Hydroxocobalamin: Physiological Retention in the Dog. (26162)

HELEN R. SKEGGS, EDWARD J. HANUS, ALICE B. MCCAULEY AND VINCENT J. RIZZO
(Introduced by D. K. Bosshardt)
Merck, Sharp & Dohme Research Division, West Point, Pa. and Merck Chemical Division, Rahway, N. J.

Therapy aimed at maintained elevation of Vit. B_{12} in the body is hampered by the rapid urinary excretion of the vitamin and consequent need for repeated administration. The therapeutic effectiveness of a single injection of a small amount of cyanocobalamin in pernicious anemia, with rapid excretion in the urine of larger amounts has been established(1). Indeed, Reisner and Weiner(2) state that individual doses of Vit. B_{12} above 50 μg are not warranted since they exceed the renal threshold value, are rapidly and quantitatively excreted, and do not prolong the period of remission. Mollin and Ross(3) find that hemapoiesis is normoblastic when serum Vit. B_{12} levels are maintained at 100 μg or more, but that megaloblasts appear in the marrow of pernicious anemia patients when serum levels fall below that figure. In the opinion of Mollin and Ross, tissue Vit. B_{12} deficiency must be extreme before serum levels fall below normal and megaloblastic anemia develops. To restore the depleted tissues to their normal range of 1 to 2 mg, considerably more Vit. B_{12} is needed than for producing hematological remission and they suggest that 5-1 mg doses, administered over a 2-week period, with retention of 20 to 30% of each injection would constitute rational initial therapy, followed by maintenance with monthly injections of 100 μg. This constitutes a more intensive therapy than recommended by Unglaub and Goldsmith(4), but is supported by reports(5,6) that therapy adequate to produce a hematological remission may be inadequate for reversing the neurological disease pattern in the Vit. B_{12} deficient individual.

Apart from its physiological role in maintaining normal metabolism, Vit. B_{12} has been reported to exert a pharmacological effect in a variety of conditions such as non-specific neurological disease and neuroblastoma in infants(2,7,8) when massive doses, 1 mg or more, are administered repeatedly. Thus, it appears that there would be a therapeutic advantage in certain disease states to maintaining an elevation of the Vit. B_{12} circulating in the body. To this end considerable effort has been directed toward the search for forms of Vit. B_{12} that upon injection would be better retained by the body and maintain elevation of blood levels for a longer period of time. This effort has met with some success in development of effective depot forms of cyanocobalamin(9,10).

We are reporting in this paper that high and prolonged serum levels are obtained in dogs after injection of aqueous solutions of hydroxocobalamin.* Since this substance is injected in true solution its retention must be due to other factors than a depot effect and, therefore, the term physiological retention has been used to describe this phenomenon

Experimental procedure. Dogs were individually caged and fed standard rations throughout the experiment. They were selected at random from animals being held for future pharmacologic study and, except for those which had received antibiotic therapy for infections, had not been on previous medication. Since this study was concerned with maintenance of elevation of serum Vit. B_{12} levels, samples were not collected until 5 hours post injection, when preliminary investigation had revealed that the levels following administration of aqueous cyanocobalamin had passed their peak and maximum rate of decline.

Solutions of the cobalamins to which 0.9% benzyl alcohol had been added as a preservative were sterilized by filtration and injected into the gluteal muscle (500 μg). Blood was obtained by venipuncture of the jugular vein

* Hydroxocobalamin has been referred to in the literature as vit. B_{12a} and vit. B_{12b}(11). According to Smith(12) it exists as aquocobalamin except in alkaline solutions.

TABLE I. Comparison of Effect of Single Injections of Hydroxocobalamin (H)* and Cyanocobalamin (C) on Vit. B_{12} Serum Levels of the Dog.

		mμg vit. B_{12} activity/ml											
		Hr after administration											
	No. dogs	0		5		24		48		96		168	
Exp.	compared	H	C	H	C	H	C	H	C	H	C	H	C
1	2	.60	.61	15.70	13.20	3.98	1.32	1.86	.91				
2	2	.33	.42	18.60	3.31	4.68	1.41	2.51	.84	1.06	.79		
3	2	.32	.47	19.10	8.51	4.22	1.60	2.54	1.43				
4	2	.28	.32	12.60	4.03	2.69	1.10						
5	2	.23	.26	34.30	3.31	4.52	1.41	1.93	.85	1.16	.62		
6	3	.16	.33	31.80	5.66	6.76	2.14	2.84	1.56	1.51	.94	.61	.52
6A†	3	.20		30.85		5.37		2.88		1.55		.57	
7	2	.29	.26	72.40	4.22	10.10	1.27	4.22	.78			1.26	.44
Avg		.28	.36	25.10	5.33	4.98	1.48	2.57	1.05	1.27	.79	.78	.48
P value				<0.05		<.001		<.001		<.025		NS	

500 μg of each preparation were inj. into the gluteal muscle of 2 (3) dogs per experiment. Blood was obtained for serum analysis at indicated interval. Values are expressed as geometric mean.

* Hydroxocobalamin in Exp. 1 through 4 analysed as 75% of total cobalamins. Purity of hydroxocobalamin in Exp. 5-7 was reported to be 90%.

† These data not included in the statistical analyses.

0, 5, 24 and 48 hours post injection. Serum was drawn off after clot formation and kept frozen until assayed.

Vit. B_{12} was determined by the microbiological assay procedure employing *L. leichmannii,* ATCC 4797(13) with the following modifications:

(1) Double strength assay medium was supplemented with 200 mg/liter each of guanosine, guanylic acid and calcium chloride, and 1 g of DL-α-alanine. The final mixture was adjusted to pH 5.8.

(2) *L. leichmannii* was carried in single strength basal medium supplemented with 0.1 mμg per ml of Vit. B_{12} for several 24-hour transfers prior to use as an inoculum, returning to stock milk culture every 2 weeks. The inoculum was prepared by washing the cells 3 times with sterile physiological saline, resuspending in saline, and diluting 1 to 5,000.

(3) Cyanocobalamin was used as the standard in the range from 10 to 100 μμg per 10 ml single strength medium. Hydroxocobalamin was equal to cyanocobalamin in microbiological activity.

Serum samples were diluted with water and assayed without further treatment. Because of serum protein precipitation in the assay tubes, assays were read titrimetrically instead of turbidimetrically after 72 hours incubation at 37.5°. All glassware employed, both in collection of blood samples and in the assay, was meticulously cleaned.

Results. A comparison of serum Vit. B_{12} activity after injection of 500 μg doses of cyanocobalamin and hydroxocobalamin is shown in Table I. All sera in a given experiment were assayed simultaneously. Although an equivalent total cobalamin dose was administered, the hydroxocobalamin samples were not chemically pure, so that in fact the first 4 experiments compare an actual hydroxocobalamin concentration of 375 and the last three 450 μg with the 500 μg dose of cyanocobalamin. Regardless of this discrepancy, the serum cobalamin concentrations following administration of hydroxocobalamin are consistently significantly higher than those following administration of cyanocobalamin. Hydroxocobalamin was administered in a stabilized solution in Exp. 5, 6 and 7. The data shown in Exp. 6A were obtained simultaneously with those in Exp. 6 on dogs injected with the same concentration of hydroxocobalamin in water and show that the stabilizing formula does not alter the inherent activity of the hydroxocobalamin.

The prolonged serum concentration observed after injection of hydroxocobalamin suggested that, in comparison with cyanocobalamin, it is more readily taken up by body receptor sites. It was therefore of interest to

TABLE II. Comparison of Total and Bound Vitamin B_{12} in Serum after Administration of Cyanocobalamin or Hydroxocobalamin to Dogs.

Hr after admin.	mμg vit. B_{12}/ml Hydroxocobalamin Total	Bound	Cyanocobalamin Total	Bound
0	.264	.160	.305	.165
5	20.6	6.25	8.81	1.05
24	5.28	1.60	1.80	.638
48	1.61	.852	.782	.590

500 μg of each preparation were inj. into gluteal muscle of 2 dogs. Avg values are given.

determine whether there was a difference in amount of bound Vit. B_{12} activity in the serum of dogs injected with the 2 cobalamin analogs. Using the charcoal absorption technic of Miller(14) to remove free Vit. B_{12}, the sera from dogs in one study group were assayed before (total) and after (bound) norit treatment. The results are shown in Table II. The amount of bound Vit. B_{12} in the serum of dogs injected with hydroxocobalamin was greater than in dogs receiving cyanocobalamin. Preliminary studies had established that the concentration of charcoal employed was adequate to remove either of the free cobalamins up to 50 mμg.

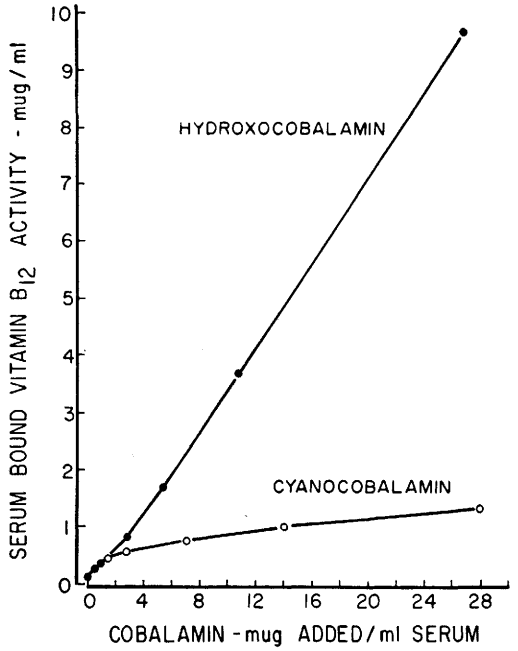

FIG. 1. Binding of cobalamins by normal dog serum.

The difference in binding capacity of dog sera for the 2 cobalamins was clearly demonstrable *in vitro*. Fig. 1 shows that when serum was incubated with varying concentrations of hydroxocobalamin and subsequently treated with charcoal to remove free cobalamin, the concentration of bound Vit. B_{12}, as determined microbiologically, increased in proportion to the original concentration to which the serum was exposed. On the other hand, serum binding of cyanocobalamin was proportionately less as concentration was increased, as Miller had found, and appeared to level off at a relatively low concentration. A possible explanation for the difference in serum binding may lie in the chemical stability of cyanocobalamin which limits the sites at which it may be bound as compared to hydroxocobalamin which readily dissociates and may equilibrate with a variety of serum constituents.

Discussion. Most of the cobalamin found in tissue is known to be bound to protein and a large part of it is present in the noncyanide form(15). The data presented here suggest that hydroxocobalamin may be more available to tissue receptor sites than cyanocobalamin. By whatever mechanism it is retained for longer periods in the circulating blood, the time interval for effective tissue saturation is increased. If the amounts bound in serum are indicative, tissue binding generally may be increased. Preliminary clinical studies(16) in normal subjects indicate that this may be the case, since there appears to be in man slower disappearance of hydroxocobalamin from site of injection, increased liver uptake and less rapid urinary excretion when compared with cyanocobalamin. Thus while hydroxocobalamin is not a "depot" compound in the usual sense of the word, it appears to have physiological "depot" activity.

It has long since been established that hydroxocobalamin is as effective as cyanocobalamin in treatment of pernicious anemia (17). If there is any virtue to the supposition that prolonging the availability of cobalamin will be of therapeutic advantage in treatment of certain neurological disease, hydroxocobalamin may prove more effective in

these conditions than cyanocobalamin.

Summary. Hydroxocobalamin, when injected intramuscularly into dogs, produces more prolonged elevation of serum Vit. B_{12} than does injection of equal quantities of cyanocobalamin. The possible physiological significance of this observation is discussed.

1. Conley, C. L., Krevans, J. R., Chow, B. F., Barrows, C., Lang, C. A., *J. Lab. and Clin. Med.*, 1951, v38, 84.

2. Reisner, E. H., Weiner, L., *Blood*, 1953, v8, 81.

3. Mollin, D. L., Ross, G. I. M., *Brit. Med. J.*, 1953, v2, 640.

4. Unglaub, W. G., Goldsmith, G. A., *J. Am. Med. Assn.*, 1956, v161, 623.

5. Holmes, J. M., *Brit. Med. J.*, 1956, v2, 1394.

6. Lereboullet, J., Pluvinage, R., *Semaine d. hop. Paris*, 1953, v29, 1849 (Abst. *J. Am. Med. Assn.*, 1953, v153, 601).

7. Bodian, M., *Pediat. Clin. North Am.*, 1959, v6, 449.

8. Surtees, S. J., Hughes, R. R., *Lancet*, 1954, v1, 439.

9. Arnold, A., Berberian, D. A., Sobell, S. D., *Am. Pract. Digest Treat.*, 1958, v9, 1835.

10. Thompson, R. E., Hecht, R. A., *Am. J. Clin. Nutrition*, 1959, v7, 311.

11. Kaczka, E. A., Wolf, D. E., Kuehl, F. A., Jr., Folkers, K., *Science*, 1950, v112, 354.

12. Smith, E. L., *Vitamin B_{12} and Intrinsic Factor*, Ed. Heinrich, pub. Stuttgart, 1957, p. 3.

13. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., Wright, L. D., *J. Biol. Chem.*, 1950, v184, 211.

14. Miller, O. N., *Arch. Biochem. and Biophysics*, 1957, v68, 255.

15. Tarr, H. L. A., *Can. J. Technol.*, 1952, v30, 265.

16. Glass, G. B. J., Int. Congress Hematol., Tokyo, Japan, Sept. 1960.

17. Schilling, R. F., Harris, J. W., Castle, W. B., *Blood*, 1951, v6, 228.

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Bile Salt Absorption at Various Levels of Rat Small Intestine.* (26163)

R. DAVID BAKER,†‡ AND GORDON W. SEARLE

Department of Physiology, College of Medicine, State University of Iowa, Iowa City

Frolicher(1), using an *in vivo* technic, showed that the terminal ileum of the rat has the ability to absorb cholates more rapidly than does the upper jejunum. The upper jejunum, in turn, was shown to be a better cholate absorber than the duodenum. The present investigation confirms and extends Frolicher's findings.

Methods. The experiments were performed on adult, male, albino rats (Holtzman Co.) which had been fasted for 24 hours with access to tap water. Extract of Ox Bile, N. F. (Wilson Laboratories) was used as a source of conjugated cholate. The bile salts in this preparation consist principally of sodium glycocholate and sodium taurocholate. An approximately 1.0% solution of Extract of Ox Bile in distilled water was used as the initial test solution. The cholate concentration of this solution in the various experiments ranged from 11.6 to 13.2 μmoles per ml.

An *in vivo* isolated loop technic was used. An attempt was made to overcome two major problems often encountered in such technics: (a) the progressive decrease in intralumenal hydrostatic pressure, and (b) the difficulty in completely removing all of the remaining test solution from the loop at end of experiment.

Each animal was anesthetized with sodium pentobarbital (about 40 mg/kg, I.P.); its abdomen was opened in the midline; and a segment of small intestine, roughly 10 to 15 cm long was cannulated at both ends. The position of the segment along the intestine varied from animal to animal. The bile duct was tied off if it entered the loop of intestine selected. The cannulae were connected

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‡ Present address: Dept. of Physiology, Univ. of Texas, Medical Branch, Galveston.