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Toadfish Serum as a Binder for In Vitro Assay of Vitamin B_{12}

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Serum from the oyster toadfish,* Opsanus tau, *has a binding capacity for cyanocobalamin 1,000 times greater than that of human serum. The binding follows the principle of isotope dilution in the physiologic range of vitamin B_{12} present in human serum. Under proper conditions of storage, this binder is stable for at least 1 year. Standard reagents and techniques used in other vitamin B_{12} competitive binding assays can be used with the toadfish serum binder. Toadfish serum offers potential advantages over intrinsic factor and human serum, the most commonly used binders in vitamin B_{12} assays.

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Radioassays for vitamin B_{12} based on competitive protein binding have been available for over 10 years, since the pioneering efforts of Barakat and Ekins (*1*) and Rothenberg (*2*). These initial workers used human serum and hog intrinsic factor as sources for the vitamin B_{12} binding protein. Since then a number of assays have been developed utilizing the specific binding substances in human serum (*3*), serum from patients with chronic myelogenous leukemia (*4*), saliva (*5*), and chicken serum (*6*). Technical problems and discrepancies between biologic assays and radioassays of serum vitamin B_{12} (*6–13*) led us to investigate a new and potentially useful binder of cyanocobalamin.

In an earlier study in this laboratory, the oyster toadfish (*Opsanus tau*) was found to have serum with a very high binding capacity for vitamin B_{12} (*14*). We report here our observations regarding the use of serum from this common estuarine fish as a binder for cyanocobalamin.

METHODS

The methods and principles of Matthews et al. (*3*), Lau et al. (*15*), and Newmark et al. (*16*) form the basis of our approach. Cobalt-57-labeled cyanocobalamin of high specific activity (130–213 μCi/μg) was obtained from Amersham/Searle (Arlington Heights, Ill.) and diluted to a concentration of 500 pg/ml with water. Standard curves were obtained by combining 50 pg of ^{57}Co-cyanocobalamin with 20–160 pg of crystalline cyanocobalamin (Elkins Sinn, Inc.) in 4 ml of 0.2 *M* carbonate–bicarbonate buffer, pH 9.5. Serum from three toadfish was pooled and diluted 1:3,000 with 1% human serum albumin, and 0.1 ml of this dilution was added to each tube. The cyanocobalamin and toadfish serum (TFS) were incubated at room temperature for 1 hr. Bound and free cyanocobalamin were separated by adding 0.5 ml of albumin-coated charcoal (*15*), mixing for 5 min, and centrifuging for 10 min. The supernatant (bound activity) was decanted and counted in a well scintillation counter. "Total" counts were obtained either by counting an untreated aliquot of the ^{57}Co-cyanocobalamin or by counting the charcoal (free cyanocobalamin) and adding these counts to those from the supernatant. A "blank" containing only ^{57}Co-cyanocobalamin and buffer was treated with charcoal and used to correct the supernatant counts in each experiment. This always represented less than 3% of the total activity.

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The rate of association of ^{57}Co-cyanocobalamin with toadfish serum was measured by incubating 600 pg of ^{57}Co-cyanocobalamin with 10 ml of acetate–KCN buffer,* 88 ml of the carbonate–bicarbonate buffer, and 2 ml of TFS (diluted 1:3,000). This solution was incubated at room temperature, and 5-ml samples were removed at various times for determination of the bound activity. After 3 hr, 10 μg of crystalline cyanocobalamin was added to 50 ml of the remaining reaction mixture and the rate of dissociation was measured over a 3-day period.

Recovery experiments were performed by incubating 0.1 ml of normal serum with 20–160 pg of stable cyanocobalamin for 1 hr and then extracting the total vitamin B_{12} by boiling the sample for 15 min with 0.5 ml of the acetate–KCN buffer.

Sera from several other species of fish were serially diluted with 0.1% human serum albumin; 0.1 ml was incubated with 50 pg of ^{57}Co-cyanocobalamin, and the above methods were used to determine the dilution that would bind 50% of the added cyanocobalamin.

RESULTS AND DISCUSSION

Toadfish serum (TFS). The diluted toadfish serum binds added cyanocobalamin according to the principle of isotope dilution (*13,15*). The bound radioactivity diminishes proportionately with increasing amounts of nonradioactive cyanocobalamin, as shown by the linear graph in Fig. 1. An extrapolation of the line intersects the horizontal axis at –50 pg, the amount of labeled material added. This further indicates that the principle of isotope dilution is being observed (*13*). Expressing the data as the ratio of total counts to bound counts, followed by extrapolation of the straight line to the amount of labeled material added, provides an internal check on the system and facilitates reading unknown sera (*6*). This principle of isotope dilution was shown not to be fulfilled when human serum or intrinsic factor was used as the binder for cyanocobalamin (*13*).

The standard curves were highly reproducible, as indicated by the standard error of the means in Fig. 1. This curve is a composite of five standard curves obtained over a period of 5 months using the frozen pooled TFS with various lots of ^{57}Co-cyanocobalamin and other reagents.

The binding protein has such a high avidity for cyanocobalamin that the binding is essentially unidirectional over the entire range of physiologic vitamin B_{12} concentrations. Diluted toadfish serum is saturable with cyanocobalamin in low concentration

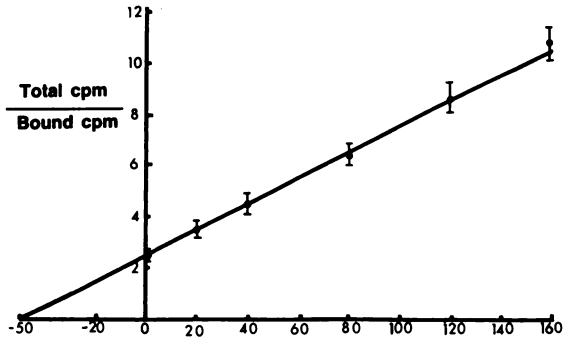

FIG. 1. Mean of five standard curves is plotted with standard error indicated. 50 pg of ^{57}Co-cyanocobalamin was added to increasing amounts of crystalline cyanocobalamin, and mixture was incubated for 1 hr at room temperature with 0.1 ml of TFS, diluted 1:3,000. Bound and free activity were separated by albumin-coated charcoal. Straight-line extrapolation to —50 pg indicates that toadfish serum binder follows principle of isotope dilution.

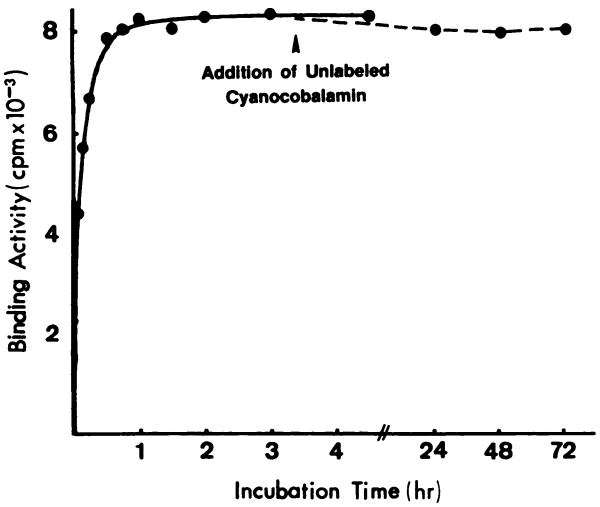

FIG. 2. Rate of association (continuous curve) and dissociation (broken curve) of toadfish serum and cyanocobalamin. ^{57}Co-cyanocobalamin was incubated with diluted TFS, samples were removed, and bound activity determined. After 3 hr, 10 μg of nonradioactive cyanocobalamin was added to determine dissociation rate by same procedure.

and the association constant was calculated to be greater than $10^{12}/M$. Assay sensitivity is only limited by the specific activity of the radioactive cyanocobalamin. The high binding capacity of toadfish serum for cyanocobalamin makes it possible to use the serum at high dilution. One milliliter of undiluted serum is enough for 30,000 assay tubes. Maximal binding of cyanocobalamin to the toadfish serum was obtained after 30 min of incubation at room temperature (Fig. 2). The dissociation rate was very slow and the reaction was essentially irreversible. The mean percent recovery of cyanocobalamin added to normal serum before extraction was 99.7 ± 7.8%

* 0.1 *M* acetate buffer, pH 4.0, containing 20 mg of KCN per liter.

TABLE 1. DILUTION AT WHICH 0.1 ml OF SERUM FROM VARIOUS SPECIES WILL BIND APPROXIMATELY 50% OF 50 pg ^{57}Co-CYANOCOBALAMIN

Species	Dilution with 0.1% human serum albumin
Human	1:3
Rainbow trout (*Salmo gairdneri*)	1:500
Chicken	1:2,000
Toadfish (*Opsanus tau*)	1:3,000
Sockeye salmon (*Oncorhynchus nerka*)	1:7,000
Striped bass (*Morone saxatiles*)	1:10,000
Coho salmon (*Oncorhynchus kisutch*)	1:50,000

($n = 14$), compared to the total of stable and serum vitamin B_{12} added. When stored frozen, the toadfish serum retained its high binding capacity for over a year. It could be stored at 4°C for at least 4 weeks when diluted with serum albumin.

Comparison of vitamin B_{12} binding of TFS and other sera. Rosenthal and Austin (*17*) had previously reported high vitamin B_{12} binding capacities in the sera of several other species of fish. To confirm this observation, we obtained sera from several northwest species. The vitamin B_{12} binding capacities of these and other sera were compared to that of *Opsanus tau*. Table 1 lists the dilution factors required for 0.1 ml of the diluted sera of several types of species to bind approximately 50% of the tracer (50 pg of ^{57}Co-cyanocobalamin).

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