THE ROLE OF BIOTINIDASE IN THE INTESTINAL UPTAKE OF BIOTIN

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To my mother and father

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ABSTRACT

The uptake of biotin in rat small intestine was examined. The uptake of the vitamin in vitro is concentration dependent. Below a concentration of 40 nM biotin the uptake proceeds by a process which shows saturation kinetics. Above this concentration of biotin the uptake proceeds linearly. Hence, the uptake of biotin in the rat small intestine, which previously was thought to proceed mainly by a passive diffusion, can also be transported by a carrier mechanism.

Biocytin relesed during the digestion of protein-bound biotin may predominate over the concentration of free biotin in the gut as the bulk of the biotin ingested is protein-bound. The uptake of [^{3}H]-biocytin in the rat small intestine was also examined. Its uptake at a given concentration expressed as moles transported per gram of tissue was one and a half-fold higher than the uptake of biotin per gram of tissue at a similar concentration.

A study was conducted to determine whether biotinidase in the rat small intestine could be involved in the transport of biotin. The protein fractions of cytosol and brush border membrane obtained from intestine by sucrose density gradient centrifugation were analyzed for biotinidase activity and biotin-binding activity. Both the cytosolic and brush border preparations contained biotinidase activity, but the amount of activity in the brush border was quite low and could arise as a cotaminant of the cytosol. However, in both fractions

biotinidase activity co-migrates with the biotin-binding activity. Hence it appears that only fractions containing biotinidase activity have biotin-binding activity. This preliminary evidence seems to suggest that biotinidase from rat small intestine can bind to [^{3}H]-biotin *in vitro*. It is speculated that intestinal biotinidase *in vivo* may have a possible role in the transport of biotin.

One form of biotin-responsive multiple carboxylase deficiency in man results in biotinidase activity being decreased in tissues. Due to this deficency biocytin could accumulate in the urine of these patients. A non-competitive assay for biocytin was developed using the serum and urine of normal human subjects. An assay for biotinidase using biocytin was also developed based upon the fluorescence reaction of lysine with 1,2-diacetylbenzene.

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ABBREVIATIONS

ACC	Acetyl coA carboxylase
BSA	Bovine serum albumin
EDTA	Ethylene diamine tetraacetic acid
HCS	Holocarboxylase synthetase
PCC	Propionyl CoA carboxylase
PC	Pyruvate carboxylase
β-MCC	β -methyl crotonyl CoA carboxylase
MCD	Biotin responsive multiple carboxylase deficiency
NCS	NCS tissue solubilizer
OCS	OCS scintillator
TLC	Silica gel thin layer chromotography

INTRODUCTION

I BIOTINIDASE: A BIOTIN RECYCLING ENZYME

a) Function of biotinidase

Biotin is the prosthetic group of the enzymes: Acetyl CoA carboxylase, pyruvate carboxylase, propionyl CoA carboxylase, and β-methyl crotonyl CoA carboxylase. All four carboxylases are synthesized as the inactive apoenzymes in which the biotin moiety is absent. The conversion of the inactive apoenzyme to the active holoenzyme is preceded by the covalent attachment of biotin to the ε-amino group of a lysine residue. This function is catalyzed by the holocarboxylase synthetase. Details regarding the covalent attachment of biotin to these carboxylases has been a subject of much research (Moss and Lane, 1971) but information regarding the release of biotin from the holocarboxylase during its turnover is limited.

Thoma and Peterson (1954) described an enzyme in hog liver and kidney and in chicken pancreas which released biotin from tryptic digests of hog liver. They called this enzyme biotinidase. In the same year Wright et al., (1954) described a similar enzyme in human plasma which was capable of hydrolyzing biocytin. Biotinidase ([E.C.3.5.1.12]) catalyzes the removal of biotin from biocytin (N-[d-biotinyl]-L-lysine), various synthetic substrates such as N-(d-biotinyl)-4-aminobenzoate, and biotinyl peptides which form the products of carboxylase degradation, but not from intact holocarboxylases (Thoma and Peterson, 1954, Wright et al., 1954). The reactions catalyzed by biotinidase are:

1. d-biocytin ----------> L-lysine + d-biotin
<-----
2. N-(d-biotinyl)-4-aminobenzoate ----------> 4-amino-
<----
benzoate + d-biotin

The rate of cleavage of biotin from biotinyl peptides is dependent on the chain length of the biocytin containing peptide, with the rate of cleavage being slower as the chain length increases, however, the rate of cleavage of the biotinylated peptides is much slower compared to cleavage of biocytin (Craft et al., 1985). The natural substrate of the enzyme in vivo is biocytin. The enzyme functions primarily as a mechanism for the recycling of endogenous biotin. Biotinidase is a ubiquitous enzyme. It occurs in microbial, avian, amphibian, and mammalian species (Knappe et al., 1963, Pispa, 1965). In mammals the highest activity is found in the liver followed by serum and kidney. Other tissues such as the adrenals, small intestine, and lungs do have enzyme activity.

b) Properties of biotinidase

Although biotinidase had been discovered over 30 years ago, it was only recently that the enzyme was characterized. A partial purification of hog serum biotinidase (1910-fold)and hog liver biotinidase (204-fold) had been obtained (Pispa, 1965) previously. The bacterial enzyme from Streptococcus faecalis and Lactobacillus arabinosus was partially purified by 700-fold (Koivusalo et al., 1963) and 135-fold (Pispa, 1965), respectively. Human plasma biotinidase was purified 4800-fold by Craft et al., (1985) using a purification procedure

involving DEAE-sephadex A-50, Bio-gel A-0.5m, Affi-gel blue, DEAE-cellulose, and PLC-TSK gel filtration. Purification to homogeneity of human serum biotinidase on DEAE-sephacel, hydroxylapatite, octyl-sepharose CL-4B, and Sephadex G-100 has recently been achieved in this laboratory (Chauhan and Dakshinamurti, 1986). Polyclonal rabbit anti-human biotinidase antibodies have also been generated. An apparent molecular weight of 76 kilodaltons has been reported for biotinidase (Craft et al., 1985). However, since glycoproteins migrate anamolously on SDS polyacrylamide gels, the true molecular weight of the enzyme was determined from Ferguson type plots to be 68 kilodaltons (Ferguson, 1964). In this procedure the relative migration of biotinidase was followed as a function of the polyacrylamide gel concentration. The enzyme is sensitive to p-hydroxymercuribenzoate, iodoacetamide, and N-bromohydroxy succinamide inhibition indicating that sulphydral group(s) and tryptophan residues are essential for enzyme activity. The inhibition of the enzyme activity with p-hydroxymercuri benzoate is reversible with thiol containing reagents. The K_m value for biocytin is 7.8 μM whereas with the synthetic substrate, N-(d-biotinyl)-4-aminobenzoate, the K_m is 10 μM. Biotin is a competitive inhibitor of the enzyme. Biotin inhibition with both substrates is greater under acidic condition. The pH optimum of the enzyme is substrate dependent. With biocytin the optimum is between 4.5-6.0 whereas with N-(d-biotinyl)-4-aminobenzoate the optimum is between 6.0-7.5

(Ebrahim and Dakshinamurti, 1986). Enzyme activity is 30% higher with biocytin when compared to the activity with the synthetic substrate (Ebrahim and Dakshinamurti, 1986).

c) Assays for biotinidase

Various assays have been used to assess the activity of the enzyme. Earlier studies of Wright and Skeggs (1944) utilized the differential growth responsiveness of the microorganism Lactobacillus arabinosus (strain ATCC 8014) to biotin and biocytin for measuring the activity of plasma biotinidase. The principle of this assay is that the growth of the microorganism is dependent on the amount of biotin released during enzymatic hydrolysis of biocytin. As this microbial assay involves a long incubation time, it is not used routinely. A more convenient assay which uses a synthetic substrate, N-(d-biotinyl)-4-aminobenzoate, was introduced by Knappe et al. (1963). In this assay the release of 4-aminobenzoate was followed colorimetrically at 546 nm following its diazotization. This assay was made more sensitive for clinical application by following the release of [^{14}C-carboxyl]-4-aminobenzoate from N(d-biotinyl)-[^{14}C-carboxyl]-4-aminobenzoate (Wolf and McVoy, 1983) after the removal of biotin and excess unreacted substrate with avidin followed by bentonite precipitation. More recently a fluorometric assay measuring the release of 6-aminoquinoline (at excitation wavelength = 350 nm and emission wavelength = 550 nm) from N-(d-biotinyl)-6-aminoquinoline has been described by Wastell et al. (1984). A bioassay for

determining biotinidase activity in human serum using holocarboxylase synthetase-deficient cultured fibroblasts has also been described (Weiner et al., 1985). Biotinidase activity has also been shown to coincide with the [^{3}H]-biotin binding activity (Dakshinamurti and Chauhan, 1986) and the binding activity shows the same pH dependence phenomenon as seen with enzyme activity with biocytin.

II THE CLINICAL SIGNIFICANCE OF BIOTINIDASE

a) Biotin-reponsive multiple carboxylase deficiency

There are four biotin-dependent carboxylase enzymes in mammalian tissues, their place in intermediary metabolism is shown in figure 1. Acetyl CoA carboxylase ACC (E.C. 6.4.1.2) is a regulatory enzyme involved in the synthesis of fatty acids; pyruvate carboxylase PCC (E.C. 6.4.1.1) catalyzes the initial commited step in gluconeogenesis; propionyl CoA carboxylase PCC (E.C. 6.4.1.3) catabolizes the branched chain amino acids valine, isoleucine, methionine and threonine, as well as odd chain fatty acids and the side chain of cholesterol; and β-methylcrotonyl CoA carboxylase β-MCC (E.C. 6.4.1.4) is involved in the catabolism of leucine. Isolated deficiencies in humans of each of the biotin-dependent carboxylases have been described (Finnie et al., 1976, DeVivo et al., 1977, Bloom et al., 1981, Wolf et al., 1981). In addition to individual deficiencies of these carboxylases, a rare condition of in born error of metabolism known as biotin-responsive multiple

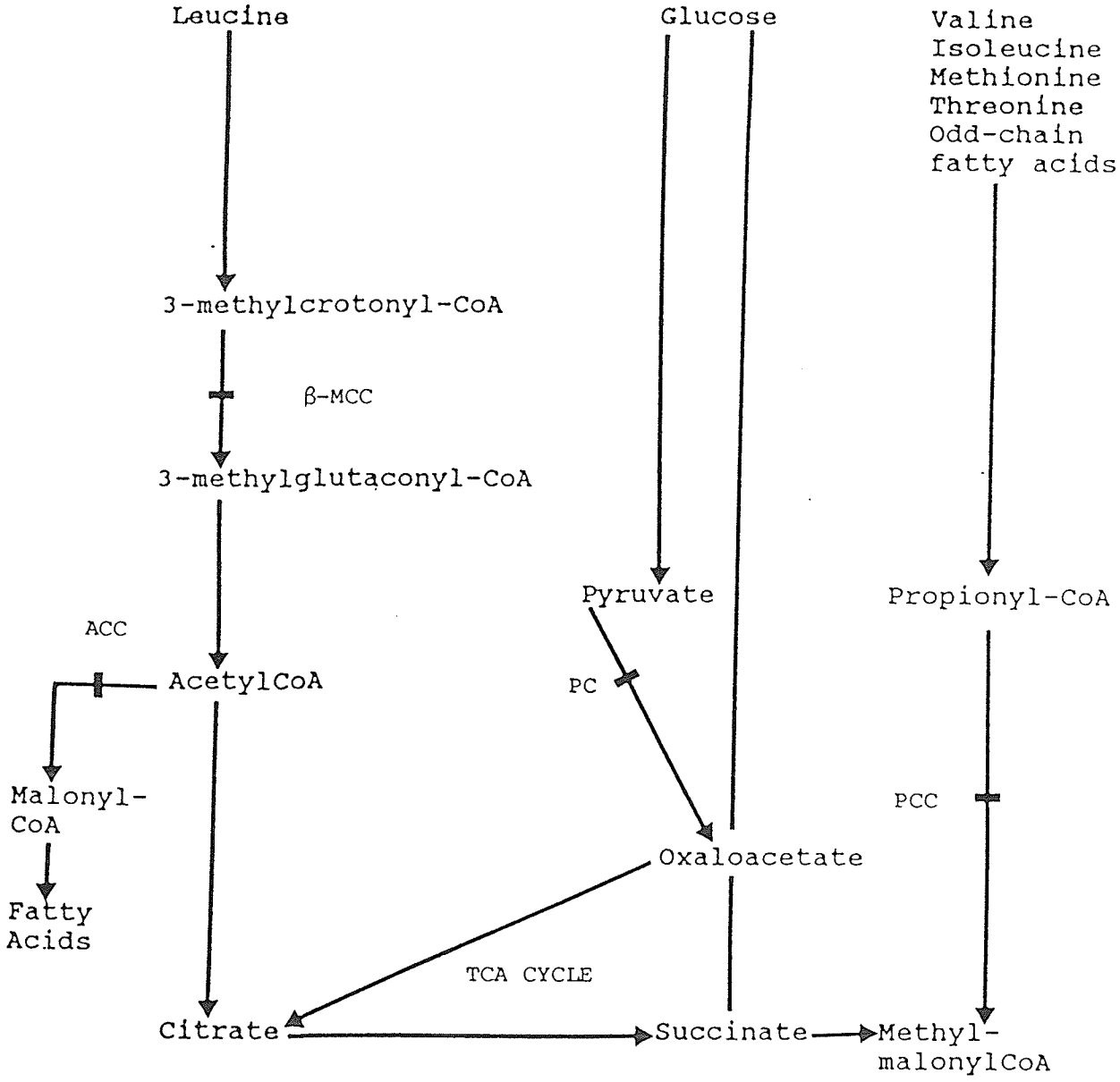


Figure 1. - Metabolic pathways involved in carboxylase deficiencies. Sites of PCC, PC, β-MCC, and ACC are indicated by solid bars.

carboxylase deficiency (MCD; Sweetman, 1981) is characterized by deficiency of the three mitochondrial biotin-containing carboxylases namely, propionyl CoA carboxylase, pyruvate carboxylase, and β-methyl crotonyl CoA carboxylase in the fibroblasts (Saunders et al., 1979) and leukocytes (Cowan et al., 1979) of patients. The general involvement of all three biotin-dependent carboxylases in this disorder suggests that the primary defect is in the metabolism of biotin.

Based on differences in clinical features and time of appearance, MCD has been classified into two forms, namely, the neonatal type and the juvenile type. Table 1 summarizes the clinical and biochemical characteristic of the two types of MCD. The neonatal type of MCD afflicts early in life and these infants present with symptoms of vomiting, ketosis, and severe metabolic acidosis. The patients with the late onset form (juvenile type) are generally asymptomatic for the first few months of life. At three months a skin rash develops, and they develop conjuctivitis, alopecia, candidiasis, ataxia and delayed motor development. The intravenous administration of a pharmacological dose of biotin to patients results in the correction of the acid-base disturbances in both type of MCD.

b) Neonatal type of MCD

The neonatal type of multiple carboxylase deficiency appears to have a defect in holocarboxylase synthetase (HCS) enzyme (Sweetman, 1981, Burri et al., 1981). This enzyme covalently attaches biotin to a number of apo-carboxylases to

Table 1 - Two forms of biotin-responsive carboxylase deficiencies.

	Neonatal Type	Late-onset Type (~ 3 mos)
Clinical features.....	Vomiting, lethargy, hypotonia	Skin rash, conjunctivitis, alopecia, candidiasis, ataxia developmental delay
Biochemical features...	Keto-acidosis, organic acidemia, hyperammonemia	Keto-acidosis, organic acidemia
Serum biotin levels....	Normal	Low
Urinary biotin levels..	Normal	Low
Leukocyte carboxylase activities:		
Before biotin.....	Deficient	Deficient
After biotin.....	Near-normal or normal	Normal
Fibroblast carboxylase activities:		
Before biotin.....	Deficient	Normal
After bitoin.....	Near-normal or normal	Normal
Defect.....	Holocarboxylase synthetase	Biotinidase Biotin absorption or transport?

form enzymatically active holocarboxylases (Achuta Murthy and Mistry, 1982). The conversion of apo-PCC to holo-PCC occurs as follows:

$$\text{Biotin} + \text{ATP} \underset{\text{HCS}}{\overset{Mg^{++}}{\rightleftharpoons}} \text{Biotinyl-5'-adenylate} + \text{PPi}$$

$$\text{Biotinyl-5'-adenylate} + \text{apo-PCC} \xrightarrow{\text{HCS}} \text{Holo-PCC} + \text{AMP}$$

Following the activation by ATP, biotin forms an amide bond with the ε-amino group of a lysine residue on the apo-PCC to form holo-PCC. Elevated K_m values of the holocarboxylase synthetase for biotin seem to be responsible for the neonatal type of MCD. The normal range of K_m value of human fibroblast holocarboxylase synthetase for biotin is 15 ± 3 nM (Sweetman et al. 1985). The K_m value of the fibroblast enzyme for biotin is elevated between 3-70 times the normal range in patients. The difference in the kinetic parameter of holocarboxylase synthetase from different patients may be indicative of age of onset or severity of symptoms. The lesion in the holocarboxylase synthetase enzyme appears to be at the biotin binding site. The K_m value of the fibroblast enzyme for ATP is unchanged in patients. The elevated Km values for biotin explains the requirement of a large dose of biotin for clinical therapy and the high concentration of biotin in culture medium needed for restoration to normal values of the carboxylase activity in the fibroblasts of these patients.

The holocarboxylase synthetases from different organisms and tissues have a broad specificity and cross-react with

numerous apoenzymes. The transcarboxylase synthetase from Propionibacterium, for example catalyzes the biotination of apopropionyl-CoA carboxylase from rat liver (Kosow et al., 1962). The studies of McAllister and Coon (1966) also showed that the holocarboxylase synthetase from rabbit liver, yeast, and P. shermanii cross-react forming the active enzymes from apopropionyl-CoA carboxylase from rat liver, from 3-methylcrotonyl-CoA carboxylase from Achromobacter, and from apotranscarboxylase from P. shermanii. The broad specificity of the holocarboxylase synthetase may indicate that the apobiotin enzyme have similar features that provide a recognition site for the synthetase and permit the precise attachment of the cofactor into the active sites of these enzymes.

Holocarboxylase synthetase has been partially purified from a variety of species (Cazzulo et al., 1971, Wood et al., 1980, and Barker and Campbell, 1981). The purification of the mammalian enzyme to homogeneity will be useful in determining whether two different enzymes are present in the cytosol and mitochondria. In man, however, there is some evidence to support the existence of only one holocarboxylase synthetase as it has been shown that a patient with neonatal MCD (Feldman and Wolf, 1981) had enzyme activity levels of both pyruvate carboxylase and acetyl coA carboxylase depressed. This may indicate that a single enzyme normally catalyzes the formation of these holocarboxylases from their respective precursors.

c) Juvenile type of MCD

The juvenile form of multiple carboxylase deficiency results in low levels of biotin in plasma and urine but the carboxylase activities are normal in fibroblast extracts regardless of the biotin concentration in the culture media. Also varying concentrations of biotin administered orally to patients with juvenile MCD did not increase plasma biotin concentrations to the same levels as in similar control healthy subjects. This was speculated to be due to an abnormality in the intestinal absorption of biotin (Munnich et al., 1981, Theone et al., 1983). However, it is now well established that juvenile type of MCD results primarily in a defect in biotinidase (Gaudry et al., 1983, Theone and Wolf, 1983, Wolf et al., 1983a, Wolf et al., 1983b, Wolf et al., 1983c). The juvenile type of MCD is a homozygous recessively inherited disease and the estimated incidence in the United States is about 1:40,000. The deficiency of the enzyme occurs in all the tissues and the percentage activity in serum which has the highest activity is about 1-4% of the level found in normal individuals.

Serum biotin concentrations of patients with late-onset type of MCD are normal or below the normal range prior to biotin treatment. Because most methods used to determine biotin quantitatively measure biotin and biocytin (Baker et al., 1962, Landman, 1976), it is possible that the serum biotin concentration reported may be spuriously high, reflecting in

part an elevation in serum biocytin. Attempts to measure biocytin using the ninhydrin method of detection has failed to detect any biocytin in plasma and urine of these patients.

The consequence of biotinidase deficiency in patients is that they suffer a rapid depletion of biotin stores after birth. The mechanism for this rapid depletion is not fully understood. The considerable loss of biocytin and biotin through the kidneys is certainly a major factor leading to the increased biotin requirement. Another factor might be the patient's inability to utilize dietary biotinyl compounds arising during digestive degradation of protein which are probably a major source of dietary biotin under normal conditions (Wolf et al., 1984). Hence children lacking biotinidase activity are unable to recycle endogenous biotin and have to depend on exogenous free biotin to prevent the clinical and biochemical features of biotin deficiency.

III The transport of biotin

a) Uptake of biotin in bacteria and yeast

The transport of biotin in microorganisms has been well studied. No specific transport process was demonstrated in bacteria such as E. coli Crookes (ATCC 8739) and in the parent strain of E. coli K-12 (Cicmanec and Lichstein, 1978) It was shown that the initial and steady-state incorporation of [^{14}C]-biotin in resting and dividing cells of these strains was temperature and energy independent and ocurred over a wide

extracellular concentration of biotin. However, in another strain of E. coli, strain Y-10, [^{14}C]-biotin was transported into the cell by a process which was energy-requiring, temperature dependent, and sensitive to analog inhibition and pH (Prakash and Eisenberg, 1974) thus indicating that active transport may be involved. A similar active transport of biotin has been demonstrated in the biotin-deficient strain of E. coli - 162 (Diffeteu et al., 1982) and in other bacteria such as L. arabinosus (Lichstein and Ferguson, 1958), L. planataram (Waller and Lichstein, 1965a, Waller and Lichstein, 1965b) and L. casei (Henderson et al., 1979).

In the yeast, Saccharomyces cerevisiae, evidence for a carrier mediated, active process for the transport of biotin has been presented (Rogers and Lichstein, 1969a) and the indication that a carrier protein may be involved (Roger and Lichstein, 1969b) has been supported by the irreversible inactivation of biotin uptake in yeast by p-nitrophenyl ester of biotin (Becker et al., 1971). The membrane bound location of the transport protein has been inferred using cold-shocked cells and spheroplasts of S. cerevisiae (Cicmanec and Lichstein, 1974).

b) Uptake of biotin in mammalian cells

In contrast, however, the mechanisms for transport of biotin in mammalian cells have not been fully characterized. A nutritional requirement of biotin for mammalian cells in culture has been established for HeLa cells, human fibroblasts,

Baby hamster kidney cells and polyoma transformed baby hamster kidney cells. The transport of biotin into HeLa cells (Dakshinamurti and Chalifour, 1981, Chalifour and Dakshinamurti, 1982a), fibroblasts (Chalifour and Dakshinamurti, 1982b) and rat liver cells (Chalifour and Dakshinamurti, 1983) has been examined. In both cases avidin [^{3}H]-biotin complex binds at 'specific' receptor sites on the cell membrane and is then internalized. The free biotin is made available after degradation of the avidin [^{3}H]-biotin complex which is presumably carried out by the hydrolytic enzymes in the lysozomes. The transport of avidin [^{3}H]-biotin complex into HeLa cells and fibroblasts occurs by absorptive pinocytosis. Free biotin is taken up by HeLa cells but at a rate which is 15 times slower compared to the uptake of avidin [^{3}H]-biotin complex and its mode of uptake is by fluid phase pinocytosis. As avidin is not a natural protein in the system studied, it was suggested that avidin was mimicking a natural ligand which could bind to biotin. This could be the biotin-carrier protein currently being characterized (Chauhan et al., 1986a).

The fully differentiated mouse 3T3-L1 cell line displays biochemical characteristics of a biotin dependent mammalian cell and it transports biotin by two processes: the first, a carrier mediated process shows a non-linear dependence on external biotin concentration, it occurs between 5 nM and 50 M and shows temperature sensitivity, saturation kinetics, and specificity for the substrate; the second process, is diffusion

driven and occurs at higher concentration of extracellular biotin i.e., above 75 M (Cohen and Thomas, 1982). Since 3T3-L1 cells are similar to other biotin dependent tissues like liver and kidney, this study may imply that uptake of biotin in these tissus occur via a carrier mechanism.

Two different studies on the uptake of biotin in rat hepatocytes maintained in culture present data that are not consistent with each other. One study with rat hepatocytes (Bowers-Komro and McCormick, 1985) shows that biotin is transported linearly over a concentration range between 2nM and 2 μ M, but at 20nM concentration of biotin which resembles the physiological concentration in rat plasma, the amount of biotin transported in hepatocytes from normal and biotin-deficient rats did not vary appreciably between these two sets and the uptake was sodium and energy dependent, sensitive to temperature and analog inhibition, and shared a transport component similar to that for the acid-anion carrier of bilirubin and cholic acid. The other study using hepatocytes from normal rats and biotin-deficient rats (Weiner and Wolf, 1985) showed that the uptake of biotin at 5nM concentration was linear for up to one hour in the case of hepatocytes from normal rats whereas the uptake of biotin in hepatocytes from biotin-deficient rats was linear for up to 24 hours and the amount taken up was sixteen times greater compared to that observed in hepatocytes from normal rats. Also the uptake of biotin by hepatocytes in these studies was reported to be

unaffected by Na^+, pH, analog or metabolic inhibitors and hence the transport of biotin in rat hepatocytes in this study was concluded to be driven by diffusion.

c) Uptake of biotin in the mammalian small intestine

The mechanism of uptake of biotin across the small intestine has not been studied in detail. The earlier studies of Turner and Hughes (1962a) using a microbial assay (Barton-Wright, 1952) for measuring the transmural flux of biotin showed that the *in vitro* uptake of biotin across everted sac preparations of rat and hamster between 1 to 7 μ M and at 2 μ M concentration of biotin, respectively did not occur against a concentration gradient in either case and in the case of the rat the mucosal to serosal flux at a given concentration was equal to that in the reverse (i.e. serosal to mucosal) direction. The authors point out the limitations in the measurement of biotin concentration in the mucosal and serosal compartments due to the limited precision of the assay by the microbial method and they concluded that the transport of biotin in hamster and rat intestine occured by passive diffusion. That the transport of biotin in rat intestine occurs by passive diffusion has been reaffirmed (Spencer *et al.*, 1962, Spencer and Brody, 1964) using the radioisotopic method ([^{3}H]-biotin and [^{14}C]-biotin) for quantitating the amount of biotin transferred from the mucosal and serosal compartments of the everted sac preparation. In the hamster, however, it has been shown using the more sensitive radioisotopic assay that the

transmural uptake of biotin across everted sac occurs against a concentration at 1 μM, 5 μM and 10 μM of biotin (Spencer et al., 1962, Spencer and Brody, 1964, Spencer and Bow, 1964), respectively. The transport occurs maximally along the small intestine of the hamster two-thirds of its length beginning from the pyloric end (Spencer et al., 1962, Spencer and Brody, 1964) and is blocked by avidin, and various analogs of biotin such as thioctic acid, desthiobiotin, diaminobiotin, biotin methyl ester and biocytin (Spencer et al., 1962, Spencer and Brody, 1964).

The in vitro uptake of biotin in segments of hamster intestine in which only the mucosal side of the tissue was exposed to the incubating fluid showed that the media to mucosal tissue uptake of the radio-labeled biotin was sodium dependent and displayed saturating kinetics with a K_m and V_{max} of 1mM and 22nmol/ml tissue water/min, respectively and an inhibition pattern with analog which indicated that the carboxyl side chain of biotin was essential for the carrier mediated transport of biotin (Berger et al., 1972).

Biotin uptake measured by the in vivo loop technique demonstrated maximal uptake in the proximal jejunum of the rat (Bowman and Rosenberg, 1986). The distal ileum and the proximal colon which are considered to be sites of supply to the host of biotin of microbial origin also show significant biotin uptake. The in vivo uptake of biotin measured by luminal disappearance of biotin in jejunal loops (Bowman et al., 1986) was linear for

10 minutes up to 5.0 μM biotin. The uptake below 5 μM biotin proceeded largely by a saturable process while at concentrations above 20 μM, non-saturable uptake predominates.

d) Role of bound forms of biotin for intestinal uptake

Eukaryotes lack the enzyme machinery for synthesizing biotin and therefore are entirely dependent on an external supply. In mammals this vitamin is supplied from two sources: the first source is from the biotin contained in the food consumed, and the second source is from the biotin that is synthesized by the enteral microflora. The relative contribution of the microflora to the pool of biotin available for uptake by the small intestine is presently unclear. Biotin in food is mostly protein-bound being attached exclusively to the ε-amino group of specific lysine residue in the protein. During the course of digestion protein is broken down into small peptides and individual amino acids by the action of specific proteases present in the stomach and small intestine. The proteolytic digestion of biotin containing proteins would result in the release of small biotinylated peptides or biocytin (ε-amino-N-[d-biotinyl]-L-Lysine). These bound forms of biotin are metabolized by biotinidase resulting in the release of free biotin.

Assuming the supply of free biotin of microbial origin to the mammalian host is small or negligible then in vivo the form of biotin available for uptake by the small intestine would predominantly be in the bound form with free biotin (of dietary

origin) comprising a very small fraction. All previous studies on transport of biotin were done with free biotin only. One study has been performed on the transport of biocytin where its uptake was measured in everted sac preparation of rat small intestine (Turner and Hughes, 1962b). Transmural transport of biocytin from the mucosal side to the serosal side at a concentration of 10 μM was measured using the microbial assay for biocytin. Although this study showed that biocytin was transported from the mucosal side and some of it was cleaved within the mucosal compartment, no firm conclusion could be drawn regarding the rate of transport of biocytin relative to biotin due to the poor precision of the microbial assays.

Biotinidase may have an important role as a protein involved in the transport of biotin. It is known that apart from the normal role in the recycling of endogenous biotin this enzyme plays an important role in the processing of dietary, protein bound biotin (Wolf *et al.*, 1985). Evidence for this comes from a study which shows that biocytin gets absorbed *in vivo* from the small intestine of patients with juvenile form of biotin responsive multiple carboxylase deficiency after an oral dose of biocytin (Baumgartner *et al.*, 1985). Two to four hours after an oral dose of 0.4 μ mol biocytin/kg body weight the plasma concentration of uncleaved biocytin becomes detectable without a change in the plasma biotin levels in these patients whereas normal healthy children given an identical dose of biocytin show an increase in the plasma levels of biotin but no

biocytin was detected.

RATIONALE FOR EXPERIMENTAL WORK

Chapter I describes the work on the fluorometric assay for biotinidase using biocytin as substrate. Until recently the only reported method for measuring biotinidase activity with biocytin was by the bioassay (Wright and Skeggs, 1944). This assay was a lengthy procedure. Although several other methods for measuring biotinidase activity are available (Knappe et al., 1968, Wastell et al., 1984), these methods use substrates other than biocytin. The assay described in chapter I was an attempt to provide a method for assaying biotinidase activity in human serum using the natural substrate. Human serum was chosen to develop the method because of its high activity and ready availabilty. The usefulness and limitations of the fluorometric assay are discussed.

The work described in the second chapter deviates a little from the main theme of this project. This chapter is devoted to the work done on the adaptation of the isotope dilution assay (Dakshinamurti et al., 1974, Dakshinamurti and Allan, 1979) for measuring biocytin. Patients with juvenile type MCD are probably the only individuals expected to have biocytin in their biological fluids. Since samples of serum and urine from these patients were not readily available, the principle of the assay could only be tested by adding known amount of biocytin to serum and urine obtained from patient samples known not to have a defect in biotin metabolism.

In chapter III the uptake of biotin and biocytin was

examined. Based on the postulated role of human serum biotinidase as a carrier protein for biotin, it was of interest to determine whether biotinidase in rat small intestine could have a possible role in the transport of biotin. A preliminary study was conducted to determine whether biotinidase or other protein(s) in the brush border or the cytosol were involved in the transport of biotin. Protein fractions from cytosol and brush border obtained by sucrose density gradient centrifugation were analyzed for biotinidase activity and biotin-binding activity.

CHAPTER I

Chapter 1

FLUOROMETRIC ASSAY FOR BIOTINIDASE

RESEARCH OBJECTIVE

The primary role of biotinidase is to cleave biocytin to biotin and lysine. The biotin released during the reaction lacks a useful distinguishing spectral characteristic therefore the assay of biotinidase activity with biocytin was restricted to a bioassay (Wright and Skeggs, 1944) in which the growth of the lactobacillus arabinosus (ATCC 8014) was directly proportional to the amount of biotin available. The growth of Lactobacillus arabinosus was unaffected by excess biocytin, but interference due to certain unspecific stimulatory substances, such as oleic acid in the presence of aspartic acid, can lead to exaggerated biotin values by this method. Another obvious disadvantage of the microbioassay is its processing time which ranges from 36 to 48 hours. The isotope dilution method (Dakshinamurti et al., 1974, Dakshinamurti and Allan, 1979) cannot be used directly for biotin determination in the biotinidase assay as excess unreacted biocytin interferes in this assay. Separation of biocytin from biotin however, can be obtained on silica gel thin layer chromatography. The objective here was to develop a biochemical enzyme assay for biotinidase with biocytin as the substrate. Lysine the second product of the reaction catalyzed by biotinidase can be determined spectrofluoro-metrically by its well known reactions with ninhydrin, O-phthaldehyde, and Fluorescamine. However, excess

biocytin as well as lysine react strongly with these reagents. This drawback was overcome by using 1,2-diacetylbenzene (DAB) which reacts strongly with lysine but not with biocytin. Biotinidase activity in human serum was determined by measuring the fluorescence of the released lysine after its derivatization with 1,2-diacetylbenzene.

MATERIALS AND METHODS.

Biocytin, lysine, 2-mercaptoethanol were purchased from Sigma. 1,2-diacetylbenzene was obtained from Aldrich (Milwauke, Wisc.) and Bio-Rad assay dye concentrate from Bio-Rad laboratories (Richmond, Calif.). All other reagents used were of analytical grade and purchased from standard commercial sources. Pooled human serum was obtained from patient (patients known not to have any known disease of biotin metabolism) samples from the Clinical Biochemistry Laboratory at the Health Sciences Centre, Winnipeg and was stored at -20°C until required for use.

a) Dialysis of human serum

Human serum was dialyzed extensively against 50 mM phosphate buffer, pH 7.0, containing 1.0 mM EDTA and 1.0 mM 2-mercaptoethanol. The dialyzed samples were stored at -20°C until required for use.

b) Determination of protein

Protein concentration was determined by the Bio-Rad assay which is based on the method described by Bradford (1976) with bovine serum albumin as a standard.

c) Biotinidase assay with N-(d-biotinyl)-4-amino benzoate

The method used was that of Knappe et al. (1963), with slight modification. The assay was carried out as follows: Test sample was added to 1.9 ml of a mixture containing 20 μ mol potassium phosphate buffer, pH 6.0, 20 μ mol EDTA, 0.5 mg serum albumin, and 0.3 mol N-(d-biotinyl)-4-aminobenzoate in a final volume of 2.0 ml. The mixture was incubated for 30 min at 37°C and the reaction terminated by adding 0.2 ml 30 % trichloro-acetic acid. The protein was removed by centrifugation and 1.5 ml of the supernatant was added to 0.5 ml distilled water. At 3.0 minute intervals, 0.2 ml 0.1 % sodium nitrite (made fresh), 0.2 ml 0.5 % ammonium sulphamate and 0.2 ml 0.1 % N-1-napthylethylenediamine hydrochloride were added in succession and incubated for 10 minutes before measuring the absorbance at 546 nm.

d) Determination of spectral properties of derivatized lysine and biocytin

The spectral characteristics of lysine and biocytin derivatized with diacetylbenzene were determined in a total derivatized volume of 1.22 ml. Biocytin and lysine (final derivatized concentration of 82 μ M each) were derivatized with 0.5 ml derivatizing buffer (0.5 M sodium carbonate buffer, pH 9.5, 0.1 % 1,2-diacetyl benzene and 3.0 mM 2-mercaptoethanol) and the excitation and emission spectra were determined after 25 minutes at room temperature in an Aminco-Bowman

spectrofuorometer. For the excitation spectra the emission wavelength was set at 355 nm whereas for the emission spectra the excitation wavelength was 455 nm.

e) Effect of derivatization time on fluorescence

The effect of derivatization incubation time on the fluorescence was studied with lysine or biocytin at a final derivatized concentration of 82 μ M (derivatized volume = 1.22 ml). This was derivatized at room temperature with 0.5 ml derivatization buffer. The fluorescence was determined at timed intervals for up to 45 minutes in an Aminco Bowman spectrofluorometer at excitation wavelength = 355 nm and emission wavelength = 455 nm.

f) Preparation of standards for biotinidase assay

Standard amount of lysine (in 0.1 M sodium acetate buffer) up to 50 nmol was derivatized, and the fluorescence determined as described previously. The standards used for biotinidase assay (total volume 0.45 ml) were prepared with 0 to 50 nmol of lysine in 0.1 M sodium acetate buffer, pH 5.5, and in addition contained 0.5 mM biocytin and the appropriate amount of denatured dialyzed serum sample. After the addition of 0.27 ml 2 M Na_2CO_3, the standards were derivatized as before and the fluorescence determined against the appropriate blank which contained the identical composition but with the lysine omitted.

g) Fluorometric procedure for biotinidase assay

The reaction was performed by adding dialyzed serum sample

to a reaction mixture containing 0.1 M sodium acetate buffer, pH 5.5, 5 mM EDTA, and 0.5 mM biocytin in a final reaction volume of 0.5 ml. After 1.0 hour incubation at 37°C, 50 µl 60 % perchloric acid was added to stop the reaction. The precipitated protein was removed by centrifugation. To 0.45 ml of the supernatant 0.27 ml 2.0 M Na_2CO_3 was added to make the medium alkaline and 0.5 ml of derivatizing buffer was added. After 25 minutes at room temperature the samples were read in an Aminco-Bowman spectrofluorometer (slit width = 10 nm; excitation wavelength = 355 nm and emmision wavelength = 455 nm.) against a blank which had an identical composition to that of the test (including buffer, biocytin, and dialyzed serum) with the exception that 50 µl 60 % perchloric acid was added at zero time. The further derivatization of the blank and test was identical. After derivatization the samples were read against their corresponding blanks.

h) Influence of pH on biotinidase activity

The effect of pH on enzyme activity was evaluated by the colorimetric and the fluorometric assays using purified biotinidase from human serum (Chauhan and Dakshinamurti, 1986). The assays were performed as described previously. The buffers used were: 0.1 M sodium citrate/ citric acid pH 3.0 to 3.5), sodium acetate (pH 4.0 to 5.5), sodium phosphate (pH 6.0 to 8.0), and sodium carbonate/ bicarbonate (pH 9.0 to 10.5).

EXPERIMENTAL RESULTS

a) Fluorescence spectra of lysine and biocytin

The fluorescence of lysine and biocytin derivatized with 1,2-diacetyl benzene is shown in figure 2. Lysine and biocytin cause excitation and emission at identical wavelengths of 355 nm and 455 nm respectively. The distinguishing feature of the biocytin spectra from the lysine spectra is that the fluorescence of biocytin at equimolar concentration is less than 5 % of that of lysine under the same condition. This property of 1,2-diacetylbenzene enabled the determination of lysine with little interference from biocytin by the use of the appropriate blanks.

b) Effect of derivatization incubation time on fluorescence yield

The fluorescence of derivatized lysine with time is shown in figure 3. The fluorescence of lysine was fairly stable with time and could be read 20 to 35 minutes after derivatization.

c) Calibration curves for lysine alone and lysine under assay conditions

Figure 4 shows the calibration curves determined for lysine. The fluorescence of lysine alone determined in buffer is linear up to 50 nmol. The fluorescence of lysine in the presence of biocytin was unaffected by biocytin excess in the reaction medium (results not shown). The fluorescence of lysine under the assay condition where substrate and denatured serum is present shows reduced fluorescence but the response is

linear up to 50 nmol of lysine.

d) Biotinidase activity as a function of serum volume

Biotinidase activity was linear up to 33 µl dialyzed human serum (figure 5), but became nonlinear at greater sample volume. The activity in 20 µl dialyzed serum was routinely determined and this resulted in the release of 0.3 nmol lysine per minute at 37°C for 20 µl of dialyzed serum.

e) Biotinidase activity as a function of incubation time

The effect of incubation at 37°C on the assay is shown in figure 6. The release of lysine is linear up to 180 minutes of incubation.

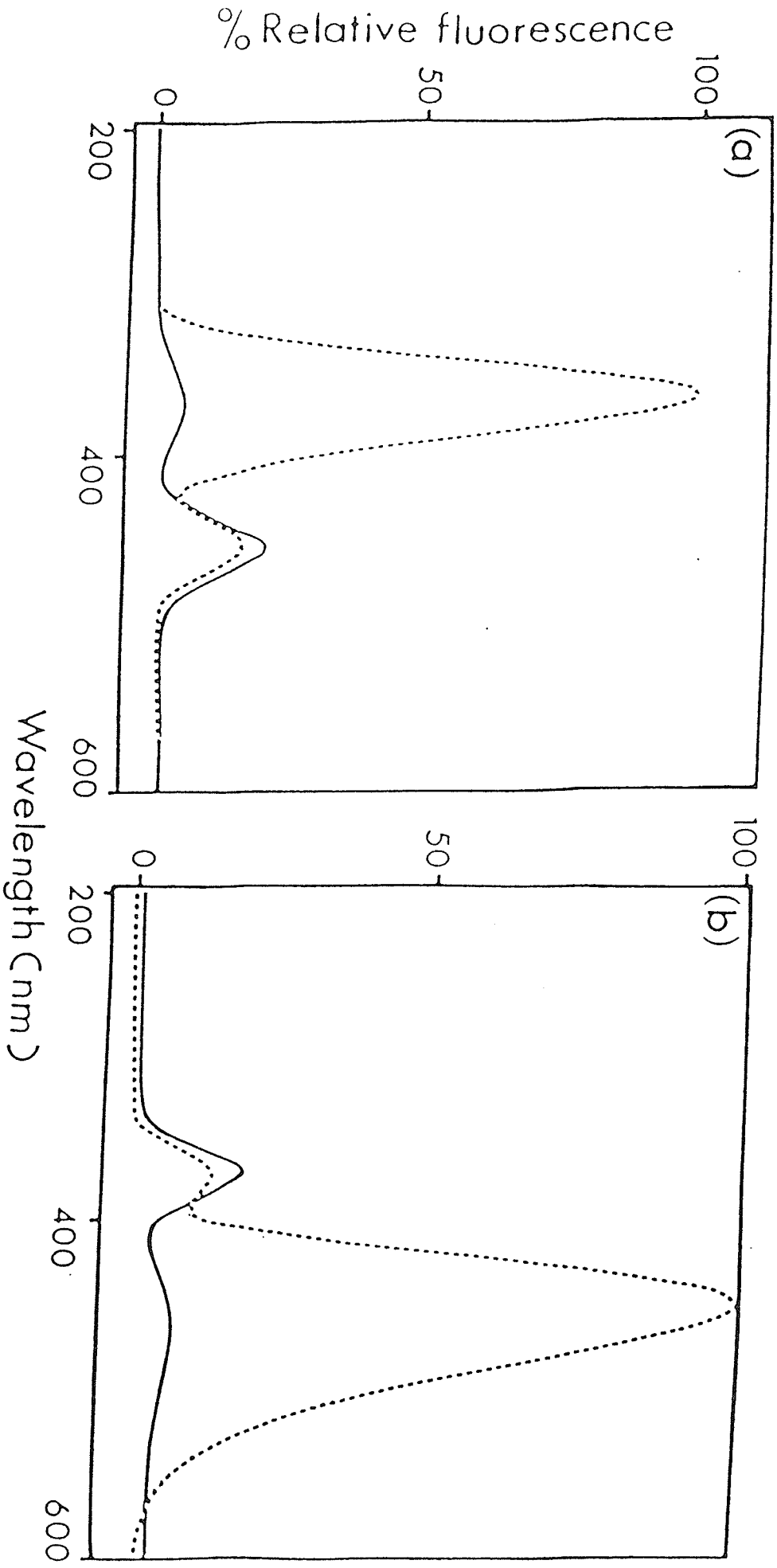
f) Effect of pH on human serum biotinidase activity

The pH profile of homogeneously purified human serum biotinidase with N-(d-biotinyl)-4-aminobenzoate and d-biocytin is shown in figure 7. The optimum pH with N-(d-biotinyl)-4-aminobenzoate was between 6.0 to 7.5 whereas with d-biocytin the optimum was between 4.5 to 6.0.

g) Comparison of biotinidase activity by the colorimetric and fluorometric assays

The activity of the dialyzed and undialyzed serum enzyme was compared by the two assay procedures (Table II). Although the activity of biotinidase with N-(d-biotinyl)-4-aminobenzoate was determined to be lower than the reported values of 4.68 to 6.75 (Wastell et al., 1984) and 4.30 to 7.54 (Wolf et al., 1983) for human serum biotinidase, the activity in dialyzed serum is 2.7-fold greater than with the undialyzed

Figure 2 - Fluorescence spectra of lysine and biocytin. The (a) excitation and (b) emission spectra were determined in a total derivatization volume of 1.22 ml as described under "methods" section with the concentration of lysine and biocytin each set at 82 μ M. (.....) fluorescence of lysine; (———) fluorescence of biocytin.


% Relative fluorescence
0
50
100
(a)
200
400
600
Wavelength (nm)
0
50
100
(b)
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400
600

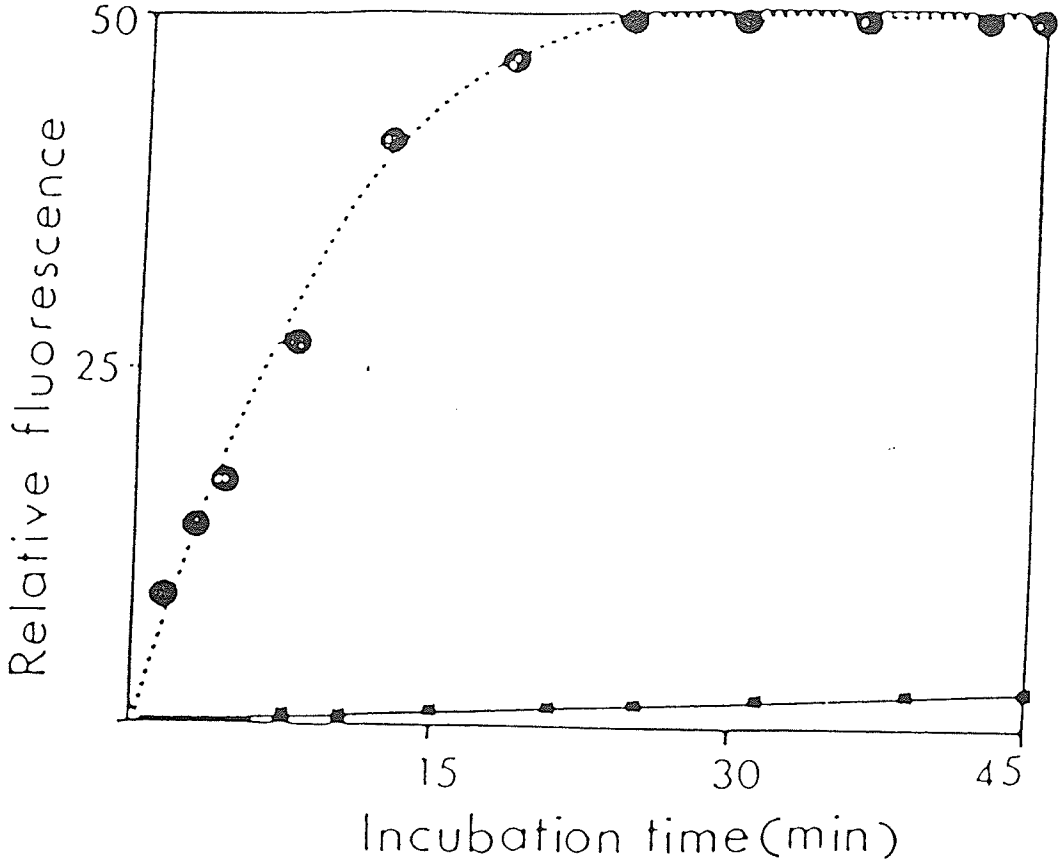


Figure 3 - Effect of derivatization incubation time on fluorescence yield. The effect of derivatization time on the fluorescence of DAB-lysine and DAB-biocytin is shown. Equimolar amounts (82 μ M) each of lysine or biocytin were derivatized for varying time up to 45 minutes with 1,2-diacetylbenzene and the fluorescence of DAB-lysine (.....) and (————) DAB-biocytin determined.

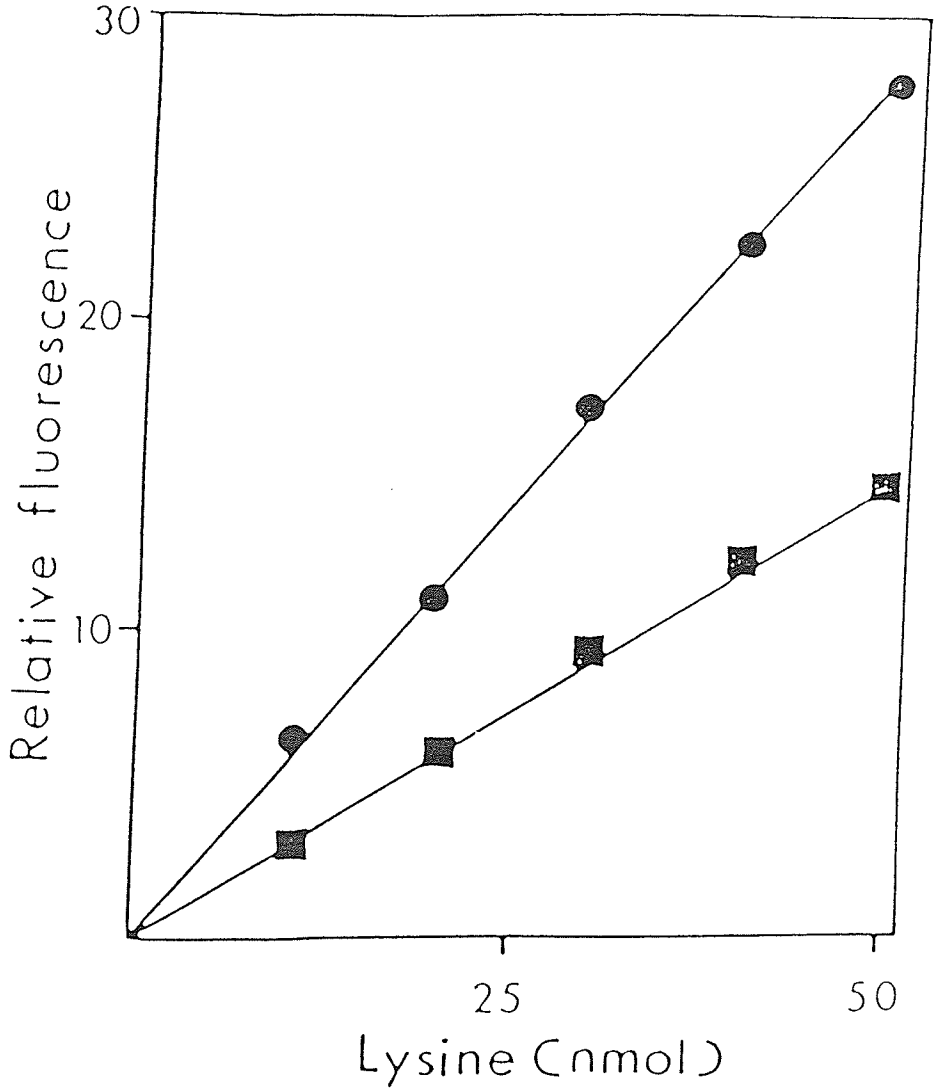


Figure 4 - Calibration curves for pure lysine and lysine under assay conditions. The fluorescence of 0-50 nmol lysine (●——●) derivatized on its own. The fluorescence of 0-50 nmol (■——■) lysine derivatized in the presence of 0.3 mM biocytin and 20 µl heat denatured dialyzed serum.

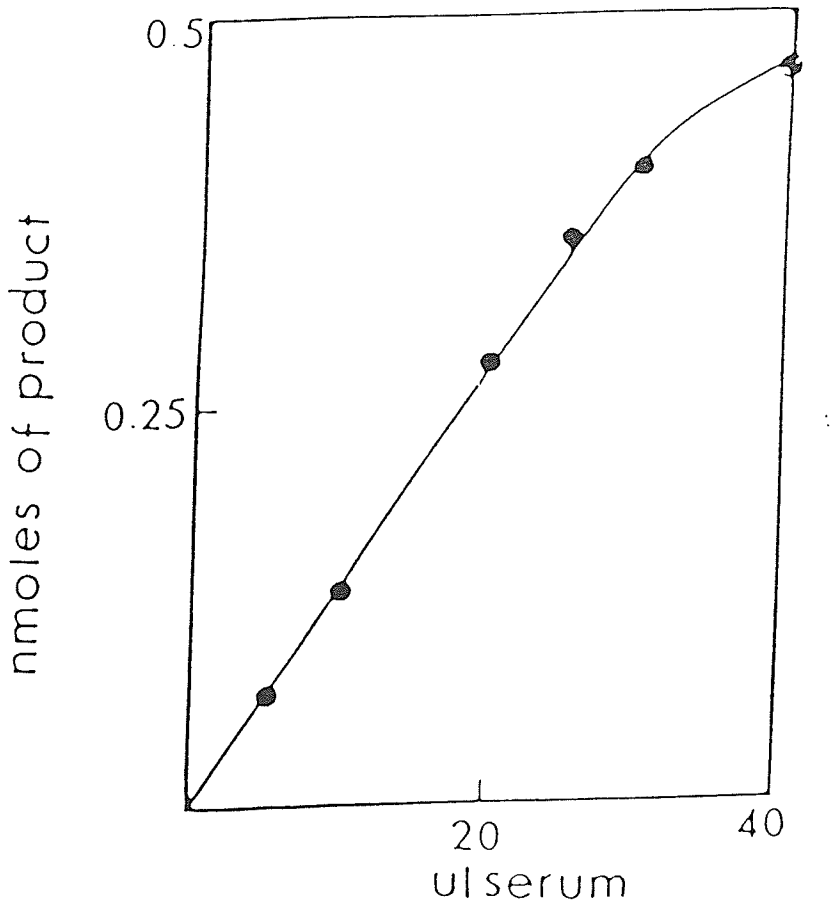


Figure 5 - Biotinidase activity as a function of serum volume. The assay was carried out using 0-40 µl of dialyzed human serum with the appropriate blank at each serum volume. Enzyme activity is expressed as nmol lysine released from biocytin per minute.

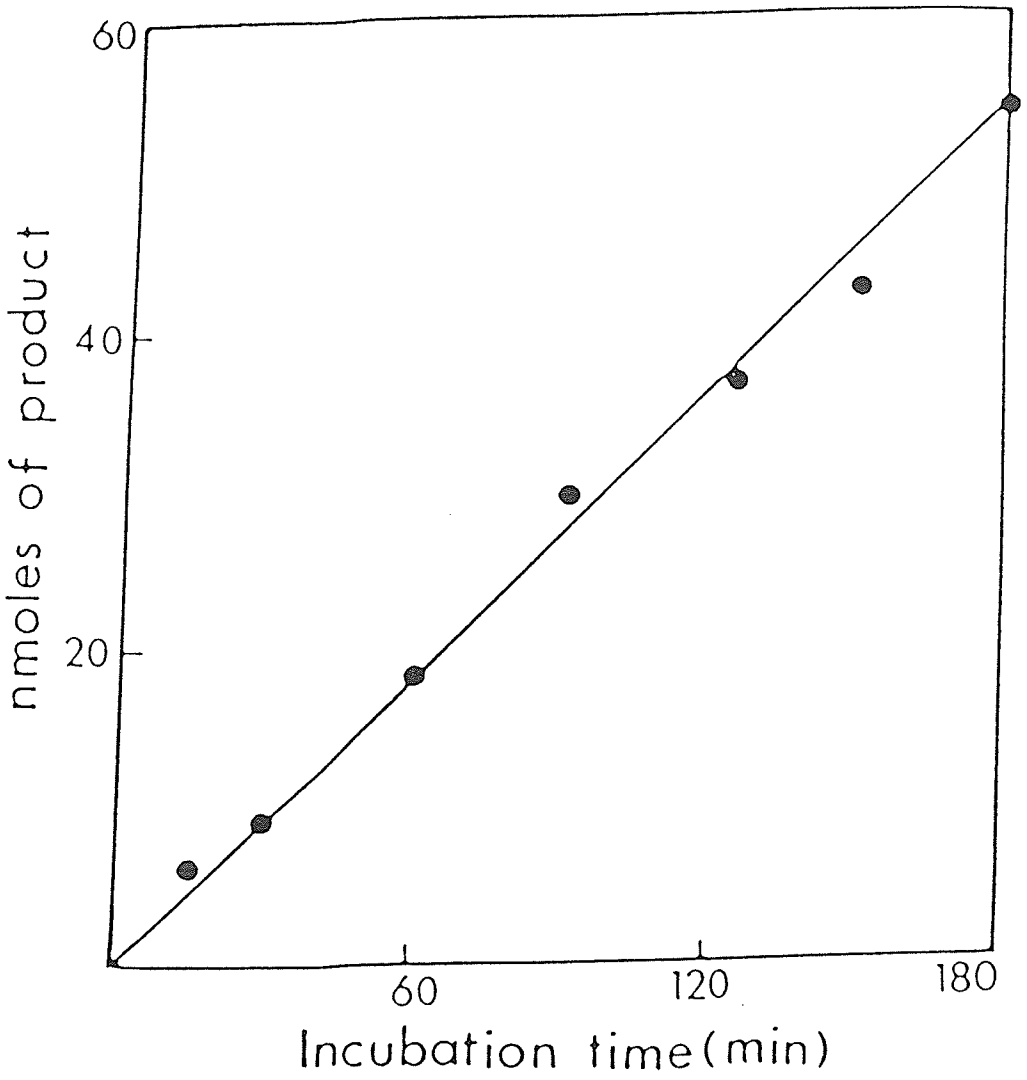


Figure 6 - Biotinidase activity as a function of incubation time. 20 µl dialyzed human serum was used to determine the activity at 37°C as a function of time. Activity is expressed as nmol of lysine released per 20 µl dialyzed serum.

Figure 7 - Effect of pH on biotinidase activity. Purified human serum biotinidase was assayed using N-(d-biotinyl)-4-aminobenzoate (·····) and d-biocytin (═══) as substrates. The pH range was between 3.5 and 10.5 using 0.1 M sodium citrate/citric acid (pH 3.0 to 3.5), sodium acetate (pH 4.0 to 5.5), sodium phosphate (pH 6.0 to 8.0) and sodium carbonate/bicarbonate (pH 9.0 to 10.5) buffers. The values for 100% activity are 18 nmol of 4-aminobenzoate released per minute per mg protein and 23.3 nmol lysine released per minute per mg protein.

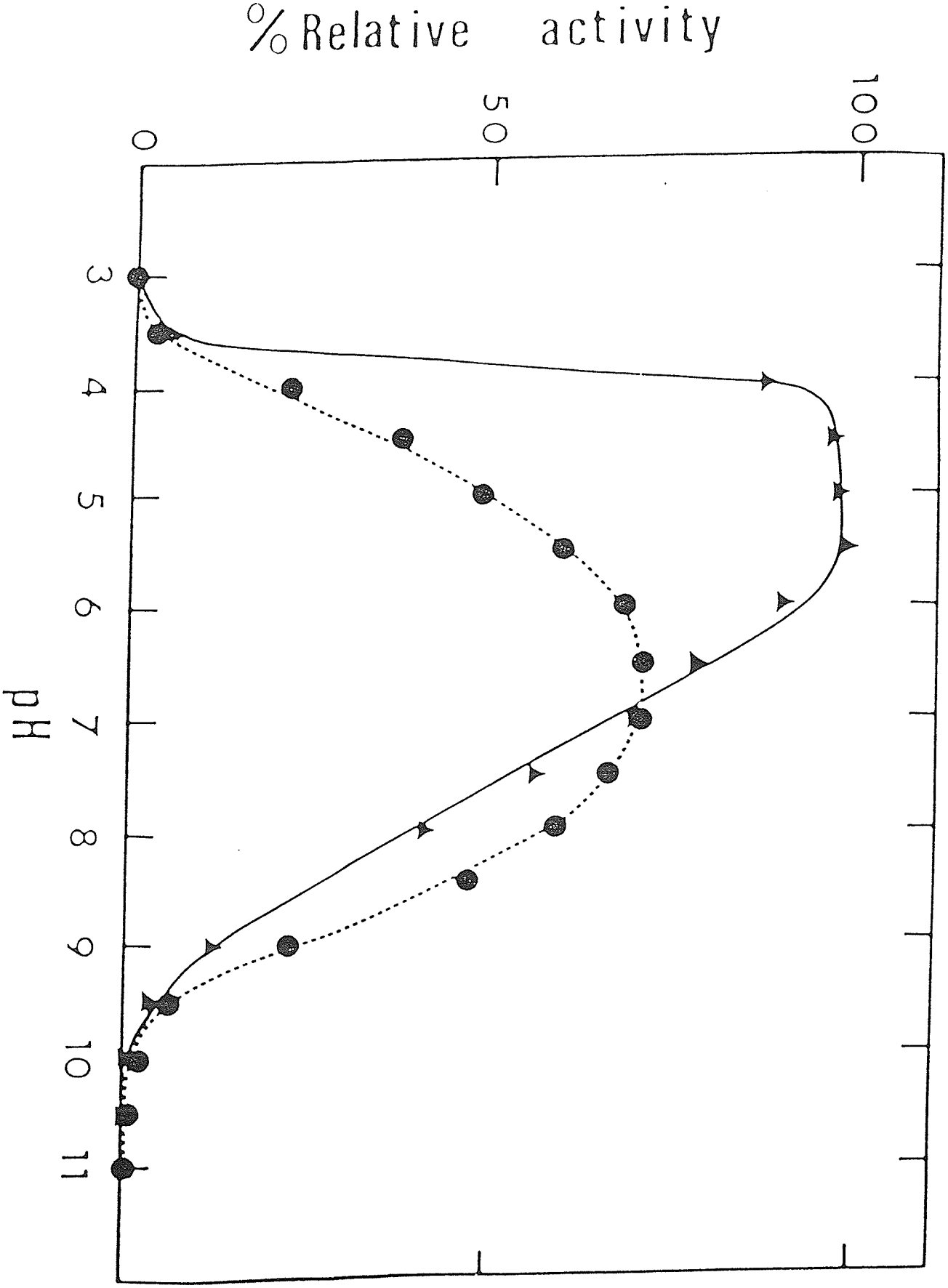

% Relative activity
0
50
100
pH
3
4
5
6
7
8
9
10
11

Table II - A comparison of the activity of human serum biotinidase by the colorimetric and fluorometric methods.

Assay substrate	Method	Serum biotinidase activity	
		undialyzed	dialyzed
N-(d-biotinyl)-4-aminobenzoate	Colorimetric	3.49 ± 0.58 (3.05 - 4.22)	9.28 ± 0.85 (8.45 - 10.80)
N-(d-biotinyl)-L-lysine	Fluorometric	nd	15.16 ± 2.24 (10.82 - 18.93)

nd = not determined

* Biotinidase activities expressed (mean ± SEM) as nmol of 4-aminobenzoate released (colorimetric method) or nmol lysine released (fluorometric method) per minute per ml serum determined respectively on the same 16 samples of human serum.

Table III - Precision of the fluorometric and colorimetric assays. Activities are expressed as nmol of the respective products released per minute per ml serum.

	Fluorometric method	Colorimetric method
Range	15.41 - 17.67	9.03 - 9.54
Mean	16.11	9.29
S.D	0.55	0.21
CV	3.39	2.26
n	18	18

serum.

h) Precision of the colorimetric and the fluorometric assays

Table III shows the precision achieved by the two assay methods. Both the colorimetric and the fluorometric assays show good precision, the fluorometric procedure displays a little more variation between runs compared to the colorimetric assay.

DISCUSION

The reaction of free amino groups with 1,2-diacetyl benzene has long been recognized (Hillman, 1943). This reagent however, gives a high fluorescence only with taurine, histamine, ornithine, and lysine. Lysine was assayed with 1,2-diacetylbenzene by a modification of the procedure of Roth (1971).In this procedure basic amino acids were derivatized with 1,2-diacetylbenzene under reducing conditions. The sensitivity of the assay has been maximized by reducing the assay volume 2.5-fold to 1.22 ml and decreasing the amount of DAB added to 0.5mg per assay. 1,2-diacetylbenzene reacts selectively with lysine therefore it is not essential to separate the excess unreacted biocytin from the reaction mixture. Lysine probably gives a strong fluorescence with 1,2-diacetylbenzene due to its two primary amino groups.

Interference due to serum, even after dialysis, does occur in this assay. This is seen when standard amount of lysine is derivatized under assay conditions where both biocytin and serum is present. The observed lowering in the fluorescence of

lysine under these conditions is due to the serum component since biocytin in the presence of lysine does not cause a lowering of the fluorescence. Although dialyzed serum causes some quenching of the fluorescence of lysine, the fluoresence response under the assay conditions is linear up to 50 nmol of lysine. Hence, apart from the use of the enzyme and substrate blanks, an additional blank containing the appropriate amount of denatured dialyzed serum was used in assessing the lysine released.

There are two important findings of this assay. The first is the observation that the pH optimum of purified serum biotinidase is substrate dependent. The pH optimium with N-(d-biotinyl)-4-aminobenzoate was between 6.0 to 7.5 whereas with d-biocytin the optimum was slightly towards the acidic range of 4.5 to 6.0. Apart from this significant difference in the pH optima another important observation is that the activity of serum biotinidase is 30 $\pm$ 5.0 % greater with d-biocytin compared to its activity with N-(d-biotinyl)-4-aminobenzoate. The observed increase in activity with d-biocytin may be due to a lower dissociation constant of this substrate. The second important observation of this assay is that biotinidase activity in dialyzed serum is 2.7-fold greater compared to its activity in undialyzed serum. The apparent increase in enzyme activity upon dialysis can be assessed only by the colorimetric assay (Knappe et al., 1963) as undialyzed serum gave a very high blank fluorescence value which was due to the basic amino

acids and small peptides present in the serum. The increase in biotinidase activity in dialyzed serum was attributed to the removal of an endogenous inhibitor of the enzyme, probabaly a biotin metabolite (Chauhan and Dakshinamurti, 1986). The value of the fluorometric assay rests with the use of biocytin, the natural substrate of the enzyme.

The biotinidase assay developed using the fluorometric reaction of lysine with 1,2-diacetylbenzene is a valuable addition to the available methods for the assay of biotinidase. The assay was developed for human serum, however after a dialysis step to remove small peptides and amines from the biological sample, this procedure could be applied for assaying the enzyme from different biological samples.

Since the fluorometric assay using biocytin has a different pH optimum compared to other assays with synthetic substrates, and also the measured activity of the enzyme is higher with biocytin, the fluorometric assay is the proper standard against which all other assays should be compared. The colorimetric assay is simpler to use but it only gives a relative value of enzyme activity. The limitation of the fluorometric assay is that samples have to be dialyzed in order to minimize interference from small peptides and amines.

CHAPTER II

Chapter II

DETERMINATION OF BIOCYTIN IN HUMAN SERUM AND URINE

RESEARCH OBJECTIVE

The late-onset type of multiple carboxylase deficiency is characterized by tissue deficiency of biotinidase (Wolf and McVoy, 1983, Wolf et al., 1983a). In this disorder there is an increased urinary loss of biotin and biocytin (Baumgartner et al., 1985). Biocytin is normally undetectable in the serum and urine of healthy individuals, but children with late-onset type of MCD accumulate biocytin in their biological fluids.

Biocytin in these patients could not be determined by the ninhydrin or O-phthaldehyde reactions due to the low sensitivities of these reactions. The determination of biocytin by measuring the 'liberated biotin' following its hydrolysis by normal human serum (used as source of biotinidase, Bonjour and Bausch, 1984) gives only an indirect estimate of the amount of biocytin present.

The objective of this research was to devise an assay for determining biocytin in human serum and urine. To develop the method human serum and urine from patient samples (known not to have any defect in biotin metabolism) were used. A known amount of biocytin was added to these samples. These samples contained endogenous biotin as well. Biotin was removed by adsorption on an anion exchange resin. The available binding sites on excess avidin were first saturated with biocytin and the residual vacant sites on the avidin were then bound with radiolabeled

biotin. Thus, the decrease in avidin-bound radioactivity is proportional to biocytin saturating the avidin sites. The recoveries of added biocytin in human serum and urine were determined.

MATERIALS AND METHODS

Avidin was purchased from Vega Biochemicals (Tucson, Arizona). [^{3}H]-biotin was a kind gift donated by Hoffman La Roche (Nutley, N.J.). All other chemicals used were of reagent grade and purchased from standard commercial sources. All solution were prepared with distilled water and were adjusted to the desired pH. Samples of pooled human serum and urine were obtained from patient (not having any known disease of biotin metabolism) at the Clinical Biochemistry Laboratory at the Health Sciences Centre, Winnipeg. Human serum was stored at-20^{o}C until required for use. Pooled human urine was kept in the cold at 4^{o}C and the precipitated material (phosphates, etc) removed by centrifugation.

a) Preparation of test serum and urine samples

Test urine samples were prepared as follows: Urine samples (0.5 ml) containing zero, 2, 4, 6 ng of added biocytin were diluted two-fold with 20 mM sodium phosphate buffer pH 6.0 and 0.25 ml aliquot of the diluted samples was treated with Bio-Rad AG1X-8 resin (one volume of resin and three volumes of 20 mM sodium phosphate buffer, pH 6.0) suspension in the acetate form and mixed on an Eppendorf shaker for half an hour. Test serum samples were prepared as follows: Serum samples (0.5 ml)

were treated with 3 mM iodoacetic acid before the addition of zero, 0.5, 1.0, and 1.5 ng of biocytin. Iodoacetic acid irreversibly inhibits biotinidase in the serum. Resin treatment of serum samples was carried out as described for the urine samples. After centrifugation for five minutes, 0.2 ml aliquot of the supernatant from each treatment was assayed by the avidin-binding assay to determine the amount of biocytin present.

b) Preparation of serum and urine biocytin standards

Blank serum and urine samples were prepared as described for the test samples with the exception that no biocytin was added. Serum biocytin standards were prepared as follows: To 0.8 ml of blank serum supernatant 2.0 ng of biocytin was added. Aliquots of this containing up to 400 picogram biocytin were taken and the final volume made up to 0.2 ml with blank serum supernatant (to which no biocytin was added). Urine biocytin standards were prepared in a similar manner as described for serum biocytin standards.

c) Avidin-binding assay for biocytin

The biocytin assay is described for test serum samples and standards. To 0.2 ml test serum supernatants and serum standards, avidin (0.4 milliunits) was added followed by 0.5 ml 0.2 M $(NH_4)_2CO_3$. After mixing these were incubated at 37°C for half an hour. Radiolabeled biotin (0.2 ml containing 60,000 DPM and 600 pg [^{3}H]-biotin) was added and the incubation continued for a further half an hour. It is of importance to note that

biocytin is allowed to bind to avidin before [^{3}H]-biotin. Following this 0.2 ml bentonite (10 mg/ml in distilled water) was added and mixed on an Eppendorf shaker for 10 minutes. The bentonite containing adsorbed avidin-[^{3}H]-biotin complex was pelleted by centrifugation and washed twice with one ml 0.2 M $(NH_4)_2CO_3$. The pellet was suspended in one millilitre distilled water and the Eppendorf tubes with their contents were transferred to scintillation vials to which 12 ml of Scintiverse II liquid scintillant was added. Radioactivity was determined in a Beckman LS-2000 counter. The assay for test urine samples and standards was performed as described for test serum samples and standards with the exception that 0.2 milliunits of avidin were used and the amount of radiolabeled biotin added was 45,000 DPM and 450 pg [^{3}H]-biotin. The recoveries of biocytin in test serum and urine samples were determined from the serum and urine standard curves.

EXPERIMENTAL RESULTS

a) Standard curves for biocytin in human serum and urine

The calibration of biocytin in serum and urine is shown in figure 8. A linear decrease in the avidin bound radioactivity is seen in both cases. The difference in the amount of radioactivity bound at zero biocytin concentration in serum and urine sample is due to a higher concentration of avidin used for serum in this case.

b) Recovery of biocytin added to human serum and urine

Table IV gives the recovery of biocytin at the determined concentrations from human serum and urine. More biocytin was added to human serum samples as there is evidence to show that the concentration of biocytin in biotinidase deficient patients may be greater in the urine compared to that found in the serum (Baumgartner et al. 1985). The amount of biocytin recovered in serum ranged between 78 to 82 % whereas the amount recovered in urine ranged between 71 to 77 %. In the case of serum more variability was observed for biocytin determined at the lower concentration.

DISCUSSION

The isotope dilution assay for measuring the amount of biotin in biological fluids (Dakshinamurti et al., 1974) is not suitable for measuring biocytin because of its low sensitivity. In the avidin-binding assay for biocytin, the biocytin is first reacted with excess available sites on the avidin and the residual sites are allowed to react with [^{3}H]-biotin. This is compared with the binding of [^{3}H]-biotin to the avidin in the absence of biocytin. Thus, the decrease in bound radioactivity is directly proportional to the amount of biocytin. This is in contrast to the isotope dilution assay (Dakshinamurti et al., 1974, and Dakshinamurti and Allan, 1979), where the fall in radioactivity occurs in an exponential manner as a result of the competition for binding to avidin

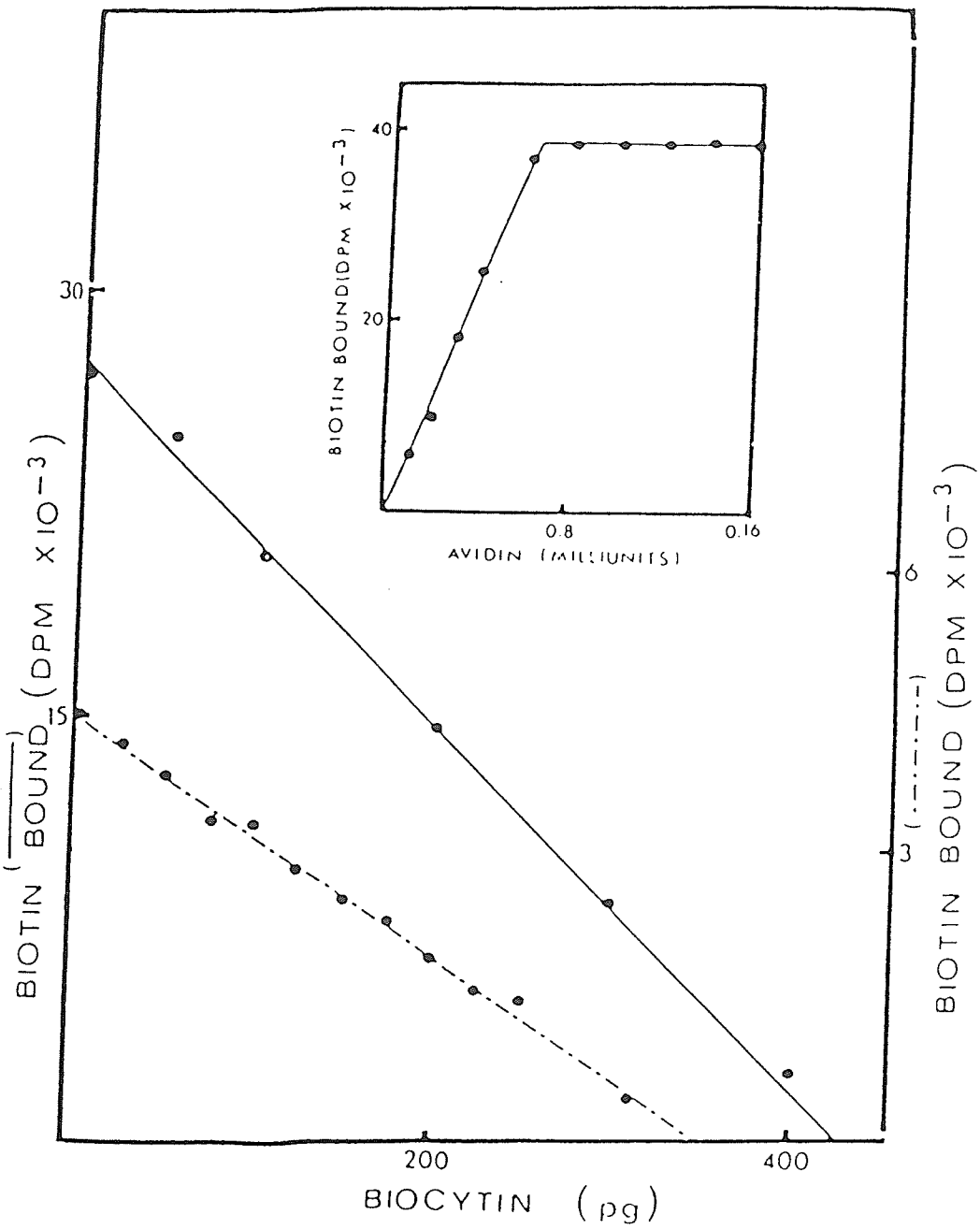


Figure 8 - Standard curves of biocytin in serum and urine. The standard curves for biocytin in serum (——————) and urine (.....) were obtained as described under "Materials and Methods". The inset shows the determination of equivalence for the binding of [^{3}H]-biotin as a function of the amount of avidin.

Table IV - Recovery of biocytin added to normal human serum and urine. The values are mean of six determinations.

	Biocytin added pg/0.5 ml	Conc. nM	% Recovery	CV
Serum:	500	2.6	77.8 ± 3.63	10.4
	1000	5.2	81.6 ± 3.19	8.7
	1500	7.8	81.8 ± 2.69	8.1
Urine	2000	10.5	71.2 ± 2.24	7.1
	4000	21.0	77.2 ± 2.73	7.9
	6000	31.5	73.0 ± 1.84	6.2

that occurs between the radiolabeled biotin and the "cold" or unlabeled ligand. The standard calibration curves of biocytin in serum and urine showed a linear decrease in the bound radioactivity with increasing amount of biocytin. The lower binding of initial radioactivity to avidin (at zero biocytin concentration) with urine samples is due to a lower amount of avidin used (0.2 miliunits) as compared to the amount used for serum (0.4 milliunits). More avidin had to be used for serum in this assay as non-specific binding of [^{3}H]-biotin to serum proteins (which remained adsorbed to the bentonite pellet) interfered by masking the biocytin dependent displacement of the radioactivity at the lower avidin concentration. The displacement in radioactivity in the assay is due to biocytin alone as the biotin in the serum and urine had been removed by the treatment with the BioRad AG1X-8 anionic exchange resin. The amount of biotin present in serum and urine before resin treatment was 1.8 nanogram/ml and 52 nanogram/ml, respectively. This was completely adsorbed on the anion exchange resin. The recovery of biocytin in serum and urine were compatible for the method to be applied to patient samples.

The non-competetive assay for biocytin should be useful in determining biocytin in serum and urine of patients with the juvenile type of multiple carboxylase deficiency. Biocytin in these biological fluids can be separated from the biotin with the BioRad AG1X-8 resin and the biocytin left free in solution can be determined by the procedure described here. The total

"biotin" (free biotin plus biocytin) can be determined by measuring the total avidin bound radioactivity following acid hydrolysis (Dakshinamurti et al., 1974) In order to measure the biocytin in the urine and serum of patients it would be necessary to use the corresponding blanks and standards developed with normal urine or serum.

The practical usefulness of the assay remains to be tested upon the availability of these samples from patients. It is also important to note that the Bio-Rad AG1X-8 resin used in this method is capable of removing about ten times the amount of biotin found in normal samples. However, patients with juvenile type MCD are usually supplemented with biotin to varying degree during the course of therapy. For this reason the performance of the Bio-Rad AG1X-8 resin in the removal of such large amounts of biotin has yet to be tested. The complete removal of biotin from these samples will be crucial in determining the small amount of biocytin present in such biological samples.

CHAPTER III

CHAPTER III

UPTAKE OF BIOTIN IN THE RAT SMALL INTESTINE

RESEARCH OBJECTIVES

The mechanisms involved in the transport of biotin in the rat small intestine are unclear. Previously it was established in mammals using [^{3}H]-biotin that the intestinal uptake of biotin showed considerable species variation (Spencer and Brody, 1964). A carrier mediated process was indicated in the case of the hamster (Berger *et al.*, 1972) but a passive diffusion process was thought to be the mode of uptake for biotin in a vast number of species, such as rabbit, guinea pig, ferret and rat. Recently, however, there is evidence to indicate that the rat in addition to transporting biotin by passive diffusion also transports it by a process showing saturation kinetics (Bowman *et al.*, 1985).

The first objective of this research was to determine whether the uptake of free biotin in the rat small intestine proceeds via these two mechanisms. This study would indicate the *in vitro* capacity of the transport system under physiological concentrations of biotin in the gut. In the past intestinal uptake studies with biotin have been performed at concentrations of biotin which are far in excess of the concentration *in vivo*. This study may also be useful perhaps in explaining why biotin-responsive disorders in man require a large pharmacological dose of biotin for the reversal of the adverse symptoms.

Dietary biotin exist primarily as biotinyl groups linked to the ε -amino group of lysine residues of protein (Pispa, 1965). Biotinidase hydrolyzes this bond, releasing free biotin. Although this enzyme's primary site of action on dietary proteins is not known, its deficiency could impair the ability to maintain normal body stores of biotin. Whether biotinidase is involved in the absorption of free biotin is not known.

The second objective was to study the uptake of biocytin in the small intestine of the rat. Biocytin represents the simplest form of the conjugate of biotin and it can predominate in vivo as it is released from protein-bound biotin during digestion. In this study N-(d-[^{3}H]-biotin)-L-lysine ([^{3}H]-biocytin) was prepared. The main purpose of this study was to determine whether biocytin was transported by the small intestine and to determine its rate of transport relative to the transport of free biotin.

The third objective was to determine whether biotinidase was involved in the transport of biotin. This study was initiated based upon two reasonings. The first was that quite a few substances, such as dissaccharides, peptides, and even bound forms of certain vitamins such as poly glutamylfolates are absorbed by the small intestine by passage through the brush border. Enzymes or specific carrier proteins located in the brush border cleave these conjugates to release their basic units which are then transported through the mucosal tissue and finally into the circulatory system after crossing the barrier

imposed by the basolateral membrane. The second reason for this study was that we have obtained evidence in our laboratory to indicate that human serum biotinidase may act as a carrier protein for biotin. In order to test a possible role of biotinidase in the intestinal transport of biotin a study was designed to determine the in vitro binding of [^{3}H]-biotin to solubilized protein fractions of brush border membranes as well as cytosol which had been previously separated based on their molecular weights by sucrose density gradient centrifugation. A correlation between [^{3}H]-biotin binding activity and biotinidase activity in these fractions was made.

MATERIALS

a) Chemicals

Biocytin, lysine, 2-mercaptoethanol, N-hydroxy succinamide, biotin, N- α -t-boc-L-lysine, and dicyclo carbodiimide were purchased from Sigma (St. Louis, MO.). Avidin was purchased from Vega Biochemicals (Tucson, Arizona). Silica gel thin layer chromatography plates were purchased from Fisher Chemical Company. [^{3}H]-biotin was a kind gift donated by Hoffman La Roche (Nutley, N.J.). [^{14}C]-inulin and [^{3}H]-galactose was purchased from New England Nuclear. All other chemicals used were of reagent grade and were purchased from standard commercial sources. All solutions were prepared with distilled water and adjusted to the desired pH.

b) Experimental Animals

Sprague-Dawley rats, 200 - 230 g were maintained on a laboratory rat chow and tap water ad libitum. The animals were maintained for normal light and dark cycles in a temperature controlled environment.

METHODS

a) General methods

i) Biotin binding activity

The assay depends on the strong noncovalent binding of [^{3}H]-biotin to biotin binding proteins. The assay contains, in a final volume of 0.3 ml, the following components: potassium phosphate buffer, pH 6.0, 100 mM, NaCl 150 mM, EDTA 1.0 mM, 2-mercaptoethanol 1.0 mM, [^{3}H]-biotin, the appropriate amount of the test protein, and 1.0 mg γ-globulin. The control in addition to the labeled biotin also contained unlabeled biotin at a final concentration of 10^{-4} M to correct for non specific binding. The reaction was incubated at 37^{o}C for 10 minutes and the protein was precipitated by the addition of 0.9 ml ice cold saturated ammonium sulphate in phosphate buffer, pH 6.0. The ammonium sulphate does not cause the noncovalently bound [^{3}H]-biotin to dissociate. After standing for 10 minutes on ice, the precipitated protein was collected by centrifugation and washed once with 1.0 ml ice cold 70 % saturated ammonium sulphate in phosphate buffer. The precipitate was collected by centrifugation and dissolved in 0.1 ml distilled water. Radioactivity was determined using 1.0 ml scintiverse II

scintillant.

ii) Alkaline phosphatase assay

The method of Forstner et al., (1968) was used. The assay consisted of 0.6 ml p-nitrophenylphosphate reagent (42 mM glycine, pH 9.2, 0.83 mM $ZnCl_2$, 4.2 mM $MgCL_2$, and 15 mM p-nitrophenylphosphate. Test samples were added (in a final volume of 0.7 ml) to start the reaction and incubated at timed intervals at 37°C for 15 minutes. Standards consisting up to 20 nmol p-nitrophenol in 0.6 ml p-nitrophenylphosphate reagent were also incubated at 37°C for 15 minutes. Two and a half ml 0.02 M NaOH was added to stop the reaction. The absorbance at 400 nm was measured after 10 minutes. One unit of alkaline phosphate releases one μmol p-nitrophenol per minute at 37°C.

b) Intestinal preparations

i) Preparation of everted jejunal segments

Male Sprague-Dawley rats were sacrificed by decapitation and a midline incision made. The duodenal and the ileocecal end were identified and cut. The intestine was separated from the connecting messentries and was transferred on to a glass dish on ice. The duodenal segment and the lower two-thirds of the ileum was cut and discarded. The entire piece of the proximal jejunum was flushed with 40 ml of ice cold 0.9 % saline. The jejunum was everted by catching one end on a glass rod (1.5 mm diameter) and everting the whole length. The whole procedure upto this point takes three minutes. The everted jejunum was washed with 0.9 % saline and cut into pieces of 2.0 cm length.

These jejunal segments were used immediately for uptake studies of [^{3}H]-biotin or [^{3}]-biocytin.

ii) Preparation of mucosal cell suspensions

Male Sprague-Dawley rats 220 g were sacrificed and the proximal jejunum removed and everted as described before. The jejunum was cut into 4.5 cm pieces and these were incubated at 37^{o}C with gentle agitation in a plastic beaker containing 20 ml isolation medium, pH 7.2 (Reiser and Christiansen, 1973). The isolation medium contained 96 mM NaCl, 8 mM KH_2PO_4, 50 mM trisodium citrate, 5.6 mM Na_2HPO_4, 1.5 mM KCl, 450 units/ml hyaluronidase, and 2.5 mg/ml bovine serum albumin. After 20 minutes of incubation the pieces were gently patted with a plastic pipette tip. The dislodged cells were filtered through a single layer of cheese cloth and the cells harvested by centrifugation 1000 X g for 1 minute. The hyaluronidase containing medium was decanted and the cells washed twice with incubation medium containing 120 mM NaCl, 20 mM tris-Cl (pH 7.4), 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 11 mM glucose. The final cell pellet was suspended in incubation medium at a concentration of 1 mg cell protein/ml.

iii) Preparation of brush border membrane vesicles

The method of Kessler et al. (1978), with modification was used for preparing jejunal brush border membrane vesicles. Figure 9 shows the flow diagram of the procedure followed. Important at all stages is to work at ice temperatures. The mucosal cell scraping was homogenized in a Waring blender at

full speed for 1 minute in 15 volumes of 50 mM mannitol, 1 mM 2-mercaptoethanol, and 10 mM phenylmethylsulfonylfluoride. An aliquot of the homogenate was retained for enzyme analysis. Working at ice temperature solid $CaCl_2$ was added to a final concentration of 10 mM. The solution was mixed gently a few times while it stood on ice for 20 minutes. The $CaCl_2$ caused a selective aggregation of the microsomes by a mechanism which is not fully understood. The low speed centrifugation step removes the unwanted cellular material along with the microsomal fraction. The supernatant fraction containing the brush borders were pelleted by a high speed centrifugation step and were resuspended by homogenization in phosphate buffer.

c) Synthesis and purification of [^{3}H]-biocytin

The procedure for the synthesis of biocytin involved a two step reaction. In the first step the active ester of biotin was formed by reacting it with N-hydroxysuccinamide. The active ester was then reacted with α-t-boc-lysine to produce α-t-boc-biocytin. The blocking group on the α-amino group was removed by hydrolyzing it with trifluoroacetic acid. N-hydroxy succinamide (28.6 mg), biotin (40 mg) and 10 mCi [^{3}H]-biotin (10 Ci/ mmol) were added to 10 ml dimethylformamide. This was stirred at 4°C overnight. The solvent was evaporated to 3 ml under vacuo. To this a mixture of 40.3 mg α-t-boc-lysine in 0.5 ml saturated sodium carbonate was added and it was left stirring at 4°C overnight. The residual solvent was removed by evaporation in vacuo. Trifluoroacetic acid (5 ml) was added and

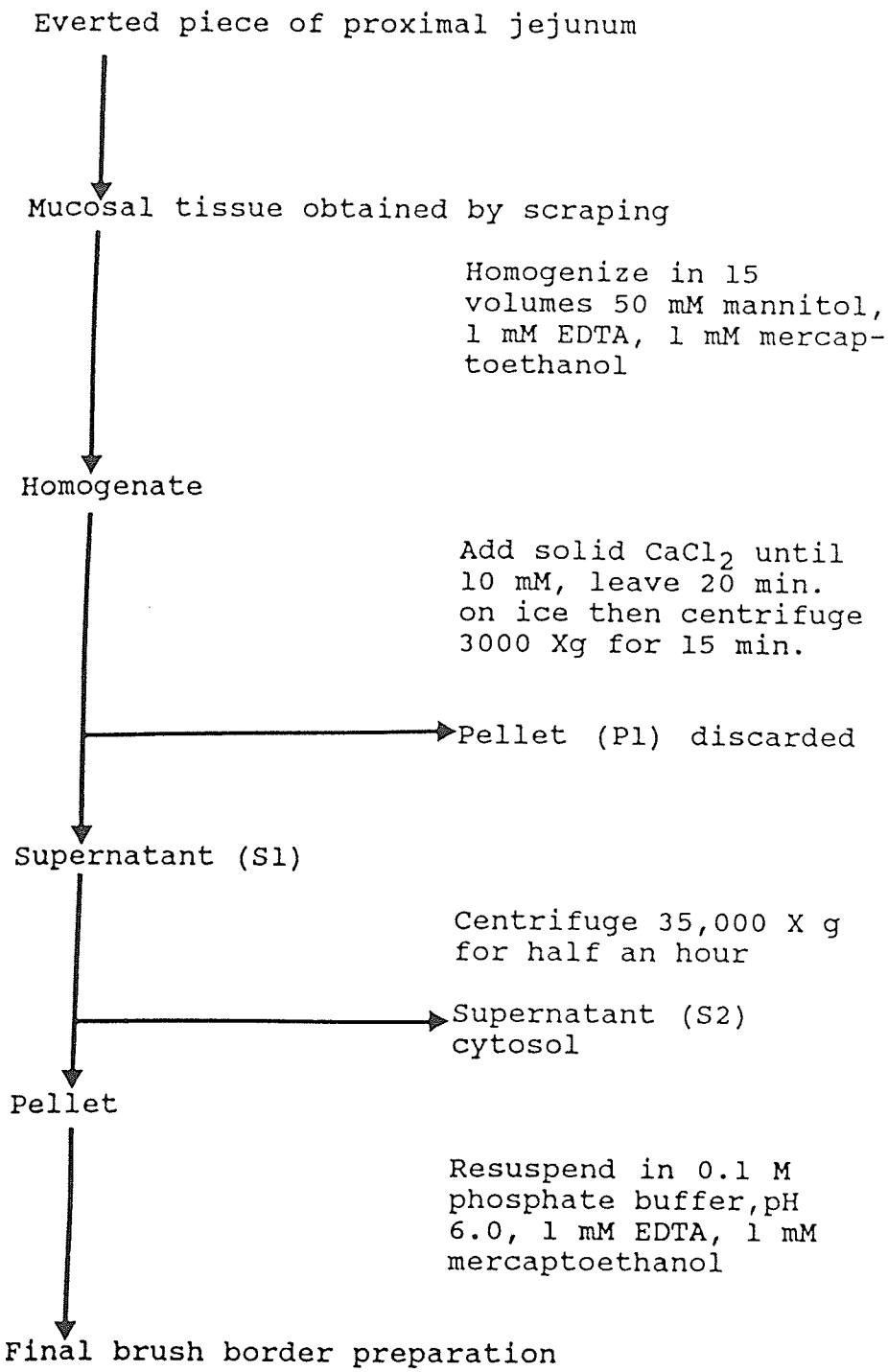


Figure 9 - Protocol for brush border membrane preparation.

the solution stirred for half an hour. Trifluoroacetic acid was removed in vacuo. To the slurry that was obtained 10 ml distilled water was added and the pH adjusted to 7.0 with potassium hydroxide. The solid material was removed by passing through scintered glass funnel. The filtrate was passed through a column (1.5 cm X 20 cm) of Bio-Rad AG1X-8 resin in the acetate form. The eluate containing [^{3}H]-biocytin was collected. The column was washed with distilled water. The eluate was freeze dried and dissolved in 10 ml distilled water. This material which was now depleted of biotin (as judged by T.L.C chromatography) was applied in small amount (usually 1 ml per plate) to preparative T.L.C silica gel plates which were run with ethanol/water (vol:vol, 1:1). The area corresponding to the migration of authentic biocytin was scrapped and packed into a column. The material was eluted with distilled water and the sample reduced to a small volume by freeze drying. The [^{3}H]-biocytin was judged to be pure based on its migration to a position occupied by authentic biocytin. The TLC plate did not show any biotin in [^{3}H]-biocytin. The specific radioactivity of [^{3}H]-biocytin was 90.4 mCi/mmol.

d) Intestinal uptake of biotin and biocytin

i) Uptake of biotin in jejunal segments

The uptake studies were performed in 20 ml plastic scintillation vials containing 12 ml of oxygenated Krebs-Ringer phosphate buffer pH 7.4, and 11 mM glucose. Varying amounts of unlabeled biotin upto the desired concentration were

added. The amount of [^{3}H]-biotin added was such that the final specific radioactivity of 1.29 Ci/mmol remained constant at each biotin concentration. The uptake studies were done at 37^{o}C in a shaking Dubnoff water bath at 90 oscillations per minute. The reaction was started by adding the 2.0 cm everted jejunal segments at timed intervals. After 10 minutes of incubation the jejunum segment was removed and placed on a single layer cheese cloth held in place by a rubber band over a wide neck flask. The piece of tissue was washed with 5 ml ice cold Krebs-Ringer phosphate buffer, pH 7.4 containing 1 µM biotin and the excess fluid drained off. The segments were weighed on a digital balance and the wet weights noted. Each piece of tissue was transferred into a plastic scintillation vial and 1.1 ml NCS tissue solubilizer added and incubated at 50^{o}C with gentle agitation for 5 hours. To the solubilized material 50 µl of benzoyl peroxide (1 g in 5 ml toluene) was added and the incubation at 50^{o}C continued for half an hour. The samples were cooled before the addition of 15 ml OCS scintillant. The vials were kept at 4^{o}C in the dark overnight and the radioactivity determined in a Beckman LS-2000 counter.

ii) Uptake of biotin in mucosal cell suspension

The uptake of biotin in cell suspension was studied in 25 ml conical flasks. The total volume of the uptake medium was 4.0 ml. This medium consisted of 120 mM NaCl, 20 mM tris-Cl (pH 7.4), 30 mM K_2HPO_4, 1 mM $MgCl_2$,1 mM $CaCl_2$, and 11 mM glucose. Varying amounts of unlabeled biotin up to the desired

concentration were added. Radiolabeled biotin was added so that the final specific radioactivity (0.25 Ci/mmole) was constant at each concentration of biotin. An aliquot (0.2 ml) of the cell suspension containing 0.194 mg cell protein was added at timed intervals and incubated at 37°C in an shaking water bath (90 oscillations/ minute). A 200 µ l aliquot was removed at timed intervals and filtered under vacuum through a Millipore GF/B filter in a Millipore filter apparatus. The filters were washed with 5 ml cold incubation medium which contained 1µ M unlabeled biotin. The filters were transferred to plastic scintillation vials and 1 ml NCS tissue solubilizer was added and incubated for 1 hour at 37°C. The vials were cooled and 15 ml OCS scintillant was added. Radioactivity was determined in a Beckman LS-2000 counter.

iii) Uptake of biocytin in jejunal segments

The uptake of biocytin was performed as described (part d (i)). The concentration of biocytin used in this study was between 7.8 nM to 200 nM. The amount of radioactive biocytin added was such that a constant specific radioactivity of 0.1 Ci/ mmol was obtained at each concentration. The uptake study was performed at 37°C for 10 minutes and the further treatment of the samples was as described in part d (i).

e) Fractionation of brush border membrane protein

Brush border membranes were solubilized in 0.3 % sodium deoxycholate on ice for half an hour. The solubilized fraction was obtained after centrifuging at 35,000 X g for half an hour.

The pellet was resolubilized in 0.1 M phosphate buffer, pH 7.0 and checked for residual alkaline phosphatase and biotinidase activity. The solubilized fraction was dialyzed overnight against 50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA, and 1 mM 2-mercaptoethanol. The dialyzed material was concentrated to 1.0 ml. Sucrose density gradients (5 to 20 %), containing 1 mM mercaptoethanol were formed with the Beckman gradient maker. The concentrated solubilized material (between 200 µl and 350 µl) was layered on top of the sucrose gradient. Bovine serum albumin (mol. wt. 68,000), aldolase (mol. wt. 158,000), and catalase (mol. wt. 232,000) were run as marker proteins. The standards and the sample were centrifuged at 35,000 X rpm in a SW40 Beckman rotor for 16 hours. 0.4 ml fractions from the gradients were collected in a fraction collector and analyzed for protein by the Bio-Rad method, biotinidase activity by the colorimetric assay, and biotin binding activity as described earlier. Biotinidase was assayed by the colorimetric assay because only a relative value of the activity in the fractions was required.

EXPERIMENTAL RESULTS

a) UPTAKE STUDIES

i) Uptake of biotin in mucosal cell suspension

Figure 10 shows the time course of the uptake of biotin in mucosal cell suspensions at different biotin concentration. The uptake of biotin has reached its maximum after 8 minutes. The relationship between the maximal uptake rate and the amount of

biotin in the medium is linear (see insert for a replot of uptake v/s concentration) suggesting that a passive diffusion driven process occurs between 0.1 μM to 3.0 μM biotin.

ii) Uptake of biotin in jejunal segments (0.05 μM to 3.0 μM)

The uptake of biotin in jejunal segments is shown in figure 11. The range of biotin concentration studied was between 0.05 μM to 3.0 μM. The relationship between the biotin concentration and the 10 minute uptake appears to be essentially linear. However at the lower biotin concentration (less than 0.25 μM) there appears to be a slight deviation from this linearity.

iii) Uptake of biotin in jejunal segments (2.5 nM to 60 nM)

The uptake of biotin at a lower concentration (2.5 to 60nM) was examined. The results are shown in figure 12. The uptake of biotin between the 2.5 nM to 40 nM biotin proceeds by a process which shows saturation kinetics. The uptake beyond a concentration of 40 nM biotin proceeds by passive diffusion as indicated by the linear relationship between uptake and concentration of biotin.

iv) Uptake of biocytin in jejunal segments

Figure 13 shows the uptake of biocytin by the jejunal segments. The amount of biocytin transported appears to plateau at about 50 nM biocytin concentration however, the uptake of biocytin above 50 nM is essentially a direct function of the

biocytin concentration. The concentration of biocytin used was between 7.8 nM and 200 nM. The amount (moles) of biocytin transported per gram wet weight of tissue does not appear to be significantly higher (one and a half-fold higher) than the amount of biotin transported at similar concentration.

b) Preparation of brush border membranes

Table IV shows the assesment of the protocol used for preparing brush border membrane. The method results in a five-fold enrichment in this fraction as determined by the alkaline phosphatase activity. The brush border membrane preparation contains a low amount of biotinidase activity. Since marker enzyme studies for cytosol were not performed it is possible that the biotindase activity in this preparation is a contaminant of the cytosol.

c) Sucrose density gradient analysis of brush border membrane protein and protein from cytosol

Starting with homogenates of rat mucosal tissue brush border membrane was prepared and the final 35,000 X rpm cytosol fraction obtained. The brush border membrane was solubilized with sodium deoxycholate. The brush border protein and the cytosol protein were separated by sucrose density gradient (5% to 20%) centrifugation. Figure 14 shows the biotinidase activity and biotin-binding profiles of the separated protein from the two preparations. In both fractions biotinidase activity migrates slightly ahead of the albumin peak and therefore rat intestinal biotinidase has an apparent molecular

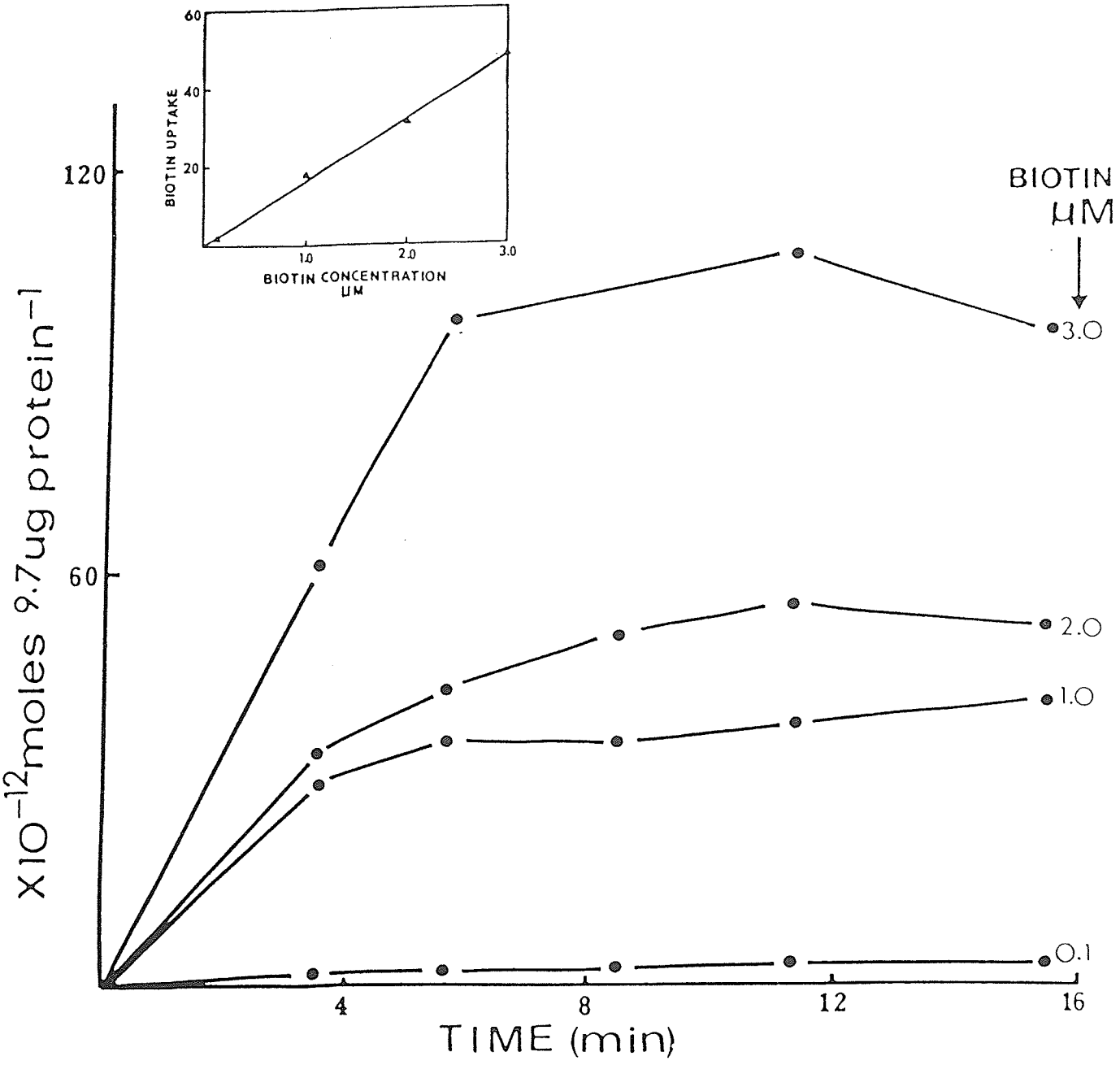


Figure 10 - Uptake of biotin by mucosal cells.The uptake of biotin by mucosal cells at the four concentrations shown was studied as a function of time. The uptake studies were performed as described under 'Methods' part d (ii). The insert is a replot of the maximal uptake rate of biotin (i.e the 10 minute uptake values) versus the biotin concentration. The data shown is derived from a single determination.

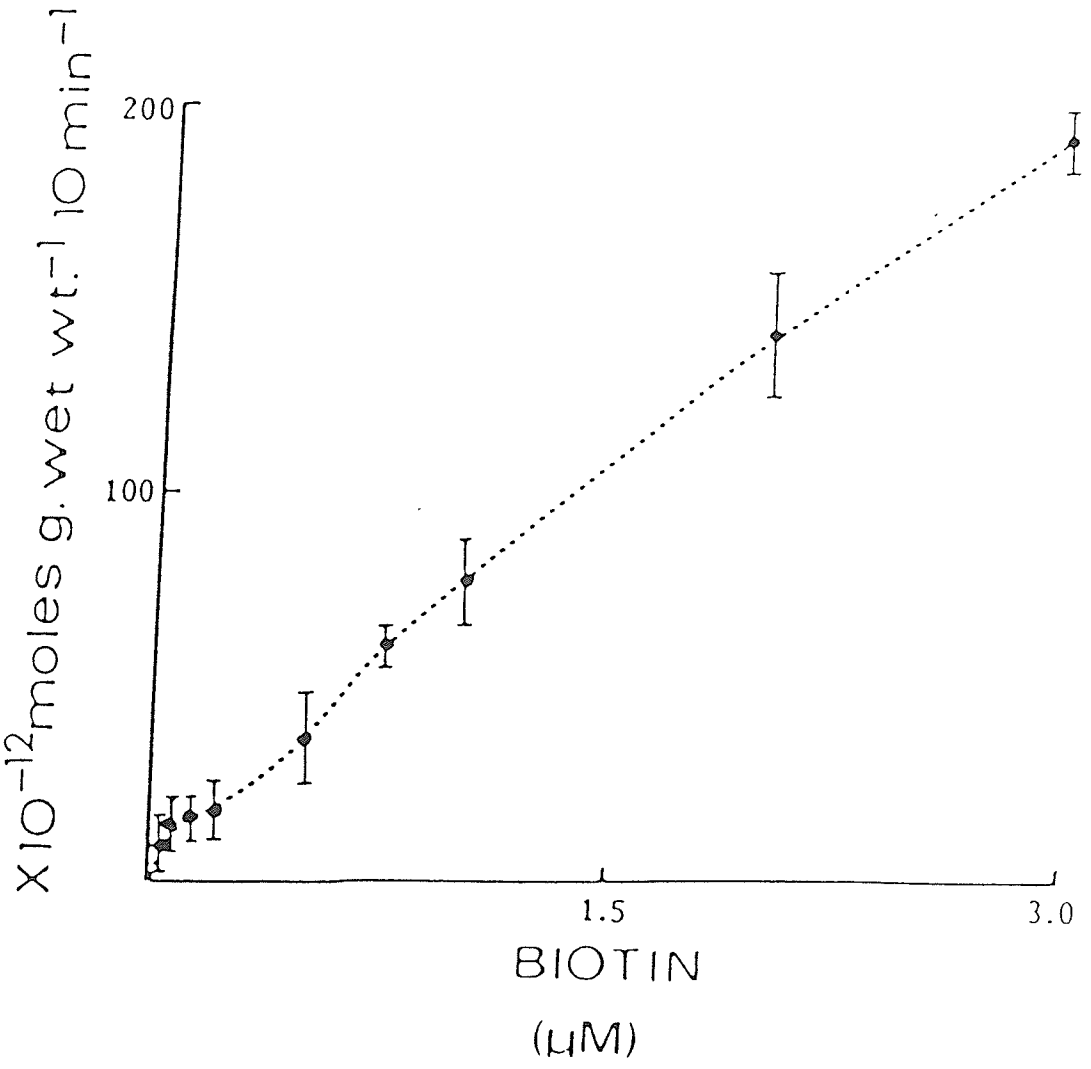


Figure 11 - Uptake of biotin in jejunal segments (0.05 μM to 3.0 μM). The uptake of biotin in 2.0 cm jejunal segments was determined as a function of the concentration of biotin. The uptake is expressed as moles of biotin transported per g wet weight of tissue in 10 minutes. The data shown is a mean of two determinations and the bars represent these values.

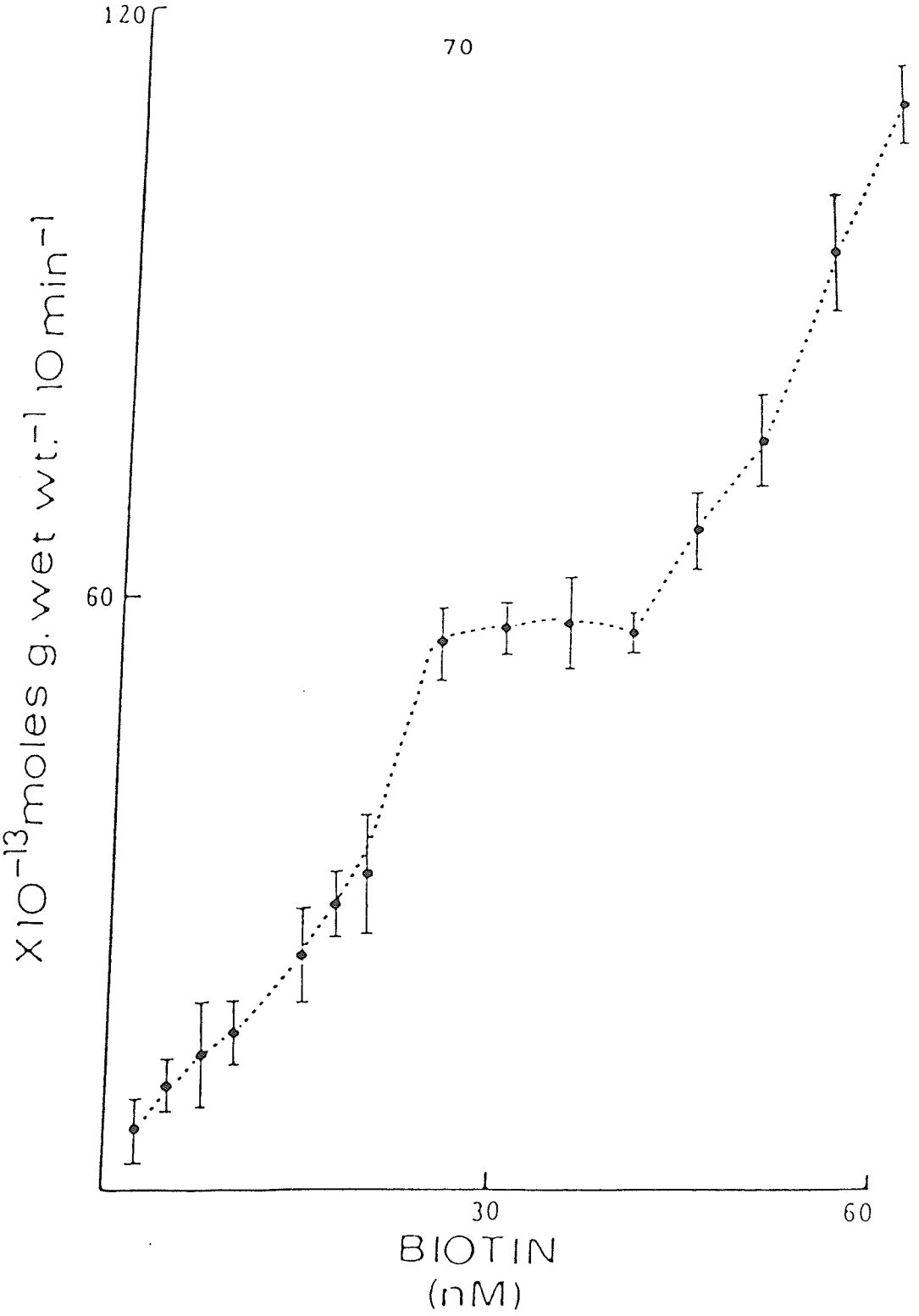


Figure 12 - Uptake of biotin in jejunal segments (2.5 nM to 60 nM). The uptake of biotin in jejunal segments was determined as a function of the concentration of biotin. Uptake was expressed as moles of biotin transported per g wet weight of tissue in 10 minutes. The data shown is a mean of two determinations and the bars represent these values.

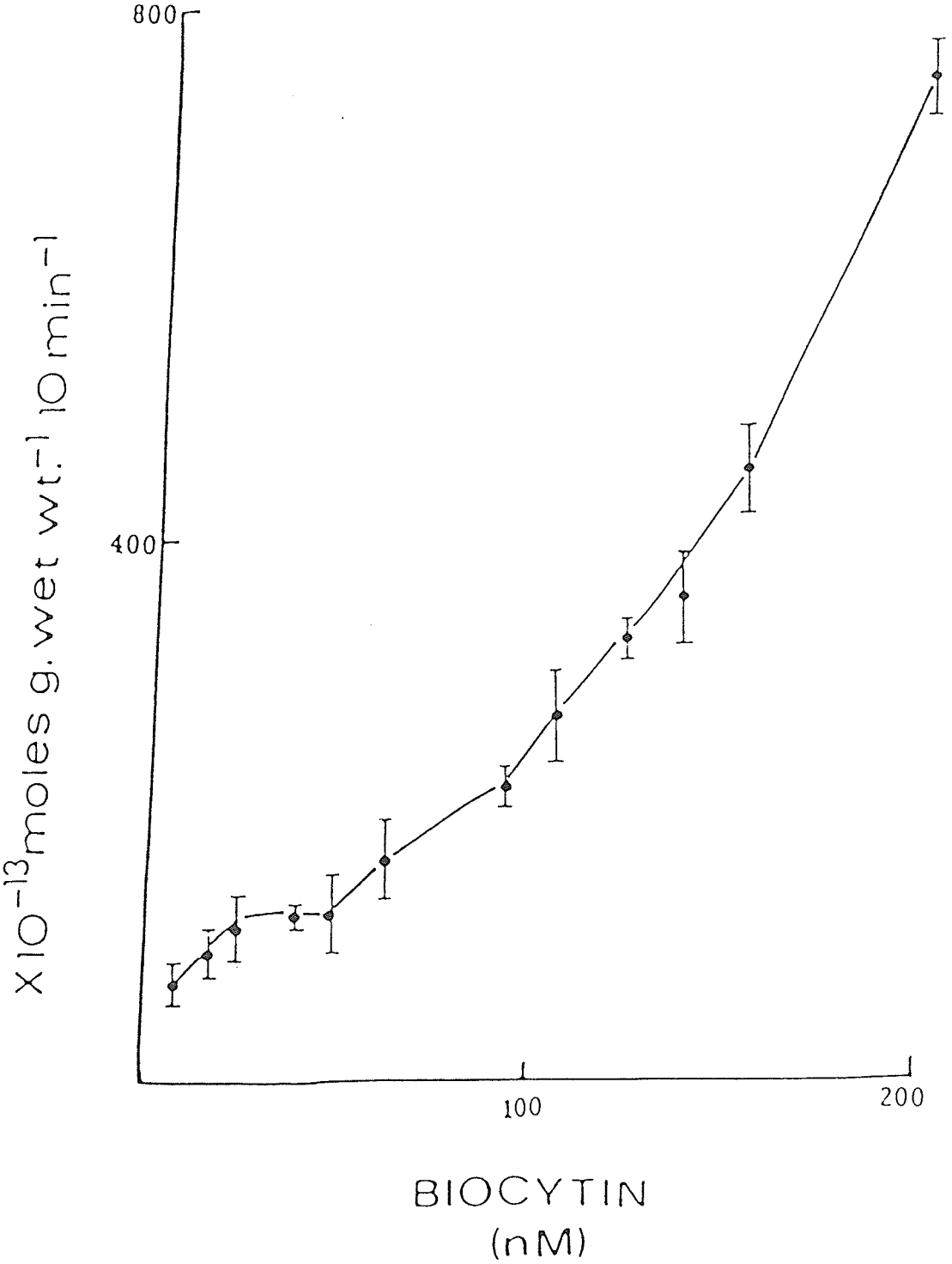


Figure 13 - Uptake of biocytin in jejunal segments. The uptake of biocytin in jejunal segments was determined as described under methods part d (iii). The uptake of biocytin was expressed as moles of biocytin transported per g wet weight of tissue in 10 minutes. The data shown is a mean of two determinations and the bars represent these values.

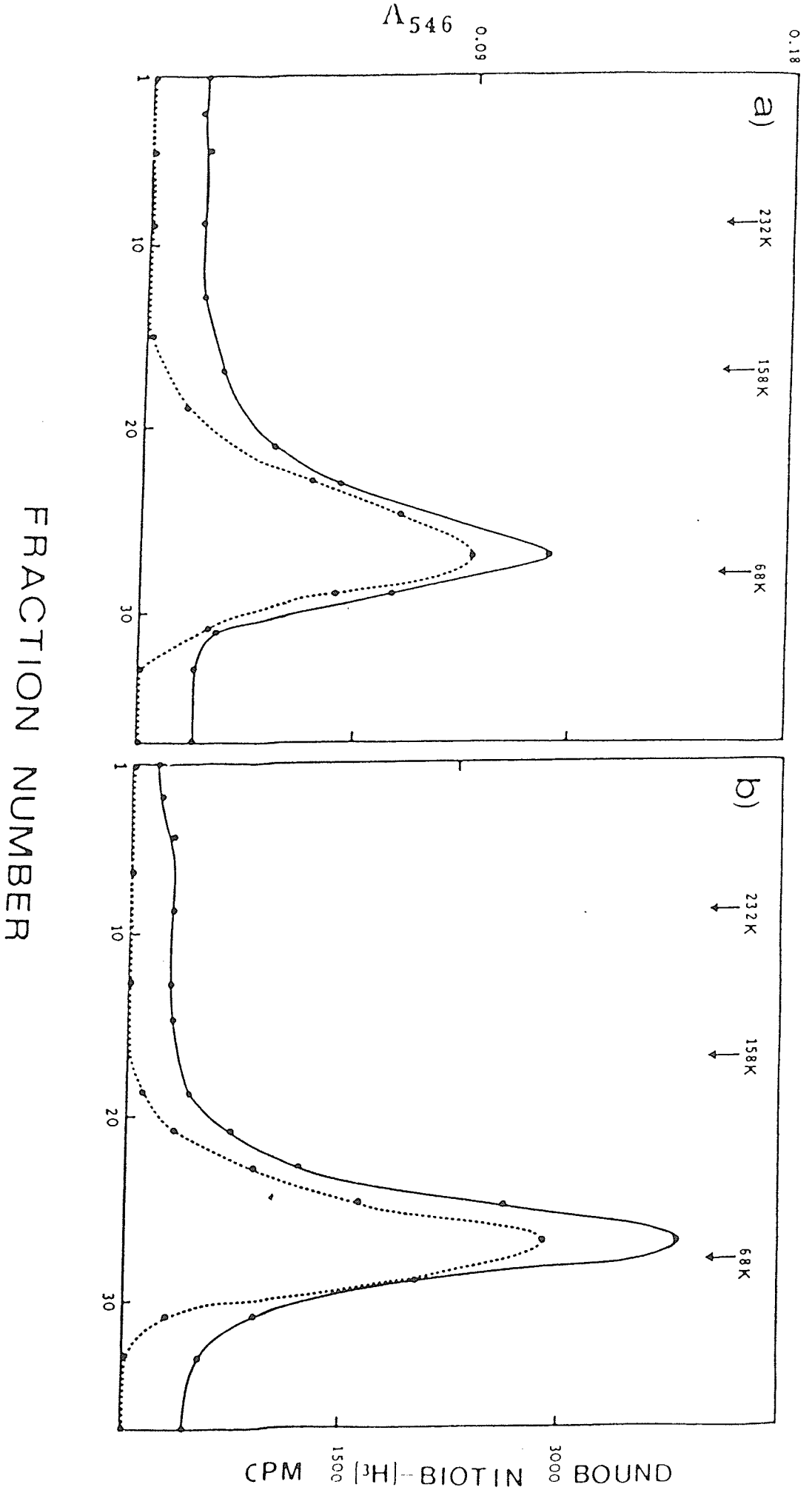
Table IV - Analysis of brush border membrane preparation

	Homogenate	X3000 supernatant	Final brush border preparation
Alkaline phosphatase:			
i) Total units	12,096	8,250	7,955
ii) Specific activity *	0.29	0.28	1.42
iii) Fold-erichment	-	0.97	5.0
Biotinidase:			
i) Total units	71.7	2.1	0.87
ii) Specific activity **	29.5	1.2	2.3
iii) Fold-enrichment	-	0.04	0.08

* µmoles p-nitrophenol released per min at 37°C per mg protein.

** pmoles p-aminobenzoate released per minute at 37°C per mg protein.

Figure 14 - Analysis of solubilized brush border membrane protein and cytosol on sucrose density gradient. Brush border membrane were prepared as described under 'methods'. A concentrated cytosol fraction (35,000 X rpm supernatant) was separated on a sucrose gradient (a). The detergent treated, dialyzed and concentrated preparation of brush border protein (b) was also separated by sucrose density gradient centrifugation. Biotinidase activity (————————) and biotin-binding activity (----------) were examined in the fractions.


A_{546}
0.09
0.18
a)
232K
158K
68K
1
10
20
30
b)
232K
158K
68K
1
10
20
30
1500
3000
CPM [3H]-BIOTIN BOUND
FRACTION NUMBER

weight of just over 68 Kilodalton. This approximate value of the molecular weight obtained here is in close agreement with the value of 68 Kilodalton obtained for human serum biotinidase and rat liver biotinidase. The biotin-binding activity in the cytosol and brush border preparation migrate to an identical position as the biotinidase peak.

DISCUSSION

Biotin uptake *in vitro* was studied in isolated mucosal cells and in small segments of the intact tissue. The uptake of biotin in the micromolar range appears to occur by a non-saturable process. This micromolar concentration of biotin is probably in vast excess of the concentration found in the gut. A saturable process for biotin uptake, however, can be demonstrated at a biotin concentration in the nanomolar range. Between 2.5 nM to 40 nM bitoin is transported in a saturable manner. Above 40 nM concentration of biotin the uptake occurs in a non-saturable manner. The biphasic transport of biotin by the jejunum is in agreement with a similar process reported for the rat *in vivo* (Bowman *et al.*, 1985). Hence provided that the appropriate concentration of biotin is used in the uptake studies, it is possible to demonstrate the saturable and non-saturable processes.

The rat jejunum *in vitro* shows a slightly enhanced uptake of biocytin compared to the uptake of biotin. For example, at a concentration of 60 nM of biotin and biocytin, respectively, the uptake values are 111 X 10^{-13} moles per g wet weight tissue

in 10 minutes and 162 X 10^{-13} moles per g wet weight tissue in 10 minutes, respectively. The uptake of biocytin proceeds essentialy in a linear manner, however, up to a concentration of 50 nM biotin there does seem to be some evidence for a saturable process. Since the [^{3}H]-biocytin prepration was not of a very high specific radioactivity, the closer examination of its uptake within this range could not be determined. The specific radioactivity of the [^{3}H]-biocytin prepared was 90.4 Ci/mmol. The manner in which biocytin could be transported is not precisely known. There are two ways by which this could occur. It could either be transported across the mucosal tissue uncleaved and then hydrolyzed afterwards or it could be hydrolyzed within the lumen first and then the biotin transported afterwards. Regardless of the mechanisms involved, it is quite clear that biocytin does have an important role in the transport of biotin from its protein-bound form.

Table IV shows that the biotinidase activity in brush border is quite low, this may be a contaminant of the cytosol. The possible role of biotinidase in the transport of biotin was determined <u>in vitro</u> by analyzing the enzyme and biotin-binding activity in the brush border and cytosol. As indicated in figure 14 both preparations showed a single biotinidase activity which co-migrates with the biotin-binding activity. This evidence is suggestive of a possible role of the cytosolic enzyme in the transport of biotin.

GENERAL DISCUSSION

The uptake of biotin in rat small intestine appears to be carried out by two processes. The first process is saturable and occurs below 40 nM biotin. The second process is nonsaturable and occurs probably by passive diffusion above 40 nM biotin. The biphasic nature of the uptake of biotin indicated by this study is in agreement with the results of a study of *in vivo* uptake of biotin in rat jejunum (Bowman *et al.*, 1985). The significance of the biphasic transport of biotin in the rat small intestine is that when the biotin concentration in the gut falls below 40 nM, the saturable uptake mechanism operate so that enough biotin is made available to the animal. If such a system indeed operated in other mammals, including man it would have tremendous advantage in the context of a fluctuating amounts of biotin ingested in the diet. In the rat gut one would expect the biotin concentration in the lumen to reach only the nanomolar rather than the micromolar range. The nanomolar concentration in the gut is based upon theoretical calculations of the plasma biotin levels (which are in the picomolar range) and the distribution of the gut contents. Hence, any study on the transport of biotin in the micromolar range will not represent a physiological situation. Although the study of the transport of biotin shows a saturable component, this is only a preliminary study and characterization of the system awaits further research.

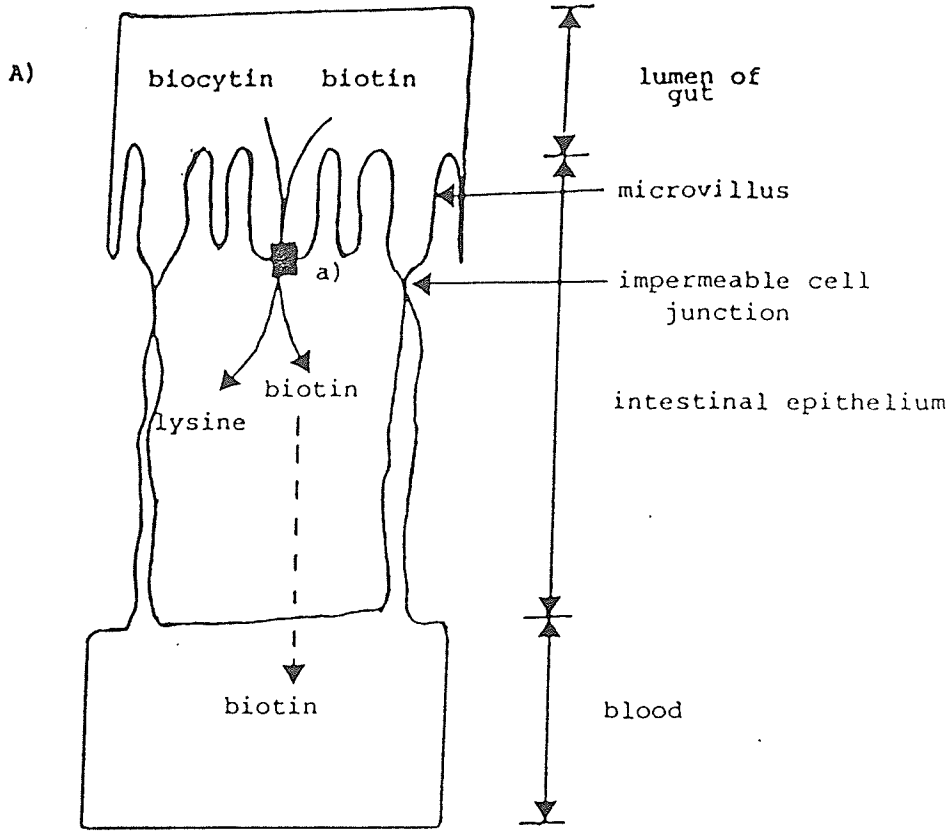
Biocytin is taken up by the jejunal segments but its transport is not appreciably high (only 1.5-fold higher) in comparison to the transport of biotin. For this reason it is considered that biocytin may not be a preferred transport form. It remains also possible that biocytin in the lumen gets hydrolyzed to lysine and biotin before it can enter the mucosal cell. This would be possible if the intestine secreted biotinidase. There is no information in the literature regarding this, although biotinidase activity is present in pancreatic juice.

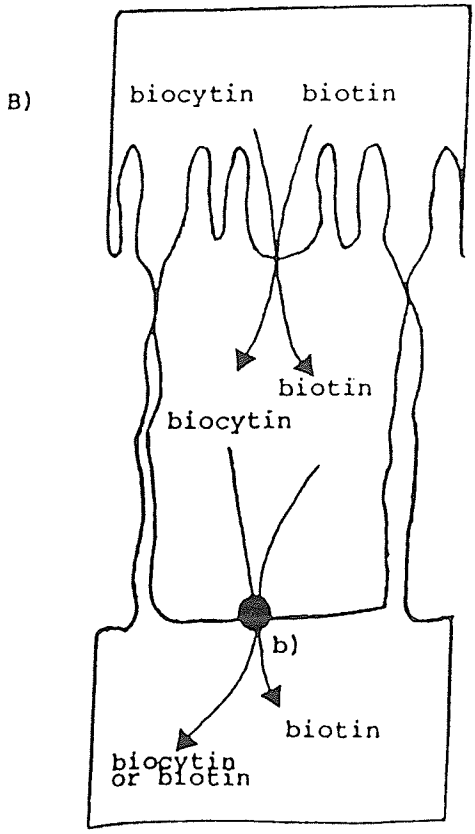
The brush border and cytosol preparations show a single biotinidase activity peak. The apparent molecular weight determined is in close agreement with the value of 68 Kilodaltons for human serum and rat liver biotinidase. The brush border membrane had very little biotinidase activity. It is possible that biotinidase in the brush border is a contaminant. The further fractionation of brush border and cytosol was carried out in the hope that if protein(s) other than biotinidase were involved in the transport of biotin, then these would have been indicated by the biotin-binding assay. The preliminary results seem to suggest that a single protein fraction in the cytosol and brush border is present in which biotinidase activity co-migrates with the biotin-binding activity. Whether this fraction contains biotinidase protein alone or a few proteins of related mass or shape remains to be answered. Based on this preliminary <u>in vitro</u> studies it is

speculated that biotinidase in vivo may have a role in the transport of biotin. The implied role of biotinidase in the transport of biotin will have to be confirmed using biotinidase antibody, this study would establish whether the saturable biotin transport system is decreased in the presence of these specific antibodies.

The different scenarios which are possible with respect to the arrangement of the hypothetical protein involved in the transport of biotin is shown in figure 15. In part A) of the figure, the hypothetical carrier protein a), is shown located within the brush border membrane (microvilli). This carrier protein could transport biotin from the lumen into the epithelial cell. If biotinidase is present in the brush border it could hydrolyze biocytin to lysine and biotin. The biotin within the epithelial cell cannot enter an adjacent cell due to the tight junction present between cells. Biotin in the epithelial cell could diffuse passively (as shown by the dotted line, figure 15 A) across the basolateral membrane and into the circulation. Figure 15 B) shows an alternative arrangement of the transport protein. The carrier is located in the basolateral membrane. Biocytin and biotin could enter the epithelial cell by simple diffusion. The carrier protein could transport the biotin across this barrier into the circulation. If this carrier is biotinidase it could hydrolyze biocytin at the same time. In figure 15 B) it remains possible for biotinidase in the cytosol to hydrolyze biocytin, but this is

Figure 15 - Hypothetical intestinal transport mechanisms for biotin and biocytin. The figure in part A), indicates the presence of the hypothetical carrier (designated by the letter a) in the brush border, whereas in part B), the carrier (designated by the letter b) is shown located in the basolateral membrane.


A)
biocytin
biotin
lumen of gut
microvillus
a)
impermeable cell junction
biotin
lysine
intestinal epithelium
biotin
blood


B)
biocytin
biotin
biotin
biocytin
b)
biotin
biocytin
or biotin

not indicated in the figure.

Some specific examples of the different mechanisms known to operate in the intestinal transport of vitamins now follow. The transport of [^{14}C]-ascorbic acid by guinea-pig ileal mucosa shows evidence of a carrier-mediated entry of ascorbic acid (Mellors et al., 1977). This study indicated a carrier mechanism exist for ascorbic acid at the brush border membrane. The transport of ascorbic acid by the carrier is coupled tightly to the external sodium ion concentration (Mellors et al., 1977, Patterson et al., 1982). The uptake of [^{14}C]-ascorbic acid by guinea-pig ileal mucosa was also inhibited by metabolic poisons such as rotenone and cynaide, and under anoxic conditions (Patterson et al., 1982).

The second example of intestinal transport of vitamin is the transport of dietary folates. The form of folates in the diet of mammals is predominantly in its bound form as polyglutamyl folates. The biological availability of folate is dependent on the digestion of folyl polyglutamates and the release of monoglutamyl folates during digestion and absorption. The intestinal mucosa of human contains one or more form of pteroylpolyglutamyl hydrolase (sometimes referred as conjugase enzyme; Reisenauer et al., 1977). The conjugase enzyme cleaves poluglutamyl foltes into monoglutamyl foltes. One form of conjugase in human small intestine is located in the brush border and the other form is located in an intracellular site. Monoglutamyl folates are absorbed by a

structure specific process in rat jejunum (Selhub and Rosenberg, 1981, Strum, 1977). The transport of monoglutamyl folates is highly sensitive to pH in the rat, with an optimum of pH 6.0. The transport of monoglutamyl folates is also dependent on temperature and cellular metabolism. The transport of monoglutamyl folates appear to occur by a specific saturable system with a K_m of 0.42 M and a V_{max} of 0.67 pmol/mg protein/0.5 min for [^{3}H]-PteGlu (Selhub and Rosenberg, 1981). An additional pathway of folate uptake by passive difussion is also demonstrable in the rat at higher folate concentrations. The involvement of of the conjugase enzyme in the digetion of polyglutamyl folates is quite clear, but its role, if any, in the transport of folates has yet to be demonstrated. Both biotinidase and conjugase enzymes hydrolyze bound forms of biotin and folates, respectively. It will be interesting to determine whether these enzymes could play a role in the transport of these vitamins.

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