The Heterogeneity of the High Molecular Weight B12 Binder in Serum

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THE FINDING that each of several different technics for separating serum proteins resolves the vitamin B12-binding properties of human serum into two discrete fractions has led to the belief that there are just two serum proteins that can bind vitamin B12 specifically. However, it has not been demonstrated that the two B12-binding protein fractions defined by each separation technic are identical.

Electrophoretic separation of B12-enriched serum has distinguished an α1-globulin and a β-globulin binding protein (α1-binder and β-binder respectively). Chromatographic separation on columns of DEAE- or CM-cellulose also yields two B12-binding components: transcobalamin I (TC I) apparently corresponds to the α1-binder, and transcobalamin II (TC II) to the β-binder. More recently, while the present studies were under way, it was reported that Sephadex gel filtration also resolved the serum B12-binders into two fractions, one having a higher molecular weight (MW) than the other. These two components were also designated TC I (the higher MW fraction) and TC II (the lower MW fraction) respectively although it was not proven rigorously that these components actually corresponded with those so named and obtained by chromatography on DEAE-cellulose.

The present studies were directed toward establishing whether the respective fractions obtained by Sephadex gel filtration (high MW and low MW) and by DEAE-cellulose column chromatography (TC I and TC II) were, in fact, identical. The results show that the low MW binder in normal serum is TC II, but that the high MW B12-binding fraction contains both TC II and TC I; that is, there are not just two but at least three serum B12-binders in normal serum, a high and a low molecular weight TC II (both β-globulins), and a high molecular weight TC I (an α1-globulin).

MATERIALS AND METHODS

A. Subjects

Normal serum was obtained from 34 healthy fasting men and women whose routine hematologic studies were normal. Subjects who had been limiting their diet in any important regard were not used in this study. Sera were used within 30 days of collection and were meanwhile stored at −20 C.
B₁₂-deficient serum was obtained from fasting patients with untreated Addisonian pernicious anemia (PA). The unsaturated B₁₂-binding capacity of serum was estimated within seven days of collection on either freshly-obtained or frozen stored serum and the stored specimens were used later for the studies reported here only if the remeasured unsaturated B₁₂-binding capacity was essentially the same as the original estimate.

B. Analytical

Radioactive Co⁵⁷B₁₂ with a specific activity (SA) of 7 μc./μg. and a concentration of 2,667 pg. Co⁵⁷B₁₂/ml. serum was used for estimating the unsaturated B₁₂-binding capacity and for the paper electrophoretic studies. The procedures were the same as reported previously except that hemoglobin-coated charcoal (100 mg. Norit-A† plus 10 mg. hemoglobin/ml.) rather than uncoated charcoal was used to adsorb the unbound Co⁵⁷B₁₂.

Gel filtration was performed by upward flow through 3.2 × 50 cm. columns of Sephadex G-200 using an LKB UltroRac fraction collector with a pump flow of 18 ml/hr., collecting 3.8 ml. (70 drops) samples. Sephadex G-200 was equilibrated with 0.075 M NaCl—0.075 M TRIS buffer, pH 7.65. The optical density (OD) at 254 μm was measured continuously with an LKB Uvicord instrument. The void volume averaged 98 ml and three peaks at OD 254 μm were recorded in samples eluted thereafter: the first represented macroglobulins, the second gamma globulins, and the third albumin. The volume of each specimen applied to the column was 5 ml. In the case of normal and many PA sera, this consisted of 3 ml. of serum tagged with Co⁵⁷B₁₂ (SA 7 μc./μg.) plus NaCl-TRIS buffer to make a total of 5 ml. When the serum unsaturated B₁₂-binding capacity was less than 35 per cent, or the amount of serum available was limited, only 1.5 ml. of serum was tagged with Co⁵⁷B₁₂ (SA 20 μc./μg.) and additional buffer was added to bring the sample volume to 5 ml. The radioactivity in each specimen was measured in a sodium iodide well counter with a counting error of less than 1 per cent for the specimens with peak radioactivity.

The low molecular weight binder was adsorbed completely from serum by adding a larger amount of uncoated charcoal (150 mg./ml.) than in the other studies. The charcoal-containing serum was mixed on a mechanical rotator for 30 minutes and then centrifuged for 20 minutes at 34,000 × g. to remove the charcoal. Co⁵⁷B₁₂ (2,667 pg./ml., SA 20 μc./μg.) was then added to aliquots of this specially “pre-adsorbed” serum and to paired aliquots of untreated serum. Free Co⁵⁷B₁₂ was then removed from each specimen by adding 75 mg. charcoal plus 7.5 mg. hemoglobin/ml. serum, mixing for 15 minutes, and then removing the hemoglobin-coated charcoal by centrifugation. One half ml. samples of each of these paired sera were then chromatographed through separate "baby columns" of DEAE-cellulose§ prepared and used as described by Retief, et al. Three and a half ml. of the remaining aliquot pairs were filtered separately through Sephadex G-200 after dilution with 1.5 ml. NaCl-TRIS buffer.

Endogenous serum vitamin B₁₂ activity was assayed by the method of Spray, the normal range being 150–900 pg./ml.

RESULTS

A. Separation of the B₁₂ β-Binder of Normal Serum into High and Low Molecular Weight Components

Electrophoresis of normal sera that had been enriched with 2,667 pg. Co⁵⁷ B₁₂/ml. and then adsorbed with hemoglobin-coated charcoal disclosed that almost all of the protein-bound Co⁵⁷B₁₂ migrated in the β-globulin and

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* E. R. Squibb and Sons, New Jersey
† Fisher Scientific Company, New York
‡ LKB Instrument, Inc., Rockville, Maryland
§ Selectacel ion exchange cellulose, #71 DEAE, Type 20, Carl Schleicher and Schull Co., Keene, New Hampshire.
Fig. 1.—The serum β-globulin Co57B12-binder consists of “high” and “low” molecular weight components. Left: paper electrophoretic separation of Co57B12-enriched normal serum showing unimodal β-globulin localization of Co57B12. Right: Sephadex G-200 gel filtration elution patterns of bound Co57B12 in two different normal sera showing localization in “high” and “low” MW fractions at 195 ml. and 250 ml. respectively. The curve of OD at 254 mλ shows the three major protein fractions.

α2-globulin zones (Fig. 1, left). Because the distribution of radioactivity was unimodal, centered in the “fast” β-globulin range, this will be referred to as indicating the binding of vitamin B12 by β-globulin.

Gel filtration of such serum through Sephadex G-200 regularly disclosed two major peaks of radioactivity (Fig. 1, right). The peak of the earlier-appearing high MW fraction (at 195 ml.) came shortly after the peak of the gamma-globulins, and the peak of the later-appearing low MW fraction (at 250 ml.) was eluted after the peak of albumin. The proportion of Co57 B12 bound by the two fractions differed among the subjects (Fig. 1, right, upper and lower curves) but was the same in any one subject studied on different days over short periods. Thus, while Co57 B12 added to normal serum migrated on electrophoresis in the β-globulin zone, gel filtration resolved this B12-binding fraction into two components that had different MW.

B. Demonstration of the Alpha1-Globulin B12-binder in the High Molecular Weight Fraction

The α1-binder was studied using B12-deficient serum in which this B12-binder is unsaturated and can therefore bind added Co57B12. Electrophoresis of Co57B12-enriched hemoglobin-coated-charcoal adsorbed serum obtained from patients with untreated PA disclosed two major peaks of radioactivity in most cases. One peak, as in normal serum, was in the β-globulin zone, and the other was in the α1-globulin zone (Fig. 2, left).
Aliquots of 10 Co\textsuperscript{57}B\textsubscript{12}-enriched PA sera were split and one portion of each was adsorbed with hemoglobin-coated-charcoal and the other with 100 mg./ml. of uncoated charcoal. The binding of Co\textsuperscript{57}B\textsubscript{12} by $\alpha_1$-globulin was the same in the paired sera, but much less Co\textsuperscript{57}B\textsubscript{12} was bound by $\beta$-globulin when uncoated charcoal was used (Fig. 2, left).

Gel filtration of Co\textsuperscript{57}B\textsubscript{12}-enriched PA serum adsorbed with uncoated charcoal also yielded a different pattern of radioactivity from that found when the same serum was adsorbed with hemoglobin-coated charcoal (Fig. 2, right). The magnitude of the high MW-peak was the same in the paired sera but the low MW Co\textsuperscript{57}B\textsubscript{12} peak was much lower when 100 mg. of uncoated charcoal per ml. of serum was used.

The lesser binding of Co\textsuperscript{57}B\textsubscript{12} by $\beta$-globulin when using uncoated rather than hemoglobin-coated charcoal could theoretically have resulted from either removal of bound Co\textsuperscript{57}B\textsubscript{12} from its $\beta$-globulin by the uncoated charcoal, or from adsorption of $\beta$-globulin binder with its bound Co\textsuperscript{57}B\textsubscript{12}. In order to distinguish between these possibilities, the pattern of Co\textsuperscript{57}B\textsubscript{12} binding was examined in paired aliquots of PA sera. Co\textsuperscript{57}B\textsubscript{12} was added directly to one sample; it was added to the other only after the serum had been first pre-adsorbed with uncoated charcoal; in each case the serum was then separated by electrophoresis (Fig. 3). Because free Co\textsuperscript{57}B\textsubscript{12} was not removed from these mixtures, there was a prominent peak of radioactivity in the gamma globulin zone. Much less Co\textsuperscript{57}B\textsubscript{12} was bound by $\beta$-globulin in the serum to which charcoal had been added before Co\textsuperscript{57}B\textsubscript{12}, indicating that the charcoal had adsorbed some of the $\beta$-globulin binder as first suggested by Gottlieb et al.\textsuperscript{15}
Fig. 3.—Paper electrophoretic patterns showing removal of most of the $\beta$-globulin $\text{Co}^{57}\text{B}_{12}$-binding protein from PA serum by "pre-adsorption" (dashed line) as compared to control specimen (solid line). Arrows refer to free $\text{Co}^{57}\text{B}_{12}$ in the $\gamma$-globulin zone (see text).

Fig. 4.—Sephadex G-200 elution patterns (left) and paper electrophoretic patterns (right) of bound $\text{Co}^{57}\text{B}_{12}$ in normal serum (dashed line) and PA serum (solid circles), adsorbed with uncoated charcoal in both cases to remove the "low" MW $\text{B}_{12}$-binding protein and free $\text{B}_{12}$. The "high" MW $\text{B}_{12}$-binding fraction in PA serum includes an $\alpha_1$-globulin as well as the "high" MW component of the $\beta$-globulin (right).
Fig. 5.—Sephadex G-200 (left) and the DEAE-cellulose “baby column” (right) elution patterns of Co\textsuperscript{57}B\textsubscript{12}-enriched normal serum adsorbed with hemoglobin-coated charcoal (solid circles) or “preadsorbed” with uncoated charcoal (open circles). Uncoated charcoal adsorbed the “low” MW B\textsubscript{12}-binding protein (left) and this corresponds to adsorption of only part of the TC II fraction (right, peak in tube no. 3). The remaining ‘high’ MW fraction in the “preadsorbed” serum (left, shaded area) corresponds to part of TC II (right, tube no. 3) and all of the TC I fraction (right, tube no. 9).

The addition of a larger amount of uncoated charcoal (150 mg./ml.) to Co\textsuperscript{57}B\textsubscript{12}-tagged-serum effected removal of all of the low MW binder and free Co\textsuperscript{57}B\textsubscript{12} without diminishing the binding by the high MW fraction (Fig. 4, left). Electrophoresis of the remaining high MW fraction of normal serum yielded a single peak of radioactivity in the \(\beta\)-globulin zone (Fig. 4, right). In contrast, the high MW fraction of serum from patients with untreated PA yielded two components, one with \(\beta\)-globulin mobility as in normal serum, and the other with \(\alpha_{1}\)-globulin mobility (Fig. 4, right).

C. DEAE-Cellulose Chromatography of the High Molecular Weight B\textsubscript{12} Binder of Normal Serum

Passage of Co\textsuperscript{57}B\textsubscript{12}-enriched normal serum through “baby” columns of DEAE-cellulose disclosed two major peaks of radioactivity as described by Retief et al.\textsuperscript{11} and designated TC II (the earlier-appearing fraction eluted with 0.06 M phosphate) and TC I (the later-appearing fraction eluted with 1 M NaCl). Ten normal and 10 PA sera were each split and half of each specimen was “pre-adsorbed” with 150 mg./ml. of uncoated charcoal before adding Co\textsuperscript{57}B\textsubscript{12} (see Methods). Gel filtration on Sephadex G-200 showed that all of the low MW B\textsubscript{12}-binding activity had been removed by this “pre-adsorption,” leaving only the high MW B\textsubscript{12}-binding fraction (Fig. 5, left). Chromatography of these high MW fractions through “baby” columns of DEAE-cellulose showed Co\textsuperscript{57}B\textsubscript{12} in two zones, TC I and TC II (Fig. 5, right). The radioactivity in the TC I zone (tube No. 9) was essentially the same as in the other portion of each specimen that had not been “preadsorbed.” In striking contrast, the radioactivity in the TC II zone (peak in tube No. 3) was
Fig. 6.—Diagrammatic representation of the relationships between the various methods of separating the Co$_{57}$B$_{12}$-binding fractions of serum.

much lower than in the sera that had not been pre-adsorbed. These findings thus indicate that the remaining high MW binding activity consisted of both TC I and TC II and that the low MW binding fraction adsorbed by the charcoal corresponded to part of the TC II fraction.

**Discussion**

Electrophoretic separation of serum delineates two fractions with B$_{12}$-binding affinity, one an $\alpha_1$-globulin and the other a $\beta$-globulin.$^{1-8}$ The $\alpha_1$-globulin, which binds the endogenous circulating vitamin B$_{12},^{1-3}$ is normally nearly saturated, so that when Co$_{57}$B$_{12}$ is added to normal serum in vitro it is bound mainly by $\beta$-globulin.$^{4-8}$ In B$_{12}$-deficient serum, however, the $\alpha_1$-globulin binder is unsaturated and hence can bind some of the added B$_{12}.$$^{6-8,18}$

Separation of Co$_{57}$B$_{12}$-enriched serum on columns of DEAE and CM-cellulose has also defined two fractions with B$_{12}$-binding activity, designated by Hall and Finkler as TC I and TC II.$^{10}$ TC I was shown to have $\alpha_1$-globulin electrophoretic mobility and TC II to have $\beta$-globulin mobility.$^{3,8-11}$ It has therefore been inferred that the electrophoretic and chromatographic procedures separate serum B$_{12}$-binding activity into essentially the same two corresponding fractions.

Filtration of Co$_{57}$B$_{12}$-enriched normal serum through Sephadex G-200 shows that what appears on electrophoresis as a 'single' $\beta$-globulin B$_{12}$-binder actually consists of two components, one having a higher molecular weight than the other (Fig. 1). Thus, there are not just two, but at least three B$_{12}$-binding proteins in serum, an $\alpha_1$-globulin and two $\beta$-globulins, one of the latter having a lower molecular weight than the other.
From the knowledge that TC I is an α₁-globulin and that TC II has β-globulin mobility, one would anticipate that TC I should be present in the high MW Sephadex eluates and that TC II should be present in both the high and the low MW Sephadex eluates. In other words, the high MW B₁₂-binding fraction should contain both TC I and TC II while the low MW fraction should contain just TC II and this prediction was verified (Fig. 5).

Electrophoresis of serum pairs, one “pre-adsorbed” and the other not, showed that removal of the low MW fraction corresponded to removal of part of the β-globulin fraction (Fig. 2). The adsorption of the low molecular weight β-binder by uncoated charcoal accounts partially for our previous observation concerning decreased β-binding activity in the serum of patients with untreated PA. Restudy of the available sera using hemoglobin-coated charcoal showed, however, that this did not account for either the virtual absence of β-binding activity observed in 3 such cases, nor for a lower average total β-binding activity in PA as compared to normal sera.

In normal sera the high MW B₁₂-binding fraction remaining after “pre-adsorption” consisted only of β-globulin whereas in PA sera it contained both α₁- and β-globulin (Fig. 4). Thus, while each of these three separation technics yields just two B₁₂-binding fractions, one of the fractions is, in each case, bipartite. That is, the high MW fraction of the Sephadex eluates consists of both TC I and TC II and the β-globulin binder has both high and low molecular weight components. The relation between the various B₁₂-binding fractions of serum as defined by each of these three technics for separating proteins is diagrammed in Figure 6. These findings show that the designation by Hom, Oleson and Lous of their high and low MW Sephadex fractions as TC I and TC II respectively is not warranted.

These studies have not answered the question of whether there may be a separate and specific α₂-globulin binder which, if it does exist, may actually be one of the two β-globulin (or TC II) fractions. It is also conceivable that part of the high MW fraction may be a polymer of the low MW β-globulin.

**SUMMARY**

The binding of vitamin B₁₂ by serum proteins was studied by separating Co<sup>57</sup>B₁₂-enriched serum by Sephadex gel filtration, column chromatography with DEAE-cellulose, and paper electrophoresis. Each method of separation yielded two discrete B₁₂-binding fractions. However, the analysis of each serum by all three separation technics indicated that one of the fractions was, in each case, bipartite.

The “high” molecular weight B₁₂-binding fraction defined by Sephadex gel filtration consisted of transcobalamin I and just part of the transcobalamin II fraction. The remaining portion of transcobalamin II was eluted from Sephadex gel in a “low” molecular weight fraction. Thus, transcobalamin II, equivalent to the β-globulin B₁₂-binder, consisted of both “high” and “low” molecular weight components.

This suggests that there are at least three serum proteins that can bind
vitamin B₁₂: two β-globulins, together comprising the transcobalamin II fraction and differing in molecular weight; and transcobalamin I.

**SUMMARIO IN INTERLINGUA**

Le ligation de vitamina B₁₂ per proteinas seral esseva studiate per medio del separation de sero inricchite in vitamin B₁₂ a C₀⁵⁷ con le uso del methodos a filtration con gel Sephadex, de chromatographia columnal con DEAE-cellulosa, e de electrophorese a papiro. Cata-un de iste methodos de separation rendeva duo discrete fractiones capace a ligar vitamina B₁₂. Tamen, le analyse de omne specimen de sero con le uso de omne le tres tecnicas de separation indicava que un del fractiones esseva—in cata-un del casos—bipartite.

Le fraction ligante vitamina B₁₂ e characterisate per un alte peso molecular, in tanto que illo esseva definit per filtration a gel Sephadex, consisteva de transcobalamina I e non plus que un minime parte del fraction de transcobalamina II. Le remanente transcobalamina II esseva eluite ab gel Sephadex in un fraction a basse peso molecular. Il seque que transcobalamina II—equivalente a globulina β ligatori de vitamina B₁₂—consisteva de componentes a pesos molecular tanto alte como etiam basse.

Isto suggestiona que il existe al minus tres proteinas seral que pote ligar vitamina B₁₂: duo globulinas β—representante insimul le fraction transcobalamina II sed characterisate per differente pesos molecular—e transcobalamina I.

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