Complexing of Transcobalamin 2 and Apparent Combination With Heparin

By Bernard A. Cooper

Most of the vitamin B₁₂ in plasma or serum is bound to proteins¹⁻³ which have been named transcobalamin 1 and 2 by Hall and Finkler.⁴ When serum which contains radioactive vitamin B₁₂ is filtered through sephadex, the radioactivity appears in the effluent in fractions corresponding to molecular weights of 121,000 and 36,000.⁵

It has been assumed that these fractions correspond to association of the B₁₂ with TC₁ and TC₂, respectively. It has been reported that the fraction of larger molecular weight is heterogeneous when chromatographed on DEAE cellulose,⁷ and that in plasma from some patients, the apparent size of TC₂ is increased, possibly due to antibody to this material.⁸ We have reported that plasma from human portal venous blood obtained through the umbilical vein during absorption of ⁵²CoB₁₂ contained no fraction with molecular weight of 36,000 when filtering through sephadex⁹ but its affinity for DEAE cellulose was found subsequently to be identical with that of TC₂. Because the portal blood contained heparin, and we have observed that the distribution of B₁₂ in fractions obtained when serum was filtered through sephadex differed from that when plasma from heparinized or EDTA blood was filtered, we have studied the effect of heparin and EDTA on the B₁₂ binders in human plasma and serum. Heparin has been reported to alter B₁₂ binding by plasma.¹⁰,¹¹

Blood was obtained from patients undergoing venepuncture for the control of anticoagulant therapy, and from volunteer normal subjects. The effect of heparin and of EDTA on the filtration patterns of pools of serum and plasma and of plasma from different individual normal subjects was similar. For convenience, all studies illustrated in the figures in this paper utilized blood from a single normal subject.

Materials and Methods

Aliquots of blood were transferred to clean test tubes, some of which contained 2 mg. of EDTA per ml. of blood, and some 1 mg. (1000 units) of heparin per ml. of blood. Blood in tubes without anticoagulant was left at 37° C for 60 minutes, centrifuged, and the serum was collected. Heparinized serum and heparinized EDTA plasma were prepared by mixing 2 mg. of heparin per milliliter of serum or EDTA plasma. Before filtration through sephadex,
each milliliter of serum or plasma was mixed with 335 pg. of $^{57}$CoB$_{12}$ (specific activity 15-20 $\mu$C./µg.), the mixture was incubated at $37^\circ$ C for 30 minutes and then applied to the sephadex column.

Filtration through sephadex utilized two columns containing sephadex G-150: one (which was used for most of the studies described here), 100 cm. high and 2.5 cm. in diameter, was maintained at $2^\circ$ C with a refrigerated water jacket; samples were applied at the bottom in 5 ml. and filtration was carried out from below upwards. The other (used for the experiments illustrated in Fig. 3) was an unrefrigerated column, 50 cm. high and 1.25 cm. in diameter; the sample was applied at the top of the column in 0.3 per cent sucrose, and filtration was carried out from above downwards using a head of pressure of 7.5-10 cm. of filtering buffer. The filtration solution was 150 mM sodium chloride containing 10 mM sodium phosphate at pH 7.0. The transmission of light at 280 mp. through the sample was monitored with a uvicord ultraviolet monitor. The relationship between filtration volume and molecular weight was standardized for the column as described previously.$^6$

Radioactivity was measured in a well-type scintillation counter. B$_{12}$ level was determined with Z strain of Euglena gracilis.$^{12}$

**RESULTS**

Blood was obtained from a single normal subject, and heparinized plasma, EDTA plasma, serum, heparinized serum, heparinized EDTA plasma, and EDTA serum were prepared. Aliquots were frozen at $-20^\circ$ C and thawed for filtration through the large, refrigerated sephadex G-150 column. The results obtained when serum, EDTA and heparinized plasma, and heparinized EDTA plasma were filtered are illustrated in Figs. 1 and 2. It is apparent that the pattern of filtration obtained was altered by the presence of EDTA and of heparin in the specimen.

When serum mixed with $^{57}$CoB$_{12}$ was filtered, the $^{57}$CoB$_{12}$ appeared in two fractions, one before, and one after albumin, corresponding to apparent molecular weights of about 120,000 and 36,000, respectively. When heparin was present in either plasma or EDTA plasma, all of the $^{57}$CoB$_{12}$ filtered in a single fraction which could not be differentiated from the one with apparent molecular weight of about 120,000. In plasma containing EDTA but no heparin, less of the $^{57}$CoB$_{12}$ filtered with the 120,000 fraction than in serum; relatively more filtering after albumin with the fraction of apparent molecular weight approximately 36,000. When heparin was added to serum, $^{57}$CoB$_{12}$ added to it filtered exactly as illustrated for heparinized plasma in Figs. 1 and 2 (data not shown).

The native vitamin B$_{12}$ in serum and plasma filtered in a manner similar to, but not identical with, that of the $^{57}$CoB$_{12}$. As reported previously, unlike the $^{57}$CoB$_{13}$, much of the native B$_{12}$ of plasma$^6$ and of serum, filtered through the sephadex columns as free vitamin B$_{12}$, appearing in the effluent immediately before the peak of free amino acids (Figs. 1 and 2). In samples containing EDTA, growth of the assay organisms was prevented by the EDTA, and the large fraction of free B$_{12}$ was not observed. A significant fraction of the native B$_{12}$ of plasma and serum was excluded from the gel, although none of the added $^{57}$CoB$_{12}$ appeared in this fraction. Addition of heparin to either plasma or serum increased the proportion of the total assayable B$_{12}$ which filtered

$^1$Purchased from Charles E. Frosst & Co., Montreal, Quebec.
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Fig. 1.—Filtration of EDTA plasma, heparinized plasma, serum, and heparinized EDTA plasma mixed with 335 pg./ml. $^{57}$CoB$_{12}$ through Sephadex G-150. Broken lines indicate transmission of light at 280 mµ; fine lines indicate the pattern of microbiologic determination of B$_{12}$; heavy lines $^{57}$CoB$_{12}$, both expressed as picograms per milliliter. Each tube contained approximately 4.5 ml. of effluent. Each picogram of $^{57}$CoB$_{12}$ corresponds to 7 counts per minute. Filtration at 0–2°C.

before albumin, and EDTA increased the proportion which filtered after albumin.

Because the two fractions of radioactivity observed in serum have been assumed to correspond to transcobalamin 1 and 2, respectively, serum, and heparinized plasma were fractionated on DEAE cellulose to determine if heparin altered the relative proportion of these B$_{12}$ binders. The elution pattern of radioactivity was identical with both materials, confirming the observation$^{13}$ that heparin does not affect the elution of these materials from DEAE cellulose (data not shown).

Aliquots of EDTA plasma from a single venepuncture were mixed with different quantities of heparin and the mixtures were filtered through a 50-ml. Sephadex column with $^{57}$CoB$_{12}$ to determine the quantity of heparin required to alter the filtration volume of transcobalamin 2. When 100 µg. (10 units) or less of heparin were used, all of the radioactivity filtered after albumin (Fig. 3). When 1000 µg. (100 units) or more of heparin were mixed per millil-
 liter of EDTA plasma, however, the peak of radioactivity in the effluent appeared before that of albumin. Because of the small volume of this gel column (36 ml.) the large and small molecular weight B₁₂ binders overlapped, but Fig. 3 clearly demonstrates that the apparent size of most of the $^{57}$CoB₁₂ binding material in EDTA serum was greater when 1000 μg. of heparin was added to it than when less heparin was used. One hundred units per milliliter of heparin is about ten times the usual therapeutic concentration. The unsaturated B₁₂ binding capacity of this serum was 1200 pg./ml. and its total binding capacity 1850 pg./ml.

Heparin did not bind $^{57}$CoB₁₂ during filtration through sephadex. In the experiment illustrated in Fig. 4, 400 units of heparin were mixed with 335 pg. of $^{57}$CoB₁₂ and applied to the column. All of the $^{57}$CoB₁₂ filtered as free B₁₂. The major anticoagulant component of the heparin preparation used for these studies filtered through the column with an apparent molecular weight of 50,000–80,000 (Fig. 5). In this experiment, 40 units of heparin were applied to the column, and 0.5 ml. of freshly-drawn blood was added to 0.1 ml. from each effluent tube. The effect on the clotting time of the blood was maximum in tubes 42–46, corresponding to the zone of apparent molecular weights between 50,000 and 80,000.

Ascitic fluid from mice bearing Ehrlich ascites carcinoma contains a B₁₂
Fig. 3.—Effect of heparin concentration on mobility of B12 binding fraction in EDTA plasma. When 10 units (100 μg.) of heparin were mixed per milliliter of plasma, most of the radioactivity filtered after the albumin peak (upper figure); when 100 units (1000 μg.) were used, most filtered before the albumin peak. Each tube contained 15 drops (about 0.7 ml.) of effluent. Filtration at 25°C.

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binder which enhances uptake of vitamin B12 by tumor cells as does transcobalamin and filters through sephadex with an apparent size identical with that of human transcobalamin 2. When heparin was added to mouse ascites fluid, the apparent size of this transcobalamin 2-like binder increased in a manner identical with the effect of heparin on the TC2 peak of normal human serum.

Combination of heparin with transcobalamin 2 would convert the latter from a small protein to a larger B12 binder containing carbohydrate. The effect of mixtures of heparin and mouse ascitic fluid, and heparin and human serum on 57CoB12 uptake by guinea pig intestinal mucosal homogenate and on absorption of 57CoB12 in a gastrectomized human subject were determined to exclude the unlikely possibility that the biologic characteristics of a heparin-transcobalamin-2 complex resembled those of intrinsic factor. A mixture of heparin and mouse ascitic fluid did not enhance uptake or absorption of 57CoB12 in these systems.

To determine the effect of EDTA on the B12-binding proteins of serum, 2 mg. of EDTA were mixed with serum and filtered through sephadex with
Fig. 4.—Failure of heparin to bind $^{57}$CoB$_{12}$ during filtration through sephadex G-150. All of the radioactivity in the effluent filtered as a small molecule. The volume of gel in the column in this experiment was smaller than in the experiments illustrated in Fig. 1, and identical with that in Figs. 4 and 5.

Fig. 5.—Clotting time of blood mixed with the effluent from sephadex G-150 column during filtration of 40 units of heparin. The blood used for tubes 15–49 was from a different subject from that used for tubes 50–88.

$^{57}$CoB$_{12}$. The filtration pattern of the $^{57}$CoB$_{12}$ in the serum was identical with that from EDTA plasma (Fig. 6). Less of the $^{57}$CoB$_{12}$ filtered in the peak corresponding to an apparent molecular weight of 120,000 and more in the 36,000 peak than in serum to which EDTA had not been added.
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Fig. 6.—Filtration of serum, EDTA plasma and EDTA serum from a single venepuncture with \(^{57}\text{CoB}_{12}\) through sephadex G-150. In the presence of EDTA, less of the \(^{57}\text{CoB}_{12}\) filtered in the first fraction, and more in the second than when no EDTA was present.

DISCUSSION

It is apparent that the two major fractions of \(^{57}\text{CoB}_{12}\) identified in serum by filtration through sephadex cannot be assumed to correspond necessarily to transcobalamin 1 and 2, respectively. The apparent shift of a portion of the first fraction into the second when EDTA was added to serum would be consistent with an association of TC2 molecules by bivalent cationic bridging, with dissociation of transcobalamin 1 by EDTA; or release of B\(_{12}\) from TC1 and its binding by TC2. Data are not available to differentiate between these possible explanations, but the demonstration\(^7\) that the large molecular weight binder is heterogeneous would make the first more likely. The effect of heparin on the pattern of filtration of vitamin B\(_{12}\) would appear to be due to combination of heparin with transcobalamin 2. A 1:1 combination of a molecule of the size indicated for heparin in Fig. 5, and of TC2 (36,000) would result in a B\(_{12}\) binder with mobility through sephadex, similar to that observed in heparinized serum and plasma. Heparin is a strong cation exchanger and might be expected to combine with a molecule such as TC2, which has a strong affinity for a cation exchanger such as carboxymethyl cellulose,\(^4\) and a weak affinity for DEAE cellulose. Combination with heparin also might not alter the affinity of TC2 for an anion exchanger such as DEAE cellulose, since the charged groups reacting with the DEAE group would differ from those reacting with heparin.

We have previously reported that human portal venous blood obtained during the absorption of \(^{57}\text{CoB}_{12}\) in vivo is associated with a protein fraction larger than TC2 when filtered through sephadex.\(^6\) This probably was due to the heparin content of the portal venous blood.

If a 1:1 combination occurs between heparin and TC2, then, assuming
that one molecule of TC2 binds one molecule of vitamin B₁₂, the data in Fig. 2 suggest that based on the total B₁₂ binding capacity of the serum, when 2000 moles of heparin were present per mole of TC2, no detectable binding occurred; when 20,000 moles were present, most of the TC2 was bound.

Heparin has been reported to combine with many biologic materials, so the demonstration that it may combine with TC2 does not indicate a unique phenomenon. Since TC2 combines with the surfaces of certain cells, its combination with heparin may be fortuitous. If, however, the role of TC2 is to combine with cell surface and to allow pinocytosis of the TC2-B₁₂ complex, as has been suggested, then the reaction of TC2 with a mucopolysaccharide such as heparin might be due to characteristics which would be desirable in such a molecule.

When radioactive cyanocobalamin was added to serum and filtered through sephadex, none of the radioactivity filtered as free vitamin B₁₂, although a large fraction of the native vitamin B₁₂ filtered in the precise position in the filtrate of free B₁₂ (Figs. 1 and 2).

Unbound native vitamin B₁₂ is not present in serum and plasma when binding is measured by electrophoresis and chromatography. Studies were not undertaken to differentiate this fraction from B₁₂ bound to a small molecule, or to a large molecule retarded by adsorption to the gel. A complex forms between two molecules when the sum of the rates of association and dissociation are appropriate. During gel filtration, dissociation is favored, since components are separated once dissociation occurs. Thus, the amount of dissociation of a complex during gel filtration is determined by the rate of dissociation at the temperature of filtration and the duration of filtration. In these studies, filtration required 24 hours at 0–2°C. This suggests that although native B₁₂ in plasma (which is not cyanocobalamin) is bound, its rate of dissociation at 0–2°C allows dissociation of much of the complex over 24 hours, whereas the rate of dissociation of cyanocobalamin protein complexes at this temperature would appear to be lower. A significant fraction of free B₁₂ also was found in portal venous blood during B₁₂ absorption. Rewarding speculation about its significance is not possible at this time, but it probably reflects the difference between the cyanocobalamin and the forms of native B₁₂ in plasma.

**SUMMARY**

⁵⁷Co cyanocobalamin mixed with serum filters through sephadex G-150 in two fractions, corresponding to apparent molecular weights of about 120,000 and 36,000, respectively. Much of the native B₁₂ also filters in these fractions, but some is excluded from the gel, and a large fraction filters in the position of free B₁₂. When heparin is mixed with serum or plasma, most of the B₁₂ filters with the 120,000 fraction. When EDTA is mixed with serum or plasma not containing heparin, less ⁵⁷CoB₁₂ filters in the 120,000 fraction, and more filters in the 36,000 fraction. The 120,000 and 36,000 fractions cannot therefore be assumed to correspond precisely with transcobalamin 1 and 2, respectively.

* Gräsheek, R.: Personal communication.
It is suggested that heparin binds transcobalamin 2 and that EDTA dissociates either TC2 complexes or some of the TC1.

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REFERENCES


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