Uptake and Metabolism of Riboflavin-5′-α-D-Glucoside by Rat and Isolated Liver Cells[1,2]

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ABSTRACT The effect of riboflavin α-D-glucoside, isolated from rat liver, on the uptake of riboflavin was studied using freshly isolated rat liver cells. The transport characteristics and metabolic fate of the glucoside were also determined using the radioactive compound. The initial (1-min) uptake of 1 μmol/L [^{3}H]riboflavin glucoside (2.90 ± 0.29 pmol/10^6 cells) was higher than that of 1 μmol/L [^{3}H]riboflavin (1.35 ± 0.30 pmol/10^6 cells). However, the accumulation of glucoside after 60 min was significantly lower than that of riboflavin. The presence of up to 30 μmol/L glucoside had no significant effect on the initial uptake of [^{3}H]riboflavin (3 μmol/L, 10^9 cells/L). Measurement of the kinetic parameters for glucoside gave an apparent K_t value of 83.4 ± 12.4 μmol/L and a V_{max} of 208.6 ± 17.9 pmol/(10^6 cells·min). Decreases in the temperature of incubation decreased uptake rate. Replacement of Na^+ with other monovalent cations did not affect uptake. The presence of D-glucose (1 μmol/L to 5.5 mmol/L) had no inhibitory effect on uptake of 1 μmol/L [^{3}H]riboflavin glucoside. The results indicate that the transport of riboflavin glucoside may not involve the transport mechanisms for riboflavin or D-glucose. Metabolic studies with isolated hepatocytes showed that the glucoside was hydrolyzed to yield riboflavin upon entry into the cell. The vitaminic efficiency of this compound was tested by feeding it to growing male rats. These experiments indicate that the glucoside and free riboflavin are comparable sources of the vitamin. J. Nutr. 125: 2194–2198, 1995.

INDEXING KEY WORDS:

- *riboflavin glucoside* • *rat hepatocytes*
- *flavin transport* • *flavin metabolism*
- *riboflavin*

Homogenates of rat liver and aqueous extracts of acetone powder prepared from rat liver were shown to produce riboflavin-5′-α-D-glucoside (RF-glucoside) when incubated with riboflavin (Whitby 1952). Riboflavin-glucoside was also detected in the urine of rats after oral administration of [2-^{14}C]riboflavin (Ohkawa et al. 1983). Thus it is probable that this metabolite is produced in the liver and may have a significant biological role, especially in the wake of findings that pyridoxine-5′-β-D-glucoside (PN-glucoside) competitively inhibits the uptake of vitamin B-6 into isolated liver cells wherein the glucoside undergoes a rate-limiting release of vitamin (Zhang et al. 1993). Hence, it was an objective of this study to determine the effect, if any, that RF-glucoside may have on the uptake of riboflavin, using liver cells freshly isolated from rats. Our aim was further to characterize the transport and metabolic aspects of RF-glucoside using the isolated hepatocytes. Finally, it was of interest to determine the effect of a diet with riboflavin glucoside instead of riboflavin on the body weight gain of rats. Feeding experiments were conducted using a riboflavin-deficient diet with or without a supplement of riboflavin or RF-glucoside.

MATERIALS AND METHODS

Chemicals. Riboflavin and collagenase (Type IV) were purchased from Aldrich Chemical (Milwaukee, WI) and Sigma Chemical (St. Louis, MO), respectively. [^{3}H]Riboflavin (1.22 GBq/μmol) was purchased from Moravek Biochemicals (Brea, CA). All other chemicals and reagents were of analytical grade. Riboflavin-5′-α-D-glucoside was prepared as described earlier (Whitby 1971), except that acetic acid rather than formic acid was used to elute flavins from the cellulose columns.

Animals and diets. Male Sprague-Dawley rats (SASCO, Omaha, NE) weighing 200–300 g were fed a nonpurified diet (Purina 5001 Rodent Laboratory Chow, Purina Mills, St. Louis, MO) and had free access to water. These were the animals from which

[1]Supported by the National Institutes of Health (DK 43005) and the Coca-Cola Foundation.

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Manuscript received 28 November 1994. Initial review completed 9 January 1995. Revision accepted 9 March 1995.

liver cells were obtained. For the feeding experiments, weaned male Sprague-Dawley rats weighing 50–60 g were divided into four experimental groups. For 3 wk, one group had free access to a powdered riboflavin-deficient synthetic diet[4] (Harlan Teklad, Madison, WI). The remaining groups were fed the riboflavin-deficient diet supplemented with *1*) 10 μg riboflavin/15 g, *2*) 30 μg riboflavin/15 g (riboflavin-sufficient), or *3*) 43 μg riboflavin-glucoside/15 g, which contains the same molar equivalence as 30 μg of riboflavin. Each rat was weighed every other day. All rats used in these experiments were cared for in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (NRC 1985).

Cellular uptake studies. Hepatocytes were isolated as described earlier (Zhang and McCormick 1992). Routinely, the cells were at least 85% viable as ascertained by exclusion of 0.2% trypan blue. Incubations (10^9 cells/L) were performed in Krebs-Henseleit buffer supplemented with 12.5 mmol/L HEPES, pH 7.4, at 37°C (unless otherwise stated) in a shaking water bath. The concentrations of riboflavin and RF-glucoside and the incubation times were varied as desired. For the studies on the effect of replacing Na^+ with other monovalent cations, the concentration of Na^+ in the incubation mixture was reduced from 142 mmol/L to 43 mmol/L. The uptake of [^{3}H]riboflavin and [^{3}H]RF-glucoside was monitored as described earlier (Aw et al. 1983) using the membrane filtration method. Radioactivity was determined using a Packard Tri-Carb 1900-TR Scintillation Counter (Packard Instrument, Downers Grove, IL). Values of K_t (equivalent to K_m) and V_{max} were obtained by fitting the data to the Michaelis-Menten equation, using the nonlinear least-squares method.

Cellular metabolism. Freshly isolated hepatocytes (25×10^9 cells/L) were incubated with 1 μmol/L riboflavin or RF-glucoside as described above. Aliquots were withdrawn after different time intervals, sonicated to disrupt the cells, heated and centrifuged to precipitate most of the proteins. Aliquots were applied on a TLC plate and developed using the solvent system: *n*-butanol-acetic acid-water (4:1:5, upper phase). Bands corresponding to FAD, FMN, RF-glucoside and riboflavin were scraped off and counted using the liquid scintillation counter.

Statistics. Uptake was measured with duplicate or triplicate samples from at least three cell preparations. Metabolic studies were based on two cell preparations. Data are expressed as means ± SD. Data were analyzed with the SPSS program (SPSS Inc., Chicago, IL). Statistical values of differences between means were determined by unpaired Student's *t* test. The differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The importing of water-soluble vitamins by mammalian cells is typically facilitated within physiologic ranges (McCormick and Zhang 1993). The transporters involved are relatively specific for different vitamins. This seems to be the case for the uptake of vitamin B-6 by the parenchymal hepatocytes at physiological concentration (Kozik and McCormick 1984). Pyridoxine-5′-β-D-glucoside was shown to inhibit the uptake of pyridoxine (Zhang et al. 1993) by freshly isolated liver cells, suggesting that the same transporter is involved in the transport of pyridoxine and PN-glucoside. Entry of riboflavin into hepatocytes was shown with rats to occur predominantly by a Na^+-independent, facilitated diffusion process followed by rapid trapping by flavokinase-catalyzed phosphorylation of FMN (Aw et al. 1983). Recent studies using Caco-2 human intestinal epithelial cells have also indicated that the uptake is Na^+ independent (Said and Ma 1994).

In the present work, we first examined the effect of various concentrations of RF-glucoside on the transport of riboflavin into isolated rat hepatocytes. The uptake of 3 μmol/L [^{3}H]riboflavin in the presence [5.15 ± 0.46 pmol/(10^6 cells·min)] or absence [5.24 ± 0.90 pmol/(10^6 cells·min)] of 1 to 30 μmol/L unlabeled riboflavin glucoside indicated no inhibition by the glucoside. The effect of RF-glucoside on the uptake of riboflavin over a period of time was also studied, using an equimolar mixture (3 μmol/L) of riboflavin and RF-glucoside (**Fig. 1**). No inhibition in the uptake of riboflavin was observed, and the accumulation at the end of 60 min was comparable, both in the absence and presence of RF-glucoside.

Because the solubility of RF-glucoside is much greater than that of riboflavin, it was suggested that this may be a natural derivative of importance in the transport of the relatively insoluble vitamin (Whitby 1954). Preliminary studies indicated that RF-glucoside is indeed transported into the cells. The uptake of RF-glucoside over a period of time was studied next. The results are shown in **Figure 2**. The uptake of an equivalent amount of riboflavin was used for comparison. The initial (1 min) uptake of 1 μmol/L RF-glucoside (2.90 ± 0.29 pmol/10^6 cells) was higher than that of 1 μmol/L riboflavin (1.35 ± 0.30 pmol/10^6 cells). However, the accumulation of glucoside after an hour was significantly lower. Because RF-glucoside does not inhibit the uptake of riboflavin, it probably enters the cell by an alternate route, bypassing the riboflavin transporter, which has been shown to exhibit relative specificity for the flavin structure in both liver (Aw et al. 1983) and proximal tubular cells of the kidney (Bowers-Komro and McCormick 1987).

[4]Composition of riboflavin-deficient diet: 56% sucrose, 19.3% vitamin-free casein, 10% cornstarch, 5% vegetable oil, 5% cellulose, 0.3% DL-methionine, 3.5% mineral mixture (AIN-76) and vitamin mixture, which is an AIN-76 modification minus riboflavin.

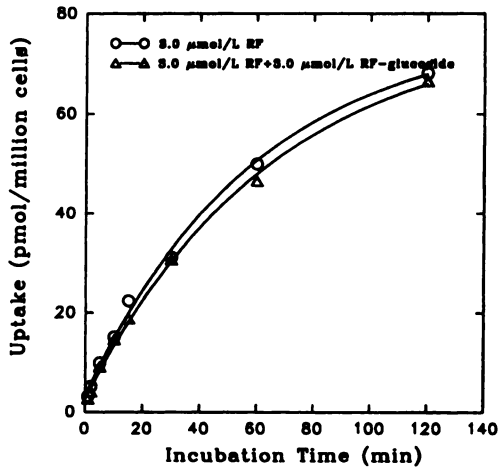

FIGURE 1 Time course of [^{3}H]riboflavin (RF) (3.0 μmol/L) uptake in the absence and presence of riboflavin-5′-α-D-glucoside (RF-glucoside). Isolated rat liver cells (10^9 cells/L) were incubated in the absence and presence of 3.0 μmol/L RF-glucoside. Experimental points are means of at least three cell preparations.

It is conceivable that the entry of the glucoside is facilitated by a glucose transporter because of the presence of the α-D-glucose moiety. The main transporter of glucose in hepatocyte is called GLUT-2 (Pessin and Bell 1992). This transporter remains associated with the plasma membrane and is not translocated to intracellular membranous vesicles. The K_m for glucose being transported into hepatocytes is 15 to 20 mmol/L. To find out whether the glucose transporter is involved in the entry of RF-glucoside, RF-glucoside was incubated with isolated hepatocytes in the presence of various concentrations of glucose. Specifically, the 1-min uptake of 1 μmol/L [^{3}H]riboflavin glucoside in the presence or absence of 1 μmol/L to 5.5 mmol/L D-glucose was measured and found to be substantially the same, with no evidence of inhibition by the latter. Neither was inhibition observed at the end of 15 min. Thus the entry of RF-glucoside apparently is not via the glucose transporter.

Because RF-glucoside does not compete with riboflavin for entry, and because glucose does not interfere with entry of the glucoside into liver cells, it is likely that the transporter systems used for these latter nutrients are not involved in delivery of the glucoside.

The effect of concentration of RF-glucoside on its uptake is shown in **Figure 3**. The uptake demonstrated saturation kinetics. Measurements of the kinetic parameters yielded an apparent K_t value of 83.4 ± 12.4 μmol/L and V_{max} of 208.6 ± 17.9 pmol/(10^6 cells·min). The uptake process was significantly affected by temperature, as typical for facilitated transport. At the end of 1 min, the uptakes at 25°C and 4°C were 90 and 28%, respectively, of the uptake at 37°C. At the end of 15 min, the values at 25°C and 4°C were 78 and 34%, respectively, of the control at 37°C. Decreasing Na^+ in the incubation mixture by replacement with other monovalent cations, namely choline, Li^+ or K^+ (chloride salts), did not affect uptake of RF-glucoside (1 μmol/L). Hence, as was previously reported for riboflavin (Aw et al. 1983), Na^+-dependent active transport does not seem to be involved.

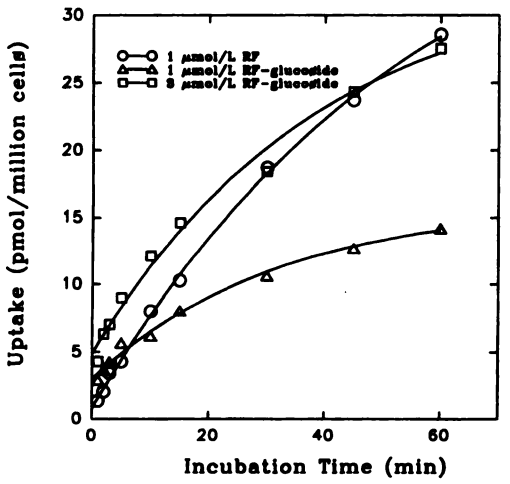

FIGURE 2 Effect of incubation time on the transport of riboflavin (RF) and riboflavin-5′-α-D-glucoside (RF-glucoside) by isolated liver cells. Incubation was performed as described in Materials and Methods with 1.0 μmol/L [^{3}H]RF, 1 μmol/L [^{3}H]RF-glucoside and 3.0 μmol/L [^{3}H]RF-glucoside. Experimental points are means from at least three cell preparations.

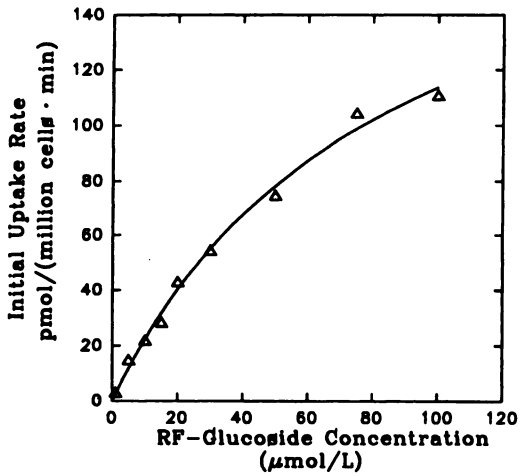

FIGURE 3 Dependence of initial rate of riboflavin-5′-α-D-glucoside (RF-glucoside) uptake on its concentration. Isolated rat liver cells were incubated with the substrate for 1 min at 37°C as described in Materials and Methods. The data shown were obtained from at least three cell preparations.

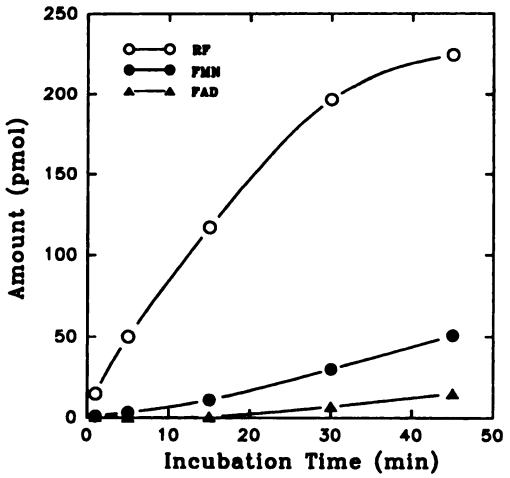

FIGURE 4 Metabolism of riboflavin (RF) in isolated rat hepatocytes. Isolated rat liver cells (25×10^9 cells/L) were incubated with 1 μmol/L [^{3}H]RF. The amounts of RF, FMN and FAD present in the cells (12.5×10^6) were determined as described in Materials and Methods. Experimental points are means of two cell preparations.

The metabolic conversion of riboflavin and RF-glucoside, in the cells, over 45 min are shown in **Figures 4** and **5**, respectively. At the end of 45 min, ~25 and 45% of riboflavin and RF-glucoside, respectively, were metabolized. Riboflavin-glucoside is hydrolyzed to yield the free vitamin, which is phosphorylated to yield FMN and FAD. The hydrolysis is presumably mediated by nonspecific α-glucosidases known to occur in mammalian tissues (Yamamoto et al. 1990). Kamimura et al. (1992) reported the formation of α-glucoside derivatives of several xenobiotics by rat liver α-glucosidases.

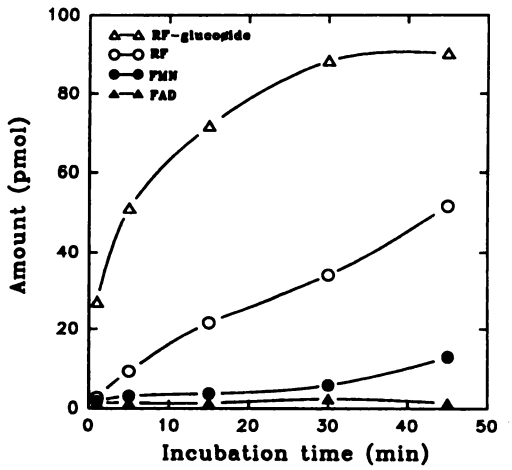

FIGURE 5 Metabolism of riboflavin-5′-α-D-glucoside (RF-glucoside) in isolated rat hepatocytes. Isolated rat liver cells (25×10^9 cells/L) were incubated with 1 μmol/L [^{3}H]RF-glucoside. The amounts of RF-glucoside, riboflavin (RF), FMN and FAD present in the cells (12.5×10^6) were determined as described in Materials and Methods. Experimental points are means of two cell preparations.

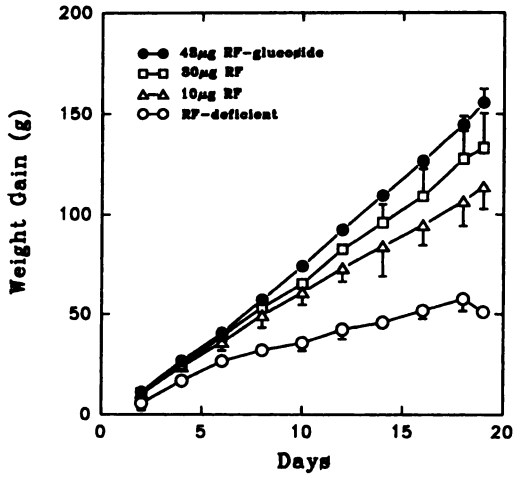

FIGURE 6 Weight gain in rats fed a riboflavin (RF)-deficient diet or a RF-deficient diet supplemented (per 15 g diet) with 10 μg RF, 30 μg RF, or 43 μg riboflavin-5′-α-D-glucoside (RF-glucoside) for 19 d. Values are means of three rats per group. The vertical bars show the SD at each experimental day. Data were analyzed by unpaired Student's *t* test. Weight gains in the 43 μg RF-glucoside group were significantly different from weight gains in the RF-deficient group ($P < 0.001$) and the 10 μg RF group ($P < 0.025$). There was no significant difference between the 43 and 30 μg RF-glucoside groups ($P > 0.05$).

Even though certain vitamins are found naturally as glucose conjugates (Kabir et al. 1983, Rubin et al. 1947, Siegel et al. 1943, Yasumoto et al. 1977), their nutritional importance is not yet clear. It has been speculated that formation of glucosides may be a means by which the organism can store vitamin in a stabilized form (Kabir et al. 1983). It was of interest to determine the effect of replacing riboflavin with RF-glucoside in the diet of rats. The results from feeding experiments over 19 d are shown in **Figure 6**. The weight gain in rats fed RF-glucoside (43 μg/15 g diet, equivalent to a riboflavin-sufficient diet of 30 μg/15 g diet) was about the same as the weight gain observed for rats consuming a riboflavin-sufficient diet. Thus, the nutritional efficacy of RF-glucoside is similar to that of the free vitamin. However, it is not clear how much of the RF-glucoside that is absorbed reaches the liver as the intact glucoside. Because the intestines are a rich source of α-glucosidases (Yamamoto 1990), it is possible that a significant amount of hydrolysis occurs in these tissues.

Our studies show that RF-glucoside is readily taken into isolated rat hepatocytes and hydrolyzed to release the free vitamin. Because it is apparent that this transport does not involve the riboflavin transporter, this would be an alternate way of delivering the vitamin, circumventing the necessity of a riboflavin transporter.

ACKNOWLEDGMENTS

We thank H. Chen for technical assistance with preparation of figures and J. Zempleni for help with statistical analysis.

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