Vitamin B₁₂ and its binding proteins in milk from cow and buffalo in relation to bioavailability of B₁₂

Sergey N. Fedosov,^{1*} Ebba Nexø,² and Christian W. Heegaard¹

¹Department of Molecular Biology and Genetics, Aarhus University, Science Park, Gustav Wieds Vej 10C 8000, Aarhus C, Denmark

²Department of Clinical Biochemistry, Aarhus University Hospital, Palle Juul-Jensens Boulevard 99, 8200 Aarhus N, Denmark

ABSTRACT

Milk is an important source of highly bioavailable vitamin B₁₂ (cobalamin) in human nutrition. In most animal products, vitamin B₁₂ is strongly bound to various specific protein carriers. The 2 vitamin B₁₂-specific proteins, predominantly transcobalamin (TC) and haptocorrin (HC), were earlier found in milk from Holstein Friesian cows and in human or sow milk, respectively. As the type of vitamin B₁₂ binders may influence bioavailability of the vitamin, we examined vitamin B₁₂ carriers in pooled milk specimens derived from European and Indian cow and buffalo herds. The total endogenous vitamin B₁₂ concentration was comparable in all milk pools (≈ 3 nM), but the vitamin carriers varied considerably: TC + caseins in Danish cows, TC + HC in Indian cows and buffaloes, and mainly HC in Italian buffaloes. Danish cow milk contained half as much TC as vitamin B₁₂, and the surplus vitamin was all attached via a single coordination bond to abundantly available histidine residues of casein. The specific binding proteins in Indian cow milk (TC + HC) approximately matched the molar content of vitamin B₁₂. Milk from the 2 buffalo breeds contained more specific binders than vitamin B₁₂, and the surplus proteins included the unsaturated TC ≈ 3 nM (Indian stock), or both TC ≈ 4 nM and HC ≈ 23 nM (Italian stock). The abundant HC of the latter sample bound nearly all endogenous vitamin B₁₂. We tested (in vitro) the transfer of vitamin B₁₂ from milk proteins to human carriers, involved in the intestinal uptake. The bovine TC-vitamin B₁₂ complex rapidly dissociated at pH 2 (time of half reaction, $\tau_{1/2} < 1$ min, 37°C) and was susceptible to digestion with trypsin + chymotrypsin (pH 7.5). Transfer of vitamin B₁₂ from the precipitated bovine casein (pH 2) to human carriers proceeded with $\tau_{1/2} \approx 7$ min (37°C) and $\tau_{1/2} \approx 35$ min (20°C). Liberation of vitamin B₁₂ from buffalo HC was hampered

because of its pH stability and slow proteolysis. Nutritional availability of vitamin B₁₂ is expected to be high in cow milk (with TC-vitamin B₁₂ and casein-vitamin B₁₂ complexes) but potentially constrained in buffalo milk (with HC-vitamin B₁₂). This especially concerns the Italian buffalo milk, where a high excess of HC was found. We speculate whether the isolated stock of Italian buffalo maintained the ancestral secretion of carriers (HC $\gg$ vitamin B₁₂, TC ≈ 0), whereas intensive crossbreeding of cows and buffaloes from other regions caused a change to TC $\leq$ vitamin B₁₂, with low or absent HC. The substitution of HC by less sturdy carriers is apparently more beneficial to human consumers as far as vitamin B₁₂ bioavailability is concerned.

Key words: vitamin B₁₂, cow, buffalo, milk, digestion

INTRODUCTION

Vitamin B₁₂, or cobalamin (**Cbl**), is an essential organometallic nutrient with the schematic structure of X-[Co³⁺]Cbl, where X stands for various exchangeable groups (Pratt, 1972; Fedosov, 2012; Kräutler and Puffer, 2012). Insufficiency of Cbl leads to several neurological and hematological disorders (Green et al., 2017). Food of animal origin usually covers the recommended daily allowance of 2.4 μ g = 1.8 nmol (Watanabe, 2007; Green et al., 2017). For example, cow milk contains 0.6 to 3.6 nmol of Cbl per 1 L (Fedosov et al., 1996; Matte et al., 2014; Gille and Schmid, 2015), providing the vitamin with a high bioavailability (Vogiatzoglou et al., 2009; Matte et al., 2014; Gille and Schmid, 2015). This implies a good digestion of its protein carriers, which might otherwise constrain the uptake (Del Corral and Carmel, 1990; Carmel, 1995).

Cobalamin in milk from different species is usually bound to the specific proteins haptocorrin (**HC**), or transcobalamin (**TC**), or both [each one having an extraordinary high affinity for Cbl (Fedosov, 2012; Kräutler and Puffer, 2012)]. Human milk contains a large quantity of unsaturated HC ≈ 20 to 180 nM, whereas the concentration of its ligand complex is low HC-Cbl ≈ 0.3 to 0.6 nM (Sandberg et al., 1981; Greibe et al., 2013). In addition, a small amount of TC ≈ 0.6 nM was

Received May 4, 2018.

Accepted February 7, 2019.

*Corresponding author: snf@mbg.au.dk

detected in this source immunologically (Sandberg et al., 1981). Milk from rats, sows, and goats demonstrated similar patterns (Gregory and Holdsworth, 1955; Ford et al., 1975; Trugo, 1988; Raaberg et al., 1989).

Cow milk clearly contrasts the above examples by exhibiting only a trace of superfluous Cbl binding capacity (e.g., $TC \leq 0.05$ nM; Fedosov et al., 1996). Another unusual property concerns the nature of Cbl-carriers, characterized as TC and caseins, both containing approximately equal fractions of Cbl (Fedosov et al., 1996). The specific carrier TC has been thoroughly described earlier (Fedosov et al., 1995, 1996, 1999). The nature of casein-Cbl interactions was recently established as metal coordination (casein-His- $[Co^{3+}]Cbl$), because the abundant aquo-form of the vitamin $H_2O-[Co^{3+}]Cbl$ (**HOCbl**) exceeds TC and easily coordinates to His, if available (Fedosov et al., 2018). In contrast, the synthetic cyanoCbl (**CNCbl**) is incapable of His- $[Co^{3+}]Cbl$ coordination due to the tightly bound CN-group, protecting the $[Co^{3+}]$ site (Pratt, 1972; Fedosov et al., 2007a). Thereby, the 2 Cbl forms exhibit the 2 types of binding in cow milk: specific + coordination for HOCbl and purely specific for CNCbl.

Human and cow milk are the only 2 dietary types of milk with the known protein association patterns of Cbl. In this work, we compare pooled milk samples from 2 Danish and Indian cow herds, as well as 2 Italian and Indian buffalo herds. These sources represent the most popular types of milk in Europe and Asia, and the choice of combinations (cows + buffaloes from Europe and cows + buffaloes from Asia) could potentially expose generic, regional, and breeding differences. We report a considerable difference in the patterns of Cbl carriers, which is of consequence for the in vitro digestion and, possibly, the bioavailability of Cbl.

MATERIALS AND METHODS

Materials

Pooled milk from Danish cows (*Bos taurus*, Holstein Friesian breed, $n > 1,000$) was obtained from the Arla dairy plant, Brabrand, Denmark. An alternative test sample represented pasteurized milk from a Danish supermarket. Pooled milk from Indian cows [a crossbreed of *Bos indicus* (Sahiwal breed) and *Bos taurus* (Holstein Friesians), $n > 1,000$], as well as Indian buffaloes (*Bubalus bubalis*, graded Murrah, $n > 1,000$), was obtained from Chitale Dairy, Maharashtra, India. Pooled milk from Italian buffaloes (*Bubalus bubalis*, Italian Mediterranean breed, $n = 8$) originated from a smaller commercial herd in mid lactation (region of Macerata, Italy). One representative sample was obtained from each of the 4 breeds of cows and buffaloes. The animals

were kept on a mixed diet including grass-based feed and commercial supplements available in the respective regions. The feed did not include vitamin B₁₂ (cobalamin) but supplied ample amounts of cobalt salts (needed for synthesis of cobalamin by rumenal bacteria). The collected milk was kept at 4°C and transported to the laboratory within 3 h for skimming by centrifugation. The specimens were stored in aliquots at -20°C (or on dry ice during air transportation) until further application.

The CNCbl, HOCbl, and dicyanocobinamide (**Cbi**) were purchased from Sigma (St. Louis, MO). Radioactive cyano-form of cobalamin $CN-[^{57}Co^{3+}]Cbl$ (**CN⁵⁷Cbl**) was manufactured by MP Biomedicals LLC (Santa Ana, CA) and had the following specifications: 1.75 $\mu Ci/mL$ and $CN^{57}Cbl \approx 4$ pmol/mL. Radioactive aquo-form of cobalamin $H_2O-[^{57}Co^{3+}]Cbl$ (**HO⁵⁷Cbl**) was prepared from $CN^{57}Cbl$ by the photo-aquation method described earlier (Kornerup et al., 2016). Both radioactive forms were, if necessary, diluted with nonradioactive CNCbl or HOCbl to get the desired specific radioactivity.

Polyclonal antibodies against recombinant bovine TC (Fedosov et al., 1999) were produced by Dako A/S (Glostrup, Denmark). Monoclonal antibodies were produced by GenScript (Piscataway, NJ). Recombinant human intrinsic factor (**IF**) from plants (Fedosov et al., 2003) and gastric human IF (Nexo, 1975) were purified as described elsewhere. Anonymous human saliva was used as a source of native human HC ≈ 64 nM. Recombinant human HC (Furger et al., 2012; Mutti et al., 2017) was employed in the experiments, where a high concentration of the protein was required.

The Sephacryl S-200 High Resolution was purchased from GE Healthcare (Chicago, IL). All standard salts and organic compounds were purchased from Sigma, including proteins: trypsin, α -chymotrypsinogen, pancreatic trypsin inhibitor (**PTI**; all from bovine pancreas), and BSA.

Gel Filtration

Separation of proteins by size exclusion chromatography was performed at room temperature on a Sephacryl S-200 column (height = 100 cm, diameter = 1.6 cm) equilibrated with 0.1 M Tris (pH 7.5) and 1 M NaCl, essentially as described earlier (Fedosov et al., 1996, 2018).

Measurement of Endogenous Cbl and Its Extraction

Total Cbl was measured by the standard procedure for Advia Centaur CP System (Siemens Healthcare Diagnostics, Ballerup, Denmark). When detecting Cbl

in the gel-filtration fractions, we prepared a specific calibration curve in the elution buffer to compensate its effect on the luminescent tracer.

Raw milk samples underwent an additional extraction procedure: 1 mM potassium cyanide (KCN), 100°C, 20 min; 2% SDS, 100°C, 40 min; 0.2 M KCl, 0°C, 5 to 10 min. The supernatant was collected (20,000 × *g*, 5°C, 5 min) and used for Cbl measurements. The same treatment was applied to the HOCbl standards (1, 2, 3, and 4 mM) in 0.05 M sodium phosphate buffer (**P_i**), pH 6.8, 15 mg/mL of BSA.

Measurement of the Cbl Binding Capacity and Its Specificity

We measured the Cbl-binding capacity by adding excess of CN⁵⁷Cbl (with a known specific radioactivity) and separating the protein-bound and unbound fractions of CN⁵⁷Cbl with help of charcoal or gel filtration. The samples were measured by gamma counting (1 h) in a 2470 Wizard2 Automatic Gamma Counter (Perkin Elmer, Waltham, MA). When the specificity for corrinoids was investigated, we preincubated the sample with some excess of Cbi (≈15 min), followed by equimolar CN⁵⁷Cbl (≈3 h), whereupon suppression of CN⁵⁷Cbl binding was assessed by gel filtration.

Immunological Measurement of TC

We used a noncertified in-house ELISA, essentially as described for human transcobalamin (Nexo et al., 2000), but using the recombinant bovine TC-Cbl instead (Fedosov et al., 1999). The measurement range for the ELISA was 7.8 to 500 pM with a detection limit of 7.5 pM. The imprecision of ≈10% was assessed from 15 runs over a period of 14 mo.

Ultracentrifugation of Milk Samples

Four-gram samples were preincubated with trace amounts of CN⁵⁷Cbl (4 pM, ≈1 h), whereupon caseins were precipitated at 100,000 × *g* (1 h, 20°C). The pellets were suspended in 4 mL of 0.1 M **P_i**-buffer (pH 6.8) and briefly centrifuged (20,000 × *g*, 5 min, 20°C). Both the dissolved pellets and the supernatants were analyzed for their radioactivity and the distribution between the protein fractions.

Purification of HC from Buffalo Milk (Italy)

We acidified skim milk (1.4 L) to pH 3 (≈16.5 mL of 37% HCl in droplets, 21°C). The sample was centrifuged

(2,500 × *g*, 10 min, 21°C), the supernatant (1 L) was collected, and NaCl was added to the concentration of 1 M. We suspended the casein pellet in 1.25 M NaCl (0.8 L), centrifuged the sample, and pooled the second supernatant (0.8 L) with the main whey fraction. Afterward, the final concentration of NaCl was adjusted to ≈1 M. The pooled sample (2 L) was neutralized to pH 7.5 (≈19 mL of 5 M NaOH in droplets), cooled, and centrifuged. The residual fat was removed by filtration through a coffee filter (4°C), clogged in advance with ≈200 mL of whey. The clear solution with HC ≈ 14 nM and TC ≈ 1 nM was applied to a small affinity column (3 mL) containing Sepharose-NH₂-[Co³⁺]Cbl at 4°C (Nexo, 1975; Fedosov et al., 2003). The column with adsorbed proteins was washed (4 × 3 mL of 1 M NaCl, 0.1 M Tris, pH 7.5, 4°C), whereupon the Cbl-saturated proteins with some free Cbl were eluted after 20 h of incubation at 37°C. We dialyzed the eluate (4 mL) against 1.2 L of water (2 d at 22°C without change) and lyophilized it. The sample was dissolved in 0.5 mL of water (final 60 mM NaCl and 6 mM Tris), briefly centrifuged (20,000 × *g*, 5 min, 22°C), and the supernatant was filtered through a CM Sepharose column (0.3 mL) to capture possible remains of TC. The content of HC-Cbl ≈18 nmol was measured by its absorbance spectrum (Fedosov et al., 2002).

We devoided HC of Cbl by denaturing-renaturing dialysis in 150 mL of 8 M guanidinium chloride as described earlier (Fedosov et al., 2002).

Effect of Low pH on the Affinity for Cbl

Liberation of CN⁵⁷Cbl from the Specific Proteins at Different pH. Recombinant bovine TC (10 μM) or purified buffalo HC (10 μM) were loaded with trace amounts of CN⁵⁷Cbl (2 nM) in 0.1 M Tris with BSA (1 mg/mL) for 15 min. Afterward, 20 μL of each mixture was added to 10 mL of 0.1 M **P_i**-buffer (21°C), pH 2.0 or pH 6.8, containing BSA (1 mg/mL) and a high excess of the chase ligand CNCbl (100 nM). At the stipulated time intervals, 1 mL of the protein mixture was collected and either neutralized (0.2 mL of 0.5 M NaOH, 2.5 M NaCl) or mixed with 0.2 mL of 2.5 M NaCl. Liberated CN⁵⁷Cbl (as well as free CNCbl) was precipitated (15–20 min) with charcoal pellets, collected from 0.5 mL of a charcoal suspension (1% in 5 mg/mL of BSA). The residual protein-bound CN⁵⁷Cbl was quantified in the supernatants by gamma counting (see above). Two sets of controls underwent all the above procedures with exclusion of either charcoal (providing the total content of CN⁵⁷Cbl) or Cbl-specific binding proteins (exposing the nonspecific free CN⁵⁷Cbl, subtracted).

Transfer of CN^{57}Cbl from Bovine TC to Human HC During Alteration of pH. Milk from Danish cows (6 mL, pH 6.6) was loaded with a trace amount of CN^{57}Cbl (20 pM, 1 h, 21°C), giving a TC- CN^{57}Cbl complex. Then we added HC (16 nM from 2 mL of human saliva) and optionally human recombinant IF (100 nM), whereupon the sample was rapidly acidified to pH 2.0 ($\approx 70 \mu\text{L}$ of 37% HCl). After 5 min, pH was adjusted to 6.6, and the supernatant (3 mL) was subjected to gel filtration to assess the transfer from TC to HC (optionally IF).

Transfer of HO^{57}Cbl from Bovine Caseins to Human HC at pH 2 Over Time. Milk from Danish cows (20 mL) was incubated for 1 h at room temperature with CNCbl (0.2 nM) to suppress a trace specific binding capacity of TC ≈ 0.02 nM. Then we interacted HO^{57}Cbl (1 nM) with caseins (20 or 37°C, 1 h) and acidified the sample to pH 1.85 ($\approx 265 \mu\text{L}$ of 37% HCl). The transfer of HO^{57}Cbl from caseins to HC was initiated by adding human saliva (6.6 mL, final HC ≈ 16 nM, pH 2.0). The reaction was carried out in a shake incubator at either 20 or 37°C, and 2-mL samples were collected, briefly centrifuged ($20,000 \times g$, 1 min), and examined for their radioactivity. The pellets were washed with 0.5 M NaCl and also counted to keep the material balance.

Proteolytic Degradation of Cbl-Binding Proteins at Neutral pH

The proteolytic mixtures contained a “donor” protein (11 nM, see the Results section) preloaded with CN^{57}Cbl (10 nM, 15 min, 22°C) in 0.1 M Tris (pH 7.5), 10 mg/mL of BSA. A Cbl-specific “acceptor” (100 nM) was chosen according to its proteolytic stability and the convenience of separation during size exclusion chromatography. The prepared mixture was exposed to chymotrypsinogen (0.1 mg/mL = 4 μM) and trypsin (0.2 mg/mL = 8.6 μM) for 30 min at 37°C. We stopped the proteolysis by an excess of PTI (0.13 mg/mL = 20 μM), centrifuged the mixture, and assessed the transfer of CN^{57}Cbl (“donor” $\rightarrow$ “acceptor”) by gel filtration.

Analogous proteolytic experiments were conducted with recombinant bovine TC in the mixture of BSA (10 mg/mL) + caseins (15 mg/mL), or natural bovine TC in milk from Danish cows, both preloaded with a tracer (0.02 nM CN^{57}Cbl , 3 h, 22°C).

Another set of proteolytic mixtures (for SDS PAGE) contained a stipulated Cbl-specific protein (≈ 1 mg/mL), either free or saturated with equimolar CNCbl. It was incubated for 30 min at 37°C in 0.1 M Tris (pH 7.5) with trypsin (0.2 mg/mL) and chymotrypsinogen (0.1 mg/mL). We terminated the proteolytic reaction by heating (≈ 5 min, 100°C) in SDS loading solution (final

0.06 M Tris-HCl, pH 7, 1% SDS, 9% glycerol, 20 mM dithiothreitol, a trace of pyronin Y). The SDS-PAGE (16%) was conducted in the standard Tricine system to assess the change of protein bands.

Statistical Procedures

The data were presented as mean $\pm$ standard error of the mean (SEM) to show precision of the result, implying that the true value finds within the indicated boundaries with probability of $\approx 68\%$. Presentation of mean $\pm$ standard deviation stressed the variability of samples, implying that any arbitrary sample picked up from this (or identical) cohort has a probability of $\approx 68\%$ to be found within the indicated boundaries. All calculations and graphics were rendered using free software KyPlot 5 (kyenslab.com).

RESULTS

Total Endogenous Cbl and the Cbl-Binding Capacity in Milk

Different milk samples contained various sets of Cbl-binding proteins, characterized by unequal resistances to denaturation. Therefore, all experimental specimens (as well as the calibration samples) underwent a pre-treatment procedure (see the Materials and Methods section). Yet, the resilient fraction of HC in Italian buffalo milk was not a trivial case because the total HC (30 nM) considerably exceeded the bound Cbl (3 nM). When Italian buffalo milk was “spiked” with 1 nM of HOCbl, it showed an inadequate increase of $+\Delta\text{Cbl} \pm \text{SEM} = +0.72 \pm 0.06$ nM ($n = 10$), even after the extraction. In contrast, the “spiked” cow milk (Denmark) gave an appropriate increment by $+\Delta\text{Cbl} \pm \text{SEM} = +0.94 \pm 0.05$ nM ($n = 10$). We conjectured that the applied denaturation procedure releases $\approx 75\%$ of endogenous Cbl from Italian buffalo milk.

The performed measurements of endogenous Cbl (Table 1, first column) showed comparable concentrations of Cbl in all milk samples. The variation within the same breed ($\text{Cbl} \pm \text{SD} = 3.2 \pm 1.1$ nM) was tested in Holstein Friesian cows by Rutten et al. (2013), and it was higher than the variation between different species and breeds ($\text{Cbl} \pm \text{SD} = 3.2 \pm 0.3$ nM or 3.5 ± 0.6 , if assuming 3.1 nM or 4.2 nM Cbl concentrations in Italian buffalo milk, respectively).

The Cbl-specific binding capacity was examined using CN^{57}Cbl , incapable of the binding to caseins (Fedosov et al., 2018). The results are shown in Table 2 (first column).

The unspecific coordination of HOCbl to caseins was potentially present in all milk specimens, if adding a

Table 1. The endogenous cobalamin (Cbl) and its association with different protein fractions in pooled milk from different breeds of cows and buffaloes

Milk	Total Cbl, ¹ mean ± SEM, nM	Casein-Cbl, ² %	HC-Cbl, ² %	TC-Cbl, ² %
Cow, DK (supermarket)	3.0 ± 0.1 (2.9)	54 (≈50)	≈0 (≈0)	46 (≈50)
Cow, IN	3.6 ± 0.1	31 (casein-TC)	38	31
Buffalo, IN	3.1 ± 0.1	26 (casein-TC)	63	11
Buffalo, IT	3.1 ± 0.1 (≈4.2) ³	≈0	89	11

¹Direct measurements in skim milk. The data present repeated tests (n) of the same milk samples, collected from dairy plants in Denmark (DK), fresh milk (n = 10) and milk from a Danish supermarket (n = 2), and India (IN; n = 10), as well as from a local herd in Italy (IT; n = 10).

²Approximate distributions of Cbl between different protein fractions based on the gel filtration profiles in Figure 1. HC = haptocorrin; TC = transcobalamin.

³Correction for the assumed incomplete extraction of Cbl (≈75%) from Italian buffalo milk.

very high concentration of HOCbl (1 mM). A deeply red pellet of precipitated casein (pH 4.5) was produced due to the color of the attached Cbl (not shown).

Gel Filtration Profiles of the Endogenous Cbl

The 4 pools of milk had comparable protein profiles during size-exclusion chromatography (Figure 1, dotted line), containing (in the order of elution) the peaks of caseins, β-LG, α-LA, and casein peptides. The profiles of endogenous Cbl (Figure 1) revealed, on the other hand, a remarkable difference, also observed after displacement of Cbl from the casein in the presence of KCN (Figure 1A).

The bovine milk from Denmark (Figure 1A) demonstrated the expected equivalent distribution of Cbl between the high-molecular casein fraction and TC-Cbl. Treatment of milk with KCN liberated Cbl from its coordination with caseins. Some residual Cbl was still present in the casein fraction. No significant amount of HC-Cbl was found, as follows from the absence of Cbl between the peaks of casein and TC (Figure 1A).

In contrast to the above result, milk from the Indian cows (Figure 1B) demonstrated 3 peaks of the protein-bound Cbl, counting here the Cbl-peak with the apparent molecular weight (M_w) of HC ($M_w \approx 140$

kDa under gel filtration). The effects of cyanide on the casein-Cbl peak was limited, suggesting that only 30 to 50% of its Cbl is directly coordinated to His-residues. The liberated Cbl did not appear as free CNCbl, presumably being dispersed along the profile.

We also observed 3 peaks of endogenous Cbl in buffalo milk from India (Figure 1C). The main portion of endogenous Cbl belonged to the assumed HC peak, whereas 2 minor peaks to the right and to the left were ascribed to TC-Cbl (either free or incorporated into the casein fraction). Treatment with KCN did not cause any redistribution, meaning the absence of casein coordination.

The profile of buffalo milk from Italy (Figure 1D) revealed one major peak corresponding to HC-Cbl and apparently a minor fraction of TC-Cbl complex.

The approximate representation of Cbl-peaks (in percent) in different milk samples is summarized in Table 1 (3 last columns).

Gel Filtration Profiles of the Cbl-Specific Binding Capacity

The unsaturated Cbl-specific proteins in the milk samples were labeled by the exogenous tracer CN⁵⁷Cbl. The profiles of radioactivity in the milk from Danish

Table 2. The synthetic cyanoCbl (CNCbl)-binding capacity and its association with different protein fractions in pooled milk from different breeds of cows and buffaloes

Milk	Total binding, ¹ mean ± SEM, nM	Casein, ² mean ± SEM, nM	HC, ² mean ± SEM, nM	TC, ² mean ± SEM, nM
Cow, DK (supermarket)	≤0.02 (≈0)	≤0.05 (≤0.01)	0 (≈0)	≤0.05 (≤0.03)
Cow, IN	≤0.02	≤0.05	0	≈0
Buffalo, IN	3.2 ± 0.1	≈0.4 (casein-TC)	≈0.2	2.6 ± 0.1
Buffalo, IT	27 ± 1	<0.1	23 ± 1	4.0 ± 0.1

¹Direct measurements in skim milk. The data present repeated tests (n) of the same milk samples, collected from dairy plants in Denmark (DK), fresh milk (n = 10) and milk from a Danish supermarket (n = 2), and India (IN; n = 10), as well as from a local herd in Italy (IT; n = 10).

²Binding capacity in different protein fractions based on the gel filtration profiles in Figure 2. HC = haptocorrin; TC = transcobalamin.

and Indian cows (Figure 2A and 2B, respectively) did not show any significant interaction between CN^{57}Cbl (10 nM) and the proteins. A minor interaction with the peaks of casein and TC was still observed in the freshly frozen milk, but the binding considerably diminished in the pasteurized milk from a Danish supermarket (Table 2). Buffalo milk from India (Figure 2C) demonstrated (1) a small peak of CN^{57}Cbl associated with caseins, (2) a very small peak with M_w of HC, and (3) a well-expressed fraction of TC. Buffalo milk from Italy differed from all above milk samples and showed one major peak, corresponding to HC (Figure 2D).

The 2 Indian samples and the buffalo milk from Italy were subjected to additional examination, to discriminate between TC and HC (Danish bovine milk was tested earlier by Fedosov et al., 1996). All samples were incubated with the saturating amounts of incomplete corrinoid Cbi, capable of suppressing the binding of CN^{57}Cbl to HC (Allen et al., 1978; Fedosov et al., 1995). The profiles of Indian cow milk (not shown) and Indian buffalo milk (Figure 2C) revealed a TC-like binding capacity (not suppressed by Cbi), though at quite different quantities (in agreement with Table 2). A small fraction of unsaturated HC (suppressed by Cbi) was

Figure 1. Gel filtration profiles of endogenous (endog.) cobalamin (Cbl) in milk from cow and buffalo breeds. The records present absorbance A_{280} : the profiles of endogenous Cbl without pretreatment and after treatment of milk with 1 mM KCN. Antibody symbols (Y, large and small) qualitatively indicate large and small quantities of bovine or buffalo transcobalamin (TC) detected immunologically in the eluted peaks. The peaks of TC, haptocorrin (HC), and casein (cas) are indicated, where necessary, with the respective tags. Profiles of (A) cow milk, Denmark; (B) cow milk, India; (C) buffalo milk, India; and (D) buffalo milk, Italy.

Figure 2. Gel filtration patterns of the specific cobalamin (Cbl)-binding capacity in milk from cow and buffalo breeds. The records present absorbance A_{280} ; the profiles of $CN^{57}Cbl$ without or with preincubation of milk with cobinamide (Cbi). Elution peaks of transcobalamin (TC), haptocorin (HC), and casein (cas) are indicated, where necessary, with the respective tags. Profiles of (A) cow milk, Denmark; (B) cow milk, India; (C) buffalo milk, India; and (D) buffalo milk, Italy.

also present in the milk from Indian buffaloes (Figure 2C). Contrasting the above results, the Italian buffalo sample revealed a markedly different picture (Figure 2D). The pretreatment with Cbi largely suppressed the binding of $CN^{57}Cbl$, implying a high content of an unsaturated HC-like protein (≈ 23 nM in the original milk). The Cbi-treated profile also clearly exposed a TC-like peak (≈ 4 nM) in the Italian buffalo milk; see Table 2 for a further quantitative assessment.

Heating of milk (10 min at $95^{\circ}C$), preloaded with small quantity of $CN^{57}Cbl$, caused release of two-thirds of the protein-bound radioactivity in the milk from

Danish and Indian cows, as well as Indian buffaloes, (all containing $CN^{57}Cbl$ bound to a TC-like carrier), whereas no release was detected in the milk from Italian buffaloes (HC-like carrier; data not shown).

Immunological Assessment of TC in Milk Samples

Presence of TC on the gel-filtration profiles of all milk samples was tested immunologically using antibodies against recombinant bovine TC (see the Materials and Methods section), very similar in its sequence to buffalo TC (National Center for Biotechnology Information

database, ABG81438.1 and XP_006069867.1). Immunological responses were traced for TC-Cbl (the antigen used for immunization), whereas all unsaturated binders (if any) were removed before gel filtration by affinity adsorption. Responses were detected in the fractions with M_w of TC (major signal) and in the casein fractions (minor signal). The TC was not visible in the casein fraction of Italian buffalo milk, probably because TC concentration fell below the detection limit. The immunological data are schematically shown in Figure 1 as large and small antibody symbols, roughly reflecting the associated amounts of TC.

Ultracentrifugation of Milk Samples and Analysis of the Obtained Fractions

Ultracentrifugation of all milk samples, preloaded with $CN^{57}Cbl$ (Figure 3), was undertaken to precipitate caseins and examine their possible association with the Cbl-specific proteins under native conditions versus a high ionic strength (1 M NaCl) under gel filtration in Figures 1 and 2. The ultracentrifugation produced whey supernatants containing the major portion of radioactivity ($\geq 89\%$, Figure 3A) irrespective of ionic strength, though addition of NaCl increased the signal

Figure 3. Ultracentrifugation of milk samples and analysis of the obtained fractions. (A) Ultracentrifugation of cow milk from Denmark (CDK) and India (CIN), as well as buffalo milk from India (BIN) and Italy (BIT). All milk samples were loaded with a trace amount of CN^{57} cobalamin (Cbl) (4 pM) before their centrifugation (intended to precipitate only caseins). Percentage of residual radioactivity in the supernatants is shown in the samples with added 0.02 M Na-phosphate buffer (P_i) pH 6.8 or 1 M NaCl (examined for BIN and BIT). Gel filtration profiles of (B) CDK supernatant without any further additives; (C) resuspended BIT pellet after addition of $CN^{57}Cbl$ (10 nM); and (D) BIT supernatant after addition of $CN^{57}Cbl$ (30 nM); all profiles showing A_{280} absorbance and percentage of radioactivity, see the key in the panel. Elution peaks of transcobalamin (TC) and haptocorrin (HC) are indicated, where necessary, with the respective tags. The question mark in panel C indicates the assumed (but not proven) peak of TC, associated with caseins.

in supernatants to $\approx 100\%$. Gel filtration of a cow milk supernatant (Figure 3B) corroborated that practically all CN⁵⁷Cbl was TC-bound, despite a very low Cbl-specific binding capacity in both cow samples.

A more thorough analysis was performed for both supernatant and pellet of Italian buffalo milk. The saturating concentrations of CN⁵⁷Cbl were added to the dissolved pellet (+10 nM CN⁵⁷Cbl) and the supernatant (+30 nM CN⁵⁷Cbl), whereupon the samples were subjected to gel filtration. The dissolved casein pellet revealed presence of the Cbl-specific proteins (Figure 3C) capable to bind ≈ 3 nM of Cbl, if expressing this value as the molar binding capacity per 1 L of unprocessed milk. We also observed a strong amplification of the TC peak (in comparison to the assumed HC peak) in Figure 3C. The presence of the Cbl-specific proteins in the pellet suggests some interaction of casein with TC and, to a lower grade, with HC. The HC- and TC-bound radioactivity in the casein fraction (Figure 3C) versus the whey fraction (Figure 3C) gives the ratios of pellet: supernatant $\approx 1:20$ for HC and $\approx 1:3$ for TC.

Purification of HC from Buffalo Milk (Italy)

The major Cbl-binding protein present in Italian buffalo milk was purified by affinity chromatography (see the Materials and Methods section). The obtained preparation revealed a single protein band on SDS-PAGE and showed the absorbance spectrum characteristic for HC-HOCbl complex (Fedosov et al., 2002); see Supplemental Figure S1 and Supplemental Text (<https://doi.org/10.3168/jds.2018-15016>). The N-terminal sequence QI(C)VVNET... of the isolated protein was identical to the sequence predicted for mature HC (transcobalamin I) from buffalo (National Center for Biotechnology Information genomic database, XP_006068125.1). The unsaturated form of HC was prepared (see the Materials and Methods section) and used in several tests concerning transfer of Cbl-ligands between different protein fractions and proteolytic degradation, see below.

Effect of Low pH on the Affinity for Cbl

Liberation of CN⁵⁷Cbl from the Specific Proteins at Different pH. We examined liberation of CN⁵⁷Cbl from its complexes with the specific proteins (recombinant bovine TC and purified buffalo HC) at pH 2 (resembling the stomach) without any proteases, because of a very limited effect of pepsin on human Cbl-binders (Allen et al., 1978). Excessive CNCbl was added as a chasing agent, and protein-free Cbl was

precipitated with charcoal. Figure 4A shows that disappearance of radioactivity from the soluble protein fraction at pH 2 was very fast for bovine TC with the time of half reaction ($\tau_{1/2}$) ≤ 1 min at 21°C. In contrast, buffalo HC efficiently retained the bound CN⁵⁷Cbl at a low pH. Both proteins did not release any significant portion of the radioactive ligand in the neutral medium.

Transfer of CN⁵⁷Cbl from Bovine TC to Human HC During Alteration of pH. A pH jump (pH 6.6 $\rightarrow$ pH 2 $\rightarrow$ pH 6.6) in cow milk (containing TC-CN⁵⁷Cbl as a donor and human saliva HC as a capturing agent) demonstrated that all radioactivity was quickly transferred from TC to HC (not shown), in accord with previous findings for human TC (Newmark and Mester, 1974; Brada et al., 2001). Presence of excessive IF did not affect this redistribution, in agreement with Allen et al. (1978).

Transfer of HO⁵⁷Cbl from Caseins to Human HC. A significant portion of Cbl in cow milk (Denmark) is directly bound to caseins via a coordination bond (Fedosov et al., 2018). Transfer of the radioactive tracer from caseins in cow milk (preloaded with HO⁵⁷Cbl at pH 6.6, and precipitated at pH 2) to human saliva HC (soluble and active at pH 2) was traced over time at 2 temperatures (Figure 4B). The process was largely accomplished after 0.5 h ($\tau_{1/2} \approx 7$ min at 37°C) and 2.5 h ($\tau_{1/2} \approx 35$ min at 20°C). Temperature coefficient of the reaction was assessed as $Q_{10} \approx 2.6$ (i.e., acceleration of the process at a 10°C increase in the temperature). These results suggest that the main part of casein-coordinated Cbl is released and binds to HC already in the stomach, but ingestion of cold cow milk would somewhat prolong this transfer.

Proteolytic Degradation of Cbl-Binding Proteins at Neutral pH

Main degradation of a Cbl-containing matrix and the uptake of Cbl take place in the small intestine (Allen et al., 1978; Carmel, 1995; Fedosov, 2012; Green et al., 2017). Therefore, we examined proteolytic resistance of Cbl-carriers (“donors” preloaded with CN⁵⁷Cbl) at physiologically relevant trypsin + chymotrypsinogen concentrations (pH 7.5, 37°C). The reaction medium also contained a resistant “acceptor” of Cbl, suitable for separation by gel filtration.

Figure 5A illustrates cleavage of the recombinant bovine TC-CN⁵⁷Cbl, whereas the unsaturated buffalo HC served as an acceptor of CN⁵⁷Cbl. A complete degradation of bovine TC occurred in less than 15 min ($\tau_{1/2} \approx 1.5$ min), and all radioactivity concentrated in the peak of buffalo HC (Figure 5A). Addition of caseins somewhat decelerated cleavage of bovine TC, showing

Figure 4. Release of ^{57}Cbl from its complexes with different milk proteins at acidic and neutral pH. (A) Release from recombinant bovine (bov.) transcobalamin (TC) and buffalo (buf.) haptocorrin (HC). Radioactive ligand [CN^{57} cobalamin (Cbl) = 4 pM] was bound to either TC or HC (1 nM) and exposed to pH 2 or pH 7 in the presence of synthetic cyanoCbl (CNCbl; 100 nM). Time-dependent records show percentage of protein-associated CN^{57}Cbl at pH 2 (TC or HC) and pH 7 (TC or HC), see the key in the panel. (B) Release from casein pellet. The radioactive ligand ($\text{HO}^{57}\text{Cbl} \approx 2 \text{ nM}$) was coordinated to caseins of milk, the solution was acidified to pH 2 and exposed to HC (16 nM) from human saliva. Downward and upward curves show the percentage of HO^{57}Cbl associated at different time intervals with casein pellet (at 37 and 20°C), respectively; see the key in the panel.

$\approx 25\%$ of CN^{57}Cbl in the TC-peak after 15 min (Figure 5A). The same result ($\approx 21\%$ of residual TC- CN^{57}Cbl after 15 min) was observed after a direct treatment of cow milk, containing the natural protein preloaded with CN^{57}Cbl (not shown).

It is expected that Cbl from an easy degradable source would be accumulated on human HC, which passes to the intestine. Therefore, we tested the proteolytic degradation of human saliva HC and transfer of CN^{57}Cbl from human HC to human recombinant IF (used as a capturing protein in Figure 5B). It appeared that human HC was quite sensitive to cleavage, and $\approx 50\%$ or $>90\%$ of radioactivity ended on a more resistant IF after 15 min or 1 h of incubation, respectively (Figure 5B).

In contrast to human HC, buffalo HC was exceptionally resistant toward a combined trypsin and chymotrypsin treatment, and the reaction mixture did not reveal any transfer of radioactivity to IF even after 1 h of proteolysis (Figure 5C). It should be noted that IF still retained Cbl after such treatment, as was tested in the control experiment (Figure 5C).

We also examined the cleavage of isolated human recombinant IF, human recombinant HC, and buffalo HC (purified from Italian milk) on reductive SDS-PAGE (Figure 5D). All proteins were loaded with CNCbl

before the treatment. Comparison of the left part of the panel (native proteins) to its right part (30 min of proteolysis) showed presence of the original human IF and buffalo HC bands plus high quantities of the major fragments with $M_w \approx 30$ and 20 kDa. These 2 fragments correspond to the N- and C-terminal domains, respectively, which still bind Cbl into a “sandwich” structure even being separated from each other, as was tested earlier on human IF fragments (Fedosov et al., 2003, 2004; Mathews et al., 2007).

The proteolytic pattern of human HC exhibited a much higher sensitivity to cleavage. No native human HC ($M_w \approx 60 \text{ kDa}$) was found after 30 min, and the upper band corresponded to a truncated molecule with $M_w \approx 50 \text{ kDa}$. The stable fragments (notated as 30 kDa and 20 kDa in Figure 5D) were not accumulated, and the faint bands with $M_w \approx 25$ and $\approx 23 \text{ kDa}$ (visible in this lane) corresponded to chymotrypsin and trypsin, respectively.

Proteolysis of the unsaturated proteins (not shown) was 3 to 10 times faster than the degradation of their respective Cbl complexes. It lead to a temporary accumulation of 30 + 20 kDa fragments in the lanes of human recombinant IF, human gastric IF, and buffalo HC, whereas destruction of human recombinant HC was nearly complete after 30 min.

Figure 5. Proteolysis of the specific cobalamin (Cbl) carriers. The Cbl-transport protein (“donor”) was loaded with CN^{57}Cbl (1 nM) and treated with trypsin + chymotrypsin (pH 7.5, 37°C, BSA solution) in the presence of a more resistant Cbl-“acceptor.” The cleavage was stopped by an inhibitor, and the mixtures were fractionated by gel filtration. The obtained records (see the key in each panel) show (A) transfer of radioactivity from the recombinant bovine (bov.) transcobalamin (TC; 10 nM) to the purified buffalo (buff.) haptocorrin (HC; 16 nM) after 0, 5, and 15 min of proteolysis, where addition of caseins (cas) decelerates cleavage; (B) transfer of radioactivity from human (hum.) saliva HC (16 nM) to the recombinant human intrinsic factor (IF; 100 nM) after 0, 15, and 60 min of proteolysis; (C) absent transfer from the purified buffalo HC (10 nM, 1 h of incubation) to the recombinant human IF (100 nM, control); and (D) analysis of proteolysis by reductive SDS-PAGE of the recombinant human IF, the recombinant human HC, and the purified buffalo HC (all in complex with Cbl). Lanes to the left show the original protein preparations (≈ 0.6 mg/mL). The central lane shows standards with the indicated molecular weights (M_w). Lanes to the right present proteolysis of the respective pure protein-Cbl complexes. The upper dashed lines align the original protein bands with the upper bands in the proteolyzed samples. The lower dotted lines indicate the approximate positions of 2 stable fragments of ≈ 30 and 20 kDa.

DISCUSSION

The necessity of Cbl from exogenous sources is ubiquitous in the animal kingdom, though herbivores and carnivores solve this problem differently. The vitamin is synthesized by microorganisms, inhabiting, for example, the digestive system of ruminants, where cellulose is processed via bacterial fermentation (Schneider and Stroiński, 1987). The excessive Cbl accumulates in the host tissues and secretions (e.g., milk), which become a source of the vitamin for the offspring, as well as carnivores and omnivores.

The content of Cbl in cow milk (approximately 3 nM ≈ 4 $\mu\text{g/L}$) is at a lower end of values, if compared with meat (≈ 20 nmol/kg) or liver (500–1,000 nmol/kg; Watanabe, 2007; Fedosov, 2012; Gille and Schmid, 2015). Nevertheless, dietary surveys in human populations show a high positive correlation between the Cbl status and the intakes of milk and dairy product (Rice et al., 2013; Wang et al., 2013; Laird et al., 2017).

We found that the milk samples from the investigated large herds of cows and buffaloes had more or less uniform Cbl concentrations (≈ 3 nM, Table 1), which agrees with the literature (Gille and Schmid, 2015). It

should be stressed, however, that the total quantity of Cbl is not identical to its biologically available amount as the resistance of Cbl carriers toward destruction by heating, acidic pH, and proteolysis is of consequence for the intestinal Cbl uptake, which requires liberation of the vitamin from the food matrix (Watanabe, 2007; Gille and Schmid, 2015).

The process of Cbl uptake (Nexø, 1998; Fedosov, 2012; Green et al., 2017) starts with partial degradation of the food matrix in the acidic medium of human stomach, where Cbl is partially liberated and binds to the first human carrier HC. The digestion continues in the intestine, resulting in proteolysis of most proteins (including human HC) and transfer of Cbl to the proteolytically stable human carrier IF. The complex IF-Cbl interacts with the intestinal receptor cubam, whereupon Cbl is transferred to blood and delivered to different tissues (see Nexø, 1998; Fedosov, 2012; Green et al., 2017, for reviews). It should be mentioned that only HC binds Cbl at pH 2 in a tight complex, whereas TC and IF have a low affinity for Cbl in the acidic mediums (Newmark and Mester, 1974; Del Corral and Carmel, 1990; Brada et al., 2001).

It was earlier found that TC and HC are present in milk of Holstein Friesian cows and humans, respectively (Burger and Allen, 1974; Fedosov et al., 1996; Greibe et al., 2013). Other species with identified Cbl carriers reveal an HC-like protein in sow milk (Trugo, 1988) and TC with the functional properties of HC in rodents (Raaberg et al., 1989; Hygum et al., 2011). The excessive and resistant Cbl binders may negatively affect bioavailability of the vitamin, and this fact prompted us to examine Cbl carriers in milk from European and Indian cows and buffaloes.

The 4 milk specimens of the current study demonstrated different sets of the endogenous Cbl-carriers, which greatly varied in the degree of their saturation (Figures 1 and 2 and Tables 1 and 2). The main Cbl-specific proteins in the milk samples from Danish cows and Italian buffaloes were respectively identified as TC (Fedosov et al., 1996) and HC (this publication) after isolation and N-terminal sequencing. Application of the chromatographic separation, the thermostability test, as well as the immunological and the ligand binding studies, allowed us further to define the Cbl-binding proteins in all samples, where an unexpectedly high diversity was found, suggesting some speculations in the context of evolution and Cbl bioavailability.

It appeared that the Italian Mediterranean buffaloes produced milk rich in HC, similar to many other species, but markedly different from the 3 other milk specimens of our study, where TC was found. We do not think that a relatively small herd of Italian buffaloes could have provided such outlying result due to its size

and regional singularity (though these causes cannot be completely excluded). We speculate instead, whether this pattern might belong to an ancestral or “wild type” milk. It is known that Mediterranean breed of buffalo remained isolated over a thousand years without cross-breeding with other breeds, whereas intensive selection took place in other regions of the world (Cockrill, 1981). In such a way, the Italian buffaloes could have retained the ancestral pattern of Cbl carriers with a high HC and a low TC. Presence of a resilient protein HC in milk of different species was physiologically rationalized in several publications (Ford et al., 1975; Trugo and Newport, 1985; and references thereof), which suggested a direct intestinal uptake of HC-Cbl complex in neonates during the first 0 to 14 d, when the specific IF-dependent uptake has not yet been developed. At a later stage of development, the prevailing mechanism requires proteolysis of HC. A hampered assimilation of Cbl bound to HC was found under supplementation of mice with buffalo milk (Indian vs. Italian), where the unsaturated carriers (TC vs. HC) were labeled with radioactive Cbl (Greibe et al., 2017). It seems that secretion of a less sturdy carrier (like TC) to milk might be advantageous, at least from the point of view of human consumers.

Indeed, the milk of other breeds (e.g., buffaloes and cows from India) exhibited a diminished content of both HC and the excessive Cbl binding capacity. The utmost grade of this transformation was found in the milk of Holstein Friesian cows (Denmark), which contained only TC, present at a concentration below the total Cbl. The surplus of the ligand (represented by HOCbl) was confined to the casein fraction, where HOCbl got coordinated to His groups of caseins (Fedosov et al., 2018). We should, however, mention that bovine colostrum contained unsaturated HC ≈ 5 nM, identified by N-terminal sequencing in a partially purified sample (S. N. Fedosov, unpublished data, 1997). Secretion of HC apparently stops at a later stage of lactation.

The ceased secretion of excessive HC on the background of scarce TC in milk is expected to simplify the intestinal uptake of Cbl in several aspects. First of all, an easy release of Cbl from TC would take place already in the stomach, if the level of acid is normal (Figure 4A). Second, TC is proteolyzed at an exceedingly high rate (Figure 5A), which can be explained by a high number of Lys and Arg residues on its surface (Wuerges et al., 2006), making this protein an easy target for trypsin. Proteolysis of several other Cbl-specific proteins showed that human HC is rather sensitive to trypsin + chymotrypsin treatment (Figure 5B), meaning that the initial capturing of dietary Cbl by human HC can hardly present a problem. The residue of Arg-83 in the N-terminal domain of human HC is appar-

ently the first target for trypsin, because this residue is particularly exposed (based on the 3-dimensional structure from Furger et al., 2013), and cleavage at this site would cause a loss of a 9.3-kDa fragment, as observed on SDS-PAGE (Figure 5D). Interestingly, the sequence of buffalo HC (National Center for Biotechnology Information genomic database, XP_006068125.1) contains a substitution at this position (Glu-83), which apparently confers some resistance against trypsinolysis. In both cases, we refer to the mature sequence numbering.

All types of HC and IF are glycoproteins (Allen et al., 1978; Gordon et al., 1991; Nexø, 1998; Fedosov et al., 2007b), where multiple sugar residues provide some protection against proteases. We can mention in this regard that both human and buffalo HC contain 6 sites with a high N-glycosylation potential (plus 2 and 1 site, respectively, with somewhat lower potentials). Yet, human HC has no protection of its N-terminal part (first glycosylation being at Asn-114 in the mature sequence), whereas glycosylation of buffalo HC starts already from Asn-6. All in all, the N-terminal part of human HC seems to be easily degradable, which provides a facilitated transfer of Cbl to proteolytically resistant human IF (Figures 5B, 5D), capable of binding Cbl even being cleaved in 2 main fragments (Fedosov et al., 2004, 2005; Mathews et al., 2007).

The Cbl carriers in milk should also be discussed in respect to association of Cbl with caseins. This feature might be of a consequence for cheese production, because presence or absence of Cbl can potentially govern the growth of microorganisms (dependent on or independent of the exogenous Cbl). The excessive Cbl-specific binding capacity of TC or HC (found in both types of buffalo milk) leads to a general abstraction of Cbl from the casein fraction. This was illustrated both earlier (Fedosov et al., 2018) and in Figure 4B. In contrast, the insufficient binding capacity (TC + HC < HOCbl, as in cow milk) leads to coordination of the free vitamin to caseins, providing a 2- to 10-fold higher Cbl in comparison to caseins of buffalo milk.

Casein and its peptides might affect the intestinal uptake of Cbl both positively and negatively. The precipitated complex of casein-Cbl is expected to transfer some amount of Cbl to human gastric HC in the stomach, though this process considerably decelerates at a low temperature (Figure 4B). In the small intestine IF gradually extracts Cbl from the casein, which may, however, delay the formation of IF-Cbl complex. Such delay is not necessarily negative, as stretching of IF-Cbl formation over time reduces the temporary overload and incapacitation of the specific absorption system. So far, our preliminary experiments in rats showed comparable uptakes of Cbl, administered as the casein-Cbl complex or the free vitamin in water (Greibe et al., 2017).

CONCLUSIONS

We analyzed Cbl-binding proteins in pooled milk samples from Danish cows (*Bos taurus*, Holstein Friesian breed), Indian cows (a crossbreed of local *Bos indicus* and Friesians), Indian buffaloes (*Bubalus bubalis*, a crossbred of local animals and Murrah breed), and Italian buffaloes (*Bubalus bubalis*, Italian Mediterranean breed). All samples had similar concentrations of Cbl (≈ 3 nM) but differed in their sets of Cbl binding proteins (HC and TC), as well as in either highly excessive or insufficient Cbl binding capacity. A high content of HC (largely exceeding the endogenous Cbl) was found in milk of Italian buffaloes. This might present some problem for assimilation of Cbl by humans, because buffalo HC is resistant to both acidic treatment (resembling the stomach conditions) and proteolytic degradation by trypsin and chymotrypsin (resembling the intestinal conditions). Even a harsh denaturing caused only a partial release of Cbl from HC. Less confined pools of Cbl were found in milk from Indian buffaloes and cows that contained the decreasing amounts of HC and the increasing amounts of the other Cbl-carrier TC, susceptible to low pH and proteolysis. A progressive loss of the surplus Cbl-binding capacity was also observed in milk from these animals, when put in the above range. Danish cows produced milk without any significant quantity of HC, and the amount of TC was not sufficient to bind all endogenous Cbl. Therefore, approximately one-half of the vitamin was attached to caseins via a single coordination bond. Neither of the Cbl-specific proteins bound to caseins to any appreciable grade, implying a low content of Cbl in the casein pellet of Italian buffaloes and a relatively high content of Cbl in the casein pellet of Danish cows. The latter fact might be of consequence for cheese production because Cbl potentially regulates growth of Cbl-dependent versus Cbl-independent microorganisms.

ACKNOWLEDGMENTS

We express our sincere gratitude to Francesco Fantuz, Dipartimento di Bioscienze e Medicina Veterinaria, Università degli Studi di Camerino, Italy, for help in acquisition of buffalo milk samples from a local farm, and Sadanand Naik, Department of Pathology, Deenanath Mangeshkar Hospital and Research Center, Pune, India, for help in acquisition of cow and buffalo milk from Chitale Dairy, Maharashtra. We greatly appreciate the excellent technical assistance of I. M. Jensen and J. F. Pedersen. This work was supported by the Danish Agency for Science, Technology and Innovation (Copenhagen, Denmark), grant number 0603-00518B, Transcobalamin in Milk (TRIM) project.

REFERENCES

- Allen, R. H., B. Seetharam, N. C. Allen, E. R. Podell, and D. H. Alpers. 1978. Correction of cobalamin malabsorption in pancreatic insufficiency with a cobalamin analogue that binds with high affinity to R protein but not to intrinsic factor. In vivo evidence that a failure to partially degrade R protein is responsible for cobalamin malabsorption in pancreatic insufficiency. *J. Clin. Invest.* 61:1628–1634. <https://doi.org/10.1172/JCI109083>.
- Brada, N., M. M. Gordon, J. Wen, and D. H. Alpers. 2001. Transfer of cobalamin from intrinsic factor to transcobalamin II. *J. Nutr. Biochem.* 12:200–206. [https://doi.org/10.1016/S0955-2863\(00\)00129-7](https://doi.org/10.1016/S0955-2863(00)00129-7).
- Burger, R. L., and R. H. Allen. 1974. Characterization of vitamin B12-binding proteins isolated from human milk and saliva by affinity chromatography. *J. Biol. Chem.* 249:7220–7227.
- Carmel, R. 1995. Malabsorption of food cobalamin. *Baillieres Clin. Haematol.* 8:639–655. [https://doi.org/10.1016/S0950-3536\(05\)80224-0](https://doi.org/10.1016/S0950-3536(05)80224-0).
- Cockrill, W. R. 1981. The water buffalo: A review. *Br. Vet. J.* 137:8–16.
- Del Corral, A., and R. Carmel. 1990. Transfer of cobalamin from the cobalamin-binding protein of egg yolk to R binder of human saliva and gastric juice. *Gastroenterology* 98:1460–1466. [https://doi.org/10.1016/0016-5085\(90\)91076-I](https://doi.org/10.1016/0016-5085(90)91076-I).
- Fedosov, S. N. 2012. Physiological and molecular aspects of cobalamin transport. *Subcell. Biochem.* 56:347–367. https://doi.org/10.1007/978-94-007-2199-9_18.
- Fedosov, S. N., L. Berglund, N. U. Fedosova, E. Nexø, and T. E. Petersen. 2002. Comparative analysis of cobalamin binding kinetics and ligand protection for intrinsic factor, transcobalamin and haptocorrin. *J. Biol. Chem.* 277:9989–9996. <https://doi.org/10.1074/jbc.M111399200>.
- Fedosov, S. N., L. Berglund, E. Nexø, and T. E. Petersen. 1999. Sequence, S–S bridges, and spectra of bovine transcobalamin expressed in *Pichia pastoris*. *J. Biol. Chem.* 274:26015–26020. <https://doi.org/10.1074/jbc.274.37.26015>.
- Fedosov, S. N., L. Berglund, E. Nexø, and T. E. Petersen. 2007a. Tetrazole derivatives and matrices as novel cobalamin coordinating compounds. *J. Organomet. Chem.* 692:1234–1242. <https://doi.org/10.1016/j.jorganchem.2006.08.081>.
- Fedosov, S. N., N. U. Fedosova, L. Berglund, S. K. Moestrup, E. Nexø, and T. E. Petersen. 2004. Assembly of the intrinsic factor domains and oligomerization of the protein in the presence of cobalamin. *Biochemistry* 43:15095–15102. <https://doi.org/10.1021/bi048924c>.
- Fedosov, S. N., N. U. Fedosova, L. Berglund, S. K. Moestrup, E. Nexø, and T. E. Petersen. 2005. Composite organization of the cobalamin binding and cubilin recognition sites of intrinsic factor. *Biochemistry* 44:3604–3614. <https://doi.org/10.1021/bi047936v>.
- Fedosov, S. N., N. U. Fedosova, B. Kräutler, E. Nexø, and T. E. Petersen. 2007b. Mechanisms of discrimination between cobalamins and their natural analogues during their binding to the specific B12-transporting proteins. *Biochemistry* 46:6446–6458. <https://doi.org/10.1021/bi062063l>.
- Fedosov, S. N., N. B. Laursen, E. Nexø, S. K. Moestrup, T. E. Petersen, E. Ø. Jensen, and L. Berglund. 2003. Human intrinsic factor expressed in the plant *Arabidopsis thaliana*. *Eur. J. Biochem.* 270:3362–3367. <https://doi.org/10.1046/j.1432-1033.2003.03716.x>.
- Fedosov, S. N., E. Nexø, and C. W. Heegaard. 2018. Binding of aquocobalamin to bovine casein and its peptides via coordination to histidine residues. *Int. Dairy J.* 76:30–39. <https://doi.org/10.1016/j.idairyj.2017.08.005>.
- Fedosov, S. N., T. E. Petersen, and E. Nexø. 1995. Binding of cobalamin and cobinamide to transcobalamin from bovine milk. *Biochemistry* 34:16082–16087. <https://doi.org/10.1021/bi00049a023>.
- Fedosov, S. N., T. E. Petersen, and E. Nexø. 1996. Transcobalamin from cow milk: Isolation and physico-chemical properties. *Biochim. Biophys. Acta* 1292:113–119. [https://doi.org/10.1016/0167-4838\(95\)00173-5](https://doi.org/10.1016/0167-4838(95)00173-5).
- Ford, J. E., K. J. Scott, B. F. Sansom, and P. J. Taylor. 1975. Some observations on the possible nutritional significance of vitamin B12- and folate-binding proteins in milk. Absorption of [58Co] cyanocobalamin by suckling piglets. *Br. J. Nutr.* 34:469–492. <https://doi.org/10.1017/S0007114575000052>.
- Furger, E., S. N. Fedosov, D. L. Lildballe, R. Waibel, R. Schibli, E. Nexø, and E. Fischer. 2012. Recombinant human haptocorrin expressed in human embryonic kidney cells is similar to native haptocorrin. *PLoS One* 7:e37421. <https://doi.org/10.1371/journal.pone.0037421>.
- Furger, E., D. C. Frei, R. Schibli, E. Fischer, and A. E. Protta. 2013. Structural basis for universal corrinoid recognition by the cobalamin transport protein haptocorrin. *J. Biol. Chem.* 288:25466–25476. <https://doi.org/10.1074/jbc.M113.483271>.
- Gille, D., and A. Schmid. 2015. Vitamin B12 in meat and dairy products. *Nutr. Rev.* 73:106–115. <https://doi.org/10.1093/nutrit/nuu011>.
- Gordon, M. M., C. Hu, H. Chokshi, J. E. Hewitt, and D. H. Alpers. 1991. Glycosylation is not required for ligand or receptor binding by expressed rat intrinsic factor. *Am. J. Physiol.* 260:G736–G742. <https://doi.org/10.1152/ajpgi.1991.260.5.G736>.
- Green, R., L. Allen, A. L. Bjørke-Monsen, A. Brito, J. L. Guéant, J. W. Miller, A. M. Molloy, E. Nexø, S. Stabler, B. H. Toh, P. M. Ueland, and C. Yajnik. 2017. Vitamin B12 deficiency. *Nat. Rev. Dis. Primers* 3:17040. <https://doi.org/10.1038/nrdp.2017.40>.
- Gregory, M. E., and E. S. Holdsworth. 1955. The occurrence of a cyanocobalamin-binding protein in milk and the isolation of a cyanocobalamin-protein complex from sow's milk. *Biochem. J.* 59:329–334. <https://doi.org/10.1042/bj0590329>.
- Greibe, E., D. L. Lildballe, S. Streym, P. Vestergaard, L. Rejnmark, L. Mosekilde, and E. Nexø. 2013. Cobalamin and haptocorrin in human milk and cobalamin-related variables in mother and child: A 9-mo longitudinal study. *Am. J. Clin. Nutr.* 98:389–395. <https://doi.org/10.3945/ajcn.113.058479>.
- Greibe, E., O. Nymark, S. Fedosov, M. S. Ostensfeld, C. W. Heegaard, and E. Nexø. 2017. Absorption of vitamin B12 administered in buffalo milk: An experimental study in rats. *Scitechz Nutr. Food Sci.* 2:1006. www.scitechz.com/nutrition-food-science/fulltext/Nutrition-17-1006.pdf.
- Hygum, K., D. L. Lildballe, E. H. Greibe, A. L. Morkbak, S. S. Poulsen, B. S. Sørensen, T. E. Petersen, and E. Nexø. 2011. Mouse transcobalamin has features resembling both human transcobalamin and haptocorrin. *PLoS One* 6:e20638. <https://doi.org/10.1371/journal.pone.0020638>.
- Kornerup, L. S., C. B. Juul, S. N. Fedosov, C. W. Heegaard, E. Greibe, and E. Nexø. 2016. Absorption and retention of free and milk protein-bound cyano- and hydroxocobalamins. An experimental study in rats. *Biochimie* 126:57–62. <https://doi.org/10.1016/j.biochi.2015.11.024>.
- Kräutler, B., and B. Puffer. 2012. Vitamin B₁₂-derivatives: Organometallic catalysts, cofactors and ligands of bio-macromolecules. Pages 133–263 in *Handbook of Porphyrin Science*. K. M. Kadish, K. M. Smith, and R. Guilard, ed. World Scientific, Singapore. https://doi.org/10.1142/9789814397605_0019.
- Laird, E., M. C. Casey, M. Ward, L. Hoey, C. F. Hughes, K. McCarroll, C. Cunningham, J. J. Strain, H. McNulty, and A. M. Molloy. 2017. Dairy intakes in older Irish adults and effects on vitamin micronutrient status: Data from the TUDA study. *J. Nutr. Health Aging* 21:954–961. <https://doi.org/10.1007/s12603-016-0845-4>.
- Mathews, F. S., M. M. Gordon, Z. Chen, K. R. Rajashankar, S. E. Ealick, D. H. Alpers, and N. Sukumar. 2007. Crystal structure of human intrinsic factor: Cobalamin complex at 2.6-Å resolution. *Proc. Natl. Acad. Sci. USA* 104:17311–17316. <https://doi.org/10.1073/pnas.0703228104>.
- Matte, J. J., M. Britten, and C. L. Girard. 2014. The importance of milk as a source of vitamin B12 for human nutrition. *Anim. Front.* 4:32–37. <https://doi.org/10.2527/af.2014-0012>.
- Mutti, E., M. Hunger, S. N. Fedosov, E. Nexø, and B. Kräutler. 2017. Organometallic DNA-B12 conjugates as potential oligonucleotide vectors: Synthesis and structural and binding studies with human cobalamin-transport proteins. *ChemBioChem* 18:2280–2291. <https://doi.org/10.1002/cbic.201700472>.

- Newmark, P., and S. Mester. 1974. Effect of pH on vitamin B₁₂ binding by transcobalamins. *Biochim. Biophys. Acta* 343:627–631. [https://doi.org/10.1016/0304-4165\(74\)90281-5](https://doi.org/10.1016/0304-4165(74)90281-5).
- Nexo, E. 1975. A new principle in biospecific affinity chromatography used for purification of cobalamin-binding proteins. *Biochim. Biophys. Acta* 379:189–192.
- Nexo, E. 1998. Cobalamin binding proteins. Pages 461–475 in *Vitamin B12 and B12-Proteins*. B. Kräutler, D. Arigoni, and B. T. Golding, ed. Wiley-VCH, Weinheim, Germany. <https://doi.org/10.1002/9783527612192.ch30>.
- Nexo, E., A. L. Christensen, A. M. Hvas, T. E. Petersen, and S. N. Fedosov. 2000. Measurement of transcobalamin by ELISA. *Clin. Chem.* 46:1643–1649. <http://clinchem.aaccjnl.org/content/46/10/1643>.
- Pratt, J. M. 1972. *Inorganic Chemistry of Vitamin B₁₂*. Academic Press, London, UK.
- Raaberg, L., E. Nexø, S. S. Poulsen, and L. Tollund. 1989. Cobalamin and its binding in rat milk. *Scand. J. Clin. Lab. Invest.* 49:529–535. <https://doi.org/10.3109/00365518909089132>.
- Rice, B. H., E. E. Quann, and G. D. Miller. 2013. Meeting and exceeding dairy recommendations: Effects of dairy consumption on nutrient intakes and risk of chronic disease. *Nutr. Rev.* 71:209–223. <https://doi.org/10.1111/nure.12007>.
- Rutten, M. J. M., A. C. Bouwman, R. C. Sprong, J. A. M. van Arendonk, and M. H. P. W. Visker. 2013. Genetic variation in vitamin B₁₂ content of bovine milk and its association with SNP along the bovine genome. *PLoS One* 8:e62382. <https://doi.org/10.1371/journal.pone.0062382>.
- Sandberg, D. P., J. A. Begley, and C. A. Hall. 1981. The content, binding and forms of vitamin B₁₂ in milk. *Am. J. Clin. Nutr.* 34:1717–1724. <https://doi.org/10.1093/ajcn/34.9.1717>.
- Schneider, Z., and A. Stroiński. 1987. *Comprehensive B12: Chemistry, Biochemistry, Nutrition, Ecology, Medicine*. Walter de Gruyter, Berlin, Germany. <https://doi.org/10.1515/9783110844795>.
- Trugo, N. M. 1988. Characterization of the vitamin B₁₂-binding protein isolated from sow's milk and its affinity for cyanocobalamin and other corrinoids. *Braz. J. Med. Biol. Res.* 21:883–894.
- Trugo, N. M., and M. J. Newport. 1985. Vitamin B₁₂ absorption in the neonatal piglet. 2. Resistance of the vitamin B₁₂-binding protein in sows' milk to proteolysis in vivo. *Br. J. Nutr.* 54:257–267. <https://doi.org/10.1079/BJN19850109>.
- Vogiatzoglou, A., A. D. Smith, E. Nurk, P. Berstad, C. A. Drevon, P. M. Ueland, S. E. Vollset, G. S. Tell, and H. Refsum. 2009. Dietary sources of vitamin B-12 and their association with plasma vitamin B-12 concentrations in the general population: The Hordaland homocysteine study. *Am. J. Clin. Nutr.* 89:1078–1087. <https://doi.org/10.3945/ajcn.2008.26598>.
- Wang, H., K. A. Livingston, C. S. Fox, J. B. Meigs, and P. F. Jacques. 2013. Yogurt consumption is associated with better diet quality and metabolic profile in American men and women. *Nutr. Res.* 33:18–26. <https://doi.org/10.1016/j.nutres.2012.11.009>.
- Watanabe, F. 2007. Vitamin B₁₂ sources and bioavailability. *Exp. Biol. Med.* (Maywood) 232:1266–1274. <https://doi.org/10.3181/0703-MR-67>.
- Wuerges, J., G. Garau, S. Geremia, S. N. Fedosov, T. E. Petersen, and L. Randaccio. 2006. Structural basis for mammalian vitamin B₁₂ transport by transcobalamin. *Proc. Natl. Acad. Sci. USA* 103:4386–4391. <https://doi.org/10.1073/pnas.0509099103>.