The Vitamin B_{12}-Binding Proteins of Some Amphibians (35391)

DAVID W. SONNEBORN[1] AND HANS J. HANSEN

Department of Biochemical Nutrition, Hoffmann-LaRoche, Inc., Nutley, New Jersey 07110

Three immunologically distinct unsaturated (without vitamin) vitamin B_{12}-binding proteins have been demonstrated in humans. Intrinsic factor, the best known, has a molecular weight of 55,000 and promotes the uptake of vitamin B_{12} by the intestinal epithelium (1, 2). Transcobalamin II, a liver-synthesized serum B_{12} binder with a molecular weight of 35,000, is apparently responsible for the uptake of the vitamin by the tissues (1, 2). A third binder, R binder or transcobalamin I, has a molecular weight of 110,000 and is found in serum, saliva, gastric juice, and a variety of other body fluids (1, 2). The function of this binder is not known.

It has been shown recently that chickens have a single unsaturated serum B_{12} binder with a molecular weight of 113,000 and an immunologically similar proventriculus B_{12} binder with a molecular weight of 96,000 (3). These binders superficially resemble the human R binders. They are approximately the same size as the human R binder, and in both cases immunologically similar forms occur in both the serum and gastric juice. However, antibody against the chicken binder does not react with human R binder (3). The present study demonstrates the presence of unsaturated B_{12} binders in amphibians. These resemble neither the human binders nor the chicken binders.

Materials and Methods. Levels of unsaturated vitamin B_{12} (B_{12}) binders were measured using zirconyl phosphate gel (Z-gel), a highly negatively charged gel that binds proteins below their isoelectric point (4, 5). B_{12}-^{57}Co was purchased from Philips-Duphar, Holland, and was diluted with unlabeled B_{12} to give solutions containing either 100 ng/ml (1000 counts/ng) or 10 ng/ml (10,000 counts/ng). B_{12}-^{60}Co was used as purchased (1 μCi/1.484 μg or 0.652 μCi/μg; E. R. Squibb and Sons, New York). Radioactivity was measured in a Packard gamma scintillation spectrometer.

Leopard frogs (*Rana pipiens*), a female bullfrog (*R. catesbeiana*) and 2 male giant toads (*Bufo marinus*) were purchased from the Lemberger Company, Oshkosh, Wisconsin. A female *Amphiuma means* and 2 female mudpuppies (*Necturus maculosus*) were purchased from the Carolina Biological Supply Company, Burlington, South Carolina.

Sephadex G-50, G-100, and G-200 were purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. Columns 2.5 × 100 cm were eluted by gravity with Tris–HCl buffer (0.1 *M* Tris) in 0.9% NaCl at pH 7. Five-ml fractions were collected.

Stomachs were ground in 5 or 10 vol (w/v) of cold 0.9% NaCl with a Sorvall Omnimixer at full speed for 5 min. Homogenates were then centrifuged at 7000*g* for 15 min at 4°. Blood was allowed to clot and serum was prepared by centrifugation in this manner.

Results. Table I shows the levels of unsaturated binders in some amphibians as determined by the Z-gel technique. These observations extend the previously published findings of Rosenthal and Austin *et al.* (6).

All of the females examined had a gastric B_{12} binder with a molecular weight of approximately 15,000. Figure 1 shows a Sephadex G-50 cochromatogram of the gastric binders of a female bullfrog (*R. catesbeiana*) and the gastric binders of a female *Amphiuma*. Cytochrome oxidase (from horse heart, type IV, Sigma Chemical Company, St. Louis, Mo.) and ^{125}I-labeled insulin (Amersham/Searle, Des Plaines, Illinois) were added as markers. The low mole-

[1] Present address: Department of Anatomy, Medical College of Virginia, Health Sciences Division of Virginia Commonwealth University, Richmond, Virginia 23219.

TABLE I. The Levels of B_{12} Binders in Sera and Stomachs of Some Amphibians (ng/ml).

Species	Serum	Stomach:homogenate (1:5; w/v)
Mudpuppy (*Necturus maculosus*)	27	496
	14	359
Amphiuma (*Amphiuma means*)	30	196
Giant toad (*Bufo marinus*)	90	47
	69	51
Bullfrog (*Rana catesbeiana*)	241	15
Leopard frog (*Rana pipiens*) (av of 8)	36	2

cular weight binder present in both these homogenates is slightly larger than cytochrome oxidase. Binders with the same molecular weight as these binders were found in the stomachs of 6 female leopard frogs (*Rana pipiens*) and 2 female mudpuppies (*Necturus maculosus*). These binders were not present in serum.

Ten male leopard frogs were studied. The stomachs of 8 of these had but a trace of this low molecular weight binder. Two male leopard frogs, however, had an amount of binder comparable to that found in the females. Two male giant toads (*Bufo marinus*) did not have any low molecular weight binder.

High molecular weight binders are also found in amphibians. The results of G-100 and G-200 chromatography of these binders are shown in Table II. The larger gastric binders in the stomachs of bullfrogs and leopard frogs may be due to the binder found in the blood and extracellular fluid. This was not true of the larger binders in the stomachs from the other species which clearly concentrated binder in this organ. The function of these is unknown and will be the subject of future investigation.

Discussion. There were high molecular weight binders in both stomachs and sera of amphibians. Specimens of *Rana pipiens* have a 140,000 (160,000 or greater in 1 animal) molecular weight binder in both sera and stomachs. In addition, binders were found in the serum with a molecular weight of approximately 250,000 and 65,000. The former was a shoulder on the main peak, and the latter occurred in very small quantities. In *Bufo marinus*, the serum and gastric binders were the same size. The differences observed in the size of the small binders with G-100 may be due to a column artifact as they chromatographed together on G-200. The shortcomings of using Sephadex for molecular weight determinations are well known but are far outweighed by the advantages, particularly for impure protein samples (7). In *Rana cates-*

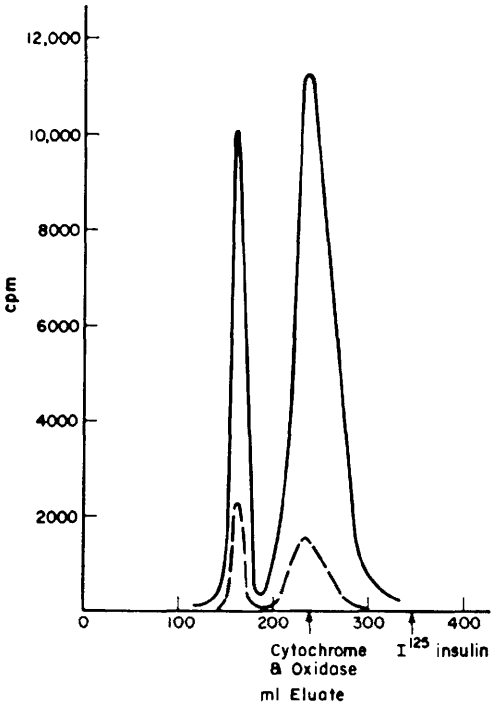

FIG. 1. Cochromatography of the B_{12} binders in the stomachs of bullfrog and amphiuma on Sephadex G-50 (coarse). The amphiuma binders were saturated with B_{12}-^{60}Co (—); while the bullfrog binders were labeled with B_{12}-^{57}Co (- -). Cytochrome oxidase and ^{125}I-labeled insulin were added as markers. The small binder has the same size in both species. Radioactivity of ^{57}Co is shown $\times 10^{-1}$.

TABLE II. Large Vitamin B_{12} Binders of Amphibians (other than the "small binder").

Species	Sephadex gel used	Mol wt of serum binders	Mol wt of gastric binders
Rana catesbeiana	G-200	260,000 65,000 (trace)	120,000–260,000[a] 60,000 (trace)
Rana pipiens	G-200	250,000 160,000	160,000
	G-100 (4 animals)	120,000–160,000 60,000– 66,000 (trace)	120,000–160,000
Bufo marinus	1. G-100	160,000 or greater 78,000	160,000 or greater 74,000
	2. G-200	260,000 75,000	260,000 75,000
Amphiuma means	G-100	115,000	115,000 68,000 (trace)
Necturus maculosus	1. G-100	110,000	120,000 60,000
	2. G-100	110,000	120,000 60,000

[a] See text.

beiana, the predominant serum binder was larger than the predominant gastric binder. The shape of the peak for the gastric binder, however, suggested more than one binder was present, and it is possible that the larger of these was the same size as the serum binder. In the mudpuppy, the serum binder was smaller than the larger of the two gastric binders, while the corresponding binders in *Amphiuma* were the same size.

The large amounts of 15,000 molecular weight binder found in all females and the small amounts of this binder found in most males suggests that this binder may be related to reproduction. It is, however, possible that the *Rana pipiens* studied were collected in different areas and that strain differences reflect the different amounts of binder. Another alternative is that the small size binder is a precursor for the other binders and that different quantities of the small binder represent different rates of synthesis in males and females. The low molecular weight binder may be produced by the action of enzymes acting more in females than in males, either as an artifact produced by homogenization or as the means by which a functioning 15,000 molecular weight binder is formed. A similar sized binder has recently been produced by the action of enzymes on porcine intrinsic factor (8). Further studies should establish the significance of these binders.

It is possible that anurans can be distinguished from urodeles by the presence of 260,000 molecular weight binder in urodele serum. A larger sample should be examined to verify this hypothesis. It will also be of great interest for our understanding of the phylogeny of this class to determine if a 15,000 molecular weight binder is found in fish. If not, the presence of this binder in both urodeles and anurans would suggest that these orders had a common ancestor. The present study extends previous studies on Vitamin B_{12}-binding proteins of amphibians (6) not only establishing their presence in additional species, but also presenting partial characterization of the proteins. The presence of these proteins suggests that as in mammals the transport of vitamin B_{12} in amphibia requires specific factors.

1. Grasbeck, R., *in* "Progress in Hematology" (E. B. Brown and C. V. Moore, eds.), Vol. 5, p. 233. Grune and Stratton, New York (1969).

2. Simons, K., *in* "Progress in Gastroenterology" (G. B. Glass, ed.), Vol. 1. Grune and Stratton, New

York (1968).

3. Sonneborn, D. W., and Hansen, H. J., Science **168**, 591 (1970).

4. Hansen, H. J., and Miller, O. N., Anal. Biochem. **7**, 129 (1964).

5. Hansen, H. J., Miller, O. N., Gallo-Torres, H. E., and Goldsmith, G. A., Anal. Biochem. **16**, 287 (1960).

6. Rosenthal, H. L., and Austin. S., Proc. Soc. Exp. Biol. Med. **109**, 179 (1961).

7. Andrews, P., Biochem. J. **91**, 222 (1964).

8. Ellenbogen, L., and Highly, D. R., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **29**, 633 (1970).

Received Oct. 16, 1970. P.S.E.B.M., 1971, Vol. 136.