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Research Article

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ENHANCEMENT BY LITHIUM AND ELIMINATION BY FLUORIDE OF IN VITRO INCREMENTS IN VITAMIN B₁₂-BINDING CAPACITY

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ABSTRACT Unsaturated vitamin B₁₂-binding capacity (UBBC) of human serum is not reproducibly measurable because it increases variably in vitro in relation to time, temperature, and, in the case of plasma, anticoagulant present before removal of cells. This variable increase proved to be due to variable release in vitro of transcobalamin III (TC III) from granulocytes. UBBC increase was greatest (up to fourfold normal levels) in the presence of lithium, which is the heparin salt used in many laboratories doing UBBC studies. In vitro increase was least when blood was collected in EDTA at 0°C and immediately centrifuged at 0°C (T₀ sample); results equivalent to T₀ were obtained at room temperature even after several hours delay when 47 mM fluoride was present; either cold temperature or 47 mM fluoride appeared to prevent TC III release from granulocytes. The measured levels of the three transcobalamins with T₀ methods of collection, which presumably reflect most closely the in vivo circulating levels, suggest that TC I and TC III in normal plasma are of the same order of magnitude and together normally comprise less than 10% of the UBBC.

Approximately 90% of the UBBC content of sonicates of peripheral blood granulocytes and of bone marrow aspirates of normal individuals appears to be TC III, with the rest being TC I. Thus, normal myelocytes, like

normal granulocytes, appear to contain mainly TC III. No TC II was present in any of the sonicates.

The general practice in most laboratories has been to determine serum UBBC. Because in vitro increments of up to 119% were found to occur in serum, this practice should be replaced by collection using methods that prevent such increments. Blood collected in EDTA-47 mM NaF had a stable, reproducible UBBC with no significant in vitro increment with time.

EDTA-NaF UBBC was 640 ± 168 (range 380–921 pg B₁₂ bound/ml plasma) for 12 normal adult men and 809 ± 232 (range 505–1208) for 10 normal adult women. It presumably approximates circulating UBBC and is substantially below the serum UBBC mean of 935 ± 262 (range 611–1506 for the same 12 men) and 1273 ± 355 (range 811–2306 for the same 10 women).

INTRODUCTION

It has become increasingly apparent that normal circulating human plasma may contain three rather than two major vitamin B₁₂ binders (1–6). Some studies (1, 4) had concluded that the binding of B₁₂ to a plasma component other than the two established binders, transcobalamin I (TC I)¹ and transcobalamin II (TC II) was due to secondary binding of B₁₂ to weak nonspecific binders and only at high concentrations of the vitamin. This view, together with the concept of the complementary roles of TC I as “storage” binder and TC II as “trans-

Preliminary reports of parts of this study have recently been published (36–38).

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¹ Abbreviations used in this paper: T, time between collection and centrifugation; TBBC, total vitamin B₁₂-binding capacity; TC, transcobalamin; UBBC, unsaturated B₁₂-binding capacity.

port" binder (7, 8), tended to exclude a third binder as of no importance. However, a third binder, designated TC III, proved capable not only of binding B_{12} at low concentrations of the vitamin (9) but also of binding B_{12} administered orally in man (10). Precluding consideration of this binder because of lack of an apparent "functional" role now also seems less valid since it appears that TC I may also lack a functional role (11), even though it carries endogenous B_{12} in the circulation.

The current study addresses itself to the following considerations pertaining to TC III: its derivation from granulocytes, factors relating to its release from granulocytes, and its relationship to TC I in normal subjects. Even though TC I and TC III have no currently known function, their interrelationship within the granulocyte and their levels in the circulation may prove of value in differential diagnosis of true from pseudomyeloproliferative disorders.

The present study reaffirms variation in the unsaturated B_{12} binding capacity (UBBC) noted previously by ourselves and others when different anticoagulants are used to collect samples (12-16) and suggests these differences are related to different degrees of release of TC III by granulocytes *in vitro*, which may be eliminated by collection of blood in sodium fluoride.

METHODS

Collection of blood samples. Blood samples were collected directly into the appropriate Vacutainer tube (Becton-Dickinson & Co., Rutherford, N. J.). The following tube types were used: "red tops" (3200) containing no anticoagulant for collection of serum; plasma samples were collected in Vacutainer tubes with a 10-ml draw, including "lavender tops" (3200XF40) containing 15 mg of EDTA (K_2)/tube; "green tops" (3208KA) containing sodium heparin, 134 USP U/tube; "grey tops" (3200XF42) containing 20 mg of sodium fluoride and 10 mg EDTA (Na_2)/tube; green tops (3200LH) containing lithium heparin 143 USP U/tube; "black tops" (3200NAX) containing 0.5 ml of 0.1 M sodium oxalate. When sodium citrate was used it was added to red-top Vacutainer tubes so as to yield a final (i.e., after blood was added) concentration of 0.4% of sodium citrate. When lithium was used other than in the lithium heparin Vacutainers mentioned above, it was added as 50 μ l/5 ml blood as a saline solution of lithium chloride of the appropriate concentration.

Subjects. Blood samples were taken from normal individuals between the ages of 25 and 45. All normal subjects had normal serum and red blood cell folate levels (17) and normal B_{12} levels (12) and had no known gastrointestinal or hematological disorders. None were receiving medication at the time of or within 2 wk before blood donation. In all instances, the nature of the study was made known to the subjects before the donation of blood, and informed consent was obtained. Most subjects were colleagues. In each instance a complete blood count and peripheral blood smear was routinely obtained.

Vitamin B_{12} . The solution of [^{57}Co]cyanocobalamin ([^{57}Co]B $_{12}$) for the column chromatography was prepared from the stock solution supplied by Amersham/Searle

Corp., Arlington Heights, Ill., at a sp act of 15.01 μ Ci/ μ g and made up to a concentration of 10 ng of B_{12} /ml in saline. For the UBBC estimation, a solution containing 500 pg/ml in isotonic saline was used.

UBBC. The UBBC was estimated as described earlier (18) with the following modification: serum (100 μ l) was added with an Eppendorf pipette (disposable plastic tip) (Brinkmann Instruments, Inc., Westbury, N. Y.) to disposable siliconized glass test tubes (Vacutainer 3200) followed by 1 ml of saline containing 500 pg of radioactive B_{12} . A control tube contained 100 μ l of saline instead of serum. All tubes were incubated at 37°C for 30 min to maximize binding (19), after which time 1 ml of hemoglobin-coated charcoal (19) was added and the tubes were centrifuged in a swing-out (not angle) head for 15 min at 1,000g. The supernate was decanted, and its radioactivity determined in a Picker Autowell Model II (Picker Corp., Cleveland, Ohio). The radioactivity was converted to picograms of B_{12} by comparison with a standard. Two pooled sera of known UBBC were routinely included in each UBBC assay for quality control. In our hands the assay showed a variation of $\pm 6.9\%$ in 20 separate estimations on one such pool and $\pm 10.6\%$ on 15 estimations on another pool. Part of this variation is decanting error. When more than 80% of the added B_{12} was bound, the estimation was repeated with 10 μ l of serum and 90 μ l of saline, instead of the original 100 μ l of serum. When this still bound more than 80% of the added B_{12} , the sample was diluted sufficiently to bind less than 80% of the quantity of added B_{12} . The latter dilutions are necessary because of the nature of the curve of binding of B_{12} to its binding proteins in serum or other binders (12, 18). Control experiments using plasma of known UBBC showed that NaF, EDTA, or lithium at concentrations up to 10 times those used in this study had no effect upon the UBBC assay *per se*. Similarly, various other metabolic inhibitors used in an attempt to suppress UBBC release had no artificial effect on the assay at the concentrations used.

Chromatography. Ion exchange chromatography was carried out on DEAE cellulose as described (2) and modified (5) previously. Since the UBBC varied over a wide range, the procedure adopted was to determine the UBBC first, as described above. Just before chromatography an amount of B_{12} that exceeded the binding capacity by 20% was added. After incubation at 37°C for 30 min, 1 ml of hemoglobin-coated charcoal was added, and the volume was adjusted to 3.0 ml with water. After centrifugation the supernate was decanted and applied to the column. Gel filtration studies of plasma samples and the pooled fractions eluted during DEAE cellulose chromatography were carried out on Sephadex G-200 as previously reported. (5).

Sonication studies. Marrow samples containing 5×10^6 cells/ml were sonicated in 5.0-ml portions in red-top Vacutainer 10-ml test tubes. Granulocytes were sonicated in a similar manner, but the preparation differed as follows: peripheral blood sonicates were prepared by centrifuging 5.0 ml of whole blood collected in EDTA at 0°C. The plasma was removed, the volume was readjusted to 10.0 ml with saline, and the content of the tube was gently mixed. The supernates were removed by centrifugation, and the volume was readjusted to 5 ml with saline. Alternatively, 0.02 M sodium phosphate buffer was used instead of saline. Both procedures gave essentially the same results. A white cell count was determined before sonication in each instance. The washing procedure removed contamination from the plasma transcobalamins, and in the case of the buffer, washing removed in addition the majority of the red cell

TABLE I
Percent Increment above NaF/EDTA T₀ Sample

	NaF/EDTA		Serum*		Citrate		EDTA		Na heparin		Na oxalate		Li heparin	
	T ₀	T ₂₄	T ₀	T ₂₄	T ₀	T ₂₄	T ₀	T ₂₄	T ₀	T ₂₄	T ₀	T ₂₄	T ₀	T ₂₄
No lithium added														
Mean	‡	10	24	95	4	49	19	49	35	146	22	173	51	243
SD	‡	7	15	70	6	16	23	14	17	61	32	102	26	88
SE	—	3	3	26	1	6	5	5	4	23	7	39	6	33
n	20	7	20	7	20	7	20	7	20	7	20	7	20	7
Lithium added, 50 meq/liter														
Mean	‡	8	26	150	15	102	20	341	35	158	5	270	34	213
SD	‡	7	16	79	7	60	25	141	12	70	10	129	21	100
SE	—	6	6	18	3	14	10	32	6	16	4	29	8	22
n	7	19	7	19	7	19	7	20	6	20	7	20	7	20

n, number of subjects.

* Whole blood with no anticoagulant added.

‡ The mean T₀ UBBC without lithium added was 731±197, and with lithium added was 708±204.

hemoglobin present. Sonication in all instances was carried out in ice in 30-s sequences for a total of 3 min. Between each sequence the tube was recooled to 0°C. At no point did temperature rise above 8°C during sonication. The instrument used was an Isonator Model 1000 (Savant Instruments, Inc., Hickville, N. Y.). The microtip probe was used for these studies with the power control set at 2.0, which gave a reference meter reading of 100. After sonication a repeat of the cell count confirmed that sonication disrupted in excess of 95% of the white cells.

Removal of TC II with uncoated charcoal. TC II was adsorbed out of plasma by a slight modification of a previously described method (3). 300 mg uncoated charcoal (Norit A)^a was added per 2 ml of plasma. The suspensions, after being agitated for a few seconds, were incubated at 37°C for 30 min in a Dubnoff shaking waterbath. Every 5 min the tubes were removed and mixed by inversion. After incubation the charcoal was removed by centrifugation at 3,400g at 4°C. If charcoal remained in the supernate it was precipitated by recentrifugation under identical conditions.

Preparation of platelets. Blood from a normal donor was collected in a plastic syringe without anticoagulant. Immediately, 9.0 ml was transferred to a siliconized tube that contained 1.0 ml of 4% trisodium citrate dihydrate. The tube was mixed gently by inversion and centrifuged at room temperature for 10 min at 160g. The top of the supernate (which was cloudy) was removed. The supernate was recentrifuged at 40g for 10 min, and the platelet-rich supernate, relatively free of white cells, was removed.

Cell counts were made of the platelet-rich supernate and the white cell-rich precipitate. The preparation showed 6,740 red cells/ml, 14 white cells/ml, and 14,300 platelets/ml.

Preparation of white cells. To 27 ml of blood collected in a plastic syringe without anticoagulant was added 3.0 ml of 4% trisodium citrate dihydrate in a siliconized tube. The tube was mixed gently by inversion, after which 15 ml of 4% Dextran 250 (Pharmacia Fine Chemicals, Inc., Pis-

cataway, N. J.) was added (20). This was allowed to stand for 1 h in ice. Most of the supernate was removed and centrifuged at 250g for 10 min. Three washings with saline/citrate (5 parts saline, 1 part 4% trisodium citrate dihydrate) were carried out by suspending the cells in 20 ml, centrifuging at 250g for 10 min, removing the supernates and repeating the process two more times. All of the above was carried out at 4°C. The original supernate and the three washes were kept on ice after preparation. UBBC estimation were carried out on the washings to ensure that no binder was lost during this process. Cell counts for red cells, white cells, and platelets were carried out on all preparations. Blood smears were also obtained from each preparation. The preparation showed (per ml): 9,580 red cells, 2,900 white cells, and 5,000 platelets.

Preparation of blood cells without granulocytes. In a plastic syringe 27 ml of blood was obtained from a severely neutropenic subjects. To this was added 3.0 ml of 4% trisodium citrate dihydrate in a siliconized tube. After being gently mixed the tube was allowed to stand at 4°C for 1 h. A preparation of cells was taken from the bottom of the tube and contained (per ml) 3,700,000 red cells, 740 white cells, and 286,000 platelets.

Each of the above cell preparations were divided in three: one third was sonicated for 3 min at 0°C; the second third was used to obtain a T₀ sample by centrifuging off the cells at 4°C immediately after preparation; the last third was used to obtain a T₂₄ plus lithium sample by incubating at room temperature for 24 h in a final concentration of 50 meq/liter lithium chloride before removal of the cells.

RESULTS

Effect of type of anticoagulant on UBBC. Blood samples collected from normal individuals under various conditions and in different routinely used anticoagulants showed widely varying UBBC. The results in representative experiments are noted in Table I. This increase in UBBC was greatest either when lithium was present

^a Pharmaceutical grade, neutral. Eimer and Amend, New York.

in the anticoagulant (lithium heparin) or was added to the anticoagulant. The lowest values were obtained when NaF was present.

The UBBC increased in vitro with time before removal of the cells over a 24-h period in all samples except those collected either in a high concentration of sodium fluoride or in those EDTA samples maintained at 0°C from the time of collection to the removal of the cells (Table II). These increases were due almost exclusively to an increase in TC III (Table II).

The increase when lithium was present appeared to be due to the lithium ion itself, as manifested by the fact that it could be effected by lithium in the presence of various anticoagulants or when no anticoagulant was present, i.e. lithium added to blood (Table I). Sodium fluoride appeared to stop the action of lithium on UBBC release effectively, even over a 24-h period, although in 24 h some release did occur (Tables I and II). Neither lithium nor sodium fluoride nor EDTA had any effect on the UBBC assay when added to control plasma or sera, even at concentrations ten times those ordinarily used in these experiments.

Comparison of UBBC values of serum as compared to plasma obtained by collection in NaF/EDTA. Since serum is the usual sample for UBBC estimation, a comparison of UBBC values of serum versus plasma collected at the same time in NaF/EDTA was made (Table III). Serum values always proved higher than those of NaF/EDTA plasma, and the serum values were unpredictable in extent above the NaF/EDTA plasma UBBC baseline (Table III).

Establishment of a new normal range for UBBC. Since previous methods of collection took no precautions to stop the release of TC III after the collection of the sample, since it was not recognized such release was substantial, all prior reported UBBC values, whether in normals or in various clinical disorders, are probably higher than circulating values. The current study therefore strove to establish a new normal mean and range, using 12 normal adult men and 10 normal adult women. The results are presented in Table III and are considerably lower than in prior reports, being 640 ± 168 pg/ml for the men and 809 ± 232 pg/ml for the women. This new "true" or "base-line" normal range presumably is a close approximation of the circulating UBBC rather than being above it, as is probably the case for all prior reports.

Determination of the concentration of lithium for maximum UBBC release from granulocytes. Lithium concentrations up to approximately 90 meq/liter of blood increased the quantity of UBBC released over a 24-h period (Fig. 1). Higher lithium concentrations were inhibitory. The exact relationship between maximal release of UBBC and lithium concentration varied

TABLE II
Effect of Fluoride and Lithium*

	Time	UBBC			
		TC I	TC II	TC III	Total
		pg/ml			
EDTA (1.0 mg/ml)	T ₀	53	1,124	64	1,241
+ NaF (2.0 mg/ml)	T ₂₄	55	1,237	73	1,365
EDTA (1.5 mg/ml)	T ₀	59	1,262	85	1,406
+ LiCl (2.14 mg/ml)	T ₂₄	94	1,309	2,888	4,291
EDTA (1.5 mg/ml)	T ₀	38	915	39	992
+ NaF (2.0 mg/ml)	T ₂₄	44	1,155	348	1,547
+ LiCl (2.14 mg/ml)					
EDTA (1.5 mg/ml), 0°C	T ₀	61	1,308	93	1,462
EDTA (1.5 mg/ml),	T ₀	62	1,274	248	1,584
room temperature	T ₂₄	52	1,233	424	1,709

* Concentrations of reagents are stated per milliliter of blood collected.

from subject to subject, but maximal release always occurred with less than 75 meq lithium/liter of blood.

In subsequent studies on lithium-stimulated release of UBBC, 50 meq lithium/liter was chosen as a suitable working concentration, since although it was below the peak area, it always effected substantial release of UBBC. In these and subsequent studies where lithium was added, EDTA was always present as an anticoagulant (15 mg/10 ml of blood). It should be noted that the high (47 mM) concentration of fluoride variably reduced the ability to measure endogenous B₁₂ by radioassay, although it had no such effect on measuring UBBC. Therefore, endogenous B₁₂ levels should be measured in specimens not exposed to high fluoride concentrations.

Time course of UBBC release in the presence and absence of lithium. UBBC release both in the presence and absence of lithium follows a sigmoidal shape curve. The action of lithium was to accelerate the process greatly (Fig. 2).

Studies on the nature of the binder released by whole blood. The type of binder released by granulocytes from whole blood, both in the presence and absence of lithium stimulation, was determined by ion exchange chromatography (Fig. 3) and gel filtration on Sephadex G-200 (Fig. 4). This was done by determining the elution profile in the starting plasma samples (T₀) and in the samples that had been allowed to release their UBBC for 24 h (T₂₄). The release both in the absence (Fig. 3a) and presence (Fig. 3b) of lithium was investigated. The UBBC increment brought about by incubating the cells with plasma either in the presence or absence of lithium was due almost exclusively to release of TC III. The other two transcobalamins, TC I and TC II, remained virtually unaltered throughout this time. Thus it appears that while collection of plasma in NaF/EDTA or at 0°C in EDTA results in very low

TABLE III
Variation of Serum UBBC above Plasma (NaF/EDTA) UBBC

Diagnosis	Sex	Number of subjects	UBBC		Serum above NaF/EDTA
			Serum	NaF/EDTA	
			<i>pg/ml</i>		<i>%</i>
Normal	Female	10	1,273±355 (811-2,036)	809±232 (505-1,208)	4-67
Normal	Male	12	935±262 (611-1,506)	640±168 (380-921)	23-119
Normal	Male and female	22	1,088±346 (611-2,036)	717±212 (380-1,208)	4-119
Cirrhosis (N. M.)	Male		1,242	971	28
Cirrhosis (A. P.)	Male		1,071	911	18
Cirrhosis (E. W.)	Male		1,036	949	9
Cirrhosis (S. G.)	Male		1,931	1,185	63
Pernicious anemia treated (W. K.)	Male		1,744	1,315	35
treated (M. B.)	Male		1,322	699	89
treated (L. F.)	Male		1,571	1,358	12
Chronic lymphatic leukemia (I. K.)	Male		1,911	1,856	3
leukemia (F. L.)	Male		590	534	11
Lung cancer (L. S.)	Male		1,920	1,846	4
Lung cancer (J. F.)	Male		2,010	1,580	27
Alcoholic (W. N.)	Male		1,057	827	28
Partial gastrectomy (J. A.)	Male		1,029	813	27
Diabetic (J. F.)	Male		1,150	785	32
Leukemoid reaction (A. D.)	Male		6,507	4,840	34
Sideroblastic anemia	Male		840	773	10
Female NaF/EDTA and male NaF/EDTA			significant $P < 0.1$		
Female serum and male serum			significant $P < 0.01$		
Female serum and female NaF/EDTA			significant $P < 0.005$		
Male serum and male NaF/EDTA			significant $P < 0.005$		
Combined male and female sera and combined male and female NaF/EDTA			significant $P < 0.001$		

values for TC I and TC III (presumably approximating the levels existing in the circulation), cells release TC III on standing at room temperature, and this release is greatly stimulated by the presence of lithium.

Sonication studies on granulocytes derived from peripheral blood. By ion exchange chromatography on DEAE cellulose and gel filtration on Sephadex G-200, normal granulocytes contained both TC I and TC III, with TC III predominating (Table IV).

Sonicates of marrow cells. Sonicates of normal human bone marrow cells were prepared, and their TC I: TC III ratio was determined by ion exchange chromatography and gel filtration on Sephadex 200. Like nor-

mal granulocytes from peripheral blood (Table V), these cells also contain a much higher content of TC III than TC I (Table V).

Effect of metabolic inhibitors is delineated in Table VI. It was found that 47 mM NaF (the amount in the commercial Vacutainer tube) completely inhibited UBBC release, even in the presence of lithium. NaF at 1 mM had less effect, permitting a substantial amount of UBBC release on lithium stimulation. Sodium arsenate at a comparable concentration was equally effective. Other inhibitors such as KCN, sodium azide, 2,4 dinitrophenol, 2 deoxyglucose, and methotrexate were ineffective in preventing lithium-stimulated UBBC release

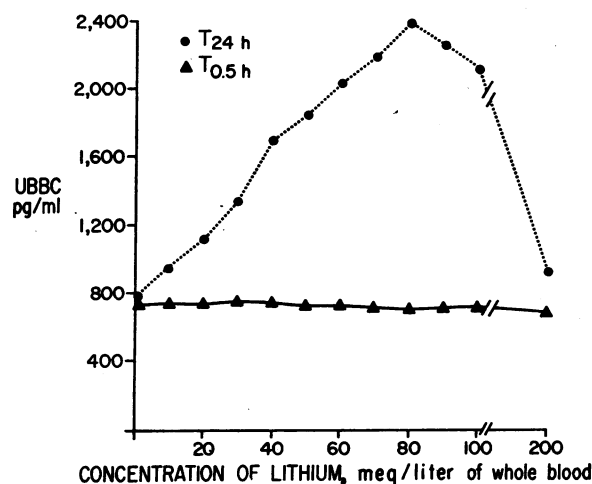


FIGURE 1 Effect of varying the lithium concentration on the release of UBBC from whole blood; cells removed after $\frac{1}{2}$ h \blacktriangle ; cells removed after 24 h \bullet . All samples were incubated at 22°C before the removal of the cells.

at a concentration of 1 mM. None of the above inhibitors had any effect on the UBBC assay when added just before assay.

Origin primarily in granulocytes of the TC III secreted in vitro. Following the protocol outlined in the Methods section, preparations of white cells, platelets, and peripheral blood cells almost devoid of granulocytes were obtained. UBBC estimations were carried out on the lithium-stimulated TC III increments of these preparations (Table VII). Only in the case of the white cell preparation was there any increase in UBBC upon incubation for 24 h with lithium. The T_0 values in each instance represent the UBBC present at the start of the incubation, i.e., the original plasma.

Demonstration that the in vitro increment is almost

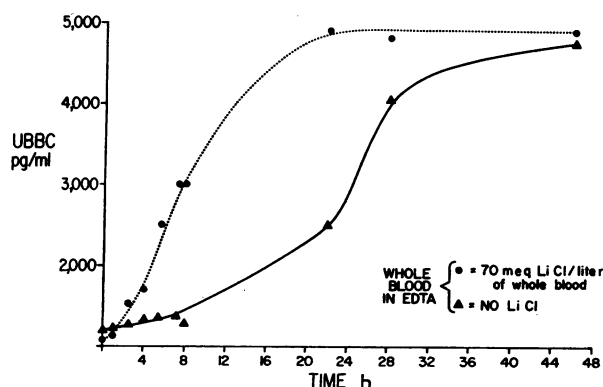


FIGURE 2 Effect of time on the release of UBBC from whole blood; \blacktriangle , no additive; \bullet , 70 meq LiCl/liter of whole blood. All samples were incubated at 22°C before the removal of the cells.

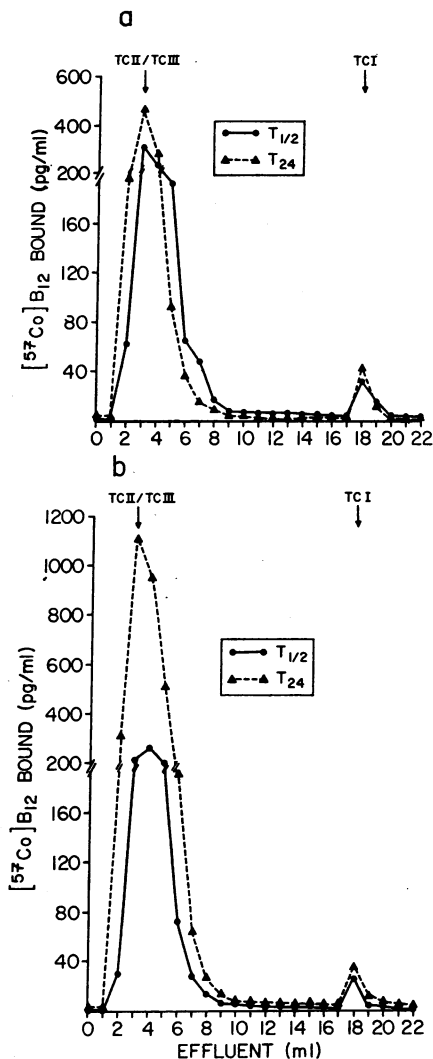


FIGURE 3 DEAE cellulose fractionation of: (a) Whole blood collected in EDTA. (b) Whole blood collected in EDTA containing lithium chloride to give a final concentration of 50 meq/liter. \bullet , cells removed at zero time; \blacktriangle , cells removed after incubation at 22°C for 24 h.

exclusively TC III. When TC II is removed by uncoated charcoal and TC I and III are then separated by DEAE-cellulose, the TC II values are subject to a 10–15% error, and it may appear that some TC II increment has occurred (Table II). This error is avoided by using both DEAE cellulose and Sephadex G-200 chromatography; by such methodology there is no increment in TC II, only a very slight increment in TC I, and the in vitro increment is almost exclusively TC III (Fig. 5).

Table VIII demonstrates that normal total B_{12} binding capacity (TBBC) is approximately 40% saturated with B_{12} when true UBBC is determined (i.e. with samples collected in fluoride) but only about 30% sat-

rated when this determination is made from blood collected without shutting off in vitro increments in UBBC.

DISCUSSION

Previous studies have shown differences in UBBC depending upon the particular anticoagulant used (12-

16). These differences were more or less clearly due to interaction between a particular anticoagulant, B_{12} , and plasma proteins resulting in an artifactual increase in apparent B_{12} binding. The present studies show that increased UBBC is due primarily to release of B_{12} -binding protein in vitro into the serum or plasma by the

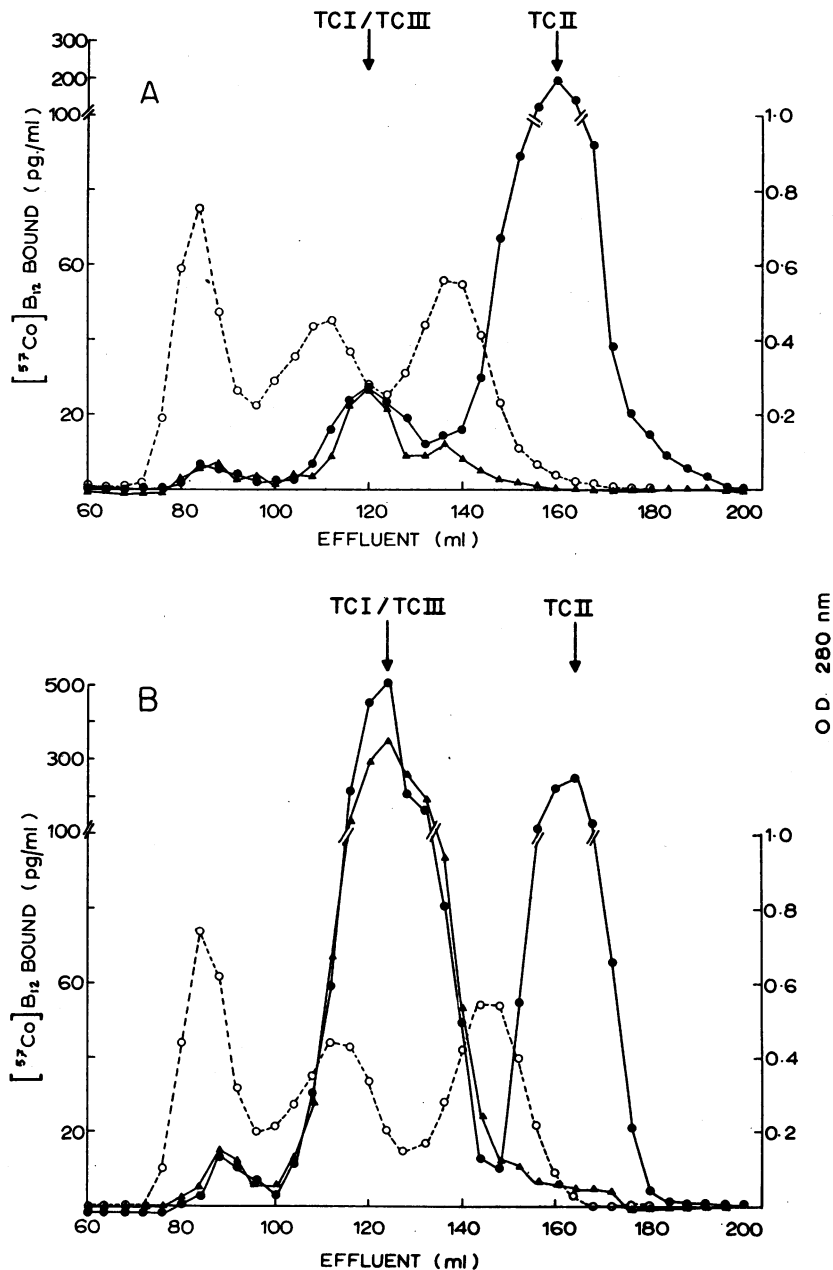


FIGURE 4 Sephadex G-200 fractionation of pooled plasma which had been incubated in 25 meq LiCl/liter of whole blood at room temperature for (a) $\frac{1}{2}$ h; (b) 24 h. The broken line (○) is the protein concentration. The continuous line represents the amount of [⁵⁷Co]B₁₂ bound. ●, untreated; ▲, where TC II was removed by treatment with uncoated charcoal before fractionation.

cellular components of normal whole blood (Table I, Fig. 2), just as similar studies had shown a similar release when isolated human chronic granulocytic leukemia granulocytes were incubated in vitro in the absence of serum or plasma (2). This binder, released in vitro, proved in the present study to be almost exclusively TC III (Figs. 3 and 4). Other workers (21) have claimed that normal leukocytes contain only TC I and that upon incubation in vitro they release only TC I. Our studies show that not only is it TC III and not TC I that is released by white cells (Table II) but that those cells contain primarily TC III and only small amounts of TC I. The amount of binder released proved to vary from one to another normal subject whether the blood was collected in sodium heparin, sodium EDTA, or sodium citrate as anticoagulant (Table I). Since the current studies also show that the lithium ion causes a very rapid release of TC III from the cells (Fig. 2, Table I), it is not surprising that the use of lithium heparin as anticoagulant results in the highest reported "normal" UBBC levels.

Unpredictable percentage increment in UBBC level was observed when blood was collected without fluoride (Tables I and III). This appears in serum to be partly dependent upon how effectively granulocytes are held within the clot and the time taken for the clot to form. This is an important consideration, since "serum UBBC" is currently the standard UBBC in many laboratories.

The current study aimed in part to develop a method for obtaining a uniform normal UBBC, because of wide variance reported from laboratory to laboratory. In our initial investigations we found that collection of the blood sample in EDTA into a tube at 0°C with rapid equilibration of the blood to that temperature and rapid centrifugation at 4°C gave the lowest UBBC. We subsequently found that sodium fluoride (with EDTA as anticoagulant) was an effective inhibitor of the release of TC III from granulocytes and provided even lower and more consistent results than collection in the cold, possibly because blood comes out of the vein at 37°C and takes time to cool even when immediately placed on ice (Table

TABLE IV
DEAE-Cellulose Fractionation of Sonicated Normal Human Granulocytes

Source	Vitamin B ₁₂	
	TC III	TC I
	pg/ml bound	
V. H.	795 (86%)	131 (14%)
R. S.	1,510 (86%)	242 (14%)
L. C.	1,541 (97%)	47 (3%)
L. F.	502 (97%)	18 (3%)

TABLE V
DEAE-Cellulose Fractionation of Sonicated Normal Human Bone Marrow Cells

Source	Vitamin B ₁₂ bound	
	TC III	TC I
	pg/ml	
J. P.	1,848 (97%)	47 (3%)
T. L.	1,473 (95%)	70 (5%)
A. P.	1,449 (93.5%)	94 (6.5%)
A. M.	1,568 (97%)	40 (5%)
J. F.	1,104 (89%)	140 (11%)

II). The action of fluoride was to inhibit effectively the release of TC III. It did not produce an artifact in the UBBC assay, since even higher concentrations of fluoride than those used in collection have no inhibitory effect on the UBBC assay. Sodium fluoride at lower concentrations (1 mM instead of 47 mM) was only partly effective (Table VI). Sodium arsenate at 1 mM was also partly effective in preventing UBBC release.

With EDTA-NaF for blood collection, 12 normal adult men showed a UBBC range of 380-921 pg/ml (mean: 640), and 10 normal adult women had a UBBC range of 505-1,208 pg/ml (mean: 809). A higher normal range was found for serum (611-2,036), similar to the ranges of the order of approximately 600-2,200 pg/ml reported previously (4, 22-26) presumably due to the release of TC III from the cells in vitro after collection in prior studies. The current studies suggest that for uniformity, reproducibility, and similarity to the levels in vivo, samples should be collected in EDTA-47 mM NaF when UBBC levels are desired. That the granulocytes were the source of the UBBC released in vitro was shown by the studies with different formed elements. It was clear

TABLE VI
The Effect of Various Metabolic Inhibitors on the Release of UBBC from Whole Blood in the Presence and Absence of Lithium Stimulation (50 meq/liter of blood)

Inhibitor	Concentration	T ₀		T ₂₄	
		-Li	+Li	-Li	+Li
	mM				
Control (no inhibitor)	—	1,005	991	1,184	1,998
Sodium fluoride	47	924	877	925	944
Sodium fluoride	1	1,015	932	1,203	1,543
2-4 Dinitrophenol	1	1,039	969	1,139	1,990
Sodium azide	1	998	900	1,122	2,089
KCN	1	940	876	1,129	1,906
2-Deoxyglucose	1	1,068	932	1,153	1,987
Sodium arsenate	1	980	938	1,137	1,419

All samples collected as 10 ml blood in 15 mg tripotassium EDTA, plus inhibitor.

TABLE VII
Cell Count and UBBC Data for Various Preparations

Preparation	Cell count				Vitamin B ₁₂ bound		
	RBC	WBC	Granulocytes	Platelets	Sonicates	T ₀	T ₂₄ lithium
			ml ⁻¹			pg/ml	
"Platelets"	6,740	14	11	14,300	178	182	196
Blood cells*	3,700,000	730	102	286,000	975	1,273	1,234
"White cells"	9,580	2,900	1,850	5,000	1,230	24	608

* From patient with gold-therapy-associated neutropenia.

that platelets, red cells, and white cells without granulocytes had no substantial capacity to release TC III even upon stimulation with lithium, but such release always occurred when granulocytes were present in substantial numbers (Table VII).

The inhibition of TC III release by fluoride, apart from representing a convenient way to obtain a UBBC that most closely approaches the circulating UBBC, is of interest in and of itself. It was previously reported that the majority of granulocyte B₁₂ binder is localized in the granules (27). It has also been found that the release of granulocyte lysosome enzymes is inhibited by fluoride (28), via inhibition of anaerobic glycolysis. Other inhibitors, including dinitrophenol, sodium azide, potassium cyanide, methotrexate, and 2-deoxyglucose, had not effect at similar concentrations. A similar pattern of inhibition was observed on release of TC III from white cells (Table VI). Interestingly, in vitro studies in human renal medulla have been reported to

show that lithium inhibits the enzyme adenylate cyclase, while fluoride stimulates it (29).

The current studies emphasize the relationship between release of B₁₂-binder from blood cells in vitro (15, 16, 27), nature of anticoagulant used in blood collection (12-16), and UBBC level. Like Gullberg (16), we found that there is less of a UBBC increment in vitro in EDTA plasma than in serum or heparinized plasma (Table I), but the current study goes on to demonstrate that the in vitro increment in UBBC in EDTA plasma is unacceptably high as compared to NaF/EDTA plasma or even citrate plasma (Table I).

The finding in the current study that TC III dominates in sonicates of normal granulocytes supports a similar earlier finding that "β-globulin" B₁₂ binder dominates in such sonicates, as measured by DEAE-cellulose chromatography (6). In such chromatography, β-globulin B₁₂ binder is TC II plus TC III, and there is no TC II in granulocytes.

The previous studies in which UBBC release by granulocytes was investigated (27) used gel filtration to identify the product released. That product was the single filtrate peak that would contain both TC I and TC III, since these two TCs are not separated by gel fractionation. In the present study, extracts of granulocytes were prepared by sonication and fractionated not only by gel filtration but also by ion exchange chromatography, which does separate TC I and TC III.

The existence of two granulocyte-derived binders may be of importance. For example, in a recent study (30) that purified the granulocyte binder, it was unclear whether this was TC I, TC III, or a mixture of the two. The finding that most of the granulocyte binder is TC III suggests that the binder purified by Allen and Majerus (30) is TC III rather than TC I, or is mainly TC III. In this context the observation that granulocytes release almost exclusively TC III in vitro has been used by us to prepare preparations of TC III free of the other two binders. This can be done conveniently by washing whole blood twice with saline and resuspending it in saline containing the appropriate concentration

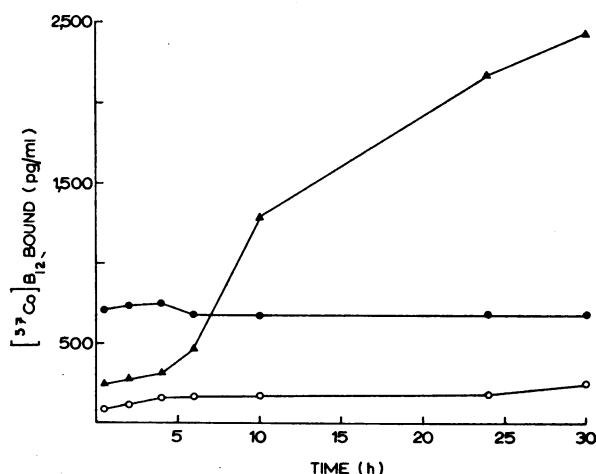


FIGURE 5 Demonstration that the in vitro increment in B₁₂-binding capacity in normal plasma is almost all TC III. DEAE-cellulose and Sephadex G-200 fractionation of plasma after incubation of lithium (25 meq/liter)—heparin whole blood for periods stated on abscissa. O, TC I; ●, TC II; △, TC III.

TABLE VIII
Normal Saturation of TBBC

Sex	Number of subjects	Serum B ₁₂	UBBC		TBBC	
			Serum	NaF/EDTA	Serum	NaF/EDTA
		<i>pg/ml</i>		<i>pg/ml</i>		<i>pg/ml</i>
Female	10	459±127 (340-692)	1,273±355 (811-2,036)	809±232 (505-1,208)	1,752±330 (1,416-2,465)	1,272±224 (993-1,737)
Male	12	460±120 (266-591)	935±262 (611-1,506)	640±168 (380-921)	1,402±279 (877-1,835)	1,103±209 (949-1,423)
Male and female	22	460±120 (266-692)	1,088±346 (611-2,036)	717±212 (380-1,208)	1,576±347 (877-2,465)	1,187±227 (949-1,737)

Results of *t* test:

male serum B ₁₂ and female serum B ₁₂	NS
male TBBC/NaF and female TBBC/NaF	NS
male TBBC/NaF and male TBBC/serum	<i>P</i> < 0.02
female TBBC/NaF and female TBBC/serum	<i>P</i> < 0.005
male TBBC serum and female TBBC/serum	<i>P</i> < 0.05
male plus female TBBC/NaF and male plus female TBBC/serum	<i>P</i> < 0.001

of lithium for 24 h at room temperature. After removal of the cells by centrifugation a preparation of TC III almost free of the other binders is obtained.

In normal subjects over 80% of the UBBC in granulocytes appears to be TC III (Table IV) and it was found by Sephadex column chromatography that no TC II was present. The relatively small amount of TC I in the granulocytes is consistent with the finding that little of it is released on in vitro incubation of peripheral blood in the presence of lithium. However, a significant amount of TC I is found in the normal circulation, equal to or greater than the amount of TC III found there. Possible explanations for this include either a greater half-life for circulating TC I or a greater production and release of TC I than of TC III in early granulocyte cell life. To examine the latter possibility we fractionated sonicates of washed marrow cells, since these represent a younger cell population than that of the peripheral blood buffy coat. Such sonicates showed essentially the same greater proportion of TC III to TC I as did the older cells (Table V), and again upon gel filtration chromatography were found to have no TC II. This finding, by making less likely the explanation of a progressively greater release of TC III at the expense of TC I as myeloid cells mature, makes more likely the possibility that the in vivo half-life of TC I is relatively long compared to that of TC III. Interpreting earlier studies (27, 31, 32) in the light of the current study, it appears probable that the amount of TC III released by granulocytes is proportional to the mass of UBBC-containing granulocyte granules, wherever they may be located (i.e., peripheral blood, bone marrow, etc.), as is the amount of TC I.

Much of the past confusion about serum B₁₂ binders is clarified by recognition of the fact that, by methodology such as used in the current paper, the buffer eluate from DEAE columns contains TC II and TC III, with the NaCl eluate containing TC I; conversely, the "large molecular weight region eluate" from Sephadex contains TC I and TC III, with the "low molecular weight region eluate" containing TC II. Thus, the three binders are clearly distinguished by using both separatory procedures. This does not preclude the possibility that the TC I and TC III, which are granulocyte-granule derived, may be only slight variants of each other physicochemically. While the granulocyte may not be the sole source of TC I and TC III, the fact that both occur in the granulocyte explains the correlation (33) of B₁₂-binding "α-globulin" (which is TC I) with blood granulocyte pool. It also explains the similar correlation to a lesser degree (33) with B₁₂-binding "β-globulin," because "beta" contains both TC III and TC II, and TC II is not found in granulocytes, but rather appears to be liver-derived (7).

The high UBBC previously reported (34) in patients taking lithium therapy may be partly due to in vivo release of UBBC from granulocytes by lithium.

Because of the wide variability of UBBC results in prior studies, it was not possible in the past to establish normal saturation of circulating TBBC, other than to estimate that normally TBBC was approximately one-third to one-fifth saturated (7). The current study, by establishing a new normal range of UBBC, makes it possible to establish more firmly the normal saturation and range of TBBC. The current study also demonstrates that UBBC is higher in adult women than in men, presumably under a similar control to that which produces

UBBC rise in pregnancy (7). The possibility of an association between TC III and glyophorin arose because that major glycoprotein of the human erythrocyte membrane is extracted by a lithium salt (35), and lithium stimulates TC III release from granulocytes. Drs. I. Kahane and V. T. Marchesi supplied us with 5 mg of freeze-dried human red blood cell (type A+) glyophorin; it proved to have negligible (5 pg/ml) B₁₂-binding capacity.

Note added after acceptance of manuscript. In this study, we noted that grey-top Vacutainer tubes (no. 3200-XF42) containing 20 mg of sodium fluoride and 10 mg EDTA (Na₂) per tube, while ideal for collecting blood samples to get true base-line levels of UBBC, could not be used for measurement of serum vitamin B₁₂ level, because, for unknown reasons, the fluoride caused B₁₂ level to appear low as measured by radioassay. In unpublished studies, we have studied this problem, and now the same sample of plasma collected in fluoride may be of use for both measurement of vitamin B₁₂-binding capacity and of plasma vitamin B₁₂ level. The cause of the problem proved to be that fluoride minimizes the partial destruction of intrinsic factor concentrate that occurs in the presence of the acid used in radioassay for vitamin B₁₂, and thus samples containing fluoride should be measured against an intrinsic factor concentrate standard that also contains fluoride, rather than against an intrinsic factor concentrate without added fluoride. Alternatively, instead of adding fluoride to the intrinsic factor concentrate, the acid can be neutralized after the heating step and before adding the intrinsic factor concentrate.

Ascorbate ingestion may also produce artifactually low serum B₁₂ levels (39) by minimizing the partial destruction of intrinsic factor concentrate which occurs in the presence of acid. Also building on the current studies, new studies have demonstrated that TC I is released by normal granulocyte precursors (40), and that Quso G32 (a microfine precipitated silica of Philadelphia Quartz Co., Philadelphia, Pa.) has a much more selective affinity than uncoated charcoal for TC II (41). In earlier work with E. Jacob, we had observed that peripheral blood cells from patients with myeloproliferative disorders released into their incubation medium a considerably greater amount of TC I in relation to TC III than did peripheral blood cells from normal persons.

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