

THE UNIVERSITY OF MANITOBA

RAT EPIDIDYMAL ADIPOSE TISSUE ACETYL CoA CARBOXYLASE

by

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to my wife Vona and my daughter Michelle

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ABSTRACT

Acetyl CoA carboxylase (acetyl CoA:CO₂ ligase (ADP) EC 6.4.1.2) was partially purified from rat epididymal adipose tissue, and its properties studied. The partially purified enzyme was shown to be devoid of malonyl CoA decarboxylase (malonyl CoA carboxy-lyase, EC 4.1.1.9) activity which interfered with the assay of acetyl CoA carboxylase in cruder enzyme preparations.

The rat adipose tissue acetyl CoA carboxylase required preincubation with citrate, magnesium, and bovine serum albumin for maximum activity. The enzyme had a pH optimum of 7.5, and showed greater catalytic activity with magnesium than with manganese. Malonyl CoA inhibited the enzyme competitively with respect to acetyl CoA and non-competitively with respect to ATP. Coenzyme A also inhibited the enzyme but to a lesser extent than malonyl CoA. The rat adipose tissue acetyl CoA carboxylase was shown to undergo pH dependent reversible cold inactivation. The over-all properties of the rat adipose tissue enzyme were found to be very similar to the properties of the rat liver enzyme.

The effect of biotin deficiency on acetyl CoA carboxylase was studied. Biotin deficiency had a greater effect on the rat adipose tissue carboxylase than on the rat liver carboxylase. Biotin deficiency caused a decrease in the rat adipose

tissue enzyme activity to one sixth the control value, whereas the rat liver enzyme activity was decreased by only one half.

The restoration of rat adipose tissue acetyl CoA carboxylase activity by biotin administration in vivo or biotin addition in vitro was also studied.

Rat liver and epididymal adipose tissue acetyl CoA carboxylase activities were increased 1.6 and 9.4 fold respectively one hour after biotin administration to biotin deficient rats. The stimulation by biotin of the acetyl CoA carboxylase activity was maximum after one hour in the liver, but increased steadily for two hours in the epididymal adipose tissue. New enzyme synthesis did not seem to be involved in the biotin effect, since prior administration of cycloheximide did not prevent the increase in enzyme activity.

Acetyl CoA holocarboxylase synthesis in vitro was shown to occur in the 20,000 Xg supernatant of biotin deficient rat epididymal adipose tissue, in the presence of d-biotin, magnesium chloride, and ATP. d-Biotin and ATP were absolute requirements for acetyl CoA holocarboxylase synthesis and for d-biotin ^{14}C incorporation into protein. Magnesium although not required for acetyl CoA holocarboxylase synthesis stimulated d-biotin ^{14}C incorporation into protein. Acetyl CoA holocarboxylase synthesis in vitro was also shown to occur in the 105,000 Xg supernatant of biotin deficient rat liver, but to a lesser extent than in the rat adipose tissue.

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ABBREVIATIONS

Tris	:	tris (hydroxymethyl) amino methane
ATP	:	adenosine triphosphate
CoA	:	coenzyme A
PPO	:	2,5 diphenyloxazole
POPOP	:	1,4-bis-2(5-phenyloxazolyl)-benzene
EDTA	:	ethylenediamine tetraacetic acid
GSH	:	glutathione (reduced)
BSA	:	bovine serum albumin
NADH	:	reduced nicotinamide adenine dinucleotide
NADPH	:	reduced nicotinamide adenine dinucleotide phosphate
$T_{\frac{1}{2}}$:	half life
Biotinyl-AMP	:	biotinyl adenylate
P _{Pi}	:	pyrophosphate
AMP	:	adenosine 5' monophosphate
TCA	:	trichloroacetic acid
$S_{20,w}$:	sedimentation coefficient corrected to water at 20°C

CHAPTER I - INTRODUCTION

Acetyl CoA carboxylase (acetyl CoA:CO₂ ligase (ADP), EC 6.4.1.2) was first isolated from avian liver by Wakil and Gibson (1). They showed that this enzyme catalysed the ATP dependent carboxylation of acetyl CoA to form malonyl CoA. This enzyme has been considered by many investigators (2-6) to be the rate limiting and regulatory enzyme of the soluble fatty acid synthesizing system. Waite and Wakil (7, 8) have shown that this enzyme contained covalently linked biotin which was required for catalytic activity.

Purpose: Dakshinamurti and Desjardins (9) have previously shown that biotin deficiency decreased lipogenesis in rat liver. They showed that this decreased lipogenesis was reflected in a 50% decrease in liver acetyl CoA carboxylase activity. They also found that biotin deficiency decreased the total lipid content of rat epididymal adipose tissue more severely than that of rat liver.

Vagelos et al. (10) have previously studied the rat adipose tissue acetyl CoA carboxylase. They showed that citrate activated the enzyme and caused it to aggregate to a faster sedimenting form. Vagelos et al. (10) did not investigate any of the catalytic properties of this enzyme. This thesis reports on the following: 1). the catalytic properties of the partially purified acetyl CoA carboxylase from rat epididymal adipose tissue compared with the already well known properties of the rat liver (4,11-14), rat mammary gland (15) and chicken liver (16-21) enzymes,

2). the effect of biotin deficiency on the epididymal adipose tissue acetyl CoA carboxylase, 3). the effect of in vivo biotin administration on restoration of liver and adipose tissue acetyl CoA carboxylase, 4). the in vitro synthesis of acetyl CoA holocarboxylase.

General Approach: Since the earlier work of Vagelos et al. (10) on the rat epididymal adipose tissue enzyme, improved assay methods have been devised for acetyl CoA carboxylase by Lane's group (22). The epididymal adipose tissue enzyme was purified using essentially the same approach as Vagelos et al. (10), and the properties of this enzyme were investigated using essentially the assay method of Chang et al. (22).

Organization of the thesis: There are three main sections to the thesis, Literature Review, Experimental and Discussion. The Literature Review is divided into five parts 1). Fatty acid synthesis, 2). Acetyl CoA carboxylase, 3). Regulation of fatty acid synthesis, 4). Biotin deficiency and acetyl CoA carboxylase, 5). Acetyl CoA holocarboxylase synthesis. The Experimental section has been divided into Methods and Results. The Experimental and Discussion sections each consist of two sub-sections, 1). Partial purification and properties of epididymal adipose tissue acetyl CoA carboxylase, 2). Biotin status and acetyl CoA carboxylase activity.

CHAPTER II - LITERATURE REVIEW

1. FATTY ACID SYNTHESIS

Many animals are intermittent eaters and store some of the chemical energy of the ingested food as a reserve for utilization in postabsorptive periods. For many animals the major foodstuff ingested is carbohydrate. Carbohydrate is a bulky substance to store in terms of calories per gram, as it is stored in an aqueous environment. Conversion of carbohydrate to fat enables animals to store the chemical energy in a more efficient way. Thus, the major function of fatty acid synthesis in an animal is to store the chemical energy of the ingested carbohydrate foodstuff as fat.

i) Tissue Localization

Over the years there has been considerable interest regarding the relative contribution of liver and adipose tissue to fatty acid biosynthesis. Prior to 1950, it was felt that the liver was the major lipogenic organ. The adipose tissue was at this time considered to be a metabolically inert fat depot. Since then a great number of workers have shown that the adipose tissue is capable of synthesizing fatty acids from acetate or glucose (23-25). Favarger and Gerlach (26-28) in a series of in vivo experiments using glucose ^{14}C as the lipid precursor showed that the brown interscapular fat of the mouse, in spite of its small weight, synthesized more fatty acids than the liver. It thus became generally accepted that the adipose tissue played a role in

fatty acid synthesis equal to if not greater than that of the liver.

Although other sites of fatty acid synthesis have been reported (29,30), from the present state of knowledge only the liver and adipose tissue would have to be considered as the major tissues involved in fatty acid biosynthesis.

ii) Pathways of Fatty Acid Synthesis

a) Mitochondrial pathway

Prior to 1958 it was thought that fatty acid synthesis occurred via the reversal of the enzymatic reactions involved in β -oxidation. This concept was generally accepted despite earlier observations by Gurin and his group (31-33) on the possible presence of two separate and distinct systems for synthetic and degradative processes. Experimental evidence supporting fatty acid synthesis via the reversal of a modified β -oxidation pathway came from two independent observations. Langdon (34) discovered an enzyme in the soluble extract of rat liver that reduced crotonyl CoA with NADPH, and Stumpf and Barber (35) showed that stearic acid synthesis from acetyl CoA and palmityl CoA was catalysed by mitochondrial enzymes in the presence of NADH and NADPH. The mitochondrial fatty acid synthesizing pathway still has not been completely elucidated, but it is known to be involved mainly in elongation of existing acyl CoA's by C_2 units derived from acetyl CoA.

b) Cytoplasmic pathway

A second pathway for fatty acid synthesis has

been discovered. Gibson et al. (36) showed that bicarbonate was required for fatty acid synthesis in a partially purified enzyme preparation obtained from the soluble fraction of avian liver. It was known that bicarbonate was not a requirement for mitochondrial fatty acid synthesis. Wakil (37) and Brady (38) both showed that bicarbonate was required for the synthesis of malonyl CoA in the avian liver enzyme preparation, and that it was malonyl CoA which contributed the C₂ units for synthesis of long chain fatty acids. Wakil et al. (39) also showed that this partially purified avian liver enzyme preparation was free of the key enzymes of the β -oxidation sequence.

Thus acetyl CoA carboxylase, and fatty acid synthetase were recognized as enzymatic components of the soluble fatty acid synthesizing system (1,7,36-39). Wakil and Gibson (1) fractionated the avian liver soluble fatty acid synthesizing system into two fractions R₁ and R₂. The R₁ fraction was shown to be acetyl CoA carboxylase. The properties of this enzyme will be discussed at some length in the next section. The R₂ fraction was found to be the fatty acid synthetase complex which was responsible for the sequential reduction, dehydration, and further reduction of the β keto acid formed from the condensation of acetyl CoA, or fatty acyl CoA with the C₂ donor malonyl CoA, for the consequent production of long chain fatty acids. The cytoplasmic fatty acid synthesizing system was shown to be responsible for the de novo synthesis of fatty acids.

c) Microsomal pathway

As previously mentioned, chain elongation of fatty acids occurs in the mitochondria, with acetyl CoA as a donor of the C₂ fragment. Stoffel (40) has shown that malonyl CoA was required for chain elongation of linoleic acid. Nugteren (41) studied the enzymic chain lengthening of γ -linolenic acid into homo- γ -linolenic acid, and found that the chain elongation enzyme was present in rat liver microsomes and that γ -linolenyl CoA was converted to homo-linolenyl CoA by malonyl CoA and NADPH. More recently Nugteren (42) has shown that chain elongation occurs via the CoA thioesters of the corresponding β -keto acid, the β -hydroxy acid, and the trans- α,β -unsaturated acid. Consequently the chain elongation takes place as four separate reactions, which have been shown to be distinct from the mitochondrial and cytoplasmic fatty acid synthesizing systems. The microsomal fatty acid synthesizing system can elongate saturated or unsaturated fatty acids (42).

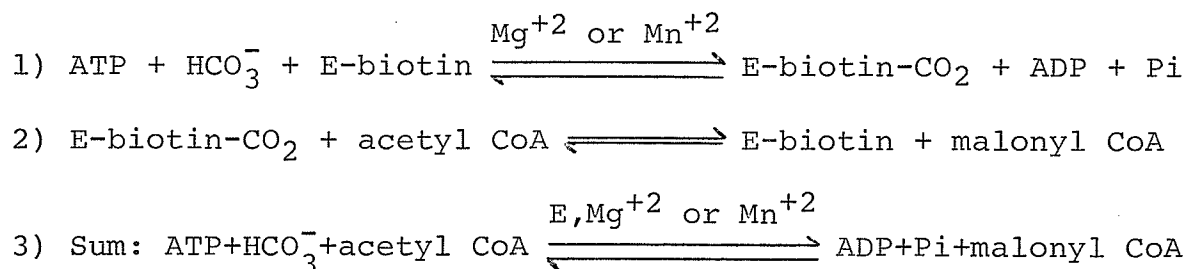
Thus there are three known fatty acid synthesizing systems which are characterized by their location in the cell as well as by the reactions they catalyze. The contribution of each fatty acid synthesizing pathway to the over-all fatty acid biosynthesis within the tissue varies according to the tissue under consideration. In the liver the cytoplasmic pathway contributes the most to fatty acid synthesis, whereas in the heart tissue the mitochondrial pathway is the predominant one (43).

In summary, the following has been shown: 1). The mitochondrial system is involved mainly in elongation of preformed fatty acids, by addition of C₂ units derived from acetyl CoA. The system uses NADH and NADPH as reducing agents. This elongation system has been shown to be different from the fatty acid oxidation system, though some steps may be catalyzed by the same enzyme, 2). The cytoplasmic fatty acid synthesizing system is involved in the de novo synthesis of long chain fatty acids, mainly palmitic, and utilizes acetyl CoA as a primer, malonyl CoA as a donor of C₂ units, and NADPH as the reducing agent, 3). The microsomal system is also an elongation system for preformed fatty acids (saturated or unsaturated) which utilizes malonyl CoA as a donor of C₂ units, and NADPH as a reducing agent.

2. ACETYL CoA CARBOXYLASE

Acetyl CoA carboxylase catalyses the ATP dependent carboxylation of acetyl CoA by bicarbonate to form malonyl CoA. Wakil and Gibson (1) have shown that this enzyme contains protein bound biotin, and that the activity of the enzyme could be inhibited by preincubating the enzyme with avidin. Avidin is a protein which has been isolated from egg-white and shown to bind biotin irreversibly (44). Green (44) determined the dissociation constant of the avidin-biotin complex to be $10^{-15}M$. Waite and Wakil (8) showed that the catalytic mechanism of acetyl CoA carboxylase could be divided into two half reactions as has been shown for

other biotin enzymes:



The biotin moiety was shown to be covalently bound to the enzyme through the ξ -amino group of a lysine residue. This was demonstrated by the isolation of biocytin (ξ -N-biotinyl-L-Lysine) from a papain digest of the carboxylase (7). Numa et al. (45) have shown the structure of the carboxylated biotin to be 1'-N-carboxybiotin.

Brady and Gurin (31) were the first to show that tri-carboxylic acid cycle intermediates stimulated fatty acid synthesis. This effect was localised at the level of acetyl CoA carboxylase (46-48). Vagelos et al. (10) working with the rat adipose tissue acetyl CoA carboxylase showed that the stimulatory effect of the tricarboxylic acid cycle intermediates, of which citrate was the most active, was through direct activation of the carboxylase, causing the enzyme to aggregate to a faster sedimenting form of greater activity. Matsushashi, Matsushashi and Lynen (4) confirmed the results of Vagelos et al. (10) using the rat liver acetyl CoA carboxylase.

Association-Dissociation. Acetyl CoA carboxylase has now been isolated and purified from many sources, but most of the physical and catalytic properties of the enzyme have

been studied with the chicken liver enzyme which has been purified to homogeneity (16,20,49,50). This enzyme has an absolute requirement for isocitrate (or citrate) for maximum activity but requires no prolonged preincubation with the activator. Gregolin et al. (16,17) have shown that the chicken liver carboxylase which was isolated as the aggregated form could be easily dissociated into protomeric subunits with concomitant loss of enzyme activity. The transformation from a catalytically inactive protomeric form (M.W. 410,000) into an active polymeric form (M.W. 7.8×10^6) was rapidly achieved by addition of tri- and dicarboxylic acids. Gregolin et al. (17) have shown that various factors can affect the equilibrium between protomer and polymer (Table I).

Kinetic properties. The reaction catalysed by chicken liver acetyl CoA carboxylase can be separated into two partial reactions as previously demonstrated by Waite and Wakil (8). Citrate or isocitrate stimulates both partial reactions. They are V_m activators, and have no effect on the K_m values for any of the substrates (20). Gregolin et al. (21) have shown that the chicken liver acetyl CoA carboxylase has one binding site per protomer (M.W. 410,000) for citrate, and another for acetyl CoA. They also showed that the acetyl CoA binding site was unaffected by the presence of citrate.

Subunits. Gregolin et al. (21) have shown that the chicken liver carboxylase can be dissociated with sodium dodecyl sulfate to give rise to subunits of molecular weight 110-114,000. The presence of non-identical subunits was

indicated by the fact that there was a single biotinyl prosthetic group and single binding sites for citrate and acetyl CoA on the 410,000 molecular weight protomer.

Electron microscopy studies. Electron microscopy of the chicken liver carboxylase has disclosed a striking correlation between the state of activation, its aggregation, and its structural characteristic (16). The disaggregated catalytically inactive enzyme was shown to be present as small particles having dimensions 70-150 Å. In the presence of isocitrate, the faster sedimenting active form assumed a filamentous form, which is 70-100 Å in width and up to 4000 Å in length. In a preliminary communication Kleinschmidt et al. (51) have recently reported that the electron microscopic, and hydrodynamic properties of the bovine perirenal adipose tissue enzyme were identical with those of the chicken liver enzyme.

Mammalian enzymes. Much of the work on mammalian acetyl CoA carboxylases has been carried out using rat tissues. The rat liver (4,45) and adipose tissue (10,48) acetyl CoA carboxylases differ from the chicken liver carboxylase in that the former enzymes are normally isolated in the "small" form (20S) while the latter enzyme is isolated in the "large" form (53-58S). Furthermore, the catalytically inactive 20S form of the chicken liver enzyme is very rapidly converted into the active "large" form in the presence of citrate (16). Hence a preincubation with citrate is not necessary. On the other hand the rat liver (4,45), adipose tissue (10,48)

and mammary gland (15) enzymes require prolonged preincubation with citrate (or isocitrate) to produce the "large" form of the enzyme. This may be due to the fact that in most of the studies cited the rat enzymes were isolated in the cold. The rat liver enzyme has been shown to undergo disaggregation from the "large" active form to the "small" inactive form at 0-4°C (11). The results of Welbourne et al. (52) seem to support this contention. They have reported that if the rat liver acetyl CoA carboxylase was isolated at 38°C, the "large" active form of the enzyme was isolated.

The catalytic properties of the rat liver (4,11,12,53) and mammary gland (15) enzymes are very similar to those of the chicken liver enzyme (20,21). This thesis will report on the properties of the rat adipose tissue enzyme.

Bacterial enzyme. Alberts and Vagelos (54) were the first to purify acetyl CoA carboxylase from Escherichia coli. They separated the enzyme into two protein fractions both of which were required for catalysis of the over-all reaction. One of these, Ea, was a biotin containing protein which formed Ea-CO_2^- in the presence of ATP, HCO_3^- and MnCl_2 . The other fraction, Eb, was required for carboxyl transfer from Ea-CO_2^- to acetyl CoA, resulting in the formation of malonyl CoA. Eb was shown to contain no biotin. Alberts, Nervi and Vagelos (55) more recently have shown that Ea could be dissociated into two subunits at pH 9.0. One subunit designated as biotin carboxylase, catalysed a model reaction, the ATP dependent carboxylation of free d-biotin.

The other subunit contained covalently linked biotin and had no known catalytic activity. It was this covalently linked biotin which was carboxylated by the biotin carboxylase subunit in the course of acetyl CoA carboxylation. The E. coli biotin carboxylase has been purified to homogeneity and shown to have a $S_{20,w}$ value of 5.7S and a molecular weight of 78,000 daltons by gel filtration on Sephadex G-200 (56). The biotin containing protein has been shown to have an $S_{20,w}$ value of 1.3S (55). It became apparent from the work of Gerwin, Jacobson and Wood (57) that the structure of E. coli acetyl CoA carboxylase was not unique for this enzyme. They found that methylmalonyl CoA-oxalacetate transcarboxylase (EC 2.1.3.1) from Propionibacterium shermanii a biotin enzyme of molecular weight 670,000, contained 6 moles of biotin per mole of enzyme, and dissociated spontaneously at low ionic strength and alkaline pH to a mixture of inactive subunits. One type of subunit contained all the biotin of the original molecule, and had an $S_{20,w}$ of 1.3S and a molecular weight of 12,000.

Alberts, Nervi and Vagelos (55) have postulated that all biotin enzymes might be composed of three different subunits: a biotin protein, a biotin carboxylase, and a subunit specifying the acceptor molecule.

3. REGULATION OF FATTY ACID SYNTHESIS

As with many metabolic pathways the control of fatty acid synthesis on a day to day basis is achieved through

regulation of the levels of acetyl CoA carboxylase and fatty acid synthetase. Synthesis and degradation of these enzymes could be altered by various effectors.

Regulation of fatty acid synthesis on a minute to minute basis is achieved by metabolites which are activators or inhibitors of the enzymes.

i) Regulation of Enzyme Concentration

It has been known for many years that fatty acid synthesis can be affected by various factors, such as starvation, diabetes, refeeding, and the fat content of the diet. In 1952 Chaikoff's group (58) reported that the ability of rat liver to convert acetate-1-¹⁴C to fatty acids was almost completely lost when rats were starved for 18 hours. Korchak and Masoro (59) confirmed these results and showed that this was due to decreased acetyl CoA carboxylase activity. Gibson's group (60,61) showed that starvation decreased carboxylase and synthetase activities, and that refeeding a fat free diet caused the levels of both enzymes to overshoot their normal values. They also showed that prior administration of puromycin or actinomycin (inhibitors of protein synthesis) prevented the elevation in activity of the carboxylase and the synthetase.

Whitney and Roberts (62) and Hill et al. (63) have shown that fat feeding decreased hepatic lipogenesis. Hill et al. (63) showed that the capacity of the liver to convert acetate carbon to fatty acids was measurably decreased when as little as 2.5 per cent fat was added to the diet. When the fat

content of the diet was increased to 15 per cent, the liver retained only 10 per cent of its original capacity to convert acetate to fatty acids. Hill et al. (64) have demonstrated that a pronounced decrease in the incorporation of acetate into fatty acids by liver slices occurs as early as one hour after fat administration to the rats. Bortz et al. (65) showed that the block in lipogenesis due to fat feeding occurred at the level of acetyl CoA carboxylase. They suggested that free fatty acids might have a direct effect on the enzyme itself.

More recently, Majerus and Kilburn (66) using the anti-serum against the homogeneous chicken liver acetyl CoA carboxylase which is known to cross-react with the rat liver acetyl CoA carboxylase, measured the half life ($T_{1/2}$) of the rat liver carboxylase under different conditions. They showed that acetyl CoA carboxylase of normal fed rats, fasted rats, and fat fed rats had $T_{1/2}$ values of 48 hours, 18 hours and 50 hours respectively. They concluded that there was increased enzyme degradation during starvation, but that fat feeding had no effect on enzyme degradation.

Majerus and Kilburn (66) have postulated that the rate of synthesis and degradation of the carboxylase regulates the level of this enzyme and hence the rate of fatty acid synthesis. However, there is no information on the nature of any existing inducer or repressor which could act on the synthesis of this enzyme.

ii) Regulation of Enzyme Activity

Acetyl CoA carboxylase has been considered by many as the rate limiting step of fatty acid synthesis (2-6,59,60). It has been mentioned in the previous section that acetyl CoA carboxylase requires citrate or isocitrate for maximum activity. Although citrate allows the enzyme to aggregate or to maintain its polymeric form, it is not known whether fluctuations of the citrate concentration in the physiological range could regulate acetyl CoA carboxylase activity. Malonyl CoA, the product of the carboxylation reaction has been shown to be a potent inhibitor ($K_i = 10^{-14} \times 10^{-6} M$) of acetyl CoA carboxylase (4,12,17). However, it is not known whether malonyl CoA accumulates in vivo to a level where it could inhibit acetyl CoA carboxylase. It has also been reported that palmityl CoA, the end product of fatty acid synthesis, inhibits acetyl CoA carboxylase (5). Palmityl CoA has also been shown to inhibit fatty acid synthetase (67-71). However Dorsey and Porter (71) have shown that palmityl CoA inhibited fatty acid synthetase by virtue of its detergent nature. They showed that the detergent sodium lauryl sulfate affected the fatty acid synthetase in a similar fashion. From this, it would appear unlikely that palmityl CoA would be a physiological agent of control. However, Levy (72) has shown a marked correlation between the rapid cessation of fatty acid synthesis, measured in rat mammary gland extracts, and the accumulation of milk in the glands during the first few hours after weaning of the young. Recently Miller et al.

(73) have shown that highly purified rat mammary gland acetyl CoA carboxylase was inhibited by milk obtained from rats 12 hours after their young had been weaned. The inhibitor was identified as a complex mixture of non-esterified fatty acids. They showed that palmityl CoA also inhibited the enzyme, in a similar manner as the milk fatty acids. Miller et al. (73) have proposed that the inhibition of acetyl CoA carboxylase by milk fatty acids serves as a regulatory mechanism to shut off fatty acid synthesis in the mammary gland. Thus the physiological relevance of all the proposed regulators remains to be established.

4. BIOTIN DEFICIENCY AND ACETYL CoA CARBOXYLASE

Early attempts (74,75) to show any in vivo effect of biotin deficiency on rat liver lipogenesis were unsuccessful. Although Wakil and Gibson (1) identified acetyl CoA carboxylase as a biotin containing enzyme in 1960, attempts to show a reduction in activity of this enzyme in the biotin deficient animal have been unsuccessful. Green and Wakil (76) reported that the acetyl CoA carboxylase activity per gram liver was the same in normal and deficient animals and that the reduction in fatty acid synthesis in these animals was brought about by a reduction in liver weight of the deficient animals. Donaldson (77) showed that biotin deficiency in chicks resulted in decreased incorporation of acetate-1-¹⁴C into carcass fatty acids, but had no significant effect on acetate incorporation into liver lipids or malonate-2-¹⁴C

incorporation into liver and carcass lipids. Schultness and Leuthardt (78) showed that biotin deficiency decreased acetate-1-¹⁴C incorporation into fatty acids in chicken liver. Puddu et al. (79) confirmed the results of Donaldson (77) and Schultness and Leuthardt (78). Dakshinamurti and Desjardins (9) have shown that the incorporation of acetate -1-¹⁴C into total liver lipids, triglycerides, phospholipids and fatty acids were decreased in biotin deficient rat liver when compared with pair-weighted controls. They showed that the decreased acetate incorporation in the biotin deficient rat was accompanied by a 50 per cent decrease in the activity of the liver acetyl CoA carboxylase. It was further shown that in biotin deficiency the lipid content of the epididymal adipose tissue was decreased to a greater extent than that of the liver. These results suggested that biotin deficiency might have a more marked effect on the metabolism of adipose tissue than that of the liver.

5. ACETYL CoA HOLOCARBOXYLASE SYNTHESIS

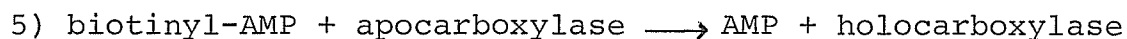
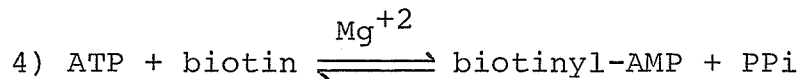
In 1962, Gilgen and Leuthardt (80) studied biotin ¹⁴C incorporation into crude liver subcellular fractions obtained from biotin deficient chickens. They showed that the greatest amount of biotin incorporation occurred in the 110,000 Xg supernatant and that ATP was required for biotin incorporation. Schultness and Leuthardt (78) later showed that crude liver extracts from biotin deficient chickens showed a decreased rate of acetate incorporation into total fatty acids. They

showed that incubation of these extracts with biotin returned acetate incorporation into fatty acids to normal levels. They suggested that this may be due to the synthesis of active acetyl CoA carboxylase from the inactive apoenzyme. Valloton and Leuthardt (81) partially purified this enzyme fraction which catalysed the incorporation of biotin ^{14}C into chicken liver protein. They showed that this enzyme fraction could also catalyse the ATP, Mg^{++} dependent incorporation of biotin into hydroxylamine to form biotin hydroxamate, and that biotinyl-AMP could replace the requirement for biotin and ATP, and could therefore be an intermediate in the reaction. Valloton et al. (82) studied some of the properties of the purified biotin incorporating enzyme using hydroxylamine as an acceptor, and measuring the radioactivity in the biotin hydroxamate formed. They also purified acetyl CoA apocarboxylase from biotin deficient chickens, and showed that the biotin incorporating enzyme could incorporate biotin ^{14}C into the apoenzyme protein, though they did not indicate whether the biotin incorporation had resulted in the synthesis of an active holoenzyme. It is also possible that the purified enzyme preparation of Leuthardt's group contained two enzymes, a biotin incorporating and a biotin activating enzyme. Recently Christner et al. (83) have isolated a biotin activating enzyme from pig liver, which forms biotinyl adenylate as an intermediate, and can transfer the biotinyl group to coenzyme A and hydroxylamine to form biotinyl CoA and biotinyl hydroxamate respectively. However this enzyme does not transfer

the biotin group to the biotin apoenzyme, and as such is different from the holocarboxylase synthetase. Lynen and Rominger (84) in a preliminary report showed that biotinyl adenylate could replace biotin and ATP for the synthesis of the holocarboxylase.

Propionyl CoA holocarboxylase synthetase has been extensively studied for many years. Kosow and Lane (85) were the first to study propionyl CoA holocarboxylase synthesis. They showed that the depressed propionyl CoA carboxylase activity in biotin deficient rat liver, could be rapidly restored to normal by biotin administration in vivo and by incubating liver slices with d-biotin in vitro. Kosow and Lane (86,87) later established a cell free system from biotin deficient rat liver which catalysed the ATP dependent synthesis of propionyl CoA holocarboxylase from the apoenzyme and biotin.

In 1965, Siegel, Foote and Coon (88) reported on the purification and properties of propionyl CoA holocarboxylase synthetase from rabbit liver. They showed that biotinyl adenylate could replace the requirement for biotin and ATP in the reaction, and suggested the following reaction scheme:



Siegel, Foote and Coon (88) suggested that the earlier failures of Kosow, Huang and Lane (87) to show that biotinyl-AMP could replace ATP and biotin in propionyl CoA holocarboxylase

synthesis may have been due to the presence of an active hydrolase in cruder enzyme preparations.

McAllister and Coon (89) have studied the specificity of rabbit liver propionyl CoA holocarboxylase synthetase, yeast acetyl CoA holocarboxylase synthetase, and Propionibacterium shermanii holotranscarboxylase synthetase toward several apoenzymes; rat liver propionyl CoA apocarboxylase, Commanonas terrigena β -methylcrotonyl CoA apocarboxylase. Significant activity was found with all synthetase-apocarboxylase combinations with the sole exception of the combination of liver synthetase-bacterial apotranscarboxylase. McAllister and Coon (89) interpreted this to indicate broad synthetase specificity and suggested that the different apocarboxylases from diverse sources have common structural features in the region of the lysine residues which accept biotin.

CHAPTER III - EXPERIMENTAL

A). METHODS

Chemicals

Acetyl CoA (Li^{+2}), coenzyme A (Li^{+2}), and malonyl CoA (Li^{+2}) (95% pure) were purchased from P. L. Biochemicals. In earlier experiments malonyl CoA was synthesized by the method of Trams and Brady (90). Quantitation of the malonyl CoA was done by determination of thiol esters by the hydroxamic acid procedure (91). The purity of the synthesized malonyl CoA was determined from the hydroxamate: adenine ratio. Adenine was determined by the method of Schmidt (92). $NaH^{14}CO_3$, malonyl ($1,3^{14}C$) CoA, and PPO (2,5 diphenyloxazole) were purchased from New England Nuclear Corporation. Avidin was purchased from Nutritional Biochemicals Corporation, toluene (certified A.C.S., spectranalysed) from Fisher Chemical Company, and POPOP (1,4-bis-2-(5-phenyloxazolyl) benzene) from Nuclear Enterprise (Winnipeg). Cycloheximide, d-biotin, ATP, and calcium phosphate gel were purchased from Sigma Chemical Company. Amino acid ^{14}C (reconstituted protein hydrolysate ^{14}C) was purchased from Schwarz Bioresearch Inc., d-biotin (carboxyl ^{14}C , specific activity 58 mCi/mM) from Amersham/Searle, and Sephadex G-25 from Pharmacia Fine Chemicals.

Animals

Normal rats. Male albino rats, 200-300 g were purchased

from the Holtzman Company and were fed Purina lab chow ad libitum. In some experiments, as indicated, normal rats were fed a low fat (5%), high carbohydrate (65%) diet, ad libitum. The composition of the diets is included in Appendix C.

Biotin deficient rats. Male albino rats (Holtzman Co.), initial weight 50-55 g, were housed in individual cages in air-conditioned quarters and placed on a powdered egg-white diet (9) for 6-8 weeks. (see Appendix C)

Pair-weighed controls (93). Biotin deficient rats were injected intraperitoneally with 100 µg biotin per week, and their food intake was carefully adjusted such that the body weight of the control rats were in the same range as that of the deficient rats. The rats were used one week after the last biotin injection.

1. PURIFICATION AND PROPERTIES OF ADIPOSE TISSUE ACETYL CoA CARBOXYLASE

i) Enzyme Preparations

a) Crude enzyme preparation

In earlier studies some of the properties of the adipose tissue acetyl CoA carboxylase were determined on crude enzyme preparations. The rats were sacrificed by decapitation and the epididymal fat pads were removed, weighed, and placed on ice. The fat pads were then minced with scissors and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle in three volumes of a buffer containing 0.06M Tris(Cl⁻), 0.15M KCl, and 0.1mM EDTA (pH 7.8 at 25°C).

The homogenate was centrifuged at 105,000 Xg for 60 minutes. The high speed supernatant was placed on a Sephadex G-25 column and eluted with the homogenization buffer. The fractions containing acetyl CoA carboxylase activity were pooled. There was 100% recovery of the protein placed on the Sephadex column.

The acetyl CoA carboxylase activity was determined on this gel-filtered cytosol.

b) Enzyme purification

Normal male Holtzman rats fed Purina lab chow were sacrificed by decapitation in batches of 30 rats each. Epididymal fat pads (70 g) were removed and immediately chilled by placing them in cold homogenization buffer containing 0.1M potassium phosphate, 0.15M potassium chloride and 0.1mM EDTA (pH 7.0 at 25°C). The fat pads were blotted dry, weighed, and homogenized in three volumes of the homogenization buffer in a Waring Blendor at low speed for 45 seconds. The homogenate was passed through double thickness of cheesecloth to remove the floating fat, and then centrifuged at 20,000 Xg for 30 minutes. The supernatant was retained.

Ammonium sulfate precipitation: It was found that most of the enzyme could be precipitated out of solution by saturating the 20,000 Xg supernatant to 45% by addition of solid ammonium sulfate. The solution was stirred continuously during the addition and then for an additional 30 minutes. The mixture was centrifuged at 13,000 Xg for 15 minutes and the supernatant discarded. The white pellet was dissolved

in one tenth the original volume with 0.01M potassium phosphate, 10mM 2-mercaptoethanol, and 0.1mM EDTA (pH 7.0 at 25°C). The enzyme solution was then dialysed against 0.01M potassium phosphate, 10mM 2-mercaptoethanol, and 0.1mM EDTA (pH 7.0 at 25°C) for three hours. The cloudy dialysate was centrifuged at 105,000 Xg for one hour, and the supernatant was retained.

Calcium phosphate adsorption: Calcium phosphate gel, 2 mg dry weight of the gel per mg protein, was added to the high speed supernatant. The mixture was stirred for 10 minutes and then centrifuged at 500 Xg for 5 minutes. The supernatant was discarded and the gel was extracted with one tenth the volume from the previous step with 0.12M potassium phosphate, 10mM 2-mercaptoethanol, and 0.1mM EDTA (pH 7.6 at 25°C), for 10 minutes and centrifuged as before. The gel was extracted once more with the phosphate buffer and again centrifuged. The supernatants were pooled and dialysed against 0.01M potassium phosphate, 10mM 2-mercaptoethanol, and 0.1mM EDTA (pH 7.0 at 25°C) for 3 hours. All of the above operations were carried out in one day, and were all done at 3-5°C. The enzyme preparation could be stored at -70°C after the calcium phosphate step for several months without any loss in activity.

The activity of the enzyme was determined after a 30 minute preincubation as described under carboxylase assay.

ii) Carboxylase Assay

Acetyl CoA carboxylase activity was determined by

the $\text{H}^{14}\text{CO}_3^-$ fixation assay of Chang et al. (22).

a) Preincubation

The rat adipose tissue enzyme requires preincubation with citrate for maximum activity (10). Preincubation was carried out for 30 or 60 minutes at 37°C in a medium containing; 60mM Tris (Cl^-), 3mM GSH, 8mM MgCl_2 , 0.1mM EDTA, 5mM potassium citrate, 0.15 mg bovine serum albumin (BSA) and the enzyme preparation, usually dissolved in 0.01-0.10M potassium phosphate buffer (pH 7.0) containing 10mM 2-mercaptoethanol, and 0.1mM EDTA. The final volume was 0.25 ml and the pH was 7.5 at 37°C.

b) Assay

Aliquots of the preincubated enzyme preparation were added to the following assay system: 60mM Tris (Cl^-), 0.1mM EDTA, 8mM MgCl_2 , 3mM GSH, 2mM ATP, 0.2mM acetyl CoA (Li^{++}), 5mM potassium citrate, 0.6 mg BSA per ml, and 10mM $\text{NaH}^{14}\text{CO}_3$ (0.2 $\mu\text{Ci}/\mu\text{mole}$), all at pH 7.5, 37°C. The final volume was 0.5 ml and the incubation time two minutes. Acetyl CoA was omitted from control tubes. The reaction was started by addition of the carboxylase and stopped with the addition of 0.1 ml 6N HCl. The reaction mixture was transferred to a liquid scintillation vial and heated under an infra red lamp (85°C) for 40 minutes. Water (200 μl) and 15 ml of toluene-ethanol scintillator was added and the radioactivity was determined. One unit of enzyme activity is defined as the amount of enzyme required to carboxylate one μmole acetyl CoA per minute at 37°C.

iii) Identification of Reaction Product

The acidified reaction mixture obtained after stopping the acetyl CoA carboxylase reaction with 6N HCl was chromatographed (15) on Whatman No. 1 paper in a solvent system containing ethanol and 1.0M acetate buffer pH 3.8 (7:3 v/v). All of the radioactivity co-chromatographed with authentic malonyl CoA. The malonyl CoA was visualised by examination under ultra violet light.

iv) Progress Curve of Acetyl CoA Carboxylase

Partially purified and crude enzyme preparations were preincubated in the preincubation medium previously described, and the progress of the reaction was followed in the assay system by stopping the reaction after various time intervals.

v) Malonyl CoA Decarboxylase Activity

From studies of the progress curve of crude preparations of the enzyme, it became quite evident that the product malonyl CoA was being destroyed. Hence malonyl CoA decarboxylase activity was measured in crude enzyme preparations. The gel filtered cytosol was prepared and preincubated with citrate as described for determination of acetyl CoA carboxylase activity. An aliquot of the preincubated enzyme preparation was used to determine acetyl CoA carboxylase activity and another to determine malonyl CoA decarboxylase activity. In some cases the enzyme preparation was preincubated in the presence of avidin (1 unit per mg gel-filtered cytosol). One unit of avidin is defined as that

amount of protein which binds 1 μ g of biotin. Malonyl CoA decarboxylase was assayed in the following system; 60mM Tris (Cl^-), 0.1mM EDTA, 8mM MgCl_2 , 3mM GSH, 3mM ATP, 0.207mM malonyl [$1,3^{14}\text{C}$]CoA (7.5 $\mu\text{Ci}/\mu\text{mole}$), and preincubated gel filtered cytosol (0.9 mg protein). The final volume was 1.0 ml and the pH 7.0 at 37°C. The control contained the complete system with only the enzyme preparation omitted. At various time intervals, aliquots (200 μl) were taken and added to tubes containing 40 μl of 6N HCl to stop the reaction. The aliquots were then transferred to liquid scintillation vials, heated for 30 minutes under an infra red lamp (85°C) and the radioactivity was measured.

During the purification of acetyl CoA carboxylase malonyl CoA decarboxylase activity was determined at each step. Malonyl CoA decarboxylase activity was measured under conditions optimal for acetyl CoA carboxylase activity and hence may not be a true evaluation of the total malonyl CoA decarboxylase enzyme present.

Malonyl CoA decarboxylase activity was measured in the same assay medium described above, except that the pH was 7.5 at 37°C. The enzyme preparation was the carboxylase enzyme preparation taken at each step of the purification and preincubated for 30 minutes in the preincubation medium as previously described. The reaction was started by the addition of the enzyme preparation and the progress curve of malonyl CoA decarboxylation was followed. The reaction was permitted to proceed to completion, that is, until no

further decrease in radioactivity was noted. The residual radioactivity (due to acetyl CoA¹⁴C), remaining after the reaction had gone to completion was subtracted from the radioactivity in the control. The control contained the same as the complete system except that the enzyme preparation was omitted. The difference in radioactivity measured was related to the total amount of malonyl CoA initially present in the assay, but which had now been decarboxylated. Malonyl CoA decarboxylase activity was expressed as μ moles of malonyl CoA decarboxylated per hour per mg protein at 37°C calculated from a 10 minute incubation, in the linear range of the reaction.

vi) Avidin Inhibition

The effect of avidin on rat epididymal adipose tissue acetyl CoA carboxylase was studied. Gel filtered cytosol was obtained from epididymal fat pads of normal rats fed Purina lab chow. The gel filtered cytosol was placed in the preincubation medium previously described, and treated with the following at 0°C for 15 minutes: 1). no treatment, 2). avidin (1 unit per mg enzyme protein, 3). avidin-biotin (1 unit avidin mixed with 10 μ g biotin (excess) per mg enzyme protein prior to addition to enzyme preparation), 4). biotin (10 μ g per mg enzyme protein). The enzyme preparations were then preincubated for 45 minutes and acetyl CoA carboxylase activity determined.

vii) Activation of Acetyl CoA Carboxylase

The partially purified enzyme was preincubated at

a concentration of 0.5 mg protein per ml in the preincubation medium previously described, except that in certain cases the following were omitted: potassium citrate (5mM), magnesium chloride (8mM), bovine serum albumin (0.6 mg per ml). Either one or two of the three were omitted at one time, and the enzyme was preincubated at 37°C for 60 minutes. At various time intervals the degree of activation was determined by assaying the acetyl CoA carboxylase activity. Care was taken to assure the proper concentrations of the constituents in the assay system.

viii) Effect of BSA on Activation of Acetyl CoA Carboxylase

The partially purified enzyme was preincubated for two hours at 37°C at a concentration of 0.98 mg protein per ml in the preincubation medium previously described in the presence and absence of 0.6 mg per ml BSA. At various time intervals during the preincubation the degree of activation was determined by assaying the acetyl CoA carboxylase activity.

ix) Effect of pH on Acetyl CoA Carboxylase Activity

The enzyme preparation (specific activity 0.225 units/mg protein) was preincubated at a concentration of 1.18 mg protein per ml for 60 minutes, pH 7.5 at 37°C. After preincubation the activity was assayed in essentially the same medium as described previously except that 60mM potassium phosphate was used as buffer for the pH range 6.0 to 7.0. Tris buffer (60mM) was used for the pH range 7.0 to 9.0. The pH of the assay medium was adjusted prior to determination

of carboxylase activity. In a separate experiment the pH of the assay system was verified during the incubation at 37°C with a pH meter.

x) Metal Requirements

The enzyme preparation (specific activity 0.259 units/mg protein) was preincubated at a concentration of 1.53 mg protein per ml for 60 minutes at 37°C in the medium previously described except that MgCl₂ and EDTA were omitted. The activity of the enzyme was then assayed at various manganese and magnesium ion concentrations with and without EDTA. The short assay time (2 minutes) and the presence of ATP, would prevent further activation of the enzyme such that the metal requirements of the assay system and not those of the activation system were studied.

xi) Cold Inactivation

The enzyme preparation (specific activity 0.259 units/mg protein) was preincubated at a concentration of 1.52 mg protein per ml at pH 7.0, 7.5 and 8.0, for 90 minutes at 37°C. An aliquot of the preincubated enzyme was exposed to cold by placing in an ice bath, and another was kept at 25°C. Enzyme activity was determined at various time intervals. All carboxylase assays were performed in a two minute incubation at pH 7.5, 37°C. The reversibility of the cold inactivation was demonstrated by re-incubating the enzyme, which had been cold inactivated (4 hours), at 37°C for one hour, and determining the carboxylase activity.

xii) Determination of Radioactivity

In all of our work a toluene-ethanol scintillator (9) was used. Toluene (600 ml), containing 0.4% PPO (2,5-diphenyloxazole) and 0.0015% POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene), was added to absolute ethanol (378 ml). Radioactivity was measured in a Packard model 3000 liquid scintillation spectrometer at the optimal settings for ^{14}C counting. External standardization was used to make corrections when quenching occurred.

xiii) Protein

All protein determinations were done according to the method of Lowry et al. (94) using crystalline bovine serum albumin as standard.

2. BIOTIN STATUS AND ACETYL CoA CARBOXYLASE ACTIVITY

i) Effect of Biotin Deficiency on Adipose Tissue Acetyl CoA Carboxylase Activity

The effects of various dietary treatments, including production of biotin deficiency, were investigated on the rat epididymal adipose tissue acetyl CoA carboxylase. Acetyl CoA carboxylase activity was determined in biotin deficient rats, pair-weighted controls, normals on Purina lab chow, and normals on a low fat, high carbohydrate diet.

a) Enzyme preparation

For this study the acetyl CoA carboxylase activity was determined on a crude enzyme preparation. The crude enzyme preparation was obtained from epididymal fat

pads as described in the first section of methods (Chapter III A,1,i,a).

b) Carboxylase assay

The assay procedure was similar to that described for the partially purified enzyme in the first section of methods (Chapter III A,1,ii) except that both the preincubation and the assay were done at pH 7.0, 37°C.

Preincubation: The gel filtered cytosol (0.5 to 1.0 mg) was preincubated for 30 minutes pH 7.0, 37°C. The final volume of the preincubation medium was 1.0 ml.

Assay: The preincubated enzyme preparation (0.25-0.50 mg) was assayed at pH 7.0, 37°C. The final volume of the assay medium was 1.0 ml. The reaction was stopped after a two minute incubation by the addition of 0.2 ml 6N HCl. Aliquots (200 μ l) were placed in a liquid scintillation vial and heated under an infra red lamp for 30 minutes and the radioactivity determined.

ii) In vivo Restoration of Liver and Adipose Tissue

Acetyl CoA Carboxylase by Biotin

a) Restoration of acetyl CoA carboxylase activity

Biotin deficient rats were injected intraperitoneally with 200 μ g of d-biotin dissolved in 0.9% NaCl. Control rats (biotin deficient) received 1.0 ml of 0.9% NaCl intraperitoneally. The biotin treated rats were sacrificed at various time intervals (0.5, 1, 2 hours) after biotin administration and acetyl CoA carboxylase activity was determined in the liver and adipose tissue.

Carboxylase assay: Acetyl CoA carboxylase activity was measured on the gel filtered high speed supernatant of the liver and adipose tissue. The liver gel filtered high speed supernatant was obtained using the procedure previously described for the adipose tissue (Chapter III A,1,i,a). Acetyl CoA carboxylase activity was measured using the same assay procedure described for the partially purified enzyme except that the preincubation was carried out for 45 minutes (Chapter III A,1,ii).

b) Effect of cycloheximide on adipose tissue protein synthesis

In vivo protein synthesis was determined in the liver and adipose tissue by measuring the incorporation of amino acid ^{14}C (reconstituted protein hydrolysate ^{14}C) into liver and epididymal adipose tissue protein. Normal rats (150 g), fed Purina lab chow, were injected intramuscularly with 3 $\mu\text{Ci}/100$ g body weight amino acid ^{14}C , and sacrificed at various time intervals (0.5, 1, 2, 3 hours) after injection of the label. The liver and epididymal fat pads were removed and homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle in three volumes of a buffer containing 0.06M Tris (Cl^-), 0.15M KCl, and 0.1mM EDTA (pH 7.5 at 25°C). The liver and adipose tissue homogenates were centrifuged at 10,000 Xg for 10 minutes, and the supernatants were retained, taking care not to include the floating fatty layer. The supernatant protein was precipitated by the addition of an equal volume of 20% TCA to an aliquot of

the 10,000 Xg supernatant. After centrifugation the precipitate was retained and successively washed, twice with 5% TCA, once with hot 5% TCA (90°C), once more with 5% TCA, twice with 70% ethanol, twice with ethanol:ether (3:1 v/v), and twice with ether. The washed protein was then dried under vacuum overnight to remove the ether. A small volume (0.4 ml) of 1N NaOH was added to the protein and the mixture was heated for 5 to 10 minutes at 60°C until the protein was completely dissolved. The specific radioactivity of the washed protein was determined. An aliquot (200 µl) of the protein solution was placed in a toluene-ethanol scintillator and the radioactivity was measured (Chapter III A,1,xii). From the progress curve it was seen that a 30 minute pulse of amino acid ^{14}C would serve to measure in vivo adipose tissue protein synthesis.

The effect of cycloheximide on the incorporation of amino acid ^{14}C into adipose tissue protein was determined by injecting rats intraperitoneally with 10 mg per 100 g body weight of cycloheximide (dissolved in 0.9% NaCl) at various time intervals (15, 30, 60 minutes) prior to the 30 minute amino acid ^{14}C pulse.

c) Effect of cycloheximide on restoration of adipose tissue acetyl CoA carboxylase activity by biotin

Biotin deficient rats were injected intraperitoneally with cycloheximide (10 mg per 100 g body weight) dissolved in 0.9% NaCl, 15 minutes prior to d-biotin administration. Rats which received d-biotin were injected

intraperitoneally with 200 µg d-biotin (dissolved in 0.9% NaCl) and sacrificed 30 minutes later. Control rats (biotin deficient) received 1.0 ml of 0.9% NaCl intraperitoneally. Cycloheximide treated rats were under the influence of cycloheximide for a total of 45 minutes. Acetyl CoA carboxylase activity was determined 30 minutes after the biotin administration (Chapter III A,2,ii,a).

iii) In vitro Adipose Tissue Acetyl CoA Holocarboxylase Synthesis

a) Acetyl CoA holocarboxylase synthetase assay

Biotin deficient rats were sacrificed by decapitation, their epididymal fat pads were removed and pooled. The fat pads were homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle in three volumes of a buffer containing 0.06M Tris (Cl^-), 0.15M KCl, and 0.1mM EDTA (pH 7.5 at 25°C). The homogenate was centrifuged at 20,000 Xg for 30 minutes, and the supernatant was retained. The supernatant was placed on a Sephadex G-25 column and eluted with the homogenization buffer.

Preincubation: The gel filtered enzyme preparation was preincubated for 60 minutes at 37°C in the following medium: 60mM Tris (Cl^-) (pH 7.5 at 37°C), 3mM GSH, 8mM MgCl_2 , 0.1mM EDTA, 5mM potassium citrate, 0.6 mg per ml bovine serum albumin. When magnesium was omitted from the preincubation medium, the citrate concentration was increased to 25 mM. The preincubation served to activate acetyl CoA carboxylase maximally.

Incubation: The preincubated enzyme preparation (0.8 ml)

was incubated for 4 hours at 30°C, pH 7.5, in the presence of 4mM ATP and 82µM d-biotin (20 µg) in a final volume of 1.0 ml. The complete reaction mixture contained 48mM Tris (Cl⁻) (pH 7.5), 2.7mM GSH, 6.4mM MgCl₂, 0.08mM EDTA, 4.0mM potassium citrate, 0.48 mg per ml bovine serum albumin, 4mM ATP, and 82µM d-biotin. When magnesium was omitted from the preincubation the citrate concentration was 20 mM in the incubation. Controls contained the complete system except that d-biotin was omitted. At the end of the four hour incubation aliquots (150 µl) were removed and the acetyl CoA carboxylase activity was assayed as previously described (Chapter III A,2,ii,a). All assays were done in duplicate. Acetyl CoA holocarboxylase synthesis is expressed as milli-units of increased enzyme activity per mg protein in the complete system after correction for controls which consisted of the complete system minus biotin.

b) Biotin ¹⁴C incorporation into protein

The gel filtered enzyme preparation was pre-incubated and incubated as described above except that d-biotin (carboxyl ¹⁴C) was used instead of cold biotin, at a concentration of 84µM (5.21 x 10⁶ dpm per 84µmoles). The reaction mixture was incubated 4 hours at 30°C and the reaction was stopped by addition of 1.0 ml 20% TCA. In the control assay (biotin omitted) the radioactive biotin was added after the reaction had been stopped with TCA. The precipitated protein was washed, dissolved in 1N NaOH, and the specific radioactivity of the protein determined as

previously described (Chapter III A,2,ii,b).

iv) In vitro Liver Acetyl CoA Holocarboxylase Synthesis

Biotin deficient rats were sacrificed by decapitation, and their livers were removed, and placed on ice. The gel filtered cytosol was prepared as before (Chapter III A,2,iii,a) except that the homogenate was centrifuged at 105,000 Xg for 60 minutes. Liver acetyl CoA holocarboxylase synthesis was determined as previously described for the adipose tissue (Chapter III A,2,iii,a).

v) Statistics

Statistical analysis of data was done using the Student's t test for unmatched groups. All calculations were done with a desk computer (Olivetti Underwood Programma 101).

B). RESULTS

1. PURIFICATION AND PROPERTIES OF ADIPOSE TISSUE ACETYL
CoA CARBOXYLASE

i) Purification

The enzyme preparation after the calcium phosphate gel adsorption step had a specific activity which varied from 0.2 to 0.4 units per mg protein (Table II). This was 4-8 times the specific activity previously reported for this tissue (10). It was not possible to determine the over-all purification accurately because of the apparent removal of an inhibitor during purification which caused a 176% increase in total enzyme units after the ammonium sulfate step. There was also a slight increase in total enzyme units after the high speed centrifugation. The nature of this inhibitor will be discussed later.

The purification of acetyl CoA carboxylase was carried out in one day, because of the instability of the enzyme at the intermediate purification steps. However, the enzyme preparation obtained after the calcium phosphate gel adsorption step could be kept in 0.01M potassium phosphate buffer, 10mM 2-mercaptoethanol, and 0.1mM EDTA at pH 7.0 and -70°C, for several months without any loss in activity. It was found that purification of the enzyme in the presence of 10-20mM citrate enhanced the stability of the enzyme in the earlier steps of the purification, but the enzyme was sometimes centrifuged out of solution by the 105,000 Xg centrifugation

and also could not be absorbed on calcium phosphate gel. The partially purified enzyme in 0.01M potassium phosphate buffer, pH 7.0, 10mM 2-mercaptoethanol, 0.1mM EDTA and 20mM citrate could also be kept at room temperature (25°C) for several days without any loss in activity. Further purification of the enzyme was attempted using DEAE cellulose, and cellulose phosphate but was unsuccessful, probably because of the small amount of protein involved in the purification and the instability of the enzyme in dilute solutions.

ii) Progress Curve of the Reaction

The progress curve of the acetyl CoA carboxylase reaction after a 30 minute preincubation using a crude enzyme preparation was linear for three minutes, and reached a plateau at 15 minutes (Fig. 1). After 20 minutes there was a rapid disappearance of the malonyl CoA already formed. The plateau region of the progress curve can be attributed to either instability of the enzyme under these conditions or to inhibition of the enzyme by the product formed. However the disappearance of the product can only be attributed to its degradation by a contaminating enzyme. Utilization of malonyl CoA for long chain fatty acid synthesis was ruled out due to the lack of NADPH. Any endogenous NADPH would have been removed during Sephadex filtration. It was also found that crude enzyme preparations from rat liver gave a similar progress curve, and thus may also contain malonyl CoA decarboxylase activity.

The progress curve of the acetyl CoA carboxylase reaction

after a 30 minute preincubation using the partially purified enzyme, in contrast to the crude enzyme preparation, was linear for nearly 20 minutes and began to plateau only after 40 minutes of incubation (Fig. 2). The activity of the partially purified enzyme was found to be concentration-dependent in the range 5 to 50 μg per 0.5 ml assay (Fig. 3).

iii) Malonyl CoA Decarboxylase Activity

The gel filtered cytosol was assayed for the presence of malonyl CoA decarboxylase, an enzyme whose presence could explain the disappearance of the malonyl CoA formed during the acetyl CoA carboxylase reaction. Malonyl CoA decarboxylase activity was found to be present in the crude enzyme preparation from normal rats fed the high carbohydrate diet (Fig. 4). Avidin had no effect on the rate of malonyl CoA decarboxylation suggesting that the reverse of the carboxylation reaction: $\text{malonyl CoA} + \text{ADP} + \text{P}_i \longrightarrow \text{acetyl CoA} + \text{ATP} + \text{CO}_2$ was not responsible for the removal of malonyl CoA. It has been shown by Gregolin et al. (20) that malonyl CoA decarboxylation, the reverse of the carboxylation reaction does not take place in the presence of avidin. The gel filtered cytosol used in the decarboxylase assay showed a typical progress curve for the crude acetyl CoA carboxylase enzyme (Fig. 1) and the enzyme activity was completely inhibited by the avidin treatment used in the decarboxylase assay. It should also be noted, that there was no significant decarboxylation during the initial two minutes of incubation (Fig. 4). This was a consistent observation and hence the

decarboxylase activity would not interfere with the initial rate studies of the carboxylase reaction. It was for this reason that the carboxylase assays consisted of only a two minute incubation when acetyl CoA carboxylase activity was determined in crude enzyme preparations.

Malonyl CoA decarboxylase activity was determined on the acetyl CoA carboxylase enzyme preparation at various steps of the purification (Table III). It can be seen that after the high speed centrifugation there was a slight increase in malonyl CoA decarboxylase specific activity, but that the relative activity of the decarboxylation reaction to the carboxylation reaction was greatly decreased. The removal of malonyl CoA decarboxylase activity could explain the increase in total acetyl CoA carboxylase units which occurred during these purification steps (Table I). There was no detectable malonyl CoA decarboxylase activity after the calcium phosphate adsorption step, under conditions which were optimal for acetyl CoA carboxylase activity.

iv) Avidin Inhibition

Avidin inhibited acetyl CoA carboxylase activity completely (Table IV). Prior incubation of the avidin with an excess of d-biotin prevented the avidin inhibition. Biotin had no effect on the acetyl CoA carboxylase activity.

v) Activation of Acetyl CoA Carboxylase

Acetyl CoA carboxylases isolated from rat tissues have been shown to require preincubation with activators for maximum activity (10,11,15). This was also true of the adipose

tissue enzyme (Fig. 5). Maximum activation was obtained in the complete system after preincubation for 60 minutes. Appreciable activation of the enzyme occurred in the absence of both magnesium and citrate. Magnesium itself did not activate the enzyme but magnesium in the presence of citrate enhanced activation. Neither magnesium nor citrate alone at these concentrations activated the enzyme in the absence of bovine serum albumin (Fig. 5). However magnesium in the presence of citrate caused appreciable activation of the enzyme even in the absence of bovine serum albumin (Fig. 6). The removal of bovine serum albumin from the preincubation decreased the rate and degree of acetyl CoA carboxylase activation. At the low protein concentrations used, the enzyme was probably protected by BSA.

vi) Effect of pH on Carboxylase Activity

The rat adipose tissue acetyl CoA carboxylase had a pH optimum of 7.5. The enzyme activity at pH 7.0 was greater in Tris buffer than in phosphate buffer (Fig. 7). The crude enzyme had a pH profile identical with that of the partially purified enzyme (Fig. 8). Both the rat and chicken liver enzymes (4,20) have similar pH optima.

vii) Metal Requirement

The adipose tissue enzyme showed optimal activity at 1.3mM manganese, and 8mM magnesium (Fig. 9). The activity with optimal manganese concentration was 55% of the activity with optimal magnesium concentration. Low concentrations of EDTA (0.1mM) had no effect on the metal requirement of

the enzyme.

viii) Substrates and Inhibitors

Optimal enzyme activity was obtained at 4mM ATP. The enzyme was inhibited by ATP at higher concentrations (Fig. 10). From a Lineweaver-Burke plot the apparent K_m for ATP was calculated to be 2.5×10^{-4} M. Since citrate was present in the preincubation the lowest citrate concentration which could be used was 0.1mM. Optimal enzyme activity was obtained at a citrate concentration of 5mM (Fig. 11).

In preliminary studies the effect of malonyl CoA was studied on the crude enzyme preparation (Table V). The malonyl CoA used was synthesized by the method of Trams and Brady (90), and was known to contain coenzyme A as an impurity. The amount of coenzyme A present could be calculated from the hydroxamate:adenine ratio. A 60% inhibition of the acetyl CoA carboxylase activity was obtained with a malonyl CoA concentration of 175 μ M. Inhibition was progressive with increasing concentration of malonyl CoA. There was also a slight inhibition of the enzyme by coenzyme A. Even at the highest concentration of malonyl CoA tested, inhibition due to coenzyme A contamination accounted for less than 15% of the inhibition attributable to malonyl CoA.

The double reciprocal plots (Fig. 12 and 13) show that the purified enzyme was inhibited by malonyl CoA. The inhibition was competitive with respect to acetyl CoA and non-competitive with respect to ATP. The malonyl CoA used was obtained commercially (95% pure). The apparent K_m for

acetyl CoA was 1.4×10^{-5} M. The K_i for malonyl CoA was 1.0×10^{-5} M. Coenzyme A also inhibited the purified enzyme although to a lesser extent than malonyl CoA (Fig. 14).

ix) Cold Inactivation

Acetyl CoA carboxylase, once maximally activated, undergoes cold inactivation (Fig. 15). Cold inactivation was relatively slight at pH 7.0, but very pronounced at pH 7.5 and 8.0. The cold inactivation at pH 7.5 and 8.0 was partially reversed by reincubating at 37°C. Thus the adipose tissue acetyl CoA carboxylase undergoes pH dependent reversible cold inactivation.

2. BIOTIN STATUS AND ACETYL CoA CARBOXYLASE ACTIVITY

i) Effect of Biotin Deficiency on Acetyl CoA Carboxylase

The effects of four different dietary treatments on the adipose tissue acetyl CoA carboxylase were studied. The rate of citrate activation, the progress curve of the carboxylase reaction, and the levels of the enzyme were investigated in the adipose tissue of normal rats on Purina lab chow, normal rats on a low fat high carbohydrate (65% dextrose) diet, biotin deficient rats, and pair-weighted controls. Preincubations and carboxylase assays were carried out at comparable concentrations of the Sephadex G-25 gel-filtered cytosol protein. The rate of citrate activation varies according to the dietary treatment (Fig. 16). The enzyme preparation from both the biotin deficient rats and those fed Purina lab chow were fully activated after 20 minutes

of preincubation with citrate. Similar enzyme preparations from pair-weighed control rats or those fed the high carbohydrate diet reached near optimal activity after 50 minutes of preincubation with citrate. The pair-weighed controls and high-dextrose fed rats exhibited higher activities than the biotin deficient rats or rats fed Purina lab chow.

A typical time course of the incorporation of $\text{H}^{14}\text{CO}_3^-$ into malonyl CoA by acetyl CoA carboxylase preparations from the different groups is shown in Fig. 17. Enzyme preparations from pair-weighed controls and high dextrose fed rats showed high initial velocities. The dextrose and fat content of the biotin deficient diet were comparable to that of the high dextrose diet and yet the enzyme preparation from deficient rats, on an equivalent protein basis, had low initial velocities suggesting low enzyme activity. All the progress curves started to plateau after 5-10 minutes of incubation. With the enzyme preparation from deficient animals there was a slight disappearance of the product formed after 10 minutes of incubation. A more marked reduction in the product formed was observed in enzyme preparations from the pair-weighed controls. The plateau region of the progress curve was attributed to inhibition of the carboxylase by malonyl CoA, and to disappearance of the product due to its degradation by a contaminating enzyme, which has been shown to be malonyl CoA decarboxylase.

Acetyl CoA carboxylase activity of the adipose tissue of rats on the different treatments were compared. The results are given in Table VI. Pair-weighed control rats

and rats fed the high carbohydrate diet both exhibited high levels of enzyme activity. The carboxylase activity of biotin deficient rat adipose tissue was less than a sixth of pair-weighted control levels. The dietary treatments imposed on the rats in these experiments were comparable to those reported earlier (9), where liver acetyl CoA carboxylase activities were studied. Biotin deficiency was previously shown to cause only a two fold decrease in liver acetyl CoA carboxylase activity under similar conditions (9).

ii) In vivo Restoration of Liver and Adipose Tissue Acetyl CoA Carboxylase by Biotin

a) Restoration of acetyl CoA carboxylase activity

Biotin deficient rats were injected intraperitoneally with 200 µg of biotin and acetyl CoA carboxylase activity was determined in the liver and adipose tissue at various time intervals after biotin administration. The in vivo restoration of acetyl CoA carboxylase activity in liver and epididymal adipose tissue of biotin deficient rats occurred at different rates (Table VII). The liver acetyl CoA carboxylase activity was maximum after one hour. The epididymal adipose tissue enzyme activity reached near maximum levels after two hours, and at three hours after biotin administration the acetyl CoA carboxylase level was 121.0 milliunits per mg protein. From this it can be calculated that 40% and 90% of the acetyl CoA carboxylase in the liver and adipose tissue respectively are in the apoenzyme form in biotin deficient rats. Thus the response of these two tissues

to biotin deficiency were quantitatively different (9).

The higher specific activities obtained for liver and epididymal adipose tissue acetyl CoA carboxylase in these experiments as compared to the values shown in Table VI, and those reported previously for the liver enzyme (9) are mainly due to the pH of the assay. In previous work (9) and in Table VI the assays were done at pH 7.0 whereas the pH optimum of the enzyme has now been shown to be 7.5.

b) Effect of cycloheximide on adipose tissue protein synthesis

The rate of amino acid ^{14}C incorporation into liver and adipose tissue protein was determined (Fig. 18). The liver and adipose tissue proteins attained the same level of specific radioactivity, but incorporation of the label occurred more rapidly in the liver. A 30 minute amino acid ^{14}C pulse was used to determine in vivo adipose tissue protein synthesis.

Cycloheximide (10 mg per 100 g body wt.) inhibited amino acid ^{14}C incorporation into epididymal adipose tissue protein by greater than 90%, when administered 15, 30, or 60 minutes prior to the 30 minute amino acid ^{14}C pulse (Table VIII). A similar inhibition of in vitro rat liver protein synthesis by cycloheximide has been reported by Korner (95).

c) Effect of cycloheximide on restoration of adipose tissue acetyl CoA carboxylase activity by biotin

The in vivo restoration of acetyl CoA carboxylase

activity by d-biotin occurred very rapidly (Table IX). Thirty minutes after biotin administration the epididymal adipose tissue acetyl CoA carboxylase activity was significantly ($p < 0.01$) stimulated. Cycloheximide at a concentration which inhibited amino acid ^{14}C incorporation into epididymal adipose tissue protein by greater than 90% did not prevent the restoration of acetyl CoA carboxylase activity in vivo. Although there was a significant ($p < 0.05$) inhibition of the restoration of acetyl CoA carboxylase activity by cycloheximide, a four fold increase in carboxylase activity was brought about by d-biotin administration.

iii) In vitro Acetyl CoA Holocarboxylase Synthesis

a) Progress curve of acetyl CoA holocarboxylase synthesis

Results of the in vitro activation of acetyl CoA apocarboxylase are given in Fig. 19. Biotin in vitro produced a 2.6 fold increase in acetyl CoA carboxylase activity during the four hour incubation. The control (biotin omitted) was stable during the first two hours of incubation at 30°C but lost 20% of the activity after 4 hours of incubation. Attempts to show holocarboxylase synthesis at 37°C were unsuccessful.

b) Effect of magnesium on acetyl CoA holocarboxylase synthesis

The effect of magnesium on acetyl CoA holocarboxylase synthesis was investigated. There was decreased acetyl CoA holocarboxylase synthesis in the presence of

magnesium (Table X). It should also be noted that acetyl CoA carboxylase activity in the control with magnesium was decreased by 44% during the four hour incubation while the control without magnesium showed only a 14% decrease. This greater loss in activity with magnesium may be due to the presence of the ATP-Mg chelate which has been shown to promote disaggregation of the chicken liver acetyl CoA carboxylase to the inactive disaggregated form (17,96). Magnesium does not seem to be required for acetyl CoA holocarboxylase synthesis. However, there may be sufficient magnesium present in this crude system to allow holocarboxylase synthesis.

c) Requirements for acetyl CoA holocarboxylase synthesis and biotin ^{14}C incorporation into protein

The ATP and d-biotin requirements for acetyl CoA holocarboxylase synthesis were studied in the absence of magnesium (Fig. 20 and 21). Half maximal holocarboxylase synthesis was attained at 2mM ATP and 2 μM d-biotin.

Acetyl CoA holocarboxylase synthesis and biotin ^{14}C incorporation into protein both required ATP and d-biotin (Table XI). In the complete system, without magnesium, there were 2.3 and 7.6 fold increases in acetyl CoA holocarboxylase activity and biotin ^{14}C incorporation respectively when compared to the controls (biotin omitted). The greater increase in biotin ^{14}C incorporation than in holocarboxylase synthesis may be due to the presence of other biotin requiring apoenzymes besides acetyl CoA apocarboxylase in the crude

enzyme preparation. There was a 2.5 fold increase in biotin ^{14}C incorporation in the presence of added magnesium when compared to the system without magnesium. Magnesium seems to inhibit acetyl CoA holocarboxylase synthesis. Holo-enzyme formation probably requires Mg^{++} as seen from biotin ^{14}C incorporation into protein but possible other effects of Mg^{++} on the enzyme itself might mask the real acetyl CoA holocarboxylase synthesis.

iv) In vitro Liver Acetyl CoA Holocarboxylase Synthesis

Acetyl CoA holocarboxylase synthesis was assayed in the liver soluble fraction (Fig. 22). The assay conditions used were the one which were found to be optimal for the adipose tissue holocarboxylase synthetase. It can be seen that very little acetyl CoA holocarboxylase synthesis occurs in the liver soluble fraction of biotin deficient rats. This could be due to the fact that the liver enzyme has different requirements than the adipose tissue enzyme. However the low acetyl CoA holocarboxylase synthesis in the liver is more likely due to the lack of sufficient acetyl CoA apocarboxylase.

CHAPTER IV - DISCUSSION

1. PURIFICATION AND PROPERTIES OF ADIPOSE TISSUE ACETYL
CoA CARBOXYLASE

Acetyl CoA carboxylase from rat epididymal fat pads has been purified to a greater specific activity than previously reported (10). The catalytic properties of the enzyme from this source have not been reported before. Crude preparations of acetyl CoA carboxylase were found to contain malonyl CoA decarboxylase activity. This enzyme interfered with the assay of acetyl CoA carboxylase activity, by removing the product formed in the carboxylation reaction. Malonyl CoA decarboxylation, the reverse of the carboxylation reaction also catalysed by acetyl CoA carboxylase, was not involved in the destruction of malonyl CoA, since avidin had no effect on malonyl CoA decarboxylase activity. Gregolin et al. (20) have shown that malonyl CoA decarboxylation the reverse of the carboxylation reaction was inhibited by avidin. Nakada et al. (97) have reported the presence of malonyl CoA decarboxylase in rat liver mitochondria. Scholte (98) has also shown malonyl CoA decarboxylase to be a mitochondrial enzyme. He has shown that it behaves as a "matrix" enzyme and thus can easily be solubilized from mitochondria, and contaminate cytoplasmic enzymes. Vagelos et al. (10) also suspected malonyl CoA decarboxylase activity in their crude enzyme preparations from rat adipose tissue. It has been shown that purification of the enzyme removes the contaminating

enzyme and that the purified enzyme preparation does not contain any malonyl CoA decarboxylase activity after the calcium phosphate adsorption step.

The rat liver (11), mammary gland (15), and adipose tissue enzymes (10), all require preincubation with activators to produce maximum enzyme activity. Greenspan and Lowenstein (53) have shown that magnesium alone could activate the rat liver enzyme in the absence of citrate, although citrate was still required for maximum activity in the assay. Similarly Miller and Levy (15) have reported that magnesium activated the rat mammary gland enzyme. Activation of the rat adipose tissue enzyme by magnesium or citrate alone was not seen when BSA was omitted from the preincubation medium. The enzyme was activated in the absence of BSA only when both magnesium and citrate were present. In the presence of BSA, magnesium or citrate alone could activate the enzyme. It was also shown that magnesium in the presence of citrate resulted in a greater activation than with citrate alone. These results indicate that activation of the enzyme was not only dependent on the presence of citrate and magnesium but that BSA also had an essential role. Swanson et al. (99) have shown that the rat liver acetyl CoA carboxylase at high protein concentrations (26-30 mg per ml) can be activated by prolonged incubation in the absence of activators. Several other workers (10,17) have also shown a dependency of the activation process on protein concentration. In addition to this Gregolin et al. (17) reported that the

presence of BSA favored the aggregate form of the chicken liver enzyme. Thus BSA has a definite enhancing effect on the activation of acetyl CoA carboxylase.

The pH profiles of both the rat liver and adipose tissue enzymes are identical, including the activation which occurs when Tris was used as a buffer instead of potassium phosphate (4).

The apparent K_m for acetyl CoA was found to be $1.4 \times 10^{-5}M$. Similar values of $3.8 \times 10^{-5}M$, $6.6 \times 10^{-5}M$ for the rat liver enzyme (4,12), $1.6 \times 10^{-5}M$ for the yeast (100) and chicken liver (20) enzymes, and $5.0 \times 10^{-5}M$ for the rat mammary gland (15) enzyme have been reported.

The apparent K_m for ATP was $2.5 \times 10^{-4}M$, which compares favorably with the value of $1.6 \times 10^{-4}M$ for the rat liver enzyme (12). The rat mammary gland enzyme was shown to have two apparent K_m 's for ATP (15). Miller and Levy (15) suggested that there may exist two different forms of the rat mammary gland enzyme with different kinetic parameters. The inhibition of acetyl CoA carboxylase activity which has been observed at higher concentrations of ATP was probably related to the fact that the molar ratio of ATP to magnesium was not maintained. Hatch and Stumpf (101) have reported that if the concentration of ATP exceeded that of magnesium, the activity of the wheat germ carboxylase was reduced by 70 to 80 per cent. The ATP-magnesium chelate has been suggested as the active species for many acetyl CoA carboxylases (15,17,102). Paradoxically the ATP-magnesium chelate has been shown to

promote disaggregation of the homogeneous chicken liver enzyme from its active polymeric state to an inactive protomeric state (17,96).

Malonyl CoA was shown to be a potent inhibitor of acetyl CoA carboxylase. The K_i for malonyl CoA was 1.0×10^{-5} M, which was comparable to 3.5×10^{-5} M for the rat liver enzyme (12), and $1.0-4.0 \times 10^{-5}$ M for the chicken liver enzyme (17). Gregolin et al. (17) have shown malonyl CoA to be a competitive inhibitor with respect to acetyl CoA of chicken liver acetyl CoA carboxylase. The rat adipose tissue acetyl CoA carboxylase was inhibited by malonyl CoA in a similar fashion, that is, competitively with respect to acetyl CoA and non-competitively with respect to ATP. Coenzyme A also inhibited acetyl CoA carboxylase but to a much lesser extent than malonyl CoA.

The rat adipose tissue enzyme can utilize Mg^{+2} or Mn^{+2} . The activity of the adipose tissue enzyme at optimal Mn^{+2} concentration was only 55% of the activity at optimal Mg^{+2} concentration. This is in agreement with the recent studies on the rat mammary gland enzyme (15) and the chicken liver enzyme (20). Scorpio and Masoro (13), however have reported that the rat liver acetyl CoA carboxylase was equally active with Mg^{+2} or Mn^{+2} once preincubated with activators, but that the un-preincubated enzyme had greater activity with Mn^{+2} . They suggested that the protomeric form of the rat liver enzyme was more active with Mn^{+2} . It should be mentioned that Scorpio and Masoro (13) used crude preparations

of the enzyme, and a 30 minute incubation time for the assay of enzyme activity. More recently Scorpio and Masoro (14) have found, again using crude enzyme preparations, that the rat liver acetyl CoA carboxylase after preincubation showed different progress curves when magnesium rather than manganese was used as the divalent cation. The progress curve with manganese was linear for the first four minutes and increased steadily up to 20 minutes of the assay. The progress curve with magnesium as the cation, levelled off after four minutes. A similar progress curve with magnesium as the cation has been shown in this study for crude enzyme preparations from the adipose tissue. It is quite possible that the preference of manganese over magnesium for the rat liver enzyme may be due to the presence of a magnesium requiring malonyl CoA decarboxylase present in crude rat liver enzyme preparations. The metal requirement of a purified rat liver acetyl CoA carboxylase free of malonyl CoA decarboxylase activity has not been determined. Nakada et al. (97) have shown that malonyl CoA decarboxylase requires magnesium for activity. It is possible that manganese does not promote as much malonyl CoA decarboxylase activity as magnesium, and thus appears to stimulate acetyl CoA carboxylase activity to a greater extent than magnesium, in crude preparations.

The rat liver (11) and adipose tissue enzymes both undergo reversible cold inactivation. The cold inactivation process for the adipose tissue carboxylase was shown to be pH dependent. The rate of cold inactivation and its

reversibility were greater at pH 7.5 and 8.0. This is in agreement with the findings of Gregolin et al. (17) for the chicken liver enzyme. Although the chicken liver enzyme is known not to be cold labile, Gregolin et al. (17) have shown that at room temperature the disaggregated form of the enzyme was favored at alkaline pH, while the aggregated form of the enzyme was favored at neutral pH. Cold inactivation has been shown to be pH dependent for the rat liver acetyl CoA carboxylase (11) and for another enzyme glycogen phosphorylase (103). Graves et al. (103) suggested that the inactivation of glycogen phosphorylase b at pH 6.0 by cold temperatures may be due to disturbance of hydrophobic bonds that are essential for maintenance of the active conformation of the enzyme. The lack of sensitivity of phosphorylase b to cold at pH 6.8, may be due to the fact that under these conditions, other forces may be as important or more important for stabilization of the active conformation of phosphorylase b. This explanation may also hold good for the rat liver and adipose tissue acetyl CoA carboxylases. The chicken liver acetyl CoA carboxylase, as mentioned, is not cold labile (17). Thus the forces involved in maintaining the enzyme in its active aggregated conformation must differ from those of the rat liver and adipose tissue enzymes.

It seems that the rat liver and adipose tissue enzymes are similar in many of their properties, such as pH optimum, affinity for substrates, inhibition by malonyl CoA, and pH dependent reversible cold inactivation. There appears to be

a slight difference in the metal requirements for these enzymes, which may be due to the presence of malonyl CoA decarboxylase activity in the rat liver preparations. In fact the catalytic properties of all animal acetyl CoA carboxylases thus far studied appear to be very similar, with respect to pH optimum, affinity for substrates, requirement for citrate or isocitrate, inhibition by malonyl CoA, preference of magnesium over manganese for optimal enzyme activity, and existence in aggregated and disaggregated forms. The only striking dissimilarity appears to be in cold sensitivity, and as explained this may be due to the fact that different forces are involved in stabilizing the active aggregated form of the enzyme in various species.

2. BIOTIN STATUS AND ACETYL CoA CARBOXYLASE ACTIVITY

Vagelos et al. (10) have shown that activation of the rat adipose tissue acetyl CoA carboxylase by citrate was dependent on the formation of an enzyme aggregate. They also showed as previously mentioned that the rate of aggregation and thus the rate of activation to be directly proportional to the enzyme concentration. A similar relationship between activity, state of aggregation, and enzyme concentration has been reported for other enzymes (104,105). The differences observed in this study, in the rate of activation of the gel filtered cytosol obtained from the adipose tissue of rats on the different diets, suggest alterations in enzyme content per ml of the gel filtered cytosol. The rats on the high

carbohydrate diet and the pair-weighed controls contained more enzyme per mg protein than rats on Purina lab chow and biotin deficient rats.

Rats fed a high carbohydrate diet showed a four fold increase in adipose tissue acetyl CoA carboxylase specific activity over similar preparations from those fed the commercial rat ration (Purina lab chow). It is significant that the acetyl CoA carboxylase activity was very low in rats fed the commercial rat ration. Purina lab chow has a lower carbohydrate content, and a higher level of fat than the synthetic diets which were used. Therefore rats fed Purina lab chow had a lower acetyl CoA carboxylase activity. Chaikoff and coworkers (63-65) have shown that inclusion of as little as 2.5% corn oil in the diet decreased the in vitro conversion of acetate into liver fatty acids. The effect of feeding Purina lab chow has been compared with that of feeding a low carbohydrate diet (93). Rats fed low carbohydrate diets and Purina lab chow have been shown to have low liver glucokinase activities (93).

It has been shown that biotin deficiency decreased the carboxylase activity of adipose tissue to less than a sixth of the pair-weighed controls. Previously it was shown that under identical conditions liver acetyl CoA carboxylase activity was reduced in the biotin deficient rat to half the pair-weighed control values (9).

The in vivo restoration of acetyl CoA holocarboxylase activity has been demonstrated for the first time in this

study. The specific activity of acetyl CoA holocarboxylase was increased 1.6 and 9.4 fold in rat liver and epididymal adipose tissue respectively, one hour after d-biotin administration to biotin deficient rats. The restoration of enzyme activity was brought about by conversion of the apoenzyme to the holoenzyme. Synthesis of new enzyme does not seem to be involved in this biotin stimulation of acetyl CoA holocarboxylase activity since cycloheximide did not prevent the restoration of enzyme activity. Biotin administration in vivo has been shown to restore the activity of two other biotin enzymes, rat liver propionyl CoA carboxylase (85,86) and pyruvate carboxylase (106).

Liver and epididymal adipose tissue lipogenesis have been shown to be affected to different extents by biotin deficiency (9). This can be explained by the fact that biotin deficiency decreased the adipose tissue acetyl CoA carboxylase activity to a greater extent than that of the liver enzyme. Jacob and Majerus (107) using antibody prepared against homogeneous chicken liver acetyl CoA carboxylase, which is known to react with the rat liver acetyl CoA carboxylase (66), have confirmed that epididymal adipose tissue of biotin deficient rats contained a large amount of catalytically inactive but immunoreactive protein. Jacob and Majerus (107) could not show any effect of biotin deficiency on the rat liver acetyl CoA carboxylase. This was probably due to the fact that their rats were not on the egg-white diet long enough. It has been shown that 40% and

90% of the liver and epididymal adipose tissue acetyl CoA carboxylase respectively are in the apoenzyme form in biotin deficient rats. The lower apoenzyme content of the liver also explains why the liver acetyl CoA carboxylase undergoes faster restoration of enzyme activity than the adipose tissue enzyme after biotin administration. The reason for the differential effect of biotin deficiency on these two tissues was not known. It was also interesting to note that rat liver propionyl CoA carboxylase (86) and pyruvate carboxylase (106) activities, both mitochondrial enzymes, are decreased to a much greater extent than the rat liver acetyl CoA carboxylase, a cytoplasmic enzyme, during biotin deficiency.

Leuthardt and his group (81,82) studied an enzyme which incorporated biotin ^{14}C into hydroxylamine, and showed that acetyl CoA apocarboxylase from biotin deficient chicken liver could also act as a substrate in that biotin ^{14}C was incorporated into the protein, but never showed that the enzyme activity was increased by the biotin incorporation. In a preliminary communication Lynen and Rominger (84) reported that biotinyl-AMP was involved in acetyl CoA holocarboxylase formation in yeast enzyme preparation. Propionyl CoA holocarboxylase synthetase, the enzyme which covalently links biotin to propionyl CoA apocarboxylase, a biotin enzyme, has been extensively studied in rat and rabbit liver (83, 87-89,108,109), but it is not known whether these synthetases are the same or homologous to acetyl CoA holocarboxylase synthetase.

The in vitro synthesis of acetyl CoA holocarboxylase has been shown to require ATP and d-biotin but no absolute requirement for magnesium could be demonstrated. d-Biotin ^{14}C incorporation into protein shows similar requirements to acetyl CoA holocarboxylase synthesis in that ATP and d-biotin were required. Magnesium was found to inhibit acetyl CoA holocarboxylase synthesis but stimulated biotin ^{14}C incorporation into protein. Kosow and Lane (87) have also reported that magnesium was not required for propionyl CoA holocarboxylase synthetase. However, Coon and coworkers (108,109) have shown that magnesium was required for the formation of biotinyl-AMP an intermediate in the propionyl CoA holocarboxylase synthetase reaction. Acetyl CoA holocarboxylase synthesis probably requires magnesium for activity, but this requirement may be masked by the following factors. The ATP-Mg^{+2} chelate has been shown to promote disaggregation of the homogeneous chicken liver enzyme from its active polymeric state to an inactive protomeric state (17,96). It is also known that ATP in the presence of magnesium or citrate inhibits the aggregation of rat liver acetyl CoA holocarboxylase to its active form (14,102). Thus the presence of magnesium would cause more of the enzyme to be in an inactive disaggregated form, and make it appear that acetyl CoA holocarboxylase synthesis does not require magnesium.

The in vitro synthesis of liver acetyl CoA holocarboxylase in biotin deficient rats has also been shown. The lower rate of liver acetyl CoA holocarboxylase synthesis probably

reflects the low apoenzyme content of the liver rather than low liver acetyl CoA holocarboxylase synthetase activity.

It has been shown that the rat liver and adipose tissue acetyl CoA carboxylases are very similar in many respects to the chicken liver enzyme except in terms of cold sensitivity. It would be interesting to investigate further the cold inactivation phenomenon of the rat liver or adipose tissue enzymes, and to see if the presence of substances such as methanol, sucrose, etc. could protect the enzyme against cold inactivation as has been shown for other enzymes (103, 110). Modification of the rat liver or adipose tissue enzymes by bifunctional reagents such as glutaraldehyde possibly could enhance the stability of the enzyme to cold and heat as has been shown for glycogen phosphorylase b (111). Studies of this modified enzyme might give an insight into 1). the forces involved in the cold inactivation process 2). the mechanism of catalysis and allosteric regulation of this enzyme. These latter investigations would require a nearly homogeneous enzyme preparation.

For further consideration it would be interesting to study the effects of palmityl CoA and malonyl CoA, inhibitors of acetyl CoA carboxylase and investigate their physiological significance in metabolic regulation.

The differential effect of biotin deficiency on the liver and adipose tissue acetyl CoA carboxylase should also be investigated. It may be that the liver contains a larger amount of a biotin binding protein than the adipose tissue,

and as such retains biotin more efficiently even in severe biotin deficiency. It may be that this biotin binding protein is acetyl CoA holocarboxylase synthetase. To conduct the above investigation it would be necessary to purify the acetyl CoA apocarboxylase free of the synthetase, so that it could be used as substrate for the synthetase. With the isolation of the apoenzyme it would be possible to determine the activity of the synthetase in the liver and adipose tissue, and confirm or reject the postulate of the biotin binding protein. It might also be possible to purify the acetyl CoA holocarboxylase synthetase from the rat liver, investigate its properties, cellular localisation, and specificity toward other biotin apoenzymes (pyruvate apocarboxylase, propionyl CoA apocarboxylase). It would indeed be interesting to determine whether one synthetase functions for all apoenzymes, or if each apoenzyme has a specific synthetase.

BIBLIOGRAPHY

1. S. J. Wakil and D. M. Gibson, *Biochim. Biophys. Acta* 41,122 (1960)
2. J. Ganguly, *Biochim. Biophys. Acta* 40,110 (1960)
3. S. Numa, M. Matsushashi, and F. Lynen, *Biochem. Z.* 334,203 (1961)
4. M. Matsushashi, S. Matsushashi, and F. Lynen, *Biochem. Z.* 340,263 (1964)
5. S. Numa, E. Ringelmann, and F. Lynen, *Biochem. Z.* 343,243 (1965)
6. W. M. Bortz, and F. Lynen, *Biochem. Z.* 337,505 (1963)
7. M. Waite, and S. J. Wakil, *J. Biol. Chem.* 238,77 (1963)
8. M. Waite, and S. J. Wakil, *J. Biol. Chem.* 238,81 (1963)
9. K. Dakshinamurti, and P. R. Desjardins, *Can. J. Biochem.* 46,1261 (1968)
10. P. R. Vagelos, A. W. Alberts, and D. B. Martin, *J. Biol. Chem.* 238,533 (1963)
11. S. Numa, and E. Ringelmann, *Biochem. Z.* 343,258 (1965)
12. P. W. Majerus, R. Jacobs, M. B. Smith, and H. P. Morris, *J. Biol. Chem.* 243,3588 (1968)
13. R. M. Scorpio, and E. J. Masoro, *Biochem. Biophys. Res. Commun.* 31,950 (1968)
14. R. M. Scorpio, and E. J. Masoro, *Biochem. J.* 118,391 (1970)
15. A. L. Miller, and H. R. Levy, *J. Biol. Chem.* 244,2334 (1969)

16. C. Gregolin, E. Ryder, A. K. Kleinschmidt, R. C. Warner, and M. D. Lane, Proc. Nat. Acad. Sc. U.S. 56,148 (1966)
17. C. Gregolin, E. Ryder, R. C. Warner, A. K. Kleinschmidt, and M. D. Lane, Proc. Nat. Acad. Sc. U.S. 56,1751 (1966)
18. E. Ryder, C. Gregolin, H. C. Chang, and M. D. Lane, Proc. Nat. Acad. Sc. U.S. 57,1455 (1967)
19. E. Stoll, E. Ryder, J. B. Edwards, and M. D. Lane, Proc. Nat. Acad. Sc. U.S. 60,986 (1968)
20. C. Gregolin, E. Ryder, and M. D. Lane, J. Biol. Chem. 243,4227 (1968)
21. C. Gregolin, E. Ryder, R. C. Warner, A. K. Kleinschmidt, H. C. Chang, and M. D. Lane, J. Biol. Chem. 243,4236 (1968)
22. H. C. Chang, I. Seidman, G. Teebor, and M. D. Lane, Biochem. Biophys. Res. Commun. 28,682 (1967)
23. B. Shapiro, and E. Wertheimer, J. Biol. Chem. 173,725 (1948)
24. D. D. Feller, J. Biol. Chem. 206,171 (1954)
25. F. X. Hausberger, S. W. Milstein, and R. J. Rutman, J. Biol. Chem. 208,431 (1954)
26. P. Favarger, and J. Gerlach, Helv. Physiol. Pharmacol. Acta 13,91 (1955)
27. P. Favarger, and J. Gerlach, Helv. Physiol. Pharmacol. Acta 16,188 (1958)

28. P. Favarger, and J. Gerlach, *Helv. Physiol. Pharmacol. Acta* 18,328 (1960)
29. J. K. Patkin, and E. J. Masoro, *Can. J. Physiol. Pharmacol.* 42,101 (1964)
30. E. J. Masoro. "Physiological Chemistry of Lipids in Mammals", page 64, editor E. J. Masoro, 1968. W. B. Saunders Co.
31. R. O. Brady, and S. Gurin, *J. Biol. Chem.* 199,421 (1952)
32. F. Dituri, and S. Gurin, *Arch. Biochem. Biophys.* 43, 231 (1953)
33. J. Van Baalen, and S. Gurin, *J. Biol. Chem.* 205,303 (1953)
34. R. G. Langdon, *J. Biol. Chem.* 226,615 (1957)
35. P. K. Stumpf, and G. A. Barber, *J. Biol. Chem.* 227,407 (1957)
36. D. M. Gibson, E. B. Titchener, and S. J. Wakil, *J. Am. Chem. Soc.* 80,2908 (1958)
37. S. J. Wakil, *J. Am. Chem. Soc.* 80,6465 (1958)
38. R. O. Brady, *Proc. Nat. Acad. Sc. U.S.* 44,993 (1958)
39. S. J. Wakil, E. B. Titchener, and D. M. Gibson, *Biochim. Biophys. Acta* 29,225 (1958)
40. W. Stoffel, *Z. Physiol. Chem.* 233,71 (1963)
41. D. H. Nugteren, *Biochem. J.* 89,28P (1963)
42. D. H. Nugteren, *Biochim. Biophys. Acta* 106,280 (1965)
43. A. F. Whereat, and M. W. Orishimo, *Am. J. Physiol.* 217,998 (1969)
44. N. M. Green, *Biochem. J.* 89,585 (1963)

45. S. Numa, W. M. Bortz, and F. Lynen, *Adv. Enzyme Regulation* 3,407 (1965)
46. M. Waite, and S. J. Wakil, *J. Biol. Chem.* 237,2750 (1962)
47. M. Matsushashi, S. Matsushashi, S. Numa, and F. Lynen, *Fed. Proc.* 21,288 (1962)
48. D. B. Martin, and P. R. Vagelos, *J. Biol. Chem.* 237,1787 (1962)
49. S. Numa, E. Ringelmann, and B. Riedel, *Biochem. Biophys. Res. Commun.* 24,750 (1966)
50. T. Goto, E. Ringelmann, B. Riedel, and S. Numa, *Life Sc.* 6,785 (1967)
51. A. K. Kleinschmidt, J. Moss, and M. D. Lane, *Science* 166,1276 (1969)
52. W. P. Welbourne, R. F. Swanson, and H. S. Anker, *Biochem. Biophys. Res. Commun.* 37,933 (1969)
53. M. Greenspan, and J. M. Lowenstein, *Arch. Biochem. Biophys.* 118,260 (1967)
54. A. W. Alberts, and P. R. Vagelos, *Proc. Nat. Acad. Sc. U.S.* 59,561 (1968)
55. A. W. Alberts, A. M. Nervi, and P. R. Vagelos, *Proc. Nat. Acad. Sc. U.S.* 63,1319 (1969)
56. M. D. Lane, P. Dimroth, R. Guchait, and E. Stoll, 160th A.C.S. National Meetings, Division of Biological Chemistry, 1970.
57. B. I. Gerwin, B. E. Jacobson, and H. G. Wood, *Proc. Nat. Acad. Sc. U.S.* 64,1315 (1969)

58. I. Lyon, M. S. Masri, and I. L. Chaikoff, *J. Biol. Chem.* 196,25 (1952)
59. H. M. Korchak, and E. J. Masoro, *Biochim. Biophys. Acta* 58,354 (1962)
60. D. W. Allman, D. D. Hubbard, and D. M. Gibson, *J. Lip. Res.* 6,63 (1965)
61. S. E. Hicks, D. W. Allman, and D. M. Gibson, *Biochim. Biophys. Acta* 106,441 (1965)
62. J. E. Whitney, and S. Roberts, *Am. J. Physiol.* 181,446 (1955)
63. R. Hill, J. M. Linazasoro, F. Chevallier, and I. L. Chaikoff, *J. Biol. Chem.* 233,305 (1958)
64. R. Hill, W. Webster, J. M. Linazasoro, and I. L. Chaikoff, *J. Lip. Res.* 1,150 (1960)
65. W. Bortz, S. Abraham, and I. L. Chaikoff, *J. Biol. Chem.* 238,1266 (1963)
66. P. W. Majerus, and E. Kilburn, *J. Biol. Chem.* 244,6254 (1969)
67. J. W. Porter, and R. W. Long, *J. Biol. Chem.* 233,20 (1958)
68. P. K. Tubbs, and P. B. Garland, *Biochem. J.* 89,25P (1963)
69. P. K. Tubbs, and P. B. Garland, *Biochem. J.* 93,550 (1964)
70. J. D. Robinson, R. O. Brady, and R. M. Bradley, *J. Lip. Res.* 4,144 (1963)
71. J. A. Dorsey, and J. W. Porter, *J. Biol. Chem.* 243,3512 (1968)

72. H. R. Levy, *Biochim. Biophys. Acta* 84,229 (1964)
73. A. L. Miller, M. E. Geroch, and H. R. Levy, *Biochem. J.* 118,645 (1970)
74. G. L. Curran, *Proc. Soc. Exptl. Biol. Med.* 75,496 (1950)
75. K. Guggenheim, and R. E. Olson, *J. Nutr.* 48,345 (1952)
76. D. E. Green, and S. J. Wakil, in K. Bloch "Lipide Metabolism", Wiley, New York, 1960, page 1.
77. W. E. Donaldson, *Proc. Soc. Exptl. Biol. Med.* 116,662 (1964)
78. F. Schultness, and F. Leuthardt, *Helv. Chim. Acta* 46,1244 (1963)
79. P. Puddu, P. Zanetti, E. Turchetto, and M. Marchetti, *J. Nutr.* 91,509 (1967)
80. A. Gilgen, and F. Leuthardt, *Helv. Chim. Acta* 45,1833 (1962)
81. M. Valloton, and F. Leuthardt, *Helv. Chim. Acta* 47,311 (1964)
82. M. Valloton, G. A. Borel, R. Schurter, and F. Leuthardt, *Helv. Chim. Acta* 47,2390 (1964)
83. J. E. Christner, M. J. Schlesinger, and M. J. Coon, *J. Biol. Chem.* 239,3997 (1964)
84. F. Lynen, and K. L. Rominger, *Fed. Proc.* 22,537 (1963)
85. D. P. Kosow, and M. D. Lane, *Biochem. Biophys. Res. Commun.* 4,92 (1961)
86. D. P. Kosow, and M. D. Lane, *Biochem. Biophys. Res. Commun.* 5,191 (1961)

87. D. P. Kosow, S. C. Huang, and M. D. Lane, *J. Biol. Chem.* 237,3633 (1962)
88. L. Siegel, J. L. Foote, and M. J. Coon, *J. Biol. Chem.* 240,1025 (1965)
89. H. C. McAllister, and M. J. Coon, *J. Biol. Chem.* 241, 2855 (1966)
90. F. G. Trams, and R. O. Brady, *J. Am. Chem. Soc.* 82, 2972 (1960)
91. F. Lipmann, and L. C. Tuttle, *J. Biol. Chem.* 159,21 (1945)
92. G. Schmidt. "Methods in Enzymology", vol. III, S. P. Colowick, and N. O. Kaplan (editors). Academic Press, 1957, page 779.
93. K. Dakshinamurti, and C. Cheah-Tan, *Can. J. Biochem.* 46,75 (1968)
94. O. H. Lowry, N. J. Rosenbrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* 193,265 (1951)
95. A. Korner, *Biochem. J.* 101,627 (1966)
96. S. Numa, T. Goto, E. Ringelmann, and B. Riedel, *European J. Biochem.* 3,124 (1967)
97. H. I. Nakada, J. B. Wolfe, and A. N. Wick, *J. Biol. Chem.* 226,145 (1957)
98. H. R. Scholte, *Biochim. Biophys. Acta* 178,137 (1969)
99. R. F. Swanson, W. M. Curry, and H. S. Anker, *Biochim. Biophys. Acta* 159,390 (1968)
100. M. Matsushashi, S. Matsushashi, S. Numa, and F. Lynen, *Biochem. Z.* 340,243 (1964)

101. M. D. Hatch, and P. K. Stumpf, J. Biol. Chem. 236,
2879 (1961)
102. M. D. Greenspan, and J. M. Lowenstein, J. Biol. Chem.
243,6273 (1968)
103. D. J. Graves, R. W. Sealock, and J. H. Wang, Biochemistry
4,290 (1965)
104. K. L. Yielding, and G. M. Tomkins, Proc. Nat. Acad.
Sc. U.S. 46,1483 (1960)
105. C. Frieden. "Regulation of Enzyme Activity and Allosteric
Interactions", Fed. European Biochem. Soc. Proc.
page 59, 1967. E. Kvamme and A. Pihl, editors,
Academic Press
106. A. D. Deodhar, and S. P. Mistry, Biochem. Biophys.
Res. Commun. 6,755 (1969)
107. R. Jacobs, and P. W. Majerus, Fed. Proc. 29,735 (1970)
108. J. L. Foote, J. E. Christner, and M. J. Coon, Biochim.
Biophys. Acta 67,676 (1963)
109. L. Siegel, J. L. Foote, J. E. Christner, and M. J. Coon,
Biochem. Biophys. Res. Commun. 13,307 (1963)
110. J. J. Irias, M. R. Olmsted, and M. F. Utter, Biochemistry
8,5136 (1969)
111. J. H. Wang, and J. I. Tu, Biochemistry 8,4403 (1969)

APPENDIX A - TABLES

TABLE I

Protomer-Polymer Equilibrium of Acetyl CoA Carboxylase*

<u>PROTOMER</u>	\rightleftharpoons	<u>POLYMER</u>
(small form)		(large form)
Mol. wt. 41×10^4		Mol. wt. $4-8 \times 10^6$
$S_{20,w} = 13.1 \text{ S}$		$S_{20,w} = 40-55 \text{ S}$
Equilibrium toward small form favored by:		Equilibrium toward large form favored by:
ATP-Mg (HCO_3^-)		Isocitrate, citrate (malonate)
Malonyl CoA		phosphate
alkaline pH		albumin
NaCl		pH 6.5-7.0
low protein concentration		high protein concentration

* Table taken from paper by Gregolin et al. (17).

TABLE II

Partial Purification of Adipose Tissue Acetyl CoA Carboxylase

	Volume (ml)	Protein (mg/ml)	Total activity units*	Specific activity**	Recovery ⁺ %
1) 20,000 Xg Supernatant	165	2.34	5.8	0.015	100
2) 0-45% Ammonium Sulfate	20.2	6.33	10.22	0.080	176
3) 105,000 Xg Supernatant	18.5	3.73	12.70	0.184	217
4) Calcium Phosphate	2.8	5.10	4.62	0.323	80

* One enzyme unit carboxylates 1 μ mole of Acetyl CoA per min. at 37°C

** Units per mg protein

+ Calculated on basis of total units

TABLE III

Malonyl CoA Decarboxylase Activity of the Carboxylase Enzyme
Preparation at Various Steps of Purification

Step of Purification	Carboxylase* Activity	Decarboxylase** Activity	Relative Activity
1) 20,000 Xg Supernatant	1.08	0.36	0.33
2) 105,000 Xg Supernatant	8.76	0.64	0.07
3) Calcium Phosphate Gel Adsorption	24.00	---***	Nil

* μ Moles Acetyl CoA Carboxylated per hour per mg Protein

** μ Moles Malonyl CoA Decarboxylated per hour per mg Protein

*** None Present Under Carboxylase Conditions

TABLE IV

Effect of Avidin on Acetyl CoA Carboxylase*

Treatment	Acetyl CoA carboxylase activity milliunits per mg protein
None	11.22
Avidin	0.00
Avidin + Biotin	10.47
Biotin	10.49

* Gel filtered cytosol was obtained from epididymal fat pads of a normal rat fed Purina lab chow. The enzyme preparation was placed in the preincubation medium, at 0°C, with 1) avidin (1 unit per mg enzyme protein), 2) avidin pretreated with excess d-biotin (1 unit avidin + 10 µg biotin per mg enzyme protein), 3) biotin (10 µg per mg enzyme protein). The enzyme was preincubated at 37°C for 30 minutes and assayed for acetyl CoA carboxylase activity (Chapter III A, 1,ii).

TABLE V

Inhibition of Acetyl CoA Carboxylase
by Malonyl CoA and Coenzyme A*

Malonyl CoA μmoles	Coenzyme A μmole	Specific enzyme activity**	Percent inhibition
0	0	66	--
175	44 ⁺	26	59.8
350	87 ⁺	16	75.6
875	219 ⁺	3	95.0
	175	66	3.1
	432	56	15.5
	875	41	37.3

* Gel filtered cytosol was obtained from epididymal fat pads of a normal rat fed the low fat high carbohydrate diet. The enzyme preparation was preincubated for 30 minutes and the acetyl CoA carboxylase activity determined in the presence of malonyl CoA and coenzyme A. The malonyl CoA concentration and the amount of coenzyme A present as a contaminant were calculated from the hydroxamate adenine ratio.

** Expressed in milliunits per mg protein.

+ Coenzyme A calculated to be present as a contaminant of malonyl CoA.

TABLE VI

Effect of Biotin Deficiency on
Adipose Tissue Acetyl CoA Carboxylase Activity

Treatment	No. of rats	Acetyl CoA carboxylase activity, milliunits*		
		per mg protein	per two fat pads	per 100 g body wt.
Biotin deficient	4	3.25 ± 1.19	28.5 ± 10.9	21.2 ± 7.4
Control (+ biotin)	6	19.13 ± 4.44	236.0 ± 21.0	133.9 ± 17.3
Purina lab chow	4	4.33 ± 2.92	44.6 ± 19.7	18.5 ± 13.1
65% dextrose diet	3	19.01 ± 4.46	194.8 ± 32.8	97.1 ± 25.6

* Values are means ± standard deviation

TABLE VII

In vivo Restoration of Liver and Adipose
Tissue Acetyl CoA Carboxylase Activity*

Treatment	Acetyl CoA carboxylase activity, milliunits per mg protein	
	Liver	Epididymal adipose tissue**
Nil	12.9 ± 4.2(7)***	8.3 ± 2.2(4)***
Biotin, 1 hour	21.3 ± 7.4(5)	77.4 ± 19.2(4)
Biotin, 2 hours	17.8 ± 5.9(5)	97.1 ± 27.3(4)

* Biotin deficient rats were injected intraperitoneally with 200 µg of d-biotin in 0.9% NaCl for various time intervals. Control rats (biotin deficient) received a 1.0 ml injection (i.p.) of 0.9% NaCl. Rats were sacrificed and the acetyl CoA carboxylase activity determined as described (Chapter III A,2,ii,a).

** Epididymal fat pads from 2 or 3 rats were pooled for each assay.

*** Values are means ± standard deviation. The number in the brackets indicates the number of rats (liver) or groups of rats (adipose tissue) on which activities were determined. Each assay was done in triplicate.

TABLE VIII

Effect of Cycloheximide on Amino Acid ^{14}C Incorporation
into Adipose Tissue Protein*

Treatment	cpm per mg protein	% Inhibition
None	51.6 \pm 0.4	-
Cycloheximide 15 min.	1.7	97
30 min.	1.0	98
60 min.	3.3	94

* Rats (150 g) were fed ad libitum on Purina lab chow. They were injected (i.p.) with 10 mg per 100 g body wt. cycloheximide, 15, 30, and 60 minutes prior to the intramuscular injection of amino acid ^{14}C (3.68×10^6 cpm per 100 g body weight). The rats were sacrificed 30 minutes after the amino acid ^{14}C injection, and the specific radioactivity of the epididymal adipose tissue protein was determined as described in "Methods".

TABLE IX

In vivo Effect of Cycloheximide on
Restoration of Acetyl CoA Carboxylase Activity by d-Biotin*

Treatment	Acetyl CoA carboxylase activity, milliunits per mg protein	
	-cycloheximide	+cycloheximide
Nil	8.3 ± 2.2 (4)**	7.5 ± 1.0 (4)**
Biotin, 30 minutes	43.4 ± 7.7 (5)	27.5 ± 6.0 (4)

* Biotin deficient rats were injected (i.p.) with cycloheximide 10 mg per 100 g body wt. in 0.9% NaCl, 15 minutes prior to d-biotin administration. The rats were under the influence of cycloheximide for a total of 45 minutes. Rats which received d-biotin were injected (i.p.) with 200 µg of d-biotin (1.0 ml) in 0.9% NaCl. Control rats (biotin deficient) received 1.0 ml of 0.9% NaCl (i.p.).

** Values are means ± standard deviation. The numbers in the brackets indicate the number of groups of rats on which activities were determined. Each assay was done in triplicate.

TABLE X

Effect of Magnesium on Acetyl CoA Holocarboxylase Synthesis*

Deletions or additions	Acetyl CoA carboxylase activity, milliunits per mg protein	
	<u>TIME (Hours)</u>	
	0	4
MgCl ₂ added	1.08	1.81
MgCl ₂ added, d-biotin omitted (control)	0.87	0.49
Holocarboxylase synthesis munits	-	1.32
None	1.28	3.36
d-biotin omitted (control)	1.37	1.18
Holocarboxylase synthesis munits	-	2.18

* The gel filtered 20,000 Xg supernatant from epididymal adipose tissue was preincubated for one hour at 37°C in the preincubation medium in the presence of 25 mM citrate, without magnesium. The preincubated enzyme preparation (1.33 mg protein) was then incubated at 30°C in the following system: 48 mM Tris, pH 7.5, 2.8 mM GSH, 0.08 mM EDTA, 20 mM citrate (K⁺), 0.48 mg/ml bovine serum albumin, 2 mM ATP, 82 μM d-biotin. When MgCl₂ was added the final concentration in the incubation was 6.4 mM. Acetyl CoA carboxylase activity was determined on aliquots of the reaction mixture. All assays were done in duplicate. Holocarboxylase synthesis is expressed in milliunits acetyl CoA carboxylase per mg protein in the complete system after correction for controls, which consisted of the complete assay system with d-biotin being omitted (± magnesium).

TABLE XI

Requirements of Acetyl CoA Holocarboxylase Synthesis
and Biotin ^{14}C Incorporation into Protein*

Deletions or additions	Acetyl CoA carboxy- lase activity, munits per mg protein	d-Biotin ^{14}C incorporation, pmoles per mg protein
None	7.40	9.2
ATP omitted	4.02	3.4
d-Biotin omitted (control)	3.18	1.2**
Holocarboxylase synthesis or biotin incorporated	4.22	8.0
MgCl ₂ added	2.90	21.6
MgCl ₂ added, d-biotin omitted (control)	1.20	1.3**
Holocarboxylase synthesis or biotin incorporated	1.70	20.3

* The gel filtered 20,000 Xg supernatant from epididymal adipose tissue was preincubated for one hour at 37°C in the preincubation medium in the presence of 25 mM citrate without magnesium. The preincubated enzyme preparation (1.11 mg protein) was then incubated at 30°C for 4 hours in the following system: 48 mM Tris, pH 7.5, 2.7 mM GSH, 0.08 mM EDTA, 20 mM citrate (K⁺), 0.48 mg/ml bovine serum albumin, 4mM ATP, 82 μM d-biotin. When MgCl₂ was added its final concentration in the incubation was 8 mM. Acetyl CoA carboxylase activity was determined on aliquots of the reaction mixture at the end of the 4 hour incubation. All assays were done in duplicate. In the d-biotin ^{14}C incorporation experiment conditions were identical as described above except 84 μM d-biotin (carbonyl ^{14}C), 5.21 x 10⁶ dpm per 84 μmoles, was used. Holocarboxylase synthesis is expressed in milliunits acetyl CoA carboxylase, and picomoles biotin ^{14}C incorporated per mg protein in the complete system after correction for controls (biotin omitted).

** In control experiment biotin ^{14}C was added to the reaction mixture after stopping the reaction with 1.0 ml 20% TCA.

APPENDIX B - FIGURES

Figure 1. Progress curve of the acetyl CoA carboxylase reaction using a crude enzyme preparation.

Gel filtered cytosol was obtained from epididymal fat pads of a pair-weighed control. The enzyme preparation was preincubated for 30 minutes and the progress of the reaction was followed.

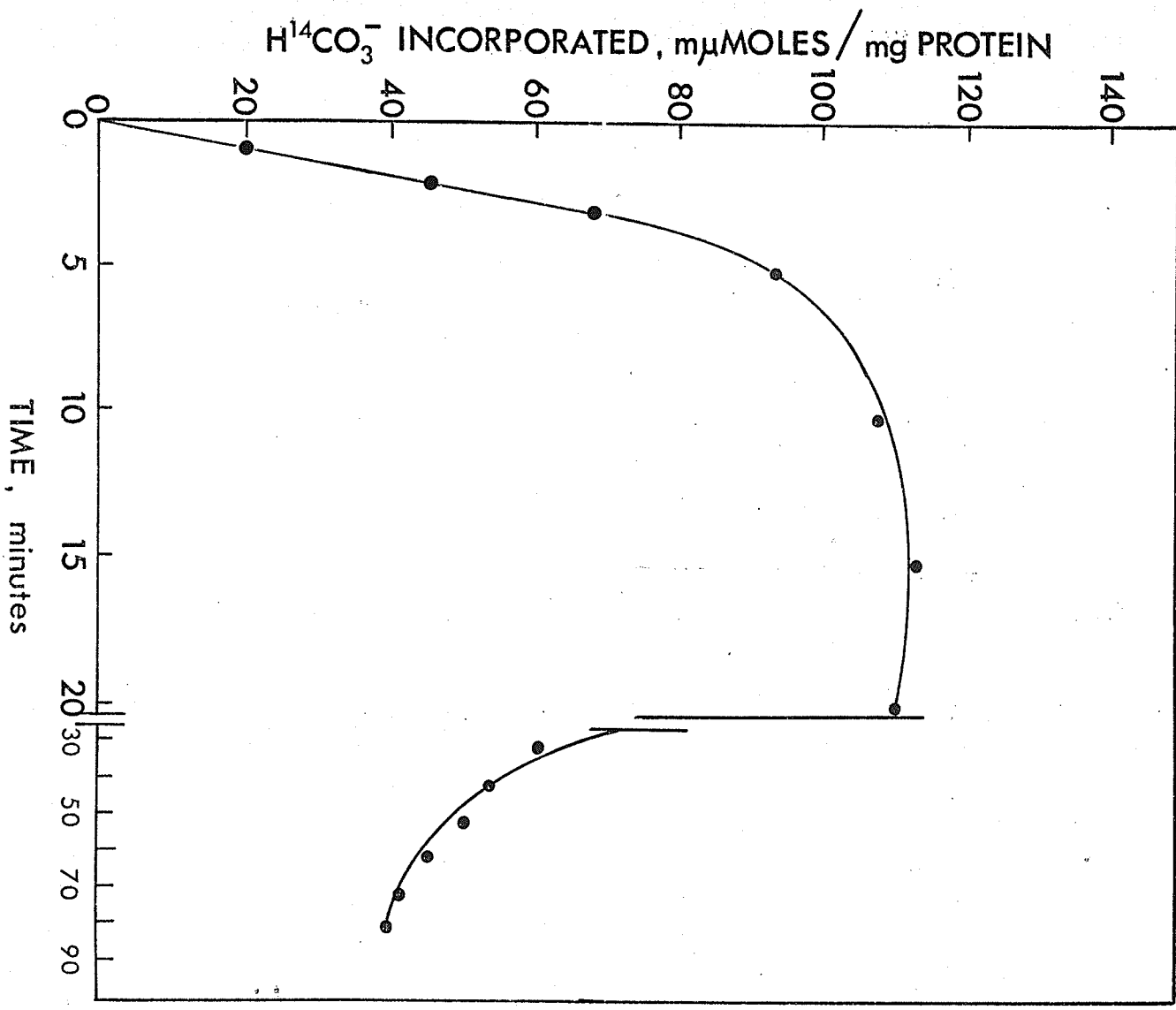


Figure 2. Progress curve of the acetyl CoA carboxylase reaction using a partially purified enzyme preparation.

The partially purified enzyme (spec. act. 0.200 units per mg protein) was preincubated in the preincubation medium for 30 minutes and the progress of the reaction was followed.

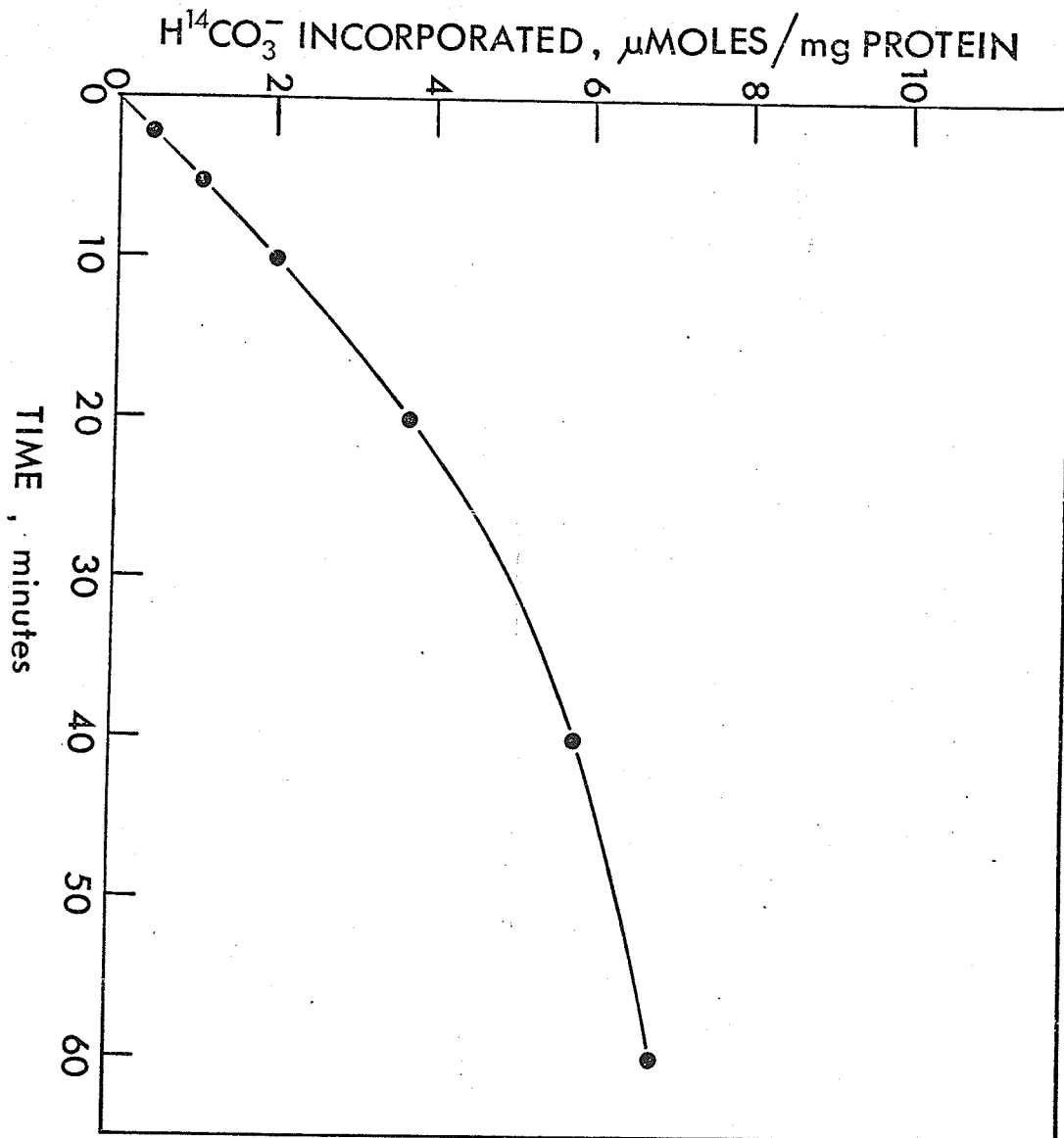


Figure 3. Enzyme concentration dependence of the acetyl CoA carboxylase assay.

The partially purified enzyme (spec. act. 0.200 units per mg protein) was preincubated in the preincubation medium for 30 minutes, and the acetyl CoA carboxylase activity was measured using varying amounts of the enzyme.

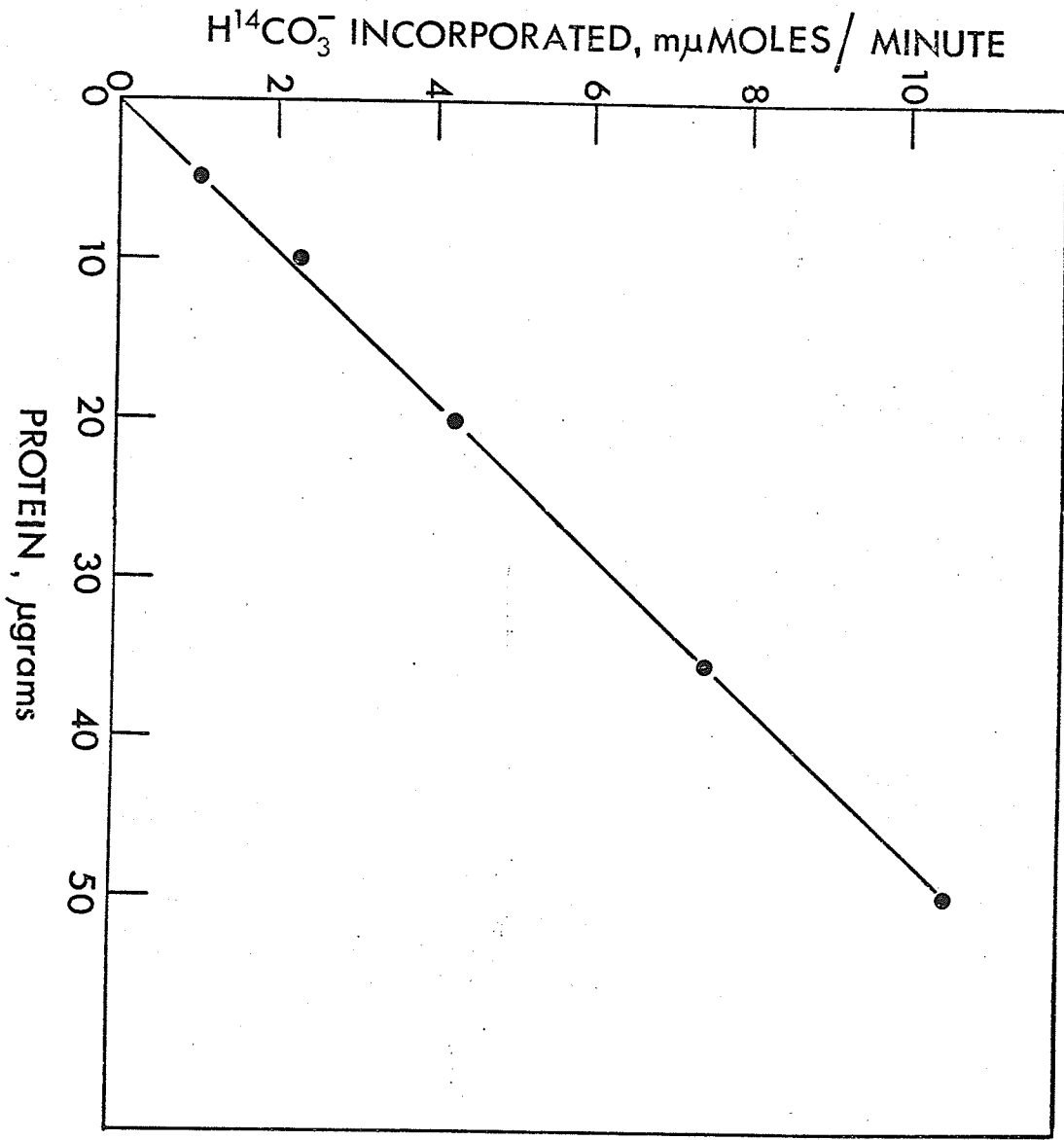


Figure 4. Malonyl CoA decarboxylase activity in crude enzyme preparations.

Gel filtered cytosol was obtained from epididymal fat pads of a normal rat fed a low fat, high carbohydrate diet. The enzyme preparation was preincubated for 30 minutes in the preincubation medium, and malonyl CoA decarboxylase activity was determined. Ordinate represents malonyl CoA left unchanged. ■—■ , control with enzyme omitted; □—□ , citrate activated gel filtered cytosol; ●—● , citrate activated gel filtered cytosol treated with avidin as described in "Methods".

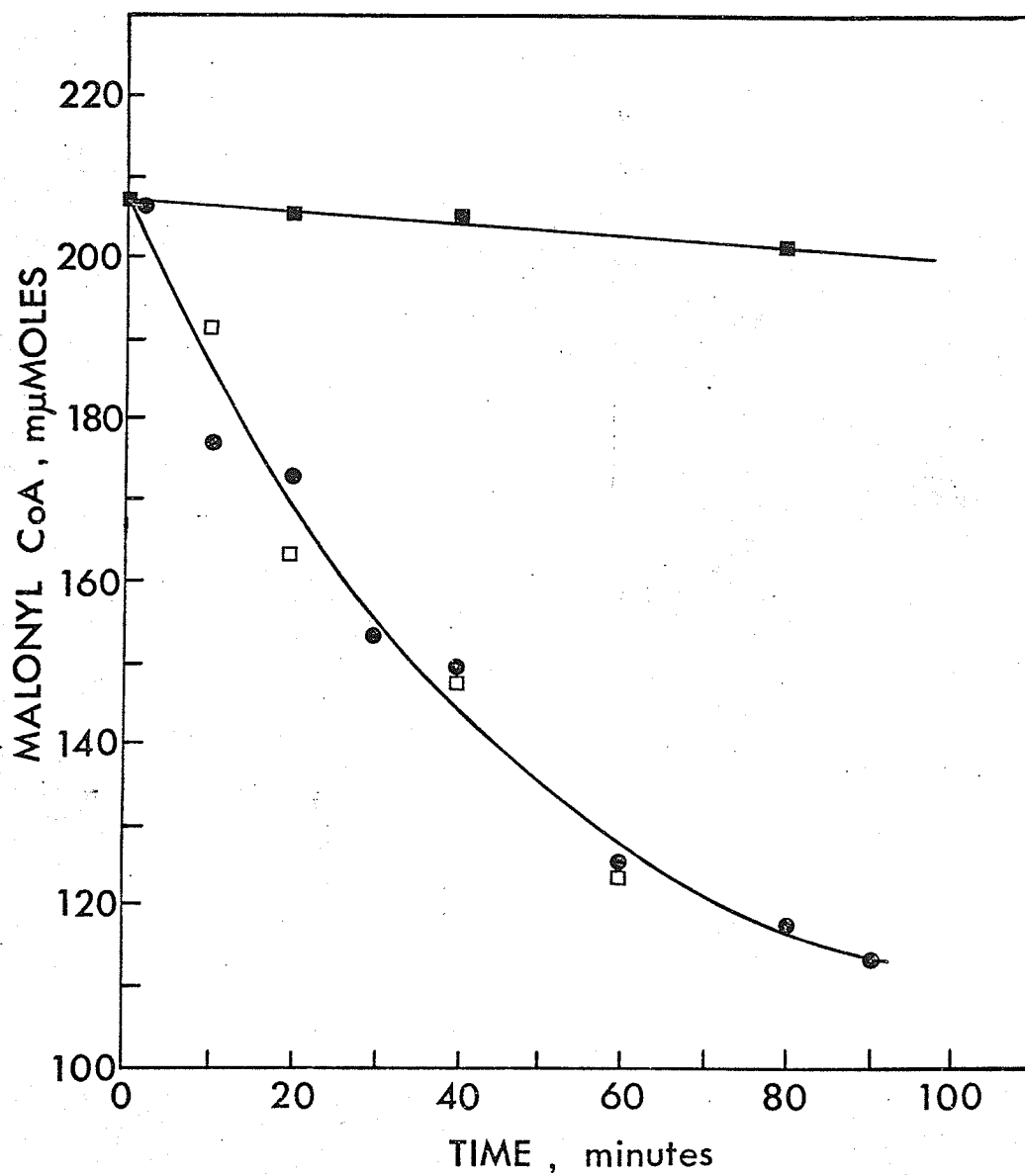


Figure 5. Effect of activators on the rate of activation of acetyl CoA carboxylase.

The partially purified enzyme was preincubated at a concentration of 0.5 mg per ml. The preincubation medium was the same as that described in "Methods" except that in certain cases (as indicated) one or two of the following effectors were omitted: 5mM citrate (K^+), 8mM $MgCl_2$, 0.6 mg per ml BSA. The degree of activation was followed using 2 minute assays. $\star \longrightarrow \star$ complete system, +cit, +Mg, +BSA; $\bullet \longrightarrow \bullet$, +cit, +BSA, -Mg; $\circ \longrightarrow \circ$ +Mg, +BSA, -cit; $\times \longrightarrow \times$, +BSA, -cit, -Mg; $\square \longrightarrow \square$, +cit, -Mg, - BSA; $\blacksquare \longrightarrow \blacksquare$, +Mg, -cit, -BSA.

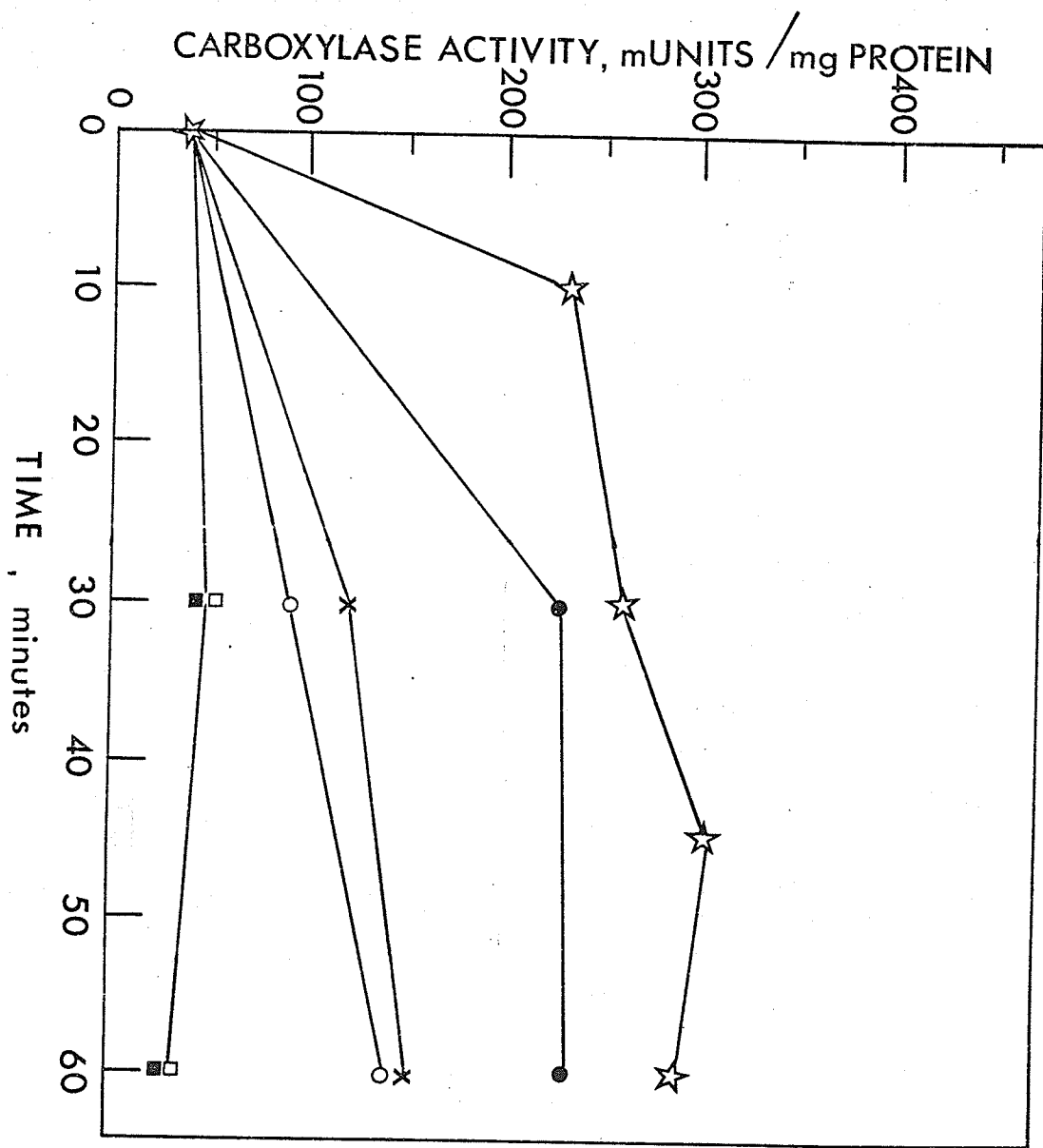


Figure 6. Effect of BSA on the rate of activation of acetyl CoA carboxylase.

The partially purified enzyme was preincubated at a concentration of 0.98 mg per ml. The preincubation medium was the same as that described in "Methods" except that in one case BSA was omitted. The degree of activation was determined by assaying acetyl CoA carboxylase activity. ●—●, complete system; ○—○ BSA omitted.

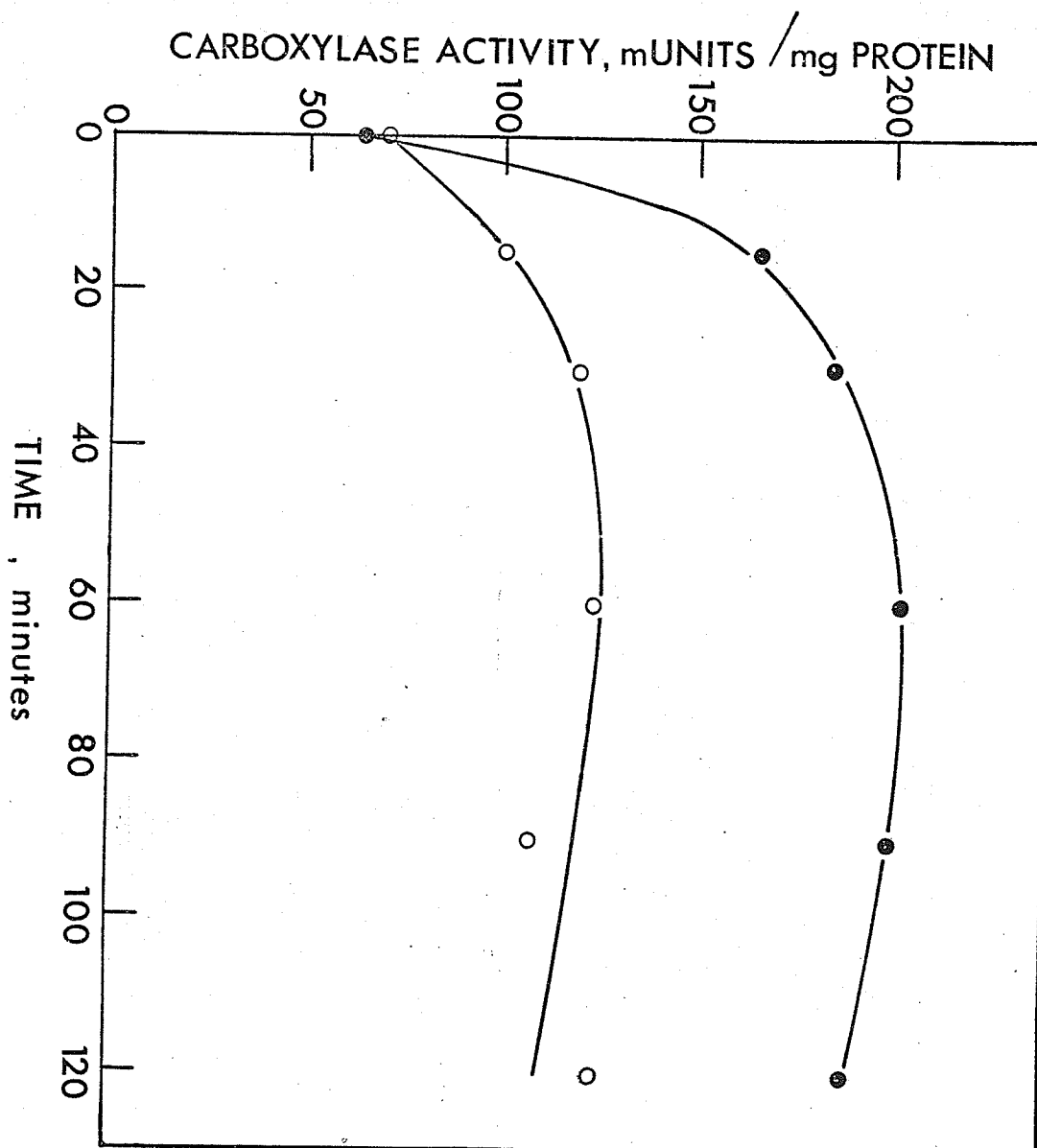


Figure 7. Effect of pH on the carboxylation reaction using a partially purified enzyme preparation.

The enzyme was preincubated and assayed as described in "Methods". ○—○ , 60mM potassium phosphate buffer; ■—■ , 60mM Tris (Cl⁻) buffer.

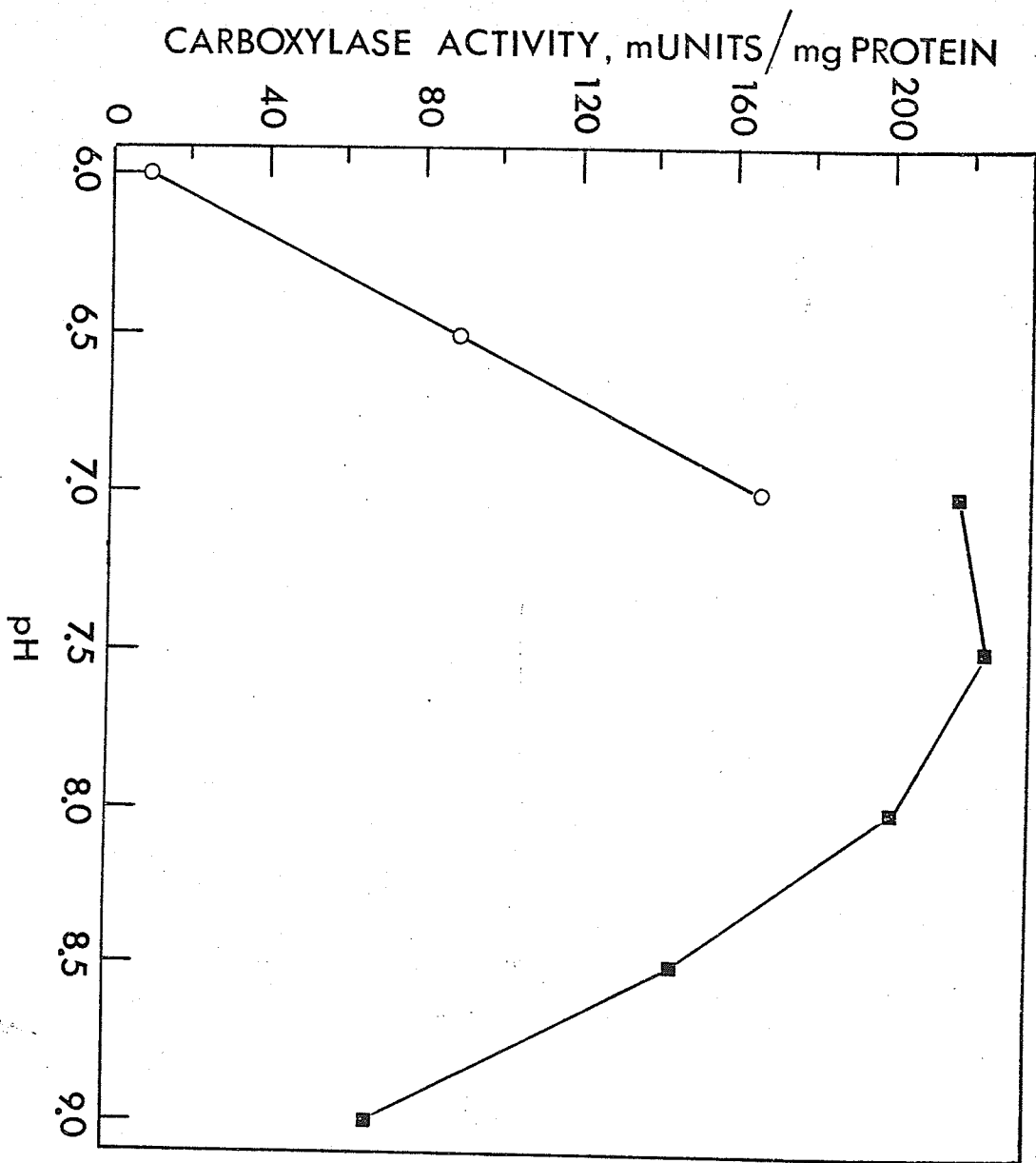


Figure 8. Effect of pH on the carboxylation reaction using a crude enzyme preparation.

The gel filtered cytosol was preincubated and assayed as described in "Methods".

○—○, 60mM potassium phosphate buffer; ■—■, 60mM Tris (Cl⁻) buffer.

CARBOXYLASE ACTIVITY, mUNITS/mg PROTEIN

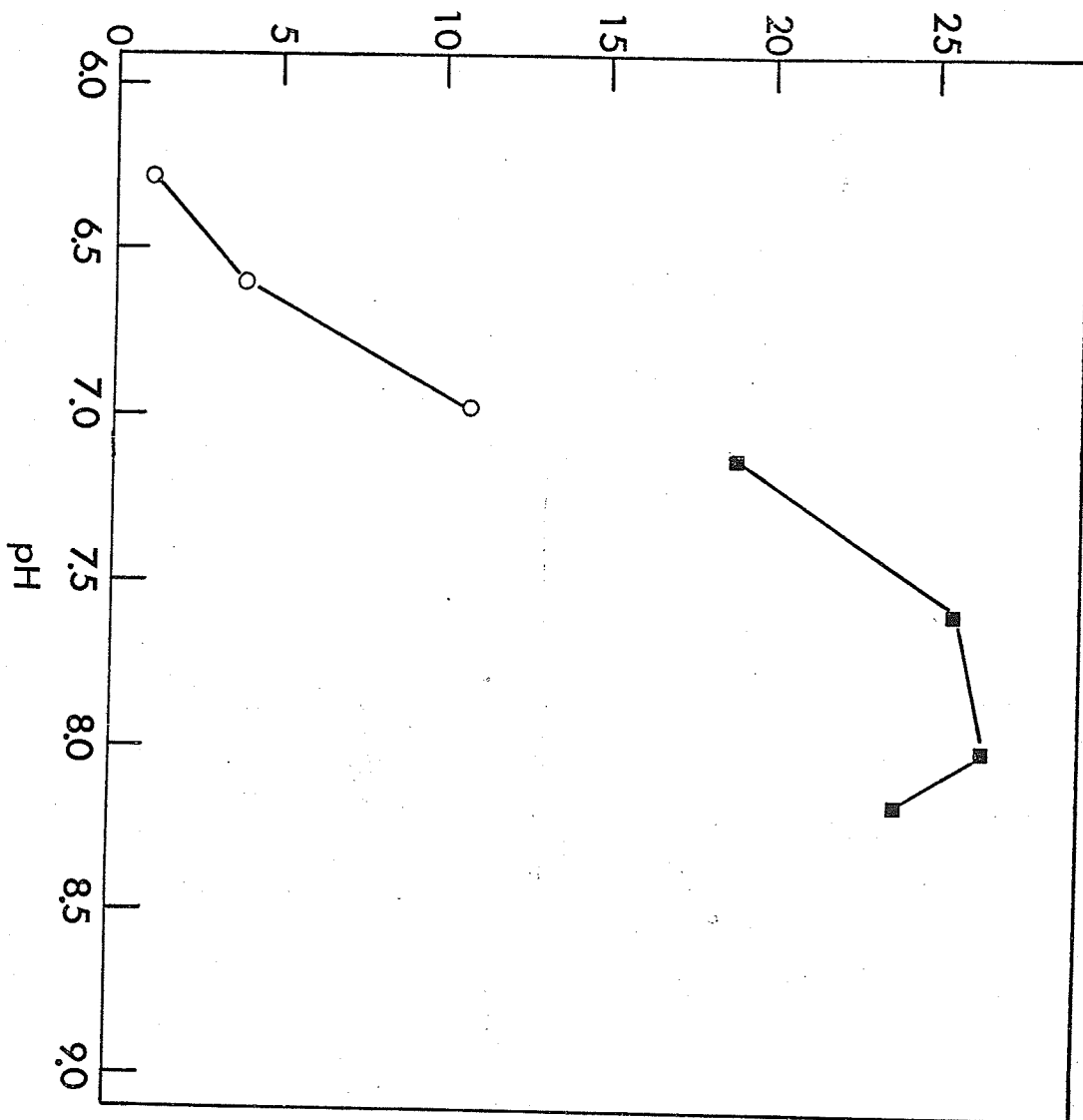


Figure 9. Metal requirement of acetyl CoA carboxylase.

The enzyme was preincubated in the absence of $MgCl_2$ and EDTA as described in "Methods". The manganese or magnesium ion concentration was varied in the assay medium with and without EDTA present. \square — \square , +manganese, -EDTA; \blacksquare — \blacksquare , +manganese, +EDTA; \circ — \circ , +magnesium, -EDTA; \bullet — \bullet , +magnesium, +EDTA.

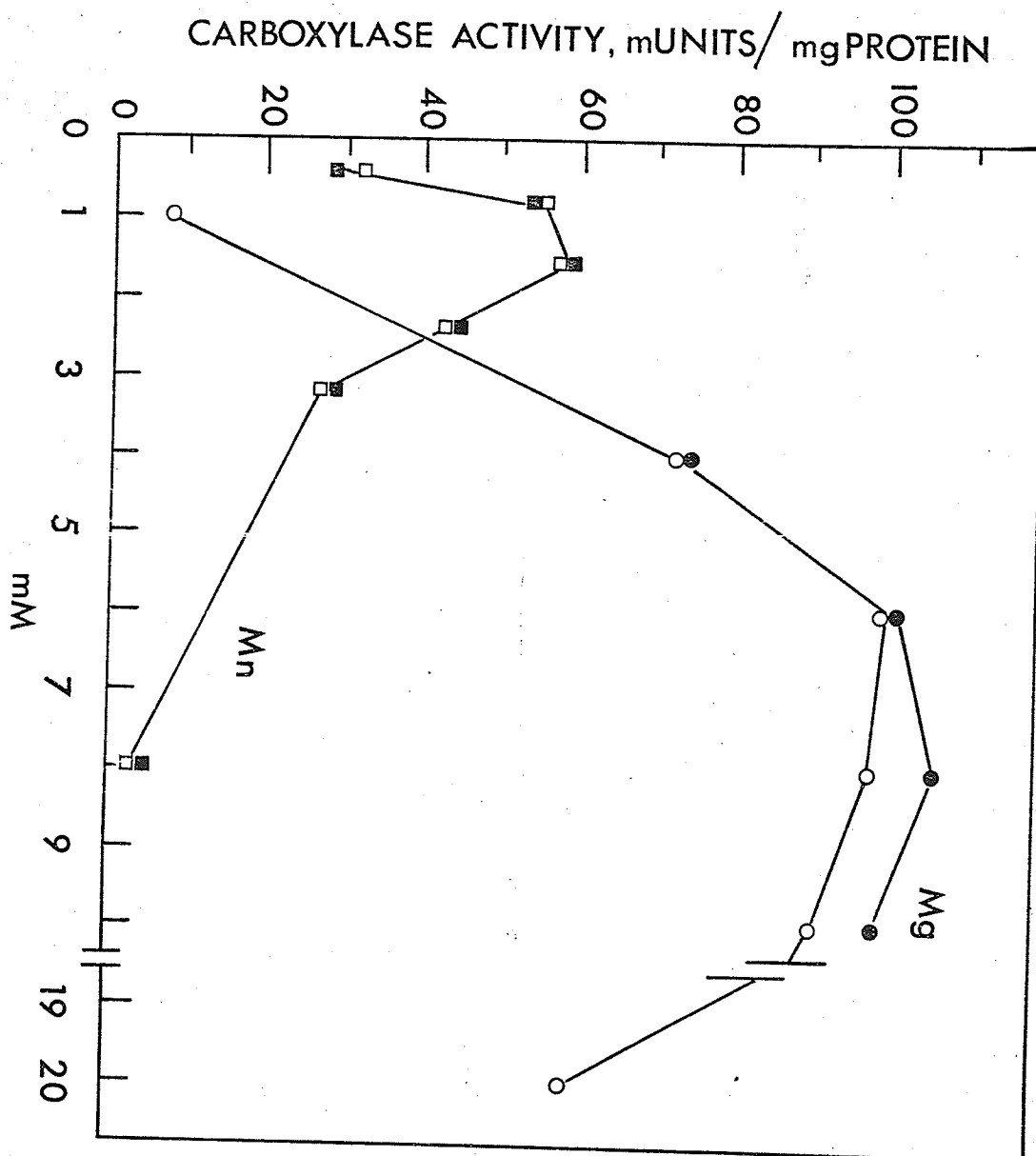


Figure 10. ATP requirement of acetyl CoA carboxylase.

There was no ATP present during the preincubation. The ATP concentration in the assay was varied and the magnesium ion concentration was kept at 8 mM. Concentrations of other constituents are as described in "Methods".

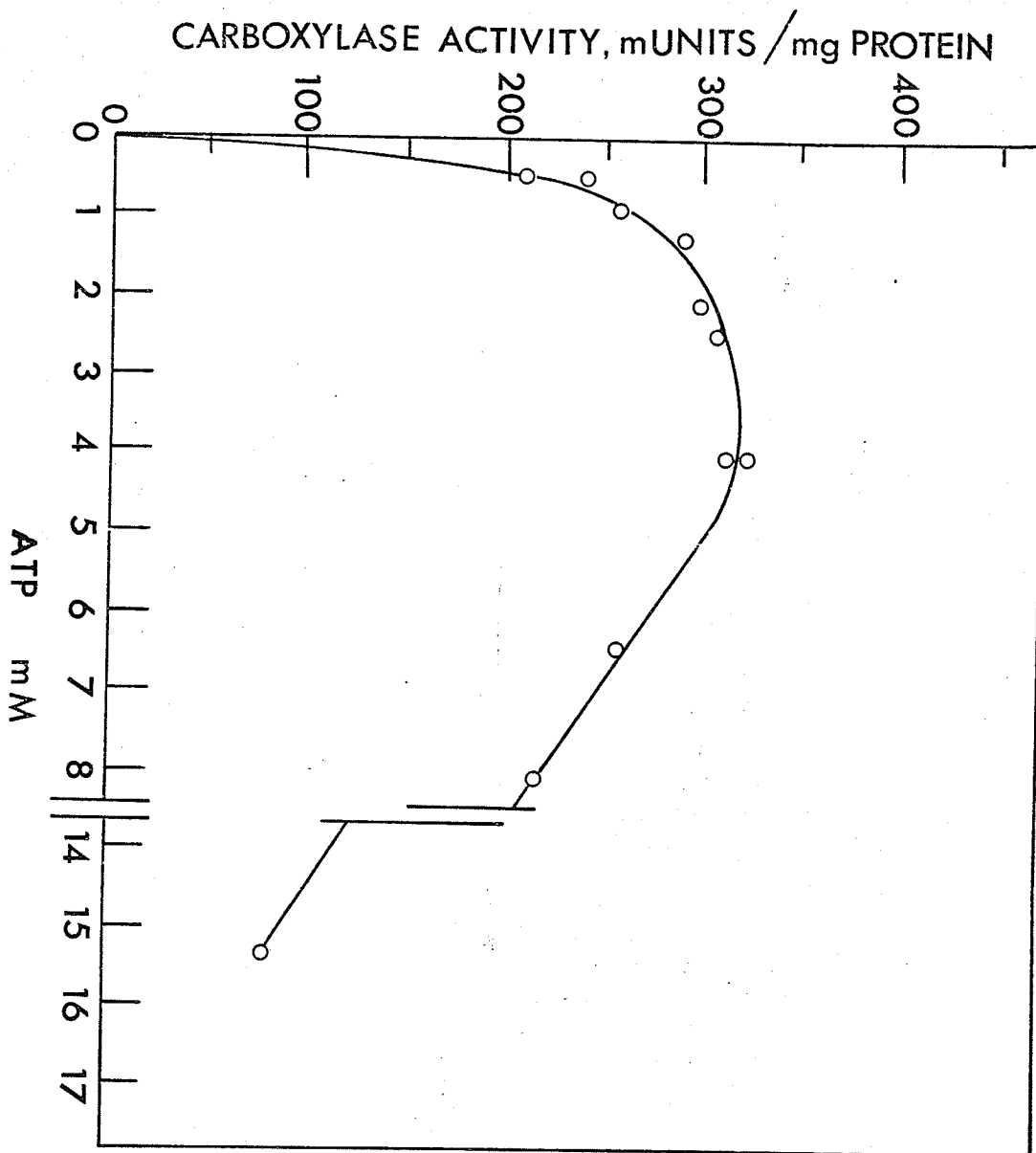


Figure 11. Citrate requirement of acetyl CoA carboxylase.

The enzyme was preincubated and assayed as described in "Methods". The citrate present in the preincubation was taken into account when the citrate concentration of the assay system was calculated.

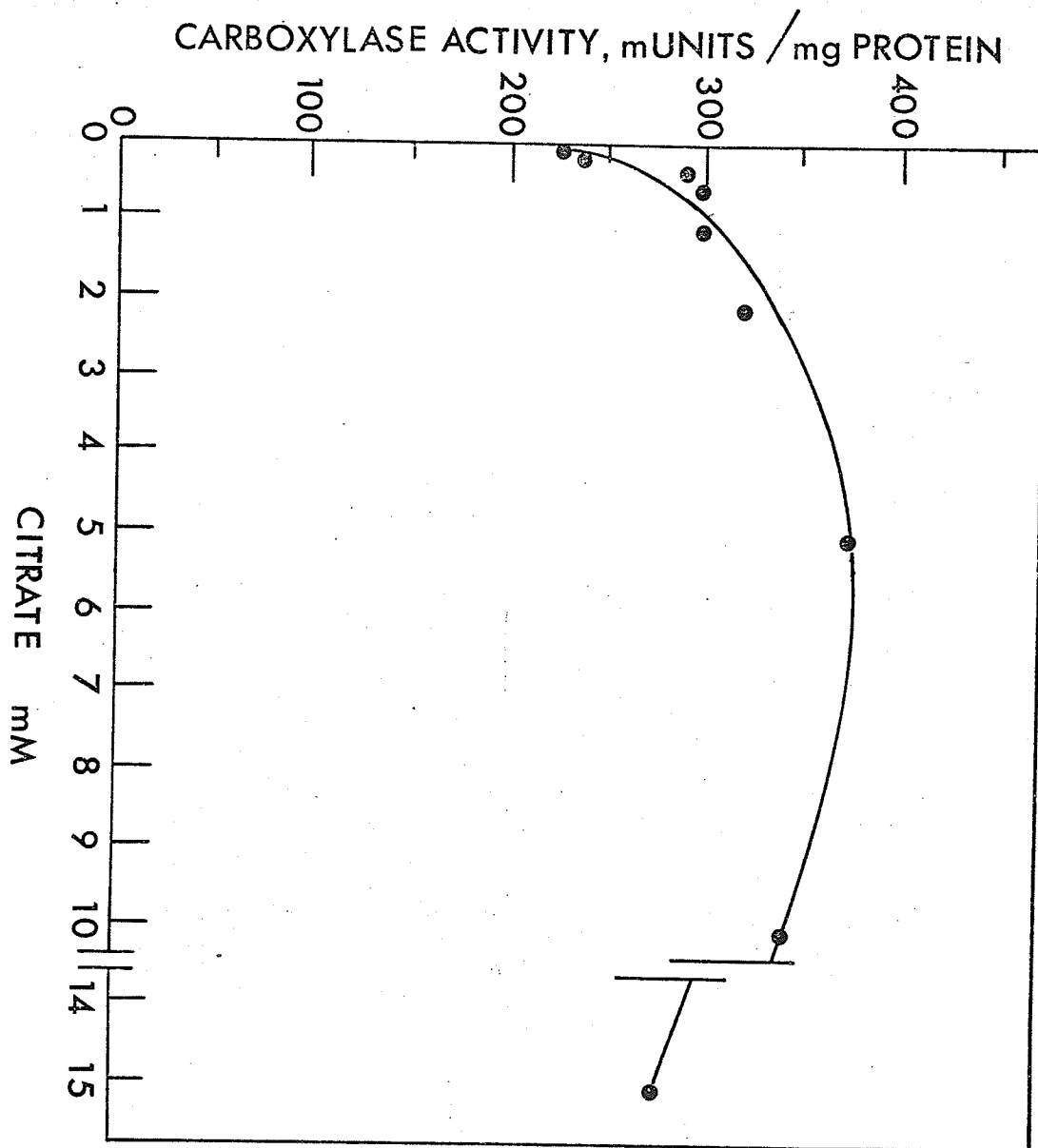


Figure 12. Malonyl CoA inhibition of the carboxylation reaction with respect to acetyl CoA.

Double reciprocal plot of the variation in velocity with changes in acetyl CoA concentration. Velocity V is expressed as units per mg protein in the assay. The enzyme was preincubated and assayed as described in "Methods". ● , no malonyl CoA; □ , 0.016 mM; ▽ , 0.033 mM; ○ , 0.061 mM; ▲ , 0.081 mM; ■ , 0.195 mM malonyl CoA.

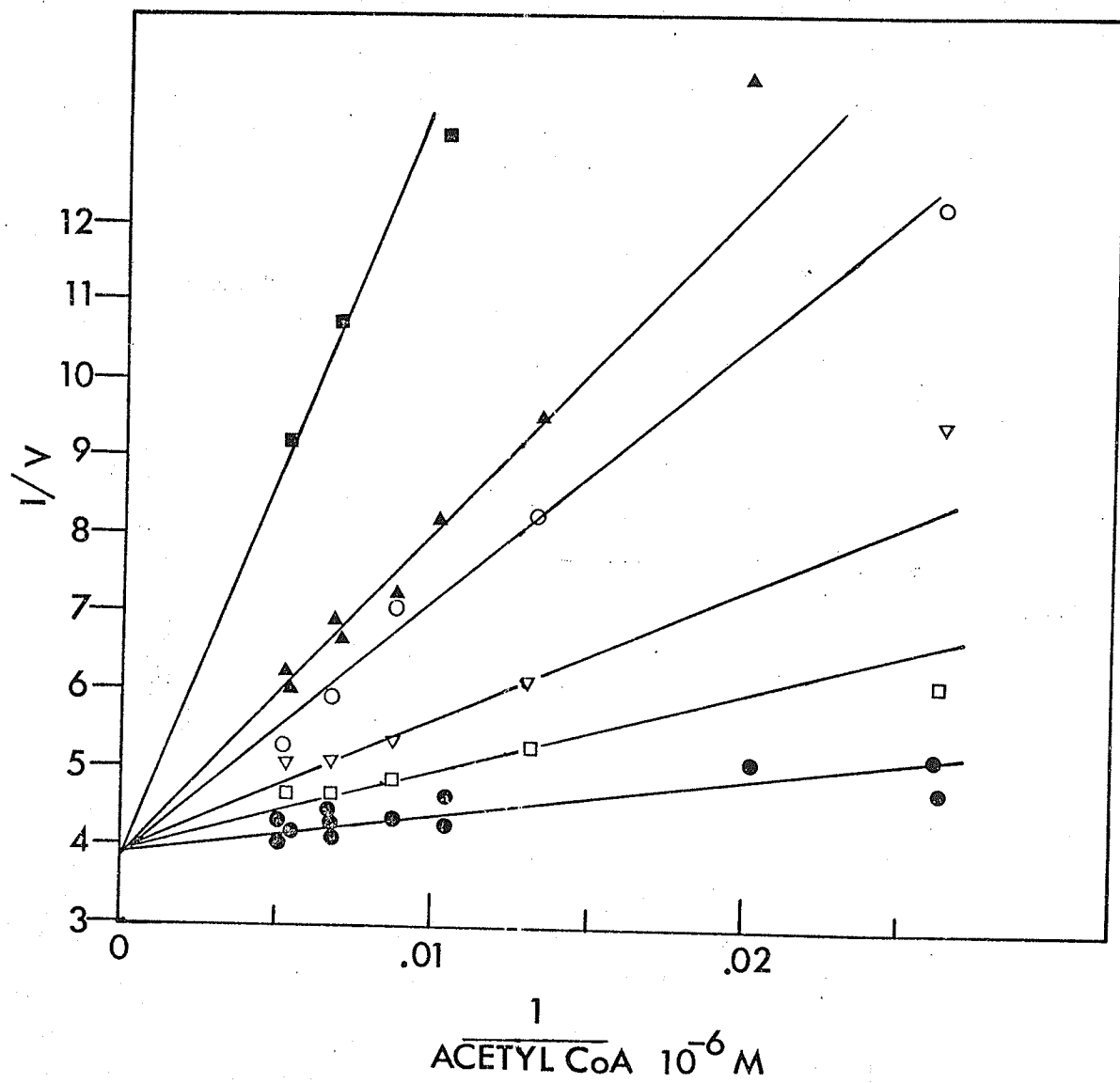


Figure 13. Malonyl CoA inhibition of the carboxylation reaction with respect to ATP.

Double reciprocal plot of the variation in velocity with changes in ATP concentration. Velocity V is expressed as units per mg protein in the assay. The enzyme was preincubated and assayed as described in "Methods". \circ , no malonyl CoA; \bullet , 0.40 mM malonyl CoA; \square , 0.82 mM malonyl CoA.

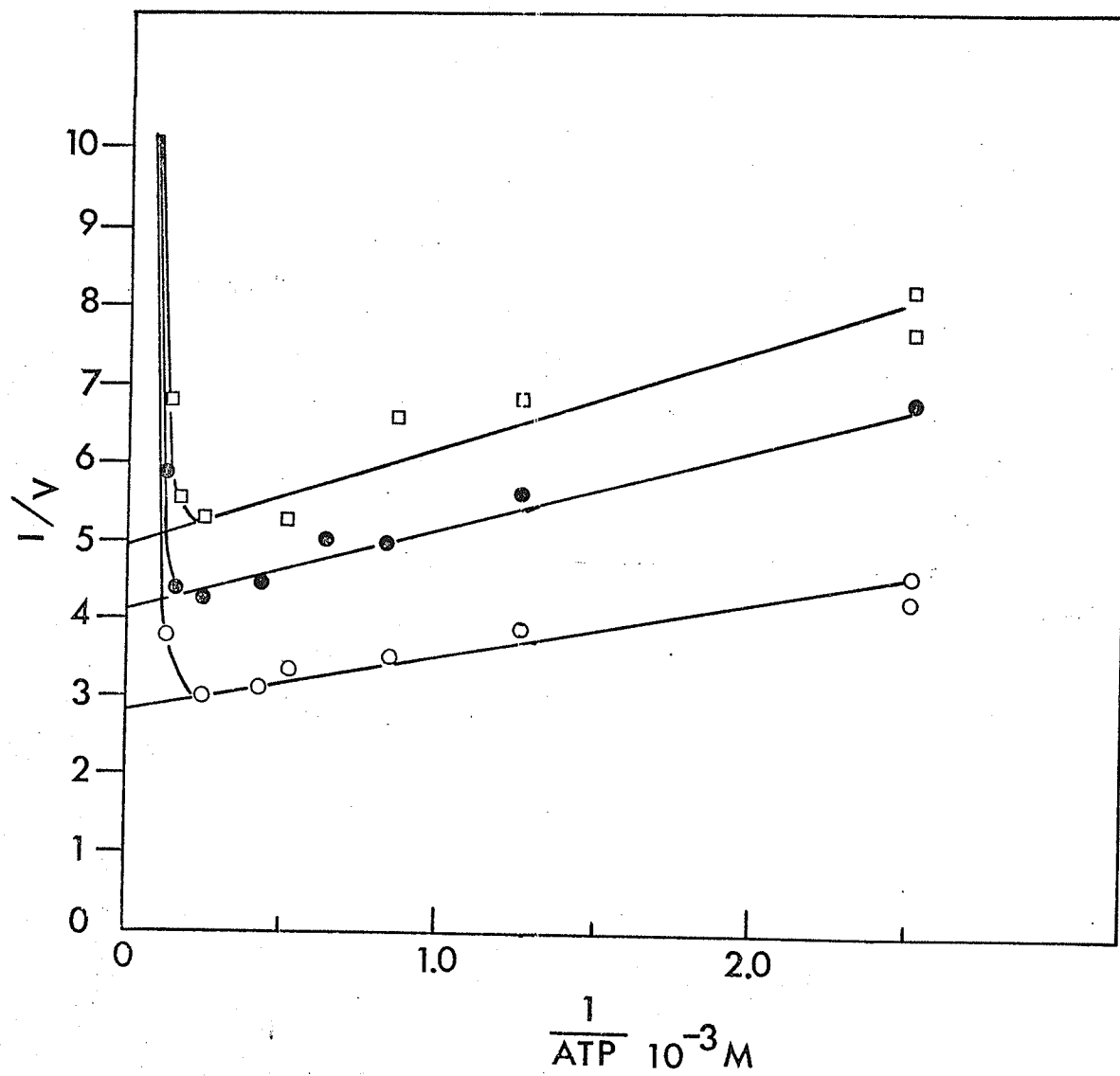


Figure 14. Inhibition of acetyl CoA carboxylase by coenzyme A.

The enzyme was preincubated and assayed as described in "Methods".

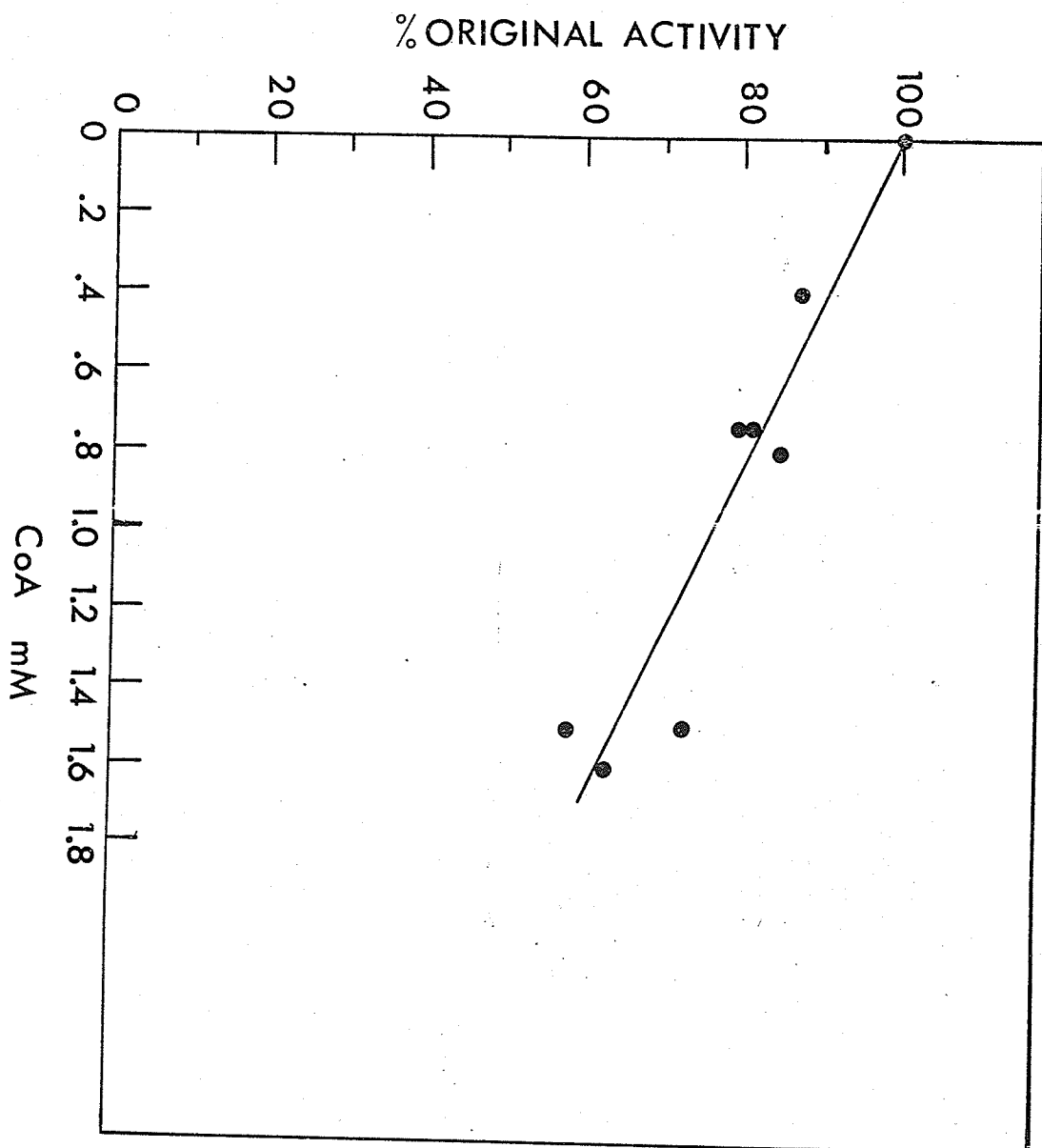


Figure 15. Effect of pH on the reversible cold inactivation of acetyl CoA carboxylase.

The enzyme was preincubated at various pH's in Tris (Cl^-) buffer and assayed in a 2 minute incubation at pH 7.5 as described in "Methods". ---- 37°C, — 0°C.
□ pH 7.0, Δ pH 7.5, ○ pH 8.0. Controls were preincubated at the various pH's for 90 minutes at 37°C and kept at 25°C for 4 hours, prior to assay, ■ pH 7.0, ▲ pH 7.5, ● pH 8.0.

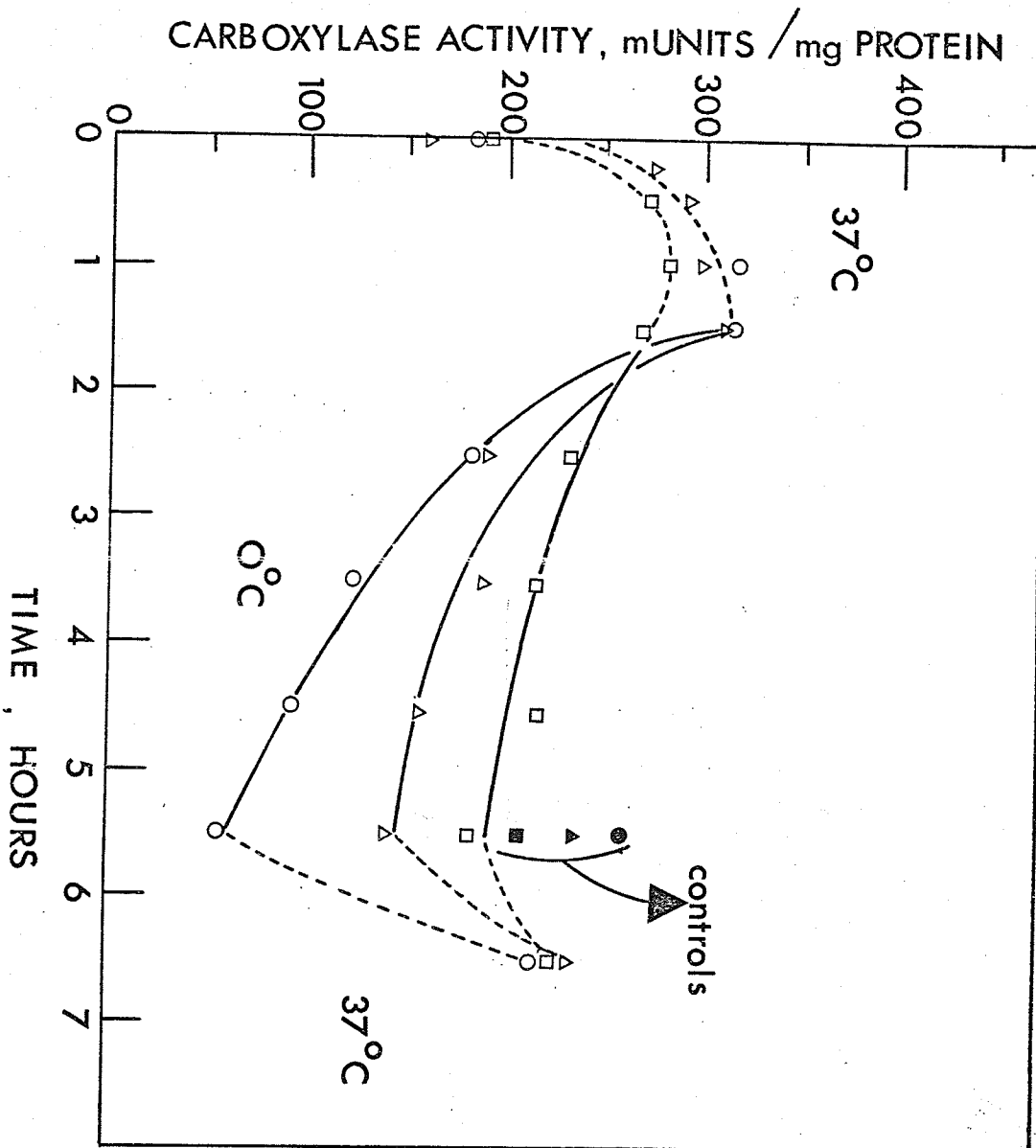


Figure 16. Effect of diet on the citrate activation of crude preparations of acetyl CoA carboxylase.

Gel filtered cytosol's were obtained from the epididymal fat pads of rats on various diets as described in "Methods". All preincubations were done at enzyme concentrations of 1.0 mg gel filtered cytosol protein per ml. Each point represents determinations on two pairs of epididymal fat pads done in duplicate. ○—○ , biotin deficient; ●—● , pair-weighed controls; ■—■ , Purina lab chow; □—□ , 65% dextrose.

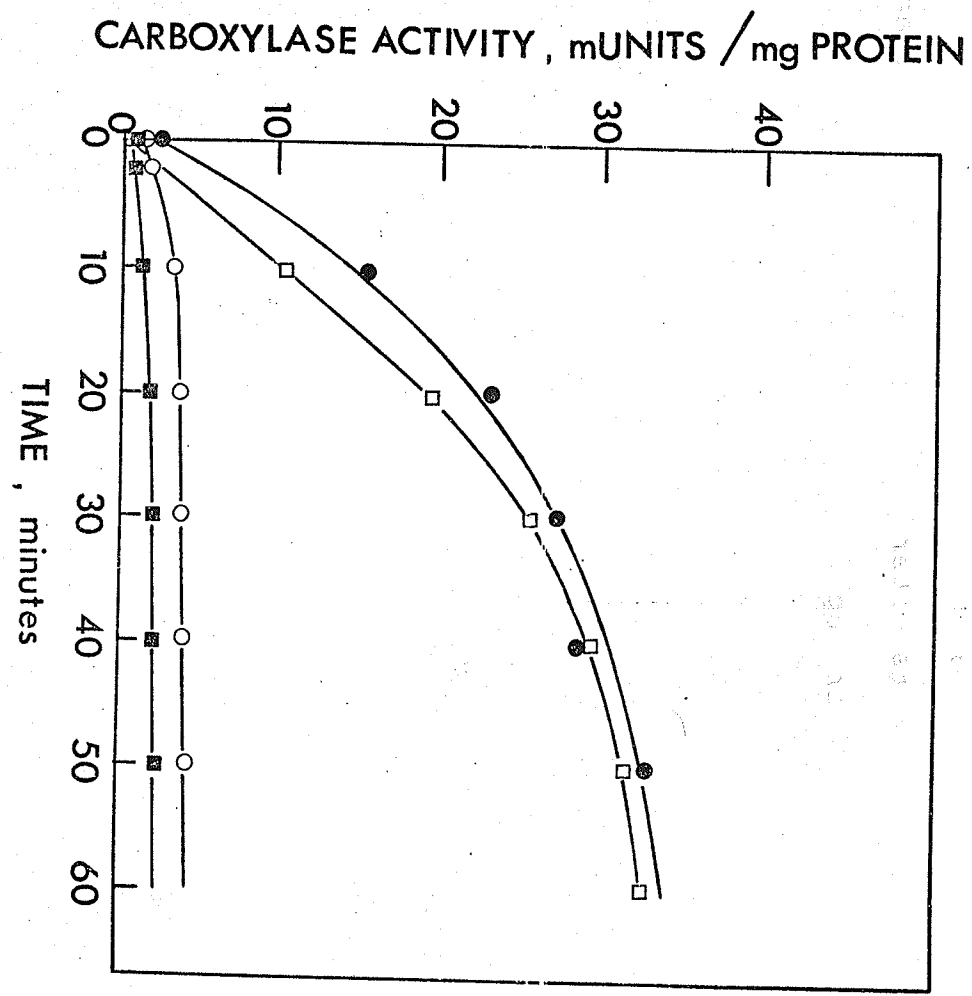


Figure 17. Effect of diet on the progress curve of crude preparations of acetyl CoA carboxylase.

Gel filtered cytosol was obtained from the epididymal fat pads of rats on various diets as described in "Methods". Each point represents determinations on two pairs of epididymal fat pads done in duplicate. O—O , biotin deficient; ●—● , pair-weighed control; ■—■ , Purina lab chow; □—□ , 65% dextrose.

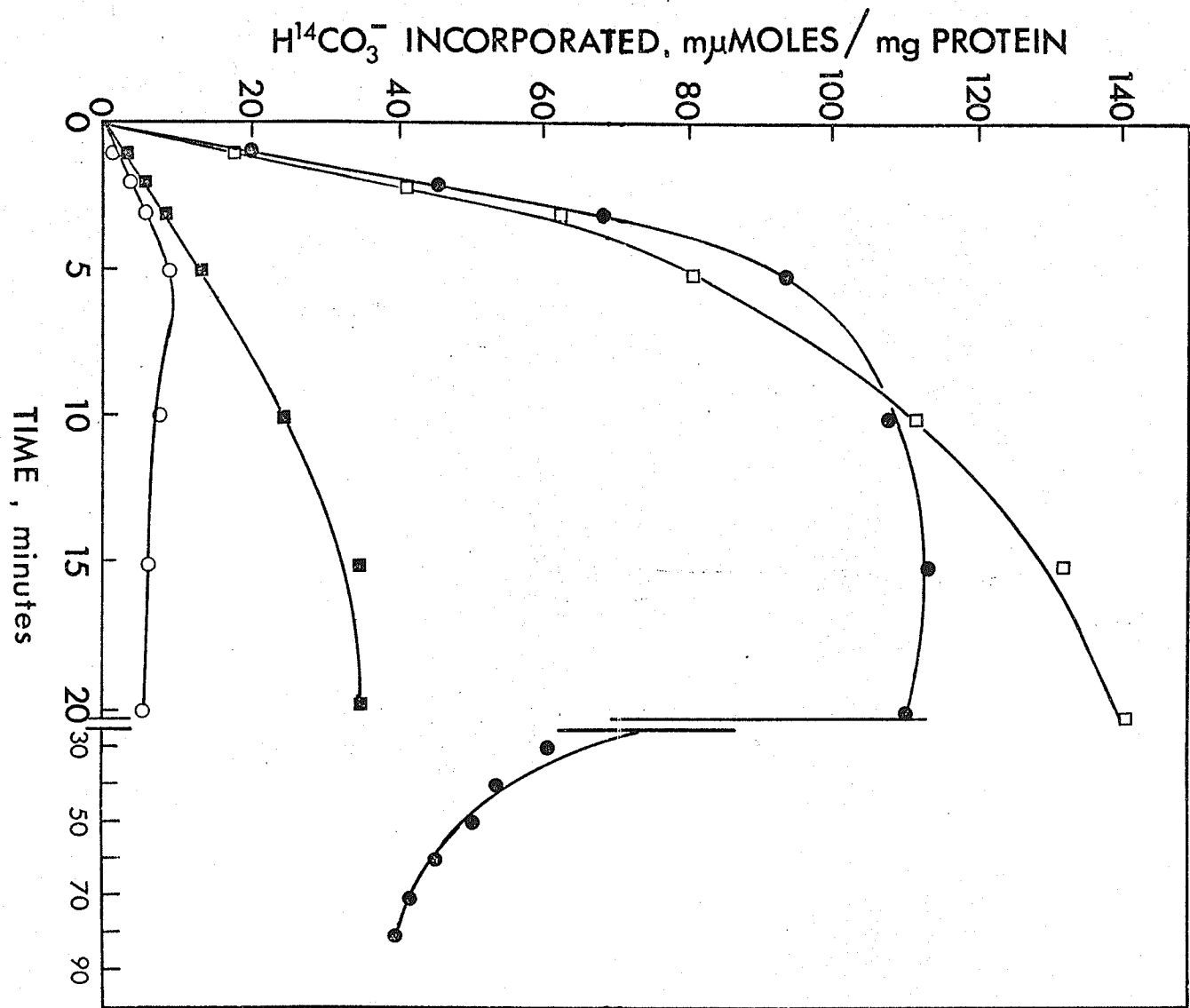


Figure 18. Progress curve of *in vivo* amino acid ^{14}C incorporation into liver and adipose tissue protein.

Normal rats on Purina lab chow were injected intramuscularly with amino acid ^{14}C and the specific radioactivities of the liver and adipose tissue protein were determined after various time intervals, as described in "Methods".

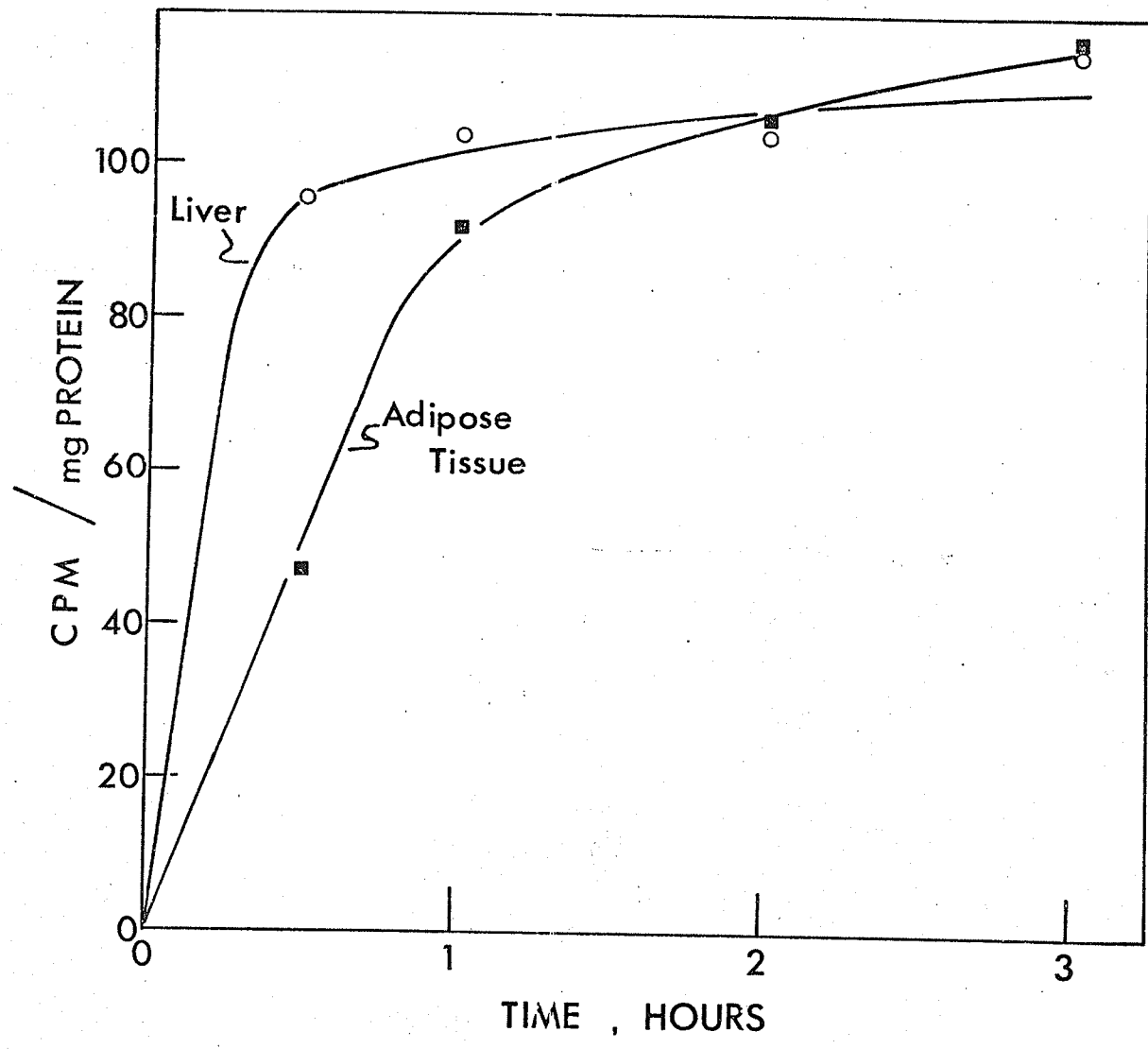


Figure 19. Progress curve of *in vitro* acetyl CoA holocarboxylase synthesis.

The gel filtered 20,000 Xg supernatant from epididymal adipose tissue was preincubated for one hour at 37°C in the preincubation medium in the presence of 25mM citrate without magnesium. The preincubated enzyme preparation (1.15 mg protein) was then incubated at 30°C for 4 hours. The complete system contained the following: 48mM Tris, pH 7.5, 2.7mM GSH, 0.08mM EDTA, 20mM Citrate (K⁺), 0.48 mg/ml bovine serum albumin, 82μM d-biotin, and 8mM ATP. Acetyl CoA carboxylase activity was determined at various time intervals on aliquots of the reaction mixture. All assays were done in duplicate.

●—● , complete system; ○—○ , complete system minus biotin (control).

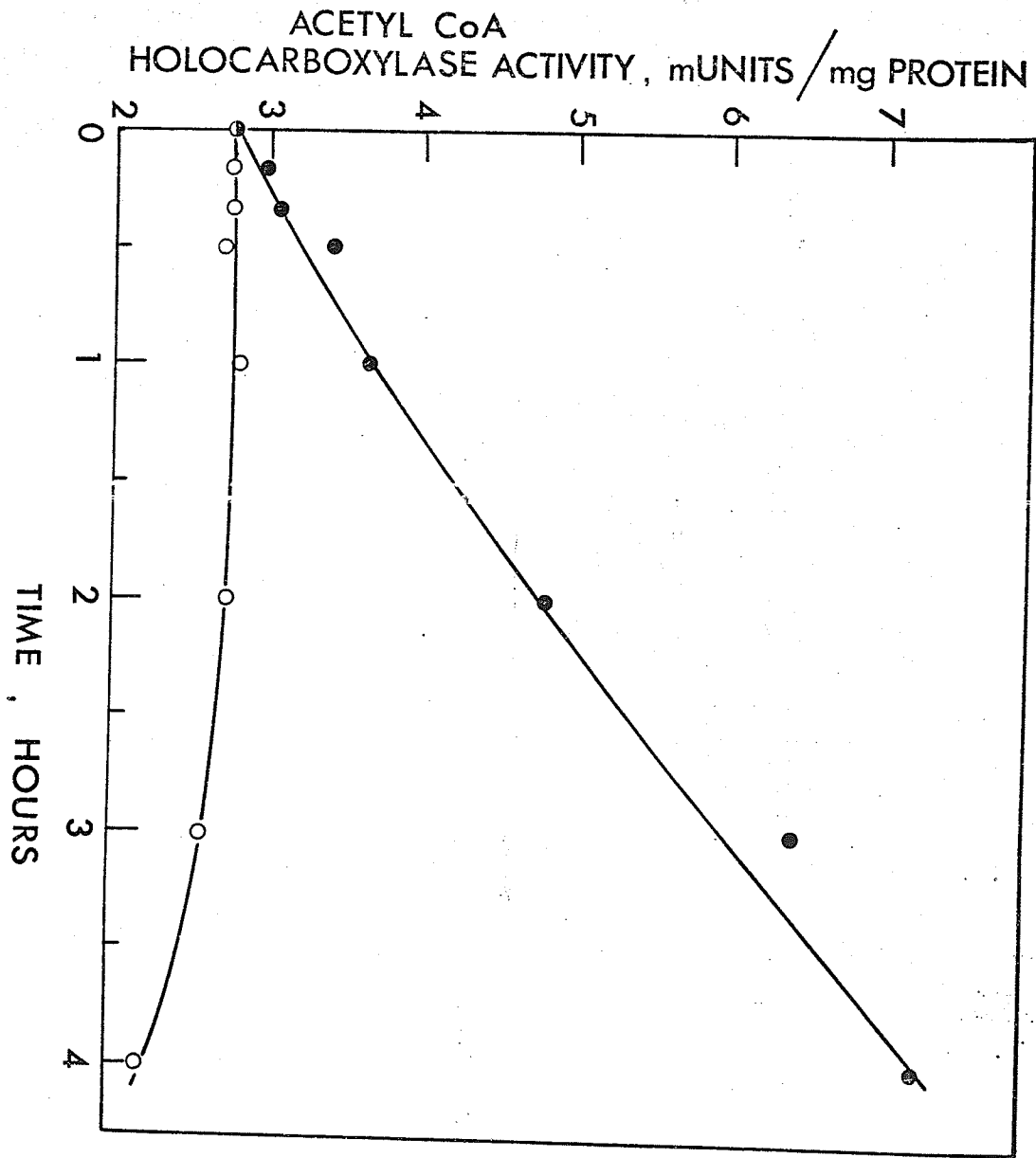


Figure 20. ATP requirement for acetyl CoA holocarboxylase synthesis.

Conditions were identical to those of Fig. 19 except that the preincubated enzyme preparation (1.15 mg protein) was incubated in the complete system with varying ATP concentrations. Holocarboxylase activity is expressed as increased acetyl CoA carboxylase activity (milliunits/mg protein) in the complete system after correction for the control (biotin omitted), in a four hour incubation at 30°C.

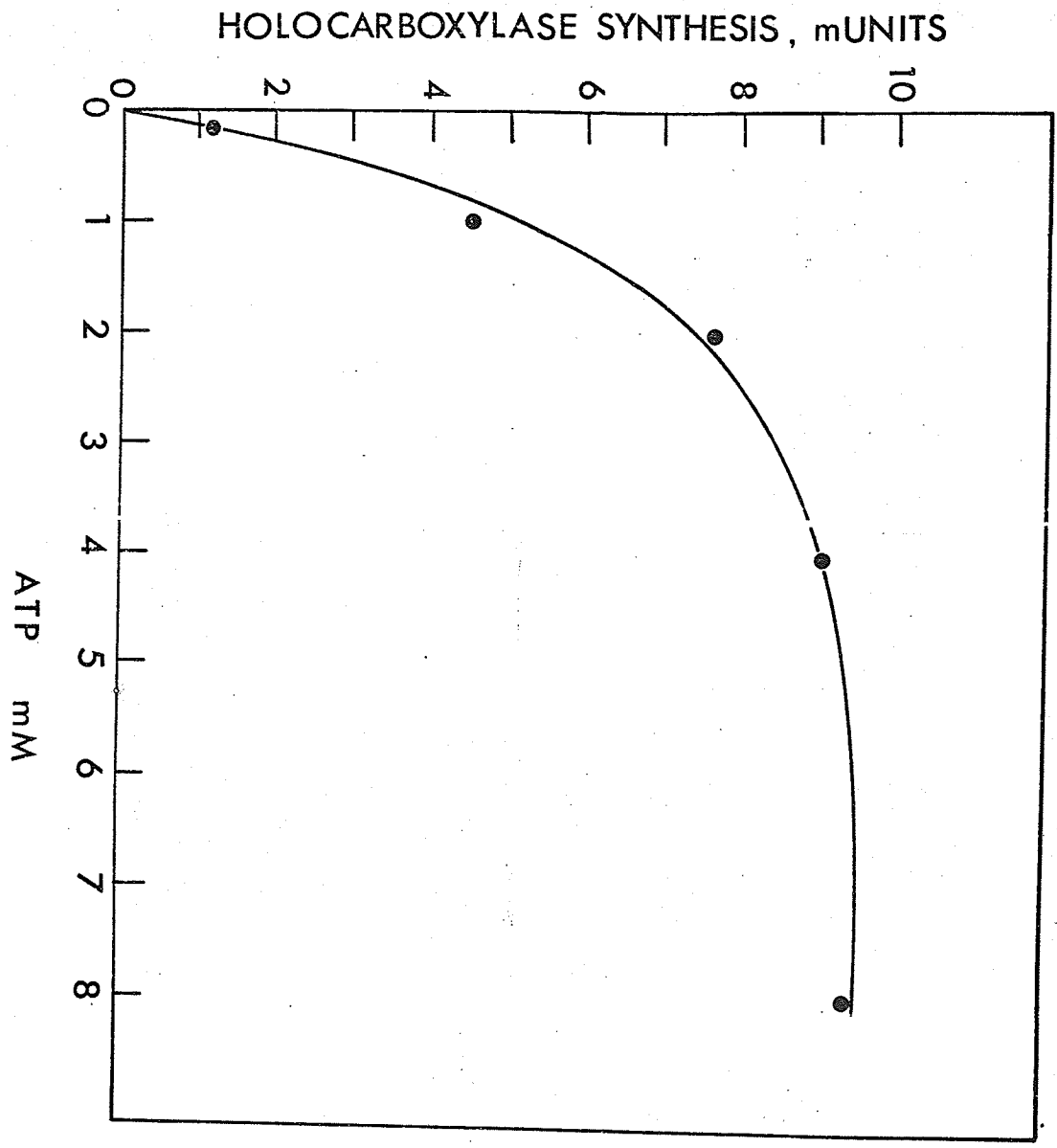


Figure 21. d-Biotin requirement for acetyl CoA holocarboxylase synthesis.

Conditions were identical to those of Fig. 19 except that the preincubated enzyme preparation (1.2 mg protein) was incubated in the complete system with varying d-biotin concentrations as indicated while ATP remained at 4 mM. Holocarboxylase synthesis is expressed in the same units as for Fig. 20.

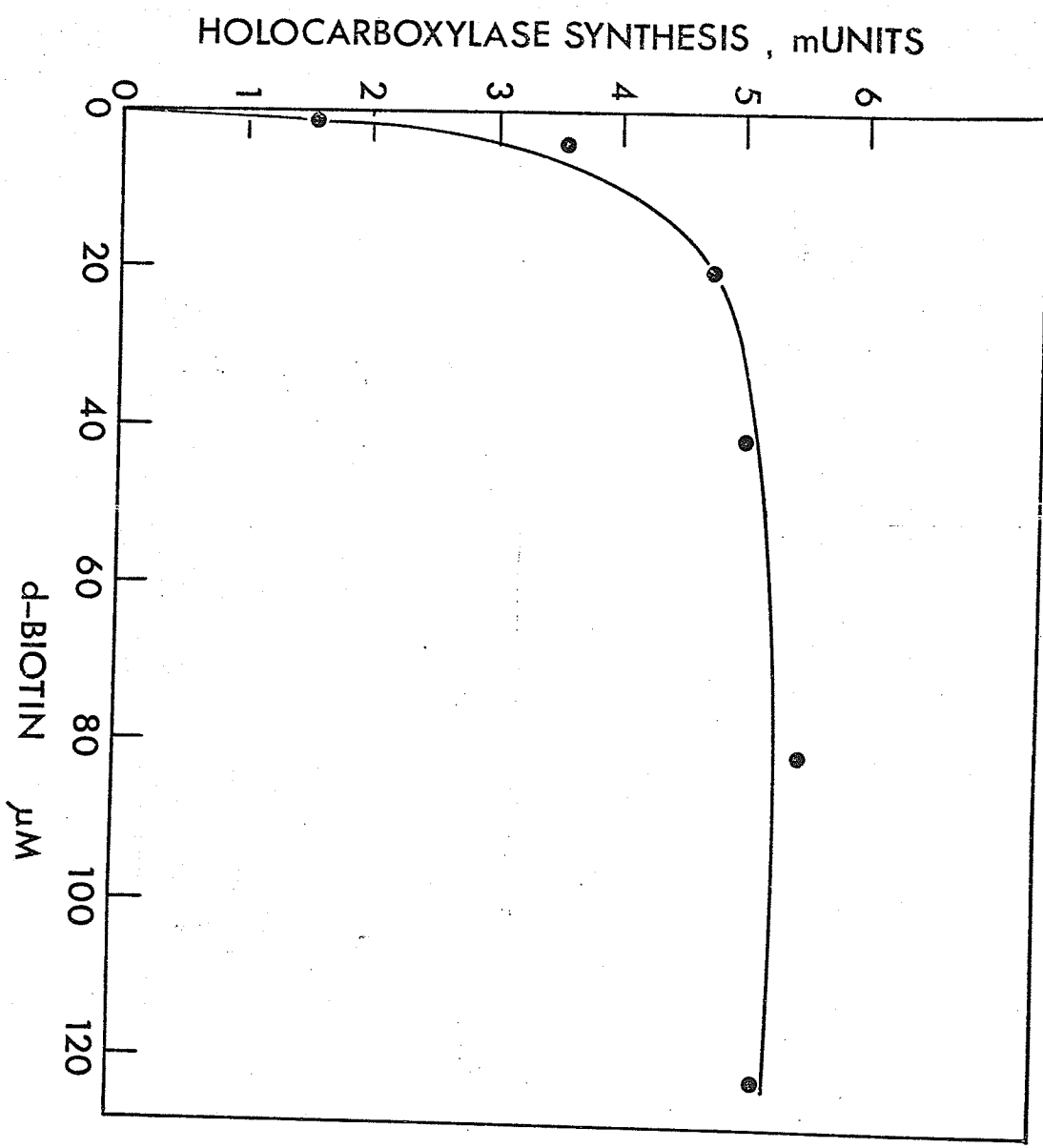
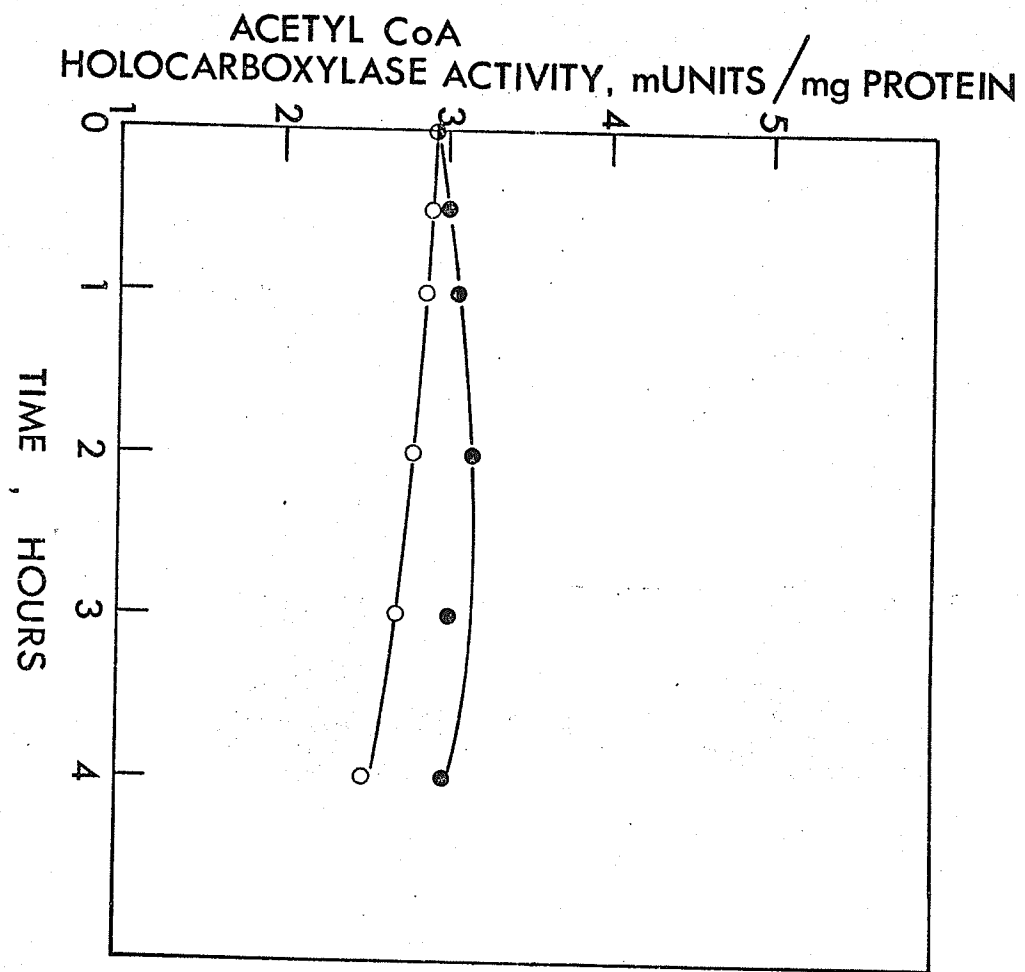


Figure 22. Progress curve of liver acetyl CoA holocarboxylase synthesis.

The gel filtered 105,000 Xg supernatant from rat liver was preincubated for one hour at 37°C in the preincubation medium in the presence of 25mM citrate without magnesium. The preincubated enzyme was then incubated with ATP and d-biotin as described in Fig. 19. Acetyl CoA carboxylase activity was determined at various time intervals on aliquots of the reaction mixture. ●—● , complete system; ○—○ , complete system minus biotin (control).



APPENDIX C - DIET AND VITAMIN MIXTURE COMPOSITIONS

Appendix C - DIET AND VITAMIN MIXTURE COMPOSITION

DIET COMPOSITION

COMPONENTS*	DIET	
	biotin deficient g/100 g diet	high carbohydrate g/100 g diet
Egg-white (powdered)	25.00	--
Casein	--	25.00
Dextrose	64.85	65.00
Corn oil	5.00	5.00
Mineral mix (#446)	4.00	4.00
Vitamin fortification mixture (General Biochemicals)	--	1.0
Vitamin mix (-biotin)**	1.0	--
Choline chloride	0.15	--

* The vitamin fortification mix was purchased from Nutritional Biochemicals, corn oil from St. Lawrence Starch Co. (Port Credit, Ont.) and choline chloride from the Sigma Chemical Co. All other components were purchased from General Biochemicals.

** This vitamin fortification mixture was prepared in the laboratory. The basic composition of both vitamin mixtures is shown in the following table.

VITAMIN MIXTURE COMPOSITION

COMPONENTS	Commercial (General Biochemicals) g/Kgm mix	-Biotin g/Kgm mix
Vitamin A (200,000 units per gram) and D (400,000 units per gram) concentrate	4.5	4.0
Alpha Tocopherol	5.0	6.0
Ascorbic Acid	45.0	50.0
Inositol	5.0	5.5
Choline Chloride	75.0	---
Menadione	2.25	2.5
p-Aminobenzoic Acid	5.0	5.0
Niacin	4.5	5.0
Riboflavin	1.0	1.0
Pyridoxine, HCl	1.0	1.0
Thiamine, HCl	1.0	1.0
Calcium Pantothenate	3.0	3.0
Biotin	0.02	---
Folic Acid	0.09	0.10
Vitamin B-12	0.001	0.001
DEXTROSE	848.1	915.9