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Studies on Vitamin B_{12} Binders of Human Serum (I) Isolation and Characterization (38345)

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In recent years, many workers have demonstrated the presence of at least three vitamin B_{12} binders in normal serum, namely, transcobalamin II (TC II), transcobalamin I (TC I) and transcobalamin III (TC III), using a combination of chromatography on DEAE-cellulose and gel-filtration on Sephadex G-200 (1–6). In earlier studies from this laboratory, Gizis and Meyer (7) described the procedure for the isolation of all the three binders on small DEAE-cellulose columns (baby columns) using a stepwise gradient of three sodium phosphate buffers. In subsequent studies in our laboratory, however, it was observed that results utilizing "baby columns" were not always reproducible. Abrupt changes of buffer concentrations used for elution of binders from DEAE-cellulose columns could possibly explain this discrepancy. Furthermore, while the three binders obtained by chromatography on a larger (3 × 60 cm) DEAE-cellulose column were characterized by electrophoresis and molecular weight studies (6), the binders obtained on small columns had not been subjected to the same analyses.

We have now standardized the technique of isolating three vitamin B_{12} binders on a small DEAE-cellulose column, using 0.5 ml of normal serum and ^{57}Co B_{12} in a five buffer system. The method can be used as a routine and rapid procedure for determining levels of three binders in serum. The three binders so obtained have been characterized by their molecular weights and electrophoretic mobilities.

Methods and Materials. Sera were obtained from 19 normal volunteers (9 females and 10 males), and stored at −20°.

DEAE-Cellulose column chromatography. (a) "Baby columns." DEAE-cellulose was prepared according to Gizis and Meyer (7). The suspension, which had been washed with molar NaOH, distilled water and starting buffer (0.0175 *M*-phosphate buffer, pH 6.3), was used as a slurry for pouring on a 0.5 cm column (5 ml pipette plugged with glass wool) to a height of 22 cm. Before use, packed columns were washed overnight with about 250 ml of starting buffer.

To 0.5 ml of serum, 250 pg of ^{57}Co B_{12} (Amersham, sp. act. 182 mCi/mg) were added and the mixture incubated at 37° for 20 min. The mixture, after counting, was dialysed overnight against a liter of starting buffer and centrifuged to remove precipitate. The supernatant was applied to the DEAE-cellulose column and elution performed with a step-wise gradient of sodium phosphate buffers, starting with 16 ml of 0.0175 *M*, pH 6.3; 8 ml of 0.03 *M*, pH 5.9; 28 ml of 0.06 *M*, pH 5.85; 16 ml of 0.1 *M*, pH 5.8; and 20 ml of 0.25 *M*, pH 5.4. Two milliliter fractions were collected and counted for two minutes each in an automatic well type scintillation counter (Nuclear-Chicago, model 1185). Relative percentage of each binder, with respect to total radioactivity recovered, was calculated by combining counts for each peak of radioactivity.

(b) Large columns. For obtaining large amounts of B_{12} binders, 30 ml of serum were incubated with ^{57}Co B_{12} (300 pg/ml of serum) for 20 min at 37°, dialyzed for 24 hr against three changes of 4 liters each of 0.0175 *M* phosphate buffer, pH 6.3, and chromatographed on a DEAE-cellulose column (3 × 80 cm) preequilibrated with 0.0175 *M* buffer, pH 6.3. Elution of binders from column was performed with sodium phosphate buffers at a flow rate of 30 ml/hr, collecting 10 ml fractions. Volumes of buffers used for elution were 350 ml of 0.0175

M, pH 6.3; 225 ml of 0.03 *M*, pH 5.9; 725 ml of 0.06 *M*, pH 5.85; 900 ml of 0.1 *M*, pH 5.8; and 650 ml of 0.25 *M*, pH 5.4. All above procedures were performed in the cold room at 4°. Three peaks of radioactivity were collected, dialyzed against large volumes of distilled water in the cold for 24–48 hr, and freeze-dried.

Molecular weight determination using Sephadex G-200 columns. Molecular weights of three vitamin B_{12} binders were determined by gel-filtration on a Sephadex G-200 column (2.6 × 33 cm, void volume 50 ml, prepared according to the manufacturers—Pharmacia), after being allowed to swell in 0.005 *M* phosphate buffer, pH 7.4 containing 1 *M* NaCl, for 1 week. A selectivity curve for proteins of known molecular weights was prepared by determining elution volumes of Ribonuclease A, Chymotrysinogen A, Ovalbumin, Aldolase and Blue Dextran 2000, using 0.005 *M* phosphate buffer, pH 7.4 containing 1 *M* NaCl for elution from the column. *Kav* values for each standard protein were calculated using the equation:

$$Kav = (Ve - Vo)/(Vt - Vo),$$

where Ve = elution volume for the protein, Vo = elution volume for Blue Dextran 2000, and Vt = total bed volume, and plotted against the logarithm of their molecular weights. The *Kav* values for Ribonuclease A, Chymotrysinogen, Ovalbumin and Aldolase were 0.80, 0.64, 0.45 and 0.26, respectively.

For molecular weight determination of fractions eluted from DEAE-cellulose columns (large and small) samples were dialyzed, freeze-dried, and subsequently dissolved in 0.005 *M* phosphate buffer, pH 7.4 containing 1 *M* NaCl. Elution volumes for each binder were determined on the same Sephadex column described in preceding paragraph and molecular weights determined by locating points on the selectivity curve corresponding to their *Kav* values.

Paper electrophoresis of TC I, TC II and TC III. Electrophoresis of the freeze-dried peaks of radioactivity obtained from large DEAE-cellulose columns, dissolved in 0.075 *M* Barbital buffer, pH 8.6, and concentrated fractions from small columns using an Amicon B_{15} micro concentrator, was performed on a Durrum-type of electrophoresis cell (Beckman/Spinco, model R, series C). Samples of TC I, TC II and TC III, and a normal serum control were applied to strips of paper (Beckman #320046) and electrophoresis performed at 75 V for 18 hr in 0.075 *M* Barbital buffer, pH 8.6. One strip of each of the binder and normal serum was stained with bromophenol blue (0.1% in methanol). Unstained strips of binders were cut at 0.5 cm intervals, identified in relation to major serum protein fractions, and counted in a well type scintillation counter.

Results. A. DEAE-cellulose column chromatography. (1) "Baby columns." Results of chromatography on "baby columns" of DEAE-cellulose are presented in Table I. Three distinct peaks of radioactivity were obtained: TC II with 0.06 *M*, pH 5.85 buffer; TC III with 0.1 *M*, pH 5.8 buffer; and TC I with 0.25 *M*, pH 5.4 buffer (Fig. 1). Percentage distribution of ^{57}Co B_{12} binders in serum of normal adults was, TC II 77 ± 4.5; TC III 15 ± 4.1, and TC I 8.5 ± 2.7. No differences in distribution of radioactivity in B_{12} binders were observed between male and female donors, and so results are presented in a combined table. Two persons (E. V. & J. M.) showed persistent high levels of TC III (over 20%) on each of two occasions (Table I).

(2) Large columns. Pattern of radioactivity distribution in three peaks is presented in Fig. 2.

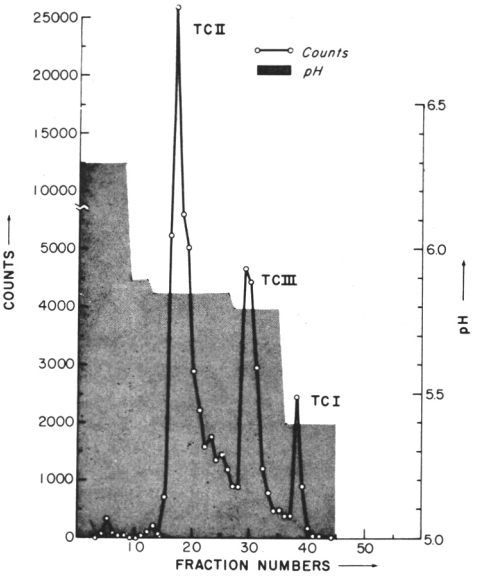

FIG. 1. Pattern of distribution of radioactivity of vitamin B_{12} binders of normal serum on "baby columns" of DEAE-cellulose. Elution was performed with increasing concentrations of sodium phosphate buffers and each fraction was counted for 2 min, as described in the text.

TABLE I. Distribution of Co^{57} B_{12} Binding Proteins in Normal Human Serum.

Donor's initials	Serum B_{12} pg/ml	Total binding* (%)	Distribution of radioactivity % of total bound		
			TC II	TC III	TC I
L.E.	300	84	74	12	14
C.B.	344	93	76	12	12
E.V.	200	91	73	22	5
A.M.	376	87	75	16	9
S.K.	216	88	74	18	8
P.R.	288	82	79	14	7
F.B.	284	88	77	18	6
L.M.	700	84	79	13	8
J.M.	340	92	71	23	6
T.	142	89	76	12	12
P.	160	81	73	18	9
R.	268	90	79	13	8
I.M.	720	91	73	14	13
L.	440	93	76	15	9
L.M.	752	89	88	7	5
S.	500	97	76	18	6
S.	1040	91	78	12	10
P.	596	89	88	8	5
K.	560	85	77	14	9
MEAN ± SD	433 ± 240	89 ± 4.1	77 ± 4.5	15 ± 4.1	8.5 ± 2.7

* The assay system contained 500 pg of $Co^{57}B_{12}$/ml of serum. The figure represents the percentage of total $Co^{57}B_{12}$ bound/ml of serum.

Arrows indicate changes of eluting buffer.

Rechromatography of TC I, TC II and TC III on "baby columns." Fractions containing maximum counts and constituting peaks corresponding to TC II, TC III and TC I were collected from six chromatographic runs on serum of a normal adult, using "baby columns." Analogous peaks from different runs were combined, radioactivity counted, and mixtures treated as follows:

(a) In one set of experiments, combined fractions for each peak were dialyzed against large volumes of distilled water for 24 hr at 4°, the dialyzed material collected and freeze-dried.

(b) In a second set, combined fractions for each peak were concentrated on an Amicon B_{15} microconcentrator. Concentrated material was collected, diluted to its original volume with distilled water, and reconcentrated. Process was repeated twice to remove as much salt as possible. Final concentrated material was collected and counted for radioactivity.

Peaks of TC I, TC II and TC III obtained by these two methods, were rechromatographed on "baby columns" of DEAE-cellulose using the procedure described earlier. More than 95% of applied radioactivity of TC I and TC III was recovered as TC I and TC III, respectively (Fig. 3). On the other hand, only about 80% of the total radioactivity of TC II was recovered as TC II on rechromatography, the remaining was found to be TC III (15%) and TC I (6%). Identical results were observed when freeze-dried material from the large DEAE-cellulose column was rechromatographed on "baby columns" of DEAE-cellulose, after dissolving in 0.0175 *M* phosphate buffer, pH 6.3.

Molecular weight determinations. Calculation of molecular weights of TC I, TC II and TC III obtained from DEAE-cellulose column chromatography, on the basis of elution from Sephadex G-200 column (2.6 × 33 cm), indicates that these binders behave as proteins with molecular weights of 40,000 (TC II), 120,000 (TC I) and 100,000 (TC III), their *Kav*

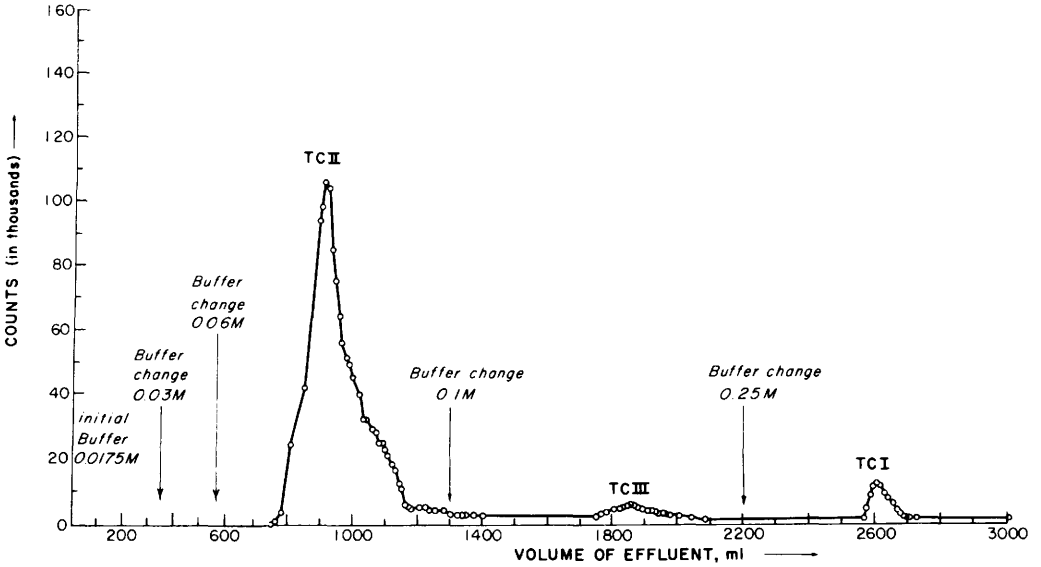

FIG. 2. Pattern of distribution of radioactivity of vitamin B_{12} binders of normal human serum on a large DEAE-cellulose column. The arrows indicate changes of eluting buffers.

values being *0.56* (TC II), *0.32* (TC I) and *0.36* (TC III).

Paper electrophoresis. Results of electrophoresis of TC I, TC II and TC III are shown in Fig. 4. While the major portion of TC II peak (70%) as determined by counting radioactivity in different areas, moved as a β-globulin (Rf=0.24), a part of it also migrated as α_2-globulin. On the other hand, TC I has a mobility between α_1 and α_2-globulins (Rf=0.67), and TC III between β and α_2-globulins (Rf=0.45).

Discussion. The present method has the distinct advantage in being able to resolve three binders as distinct peaks. It is reproducible and can be used routinely for determining distribution of binders in human serum.

As reported earlier (7), TC II obtained from "baby columns" has an approximate molecular weight of 40,000 and TC III and TC I have similar molecular weights, namely, 100,000 and 120,000 respectively. Present results are also in agreement with findings of other workers that TC II moves as a β-globulin, TC I between α_1 and α_2-globulin, and TC III between α_2 and β-globulin on electrophoresis.

On rechromatography, almost all TC III and TC I are recovered as single peaks at the same

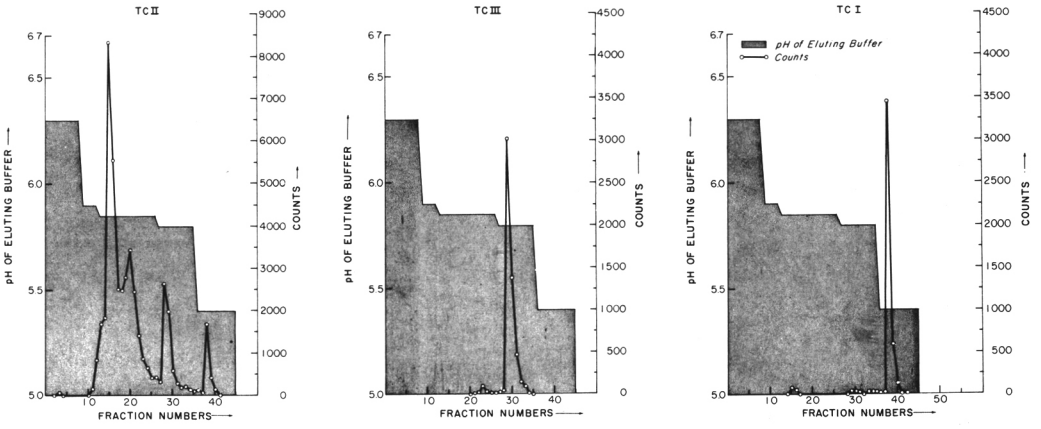

FIG. 3. Rechromatographic patterns of TC II, TC III and TC I on "baby columns" of DEAE-cellulose.

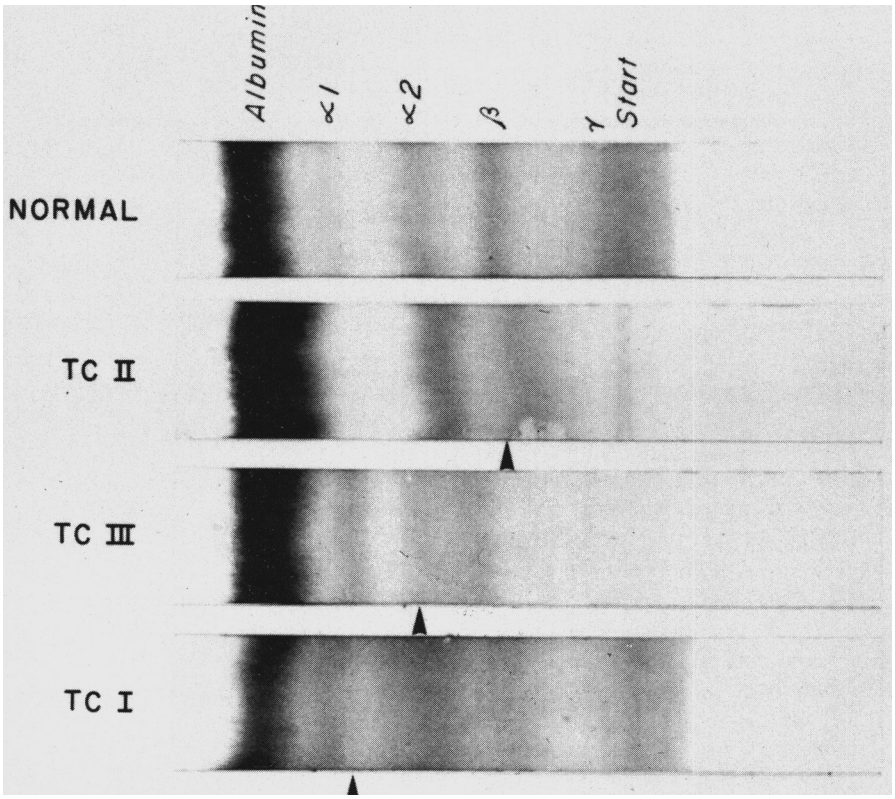

FIG. 4. Paper electrophoretic patterns of TC II, III and I with normal serum as reference. Arrows indicate areas where maximum radioactivity of each binder was localized.

positions on small DEAE-cellulose columns, while part of TC II is recovered also as TC III and TC I. Whether this is only a contamination or due to aggregation of TC II (8) can only be surmised at present.

Summary. A DEAE-cellulose column chromatographic technique for rapid separation of TC II, TC III and TC I from 0.5 ml of normal human serum is described. The molecular weights of TC II, TC III and TC I, as determined by gel-filtration on Sephadex G-200 columns were found to be 40,000, 100,000 and 120,000 respectively. On electrophoresis, TC II moves as a β-globulin, TC I between α_1 and α_2-globulin, and TC III between β and α_2-globulin.

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