Separation of Transcobalamins on Small DEAE-cellulose Columns (36451)

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Three vitamin B_{12} binders have been isolated from normal serum by DEAE-cellulose column chromatography utilizing 3 × 60 cm columns (1–3). These binders have been designated as transcobalamin II (TCII), main protein peak binder (MPPB), and transcobalamin I (TCI) (2, 3). TCII has a molecular size of approximately 36,000, disappears rapidly from the circulation following intravenous injection into normal human subjects, and is bound to rat liver when perfused through the isolated organ (3, 4). MPPB has a molecular size of about 120,000 and acts like TCII when injected intravenously into human subjects or when it is perfused through the isolated rat liver (3, 4). TCI has a molecular size of about 121,000, but differs from TCII and TCI in that clearance from plasma is slow following intravenous injection into normal human subjects, and is poorly bound to isolated rat liver when perfused through the organ (3, 4).

In the present study DEAE-cellulose column chromatographic technique was adapted to small scale separation of vitamin B_{12} binders in 1 ml of serum.

Materials and Methods. Preparation of the adsorbent. The procedure has been described elsewhere (1). Forty grams of dry DEAE-cellulose (Schleicher and Schuell, No. 70, 0.90-0.94 mEq/g) were allowed to sink in 2 liters of 1 *N* NaOH. After 30 min, suspension was filtered through a coarse fritted-glass filter funnel and washed with additional 1 *N* NaOH until filtrate was colorless. Filter cake was suspended in distilled water, filtered through the funnel, and washed with distilled water to pH 7.0. The cake was resuspended in 4 vol of 0.0175 *M* sodium phosphate buffer (pH 6.3), and enough 0.0175 *M* solution of $NaH_2PO_4 \cdot H_2O$ added to bring the pH to 6.2. DEAE-cellulose was washed in funnel with 0.0175 *M* sodium phosphate buffer (pH 6.3) until pH of suspension was 6.3. Cellulose was transferred to a beaker where it was resuspended in sufficient buffer to make about 50 ml of suspension for each gram of dry cellulose used.

Suspension was packed into 0.6 × 25 cm columns (5 ml pipettes plugged with glass wool). Columns were equilibrated overnight with 70 ml of 0.0175 *M* sodium phosphate buffer (pH 6.3) at room temperature. Tubing connecting the buffer reservoir (250 ml separatory funnel) with column was at a lower level than tip of column, so that column remained moist.

Preparation of the serum. Serum was kept frozen at —20°. The following quantities of ^{57}Co B_{12} (Amersham, sp act ca. 100 μCi/μg) were added per milliliter of thawed serum: 250, 500, 1000, 2500 and 5000 pg. Radioactivity was counted in a well-type scintillation spectrometer. Solution was allowed to stand at 37° for 15 min and transferred into dialysis tubing. Residual activity in test tube was counted. Labeled serum was dialyzed at 4° against an excess of 0.0175 *M* buffer (pH 6.3) for 24 to 48 hr. At the end of this period dialysis tubing was placed in a 16 × 150 mm test tube and nondialyzable activity was recorded.

Chromatographic procedure. Any solution remaining above the column after equilibration with initial buffer was allowed to sink into adsorbent and serum sample pipetted onto the surface. Test tubes, 13 × 100 mm, marked to 2 ml volume, were used for collection of eluate. Residual activities in test tube and dialysis tubing were counted for radioactivity. When entire sample entered adsorb-

TABLE I. Chromatographic Separation of Normal Sera (10 Persons) and CML Sera (2 Patients) Following Addition of Indicated Amount of ^{57}Co B_{12} per Milliliter of Serum.

Added ^{57}Co B_{12} (pg): Fraction	250	500	1000	2500	5000
		Normals (%)			
Bound	82 ± 6[a]	85 ± 9	83 ± 9	51 ± 17	29 ± 5
TCII	70 ± 8	68 ± 7	70 ± 7	70 ± 8	69 ± 7
MPPB	23 ± 8	24 ± 7	23 ± 6	22 ± 6	23 ± 6
TCI	7 ± 2	8 ± 1	7 ± 2	8 ± 2	8 ± 1
Recovery	89 ± 3	87 ± 9	85 ± 9	92 ± 5	89 ± 5
		Patients[b] (%)			
Bound	99	93	94	99	95
	99	99	99	99	99
TCII	12	12	12	12	12
	6	5	5	6	6
MPPB	44	52	46	45	54
	37	32	49	43	40
TCI	44	36	42	43	34
	57	63	46	51	54
Recovery	86	91	92	74	90
	77	83	87	78	77

[a] SD of the mean.

[b] Top line, patient H.; second line patient G.

ent, top of column was rinsed with 3 small portions of 0.0175 *M* buffer. Thereupon empty part of tube was filled with the initial buffer and the column was connected to a 250 ml separatory funnel containing 0.0175 *M* sodium phosphate buffer (pH 6.3). Six 2 ml samples were collected with this buffer. After sixth 2 ml sample was obtained, buffer reservoir was quickly disconnected and a new one containing 0.04 *M* sodium phosphate buffer (pH 5.9) was used for elution of twenty 2 ml samples. In same manner six 2 ml samples were collected with 0.1 *M* sodium phosphate buffer (pH 5.8), and seven 2 ml samples with 0.4 *M* sodium phosphate buffer (pH 5.2). Buffers were prepared in distilled water containing 0.09% methylparaben and 0.01% propylaraben (1). Tubes containing eluate were counted for radioactivity. TCII was eluted with the 0.04 *M* sodium phosphate buffer (pH 5.9) (1, 3); MPPB with 0.1 *M* sodium phosphate buffer (pH 5.8) (1, 3); TCI with 0.4 *M* sodium phosphate buffer (pH 5.2) (1, 3). Any free ^{57}Co B_{12} present in the serum was eluted with 0.0175 *M* sodium phosphate buffer (pH 6.3).

Results and Discussion. Table I indicates results obtained from separation of vitamin B_{12} binders in serum of 10 normal persons. The percentage bound ^{57}Co B_{12} was practically constant for amounts of added ^{57}Co B_{12} up to 1000 pg/ml. At 2500 and 5000 pg/ml levels of added ^{57}Co B_{12} percentage bound vitamin decreased, although absolute amount bound increased. Percentage of ^{57}Co B_{12} bound to each of three binders remained essentially constant for quantities of added vitamin B_{12} used in this study. Significantly more ^{57}Co B_{12} was bound to MPPB than to TCI in all 10 sera.

Table I indicates results obtained from separation of the binders in serum of two patients with chronic myelocytic leukemia. Percentage bound ^{57}Co B_{12} was increased. The percentage of ^{57}Co B_{12} bound to TCII decreased significantly in both cases with a corresponding increase in activity bound to MPPB and TCI. This disease is evidently associated with a significant increase of MPPB and TCI in the serum. Because of similarity in molecular size of MPPB and TCI (3), gel filtration does not separate these two binders and results obtained by this method show a high TCI content. Since

TCII has the tendency to aggregate (5), it is possible that MPPB is an aggregate of TCII. Conditions under which TCII undergoes aggregation are not well understood.

It thus appears that MPPB is normally present in human serum and can be separated from other B_{12} binders by DEAE-cellulose column chromatography, provided a suitable elution system is used.

Summary. A DEAE-cellulose column chromatographic technique was used for separation of TCII, MPPB and TCI in 1 ml of human serum. The percentage of vitamin bound to each binder remained practically constant at levels of added vitamin varying from 250 to 5000 pg ^{57}Co B_{12}/ml of serum. The percentage ^{57}Co B_{12} bound to MPPB and TCI in chronic myelocytic leukemia serum was significantly higher than in normal serum.

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