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THIAMINE TRANSPORT IN THIAMINE-DEFICIENT RATS
ROLE OF THE UNSTIRRED WATER LAYER

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SUMMARY

As part of a systematic study of alcoholism and thiamine absorption, the effect of diet-induced thiamine deficiency and the role of the unstirred water layer on thiamine transport were investigated. Using ^{3}H-labeled dextran as a marker of adherent mucosal volume, jejunal uptake of ^{14}C-labeled thiamine hydrochloride was measured, in vitro, in thiamine-deficient rats and pair-fed controls. Uptake of low thiamine concentrations (0.2 and 0.5 μM) was greater in the thiamine-deficient rats than in the controls. In contrast, uptake rates for high thiamine concentrations (20 and 50 μM) were similar in both groups. While $^{*}J_{max}$ was unaltered, $^{*}K_{m}$ was decreased in thiamine deficiency, suggesting a decrease in unstirred water layer thickness. Accordingly, the thickness of the water layer was measured in both groups of animals and correlated with $^{*}J_{max}$ and $^{*}K_{m}$ under unstirred and stirred conditions. Without stirring, there was no difference in $^{*}J_{max}$ between the two groups. In contrast, both $^{*}K_{m}$ and the water layer were reduced in the thiamine-deficient rats. With stirring, $^{*}J_{max}$ was not affected, but both $^{*}K_{m}$ and the water layer thickness were reduced to similar values in both groups. Reversal of thiamine deficiency resulted in the return of thiamine uptake and the unstirred water layer thickness to control values. These data support the concept of a dual system of thiamine transport and emphasize the role of the unstirred water layer as an important determinant of transport kinetics not only under physiologic situations but also in diet-induced rat thiamine deficiency, a model for a clinical pathological state. The decrease in the unstirred water layer thickness in thiamine deficiency may be also viewed as a possible adaptive mechanism to facilitate absorption of meager supplies of thiamine.

INTRODUCTION

The rate of absorption of a probe molecule into the intestinal mucosal cell is determined by two major diffusion barriers, an unstirred water layer adjacent to the cell membrane and the lipid cell membrane, itself [1]. In vitro studies have shown that

the aqueous diffusion barrier in the small intestine may lead to significant underestimations of passive permeability coefficient, P, [2, 3] and cause marked overestimation of the true Michaelis constant (K_m) for active transport [4, 5]. Indeed, the unstirred water layer, and not the cell membrane, may be rate limiting for absorption of such physiologically important compounds as bile acids and fatty acids. Mechanical stirring of the bulk water phase decreases the thickness of the diffusion layer, thereby facilitating the transport of such substances [2, 3, 6, 7]. The effect of the unstirred water layer on transport has been determined only under in vitro physiological situations, and until now its possible role in specific pathological conditions has not been explored.

In an attempt to understand better the pathogenesis of thiamine deficiency in chronic alcoholism, the characteristics of normal thiamine transport across the rat intestine were recently investigated [8]. The results indicate that there exists a dual system of thiamine transport. At low thiamine concentrations ($< 1.0\ \mu M$), transport is a saturable, carrier-mediated, energy-requiring active process, while at high concentrations ($> 1.0\ \mu M$), transport proceeds by simple diffusion. These studies further revealed that for low thiamine concentrations stirring of the water layer reduced the apparent Michaelis constant ($^*K_m$) while the apparent maximal transport capacity ($^*J_{max}$) remained unchanged. The passive permeability coefficient (P) for high thiamine concentrations was not affected by stirring. Subsequent investigations showed that ethanol inhibits the active but not the passive component of thiamine transport apparently by blocking the transfer of low thiamine concentrations from the cell interior to the serosal compartment [9]. Moreover, various vitamin deficiencies accompany alcoholism [10] and folate deficiency has been suspected [11] and later observed [12] to reduce active transport of thiamine. In the present study the effect of a diet-induced thiamine deficiency on thiamine transport was assessed. Furthermore, the role of the unstirred water layer was determined in this particular pathologic entity.

MATERIALS AND METHODS

Preparation of experimental animals

Pairs of female Sprague-Dawley rats, weighing 60–80 g, were placed in individual metabolic cages. One member of each pair was fed a special synthetic diet completely devoid of thiamine (Nutritional Biochemical Corp., Cleveland, Ohio), while the other received the same diet replete with thiamine hydrochloride (1 g/100 lb). Rats fed the thiamine-deficient diet gradually developed anorexia and lost weight. To correct for this, the daily food intake of the pair-fed control rat was adjusted to the previous day's consumption of the rat fed the thiamine-deficient food. Water was provided ad libitum to both animals. With this method [13], neurological signs of thiamine deficiency developed in 4–6 weeks and consisted of ataxia and sometimes convulsions. When the neurological signs became evident, the rate of intestinal uptake of thiamine was measured. The control rats showed no neurologic signs and their brain thiamine content was found to be essentially normal [13].

Measurement of unidirectional uptake rate

Using a method previously described [8, 14], the rate of unidirectional jejunal uptake of thiamine ((2-^{14}C)-labeled thiazole hydrochloride) (Amersham/Searle Corp.,

Arlington Heights, Ill.) was measured. ^{14}C-Labeled thiamine hydrochloride (spec. act. 18.9 Ci/mol) was determined to be at least 90 % radiochemically pure by paper chromatography using a propanol/acetate buffer/water system (70 : 10 : 20). The rats were killed by a blow on the back of the head and bled. The jejunum was removed and internally rinsed with iced physiologic saline. Sacs, 1.0–1.5 cm long, were prepared and filled with oxygenated (95 % O_2/5 % CO_2) Krebs bicarbonate buffer, pH 7.4. Measurement of unidirectional mucosal uptake was carried out by incubating the sacs at 37 °C in 20 ml of oxygenated buffer solution containing a known concentration of ^{14}C-labeled thiamine hydrochloride as the probe molecule and 3H-labeled dextran as a marker of adherent mucosal volume. At the end of the incubation period the sacs were quickly removed, rinsed in cold buffer and placed on moistened filter paper. The ends of each sac were cut off and discarded while the central cylinder was placed in a preweighed scintillation vial, oven dried at 93 °C overnight, cooled and the dry tissue weight determined. The tissue was saponified with 0.8 ml of 0.75 M NaOH, after which 15 ml of scintillation fluid was added and the solution agitated with a vortex mixer. The scintillation fluid contained: 7 g of PPO, 0.1 g of 1,4-bis-2-(5-phenyl-oxazolyl)-benzene (POPOP), 1 l toluene, 500 ml Triton X-100 and 80 ml 2.5 M HCl. Counting was carried out in a Packard Tri-Carb liquid scintillation spectrometer 2003 with automatic external standardization. The formulas for calculations are as given by Sallee et al. [14]. Thiamine-uptake rates were determined in thiamine-deficient rats with neurologic signs and in the pair-fed controls as well as in another group of deficient and control rats in which the neurologic signs of thiamine deficiency were reversed with daily intraperitoneal injections of thiamine, 500 μg/day for two days.

Measurement of water layer thickness

The thickness of the water layer was determined according to the method of Diamond [15] as applied by Wilson and Dietschy [4]. The animals used in these studies were probably too small to be suitable for the method recently described [16, 17]. The method chosen involved measuring the halftime ($t_{\frac{1}{2}}$) required for a change in streaming or diffusion potential to develop across a biological membrane [4]. An everted jejunal sac, 5–8 cm long, was closed at one end and tied to the extended side arm of a glass cannula, while the proximal end of the sac was slipped over and tied around the open end of the cannula. The serosal compartment of the sac was filled with oxygenated Krebs bicarbonate buffer, pH 7.4. The mucosal compartment consisted of 100 ml of buffer or an equal amount of sucrose 100 mM in buffer. Both solutions were maintained at 37 °C and a 95 % O_2 and 5 % CO_2 mixture was bubbled through a small polyethylene tube so that minor agitations even in "unstirred" experiments were unavoidable. Electrical transients were generated by quickly transferring the sac from one mucosal solution to the other. With a KCl/agar bridge placed in the serosal compartment and another in the mucosal compartment electrical transients were recorded using a potentiometric strip chart recorder (Sargent Welch Model SRG). Stirring was accomplished with a magnetic stirrer and a 5/8 inch stirring disc. The water layer thickness (d), under unstirred and stirred conditions, was then calculated using the formula $d = (D \cdot t_{\frac{1}{2}}/0.38)^{\frac{1}{2}}$ where D is the diffusion constant of sucrose at 37 °C ($0.69 \cdot 10^{-5}$ cm$^2 \cdot$ s^{-1}) and $t_{\frac{1}{2}}$ the measured half-time. The thickness of the water layer under unstirred and stirred conditions, in thiamine-deficient and pair-fed control rats, were compared and subsequently correlated with $^*J_{max}$ and

*K_m, for active thiamine transport that had been obtained under similar experimental conditions. In addition, the unstirred water layer thickness was measured in thiamine-deficient rats whose neurologic signs were reversed with thiamine.

Statistical analysis

The data were examined by paired t analysis or by the nonparametric Mann-Whitney U test.

RESULTS

The effect of thiamine deficiency on thiamine transport is shown in Fig. 1. As indicated in the left panel, the unidirectional jejunal mucosal uptake of low concentrations of thiamine (0.2 and 0.5 μM) under unstirred conditions in thiamine-deficient rats was greater than in the pair-fed controls, by 39 and 26 %, respectively ($p < 0.01$). In contrast, as shown in the right panel, the uptake rates of high thiamine concentrations (20 and 50 μM) were similar in both the thiamine-deficient and the control animals.

To determine the mechanism of the selective enhancement of uptake in thiamine deficiency, the rate of unidirectional mucosal uptake under unstirred conditions was examined in relation to a series of low thiamine concentrations, ranging from 0.1 to 1.0 μM. As can be seen in Fig. 2, in both thiamine-deficient and control animals, transport was a saturable process similar to that previously observed [8]. However, uptake of thiamine 0.1–0.5 μM was significantly greater in thiamine-deficient than in the control rats, while no statistical difference was evident in the uptake of thiamine 1.0 μM. These findings suggested a decrease in the thickness of the unstirred water

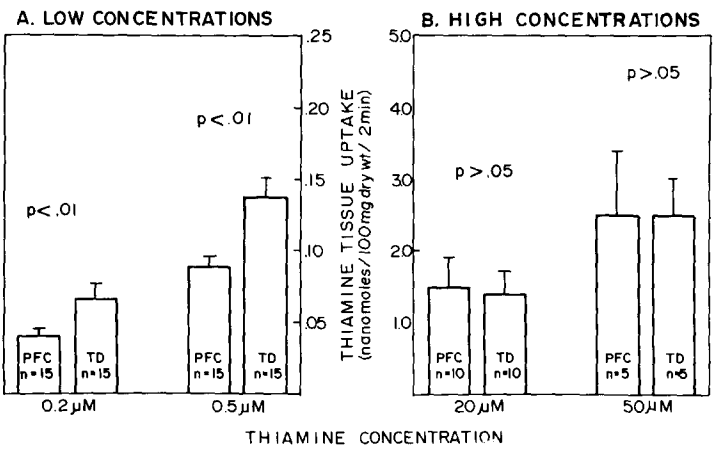

Fig. 1. Effect of thiamine deficiency on rates of jejunal uptake of low and high thiamine concentrations under unstirred conditions. The panel on the left shows that for low thiamine concentrations (0.2 and 0.5 μM), uptake rates were greater in the thiamine-deficient (TD) rats than in the pair-fed controls (PFC). In contrast, as shown in the right panel, thiamine deficiency did not influence the uptake rates of high thiamine concentrations (20 and 50 μM). The effect of thiamine deficiency was, therefore, on the active and not the passive component of thiamine transport. The bars indicate the means $\pm$ standard errors for the number of pairs of animals shown in the bottom of each bar.

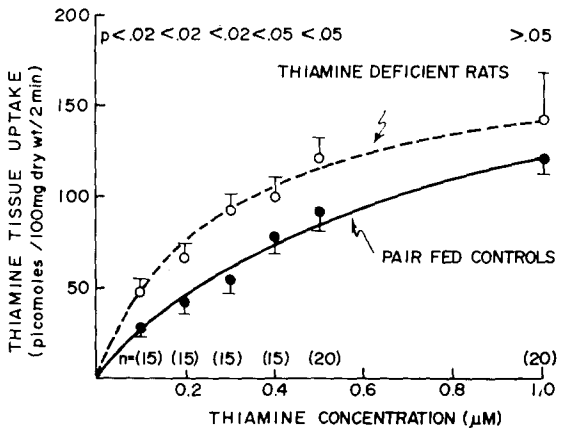

Fig. 2. Selective enhancement of uptake of low concentrations of thiamine in thiamine deficiency under unstirred conditions. Uptake in both thiamine-deficient and pair-fed control rats show a saturable process. However, uptake of thiamine 0.1–0.5 μM was consistently greater in thiamine-deficient than in the control animals, but was not significantly different for 1.0 μM thiamine. These results suggest that in deficient rats $*K_m$ is reduced, while $*J_{max}$ remains essentially unchanged. The number of pairs studied appears in parentheses and the mean uptake rates $\pm$ standard errors are given.

layer [8] or an increase in intestinal affinity for thiamine. We chose to examine the first possibility at this time. Accordingly, the role of the unstirred water layer in thiamine deficiency was investigated in two ways: indirectly by determining the influence of stirring on thiamine uptake and directly by measuring the thickness of the water layer, under unstirred and stirred conditions, in both thiamine-deficient and control rats. As shown in Fig. 3, $*J_{max}$, $*K_m$ and water layer thickness were compared between the two groups of animals. Without stirring, upper panels, there was no difference in $*J_{max}$ between the thiamine-deficient and the control rats. In contrast, $*K_m$ was significantly reduced in the deficient rats. Moreover, this reduced $*K_m$ correlated with a decreased water layer thickness in the deficient animals. With stirring, lower panels, $*J_{max}$ was not affected. On the other hand, in the thiamine-deficient rats as well as in the pair-fed controls, both $*K_m$ and the water layer thickness were significantly reduced by stirring (with the reduction being greater in the controls than in the deficient rats), to the extent that the difference in $*K_m$ and water layer thickness between the two groups disappeared. It should be noted that the water layer thickness observed here for the pair-fed control animals, 213.2 μm (unstirred) and 159.4 μm (stirred), are comparable to the values of 198–217 μm (unstirred) and 141–159 μm (stirred) reported elsewhere for the normal rats [2].

Intraperitoneal injections of thiamine hydrochloride to thiamine-deficient rats reversed the neurological signs and resulted in the return of intestinal thiamine uptake to control values, as shown in Fig. 4. Similarly, as indicated in Fig. 5, treatment of thiamine deficiency increased $*K_m$ and the unstirred water layer thickness to values which no longer differed statistically from those of the pair-fed controls.

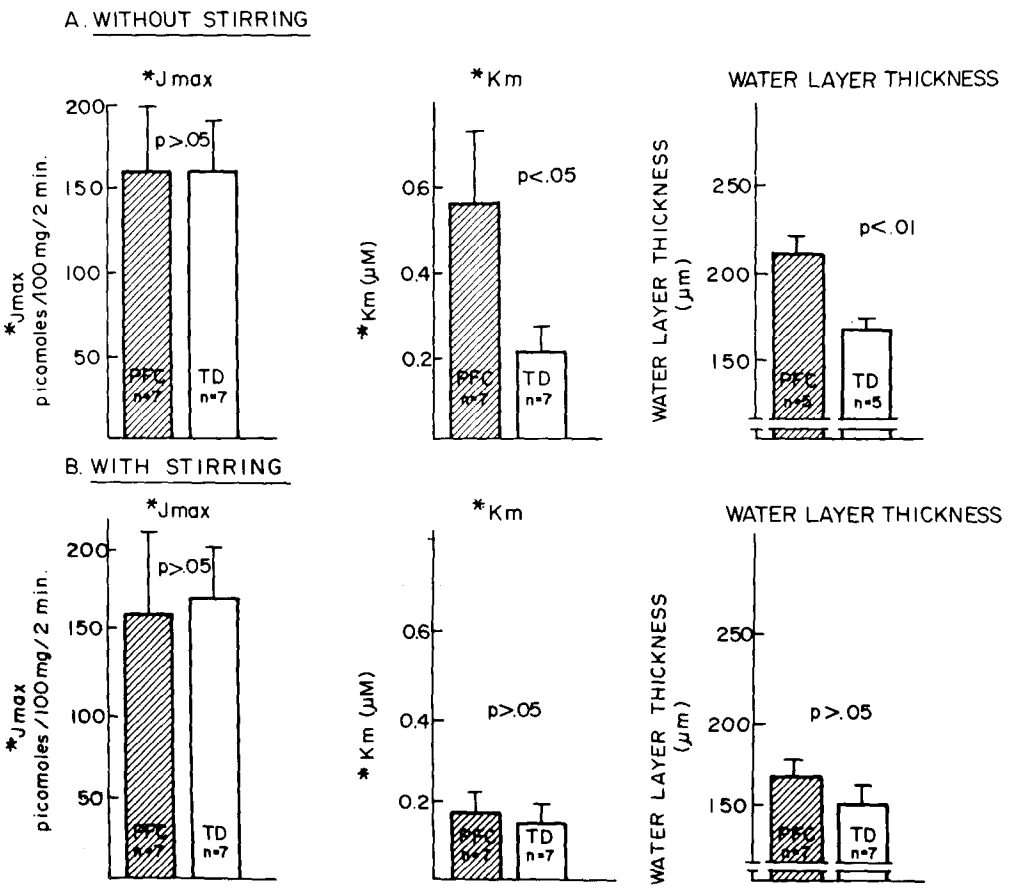

Fig. 3. Effect of thiamine deficiency on $*J_{max}$, $*K_m$ and the water layer thickness under unstirred and stirred conditions. Without stirring (upper panels) $*J_{max}$, shown on the left panel, was unaffected. In contrast, there was a parallel decrease in $*K_m$ (middle panel) and the water layer thickness (right panel) in thiamine-deficient (TD) rats as compared to pair-fed controls (PFC). Stirring (lower panels) did not change $*J_{max}$ from unstirred values but significantly reduced $*K_m$ and the water layer thickness to values which were statistically similar in both groups. The number of pairs ± standard errors of the mean are given.

DISCUSSION

In previous studies in the rat, intestinal transport of low concentrations of thiamine was shown to be a saturable process which was impaired by anoxia, low temperature, sodium lack, a structural analog (pyrithiamine) and such metabolic inhibitors as dinitrophenol, *N*-ethyl maleimide and ouabain [8]. On the other hand, these factors or agents exerted little or no effect on the transport of high concentrations of thiamine [8]. These findings suggest that at low thiamine concentrations transport of this vitamin is an active process, but at high thiamine concentrations, transport is predominantly passive. The concept of a dual system of thiamine transport [8] is further supported by the past finding that folate deficiency in rats depressed the in vivo

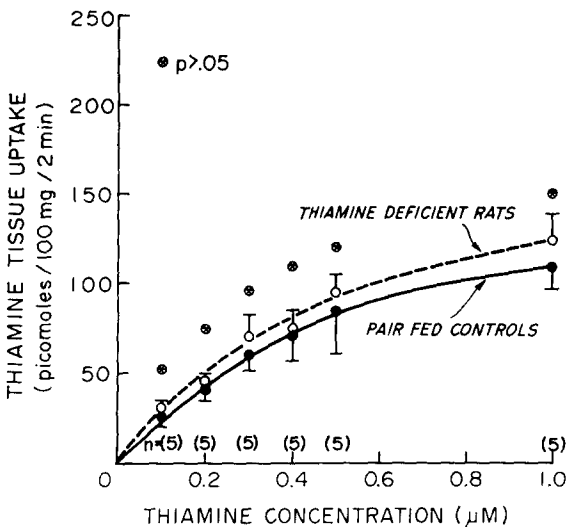

Fig. 4. Rates of jejunal thiamine uptake 48 h after reversal of overt thiamine deficiency. Rats rendered thiamine deficient (with neurologic signs) were given thiamine intraperitoneally (see Materials and Methods) to reverse the deficiency state. Studies 48 h later revealed reversal of neurologic signs and return of uptake rates to levels not significantly different from those in pair-fed controls. Means ± standard errors for 5 pairs of animals are given.

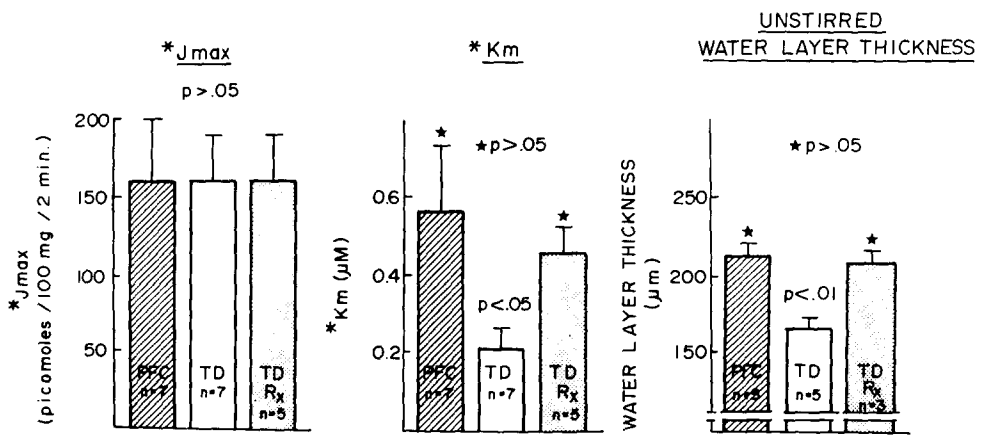

Fig. 5. Effect of treatment of thiamine deficiency (TD) on $*J_{max}$, $*K_m$ and the unstirred water layer thickness. Treatment of thiamine deficient rats (TD-R_x, stippled bars) by intraperitoneal injection of thiamine hydrochloride, while having no effect on $*J_{max}$ (left panel), resulted in increase of $*K_m$ (middle panel) and in the unstirred water layer thickness (right panel) to values which were statistically similar to those of the pair-fed controls (PFC). The star before the P values indicate comparison between the pair-fed controls (PFC) and the treated thiamine-deficient rats (TD-R_x); P values without a star indicate comparison between PFC and TD rats or between TD and TD-R_x.

intestinal absorption of 0.5 μM (low concentration) but not of 17.5 μM (high concentration) thiamine hydrochloride [12] and by the present observation that in vitro jejunal mucosal uptake of low, but not of high, thiamine concentrations was enhanced in thiamine-deficient rats. In addition, the present data show that in thiamine deficiency $^*K_m$ is reduced while $^*J_{max}$ remains unchanged, suggesting a decrease in the thickness of the unstirred water layer, a transport barrier previously shown to affect active thiamine absorption [8]. A brief consideration of the unstirred water layer may aid in understanding the basis for the present observations.

In the presence of a significant unstirred water layer the concentration of the probe molecule at the aqueous-lipid interface, C_2, is reduced below concentrations of the molecule in the bulk incubation solution, C_1. The degree of this reduction is given by the expression:

$$C_2 = C_1 - J\left(\frac{d}{D}\right) \tag{1}$$

where J is the flux rate of the probe molecule across the unstirred layer, d equals the thickness of the unstirred water barrier and D is the free diffusion coefficient for the molecule in water [4]. Consequently, according to a modification [4] of the formulation of Winne [18], a rather complicated expression for the active transport rate is obtained:

$$J = 0.5\frac{D}{d}\left[K_m + C_1 + \frac{J_{max}d}{D} \pm \sqrt{\left(K_m + C_1 + J_{max}\frac{d}{D}\right)^2 - 4\left(\frac{d}{D}\right)(J_{max}C_1)}\right] \tag{2}$$

where K_m and J_{max} represent the true Michaelis constant and maximal transport velocity, respectively, for the membrane carrier.

In previous studies, experimental data confirmed certain theoretical predictions derived from Eqn. 2. First, reduction of the water diffusion barrier should not open new sites for active transport. Thus, altering d does not change $^*J_{max}$. Second, reduction of d should facilitate a higher concentration of solute at C_2 for a given concentration at C_1; the higher concentration at C_2 results in enhanced mucosal uptake (J) across the mucosal cell membrane. Indeed, $^*K_m$ values vary in the same direction as d [4].

From these observations the mechanism for the enhanced mucosal uptake of thiamine becomes apparent for the thiamine-deficiency state. While the maximal transport capacity ($^*J_{max}$) remained unchanged, the Michaelis constant ($^*K_m$) was significantly reduced in thiamine deficiency. These findings are entirely consistent with the measured decrease in the thickness of the unstirred water layer in thiamine deficiency. On the other hand, the unstirred water layer was shown in previous studies to have no effect on passive thiamine transport [8]. This finding suggests that diffusion of thiamine across the unstirred water layer is very rapid relative to the rate of passive membrane permeation. In this situation, C_2 approximately equals C_1, so that the rate of jejunal uptake essentially equals the product $(C_1)(P)$, where P is the permeability coefficient of the probe molecule. It is not surprising, therefore, that thiamine deficiency and the associated reduction in unstirred water layer thickness did not influence the passive uptake of high thiamine concentrations.

Treatment and reversal of the deficiency state caused a return to normal not

only of the mucosal uptake rate and $*K_m$ of thiamine but also of the thickness of the unstirred wafer layer. The reversibility of these changes provides further evidence that the unstirred water layer was altered in thiamine deficiency and that, in this pathological condition, it played an important role in active thiamine transport.

It is not clear what component of the unstirred water layer is altered in thiamine deficiency. According to Westergaard and Dietschy [17], the unstirred water layer consists of two components: a superficial layer overlying the upper portions of the intestinal villi and a deeper component of water located between the lower portions of the villi. From their observations, it is reasonable to assume that the superficial component is relatively more accessible to the effect of external stirring than is the deeper layer [17]. Therefore, differences in $*K_m$ due to alterations in the superficial component of the unstirred water layer are more likely to disappear on stirring, while differences in $*K_m$ due to changes in the deeper component are less likely to be so affected. In the present studies, stirring brought about a further decrease in $*K_m$ and the water layer thickness so that the differences between the thiamine-deficient rats and the pair-fed controls disappeared, suggesting that thiamine deficiency caused a change mainly in the superficial rather than the deep component of the unstirred water layer.

However, the precise mechanism for the decrease in the thickness of the unstirred water layer in thiamine deficiency remains to be established and requires further investigations. In addition, it should be noted that the diet-induced experimental thiamine deficiency model is complex and, despite pair feeding of controls, may be associated with an abnormal assimilation and utilization of nutrients or with other as yet obscure changes which may influence absorption [19]. However, it resembles very closely human thiamine deprivation. In the thiamine-deficient rat the selective enhancement of thiamine intestinal uptake is probably not an isolated phenomenon, so that it may be of interest to measure in this animal model transport of other essential nutrients. Nevertheless, although incompletely understood, the present observations serve to emphasize the role of the unstirred water barrier as an important determinant of transport kinetics in the diet-induced model of thiamine deficiency and for the first time extend its significance from the purely physiological situations to a pathologic condition. The reduced unstirred water layer thickness in thiamine deficiency may be further viewed as a possible adaptive mechanism which allows the afflicted animal to absorb more efficiently whatever meager supply of thiamine may be available. Finally, it is possible that changes in the intestinal unstirred water layer may be found in the future to have certain clinical implications.

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