

RED CELL STROMA PROTEIN RICH IN VITAMIN B₁₂ DURING
ACTIVE REGENERATION

ANEMIA STUDIES USING RADIOACTIVE COBALT B₁₂ IN DOGS*

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The two preceding papers on red cell stroma furnish the necessary background for the use of vitamin B₁₂ with radioactive cobalt.¹ The most active red cell stroma production is found during the 3 to 4 days after the red cells emerge from the bone marrow (second paper). It has been shown that in active regeneration the red cells remain about 3 to 4 days in the marrow as they grow toward maturity. Evidence was given (first and second papers) to indicate that stroma is produced a little ahead of the new hemoglobin and that peak production of stroma protein in hemorrhage anemia anticipates the maximum for hemoglobin production by 2 to 4 days.

In the experiments described below, various types of anemia were induced by bleeding or blood destruction. The dogs were then fed a diet favorable for active blood regeneration and vitamin B₁₂ Co⁶⁰ was given subcutaneously over 3 to 7 days as indicated. Shortly before and after the final B₁₂ injections, red blood cell stroma samples showed high levels of radioactive cobalt B₁₂ concentration. The radioactivity of the stroma protein falls rapidly, and was close to zero in a week or two as blood regeneration brought the dogs back to normal.

At autopsy the organs and tissues showed some rather unexpected concentration of vitamin B₁₂ radioactive cobalt. Unlike the concentration of B₁₂ in the stroma, which was transient and found only during the active red cell regeneration, the radioactive cobalt content of the organs lasted many weeks and probably months. High values of radioactive cobalt B₁₂ were noted in the spleen, pancreas, stomach, heart, and ductless glands. Cobalt escaped in the urine in small amounts even 7 weeks after the injections. The kidney cortex values were quite high as a result. Brain tissue had significant amounts of the radioactive cobalt B₁₂.

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¹The vitamin B₁₂ labeled with radioactive cobalt was purchased from Merck and Company.

Methods

The preparation of stroma protein has been described in the first paper.

Radioactivity measurements were made by placing approximately 1 gm. portions of organs, or the whole organ if it weighed less than 1 gm., in a weighed 13 × 100 ml. pyrex culture tube which was then inserted into a well-type scintillation counter similar to that described by Anger (1). The tube plus organ was then weighed and net weight obtained by difference. Measurements are in terms of a known portion of the injected material, diluted to 1 ml. volume and used as a standard for radioactivity.

For dry weights, tissues obtained at autopsy were dried to constant weight at 84°C. Figures in Experiments 1 to 3 are given for 1 gm. dry weight of stroma or organs.

EXPERIMENTAL OBSERVATIONS

The first experiment (dog 50-72) was a preliminary test and it gave information making for better subsequent experiments. Some concentration of radioactive cobalt⁶⁰-vitamin B₁₂ was noted in stroma protein on the 2nd and 5th days following injection of the cobalt B₁₂. The amounts of vitamin B₁₂-Co⁶⁰ given were less than in subsequent experiments but the doses were all concentrated in 3 days. Meanwhile the diet of liver for active blood regeneration was given for only 2 days. This probably explains the lower figures for radioactivity in stroma protein in Experiment 1 as compared with Experiments 2 and 3. The stroma radioactivity fell close to zero between April 4 and April 21. Possibly the B₁₂ participates in the stroma protein regeneration in the young red cell but does not become a fixed part of this protein in the fully matured cell.

ANEMIA DUE TO BLOOD LOSS

B₁₂ Vitamin (Radioactive Cobalt) Appears in Stroma Protein

Experiment 1—Dog 50-72.—

May 1954 to Mar. 21, 1955, continuous *anemia* due to *blood loss*.

Mar. 21, vitamin B₁₂-radioactive cobalt—Bread diet. Hemoglobin level 6.7 g.m per cent.

Mar. 22, vitamin B₁₂-radioactive cobalt—*Liver*, liver extract and bread diet.

Mar. 23, vitamin B₁₂-radioactive cobalt—*Liver*, liver extract and bread diet.

Total B₁₂-radioactive *cobalt* given subcutaneously = 4.5×10^6 counts per minute.

Mar. 24, blood sample	}	Hemoglobin 52 counts per minute per gm.
		<i>Stroma</i> protein 400 counts per minute per gm.

Mar. 24 to Apr. 7, bread diet—slow blood regeneration.

Mar. 28, blood sample	}	Hemoglobin 29 counts per minute per gm.
		<i>Stroma</i> protein 181 counts per minute per gm.

Apr. 4, blood sample	}	Hemoglobin level 6.2 gm. per cent.
		<i>Stroma</i> protein 130 counts per minute per gm.

Apr. 7, hemoglobin level 7.7 gm. per cent. Liver diet and rapid regeneration of red cells to normal.

Apr. 21, blood sample	}	Hemoglobin 17 counts per minute per gm.
		<i>Stroma</i> protein 50 counts per minute per gm.
		Hemoglobin level 12.7 gm. per cent.

Apr. 26, blood sample	}	<i>Stroma</i> protein 47 counts per minute per gm.
		Hemoglobin level 15.7 gm. per cent.

All counts are net above background counts.

ANEMIA DUE TO BLOOD LOSS

*Stroma Protein Content of B₁₂-Radioactive Cobalt High during Blood Regeneration Recovery Period Followed by a Second Period of Anemia due to Bleeding**Experiment 2—Dog 46-10.—*

Sept. 24, 1947 to Aug. 1, 1949, anemia and hypoproteinemia with interspersed recovery periods. Continuous anemia Nov., 1953 to May 9, 1955.

May 9 and 10, vitamin B₁₂-radioactive cobalt (Co⁶⁰) 3 doses daily, subcutaneously. Diet, salmon bread, 350 gm., salmon, 100 gm., klim, 20 gm. Hemoglobin level, 6.1 gm. per cent.

May 11, active *blood regenerating* diet of cooked pig liver, 400 gm., bread, 250 gm., klim, 20 gm., lextroon including iron, 7.0 gm.

May 13, blood sample } Stroma protein 364 counts per minute per gm.
Hemoglobin 54 counts per minute per gm.

May 16, vitamin B₁₂-radioactive cobalt (Co⁶⁰) injections concluded, total 21 doses, 6.1×10^6 counts per minute.

Blood sample } Stroma protein 1,290 counts per minute per gm.
Hemoglobin 175 counts per minute per gm.

Dog maintained on liver diet until return to hemoglobin level of 13.5 gm. per cent, weight 20.4 kilos on June 1.

June 1, blood sample } Stroma protein 25 counts per minute per gm.
Hemoglobin 16 counts per minute per gm.

June 2, *hemorrhage anemia* produced, continued to July 6, 1955. Salmon bread diet.

July 6, dog killed, ether anesthesia, bleeding, hemoglobin level of 7.1 gm. per cent.

Autopsy (Dog 46-10) Showed no Significant Organ Abnormalities

Autopsy—counts per min. per gm. dry weight—radioactive cobalt

Stroma	0	Lymph node	816	Liver	2,790
Hemoglobin	0	Pancreas	6,996	Hypophysis	7,134
Urine	449	Heart ventricle	4,755	Adrenal	3,409
Kidney cortex	4,425	Skeletal muscle	383	Thyroid	1,013
Kidney medulla	1,272	Bladder	629	Testis	3,652
Rib marrow	644	Stomach	6,117	Lung	1,091
Rib marrow	727	Small intestine	866	Brain	1,601
Spleen	2,632	Large intestine	682	Skin	138

Experiment 2 with anemia due to blood loss was much more complete than Experiment 1. Dog 46-10 had been used for anemia experiments since 1947, with recovery and rest periods interspersed. Anemia was continuous from November, 1953, to the present experiment. Anemia levels = hemoglobin approximately 7 gm. per cent. Vitamin B₁₂-radioactive cobalt was given subcutaneously in divided doses over 7 days, May 9 to May 16—a total of 6.1×10^6 counts per minute.

Active blood regeneration began May 11 with a diet of liver 400 gm., bread 250 gm., klim 20 gm., and liver extract plus iron. This diet continued for 3 weeks up to a near normal hemoglobin level of 13.5 gm. per cent. We can assume a *very active red cell production* under these circumstances.

Four days after start of B₁₂ injection, the stroma protein showed 364 counts per minute per gm. At the completion of the B₁₂ injection, the stroma protein showed 1,290 counts per minute per gm. dry weight. Two weeks later the stroma protein showed only 25 counts per minute per gm. At this time the hemoglobin level was 12.5 gm. per cent. The dog returned to near normal on June 1, 1955.

A second anemia period was then induced by heavy bleeding to a level of hemoglobin of 7.1 gm. per cent. It was thought that a second period of anemia might mobilize B₁₂ from the stores in the body organs and tissues. Obviously this did not occur, as noted in the autopsy table (dog 46-10). The stroma shows *zero* B₁₂ cobalt while the organs retain much activity. Tissue net weight radioactivity calculations show that most of the administered radioactivity was still retained in the body. Some cobalt activity also appeared in the urine, so there was continued movement of the radioactive element. Does this mean obsolescence of B₁₂? Do the organs contain just the cobalt label but not the vitamin B₁₂? If the great bulk of the cobalt activity was observed in the reticuloendothelial system, we might believe that the B₁₂ had disintegrated. Much more work with the urine and other organs must be done before these and many other questions can be answered.

The high values of the stomach, pancreas, heart, and ductless glands suggest interesting possibilities. The brain contained significant amounts of radioactive material.

HEMOLYTIC ANEMIA

Stroma Protein Content of B₁₂-Radioactive Cobalt Very High Early in Blood Regeneration Experiment 3—Dog 54-50.—

Apr. 7 to May 16, 1955, phenylhydrazine anemia. Dosage 50 mg. to 200 mg. daily subcutaneously. Weight 18.4 kilos, hemoglobin 19.1 gm. per cent at start. Diet, salmon bread.

May 16, blood *stroma*, plasma, and hemoglobin show zero counts per minute.

May 16 and 17, vitamin B₁₂-radioactive cobalt (Co⁶⁰) subcutaneously, 3 doses daily. Diet, salmon bread 350 gm., salmon 75 gm., klim 20 gm. Hemoglobin level 7.1 gm. per cent.

May 18, active blood-regenerating diet of cooked pig liver 400 gm., bread 250 gm., klim 20 gm., lextroon including iron 7.0 gm., vitamin B₁₂-radioactive cobalt (Co⁶⁰) 3 doses daily continued.

May 20, blood *stroma* protein 1,279 counts per minute per gm. Hemoglobin level 9.5 gm. per cent.

May 23, total injection period 21 doses in 7 days. Total amount 6.099×10^6 counts per minute per gm. Hemoglobin level 9.5 gm. per cent. Phenylhydrazine continued during entire period. Blood *stroma* protein 2,570 counts per minute per gm.

May 25, blood *stroma* protein 2,632 counts per minute per gm. Hemoglobin level 10.5 gm. per cent.

May 26, dog killed, ether anesthesia, bleeding. Final weight 18.7 kilos, hemoglobin level 7.9 gm. per cent.

Autopsy (Dog 54-50) Showed no Significant Organ Abnormalities

Autopsy counts per min. per gm. dry tissue					
Stroma	2,632	Lymph node	1,951	Liver	6,275
R.B.C. (washed)	1,333	Pancreas	6,254	Hypophysis	5,134
Urine	1,200	Heart	4,686	Adrenal	5,514
Kidney cortex	13,661	Skeletal muscle	866	Thyroid	3,029
Kidney medulla	3,164			Ovary	572
Rib marrow	5,691	Stomach	4,210	Lung	6,029
Rib marrow	4,595	Small intestine	2,005	Brain	1,074
Spleen	12,200			Skin	265

Experiment 3 records a hemolytic anemia of severe type but relatively short duration. The vitamin B₁₂ with radioactive cobalt was given during a week of very active blood regeneration on a diet of liver, liver extract, and iron. The dog was killed 3 days after completion of the B₁₂ injections. The red cell stroma protein was rich in the B₁₂-cobalt vitamin. Organs were rich in B₁₂-cobalt and the urine also contained radioactive material.

The first and second papers give evidence that the stroma protein is about twice as abundant during rapid blood regeneration following hemolytic anemia as in simple anemia due to blood loss. The concentration of B₁₂-cobalt in Experiment 3 indicates a very active production of new stroma labeled with cobalt. A part of this rapid regeneration in hemolytic anemia may be due to readily available parts of destroyed red cells (including stroma). The very high values from the spleen are probably a part of this picture of active blood destruction.

The high values for bone marrow might suggest a large component of rapidly growing red cells rich in B₁₂-radioactive cobalt. These marrow figures are much higher than those recorded in Experiment 2, by a factor of 7. In Experiment 2 there were no labeled red cells after an interval of 6 weeks from B₁₂ injection. High values for the kidney cortex possibly are due to elimination of radioactive material in the urine. High values recorded for the stomach, pancreas, heart and ductless glands resemble the findings in Experiment 2 and bring up the large question of the internal metabolism of B₁₂. This distribution does not suggest a separation of the label from a disintegrated B₁₂ complex. Many important questions must await further observations.

DISCUSSION

Radioactive cobalt as chloride has been administered intravenously in mice by Lawrence (6). There was a very rapid escape of about 50 per cent of the material in the urine during the first 24 hours or less. The distribution of the cobalt in normal mice was somewhat different from the distribution of B₁₂-radioactive cobalt in dogs, as now determined. After 2 weeks, a considerable portion of the remaining radioactivity was found in the mouse pancreas, heart, bone, lungs, liver, spleen, and kidneys, but because of continuing high excretion it added up to only a very small fraction of the original Co₆₀ administered.

Radioactive cobalt chloride was given intravenously to calves by Comar (2). During the first 24 hours or less, large amounts escaped in the urine (about 50 per cent). The distribution after 17 hours was scarcely comparable with our experiments in dogs but showed relatively high values in liver and bile. The intestines and kidneys showed high values.

Vitamin B₁₂-radioactive cobalt was given to normal rats intramuscularly (7). The rats were killed in 4 days. The kidneys showed very high values. Liver, spleen, and heart also showed high values. It was given as well to normal rats subcutaneously (5). Old rats were compared with young ones and no difference in distribution of B₁₂ was found. Other workers also gave it to normal adult rats subcutaneously (4). Observations showed that the radioactivity persisted throughout 90 days in the kidneys, liver, and pancreas. A rapid fall was noted thereafter in the kidneys.

Pernicious anemia has been studied by pathologists with acute interest over the years. Many hypotheses have been proposed and discarded. One of us as early as 1922 (9) expressed the belief that in this disease there was a "scarcity of stroma building material and a surplus of hemoglobin." Again that there was a "lack of stroma building material" (8). The experiments with vitamin B₁₂-Co₆₀ recorded above give support to this hypothesis.

Experimental pernicious anemia has never been produced in animals in spite of numerous attempts. Many of the interesting questions relating to pernicious anemia could be resolved if the disease could be produced in animals. The spectacular response of human pernicious anemia to small doses of B₁₂ is well known. Participation of B₁₂ in the production of new red cell stroma is indicated by these experiments and would fit with the facts as known for pernicious anemia. Observations using B₁₂ labeled with radioactive cobalt can be made in the clinic if this material can be used with safety (3).

Vitamin B₁₂ appears to have some influence upon certain central nervous system abnormalities in pernicious anemia. The accumulation of B₁₂ in the brain at rather high levels is to be noted in Experiments 2 and 3.

Megaloblastic anemias of certain types (other than pernicious anemia) respond to B₁₂ and observations in such cases might be possible using cobalt-labeled B₁₂. It is obvious that much information can be gained by continued experiments dealing with experimental anemia and the utilization of B₁₂-radioactive cobalt.

SUMMARY

During active blood regeneration in anemia in dogs an increase occurs in the stroma protein of the red cells.

When vitamin B₁₂ with radioactive cobalt is given at the start of this blood regeneration one finds *concentration of labeled B₁₂ in the stroma protein* but not in the hemoglobin.

After the acute phase of red cell regeneration is ended the concentration of B₁₂ in stroma protein falls rapidly to very low levels within 2 weeks. Subsequent episodes of red blood cell regeneration seems not to cause remobilization of radioactive cobalt into red cells from other body stores.

It appears that the vitamin B₁₂ is a factor of importance in the first steps of stroma protein formation in the first few days of the life of the red cell in the dog.

This response in dogs and the response in *pernicious anemia* to vitamin B₁₂ may have some points in common.

Distribution of the B₁₂-radioactive cobalt in the organs and tissues at autopsy has been recorded. Some very suggestive localizations were noted and some variation 1 week and 7 weeks after B₁₂ injections.

Radioactive cobalt escapes in the urine during the weeks following B₁₂ injections.

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