Biochimica et Biophysica Acta, 1148 (1993) 263–268

BBAMEM 75967

Uptake of riboflavin by intestinal basolateral membrane vesicles: a specialized carrier-mediated process

Hamid M. Said [a,b], Daniel Hollander [b] and Reza Mohammadkhani [a,b]

[a] *Medical Research Service, V.A. Medical Center, Long Beach, CA (USA) and* [b] *Departments of Medicine, Pediatrics and Physiology / Biophysics, University of California-School of Medicine, Irvine, CA (USA)*

(Received 16 October 1992)

Key words: Riboflavin transport; Basolateral membrane; Water-soluble micronutrient transport; Intestinal transport; (Rabbit small intestine)

The mechanism of riboflavin (RF) uptake by intestinal basolateral membrane vesicles (BLMV) was examined in this study. BLMV were isolated by an established Percoll-gradient methodology from rabbit small intestine. Uptake of riboflavin was mainly the result of transport of the substrate into an osmotically active intravesicular space with less binding to membrane surfaces. Uptake of RF with time was similar in the presence of a Na^+ and a K^+ gradient (out > in) and was not significantly influenced by changes in incubation buffer pH. The initial rate of uptake of riboflavin as a function of concentration was saturable in both jejunal and ileal BLMV and occurred with apparent K_m values of 5.0 μM and 4.4 μM and V_{max} values of 91.6 and 60.8 pmol/mg protein per 5 s, respectively. Unlabeled riboflavin and the structural analogues lumiflavin, isoriboflavin and 8-aminoriboflavin all caused significant inhibition (but to different degrees) in the uptake of [^{3}H]riboflavin. On the other hand, 8-hydroxyriboflavin, lumichrome, lumazine and D-ribose failed to inhibit [^{3}H]riboflavin uptake. Trans-stimulation of [^{3}H]riboflavin efflux from preloaded BLMV by unlabeled riboflavin or lumiflavin was also observed. Altering transmembrane electrical potential by anion substitution and valinomycin-induced K^+ diffusion did not affect the riboflavin uptake process. These results demonstrate the existence of a specialized carrier-mediated mechanism for riboflavin uptake by intestinal BLMV. Furthermore, the system appears to transport the vitamin by a process which is Na^+- and pH-independent, and electroneutral in nature.

Introduction

The micronutrient riboflavin (RF), a member of the water-soluble B-family of vitamins, is essential for normal cellular functions, growth and development. Humans and other mammals cannot synthesize RF, therefore, they must obtain the vitamin from the diet by intestinal absorption. Absorption of RF from the intestinal lumen into the blood represents the vectorial movement of the vitamin across the highly polarized enterocyte. This process represents transport of the vitamin across two membranes that have different structure, composition and permeability – namely the brush-border membrane (BBM) and the basolateral membrane (BLM). A number of previous studies, including those from our laboratory, have examined the overall absorption process of RF [1–5], but limited information is available regarding the mechanism(s) and regulation of the individual transport event of the absorption process. Such studies are crucial for detailed understanding of the physiology of the absorption process of RF and of the factors that control and interfere with it. Recent investigations from our laboratory and others have begun to address these issues and have shown the existence of a specialized carrier-mediated system for RF transport across the BBM of human, rabbit and rat enterocytes [6–8]. Nothing, however, is known about the mechanism of exit of RF out of the absorptive cell, i.e., transport across the BLM. The aim of the present study was, therefore, to address this issue using BLM vesicles (BLMV) isolated from rabbit intestine by a well established procedure.

Correspondence to: H.M. Said, Medical Research Service (151), V.A. Medical Center, Long Beach, CA 90822, USA.

Materials and Methods

Materials. [G-^{3}H]RF with specific activity of 40 Ci/mmol and radiochemical purity of 98% was purchased from American Radiolabeled Chemical, St. Louis, MO. 8-Aminoriboflavin (8-NH_2-RF), isoriboflavin (isoRF) and 8-hydoxyriboflavin (8-OH-RF) were a generous gift from Dr. Vincent Massey of the University of Michigan-School of Medicine. Filters

(cellulose nitrate, pore size 0.45 μm) were obtained from Milipore, Bedford, MA; other chemicals and reagents were of analytical quality and was obtained from commercial sources.

Methods. Intestinal BLMV were isolated from the jejunum (or ileum) of a 2.5–3.5 kg adult New Zealand white rabbits using a self-generating Percoll-gradient method [9] as described in detail by us previously [10–12]. Isolated BLMV were suspended in a transport 'intravesicular' buffer of 280 mM mannitol and 20 mM Hepes-Tris (pH 7.4) to achieve a final protein concentration of 3 to 7 mg/ml. Transport studies were performed on freshly prepared BLMV using a rapid-filtration technique [13] as described before [10–12]. The reaction was started by mixing 20 μl of vesicle suspension with 80 μl of incubation buffer (final concentrations: 100 mM KCl or NaCl, 80 mM mannitol and 20 mM Hepes-Tris (pH 7.4); when the study was done at pH 6.5 and lower, Mes-Tris was used as the buffering system) containing labeled and unlabeled RF and other constituents. The reaction was stopped at the desired period by adding 1 ml of ice-cold stop solution (100 mM NaCl, 100 mM mannitol and 10 mM KH_2PO_4, pH 7.4), vortexed, and filtered. The filter was then washed with ice-cold stop solution, transferred to a scintillation vial, and counted for radioactivity.

In one study, BLMV were preloaded with [^{3}H]RF (1.12 μM) for 10 min at room temperature, pelleted, washed twice with 20 ml of ice-cold buffer, then incubated in a K^+-containing incubation buffer in the absence and presence of 20 μM unlabeled RF and lumiflavin to study possible trans-stimulation in [^{3}H]RF transport across the intestinal BLM [14].

Uptake results are presented in this paper as mean $\pm$ S.E. and are expressed in either pmol/mg protein per unit time or as a percentage relative to simultaneously performed controls. Data are from multiple transport determinations from 2–4 different BLMV preparations from different rabbits. As seen before with this membrane preparation [10–12], slight quantitative variations in the absolute amount of RF uptake were noticed in some BLMV preparations. For this reason, simultaneously performed controls were run with each study to permit proper comparison of the results. Data were analyzed using regression analysis and the Student's t-test. Transport kinetics parameters of the observed saturable component of RF uptake (i.e., the V_{max} and the apparent K_m) were calculated (following subtraction of the diffusion component, determined from the slope of the uptake lined between the point of origin and 100 μM, from total uptake) using a computerized model of the Michaelis-Menten equation as described by Wilkinson [15]. Protein concentrations of BLMV preparations were determined using the method of Lowry et al. [16]. Purity of rabbit intestinal BLMV used in this study was confirmed by demonstrating an average enrichment of 10.86- and 0.73-fold in the activities of the BLM marker K^+-stimulated phosphatase and the BBM marker enzyme enzyme leucine aminopeptidase, respectively, in the final vesicular preparations compared to initial mucosal homogenates. These results are similar to those found for rat and human intestinal BLMV [10–12].

The purity of the stock [^{3}H]RF and the determination of the metabolic form of the radioactivity taken up by the vesicles following incubation with [^{3}H]RF were determined using silica-gel precoated thin-layer plates and a solvent system of ethanol/water (9 : 1, v/v) [3].

Results

Uptake of RF by intestinal BLMV: effect of time, osmolarity and incubation buffer pH

Fig. 1A depicts the uptake of a physiological concentration (0.11 μM) of [^{3}H]RF as a function of time in the presence of a Na^+ and a K^+ gradient (outside 100 mM, inside 0 mM). Uptake of RF increased linearly with time for up to 20 sec; following that uptake rate slowed reaching a plateau at approx. 40 s and stayed at that level for the rest of the 60 min incubation. No significant difference was seen in RF uptake in the presence of a Na^+ and a K^+ gradient. Similar results were obtained for the time dependent uptake of 20 μM RF (Fig. 1B).

In another study, we examined the effect of varying the incubation medium osmolarity (using mannitol) on equilibrium uptake of RF (i.e., uptake at 60 min). The aim of this study was to differentiate between uptake that occurs due to transport of the substrate into the intravesicular space and that due to membrane binding. The results (Fig. 2) showed an inverse and linear ($r = 0.97$) relationship between RF uptake and medium osmolarity. Extrapolating the uptake line to infinite osmolarity yielded a y-intercept of 0.75 pmol/mg protein per 60 min. From these findings, it was calculated that uptake of RF at equilibrium and under isoosmotic condition was mainly (73%) the result of transport of the vitamin into the intravesicular space and the remainder was due to binding to membrane surfaces.

The effect of varying the incubation buffer pH on RF uptake was also examined in jejunal BLMV loaded with transport buffer pH 7.4 and incubated in K^+ incubation buffer (outside 100 mM, inside 0 mM) at different pH values. No significant difference in RF uptake was seen at the different incubation buffer pH values (uptake of 1.84 ± 0.24 ($n = 5$), 1.96 ± 0.23 ($n = 6$), 2.12 ± 0.17 ($n = 6$), 2.07 ± 0.23 ($n = 5$), 1.8 ± 0.35 ($n = 3$) and 1.95 ± 0.18 ($n = 6$) pmol/mg protein per 5 s, was found at incubation buffer pH of 5.0, 5.6, 6.4, 6.9, 7.4 and 7.8, respectively).

We also examined the metabolic form of the transported radioactivity into jejunal BLMV following incu-

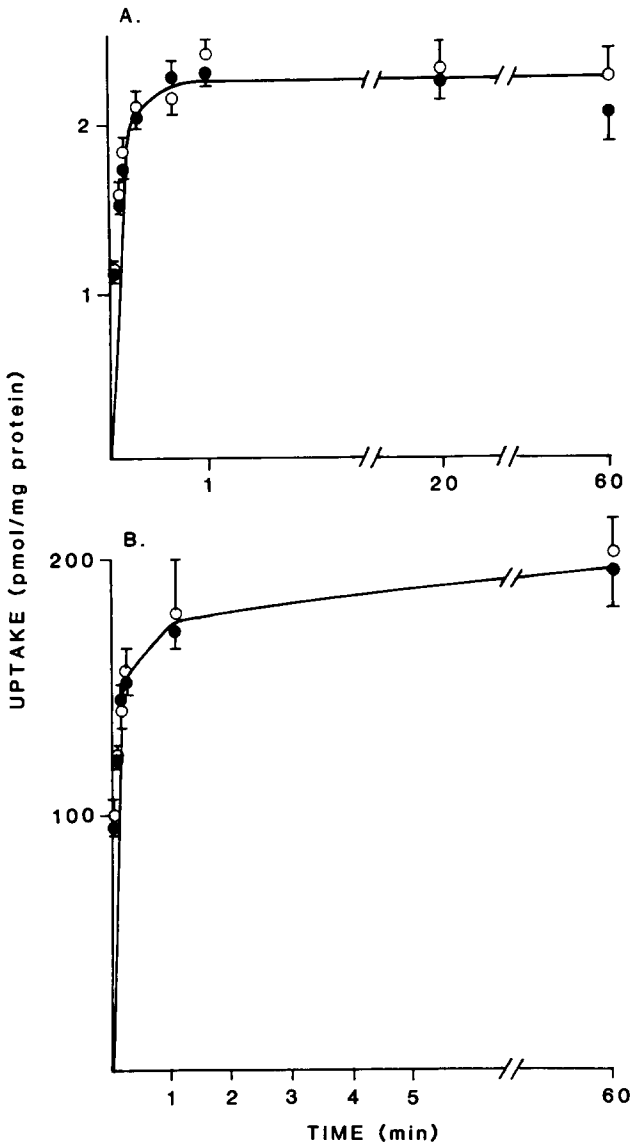


Fig. 1. Uptake of [^{3}H]RF by rabbit jejunal BLMV with time in the presence of an inwardly directed Na^+ and a K^+ gradient. BLMV were preloaded with a buffer of 280 mM mannitol and 20 mM Hepes-Tris (pH 7.4). Incubation was done in a buffer of 100 mM NaSCN (closed circles) or KSCN (opened circles) or, 80 mM mannitol and 20 mM Hepes-Mes (pH 7.4). RF at 0.11 μM (A) and 20 μM (B) was added to the incubation medium at the beginning of the study. Each point is the mean ± S.E. of 4–9 separate transport determinations from two or three different BLMV preparations from different rabbits. Osmolarity is in mosmol/l.

bation of vesicles with 0.11 μM [^{3}H]RF for 60 min as described in Methods. The results showed that 98% of the radioactivity transported into the intravesicular space to be in the form of intact RF.

Uptake of RF as a function of concentration

Initial rate of uptake of RF was examined as a function of concentration (0.11–20 μM) in jejunal and ileal BLMV isolated from the same rabbits. In both areas of the small intestine, uptake of RF was found to include a saturable component (Fig. 3). Kinetic parameters of the saturable component were calculated as described in Methods and found to be 5.0 μM and 4.4 μM for the apparent K_m and 91.6 and 60.8 pmol/mg protein per 5 s for the V_{max} in jejunal and ileal BLMV, respectively.

Effect of unlabeled RF and related components on the uptake of [^{3}H]RF

In these experiments, we examined the effect of adding to the incubation medium different concentrations of unlabeled RF and related compounds (see Fig. 4 for structure) on the initial rate of uptake (5 sec) of 0.11 μM [^{3}H]RF by jejunal BLMV. The results (Table I) showed that 25 and 50 μM unlabeled RF and lumiflavin cause similar and significant inhibition in [^{3}H]RF uptake. IsoRF and 8-NH_2-RF also caused significant inhibition in RF uptake both at 25 and 50 μM but the degree of inhibition was less than that seen with unlabeled RF and lumiflavin. Lumichrome, 8-OH-RF, lumazine and D-ribose did significantly inhibit RF uptake.

We also examined possible trans-stimulation of [^{3}H]RF movement across the BLM by unlabeled RF and lumiflavin. This was done as described before [14] by examining the efflux of [^{3}H]RF from preloaded jejunal BLMV (see Methods) incubated in the absence and presence of 20 μM unlabeled RF or lumiflavin. The results showed significantly lower [^{3}H]RF content in BLMV incubated in medium containing unlabeled RF and lumiflavin compared to simultaneously performed control BLMV incubated in the absence of

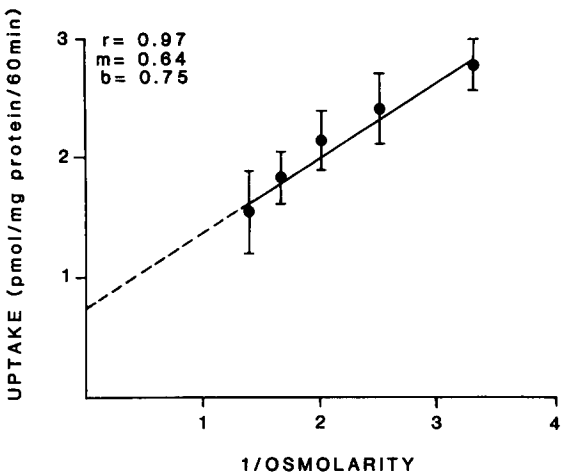


Fig. 2. Effect of incubation medium osmolarity on the uptake of RF by jejunal BLMV. BLMV were preloaded with a buffer of 280 mM mannitol and 20 mM Hepes-Tris (pH 7.4), and incubated for 60 min in a buffer of 100 mM NaSCN, 20 mM Hepes-Mes (pH 7.4) and different amount of mannitol to give the indicated osmolarity. RF (0.11 μM) was added to the incubation medium at the beginning of the study. Each point is the mean ± S.E. of five or six separate transport determinations from two BLMV prepared from different rabbits.

unlabeled RF and lumiflavin. (A vesicle content of [^{3}H]RF of 9.24 ± 1.49 (n = 4), 3.94 ± 0.25 (n = 6) and 3.65 ± 0.24 (n = 6) pmol/mg protein following 30 s incubation was found for control and in the presence of unlabeled RF and lumiflavin in the incubation medium, respectively.)

Effect of membrane transport inhibitors on [^{3}H]RF uptake

The effect of the membrane transport inhibitors 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) (both at 1 mM) and that of folic acid (100 μM) on the uptake of 0.11 μM [^{3}H]RF by jejunal BLMV was examined. Incubation was done for 5 s at 37°C. None of the compounds tested were found to affect RF uptake into BLMV. (The uptake expressed as percentage relative to simultaneously performed controls of 100 was, 94.3 ± 5.18 (n = 5), 94.3 ± 9.33 (n = 6) and 96.9 ± 4.31 (n = 6) for DIDS, SITS and folic acid, respectively.)

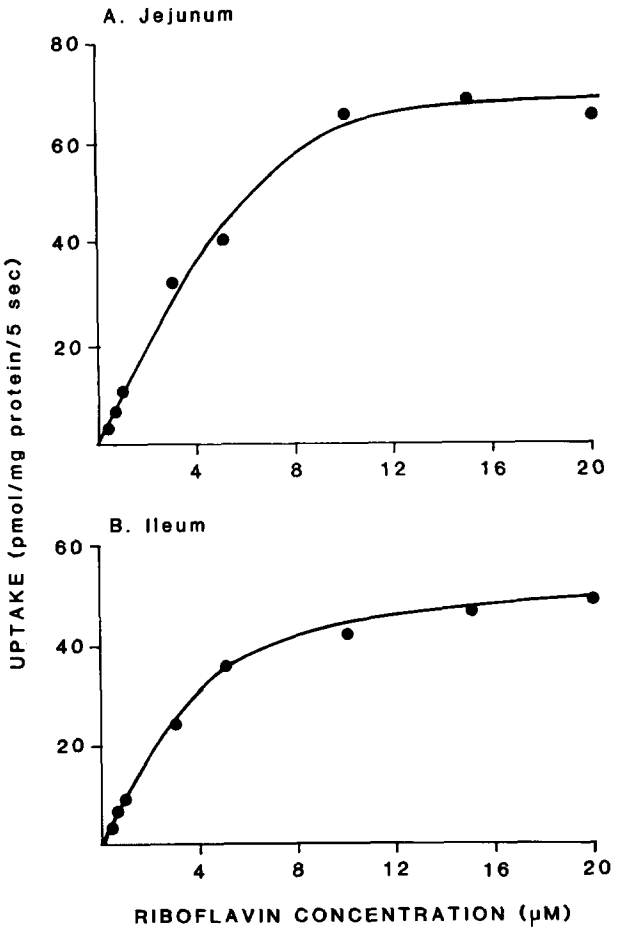


Fig. 3. Initial rate of RF uptake as a function of concentration in jejunal (A) and ileal (B) BLMV. BLMV were isolated simultaneously from the jejunum and the ileum of the same rabbits and were preloaded with a buffer of 280 mM mannitol and 20 mM Hepes-Tris (pH 7.4). Incubation was done for 5 s (initial rate) in a buffer of 100 mM NaSCN, 80 mM mannitol and 20 mM Hepes-Mes (pH 7.4). Different concentrations of RF (1–20 μM) were added to the incubation medium at the onset of incubation. Data represent uptake by the carrier-mediated process determined as described in Methods. Three to six separate transport determinations were done on two different BLMV prepared from different rabbits.

TABLE I

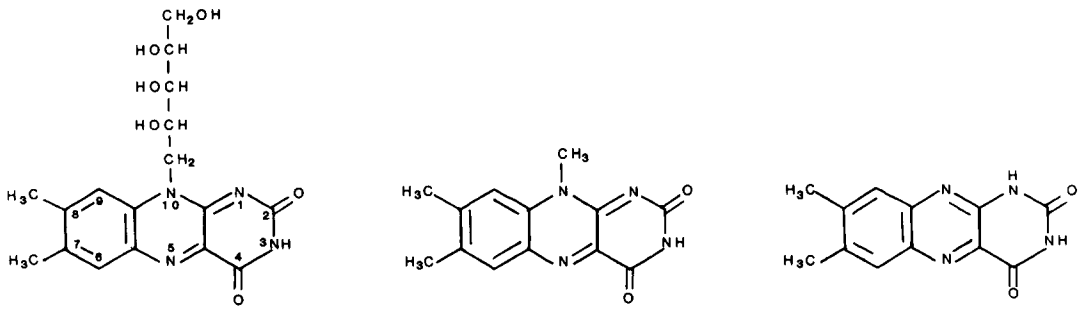
Effect of RF related compounds on the initial rate of uptake of [^{3}H]RF by jejunal BLMV

BLMV were preloaded with 280 mM mannitol and 20 mM Hepes-Tris (pH 7.4) and incubated for 5 s in a buffer of 100 mM NaSCN, 80 mM mannitol and 20 mM Hepes-Tris (pH 7.4), containing 0.11 μM [^{3}H]RF and the compound under investigation. Results were calculated in terms of percentage relative to simultaneously performed controls. The number in parentheses is the number of separate transport measurements from two or three separate BLMV preparations from different rabbits. The P values were calculated using the Student's t-test. n.s., not significant.

Condition	Concn. (μM)	Uptake (% of simultaneously performed controls	P
Control		100	
Unlabeled RF	25	48.30 ± 4.35 (5)	< 0.01
	50	37.70 ± 4.35 (5)	< 0.01
Lumiflavin	25	42.40 ± 3.26 (5)	< 0.01
	50	34.80 ± 4.35 (5)	< 0.01
Iso-RF	25	62.90 ± 8.30 (7)	< 0.01
	50	50.30 ± 4.84 (7)	< 0.01
8-NH_2-RF	25	77 ± 11.10 (5)	< 0.01
	50	65.40 ± 7.86 (7)	< 0.01
Lumichrome	25	95.10 ± 10.90 (4)	n.s.
	50	90.30 ± 7.61 (6)	n.s.
8-OH-RF	25	107 ± 10.10 (8)	n.s.
	50	92.20 ± 6.54 (7)	n.s.
Lumazine	25	101 ± 6.29 (6)	n.s.
	50	91.20 ± 8.81 (6)	n.s.
D-Ribose	25	104 ± 15.70 (5)	n.s.
	50	97.50 ± 13.20 (5)	n.s.

Effect of transmembrane potential on uptake of RF

The effect of altering transmembrane electrical potential on the uptake of RF by jejunal BLMV was examined using anion substitution and valinomycin induced K^+-diffusion methodologies [10–12,17–20]. In the anion substitution method, the initial rate of RF uptake was examined in the presence of anions of different membrane permeability (SCN^- > Cl^- > gluconate$^-$) (incubation with a permeable anion would create a greater transient negative intravesicular electrical potential than a less permeable anion, thereby affecting any potential sensitive component of RF uptake). The results showed similar uptake of RF in the presence of the different anions (1.59 ± 0.16 (n = 8), 1.40 ± 0.06 (n = 7) and 1.50 ± 0.12 (n = 8) pmol/mg protein per 5 s for SCN^-, Cl^- and gluconate$^-$, respectively, indicating that the RF uptake process is electroneutral in nature. This conclusion was further confirmed by the findings of the valinomycin-induced K^+ diffusion method which showed similar uptake of RF



	6	7	8
Riboflavin	-H	$-CH_3$	$-CH_3$
Isoriboflavin	$-CH_3$	$-CH_3$	-H
8-amino-riboflavin	-H	$-CH_3$	$-NH_2$
8-Hydroxy-riboflavin	-H	$-CH_3$	-OH

Fig. 4. Structure of riboflavin and certain structural analogues.

following induction of a positive intravesicular space compared to 'voltage clamp' condition (Table II).

Discussion

The present study examined the mechanism of uptake of RF by BLMV isolated form rabbit intestine. Uptake of RF with time was found to be similar in the presence of a Na^+ and a K^+ gradient. Uptake was also found to be largely the result of transport of RF into an osmotically active intravesicular space with less binding to membrane surfaces. In addition, the uptake process was found to be independent of the pH of the incubation medium. No metabolic alteration was found in the transported radioactivity following incubation of BLMV with [^{3}H]RF.

RF uptake in both jejunal and ileal BLMV was found to be saturable as a function of concentration indicating the involvement of a carrier-mediated system. The similar apparent K_m (5.0 and 4.4 μM, respectively) of the uptake systems in jejunal and ileal BLMV suggests that these systems are similar. If this is the case, then the higher (50%) the V_{max} of the putative uptake carrier in the jejunum compare to the ileum (91.6 vs. 60.8 pmol/mg protein per 5 s, respectively) may suggest a greater distribution (and/or activity) of the RF carrier in the jejunum.

The existence of a carrier-mediated system for RF uptake in jejunal BLMV was further confirmed by the findings that unlabeled RF and certain structural analogues caused significant inhibition in [^{3}H]RF uptake when added to the incubation medium and by the observed trans-stimulation in [^{3}H]RF efflux from preloaded vesicles by unlabeled RF and lumiflavin. The study with different analogues also provided important information about the structural requirement in the RF molecule that are needed for recognition and efficient interaction with the uptake carrier. First, replacing the ribityl side chain at position 10 of the RF molecule by a methyl group appeared to have no effect on the ability of the analogue compound (lumiflavin) to interact with the carrier system as indicated by the similar degree of inhibition caused in [^{3}H]RF uptake by unlabeled RF and lumiflavin. On the other hand, total removal of the side chain led to disappearance of significant interaction as indicated by the inability of lumichrome to induce significant inhibition in [^{3}H]RF uptake. These findings demonstrate the importance of having a group at position 10 of the isoalloxazine ring for proper ligand-carrier interaction. Second, having a $-CH_3$ group at position 8 also appeared to be somewhat important for efficient interaction with the uptake carrier as removal of this group or its replacement with an amino group led to a decrease in the degree of inhibition by the new compounds (isoRF and 8-NH_2-RF) of [^{3}H]RF uptake compared to unlabeled RF. On the other hand, replacing this $-CH_3$ group with a hydroxyl group led to disappearance of the ability of

TABLE II

Effect of transmembrane electrical potential on RF uptake by jejunal BLMV

BBMV were preloaded with a buffer of 50 mM K-gluconate, 80 mM mannitol and 20 mM Hepes-Tris (pH 7.4) (voltage clamp) or 280 mM mannitol and 20 mM Hepes-Tris (pH 7.4) (positive membrane potential) and were preincubated with valinomycin (10 μg/mg protein) for 10 min at room temperature. Incubation was performed at 37°C in an incubation buffer of 90 mM Na-gluconate, 50 mM K-gluconate and 20 mM Hepes-Tris (pH 7.4). [^{3}H]RF (0.11 μM) was added to the reaction mixture at the start of the experiment. The number in parentheses is the number of separate transport assays from three separate BBMV preparations from different rabbits.

Time (s)	Uptake (pmol/mg protein)	
	voltage clamp	positive membrane potential
5	1.53 ± 0.09 (9)	1.51 ± 0.09 (8)
20	1.87 ± 0.06 (8)	2.04 ± 0.19 (6)

the new compound (8-OH-RF) to inhibit the uptake of [^{3}H]RF. The inability of 8-OH-RF to inhibit [^{3}H]RF uptake may be due to the changes in the physiochemical property of 8-OH-RF due to ionization of the -OH group (the pK_a of RF is 10.2 and becomes 4.8 when the compound is converted to 8-OH-RF [21]).

It has been suggested that RF might behave as an anion with regard to its transport across biological membranes [22]. For this reason we examined the effect of the anion transport inhibitors DIDS and SITS on the vitamin uptake by BLMV. Neither inhibitor altered the RF uptake process. Similarly, the anionic folic acid (which has been shown to interact with RF transport across biological membranes [23]) was found to have no effect on RF uptake. In other studies, the effect of altering the transmembrane electrical potential on RF uptake by jejunal BLMV was examined using well established methodologies of anions substitution and valinomycin K^+-induced electrical potential. Neither method, however, was found to affect RF uptake indicating that the uptake process is electroneutral in nature.

In summary, the present study shows for the first time the involvement of a specialized carrier-mediated system or transport of RF across the BLM of rabbit intestine. This system is independent of Na^+ and pH, and moves the substrate by an electroneutral process.

Acknowledgements

This study was supported by a merit review grant (H.M.S.) from the Medical Research Service of the Department of Veterans Affairs.

References

1 Akiyama, T., Selhub, J. and Rosenberg, I.H. (1982) J. Nutr. 112, 263–268.
2 Daniel, H., Wille, U. and Rehner, G. (1983) J. Nutr. 113, 636–643.
3 Hegazy, E. and Schwenk, M. (1983) J. Nutr. 113, 1702–1707.
4 Said, H.M., Ghishan, F.K., Green, H.L. and Hollander, D. (1985) Pediatr. Res. 19, 1175–1178.
5 Middleton, H.M. (1990) J. Nutr. 120, 588–5939.
6 Said, H.M. and Arianas, P. (1990) Gastroenterology 100, 82–88.
7 Said, H.M., McCloud, E. and Mohammadkhani, M. (1993) Proc. Soc. Exp. Biol. Med. 202, 428–434.
8 Daniel, H. and Rehner, G.I. (1992) J. Nutr. 122, 1454–1461.
9 Scalera, V., Storelli, C., Storelli-Joss, C., Haase, W. and Mures, H. (1980) Biochem. J. 186, 177–181.
10 Said, H.M. and Redha, R. (1987) Biochem. J. 247, 141–146.
11 Said, H.M., Redha, R. and Nylander, W. (1988) Gastroenterology 94, 1157–1163.
12 Said, H.M. (1991) Biochem. J. 279, 671–674.
13 Hopfer, U., Nelson, K., Preotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32.
14 Said, H.M., Hollander, D. and Korchid, S. (1991) Gastroenterology 101, 1094–1101.
15 Wilkinson, G.N. (1961) Biochem. J. 80, 324–332.
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
17 Said, H.M., Ghishan, F.K. and Redha, R. (1987) Am. J. Physiol. 252, G229–G236.
18 Ghishan, F.K., Said, H.M., Leonard, D., Pietsch, J. and Abumrad, N. (1989) Biochim. Biophys. Acta 979, 77–81.
19 Meier, P.J., Abt, A.S., Barrett, C. and Boyer, J.L. (1984) J. Biol. Chem. 259, 10614–10622.
20 Schron, C.M., Knickelbein, T.G., Aronson, P.S. and Dobbins, J.W. (1987) Am. J. Physiol. 253, G404-G410.
21 Ghilsa, S. and Mayhew, S.G. (1976) Eur. J. Biochem. 63, 373–390.
22 Lowy, R.J. and Spring, K.R. (1990) J. Membr. Biol. 117, 91–99.
23 Lichtestein, N.S. and Goldman, D.I. (1970) Biochem. Pharmacol. 191, 229–1239.