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Riboflavin uptake by native *Xenopus laevis* oocytes

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Abstract

The existence of a membrane-associated uptake carrier for riboflavin (RF) is demonstrated in *Xenopus* oocytes. Uptake of low (0.017 μM) and high (3 μM) concentrations of RF was linear with time for up to 2 hours, and occurred with little initial binding to oocytes, and little metabolism. Uptake of RF was found to be independent of extracellular pH and Na^+. The initial rate of RF uptake was saturable as a function of concentration with an apparent K_m of 0.41 ± 0.02 μM and a V_{max} of 2.86 ± 0.04 fmol/oocyte per h. Uptake of ^{3}H-RF was inhibited by unlabeled RF and by the structural analogs lumiflavin, isoriboflavin (iso-RF), 8-aminoriboflavin (8-NH_2-RF), 8-hydroxyriboflavin (8-OH-RF), and lumichrome, but was not affected by flavin adenine dinucleotide (FAD), D-ribose or lumazine. Uptake of RF was significantly retarded by the metabolic inhibitor 2,4-dinitrophenol. The sulfhydryl group-modifying reagents *p*-chloromercuriphenylsulfonate (pCMPS), *p*-chloromercuribenzoate (pCMB), *N*-ethylmaleimide and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) all caused significant inhibition in RF uptake. The inhibitory effect of pCMPS was completely reversed by treatment of pCMPS-pretreated cells with reducing agents. While the transmembrane transport inhibitors 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and furosemide had no effect on RF uptake, amiloride and probenecid suppressed RF uptake in a dose-dependent fashion. Closer examination of the inhibition mediated by amiloride showed that it was competitive in nature with an apparent K_i of ~1.8 mM, whereas the inhibition induced by probenecid was nonspecific. Together, these findings indicate that *Xenopus* oocytes possess an endogenous, specific, membrane-associated carrier-mediated uptake system for RF. The results also demonstrate the usefulness of *Xenopus* oocytes as a model system with which to study the RF transport event across biological membranes, which should further our present understanding of RF uptake by various vertebrate cells.

Keywords: Oocyte; Riboflavin uptake; (*X. laevis*)

1. Introduction

Riboflavin (RF) is a water-soluble vitamin that is centrally important to a number of biological processes, including lipid, protein and carbohydrate metabolism [6,17,19]. Mammals can not synthesize RF but obtain the vitamin from diet; the vitamin is then transported into different cells for utilization. Thus, studies by us and others have been focused on understanding the mechanisms of RF transport across different biological membranes [2,9,14,15,18,20--24].

The *Xenopus* oocyte has proven to be an attractive model system for exploring many biological processes at the single cell level, including neurotransmitter receptor function, ion channels, and membrane transport of nutrients [5,8,13,25,28,29]. Furthermore, the oocyte has been widely used for expression of membrane transporters encoded by foreign mRNA such as the intestinal Na^+/glucose cotransporter [13], the hepatic Na^+/bile acid cotransporter [12] and the intestinal high-affinity glutamate transporter [16]. Therefore, through expression of the RF transporter encoded by intestinal RNA, *Xenopus* oocytes might also greatly facilitate the cloning of the mammalian RF transporter(s). This application of oocytes requires a prior detailed knowledge of the oocyte's endogenous RF transport mechanism(s). To determine the suitability of oocytes both as a unicellular in vitro model system for studying transport of RF across biological membranes in general, and ultimately as an expression system for the cloning of mammalian RF transporters, we have characterized the mechanism of RF uptake of the *Xenopus* oocyte.

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2. Materials and methods

^{3}H-RF (specific activity 44 Ci/mmol) was obtained from American Radiolabeled Chemicals, St. Louis, MO. Unlabeled RF and RF-related compounds were purchased from Sigma (St. Louis, MO). Isoriboflavin (iso-RF), 8-aminoriboflavin (8-NH_2-RF) and 8-hydroxyriboflavin (8-OH-RF) were a generous gift from Dr. Vincent Massey of the University of Michigan School of Medicine at Ann Arbor, MI. All other chemicals and reagents used in this study were obtained commercially and were of analytical quality. The radiochemical purity of ^{3}H-RF was found to be greater than 98% as assessed by thin layer chromatography using precoated silica gel plates and a solvent system of 2% anhydrous Na_2HPO_4 [10]. ^{3}H-RF was periodically checked for purity and was repurified using the same chromatographic procedure when purity was less than 95%.

Oocytes used in these studies were obtained from healthy *Xenopus laevis* clawed frogs purchased from Xenopus I (Ann Arbor, MI) following methods described elsewhere [11]. Briefly, frogs were anesthetized by immersion in a solution of ethyl-3-aminobenzoate (MS-222; Sigma, St. Louis, MO) at a concentration of 1.5 g/l. Portions of the ovary were surgically removed from selected donors through a small incision in the abdomen, and then treated with 1.4 mg/ml of collagenase (Type A; Boehringer-Mannheim, Indianapolis, IN) for 30 to 40 min in Ca^{2+}-free ovarian Ringer solution (in mM: 96 NaCl; 2 KCl; 1 $MgCl_2$; 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.5). After collagenase treatment, cells were rinsed several times in Ca^{2+}-free medium, and then transferred to Barth's solution (in mM: 88 NaCl, 1 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 2.4 $NaHCO_3$, 10 Hepes, pH 7.6). Stage V-VI oocytes were then manually defolliculated and used for uptake studies by incubating 5–8 cells in 200 μl of Ringer solution (in mM: 120 NaCl, 2 KCl, 1.8 $CaCl_2$, 5 Hepes, pH 7.0) containing 17 nM of labeled and different concentrations of unlabeled RF and/or other compounds. Unless otherwise stated, all uptake incubations were performed at room temperature. At the end of incubation, uptake was terminated by the addition of 5 ml of ice-cold Ringer solution followed by four successive washes with the same buffer. Cells were then transferred individually to scintillation vials, dissolved in 100 μl of 1 N NaOH at 70°C for 1 h. Basic cell extracts were neutralized with 100 μl of 1 M HCl, following which 5 ml of scintillation fluid was added and radioactivity was counted in an LS100C Beckman scintillation counter (Beckman Instruments, Irvine, CA). Uptake results presented in this paper are from multiple oocytes obtained from different donors, and are expressed as mean ± S.E. in fmol/oocyte per unit time unless otherwise stated. The V_{max} and K_m of the uptake process were determined using a computer program model of the Michaelis-Menton equation as described previously [30]. Data were analyzed using the Student's *t*-test and regression analysis. No significant differences in RF uptake per oocyte were observed when one or eight oocytes were incubated together in 200 μl of incubation buffer. Oocytes taken from different frogs showed some quantitative, but not qualitative, variability in RF uptake; however, the results from individual donors were consistently homogenous.

For studies of ^{3}H-RF binding to membranous fraction of oocytes, 100 oocytes were homogenized in 5 ml of Ringer solution containing Aprotinin (20 μg/ml) and phenylmethylsulfonyl fluoride (0.1 mM). The homogenate was centrifuged at 100 000 × *g* for 1 h at 4°C, and the pellet was suspended in 100 μl of the homogenization buffer. Half of this material was then incubated with 450 μl of Ringer solution containing ^{3}H-RF (40 nM) and the other half was incubated in the presence of ^{3}H-RF (40 nM) plus unlabeled RF (90 μM). Incubation was done at room

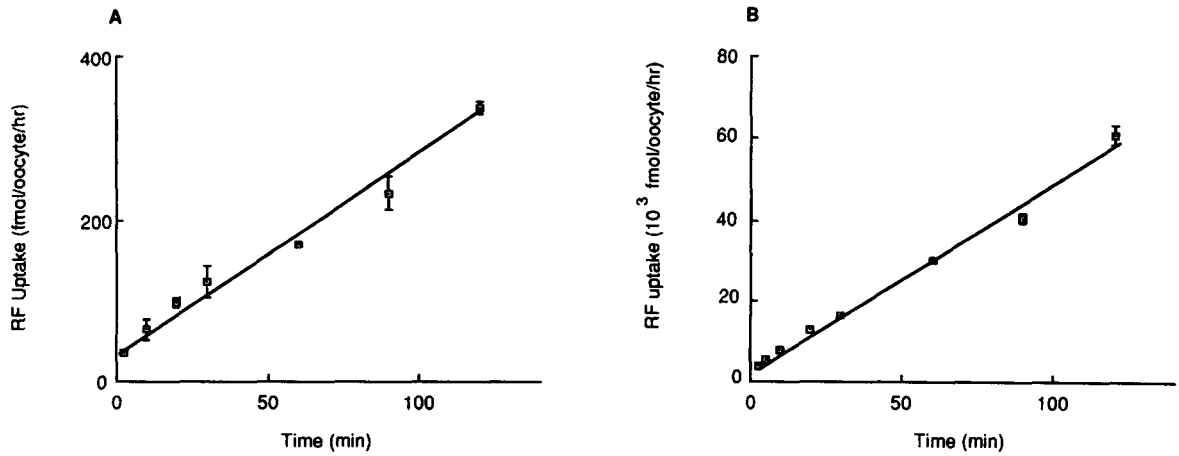

Fig. 1. Time-dependent uptake of low (A: 0.017 μM) and high (B: 3.0 μM) concentrations of RF by *Xenopus* oocytes. Cells were incubated in Ringer buffer at room temperature for varying periods of time after the addition of labeled and unlabeled RF to the incubation medium at the start of incubation. Results are mean ± S.E. of 5–10 separate uptake determinations. A: $y = 2.4x + 34$; B: $y = 4.24x + 385.3$.

temperature for 30 min. Incubation was terminated by a rapid filtration technique as described by us before [20,22] onto 0.45 μm nitrocellulose filters (Millipore, Bedford, MA) followed by washing with 5 ml of ice-cold Ringer solution. Radioactivity was then determined by liquid scintillation counting.

3. Results

3.1. General aspects of RF uptake

The ability of oocytes to take up low (17 nM) and high (3 μM) concentrations of RF was tested. As shown in Fig. 1, the uptake of RF was linear at both concentrations for up to 2 h ($r = 0.98$ and 0.99, respectively), and occurred at a rate of 2.4 and at 424.6 fmol/oocyte per min at low and high RF concentrations, respectively. All subsequent uptake studies were carried out for 1 hr because the rate of RF uptake at this time was representative of the initial rate of uptake, and was readily detectable.

To determine whether the uptake of RF in follicle-enclosed cells was different than in defolliculated cells, RF uptake was tested in both cases. It was found that RF uptake in follicle-enclosed cells was significantly less ($P \leq 0.05$) than in defolliculated cells (72 ± 2 versus 90 ± 4 fmol/oocyte per h, respectively), indicating that follicle cell layers surrounding the oocyte may act as a barrier to the uptake of ^{3}H-RF, and that the major site of RF uptake is the oocyte itself.

To verify the identity of the radiolabeled compound taken up into the oocyte, extracts of cells were subject to thin layer chromatography. For this, oocytes were incubated in Ringer solution containing 17 nM of ^{3}H-RF for 1 h. At the end of the incubation, cells were homogenized, and extracted with 95% ethanol. Particulate material was pelleted by centrifugation at $14\,000 \times g$ for 10 min. The supernatant was applied to a silica gel-precoated TLC plate, and run in a solvent system of 2% anhydrous Na_2HPO_4. Stock ^{3}H-RF was run simultaneously as a standard. Results indicated that ~93% of the extracted radioactivity was in the form of intact RF.

To determine whether the RF uptake mechanism in the native oocyte was sensitive to extracellular pH, the effect of varying the pH of the incubation buffer on ^{3}H-RF (17 nM) uptake was tested. Varying extracellular pH from 5.5 to 8.5 did not affect on RF uptake (uptake of 82 ± 2, 95 ± 4, 84 ± 5, 77 ± 2 and 84 ± 3 fmol/oocyte per h, at pH 5.5, 6.0, 7.0, 7.5 and 8.5, respectively). In contrast, the RF uptake mechanism was found to be temperature-dependent. For this experiment, cells were incubated for 1 h at either room temperature or at 4°C. ^{3}H-RF (17 nM) uptake was found to be inhibited approx. 4-fold in cells incubated at 4°C compared to the control (18 ± 2 vs. 110 ± 15 fmol/oocyte per h).

3.2. Na^+ dependence of RF uptake

The dependence of RF uptake in oocytes on extracellular Na^+ was investigated by isosmotically replacing Na^+

Table 1
Effect of unlabeled RF and related compounds on the uptake of ^{3}H-RF by *Xenopus* oocytes

Condition	Concentration (μM)	Uptake (fmol/oocyte per h)	P values *
(A)			
Control		101 ± 8 (9) **	
Unlabeled RF	1	68 ± 2 (5)	< 0.01
	10	32 ± 3 (5)	< 0.01
	25	16 ± 0.8 (5)	< 0.01
Lumiflavin	1	79 ± 5 (4)	< 0.01
	10	27 ± 2.6 (9)	< 0.01
	25	11 ± 0.9 (9)	< 0.01
Iso-RF	1	100 ± 6 (8)	NS
	25	20 ± 1.2 (9)	< 0.01
8-NH_2-RF	1	104 ± 0.8 (9)	NS
	25	24 ± 4 (8)	< 0.01
8-OH-RF	1	108 ± 4 (9)	NS
	25	64 ± 8 (9)	< 0.01
Lumichrome	1	105 ± 7.5 (9)	NS
	25	61 ± 8 (9)	< 0.01
(B)			
Control		117 ± 5 (9)	
FAD	25	115 ± 7 (5)	NS
Lumazine	100	125 ± 2 (7)	NS
D-Ribose	100	124 ± 4 (9)	NS

Oocytes were incubated at room temperature for 1 h in Ringer buffer in the absence (control) or the presence of the compound under study and ^{3}H-RF (17 nM).

* P values were calculated using the Student's t-test; comparisons were made relative to simultaneously-performed controls. NS means not significant (i.e., $P > 0.05$).

** Number of oocytes.

Table 2
Effect of metabolic inhibitors on ^{3}H-RF uptake by *Xenopus* oocytes

Condition	Uptake (fmol/oocyte per h)	P values *
Control	171 ± 7 (8) **	
2,4-Dinitrophenol (5 mM)	74 ± 2 (8)	< 0.01
Ouabain (1 mM)	177 ± 6 (8)	NS
Gramicidin (0.01 mM)	161 ± 4 (8)	NS

Oocytes were pre-incubated at room temperature for 10 min in Ringer buffer in the absence (control) or in the presence of the compound under investigation. ^{3}H-RF (17 nM) was then added and incubation continued for 1 h.

* P values were calculated using the Student's t-test; comparisons were made to simultaneously-performed controls. NS means not significant (i.e., $P > 0.05$).

** Number of oocytes.

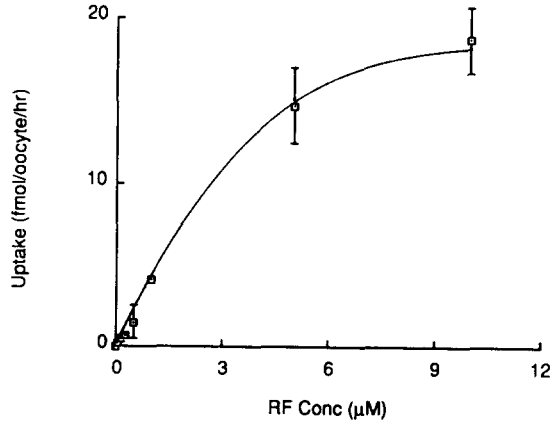

Fig. 2. Initial rate of uptake of RF by *Xenopus* oocytes as a function of concentration. Oocytes were incubated at room temperature for 1 h in Ringer buffer containing different concentrations of labeled and unlabeled RF. Results are mean ± S.E. of 5–10 separate uptake determinations. Uptake kinetic parameters were determined as described in Materials and methods.

ions in the incubation medium with other monovalent cations, including K^+, choline, Tris and NH_4^+, and with the inert, uncharged compound mannitol. In all cases, the replacement of Na^+ with different monovalent cations, or with mannitol, did not significantly affect RF uptake (uptake of 90 ± 4, 84 ± 4, 91 ± 4, 81 ± 3, 92 ± 9 and 81 ± 2 fmol/oocyte per h for control (i.e., in the presence of Na^+) and with Na^+ substituted by K^+ choline, Tris, NH_4^+ and mannitol, respectively).

3.3. Uptake of RF as a function of concentration

In these experiments, RF uptake by oocytes was examined as a function of increasing RF concentration ranging from 17 nM to 100 μM. Incubations were performed in Ringer buffer for 1 h at room temperature, as described in Materials and methods. Uptake of RF was found to include a saturable component. Uptake by this component was determined by subtracting non-specific uptake (calculated from the slope of the uptake line between the point of origin and 100 μM RF) from the total uptake (Fig. 2). The kinetic parameters of the carrier-mediated uptake process were then determined using a non-linear method previously described by Wilkinson [30]. An apparent K_m and V_{max} of 0.41 ± 0.02 μM and 2.86 ± 0.04 fmol/oocyte per h were found, respectively.

3.4. Effect of RF structural analogs on ^{3}H-RF uptake

To characterize the degree of specificity of the RF uptake mechanism in the oocyte, the effect of RF structural analogs and related compounds on the initial rate of ^{3}H-RF (17 nM) uptake was examined. The results (Table 1) showed that unlabeled RF, lumiflavin, iso-RF, 8-NH_2RF, 8-OH-RF and lumichrome inhibited the uptake of ^{3}H-RF

Table 3
The effect of sulfhydryl group-modifying reagents on uptake of ^{3}H-RF by *Xenopus* oocytes

Condition	Uptake (fmol/oocyte per h ± S.E.)	P values *
(A)		
Control	107 ± 18 (9) **	
pCMPS (0.5 mM)	33 ± 4 (10)	< 0.01
pCMB (0.5 mM)	18 ± 1 (7)	< 0.01
N-Ethylmaleimide	16 ± 1 (7)	< 0.01
NBD-Cl (0.5 mM)	60 ± 9 (5)	< 0.01
(B)		
Control	122 ± 11 (9)	
pCMPS (0.1 mM)	70 ± 6 (11)	< 0.01
pCMPS (0.1 mM) then dithiothreitol	122 ± 11 (9)	NS
pCMPS (0.1 mM) then 2-mercaptoethanol	120 ± 12 (8)	NS
pCMPS (0.5 mM) then 2,4-dimercaptopropanol	117 ± 6 (8)	NS

Oocytes were pretreated with the compound under study for 10 min at room temperature in 200 μl of Ringer buffer. For reversibility studies, cells were incubated for an additional 10 min with 10 mM of either dithiothreitol, 2-mercaptoethanol, 2,4-dimercaptopropanol. Uptake was initiated by the addition of ^{3}H-RF (17 nM).

* P values were calculated using the Student's t-test; comparisons were made relative to simultaneously-performed controls. NS means not significant (i.e., $P > 0.05$).

** Number of oocytes.

to different degrees. In contrast, FAD, lumazine, and D-ribose did not significantly affect ^{3}H-RF uptake.

3.5. Effect of metabolic inhibitors and group specific reagents on RF uptake

To determine if metabolic energy is required for RF uptake by oocytes, the effect of 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, on ^{3}H-RF (17 nM) uptake was tested. This compound significantly suppressed RF uptake compared to control (Table 2). We also examined the effect of ouabain (1 mM), a Na^+/K^+-ATPase inhibitor, and that of gramicidin (0.01 mM), a Na^+ ionophore. Neither compound, however, showed an effect on substrate uptake (Table 2).

In order to gain some insight into the structure-activity relationships of the putative protein involved in the transmembrane uptake of RF, we examined the effect of sulfhydryl group-specific reagents on the uptake of ^{3}H-RF by oocytes. We tested the effects of pCMPS, *N*-ethylmaleimide and NBD-Cl on ^{3}H-RF (17 nM) uptake by treating oocytes with the agent under study for 10 min prior to performing the RF uptake assay. As shown in Table 3, pretreatment of oocytes with any of these compounds significantly inhibited RF uptake compared to controls. Furthermore, the 43% inhibition induced by pCMPS was fully reversed when pCMPS-treated cells were subsequently incubated in the presence of the reducing agents dithiothreitol, 2-mercaptoethanol, or 2,4-dimercaptopropanol before RF uptake assay.

3.6. Effect of membrane transport inhibitors on RF uptake

To further characterize the mechanism by which RF is taken up by oocytes, the effect of the membrane transport inhibitors SITS, DIDS, furosemide, amiloride and probenecid was tested. As shown in Table 4, SITS, DIDS and furosemide had no noticeable effect on ^{3}H-RF uptake. In contrast, amiloride caused a significant inhibition of RF uptake, but did not affect the uptake of the structurally unrelated biotin which is taken up by *Xenopus* oocytes by a specialized carrier-mediated process (uptake of 16 nM of biotin was 83 ± 13 and 104 ± 19 fmol/oocyte per h for control and amiloride-treated cells, respectively). Analysis of amiloride inhibition of RF uptake by the Dixon method indicated that the inhibition is competitive in nature with a K_i value of ~ 1.8 mM (Fig. 3). Similarly, probenecid (5 mM) was found to significantly inhibit ^{3}H-RF uptake (Table 4), however, this inhibition was not limited to RF but also affected the uptake of biotin (uptake of 16 nM biotin was 93 ± 14, 20 ± 5 and 5 ± 1 fmol/oocyte per h for control and cells treated with 1 and 5 mM probenecid, respectively).

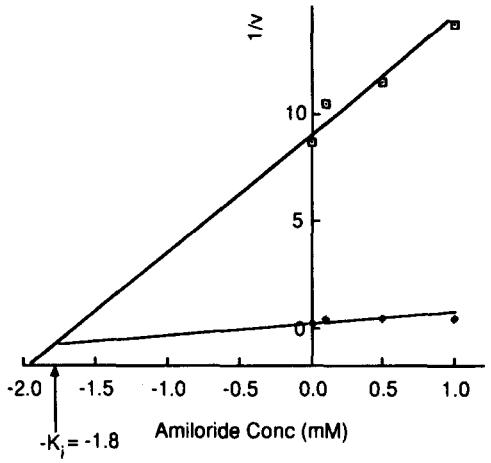

Fig. 3. Dixon plot of the effect of different concentrations of amiloride on the uptake of 0.017 μM (open symbols) and 1.7 μM RF (closed symbols) by *Xenopus* oocytes. Each data point represents the mean ± S.E. of 5–10 separate uptake determinations.

Table 4
The effect of membrane transport inhibitors on ^{3}H-RF uptake by *Xenopus* oocytes

Condition	Uptake (fmol/oocyte per h)	*P* values *
Control	117 ± 13 (9) * *	
SITS (1 mM)	115 ± 12 (9)	NS
DIDS (1 mM)	118 ± 4 (9)	NS
Furosemide (0.1 mM)	100 ± 20 (7)	NS
Amiloride (1 mM)	70 ± 2 (4)	< 0.01
Probenicid (1 mM)	16 ± 1 (4)	< 0.01
(5 mM)	5 ± 4 (5)	< 0.01

Oocytes were incubated at room temperature for 1 h in Ringer buffer containing 17 nM ^{3}H-RF and the compound being tested.
* *P* values were calculated using the Student's *t*-test; comparisons were made to simultaneously-performed controls. NS means not significant (i.e., $P > 0.05$).
* * Number of oocytes.

4. Discussion

The object of this study was to characterize the RF uptake mechanism in native *Xenopus* oocytes. The uptake of both low and high concentrations of RF was found to be linear with time for at least 2 h, and occurred with little metabolic alteration of the RF molecule. RF uptake was markedly inhibited (~ 84%) during incubation at low temperature (4°C) compared to uptake at room temperature demonstrating the temperature-dependence of the uptake process. RF uptake was pH- and Na^+-independent, as demonstrated by the lack of effect of incubation buffer pH and by the study on the lack of effect of Na^+ replacement with K^+, choline, Tris, NH_4^+ or mannitol, on the substrate uptake, respectively. This conclusion was further supported by the findings that neither ouabain, a cardiac glycoside inhibitor of Na^+/K^+-ATPase, nor gramicidin, a monovalent cation ionophore, had any effect on RF uptake by oocytes. The finding that the RF uptake process in *Xenopus* oocytes is Na^+-independent is similar to the

recent finding by us and others on the substrate transport across plasma membranes of mammalian cells [2,22–24], and may suggest a universal property of this nutrient transport process.

The process of RF uptake by the oocyte was found to be saturable, with an apparent K_m and V_{max} of 0.41 ± 0.02 μM and 2.86 ± 0.04 fmol/oocyte per h, respectively, indicating that RF uptake may occur via a carrier-mediated system. This conclusion was further supported by the findings that a variety of RF structural analogs inhibited the uptake of ^{3}H-RF. The degree of inhibition by the unlabeled RF and structural analogs varied and in general decreased in the following order: RF ≈ lumiflavin; iso-RF ≈ 8-NH_2-RF; 8-OH-RF ≈ lumichrome. These findings show that different parts of the RF molecule affect ligand–protein interactions differently. The inability of lumazine and D-ribose to inhibit ^{3}H-RF uptake suggests that the uptake process is specialized in nature.

Uptake of RF was found to be dependent on intracellular energy metabolism as indicated by the significant inhibition in the substrate uptake by the metabolic inhibitor 2,4-dinitrophenol. Experiments with sulfhydryl-group modifying reagents provided some insight into possible relationships between the structure and the function of the putative RF transmembrane carrier. It was found that pCMPS, pCMB, *N*-ethylmaleimide and NBD-Cl severely inhibit the uptake of RF by oocytes. Furthermore, the inhibitory effect of pCMPS on RF uptake was completely reversed by treating pCMPS-pretreated cells with the reducing agents dithiothreitol, 2-mercaptoethanol or 2,4-dimercaptopropanol. This further confirms that pCMPS was indeed interacting with sulfhydryl groups. It is interesting to note that the membrane-permeant mercuri-derivative pCMB [26] caused only a slightly higher inhibition in RF uptake compared to the membrane-impermeant mercuri-derivative pCMPS [26], indicating that most of the sulfhydryl groups with which these compounds are interacting are located at the exofacial domain of the oocyte membrane.

It has been suggested that RF behaves as an anion when it comes to transport across biological membranes [15,17,27]. Therefore, the effects of the anionic transport inhibitors SITS and DIDS on RF uptake were tested. These compounds were ineffective in blocking RF uptake, arguing against that possibility. Similar observations have been made on the effect of these compounds on RF transport across mammalian intestinal brush-border membranes [23]. Although the anionic probenecid significantly reduced RF uptake by oocytes, this inhibition was apparently nonspecific in nature because probenecid also inhibited the uptake of biotin, a compound whose structure is unrelated to RF, and whose transport is mediated by a different specialized carrier-mediated system [25].

The pyrazine diuretic amiloride demonstrated a specific and competitive inhibition of the RF uptake process. This inhibition was similar to that demonstrated by amiloride for RF uptake in the human-derived Caco-2 cultured intestinal epithelial cells [24]. Amiloride is known to inhibit a number of Na^+-dependent transport processes in epithelial cells [3,7,28], presumably through a direct interaction with the transport protein. The effect produced by amiloride on the Na^+-independent RF transport in the oocyte might therefore likewise reflect a direct interaction with the RF transporter. However, further study will be needed to clarify the mechanism of this inhibition in the oocyte.

It is well known that oocytes of different species contain an intracellular RF binding protein [1,4]. Thus, it might be argued that the findings observed in the present study are due to binding of RF to such a putative intracellular binding site in *Xenopus* oocytes, and not to the existence of a specific membrane-associated RF uptake carrier. This possibility, however, appears to be unlikely because: (1) RF is a water-soluble substrate which must first cross the lipid plasma membrane of the oocyte in order to reach any intracellular binding site; translocation of such a substrate by simple diffusion across the cell membrane is thermodynamically unfavorable; (2) our results on ^{3}H-RF binding to membranous fraction of fractionated oocytes indicated the existence of a specific RF binder (unpublished observation); (3) binding of a ligand to its target is usually temperature-independent, while the uptake process of RF by oocytes was highly temperature-dependent; (4) the apparent K_m value of the RF uptake process in *Xenopus* oocytes (414 nM) is more than 300-fold greater than the apparent K_d value (14 nM) reported for the egg-white RF binding protein [4]; (5) the RF derivative FAD, which binds to the RF binding protein of avian egg-white ($K_d = 14$ μM [4]), did not affect the uptake of RF by *Xenopus* oocytes, and (6) RF uptake was severely inhibited by the membrane-impermeant sulfhydryl group reagent pCMPS, an inhibition which was reversed by reducing agents.

In summary, this study indicated that RF uptake by *Xenopus* oocytes occurs through a pH- and Na^+-independent, specialized membrane-associated carrier-mediated process that is temperature- and energy-dependent. These features of RF uptake by *Xenopus* oocytes are similar to those seen in human-derived Caco-2 intestinal cells, suggesting that *Xenopus* oocytes may represent an accessible in vitro, single-cell model system with which to identify the finer details of RF uptake mechanisms across biological membranes.

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