VITAMIN $B_{12}Co^{60}$ DISTRIBUTION IN DOG TISSUES DURING MANY MONTHS

RED CELL STROMA WITH LABELED B_{12} IN HEMOLYTIC ANEMIA*, ‡, §

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(Received for publication, March 17, 1958)

Published observations from this laboratory (Whipple, Robscheit-Robbins, and Bale) (1) give the concentration of isotopically labeled vitamin B_{12} in various organs and tissues of the dog in short term experiments, 3 to 51 days. Dog 50-72, which remained from these initial experiments, was sacrificed at the end of 9 months. Because of the unexpected distribution of radioactive material, it seemed wise to repeat the procedure in normal and anemic dogs. The concentration and distribution of B_{12} on a per gram basis and for total organ weight are given. The heart, liver, gastric mucosa, and spleen contain large amounts of $B_{12}Co^{60}$ in these long term experiments. The concentration in certain organs is not satisfactorily explained but offers interesting possibilities which are discussed below.

Repeated periods of hemolytic anemia in two dogs (Table 5) result in the appearance, during the period of recovery from anemia, of the isotope labeled B_{12} in red cell stroma. As the non-anemic dog contains little or no $B_{12}Co^{60}$ in the marrow, it is apparent that the material is called forth from the other organs along with non-labeled vitamin B_{12} and used by the marrow during recovery from anemia. Dietary restrictions were not imposed on these dogs: thus it may be assumed that both exogenous and endogenous vitamins are utilized for recovery in these phenylhydrazine anemia periods.

These findings in the dog are in variance with observations in the rat by other investigators (Rosenblum *et al.*) (2). The species differences are further brought out by Miller *et al.* (3), using mice, hamsters, and guinea pigs. The distribution of the isotope-labeled vitamin differs from that of cobalt or cobalt salts as described by Stokinger (6).

* This work was supported by Army Contract DA 49-007 MD 451, with The University of Rochester.

‡ We acknowledge aid from the Ernest L. Woodward Fund.

§ The vitamin B_{12} labeled with radioactive cobalt was received from Merck and Company.

Methods

Red cell stroma protein was prepared as described by Tishkoff *et al.* (4).

All the animals were sacrificed with ether anesthesia, and organs, tissues, blood, bile, and urine were saved and sampled for Co^{60} activity. For measurements of radioactivity 1 gm. portions or the whole organ (when weight was less than 1 gm.) or 1 cc. of fluid were put in 13 × 100 ml. pyrex culture tubes and placed in a well type scintillation counter, similar to that described by Anger (5). The net weight of the organ sample was determined by subtracting weight of tube from weight of organ plus tube. Measurements are expressed as counts per minute per gram of wet tissue. One ml. standards were made on each sample of vitamin $B_{12}Co^{60}$.

Anemias were produced by the subcutaneous injection of acetylphenylhydrazine in saline.

TABLE 1

Anemia Due to Bleeding and Recovery

Dog 50–72. Dose of $B_{12}Co^{60}$, 4.5×10^6 counts per minute.

Autopsy—C.P.M. per gm. wet weight—radioactive cobalt					
Heart	1896	Pancreas	446	Stomach mucosa	985
Skeletal muscle	174	Pituitary	360	Stomach muscle	186
Brain	580	Adrenal	376	Small bowel	91
Spinal cord	67	Thyroid	216	Colon	42
Liver	559	Ovary	174	Uterus	62
Bile	30	Kidney cortex	158	Tubes	173
Spleen	309	Kidney medulla	123	Bladder	101
Lymph node	96	Urine (100 cc.)	19	Skin	154
Bone marrow	25	Lung	87	Aorta	52
Bone	13				

EXPERIMENTAL OBSERVATIONS

The first dog (50-72) was one of three animals used in the previous short term anemia experiments (Whipple *et al.*) (1). The anemia in this dog was produced by bleeding and total vitamin $B_{12}Co^{60}$ received subcutaneously by the animal approximated 4.5×10^6 counts per minute, given in three divided doses. During the subsequent 9 months' period the animal was confined to a cage with no further bleeding. At the end of the 9 months' period the dog was anesthetized (ether) and exsanguinated. All organs were removed immediately, weighed, and samples taken for measurement of radioactivity. The most striking feature of the distribution in this animal was the very high activity of the cardiac muscle per gram as compared to the other tissues. The stomach mucosa also had a very high order of radioactivity but its smooth muscle coat was quite low. The liver showed a high count per gram and because of its weight, a *very high total activity*. A striking difference was noted between the stomach and small and large bowel. The brain showed a high activity, 580 counts per minute per gm., total activity 55,100. The spinal cord read only 67 counts per gm. The bone marrow showed a very low order of activity. The sera and red blood cells had no detectable radioactivity. A 24 hour urine, 1240 cc. from which a 100 cc. sample was

dried and the residue measured for radioactivity, revealed 19 counts per minute. Bile, 1 cc., contained 30 counts.

A second dog (50-64) had multiple periods of anemia produced by phenylhdrazine, and was sacrificed 9 months after the subcutaneous injection of B_{12} (13×10^6 counts per minute). The isotope-labeled vitamin was given prior to the production of hemo-

TABLE 2

Anemia Due to Phenylhydrazine

Dog 50–64. Dose of $B_{12}Co^{60}$, 13×10^6 counts per minute.

Autopsy—c.p.m. per gm. wet weight—radioactive cobalt					
Heart	692	Pancreas	333	Stomach mucosa	611
Skeletal muscle	144	Pituitary	775	Stomach muscle	71
Liver	115	Adrenal	283	Small bowel	51
Spleen	706	Thyroid	46	Large bowel	35
Lymph node	60	Ovary	188	Tubes	61
Bone marrow	10	Kidney cortex	229	Parotid	130
		Kidney medulla	75		
		Lung	40		

TABLE 3

Normal Control

Dog 49–40. Dose of $B_{12}Co^{60}$, 5.7×10^6 counts per minute.

Autopsy—c.p.m. per gm. wet weight—radioactive cobalt					
Heart	1344	Pancreas	241	Stomach mucosa	?
Skeletal muscle	42	Pituitary	282	Tubes	46
Diaphragm	223	Adrenal	184	Uterus	24
Liver	179	Thyroid	73	Bladder	112
Spleen	467	Ovary	54	Esophagus	53
Bone marrow	21	Kidney cortex	195	Skin	12
Bone	0	Kidney medulla	67		
Brain	483	Lung	50		

lytic anemia. There were no diet restrictions or attempts to alter exogenous sources of B_{12}. Even though multiple periods of phenylhydrazine anemia were produced, the tissues at autopsy still contained considerable radioactivity. The heart, liver, stomach mucosa, and spleen showed the highest readings. This splenic activity resulted, in part, from the hydrazine anemia with the accumulation of damaged red cells and stroma within the splenic pulp. Again a striking feature of this long term dog was the very high activity of the gastric mucosa and heart muscle. The small and large bowel had very small amounts of activity. The adrenal and pituitary had high orders of activity per gram but low total activity because of small size. The thyroid showed very low counts. The pancreas showed a medium concentration of counts per gram, but the parotid showed about one-third as much (Table 2). The accumulation of

B_{12} within the pancreas is perhaps explained, in part, by the affinity of cobalt for ductless glands. The urine and bile were not collected.

The third dog (49-40) was sacrificed 9 months after a single subcutaneous injection of vitamin $B_{12}Co^{60}$ (5.7×10^6 counts). This was a normal adult on a stock diet. The distribution of isotope again was quite high in the heart, liver, spleen, and brain. The pituitary in this animal was similar to the other endocrines. The thyroid again showed low activity. The brain deserves some consideration as it showed relatively high activity in two dogs—especially the total activity due to its weight. The bone marrow in this non-anemic dog showed the same low counts observed in those animals which were stimulated by multiple periods of active red cell regeneration. The stomach showed unsatisfactory readings, but owing to loss of specimen it could not be checked.

TABLE 4

Total Organ Counts and Weight at Autopsy

Organ	Dog 49–40 normal		Dog 50–72 anemia bleeding		Dog 50–64 anemia hydrazine	
	5.7×10^6 C.P.M. given		4.5×10^6 C.P.M. given		13×10^6 C.P.M. given	
	gm.	*C.P.M.*	*gm.*	*C.P.M.*	*gm.*	*C.P.M.*
Heart	150	201,600	160	303,360	150	103,800
Skeletal muscle	7600	318,200	7600	1,324,000	7600	1,094,400
Liver	585	104,715	510	285,090	620	71,300
Pancreas	47	11,327	60	26,760	40	13,320
Spleen	70	32,690	50	15,450	50	35,300
Kidney	50	9,750	50	7,900	50	11,450
Stomach	—	—	160±	150,000±	160	97,760
Brain	72	34,776	95	55,100	—	—

The summary in Table 4 adds some facts of interest as it brings out more clearly the magnitude of the amount of total radioactive material present in various tissues and organs than does the data showing amount per gram only. The dogs weighed approximately 20 kg. and the skeletal muscle was estimated on the basis of other observations at 38 per cent total body weight. This total figure for all skeletal muscle is admittedly a rough estimate, but indicates, at least, that it has retained much radioactive material. High figures for the heart, liver, gastric mucosa stand out, but it is apparent that other organs also retain significant amounts. In the organs listed, approximately 10 per cent ± 2 per cent of the initially injected isotope still remains after a 9 months' period.

In Dogs 50-64 and 50-71, repeated periods of phenylhydrazine anemia were induced. The total number of hemolytic periods was three for dog 50-64 and five for dog 50-71. The total vitamin $B_{12}Co^{60}$ for dog 50-64, 13×10^6 counts per minute and dog 50-71, 13.1×10^6 counts per minute. The dosage of phenylhydrazine ranged between 150 to 200 mg. per injection.

Vitamin $B_{12}Co^{60}$ was administered to dog 50-64 before any anemia periods, followed by immediate sampling (as given in Table 5), whereas dog 50-71 received vita-

TABLE 5

Isotope Activity in Red Cell Stroma Protein during Recovery from Repeated Phenylhydrazine Anemia Periods

Dog 50–64. Total counts give 13 × 10^6. Dog 50–71. Total counts give 13.1 × 10^6.

Time from start	Days in experiment	c.p.m./*gm.*	Time from start	Days in experiment	c.p.m./*gm.*
Nov. 1, 1956	0	0	Jan. 4, 1957	0	0
	2	22		10	1224
0	5	129	0	12	1228
Non-anemic control	9	494	Anemia	17	1220
				49	213
Mar. 3, 1957	0	0	Mar. 20, 1957	0	14
	15	571		14	159
43 days	22	188	64 days	22	63
Anemia	27	125	Anemia	26	99
	36	0		29	45
				40	129
June 4, 1957	0	0	July 1, 1957	0	0
	8	57		22	17
113 days	16	203	188 days	37	63
Anemia	21	54	Anemia	44	47
	23	32		57	92
	30	0		59	40
				71	135
				86	72
Sept. 10, 1957	0	0	Dec. 26, 1957	0	0
	8	30		12	33
218 days	10	51	356 days	14	20
Anemia	14	28	Anemia	16	18
	16	58		19	11
	27	5		21	18
	29	0			
			Repeated hydrazine injection Jan. 27, 1958	46	63
				50	54
				54	14
				57	9
				64	9

min $B_{12}Co^{60}$ at the start of the destruction of red cells and at the peak of red cell destruction of the first phenylhydrazine anemia period. As an index of red cell destruction, hematocrit values were determined before each injection of phenylhydrazine. Blood samples for red cell stroma were obtained before each of the anemia periods (zero time) and when the hematocrit value was down in the range of 20 to 25 per cent, stroma sampling was begun, on about the 10th day. The days of experimental period are indicated in Table 4. Phenylhydrazine administration was discontinued

following the establishment of a satisfactory anemia. Prompt recovery from the anemia then followed.

The red cell stroma was prepared from 50 cc. samples of whole blood obtained by vena puncture. The extracted protein stroma samples were dried and weighed and then counted. The initial samples counted from dog 50-64 were 100 mg. approximately, whereas those of dog 50-71 averaged 500 mg. in the last three experimental periods in order to decrease errors in counting due to small sample size.

The initial activity of the red cell stroma is relatively high, but with repeated anemic periods the activity per gram falls off and there is detectable activity (during recovery from anemia) in the red cell stroma up to one year following the initial injection of vitamin $B_{12}Co^{60}$ (dog 50-71). Isotope activity is found in the stroma for a variable period of time. Although the per gram activity in each sample is not extremely high, the evident repeated rise and fall of isotope activity, with the repeated anemia periods in these two animals, indicates the calling forth from body stores of the isotope and vitamin, as demonstrated in distribution tables. Urine and feces were not collected during these periods.

DISCUSSION

The unexpected distribution of radioactivity in various organs in these 9 month experiments is easily recorded, but the correct explanation will be the subject of debate by various investigators. This distribution certainly does not suggest a cleavage of $B_{12}Co^{60}$ and storage of the radioactive radical as a physiologically inert substance.

Its concentration within the *heart muscle* speaks against any such cleavage, but why is it found here 9 months after its injection? The heart muscle cell does not pick up inert material. One of the substances that varies within the heart muscle is the muscle hemoglobin (myoglobin). It increases with work and training. The energy production in heart muscle is very high. In contrast to the heart muscle we note the *skeletal* striated muscle per gram contains small amounts of radioactivity—approximately one-tenth as much as the heart. Smooth muscle likewise is low in radioactivity.

The gastric mucosa which initially contains much of the isotope labeled vitamin seems to retain this with the passage of time, and, on the basis of total weight, ranks along with the heart and liver. This persistence of the vitamin is, perhaps, related to Castle's intrinsic factor.

The liver content on a per gram basis is not as great as either the heart or gastric mucosa, but on a total weight basis is seen to have a large amount. This can be explained by the known relationship of liver and erythrocyte maturation. An additional factor may be the reported role of vitamin B_{12} in methyl transfer, and the utilization of homocystine in the presence of B_{12} as a source of methionine (7).

The brain has a high total content of $B_{12}Co^{60}$ and again this cannot be attributed to storage of an inert fraction. The brain in megaloblastic anemias

may show functional disturbance which will clear up following administration of B_{12}. Presumably, its storage in the normal dog brain would indicate some functional importance.

The spleen and liver may accumulate cell fragments and resistant material from the blood—for example, malarial pigment. This material, as a rule, will be metabolized and eliminated or used over within the body pool. When the isotope label is found in these organs, one might suspect that a cleavage fraction of the whole B_{12} is being analyzed. Other evidence speaks against this explanation.

Although there is apparently abundant labeled material in the various endocrine organs (pituitary and adrenal), the reason is not clear but, perhaps, is related to the affinity of cobalt for the hormones contained within these areas. Its role in the metabolism of these organs is not clear. The thesis of cobalt-hormone affinity may be best brought out by the comparison of the pancreas and parotid glands which have excretory function. The pancreas has the additional function of producing insulin.

The bone marrow, during normal periods, contains small amounts of the isotope labeled material, but with stress, such as produced by phenylhydrazine anemia, there is an increased need for vitamin B_{12}. Thus there is transfer from other organ sites to the marrow for use in red cell production, occurring even though the animals have normal diets. Because of localization in the stroma, it is our feeling that we are dealing with isotopically labeled vitamin B_{12} and not fragments of the vitamin containing this label.

The dog initially excretes in urine large quantities of Vitamin $B_{12}Co^{60}$. With time this is greatly diminished but continues at a very low rate. Bile is apparently another pathway of excretion and this may represent a considerable quantity, as at the end of 9 months 1 cc. of bile contains more isotope than 100 cc. of urine.

The persistence, in these long term experiments, of radioactive cobalt in dog tissues raises the question of the advisability of the use of $B_{12}Co^{60}$, or other cobalt isotopes, as diagnostic procedures in studies of anemias in humans.

SUMMARY

Experiments dealing with the distribution of $B_{12}Co^{60}$ in the dog indicate that with time (9 months after administration) there is a shift in the distribution of the vitamin as compared to the short term experiments, as well as prolonged retention of the vitamin within various dog tissues.

The heart, gastric mucosa, liver, spleen, and brain show high concentrations of the isotope in long term experiments.

This distribution, in the heart for example, does not fit with an hypothetical

breakdown of $B_{12}Co^{60}$ complex and storage of a physiologically inactive fraction.

Repeated periods of anemia produced by phenylhydrazine make it possible to demonstrate radioactive material in red cell stroma of dogs that have previously received vitamin $B_{12}Co^{60}$. This radioactive material must have come from other body stores, such as liver and stomach.

The high concentration of $B_{12}Co^{60}$ in the gastric mucosa suggests a relationship between it and the intrinsic factor as described by Castle.

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