Stability of Injected Vitamin B_{12}-Co^{60} and Vitamin B_{12} Content of Dog Liver. (26038)

C. ROSENBLUM, D. A. WILLIGAN, H. T. MERIWETHER AND E. P. CRONKITE
Merck Sharp & Dohme Research Laboratories, Rahway, N. J. and Brookhaven National Laboratory, Upton, L. I.

Interpretations of biological studies with cobalt-labeled Vit. B_{12} customarily assume that the administered tracer remains in tissue as the intact vitamin. The need for experimental support for this assumption has been recognized by many investigators(1,2). Scattered information indicates that orally administered Vit. B_{12}-Co^{60} exists in the human, at least initially and in part, as undegraded vitamin(3). A similar conclusion was drawn from examination of urine of rats receiving comparatively large doses of the labeled vitamin subcutaneously(4). The stability of Vit. B_{12} injected at physiological doses and retained by tissue for prolonged periods has not received much attention. Reports of examination of a single dog liver receiving 8.25 μg of labeled vitamin would indicate that Vit. B_{12} is stored at least partially (up to $\simeq$ 64%) intact(2,5). The present communication presents results of an investigation of livers from 5 dogs receiving 3.6-14 μg (specific activity $\simeq$ 1 mc/mg) of Vit. B_{12}-Co^{60} intravenously. The dogs were sacrificed 5-71 days following injection, and tissues were immediately stored in the frozen state for 10 months until assayed for cyanocobalamin and for non-cyano forms by isotope dilution methods (6). By this means it was possible to demonstrate that the administered labeled vitamin retained in the livers was present as intact cobalamin and probably as cyanocobalamin. It was further shown by a reverse isotope dilution experiment(6), that amount of free cobalt produced was negligible. Finally, an estimate of total cobalamin concentration was made by direct isotope dilution assay (6,7,8,9,10) employing the *in situ* radioactive cobalamin-Co^{60} as tracer, and in certain instances, with added Vit. B_{12}-Co^{57}. The isotope dilution method for assaying biological and pharmaceutical preparations for total cobalamins is amenable to modification by omission of initial cyanide treatment, so as to yield a value for cyanocobalamin alone.

Experimental. Animals. One of the dogs involved (282) was the subject of an extensive study of biliary excretion of Vit. B_{12}-Co^{60} reported recently(1) by several of the present authors. Three of the remaining animals were employed for an irradiation study. Dog 274 was normal. Relevant information such as weights, vitamin doses, and vitamin tissue content is compiled in Table I. The radioactive vitamin constituted but a negligible part of total Vit. B_{12} activity of these livers.

Procedures. In the reverse isotope dilution assay for total cobalamin, 50 g of tissue was homogenized with water in a Waring blendor to a total volume of $\simeq$ 250 ml, and $\simeq$ 10 mg (known amount) of crystalline cyanocobalamin added as carrier. To $\simeq$ 125 ml were added $\simeq$ 600 mg of $NaNO_2$ and 250 mg of KCN, and the whole heated to boiling for an hour before proceeding with the isolation procedure(9,10) for purified Vit. B_{12}. In addition to the standard procedure a zinc defecation step(7) and more rigorous acid washing were introduced. When only cyanocobalamin was sought, initial KCN was omitted. Carrier was of course omitted in direct isotope dilution assays with *in situ* Vit. B_{12}-Co^{60} or with added Vit. B_{12}-Co^{57}. In these analyses 100-200 g samples of tissue were employed, and both nitrite and cyanide were added. Cobalt chloride was employed as carrier (10 mg Co^{++}) for the reverse dilution assay for freely exchangeable or ionic cobalt. Approximately 50 g of homogenized liver containing the carrier cobalt was equilibrated by long standing at room temperature and by heating for an hour. The homogenate was then filtered, and ionic cobalt was extracted as the thiocyanato complex by means of secondary amyl alcohol. Cobalt was determined colorimetrically by the thiocyanate method(11). Cyanocobalamin content of carrier solutions and of mixed iso-

TABLE I. Experimental Animals (Males)—IV Administration.

Dog	Wt (lb)	Vit. B_{12}-Co^{60} dose (μg)	Days post-inj. at sacrifice	Liver wt (g)	Vit. B_{12} in liver: μg vit. B_{12}-Co^{60}	Vit. B_{12} in liver: Total μg	Comments
282	52	3.6	5	906	.7	400	Biliary fistula experiment
274	32	14.3	25	650	3.4	400	Normal
276	22	3.6	70	304	.3	73	Received 890 R x-radiation unilaterally
328	35	3.6	71	420	.4	135	
320	31	3.6	71	400	.4	84	Bilateral irradiation, total dose 290 R

topic products isolated from liver was determined spectrophotometrically. Radioactivity was measured by gamma ray scintillation counting in a well-type NaI.T1 crystal. When Co^{57} and Co^{60} labeled tracers were both present, gamma scintillation spectrometry was employed to distinguish between modifications.

Confirmatory evidence of the identity of recovered radioactivity with cyanocobalamin was obtained in all cases. In 2 instances, identification was made by chromatography on paper(12) using 0.01% KCN in secondary butanol (water saturated) as a developing solvent. This correspondence was established in all cases by determining radioactivity and color (where possible) distribution coefficients between the immiscible solvent pair water-butanol.

Results and discussion. Table II is a summary of results of reverse isotope dilution assays of 5 livers. This table contains percent recovery of radioactivity as cyanocobalamin alone and as total cobalamins. In all cases but the liver of dog #276, both total and cyanocobalamin account for approximately 100% of total radioactivity suggesting that the administered Vit. B_{12}-Co^{60} which resides in these tissues is still present as intact cyanocobalamin. Paper chromatography of total cobalamin isolate from a sample of liver from dog #274 revealed but one component in which radioactivity and Vit. B_{12} color (visible and spectrophotometric assay) coincided. Final confirmation of the cobalamin nature of the radioactive isolate is seen in the values of distribution coefficients (Columns 4 and 5, Table II) determined colorimetrically and radiometrically. The essential equality of these 2 values (in a given isolate) supports the common origin of color and radioactivity, *i.e.*, cyanocobalamin. It was also noted, in agreement with an earlier report(2), that radioactivity was distributed uniformly throughout the liver since different samples of a given tissue exhibited comparable (within 10%) cpm/g.

TABLE II. Recovery of Injected Cyanocobalamin-Co^{60} from Dog Livers.

Dog	% Co^{60} recovered: As cyanocobalamin	% Co^{60} recovered: As total cobalamins	Dist. coeff.: R'act.	Dist. coeff.: Color
282	99	103	1.18	1.21
	100	100	1.25	1.21
274	98	98	1.23	1.18
	92	104	1.26	1.16
276	56	97	1.34	1.20
	66	98	—	—
328	96	95	1.26	1.24
320	94	95	1.25	1.21

Reverse dilution assay of one liver sample (dog #274) for free or readily exchangeable cobalt yielded a value of $\simeq$ 1.0% of total radioactivity. Further purification would probably lower this value still further. Obviously, appreciable degradation of Vit. B_{12} to low molecular weight (ionic) cobalt compounds does not occur.

In view of the apparent stability of injected Vit. B_{12}-Co^{60} retained by liver, this endogenous labeled vitamin was utilized as an *in situ* tracer for the isotope dilution assay of liver for total cobalamin content. Isolation was performed as described in the reverse dilution procedure for total cobalamins. No attempt was made to distinguish between cyano and non-cyanocobalamins. Values of total cobalamins are listed in Table III. Radioactiv-

TABLE III. Vitamin B_{12} Content of Dog Livers.

Dog	Time lapse, mo*	μg vit. B_{12}/g Isotope Dilution Co^{60}	Co^{57}	$K_{dist.}$ (Co^{60})
282	10	.44	—	1.21
	22	.10	.08	
		.06	.07	
274	10	.62	—	—
	22	.14	.14	
276	10	.24	—	1.21
328	10	.32	—	1.28†
	22	.28	.24	
320	10	.21		1.29
Avg of initial assays		.36		

* Between sacrifice of animal and sampling for assay.

† Color distribution coefficient was 1.21.

ity (Co^{60}) distribution coefficients (K_{dist}) reported in column 5 again support the supposition that the isolate is authentic Vit. B_{12}. Furthermore, a paper chromatogram after addition of carrier vitamin to the isolate from dog #274 consisted of a single radioactive spot which coincided with the location of visible color. Using initial (10 months in frozen state) assays, the isotope dilution method gives an average cobalamin concentration of 0.36 μg/g of liver. This is of the order of Vit. B_{12} concentrations reported for mammalian livers(13,14). The drop in successive assay values for the dog livers is attributed to a gradual decrease in tissue cobalamin content, despite storage in the frozen condition during the over-all lapsed time of $\simeq$ 2 years. A similar loss in activity was observed by microbiological assay(15) with *L. leichmannii*. This may be due to microbial infestation while specimens were thawed for sampling. That retained Vit. B_{12}-Co^{60} constitutes an authentic tracer is demonstrated by the agreement with values (Table III) obtained from dilution experiments with Vit. B_{12}-Co^{57} in the cases of livers 282, 274 and 328.

No attempt was made, in performing liver assays by direct isotope dilution, to differentiate between cyano- and non-cyano forms, only total cobalamins being determined. Vit. B_{12} activity appears to exist in tissue, at least in part, as hydroxocobalamin or other non-cyano analogues(16,17). This is in contrast to the observation that injected cyanocobalamin is retained by liver essentially unchanged. It is unlikely that digestion with nitrite alone would generate sufficient cyanide ion to convert non-cyanocobalamin-Co^{60} to the cyano form. It is more likely that the vitamin B_{12} present in tissue, being derived from exogenous sources, is ingested as a non-cyano analogue, whereas injected cyanocobalamin is retained intact. That residence times in tissue between 5 and 71 days yielded the same complete cyano-cobalamin-Co^{60} recoveries indicate the great stability of this compound in tissue.

Summary. Vit. B_{12}-Co^{60} injected intravenously into dogs is retained by dog livers as an intact cobalamin, probably as cyanocobalamin. This was demonstrated by the method of reverse isotope dilution. Direct isotope dilution indicates an average total cobalamin concentration of 0.36 μg/g of dog liver. The authenticity of retained Vit. B_{12}-Co^{60}, introduced by injection, as an isotope dilution tracer was demonstrated by simultaneous analysis with Vit. B_{12}-Co^{57}. Degradation of Vit. B_{12} to ionic cobalt is negligible.

The authors are greatly indebted to Mrs. Helen R Skeggs, Merck Sharp & Dohme Research Laboratories, for microbiological assays. They wish to acknowledge the assistance of Mr. P. G. Gethard from these same laboratories for certain isolations and measurements contributing to Table II.

1. Willigan, D. A., Cronkite, E. P., Meyer, L. M., Note, S. L., PROC. SOC. EXP. BIOL. AND MED., 1959, v89, 81.

2. Glass, G. B. J., Mersheimer, W. L., Laughton, R. W., Schaffer, H., Schwartz, G., *J. Lab. Clin. Med.*, 1958, v52, 860.

3. McLean, L. D., Bloch, H. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 171.

4. Rosenblum, C., Chow, B. F., Condon, G. P., Yamamoto, R. S., *J. Biol. Chem.*, 1952, v198, 915.

5. Wolf, F. H., Weston, R. H., Glass, G. B. J., in Glass, G. B. J., *Gastroenterology*, 1959, v36, 180.

6. Rosenblum, C., *Anal. Chem.*, 1957, v29, 1740.

7. Bacher, F. A., Boley, A. E., Shonk, C. E., *ibid.*, 1954, v26, 1146.

8. Chaiet, L., Miller, T., Bolley, A. E., *J. Agr. Food Chem.*, 1954, v2, 784.

9. Bruening, C. F., Neuss, J. D., Numeroff, P., Kline, O. L., *J. Am. Pharm. Assn. Sci. Ed.*, 1957, v46, 66.

10. U. S. Pharmacopeia XV. First Suppl., 1956, 21.
11. Sandell, E. B., *Colorimetric Metal Analysis,* Interscience Publishers, N. Y., 3 Ed. 1959, p423.
12. Friedrich, W., Bernhauer, K., *Z. Naturforschg.,* 1955, v10B, 6.
13. Shenoy, K. G., Ramasarma, G. B., *Arch. Biochem. Biophysics,* 1954, v51, 371.
14. Wolff, R., Vitamin B_{12} and Intrinsic Factor. 1. Europäisches Symposium Hamburg 1956, Ferdinand Enke Verlag, Stuttgart, 1957, 519.
15. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., Wright, L. D., *J. Biol. Chem.,* 1959, v184, 211.
16. Cooley, G., Ellis, B., Petrow, V., Beavan, G H., Holiday, E. R., and Johnson, E. A., *J. Pharm. Pharmacol.,* 1951, v3, 271.
17. Tarr, H. L. A., *Can. J. Technol.,* 1952, v30, 265.

Received July 15, 1960. P.S.E.B.M., 1960, v105.

Vitamin B_{12} Activity of 5,6-Dimethylbenzimidazolylcobamide Coenzyme for the Chick. (26039)

M. R. SPIVEY FOX, WALTER J. LUDWIG, H. A. BARKER AND H. WEISSBACH
Lab. of Nutrition and Endocrinology, Nat'l Inst. of Arthritis and Metabolic Diseases, Bethesda, Md.; Dept. of Biochemistry, University of California, Berkeley; and Lab. of Clinical Biochemistry, Nat. Heart Inst., Bethesda, Md.

The isolation and properties of 5,6-dimethylbenzimidazolylcobamide coenzyme (DBC coenzyme), which is structurally similar to Vit. B_{12}, have been described(1,2). This coenzyme is required for conversion of glutamate to β-methylaspartate by cell-free extracts of *Clostridium tetanomorphum*(2), and also for isomerization of methylmalonyl-CoA to succinyl-CoA(3-5). The DBC coenzyme has activity similar to that of Vit. B_{12} for supporting growth of *Ochromonas malhamensis* and the Vit. B_{12}-requiring mutant of *Escherichia coli*(2). Since presence of the coenzyme has been demonstrated in rabbit liver, it is reasonable that this compound may also be a coenzyme form of Vit. B_{12} for higher animals. In the present study, activity of the DBC coenzyme was compared with that of Vit. B_{12} for supporting growth of young chicks. A second parameter used for assessing DBC coenzyme activity was suppression of formiminoglutamic acid (FGA) excretion by Vit. B_{12}-deficient chicks. This metabolite of histidine, which is excreted in negligible quantities by normal animals, was found to be excreted in large amounts in urine of Vit. B_{12}-deficient rats(6). The same finding has been observed in Vit. B_{12}-deficient chicks (unpublished data).

Methods. Day-old female New Hampshire chicks were distributed into groups of 8 chicks each and maintained for a period of 4 weeks in electrically heated batteries with screen wire floors. The chicks were weighed at weekly intervals. The diet was fed *ad libitum.*

Diet C62, which was used throughout this study, had the same composition as Diet C47 (7) except that 200 g of the glucose was replaced by an equal weight of hydrogenated vegetable oil (Crisco) in each kg of diet. Also, the Salts A used in Diet C47 was replaced by an equal quantity of Salts N(8). Vit. B_{12} was incorporated in this diet at levels shown in Table I.

Since the DBC coenzyme is destroyed by light, it could not be given in the diet. The chicks that received either Vit. B_{12} or the DBC coenzyme by injection received no Vit. B_{12} in the diet. In each case, aqueous solutions of supplements were injected intraperitoneally. The chicks fed a suboptimal level of Vit. B_{12}, 5 μg/kg of diet, served as the reference group in determining quantity of either Vit. B_{12} or DBC coenzyme to be injected. Food intake of this group was measured 3 times weekly and the groups receiving the 5 μg level of "supplement" by injection received exactly the same amount of Vit. B_{12} or DBC coenzyme as Vit. B_{12} eaten by the reference group. For convenience, in tables and text reference is made to weights of Vit. B_{12} and