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Hydrophobic Interactions of Transcobalamin II (TC II) from Mammalian Sera (41572)

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Abstract. The hydrophobic properties of mammalian transcobalamin IIs (TC II) were studied by chromatography of radioactive cyanocobalamin (CN[^{57}Co]Cbl)-labeled serum on phenyl-Sepharose CL-4B. Mammalian holo TC IIs (CN[^{57}Co]Cbl–TC II) exhibited species variability in their affinity for the hydrophobic matrix in the order: dog > mouse > human > rat > rabbit. Phenyl-Sepharose chromatography of the isolated CN[^{57}Co]Cbl–TC II peaks from gel filtration of dog and rat serum showed no hydrophobic change in dog TC II, but an increase in hydrophobicity of rat TC II. Phenyl-Sepharose chromatography of CN[^{57}Co]Cbl-labeled rabbit serum (holo TC II) and the unlabeled serum (apo TC II) showed apo TC II to be more hydrophobic than holo TC II as has been shown for human TC II (Begley *et al.*, Biochem Biophys Res Commun **103**:434–441, 1981). Thus mammalian holo TC IIs differ in their hydrophobic properties and apo TC II, in man and rabbit, is more hydrophobic than holo TC II. In addition, isolation of the TC II in some animal sera by gel filtration may result in a TC II that is more hydrophobic than the native molecule.

Human and other mammalian sera contain vitamin B_{12} (cobalamin, Cbl) binding proteins which circulate bound to endogenous Cbl (holo binder) and can be detected by assay of that bound Cbl (1). These binders also circulate free of Cbl (apo binder) and can be detected by their ability to bind Cbl *in vitro* (2, 3). Human serum contains three Cbl binding proteins as detected by gel filtration: transcobalamin O (TC O), R-type binder, and transcobalamin II (TC II). Their function and physiochemical properties have been thoroughly reviewed (4). In most mammals, TC II, that is, a Cbl binding protein with a molecular radius similar to human TC II, is the major Cbl binder (2). Unlike human serum, in which only approximately 30% of the endogenous Cbl is bound to TC II, other mammals have the majority of their endogenous Cbl bound to TC II (1).

We have shown previously that human TC II is more hydrophobic than the other Cbl binders (5). In addition, human apo TC II shows greater hydrophobic properties than holo TC II. In the present study we report that other mammalian TC IIs exhibit a species difference in their TC II hydrophobicities and that the apo and holo form of TC II within a species are hydrophobically distinct.

Materials and Methods. Phenyl-Sepharose CL-4B was purchased from Sigma Chemical Company and stored at 4°C. Radioactive cyanocobalamin (CN[^{57}Co]Cbl) was obtained from the Radiochemical Center, Amersham, England and diluted with nonradioactive CN–Cbl (Sigma Chemical Company) and stored at 4°C. All CN[^{57}Co]Cbl solutions were assayed for content and purity by bioassay (6) and column chromatography (7). Animal sera were obtained from healthy laboratory animals housed at this facility. Gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals) was performed as described (8). *In vitro* CN[^{57}Co]Cbl binding ability was determined by the albumin-coated charcoal method (9).

Phenyl-Sepharose CL-4B was packed by gravity at 4°C to a height of 12 cm in 0.9 × 16.7-cm glass columns and equilibrated with 10 column volumes of 50 m*M* sodium phosphate buffer ($NaPO_4$), pH 7.4, containing 150 m*M* sodium chloride (NaCl). Serum (0.5 ml) was mixed with an amount of CN[^{57}Co]Cbl sufficient to saturate Cbl binders. An equal vol of 100 m*M* $NaPO_4$ buffer, pH 7.4, containing 300 m*M* NaCl was added and the samples incubated for 30 min at 37°C followed by an additional 30 min at 4°C. The sample was then applied to the phenyl-Sepharose column and washed with the follow-

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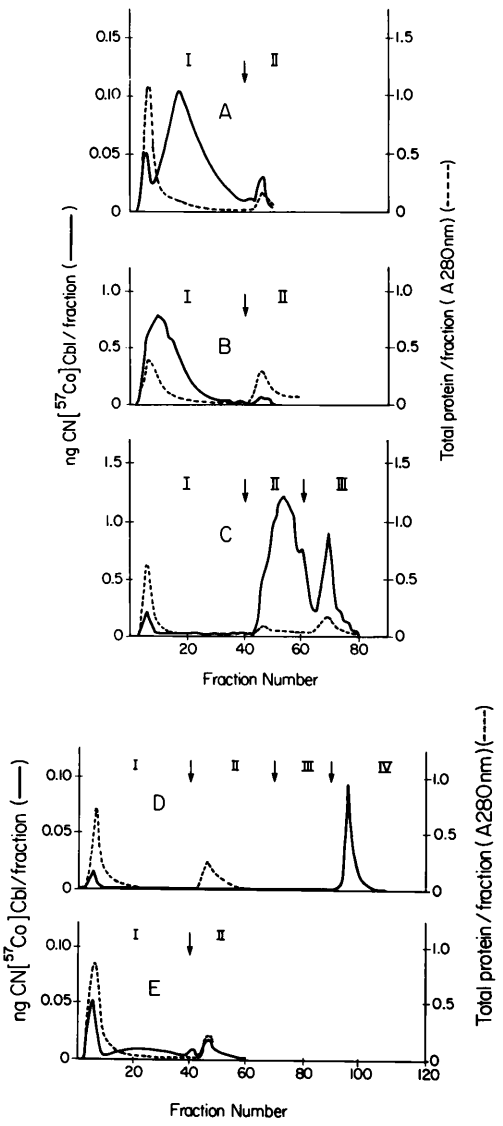

FIG. 1. Phenyl-Sepharose hydrophobic chromatography of rat (A), rabbit (B), mouse (C), dog (D), and human (E) serum labeled with CN[^{57}Co]Cbl. Columns where indicated were eluted with: I. 50 mM sodium phosphate, pH 7.4, containing 150 mM sodium chloride, II. 50% glycerol in buffer I, III. Glass distilled water, IV. 50% v/v absolute ethanol and water. Each fraction was assayed for ^{57}Co (——) and protein (- - -). Vertical arrows indicate change of buffer.

ing sequence: (a) 50 mM $NaPO_4$, pH 7.4, containing 150 mM NaCl, (b) 50% glycerol in 50 mM $NaPO_4$, pH 7.4, 150 mM NaCl, (c) glass distilled water, and (d) 50% (v/v) absolute ethanol in glass distilled water (ETOH/H_2O). Two milliliter fractions were collected and assayed for ^{57}Co and protein (absorbance at 280 nm). Where indicated, individual or pools of fractions were assayed for CN[^{57}Co]Cbl binding ability (9) and endogenous Cbl (6).

Results. Figure 1 and Table I show the fractionation of CN[^{57}Co]Cbl-labeled rat, rabbit, mouse, dog, and human serum by phenyl-Sepharose columns. With rat, rabbit, and human serum (Fig. 1A, B, and E) the majority of ^{57}Co eluted with 50 mM $NaPO_4$ buffer, pH 7.4, containing 150 mM NaCl. With rat and rabbit serum, neither of the major CN[^{57}Co]Cbl peaks (fractions 17 and 10, respectively) coeluted with the major protein (fraction 6). For human serum, 31% of the radioactivity eluted with the main protein peak. Analysis of this peak by gel filtration on Sephadex G-200 showed it to consist of R-binder (80%), TC II (13%), and TC O binder (7%). The remainder of the radioactivity from the phenyl-Sepharose column eluted with the $NaPO_4$ buffer in a broad area (fractions 9–40), the tailing area being rapidly eluted by 50% glycerol in $NaPO_4$ buffer (fractions 45–60). On smaller columns of phenyl-Sepharose (5) human holo TC II eluted with 50% glycerol as a sharp peak. This was due to the high affinity human TC II has for the matrix in the phosphate buffer with subsequent facilitation of elution by the glycerol buffer. The ratio of buffer to bed volume of phenyl-Sepharose in the present study was increased twofold in order to completely elute the rat TC II–B_{12}. Six percent of the radioactive CN[^{57}Co]Cbl-labeled mouse serum (Fig. 1C) eluted with $NaPO_4$ buffer, half of which formed a peak of radioactivity coinciding with the main protein peak. Sixty-two percent and 32% eluted with 50% glycerol and H_2O, respectively. The latter peak may represent facilitated elution of the tailing edge of the glycerol area. Dog serum (Fig. 1D) showed a binder of Cbl with the greatest hydrophobicity, 73% of the radioactivity eluting with ETOH/H_2O. The total protein in this area was not analyzed. Gel filtration of the radioactivity in the ETOH/H_2O eluate resulted in a single peak in the general vicinity of R-type binder (data not shown). Analysis of dog serum by gel filtration showed only minor radioactivity in this area (4%). The ETOH/H_2O-eluted ^{57}Co may represent aggregates of dog

TABLE I. PERCENTAGE DISTRIBUTION OF CN[^{57}Co]Cbl IN ELUATES FROM PHENYL-SEPHAROSE HYDROPHOBIC CHROMATOGRAPHY

Species	PBS (I)[a]	Glycerol (II)[b]	H_2O (III)[c]	ETOH/H_2O (IV)[d]	% Recovery
Rat[e]	93	7	—	—	98
Rabbit[e]	95	5	—	—	100
Mouse[e]	6	62	32	—	82
Dog[e]	13	7	7	73	91
Human[e]	74	26	—	—	98
Dog TC II[f]	3	2	2	93	75
Rat TC II[f]	33	66	1	0	92

[a] 50 m*M* $NaPO_4$, pH 7.4, containing 150 m*M* NaCl.
[b] 50% glycerol (by volume, final concentration) in $NaPO_4$ buffer (50 m*M*), pH 7.4, containing 150 m*M* NaCl.
[c] Glass distilled water.
[d] 50% (v/v) absolute ethanol in glass distilled water.
[e] CN[^{57}Co]Cbl-labeled serum. Data obtained from one serum sample from each species.
[f] CN[^{57}Co]Cbl–TC II from gel filtration of the above respective serum.

TC II. Analysis of the G-200 isolated dog CN[^{57}Co]Cbl–TC II peak by phenyl-Sepharose chromatography again showed binding and elution with ETOH/H_2O (Table I).

Figure 2 is a composite plot of 0.5 ml rabbit serum (10.5 ng Cbl binding ability) labeled with CN[^{57}Co]Cbl and the same serum without a label. Fractions (0.5 ml) of the latter were analyzed for CN[^{57}Co]Cbl binding ability to detect apo TC II. Ninety-eight percent of the applied holo TC II (CN[^{57}Co]Cbl–TC II) eluted with $NaPO_4$ buffer, only 9% of the CN[^{57}Co]Cbl binding ability (apo TC II) was detected in these fractions. Analysis of the pooled and concentrated fractions from the apo TC II column showed a CN[^{57}Co]Cbl binding ability of 0.4 ng with a total Cbl by bioassay of 15.6 ng (endogenous holo TC II). This data indicates that like human TC II (5), rabbit apo TC II is more hydrophobic than the holo form of the protein.

Figure 3 and Table I show the hydrophobic interaction of dog and rat CN[^{57}Co]Cbl–TC II that had been isolated by gel filtration. Ninety-three percent of the dog TC II radioactivity eluted with the ETOH/H_2O (Fig. 3A), similar to the pattern obtained with whole dog serum (Fig. 1D). The gel-filtration isolated rat CN[^{57}Co]Cbl–TC II (Fig 3B) did not interact as it did in whole serum (Fig. 1A). No radioactive peak was detected at fraction 17 and only 33% was eluted with the phosphate buffer. Glycerol eluted 66% of the radioactivity which appeared to represent facilitated elution of the potentially broad buffer peak if the buffer had been continued. It appears that the isolated rat CN[^{57}Co]Cbl–TC II from gel filtration exhibited a greater interaction with the hydrophobic matrix than the same TC II in whole serum.

Discussion. The major Cbl binders in the sera of the present study have a mol wt of between 37,000 and 42,000 (2) and all are

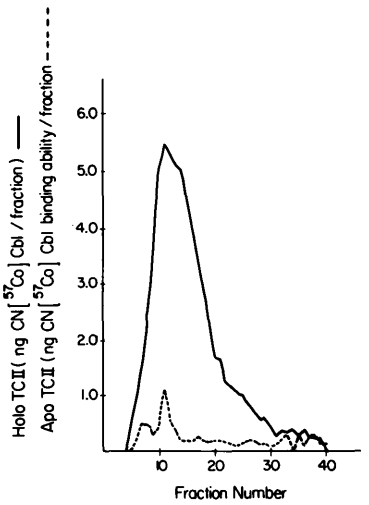

FIG. 2. Hydrophobic chromatography of rabbit holo and apo TC II. Aliquots (0.5 ml) of serum with (——) and without (- - -) the addition of CN[^{57}Co]Cbl were chromatographed separately using 50 m*M* sodium phosphate buffer, pH 7.4, containing 150 m*M* sodium chloride. Fractions from the CN[^{57}Co]Cbl-labeled sample were assayed for radioactivity (holo TC II). Aliquots (0.5 ml) of the fractions from the unlabeled sample were assayed for CN[^{57}Co]Cbl binding ability (apo TC II) using albumin-coated charcoal (9).

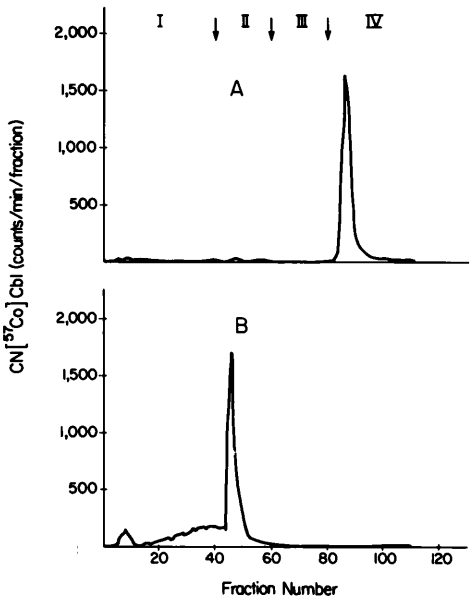

FIG. 3. Phenyl-Sepharose chromatography of the CN[^{57}Co]Cbl–TC II peak from gel filtration of dog (A) and rat (B) serum. Gel-filtration peaks were concentrated to 6 ml and dialyzed overnight against 50 m*M* sodium phosphate buffer, pH 7.4, containing 150 m*M* sodium chloride. Aliquots (3 ml) of each was applied to the columns and eluted as in Fig. 1.

presumably functionally related to human TC II since they all facilitate the uptake of Cbl in cell culture in a species-specific manner (10). In addition, antibody to human TC II will cross react either completely or partially with these animal TC IIs (10). The present studies show that mammalian TC IIs exhibit marked species variability in their affinity for a hydrophobic matrix in the order: dog > mouse > human > rat > rabbit. Most proteins contain hydrophobic areas composed of regions of nonpolar amino acids in both the interior and exterior parts of the molecule (11). These hydrophobic regions are thought to be a contributing factor in the formation and stabilization of a protein's secondary structure (12). The difference in affinity of mammalian holo TC IIs is most likely an expression of species variability in the number and size of these hydrophobic regions as well as their accessibility to the particular solvent.

On binding Cbl, human TC II exhibits a decreased interaction with phenyl-Sepharose (5). The present study shows that this phenomenon is not only a characteristic of human TC II. Rabbit holo and apo TC II also interact differently toward the hydrophobic matrix (Fig. 2), apo TC II being more hydrophobic than holo TC II. Hippe has shown that human TC II, on binding Cbl, exhibits a decrease in the Stokes radius which they attributed to a conformational change resulting in a more compact structure (13). Our results with human (5) and rabbit (Fig. 2) TC II indicate that on binding Cbl, TC II changes in conformation such that less surface area, including hydrophobic regions, are accessible to solvent. As a result, holo TC II is less hydrophobic than apo TC II.

The hydrophobic interactions which a particular species' TC II exhibits may be influenced by its immediate environment. Dog holo TC II showed no detectable difference in hydrophobicity whether it was from the unfractionated serum or post gel filtration. Rat holo TC II isolated by gel filtration, however, showed a marked increase in affinity for the hydrophobic matrix (Fig. 3B) as opposed to TC II in serum. The significance of this observation needs to be explored further.

Preliminary studies reported by Kapadia *et al.* (14) may have some bearing on the present observations. They show that Quso, a microfine silica powder, did not adsorb the TC II from rabbit, rat, guinea pig, and monkey serum, but did from dog and human serum. When their isolated TC II fractions from gel filtration were exposed to Quso, all of the species' TC II adsorbed. The authors hypothesized the presence of a plasma factor(s) which prevented the TC II in plasma from adsorbing to Quso, but which was removed by gel filtration. Quso was originally used to adsorb out the TC II from human serum (15), although the mechanism was not known. In unpublished studies we have shown that human holo TC II adsorbed by Quso is more easily eluted with substances that disrupt hydrophobic bonds: Triton X-100 > glycerol > 1 M NaCl > buffered saline. We therefore concluded that at least part of the mechanism of TC II adsorbtion to Quso is by hydrophobic interaction. Human and dog TC II were the most hydrophobic mammalian TC IIs of the present study, and Kapadia *et al.* (14) observed that these did adsorb to Quso from plasma. However, rabbit and rat TC II, which

are less hydrophobic by the present study, did not. Isolated rat TC II obtained by gel filtration was more hydrophobic than when in serum, and it did bind to Quso (11). More detailed studies are needed to clarify this relationship.

Further studies are necessary in order to ascertain the role hydrophobicity plays in the binding, uptake, and intracellular catabolism of TC II, as well as on the fate of newly synthesized TC II in animal species. In addition, the use of silica (15) and nitrocellulose (16) in the isolation and identification of nonhuman TC II should be carefully evaluated. These substances can bind proteins via hydrophobic interactions (17) and may not react with the less hydrophobic forms of TC II.

1. Linnell JC, Collings L, Down MC, England JM. Distribution of endogenous cobalamin between the transcobalamins in various mammals. Clin Sci **57**:139–144, 1979.
2. Hippe E, Jorgensen FS, Olesen H. Cobalamin binding proteins in stomach and serum from various animal species. Data for B_{12} binding capacities and molecular sizes of the binding proteins. Comp Biochem Physiol **56B**:305–309, 1977.
3. Green R, Keep ME, Colman N, Metz J. Vitamin B_{12} and its binding proteins in the serum of some wild game species. S Afr J Med Sci **40**:9–13, 1975.
4. Jacob E, Baker SJ, Herbert V. Vitamin B_{12} binding proteins. Physiol Rev **60**:918–960, 1980.
5. Begley JA, Heckman SM, Hall CA. Hydrophobic interactions of the apo and holo forms of human cobalamin binding proteins. Biochem Biophys Res Commun **103**:434–441, 1981.
6. Anderson B. Investigations into the Euglena method for the assay of the vitamin B_{12} in serum. J Clin Pathol **17**:14–26, 1964.
7. Begley JA, Hall CA. Overestimation of cyanocobalamin due to coelution of the sulfitocobalamin on SP-Sephadex C-25. J Chromatogr **177**:360–362, 1979.
8. Hall CA, Hitzig WH, Green PD, Begley JA. Transport of therapeutic cyanocobalamin in the congenital deficiency of transcobalamin II (TC II). Blood **53**:251–263, 1979.
9. Gottlieb C, Lau KS, Wasserman LR, Herbert V. Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B_{12} binding capacity, antibody to IF and serum unsaturated B_{12} binding capacity. Blood **25**:875–884, 1965.
10. Haus M, Green PD, Hall CA. Species specificity in the immunologic reactions and biological functions of transcobalamin II. Proc Soc Exp Biol Med **162**:295–298, 1979.
11. Hofstee BHJ. Accessible hydrophobic groups of native proteins. Biochem Biophys Res Commun **63**:618–624, 1975.
12. Rose GD, Roy S. Hydrophobic basis of packing in globular proteins. Proc Nat Acad Sci USA **77**:4643–4647, 1980.
13. Hippe E. Changes in Stokes radius on binding of vitamin B_{12} to human intrinsic factor and transcobalamins. Biochem Biophys Acta **208**:337–339, 1970.
14. Kapadia CR, Voloshin KW, Donaldson RM. Marked species differences in transcobalamin II. Clin Res **29**:519A, 1981.
15. Jacob E, Herbert V. Measurement of unsaturated "granulocyte-related" (TC I and TC II) and "liver-related" (TC II) B_{12} binders by instant batch separation using a microfine precipitate of silica (Quso G32). J Lab Clin Med **86**:505–512, 1975.
16. Selhub J, Rachmilewitz B, Grossowicz N. Fractionation of serum transcobalamins on charged cellulose filters. Proc Soc Exp Biol Med **152**:204–209, 1976.
17. Fabry M, Kolvoda L, Rychlik I. Hydrophobic interactions of *Escherichia coli* ribosomes. Biochem Biophys Acta **652**:139–150, 1981.

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