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# VITAMIN $B_{12}$-BINDING PROTEINS OF THE HORSESHOE CRAB LIMULUS POLYPHEMUS

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SUMMARY: Vitamin $B_{12}$-binding proteins were detected in the body fluids and/or tissues of horseshoe crabs (Limulus polyphemus), clams and sponges. Among the biological specimens tested the Limulus plasma was especially rich in free $B_{12}$-binding proteins. Gel filtration experiments revealed that Limulus plasma contains two classes of $B_{12}$-binding proteins. One class of proteins, molecular weight in excess of 100,000, bind $B_{12}$ preferentially with affinity constant of $5 \times 10^{11} M^{-1}$. The second type of proteins, molecular weights around 50,000, bind $B_{12}$ with specificity approaching that of mammalian intrinsic factors. The binding constant of these proteins for $B_{12}$ is around $10^{11} M^{-1}$. 

The concentration levels of vitamin $B_{12}$ and its analogs in ocean waters vary both seasonally and geographically. These variations range from 10 - 0.1 pg/mL for $B_{12}$ and 40 - 2 pg/mL for the analogs (1,2). Although these concentrations are extremely low, many marine organisms are able to satisfy their biological needs for $B_{12}$ or its analogs by absorbing them from ocean waters (3). In order to accomplish this remarkable feat, these organisms, like terrestrial mammals (4), must have proteins which bind $B_{12}$ and its analogs differentially with high degree of avidity. We have obtained conformation of this hypothesis by discovering that $B_{12}$-binding proteins are present in the body fluids and/or tissues of horseshoe crabs, clams and sponges.

We decided to conduct detailed studies on the $B_{12}$-binding proteins of the horseshoe crab, Limulus polyphemus, because of several reasons. First, Limulus is among the most primitive animals known (5). A comparative study of the $B_{12}$-binding proteins of this animal and highly advanced terrestrial mammals should reveal the structural changes that might have occurred in these proteins during the course of evolution. Second, the horseshoe crab may serve as a model for understanding the

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mechanisms which facilitate the uptake of $B_{12}$ by cells. The uptake of $B_{12}$ by cells is mediated by $B_{12}$-binding proteins but the mechanisms involved are not completely defined (4). Finally, the Limulus blood plasma and amebocytes are very rich in $B_{12}$-binding proteins. These materials may serve as commercially preferred source of $B_{12}$-binding proteins which are used in radioassay kits for the determination of $B_{12}$ in biological fluids. Currently, Limulus plasma and amebocyte ghosts are the waste products of Limulus Lysate industries.

In this communication we describe the results of our preliminary investigations on the free $B_{12}$-binding proteins present in the blood plasma of Limulus polyphemus.

## MATERIALS AND METHODS

Materials: Dextran-charcoal suspensions; solutions of $^{57}$Co-labelled $B_{12}$ containing KCN plus human serum albumin were donated by Becton Dickinson Immunodiagnostics, Mountain View Avenue, Orangeburg, N.Y. 10962. Cobinamide dicyanide was purchased from Sigma Chemical Co. Scintiverse II cocktail was obtained from Fisher Scientific, N.J. A Packard Liquid Scintillation Spectrometer equippped with a teletypewriter was used for measuring radioactivities.

Limulus whole blood was generously supplied by Mr. James J. Finn, President, Marine Biologicals, Inc., Seaville, N.J. Hard clams (Mercinaria mercinaria) and some horseshoe crabs (Limulus polyphemus) were collected by Tom Farrel of New Jersey Marine Sciences Consortium, Seaville, N.J. Three species of marine sponges used in the investigations are: Hymeniacidon heliophila (sun sponge), Cliona celata (sulfur sponge) and Microciona prolifera (red beard sponge). These sponges were collected with the help of Dr. Vincent G. Guida and Mr. Dominic Dragotta of Lehigh University's Wetlands Institute at Stone Harbor, N.J. The collection site was Great Channel behind Seven Beach, Stone Harbor, N.J.

Methods: Protein concentrations were determined by Lowry method. All extraction and fractionation procedures were carried out at 4°C. Biological specimens, extracts and chromatographic fractions were stored frozen below 0°C. $B_{12}$-binding studies were conducted in disposable polypropylene tubes measuring 12x75 mm.

Blood from horseshoe crabs was obtained aseptically by following known techniques (6). Whole blood, 400 mL, from 5 Limuli was collected in 70 mL of a sterile solution containing 0.8% citric acid, 2.2% sodium citrate and 2.5% dextrose. Cell free plasma was obtained by centrifuging whole blood at 10,000xg in a refrigerated centrifuge for 30 min. The supernatant plasma was decanted and tested for the presence of $B_{12}$-binding proteins.

Buffer extracts of amebocyte pellet, clam tissues and sponges were prepared by homogenizing each wet biological specimen (50 g) with 100 mL of 0.02 M phosphate buffer (pH 7.2) in a blender. After centrifugation, supernatants were decanted and tested for the presence of $B_{12}$-binding proteins.

Detection of $B_{12}$-binding Proteins: A solution of $^{57}$Co-labelled $B_{12}$ containing around 40 pg $B_{12}$ and 16000 cpm/mL was used for the detection of $B_{12}$-binding proteins in body fluids, tissues extracts, chromatographic fractions, etc. Small volumes (100 to 500 µL) of the test samples were incubated with 0.4 mL aliquots of the radiolabelled $B_{12}$ solution for 30 min. at room temperature. Separation of bound and free $B_{12}$ was achieved by contact for 10 min. with 0.4 mL aliquots of a dextran-coated charcoal suspension. After centrifugation, the bound $B_{12}$ in each supernatant solution was decanted and mixed with scintillation cocktail in a vial. Bound radioactivities were measured in the liquid scintillation counter. Test samples

capable of binding at least 200 cpm above background counts were considered to contain $B_{12}$-binding proteins.

Specific activities: The samples were diluted with 0.02 M phosphate buffer pH 7.2 so that 100 µL aliquots of the diluted solutions bind 50±10% of the radioactivity when incubated with 1 mL volumes of a $^{57}$Co-labelled $B_{12}$ solution containing 42 pg $B_{12}$/mL (cpm 16000). From the bound cpm and protein concentrations of the test solutions the amount of $B_{12}$ bound/mg protein was calculated.

Purification of Limulus plasma $B_{12}$-binding proteins: The precipitate obtained by increasing the concentration of ammonium sulfate in Limulus plasma from 40-75% was collected by centrifugation. The pellet was dissolved in distilled water and the solution was dialyzed against two changes of 2 liter distilled water over a 24 hour period. The solution in the dialysis tubing was freeze-dried. A known amount (2 g) of the lyophilized material was dissolved in 100 mL of 0.02 M phosphate buffer (pH 7.2). This solution was analyzed for protein concentration and $B_{12}$-binding activity. A 2.0 mL aliquot (26 mg protein) of the solution was chromatographed over a precalibrated Sephadex G-150 column (1.8x90 cm) equilibrated with 0.1 M $NH_4HCO_3$ solution. The column was eluted with $NH_4HCO_3$ solution. The flow rate was 18 mL/hr. and 3 mL fractions were collected. The $B_{12}$-binding activities and absorbance at 280 nm of the fractions are plotted in Fig. 1 (bottom).

Fractions 24-36 and 43-56 of peaks A and B respectively were pooled in two separate dialysis tubings with molecular weight cut off limit of 7000. The contents of the tubes were dialyzed against two changes of distilled water over a 24 hr. period. The solutions after dialysis were freeze-dried. The freeze-dried materials were dissolved in 0.02 M phosphate buffer and the volumes were made to 2.0 mL. These solutions were called fractions A and B. These fractions were analyzed for total proteins, binding activities, binding specificities, and affinity constants for $B_{12}$.

Saturation curves, Fig. 2 and 3: Fractions A and B were diluted with 0.02 M phosphate buffer so that 100 µL aliquots of the dilute solutions bind 40±10% radioactivity when incubated with 1 mL volume of a $^{57}$Co-labelled $B_{12}$ standard solution containing 42 pg $B_{12}$/mL. The data for the saturation curves were obtained in duplicate by incubating 100 µL aliquots of the dilute solutions with progressively increasing amounts of $^{57}$Co-labelled $B_{12}$ for 30 min. at room temperature. The bound and free radioactivities were separated by contact for 10 min. with coated charcoal. After centrifugation, the supernatants were decanted and bound radioactivities were determined in the liquid scintillation counter. The curves shown in Figs. 2 and 3 were obtained by plotting total cpm versus bound cpm. From these graphs free cpm (total - bound) at half saturation were determined. Free cpm were converted into concentrations of free $B_{12}$ at half saturations. Reciprocal of these concentrations gave the affinity constants of fractions A and B for vitamin $B_{12}$.

Specificity studies: Mixtures of progressively increasing amounts of cobinamide dicyanide with a constant amount of $^{57}$Co-labelled $B_{12}$ were incubated with 100 µL aliquots of the dilute solution of fraction A or fraction B for 30 minutes at room temperature. The bound radioactivities were determined by coated charcoal techniques. The results are presented in Tables 1 and 2.

## RESULTS AND DISCUSSION

Our investigations revealed that the body fluids and/or tissues of horseshoe crabs, hard clams and sponges exhibit vitamin $B_{12}$-binding activities. The results of our initial attempts to isolate the $B_{12}$-binding principles (proteins) present in the blood plasma of the horseshoe crab, Limulus polyphemus, are discussed below.

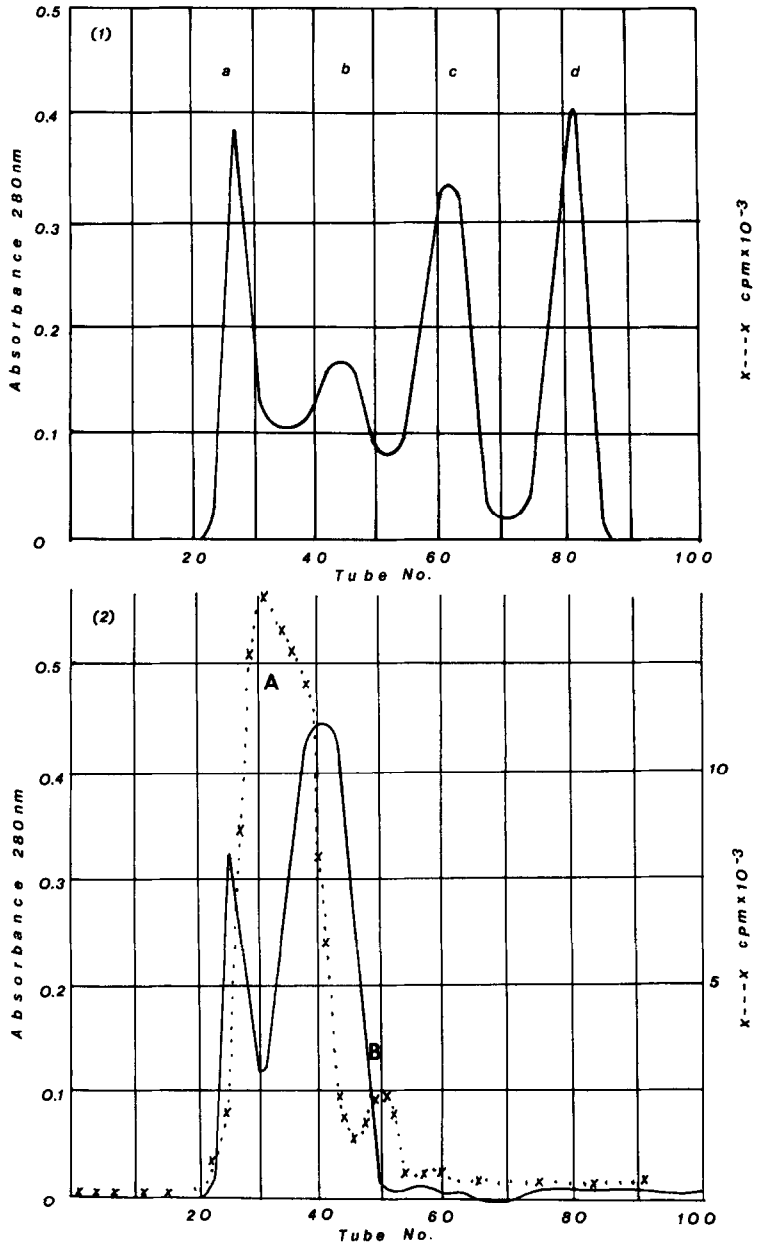


Fig. 1. Top curve: Calibration of the 1.9x90 cm Sephadex G-150 column. Peaks a, b, c and d represent the elution profiles of blue dextran ($2x10^6$), serum albumin (6700) ribonuclease (13,700) and DNP-glycine (241) respectively. Curve numbers in parentheses are molecular weights.

Bottom curve: Gel Filtration of 40-70% ammonium sulfate fraction over the precalibrated Sephadex column. Eluent 0.1 M $NH_4HCO_3$. Solid curve represents absorbance at 280 nm of the fractions. Dotted curve is the $B_{12}$-binding capacity of the fractions.

Limulus blood plasma obtained from whole blood collected in citric acid/citrate/dextrose solution, contained 47 mg protein/mL of the fluid. The protein concentra-

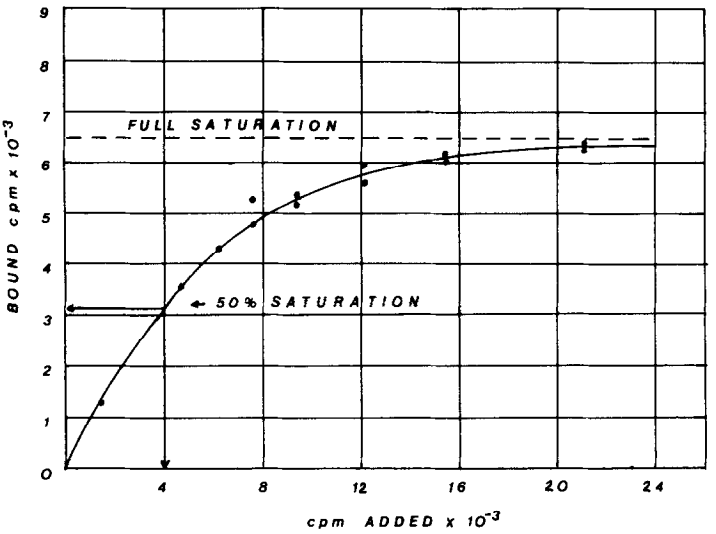


Fig. 2. Saturation of fraction A with vitamin $B_{12}$. Total reaction volume = 1.1 mL. 15215 cpm = 42 pg $B_{12}$. Free cpm at 50% saturation; 4100-3200 = 900 cpm. Conc. of free $B_{12}$ at 50% saturation = $1.7 \times 10^{-12}$M. Affinity constant $K = \frac{1}{1.7 \times 10^{-12}} = 5.8 \times 10^{11} M^{-1}$.

tion of the plasma supplied by the Marine Biological, Inc. was 27 mg/mL. The higher protein concentration of the citrate plasma is due to clumping and lysing of amebocytes during transportation of the whole blood from the collection site, to our laboratories. Marine Biological Laboratories use a special anticoagulant (proprietary information) which apparently prevents cell clumping and lyses. Nevertheless, both plasmas were found to be rich in $B_{12}$-binding proteins, and further work was carried out with citrate plasma only.

The citrate plasma was found to bind 3 ng $B_{12}$/mg protein. Assuming that molecular weight of $B_{12}$-binding proteins is around 50,000 (see later) and one protein molecule binds one $B_{12}$ molecule then the concentration of free $B_{12}$-binding proteins in the plasma is calculated to be 5 mg/L.

The proteins in Limulus plasma were fractionated by ammonium sulfate precipitation procedure. The precipitate obtained by increasing the ammonium sulfate concentration in plasma from 40 to 75% was found to bind 3.7 ng $B_{12}$/mg protein. The specific activity of plasma was 3.5 ng $B_{12}$/mg protein. The ammonium sulfate precipi tation procedure, therefore, achieved little concentration of $B_{12}$-binding proteins.

The results of the fractionation of 40-75% ammonium sulfate fraction over a precalibrated Sephadex G-150 column are shown in Fig. 1. This chromatogram shows that

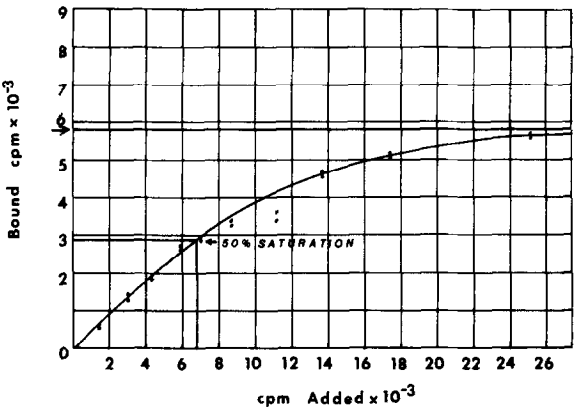


Fig. 3. Saturation of fraction B with vitamin $B_{12}$. Total reaction volume = 1.1 mL. 13602 cpm = 37.5 pg $B_{12}$. Free cpm at 50% saturation = 6800 - 2900 = 3900 cpm. Conc. of free $B_{12}$ at 50% saturation = $7.2 \times 10^{-12}$M. Affinity constant = $\frac{1}{7.2 \times 10^{-12}\text{M}} = 1.4 \times 10^{11}\text{M}^{-1}$.

Limulus plasma contains two types of $B_{12}$-binding proteins. These proteins appear in fractions comprising peaks A and B of the chromatogram. The $B_{12}$-binding proteins corresponding to peak A have molecular weights in excess of 100,000 while the ones corresponding to peak B have molecular weights approaching 50,000. To obtain materials with high specific activity fractions 24-36 of peak A and fractions 43-56 of peak B were pooled. These fractions were made free of small molecules and concentrated by dialyzing, freeze-drying and dissolving the freeze-dried materials in 2.0 mL of 0.02 M phosphate buffer ph 7.2. The concentrated fractions A and B were analyzed for total proteins, binding specificities and affinity constants for $B_{12}$. The affinity constants were estimated from the saturation curves shown in Fig. 2 and 3.

TABLE 1. Specificity of Fraction A for NC-$B_{12}$

| Cobinamide Dicyanide (pg) | $^{57}$Co-$B_{12}$ (pg) | Bound (CPM) | % Bound (CPM) |
|---|---|---|---|
| 0 | 40 (CPM 15027) | 8959 | 100 |
| 50 | " | 8293 | 92.6 |
| 100 | " | 7058 | 78.8 |
| 200 | " | 5676 | 63.3 |
| 500 | " | 3942 | 44.0 |
| 1000 | " | 2413 | 27.0 |

TABLE 2. Specificity of Fraction B for NC-$B_{12}$

| Cobinamide Dicyanide (pg) | $^{57}Co$-$B_{12}$ (pg) | Bound (CPM) | % Bound (CPM) |
|---|---|---|---|
| 0 | 39 (CPM 14596) | 6190 | 100 |
| 100 | " | 6145 | 99.2 |
| 200 | " | 5993 | 96.8 |
| 500 | " | 5965 | 96.3 |
| 1000 | " | 5796 | 93.6 |
| 2000 | " | 5650 | 91.3 |

Fractions A and B were found to contain 2.7 and 1.7 mg total proteins respectively. The specific activity of fraction A was 66 ng $B_{12}$/mg protein while that of fraction B was about 1 ng $B_{12}$/mg protein.

Fraction A binds vitamin $B_{12}$ preferentially from mixtures of a constant amount of CN-$B_{12}$ with progressively increasing amounts of cobinamide dicyanide (Table 1). Fraction B, on the other hand, binds CN-$B_{12}$ with specificity reminiscent of mammalian intrinsic factors (Table 2). The binding constant of fraction B for $B_{12}$ is $10^{11}M^{-1}$ which is an order of magnitude greater than the affinity constant of $10^{10}M^{-1}$ for the hog intrinsic factor (4). Fraction A binds $B_{12}$ with affinity constant of $5x10^{11}M^{-1}$ which is greater than the association constant of $10^{11}M^{-1}$ for the human vitamin $B_{12}$ transport proteins (4).

Fractions A and B are by no means homogenous. Further work on the purification of the $B_{12}$-binding proteins present in these fractions is in progress.

It is conceivable that Limulus plasma contains proteins that are already saturated with vitamin $B_{12}$. Such holo proteins if present could not be dissociated by varying the pH of the plasma in the range of 2 to 12. This aspect of the project is also being further investigated.

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