Binding of aquocobalamin to bovine casein and its peptides *via* coordination to histidine residues.

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Abbreviations. AdoCbl, 5'–deoxy–5'–adenosylcobalamin; AU, absorbance units; B_{12}, vitamin B_{12}; Cbl, cobalamin, active group not specified; Cas/Pep/HisCbl, complexes of casein, casein peptides or histidine with Cbl; CasHis, active His in caseins; CNCbl, cyanocobalamin; CN^{57}Cbl, radioactive $CN[^{57}Co^{3+}]$Cbl; DMB, 5,6–dimethylbenzimidazole; DU, dilution units; HOCbl, aquocobalamin (both H_2OCbl^+ and $HOCbl^0$); HO^{57}Cbl, radioactive $HO[^{57}Co^{3+}]$Cbl; IF, intrinsic factor; MeCbl, methylcobalamin; PepHis, active His in casein peptides; P_i, inorganic phosphate or Na–phosphate buffer; TC, transcobalamin; XHis, unspecified His–containing compound.

Abstract

Vitamin B_{12} (cobalamin, Cbl) is an essential nutrient of bovine milk bound to the casein fraction and the Cbl–specific protein transcobalamin (TC) at a ratio of 50:50. The current work aims to elucidate the mechanism of interaction between Cbl and the caseins. We found that the isolated caseins bind aquocobalamin (HOCbl) *via* histidine–[Co^{3+}]Cbl coordination. The casein–Cbl complex slowly dissociates in the presence of KCN due to formation of CNCbl. The "active" His–groups (5.7 mM measured in 36 mg·mL^{-1} caseins) accumulate HOCbl≤3 mM at a high rate ($t_{½}$≈10 min, 20 ºC) and with a high affinity (K_d=0.1 mM). Low pH hinders the binding but does not affect a very slow dissociation ($t_{½}$≈8 h). Increased temperature and/or the presence of the specific Cbl–binding proteins accelerate the dissociation. The consequences of casein–Cbl interactions for the intestinal uptake of Cbl remain unclear.

1. Introduction

Vitamin B_{12} or cobalamin (Cbl) is an important nutrient synthesized by bacteria and obtained by animals *via* the food chain. The vitamin has a complex core structure (Fig. 1A) including a corrin ring and a nucleotide base 5,6–dimethylbenzimidazole (DMB) coordinated to the central cobalt ion at the lower plane [Co^{3+}]–DMB (Kräutler & Puffer, 2012; Pratt, 1972). The upper plane R–[Co^{3+}] contains an exchangeable coordination group "R", e.g., 5′–deoxyadenosyl (Ado), methyl (Me), water (H_2O/HO^-) or cyanide (CN^-). Animal cells produce the catalytically active cofactors (AdoCbl and MeCbl) from most Cbl–species, including the widely used vitamin form CNCbl. The two Cbl–cofactors lose their active groups under exposure to light or after an abnormal termination of the catalytic cycle caused by oxidation of the reduced intermediates [Co^{2+}] or [Co^{1+}]Cbl (Kräutler & Puffer, 2012; Pratt 1972). These conversions produce aquoCbl, which exists in a fast protonation equilibrium $H_2OCbl^+ \leftrightarrow H^+ + HOCbl^0$ with pK 7.5 – 7.8 (Pratt, 1972). In the present paper we will use the abbreviation HOCbl to cover all aquo–species irrespective of pH. The exchangeable groups "R" vary in their affinity for [Co^{3+}]Cbl, and the stronger ligands (e.g., CN^-) displace the weaker ligands (e.g., H_2O) (Pratt, 1972). Each particular [Co^{3+}]–ligand affects the conjugated bonds of the corrin ring and markedly changes the absorbance spectrum of Cbl, which in tern facilitates the identification of the [Co^{3+}]–coordinated groups (Pratt, 1972).

Cobalt–sufficient herbivores have a free access to Cbl due to microbiological fermentation of cellulose in their digestive system, accompanied by intensive synthesis of Cbl and its release from the degrading bacterial cells (Fedosov, 2012). Carnivores and omnivores, including humans, obtain Cbl from the food of animal origin. Milk is an important source of the vitamin, and bovine milk has been notably reported to provide highly bioavailable Cbl in comparison to other food items (Matte, Britten, & Girard, 2014; Vogiatzoglou et al., 2009). Most animal products (including milk) contain considerable quantities of HOCbl (Farquharson & Adams, 1976; Fie, Zee, & Amiot, 1994; Gimsing

& Nexo, 1983), since both AdoCbl and MeCbl convert to this form upon exposure to light. The exact levels for different Cbl–species in shelf products are, however, debated.

Milk from different species usually contains varying amounts of endogenous Cbl plus some excessive binding capacity associated with the specific high–affinity proteins of Cbl transport. Bovine milk practically lacks such specific binding capacity ($\leq$ 0.05 nM) but contains approximately 3 nM of endogenous Cbl (Fedosov, Petersen, & Nexo, 1996), which equally separates into two protein fractions ($M_w \geq$ 200 kDa and 40 kDa) upon gel–filtration.

The Cbl–carrier from the 40 kDa fraction of bovine milk was purified (Fedosov et al., 1996), cloned (Fedosov, Berglund, Nexo, & Petersen, 1999), characterized (Fedosov et al., 1999; Wuerges et al., 2006) and classified as transcobalamin (TC), a 43 kDa protein from a small family of extracellular Cbl–transporters responsible for preservation, uptake and targeted delivery of the vitamin (Fedosov, 2012; Gimsing & Nexo, 1983). Other representatives of this group are intrinsic factor (IF) and haptocorrin (HC). All three types of Cbl–specific proteins have an exceptional affinity for Cbl with $K_d \leq 10^{-14}$ M (Fedosov, Fedosova, Kräutler, Nexo, & Petersen, 2007) caused by an intricate scaffolding of protein – ligand bonds, found in their 3D–structures (Wuerges et al., 2006; Furger, Frei, Schibli, Fischer, & Prota, 2013 and references thereof). IF is produced in gastric juice, selectively binds Cbl and ensures its intestinal uptake. HC is the dominating Cbl–carrier in blood plasma and most extracellular fluids, e.g., human milk (Sandberg, Begley, & Hall, 1981) and saliva (Nexo, Hansen, & Konradsen, 1988). The presence of TC in bovine milk was rather unexpected, because HC is usually secreted to exocrine fluids (Morkbak, Poulsen, & Nexo, 2007; Nexo et al., 1988; Sandberg et al., 1981).

The high molecular Cbl fraction in bovine milk co–eluted with the casein micelle (Fedosov et al., 1996). Casein in milk is found as large amorphous aggregates of four small proteins: α_{s1}–, α_{s2}–, β– and κ–caseins (M_w = 18 – 24 kDa) present at the approximate overall concentration of 1.2 mM. They form a microporous network (Fig. 1B) that dynamically changes its structure according to

several conflicting models of micelle / submicelle organization (Farrell, Malin, Brown, & Qi, 2006). The nature of Cbl – casein interactions has remained unexplored since our original publication (Fedosov et al., 1996). Several possible binding mechanisms are sketched out in Fig. 1B (see Discussion section for more details). One of them (Scheme 4, direct coordination of HOCbl to a protein group) stands apart from all other mechanisms, because this is the only type of interaction sensitive to cyanolysis (Pratt, 1972).

Here we aim to reach a better understanding of the nature of the casein – Cbl interactions, because this may be relevant to the bioavalability and quantity of the vitamin in milk and milk products. We present a detailed characterization of the interactions between Cbl (endogenous or exogenous) and the casein fraction of bovine milk (existing as a part of the milk matrix or purified). We demonstrate that very high quantities of HOCbl can be loaded into caseins *via* coordination to their histidine (His) residues, and that caseins retain all HOCbl above the specific binding capacity of TC, IF or HC (if any of those is present).

2. Materials and Methods

2.1. Materials

Fresh bovine milk was obtained from a local farmer (Denmark, Holstein cows). The milk was neither specifically protected from light nor decidedly subjected to illumination, resembling in this regard the ordinary product delivered to dairies. The sample was skimmed by centrifugation and stored at 4 ºC or –20 ºC. Polyclonal antibodies against recombinant bovine TC, notated as № 1 and № 2 (Fedosov et al., 1999), were produced by Dako A/S (Denmark). Radioactive CN[$^{57}Co^{3+}$]Cbl was manufactured by MP Biomedicals, LLC (USA). Radioactive HO[$^{57}Co^{3+}$]Cbl was produced from CN[$^{57}Co^{3+}$]Cbl by the photo–aquation method described earlier (Kornerup et al., 2016). Chymosin solution was kindly provided by Arla Foods (Denmark). Recombinant intrinsic factor (IF) was produced and purified from plants as described earlier (Fedosov et al., 2003). Anonymous

107 human saliva was used as a source of native HC (concentration of 40 nM). Sephacryl^{TM} S–200
108 High Resolution was purchased from GE Healthcare (USA). All standard salts and organic
109 compounds were purchased from Sigma (USA).
110
111 *2.2. Isolation of casein from bovine milk*
112 A sample of skim milk (200 mL) was put on ice and 2.1 mL of 37% HCl was added in 0.1 –
113 0.2 mL portions under constant mixing until pH decreased to 2.0. The suspension was centrifuged
114 (15 000 g, 10 min), and the effectiveness of precipitation was corroborated by gel–filtration of the
115 supernatant, where the content of soluble caseins decreased from 20 – 24 mg·mL^{-1} in the original
116 milk to 0.2 – 0.4 mg·mL^{-1} in the whey fraction. The casein pellet was collected (with loss of 15 –
117 20 % under transfer between test tubes) and washed twice by suspending it in 2 × 130 mL of a cold
118 salt solution (0.3 M NaCl + 5 mM HCl) and precipitating the protein by centrifugation. The washed
119 pellet was finally suspended in 60 mL of water (room temperature) and neutralized by adding
120 approximately 400 μL of 12 M NaOH in 20 – 50 μL portions until pH increased to 6.6 – 6.8 and
121 stabilized (after approximately1 h at room temperature). The neutralized sample was centrifuged to
122 remove some remains of insoluble protein (15 – 20 %), and the supernatant was stored frozen until
123 its application (85 mL, 36 mg·mL^{-1}, with titrated active [CasHis] = 5.70 mM, Section 3.3).
124 Reconstitution of the high–molecular casein structure was tested by gel–filtration (Section 3.2), and
125 the protein composition of the isolated preparation did not differ from that of the native milk caseins
126 (based on SDS PAAGE, not shown).
127
128 *2.3. Preparation of casein peptides*
129 A sample of casein (28 mL, 36 mg·mL^{-1}) was mixed with trypsin (final concentration of 0.14
130 mg·mL^{-1}) and chymotrypsinogen (0.07 mg·mL^{-1}), and the mixture was incubated for 20 h at 37 ºC.
131 The remaining proteolytic activity was inactivated by heating the sample at 85 – 95 ºC for 1 h. The

peptide mixture was cooled down and briefly centrifuged. No residual proteolytic activity was found when testing with N–benzoyl–L–Arginine–p–nitroanilid (0.5 mM). The sample was concentrated by lyophilization and stored as a stock solution (12 mL, 65 $mg \cdot mL^{-1}$, total [PepHis] = 8.7 mM assessed from the total His content in the protein sequences, Section 3.4).

2.4. Gel–filtration

Separation of proteins by their hydrodynamic size was performed at room temperature on a Sephacryl S–200 column (h = 100 cm, d = 1.6 cm) equilibrated with 0.1 M Tris pH 7.5, 1 M NaCl (filtration rate of 0.15 ml/min, 2.8 – 3 mL per fraction). Samples of milk (3.5 mL) or casein (3.5 mL) were briefly centrifuged, then the supernatant (3 mL) was collected and preincubated with or without reactants for 1 – 3 h at 37 ºC. The sample was loaded onto the column after an additional centrifugation. The protein profiles were recorded at A_{280} or $A_{280} - A_{350}$ in the case of turbidity. The elution peaks of proteins with known molecular masses were used as the reference points: IgM of blood plasma ($M_w \approx 900$ kDa, $V_0 = 75$ mL), IgG (≈ 160 kDa, $V_e = 85$ mL), bovine / human albumins (67 kDa, 105 mL), β–lactoglobulin (dimer 37 kDa, 115 mL), α–lactalbumin (14 kDa, 135 mL), small peptides ($V_0 + V_i = 195$ mL).

2.5. Measurement of endogenous Cbl and Cbl–binding capacity

Total Cbl was measured by the standard procedure for ADVIA Centaur CP immunoassay System (Siemens Healthcare Diagnostics, Denmark). When detecting Cbl in the gel–filtration fractions, a specific calibration curve was prepared using Cbl dissolved in the corresponding buffer (0.1 M Tris, pH 7.5 and 1 M NaCl) because of its effect on the luminescent signal of the tracer (a Cbl–conjugate). Cbl–binding capacity was measured by incubating the sample with excess of CN[^{57}Co]Cbl with a known specific radioactivity followed by precipitation of the unbound CN[^{57}Co]Cbl with hemoglobin–coated charcoal essentially as previously described (Gottlieb, Lau,

157 Wasserman, & Herbert, 1965). Radioactivity was measured by gamma counting in 2470 Wizard2
158 Automatic Gamma Counter, (Perkin Elmer, USA).
159
160 *2.6. Immunological measurement of TC*
161 Detection of total TC in bovine milk or its fractions was based on an in house immunoassay
162 performed essentially as previously described for total human TC (Nexo, Christensen, Hvas,
163 Petersen, & Fedosov, 2000).
164
165 *2.7. Absorbance measurements and monitoring of binding – dissociation*
166 The concentrations of reactants were determined using the coefficients of molar absorbance:
167 ε_{351} = 26 200 AU·M^{-1} (HOCbl pH < 6.6), ε_{351} = 28 080 AU·M^{-1} (CNCbl), ε_{351} = 30 700 AU·M^{-1}
168 (diCNCbl, 10 mM KCN, pH 12) (Pratt, 1972), ε_{212} = 5 700 AU·M^{-1} (His, pH 2.0) (Saidel, Golfarb,
169 & Waldman, 1952), $E_{280} \approx 1$ AU·ml·mg^{-1} (for a protein mixture).
170 Equilibrium binding of HOCbl (0.205 mM and 1.06 mM) to caseins (at different dilutions of
171 the latter with 0.1 M P_i–buffer, pH 6.8) was examined by optical response of HOCbl after a
172 prolonged incubation of the two interacting species (18 h at 37 ºC followed by 4 h at 20 ºC). We
173 employed wavelengths at downward (470 nm) and upward (560 nm) shifts in absorbance (e.g., A_{560}
174 – A_{470}) to counteract possible unspecific changes of the signal (e.g., light scattering of the
175 concentrated protein samples). The absorbance characteristics of pure HOCbl were set as zero. All
176 samples with 1 mM Cbl were additionally diluted 1/5 before the measurements to match the
177 absorbance of 0.2 mM Cbl. The degree of immediate dissociation of XHisCbl species was
178 considered to be insignificant within 1 min after the dilution.
179 Time–dependent binding (HOCbl + XHis) was started by adding 4 µL of 1.06 mM HOCbl to
180 200 µL of XHis–containing solution and monitoring the change of absorbance $A_{358} - A_{351}$ over
181 time. The absorbance difference for pure HOCbl was set as zero. Mediums with different pH

included: 0.1 M P_i–buffer, pH 6.8; 0.02 M Na–acetate + 0.1 M P_i, pH 4.6; and 0.1 M P_i–buffer, pH 2.0. In all cases, pH was verified after addition of His and adjusted, if necessary. All curves (here and in other kinetic experiments) were recorded with 2 or 3 repeats, whereupon the average curves were presented in the figures.

In the dissociation experiments, all Cbl complexes were pre–formed in advance by incubating 1.06 mM HOCbl with a His–containing reactant (e.g., caseins 7.2 $mg \cdot mL^{-1}$ = 1.14 mM of CasHis or 10 mM of pure His) for 20 h at pH 6.8. Dissociation in the neutral medium was initiated by a 1/50 dilution with 0.1 M P_i–buffer, pH 6.8. The process was monitored at $A_{358} - A_{351}$ (no further additives), $A_{350} - A_{362}$ (in the presence of 0.1 – 10 mM KCN), or $A_{358} - A_{352}$ (in the presence of 40 μM IF). In some experiments, KCN (1 – 10 mM) was added to the undiluted complex of XHisCbl ([XHis] = 3.1 – 3.6 mM). The dissociation rates at different pH were examined for HisCbl. Firstly, the complex was formed by incubating 1 mM HOCbl + 10 mM His for 20 h at pH 6.8. Then, the original solution was diluted 1/50 with one of the mediums: (i) 0.1 M P_i–buffer, pH 6.8; (ii) 0.02 M Na–acetate buffer + 0.1 M P_i, pH 4.6; or (iii) 0.1 M P_i–buffer, pH 2.0. The changes in absorbance were monitored at $A_{358} - A_{351}$ immediately after dilution.

2.8. Interaction between casein pellet and HOCbl

Casein was precipitated from a milk sample of 2 mL by (i) 2% chymosin treatment + 20 mM $CaCl_2$, 37 ºC, 30 min; or (ii) acidification to pH ≈ 4 (+ 0.06 M HCl). Pellets were collected by a short centrifugation (20 000 g, 5 min) and washed three times with excess of (i) 0.3 M NaCl followed by 0.1 M P_i–buffer, pH 6.8 for the chymosin sample; or (ii) 0.2 M Na–acetate, pH 4.6 followed by 0.02 M Na–acetate, pH 4.6 + 0.15 M NaCl for the acidified sample. The washed casein pellets were suspended in 0.1 M P_i–buffer pH 6.8 (after chymosin) or 0.02 M Na–acetate + 0.15 M NaCl pH 4.6 (after low pH) whereupon 0.1 – 1 mM HOCbl was added. The suspensions were incubated under constant agitation at room temperature. Change of color in the pellet was

monitored over time by visual observations, and content of Cbl in the supernatant was assessed by spectral measurements.

Dissociation of HOCbl from the casein pellet was examined similarly with a few modifications, the soluble casein or a milk sample was exposed to 0.1 – 1 mM HOCbl (1 h, room temperature), whereupon casein was precipitated by either chymosin or low pH and washed as described above. The red / black casein–Cbl pellets were incubated in suspension, and appearance of Cbl in the supernatants was monitored over time by spectral records.

2.9. Transfer of Cbl from milk caseins to IF

A milk sample (2 mL) containing endogenous Cbl was incubated with 100 nM of apoIF (2 h, 37 ºC) whereupon the sample was acidified on ice (+20 µL of 37% HCl in portions) and centrifuged (20 000 g, 5 min, 5 ºC). Then the casein pellet was washed 3 times by suspending it in cold 0.3 M NaCl (3 × 2 mL) and repeating the centrifugation. The final pellet was neutralized by adding small quantities of 12 M NaOH (≈ 4 µL in total) until pH became neutral according to the indicator paper. Possible traces of IF were inactivated by heating (30 min, 95 ºC). The sample was centrifuged and the supernatant tested for its Cbl content. The control sample underwent the same treatment, excluding exposure to IF. A variant of this procedure included incubation with HC (6 h at 37 ºC) added with human saliva (0.6 mL to 1.4 mL of milk) to get ≈ 10 nM of the final apoHC binding capacity. The other procedures were identical to the IF–treatment.

2.10. Analysis of binding – dissociation kinetics

All binding – dissociation interactions of the current study obeyed a bimolecular reversible reaction A + B ↔ C at comparable concentrations of the reactants. The theoretical background of this scheme was considered earlier (King, 1982). The relevant equations and fitting details are presented in the Supplementary materials.

3. Results

3.1. Distribution of Cbl and TC in bovine milk

The milk fractions were separated by gel–filtration and analyzed for presence of endogenous Cbl and TC (Fig. 2A, Δ–gray). The concentration of Cbl in the pooled 40 kDa fraction corresponded to 1.5 nM, which was almost equivalent to 1.2 nM of TC found by an immunological assay. The pooled casein fraction contained TC < 0.1 nM, which did not match the 1.3 nM of Cbl found in this fraction (both values refer to the original milk concentrations). Acidification and neutralization of milk (pH 6.6 → pH 2 → pH 6.6) completely stripped caseins of the associated TC, but did not release any considerable amount of the endogenous Cbl bound to this fraction. This discrepancy prompted us to scrutinize the nature of the Cbl binding to casein.

3.2. Cbl in milk fractions and the effect of KCN.

An aliquot of bovine milk ([Cbl] = 2.8 nM, binding capacity for [CNCbl] ≈ 30 pM) was incubated for 3 h at 37 ºC without any additives and subjected to gel–filtration. The observed profile of the endogenous Cbl (Fig. 2A, ▲–red) showed a 40 : 60 distribution between the casein fraction and TC. Incubation of an identical aliquot of milk with 1 mM KCN prior to gel–filtration (Fig. 2A, □–magenta) caused a major decrease in Cbl eluted together with caseins, a small increase in Cbl eluted with TC, and the appearance of a noticeable peak of free Cbl (absent in the original milk sample). This is a strong indication that Cbl is attached to a component of the casein fraction *via* a single coordination bond R–[Co^{3+}]Cbl, which dissociates in the presence of exceptionally potent [Co^{3+}]–coordinating agent CN^{-}.

Incubation of milk with a very small concentration of exogenous radioactive HO^{57}Cbl (0.02 nM, below the binding capacity of TC) lead to incorporation of the label into ≥ 200 kDa and 40 kDa protein fractions at a changing proportion. After 1 h of incubation, the ratio between the two peaks

257 was in favor of casein (Fig. 2B, ▲–red) with a 69 : 29 ratio of peak areas, but after 3 h the balance
258 of Cbl was changing in favor of TC (Fig. 2B, ▼–green) with a 51 : 45 ratio. This observation
259 indicates prevailing initial formation of a casein–Cbl complex, whereupon the ligand is gradually
260 transferred to a much stronger binder TC until its binding capacity is saturated.

261 When the concentration of either HO^{57}Cbl (50 nM) or CN^{57}Cbl (50 nM) considerably
262 exceeded the binding capacity of TC in milk (≈ 0.03 nM), two completely different profiles of
263 radioactivity were observed (Fig. 2C). The major peak of HO^{57}Cbl counts (▲–red) was in the high–
264 molecular casein fraction. In contrast, no considerable amount of CN^{57}Cbl bound to milk proteins,
265 but the ligand was eluted in its free form (□–magenta). The material balance of HO^{57}Cbl and
266 CN^{57}Cbl profiles showed disappearance of 40 – 50 % of the label in the first case. A separate test
267 revealed that the column matrix (Sephacryl S–200) has some affinity for HOCbl, probably due to
268 the presence of NH═ and NH_2–groups. In this regard, the profile of HO^{57}Cbl (Fig. 2C, ▲–red)
269 should be interpreted qualitatively rather than quantitatively.

270 The analogous experiment (casein + 50 nM HO^{57}Cbl) was performed with an isolated casein
271 fraction stripped of the endogenous TC and Ca^{2+} by acidic precipitation at pH 2 (Section 2.2). The
272 neutralized and dissolved casein preparation gave a transparent aqueous phase (convenient for the
273 planned optical measurements). The gel–filtration profiles of the original casein in milk (Fig. 2C)
274 and its Ca^{2+}–depleted transparent counterpart (Fig. 2D) were similar in terms of both HO^{57}Cbl
275 binding (▲–red) and the apparent size of protein micelle / submicelle (····· dark blue). This
276 observation points to similar properties of the two variants of casein in terns of their overall
277 structure and affinity for HOCbl.

278

279 *3.3. Equilibrium binding of HOCbl to casein and the accompanying spectral shift*

280 Incubation of optically detectable quantities of HOCbl (20 μM) with casein (18 mg·mL^{-1}, pH
281 6.8) was accompanied by a pronounced spectral shift (Fig. 3A). This indicates coordination of a

new axial ligand differing from the original water. When HOCbl was preincubated with 20 mM histidine (His), the recorded spectrum (Fig. 3A, ---- blue) was identical to that of the casein + Cbl sample (both showing absorbance maximums at 358 nm). Some deviation in the near ultraviolet (300 – 330 nm in Fig. 3A) was ascribed to imprecision of the baseline because of its high value within this region. This deviation was ignored, and the active groups involved into Cbl–coordination were assumed to be His–residues. Other relevant coordination species have incompatible absorbance maximums: NH_3Cbl (356 nm), CysSCbl (370 nm), CH_3COOCbl (352 nm) (Pratt, 1972). Addition of cyanide to HOCbl or HisCbl samples caused a spectral transition to CNCbl (Fig. 3A, ···· faint magenta, maximum at 361 nm) that occurred rather quickly for HOCbl but slowly for HisCbl. The registered spectral differences (Fig. 3A) were used to follow casein + HOCbl interactions as well as cyanolysis of casein–Cbl bond.

Further equilibrium binding experiments were conducted at two very high concentrations of HOCbl (0.2 mM and 1 mM) in attempt to assess the amount of the active His–residues in caseins. The changes in absorbance were measured after a prolonged incubation of HOCbl with casein at different dilutions (24 h, pH 6.8, 20–22ºC), and the absorbance shifts were plotted versus the dilution units DU = [Diluted] / [Stock] (Fig. 3B). To bring the absorbances of 1 mM and 0.2 mM Cbl within the same scale of absorbance units (AU), all samples with 1 mM Cbl were diluted 1 : 5 just before the measurements (no immediate dissociation of CasCbl accompanied this fast change of concentration). The two curves have different shapes, because a high concentration of HOCbl changes the hyperbolic saturation curve (valid for low concentrations of binding sites) to the universal square root expression (Supplementary materials, Eq. (1)). The graphical expressions of the total HOCbl concentration ($[Cbl]_0$) and the dissociation constant (K_d) are shown according to Dixon (1972) with help of dashed lines in Fig. 3B. The lines are drawn through 0 and ½ (or ⅔, or ¾ etc) saturation points and after extrapolation they cut segments at the maximal saturation level. These segments are projected onto the X–axis and visualize $[Cbl]_0 + K_d$ and K_d alone by the

distances between the secant lines (Dixon, 1972). The segments on the X–axis are expressed in DU but can be directly related to the known Cbl concentrations of 0.2 mM or 1 mM. The more precise data fitting was performed using the supplementary Eq. (1) and gave the values of total HOCbl ($[Cbl]_0$) and the dissociation constant (K_d), both expressed in DU. For example, the curve of best approximation at $[Cbl]_0$ = 1.06 mM had parameters of $[Cbl]_{0,DU}$ = 0.18 ± 0.013 DU and $K_{d,DU}$ = 0.0206 ± 0.0074 DU. Since $[Cbl]_0$ is expressed as both 0.18 DU and the known true concentration of 1.06 mM, a few simple calculations easily produce 1 DU = [Stock–His] = 5.88 mM and K_d = 0.0206 DU = 0.121 mM. The average values were obtained from the two curves in Fig. 3B and the final levels in Fig. 4A giving an estimate of [Stock–His] = 5.70 ± 0.37 mM (at 36 $mg \cdot mL^{-1}$ of casein) and K_d = 0.107 ± 0.011 mM at pH 6.8, 20–22 ºC. The total concentration of all His residues in a casein solution of 36 $mg \cdot mL^{-1}$ was estimated as 7.14 mM based on the known sequences and the realistic concentrations of all caseins (α_{s1} = 16.2 $mg \cdot mL^{-1}$, α_{s2} = 4.2 $mg \cdot mL^{-1}$, β = 12 $mg \cdot mL^{-1}$ and κ = 3.6 $mg \cdot mL^{-1}$). This calculation predicts that 80 % of all His residues in casein micelle / submicelle are available for coordination of HOCbl. One "active" residue binds to one Cbl molecule, because only a single coordination connection is available for external ligands (Fig. 1A, exchangeable group R).

3.4. Kinetics of HOCbl binding to casein, casein peptides and pure histidine

Different concentrations of "active" His in casein (CasHis) were added to a constant concentration of HOCbl (20.5 μM), whereupon the absorbance shifts were used to monitor interaction of the reactants over time. An example of records for casein is shown in Fig. 4A. The fitting analysis of rate constants is shown in the Supplementary material, and the results of calculations are presented in Table 1. The dissociation constant of the CasCbl complex was generally the same ($K_d \approx$ 0.10 mM) at all protein concentrations. Yet, both forward and backward rate coefficients proportionally decreased at high protein concentrations, because of the increasing

viscosity of the medium. Similar observations have been reported earlier when studying interactions of HOCbl with a bacterial protein HbpS (Lucana, Fedosov, Wedderhoff, Che, & Torda, 2014). The temperature coefficients of both binding and dissociation were high (Table 1).

Interactions between HOCbl and casein peptides (obtained after trypsin + chymotrypsin treatment, Fig. 2, ---- blue) were performed in a similar way. The content of "active" His in casein peptides is expected to be from 80 % (as in the assembled casein complex) to 100 % (as in the free His amino acids), and the latter value was chosen as a fair assumption. We found that the rate constants in the peptide mixture are somewhat higher than the respective values in the casein solution: $k_{Pep}/k_{Cas} \approx 2$ (0.7 mg·mL^{-1}) and $k_{Pep}/k_{Cas} \approx 1.4$ (30 mg·mL^{-1}), see Table 1. This can be ascribed to loss of the quaternary structure, providing a facilitated access to His–residues in the casein peptides compared to the organized casein aggregate.

The binding experiments with pure histidine showed k_+ values close to those of caseins but somewhat lower than k_+ of peptides (Table 1). On the other hand, all k_- values of pure His were clearly higher. Comparison of the rate constants in solutions of equal density (≈ 0.7 mg·mL^{-1}) and viscosity is shown in Table 1 for caseins, their peptides and His.

3.5. Direct dissociation experiments

A series of dissociation experiments was conducted to assess k_- in a direct assay. For this purpose, caseins were first loaded with HOCbl and then diluted 1 : 50 with a buffer at 20 ºC or 37 ºC (with or without an excess of the specific binding protein IF). Dissociation was monitored by changing absorbance, and the process was accelerated at increased temperature and/or the presence of IF (Fig. 4B). The absorbance signals in Fig. 4B were normalized (100 % = initial CasCbl, 0 % = full dissociation) to simplify comparison of the curves. Dissociation of CasCbl in the presence of IF was regarded as irreversible because of a very strong Cbl binding to IF (Fedosov et al., 2007).

Dissociation by dilution (without IF) was a reversible process, and the assessed k_- value matched the values from Section 3.4, if extrapolating the results to a low protein concentration.

The temperature responses of k_+ and k_- (Table 1) allowed assessment of K_d values at e.g., 37 ºC (0.152 mM) and 3 ºC (0.061 mM), relevant for physiology of milk and its storage.

Dissociation of CasCbl, PepCbl and HisCbl complexes in the presence of 1 mM KCN was examined (Fig. 4C), where formation of CNCbl was monitored *via* changing absorbance (Fig. 3A). This process is irreversible and facilitates experiments at high "physiological" protein concentrations. The molar concentrations of "active" His–groups were maintained at a similar level (3.1 – 3.6 mM) in all experiments, but the total masses of dissolved solids ($mg \cdot mL^{-1}$) were different (high for CasCbl and PepCbl, but low for HisCbl), giving a somewhat different viscosity. The major slow phase of the process (XHisCbl → XHis + HO/CNCbl) corresponded to the "ordinary" dissociation constant k_- (e.g., Fig. 4B *vs* Fig. 4C). Change of KCN in the range of 0.1 – 10 mM did not affect the slow phase (not shown). It can be concluded that CN^- does not directly attack the bond XHis–Cbl but binds to Cbl only after its dissociation from the coordination center.

3.6. Binding and dissociation in a model system (His + HOCbl) at different pH

Binding and dissociation in the mixture of XHis + HOCbl is expected to depend on pH because both compounds can be protonated and deprotonated. The relevant pK–values of His in the neutral – acidic region are pK 6.0 (imidazole group) and pK 1.8 (carboxylic group); whereas HOCbl features pK 7.6 (Co^{3+}–coordinated water) and pK ≈ 2 (phosphate of HOCbl). All experiments were conducted using pure His, because caseins and their peptides precipitate at pH < 5. The main forms of aquoCbl at pH < 7 included H_2OCbl^+ (pH 6.8, pH 4.6) and a mixture of $H_2OCbl^+ \leftrightarrow H_2OCbl(H)^{2+}$ (pH 2.0), whereas His changed its charge from His^0 (pH 6.8) to $HisH^+$ (pH 4.6) and the mixed compound $HisH^+ \leftrightarrow HisHH^{2+}$ (pH 2.0). The data in Fig. 5 demonstrate that the binding rate was critically affected by the change of pH with nearly no binding at pH 2. At the

same time, no particular variation in the dissociation rates was observed (all of them being low irrespective of pH).

3.7. Interactions of HOCbl with casein in milk and precipitated casein

When 1 µmol HOCbl was added to 1 mL of milk (1 h, 37 ºC) and the mixture was adjusted to pH 2, a red / black pellet of CasCbl formed. Distribution of Cbl corresponded to 75 % in the pellet and 25 % in the supernatant. The analogous experiment with the isolated casein (36 $mg \cdot ml^{-1}$) gave the distribution of Cbl 80 % to 20 %, where Cbl in the supernatant was bound to His–containing compounds according to spectral measurements (not shown). High quantities of the bound Cbl apparently increase acidic solubility of the precipitated casein–Cbl complex due to additional positive charges of the attached $–Cbl^{+}$ and $–Cbl(H)^{2+}$.

White casein pellets (≈ 28 mg, prepared in advance from 1 mL of milk in course of enzymatic or acidic treatment) were incubated with red solutions of HOCbl (0.1 – 1 mM, 1 mL) for 1 – 2 hours of incubation. An intensely red / black precipitate was formed at pH 6.8, while a faintly pink precipitate was observed at pH 4.6. This experiment shows that (i) the casein pellet (with or without Ca^{2+}) matches the soluble casein in its Cbl binding potency; and (ii) the pH–dependent interactions of casein and HOCbl resemble those of His + HOCbl.

Dissociation of endogenous or added HOCbl from the precipitated casein (pH 4.6) was very slow (≤ 1 % after 1 h at 22 ºC) and probably hampered in comparison to the dissolved protein.

3.8. Dissociation of endogenous Cbl from caseins by IF / HC at low physiological concentrations

Incubation of a milk sample with IF (100 nM, 2 h at 37 ºC) was accompanied by a considerable transfer of the endogenous Cbl from caseins to IF equal to 75 – 85 % (based on measurement of the residual Cbl in precipitated casein). Other tested setups with a longer incubation time (6 h at 37 ºC with 100 nM IF or 10 nM HC) showed a major transfer of Cbl (>

406 90%) from casein to IF or HC. Further work is planned concerning more accurate analysis of this
407 exchange.
408
409 **4. Discussion**
410 The nature of a high–molecular Cbl binder in bovine milk (containing one half of the vitamin)
411 remained elusive for a long time. Fig. 1B presents the four most probable mechanisms of interaction
412 including: (i) adsorption of the positively charged TCCbl complex on the negatively charged casein
413 micelle followed by a deeper entrance of TCCbl into the matrix; (ii) capturing of TCCbl by a
414 receptor, e.g., the solubilized CD320 (Abuyaman, Andreasen, Kronborg, Vittinghus, & Nexo, 2013)
415 integrated into the micelle; (iii) non–specific microporous adsorption of free Cbl; and (vi)
416 coordination of HOCbl to a protein residue (the bond is sensitive to cyanide treatment).
417 We suggested in our early publication (Fedosov et al., 1996) that the TCCbl complex is
418 captured inside the casein micelle (mechanisms № 1 and № 2 in Fig. 1B). This was deduced from
419 the lack of effect of KCN treatment in cold milk (interpreted as the lack of coordination patterns R–
420 Cbl). In the current work, we directly demonstrated that less than 10 % of total TC in bovine milk is
421 associated with caseins according to the immunological assay (Fig. 2A, Δ–gray). The current data
422 also showed that the cyanolysis of CasCbl complex is possible but the process goes very slowly
423 even at 20 ºC, and an additional 9–fold deceleration can be predicted at 4 ºC (Table 1, Fig. 4B). This
424 explains the previous failure of KCN treatment. Dissociation of CNCbl from a protein complex is
425 the crucial characteristic of mechanism № 4 in Fig. 1B, where the pre–formed coordination bond
426 between HOCbl and a casein group gets broken by CN^-.
427 A number of amino acid residues can act as HOCbl–coordination agents (Fig. 1B), but
428 imidazoles and thiols are the most likely candidates because of their relatively high affinity for
429 HOCbl at neutral pH (Pratt, 1972) combined with a reasonably high frequency of these groups in
430 protein sequences. Analysis of the absorbance spectra in the casein + HOCbl mixtures revealed that

the “active” groups are His–residues (Fig. 3A). No visible competition from other groups was detected, which can be explained by low affinities of XNH_3^+ and $XCOO^-$ compared to XHis (Pratt, 1972), as well as by an inactive state of thiols in milk proteins, either masked or bound into S–S–bridges (Owusu–Apenten & Chee, 2004). Kinetic characteristics of casein + HOCbl and His + HOCbl interactions resembled each other (Table 1), including the pH–dependent effects (Sections 3.6 and 3.7). This corroborates the conclusion about “active” His in caseins (reached from spectral analysis in Fig. 3A).

Ability of HOCbl to interact with His–residues of different proteins is a known phenomenon (Gimsing & Nexo, 1983). Nevertheless, it was rather surprising to observe the efficiency of the binding, where the millimolar concentrations of HOCbl could be loaded into caseins. This efficiency is explained by a high ratio [CasHis]/K_d, where the “active” His–residues (e.g., 4.4 mM in a casein solution of 28 $mg \cdot mL^{-1}$) are related to the dissociation constant (K_d = 0.1 mM, pH 6.8, 20 ºC). It can be calculated that more than 95 % of any relevant amount of HOCbl (≤ 3 mM) will bind to caseins of milk (≈ 1.2 mM) with the half reaction time below 10 min. The feasible loads can be estimated as up to 100 nmol of Cbl per 1 mg of caseins.

Micellar / submicellar casein, casein peptides and pure His appear to be HOCbl–coordinating agents of similar potency (Table 1). Comparable characteristics of casein and its peptides show that the supramolecular structure of native casein is irrelevant for the binding and retention of HOCbl, except for some constrains of Cbl diffusion within the micelle. Comparison of casein peptides with pure His shows that the peptides coordinate HOCbl approximately 2–fold faster and their dissociation rate is 3–fold slower. This suggests that the neighboring amino acid residues of an average peptide assist the “active” His–group in capturing and retention of HOCbl, for example *via* hydrophobic interactions with the amide side chains of Cbl, observed, for example, in the high-affinity specific protein–Cbl complexes (Wuerges et al., 2006).

455 The heterogeneous pools of caseins and peptides apparently bind HOCbl in a uniform manner
456 (e.g., Fig. 3B), at least if using the micromolar and millimolar concentrations of Cbl. There are,
457 however, indications that the physiological nanomolar concentrations of Cbl might bind slightly
458 stronger. For example, incubation of caseins (2 h at 37 ºC) with a considerable excess of IF
459 removed 75 – 85 % of endogenous Cbl (Section 3.8) instead of > 95 % observed at the high
460 concentrations (Fig. 4A).
461 It is obviously of interest to estimate the interplay between caseins and the specific Cbl–
462 binders under physiological conditions. It seems that secretion of Cbl from mammary glands to
463 bovine milk exceeds that of TC and proceeds independently. The exceptionally potent binder TC
464 accumulates Cbl until a nearly complete saturation. High molar excess of caseins cannot counteract
465 the final saturation of TC by Cbl because the ratio of affinities is very much in favor of TC (> 10^{10}).
466 The rest of Cbl becomes attached to caseins. The absence of free Cbl means that the prevailing
467 vitamin form in milk is HOCbl, because only HOCbl binds to caseins.
468 Bovine milk is an excellent source of dietary Cbl and possibly outperforms the supplements
469 and meat (Matte, Britten, & Girard, 2014; Vogiatzoglou et al., 2009). Digestion of milk starts with
470 its exposure to the acidic medium of the stomach (pH 2) containing pepsin. The TCCbl complex is
471 expected to dissociate at low pH (Newmark & Mester, 1974), whereupon the released Cbl binds to
472 human proteins involved in Cbl uptake (Fedosov, 2012, Gimsing & Nexo, 1983). A considerable
473 portion of the casein–Cbl complex will resist acidification because of a low dissociation rate (see a
474 model experiment with HisCbl at pH 2 in Fig. 5C). Yet, this process accelerates in the presence of
475 any specific Cbl–binder and at the elevated temperature (Fig. 4B). All in all, casein and its peptides
476 might become the active players under the intestinal uptake of Cbl, where both a positive and a
477 negative role can be conjectured. For example, the emulsifying properties of amphiphilic casein
478 peptides (possibly in combination with hydrolyzed emulsion of milk fats) may facilitate non–
479 specific crossing of cell walls for the coordinated Cbl, thereby improving uptake of the vitamin. On

the other hand, the role of caseins can be partially negative, because transition to the intestinal binder IF can be delayed. Yet, a delayed uptake might provide time for recycling of the IFCbl receptors, leading to a higher overall absorption of Cbl from the intestine.

5. Conclusions

In this work, it is demonstrated that caseins in bovine milk bind high quantities of HOCbl due to formation of CasHis–Cbl coordination bonds. Kinetic characteristics of this interaction are established at relevant pH values and temperatures. The His–Cbl bond is sufficiently stable to pass the acidic medium of stomach raising a question about the active role of casein and its peptides (alone or together with fats or/and the specific Cbl–binding protein) in relation to the uptake of Cbl by intestinal cells.

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571 **Figure legends.**

572

573 **Fig. 1.** Vitamin B_{12} (cobalamin, Cbl) structure and its possible interactions with casein. (**A**)
574 Structure. The exchangeable "upper" group is notated as R. DMB stands for 5,6–
575 dimethylbenzimidazole nucleotide base. (**B**) Possible interaction patterns between Cbl (or TCCbl
576 complex) and casein: (1) Nonspecific adsorption of TCCbl on the casein surface and incorporation
577 of TCCbl into micelle; (2) Binding of TCCbl to a specific receptor encapsulated within the casein
578 micelle; (3) Nonspecific adsorption of Cbl onto microporous surface of the casein micelle; (4)
579 Coordination of HOCbl (with substitution of water) to an amino acid residue of casein.

580

581 **Fig. 2.** Gel–filtration profiles for bovine milk and its fractions (**A**) Milk and endogenous Cbl.
582 The figure presents protein (·····, dark blue), endogenous TC (Δ, gray, nM scale), endogenous Cbl
583 (nM scale) in either the untreated milk (▲, red) or after incubation with 1 mM KCN for 3 h at 37 ºC
584 (□, magenta). Arrows highlight the changes in Cbl–protein association patterns. Peaks of casein, β–
585 lactoglobulin, α–lactalbumin and TC are notated. (**B**) Milk and added radioactive Cbl (low
586 concentrations). The profiles show elution of $HO^{57}Cbl$ (0.02 nM) incubated with milk for 1 h at 37
587 ºC (▲, red) or 3 h at 37 ºC (▼, green). (**C**) Milk and radioactive Cbl (high concentrations). The
588 profiles show elution of 50 nM $HO^{57}Cbl$ (▲, red) or 50 nM $CN^{57}Cbl$ (□, magenta) incubated with
589 milk for 3 h at 37 ºC. (**D**) Casein and radioactive Cbl. The profiles show protein (·····, dark blue)
590 and $HO^{57}Cbl$ (▲, red) after incubation of casein with $HO^{57}Cbl$ (50 nM) for 3 h at 37 ºC. Cleavage
591 of casein with trypsin and chymotrypsin causes a shift in the protein profile (-----, light blue).

592

593 **Fig. 3.** Equilibrium binding of HOCbl to bovine casein followed by changes in the absorbance
594 spectrum of Cbl. (**A**) Absorbance spectra. HOCbl (20.5 μM) was incubated in 0.1 M P_i–buffer pH
595 6.8 (6 h, 20 – 22 ºC) without any additives (—, red) or with 20 mM His (- - -, blue), 18 $mg \cdot mL^{-1}$

596 casein (—, cyan), 1 mM KCN (⋯, faint magenta); all baselines are subtracted. Arrows show the
597 wavelengths chosen for the monitoring of ligand – Cbl interactions for the remaining part of the
598 studies. (**B**) Saturation curves. The constant concentrations of either 0.205 mM HOCbl (○, dark red)
599 or 1.06 mM HOCbl (▲, dark green) were incubated for 18 h with casein (36 mg·mL^{-1}) diluted 1/40
600 – 1/2 with 0.1 M P_i–buffer pH 6.8. Samples with high HOCbl were additionally diluted 1/5
601 immediately before the absorbance measurements to decrease absorbance. The Y–axis shows
602 optical response observed at X casein dilution units, 1 DU = undiluted casein. The least square
603 fitting by the supplementary Eq. (1) (—) provided the values of $[Cbl]_0$ and K_d in DU. Dashed and
604 dotted lines show graphical interpretation of $[Cbl]_0$ and K_d according to Dixon (1972) (presented
605 with more details for 1 mM HOCbl).
606
607 **Fig. 4.** Kinetics of interaction between HOCbl and bovine casein (**A**) Time records for casein.
608 The absorbance shift $A_{358} - A_{351}$ (—, light colors) was recorded upon incubation of 20.5 μM HOCbl
609 with different concentrations of "active" His–residues (CasHis) present in casein. Acceleration of
610 the binding upon increasing temperature (from 20 ºC to 37 ºC) for the sample containing 2.85 mM
611 CasHis is indicated by an arrow. The level "max" corresponds to the signal at an infinite casein
612 concentration. The fitting by the supplementary Eq. (2) (—, dark colors) employed the coefficient
613 of molar absorbance ε = 11.3664 AU·mM^{-1} (**B**) Dissociation of the casein–Cbl complex shown in
614 %. Casein (7.2 mg·mL^{-1}, [XHis] = 1.14 mM) and HOCbl (1.06 mM) were preincubated and diluted
615 1/50 with 0.1 M P_i–buffer pH 6.8 (20 ºC or 37 ºC) with 40 μM of Cbl–binding capacity (+IF) or
616 without IF, see notations in the panel. Light and dark curves (—) show the measured data (CasCbl
617 in % over time) and the fitting by the supplementary Eq. (3), respectively. The dashed curve shows
618 prediction of the dissociation pattern in a concentrated protein sample (30 mg·mL^{-1}) (**C**) Optical
619 response to dissociation of Cbl complexes in the presence of KCN. The samples ([XHis] = 3.1 mM
620 in bovine casein, 3.6 mM in casein peptides and His) were first preincubated with 20.5 μM HOCbl

as indicated above and then exposed to 1 mM KCN (pH 6.8, 20 ºC) with a negligible dilution of the samples.

Fig. 5. Association and dissociation of His and HOCbl at different pH at 20 – 22 ºC. (**A**) Interactions at pH 6.8 (0.1 M P_i–buffer). The association curve (red) was recorded after mixing His (3.6 mM) and HOCbl (20.5 μM). The dissociation curve (blue) was recorded for the preincubated mixture His (3.6 mM) + HOCbl (1.06 mM), 20 h at pH 6.8, diluted 1/50 with the medium at pH 6.8. (**B**) Interactions at pH 4.6 (0.02 M Na–acetate buffer, 0.1 M P_i). Association was recorded after mixing His (10 mM) and HOCbl (20.5 μM). Dissociation was followed for the preincubated mixture His (10 mM) + HOCbl (1.06 mM), 20 h at pH 6.8, diluted 1/50 with the medium pH 4.6. (**C**) Interactions at pH 2.0 (0.1 M P_i–buffer). All other conditions as in panel B.

Table 1. Interactions of HOCbl with casein and histidine under different conditions (20 ºC).

Compound	pH	k_+, $M^{-1}s^{-1}$	k_-, s^{-1}	K_d, mM
casein (≈ 30 mg/mL)	6.8	0.220 ± 0.025 Q_{10} = 3.0 ± 0.1	(2.07 ± 0.35) · 10^{-5} Q_{10} = 3.9 ± 0.1	0.094 ± 0.019 Q_{10} = 1.3 ± 0.05
casein (≈ 0.7 mg/mL)	6.8	0.358 ± 0.033	(3.45 ± 0.47) · 10^{-5}	0.096 ± 0.016
casein peptides (≈ 30 mg/mL)	6.8	0.316 ± 0.021 Q_{10} = 3.0	(3.68 ± 1.19) · 10^{-5}	0.116 ± 0.038
casein peptides (≈ 0.7 mg/mL)	6.8	0.724 ± 0.016	(7.96 ± 0.91) · 10^{-5}	0.110 ± 0.013
His (≈ 0.7 mg/mL)	6.8	0.361 ± 0.017 Q_{10} = 2.8 ± 0.1	(1.28 ± 0.04) · 10^{-4} Q_{10} = 3.8 ± 0.2	0.354 ± 0.020 Q_{10} = 1.3 ± 0.1
His	4.6	0.0148	1.19 · 10^{-4}	8.06
His	2.0	≈ 4.4 · 10^{-4}	1.65 · 10^{-4}	≈ 380

Figure 1, casein-Cbl (color, Web)
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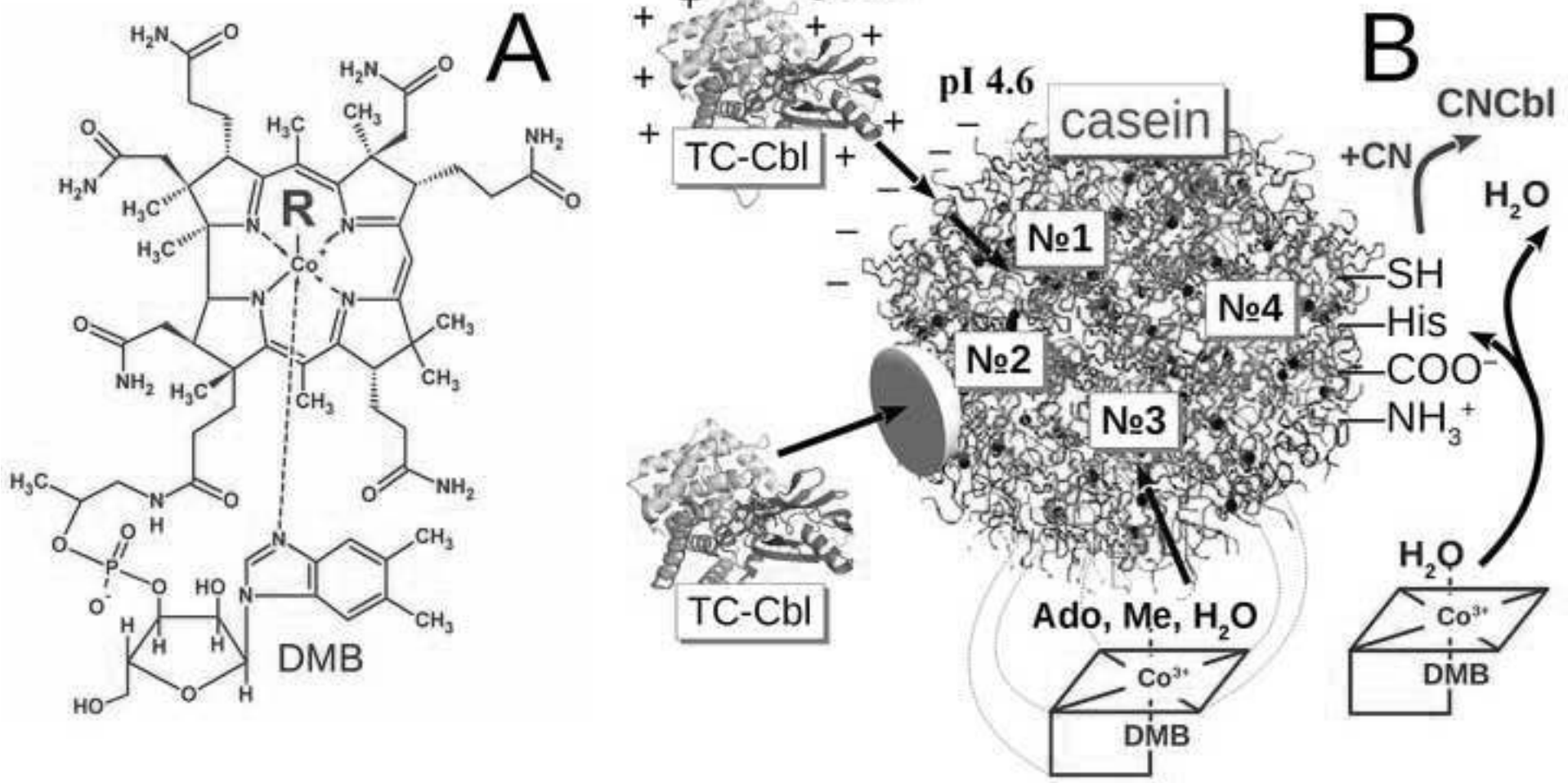

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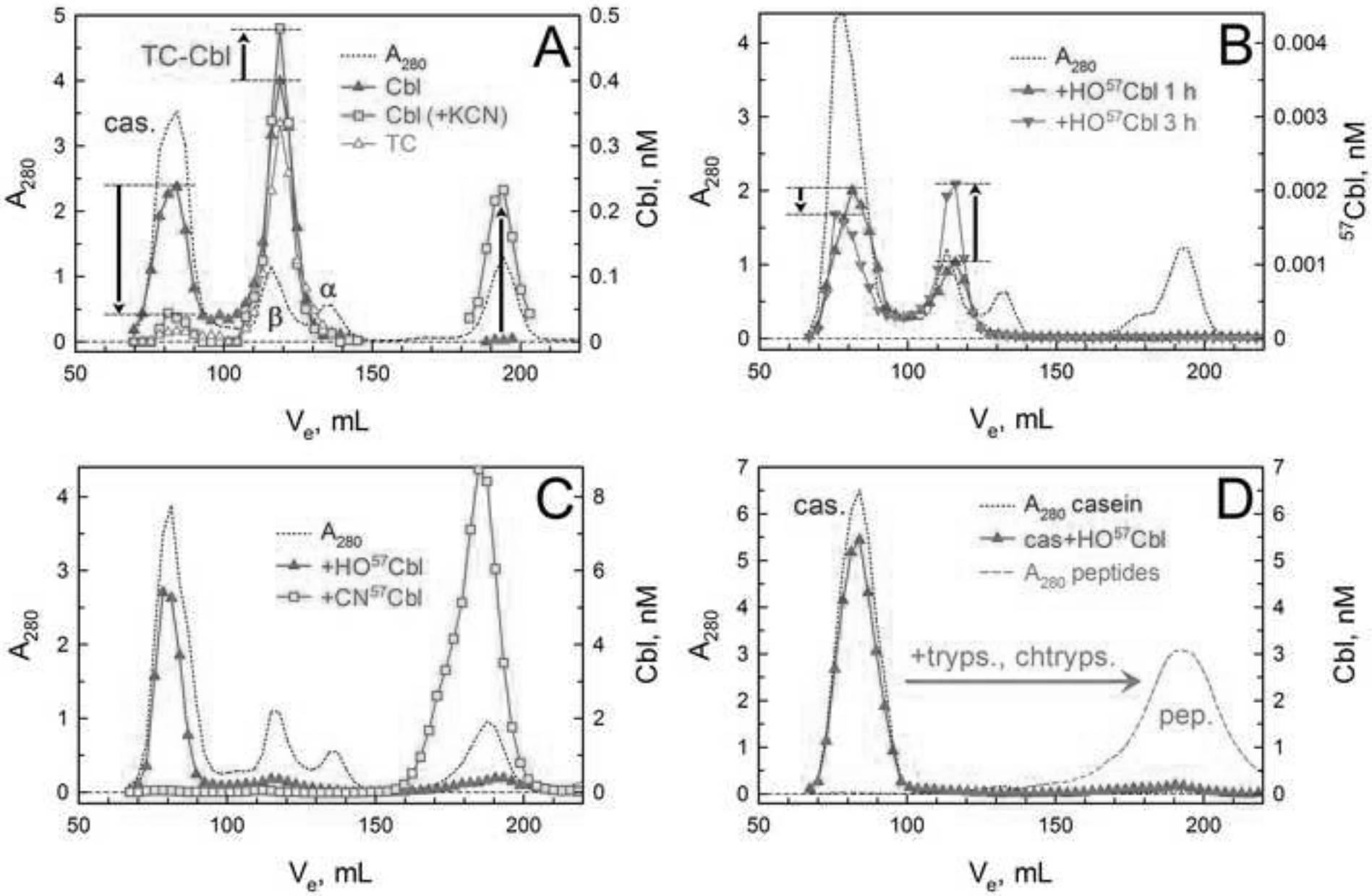

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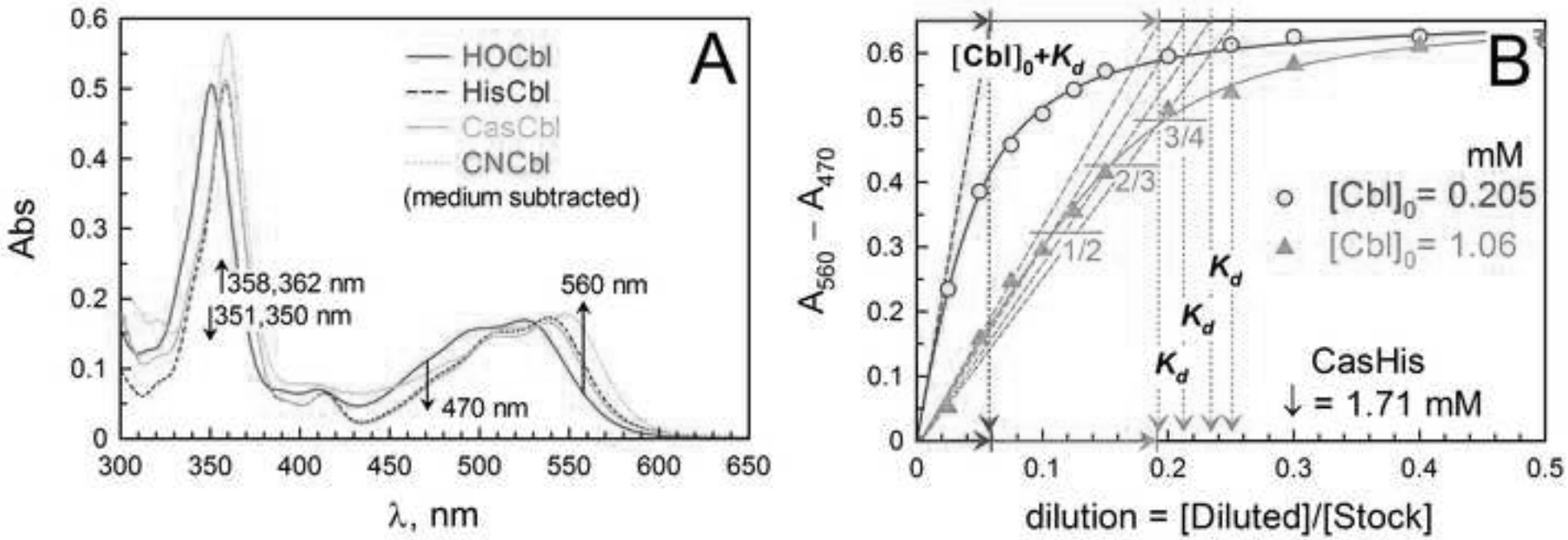

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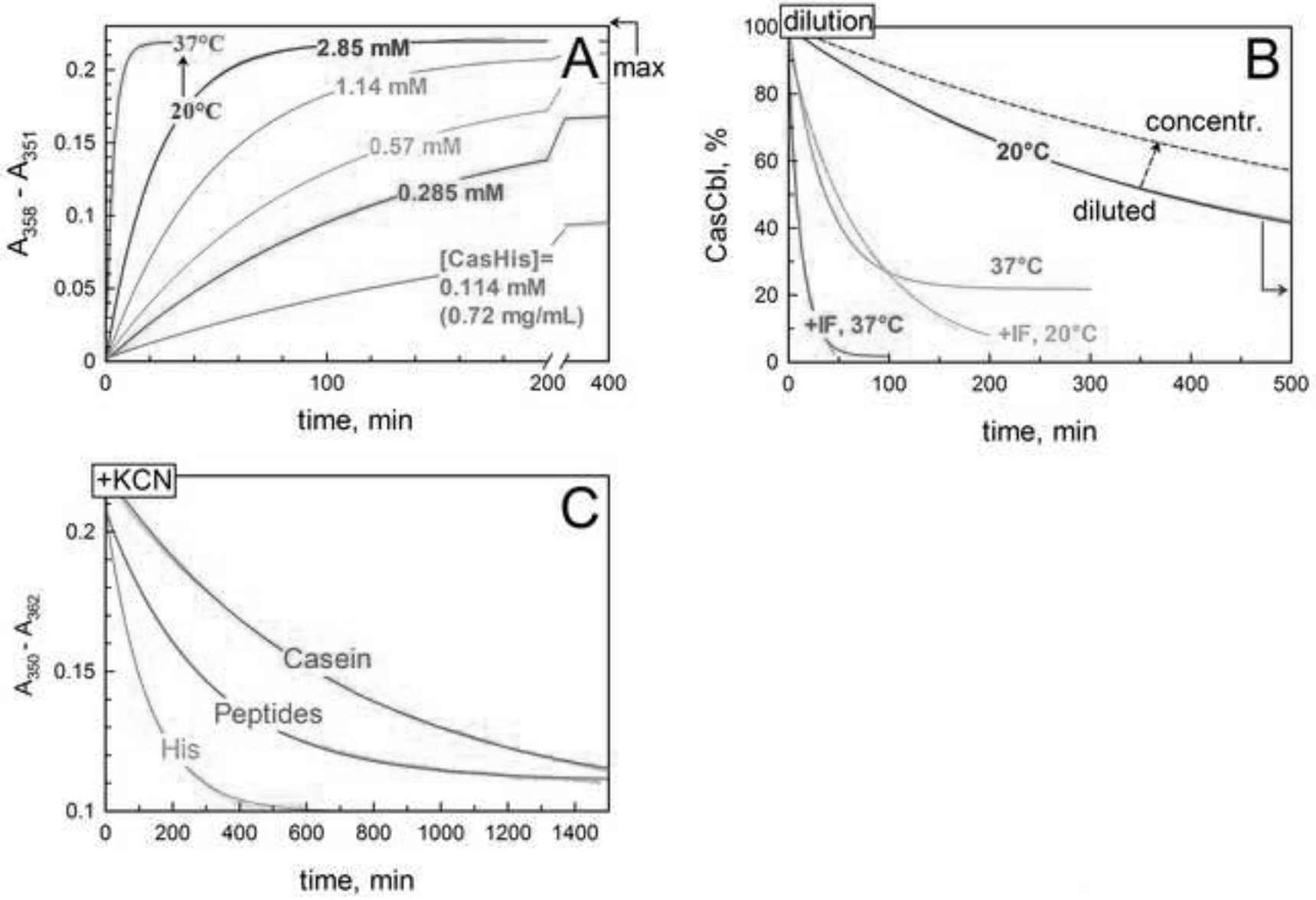

Figure 5, His & Cbl pH (color, Web)
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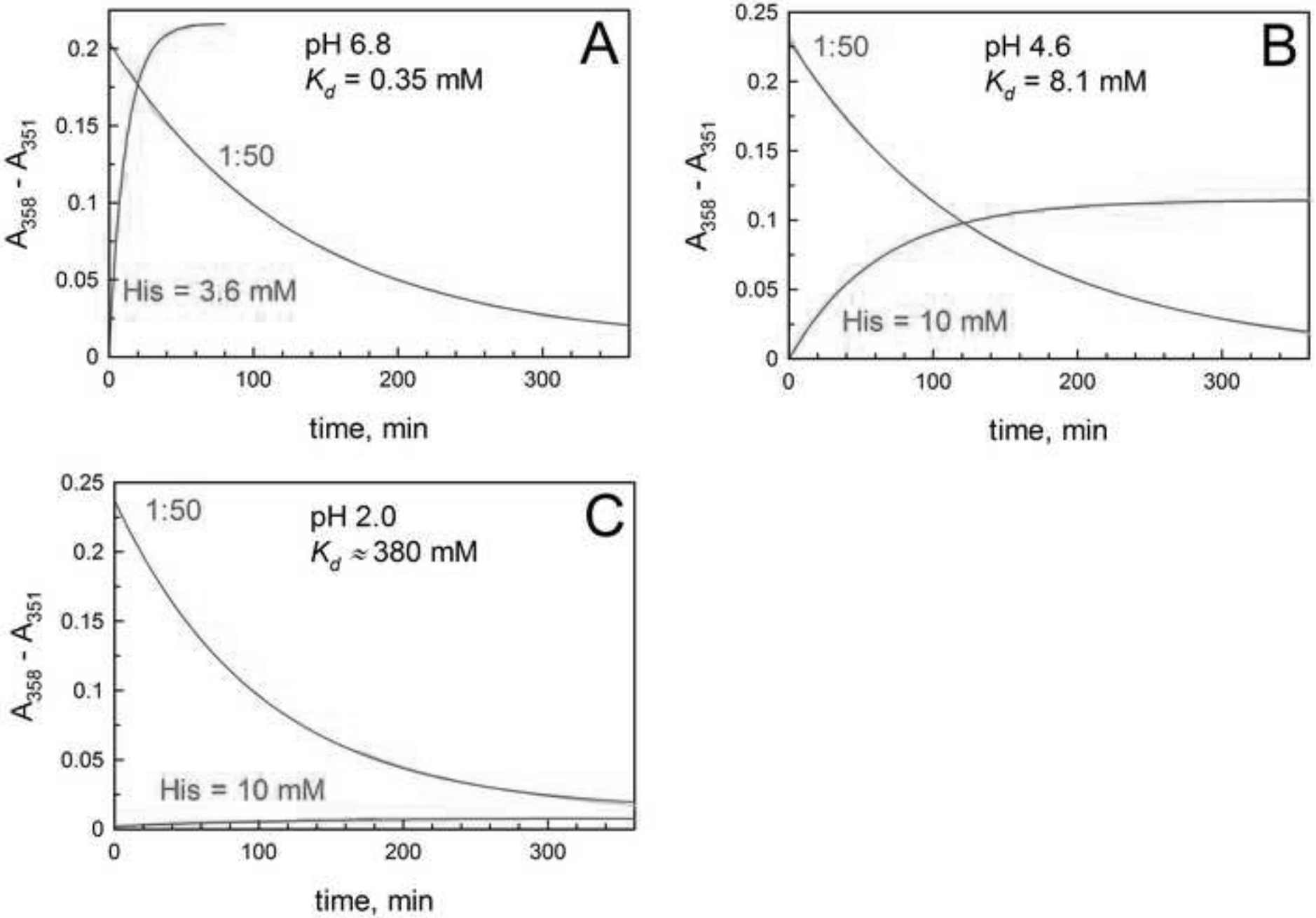

Supplementary Materials (DOC)

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