8-1968

The counteraction effects of vitamin b12 on the hemolytic effects of methonine in rats

Mayme Novella Payne

THE COUNTERACTION EFFECTS OF VITAMIN B_{12} ON THE HEMOLYTIC EFFECTS OF METHIONINE IN RATS

A THESIS

SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

BY

MAYME NOVELLA PAYNE

DEPARTMENT OF BIOLOGY

ATLANTA, GEORGIA

AUGUST 1968

ACKNOWLEDGEMENTS

As I walked down the streets of life, three people stood at the corners and showed me the way:

My mother, my father and my brother

Archie

I am grateful for their kind words of encouragement which constantly inspired me to continue in this endeavor. My deepest gratitude to Dr. George Riley for his kind assistance and to Dr. Clyde Johnson whose helpful suggestions made the completion of this work possible. I continue to be indebted to Mr. Jerry Bennett for the many courtesies extended to me during the course of my research.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

Cyanocobalamin, better known as vitamin B_{12}, is by far one of the most important hemopoietic substances yet discovered. Necessary for red blood cell maturation, it was found by Chalmers and Shinton (1958) to be effective in millionths of grams when administered intravenously, but when administered orally, it was only effective in larger dosages.

Excess dietary methionine was found by Klavins and Peacocke (1964) to be an inhibitory factor for it stunted growth in the physiological system by destroying body tissues and organs.

Mengel and Klavins (1967) performed an experiment on male albino rats hoping to reveal the exclusive role of methionine on erythropoiesis. Their daily basal diet, administered by mouth, was supplemented with varying degrees of methionine but was comprised of only that minute amount of vitamin B_{12} found as a component in 1/1000 ml of halibut liver oil. They concluded that this dietary amino acid in excess amounts resulted in hemolytic anemia. In addition to a minute vitamin B_{12} source, their basal diet was deficient in vitamin C which was found by Moore (1955) and Parsons and Smallwood (1935) to be necessary in stimulating the bone marrow to release some unknown factor capable of enabling vitamin B_{12} to be utilized.

The purpose of my experiment is to demonstrate that the hemolytic

effects of excess methionine can be prevented with increased levels of vitamin B_{12}.

CHAPTER II

REVIEW OF LITERATURE

Wintrobe (1967) reported how hemolytic anemia may eventuate. The total red blood cell mass is replaced approximately every four months. Accordingly, if red cell destruction is increased, red cell production is also increased for the amount of blood in a system represents the balance between production and destruction. When destruction exceeds production, thereby altering the balance of equilibrium so that less than the normal number of red blood corpuscles is found in circulation, anemia results. When the average life span of red corpuscles has been reduced from the normal 120 days to less than 15-17 days as a result of increased blood cell destruction, hemolytic anemia develops.

Wintrobe (1967) asserted that amino acids are essential for blood corpuscles construction although the specific function of individual amino acids on erythropoiesis is not known.

Brown and Allison (1948) experimentally established the view that excess methionine is destructive to tissues and organs, thus causing suppression of growth and weight loss.

Klavins, Kinney and Durham (1963) demonstrated that of all the amino acids, excess dietary methionine caused the greatest depression in tissue growth and consequently a loss in weight.

Klavins and Peacocke (1964) investigated the precise role of sulfur containing amino acids in the system. Methionine was fed in excessive amounts with no other controlling factor and was found to inhibit growth by destroying body tissues and organs. There was a large percentage of weight loss and anatomical changes in the pancreas, salivary glands and gastrointestinal tract. When methionine was administered with equimolar amounts of glycine and arginine, growth inhibition was alleviated.

Mengel and Klavins (1967) investigated the role of excess methionine on erythropoiesis. They fed a basal diet which contained 0.001 ml of halibut liver oil as a vitamin B_{12} source and omitted vitamin C. They allotted high levels of methionine and concluded that increased methionine caused hemolytic anemia.

Copp (1941) experimented with radioactive cobalt and found that 45 percent of a small amount of cobalt (10 µg) was absorbed when fed. In contrast, very little was retained when given intravenously.

Shorb (1948) isolated vitamin B_{12} and showed that large dosages were necessary to be active against anemia when administered orally.

Israels (1943) served a vitamin C deficient diet and proved that vitamin B_{12} is valueless unless there is an adequate source of vitamin C.

Parsons and Smallwood (1935), Parsons (1938), and Moore (1955) disclosed that vitamin C played a part in stimulating the bone marrow to release some unknown factor capable of enabling vitamin B_{12} to be

utilized, thus denoting that a vitamin C deficiency is associated with anemia. When 10 µg of vitamin C were administered daily, the anemia was overcome.

CHAPTER III

MATERIALS AND METHODS

Twenty-four mature male rats of the Long Evans strain were divided into 4 groups of 6 animals each and housed individually in wire mesh cages. The diet was distributed daily as follows:

Group I -- Control - received basal diet only

Group II -- Experimental - received basal diet supplemented with 15 µg of vitamin B_{12}

Group III -- Experimental - received basal diet supplemented with 150 mg of methionine

Group IV -- Experimental - (a) 3 animals received basal diet supplemented with 150 mg of methionine and 15 µg of vitamin B_{12}; (b) 3 animals received basal diets supplemented with 300 mg of methionine and 15 µg of vitamin B_{12}.

The daily basal diet for each animal consisted of 36.65 grams of the following ingredients and 4 grams of a salt mixture in the given ratio:

(In grams) glucose, 6.7; casein, 18.0; corn oil, 11.0; choline chloride, 0.3.

(In milligrams) thiamine hydrochloride, 100; riboflavin, 100; vitamin C, 200; pyridoxine HCl, 4; Ca pantothenate, 15; and cod liver oil, .001 ml.

Salt mixture

(In milligrams) $CaCO_3$, 600; K_2HPO_4-dibasic, 645; $CaHPO_4 \cdot 2H_2O$, 150;

$MgSO_4 \cdot 7H_2O$, 204; NaCL, 335; Fe $(C_6H_5O_7)_2 \cdot 6H_2O$, 55; KI, 1.6; $MnSO_4 \cdot 4H_2O$, 10; $ZnCl_2$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.6. Water was administered ad libitum.

Vitamin B_{12} and the various concentrations of dietary methionine were weighed on the Sartorius analytical balance and administered individually to the proper experimental animals daily and cod liver oil was separately delivered by means of a micro pipette.

Cardiac punctures were performed once a week and the blood was collected in tubes containing the anticoagulant EDTA. Samples were examined for hemoglobin as cyanomethmoglobin and hematocrit determinations. Hematocrit values were obtained by the micro hematocrit method in a micro hematocrit centrifuge at 12,500 rev/min for 5 minutes at 14,848 force gravities.

Reticulocyte counts were conducted and specimens were screened for the osmotic fragility of erythrocytes according to Sanford's method. If hemolytic anemia was indicated by preliminary screening, then the fragility test was carried out extensively with the final readings being made in a Spectronic 20 colorimeter. Smears for the detection of reticulocyte percentages and morphology of cells were stained with New methylene blue and Wright's stain respectfully.

Animals were anaesthetized with nembutal sodium injections and weighed prior to cardiac punctures. The dosage allowed was 35 mg/kg. The experiment lasted 5 weeks.

CHAPTER IV

EXPERIMENTAL RESULTS

Results of individual group analysis are shown in Tables 1-5. Table 1 illustrates a definite loss in weight in both the experimentals and controls after 1 week with no significant differences among the groups. The succeeding weeks disclosed a period of fluctuations again denoting no contrasting variations. By the termination of the fifth week there was significant evidence of a varying but gradually decreasing pattern of weight loss in group III and to a similar but lesser extent in group IV, whereas a gradual increase resulted in groups I and II.

Hemoglobin and hematocrit readings dropped at onset in all instances with some individuals remaining within and others below the normal range. Later major group differences were delineated as shown in Tables 2 and 3.

Group I, the controls which were fed the basal diet only remained normal. Three members which developed anemia at onset later made a steady gain. Group II, the experimental group, furnished the basal diet and vitamin B_{12} had 2 members lag before attaining normal hematological values. The others were either average or showed erythrocytosis. The entire group III showed evidence of anemia which persisted throughout the study. The 3 animals in part A of group IV (IV-1, IV-2, IV-3) which were allotted the basal diet, vitamin B_{12} and 150 mg of methionine,

Table 1. Comparative changes in body weight in grams of experimental and control rats in 3 studies over a 5 week period.

	Weeks				
Experiment 1	1	2	3	4	5
* I - 1	380.1	371	368	373	365
I - 2	310	298	325	408	409
II - 1	283.1	272	272	280	300
II - 2	297.2	265	307.3	287.1	314.5
III - 1	400.3	377	363.7	270	342.4
III - 2	306.1	299.5	260	259	241
IV - 1	409.1	389	348.6	351.5	348
IV - 2	355.5	318	320.7	319	310
Experiment 2					
I - 3	309.1	298	250	273	315
I - 4	319.1	300	282	273.5	306
II - 3	358	355	320	308	325
II - 4	364.2	342	305	295	330
III - 3	332.1	216	208	210	201
III - 4	340.1	330	326	316	258
IV - 3	314.1	294.6	287	274.5	267.1
IV - 4	264.7	193.8	179	200	234

Table 1 (Continued)

Experiment 3	Weeks 1	2	3	4	5
* I - 5	343.4	320.4	315	314	212
I - 6	373.6	360	351	314	325.5
II - 5	316.9	295.5	296	295	330
II - 6	279.1	262	270	269	257.7
III - 5	366	341.5	286	309.5	330
III - 6	314	268.1	258.2	272.5	248
IV - 5	346.1	324	371	372	285
IV - 6	353.1	291.5	270	269.5	289.5

*The Roman numeral is the group number, the second figure is the animal's number.

Table 2. Comparative hematocrit percentages (cells vs. plasma) of control and experimental rats in 3 studies over a 5 week period.

	Weeks				
Experiment 1	1	2	3	4	5
* I - 1	46	39	42	31	34
I - 2	42	34	39	37	41
II - 1	42.5	34	46	45	51
II - 2	47	44	36	43	45
III - 1	47.5	31	24	19	23
III - 2	40.4	27	63	24	19
IV - 1	36.5	29	41	38	45
IV - 2	38	24	20	41	37
Experiment 2					
I - 3	46.5	38	36	40	40
I - 4	39	40	40	42	37
II - 3	42	38	37	42	40
II - 4	49	48	40	19	46
III - 3	36	25	24	19	21
III - 4	40	27	22	24	24
IV - 3	40	24	32	38	51
IV - 4	39.5	30	19	18	32

Table 2 (Continued)

Experiment 3	Weeks 1	2	3	4	5
* I - 5	42	32	31	37	35
I - 6	46	23	40	40	39
II - 5	37	31	31	35	39
II - 6	42	42	40	32	33
III - 5	40	30	31	29	29
III - 6	44	23	19	21	20
IV - 5	46	27	25	25	29
IV - 6	44	34	38	39	41

*The Roman numeral is the group number, the second figure is the animal's number.

Table 3. Comparative hemoglobin values in grams per 100 cc of blood of control and experimental rats in 3 studies over a 5 week period.

	Weeks				
Experiment 1	1	2	3	4	5
* I - 1	15.2	13	14	10.4	11.3
I - 2	14	11.3	13	12.2	13.7
II - 1	14	11.4	15.2	15	17
II - 2	15.5	14.7	12	14.4	15
III - 1	15.7	10.2	8	6.5	7.5
III - 2	13.4	9	7.5	8.1	6.8
IV - 1	12.2	9.6	13.6	12.8	15
IV - 2	12.5	8	6.7	13.6	12.4
Experiment 2					
I - 3	15.1	11.5	12	13.6	13.2
I - 4	13	14	13.4	14	12.2
II - 3	14	12.6	12.2	14	13.2
II - 4	16.3	16	13.4	16.3	15.5
III - 3	12	8.3	8	6.4	7
III - 4	13.2	9	7.3	8	8
IV - 3	13	8	10.7	12.8	17
IV - 4	13	10.1	6.4	6.1	10.7

Table 3 (Continued)

Experiment 3	Weeks 1	2	3	4	5
* I - 5	14	10.7	10.2	12.3	11.8
I - 6	15.4	7.6	13.3	13.4	13
II - 5	12.3	10.3	10.2	11.5	13
II - 6	14	14	13.2	10.6	11
III - 5	13	10	10.2	9.7	9.8
III - 6	14.6	7.5	6.7	7	6.6
IV - 5	15.2	9.1	8.4	8.4	9.8
IV - 6	14.6	11.4	12.5	13	13.6

*The Roman numeral is the group number, the second figure is the animal's number.

Table 4. Comparative reticulocyte percentages of experimental and control rats in 3 studies over a 5 week period.

	Weeks				
Experiment 1	1	2	3	4	5
* I - 1	.8	1.7	4.7	3.9	3.6
I - 2	.5	3.8	6.6	1.8	5.9
II - 1	.9	1.7	2.0	5.8	6.7
II - 2	.57	2.2	4.3	2.9	5.5
III - 1	.61	.92	8.8	11	12.2
III - 2	.88	7.0	8.0	10.4	13.3
IV - 1	.9	4.5	5.4	3.3	4.0
IV - 2	.79	6.2	7.1	6.0	3.7
Experiment 2					
I - 3	.49	2.6	2.7	3.6	5.0
I - 4	.62	2.8	2.4	1.7	2.0
II - 3	.83	4.0	3.5	5.6	2.9
II - 4	2.6	6.2	2.9	1.3	3.1
III - 3	.72	7.6	9.0	12	8.4
III - 4	.7	7.6	7.5	10	11.0
IV - 3	.54	4.0	5.0	6.2	4.9
IV - 4	.5	7.1	9.2	10.1	8.7

Table 4 (Continued)

Experiment 3	Weeks 1	2	3	4	5
* I - 5	.55	7.0	5.4	3.5	4.4
I - 6	.58	1.8	7.2	6.0	5.9
II - 5	.6	1.1	2.5	.9	1.2
II - 6	.76	.97	4.3	6.4	3.0
III - 5	1.1	8.0	5.5	9.1	11.4
III - 6	.54	9.3	11	12.7	14.8
IV - 5	.87	5.0	7.6	6.0	12.4
IV - 6	.73	.82	5.4	4.1	10.9

*The Roman numeral is the group number, the second figure is the animal's number.

Table 5. Comparative osmotic fragility percentages (salt concentrations) of erythrocytes in 3 studies on control and experimental rats over a 5 week period.

	Weeks				
Experiment 1	1	2	3	4	5
I - 1	*	*	*	*	*
I - 2	*	*	*	*	*
II - 1	*	*	*	*	*
II - 2	*	*	*	*	*
III - 1	*	.46, .36	.50, .36	.48, .36	.50, .40
III - 2	*	.50, .36	.46, .32	.50, .40	.50, .34
IV - 1	*	.46, .34	*	*	*
IV - 2	*	.50, .34	.50, .40	*	*
Experiment 2					
I - 3	*	*	*	*	*
I - 4	*	*	*	*	*
II - 3	*	*	*	*	*
II - 4	*	*	*	*	*
III - 3	*	.50, .34	.50, .32	.50, .36	.50, .34
III - 4	*	.50, .32	.50, .32	.50, .34	.48, .38
IV - 3	*	.48, .34	.46, .32	*	*
IV - 4	*	.46, .36	.50, .36	.50, .38	*

Table 5 (Continued)

Experiment 3	Weeks 1	2	3	4	5
I - 5	*	*	*	*	*
I - 6	*	*	*	*	*
II - 5	*	*	*	*	*
II - 6	*	*	*	*	*
III - 5	*	*	*	.46, .32	.50, .38
III - 6	*	.48, .34	.50, .36	.50, .40	.50, .40
IV - 5	*	.50, .34	.50, .34	.50, .38	.48, .34
IV - 6	*	.46, .32	*	*	*

* = normal blood (have an initial hemolysis at .44 and complete at .34). In all other cases the 1st value indicates initial hemolysis and the 2nd denotes complete hemolysis.

The Roman numeral is the group number, the second figure is the animal's number.

slumped below normal initially and then demonstrated elevated blood pictures the ensuing weeks with 1 individual revealing moderate erythrocytosis. In part B of group IV, the group which received the basal diet, vitamin B_{12} and 300 mg of methionine, experimentals IV-4 and IV-5 contracted prolonged anemia while member IV-6 maintained normalcy.

Reticulocytes increased in all groups as observed in Table 4. Groups I and II had the lowest percentage, group IV was next and group III the highest.

The osmotic fragility test (Table 5) indicated normal erythrocytes in groups I and II throughout. Group III revealed a persistent increase in the fragility response. Excluding one member in group IV B who showed continuous hemolysis, groups IV A and IV B showed signs of anemia at onset only.

Blood smears displayed spherocytes and hypochromic cells in anemic individuals.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Former studies by Klavins, Kinney and Durham (1963), Klavins and Peacocke (1964), Brown and Allison (1948), and Mengel and Klavins (1967) disclosed that excess methionine in the diet with no other controlling factor resulted in definite losses in weight for it impaired tissues and organs. Changes in weight in the present analysis parallel those of previous investigators. Each of the experimental groups lost weight initially with the methionine fed groups persisting and the non-methionine fed gradually regaining.

There were several significant differences among the treatment groups of the present study and those of Mengel and Klavins' work. The control groups of both inquiries which received the basal diet only exhibited normal blood conditions. Since there were no apparent dissimilarities, it is reasonable to assume that the ingredients in the basal diet alone are sufficient for maintaining normal erythrocytes in rats with or without the addition of vitamin C which was deficient in Mengel and Klavin's experiment.

In contrast to the controls, group II of the present study revealed mild erythrocytosis after the administration of 15 µg of vitamin B_{12} to the basal diet which denotes that increased vitamin B_{12} instigates red blood cell production.

The animals which received 150 mg of methionine concomitant with

the basal diet in the two studies manifested definite anemia as unfolded by an increased reticulocyte and osmotic fragility determination, and a decreased hemoglobin and hematocrit value. The individuals in Mengel and Klavins' group which received 300 mg of methionine acquired a more intense anemia.

To compare differences and test the potency of vitamin B_{12}, 15 µg were added to diets containing 150 mg and 300 mg of methionine. In the lower concentration diet, anemia was prevalent at onset only. The higher concentration diet provoked anemia in 2 animals, the 3rd sustaining normalcy.

It is perceivable that 15 µg of vitamin B_{12} are sufficient to offset the hemolytic effects of 150 mg of methionine but not of 300 mg. There is the indication that certain concentrations of methionine are too cogent to be counteracted by certain concentrations of vitamin B_{12}.

Perhaps slightly greater strengths of this vitamin could neutralize 300 mg or more of methionine. The animal which remained normal in the high concentration group may have been more resistant than the others or failed to consume all of the methionine administered since it is not stored.

Resultant fluctuations among the groups may be attributed to the fluid intake and diet. Absorption of large amounts of water may result in a decreased hemoglobin and hematocrit, whereas dehydration encourages increased estimations.

Methionine is only hemolytic when it is present in the absence of other controlling factors. Since it is a substance causing deteriora-

tion, it could possibly be involved in red cell destruction which is necessary for maintaining the blood's equilibrium. The overall average results of body weights and blood values are illustrated in Tables 6 and 7.

Table 6. Overall average of hematologic values in control and experimental animals of all groups after 5 weeks of treatment.

Blood Tests		Group I	Group II	Group III	Group IV
Hematocrit readings		38.26	39.88	27.53	34.03
Hemoglobin in grams per 100 cc blood		12.72	13.59	9.16	11.34
Reticulocyte percentage		3.31	2.90	7.7	5.02
Osmotic fragility percentage	Initial hemolysis	.44	.44	.48	.45
	Complete hemolysis	.34	.34	.35	.34

Table 7. Overall average changes of body weight in grams of control and experimental groups.

Controls	Group I	328.72
Experimental	Group II	301.72
Experimental	Group III	298.34
Experimental	Group IV	304.99

CHAPTER VI

SUMMARY

Since methionine in excessive amounts causes hemolytic anemia only in the absence or presence of minute quantities of vitamin B_{12} and not when larger portions are present through oral intake, it stands to follow that Mengel and Klavins' work (1967) indicates that methionine was found to be hemolytic only under the conditions they chose to run their experiment, with no controlling factors. This is evidenced by the fact that group III, the methionine only fed group, was the sole abnormal group. Since hemolytic anemia did not result with increased vitamin B_{12}, then it is apparent that methionine in excess is ordinarily not a definite cause of hemolytic anemia. Instead, Mengel and Klavins' work and the information derived from the results of group III in this study seem to suggest that hemolytic anemia may result from an improper balance of this dietary amino acid and vitamin B_{12}. This postulation is supported by Cantarro and Schepartz (1967) who reported, "When the diet contains only minimally adequate amounts of certain B vitamins, addition of an excess of certain amino acids may precipitate or exaggerate the vitamin deficiency."

Due to the fact that methionine has experimentally been proven to be a destructive agent and increased methionine upsets the balance of erthrocyte production and destruction resulting in anemia, (Wintrobe, 1967), it is perceivable that methionine could play the part of main-

taining the red blood cell equilibrium since production equals destruction or vitamin B_{12} could prevent methionine from having any detrimental influence on blood cells.

LITERATURE CITED

Brown, J. and J. Allison. 1948. Effects of excess dl-methionine and/or l-arginine in rats. Proc. Soc. Biol. Med. 69: 196-200.

Cantarro, A. and B. Schepartz. 1967. Biochemistry, 4th ed. W. B. Saunders Co., Philadelphia and London. 849 p.

Chalmers, J. and K. Shinton. 1958. Absorption of orally administered vitamin B_{12} in pernicious anemia. Lancet 2: 1298-1302.

Copp, D. 1941. Studies in mineral metabolism with the action of artificial radioactive isotopes. Proc. Nat. Acad. Sci. 27: 153-156.

Israels, N. 1943. Erythropoiesis in scurvy. Lancet 1: 170-172.

Klavins, J., T. Kinney and N. Durham. 1963. Histopathologic changes in methionine excess. Arch. Path. 75: 661-673.

Klavins, J. and T. Peacocke. 1964. Effects of administration of excessive amounts of sulfur containing amino acids: methionine with equimolar amounts of glycine and arginine. Brit. J. Exptl. Path. 45: 533-542.

Mengel, C. and J. Klavins. 1967. Development of hemolytic anemia in rats fed methionine. J. Nutr. 92: 104-110.

Moore, C. 1955. The importance of nutritional factors in the pathogenesis of iron deficiency anemia. Amer. J. Clin. Nutr. 3: 3-10.

Shorb, M. 1948. Activity of vitamin B_{12} for the growth of Lactobacillus lactis. Science 107: 397-398.

Parsons, L. 1938. Nutrition and nutritional diseases. Lancet 1: 65-70.

Parsons, L. and W. Smallwood. 1935. Studies in the anemia of infancy and early childhood. Arch. Dis. Childh. 10: 327:336.

Wintrobe, M. 1967. Clinical hematology, 6th ed. 1239. p.