PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 204-209 (1976)

Fractionation of Serum Transcobalamins on Charged Cellulose Filters (39361)

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The three transcobalamins (i.e., vitamin B_{12}-binding proteins), TCI, TCII, and TCIII, known to be present in human serum, are difficult to determine by current procedures. The difficulties stem from the unique properties of TCIII, which resembles TCII in its electrophoretic mobility and behavior on DEAE-cellulose batch separation, while immunologically and in its molecular weight it is similar to TCI (1-4). The determination of the three transcobalamins requires therefore a combination of two procedures, a DEAE-cellulose column to separate TCI from TCII and TCIII on one hand, and a sephadex G-200 column to separate TCII from TCIII on the other (1-4). Alternatively, the selective removal of TCII either by precipitation with ammonium sulfate (5, 6) or by adsorption to uncoated charcoal (2) and to silica powder (7), enabled the use of DEAE-cellulose chromatography alone. A DEAE-cellulose column was used to separate all the three binders by changing the eluting buffer several times (8, 9); however, such a method seems too cumbersome for adaptation to the clinical laboratory.

It is already recognized that certain pathological conditions are associated with significant and specific changes in concentrations of the serum transcobalamins. Low unsaturated B_{12}-binding capacity (UBBC) and increase in serum B_{12} concentration bound mostly to TCII is characteristic of hepatocellular damage (10-12). Marked increases in TCI and elevated serum B_{12} are associated with chronic myelogenous leukemia (CML) and acute promyelocytic leukemia (APL) (13-23). Elevated TCIII and normal serum B_{12} values are found in polycythemia vera (PV) and clinical conditions associated with leukocytosis other than leukemia (1, 4, 24).

A rapid and simple procedure for fractionation of the transcobalamins should therefore be of value in the diagnosis of various pathological conditons.

A simple method for the quantitative determination of TCI and TCIII was recently reported by us (25). It is based on the selective adsorption of these transcobalamins to charged filter disks of DEAE-cellulose and cellulose-nitrate, respectively. In the present study the method was modified to determine simultaneously all three B_{12} binders.

Materials and methods. Serum preparation. Sera from normal subjects and from patients suffering from chronic myelogenous leukemia (CML) and polycythemia vera (PV) were rapidly separated and stored at −20°. A large batch of pooled normal serum (100 ml) was incubated for 30 min with excess (5 ng/ml) of nonradioactive vitamin B_{12} and dialyzed overnight against distilled water. This serum will be referred to as B_{12}-saturated serum and was used as an additional coating procedure for the charcoal (see below) to prevent adsorption of TCII from incubation mixtures containing small amounts (0.01 ml) of serum.

^{57}Co B_{12}. ^{57}Co B_{12} of high specific activity between 135-200 μCi/μg (The Radiochemical Centre, Amersham, Bucks, England) was used. Batches of 10 μCi were diluted with water to a final concentration of 10,000 pg B_{12}/ml and stored in the refrigerator when not in use.

Determination of UBBC and transcobalamin distribution by the filter stack. The filter stack is an assortment of wet charged cellulose disks of 25 mm in diameter (25) placed on a millipore type filter holder apparatus (The Tamar Co., Jerusalem, Israel). The stack is composed of a single cellulose-nitrate filter (Schleicher and Schüll, Dassel, Germany) which is placed on top of three DE-81 filters (The Whatman Biochemicals Ltd., Maidstone, Kent, England) rather than two as used previously (25), as it enables a complete recovery of TCI and TCIII

fractions. Buffer solutions for the filter stacks (see below) were prepared using glass distilled water and filtered through a cellulose-nitrate filter to remove particles which may interfere with the assay.

Duplicate samples of the serum (0.01 ml each) were incubated for 30 min at 37° with excess ^{57}Co B_{12} (50–100 pg/0.01 ml), 0.2 ml of 0.1 *M* sodium borate buffer (pH 8.5) and water to a final volume of 0.4 ml; alternatively, larger aliquots of sera and radio B_{12} diluted 1:10 or so may be taken. After incubation, each reaction mixture was diluted with 10–12 ml of the borate buffer and passed, by applying vacuum, through the filter stack described above; a 1/3 HP air vacuum pump and a trap for collecting the excess of unbound ^{57}Co B_{12} was used. Traces of unbound radio-vitamin were removed by washing the filter stack twice, with 10 ml of the same buffer. One washing with 10 ml of buffer was usually sufficient to remove all unbound radioactivity as assayed directly in the filtrate; however, to assure constant and complete removal of the unbound ^{57}Co B_{12} two additional washings are recommended. The unsaturated B_{12}-binding capacity (UBBC), expressed as amount of picograms of ^{57}Co B_{12} bound per milliliter of serum, was calculated from the radioactivity retained by the stack. The percentage distribution of the binders was determined from the radioactivities retained by each filter-type before and after subjecting the stack to further washing with acid potassium phosphate buffer as detailed in the text.

Determination of UBBC by the coated-charcoal adsorption method. The reaction mixture and incubation conditions were as described above. After the incubation period, 0.5 ml of the "B_{12}-saturated serum" mentioned above was added to the reaction mixture. Excess of unbound ^{57}Co B_{12} was then removed by adding of 0.5-ml suspension of Dextran 80-coated charcoal (1 g of Dextran 80/5 g of Norit A/100 ml of salt solution) (26). After centrifugation the UBBC was determined by measuring the radioactivity of the supernatant.

Selective removal of TCII and estimation of TCI and TCIII by chromatography. Transcobalamin II was selectively removed from the sera by treatment with Quso G32 (a microfine precipitated silica which was generously supplied by Dr. Victor Herbert), by adding 20 mg of the silica per 1 ml of serum and shaking the suspension for 30 min (7). After centrifugation, the supernatant was used to determine either the total concentration of TCI and TCIII or their individual components after separation on a DE-52 column or by specific elution from the DE-81 filter stacks as described in the Results.

The separation of TCII from TCI and TCIII was also carried out using the Sephadex G-200 column chromatography described by Hom *et al.* (19).

Results. Adsorption of ^{57}Co B_{12}-transcobalamin complexes to charged cellulose filters. A prerequisite for determination of the three transcobalamins is that they adsorb both quantitatively and selectively to the filter stack. The UBBC values of various sera containing quantitatively different assortments of transcobalamins were therefore determined by the filter stack procedure and the results compared with those obtained by the coated-charcoal adsorption method. The good correlation obtained (Table I) indicates that the three binders are quantitatively adsorbed to the filters.

The selective retention of TCII by the cellulose-nitrate filters is shown in Tables I and II. Removal of TCII with Quso G32 resulted in reduction of 90% or more of the radioactivity retained by the cellulose-nitrate filter (Table I). Furthermore, the radioactivities of fractions from Sephadex G-200 column containing TCII only were retained exclusively by the cellulose-nitrate filters (Table II). On the other hand, Quso G32 treatment did not reduce significantly the radioactivities adsorbed to the DE-81 filters, except for the serum from one CML patient (C.G.), to be dealt with separately (Table I). Also the radioactivities of the Sephadex G-200 fractions containing both TCI and TCIII were adsorbed quantitatively to the DE-81 filters (Table II).

The capacities of each filter-type to retain the respective transcobalamins from increasing amounts of sera is shown in Fig. 1. As seen, a quantitative and selective adsorption was obtained with up to 0.015 ml of serum; when higher amounts of serum were

used the retention was not as quantitative due to the exclusion of the B_{12} binders from the filters by the serum proteins.

Selective desorption of TCIII from the DE-81 filters. The DE-81 filters to which both TCI and TCIII adsorb are chemically similar to the granular DE-52, which is used for the separation of the two. However, since the retention of TCIII by DEAE-cellulose is weaker than that of TCI, conditions were explored for the selective desorption of TCIII from the DEAE filters. Several filter stacks to which the transcobalamins from sera of patients with CML and PV were adsorbed, were subjected to further washing with phosphate solutions of varying pH and molarity.

While TCII was strongly retained by the cellulose-nitrate filter both TCI and TCIII were desorbed to a different degree from the DE-81 filters. The data (Figs. 2 and 3)

TABLE I. UNSATURATED B_{12} BINDING CAPACITY (UBBC) OF VARIOUS SERA AS DETERMINED BY THE FILTER STACK AND THE CHARCOAL ADSORPTION METHOD.[a]

		UBBC (pg/ml serum) Filter stack			
Subject	Quso G32 treatment	Cellulose-nitrate (CN)	DE-81	Total (CN + DE-81)	Charcoal
H.I. (normal)	−	1750	110	1860	1800
	+	150	110	260	300
S.I. (normal)	−	715	1100	1815	1900
	+	85	900	985	900
C.G. (CML)	−	850	1800	2650	2800
	+	90	1400	1490	1490
R.P. (CML)	−	370	5500	5870	5700
	+	0	5600	5600	5650
E.R. (PV)	−	800	3500	4300	4500
	+	70	3560	3630	3540
G.I. (PV)	−	770	3900	4760	4800
	+	35	3800	3835	3750

[a] Sera (0.02 ml) were incubated for 30 min at 37° with ^{57}Co B_{12} (150 pg) and 0.1 *M* sodium borate buffer (pH 8.5) in a total volume of 0.4 ml. Half of the incubation mixture was tested for UBBC by removing unbound radioactivity with the coated charcoal (See Materials and Methods). The other half was assayed by the filter stack procedure as described. Additional samples of these sera were treated with Quso G32 prior to incubation and UBBC determination.

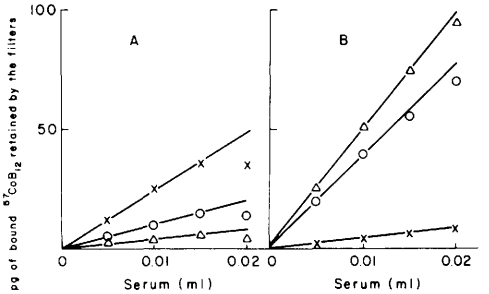

FIG. 1. Retention of transcobalamins by the filters of cellulose-nitrate and DE-81 as a function of serum concentration. Samples of sera outlined below (0.08 ml) were incubated each with 800 pg of ^{57}Co B_{12}. After adjusting the volume to 8 ml with 0.1 *M* sodium borate buffer (pH 8.5), aliquots from 0.25 to 2 ml were diluted with the borate buffer and passed through the filter stacks as outlined in Materials and Methods. (A) ^{57}Co B_{12} retained by the cellulose-nitrate filter; (B) ^{57}Co B_{12} retained by the DE-81 filters. The following sera were used: A.I. (normal), x; C.G. (CML), △; and E.R. (PV), ○,

TABLE II. ADSORPTION OF ^{57}Co B_{12}-TRANSCOBALAMIN COMPLEXES TO CELLULOSE-NITRATE AND DE-81 FILTERS.[a]

		Radioactivity (cpm)		
			Retained	
Subject	Transcobalamin types	Added	Cellulose-nitrate	DE-81
R.G. (Normal)	TCII	9000	8550	100
R.P. (CML)	TCII	8700	8600	0
W.B. (PV)	TCII	5600	5600	0
R.G. (Normal)	TCI + TCIII	2000	0	2000
R.P. (CML)	TCI (mainly)[b] + TCIII	9300	0	9200
W.B. (PV)	TCIII (mainly)[c] + TCI	5900	0	5900

[a] The sera (1 ml) were incubated with a subsaturating concentration of ^{57}Co B_{12} (300 pg/ml) and fractionated on columns of Sephadex G-200 by the procedure of Hom *et al.* (19). Pooled fractions from regions of high-(TCI and TCIII) and low-(TCII) molecular weight were dialyzed; the contents of bags were concentrated by aeration to about 2 ml, then redialyzed against distilled water. Aliquots of 0.1 or 0.2 ml, depending on the radioactivity of the fractions, were passed through filter stacks at pH 8.5, and the radioactivity of each filter type was determined.

[b] 90% and [c] 80% as determined by DE-52 column chromatography.

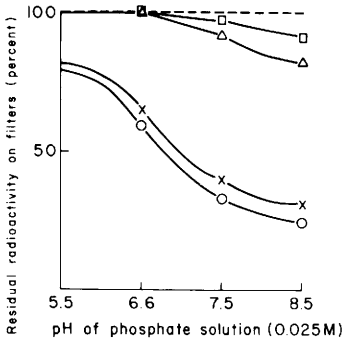

FIG. 2. Effect of pH of the phosphate solution on elution of transcobalamins from the filter stack. Samples of sera (0.1 ml) were incubated with 1000 pg of ^{57}Co B_{12} in presence of borate buffer. Final volume was 2.0 ml. Aliquots of 0.2 ml were diluted with the borate buffer (pH 8.5) and passed through filter stacks as described. One stack, which served as control, was washed further with 15 ml of the same buffer solution. The others were washed each with a 15-ml solution of a different pH value (varying from 5.5 to 8.5) of 0.025 *M* potassium phosphate. The residual radioactivity of each filter type was compared to that of the control (100% retention). The dashed line in the figure represents the residual radioactivity on the cellulose-nitrate filters of all sera tested. Solid lines represent residual radioactivities on the DE-81 filters of the following sera: W.B. (PV), ○; G.L. (PV), x; R.P. (CML), □; and C.D. (CML) △.

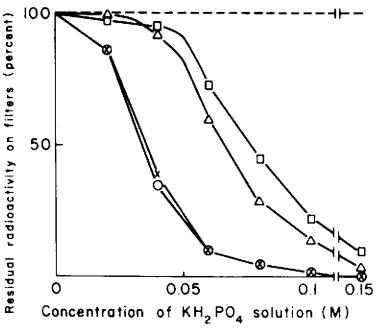

FIG. 3. Effect of phosphate (KH_2PO_4) concentration on elution of transcobalamins from the filters. The experimental procedure and symbols used were as described in the Legend to Fig. 2, except that increasing concentrations of KH_2PO_4 (pH 4.6) were used as eluant.

show that the retention of the binder which predominates in the PV sera (TCIII) was much weaker than that of TCI, the main B_{12} binder in CML. Employing a pH, at which the degree of ionization of the phosphates is minimal (i.e., KH_2PO_4, pH 4.6), the effect of increase in the concentration of the wash solution on the retention of the B_{12} binders was tested (Fig. 3). The data in this figure suggested that TCIII was selectively desorbed from the filters by a 0.05 *M* KH_2PO_4 solution; at this concentration most of the binder from the PV serum was removed, yet that of the CML serum was mostly retained. Confirmation of the above is shown in Table III. Sera from normal subjects and from patients with CML and PV were treated with Quso G32 and its TCI and TCIII components were determined after DE-52 column chromatography. The values obtained were either similar to or identical with those determined by the filter stack using 0.05 *M* KH_2PO_4 solution for desorption of TCIII.

The UBBC values and the relative distribution of the three B_{12} binders in normal, CML, and PV sera as determined by the filter stack are listed in Table IV. As seen, these sera are clearly distinguishable when assayed by the described filter stack procedure.

TABLE III. DISTRIBUTION OF TCI AND TCIII AFTER REMOVAL OF TCII BY QUSO G32.[a]

	Distribution of transcobalamins (percentage)			
	DE-52 column		Filter stack	
Subject	TCI	TCIII	Retained	Desorbed
B.R. (normal)	23	77	22	78
B.B. (normal)	46	54	42	58
Z.M. (normal)	43	57	44	56
R.P. (CML)	95	5	91	9
E.X. (CML)	91	9	90	10
C.D. (CML)	88	12	81	19
W.B. (PV)	19	81	22	78
J.J. (PV)	25	75	24	76
G.L. (PV)	21	79	26	74

[a] All sera (1 ml each) were treated with Quso G32; 0.5-ml aliquots were incubated with 150 pg of ^{57}Co B_{12} and the TCI and TCIII distribution was determined after DE-52 column chromatography. Duplicate samples of the same sera (0.01 ml each) were also incubated with ^{57}Co B_{12} (100 pg/0.01 ml), then passed through the filter stack, and washed with borate buffer. One stack from each duplicate was further washed with 15 ml of 0.05 *M* KH_2PO_4 (pH 4.6). There was no detectable radioactivity on the cellulose-nitrate filters. The percentage of transcobalamin retained by the DE-81 filters was calculated from the radioactivities obtained on these filters prior and after the elution with the acid phosphate solution.

TABLE IV. UBBC AND PERCENTAGE DISTRIBUTION OF THE THREE TRANSCOBALAMINS OF VARIOUS SERA AS DETERMINED BY THE FILTER STACK.[a]

Subject	UBBC (pg B_{12}/ml serum)	TCII	TCI (percentage)	TCIII
B.R. (normal)	2300	70	7	23
B.B. (normal)	1760	81	8	11
Z.M. (normal)	1900	75	11	14
H.Z. (CML)	4430	17	80	3
E.X. (CML)	7000	30	66	4
C.D. (CML)	6660	24	65	11
E.N. (PV)	8600	14	19	67
W.B. (PV)	7000	12	14	74
H.S. (PV)	6300	14	10	76

[a] The experimental procedures were as described in Table III except that treatment with Quso G32 was omitted.

Discussion. The fractionation of the three transcobalamins found in human serum by the described filter stack can be regarded as a combination of several procedures which were scaled down for use of small amounts of sera.

The adsorption of TCII to the cellulose-nitrate filter is due probably to nonelectrostatic attraction of the binder by the filter. The pH (8.5) of the filtration solution is too high for TCII (p*I*, 5.4–6.6) (27) to be attracted by electrostatic forces to the negatively charged filter. Consistent with the above was the failure of potassium phosphate solutions of varying pH to elute TCII from the filter (Figs. 2 and 3).

The treatment with solid adsorbing agents such as Quso G32 yielded similar results, although the mechanism of adsorption of TCII is probably not identical. In some cases, of which one is given in Table I (C.G.), the treatment with Quso G32 resulted in the adsorption of more transcobalamin than observed with the cellulose-nitrate filter. Further information, not brought here, indicates that the extra binding adsorbed to the Quso G32 was definitely not due to TCII and it represents rather the TCI-TCIII group.

Contrary to the strong adsorption of TCII to the cellulose-nitrate filter, that of TCI and TCIII to the DEAE-cellulose filters is due to electrostatic attraction. As in the case of DEAE-cellulose column chromatography specific desorption of TCIII is achieved because of the weaker retention of the latter (p*I*, 3.4–4.0) (6, 27) by the DEAE filters; thus, a solution of moderate ionic strength, such as 0.05 *M* KH_2PO_4 enables TCIII desorption without eluting TCI (p*I*, 2.9–3.1) (6, 27).

While TCII is a clearly defined and distinct protein entity (6), TCI and TCIII may be fractions of the same protein, varying in their isoelectric mobilities (27) and perhaps in additional properties. Stenman (27) suggests that the latter transcobalamins be named cobalophilins rather than R binders, as no definite transport function can as yet be ascribed to them and they do not necessarily show rapid electrophoretic mobility.

The filter stack method, in spite of its simplicity, gave similar results when compared with the more laborious procedures such as the combination of Quso G32 and DEAE-cellulose chromatography. DEAE chromatography alone was inferior in our hands as TCII was partly retained by the column and was eluted together with TCI. The method described is not only simple, but also rapid, and over 100 samples can be tested daily. The quantitative distribution of the B_{12} binders in sera from various pathological conditions is now being investigated on a larger scale. Also effects of different drugs currently used in the therapy of various myeloproliferative diseases on the distribution of the transcobalamins will be studied.

Summary. A simple and rapid fractionation procedure of the three transcobalamins, TCI, TCII, and TCIII, of human serum was achieved by filtration through a stack of charged cellulose filters composed of one cellulose-nitrate and three DEAE-cellulose (DE-81) disks.

A reaction mixture containing microliter amounts of serum was incubated with excess of ^{57}Co B_{12} of high specific activity, diluted with 0.1 *M* sodium borate buffer (pH 8.5), and passed through the filter stack by applying vacuum. Under these conditions TCII is selectively and quantitatively adsorbed to the cellulose-nitrate filter while both TCI and TCIII adsorb to the DE-81 filters. In the second step TCIII is selectively de-

sorbed from the latter filters by a 0.05 *M* monopotassium phosphate solution of pH 4.6.

Using sera of different distribution of transcobalamins the data obtained were comparable to those determined by the more laborious methods employing DE-52 column chromatography combined with procedures to remove TCII.

This work was supported in part by Grant 015.0187 from the Joint Research Fund of The Hebrew University and Hadassah, and by a grant from The Ministry of Health of the Government of Israel.

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Received January 19, 1976. P.S.E.B.M. 1976, Vol. 152.