Biochimica et Biophysica Acta, 1105 (1992) 271–277

BBAMEM 75585

Age-related thiamin transport by small intestinal microvillous vesicles of rat

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(Received 26 November 1991)

Key words: Aging; Thiamin; Intestinal transport; Microvillous membrane vesicle; (Rat)

The effect of aging on the intestinal transport of thiamin was studied using small intestinal microvillous vesicles prepared from groups of rats aged 1, 2, 6, 12 and 24 months, respectively. The vesicles (enrichment 14.6–17.8-fold) were incubated with 0.125 to 12.5 μM tritiated thiamin and the radioactivity taken up was measured radiometrically after rapid filtration. The time course and cumulative uptake curves of thiamin and the inhibiting potency of the thiamin structural analogs pyrithiamin, amprolium and oxythiamin on the saturable component of thiamin transport were determined. The vesicle diameter was measured by using a computerized morphometric procedure, and found to be decreased in aged rats. The K_m and J_{max} values of the saturable component of transport increased with increasing age, the difference with younger groups being statistically significant at 24 and 12 months. The inhibitory potencies of pyrithiamin and amprolium gradually decreased with increasing age, while oxythiamin was devoid of significant inhibitory activity. Passive permeability coefficients decreased with increasing age, reaching their lowest value at 24 months. These results show that aging is associated with intrinsic alterations of the enterocytic plasma membrane resulting in a decrease of the affinity for thiamin, associated with a faster rate of the saturable component of thiamin transport, and with a significant depression of the non-saturable component.

Introduction

Aging is associated with several functional and morphological changes in the gastrointestinal tract [1]. These include modifications in the intestinal absorption of different substrates, and alterations in the composition and fluidity of the brush border membranes [2], which can influence their function. The intestinal absorption of thiamin also appears to be modified by aging, but the results reported in the literature are controversial. Some authors described a reduction in thiamin absorption in man [3,4] and rat [5,6], while others did not find any modification [7,8]. These studies were performed in vivo in the intact rat, a situation which does not allow to exclude the effect of metabolic and circulatory changes induced by age. In addition, the doses of thiamin used in the experiments mentioned above were rather high (> 2 μM), within a range in which thiamin intestinal transport is predominantly a diffusion process [9,10].

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The aim of the present investigation was to assess whether aging can modify the intestinal absorption of thiamin by inducing intrinsic alterations of the enterocytic plasma membrane. Experiments were carried out in vitro by using brush border membrane vesicles of rat small intestine, a well known preparation which is capable of transporting efficiently substances in the absence of metabolic and circulatory influences. We have previously found that thiamin transport in this preparation is a membrane process accounted for by a dual mechanism, which is saturable at thiamin concentrations lower than approx. 2 μM, and non-saturable at higher concentrations [9,10].

Rats of different ages ranging from 1 to 24 months were used to evaluate: (1) the diameter of the microvillous vesicles, as an index of the physical characteristics of the brush border membrane; (2) the time course and the rate of thiamin transport in the vesicles; (3) the apparent kinetic constants K_m and J_{max} of the saturable transport component and the inhibitory potency of the structural analogs pyrithiamin, amprolium and oxythiamin; (4) the apparent passive permeability coefficients (K_d) of the non-saturable component.

A preliminary report of some data has been published (Gastaldi et al. [11]).

Materials and Methods

Animals

Male albino rats of the Wistar strain, reared on the standard complete diet of Randoin and Causeret [12], which contained 12 μg/g of thiamin, were used. The animals were divided into five age groups, which were classified as: very young (1 months of age, weighing 80.6 ± 2.8 g); young (2 months of age, weighing 244.8 ± 7.6 g); adult (6 months of age, weighing 466.0 ± 11.2 g); mature (12 months of age, weighing 557.0 ± 8.2 g), old (24 months of age, weighing 525.0 ± 10.9 g). This classification resembles that proposed by Esposito et al. [13].

Preparation of vesicles

The animals were killed by decapitation after about 12 h fasting with water ad libitum. The small intestinal microvillous vesicles were prepared at 0–4°C by using the Ca^{2+}-precipitation method of Kessler et al. [14] with minor modifications. The purity of microvillous membranes was estimated by assessing the degree of enrichment of individual preparations expressed as the increment in saccharase activity [15] of the final preparation with respect to the initial mucosal homogenate. Protein content was determined according to Lowry et al. [16], using bovine serum albumin as standard. Spectrophotometric measurements were carried out by using a Beckman DU5 spectrophotometer (Irvine, CA, USA).

Uptake determination

In the experiments designed to evaluate the time course of thiamin uptake, the vesicles were incubated at 25°C with 0.25 μM [^{3}H]thiamin (750 mCi/mmol, Amersham International p.l.c., Amersham, UK), for time intervals ranging from 30 s to 30 min.

For the evaluation of K_m and J_{max}, the vesicles were incubated for 4 s at 25°C with [^{3}H]thiamin at concentrations ranging from 0.25 to 12.5 μM. In inhibition experiments, the structural analogs of thiamin were added initially at concentration 10 times as high as that of thiamin (0.5 μm), and the incubation was carried out for 30 s at 25°C. In these experiments, the non-saturable component was evaluated by incubating the vesicles at 0°C. A STRUMA short-time incubation apparatus (Innovativ-Labor AG, Adliswil, Switzerland) was used to measure rates of reaction according to the following procedure. 20 μl of vesicular suspension were mixed with 20 μl of incubation medium containing (mM, final concentrations): 100, D-mannitol; 2, $MgSO_4$; 10, Tris-Hepes (pH 7.5); 100, NaCl and [^{3}H]thiamin at the selected concentration. The reaction was stopped at the fixed times by adding 1.5 ml of cold (approximately 3°C) 'stopping' solution containing (mM, final concentrations): 150, NaCl and 1, Tris-Hepes (pH 7.5). The amount of labelled thiamin taken up by the vesicles was measured by a rapid filtration procedure, using cellulose nitrate microfilters (Microfiltration System, Dublin, CA, USA) with 0.65 μm pore diameter, previously saturated with unlabelled thiamin [9]. The radioactivity of labelled thiamin non-specifically adsorbed on the microfilter was evaluated in each experiment by using appropriate blanks, the values of which were subtracted from the total radioactivity retained on the filter. All radioactivity measurements were carried out by using a Packard Tri-Carb 2000 CA Analyzer (Packard Instrument Co., Inc., Downer Grove, IL, USA).

Concentration curves

Plots of thiamin uptake versus thiamin concentrations (concentration curves) were obtained by fitting the experimental points by computerized least-squares regression. The saturable and non-saturable components were separated from cumulative curves by using the graphical procedure described by Gastaldi et al. [17], while the respective K_m and J_{max} of the saturable component and the K_d constant of the non-saturable component were calculated by using the computerized Graphpad program (ISI, 1987).

Morphometric analysis

For morphometric analysis, a vesicle suspension was mixed with fixative solution containing 3% (v/v) distilled glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.35). After 30 min prefixation, thin pellets were obtained by centrifugation at 100000 × g for 60 min and left in the same solution for 2 h. Pellets were then cut into tiny blocks, washed and stabilized osmotically in 0.1 M Na-cacodylate buffer (pH 7.35) containing 2.5% (w/v) saccharose (315 mosmol/kg H_2O). Postfixation was carried out in 1.33% (w/v) OsO_4 s-collidine (Merck, Darmstadt, Germany) buffered solution (pH 7.35). The tiny blocks were subsequently processed according to conventional electron microscopy technique. Electron microscopic observations and micrographs were made by using a Zeiss EM 109 transmission electron microscope. Interactive/automated morphometric analysis was carried out by using a Zeiss-Kontron IBAS 2 computerized image analyzer, provided with a TV camera stand for the input of images directly from negative films, on negative pictures (56 × 72 mm^2) of ultrathin sections obtained at 20000 × electron magnification. Particular care was taken in the selection of the vesicles to be used for morphometric analysis. The following selection criteria were used, as recommended by Hunter et al. [18]: (a) the vesicle membrane must be clearly visible around the whole vesicular perimeter, (b) vesicles must appear as single

structures, without a dense core and devoid of any smaller vesicle inside. The morphometric parameters determined were the diameter D (nm) of the area-equivalent circle and the shape factor (D_{min}/D_{max}), in order to assess the 'sphericity' of the vesicles and to calculate the corresponding volume. Further details about the procedures used for sample preparation and morphometric analysis have been described by Gastaldi et al. [19].

Statistics

Significance of the differences among kinetic constants, among inhibition percentages and among vesicular diameters was tested by analysis of variance followed by Newman-Keuls's Q-test [20].

Materials

All reagents were of analytical grade and supplied by Sigma Chemical Co. (St. Louis, MO, USA) and BDH Ltd. (Poole, UK). Pyrithiamin bromide hydrobromide and 4'-oxythiamin chloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Amprolium was obtained from Merck, Sharp and Dohme (Pavia, Italy).

Results

The enrichment in brush border membranes in rats from different age groups ranged from 14.6- to 17.8-fold, without any statistically significant difference among the various groups. Examination of morphometric parameters (Table I) showed that the vesicles were consistently spherical, their shape factors being approximately 1. Vesicle diameters and volumes showed a progressive and significant decrease with increasing age. The smallest diameter, which was observed in old rats (24 months of age), was 69% of the value observed in very young animals (1 month of age).

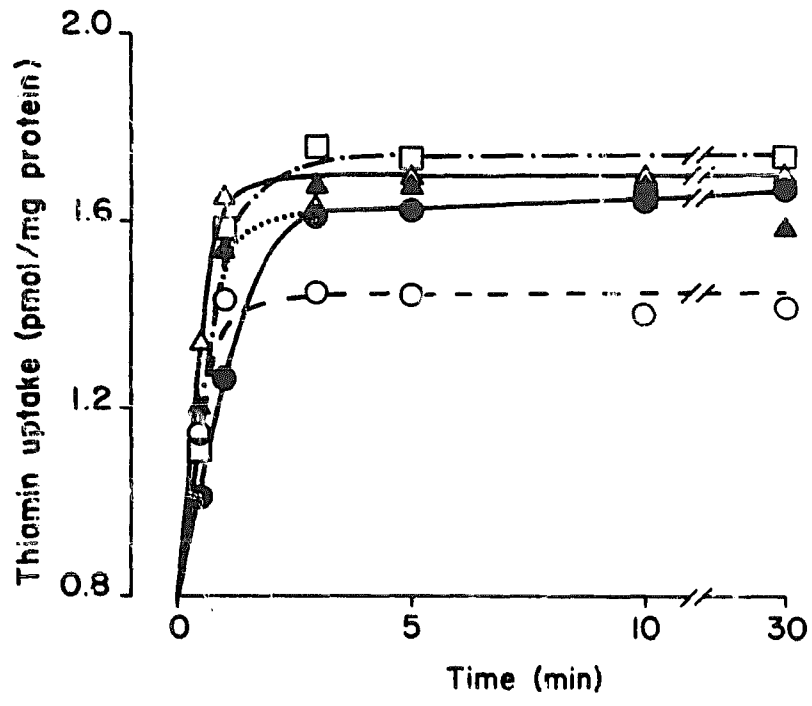


Fig. 1. Time course of thiamin uptake by small intestinal microvillous vesicles from rats of different ages. Uptake was measured in the presence of 0.25 μM [^{3}H]thiamin (specific activity, 750 mCi/mmol) and of an initial gradient of 100 mM NaCl (out). Incubation medium (mM, final concentration): 100 D-mannitol; 2 $MgSO_4$; 10-Tris-Hepes (pH 7.5). Symbols represent the means of determinations (in replicates of at least 3) for five different preparations, each including intestinal mucosa from 2–6 rats, according to age. S.E. values (omitted for clarity) were within 10%. Age (months): ●, 1; ▲, 2; □, 6; ○, 12; △, 24.

The time-course profiles of thiamin uptake by vesicles were similar for all age groups (Fig. 1). In spite of the differences in vesicle diameters, no statistically significant difference was found among the various curves.

The profiles of the concentration curves for the cumulative uptake of thiamin were qualitatively similar for the vesicles of all age groups, but the uptake rates differed according to age, being lowest in very young rats (1 month of age) (Fig. 2 and upper portion A). Each of the curves could be resolved into a linear and

TABLE I

Morphometric parameters of small intestinal microvillous vesicles from rats of different ages

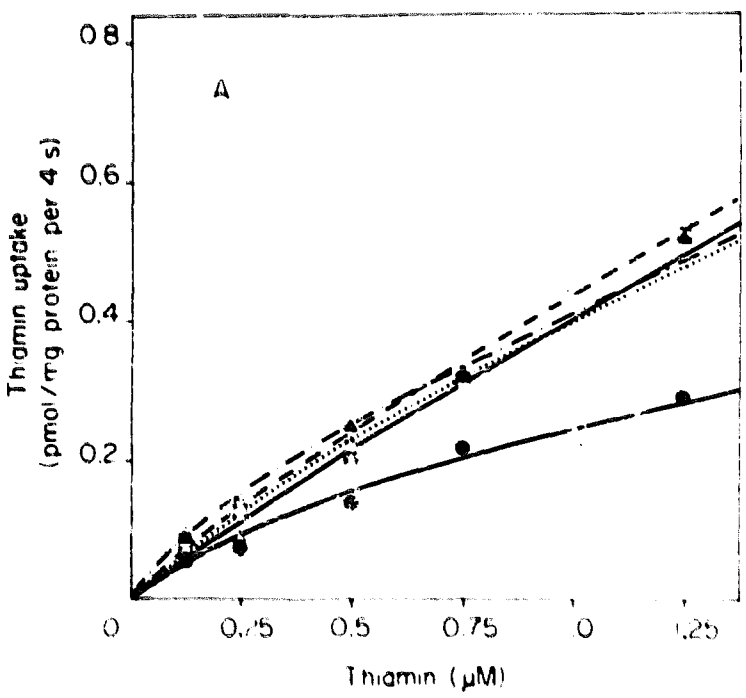
Data represent means ± S.E.

Age (months)	Microvillous vesicles				
	Number	Diameter [a,b] (nm)	Shape factor [c,d]	Area [d] (nm^2)	Volume [d] (nm^3)
1	58	114.54 ± 2.09 [e]	0.895	41216	786811
2	74	116.31 ± 1.93 [e]	0.917	42499	823853
6	66	89.69 ± 1.95 [f]	0.884	25272	377773
12	110	84.14 ± 2.04	0.879	22241	311893
24	110	79.71 ± 1.14	0.886	19961	265178

[a] Diameter of the area-equivalent circle.
[b] Value measured directly with an automated system (see Materials and methods).
[c] Ratio between the smaller and the larger diameter.
[d] Calculated value.
[e] $P \leq 0.05$ versus 6, 12 and 24 months. Post-variance test: Newman-Keuls's Q test [20].
[f] $P \leq 0.05$ versus 12 and 24 months. Post-variance test: Newman-Keuls's Q test [20].

a non-linear component, as indicated above [17]. This confirmed that, irrespectively of age, a dual mechanism is involved in thiamin membrane transport: a saturable component at low (< 2 μM), physiological concentrations, and a non-saturable component at higher concentrations. All the concentration curves of the saturable component showed a clearly hyperbolic course, transport rates being higher in the older groups (rats aged 12 and 24 months) (Fig. 3 and upper portion A).

For the calculation of the apparent kinetic constants (K_m and J_{max}), 4 s was chosen as the earliest time point which could be reliably used in the concentration curves in all age groups. To validate the use of this time point for this purpose, we had established in previous experiments that the time courses are reasonably linear for up to at least 6 s in vesicles from all age groups, and that the extrapolation of the curves to zero



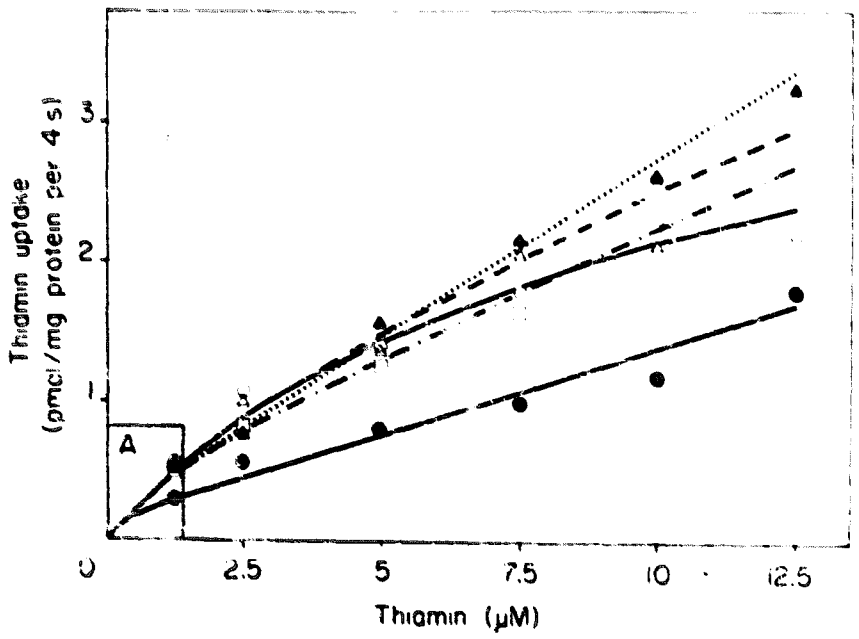
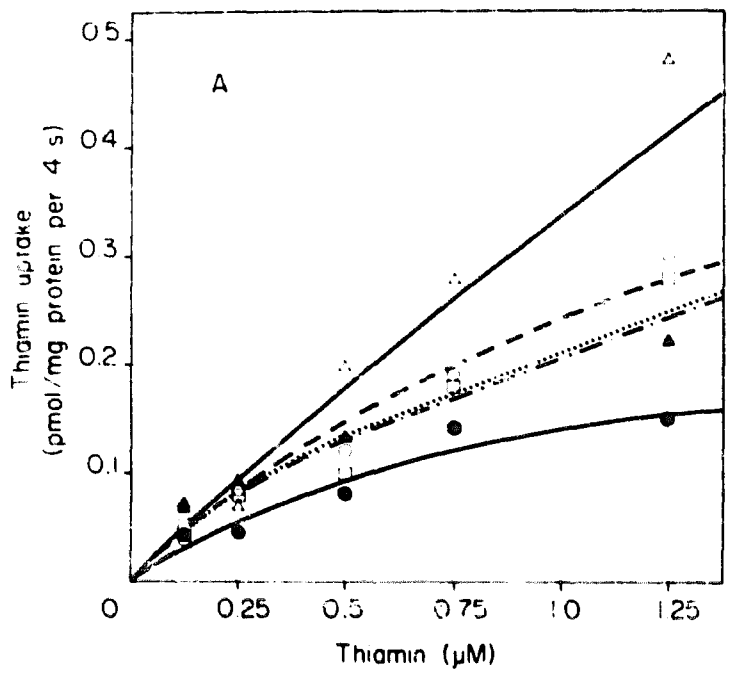


Fig. 2. Cumulative uptake of thiamin by small intestinal microvillous vesicles from rats of different ages. Uptake was measured after 4 s incubation with different thiamin concentrations. The initial part of the curves (A), which refers to physiological concentrations, is shown in detail with expanded scale in the upper portion of the figure. Incubation medium, number of determinations and symbols as in Fig. 1. S.E. values averaged 12% of the mean.



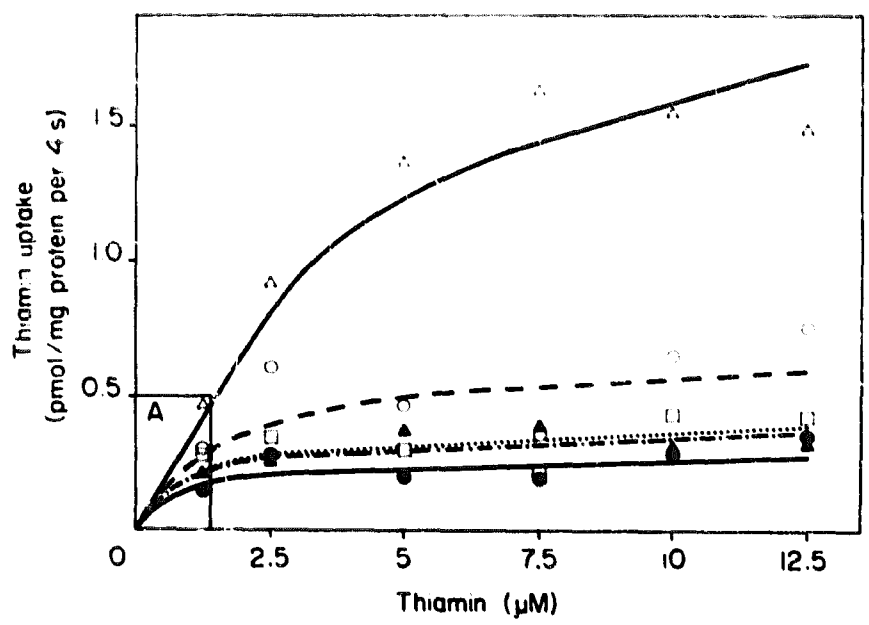


Fig. 3. Saturable component of thiamin uptake by small intestinal microvillous vesicles from rats of different ages. The initial part of the curves (A), which refers to physiological concentrations, is shown in detail with expanded scale in upper portion of the figure. The plot was obtained graphically from the cumulative uptake curve shown in Fig. 2. Symbols as in Fig. 1.

time goes through the origin (data not shown). Under these conditions, the apparent kinetic constants of the saturable component were found to increase with increasing age. However, the differences were statistically significant only for the mature (12-month old) and old (24 months) animals as compared to the other groups. A statistically significant difference was also found between the old and the mature groups (Fig. 4, A and B).

Among the thiamin structural analogs tested, pyrithiamin and amprolium were found to be active in inhibiting the saturable component of thiamin uptake, whereas oxythiamin was virtually inactive (Fig. 5). The inhibitory potency of each of the active analogs decreased significantly during aging and reached in 24-month old rats values which were 35% (for pyrithiamin) and 20% (for amprolium) of those found in very young animals. No statistically significant differences, however, were found between the activity of pyrithi-

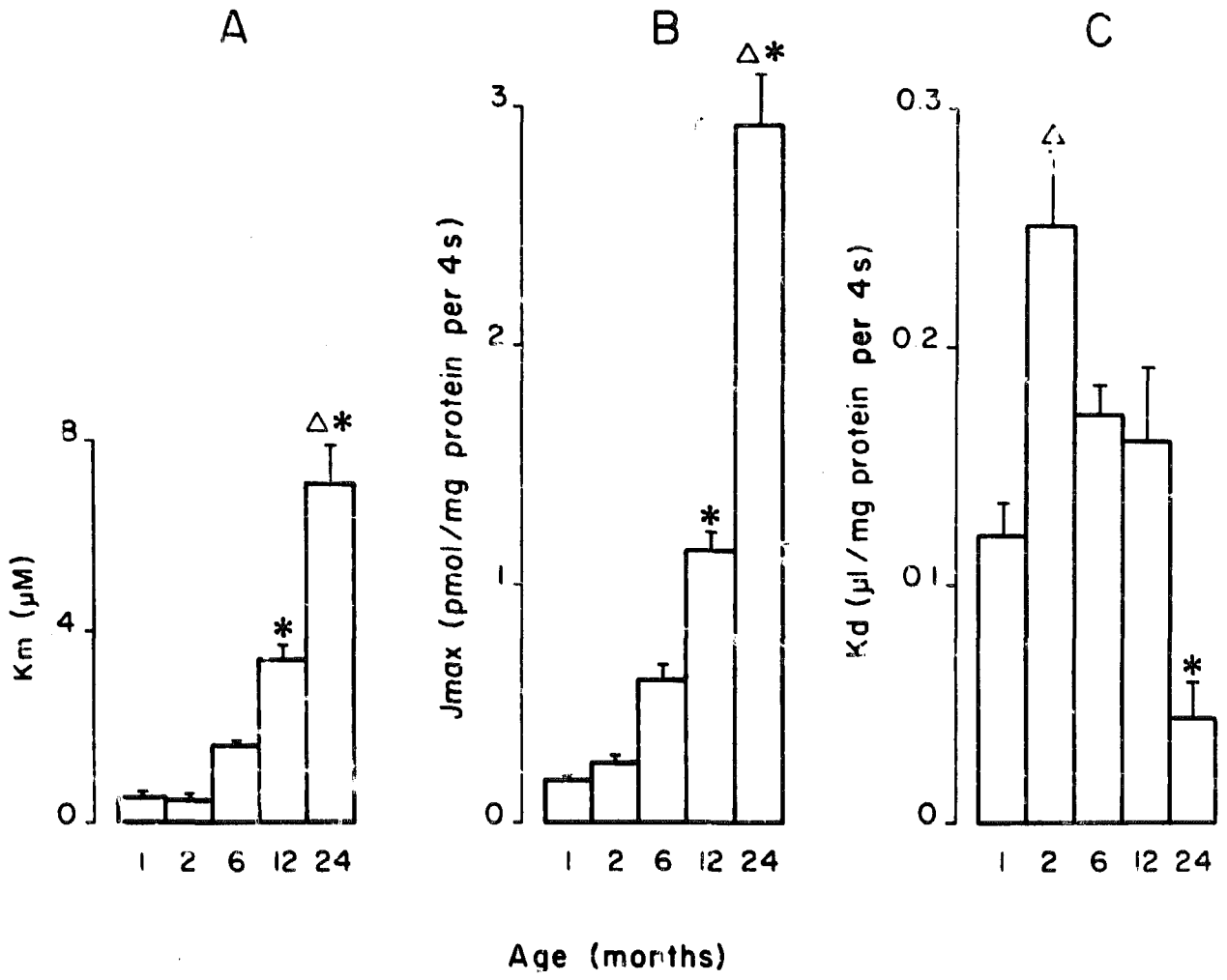


Fig. 4. Kinetic parameters of thiamin uptake by small intestinal microvillous vesicles from rats of different ages. A and B, K_m and J_{max} values of the saturable component; C, K_d values of the non-saturable component. The apparent kinetic constants were calculated from the data shown in Fig. 2 by using the computerized Graphpad program (ISI, 1987). Bars represent means ± S.E. In A and B: *, $P \leq 0.05$ vs. 1-, 2-, 6-month old rats; ^, $P \leq 0.05$ vs. 12-month old rats. In C: *, $P \leq 0.05$ vs. 1-, 2-, 6-, 12-month old rats; ^, $P \leq 0.05$ vs. 1-, 6-, 12-month old rats. Post-variance test: Newman-Keuls's Q-test [20].

amin and that of amprolium in vesicles from the same age group.

The non-saturable component of thiamin transport was characterized by calculating the slope of the linear portion of the concentration curves (Fig. 2). According to Thomson [21], the slope represents the apparent passive permeability coefficient of the non-saturable component of transport and provides an index of the passive permeability of the microvillous membrane. The greatest passive permeability to thiamin was observed in the brush border membrane from young rats (2-month old) (Fig. 4, C). Permeability coefficients decreased progressively and significantly with increasing age, reaching in old (24 months) rats a value which was 18% of that observed in young (2-month old) animals (Fig. 4, C).

When the two components of thiamin transport were compared, the transport rate of the saturable component was markedly higher than that of the non-saturable component in old rats. In particular, at a 2 μM initial concentration of thiamin in the incubation medium, which is in the physiological range, the saturable component accounted for 94% of the cumulative uptake in old (24 months) rats, while in the other groups the saturable component was consistently about 50% (Figs. 2 and 3). Overall, the microvillous membrane vesicles from old (24 months) rats were characterized, in comparison to those from younger animals, by a higher saturable (specific) component of transport, associated with a sharp decrease in passive permeability to thiamin. Based on the data shown in Fig. 2, it can

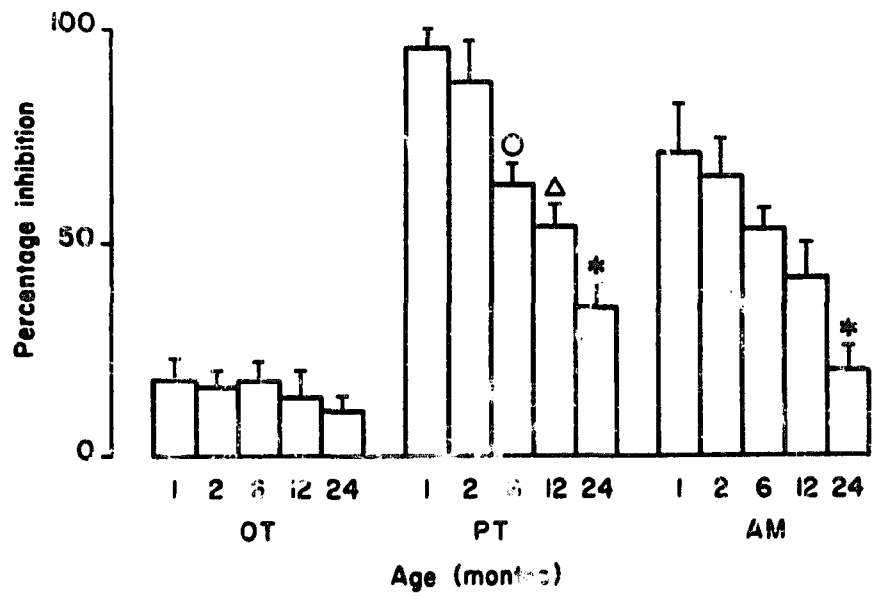


Fig. 5. Potency of various thiamin analogs (OT, 4'-oxythiamin; PT, pyrithiamin; AM, amprolium) in inhibiting the saturable transport of thiamin in small intestinal microvillous vesicles from rats of different ages. Thiamin analogs were added in the incubation medium at an initial concentration 10 times as high as that (0.5 μM) of [^{3}H]thiamin. The saturable component of thiamin transport was measured at 30 s by subtracting the diffusional component (measured at 0°C) from the cumulative uptake (measured at 25°C). Incubation medium and number of determinations as in Fig. 1. Bars represent means of percentage inhibition ± S.E. *, $P \leq 0.05$ vs. 1-, 2-, 6-month old rats; ^, $P \leq 0.05$ vs. 1-, 2-month old rats; °, $P \leq 0.05$ vs. 1-, 2-month old rats. Post-variance test: Newman-Keuls's Q test [20].

be calculated that, at the highest thiamin concentration used (12.5 μM, much higher than K_m values), the passive permeability rates accounted for 87–90% of the cumulative transport in the vesicles from the 1- to 12-month old groups, and for only 30% of the cumulative transport in the vesicles from the 24-month old groups.

Discussion

It is well established that aging is associated with changes in the composition and structure of the brush border membrane of the small intestine. In the microvillous intestinal membranes of old rats higher cholesterol/phospholipid and protein/lipid ratios as well as lower double bond index for fatty acids have been reported in comparison to membranes from young rats [22]. Similar alterations have been found in the small intestine of old rabbits [2,23,24], so that the microvillous membrane of young animals can be considered to be inherently more fluid than that of old animals. These structural features could conceivably account in our study for the larger volumes of the microvillous vesicles from young rats.

Intestinal absorption is also frequently affected by aging, though age-related absorption changes differ from one substance to another [1]. Using brush border membrane vesicles, Esposito et al. [13] reported a reduced D-glucose entry in old (12–24 months) as compared to young (45–60 days) and adult (2–12 months) rats, a finding confirmed by Doubek and Armbrecht, who reported a reduction of J_{max} only [25], by Freves et al. [26] and, in part, by Hirst and Wolfis [27]. In guinea pig ileum brush-border vesicles, however, aging enhances the transport of L-proline (but not that of L-leucine) [28]. The kinetics of L-proline transport in vesicles of aged animals shows a decrease in K_m value, while J_{max} is unaltered as compared to younger animals.

Present findings show that thiamin transport is also affected by aging: both the J_{max} and the K_m values of the saturable component appear to be increased, while passive permeability is reduced.

As far as the saturable component is concerned, our data may indicate that in aged microvillous membranes the number (or the activity) of thiamin carriers is increased (resulting in a higher J_{max}), while the affinity for thiamin is reduced (resulting in a higher K_m). This suggests that in aged rats the characteristics of the carriers differ from those observed in young animals. The occurrence of an age-related alteration in the characteristics of the carriers is also indicated by the results of inhibition experiments: the inhibitory potency of pyrithiamin and amprolium, two well known competitive inhibitors of the saturable component of thiamin transport [9], was reduced by aging, a finding which suggests a lower affinity of the thiamin carrier for these compounds. On the contrary, oxythiamin, a thiamin analog virtually inactive on thiamin transport [9], did not influence the saturable component, irrespectively of the age group in which it was tested.

Since the enrichment in the marker enzyme of microvillous membranes was the same for all age groups, the observed differences in K_m and J_{max} values should be considered as a reliable finding. Aging is associated with a delayed generation time of epithelial cells and with a reduced extrusion of these cells from the villus tip. Therefore, the cells lining the villus are more mature and may display enhanced functional abilities, including enhanced enzymic activities and substrate transport [1,2,29]. The age-dependent changes in kinetic parameters observed in our study may be related to alterations in microvillous membrane composition, especially lipid concentration and type, but also content in extrinsic (enzymes) and intrinsic (transport, etc.) proteins, possibly modulated by the fluidity state of lipids [30].

As far as the non-saturable component of transport is concerned, our data show that microvillous vesicles from old rats exhibit a greatly reduced passive permeability to thiamin. This could be related to the general trend of the passive permeability of small intestinal mucosa to decrease with increasing age, as shown for many substances including water, electrolytes, glucose, L-valine, and macromolecules [21,23,31,32]. Since there was no gross correlation between apparent passive permeability coefficients and vesicular volumes (Fig. 4C and Table I), the reduction in permeability in vesicles from mature (12 months) and old (24 months) animals is likely to result mainly from changes in membrane structure and composition. Indeed, as mentioned above, microvillous membranes from aged rats (and rabbits) are characterized by higher protein/lipid and cholesterol/phospholipid ratios [22,23]. These membranes also show an increased protein, fatty acids and total cholesterol content as well as qualitative changes in fatty acid and phospholipid composition [2,22], while their glycolipid content is reduced [23]. The decreased passive permeability of vesicular membranes to thiamin, as shown by the changes in K_d values, may reduce considerably the transport rate of high concentrations of vitamin ($\gg K_m$), for which passive diffusion is the main mechanism of intestinal absorption [9,10]. A decreased diffusion of thiamin, possibly associated with an age-induced decrease in small intestinal blood flow [33,34], may explain the reduced thiamin absorption reported in vivo in old rats [5,6]. In fact, the doses of thiamin used in these in vivo experiments were well above those which can be handled by the saturable (specific) transport system, so that under those conditions passive diffusion could be considered as the main process involved in the intestinal absorption of the vitamin.

Interestingly, an in vitro study on D-glucose transport by rabbit intestine found aging to be associated with increased J_{max} and K_m values, and with a decreased passive permeability [21], a finding similar to that observed in our investigation on thiamin vesicular transport. The increase in J_{max} for glucose transport was considered to represent a compensatory mechanism for the lower affinity and permeability typical of old brush border membranes, an interpretation which may also apply to present findings.

Acknowledgements

This work was supported by a grant from the Ministero P.I. 40% (Rome, Italy). We wish to express our gratitude to Prof. E. Perucca (Department of Internal Medicine, Pharmacology Division, University of Pavia) for revising the English. Thanks are expressed to Mrs. P. Vai Gatti and M. Agrati Greco for their excellent typing.

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