Separation of Vitamin B₁₂-Binding Proteins of Serum, Gastric Juice and Saliva by Rapid DEAE Cellulose Chromatography

By François P. Retief, Chester W. Gottlieb, Shaul Kochwa, Peter W. Pratt and Victor Herbert

Normal serum contains at least two binders of vitamin B₁₂, one migrating electrophoretically as an alpha-globulin and the other as a beta-globulin. There is evidence that these substances have different metabolic functions. Whereas native B₁₂ is bound to alpha-globulin, the beta-globulin binder probably acts as a temporary B₁₂ carrier. Certain disease states, and chronic myeloid leukemia (CML) in particular, are characterised by a striking increase of alpha-globulin binder. This may be of some diagnostic value in differentiating between the myeloproliferative syndromes. Separation of the B₁₂-binders thus has clinical implications in addition to its obvious value in the study of B₁₂ kinetics.

We wish to describe a separation technic based on DEAE cellulose column chromatography which can be completed in approximately 2 hours. This “baby column” method is a modification of the procedure reported for isoagglutinin separation by Kochwa et al. Briefly, it consists in (1) adding an excess of Co²⁺B₁₂ to 1 ml. of serum to saturate B₁₂ binders; (2) removing free Co²⁺B₁₂ with hemoglobin-coated charcoal; (3) adding 2 ml. distilled water to the 1 ml. serum; (4) passing the 3 ml. sample through a 5 ml. pipette packed with cellulose, collecting 6 samples (of 2 ml. each) using a buffer eluant and 5 more using 1 M NaCl as eluant. The 0.06 M, pH 6.3, phosphate buffer elutes gamma globulin, β₂λ, β₁, B₁₂-binding β, and...
half the albumin; 1 M NaCl then elutes the remaining albumin, plus alpha globulin and macroglobulin. Fractions 5 through 7 have most of the albumin, 4 has most of the beta, and 8 has most of the alpha.

**Material and Methods**

**Specimens**

Serum from normal persons and patients with CML and pernicious anemia (PA) was frozen immediately after collection and stored at −20 C. Heparinized plasma gave separation patterns essentially similar to serum. Gastric juice (GJ) from PA and non-PA subjects was collected by incubation after maximal histamine stimulation,12 depepsinized and stored at −20 C at pH 7.0, as previously described.13 Saliva from normal persons was stored at −20 C. Serum from PA patients with high titers of autoantibody to intrinsic factor (IF antibody) was supersaturated with nonradioactive B12, incubated at 37 C for 15 min, and treated with a 50 mg. pellet of hemoglobin-coated charcoal11 to remove unbound B12. A 50 mg. pellet, produced by centrifugation of 2 ml. 2.5 per cent suspension of hemoglobin-coated charcoal adsorbs at least 1 mg. free B12. The B12-saturated serum stored at −20 C. was used as such, as a source of IF antibody.

**Preparation of Serum, GJ and Saliva for Chromatography**

1. One ml. serum was saturated with radioactive B12 (Co57B12 or Co58B12, with specific activities of 20–40 μc./μg. and 35 μc./μg., respectively) in amounts in excess of the unsaturated B12-binding capacity (UBBC) as determined by the method of Gottlieb et al.14 A working solution of 10 ng. radio-B12/ml. saline was added to serum as needed to slightly exceed UBBC, as follows (UBBC in ng./ml.): when UBBC < 2.5, 0.25 ml. (2.5 ng.) was added; when UBBC was 2.5 to 4.9, 0.5 ml. (5 ng.) was added; when UBBC 5.0 to 7.4, 0.75 ml. (7.5 ng.) was added. With UBBC of 7.5 to 25 ng./ml. 1.25 ml. radio-B12 (12.5 ng.) was added to 0.5 ml. serum; rare sera with UBBC < 25 ng./ml. were saturated with radio-B12 solutions of higher concentrations. The total volume of the radio-B12 saturated serum sample thus varied between 1.25 and 1.75 ml.; variations of this order did not affect elution patterns significantly. Free radio-B12 was removed from these samples by mixing with a 50 mg. hemoglobin-coated charcoal pellet, following incubation at 37 C. for 30 min. Charcoal was removed by centrifugation and supernatant radioactivity counted in a well-type scintillation detector. The supernatant was dialyzed overnight (or for a minimum of 4 hours) in Visking casing* against 0.02 M phosphate buffer, pH 6.3, at 4 C. The dialysis bags were emptied and the radioactivity of their contents determined before application to the column. No measurable activity normally diffused into the buffer because free B12 had been previously removed by coated charcoal. A variable amount (11.3–14.4 per cent) was lost from the dialysis bag (Table 1). In subsequent studies, we found that almost as good separations could be achieved by adding 2 ml. distilled water to the 1 ml. serum and applying the 3 ml. of mixture directly to the column, instead of dialyzing overnight against buffer prior to applying serum to the column (Fig. 1); in this modification it is necessary to collect 11 rather than 10 aliquots of 2 ml. eluate. changing eluant after collection of tube 6 rather than after tube 5.

2. Because of the lesser UBBC of serum,15 0.1 ml. GJ and saliva were compared to 1.0 ml. serum. Specimens were again saturated with radio-B12 (10 ng./ml. solution) according to their UBBC (in ng./0.1 ml.): UBBC < 5.0, 0.5 ml. (5.0 ng.) was added; when UBBC 5.0–7.4, 0.75 ml. (7.5 ng.) was added; when UBBC was 7.5–12.4, 1.25 ml. (12.5 ng.)

*Visking casing, supplied in rolls of 100 ft length and 8/32 inch cross-section; Union Carbide Corp., 6733 West 65th Street, Chicago, Ill. 60638. Prior to use, the casing is boiled for 20 minutes in 0.1 M Na2CO3 to remove a yellow contaminant which damages IF antibody function.
Table 1.—"Loss" of Radioactive B₁₂ from Test Samples

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>Percentage Sample Radioactivity After:</th>
<th>a) Dialysis against buffer*</th>
<th>b) Passage through DEAE cellulose†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum</td>
<td>87.6 ± 0.7</td>
<td>88.0 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>PA serum</td>
<td>85.6 ± 1.5</td>
<td>87.8 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>CML serum</td>
<td>88.7 ± 1.2</td>
<td>87.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Normal GJ</td>
<td>88.7 ± 0.8</td>
<td>81.8 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>PA GJ</td>
<td>86.8 ± 1.3</td>
<td>81.9 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Saliva</td>
<td>76.5 ± 5.3</td>
<td>65.0 ± 2.2</td>
<td></td>
</tr>
</tbody>
</table>

*Radioactivity of sample after buffer dialysis
Radioactivity of sample before buffer dialysis × 100

†Radioactivity eluted
Radioactivity applied × 100

Values recorded as means ± standard errors of 5–17 observations.
(a) All "lost" B₁₂ was retained in and/or on the dialysis bag.
(b) All "lost" B₁₂ was recoverable from the container which delivered the B₁₂ to the column (approximately 20 per cent) and from the column (approximately 80 per cent).

was added. With UBBC was 12.5–24.9. 0.25 ml. 100 ng./ml. radio-B₁₂ (25 ng.) was added. In all instances the final sample volume was adjusted to 1.25–1.5 ml. with 0.9 per cent saline. Specimens were treated with hemoglobin-coated charcoal and dialyzed, as described for serum. The effect of IF antibody pretreatment on elution patterns of GJ was tested by incubating GJ with 0.1 ml. IF antibody serum at 37 C. for 15 minutes, before adding radio-B₁₂.

**DEAE Cellulose Chromatography Procedure**

The apparatus is depicted in Figure 2.

A 250/5.5 mm. glass column was prepared from a 5 ml. serologic pipette by removing the narrow mouthpiece, dropping in a tiny plug of glass wool, and filling to a height of 220 mm. with DEAE cellulose* suspended in 0.02 M phosphate buffer, pH 6.3. The column was connected with 6.0 mm. rubber tubing to a 125 ml. glass separating funnel containing 0.02 M phosphate buffer, pH 6.3. The cellulose was packed with this buffer at approximately 20 cm. hydrostatic pressure, and allowed to equilibrate for at least 4 hours. The supernatant buffer was removed (or permitted to drain away); the sample was then applied to the top of the column and collection of eluate started in a series of 7 ml. glass tubes, each marked to take 2 ml. fluid. Immediately after the sample was absorbed, the column, tubing and funnel were filled with 0.06 M phosphate buffer, pH 6.3, as initial eluant, and the flow rate set to 1 ml. per 5–8 minutes, by adjusting the height of the funnel. The collecting tubes, each containing 2 ml. eluate, were changed by hand every 10–15 minutes. After collection of tube 5, the rubber tube was removed and remaining buffer removed from above the cellulose, and replaced with 1 M sodium chloride. Elution with 1 M NaCl

*Selectacel ion exchange cellulose. #70 DEAE Standard, Carl Schleicher & Schull Co., Keene, New Hampshire. 100 gm. of the dry cellulose is added to 2 L. 1 N NaOH, mixed, allowed to stand for 1 hour, poured slowly onto a Buchner funnel, allowed to stand for 5 minutes, the liquid then sucked thru the funnel, and the cellulose again washed with about 10 2 L aliquots of distilled water until pH 7. The cellulose is then left overnight in 2 to 3 L. 0.02 M pH 6.3 phosphate buffer, following which it is washed with about 10 2 L aliquots of the buffer until pH 6.3, and left suspended in the final 2L. For addition to columns, each 1 ml. of this stock slurry (about 25 per cent cellulose by volume) is diluted with 2 ml. of buffer (0.02 M phosphate, pH 6.3).
was continued for a further 5 tubes, giving a total eluate of 20 ml. in 10 tubes. The void volume of the column is approximately 2.0 ml. Fractions 1–6 were referred to as “buffer eluates,” and 7–10 as 1 M NaCl eluates or “saline eluates.” (In the modified procedure, fractions 1–7 are “buffer eluates” and 8–11 are “saline eluates.”) Eluates were investigated as follows:

1. Radioactivity was determined, and converted to pg. radioactive B₁₂ per eluate, by comparing the counting rate with that of a radioactive B₁₂ standard.
2. Optical density (OD) at 280 nm was determined in a Beckman DU spectrophotometer, as an index of total protein present. Serum eluates were diluted 1:10 with saline prior to testing, but GJ and saliva were read as such.
3. B₁₂ content of eluates from sera not presaturated with radio-B₁₂ was assayed with the hemoglobin-coated charcoal radio-dilution method of Lau et al.¹³
4. Cellulose acetate strip electrophoresis in veronal buffer, pH 8.6, was performed on the full spectrum of eluates from representative specimens. In selected instances where samples had been labeled with Co₂⁵⁸B₁₂, the tubes with maximal radioactivity in the “buffer eluate” and “saline eluate” regions were concentrated tenfold by ultrafiltration* and then electrophoresed on 1 per cent agar gel at pH 8.6 on 4" × 5" glass plates at 110 volts for 2 hours; application sample was 0.05 ml. Autoradiographs were produced by exposing samples to photograph film† for 72 hours. Shaking with hemoglobin-coated charcoal

*Collodion Bags CB, porosity < 5 millimicrons, size 8 ml.; Membran-Filtergesellschaft, 34 Gottingen, Germany.
†Kodak medical x-ray film, no screen.
demonstrated no free $\text{B}_{12}$ in the eluates. Passage of free $\text{Co}^{67}\text{B}_{12}$ through the column showed that it came through in fraction 2.

**RESULTS**

**Serum**

The typical pattern of radioactive $\text{B}_{12}$ elution from 5 normal, PA and CML sera is represented in Figure 3. In normal and PA serum, radioactivity ap-
peared predominantly in the "buffer eluates"; in CML, radio-B₁₂ was maximally eluted by 1M NaCl. Maximal radioactivity was usually found in tubes 3 and 7, but occasionally in tubes 4 and 8. The reproducibility of the method is demonstrated in Figure 4; the total radio-B₁₂ in the "buffer eluates" (tubes 1-6) and 1 M NaCl eluates (tubes 7-10) agreed closely in duplicate determinations.

Agar gel electrophoresis (Fig. 5) showed that 1 M NaCl eluate number 7 (with maximal radioactivity) contained albumin, α₁-globulins, and α₂-globulins, but no β-globulins. Buffer eluate number 3 (with maximal radioactivity) contained β-globulins, in addition to α₂-globulins, albumin and γ-globulin, but apparently lacked α₁-globulins. This held true for both normal and CML sera. Cellulose acetate strip electrophoresis confirmed the absence of α₁-globulins from all buffer eluates, but showed a trace of β-globulins in the 1 M NaCl eluates. However, autoradiography (Fig. 5) demonstrated that radioactivity in the 1 M NaCl eluates was associated only with material of α₁ mobility and in the buffer eluates with material of β and α₂ mobility.

Hemoglobin-coated charcoal assay eluates from normal and CML sera not presaturated with radioactive B₁₂ showed endogenous B₁₂ activity only in 1 M NaCl eluates. With acute hepatitis serum (B₁₂ = 1360 pg./ml., UBBC = 1228 pg./ml.), however, 16.5 per cent of endogenous B₁₂ present in the eluates, was found in the buffer fractions.

In Figure 6, OD readings are compared with radioactive B₁₂ content.
Fig. 4.—"Baby" DEAE column radioactive B₁₂ elution patterns from 3 normal sera and a CML serum chromatographed in duplicate; good reproducibility is confirmed by comparison of total α-globulin and β-globulin B₁₂ binders calculated from the graphs. Saline eluate means eluate with 1 M NaCl.

of serum, CJ and saliva. The great discrepancy in total protein values with comparable B₁₂ contents is not unexpected as B₁₂-binders are trace proteins.₁⁶

The loss of radioactivity at various stages in the separation procedure is summarized in Table 1. During dialysis against buffer, the mean UBBC decrease was 11.3–14.4 per cent, as judged by loss of radioactive B₁₂. Passage through the DEAE cellulose decreased radioactive B₁₂ by a further 12–12.6 per cent. There was no significant difference in per cent loss of UBBC between normal, PA and CML serum. When serum was passed through the column, without prior saturation with B₁₂, the loss of measurable binding protein was much greater than when the serum had been presaturated with radioactive B₁₂ (Fig. 7). Binding protein was determined quantitatively by estimating unsaturated B₁₂ binding capacity of original sample and total eluates. The average and range of unsaturated B₁₂-binding capacity and distribution between alpha and beta globulin obtained using the "baby column" method has been alluded to in reference 9 and is delineated in Table 2.

**Gastric Juice**

In Figure 8, the radioactive B₁₂ elution patterns obtained from two normal gastric juices with different binding capacity and percentage IF, and non-IF
Table 2.—Distribution of Human Serum Unsaturated $B_12$ Binding Capacity Between $\alpha$ and $\beta$ Globulin Fractions of "Baby Column"

<table>
<thead>
<tr>
<th>Category</th>
<th>Number of Subjects</th>
<th>Average Serum Vitamin $B_12$ Level</th>
<th>Per Cent of UBBC* $\alpha$</th>
<th>Per Cent of UBBC* $\beta$</th>
<th>Total $\alpha$ Expressed as pg. of $B_12$</th>
<th>Total $\beta$ Expressed as pg. of $B_12$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5</td>
<td>386</td>
<td>12.1--29.1</td>
<td>70.9--87.2</td>
<td>119--301</td>
<td>731--1093</td>
</tr>
<tr>
<td>Pernicious anemia (PA)</td>
<td>11</td>
<td>54</td>
<td>18.4--55.8</td>
<td>41.2--81.6</td>
<td>214--305</td>
<td>516--2867</td>
</tr>
<tr>
<td>$B_12$ deficient (not PA)</td>
<td>5</td>
<td>47</td>
<td>10.2--45.7</td>
<td>51.3--89.6</td>
<td>254--400</td>
<td>507--1344</td>
</tr>
<tr>
<td>Treated PA</td>
<td>6</td>
<td>459</td>
<td>20.8--41.2</td>
<td>50.8--70.2</td>
<td>242--671</td>
<td>652--1305</td>
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<tr>
<td>Liver disease (cirrhosis)</td>
<td>9</td>
<td>927</td>
<td>13.8--30.7</td>
<td>69.3--66.2</td>
<td>208--656</td>
<td>734--1736</td>
</tr>
<tr>
<td>Leucocytosis</td>
<td>4</td>
<td>412</td>
<td>11.5--31.5</td>
<td>68.5--85.8</td>
<td>232--335</td>
<td>1054--2730</td>
</tr>
<tr>
<td>Leucopenia</td>
<td>5</td>
<td>673</td>
<td>15.9--35.1</td>
<td>64.9--84.1</td>
<td>108--355</td>
<td>442--991</td>
</tr>
<tr>
<td>Uremia</td>
<td>6</td>
<td>745</td>
<td>18.6--52.4</td>
<td>47.6--81.4</td>
<td>375--1017</td>
<td>923--1848</td>
</tr>
<tr>
<td>Polycythemia vera (uncontrolled)</td>
<td>8</td>
<td>1441</td>
<td>19.7--60.2</td>
<td>39.8--80.3</td>
<td>189--1435</td>
<td>835--7348</td>
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<tr>
<td>Acute myeloid leukemia</td>
<td>13</td>
<td>1963</td>
<td>21.2--82.1</td>
<td>17.9--78.8</td>
<td>240--5650</td>
<td>763--3158</td>
</tr>
<tr>
<td>Di Guglielmo syndrome</td>
<td>3</td>
<td>826</td>
<td>29.8--34.9</td>
<td>65.1--70.2</td>
<td>192--278</td>
<td>358--638</td>
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<tr>
<td>Acute myeloid leukemia No therapy</td>
<td>8</td>
<td>1106</td>
<td>12.4--61.8</td>
<td>48.2--87.6</td>
<td>192--540</td>
<td>786--1957</td>
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<tr>
<td>Chronic myeloid leukemia No therapy</td>
<td>9</td>
<td>918</td>
<td>20.4--50.3</td>
<td>49.7--79.6</td>
<td>242--2248</td>
<td>893--1627</td>
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<tr>
<td>Chronic myeloid leukemia Therapy</td>
<td>5</td>
<td>4480</td>
<td>68.6--97.3</td>
<td>2.7--31.4</td>
<td>6760--17,170</td>
<td>746--5964</td>
</tr>
<tr>
<td>Blastic</td>
<td>7</td>
<td>3229</td>
<td>42.8--89.3</td>
<td>10.7--57.2</td>
<td>460--7880</td>
<td>610--2272</td>
</tr>
</tbody>
</table>

*UBBC = Unsaturated $B_12$ binding capacity.

†Assuming that all endogenous serum $B_12$ is on $\alpha$. This is not so in liver disease.
Fig. 5.—(a) Agar gel electrophoresis of normal and CML sera, accompanied by electrophoresis of "baby" column fractions with maximal B₁₂-binder, in buffer (#3) and 1 M NaCl (#7) eluates obtained from these sera.

(b) Autoradiographs of same sample; sera presaturated with Co⁵⁸B₁₂. Radioactivity in eluates #3 associated with α₂ and β-globulin, and in eluates #7 with α₁-globulin.

binder, are compared with that of one PA GJ and a basal non-PA juice which lacked IF by in vitro assay.¹⁴ Radioactivity was eluted in both the buffer and 1 M NaCl eluates of all samples but with non-PA GJ, lacking IF, the buffer eluate showed a very small peak of activity. Pretreatment with IF antibody greatly reduced the buffer peak in normal GJ, but had no effect on the buffer peak of PA GJ; the 1 M NaCl peak remained unaffected in normal GJ and PA GJ.

The percentage of radioactivity lost from GJ during dialysis against buffer and passage through the cellulose column is comparable to that lost from serum (Table 1), with no significant difference between normal and PA GJ.

*Very little IF is occasionally found in otherwise normal basal gastric juice.¹³
Fig. 6.—Comparison of radioactive B₁₂ and total protein content (O.D. at 280 m₀) of “baby” DEAE column eluates of normal and CML serum, GJ, and saliva.

As with serum, the loss of B₁₂-binder in the column was much greater when GJ was unsaturated than when saturated with B₁₂.

Saliva

The radioactive B₁₂ distribution in eluates from three normal salivas shows marked variation in total B₁₂ binding capacity, but similar general patterns of maximal elution in the 1 M NaCl fractions with a minor buffer eluate peak (Fig. 9). This is similar to the graph obtained with non-PA GJ, lacking IF.

In Table 1 it is shown that the amount of radioactivity lost from saliva during buffer dialysis was slightly more than that lost from GJ and serum, and more radioactive B₁₂ was retained by the cellulose than with the other two materials.

DISCUSSION

Previous workers have separated serum B₁₂ binding proteins chromatographically with DEAE-cellulose⁶,¹⁷ or CM-cellulose,¹⁸ these conventional technics are time-consuming and unsuited to a routine laboratory. The “baby column” DEAE cellulose method here described may be performed rapidly as a routine procedure.

Pitney et al.² reported in 1954 that the alpha-globulin carrying endogenous B₁₂ had very little additional ability to bind B₁₂ added in vitro. Miller⁴ showed by paper electrophoresis that approximately 70 per cent of added B₁₂ migrated with β or α₂-globulins when the ratio was 15 ng. added B₁₂ to 1 ml.
Fig. 7.—Comparison of “loss” of B₁₂ binding capacity of normal serum and GJ when passed thru column after vs. before addition of Co⁵⁷B₁₂. Left-hand column is unsaturated B₁₂ binding capacity of an aliquot of sample, determined by adding Co⁵⁷B₁₂. Other dark columns represent portions of this aliquot appearing in saline, buffer and total eluate after passage (saturated with Co⁵⁷B₁₂) through column. Light columns represent a different aliquot of the same sample, which was passed thru the column without added Co⁵⁷B₁₂. Much more of the measurable B₁₂ binding capacity was lost from samples passed through the column without previous addition of a saturating quantity of B₁₂.

serum. Hall and Finkler differentiated by extensive DEAE chromatography between the two main B₁₂ binders in normal serum. Their tables show fair agreement with our rapid “baby column” separation, illustrated in Figure 2, where approximately one-fifth of a saturating dose of radioactive B₁₂ eluted with alpha-globulin (1 M NaCl eluate) and four-fifths with the beta-globulin binder (buffer eluate). The total amount of beta-globulin B₁₂-binder in a test serum may be estimated by totalling the amount of radioactive B₁₂ eluted by 0.06 M phosphate buffer (tubes 1–6). The alpha-globulin component of the UBBC is derived from the total 1 M NaCl eluate (tubes 7–10); by adding the endogenous serum B₁₂ value to this figure, the total alpha-globulin B₁₂-binder in normal and CML serum can be calculated.

This method is based on the fact that beta-globulins are maximally eluted
from cellulose at a molarity of less than 0.06, while alpha-globulins elute at a much higher ionic strength, in the range of 0.15 M. Initial washing with 0.06 M buffer thus separate beta-globulins from alpha-globulins, and the latter proteins are subsequently eluted by 1 M NaCl in nonspecific manner.

Gastric juice was fractionated as a buffer eluate containing all the IF (and also, a significant amount of non-IF), and a 1 M NaCl eluate containing only non-IF B₁₂ binders. The physicochemical basis for this useful separation is unclear. PA GJ also produced two peaks of radioactivity, but pretreatment with IF antibody showed that neither contained IF. Saliva produced a major 1 M NaCl peak and a pattern rather similar to that for non-PA GJ, lacking IF (Fig. 8). Simons reported that the non-IF “R-binder” of GJ was chromatographically and immuno-electrophoretically similar to the main B₁₂-binder of saliva and the serum binder not carrying endogenous B₁₂ (i.e., the beta-globulin binder). Although our separation technic does not yield discrete individual proteins, our elution patterns tend to confirm the similarity between the GJ non-IF binder and the salivary B₁₂-binder, both of which are found in the 1 M NaCl eluate. However, the beta-globulin serum binder was eluted from the “baby column” by 0.06 M buffer instead of 1 M NaCl, and therefore, as others have also indicated, it may not be the same binder as those in GJ and saliva. Dr. Gräsbek kindly sent us small aliquots of Co⁵⁷B₁₂ tagged fractions S, I, and R, sufficient for a run through the baby column.” Most of the Co⁵⁷B₁₂ of each of these three fractions was eluted by 0.06 M buffer (792 of 852 pg, S, 1058 of 1078 pg, I, 1099 of 1274 pg, R).
During dialysis against buffer, radioactivity lost from serum, GJ, and saliva represented primarily adsorption to Visking casing. Passage of test samples through the cellulose column resulted in an equal mean decrease of radioactivity from normal, PA and CML serum. As normal serum UBBC consists predominantly of beta-globulin binder and CML serum of alpha-globulin binder, these findings suggest that alpha-globulin and beta-globulin B₁₂ binders are retained in random manner. Mean loss of activity was similar from normal (18.2 per cent) and PA GJ (18.1 per cent); the greatest loss of binder occurred from saliva (35.0 per cent).

With normal and CML serum, B₁₂ activity originating from endogenous B₁₂ was found only in the 1 M NaCl eluates. This is the expected finding, as B₁₂ is normally bound to an alpha₁ globulin in serum.²,⁸ The presence of B₁₂ in buffer and 1 M NaCl eluates of hepatitis serum, however, suggests that
endogenous B\textsubscript{12} in this specimen was bound to both beta-globulin and alpha-globulin. The previous finding of Herbert et al.\textsuperscript{21} that serum from patients with liver disease, transferred B\textsubscript{12} inefficiently to reticulocytes, suggested atypical B\textsubscript{12} binding in this condition.

It is not clear why presaturation with B\textsubscript{12} “protects” B\textsubscript{12} binding protein during passage through the cellulose column (Fig. 7). The absence of demonstrable B\textsubscript{12} from all eluates except the alpha-globulin-containing tubes\textsuperscript{7,8} precludes the possibility that contaminating “cold” B\textsubscript{12} in the cellulose might saturate the binder and thus produce an apparent decrease in UBBC when subsequently tested with radio-B\textsubscript{12}. It is known that B\textsubscript{12} may “protect” binders in G\textsuperscript{22} and sow’s milk\textsuperscript{23} against enzymatic degradation, but a similar “protection” against retention of binder by cellulose has to our knowledge not been described. The reactivity of the B\textsubscript{12} binders with B\textsubscript{12} may have been changed by one or more of the conditions of cellulose chromatography, including exposure to varying pH and ionic strength.

The technic fills a gap in that it allows rapid routine evaluation of large numbers of serum specimens for distribution of B\textsubscript{12} binders as an aid in differential diagnosis of CML vs. myeloid metaplasia.\textsuperscript{9} It may also prove useful in situations where rapid separation of alpha- from beta-globulins may be of value, as in the separation of anti-hemophilic globulin from clottable fibrinogen and gamma globulin.\textsuperscript{24}

Previous work, summarized by Simons and Weber,\textsuperscript{25} suggests that the granulocytes are one source of serum vitamin B\textsubscript{12} binding protein, of the type labeled “R” by Gräsbeck and Simons. Since labeled R-binder, supplied by Dr. Gräsbeck, appears to be almost exclusively in the beta globulin fraction in our baby column system, and since it was found (Table 2) that B\textsubscript{12} binding beta globulin is also elevated in chronic myeloid leukemia and appears reduced in chronic leukopenia (all of whom had leukopenia), we would suggest that granulocytes are one source of B\textsubscript{12} binding beta globulin as well as of B\textsubscript{12} binding alpha globulin. Further evidence is the relatively greater rise in B\textsubscript{12} binding beta than in alpha globulin in leukocytosis not due to chronic myeloid leukemia; this finding also suggests that the myeloid leukemia granulocyte gives rise to more B\textsubscript{12} binding alpha globulin and less B\textsubscript{12} binding beta globulin than do normal granulocytes.

SUMMARY

A method of DEAE cellulose chromatography is described whereby the main vitamin B\textsubscript{12} binders in serum, gastric juice and saliva may be separated into two distinct fractions, one containing alpha-globulin binder and the other containing beta-globulin binder, in less than 2 hours. Typical elution patterns of alpha-globulin and beta-globulin B\textsubscript{12} binders from normal (\% beta; \% alpha), pernicious anemia (\% beta; \% alpha), and chronic myeloid leukemia (CML) (\% alpha) sera are presented. Chromatography of normal gastric juice produced two peaks of B\textsubscript{12}-binding material, one containing all the intrinsic factor (IF) B\textsubscript{12} binder (as well as some non-IF binder), and the other consisting almost exclusively of non-IF B\textsubscript{12} binders. Pernicious anemia gastric
juice also produced two peaks, but IF was absent. Saliva had one main peak of B₁₂-binding material which seemed to correspond to the non-IF binder of normal gastric juices; both of these eluted with the alpha-globulin fraction.

**SUMMARIO IN INTERLINGUA**

Es describite un methodo de chromatographia a cellulosa diethylaminio-ethanolic le qual permitte separar duo distincte fractiones in le major ligatores de vitamina B₁₂ in sero, succo gastric, e saliva. Le separation pote esser compite in minus que 2 horas. Un del resultante fractiones contine le ligator globulina alpha, le altere le ligator globulina beta. Es presentate typic configurationes de elution del ligatores de vitamina B₁₂ a globulina alpha e a globulina beta ab subjectos normal (beta %; alpha %), ab subjectos con anemia perniciose (beta %; alpha %), e ab subjectos con chronic leucemia myeloide (alpha %). Le chromatographia de normal succo gastric produceva duo culmines de material capace a ligar vitamina B₁₂. Un de illos contineva le totalitate del ligator de vitamina B₁₂ del typo de factor intrinsec e etiam un certe quantitate de ligator altere que factor intrinsec, durante que le altere culmine consisteva quasi exclusivemente de ligatores de vitamina B₁₂ non del typo de factor intrinsec. Le succo gastric ab patientes con anemia perniciose etiam produceva duo culmines, sed factor intrinsec esseva absent. Saliva habeva un sol major culmine de material capace a ligar vitamina B₁₂. Illo pareva corresponder al ligator altere que factor intrinsec in normal succes gastric.

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Separation of Vitamin B$_{12}$-Binding Proteins of Serum, Gastric Juice and Saliva by Rapid DEAE Cellulose Chromatography

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