Isolation of Gastric Vitamin B₁₂-binding Proteins Using Affinity Chromatography

II. PURIFICATION AND PROPERTIES OF HOG INTRINSIC FACTOR AND HOG NONINTRINSIC FACTOR*

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SUMMARY

Two vitamin B₁₂-binding proteins, designated hog intrinsic factor and hog nonintrinsic factor, have been isolated from hog gastric mucosa. Affinity chromatography on vitamin B₁₂-Sepharose resulted in the removal of the bulk of protein present in a crude extract of hog gastric mucosa. The two vitamin B₁₂-binding proteins were separated subsequently by a method of “selective” affinity chromatography with an affinity adsorbent containing covalently bound derivatives of vitamin B₁₂ that lack the nucleotide portion of the native vitamin. Under appropriate conditions hog nonintrinsic factor was adsorbed to a column of this material, while hog intrinsic factor was not. After additional purification both proteins were isolated in homogeneous form based on polyacrylamide disc gel electrophoresis, sedimentation equilibrium ultracentrifugation, and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Hog intrinsic factor (24 μg) corrected vitamin B₁₂ malabsorption when given to a patient with pernicious anemia, while hog nonintrinsic factor (49 μg) had no effect.

Hog intrinsic factor binds 30.3 μg of vitamin B₁₂ per mg of protein. Molecular weight values of 52,300 to 58,600 were obtained by sedimentation equilibrium ultracentrifugation and amino acid and carbohydrate analysis. The protein contains 17.5% carbohydrate which accounts for the elevated molecular weight values (66,000 to 75,000) obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration. In the presence of vitamin B₁₂ hog intrinsic factor aggregates to form dimers and higher molecular weight oligomers.

Hog nonintrinsic factor binds 25.1 μg of vitamin B₁₂ per mg of protein. Molecular weight values of 61,000 to 66,000 were obtained by sedimentation equilibrium ultracentrifugation and amino acid and carbohydrate analysis. The protein contains 35.5% carbohydrate which accounts for the elevated molecular weight values (100,000 to 130,000) obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration. Hog nonintrinsic factor retains its monomeric form in the presence of vitamin B₁₂ under conditions in which hog intrinsic factor aggregates.

Hog intrinsic factor and hog nonintrinsic factor differ significantly from each other in their amino acid and carbohydrate composition. These differences, together with differences in other parameters, demonstrate that these two proteins are distinct and separate species.

Crude extracts of hog gastric mucosa have a vitamin B₁₂-binding activity of approximately 0.05 μg of vitamin B₁₂ bound per mg of protein, and these extracts also contain intrinsic factor activity since approximately 60 mg of dry material are able to correct vitamin B₁₂ malabsorption by patients with pernicious anemia as judged by the results of Schilling tests (1). All of the vitamin B₁₂-binding activity in these crude extracts is not attributable to hog IF, however, for a second vitamin B₁₂-binding protein is present that lacks IF activity in Schilling tests (1, 2). This second vitamin B₁₂-binding protein has been referred to as hog NIF.

Studies using extracts of hog gastric mucosa or partially purified preparations of these two proteins are somewhat difficult to interpret, but they suggest that hog IF and hog NIF also differ from each other in other ways. These differences include: (a) anti-IF antibody obtained from the serum of certain patients with pernicious anemia blocks some of the vitamin B₁₂-binding activity present in crude extracts of hog gastric mucosa. Irvine (3) has demonstrated that the amount of anti-IF antibody-mediated decrease in vitamin B₁₂-binding activity is a more reliable guide to the Schilling test IF activity of crude hog gastric mucosal preparations than is the level of total vitamin B₁₂-binding activity. This observation suggests that the vitamin B₁₂-binding ability of hog IF is blocked by the antibody, while that of hog NIF is not. (b) Sera from rabbits immunized with hog leukocyte extracts contain antibodies which appear to bind to hog NIF but not to hog IF (4). (c) Hog IF appears to facilitate

* This work was supported by Grants AM 16668, AM 10550, and HE 00022 and Special Research Fellowship AM 51261 from the National Institutes of Health.

1 The abbreviations used are: IF, intrinsic factor vitamin B₁₂-binding protein; NIF, nonintrinsic factor vitamin B₁₂-binding protein; pseudo-vitamin B₁₂, α-(adenyl)-cobamide cyanide.
calcium-dependent vitamin B₁₂ binding by guinea pig ileal mucosal homogenates, while hog NIF does not (5). (d) Hog NIF appears to facilitate calcium-dependent vitamin B₁₂ binding by rat ileal homogenates, while hog IF does not (5). (e) Certain structural analogs of vitamin B₁₂ are bound by hog NIF to a greater extent than they are by hog IF (1).

Numerous attempts have been made to isolate hog IF and hog NIF in homogeneous form (6). Holdsworth (2) and Ellenbogen et al. (1, 7) have isolated vitamin B₁₂-binding proteins from hog gastric mucosa in highly purified form and were able to separate the vitamin B₁₂-binding protein into two fractions using ion exchange chromatography. In both studies one fraction contained IF based on Schilling tests, while the second fraction did not. Immunologic and ileal and liver homogenate binding studies were not performed, but Ellenbogen et al. (1) did demonstrate that their IF active fraction bound only 10% as much pseudo-vitamin B₁₂ as native vitamin B₁₂, while their IF inactive fraction bound 90% as much pseudo-vitamin B₁₂ as native vitamin B₁₂.

Ellenbogen et al. (1, 7) observed a molecular weight of approximately 50,000 for both of their protein fractions in the absence of vitamin B₁₂ using sedimentation velocity ultracentrifugation. In the presence of vitamin B₁₂, molecular weight values of 100,000 were obtained for both fractions suggesting that the protein in both fractions dimerized in the presence of the vitamin. Holdsworth (2) also observed molecular weight values of approximately 50,000 for both of his protein fractions, but he did not observe any significant increase when vitamin B₁₂ was present. Neither Ellenbogen et al. nor Holdsworth were able to detect any significant differences in the amino acid compositions of their IF active and IF inactive fractions. The same type of carbohydrate residues were observed in both fractions. Ellenbogen et al. did note that their IF active fraction contained twice as much galactose as their IF inactive fraction, but this difference was not observed by Holdsworth who noted that hog IF contained approximately 30% less of each type of carbohydrate residue when compared with hog NIF. Thus immunologic studies, ileal and liver homogenate binding studies, and analog binding studies all suggest that hog IF and hog NIF are structurally dissimilar. On the other hand, studies of molecular weight, protein aggregation, and amino acid and carbohydrate composition have failed to reveal any major and consistent differences between hog IF and hog NIF. It is thus unclear whether hog NIF is (a) azymogen-like precursor of hog IF, (b) a limited degradation product of hog IF, or (c) structurally unrelated to hog IF.

We have previously reported (8) that the hog gastric mucosal vitamin B₁₂-binding proteins can be isolated in high yield using affinity chromatography on vitamin B₁₂-Sepharose which is prepared by covalently attaching monocarboxylic acid derivatives of vitamin B₁₂ to a substituted Sepharose using a carbodiimide. Analysis of the vitamin B₁₂-binding protein obtained after affinity chromatography revealed that only 30% of the vitamin B₁₂-binding protein is IF based on assays using anti-IF antibody obtained from the serum of a pernicious anemia patient. Attempts to separate hog IF from hog NIF using ion exchange chromatography have been unsuccessful. This difficulty has been circumvented, however, and hog IF and hog NIF have been resolved using a method of "selective" affinity chromatography in which carboxylic acid derivatives of vitamin B₁₂ that lack the nucleotide portion of the vitamin are covalently coupled to Sepharose. Under appropriate conditions hog NIF is adsorbed to a column of this material, while hog IF is not. This report is concerned with the isolation, separation, and properties of hog IF and hog NIF.

**EXPERIMENTAL PROCEDURES**

**Materials**

Hog intrinsic factor concentrate, type S, 80 mesh, Lot 48744-414, was obtained from Lederle. This material is a crude water extract of hog pyloric mucosa that was spray-dried without additional purification. We have stored this material in the dry state at 4° for up to 1 year without noting any loss of vitamin B₁₂-binding activity. All other materials were obtained as described in the accompanying paper in this series (9).

**Methods**

**Preparation of Sepharose-bound Derivatives of Vitamin B₁₂ Lacking the Nucleotide—**Vitamin B₁₂, 10.0 g, containing 2 µCi of [³²P]vitamin B₁₂, was dissolved in 500 ml of 11N HCl and incubated at 70° for 1 hour in the dark. The solution was subsequently lyophilized, dissolved in 500 ml of H₂O, and lyophilized again. The dry material was dissolved in water to give a concentration of vitamin B₁₂ derivative of 25.0 mg per ml and stored at -20°.

These derivatives of vitamin B₁₂ were covalently attached to 3,3'-diaminodipropylamine-substituted Sepharose at pH 5.6 using 1-ethyl-3-(3-diethylaminopropyl)-carbodiimide. The concentration of vitamin B₁₂ derivatives in the coupling reaction prior to the addition of the carbodiimide was 12.5 mg per ml. The coupling reaction and subsequent washing and storage of the Sepharose substituted with vitamin B₁₂ derivatives were performed under the same conditions used for the preparation of vitamin B₁₂-Sepharose (8). The washed Sepharose containing the 11N HCl hydrolysis derivatives of vitamin B₁₂ had a vitamin B₁₂ derivative content of 0.22 µmole per ml of packed Sepharose based on measurements of radioactivity.

Paper chromatography of vitamin B₁₂ and its derivatives was performed as previously described (8). The amount of individual components was determined by cutting out individual spots and counting them in a Packard γ scintillation counter.

All other methods were performed as described in the accompanying paper in this series (9).

**Purification of Hog IF and Hog NIF**

**Step 1: Preparation of Hog Gastric Mucosal Extract—**All procedures were performed at 4° unless specifically noted. Hog intrinsic factor concentrate, 50 g, was added to 1.0 liter of 0.1 M Tris-acetate, pH 9.2, and stirred for 30 min. The suspension was centrifuged at 20,000 × g for 30 min, and the turbid supernatant was decanted. The supernatant was filtered with vacuum suction through Celite using a Buchner funnel containing a coarse scintillation glass disc. The filtrate was centrifuged at 20,000 × g for 30 min, and the supernatant was immediately subjected to affinity chromatography on vitamin B₁₂-Sepharose.

**Step 2: Affinity Chromatography on Vitamin B₁₂-Sepharose—**A column (2.5 × 7.5 cm) of vitamin B₁₂-Sepharose containing 12.0 mg of covalently bound vitamin B₁₂ was prepared and washed with 200 ml of 0.1 M glycine-NaOH, pH 10.0, followed by 100 ml of 0.1 M potassium phosphate, pH 7.5, immediately prior to the sample application to remove any hydrolyzed vitamin B₁₂. The flow rate was 100 ml per hour. After the entire sample had passed onto the column, the column was washed with 25 ml of 0.1 M potassium phosphate, pH 7.5. The first 900 ml of effluent were collected in their entirety. The column was
**TABLE I**

**Affinity chromatography of hog gastric vitamin B12-binding proteins**

| Item | Volume (ml) | Vitamin B12-binding activity (µg/ml) | Total µg | % IF | Protein (mg/ml) | Total µg | Flow rate (ml/hr) |
|------|-------------|-------------------------------------|----------|------|----------------|----------|------------------|
| Hog gastric mucosal extract applied to vitamin B12-Sepharose | 875 | 2.99 | 2620 | 29.6 | 27.0 | 23,600 | 100 |
| Initial vitamin B12-Sepharose effluent | 900 | 0.0042 | 3.36 | 70.2 | 25.0 | 22,500 | |
| Further elutions of vitamin B12-Sepharose | | | | | | | |
| 1. 0.1 M potassium phosphate, pH 7.5 | 500 | 0.00034 | 0.17 | 64.7 | 0.87 | 435 | 150 |
| 2. 0.1 M glycine-NaOH, pH 10.0, 0.1 M glucose, 1.0 M NaCl | 1,000 | 0.00000 | 0.00 | 0.03 | 0.06 | 30 | 150 |
| 3. 0.1 M potassium phosphate, pH 7.5 | 500 | 0.00075 | 0.39 | 59.7 | 0.02 | 10 | 150 |
| 4. 0.1 M potassium phosphate, pH 7.5, 7.5 M guanidine-HCl | | | | | | | |
| a. Initial eluate | 65 | 32.8 | 2130 | 24.4 | 2.68 | 174 | 40 |
| b. Eluted 17 hours after 4a | 65 | 0.08 | 5.2 | 27.6 | 0.00 | 0.00 | 40 |

Then eluted as follows: (a) 500 ml of 0.1 M potassium phosphate, pH 7.5; (b) 1000 ml of 0.1 M glycine-NaOH, pH 10.0, containing 0.1 M glucose and 1.0 M NaCl; and (c) 500 ml of 0.1 M potassium phosphate, pH 7.5. The fourth elution solution consisted of 0.1 M potassium phosphate, pH 7.5. The fourth elution solution consisted of 7.5 M guanidine HCl. When 10 ml of this solution had passed through the column, flow was stopped. After 1 hour an additional 55 ml of eluate were collected, pooled with the first 10 ml, and designated as Eluate 4a. Flow was stopped for an additional 17 hours and then an additional 65 ml of eluate were collected and designated as Eluate 4b.

The starting material, initial column effluent, and each of the column eluates were assayed for vitamin B12-binding activity, IF activity, and protein content. The results are summarized in Table I.

Eluate 4a was dialyzed against 6.0 liters of distilled water for 24 hours with dialysis changes at 4 and 16 hours. The sample was concentrated with an Amicon ultrafiltrator equipped with a Diaflo UM-10 membrane and centrifuged at 20,000 X g for 10 min. The supernatant, 8.5 ml, was decanted and 1.10 ml of 1.0 M sodium acetate, pH 5.0, followed by 130 ml of 7.5 M sodium acetate, pH 5.0, and finally with 300 ml of 1.0 M sodium acetate, pH 5.0, and 1.47 ml of 7.5 M potassium phosphate, pH 7.5. The fourth elut solution consisted of 0.1 M potassium phosphate, pH 7.5, containing 0.75 M NaCl.

Further Puriification of Hog IF

Repeat Affinity Chromatography on Vitamin B12-Sepharose—Fractions 11 to 13 from Step 3 were pooled and dialyzed for 24 hours against 2.0 liters of 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl with dialysate changes at 4 and 16 hours. The sample was applied to a column (0.9 x 18 cm) of substituted Sepharose containing covalently bound derivatives of vitamin B12 that lacked the nucleotide portion of the vitamin was prepared at room temperature and equilibrated with 0.1 M sodium acetate, pH 5.0, containing 1.0 M guanidine HCl. The sample (from purification Step 2) consisted of a mixture of hog IF and hog NIF in 11.0 ml of equilibrating buffer. The column was eluted initially with equilibrating buffer followed, as indicated by the position of the arrow, by 0.1 M sodium acetate, pH 5.0, containing 7.5 M guanidine HCl. The flow rate was 50 ml per hour and 9.0-ml fractions were collected.

**Fig. 1.** Selective affinity chromatography (purification Step 3) of hog IF and hog NIF. A column (3.5 x 18 cm) of substituted Sepharose containing covalently bound derivatives of vitamin B12 that lacked the nucleotide portion of the vitamin was prepared at room temperature and equilibrated with 0.1 M sodium acetate, pH 5.0, containing 1.0 M guanidine HCl. The sample (from purification Step 2) consisted of a mixture of hog IF and hog NIF in 11.0 ml of equilibrating buffer. The column was eluted initially with equilibrating buffer followed, as indicated by the position of the arrow, by 0.1 M sodium acetate, pH 5.0, containing 7.5 M guanidine HCl. The flow rate was 50 ml per hour and 9.0-ml fractions were collected. O, A185; $\Delta$, percentage of vitamin B12-binding activity attributable to hog IF; $\bullet$, hog IF; $\sigma$, hog NIF.
hours with dialysate changes at 4 and 16 hours. The sample at 20,000
was concentrated to 6.0 ml as described in Step 2 and centrifuged
at 20,000 x g for 10 min. The supernatant was applied to a
column (2.0 x 90 cm) of Sephadex G-150, fine grade, that had
been equilibrated with 0.05 M potassium phosphate, pH 7.5, containing
0.75 M NaCl. The flow rate was 20 ml per hour, and 4.0-ml
fractions were collected. Appropriate fractions were assayed for:
\( A_{280} \), \( A_{320} \), and \( \beta \), vitamin B\(_{12}\)-binding ability.

Fig. 2. Sephadex G-150 chromatography of hog NIF. The
material present in Fractions 33 and 34 from selective affinity
chromatography (purification Step 3) was applied to a column
(2.0 x 98 cm) of Sephadex G-150 equilibrated with 0.05 M potas-
sium phosphate, pH 7.5, containing 0.75 M NaCl. The sample
volume was 6.0 ml, and 4.0-ml fractions were collected. Approp-
riate fractions were assayed for: \( \phi \), \( A_{280} \); \( \Delta \), \( A_{195} \); and \( \theta \), vita-
mín B\(_{12}\)-binding activity.

It is important that selective affinity chromatography be
carried out as described in Step 3 of the purification scheme (see
Fig. 1) since deviations in flow rate, pH, temperature, initial
guanidine concentration, and column size (i.e. total vitamin B\(_{12}\)
derivative content) relative to sample size and content can
result in decreased separation ofhog IF from hog NIF. Thus,
slower flow rates, neutral pH, decreased temperature, lower
initial guanidine concentrations, increases in column size relative
to the amount of hog IF and hog NIF applied, and substitution
of vitamin B\(_{12}\)-Sepharose for the selective affinity adsorbent all
have the effect of increasing the amount of hog IF that is ad-
sorbed to the column without affecting the adsorption of hog
NIF. Increases in flow rate and initial guanidine concentra-
tion as well as decreases in relative column size have the effect
of decreasing the adsorption of both hog IF and hog NIF.

The first 7.5 cm guanidine eluate (Eluate 4a) from the initial
vitamin B\(_{12}\)-Sepharose column (see Table I) has a visible yellow
color and broad but significant absorption that declines gradually
from 300 to 500 nm. This colored material co-chromatographs
with hog NIF during selective affinity chromatography since
fraction by fraction correspondence with hog NIF could be
demonstrated visually and by measurements of \( A_{280} \). This
unbound vitamin B\(_{12}\) is removed under these conditions. The
proteins were stored at \(-20^\circ\).
Removal of Vitamin B₁₂—Greater than 99% of bound vitamin B₁₂ can be removed from hog IF and hog NIF by dialysis at 22° for 72 hours against 15 volumes of 0.1 M phosphate buffer in the presence of sodium dodecyl sulfate. Studies based on sedimentation equilibrium ultracentrifugation and renaturation of these two proteins from guanidine and suggests that the presence of vitamin B₁₂ is not required to achieve renaturation of vitamin B₁₂-binding activity. In other experiments, guanidine was first removed by dialysis prior to the addition of excess vitamin B₁₂ and subsequent dialysis to remove unbound vitamin. In these experiments, hog IF and hog NIF both bound more vitamin B₁₂ than when the two proteins were diluted 1:10,000 in guanidine-free buffer and assayed directly for vitamin B₁₂-binding activity. Other factors such as protein concentration and the rate of renaturation may play important roles. Detailed studies concerning the renaturation process have not been conducted for hog IF and hog NIF.

Schilling Test—The results of Schilling tests performed on a single pernicious anemia patient are presented in Table III and demonstrate that 24 μg of hog IF were able to restore vitamin B₁₂ absorption to a normal level. Hog NIF, at a dose of 49 μg, was ineffective.

Colored material was separated from hog NIF by gel filtration on Sephadex G-150 (see Fig. 2) where A₂₈₀ co-chromatographed with the small A₁₃₀ peak that was eluted prior to the major A₂₈₀ peak that coincided with the single peak of vitamin B₁₂-binding activity. The nature of this yellow material is unknown.

A summary of the purification of hog IF and hog NIF is presented in Table II. Hog IF has been purified 920-fold with a yield of 26%. Hog NIF has been purified 320-fold with a yield of 19%. Hog NIF contains 25.1 μg of vitamin B₁₂ and has an A₂₈₀ of 1.56, an A₁₃₀ of 0.74, and a ratio of A₁₃₀/A₂₈₀ of 2.13. The final preparations of both proteins are homogeneous based on sedimentation equilibrium ultracentrifugation and polyacrylamide gel electrophoresis performed in the presence and absence of sodium dodecyl sulfate. Studies (see below) employing anti-IF antibody and pseudo-vitamin B₁₂ indicate that purified hog IF and hog NIF are free of significant contamination with each other.

Removal of Vitamin B₁₂—Greater than 99% of bound vitamin B₁₂ can be removed from hog IF and hog NIF by dialysis at 22° for 72 hours against 15 volumes of 0.1 M phosphate buffer in the presence and absence of sodium dodecyl sulfate. Studies (see below) employing anti-IF antibody and pseudo-vitamin B₁₂ indicate that purified hog IF and hog NIF are free of significant contamination with each other.

Renaturation of Hog IF and Hog NIF—The data presented in Table II (see Steps 3A₂, 3A₃, 3B₄, and 3B₅) indicate that when an excess of vitamin B₁₂ was added to hog IF and hog NIF, it was ineffective.

### Table II

| Step | Volume (ml) | Vitamin B₁₂-binding activity (μg) | Protein (mg) | Specific activity (μg vitamin B₁₂ bound/mg protein) | A₁₃₀/A₂₈₀ |
|------|-------------|----------------------------------|--------------|-----------------------------------------------|-------------|
| 1. Hog gastric mucosal extract | 875 | 2,620 | 29.6 | 23,600 | 0.111 |
| 2. Affinity chromatography on vitamin B₁₂-Sepharose | 65.0 | 2,130 | 24.4 | 174 | 12.2 |
| 3. Selective affinity chromatography | | | | | |
| A. Hog IF | | | | | |
| A₁: fractions (11 to 13) from selective affinity chromatography | 27.0 | 148 | 92.7 | 8.65 | 17.1 |
| A₁: repeat affinity chromatography on vitamin B₁₂-Sepharose | 9.9 | 122 | 97.3 | 4.89 | 24.9 |
| A₁: after the addition of 360 μg vitamin B₁₂ to A₁ followed by dialysis | 16.3 | 148* | 97.3 | 4.89 | 30.3 |
| B. Hog NIF | | | | | |
| B₁: fractions (33 to 34) from selective affinity chromatography | 18.0 | 1,010 | 7.6 | 67.4 | 15.0 |
| B₂: Sephadex G-150 | 22.0 | 366 | 2.1 | 28.9 | 12.7 |
| B₃: 3,3'-diaminodipropylamine-Sepharose | 45.0 | 307 | 5.3 | 25.2 | 12.2 |
| B₄: repeat affinity chromatography on vitamin B₁₂-Sepharose | 28.0 | 308 | 3.6 | 19.1 | 16.1 |
| B₅: after the addition of 900 μg vitamin B₁₂ to B₅ followed by dialysis | 43.7 | 480* | 3.6 | 19.1 | 25.1 |

* Vitamin B₁₂ content.

### Table III

| Protein ingested | [¹⁵⁸⁷Co]Vitamin B₁₂ ingested mg | Volume (ml) | [¹⁵⁸⁷Co]Vitamin B₁₂ content (% ingested) |
|------------------|--------------------------------|-------------|--------------------------------------|
| None | | | |
| 501 | 24 μg Hog IF | 1400 | 13 | 1.4 |
| 501 | 49 μg Hog NIF | 1250 | 13 | 2.5 |

Urine collected for 24 hours following the ingestion of [¹⁵⁸⁷Co]vitamin B₁₂.
Inhibition of Vitamin B₁₂ Binding by Anti-IF Antibody—The results of experiments performed to determine the ability of anti-IF antibody to inhibit vitamin B₁₂ binding by hog IF, hog NIF, and human IF are presented in Fig. 3. At a level of 200 μl of antibody, hog IF and human IF were inhibited approximately 98%, while only approximately 3% inhibition was noted with hog NIF. The inhibition curves obtained suggest that this particular antibody has a significantly lower affinity for hog IF than for human IF.

Interaction with Pseudo-vitamin B₁₂—Samples of hog IF, hog NIF, and human IF in 7.5 M guanidine HCl were diluted 1:10,000 in 0.1 M potassium phosphate, pH 7.5, and utilized to study the ability of pseudo-vitamin B₁₂ to block vitamin B₁₂ binding by these proteins at 4°C. The results are presented in Table IV, and the 22-hour data indicate that hog IF and hog NIF contain less than 5% contamination with each other. The fact that prior incubation with pseudo-vitamin B₁₂ results in a definite decrease in the rate of [⁶⁷Co]vitamin B₁₂ binding by hog IF indicates that this protein does bind pseudo-vitamin B₁₂, although with a lower affinity than for vitamin B₁₂. The fact that prior incubation with pseudo-vitamin B₁₂ slows the rate of subsequent [⁶⁷Co]vitamin B₁₂ binding by human IF to a lesser degree than for hog IF suggests that human IF may bind pseudo-vitamin B₁₂ with a lower affinity relative to vitamin B₁₂ than does hog IF. No definite conclusion can be reached in regard to this latter point, however, since association and dissociation rates will obviously influence the time course data presented in Table IV.

Polyacrylamide Disc Gel Electrophoresis—The results of the polyacrylamide disc gel electrophoresis experiments are presented in Fig. 4. Single protein bands were observed in the absence of vitamin B₁₂ with 25 μg of hog IF and 25 μg of hog NIF (Gels A and C, Fig. 4). When 25 μg of hog IF saturated with vitamin B₁₂ were studied, a series of protein bands was observed that appeared closer and closer together as one approached the top of the gel (Gel B, Fig. 4). This finding suggests that hog IF aggregates in the presence of vitamin B₁₂.

### Table IV

| Item         | Nonradioactive compound present during 30 min preincubation | [⁶⁷Co]Vitamin B₁₂ bound at different time periods following the addition of 1000 pg | % |
|--------------|-----------------------------------------------------------|-----------------------------------------------------------------|---|
|              |                                                           | 0.4 min | 1.0 min | 2.0 min | 5.0 min | 30 min | 2 hr | 22 hr |
| Hog IF       | None                                                      | 176     | 323     | 341     | 401     | 533     | 548     | 690   | 100.0 |
| Hog IF       | 1500 pg pseudo-vitamin B₁₂                                | 93      | 107     | 126     | 134     | 303     | 456     | 602   | 95.3  |
| Hog IF       | 1500 pg vitamin B₁₂                                       | 0       | 0       | 1       | 0       | 6       | 12      | 46    | 7.9   |
| Hog NIF      | None                                                      | 255     | 307     | 516     | 657     | 802     | 906     | 972   | 100.0 |
| Hog NIF      | 1500 pg pseudo-vitamin B₁₂                                | 0       | 0       | 1       | 0       | 0       | 7       | 19    | 1.9   |
| Hog NIF      | 1500 pg vitamin B₁₂                                       | 0       | 0       | 0       | 1       | 0       | 10      | 1    | 1.0   |
| Human IF     | None                                                      | 148     | 160     | 221     | 305     | 550     | 548     | 613   | 100.0 |
| Human IF     | 1500 pg pseudo-vitamin B₁₂                                | 95      | 127     | 168     | 291     | 477     | 537     | 594   | 97.0  |
| Human IF     | 1500 pg vitamin B₁₂                                       | 0       | 1       | 1       | 1       | 9       | 16      | 50    | 8.1   |

Fig. 3. Anti-IF antibody studies. Constant amounts of hog IF, hog NIF, and human IF were incubated at 22°C for 30 min with varying amounts (0 to 200 μl) of anti-IF antibody obtained from the serum of a patient with pernicious anemia. At the end of a 30-min incubation period, 1000 pg of [⁶⁷Co]vitamin B₁₂ were added; after an additional 20 min, the amount of vitamin bound was determined using the charcoal adsorption method. The amount of [⁶⁷Co]vitamin B₁₂ bound was plotted versus the amount of antibody present. ●, hog NIF; ○, hog IF; △, human IF.

Fig. 4. Polyacrylamide disc gel electrophoresis of hog IF and hog NIF in the absence and presence of vitamin B₁₂. Protein samples devoid of vitamin B₁₂ were renatured from guanidine by dialysis against H₂O for 72 hours at 4°C. Protein samples saturated with vitamin B₁₂ were renatured as described above except that excess vitamin B₁₂ was added prior to dialysis. Gel A, 25 μg of hog IF devoid of vitamin B₁₂; Gel B, 25 μg of hog IF containing 0.76 μg of bound vitamin B₁₂; Gel C, 25 μg of hog NIF devoid of vitamin B₁₂; Gel D, 25 μg of hog NIF containing 0.63 μg of bound vitamin B₁₂. All four gels were subjected to electrophoresis at the same time. Electrophoresis was terminated when the tracking dye approached the bottom of the gels.
When a sample of hog NIF saturated with vitamin B₁₂ was studied under similar conditions, straight lines were obtained for plots of ln $A_{280}$ versus $R^2$ and ln $A_{280}$ versus $R^3$ (Fig. 5D). Molecular weight values of 63,100 and 61,300 were obtained for the hog NIF-vitamin B₁₂ complex using the $A_{280}$ and $A_{320}$ data, respectively. These two values are not significantly different from each other and demonstrate correspondence between protein and vitamin B₁₂. The molecular weight values obtained for the hog NIF-vitamin B₁₂ complex (63,100 and 61,300) are not significantly different from the value of 66,000 obtained for hog NIF devoid of vitamin B₁₂. This fact indicates that hog NIF did not aggregate in the presence of vitamin B₁₂ under the conditions in which this experiment was performed.

When a sample of hog IF saturated with vitamin B₁₂ was studied, plots of ln $A_{280}$ versus $R^2$ and ln $A_{280}$ versus $R^3$ revealed significant curvature (Fig. 5B). The fact that the degree of curvature is the same for both plots indicates correspondence between protein and vitamin B₁₂. When portions of the two curves were used for molecular weight calculations, values ranging from 60,000 to 62,000 were obtained in each case. This range of molecular weight values indicates that the hog IF-vitamin B₁₂ complex existed as a mixture of monomers and higher molecular weight oligomers under the conditions in which this experiment was performed.

**Amino Acid and Carbohydrate Composition**—The results of the amino acid and carbohydrate analyses are presented in Table V. The values presented represent the average of two separate analyses for each protein. Hog IF contains 17.5% carbohydrate and hog NIF contains 35.5%. Based on the molecular weights of the individual amino acids and carbohydrates determined, hog IF contains 58,600 g of amino acid and carbohydrate per mole of bound vitamin B₁₂. A value of 64,000 g of amino acid and carbohydrate per mole of bound vitamin B₁₂ was obtained for hog NIF. These values are close to the molecular weight values obtained for hog IF (52,300) and hog NIF (63,500) by sedimentation equilibrium ultracentrifugation (see above) and indicate that both proteins contain single vitamin B₁₂-binding sites.

The amino acid and carbohydrate compositions of hog IF and hog NIF are quite similar, but significant differences do exist. Hog IF contains significantly greater amounts of threonine, proline, and methionine than hog NIF, while the latter protein contains significantly more of each of the six carbohydrates that are present in both proteins.

The sullhydrolyl group content of hog IF and hog NIF was assayed in 0.1 M potassium phosphate containing 7.5 M guanidine HCl. No free sulfhydryl groups were detected (<0.1 residue per mole), suggesting that all of the cysteine residues present in these proteins are involved in disulfide bonds.

**Molecular Weight Determinations by Gel Filtration**—When samples of hog IF and hog NIF devoid of vitamin B₁₂ were subjected to gel filtration on Sephadex G-150, single peaks of vitamin B₁₂-binding activity were observed for both proteins (Fig. 6, A and C). Based on the elution positions of the two proteins, an apparent molecular weight of 74,000 was calculated for hog IF and a value of 130,000 was calculated for hog NIF. These values are significantly higher than the molecular weights obtained for these two proteins using sedimentation equilibrium ultracentrifugation and amino acid and carbohydrate analyses (see above). Protein aggregation appears to be an unlikely explanation for the discrepancies since gel filtration was performed at lower protein concentrations and in the same buffer as was employed for sedimentation equilibrium ultra-
centrifugation. A more likely explanation is that the gel filtration molecular weight values are falsely elevated due to the fact that hog IF and hog NIF contain, respectively, 17.5% and 33.5% carbohydrate. This phenomenon has been reported for other glycoproteins (9, 11, 12).

When a sample of the hog NIF-vitamin B_{12} complex was studied by gel filtration, the result presented in Fig. 6D was obtained. Under these conditions, hog NIF eluted as a single symmetrical peak with an apparent molecular weight of 130,000, which is essentially the same as the value of 130,000 that was obtained when the protein was eluted in the absence of vitamin B_{12} (Fig. 6C). This observation indicates that hog NIF did not aggregate in the presence of vitamin B_{12} under the conditions in which this experiment was performed.

When the hog IF-vitamin B_{12} complex was studied (Fig. 6B), two peaks of [^{57}Co]vitamin B_{12} were observed with apparent molecular weights of 75,000 and 160,000. This indicates that a mixture of hog IF monomers and dimers was present, and that aggregation in the presence of vitamin B_{12} had occurred. The fact that a shoulder is present on the leading edge of the 160,000 molecular weight peak (Fig. 6B) suggests that trimers or tetramers of hog IF may also have been present.

The experiments presented in Fig. 6, B and D were repeated under identical conditions except that the protein samples were incubated in 0.5 ml of gel filtration buffer at 37°C for 8 hours prior to being cooled to 4°C and placed on the Sephadex G-150 column. In both cases, no change in the elution pattern of [^{57}Co]vitamin B_{12} was observed.

**Equilibrium Dialysis**—The results of equilibrium dialysis experiments are presented in Fig. 8. A value of 1.5 \times 10^{9} M^{-1} was obtained for the association constant, K_{a}, for hog IF and vitamin B_{12}. A value(551,964),(688,982) of 1.3 \times 10^{9} M^{-1} was obtained for hog NIF and vitamin B_{12}. These two values are not significantly different and are similar to the value of 1.5 \times 10^{9} M^{-1} obtained for human IF and vitamin B_{12} under the same conditions (9).

**Absorption Spectra**—The spectra of equal concentrations of the hog IF-vitamin B_{12} complex, the hog NIF-vitamin B_{12} complex, and unbound vitamin B_{12} are presented in Fig. 9. When vitamin B_{12} is bound to either of these proteins, the 361 nm spectral maximum for unbound vitamin B_{12} shifts to 362 nm.

When vitamin B_{12} binds to either hog IF or hog NIF, there is general enhancement of the vitamin B_{12} spectrum above 320 nm since the spectra of the two proteins devoid of vitamin B_{12} are those of typical proteins with insufficient absorption above 320 nm to account for the differences between the protein-bound vitamin B_{12} spectra and that of unbound vitamin B_{12} (see Fig. 9) in the region above 320 nm. This observation is supported by experiments in which subsaturating aliquots of vitamin B_{12} were added to buffer containing hog IF and hog NIF and the increase in absorption at 361 nm was noted to be approximately 30% greater than when aliquots of vitamin B_{12} were added to buffer alone.

**DISCUSSION**

Hog IF and hog NIF have been isolated from a crude extract of hog pyloric mucosa. Affinity chromatography on vitamin B_{12}-Sepharose served to remove most proteins that do not bind vitamin B_{12}, but extreme difficulty was encountered in attempts to separate the two vitamin B_{12}-binding proteins from each other. Holdsworth (2) and Ellenbogen et al. (1, 7) have succeeded in at least partially separating these two proteins by the use of ion exchange chromatography, but we and other investigators (4) have not had success with this technique. The reasons for this discrepancy are not clear, but they may involve the fact that both Holdsworth and Ellenbogen et al. employed incubation with proteolytic enzymes at early stages in their purification schemes since it is possible that limited proteolysis changes the chromatographic behavior of hog IF and hog NIF.

Several reports (1, 15) suggest that hog IF and hog NIF differ

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**Table V**

**Amino acid and carbohydrate composition of hog IF and hog NIF**

Amino acid analysis was performed on duplicate 22-hour hydrolysates, and carbohydrate analysis was performed by gas- liquid chromatography.

| Item            | Hog IF | Hog NIF |
|-----------------|--------|---------|
| Amino acid      |        |         |
| Lysine          | 17     | 21      |
| Histidine       | 9      | 8       |
| Arginine        | 12     | 12      |
| Aspartic acid   | 50     | 47      |
| Threonine       | 37     | 19      |
| Serine          | 36     | 29      |
| Glutamic acid   | 48     | 42      |
| Proline         | 31     | 15      |
| Glycine         | 25     | 23      |
| Alanine         | 32     | 24      |
| Valine          | 26     | 24      |
| Isocitric acid  | 25     | 25      |
| Lactic acid     | 43     | 36      |
| Tyrosine        | 9      | 11      |
| Phenylalanine   | 14     | 12      |
| Methionine      | 10     | 9&superscript;6&superscript; | 9&superscript;6&superscript; |
| Half-cystine     | 9      | 10      |
| Tryptophan      | 6&superscript;8&superscript; | 6&superscript;8&superscript; |
| Carbohydrate    |        |         |
| Fucose          | 7      | 25      |
| Galactose       | 8      | 10      |
| Mannose         | 15     | 26      |
| Galactosamine   | 8&superscript;7&superscript; | 16&superscript;9&superscript; | 16&superscript;9&superscript; |
| Glucosamine     | 12&superscript;13&superscript; | 30&superscript;25&superscript; | 30&superscript;25&superscript; |
| Sialic acid     | 5&superscript;7&superscript; | 10&superscript;8&superscript; | 10&superscript;8&superscript; |

&superscript;1&superscript; Determined by the thiobarbiturate method.

&superscript;2&superscript; Determined as methionine sulfoxide after performic acid oxidation.

&superscript;3&superscript; Determined as carboxymethylcysteine. Accurate quantitation as cysteic acid was not possible since ninhydrin-positive material was present in the cysteic acid region in the absence of performic acid oxidation.

&superscript;4&superscript; Determined spectrophotometrically.

&superscript;5&superscript; Values in parentheses were determined using the amino acid analyzer.

&superscript;6&superscript; Determined by the thioarbiturate method.
FIG. 6. Gel filtration studies of hog IF and hog NIF in the absence and presence of vitamin B₁₂. Experiments were performed at 4°C using a column (2.0 x 95 cm) of Sephadex G-150 equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. Protein samples devoid of vitamin B₁₂ were renatured from guanidine by dialysis against Sephadex G-150 buffer for 72 hours at 4°C. Protein samples saturated with vitamin B₁₂ were renatured as described above except that excess [⁵⁷Co]vitamin B₁₂ was added prior to dialysis. A, the sample contained 21 μg of hog IF devoid of vitamin B₁₂. A molecular weight of 74,000 was obtained for hog IF based on the elution position of the single peak of vitamin B₁₂-binding activity. B, the sample contained 21 μg of hog IF and 0.64 μg of [⁵⁷Co]vitamin B₁₂. Molecular weights of 75,000 and 160,000 were obtained for hog IF based on the elution position of the two peaks of [⁵⁷Co]vitamin B₁₂. C, the sample contained 36 μg of hog NIF devoid of vitamin B₁₂. A molecular weight of 130,000 was obtained for hog NIF based on the elution position of the single peak of vitamin B₁₂-binding activity. D, the sample contained 36 μg of hog NIF and 0.90 μg of [⁵⁷Co]vitamin B₁₂. A molecular weight of 128,000 was obtained for hog NIF based on the elution position of the single peak of [⁵⁷Co]vitamin B₁₂. The amount of vitamin B₁₂-binding activity, and [⁵⁷Co]vitamin B₁₂, recovered after gel filtration ranged from 72 to 92% of the amounts applied in these experiments.

FIG. 7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of hog IF and hog NIF. Gel A, 30 μg of hog IF. A molecular weight of 66,000 is indicated by the mobility of the protein band. Gel B, 30 μg of hog NIF. A molecular weight of 100,000 is indicated by the mobility of this protein band. The arrow indicates the direction of mobility.

from each other in their affinities for vitamin B₁₂ analogs that contain absent or structurally modified nucleotides. We have taken advantage of these observations and have synthesized an affinity adsorbant that contains covalently bound vitamin B₁₂ derivatives that lack the nucleotide portion of the native vitamin. Hog IF has a marked affinity for this adsorbant but under certain conditions this affinity is less than that of hog NIF, and this fact has enabled us to separate these two proteins.

We have demonstrated that hog IF contains significantly more threonine, proline, and methionine and significantly less fucose, mannose, galactose, galactosamine, glucosamine, and sialic acid than does hog NIF. The differences between our results and those of previous investigators (see introductory section) are probably attributable to the fact that previous preparations of hog IF (1, 2, 7) appear to have contained significant contamination with other proteins. This suggestion is supported by the fact that previous preparations of hog IF had lower specific activities than ours.

Under certain conditions, hog IF appears to exist as a mixture of monomers, dimers, and other higher molecular weight oligomers. This phenomenon has been observed in studies employing polyacrylamide disc gel electrophoresis, sedimentation equilibrium ultracentrifugation, and gel filtration on Sephadex G-150. The presence of vitamin B₁₂ appears to be a requirement for oligomer formation. Similar studies have failed to provide evidence for significant oligomer formation by hog NIF. This observation appears to conflict with the observations of Ellenbogen et al. (1, 7) since they obtained ultracentrifugational evidence that both hog IF and hog NIF were capable of forming oligomers in the presence of vitamin B₁₂. We do not have any definite explanation for this discrepancy, but it is possible that hog NIF is capable of forming oligomers only with different salt or protein concentrations than we have employed. Ellenbogen et al. employed incubation with trypsin and chymotrypsin as part of their purification scheme and it is also possible that limited proteolysis of hog NIF must occur before this protein is capable of oligomer formation.
FIG. 8. Determination of the association constant, $K_a$, for hog IF and vitamin $B_12$ (A) and for hog NIF and vitamin $B_12$ (B). The experimental points were obtained using equilibrium dialysis in 0.1 M potassium phosphate, pH 7.5, at 4°C.

FIG. 9. Absorption spectra. $\cdots$, 540 µg per ml of hog IF containing 16.4 µg per ml of vitamin $B_12$; $\cdots$, 653 µg per ml of hog NIF containing 16.4 µg per ml of vitamin $B_12$; $\cdots$, 16.4 µg per ml of vitamin $B_12$. All spectra were obtained at room temperature in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl.

Hog IF and hog NIF have a number of properties in common but the two proteins also differ in a number of respects. Similar properties include: (a) both proteins have single vitamin $B_12$-binding sites; (b) both proteins have affinity constants for vitamin $B_12$ in the range of $1.3 \times 10^9$ M$^{-1}$; (c) both proteins contain single polypeptide chains; (d) neither protein has any demonstrable free sulfhydryl groups; (e) when vitamin $B_12$ binds to either protein, there is general enhancement of the vitamin $B_12$ spectrum above 320 nm; the spectra of the two protein-vitamin $B_12$ complexes are indistinguishable in this region; (f) the 361 nm spectral maximum for unbound vitamin $B_12$ shifts to 362 nm when the vitamin is bound to either protein; (g) both proteins have molecular weights close to 60,000, although the molecular weight of hog IF is slightly less than that of hog NIF; (h) hog IF and hog NIF are both glycoproteins and contain the same kind of carbohydrate residues.

Differences between hog IF and hog NIF include: (a) the vitamin $B_12$-binding ability of hog IF is inhibited by antibody obtained from a patient with pernicious anemia, while that of hog NIF is not. (b) Hog IF has a lower affinity for pseudo-vitamin $B_12$ relative to vitamin $B_12$ than does hog NIF. (c) Hog IF is able to correct vitamin $B_12$ malabsorption in a patient with pernicious anemia, while hog NIF cannot. (d) Hog IF facilitates vitamin $B_12$ binding to homogenates of human and guinea pig distal small intestine, while hog NIF does not. (e) Hog IF and hog NIF have markedly different apparent molecular weights when determined by gel filtration. Both values are falsely elevated but the degree of false elevation is greater for hog NIF. (f) The two proteins also have significantly different apparent molecular weights when determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The degree of false elevation in these values is also greater for hog NIF. (g) Hog IF aggregates in the presence of vitamin $B_12$ under conditions in which hog NIF does not. (h) The hog IF-vitamin $B_12$ complex contains less absorption from 250 to 290 nm than the hog NIF-vitamin $B_12$ complex. (i) Hog IF contains significantly greater amounts of threonine, proline, and methionine than hog NIF. (j) Hog IF contains 17.5% carbohydrate, while hog NIF contains 35.5%. Hog IF contains significantly lower amounts of fucose, galactose, mannos, galactosamine, glucosamine, and sialic acid than hog NIF.

The differences observed between hog IF and hog NIF demonstrate that these two proteins are distinct entities. The fact that hog NIF has a slightly larger molecular weight than hog IF and contains approximately twice as much carbohydrate rules out the possibility that hog NIF is a limited degradation product of hog IF. The fact that hog IF contains significantly greater amounts of threonine, proline, and methionine rules out the possibility that hog NIF is a zymogen-like precursor of hog IF. It is conceivable that both proteins are derived from a common, larger molecular weight glycoprotein, but we are not aware of any evidence to suggest such a possibility.

The function of hog NIF is unknown and our studies have not provided any clues concerning this question. Aro and Gräbe (4) have demonstrated that hog NIF reacts with antibodies prepared against crude hog granulocyte extracts and have suggested that hog NIF is a R type vitamin $B_12$-binding protein since members of this group of proteins have immunologic similarities and are present in granulocytes and a variety of body fluids. It is of interest in this regard that hog NIF does have many properties in common with the human granulocyte vitamin $B_12$-binding protein (12).

$^2$ Unpublished experiments performed in collaboration with Mr. David Hooper and Dr. David Alpers of Washington University School of Medicine.
Hog IF and human IF (9) have many common properties, but the two proteins do differ in amino acid and carbohydrate composition, molecular weight, and in their interactions with anti-IF antibody and pseudo-vitamin B₁₂. The physiologic significance of these differences is unclear and they may reflect merely a species difference. It is important to note, however, that hog IF was isolated from gastric mucosa, while human IF was isolated from gastric juice. This difference could be important if IF is altered by proteolytic enzymes, or other factors, prior to, during, or after it is secreted into a gastric juice.

Acknowledgments—In addition to the people acknowledged in the accompanying paper (9), we would like to thank Dr. Leon Ellenbogen for his help in obtaining information concerning intrinsic factor concentrate.

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Isolation of Gastric Vitamin B\textsubscript{12}-binding Proteins Using Affinity Chromatography: II. PURIFICATION AND PROPERTIES OF HOG INTRINSIC FACTOR AND HOG NONINTRINSIC FACTOR

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*J. Biol. Chem.* 1973, 248:3670-3680.

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