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Received November 17, 1965. P.S.E.B.M., 1966, v121.

Distribution of $Co^{60}B_{12}$ in Blood of Chickens and Rabbits.* (30981)

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Doran and Gregory(1) recently reported that 43 to 91% of chicken blood vit B_{12} is present in the plasma. In contrast to these results, Rosenthal and Brown(2) found that only 12% of chicken blood vit B_{12} is present in the plasma, while in mammals the vitamin is almost equally distributed between the cells and the plasma. Baker *et al*(3,4) found that red cells from normal human subjects contained about 20% of the blood vit B_{12}. Furthermore, Yamamoto *et al*(5) indicated that dog erythrocytes contain no microbiological B_{12} activity while Whipple *et al*(6) found dog red cell stroma protein to be rich in radioactivity following injection of Co^{60} cyanocobalamin during active blood regeneration.

The present study reports on the distribution of fed and injected Co^{60} cyanocobalamin in blood of rabbits and chickens in an effort to resolve the discrepancies between various laboratories by a method which is essentially unequivocal.

Materials and methods. Exp. 1. Two White Rock pullets (1.5 kg) and one Rhode Island Red hen (2.4 kg) were injected I.M. daily with 0.1 ml $Co^{60}B_{12}$† for 18 days while being fed a vit B_{12}-deficient pelleted diet.‡ Three days after the last injection, blood was obtained by heart puncture.

Exp. 2. Sixteen newly-hatched chicks of mixed sex and mixed Red and White Rock strains were obtained from a commercial hatchery. The chicks were placed on a ground purified vit B_{12}-deficient diet‡ supplemented with 8 μg $Co^{60}B_{12}$/kilo diet for 11 weeks. Two chicks died during the first 2 weeks. Seven chicks were exsanguinated by heart puncture for an initial estimation of radioactivity. The remaining 7 chicks were fed the deficient diet without added B_{12} for an additional 16 days in order partially to deplete the vit B_{12} stores and a final blood sample was obtained.

Exp. 3. Seven 30-day-old New Zealand white rabbits of mixed sexes weighing 500 g were fed a ground commercial rabbit diet (Purina Rabbit Checkers) supplemented with 2 μg $Co^{60}B_{12}$/kilo diet for 8 weeks. Two additional rabbits were fed a commercial vit B_{12}-deficient diet‡ supplemented with 2 μg $Co^{60}B_{12}$/kilo. Twenty ml blood was obtained by cardiac puncture for an initial determination. The animals were then placed on commercial pelleted rabbit diet for an additional 14 days with no added $Co^{60}B_{12}$ and a final blood sample was obtained.

All blood samples were heparinized. Whole blood and plasma radioactivity was determined in a well-type scintillation detector,

* Supported in part by Grant P-345A from Am. Cancer Soc.

† The activity of $Co^{60}B_{12}$ (cyanocobalamin) used throughout this study was about 1$\mu c/\mu g$/ml, and was generously supplied by Dr. Elmer Alpert of Merck, Sharp & Dohme Research Laboratories, West Point, Pa.

‡ General Biochemicals.

TABLE I. Distribution of Co^{60} Cyanocobalamin in Blood of Rabbits and Chickens During Vitamin B_{12} Alimentation and Depletion.

Exp	Animal	Condition*	No.	Hct	Plasma, mγ/ml ± S.E.	Blood, mγ/ml ± S.E.	% in plasma
1	Chicken	Initial	3	31	4.72 ±1.88	4.03 ±1.38	81
2	"	Initial	6	30	4.32 ± .55	3.48 ± .43	87
		Final	7	23	2.03 ± .42	1.85 ± .33	84
3	Rabbit	Initial	9	37	1.89 ± .87	1.09 ± .41	109
		Final	9	36	.20 ± .07	.14 ± .04	91

* Initial bleeding indicates samples obtained after $Co^{60}B_{12}$ feeding for 8 weeks (rabbit) and 11 weeks (chicken). Final bleeding obtained after vit B_{12} depletion for 14 days (rabbits) and 16 days (chicken).

TABLE II. Co^{60} Cyanocobalamin in Red Cells of Chickens and Rabbits During Vitamin B_{12} Alimentation and Depletion.

				Red cell conc†		
				Calculated	Found	
Exp	Animal	Condition*	No.	pg/ml ± S.E.		%
1	Chicken	Initial	3	219 ± 26	176 ± 54	80
2	Chicken	Initial	5	127 ± 25	113 ± 25	89
		Final	7	123 ± 25	123 ± 28	100
3	Rabbit	Initial	7	55 ± 23	57 ± 6	104
		Final	7	17 ± 8	14 ± 3	82

* See legend for Table I.
† Calculated from blood and plasma radioactivity and hematocrit before washing. Found concentration only for cells washed 4 times.

and Wintrobe hematocrit determinations were made. The remaining blood samples were distributed in 4 calibrated centrifuge tubes in equal amounts ranging from 4-7 ml for washing. The cells were packed by centrifugation and, after removal of plasma, the cells were washed 4 times with sufficient saline to reconstitute the initial blood volume. After each washing a tube of red cells was hemolyzed with distilled water and an aliquot taken for determination of radioactivity. The data obtained for both sexes were averaged because no significant differences were apparent.

Results. As shown in Table I, the major portion of blood Co^{60} cyanocobalamin is present in the plasma of rabbits and chickens even after the animals were removed from Co^{60} cyanocobalamin for 2 weeks and the distribution is independent of the mode of administration. The blood and plasma levels of Co^{60} cyanocobalamin for chicks on a vit B_{12}-deficient diet for 16 days (Exp. 2) decreased about 50% with little change in distribution of the Co^{60} cyanocobalamin. The blood and plasma vitamin levels of rabbits fed a commercial diet (Exp. 3) for 14 days was about 12% of the initial values with no change in the distribution.

The Co^{60} cyanocobalamin in the red cell reaches a constant level after 2 or 3 successive saline washes. More than 80% of the red cell radioactivity is firmly bound and cannot be removed after being washed at least 4 times (Table II).

Discussion. It is apparent from these experiments that the major portion of blood vit B_{12} is present in plasma in accord with the results of Doran and Gregory(1) but does not agree with the previous data of Rosenthal and Brown(2).

Doran and Gregory liberated the protein bound vitamin by digesting blood and plasma samples with papain, while Rosenthal and Brown used autolysis. Both groups of investigators determined the vit B_{12} content of the digests by microbiological assay using *L. leichmanii*. Papain digestion appears to be more elegant for the release of bound vit B_{12} because interfering deoxyribosides are not liberated by the enzyme while large amounts of deoxyribosides are released during autolysis(7). The autolytic procedure, therefore, requires correction for the microbiological activity contributed by the deoxyribosides. This correction is performed by an alkaline treatment that inactivates vit B_{12}, but the deoxyribosides are stable under these conditions (8). Although an explanation for the divergent results between Doran and Gregory(1) and the present study with the previous data of Rosenthal and Brown(2) is obscure, it appears unlikely that interference from deoxyribosides is the sole cause of the discrepancy for at least two reasons. First, alkaline treatment of autolyzed extracts of chicken blood (2) inactivated 75% of the total microbiologically active material while the deoxyribosides would be expected to be stable to such treatment(8). Second, mammalian blood samples that were not autolyzed also contained between 65-95% microbiologically active material which was inactivated by alkaline treatment, but which is apparently not vit B_{12}. It appears more likely, therefore, that red cells of birds and mammals contain unknown alkali-labile substances with vit B_{12} activity for *L. leichmanii* that are not vit B_{12} or known deoxyribosides *per se*. The fact that treatment with proteolytic enzymes, such as papain, tends to eliminate interference from these substance(s) suggests the possibility that these substances may contain a peptide bond.

Although the use of Co^{60} cyanocobalamin as a tracer for vit B_{12} in the present experiments appears to be unequivocal, it is conceivable that small amounts of Co^{60} cyanocobalamin could be degraded to release inorganic or non-cyanocobalamin cobalt-60, thereby invalidating the interpretation of the present experiments. This does not appear to be tenable because Rosenblum *et al*(9) have convincingly shown that Co^{60} cyanocobalamin may remain in animal tissues for long periods and that negligible amounts of degradation occur. Furthermore, about 50% of an injected dose of inorganic radioactive cobalt is excreted in urine within the first 24 hours or less following administration to mice (10), calves(11), and rabbits(12).

Summary. The distribution of radioactivity between plasma and erythrocytes was determined in 30-day-old rabbits and newly-hatched chicks fed Co^{60} cyanocobalamin supplemented diets for 8 and 11 weeks respectively. In chick blood, 87% of the radioactivity was found in plasma, and all of the radioactivity was present in plasma of the rabbits. The distribution was not altered when the animals were depleted of vit. B_{12} for 2 weeks. The small amount of radioactivity present in erythrocytes was firmly bound and less than 20% was removed by washing the cells 4 times with saline.

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Received November 18, 1965. P.S.E.B.M., 1966, v121.