase and inactivated. However, Lent and Kim (1983) have presented data showing that purified rat liver ACC could not be phosphorylated or inactivated by the cAMP-dependent protein kinase.

Adipose tissue is highly specialized for triacylglecerol synthesis as is the lactating mammary gland, thus, it was of interest to investigate if adipose tissue ACC was regulated by phosphorylation in a manner similar to that proposed for the mammary gland ACC. One would expect to see a similar role for phosphorylation in the regulation of acetyl-CoA carboxylase in tissues other than lactating rat and rabbit mammary gland. We, therefore

purified ACC from porcine adipose tissue to assess the role of in vitro phosphorylation by the catalytic subunit of cAMP-dependent protein kinase on the activity of porcine adipose tissue ACC.

(a) Materials

Avidin was purchased from Vega Biochemicals (Tucson, Az.). Phosphorylase a, phosphorylase b, the purified catalytic subunit of cAMP-dependent protein kinase and guanidine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, Mo). CNBr activated Sepharose 4B and the high molecular weight protein marker kit was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Guanidine thiocyanate was purchased from Fluka Chemicals Corp. (Hauppauge, N.Y.). Fiske-Subbarow reagent (1-amino-2-naphthol-4 Sulfonic acid) was obtained from Fisher Scientific Co. (Fairlawn, N.J.). [γ-^{32}P] ATP (S.A. 10-40 Ci/mmol) was purchased from New England Nuclear Corp. (Montreal, Canada). The source of other reagents used has been mentioned previously.

(b) Acetyl-CoA Carboxylase Assay

Acetyl-CoA carboxylase was assayed using the ^{14}C-bicarbonate fixation assay as previously described in section IV (A).

(c) Protein Determination

Protein was determined by the dye-binding method using the reagent supplied by Bio-Rad (Bradford, 1976;Biorad Technical Bulletin, 1977). Bovine serum albumin was used to construct the standard curve.

(d) Preparation of Monomeric Avidin-Sepharose

Monomeric avidin-Sepharose for affinity chromatography was prepared according to the procedure of Beaty and Lane (1982). 40 ml of CNBr activated Sepharose was washed with three volumes of 0.01 M $NaPO_4$ (pH 7.0) and resuspended in 40 ml of the same buffer containing 100 mg of avidin (S.A. 13-15 units/mg). This suspension was shaken gently for 24 hr. The gel was filtered and resuspended in 40 ml of 0.1 M 2-aminoethanol (pH 7.0) to block any unreacted sites on the resin. The gel was then extensively washed with phosphate-buffer (0.01 M, pH 7.0) followed by three washes with 20 ml of 6 M guanidine-HCl containing 0.2 M KCl-HCl (pH 1.5) to dissociate covalently bound avidin tetramers into monomers and elute avidin monomers not covalently linked to the resin. The gel was re-equilibrated in phosphate-buffer (0.01 M, pH 7.5), packed into a column (1.5 x 20 cm) and washed with 1 column volume of guanidine thiocyanate (3 M, containing 0.2 M KCl-HCl, pH 1.5) to complete the avidin subunit dissociation. The monomeric avidin-Sepharose was then equilibrated in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl, 1.0 mM EDTA, 0.1 mM dithiothreitol and 5% glycerol (column buffer). The column was washed with column buffer containing 0.8 mM biotin which binds to both low and high affinity biotin binding sites on the avidin-Sepharose column. Biotin bound at the low affinity sites, presumably avidin monomer is removed by washing the column with glycine-HCl (0.1 M, pH 2.0) as the affinity of avidin monomer for biotin is lower (K_a 10^8 M) than the affinity of tetrameric avidin for biotin (K_a 10^{15}). After re-equilibration with column buffer, the column was ready for use. Subsequent regeneration of the column merely requires washing the column with 0.1 M glycine-HCl (pH 2.0) and re-equilibration with column buffer. All these steps were carried out at 4^oC.

The exchangeable biotin-binding capacity of the final avidin-Sepharose

column was determined by charging the column with an excess of [^{3}H] biotin of known specific activity, followed by elution with 0.1 mM unlabelled biotin and quantitation of the eluted [^{3}H] biotin. The exchangeable biotin-binding capacity of a typical preparation of avidin-Sepharose was approximately 17 nmol of biotin/ml of packed gel.

(e) Polyacrylamide Gel Electrophoresis in the Presence of SDS (SDS-PAGE)

SDS-PAGE slab gels were run according to the method of Laemmli (1970) as previously described (Section IV A). To detect the biotin-containing subunit of ACC, the procedure of Lau et al (1979) was followed. Purified ACC was treated with avidin for 10 min at 37°C and the sample denatured in the presence of 1% SDS and 5% β-mercaptoethanol at 56°C for 15 min. The denatured sample was then analyzed on polyacrylamide gels as mentioned above.

The molecular weight of purified ACC was determined by comparing its mobility on polyacrylamide gels to that of proteins with known molecular weight.

(f) Purification of Porcine Adipose Acetyl-CoA Carboxylase

(i) Homogenization and PEG Precipitation

Porcine perirenal adipose tissue was obtained from Burn's Meat Co. (Winnipeg, Manitoba) and transported immediately to the laboratory so as to prevent solidification of the fat. After removal of connective tissue and blood vessels, 500gm batches of the adipose tissue were homogenized in 750 ml of 50 mM phosphate-buffer(pH 7.5) containing 1 mM EDTA and 5 mM β-mercaptoethanol for 30 sec in a Waring blender at room temperature. The

homogenate was allowed to stand at -20°C for 1 hr so as to solidify the fat. The solidified fat was removed by sqeezing through 2 layers of cheesecloth. The extract was centrifuged at 10,000 x g for 30 min at 4°C and the supernatant filtered through 2 layers of cheesecloth. The filtered supernatant was made 6% in PEG by the slow addition with stirring of a 50% stock PEG solution. The solution was allowed to stand at room temperature for 1 hr. The precipitated protein was collected by centrifugation at 10,000 x g for 30 min at 4°C. The precipitate was resolubilized in the homogenization buffer and centrifuged at 10,000 x g for 20 min to remove any insoluble material.

(ii) DEAE-Cellulose Chromatography and PEG Precipitation

The supernatant obtained in step (i) was applied to DEAE-cellulose column (5 x 20 cm) which had previously been equilibrated with homogenization buffer. The column was then washed with homogenization buffer until A_{280} of effluent reached zero. The column was then washed with 50-600 mM phosphate-buffer (pH 7.5) gradient containing 0.1 mM EDTA, 5 mM β-mercaptoethanol and 10 ml fractions collected at a flow rate of 1 ml/min. The fractions were assayed for ACC activity, absorbance at 280 nm and conductance. Fractions containing enzyme activity were pooled and brought to room temperature. The solution was then made 6% PEG by the slow addition with stirring of a 50% stock PEG solution. The solution was allowed to stand at room temperature for 2 hr and the precipitated protein collected by centrifugation at 10,000 x g for 30 min. The precipitate was resolubilized in 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM EDTA , 5 mM β-mercaptoethanol and 30% glycerol. After removal of insoluble material by centrifugation the final enzyme preparation was stored at -20°C.

The enzyme was also prepared as described above except that all buffers contained 50 mM sodium fluoride.

(iii) Purification using Monomeric Avidin-Sepharose Chromatography

The precipitate obtained in step (i) after PEG precipitation was resolubilized in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 5 mM β-mercaptoethanol and 5% glycerol (column buffer). After the removal of any insoluble material the supernatant was applied to a monomeric avidin-Sepharose column (1.5 x 20 cm) which had been previously equlibrated with the column buffer. The column was then washed with the column buffer until A_{280} of the effluent reached zero. The carboxylase was eluted by washing with column buffer containing 0.1 mM biotin and 2 ml fractions collected. Fractions containing enzyme activity were pooled and stored at -20°C as the enzyme source.

(g) Determination of K_m value for Substrate

The K_m value for the substrates acetyl-CoA, ATP and HCO_3^- were determined using the double reciprocal plots.

(h) Determination of K_a for Citrate

The final preparation of purified porcine ACC was dialyzed overnight against 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM EDTA, 5 mM β-mercaptoethanol, 0.5 M NaCl and 30% glycerol. The enzyme was then assayed as previously described except that the citrate concentration in the assay mixture was varied from 0 to 10 mM. The activation constant (K_a) for citrate

was defined as the concentration of citrate required to achieve half-maximal velocity (1/2 V_{max}).

(i) Determination of Biotin Content

The biotin content of purified porcine ACC was determined using the isotope-dilution method (Dakshinamurti and Allan, 1979). The experimental details of this assay are covered in section VI.

0.5 mg of the pure enzyme was hydrolyzed in 4.5 N H_2SO_4 by autoclaving at a pressure of 15 psi for 1 hr. The hydrolyzed sample was neutralized with NaOH and filtered. The filtrate was used for the determination of biotin.

(j) Determination of Alkali-labile Phosphate Content

Alkali-labile phosphate content of the purified porcine ACC was determined according to the procedure of Nimmo and Cohen (1976) using Fiske-Subbarow reagent (Bartlett, 1959).

5 mg of purified ACC, phosphorylase a and phosphorylase b respectively were dialyzed overnight against H_2O. The dialyzed samples were then made 5% in TCA, left at 4°C for 10 min and centrifuged. The pellets were suspended in 1 ml of 0.1 N NaOH, precipitated by the addition of 1 ml of 10% TCA, left at 4°C for 10 min and centrifuged. 0.3 ml of 1 N NaOH was added to each of the precipitates and allowed to hydrolyze overnight at 37°C. 0.2 ml of 50% TCA was added to the hydrolyzate and the sample used for phosphate determination. The assay medium for phosphate determination contained: 0.3 ml 10 N H_2SO_4, 0.2 ml 5% $(NH_4)_2$ molybdate, 0.05 ml Fiske-Subbarow reagent and hydrolyzed sample plus water to bring the volume up to 1.0 ml. The assay mixture was then boiled for 7 min, cooled and the absorbance read at 830 nm. The standard

curve was prepared using a stock solution of KH_2PO_4 dissolved in 25% TCA.

(k) Phosphorylation by the cAMP-dependent protein kinase

Phosphorylation was carried out at 37°C in the presence and absence of the purified cataltytic subunit of cAMP-dependent protein kinase (50 units/ml). The assay medium contained magnesium acetate (1 mM), [γ-^{32}P] ATP (0.5 mM;1000 dpm/pmol) and the purified porcine adipose tissue ACC. Samples were removed at indicated times and the incorporation of [^{32}P] into protein was determined according to the procedure of Butcher (1971). A sample (28 min sample) of the phosphorylated enzyme was also analyzed by SDS-PAGE as described previously. The gel was dried and an autoradiogram developed using Fuji R_x film.

In an attempt to correlate phosphorylation of the enzyme with changes in enzyme activity, the purified ACC was incubated in the presence and absence of the protein kinase as mentioned above except that unlabelled ATP was substituted for [γ-^{32}P] ATP in the assay medium. Aliquotes were removed at indicated times to assay for ACC activity as previously mentioned except that citrate concentration in reaction mixture was kept at 0.5 mM.

(i) Purification of ACC from Porcine Adipose Tissue

Earlier attempts to purify acetyl-CoA carboxylase from porcine adipose tissue using the standard procedure of ammonium sulfate precipitation as the first step after homogenization were unsuccessful. ACC from porcine adipose tissue was susceptible to inactivation during ammonium sulfate precipitation and the subsequent dialysis steps. The yield of enzyme at the ammonium sulfate step was also very low. Therefore, ammonium sulfate precipitation as the initial step in the purification of porcine ACC was abandoned in favor of polyethylene glycol (PEG). Polyethylene glycol precipitation involves nondenaturing conditions for the protein precipitation and thus avoids the dialysis step which is usually employed in protein precipitated by the use ammonium sulfate.

Using polyethylene glycol precipitation and DEAE-cellulose chromatography, acetyl-CoA carboxylase from porcine adipose tissue was purified to apparent homogeneity. The enzyme was purified seperately, both in the absence and presence of sodium fluoride (50 mM) in all the buffers. When ACC was purified in the absence of F^- the final enzyme preparation had a specific activity of 3.2 units/mg of protein (Table 4). Similar results were obtained when the enzyme was purified in the presence of 50 mM sodium fluoride (Table 5). This represents an approximately 250-fold purification of the enzyme from the starting material. The enzyme purified using monomeric avidin-Sepharose chromatography had the same specific activity as of the above preparation.

TABLE 4

PURIFICATION OF PORCINE ADIPOSE ACC IN THE ABSENCE OF F^-

STEP	TOTAL ACTIVITY (UNITS)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (Units/mg)
HOMOGENATE[a]	208	17,482	0.012
6% PEG PPTN.	145	854	0.17
DEAE-CELLULOSE POOL	66	240	0.27
6% PEG PPTN.	42	13	3.20

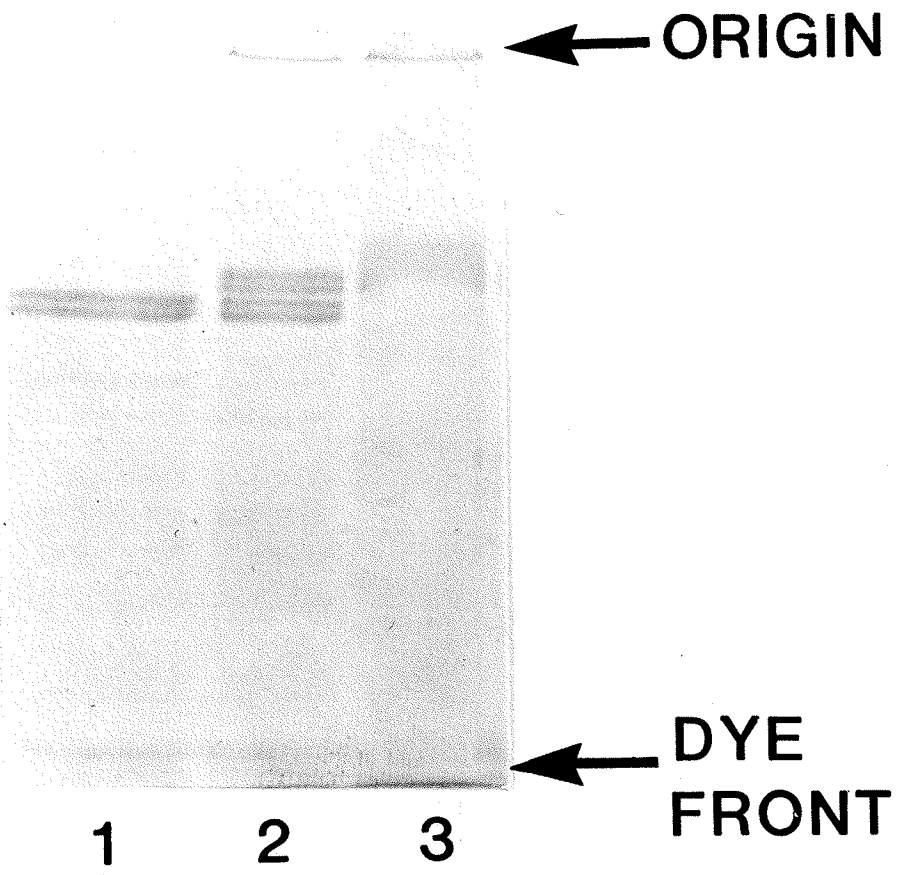
[a]: from 10 kg of porcine adipose tissue.

TABLE 5

PURIFICATION OF PORCINE ADIPOSE ACC IN THE PRESENCE OF F^-

STEP	TOTAL ACTIVITY (UNITS)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (Units/mg)
HOMOGENATE[a]	82.8	9,040	0.09
6% PEG PPTN.	66.2	552	0.12
DEAE-CELLULOSE POOL	24.8	73.7	0.33
6% PEG PPTN.	16.4	5.4	3.00

[a]: from 5 kg of porcine adipose tissue.



SDS-PAGE ANALYSIS OF PURIFIED PORCINE ADIPOSE ACC

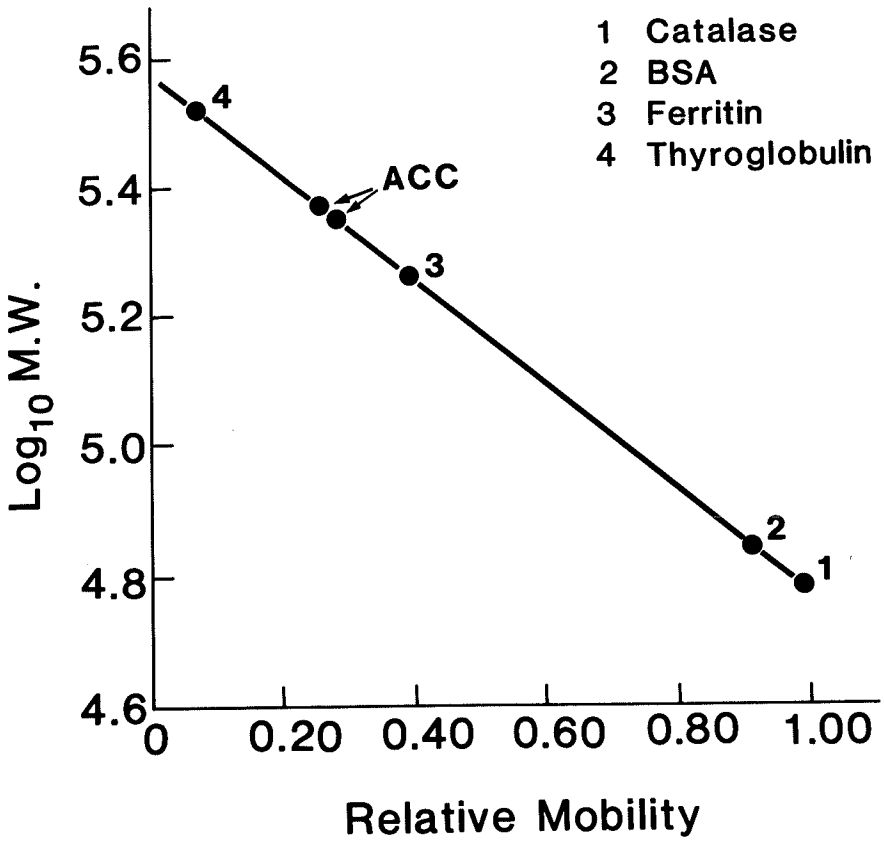
Fig. 9. 7 μg of purified ACC was denatured by heating at 100°C for 2 min in the presence of 1% SDS and 5% β-mercaptoethanol (lane 1). Lanes 2 and 3 represent ACC treated with avidin (1 μg and 10 μg respectively) and then denatured at 56°C for 15 min in the presence of 1% SDS and 5 % β-mercaptoethanol.

(ii) Purity and Physicochemical Characterization

Two protein staining bands were observed when the purified preparation of porcine ACC was analyzed by SDS-PAGE (Fig. 9, lane 1). When the preparation was treated with avidin and analyzed by SDS-PAGE, both protein staining bands showed decreased mobility indicating the presence of biotin (Fig. 9, lanes 2 and 3). In the presence of excess avidin, all the protein (ACC) was bound to avidin and showed decreased mobility as compared to its mobility in the absence of avidin (Fig. 9, lanes 1 and 3). The purified preparation when analyzed by SDS-PAGE in the presence of marker proteins, provided an apparent molecular weight of 240,000 and 234,000 daltons for the top and lower protein staining bands (Fig. 10). The alkali-labile phosphate content was found to be 0.80 ± 0.25 and 0.75 ± 0.10 mole P_i/mole subunit of enzyme for ACC purified in the absence and presence of 50 mM F^- respectively. The biotin content of the purified ACC was found to be 1.1 ± 0.12 mole of biotin per mole subunit of enzyme. Purified porcine ACC was inactivated by antibody raised against purified chicken liver ACC suggesting similarity of antigenic sites between the two enzymes.

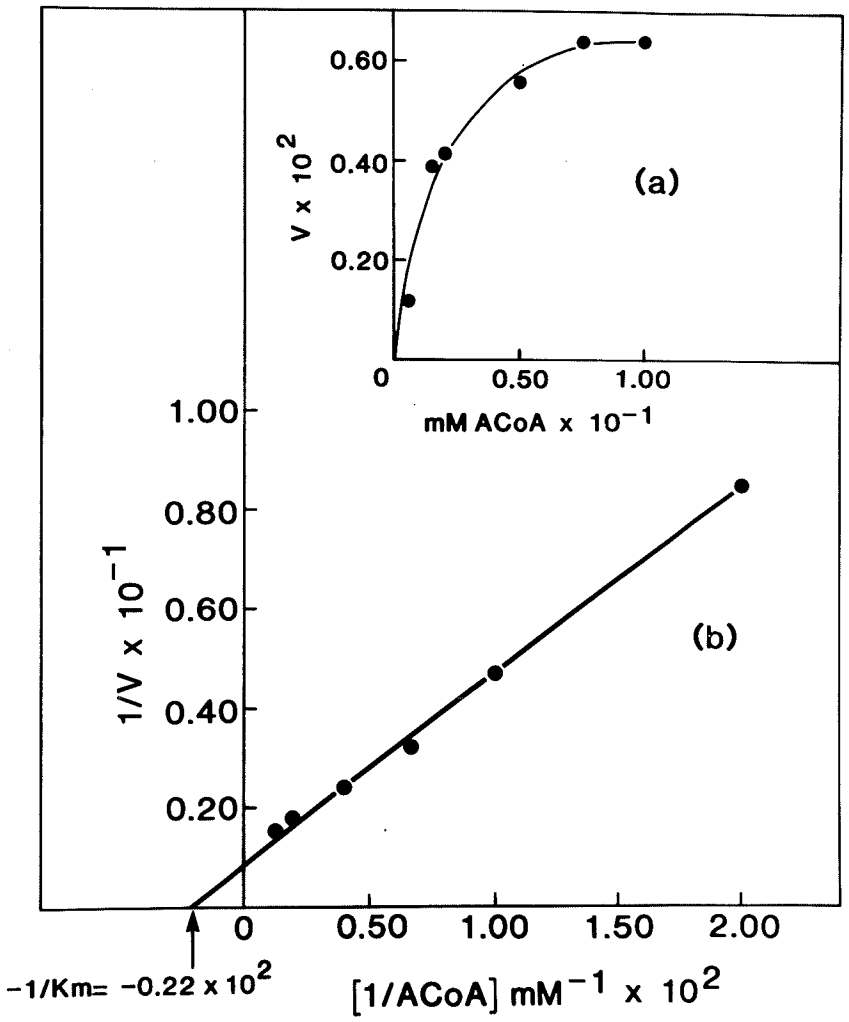
(iii) Kinetic Properties of Porcine Adipose ACC

The K_m values as determined from the double reciprocal plots were 0.038 mM, 0.36 mM and 7.7 mM for acetyl-CoA, ATP and HCO_3^- respectively (Fig. 11, 12, 13). The purified preparation of porcine ACC was sensitive to activation by its well known allosteric activator, citrate. The activity of the enzyme was stimulated 10-fold when the enzyme was preincubated in the presence of citrate (10 mM). The apparent K_a determined for citrate was 0.90 mM (Fig. 14).



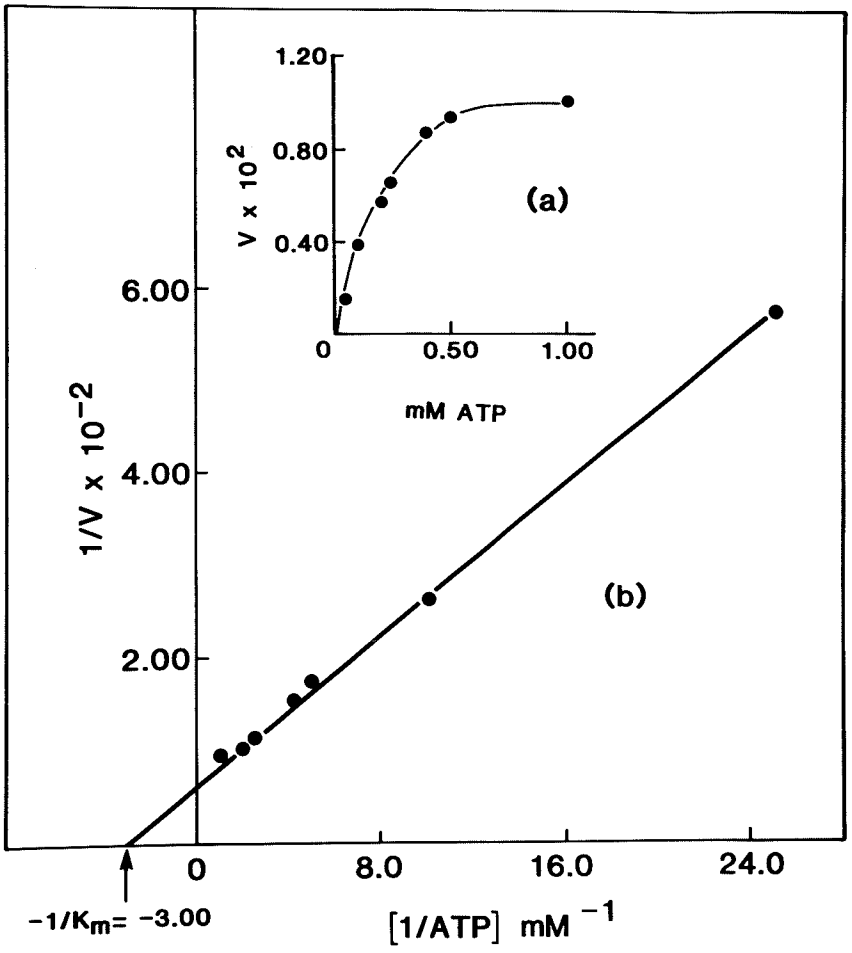
SUBUNIT MOLECULAR WEIGHT DETERMINATION BY SDS-PAGE

Fig. 10. A plot of $\log_{10}$ molecular weight vs relative mobility. The marker polypeptides used to prepare the calibration plot were: catalase, 60K; albumin, 67K; partially denatured ferritin, 220K; thyroglobilin, 330K. The arrows indicate the relative mobility of dissociated porcine adipose ACC.



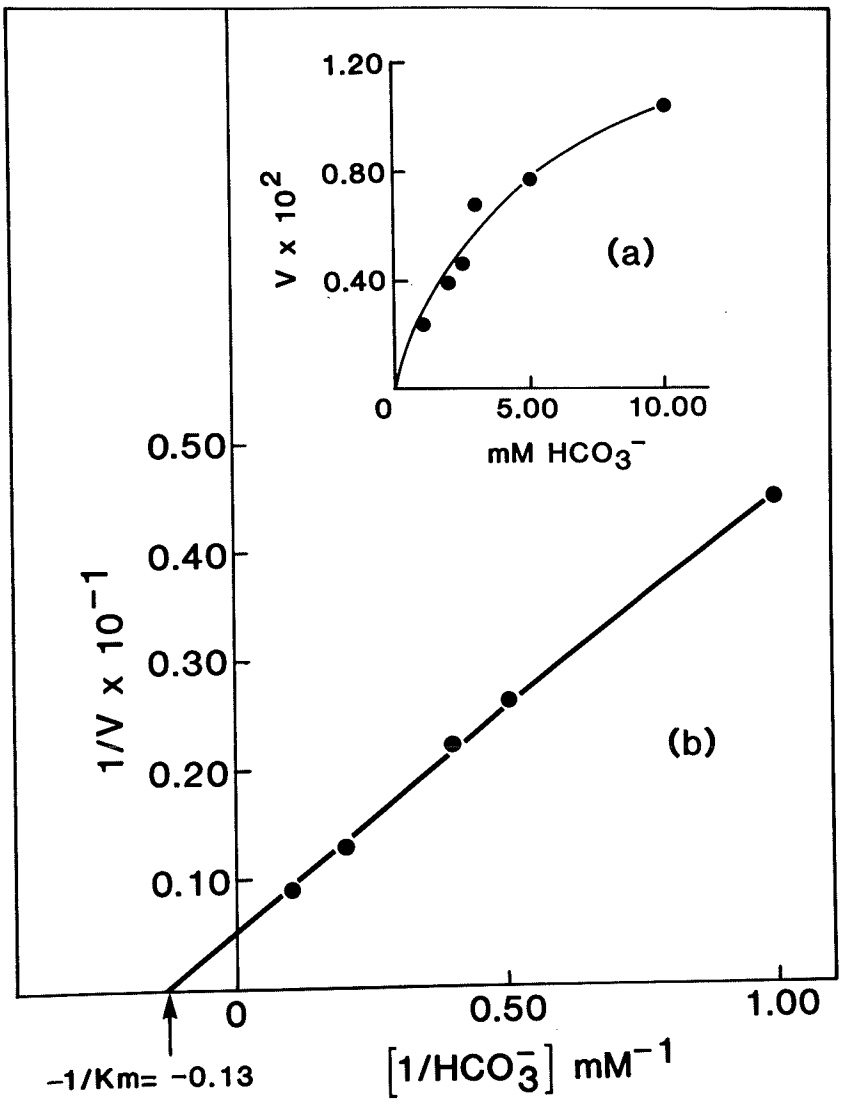
DETERMINATION OF K_m FOR ACETYL-CoA

Fig. 11. (a) Substrate saturation curve for acetyl-CoA. (b) Double reciprocal plot of the variation in velocity with changes in acetyl-CoA concentration. Velocity (v) is expressed as munits/ml enzyme (concn. 40 µg/ml).



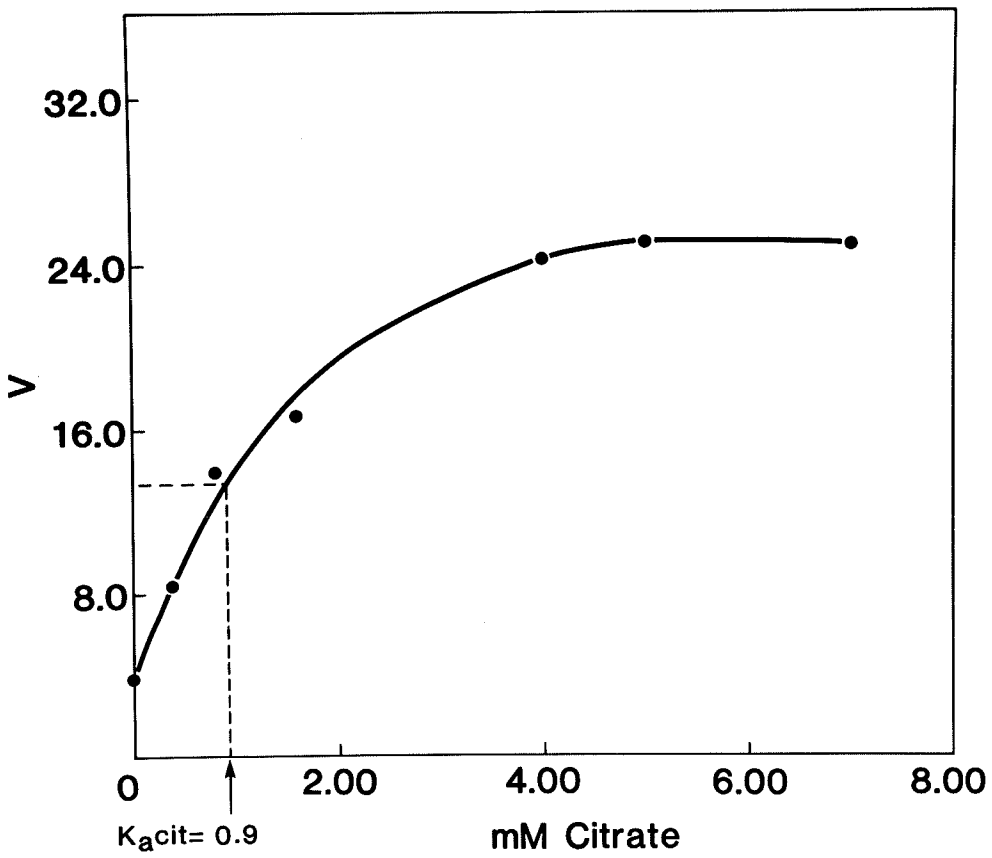
DETERMINATION OF K_m FOR ATP

Fig. 12. (a) Substrate saturation curve for ATP. (b) Double reciprocal plot of the variation in velocity with changes in ATP concentration. Velocity (v) is expressed as munits/ml enzyme (concn. 40 µg/ml).



DETERMINATION OF K_m FOR HCO_3^-

Fig. 13. (a) Substrate saturation curve for HCO_3. (b) Double reciprocal plot of the variation in velocity with changes in HCO_3 concentration. Velocity (v) is expressed as munits/ml enzyme (concn. 40 µg/ml).

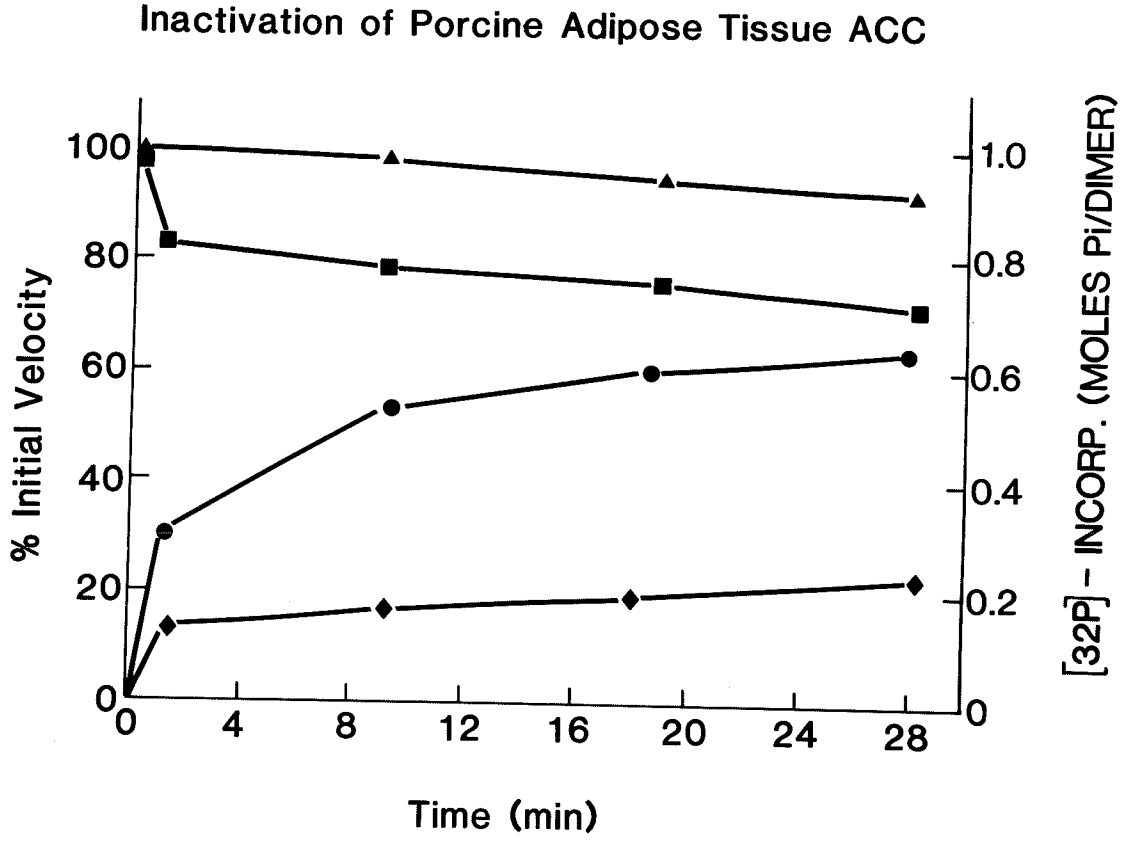


DETERMINATION OF K_a FOR CITRATE

Fig. 14. The dependence of ACC activity on citrate concentration. Velocity (v) is expressed as munits/ml enzyme (concn. 7 µg/ml).

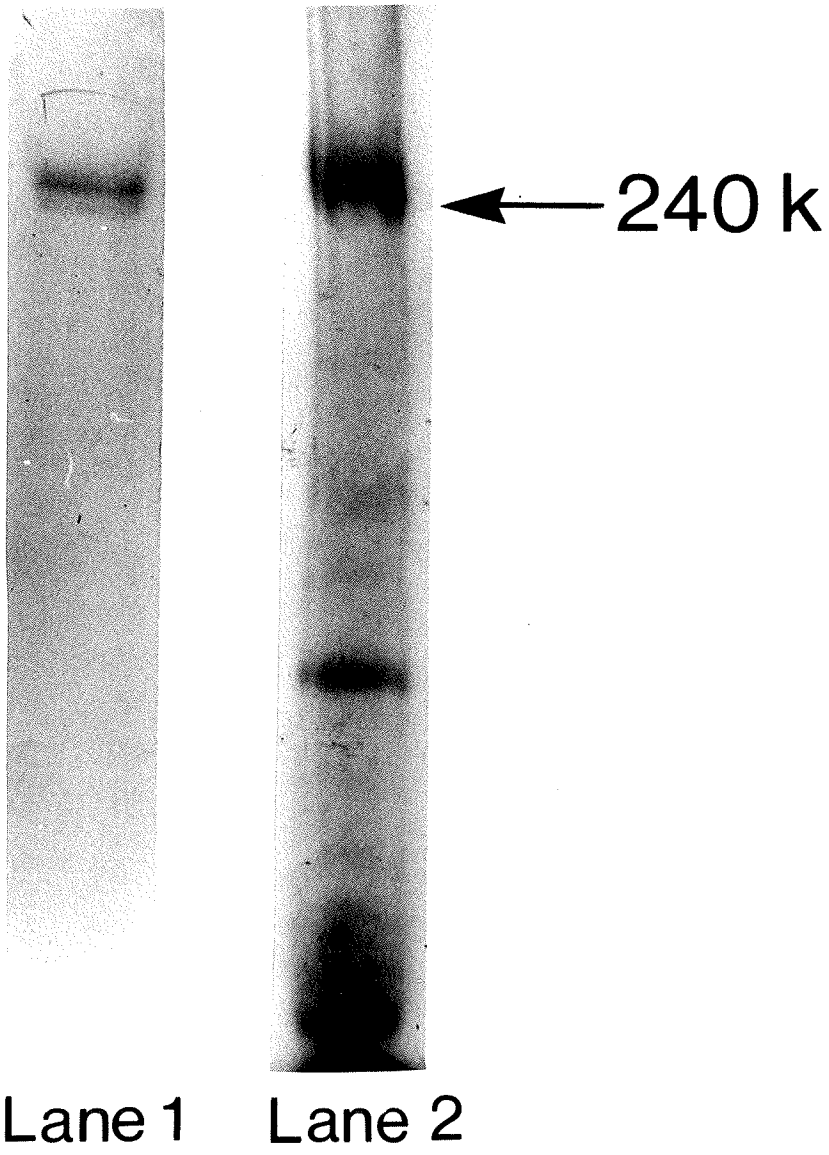
(iv) Effect of Phosphorylation on ACC Activity

When porcine ACC was incubated with Mg^{2+}-ATP and the purified catalytic subunit of cAMP-dependent protein kinase, the enzyme activity was reduced to 80% of control value by 5 min of incubation (Fig. 15) and further incubation did not cause anymore decrease in enzyme activity. When the enzyme was incubated with Mg^{2+}-ATP in the absence of the protein kinase no decrease in enzyme activity was observed (Fig. 15). To correlate this inactivation with phosphorylation, the enzyme was incubated in the presence of [γ-^{32}P] ATP. A very rapid incorporation of phosphate (0.15 mole per mole of enzyme, Fig. 15) occured in the presence of protein kinase, reaching a maximum value of 0.40 mole phosphate incorporated per mole of enzyme by 28 min. In the absence of the protein kinase endogenous phosphorylation also occured reaching a maximum value of 0.25 mole of phosphate incorporated per mole of enzyme (Fig. 15). The small decrease observed (20%) in enzyme activity in the presence of cAMP-dependent protein kinase correlated with phosphorylation only for the initial 5 min of the reaction. After this time phosphorylation was still proceeding while no further inactivtion of the enzyme occured. To confirm that radioactive phosphate was associated with ACC, SDS-PAGE analysis and autoradiography of the phosphorylated ACC (28 min sample) was done. It is clear from Fig. 16 that in the above incubations radioactive phosphate was associated with ACC. In the absence of cAMP-dependent protein kinase in the medium phosphorylation of ACC was also taking place (Fig. 16, lane 1). The presence of the protein kinase in the medium augmented phosphorylation (Fig. 16, lane 2). Autophosphorylation of the cAMP-dependent protein kinase was also seen (Fig. 16, lane 2;bands below the 240K dalton ACC band;Walsh et al, 1972).



PHOSPHORYLATION AND EFFECT ON ACC ACTIVITY

Fig. 15. Phosphorylation was carried out at 37°C in the presence and absence of the purified catalytic subunit of the cAMP-dependent protein kinase. Aliquotes were removed at indicated times to measure ^{32}P incorporation in the presence (●——●) and absence (◆——◆) and activity in the presence (■——■) and absence (▲——▲) of the kinase as described in experimental procedures.



SDS-PAGE ANALYSIS OF PHOSPHORYLATED ACC

Fig. 16. Purified ACC was phosphorylated for 28 min in the absence (lane 1) and presence (lane 2) of the cAMP-dependent protein kinase. The samples were analyzed by SDS-PAGE. The gel was dried and an autoradiogram developed. A picture of an autoradiogram is shown above.

DISCUSSION

Limited proteolysis has been a serious problem in the isolation of mammalian acetyl-CoA carboxylases. Inoue and Lowenstein (1972) purified ACC from rat liver and detected three protein staining bands (215K, 125K and 118K daltons) by SDS-PAGE analysis. This led to the suggestion that mammalian ACC consisted of three subunits. However, later studies confirmed that mammalian ACC consists of two identical subunits (M_r 220K to 260K dalton per subunit) and that the smaller fragments are produced by proteolysis of the native subunit (Mackall and Lane, 1977).

We have purified ACC from porcine adipose tissue using polyethylene glycol and this preparation is free of any major proteolytic degradation which has often been observed in the purified avian and mammalian liver enzymes. The purified preparation on SDS-PAGE analysis showed two protein staining bands. This would suggest the possibility of porcine adipose tissue ACC possessing two nonidentical subunits since both protein staining bands contained biotin as evidenced by SDS-PAGE analysis in the presence of avidin. However, it is more likely that the lower protein staining band was produced from the top band by proteolytic degradation. Such minor proteolytic degradation of ACC from rabbit mammary gland has also been observed (Hardie and Cohen, 1978b). Also in some of our preparations of ACC which were not homogenous only one major band for ACC with a subunit molecular weight of 240K daltons was observed. The lack of proteolysis in case of porcine ACC may be due to the relative lack of proteinase activity in the adipose tissue compared to the liver.

The subunit molecular weight of 240K dalton obtained for the porcine adipose ACC is in agreement with the value reported for rat liver (Tipper and

Witters, 1982) and rat mammary gland ACC (Hardie and Guy, 1980). However, it is smaller than the value of 252K dalton reported for rabbit mammary gland ACC (Hardie and Cohen, 1978b) and larger than the value of 225K dalton reported for chicken liver ACC (Beaty and Lane, 1982). Recently, it has been reported that crude rat liver cytosol contains two larger molecular weight (257K and 260K dalton) forms of acetyl-CoA carboxylase and that during purification the enzyme gets degraded into smaller molecular weight (241K and 252K dalton) forms (Goodson et al, 1984). Thus, it is possible that the subunit molecular weight determined for porcine adipose tissue ACC may not reflect that of the native enzyme because the purification process may alter its structure (e.g. proteolytic degradation) or favor the purification of one form preferentially. Goodson et al (1984) have shown that monomeric [^{14}C-methyl] avidin binds to SDS-denatured biotinyl proteins and remains bound through polyacrylamide gel electrophoresis which allowed their detection by fluorography. Using this approach they have shown that crude rat liver cytoplasm contained two larger molecular weight forms of ACC (257K and 260K daltons) which undergo proteolysis during purification. A similar procedure might help in the determination of the native subunit molecular weight of porcine adipose tissue ACC in crude cytoplasm.

The specific activity of the final enzyme preparation was 3.2 units/mg enzyme protein. This value is lower than that reported in earlier literature for preparations of ACC from chicken liver, rat liver and rat mammary gland (Table 6). However, it is in close agreement with the value recently reported for preparations of chicken liver and rabbit mammary gland (Table 6). The higher enzyme specific activity of these earlier preparations has been associated with proteolytic degradation of the enzyme during purification, as trypsin treatment of the purified enzyme leads to an increase in activity (Iritani et al, 1969). Trypsin treatment has been shown to cause degradation

of the native rat liver subunit of 230K dalton into 124K and 118K dalton components and an increase in enzyme specific activity (Tanabe et al, 1975). Similar results have been reported for ACC from rabbit mammary gland (Guy and Hardie, 1981). It is of interest to note that chicken liver ACC purified in section IV A is proteolytically degraded and has a high enzyme specific activity (9.3 units/mg protein). Thus, it would appear that higher enzyme specific activity does not necessarily mean a very pure enzyme preparation but it might suggest the possibility of extensive proteolytic degradation of ACC during purification.

The K_m values determined for acetyl-CoA, ATP and HCO_3^- (0.038 mM, 0.36 mM and 7.7 mM respectively) are in close agreement with the values reported in literature for ACC purified from other sources (Miller and Levy, 1969;Moss et al, 1972;Inoue and Lowenstein, 1972). The K_a value of 0.90 mM determined for citrate is in close agreement with the value reported for ACC purified from other tissue sources. The biotin content of purified porcine ACC was 1.1 mole of biotin per mole of enzyme subunit. This corresponds to approximately 2 moles of biotin per mole of enzyme which is identical to values in literature for ACC from other sources.

The major difference between ACC isolated from porcine adipose tissue and ACC from other sources is in its low alkali-labile phosphate content and regulation by further phosphorylation. The alkali-labile phosphate content of ACC reported in literature varies considerably (Table 6). Hardie and Cohen (1979) reported that if F^- was ommitted during the isolation of rabbit mammary gland ACC, the phosphate content fell from 6.2 (in the presence of F^-) to 4.8 moles P_i/mole subunit of enzyme and the enzyme specific activity increased from 1.2 (in the presence of F^-) to 6.2 units/mg enzyme protein. This change in the alkali-labile phosphate content of ACC has been used to support the concept of regulation of ACC by a phosphorylation-dephosphorylation mechanism.

However, in the purification of porcine ACC the inclusion or ommission of F^- during the isolation procedure resulted in no significant change in the alkali-labile phosphate content or in the enzyme specific activity. These results indicate that porcine adipose tissue ACC is not phosphorylated to the same extent as the rabbit mammary gland ACC. If ACC from porcine adipose tissue was dephosphorylated during the isolation procedure, the phosphatase responsible would appear to be F^- insensitive. One would also expect a higher enzyme specific activity under these circumstances if dephosphorylation is associated with increase in enzyme activity. But, one cannot exclude the possibility that the site(s) containing the alkali-labile phosphate might have been lost during the purification as porcine adipose ACC has undergone minor proteolytic degradation. However, it is not likely to be the case as preparations of ACC from other sources containing high alkali-labile phosphate content show similar proteolytic degradation.

TABLE 6

PHOSPHATE CONTENT AND SPECIFIC ACTIVITY OF ACC FROM VARIOUS SOURCES

SOURCE	Enzyme S.A.[a]	PHOSPHATE[b] CONTENT	REFERENCE
Chicken Liver	11.0	NR[c]	Gregolin et al(1968)
Chicken Liver	5.2	NR	Beaty & Lane (1982)
Rat Liver	15.0	2.1	Inoue & Lowenstein (1972)
Rat liver	1.2	5.5	Witters & Vogt (1981)
Rat Liver	1.17	3.0	Tipper & Witters (1982)
Rat Mammary	15.0	6.0	Ahmad et al (1978)
Rat Mammary	6.7	3.1	Hardie & Guy (1980)
Rabbit Mammary	4.2	3.2	Hardie & Cohen (1978b)
Rabbit Mammary	1.2	6.2	Hardie & Cohen (1979)
Rabbit Mammary	3.0	4.8	Hardie & Cohen (1979)

a: Specific Activity (S.A.) expressed as units/mg protein.

b: Expressed as moles P_i/mole subunit enzyme.

c: Not Reported (NR).

Phosphorylation-dephosphorylation as a mechanism for the acute regulation of ACC activity has received much attention over the past 10 years. Carlson and Kim (1974) using a crude rat liver ACC preparation reported that it was inactivated upon incubation with Mg^{2+}-ATP. This was followed by the report from Hardie and Cohen (1979) that pure lactating rabbit mammary gland ACC isolated in a highly phosphorylated form with low enzyme specific activity could be converted into a higher specific activity enzyme upon incubation with protein phosphatase. Witters et al (1979) suggested that in rat hepatocytes, glucagon inhibits ACC activity through increasing phosphorylation of the enzyme while insulin stimulates ACC activity by decreasing its state of phosphorylation. Witters et al (1979) also pointed out that cAMP-dependent phosphorylation played a major role in the regulation of rat liver and adipose tissue ACC.

However, other reports (Halestrap and Denton, 1974;Desjardins and Dakshinamurti, 1978;Pekala et al, 1978;Gillevet and Dakshinamurti, 1983) have shown that cAMP did not affect enzyme activity in rat liver, adipose tissue or chicken hepatocyte cultures. Kim (1983) has pointed out that the principal anamoly in the concept of regulation through cAMP-mediated phosphorylation was that ACC from rat liver or epididymal tissue was not affected in vitro by cAMP nor was ACC phosphorylation inhibited by the presence of the inhibitor protein of cAMP-dependent protein kinase. Yeh et al (1980) presented evidence to show that 5' AMP stimulated phosphorylation and inactivation of rat liver ACC and that cAMP only mimicked the 5' AMP effect. Ly and Kim (1981) have reported that the effect of epinephrine on ACC activity might not be mediated through the β-adrenergic receptor mechanism but through a Ca^{2+} responsive α-receptor. However, this has been refuted by Allred et al (1983). Hardie and Guy (1980) working with purified rat mammary gland ACC showed that it could be phosphorylated and inactivated by ATP in the presence of cAMP-dependent

protein kinase. They reported a 65% decline in enzyme activity with incorporation of 1.5 mol of phosphate per mol subunit of the enzyme after 50 min of incubation. Tipper and Witters (1982) working with purified rat liver ACC showed that it was also inactivated (65%) and phosphorylated (1.09 mol phosphate per mol of enzyme after 90 min incubation) upon incubation with ATP in the presence of cAMP-dependent protein kinase. In this study in the absence of the cAMP-dependent protein kinase, purified rat liver ACC incorporated 0.26 mol of phosphate per mol of enzyme accompanied by a 43% decline in enzyme activity. Thus, cAMP-dependent protein kinase mediated inactivation of the enzyme was only 22%. In these studies phosphorylation was still taking place at a linear rate while no further decline in enzyme activity was observed (Hardie and Guy, 1980;Tipper and Witters, 1982). Lent and Kim (1983) also working with the purified rat liver enzyme showed that it could not be phosphorylated and inactivated by the catalytic subunit of the cAMP-dependent protein kinase, thus refuting the results of Tipper and Witters (1982). Results obtained by us with the purified porcine adipose tissue ACC indicate that cAMP-dependent protein kinase does not phosphorylate and inactivate this enzyme to the same degree that has been observed in the case of lactating rat mammary gland ACC (Hardie and Guy, 1980) and rat liver ACC (Tipper and Witters, 1982). The lack of phosphorylation of porcine adipose tissue ACC by the cAMP-dependent protein kinase and the subsequent inactivation of the enzyme cannot be ascribed to the degree of proteolytic degradation of the enzyme preparation used here. Other investigators who have shown inactivation of ACC by the cAMP-dependent protein kinase mediated phosphorylation used enzyme preparations that have undergone proteolysis to a similar or even higher degree when compared to porcine adipose tissue ACC used here (Hardie and Guy, 1980;Tipper and Witters, 1982).

In addition to the cAMP-dependent protein kinase which phosphorylates

ACC, another enzyme which phosphorylates ACC similarily has been isolated from lactating rabbit mammary gland and is referred to as ACC kinase-2 (Munday and Hardie, 1984). However, this enzyme can be distinguished from the free catalytic subunit of cAMP-dependent protein kinase by its molecular mass, its substrate specificity and by its complete lack of sensitivity to the inhibitor protein of cAMP-dependent protein kinase (Hardie and Guy, 1984). Furthermore, insulin which causes an increase in ACC activity has also been shown to cause phosphorylation of ACC (Witters, 1981;Witters et al, 1983;Brownsey et al, 1984). Two more cAMP-independent protein kinase (A & B) have been isolated from rat liver and are the equivalent of casein kinases (Brownsey and Denton, 1982). The sites on ACC phosphorylated by casein kinases seem to be similar to those phosphorylated by insulin, but this phosphorylation has no effect on enzyme activity (Witters et al, 1983;Tipper et al, 1983).

Studies to demonstrate correlation between the inactivation of ACC in vitro and the incorporation of [γ-^{32}P] ATP into the enzyme have not been very successful. Results presented here and those in literature indicate that the relatively small extent of inactivation of the enzyme and phosphorylation correlate only for the first 5 min of the reaction after which time phosphorylation is still proceeding without any effect on enzyme activity (Tipper and Witters, 1982;Gillevet and Dakshinamurti, 1983;Munday and Hardie, 1984). Studies done with hepatocytes in culture indicate that glucagon is able to inhibit (40%) acetyl-CoA carboxylase activity within 15 min after addition to the medium (Witters et al, 1979). However, studies carried out in vitro with pure rat liver ACC indicate that phosphorylation by the cAMP-dependent protein kinase for 15 min causes only about 20% inhibition of enzyme activity (Tipper and Witters, 1982). Similar results have been obtained with the rat mammary gland ACC (Hardie and Guy, 1980). The inactivation of ACC observed in vitro by phosphorylation does not seem to

correlate with the in vivo results. One of the classical examples of an enzyme that is regulated by phosphorylation-dephosphorylation is the pyruvate dehydrogenase (PDH) complex (Randle, 1981). Bovine kidney mitochondria PDH was inactivated (98%) upon incubation with ATP for 8 min and by this time phosphorylation of the enzyme had reached its maximum as well (Reed, 1981). Incubation of the phosphorylated enzyme with 10 mM $MgCl_2$ resulted in complete recovery of enzyme activity (Linn et al, 1969). The activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) has been shown to be modulated by a phosphorylation-dephosphorylation mechanism (Beg and Brewer, 1981;Beg et al, 1985). Maximal phosphorylation (1.05 mol of phosphate per mol of enzyme after 40 min) of the enzyme by the Ca^{2+}-dependent protein kinase C was associated with maximal loss in enzyme activity (80%) while dephosphorylation by the protein phosphatase resulted in complete recovery of enzyme activity (Beg et al, 1985). No such correlation can yet be established for ACC. Phosphorylation of an enzyme does not assure a role for phosphorylation in the regulation of enzyme activity. ATP-citrate lyase can be phosphorylated (0.9-1.0 mol of phosphate per subunit of enzyme) by the cAMP-dependent protein kinase, however, this phosphorylation had no effect on enzyme activity (Ramakrishna et al, 1983).

Analyzing the data presented here and by many other laboratories it is now clear that there is no controversy about ACC being phosphorylated. The general acceptance of the concept of regulation of ACC through phosphorylation-dephosphorylation is derived from the simplicity of the concept and its analogy to other systems like glycogen synthesis and breakdown as well as to lipolysis (Hardie, 1981). However, we now recognize that the observations regarding the correlation between phosphorylation of ACC and enzyme activity are too varied. There is controversy regarding the role of phosphorylation of ACC in the innate enzyme activity and the hypothesis that

phosphorylation-dephosphorylation results in the inactivation-activation respectively of ACC. It is apparent that a hypothesis that would explain all the observations does not exist. It is possible, as has been reported by Carlson and Kim (1974) that enzyme phosphorylation results in changes in the affinity of ACC for citrate and long chain acyl-CoA. In addition, phosphorylation-dephosphorylation state of ACC has been implicated in the dissociation-association of the lipogenic enzymes (Gillevet and Dakshinamurti, 1982). It has recently been reported that colchicine decreases ACC activity in crude preparations, suggesting that the shift in the protomer-polymer equilibrium of ACC may be important in the regulation of its activity (Buechler et al, 1984). However, there is still no answer to the question as to what regulates the site and extent of phosphorylation of ACC and the significance of phosphorylation(s) on the enzyme specific activity.

SECTION VI

BIOTIN: ROLE IN CELLULAR FUNCTIONS

As discussed in section III, the most well understood role of biotin in metabolism is as the prosthetic group of carboxylases. However, biotin has been implicated in several other areas of metabolism where its role is not well understood.

(a) Oxidative Phosphorylation

Pilgrim et al (1942) observed that biotin-deficient rat liver homogenates show only one third of the normal capability to oxidize pyruvate suggesting that biotin may play a role in the synthesis of dicarboxylic acid. The activity of rat liver acetyl-CoA carboxylase as well as the in vivo incorporation of [1-^{14}C] acetate into liver phospholipids are decreased to less than 50% of normal levels in biotin-deficiency (Dakshinamurti and Desjardins, 1968). Since lipids play a vital role as components of membranes, Dakshinamurti and co-workers investigated the effect of biotin-deficiency on mitochondrial function (Dakshinamurti et al, 1970a;Bhuvaneswaran and Dakshinamurti, 1971). They found no morphological differences between biotin-deficient and normal rat liver mitochondria. The evidence suggested a defect specifically at energy conservation site I (Bhuvaneswaran and Dakshinamurti, 1970).

(b) Carbohydrate Metabolism

A defect in the utilization of glucose by the biotin-deficient rat was reported by Dakshinamurti et al (1962). They showed that biotin-deficient

rats can use fructose or sorbitol better than glucose to generate energy for synthetic processes. The incorporation *in vivo* of [2-^{14}C] leucine into liver microsomal proteins of the deficient animal was restored to the level of control animals receiving biotin (Mistry and Dakshinamurti, 1964). Biotin-deficient animals showed poor tolerance to oral or intravenous glucose administration (Singh *et al*, 1963) and a decrease in glycogen synthesis (Mistry *et al*, 1962). Administration of insulin to the deficient animals restored liver glycogen and incorporation *in vivo* of [U-^{14}C] leucine in liver microsomal proteins to the normal level indicating that without insulin treatment the biotin-deficient animal was not able to utilize glucose as readily as fructose or sorbitol (Mistry *et al*, 1962). Analysis of some of the liver enzymes of the glycolytic and pentose pathways showed that hexokinase activity was decreased significantly during late stages of biotin-deficiency (Mistry *et al*, 1962) while glucose-6-phosphatase activity showed a slight increase in the deficient animal (Mistry and Dakshinamurti, 1964). The decrease in hexokinase activity in biotin-deficiency resulted in a decrease in the concentration of NADPH in liver (Dakshinamurti and Mistry, 1962). Analysis of the activities of $NADP^+$-dehydrogenases involved in the metabolism of glucose-6-phosphate indicated that the activity of glucose-6-phosphate dehydrogenase was increased while 6-phosphogluconate dehydrogenase activity was not significantly affected in the liver of biotin-deficient rats (Mistry and Dakshinamurti, 1964). As the activity of glucose-6-phosphate dehydrogenase is increased in biotin-deficiency, and since decreased fatty acid synthesis would result in a decreased utilization of NADPH, one would expect to find an increase in NADPH level rather than a decrease unless glucose-6-phosphate was not available for metabolism. This was found to be the case as glucose labelled in the 1- or 6-position was oxidized to [^{14}C] CO_2 at a considerably reduced rate in the deficient rat although the same animal

showed a marked increase in glucose-6-phosphate dehydrogenase activity of liver (Dakshinamurti and Mistry, 1962). Thus, formation of glucose-6-phosphate appeared to be the rate limiting step in the metabolism of glucose by the deficient animal and explained the beneficial effect of fructose or sorbitol substitution when glucose was the sole source of carbohydrate in the biotin-deficient diet.

Rat liver contains two glucose phosphorylating enzymes, glucokinase and hexokinase. They differ from each other in their affinity for the substrate. Glucokinase is highly responsive to glucose concentration in the physiologic range. As discussed previously, glucose phosphorylation by hexokinase was significantly reduced in biotin-deficiency. Dakshinamurti and Cheah-Tan (1968a) working with glucokinase showed that its activity was reduced 40-45% in the biotin-deficient rat liver and administration of biotin or insulin or both to the biotin-deficient rats restored liver glucokinase activity to the control level within 24 hr. They further showed that biotin administration to animals that were not biotin-deficient resulted in a 3-5 fold stimulation of glucokinase activity and inhibitors of protein synthesis inhibited the increase in enzyme activity suggesting that the action of biotin might be mediated through enzyme induction (Dakshinamurti and Cheah-Tan, 1968b). Dakshinamurti and Hong (1969) showed that in 14-18 day old suckling rat, injection of biotin caused a significant and precocious increase in the activity of glucokinase, phosphofructokinse and pyruvate kinase. Dakshinamurti et al (1970b) showed that biotin also enhanced liver glucokinase, phosphofructokinase and pyruvate kinase activity in the diabetic rat. Using inhibitors of protein and RNA synthesis they showed that increase by biotin in glucokinase activity in the diabetic rat was mediated through change in de novo protein synthesis even though glucokinase does not contain any biotin. Vesley (1982) showed that biotin and its analogues enhanced

soluble guanylate cyclase (E.C.4.6.1.2) activity 2-3 fold in rat liver, kidney, colon, cerebellum and heart. Recently, Spence and Koudelka (1984) reported that addition of biotin (10^{-6}M) to hepatocyte cultures caused a 3-fold increase in cGMP level and glucokinase activity was increased 4-fold suggesting that the effect of biotin on glucokinase might be mediated through changes in the intracellular level of cGMP.

(c) Ascorbic Acid and Folic Acid Metabolism

D-Glucose is converted to L-ascorbic acid via the intermediate D-glucuronic acid and L-gulonic acid. As previously discussed, the utilization of glucose is impaired in biotin-deficiency. It was found that the synthesis of L-ascorbic acid from glucose or L-γ-gulonolactone was severly decreased in biotin-deficiency due to reduction in glucose utilization and L-γ-gulonolactone oxidase activity, the enzyme which converts L-γ-gulonolactone to L-ascorbic acid (Dakshinamurti and Mistry, 1962). Biotin-deficient rats have also been reported to have lower stores of folate derivatives in the liver, as biotin has been shown to stimulate the conversion of folic acid into folate derivatives (Marchetti et al, 1965).

(d) Protein and RNA Synthesis

As long ago as 1956, Kristman et al (1956) observed that the incorporation of [^{35}S] methionine into protein in the biotin-deficient rats was reduced as compared to normal animals. Ravel et al (1961) showed that biotin was involved in the synthesis of ornithine transcarbamylase through the synthesis of a 4 carbon unit. Mistry and Dakshinamurti (1961) observed that incorporation in vivo of [1-^{14}C] leucine was decreased considerably in

microsomal, mitochondrial and nuclear proteins of biotin-deficient animals. Dakshinamurti and Mistry (1963b) showed that incorporation of [^{14}C] methionine into tissue proteins in vivo was reduced 20 to 40% in biotin-deficiency. This was related to alterations in the nature of the soluble ribonucleic acid which is involved in amino acid activation and transfer. They further suggested that when 10% succinate was added to the biotin-deficient diet, the amino acid incorporation into various cellular fractions in vivo was restored to normal indicating that the decrease in amino acid incorporation in biotin-deficiency was due to the result of a reduction in the synthesis of dicarboxylic acids. Dakshinamurti and Litvak (1970) showed that administration of biotin to biotin-deficient rats resulted in increased incorporation of [^{14}C] amino acids into the proteins of all subcellular fractions with greatest stimulation seen in the microsomal fraction. The effect was quite pronounced 12 hr after biotin administration. Boeckx and Dakshinamurti (1974) showed that administration of biotin to biotin-deficient rats resulted in stimulation by more than 2-fold of amino acid incorporation into protein, both inn vivo and in vitro, in rat liver, pancreas, intestinal mucosa and skin. Furthermore, dual-labelling analysis of amino acid incorporation indicated that the synthesis of some protein was stimulated more than 2-fold, but others were not stimulated at all suggesting a specificity in the stimulation of protein synthesis mediated by biotin (Boeckx and Dakshinamurti, 1974). As discussed previously, biotin has been shown to stimulate de novo synthesis of glucokinase (Dakshinamurti and Cheah-Tan, 1968b;Dakshinamurti and Hong, 1969;Dakshinamurti et al, 1970b;Spence and Koudelka, 1984).

It has been suggested that stimulation of protein synthesis by biotin in biotin-deficient animals is preceded by stimulation of RNA synthesis (Dakshinamurti and Litvak, 1970). They showed that biotin-treated deficient or normal rats incorporated [6-^{14}C] orotate into nuclear RNA to same extent

and actinomycin D injection 1 hr prior to biotin administration to the deficient animal abolished the stimulation in RNA synthesis (Dakshinamurti and Litvak, 1970). Later, Boeckx and Dakshinamurti (1975) showed that a single injection of biotin to the deficient rat produced a 2-fold increase in the incorporation, both in vivo and in vitro, of precursors into nucleic acids as early as 2 hr after biotin treatment and also caused a marked decrease in the messenger-free ribosome in rat liver.

In summary, biotin causes increase in activity of various enzymes that do not contain biotin through new protein synthesis as inhibitors of protein synthesis abolish the observed increase. Biotin also appear to cause increased synthesis of proteins which have not been characterized. Under the influence of biotin, mRNA synthesis and polysomal aggregation also seem to occur. The observed effects upon biotin administration to deficient animal appear to be direct as they become apparent within a short time interval (2 hr) after biotin administration.

However, it is possible that even at these short times after biotin administration to biotin-deficient animals, hormonal changes have occured under the influence of biotin and the effect on protein synthesis is secondary to the hormonal changes. There is no indication as to what these hormonal changes might be or to their identity. To avoid the possibility that hormonal changes are responsible for the effect of biotin on protein synthesis, one can investigate the effect of biotin on protein synthesis using cells in culture. This provides one with an opportunity to investigate the role of biotin in protein synthesis without the effect of any hormonal changes that may occur in the case of an animal.

(e) Biotin Requirement of Cells in Culture

That biotin should constitute an essential requirement for cells in culture would be expected from its obligatory involvement in carbohydrate and lipid metabolism. The requirement for biotin by cells in culture is satisfied by the serum supplement added to culture medium. Thus, studies carried out to investigate the biotin requirement for cells in culture have utilized culture media that is serum-free or contained serum depleted of biotin by different methods.

Initial studies utilizing serum that had been dialyzed extensively prior to addition to culture media indicated no biotin requirement for cells in culture (Eagle, 1955;Holmes, 1959;Dupree et al, 1962). Keranen (1972) included avidin, a biotin-binding protein, in culture medium on the rationale that avidin bound biotin will be unavailable to cells and showed that HeLa cells had no requirement for biotin and that HeLa cells were capable of synthesizing biotin.

However, other studies have demonstrated a requirement for biotin by cells in culture. A number of cell lines maintained in continuous culture in defined serum-free media were shown to require biotin for cell growth (Takaoka and Katsuta, 1971;Higuchi and Robinson, 1973). Pienskowska and Koziorowska (1975) using avidin in culture medium demonstrated a biotin requirement for mouse leukemic cells. Hatten et al (1977) and Cornell et al (1977) found that in the presence of avidin in culture media, cells accumulated in G1 phase of cell cycle and only the addition of lipid or biotin to the culture media allowed continuation of the cell cycle past G1. Messmer and Young (1976) using a low concentration of serum (0.20%) in the media showed that the growth of SV3T3 cells was enhanced by the addition of biotin or certain unsaturated fatty acids to the culture media. Moskowitz et al (1980) and Young et al

(1980) showed that biotin stimulated the growth of BHK (Baby Hamster Kidney) and SV40-transformed 3T3 cells. Dakshinamurti and Chalifour (1981) and Chalifour and Dakshinamurti (1982) using serum that was rendered biotin-deficient by avidin-Sepharose chromatography, showed a biotin requirement for HeLa cells, BHK and transformed BHK cells and human fibroblasts in culture and further showed that avidin-biotin complex added to culture medium was internalized by these cell lines. The internalized avidin-biotin complex is degraded and biotin made available to be used as the prosthetic group of biotin containing enzymes as the activity of propionyl-CoA carboxylase increases upon the addition of avidin-biotin to the deficient cell culture (Dakshinamurti and Chalifour, 1981;Chalifour and Dakshinamurti, 1982). This would cast doubt on studies which use avidin in culture medium to deplete medium biotin.

Cell culture provides one an opportunity to investigate effect of the addition or depletion of constituents on cellular processes. Using HeLa cells we have looked at the effects of biotin-deficiency and subsequent biotin addition to deficient cells on cellular processes that are related to cell growth.

(i) Materials

Eagle's Minimal Essential Medium (EMEM), fetal bovine serum (FBS), Earle's balanced salt solution, Penicillin G, Fungizone and trypsin solution (2.5%) were purchased from Grand Island Biological Co. (St. Louis, Mo.). L-[U-^{14}C] leucine (S.A. 336 mCi/mmol), L-[4,5-^{3}H] leucine (62 Ci/mmol), [8(n)-^{3}H] puromycin dihydrochloride (8 Ci/mmol), NCS tissue solubilizer and OCS scintillation cocktail were obtained from Amersham-Searle Corp. (Oakville, Canada). [1-^{14}C] sodium acetate (0.2 mCi/mmol), [methyl-^{3}H] thymidine (53 Ci/mmol) and Aquasol-2 scintillation cocktail were purchased from New England Nuclear Corp. (Montreal, Canada). [^{3}H] biotin (40 Ci/mmol) was a gift from Hoffman La-Roche Inc. (Nutley, N.J.). Avidin was obtained from Vega Biochemicals (Tucson, Az.). All electrophoresis reagents were obtained from Bio-Rad Laboratories (Mississauga, Canada). CNBr activated Sepharose 4B and high molecular weight protein marker kit were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Millipore filters (0.20 µm and 0.22 µm) were purchased from Millipore Ltd. (Mississauga, Ontario). Cordycepin and all other chemicals used were obtained from Sigma Chemical Co. (St. Louis, Mo.).

(ii) Preparation of Biotin-Deficient Fetal bovine serum

Fetal bovine serum was rendered biotin-deficient (BDFBS) using avidin-Sepharose affinity chromatography. Avidin was coupled to CNBr-Sepharose 4B according to the procedure of Dakshinamurti and Chalifour (1981). CNBr-Sepharose 4B (2-3 gm) was allowed to swell on a sintered glass

filter in 30 ml of 1 mM HCl and then washed with 200 to 300 ml of 1 mM HCl. The gel was suspended in 20 ml of coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3), 20 mg of avidin (S.A. 13-15 units/mg protein) added and incubated overnight in an end-over-end mixer. The gel was then washed with 100 ml of coupling buffer and reincubated for 2 hr at 4°C in 20 ml of 1 M ethanolamine (pH 8.0) to block any unreacted sites on the gel. The gel was washed alternatively with 100 ml of coupling buffer and 0.1 M Na-acetate containing 0.5 M NaCl (pH 4.0), ending with coupling buffer. This completed the preparation of avidin-Sepharose gel.

To make the fetal bovine serum biotin-deficient, 100 ml of FBS was incubated with avidin-Sepharose gel overnight at 4°C in an end-over-end mixer. After filtration on a sintered glass filter to remove the avidin-Sepharose gel, the biotin content of FBS before and after avidin-Sepharose gel treatment was determined.

(iii) Determination of Biotin Content

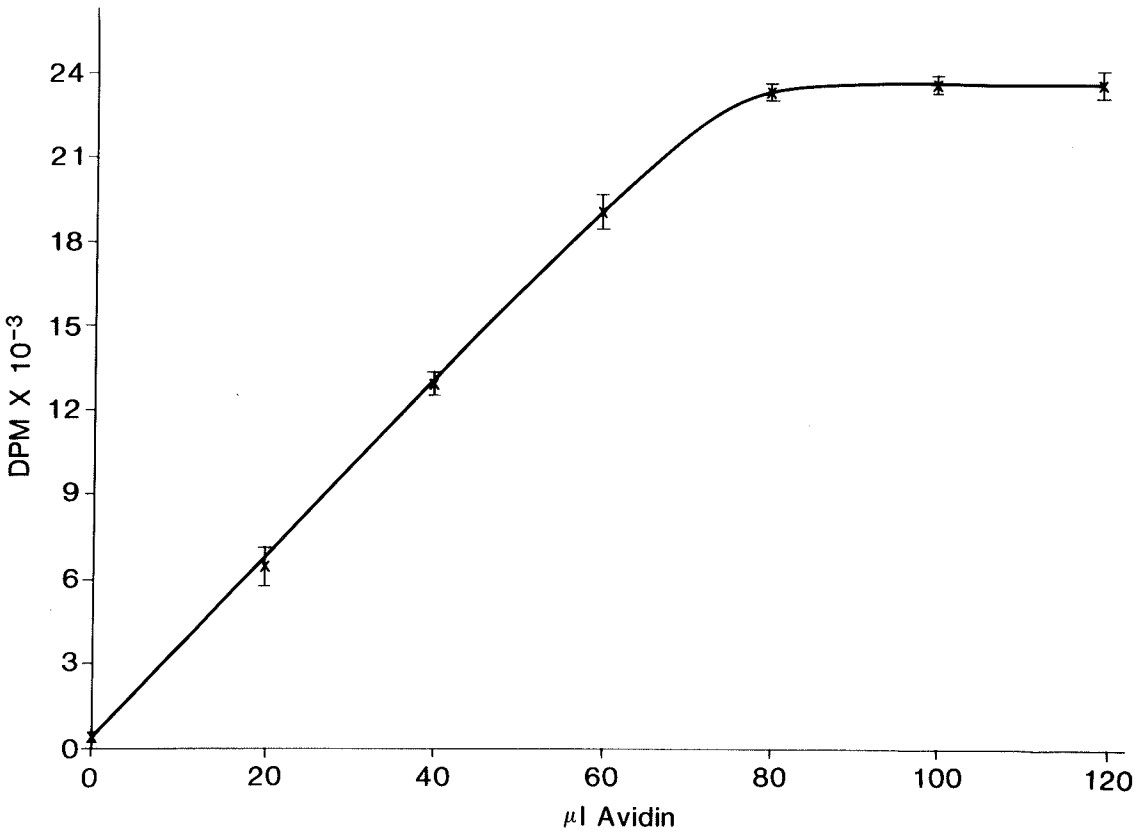
The biotin content was determined according to the isotope-dilution method of Dakshinamurti and Allan (1979).

(a) Preparation of Bentonite Suspension: The method is essentially the same as that of Fraenkel-Conrat et al (1961). 20gm of bentonite was suspended in 200 ml of distilled water and stirred vigorously overnight. The suspension was centrifuged at 3,000 x g for 15 min and the supernatant further centrifuged at 10,000 x g for 15 min. The pellet was collected and suspended in 0.2 M ammonium carbonate. Bentonite concentration was determined by drying an aliquot of the suspension at 60°C and weighing the residue. Suitable dilution was made to give a final concentration of 10 mg bentonite per ml.

(b) Determination of Equivalence between Avidin and [^{3}H] Biotin solution: In a

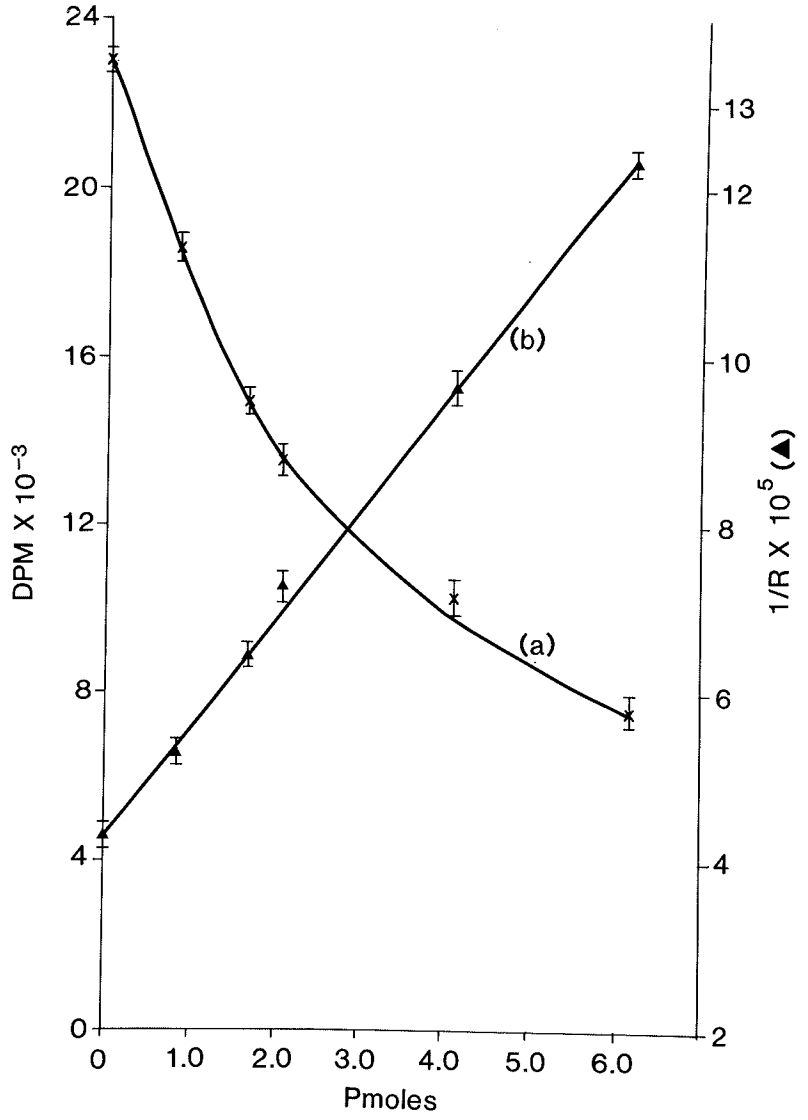
series of Eppendorf centrifuge tubes were placed: 25 µl [^{3}H] biotin (24,000 dpm) solution, 100 µl 1.55 M sodium sulfate solution and 300 µl 0.2 M ammonium carbonate solution. Varying volumes (0-120 µl) of the standard avidin (0.02 unit/ml) solution were added and the tubes allowed to mix for 15 min in an Eppendorf shaker at room temperature. 0.3 ml of bentonite (10 mg/ml) suspension was added, the tubes were allowed to mix in the shaker for 15 min after which time the solution was filtered on a Millipore filter (0.22 µM). The filter was washed with 5 ml of 0.2 M ammonium carbonate, dried and transferred to a scintillation vial containing 10 ml of Aquasol-2 scintillant. The vials were left overnight in the dark and radioactivity determined using a Beckman LS 2800 liquid scintillation spectrometer. Figure 17 is a representative curve obtained for the determination of equivalence between avidin and biotin. From the curve it is seen that 80 ul of the standard avidin (0.02 unit/ml) solution completely complexes with [^{3}H] biotin in 25 µl of the solution added.

(c) Biotin Calibration Plot: To a series of Eppendorf centrifuge tubes 300 µl of 0.2 M ammonium carbonate, 100 µl of 1.55 M sodium sulfate, 25 µl [^{3}H] biotin and increasing amounts (0-1.5 ng) of cold biotin were added. After mixing, 80 µl of the standardized avidin solution were added. After mixing in a shaker for 15 min, 0.3 ml of bentonite suspension was added and mixed for another 15 min. The precipitate of the avidin-biotin complex adsorbed on the bentonite was collected by filtration and the radioactivity determined as described previously. Figure 18 is a representative plot for the biotin calibration experiment. Curve (a) is a plot of the radioactivity of avidin-biotin-bentonite pellet against picomoles of biotin. However, the calibration curve is better represented as a semireciprocal plot of 1/R vs picomoles of cold biotin (Fig. 18 (b)) where R represents the radioactivity associated with avidin-biotin-bentonite complex. Curve (b) also shows that



EQUIVALENCE POINT BETWEEN AVIDIN AND [^{3}H] BIOTIN

Fig. 17. Varying volumes of standard avidin solution (0.02 units/ml) were added to a constant amount of [^{3}H] biotin (25 µl). Radioactivity associated with avidin-biotin-bentonite complex was determined as described in "Experimental" section.



CALIBRATION PLOT FOR BIOTIN

Fig. 18. (a) Varying amounts of cold biotin were added to a constant amount of [^{3}H] biotin. Radioactivity associated with the pellet was determined as described in "Experimental" section. (b) Represents a semireciprocal plot for dpm associated with the pellet as a function of amount of cold biotin present.

the assay procedure for biotin determination is linear for the concentrations of cold biotin utilized (0-1.5 ng).

(d) Determintion of biotin content of FBS and BDFBS: 1 ml of FBS or BDFBS was hydrolyzed in 4.5 N H_2SO_4 by autoclaving for 1 hr at 15 psi. The hydrolyzed sample was neutralized with NaOH and filtered. The filtrate was used for the determination of biotin.

(iv) Preparation of Dialyzed Fetal bovine serum

Dialyzed fetal bovine serum was prepared according to the method of Allen and Moskowitz (1978a). 1 vol of FBS or BDFBS was dialyzed against 10 vol of Earle's balanced salt solution for 48 hr at 4°C with buffer changes every 12 hr. The serum was then dialyzed at 4°C for 12 hr against 10 vol of EMEM. The dialyzed serum was sterilized by filtration through a 0.22 µm Millipore filter and used in experiments.

(v) Cell Culture Method

HeLa 229 cells (Nelson-Rees and Flandmeyer, 1976) were routinely cultured as a monolayer in EMEM supplemented with 5% FBS, 100 units/ml penicillin G, 100 µg/ml streptomycin and 2.5 µg/ml fungizone. To subculture, the growth medium was poured off and 2 ml of 0.25% trypsin solution added to the culture dish. After a short incubation at 37°C, the cells were gently shaken and a suitable aliquot of the cell suspension transferred to a culture dish containing fresh growth medium.

HeLa cells were rendered biotin-deficient by passaging four times in EMEM supplemented with 5% biotin-deficient fetal bovine serum.

The viability of the cells was determined using the Trypan blue exclusion test of Phillips (1973). 0.1 ml of the cell suspension was mixed with 0.4 ml of Trypan blue and allowed to incubate at 37°C for 5 min. A drop of this cell suspension was examined under a light microscope and the percent of dye-filled cells determined. The dye-filled cells were considered non-viable.

(vi) Definitions

The terms control, biotin-deficient and biotin-supplemented HeLa cells are used frequently and represent the following:

Control HeLa Cells: Refers to HeLa cells cultured routinely in EMEM supplemented with 5% FBS.

Biotin-Deficient HeLa Cells: Refers to HeLa cells cultured in EMEM supplemented with 5% BDFBS. Experiments where biotin-deficient HeLa cells were used, cells had been passaged four times in the biotin-deficient medium.

Biotin-Supplemented HeLa Cells: Refers to biotin-deficient cells (after the 4^{th} passage) that were supplemented with exogenous biotin (2 ng/ml).

(vii) Assay for Acetyl-CoA Carboxylase in HeLa cells

The assay for ACC in HeLa cell extract was performed according to the procedure described in section IV B. ACC activity was measured in control, biotin-deficient and biotin-supplemented HeLa cells.

(viii) Determination of Protein Content

Protein content of HeLa cell extract was determined according to the procedure of Lowry et al (1951). Bovine serum albumin was used to construct the standard curve.

(ix) ^{14}C-Acetate Incorporation into Total Cellular Lipids

Biotin-deficient HeLa cells were trypsinized, washed three times in phosphate-buffered saline (PBS, pH 7.5), suspended in biotin-deficient medium and divided into three equal parts. To each part [^{14}C] acetate (2.5 µCi/ml) was added except that in addition part II contained exogenous biotin (2 ng/ml) and part III contained exogenous biotin (2 ng/ml) plus cycloheximide (200 µg/ml). The cells were incubated at 37°C and at indicated times a sample was taken from each part and washed three times with cold PBS. Total lipid extraction was carried out according to the procedure of Bligh and Dyer (1959) as described by Cheng and Moskowitz (1982). The cell pellet was extracted at 0°C for 2 min with 1.5 ml of methanol-chloroform (2:1, v/v). 0.5 ml of chloroform was added followed 30 sec later by 0.5 ml of 0.04% calcium chloride solution. This was mixed and allowed to stand at 0°C for 30 min for phase seperation. The top alcohol layer was removed, 0.6 ml of the lower chloroform layer was transferred to a scintillation vial and evaporated under a stream of nitrogen. The residue was resuspended in 0.5 ml of NCS tissue solubilizer. 20 µl of glacial acetic acid followed by 10 ml of OCS scintillant were added and radioactivity determined in a Beckman LS 2800 liquid scintillation spectrometer.

(x) Protein Synthesis in HeLa Cells

To investigate cellular protein synthesis, control and biotin-deficient HeLa cells were removed from culture dishes, washed three times with PBS and resuspended seperately in 1.0 ml of leucine free EMEM. The cells were incubated for 20 min at 37°C in the presence of L-[U-^{14}C] leucine (10 µCi/ml). At the end of this incubation period cells were washed three times with cold PBS, resuspended in 0.3 ml of 10 mM Tris-HCl (pH 7.2) containing 0.15 M NaCl and sonicated in a Bronwill sonicator. A portion of the sonicate was centrifuged at 10,000 x g for 10 min to obtain the cytosolic fraction. An aliquot of sonicate and cytosol were placed on Whatmann 3MM filter paper, washed successively with cold 10% Trichloroacetic acid (TCA), 5% TCA (2x) and ethanol. The filter papers were dried, transferred to scintillation vial containing 10 ml of Aquasol-2 scintillant and radioactivity determined in a Beckman LS 2800 liquid scintillation spectrometer.

(xi) Dual-Labelling Analysis of Protein Synthesis

The effect of biotin-deficiency on protein synthesis in HeLa cells was examined using the dual-labelling technique (Boeckx and Dakshinamurti, 1974). Control and biotin-deficient HeLa cells were removed from culture dishes by trypsinization, washed three times in PBS and counted in a Coulter counter. Equal number of control and biotin-deficient HeLa cells were seperately resuspended in 1.0 ml of leucine free EMEM and incubated with L-[U-^{14}C] leucine (40 µCi/ml) and L-[4,5-^{3}H] leucine (100 µCi/ml) respectively for 20 min at 37°C. At the end of this incubation period, cells were washed three times with cold PBS, combined and suspended in 0.3 ml of 10 mM Tris-HCl (pH 7.2) containing 0.15 M NaCl. The cells were sonicated and the cytosolic

fraction prepared as previously described (x). The cytosolic fraction was analyzed using 10% polyacrylamide gels in the presence of sodium dodecyl sulfate according to the procedure of Laemmli (1970). The gel was sliced (2 mm), transferred to scintillation vials containing 0.9 ml NCS tissue solubilizer and incubated overnight at 56°C. 100 µl of water, 50 µl of glacial acetic acid, 10 ml of OCS scintillant was added and radioactivity determined in a Beckman LS 7500 liquid scintillation spectrometer. The radioactivity data was analyzed using computer program SCINT II (Wrogemann et al, 1977) to obtain the ratio of picomoles of leucine incorporated into proteins in control cells/picomoles of leucine incorporated into proteins in biotin-deficient cells. The molecular weight of proteins was determined by using the high molecular weigt protein marker kit.

(xii) Cellular Protein Synthesis in Biotin-Supplemented HeLa Cells

Biotin-deficient HeLa cell cultures were supplemented with exogenous biotin (2 ng/ml) while an identical culture was left in the biotin-deficient medium. At various times, cells were removed from the respective culture dishes by trypsinization, washed with PBS, resuspended in 1.0 ml of leucine free EMEM and incubated with L-[U-^{14}C] leucine (10 µCi/ml) for 20 min at 37°C. At the end of this incubation period, cells were washed three times with cold PBS and radioactivity associated with cytosolic proteins of biotin-supplemented and biotin-deficient cells determined as previously described (x).

(xiii) Effect of Additions to the Incubation Medium on Protein Synthesis

Biotin-deficient HeLa cell cultures were exposed to asparagine (100

μg/ml) and aspartic acid (100 μg/ml) respectively. 6 hr later the cells were removed from culture dishes by trypsinization, washed three times with PBS, resuspended in 1.0 ml of leucine free EMEM and incubated with L-[U-^{14}C] leucine (10 μCi/ml) for 20 min at 37°C. At the end of this labelling period, radioactivity associated with the cytosolic proteins was determined as described previously (x).

(xiv) Peptidyl-Puromycin Formation

For the determination of rate of peptidyl-puromycin formation in cell culture, biotin-deficient, biotin-supplemented and control HeLa cells were removed from respective culture dishes, resuspended after washing with PBS in 1.0 ml of EMEM and incubated for 30 min at 37°C in the presence of [8(n)-^{3}H] puromycin dihydrochloride (2 μCi/ml). At the end of this labelling period cells were washed with cold PBS, resuspended in 0.5 ml of 10 mM Tris-HCl (pH 7.2) containing 0.15 M NaCl and sonicated in a Bronwill sonicator. An aliquot of the sonicate was made 5% in TCA and the precipitated protein collected on 0.20 μm Millipore filter. The precipitate was washed with cold 5% TCA and all the washes were collected. The pellet was solubilized in 0.7 ml of 88% formaldehyde (Nakano and Hara, 1979). Radioactivity associated with free puromycin (pooled washes) and solubilized pellet was determined in a Beckman LS 2800 liquid scintillation spectrometer.

For incorporation of [^{3}H] puromycin in a cell-free system control, biotin-deficient and biotin-supplemented HeLa cells were removed from culture dishes and resuspended in 0.5 ml of 10 mM Tris-HCl (pH 7.5) containing 10 mM magnesium acetate, 100 mM ammonium chloride and 1 mM dithiothreitol. The cells were sonicated and the sonicate centrifuged for 20 min at 10,000 x g. The supernatant containing the ribosomes was used to determine

peptidyl-puromycin formation. The reaction mixture contained in a volume of 210 µl the following components: 10 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 100 mM ammonium chloride, 1 mM dithiothreitol, [^{3}H] puromycin (10 µCi) and ribosome fraction. Incubation was carried out at 37°C for 30 min after which time 50 µl aliquot of the reaction mixture was placed on Whatmann 3MM filter paper and washed successively in 10% TCA, 5% TCA (2x) and ethanol to remove any unreacted puromycin. The filter paper was dried and radioactivity determined after the addition of 10 ml of Aquasol-2 in a Beckman LS 2800 liquid scintillation spectrometer.

(xv) Effect of Cordycepin on Protein Synthesis

Control, biotin-deficient and biotin-supplemented HeLa cells were exposed to cordycepin (25 µg/ml) for 6 hr while identical cultures of control, biotin-deficient and biotin-supplemented cells were left in medium containing no cordycepin. At the end of this time period, cells were removed from respective culture dishes, washed with PBS and resuspended in 1.0 ml of leucine free EMEM. The cells exposed to cordycepin during culture conditions were supplemented with cordycepin at this stage also. The cells were labelled with [^{14}C] leucine and radioactivity associated with cytosolic proteins determined as previously described (x).

(xvi) Thymidine Incorporation

HeLa cells were grown for three passages in EMEM supplemented with 5% BDFBS. During the fourth passage, cells were subcultured and left in EMEM supplemented with 5% BDFBS for 6 hr. At the end of this time period, the medium was poured off and cells rinsed with warm EMEM. The cells were then

placed for 24 hr in EMEM supplemented with 5% dialyzed BDFBS to achieve G1 arrest of cell cycle (Allen and Moskowitz, 1978a). At the end of this G1 arrest time period, medium was poured off and cells allowed to grow for 2 days in EMEM supplemented with 5% BDFBS. The test cells were then supplemented with exogenous biotin (2 ng/ml) while an identical culture dish was left in the biotin-deficient medium. At various times, cells were removed from respective culture dishes by trypsinization, washed with PBS, counted in a Coulter counter and resuspended in 1.0 ml of EMEM. The cells were then labelled for 20 min at 37°C with [methyl-^{3}H] thymidine (10 µCi/ml). At the end of the labelling period cells were washed three times with cold PBS. To the cell pellet 20 µl of FBS was added to serve as a carrier, followed by 1.0 ml of 5% TCA and left to stand at 4°C overnight (Allen and Moskowitz, 1978b). The precipitate was collected by centrifugation and washed twice with cold 5% TCA. The precipitate was resuspended in 0.4 ml of NCS tissue solubilizer and radioactivity determined after the addition of 20 µl glacial acetic acid followed by 10 ml of OCS scintillant in a Beckman LS 2800 liquid scintillation spectrometer.

(xvii) Effect of Cycloheximide on Thymidine Incorporation

Biotin-deficient HeLa cells during the fourth passage were arrested in the G1 stage of the cell cycle as described previously (xvi). The cells were then allowed to grow for 2 days in EMEM supplemented with 5% BDFBS. Cells were then placed for 6 hr in EMEM containing exogenous biotin (2 ng/ml) or exogenous biotin (2 ng/ml) plus cycloheximide (100 µg/ml). An identical culture dish of biotin-deficient cells was also supplemented with cycloheximide. At the end of this time period cells were removed by trypsinization, washed with PBS, counted in a Coulter counter, resuspended in

1.0 ml of EMEM. Cells exposed to cycloheximide during culture conditions were supplemented with cycloheximide (100 µg/ml) at this stage also. The cells were then labelled with [methyl-^{3}H] thymidine and radioactivity determined as previously described (xvi).

(xviii) Assay Procedure for Cell Growth Determination

HeLa cells after the third passage in biotin-deficient medium were removed from culture dishes by trypsinization and counted in a Coulter counter. $4x10^{5}$ cells were inoculated into 60 mm culture dishes and cells synchronized as described previously (xvi). The cells were then supplemented with exogenous biotin (2 ng/ml) while an identical culture dish was left in biotin-deficient medium. At indicated times, cells were removed from respective culture dishes by trypsinization and counted in a Coulter counter. The growth curve for control cells was also determined as above except that FBS was substituted for BDFBS.

RESULTS

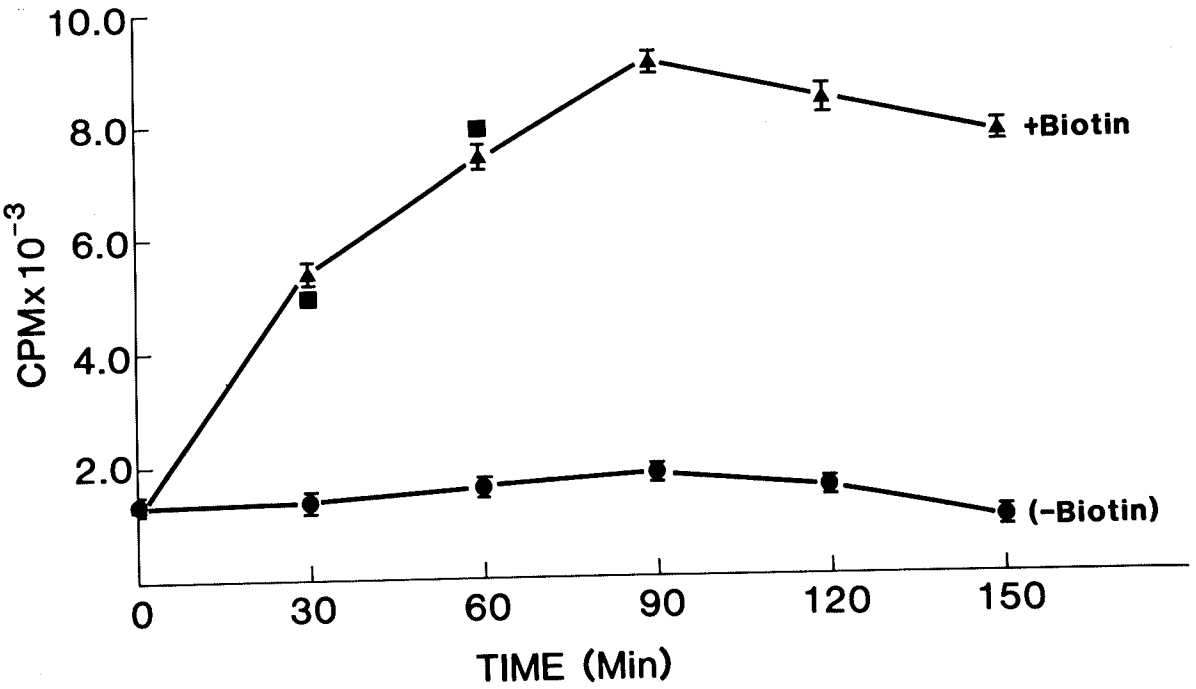
(i) Biotin Content of FBS and BDFBS

The biotin content of fetal bovine serum was determined to be 44.2 ± 2.5 ng/ml (mean ± S.D. of 6 determinations). After treatment with avidin-Sepharose the biotin content dropped to 3.20 ± 0.40 ng/ml (mean ± S.D. of 6 determinations). Only rarely was the biotin completely removed from FBS by avidin-Sepharose treatment. Thus, when serum used at a 5% concentration (v/v), the effective concentration of biotin in the media for cells cultured in FBS was 2.2 ng/ml and 0.16 ng/ml for cells cultured in BDFBS.

(ii) [^{14}C] Acetate Incorporation and ACC Activity

Biotin-deficient HeLa cells incorporated [^{14}C] acetate at a lower rate as compared to the biotin-supplemented cells (Fig. 19). Addition of exogenous biotin (2 ng/ml) to the biotin-deficient cells resulted in a rapid increase in the rate of [^{14}C] acetate reached a maximum by 90 min. Biotin-deficient cells showed no increase during the same time period. To investigate if protein synthesis was a prerequisite for this observed increase, [^{14}C] acetate incorporation was carried out in the presence of cycloheximide (200 µg/ml). The addition of cycloheximide to the biotin-supplemented cells did not inhibit the observed increase in [^{14}C] acetate incorporation (Fig. 19).

The acetyl-CoA carboxylase activity of biotin-deficient HeLa cells was 0.12 ± 0.04 munits/mg protein and 0.30 ± 0.05 munits/mg protein for the control cells. When biotin-deficient cells were exposed to exogenous biotin



[^{14}C] ACETATE INCORPORATION

Fig. 19. Biotin-deficient (●——●), biotin-supplemented (▲——▲) and biotin supplemented plus cycloheximide (■——■) HeLa cells were labelled with [^{14}C] acetate. At indicated times, a cell sample was taken and radioactivity incorporated into total cellular lipids determined according to procedure described in "Experimental" section.

(2 ng/ml) for 1 hr, the acetyl-CoA carboxylase activity increased to 0.43 ± 0.06 munits/mg protein, due to the conversion of apo acetyl-CoA carboxylase into holo acetyl-CoA carboxylase by the addition of biotin.

(iii) Effect of Biotin-Deficiency on Protein Synthesis

Biotin-deficient HeLa cells showed a significant decrease in rate of protein synthesis as measured by radioactive leucine incorporation into cellular proteins (Table 7). The observed decrease in the rate of protein synthesis was not due to changes in the uptake of leucine as the internal radioactive leucine pool was found to be similar in biotin-deficient and control HeLa cells (0.043 picomoles leucine/biotin-deficient cell and 0.042 picomoles leucine/control cell).

When protein synthesis was investigated using the dual-labelling technique, it was observed that polypeptides in the molecular weight range of 90-110K daltons showed a greater decrease in leucine incorporation compared to other molecular weight range polypeptides (Fig. 20) in the biotin-deficient cells as compared to control cells.

(iv) Effect of Additions to Biotin-Deficient HeLa Cells

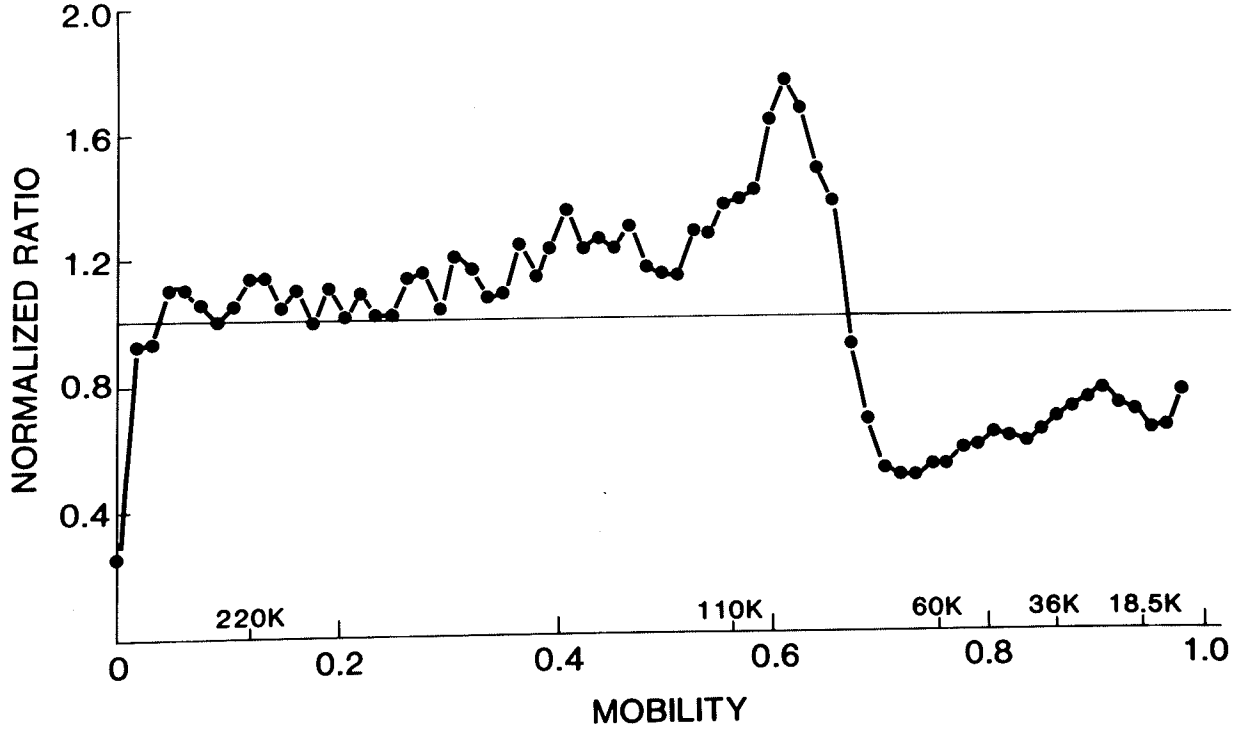
When HeLa cells cultured in biotin-deficient media were supplemented with exogenous biotin (2 ng/ml), the incorporation of radioactive leucine into proteins was stimulated about 2-fold (Fig. 21). This increased rate of protein synthesis reached a plateu by approximately 6 hr after the addition of exogenous biotin. The new steady state rate of protein synthesis in the biotin-supplemented cells was similar to that observed for the control cells (Table 7 and Fig. 21). The rate of protein synthesis in the biotin-deficient

TABLE 7

EFFECT OF BIOTIN-DEFICIENCY ON PROTEIN SYNTHESIS

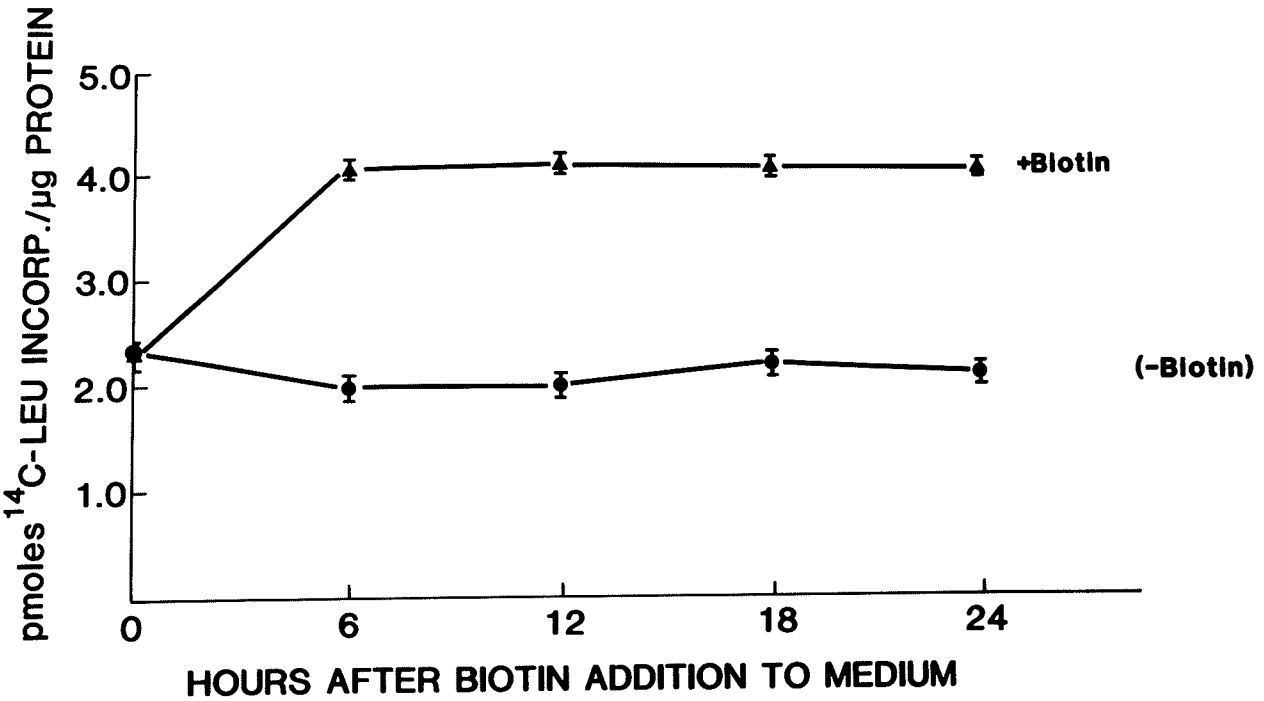
	picomoles ^{14}C-Leucine Incorporated per µg protein		
	Control	Biotin Deficient	% Decrease
HOMOGENATE	5.3 ± 0.3	3.4 ± 0.4	36
CYTOSOL	4.1 ± 0.3	2.8 ± 0.3	32

Control and biotin-deficient HeLa cells were incubated for 20 min at 37°C in the presence of L-[^{14}C] leucine. Protein associated radioactivity in the homogenate and cytosol fractions was determined as described under "Experimental" section. Values represent mean ± S.D. of four determinations in each group.



DUAL LABELLING ANALYSIS

Fig. 20. Equal number of control and biotin-deficient cells were labelled respectively with L-[U-^{14}C] and L-[4,5-^{3}H] leucine for 20 min at 37°C. After combining the cells, cytosolic fraction was analyzed by 10% SDS-PAGE. Gel was sliced (2 mm) and radioactivity determined as described in "Experimental" section. Radioactivity data were converted by the use of a computer program to obtain a ratio of the picomoles leucine incorporated in protein of control cells/picomoles of leucine incorporated in protein of biotin-deficient cells.



TIME RESPONSE CURVE FOR PROTEIN SYNTHESIS AFTER BIOTIN ADDITION

Fig. 21. Biotin-deficient cells were supplemented with exogenous biotin (2 ng/ml) while an identical culture was left in biotin-deficient medium. At indicated times, cells were removed from the respective culture dishes and incorporation of radioactive leucine into cell proteins determined as described in "Experimental" section. The results represent mean ± S.D. of three seperate determinations at each time point.

TABLE 8

EFFECT OF ADDITIONS TO BIOTIN-DEFICIENT CULTURE ON PROTEIN SYNTHESIS

Additions	pmoles leucine incorporated per µg protein
None	2.4 ± 0.3
Asparagine (100 µg/ml)	2.2 ± 0.4
Aspartic acid (100 µg/ml)	2.3 ± 0.4

To seperate culture dishes of biotin-deficient HeLa cells were added respectively, asparagine and aspartic acid at concentrations indicated. 6 hr later cells were removed and L-[^{14}C] leucine incorporation into cytosolic proteins determined as described in "Experimental" section. Values represent mean ± S.D. of four determinations in each group.

cells remained unaffected during the same time period. When biotin-deficient cells were supplemented with asparagine (100 µg) or aspartic acid (100 µg), the rate of protein synthesis did not change from that observed in the biotin-deficient cells (Table 8).

(v) Peptidyl-Puromycin Formation

An alternative approach that can be used to determine the rate of protein synthesis is by using puromycin. Puromycin binds to the C-terminal of the growing peptide chain on ribosomes and thus stops further chain elongation (Kersten, 1971). Thus, if one can determine the number of puromycin molecules attached to the nascent peptide chain by using radioactive puromycin, an estimate of the number of growing peptide chains can be obtained which in turn reflects the rate of protein synthesis as well as the number of ribosomes active in protein synthesis. This approach has been used for *in vivo* and *in vitro* incorporation of [^{3}H] puromycin into peptidyl-puromycin (Clarke and Ward, 1983).

Results using cultured cells indicate a significantly lower peptidyl-puromycin formation in biotin-deficient HeLa cells as compared to the control cells (Table 9). When biotin-deficient cell cultures were supplemented with exogenous biotin (2 ng/ml), the rate of peptidyl-puromycin formation was increased to the level observed in control cells (Table 9). Peptidyl-puromycin formation was also investigated using cell free system. This method does not depend on the maintenance of polysomal integrity. Results obtained with cell free system confirm the cell culture observations. The number of active ribosomes carrying peptidyl-tRNA was significantly decreased in biotin-deficient cells as compared to the control cells (Table 9). Upon the addition of exogenous biotin (2 ng/ml) to biotin-deficient

TABLE 9

INCORPORATION OF [8(n)-^{3}H] PUROMYCIN DIHYDROCHLORIDE

Status	Cell Culture*	Cell Free**
Control***	110 ± 4	4.22 ± 0.18
Biotin-deficient	80 ± 3	2.63 ± 0.25
Biotin-supplemented****	120 ± 6	4.01 ± 0.10

HeLa cells were labelled with radioactive puromycin for in vivo and in vitro incorporation as described in methods. Values represent mean ± S.D. of three determinations in each group.

* Results expressed as bound DPM/free DPM per mg protein.

** Results expressed as nmoles puromycin incorporated per µg protein.

*** Refers to HeLa cells cultured continuously in biotin containing medium

**** Refers to biotin-deficient cells that were exposed to 2ng/ml of exogenous biotin for 12h.

cells, the number of active ribosomes carrying peptidyl-tRNA was similar to that observed in control cells (Table 9).

(vi) Effect of Cordycepin on Protein Synthesis

Cordycepin (3' deoxyadenosine), an analogue of adenosine has been shown to inhibit RNA synthesis in cells in culture (Guarino, 1977). When biotin-deficient HeLa cells were supplemented with biotin in the presence of cordycepin (25 µg/ml), no increase in protein synthesis was observed (Table 10). However, when biotin-deficient cells were exposed to biotin in the absence of cordycepin, an increase in the rate of protein synthesis was observed which matched the rate observed in control cells (Table 10). The concentration of cordycepin used in this experiment did not have an adverse effect on protein synthesis as control cells have identical rate of protein synthesis in the presence and absence of cordycepin (Table 10).

(vii) Effect of Biotin-Deficiency on Rate of DNA Synthesis

HeLa cells cultured in biotin-deficient media have a reduced rate of DNA synthesis as measured by radioactive thymidine incorporation. When biotin-deficient cells were supplemented with exogenous biotin (2 ng/ml), there was a significant increase in rate of DNA synthesis reaching a maximum by 4 hr (Fig. 22). This new rate of DNA synthesis in the biotin-supplemented cells was maintained for approximately 24 hr (results not shown).

To investigate if protein synthesis was a prerequisite for the observed increase, thymidine incorporation was carried out in the presence of cycloheximide (100 µg/ml). Under these conditions, a small but significant increase in thymidine incorporation was observed in the biotin-supplemented

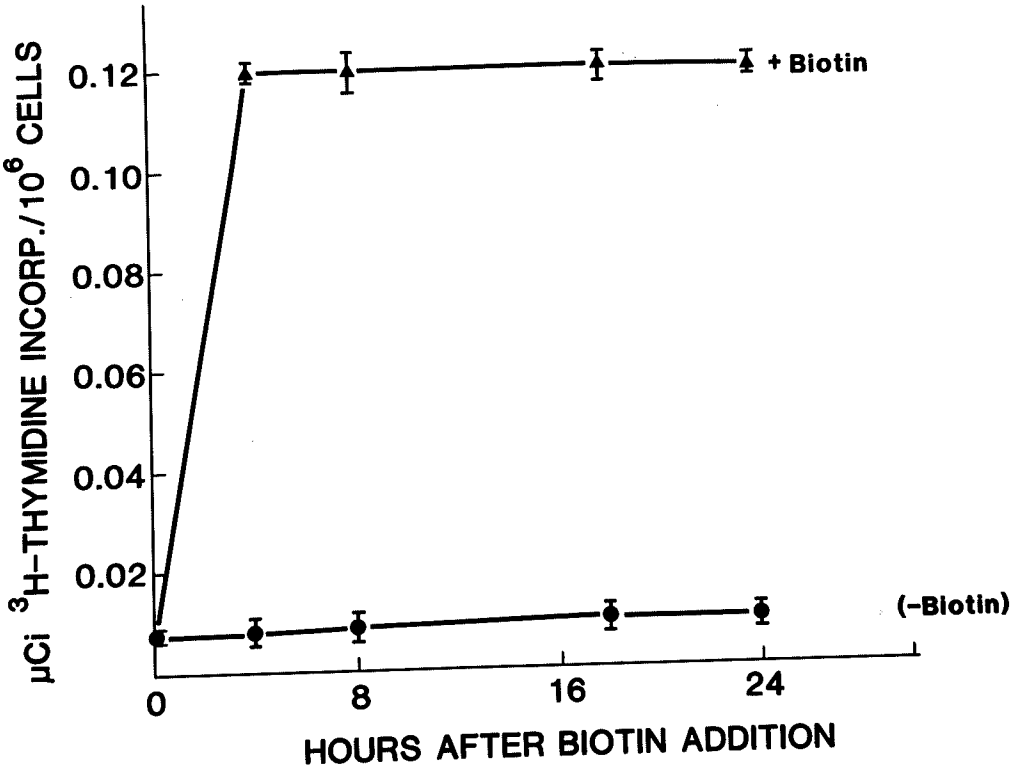
TABLE 10

PROTEIN SYNTHESIS IN THE PRESENCE OF CORDYCEPIN

Status	pmoles ^{14}C leucine incorporated/µg protein
Control*	3.9 ± 0.2
Control+cordycepin	3.9 ± 0.2
Biotin-deficient (BD)**	2.8 ± 0.3
BD+cordycepin	2.8 ± 0.2
Biotin-supplemented (BS)***	3.8 ± 0.2
BS+cordycepin	2.8 ± 0.3

Cordycepin at a concentration of 25 µg/ml was added to the culture dishes for 6 hr. Values represent mean ± S.D. of 3 determinations in each group.

* Refers to HeLa cells cultured continuously in biotin containing medium.
** Refers to HeLa cells cultured in 5% BDFBS for four passages.
*** Refers to biotin-deficient HeLa cells exposed to 2ng/ml exogenous biotin for 6 hr.



[METHYL-^{3}H] THYMIDINE INCORPORATION

Fig. 22. Synchronized biotin-deficient cells were exposed to exogenous biotin (2 ng/ml) while an identical culture was left in biotin-deficient medium. At indicated times, cells were removed from respective culture dishes and [methyl-^{3}H] thymidine incorporation determined as described in "Experimental" section. The results represent mean ± S.D. of three seperate determinations at each time point.

cells (Table 11). However, in the presence of cycloheximide the rate of thymidine incorporation does not reach the same rate as that observed in biotin-supplemented cells not exposed to cycloheximide (Table 11).

(viii) Growth of HeLa Cells Under Different Conditions

HeLa cells cultured in biotin-deficient media had a reduced rate of growth as compared to biotin-supplemented and control cells (Fig. 23). The difference in growth between biotin-deficient and biotin-supplemented cells became more pronounced after approximately 40 hr (Fig. 23). The growth of biotin-supplemented cells matched that of control cells.

TABLE 11

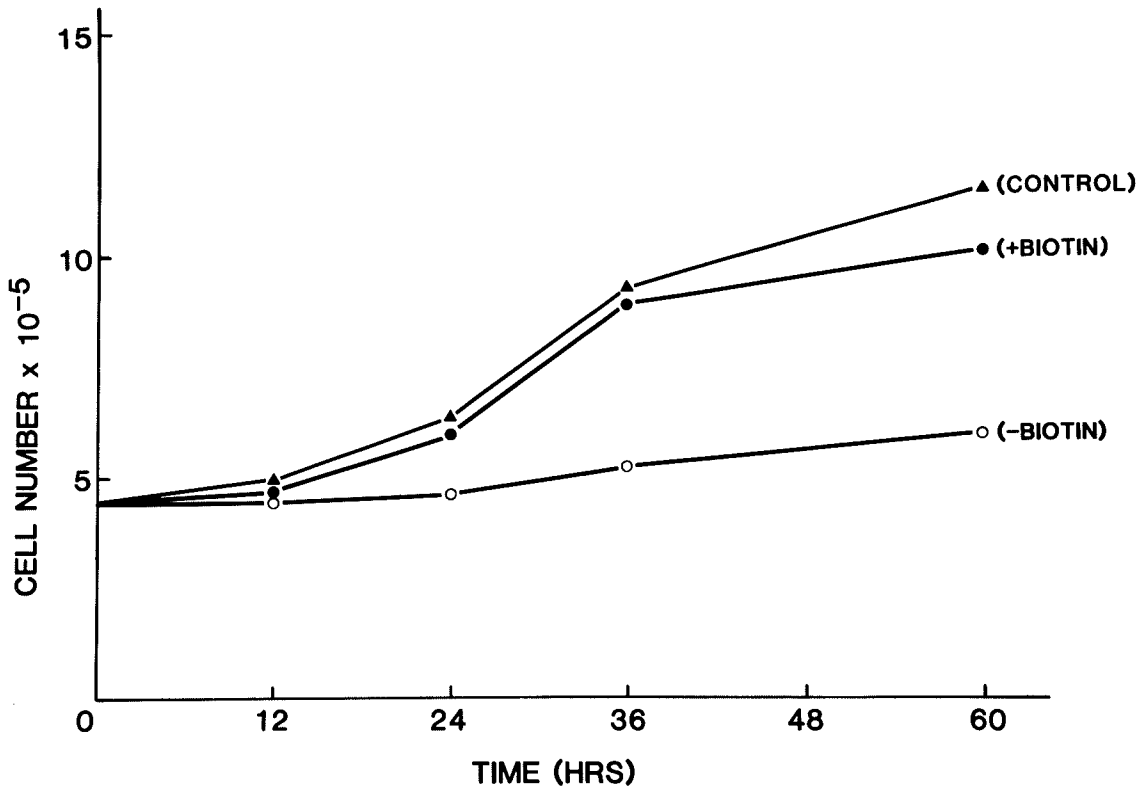
[METHYL-^{3}H] THYMIDINE INCORPORATION IN THE PRESENCE OF CYCLOHEXIMIDE

Status	μCi thymidine incorporated /10^6 cells
Biotin-supplemented*	0.12 ± 0.0010
Biotin-supplemented + cycloheximide	0.05 ± 0.0008
Biotin-deficient	0.02 ± 0.0005
Biotin-deficient + cycloheximide	0.02 ± 0.0005

Biotin-deficient and biotin-supplemented HeLa cells respectively were exposed to 100 μg/ml of cycloheximide for 6 hr. At the end of this incubation cells were labelled with [methyl-^{3}H] thymidine and radioactivity determined as described in methods.

Values represent mean ± S.D. of 3 determinations in each group.

* Refers to biotin-deficient cells exposed to 2ng/ml exogenous biotin for 6h.



GROWTH OF HeLa CELLS IN DIFFERENT CULTURE MEDIUM

Fig. 23. Control and biotin-deficient cells were synchronized as described in "Experimental" section. Biotin-deficient cells were either supplemented with exogenous biotin (2 ng/ml) or left in the biotin-deficient medium. At indicated times control (▲——▲), biotin-deficient (○——○) and biotin-supplemented (●——●) cells were removed from respective culture dishes and counted in a Coulter cell counter. The figure above represents average of three culture plates at each time point.

DISCUSSION

Biotin plays an important role in carbohydrate and lipid metabolism as it is the prosthetic group of the carboxylases (Mistry and Dakshinamurti, 1964;Wood and Bardeen, 1977). Of the biotin-dependent carboxylases of significance, acetyl-CoA carboxylase is required for lipogenesis and hence membrane genesis. Pyruvate carboxylase is essential for the generation of oxaloacetate and the maintenance of tricarboxylic acid cycle (Nakano et al, 1982) and for gluconeogenesis. Propionyl-CoA carboxylase and β-methyl crotonyl-CoA carboxylase are required for the further metabolism of certain amino acid residues. Thus, under culture conditions biotin should subserve the lipogenic and dicarboxylic acid needs of the cell.

However, earlier studies indicated no biotin requirement for cells in culture (Eagle, 1955;Holmes, 1959;Dupree et al, 1969;Keranen, 1972). The presence of protein-bound biotin in the serum supplement of culture media used by these investigators may have masked the biotin requirement of cells in culture as other studies indicated a biotin requirement for cells in culture (Takaoka and Katsuta, 1971;Higuchi and Robinson, 1973;Pienskowska and Koziorowska, 1975;Messmer and Young, 1976;Hatten et al, 1977;Cornell et al, 1977;Moskowitz et al, 1980;Young et al, 1980). Dakshinamurti and Chalifour (1981) and Chalifour and Dakshinamurti (1982) using serum that was rendered biotin-deficient by avidin-Sepharose chromatography, showed a biotin requirement for HeLa cells and human fibroblasts in culture and that avidin-biotin complex added to the culture medium was taken up by cells and once internalized the biotin was made available to the cell to be incorporated as the prosthetic group of biotin enzymes.

In the present work we have used serum in the culture medium that was

rendedred biotin-deficient by the use of avidin-Sepharose chromatography. Biotin-deficient HeLa cells incorporate [^{14}C] acetate into lipids at a reduced rate as compared to the control cells. Once supplemented with biotin, there is a 4-fold increase in [^{14}C] acetate incorporation into lipids. Cycloheximide, a potent inhibitor of protein synthesis, did not abolish the observed increase in acetate incorporation into lipids indicating that new protein synthesis is not required for the effect. This increased lipid synthesis in the biotin-supplemented cells is due to the conversion of apo acetyl-CoA carboxylase into holo acetyl-CoA carboxylase, the rate limiting enzyme for fatty acid synthesis (Volpe and Vagelos, 1976). This suggestion is confirmed by the observed increase in activity of acetyl-CoA carboxylase in the biotin-supplemented cells. However, the decrease in acetyl-CoA carboxylase activity in biotin-deficient HeLa cells is not of the same magnitude as has been reported for propionyl-CoA carboxylase in biotin-deficient HeLa cells and fibroblasts (Dakshinamurti and Chalifour, 1981;Chalifour and Dakshinamurti, 1982). It is possible that any minute quantities of biotin present in the deficient culture medium once taken up by the cells are utilized in the cytosol for the conversion of apo acetyl-CoA carboxylase into holo acetyl-CoA carboxylase. As propionyl-CoA carboxylase is a mitochondrial enzyme, less of the biotin under biotin-deficient culture conditions might be available to mitochondria and thus a greater decrease in the activity of propionyl-CoA carboxylase as compared to acetyl-CoA carboxylase would be expected.

Although each of the biotin containing enzymes has a specific role in the operation of the various metabolic pathways in the cell, only acetyl-CoA carboxylase seems to be of significance under cell culture conditions in the synthesis of fatty acids for membrane genesis (Horwitz et al, 1974). However, serum lipids are taken up by cells (Wisniesky et al, 1973) and saturated fatty

acid are without growth-promoting activity for SV3T3 cells under conditions of biotin-deficiency (Messmer and Young, 1976). In our experiments we specifically removed biotin from FBS by avidin-Sepharose chromatography, thus other components of serum including lipid are unaltered. Under these conditions a requirement for biotin is demonstrated by HeLa cells. The addition of biotin to biotin-deficient cells resulted in an increase in rate of protein synthesis to the level observed in control cells. The amount of exogenous biotin (2 ng/ml) added to the biotin-deficient HeLa cell cultures was similar to that used in media for the culturing of control cells.

Pienskowska and Koziorowska (1975) have shown that mouse-leukemic cells grown in a biotin-deficient medium had a decreased content of aspartic acid and Nakano et al (1982) suggested that biotin is critical for maintaining the Kreb's cycle through its role in oxaloacetate formation. However, not all intermediates of Kreb's cycle were growth-promoting in their experiments. Adler et al (1981) reported that aspartic acid and fatty acids are unable to replace the biotin requirement of Saccharomyces cerevisiae and Aspergillus nidulans. Our results indicate that the effect of biotin-deficiency on protein synthesis in HeLa cells is not mediated through changes in the aspartate content as addition of L-aspartic acid or asparagine to biotin-deficient HeLa cells does not cause an increase in the rate of protein synthesis while the addition of biotin does.

Puromycin binds to the C-terminal of the growing peptide chain and thus stops further chain elongation (Kersten, 1971). Thus, by using radioactive puromycin one can estimate the number of growing peptide chains which in turn reflects the rate of protein synthesis as well as the number of ribosomes active in protein synthesis. This approach has been used for in vivo and in vitro incorporation of [^{3}H] puromycin into peptidyl-puromycin (Clarke and Ward, 1983). The in vitro system reflects the protein synthesizing activity

of the cells correlating directly with the level of polysomes (Aharonowitz and Zon, 1975) and furthermore, the *in vitro* assay of ribosome activity does not depend on the polysome integrity (Unsworth and Pain, 1983). Nakano and Hara (1979) using this *in vivo* approach showed that both starvation and feeding of a protein-free diet to rats caused marked decreases in the relative rate of formation of peptidyl-puromycin, which reflects the protein synthesis activity *in vivo*, in liver, skeletal muscle, heart, spleen, lung, testis, kidney and intestine. Clarke and Ward (1983) using the *in vivo* approach showed that cardiac protein synthetic activity in rats, as determined by the rate of peptidyl-puromycin formation, was increased by both tri-iodothyronine and isoproterenol, implying an increase in the number of functional ribosomes. They further showed that no increase in protein synthetic rate was detected when [^{14}C] tyrosine incorporation into proteins was used as a measure for changes in protein synthetic activity indicating that the use of [^{3}H] puromycin was a more sensitive method for detecting changes in rate of protein synthesis (Clarke and Ward, 1983). Results obtained using both approaches indicate a decrease in the number of functional ribosomes in the biotin-deficient HeLa cells. Addition of biotin causes an increase in the level of functional ribosomes to that observed in control cells.

The observed increase in rate of protein synthesis can be due to either increased rate of translation of preformed mRNA or due to the synthesis of new mRNA. One can differentiate between these two hypotheses by using an inhibitor of RNA synthesis. Cordycepin, an analogue of adenosine, prevents the appearance of newly synthesized mRNA into the cytoplasm but does not interfere with the synthesis of hnRNA, the putative precursor of cytoplasmic mRNA (Adesnik *et al*, 1972). In HeLa cells, DNA and protein synthesis as well as hnRNA synthesis are insensitive to cordycepin while rRNA and mRNA formation is prevented (Siev *et al*, 1969). Results presented here indicate that

appearance of newly synthesized RNA in the cytoplasm is essential for the observed increase in rate of protein synthesis in biotin-supplemented cells. The inability of cordycepin treated HeLa cells to show an increase in rate of protein synthesis is due to the lack of appearance of newly synthesized RNA in the cytoplasm. The defect can be at the level of synthesis and transport of hnRNA or just at the level of transport from the nucleus into cytoplasm. The results presented here do not allow us to differentiate between these two events. But, one can say that appearance of newly synthesized RNA in the cytoplasm is an event brought about when biotin-deficient cells are supplemented with biotin.

Moskowitz and Cheng (1979) presented evidence that Rous sarcoma virus-transformed BHK (Baby Hamster Kidney) cells cultured in the absence of biotin show reduced DNA synthesis and are blocked in G1 stage of cell cycle. Later, Cheng and Moskowitz (1982) showed that G1 arrested Rous sarcoma virus transformed BHK cells multiplied rapidly in basal media supplemented with dialyzed fetal calf serum but did not multiply in media containing delipidized serum. From these studies they concluded that although lipids were necessary for continuous growth, the stimulation of the synthesis of growth factors by biotin are also required for continuous cell multiplication. However, in their studies the exact biotin status of the cells is not clear as they used avidin to deplete the media of biotin. As Dakshinamurti and Chalifour (1981) and Chalifour and Dakshinamurti (1982) have shown that cells are able to internalize the avidin-biotin complex and once internalized can utilize the biotin, use of avidin in the medium might not be a good technique to investigate the biotin requirement of cells in culture. In our investigation using biotin-depleted serum, HeLa cells cultured in lipid containing medium showed a decreased growth rate and addition of biotin restored the growth of cells to the level observed in control cells. Protein synthesis is necessary

for the increased DNA synthesis observed in biotin-supplemented HeLa cells as cycloheximide, a potent inhibitor of protein synthesis (Gautschi et al, 1973), partially abolishes the observed increase. It is significant to note that the incorporation of radioactive thymidine into DNA reaches a maximum within 4 hr after addition of biotin to the biotin-deficient cells and by this time there has already been stimulation of protein synthesis. One could speculate that these two events are related and that the growth-promoting effect of biotin might be achieved through stimulation of the synthesis of yet uncharacterized protein(s).

SECTION VII

BIOTIN: PRESENCE IN NUCLEUS

Dakshinamurti and Mistry (1963a) investigated the uptake of radioactively labelled biotin into various tissues of rat and chick. Upon injection of [^{14}C-carboxyl] biotin to biotin-deficient rats, 15-30 % of the radioactivity was recovered in the liver and kidney, approximately 10% was recovered in muscle and viscera, 7-27 % remained unabsorbed in the breast muscle and about 30% of the dose was excreted (Dakshinamurti and Mistry, 1963a). When the distribution of radioactive biotin in intracellular fractions of rat liver was investigated, 52% of the radioactivity was found in the supernatant of rat liver homogenate, another 18% in the mitochondria, 15% in the nuclei and a very small amount in microsomes (Dakshinamurti and Mistry, 1963a). Furthermore, it was reported that 89% to 99% of the biotin was in the bound form (Dakshinamurti and Mistry, 1963a). Boeckx (1973) reported that the normal rat liver contained about 2 µg of biotin per gram fresh tissue and about 9% (0.18 µg) of this biotin was localized in the nucleus. Biotin-deficient rat liver contained only 0.162 µg of biotin per gram fresh tissue and of this 19% (0.03 µg) was localized in the nucleus suggesting that nuclear biotin was conserved in biotin-deficiency (Boeckx, 1973). When [^{14}C-carboxyl] biotin uptake by liver <u>in vivo</u> was investigated 30 min after injection of the radioactive biotin, it was found that 15% of the radioactive biotin was associated with the nucleus, all of which was protein bound, while the cytosol only contained 5% of the radioactive biotin in the protein bound form (Boeckx, 1973). This suggested that nucleus was rapidly incorporating biotin during this time although no function for biotin in the nucleus has been reported. Chalifour (1982) using various methods for subcellular fractionation investigated the radioactive biotin distribution in rat liver.

Considerable amount of radioactive biotin was found associated with nucleus in protein bound form (Chalifour, 1982). When incorporation of radioactive biotin into subcellular fractions of HeLa cells was investigated, it was found that supplementing the biotin-deficient HeLa cells with avidin-[^{3}H] biotin complex resulted in a higher amount of radioactive biotin associated with nuclei (9.8 pmole/mg protein) as compared to cells supplemented with [^{3}H] biotin (0.42 pmole/mg protein). Uptake of the avidin-[^{3}H] biotin complex by HeLa cells and human skin fibroblasts was reported to be through a receptor mediated endocytosis mechanism and pretreatment of cells with cycloheximide inhibited binding and uptake of the avidin-biotin complex (Chalifour and Dakshinamurti, 1982a;1982b). The uptake of biotin by 3T3-L1 cells was shown to occur via a specific membrane carrier (Cohen and Thomas, 1982). Even though avidin appears to bind to the cell surface, it is unlikely that a natural receptor for avidin on cell membrane exists. Avidin is not a normal circulatory protein. It is more likely that avidin is mimicking a circulatory protein that might be responsible for the transport of biotin. Recently in our laboratory, protein(s) have been isolated from rat and human serum that appear to bind biotin in a reversible manner and thus may play role in biotin transport and eventual uptake by various tissues.

Although the presence of biotin in the nuclei is now well established, its role in the nucleus is not clear. Nuclei do not contain any of the known biotin carboxylases. Thus, biotin is not required in the role of the prosthetic group of biotin carboxylases. The fact that biotin in the nucleus is associated with protein(s) led us to investigate the protein(s) involved in this binding. The work presented in this section deals with the isolation of a protein from rat liver nuclei that binds biotin <u>in vitro</u>.

EXPERIMENTAL

(a) Materials

Piperazine-N,N'-bis (2-ethane sulfonic acid) (PIPES), iodoacetamide, phenylmethylsulfonyl fluoride and agarose-biotin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Hexylene glycol, thiodiglycol and sodium butyrate were obtained from Fisher Scientific Co. (Fairlawn, N.J.). Percoll and molecular weight marker kit were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The source of other reagents has been described previously.

(b) Preparation of Rat Liver Nuclei

Rat liver nuclei were prepared according to the procedure of Wray (1975) with some modifications. A rat was sacrificed, the liver removed and rinsed in ice-cold phosphate-buffered saline (pH 7.5). The liver was homogenized in 10 vol of buffer A (1M hexylene glycol, 10 mM PIPES, pH 7.5, 2 mM $MgCl_2$, 1% thiodiglycol, 30 mM sodium butyrate and 10 mM iodoacetamide) using a Virtis homogenizer for 1 min. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 2,200 x g for 10 min. The pellet was resuspended in buffer A and centrifuged at 2,200 x g for 10 min. The resulting pellet was resuspended in buffer B (1M hexylene glycol, 10 mM PIPES, pH 7.0, 2 mM $MgCl_2$, 1% thiodiglycol, 30 mM sodium butyrate) containing 0.2% Nonidet P-40 and centrifuged at 2,200 x g for 10 min. The pellet was resuspended in buffer B containing 0.2% Nonidet P-40 and centrifuged at 1,200 x g for 10 min. The pellet was resuspended in buffer B and centrifuged at 600 x g for 10 min. The resulting pellet was resuspended in 10 ml of buffer B

containing 1 mM $CaCl_2$ and loaded onto a preformed Percoll gradient (15 ml). The percoll gradients (75% Percoll in buffer B containing 1 mM $CaCl_2$) were prepared by centrifugation at 27,000 x g for 60 min. The nuclei were recovered from near the bottom of the gradient after centrifugation for 8 min at 5,000 x g in a Sorvall HB-4 rotor. The nuclei were diluted with buffer B and collected by centrifugation at 2,200 x g for 10 min. 1 mM phenylmethylsulfonyl fluoride was included in all the buffers used.

(c) Assay for Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase activity was measured after preincubation according to the procedure previously described (section IV). The enzyme activity was measured in the homogenate and nuclei. Nuclei prepared from rat liver were sonicated in 50 mM phosphate-buffer (pH 7.5) containing 1 mM EDTA and 5 mM β-mercaptoethanol. The nuclear sonicate was used as the source of enzyme.

(d) Assay for Propionyl-CoA Carboxylase

Propionyl-CoA carboxylase activity was measured in the rat liver homogenate and nuclear sonicate prepared according to the procedure described previously (c). The assay medium contained in a final volume of 1 ml:Tris-HCl (pH 8.5), 100 mM;glutathione reduced, 2 mM;$MgCl_2$, 6 mM;KCl, 100 mM;ATP, 3 mM;propionyl-CoA, 0.5 mM;$NaH^{14}CO_3$ (1000 cpm/μmol), 10 mM and homogenate or nuclear sonicate. The reaction was carried out at 37°C for 5 min and stopped by the addition of 200 μl 6N HCl. After centrifugation, 200 μl of the supernatant was transferred to a scintillation vial and taken to dryness under a fumehood. 500 μl of H_2O followed by 10 ml of Aquasol-2 scintillant were

added to the vial and radioactivity determined in a Beckman LS 2800 liquid scintillation spectrometer.

One unit of enzyme activity is defined as that amount of enzyme that catalyzes the carboxylation of 1 μmol of propionyl-CoA per min at 37°C into acid stable product.

(e) Protein Determination

Protein was determined according to the procedure of Lowry et al (1951) using bovine serum albumin as the standard.

(f) Uptake In Vivo of [^{3}H] Biotin

Rats were made biotin-deficient by feeding them a diet containing spray dried egg white for 6 weeks (Dakshinamurti and Cheah-Tan, 1968b). To investigate the uptake in vivo of biotin, biotin-deficient rats were injected with [^{3}H] biotin (5 μCi; 5 μg). 24 hr later rats were sacrificed and liver nuclei prepared as described previously (b). Radioactivity associated with nuclei was determined by solubilizing a portion (50 μl) of nuclei at 56°C in the presence of 0.9 ml of NCS. To the solibilized nuclei, 50 μl of glacial acetic acid followed by 10 ml OCS scintillant were added and radioactivity determined in a Beckman LS 2800 scintillation spectrometer.

To determine if [^{3}H] biotin was present in bound form, nuclei were lysed by the addition of 0.1 mM EDTA. A sample (100 μl) of the lysed nuclei was dialyzed against 0.1 mM EDTA. After dialysis radioactivity associated with lysed nuclei was determined as described above.

(g) Purification of Biotin-Binding Protein From Rat Liver Nuclei

Rat liver nuclei were prepared as described previously. The final nuclear pellet was resuspended in 50 mM phosphate-buffer (pH 7.5) so as to lyse the nuclei. The nuclear lysate was centrifuged at 1,000 x g for 10 min and the supernatant loaded onto an agarose-biotin column (1.5x20 cm) that had previously been equilibrated in 50 mM phosphate-buffer (pH 7.5). The column was washed with 50 mM phosphate-buffer (pH 7.5) until A_{280} reached zero. The protein(s) bound to the column was(were) eluted by washing the column with 50-200 mM phosphate-buffer (pH 7.5) gradient and 2.5 ml fractions collected.

(h) Polyacrylamide Gel Electrophoresis in the Presence of SDS (SDS-PAGE)

SDS-PAGE was performed according to the procedure of Laemmli (1970) using 10% polyacrylamide gels. The molecular weight of the biotin-binding protein under denaturing conditions was determined by comparing its mobility to that of proteins of known molecular weight.

(i) Sucrose Gradient Analysis

The molecular weight of the biotin-binding protein under nondenaturing conditions was estimated by comparing its sedimentation in a sucrose gradient to that of proteins of known molecular weight. 200 µl aliquot of the purified protein was centrifuged through a 5% to 20% sucrose gradient (12 ml) for 22 hr at 100,000 x g in a Beckman SW 40.1 Ti rotor. At the end of the run 250 µl fractions were collected from the bottom of the gradient. The presence of the biotin-binding protein was detected by analyzing the fractions on 10% SDS-PAGE. The protein standards used were:catalase ($S_{20,w}$, 11.3), rabbit

liver aldolase ($S_{20,w}$, 7.4), bovine serum albumin ($S_{20,w}$, 4.6) and lysozyme ($S_{20,w}$, 1.9). The molecular weight of the biotin-binding protein was determined according to the procedure of Martin and Ames (1961).

(j) In Vitro [^{3}H] Biotin Binding to Purified Protein

Equilibrium dialysis procedure was used to investigate the binding of biotin to purified protein in vitro (Eisen and Siskind, 1964). The method is based on the principle that the dialysis membrane allows only the biotin molecule to pass through freely into and out of the dialysis tubing while the protein molecule is contained within the tubing due to its large size. At equilibrium, the concentration of free or unbound biotin outside and inside the dialysis membrane is the same; however, there are a greater number of biotin molecules inside the dialysis tubing because in addition to the free biotin molecules there are biotin molecules which are bound to the protein. By determining the concentration of free biotin and bound biotin, one can calculate the dissociation constant, K_d, for biotin.

0.50 µg of purified protein in 50 mM phosphate-buffer (pH 7.5) was placed inside dialysis tubing and ends of the tubing secured with a thread. The dialysis tubing was placed in test tubes containing varying concentrations (50-1000 nM) of [^{3}H] biotin. The test tubes were then mixed overnight in an end-over-end mixer. At the end of this time period, dialysis tubing was removed and radioactivity associated with protein as well as with the solution in test tube was measured. Equal volumes (50 µl) of the protein solution or the dialysate were added to 10 ml of Aquasol-2 and radioactivity determined in a Beckman LS 2800 liquid scintillation spectrometer. The difference between dpm associated with protein solution and those associated with the dialysate represented total radioactivity associated with protein.

To determine non-specific binding of [^{3}H] biotin to the purified protein, [^{3}H] biotin binding was carried out as described above except that now at each concentration of [^{3}H] biotin used a 1000-fold excess of cold biotin was also present. Radioactivity associated with protein under these conditions was deemed to bind non-specifically to the protein.

The amount of biotin bound specifically to the protein was determined by subtracting the non-specific bound biotin from total biotin bound to the protein. Scatchard plot of the specific binding data was used to determine dissociation constant, K_d, and maximum binding, B_{max} (Scatchard, 1949).

RESULTS

(i) Purity of Nuclei

The procedure used yielded rat liver nuclei that were essentially free of contamination by other cellular organelles. When the preparation was visualized under a phase-contrast microscope, nuclei appeared intact and free of any major contamination by membrane fragments and cellular organelles. To further assess the purity of nuclei, activity of acetyl-CoA carboxylase (ACC, a cytosolic enzyme) and propionyl-CoA carboxylase (PCC, a mitochondrial enzyme) was measured in the homogenate and the final nuclear preparation. As indicated in Table 12, the activities of ACC and PCC were measurable in the homogenate while neither enzyme activity could be detected in the final nuclear preparation.

(ii) [^{3}H] Biotin in Nuclei

Liver nuclei prepared from [^{3}H] biotin injected biotin-deficient rats contained a significant amount of radioactivity (Table 13). To investigate if radioactivity associated with nuclei was in bound or free form, nuclei were lysed and a sample dialyzed overnight against 0.1 mM EDTA. Dialysis did not result in any loss of radioactivity from the nuclear fraction indicating that the radioactivity associated with nuclei was in bound form. To determine if radioactivity associated with nuclei was [^{3}H] biotin, the nuclear lysate was treated with avidin-Sepharose gel. This treatment resulted in the loss of radioactivity from the supernatant to the pellet (0.07 pmoles biotin/mg protein). This suggested that radioactivity associated with nuclei is due to

TABLE 12

ACTIVITY OF BIOTIN CONTAINING ENZYMES

Fraction	Enzyme Specific Activity*	
	ACC	PCC
Homogenate	1.13 ± 0.20	9.28 ± 0.55
Nuclei	0	0

The activity of acetyl-CoA carboxylase (ACC) and propionyl-CoA carboxylase (PCC) was measured in the homogenate and nuclei according to the procedure described in "Experimental" section. The values represent mean ± S.D. of 6 seperate determinations.

*Enzyme specific activity expressed as munits/mg protein.

TABLE 13

DISTRIBUTION OF [^{3}H] BIOTIN IN RAT LIVER

FRACTION	pmoles [^{3}H] Biotin/mg protein
Homogenate	0.85 ± 0.15
Nuclei	0.07 ± 0.01

Liver nuclei were prepared from 6gm of biotin-deficient rat injected with [^{3}H] biotin (5 µci) according to the procedure described in "Experimental" section. The values represent mean ± S.D. of four seperate preparations.

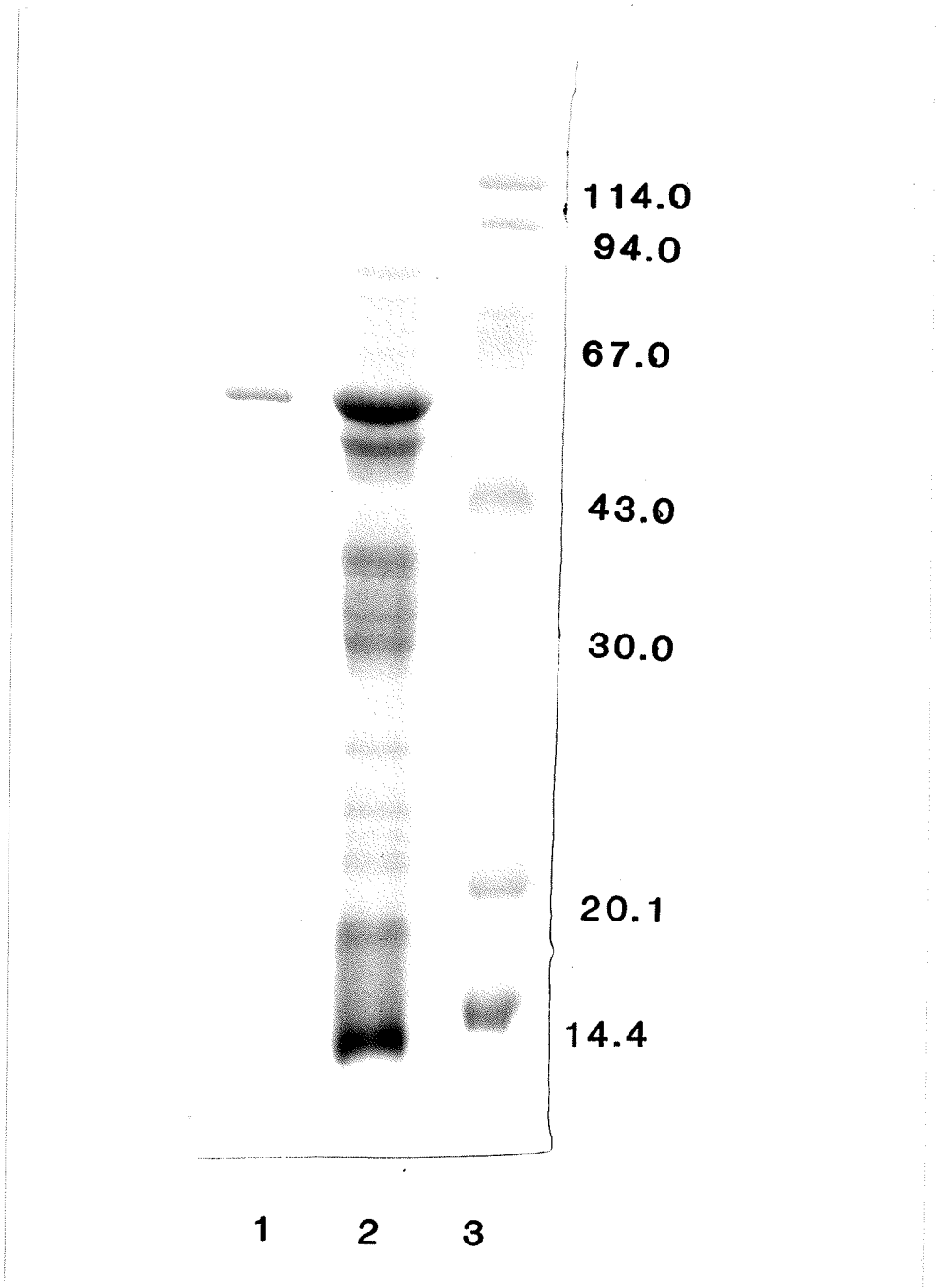
the presence of [^{3}H] biotin. Furthermore, when [^{3}H] biotin containing nuclear lysate was dialyzed against medium containing 1 mM non-radioactive biotin, no radioactivity in the nuclear lysate could be detected suggesting that [^{3}H] biotin present in the nuclear lysate was non-covalently bound and could be displaced by unlabelled biotin.

(iii) Purification of Biotin-Binding Protein

The biotin-binding protein from rat liver nuclei was purified with the use of agarose-biotin affinity column chromatography. The biotin-binding protein was eluted off the agarose-biotin column as a sharp peak. When the protein was analyzed by SDS-PAGE, a single protein staining band with a subunit molecular weight of 60K dalton was observed (Fig. 24). When the molecular weight of the protein was determined under non-denaturing conditions, a value of 240K dalton was obtained suggesting that the native protein exists as a tetramer (Fig. 25).

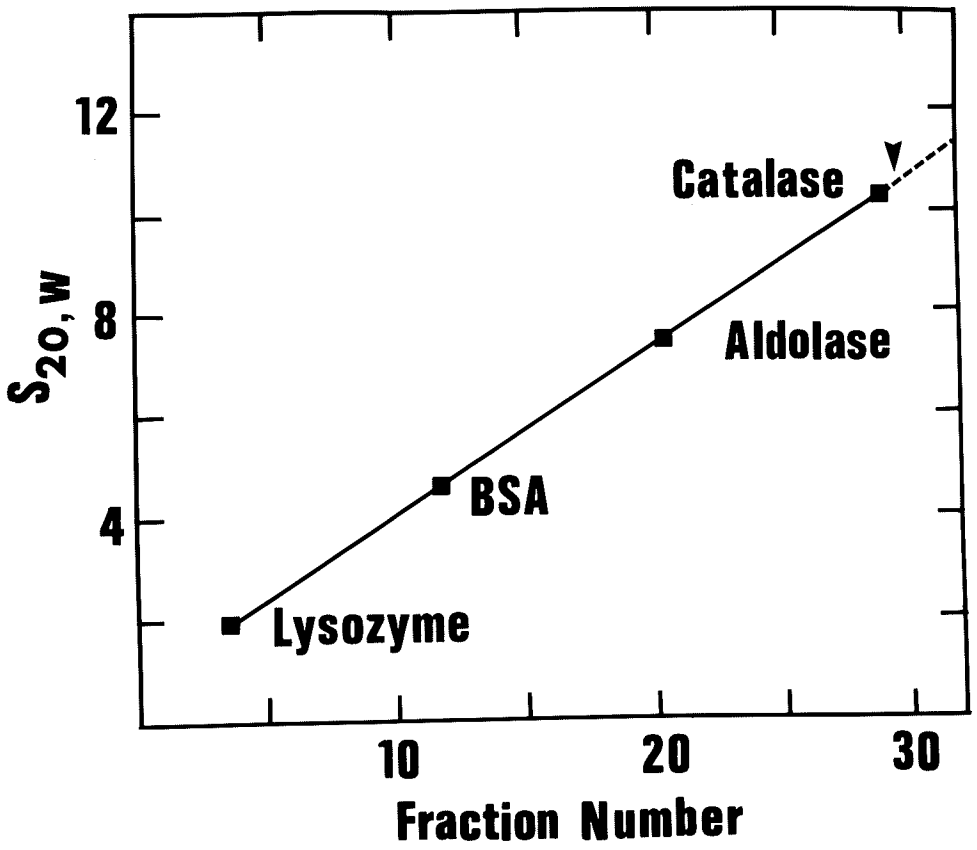
(iv) In Vitro Biotin Binding to Purified Protein

The binding of biotin in vitro to the purified protein was investigated using [^{3}H] biotin. The binding data shows that [^{3}H] biotin binding to the purified protein is saturable (Fig. 26). The specific binding data when analyzed using the Scatchard plot (1949) shows a single high affinity binding site (Fig. 27) with a dissociation constant, K_d, for biotin of 0.22 µM and a maximum binding, B_{max}, of 3.54 pmoles of biotin per ug protein. This corresponds to 0.85 mole of biotin bound per mole of protein (240K dalton used as the molecular weight of protein). Addition of biocytin (1 µM) in the presence of 50 nM [^{3}H] biotin did not inhibit binding of biotin to the purified protein.



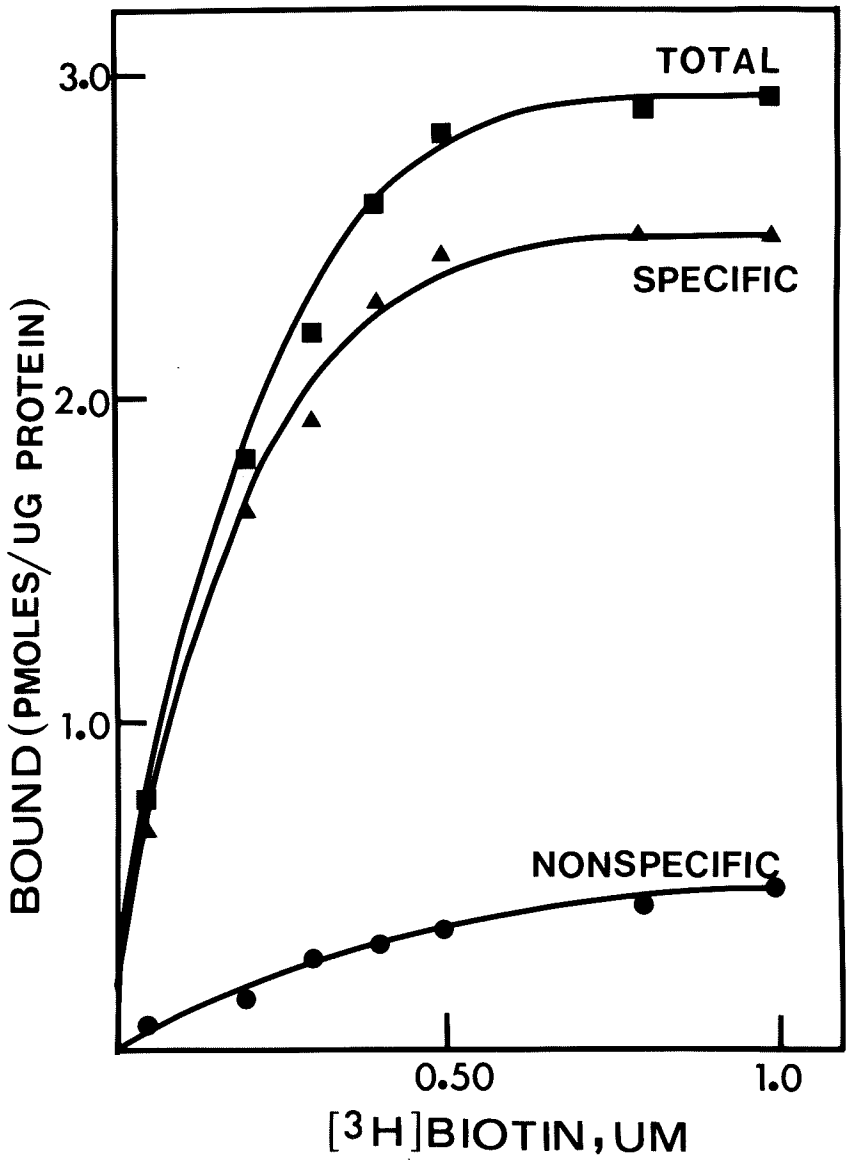
SDS-PAGE ANALYSIS OF PURIFIED BIOTIN-BINDING PROTEIN

Fig. 24. Purified biotin-binding protein, (5 µg; lane 1) and nuclei lysate (lane 2) was denatured by heating at 100°C for 2 min in the presence of 1% SDS and 5% β-mercaptoethanol. Lane 3 shows the protein standards used in the determination of the molecular weight.



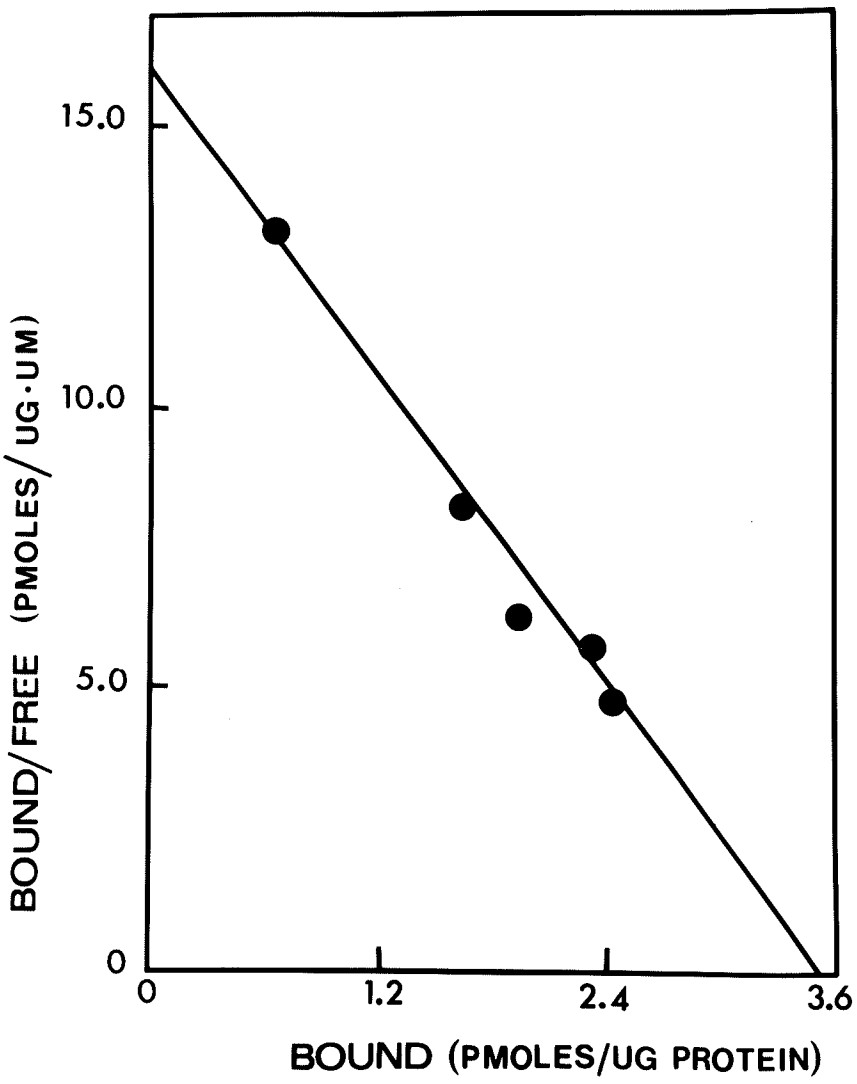
SUCROSE DENSITY GRADIENT ANALYSIS

Fig. 25. Biotin-binding protein (10 µg) was layered on top of a 5 to 20% gradient of sucrose and centrifuged at 100,000 xg for 22 hr in a Beckman SW 40.1 Ti rotor. At the end of the run fractions (250 µl) were collected and analyzed by SDS-PAGE according to the procedure described in "Experimental" section. The arrow represents the position of biotin-binding protein.



[^{3}H] BIOTIN BINDING TO PURIFIED PROTEIN

Fig. 26. Purified biotin-binding protein (0.50 µg) was incubated with increasing concentrations of [^{3}H] biotin. A parallel set contained the same amount of [^{3}H] biotin plus a 1000-fold excess of cold biotin. The data was analyzed according to the procedure described in "Experimental" section.



SCATCHARD PLOT OF SPECIFIC BINDING

Fig. 27. The specific binding of [^{3}H] biotin to the biotin-binding protein from Fig. 26 was plotted in the Scatchard manner. The best fit line was obtained using linear regression analysis.

DISCUSSION

As the prosthetic group of several carboxylases, biotin is involved in the metabolism of fatty acids and amino acids. But as mentioned in section VI, biotin has been implicated in other areas of metabolism where its role is not explained on the basis of its function as the prosthetic group of the known biotin-containing enzymes. One such area deals with the role of biotin in the nuclei. Biotin is taken up by the nuclei *in vivo* even though nuclei do not possess any CO_2-fixing capability, indicating that biotin in nuclei does not serve the purpose of a prosthetic group (Dakshinamurti and Mistry, 1963a;Boeckx and Dakshinamurti, 1973). During biotin-deficiency nuclear biotin seems to be conserved while it is preferentially lost from other cellular organelles (Boeckx and Dakshinamurti, 1973). Results presented in Section VI suggested that biotin stimulates protein synthesis in biotin-deficient HeLa cells through an increase in RNA synthesis and that rate of DNA synthesis is increased as well. This led us to investigate the function of biotin in nuclei.

Results presented in this section indicate that biotin present in the nuclei is indeed bound to protein in non-covalent manner. We have been able to purify a protein from rat liver nuclei that binds reversibly with biotin *in vitro*. SDS-PAGE analysis indicated a molecular weight of 60K dalton while under non-denaturing conditions a molecular weight of 240K dalton was obtained. This would suggest that the protein purified is a nonhistone protein as the largest histone protein has a molecular weight of 21K dalton under denaturing conditions (Spelsberg *et al*, 1972). Nonhistone proteins from rat liver nuclei have been shown to range in molecular weight under denaturing conditions from 5,000 to more than 100,000 dalton (Elgin and Bonner, 1970). The molecular weight obtained for the purified rat liver nuclear

biotin-binding protein suggests a tetrameric structure. The binding of biotin (0.85 mole/mole protein) to the purified protein is not inhibited by biocytin suggesting that the binding is specific for biotin. Studies on the transport of biotin in 3T3-L1 cells by a carrier mediated mechanism has been shown not to be inhibited by the presence of biocytin indicating that the carrier is specific for biotin (Cohen and Thomas, 1982).

At presnt, it is speculative to ascribe a physiological role for this biotin-binding protein in the observed effects of biotin. However, one can use the analogy of the role of steriod hormones which induce the transcription of specific genes. After the original discovery of Jensen and Jacobson (1962) that radioactively labelled estrogenic compounds rapidly accumulated in certain target-cell nuclei, much work has gone into the elucidation of the mechanism by which steroid hormones induce transcription of specific genes. In 1964, it was found that in the uterus high-affinity hormone binding components identified as protein molecules existed in the cytosol (Talwar et al, 1964). These experimental findings led to the advent of a two-step hypothesis of a cytoplasmic-nuclear traslocation event proposed by Jensen et al (1968) which is generally used to explain the regulation of gene expression by steroid hormones. According to this model, steroid hormones interact with the high-affinity cytoplasmic receptor causing a conformational change in the receptor-hormone complex such that it is rapidly translocated into the nucleus where they are thought to interact with a variety of nuclear components and in particular with the genome itself to induce or modulate the expression of a few specified genes (Cato, 1983). This general model seems to hold for all the major steroid hormones examined, including estrogens (Gorski, 1964;Raynaud-Jammet et al, 1971), progesterone (McGuire and O'Malley, 1968;Schrader and O'Malley, 1971), glucocorticoids (Dukes et al, 1966;Sajdel and Jacob, 1971), aldosterone (Feldman et al, 1972;Swancek et al, 1970) and

vitamin D (Zerwekh et al, 1974). However, there is recent evidence suggesting that prior to cell disruption, unoccupied steroid receptors reside primarily in the nucleus and that the presence of cytosolic receptor represents an artifact of isolation procedure (King and Greene, 1984;Welshous et al, 1984).

The steroid receptor from chick oviduct has been purified and shown to be a dimer composed of A and B subunits (Vedeckis et al, 1978). The A protein has a molecular weight of 79,000 and the B protein, 117,000 dalton. However, the size of the native receptor has been a matter of controversy. Molecular weights of 30,000-40,000 (Litwack and Rosenfield, 1975;Westphal et al, 1981), 60,000-70,000 (Puca et al, 1980;Katzenellenbogen et al, 1983), 80,000-100,000 (Norden et al, 1981;Housley and Pratt, 1983) and 100,000-200,000 dalton (Andre et al, 1978;Notides et al, 1981) have been reported for various cytosol receptors under different conditions. However, under conditions where the receptor was stabilized by the addition of sodium molybdate, a strikingly similar structure with M_r in the range of 280,000-330,000 dalton for cytosolic receptors for several classes of steroids was obtained (Colvard and Wilson, 1981;Redeuilh et al, 1981;Vedeckis, 1983;Holbrook et al, 1983;Raaka and Sameuls, 1983). Now, it is believed that the untransformed steroid receptor is an olgimer, probably a tetramer, with an M_r of 330,000 and that smaller forms of the native receptor are derived by dissociation and/or proteolysis of the native receptor (Sherman et al, 1983). The biotin-binding protein purified from rat liver nuclei also appears to be a tetramer with an M_r of 240,000.

The chick oviduct receptor for progesterone shows a very high-affinity (K_d, $8x10^{-10}$ M) for the steroid (O'Malley et al, 1971). Gschwendt and Kittstein (1974) reported that estradiol binds directly to rooster liver chromatin with high-affinity (K_d, 10^{-9} M). In Xenopus liver cytosol, high-affinity (K_d, 10^{-8} M) binding sites for estradiol have also been reported

(Tata and Smith, 1979). Nuclear receptors for thyroid hormones show a high-affinity (K_d, 10^{-9} M) binding as well (Latham et al, 1976). The high-affinity taurine receptors on rat heart sarcolemma were shown to bind taurine with a K_d value of 3.5×10^{-4} M (Kulakowski et al, 1978). The biotin-binding protein isolated from rat liver nuclei binds biotin in vitro with a K_d value of 2.2×10^{-7} M. This value is higher than that reported for the steroid binding to steroid receptors.

The concentration of biotin used (80 nM) in vitro in cell culture medium to observe its effects is in the same range as that used for observing the effects of estradiol (10 nM) in vitro when added to cell culture medium (Wangh and Knowland, 1975;Green and Tata, 1976). One would expect a higher concentration of biotin required to observe its effects on protein and DNA synthesis as it serves as the prosthetic group of carboxylases in addition to its role in protein and DNA synthesis. The effective concentration of biotin available to the nuclei thus would be much lower than what is present in the culture medium. In the case of steroids, 90% of the injected estrogen was found in the nuclei suggesting that the effective concentration of steroid in the nuclei is about the same as that added to the culture medium (O'Malley and Means, 1974).

The receptor-steroid complex once bound to nuclear acceptor sites markedly alters RNA synthesis (Hamilton et al, 1968;Knowler and Smellie, 1971) and the transcription of specific genes (Chan et al, 1973;Harris et al, 1975;Chan et al, 1976;Spelsberg et al, 1976). On a chronological basis, these events occur rapidly. Within 1 to 2 min after injection of labelled progesterone into the chicks, which have been primed with estrogen, the steroid is located in the cytosol bound to its receptor and 10 min post-injection, the steroid-receptor complex accumulates in the nucleus, followed closely by changes in RNA synthesis and chromatin template activity

(Spelsberg et al, 1976). After 1 to 2 hr specific mRNAs appear whose coded proteins begin to accumulate within 3 to 6 hr (Gorski and Gannon, 1976). In case of biotin, 30 min after injection of radioactive biotin to the deficient animal, 15% of the radioactive biotin was found to be associated with the nucleus all of which was protein bound (Boeckx, 1973). As discussed in section VI biotin stimulated DNA synthesis 4 hr after biotin addition to biotin-deficient HeLa cells and rate of protein synthesis was enhanced to control value within 6 hr after biotin addition. Thus the effects of biotin are observed in a similar time span as for the steroids.

While the results presented in this section help in the identification of a protein in the nuclei that binds biotin in vitro, many questions remain unanswered. It is not clear at the present time how biotin is transported into the nuclei. Purified rat liver nuclei do not take up biotin in vitro under a variety of conditions (Boeckx, 1973). A similar situation has been observed with estradiol. When preparations of purified nuclei were incubated with [^{3}H] estrdiol, no radioactivity could be detected in the nuclei (Jensen et al, 1968). It is not clear at the present time as to what role biotin-binding protein plays in the effects observed when biotin-deficient animals are supplemented with biotin. The biotin-binding protein from nuclei has a K_d value which does not compare favourably with the K_d for steroids for their receptor. The K_d value obtained for the binding of biotin to the purified nuclear biotin-binding protein is in close agreement with the values reported for the binding of other vitamins to their respective binding proteins. Thiamine-binding protein binds thiamine with a K_d for thiamine of 5.5×10^{-7}M (Nishimura et al, 1984). A corticosteroid-binding globulin from rat serum was shown to bind corticosterone with a K_d for corticosterone of 2.8×10^{-7}M (Chader and Westphal, 1968). However, these binding proteins are involved in the transport of vitamins and steroids in the serum while the

biotin-binding protein under study here is located in the nuclei and thus may be involved in the expression of effects observed when biotin is administered to biotin-deficient animals or cells. Further work may help in the elucidation of the role of the nuclear biotin-binding protein.

SECTION VIII

GENERAL DISCUSSION

Acetyl-CoA carboxylase, the regulatory enzyme in fatty acid synthesis is regulated through the combined effects of allosteric regulation, phosphorylation-dephosphorylation and enzyme synthesis. Changes in rate of enzyme synthesis are operative during long-term regulation while allosteric regulation and phosphorylation-dephosphorylation are believed to be important in the short-term regulation of acetyl-CoA carboxylase activity. The present investigation has dealt with the changes in enzyme activity and synthesis in HeLa cells under the influence of glucagon as well as the regulation of purified porcine adipose tissue acetyl-CoA carboxylase activity by cAMP-dependent protein kinase mediated phosphorylation.

There is substantial evidence suggesting that glucagon decreases lipogenesis *in vivo*. In the present investigation we have demonstrated that glucagon causes a decrease in ACC activity and fatty acid synthesis, and this decrease is due to a change in the synthesis of ACC. During short-term regulation, glucagon acts through cAMP in decreasing lipogenesis *in vivo*. However, it is not clear whether the same second messenger mechanism is operative during long-term regulation of ACC activity and fatty acid synthesis.

Several laboratories have demonstrated that crude as well as pure preparations of ACC can be phosphorylated at significant rates *in vitro* and in some instances cAMP-dependent phosphorylation has been shown to inactivate the enzyme. To be certain that an enzyme is regulated by a phosphorylation-dephosphorylation mechanism, the following criteria must be fulfilled:

(1) Demonstration that stoichiometric phosphorylation and dephosphorylation occurs *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.

Glucagon, acting through cAMP has been shown to decrease lipogenesis *in vivo* and this is believed to be due to the phosphorylation of ACC. Insulin, which causes an increase in lipogenesis *in vivo* has also been shown to cause an increase in the phosphorylation of ACC. The hypothesis that ACC is regulated directly by a phosphorylation-dephosphorylation mechanism mediated by cAMP has only been demonstrated for ACC from lactating rat and rabbit mammary gland. Furthermore, phosphorylation and inactivation of ACC *in vitro* by the cAMP-dependent protein kinase does not correlate with the changes observed in lipogenesis *in vivo* in the presence of glucagon. 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.

Apart from its role as the prosthetic group of the carboxylases, biotin has been implicated in protein and RNA synthesis. Cell culture studies provide an opportunity to investigate the direct effect of the addition or depletion of medium constituents on cellular processes excluding secondary influences like that of hormonal system. HeLa cells grown in biotin-deficient serum showed decreased protein synthesis and cellular growth. The specificity of biotin effect was established in experiments where the addition of biotin to the medium of biotin-deficient HeLa cells resulted in restoration of protein synthesis and cellular growth. Although culture medium used in these

experiments contained serum lipids, HeLa cells still required biotin for growth. The mechanism of action of biotin involves the appearance of new RNA in cytoplasm resulting in increased protein synthesis. It is of interest to note that biotin is present in the cell nucleus although no known biotin containing enzymes are associated with the nuclear fraction. A "biotin-binding protein" has also been isolated from rat liver nuclei. This protein binds biotin in vitro in a reversible manner. Drawing an analogy to the steroid hormone action, it is speculated that biotin might bring about its effects on protein synthesis through the stimulation of transcription of specific genes. The biotin-binding protein may play a role in this process of gene activation. Studies on the association of biotin with DNA and the role of the biotin-binding protein in this association may help in the elucidation of the role of biotin in nucleus. It is possible that the effect of biotin is not due to direct derepression of specific genes, but may be due to blocking either the expression or the action of a repressor gene product. This repressor gene product may block the transcription of certain genes during biotin-deficiency resulting in decreased protein synthesis. It would indeed be of interest to determine which proteins are direectly affected by biotin-deficiency and the role of these proteins in cellular metabolism.

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