PURIFICATION AND CHARACTERIZATION OF RAT LIVER BIOTINIDASE

A Thesis Presented to the

Faculty of Graduate Studies

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In Partial Fulfilment

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for the Degree of

MASTER OF SCIENCE

by

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To my parents, my brother,

and my sister

SUCCESS

To laugh often and much; to win the respect of intelligent people and the affection of children; to earn the appreciation of honest critics and endure the betrayal of false friends; to appreciate beauty, to find the best in others; to leave the world a bit better, whether by a healthy child, a garden patch or a redeemed social condition; to know even one life has breathed easier because you have lived. This is to have succeeded.

- Ralph Waldo Emerson

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ABSTRACT

Biotinidase (E.C. 3.5.1.12) hydrolyzes biocytin (ϵ-N-biotinyllysine), product of proteolytic digestion of biotin-containing proteins, to the vitamin biotin and lysine. Among the rat tissues determined for biotinidase activity, serum had the highest specific activity. Liver, kidney, and intestine also had relatively high biotinidase activity whereas activities in heart and brain were low but detectable. Rat liver biotinidase was found to be completely membrane-associated with specific activity highest in the microsome. The enzyme was purified 25,000-fold with a 12 % recovery using an 8-step procedure. After obtaining membrane fraction by centrifugation, the pellet was resuspended and biotinidase was solubilized by sodium deoxycholate. The solution was then centrifuged and the supernatant was heated to 60 °C for 12 min. The sample was then centrifuged to remove denatured proteins. The pellet was discarded and the supernatant was fractionated by ammonium sulfate cut (30-55%). The enzyme was further purified using DEAE-Sephacel, Phenyl-Sepharose, and Sephacryl 100HR columns. The purified enzyme had a specific activity of 1,800 nmol/min/mg and showed a single silver staining band with polyacrylamide gel electrophoresis under both denaturing and non-denaturing conditions and had a molecular weight of 61 KDa using SDS-PAGE. This suggests that rat liver biotinidase is a single polypeptide chain. Gel filtration analysis using Sephacryl S200 gave a molecular weight of 70 KDa. Rat liver biotinidase has a broad pH optimum (pH 5 -

7.5) with N-(d-biotinyl) p-aminobenzoate as substrate. Kinetic studies with this substrate revealed a *Km* value of 33 uM. Rabbit antibody was raised against the purified rat liver biotinidase. Its specificity was demonstrated by the ability to inhibit biotinidase activity. This antibody may be used to study the biosynthesis and secretion of biotinidase in rat tissues.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ADP :	Adenosine-5′-diphosphate
ATP :	Adenosine-5′-triphosphate
BBP :	Biotin binding protein
BCIP :	5-bromo-4-chloro-3-indoyl phosphate-p-toluidine salt
cGMP :	Cyclic guanine monophosphate
CoA :	Coenzyme A
DEAE :	Diethylaminoethyl
DNA :	Deoxyribonucleic acid
EDTA :	Ethylenediaminetetraacetic acid
GMP :	Guanine monophosphate
IgG :	Immunoglobulin G
KDa :	Kilodalton
mRNA :	messenger RNA
NBT :	p-nitro blue tetrazolium chloride
NP-40 :	Nonidet P-40
PAGE :	Polyacrylamide gel electrophoresis
Pi :	inorganic phosphate
PMSF :	Phenyl methyl sulfonyl fluoride
RNA :	Ribonucleic acid
S.D. :	Standard deviation
SDS :	Sodium dodecyl sulfate
Tris :	Tris(hydroxymethyl)aminomethane

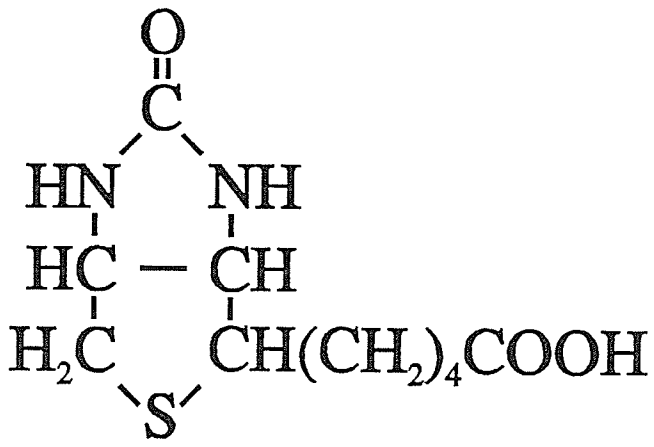
CHAPTER I

LITERATURE REVIEW

1. Biotin : Its discovery, structure and biogenesis

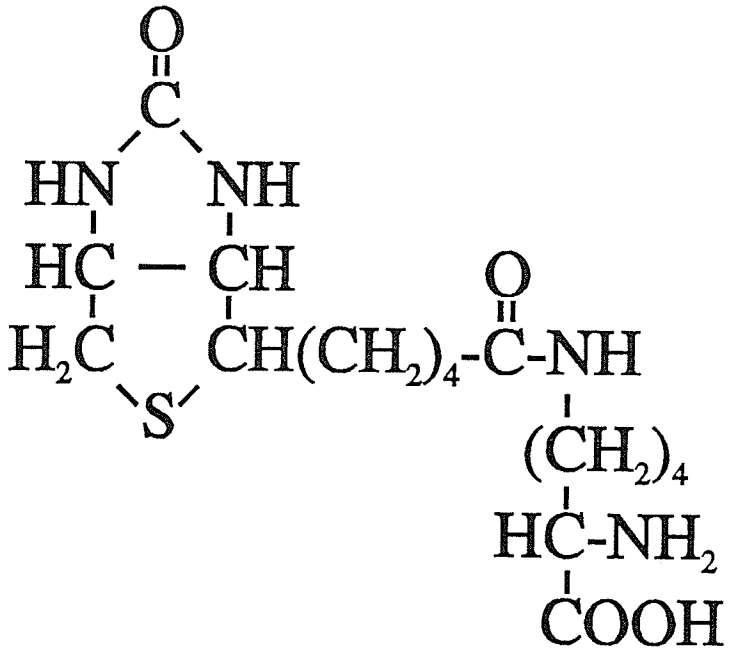
As early as 1901, Wilders (1901) found that yeast could only be grown in salt and sugar solutions if small amounts of beef extract or peptone was added. He proposed that an active factor is present which can promote yeast growth and named it "bios". It was subsequently discovered that a diet high in egg white resulted in dermatitis, hair loss, and neurological abnormalities in rats (Boas, 1924, 1927; Parsons, 1931; Parsons and Kelly, 1933) and was designated as "egg-white injury" syndrome. This syndrome could be prevented by a compound present in potato starch, yeast, egg yolk, milk, and liver. Kögl and Tönnis (1936) isolated this compound and named it biotin. The chemical structure of biotin was later determined by Du Vigneaud *et al.* (1942). Biotin was first synthesized chemically in 1943 (Harris *et al.*, 1943) and its crystal structure was eventually determined (Traub, 1956). The stereospecific synthesis of the natural isomer, d-(+)-biotin, was identified in 1975 (Confalone *et al.*, 1975; Ohrui and Emoto, 1975) and biotin was shown to be a (+)-cis-hexahydro-2-keto-1,H-thieno-(3,4)-imidazole-4-valeric acid (Figure 1a). Enzymatic digestion of biotin containing proteins releases biocytin (Figure 1b) which is a biotin molecule covalently attached to the ϵ-amino group of lysine (Lynen, 1967). Biotin is degraded by microorganisms (Christner *et al.*, 1964) forming urea, ammonia, bicarbonate, and L-cysteine (Brady *et al.*, 1966). In animals biotin seems to be excreted undegraded in the form of biotin or biotin sulfoxide in urine (Baxter and Quastel, 1953; Mistry and Dakshinamurti, 1964).

a)



Biotin

b)



Biocytin

Figure 1. Structure of biotin and biocytin.

2. Functions of biotin

2.1. Prosthetic group functions

2.1.1. General

Biotin is best known for its classical role as the prosthetic group of biotin containing enzymes. It is essential for a small number of enzymes that carry out very different metabolic functions. Biotin generally serves as a carbon dioxide carrier. It is covalently linked to the ϵ-amino group of a lysine residue in biotin enzymes. Biotin-containing enzymes can be divided into three classes: Class I biotin enzymes, carboxylases that carry out ATP-dependent carbon dioxide fixation into an acceptor such as pyruvate, acetyl-CoA, propionyl-CoA, 3-methylcrotonyl-CoA, geranyl-CoA, and urea; Class II biotin enzymes, decarboxylases that facilitate sodium transport in anaerobes coupled to the removal of CO_2 from ß-keto acids and their thioesters, including oxalacetate, methylmalonyl-CoA, and glutaconyl-CoA. Class III biotin enzyme, transcarboxylase which transfers a carboxyl group from a donor (oxalacetate) to an acceptor (propionyl-CoA). Mechanisms of biotin enzymes were reviewed in detail by Wood (1977) and Jeremy (1989).

2.1.2. Biotin enzymes in mammalian tissues

Only four biotin enzymes have been identified in mammals. They are acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase, and 3-methylcrotonyl-CoA carboxylase. Each of these carboxylases carries out an ATP-dependent CO_2 fixation reaction. Figure 2 depicts the involvement of these four

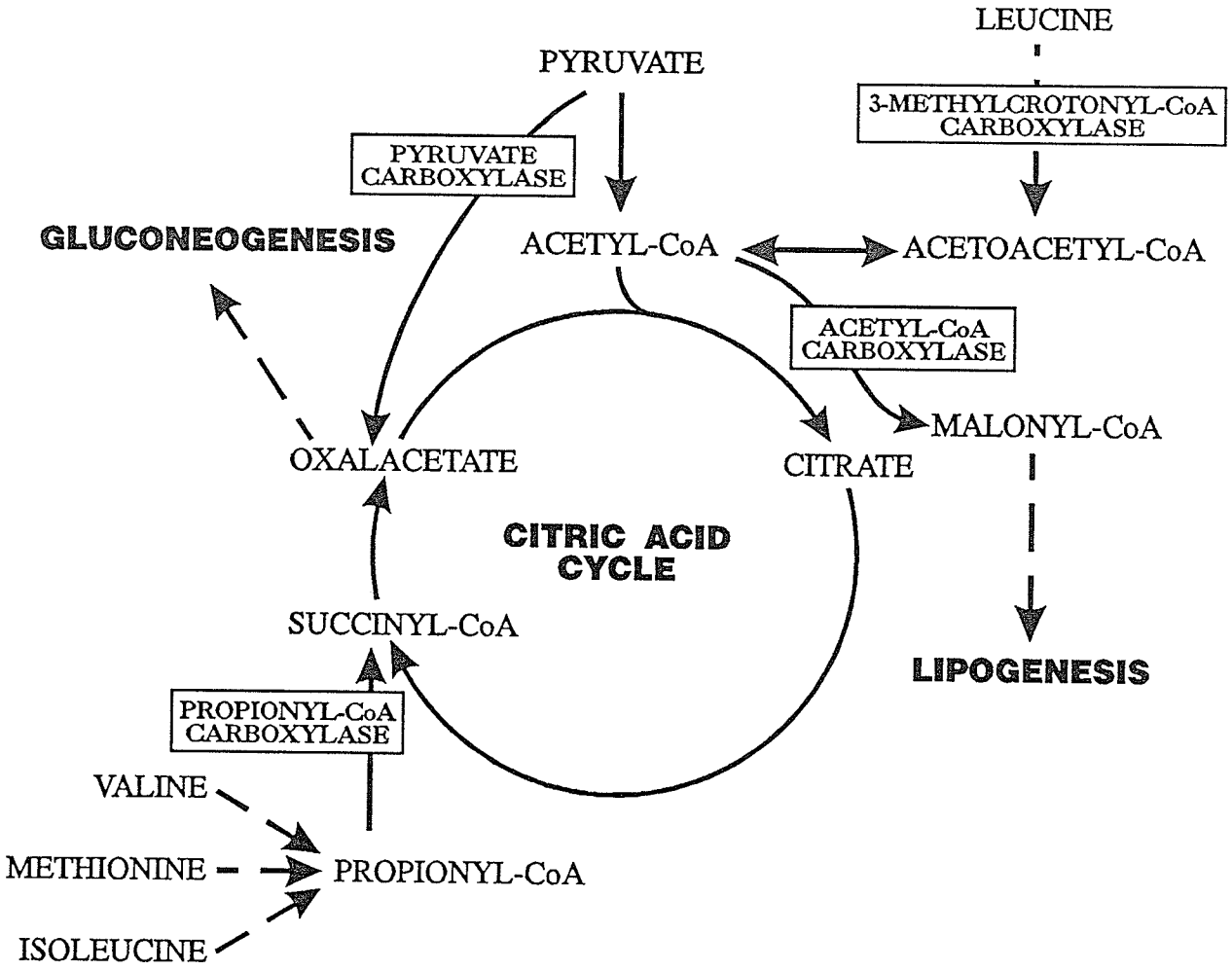


Figure 2. Biotin-containing carboxylases in cellular metabolism. (Adapted from Dakshinamurti and Chauhan, 1985.)

biotin enzymes in cellular metabolism. Acetyl-CoA carboxylase is the only cytosolic enzyme whereas the other three are located in mitochondria. The enzymes acetyl-CoA carboxylase and pyruvate carboxylase are regulated *in vivo* whereas propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase are not (Dakshinamurti and Chauhan, 1988). The functions of these four carboxylases are discussed below.

2.1.3. Acetyl-CoA carboxylase

Acetyl-CoA carboxylase (E.C. 6.4.1.2) is an important enzyme in lipogenesis. It catalyzes the committed step in fatty acid synthesis that converts acetyl-CoA to malonyl-CoA:

$$\text{Acetyl-CoA} + \text{ATP} + \text{HCO}_3^- \rightleftharpoons \text{Malonyl-CoA} + \text{ADP} + \text{P}_i$$

This enzyme has been purified from various sources such as bovine adipose tissue (Moss and Lane, 1972), rat liver (Inoue and Lowenstein, 1972), rat mammary gland (Ahmad *et al.*, 1978), rabbit mammary gland (Hardie and Cohen, 1978), chicken liver (Beaty and Lane, 1982), and rat adipose tissue (Ramakrishna and Benjamin, 1983). Purified acetyl-CoA carboxylase from these tissues is a protomer consisting of two identical subunits of molecular weight 220-260 KDa. Moss and Lane (1972) showed that this enzyme exists in equilibrium between the inactive protomeric form and a high molecular weight active polymeric form (4,000-10,000 KDa). Acetyl-CoA carboxylase activity is under both long-term and short-term controls.

Short-term control involves the regulation of the enzyme activity by a variety of factors. Citrate was shown to increase acetyl-CoA carboxylase activity *in vitro*

by inducing a conformational change in the enzyme and subsequent polymerization of the enzyme molecules (Beaty and Lane, 1983a, 1983b). Carboxylation of the biotinyl group of the polymerized enzyme leads to depolymerization. Furthermore, long-chain fatty acyl-CoA was found to inhibit enzyme activity by promoting depolymerization (Ogiwara *et al.*, 1978). Therefore, the equilibrium between active polymer and inactive protomer is affected by the concentrations of citrate, fatty acyl-CoA, and malonyl-CoA. Coenzyme A also activates the enzyme by lowering the *Km* for acetyl-CoA (Yeh *et al.*, 1981).

Long-term control involves the regulation of the amount of the enzyme protein by hormonal, nutrient, and dietary conditions (Dakshinamurti and Chauhan, 1988). Insulin and glucagon were the most studied effectors of acetyl-CoA carboxylase. In rat hepatocyte this enzyme was stimulated two-fold with 0.1 uM insulin and the increase in enzyme activity was prevented by amanitin or cordycepin (Katz and Ick, 1981; Giffhon and Katz, 1984). This suggests that acetyl-CoA carboxylase is regulated at the transcriptional level by insulin. Stimulation of acetyl-CoA carboxylase synthesis by insulin has also been shown in human skin fibroblast (Shafrir and Bierman, 1981) and chicken liver cells (Fisher and Goodridge, 1978). In addition, insulin and epidermal growth factor were found to stimulate lipogenesis and acetyl-CoA carboxylase activity (Haystead and Hardie, 1986). Bhuller and Dakshinamurti (1985a) showed that addition of glucagon resulted in a decrease of 50 % of acetyl-CoA carboxylase activity in HeLa cells and this was accompanied by a corresponding decrease in the enzyme protein. Acetyl-CoA carboxylase has also

been shown to be regulated by phosphorylation/deposphorylation (Dakshinamurti and Chauhan, 1988). Phosphorylation causes inactivation of the enzyme.

2.1.4. Pyruvate carboxylase

Pyruvate carboxylase (E.C. 6.4.1.1) is a key enzyme of gluconeogenesis in liver and kidney. It catalyzes the following reaction:

$$\text{Pyruvate} + \text{ATP} + HCO_3^- \rightleftharpoons \text{Oxalacetate} + \text{ADP} + P_i$$

This enzyme also has an anapleurotic role in replenishing oxalacetate in the citric acid cycle. Pyruvate carboxylase was found in other lipogenic tissues including adipose, lactating mammary gland, and adrenals and it participates in fatty acid synthesis by transporting citrate and malate from mitochondria to cytosol. This enzyme has been isolated from various sources. It is a homopolymer of identical subunits, with a subunit molecular weight of 125 KDa (Barden *et al.*, 1975).

Pyruvate carboxylase activity is affected by a number of compounds. Acetyl-CoA, the substrate pyruvate, Mg^{2+}, and K^+ activate the enzyme (Utter and Keech, 1963; McClure and Lardy, 1971a, 1971b; Scrutton and Fung, 1972) whereas ADP and SO_4^{2-} inhibit the enzyme activity (Scrutton and Fung, 1972). Long-term regulation of pyruvate carboxylase is dependent on the insulin status of the animal. The amount of enzyme protein was shown to increase in the liver of diabetic and hyperthyroid rats and to decrease in thyroidectomized rats (Weinberg and Utter, 1979, 1980).

2.1.5. Propionyl-CoA carboxylase

Propionyl-CoA carboxylase (E.C. 6.4.1.3) is important in the degradation of odd-chain fatty acids and branched-chain amino acids including isoleucine, leucine, threonine, methionine, and valine (Rosenberg, 1983). This enzyme catalyzes the following reaction:

$$\text{Propionyl-CoA} + \text{ATP} + HCO_3^- \rightleftharpoons \text{Methylmalonyl-CoA} + \text{ADP} + P_i$$

Methylmalonyl-CoA is then converted to succinyl-CoA and enters the citric acid cycle. Propionyl-CoA carboxylase allows propionate to be utilized in the citric acid cycle. This enzyme has been purified from human liver (Gravel *et al.*, 1980; Kalousek *et al.*, 1980) and sheep liver (Goodall *et al.*, 1985). The human enzyme has a molecular weight of 540 KDa and a subunit structure of $\alpha_4\beta_4$ whereas the sheep enzyme has a molecular weight of 730-840 KDa and a proposed $\alpha_6\beta_6$ subunit configuration.

2.1.6. 3-methylcrotonyl-CoA carboxylase

3-methylcrotonyl-CoA carboxylase carries out the reaction below:

$$\text{3-methylcrotonyl-CoA} + \text{ATP} + HCO_3^- \rightleftharpoons \text{3-methylglutaconyl-CoA} + \text{ADP} + P_i$$

This enzyme is involved in the degradation of leucine and some isopreniod compounds. It has been purified from bovine kidney (Hector *et al.*, 1980) and *Achromobacter* (Schiele *et al.*, 1975). The kidney enzyme has two subunits of 62 KDa

and 80 KDa. The *Achromobacter* enzyme is a tetramer of two non-identical subunits with molecular weights 78 KDa and 96 KDa, respectively.

2.2. Non-prosthetic group functions

2.2.1. Presence of biotin in nuclei

Dakshinamurti and Mistry (1963a, 1963b) showed that a significant amount of biotin is associated with the nuclear fraction in various tissues of rat and chicken. Boeckx and Dakshinamurti (1974, 1975) later demonstrated that while biotin content of biotin-deficient rat liver is 10 % of the normal amount, 20 % of this residual biotin is present in the nuclear fraction. The relatively high concentration of biotin in the nuclear fraction of rat liver and HeLa cells was further confirmed by Chalifour (1982) and biotin was found to be protein bound. This biotin-binding protein in nuclei was subsequently isolated (Bhuller, 1985). Activity of any of the biotin carboxylases was not detected in the nuclear fraction. The presence of significant amount of biotin in the nuclei, the absence of biotin carboxylase activity in the nuclei and the conservation of nuclear biotin in the biotin deficient rat liver suggest that biotin may have some function other than that of a prosthetic group of the biotin carboxylases.

2.2.2. Role of biotin in cell growth

In view of the involvement of biotin enzymes in several major metabolic pathways including gluconeogenesis, lipogenesis, and catabolism of certain amino

acids, it would be expected that biotin is required for cells growing in culture. However, some early reports suggested that biotin is not required for cells in culture (Eagle, 1955; Swim and Parke 1958; Holmes, 1959; Dupree *et al.*, 1962). Keranen (1972) found that HeLa cells growing in biotin-deficient medium contained more biotin than those growing in biotin-supplemented medium. He speculated that transformed cells may acquire the ability to synthesize biotin *de novo*.

Dakshinamurti and Chaulifour (1981) and Chaulifour and Dakshinamurti (1982a, 1982b) showed that there is an obligatory biotin requirement for cultured cells. They demonstrated the requirement for biotin for HeLa cells, human fibroblasts, and Rous Sarcoma virus-transformed baby hamster kidney cells based on cell viability, biotin content, and activities of both biotin-dependent and -independent enzymes. Bhuller and Dakshinamurti (1985b) further substantiated the biotin requirement by demonstrating a significant decrease in the rate of protein, RNA, and DNA synthesis in biotin-deficient HeLa cells which could be restored by the addition of biotin to the medium. In view of the growth promoting effects of biotin, including protein synthesis, DNA synthesis, and cell growth in HeLa cells, biotin may stimulate production of certain proteins which in turn stimulate cell growth. The involvement of biotin in cell growth was also supported by the discovery of Collins *et al.* (1988) that biotin is required for the expression of asialoglycoprotein receptor in HepG2 cells. Asialoglycoprotein receptor mediates endocytosis of asialoglycoproteins which lead to their lysosomal degradation (Stockert and Morell, 1983). The expression of this receptor has been shown to be dependent on the differentiated state of

hepatocytes. In rats and mice, development of the receptor occurs late in primary differentiation. However, the amount of receptor was 2-3 fold higher in pregnant mouse liver just before parturition than in normal adult mouse liver (Collins *et al.*, 1984).

Shaw and Phillips (1942) reported that testes of biotin-deficient rats were visually much smaller than those of the normal rats. In addition, seminiferous tubules were very small and showed signs of degeneration. A more systematic comparison was done by Delost and Terroine (1956) who found that the testes size was smaller and weighed less in the biotin-deficient animals. They also observed a delayed spermatogenesis and a reduction in the number of spermatozoa. Terroine (1960) later suggested that these changes were due to biotin alone since animals fed on restricted but normal diet did not show these disturbances. Paulose *et al.* (1987) found that levels of testosterone in serum and testes were greatly decreased in biotin-deficient rats. The testosterone level was increased by administration of luteinizing hormone or biotin. The morphological abnormalities of the biotin-deficient rat testes were reversed by biotin treatment alone, whereas continuous administration of testosterone produced no such effect. Thus, biotin may have non-prosthetic group function in the development of testes.

2.2.3. Effect of biotin on induction of specific proteins

Guanylate cyclase and its product cyclic GMP has been shown to be involved in cell growth (Kram and Tompkins, 1973; Vesely *et al.*, 1976). Cyclic GMP has been

shown to stimulate DNA (Seifert and Rudland, 1974; Weinstein *et al.*, 1974; Kram and Tompkins, 1973), RNA (Goldberg *et al.*, 1974), and protein synthesis (Varrone *et al.*, 1973; Watson *et al.*, 1973). Vesely (1981, 1982, 1984) reported that biotin and its analogs enhanced guanylate cyclase activity in various rat tissues at micromolar concentration. This suggests that biotin may play a role in activating the enzyme. Spence and Koudelka (1984) further substantiated the stimulation of guanylate cyclase activity by demonstrating an increase in intracellular cyclic GMP and guanylate cyclase activity levels in primary cultures of rat hepatocyte by biotin. The concentrations of biotin used for the above experiments were at micromolar range which is about two orders of magnitude higher than the normal biotin concentration in cells. Singh and Dakshinamurti (1988) showed that biotin stimulates cyclic GMP and guanylate cyclase activity at a physiological concentration of biotin, i.e. in the nanomolar range.

RNA polymerase II catalyzes the formation of heteronuclear RNA which is the precursor of mRNA. Increased RNA polymerase II activity has been found in rapidly growing mammalian cells. cGMP stimulation of nuclear DNA-dependent RNA polymerase has been shown in lymphocytes (Johnson and Haddon, 1975), rat mammary gland (Anderson *et al.*, 1975), and in fetal calf liver cells cultured in the presence of sheep erythropoietin (Canas and Congote, 1984; White and George, 1981). Singh and Dakshinamurti (1988) found that RNA polymerase II activity was significantly lower in nuclear lysates of biotin-deficient HeLa cells and fibroblasts

than in those of the biotin-supplemented cells. Addition of 10^{-8} M biotin caused a 60-70 % increase in enzyme activity within 4 hours.

Glucokinase (E.C. 2.7.1.1) is one of the isoenzymes of mammalian hexokinase (ATP:D-hexose-6-phosphotransferase) which catalyzes the first reaction of glucose metabolism (Meglasson and Matschnisky, 1984). Glucokinase has a relatively high *Km* (6-10 mM) for glucose and it is not subjected to feedback inhibition by physiological levels of glucose-6-phosphate (Lynedjian *et al.*, 1986; Hers and Hue, 1983; Meglasson and Matschnisky, 1984; Vischer *et al.*, 1987). The enzyme is expressed in a tissue-specific fashion and is found only in mature liver parenchymal cells and pancreatic ß cells (Meglasson *et al.*, 1986). The liver glucokinase is involved in the utilization of excess circulatory glucose and is subjected to hormonal control. The activity is decreased in diabetes and increased after insulin treatment or after carbohydrate refeeding (Spence, 1983; Lynedjian *et al.*, 1987, 1988). Glucokinase in the pancreatic ß cells may act as a sensor for circulating glucose which may regulate the levels of glucose-induced insulin secretion (Meglasson and Matschnisky, 1984; Spence, 1983). Chauhan and Dakshinamurti (1991) have recently demonstrated that administration of biotin caused marked and rapid induction of glucokinase mRNA in starved rats and this effect is due to the regulation of the transcription of the glucokinase gene. This is so far the first report that provides direct evidence that a water-soluble vitamin regulates gene expression.

Two biotin-binding protein, BBP1 and BBP2, were isolated from egg yolk (Meslar *et al.*, 1978; White and Whitehead, 1987) which are distinct from avidin, a

high affinity biotin-binding protein present in egg white. White and Whitehead (1987) showed that the concentrations of BBP1 and BBP2 in egg yolk were directly related to the amount of dietary biotin. BBP1 has been shown to be induced by estrogen (Murty and Adiga, 1985). The induction of BBP1 and BBP2 by biotin suggests that it may regulate the amounts of these binding proteins by transcriptional control similar to the action of sex hormones. The ability of biotin to induce production of certain proteins which have regulatory roles highlights the non-prosthetic group functions of biotin.

3. Enzymes of biotin metabolism

In addition to the four biotin containing enzymes, two enzymes are important in the metabolism of biotin. They are biotin holocarboxylase synthetase and biotinidase. Biotin holocarboxylase synthetase is responsible for the synthesis of biotin holoenzymes whereas biotinidase releases biotin from the proteolytic degradation products of biotin enzymes. Figure 3 shows a hypothetical model of the interaction between these two enzymes. Both enzymes are widely distributed and it is fairly well established that holocarboxylase synthetase carries out its function intracellularly. However, the site of action of biotinidase remains unclear since it is present in various tissues with serum having the highest activity. These two enzymes are discussed in greater detail below.

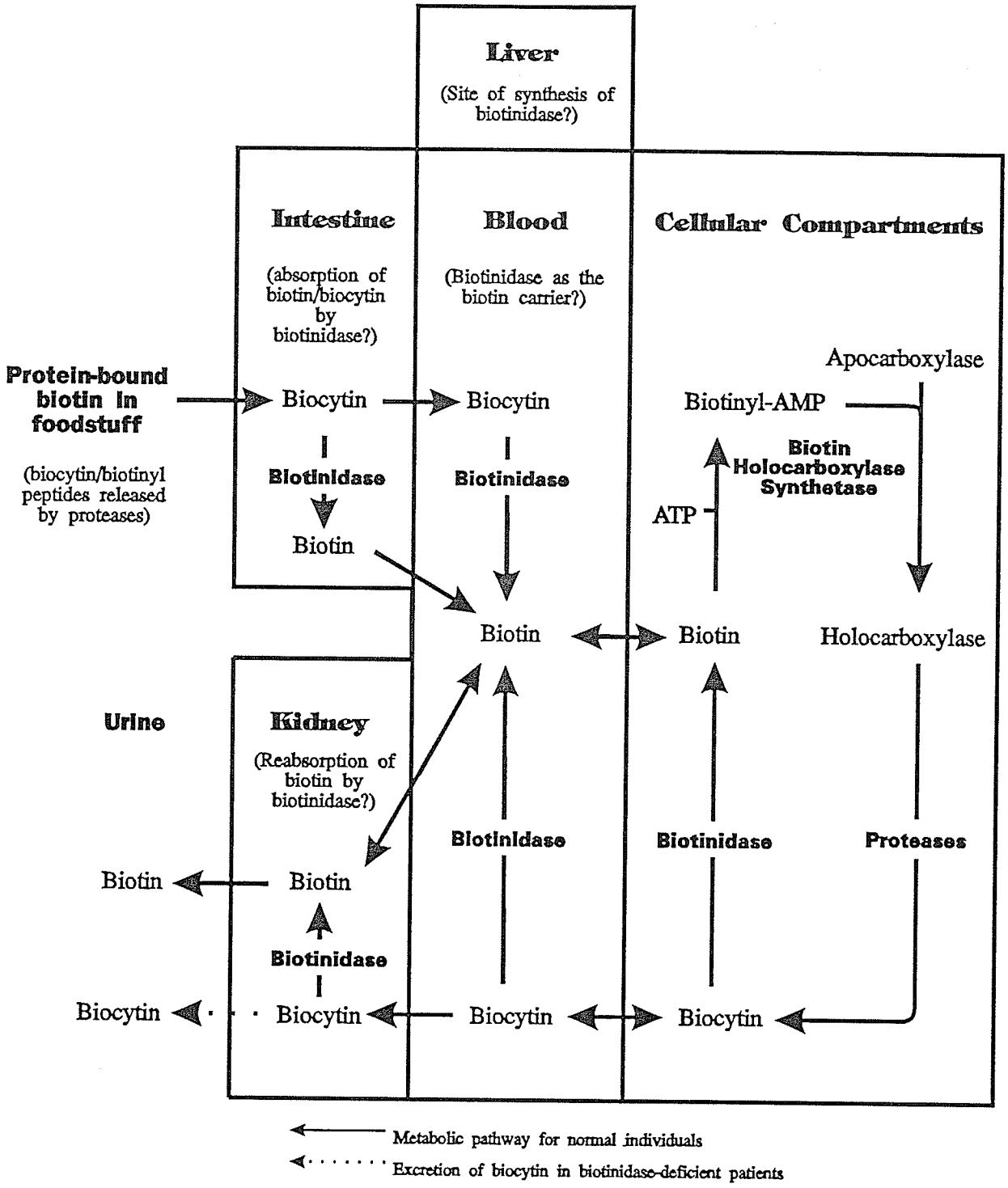


Figure 3. Hypothetical model of the functions of biotin holocarboxylase synthetase and biotinidase.

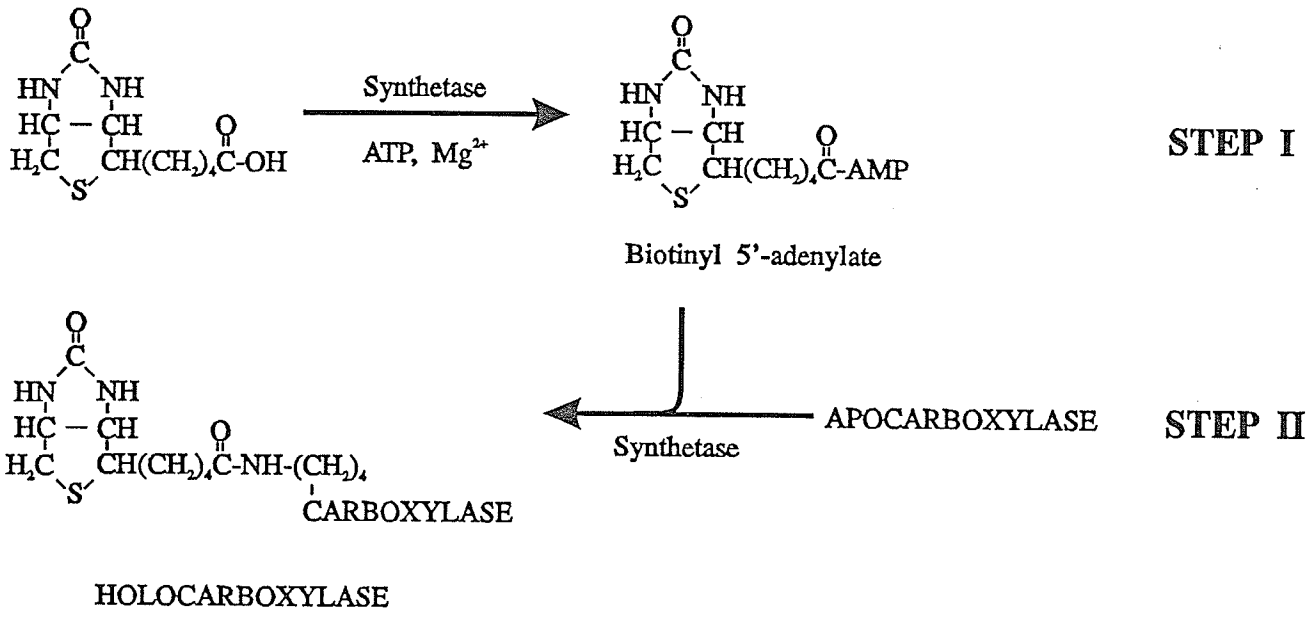
3.1. Biotin holocarboxylase synthetase

The synthesis of active holocarboxylase requires the post-translational attachment of biotin to the apocarboxylase. The enzyme biotin holocarboxylase synthetase catalyzes this step. It attaches biotin to the ϵ-amino group of a lysine residue of the apocarboxylase via a two-step reaction (Figure 4a). Holocarboxylase synthetases from the corresponding sources to the apoenzymes have been shown to attach biotin to apo-forms of pyruvate carboxylase (Cazzulo *et al.*, 1971), acetyl-CoA carboxylase (Lynen and Rominger, 1963), propionyl-CoA carboxylase (Siegel *et al.*, 1963), 3-methylcrotonyl-CoA carboxylase (Höpner and Knappe, 1965), and transcarboxylase (Lane *et al.*, 1964).

Holocarboxylase synthetase appears to have a broad specificity towards its substrate. Transcarboxylase synthetase from propionibacteria was shown to biotinylate rat liver apopropionyl-CoA carboxylase (Kosow *et al.*, 1962). McAllister and Coon (1966) have also demonstrated that synthetases from *Propionibacterium shermanii*, yeast, and rabbit liver were able to biotinylate rat liver apopropionyl-CoA carboxylase, bacterial apo-3-methylcrotonyl-CoA carboxylase, and apotranscarboxylase with only one exception. Holocarboxylase synthetase from rat liver did not biotinylate apotranscarboxylase.

In organisms that cannot synthesize biotin, it has to be obtained from external sources. Deodhar and Mistry (1969) demonstrated that the amount of holocarboxylase increased rapidly after administration of biotin and the effect was not due to synthesis of new protein because actinomycin D and puromycin did not

a) Biotin holocarboxylase synthetase



b) Biotinidase

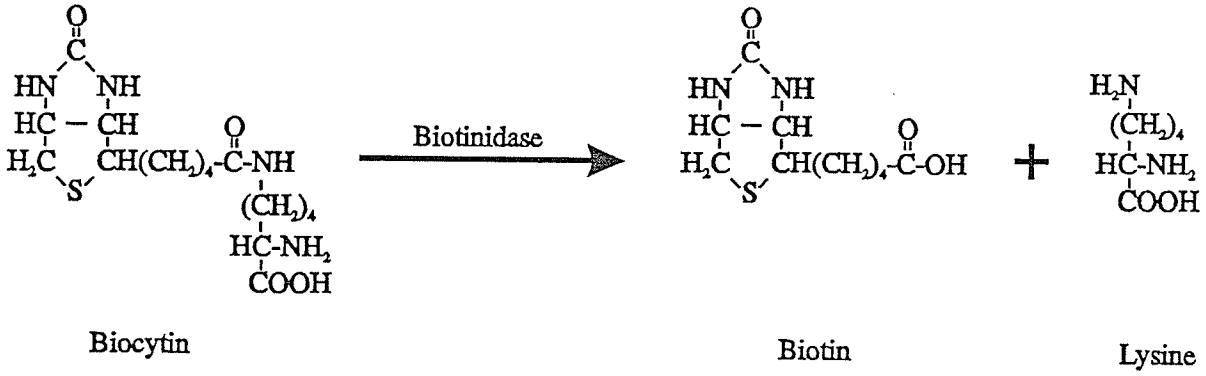


Figure 4. Reactions catalyzed by biotin holocarboxylase synthetase and biotinidase.

block the increase in holocarboxylase. Freytag and Utter (1980) also showed that pyruvate carboxylase level was low in avidin-treated 3T3-L1 cells but was restored with addition of biotin. The amount of immunoprecipitable pyruvate carboxylase was similar in cells treated or not treated with avidin. Therefore, it appears that biotinylation is the limiting step. Apoenzyme is synthesized without the presence of biotin and attachment of biotin to existing apoenzyme occurs when biotin is available. Wood *et al.* (1980) also showed that intact apoenzyme is not required for biotinylation. *Propionibacterium shermanii* synthetase biotinylates both the aposubunit and intact apotranscarboxylase.

Biotin holocarboxylase synthetase was first purified to homogeneity from *Escherichia coli* (Eisenberg *et al.*, 1982) and was also found to function as the repressor for the *bio* operon consisting of 5 genes. The products of these genes allow *E. coli* to synthesize biotin *de novo*. The *E. coli* holocarboxylase synthetase is a single polypeptide with a molecular weight of 34 KDa. Holocarboxylase synthetase has also been purified from *Propionibacterium shermanii* (Shenoy and Wood, 1988). It has a molecular weight of 30 KDa. Little is known about holocarboxylase synthetase in eukaryotes, even information regarding molecular weight and intracellular localization. This enzyme was found in both mitochondria and cytosol (Cohen *et al.*, 1985) but it is not clear if the synthetase activities located in these two subcellular fractions belong to a single enzyme species. The interest in biotin holocarboxylase synthetase has recently been rekindled due to its involvement in a genetically

transmitted disease called multiple carboxylase deficiency. Its role in the neonatal form of this disease will be discussed in Section 4.

3.2. Biotinidase

3.2.1. Discovery

Thoma and Peterson (1954) described an enzyme in hog liver that liberates biotin from peptic digest of hog liver and they named it biotinidase (biotin-amide amidohydrolase, E.C. 3.5.1.12). They also found that this enzyme hydrolysed biocytin and a synthetic compound N-(d-biotinyl)-p-aminobenzoate. Wright and Peterson (1954) discovered an enzyme in human blood that hydrolysed biocytin. They pointed out that this enzyme is enriched in plasma. Koivusalo *et al.* (1963) partially purified this enzyme from *Streptococcus faccalis* and showed that it is specific for the biotin moiety of simple biotin esters and amides and released equimolar amounts of biotin and lysine from biocytin. Using biocytin as a substrate, they also found that biotinidase is widely distributed in various rat tissues including liver, kidney, heart, brain, spleen, intestine, and plasma. In addition, biotinidase activity was found in yeast, *Propionibacterium shermanii*, and *Streptococcus faccalis*. In another report, Koivusalo and Pispa (1963) demonstrated the presence of biotinidase activity in sera from human, rat, guinea pig, and rabbit, liver and intestine from rabbit, using biocytin as substrate. Figure 4b describes the reaction catalyzed by this enzyme.

3.2.2. Role of biotinidase in releasing protein-bound biotin

Biotin cannot be synthesized by mammals. It is one of the water-soluble vitamins. Therefore, biotin is thought to be made available to cells through three major sources. First, biotin can be obtained from foodstuff. Second, biotin can be recycled from degraded biotin enzymes. Third, biotin can be obtained from colonic microflora which are capable of synthesizing biotin *de novo*. It has been reported that biotin transport is higher in jejunum than in ileum and is minimal in colon (Bowman *et al.*, 1986; Said and Redha, 1987). Furthermore, Sorrell *et al.* (1971) showed that biotin was better absorbed when it was taken orally than when it was introduced directly into colon. This seems to rule out the possibility that microorganisms in the colon provide a significant amount of biotin to the host (Dakshinamurti and Chauhan, 1989).

Biotin is protein-bound in foodstuff such as cereals and meat (György, 1939; Thompson *et al.*, 1941; Pispa, 1965; Scheiner and DeRitter, 1975). Thoma and Peterson (1954) demonstrated that while acid hydrolysis releases free biotin from hog liver, proteolytic digestion produces biotinyl peptides and biocytin. This would suggests that digestion in the gut releases biocytin and biotinyl peptide rather than free biotin. Craft *et al.* (1985) showed that plasma biotinidase hydrolysed biocytin at a rate 83-fold higher than the biotinyl peptides (5-13 residues in length). Further increase in peptide length to 65-123 residues reduced the rate of hydrolysis to 1/1200 of that of biocytin. These biotinyl peptides were obtained from specific proteolytic digestion of the biotinyl subunit of transcarboxylase. Chauhan and Dakshinamurti

(1986) attached ^{3}H-biotin covalently to ribonuclease and different polylysines with molecular weights ranging between 3 KDa and 195 KDa. They found no release of ^{3}H-biotin using human serum biotinidase. Thus, it is likely that biocytin is the primary substrate for biotinidase.

3.2.3. Role of biotinidase in recycling of biotin

Proteolytic digestion of biotin enzymes produces biocytin and biotinyl peptides. Biotinidase is the only enzyme that releases biotin from biocytin and biotinyl peptides. Therefore the presence of biotinidase in the gastrointestinal tract confers partial independence from dietary biotin requirement. Biotinidase is considered a recycling enzyme (Wolf *et al.*, 1985). Patients with defects in biotinidase require ingestion of high doses of free biotin. This may be in part due to the inability of patients to recycle biotin from degraded biotin containing enzymes.

The primary site of action by this enzyme is unclear. Biotinidase is present in various tissues. Highest specific activity of biotinidase was found in serum (Koivusalo and Pispa, 1963; Pispa 1965; Chauhan and Dakshinamurti, 1986). Activities are high in liver, kidney, and adrenal glands (Pispa, 1965; Suchy *et al.*, 1985). Biotinidase activity was detected in the brain, but its activity is low. In addition, biotinidase activity has been found in fibroblasts, leukocytes, pancreatic juice, and zymogen granules (Wolf, *et al.* 1984; Heard, *et al.*, 1985). Since biotinidase is so widely distributed, it is likely that the degradation of biotin enzymes takes place intracellularly. Pyruvate carboxylase has been demonstrated to be degraded in the

lysosome (Chandler and Ballard, 1983). However, subcellular fraction studies showed that biotinidase is not enriched in the lysosome (Pispa, 1965; Heard *et al.*, 1985). Various reports have indicated biotinidase is enriched in the microsomal fraction of rat and guinea pig liver (Heard *et al.*, 1985; Oizumi and Hayakawa, 1989). This suggests that biotinidase is not a lysosomal enzyme. Biotinidase is most abundant in serum which implies that the enzyme may function primarily extracellularly. Chandler and Ballard (1983) followed the degradation of ^{3}H-biotin labelled pyruvate carboxylase in 3T3-L1 adipocytes. They showed that the percentage degradation of pyruvate carboxylase remained the same over a 6 hour period in the presence or absence of cyclohexamide. They suggested that ^{3}H-biotin containing degradation products are released into the medium rather than re-utilized for incorporation into apocarboxylases. While it is likely that biotinidase cleaves biotin from biocytin or biotinyl peptides both intracellularly and extracellularly, the primary site of action for recycling biotin is not established. Further studies are needed to clarify this matter.

3.2.4. Role of biotinidase in intestinal absorption of biotinidase

It is now fairly well established that biotin transport in the intestine is a carrier-mediated process. However, little has been done to characterize the transporter itself and the mechanism of transport. Chaulifour and Dakshinamurti (1983) studied uptake of biotin-avidin complex in HeLa cells and rat liver plasma membrane and suggested that biotin uptake may proceed through adsorptive pinocytosis. The role of biotinidase in the intestinal uptake of biotin was studied by

Dakshinamurti *et al.* (1987). Solubilized brush-border membrane and cytosol were fractionated by sucrose density gradient centrifugation. The biotin-binding and biotinidase activities coincided in both preparations.

As late as 1986, uptake of biotin in the intestine was regarded as a passive diffusion process (Goré *et al.*, 1986). During the same year, Bowman *et al.* (1986) studied the intestinal absorption of biotin in rat using *in vivo* intestinal loop technique and suggested the presence of saturable and non-saturable components of biotin uptake. They concluded that intestinal absorption of biotin proceeds through a saturable process when biotin concentration was less than 5 uM. Dakshinamurti *et al.* (1987) showed that in rat intestine uptake of biotin and biocytin proceeds through a saturable process at 40 nM biotin or 50 nM biocytin which is the normal expected *in vivo* concentration. However, at higher unphysiological concentrations, passive diffusion predominates. Goré and Hoinard (1987) also demonstrated that biotin transport in isolated hamster intestinal cells proceeded with a saturation kinetics and was inhibited by biotin and biocytin suggesting specific structural recognitions were required for the uptake. Intestinal absorption of biotin was studied in rat using everted sac technique (Said and Redha, 1987) and brush-border membrane vesicle technique (Said and Redha, 1988). The transport of biotin at physiological concentrations was Na^+-, energy-, and temperature-dependent. In addition, biotin uptake was saturable and proceeded against a concentration gradient. Structural analogs of biotin such as biotin methyl ester and desthiobiotin were found to inhibit the uptake of biotin. Their findings support the suggestion that the uptake of biotin

in the intestine is a carrier-mediated process. Transport of biotin in human intestine has also been studied by Said *et al.*(1987, 1988a, 1988b). They demonstrated that uptake of biotin in the intestine of human is remarkably similar to that of the rat. Biotin absorption in the human intestine is saturable, Na^+-dependent, and inhibited by structural analogs of biotin (Said *et al.*, 1987, 1988a). The similarities of biotin uptake in the intestine between rat and human make the rat a good model system for studying intestinal absorption of biotin. Said *et al.* (1988b) found that transport of biotin by the Na^+-dependent process was higher in the duodenum than jejunum, which was in turn higher than ileum (Said *et al.*, 1988b). The decrease in biotin uptake appeared to be due to a decrease in the concentration of the biotin carriers since the *Km*'s for biotin uptake were similar in various regions of the intestine whereas *Vmax* decreased distally along the intestine. The intestinal absorption of biotin was found to be under developmental changes (Said *et al.*, 1990a, 1990b; Said and Redha, 1988). *Vmax* of the carrier-mediated transport system of biotin showed progressive decrease with maturation whereas *Km* for biotin remained the same. The results suggested that maturation is associated with a decrease in the number (and/or activity) but not the affinity of the transport carriers. The amount of transporter appeared to be regulated by biotin (Said *et al.*, 1989a). Biotin transport was increased in biotin-deficient rats through an increase in the *Vmax* in the uptake of biotin with little apparent change in *Km* comparing to biotin-sufficient rats. Administration of biotin caused decrease in biotin transport.

3.2.5. Role of biotinidase as a serum biotin carrier

The studies on HeLa cells (Dakshinamurti and Chaulifour, 1981) and human fibroblasts (Chaulifour and Dakshinamurti, 1983) suggested that biotin uptake by mammalian cells may involve adsorptive pinocytosis through the action of specific circulating protein. The specific binding of avidin-biotin complex suggests that avidin-biotin complex may mimic a natural biotin carrier protein since avidin is an egg-white biotin-binding protein which is not present in mammalian cells. The presence of a biotin carrier was confirmed by Cohen and Thomas (1982) using fully differentiated 3T3-L1 cells.

The decrease in plasma biotin and increase in renal excretion of biotin and biocytin in biotinidase-deficient patients lead Baumgartner *et al.* (1985) to suggest the existence of a biotin carrier in plasma. Other indirect evidence came from the study of epileptic patients on anticonvulsants treatment for a long period of time (Krause *et al.*, 1985). The biotin levels in sera of these patients are lower than in normal individuals. The anticonvulsants have the same cyclic carbamide group as biotin and it is this structure that is involved in protein binding (Green, 1975). Using purified serum biotinidase, Chauhan and Dakshinamurti (1988) demonstrated that anticonvulsants compete with biotin for binding to biotinidase. Said *et al.* (1989b) also found that these anticonvulsants inhibited biotin transport in the human intestine. The results suggest that biotinidase may be the only protein that binds biotin in the intestine and may function *in vivo* in the intestinal absorption of biotin. The concentration of biotinidase in human serum was estimated to be about 0.025 uM

(Chauhan and Dakshinamurti, 1986) which is over 10 fold higher than the concentration of biotin (free and bound). The excess of enzyme concentration compared to that of biotin indicates that biotinidase may have functions other than hydrolysing biocytin.

Initial studies found that human albumin, α- and ß-globin can bind biotin (Frank *et al.*, 1970). It was later demonstrated that this association is a non-specific one since no biotin binding was observed at nanomolar concentrations of biotin (Chauhan and Dakshinamurti, 1988). A glycoprotein that binds biotin was reported in human serum (Vallotton *et al.*, 1965; Gehrig and Leuthardt, 1976). Dakshinamurti *et al.* (1985) isolated this protein using an agarose-biotin column and showed that the protein does not bind biotin specifically. Chauhan and Dakshinamurti (1988) fractionated human serum with Sephadex G-150 column and analyzed the fractions for biotinidase and biotin binding activities. They found that both activities coincided and concluded that biotinidase is the only protein that binds biotin in human serum. This suggests that biotinidase is probably the biotin carrier in plasma. Baur *et al.* (1990) demonstrated that biotin transport in rat kidney is specific, carrier-mediated, and Na^+-dependent and suggested the existence of a reabsorption mechanism in the kidney cortex. The relatively high abundance of biotinidase in kidney and the renal loss of biotin and biocytin by biotinidase-deficient patients would imply that biotinidase may be involved in this reabsorption mechanism as well.

3.2.6. Site of synthesis of serum biotinidase

Many serum proteins such as albumin and transferrin are secreted from liver (Fishman and Doellgast, 1977). Treatment with carbon tetrachloride results in fatty infiltration of hepatic cells (Stoner and Magee, 1957) and reduced protein synthesis in liver. Pispa (1965) demonstrated that liver biotinidase activity was reduced by 50 % and serum biotinidase activity was decreased by 30 % in carbon tetrachloride-treated rats. Hepatectomized rats had a 30 % decrease in serum biotinidase activity. He suggested that biotinidase is synthesized in liver and subsequently secreted into plasma. Grier *et al.* (1989) studied serum biotinidase activity in patients with cirrhosis which compromises their ability to synthesize proteins in liver. They found that biotinidase activity in serum correlated strongly ($r = 0.87$) with the amount of albumin present in these patients. Albumin is regarded as being secreted entirely from the liver. This further supports the contention that the liver is a major site of synthesis of biotinidase. Using cultured rat hepatocytes Weiner *et al.* (1987) found that biotinidase activity in the culture medium increased over time. This was reduced by 28-62 % when cells were pre-treated with cyclohexamide. Biotinidase is enriched in the microsomal fraction in rat liver (Heard *et al.*, 1985) which is consistent with the notion that the enzyme is synthesized in the endoplasmic reticulum, transferred through Golgi and eventually secreted outside the cell.

There are nevertheless questions to be answered about the secretion of biotinidase. First, The results from the experiments described above are indirect. No studies have been done to follow the synthesis of biotinidase. While studies on the

synthesis and secretion of biotinidase are limited, there are no reports investigating the secretion of biotinidase in other organs or cells that have biotinidase activity. Kidney has relatively high biotinidase activity compared to other organs although not as high as serum and liver. It is not known whether this organ is also responsible for secretion of biotinidase into serum. Second, since biotinidase is found in various tissues, it is not certain whether a portion of biotinidase is secreted into serum whereas the rest functions intracellularly for recycling biotin enzymes. If this were to be the case, how is this partition between these pools achieved?

4. Clinical aspects of multiple carboxylase deficiencies

Inherited disorders relating to each of the four biotin enzymes have been identified in humans. These result in the synthesis of a defective carboxylase and the patients do not respond to pharmacological doses of biotin. The defects in these enzymes are reflected in the accumulation of secondary metabolites in blood and urine (Figure 5). Later, disorders were identified which are defective in one or more biotin carboxylase activities (Sweetman and Nyhan, 1986). Unlike the individual carboxylase deficiencies which are due to defects in the carboxylase genes, multiple carboxylase deficiency responds to pharmacological doses of biotin. The causes of abnormalities in these patients were eventually elucidated. Multiple carboxylase deficiency has two distinct forms. The neonatal form is due to defects in biotin holocarboxylase synthetase whereas the late-onset form is due to defect in biotinidase. These two forms of multiple carboxylase deficiency responded to the

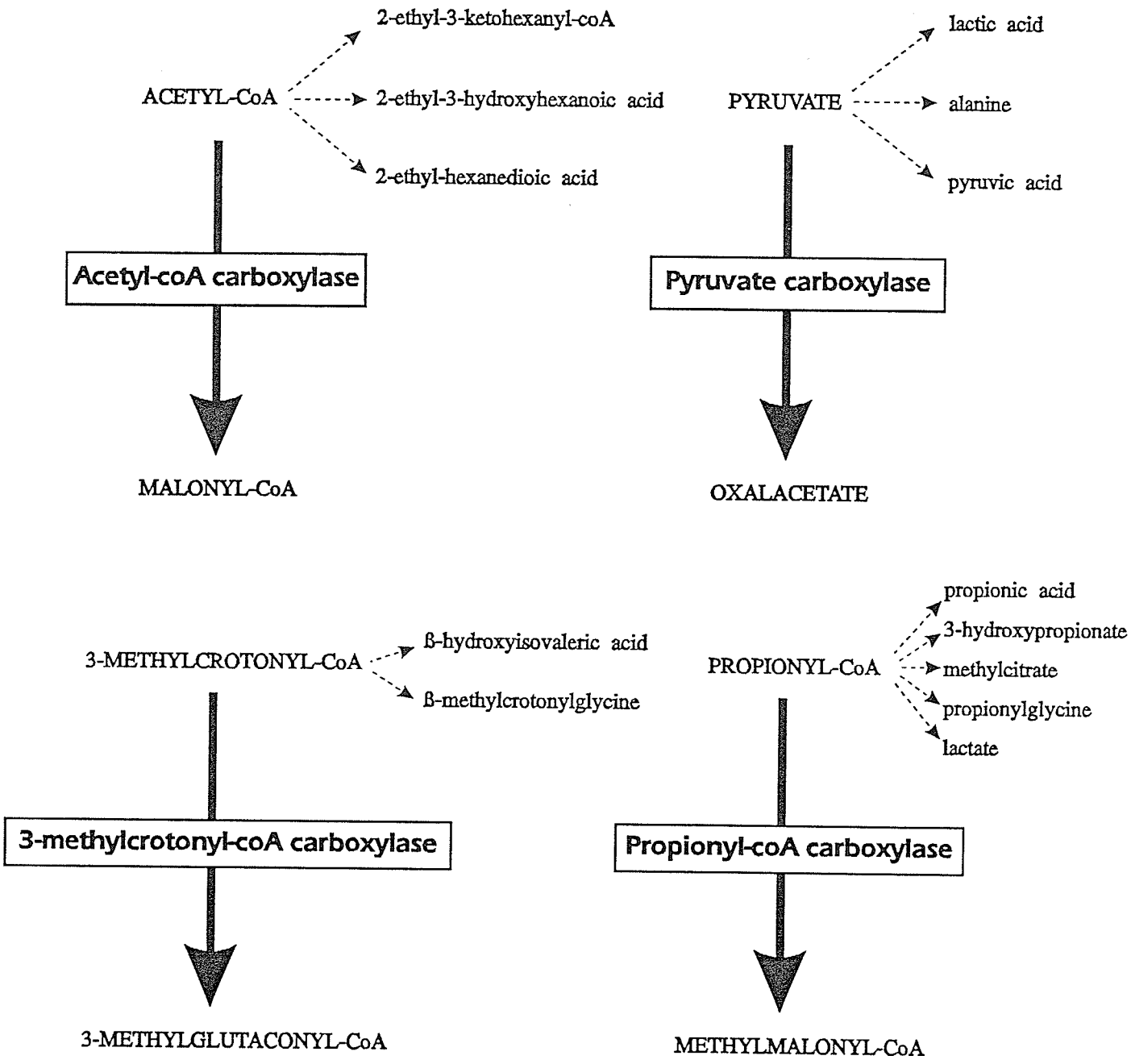


Figure 5. Accumulation of secondary metabolites in single carboxylase deficiency. Normal metabolites, ⟶; metabolites in carboxylase-deficient patients ----➤. (Adapted from Dakshinamurti and Chauhan, 1989.)

treatment with biotin (5-10 mg daily). The neonatal and late-onset forms of multiple carboxylase deficiency are described below.

4.1. Neonatal multiple carboxylase deficiency (Biotin holocarboxylase deficiency)

This disease, as its name suggests, usually develops during the first days of life. The disease manifests itself as episodes of severe vomiting, massive ketosis, profound metabolic acidosis, hyperammonemia, irregular breathing, convulsion, and lethargy (Nyhan, 1988). If the patient is not treated immediately, death results. Surviving patients often have dermatitis and hair loss. In most patients, the disease typically appears within the first 6 weeks (Burri *et al.*, 1985). However, a few patients have developed this disorder as late as 15 months (Sherwood *et al.*, 1982). Therefore, the time of onset is not an absolute criteria for distinguishing between these two forms.

Biotin holocarboxylase synthetase is responsible for attaching the biotin moiety to the apocarboxylase to form the active holo-enzyme. Synthetase activities in patients were assayed using partially purified rat apopropionyl-CoA carboxylase (Burri *et al.*, 1981). The *Km* for biotin was about 8 nM in normal fibroblasts whereas the *Km* for biotin was about 60-fold higher in the patients' fibroblasts. The *Vmax* of the enzyme was 30-40 % of normal. Patients with higher *Km* appeared to have more severe symptoms and were less responsive to various concentrations of biotin in their cultured fibroblasts. The age of onset was also found to correlate positively with the *Km* for biotin of the holocarboxylase synthetase (Burri *et al.*, 1985). Therefore, a

lesion in the biotin binding site of holocarboxylase synthetase seems to be the cause of this disease.

Prenatal diagnosis of holocarboxylase deficiency has been demonstrated. Diagnosis was based on biotin-responsive deficiencies of cultured amniocytes and the presence of 2-methylcitric acid or 3-hydroxyisovaleric acid in amniotic fluid obtained by amniocentesis (Packman *et al.*, 1982; Jakcobs *et al.*, 1984). Prenatal therapy of holocarboxylase deficiency has been accomplished. In the first case of such treatment, the mother who was suspected of holocarboxylase deficiency refused a prenatal diagnosis. However, 10 mg per day of biotin was given orally from the 34th week of pregnancy (Roth *et al.*, 1982). Healthy fraternal twins were born with normal urine organic acid profiles and 4- to 7-fold higher biotin concentration in cord blood. Carboxylase assays and genetic complementation studies on fibroblasts from the infants showed that one of them had multiple carboxylase deficiency, although he was clinically and biochemically normal throughout the neonatal period.

4.2. Late-onset multiple carboxylase deficiency (Biotinidase deficiency)

The late-onset form of multiple carboxylase deficiency usually appears as early as three months or as late as four years of age (Bartlett *et al.*, 1980; Sweetman, 1981). Similar to the holocarboxylase deficiency, the patient may also experience life-threatening episodes of acidosis and ketosis (Cowan, *et al.*, 1979; Munnich *et al.*, 1981; Wolf and Secor McVoy, 1983) but it is less frequent than in the neonatal form. This disorder also has symptoms including skin rashes, conjunctivitis, alopecia,

hypertonia, and developmental delay. Neurological abnormalities including ataxia, delayed intellectual development are more common in the late-onset form of the deficiency (Thoene *et al.*, 1981; Sander, *et al.*, 1980). Optical and auditory nerves may suffer permanent damage in biotinidase-deficient patients but not those with deficient holocarboxylase synthetase. While most symptoms of multiple carboxylase deficiency are reversed with high doses of biotin, these neurological disturbances are not alleviated by biotin therapy (DiRocco *et al.*, 1984; Taitz *et al.*, 1985; Thuy, *et al.*, 1986; Wolf *et al.*, 1983a). It has been suggested that the neurological abnormalities may due to a combination of biotin deficiency and biocytin excess (Wolf *et al.*, 1983b).

Wolf *et al.* (1983c) identified that the late-onset form of multiple carboxylase deficiency is due to a deficiency of biotinidase. Biotinidase catalyzes the release of biotin from biocytin or biotinyl peptides. Patients have low levels of biotin in blood and urine (Thoene *et al.*, 1981). The defect in this enzyme results in the accumulation of biocytin and biotinyl peptides. Loss of biocytin in urine is elevated in biotinidase-deficient patients (Suormala *et al.*, 1988). The levels of biotinidase activity in these patients correlate negatively with the amount of biocytin excretion in urine. Loss of biotin into urine was also increased (Baumgartner *et al.*, 1985). Wolf *et al.* (1987) compared sera from normal individuals and biotinidase-deficient patients (< 10 % mean normal activity) using antiserum against human serum biotinidase and could not detect any biotinidase protein in these patients. Since biotinidase was found in various tissues, they discarded the possibility of defective secretion and concluded

that a failure to synthesize the enzyme protein or mutant biotinidase that has a markedly altered structure results in this disorder. The identification that biotinidase is the only biotin-binding protein in plasma (Chauhan and Dakshinamurti, 1988) would indicate that patients with late-onset form of multiple carboxylase deficiency are not able to absorb and transport the physiological amounts of biotin from their diet. The possible presence of a mutant defective biotinidase in these patients would require a much higher level of biotin for its transport (*Km* defect of biotinidase in plasma).

Screening for biotinidase was established in 12 countries (Wolf and Heard, 1990) using a simple semi-quantitative colorimetric test on blood. Biotinidase deficiency was detected in 72 newborns. 32 of them had profound biotinidase deficiency (< 10 % of mean normal serum biotinidase activity) and 40 had partial deficiency (10 - 30 % of mean normal activity). The frequency was calculated to be 1 in 61,067 newborns. This places this disorder well within the incidence of other disorders for which newborns are tested.

5. Isolation of Biotinidase

5.1. Assay methods

Early studies of biotinidase utilized the differential growth response of *Lactobacillus arabinosus* (strain ATCC 8014) which can grow on biotin but not on biocytin (Wright and Skegg, 1944). This assay, however, requires a long period of incubation and has a low sensitivity. Later, Knappe *et al.* (1963) devised a more rapid

and simple assay using a synthetic substrate, N-(d-biotinyl) p-aminobenzoate. Biotinidase releases p-aminobenzoate from N-(d-biotinyl) p-aminobenzoate which can be quantitated colorimetrically by the Bratton-Marshall reaction (Bratton and Marshall, 1939). This method has since been used most often in determining biotinidase activity.

Variations of this method have also been developed. Heard *et al.* (1984) determined biotinidase colorimetrically using dried samples of blood spot. This assay was modified to allow the quantitation to be done using microtiter plates and a plate reader (Yamaguchi *et al.*, 1987; Dove Pettit *et al.*, 1989). An automated procedure was introduced to assay biotinidase in serum samples which is based on colorimetric development of p-aminobenzoate (Weissbecker *et al.*, 1989). A more sensitive radioassay was developed which determines the release of [^{14}C-carboxyl]-p-aminobenzoate from biotinyl-[^{14}C-carboxyl]-p-aminobenzoate (Wolf and Secor McVoy, 1983). Hayakawa and Oizumi (1986) developed an assay which quantitated the amount of p-aminobenzoate produced from biotinyl p-aminobenzoate using a HPLC method and a fluorometric detector to measure p-aminobenzoate. Artificial substrate other than biotinyl p-aminobenzoate have also been used. Wastell *et al.* (1984) used a fluorometric method for biotinidase using a fluorigenic substrate, biotinyl-6-aminoquinoline.

Two assays have been developed to determine biotinidase using its natural substrate, biocytin. Ebrahim and Dakshinamurti (1986) measured the activity using biocytin. Lysine, which is a product of cleavage by biotinidase, was quantitated

fluorometrically following derivatization with 1,2-diacetylbenzene. Biotinidase activity in human plasma was also assayed using [^{14}C]-biocytin. The [^{14}C]-biotin released was measured after separation from [^{14}C]-biocytin with Bio-Rad AG1-X2 anion exchange resin.

5.2. Purification of biotinidase

Biotinidase has been partially purified from various sources (Table 1). Chauhan and Dakshinamurti (1986) first purified this enzyme from human serum to homogeneity. They obtained a purified enzyme preparation with a specific activity of 1900 unit/mg and a 28 % recovery of enzyme activity. Although Oizumi and Hayakawa (1989, 1990) have purified biotinidase from guinea pig liver (12 unit/mg) and porcine cerebrum (98 unit/mg), the low specific activities of the purified enzymes raises some doubts whether their preparations are indeed homogeneous even though they showed a single protein band in each case using Comassie Blue R-250 stain after SDS-PAGE.

6. Research Objectives

Section 3.2 has discussed the various roles biotinidase may play. This includes releasing protein-bound biotin, recycling biotin, absorption of biotin in the intestine, and being a serum biotin carrier. The clinical significance of biotinidase has been demonstrated. It is the cause of the late-onset form of multiple carboxylase deficiency. Majority of work done on this enzyme has been on its relations to the

Table 1. Purification of biotinidase from various sources.

Source	Degree of Purification	Specific Activity (unit/mg)	Authors	Year
Streptococcus faecalis	700	18	Koivusalo *et al.*	1963
Lactobacillus casei	135	292	Knappe *et al.*	1963
Hog kidney	3,000	187	Knappe *et al.*	1963
Hog liver	204	6	Pispa J.	1965
Hog serum	1,910	210	Pispa J.	1965
Human plasma	4,819	361	Craft *et al.*	1985
Human serum	21,411	1,927	Chanhan J. and Dakshinamurti, K.	1986
Guinea pig liver	682	13	Oizumi, J. and Hayakawa, K.	1989
Porcine cerebrum	22,182	98	Oizumi, J. and Hayakawa, K.	1990

multiple carboxylase deficiency. Biotinidase has been found in various tissues (see Section 3.2.3) with serum having the highest specific activity. The site of synthesis of biotinidase however remains unclear although liver seems to be the primary site of synthesis (see Section 3.2.6). Biotinidase appears to be membrane-bound in liver. Oizumi and Hayakawa (1989) showed that 80 % of biotinidase activity in guinea pig liver was membrane-associated and again, the activity was enriched in the microsome. They however, demonstrated that 58 % of biotinidase activity in porcine cerebrum was in the soluble fraction (Oizumi and Hayakawa, 1990). It is not clear whether biotinidase is secreted from the liver, i.e the enzyme is synthesized in the endoplasmic reticulum and transported through Golgi apparatus, plasma membrane and eventually secreted out of the cell. Kidney with its relatively high biotinidase activity may also play a role in the secretion of biotinidase. Because of the presence of biotinidase in various cell types, it is not known if a portion of biotinidase remains inside the cell while the rest is secreted. Biotinidase has also been demonstrated to be the only protein which binds biotin in the intestine (Dakshinamurti *et al.*, 1987). However, the involvement of biotinidase in the absorption of biotin in the intestine has not been directly proven. Therefore, it is of interest to follow the synthesis and secretion of biotinidase. In order to monitor the synthesis and secretion of biotinidase, antibody specifically against biotinidase is required to immunoprecipitate the intermediates of the pathway for subsequent analysis. Antibody against biotinidase can also be used to find out if it can block the absorption of biotin in the

intestine. This would prove the involvement of biotinidase in the intestinal absorption of biotin.

Rat would be a good model system because it has been used to study the absorption of biotin in the intestine which shares characteristics with absorption of biotin by the human intestine (see Section 3.2.4). In addition, it allows direct manipulation of experimental conditions and the synthesis and secretion of biotinidase can be followed in the rat. The aim of this thesis is to purify biotinidase to homogeneity from rat liver and compare its physical and kinetic properties with those of the human serum enzyme. Antibody specifically against the rat liver biotinidase will be raised which can be used as a tool in studying the biosynthesis and secretion of biotinidase in rat liver as well as in the intestinal absorption of biotin.

CHAPTER II

EXPERIMENTAL PROCEDURES

1. Tissue and subcellular distribution of rat liver biotinidase

1.1. Materials

Male Sprague-Dawley rats (100-150 g) were purchased from Charles River Canada (St. Constant, PQ). N-(d-biotinyl) p-aminobenzoate was purchased from Sigma Chemical Co. (St. Louis, MO). Bio-Rad assay dye concentrate was purchased from Bio-Rad Laboratories (Richmond, CA). All reagents used were of analytical grade and purchased from CANLAB (Winnipeg, MAN).

1.2. Assay for biotinidase activity

Biotinidase activity was determined using the method of Knappe, *et al.* (1963) with some modifications. The enzyme assay was initiated by the addition of 0.1 ml of enzyme solution to 0.8 ml of a mixture containing 0.1 M potassium phosphate buffer, pH 7.0, 10 mM EDTA, 0.5 mg bovine serum albumin and 0.3 mM N-(d-biotinyl) p-aminobenzoate. The mixture was incubated for 1 hour at 37 °C. The reaction was then stopped by addition of 0.1 ml of 30 % trichloroacetic acid. The mixture was centrifuged at 12,000 g for 5 minutes. The following reagents were added to 0.8 ml of supernatant at room temperature at 3 minute intervals, 0.1 ml of sodium nitrite (0.2 %), 0.1 ml of ammonium sulfamate (1 %), and 0.1 ml of N-(1-napthyl) ethylenediamine dihydrochloride (0.2 %). Absorbance at 546 nm was determined after 15 minutes. An assay mixture without enzyme was used as a control. A standard curve was established with p-aminobenzoate.

1.3. Definition of enzyme unit

One enzyme unit corresponds to the amount which catalyzes the release of 1 nmol of p-aminobenzoate per minute at 37 °C from N-(d-biotinyl) p-aminobenzoate.

1.4. Determination of protein

Protein concentration was determined by the method of Lowry *et al.* (1951). Bovine serum albumin was used as a standard. The absorbance was linear between 10 and 100 ug of bovine serum albumin.

1.5. Preparation of rat serum

Male Sprague-Dawley rat was anaesthetized with pentobarbitone (60 mg/Kg) injected intraperitoneally. After exposing the right carotid artery, it was carefully separated from the vagus nerve and extraneous tissue. A catheter (PE 50) was introduced into the right carotid artery after an incision and securely tied to the artery with thread. Blood was drawn from the catheter with a syringe and was allowed to clot at 37 °C for 1 hour. Serum was obtained from the clot by centrifugation at 10,000 g for 10 minutes.

1.6. Preparation of various rat organ tissues

After blood was drawn from the rats as described above, they were sacrificed by decapitation. The brain, liver, kidney, heart and small intestine were removed. The interior of the small intestine was rinsed thoroughly with saline.

1.7. Tissue homogenization

Tissues were homogenized with a Potter-Elvehjem homogenizer (4 volumes of buffer per gram of tissue) with a motor-driven teflon pestle in ice-cold solution containing 0.3 M sucrose, 20 mM phosphate pH 7.0, 1 mM EDTA and 1 mM ß-mercaptoethanol. All homogenates were used immediately.

1.8. Preparation of subcellular fractions from rat liver

Differential centrifugation was used to prepare subcellular fractions as described by Stal (1968) and Albers *et al.* (1965). Male Sprague-Dawley rats were killed by decapitation. Livers were excised and homogenized as previously described. All procedures were performed at 4 °C. Pellets containing unbroken cells and nuclei were obtained by centrifugation at 1,000 g for 10 minutes. Centrifugation at 10,000 g for 15 minutes yielded the mitochondrial pellet. The supernatant was then centrifuged at 100,000 g for 1 hour to yield the cytosol (supernatant) and the microsomal fraction (pellet). Pellets were resuspended with the same buffer used in tissue homogenization. Biotinidase activity and protein concentration in each fraction were determined as previously described.

2. Purification of Rat Liver Biotinidase

2.1. Materials

DEAE-Sephacel, Phenyl-Sepharose and Sephacryl 100HR were purchased

from Pharmacia-LKB (Montreal, PQ). p-chloromercuribenzoate, N-bromosuccinimide, iodoacetamide, phenyl methyl sulfonyl fluoride, Triton X-100 and N-(d-biotinyl) p-aminobenzoate were purchased from Sigma Chemical Co. (St. Louis, MO). Bio-Rad assay dye concentrate, silver staining reagents, SDS-PAGE standards, Mini Protean II gel electrophoresis apparatus were purchased from Bio-Rad Laboratories (Richmond, CA). Centriprep-10 and Centriprep-30 concentrators were bought from Amicon (Danvers, MA). All other reagents used were of analytical grade and purchased from CANLAB (Winnipeg, MAN). Male Sprague-Dawley rats (weight between 400 g and 450 g) were purchased from Charles River Canada (St. Constant, PQ).

2.2. Assay for biotinidase activity

Biotinidase activity was determined as described before.

2.3. Protein determination

The method of Lowry *et al.* (1951) was used for fractions before sodium deoxycholate was added. These included the initial homogenate and membrane preparation. After biotinidase was solubilized, Bio-Rad protein assay which is based on the method by Bradford (1976) was used. Bovine serum albumin was used as a standard. The standard Bio-Rad assay was used for solubilized membrane preparations, heat-treated membrane preparations and 30-55 % ammonium sulfate

fractions. Absorbance was linear between 10 and 100 ug of bovine serum albumin. The Bio-Rad microassay was used for subsequent steps. Absorbance was linear between 2 and 10 ug of bovine serum albumin.

2.4. Polyacrylamide gel electrophoresis at pH 8.8

The degree of homogeneity of the purified rat liver biotinidase was assessed by 7.5 % SDS-PAGE or 7.5 % non SDS-PAGE at pH 8.8, followed by localization of proteins by silver staining. The SDS-PAGE system was that of Laemmli (1970). Non-SDS-PAGE was as described by Davis (1964). 0.75 mm gel slab was used and electrophoresis was performed with Bio-Rad Mini Protean II Gel Electrophoresis apparatus. Gels were silver-stained using the Bio-Rad Silver Stain derived from the method of Merril *et al.* (1979). The following standards were used for calibration: myosine (200 KDa), ß-galactosidase (116 KDa), phosphorylase b (97 KDa), bovine serum albumin (66 KDa), and ovalbumin (43 KDa).

2.5. Purification of rat liver biotinidase

2.5.1. Homogenization

Male Sprague-Dawley rats were fasted overnight and were killed by decapitation. Livers were immediately excised. 250 gram of rat livers were homogenized with a Potter-Elvehjem homogenizer (4 milliliter of buffer per gram of tissue) with a motor-driven teflon pestle in an ice-cold buffer containing 0.3 M

sucrose, 20 mM phosphate, pH 7.0, 1 mM EDTA, and 1 mM ß-mercaptoethanol. All procedures were performed at 4 °C unless stated otherwise.

2.5.2. Preparation of membrane fraction

The homogenate was centrifuged at 100,000 g for 1 hour. The supernatant was discarded. The pellet was resuspended with one liter of the above buffer using Potter-Elvehjem homogenizer.

2.5.3. Solubilization with sodium deoxycholate

The membrane fraction was brought to 0.25 % sodium deoxycholate and left stirring for one and a half hour. The solution was then centrifuged at 100,000 g for 1 hour. The pellet was discarded.

2.5.4. Heat treatment of membrane preparation

Aliquots of the supernatant from the previously step (150-200 ml) were heated in a 60 °C water bath for 12 minutes with constant swirling. The samples were then centrifuged at 100,000 g for 1 hour. The pellets were discarded.

2.5.5. Ammonium sulfate fractionation

Solid ammonium sulfate was added to the supernatant obtained from the previous step until 30 % saturation was reached. The sample was left stirring for

another hour and then centrifuged at 10,000 g for 1 hour. The pellet was discard. The supernatant was brought to 55 % saturation with ammonium sulfate as before. The sample was centrifuged at 10,000 g for 1 hour. The supernatant was discarded. The pellet was resuspended in 35 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM ß-mercaptoethanol, and 10 % glycerol and was dialyzed overnight with two changes of 4 liters of the same buffer.

2.5.6. Fractionation on DEAE-Sephacel column

The dialyzed 30-55 % ammonium sulfate fraction was loaded at a flow rate of 50 ml/hr onto a DEAE-Sephacel column (2.6 x 50 cm) previously equilibrated with 35 mM phosphate, pH 7.0, containing 1 mM EDTA, 1 mM ß-mercaptoethanol, and 10 % glycerol. The column was washed with about 1 liter of the above buffer and then eluted with a linear gradient of 35 mM to 0.15 M phosphate, pH 7.0 (a total volume of 1.4 liter). 10 ml fractions were collected and assayed. Fractions with biotinidase activity greater than 15 unit/mg were pooled for the next step.

2.5.7. Fractionation on Phenyl-Sepharose column

Pooled fractions from the DEAE-Sephacel column were brought to 25 % ammonium sulfate saturation and applied to a Phenyl-Sepharose column (2.5 x 20 cm) previously equilibrated with 50 mM phosphate pH 7.0 containing 1 mM EDTA, 1 mM ß-mercaptoethanol, 25 % ammonium sulfate, and 10 % glycerol at a flow rate

of 50 ml/hr. The column was eluted with a gradient of simultaneously decreasing ammonium sulfate (25 - 0 %) and increasing ethylene glycol (0 - 50 %). 10 ml fractions were collected and those with biotinidase activity greater than 250 unit/mg were pooled.

2.5.8. Ultrafiltration

Enzyme solution from the Phenyl-Sepharose column was diluted with 4 volumes of 0.1 M phosphate buffer, pH 7.0, containing 1 mM EDTA, 1 mM ß-mercaptoethanol and 10 % glycerol. The solution was concentrated to about 10 ml with Centriprep-30 concentrator. The concentrated solution was again diluted to the original volume with the same buffer and then concentrated as before until a final volume of less than 10 ml.

2.5.9. Gel filtration on Sephacryl 100HR column

Concentrated enzyme solution was separated on Sephacryl 100HR column (2.5 cm x 100 cm) pre-equilibrated with 0.1 M phosphate, pH 7.0, containing 1 mM EDTA, 1 mM ß-mercaptoethanol, and 10 % glycerol at 10 ml/hr. Fractions (2 ml) were collected and assayed for activity and those with biotinidase activity greater than 1500 unit/mg were pooled.

2.6. Amino acid analysis

The purified enzyme was hydrolyzed in 6 N hydrochloric acid and 0.1 % ß-mercaptoethanol at 150 °C *in vacuo* for 1, 2 or 4 hours for the standard amino acid analysis. For the determination of tryptophan, the enzyme solution was hydrolyzed in 6 N hydrochloric acid and 4 % thioglycolic acid at 150 °C *in vacuo* for an hour according to procedures by Matsubara and Sasaki (1969). Analysis of cysteine and cystine requires the oxidation to cysteic acid. The enzyme sample was treated with performic acid as described by Hirs (1967) and then hydrolyzed in 6 N hydrochloric acid and 0.1 % ß-mercaptoethanol at 150 °C *in vacuo* for 1, 2 or 4 hours. Norleucine was added to the samples prior to analysis as an internal standard.

The hydrolysates were dried under vacuum and subsequently redissolved in an appropriate volume of sodium citrate buffer pH 2.2, and a suitable aliquot was applied to the Beckman Amino Acid Analyzer. Amino sugars analysis was performed with the Beckman Amino Sugar Analysis System.

3. Preparation of polyclonal antibody

3.1. Materials

A New Zealand white rabbit (3 months) was obtained from Blue Farms (Winnipeg, MAN). Glass syringes and two-way adapters were bought from CANLAB (Winnipeg, MAN). Freund's complete adjuvant was purchased from Calbiochem

(LaJolla, CA) and Freund's incomplete adjuvant was obtained from Difco Laboratories (Detroit, MI). Dot-blot apparatus, Trans-Blot nitrocellulose membrane, alkaline phosphatase-conjugated goat anti-rabbit IgG antibody, color development agents (5-bromo-4-chloro-3-indoyl phosphate-p-toluidine salt and p-nitro blue tetrazolium chloride) were purchased from Bio-Rad (Richmond, CA).

3.2. Preparation of antigen solution

10 ug of purified biotinidase was brought to a sodium dodecyl sulfate concentration of 0.001 % and heated at 95 °C for 10 minutes. The heated sample was mixed with the 30 ug non-heat treated purified biotinidase. The protein solution was emulsified with equal volume of Freund's complete or incomplete adjuvant using two glass syringes. The syringes were connected through a two-way valve and the plunger was depressed from the aqueous solution first, driving the antigen into the oil of the adjuvant. The plungers were depressed alternatively to mix the antigen solution and adjuvant until a thick emulsion developed. The emulsion was transferred to one of the syringes for injection.

3.3. Immunization of rabbit

Antibody to rat liver biotinidase was prepared in a New Zealand white rabbit. The rabbit was first injected subcutaneously with Freund's complete adjuvant. Subsequent injections were performed with Freund's incomplete adjuvant at two

week intervals. The rabbit was bled before the first injection and 10 days after every injection. Serum was prepared and antibody titer was determined using dot-blot apparatus (Bio-Rad). Serum obtained before the first injection of antigen was used as a control.

3.4. Test bleed on rabbit

The rabbit was placed in a restraining device and the ear was warmed under a lamp. A small incision was made just through the top of the marginal vein with a scalpel. Blood was collected by allowing it to drip into a clean glass tube. When the desired amount of blood was obtained, blood flow was stopped by gentle pressure to the cut for 10 to 20 seconds with a piece of cotton.

3.5. Preparation of rabbit serum

The blood collected was allowed to clot for an hour at 37 °C. The clot was then separated from the sides of the glass tube using a glass rod and was left overnight at 4 °C to allow it to contract. The serum was obtained by centrifugation at 10,000 g for 10 minutes.

3.6. Detection of antibody

A piece of Trans-Blot nitrocellulose membrane (8 cm x 12 cm) which had been previously washed and blotted dry was placed in a Bio-Rad dot-blot apparatus.

The apparatus was filtered once with TBS (20 mM Tris, 0.5 M NaCl, pH 7.5) under vacuum. Vacuum was then disconnected and appropriate wells were loaded with 100 ul TBS containing various amounts of the purified biotinidase. With vacuum off but flow valve open, entire sample was allowed to flow through the membrane by gravity. After filtration had completed, vacuum was applied to completely dry the wells. The membrane was removed from the dot-blot apparatus and was blocked with 5 % bovine serum albumin for 1 hour at room temperature. The membrane was then washed with two changes of washing solution (20 mM Tris, 0.5 M NaCl, 0.05 % Tween-20, pH 7.5) for 5 minutes.

The membrane was cut into various strips and each strip was incubated with 10 ml of appropriately diluted immune or control serum (in TBS containing 1 % bovine serum albumin). The incubation was performed at room temperature overnight using a rotating table. The strips were subsequently washed with two changes of washing solution and incubated at room temperature for one hour with alkaline phosphatase-conjugated anti-rabbit IgG (in TBS containing 1 % bovine serum albumin). The strips were then washed with two changes of washing solution. The presence of antibody against rat liver biotinidase was visualized by color development using the alkaline phosphatase substrate BCIP/NBT as described by the supplier.

3.7. Quantitation of antibody titer

The intensity of color development on the nitrocellulose blot depends on the amount of antibody against the rat liver biotinidase present which allows quantitation of antibody titer. A Hewlett Packard ScanJet II, which is capable of scanning 256 grey scales, was used to assess the intensity of color developed on the blot. The intensity of each sample was integrated on a MacIntosh Si computer using the program Image 1.37.

3.8. Partial purification of IgG from serum

IgG was partially purified from rabbit serum using the Econo-Pac Serum IgG purification kit (Bio-Rad). The purification was carried out according to the procedures that accompanied the kit. The separation was performed with 3 ml aliquots of immune or pre-immune serum. The purified sample was concentrated by Centriprep-10 concentrators.

CHAPTER III

RESULTS

1. Tissue and subcellular distributions of biotinidase in rat

The specific activities of biotinidase in various rat tissues were determined (Figure 6). Serum has the highest activity. Relatively high biotinidase activities were found in intestine, liver, and kidney. While activities in the heart and the brain were relatively low, they were nevertheless detectable. The pattern of distribution is similar to that reported by Koivusalo *et al.* (1963) except that they found that the specific activity of biotinidase in rat liver homogenate (0.021 unit/mg) was twice as much as that of the kidney (0.012 unit/mg) using the microbiological method. The specific activity of biotinidase in rat serum is roughly the same as the activity in human serum (0.138 unit/mg) (Craft *et al.*, 1985) using the biotinyl p-aminobenzoate as substrate.

The subcellular localization was subsequently studied in rat liver (Figure 7). Biotinidase activity was found to be present in all of the membrane fractions. However, no biotinidase activity was detected in the soluble fraction suggesting that biotinidase is completely membrane-associated. The microsomal fraction was most enriched with biotinidase. A relatively large amount of biotinidase activity was detected in the mitochondrial fraction but its significance is not known. This finding is consistent with that of Heard *et al.* (1985). Oizumi and Hayakawa (1989) also demonstrated that biotinidase was most enriched in the microsomal fraction of guinea pig liver with 80 % of the total biotinidase activity being membrane-bound. The association of biotinidase activity with the membrane fraction appears to be a loose one rather than biotinidase being an integral membrane protein. When

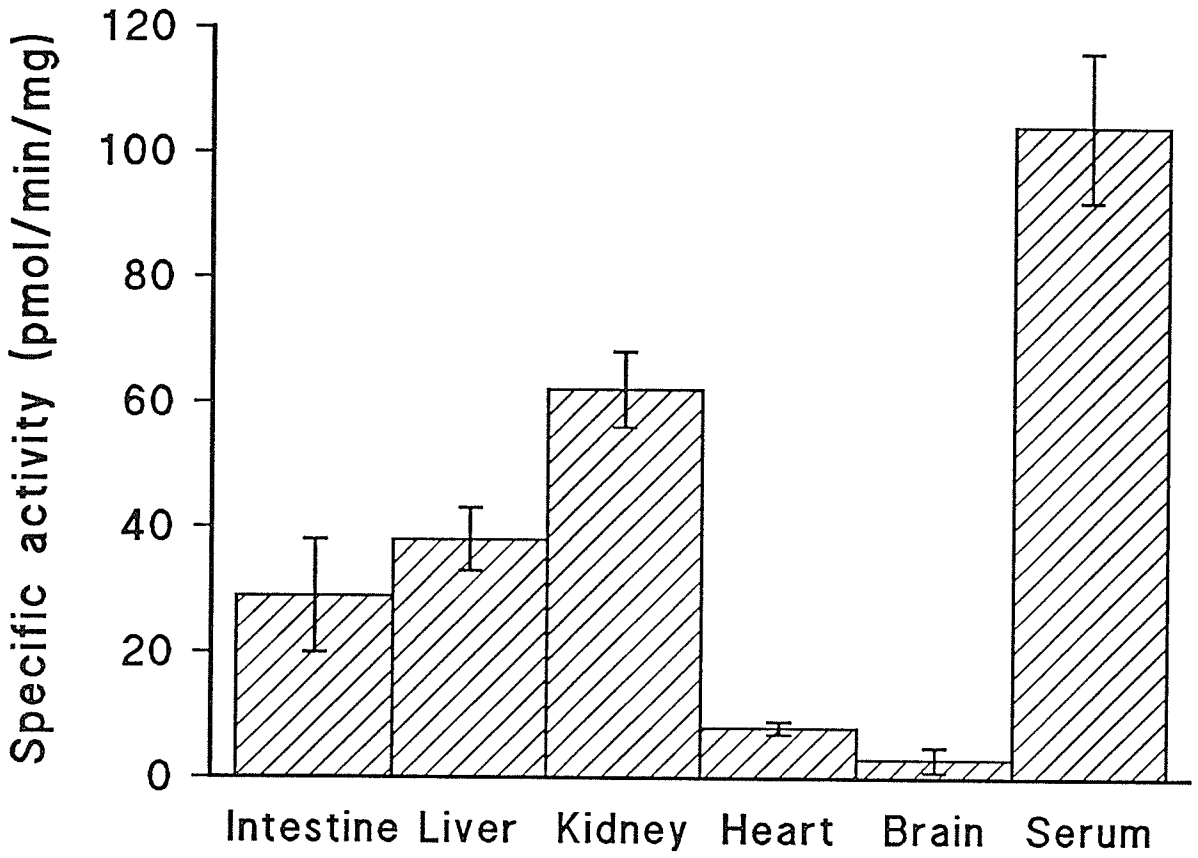


Figure 6. Tissue distribution of biotinidase in rat. Biotinidase activity in the indicated tissues was determined as described under the Experimental Procedures. Each value presents the mean ± S.D. of 12 determinations on each tissue from 4 rats.

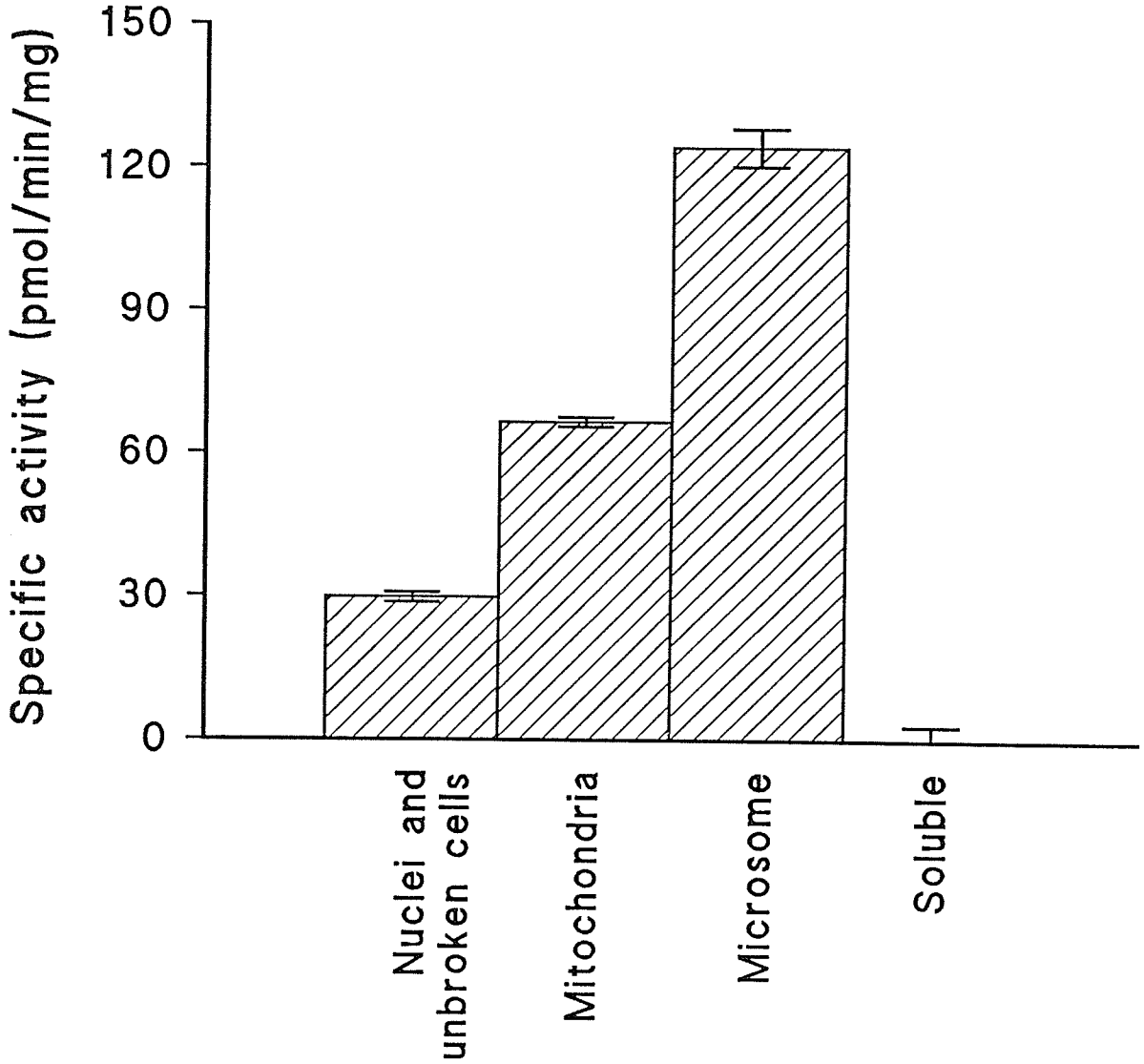


Figure 7. Subcellular localization of rat liver biotinidase.
Biotinidase activity in the indicated subcellular fractions was determined as described under the Experimental Procedures. Each value represents the mean ± S.D. of 4 determinations from two rats.

subcellular localization was performed on rat livers previously frozen at -20 °C, about half of the total biotinidase was found in the soluble fraction. This leads to some suspicion of the findings of Oizumi and Hayakawa (1990) that 60 % of total biotinidase activity was present in the soluble fraction of porcine cerebrum. They freeze-thawed the porcine tissues twice (once at -20 °C and once at -80 °C) which may have released biotinidase into the soluble fraction even though the tightly bound subcellular marker enzymes remained in their corresponding subcellular fractions. Since membrane-associated biotinidase in rat liver could be released into cytosol with repeated freezing and thawing, only fresh rat liver tissues were used in the purification of biotinidase.

2. Purification of biotinidase from rat liver

The specific activity of biotinidase in crude rat liver homogenate was usually between 0.05 and 0.08 unit/mg which is higher than the values shown in Figure 1 which is between 0.03 and 0.05 unit/mg. The discrepancy is probably due to the fact that rats of different weights were used. The subcellular and tissue localizations were determined in 100-150 gram rats whereas rats weighing 400-450 grams were used for the purification of liver biotinidase. The reason is that since large amount of rat livers were required for purification (250 gram), it was more economical to use larger rats. The enzyme activity per unit volume appeared to be similar for homogenates obtained from both 100-150 gram and 400-450 gram rats whereas the protein concentration was lower in the 400-450 gram rats. This is probably due to the

presence of the more insoluble connective tissue in their livers. 10 % glycerol, 1 mM ß-mercaptoethanol, and 1 mM EDTA appeared to stabilize the enzyme and were, therefore, incorporated in all of the buffers used for the purification.

The results of a typical purification of rat liver biotinidase are shown in Table 2. This is an eight-step procedure. The first half of the purification steps required extensive use of ultracentrifuge. The membrane portion of the liver homogenate was separated from the soluble portion using ultracentrifugation. The membrane-bound biotinidase was released with 0.25 % sodium deoxycholate. The effect of sodium deoxycholate was checked. It showed no activation or inactivation of biotinidase at this concentration. This procedure also required the heating of the sample at 60 °C for 12 minutes. The heating step was used because of the high heat stability of this enzyme. It is a crucial step because it not only removed a large amount of contaminating proteins but also clarified the preparation to allow better loading onto subsequent column chromatography. After heat treatment, the sample was subjected to 30-55 % ammonium sulfate cut. Upon removal of ammonium sulfate by dialysis, the sample was loaded onto a DEAE-Sephacel column (Figure 8). The unbound protein formed a readily visible sharp band which moved slowly down the column. This is probably due to the hydrophobic nature of the preparation. Fractions collected from DEAE-Sephacel were loaded onto a Phenyl-Sepharose column (Figure 9). Rat liver biotinidase appeared to be less hydrophobic than most of the proteins present in the sample and eluted early. No washing of this column was required. The next step was concentration by Centriprep-30 concentrator. It

Table 2. Purification of rat liver biotinidase.

Fraction	Total Volume (ml)	Total Protein (mg)	Total Activity (unit)	Specific Activity (unit/mg)	% Recovery	Degree of Purification
Homogenate	1,240	66,439	4,790	0.07	100	1
Membrane preparation	1,000	36,815	3,677	0.10	77	1
Solubilized membrane	890	26,888	3,583	0.13	75	2
Heat treatment	750	2,686	3,020	1.1	63	16
30-55% ammonium sulfate cut	180	995	2,305	2.3	48	32
DEAE-Sephacel	120	38	1,174	31	25	424
Phenyl-Sepharose	104	3.1	1,076	344	22	4,774
Ultrafiltration	5	1.5	719	467	15	6,480
Sephacryl 100HR	20	0.3	598	1,800	12	24,956

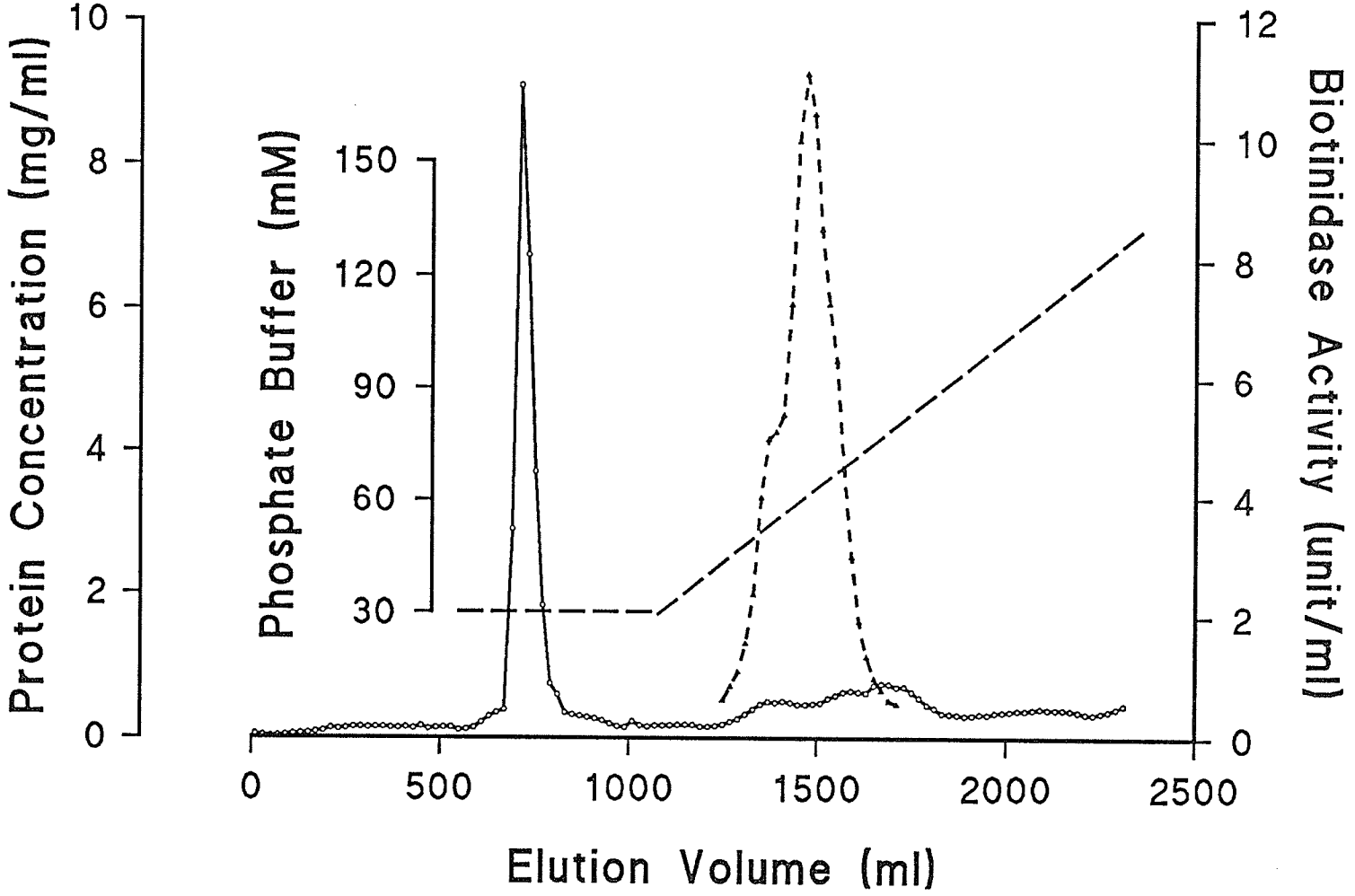


Figure 8. DEAE-Sephacel column chromatography of rat liver biotinidase. Dialyzed 30-55 % fraction was loaded onto DEAE-Sephacel column. The column was washed and eluted with a phosphate concentration gradient as described under the Experimental Procedures. Aliquots of column fractions were assayed for biotinidase activity (▲) and protein (o) as described under the Experimental Procedures.

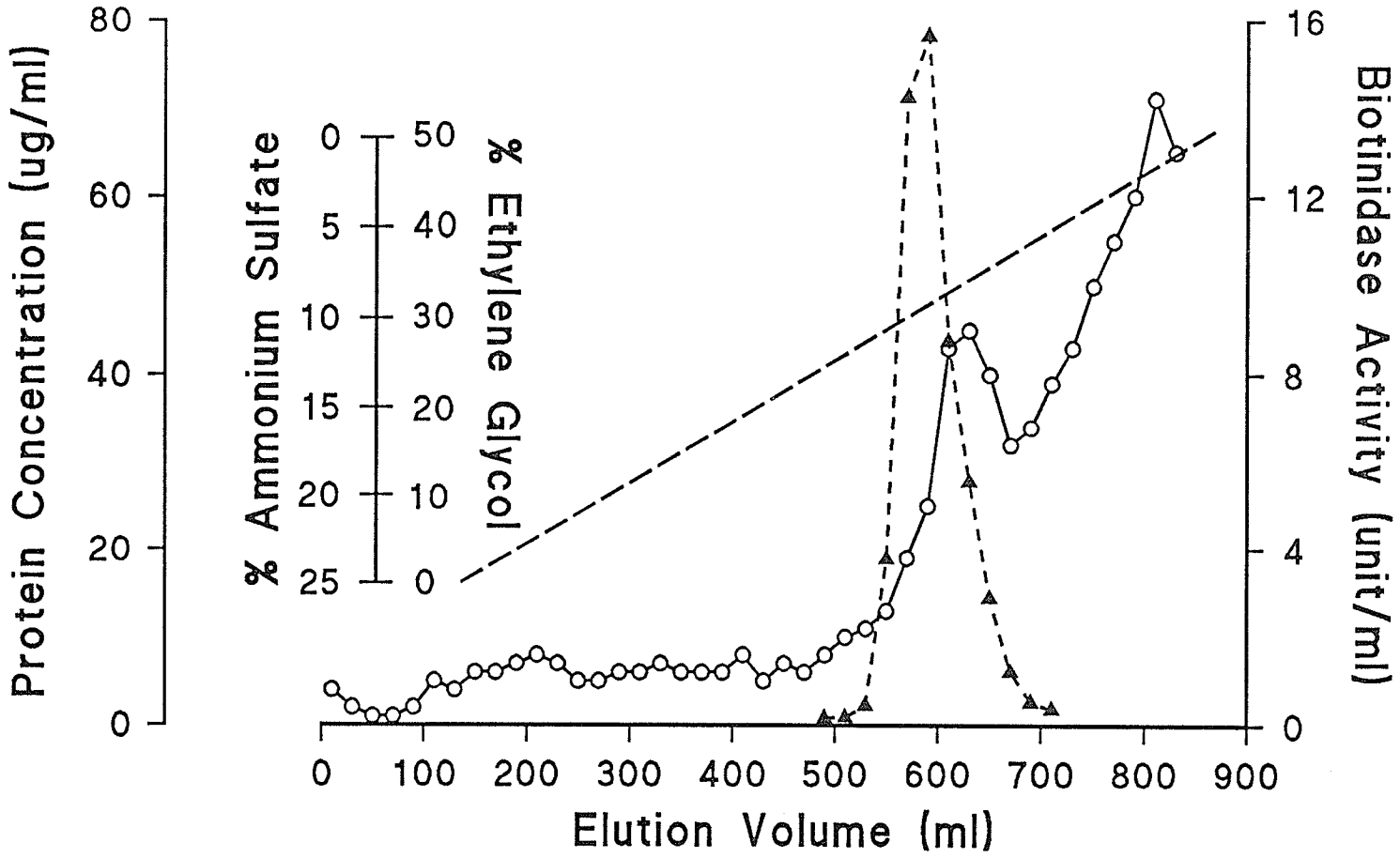


Figure 9. Phenyl–Sepharose column chromatography of rat liver biotinidase. The eluate from DEAE–Sephacel was brought to 25 % ammonium sulfate saturation and loaded onto a Phenyl–Sepharose column. The column was then eluted with a linear gradient of simultaneously decreasing ammonium sulfate and increaing ethylene glycol concentrations as described under the Experimental Procedures. Columnn fractions were analyzed for biotinidase activity (▲) and protein (○) as described under the Experimental Procedures.

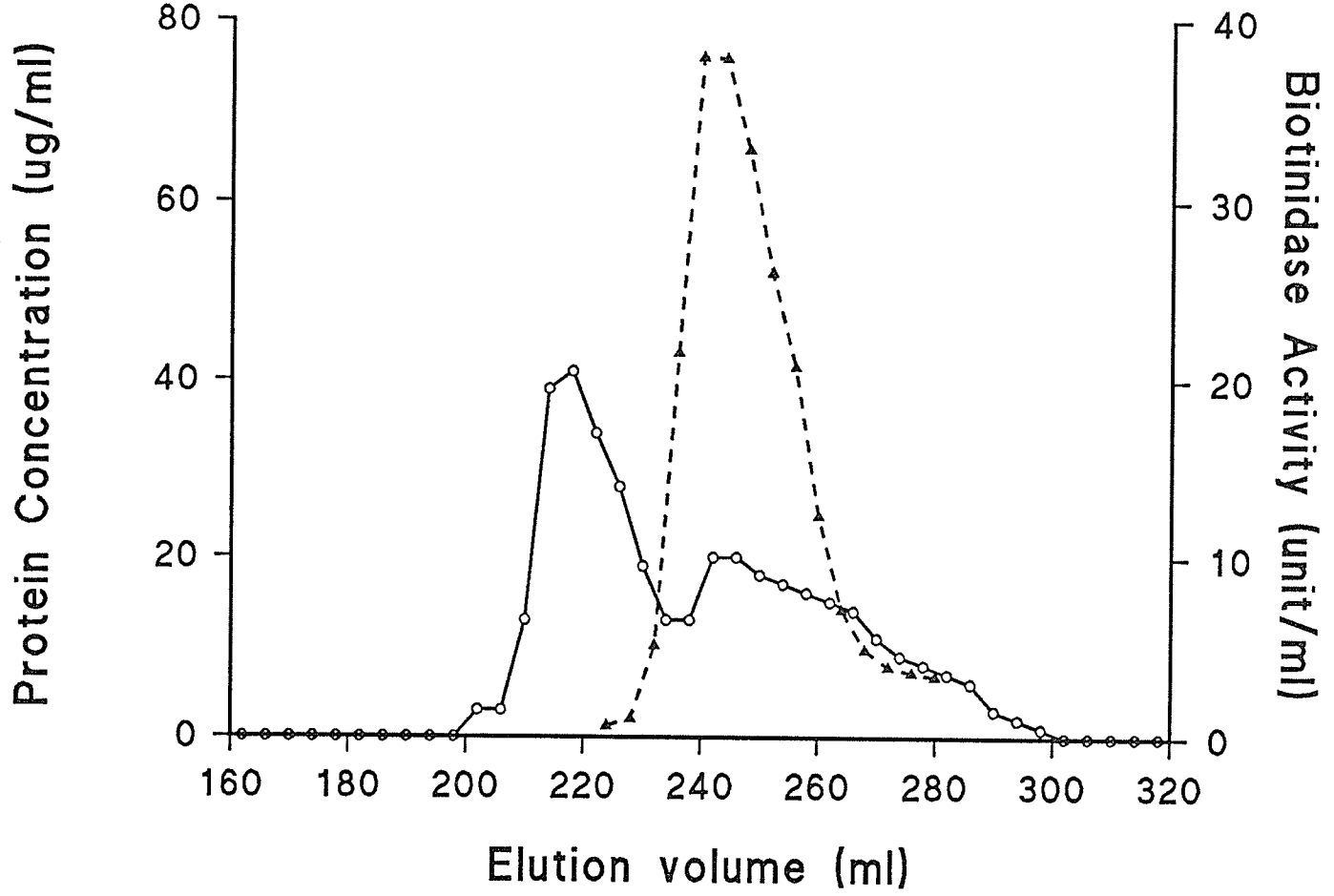


Figure 10. Sephacryl 100HR column chromatography of rat liver biotinidase. Concentrated sample from the Phenyl-Sepharose column was chromatographed on Sephacryl 100HR column as described under the Experimental Procedures. Elutant was assayed for biotinidase activity (▲) and protein (o) as described under the Experimental Procedures.

performed three functions simultaneously. First, it removed the highly viscous ethylene glycol present in the elutant of Phenyl-Sepharose column which may interfere with separation on the Sephacryl 100HR column. Second, it reduced the sample volume for loading onto the gel filtration column. Third, it eliminated some low molecular weight protein species which resulted in further purification. The final step was the separation of biotinidase on a Sephacryl 100HR column (Figure 10). The purified biotinidase had a specific activity of 1,800 unit/mg with a 25,000-fold purification from the crude homogenate and a 12 % recovery of total enzyme activity. The entire procedure usually took 7-8 days. Purified biotinidase was aliquoted and stored at -80 °C. It suffered little loss in activity for at least 3 months.

3. Characterization of purified rat liver biotinidase

3.1. Determination of purity and molecular weight of rat liver biotinidase

The purity of the enzyme was assessed by SDS-PAGE. Purified rat liver biotinidase showed a single band which was estimated to have a molecular weight of 61 KDa (Figure 11). Non-denaturing PAGE also showed a single band. Therefore, the purified enzyme probably consists of a single polypeptide with molecular weight of 61 KDa.

The molecular weight of rat liver biotinidase was estimated using Sephacryl S200 column. It was done by finding the *Kav* values of biotinidase and the protein standards using the following formula to construct a standard curve:

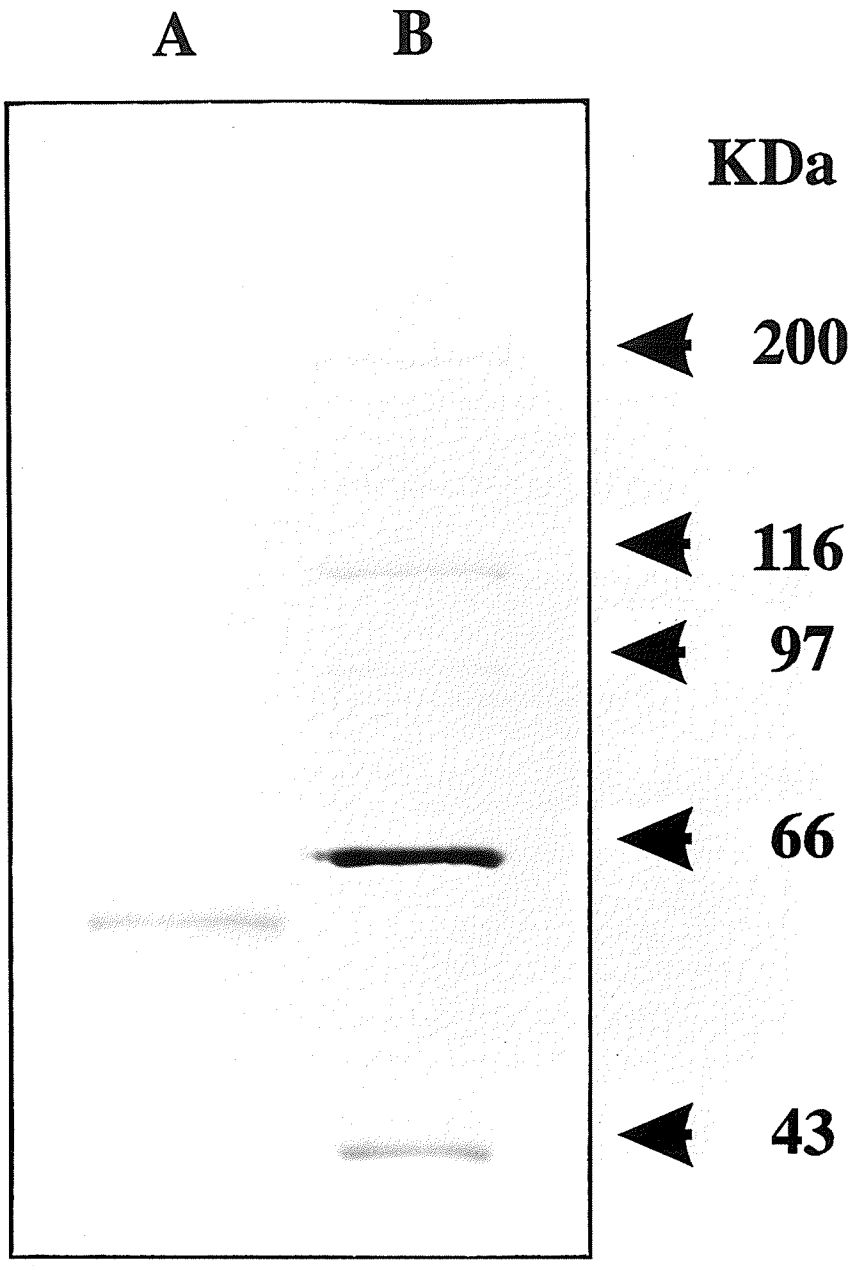


Figure 11. SDS-polyacrylamide gel electrophoresis of purified rat liver biotinidase. SDS-polyacrylamide gel electrophoresis was performed in a 7.5 % acrylamide gel. Lane A contains 0.4 ug of purified rat liver biotinidase. Lane B contains molecular weight markers. The gel was visualized by silver-staining.

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

where Ve = elution volume for the protein

Vo = column void volume = elution volume for Blue Dextran 2000

Vt = total bed volume

Molecular weight of rat liver biotinidase was estimated to be 71 KDa (Figure 12). The value is lower than that of hog liver and serum biotinidase (115 KDa) on Sephadex G-100 and Sephadex G-200 (Pispa, 1965). The human biotinidase is about 110 KDa on both Sephacryl S200 and Sephadex G-100 columns (Chauhan and Dakshinamurti, 1986).

3.2. Effect of pH on rat liver biotinidase

Rat liver biotinidase has a broad pH optimum using N-(d-biotinyl) p-amino-benzoate as a substrate (Figure 13). The enzyme activity dropped sharply at pH lower than 4 and at pH higher than 7.5. The maximum enzyme activity was observed at pH 6.5. The pH profile is similar to that of the human serum biotinidase (Chauhan and Dakshinamurti, 1986). Subsequent characterizations were carried out at pH 6.5.

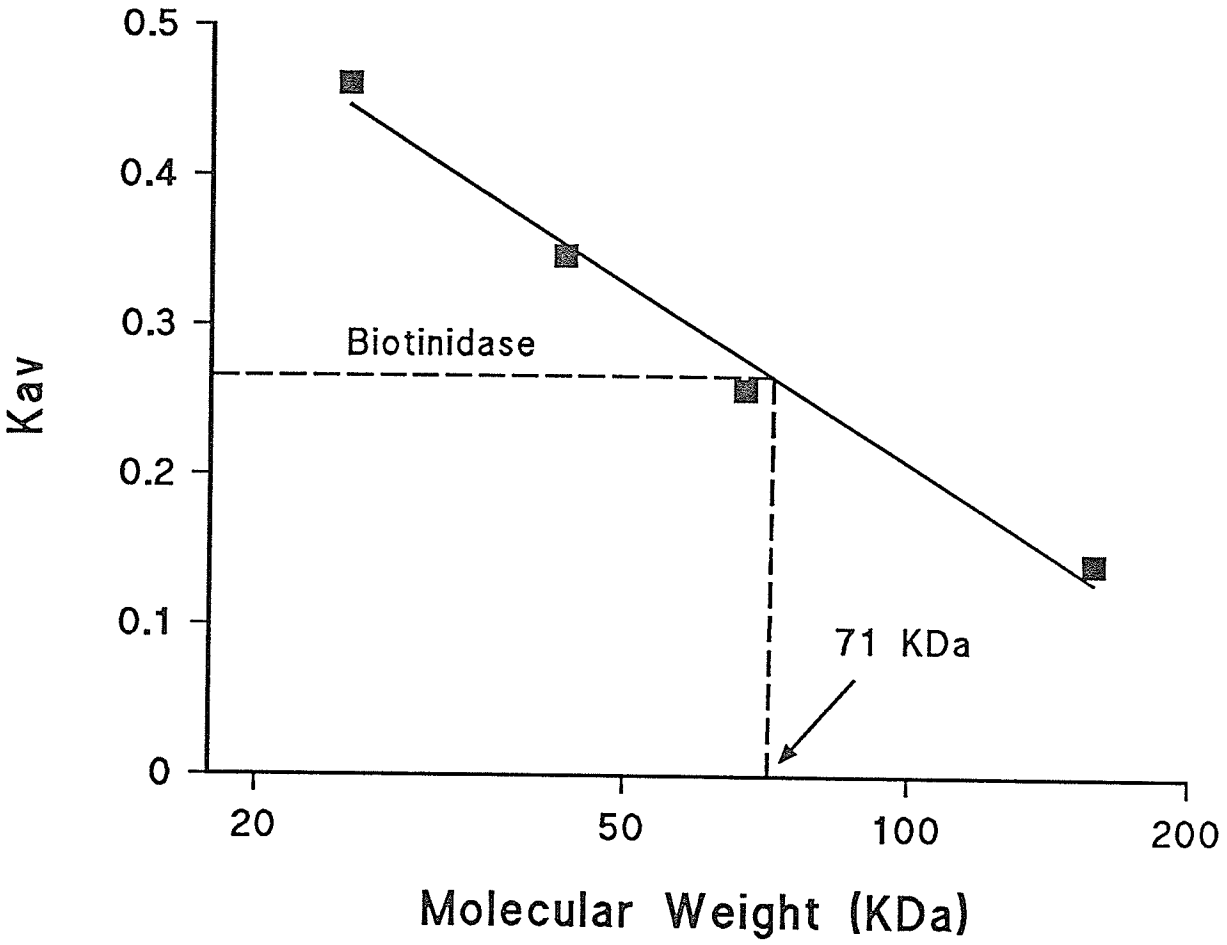


Figure 12. Molecular weight determination of purified native rat liver biotinidase by gel filtration. Purified biotinidase and protein standards were chromatographed on a Sephacryl S200 column at 4 °C in a buffer containing 0.1 M phosphate, pH 7.0, 1 mM EDTA, and 1 mM β–mercaptoethanol. The position of biotinidase was determined by assay with d–biotinyl p–amino–benzoate. The protein standards used were: aldolase (158 KDa), Albumin (67 KDa), Ovalbumin (43 KDa), Chymotrypsinogen A (25 KDa). The molecular weight of rat liver biotinidase was determined to be 71 KDa.

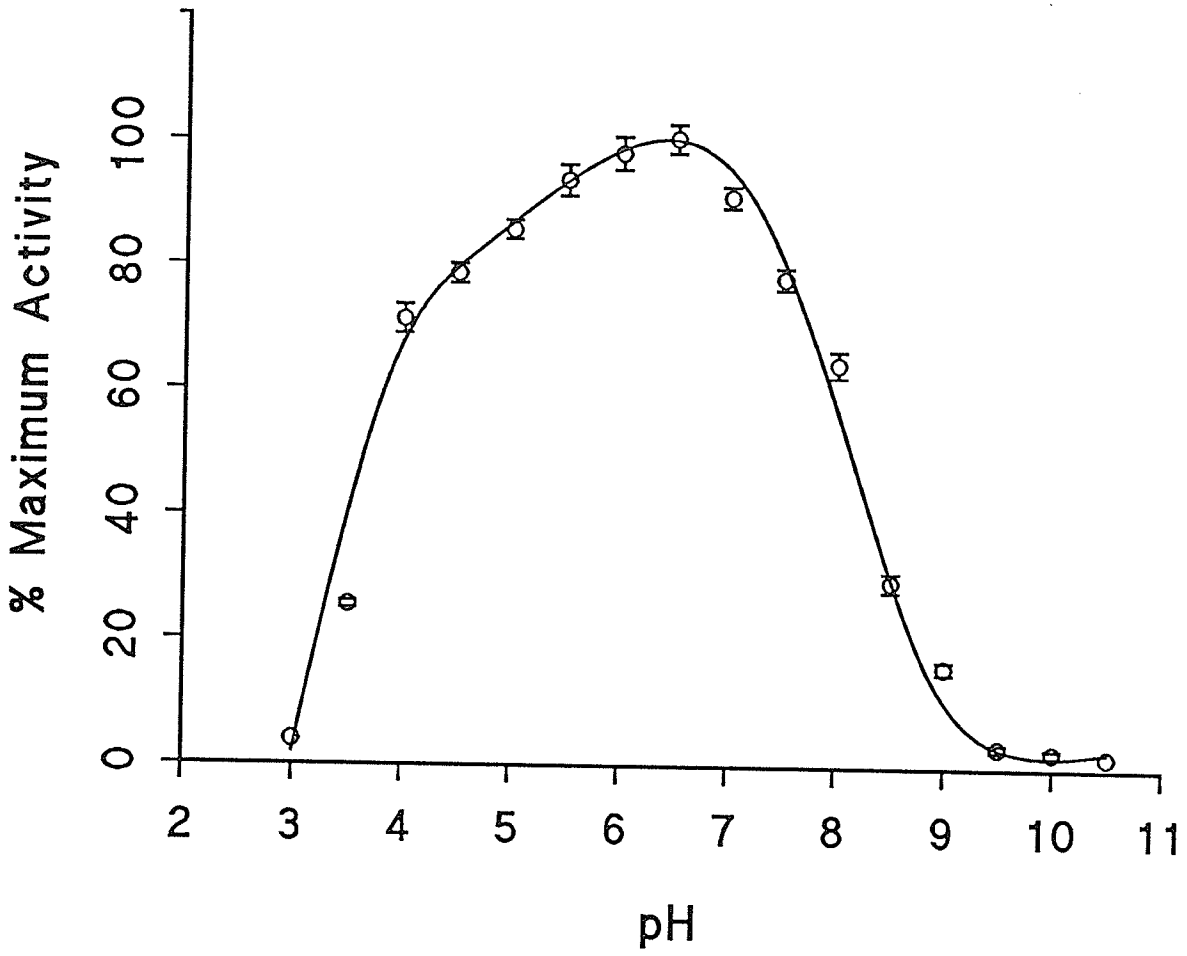


Figure 13. Effect of pH on Rat Liver Biotinidase. 100 ng of rat liver biotinidase was assayed as described under the Experimental Procedures except the following buffers were used : 0.1 M citrate/sodium citrate (pH 3.0 – 3.5) acetate/sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0 – 8.5), bicarbonate (pH 9.0–10.5). Each value represents the mean ± S.D. of 3 samples.

3.3. Kinetic properties of rat liver biotinidase

100 ng of Purified biotinidase was incubated with various concentrations of N-(d-biotinyl) p-aminobenzoate (Figure 14). The double-reciprocal plot (Figure 14 inset) gave a *Vmax* of 2,387 unit/mg and a *Km* of 33 uM. The *Km* of rat liver biotinidase was similar to the values obtained from human serum (Chauhan and Dakshinamurti, 1986) and guinea pig liver (Oizumi and Hayakawa, 1989) which were 35 uM and 31 uM, respectively. The *Km* for N-(d-biotinyl) p-aminobenzoate for biotinidase from human plasma is 10 uM (Craft *et al.*, 1985) and the hog serum biotinidase has a *Km* of 55 uM (Pispa, 1965).

3.4. Amino acid composition

The amino acid composition of rat liver biotinidase is shown in Table 3 along with that of human serum biotinidase for comparison. The high relative abundance of aspartic acid/asparagine (12.24 mole %) and glutamic acid/glutamine (10.88 mole %) indicates the acidic nature of the enzyme protein. In addition, slightly higher levels of valine, phenylalanine, and leucine suggest that this enzyme is relatively hydrophobic. The rat liver biotinidase has 60 % of the serine content of the human serum biotinidase (Chauhan and Dakshinamurti, 1986) and the tyrosine and cysteine content is about half of the human serum biotinidase. Amino sugar analysis detected the presence of N-acetylglucosamine but not N-acetylgalactosamine. The presence of amino sugars suggests that the rat liver biotinidase is a glycoprotein.

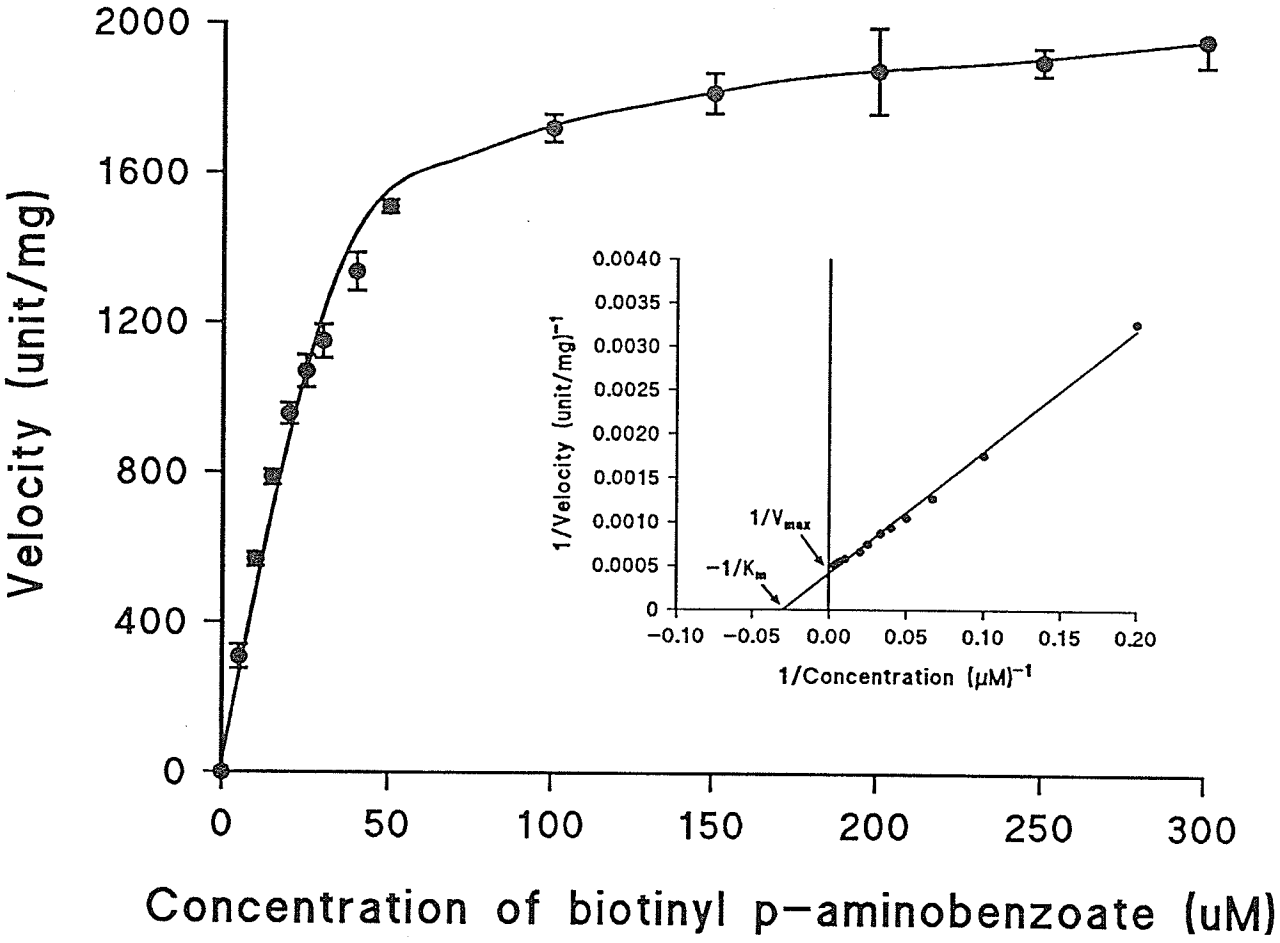


Figure 14. Effect of substrate concentration on biotinidase. 100 ng of purified biotinidase was incubated with the indicated concentrations of biotinyl p−aminobenozate as described under the Experimental Procedures. Km and V_{max} values of purified biotinidase were determined using Lineweaver−Burk plot (inset). Each data point represents the mean ± S.D. of triplicate determinations.

Table 3. Amino acid compositions of purified biotinidase from rat liver and human serum.

Amino Acid	Rat Liver Biotinidase (Mole %)	Human Serum Biotinidase (Mole %)
Aspartic Acid/Asparagine	12.24	9.10
Threonine	5.00	5.84
Serine	4.50	7.49
Glutamic Acid/Glutamine	10.88	10.97
Proline	7.31	6.37
Glycine	9.98	11.23
Alanine	7.47	6.75
Valine	6.49	5.80
Methionine	1.33	1.53
Isoleucine	3.63	3.67
Leucine	10.31	8.30
Tyrosine	1.96	3.84
Phenylalanine	5.91	5.03
Histidine	2.31	3.50
Lysine	4.18	3.42
Arginine	4.11	3.45
Cysteine	1.68	3.17
Tryptophan	0.72	0.97

3.5. Effect of various salts on rat liver biotinidase

The effect of various salts on the purified rat liver biotinidase is shown in Table 4. Liver biotinidase activity was not affected by the following salts: $ZnCl_2$ and $FeCl_3$ at 0.1 mM; $MgCl_2$, $MnCl_2$, $CaCl_2$, and $NiSO_4$ at 1 mM; NaCl and KCl at 0.1 M. $ZnCl_2$ at 1 mM caused a slight inhibition of biotinidase activity (16 % inhibition) whereas $HgCl_2$ and $Cu(CH_3COO)_2$ at 0.1 mM completely inhibited biotinidase activity. The inhibition of biotinidase activity by copper chloride (40 % inhibition at 0.5 mM) and zinc chloride (30 % inhibition at 1 mM) has been demonstrated in human serum (Chauhan and Dakshinamurti, 1986).

3.6. Effect of some chaotropic agents on rat liver biotinidase

Two chaotropic agents were assessed for their abilities to inhibit biotinidase activity (Table 5). The inhibitions of biotinidase of rat liver by 0.5 M urea (29 % inhibition) and by 0.5 M guanidine hydrochloride (37 % inhibition) are slightly less than that of the human serum biotinidase (Chauhan and Dakshinamurti, 1986). 0.5 M Urea or 0.5 M guanidine hydrochloride inhibited the serum enzyme by 50 %.

Hayakawa and Oizumi (1987) showed that human serum biotinidase activity was enhanced by increasing concentrations (up to 2 %) of non-ionic detergents Nonidet P-40 (NP-40) and Triton X-100. They showed that the enzyme activity was increased by approximately 70 % and 100 % for NP-40 and Triton X-100, respectively. The effects of these two detergents were studied on the purified rat liver biotinidase (Table 5). Activity of the rat liver biotinidase was not affected by NP-40

Table 4. Effect of various salts on purified rat liver biotinidase.

Salts	Concentration (mM)	Biotinidase Activity[a] (% of control)
$MgCl_2$	1.0	103 ± 4
$MnCl_2$	1.0	102 ± 2
$CaCl_2$	1.0	103 ± 2
$ZnCl_2$	0.1	100 ± 2
$ZnCl_2$	1.0	82 ± 9
$NiSO_4$	1.0	102 ± 1
$FeCl_3$	0.1	102 ± 3
$HgCl_2$	0.1	2 ± 1
$Cu(CH_3COO)_2$	0.1	3 ± 1
NaCl	100	104 ± 3
KCl	100	107 ± 1

[a]Aliquots of purified biotinidase (100 ng) were assayed in incubation buffer containing 0.1 M potassium phosphate, pH 6.5, 0.063 % bovine serum albumin, and the indicated concentrations of salts at 37 °C for 1 hour. Incubation buffer which did not contain any of the indicated salts was used as a control. Each value represents the mean ± S.D. of 3 determinations.

Table 5. Effect of some chaotropic agents on purified rat liver biotinidase.

Salts	Concentration	Biotinidase Activity[a] (% of control)
Triton X-100	0.5 %	100 ± 2
	1.0 %	96 ± 2
	2.0 %	81 ± 3
Nonidet P-40	0.5 %	102 ± 1
	1.0 %	100 ± 1
	2.0 %	86 ± 3
Urea	0.1 M	96 ± 1
	0.5 M	71 ± 5
Guanidine hydrochloride	0.1 M	80 ± 3
	0.5 M	63 ± 4

[a]Aliquots of purified biotinidase (100 ng) were assayed in incubation buffer containing 0.1 M potassium phosphate, pH 6.5, 10 mM EDTA, 0.063 % bovine serum albumin, and the indicated concentrations of agents at 37 °C for 1 hour. Incubation buffer which did not contain any indicated agents was used as a control. Each value represents the mean ± S.D. of 3 determinations.

or Triton X-100 at a concentration of 1 % whereas it was slightly inhibited at a concentration of 2 %. These effects are opposite to the findings by Hayakawa and Oizumi (1987).

3.7. Effect of some enzyme inhibitors on rat liver biotinidase

Figure 15a shows the effect of iodoacetamide on purified rat liver biotinidase. Biotinidase activity was inhibited by iodoacetamide (84 % inhibition at 1mM) that appears to be similar to that of human serum biotinidase, which showed 85 % inhibition at 0.5 mM (Hayakawa and Oizumi, 1988) and human plasma biotinidase, which showed 99 % inhibition at 0.5 mM (Craft *et al.*, 1985). The results suggest that sulfhydryl group(s) may be important in catalyzing the enzyme reaction.

Enzyme activity was inhibited by N-bromosuccinimide (Figure 15b). The human serum biotinidase was found to be more sensitive to N-bromosuccinimide in that it was completely inhibited by 10 uM N-bromosuccinimide (Chaunhan and Dakshinamurti, 1988). The inhibition of rat liver biotinidase activity by N-bromosuccinimide suggests that tryptophan residues may be involved in the catalysis.

Rat liver biotinidase was not inhibited by phenyl methyl sulfonyl fluoride (PMSF) up to 1 mM (Figure 15c). Chauhan and Dakshinamurti (1986) found that PMSF had no effect on human serum biotinidase at a concentration of 1 mM. Porcine cerebrum biotinidase was also not affected by PMSF at a concentration of 50 uM (Oizumi and Hayakawa, 1990). However, Craft *et al.* (1985) found that 99 % of the human plasma biotinidase activity was inhibited at 0.1 uM of PMSF.

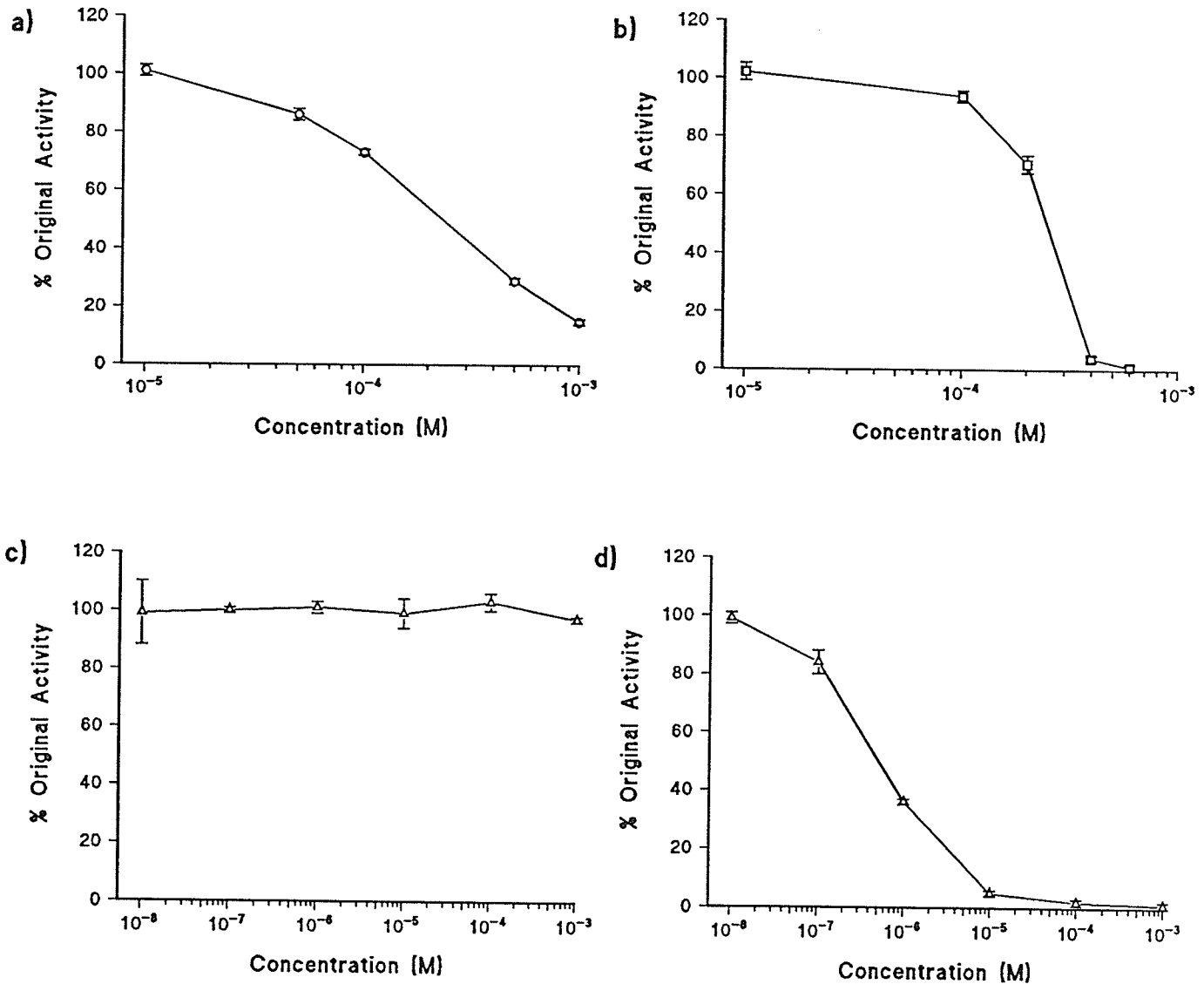


Figure 15. Effect of some enzyme inhibitors on purified rat liver biotinidase. 100 ng of purified biotinidase was assayed in a buffer containing 0.1 M phosphate, pH 6.5, 10 mM EDTA, 0.063 % bovine serum albumin, and the indicated concentration of iodo-acetamide (a), N-bromosuccinimide (b), phenyl methyl sulfonyl fluoride (c), or p-chloromercuribenzoate (d). The reaction was performed as described under the Experimental Procedures. Incubation buffer which did not contain any inhibitors was used as a control. Each value represents the mean ± S.D. of 3 determinations.

The lack of effect by PMSF suggests that serine hydroxyl group(s) is probably not essential in the enzyme reaction of rat liver biotinidase.

Rat liver biotinidase activity was completely inhibited by p-chloromercuribenzoate at 10 uM (Figure 15d) suggesting cysteine residue(s) may be involved in catalysis. Complete inhibition by p-chloromercuribenzoate was also observed in human serum (Chauhan and Dakshinamurti, 1988), human plasma (Craft *et al.*, 1985), and hog kidney (Knappe *et al.*, 1963) biotinidases at the same concentration. Hog serum and hog liver (Pispa, 1965) and guinea pig liver (Oizumi and Hayakawa, 1989) biotinidases were inhibited totally by p-chloromercuribenzoate at 0.1 mM, 50 uM, respectively. Porcine cerebrum biotinidase was inhibited by 80 % at 50 uM of p-chloromercuribenzoate (Oizumi and Hayakawa, 1990). However, *Lactobacillus casei* and *Streptococcus faecalis* were not inhibited at concentration up to 0.1 mM p-chloromercuribenzoate (Koivusalo et al., 1963; Knappe *et al.*, 1963).

3.8. Heat inactivation of rat liver biotinidase

Purified rat liver biotinidase was inactivated 15 and 25 % after heating at 70 °C for 15 and 20 minutes, respectively (Figure 16). It, therefore, seems more heat stable than the purified human serum biotinidase which was denatured 60 % after 15 minutes at 60 °C (Chauhan and Dakshinamurti, 1986). Rat liver biotinidase was completely denatured after 10 minutes at 80 °C.

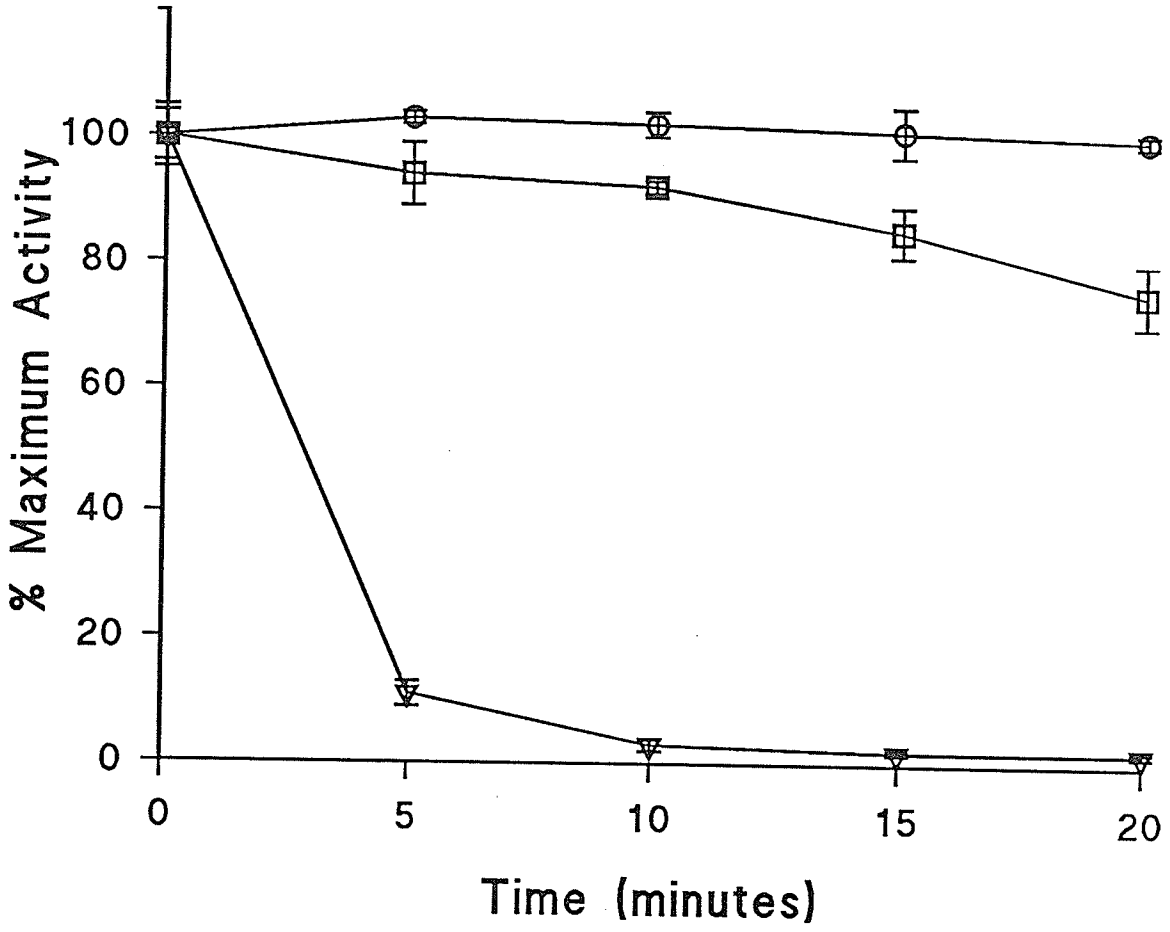


Figure 16. Heat stability of purified rat liver biotinidase. 100 ng of purified biotinidase in 100 ul of 0.1 M phosphate buffer, pH 6.5 was incubated at 60 °C (○), 70 °C (□), 80 °C (▽) and subsequently assayed for biotinidase activity as described under the Experimental Procedures. Each value represents the mean ± S.D. of 3 determinations.

4. Characterization of antibody

4.1. Antibody titer

Various dilutions of antiserum between 1:100 and 1:800 were used to find the best signal with lowest background. 1:400 dilution of antiserum gave the lowest background with highest sensitivity for the detection of antigen. Therefore, this dilution was used for subsequent studies. Figure 17 shows the dose-response curve of different amounts of purified rat liver biotinidase. Intensity was linear between 10 and 100 ng of enzyme protein. 1:400 dilution of antiserum was able to detect 10 ng of biotinidase protein using alkaline phosphatase-conjugated second antibody.

4.2. Immunoinhibition of purified rat liver by polyclonal antibody raised against it

The specificity of the antibody raised against the purified rat liver biotinidase was demonstrated (Figure 18). The partially purified IgG from pre-immune serum did not inhibit biotinidase activity (100 ng purified enzyme protein) at an amount up to 70 ug. However, biotinidase activity was inhibited sharply by the partially purified IgG from immune serum between 0 and 20 ug of IgG. The degree of inhibition was reduced with further increase in the amount of the immune IgG. About 70 % of the enzyme activity was inhibited with 70 ug of immune IgG.

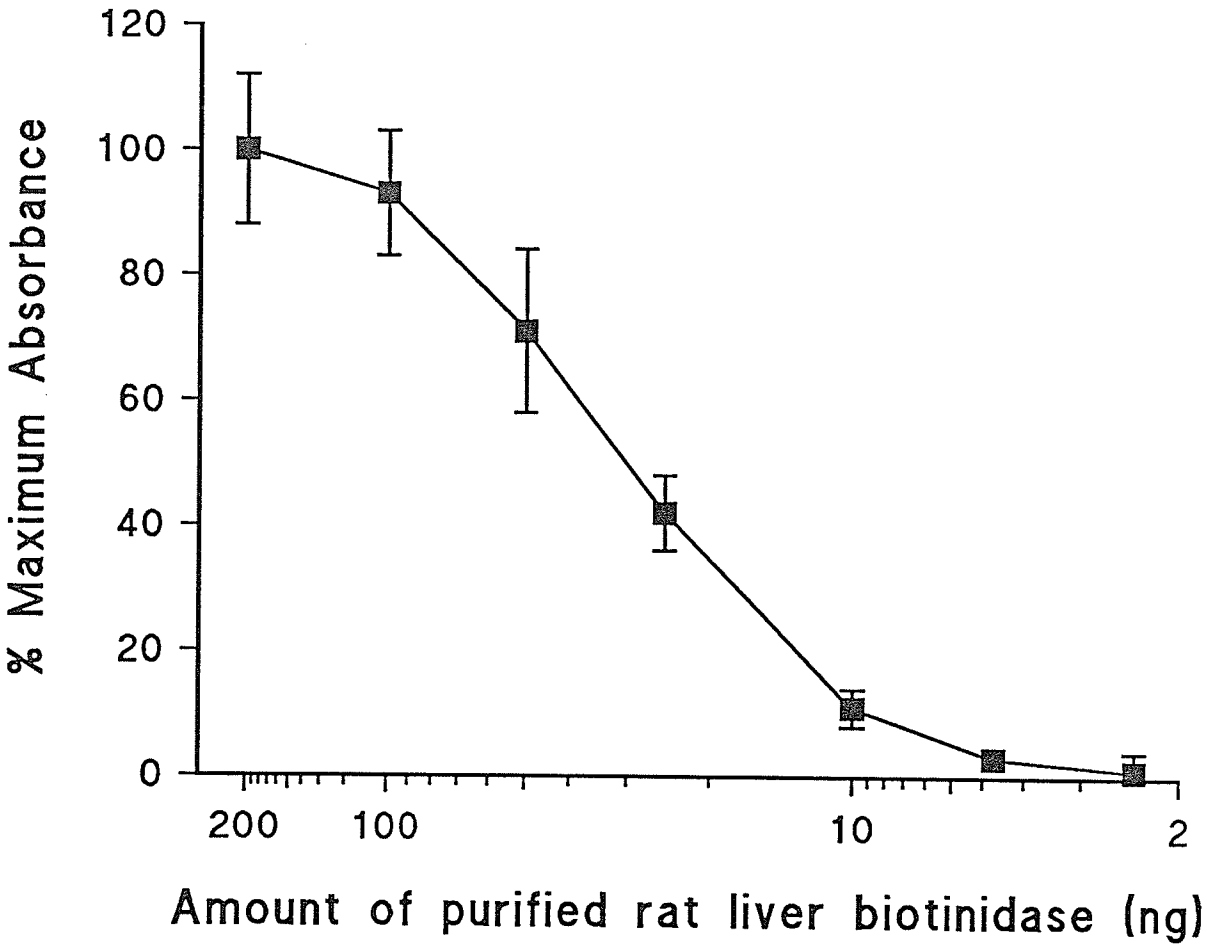


Figure 16. Densitometric determination of antiserum titer. Various amounts of purified rat liver biotinidase were blotted onto a nitrocellulose membrane and were assayed as described under the Experimental Procedures. 1:400 dilution of the anti-serum was used as the first antibody. The intensity of each dot was determined as described under the Experimental Procedures. Each value represents the mean ± S.D. of 3 determinations.

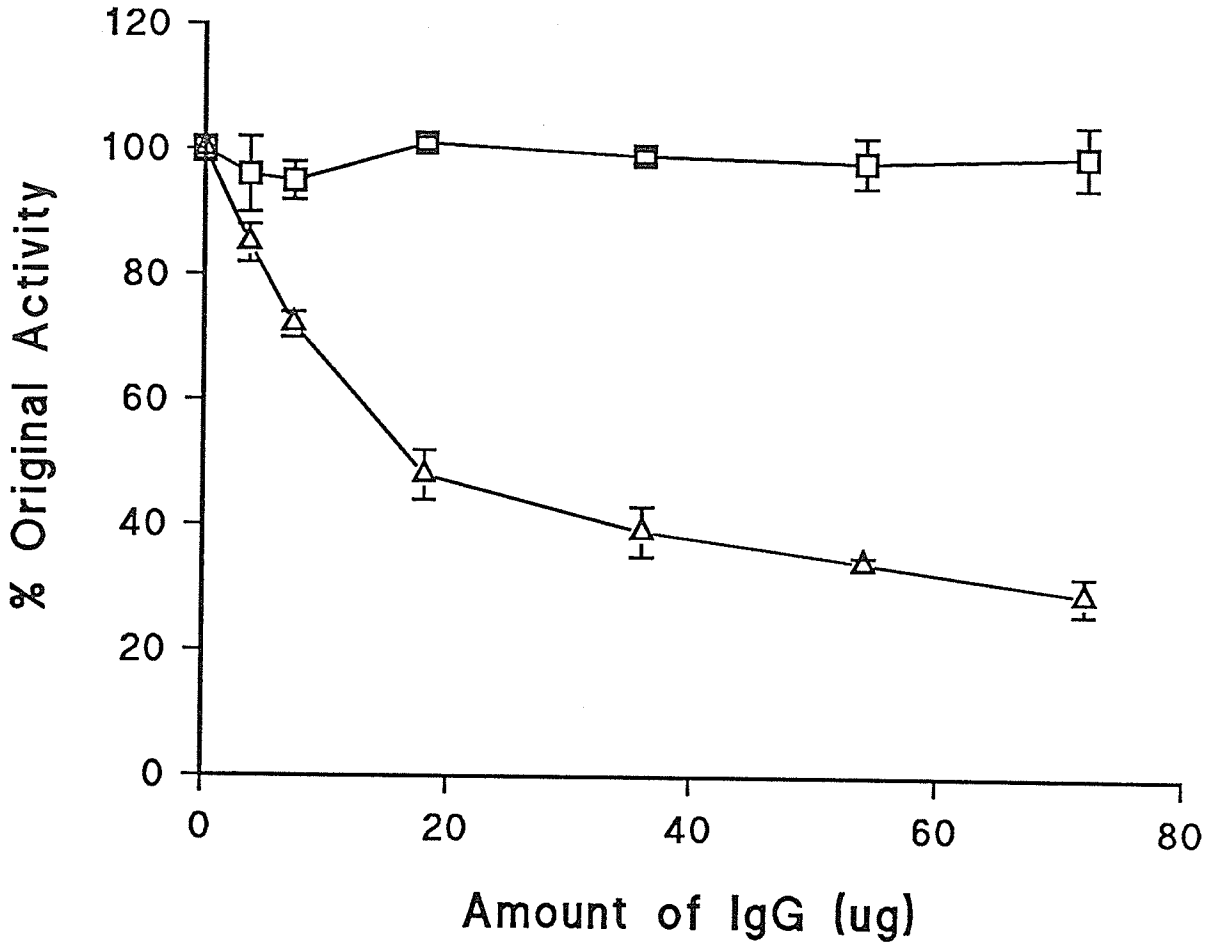


Figure 18. Immunoinhibition of rat liver biotinidase. 100 ng of purified biotinidase was incubated with the indicated amounts of IgG obtained from pre-immune serum (□) or immune serum (△) in 100 ul of buffer containing 0.1 M phosphate, pH 7.0, for 24 hours at 4 °C. The samples were then assayed for biotinidase activity as described under the Experimental Procedures. Samples with only the purified biotinidase were used as a control. Each value represents the mean ± S.D. of 3 determinations.

CHAPTER IV

DISCUSSION

This study on biotinidase activities in various rat tissues indicated that the highest specific activity was found in serum. Other tissues including liver, kidney, and intestine also had a relatively high enzyme specific activity. In rat liver, biotinidase is completely membrane-bound. It can be solubilized from the membrane fraction. Oizumi and Hayakawa (1989) suggested that biotinidase from guinea pig liver is an integral membrane protein which implies that this enzyme is tightly embedded in the membrane. They based this suggestion on the observation that 80 % of the total biotinidase activity in guinea pig liver was associated with membrane fraction and was released by 0.1 % of the detergent NP-40.

The low concentration of detergent required to solubilize biotinidase from rat liver membrane preparations with relatively little loss in enzyme activity and the release of biotinidase to the soluble fraction when the liver tissues were frozen and thawed suggest that biotinidase is loosely associated with membrane, but not an integral membrane protein. This finding is consistent with the notion that biotinidase in serum is synthesized in organs such as liver and then secreted into the serum. It is possible that biotinidase is synthesized in its active form in the endoplasmic reticulum since specific activity of the purified rat liver biotinidase was comparable to that of the human serum enzyme. Post-translational attachment and modification of carbohydrate moiety to the enzyme may occur while the enzyme is transported through Golgi apparatus, plasma membrane, and eventually into serum. Biotinidase present in the intestine is probably synthesized and secreted by the epithelial cells.

Biotinidase in rat liver was purified 25,000-fold to a specific activity of 1,800 unit/mg with a 12 % recovery of total enzyme activity. It has also been partially purified from various other sources such as *Streptococcus faecalis*, *Lactobacillus casei*, and kidney, liver, and serum from hog. Human serum biotinidase was the first to be purified to homogeneity (Chauhan and Dakshinamurti, 1986). The purified human serum enzyme had a specific activity of 1,900 unit/mg. Oizumi and Hayakawa (1989, 1990) purified biotinidase from guinea pig liver and porcine cerebrum to specific activities of 13 and 98 unit/mg, respectively. They stated that these enzyme preparations were pure based on Comassie Blue stain of SDS-PAGE.

The purified rat liver biotinidase gave a single protein band with both SDS and non-SDS-PAGE indicating that rat liver biotinidase consists of a single polypeptide. SDS-PAGE analysis of purified rat liver biotinidase gave a molecular weight of 61 KDa. Purified human serum biotinidase was shown to have a molecular weight of 80 KDa (Chauhan and Dakshinamurti, 1986) using the SDS-PAGE system of Laemmli (1970). Assays using Ferguson plot for starch gel analysis and sucrose density gradient estimated the molecular weight of human serum biotinidase to be 68 KDa. Thus, the molecular weight of the rat liver enzyme is significantly smaller than that of the human serum enzyme. Oizumi and Hayakawa (1989, 1990) showed that biotinidase from both guinea pig liver and porcine cerebrum have a molecular weight of 68 KDa using SDS-PAGE system. A major protein band of about 70 KDa was identified in this study during the later stages of purification of rat liver

biotinidase. This band was not biotinidase. Oizumi and Hayakawa assessed the purity of the purified enzyme preparations by Comassie Blue stain which is about 50 times less sensitive than silver-stain. Therefore, relatively small amounts of protein may not be detectable as a visible protein band using this method. In addition, the specific activities of these "purified enzyme preparations" were very low compared to the values of biotinidase from human serum and rat liver. Furthermore, the recoveries of enzyme activities were relatively high. They were 14 % for guinea pig liver and 36 % for porcine cerebrum. This suggests that the low specific activities of the enzyme preparations were not because of extensive loss or inactivation of enzyme activities during the purification process. As a result, it is not certain if the protein bands they showed were indeed biotinidase.

Sephacryl S200 column chromatography was also used to determine the native molecular weight of rat liver biotinidase which was estimated to be about 70 KDa. Chauhan and Dakshinamurti (1986) found that human serum biotinidase had a molecular weight of 110 KDa using the Sephacryl S200 column chromatography. They attributed the larger molecular weight obtained using gel filtration compared to that using SDS-PAGE, to a larger stroke radius due to its glycoprotein nature. Therefore, it eluted earlier than expected. The molecular weight of rat liver biotinidase is smaller than that of the human serum biotinidase using either SDS-PAGE or gel filtration. The presence of N-acetylglucosamine in rat liver biotinidase indicated that it is also a glycoprotein.

The human serum biotinidase has been extensively characterized (Chauhan and Dakshinamurti, 1986). The rat liver biotinidase shares many of the properties of the human serum enzyme. Both rat liver biotinidase and human serum biotinidase have a broad pH profile with maximum activity at pH 6.5, using d-biotinyl p-aminobenzoate as substrate. The *Km* and specific activities of these two enzymes are virtually identical. The purified rat liver biotinidase has a *Km* of 33 uM and a specific activity of 1,800 unit/mg. The purified human serum enzyme has a *Km* of 35 uM and a specific activity of 1,900 unit/mg. With the exception of few amino acids such as serine, tyrosine, and cysteine, the amino acid composition of these two enzymes are similar. They both appear to be acidic and slightly hydrophobic. Significant differences were found in the contents of serine, tyrosine, and cysteine, all of which are at a lower percentage composition in rat liver biotinidase. Metal ions such as Cu^{2+} and Zn^{2+} inhibited activities of both enzyme. The degree of inhibition of these two enzyme activities by chaotropic agents, such as urea and guanidine hydrochloride, appeared to be similar. Both enzymes were also inhibited by p-chloromercuribenzoate, N-bromosuccinimide, and iodoacetamide. Phenyl methyl sulfonyl fluoride had no effect on these two enzymes at a concentration of 1 mM. The results indicated that sulfhydryl group(s), tryptophan residue(s), and cysteine residue(s) may be important in enzyme catalysis by biotinidase. The similar effects of various agents on rat liver biotinidase and human serum biotinidase and the almost identical *Km* and specific activity of these two enzymes suggest that they may

possess a very similar active site. There are, however, some major differences between the two enzymes. As mentioned earlier, there is a significant difference in molecular weight between the enzymes, perhaps, due to the different polypeptide chain lengths. It is also possible that the rat liver enzyme is less glycosylated than the human serum biotinidase. Rat liver biotinidase also appeared to be more heat stable. In addition, rat liver biotinidase is membrane-bound whereas the serum biotinidase is a soluble enzyme. The similar kinetic properties of these two enzymes may suggest that the soluble and the membrane-bound forms of biotinidase are very closely related. More definite proof will come from comparison of the liver and serum enzymes within the same species. Nevertheless, the similar kinetic properties indicate that biotinidase catalytic site is highly conserved between rat and human.

Polyclonal antibody against rat liver biotinidase was produced in the rabbit. The specificity of the antibody towards the enzyme was demonstrated by its ability to inhibit enzyme activity while pre-immune serum did not affect the enzyme activity. However, biotinidase activity was not completely inhibited by this antibody. Depending on the location and the affinity of the epitope(s) on the enzyme, the antibody may not bind exactly on the active center of the enzyme that causes complete inhibition of biotinidase activity. Instead, the antibody may bind to epitope(s) near the active center of the enzyme which interferes with the reaction, but does not completely inhibit biotinidase activity. The apparent biphasic nature of the inhibition curve suggests another possibility. Chauhan and Dakshinamurti (1988)

demonstrated the presence of different types of active sites in human serum biotinidase, a high affinity biotin-binding site and a low affinity biotin-binding site. The similar kinetic properties between human serum and rat liver biotinidases suggest that these two different active sites may also be present in the rat liver biotinidase. The antibody may be able to effectively inhibit one of these two active sites more than the other one.

This antibody could be used to study biosynthesis and secretion of biotinidase in rat tissues. Methods such as those employed in studying the secretion of transferrin in the rat liver by immunoprecipitating the intermediates of biosynthesis and secretion may be used (Schreiber et al., 1979; Morgan and Peters, 1985).

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