Binding Form of Vitamin B$_2$ in Bovine Milk: Its Concentration, Distribution and Binding Linkage

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(Received April 20, 1990)

Summary The contents of total, free, and bound vitamin B$_2$ (B$_2$) in bovine milk and their distribution in four separate milk fractions, including milk during the early lactation stage, were estimated. The total B$_2$ content in whole mature milk was $179 \pm 25 \mu g/100 g$ ($n = 16$), and its distribution in the cream, whey, skim milk membrane, and casein fractions was 6, 67, 9, and 18%, respectively. The amount of flavins bound to protein in the total B$_2$ was 13.6% in whole milk and rich in membrane fraction. The total B$_2$ content ($\mu g/100 g$ of milk) was higher in colostrum at 1-3 days ($287 \pm 120$) than in colostrum at 4-7 days ($173 \pm 27$), in transitional milk ($182 \pm 33$), and in mature milk ($179 \pm 44$). The bound flavin content decreased slightly as lactation progressed (20-30 $\mu g/100 g$), but the ratio of bound/total B$_2$ did not vary (12-15%). Milk fat globule membrane (MFGM) contained $414 \pm 65 \mu g$ of B$_2/g$ of protein, most of it being bound to protein (92%). Market milks contained as much total B$_2$ as raw whole milk, but the amount of bound form was only 2%. Guanidine HCl, urea, sodium dodecyl sulfate, pH at 3.0-3.5, delipidation, and boiling released most of the B$_2$ bound to protein, suggesting that bound flavins bind to milk proteins by a hydrophobic linkage.

Key Words riboflavin, vitamin B$_2$, bovine milk, colostrum, lactation, milk fat globule membrane, skim milk membrane

Bovine milk is a very rich source of vitamin B$_2$, which occurs as riboflavin (RF), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). Much RF in milk is contained in the free form, while a small part is FMN and FAD (1,2). For example, xanthine oxidase [EC 1.2.3.2], which is abundant in bovine milk fat globule membrane (MFGM) (3), is a flavoprotein which binds two molecules of FAD per molecule of protein (4). The ratio and distribution of RF in milk are variable among the reported papers, the reason for this being obscure.
During investigations of MFGM, we have observed that a water-soluble, yellow material was released by delipidating MFGM. We considered that RF was bound to MFGM proteins. We have paid special attention to RF bound to milk proteins, since RF-binding proteins have been reported in various sources. In addition, the state of vitamin B2 in milk may be important for cheese manufacture with respect to the nutritional aspect of vitamin B2, because most free vitamin B2 will be drained with cheese whey and be not retained in cheese curd. The vitamin B2 content in cheese is very low compared with that of the bovine milk used in its production. Vitamin B2 bound to milk proteins such as MFGM and casein, which are incorporated into the formation of cheese curd, may contribute to the retention of vitamin B2 in cheese.

The present study was undertaken to determine the amounts of vitamin B2 bound to milk protein, and the distribution of total, free and bound vitamin B2 in four milk fractions, including milk during the early lactation stage, and the linkage of vitamin B2 to milk proteins.

MATERIALS AND METHODS

Materials. Fresh bovine milk (morning milk) was obtained from Holstein cows of the University herds and from dairy farms in the Nasu district (Tochigi prefecture). Riboflavin, sodium dodecyl sulfate (SDS), diastase, Triton X-100, and Tween 20 were purchased from Wako Pure Chemical (Tokyo). Nonidet P-40 was from Nakarai Kagaku (Kyoto), and sodium cholate was from Tokyo Kasei Kogyo (Tokyo), Sodium deoxycholate was from Difco Lab. (Detroit, MI). RF recrystallized from 2N acetic acid was dried over P2O5 in a desiccator for 72h. The other chemicals were of analytical grade, and organic solvents were redistilled before use.

Fractionation of milk. Milk was centrifuged for 90 min at 100,000×g and at 4°C on a swing rotor (Hitachi RPS-27-2) and fractionated into four fractions: a floating cream, subnatant (whey), and a fluffy layer (skim milk membrane) just over a casein micellar pellet. The skim membrane fraction was collected immediately by gentle suction. The packed cream layer was next carefully collected by scooping up with a spatula, and the whey was then collected by gentle suction. The cream was dispersed in 2.5 volumes of deionized water, and the casein pellet was dispersed in 3 volumes of 10mM phosphate buffer (pH 6.8) in a Potter-Elevehjem type of homogenizer. The dispersed cream was chilled and churned by hand shaking, buttermilk being collected after churning. Market milks also were treated similarly. The whey fraction after dialysis was concentrated by ultrafiltration on TCF-10 equipment with a PM 10 membrane (Amicon, U.S.A.). The average weight ratios of the milk fractions from raw mature milk and colostrum were 4.4 and 7.0% in cream, 80.2 and 75.5% in whey, 6.0 and 7.2% in the skim milk membrane fraction, and 8.0 and 9.1% in the casein pellet, respectively.

Preparation of MFGM. This was prepared from washed cream by the method B of the procedures of Kanno and Kim (5).
Treatment with various agents. The milk fractions (concentrated whey, MFGM and casein) were each mixed with an equal volume of 2% (w/v) detergent or 2 M KCl and NaCl. In the case of urea (8 M), guanidine HCl (6 M), and ammonium sulfate (100, 80, 60, 40, and 20% saturation), solid chemicals were added directly into each milk fraction to give the final concentration. The samples were then stirred for 1 h at room temperature. For delipidation, two volumes of a mixture of methanol and chloroform (2:1, v/v) were added to MFGM and, after stirring for 60 min at 4°C, the mixture was centrifuged and the lower solvent layer removed. The milk fractions were heated for the indicated time in a boiling water bath and cooled in tap-water.

Chemical measurements. Total RF was estimated by the procedure of Rettenmaier and Vuilleumier (6). Briefly, a milk sample (2.0 g) in a Corex tube enveloped with aluminum foil was mixed with 0.25 N sulfuric acid, adjusted to pH 4.5, hydrolyzed with diastase for 90 min at 45°C, centrifuged, and then filtered (0.45 μm). The clear solution was measured in a spectrofluorometer (Shimadzu RF-540) at 462 nm of excitation and 520 nm of emission wavelength. RF was used as the standard. A blank was made by adding sodium hydrosulfite to the measured sample.

The milk fractions and agent-treated samples were dialyzed against 0.01 M sodium phosphate buffer (pH 6.8) containing 0.02% NaN₃ for 48 h at 4°C exchanging at 12-h intervals. The RF in samples before and after dialysis was measured.

Protein was measured in the samples before and after dialysis by the method of Markwell et al. (7), using bovine serum albumin as the standard.

In this work, total RF was defined as total vitamin B₂, since FMN and FAD were converted into RF by diastase method. The bound vitamin B₂ is defined as the remaining vitamin B₂ after dialysis. Free vitamin B₂ was calculated by subtracting the bound vitamin B₂ from the total vitamin B₂. All experiments were carried out under dim light and/or by using brown glassware.

Statistics. The differences between groups were calculated with Tukey’s q test.

RESULTS

Riboflavin extracted from the milk and milk fractions (6) was identified spectrofluorometrically. The total and bound RF in whole milk, whey, cream, skim milk membrane, casein, MFGM, and market milk had the same absorption spectra at emission and excitation as that of authentic RF (data not shown).

Content and distribution of vitamin B₂ in mature milk

The contents of total, free, and bound vitamin B₂ were first determined in mature milk and their milk fractions. The total vitamin B₂ content was about 179 ± 25 μg/100 g of whole milk (Table 1). The bound vitamin B₂ content was 24 ± 4 μg/100 g, the remainder being in the free form. Of the total vitamin B₂, 14 ± 3% was
Table 1. Contents (A) and distribution (B) of total, free, and bound vitamin B2 in raw whole milk and milk fractions.

| Milk and fraction | Total vitamin B2 (A) | Bound vitamin B2 (B) | Free vitamin B2 (B) | Percentage of bound flavin (B) (%) |
|------------------|----------------------|----------------------|---------------------|-----------------------------------|
| Whole milk       | 178.9±25.4           | 23.7±3.5             | 153.0±25.4          | 13.6±2.5                          |
| Cream            | 9.4±1.9              | 5.5±1.1              | 3.9±0.3             | 340.0±50                          |
| Whey             | 16.6±2.0             | 6.6±1.7              | 10.0±1.3            | 25.0±4.9                          |
| Skim milk membrane | 14.7±4.9          | 8.8±1.5              | 6.9±0.2             | 107.5±18.2                        |
| Casein           | 30.8±7.6             | 17.8±3.9             | 13.0±2.4            | 121.3±3.6                         |

Content is expressed as mean±SD of vitamin B2 (μg) per 100 g of milk (n=16).
Table 2. Contents of total, free, and bound vitamin B₂ in bovine milk fat globule membrane.

| Preparation | Vitamin B₂ (μg/g of protein) | Percentage of bound flavin |
|-------------|-----------------------------|---------------------------|
|             | Total | Bound | Free |                  |                          |
| 1           | 555.8 | 538.6 | 17.2 | 96.9              |
| 2           | 329.6 | 322.0 | 7.6  | 97.7              |
| 3           | 406.2 | 385.1 | 21.1 | 94.8              |
| 4           | 411.4 | 396.9 | 14.5 | 96.5              |
| 5           | 428.1 | 418.3 | 9.9  | 97.7              |
| 6           | 371.0 | 305.2 | 65.8 | 82.3              |
| 7           | 357.5 | 331.0 | 26.5 | 92.6              |
| 8           | 448.6 | 343.8 | 104.8| 76.7              |
| Average     | 413.5 | 380.1 | 33.4 | 91.9              |
| ±SD         | 64.9  | 70.4  | 32.0 | 7.5               |

SD, n = 8.

in the form bound to protein.

When whole milk was separated into four fractions, most of the total vitamin B₂ was found in the whey fraction (67%). The relative amount of total vitamin B₂/100 g of milk was lower in the cream (5.5%) than in the skim milk membrane (8.6%) and casein fractions (17.8%). The latter two fractions still included vitamin B₂ to be distributed to whey, however. Vitamin B₂ in the whey fraction was predominantly in the free form (92.4%) and the remainder was in bound form (7.6%), which accounted for 45% of the total bound vitamin B₂. In contrast, most of the vitamin B₂ in the cream fraction was in the bound form (72.4%). The casein fraction did not contain as much bound vitamin B₂ as that in whey, and the skim milk membrane fraction contained 15% of bound vitamin B₂. The total vitamin B₂ content of MFGM, which is a major constituent of cream, was 414 ± 65 μg/g of protein (Table 2), most of which was in the bound form with free form of only 8%.

Content and distribution of vitamin B₂ in market milk

The total vitamin B₂ content of market milks was 163 ± 11 μg/100 g (Table 3) and similar to that of fresh whole milk (Table 1). The amount of bound vitamin B₂ was only 2% in whole milk. Most of the bound vitamin B₂ was present in the whey and casein fractions. This distribution differed from that of raw milk.

Variation of vitamin B₂ content during lactation stages

1. Whole milk. The content and distribution of the total and bound vitamin B₂ in whole milk obtained from cows during the early lactation stage, which is classified into colostrum (1–7 days postpartum), transitional milk (8–14 days), and mature milk (15 days or more), were measured (Fig. 1).

The total vitamin B₂ content was highest on the first day postpartum (457 ±
Table 3. Contents (A) and distribution (B) of total, and bound vitamin B$_2$ in market milk.

| Milk fraction     | Vitamin B$_2$ (µg/100 g) | Percentage of bound flavin |
|-------------------|---------------------------|-----------------------------|
|                   | Total         | Bound         |                             |
|                   | A  | B (%) | A  | B (%) |                             |
| Whole milk        | 163.0±10.5 | 100.0         | 3.0±2.2 | 100.0 | 1.6                         |
| Cream             | 6.0±0.8 | 3.7            | 0.4±0.4 | 13.3 | 6.5                         |
| Whey              | 112.9±4.4 | 69.3 | 1.7±1.1 | 56.7 | 1.5                         |
| Skim milk membrane| 9.1±0.7 | 5.6            | 0.2±0.2 | 6.7  | 1.9                         |
| Casein            | 28.1±4.3 | 17.2 | 0.7±0.4 | 23.3 | 2.3                         |

Numerals indicate the mean±SD ($n=7$).

Fig. 1. Variation of the content of total and bound vitamin B$_2$, and the ratio of bound vitamin B$_2$ in whole milk during the early lactation stages of cows. ●, total vitamin B$_2$; ○, bound vitamin B$_2$; ■, ratio of bound/total vitamin B$_2$.

131 µg/100 g, $n=4$), decreased linearly up to the fourth day (168±39 µg/100 g, $n=3$), and reached an almost constant level at the latter half of colostrum. The vitamin B$_2$ content of colostrum at 1–3 days postpartum was 1.5 times higher than that at the latter half of colostrum (4–7 days), as summarized in Table 4. On the other hand, the bound vitamin B$_2$ content and the ratio of bound/total vitamin B$_2$ in whole milk did not vary during lactation (Fig. 1 and Table 4) and their values were almost similar to those of normal milk. A significant statistical difference of vitamin B$_2$ content ($p<0.01$) was observed between the first half and the latter half of colostrum, but not among the latter half of colostrum, transitional milk, and mature milk, and then not for the bound vitamin B$_2$ among milks during the early

*J. Nutr. Sci. Vitaminol.*
Table 4. Concentration and distribution of total and bound vitamin B$_2$ (B$_2$) in bovine whole milk and milk fractions during lactation stages.

| Lactation stage | Fraction         | Total B$_2$ (µg/100 g of milk) | Bound B$_2$ | Percentage of bound flavin |
|-----------------|------------------|--------------------------------|-------------|-----------------------------|
| Milk before parturition | Whole milk | 492.5±20.3 | 37.4±18.2 | 7.6±3.6 |
| $n=4$ | Cream | 8.3±5.4 | 4.1±2.2 | 51.9±18.3 |
| 2 cows | Whey | 337.0±43.9 | 16.6±3.4 | 4.9±0.8 |
| | SK membrane | 37.2±7.1 | 5.1±3.3 | 13.1±7.1 |
| | Casein | 40.1±18.7 | 3.7±2.9 | 8.9±3.4 |
| Colostrum (1-3 days) | Whole milk | 287.3±119.5* | 29.9±9.6 | 11.5±5.1 |
| $n=18$ | Cream | 15.0±7.5 | 9.1±4.8 | 61.6±12.2 |
| | Whey | 184.7±90.1* | 7.9±4.1 | 4.4±1.7 |
| 4 cows | SK membrane | 27.0±9.3 | 2.8±0.8 | 11.2±4.4 |
| | Casein | 36.7±14.1 | 3.1±2.5 | 8.3±4.4 |
| Colostrum (4-7 days) | Whole milk | 173.3±26.5* | 26.5±9.2 | 15.3±4.5 |
| $n=14$ | Cream | 16.3±7.3 | 11.2±5.4 | 67.6±8.3 |
| | Whey | 103.7±15.1* | 4.1±2.1 | 4.1±2.3 |
| 4 cows | SK membrane | 12.9±3.4 | 2.6±0.7 | 22.1±8.9 |
| | Casein | 34.0±6.9 | 3.3±0.9 | 10.1±3.3 |
| Transitional milk | Whole milk | 181.9±33.4 | 23.0±6.7 | 13.2±4.9 |
| $n=12$ | Cream | 10.4±4.2 | 7.1±3.8 | 66.9±14.3 |
| | Whey | 114.9±22.0 | 2.4±0.3 | 2.2±0.6 |
| 3 cows | SK membrane | 11.1±2.4 | 2.3±0.6 | 20.9±6.1 |
| | Casein | 34.2±11.3 | 2.5±1.2 | 7.6±2.8 |
| Mature milk | Whole milk | 179.2±44.3 | 20.1±3.3 | 12.1±4.5 |
| $n=5$ | Cream | 10.0±3.4 | 6.3±2.7 | 62.3±10.5 |
| | Whey | 115.5±27.5 | 4.0±1.8 | 3.6±1.8 |
| 3 cows | SK membrane | 8.8±1.3 | 2.0±0.5 | 23.0±4.1 |
| | Casein | 33.8±9.0 | 2.2±0.5 | 6.7±1.1 |

Numerals shows mean±SD. *Skim milk membrane fraction. *$p<0.01$ between 1-3 days and 4-7 days of colostrum.

lactation stages (Table 4). Milk from cows before parturition contained a higher total and bound vitamin B$_2$ (Table 4). This amount was 1.7 times higher than that of the first half of colostrum. The bound/total vitamin B$_2$ ratio was low in milk before parturition (7.6±3.6%).

The content of free form increased markedly in the first half of colostrum, whereas that of bound form did not vary during early lactation.

2. Milk fractions. The distribution of total and bound vitamin B$_2$ in the milk fractions was then compared (Fig. 2 and Table 4). The variation of total vitamin B$_2$ content in the whey and skim milk membrane fractions showed a very similar tendency to that of whole milk in Fig. 1 (data not shown). In general, 62-69% of the total vitamin B$_2$ was found in the whey fraction throughout lactation, excepting
The distribution of the total and bound vitamin B₂ in milk fractions during lactation stages. A, milk before parturition; B, colostrum at 1–3 days after parturition; C, colostrum at 4–7 days after parturition; D, transitional milk at 8–14 days; E, normal milk at 20–30 days.

milk before parturition (80%). The total vitamin B₂ content in the cream and casein fractions did not vary significantly during lactation (Table 4).

The bound vitamin B₂ content and the ratio of bound/total vitamin B₂ in the milk fractions also did not vary throughout early lactation (Fig. 2 and Table 4). However, there was a tendency that the content of bound form in whey decreased from milk before parturition to transitional milk. In agreement with the results for normal milk (Table 1), the ratio of bound/total vitamin B₂ was higher in the cream and skim milk membrane fractions than in the other fractions.

The distribution of bound form in whey was 34% in colostrum at 1–3 days and 19–27% at the latter half of colostrum, and in transitional and mature milk, excepting milk before parturition (59%) (Fig. 2). Bound form in the cream fraction increased from 41 to 51% in the colostrum period, whereas it was 13% in milk before parturition. The distribution of bound vitamin B₂ in the skim milk membrane and casein was not influenced by the lactation stage, the values being 13–16% and 13–19%, respectively.

**Binding form of bound flavins**

The binding of flavins to whey protein, MFGM and casein was investigated by using various agents, delipidation, and heat treatment (Table 5). Most of the flavins bound to MFGM and whey protein were not dissociated by KCl, NaCl, nonionic detergents such as Triton X-100, Nonidet P-40, and Tween 20, and mild anionic detergents such as sodium cholate and sodium deoxycholate. Ammonium sulfate, which dissociated flavins from flavoprotein (8, 9), did not have a profound effect on the liberation of flavins from MFGM and whey proteins. With urea, half the amount of flavins bound to MFGM was liberated, whereas the flavins bound to whey protein were not. With SDS, most of the flavins were dissociated from...
Table 5. Effect of various agents on the release of bound flavins from milk fat globule membrane (MFGM), whey, and casein fractions.

| Reagent                  | Concentration | Remaining flavins (%) | MFGM* | Whey* | Casein* |
|--------------------------|---------------|-----------------------|-------|-------|---------|
| KCl                      | 1 M           | 102.0                 | 126.9 | 63.6  |
| NaCl                     | 1 M           | 97.0                  | 100.4 | 71.4  |
| Urea^                   | 8 M           | 55.9                  | 93.2  | 36.6  |
| Guanidine HCl^           | 6 M           | 1.4                   | 13.3  | 19.7  |
| Triton X-100             | 1%            | 91.1                  | 105.9 | 74.1  |
| Nonidet P-40             | 1%            | 91.5                  | 101.4 | 78.8  |
| Tween 20                 | 1%            | 92.1                  | 133.3 | 91.5  |
| Sodium cholate           | 1%            | 105.0                 | 96.2  | 74.4  |
| Sodium deoxycholate      | 1%            | 82.0                  | 108.4 | 57.5  |
| Sodium dodecyl sulfate   | 1%            | 2.5                   | 106.3 | 35.2  |
| Ammonium sulfate^        |               |                       |       |       |         |
| (saturation)             | 100%          | 96.4                  | 90.8  | —     |
|                          | 80%           | 93.6                  | 85.9  | —     |
|                          | 60%           | 91.9                  | 97.1  | —     |
|                          | 40%           | 92.9                  | 120.5 | —     |
| Delipidation             |               | 14.2                  | —     | —     |
| Heating (100°C)          | 10 min        | 1.4                   | 2.4   | 5.1   |
|                          | 20 min        | 1.3                   | 2.4   | 5.6   |
|                          | 30 min        | 1.5                   | 2.4   | 4.5   |

*Total flavin contents before treatment were 477 μg/g of protein in MFGM, 5.64 μg/g of protein in concentrated whey, and 2.3 μg/g of protein in casein. ^Destruction of flavins by these reagents was corrected.

MFGM, but did not affect the flavins bound to whey protein. Guanidine HCl, which is a chaotropic agent for the perturbation of biological macrostructures (10), liberated flavins from both MFGM and whey proteins. As flavins, probably RF, were decreased by the increase in concentration of guanidine HCl, urea and ammonium sulfate, but not by the others (data not shown), contents were corrected by the ratio obtained for authentic RF and these reagents. The bound flavins of MFGM were also dissociated by delipidation with a mixture of methanol and chloroform. These results suggest that the binding of flavins with protein is noncovalent and hydrophobic for the flavin-MFGM complex.

About 20-40% of the flavins bound to casein was liberated by various agents which did not dissociate MFGM-flavins. This suggests that flavins trapped in casein micelles were removed by dialysis. Accordingly, the remainder seems to correspond to flavins truly bound to casein. Guanidine HCl and SDS released flavins from casein more effectively than the other agents, suggesting that the casein-flavin complex also involved hydrophobic interaction.

By heating in a boiling water bath, most of the bound flavins were liberated from MFGM, whey protein and casein. The flavins were not destroyed by heating.
Fig. 3. Effect of pH on the dissociation of vitamin B$_2$ bound to milk fat globule membrane (MFGM) and whey. Vitamin B$_2$ content was 349.3 µg/g of protein for MFGM (●) and 2.1 µg/g of protein for concentrated whey (○). pH 3.0 and 3.5, glycine-HCl buffer; pH 4.0, 4.5, 5.0, and 5.5, 0.2M acetate buffer; pH 6.0 and 6.8, 10mM phosphate buffer.

**under neutral conditions, judging from their fluorescence spectra.**

**Effect of pH on dissociation of bound flavins**

The flavins in the MFGM were stable in a range of pH from 4.0 to 10.0, but dissociated drastically at pH 3.5 to 3.0 (Fig. 3). At pH 4.8, which is the isoelectric point of MFGM (5), most of the bound flavins were not dissociated. The flavins bound to whey protein were also dissociated at pH 3.0, at which 90% of the flavins dissociated. The flavins associated to casein were influenced by pH (data not shown), about 50 to 60% of flavins being dissociated in the alkaline (pH 8.0–10.0) and acid pH regions (3.0–4.6). However, there was no direct relationship with pH.

**DISCUSSION**

Most investigators reported that from 65 to 95% of the RF in bovine milk was in the free form (1), and the rest was present as FAD, whereas none occurred as FMN (4,13). However, Funai reported 21% in the form of FMN, with 14% as FAD and the remainder in the free form (RF) (11). Nagasawa et al. also found 94–95% of the RF free with the remainder bound at about 60% as FMN and 40% as FAD in skim milk (12). Furthermore, Roughhead and McCormick (13) reported the occurrence of 10-(2'-hydroxyethyl)flavin (11–19%) in addition to FAD (13–46%) and RF (35–73%) in bovine milk by HPLC. However, we could not detect 10-(2'-hydroxyethyl)flavin in bovine milk by HPLC method of Kanno et al. (14). Thus, there is a great difference in the form of flavins in bovine milk and these studies have been concerned in the ratio of RF, FMN, and FAD. In this study, our
special attention was paid to whether RF including FAD and FMN was present in
the free form or in the form bound to protein in bovine milk.

The total vitamin B<sub>2</sub> content in mature whole milk was close to the average
value (1.74 mg/kg, ranging 0.81–2.58 mg/kg) reviewed by Hartman and Dryden (1, 2). The variation of total vitamin B<sub>2</sub> content in milks during the lactation stage, especially in the first half of colostrum, is very similar to the results of Nagasawa et al. (12). However, the total vitamin B<sub>2</sub> content (457 µg/100 g) in milk just after parturition was higher in this study than in the results (354 µg/100 g of milk) of Nagasawa et al. (12). The content in whey also agrees well with the average value (1.2 mg/kg) (1).

There is less known about the amount of vitamin B<sub>2</sub> bound to protein, the
ccontent of bound vitamin B<sub>2</sub> and the distribution of vitamin B<sub>2</sub> in mature milk
fractions being the first reported in this study. Of the total vitamin B<sub>2</sub>, about 13±
4% was in the bound form throughout lactation. In particular, flavin bound to
protein was abundant in the cream fraction (66±10%, Table 4) and most of
vitamin B<sub>2</sub> in the MFGM prepared from washed cream was in the bound form
(Table 2). The free form (FMN and RF) in the MFGM may be released from
FAD bound to MFGM by FAD-pyrophosphatase and monophosphatases (unpub-
lished data). A high amount of bound form (15%) contained in the skim milk
membrane fraction reflects identical origin to MFGM (15, 16). It is still unknown
from this study whether all of the bound vitamin B<sub>2</sub> was present as a cofactor for
flavin-containing enzymes such as xanthine oxidase and dihydrolipoamide reduc-
tase [EC 1.6.4.3]. On the other hand, the decrease of bound form in market milk
seems to result from pasteurization and homogenization under high pressure, as
seen in the accelerated release of flavins by boiling (Table 5).

The effects of urea and SDS on the dissociation of flavins bound to MFGM
were different from those of whey protein (Table 5). Urea might release flavins by
cleavage of the hydrogen bond of flavin and protein. Most of bound flavins in both
samples were liberated by boiling or denaturation of the protein, while a small
amount of flavins did not, suggesting the presence of covalent peptidyl flavins (17).
RF could not bind to apo-RF-binding protein denatured with 4 M guanidine HCl,
although the binding to native apo-RF-binding protein was a reversible reaction
(18). The results suggest that the binding of flavins to protein is attributable to
hydrophobic interaction of the flavin ring system with non-polar groups on the
protein, and hydrogen bonding of the hydroxyl groups on the N-10 side chain of
flavin (19). In most flavoproteins, FAD and FMN are held by noncovalent
linkages and were liberated as free flavin nucleotides upon denaturation of
the protein (17). Most flavin-protein complexes were readily destroyed by acidic pH
and high temperature (20), suggesting that the flavin-MFGM complex is formed by
noncovalent bonding.

The presence of proteins binding RF as the prosthetic groups is known, in
addition to flavoenzymes. RF-binding protein has been found in chicks (21) and
eggs (22, 23), and RF-carrier proteins in chicks (24), pregnant rats (25, 26), bovine
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blood (27), pregnant women (28), and human blood (29). RF-binding protein was produced by laying hens for plasma transport and the storage in eggs of adequate supplies of RF for the developing embryo (24, 30). RF-carrier protein in pregnancy plays a vital role in the transport of RF from the mother to fetus, with intraembryonic events in the rat fetus that culminated in fetal death after immunizing the mother with an antiserum to RF-binding protein (25, 26). Whey protein binding RF may be such an RF-binding protein.

This work was supported in part by the Grant-in-Aid for Special Project Research (60216004) from the Ministry of Education, Science and Culture of Japan and by The Food Science Institute Foundation (Ryoushoku Kenkyukai).

REFERENCES

1) Hartman, A. M., and Dryden, L. P. (1965): Vitamin in Milk and Milk Products, American Dairy Science Association, pp. 24–33.
2) Hartmann, A. M., and Dryden, L. P. (1974): The vitamin in milk and milk products, in Fundamentals of Dairy Chemistry, 2nd Ed., ed. by Webb, B. H., Johnson, A. H., and Alford, J. A., AVI Publishing Inc., Connecticut, pp. 325–441.
3) Briley, M. S., and Eisenthal, R. (1975): Association of xanthine oxidase with the bovine milk fat globule membrane. Biochem. J., 147, 417–423.
4) Modi, V. V., and Owen, E. C. (1956): Riboflavin in milk. Nature, 178, 1120.
5) Kanno, C., and Kim, D.-H. (1990): A simple procedure for the preparation of bovine milk fat globule membrane and a comparison of its composition, enzymatic activities, and electrophoretic properties with those prepared by other methods. Agric. Biol. Chem., 54, 2845–2854.
6) Rettenmaier, R., and Vuilleumier, J. P. (1983): A simple method for the determination of riboflavin in human milk. Intern. J. Vit. Nutr. Res., 53, 32–35.
7) Markwell, M. A. K., Haas, S. M., Bieber, L. L., and Tolbert, N. E. (1978): A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem., 87, 206–210.
8) Tsuge, H. (1985): Interaction between flavin coenzyme and apoprotein in flavoenzymes. Vitamins (J. Vitam. Soc. Jpn.), 59, 109–119.
9) Swoboda, B. E. P. (1969): The relationship between molecular conformation and the binding of flavin-adenine dinucleotide in glucose oxidase. Biochim. Biophys. Acta, 175, 365–379.
10) Hatefi, Y., and Hanstein, W. G. (1974): Destabilization of membranes with chaotropic ions. Methods Enzymol., 31, 770–790.
11) Funai, Y. (1957): Studies on riboflavin in milk. 4. On riboflavin content in cow’s, goat’s and dried milk. Tokushima J. Exp. Med., 3, 201–206.
12) Nagasawa, T., Kuzuya, Y., and Shigeta, N. (1961): Studies on riboflavin in milk. II. Partition of riboflavin in colostrum, mid- and late-lactation milk. Jpn. J. Zootech. Sci., 32, 240–244.
13) Roughead, Z. K., and McCormick, D. B. (1990): Qualitative and quantitative assessment of flavins in cow’s milk. J. Nutr., 120, 382–388.

J. Nutr. Sci. Vitaminol.
14) Kanno, C., Shirahoji, K., and Hoshi, T. (1991): A simple method for the separate determination of three flavins in bovine milk by high-performance liquid chromatography. *J. Food Sci.*, 56, accepted.

15) Kanno, C. (1980): Recent studies on milk fat globule membrane with special reference to the constituent proteins. *Jpn. J. Zootech. Sci.*, 51, 75–88.

16) McPherson, A. V., and Kitchen, B. J. (1983): Reviews of the progress of dairy science: the bovine milk fat globule membrane—its formation, composition, structure and behaviour in milk and dairy products. *J. Dairy Res.*, 50, 107–133.

17) Singer, T. P., Salach, J., Hemmerich, P., and Ehrenberg, A. (1971): Flavin peptides. *Methods Enzymol.*, 18 (Part B), 417–427.

18) Nishina, Y., Horiike, K., Shiga, K., and Yamano, T. (1977): A fluorescence study of egg white riboflavin-binding protein. *J. Biochem.*, 82, 1715–1721.

19) Choi, J.-D., and McCormick, D. B. (1980): The interaction of flavins with egg white riboflavin-binding protein. *Arch. Biochem. Biophys.*, 204, 41–51.

20) Koziol, J. (1971): Fluorometric analysis of riboflavin and its coenzymes. *Methods Enzymol.*, 18 (Part B), 253–285.

21) Froehlich, J. A., Merrill, A. H., Jr., Clagett, C. O., and McCormick, D. B. (1980): Affinity chromatographic purification and comparison of riboflavin-binding proteins from laying hen liver and blood and from egg yolk. *Comp. Biochem. Physiol.*, 66B, 397–401.

22) Rhodes, M. B., Bennett, N., and Fenney, R. E. (1959): The flavoprotein-apoprotein system of egg white. *J. Biol. Chem.*, 234, 2054–2060.

23) Hamazume, Y., Mega, T., and Ikenaga, T. (1984): Characterization of hen egg white-and yolk-riboflavin binding proteins and amino acid sequence of egg white-riboflavin binding protein. *J. Biochem.*, 95, 1633–1644.

24) Hammer, C. H., Buss, E. G., and Clagett, C. O. (1973): Avian riboflavinuria. 8. The rate of the riboflavin-binding protein—riboflavin complex during incubation of hen's eggs. *Poult. Sci.*, 52, 520–530.

25) Krishnamurthy, K., Surolia, N., and Adiga, P. R. (1984): Mechanism of fetal wastage following immunoneutralization of riboflavin carrier protein in the pregnant rat: disturbances in flavin co-enzyme levels. *FEBS Lett.*, 178, 87–91.

26) Surolia, N., Krishnamurthy, K., and Adiga, P. R. (1985): Enzymatic basis of deranged fetal flavin-nucleotide metabolism consequent on immunoneutralization of maternal riboflavin carrier protein in the pregnant rat. *Biochem. J.*, 230, 363–367.

27) Merrill, A. H., Jr., Froehlich, J. A., and McCormick, D. B. (1979): Purification of riboflavin-binding proteins from bovine plasma and discovery of a pregnancy-specific riboflavin-binding protein. *J. Biol. Chem.*, 254, 9362–9364.

28) Murthy, C. V. R., and Adiga, P. R. (1982): Isolation and characterization of a riboflavin-carrier protein from human pregnancy serum. *Biochem. Int.*, 5, 289–296.

29) Innis, W. S. A., McCormick, D. B., and Merrill, A. H., Jr. (1985): Variation in riboflavin binding by human plasma: identification of immunoglobulins as the major proteins responsible. *Biochem. Med.*, 34, 151–165.

30) Miller, M. S., Bruch, R. C., and White, H. B., III (1982): Carbohydrate compositional effects on tissue distribution of chicken riboflavin binding protein. *Biochim. Biophys. Acta*, 715, 126–136.