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# Riboflavin Uptake by Rat Small Intestinal Brush Border Membrane Vesicles: A Dual Mechanism Involving Specific Membrane Binding

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**Abstract.** The first step of riboflavin absorption was studied by determining the uptake of the vitamin by rat small intestinal brush border membrane vesicles. Vesicles were incubated at 25°C in the presence of [$^3$H]-riboflavin at concentrations within the physiological intraluminal range for rat. The time course of [$^3$H]-riboflavin uptake was unaffected by $Na^+$ or $K^+$ gradients. The 5 sec uptake rate plotted as a function of the initial concentration of [$^3$H]-riboflavin in the medium (0.125 to 7.5 $\mu$M) revealed the presence of a dual mechanism, with a saturable component (apparent kinetic constants: 0.12 $\mu$M for $K_m$ and 0.36 pmol · mg$^{-1}$ protein · 5 sec$^{-1}$ for $J_{max}$) prevailing at low concentrations (<2 $\mu$M), and a nonsaturable component prevailing at higher concentrations. The presence of a carrier-mediated system for riboflavin was validated by countertransport experiments. At equilibrium, uptake was almost completely accounted for by membrane binding, whereas at earlier times the transport component accounted for about 30% of total uptake. The plot of [$^3$H]-riboflavin binding at equilibrium as a function of its concentration in the medium was quite similar to that of the 5 sec uptake rate in both intact and osmotically shocked vesicles and demonstrated the occurrence of a saturable component: binding constants were 0.07 ($K_d$ in $\mu$M), 0.54 ($B_{max}$ in pmol · mg$^{-1}$ protein), and 0.11 ($K_d$), 1.13 ($B_{max}$), respectively, indicating the existence of specific riboflavin binding sites. The specificity of riboflavin binding to the membrane was confirmed by preliminary studies with structural analogues. Specific binding could represent the first step of a specific riboflavin entry mechanism in enterocytes.

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**Key words:** Intestinal brush border vesicles—Riboflavin—Transport—Binding

## Introduction

The intestinal transport of riboflavin (RF) has been studied in vitro by using whole tissue preparations (Meinen, Aeppli & Rehner, 1977; Akiyama, Selhub & Rosenberg, 1982; Daniel, Wille & Rehner, 1983; Said, Hollander & Duong, 1985*b*; Middleton, 1990), and isolated enterocytes (Hegazy & Schwenk, 1983). In these investigations, the transport of RF at physiological intraluminal concentrations has been shown to be specific, saturable, $Na^+$, temperature- and energy-dependent, and possibly coupled to cellular metabolism (Yoshimine, 1984; Kasai et al., 1988, 1990; Bowman, McCormick & Rosenberg, 1989; McCormick, 1989). More recent studies on the first step of tissue uptake, i.e., membrane transport, carried out by using intestinal sacs and short incubation times (Middleton, 1990) or brush border membrane vesicles (BBMV) (Casirola et al., 1989; Casirola & Ferrari, 1991; Said & Arianas, 1991; Elbert, 1987 *quoted by* Feder, Daniel & Rehner, 1991), have produced controversial results. In this paper, RF uptake by BBMV was investigated further, particularly in respect to membrane binding[1].

A partial account of this work has been published previously (Casirola & Ferrari, 1991).

[1] After submission of this paper, Daniel and Rehner (*J. Nutr.* 1992, **122:**1454–1461) reported that the uptake of riboflavin by brush border membrane vesicles of rat small intestine is predominantly a $Na^+$-dependent carrier-mediated transport into the intravesicular space.

## Materials and Methods

### Vesicle Preparation

Vesicles were prepared from the small intestines of 5–6 Wistar albino rats of either sex, about 150 g body weight, killed by decapitation after 15 hr fasting with water *ad libitum*. The preparation method was based on $Ca^{2+}$ precipitation according to Kessler et al. (1978), with minor modifications. The purity of the preparation was assessed by determining the enrichment in sucrase activity (Dahlqvist, 1964) of BBMV compared to the initial mucosal homogenate. Proteins were determined according to Lowry et al. (1951), using bovine serum albumin as a standard. Spectrophotometric determinations were carried out by using a Beckman DU5 spectrophotometer (Irvine, CA).

### Uptake Measurements

#### *General Procedures*

In the uptake studies, vesicles were incubated at 25°C in the presence of [$^3$H]-riboflavin ([$^3$H]-RF) (Amersham International, Amersham, UK: specific activity, 900 mCi/mmol, special synthesis). The radiochemical purity of [$^3$H]-RF was checked periodically and found to be 98% as determined by using silica gel plates (E. Merck, precoated thin-layer chromatographic plate No. 5721) in the following solvent system: butanol-acetic acid-water (20:1:4 by volume) (Kasai et al., 1988). Details of incubation media and experimental conditions are given in the figure legends. At the end of the incubation, BBMV were separated from the medium by using a rapid filtration procedure and cellulose nitrate microfilters (Microfiltration System, Dublin, CA) with 0.65 $\mu$m pore diameters (for details, *see* Casirola et al., 1988). Uptake was determined by measuring the amount of radioactivity retained on the filters, using a Packard TriCarb 2000 CA analyzer (Packard Instruments, Downers Grove, IL). Nonspecific binding of radioactivity to filters was accounted for by using appropriate blanks.

#### *Binding and Transport*

The relative contributions of binding and transport to total RF uptake were evaluated by determining the effect of osmotic perturbations on [$^3$H]-RF uptake. The desired medium osmolarities (*see* legend of Figs. 5–7) were obtained by adding appropriate amounts of sucrose or D-mannitol to the standard incubation medium. The plot of [$^3$H]-RF uptake *vs.* the inverse of osmolarity (henceforth defined as "osmoplot") was obtained by computerized linear regression (Graphpad Software, San Diego, CA, 1992); the y-axis intercept represents the amount of membrane-bound [$^3$H]-RF.

"Osmoplots" were used for all binding characterizations in this paper.

### Calculation of Kinetic and Binding Constants

Kinetic and binding constants were obtained by fitting the experimental points to a Michaelis-Menten equation with an additional linear term:

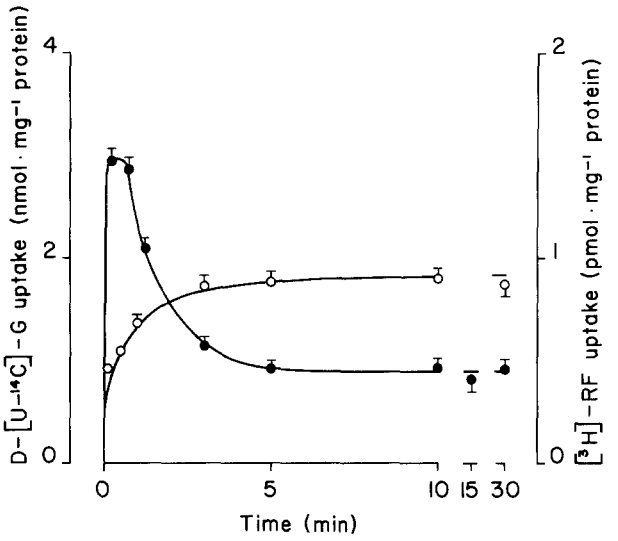


**Fig. 1.** Time course of D-glucose and riboflavin uptake by rat small intestinal brush border membrane vesicles. Uptakes of: (●) 1 mM D-[U-$^{14}$C]-glucose (D-[U-$^{14}$C]-G) and (○) 0.25 $\mu$M [$^3$H]-riboflavin ([$^3$H]-RF) were measured at 25°C, in the presence of a 100 mM (out) $Na^+$ gradient. Incubation medium (mM): 100 D-mannitol, 2 $MgSO_4$, 10 TRIS-HEPES, pH 7.5, and 100 NaCl. Symbols represent means ± SEM of triplicate determinations for each of at least five different preparations. When not shown, SEM were within symbol area.

$$y = \frac{A \cdot x}{B + x} + C \cdot x,$$

where $x$ = RF concentration ($\mu$M) in the incubation medium, $y$ = RF uptake (pmol/mg protein) after incubation, $A$ = maximal rate of transport or binding, $B$ = dissociation constant, $C$ = permeability constant. Nonlinear regression analysis was performed by a computerized method (Graphpad Software, San Diego, CA, 1992).

### Materials

All reagents were of analytical grade and supplied by Sigma (St. Louis, MO) and BDH (Poole, UK). Unlabeled riboflavin was obtained from Prodotti Roche, Milan, Italy; lumichrome, 8-demethyl-riboflavin and 3-methyl-riboflavin were synthesized by S. Kasai.

## Results

Enrichment in sucrase activity in BBMV was consistently about 14-fold compared to the initial mucosal homogenate.

Prior to use, the vesicle preparation was evaluated functionally by determining the uptake profile of 1 mM D-[U-$^{14}$C]-glucose (Amersham International, Amersham, UK: specific activity, 275 mCi/mmol) in the presence of a 100 mM (out) NaCl gradient. The time course of uptake showed the typical peak at about 30 sec and equilibrium at 10 min (Fig. 1).

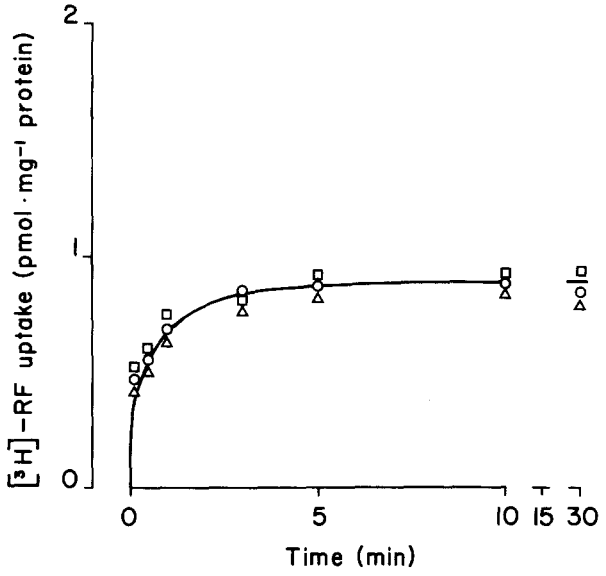


**Fig. 2.** Time course of riboflavin uptake by rat small intestinal brush border membrane vesicles. Uptake of 0.25 $\mu$M [$^3$H]-riboflavin ([$^3$H]-RF) was measured in the presence of: (○) 100 mM (out) NaCl; (□) 100 mM (out) KCl or (△) no alkaline ions, osmotically replaced by D-mannitol. Incubation medium (mM): 100 D-mannitol, 2 $MgSO_4$, 10 TRIS-HEPES, pH 7.5. Number of experiments for each symbol as in Fig. 1. SEM were within 10% of the mean values.

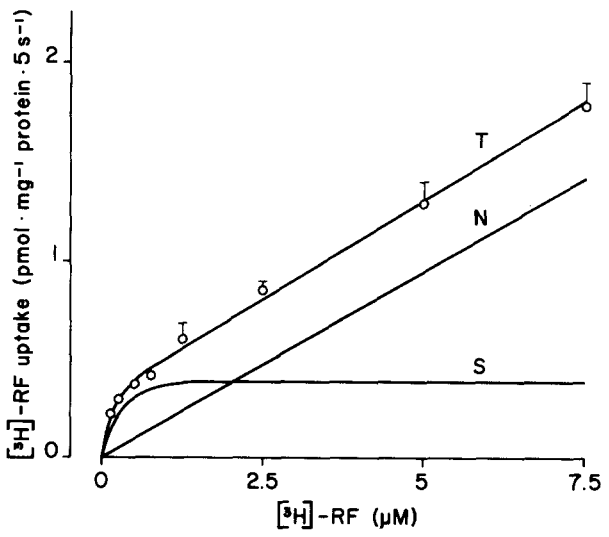


**Fig. 3.** Short time uptake of riboflavin by rat small intestinal brush border membrane vesicles as a function of riboflavin concentration in the medium. [$^3$H]-riboflavin ([$^3$H]-RF) uptake was measured after 5 sec incubation at 25°C and at different [$^3$H]-RF concentrations (0.125–7.5 $\mu$M). Incubation medium and symbols as in Fig. 1. When not shown, SEM were within symbol area. The curve of total uptake ($T$) was obtained by computerized nonlinear regression (Graphpad Software, San Diego, CA, 1992) and resolved into a saturable ($S$) and a nonsaturable ($N$) component.

## General Features of Riboflavin Uptake

### *Time Course and Ion Gradients*

In the time course experiments, BBMV were incubated with 0.25 $\mu$M [$^3$H]-RF in the presence of an initial 100 mM (out) NaCl gradient. The time course showed no peak and reached equilibrium after about 10 min incubation (Fig. 1).

To investigate the effect of $Na^+$ on the time course of [$^3$H]-RF uptake, 100 mM NaCl was subsequently replaced with 100 mM KCl or with isosmotic D-mannitol. The uptake profile was virtually unaffected under the latter conditions (Fig. 2).

### *Concentration Curve with 5 sec Incubation*

[$^3$H]-RF uptake as a function of its concentration in the medium (0.125 to 7.5 $\mu$M) was measured after 5 sec incubation by using a "STRUMA" short time incubation apparatus (Innovativ-Labor, Adliswil, Switzerland). To validate the use of this time point, we established in earlier experiments that the time course of uptake is reasonably linear for up to at least 6 sec and that at zero time the curve passes through the origin (*data not shown*). The 5 sec uptake of [$^3$H]-RF as a function of its concentration in the medium was characterized by a biphasic curve which was resolved into two components: a nonlinear (saturable) component, prevailing at concentrations $< 2$ $\mu$M, and a linear (nonsaturable) component, prevailing at higher concentrations (Fig. 3). The apparent kinetic constants of the saturable component were 0.12 $\mu$M for $K_m$ and 0.36 pmol · mg$^{-1}$ protein · 5 sec$^{-1}$ for $J_{max}$.

### *Countertransport at Equilibrium*

Countertransport at equilibrium was measured as follows: vesicles were preincubated with 12.5 $\mu$M unlabeled RF, 11-fold diluted and then incubated with 0.25 $\mu$M [$^3$H]-RF. As control, a sample of vesicles from the same preparation was preincubated without unlabeled RF, 11-fold diluted and incubated with 0.25 $\mu$M [$^3$H]-RF. For the preincubation and incubation media, *see* legend of Fig. 4. [$^3$H]-RF uptake was higher in RF-preloaded than in control vesicles. The differences were statistically significant ($P < 0.001$ by Student's $t$-test) from 30 sec to 30 min of incubation (Fig. 4).

The results are in accordance with those of former experiments in which vesicles preincubated with 0.25 $\mu$M [$^3$H]-RF were incubated with or without 12.5 $\mu$M unlabeled RF. After 5 min incubation, 50 and 10% of the initial [$^3$H]-RF content of BBMV

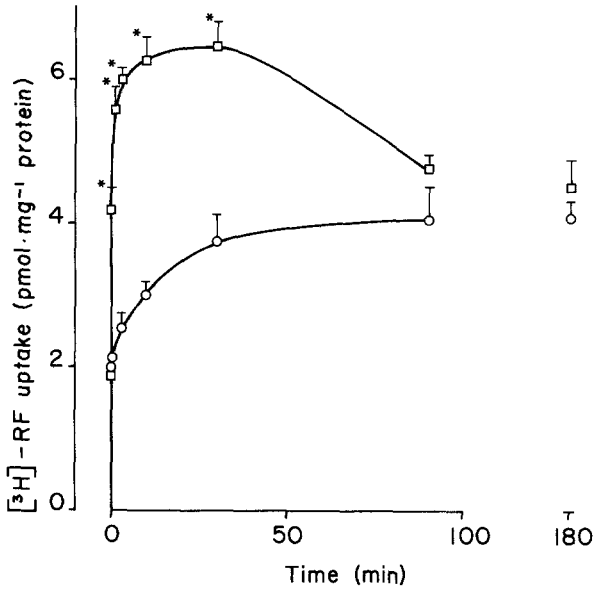


**Fig. 4.** Countertransport at equilibrium in rat small intestinal brush border membrane vesicles. Vesicles were preincubated at 25°C for 30 min in a medium containing (mM): (□) 280 D-mannitol, 2 $MgSO_4$, 10 TRIS-HEPES (pH 7.5), and 12.5 $\mu$M unlabeled riboflavin; (○) in the same medium without unlabeled riboflavin (control). Vesicles were 11-fold diluted and incubated in a medium containing (mM): 280 D-mannitol, 2 $MgSO_4$, 100 NaCl, 10 TRIS-HEPES, pH 7.5, and 0.25 $\mu$M [$^3$H]-riboflavin ([$^3$H]-RF). Symbols represent means ± SEM of triplicate determinations for each of three different preparations. When not shown, SEM were within symbol area. (*) $P < 0.001$ *vs.* control (Student's *t*-test).

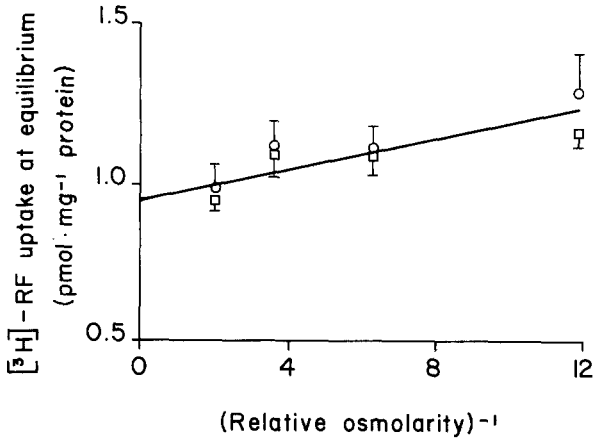


**Fig. 5.** Effect of increasing medium osmolarity on riboflavin uptake by rat small intestinal brush border membrane vesicles at equilibrium. The uptake of 0.25 $\mu$M [$^3$H]-riboflavin ([$^3$H]-RF) was measured at 25°C after 20 min incubation in media with different osmolarities. The osmolarity of the media with respect to nonpenetrating nonelectrolytes (D-mannitol and sucrose) was expressed as relative osmolarity. Incubation medium (mM): 50 D-mannitol, 2 $MgSO_4$, 25 NaCl, 10 TRIS-HEPES, pH 7.5 and sufficient amounts of: (○) D-mannitol or (□) sucrose to yield the indicated relative osmolarities. Number of experiments for each symbol as in Fig. 1. The line was obtained by linear regression analysis ($r = 0.96$) (Graphpad Software, San Diego, CA, 1992).

had been displaced by the addition of unlabeled RF and RF free incubation medium, respectively, (*data not shown*).

## Binding and Transport

### *Binding and Transport at Equilibrium*

At equilibrium (20 min incubation), binding in the presence of either D-mannitol or sucrose accounted for about 90% of uptake under isosmotic conditions (Fig. 5).

### *Binding and Transport as a Function of the Incubation Time*

To determine the extent of membrane binding before the onset of equilibrium, the vesicles were preincubated (20 min), and then incubated (5 sec to 30 min), in the same media with different osmolarities. For media composition, *see* legend of Fig. 6. Up to 6 sec, both membrane binding and transport curves were linear, with transport accounting for about 30% of total uptake. Afterward, the curves steadily increased, although at different rates, reaching equilibrium at 10 min (Fig. 6).

## Characterization of Binding at Equilibrium

### *Specificity of Binding*

The specificity of [$^3$H]-RF binding to the membrane was studied by using two different experimental approaches: (i) binding at equilibrium (20 min) was determined in intact BBMV by means of the "osmoplot" at each of six different [$^3$H]-RF concentrations, and mean binding values at each concentration were plotted as a function of [$^3$H]-RF concentrations in the medium; (ii) binding at equilibrium at different [$^3$H]-RF concentrations in the medium was determined by measuring the radioactivity retained by BBMV osmotically shocked with 20 mM TRIS-HEPES, pH 7.5, after rinsing away the internal fluid with the shocking solution (Li et al., 1990). The mathematical handling of data was the same for both conditions (i) and (ii).

The curves were resolved into two components: a saturable and a nonsaturable component. Under condition (i), the saturable component prevailed at concentrations < 0.3 $\mu$M. The binding constants $K_d$

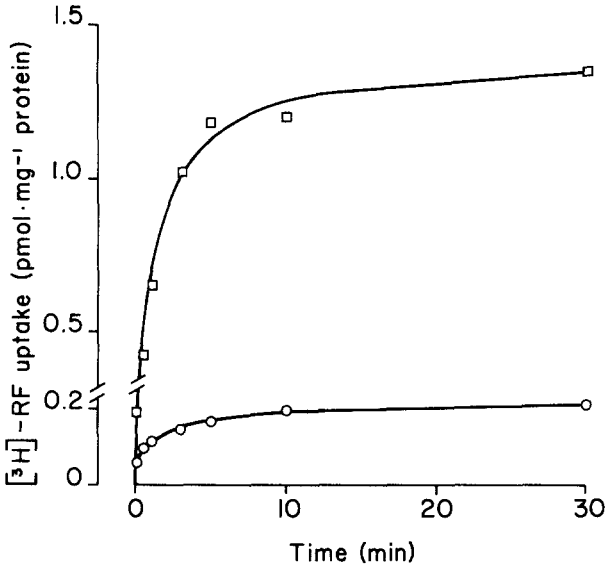


**Fig. 6.** Time course of riboflavin binding and transport by rat small intestinal brush border membrane vesicles. Vesicles were preincubated at 25°C for 20 min in media with different osmolarities (84, 280, 500 mOsmM). Total uptake was measured incubating the vesicles with 0.25 $\mu$M [$^3$H]-riboflavin ([$^3$H]-RF]). At each time point, binding (□) was determined as in Fig. 5 (*see also* Materials and Methods); transport (○) was the difference between total uptake and binding. Preincubation and incubation media (mM): 50 D-mannitol, 2 $MgSO_4$, 25 NaCl, 10 TRIS-HEPES, pH 7.5 and sufficient amounts of sucrose to yield the indicated osmolarities. Symbols represent means of triplicate determinations for each of two different preparations.

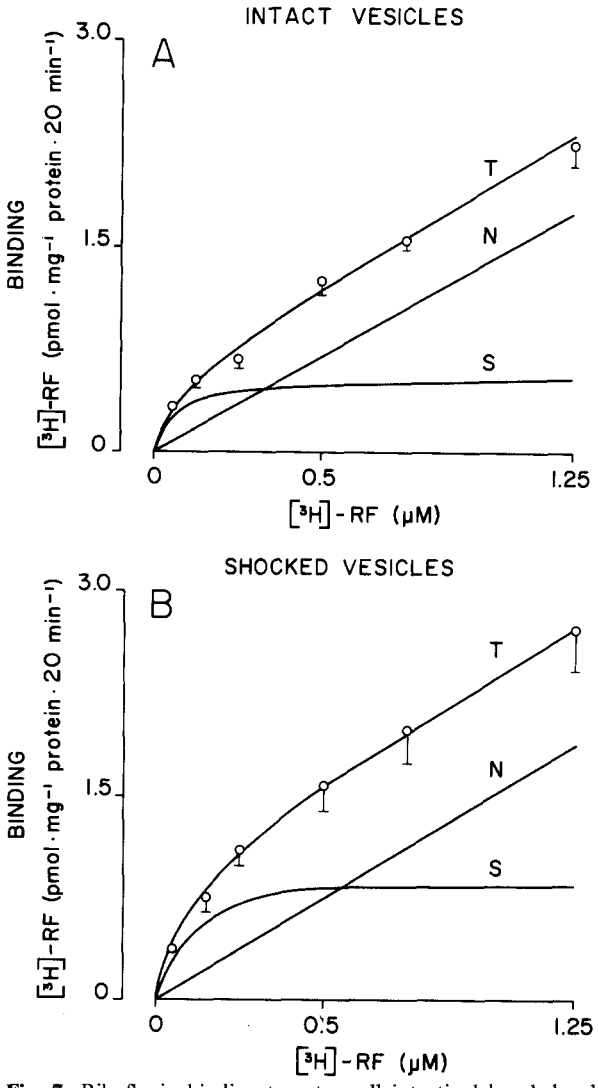


**Fig. 7.** Riboflavin binding to rat small intestinal brush border membrane vesicles at equilibrium as a function of riboflavin concentration in the medium. (*A*) Each point represents the mean ± SEM of five different binding values determined from the plot of uptake *vs.* osmolarity (*see* legend of Fig. 5) at each [$^3$H]-riboflavin ([$^3$H]-RF) concentration. (*B*) Each point represents the mean ± SEM of five different binding values determined as the amount of [$^3$H]-RF retained after osmotic shock of the brush border membrane vesicles (*see text*) at different [$^3$H]-RF concentrations. Incubation medium as in Fig. 5. Mathematical processing of data and T, S, N, as in Fig. 3. When not shown, SEM were within symbol area.

(apparent dissociation constant) and $B_{max}$ (maximal binding, which indicates the number of binding sites) were 0.07 $\mu$M and 0.54 pmol · mg$^{-1}$ protein, respectively. At 0.25 $\mu$M [$^3$H]-RF, the saturable component accounted for about 58% of total binding (Fig. 7*A*). Under condition (ii), the saturable component (binding constants: $K_d$, 0.11 $\mu$M and $B_{max}$ 1.13 pmol · mg$^{-1}$ protein) prevailed at [$^3$H]-RF concentrations < 0.5 $\mu$M (Fig. 7*B*).

### *Group Specificity of Binding*

The specificity of membrane sites for the molecular groups of RF was assessed by using some potential inhibitors, including: unlabeled RF and structural analogues monosubstituted at the ribityl side chain (lumichrome) and in position 3 (3-methyl-RF, synthesized according to Kasai et al., 1988) or 8 (8-demethyl-RF, synthesized according to Matsui, Sugimoto and Kasai, 1982) of the isoalloxazine moiety, respectively. Incubations were carried out for 20 min after adding unlabeled RF or its structural analogues to the standard incubation medium containing 0.25 $\mu$M [$^3$H]-RF, in a concentration ratio of 10 to 1. The inhibitory potencies of unlabeled RF and its structural analogues, expressed as percent inhibition of the saturable component of 0.25 $\mu$M [$^3$H]-RF binding at equilibrium, are shown in Fig. 8. Unlabeled RF showed greatest inhibitory potency (100%) of the saturable component, while 8-demethyl-RF inhibited the saturable component by about 50% and lumichrome and 3-methyl-RF had almost no effect.

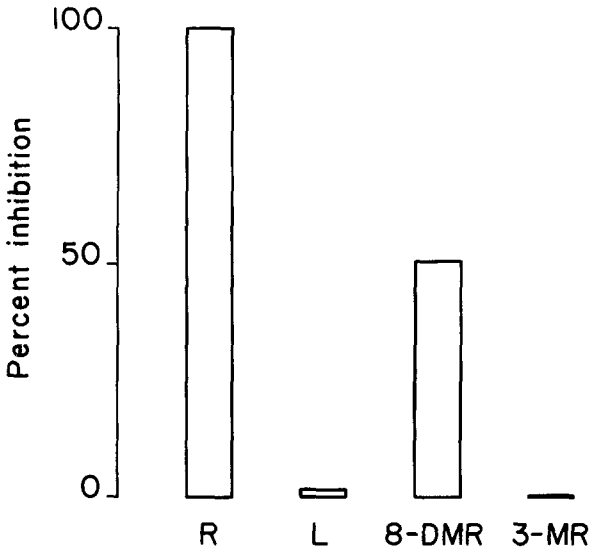


**Fig. 8.** Potencies of unlabeled riboflavin and riboflavin analogues in inhibiting the saturable component of riboflavin binding to rat small intestinal brush border membrane vesicles at equilibrium. Uptake of 0.25 $\mu$M [$^3$H]-riboflavin ([$^3$H]-RF) was measured at 25°C after 20 min incubation. Unlabeled riboflavin and analogues were added to the incubation medium at an initial 2.5 $\mu$M concentration. Bars represent means of percentage inhibition. Incubation medium and number of experiments as in Fig. 1. (*R*) unlabeled riboflavin; (*L*) lumichrome; (*8-DMR*) 8-demethyl-riboflavin; (*3-MR*) 3-methyl-riboflavin.

## Discussion

Both enrichment of sucrase activity of vesicles and time course profile of their D-glucose uptake (Fig. 1) indicated that the preparation was sound and capable of ensuring an efficient transport.

While in whole tissue (Meinen et al., 1977; Daniel et al., 1983; Said et al., 1985*b*) and in isolated enterocytes (Hegazy & Schwenk, 1983) the uptake of RF is known to be $Na^+$ dependent, no influence of alkaline ion gradient could be demonstrated in BBMV (Fig. 2), indicating that the $Na^+$-dependent mechanism is not located in the brush border membrane. As suggested by McCormick (1989), an $Na^+$-$K^+$-ATPase-dependent transport system for RF located in the basolateral membrane could easily account for the $Na^+$ dependence of RF transport in intact intestinal tissue.

After 5 sec incubation, the total uptake of RF was accounted for partly by a specific, saturable, high affinity and low capacity component, which prevailed at RF concentrations up to 1.25 $\mu$M, i.e., within the physiological intraluminal range for rat (0.1–4 $\mu$M) (Said et al., 1985*a*, Feder et al., 1991), and partly by a nonspecific, nonsaturable component, which prevailed at higher concentrations (Fig. 3). A similar dual mechanism has been described in isolated rat jejunal segments (Daniel et al., 1983), everted jejunal sacs (Said et al., 1985*b*; Middleton, 1990), isolated enterocytes (Hegazy & Schwenk, 1983), and also in the in vivo rat (Feder et al., 1991).

The presence of a carrier-mediated process for RF transport was confirmed by countertransport experiments: in BBMV both inflow and outflow [$^3$H]-RF were substantially enhanced by unlabeled RF.

At equilibrium, RF was largely membrane bound (Fig. 5). The high binding capacity for RF confirms our previous findings (Casirola et al., 1989; Casirola & Ferrari, 1991) as well as those recently reported in the in vivo rat (Feder et al., 1991) and in rat BBMV (Elbert, 1987 quoted by Feder et al., 1991). By contrast, only 10% of the total RF taken up by human intestinal BBMV is membrane bound (Said & Arianas, 1991).

Evaluation of binding and transport as a function of time indicated that at early time points membrane binding was predominant with respect to transport. Membrane binding and transport as well increased up to 10 min, although with different rates, and reached equilibrium thereafter (Fig. 6). A time dependence of binding has also been shown by McNamara, Pepe and Segal (1981) for L-cystine in rat kidney BBMV, by Reisenauer, Chandler and Halsted (1986) and Bhandari, Joshi and McMartin (1988) for folate in pig intestine and rat kidney BBMV, respectively.

RF binding at equilibrium in both intact and shocked BBMV (Fig. 7*A* and *B*) exhibited a saturable component, which indicates the presence of membrane sites specific for RF. This saturable component prevailed at low RF concentrations (<0.3–0.5 $\mu$M), and both $K_d$ values were small and similar for both intact and shocked BBMV. The similarity of the $K_m$ and of the $K_d$ values would suggest that a single membrane site could be responsible for RF binding and transport in vivo.

The specific RF binding component in the membrane was tentatively characterized by preliminary studies of RF molecular group specificity (Fig. 8). The inhibitory effects of RF structural analogues indicated that: (i) the side chain seems to be essential for recognition by the binding sites; (ii) substituents at position 8 of the RF molecule seem to enhance, and those at position 3 to decrease binding. The molecular specificity of the membrane appears to be similar to that of the intracellular phosphorylating enzyme flavokinase (Kasai et al., 1990).

Since RF is transported efficiently across the epithelium from the mucosal to the serosal side (Meinen et al., 1977; Daniel et al., 1983; Said et al., 1985*b*; Middleton, 1990), and the $K_m$ or $K_d$ values observed in the present study are very similar to the apparent $K_m$ or $K_t$ values reported for rat intestinal

preparations in vitro (Daniel et al., 1983; Middleton, 1990) and in vivo (Feder et al., 1991), the binding of RF to specific membrane sites in the rat intestine may account at least in part for the specificity of the entry mechanism in whole tissue preparations, internalization and exit of RF being coupled to intracellular metabolic trapping (phosphorylation) (Choi & McCormick, 1980; Aw, Jones & McCormick, 1983; Hegazy & Schwenk, 1983; Bowers-Komro & McCormick, 1987; Kasai et al., 1988; McCormick, 1989; Bowman et al., 1989) and basolateral membrane events.

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