THE EFFECT OF VITAMIN B_{12} CONCENTRATE AND COBALT ON THE ERYTHROCYTE COUNT AND BLOOD HEMOGLOBIN LEVEL OF THE ANEMIC RABBIT

by

ARCHIE LEROY GOOD

V. M. D., University of Pennsylvania, 1943

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Physiology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1950

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INTRODUCTION

Recent evidence has shown that another mineral, cobalt, must be included among those elements essential to the health and well being of animals, especially ruminants. Also of recent interest, has been the discovery of vitamin B_{12}. Investigation has proved cobalt to be a part of the vitamin B_{12} molecule. Realizing that the metabolism of one may also be intimately related with the other, this experiment was set up to attempt to gain evidence as to whether cobalt was used directly by the rabbit or whether B_{12} was a step in the metabolism of cobalt in the animal body.

REVIEW OF LITERATURE

Cobalt

Deficiency Diseases. There has been very sufficient evidence to warrant the placing of cobalt among those elements considered to be dietary essentials. Dukes (9) has mentioned that there has occurred in Florida a nutritional anemia known as salt lick that responded to cobalt administration. In addition, cobalt deficiency in cattle or sheep has been reported in Michigan (17), Wisconsin (12) and Australia (6). Other locations mentioned by Dukes include New Hampshire, North Carolina and New York in this country and New Zealand. In these areas the soil has been found to be deficient in cobalt or the element is in a form which is unavailable to plants. The

administration of cobalt in all cases reported, greatly alleviated the deficiency symptoms or caused them to disappear completely. The symptoms of cobalt deficiency as noted by Killhan (17) are extreme emaciation, lack of normal appetite, pica, stary hair coat and anemia. The oral administration of cobalt to these animals produced a remarkable response in that the appetite and appearance improved in three to ten days. The hemoglobin levels at first decreased but gradually climbed to normal. It was suggested that the hemoglobin had not actually been reduced, but that the percentage decrease was due to the increase in blood volume resulting from greater intake of feed and water.

The reported cases of cobalt deficiency have thus far been confined to ruminants. However, there is some evidence that the dog also needs the element. Frost, Elvehjem and Hart (11) working with dogs, noted that about half of their experimental animals showed a cobalt deficiency as evidenced by the hematopoietic response to the administration of cobalt at a minimum level of 0.1 mg per day. In some cases supplementation of milk with iron and copper was sufficient for normal hemoglobin building but the addition of cobalt stimulated hematopoiesis in certain dogs in which blood formation appeared unusually slow.

Role in the Body. The exact role of cobalt in the animal body is at present unknown. In 1928, Titus, Cave and

Hughes (37) reported the role of manganese, copper and iron in the building of hemoglobin. To these, cobalt must now be added, as is evidenced in animals showing the symptoms of cobalt deficiency. Ray et al. (26) among others have postulated that cobalt in minute quantities is related to the proper functioning of rumen bacteria in their synthesis of various B complex vitamins. These workers have called attention to the fact that symptoms of a cobalt deficiency are also manifestations of certain vitamin B complex deficiencies. The anorexia observed in cobalt deficiency of animals may also be seen in the case of insufficient thiamin, and anemia is in turn related to folic acid, pyridoxine, riboflavin, biotin, and pantothenic acid deficiencies. The normal ruminant is not dependent on a dietary source of these vitamins. It is therefore possible that in the absence of cobalt these vitamins may not be produced in sufficient quantities to maintain normal body weight and prevent the other symptoms observed in cases of cobalt deficiency. The fact that cobalt administered orally to ruminants is more effective than when given parenterally (26, 1) adds some weight to the afore mentioned theory. However, this theory does not, by any means, enjoy complete experimental support.

In the attempt to gain further information on cobalt metabolism, radioactive cobalt has been used (5, 14). By this means it was found that when labeled cobalt was

administered orally to rats about 80 per cent of the dose was eliminated in the feces, 10 per cent rapidly eliminated in the urine and very little being retained in the tissue with only the liver consistently accumulating significant amounts. When the labeled cobalt was given orally to a steer, very little was found in the tissues 10 days later. The largest amount was found in the liver which contained 0.25 per cent of the administered dose. When given by injection, there was general distribution throughout the tissues, 5 per cent being retained after 10 days, of which 1 per cent was retained by the liver. Also of interest is the fact that significant amounts of injected, labeled cobalt appeared in the abomasal contents, while none was found in the contents of the three remaining compartments of the ruminant stomach.

The work of Chamberlain et al. (4) reveals that administered cobalt is partially eliminated in the milk of ewes when fed at the level of 100 mg daily per ewe. These experiments also point to the possibility that there may be some placental transfer of cobalt.

The experiments of Kleinberg, Gordon and Charipper (18) tend to prove that cobalt has to do with erythropoiesis at least in the rabbit. These workers produced anemia in the experimental rabbits by bleeding and injections of benzol. When the red corpuscle counts had dropped from approximately

6 million to 2½ to 3 million per cu mm, injections of 50 mg cobalt nitrate daily was begun. The counts of these animals returned to normal in about 15 days whereas when cobalt was not given 25-30 days were required for the counts to return to the normal level. The conclusion drawn from this work was that cobalt in some way stimulates the erythrogenic precursors in the bone marrow.

Toxicity. Though cobalt seems to be specific in cases of apparent cobalt deficiency, the indiscriminate use of large amounts of the element is definitely harmful. The report of Ely et al. (10) substantiates this. These workers found that when cobalt was fed to dairy calves in excess of 40 mg per 100 pounds body weight daily toxicity symptoms were produced. Equivalent amounts of cobalt fed as the sulfate, chloride or carbonate were equally toxic and increasing the methionine content of the grain mixture by the addition of casein did not reduce this toxicity.

Vitamin B_{12}

For more than twenty years prior to the early part of 1948, pernicious anemia in the human being had been treated by dietary means, the eating of whole liver (41). The requirement of liver was one pound per day. It was natural that this treatment should be distasteful to patients, for the consumption of four ounces of liver once weekly is a

commendable dietary practice for the normal adult.

Isolation of and Crystallization. During the years that followed, chemists succeeded in extracting from liver extracts those portions which were responsible for the alleviation of the symptoms of pernicious anemia. By 1943 (41) the extracts from liver were so well concentrated that patients could now survive by taking one milligram of liver extract instead of 400,000 milligrams of liver. The specific constituent of these extracts was unknown until April of 1948 when Rickes et al. (28) isolated a red crystalline compound from liver extracts which gave positive responses in cases of addisonian pernicious anemia. This compound they called vitamin B_{12}.

Chemistry. Vitamin B_{12} crystallizes in the form of small red needles and was soon found to contain cobalt as a part of its molecule (29). The molecular weight was estimated to be between 1550 and 1750. Probably the most significant finding has been the intimate relationship of vitamin B_{12} with cobalt which, as has been mentioned, must now be recognized as a nutritional essential in ruminants in which cobalt dificiency results in typical anemia.

Vitamin B_{12} has been found to be biologically active in extremely minute quantities. Prior to the isolation of vitamin B_{12}, liver extracts had been assayed by using the organism Lactobacillus lactis Dorner. Using this method of assay, Shorb (33) compared vitamin B_{12} with liver concentrate

and found the latter to be approximately 11,000 times more active than the liver concentrate.

Relationship with Animal Protein Factor. The close relationship or possible identity of vitamin B_{12} with the "animal protein factor" has been postulated by a number of authors (2, 21, 23, 34, 41). Important evidence in this direction has been afforded by the work of Ott et al. (21). These workers reported that when crystalline vitamin B_{12} was added to purified basal diets with 40-70 per cent soybean meal as the only source of protein it exerted animal protein factor activity in chicks from hens on an all plant ration. The chicks responded to various supplements known to be sources of the animal protein factor and also to vitamin B_{12} in doses as small as 6 micrograms per kilo of diet. The authors concluded that since the crystalline substance elicited growth responses comparable to the crude sources of the animal protein factor, it was possible that vitamin B_{12} is identical with or very closely related to this factor.

Assay. Various and numerous methods have been used as assay procedures for the potency of vitamin B_{12} (8, 21, 22, 27, 33, 34). Some are microbiological using different lactobacilli and some are biological using chicks or rats. In one case (15) the algal flagellate Euglena gracilis was used as the assay organism. Shive et al. (31) have shown by assay procedures with Lactobacillus lactis Dorner and Lactobacillus

leichmannii that B_{12} and purines (thymidine) are probably interrelated in some way in that thymidine, under certain conditions, is able to replace vitamin B_{12} in the nutrition of the assay organisms.

In so far as growth is concerned Hartman, Dryden and Cary (13) report that vitamin B_{12} occurs in milk, nonfat milk solids, cheese, commercial casein, liver extracts, and leafy foods and feeds (roughages) but is practically absent in yeast and grains. Using a biological assay procedure with rats, Lewis et al. (19) report that fish solubles, streptomycin "slops", sheep rumen contents and glandular meats are excellent sources of the vitamin. Muscle tissue, eggs and milk products contain lesser amounts, whereas plant materials show no measureable activity. Rickes et al. (30) report that a red crystalline compound has been found in the broth cultures of Streptomyces griseus. This compound has been fairly definitely proven to be identical with vitamin B_{12}.*

Use in Treating Pernicious Anemia. One of the earliest reports of the use of vitamin B_{12} in treating pernicious anemia of man was that of West (39). Three patients showing clinical symptoms of pernicious anemia were treated with

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* This is now a commercial source of vitamin B_{12} concentrate (Squibb's Rubramin).

crystalline vitamin B_{12} resulting in an increase in blood hemoglobin, red blood corpuscle count and accompanying reticulocytosis.

Spies et al. (36) found that crystalline vitamin B_{12} is effective in producing a hematological response in persons who have pernicious anemia, nutritional macrocytic anemia and tropical sprue. According to these workers vitamin B_{12}, per unit of weight, is the most effective antianemic substance known. Folic acid was found to produce a positive hematological response to pernicious anemia but did not alleviate or prevent the neurological complications common to persons afflicted with pernicious anemia, whereas these symptoms responded promptly to vitamin B_{12} therapy. In these cases, the crystalline vitamin is usually administered parenterally since Spies et al. (35) have found that though there is a positive response following oral administration, the effective oral dose is 30-50 times greater than the parenteral dose. Davis et al. (7) have reported a case of pernicious anemia in a 6 year old girl which responded favorably to vitamin B_{12} administration.

Role in the Body. The exact physiological role of vitamin B_{12} in the body is unknown. It undoubtedly is essential for proper growth, reproduction, and lactation according to Hartman, Dryden and Cary (13). These workers also mention its relationship to the utilization of high protein diets in

the normal mammal. In addition, these authors mention the fact that ordinarily cattle may synthesize enough B_{12} to maintain the B_{12} potency of the milk, and the work of Lewis et al. (19) would tend to substantiate this. Shive et al. (31) have found that with certain assay procedures, thymidine, may replace vitamin B_{12}. Wright, Skeggs, and Huff (42) have also found that thymidine may replace vitamin B_{12} as a growth factor for certain lactobacilli. They interpreted the data obtained from their experiments as indications that vitamin B_{12} functions as a coenzyme in carrying out reactions concerned with the conversion of thymine to thymidine. Furthermore these authors postulated that the primary biochemical defect in pernicious anemia may very well be the inability to synthesize certain nucleosides, particularly thymidine, from parent purines or pyrimidines. However, thymidine, though able to replace vitamin B_{12} in certain assay procedures, has been found by Ungley (38) to be unable to produce positive hematogenic responses in pernicious anemia.

A possible additional role of vitamin B_{12} in the body may well be in the metabolism of fat. Drill, and McCormick (8) have found that the vitamin exerts a lipotropic effect in rats receiving a high fat diet. The nature of this lipotropic action is unknown. These workers point to the possibility that the action of B_{12} in this respect may be related with that of choline particularly since choline exerts a

sparing action on vitamin B_{12} as measured by the growth of the chick.

Since vitamin B_{12} has been found to be a cobalt complex (29), the possibility existed that it may be an intermediate step in the metabolism of cobalt. Becker, Smith and Loosli (1) working with cobalt deficient sheep, however, were able to give no experimental support to this theory.

That vitamin B_{12}, along with folic acid, is essential for normal hemopoiesis in the pig, is suggested by Cartwright et al. (3). These authors produced a severe macrocytic anemia in pigs by placing them on a folic acid deficient diet. These animals showed some response to the administration of vitamin B_{12} in crystalline form.

MATERIALS AND METHODS

The 12 rabbits used for the experiment were purchased from Mr. G. W. Welch of Manhattan, Kansas. They were about 6 weeks of age at the time of purchase and to all appearances, were in good health. The rabbits were from two litters of six each, both of which were sired by the same buck and the does were mother and daughter. The experimental animals were therefore closely related genetically, tending to insure uniformity in response to experimental treatment.

The feed for the animals prior to purchase consisted of the following mixture:

Kafir corn	100 pounds
Wheat	100 pounds
Whole Oats	50 pounds
Soybean pellets	50 pounds

In addition chopped alfalfa, salt, and water were fed ad libitum.

These rabbits were then divided into four groups of three each and put on a low cobalt containing ration which consisted of the following:

Ground yellow corn	70 pounds
Whole milk powder	15 pounds
Purified Casein	10 pounds
Corn Starch	5 pounds

Two mineral supplements were made of high purity chemicals, both being identical in composition except that one contained cobalt whereas the other did not. These supplements supplied the following daily allowances:

Iron, as ferric ammonium citrate	2.0 mg
Copper, as copper (ous) sulfate	0.2 mg
Manganese, as manganous sulfate	2.0 mg
Potassium iodide	0.01 mg
Sodium chloride	200.0 mg
Cobalt, as cobalt (ous) sulfate	0.04 mg

The supplement was given to each animal weekly on the feed, the control animals (group I) only receiving the cobalt mixture. When the mineral was administered, the amount of feed given was reduced to insure complete consumption.

Realizing that the cobalt requirement for rabbits must be very low, great care was taken to see that there was no contamination of the feed and water given to the animals in groups II, III, and IV. The feed and water containers of group I animals were washed by hand when necessary and returned to the same animals so that they could not be given to any animal in any of the other remaining groups. The feed given the rabbits was, after being carefully but thoroughly mixed, stored in a covered container to prevent contamination with dust.

For a period of eight weeks the experimental animals were observed and closely studied. The red blood corpuscle count, hemoglobin and body weights of each animal were recorded weekly during this time. In this way, an accurate picture of all normal, recorded values was obtained. All animals, including the controls, were then bled to produce anemia. Each animal was bled five times with three to four days intervening between bleedings. At each time 15 to 30 cc of blood were taken, the actual amount being dependent on body weight and general condition of the individual rabbit.

The bleeding was carried out by making a small

longitudinal incision in one of the marginal ear veins and allowing the blood to flow into a glass graduated cylinder. During the bleeding period red corpuscle counts and hemoglobin values were recorded more often so that no animal was bled excessively and the approximate status of each animal in this regard was known at all times.

After the fifth bleeding, the red cell count of all animals had dropped well below four million per cubic millimeter with two animals having a count below three million per cubic millimeter. Treatment was then begun as follows: groups I and II received 1 cc sterile distilled water intravenously, group III 0.1 mg cobalt chloride intravenously and group IV 10 micrograms of vitamin B_{12} (Squibb's Rubramin) intravenously. These injections were administered twice weekly over a period of 2 weeks and during this time red cell counts and hemoglobin values were recorded every third or fourth day. To be assured of accuracy, each count was carried out by two persons and the average taken. In cases of poor agreement between the counts of the two individuals, the counts were rechecked.

Throughout the experiment hemoglobin values were determined by means of a Coleman spectrophotometer, standardized by blood-iron analysis by the method of Wong (40) as modified by Ponder (24).

RESULTS

In Tables 1, 2, 3 and 4 are shown the average body weights, erythrocyte counts, and hemoglobin values of the four groups of animals throughout the experimental period. Figures 1, 2 and 3, in graphic form, serve to compare the body weights of all groups (Fig. 1), the erythrocyte counts of all groups (Fig. 2) and the hemoglobin values of all groups (Fig. 3).

At first, the animals not being accustomed to the semi-purified diet, ate little of it and therefore did not gain rapidly in weight. However, by the third week of the experimental period, they had begun to eat well and this was reflected in their steady gain in weight up to the eighth week at which time bleeding was begun. This caused a reduction in gains of body weight and in one case (Group III) apparently caused a slight loss until the eleventh week at which time a gain was recorded.

The erythrocyte counts, after the third week, fluctuated usually between six and seven million per cubic millimeter of blood until the eighth week. The average erythrocyte count for all groups of animals during this eight week interval was 6,480,000 per cubic millimeter of blood. At the eighth week bleeding was begun and the counts of all groups dropped rapidly to between three and four million, with one group dropping below three million. After the

bleeding was completed (between tenth and eleventh week) all counts began to rise and rose rapidly until the twelfth week. At this time, the average counts of groups III and IV were within normal ranges, with that of group II being almost 6,000,000. Cobalt and vitamin B_{12} therapy was begun between the tenth and eleventh week (December 22, 1949). Group III received the cobalt and group IV the vitamin B_{12}. Both of these groups showed a greater increase in erythrocyte count than did the other two, with the cobalt group having a slightly higher average count than did the group receiving vitamin B_{12}. One animal in group I was responsible for the fall in the average erythrocyte count for the group.

As can be seen upon examination of Fig. 3, the fluctuations of the average hemoglobin values correspond well with the fluctuations of the average erythrocyte counts so that what has been said concerning the erythrocyte counts applies as well to those recorded values for hemoglobin. The average value of hemoglobin for all animals for the first eight weeks of the experiment was 13.14 grams per 100 cc blood.

Table 1. Average body weights, erythrocyte counts and hemoglobin values of Group I (controls) during the period of the experiment.

Week	Body weight in grams	Erythrocyte count millions/cu mm	Hemoglobin grams/100 cc
1	930		11.8
2	958	5.71	13.21
3	998	6.79	14.8
4	1133	6.84	13.6
5	1243	6.85	13.7
6	1372	6.36	12.7
7	1474	6.49	12.5
8	1653	6.14	12.3
		4.51*	
9	1749	4.11	9.1
10	1810	3.59	8.7
		3.57**	
11	1831	4.62	10.2
		5.33***	
12	1830	4.49	10.25

* 12-9-49.
** 12-21-49.
*** 12-29-49.

Table 2. Average body weights, erythrocyte counts and hemoglobin values of Group II (received only the low-cobalt ration) during the period of the experiment.

Week	Body weight in grams	Erythrocyte count: millions/cu mm	Hemoglobin grams/100 cc
1	923		11.3
2	966	4.88	12.3
3	966	6.20	14.18
4	1032	6.36	14.1
5	1160	6.73	13.3
6	1227	6.30	13.2
7	1301	6.95	11.6
8	1335	6.39	12.4
		5.42*	
9	1870	4.75	10.6
10	1867	4.28	8.6
		2.62**	
11	1930	4.36	10.3
		5.19***	
12	1930	5.88	12.2

* 12-9-49.
** 12-21-49.
*** 12-29-49.

Table 3. Average body weights, erythrocyte counts and hemoglobin values of Group III (received cobalt intravenously beginning 12-22-49) during the period of the experiment.

Week	Body weight in grams	Erythrocyte count: millions/cu mm	Hemoglobin grams/100 cc
1	867		11.8
2	934	6.31	13.2
3	1020	6.62	13.98
4	1179	6.15	13.9
5	1347	6.74	13.7
6	1471	6.25	12.9
7	1577	6.12	12.6
8	1718	6.26	11.9
		4.29*	
9	1719	4.40	10.3
10	1712	3.60	8.7
		3.17**	
11	1696	5.19	11.05
		5.63***	
12	1757	6.96	13.2

* 12-5-49.
** 12-21-49.
*** 12-29-49.

Table 4. Average body weights, erythrocyte counts and hemoglobin values of Group IV (received vitamin B_{12} intravenously beginning 12-22-49) during the period of the experiment.

Week	Body weight in grams	Erythrocyte count: millions/cu mm	Hemoglobin grams/100 cc
1	704		10.9
2	773	5.84	12.4
3	917	6.60	14.05
4	985	6.35	15.1
5	1151	7.37	14.9
6	1268	7.38	14.8
7	1409	7.42	13.5
8	1548	7.16	14.2
		4.13*	
9	1673	4.30	9.4
10	1709	3.31	7.95
		3.08**	
11	1806	5.17	10.5
		5.57***	
12	1896	6.67	12.5

* 12-5-49.
** 12-21-49.
*** 12-29-49.

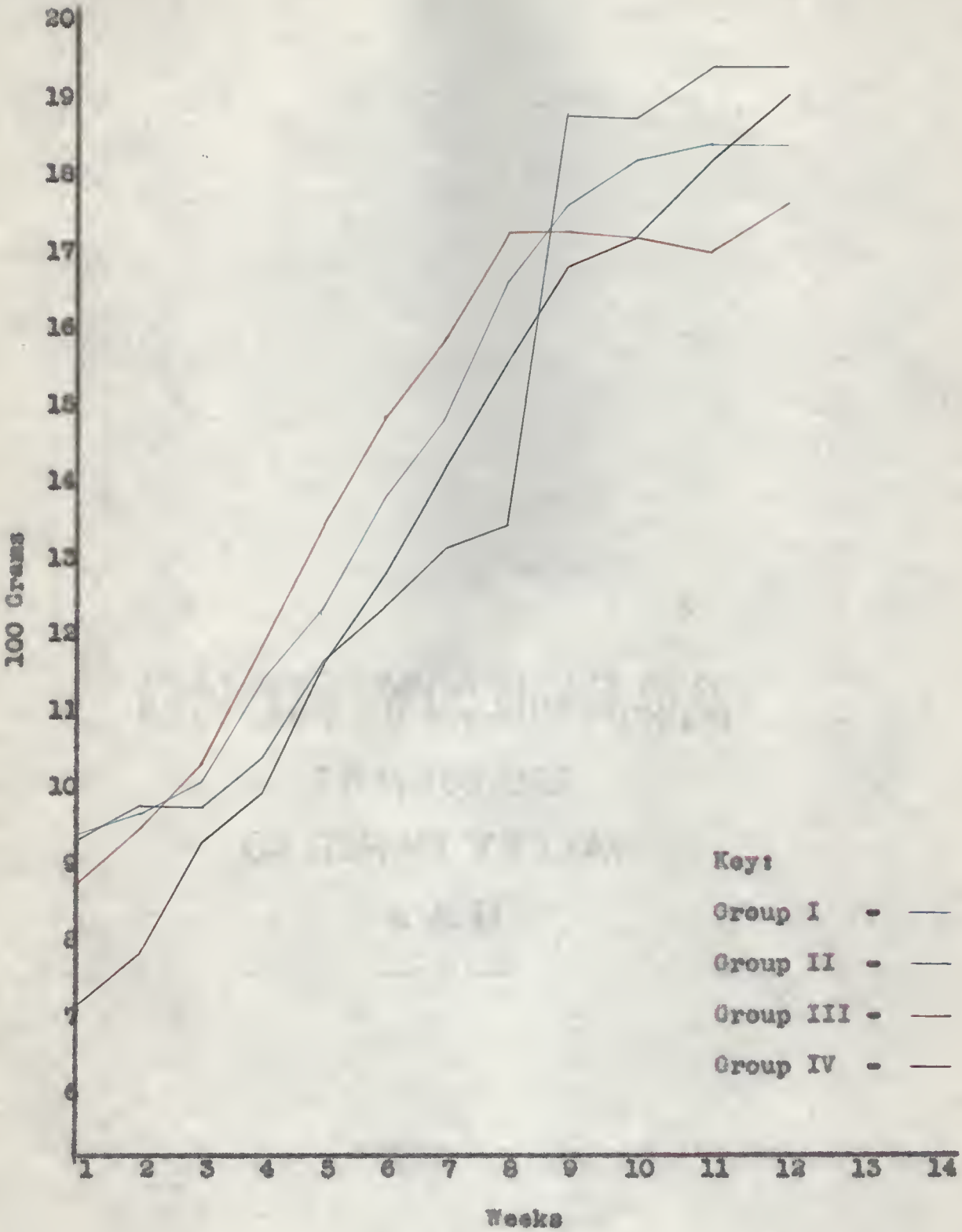

Fig. 1. Group averages of body weights during the experiment.

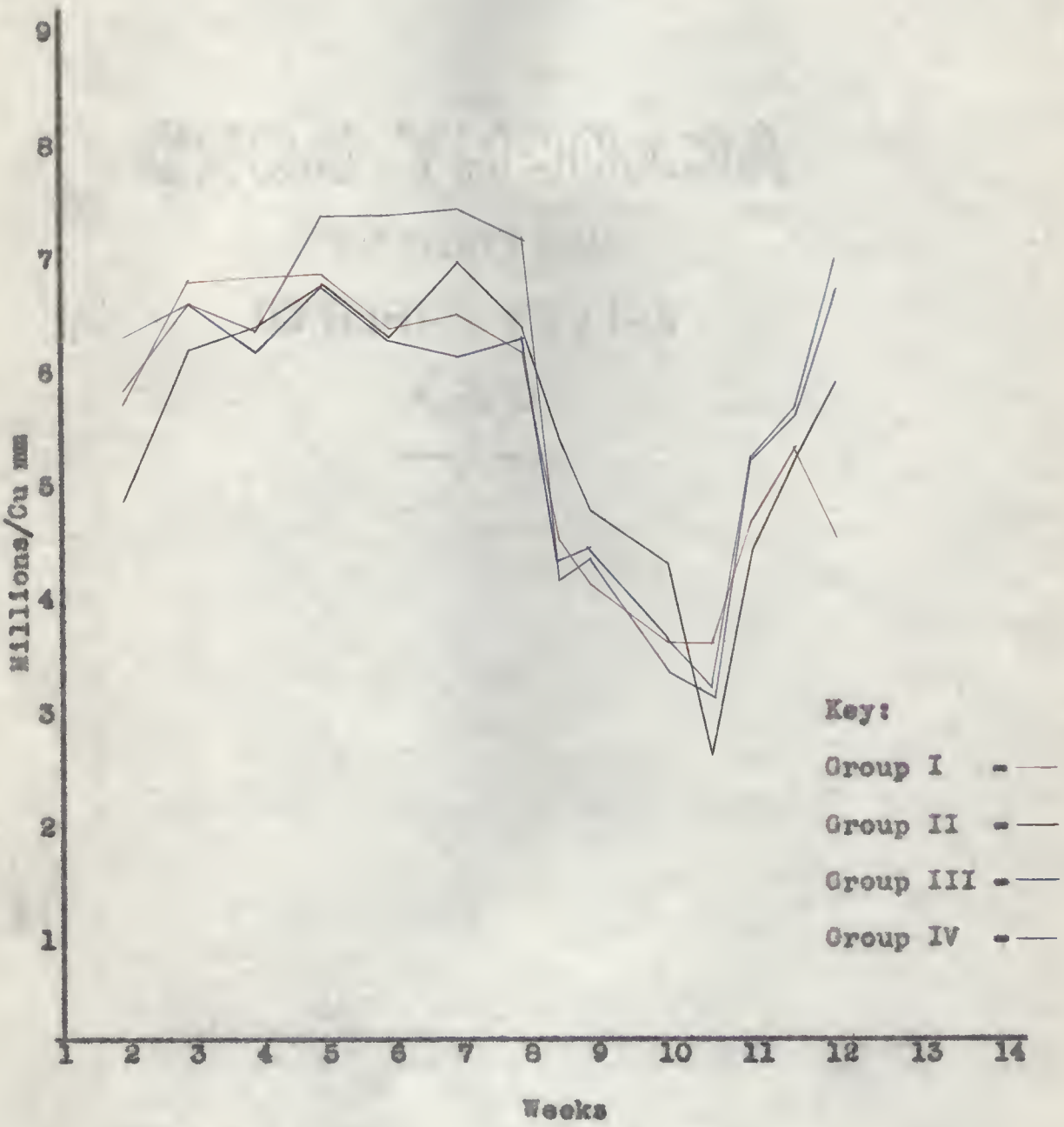

Fig. 2. Group averages of erythrocyte counts during the experiment.

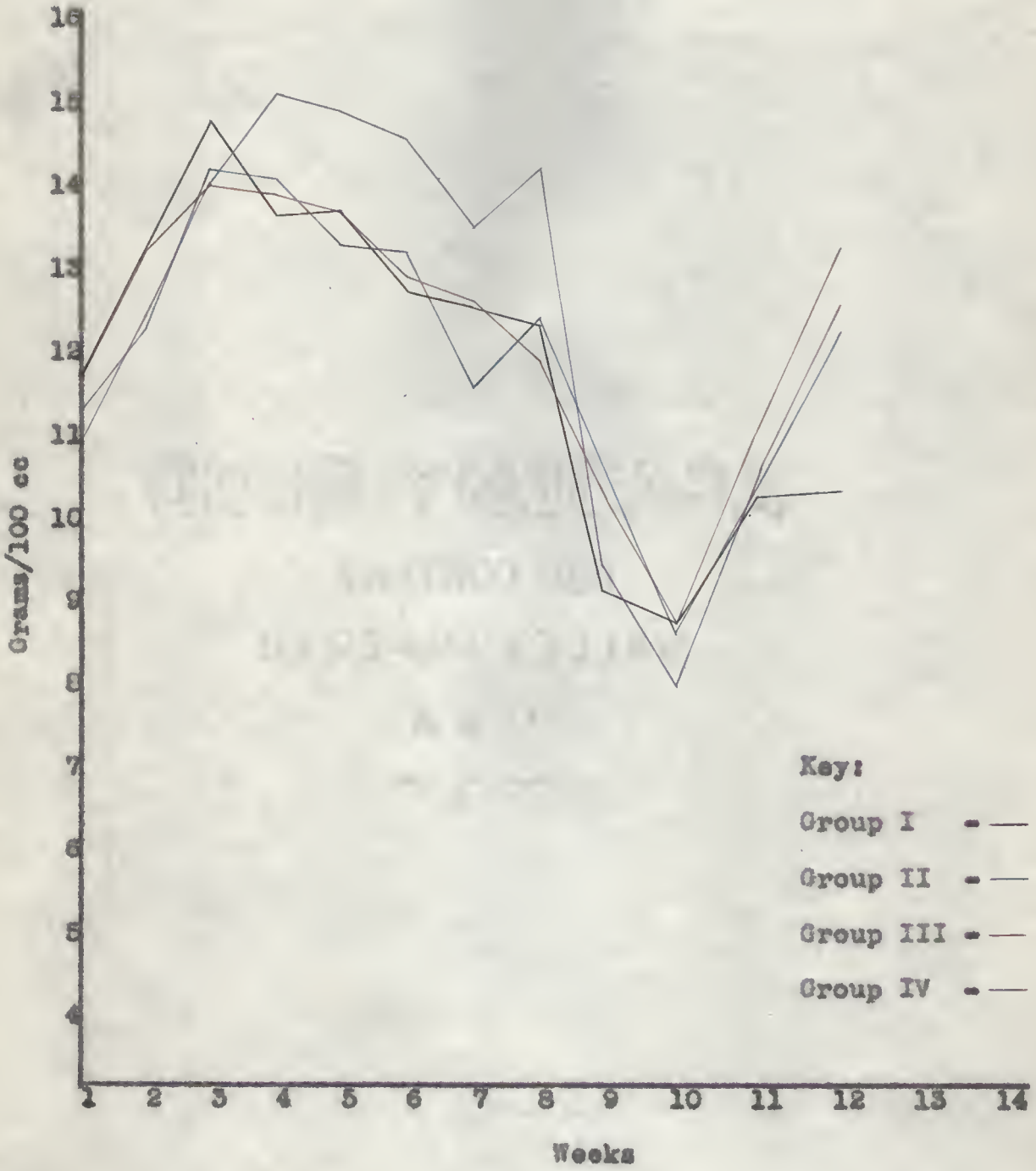

Fig. 3. Group averages of hemoglobin levels during the experiment.

DISCUSSION

It is fairly obvious to note, by examination of Figs. 2 and 3 that the administration of cobalt and vitamin B_{12} under the experimental conditions, caused a definite increase in erythrocyte count and hemoglobin values as compared to the control animals. This difference is apparent upon examination of Tables 1, 2, 3 and 4. At the twelfth week the control animals (group I) had an average red cell count of 4.49 million and a hemoglobin level of 10.25 grams per 100 cc blood and group II (received only the low-cobalt ration) a red cell count of 5.88 million whereas the animals that had received cobalt showed a red cell count of 6.96 million and a hemoglobin level of 13.2 grams. The animals receiving vitamin B_{12} (group IV) were not far below those that had received the cobalt. They had an average red cell count of 6.67 million and 12.5 grams of hemoglobin. Reference should be made here to the work of Kleinberg, Gordon and Charipper (18) who carried out similar experiments with rabbits. At the time of Kleinberg's report, vitamin B_{12} had not been discovered and the effect of cobalt only, on erythropoiesis was reported. These workers administered cobalt nitrate by injections in doses of 50 mg daily. Under those conditions it required about 15 days for the erythrocyte counts of the cobalt treated animals to return to normal. In the

experiment carried out here the erythrocyte counts were normal in 10-11 days when cobalt was administered in the form of the chloride. This difference in the time required for the animals to return to normal becomes more striking when one considers the great difference in the amount of cobalt administered. Kleinberg administered 350 mg of cobalt nitrate weekly whereas in this experiment only 0.2 mg was given over a period of seven days. However, it is possible that the age of the experimental animals would make some difference in the response to cobalt therapy. The animals used here were very young whereas the animals used by Kleinberg may have been adults. There is no mention of the ages of the experimental animals in his report.

According to the report of Becker, Smith and Loosli (1), cobalt deficient lambs did not respond to injections of cobalt but did when it was administered orally. In this experiment there was a definite response, in fact those animals given cobalt intravenously had higher erythrocyte counts than any of the other groups. Furthermore, Becker, Smith and Loosli obtained no response from the administration of vitamin B_{12} to cobalt deficient lambs whereas in our experiment, the response to vitamin B_{12}, so far as hemoglobin and erythrocyte counts were concerned, was not significantly different from that obtained with cobalt. From these data, it may be postulated that possibly rabbits respond differently to cobalt

and vitamin B_{12} administration than do cobalt deficient sheep. The rabbit seems to respond to vitamin B_{12} administration as do pernicious anemia patients.

Since actually what was produced in the rabbits in the course of this experiment was a hemorrhagic anemia, it seems possible that animals which have suffered from severe hemorrhage because of traumatic injury might be benefited by the administration of cobalt or vitamin B_{12}, or both.

It is fully realized that this is more or less of a preliminary experiment, and that it should actually be repeated using a greater number of experimental animals. For more conclusive evidence, studies of whole blood smears, bone marrow examinations, and hematocrit readings should be included in the experimental procedures.

SUMMARY AND CONCLUSIONS

An investigation was carried to attempt to gain information concerning the possibility that vitamin B_{12} may be an intermediary step in the metabolism of cobalt in the rabbit. The animals were placed on a low-cobalt ration then after a preliminary control period were bled to produce anemia. Vitamin B_{12} and cobalt were then administered and the effects upon body weight, erythrocyte count and hemoglobin values noted.

The experimental data obtained does not lend support to the postulate that vitamin B_{12} is an intermediary step in the metabolism of cobalt in the rabbit, since the effects of these substances were not significantly different in regard to erythrocyte count and hemoglobin values. At the twelfth week the animals receiving cobalt had an average red cell count of 6.96 million and 13.2 grams of hemoglobin and those animals receiving vitamin B_{12} had an average red cell count of 6.67 with 12.5 grams of hemoglobin per 100 cc of blood.

Vitamin B_{12} and cobalt chloride administered intravenously to the rabbit apparently stimulate erythropoiesis in the rabbit in some way, possibly by influencing the blood forming centers in the bone marrow.

If the rabbit depends on a dietary source of cobalt, its requirements must be very low since the ration, by analysis, contained only 0.527 micrograms of cobalt per gram of feed (unpublished work now being carried out by the Department of Physiology).

ACKNOWLEDGMENT

The author is much indebted to Dr. G. K. L. Underbjerg, major instructor, for valuable advice and encouragement, and to Mr. Elliott L. Hix who compounded the low-cobalt ration. Mr. Hix also made up the mineral mixtures and gave valuable technical assistance throughout the experiment.

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