UNIVERSITY OF CINCINNATI

May 27, 1936.

I hereby recommend that the thesis prepared under my supervision by Robert Reed McNary

entitled THE EFFECT OF PHYLLOERYTHRIN ON BLOOD FORMATION

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy.

Approved by:

Albert P. Mathews

Form 668—G. S. and Ed.—1M—7-31

THE EFFECT OF PHYLLOERYTHRIN ON BLOOD FORMATION

A dissertation submitted to the

Graduate School
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

1936

by

Robert Reed McNary

Ch.E. University of Cincinnati 1926
A.M. Antioch College 1933

Table of Contents.

THE EFFECT OF PHYLLOERYTHRIN ON BLOOD FORMATION

by

Robert Reed McNary

Department of Biochemistry, University of Cincinnati.

Introduction.

For many years it has been common knowledge among dietitians, physicians and the public in general that certain green vegetables such as cabbage, beet greens, lettuce, etc., have merit in a diet. The reason for this was not clear until about twenty-five years ago, when vitamins were discovered. These vitamins, which are necessary for normal growth, reproduction and health, are found, in varying amounts, in nearly all fresh green vegetables and fruits. The greenness of these vegetables, however, is not due to the vitamins but to chlorophyll; and while certain vitamins, such as A and C seem to be associated in some way with chlorophyll, chlorophyll itself is not a necessary constituent of the diet. Animals, including man, can exist, grow and reproduce in a perfectly normal manner on a diet free from chlorophyll although all such diets contain the precursors of chlorophyll and also porphyrins, closely allied in nature to part of the chlorophyll

molecule. But chlorophyll itself cannot be called a vitamin or a necessary food substance.

However, the possibility still remains that chlorophyll might have a definite influence on either normal or pathological metabolism. More especially, since chlorophyll does have a close chemical relationship to heme, the red pigment material in mammalian blood, it is conceivable that chlorophyll might, in some way, exert a beneficial influence on blood metabolism and might possibly contribute some part of itself to make heme. Indeed, the possible food value of chlorophyll has been the subject of considerable study as will be outlined later.

Since chlorophyll has never been known to be absorbed unchanged by any mammal, and since certain decomposition products of chlorophyll are known to be absorbed, it would seem reasonable to suspect that if chlorophyll had any nutritive action it would be in consequence of these absorbed products of chlorophyll decomposition. This concept formed the basis upon which the present research was carried out.

I. The Physiological Effects of Chlorophyll.

Koichi Miyadera (1921) stated that chlorophyll dissolved in olive oil produces a slight brachycardia in the

heart of the frog. The chlorophyll was extracted from nettle leaves with ether, the carotinoids separated by treatment of the ether solution with aqueous alcohol and the residue from the evaporation of this solution was dissolved in olive oil. The thorax of the frog was opened and the pericardium laid bare. The point of the heart was attached to a heart lever, the excursions of which were recorded on a kymograph. The chlorophyll in olive oil was allowed to drop slowly onto the surface of the heart.

¶ Koenigsfeld (1922) found that the excretion of phosphates and sulphates in the urine in man is slightly increased after the ingestion of chlorophyll in the form of chlorosan-Bürgi pills which contain extracted chlorophyll. The urinary nitrogen values were not increased but the nitrogen excretion in the feces was increased. Gordonoff (1925), as the result of his experiments concluded that chlorophyll has a stimulating effect on all the body organs, not merely on the blood-regenerating tissue. Gordonoff and Hosokawa (1925) reported that the addition of colloidal chlorophyll or of chlorophyll-sodium to Ringer's solution increases the activity of a frog nerve-muscle preparation, the result being the same whether the chlorophyll is applied to the muscle or to the nerve. An exhausted nerve-muscle preparation commences to contract again when colloidal

chlorophyll or chlorophyll-sodium is added. Gordonoff (1927) also found that chlorophyll increases the contractility of the isolated frog heart in concentrations of 1:20,000, the action being on the heart muscle. There is a similar action on intestine and muscle preparations. Rydin (1928) reported that the subcutaneous injection of 0.01 gram of sodium chlorophyllate dissolved in physiological salt solution increased the elimination of carbon dioxide and the absorption of oxygen in both normal and thyroidectomized rats. His opinion was that chlorophyll acted as a general cellular stimulant having the power of increasing the fundamental metabolism of the cells. In comparison with thyroxine the stimulation of respiratory metabolism by chlorophyll was slight and it did not affect the body weight.

Bürgi (1927) used his chlorophyll preparation (chlorosan-Bürgi) in the treatment of arteriosclerosis with the result that a lowered blood pressure and a more normal blood picture were obtained. Lorenzi (1931) found that chlorophyll caused a constant decrease of the blood pressure in hypertension and this was associated with dilation of the blood vessels.

The most thorough investigation of the pharmacological action of chlorophyll was published by Rentz (1927, 1929a). His summary and conclusions are as follows:

1. "Chlorophyll in weak or moderate (for example 1:125,000, dissolved in Tyrode solution) doses has a motor effect on the intestine, uterus and heart. Larger doses (1:2400) have an inhibitory action on the intestine of the rabbit, resulting in a paresis. On the uterus of the rabbit even the strongest doses tested have a decided motor effect, entailing a marked increase in the tonus and as a rule a more vigorous automatism.

"Chlorophyll introduced into the frog's heart causes a brief primary reduction of the volume of contraction, followed by an increase, which is often combined with a slight diminution of frequency.

2. "Atropinization of the preparation from the intestine or uterus reduces very slightly the motor action of the chlorophyll, whence it may be presumed that this action mainly affects the muscles. Nor does "ergotaminization" of the frog's heart affect the stimulative action of the chlorophyll on the latter, which indicates that in this case also chlorophyll acts principally on the muscles. Seeing that chlorophyll does not change the effect of parasympathetic excitants on the intestine, it seems that in the dosage used, chlorophyll does not change the stimulus of the parasympathetic innervation.

3. "The inhibitory action of adrenalin on the intestine of the rabbit, as well as its motor effect on the

uterus of the rabbit, is greatly prolonged by the simultaneous addition of chlorophyll. On the other hand, moderate doses of chlorophyll do not change the effect of adrenaline on the frog's heart. Strong doses of chlorophyll, however, neutralize the powerfully stimulative effect of adrenaline on the heart, due to action on the sympathetic nerve, as well as the vagotrope effect, due to irritation of the vagus nerve. The reaction is, however, reversible.

4. "Chlorophyll has a powerful dilating effect on the blood vessels of the frog. Nevertheless chlorophyll has a capacity for strongly potentizing the constrictive action of adrenalin on the vessels, and indeed is able to convert weak doses of adrenalin, which otherwise have a dilating action on the vessels, into constrictive agents. Seeing that the effect of barium chloride on the vessels is not analogously potentized by the simultaneous addition of chlorophyll, the cause of the potentizing of the adrenalin must be looked for in a change of the elements on which adrenalin has an irritating action.

5. "It may therefore be presumed that chlorophyll produces increased irritation - - without itself having any appreciable irritating effect - - on the sympathetic nerve-endings in the intestine and uterus of the rabbit, and in the blood vessels of the frog.

6. "The effect of chlorophyll on the increase of the blood-pressure is rather insignificant: very large doses actually entail a marked reduction of the blood-pressure. The respiration is affected very slightly, moderate doses having a weakly stimulative effect, very strong doses a paretic effect. The blood-vessels of the intestine and kidney are dilated by weak and moderate doses, whereas very strong doses may contract them. The blood-vessels of the extremities, on the other hand, are as a rule scarcely affected at all; where there is any appreciable effect, it is of a constrictive nature.

7. "Whereas in the rabbit chlorophyll is unable to make any change in the efficacy of a following injection of adrenalin, the reverse is the case with the cat. In the latter chlorophyll entails a marked enhancement of the effect of a subsequent injection of adrenalin on the increase of the blood-pressure and the contraction of the blood-vessels."

The effect of chlorophyll on the blood of normal rabbits also was studied by Rentz (1927, 1928, 1929b). He says: "Subcutaneous injections of chlorophyll -- non-recurring or repeated -- into the rabbit produce very remarkable changes in the blood picture. They gradually give rise to a very pronounced thrombocytosis, which reaches extremely

high figures, and is of rather long duration. The intravenous injections entail analogous effects, but are attended by a primary thrombopenia, which is developed in the course of the first hour after the injection, and then passes into thrombocytosis. The number of leukocytes is likewise increased, though not in anything like as marked a degree as that of the thrombocytes; moreover only the stronger dosages appear to be effective in this respect. Both polymorphonuclear and mononuclear leukocytes take part in this leukocytosis, sometimes chiefly or solely the former.

"Chlorophyll thus brings about a strong irritation of that part of the bone marrow which produces the thrombocytes (megakaryocytes), as is also indicated by the increase in the number of the polymorphonuclear leukocytes. But also other organs belonging to the leukopoietic system are apparently affected by chlorophyll."

Besides the effect of chlorophyll on the leukocytes and thrombocytes, it has been claimed that this substance stimulates erythrocyte production and has growth-promoting properties. The chief protagonist on this subject is Emil Bürgi. He, and his followers, recommend that chlorophyll be used as an excellent specific for influencing anemias of various kinds, for bettering the general condition of health, for improving the action of the heart, -

for reducing the blood-pressure in cases where it is abnormally high, etc.

Bürgi's publications on this subject began in 1916. In this paper he describes the difficulty of adding chlorophyll to the diet so that it can be readily assimilated, since it is totally insoluble in aqueous solvents.

> "After much difficulty, we succeeded in obtaining a pharmacologically unobjectionable chlorophyll from a uniformly superior plant material by means of an especial extraction procedure, which shall be reported in another place."

However, a search of any publication since then has failed to reveal the details of the process. In most of Bürgi's work chlorophyll was administered in the form of pills which were given the trade name of Chlorosan-Bürgi. These contained 0.03 gram of chlorophyll and 0.005 gram of iron per pill; and were enclosed in a green-colored sugar coating (cf. Bürgi, 1918). In some of his early experiments the iron was omitted from the pills. Löffler (see Kitahara, 1925) estimated the amount of pure chlorophyll in chlorosan-Bürgi to be 1.25 mg. per pill and indicated that Bürgi's value also included chlorophyll decomposition products. The greater absorption of the chlorophyll from these pills was shown by Kitahara in 1925. The appearance of porphyrins in the urine (of men) was taken as an indication of appreciable absorption. No porphyrins were found after feeding either raw or cooked spinach or

cabbage. A positive test was obtained after the ingestion of the fluid of macerated, uncooked spinach or cabbage, but not from cooked. The ingestion of 0.2 gm of pheophytin or of 12 chlorosan pills gave a positive test. Six pills were insufficient to give a positive test. The amount of chlorophyll contained in the spinach or cabbage which was consumed far exceeded the amount in the 12 chlorosan pills. Thus the absorption of chlorophyll from ingested green leaves is very slight and is probably due to the fact that the pigment is enclosed in the cellulose cell-walls and is not released from the cells during digestion. The absorption of chlorophyll from the juice of macerated leaves or from extracted chlorophyll is many times greater.

Grigoriew (1919) published his results on the effect of chlorophyll and iron on the red blood cell count of normal and naturally anemic rabbits. He found that chlorophyll in the form of pheophytin had an erythropoietic effect, as did iron, and the combination of the two was more effective than either alone. At the same time Bürgi and Traczewski (1919) investigated the effect of chlorophyll on rabbits made anemic by bleeding and by injection of phenylhydrazine. The pigment was administered as unchanged chlorophyll prepared by Willstätter's extraction procedure, as pheophytin and as chlorosan-Bürgi. It was found that chlorophyll as well as iron, when given in large doses

possessed a blood-forming capacity, and were equally effective in the two types of anemia studied. Small doses of chlorophyll were found to possess a "sensitizing" effect on the action of iron in the production of blood cells.

From his anemia studies, Bürgi thought at first that chlorophyll could be transformed by the animal body into the red blood pigment because the two pigments have a remarkably close chemical resemblance, and since he found that chlorophyll increased the production of red blood cells. It was necessary, however, in a later paper (1928) for him to modify this theory to that of a stimulation effect of chlorophyll on hematopoiesis.

In 1930, Bürgi associated chlorophyll with vitamin A. Rats on a vitamin A-free diet were said to immediately increase in weight and size after the administration of pure chlorophyll, pheophytin, chlorophyll salts, chlorosan, and phyllosan. The effect was exactly analogous to the effect of butter. The control animals died. However, chlorophyll is always associated with the carotinoid pigments in plants and those who have had experience in purifying chlorophyll realize the great difficulty with which the last traces of carotene and xanthophyll are removed, since it is now known that carotene is the precursor of vitamin A, it is not surprising that Bürgi's chlorophyll preparations showed vitamin A properties. Bürgi

acknowledged the possibility that the vitamin might be adsorbed on his various preparations.

Zih has published three papers corroborating Bürgi on the erythropoietic effect of chlorophyll. In the first paper (1930) a chlorophyll-free diet was compared with a chlorophyll-rich diet, using rabbits as the test animal. The chlorophyll-free diet consisted of turnips, oats, corn and cow's milk. The chlorophyll-rich diet consisted of fresh, green grass, oats and corn. Zih found a definite erythropoietic effect with the chlorophyll-rich diet. However, it is difficult to interpret the results on the basis of the presence or absence of chlorophyll in the diet since the two diets were so different in composition. Factors other than chlorophyll might be the cause of the changes in the red cell count. Zih did say, however, that the erythropenia on the chlorophyll-free diet was cured by the addition of chlorophyll alone. As stated before, chlorophyll is very difficult to prepare in an absolutely pure condition and the impurities may well have been the active material.

In 1933, Zih published similar experiments on white rats. The rats were kept on a diet of white bread, corn, milk and cheese. A 13 to 15 per cent rise in the red cell count was obtained upon the addition of 5 mg. of "pure" chlorophyll to the diet. Excessive doses, e.g. 20 mg.,

caused a reduction in the red cell count by approximately the same amount. Raw green fodder given at the rate of 5 grams per day caused no essential change in the blood count. The same amount, cooked slowly, increased the erythrocyte count 15 to 20 per cent in three to four weeks with a return to the original value in 8 to 12 days after cessation of the green feeding. It should be noted that Zih found that the copious addition of vitamins annulled the erythropenic effect of the chlorophyll-free diets.

Hughes and Latner (1936) studied the effect of feeding chlorophyll to rabbits rendered anemic by repeated daily hemorrhages of 5 to 20 cc. When the hemoglobin of the animals was reduced to approximately 40 per cent of the normal value, chlorophyll in olive oil was fed by mouth. With large doses (0.33 grams per day), the recovery from the anemia was unaffected; with smaller doses, for example 0:05 grams of chlorophyll per day, or even better, 0.015 grams per day, a favorable effect of chlorophyll on blood regeneration was obtained. A magnesium-free, water-soluble chlorophyll derivative (sodium pheophorbide ?), which they did not describe, was also effective when fed at the rate of 1.0 gram daily, but was ineffective when injected subcutaneously at the rate of 0.25 gram. Large doses, 1.0 gram per day, of crude chlorophyll acted favorably. The authors consider these results as evidence that the

animal body is capable of converting chlorophyll into hemoglobin and that the failure of the large doses of chlorophyll is due to its toxic action on the bone marrow but in the case of the crude chlorophyll, the presence of some other substance neutralized the toxic effect. The failure of the subcutaneous injections of soluble chlorophyll was interpreted to mean that probably the chlorophyll must be altered in the digestive tract before it becomes active in hematopoiesis.

Scott (1923) studied the influence of diet on the occurrence of secondary anemia following repeated hemorrhage in rats. The basal diet was white bread and whole milk, to which cabbage was added for the control group. The rats receiving cabbage recovered more rapidly from the anemia than those on the basal diet. Scott, however, made no claims as to what was the active constituent of the cabbage.

In contrast to the above results, Edwards and Holley (1932) studied the effect of chlorophyll in the diet on the body weight of rats and found no significant difference. Three comparable groups of six rats each were fed Sherman's diet B for 56 days. The first group received no additional chlorophyll; the second group received 10 mg. of purified chlorophyll per animal daily; the third group received 30 mg. daily. It was found that there was a slight lowering of the feed requirements per unit gain in weight as the chlorophyll feeding level was raised. These authors made no

study of the changes, if any, in the blood picture.

Robscheit-Robbins and Whipple (1925a) maintained dogs in a state of chronic anemia by periodic bleedings, the size of the bleedings being adjusted so as to keep the hemoglobin level at about one third normal. They believe this procedure to cause maximal stimulation of the blood forming tissues. When supplements were added to the basal diet, the increased amount of blood removal necessary to maintain hemoglobin at one third normal was taken as a measure blood forming power of the supplement. With green vegetables as supplements only a moderate response was obtained and this response is attributed by Whipple to the salts present in the vegetables. The vegetables tried were spinach, beet greens, lettuce and asparagus (1925b), and spinach, white and red cabbage, and onions (1930).

Patek (1936) investigated the effect of chlorophyll, pheophytin, pheophorbide, sodium chlorophyllin and phytochlorin e on 15 patients with chronic hypochromic anemia. While this type of anemia responds to iron treatment alone, Patek considered it a suitable disease with which to investigate the hematopoietic properties of chlorophyll derivatives. No effect was obtained by the administration of these substances alone. Very large doses of chlorophyll with very small doses of iron likewise had no effect. However, after a period of medication with suboptimal

amounts of iron, the administration of chlorophyll products "was followed by an orderly and significant increase in the concentration of hemoglobin and a second reticulocyte response. This combined effect was produced when the materials were given parenterally as well as orally. The studies suggested that the body can use preformed pyrrole substances for the building of hemoglobin."

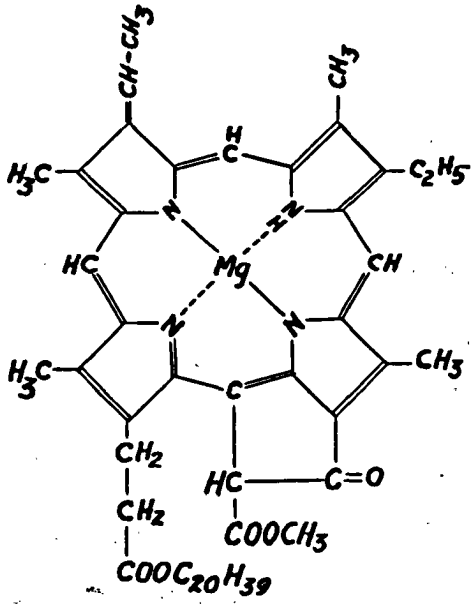
Gsell (1929) published the case histories of 21 patients treated with chlorosan-Bürgi. This series included secondary anemia of different origins, seven cases; pernicious anemia (treated with chlorophyll, vigantol and liver), one case; secondary syphilis, one case; paratyphoid, one case; lymph gland tumor, one case; pulmonary tuberculosis, two cases; cyanosis, one case; pneumonia, one case; febrile bronchitis, one case; angina pectoris, one case; spontaneous abortion, one case; paresis of arm and leg, one case; chronic nephritis with secondary heart dilation, one case; hemorrhoids, one case. Gsell claimed that chlorophyll acted as a general tonic in these cases. These results are of doubtful value, however, because of the small number of cases in each disease.

Dr. J. H. Kellog (cf. Rudolph, 1930), medical director of the Battle Creek Sanitarium studied the effect of chlorophyll injections and reported as follows:

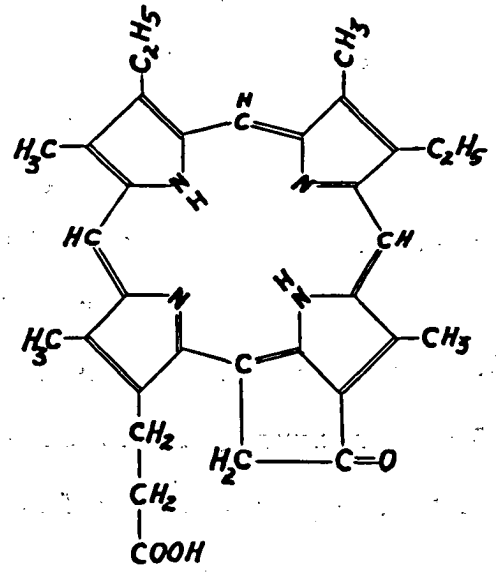
"Care was taken to see that no other treatment was used while the chlorophyll was being employed. Cases were selected which had been in the hospital for some time. The cases studied were those of secondary anemia and tuberculosis of the lungs. The red cell count, before treatment, was, on the average, four million; and after hypodermic injection of chlorophyll, daily for six days, five million. The patients felt much improved, the skin was clearer, the color better and there was less fatigue. In a month the weight increased as much as 16 pounds."

II. The Degradation of Chlorophyll in the Digestive Tract.

The removal of the magnesium in chlorophyll with the formation of pheophytin has been shown to take place under the influence of the gastric juice of dogs by Kortschagin (1924). In the stomachs of cows, a further breakdown into phylloerythrin, rhodo-, phyllo-, and pyrroporphyrins was found by Rothemund and Inman (1932), and in the stomachs of sheep, phylloerythrin and probophorbides were found. A little later, Rothemund, McNary and Inman (1934) also found phaeopurpurin 18 and pheophytin in the cow's stomach contents. Phylloerythrin was first discovered in the fresh feces of cows fed on green food by Marchlewski (1903). Kémeri (1924) obtained a porphyrin from human feces which Fischer and Hilmer (1925) found to be phylloerythrin. Fischer and Hendschel (1933a) found phylloerythrin in elephant feces but no probophorbides like that occurring in sheep feces. The same authors investigated human feces after a vegetable diet mainly of



Chlorophyll a.



Phylloerythrin.

spinach. They obtained phylloerythrin and probophorbide a, hence, in man the breakdown of chlorophyll is fundamentally the same as that in ruminants. In silkworms, the degradation was found by the same authors (1931) to follow a different course. They isolated from silkworm feces a crystalline substance with the empirical formula $C_{34}H_{36}O_6N_4$ and which contains no magnesium, phytol or methoxyl group. They gave it the name phyllobombycin, which in a later paper (1933b) was shown to be a molecular compound of pheophorbide a and pheopurpurin 7.

Not only is chlorophyll broken down in the animal digestive tract; the absorption of some of these derivatives has been shown to take place. Thus MacMunn (1885)

measured the spectrum of a pigment occurring in the bile of herbivorous animals and called it cholehematin. He thought it was derived from hematin. Loebisch and Fischler (1903) isolated from ox bile a pigment which they called bilipurpurin. Marchlewski (1924) proved that these two substances were identical with phylloerythrin and that this was derived from chlorophyll and not from blood pigment. Isolation from ox bile and the in vitro degradation of chlorophyll are the two principal methods of preparing phylloerythrin.

Rothemund, McNary and Inman (1934) found in the mucosa of the third and fourth stomachs of the cow, traces of phylloerythrin, another porphyrin whose spectrum closely resembles that of rhodoporphyrin, and a third unidentified porphyrin, as well as, pheophorbide a and pheopurpurin 18.

As mentioned before, when extracted chlorophyll is fed to men in sufficient amounts, porphyrins appear in the urine (Kitahara; 1925).

Chlorophyll, eosin and certain derivatives of hemoglobin, especially hematoporphyrin, are known to produce a sensitivity to light when injected into animals (cf. Laurens, 1933). In a certain region of South Africa, difficulty was experienced with grazing sheep becoming photosensitized. This was investigated by Rimington and

Quin (1934) who stated that the pigment responsible for the photosensitivity in "geeldikkop" (poisoning of small stock by excessive feeding on Tribulus during certain seasons of the year), and also that developing after operative ligature of the bile duct in sheep (experimental icterus), was isolated and identified as phylloerythrin. In the absence of chlorophyll from the diet, experimental animals neither became photosensitive nor could phylloerythrin be isolated from the bile, serum or feces. In a later paper, Quin, Rimington, and Roets (1935) reported that the phylloerythrin content of the feces of sheep fed only on Tribulus, which is particularly rich in chlorophyll, was considerably higher than that of the feces of sheep fed on lucerne. Furthermore, the symbiotic infusoria and bacteria present in the digestive tracts of the animals were thought to influence the amount of phylloerythrin production.

III. Nutritional Anemia in the Rat.

Abderhalden in 1899 demonstrated the production of nutritional anemia in the white rat fed solely on a milk diet. Bunge (1900) a year later showed that milk was notoriously low in iron content. This anemia was curable by adding ferrous salts to the diet. Hart, Steenbock, Elvehjem and Waddell reported in 1925 that iron alone was

ineffective in curing the anemia unless accompanied by lettuce or cabbage. In 1927, Hart, Elvehjem, Waddell and Herrin reported that the ash of lettuce and cabbage with iron was sufficient for cure. In 1928, Hart, Steenbock, Waddell and Elvehjem found that copper was the necessary adjunct to iron in the remission of nutritional anemia on a milk diet. Since that time a considerable volume of literature has been published on the action of copper in this type of anemia. Some authors contended that iron alone was sufficient and others claimed other metals could replace copper in this respect. However, in each case, these contrary results have not been generally confirmed in later publications. Hence, it seems probable at present that both copper and iron are necessary for the prevention or cure of milk anemia. An excellent review of this literature was published by Elvehjem (1935) which may be consulted for further details.

The explanation of the contrary results might possibly lie in faulty technic in one or several of the following respects: The use of impure salts as supplements; the use of milk which had had contact with metals; the use of cages which were not well galvanized, or in which coprophagy could take place; or the rats obtained some copper or iron or both while they were being handled — as for instance when they are removed from the cages for weighing

or for obtaining a blood sample. It is obvious, therefore, that all of these factors should be carefully controlled in this type of work.

The fact that iron and copper alone are necessary does not exclude the possibility that other substances may have a beneficial effect upon rats on a milk diet. Indeed, the addition of traces of manganese was found necessary for normal growth by Kemmerer, Elvehjem and Hart (1931); furthermore, Rider (1933) claimed that glutamic acid, when added to iron and copper as a supplement, caused a slightly quicker recovery from the anemia. Studies by Elvehjem, Hart, Jackson and Weckel (1934) indicate that growth and reproduction depend to a large extent upon seasonal variations in milks used. When goat's milk is used instead of cow's milk, an exactly analogous anemic condition is produced which is curable by iron and copper (Kohler, Elvehjem and Hart, (1935). The growth, however, is inferior to that obtained when cow's milk is used and the deficiency was not corrected by the addition of cod-liver oil, yeast, liver, or crystalline vitamin B_1. Normal growth was obtained by the addition of brain tissue. The addition of a better grade of alfalfa hay to the diet of the goats definitely improved the growth-promoting properties of the milk.

Rothemund, McNary and Inman (1934) reported that

porphyrins derived from chlorophyll had a definite hematopoietic effect when fed to rats with milk anemia. However, variations in the response to porphyrins were obtained which could not be explained. In commenting on this report, it may be stated that the number of animals available was too small for conclusive results, reconstituted milk from dried milk powder was used for the diet, and the water used in the preparation of the supplements and of the reconstituted milk was not re-distilled through glass apparatus.

The variability of the results of porphyrins on anemia led the author to repeat them under more carefully controlled conditions in order to obtain, if possible, an explanation of the porphyrin action. Phylloerythrin was selected from among the porphyrins derived from chlorophyll for several reasons: First, it is the chlorophyll derivative normally absorbed by the body in the largest quantity; second, its hematopoietic action according to the experiments cited in the preceding paragraph was second only to pheophytin; third, it is the most readily prepared of the chlorophyll porphyrins in sufficient quantities; fourth, its method of preparation is rigorous enough to remove all metallic impurities and vitamins.

IV. Experimental.

The animals used in this experiment were weanling white rats 18 to 20 days old obtained from the breeder at Bloomfield, Indiana.

Diet. The diet of the rats consisted solely of fresh whole milk obtained from Henkenbern's dairy farm at 4646 Kirby Road, Cincinnati. The milk was collected in glass and brought directly to the laboratory, thus it had no chance to pick up additional iron or copper before being fed to the rats. Since difficulty was encountered with diarrhea in some of the rats when they were small, the milk was pasteurized during the latter part of the experiment by heating in a Pyrex flask for 30 minutes at 62° C. When given to the rats, the milk soured before it was entirely consumed and in order to prevent a curd from forming, six cc. of saturated sodium citrate were added to each liter of milk. It was found that the rats consumed the milk better when no curd was formed.

Milk is low in its manganese content. In order to make up this deficiency and to make certain that any abnormal growth would not be due to a lack of this element, one cc. of a solution of manganese chloride containing 4.5 g. $MnCl_2.4H_2O$ per 100 cc. was added to each liter of milk.

The possibility that the milk was deficient in vitamin A was considered. Crystalline carotine was dissolved in ethyl acetate to saturation and 0.5 cc. this solution was emulsified with 3.0 liter of the milk by vigorous shaking. However, no difference was observed upon the addition of carotine and its use was discontinued.

The Copper and Iron Supplements. These were added as solutions of copper sulphate and ferric chloride. During the first five weeks of the experiment when copper alone was added, an amount of copper sulphate solution equivalent to 0.01 mg. of copper was mixed with the milk given to each rat at each morning feeding. During the latter part of the experiment, an amount of solution of copper sulphate and ferric chloride equivalent to 0.03 mg. of copper and 0.5 mg. of iron was administered daily in the same manner.

Cages. Since the metal cages available were not well galvanized, the rats were kept in glass museum jars approximately 10 inches in diameter and 8 inches deep. A false bottom was provided which was made of aluminum wire strung on an aluminum hoop. The wires were spaced 1/2 inch apart and 2 inches from the bottom of the jar. The top was covered with 1/2 inch mesh galvanized iron screening. When the rats were about six weeks old they gnawed

through the aluminum screen and obtained access to their feces. It was necessary, therefore, to replace the aluminum screen with galvanized iron. The milk was placed in 35 cc. glass dishes recessed in the bottom screen so that they could not be upset.

Hemoglobin Determinations. These were made weekly on a drop of blood obtained by snipping a very small piece from the tip of the tail with a razor blade. The blood was taken up in a Trenner automatic filling pipette which was made especially for this purpose. The blood was diluted with 1:100 hydrochloric acid and the acid hematin color produced was compared in a Bausch and Lomb colorimeter with a Newcomber glass standard.

Phylloerythrin. The phylloerythrin was prepared from chlorophyll by the method of Fischer and Bäumler (1929). Essentially, the method is as follows: The chlorophyll (0.5 gm.) is dissolved in 75 cc. of glacial acetic acid to which 10 cc. of hydriodic are added. The solution is heated for seven minutes in a boiling water bath, cooled, and added to 1 1/2 liters of ether in a separatory funnel and diluted with water. The mixture is made very weakly alkaline with ammonia, forcing the porphyrin into the ether upon shaking. The water layer is discarded. The porphyrin solution in ether is extracted with 15% hydrochloric acid until further extraction removed no more porphyrin by

spectroscopic test. The porphyrin in the acid extract is then transferred portionwise to fresh ether by dilution and neutralization of the acid with 20% sodium hydroxide until neutral to congo. Considerable ether is necessary for this step. The red ether solution of the phylloerythrin, which is slightly acid, is washed several times with redistilled water and then with extremely dilute sodium hydroxide solution (a few drops of N/10 NaOH per 100 cc.) until the washings give a slight green color with brom thymol blue. This removes all excess acid from the ether solution. The phylloerythrin is now extracted with a minimal quantity of N/10 sodium hydroxide. This gives a solution of the sodium salt of the porphyrin.

The solution of the sodium salt is now assayed by transferring one cc. of the solution to a separatory funnel containing 150 to 200 cc. of ether. About 50 cc. of redistilled water is added and the mixture made slightly acid by the addition of a few cc. of 2% hydrochloric acid. Upon shaking, the neutral porphyrin is obtained in the ether layer. The water layer is discarded and the ether layer is washed several times with re-distilled water. The ether solution is then evaporated to dryness in a weighed dish on the steam bath. From the weight of the residue, the concentration of the phylloerythrin-sodium salt solution is calculated. The phylloerythrin-sodium salt solution is then

added to a M/15 buffer solution of pH 7.0 made from a mixture of NaH_2PO_4 and Na_2HPO_4 (cf. Mathews) in such an amount that the final concentration of phylloerythrin was one mg. per cc. of solution.

A fresh solution of phylloerythrin was made up approximately every two weeks.

A mixture of N/10 sodium hydroxide and phosphate buffer was made up exactly similar to the phylloerythrin solution except that the porphyrin was omitted. This was fed to the control rats in order that the added salts and the traces of iron and copper that they might contain would make no difference in the results.

Procedure in the tests. The 18 to 20 day old rats were placed in individual cages and fed twice daily with citrated fresh, whole milk. When their hemoglobin level had reached approximately one third normal (in about six weeks) they were divided into two groups. In the preliminary experiment each group consisted of two animals. To the first group was given 0.5 mg. of phylloerythrin daily, mixed with the milk at the evening feeding. The control group was given the N/10 NaOH-buffer mixture in the same volume as the phylloerythrin solution. The rats were given as much milk as they would completely consume before the next feeding. They were weighed at two to three day intervals and their hemoglobin determined at weekly

intervals. The experiment was continued for 23 days, by which time three of the four rats had died.

Since, in the preliminary experiment, no response to the porphyrin treatment was obtained, it was decided in the second experiment to double the dose of phylloerythrin and to add to the diet a minimal quantity of copper, which, if sufficient iron were also added, would allow recovery to take place. This change was based upon the hypothesis that the addition of a porphyrin might influence the iron metabolism (for example, increase the absorption and decrease the excretion of the small amount of iron that the rat obtains from the milk), and that the cause of the lack of response in the preliminary experiment was the deficiency in copper.

Accordingly, in the second experiment, fifteen rats were made anemic by the same method. When their hemoglobin was reduced to about one third normal they were divided into two groups. The first group, consisting of eight rats, was given 0.01 mg. of copper per rat per day at the morning feeding, and 1.0 mg. of phylloerythrin per rat per day at the evening feeding. The control group was given the same amount of copper at the morning feeding, and at the evening feeding the mixture of N/10 sodium hydroxide and phosphate buffer in the same volume as the phylloerythrin solution was supplied the other group. The amount was adjusted so those rats having the least appetite would

have completely consumed their ration of milk before the time of the next feeding.

At the end of five weeks both groups were given sufficient iron and copper to permit complete recovery from the anemia. The amounts were 0.5 mg. of iron and 0.03 mg. of copper, which were mixed with the milk at the morning feeding. This amount of iron and copper was so large that the slight excess of these metals which a rat might obtain by consuming a little more milk than the others would make no difference. Therefore, from the time that iron and increased copper was added to the diet, each rat was given as much milk as he would totally consume before the next feeding. As the rats grew larger it was necessary to feed them three times a day since they consumed more than could be placed in their dishes during two feedings. No supplement was added to the third feeding in the afternoon.

It will be noted that the copper (or copper and iron) and phylloerythrin supplements were given at different times (morning, evening). This was done in order that there would be little likelihood that an inert copper-porphyrin complex would be formed which would pass through the animal unchanged as was found by Schultze, Elvehjem and Hart (1934) in the case of copper-hematoporphyrin complex.

Thrombocytosis. A third experiment was performed the purpose of which was to confirm the work of Rentz (1927)

on the action of chlorophyll on the increase of thrombocytes in the normal rabbit. The erythrocyte and platelet counts were made on four albino rabbits until their normal levels were established. Phylloerythrin-sodium salt solution was injected subcutaneously into two of the rabbits and sodium chlorophylline into the other two. A total of five injections were made in each rabbit. The erythrocyte and platelet counts were made during and after the course of the injections on a drop of blood removed from the ear vein. The platelets were counted by the direct method of Rees and Ecker (1923).

Sodium chlorophylline is chlorophyll in which the phytol alcohol radical is replaced by sodium. It was prepared by shaking an ether solution of chlorophyll with a 10% methyl alcoholic solution of sodium hydroxide, the chlorophylline going into the alcohol layer. The alcohol solution was placed with fresh ether and exactly neutralized with hydrochloric acid, the chlorophylline returning to the ether layer. This ether solution was then extracted with a minimal quantity of 2/10 sodium hydroxide and the resulting extract was assayed for its chlorophylline content in the same manner as the phylloerythrin solution was assayed.

V. Results.

Anemia Experiments. The results of the anemia experiments are best presented in graphical form to which the reader is referred. Where the death of an animal occurred it is marked on the curves by a †.

Figure I gives the growth and hemoglobin curves of the preliminary anemia experiment.

Figure II gives the hemoglobin curves of the phyllo-erythrin rats in the second experiment. For explanation of note (a) see Discussion.

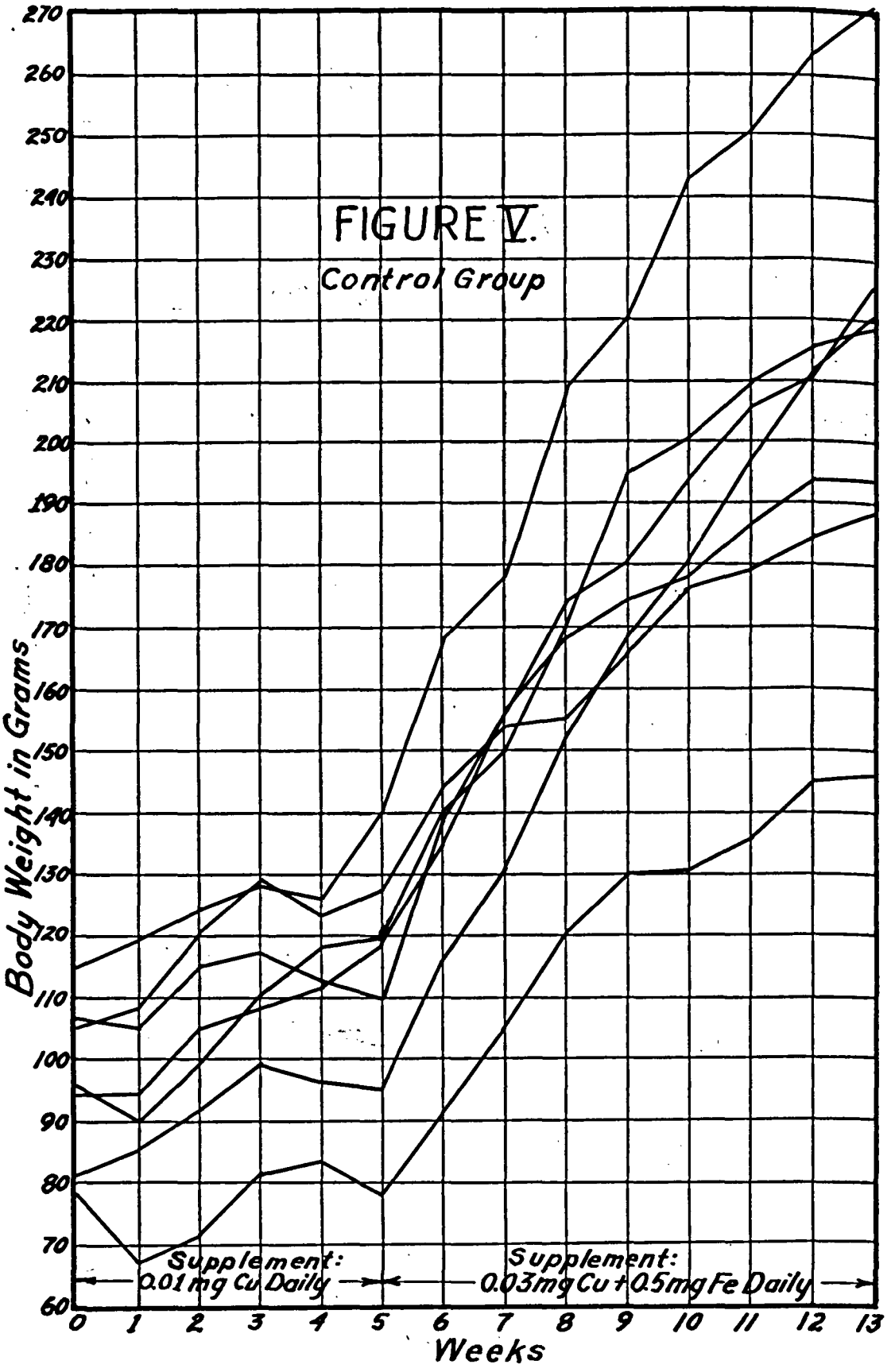
Figure III gives the hemoglobin curves of the control rats in the second experiment. For explanation of note (b) see Discussion.

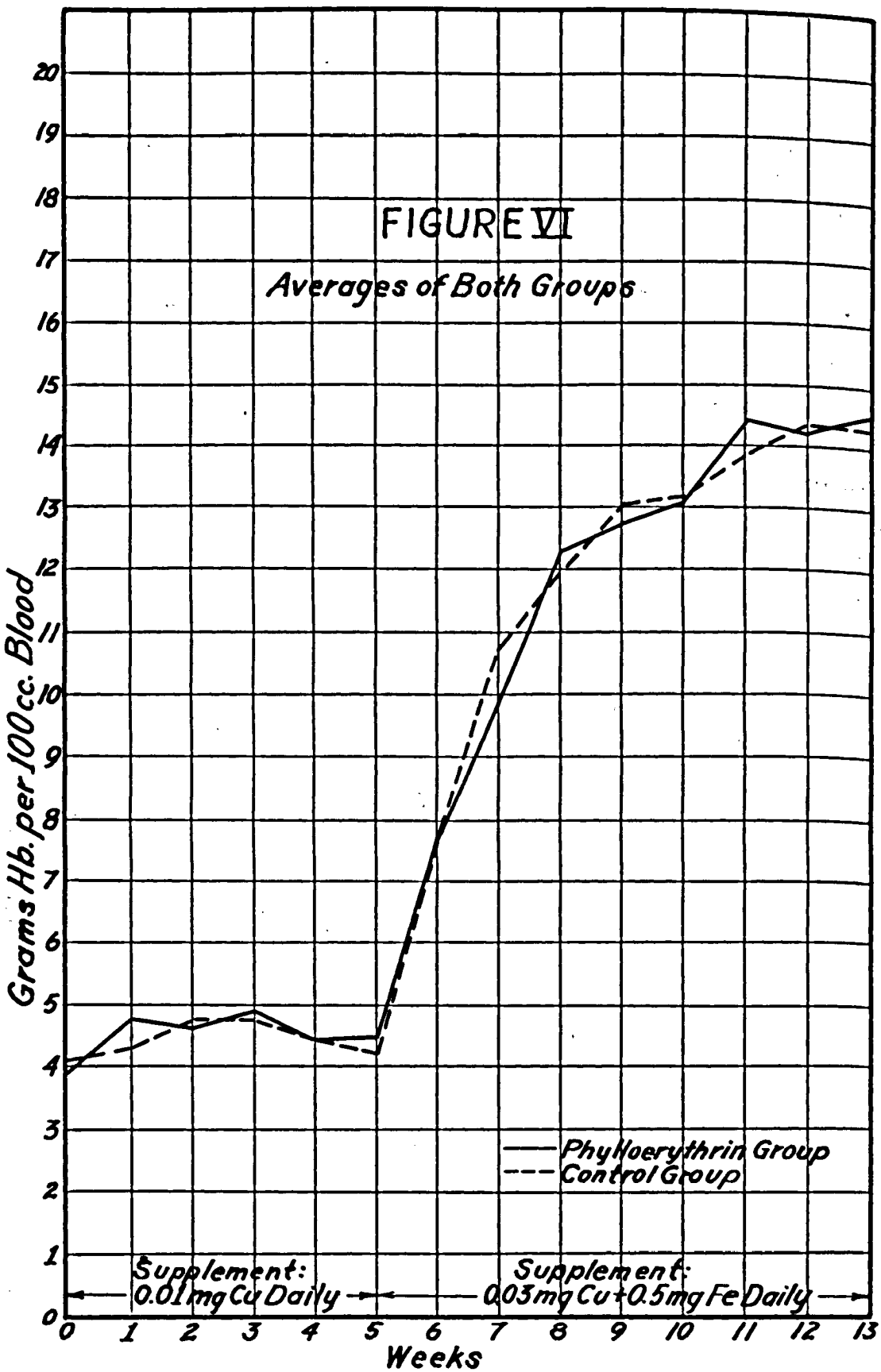
Figure IV gives the growth curves of the phyllo-erythrin rats in the second experiment.

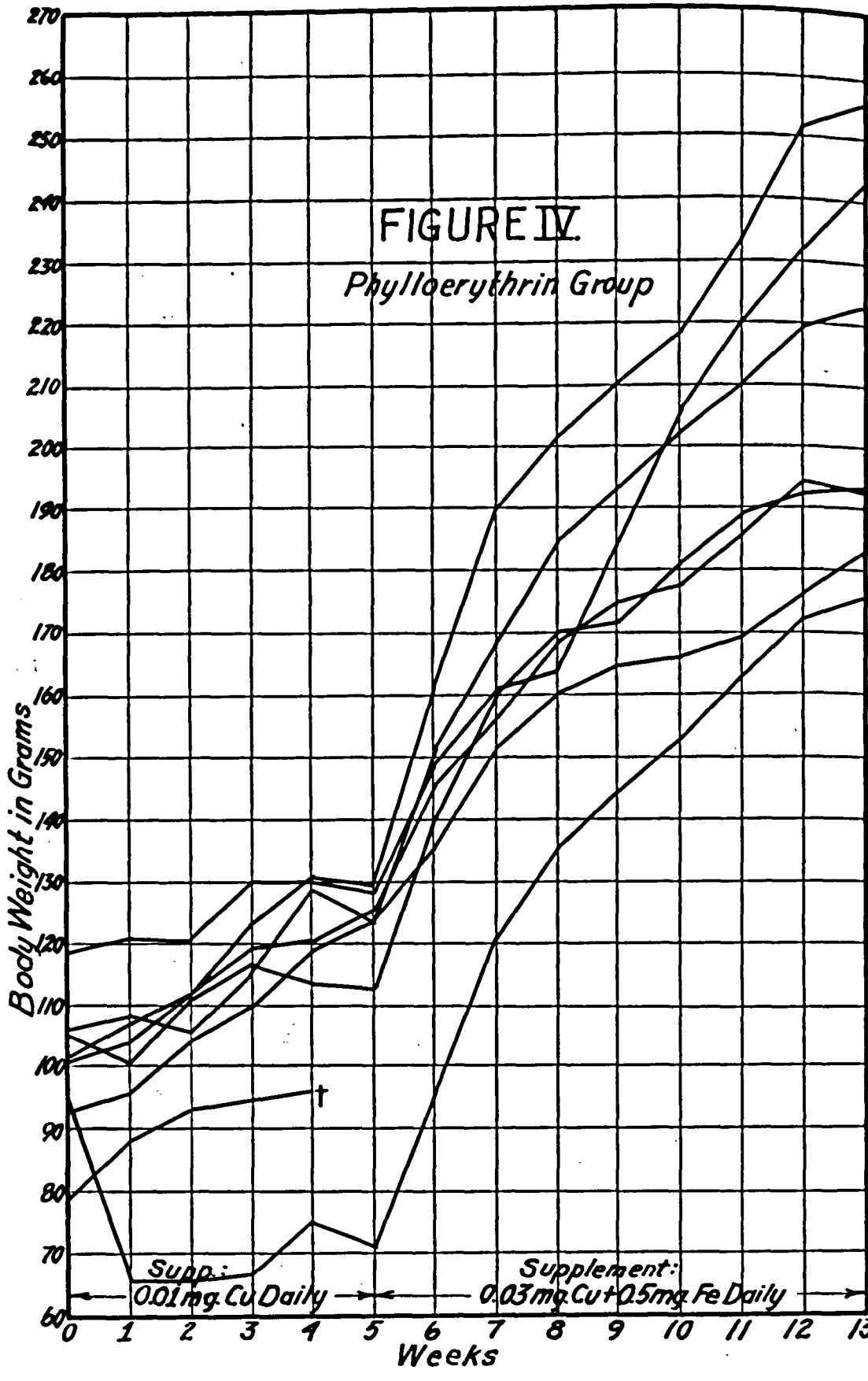
Figure V gives the growth curves of the control rats in the second experiment.

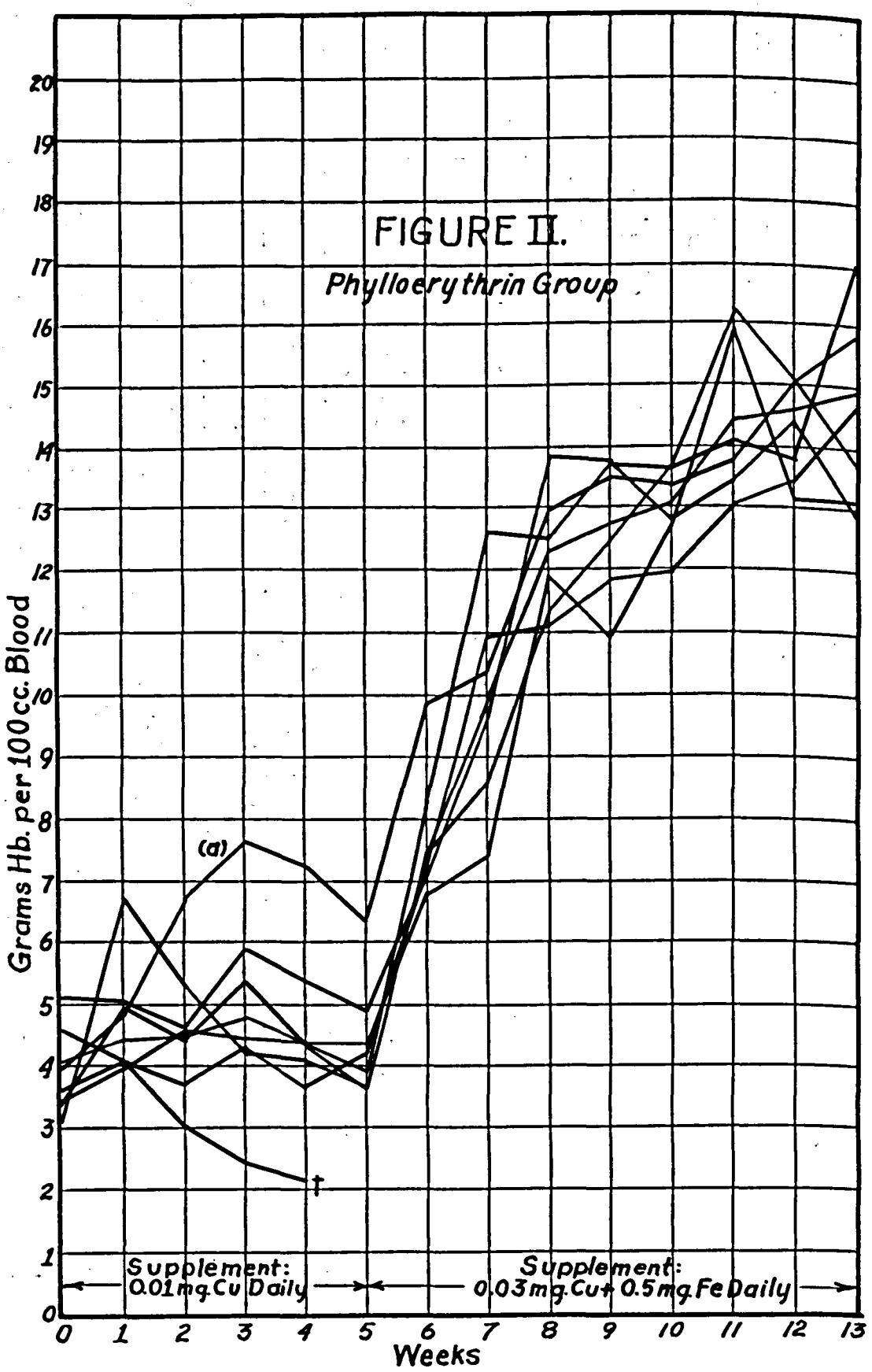
Figure VI gives a comparison of the average hemoglobin curves of both groups in the second experiment.

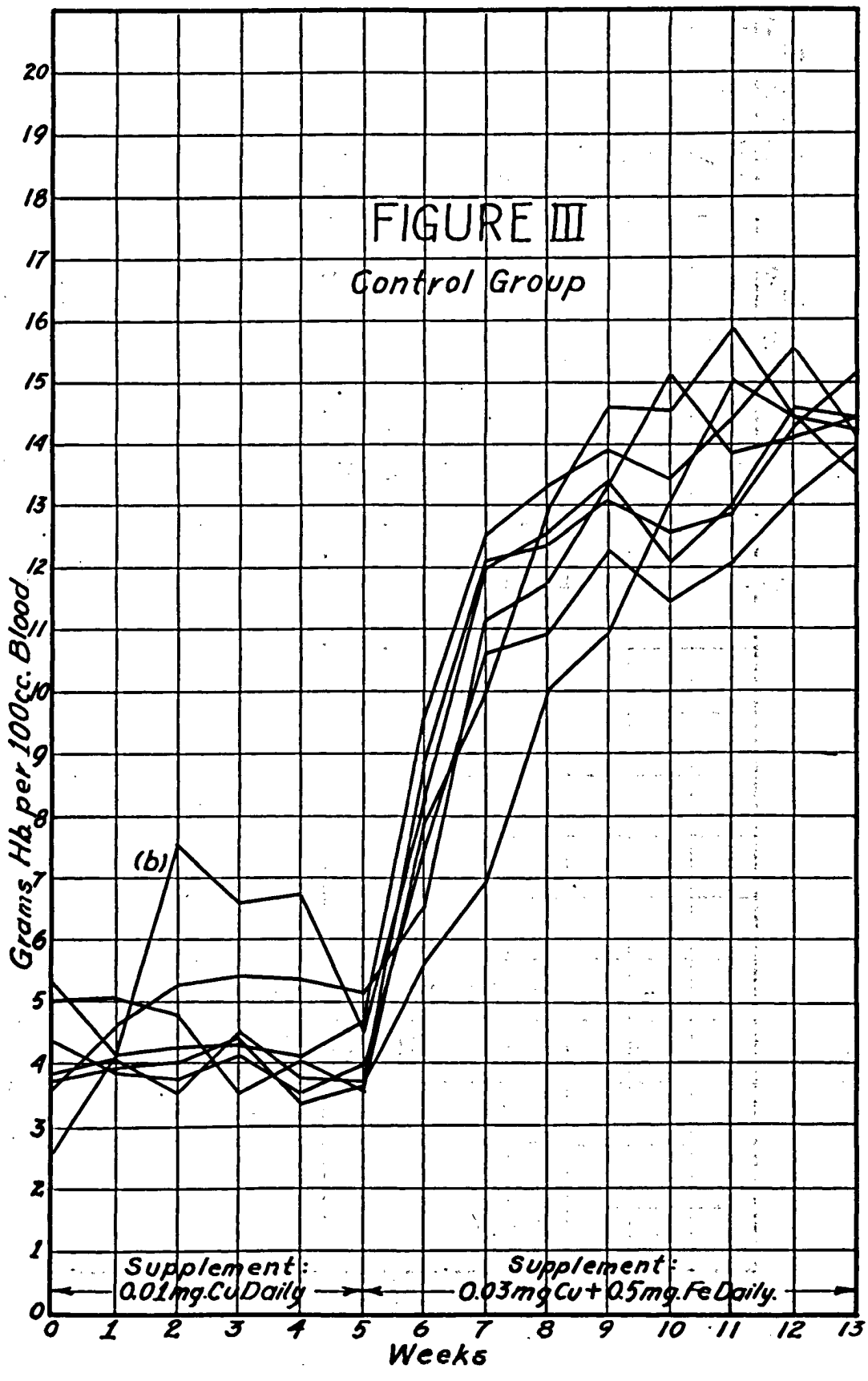
Figure VII gives a comparison of the average growth curves of both groups in the second experiment.

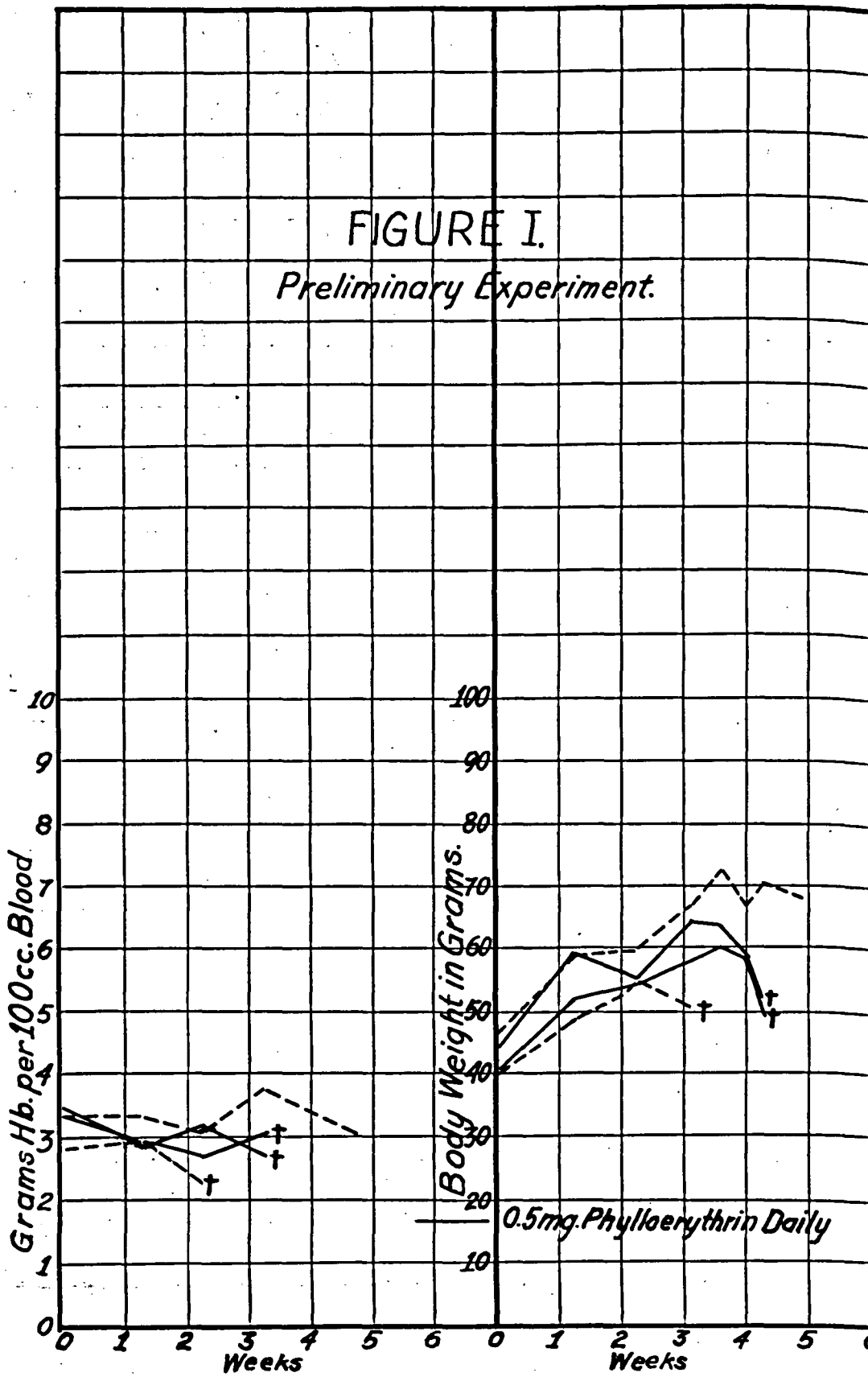


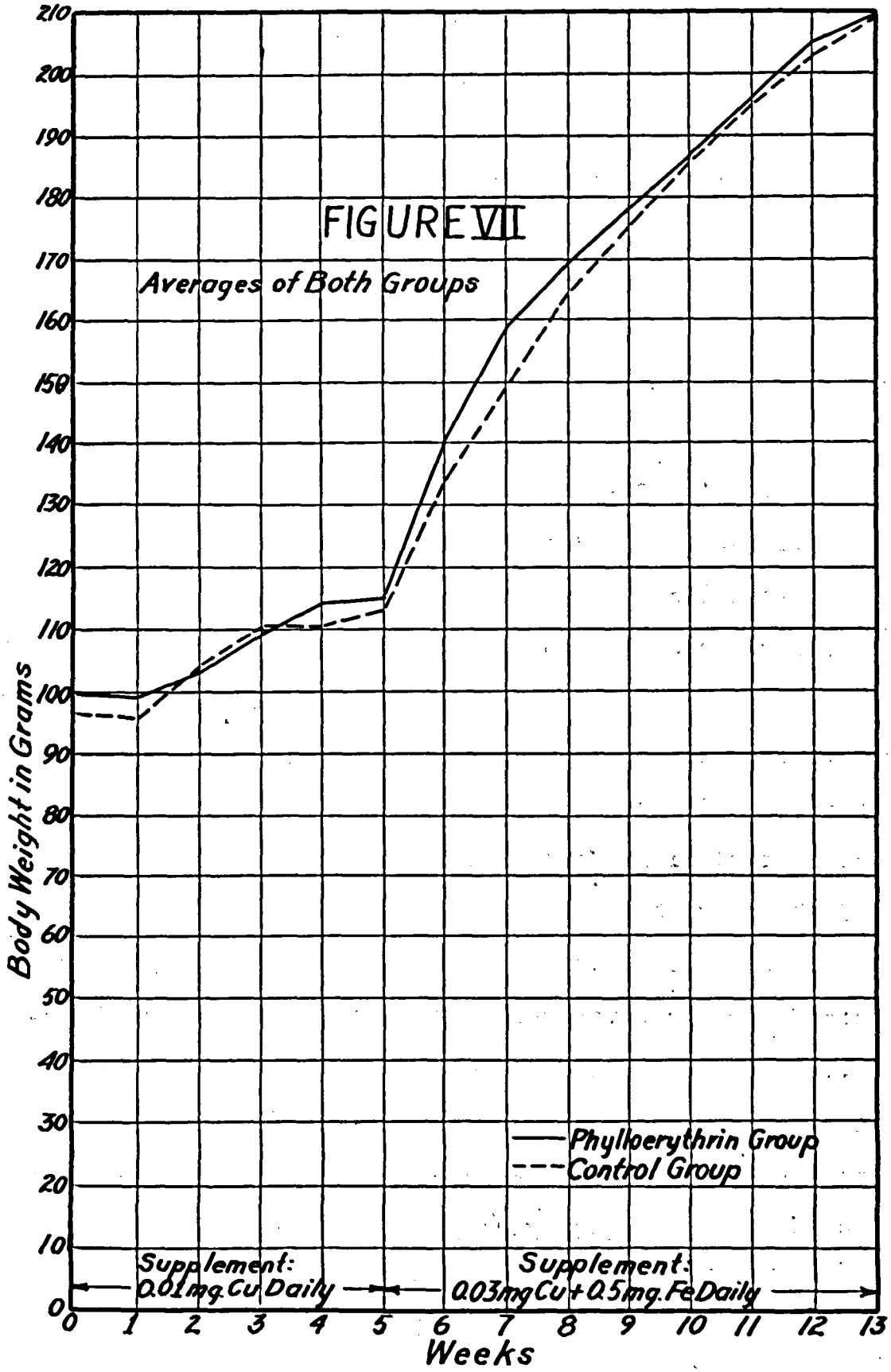












Thrombocytosis Experiments. The following table gives the results of the thrombocytosis experiments on normal rabbits. On the days in which an injection was given the blood count was made before the injection.

(Insert Table I.)

VI. Discussion.

In the preliminary anemia experiments the number of experimental animals was obviously too small to allow definite conclusions to be drawn. However, the author believes it significant that none of the animals showed any signs of recovery from the anemia; on the contrary three of the four died within a short time.

In the second anemia experiment the effects of two slips in the technic are shown. In Figure II a decided increase in the hemoglobin of one of the animals is marked by (a). This increase probably was caused by the faulty galvanizing of the bottom screen of the cage. The rat tore off a small portion of the zinc covering and obtained access to the iron wire underneath. It will be observed that the hemoglobin of this animal immediately decreased when it was transferred to another cage. In Figure III another increase in hemoglobin took place which is marked

Table I.

Rabbit No.		1		2		3		4	
Day	Dosage	Phylloerythrin		Phylloerythrin		Na-chlorophylline		Na-chlorophylline	
		Red Cells millions	Thrombo-cytes thousands	Red Cells millions	Thrombo-cytes thousands	Red Cells millions	Thrombo-cytes thousands	Red Cells millions	Thrombo-cytes thousands
Normal		6.500	361	7.473	372	7.423	720	8.067	352
1	1 mg./kg.	6.690	424	7.900	266	7.270	740	9.070	280
2		7.980	352	7.780	496	6.660	676	8.280	360
3	2 mg./kg.	6.390	292	6.410	514	7.550	770	7.430	480
4		6.520	464	7.160	362	6.100	700	7.440	246
5	2 mg./kg.	6.450	404	7.300	272	7.200	812	8.000	476
6	2 mg./kg.	6.290	516	6.620	430	6.630	980	7.650	546
7	2 mg./kg.	6.410	452	7.570	562	7.130	900	8.170	464
8		6.470	648	7.310	454	6.410	982	6.790	492
9		6.000	562	6.130	518	5.840	924	6.850	422
11		5.760	588	7.010	598	6.930	784	6.630	390
15		5.920	374	7.280	422	5.310	648	6.920	458
Maximum increase			80%		47.5%		36.5%		55%

by (b). This animal broke loose from the author while a blood sample was being removed from its tail. Before it was recaptured and returned to its cage it had become covered with dirt from under the laboratory table. By licking off this dirt the rat may have obtained enough iron and copper to give the response shown, after which, the hemoglobin again decreased. These two errors once more indicate the care with which nutritional anemia experiments must be carried out.

The average hemoglobin curves for the two groups of rats (Figure VI) lie closely parallel to each other throughout the experiment and at no time are separated from each other by more than the probable experimental error. The author feels justified in concluding, therefore, that phylloerythrin does not have a hematopoietic effect in this type of anemia.

Since phylloerythrin is the decomposition product which is absorbed in largest amounts, the probability also exists that neither chlorophyll nor its other decomposition products would be active in this respect.

Furthermore, it should be noted that one animal receiving phylloerythrin died at the end of four weeks (Figure II) with a steadily decreasing hemoglobin level. In this instance, the porphyrin was ineffective in maintaining the hemoglobin level constant. With the exception of the

anemic condition, this animal was apparently normal and healthy.

Examination of the average growth curves of the two groups (Figure VII) reveals the fact that the phylloerythrin group increased in body weight faster than the control group after iron and increased copper were added to the diet. By the end of the experiment, however, the control group had caught up with the phylloerythrin group. As mentioned under "Procedure", during the first five weeks of the experiment the amount of milk fed to each rat was adjusted to the needs of those with the least appetite and during the ensuing eight weeks each animal was given as much milk as it would consume. Hence, no appreciable difference in the growth curves of the two groups can be observed in the first five weeks. However, from the end of the five week period the author believes there is a significant difference between the two curves. In addition it was noticed that the animals in the phylloerythrin group consumed their milk with a definitely greater avidity and rapidity than the controls. This difference became noticeable at the end of the third week and lasted until about the tenth week of the experiment.

Assuming that the disparity between the two groups as regards appetite and growth is of some consequence, several very interesting deductions may be made, although

these deductions were not confirmed experimentally by the repetition of the exact procedure used by Rothemund, McNary and Inman (1934). A possible explanation of the hematopoietic effect of chlorophyll porphyrins as reported by these authors might be made as follows: The milk used in those experiments was reconstituted milk prepared by adding water to commercial dried milk powder. Elvehjem (1935) has shown that dried milk powder contains more iron and copper than can be accounted for in the original milk, contamination having taken place during the processing. Supplee, Dow, Flanigan and Kahlenberg (1932) found that milk dried by the roller process produced definite improvement when fed to anemic rats. It may be stated, therefore, that the anemia produced by commercial dried milk would be more subject to remission than that produced by uncontaminated fresh milk because the deficiency of the essential metals in the powder is only a little less than the amount necessary for recovery. Any factor which would increase only slightly the amount of the metals that the rats obtain would then cause a definite response in the hemoglobin level. Such a factor might, for instance, be an increased appetite which would enable the rats to obtain more iron and copper from the extra amount of milk consumed and consequently show a recovery response. It is believed by the author that this is what took place in

the anemia experiments conducted at Antioch College--the porphyrin supplements increased the consumption of the reconstituted milk diet which caused a recovery response because of the extra iron and copper thereby obtained. A contributing factor might also be the presence of traces of iron and copper in the porphyrin supplements used, since water redistilled in glass was not used in their preparation. In the experiments reported here, this latter source of error was eliminated by redistilling all the water in glass and by the addition to the diet of the control rats a solution prepared exactly similar to the porphyrin supplement, with the exception that the phylloerythrin was omitted.

As mentioned in an earlier part of this paper, Bürgi asserts that chlorophyll feeding produces, among other things, an increased appetite and a bettering of the general health, especially in certain enervating diseases and more particularly in anemias of various kinds. Since phylloerythrin is one of the chief absorption products of chlorophyll and, since phylloerythrin was found to have a stimulating effect upon the appetite of the anemic rats, this opinion of Bürgi's is therefore confirmed. However, the claims of Bürgi that chlorophyll feeding, per se, produces a stimulation of the blood-forming tissues, leading

to an increased erythropoiesis, is not confirmed. The possibility still exists, nevertheless, that chlorophyll does have a hematopoietic effect under the conditions of Bürgi's experiments.

The claims of Hughes and Latner (1936) that the animal organism can transform chlorophyll into hemoglobin likewise have not been confirmed. However, the milk diet may possess an adequate amount of the precursors of the blood pigment and the phylloerythrin added would then be superfluous and unused.

The subcutaneous injection of phylloerythrin and of sodium chlorophylline into normal rabbits produces a thrombocytosis as is shown in Table I. The number of thrombocytes in rabbit's blood show considerable daily variation and consequently minor fluctuations are without meaning. However, rabbit number one showed an 80 per cent increase on the eighth day after the beginning of the injections of phylloerythrin and rabbit number two showed a 47.5 per cent increase on the seventh day. On the eighth day after injections of sodium chlorophylline were begun, rabbit number three showed an increase of 36.5 per cent and on the sixth day, rabbit number four showed an increase of 55 per cent. These increases are outside the limits of normal variation. The injections

were started at a level of 1.0 mg. per kg. of body weight and with an interval of two days before the second injection was made in order to see if the initial injection would have any effect. No appreciable change was observed. The amount was then doubled and again a two day interval was allowed, but no significant increase was forthcoming. The third, fourth and fifth injections of 2.0 mg. per kg. were made on succeeding consecutive days, and these produced the significant increases already noted.

These results confirm the work of Rentz on the thrombocytosis produced by the injection of chlorophyll-sodium into rabbits. Rentz found an increase of 85 per cent after seven daily injections of 1.0 mg. per kg. While the results given above are not strictly comparable owing to the difference in the course of injections, the author believes the difference to be quantitative but not qualitative. Furthermore, the fact that phylloerythrin produces a similar response to that of chlorophyll-sodium indicates that the porphyrin nucleus is the active part of the chlorophyll molecule in the production of thrombocytosis.

The erythrocyte count of the rabbits was not affected to any considerable extent by the injections of phylloerythrin or of sodium chlorophylline.

VII. Summary and Conclusions.

From the results presented above it may be concluded that:

1. Phylloerythrin does not have an hematopoietic effect when added to the diet of albino rats with milk anemia.

2. Phylloerythrin does produce a stimulation of the appetite when added to the diet of anemic rats.

3. The hematopoietic effect in anemic rats as reported by Rothemund, McNary and Inman when chlorophyll porphyrins were added to the diet may be explained by the increased appetite which permits the animals to consume more milk. Since this milk was reconstituted from commercial dried milk powder, and since this powder contains more iron and copper than uncontaminated fresh milk, the extra amounts of these metals which the rats obtained are probably the cause of the hematopoietic response.

4. The appetite stimulation of phylloerythrin is supporting evidence for the similar appetite stimulation of chlorophyll as claimed by Bürgi. The assertion made by Bürgi that chlorophyll possesses an antianemic power is not similarly supported.

5. Repeated subcutaneous injections of phylloerythrin and of sodium chlorophylline produce a thrombocytosis in normal rabbits. The analogous results of

Rentz with chlorophyll-sodium are thus confirmed and the active part of the chlorophyll molecule in this respect is shown to be the porphyrin nucleus.

Bibliography.

(1). Abderhalden, E.: Z. physiol. Chem. 26, 498 (1899).

(2). Bunge (1900); cf. Editorial, J. Am. Med. Assoc. 98, 320 (1932).

(3). Bürgi, E.: Corresp.-Bl. f. Schweiz. Aerzte N:O 15, (1916).

(4). Bürgi, E.: Therapeutische Monatshefte 32, 1-11 (1918); Ibid 33-42.

(5). Bürgi, E.: Münchener med. Wochschr. 47, 2019 (1927).

(6). Bürgi, E.: Medizinische Welt. Arztliche Wochschr. 17, (1928).

(7). Bürgi, E.: Klinische Wochschr. 9, 789-90 (1930); Deut. med. Wochschr. 56, 1650-2 (1930).

(8). Bürgi, E. and C. v. Traczewski: Biochem. Z. 98, 256-84 (1919).

(9). Edwards, F. and K. Holley: Ga. Agr. Expt. Sta. Bull. 173, 3-11 (1932).

(10). Elvehjem, C.: Physiol. Rev. 15, 471-507 (1935).

(11). Elvehjem, C., E. Hart, H. Jackson and K. Weckel: J. Dairy Sci. 17, 763 (1934).

(12). Fischer, H. and R. Bäumler: Sitzb. math.-naturw. Abt. bayer. Wiss. München 77-83 (1929).

(13). Fischer, H. and A. Hendschel: Z. physiol. Chem. 198, 33-42 (1931).

(14). Fischer, H. and A. Hendschel: Z. physiol. Chem. 216, 57-67 (1933a).

(15). Fischer, H. and A. Hendschel: Z. physiol. Chem. 222, 250-60 (1933b).

(16). Fischer, H. and H. Hilmer: Z. physiol. Chem. 143, 1-8 (1925).

(17). Gordonoff, T.: Klin. Wochschr. 4, 409 (1925).

(18). Gordonoff, T.: Z. ges. exptl. Med. 54, 294-312 (1927).

(19). Gordonoff, T. and T. Hosokawa: Z. ges. exptl. Med. 46, 454-8 (1925).

(20). Grigoriew, R.: Biochem. Z. 98, 284-94 (1919).

(21). Gsell, E.: Schweiz. Med. Wochschr. 59, Nr. 24 and 25 (1929).

(22). Hart, E., C. Elvehjem, J. Waddell and R. Herrin: J. Biol. Chem. 72, 299-320 (1927).

(23). Hart, E., H. Steenbock, C. Elvehjem and J. Waddell: J. Biol. Chem. 65, 67-80 (1925).

(24). Hart, E., H. Steenbock, J. Waddell and C. Elvehjem: J. Biol. Chem. 77, 797-812 (1928).

(25). Hughes, J. and A. Latner: J. Physiol. 86, 388 (1936).

(26). Kémeri, D.: Biochem. Z. 151, 438 (1924).

(27). Kemmerer, A., C. Elvehjem and E. Hart: J. Biol. Chem. 92, 623.(1931).

(28). Kitahara, Y.: Biochem. Z. 155, 97-112 (1925).

(29). Koenigsfeld, H.: Klin. Wochschr. 1, 322 (1922).

(30). Kohler, G., C. Elvehjem and E. Hart: Am. J. Physiol. 113, 279-84 (1935).

(31). Kortschagin: Biochem. Z. 153, 510 (1924).

(32). Laurens, H.: Book: The Physiological Effects of Radiant Energy.

(33). Loebisch and Fischler: Monatsh., 335-350 (1903).

(34). Lorenzi, A.: Riforma medica 47, No. 3 (1931).

(35). MacMunn,: J. of Physiol. 6, 22 (1885).

(36). Marchlewski, L.: Bull. intern., acad. polonaise des sciences et des lettres, A 638-642 (1903).

(37). Marchlewski, L.: Bull. soc. chim. biol. 6, 464-72 (1924).

(38). Mathews, A.P.:Book: Physiological Chemistry, 5th edition, p. 559.

(39). Miyadera, K.: Berl. klin. Wochschr. 39, 1159 (1921).

(40). Patek, A.: Arch. Int. Med. 57, 73 (1936).

(41). Quin, J., C. Rimington and G. Roets: Onderstepoort J. Vet. Sci. 4, 463-78 (1935).

(42). Rees, H., and E. Ecker: J. Am. Med. Assoc. 80, 621 (1933).

(43). Rentz, E.: Upsala Läkareförenings Förhandlingar N. F. 33, 371-92 (1927).

(44). Rentz, E.: Compt. rend. soc. biol. 98, 814-15 (1928).

(45). Rentz, E.: Skand. Arch. f. Physiol. 56, 36-117 (1929a).

(46). Rentz, E.: Skand. Arch. f. Physiol. 57, 121-137 (1929b).

(47). Rider, T.: J. Biol. Chem. 100, 243-8 (1933).

(48). Rimington, C. and J. Quin: Onderstepoort J. Vet. Sci. 3, 137-57 (1934).

(49). Robscheit-Robbins, F. and G. Whipple; Am. J. Physiol. 72, 395 (1925a).

(50). Robscheit-Robbins, F. and G. Whipple: Am. J. Physiol. 72, 431 (1925b).

(51). Robscheit-Robbins, F. and G. Whipple: Am. J. Physiol. 92, 400 (1930).

(52). Rothemund, P. and O. Inman: J. Am. Chem. Soc. 54, 4702 (1932).

(53). Rothemund, P., R. McNary and O. Inman.: J. Am. Chem. Soc. 56, 2400 (1934).

(54). Rudolph, C.: Clin. Med. and Surg. 37, 119-121 (1930).

(55). Rydin, H.: Compt. rend. soc. biol. 99, 1683-5 (1928); Ibid 1687-8.

(56). Scott, J.: Biochem. J. 17, 157 (1923).

(57). Supplee, G., O. Dow, G. Flanigan and O. Kahlenberg: Lait 12, 1-17 (1932).

(58). Zih, A.: Arch. ges. Physiol. (Pflügers) 225, 728-36 (1930).

(59). Zih, A.: Arch. ges. Physiol. (Pflügers) 231, 510-13 (1933).