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The Conversion of Acetyl-CoA Apocarboxylase into the Active Holocarboxylase by Acetyl-CoA Holocarboxylase Synthetase

by

Amiram Dan Landman

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"THE CONVERSION OF ACETYL-CoA APOCARBOXYLASE INTO THE ACTIVE HOLOCARBOXYLASE BY ACETYL-CoA HOLOCARBOXYLASE SYNTHETASE"

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AMIRAM DAN LANDMAN

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

DOCTOR OF PHILOSOPHY

To my parents,

who dedicated themselves to my career

and to my wife Judith and daughter Shirley

who helped me along.

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ABSTRACT

The conversion of acetyl-CoA apocarboxylase into the active holocarboxylase by acetyl-CoA holocarboxylase synthetase

By Amiram Dan Landman

Acetyl-CoA apocarboxylase and acetyl-CoA holocarboxylase are two proteins with virtually indistinguishable structures. Their separation, which is required for a study of the apo-holo conversion system, was obtained by affinity chromatography on a Sepharose-avidin column. The efficiency and specificity of this column in obtaining holo-free apocarboxylase preparations are demonstrated.

Acetyl-CoA holocarboxylase has a low intrinsic activity as a protomer. In the presence of citrate it aggregates; this is followed by a hundred fold increase in enzyme activity. Using techniques of selective sedimentation by a preparative ultracentrifuge and separation on Sepharose-4B, it was shown that the apocarboxylase which lacks enzymatic activity, does not have the ability to aggregate, thus pointing to the microenvironment around biotin as the site which triggers the aggregation and activation simultanously.

In view of the difficulties in obtaining the apocarboxylase, the assay of acetyl-CoA holocarboxylase synthetase was tried by replacing the apocarboxylase with artificial substrates rich in lysine residues. As these were unsuccessful, a partial reaction of the holocarboxylase synthetase which involves the exchange of labelled PP_i with ATP was devised for its assay.

"Biotin activating enzyme" was partially purified and some of its properties studied. Addition of the partially purified "Biotin activating enzyme" to cytosolic preparations of adipose tissue of biotin deficient rats did not enhance the rate of incorporation of biotin into endogenous acetyl-CoA apocarboxylase, which indicated that "Biotin activating enzyme" may be distinct from acetyl-CoA holocarboxylase synthetase.

A microbiological method has generally been used for the assay of biotin. Although the assay is accurate and reproducible it is time consuming. The radioisotope dilution assay for biotin described here has the sensitivity required to measure the levels of biotin found in biological material and is simple and rapid. The procedure was applied for the assay of biocytin, a biotin derivative which participates in the catabolism of biotin-containing-enzymes.

LIST OF ABBREVIATIONS

AMP	:	Adenosine 5' monophosphate
ADP	:	Adenosine 5' diphosphate
ATP	:	Adenosine 5' triphosphate
Acetyl-CoA	:	Acetyl-Coenzyme A
ACHS	:	Acetyl-Coenzyme A holocarboxylase synthetase
ACAP	:	Acetyl-Coenzyme A apocarboxylase
BAE	:	Biotin activating enzyme
BSA	:	Bovine serum albumin
b-AMP	:	Biotinyl-AMP
CoA	:	Coenzyme A
DCC	:	Dicyclohexylcarbodiimide
DEAE	:	Diethylaminoethyl
EDTA	:	Ethylenediamine tetraacetic acid
E	:	Molar extinction coefficient
GSH	:	Glutathione (reduced)
HABA	:	2(4' Hydroxyazobenzene) benzoic acid
I.U.	:	International units
P_i	:	Inorganic phosphate
PP_i	:	Pyrophosphate
PPO	:	2,5 Diphenyloxazole
POPOP	:	1,4-bis-2(5-phenyloxazolyl)-benzene
Tris	:	Tris (hydroxymethyl) amino methane
TCA	:	Trichloroacetic acid

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INTRODUCTION

Acetyl-CoA carboxylase is a biotin containing enzyme. The enzyme exists in both the apo and holo forms. The apoenzyme has no catalytic activity. The conversion of acetyl-CoA apocarboxylase to the holoenzyme is catalyzed by an enzyme, acetyl-CoA holocarboxylase synthetase. The purpose of this thesis was to study the interaction between acetyl-CoA holocarboxylase synthetase and the apocarboxylase,as well as to investigate further the relationship that biotin has with acetyl-CoA carboxylase.

Objectives: The first objective was to investigate rat liver acetyl-CoA holocarboxylase synthetase. In a series of reports Dakshinamurti and Desjardins (1969, 1970, 1971) have reported on the in vivo and in vitro conversion of acetyl-CoA apocarboxylase to the holoenzyme form in rat liver and adipose tissue. In their study they utilized an assay system which was encumbered by the presence of substantial holocarboxylase activity. Methods were developed in this thesis for an improved assay of acetyl-CoA holocarboxylase synthetase activity by the removal of this endogenous acetyl-CoA holocarboxylase by affinity chromatography. In addition,attempts were made to measure acetyl-CoA carboxylase synthetase in pig and rat liver using the colorimetric assay described by Coon et al. (1964). In their assay the formation of biotinyl-AMP was measured colorimetrically after its conversion to biotinylhydroxamate.

The second objective of the thesis was to investigate the role of the covalently bound biotin on acetyl-CoA carboxylase in the aggregation and disaggregation of the enzyme.

Organization of the thesis: The thesis is divided into two major sections. The first section presents the background information for this thesis and takes the form of a literature review. The next section is divided into five chapters. Each chapter contains appropriate introduction, experimental methodology, results and discussion. The first chapter deals with the isolation of acetyl-CoA apocarboxylase free of holocarboxylase activity. The next chapter describes the investigation into the role of biotin in the aggregation of the holoenzyme. The next two chapters describe attempts to improve the methodology for the assay of acetyl-CoA holocarboxylase synthetase and in the last chapter a radioisotope dilution assay for biotin is described.

LITERATURE REVIEW

A. Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase was initially recognized by Wakil and coworkers (1958) as fraction R_1 which in the presence of the complementary fraction R_2 catalyzed the formation of fatty acids in the soluble fraction of crude pigeon liver extracts. Fraction R_2 was later identified as the fatty acid synthetase complex (Wakil and Ganguly, 1959). Further investigation into the nature and role of fraction R_1 showed that bicarbonate was required for its activity and malonyl-CoA was identified as one of the products (Formica and Brady, 1959). It has now been shown (Wakil, 1961) that fraction R_1 catalyzes the following reaction:

$$\text{Acetyl-CoA} + HCO_3^- + \text{ATP} \xrightleftharpoons{Mg^{+2}} \text{Malonyl-COA} + \text{ADP} + P_i$$

Subsequently, acetyl-CoA carboxylase was identified as a biotin containing enzyme (Wakil and Gibson, 1960).

Brady and Gurin (1952) were the first to show that certain tricarboxylic acid cycle intermediates such as citrate stimulated fatty acid synthesis. Martin and Vagelos (1962, 1963) showed that citrate stimulated fatty acid synthesis at the acetyl-CoA carboxylase step. They also showed that activation of acetyl-CoA carboxylase by citrate resulted in an aggregation of the enzyme to a heavier sedimenting form. Lane and coworkers (1968, 1968a) have confirmed and extended the findings of Martin and Vagelos by using avian liver acetyl-CoA carboxylase purified to homogeneity. Subsequent studies by numerous investigators (Moss and Lane, 1971; Lane, Moss and Polakis 1974; Numa and Yamashita, 1974) have elucidated

the structure, function and the reaction mechanism of this enzyme. The acetyl-CoA carboxylase reaction was shown to be regulated by a variety of biochemical and physiological determinants and is considered by many as the rate limiting and regulatory step of fatty acid synthesis.

i. Physical properties of acetyl-CoA carboxylase

Acetyl-CoA carboxylase has been purified to homogeneity from chicken liver (Lane et al. 1968, 1968a), bovine adipose tissue (Moss et al. 1972) and rat liver (Inoue and Lowenstein, 1972). The enzyme from these various sources has similar physico-chemical properties. The enzyme exists in both the protomeric and the polymeric forms,and the equilibrium between these forms is affected by various factors such as protein concentration, pH, citrate, malonyl-CoA, phosphate etc. The protomeric form has $S_{20,w}$ of 13-15 which corresponds to a molecular weight of 410,000-550,000 daltons while the polymeric form has a molecular weight of 4-5 million daltons.

The substructure of the rat liver enzyme was analyzed by Inoue and Lowenstein (1972). Examination of rat liver acetyl-CoA carboxylase on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate dissociated the enzyme into two subunits of 215,000 daltons which could further be dissociated into nonidentical polypeptide chains of 118,000 and 125,000 daltons by prolonged and repeated treatment of the enzyme with sodium dodecyl sulfate at an elevated temperature. These investigators concluded that the enzyme contains pairs of unlike polypeptide chains which remain associated to a considerable degree in sodium dodecyl sulfate at room temperature, but which can be separated after more drastic treatment. Guchhait, Zwergel and Lane (1974) have demonstrated

that chicken liver acetyl-CoA carboxylase in the presence of 6 M urea and 0.1% sodium dodecyl sulfate dissociates into three polypeptide chains of 117,000, 129,000 and 139,000 daltons. The ratio of these chains was found to be 2:1:1 respectively. The difference in the structures of the chicken and rat carboxylases is reflected in their biotin content. While the rat liver enzyme is reported to contain two biotin molecules, presumably one for each 215,000 dalton subunit, the chicken acetyl-CoA carboxylase is reported to possess only one molecule of biotin per protomer which is presumeably attached to the 117,000 dalton polypeptide chain. More data is required to clarify whether the differences in structure and biotin content of the chicken and rat carboxylases as reported by Lane's and Lowenstein's groups is inherent to these species, or whether it is a result of different technical procedures.

ii. Effect of citrate on acetyl-CoA carboxylase

Citrate is an allosteric effector of the Vmax type (Numa, Ringelmann and Lynen, 1965). The activity of the protomeric form of the enzyme from various sources is within the range of 0.15-0.30 units/mg (one unit is defined as an activity which will catalyze the formation of malonyl-CoA in a rate of 1 μM/min.), while in the presence of citrate the enzyme aggregates and the activity is increased to 10-15 units/mg (Moss and Lane, 1971). Rat liver and rat adipose tissue are activated by citrate only after incubation at 37°C for 30-60 minutes (Dakshinamurti and Desjardins, 1969; Desjardins and Dakshinamurti, 1970). However, under appropriate conditions and in the presence of citrate or isocitrate, chicken acetyl-CoA carboxylase will aggregate within 10 seconds. Moss and Lane (1972) investigated the nature and interrelationship between the

activation and aggregation of the chicken enzyme by cooling the enzyme to 2°C, a temperature at which both processes were slowed to a considerable extent and therefore could be studied with relative ease. The enzyme was incubated in presence of phosphate in order to keep it in its aggregated state (this however did not increase the enzymic activity), and then transferred into a medium which contained the components of the assay mixture with the exception of citrate. Disaggregation of the enzyme was followed by measuring viscosity. The rate of disaggregation paralleled the rate of loss in enzyme activity. From this study it was concluded that the aggregates are "open structures" in which the number of protomers on the oligomer is not defined, and may vary depending on the pH, protein concentration and other parameters defined by Gregolin et al. (1968, 1968a), and that the enzymic activity is possibly a function of the size of the structures. It has been suggested that the "raison d'être" for the changes in the supraquaternary structure is the intimate coordination between acetyl-CoA carboxylase and other lipogenic enzymes, mainly fatty acid synthetase. Thus the macromolecular changes in the enzyme during aggregation may provide a delicate matrix for the stereospecific assembly of the fatty acid synthetase (Moss and Lane 1971, 1972).

Avidin is a glycoprotein of a molecular weight of 68,000 which exhibits a very strong affinity to free or protein-bound biotin. The dissociation constant between the two components $K_D = 10^{-15}$ reflects this affinity (Green, 1963; 1965). The glycoprotein inhibits acetyl-CoA carboxylase by irreversibly binding with the prosthetic group biotin. Moss and Lane (1972) have shown that this inhibition will occur only in the absence of citrate, but not in the presence of the activator. This

led to the conclusion that the structural changes induced by citrate involve the microenvironment surrounding the active site which will protect the prosthetic group from the inhibitor. Apparently aggregation per se does not provide this protection, since when the polymerization is induced by tricarballylate which is not coupled with increased enzyme activity, the rate of binding of avidin to the biotin is similar to the binding under conditions favoring its protomeric form.

Further evidence in regard to the structural changes induced by citrate come from model experiments on the carboxylation of free biotin (Lane, Moss and Polakis, 1974). When acetyl-CoA carboxylase was incubated with biotin and the system supplemented with Mg^{++}, ATP and HCO_3^-, the free biotin was carboxylated. However, on addition of citrate to this mixture the Km for the biotin was highly increased. This implied that the changes induced by the activator help to orient the enzyme bound biotin in a position which will favor its carboxylation but will hinder the free biotin from approaching the carboxylation site.

Moss and Lane (1972) have demonstrated that phosphate which is an aggregator is also capable of activation but that the activity is lost rapidly and can be detected only by specially designed experiments. It is generally accepted that phosphate and tricarballylate are aggregators while citrate and isocitrate are "true activators." It was postulated that the carboxylation of the biotin at the active site, causes structural strain near the active site. This strain causes further structural changes in the quartenary structure which will result in the disaggregation of the polymer. Only the "true activators" are capable of withstanding this strain, keeping the catalytic site in its

active state, and allowing the carboxyl group to be transferred to its acceptor with maximum efficiency as the enzyme is kept as an aggregate.

It is assumed that the conformational changes induced by citrate are required for the reactivation of the intermediate N-carboxybiotin (Lane, Moss and Polakis, 1974). The group which is a weak electrophile will interact with the carboxyl acceptor which is a strong nucleophile only after changes in the microenvironment around the active site will compensate for its being a weak electrophile, or change the normal angles and strength of bonds within the carboxybiotin moiety to make it a stronger electrophile. The K_D for the binding of citrate is about 10^{-3} mM while K_A is about 2-3 mM. It is suggested that the difference in these constants represents the thermodynamic parameters which are required to keep the enzyme in its active form while the enzyme is found in the constrained carboxy-biotin form (Moss and Lane, 1971).

iii. Regulation of acetyl-CoA carboxylase by other effectors

ATP and Mg^{++} are ligands which are required by acetyl-CoA carboxylase for its activity. However, at concentrations above optimum ATP becomes inhibitory. Inoue and Lowenstein (1972) reported that four phosphate molecules were bound covalenty to the homogeneous enzyme. This was followed by reports by Kim et al. (1973, 1973a, 1974) that the enzyme is inactivated by its phosphorylation and activated by dephosphorylation. Phosphorylation-dephosphorylation has been suggested to be an intermediate step between the action of some hormones and their apparent effect on lipogenesis (Lee, Thrall and Kim, 1973). Allred and Roehrig (1973) postulated that the phosphorylation may be mediated by c-AMP. Further investigations will be required to clearly evaluate these

results.

Coenzyme A derivatives of long chain fatty acids are strong inhibitors of acetyl-CoA carboxylase. Their inhibition constant $K_i \simeq 10^{-7}$, implied that these compounds may be involved in the fine regulation of the enzyme. Although fatty acid derivatives are known to function as detergents and thereby to inhibit nonspecifically a variety of enzymes (Taketa and Pogell, 1966), it was shown by Haleford and Denton (1973, 1974) that the inhibition of Acetyl-CoA carboxylase by these compounds is reversible, thus implying that their interaction with the enzyme is of a more specific nature. Data by Goodridge (1972) substantiate this finding and imply that the CoA derivatives of fatty acid may actually compete with citrate on the binding site. Since albumin binds strongly with fatty acids and their CoA derivatives it is conceivable that its interaction with these compounds may affect acetyl-CoA carboxylase activity.

iv. Variations in cellular levels of the enzyme

Acetyl-CoA carboxylase levels in various tissues are dependent on the physiological conditions, particularly on the type of diet (Desjardins and Dakshinamurti 1969; Liu and Donaldson, 1973). On low fat diet the enzyme content is increased. Maximal levels of acetyl-CoA carboxylase were obtained when the fat content of the diet was lowered to 1%. Substantial increase in enzymic contents also occurs on feeding a high carbohydrate diet. The changes in the cellular levels of acetyl-CoA carboxylase may be attributed to changes in the rate of synthesis and degradation of the enzyme. Intensive studies showed that by changing the physiological conditions, the rates of synthesis and degradation

of the enzyme could vary independently (Numa and Yamashita, 1974) and there is substantial evidence to indicate that these changes are mediated by hormones particularly insulin and epinephrine (Haleford and Denton, 1974).

B. Conversion of Acetyl-CoA Apocarboxylase Into The Holoform

Kosow and Lane (1961, 1961a, 1962, 1962a, 1962b) and Coon and coworkers (1963, 1965, 1966) have demonstrated that the attachment of biotin to the apocarboxylase is catalyzed by an enzyme referred to as holocarboxylase synthetase. Restoration of the activity of any biotin containing enzyme which is obtained from biotin deficient animals can be achieved by incubation of the enzymic preparation, usually a mixture of the active holoform and the inactive apoform, with ATP, biotin and the holocarboxylase synthetase.

i. Interaction of the apocarboxylase with the synthetase

The information on the interaction of the apoenzymes with the appropriate holocarboxylase synthetase is sparse. Some elucidation of the properties of the apocarboxylases can be done only with the arbitrary assumption that, although they differ in size and function, they behave similarly when interacting with the synthetase. There is some experimental evidence to substantiate this assumption. Biotin containing enzymes are essentially similar in structure, having a very high molecular weight and being composed mainly of four subunits (Moss and Lane, 1971). In lower species several biotin containing enzymes migrate on disc gel electrophoresis in identical bands thus indicating even higher similarity in structure. In conjunction with the

requirement of small ligands in order to trigger their activity, and their common carboxylation mechanism (Moss and Lane, 1971) it may be implied that the biotin receptor sites of the enzymes are similar. With this implication one can assume that the same holocarboxylase synthetase may interact with the apoform of any biotin containing enzyme. It is conceivable, however, that the peripheral regions of each enzyme may exert their specificity and express it in subtle differences in the active site, and as a result each apoenzyme may require specifically its own holocarboxylase synthetase. Cazzulo et al. (1971) have shown that the apo and holo forms of pyruvate carboxylase have identical bands on disc gel electrophoresis, indicating that the differences in the active sites between the two forms are not reflected in their tertiary or quartenary structures.

Propionyl-CoA carboxylase contains 84 lysine residues, however the synthetase will recognize and bind biotin to a specific lysine within the active site (Hopner and Knappe 1965). Lane et al. (1974) have shown that in the bacterial acetyl-CoA carboxylase biotin is bound to the lysine at the end of a 14Å polypeptide chain, flipping within the active site. However, whether such chain is found in acetyl-CoA carboxylase of animal species or in other biotin containing enzymes is not known.

Hopner and Knappe (1965) could not ascertain whether biotin is incorporated into the accomplished apocarboxylase or into the subunits which, subsequent to the binding of biotin, aggregate to form a complete holoenzyme. They have further followed the process of incorporation of biotin into bacterial β-methylcrontonyl-CoA apocarboxylase and showed that the rate at which the ligand is incorporated into the receptor, de-

creases parallel to the loss of activity of the active enzyme when it is exposed to conditions which cause denaturation. It seems therefore that the changes which occur in the peripheral regions of the enzyme are transmitted to the active site and make the receptor lysine inaccessible to biotin.

ii. Cellular levels of apocarboxylases and the synthetase

Dakshinamurti and Desjardins (1969) and Jacobs, Kilburn and Majerus (1970) have demonstrated that the relative amounts of the apo and holo forms of acetyl-CoA carboxylase in adipose tissue and liver vary under different physiological conditions to different extents. These findings were confirmed by Landman and Dakshinamurti (1973). Ryder (1972) has shown that in chick liver the apoenzyme is synthesized in response to hatching and that the active holoenzyme is provoked by feeding. It is possible that even in normal animals the conversion of the apo- into holo-enzyme might be a site of regulation in addition to the protomer-polymer transition of holo- acetyl-CoA carboxylase .

Quantitative evaluation of the amounts of apocarboxylase in various biological samples shows the respective form of acetyl-CoA apocarboxylase in rat liver to be of several picomoles per mg protein (Desjardins and Dakshinamurti, 1971), and in the same order of magnitude for the respective form of pyruvate carboxylase (Madappally and Mistry, 1970). In bacteria the biotin receptor content was reported to be several orders of magnitude higher.

In animals the total amounts of each of the biotin enzymes, apo plus holo forms, remain during biotin deficiency within the same order of magnitude (Desjardins and Dakshinamurti, 1971; Chiang and Mistry,

1974). In bacteria, it was reported by Hopner and Knappe (1965) that the absolute amounts of the enzyme β-methylcrotonyl-CoA carboxylase, which is the sum of the amounts of the apo and holoforms,do not change when achrombacter is grown on biotin deficient medium. Although in animals there is no evidence to show any detectable amounts of apo form of any carboxylase when the animal is grown under normal conditions, this may not be the case with bacteria. It was reported by Hopner and Knappe (1965) that in P. shermanii about 20% of the β-methylcrotonyl-CoA carboxylase was found in the apoform despite being grown on a fully supplemented medium.

The interaction of the apocarboxylase with the synthetase was studied in a crude system or after separation and further remixing of the two components. Although investigating holocarboxylase synthesis from animal preparations is technically a difficult task, bacterial harvests low in holocarboxylase activity and with high apocarboxylase content are achieved with relative ease (Hopner and Knappe, 1965). In addition,bacteria seem to contain relatively more synthetase than animal preparations (Lane, Young and Lynen, 1964). For these reasons most studies reported on biotin incorporation have been performed with bacterial preparations.

iii. Separation and partial purification of the synthetase

The apoenzymes are separated from the synthetase using the conventional procedures for enzymic purification. Desjardins and Dakshinamurti (1971) separated the rat adipose tissue synthetase and acetyl-CoA apocarboxylase by calcium phosphate gel. Bacterial β-methylcrotonyl-CoA carboxylase was separated from the synthetase by Hopner and Knappe

(1965) by gel filtration on Sephadex G-100. Separation of the synthetase from pyruvate carboxylase was obtained on Sephadex G-200, and chromatography of the synthetase on DEAE cellulose was used effectively in a later step of purification (Cazzulo et al., 1971). While a bacterial pyruvate apocarboxylase was purified nearly to homogeneity, the highest purification for the bacterial pyruvate holocarboxylase synthetase was only 150 fold. Its molecular weight has been estimated to be 40,000 daltons (Cazzulo et al., 1971). Estimation of 50,000 to 100,000 is given for the synthetase which converts the respective form of β-methylcrotonyl-CoA apocarboxylase to the holoform (Hopner and Knappe, 1965).

iv. Multiple forms of the synthetase

The issue whether a single synthetase operates on apoforms of all carboxylases or if multiple forms of synthetases exist which are specific for each apoenzyme is still open. P. shermanii that does not possess propionyl-CoA carboxylase activity elaborates an enzyme that catalyzes the attachment of d-biotin to propionyl apocarboxylase of rat. This suggests the possibility that other apocarboxylases common to P. shermanii closely resemble propionyl-CoA apocarboxylase from other organisms in the region of the acceptor site, and that there is a single synthetase (Kosow, Huang and Lane, 1962). Rabbit liver propionyl-CoA holocarboxylase synthetase was active with bacterial β-methylcrotonyl-CoA apocarboxylase as well as with the propionyl-CoA apocarboxylase of the same origin (Siegel, Foote and Coon, 1965). However, evidence reported recently by Achuta Murthy and Mistry (1974) indicates that mitochondrial and cytosolic synthetases may be different.

v. Mechanism of biotin incorporation by the synthetase

The holocarboxylase synthetase has been shown to catalyze the formation of biotinylhydroxamate (Valloton et al. 1964). Formation of this product requires b-AMP as an intermediate. Coon (1964), Lane (1964) and Lynen (1963) and coworkers have reported that b-AMP could replace biotin and ATP. This provided the proof that the initial step in the mechanism of biotin incorporation into the apoenzyme is

$$\text{I} \quad \text{biotin} + \text{ATP} \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} \text{biotinyl-AMP} + PP_i$$

the next step in the incorporation is

$$\text{II} \quad \text{b-AMP} + \text{apocarboxylase} \longrightarrow \text{holocarboxylase} + \text{AMP}$$

Although it is conceivable that the two reactions are catalyzed by a single enzyme, it is also possible that two enzymes are involved. Coon et al. (1965) raised the possibility that the two reactions may be catalyzed by two different subunits. The reaction of b-AMP with hydroxylamine to form biotinyl hydroxamate indicated that it exists as a true intermediate. Coon et al. (1963), have suggested that, in order to prevent b-AMP from hydrolysis, the compound is strongly bound to the enzyme during the catalytic process.

vi. Conditions and requirements for the incorporation

Conditions used by various investigators for the assay of the holocarboxylase synthetase differ considerably. Holocarboxylase synthetase can be assayed by two methods; one in which the incorporation of radioactive biotin into the apocarboxylases is monitored, and a second in which the increase in activity after the conversion of the apoform into the holoform is assayed. Both methods are commonly used. The first

method is more sensitive although it is less specific. By the second method the assay can be directed towards a specific enzyme but it seems to be less sensitive. Madappally and Mistry (1970) showed a close correlation between biotin incorporation into a crude system and the increase in activity of pyruvate carboxylase. More conceivable, however, is the view that the correlation is not that simple, since in a crude system biotin is incorporated into several other enzymes. Desjardins and Dakshinamurti (1971) have shown considerable deviations between the amount of biotin incorporated and the increase in acetyl-CoA holocarboxylase activity in crude preparations. They determined the affinities for ATP and biotin during the synthesis of acetyl-CoA holocarboxylase to be 1.1×10^{-3}M and about 1.1×10^{-6}M, respectively. Coon et al. (1965) determined the corresponding affinities for ATP and biotin for propionyl-CoA carboxylase to be 1.3×10^{-6}M and 4.7×10^{-9}M, respectively. Such differences in various reports could be ascribed to the different assay conditions as well as the nature of the enzyme studied. Lane et al. (1962) have reported that Mg^{++} is inhibitory for the biotin incorporation. Although this is inconsistent with the formation of b-AMP as an intermediate, this finding was substantiated by Desjardins and Dakshinamurti (1971) and Ryder (1972). Mg^{++} is used, however, in most biotin incorporating systems, and it is conceivable that the concentration of endogenous Mg^{++} is sufficient for the reaction. Considerable differences in the pH profiles of the biotin incorporation and in the incubation temperatures have been reported. Lane et al. (1962) reported that optimal incorporation of biotin into propionyl-CoA apocarboxylase occurs at 30°C, and at 37°C one or both enzymes are denatured rapidly. This was shown by Desjardins

and Dakshinamurti (1971) to occur similarly during the synthesis of acetyl-CoA holocarboxylase. In most systems,however,the higher temperature is still used. Cazzulo et al. (1969, 1971) reported that the incorporation of biotin into bacterial carboxylase is affected by acetyl-CoA and aspartate in allosteric fashion. Although it is implied that these ligands effect the synthetase, their effect on the apocarboxylase which is the substrate rather than the synthetase itself cannot be excluded.

There are indications that other nucleotides can replace the requirement for ATP although the incorporation is not as efficient (Achuta Murthy and Mistry, 1974; Kosow, Huang and Lane, 1962). Achuta Murthy and Mistry (1974) reported that ADP and AMP are inhibitors of the synthetase. Of the various analogs of biotin tested none, including L-biotin, could replace D-biotin in the synthetase reaction, indicating the strong specificity of the synthetase towards its substrates (Lane, Rominger and Young, 1964).

The purpose of this thesis was to investigate the interaction between acetyl-CoA holocarboxylase synthetase and acetyl-CoA apocarboxylase. Among the aspects which were attempted within this context are the isolation of acetyl-CoA apocarboxylase free of the holoenzyme, isolation of the holocarboxylase synthetase, the methodology for the assay of this enzyme and the involvement of biotin in the aggregation of the holoenzyme.

MATERIALS

D-biotin (carbonyl-^{14}C) 46 mCi/mmole was obtained from Amersham Searle Corporation.

Sodium pyrophosphate $^{32}p^{32}p_i$, 1Ci/mmole, and sodium bicarbonate, $NaH^{14}CO_3$, 10 mCi/mmole, were purchased from New England Nuclear Corp.

2,5-Diphenyloxazole (PPO) and 1,4-bis 2(5-pheny)-oxazolyl benzene (POPOP) were purchased from Beckman Instruments Inc.

Sephadex derivatives; G-10, G-25, G-100 and G-200, DEAE-Sephadex, Sepharose 4B and CNBr activated Sepharose are the products of Pharmacia Fine Chemicals.

Avidin, egg white, vitamins and other nutrients of which the biotin deficient diet is composed were obtained from Nutritional Biochemicals Corp.

Biocytin was a gift of Merck Sharp and Dohme Research Laboratories, Rahway, N.J.

Ferric chloride, hydroxylamine and trichloroacetic acid were purchased from J.T. Baker Chemical Company, Philipsburg, N.J.

Carbowax is a product of Union Carbide Corporation.

Nonlabelled sodium pyrophosphate and d-biotin, and histones, polylysines, HABA, bentonite, calcium phosphate and all other chemicals were obtained from Sigma Chemical Company (St. Louis, Mo.).

METHODS

a) Obtaining biotin deficient animals

The major source for acetyl-CoA apocarboxylase throughout this research project was the adipose tissue of Holtzman male rats in which biotin deficiency was produced by dietary means. The rats, 40-50g in weight when received, were fed ad libitum with a biotin-deficient diet originally used by Dakshinamurti and Chea-Tan (1968) with a composition given in Tables 1a and 1b. Symptoms of the deficiency syndrome appear after 4 to 5 weeks of feeding on the diet, and the apoenzyme was usually extracted at this stage. On feeding the diet for 1 to 4 more weeks the symptoms of the deficiency syndrome persisted; however,since at this stage the adipose tissue became depleted or virtually disappeared, and since the mortality rate at this period increased drastically, obtaining more apocarboxylase by keeping the animals on the diet for time periods longer than 5 weeks was impossible. Animals were sacrificed by decapitation and the epididymal fat pads were removed immediately and rinsed with saline, after which the adipose tissue was homogenized in the appropriate buffer.

b) Counting radioactive samples

Counting of radioactive samples was done by mixing 0.05 to .5 ml of the analyzed solution with 15 ml of a scintillation liquid which was made of 0.25% PPO, 0.009% POPOP, and 31% of ethanol in toluene. The advantage of this type of scintillator over scintillation liquids based only on toluene or dioxane is its relatively high capacity for water; up to 0.5 ml. Counting was performed in Beckman LS 250 spectrometer using the appropriate isosets.

Table 1a: Composition of the Biotin-Deficient Diet

Component	Grams per Kilogram Diet
Egg white powder	250.0
Dextrose	648.5
Salt Mix[a]	40.0
Corn Oil	50.0
Vitamin mix[b]	10.0
Choline chloride	1.5

[a]Salt mix #446 from Nutritional Biochemicals Corp.

[b]See Table 1b.

Table 1b: Composition of The Vitamin Mixture

Component	Grams per Kilogram Vitamin Mix
Ascorbic acid	99.2
p-Aminobenzoic acid	11.0
Biotin	0.0
Calcium pantothenate	6.6
Folic acid	4.6
Inositol	11.0
Menadione	5.0
Niacin	10.0
Pyridoxine HCl	2.2
Riboflavin	2.2
Thiamine	10.2
DL-α-Tocopherol acetate	11.0
Vitamine A palmitate[a]	9.0
Vitamine B_{12}[b]	3.0
Vitamin D_2[c]	0.45
Dextrose	814.55

[a]Vitamine A palmitate = 250,000 IU/g.

[b]Vitamin B_{12} is a 0.1% triturate in mannitol.

[c]Vitamin D_2 = 500,000 IU/g.

c) Determination of protein concentrations

i. Enzymic preparations with protein concentration in range of 1-20 mg/ml were assayed by the Biuret method (Rendina, 1971) with bovine serum albumin as the standard. 1 ml of sample was mixed with 4 ml of Biuret reagent (0.15% $CuSO_4 \cdot 5H_2O$ and 0.6% sodium potassium tartrate in aqueous solution containing 40 ml per liter of saturated NaOH). Full coloration developed within 30 minutes after which absorbance was read at 540 nm using Beckman DB-G spectrophotometer.

ii. Enzymic preparations with a protein concentration up to 1 mg/ml were determined by the method of Lowry et al. (1951). To 1 ml of sample were added five ml of copper reagent (2% Na_2CO_3, 0.01% $CuSO_4 \cdot 5H_2O$, and 0.02% potassium tartrate). The tubes were allowed to stand for 12 minutes after which 0.5 ml of Folin reagent was added. After 30 more minutes the tubes were read at 540 nm using Beckman DB-G spectrophotometer.

d) Assay for malate dehydrogenase (Kitto, 1969)

The reaction mixture for malate dehydrogenase contains 3.3 mM malate, 0.41 mM NAD (nicotine amide adenine dinucleotide) and enzyme preparation in 90 mM sodium glycinate buffer, pH 10.0, in a final volume of 3 ml. The reaction was started by addition of either malate or the enzyme. Readings of optical density at 340 nm were made against a blank containing all the assay components except NAD. The activity was determined from the initial slope of a paper recording of this assay.

e) Assay for acetyl-CoA carboxylase

The enzymic preparation to be assayed was preincubated in 60 mM Tris, 8 mM $MgCl_2$, 5 mM sodium citrate, .6 mg/ml BSA, .1 mM EDTA, 3 mM GSH,

for 1 hour, after which aliquots of 100 to 300 µliter were transferred to be assayed. The assay medium contained 60 mM Tris, 3 mM GSH, 2 mM ATP, 8 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ with specific activity of 0.2 mCi/mmole and .1 mM EDTA, pH 7.5, in a total volume of 500 µliter. After 2 to 10 minutes incubation at 37°C the assay was terminated with 100 µliters of 6N HCl. Aliquots of 200 µliter were transferred into scintillation vials which were then placed in vacuum-oven at 60°C for 2 hours. 10-15 ml of scintillation liquid was added and the vials were counted in Beckman LS-250 spectrometer.

CHAPTER 1: ISOLATION OF ACETYL-CoA APOCARBOXYLASE

In the course of this study we required enzymic preparations which would contain acetyl-CoA apocarboxylase without any contamination of acetyl-CoA holocarboxylase. The apo-acetyl CoA carboxylase component in earlier work (Desjardins and Dakshinamurti, 1971, 1971a; Jacobs, Kilburn and Majerus, 1970) has always been calculated by difference in view of the fact that the holo- and apoenzymes are not separated by the usual methods of enzyme purification. Thus, Cazzulo et al. (1971) have demonstrated that the holo- and apoenzymes give identical bands on disc gel electrophoresis, which makes it difficult to separate them on the basis of differences in charge or molecular weight. We have therefore attempted to isolate acetyl-CoA apocarboxylase free of endogenous holoenzyme by using the affinity chromatography technique in which avidin which is bound to the Sepharose 4B matrix will bind biotin-containing proteins which are applied to this column.

Experimental

a) Preparation of Sephadex G-25 eluates of rat liver and epididymal adipose tissue cytosols: Biotin deficient rats were used as a tissue source for the apocarboxylase. Male Holtzman rats (40-50g) were placed on a biotin deficient diet for 6 weeks, and sacrificed by decapitation. The liver and epididymal fat pads respectively were removed immediately, rinsed twice in cold saline and then homogenized in 3 volumes of buffer A (0.1 M Tris (Cl^-) pH 7.5, 0.15M KCl, 0.1 mM EDTA and 5 mM mercaptoethanol). The crude homogenates were centrifuged successively at 10,000 x g for 10 min and 100,000 x g for 60 min. The final supernatants were

passed through Sephadex G-25 columns and eluted with the homogenizing buffer. The eluates were placed in dialysis bags and covered with carbowax to concentrate the protein.

b) Preparation of Sepharose-avidin column: The affinity column was prepared essentially as recommended by the manufacturer (Pharmacia Fine Chemicals, technical bulletin, 1972). CNBr-activated Sepharose 4B (0.7 g) was allowed to swell in 10 ml of 1.0 mM HCl for 10 min and then filtered on sintered glass and washed respectively with 40 ml of 1.0 mM HCl, and 50 ml of the bicarbonate solution containing 10 mg avidin (12.0 units/mg), mixed for 4 hours at room temperature and filtered. The filtrate did not show any absorbance at 280 mμ. The Sepharose resin was again suspended in 5 ml of the bicarbonate buffer containing 200 mg bovine serum albumin and allowed to mix overnight. It was filtered and washed with the bicarbonate buffer until no trace of protein could be detected in the washings. The coupled Sepharose was placed in a 0.8 x 2.0 cm column. Sephadex G-25 eluates prepared from rat liver (20 mg protein in 1 ml) or adipose tissue (5 mg protein in 1 ml) were applied on the Sepharose-avidin column and eluted with buffer A. The eluate, 5 ml, was concentrated to its original volume using carbowax.

c) Preparation of in vivo labelled biotin-protein: Biotin deficient rats, 5 weeks on the avidin diet, were given daily intraperitoneal injections of 10 μci of D-(carbonyl ^{14}C)-biotin (25 mci/mmole) in saline for two days. They were killed by decapitation 16 hours following the last injection. The livers were quickly removed, rinsed in cold saline and homogenized in 3 volumes of medium containing 0.24 M sucrose, 0.025 M

KCl, 0.001 M $MgCl_2$ in 0.05 M Tris HCl, pH 7.6. The crude homogenate was centrifuged at 1000 x g for 10 min. The post-nuclear supernatant was sonicated with a Bronwell Biosonic probe (20 kc delivering 120 w) in 4 steps of 10 sec each with 50 sec ice-cooling interval between consecutive steps. The sonicate was centrifuged at 105,000×g for 60 min. The supernatant was placed on a Sephadex G-25 column and eluted with the buffer A. The tubes with radioactivity were pooled and fractionated with ammonium sulfate. The 0 - 60% ammonium sulfate fraction was collected, dissolved in buffer A and dialyzed against this buffer for 4 hours. This fraction which contained 93% of the supernatant radioactivity was concentrated to a small volume using carbowax.

d) Binding and release of biotin-protein: d-(carbonyl ^{14}C) -biotin-protein (∿9000 dpm in 10 mg) was applied to the Sepharose-avidin column and eluted with 5 ml of 0.2 M ammonium carbonate solution. Fractions of 12 drops each were collected and tested for radioactivity. The column was then eluted with 5 ml of 6 M guanidine hydrochloride, pH 1.5. The eluate was neutralized with 6 M NaOH. Aliquots of 0.2 ml were taken to be counted for radioactivity while the rest was dialyzed against distilled water for 16 hours. The material in the dialysis bag was collected and its radioactivity determined.

e) Separation of L-malate dehydrogenase and biotin-protein on the Sepharose-avidin column: The efficiency of separation of a biotin protein from other enzyme proteins was tested by applying a mixture of malate dehydrogenase (0.45 units) and d-(carbonyl ^{14}C)-biotin-protein (∿9000 dpm in 10 mg) on the Sepharose-avidin column and eluting it with 5 ml of 0.2 M ammonium carbonate. Radioactivity and L-malate dehydrogenase

activity were assayed in the eluate. The column was further eluted with 5 ml of 6 M guanidine hydrochloride (pH 1.5) to release the biotin-protein. The eluate was neutralized with 6 M NaOH, dialyzed for 16 hours against distilled water and the protein-bound radioactivity determined.

f) Assay for acetyl-CoA apocarboxylase: The assay was performed according to Desjardins and Dakshinamurti (1969). It is composed of three incubation periods. The first incubation with citrate was designed to bring the apo and holoforms into an homogeneous state of aggregation. In the second incubation the enzyme was incubated in the presence of biotin and ATP. The third incubation measured the increase in acetyl-CoA holocarboxylase activity arising from the conversion of the apoenzyme to the active holoenzyme form.

The gel filtered cytosol was preincubated for 60 minutes at 37°C, in the following medium: 60 mM Tris (Cl^-) (pH 7.5 at 37°C), 3 mM GSH, 8 mM $MgCl_2$, 0.1 mM EDTA, 5 mM potassium citrate, 0.6 mg per ml bovine serum albumin. The preincubated enzyme was incubated further for 4 hours at 30°C, pH 7.5, in the presence of 4 mM ATP and 82 µM d-biotin in a final volume of 1.0 ml. The complete reaction mixture contained 48 mM Tris (Cl^-) (pH 7.5), 2.7 mM GSH, 6.4 mM $MgCl_2$, 0.08 mM EDTA, 4.0 mM potassium citrate, 0.48 mg per ml bovine serum albumin, 4 mM ATP, and 82 µM d-biotin. At the end of the four hours incubation, aliquots (100 µliter) were removed and the acetyl-CoA carboxylase activity was assayed. The assay was performed in a medium containing 2 mM acetyl-CoA, 2 mM ATP, 8 mM Mg^{++}, 10 mM $NaH^{14}CO_3$ (0.2 µCi/mmole), 0.1 mM EDTA, 5 mM mercaptoethanol, 0.6 mg/ml BSA, in a final volume of 0.5 ml. After incubation for

2 minutes the assay was terminated with 100 µliter 6N NCl. Aliquots (0.2 ml) were transferred into vials and heated at 80°C in an oven under partial vacuum to dryness. Two hundred µliter of water was added to each vial followed by 15 ml of scintillation fluid. The vials were then counted. Acetyl-CoA holocarboxylase synthesis is expressed as milliunits of increased enzyme activity per mg protein in the complete system, after correction for controls which consisted of the complete system minus biotin.

Results

The specificity of the avidin-biotin binding is well known. This has been made use of in the preparation of Sepharose-avidin for affinity chromatography. Results presented in Table 2 show that protein-bound biotin binds to avidin of the column and is not eluted by 0.2 M ammonium carbonate. However, 6 M guanidine hydrochloride pH 1.5 releases the biotin protein from the avidin-biotin complex. In this process, the biotin is maintained in the protein bound state. These observations are confirmed and enlarged in the results presented in Table 3 which show that when a mixture of proteins is applied on the Sepharose-avidin column the biotin-proteins are retained on the column and those that do not contain biotin could be separated from the biotin-proteins. Thus, the malate dehydrogenase applied on the column is recovered completely in the ammonium carbonate eluate.

This principle of affinity binding of biotin-protein to the avidin on the Sepharose matrix has been applied to the separation of the biotin containing acetyl-CoA holocarboxylase from its apocarboxylase which lacks the biotin prosthetic group. The results are presented in Table 4. A

Table 2: Chromatography of d-[carbonyl ^{14}C]-biotin proteins on a Sepharose-avidin column

Treatment	Radioactivity dpm
^{14}C-Biotin protein applied on the Sepharose-avidin column	9045
0.2 M ammonium carbonate eluate	Nil
6 M guanidine HCl eluate	9140
Dialysed guanidine HCl eluate	8918

^{14}C-labelled proteins, prepared from biotin deficient rats, were passed through Sepharose-avidin column (0.8 x 2.0 cm). The column was then eluted with 0.2 M ammonium carbonate and 6 M guanidine - HCl respectively. The guanidine - HCl eluent was dialyzed against water and radioactivity was determined in aliquots of the dialyzate.

Table 3: Chromatography of malate dehydrogenase and d-[carbonyl ^{14}C] -biotin labelled proteins on a Sepharose-avidin column

Treatment	^{14}C-biotin protein dpm	L-Malate dehydrogenase units
Amount applied on the column	8740	0.45
0.2 M ammonium carbonate eluate	Nil	0.45
6 M guanidine HCl eluate	8610	Nil

A mixture of malate dehydrogenase and ^{14}C-biotin labelled proteins was applied to a Sepharose-avidin column. The column was eluted with 0.2 M ammonium carbonate and 6 M guanidine-HCl. Malate dehydrogenase and radioactivity were determined in the eluents.

Table 4: Isolation of acetyl-CoA apocarboxylase by affinity chromatography on a Sepharose-avidin column

Fraction	Acetyl-CoA holocarboxylase activity (d.p.m. $NaH^{14}CO_3$ incorporated/mg protein)					
	In the absence of biotin in incubation medium			In the presence of biotin in incubation medium		
	EXPT.1	EXPT.2	EXPT.3	EXPT.1	EXPT.2	EXPT.3
Epididymal fat pad						
Sephadex G-25 eluate	524	598	1684	2018	2414	5522
Sepharose-avidin eluate	18	16	24	1206	1348	4072
Liver						
Sephadex G-25 eluate	1952	2036	4908	2868	2914	7244
Sepharose-avidin eluate	24	26	25	990	1060	2136

Sephadex G-25 gel filtered cytosols of liver and adipose tissue respectively of biotin deficient rats, were passed through a Sepharose-avidin column and eluted with buffer A and fractions of 0.3 ml collected. Aliquots of the samples applied on the column and of the eluate were assayed respectively for acetyl-CoA apo and holocarboxylase activities.

Sephadex G-25 eluate of epididymal fat pad contains both acetyl-CoA holo and apo-carboxylases. When this is passed through the Sepharose-avidin column the holocarboxylase is effectively retained on the column. Thus, the apocarboxylase can be separated from the holoenzyme in crude or purified enzyme preparations. The results also indicate that in the biotin deficient rat the liver contains more of the holoenzyme and less of the apoenzyme, whereas the opposite is true of the adipose tissue. This is in keeping with earlier observations (Dakshinamurti and Desjardins, 1969) and suggests a preferential depletion of biotin from the adipose tissue of the biotin-deficient rat.

Discussion

The feasibility of affinity chromatography technique for any biochemical system has to be considered in the light of several pre-requisites defined by Cuatrecasas and Anfinsen (1971, 1971a).

(1) Strong binding between the bound compound and its adsorbant;

(2) Steric considerations require a spacer group between the two components (Stevenson and Landman, 1971);

(3) The binding compound has to be stable particularly under pH and ionic strength required during its binding to the matrix;

(4) The binding ligand should contain enough amino groups to make it available for binding.

Avidin-biotin and avidin-biotin-containing-protein complexes meet these requirements favourably. The binding between avidin and biotin is very strong. Avidin is a remarkably stable protein and having strongly basic isoelectric point (Fraenkel-Conrat, 1951) it possesses many amino moieties available for binding. The steric hindrance of the

matrix is overcome by the fact that avidin is composed of four subunits, each capable of binding biotin independently (Toms and Green, 1973).

Sepharose-avidin affinity column was used for the study of the kinetics of the binding between biotin and avidin (Toms and Green, 1973), as well as for the binding of nonspecific biotin-containing peptides (Bodanszki and Bodanszki, 1970) on a Sepharose-avidin column and the biotin containing subunit of acetyl-CoA carboxylase (Lane et al. 1974). Affinity chromatography on biocytin-Sepharose columns was reported by Cuatrecasas and Wilchek (1968) for a single step purification of avidin. By passing a mixture of acetyl-CoA holocarboxylase acetyl-CoA apo-carboxylase and acetyl-CoA holocarboxylase synthetase through a column of Sepharose 4B into which avidin was attached,the holocarboxylase remained tightly bound to the column while the other components passed through unaffected. The apocarboxylase obtained in this way was virtually free of holocarboxylase and was used for further study of its properties in its isolated form and its interaction with the holocarboxylase synthetase.

Certain bacterial strains give high yields of apoenzyme with only traces of the holoenzyme. However, in mammalian species, even highly deficient animals contain substantial amounts of holoenzyme which is an avoidable interference in the holoenzyme-synthetase system. The Sepharose-avidin column described here is of much value for the preparation of a pure substrate for the holoenzyme-synthetase reaction. It is also useful in the assay of the relative amounts of apo- and holoenzymes under a variety of conditions (Dakshinamurti and Desjardins, 1969). The Sepharose-avidin column has a finite capacity for binding with the biotin containing holoenzyme and could be used repeatedly only to this extent.

However, a new column with excess of avidin is highly efficient in the separation of apo and holo biotin enzymes.

Achuta Murthy and Mistry (1974) have also attempted to remove endogenous acetyl-CoA holocarboxylase activity. They masked the initial holocarboxylase activity by incubating the system with avidin. This protein inhibited irreversibly all active biotin containing holoenzymes and was in turn inactivated by addition of an excess of biotin. The biotin was further removed by dialysis. Such a procedure although inhibiting initial holocarboxylase activity did not remove it physically and did not eliminate the possibility of further interference between the inhibited holoenzyme and the apocarboxylase. In addition this procedure introduced avidin and biotin as interfering compounds into the system. The use of affinity column seems to overcome such problems in an elegant fashion.

CHAPTER 2: ACETYL-CoA CARBOXYLASE AND THE ROLE OF BIOTIN IN HOLOCARBOXYLASE AGGREGATION

The catalytic activity of acetyl-CoA carboxylase appears to be intimately associated with its state of aggregation in that the enzyme is capable of oscillating between the polymeric form of high activity and the protomeric form of low activity. Vagelos and coworkers (Martin and Vagelos, 1962; Vagelos et al. 1963) were the first to show that the stimulatory effect of citrate on fatty acid synthesis is through activation of acetyl-CoA carboxylase. This was shown to be associated with an increase in the sedimentation velocity of the enzyme (Vagelos et al. 1963; Matsuhashi et al. 1964; Numa et al. 1965). The conditions favouring respectively the aggregation and disaggregation of acetyl-CoA carboxylase have been listed by Gregolin et al. (1972). In view of the correlation between citrate-mediated catalytic activity and citrate-mediated molecular aggregation, Lane and coworkers (Gregolin et al. 1972; Moss and Lane, 1972) have suggested that changes in cellular citrate concentrations control the rate of the carboxylase reaction. It was therefore pertinent to investigate the role of the biotin prosthetic group in the transition of the protomeric to the polymeric form of the enzyme.

The question whether the biotin prosthetic group has a role in the aggregation of the holoenzyme and whether the apocarboxylase is capable of aggregation has a direct implication as to the nature of the interaction between the holocarboxylase synthetase and the apocarboxylase, since the holocarboxylase synthetase may interact with the aggregated

and disaggregated forms of the apoenzyme in completely different fashions. Assuming that acetyl-CoA apocarboxylase could aggregate, it is possible that the protomers adjacent to the one interacting with the synthetase could sterically hinder the interaction. On the other hand, it is possible that aggregation of acetyl-CoA apocarboxylase could actually enhance the incorporation of biotin into the apoenzyme by the synthetase, since the synthetase could then introduce biotin into the filaments sequentially, starting from one end of the filament and finishing at the other end. If one now assumes that the apocarboxylase lacks the ability to aggregate, the biotin receptor sites on the apocarboxylase may be openly available for the synthetase. However, in this case it would be necessary that both proteins which are found in the cytosol in low concentrations are in close proximity to each other probably within the same microcompartment. Desjardins and Dakshinamurti (1968) have predicted that the apocarboxylase aggregates to a lesser degree than the holocarboxylase. It seems that, for the understanding of the interaction between acetyl-CoA apocarboxylase and the holocarboxylase synthetase and further insight into the biotin incorporation mechanism, a study of the ability of the apocarboxylase to aggregate is a prerequisite.

Experimental

a) Assay for the conversion of acetyl-CoA apocarboxylase to holoenzyme in crude enzyme preparations: Holocarboxylase synthetase activity was measured in gel filtered cytosols of rat adipose tissue. In experiments where the acetyl-CoA apocarboxylase and holoenzyme synthetase were separated, the assay was performed by remixing of these components.

An aliquot of the enzyme preparation was incubated for 4 hours

at 30°C in a medium containing the following in a volume of 0.95 ml: 60 mM, Tris pH 7.5, 3.6 mM $MgCl_2$; 9.9 mM potassium citrate 0.72 mg/ml BSA; 4 mM ATP and 82 µM biotin. At the end of this period acetyl-CoA and $NaH^{14}CO_3$ (1 μC_i) were added in final concentrations of 0.2 mM and 10 mM respectively and further incubated for 10 min. The reaction was terminated by 0.05 ml 6N HCl and the acid stable radioactivity determined.

b) Preparation of holoenzyme synthetase free of holoenzyme: Cytosolic fractions were prepared respectively from the adipose tissue of biotin deficient or normal rats. Four ml of this fraction were passed through a Sepharose-avidin column. The protein eluate was concentrated with carbowax to a concentration of 14 mg/ml protein, dialyzed against a buffer containing 0.025 potassium phosphate, 10 mM β-mercaptoethanol, 0.1 mM EDTA, pH 7.0, for 8 hours, and the volume adjusted to 10 ml with the same buffer. To this preparation 405 mg calcium phosphate (14% solid, 5.1 mg gel per mg protein) were added and dispersed by gentle stirring until homogeneous It was stirred for an additional 30 minutes, and then centrifuged at 1000 x g for 15 minutes. The supernatant (S_1) was retained and acetyl-CoA carboxylase activity was measured before and after conversion of any apoenzyme to the holoenzyme form as described in the previous section. The pellet was extracted once with 6 ml of buffer containing 0.12 M potassium phosphate, pH 7.6, 10 mM potassium citrate, and 10 mM β-mercaptoethanol, and twice more with the same buffer but containing in addition 0.2M NaCl. The three last extracts were pooled and concentrated on carbowax. The concentrated extracts will be referred to as S_2.

c) Separation of protomeric and polymeric acetyl-CoA carboxylase on a Sepharose 4B column: The epididymal adipose tissue (8.4 g) was homogenized in 3 volumes of buffer A (0.1 M Tris, pH 7.5, 0.15 M KCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol) and the high speed supernatant (105,000 x g, 60 min) was prepared. The high speed supernatant was passed through a Sephadex G-25 column (2.5 x 45 cm) equilibrated with the buffer A. The fractions containing the major portion of the protein were pooled and brought to 45% ammonium sulfate saturation. After standing for 30 min the suspension was centrifuged for 45 min. at 13,000 x g. The precipitated protein was divided into two fractions, one of which was dissolved in buffer B (0.1 M Tris, pH 8.2; 0.15 KCl; 0.1 mM EDTA; 5 mM β-mercaptoethanol) and the other in buffer C (0.1 M potassium phosphate, pH 6.9; 0.1 mM EDTA β-mercaptoethanol, 10 mM citrate). The protein fraction which was dissolved in buffer C, was incubated at 37°C for 1 hour, and was subjected to column chromatography in a Sepharose 4B column (2.5 x 90 cm) equilibrated with buffer C. The protein fraction which was dissolved in buffer B was subjected to column chromatography on a Sepharose 4B column (2.5 x 90 cm) which was equilibrated with buffer B. The columns were then eluted with the buffer with which they were equilibrated respectively, and 3.0 ml fractions were collected.

d) Preparation of apocarboxylase: The 0-45% ammonium sulfate fraction of the gel filtered cytosols of biotin deficient rat adipose tissue was prepared as described in Section c, dissolved in 3 ml of buffer B to give a protein concentration of 28 mg per ml, passed through a Sepharose-avidin column (0.8 x 2.0 cm) and eluted with buffer B in fractions of 0.5 ml. The eluate (12 ml) was pooled and concentrated to

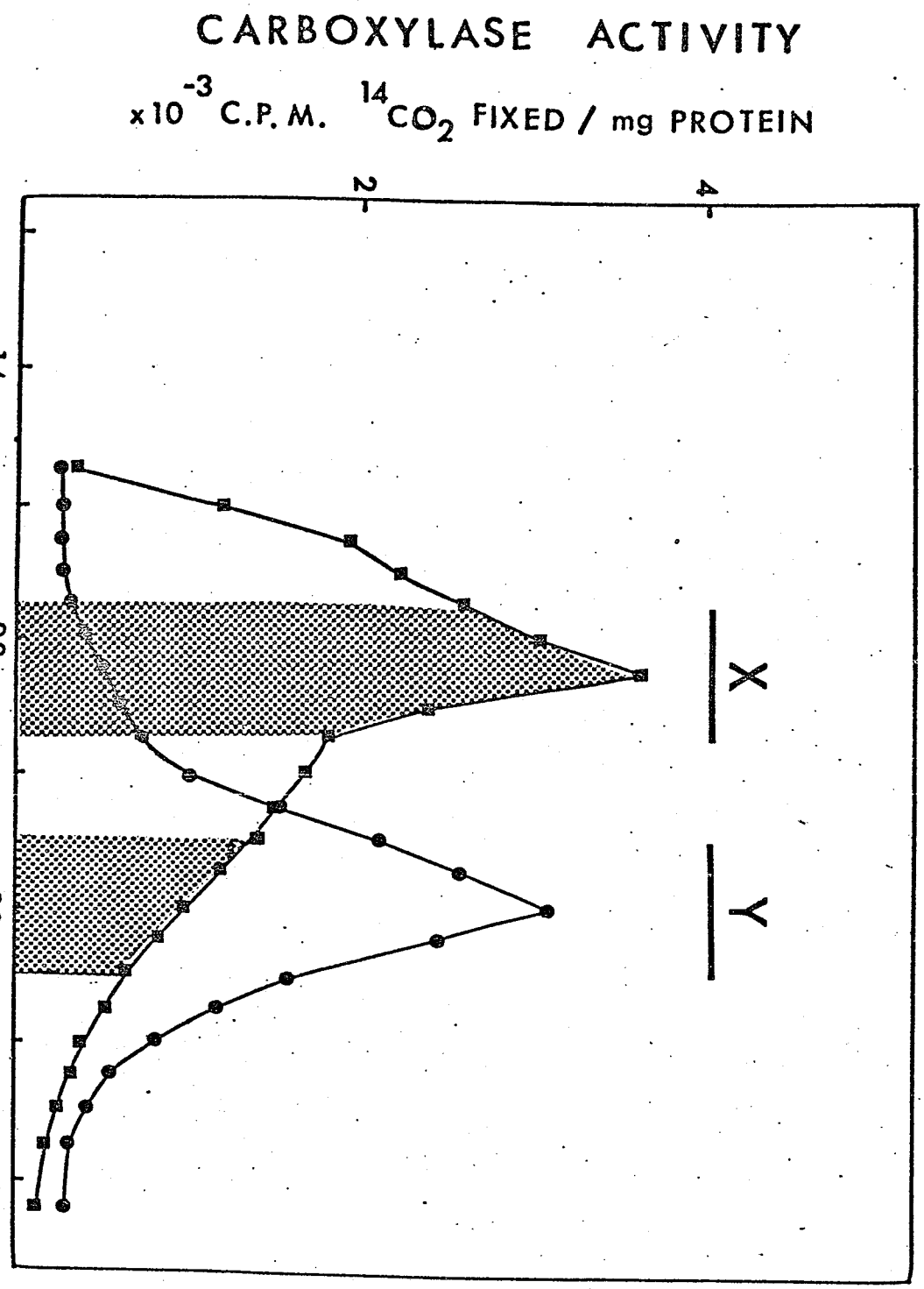
4 ml using carbowax. The apoenzyme preparation had a protein concentration of 17 mg/ml.

e) Sepharose 4B chromatography of the apoenzyme preparation: A Sepharose 4B column (2.5 x 85 cm) was equilibrated with buffer B. The column was standardized by chromatographing acetyl-CoA carboxylase under protomeric and polymeric conditions,respectively. The apoenzyme preparation described in Section d was chromatographed under conditions favoring the protomeric form (buffer B), and the fractions (3 ml) were collected. Acetyl-CoA carboxylase activity was measured in these fractions in the presence of biotin and holocarboxylase synthetase. Fractions corresponding to the position of the apoenzyme (Figure 1) were pooled and concentrated, using carbowax, to a protein concentration of 3.1 mg per ml.

f) Preparation of ^{14}C-biotin labelled acetyl-CoA holocarboxylase enzyme: The apoenzyme preparation was incubated for 4 hours at 30°C in the following medium: 60 mM Tris, pH 7.5, 4 mM ATP, 8 mM $MgCl_2$, 81 µM D(+) biotin [carbonyl-^{14}C,] 18.1 x 10^6 d.p.m./88 nmoles, apoenzyme 12.4 mg, and holocarboxylase synthetase 1.6 mg. The incubation mixture was then dialyzed against 4 liters of buffer B (favouring the protomeric form of the enzyme) for 8 hours. It was then gel filtered on a Sephadex G-10 column (0.9 x 50 cm) with buffer B. The protein eluate was brought to 50% ammonium sulfate saturation and the precipitate was dissolved in 1 ml buffer B. The enzyme preparation was then chromatographed on a Sepharose 4B column with buffer B as described in Section e. Aliquots were taken from the fractions and radioactivity was measured. The radioactivity was found to eluate in fractions 28-32. These fractions were

Figure 1: Elution of acetyl-CoA apo and holocarboxylase under conditions favoring the protomeric and polymeric forms

A column (2.5 x 85 cm) of Sepharose 4B was used and 3 ml fractions of the respective eluent were collected. All fractions were assayed for acetyl-CoA carboxylase as described in the Experimental section. (—■—), Enzyme preparation under protomeric condition (X) was eluted with buffer B, (—●—), Enzyme preparation under polymeric conditions (Y) was eluted with buffer C.



SEPHAROSE 4B CHROMATOGRAPHY OF ACETYL CoA CARBOXYLASE

pooled. Acetyl-CoA carboxylase (12.4 mg) in its protomeric form, prepared as described in Section c was added to the pooled fractions and dialyzed against 4 liters of buffer C for 8 hours at room temperature. These conditions favour aggregation of acetyl-CoA carboxylase to its polymeric form. The dialyzate was concentrated to 1 ml using carbowax, applied to a Sepharose 4B column and eluted with buffer C. Fractions of 3 ml were collected and radioactivity measured.

g) Selective sedimentation of acetyl-CoA holo and apocarboxylases: Liver and adipose tissue were obtained from biotin deficient rats. Gel filtered Sephadex G-25 cytosols of the liver and adipose tissue were prepared as described in Section c. They were brought to 50% saturation with solid ammonium sulfate, and the precipitated proteins were collected respectively by centrifugation. Aliquots of the pellets were dissolved in buffer B or buffer C. The enzyme fractions (10 ml) in either buffer B or C were centrifuged at 105,000 x g for 4 hours in Beckman Model L2 ultracentrifuge, 40.1 rotor, in 12 ml polycarbonate tubes. The supernatants were siphoned off in 3 ml fractions and the pellets were resuspended in 1 ml buffer C and assayed for acetyl-CoA apo and holocarboxylase activities.

Results

a) Acetyl-CoA holocarboxylase synthetase activity: Desjardins and Dakshinamurti (1969) have described a method for the measurement of acetyl-CoA holocarboxylase synthetase activity in crude enzyme preparations. This method comprises of three incubations; a preincubation period with citrate designed to aggregate the acetyl-CoA apo and holocarboxylase, a four hour incubation period with biotin and ATP for the conversion of the

apoenzyme to the holoenzyme form, and a third and final incubation for measuring the increase in acetyl-CoA holocarboxylase activity which occurred due to the conversion of the apoenzyme to the holoenzyme during the second incubation.

An attempt was made to investigate whether the first preincubation in the presence of citrate described by Desjardins and Dakshinmurti (1969) had any significant effect on holoenzyme formation. Holoenzyme formation was measured under three sets of conditions as described in Table 5. Condition i) consisted of incubating the reaction mixture for 4 hours with citrate and ATP but without biotin, and basically very little acetyl-CoA holocarboxylase activity was detected. Condition ii) consisted of incubating the reaction mixture for 4 hours with citrate, ATP and biotin which resulted in holoenzyme formation. Condition iii) consisted of incubating the reaction mixture for 3 1/2 hours with biotin and ATP. Citrate was added for the last 1/2 hour. In case where the system was incubated first with citrate (condition ii), the apocarboxylase may aggregate and the synthetase will use its substrate in this form. When preincubated with biotin first (condition iii), the synthetase may operate on the apocarboxylase in its protomeric unaggregated form. Due to the steric differences between the apocarboxylase in the aggregated and nonaggregated states,it can be assumed that differences in the rate at which biotin is incorporated might be observed. Table 5 indicates that changing the sequence of incubations did not have any apparent effect. Since the 4 hours incubation at 30°C is optimal for activation of acetyl-CoA holocarboxylase and since the one hour preincubation with citrate which was designed to bring the apo and holoenzyme into homogeneous state

Table 5: The effect of preincubation sequence on holocarboxylase synthetase

Conditions	Preincubation sequence	Total holocarboxylase activity mu/mg	Holocarboxylase synthesized
i	A preparation containing ACAP and ACHS was incubated without biotin for 4 hours.	0.05	
ii	A preparation containing ACAP and ACHS was incubated in presence of biotin, ATP, and citrate for 4 hours.	8.48	8.43
iii	A preparation containing ACAP and ACHS was incubated in presence of biotin and ATP for 3 1/2 hours and citrate added during the last 1/2 hour of incubation.	9.21	9.16

Sephadex G-25 gel filtered cytosol of adipose tissue of biotin deficient rats was incubated under three different conditions. The rate of conversion of the apoform to the holoenzyme by acetyl-CoA holocarboxylase synthetase was assayed.

did not have apparent effect on the activity of the synthetase, this incubation was omitted from the assay, and the assay was modified as described in Experimental Section a. It includes only an incubation with biotin and ATP followed by a shorter incubation with citrate.

b) Partial separation of acetyl-CoA holocarboxylase synthetase and acetyl-CoA apocarboxylase: This is based on the method devised by Desjardins and Dakshinamurti (1969), in which high speed supernatant of adipose tissue of biotin deficient animals was fractionated on calcium phosphate gel. After the supernatant was adsorbed on the gel, it was eluted with media of increasing salt concentrations. By applying enzyme preparation from normal rat adipose tissue on the Sepharose avidin column, the holocarboxylase activity was eliminated and the synthetase was partially isolated (Table 6).

c) Elution of apocarboxylase on Sepharose 4B under different conditions: Molecular sieving using chromatography on a Sepharose 4B column was used to separate the protomeric (M.W., 4×10^5 daltons) form of acetyl-CoA holocarboxylase from the polymeric form of the enzyme (M.W. $< 4 \times 10^6$ daltons). The rats used in this experiment were in a moderate state of biotin deficiency (4 weeks on the avidin diet) so that the adipose tissue contained both the holo and apoenzymes. Chromatography of the enzyme at a pH of 8.2 and in the absence of tricarboxylic acid effectors like citrate caused the enzyme to elute as a single peak (X) (Figure 1). Under conditions favouring aggregation, pH 6.9 and 10 mM citrate, there was a shift in the elution profile with the appearance of a large molecular weight peak (Y) (Figure 1). The enzyme preparation from biotin deficient rats was fractionated on Sepharose 4B under conditions

Table 6: Fractionation of holocarboxylase synthetase free of holoenzyme with calcium phosphate gel

Fraction	Normal rat adipose tissue			Biotin-deficient rat adipose tissue		
	Holo-carboxylase activity (-biotin)	Holo + Apo-carboxylase activity (+biotin)	Apocar-boxylase activity	Holo-carboxylase activity (-biotin)	Holo + Apo-carboxylase activity (+biotin)	Apocar-boxylase activity
	c.p.m. $^{14}CO_2$ fixed/mg protein			c.p.m. $^{14}CO_2$ fixed/mg protein		
Gel filtered cytosol before passing through Sepharose-avidin column	2271	2022	Nil	599	1606	607
S_1 - Elution by 0.025M phosphate buffer	71	90	Nil	10	479	460
S_2 - Elution by 0.12 M phosphate buffer	167	146	Nil	23	44	21
S_1+S_2 - Combination of S_1 and S_2	252	212	Nil	39	608	469

Preparations of adipose tissue derived from normal and biotin deficient rats respectively were passed through Sepharose-avidin column and then were subjected to calcium phosphate extraction. After elution with various buffers the fractions obtained were assayed for acetyl-CoA holocarboxylase and acetyl-CoA apocarboxylase respectively.

favouring aggregation, and fractions corresponding to locations of the protomeric and polymeric peaks were individually pooled. Acetyl-CoA carboxylase activity was assayed in the pooled fractions X and Y both in the presence and absence respectively of biotin and holocarboxylase synthetase. The amount of apocarboxylase activity was calculated from the difference. The results are given in Table 7. The fact that in the presence of biotin and holocarboxylase synthetase the carboxylase activity of pooled fraction Y increased almost nine fold with no increase at all in fraction X indicates that the apoenzyme is eluted from the column almost entirely along with the protomeric form of acetyl-CoA holocarboxylase. Thus, under conditions which favour holoenzyme aggregation, the apoenzyme does not aggregate.

To confirm this, acetyl-CoA apocarboxylase completely free from the holoenzyme was prepared by Sepharose-avidin affinity chromatography from the epididymal adipose tissue of rats in an advanced state of biotin deficiency (6 weeks on the avidin diet). It has been shown earlier (Desjardins and Dakshinamurti, 1969, 1970) that the apocarboxylase is not depleted under these conditions. The apoenzyme was converted *in vitro* into the holoenzyme by incorporating d-[carbonyl-^{14}C]-biotin. The radioactive holoenzyme, under conditions favouring the protomeric form, was eluted from the Sepharose 4B column precisely at the position of the apoenzyme; however, under conditions favouring the polymeric form the peak corresponding to the labelled holoenzyme shifted to a higher molecular weight region (Figure 2). This shift in elution profile indicates that the transition from the protomeric into polymeric form is possibly only in the presence of the prosthetic biotinyl group.

Table 7: Separation of apo and holocarboxylase on Sepharose-4B

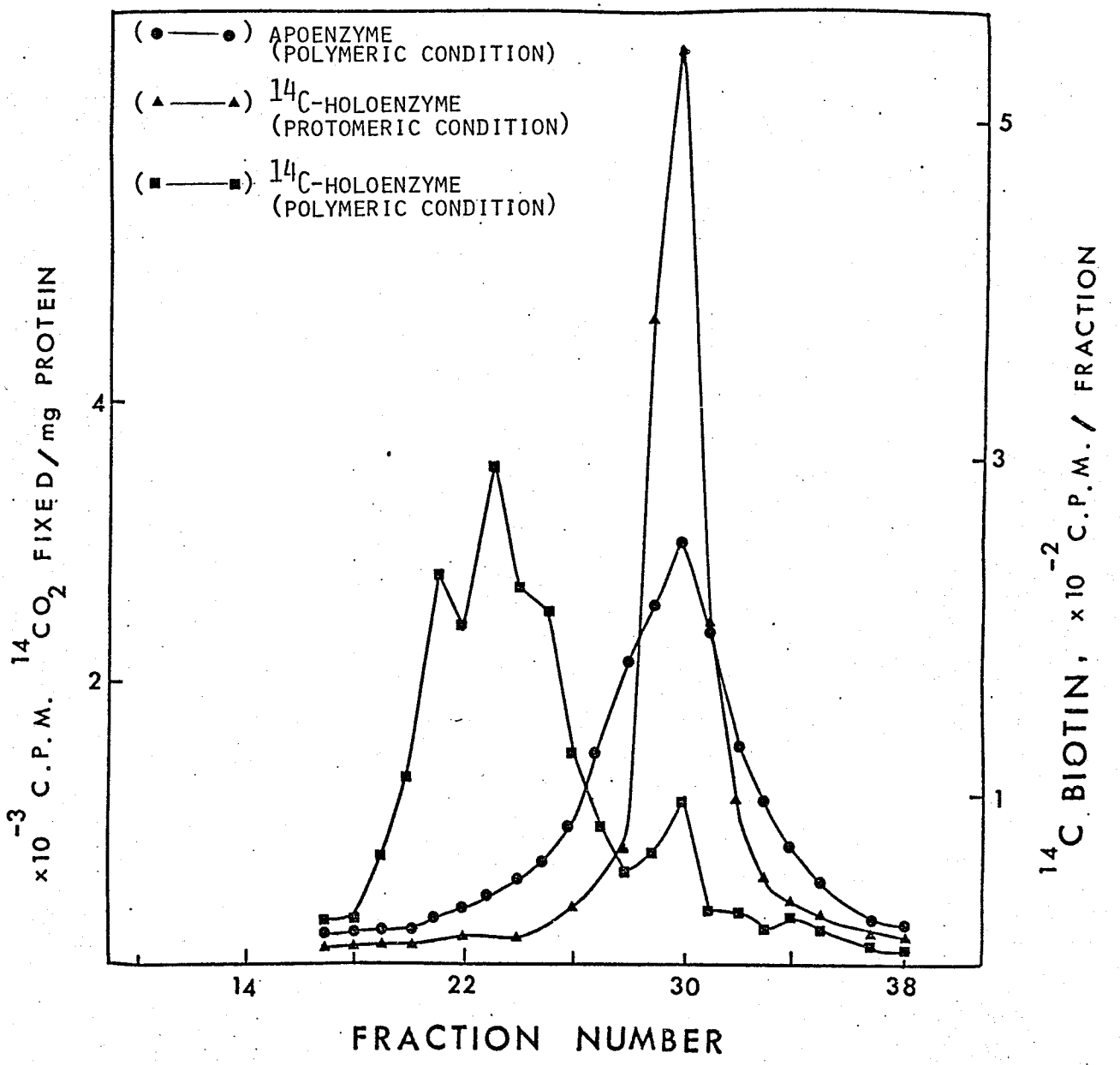
Contents of Incubation Medium	Holocarboxylase Activity (- Biotin) mU/mg protein	Holo + Apocarboxylase Activity (+ Biotin) mU/mg protein	Net Apo-Carboxylase Activity mU/mg protein
Fraction X	2.72	2.83	0.11
Fraction Y	0.72	0.72	Nil
Holocarboxy-lase Synthe-tase (ACHS)	Nil	Nil	Nil
Fraction X + ACHS	3.05	3.15	0.10
Fraction Y + ACHS	0.75	6.24	5.49

Fractions of the Sepharose-4B eluate which corresponded to peaks X and Y in Figure 1, and which were eluted from the column under conditions favouring the protomeric and polymeric forms respectively, were mixed with partially purified holocarboxylase synthetase and assayed for acetyl-CoA apocarboxylase and holocarboxylase respectively.

Figure 2: Elution of labelled acetyl-CoA carboxylase on Sepharose 4B column

A column (2.5 x 85 cm) of Sepharose 4B was used. (-▲-), apoenzyme preparation, under polymeric conditions, was eluted with buffer C and 3 ml fractions collected and assayed for acetyl-CoA carboxylase in presence of biotin and holocarboxylase synthetase; (-●-), ^{14}C-labelled holoenzyme, under protomeric conditions, was eluted with buffer B. 3 ml fractions were collected and radioactivity determined; (-■-), ^{14}C-labelled holoenzyme, under polymeric conditions, was eluted with buffer C. 3 ml fractions were collected and radioactivity determined.

SEPHAROSE 4B CHROMATOGRAPHY OF ^{14}C-LABELED ACETYL CoA CARBOXYLASE



d) Selective sedimentation of apocarboxylase and holocarboxylase mixture: Krebs et al. (1964) have used direct sedimentation in the ultracentrifuge in their purification of phosphorylase b kinase. Crude preparations containing acetyl-CoA carboxylase were prepared, incubated and centrifuged under conditions which favoured either aggregation or disaggregation. Table 8 demonstrates that under conditions favouring aggregation the enzyme sedimented faster than when centrifuged under conditions favouring the protomeric form and more enzymic activity appeared in the pellet. This principle of selective sedimentation of the high molecular weight species was applied to a gel filtered cytosol of adipose tissue from biotin-deficient rats. Under conditions favouring aggregation, the pellet showed a four fold enrichment of the holocarboxylase activity while the apocarboxylase activity was increased very slightly (Table 9),indicating again lack of ability of the apocarboxylase to aggregate.

Discussion

The role of tricarboxylic acid effectors in activation of acetyl-CoA carboxylase by favouring the transition of the protomeric to the polymeric enzyme is now well established. The results presented here show that acetyl-CoA apocarboxylase is not capable of aggregating to the polymeric state. This and the report of Moss and Lane (1972) that avidin cannot bind to acetyl-CoA holocarboxylase in the aggregated form show that the biotinyl prosthetic group is the site of the effector-mediated protomer-polymer transition of the enzyme.

Earlier work by Dakshinamurti and Desjardins, (1969) and by Jacobs et al. (1970, 1970a) have demonstrated that the catalytic efficiency

Table 8: Selective sedimentation of acetyl-CoA carboxylase by a preparative ultracentrifugation

Fraction	Sedimentation under conditions favouring aggregation		Sedimentation under conditions favouring disaggregation	
	*ACC activity c.p.m. of $^{14}CO_2$ fixed	% of total c.p.m. content	*ACC activity c.p.m. of $^{14}CO_2$ fixed	% of total c.p.m. content
Top 1/4 of tube	357	11.5	646	20.8
Fraction 2	472	15.2	562	18.1
Fraction 3	448	14.6	679	29.0
Bottom 1/4 of tube	699	22.6	1054	34.0
Pellet	1109	35.9	157	5.1

Gel filtered cytosol of normal rat liver was run on a preparative ultracentrifuge under conditions favouring aggregation* or the protomeric form**. After the run the contents of the centrifuge tubes were siphoned off and fractionated into four fractions. The pellets were dissolved in 1 ml buffer A. All fractions were than assayed for acetyl-CoA carboxylase activity.

Table 9: Selective ultracentrifugation of acetyl-CoA holo and apocarboxylase

Fraction	Holocarboxylase activity No biotin in preincubation medium mU/mg	Holo+Apocarboxylase activity. Biotin present in incubation medium mU/mg	Apocarboxylase synthesized mU/mg
Enzyme preparation before ultracentrifuge run	11.4	21.8	10.4
Supernatant after ultracentrifugation	8.1	19.2	11.1
Pellet after ultracentrifugation	46.0	60.2	14.2

Gel filtered cytosol from biotin deficient rat adipose tissue was subjected to ultracentrifugation under conditions favouring the protomeric and polymeric forms respectively. After the run the supernatant was siphoned off. The pellet and the supernatant were assayed for acetyl-CoA holocarboxylase and apocarboxylase respectively.

of acetyl-CoA carboxylase is regulated by the ratio of holoenzyme to apoenzyme in tissues of biotin-deficient rats. Ryder (1971) has shown that in chick liver the apoenzyme is synthesized in response to hatching and that the active holoenzyme synthesis is provoked by feeding. It is possible that in normal animals, under certain conditions, the conversion of the apo to holoenzyme might be a site of regulation of the expression of the fatty acid synthetic pathway.

Acetyl-CoA apo and holocarboxylases are not separated by the usual methods of enzyme purification. The results presented here show that under conditions favouring holocarboxylase aggregation, the apocarboxylase can essentially be separated from the polymeric holocarboxylase. This, in association with the Sepharose-avidin affinity chromatography offers an effective method of preparing large amounts of acetyl-CoA apocarboxylase.

CHAPTER 3: METHODOLOGY FOR STUDYING HOLOCARBOXYLASE SYNTHETASE

Acetyl-CoA carboxylase synthetase catalyzes the covalent attachment of biotin to acetyl-CoA apocarboxylase to form an active holoenzyme. Under normal dietary conditions biotin is in excess and the acetyl-CoA carboxylase is found only as the holoenzyme. In order to investigate acetyl-CoA holocarboxylase synthetase, an adequate source of apocarboxylase is required. The study of the acetyl-CoA holocarboxylase synthetase is partly limited by technical difficulties in obtaining the apocarboxylase. The only adequate source of mammalian apoenzyme to date has been the adipose tissue of biotin deficient rats (Dakshinamurti and Desjardins, 1971). Needless to say that there is a restriction in the quantity of tissue available. The requirement for an adequate source of the synthetase and apocarboxylase make the technical problems involved with this system considerably complex. Attempts were made to improve the assay for acetyl-CoA holocarboxylase synthetase and to develop an assay which could be used routinely and would not require acetyl-CoA apocarboxylase.

One approach which was pursued was the use of lysine and lysine derivatives as substrates for acetyl-CoA holocarboxylase synthetase. This enzyme has been claimed to be nonspecific with regard to its substrate in the sense that it would react with apocarboxylase from a variety of species (Moss and Lane, 1971). Since biotin is known to be incorporated into the terminal lysine of a 14Å polypeptide chain flipping freely within the carboxylase active site (Guchhait et al. 1974), it was of interest to see if the lack of specificity could be pursued to

such extent that the biotin could be incorporated into lysine and poly-lysine or into lysine rich histones.

Another attempt to achieve a simplified assay for the synthetase was to utilize one of the partial reactions which occur while biotin is being incorporated into its receptors. The mechanism of this incorportion is described as follows:

$$\text{biotin} + \text{ATP} \rightleftharpoons \text{biotinyl} - \text{AMP} + PP_i$$

$$\text{biotinyl} - \text{AMP} + \text{apocarboxylase} \longrightarrow \text{holocarboxylase} + \text{AMP}$$

and apparently allows a biotin dependent ATP-PP_i exchange. Although such an exchange was reported by Lane and coworkers (1964) for the partially purified synthetase it has not been used as a routine assay for the enzyme.

Experimental

a) ^{14}C-d-biotin incorporation into proteins: The assay was performed in Eppendorf tubes. The complete assay mixture contained 100 mM Tris, pH 7.4, 4 mM ATP, 4 mM Mg^{++}, 2.5 mM β-mercaptoethanol, 4 mμM biotin (2.5×10^4 c.p.m./mμM of ^{14}C-d-biotin) and enzyme in concentration as specified in the particular experiment. The reaction mixture was incubated for 30 minutes at 37°C and then terminated by the addition of 50 μliter of 100% trichloroacetic acid. An additional 0.5 ml of 10% trichloroacetic acid was added and the tubes were centrifuged for 10 seconds in an Eppendorf 3200 centrifuge. The supernatant was siphoned off with a pasteur pipet and the pellet was washed 5 times each with 1 ml 10% trichloroacetic acid. The washed pellet was suspended in 0.2 ml 1N KOH, transferred into liquid scintillation vials and counted after the addition of a scintillation solution.

b) Biotin dependent ATP-$^{32}P^{32}P_i$ exchange: The assay system contained 0.05 M Tris, pH 7.4, 4 mM ATP, 4 mM Mg^{+2}, 0.4 mM $^{32}P^{32}P_i$ (0.25 μC_i), and enzyme in concentration as indicated in each particular experiment, in a final volume of 0.25 ml. The incubation was performed in Eppendorf tubes, inserted in an Eppendorf thermostat 3401 kept at 37°C. The incubation was terminated by the addition of 25 µliter of 6N HCl followed by the addition of 200 µliter of 0.05N HCl. The tubes were shaken for 1 minute on an Eppendorf Rotary Shaker 3300, after which they were centrifuged for 2 minutes on Eppendorf Centrifuge 3200. The supernatant was transferred into another Eppendorf tube, the precipitate was washed with 200 µliter 0.05N HCl and the supernatants were combined. Norit A (0.5 ml of 100 mg/ml solution) was added to each tube. The tubes were shaken on a vortex and placed on an Eppendorf shaker for 30 minutes. The tubes were centrifuged for 2 minutes and the supernatants were removed with a Pasteur pipet. The charcoal pellet which contained the adsorbed ATP was washed with 1 ml of 10 mM PP_i in 0.05N HCl nine times to remove the labelled PP_i. After the final wash, 1 ml of 6N HCl was added and the tubes were mixed and inserted in the Thermostat 3401, at a temperature of 95°C for 30 minutes. The tubes were then centrifuged for 2 minutes and the supernatant was transferred quantitatively into a liquid scintillation vial. The vials were placed in a Beckman LS 250 Spectrometer and counted by Cerenkov radiation using the wide tritium channel or a wide carbon 14 channel respectively (Clausen, 1968).

Results

a) Biotin incorporation into artificial receptors: The detection of acetyl-CoA holocarboxylase synthetase and the apocarboxylase by the

incorporation of biotin into TCA precipitable material has been described by Dakshinamurti and Desjardins (1969) and Ryder (1971). The assay was initially tested by following the time course of biotin incorporation into gel filtered cytosol of biotin deficient rat adipose tissue (Figure 3). Subsequently the same procedure was applied in an attempt to incorporate biotin into polylysine of varying lysine contents and several histone fractions all of which possess ε-amino group of lysine accessible for interaction with biotin (results not presented). Following the washing of the free biotin, the radioactivity of the TCA precipitated material remained within the background level and indicated that apparently biotin was not incorporated into any of the substances used.

The attempt to bind biotin and lysine by an enzyme found in a rat liver preparation was approached in a different way. A column of Sephadex G-10 (1.5 x 85cm) was equilibrated with Tris buffer 0.05M, pH 7.4, and then was calibrated with biocytin. The eluent of this run was analyzed by the radioisotope dilution assay as described in Chapter 5, and revealed two peaks which corresponded to that of the biocytin and biotin (the biotin originates probably from the degradation of biocytin during prolonged storage) (Figure 4). After the incubations of rat liver gel filtered cytosol with labelled biotin and lysine under conditions described in Table 10 the reaction mixture was passed through the Sephadex G-10 column. The covalent binding of biotin and lysine would have resulted in the formation of labelled biocytin which could be identified by its location as determined during the calibration with the authentic biocytin. Since in none of the incubations (Table 10) could radioactivity

Figure 3: Time course of biotin incorporation into liver or adipose tissue cytosols of biotin deficient rats

Gel filtered cytosol of liver or adipose tissue of biotin deficient rats were assayed for biotin incorporation into TCA precipitable material. The assay mixture contained 100 mM Tris, pH 7.4, 4 mM ATP, 4 mM Mg^{++} and 4 m M biotin (2.5×10^4 cpm/mM ^{14}C-d-biotin). The assay was terminated with TCA, the unbound biotin was washed off and the ^{14}C-biotin incorporated into the protein was counted. (—●—) liver preparation. (—▲—) adipose tissue preparation.

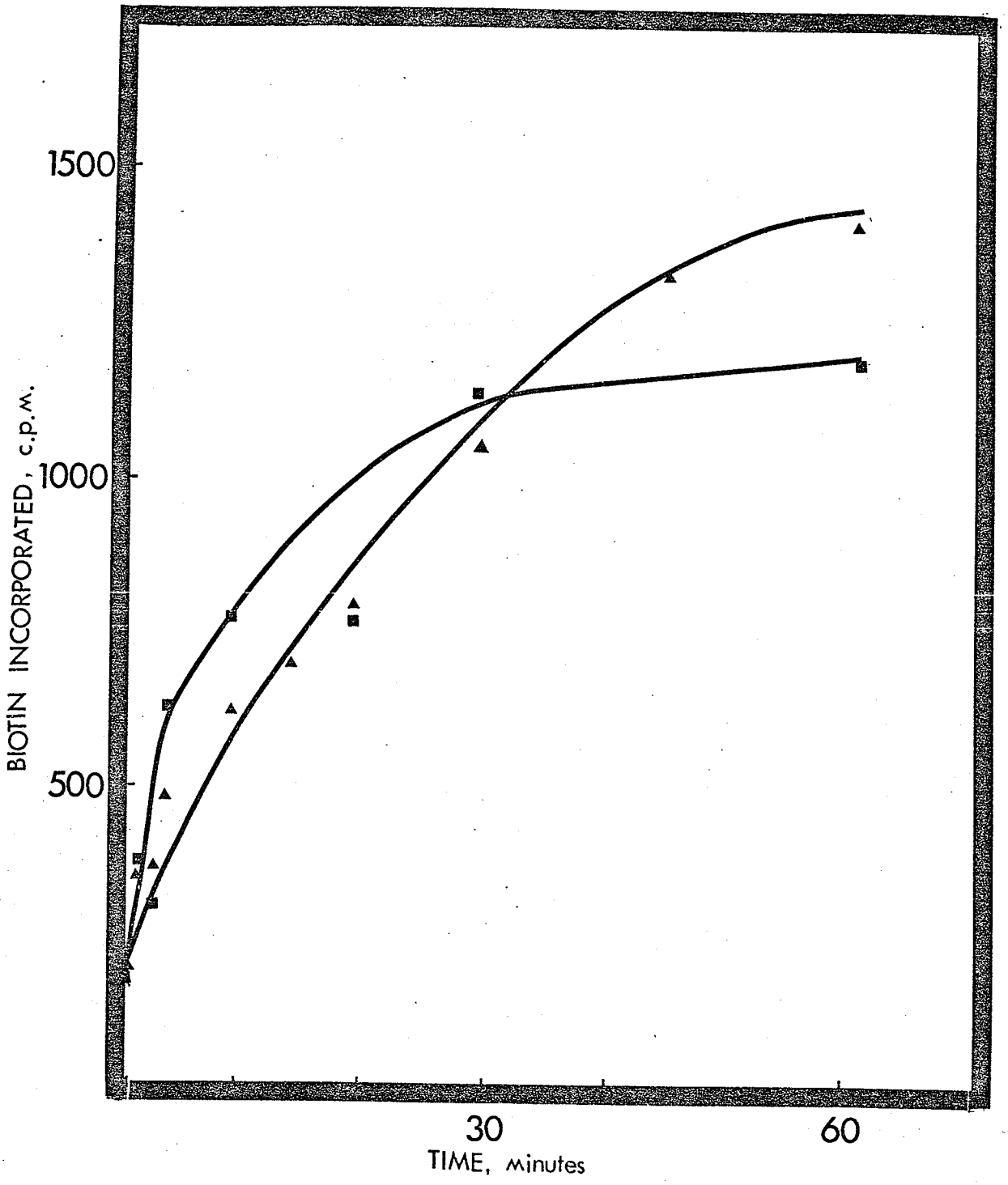

1500
1000
500
BIOTIN INCORPORATED, c.p.m.
30
60
TIME, minutes

Figure 4: Elution profile of biotin and biocytin on Sephadex G-10 column

10 µg of biocytin were suspended in 1 ml phosphate buffer 0.1 M, pH 7.4, applied on Sephadex G-10 column 1.5 x 80 cm and then eluted with the same buffer. Fractions of 2.9 ml were collected of which 10 µliter were taken to be assayed for biocytin and biotin by the radioisotope dilution assay (Chapter 5).

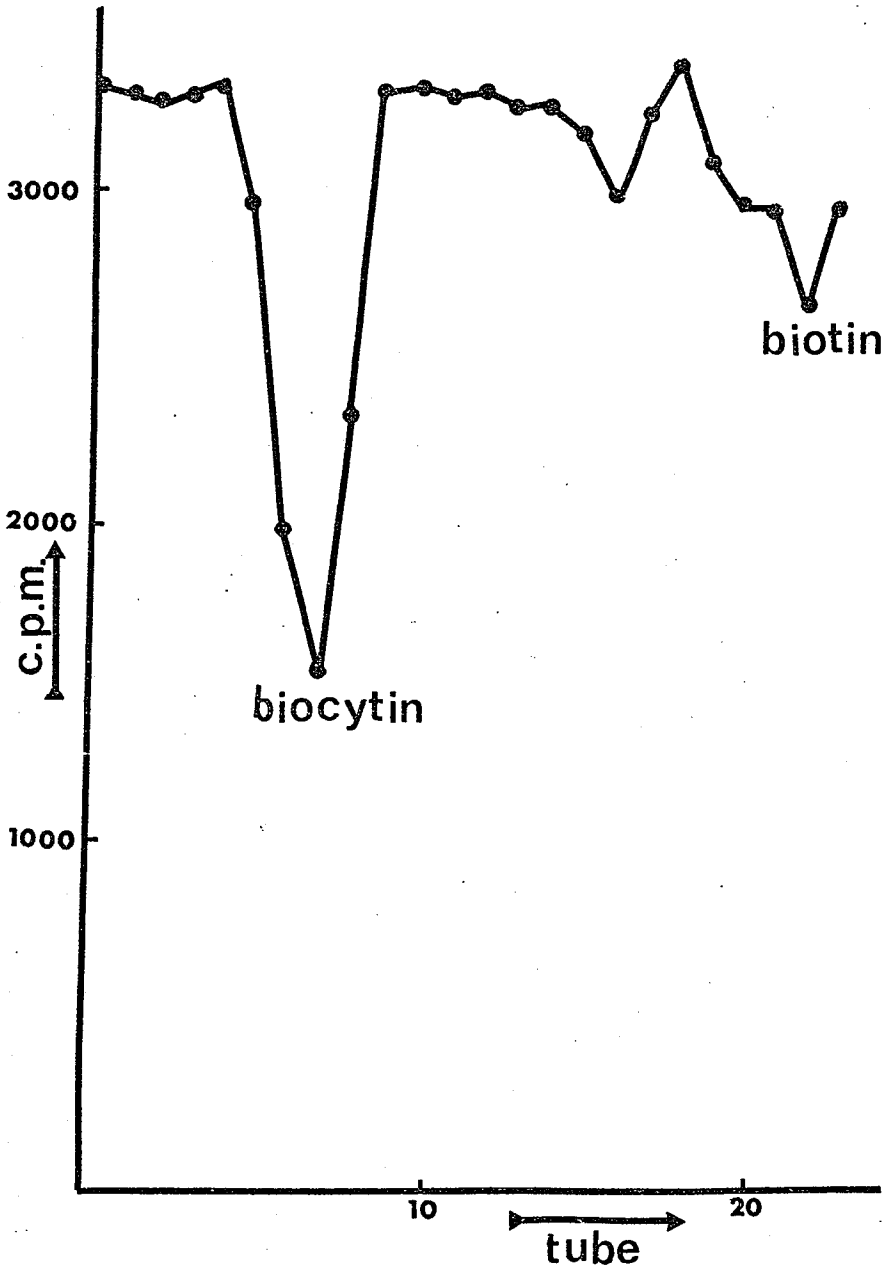

3000
2000
1000
c.p.m.
biocytin
biotin
10
20
tube

Table 10: Varying conditions for the binding of biotin and lysine

Biotin	ATP, mM	Mg^{+2}, mM	Lysine, mM	Incubation Period
40 µM, 0.8 μC_i	4	3	10	2 min.
40 µM, 0.8 μC_i	4	3	10	30 min.
40 µM, 0.8 μC_i	4	3	10	4 hours
40 µM, 0.8 μC_i	4	-	10	1 hour
40 µM, 0.8 μC_i	4	3	1	1 hour
40 µM, 0.8 μC_i	4	3	10	1 hour
40 µM, 0.8 μC_i	4	3	100	1 hour

Biotin and lysine of varying concentrations were incubated with ATP and Mg^{++} under conditions applicable for the holocarboxylase synthetase. At the end of the incubation period they were applied on a Sephadex G-10 and eluted under conditions similar to that applied for biotin and biocytin standards.

Table 11: Selective adsorption of ATP on Norit A

Component \ Channel	Wide ^{3}H, C.P.M.	$^{32}P^{32}P_i$ C.P.M.	Wide ^{14}C, C.P.M.
Labelled ATP added to the system	53542	180	53921
Labelled ATP recovered after washing and heating 60 min. at 100°C (6N HCl)	50865	173	51764
Labelled PP_i added to the system	57461	190	58661
Labelled PP_i recovered after washing and heating 60 min. at 100°C (6N HCl)	2580	16	2607
Blank vials	38	2	43

Identical amounts of labelled $^{32}P^{32}P_i$ or ATP (0.26 μC_i) were adsorbed respectively on Norit A. The adsorbant was then washed to remove the unbound ligand and then subjected to heating for 60 minutes, at 100°C, in presence of 1 ml 6N HCl. The charcoal was then cooled, centrifuged and the supernatant counted.

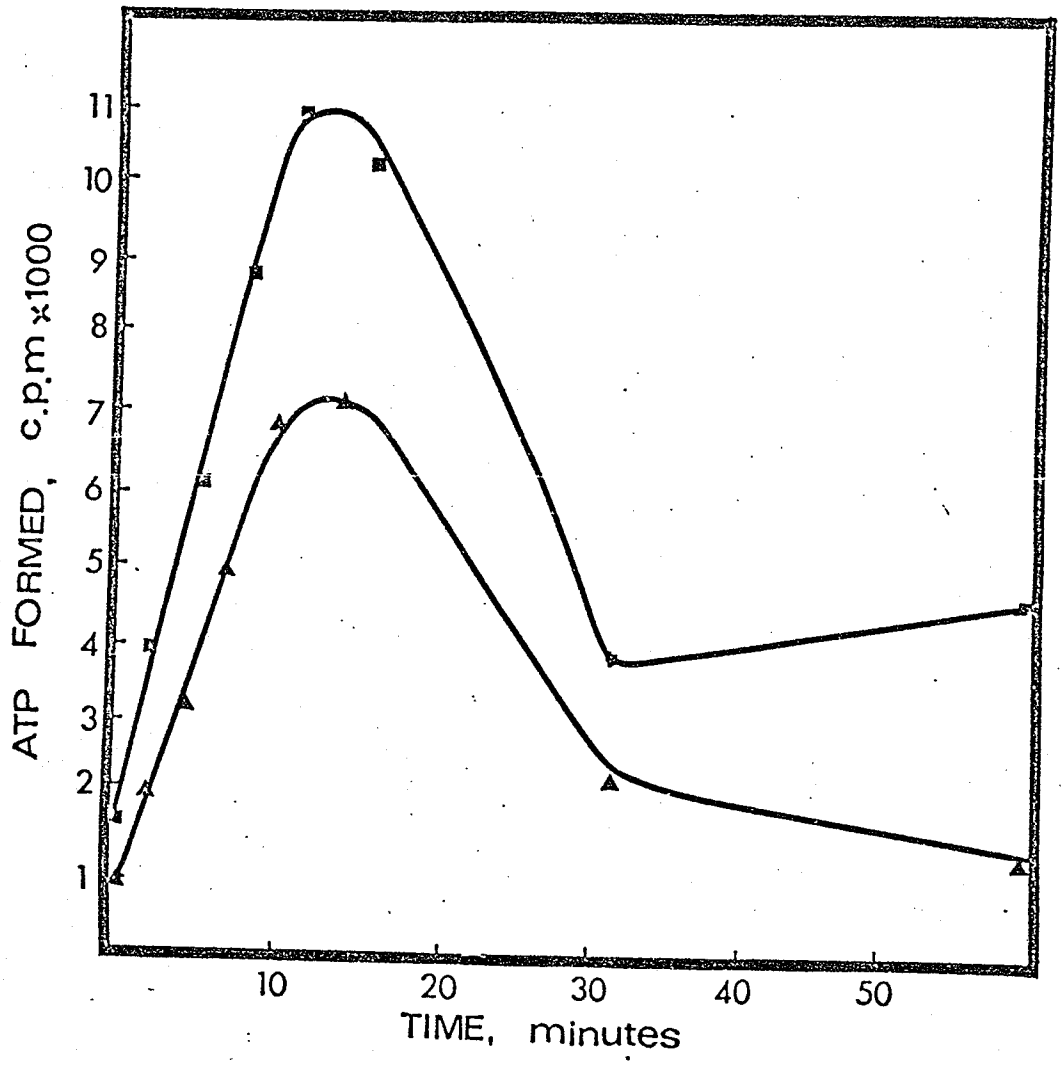
Table 12: Efficiency of counting based on Cerenkov radiation

Channel / Medium	Isoset for tritium C.P.M.	Isoset of ^{32}P C.P.M.	Isoset for ^{14}C C.P.M.
Scintillation liquid	29500	544840	447580
1 ml 6N HCl	240133	276	240857
15 ml 6N HCl	258560	1610	262016

Identical amounts of $^{32}P^{32}P_i$ (0.26 μC_i) were distributed into scintillation vials. 1 ml or 15 ml of water or scintillator were added respectively to the vials which were then counted in different channels with the appropriate isosets.

Figure 5: Time course of ATP-PP_i exchange

Porcine liver gel filtered cytosol was assayed for ATP-PP_i exchange. The assay mixture contained 0.05 M Tris, pH 7.4, 4 mM ATP, 4 mM Mg^{+2} and 0.4 mM $^{32}P^{32}P_i$ ($0.25C_i$) in a final volume of 0.25 ml. The assay was carried out for various periods after which it was terminated by the addition of 6N HCl. The ATP was then adsorbed on Norit A and hydrolyzed by 6N HCl. The $^{32}P_i$ released as a result was counted in water. (—■—) biotin dependent exchange. (—▲—) biotin independent exchange.


ATP FORMED, c.p.m x1000
11
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
TIME, minutes

be detected at this location, it can be concluded that apparently liver preparations do not contain an enzyme capable of binding biotin and lysine and obviously the holocarboxylase synthetase binds biotin only to lysine which is sterically oriented into an appropriate position by the surrounding residues of the apoenzyme.

b) ATP - $^{32}P^{32}P$ exchange: The mechanism by which biotin is incorporated into the apocarboxylase implied that the synthetase is capable of catalyzing biotin dependent PP_i - ATP exchange, and in fact such exchange was demonstrated for bacterial holocarboxylase synthetase by Lane and coworkers (1964). However, before this reaction could be adapted into a routine assay for the synthetase some technical aspects had to be resolved. Adsorption of ATP formed during the exchange, by the charcoal, was found to be quantitative; approximately 95% of an aliquot of radioactive ATP which was processed under conditions of the actual assay procedure were found to be adsorbed by Norit A (Table 11). The total ATP adsorbed was found subsequently to be hydrolyzed by 6N HCl and released from the Norit A into the supernatant (Table 11).

The ATP is hydrolyzed by suspending the charcoal adsorbed ATP in 1 ml 6N HCl, and the PP_i which is released is recovered in the supernatant after appropriate centrifugation. Detection of the labelled phosphate is routinely done by the addition of a scintillation cocktail and counting in the ^{32}P channel, yet since the maximal capacity of the cocktail used in our laboratory is 0.5 ml of water or equivalent per vial, the procedure would require concentration of the supernatant by heating in vacuum. This problem was circumvented by taking advantage

of Cerenkov effect; i.e. the emission of visible light by water or other transparent media when exposed to hard radiation. Thus, the hard β radiation of the phosphate is detected by counting it in water in the wide tritium or carbon 14 channel (Clausen, 1968). Table 12 indicates that the efficiency of this type of counting is approximately 40% both in absolute terms and in counting in the ^{32}P channel. The use of Cerenkov effect in the counting of phosphate simplifies the assay procedure and means further significant simplification in the counting procedure as well.

Under the conditions described in the Experimental section the optimal ATP-PP_i exchange was obtained in the crude extract within 15 minutes, after which reversal of the exchange is observed (Figure 5).

Discussion

The nature of this study required that substantial efforts be spent on methodology. The assay for the synthetase established earlier in our laboratory (Dakshinamurti and Desjardins, 1969) in which acetyl-CoA carboxylase activity was measured before and after complete conversion of the apo into the holoform, was directed toward the study of the substrate which is acetyl-CoA apocarboxylase, and for this reason the assay required adipose tissue in which acetyl-CoA carboxylase is a major constituent. The assay devised here, by which the synthetase is studied by the amount of radioactive biotin incorporated into TCA precipitable material, is sensitive enough to be applied for biotin deficient liver preparations. Since liver contains several apocarboxylases and can be obtained in larger quantities than the adipose tissue, it seems that the new assay is particulary suitable for the study of the synthetase.

A rat liver preparation was not capable of binding biotin either to free lysine or to polylysine and histones. This however did not eliminate the possibility that only a small portion of the apoenzyme participates in the orientation of the terminal lysine towards a favoured position for the binding with biotin, and that lysine bound to other proteins or polypeptides can be used as an appropriate substrate. In fact it is conceivable that the polypeptide chain within the acetyl-CoA carboxylase molecule in which lysine is its terminal amino acid (Dimroth et al. 1971) may be a good receptor for biotin. However, the problems involved in isolating or synthesizing such a peptide are of no less complexity than that of the present assay for the holocarboxylase synthetase, and it seems therefore that the approach of binding biotin to lysine derivatives cannot be used routinely.

More promising as an alternative assay for the synthetase is the biotin dependent ATP - $^{32}P^{32}P_i$ exchange. This assay is simple, reproducible and convenient. It suffers however from the complication that a crude enzyme preparation, even gel filtered, contains several enzymes which catalyze either independent ATP - PP_i exchange, or exchanges which are dependent on other endogenous substrates found in the preparation in minute amounts, and these may interfere strongly with the biotin dependent exchange. It seems, therefore, that the biotin dependent exchange can be utilized as an assay for the synthetase only after an initial treatment which will partially isolate the holocarboxylase synthetase.

CHAPTER 4: INVESTIGATION INTO THE POSSIBLE IDENTITY OF THE "BIOTIN ACTIVATING ENZYME" WITH ACETYL-CoA HOLOCARBOXYLASE SYNTHETASE

Coon et al. (1964) and Valloton et al. (1964) have isolated enzymes from porcine and chicken livers which could catalyze the formation of biotinyl-AMP from biotin and ATP. Since the possible physiological role of this enzyme is not known it was defined according to its function; "Biotin activating enzyme". The formation of the product by the enzyme is detected by converting the b-AMP to biotinylhydroxamate in the presence of hydroxylamine (see reaction II). The biotinylhydroxamate can be reacted with an acidic solution of ferric chloride to give a coloured complex.

"Biotin activating enzyme"

I $\quad$ biotin + ATP $\rightleftharpoons$ biotinyl-AMP + PP_i

II $\quad$ biotinyl-AMP + $NH_2OH \longrightarrow$ biotinylhydroxamate + AMP

Holocarboxylase synthetase

III $\quad$ biotin + ATP $\rightleftharpoons$ biotinyl-AMP + PP_i

IV $\quad$ biotinyl-AMP + apocarboxylase $\longrightarrow$ holocarboxylase + AMP

The similarity of reactions I and II to the mechanism by which biotin is being incorporated into the apocarboxylase as described in reactions III and IV, and particulary the common intermediate biotinyl-AMP, raised the possibility that the pig liver enzyme was identical with the acetyl-CoA holocarboxylase synthetase. Such identity would greatly facilitate the study of the synthetase, since the assay for the activating enzyme is relatively simple. Therefore it was of prime interest to confirm or negate this presumed identity.

Experimental

a) i. Partial purification of porcine "Biotin activating enzyme"

Two hundred grams of frozen porcine liver were thawed at room temperature, cut into small pieces and homogenized in a Waring blendor at medium speed for 2 minutes with 300 ml 0.5M KCl. Ice cold 0.1M KCl in 60% ethanol (400 ml) was added slowly with stirring to the homogenate. The solution was left stirring for an additional 30 minutes at 4°C. The preparation was then centrifuged in an International Refrigerated Centrifuge, Model PR6, at 2000 r.p.m. for 15 minutes at -10°C. The supernatant was dialyzed overnight against 20 liters of 0.05M $KHCO_3$, with two changes of the dialyzing buffer.

ii. Ammonium sulfate fractionation

Ammonium sulfate was added slowly to the dialyzate at 4°C to 40% saturation with respect to ammonium sulfate. The solution was stirred for an additional 30 minutes and centrifuged in a RC2 Sorvall centrifuge (GSA rotor) for 15 minutes at 6000 r.p.m. (4°C). The pellet was discarded and the supernatant was brought to 0.7 saturation, at 4°C, by slow addition with stirring of ammonium sulfate. The solution was centrifuged in a Sorvall RC2 centrifuge (GSA rotor) as above, and the supernatant was dialyzed overnight against 4 liters of 0.05M Tris, 10 mM β-mercaptoethanol, pH 7.4.

iii. Column chromatography

The enzyme preparation was passed through a Sepharose 4B column (5 x 80 cm) which was equilibrated and eluted with buffer D containing 0.05M Tris, 10 mM β-mercaptoethanol, pH 7.4. Fractions (14 ml) were collected, and aliquots (0.5 ml) were taken and assayed for "Biotin

activating enzyme" activity. The active fractions were pooled and concentrated by ammonium sulfate precipitation at 70% saturation. The pellet was suspended in a minimal volume of 0.05M Tris, 10 mM β-mercaptoethanol, pH 7.4, and dialyzed against 4 liters of the same buffer for 4 hours.

The dialyzed preparation was then subjected to chromatography on a Sephadex G-100 column (5 x 80 cm) and eluted with buffer D. Fractions of 14.0 ml were collected and assayed for the "Biotin activating enzyme" activity. The active fractions were pooled and concentrated in a procedure similar to that described for the Sepharose 4B eluate.

Further purification was achieved by chromatographing the above enzyme preparation on a DEAE-Sephadex column (2.5 x 40 cm). The column was equilibrated with buffer D and eluted with a linear gradient of 0.01 - 0.3 M NaCl (in buffer D). Fractions of 3 ml were collected and aliquots of 0.3 ml were assayed for activity. The active fractions were pooled and processed as in the earlier run.

b) Assay for "Biotin activating enzyme": The assay mixture contained 50 mM Tris, 10 mM β-mercaptoethanol, 10 mM ATP, 10 mM Mg^{++}, 20 mM biotin and 2.4 M NH_2OH, pH 7.4, in a volume of 1 ml. After incubation at 37°C for 30 to 60 minutes the assay was terminated with 1 ml 40% trichloroacetic acid followed by the addition of 3 ml of acidic $FeCl_3$ solution. The assay tubes were permitted to stand 15 minutes to allow colour development, and the mixture was filtered using a Whatman paper (No. 41). The optical density of the filtrate was measured at 540 nm on a Spectronic 20 spectrophotometer (Bausch and Lomb). A unit of activity is defined as the formation of 1 μM biotinylhydroxamate per

hour.

Acidic ferric chloride reagent contained 0.46 M $FeCl_3 \cdot 6H_2O$ dissolved in 0.67 M HCl containing 5% trichloroacetic acid. Salt free hydroxylamine was prepared by titration of saturated solution of $(NH_2OH)_2 \cdot H_2SO_4$ (approximately 1 M) with saturated solution of $Ba(OH)_2$ (approximately 3 M). The titration was done by vigorous stirring in a heated beaker. After neutralization to pH 7.0 the cloudy mixture was centrifuged for 10 minutes at 10,000 x g. The supernatant was concentrated in a flash evaporator under reduced pressure. The concentrated solution was then diluted with water to the fifth of the original volume thereby giving a final concentration of 5 M with respect to hydroxylamine.

c) Synthesis of biotinyl-AMP: Biotinyl-AMP was synthesized using essentially the method described by Lynen et al. (1963). 244 mg d-biotin was dissolved in 2 ml 75% pyridine in water by stirring and gentle heating. 0.16 μM ^{14}C-d-biotin (46 μCi/mmole) were added to this solution. It was then mixed with 2 ml of 75% pyridine in water containing 347 mg AMP. The beakers were washed with 4 ml of 75% pyridine in water and the wash was added to the original mixture. 4g of DCC (dicyclohexylcarbodiimide) was dissolved in 4 ml absolute pyridine, and added slowly with vigorous stirring to the biotin and AMP mixture at 4°C. The final mixture was stirred overnight at 4°C, and filtered on fine sintered glass to remove insoluble urea derivatives produced. The upper layer of water was removed from the filtrate with a Pasteur pipet, and 100 ml of pre-cooled acetone was added to the lower phase. The cloudy solution was left at -40°C overnight, and then it was centrifuged at 4°C, at 10,000 x g for 30 minutes. The supernatant was removed and discarded and

the pellet was suspended in 4 ml of water. The unreacted DCC and biotin are relatively insoluble in water and were essentially removed by centrifugation at 4°C, 10,000 x g for 30 minutes. The supernatant was applied on Sephadex G-10 column (2.5 x 90 cm) and eluted with water (Figure 6). Fractions (6.4 ml) were collected and aliquots (0.5 ml) were assayed for biotinyl-AMP by incubating with hydroxylamine for 10 minutes, followed by the addition of acidic ferric chloride. The tubes containing b-AMP were pooled, freeze dried and the residue was dissolved in 8.0 ml water. The yield was 172 mg of b-AMP.

d) Characterization of biotinyl-AMP

i. Paper chromatography

The purity of the b-AMP synthesized was determined by paper chromatography. This was performed in a Shandon Unikit tank 27 x 11 cm in size. Paper strips 24 x 10 cm were equilibrated in the tank in presence of the solvent about 1 hour prior to the run, after which 50 µliter of the b-AMP was spotted on the paper in 10 µliter aliquots. The descending chromatography lasted about 7 hours after which the paper was removed from the tank, dried by a stream of hot air and the spots corresponding to b-AMP were detected by a U.V. lamp (Figure 7).

ii. Scanning for optimal spectra

Scanning b-AMP and AMP for absorption spectra in the ultraviolet range was performed on Beckman DB-G spectrophotometer. Both compounds showed an optimum at 260 nm respectively (Figure 8).

e) Characterization of biotinhydroxamate: The maximal optical density values of the "Biotin activating enzyme" assay, read at 540 nm, were usually 0.4. Readings at lower wavelengths gave higher O.D. values

Figure 6: Separation of b-AMP and AMP on Sephadex G-10 column

The product of the reaction in which b-AMP was synthesized, containing labelled b-AMP and unlabelled AMP was applied on Sephadex G-10 column 2.5 x 80 cm. The elution was done with water and fractions of 6.4 ml were collected. Aliquots of 0.5 ml were assayed with NH_2OH and ferric chloride for color formation at 540 nm. Other aliquots of 100 µliter were diluted in water to 30 ml and read at 260 nm. ^{14}C-d-biotin (0.8 μC_i) was run separately to mark its location in the eluent.

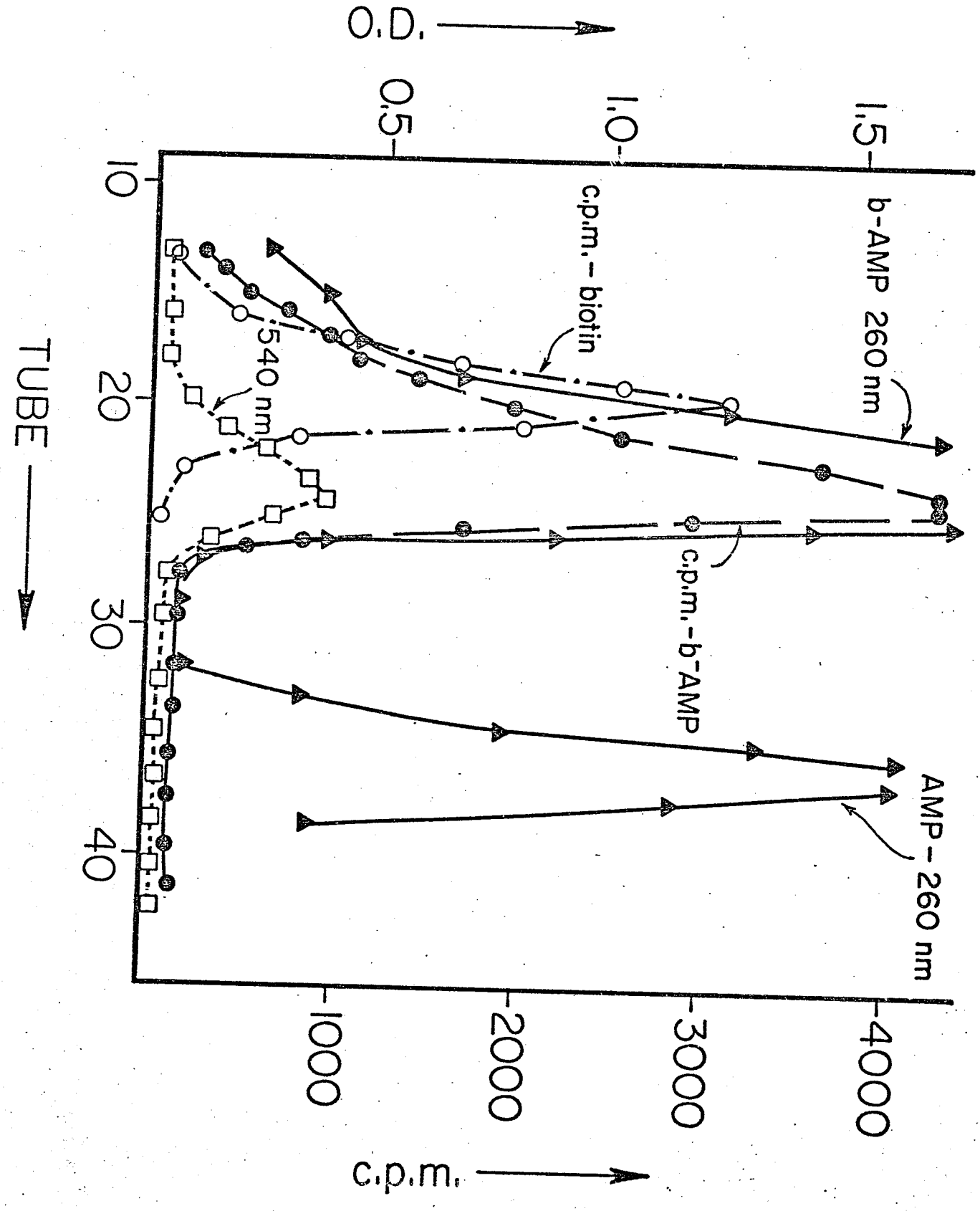

O.D.
0.5
1.0
1.5
b-AMP 260 nm
c.p.m. – biotin
540 nm
c.p.m. – b-AMP
AMP – 260 nm
10
20
30
40
TUBE
1000
2000
3000
4000
c.p.m.

Figure 7: Paper chromatogram of AMP and b-AMP

50 µliter of the b-AMP synthesized, which was eluted on Sephadex G-10, was applied on a chromatographic paper 3 MM 24 x 10 cm in size which was subjected to the chromatographic procedure described in the Experimental section. The elution was performed in a medium of isopropyl alcohol; acetic acid; water: NH_4OH (50/28/20/2, V/V). Location of authentic AMP and the synthesized b-AMP was detected by a U.V. lamp.

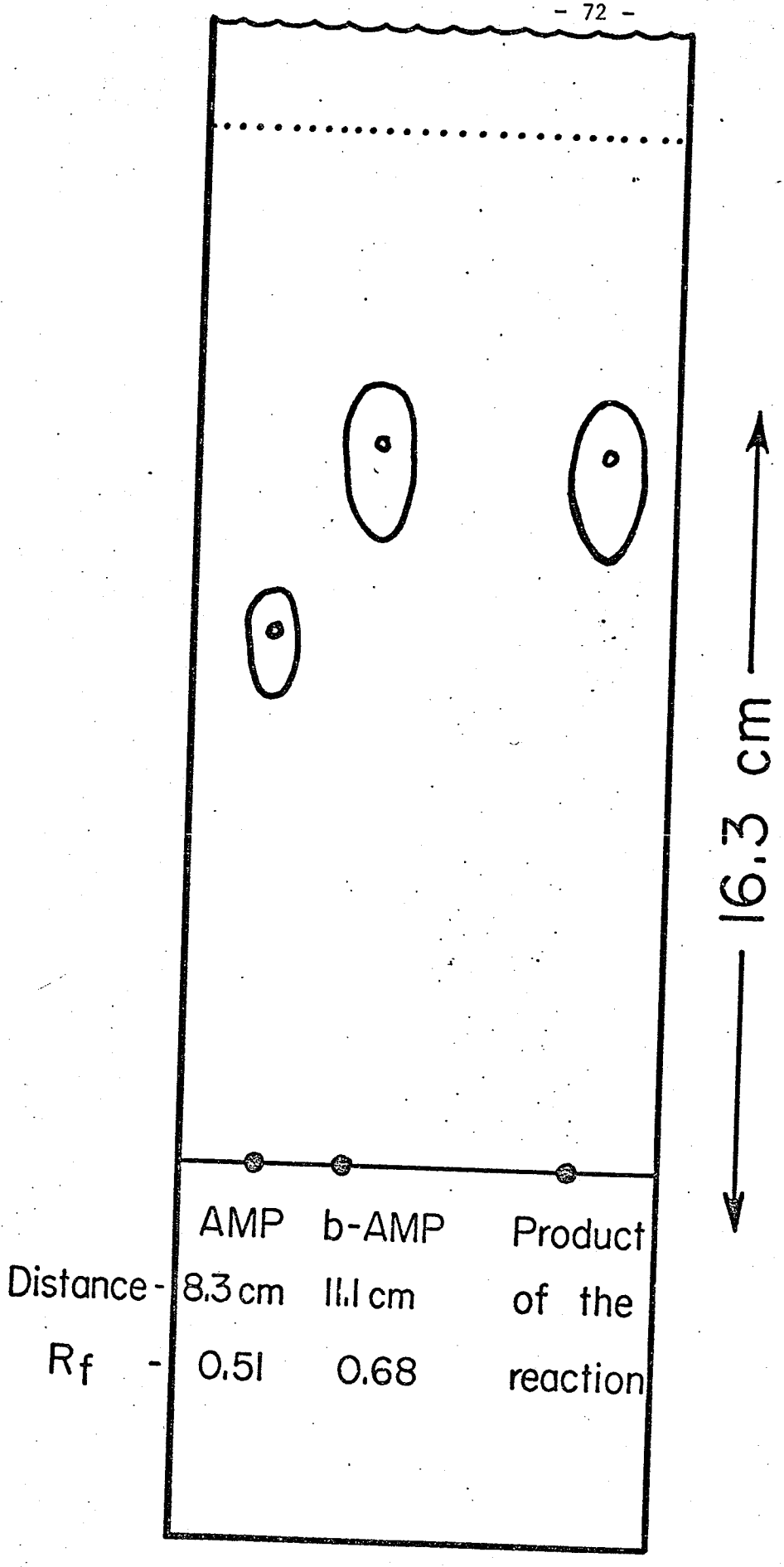

16.3 cm
AMP
b-AMP
Product
Distance -
8.3 cm
11.1 cm
of the
R_f -
0.51
0.68
reaction

Figure 8: Scanning for optimal spectra of b-AMP

Authentic AMP, and b-AMP synthesized which showed apparent purity on paper chromatogram, were scanned for optimal wavelength of absorption in the range of 320 to 210 nm. The scanning was performed on Beckman DB-G spectrophotometer equipped with 10" recorder.

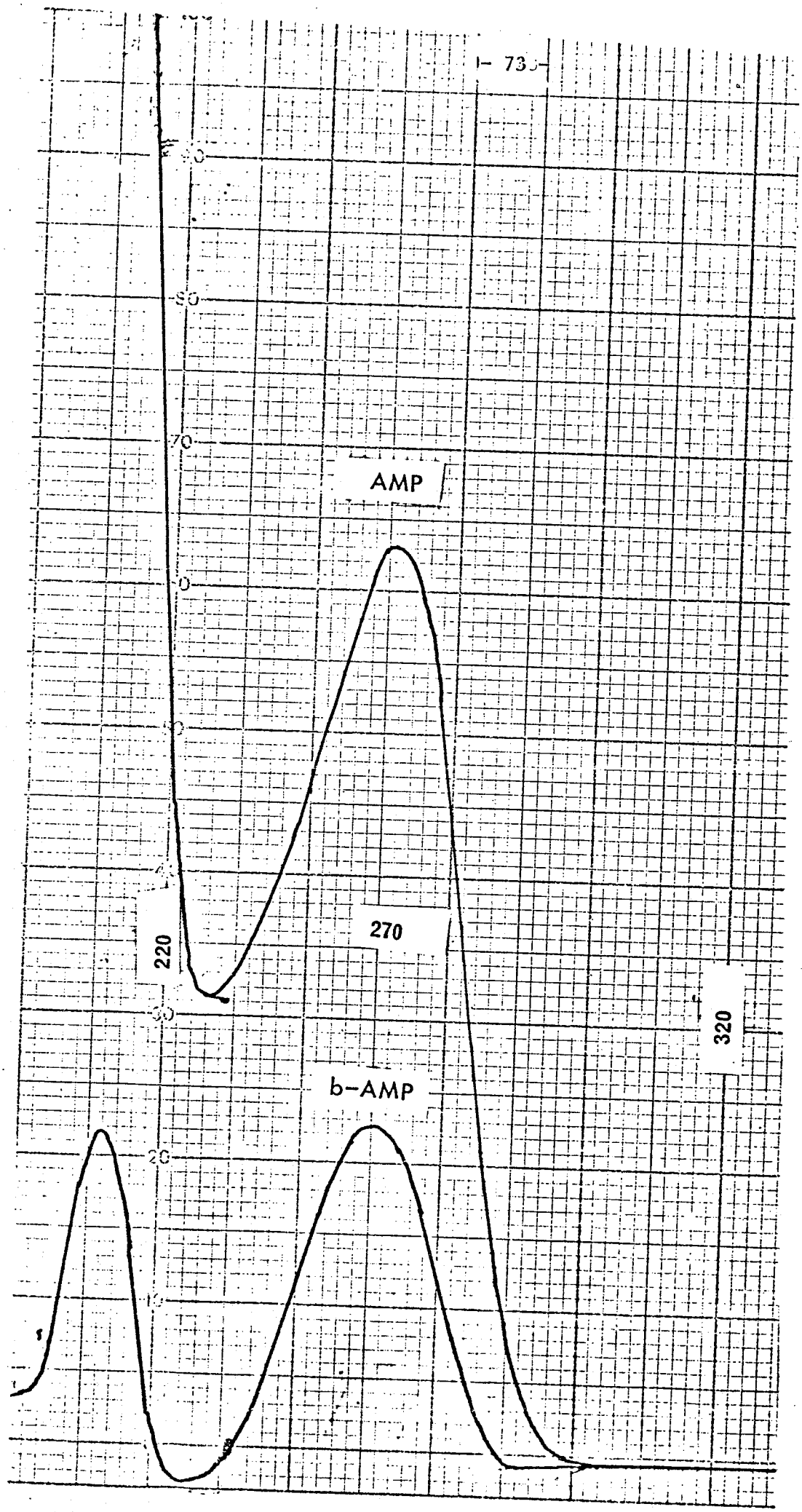
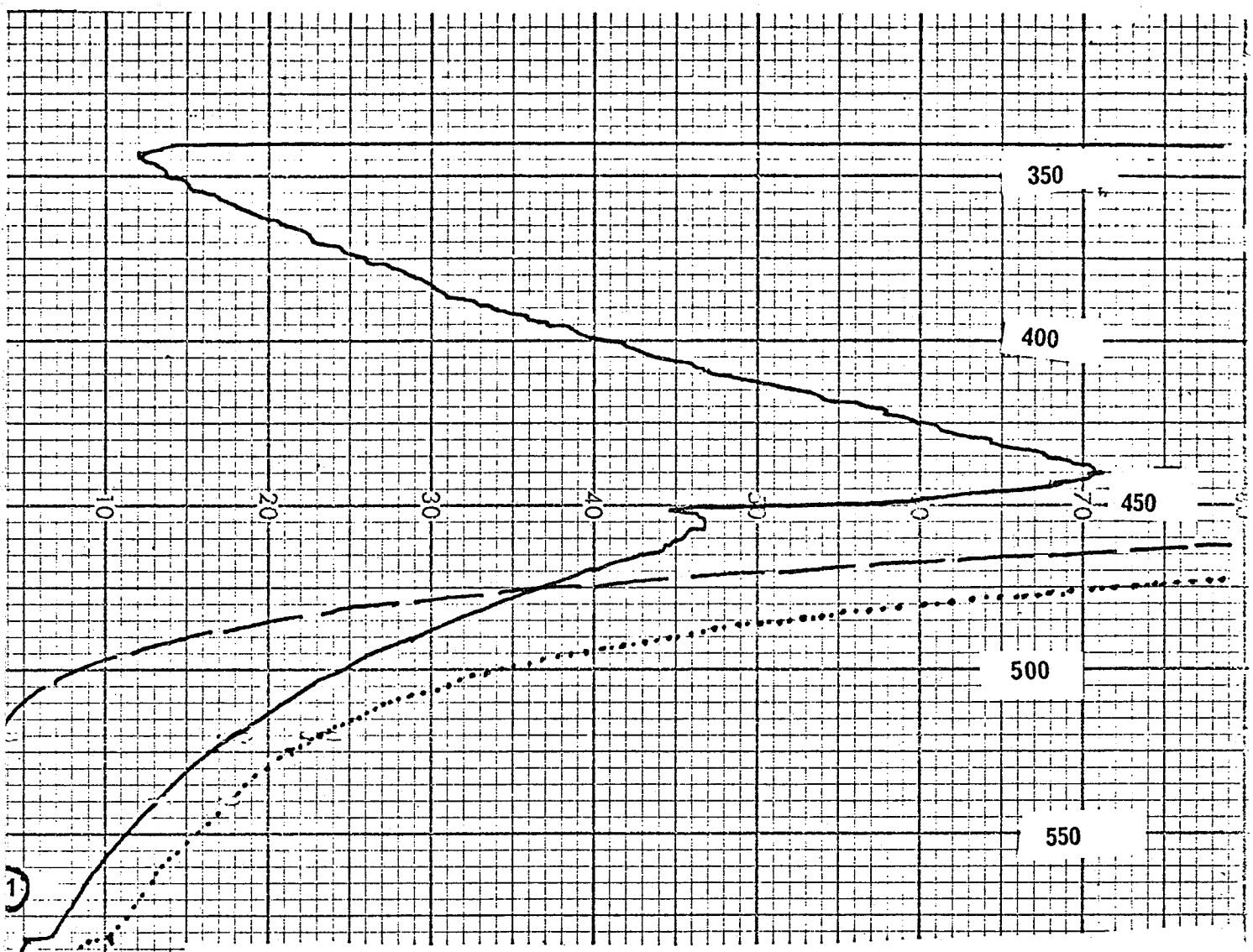

AMP
220
270
320
b-AMP

Figure 9: Absorption spectra of biotinyl hydroxamate

Acidic Ferric chloride solution mixed with NH_2OH, or with biotin hydroxamate, was scanned respectively against buffer Tris 0.05 M as a blank, and then scanned for difference spectra against each other. The scanning range was 600-300 nm and was performed on Beckman DB-G spectrophotometer equipped with 10" recorder.



1 blank, $FeCl_3$ in TCA

2 biotin hydroxamate – $FeCl_3$ complex

3 difference spectra

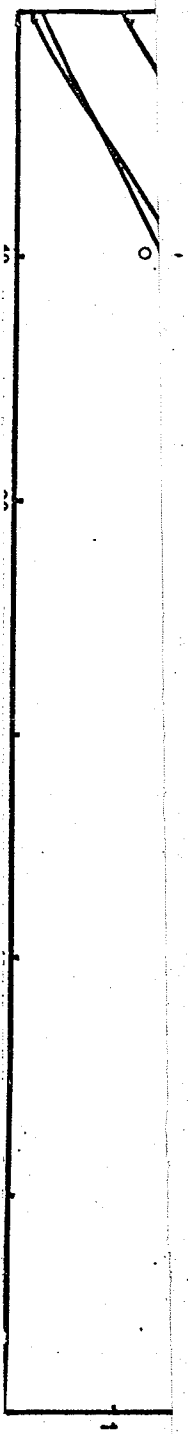
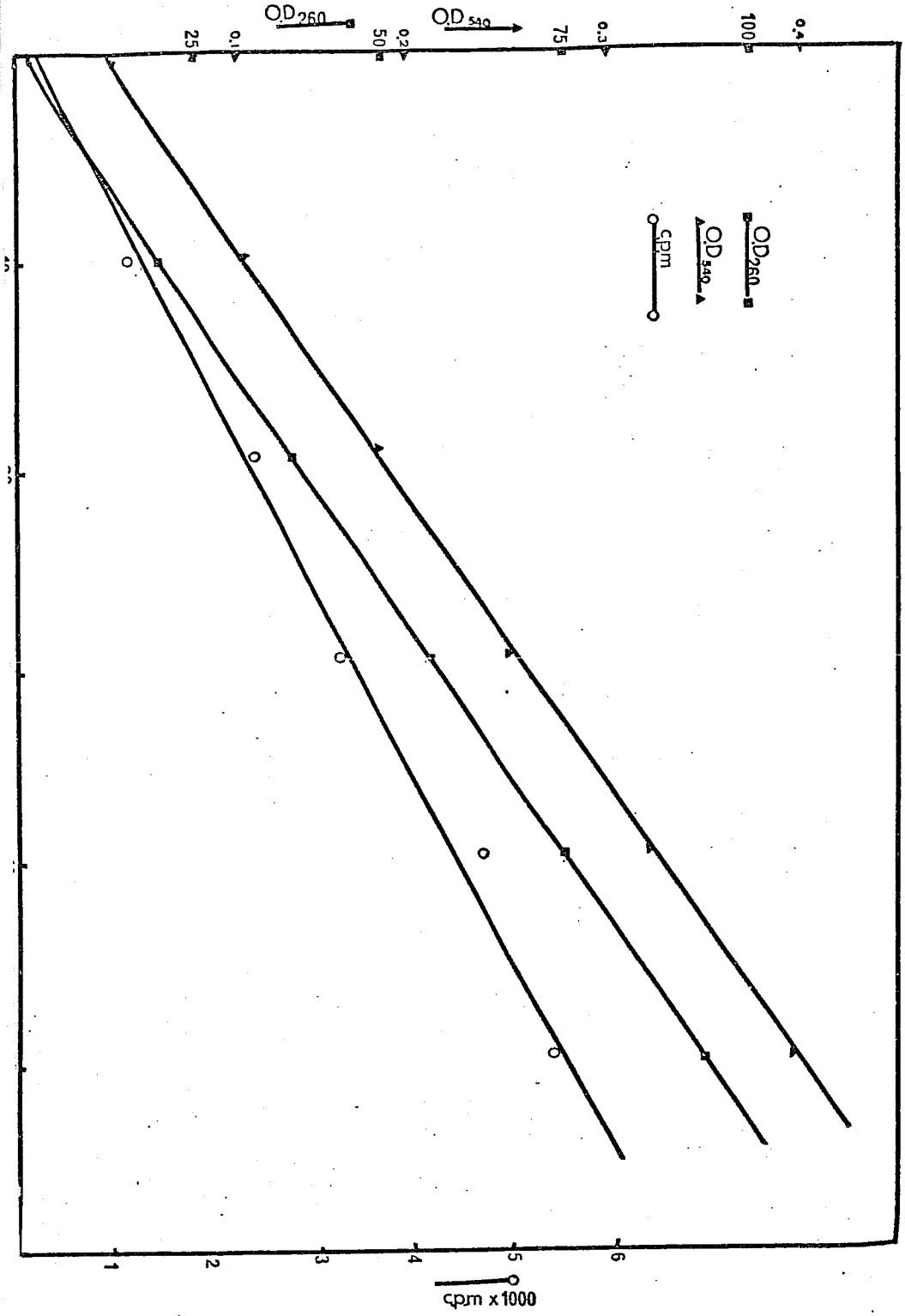


Figure 10: Determination of the extinction coefficient of b-AMP

Aliquots of the b-AMP synthesized were taken and counted for radioactivity. Other aliquots were read at 260 nm in Beckman DB-G spectrophotometer, while into a third set of samples NH_2OH and acidic solution of ferric chloride was added to give biotinylhydroxamate-ferri chloride complex which was read at 540 nm.


OD260
OD549
25
50
75
100
0.1
0.2
0.3
0.4
OD260
OD549
cpm
1
2
3
4
5
6
cpm x 1000

which could be used to increase the sensitivity of the assay, however the spectra of a ferric chloride solution and biotinhydroxamate which was synthesized enzymatically, against a reagent blank (Figure 9) indicated that,although higher values are obtained at lower wavelengths, 540 nm is the wavelength at which the difference between the readings obtained for the sample and the blank are optimal in accuracy and sensitivity.

Expression of O.D. as the amount of substrate converted into the product was done by calibration of the synthesized labelled biotinyl-hydroxamate (Figure 10). The amount of b-AMP was calculated from the radioactivity in the b-AMP, since the specific activity of biotin was known. A simple calculation based on Figure 10 shows the extinction coefficient of biotinylhydroxamate in ferric chloride solution to be $E_{540} = 800$. The extinction coefficient of biotinyl-AMP in water was determined to be $E_{260} = 21,410$.

Results

The biotin activating enzyme was partially purified from pig liver using a modification of the method described by Coon et al. (1964). The enzyme was purified 19 fold (Table 13) to a specific activity of 2.06 µmoles biotinyl-AMP formed per mg protein in 30 minutes under conditions described in the experimental section. The enzyme could be stored at -20°C for 1-2 weeks without any loss in activity.

The formation of biotinyl-AMP was linear for 15-20 minutes (Figure 11) under assay conditions described in the experimental part. The reaction rate displayed a typical parabolic response to increasing concentrations of ATP, magnesium and biotin (Figures 12 a,b,c). Half

Table 13: Partial purification of "Biotin activating enzyme"

Step	Volume ml	Activity µmole/ml	mg protein per ml	Total Activity µmole	Total Protein in mg	Specific Activity units	Yield %	Fold Purification
Alcohol step, after dialysis	565	1.5	13	807	7345	0.11	100	Nil
0.4-0.7 ammonium sulfate fraction	75	9.8	66	737	4950	0.14	87	1.27
Sepharose 4B, after concentration and dialysis	50	11	41	550	2050	0.26	64	2.36
Sephadex G-100 after concentration and dialysis	9.2	35	46	320	432	0.75	37	6.81
DEAE-Sephadex A-50	100	1.25	0.6	135	64	2.06	15	18.7

Details of the purification procedure are given in the experimental section. A unit is defined as the formation of µmole of biotinylhydroxamate per mg protein during an incubation period of 30 minutes.

Figure 11: A time course of biotinylhydroxamate formation

Sample of 0.3 mg of the DEAE-Sephadex Eluted "Biotin activating enzyme" was incubated in a complete incubation mixture, containing 50 mM Tris, pH 7.4, 10 mM ATP, 10 mM Mg^{+2}, 20 mM biotin and 2.4 M salt free NH_2OH. The incubations were terminated at various periods by addition of TCA followed by ferric chloride solution. The assay mixtures were then filtered through a filter paper and read at 540 nm.

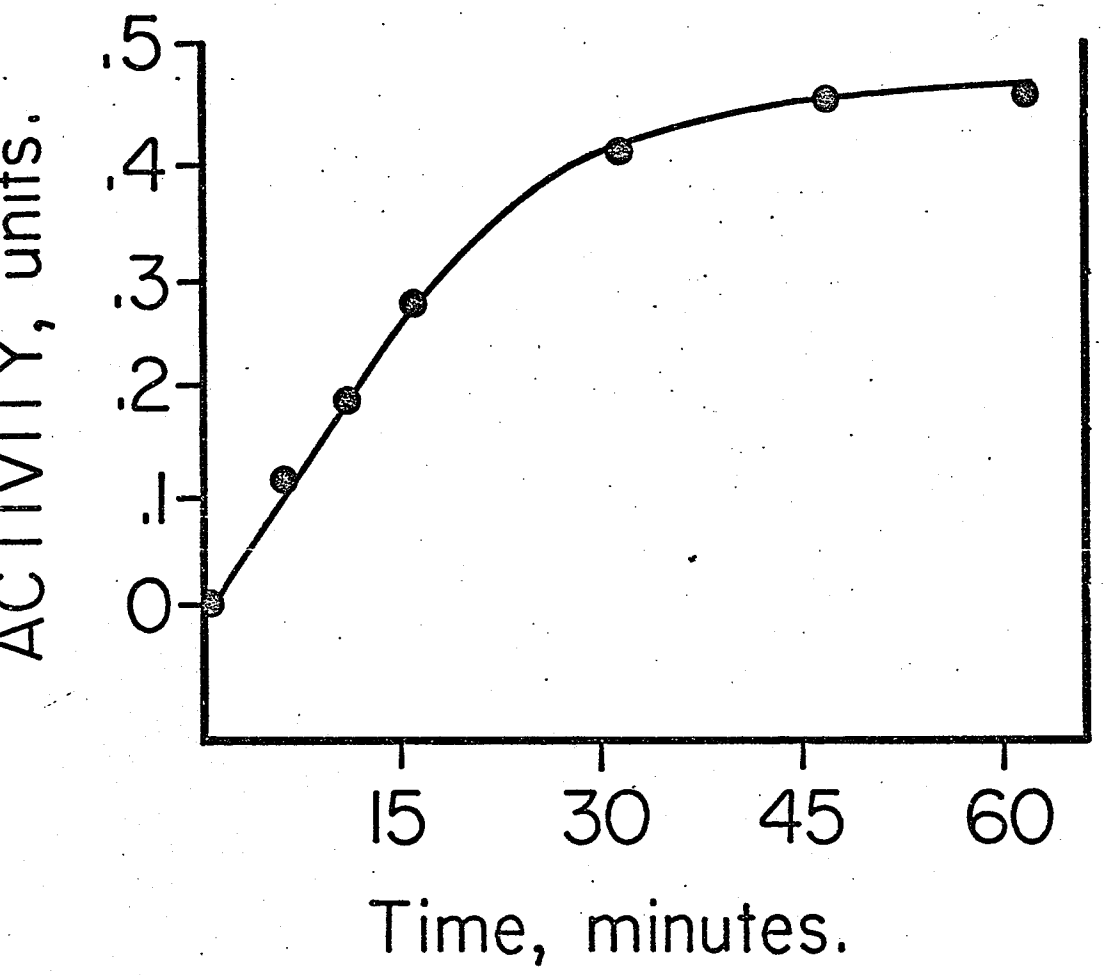
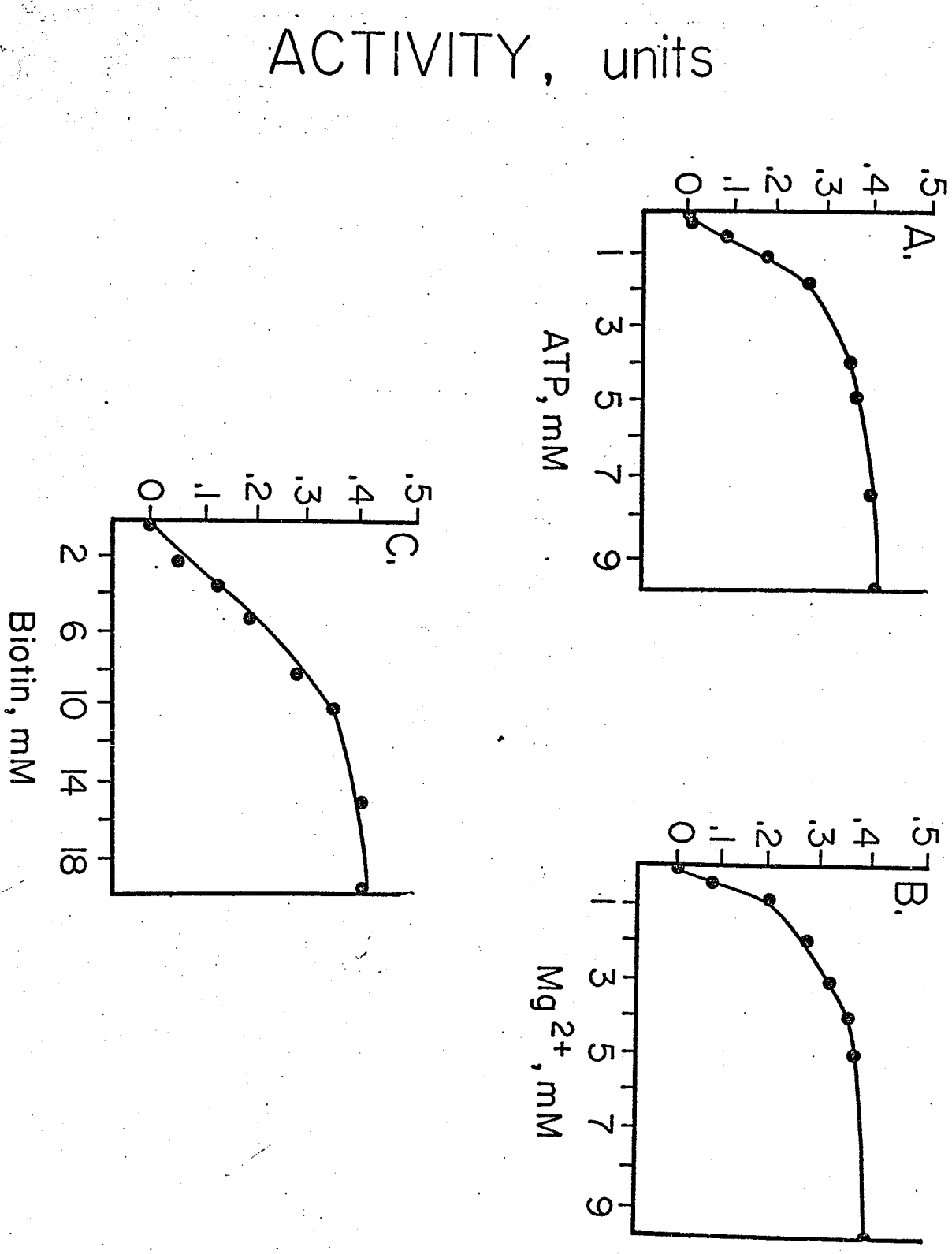

ACTIVITY, units.
.5
.4
.3
.2
.1
0
15
30
45
60
Time, minutes.

Figure 12: Determination of optimal conditions of the assay for biotin activating enzyme

0.3 mg of "Biotin activating enzyme" eluted from DEAE-Sephadex column was incubated with ATP, Mg, biotin and salt free hydroxylamine for 30 minutes at 37°C, after which it was terminated as described in the Experimental section. The determination was done by keeping the three substrates at saturating concentrations (determined in preliminary experiments) and varying the concentration of the fourth. The requirement for these substrates is expressed in Michaelis-Menten plots.


ACTIVITY, units
A.
ATP, mM
B.
Mg2+, mM
C.
Biotin, mM

maximal activity was reached at concentrations of 1.2 mM, 1.1 mM and 4.9 mM for ATP, magnesium and biotin,respectively. Maximal activity was obtained at concentrations of 10 mM, 10 mM and 20 mM for ATP, magnesium and biotin,respectively.

Biotin hydroxamate formation is dependent on two reactions which were previously described. The first reaction, the formation of biotinyl-AMP from ATP and biotin, is catalyzed by the pig liver enzyme. The second reaction, the formation of biotinylhydroxamate from hydroxylamine and biotinyl-AMP is thought to be nonenzymatic. In order to confirm this latter point the rate of formation of biotinylhydroxamate from b-AMP and hydroxylamine was investigated in the presence and absence of the pig liver enzyme (Figure 13). Biotinylhydroxamate formation occurs rapidly and is almost complete after 5 minutes. The pig liver enzyme had no effect on the rate of biotinylhydroxamate formation from b-AMP. It was of interest to pursue the role of hydroxylamine in this reaction further. The rate of biotinylhydroxamate formation is directly proportional to the concentration of hydroxylamine in the assay up to concentration of 2.8 M, after which it began to plateau (Figure 14). In fact the presence of hydroxylamine in the assay is obligatory. Biotinyl-AMP formation was investigated as a function of time in the presence and absence of hydroxylamine (Table 14). When hydroxylamine was omitted from the reaction it was added in the last five minutes of the reaction to convert all b-AMP into biotinylhydroxamate. No hydroxamate formation could be detected in the absence of hydroxylamine during the assay.

It was thought that the absolute requirement for hydroxylamine in the assay could be due to the presence of hydrolases which were specific

for b-AMP but were ineffective with biotinylhydroxamate, and as such the hydroxylamine acted as a trap. To confirm this, biotinyl-AMP was incubated for various time periods in the presence and absence of the pig liver enzyme. After this initial incubation, hydroxylamine was added and the amount of biotinyl-AMP remaining was measured (Table 15). It can be seen that by omitting the activating enzyme, b-AMP disappears rapidly, confirming the presence of hydrolase activity in the preparation.

The catalytic activity of the "Biotin activating enzyme" with various amino acids and short chain fatty acids like formic and acetic acid was measured (Table 16). None of the substrates tested gave any reaction. The optical density at 540 nm seen in the control with glutamine is due to reaction of the amide linkage.

In some experiments the enzyme appeared to be unstable at 37°C but this was not reflected by the long incubation time utilized in the assay of the enzyme (Figure 11). The effect of ATP and Mg^{++} on the stability of the enzyme at 37°C (Figure 15) was determined. ATP and Mg^{++} protect the enzyme from inactivation; in their absence the enzyme is very labile at 37°C, losing all of its activity within 15 minutes.

In preliminary experiments, attempts were made to measure the "Biotin activating enzyme" in rat liver and adipose tissue. No activity was detectable. It was therefore pertinent to see if the apparent lack of activity was due to high concentrations of hydrolases which prevented the formation of biotinyl-AMP. When porcine and rat liver gel filtered cytosols were mixed in equal amounts, the resultant activity was found to be additive (Table 17). If the lack of activity in the rat liver was due to the action of hydrolases, then the activity in the mixture would

not have been additive, and lower values would have been measured. Therefore, the low levels of activity in rat liver and adipose tissue may be a true reflection of the low enzymatic activity in these tissues.

^{14}C-Biotin incorporation into endogenous acetyl-CoA apocarboxylase

Since both the "Biotin activating enzyme" and acetyl-CoA holocarboxylase synthetase catalyze the synthesis of b-AMP, it was of interest to see if the "Biotin activating enzyme" could enhance the rate of acetyl-CoA holocarboxylase synthesis in crude gel filtered cytosols from biotin deficient rat liver and adipose tissue. The incorporation of ^{14}C-biotin into trichloroacetic acid precipitable protein was used as a measure of acetyl-CoA holocarboxylase formation. ^{14}C-Biotin incorporation into endogenous apoenzyme was measured in both biotin deficient rat adipose tissue and liver (Figure 16). The rate of incorporation was time dependent and required ATP. The rate of incorporation was similar in both liver and adipose tissue, although biotin incorporation reached a plateau after 30 minutes in the liver whereas it increased steadily up to 60 minutes in the adipose tissue.

Because the "Biotin activating enzyme" has a relatively high apparent Km for biotin,it was imperative to have a comparable estimate of the apparent Km for biotin incorporation into endogenous apocarboxylase, in both liver and adipose tissue (Figure 16). It can be seen that half maximal biotin incorporation into endogenous apoenzyme occurs at 0.8 μM in liver and 0.4 μM in adipose tissue.

Next, the effect of purified pig liver "Biotin activating enzyme" on the incorporation of biotin into endogenous apoenzyme was investigated in gel filtered cytosol from adipose tissue of biotin deficient rat

(Figure 17). It can be seen that in the presence of ATP there is a time dependent incorporation of biotin into protein and that the addition of the pig liver enzyme failed to enhance the rate of incorporation significantly.

Analysis of the partial reactions of both the "Biotin activating enzyme" and the holocarboxylase synthetase implied that both enzymes can catalyze ATP-PP_i exchange. It was therefore of interest to investigate if the two enzymes could be separated by a molecular sieve and identified by this exchange. Porcine liver cytosol was applied on Sephadex G-100 and the eluent was assayed first for "Biotin activating enzyme" activity. This showed one peak (B) (Figure 18) corresponding to the location of "Biotin activating enzyme" as identified during previous routine work with this enzyme, and a second peak (D) which contained nonspecified compounds which interacted with the reagents of the assay chemically. The chemical nature of the interaction of this peak was confirmed when color development still occured with fractions of peak D which had been boiled for 1 hour prior to the assay. The biotin dependent pyrophosphate exchange assay gave peaks A and C. It is conceivable that the overlapping region of peaks B and C which is capable both of activating biotin and exchange PP_i and ATP may contain the holocarboxylase synthetase.

Discussion

Particular emphasis was laid on the study of "Biotin activating enzyme" and the possibility of its identification with the holocarboxylase synthetase. First the possible identity of this enzyme with other enzymes which have a physiologically more defined function was explored. Bacteria

Table 14: Effect of hydroxylamine on enzymatic synthesis of biotinylhydroxamate

Incubation period, minutes	30	90	180
Synthesis during omission of NH_2OH, $O.D._{540}$	0.09	0.09	0.09
Hydroxamate formed by a complete system, $O.D._{540}$	0.17	0.30	0.47

12 mg of "Biotin activating enzyme", purified at the ammonium sulfate step, were incubated in a complete assay mixture containing 10 mM ATP, 10 mM Mg^{++}, 20 mM biotin and 2.4 M hydroxylamine and in a control mixture respectively which was of similar composition except that the hydroxylamine was omitted. Formation of hydroxamate in the control mixture was achieved by 5 minutes incubation with 2.4 M hydroxylamine after the first incubation. In both mixtures the hydroxamate formed was detected with acidic solution of ferric chloride.

Table 15: Hydrolytic activity in partially pure preparations of "Biotin activating enzyme"

Incubation period, min.	0	5	10	15	30	60
b-AMP retained, $O.D._{540}$ no enzyme	0.39	0.39	0.37	0.38	0.37	0.37
b-AMP retained after incubation with enzyme $O.D._{540}$	0.39	0.22	0.16	0.10	0.08	0.08

0.83 mg of highly purified biotinyl-AMP was incubated with 12 mg of a preparation of "Biotin activating enzyme" after its ammonium sulfate step. The amounts of b-AMP remaining after incubation at various periods, was assayed by the addition of 2.4 M hydroxylamine followed by acidic solution of ferric chloride.

Table 16: Enzymic formation of hydroxamates from amino acids and short chained fatty acids

Compound	O.D. at 540 nm		
	Control (-ATP)	10 mM	100 mM
Lysine	0.08	0.08	0.08
Methionine	0.08	0.08	0.08
Glutamic acid	0.08	0.08	0.08
Glutamine	0.32	-	0.32
Aspartic acid	0.08	0.08	0.08
Glycine	0.08	0.08	0.08
Formate	0.08	0.08	0.08
Acetate	0.10	0.08	0.10
Biotin	0.08	0.53	0.62

6.2 mg of "Biotin activating enzyme" after the ammonium sulfate step was assayed in presence of ATP, Mg, hydroxylamine, and 10 mM or 100 mM of the acid tested. In the blanks 10 mM of acid was used, however, ATP was omitted. Following an incubation period of 30 minutes at 37°C, acidic ferric chloride was added to detect the possible formation of hydroxamate.

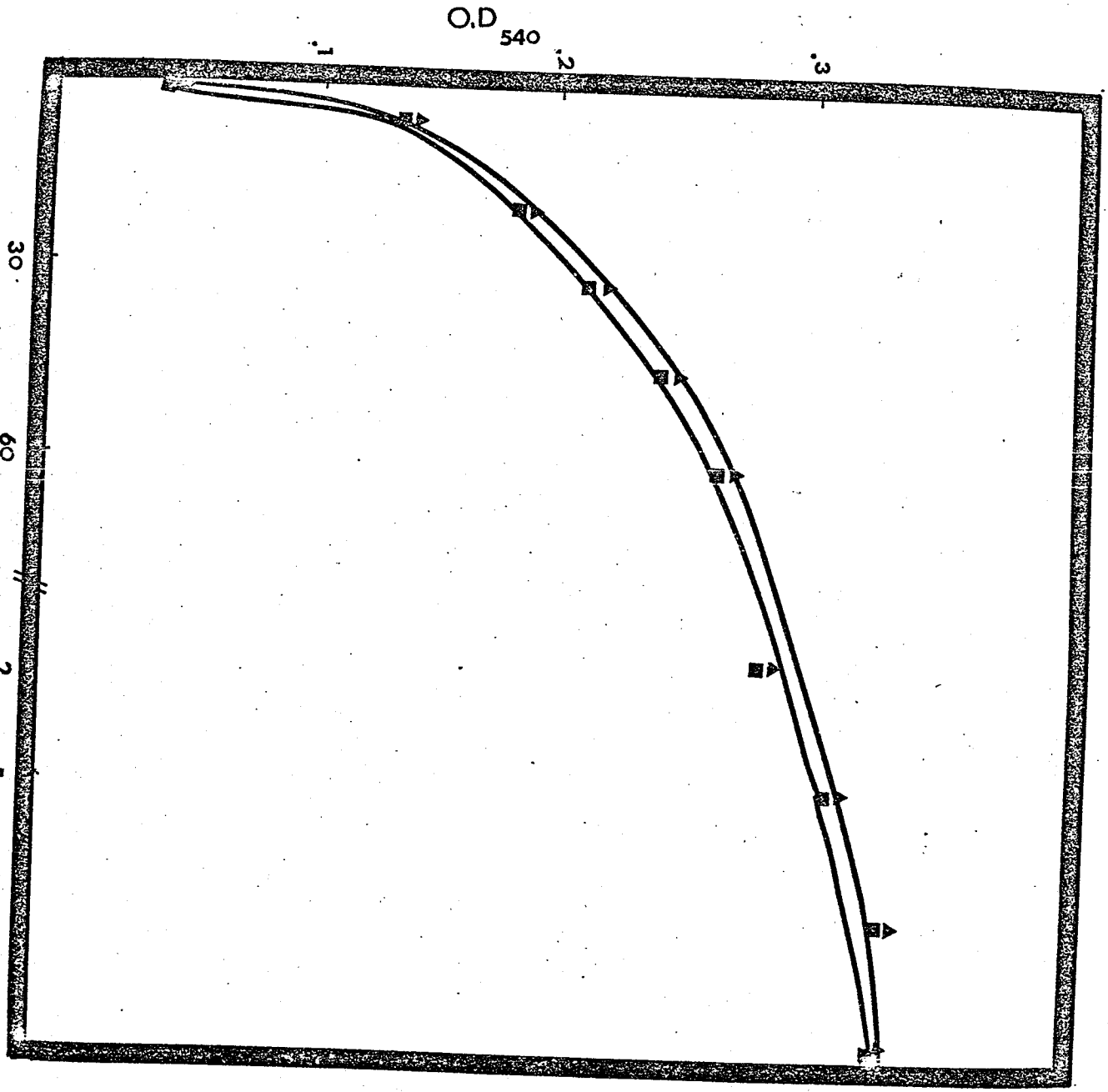
Table 17: Hydroxamate formation in porcine and rat mixed preparation

Time, minutes	0	90	240
Rat liver cytosol $O.D._{540}$	0.05	0.05	0.05
Porcine liver cytosol $O.D._{540}$	0.05	0.27	0.47
Mixture of rat and porcine preparations $O.D._{540}$	0.05	0.18	0.27

A time course for biotinylhydroxamate was performed using G-25 gel filtered cytosols of rat and porcine livers in protein concentrations of 4.4 and 7.4 mg respectively. This was compared to the time course of a sample which contained a mixture of rat and porcine cytosols in protein concentration of 2.2 and 3.7 mg respectively. The assay mixture contained 10 mM ATP, 10 mM Mg^{++}, 20 mM biotin and 2.4 M NH_2OH, pH 7.4. Incubations were terminated after 90 and 240 minutes respectively by the addition of ferric chloride solution.

Figure 13: Involvement of the "Biotin activating enzyme" in the formation of biotinyhydroxamate from b-AMP

0.83 mg of Sephadex G-10 eluted b-AMP was incubated with hydroxylamine in concentration of 2.4 M and in the presence or absence of 6.3 mg "Biotin activating enzyme" (after the ammonium sulfate step). A time course of the formation of the hydroxamate was followed. (-●-)in presence of BAE (-▲-)BAE omitted.


O.D 540
·1
·2
·3
TIME
30 seconds
60
2
minutes
5

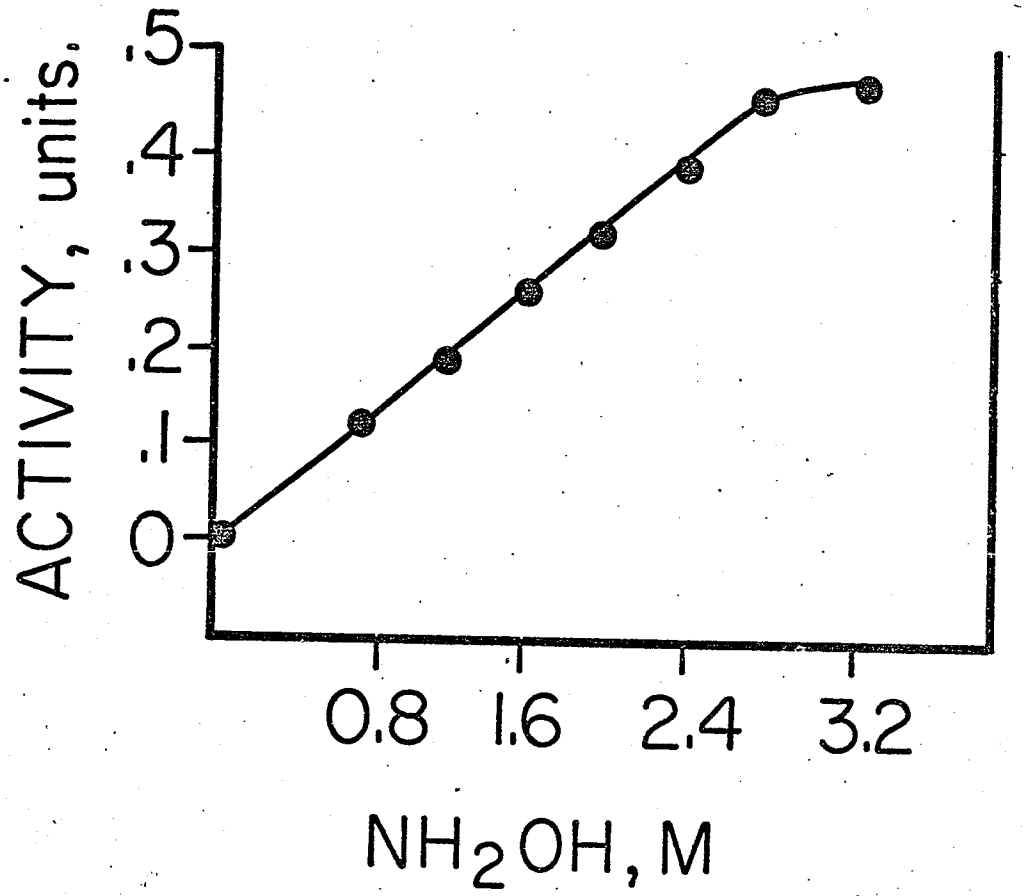


Figure 14: The requirement for hydroxylamine in the assay for "Biotin activating enzyme"

See legend of figure 12 for details.

Figure 15: The stability of "Biotin activating enzyme"

12 mg of the "Biotin activating enzyme" after the ammonium sulfate step were incubated at 37° in buffer Tris 0.05 M, pH 7.5, in the presence (●) and absence (▲) respectively of 10 mM ATP and 10 mM Mg^{++}. At various time periods aliquots of 1 ml were taken and assayed for "Biotin activating enzyme".

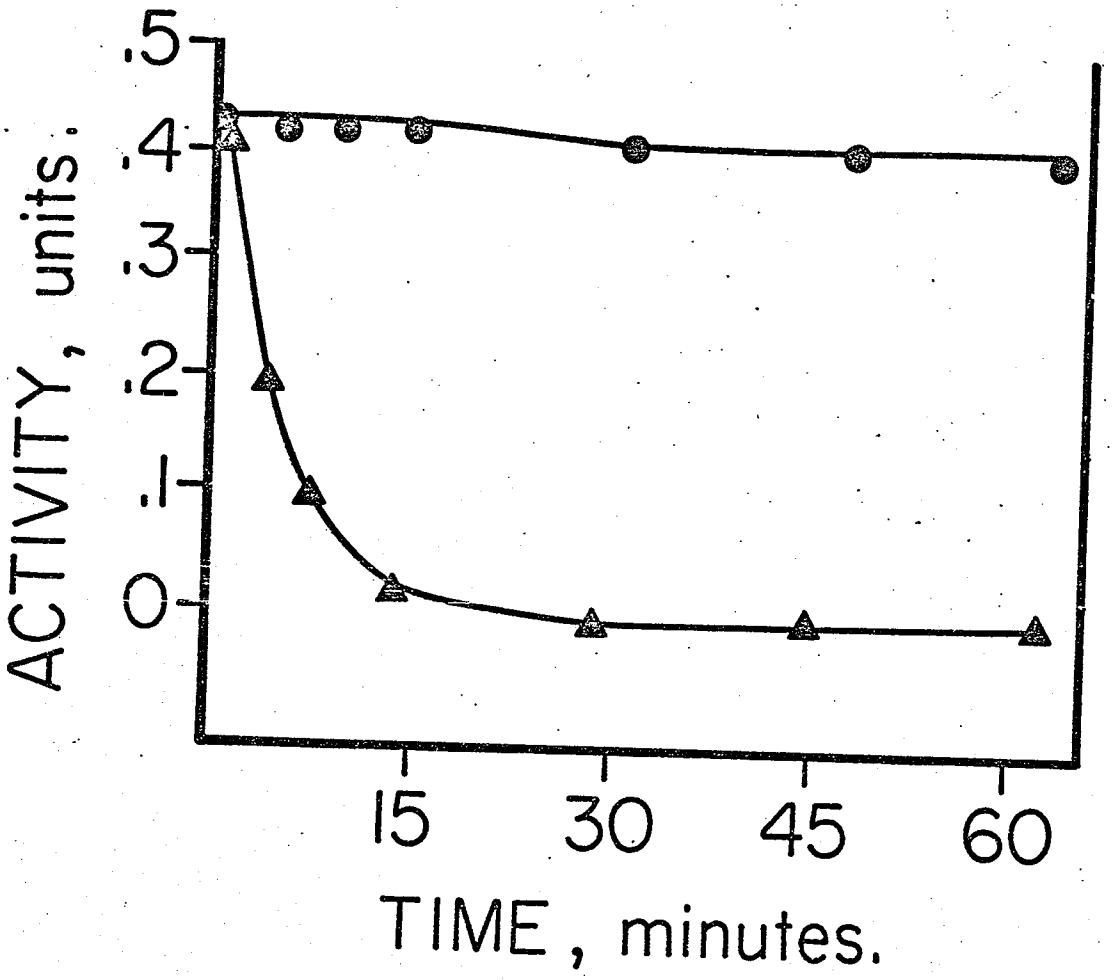

ACTIVITY, units.
.5
.4
.3
.2
.1
0
15
30
45
60
TIME, minutes.

Figure 16: The incorporation of ^{14}C-biotin into endogenous proteins by rat liver or adipose tissue cytosol preparation of biotin deficient rats

Rat liver and adipose tissue gel filtered cytosols in protein concentration of 1.55 and 2.7 mg respectively were incubated in the presence of 2 mM ATP-Mg^{+2} and increasing amounts of ^{14}C-biotin. The assay was terminated by 10% TCA after which the free biotin was washed away and the radioactive biotin incorporated into TCA precipitable protein was determined. (-▲-) liver cytosol. (-■-) adipose tissue cytosol.

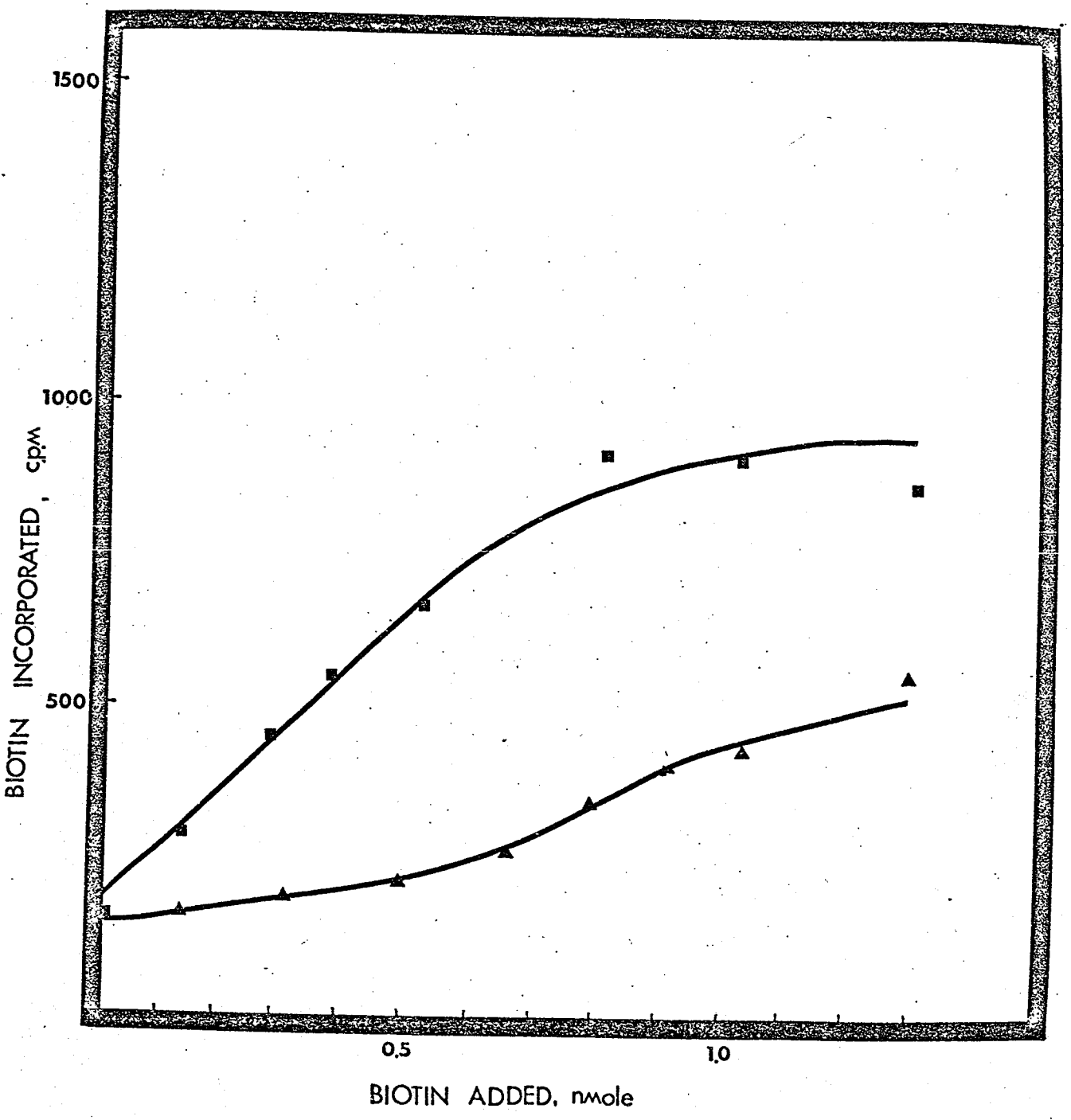

1500
1000
500
BIOTIN INCORPORATED, c.p.m.
0.5
1.0
BIOTIN ADDED, nmole

Figure 17: Incorporation of labelled biotin into cytosolic endogenous receptors in presence of "Biotin activating enzyme"

A time course for biotin incorporation into gel filtered cytosol of adipose tissue of biotin deficient rats was carried out in the presence (-●-) or absence (-▼-) of "Biotin activating enzyme". The proteins were in concentrations of 1.55 mg and 0.60 mg respectively.

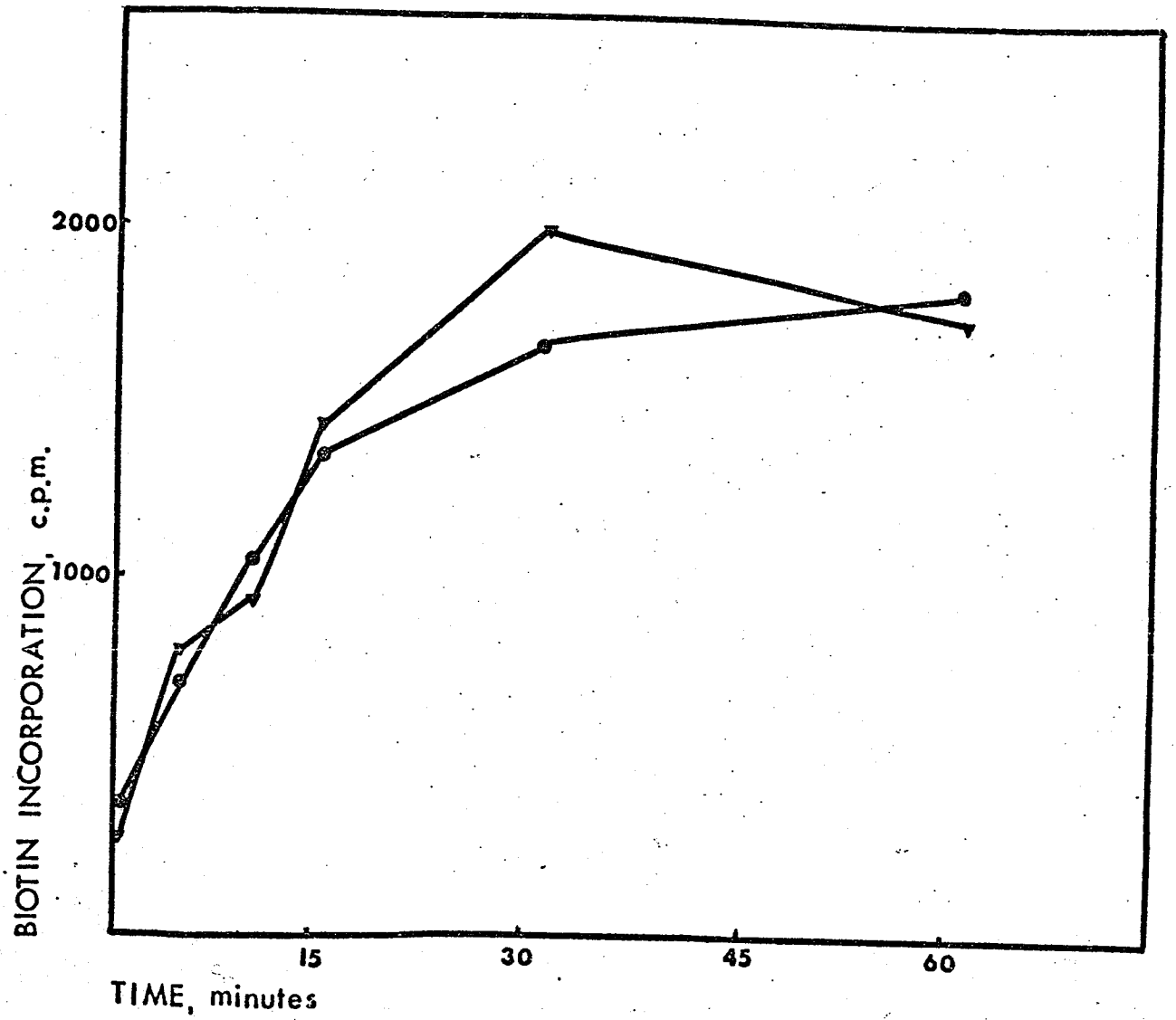
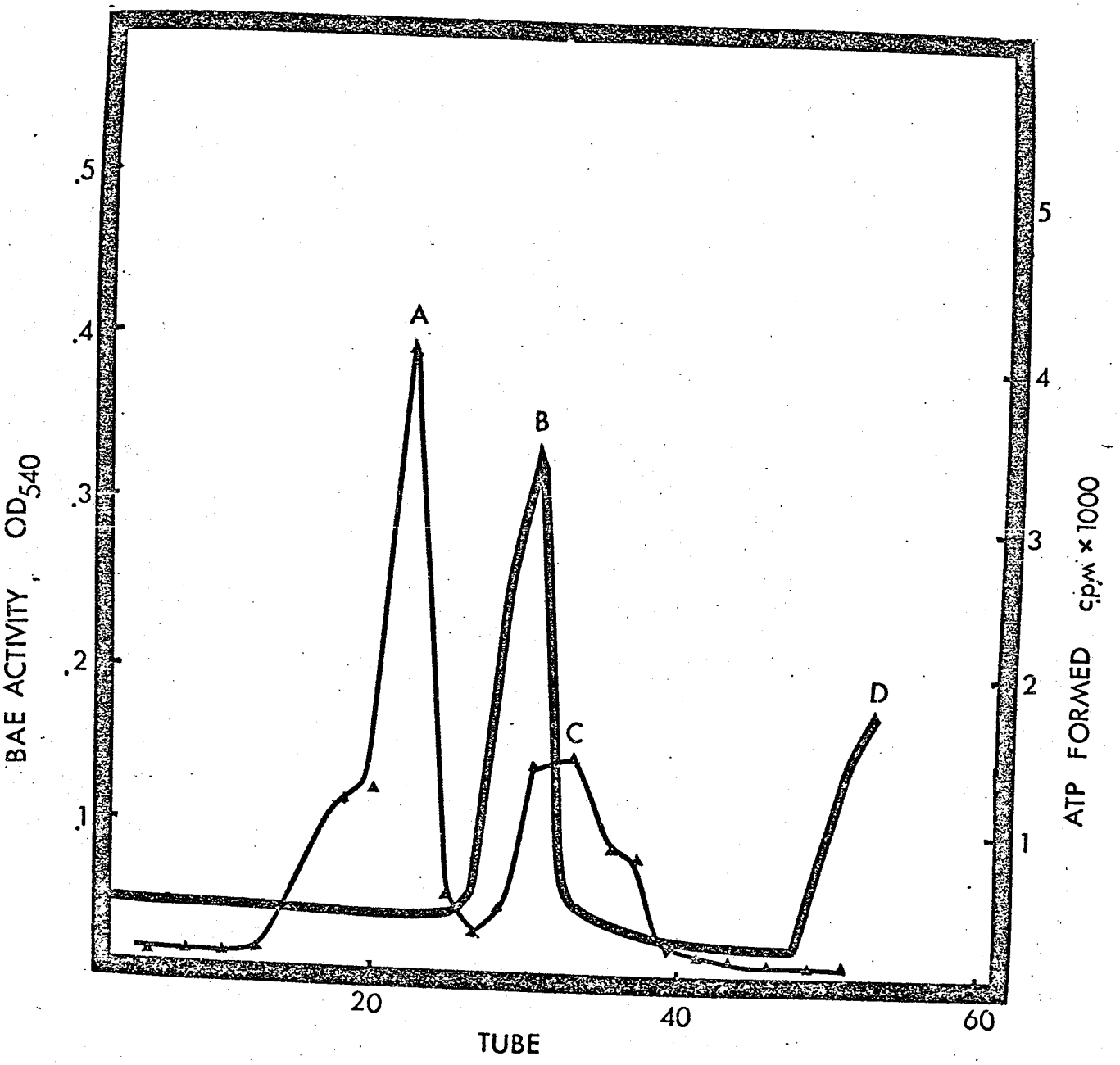

2000
1000
BIOTIN INCORPORATION, c.p.m.
15
30
45
60
TIME, minutes

Figure 18: ATP-PP_i exchange activity in porcine liver G-100 gel filtered cytosol

Porcine liver high speed supernatant was run on Sephadex G-100 2.5 x 85 cm in size. The column was eluted with a buffer containing 0.05 M Tris, 10 mM mercaptoethanol, 0.1 mM EDTA pH 7.5. Fractions were assayed for BAE (▬) and for ATP-PP_i exchange (——).


BAE ACTIVITY, OD_{540}
.5
.4
.3
.2
.1
A
B
C
D
20
40
60
TUBE
ATP FORMED c.p.m. × 1000
5
4
3
2
1

and fungi contain a set of enzymes that use naturally occuring hydroxylamine as a true substrate (Emery, 1971), this kind of enzyme has never been reported in mammals. The observation that the hydroxylamine used in the assay has only a chemical function, eliminates the possibility that this enzyme has a role similar to the fungal or bacterial enzymes. Similarly the fact that glutamic and aspartic acids did not interact in the presence of the "Biotin activating enzyme" with hydroxylamine, eliminates the possible identification of "Biotin activating enzyme" with glutamine synthetase and aspargine synthetase. These two enzymes can substitute their natural substrate, ammonia, with hydroxylamine, a chemical normally not occuring in the animal organism, to form glutamic and aspartic hydroxamates rather than aspargine and glutamine.

Ligases which incorporate acids with acceptors via AMP, are found in a variety of metabolic pathways. The fact that none of the amino acids tried were activated by the partially purified "Biotin activating enzyme", eliminates its possible identification with the amino acid activating enzyme (Berg, 1956). Similarly, its identity as the acetate activating enzyme was excluded. This is in line with the earlier conclusion by Coon et al. (1963) that "Biotin activating enzyme" is probably not the already known short and medium size fatty acid activating enzyme.

Hofner and Knappe (1965) have shown that highly purified bacterial holocarboxylase synthetase is capable of producing biotin hydroxamate via biotin adenylate. With the reservation that "pertinent proof is still missing" they make a distinction between this enzyme biotin:

apocarboxylase ligase (AMP) (E.C. 6.3.2) and the biotin activating enzyme defined as biotin: CoA ligase (AMP) (E.C. 6.2.1). With similar reservations Coon et al. (1963) postulate these two enzymes to be different, and attributed to the "Biotin activating enzyme" a function of forming biotinyl-CoA which is the initial compound in the degradative metabolism of biotin in bacteria.

Bacteria synthesize most of the biotin found in nature, and in most strains, the synthesizing capability is coupled with the ability to metabolize and degrade biotin to simpler organic compounds. This property however is not found in animals in which most of the biotin is excreted in the urine and feces essentially as free biotin. It is therefore difficult to attribute to the activating enzyme a function of forming biotinyl-CoA in animals, since it seems that this compound will not be metabolized further.

Literature data and results obtained in this study show several similarities between the synthetase and the activating enzyme. The activating enzyme is eluted on Sephadex G-100 in the same position as that of BSA which has a molecular weight of 68,000. This is within the range of the molecular weight of the bacterial holocarboxylase synthetase reported to be about 40,000 (Cazzulo et al. 1961), and 50,000-100,000 for the animal holocarboxylase synthetase as suggested by Hofner and Knappe (1965). Both enzymes can use nucleotides other than ATP as source of energy (Achuta Murthy and Mistry, 1974; Coon et al. 1964) although with less efficiency, and the affinity for ATP itself obtained in this study, 1.2 mM, is similar to that of 1.1 mM reported by Desjardins and Dakshinamurti (1971) for the rat holocarboxylase synthetase. Both enzymes

catalyze the exchange of ATP-PP_i and the formation of biotinyl hydroxamate via a similar mechanism. The fact that the partially purified activating enzyme could not compete or substitute for the synthetase, as demonstrated in Figure 17 seems to be a pertinent direct evidence that, regardless of any similarity, the holocarboxylase synthetase and the "Biotin activating enzyme" are distinct and separate enzymes. The results which show that the "Biotin activating enzyme" and the holocarboxylase synthetase have about 1000 fold difference in their affinities toward biotin seem to confirm this conclusion.

Although the synthetase is capable of forming biotinyl-AMP, the fact that the activity in rat and other animals is low and virtually undetectable by the hydroxamate method, and that unless highly purified enzyme is used, the strong hydrolytic activity will hinder the possible formation of hydroxamate, seem to exclude the use of biotinyl-AMP formation in the measurement of acetyl-CoA holocarboxylase synthetase activity.

CHAPTER 5: RADIOISOTOPE DILUTION ASSAY FOR BIOTIN

Before investigating the feasibility of lysine and lysine-rich histones acting as substrate for the acetyl-CoA holocarboxylase synthetase, it became apparent that an adequate method would have to be available for the determination of biotin and biocytin. A competitive protein binding assay was developed.

The assay used hitherto for the assay of biotin is a microbiological one which takes advantage of the stringent growth requirement of certain bacterial strains, particulary Saccharomyces Cerevisiae for biotin. This method is sensitive (0.1 to 1.0 mg/assay tube) but has the disadvantage of being time consuming. On the other hand the assay devised by Green (1963) which employs the dye HABA is quick and convenient; however, its sensitivity is several orders of magnitudes less and hence is unsuitable for the assay of the biotin content in bacteria or animal tissues. The method of radioisotope dilution assay was found effective as a sensitive and convenient assay for biotin as well as for biocytin.

In principle the assay takes advantage of the strong affinity between biotin and avidin. Unlabelled biotin in the analyzed sample competes with ^{14}C-biotin for the avidin. The avidin-biotin complex is then removed by adsorption on bentonite and centrifuging. The supernatant after bentonite treatment contains the unbound biotin. The radioactivity of the bentonite pellet containing the avidin-biotin complex is determined.

Experimental

a) Preparation of bentonite suspension: The problem of separating the biotin from the avidin-bound-biotin was explored in initial experiments

and adsorption on bentonite was found appropriate for this purpose. The method used was essentially that of Frankel-Conrat, Singer and Tsugita (1961). Bentonite (20 g) was suspended in 500 ml distilled water and stirred vigorously for 1-2 hours until a homogeneous suspension was obtained. This was then centrifuged at 3000 x g for 15 minutes. The supernatant was then centrifuged at 10,000 x g for 15 minutes and the pellet suspended in 20 ml of 0.2 M ammonium carbonate. Bentonite concentration was determined by drying an aliquot of the suspension at 60°C overnight and weighing the residue. Suitable dilution was made to give a suspension containing 10 mg bentonite per ml. The suspension can be kept for several weeks and did not precipitate.

b) Standardization of avidin: The possibility of loss of activity of the avidin preparation on prolonged storage, or even a short period of storage at low protein concentration, requires an accurate determination of the specific activity of the avidin preparation. The dye binding method of Green (1963) was used. Avidin (∿12 U/mg) was dissolved in 0.02 M potassium phosphate, pH 7.0 to a concentration of ∿1.2 U/ml. In a cuvette were combined 2.5 ml of 10 µM HABA in 0.02 M potassium phosphate, pH 7.0 and 0.5 ml avidin solution. The absorbance at 500 nm was read using a Beckman DB-G double beam spectrophotometer against a blank of the buffer. 10 µl aliquots of a solution of d-biotin (100 µM in 0.02 M potassium phosphate, pH 7.0) were added until no further decrease in absorbance at 500 nm was observed. The titration was repeated with the buffer alone to allow correction for dilution. From the amount of biotin required, the specific activity of the avidin solution was calculated to be 11.6 units. A working standard solution of avidin was made to a concentration of 0.232 units/ml.

c) Determination of the equivalence between the Avidin and ^{14}C-Biotin solutions: In a series of Eppendorf centrifuge tubes were placed 100 µl of 0.2 M ammonium carbonate solution and 60 µl of ^{14}C-biotin (46 mCi/m mole) solution (∿4200 cpm) and mixed well. Varying volumes (0-100 µl) of the standard avidin solution prepared as above were added to the tubes and allowed to mix for 5 minutes in an Eppendorf shaker at room temperature. Following this 0.3 ml of the bentonite suspension was added and the tubes were kept in the shaker for another 5 minutes. The tubes were then centrifuged in an Eppendorf centrifuge for 20 seconds. The supernatant was discarded. The pellet was washed with 0.5 ml of 0.2 M ammonium carbonate solution and quantitatively transferred to a scintillation vial using two aliquots respectively of 0.5 and 0.2 ml absolute ethanol. Fifteen ml of the scintillator containing 0.25% PPO (2,5-diphenyloxazole), 0.009% POPOP (1,4-bis 2(5phenyl oxazolyl) benzene) and 31% ethanol in toluene was added to the vial for determining the radioactivity in a Beckman LS 250 Liquid Scintillation Spectrometer.

d) Biotin calibration plot: To a series of Eppendorf centrifuge tubes were added 100 µl of 0.2 M ammonium carbonate, 60 µl of ^{14}C-biotin (4360 dpm and 11.6 ng) and increasing amounts (0-20 ng) of cold biotin in a volume of 50 µl. After thorough mixing in a shaker for 5 minutes 0.3 ml of bentonite suspension was added. The precipitation of the avidin-biotin complex with the bentonite, washing, quantitative transfer to scintillation vials and determination of radioactivity were done as described above.

We found that increased salt concentration in the medium consistently increased the radioactivity associated with the avidin-biotin

complex. Hence for all subsequent calibration plots we added 100 µl of a vitamin-free casein hydrolysate (150 mg vitamin-free casein hydrolyzed using the same procedure for tissues) before adding the non-radio active biotin. Thus, the conditions of calibration and assay of tissue hydrolysates were identical.

e) Hydrolysis of tissue to release biotin completely: Varying amounts (150-200) mg) of freeze-dried tissue containing 100-1000 ng biotin were hydrolyzed in 4 ml of 8N H_2SO_4 by autoclaving at 15 lbs pressure for 2 hours. The hydrolysates were neutralized with saturated NaOH, made up to 10 ml and filtered on a "course" grade filter paper.

f) Application of the assay procedure to tissue or biotin-protein hydrolysate: To a triplicate set of tubes were added 100 µl of 0.2 M ammonium carbonate, 60 µl (4360 dpm and 11.6 ng) of carbonyl ^{14}C-biotin and 100 µl of the tissue or protein hydrolysate. The contents were mixed thoroughly before adding 50 µl of the avidin solution. The tubes were left in a shaker for 5 minutes. All further operations were performed as described earlier. The actual amount of biotin in the sample was calculated by reference to the calibration plot. Although there is little variation from day to day, in practice, a calibration plot was obtained whenever a set of unknown samples are assayed.

g) Recovery of biotin: To a set of 4 tubes in duplicate were added 100 µl of liver hydrolysate and 50 µl of biotin solution containing respectively 2,4 and 6 ng biotin. Another duplicate set containing only the protein hydrolysate served as the control. Biotin was assayed in all the tubes and the recovery calculated.

Replicate sets of freeze-dried rat liver preparations were treated

as follows: to three sets were added respectively 200, 300 and 400 ng biotin and the fourth served as the control. Hydrolysis and assay of the biotin content were done as described above. From the difference between the sets, the recovery of added biotin was calculated.

Results and Discussion

The standardization of avidin (including commercially purchased material of declared specific activity) and ^{14}C-biotin solutions to obtain their exact specific activities and equivalence is a necessary pre-requisite for the success of this procedure. The specific activity of the avidin solution is first determined with reference to a sample of biotin using the dye (HABA) method. Figure 19 is a representative curve used to determine the equivalence between the avidin and ^{14}C-biotin. From the curve the concentration of the ^{14}C-biotin solution was calculated to be 193 ng/ml.

The calibration curve for biotin, plotted on a semi log basis is given in Figure 20. The avidin-biotin complex is more stable in a medium of high salt concentration (Wei and Wright, 1964). This is seen in a calibration plot which is essentially parallel except that it is higher, i.e. counts associated with avidin-biotin complex are higher, indicating greater stability in the medium of higher salt concentration.

Recovery of biotin added to the hydrolysate ranged between 97.5 to 105% as seen in Table 18. The recovery of biotin added to the tissue preparation prior to hydrolysis is in the same range (Table 19). Biotin assays were done on aliquots of the same sample of rat liver. The average of 6 determinations using the isotope dilution assay are 1850 ± 65 ng/per g liver.

Authentic biocytin was assayed by the same method in parallel with biotin. The pattern obtained was similar (Figure 21) and indicates that the assay can be applied for this biotin derivative.

Figure 19: Equivalence between avidin and ^{14}C-biotin

Varying amounts of avidin were titrated with ^{14}C-biotin after which the avidin-biotin complex was adsorbed on bentonite. The mixture was then centrifuged. The supernatant containing free biotin was discarded while the pellet containing the avidin-biotin complex was counted.

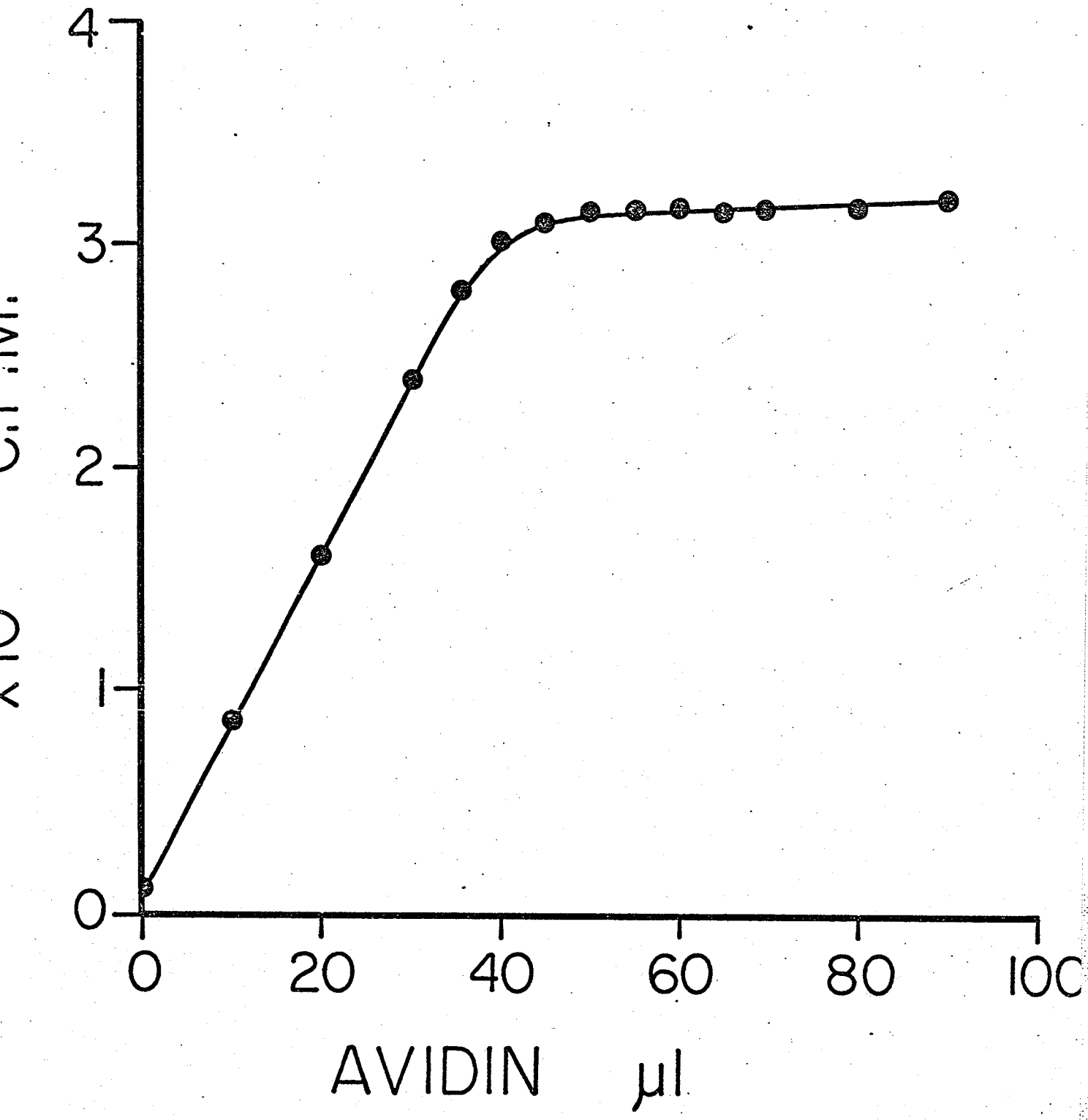

4
3
2
1
0
0
20
40
60
80
100
AVIDIN µl

Figure 20: Biotin calibration plot

Varying amounts of unlabelled biotin were added to a series of tubes which contained avidin and labelled biotin. The tubes were than treated as described in the Experimental section. Curve A (—●—) represent an assay performed in $(NH_4)_2CO_3$ as medium, curve B (—■—) represent assay performed in presence of casein hydrolyzate.

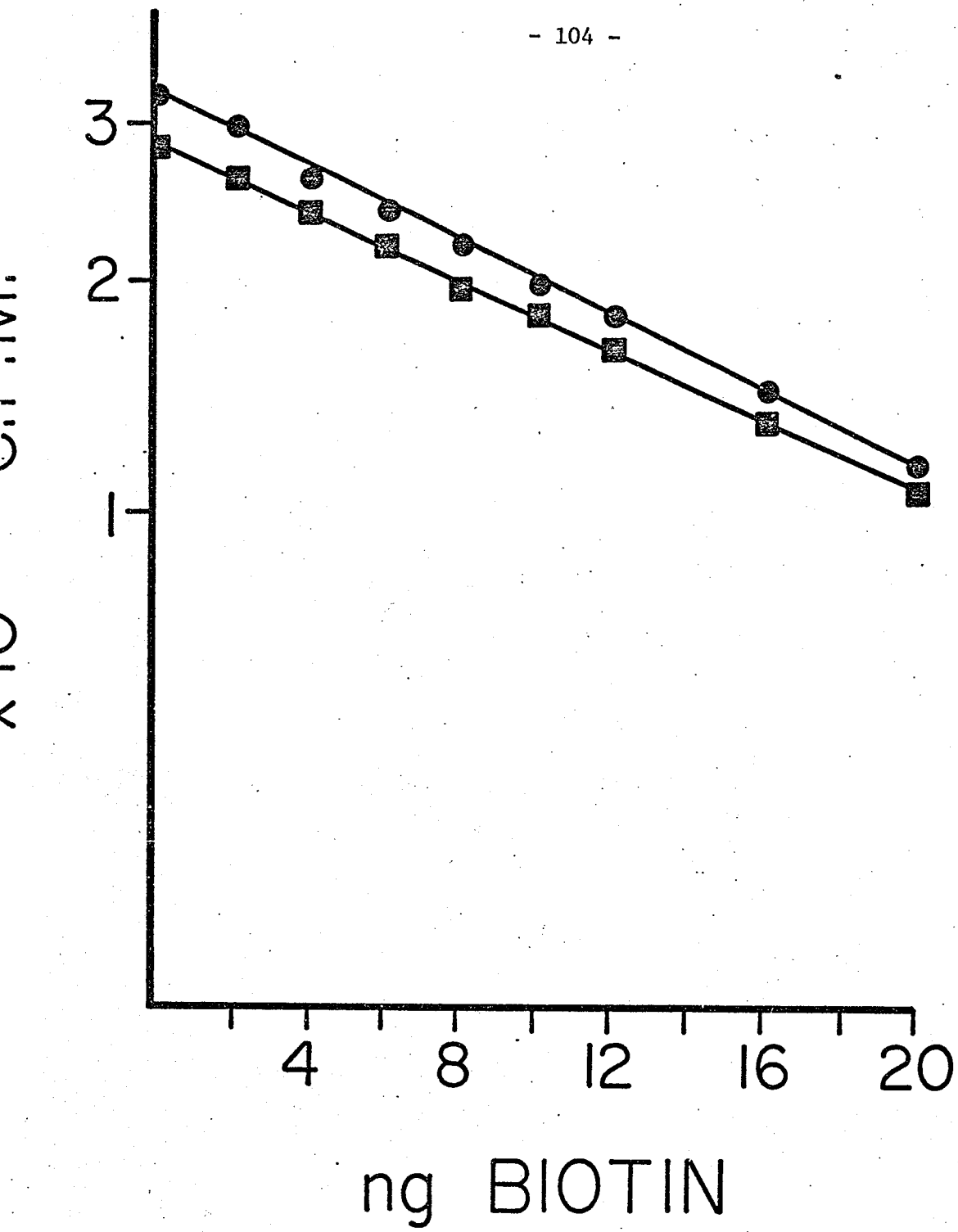

3
2
1
4
8
12
16
20
ng BIOTIN

Figure 21: Calibration curves for the assay of biotin and biocytin

Biotin or biocytin were diluted to give concentrations of 20 ng per 50 µliter. Increasing amounts of these preparations were mixed with 50 µliter (C^{14}-carbonyl) D-biotin 56 mC_i/mmole, containing 3740 c.p.m. This was followed by 200 µliter ammonium acetate 0.5 M, and 30 µliter avidin containing 0.02 units, the mixture was processed as described in the text.

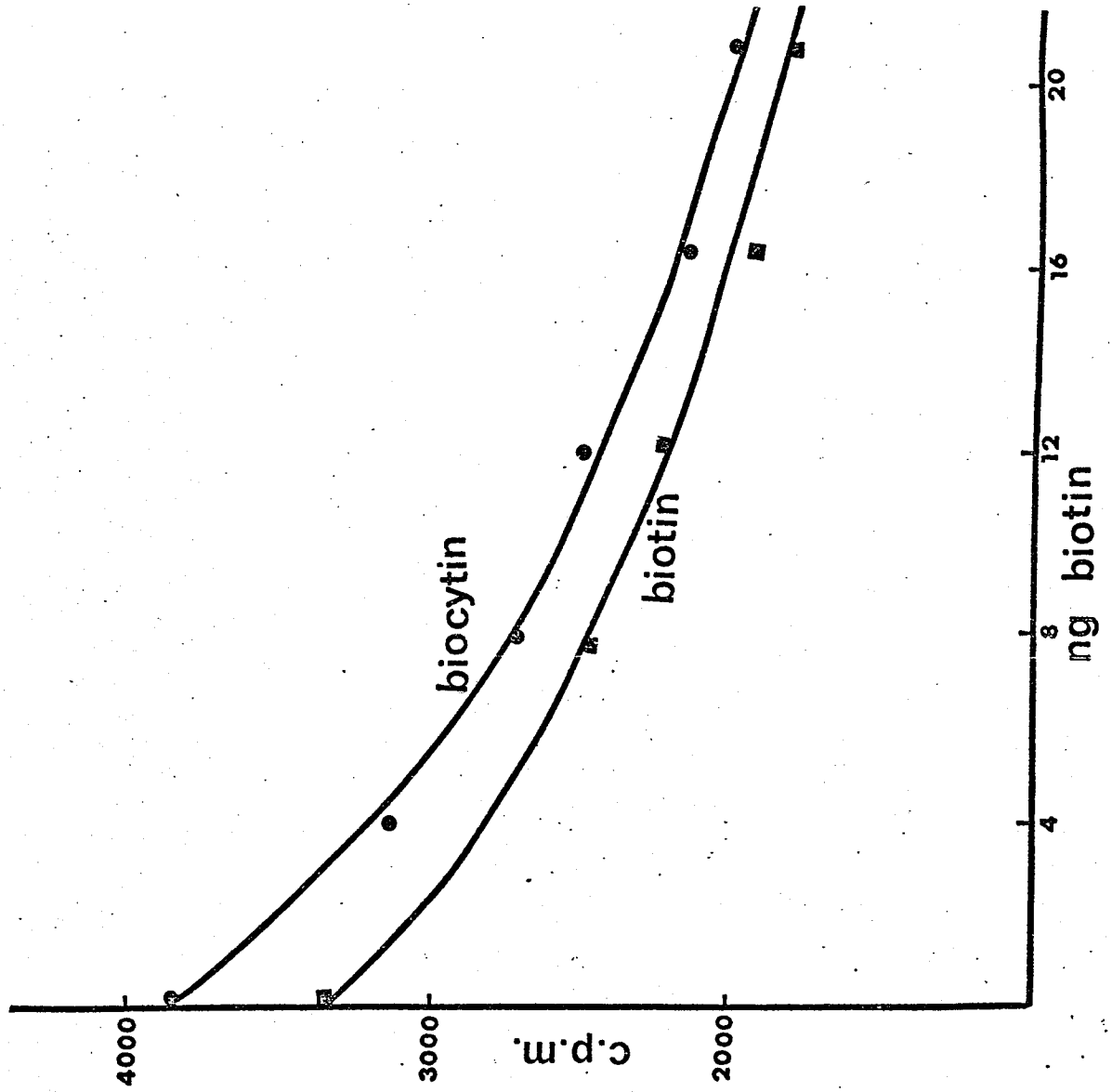

biocytin
biotin
c.p.m.
4000
3000
2000
4
8
12
16
20
ng biotin

Table 18: Recovery of biotin added to tissue before hydrolysis

Sample No.	Biotin added ng	C.P.M. biotin-avidin	ng biotin from calibration plot	Recovery of added biotin %
1	0	2083	7.7	
2	200	1905	9.9	110
3	300	1872	10.6	97.5
4	400	1732	11.8	102.5

Various amounts of biotin were added into a liver homogenate which was subsequently hydrolyzed with 6N H_2SO_4. The hydrolyzate was than neutralyzed with NaOH and assayed for biotin as described in the experimental part.

Table 19: Recovery of biotin added to a tissue hydrolyzate

Sample No.	Biotin added to hydrolysate ng	C.P.M. biotin-avidin complex	ng biotin from calibration plot	Recovery of added biotin	
				ng	%
1	0	2083	7.7		
2	2.0	1909	9.8	2.1	105
3	4.0	1778	11.6	3.9	97.5
4	6.0	1610	13.7	6.0	100

Various amounts of biotin were added to rat liver homogenate which was hydrolyzed earlier. Aliquots of the samples were assayed for biotin as described in the Experimental part.

GENERAL DISCUSSION AND CONCLUSION

The nature of this study required that a substantial part of the investigation be oriented towards methodology. The basic issues were the isolation of acetyl-CoA apocarboxylase free of active holocarboxylase and the search for a convenient method to identify and assay acetyl-CoA holocarboxylase synthetase which could be used on a routine basis.

Isolation of acetyl-CoA apocarboxylase was obtained by affinity chromatography on Sepharose-avidin column, which binds the active holocarboxylase through the biotin prosthetic group while permitting the apocarboxylase to be eluted freely. The properties of the complex avidin-biotin appear to be particularly suitable for this technique.

Finding a convenient assay for acetyl-CoA holocarboxylase synthetase represented a more complex problem since the available information indicated, that the enzyme has specific requirements with regard to its substrates, in that it will incorporate biotin only to the appropriate apocarboxylase. The approach taken to devise an assay was to use the partial reactions by which the biotin is being incorporated into its receptors. It has been reported that an enzyme described as "Biotin activating enzyme" is capable of binding biotin with ATP to form biotinyl-AMP. Since the physiological role of this enzyme was not clear it was thought that an investigation into the possible identity of this enzyme with the holocarboxylase synthetase may provide pertinent information. The results obtained demonstrate, however, that although the two enzymes share a common reaction they seem to be distinct from each other. Further attempts to elucidate the physiological role of the "Biotin activating enzyme" were not successful.

The catalytic mechanism of acetyl-CoA holocarboxylase synthetase allows the biotin dependent ATP-PP_i exchange. This reaction was utilized to assay the enzyme and some of the technical problems involved with the conversion of this partial reaction into a routine assay were solved. The limitation of the assay which was developed appears to be the interference of some exchanges of ATP-PP_i which are dependent on endogenous metabolites, and the method can therefore be used only in purified preparations where the enzymes involved with these exchanges have been removed.

The specificity of the holocarboxylase synthetase was further demonstrated when attempts were made to utilize histones and polylysine, which are rich with lysines, as substrates for the binding with biotin. These attempts which failed further indicated that the incorporation of biotin into the apocarboxylase through the ϵ-amino group of lysine requires that functional groups adjacent to the lysine will orient the holocarboxylase towards a position favouring the incorporation of the prosthetic group.

Another analytically oriented study undertaken through this thesis, was devising a convenient and accurate assay to measure biotin and biocytin. The radioisotope dilution assay which was developed appears to be advantageous with regard to convenience and speed over the microbiological methods used until now for the assay of biotin. However, since the sensitivity of the assay is dependent on the specific activity of the tracer used, it appears that the sensitivity range of the developed method is less than that of the microbiological assay.

Acetyl-CoA carboxylase exists in two enzymic forms. It is inactive

as a protomer and highly active when aggregated. It is conceivable that if the inactive apoform was able to fluctuate similarly between the two forms, then it may interact with the holocarboxylase synthetase in two different fashions. Results obtained in this study demonstrate that the apocarboxylase is not capable of aggregation and therefore it is concluded that the interactions between the holocarboxylase synthetase and its substrate have only a single form. It may be of interest to investigate further if the incorporation of biotin into other biotin containing enzymes which possess multiple sites of biotin, takes place while the individual subunits of these enzymes interact with the holocarboxylase synthetase prior to their aggregation, or whether the incorporation of the prosthetic group is performed subsequent to the aggregation of the subunits not containing biotin.

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