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MECANISMOS DE CAPTACION INTESTINAL DE BIOTINA Y BIOCITINA Y SU RELACION CON LA ACTIVIDAD DE BIOTINIDASA PANCREATICA EN HAMSTER Y RATA

T E S I S

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INTRODUCCION GENERAL

La biotina es cofactor de carboxilasas (1), en las que se encuentra unida covalentemente a un residuo de lisina. Esta vitamina tiene un papel importante en gluconeogénesis, anaplerosis del ciclo del ácido cítrico y en el catabolismo de varios aminoácidos de cadena ramificada (2,3). Hay evidencia que indica que la biotina podría también estar involucrada en la síntesis de ADN y ARN (4,5).

Debido a su importancia, esta vitamina es reciclada continuamente en el organismo durante el recambio de las carboxilasas. Al ser degradadas las holoenzimas a las que esta unida, se libera biocitina (biotinil-lisina). Para que la biotina pueda ser nuevamente utilizada, la biocitina tiene que ser hidrolizada por la enzima biotinidasa.

Los mamíferos no pueden sintetizar biotina por lo que deben obtenerla a partir de los alimentos en los que, a pesar de estar ampliamente distribuída, se encuentra en muy bajas concentraciones. Por esta razón, para que los organismos puedan utilizar a la vitamina contenida en la dieta deben contar con un eficiente sistema de absorción intestinal.

Es probable que la biotinidasa también sea importante en el procesamiento de la vitamina contenida en los alimentos, ya que

la biotina se encuentra en mayor proporcion unida a proteínas (6). Sin embargo, la identidad del sitio donde se liberaría a la biotina exógena no es del todo clara, ya que se han reportado diferencias significativas en la actividad de biotinidasa entre diferentes especies así como entre diferentes órganos. Los carnívoros y omnívoros poseen actividad mayor que los herbívoros en suero, hígado y riñón (7).

En la rata, la actividad de biotinidasa pancreática es de solo 1/30 de la actividad encontrada en plasma.
Hasta ahora no se han reportado diferencias interespecíficas en cuanto a la actividad de biotinidasa en páncreas y plasma; sin embargo, es posible que la cantidad de biotina que fuera absorbida libre o unida a lisina en el intestino delgado dependiera de la magnitud de la actividad de biotinidasa en páncreas.

Es posible que existan también diferencias interespecíficas en cuanto a los mecanismos de absorción intestinal de biotina. Los estudios previos a este trabajo indican que en hámster, el transporte de biotina parece ser un proceso mediado por acarreador aunque no esta claro si se trata de un transporte activo o un fenómeno de difusión facilitada (8,9,10). Por otro lado, en rata hay resultados contradictorios, pues existen reportes en los que se afirma demostrar la existencia de un acarreador mientras que otros reportan que el transporte de la

vitamina en esta especie es debido a difusión simple (11,12,13,14,15,16).

En la primera fase de este trabajo se describe la relación que existe entre los mecanismos de liberación y absorción intestinal de biotina; comparando hámster y rata en cuanto a la actividad de biotinidasa en plasma y páncreas y la captación de biotina y biocitina por anillos evertidos de intestino delgado.

En la segunda fase se caracterizan los diferentes mecanismos de captación intestinal de biotina y se profundiza en el estudio de la naturaleza y requerimientos del acarreador intestinal de la vitamina en el hamster, realizando experimentos de captación en concentraciones 50- 1000 nM por vesiculas de membrana en borde estriado de enterocitos. Este método no había sido empleado con anterioridad para estudiar fenómenos de trasnporte intestinal en hámster.

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Association of Pancreatic Biotinidase Activity and Intestinal Uptake of Biotin and Biocytin in Hamster and Rat[1]

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Key Words. Biotin · Biocytin · Biotinidase · Pancreas · Intestinal uptake · Hamster · Rat

Abstract. Pancreatic biotinidase activity was higher in hamster than in rat; these results were reversed in plasma. Uptake was studied in everted intestinal rings. Saturation kinetics at 37 °C were observed for biotin in hamster and for biocytin in rat, with a V_{max} of 1.83 and 1.05 nmol min^{-1} ml^{-1} and an apparent K_t of 25.14 and 40.7 μM, respectively. Biotin uptake by hamster intestine was reduced at 4 °C and when choline or potassium replaced sodium; it was inhibited by biocytin only at very high concentrations. Biocytin uptake in the rat was small compared to passive diffusion and was not influenced by sodium or temperature; it was not inhibited by biotin. We observed only passive diffusion of biotin in rat and of biocytin in hamster. Our results suggest that protein-bound biotin may be absorbed mainly in its free form in the hamster. In the rat, on the other hand, at least part of the dietary biotin may be absorbed lysine-bound, as biocytin.

Introduction

The vitamin biotin is the cofactor of carboxylases, to which it is covalently bound to a lysine residue of the apoenzymes [1]. As such, it plays an important role in gluconeogenesis, citric acid cycle anaplerosis, fatty acid synthesis and amino acid catabolism. Biocytin (biotinyl-lysine), a product of carboxylases breakdown, must be hydrolysed by means of biotinidase for biotin to be reused [2, 3]. Biotinidase is probably also important for the assimilation of dietary biotin, which is frequently protein-bound [4]. Although widely distributed in foods, biotin is present in very small amounts [1, 5, 6]. Therefore, for most dietary biotin to be of profit it should be freed by biotinidase; it also must be efficiently absorbed through the intestine.

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Biotinidase activity varies greatly among different animal species and also among their different organs [2, 7]. Carnivores or omnivores (such as the rat) tend to have higher activities than herbivores in serum, liver and kidney [2]. There is some activity in the rat pancreas [2, 4], but interspecies differences with respect to the pancreatic enzyme have not been investigated. It has been proposed that pancreatic biotinidase may play a critical role in the processing of dietary, protein-bound biotin [4]. However, its digestive role in the rat is not clear since its activity in pancreatic juice is only one thirtieth of that in serum [2, 4]. How much biotin is absorbed free or lysine-bound in a given species may depend on the level of the pancreatic enzyme.

Interspecies differences may also exist with respect to biotin intestinal absorption. It is a carrier-mediated process in the hamster [8–10], whereas there is conflicting information on the uptake mechanism in the rat [8, 11–13].

In the present investigation we compared the hamster with the rat with respect to: (1) pancreatic and plasma biotinidase activities and (2) the intestinal uptake of free and lysine-bound forms of biotin, in order to better define the mechanisms by which dietary protein-bound biotin is utilized in these species.

Materials and Methods

Biotinidase Assays

Male golden hamsters 100–180 g and male Wistar rats 200–300 g obtained from our animal house were used. For biotinidase assays the animals were lightly anesthetized with ether and were sacrificed by exsanguination by cardiac puncture; blood was collected in heparin-containing tubes. The pancreas was immediately removed, washed with ice-cold saline and homogenized in buffer KH_2PO_4, 100 m*M*, pH 6.0, containing Triton X-100 (Sigma Chemical, St. Louis, Mo.) 0.1% (4 ml/mg of tissue) at 4 °C with Polytron (Kinematica, Switzerland). The homogenate was centrifuged at 1,500 *g* for 10 min and the supernatant stored at −20 °C. Biotinidase activity was measured by Wolf colorimetric assay [14]. The reaction mixture contained 100 µl of pancreas homogenate or 50–100 µl of plasma, KH_2PO_4 100 m*M*, ethylenediaminetetraacetic acid 10 m*M*, bovine serum albumin (Sigma Chemical) 250 µg/ml and biotin-4-amidobenzoic acid (Sigma Chemical) 150 n*M*, pH 6.0 in 2 ml final volume. The tubes were preincubated at 37 °C for 10 min before the addition of the samples. After incubation for 60 min at 37 °C, 100 µl of trichloroacetic acid 30% were added to stop the reaction. The tubes were centrifuged at 1,500 *g* for 10 min and 1.5 ml of the supernatant were added to 0.5 ml distilled water to restore the 2 ml vol. $NaNO_2$ 0.1%, ammonium sulfamate 0.5% and N-1-naphthyl-ethylenediamine 0.1%, 200 µl of each were then added and the absorbance was determined at 546 nm.

Intestinal Uptake Experiments

Intestinal uptake was measured by the method of Mathews et al. [15]. The animals were anesthetized with ether. The abdomen was opened and the small intestine removed, stripped of mesentery and placed on a filter paper moistened with Tris-phosphate buffer: NaCl 118 m*M*, Tris-HCl 25 m*M*, KCl 4.7 m*M*, $CaCl_2$ 2.5 m*M*, $MgSO_4$ 1.2 m*M*, KH_2PO_4 1.2 m*M*, pH 7.4. The duodenum was selected, rinsed with buffer and everted over a glass rod; preliminary experiments showed that biotin uptake is higher in duodenum than in jejunum or ileum (data not shown). Rings of 10–20 mg were cut from the mid-region of the piece. The rings were incubated (one ring/tube) in 0.5 ml of the Tris-phosphate buffer pH 7.4, containing either ^{3}H-biotin 33 Ci/mmol (NEN Research Products, Boston, Mass.) or ^{3}H-biocytin 364 mCi/mmol, a gift from Dr. Lawrence Sweetman, University of California, San Diego, Calif. The incubation medium was prewarmed at 37 °C for 10 min and gased with 93% oxygen 5% carbon dioxide before the addition of the rings. Short incubation times (3 min) were used to study only the influx. We had previously shown that uptake is linear with time for at least 5 min (data not shown). After the incubation period the rings were rinsed in 500 ml NaCl 154 m*M* at 4 °C and the excess water was eliminated by blotting with filter paper

Table 1. Pancreas and plasma biotinidase activity (pmol min^{-1} mg protein^{-1}) in hamster and rat

Species	n	Pancreas[1]	Plasma[2]
Hamster	6	345.45 ± 10.73	40.38 ± 3.35
Rat	3	12.30 ± 4.25	103.30 ± 16.60

Values expressed as means ± SEM.
[1] $F = 637.59$; $p < 0.001$.
[2] $F = 8.83$; $p = 0.03$.

(Whatman 50). The rings were weighed and placed in 1 ml sulfosalicylic acid (60 g/l) at 100 °C for 5 min to extract the biotin or biocytin taken up by the tissue. After centrifugation (5,000 *g* for 5 min) the radioactivity in the supernatant was measured using a liquid scintillation counter Packard Tricarb 4000. Extracellular fluid space was determined using inulin to estimate the proportion of substrate remaining in the extracellular fluid space and in any fluid adherent to the tissue and which was not removed by rinsing and blotting. The intestinal rings were incubated in 0.5 ml Tris-phosphate buffer pH 7.4 containing 1.25 μCi ^{14}C-Inulin (Amersham International, Buckinghamshire), 13.4 Ci/mol at 37 °C under the above-mentioned incubation conditions. At the end of the incubation period the tissues were treated as described earlier and the radioactivity taken up measured. In order to estimate the carrier-mediated uptake of biotin or biocytin, intestinal rings were incubated at 4 °C and the results of these experiments were subtracted from uptake at 37 °C. Results are expressed as nmoles of substrate taken up per minute per milliliter cell water. Cell water was estimated as 80% of the wet weight of the tissue. To investigate the specificity of transport of the free and lysine-bound forms of biotin, intestinal rings were incubated with one form ^{3}H-labeled, at a concentration near its K_t, in the presence of increasing concentrations of the other form of the vitamin, unlabeled. The effect of sodium on the uptake was investigated by replacing NaCl with KCl or with choline Cl so as to maintain unchanged the cation concentration of the incubation mixture.

Statistical Analysis

Data were analyzed by one-way analysis of variance. Level of significance was $p < 0.05$.

Results and Discussion

Pancreatic biotinidase activity was significantly higher in hamster than in rat. These results were reversed in plasma, biotinidase being more active in rat than in hamster (table 1). Pancreas may be the major source of biotinidase in the intestinal lumen; Wolf et al. [4] reported measurable activity in rat pancreatic juice – although only one thirtieth of that present in serum, per unit volume – but none in the brush border membrane. No similar studies have been performed in other species, including the hamster. Assuming that pancreatic biotinidase activity is an accurate indicator of biocytin hydrolysis in the intestinal lumen, it is likely that after protein digestion more biotin may be free in the intestinal lumen of the hamster than of the rat. It is interesting that plasma biotinidase activity is substantially higher in this last species than in the hamster. Since there is also some intracellular enzyme activity in the rat intestinal mucosa [2, 4], absorbed dietary biocytin may be hydrolysed either in the mucosa or in the bloodstream in species with low intestinal (pancreatic) biotinidase activity. Previously, Pispa [2] reported a tendency for carnivores and omnivores, such as the rat, to have higher serum biotinidase activity than herbivores. He did not carry this comparison to the pancreatic enzyme, but studied its activity only in the rat pancreas, where it was quite low, compared to serum and to most other organs. Pancreatic biotinidase has not been previously studied in other herbivores; it is possible that they share the pattern of high pancreatic and low serum biotinidase that we have observed in the hamster.

To examine whether these species differences in dietary biotin release are associated

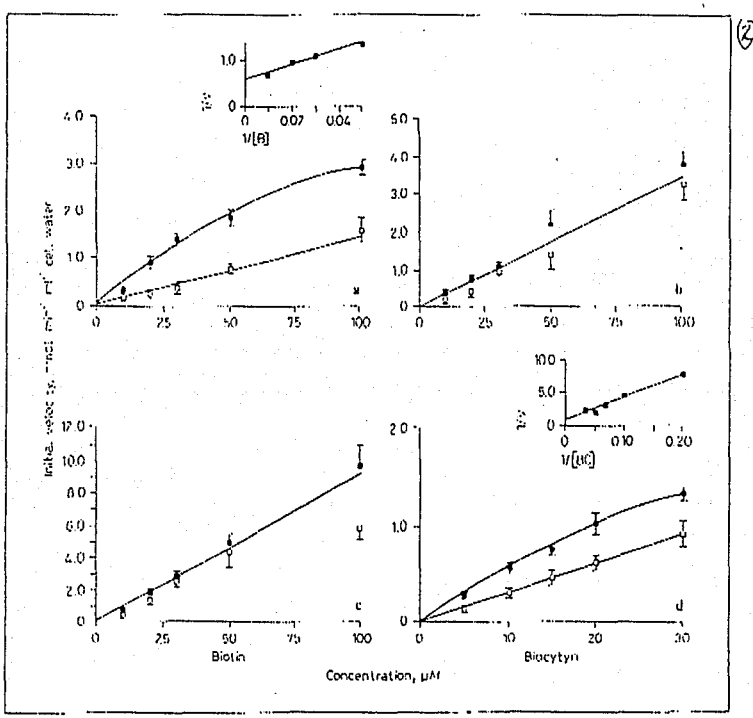

Fig. 1. Relationship between initial rate of substrate uptake and substrate concentration in the incubation medium at 4 °C (○) and at 37 °C (●). The kinetic parameters of biotin uptake in hamster (V_{max} 1.83 nmol min^{-1} ml^{-1}; K_t 25.14 μM) and of biocytin uptake in rat (1.05 nmol min^{-1} ml^{-1}; 40.7 μM) were obtained after subtracting the results at 4 °C from those at 37 °C. Values are means ± SEM; n = 3 for each point.

to the way biotin is transported through the intestinal epithelium, we measured the uptake by intestinal rings of biotin and biocytin in a range between 0.1 and 200.0 μM. Biotin uptake in the hamster duodenum showed saturation kinetics with a V_{max} of 1.83 nmol min^{-1} ml^{-1} and an apparent K_t for biotin of 25.14 μM; it was reduced at 4 °C compared to 37 °C (fig. 1a). On the other hand, biocytin uptake in this species was not appreciably affected by temperature and did not show saturation kinetics (fig. 1b). In the rat, biotin uptake was linear with increasing substrate concentration and this process was not af-

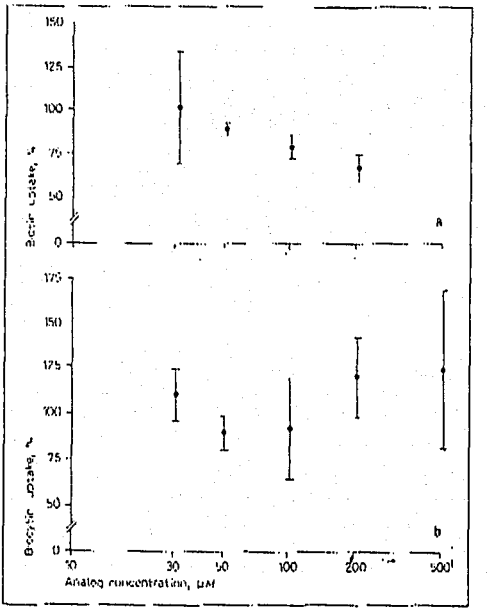

Fig. 2. Effect of nonlabeled biocytin on uptake of ^{3}H-biotin 30 μM by hamster intestinal rings (a) and of nonlabeled biotin on uptake of ^{3}H-biocytin 30 μM by rat intestinal rings (b). Values are means + SEM; n = 3 for each point.

fected by temperature (fig. 1c). Biocytin uptake in rat was also apparently linear, but when the range was narrowed down to 0.1–30 μM biocytin uptake proceeded by a process which showed saturation kinetics at 37 °C but not at 4 °C (fig. 1d), with a K_t of 40.7 μM and V_{max} of 1.05 nmol min^{-1} ml^{-1}. Tritiated biotin uptake by hamster intestinal rings was not inhibited by biocytin at concentrations near its K_t, and was reduced by 26% at biocytin concentrations 7-fold higher than the K_t for biotin (fig. 2a); ^{3}H-biocytin uptake by rat duodenum was not affected when cold biotin was added to the incubation medium at concentrations as high as 12.5-fold higher than the K_t of biocytin (fig. 2b). These results suggest the existence of specific carriers for biotin in hamster and for biocytin in rat. Biotin uptake by hamster intestine was sodium-dependent; it was reduced when choline or potassium replaced sodium in the incubation medium. In contrast, this effect was not observed for biocytin in rat intestine (table 2).

How our results are related to the dietary history of the ferine ancestors of the laboratory hamster and rat we can only speculate. The hamster in the wild is mainly a grain eater, thus consuming predominantly protein-bound biotin. We have shown that it has

Table 2. Effect of sodium on the uptake of biotin and biocytin (nmol ml^{-1} cell water for 20 min)

Incubation medium	Hamster[1] (biotin)	Rat[2] (biocytin)
NaCl	10.88 ± 0.86 (6)	7.43 ± 0.73 (4)
KCl	9.06 ± 0.66 (7)	ND
Choline Cl	7.59 ± 0.36 (6)	6.97 ± 0.33 (4)

Values expressed as means ± SEM. Numbers in parentheses represent number of experiments. ND = Not determined.

[1] NaCl compared with KCl: $F = 3.34$; $p = 0.092$. NaCl compared with choline Cl: $F = 7.46$; $p = 0.02$.

[2] NaCl compared with choline Cl: $F = 0.39$; $p = 0.56$.

quite substantial pancreatic biotinidase activity, so most dietary biotin must be released in the intestinal lumen. A sodium-dependent, specific carrier for free biotin in the hamster intestine thus makes sense. Our results were obtained in intestinal rings and coincide with those of Spencer and Brody [8] who used the everted sac technique, Berger et al. [9] who exposed only the mucosal face to the biotin-containing medium, and Goré and Hoinard [10] who studied this vitamin uptake by hamster enterocytes. On the other hand, we observed only passive diffusion of biocytin in the hamster intestine, but this may be physiologically unimportant in this species if this compound is indeed hydrolysed to biotin by pancreatic biotinidase. This is the first report of biocytin transport in hamster intestine.

There was considerably less pancreatic biotinidase activity in the rat. This is an omnivorous, coprophagic animal and as such its diet probably contains more free biotin and more abundantly too. Free biotin is produced by the colonic flora and it may be nutritionally important in a coprophagic species. There have been conflicting reports regarding the mechanism of biotin uptake by rat intestine. Neither Spencer and Brody [8], Goré et al. [11] nor us, using such different experimental systems as everted sacs, enterocytes and intestinal rings, found evidence of carrier-mediated uptake but observed instead considerable passive diffusion. On the other hand, Said and Redha [12] and Bowman et al. [13], employing everted sacs and in vivo intestinal loops, reported results suggesting a carrier for biotin in rat intestine with a K_t of 3.7 and 9.6 μM, respectively. Further studies are needed to clarify this matter. With respect to biocytin, our results are consistent with a specific carrier-mediated uptake in the rat intestine, but its physiological significance is not clear because it was quantitatively minor compared to passive diffusion at the substrate concentrations studied and because it was not significantly influenced by sodium or temperature.

We studied the uptake of the vitamin in a range between 0.1 and 200 μM; physiological concentrations of biotin and biocytin in the intestinal lumen are not known, although Goré et al. [11] estimated that it is 0.6 μM for biotin in the rat. This concentration must have been quite variable in the ferine ancestors of the laboratory hamster and rat. Biotin is widely distributed in nature but is present in very small amounts in most food sources and its bioavailability is also very variable. Since its blood concentration is also quite low [approximately 0.01 μM, 2], it is possible that carrier-mediated transport of biotin and biocytin is particularly significant in the nanomolar range, where passive diffusion may not be sufficient to supply enough vitamin to the organism. After we had com-

pleted most of the present work, the paper by Dakshinamurti et al. [16] on biotin and biocytin transport in rat intestine using the everted sac technique came to our attention; they found saturation kinetics at the nanomolar range with a K_t of 0.017 μM both for biotin and for biocytin.

In summary, our results show that in the hamster, a herbivore with high pancreatic and low plasma biotinidase activities, there is an intestinal biotin-specific carrier, but not one for biocytin. In the rat, an omnivore, these results are reversed, biotinidase activity is low in pancreas but high in plasma, and there is an intestinal carrier for biocytin, although it is not clear if there is one for biotin. Thus dietary protein-bound biotin may be absorbed mainly in its free form in the former species but lysine-bound in the latter. These results may be physiologically significant when these animals are on a relatively high biotin diet.

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MECHANISMS OF BIOTIN UPTAKE BY BRUSH-BORDER MEMBRANE VESICLES OF HAMSTER ENTEROCYTES.

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INTRODUCTION

The vitamin biotin is a cofactor of carboxylases, enzymes involved in fatty acid synthesis, gluconeogenesis, citric acid cycle anaplerosis and aminoacid catabolism (1). Physiological concentrations of biotin in the intestinal lumen are not precisely known, although they have been estimated in the nanomolar range (2). Biotin is widely distributed in nature but is present in very small amonts in most food sources and its bioavailability is very variable (3). Since its blood concentration is also quite low (4), it is possible that carrier-mediated transport of biotin is particularly significant in the nanomolar range, where passive diffusion may not be sufficient to supply enough vitamin to the organism. However, most studies on intestinal biotin transport have been performed in the micromolar range. Furthermore, there are conflicting reports regarding the mechanisms responsible for it (5,6,7). Some of these difficulties may be associated with the complexity of the preparations used

for its study (everted sacs, intestinal rings, isolated enterocytes).

Brush border membrane vesicles (BBMV) are relatively simple structures which permit the study of transport without the interference of cellular metabolism; they facilitate the use of low vitamin concentrations and provide an easier manipulation of experimental variables.

We recently showed that in the hamster, a herbivore with high pancreatic and low plasma biotinidase activities, there is an intestinal biotin-specific carrier (7). Thus dietary protein-bound biotin may be absorbed mainly in its free form. However, since these results were obtained using biotin concentrations in the micromolar range, they may be significant only when the animals are on a relatively high biotin diet. Although several studies have been done on biotin intestinal transport in the laboratory hamster, none has employed BBMV.

We now report studies using hamster BBMV which show that there is a carrier-mediated, Na-dependent active transport process which operates at lower, more physiological nanomolar concentrations. We have employed a novel approach to estimate the different components of the intestinal uptake.

MATERIALS AND METHODS.

The brush border membrane vesicles were prepared by the calcium precipitation procedure (8).

Golden hamsters 100-180g were used, 4 to 6 in each experiment. Prior to their sacrifice they were allowed water and laboratory chow ad libitum. The animals were sacrificed by a chloroform overdose. Then the intestines were removed and flushed with ice-cold saline (0.9% NaCl,J.T.Baker de Mexico,Xalostoc,Mex.) solution containing 1 mM PMSF (phenyl-methyl-sulphonyl-fluoride,Sigma Chem.Co.,St.Louis Mo.). Intestines were cut into manageable pieces and everted . A glass microscope slide was used to scrape the cells from the intestinal mucosa. The mucosal scrapes were put in a glass beaker on ice, and 100 ml of Mannitol buffer consiting of 300 mM mannitol (J.T.Baker), 1mM dithiotreithol (Sigma Chem.Co.), 0.1 mM PMSF and 1mM Tris-Cl (E.Merck,Darmstadt,W.Germany) pH 7.0 was added.

The mucosa was homogenized with a Polytron (Kinematica, Kriens-Luzern) for 30 sec at setting 9 , and then spun at 500 x g. The supernatant was aspired into a chilled flask on ice and then made up to 250 ml with mannitol buffer containing 10mM CaCl2 (J.T.Baker). The solution was left in the cold room (4°C) under constant shaking for 45 min before it was centrifuged at 5700 x g x 5 min. The supernatant was then centrifuged at 37,500 x g x 45 min. The pellets were resuspended in a 300mM mannitol, 10mM Hepes (Sigma)-Tris pH 7.4 buffer and centrifuged at 43,140 x g x 60 min. The resulting pellets were resuspended in 0.5 ml of the aforementioned buffer.

The brush border marker enzyme, alkaline phosphatase (P-nitrophenyl-phosphatase E.C.3.1.3.1), was routinely assayed (9) to verify the purity of the preparations. The specific enzyme activity was enriched approximately 18-fold in the vesicles' suspension as compared to the crude intestinal mucosa homogenate.

Differences in biotin uptake between BBMV incubated with NaCl or KCl gradients, were not observed when the vesicles had been previously frozen (Data not shown). For this reason it was assumed that BBMV had lost their functional integrity and therefore a fresh new BBMV preparation was made for each experiment.The total protein concentrations of BBMV were assayed by the method of Lowry et al. using bovine .serum albumin as a standard.

Biotin uptake assays: 10 ul of the BBMV preparation were incubated at 37°C with 20 ul of incubation buffer which contains 150 mM NaCl, KCl, LiCl or Choline-Cl (depending on the assay purpose), 3H-Biotin 33 Ci/mmol (NEN Research Products, Boston, Mass.) enough to provide the desired vitamin concentrations (50-500 nM) and 20 mM Hepes-Tris pH 7.4.

At the end of the incubation time, 1 ml of ice cold quench solution containing 150 mM KCl, 12 uM cold biotin (Sigma Chem.Co.) and 10mM Hepes-Tris pH 7.4 was added to stop the reaction. Of this, 900 ul were filtrated under vacuum through a 0.45 um prewet nitrocellulose filter (Schleicher & Schuell,Dassel, W.Germany). The filters were washed with quench solution, removed and put into scintillation vials and disolved with 1 ml of butyl-

acetate. 10 ml of Bray scintillation cocktail were added and the biotin taken up by the BBMV was measured using a Tri-Carb 4000 liquid scintillation counter (Packard Instruments,Downers Grove,IL. USA).

In the experiments where the biotin uptake as a function of incubation time or biotin concentration were studied, the values obtained in the presence of a KCl gradient were substracted to those obtained with a NaCl gradient in order to evaluate only the sodium-dependent component of the vitamin uptake. The kinetic constants were calculated by the Lineweaver-Burk and Michaelis-Menten methods.

Effect of osmolarity on biotin uptake: BBMV physical and functional integrity as well as biotin binding to vesicles were assayed studying the 200 nM biotin uptake as a function of osmolarity (the desired osmolarities were obtained by varying the mannitol concentration in the incubation buffer.).The uptake values are expressed as a function of 1/Osmolarity.

Tripsinization of BBMV: BBMV were subjected to proteolysis with trypsin (Difco Laboratories,Detroit,Mich.U.S.A.) (1mg trypsin / 20 mg of BBMV protein) for different periods of time (1-30 min). Biotin (200 nM) uptake by these different-time trypsinised BBMV was then studied incubating them in the presence of NaCl or KCl gradients for 1 min as previously described. Results are expressed as a percentage of the NaCl control value (i.e. the vitamin uptake by non-previously trypsinised BBMV incubated in a Na gradient).

RESULTS

Effect of osmolarity.

The response of biotin uptake to changes in the medium osmolarity was studied to determine the BBMV integrity and to differentiate between binding of the substrate to membrane surface and transport into the intravesicular space (Fig 1). There was a linear relationship between reciprocal osmolarity and uptake, which demonstrate that BBMV are osmotically-responsive structures. Extrapolating the line to infinite osmolarity shows minimal uptake (intercept = 0.). These results indicate that at 1 min and under isotonic conditions, 78% of biotin taken up by BBMV is the result of transport into the intravesicular space and the remaining 22% represents binding to the membrane surface.

Effect of cation gradients on biotin uptake.

It was observed that biotin uptake was higher when the vesicles were incubated in a NaCl gradient (out > in) than when subjected to gradients of other cations such as K, Li or choline (Fig 2). The cation gradient also influenced the time course of biotin uptake. In the presence of a Na gradient the transport of biotin was rapid and linear during the first 40 sec of incubation with a distinct "overshoot" phenomenon. At this point biotin was accumulated in the vesicles to approximately 2 times the equilibrium value. After that, accumulation of biotin decreased reaching the equilibrium value at ca. 180 sec of incubation indicating efflux from vesicles. In the presence of a K gradient

the initial rate of biotin transport was slower, no overshoot was observed, and equilibrium was reached after 3 min of incubation (fig 3). It is apparent that biotin is accumulated in the vesicles against a chemical gradient (intravesicular/extravesicular) by a Na-stimulated mecchanism.

Kinetics of Na-dependent biotin transport.

To determine the kinetic parameters of biotin uptake by BBMV, these were incubated in media which contained different vitamin concentrations (50-500 nM) and with either Na or K gradients. In the latter there was a linear relationship between uptake and biotin concentration, whereas in the Na medium this relation was hyperbolic, thus indicating saturation kinetics. Figure 4 depicts these results after substracting those in K from the ones observed in Na gradients. Na-dependent biotin uptake in hamster BBMV is therefore carrier-mediated with an apparent Kt and Vmax of 106.4 nM and 0.1302 pmol min^{-1} mg^{-1} respectively.

Trypsin pretreatment of BBMV

In order to explore the nature of the biotin carrier, 1 min uptake was measured after the vesicles were treated with trypsin during varying times. Na-dependent uptake was markedly reduced by trypsinization (Fig. 5). Furthermore, this treatment abolished the overshoot phenomenon (Fig. 6), indicating the protein nature of the carrier-mediated, Na-dependent active transport.

Since uptake in K gradient --where no evidence was found of a fuctional carrier-- was also diminished after trypsinization, although to a much lesser extent, part of the "total" uptake (i.e., uptake in Na gradient without trypsinization) is probably the result of binding of biotin to proteins on the vesicles surface, not transported to their interior. This binding corresponds to approximately 16% of total uptake; therefore 84% of total uptake is transported into the intravesicular space.

Both in Na and in K gradients, trypsin pretreatment diminished biotin uptake to about 30% of the total (Fig. 5); most of it likely represents passive diffusion, since active transport and non specific binding have been already accounted for. Assuming this is so, of the 84% of total uptake which is internalized, 64% is by active transport and 36% via passive diffusion.

DISCUSSION

Biotin intestinal transport has been studied since Turner and Hughes published their results in 1961; many of these studies have also been done in hamsters, using complex systems such as everted intestinal sacs or intestinal rings. This is the first time that biotin uptake has been studied in hamster BBMV; this technique makes it possible to study transport in preparations containing only the cellular entity responsible for this phenomenon and lacking cellular metabolism of the transported vitamin. Furthermore, these vesicles allow the study of uptake at lower concentrations; previous studies have used biotin concentrations in the micromolar range.

Although widely distributed in nature, biotin is present in very low amounts in most foods; Goré (2) has estimated about 600 nanomolar its concentration in the rat intestinal lumen. Its plasma concentration in mammals, including the rat and the human, is near 10 nanomolar. We have now shown that biotin uptake is a high affinity process, with a Kt of 106 nM, only one order of magnitude above the normal plasma concentration of the vitamin.

There are conflicting reports concerning the mechanism of biotin uptake in the hamster intestine. Berger and Semenza (11), with a preparation exposing the mucosa to the incubation medium, concluded that it is an active transport process. On the other

hand, Goré (6) proposed facilitated diffusion to account for his results on hamster enterocytes. Our observation of an overshoot shows that biotin uptake in hamster BBMV is to a large extent an active process, driving the vitamin against a concentration gradient. Its sensitivity to trypsin pretreatment further attests its existence. Transport of the vitamin from the epithelium to the bloodstream across the basolateral membrane, on the other hand, seems to be a facilitated diffusion process (12).

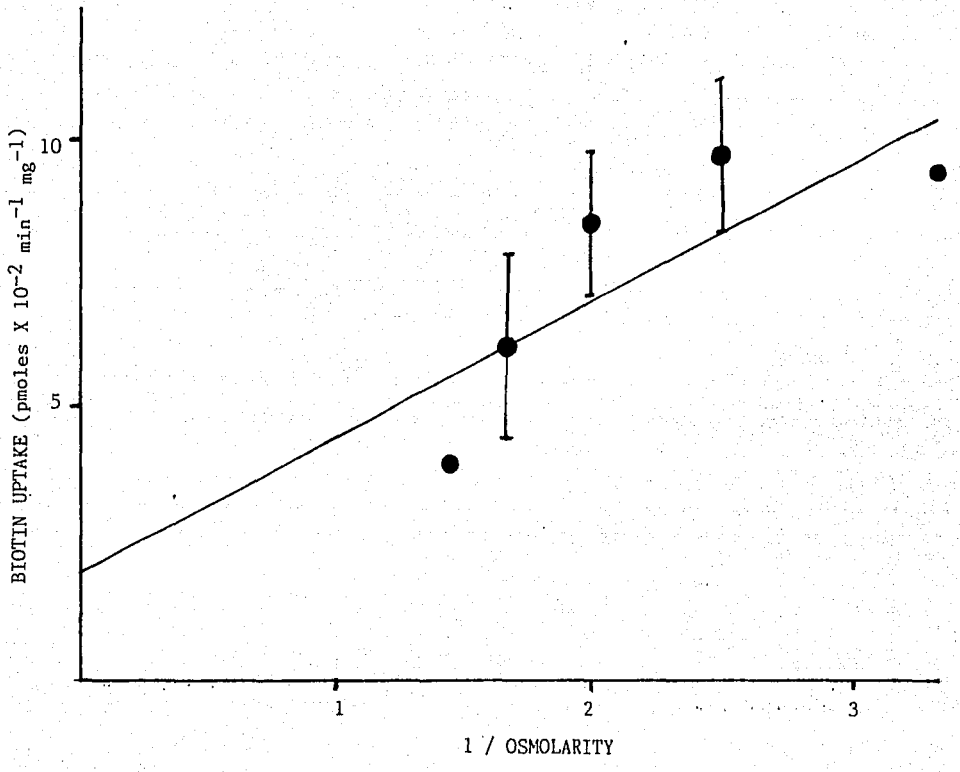
Active transport seems to be the most important component in the intestinal uptake of biotin, compared to passive diffusion or to mere surface binding. We could estimate binding by two independent approaches: uptake extrapolated at infinite osmolarity (Figure 1) and uptake in K gradient sensitive to trypsin (Fig. 5). These results are similar, 20 and 16% of total uptake, respectively. Therefore only about 80% of biotin taken up by BBMV is actually internalized. Trypsin pretreatment of BBMV allows to estimate how much of it is actively transported (64%) and how much passively diffuses into the intravesicular space (36%).

The amount of vitamin bound to the vesicles surface increased significantly the uptake values and must be taken into account whenever the actual quantity of vitamin that is transported to the intravesicular space is to be determined. Estimation of the uptake components from the results of trypsin pretreatment of

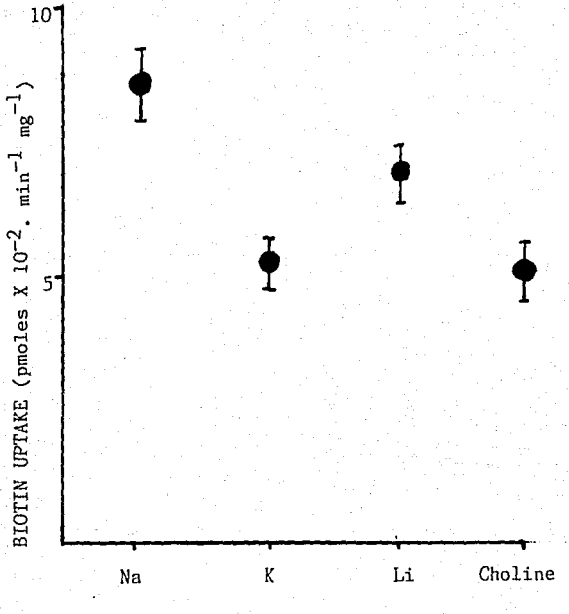
BBMV as described above is a useful approach; to our knowledge it has not been used previously.

Our results also confirm the Na dependence of biotin transport, which has been observed by several authors using such diverse preparations as everted sacs (5), exposed intestinal mucosa (11) and intestinal rings (7) and which was not found by Goré using isolated enterocytes (6). It may be that sodium, moving down its electrochemical gradient, provides the energy to move biotin against its own chemical gradient. In vivo, the Na-K ATPase pump, which is located in the basolateral membrane of the enterocytes, would mantain the sodium gradient across the cellular membrane; the gradient would then propell the vitamin transport across the intestinal epithelium.

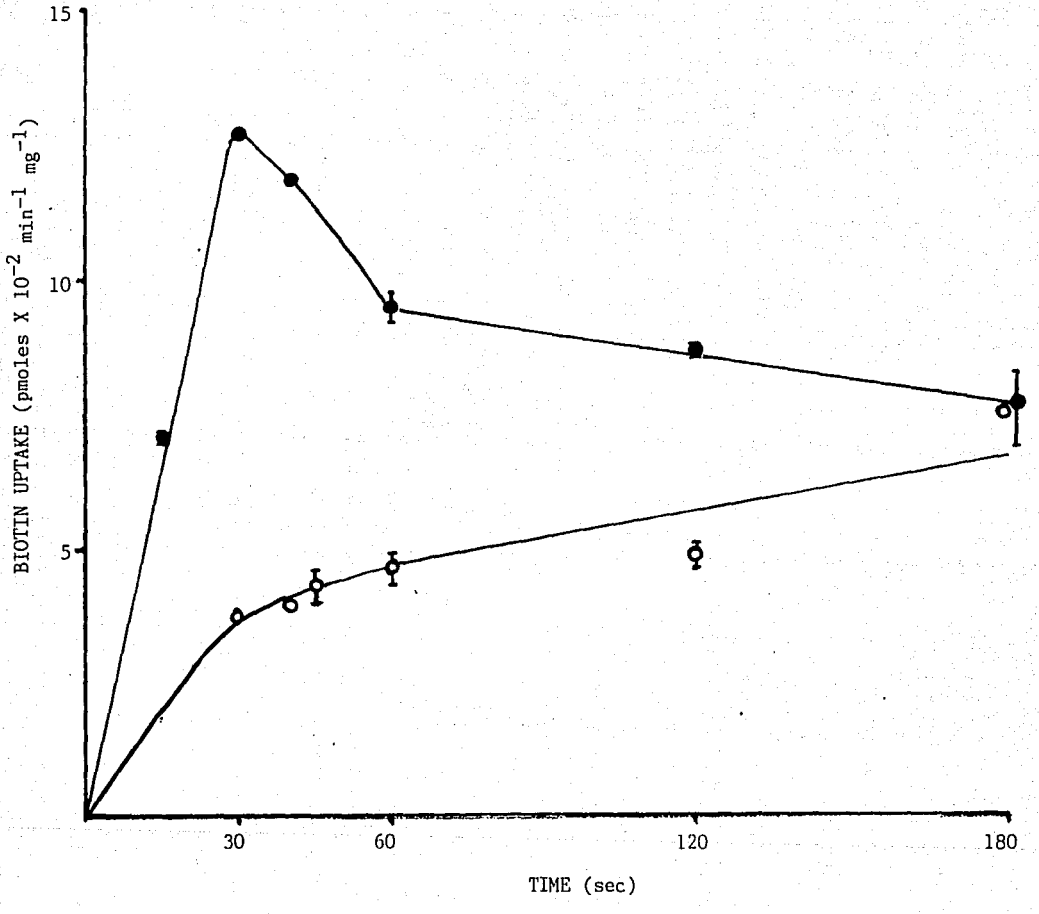
It has been suggested that biotinidase may have a role in the intestinal transport of biotin (13). We assayed biotinidase in BBMV and found it to be below detection limits (data not shown).



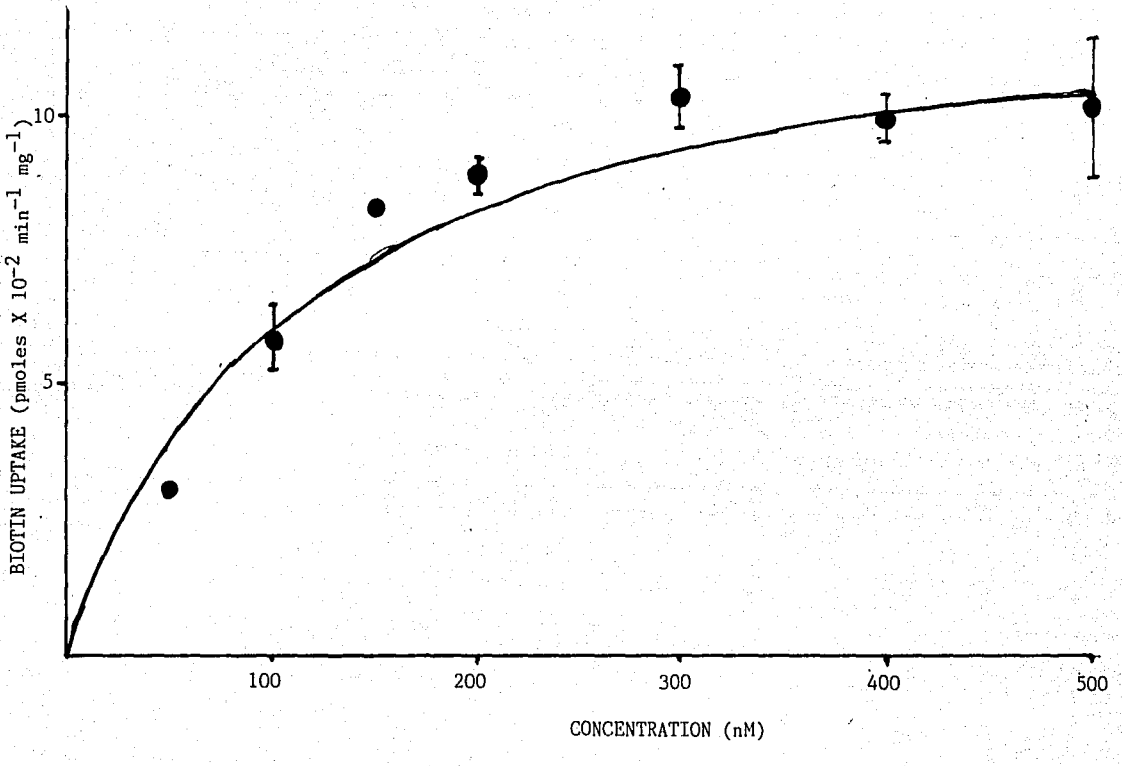
(Fig, 1) Effect of incubation medium osmolarity on biotin uptake by hamster BBMV.



(Fig, 2) Effect of different cation gradients on biotin uptake by BBMV



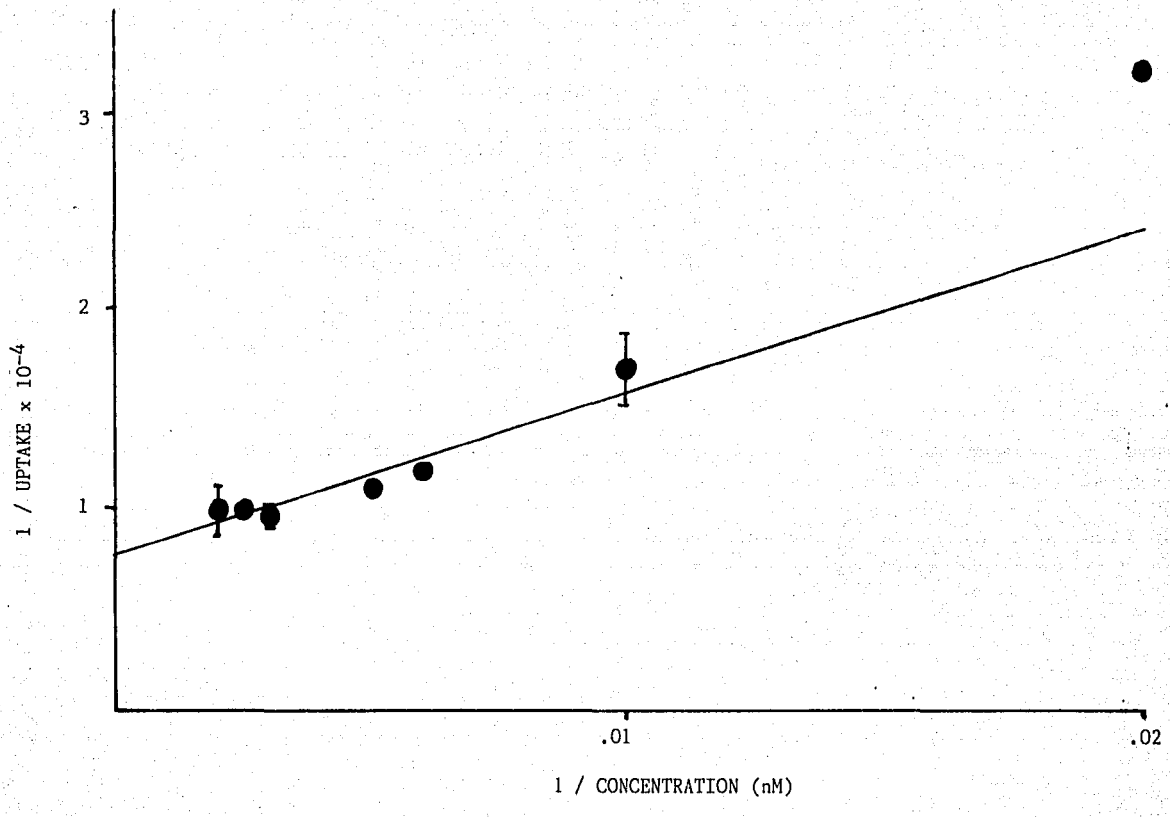
(Fig, 3) Transport of biotin as a function of time in hamster BBMV in the presence of a Na gradient●, or a K gradient ○, (Out In)



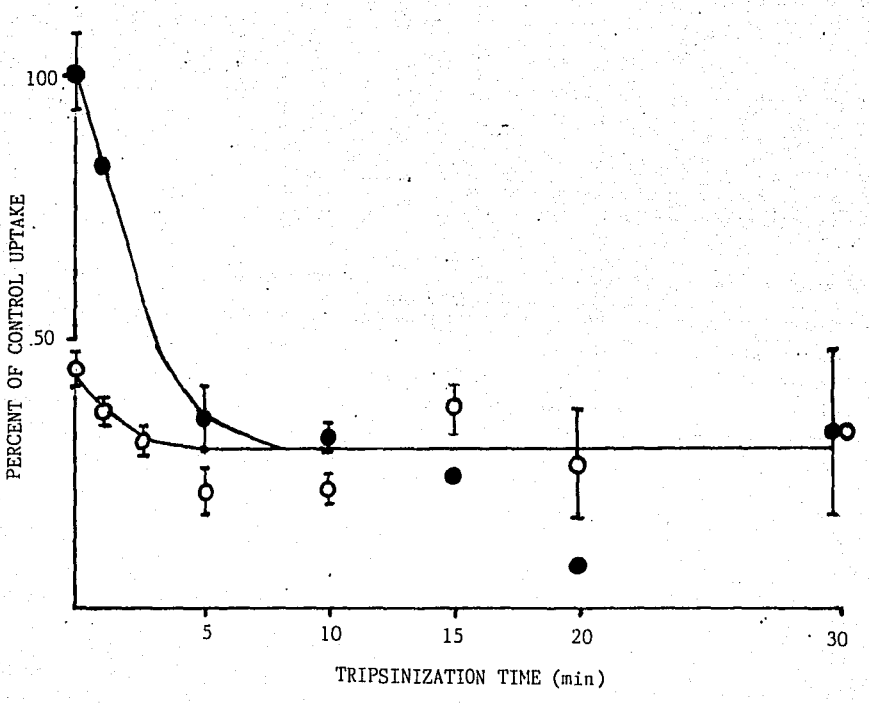
(Fig, 4) Biotin uptake by BBMV as a function of concentration

NaCl

KCl



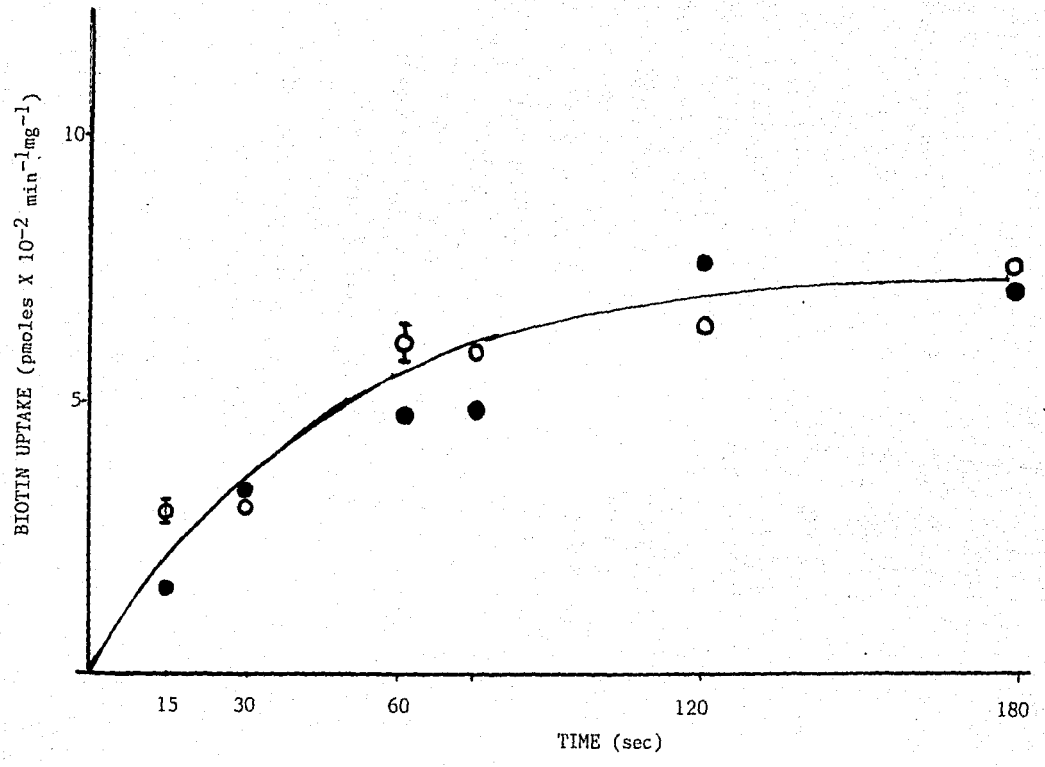
(Fig, 4B) Lineweaver-Burk plot



(Fig, 5) Effect of tripsin pretreatment on biotin uptake by BBMV

● NaCl

○ KCl



(Fig, 6) Effect of tripsin pretreatment on time-course of biotin uptake

● NaCl

○ KCl

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DISCUSION GENERAL

Los resultados obtenidos sugieren la existencia de una relación entre los mecanismos de liberación y absorción intestinal de biotina. En el hámster, especie que posee elevada actividad de biotinidasa pancreática, existe un acarreador específico para biotina pero no para biocitina, mientras que en la rata , en la que la actividad de biotinidasa es mayor en plasma que en páncreas, existe un acarreador para biocitina pero no para biotina.

La alimentación natural del hámster está constituída principalmente por granos y semillas, por lo que es probable que consuma predominantemente biotina unida a proteínas (17). Si aceptamos que la actividad de biotinidasa pancreática es un buen indicador de la capacidad de un organismo para liberar biotina, entonces en el hámster la mayor parte de la vitamina contenida en la dieta sería liberada en el lumen intestinal. Este hecho haría entonces razonable la idea de la existencia de un acarreador específico para biotina.

Los resultados obtenidos de los experimentos con anillos evertidos de intestino de hámster coinciden con los resultados de Spencer y Brody (8) en los que se utilizaron sacos evertidos de intestino, y con los de Berger et al. (10) quienes estudiaron

el transporte de biotina exponiendo únicamente la mucosa intestinal al medio de incubación.

Por otro lado, en la rata, un omnívoro coprofágico cuya dieta probablemente contiene mayores cantidades de biotina y que posee una mucho menor actividad de biotinidasa pancreática, la vitamina sería absorbida como biocitina y posteriormente liberada en el plasma o en otros tejidos, donde se ha encontrado actividad de biotinidasa. Cabe hacer notar que ni Spencer y Brody (7), Goré et al (11), ni nosotros (18) estudiando la captación de la vitamina en el orden uM hallamos evidencia de un acarreador de biotina en intestino de rata. Sin embargo, recintemente Dakshinamurti et al. (13)utilizando sacos evertidos de intestino delgado, reporta un transporte saturable en el orden nanomolar para biotina y biocitina con una Kt de 0.017 uM para ambos compuestos.

A partir de nuestros resultados parece que los acarreadores de biotina en hámster y de biocitina en rata son entidades independientes y no se trata de un acarreador común. Ambos acarreadores se ven afectados por la temperatura; sin embargo, el transporte de biotina es estimulado por sodio mientras que el de biocitina no es afectado por la composición iónica del medio de incubación. La identidad de ambos acarreadores fué posteriormente confirmada cuando se observó que no existe competencia significativa de ambas especies moleculares de la

vitamina por sus respectivos acarreadores, al menos en las concentraciones estudiadas.

Utilizando el método de anillos evertidos de intestino delgado y estudiando la captación de biotina y biocitina en concentraciones de 0.1 a 200 uM, se encontró que un importante porcentaje de la captación total, se debe a un proceso no saturable, lo que nos hizo cuestionarnos acerca de la importancia fisiológica del acarreador.

La concentración de biotina y biocitina en el lumen intestinal no se conoce, aunque Goré (11) calcula que es de alrededor de 600 nM para biotina en la rata. Ya que la concentración de biotina en sangre es también muy baja (0.01 uM) esto hace suponer que el transporte mediado por acarreador de biotina o biocitina es particularmente importante en el orden nanomolar, cuando la difusión simple no pudiera suministrar suficiente vitamina al organismo.

Con objeto de estudiar lo anterior y caracterizar al acarreador, se estudió la captación de biotina en concentraciones del orden nanomolar (50-1000 nM), por vesículas de membrana de borde estriado de enterocitos de hámster. Esta técnica hace posible estudiar el proceso de transporte a través de membranas sin la interferencia del metabolismo celular.

Al eliminar el acarreador de la vitamina tratando a las vesículas con tripsina se determinó que de la captación total de

biotina, aproximadamente el 60% es debida a un transporte mediado por acarreador, dependiente de sodio, 20% a difusión simple y el restante 20% es la cantidad de vitamina unida a componentes de la membrana sensibles a tripsina. De los tres componentes de la captación de biotina únicamente el transporte mediado por acarreador y la difusión simple podrían contribuir al suministro intracelular de la vitamina, siendo el acarreador el principal responsable del transporte de biotina al menos in vitro.

Nuestros datos confirman que el transporte de biotina en hámster es mediado por un acarreador que depende de un gradiente transmembranal (fuera>dentro) de sodio. Este acarreador es capaz de concentrar biotina en contra de su gradiente químico por lo que el sistema de transporte puede clasificarse como activo.
Nuestras observaciones refutan el modelo presentado por Goré (9), que afirma que el transporte de biotina en hámster es debido a un proceso de difusión facilitada y nos permiten proponer un modelo que explica el transporte de biotina en intestino de hámster.

La bomba de Na-K localizada en la membrana basolateral de los enterocitos mantiene un gradiente transmembranal de Na. Este catión, al desplazarse a favor de su gradiente electroquímico a través de la membrana celular, proveería la energía necesaria

para impulsar el transporte de biotina mediado por un acarreador localizado únicamente en la membrana de borde estriado.
El transporte intestinal de biotina en hámster contrastaría con el de biocitina en intestino de rata, el cual, según la evidencia presentada en la primera parte de este trabajo, parece llevarse a cabo por un proceso de difusión facilitada independiente de Na.

Es necesario realizar experimentos de captación de biocitina en el orden nanomolar por vesículas de enterocitos de rata para comprobar lo anterior así como para asegurar que no existe un acarreador de biotina en esta especie que funcione en concentraciones menores a las estudiadas empleando anillos evertidos de intestino delgado.

Nuestros resultados evidencian la existencia de un acarreador de biotina adaptado para funcionar en concentraciones de biotina del orden nanomolar. La Kt calculada (106 nM) es de solo un orden de magnitud mayor a la concentración de la vitamina en plasma. Es necesario hacer notar que al menos en hámster, la presencia de un acarreador intestinal de biotina capaz de transportar a la vitamina en contra de su gradiente de concentración crearía las condiciones ideales para que una vez dentro del enterocito, la biotina alcanzara la sangre por un proceso de difusión facilitada como fué sugerido por Said et al. (19)

Hasta donde sabemos esta es la primera vez que se utiliza el sistema de vesiculas de borde estriado de enterocitos para estudiar fenómenos de transporte intestinal en hámster. De igual modo, esta es la primera vez que se trata de dar una explicación a las diferencias interespecificas que han aparecido en la literatura con respecto a los mecanismos de transporte intestinal de biotina.

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