Isolation of Vitamin B₂₆-binding Proteins Using Affinity Chromatography

I. PREPARATION AND PROPERTIES OF VITAMIN B₂₆-SEPHAROSE

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SUMMARY

A method of affinity chromatography which is a potent tool for isolation of the trace vitamin B₁₂ binding proteins has been developed. The affinity ligand was prepared by partial acid hydrolysis (0.4 N HCl, 64 hours, room temperature) of the amide groups of the unsubstituted propionamide side chains of the corrin ring of vitamin B₁₂. The resultant mixture of mono-, di-, and tricarboxylic vitamin B₁₂ derivatives was separated by chromatography on QAE-Sephadex. The monocarboxyl derivatives of vitamin B₁₂ were coupled covalently to the free amino group of 3,3'-diaminodipropylamine-substituted Sepharose using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, thereby regenerating native vitamin B₁₂ stably coupled to Sepharose (yield = 0.7 µmole of vitamin B₁₂ per ml of packed Sepharose). The vitamin B₁₂-Sepharose bound the vitamin B₁₂ binding proteins (>90%) of human granulocytes, human plasma, human gastric juice, an extract of hog gastric mucosa, and a partially purified preparation of human transcobalamin II prepared from Cohn Fraction III of human plasma. The capacity of the vitamin B₁₂-Sepharose to bind vitamin B₁₂ binding proteins was at least 22% of the total vitamin B₁₂ bound to Sepharose assuming 1 mole of vitamin B₁₂ is bound per mole of vitamin B₁₂ binding protein.

Vitamin B₁₂ is present in nature in very small amounts and is synthesized only by microorganisms. Because of this limited supply, many animals including man have developed elaborate mechanisms for the absorption, transport, and conservation of this trace substance. Specific proteins which bind vitamin B₁₂ appear to play an important role in all of these processes. In many animals the stomach synthesizes a glycoprotein known as intrinsic factor which binds vitamin B₁₂ and facilitates its absorption in the intestine in far greater amounts than is possible by diffusion of free vitamin B₁₂ at the levels found in a normal diet. In human plasma, vitamin B₁₀ is tightly bound to at least two different proteins known as transcobalamin I and transcobalamin II. These proteins appear to function in transporting the vitamin as well as preventing the rapid urinary loss of vitamin B₁₂ which occurs if the vitamin is present in plasma in unbound form (for review see Grasbeck (2)).

Studies of structure and function of these proteins have been severely hampered by the difficulties encountered in their purification. Attempts to purify intrinsic factor have met with the most success, but there have been severe limitations. Grasbeck (2) succeeded in 1966 in purifying human intrinsic factor from human gastric juice to homogeneity, but he obtained only slightly more than 10 mg of homogeneous protein from 40 liters of Histolog-stimulated gastric juice collected from over 300 individuals. More than 10 separate column chromatographic steps were included in this purification scheme, and the small amount of material obtained precluded many of the physiological studies that might have been done. Ellenbogen and Highley have also isolated intrinsic factor from hog gastric mucosa (3). Many other purification schemes for intrinsic factor are present in the literature, but they, too, are laborious and the final product in most cases is not homogeneous (4).

The situation with respect to transcobalamin I and transcobalamin II is even more difficult; neither of these proteins has been purified to homogeneity. The enormity of the problem is readily apparent when one considers that 1000 liters of human plasma contain only approximately 80 and 20 mg, respectively, of each of these proteins. This estimate is based on molecular weights determined by gel filtration, assuming one vitamin B₁₂ binding site per molecule of protein (2). To achieve homogeneous protein preparations, transcobalamin I must be purified almost 1 million-fold and transcobalamin II at least 2 million-fold. Purification of this degree with reasonable yields is beyond the scope of conventional purification techniques.

Affinity chromatography is an attractive method for consideration, but unfortunately vitamin B₁₂ does not contain any primary amino, free carboxyl, phenolic, imidazole, or sulfhydryl groups which have been utilized in the case of other ligands to achieve covalent attachment either directly to cyanogen bromide-activated Sepharose or to various substituted Sepharoses (6).
We report herein the preparation of a derivative of vitamin B12 which differs from the native vitamin only in that it lacks a single amide group on one of the three propionamide side chains adjacent to rings A, B, and C of vitamin B12 (Fig. 1). The derivative was obtained by mild acid hydrolysis of the amide groups involved and is based on work from the laboratory of E. L. Smith on the structure of vitamin B12 (6, 7). The monocarboxylic acid derivatives of vitamin B12 were separated from vitamin B12 and other polybasic acid derivatives by ion exchange chromatography, and gram amounts of the monocarboxylic acid derivatives have been obtained. The monocarboxylic acid derivatives were coupled to the free amino group of 3,3'-diaminodipropylamine by the method of Cuatrecasas (5). Sepharose 4B and QAE-Sephadex were obtained from Pharmacia. Hydrochloride, Ultra Pure, was obtained from Mann Research Laboratories. 

** EXPERIMENTAL PROCEDURE **

Materials—Crystalline vitamin B12 and bovine serum albumin grade V were obtained from Sigma Chemical Company. Ethyl-3-(3-dimethylaminodipropyl)carbodiimide-HCl was obtained from Pierce Chemical Company. 3,3'-Diaminodipropylamine was coupled to cyanogen bromide-activated Sepharose in the Buchner funnel. The structure of vitamin B12 is shown in Fig. 1.

3,3'-Diaminodipropylamine-substituted Sepharose with a water-soluble carbodiimide, thus regenerating the structure of native vitamin B12 linked covalently to the substituted Sepharose. The vitamin B12-Sepharose so prepared is an effective affinity adsorbent for the vitamin B12-binding proteins from a variety of sources.

** Assay for Vitamin B12 Binding Ability **

Vitamin B12 binding ability was assayed by the charcoal absorption method of Gottlieb et al. (8) as modified in the following way. Samples to be assayed (0.01 to 0.4 ml) were adjusted to a volume of 2.0 ml with 0.1 m potassium phosphate, pH 7.5, containing 1 mg per ml of bovine serum albumin. One milliliter of [37Co]vitamin-B12 (1 ng per ml) was added, and the mixture was allowed to stand at room temperature for 15 min.

** Protein Assay **

Protein was assayed by the method of Lowry et al. (9) using bovine serum albumin as a standard.

** Preparation of Extract of Human Granulocytes **

Human granulocytes were isolated by dextran sedimentation and centrifugation as described previously (10). The granulocytes were frozen at -70° prior to use, then thawed, disrupted by sonication (10), and centrifuged at 20,000 × g. The supernatant was employed to test the ability of the vitamin B12-Sepharose to serve as an affinity adsorbent and ultimately for isolation of a granulocyte vitamin B12-binding protein (11).

** Human Plasma **

Human plasma was obtained from fresh human blood collected in 67.5 ml of 0.8% citric acid, 2.2% sodium...
citrate, and 2.45% dextrose per 450 ml of whole blood by centrifugation at 10,000 \( \times g \) for 20 min. The supernatant plasma was decanted and stored at \(-20^\circ\)C prior to use.

Preparation of Transcobalamin II—Transcobalamin II was the major vitamin \( B_12 \)-binding protein found in Cohn Fraction III from human plasma. Transcobalamin II was partially purified from Cohn Fraction III by batch elution from CM-Sephadex with 1 M NaCl containing 0.02 \( \mu \)M sodium phosphate, pH 5.8. This fraction contained transcobalamin II as the sole vitamin \( B_12 \)-binding protein and was further purified by affinity chromatography as described elsewhere (1, 12).

Human Gastric Juice—Gastric juice was collected by nasogastric suction from a normal human volunteer. The material utilized in these studies represents the 30- to 60-min collection following Histolog stimulation. The sample was collected on ice and the pH raised to 10.0 with 1.0 M sodium hydroxide. After the preparation had stood for 10 min at room temperature to reduce pepsin activity, the pH was reduced to 7.0 with 0.05 M HCl and the material was stored at \(-20^\circ\)C prior to use. This material was diluted in 10 volumes of 0.1 M NaCl and centrifuged at 20,000 \( \times g \) for 30 min prior to use in the vitamin \( B_12 \)-Sepharose affinity studies.

Preparation of Affinity Ligand

Acid Hydrolysis of Vitamin \( B_12 \)—Crystalline vitamin \( B_12 \) (4340 \( \mu \)mol) was dissolved in 400 ml of distilled water at room temperature and 1 \( \mu \)Ci of \( ^{57} \)Co vitamin \( B_12 \) was added. This solution was adjusted to contain 0.4 \( \times \) HCl in a volume of 300 ml then was covered and placed in the dark at room temperature for 64 hours.

A column (4.5 \( \times \) 60 cm) of BioRex AG1-X8 (acetate), 100 to 200 mesh, was prepared at 4\( ^\circ \)C and washed with 2.0 liters of 1 M sodium acetate followed by 4.0 liters of H2O. The acid hydrolysate from the preceding step was applied to the column at a flow rate of 200 ml per hour. This column was then eluted with 0.025 M acetic acid. The elution pattern could be followed visually due to the deep red color of the vitamin \( B_12 \) and its derivatives. The bulk of this red material was not significantly retarded by the column and was followed closely by a red band of moderate intensity. The first 880 ml of red eluate contained 95.5% of the radioactivity applied to the column and included the nonretarded material as well as the partially retarded red band of moderate intensity mentioned above. This fraction, designated as the AG1-X8 eluate, was free of chloride as determined by AgNO\(_3\) precipitation. At the end of this elution the entire column had a faint pink tint and several faint red bands were present near the top of the column. These were not investigated further.

The AG1-X8 eluate was taken to dryness by rotary evaporation with the water bath at a temperature of 40\( ^\circ \). The dry material was dissolved in 300 ml of water and the pH was measured at 4.8. Pyridine (55 ml) was added and the pH rose to 7.5. The pH was then adjusted to 9.0 with concentrated NH\(_2\)OH (<1 ml). The volume at this point was 385 ml, and the material was chromatographed on QAE-Sepharose.

Chromatography of AG1-X8 Eluate on QAE-Sepharose—Sixty grams of dry QAE-Sepharose A-25 (chloride form) were added to 2.5 liters of 1.0 M NH\(_4\) acetate and gently stirred at room temperature for 48 hours. The QAE-Sepharose was allowed to settle and the supernatant was discarded. After addition of 1.0 liter of 0.2 M pyridine to the settled QAE-Sepharose, the pH was adjusted to 9.0 with 1.0 M NH\(_2\)OH. The QAE-Sepharose was then washed with 1.0 liter of 0.2 M pyridine three times. A column (2.5 \( \times \) 56 cm) of QAE-Sepharose was then prepared at 4\( ^\circ \)C and washed with 300 ml of 0.2 M pyridine that was pumped through the column with a flow rate of 100 ml per hour. The AG1-X8 eluate from the preceding step was applied at the same flow rate, and 15-ml fractions were collected. The column was initially eluted with 600 ml of 0.4 M pyridine. At the end of this elution the flow rate was decreased to 50 ml per hour, and the column was eluted with a linear acetate gradient in which the mixing chamber contained 1.0 liter of 0.4 M pyridine and the reservoir contained 1.0 liter of 0.4 M pyridine 0.16 M acetic acid. Aliquots from fractions were assayed for radioactivity. The elution profile of the vitamin \( B_12 \) derivatives is shown in Fig. 2. The following fractions from the QAE-Sepharose column chromatography were pooled and designated as follows: Fractions 10 through 45, QAE (10-45); Fractions 91 and 92, QAE (91-92); Fractions 121 through 140, QAE (121-140); Fractions 204 through 220, QAE (204-220); Fractions 244 through 250, QAE (244-250). The amounts of vitamin \( B_12 \) derivatives present in each pooled fraction are presented in Table I.

Analysis of Fractions Obtained by QAE-Sepharose Chromatography

High Voltage Electrophoresis—Aliquots of the various QAE-Sepharose pooled fractions listed above as well as aliquots of the acid hydrolysate, AG1-X8 eluate, and an aqueous solution of vitamin \( B_12 \) were taken to dryness in a shaking evaporator with the water bath at 40\( ^\circ \). The dry residues were redissolved in 2 ml
of water, taken to dryness again, and then dissolved in 2 ml of water, taken to dryness again, and then dissolved in sufficient water to yield a concentration of between 13 and 24 μmoles of vitamin B₁₂ derivative per ml (based on radioactivity). Aliquots of each fraction (0.01 to 0.03 μmole) were then subjected to high voltage paper electrophoresis using Whatman No. 3MM paper at 1100 volts at room temperature. The buffer used was 0.05 M potassium phosphate, pH 6.5, containing 0.1 g of KCN per liter. Results of a typical electrophoresis are illustrated in Fig. 3.

Analysis by Descending Paper Chromatography—Aliquots of samples (0.02 to 0.03 μmole) prepared for high voltage electrophoresis were also chromatographed by descending chromatography using Whatman No 3MM paper. The chromatograms were developed at room temperature for 16 to 18 hours in dim light using 2-butanol (440 ml), H₂O (150 ml), glacial acetic acid (4.1 ml), and KCN (400 mg). A typical chromatogram is presented in Fig. 4.

### Table I

Yield of vitamin B₁₂ derivatives

The amount of vitamin B₁₂ derivative present in the initial acid hydrolysate (see “Experimental Procedure”) and in the pooled fractions from QAE-Sephadex chromatography (see Fig. 2).

<table>
<thead>
<tr>
<th>Vitamin B₁₂ derivatives</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid hydrolysate</td>
<td>100.0</td>
</tr>
<tr>
<td>AG1-X8 eluate</td>
<td>96.5</td>
</tr>
<tr>
<td>QAE (10-45)</td>
<td>50.7</td>
</tr>
<tr>
<td>QAE (91-92)</td>
<td>0.2</td>
</tr>
<tr>
<td>QAE (121-140)</td>
<td>35.0</td>
</tr>
<tr>
<td>QAE (204-220)</td>
<td>10.7</td>
</tr>
<tr>
<td>QAE (244-250)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Fig. 3. High voltage electrophoresis of the isolated vitamin B₁₂ derivatives present in the fractions of Table I. Electrophoresis was performed at 20°C on a sheet (46 × 56 cm) of Whatman No. 3MM paper in 0.05 M potassium phosphate, pH 6.5, containing 0.1 g of KCN per liter. Electrophoresis was at 1100 volts for 4 hours. The profile is presented as it appeared while the paper was still moist. •, major red spot; ○, minor red spot; □, minor purple spot. After drying, the purple spots appeared pale orange in color. The red spots retained their original color.

Fig. 4. Paper chromatogram of the isolated vitamin B₁₂ derivatives present in the fractions from Table I. Chromatography was performed on a sheet (23 × 56 cm) of Whatman No. 3MM in a solvent system consisting of 2-butanol (440 ml), H₂O (150 ml), glacial acetic acid (4.1 ml), and KCN (400 mg). The chromatogram was developed for 16 hours at room temperature in dim light. The chromatogram is presented as it appeared while still moist. •, major red spot; ○, minor red spot; □, minor purple spot. After drying, the original purple spots appeared pale orange in color. The red spots retained their original color.
Thirty milliliters of this solution containing 4.3 ml of water
were added to a beaker containing 30 ml of packed 3,3'-diamino-
dipropylamine-substituted Sepharose and mixed with a
magnetic stirrer at 22°. The pH was increased from 3.9 to 5.6 with
0.2 M NaOH (12.3 ml); while stirring continued, 3.0 ml of 1-
ethyl-3-(3-diethylaminopropyl)carbodiimide, 50 mg per ml,
were added in 0.6-ml aliquots at 1-min intervals. Gentle stirring
was continued at 22° in the dark for an additional 18 hours.
The substituted Sepharose was collected by vacuum filtration
in a Buchner funnel and washed at 22° successively with 300 ml
of H2O, 600 ml of 0.1 M glycine-NaOH, pH 10.0, 300 ml of H2O,
and 300 ml of 0.1 M potassium phosphate, pH 7.0. The washed
substituted Sepharose was deep red in color and contained 0.68
μmoles of vitamin B12 per ml of packed Sepharose as determined
by the content of radioactivity.

RESULTS

Initial experiments were directed at finding suitable conditions
of mild acid hydrolysis for producing derivatives of vitamin B12
which had a single amide group hydrolyzed from one of the un-
substituted propionamide groups of the corrin ring of vitamin
B12. The conditions finally used (0.4 N HCl, 64 hours, room
temperature) yielded about 35% of these monocarboxylic deriva-
tives of vitamin B12.

Fractionation of Vitamin B12 Acid Hydrolyzate on QAE-Sepha-
dex—Our interpretation of the nature of the vitamin B12 deriva-
tives present in each of the QAE-Sephadex fractions listed above
(see “Experimental Procedure”) is based on the observations of
Armitage et al. (6). They used similar methods of paper chro-
matography and high voltage electrophoresis to analyze the
products obtained by mild acid hydrolysis of vitamin B12. Their
conclusion that the major carboxylic acid derivatives of vitamin
B12 formed by mild hydrochloric acid hydrolysis result from the
loss of amide groups from the propionamide side chains adjacent
to rings A, B, and C is supported by more recent studies (13, 14)
utilizing x-ray crystallography and neutron diffraction analysis.
These studies demonstrated that the major monocarboxylic acid
isomer formed under these conditions differs from native vitamin
B12 only in the loss of the amide group on the propionamide side
chain adjacent to ring C.

On the basis of these observations and our paper chromatog-
ographic and electrophoretic studies we believe that QAE (121-
140) consists of the monocarboxylic acid isomer derivatives of
vitamin B12 which result from the loss of a single amide group
from any one of the propionamide side chains adjacent to rings
A, B, or C (Figs. 3 and 4). This conclusion is supported by
experiments in which QAE (121-140) was titrated from pH 4.0
to 7.0 using sodium hydroxide to determine the carboxylic acid
content. When corrected for titratable groups found on native
vitamin B12, QAE (121-140) was found to contain 0.98 titratable
group per mole of derivative. We conclude that QAE (204-
220) and QAE (244-250) consist, respectively, of the correspond-
ing di- and tricarboxylic acid derivatives formed by the loss of
two and three propionamide amide groups (Figs. 3 and 4).
QAE (10-45) consists primarily of unmodified vitamin B12 and
a small amount of the propionamide monocarboxylic acid deriva-
tives that lack the nucleotide portion of the vitamin. The
nature of QAE (91-92) is unknown.

Preparation of Vitamin B12-Sepharose—The monocarboxylic
vitamin B12 derivatives in QAE (121-140) were coupled to 3,3'-
diaminodipropylamine-substituted Sepharose as described under
“Experimental Procedure.” The yield was 8.1% with respect
to the vitamin B12 derivative using these conditions. Similar
yields were obtained with coupling between pH 5.0 and 6.0,
but decreased yields were obtained at lower pH values. When
native vitamin B12 was used in the coupling reaction, no coupling
occurred suggesting that coupling was through the carboxylic
acid group of the vitamin B12 derivative. This was further
supported by the fact that removal of the nucleotide from mono-
carboxylic acid derivatives using 11 N HCl did not impair the
coupling to Sepharose.

Since the coupling of the free amino group of the derivatized
Sepharose to the single free carboxyl group of the monocarboxylic
vitamin B12 derivative presumably regenerates the amide struc-
ture of the vitamin B12 molecule attached to Sepharose, we here-
after refer to this product as vitamin B12-Sepharose.

The vitamin B12-Sepharose was stored at 4° in an equal volume
of 0.1 M potassium phosphate, pH 7.0. Some hydrolysis of the
vitamin B12 derivative from the Sepharose occurs under these
conditions, but the amount is small since less than 5% of the
original bound vitamin B12 could be eluted in the filtrate after
storage for 3 months and washing as described under “Experi-
mental Procedure.” The amount of hydrolysis is greater if the
vitamin B12-Sepharose is stored at acidic or basic pH.

Binding of Vitamin B12-binding Proteins to Vitamin B12-Sepha-
rosee—Vitamin B12-Sepharose suspended in 0.1 M potassium
phosphate, pH 7.0, was used to prepare columns 1.0 to 6.0 cm
in height and ranging from 0.9 to 2.5 cm in diameter. These
columns were washed with 10 column volumes of 0.1 M glycine-
NaOH, pH 10.0, immediately prior to use to remove any free
vitamin B12 which might have hydrolyzed from the Sepharose.
Next the columns were washed with 5 column volumes of a buffer
appropriate to the sample that was to be passed over the column.
It was essential to remove any free vitamin B12 from the column
prior to use since the column can only bind vitamin B12-binding
proteins which are not saturated with the vitamin. Any free
vitamin decreases the yield of protein which binds to the column.
The ability of these columns to adsorb the vitamin B12-binding
proteins from a variety of sources was tested by passing various
solutions over the vitamin B12-Sepharose columns and comparing
the vitamin B12-binding activity of the original solution with
that of the solution after it had passed over the vitamin B12-
Sepharose column. The following materials were utilized: (a)
an extract of human granulocytes, (b) human plasma, (c) trans-
cobalamin II partially purified from Cohn Fraction III of human
plasma, (d) human gastric juice, and (e) an extract of hog gastric
mucosa. Vitamin B12-Sepharose bound >90% of the unsatu-
rated vitamin B12-binding protein present in each case, as shown
in Table II.

Capacity of Vitamin B12-Sepharose for Vitamin B12-binding
Protein Adsorption—An extract of intrinsic factor concentrate
from hog gastric mucosa with a vitamin B12-binding content of
2.66 μg per ml was applied to a column (1.5 × 6 cm) of vitamin
B12-Sepharose with a total vitamin B12 content of 10.0 mg. The
results of this experiment are shown in Table III. Early in the
sample application >98% of the vitamin B12-binding protein
present was removed from the solution as it passed over the
vitamin B12-Sepharose. Near the end of the sample application
the percentage of adsorption fell to 77%. Ninety-four per cent
of the vitamin B12-binding protein of the entire sample was bound
by the vitamin B12-Sepharose. Therefore, at least 22% of the
vitamin B12 covalently bound to the substituted Sepharose is
capable of binding the vitamin B12-binding protein employed,
assuming that there is one vitamin B12-binding site per molecule
of vitamin B12-binding protein. This value of 22% for the func-
tional vitamin B12-binding activity of the column is a minimum.
TABLE II

Adsorption of vitamin B12-binding proteins on vitamin B12-Sepharose

Vitamin B12-binding proteins from a variety of sources were applied to columns of vitamin B12-Sepharose. The percentage of adsorption of vitamin B12-binding protein was determined as the decrease in vitamin B12-binding activity after each item had passed through its column of vitamin B12-Sepharose. Details concerning the nature of the items tested are contained in the text under "Experimental Procedure." (Additional data concerning Item 5 are present in Table III.)

<table>
<thead>
<tr>
<th>Item applied to vitamin B12-Sepharose</th>
<th>Volume</th>
<th>Vitamin B12-binding activity</th>
<th>Column size</th>
<th>Flow rate</th>
<th>Vitamin B12-binding protein adsorption %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Human granulocyte extract..........</td>
<td>625 ml</td>
<td>35 mg/ml</td>
<td>2.0 x 1.5 cm</td>
<td>100 ml/hr</td>
<td>92.7</td>
</tr>
<tr>
<td>2. Human plasma.................</td>
<td>455 ml</td>
<td>0.678 mg/ml</td>
<td>0.3 x 1.0 cm</td>
<td>20 ml/hr</td>
<td>90.0</td>
</tr>
<tr>
<td>3. Transcobalamin II................</td>
<td>19,700</td>
<td>22.8 mg/ml</td>
<td>2.0 x 2.5 cm</td>
<td>500 ml/hr</td>
<td>92.3</td>
</tr>
<tr>
<td>4. Human gastric juice..............</td>
<td>180 ml</td>
<td>1.89 mg/ml</td>
<td>0.9 x 1.0 cm</td>
<td>20 ml/hr</td>
<td>99.1</td>
</tr>
<tr>
<td>5. Hog gastric mucosa................</td>
<td>985 ml</td>
<td>2,600 mg/ml</td>
<td>6 x 1.5 cm</td>
<td>120 ml/hr</td>
<td>94.4</td>
</tr>
</tbody>
</table>

 estimate since the column was clearly not saturated with vitamin B12-binding protein. In other experiments utilizing small columns of vitamin B12-Sepharose we have obtained values for the functional vitamin B12-binding activity of up to 50%. Table III also illustrates that the vitamin B12-binding protein is firmly bound to the vitamin B12-Sepharose only 4% of the original vitamin B12-binding protein adsorbed is removed during extensive washing with a variety of buffers of varying ionic strengths and composition.

Elution of Vitamin B12-binding Proteins from Vitamin B12-Sepharose—The elution of vitamin B12-binding proteins adsorbed onto vitamin B12-Sepharose has been studied in detail only with respect to the granulocyte vitamin B12-binding protein, transcobalamin II from human plasma, and the hog gastric mucosa vitamin B12-binding proteins. In each case we have been able to elute greater than 85% of the adsorbed vitamin B12-binding proteins with 7.5 mM guanidine HCl containing 0.1 M potassium phosphate, pH 7.5. Further details concerning the elution of the granulocyte vitamin B12-binding protein are contained in the accompanying paper which concerns the purification of this vitamin B12-binding protein to homogeneity utilizing the affinity chromatography technique as the sole purification procedure (11). The affinity chromatography technique has also been used to isolate transcobalamin II (12) and hog gastric mucosa vitamin B12-binding proteins.

DISCUSSION

Isolation of the trace vitamin B12-binding proteins of mammalian tissues by conventional techniques has proven difficult because of the extremely small amounts of these proteins present in most tissues. Thus, even with relatively high yields in each step of a complex purification scheme the overall yield remains poor in the case of intrinsic factor, and isolation of transcobalamin I and transcobalamin II from plasma has proven impossible (2). The vitamin B12-Sepharose which we have prepared circumvents most of these difficulties since vitamin B12-binding proteins can be removed from crude extracts of tissues or from plasma with nearly quantitative yields. We have studied the elution of three proteins from columns of vitamin B12-Sepharose including a human granulocyte vitamin B12-binding protein, human transcobalamin II, and the vitamin B12-binding proteins from hog gastric mucosa. In each case we have been able to recover over 80% of the native vitamin B12-binding protein contained in these extracts based on their capacity to bind the vitamin. We have eluted the proteins from vitamin B12-Sepharose with guanidine-HCl (7.5 mM) and have been able to renature the eluted proteins by dialysis of the guanidine eluates in the presence of an excess of vitamin B12. Preliminary experiments suggest that it may also be possible to elute these proteins with vitamin B12 itself, although thus far this has not been necessary.

Other potential uses of vitamin B12-Sepharose would be to serve in isolation of various enzymes which carry vitamin B12 derivatives as prosthetic groups. In this case the enzymes might be eluted from columns by any technique which removes the prosthetic group.

1 R. H. Allen, unpublished experiments.

TABLE III

Binding of hog gastric mucosa vitamin B12-binding proteins to vitamin B12-Sepharose

A hog gastric mucosa extract was applied to a column (1.5 x 6 cm) of vitamin B12-Sepharose. Fractions of 16.5 ml were collected. The column was then eluted with six solutions of different composition. The material eluted with each solution was collected separately. The total vitamin B12-binding activity adsorbed and retained by the vitamin B12-Sepharose was calculated from the difference between the amount applied to the column and the amount present in the initial effluent and the further eluates.

<table>
<thead>
<tr>
<th>Item applied to vitamin B12-Sepharose</th>
<th>Volume</th>
<th>Vitamin B12-binding activity</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Hog gastric mucosa extract applied</td>
<td>985 ml</td>
<td>2,660 mg/ml</td>
<td>100.0</td>
</tr>
<tr>
<td>B. Vitamin B12-Sepharose eluent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 10........................</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 20........................</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 50........................</td>
<td>297</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total of pooled Fractions 1 to 60...</td>
<td>985</td>
<td>148 mg/ml</td>
<td>5.6</td>
</tr>
<tr>
<td>C. Further Vitamin B12-Sepharose eluates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 0.1 M Pyridine + 1.0 M NaCl.......</td>
<td>1,280</td>
<td>50 mg/ml</td>
<td>0.004</td>
</tr>
<tr>
<td>2. 0.1 M Sodium acetate, pH 4.8......</td>
<td>108</td>
<td>2 mg/ml</td>
<td>0.02</td>
</tr>
<tr>
<td>3. 0.1 M Glycine-NaOH, pH 10.0........</td>
<td>100</td>
<td>23 mg/ml</td>
<td>0.002</td>
</tr>
<tr>
<td>4. 0.1 M Glycine-NaOH, pH 10.0 + 0.2 M glucose.</td>
<td>100</td>
<td>42 mg/ml</td>
<td>0.002</td>
</tr>
<tr>
<td>5. 0.1 M Glycine-NaOH, pH 10.0 + 0.9 M NaCl.</td>
<td>110</td>
<td>47 mg/ml</td>
<td>0.004</td>
</tr>
<tr>
<td>6. 0.1 M Potassium phosphate, pH 7.5</td>
<td>90</td>
<td>9 mg/ml</td>
<td>0.004</td>
</tr>
<tr>
<td>Total of further eluates................</td>
<td>76,400</td>
<td>2.9 mg/ml</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Total adsorbed and retained........... 2,390,000 mg/ml 91.5%
Previous attempts to utilize affinity chromatography for isolation of vitamin B12-binding proteins have met with limited success. Gräsbäck, et al. (15) were unable to elute transcobalamin II from a derivative of vitamin B12 attached to cellulose and Sephadex. Recently, Olesen et al. (16) reported the preparation of an affinity adsorbent in which vitamin B12 was covalently attached to albumin using a carbodiimide and this derivative was attached subsequently to bromoacetylcellulose. The yield of vitamin B12 bound was only 0.065 pmole of vitamin B12 attached per g of cellulose, which is over 10-fold less than that obtained in our preparation of vitamin B12-Sepharose. The linkage of the vitamin B12 to albumin was presumably through the phosphate group of the nucleotide moiety of vitamin B12. This derivative was tested as an affinity adsorbent using gastric juice and serum and was found to bind 30% and 50%, respectively, of the vitamin B12-binding protein in these extracts. The recovery after elution from the affinity adsorbent was only 12% for the gastric binder and 38% for the serum vitamin B12-binding proteins. One possible explanation for the apparent poor binding activity using this derivative is that the vitamin was attached directly to albumin which may hinder the binding site for the vitamin B12-binding proteins. The vitamin B12-Sepharose which we have prepared is removed from the bulk of the Sepharose particles by the 3,3'-diaminodipropylamine sidearm, thus facilitating binding of protein to the vitamin. The capacity of vitamin B12-Sepharose for binding vitamin B12-binding proteins is illustrated by the fact that a 2.5 cm diameter by 2.0 cm height column can bind all of the unsaturated transcobalamin II contained in at least 3000 liters of human plasma (approximately 90 mg of transcobalamin II).

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REFERENCES