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The role of inorganic substances and amino acids in the regeneration of hemoglobin in the rat

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THE ROLE OF INORGANIC SUBSTANCES AND AMINO
ACIDS IN THE REGENERATION OF
HEMOGLOBIN IN THE RAT

by

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INTRODUCTION

A Brief Discussion of Blood Formation and Anemia

The following recognized dietary essentials: proteins,
carbohydrates, fats, minerals, and vitamins, are necessary in
order to keep the animal in a normal, healthy state. These
food factors are required in appropriate amounts to build up
tissues, to furnish both heat and muscular energies, and thus
to carry on proper physiological activity. Heat and muscular
energy are both indispensable, and are derived from the oxi-
dation of organic compounds within the tissue. The exact
mechanism of the tissue oxidation of food materials is not
known, but we do know that oxygen is the primary agent invol-
ved in such a chemical reaction, and that it is transported
through the blood stream to the body cells by means of the
red blood corpuscles.

Within the red blood cells there is found a red pigment
called hemoglobin, which confers upon the blood both its
color and its oxygen-carrying capacity. Hemoglobin has been
shown to be a complex organic compound consisting of a pros-
thetic group, hemin, and a protein, globin. To the hemin
portion is ascribed the ability of taking up oxygen in the

lungs and releasing it to the tissues in a readily available form. This property of loose chemical combination with oxygen is supposed to be primarily due to the iron atom in the nucleus of the molecule. It then follows that if normal oxidative processes are to go on, an ever-ready supply of red blood cells must circulate in the blood stream, for they are the only bodies capable of carrying hemoglobin. Furthermore, since both the cells and hemoglobin are of an expendable nature, there must be a constant source of supply.

Our present knowledge of the blood tells us that erythrocytes are formed within the reticulo-endothelial system which is found widely scattered throughout the body, and especially in the red marrow of the long bones. The precursor of the erythrocyte is a nucleated cell or megaloblast which extrudes its nucleus to become a red blood corpuscle. After circulating through the blood vessels for about four weeks, these corpuscles are fragmented within the liver, spleen, bone marrow, and lymph nodes. The iron-containing hematosiderin goes back to the long bones to form new cells, while the iron-free hematoporphyrin is excreted in the form of bile pigments.

A deficiency of blood or corpuscles is known as anemia. According to our present knowledge of human anemias, this general term may be subdivided into several types, differing not only in etiology and clinical symptoms, but also in the treat-

ment necessary for their alleviation. Some anemias may be
entirely cured by medical and dietary means, while others
are arrested only as long as beneficial administration con-
tinues.

Aplastic anemias, showing typical bone marrow lesions,
are possibly the most prevalent. They are produced by inade-
quate blood formation, and are characterized by the appear-
ance of abnormal cells within the blood stream. In advanced
stages megaloblasts, which are primitive erythrocytes, appear
in large numbers in the blood. Pernicious anemia is of the
aplastic type, usually found to be associated with Addison's
disease, sprue, removal of the stomach, gastric cancer,
tumors, diseases of the bone, or radium poisoning. Reticulo-
cytosis and hematopoiesis may be prompted by feeding liver
extracts, gastric digests of beef muscle, or desiccated hog
stomach. However, the patient must continue treatment or a
relapse occurs.

Posthemorrhagic anemia, as the name implies, develops
after external bleeding and includes hemorrhage into the lu-
men of the gastro-intestinal tract, and the respiratory and
genito-urinary passages; the blood picture shows the regular
number of red blood cells, although hemoglobin is not present
in the normal amount. Therefore, a building of hemoglobin
alone is essential. This latter may be accomplished by iron
administered in large doses. There is apparently no need for
the supplementary action of copper.

Hemolytic anemias result from a destruction of blood within the body. Toxins produced by snake venom, nephritis, jaundice, and pregnancy break down the erythrocytes, thus allowing the hemoglobin to be destroyed. Other destructive agents arise from infections such as sepsis, malaria, syphilis, and parasitic worms. Curing the primary cause stamps out the anemia. Familial hemolytic jaundice exhibits sickle cells in the blood due to the fragility of the erythrocytes. A spleenectomy offers permanent relief in this case.

The majority of human anemias fall within the scope of the three classes given. All others are grouped together and called miscellaneous anemias. Of these we might form a class to include those of unknown pathogenesis, such as are found in cases of cancer, hypothyroidism, cirrhosis of the liver, and intestinal obstruction. Both hemoglobin and red cell regeneration are required, and these stubborn types improve only upon a general improvement of the condition of the patient.

Since the modern classification does not admit those anemias which are of dietary origin, we are obliged to resort to the older and more widely used differentiation. In it anemias are defined according to their etiology, thus:

> 1. Primary anemias are those in which the cause is unknown.
> 2. Secondary anemias include all those due to some ascertained cause.

Low fat anemia, which is caused by an inadequate fat intake, falls under the head of secondary anemias. The symptoms are a low hemoglobin level and erythrocyte count. Complete recovery ensues if cod-liver oil be ingested.

Nutritional anemia, characterized by a low hemoglobin level, is caused by a lack of copper or iron, or both, in the diet. Therefore, it is a deficiency disease, and is classed by some with the pernicious anemias. It is similar in that it responds to liver therapy, but dissimilar in the blood picture and condition of the marrow of the long bones. Nutritional anemia does not show an incapacitated reticulo-endothelial system or an aplastic bone marrow. At present there is no place for it unless one considers the older classification, where it would be listed among the secondary anemias.

Statement of the Problem

It was previously thought that nutritional anemia in rats could be cured by the administration of iron alone, and in the minds of some investigators this opinion still exists. Others admit the effectiveness of copper as a supplement to iron in hemoglobin building, but find that many inorganic substances may be substituted in lieu of copper. Amino acids have been valued as hematopoietic agents by another faction. Since much conflicting evidence exists concerning the sub-

stances necessary for iron utilization in nutritional anemia, and because only very minute amounts of copper function in this process, it becomes necessary to use highly purified materials in investigating the problem.

The rat lends itself very well to such research, because its physiology approximates that of man. These small animals are easily procured and adapt themselves readily to laboratory conditions. Their food consumption is low, which makes them desirable where highly purified materials must be used. Furthermore, the rat's life is only about one-thirtieth that of the human being; consequently, results may be obtained in a relatively short length of time.

Unpasteurized, whole cow's milk furnishes a food containing nearly all the necessary food essentials, but fails to supply enough iron and copper to keep up a normal hemoglobin level. Rats maintained under proper conditions upon a milk diet develop a severe nutritional anemia within a period of six weeks.

The experiments described in this thesis were designed to throw further light upon the factors involved in hemoglobin synthesis in rats suffering from nutritional anemia. During this investigation an endeavor was made to use the most refined technique both in the care of the animals and in the preparation of the various substances administered.

All work was carried out upon a highly inbred strain of rats, procured from the Iowa State College animal colony, which had been rendered anemic by feeding a basal ration of unpasteurized cow's milk. Answers were sought to the following specific questions:

1. Can nutritional anemia be induced in rats fed upon market milk, when housed in galvanized iron wire cages with shavings for bedding?

2. Do inorganic salts stimulate hemoglobin formation when administered orally with a market milk and iron diet?

3. If high dosages of iron be added to a ration of low-copper milk, will anemic animals regenerate their hemoglobin to a normal value?

4. Will copper feeding at levels higher than the recognized optimum effect a more rapid hemoglobin regeneration?

5. Do inorganic salts, aside from copper salts, supplement iron when low-copper milk is used, or is copper unique in its effect?

6. Does the addition of zinc or magnesium hasten the onset of a severe nutritional anemia?

7. Could inorganic salts, aside from copper salts, be utilized for blood regeneration if given intraperitoneally?

8. Are additional amino acids necessary for hematopoiesis?

9. In animals receiving the recognized optimum amount

of iron, what is the minimum amount of injected copper sulfate needed to restore a normal hemoglobin titer?

10. Have iron compounds, if injected alone, curative properties?

11. Will injected hydrochloric acid act as a hematinic?

12. How much copper and iron, injected together, is essential to provide an adequate amount of hemoglobin?

13. Can the animal body utilize insoluble iron compounds to advantage with or without copper supplements?

14. How does the hematopoietic potency of insoluble copper compounds compare with copper sulfate?

15. Do colloidal hydroxides of copper and iron play a role in hemoglobin building?

16. Can the effect of nutritional anemia on metabolism be shown by means of urine analysis?

REVIEW OF LITERATURE

Investigators have been concerned with mineral metabolism for at least fifty years. Hemoglobin was known to be a vital iron-containing compound of an expendable nature and, with this in mind, great efforts were put forth to ascertain just which forms of iron lent themselves most readily to the synthesis of hemoglobin within the body. Doubtless, research workers had assumed milk to be the most adequate food known because it was a natural nutrient for young animals, and they were striving to account for the fact that adults could not exist for long periods of time if restricted to it exclusively. Since milk was known to contain some iron, a recognized food factor, the question arose whether inorganic iron could be utilized as efficiently as organic iron by the animal body.

The first work of this kind was reported by von Bunge (8) in which he says, "We must ask ourselves the question: under normal circumstances, in which form is iron resorbed and assimilated?" His attempts to extract the iron from milk and hemoglobin by the use of solvents such as ether and alcohol led him to conclude that the iron in our food is in the form of complex organic compounds.

Six years later, Abderhalden (1)(2), a student working

in von Bunge's laboratory on dogs, cats, and rats, and employing a basal milk-rice diet, supplemented with individual substances such as meat, albumin, hemoglobin, hematin, and iron, summarized his results as follows:

> 1. That in the normal food there is enough iron to keep up the hemoglobin level if it be assimilated.
>
> 2. That an iron-poor food plus an addition of inorganic iron supported life but did not build as much hemoglobin as a normal food.
>
> 3. That the hematin-held iron gave a greater response than the iron contained in hemoglobin; therefore, the solution of the problem lay in the formation of hematin.

At this early date, he postulated a possible benefit to the animal due to inorganic salts of arsenic, mercury, zinc, and copper.

Nearly twenty-five years had elapsed since Abderhalden's extensive work when Williamson and Ets (70) pointed out that rats could be rendered anemic by the prolonged feeding of rice and milk and could not be cured by the addition of ferric chloride. Analyses of the carcasses showed an increase in iron content of those fed the iron salt above that of the controls.

Hart, Steenbock, Elvehjem, and Waddell (30) were able to prove that rabbits fed cow's milk exhibited a low hemoglobin content. They did not respond to ferric oxide therapy but showed marked regeneration when given fresh cabbage or its iron-free alcoholic extract, along with inorganic iron. They

obtained similar results with chlorophyll and ferric oxide.
From these findings they proposed the need in nutrition of
compounds containing pyrrole rings.

Within the same year, Robscheit-Robbins and Whipple (52)
(51), in their work with dogs suffering from hemorrhagic
anemia, did not get marked improvement by feeding spinach
and lettuce. Their conclusion was, therefore, that dogs
were unable to utilize chlorophyll. A large increase in
both the number of erythrocytes and also the hemoglobin per-
centage followed the administration of liver. Heart and
skeletal muscle were of lower regenerative value. Whipple
and Robscheit-Robbins (67) next compared a commercial iron
preparation with beef liver. The liver proved to be the
most regenerative. Riecker (50), in an attempt to check
the results of Whipple and Robscheit-Robbins, obtained bet-
ter hemoglobin synthesis with iron alone, which convinced
him that liver was beneficial only for its iron content.

Workers on pernicious anemia were beginning to observe
increased reticulocyte counts followed by a rise in the
hemoglobin level of anemic patients who ate liver. Minot,
Murphy, and Stetson (40) first reported liver therapy as
beneficial in humans suffering from pernicious anemia. Now
that pernicious anemia had fallen into the class of dietary
deficiency diseases, it remained for Castle, Heath, and
Strauss (11) to point out the etiology. They postulated the

absence of an anti-anemic principle in the gastric juice of
a person suffering from pernicious anemia. For, when beef
was allowed to undergo partial gastric digestion within the
stomach of a normal person and then transferred to the sto-
mach of the anemic individual, a marked reticulosis occurred.

Elvehjem, Herrin, and Hart (20) tested the transfer of
iron from mother to young by feeding the oxide and sulfate
to goats in amounts large enough to increase the intake of
this element five-fold above the amount embodied in the ration.
Analyses revealed no increase in the iron content of the milk.
Even the addition of fresh green cabbage to the above ration
failed to affect the milk. Seeking further light on the sub-
ject, dairy cattle were maintained on alfalfa hay twice as
high in iron as the timothy fed to the controls. All at-
tempts to cause the iron to pass into the milk failed.

Mitchell and Schmidt (43) tried various foods compara-
tively rich in iron, such as spinach, egg yolk, molasses,
dates, raisins, and meat, as well as the oxide, chloride, and
carbonate of iron, on rats made anemic by milk feeding. All
additions caused a regeneration of hemoglobin, but the oxide
and carbonate acted at a much slower rate. These results
prompted them to say, "The question should be soluble versus
insoluble rather than organic versus inorganic iron." Several
iron salts were listed by Mitchell and Vaughn (44) in the
order of their anti-anemic potency.

About this time word came from Hart, Elvehjem, and Waddell (29) that not only the alcoholic extracts of cabbage and lettuce, but also the ash of these solutions, were hematopoietic. Ferric sulfate marked C. P. by the manufacturer gave positive results, thus proving that the repurification of minerals was necessary. Later experiments by Waddell, Elvehjem, Steenbock, and Hart (64) substantiated this latter conclusion. The purified iron salts, when fed alone as chlorides, sulfates, acetates, citrates, and phosphates, did not prove regenerative. However, if the ash of beef liver, lettuce, or yellow corn were supplied along with the iron, complete recovery ensued. Hart, Steenbock, Waddell, and Elvehjem (31) then attempted a separation of the elements contained in the ash of beef liver, by bubbling hydrogen sulfide through the acidified solution of the ash. Both the precipitate and filtrate were then fed separately, while those of the precipitate proved positive. The curative properties of the filtrate were negative, while those of the precipitate proved positive. Upon examination of the precipitate, they found the principle constituent to be copper. Consequently, a milk, copper, and iron ration was fed at once to anemic rats. This treatment resulted in the attainment of a normal hemoglobin level within five weeks. Simultaneously with the appearance of the above paper, McHargue, Healy, and Hill (37) published a similar conclusion; namely, that copper was a necessary supplement to iron in the prevention of nu-

tritional anemia in rats.

Evvard, Nelson, and Sewell (24), in studying the role of copper in the nutrition of rats and swine, noted that the greater part of the animal's copper stores were confined to the liver. They made the following statement: "It is possible that the medicinal and nutritive value of liver and its proper functioning may be somehow related to this element."

Investigations on dogs made anemic by bleeding proved to Whipple, Robscheit-Robbins, Elden, and Sperry (68)(19), that a different type of anemia prevailed in this case. Their animals failed to regenerate as well on copper and iron fed together as on iron alone. Likewise the ash of liver, kidney, and apricots did not produce the same beneficial response as did the fresh material.

Opinions began to differ at this time, and many controversies arose as to whether or not copper was unique in its physiological action, or if inorganic iron was adequate in itself to build hemoglobin. Some investigators held to the opinion that certain food substances were sufficient if iron were administered simultaneously. Cartland and Koch (10) found that rats made anemic by bleeding were cured on wheat gluten and casein, and that the lack of vitamins A, B, and E have no effect on anemia. Whitehead and Barlow (69) obtained an anemia in rats by feeding boiled polished rice together

with Osborn and Mendel's salt mixture. They state that copper, iron, and cod-liver oil were ineffective, but lean beef and liver proved valuable. A synthetic diet made from purified feedstuffs by Drabkin and Waggoner (17)(18), and supposedly copper-free, possessed curative properties.

Fontes and Thivolle (25)(26) injected tryptophane and histidine into anemic dogs with an immediate response both in hemoglobin and erythrocytes. Later Matsuoka and Nakao (38) and Okagawa and Tatsui (46) were able to check the work of Fontes and Thivolle. Drabkin and Miller (15)(16) stated that arginine, tyrosine, and glutamic acid assisted iron in hematopoiesis. They mention milk as being too low in these three amino acids to support hemoglobin synthesis. Stueky (53) supported Drabkin and Miller as to the effect of glutamic acid, while Elvehjem, Steenbock and Hart (22) did not find that the addition of pure glutamic acid to a milk and iron ration increased the hemoglobin of anemic rats. Sodium hydrogen glutamate was fed by Rider (49) along with copper and iron, both singly and in combination. The outcome was a marked increase due to the amino acid salt, if copper and iron were present, above that obtained with the latter elements alone.

Supplee, Dow, Flanigan, and Mahlenberg (55), in comparing the hematopoietic value of liquid milk with that of re-constituted roller-dried milk of equal copper concentration,

found the latter milk to be the best. This led them to state that the changes wrought by desiccation, as in roller drying, gave anti-anemic properties to the milk and that copper was not always a vital factor in preventing nutritional anemia.

McHargue (36) had shown a high copper content in the meat of the codfish, but at that time the nutritive value of copper was unknown. Seven years later, Coulson, Levine, and Remington (12) tested sea foods biologically and suggested that they be used as a substitute for liver in pernicious anemia.

The effect of avitaminosis on hematopoietic function was determined by Sure, Kik, and Walker (57)(58)(59), by feeding a purified vitamin A-deficient ration. The work was incomplete as given, but indicated no change in the amount of respiratory pigment. Polyneuritic rats produced by a vitamin B-free ration exhibited an anhydremia toward the peak of the disease. Continued research on a vitamin E-free ration led them to conclude that the hemoglobin and red blood cells do not change in the female rat during the reabsorption of the fetus. Shortly after the above work, Sure, Kik, and Smith (56) discovered an anemia associated with vitamin G deficiency. This was not attributed to a mineral deficiency, since no response was obtained by feeding the ash of yeast. Yeast obtained from different sources varies in its regenerative properties, as reported by Krauss (34).

Mitchell and Miller (41) were able to add spinach to the list of green foods previously mentioned as hematinics. The juices from cooked spinach revealed the presence of copper, antimony, tin, iron, aluminum, zinc, manganese, strontium, calcium, magnesium, sodium, and potassium, and led these authors to conclude that a group of elements rather than any single one was concerned with hemoglobin building.

The unpublished data of Titus, Cave, and Hughes, accumulated from experiments with rabbits, in 1928, indicated that manganese might be used as a substitute for copper in curing nutritional anemia. Their later work, performed on rats, confirmed this belief. In the report by Titus, Cave, and Hughes (62), they state that a manganese-copper-iron complex is necessary, but that manganese plus iron regenerates almost as well. In an attempt to clear up divergence of opinion, Krauss (33) obtained solutions of manganese and iron from several laboratories in order to test their potency as hematinics. After attempting to cure nutritional anemia with these preparations, he suggested that the discrepancies in data might be due to differences in the technique of housing and feeding.

Beard and Myers (5) claimed positive results not only with iron alone, but also on addition of nickel, germanium, cobalt, and sodium germanate to the iron and milk diet. To prove that copper was unique, Waddell, Steenbock, and Hart (66)

ran a series of tests upon rats, feeding the salts of zinc, chromium, germanium, nickel, cobalt, lead, antimony, tin, cadmium, mercury, arsenic, and manganese separately in the milk and iron ration. The twelve elements were then fed all together. Every attempt to stay the oncoming anemia was fruitless until copper was added. They reported the optimum dosage of iron as 0.5 mg., and that of copper as 0.05 mg. Again Beard and Myers (6)(7) contended that the Wisconsin workers were in error since they obtained complete regeneration with arsenic, titanium, zinc, rubidium, chromium, selenium, mercury, vanadium, and cobalt. The two latter elements had produced a marked polycythemia when fed in a mixture with the stock ration. Other papers by Myers and Beard (45), Beard, Baker, and Myers (4) and Beard (3), show regeneration on iron alone if it were fed at a level above 0.25 mg. daily.

Underhill, Orten, and Lewis (63)(47) described experiments carried out in glass cages on the elements cobalt, nickel, zinc, and manganese. They contended that the effect of copper was unique. Electrolytic iron, fed by Lewis, Weichselbaum, and McGhee (55), did not regenerate hemoglobin. Either cobalt or manganese added to the milk and iron produced similar results.

Different levels of iron, copper, and manganese administered by Mitchell and Miller (42) strengthened their belief

that iron alone was adequate for hemoglobin building, and
that the amount formed was in proportion to the metallic
dosage. Waddell, Steenbock, and Hart (65) fed pure iron
alone at twenty times the optimum dosage, but the animals
succumbed to anemia.

Since workers situated in different parts of the country
produced such a variety of conflicting results, Elvehjem,
Steenbock, and Hart (21) thought that cow's milk might be
shown to vary in its copper content. Holstein cows were
given copper sulfate in their rations at an increase of five-
fold above that normally present. Copper analyses made on
the milk did not support the theory of a variation in copper
content. The chemical analyses of horse blood by Elvehjem,
Steenbock, and Hart (23) disclosed that copper was not a com-
ponent of the hemoglobin molecule, but that the corpuscle
fraction of blood contains the major portion of this element.

Cunningham (13), after both feeding and injecting puri-
fied iron, stated that regeneration occurred in both cases.
He expressed doubt as to whether or not a true nutritional
anemia could be produced on milk alone, since the animals
showed a hyperplastic bone marrow instead of the aplastic
marrow typical of an iron deficiency. When rabbits were made
anemic by bleeding, Grulee and Sanford (28) showed that they
did not build hemoglobin when injected intraperitoneally with
ferric hydroxide. These authors noted that there was a marked

iron storage, chiefly in the liver. A similar experiment was performed by Detre (14) with mineral acids instead of an iron salt. He was able to produce a rise both in the erythrocyte count and the hemoglobin percentage by administering these acids to rabbits made anemic by bleeding.

Williamson and Ewing (71) compared copper-iron therapy on rats suffering with nutritional and hemorrhagic anemias. They observed a remission in the nutritional type, but the rats made anemic by bleeding did not respond favorably. A low salt anemia exhibited by rats on rations low in inorganic constituents is described by Swanson and Smith (60)(61). This anemia is characterized by a true polycythemia accompanied by a drop in the hemoglobin titer. It can be relieved by supplying a standard salt mixture. Orten, Underhill, Mugrage, and Lewis (48) reported a polycythemia in rats maintained upon a copper, iron, and milk diet, if traces of cobalt were added. Neither copper nor iron added singly with cobalt produced the same effect. A difference in the utilization of crystalloids and colloids by the animal body is apparent in the researches of Goerner (27). He found that all combinations of crystalline manganous chloride and copper sulfate with ferric chloride caused hematopoiesis. When the above elements were used in the form of their colloidal hydroxides, the rats died of anemia.

EXPERIMENTAL

Preparation of Materials

Market milk

A good grade of pasteurized milk obtained from the Iowa State College dairy was used without subsequent treatment. Periodic copper analyses performed on this milk revealed an average copper content of 0.4 mg. per liter.

Low-copper milk

This milk was collected in one-gallon glass jugs, from pure-bred Holstein cows fed a standard dairy ration. A glass funnel was inserted in the neck of the jug through which the hand-drawn milk passed directly into the container, without touching any metallic surfaces. The fat content of this milk ranged from 3.3 to 3.5%. A fresh supply from the morning milking was procured daily. This milk showed, upon analysis, an average copper concentration of 0.2 mg. per liter.

Copper and iron-free water

At the beginning of the investigation, distilled water

from the Iowa State College laboratory supply was redistilled
over a gas flame from a five liter pyrex balloon flask, and
the vapor passed through a Liebig condenser. The receiving
flask was a two liter Erlenmeyer of pyrex glass. Since the
above procedure proved to be too slow to keep up with the de-
mand for the water, it was decided to use steam from the lab-
oratory high pressure steam line. A suitable trap for re-
moving line condensate was made from a Claisen distilling
flask attached by one neck to the steam outlet, the other
leading to a Liebig condenser. A bent glass tube inserted
into the flask served as a siphon to carry away the water
formed on cooling, so that only live steam was allowed to
pass through the condenser. An illustration of the apparatus
is shown in chart 1.

Copper and iron-free hydrochloric acid

Magnesium chloride, C. P., was decomposed by sulfuric
acid at room temperature, and the resulting hydrogen chloride
passed through glass wool to remove any mechanically held im-
purities. Thence it was bubbled through concentrated sul-
furic acid for drying purposes, before it was absorbed by the
copper and iron-free water.

Copper-free nitric acid

Concentrated C. P. nitric acid was heated in a pyrex re-

tort and the vapor condensed in a pyrex, sealed-in, Liebig
condenser.

Copper-free ferric chloride

Baker's Analyzed iron wire, designed for standardization,
was dissolved in a 1:1 solution of hydrochloric acid and
water. The iron solution was then evaporated to dryness in
order to lower the acidity, after which the ferrous chloride
crystals were put into a 2% copper-free hydrochloric acid so-
lution in an Erlenmeyer flask. A rapid stream of hydrogen
sulfide was bubbled through the iron solution for thirty min-
utes, and the flask was then stoppered and allowed to stand
for twenty-four hours. After filtering off the precipitate,
copper-free hydrochloric and nitric acids were added, and the
mixture boiled to convert the ferrous to ferric chloride.
The solution, upon cooling, was made ammoniacal with ammonium
hydroxide. A Büchner suction-filter apparatus served to re-
move the water and soluble ammonium chloride from the floccu-
lent ferric hydroxide. The precipitate was washed with re-
peated additions of copper-free water until approximately
three liters of water had been used. The ferric hydroxide
was suspended in copper-free water and a stream of hydrogen
chloride, prepared as previously described, passed through it
until all cloudiness had disappeared. The resulting ferric

chloride solution was made up to a known volume and kept in
a glass-stoppered bottle, which had previously been painted
black to prevent light from entering and causing precipitation.

The colorimetric potassium thiocyanate method of iron
analysis was used to determine the concentration of iron pre-
sent in this and all other iron solutions employed. Spec-
trographic analyses were made upon the various substances
mentioned unless otherwise stated.

Copper-free ferric hydroxide

The preparation of this compound has been described pre-
viously as one step in the formation of copper-free ferric
chloride.

Copper-free ferric citrate

A ferric hydroxide suspension, free from copper, formed
a solution of pure ferric citrate after digestion at about
80° C. on a steam hot plate with copper-free citric acid.
Three recrystallizations from hot water removed any copper
present in the citric acid. The excess ferric hydroxide was
filtered off, and the filtrate was evaporated just to dry-
ness and shaken with absolute ethyl alcohol to remove any un-
combined citric acid. Copper-free water was used to dissolve
the ferric citrate, and after making up to volume, the iron
content of the citrate solution was determined.

Copper-free colloidal ferric hydroxide

If a concentrated solution of ferric chloride be added slowly to boiling water, a deep ruby red color results, due to the formation of a colloidal ferric hydroxide. Hydrochloric acid will be present from the hydrolysis of the ferric chloride, but it can be removed by dialysis. This procedure was followed in the preparation of colloidal ferric hydroxide from copper-free materials. Complete removal of the chloride ion required about three weeks, if the dialyzate were removed daily. Analyses for iron were made after the colloid sample had been pectized by boiling with nitric acid.

Iron-free copper sulfate

Repeated recrystallization of C. P. copper sulfate does not remove all of the iron contained in it. Therefore, it becomes necessary to select the purest form of metallic copper available and make from it the copper salt. A copper electrode, spectrographically pure, was dissolved in purified nitric acid, evaporated to dryness, and the resulting copper nitrate taken up in dilute sulfuric acid; this gave an iron-free solution of copper sulfate. The iron-free salt was isolated by concentrating the solution and permitting it to stand until rhombic, blue crystals formed on the bottom of the container. Copper sulfate loses water of crystalli-

zation if heated, so that it is desirable to wash the crystals first in water and dry between filter papers. The above processes were used to obtain an iron-free copper salt. Dilute copper solutions were analyzed by the colorimetric sodium diethyldithiocarbamate method. All subsequent copper analyses were made by the same method.

Copper hydroxide

A 10% solution of iron-free copper sulfate was poured into a beaker containing a dilute solution of potassium hydroxide. Immediately following the addition, a flocculent precipitate of cupric hydroxide appeared. The process of dialysis served to free this suspension of ionizable copper and other salts. Tests for copper in the dialyzate showed it to be free of that ion after a period of three weeks, when the suspension was diluted to one liter and analyzed for its copper content.

Copper sulfide

Hydrogen sulfide bubbled through a solution of iron-free copper sulfate threw down a fine precipitate of cupric sulfide. The precipitate was removed and suspended in copper-free water in a dialyzer for a period of three weeks. When tests for copper showed the dialyzate to be free, the cupric sulfide was made up to one liter and its cupric ion content

determined.

Cuprous oxide and iodide

Cuprous oxide and cuprous iodide were purchased from the
Baker Chemical Company. Since their solubility is very low,
they were dialyzed for three weeks in order to remove the more
soluble copper salts should they be present. Suspensions
were made of the compounds, and a quantitative copper analysis
run on them.

Titanium

A weighed amount of Baker's C. P. titanium dioxide was
boiled with copper-free aqua regia, cooled, and made up to a
known volume. This procedure gave a milky suspension which
demanded shaking before administration.

Manganese, nickel, and cobalt

These three elements, as their chlorides, were each dis-
solved in 2% hydrochloric acid, and were purified by passing
hydrogen sulfide through these solutions. The filtrates were
boiled to expel all of the hydrogen sulfide, concentrated by
digestion, and the crystals allowed to settle out upon stand-
ing. Desirable quantities of the dry salts were made up to
the required volumes. Baker and Adamson's C. P. salts were
used.

Vanadium

A known amount of vanadium as the pentoxide, dissolved
in a small amount of pure hydrochloric acid, and diluted to
one liter with copper-free water, served as a source of this
element. The vanadium compound was obtained from the
Mallinckrodt Chemical Company.

Arsenic

Arsenic trioxide, bearing the label of Baker and Adamson,
was resublimed by heating the dry salt in a porcelain dish
covered with a flask containing cold water. The sublimate
formed upon the bottom of the flask and was removed by scrap-
ing. Copper-free water was employed in making up the solu-
tions.

Germanium

Spectrographically pure germanium dioxide purchased from
A. D. Mackay, New York, was weighed and digested overnight
in copper-free water over a steam hot plate. Complete solu-
tion resulted.

Zinc, mercury, chromium, and tin

Zinc chloride, mercurous nitrate, chromium chloride, and
stannous sulfate were first examined spectrographically for

traces of copper. Their initial purity enabled them to be used without subsequent purification. Weighed amounts of each were made up to definite volumes in copper-free water. All of the elements gave perfect solutions with the exception of tin. The suspension of stannous sulfate required shaking before feeding. Baker and Adamson's Chemical Company supplied the above salts.

Selenium

Baker and Adamson's C. P. selenic acid was measured out in a volumetric pipette in the amount desired, as calculated from the data given on the bottle. It was made up to volume with copper-free water.

Rubidium

Rubidium chloride was weighed and dissolved in the required volume of copper-free water. The material was obtained from A. D. Mackay, of New York.

Gold, silver, and magnesium

Gold chloride, magnesium chloride, and silver nitrate furnished the sources of these elements. They were procured from the Baker Chemical Company, weighed, and diluted to an appropriate volume with copper-free water. The purity of these salts was not confirmed.

Arginine and aspartic acid

These two amino acids were obtained from the Eastman Kodak Company. Solutions of a known concentration were made by dissolving them in dilute hydrochloric acid and making up to known volumes with copper-free water.

Glutamic acid

Glutamic acid was isolated from wheat gluten by digesting it with hydrochloric acid on a water-bath, and subsequently clarifying the mixture by adding charcoal and refluxing. The liquid portion was filtered off and evaporated in vacuo, and saturated with hydrogen chloride which caused the crystals to precipitate. Purification of the crude crystals was accomplished by a second precipitation from ice-cold alcohol.

Tyrosine

This amino acid was prepared from a tryptic digest of casein. Fresh pancreas furnished the enzyme, and sodium carbonate was used to adjust the alkalinity to a pH of about eight. The addition of toluene prevented putrefaction. After standing for ten days at a temperature of 40° C., the precipitate was removed and its tyrosine extracted with sulfuric acid. Upon neutralization with ammonium hydroxide, and

boiling, the tyrosine crystals settled out and were recrystallized from hot copper-free water.

Tryptophane

Tryptophane was isolated from a tryptic digest of casein, carried out in an 8% solution of sodium carbonate with fresh, minced pancreas. Chloroform and a little sodium fluoride prevented putrefaction in the mixture. After standing for seven days at 35° C., the preparation was heated to 80° C., cooled, and filtered. Sufficient sulfuric acid was added to the filtrate to give a 5% acid solution, and the calcium sulfate formed was removed by filtration. A 10% solution of mercuric sulfate in 5% sulfuric acid was next stirred into the filtrate, and after standing for twelve hours the mercury salts of tyrosine and tryptophane separated. The precipitate was washed with 5% sulfuric acid to remove the tyrosine. Hydrogen sulfide was employed to decompose the mercury salt of tryptophane, and the excess sulfuric acid was removed with barium hydroxide. Crystals of crude tryptophane were purified by boiling with animal charcoal in a 90% solution of ethyl alcohol and recrystallized from hot 90% alcohol.

Steenbock's ration

The above ration was prepared by mixing 76% ground yel-

low corn, 16% linseed oil meal, 5% crude casein, 2% alfalfa
meal, 0.5% sodium chloride, and 0.5% calcium carbonate.

Analytical Methods

Copper in milk

A colorimetric method for quantitative copper analysis
in biological materials was evolved from a published proced-
ure by Callan and Henderson (9) which described the use of
sodium diethyldithiocarbamate as a delicate reagent for cop-
per. This reagent had been used to determine quantitatively
the copper content of dyestuffs, acids, and alkalies by first
filtering off the iron as ferric hydroxide from an ammoniacal
solution, and then adding the sodium diethyldithiocarbamate
reagent. A delicate golden brown color which could be detec-
ted with one part of copper in fifty million parts of solu-
tion resulted. The color deepened in proportion to the cop-
per present, so that quantitative determinations were made
by comparing the color given by the unknown solution against
one of known copper content. Nessler tubes were employed
for matching the colors developed.

Fifty cubic centimeters of milk in a Sillimanite porce-
lain casserole were evaporated to dryness on a steam plate.
The residue was then burned to a char over a Bunsen burner
and subsequently reduced to a white ash in an electric muffle

at about 565° C. It required approximately six hours to completely ash the sample at this low temperature. The ash was dissolved in 5 cc. of concentrated hydrochloric acid and diluted to twice the volume with copper-free water. It was next boiled for two minutes, diluted to about 50 cc., and allowed to cool. Concentrated ammonium hydroxide was added until complete precipitation of iron resulted. Filtration through Schleicher and Schüll no. 589 white ribbon filter paper removed a heavy white precipitate which included any iron present. The precipitate was redissolved in a hot 5% hydrochloric acid solution and allowed to cool, after which concentrated ammonium hydroxide was added to reprecipitate. The precipitates were washed well in both cases. The filtrates from the first and second precipitations were combined, evaporated to 50 cc., and poured into a 100 cc. Nessler tube. To the unknown solutions in the Nessler tube, were added 1 cc. of concentrated ammonium hydroxide, 10 cc. of a 0.1% aqueous solution of sodium diethyldithiocarbamate, and the volume made up to 100 cc. with copper-free water. The golden color of the unknown solution was matched against a standard prepared by placing in a 100 cc. Nessler tube a suitable amount of a standard copper sulfate solution, 1 cc. of ammonium hydroxide, and 10 cc. of the same carbamate solution used for the sample. A stock copper sulfate solution was made by dissolving enough of the iron-free salt in copper-free water so

that each cc. contained 1.0 mg. of copper. Portions of the
stock solution were removed as needed and diluted with cop-
per-free water until 1 cc. of the standard solution contain-
ed 0.01 mg. of copper. Empty casseroles served as control
determinations to take care of unavoidable copper contamina-
tion.

Copper in rats

The rats were killed, their digestive tracts removed,
and the carcasses dried in Sillimanite casseroles of porce-
lain. They were then subjected to the same procedure as were
the milk samples. The acidified solution of animal ash was
made up to a 100 cc. volume and a 50 cc. aliquot taken for
subsequent copper analysis.

Iron in rats

The colorimetric method of Stugart (54) for the deter-
mination of iron in dried milk, with appropriate modifications,
was used in the analysis of rat ash for iron. By the ashing
procedure previously given in the copper analyses of both
milk and rats, a white ash was obtained. The ash of each in-
dividual was dissolved in enough hydrochloric acid to effect
complete solution after boiling. The acid solution was cooled
and made up to 100 cc. in a volumetric flask. One cubic cen-

timeter of this volume was accurately pipetted into a 250 cc. pyrex beaker, along with 5 cc. of concentrated hydrochloric acid, and boiled slowly for twenty minutes. Distilled water was added from time to time in order to avoid evaporation to dryness. After cooling, one or two drops of concentrated nitric acid were added to each beaker. Next, the contents were rinsed into eight-inch pyrex glass tubes. One cubic centimeter of hydrochloric acid and a drop or two of a 0.1 normal potassium permanganate solution were added to each tube until a faint pink color persisted for twenty seconds. Ten cubic centimeters of isoamyl alcohol and 5 cc. of a 20% solution of potassium thiocyanate were added. The tubes were then stoppered and shaken for thirty seconds, and the rose-colored isoamyl alcohol layer was siphoned off into a Bausch and Lomb colorimeter cup for reading. Standard iron solutions for comparison were made by treating a desired amount of a standard ferric chloride solution in exactly the same manner as was employed in the treatment of the sample.

Analysis of rat urine

The methods used for these analyses were taken almost entirely from the tenth edition of Practical Physiological Chemistry by Hawk and Bergeim (32). Unless otherwise stated, the page number given refers to this book. Since the procedures were intended for human samples, it was necessary to make

slight modifications in some cases to better adapt them to rat urine analysis.

Sugar. The qualitative tests by Benedict (32, p. 747) and Nylander (32, p. 748) were used for the detection of glucose in the urine.

Albumin. The method of Esbach (32, p. 856) was used for the determination of albumin.

Acetone bodies. These were analyzed for by the quantitative procedure of Van Slyke (32, p. 858).

Indican. Parker's modification of Askenstedt's method (32, p. 864) served in this analysis.

Chlorides. A quantitative method as outlined by Volhard and Arnold (32, p. 879) was employed.

Phosphorus. The uranium acetate method, (32, p. 876) afforded a rapid means of analysis.

Total sulfates. Folin's method (32, p. 869) was used for the quantitative determination of sulfates.

Etherial sulfates. The procedure of Folin, (32, p. 871) was followed in the determination of conjugated sulfates.

Total nitrogen. The urine was heated to boiling and acidified with acetic acid to coagulate the albumin present. After filtering, the total nitrogen was determined on the filtrate by the direct Nesslerization method of Koch and McMeekin (32, p. 820). The color standard contained 1.0 mg. of nitrogen.

Ammonia nitrogen. One cubic centimeter of urine was

taken for analysis by the Permutit method (32, p. 829). The color standard contained 0.8 mg. of nitrogen.

Creatinine. Folin's colorimetric method (32, p. 833) was employed for the quantitative determination of creatinine.

Creatine. Creatine was analyzed by the procedure given by Folin and Benedict (32, p. 835).

Urea nitrogen. The direct Nesslerization method of Folin and Youngburg (32, p. 825) allowed a rapid and accurate determination of urea. The color standard contained 0.6 mg. of nitrogen.

Uric acid. The direct colorimetric method of Benedict and Franke (32, p. 837) was employed in uric acid determinations. A uric acid standard was made by dissolving 0.04 gm. of uric acid and 0.5 gm. of urotropin in a little water. To this a few drops of sodium hydroxide were added, and the volume made up to 100 cc.

Amino acids. The method of Ronchese-Sörensen (39) was used in the quantitative determination of amino acids.

Spectrographic Technique

The purity of the inorganic substances used was controlled by qualitative spectrographic analysis. For this purpose a Hilger model E 1 quartz prism spectrograph was employed, and condensed spark spectrograms were made by the use of an alter-

nating current. Carbon electrodes of the lowest obtainable
copper content were used in this work. The lower electrode
was hollowed out sufficiently to contain about three or four
drops of the solution to be examined, or a portion of the
dampened salt; the shape of the upper electrode proved sat-
isfactory without alteration. Careful control of both line
voltage and amperage was observed during all exposures, in
order that comparable results might be procured. The imposed
E. M. F. measured 110 volts, while the exciting current was
two amperes. This line voltage was stepped up, by means of
a transformer, to a value of fifteen thousand volts between
electrodes. Instrument adjustments were made from time to
time in accordance with the recommendations of the depart-
ment of physical chemistry. Wratten and Wainwright panchro-
matic plates, four by ten inches in dimension, were exposed
to the emission spectrum for a period of three minutes,
which proved to be the optimum time for the development of
the ultimate copper lines. At one-minute intervals during
exposures, the solution in the electrode cup was replen-
ished.

Copper gives several lines in its spectrum, but the ul-
timate rays produce lines on the plate at wave lengths of
3273.967 and 3247.55 Angstrom units. When these lines were
not in evidence, the sample was considered copper-free enough
for biological work. A Hartman diaphragm inserted over the

slit allowed three pictures to be taken one above the other
in juxtaposition. A practice was made of first exposing the
carbon electrodes only, then these same electrodes with the
solution in question added to the lower one were exposed, and
finally the spectrum of pure copper was obtained. This is
the standard method of spectrographic analysis, and it proved
most convenient for comparing the solution in question with
both the blank carbon electrode, and the reference electrodes
of copper. As stated before, the carbon electrodes were of
the purest material obtainable, for this type of work. How-
ever, slight traces of copper in the carbon could be demon-
strated by the spark spectrum. All spectrograms, therefore,
were viewed under a microscope so that minute increases in
line density between the electrode lines and those of the e-
lectrode plus solution would not be missed.

Care of Animals

The experimental animals were weaned at thirty days of
age from mothers receiving the Steenbock ration. Groups of
ten were then placed in cages composed entirely of galvanized
iron wire. The anemia-producing diet consisted of market
milk exclusively. This was fed until the hemoglobin level
had fallen sufficiently low to denote a condition of severe
nutritional anemia. From four to six weeks of milk feeding

caused the eyes of albino rats to become a pale pink, and the feet and ears to lose their normal red coloring. These external signs were observed in selecting animals for hemoglobin tests preparatory to placing them on experiment.

When the hemoglobin level had fallen below 7.5%, and preferably to 4 or 5%, the animals were weighed, ear marked, housed in individual cages constructed solely of galvanized iron wire, and kept in a room removed from the large rat colony. Each battery of cages consisted of from five to six individual cells formed by wire partitions dividing the battery into sections. Outside dimensions of the batteries were twelve inches in width, twenty-four inches in length, and ten inches in height. Glass rods placed across pans containing shavings served to support the batteries and held them at a sufficient height to prevent coprophagy. The battery opened from the top with a door of galvanized iron wire covering the entire cage. Once every week the pans under the cages were thoroughly cleaned and fresh shavings supplied.

Small porcelain mortars two and a quarter inches in diameter were used for food containers. These vessels proved very efficient, since it was possible for an animal to reach every surface and still be unable to tip out the contents. Another advantage was the ease of keeping the dishes clean and free from copper contamination. At the beginning of the investigation, copper-free water was placed in the cages;

but this was soon discontinued because rats on a milk diet
refuse to drink water. Each morning the food dishes were re-
moved, scrubbed with a brush under a stream of tap water, and
then inverted upon a wire drying rack to drain. Immediately
after drying they were rinsed both inside and out in a stream
of copper-free water from a wash bottle.

All animals were fed milk twice daily. The morning feed-
ing included the various substances to be administered orally.
These additions were accurately measured out in graduated
pipettes, and thoroughly mixed with the milk contained in the
mortars. At this time the amount of milk offered was limit-
ed in order to insure total consumption. Milk alone was
liberally supplied at the evening feeding.

Graduated pipettes used to measure mineral supplements
were labeled with the name of the element above the gradua-
tions and never was a pipette used in more than one solution
during an experiment. All pipettes were placed in a covered
receptacle directly after use. A pasteboard box, so con-
structed that the pipettes could not touch one another,
served to hold them in an inclined position which insured
perfect drainage. Strips of absorbent cotton placed just
under the tips removed the solution and thus prevented con-
tamination of one from another.

One-liter ground glass-stoppered bottles appropriately

labeled held the mineral supplements. It was found that a
ferric chloride solution does not hydrolyze as readily if
light be excluded. Hence, the iron solutions were kept in
black painted bottles with only the bottoms left exposed in
order that one might detect precipitation.

Weekly hemoglobin determinations were recorded on the
experimental rats. Blood was secured from a point about one-
half inch from the tip of the tail, through a small incision
in the caudal vein. The animals were allowed to stand on a
table with their tails pointing toward the right hand. They
were held by the left hand in this manner: the palm of the
hand rested upon the rat's back while the fingers partially
encircled the body, which brought the tip of the little fin-
ger under the throat. This hold is not harmful to the rat,
but does prevent biting. The tail was stroked toward the tip
until a pink appearance denoted a goodly supply of blood
there. After puncturing the vein, the first drop of blood
was discarded, and a measured amount drawn up into a diluting
pipette which was then filled to the mark with a 1% solution
of hydrochloric acid. The acid hematin solution formed was
transferred to a labeled test tube and permitted to stand for
at least twenty minutes before reading. All hemoglobin de-
terminations were made with a Bausch and Lomb colorimeter, e-
quipped with a calibrated color disc, so that no standard acid

hematin solution was necessary. This instrument was designed
exclusively for the Newcomer acid hematin method of hemoglo-
bin determination, and reads directly in per cent of hemoglo-
bin.

At the conclusion of an experiment, the animals employ-
ed were in most cases killed with chloroform. However, it
was found that some of the larger ones could be utilized a-
gain for intraperitoneal injections, since this treatment is
not tolerated as well by smaller rats. Injections were made
with a 25 gauge chromium plated hypodermic needle attached to
a glass syringe having a capacity of two cubic centimeters.
The animals were held in the palm of the left hand with the
ventral side uppermost, and the needle was passed just through
the abdominal wall when the measured dosage was discharged
into the peritoneal cavity. It was observed that rats were
more sensitive to injections along the median line of the ab-
domen; therefore, care was taken not to puncture this region.

For the metabolism experiments to be described, cylin-
drical cages made entirely from galvanized iron wire were
used. These cages rested about an inch below the surface of
large glass funnels supported by a wooden stand. Screens of
iron wire served to collect all feces, allowing only urine
to pass into glass receiving vessels. The tops of the cages
were removable to facilitate the transference of animals.
Five hundred cubic centimeter wide-mouthed gas bottles,

equipped with glass watering tubes and wired to the sides of
the cages, allowed a ready access to the milk at all times.
A drawing illustrating the metabolism cage will be found in
chart 1.

Presentation of Data

The experiments on hemoglobin regeneration described in
this thesis, unless otherwise stated, were performed on groups
of six rats each. Equal numbers of females and males were
employed in order to rule out differences due to sex in the
composite data. To save space in the presentation of these
data, it was decided that the amount of any material mention-
ed could be considered as that dosage which was received
daily by each individual rat in a lot. The normal hemoglo-
bin level for a rat was placed at 15%, since animals from
the Iowa State College stock colony showed this average value.

In work of this kind, it seemed most expedient to show
the performances of rats, with reference to growth and hemo-
globin content of the blood, by graphic means. Curves repre-
senting changes in body weight are portrayed by solid lines,
and the hemoglobin values are represented by broken lines.
Growth and hemoglobin values are plotted as ordinates, while
time intervals are measured on the abscissa. Data from rep-
resentative males and females respectively make up the single

curves. The averaged results include all animals on individual experiments, and are shown by means of composite curves.

Ten rats, 30 days of age, were housed in a galvanized iron wire cage in which a pan of shavings served as a floor. Market milk was fed ad. lib. in an effort to produce nutritional anemia. Chart 2 shows that the hemoglobin level remained nearly constant for twelve weeks, after which a gradual rise brought the hemoglobin to a slightly subnormal value at eighteen weeks. The cage was cleaned weekly so that regenerative agents could enter only through the food and shavings. Growth was slow during the entire period of observation.

The animals represented in charts 3 and 4 were first rendered anemic, and market milk plus 0.5 mg. of iron as ferric chloride was then fed daily as a basal ration. The experimental groups received 0.05 mg. respectively of manganese and nickel as chlorides, arsenic as arsenious oxide, and copper as copper sulfate. The control group was maintained upon the basal ration without further mineral addition. Regeneration of hemoglobin occurred in all cases, but it was noted that the maximum value was reached later by the lots receiving manganese, nickel, arsenic, and by the controls. Furthermore, the hemoglobin content did not reach the recognized normal value of 15%. Those animals which were fed copper exhibited a much more rapid hemoglobin rise and reached a normal value after four weeks. The growth curves showed

a steadier rise also in the copper-fed rats.

For the purpose of ascertaining whether or not a higher level of copper would give a greater stimulation to hematopoiesis, the experiments shown on chart 5 were performed on rats suffering with nutritional anemia. Copper as copper sulfate was administered orally in amounts of 0.05, 0.5, and 1.0 mg. daily along with low-copper milk plus 0.5 mg. of iron as ferric chloride. The 0.05 mg. level of copper required five weeks for total recovery, while the hemoglobin curves of those on 0.5 and 1.0 mg. reached the same point in slightly under two weeks. It was observed that the low-copper milk gave a slower response when 0.05 mg. of copper was fed than did the market milk in the previous experiment (chart 4). Also the 1.0 mg. level of copper did not regenerate any faster than the 0.5 mg. dosage.

When purified iron as ferric chloride was added to low-copper milk at the rate of 0.5 mg. per rat per day, the onset of nutritional anemia was not stayed. The question then arose whether experiments on the same milk with larger iron additions would produce different effects. Consequently, dosages of 1.0, 5.0, and 10.0 mg. respectively of iron as ferric chloride were tried on three lots of rats, as represented in chart 6. Normal animals were placed on experiment at weaning time in order to secure sufficient data before death occurred,

in the event that anemia developed. All of the rats failed
in hematopoiesis, but upon a study of the hemoglobin curves
it was evident that the lots receiving 5.0 and 10.0 mg. were
in a measure protected from anemia, since the slope was not
so abrupt. The growth curves tended to fall off markedly.
However, these results are in sharp contrast with those of
the control group in chart 4, where market milk was employed.

Since market milk plus 0.5 mg. of iron as ferric chloride
had given regeneration either with or without mineral supple-
mentation, whereas ferric chloride in amounts large enough
to provide 10.0 mg. of iron, when fed in low-copper milk did
not protect against anemia, it was decided to feed a greater
variety of elements along with low-copper milk plus 0.5 mg.
of iron as ferric chloride. Three lots of anemic rats were
placed on experiment and fed low-copper milk plus 0.5 mg. of
iron as ferric chloride daily. In addition to this ration,
one lot received 0.05 mg. nickel as nickelous chloride,
another 0.05 mg. of manganese as manganous chloride, and the
third 0.05 mg. of titanium as the dioxide. Chart 7 depicts
a falling of the hemoglobin level and a retarded growth rate.
At the end of the fourth week the amounts of mineral supple-
mentation, exclusive of iron, were doubled to insure a suffi-
ciency. The increased dosage had no effect, and many of the
animals succumbed to anemia before the eighth week of the test
was reached.

Vanadium as the pentoxide, arsenic as arsenious oxide, and germanium as the dioxide were fed in low-copper milk along with 0.5 mg. of iron as ferric chloride. The anemic animals consumed the single elements at the rate of 0.05 mg. until the fourth week of the experiment; then the amount was increased to 0.1 mg. Chart 8 pictures the ineffectiveness of the elements fed to protect rats against nutritional anemia. There was a slow growth coupled with a decrease in the hemoglobin content of the blood. Both of these are typical in cases of anemia. This condition was not improved in three other lots fed the same basal diet; namely, low-copper milk plus 0.5 mg. of iron as ferric chloride daily, and supplemented with the salts of the elements zinc, chromium, and tin respectively. These three elements were administered as zinc chloride, chromium chloride, and stannous sulfate in amounts representing 0.05 mg. of the single element. After four weeks the level was raised to 0.1 mg. Both hemoglobin and growth curves are shown in chart 9.

Other elements to be tested for hematopoietic potency were fed to anemic rats along with low-copper milk plus 0.5 mg. of iron as ferric chloride. Mercury was administered as mercurous nitrate, cobalt as cobaltous chloride, and copper as copper sulfate. The dosage employed was 0.05 mg. of the separate elements. Chart 10 shows a sharp contrast of results in the growth and hemoglobin regeneration of the rats receiv-

ing copper as compared with those animals fed either cobalt
or mercury; even though the original dosage of these elements
was doubled at the end of the first four weeks, while the
copper feeding was not increased. Individuality was observed
in the copper-fed animals, as the single male hemoglobin
curve demonstrates a return to a normal hemoglobin level one
week previous to that of the group average.

Silver as silver nitrate and gold as auric chloride
were administered orally to animals suffering from nutrition-
al anemia. The elements were fed at levels of 0.1 mg. in a
basal ration consisting of low-copper milk plus 0.5 mg. of
iron as ferric chloride. A third lot was taken directly
from the mothers at 30 days of age and fed the basal diet un-
supplemented. This group is referred to as a control in
chart 11, where the hematopoietic and growth-promoting values
of the elements mentioned are shown. All of the animals be-
came severely anemic and failed to grow normally.

Chart 12 shows that nutritional anemia did not develop
more rapidly if large amounts of zinc or magnesium were fed
at levels of 1.0 mg. along with a low-copper milk diet, to
which 0.5 mg. of iron as ferric chloride had been added.
Both zinc and magnesium were administered in the form of
their chlorides. The control group on the basal ration, con-
sisting of 0.5 mg. of iron as ferric chloride plus low-cop-
per milk, showed the same slope in their hemoglobin curves

as the animals receiving either zinc or magnesium chloride
in the basal ration. A review of charts 7 to 12 inclusive
reveals the fact that of the various elements added to a low-
copper milk and iron ration, copper alone stimulated hemato-
poiesis.

During the onset of severe nutritional anemia, it was
noticed that animals lost considerable weight. This condi-
tion was thought to be primarily due to a loss of appetite
coupled with poor intestinal absorption. If the latter con-
dition existed, then oral dosages of elements would be largely
excreted instead of reaching the blood stream. Hence, it was
thought prudent to administer the small amounts of minerals
by intraperitoneal injection so that they could be taken up
by the subserous lymphatics within the peritoneum and thus
subsequently reach the blood stream. Daily injections of the
desired mineral dosages were tried at first, but since this
caused discomfort to the animals it was deemed advisable to
inject them every other day with twice the daily allowance.
Low-copper milk plus 0.5 mg. of iron as ferric chloride was
fed as a basal ration to all lots.

Nickel and zinc as chlorides and germanium as the diox-
ide were injected intraperitoneally into three different lots
of rats, maintained on a low-copper milk plus iron diet, and
previously rendered anemic by milk feeding. The daily levels

were 0.05 mg. of the elements for the first four weeks, after which the dose was increased to 0.1 mg. The results shown in chart 13 do not support the use of these minerals as hemotinics. None of the elements exhibited toxic properties. Growth was noticeably impaired in the experiment using germanium. Other animals, which were previously made anemic, are represented in chart 14. They showed no continued hemoglobin regeneration when injected intraperitoneally first with 0.05 mg., and a month later with 0.1 mg. of manganese as manganous chloride, while they were fed low-copper milk plus 0.5 mg. of iron as ferric chloride. Vanadium as the pentoxide and arsenic as arsenious oxide, when used to replace manganese, gave comparable results. Arsenic gave a transient relief, but although the injected dosage was raised to 0.2 mg. many of the rats died of anemia.

Three groups of rats were selected at weaning time and were immediately placed on experiment. It can be noticed in chart 15 that the initial hemoglobin was about 15% on the average. They were fed low-copper milk and received intraperitoneally 0.05 mg. of titanium, selenium, and mercury respectively. The compounds of the elements used were as follows: titanium dioxide, selenic acid, and mercurous nitrate. Since anemia progressed from the beginning of the experiment, the levels of the injected elements were raised to 0.1 mg. However, this treatment did not curb the severity of the on-

coming anemia. Growth continued for a time, but finally the curve reached a plateau and then took a downward trend. Rubidium and chromium as their chlorides, when substituted for titanium and selenium in different lots of rats, showed the same general results, as evidenced in chart 16. But when copper as copper sulfate was injected intraperitoneally into anemic rats on a low-copper milk and iron diet, at a level of 0.005 mg. of copper, hemoglobin building went on at a rapid rate. It was interesting to note that nickel, zinc, germanium, manganese, vanadium, arsenic, titanium, selenium, mercury, rubidium, and chromium failed to prevent the onset of nutritional anemia when dosages of as high as 0.1 mg. were injected intraperitoneally; whereas copper at a level of 0.005 mg. or one-twentieth of the above-mentioned dose, cured severe nutritional anemia within a period of three weeks.

Milk has been thought of in the past as a perfect food, supplying all of the dietary essentials. But it has recently been demonstrated that adequate amounts of both copper and iron are lacking, and nutritional anemia can be produced on a milk diet, due to this deficiency. Also casein, the major protein in milk, had been listed as a protein which was complete in its amino acid content from the nutritional standpoint. If however, an animal suffering from nutritional anemia needed an excess of certain amino acids to assist in hemoglobin formation, then an increase of that amino acid in

the diet should be marked by an increased hemoglobin content in the blood.

In order to investigate a possible amino acid deficiency in milk, six lots of rats were fed a low-copper milk diet until their hemoglobin values had reached a level far below the normal. They were then given low-copper milk plus 0.5 mg. of iron as ferric chloride. One group was allowed to remain upon the milk-iron ration and served as a control. To the other five lots, receiving the same milk-iron diet, were added respectively 100 mg. daily of the following amino acids: tyrosine, tryptophane, arginine, glutamic acid, and aspartic acid. Since these amino acids were put into solution by the addition of hydrochloric acid, they could be considered as existing in the form of the amine hydrochlorides. Results in chart 17 do not reveal a regenerative property in the amino acids tested. All of the rats exhibited a poor nutritional state with diarrhea constantly present. Glutamic acid produced a severe form of diarrhea and a marked inanition. A comparison of the growth curves of the control with those of the rats which had received amino acids showed that the latter were stunted in growth.

It has been shown in charts 5 and 10 that 0.05 mg. of copper as copper sulfate, when added to a low-copper milk and iron ration, gives excellent hemoglobin regeneration. Chart

16 reveals similar results when the copper as copper sulfate
was injected intraperitoneally at a 0.005 mg. level, if low-
copper milk together with 0.5 mg. of iron as ferric chloride
was fed. The question then arose as to what amount of copper
would satisfy a rat's requirement for this element. Anemic
animals offered an excellent opportunity for this quantita-
tive study, for a constant quantity of iron could be fed and
varying amounts of a soluble copper salt could be injected.
The trend of the hemoglobin curves should denote any insuf-
ficiency in copper administration. The criterion for the
optimum copper dosage was taken as the smallest amount of
copper as copper sulfate which would build the hemoglobin
content of an anemic rat's blood up to 15%, regardless of
the length of time required.

In the following experiments, dealing with intraperiton-
eally injected copper, all of the rats were made anemic by
milk feeding prior to beginning the injections. Low-copper
milk was fed ad. lib. along with 0.5 mg. of iron as ferric
chloride per rat per day. Intraperitoneal injections of
copper as copper sulfate were made every other day, employ-
ing twice the dose which will be referred to as the daily
intake. Chart 18 proves that 0.0005 mg. of copper was not
enough to supply the animals with the necessary amount of
hemoglobin. When the former level was doubled to give 0.001

mg. there was a slow rise in the hemoglobin curve, but a plateau showed that complete recovery was doubtful, since the average maximum reached was 8.4%. A slightly higher level (chart 19) caused the peak of the composite hemoglobin curve to reach nearly 9.0%, while the administration of 0.00125 mg. of copper brought the average hemoglobin level to 11.0%. Charts 20 and 21 show a fairly rapid rise in the first five weeks, due to 0.0015 mg. of copper injected, and it was thought that this amount was the optimum. However, hematopoiesis slowed down, and after twenty-six weeks the hemoglobin value was just 12.5%. Then the injected dosage of this same lot of rats was raised to 0.002 mg. of copper, which resulted in the attainment of a normal hemoglobin titer within six weeks. When 0.00175 mg. of copper was administered (chart 22), an average hemoglobin level of 12.0% was reached in about three weeks, whereas it required almost eleven weeks to attain this same value on the 0.0015 mg. level. Regeneration was not complete on 0.00175 mg. of copper, but an increase of 0.00025 mg. successfully cured the anemic condition. The next experiment (chart 23) proved that complete hemoglobin regeneration could be accomplished within six weeks upon the intraperitoneal injection of 0.002 mg. of copper as copper sulfate daily. A higher injected dosage of copper, namely, 0.005 mg., caused the hemoglobin content of the blood to reach 15% in about three weeks.

Pure iron as ferric chloride, when fed alone to anemic rats receiving a low-copper milk diet, did not produce curative effects even at extremely high dosages (chart 6). It might be inferred from these data that in nutritional anemia the iron is not assimilated properly if administered orally. Therefore, a series of experiments was designed to test the availability of iron for hemoglobin building when it was injected alone intraperitoneally. Pure ferric chloride was injected into the peritoneal cavities of anemic rats fed a low-copper milk ration. Larger animals were employed in these experiments than had been used on previous trials for it had been found in preliminary work that ferric chloride, due to its acidity, caused necrosis and a sloughing off of hair and skin at the point of injection. Post mortem examinations performed on the iron-injected rats showed a general inflammation of the intestines so that the blood vessels were plainly visible. Adhesions of the intestines to the peritoneum, dark red splotches on the liver, and enlarged spleens were other abnormalities noted. The above symptoms were distinctly evident when 1.5 mg. of iron as ferric chloride in aqueous solution was injected intraperitoneally.

The injection of 3.0 mg. of the iron every other day, which would give a daily intake of 1.5 mg., did not produce such an outstanding necrotic condition, although the rats lost weight and within five weeks many died. Therefore, a

practice of injecting twice the iron dosage, as shown in
charts 24 and 25, every other day was followed. In chart 24
it was clearly shown that 1.5 mg. of iron as ferric chloride
will regenerate hemoglobin up to about 11.5% on an average,
and there have been some animals which reached levels as
high as 14.5%. However, all rats were in a poor physical
condition, as the growth curves denoted a loss in weight.
When 1.0 mg. of iron as an aqueous solution of ferric chlor-
ide was injected, the composite hemoglobin curves rose to
nearly 12.0%. These animals were not in such poor physical
condition as were those receiving the 1.5 mg. level. At the
end of about four weeks the animals started to become anemic,
and many succumbed to the disease. Ferric chloride dissolved
in glycerol did not possess such acid properties as did the
aqueous solution. This method of injecting ferric chloride
was therefore adopted because it was possible to maintain the
animals for longer periods of time. A 0.5 mg. level of iron
as ferric chloride in glycerol was injected with beneficial
results for about eight weeks, after which nutritional
anemia developed, as shown by the drooping hemoglobin curves
(chart 24). However, it was possible to keep the animals
alive for longer periods of time than in the cases of higher
iron administration.

Chart 25 discloses the fact that some hematopoietic pro-

perties are present in aqueous solutions of ferric citrate, when injected intraperitoneally, and that it is possible to reach average hemoglobin levels as high as 12.0%. This rise was transitory in all cases and the maximum point in the hemoglobin curves was reached in five weeks when either 0.5 of 0.2 mg. of iron as ferric citrate was injected. The 0.1 mg. dosage gave a slight response which lasted only a short length of time. There were no outward indications of lesions in any of the rats receiving the various levels of iron as ferric citrate intraperitoneally. A ferric hydroxide suspension was injected intraperitoneally into an anemic lot of rats in order to test the purity of the iron, but no hemoglobin synthesis took place when the level of iron was administered at 0.5 mg.

The results represented in chart 26 demonstrate the ability of a weak solution of hydrochloric acid, injected intraperitoneally, to cause hemoglobin regeneration in anemic rats. Three lots of rats were used after first being rendered anemic by milk feeding. In the first experiment, 0.5 mg. of iron as ferric chloride was added to a low-copper milk ration, and 1 cc. of a 0.033 mormal hydrochloric acid solution was injected every other day. The progression of nutritional anemia was checked somewhat, but the animals were not cured. To another lot, fed low-copper milk plus 0.5 mg.

of iron as ferric hydroxide, 1 cc. of 0.092 normal hydro-

chloric acid was given by daily injection into each rat. An

improvement resulted with the average hemoglobin value reach-

ing 8.5% within one month. The rise was transient, however,

and the animals lapsed almost into their initial condition

after another four weeks. Lesions appeared at the point of

injection and the animals declined in body weight. When 1.0

mg. of iron as ferric chloride was fed in low-copper milk to

the third lot, and at the same time 1 cc. of 0.092 normal

hydrochloric acid was injected every other day, no necrosis

of the skin appeared. The hemoglobin rise was not as rapid

as in the former lot but was more enduring. A hemoglobin

level of 8.0% was reached on the composite curve. Growth

curves did not show a marked rise, but the rats appeared to

be in a good state of nutrition.

Nutritional anemia was cured in rats receiving 0.5 mg.

of iron as ferric chloride in low-copper milk, if 0.002 mg.

of copper as copper sulfate were injected intraperitoneally.

Charts 21, 22, and 23 are explanatory of the several lots of

animals tested. Therefore 0.002 mg. of copper was considered

to be the animal requirement when 0.5 mg. of iron as ferric

chloride was fed. In the following experiments, the copper

injections were held constant and varying amounts of ferric

iron, both as the chloride and the colloidal hydroxide, to-

gether with the copper solution, were injected intraperiton-
eally into rats fed low-copper milk. All iron and copper in-
jections were administered every other day in levels of twice
the amounts stated, in order to save the animals the discom-
fort of daily handling. Chart 27 shows that 0.002 mg. of
copper as copper sulfate and 0.05 mg. of iron as ferric chlor-
ide, when injected intraperitoneally into anemic rats, did
stimulate hematopoiesis for about nine weeks; but the iron
level was too low to permit a normal hemoglobin value to be
reached. In the same chart (27) the former experiment was
repeated, with the iron injection increased to 0.1 mg. of
iron as the colloidal hydroxide, while 0.002 mg. of copper
as copper sulfate accompanied the iron solution into the
peritoneal cavity. Inside of twelve weeks, the normal ave-
rage hemoglobin value of 15% was reached by the animals.

Ferric chloride, if injected intraperitoneally at
levels as low as 0.1 mg. of iron, caused lesions at the
point of injection, and also adhesions of the intestines
to the peritoneum. Therefore, the animals did not grow
normally. However, hematopoiesis had been demonstrated
with injected ferric chloride, even though the rats exhi-
bited retarded growth (chart 24). In chart 27 it has been
proven that complete regeneration occurred when 0.002 mg.
of copper as copper sulfate plus 0.1 mg. of iron as the

colloidal hydroxide were injected intraperitoneally. Chart
28 shows that 0.002 mg. of copper as copper sulfate plus 0.1
mg. of iron as ferric chloride injected intraperitoneally
did not cure nutritional anemia in eleven weeks. But when
the iron as ferric chloride was administered intraperiton-
eally at a 0.15 mg. level together with 0.002 mg. of copper
as copper sulfate to these same animals, they were cured of
anemia. A comparison of charts 28 and 27 shows a better
physical condition in the latter than in the former, if
growth curves are used as a criterion.

Neither intraperitoneal nor oral administrations of
iron as ferric hydroxide at the rate of 0.5 mg. of iron
daily served to build hemoglobin in anemic rats on a low-
copper milk diet. However, if 0.05 mg. of copper as copper
sulfate were fed along with 0.5 mg. of iron as a ferric hy-
droxide suspension (chart 29), the results were positive in
curing anemia. It was also demonstrated that 0.5 mg. of
iron in a ferric hydroxide suspension could be injected
intraperitoneally daily without harm to the animals, and when
low-copper milk plus 0.05 mg. of copper as copper sulfate were
fed in conjunction a normal hemoglobin level resulted. (Chart
29). Complete recovery, obtained in the latter case where
iron was injected, required about ten weeks; while the orally
administered iron took approximately five weeks to give the
same results.

Charts 30 and 31 express results obtained when insolu-
ble copper compounds, as sulfides or hydroxides, were injec-
ted every other day into anemic rats maintained on a basal
diet of low-copper milk plus 0.5 mg. of iron as ferric chlor-
ide. Twice the dosages mentioned were injected intraperiton-
eally every other day in order to minimize the handling of
the animals. Both the copper sulfide and copper hydroxide
were in a finely divided state, and a thorough mixing was
practiced before filling the syringe. Data shown in chart
30 point out the fact that 0.02 mg. of copper as copper sul-
fide was required to cause animals to reach a hemoglobin
value of 15%. Copper hydroxide proved to be a better hemo-
tinic (chart 31), since 0.005 mg. of copper as the hydrox-
ide resulted in complete recovery within fourteen weeks. A
sharp contrast was evident between the hematopoietic values
of the soluble copper sulfate (chart 23) and either the
copper sulfide or the hydroxide. The former caused complete
regeneration in six weeks with a 0.002 mg. injection of cop-
per, while the latter required 0.02 and 0.005 mg. of copper
respectively, injected over a period of fourteen weeks.

Monovalent copper in the insoluble form of either cup-
rous oxide or cuprous iodide built hemoglobin in anemic rats.
Two lots of animals were fed a low-copper milk ration with
0.5 mg. of iron as ferric chloride. One lot was injected

intraperitoneally every other day with 0.02 mg. of copper as
cuprous oxide, while the other group received the same dosage
of cuprous iodide similarly injected. This procedure proved
harmless to the animals and allowed an average daily adminis-
tration of 0.01 mg. of copper per rat. From the results of
chart 32 it appears that there is no difference in the he-
matopoietic potency of the copper compounds. The insolubi-
lity of cuprous oxide and cuprous iodide made it necessary
to shake the solution well before each injection.

It has been demonstrated that iron can be utilized in
hemoglobin building when in the form of a suspension (charts
23 and 29), or as a colloidal hydroxide (chart 27). Like-
wise, copper was available for hematopoiesis in several in-
soluble compounds (charts 30, 31, and 32). It then remained
to show whether or not colloidal solutions of both iron and
copper as the hydroxide could be utilized if administered
simultaneously. The first step was to feed the hydroxides to
anemic rats on a low-copper milk diet. Iron as colloidal fer-
ric hydroxide was fed at a level of 0.5 mg. of iron per rat
per day, and copper as colloidal cupric hydroxide was fed in
the amount of 0.05 mg. of copper per animal daily. Chart 33
illustrates the performance of rats on this experiment, where
it was found that fourteen weeks were required to completely
cure the anemia. Intraperitoneal injections of the above
compounds into anemic rats gave slower regeneration, as demon-

strated in chart 34, but after eighteen weeks the animals had regained a normal hemoglobin content. Injections were performed every other day on the rats, using twice the daily dosages mentioned. Copper as colloidal cupric hydroxide at the level of 0.002 mg. of copper and 0.1 mg. of iron as the colloidal ferric hydroxide were simultaneously injected through the abdominal wall.

Animals fed exclusively upon a milk diet developed anemia after four to six weeks. The hemoglobin fell readily to a level of 5 or 6%, after which the decline was much slower until the rats finally became too weak to eat, and death resulted. Hemoglobin values as low as 2% have been attained in some instances, but as a rule the animals died before reaching this low level. Some rats showed an ability to live for as long as twelve weeks on milk alone and were analyzed for their copper and iron content in order to determine whether or not their body stores of these elements had been depleted in an effort to synthesize sufficient hemoglobin to prolong life. These data also show the lowest copper and iron values obtainable in rats fed milk which contains only the natural copper content. The analytical data are shown in table I.

Another lot of rats were first rendered anemic by feeding low-copper milk and were continued on the same diet for eight weeks with daily intraperitoneal injections equivalent

to 0.5 mg. of purified iron as ferric chloride dissolved in copper-free glycerol. At the end of this time they were analyzed for copper and iron. The results are tabulated in table II.

A third group of anemic rats were fed a low-copper milk ration plus 0.5 mg. of purified iron as ferric chloride. Intraperitoneal injections of 0.092 normal hydrochloric acid were administered every other day. After eight weeks on experiment, the animals were analyzed for copper and iron. The findings are shown in table III.

A fourth lot of animals, which had been previously made anemic by milk feeding, were fed 0.5 mg. of purified iron as ferric chloride, and were injected intraperitoneally with 0.005 mg. of copper as copper sulfate. They were continued on experiment for eight weeks and were then analyzed for copper and iron. Table IV shows the results obtained from this experiment. This level of copper and iron gives a hemoglobin response similar to a ration made up of natural foodstuffs. This lot was run as a control group to give normal copper and iron values for animals of the same age as those shown in tables II and III.

Twelve unbred female rats were selected from a group which was fed the Steenbock stock ration. They were equally divided into two lots of six animals. The one lot was fed

market milk ad. lib. together with 0.5 mg. of iron as ferric
chloride and 0.05 mg. of copper as copper sulfate, and the
second lot was fed market milk alone. Hemoglobin determina-
tions were run periodically until the group maintained on
milk alone developed nutritional anemia to such a degree
that it was deemed advisable to start the metabolism experi-
ment. Two galvanized iron wire metabolism cages were employ-
ed (chart 1). The normal rats were continued on the market
milk, copper, and iron diet, while the anemic ones received
market milk alone. The urine was collected under toluene
during twelve hour periods of time, and was analyzed immedi-
ately by approved methods mentioned in the section of this
thesis devoted to analytical methods. Triplicate samples
were run in all cases, and these were repeated in order that
a fair average might be obtained. Results of the analyses
are shown in table V.

Table I

Analytical Data for Copper and Iron Content of Rats
Fed a Milk Ration for Twelve Weeks
(Ave. Hemoglobin = 3.7%)

Rat	Wet tissue minus intestinal tract (gms.)	Total iron (mg.)	Total copper (mg.)	Mg. iron per 100 gm. wet tissue	Mg. copper per 100 gm. wet tissue
w♀	36	1.062	0.02	2.95	0.0555
w♀ no.1	37	1.042	0.024	2.81	0.065
w♀ no.2	43	1.305	0.020	3.03	0.0465
w♀ no.3	42	1.137	0.016	2.70	0.0381
w♀ no.4	40	1.000	0.022	2.50	0.055
w♂	31	1.205	0.032	3.90	0.103
w♂ no.1	35	0.970	0.018	2.77	0.0514
w♂ no.2	43	1.407	0.024	3.27	0.056
w♂ no.3	35	1.400	0.012	4.00	0.0342
w♂ no.4	36	1.125	0.016	3.12	0.044
ave.	37.8	1.165	0.0204	3.10	0.0487

Table II

Analytical Data for Copper and Iron Content of Rats
Fed Milk and Injected with 0.5 mg. of Iron as
\ Ferric Chloride in Glycerol for Eight Weeks
(Max. Hemoglobin = 11.2%)

Rat	Wet tissue minus intestinal tract (gms.)	Total iron (mg.)	Total copper (mg.)	Mg. iron per 100 gm. wet tissue	Mg. copper per 100 gm. wet tissue
wo̅ no.1	92	9.0	0.026	9.75	0.0282
wo̅	68	13.8	0.026	20.10	0.0379
wo̅	83	9.5	0.028	11.47	0.0338
go̅	102	12.0	0.025	11.80	0.0246
wo̅ no.1	95	11.8	0.026	12.40	0.0273
wo̅	97	17.4	0.028	17.95	0.0289
w♀	98	14.8	0.026	15.20	0.0267
w♀ no.1	60	12.4	0.028	20.65	0.0536
ave.	79.37	12.58	0.0266	14.90	0.0326

Table III

Analytical Data for Copper and Iron Content of Rats Fed
Milk + 0.5 mg. Iron as Ferric Chloride and Injected
with 0.092 N Hydrochloric Acid for Eight Weeks
(Max. Hemoglobin = 8.0%)

Rat	Wet tissue minus intestinal tract (gms.)	Total iron (mg.)	Total copper (mg.)	Mg. iron per 100 gm. wet tissue	Mg. copper per 100 gm. wet tissue
w♀	60	3.13	0.05	5.22	0.0832
gs♂	141	4.40	0.07	3.12	0.0497
gs♂ no.1	174	4.75	0.10	2.73	0.0575
w♂	108	3.12	0.04	2.89	0.0370
w♀ no.1	112	3.20	0.04	2.85	0.0357
w♀ no.2	78	3.85	0.056	4.93	0.0700
w♂	78	3.40	0.06	4.35	0.0770
w♂	91	5.88	0.036	6.45	0.0396
gs♀	95	4.85	0.06	5.10	0.0630
ave.	104.1	4.06	0.0457	4.18	0.0569

Table IV

Analytical Data for Copper and Iron Content of Rats
Fed Milk + 0.5 mg. Iron as Ferric Chloride and
Injected with 0.005 mg. Copper as Copper
Sulfate for Eight Weeks
(Max. Hemoglobin = 15.5%)

Rat	Wet tissue minus in-testinal tract (gms.)	Total iron (mg.)	Total copper (mg.)	Mg. iron per 100 gm. wet tissue	Mg. copper per 100 gm. wet tissue
w♂ no.1	123	5.839	0.155	4.75	0.126
w♂	131	6.138	0.159	4.67	0.121
w♂ no.2	126	5.590	0.144	4.43	0.114
w♀ no.1	111	5.644	0.148	5.07	0.133
w♀ no.2	90	4.372	0.140	4.85	0.155
w♀ no.3	93	5.537	0.131	5.95	0.141
ave.	112	5.686	0.146	4.96	0.131

Table V

Analytical Data Showing Comparative Composition
of Urine of Normal and Anemic Rats
at Six Months of Age

	Normal	Anemic
Ave. body weight	175.7 gm.	153.6 gm.
Ave. hemoglobin	16.1 %	6.8 %
Ave. vol. urine per rat per 24 hrs.	25.05 cc.	35.52 cc.
Reactions of urine to phenolphthalein	alkaline	sl. acid
Specific gravity	1.0124	1.012

Substance	Mg. per rat per 24 hrs.		% of total nitrogen	
	Normal	Anemic	Normal	Anemic
Sugar	none	none		
Albumin	2.57	2.66		
Acetone bodies	2.549	2.41		
Indican	0.32	0.382		
Chlorides	49.344	51.84		
P_2O_5	22.59	31.99		
Total SO_3	11.74	24.05		
Etherial SO_3	0.0444	1.0854		
Total nitrogen	203.5	190.8	100.00	100.00
Urea nitrogen	158.13	163.1	77.70	85.80
NH_3 nitrogen	31.35	12.67	15.40	6.66
Creatinine nitrogen	2.155	1.52	1.059	0.80
Creatine nitrogen	1.005	0.69	0.493	0.362
Uric acid nitrogen	0.453	0.426	0.222	0.224
Amino acid nitrogen	0.379	0.372	0.186	0.195
Indican nitrogen	0.0178	0.0213	0.0087	0.0112
Nitrogen unaccounted for	10.01	11.28	4.91	5.93

DISCUSSION OF RESULTS

Chart 2 shows that it is impossible to produce anemic animals when a pan of shavings is used as a cage floor. This experiment proved that a rat may satisfy its body requirements by using certain excreted materials over and over, since anemia did not develop in a period of eighteen weeks.

Specially collected milk of low copper content (0.2 mg. of copper per liter) did not regenerate hemoglobin when fed together with purified ferric chloride to anemic rats. The animals were not improved by a further addition of titanium, manganese, nickel, vanadium, arsenic, germanium, zinc, chromium, tin, mercury, cobalt, silver, and gold to the low-copper milk and iron ration. Charts 7 to 11 inclusive show that the hemoglobin values declined steadily. Copper was the only element that could supplement iron in hemoglobin building. These results do not harmonize with those of Titus, Cave, and Hughes (62), Beard and Myers (5)(6)(7), Myers and Beard (45), Beard, Baker, and Myers (4), and Beard (3). Since these investigators employed certified milk of an unknown copper content, it was assumed that a sufficient amount of this element must have been present to cause the complete recoveries which they reported in anemic animals.

In an attempt to prove this assumption, market milk plus purified iron as ferric chloride was fed to anemic rats. Other lots on the same diet were given manganese, nickel, arsenic, and copper in addition. Charts 3 and 4 showed that market milk, which analyzed 0.35 to 0.44 mg. of copper per liter, was capable of building hemoglobin if iron alone were administered simultaneously. However, a normal hemoglobin level was not attained in any lot excepting those fed copper, which would show that there must have been other sources of copper contamination aside from milk, in the experiments of the above-mentioned investigators. Housing in defective galvanized iron wire cages, the feeding of impure ferric chloride, coprophagy, and the use of copper-contaminated water for washing cages and food dishes, would all tend to increase the copper intake.

Purified iron as ferric chloride, when fed daily to either normal or anemic rats at a level of 0.5 mg. in low-copper milk, produced a severe anemia. This fact is contrary to the findings of Cunningham (13), Mitchell and Schmidt (43), Mitchell and Vaughn (44), Mitchell and Miller (41)(42), Beard and Myers (5)(6)(7), Beard (3), Drabkin and Waggoner (17)(18), and Drabkin and Miller (15)(16), who got normal regeneration on the addition of 0.5 mg. of pure iron to a low-copper food. Certain investigators, namely, Drabkin and Waggoner (17), Myers and Beard (45), Beard,

Baker, and Myers (4), Beard (3), and Mitchell and Miller
(42), stated that 0.25 mg. of pure iron as ferric chloride
alone did not regenerate hemoglobin, because it was below the
animal requirement. But if this amount (0.25 mg.) were
slightly increased, the hemoglobin rose to a normal value.
These authors were thus led to believe that 0.5 mg. was
greatly in excess of a rat's daily need. The curves in
chart 6 show that regeneration was impossible when as high
as 10.0 mg. of pure iron as ferric chloride was fed daily.
This level is forty times the border-line dosage of 0.25 mg.
employed by other workers; and while it undoubtedly was a-
bove the daily iron requirement, it showed conclusively that
iron alone could not build hemoglobin.

Myers and Beard (45) have reported that either zinc or
magnesium in 0.5 mg. dosages, when added to a diet consist-
ing of certified milk plus iron as ferric chloride, retard
the formation of hemoglobin. If this be true, the addition
of either zinc or magnesium in 1.0 mg. dosages to a low-cop-
per milk plus iron ration should cause a more rapid develop-
ment of nutritional anemia when compared to a control lot
maintained on the same ration but without zinc or magnesium.
Chart 12 gives the results of such an experiment and proves
that neither zinc nor magnesium has an effect upon the lower-
in of the hemoglobin content of rats. Young rats at thirty

days of age were selected from the growing colony and put on
the diets without previously having been rendered anemic, in
order to rule out any abnormality which might enter in during
the time required to produce nutritional anemia.

The oral administration of various elements to anemic
rats on a low-copper milk and iron diet had failed to increase
the hemoglobin value. Other investigators, previously men-
tioned, had obtained hematopoiesis with the same elements fed
along with a low-copper and iron ration. In view of these
conflicting opinions, it seemed expedient to inject the me-
tallic salts intraperitoneally and in this way overcome any
possibility of poor absorption from the intestinal tract,
which might prevail in severe anemia. Rats were found to a-
dapt themselves very well to intraperitoneal injections,
when the solutions of the salts were not highly acid, and no
abnormal conditions were apparent. The basal ration employ-
ed consisted of low-copper milk plus 0.5 mg. of iron as fer-
ric chloride added daily. Compounds of nickel, zinc, ger-
manium, manganese, vanadium, arsenic, titanium, selenium,
mercury, rubidium, and chromium, injected intraperitoneally,
did not reduce the severity of the oncoming nutritional an-
emia. Charts 13 to 16 inclusive show that copper was the
only element tested that cured anemic rats. It was of in-
terest to note a primary stimulation in the case of arsenic.

However, it was of short duration and could not be prolonged by large increases in the amount injected.

Fontes and Thivolle (25)(26), Matsuoka and Nakao (38), Okagawa and Tatsui (46), Drabkin and Miller (15)(16), and Stucky (53) reported hemoglobin regeneration when iron in the ration was supplemented with certain amino acids. Tryptophane, histidine, arginine, tyrosine, and glutamic acid were named as hemotinics. Drabkin and Miller postulated the formation of pyrrole rings from the amino acids which would form the prosthetic group in the hemoglobin molecule. They termed it pyrrologenesis, and stated that glutamic acid formed the rings most readily, and was therefore the most regenerative of the amino acids. Milk was used as a basal ration by Drabkin and Miller, who contended that animals restricted to it alone did not receive a sufficient amount of arginine, tyrosine, and glutamic acid to permit normal hematopoiesis, but that the addition of 70.0 mg. of any of these amino acids to a milk and iron ration made a complete diet for hemoglobin regeneration. The curves in chart 17 show that tyrosine, tryptophane, arginine, glutamic acid, and aspartic acid, when fed at 100.0 mg. levels in a low-copper milk and iron diet, did not regenerate hemoglobin in anemic rats.

Iron and copper have been shown to be essential to keep up the normal hemoglobin level in the animal body. Waddell,

Steenbock, and Hart (66) reported the optimum oral dosage of iron to be 0.5 mg. and of copper to be 0.05 mg. for the rat. Mitchell and Miller (42) find the optimum oral dosage to be 0.25 mg. along with 0.1 mg. of copper. They also stated that the copper dosage could be cut down provided the iron was increased, and also that the iron could be diminished if the copper level were raised, in order to obtain optimum amounts necessary for normal hemoglobin regeneration. Since the animal body excretes a large portion of the ingested copper and iron, it was thought that intraperitoneal administration would more nearly show the absolute requirement of both elements. To accomplish this, the iron dosage was first held constant and fed as the chloride in low-copper milk, while the varying amounts of copper as copper sulfate were injected intraperitoneally. When the lowest level of copper that would bring the hemoglobin to a normal level was found, the resulting copper value was held constant and the iron was injected in varying amounts. Charts 18 to 23 inclusive, show that 0.002 mg. of copper as copper sulfate was necessary to bring the hemoglobin level of anemic rats to 15%, although 0.001 mg. of copper was sufficient to stimulate regeneration. It was of interest to note that a copper level of 0.00175 mg. sufficed to raise the hemoglobin value to 12.5%, after which no further gain was possible, although the animals appeared

in a normal, healthy condition.

Ferric chloride, if injected at a 0.1 mg. level, caused necrosis at the point of injection and adhesions of the intestines to the peritoneum, so that it was necessary to use iron in the form of the colloidal hydroxide for injection purposes. Charts 27 and 28 show that 0.002 mg. of copper injected intraperitoneally with 0.1 mg. of iron are the lowest dosages of these elements which will give complete hemoglobin regeneration. The animals showed a normal, healthy condition, and the dark-coated ones, which had faded on becoming anemic, regained their natural color. Females reproduced to give normal litters of living young. Growth approached the normal for a milk, copper, and iron diet. All of these observations tended to show that the rats were getting sufficient copper and iron to carry on a normal life.

Pure iron injected intraperitoneally, either as the chloride or citrate, stimulated hematopoiesis in anemic animals. However, regeneration was not complete, and after the fifth week they suffered a decline resulting in death due to anemia. Ferric hydroxide suspensions, either fed or injected, had no regenerative value, although they did not produce injurious effects in the rats. On the other hand, ferric chloride in aqueous solutions caused necrosis of the skin and adhesions of the intestines to the peritoneum. Inanition was prevalent, especially at the 1.5 mg. level. Ferric citrate

or a glycerol solution of ferric chloride provided a source
of iron which did not give apparent detrimental effects due
to acidity, but in none of the lots did the animals grow
normally. Charts 24 and 25 show that 0.5 mg. of purified
iron injected either as the chloride or citrate, gave a
maximum hemoglobin rise to about 12% in a period of six
weeks, indicating an identical hematopoietic action.

Castle, Heath, and Strauss (11) found that a large per-
centage of the cases of pernicious anemia in man showed a
low hydrochloric acid concentration in the stomach contents.
From the experiments described in chart 6 it was obvious
that 10.0 mg. of iron as ferric chloride given orally did
not regenerate hemoglobin, although the solution was so acid
as to coagulate the milk. Also the feeding of amino acids
in hydrochloric acid solutions did not stimulate hematopoi-
esis. These facts show that the feeding of hydrochloric acid
does not relieve nutritional anemia. However, pure hydro-
chloric acid solutions, intraperitoneally injected into ane-
mic rats receiving a diet of low-copper milk with either
ferric chloride or ferric hydroxide added, gave a rise in
the hemoglobin value. The improvement was transient (chart
26) and reached a maximum in four weeks, after which the
animals declined and died of anemia. The insoluble ferric
hydroxide gave the same improvement as the highly soluble

ferric chloride, when fed in the milk simultaneously with the acid injections.

Ferric hydroxide, administered either intraperitoneally or orally, gave normal regeneration when low-copper milk with copper added as copper sulfate was fed. This experiment proved that insoluble iron could be utilized by the animal body to build hemoglobin. A comparison of the hemoglobin curves in chart 29 with those of chart 5 reveals a more rapid regeneration when ferric chloride was substituted for ferric hydroxide in the above experiment.

Insoluble copper compounds including cupric sulfide, cupric hydroxide, cuprous oxide, and cuprous iodide, when injected intraperitoneally into anemic rats fed on low-copper milk with iron as ferric chloride added, cured nutritional anemia. Results found in charts 30 to 32 inclusive showed that copper in the form of a sulfide required four times the dosage of copper hydroxide. It seemed possible that the stability of copper sulfide might account for the difference, if the body were required to convert it to another copper compound preparatory to its utilization. Cuprous copper, either as the oxide or iodide, gave results comparable with cupric hydroxide; this shows that either the body can oxidize copper to a divalent state, or that monovalent copper can function in assisting iron to form hemoglobin.

The work of Goerner (27) showed that the colloidal forms
of copper and iron cannot be utilized for hemoglobin forma-
tion in anemic rats. He stated that crystalline salts of
either copper or manganese supplemented ferric chloride in
building hemoglobin. Results shown in charts 33 and 34 do
not coincide with the data submitted by Goerner, for when
the colloidal hydroxides of copper and iron were either fed
or injected intraperitoneally, a normal hemoglobin level was
attained. However, optimum injected amounts of copper sul-
fate and colloidal ferric hydroxide, as shown in chart 27,
required twelve weeks to bring the hemoglobin to 15%, whereas
the substitution of copper sulfate by colloidal cuprie hy-
droxide slowed down hematopoiesis, so that eighteen weeks
were necessary to produce the same results.

A comparison of the analytical data presented in table
I with that of table IV shows that animals can be made to
deplete in a measure their copper and iron stores by with-
holding these elements from the basal diet. Table II points
out the fact that intraperitoneally injected iron is stored
by the animal body, and that the copper content falls to a
low level when hemoglobin is regenerated upon a low-copper
milk diet. In table III the iron storage is not as great
when administered orally and the intraperitoneal hydro-
chloric acid injections do not lower the copper content as

much as iron administered in a similar manner. Neither was
the hemoglobin titer as high as in the case of intraperiton-
eal iron injections.

Table V shows a higher output of phosphorus, total sul-
fates, and etherial sulfates in the urine of milk-fed anemic
rats than in that of animals of a normal hemoglobin content
maintained on milk plus copper and iron. The excreted phos-
phorus comes from the food and also from tissue catabolism.
In anemia the animals tend to become emaciated with a notice-
able wasting away of the muscles, which would give rise to a
large phosphorus excretion from the metabolism of nucleic
acids. Sulfate excretions likewise arise from increased de-
composition of body proteins, and would be expected to rise
during a loss of weight in an animal. Etherial sulfates
sometimes indicate intestinal putrefaction, which gives rise
to aromatic bodies and, later, conjugated sulfates.

SUMMARY AND CONCLUSIONS

Nutritional anemia can be induced within a period of six weeks in rats housed in galvanized iron wire cages with screen floors, and fed a milk diet. Anemia does not develop in eighteen weeks if a pan of shavings is used instead of a screen floor, since the animals utilize the excreted iron and copper over and over.

Market milk cannot be used as a low-copper ration in studies on nutritional anemia, since its average copper content of 0.4 mg. per liter is high enough to give partial hematopoiesis. Special milk, collected directly into glass containers, with an average copper content of 0.2 mg. per liter furnishes a successful diet for animals used in anemia experiments.

Pure iron salts will not regenerate hemoglobin in anemic rats when fed at twenty times the optimum (0.5 mg.) oral dosage. However, if 0.5 mg. of iron as ferric chloride plus 0.05 mg. of copper as copper sulfate is added to a ration of low-copper milk, complete regeneration results inside of six weeks. Furthermore, the speed of hematopoiesis is increased by the amount of copper ingested when 0.5 mg. of iron as ferric chloride is fed, for if the copper level is increased to

0.5 mg. a normal hemoglobin value results in two weeks. One milligram of copper as copper sulfate will give the same results as 0.5 mg. of that element.

Inorganic compounds of titanium, manganese, vanadium, arsenic, germanium, zinc, chromium, tin, mercury, cobalt, silver, and gold, when fed daily at levels of either 0.05 or 0.1 mg. of the element together with 0.5 mg. of iron as ferric chloride in low-copper milk, do not act as hemotinics.

Neither zinc nor magnesium, when added at levels of either 0.5 mg. or 1.0 mg. to a basal ration of low-copper milk and iron, hastens the onset of nutritional anemia. Therefore, it is concluded that the elements zinc and magnesium do not retard hemoglobin formation.

Intraperitoneally injected inorganic compounds of nickel, zinc, germanium, manganese, vanadium, arsenic, titanium, selenium, mercury, rubidium, and chromium will not cause hemoglobin formation. Of all the elements tested either by oral or intraperitoneal administration with sufficient iron furnished orally, copper was the only element which could supplement iron in hematopoiesis. Since none of the elements which have a periodic relationship to copper can replace it as a supplement to iron in hemoglobin regeneration, it is concluded that copper occupies a unique position in this respect.

The addition of tyrosine, tryptophane, arginine, glutamic

acid, or aspartic acid to a low-copper milk and iron diet will not improve the condition of anemic rats. From these results, it is evident that milk is not lacking in amino acids essential for protection against nutritional anemia.

A daily intake of 0.002 mg. of copper as copper sulfate given intraperitoneally is the minimum amount of this element which will restore a normal condition in anemic rats maintained on a diet of low-copper milk with a daily addition of 0.5 mg. of iron as ferric chloride.

Intraperitoneal injections of iron alone, either as the chloride or citrate, will stimulate hemoglobin regeneration for a time in anemic rats fed exclusively on low-copper milk. The improvement is not permanent, and the rats die of anemia. Ferric hydroxide similarly administered does not regenerate.

Solutions of hydrochloric acid show regenerative properties when injected intraperitoneally into rats suffering with nutritional anemia, and fed on a low-copper milk and iron ration. However, the hemoglobin value declines after four weeks, and there is a lapse into the former condition.

The rat requirement of copper and iron, when both are injected intraperitoneally and simultaneously, is 0.002 mg. of copper as copper sulfate and 0.1 mg. of iron as colloidal ferric hydroxide daily. Anemic animals on a low-copper milk

diet are rendered normal in six weeks after beginning the injections. Further proof that this dosage is adequate is the fact that dark colored animals, which have faded in color due to anemia, regain the natural pigmentation, and reproduction takes place.

Ferric hydroxide, either as a suspension or in colloidal solution, is utilized as a source of iron in anemic rats when injected intraperitoneally, if small amounts of copper are administered at the same time by injection or feeding. The iron compounds may be fed, and copper given intraperitoneally or orally to cure nutritional anemia.

Cupric sulfide, cupric hydroxide, cuprous oxide, and cuprous iodide are all insoluble forms of copper and, when injected into anemic rats fed on a low-copper milk and iron diet, will cure the anemia. They are not as available as copper in the form of the soluble sulfate, since a longer time is necessary to bring back a normal condition.

Colloidal cupric hydroxide, fed along with colloidal ferric hydroxide in optimum amounts to anemic rats on a low-copper milk diet, will bring the hemoglobin value to normal. These same compounds when injected intraperitoneally completely cure nutritional anemia at daily levels of 0.002 mg. of copper and 0.1 mg. of iron. However, colloidal copper and iron solutions give a slower rate of regeneration than do

the crystalloidal solutions of the same concentrations of these elements.

The copper and iron content of rats made anemic on a low-copper milk diet is much lower than that in animals which have been fed a normal intake of the elements.

Iron is stored to a greater extent by anemic rats when injected intraperitoneally. It seems that greater absorption takes place through the peritoneal wall than through the intestinal wall.

When pure iron as ferric chloride or citrate is injected intraperitoneally into anemic rats, the hemoglobin rises but the copper content of the animal body falls. This gives rise to a hypothesis that the body utilizes the copper present to aid in the synthesis of hemoglobin. The hemoglobin falls when the stored copper has been depleted.

Quantitative analyses of the urine excreted by anemic rats on a low-copper milk diet, as compared with those fed iron and copper along with the milk, disclose a significant difference only in the amounts of phosphorus, total sulfates, and etherial sulfates. End products containing nitrogen do not vary remarkably between the two lots.

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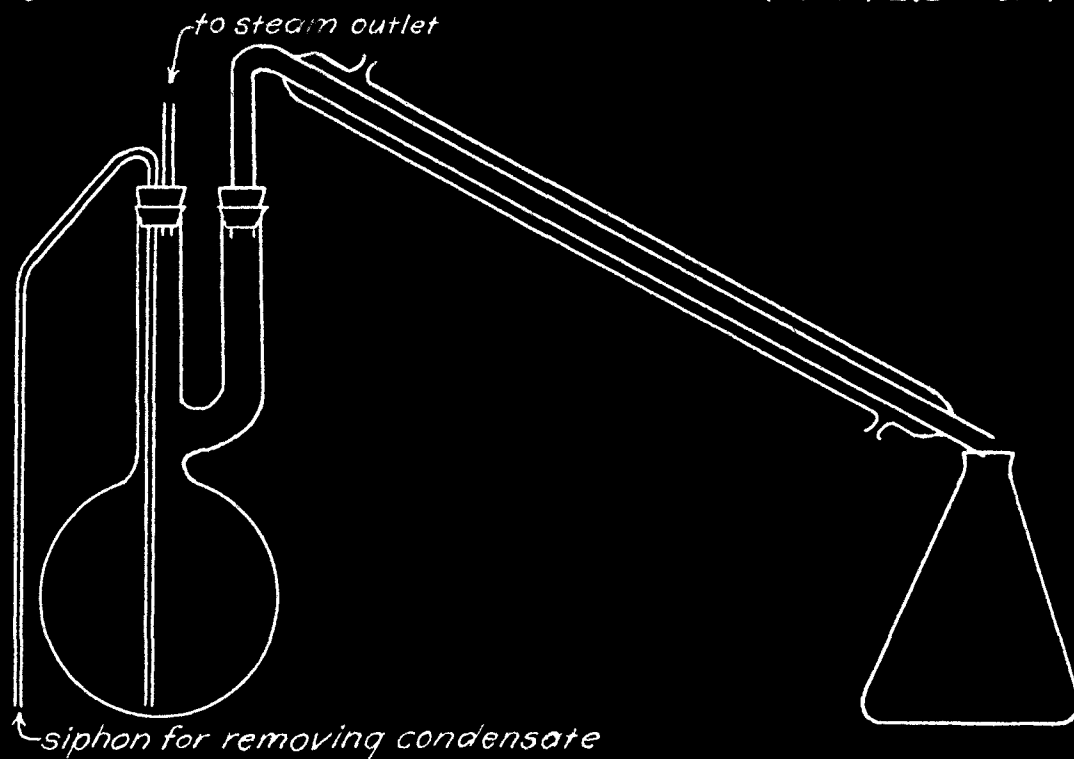
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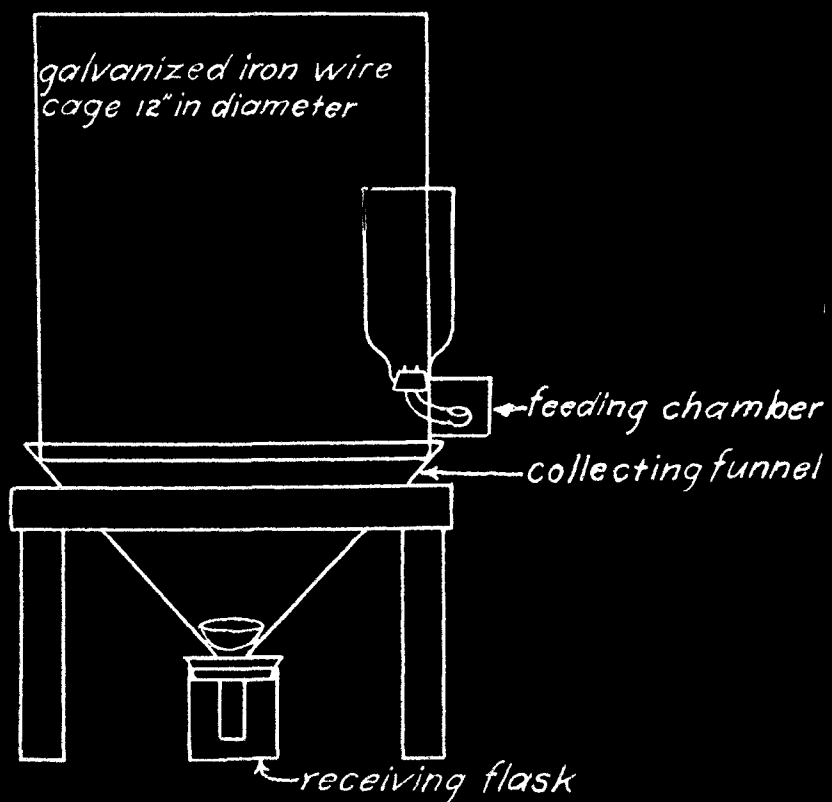
STEAM TRAP FOR COPPER-FREE WATER

to steam outlet

siphon for removing condensate

METABOLISM CAGE

galvanized iron wire
cage 12" in diameter

feeding chamber

collecting funnel

receiving flask

Chart I

ATTEMPTS TO PRODUCE ANEMIA IN RATS ON SHAVINGS

Weight in Grams ——
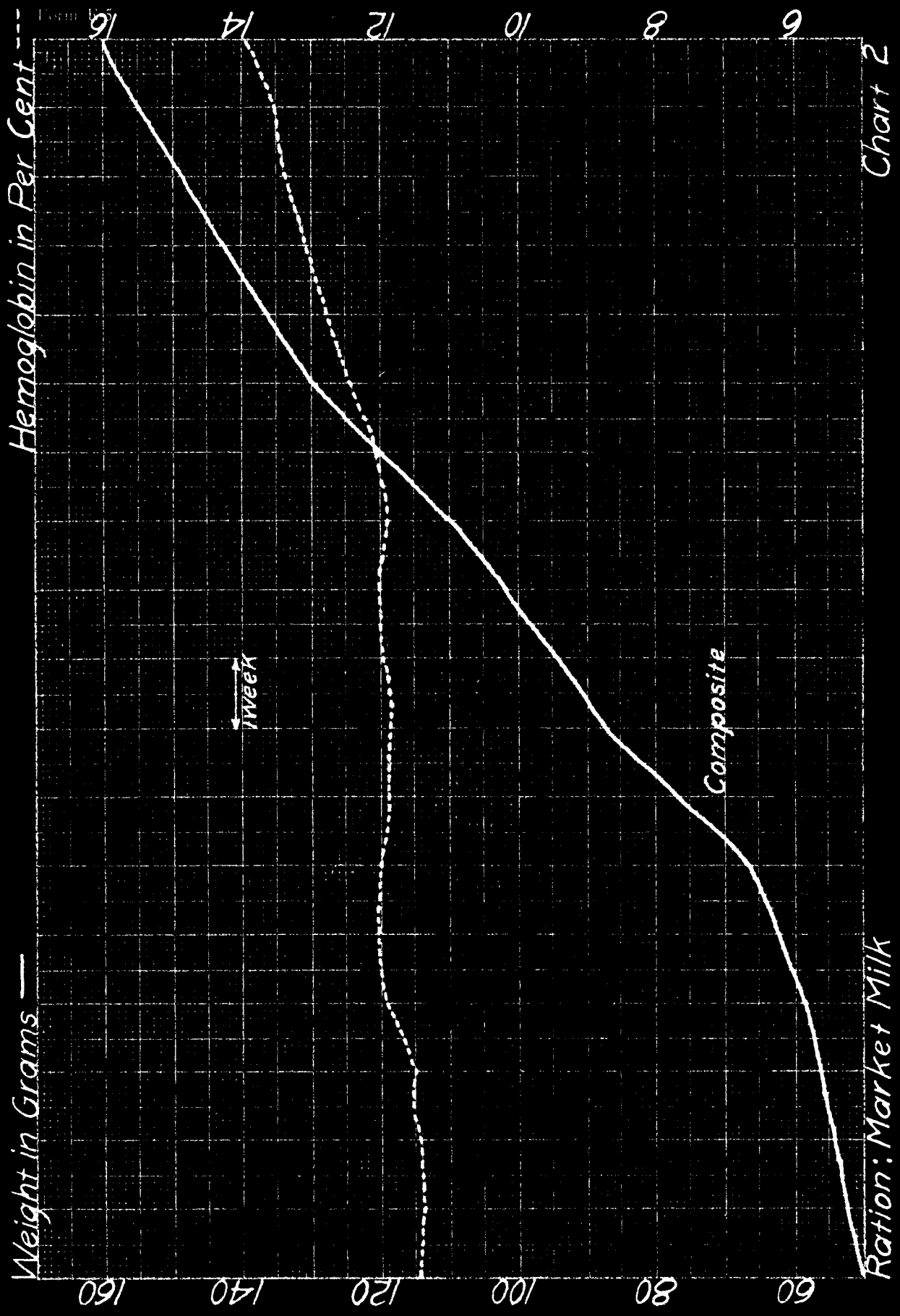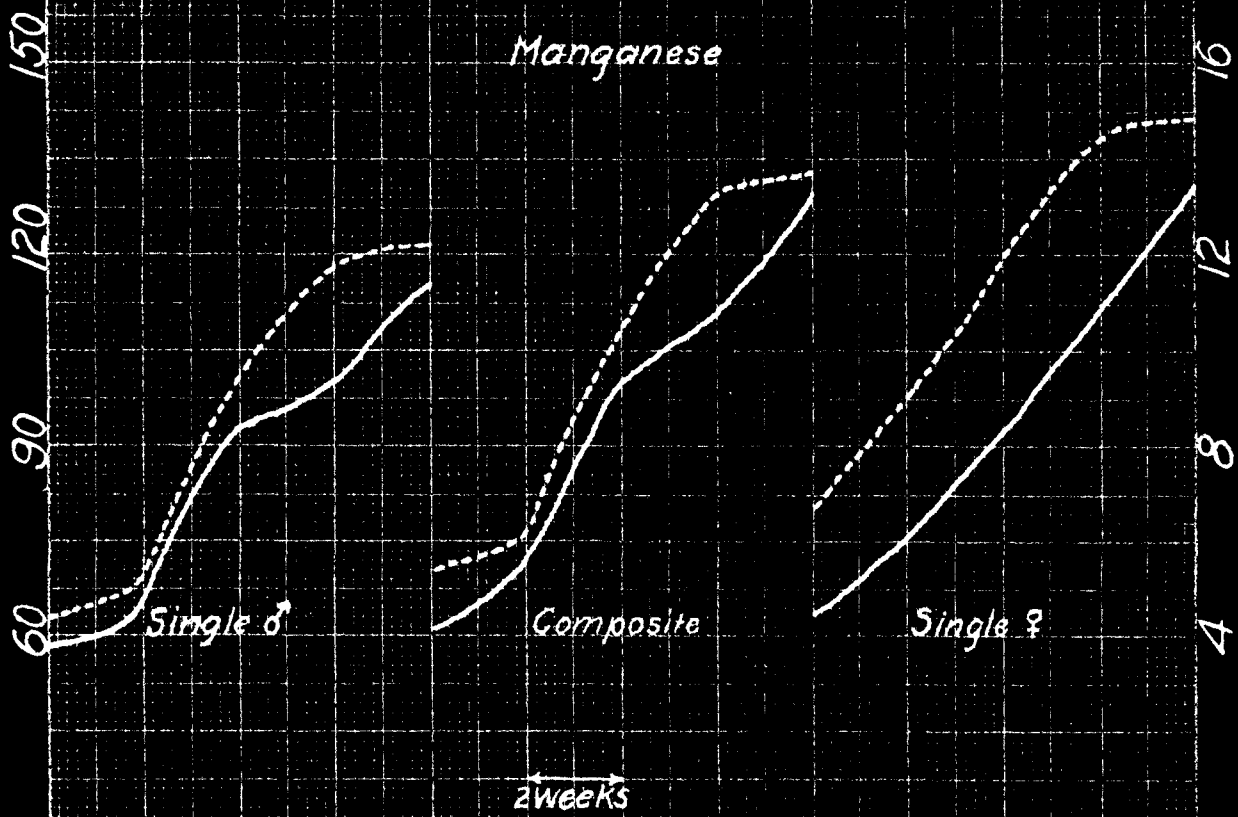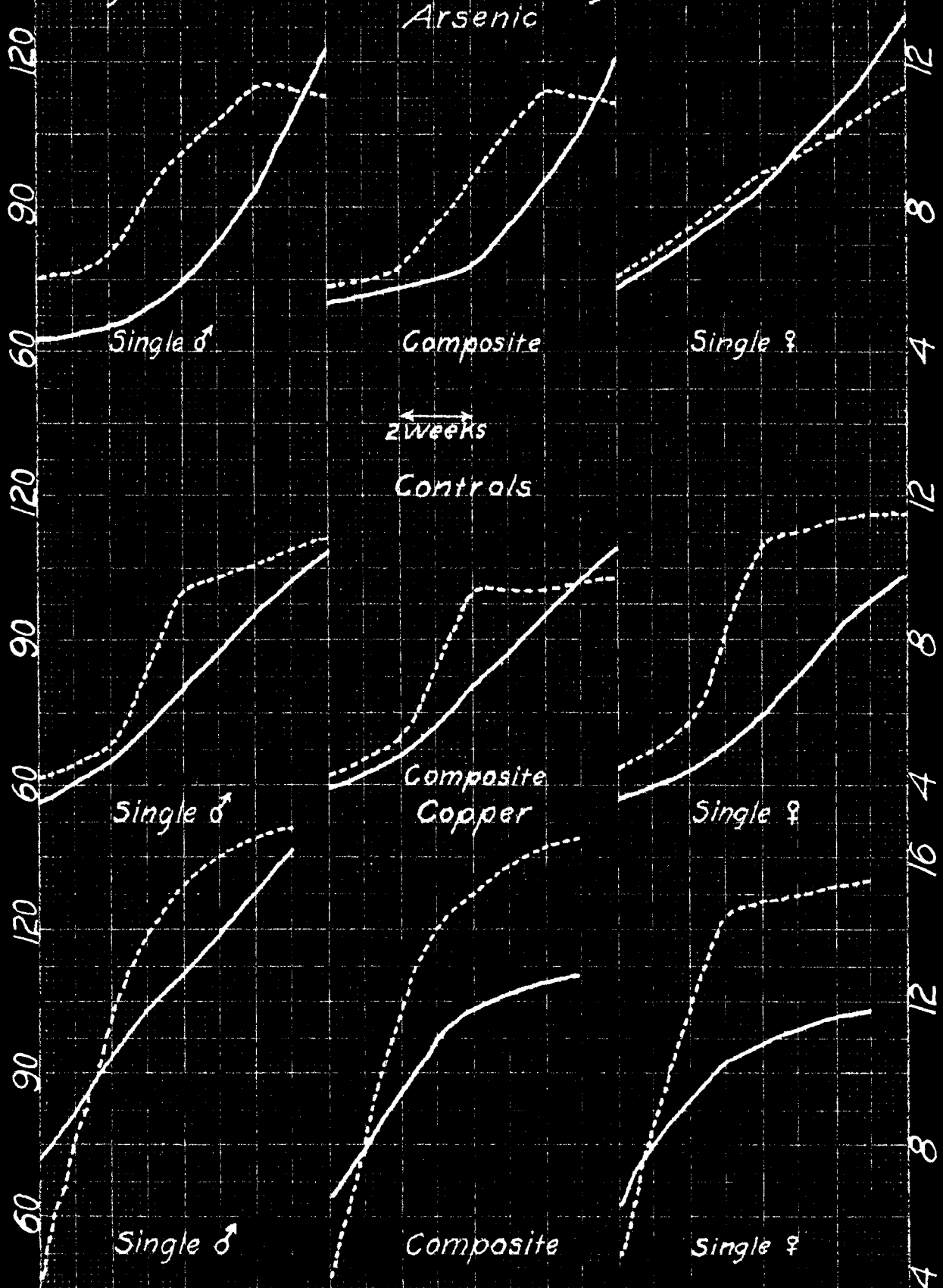Hemoglobin in Per Cent ----

Composite

Ration: Market Milk

Chart 2

RESULTS ON MINERALIZED MARKET MILK

Weight in Grams ——— Hemoglobin in Per Cent ---

Manganese

Single ♂   Composite   Single ♀

2 weeks

Nickel

Single ♂   Composite   Single ♀
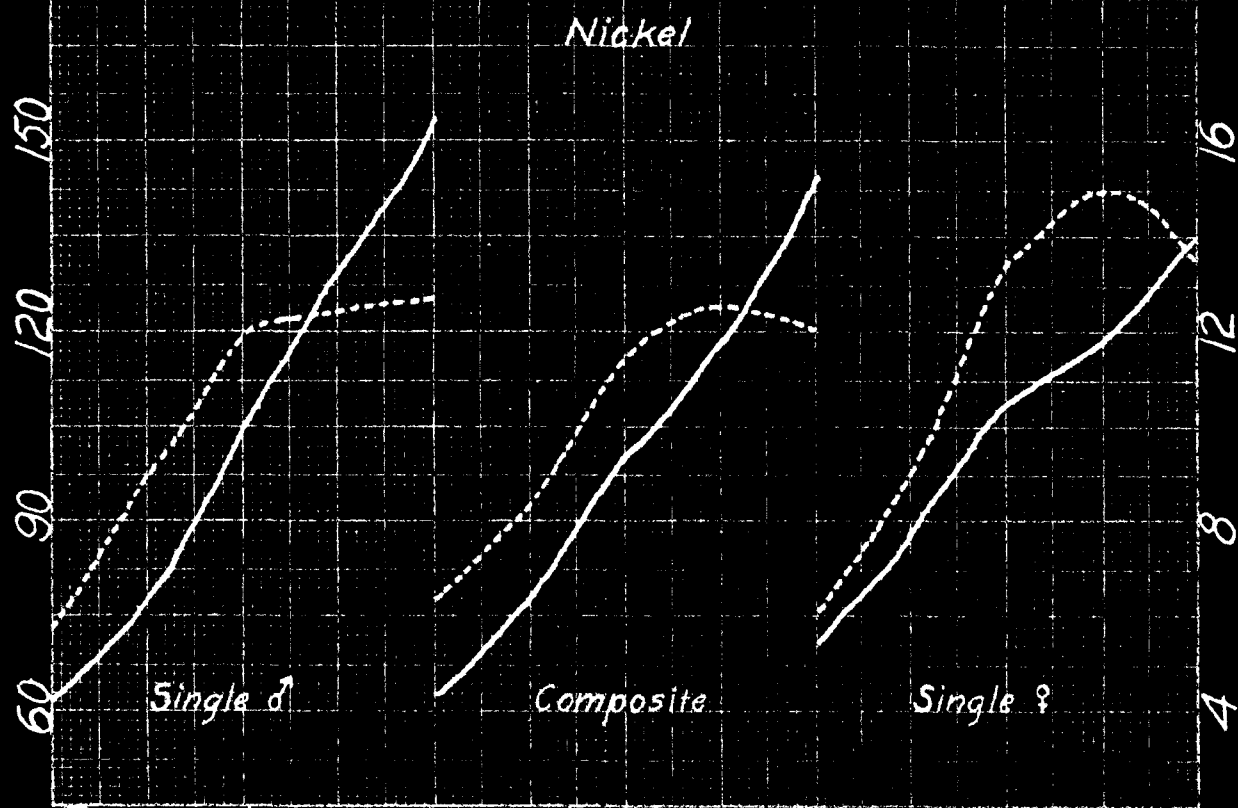
Ration: Market Milk plus Fe as FeCl₃   Chart 3

RESULTS ON MINERALIZED MARKET MILK

Weight in Grams — Hemoglobin in Per Cent ---

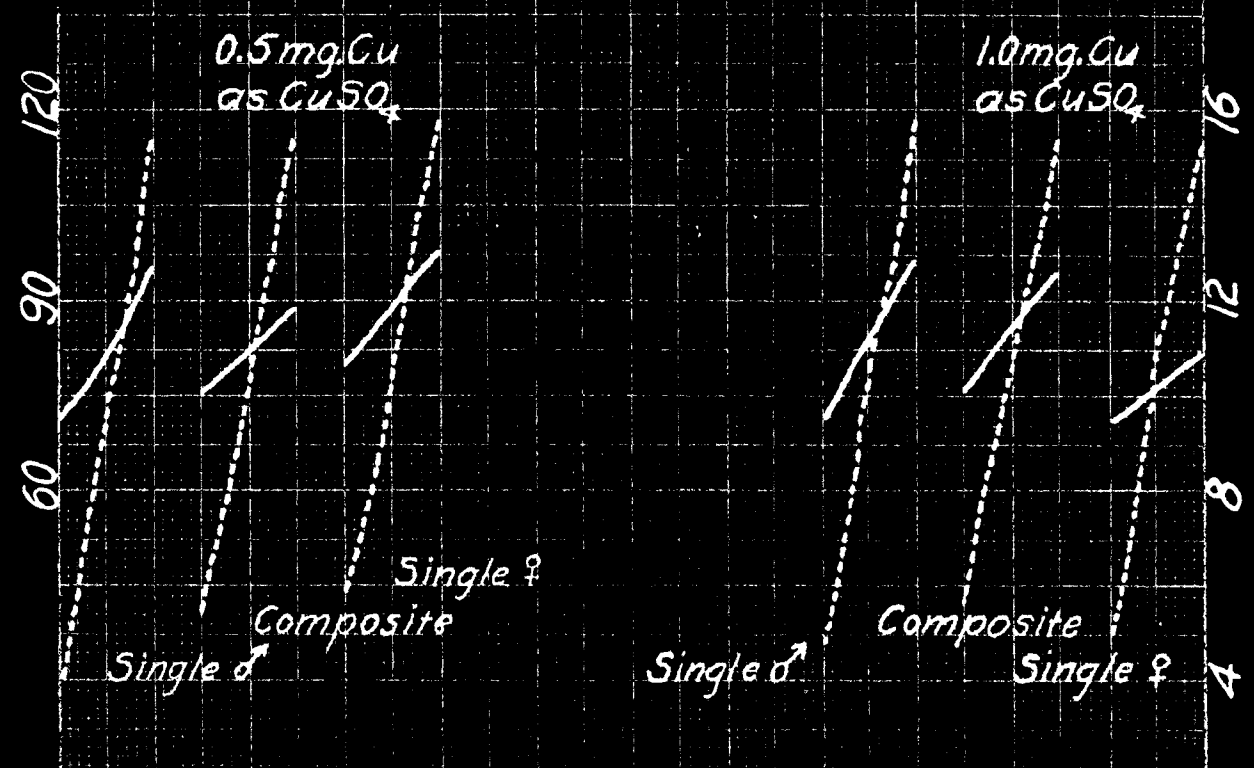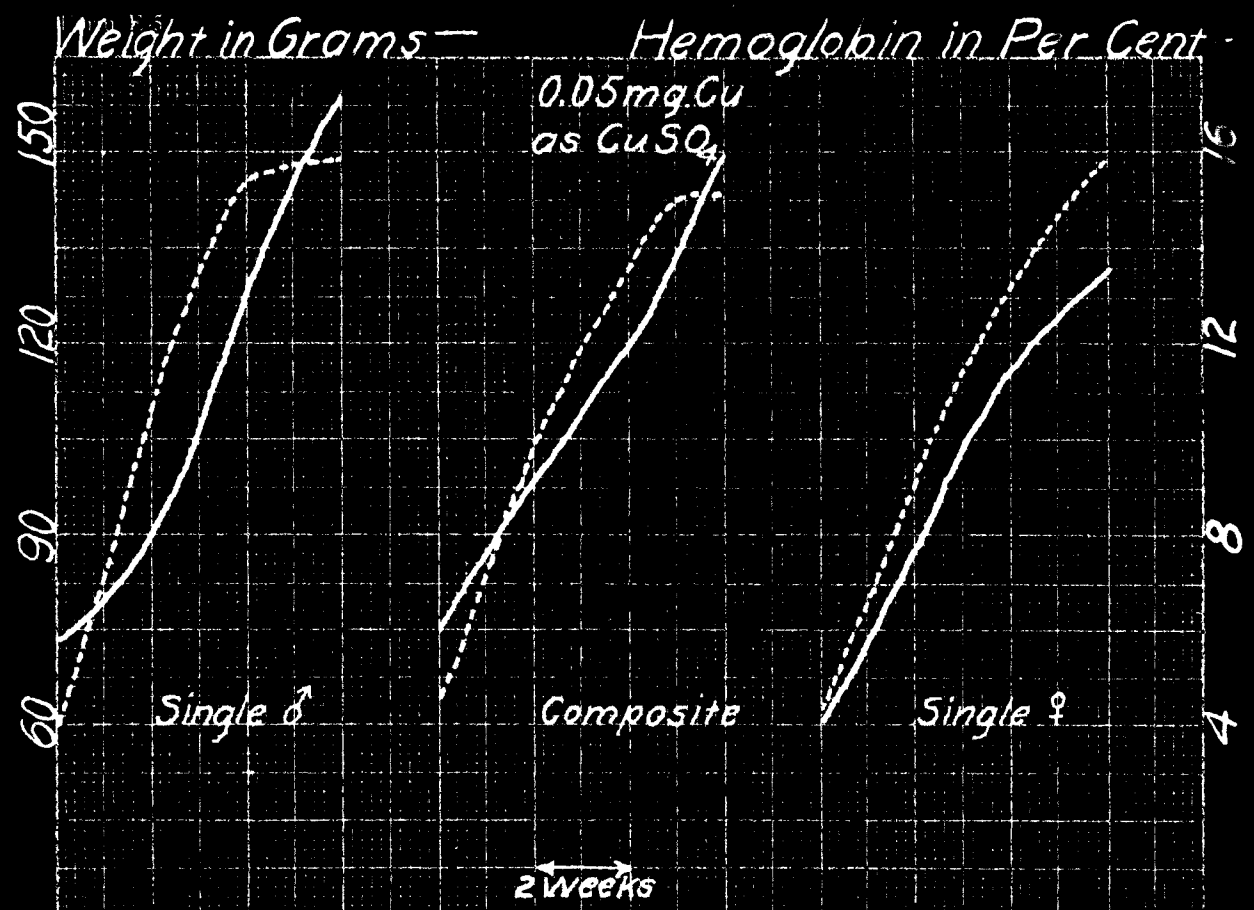Arsenic

Single ♂   Composite   Single ♀

2 weeks

Controls

Single ♂   Composite   Single ♀

Copper

Single ♂   Composite   Single ♀

Ration: Market Milk plus Fe as FeCl₃   Chart 4

RESULTS OF COPPER ADDITIONS

Weight in Grams — Hemoglobin in Per Cent.

0.05 mg. Cu as CuSO₄

Single ♂   Composite   Single ♀

2 Weeks

0.5 mg. Cu as CuSO₄   1.0 mg. Cu as CuSO₄

Single ♀   Composite   Single ♂   Composite   Single ♀
Single ♂

Ration: Milk plus Fe as FeCl₃   Chart 5

RESULTS OF IRON FEEDING

Weight in Grams————   Hemoglobin in Per Cent----

1.0 mg. Fe as FeCl₃

Single ♂   Composite   Single ♀

⟵ 2 weeks

5.0 mg. Fe as FeCl₃

Single ♂   Composite   Single ♀

10.0 mg. Fe as FeCl₃

Single ♂   Composite   Single ♀

Ration : Milk   Chart 6

RESULTS OF INORGANIC ADDITIONS

Weight in Grams —   Hemoglobin in Per Cent.

Titanium

Single ♂   Composite   Single ♀

← 2 weeks →

Manganese

Single ♂   Composite   Single ♀
                  Nickel
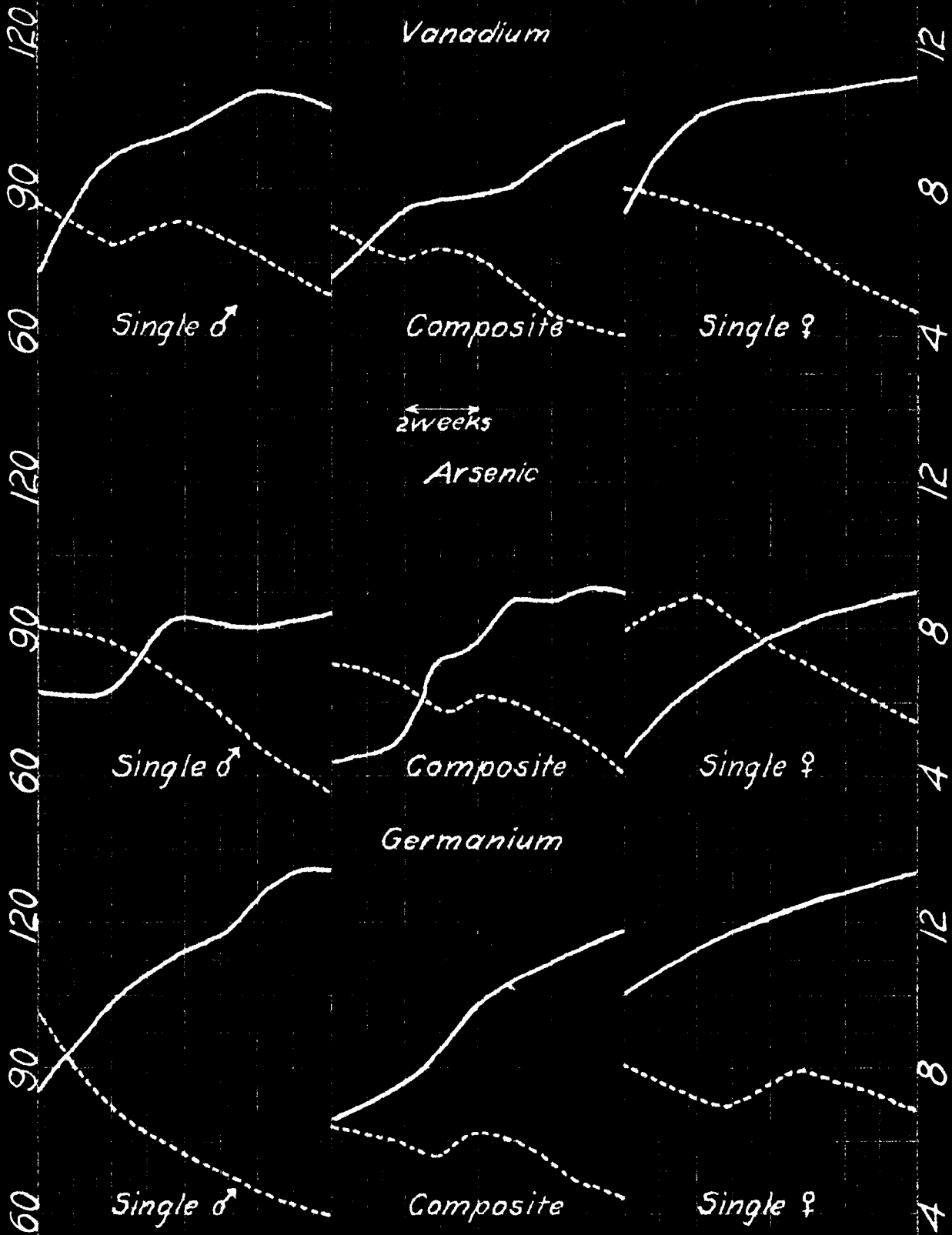
Single ♂   Composite   Single ♀

Ration: Milk plus Fe as FeCl₃   Chart 7

RESULTS OF INORGANIC ADDITIONS

Weight in Grams —   Hemoglobin in Per Cent

Vanadium

Single ♂   Composite   Single ♀

←→ 2 weeks

Arsenic

Single ♂   Composite   Single ♀

Germanium

Single ♂   Composite   Single ♀

Ration: Milk plus Fe as FeCl₃   Chart 8

Weight in Grams — Hemoglobin Per Cent

Zinc

Single ♂   Composite   Single ♀

←2 weeks→

Chromium

Single ♂   Composite   Single ♀

Tin

Single ♂   Composite   Single ♀
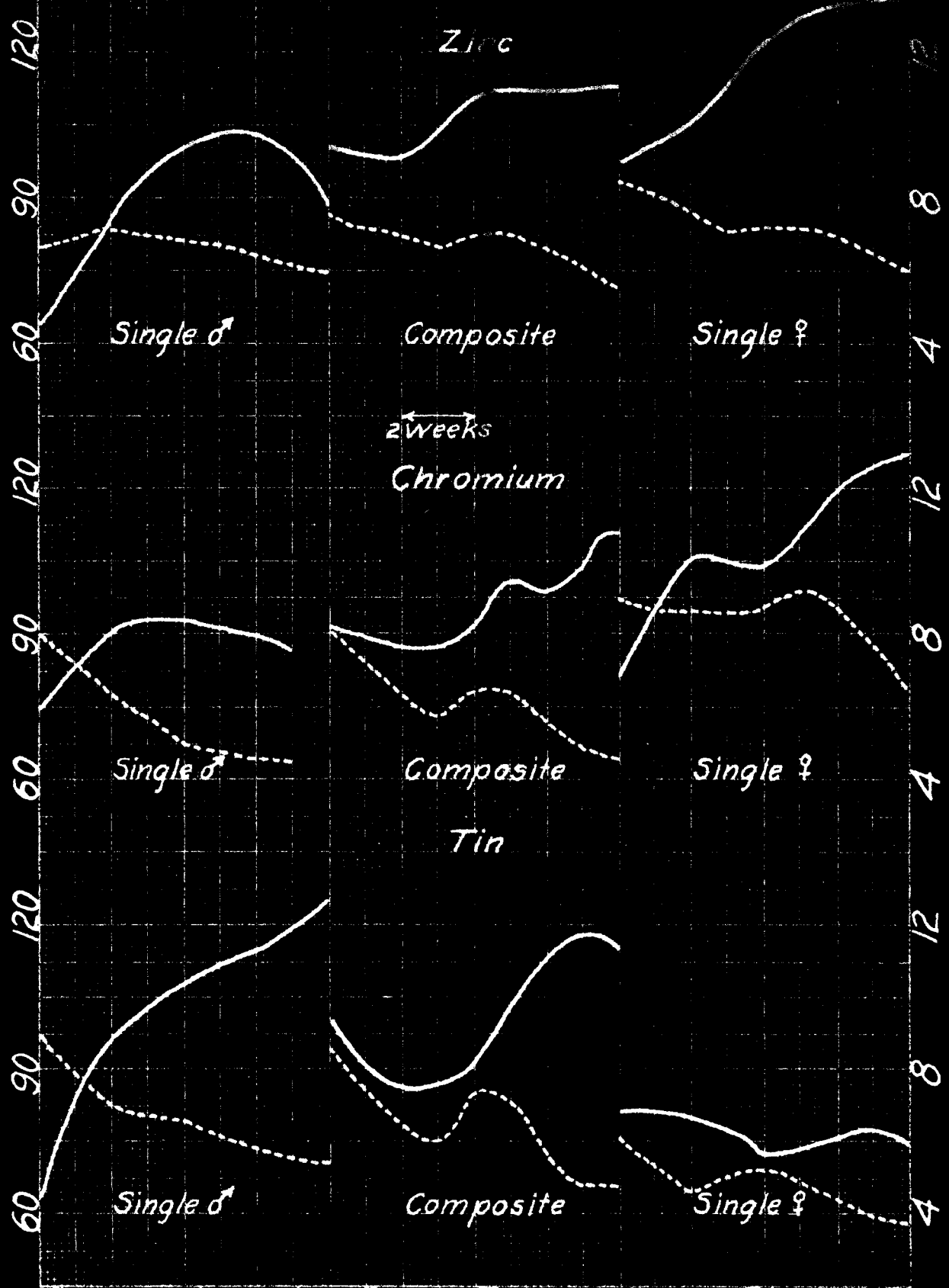
Ration: Milk plus Fe as FeCl₃   Chart 9

RESULTS OF INORGANIC ADDITIONS

Weight in Grams —   Hemoglobin in Per Cent --

Mercury

Single ♂   Composite   Single ♀

←→ 2 weeks

Cobalt

Single ♂   Composite   Single ♀

Copper

Single ♂   Composite   Single ♀
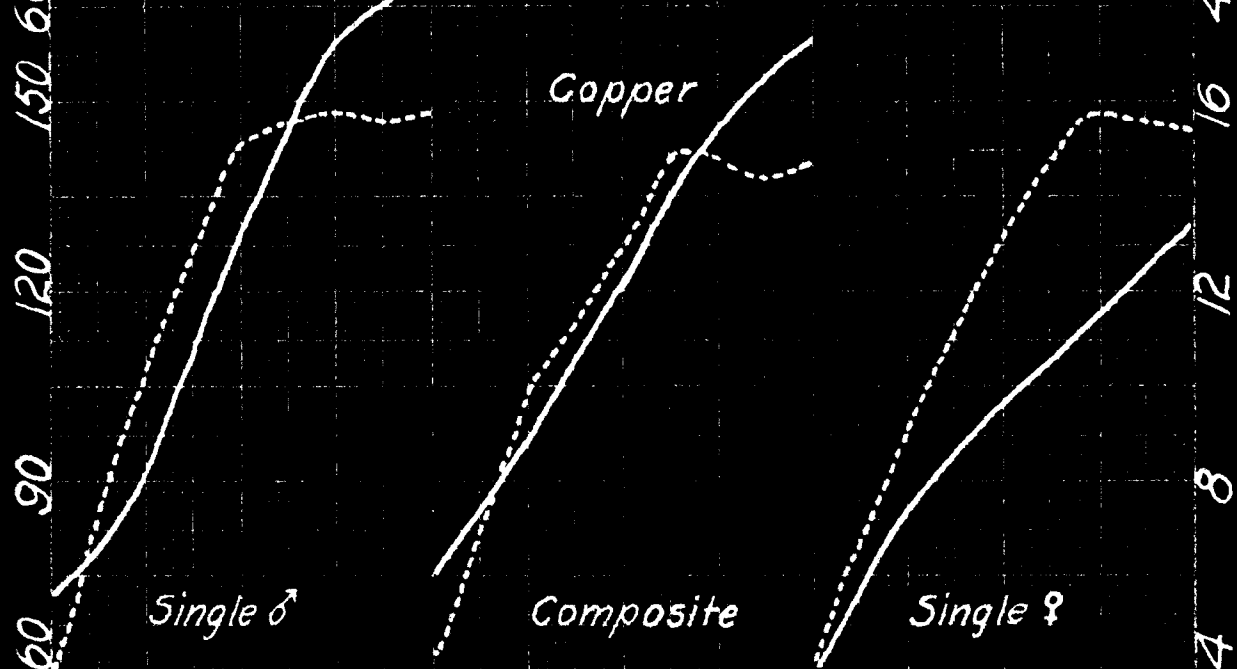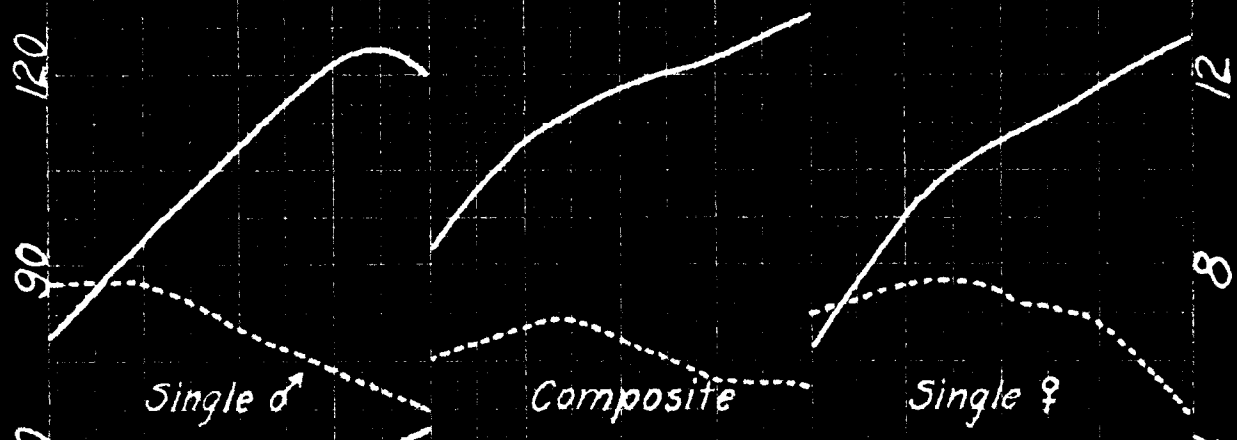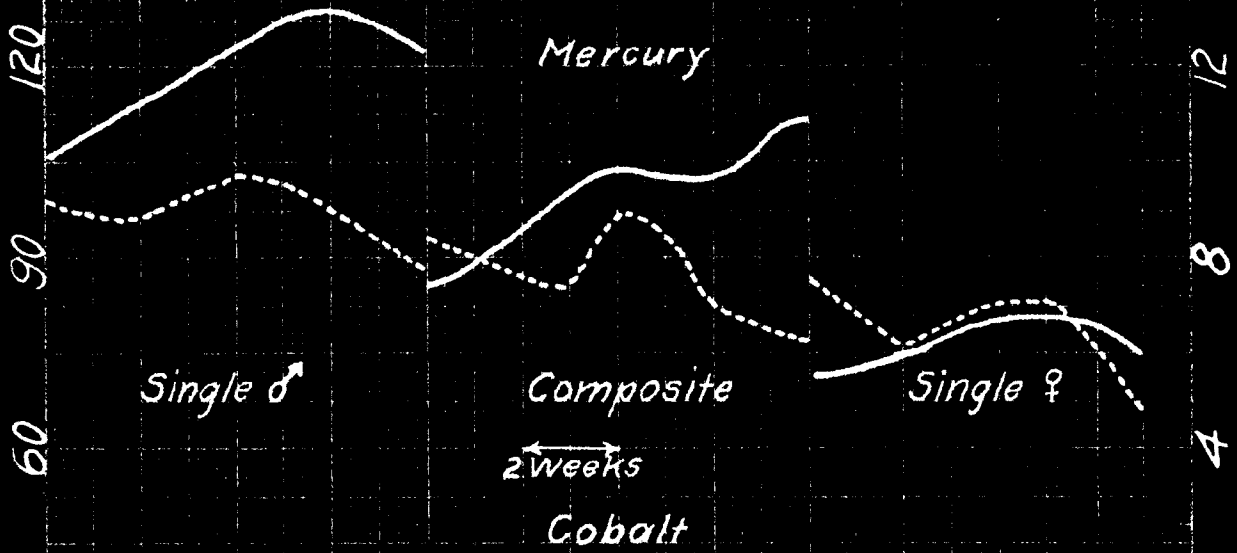
Ration: Milk plus Fe as FeCl₃   Chart 10

RESULTS OF INORGANIC ADDITIONS

Weight in Grams —   Hemoglobin in Per Cent --

Silver

Single ♂   Composite   Single ♀

←2weeks→

Gold

Single ♂   Composite   Single ♀

Controls

Single ♂   Composite   Single ♀

Ration: Milk plus Fe as FeCl₃   Chart II

Weight in grams —   Hemoglobin Per Cent --

90 *60* *30*

12 *8* *4*

Single ♂   Composite
Iron   Single ♀

2 weeks

90 *60* *30*

12 *8* *4*

Single ♂   Composite
Zinc   Single ♀

90 *60* *30*

12 *8* *4*

Single ♂   Composite
Magnesium   Single ♀
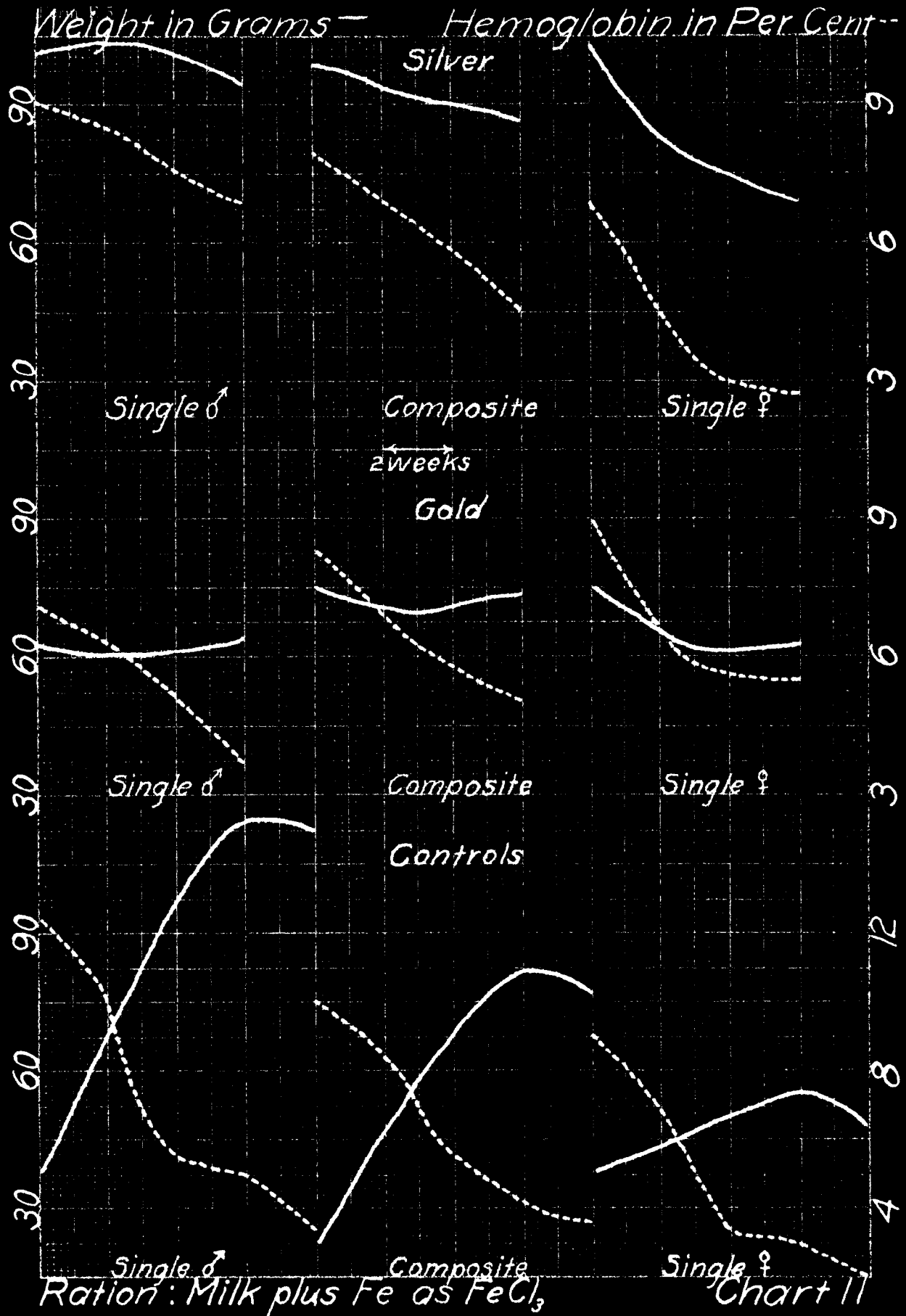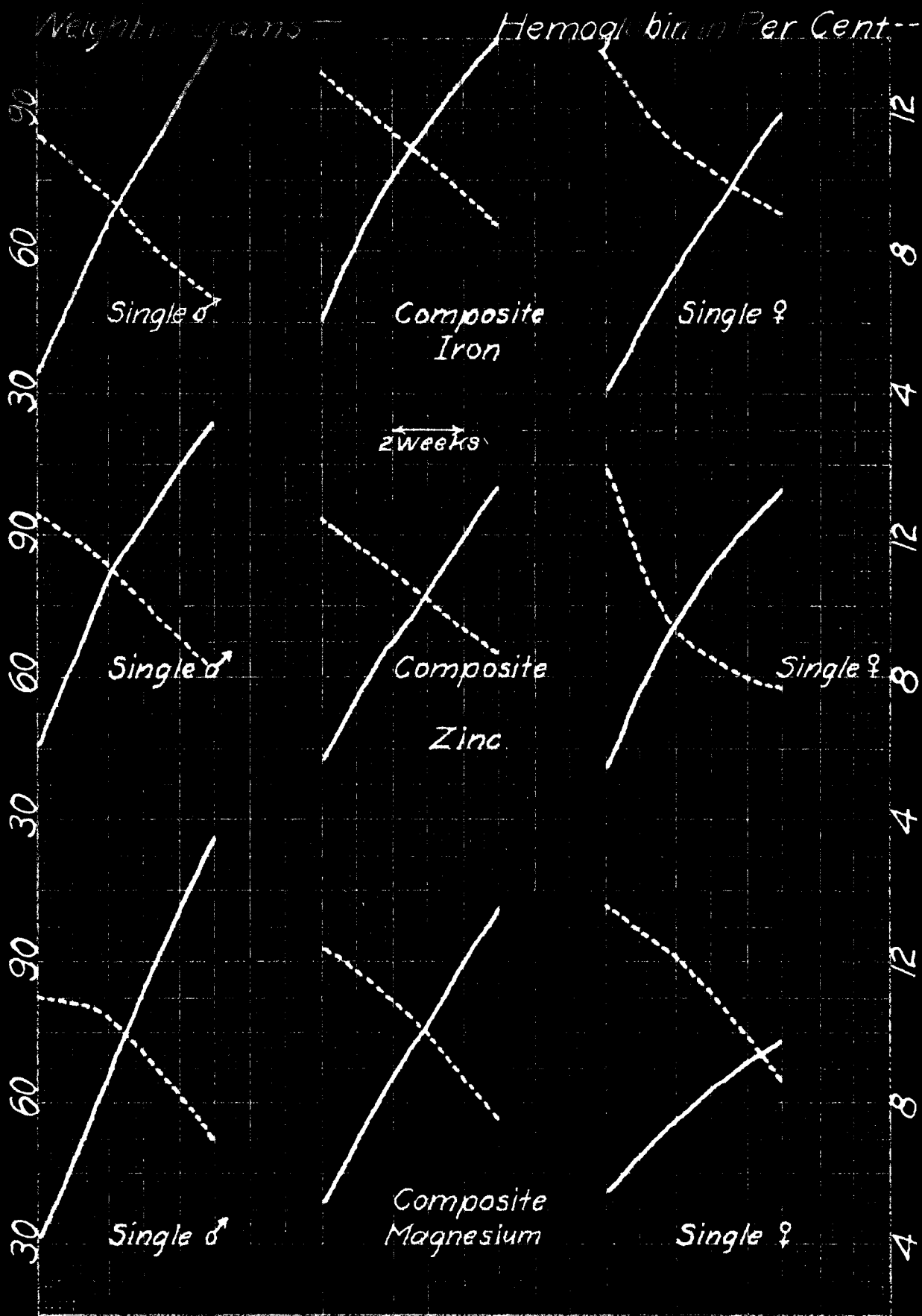
Ration: Milk plus Fe as FeCl₃   Chart 12

RESULTS OF INORGANIC INJECTIONS

Weight in Grams —   Hemoglobin in Per Cent

Nickel

Single ♂   Composite   Single ♀

Zinc

←2weeks→

Single ♂   Composite   Single ♀

Germanium

Single ♂   Composite   Single ♀
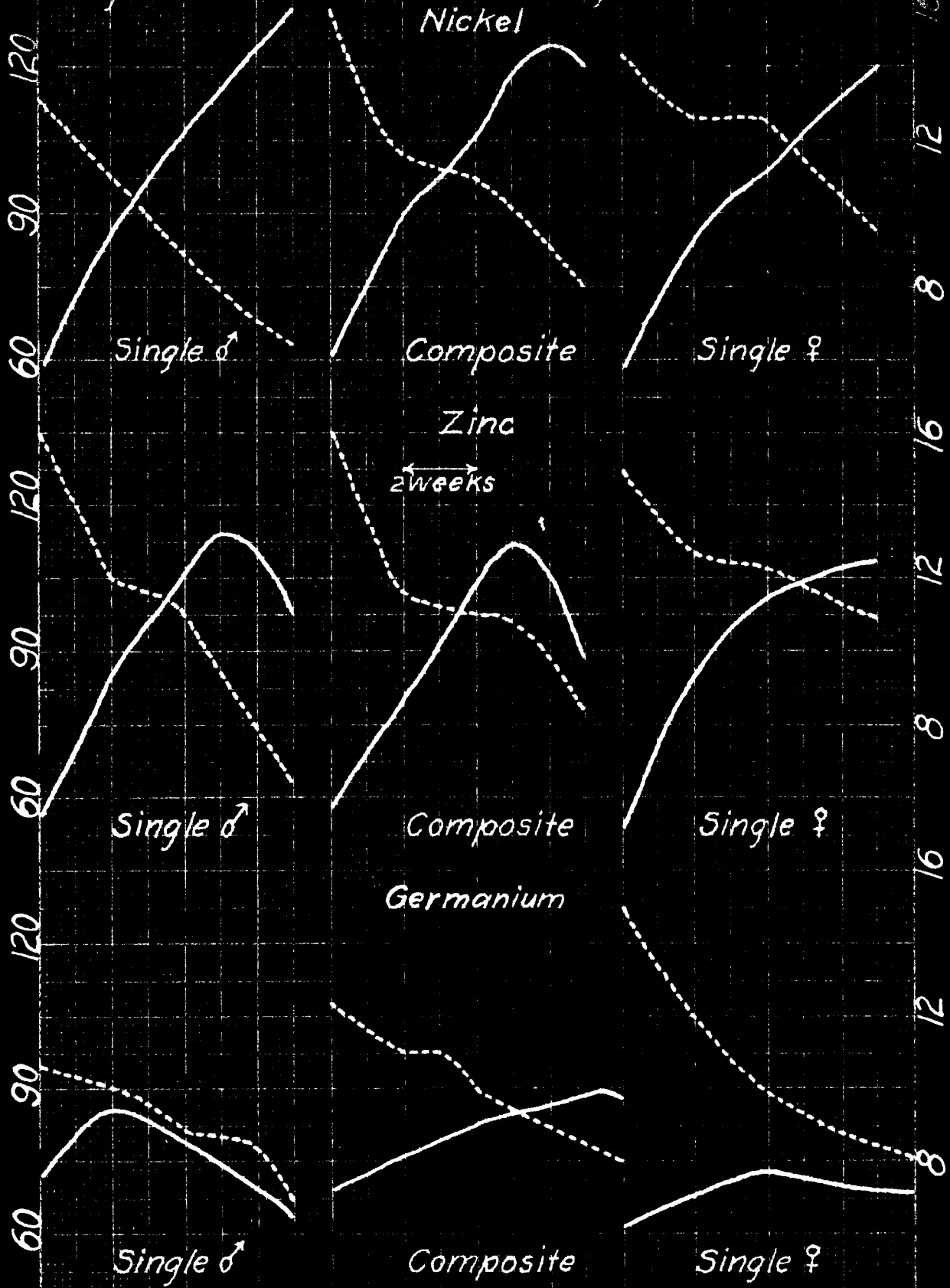
Ration: Milk plus Fe as FeCl₃   Chart 13

RESULTS OF INORGANIC INJECTIONS

Weight in Grams —   Hemoglobin in Per Cent

Manganese

Single ♂   Composite   Single ♀

Vanadium

2weeks

Single ♂   Composite   Single ♀

Arsenic

Single ♂   Composite   Single ♀
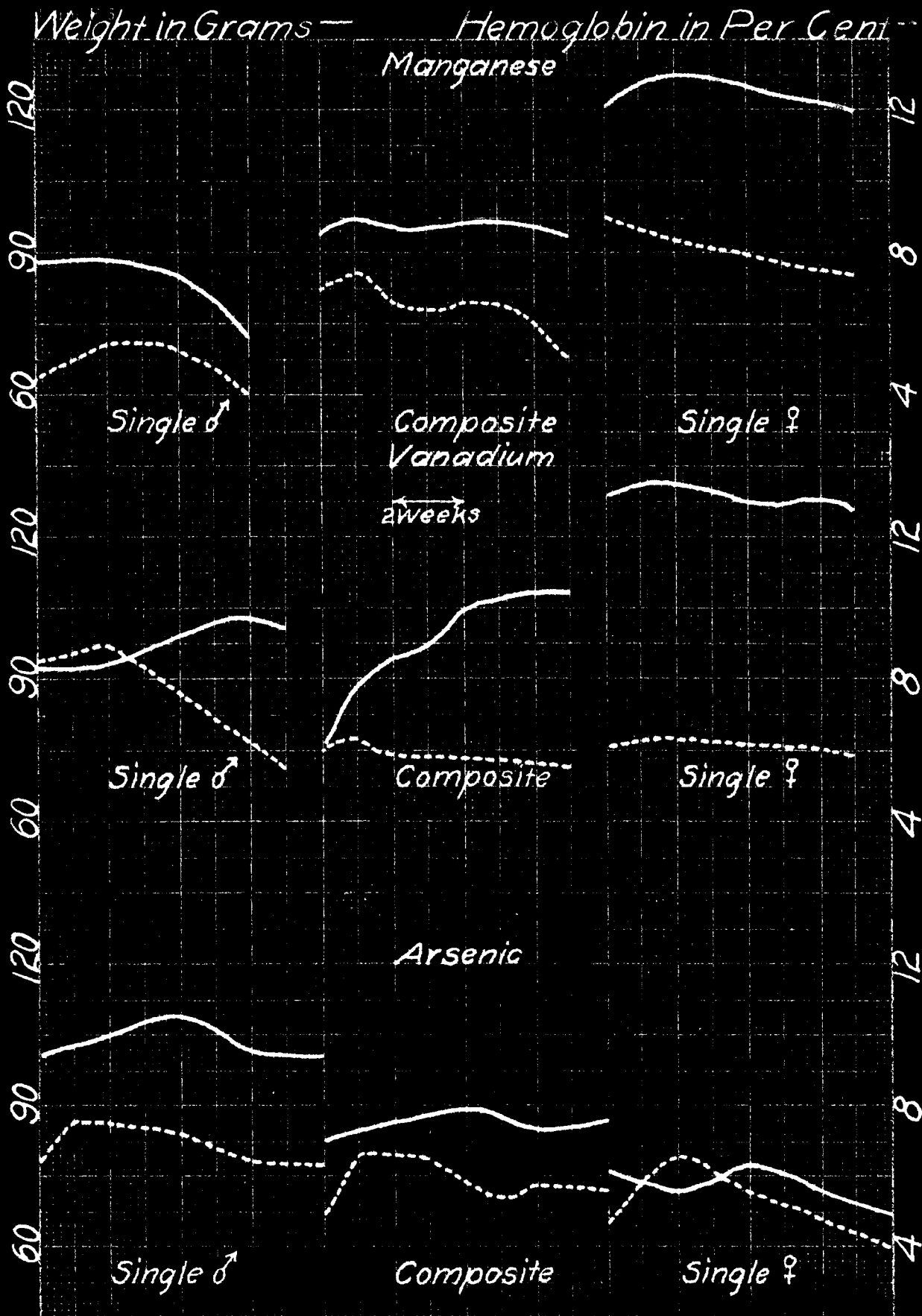
Ration: Milk plus Fe as FeCl₃   Chart 14

RESULTS OF INORGANIC INJECTIONS

Weight in Grams — Hemoglobin in Per Cent --

Titanium

Single ♂   Composite   Single ♀

← 2 weeks →

Selenium

Single ♂   Composite   Single ♀

Mercury

Single ♂   Composite   Single ♀
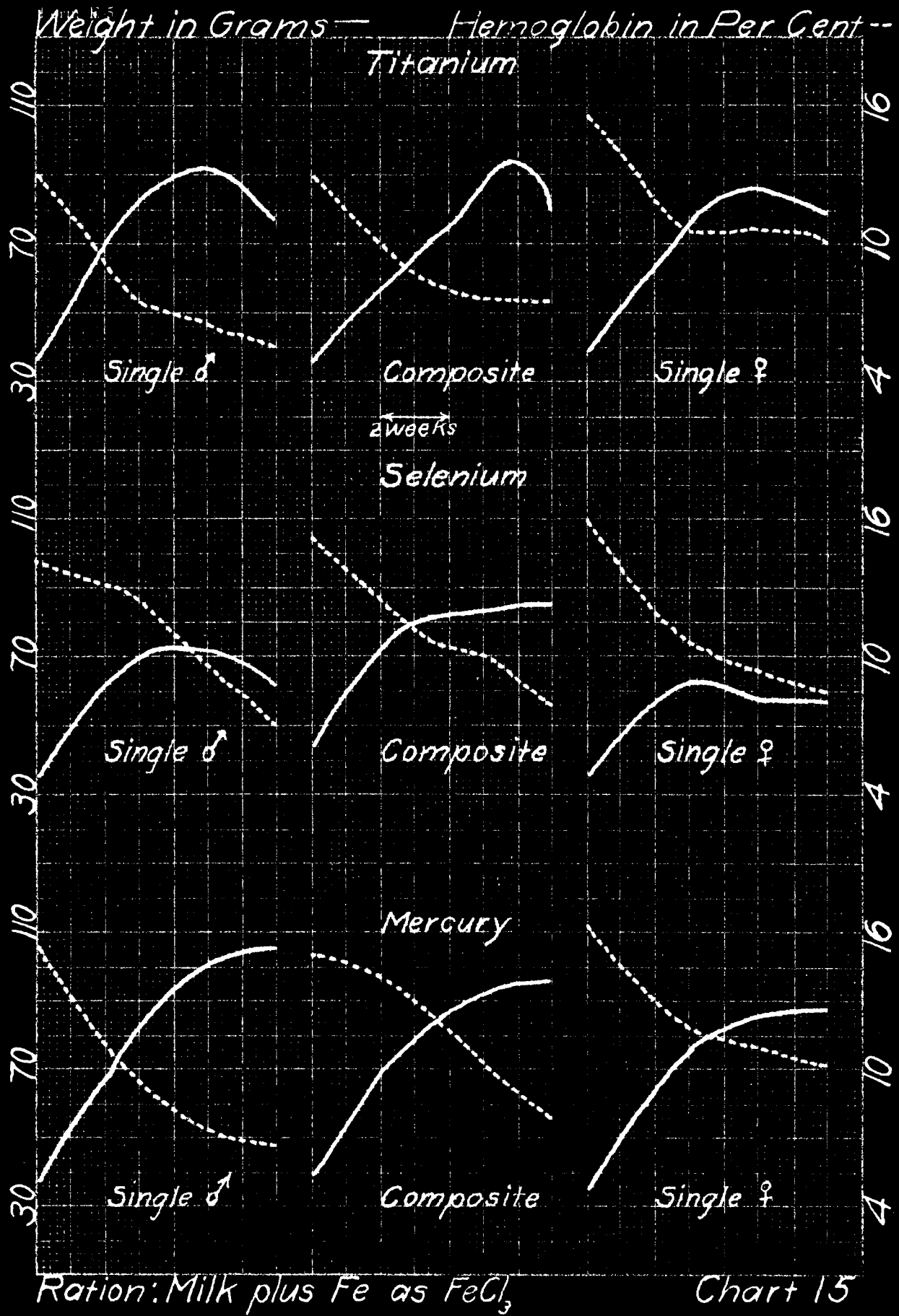
Ration: Milk plus Fe as FeCl₃   Chart 15

RESULTS OF INORGANIC INJECTIONS

Weight in Grains _____ Hemoglobin in Per Cent ----

Rubidium

Single ♂   Composite   Single ♀

←2weeks

Chromium

Single ♂   Composite   Single ♀
                  Copper
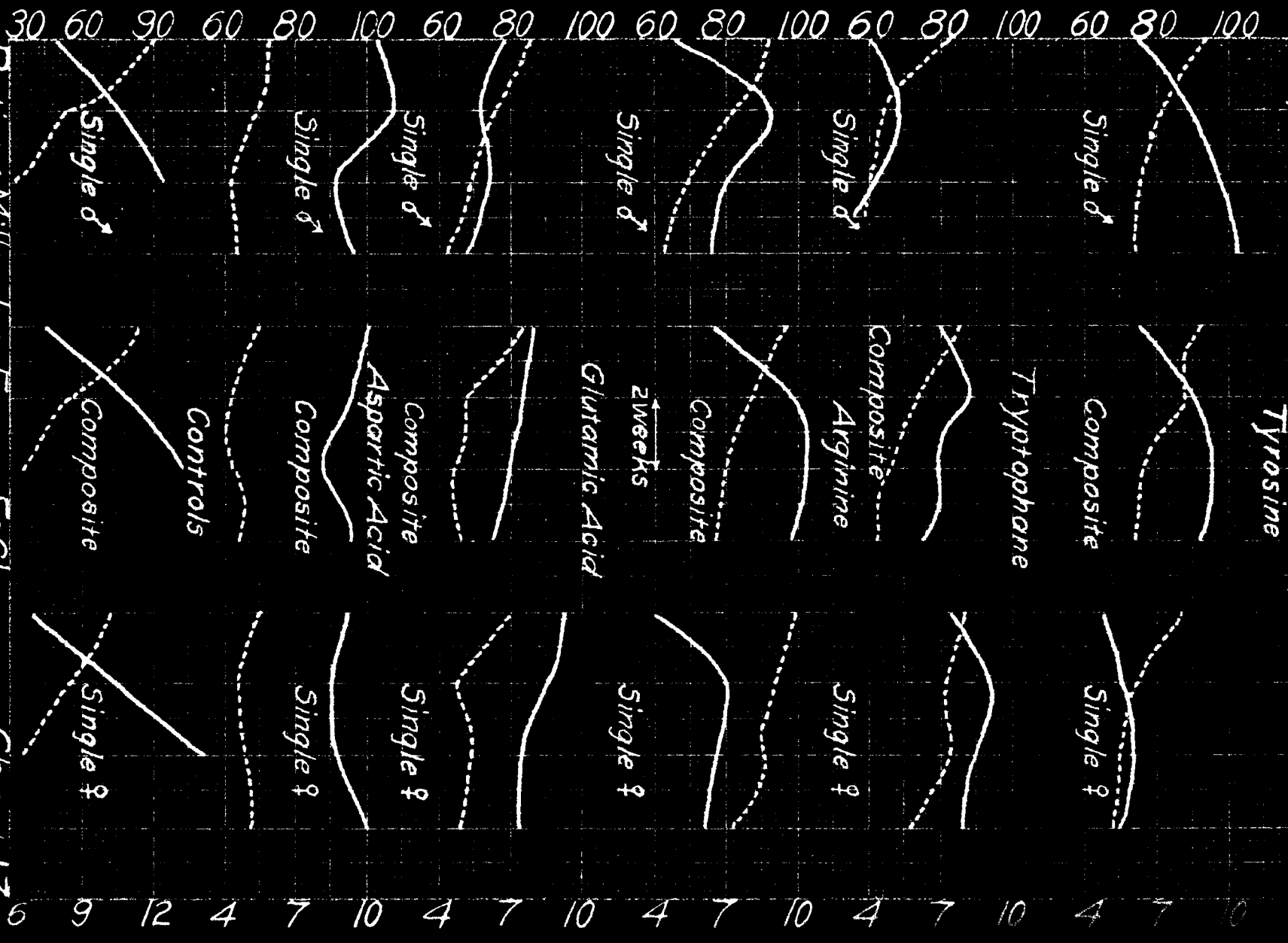
Single ♂   Composite   Single ♀
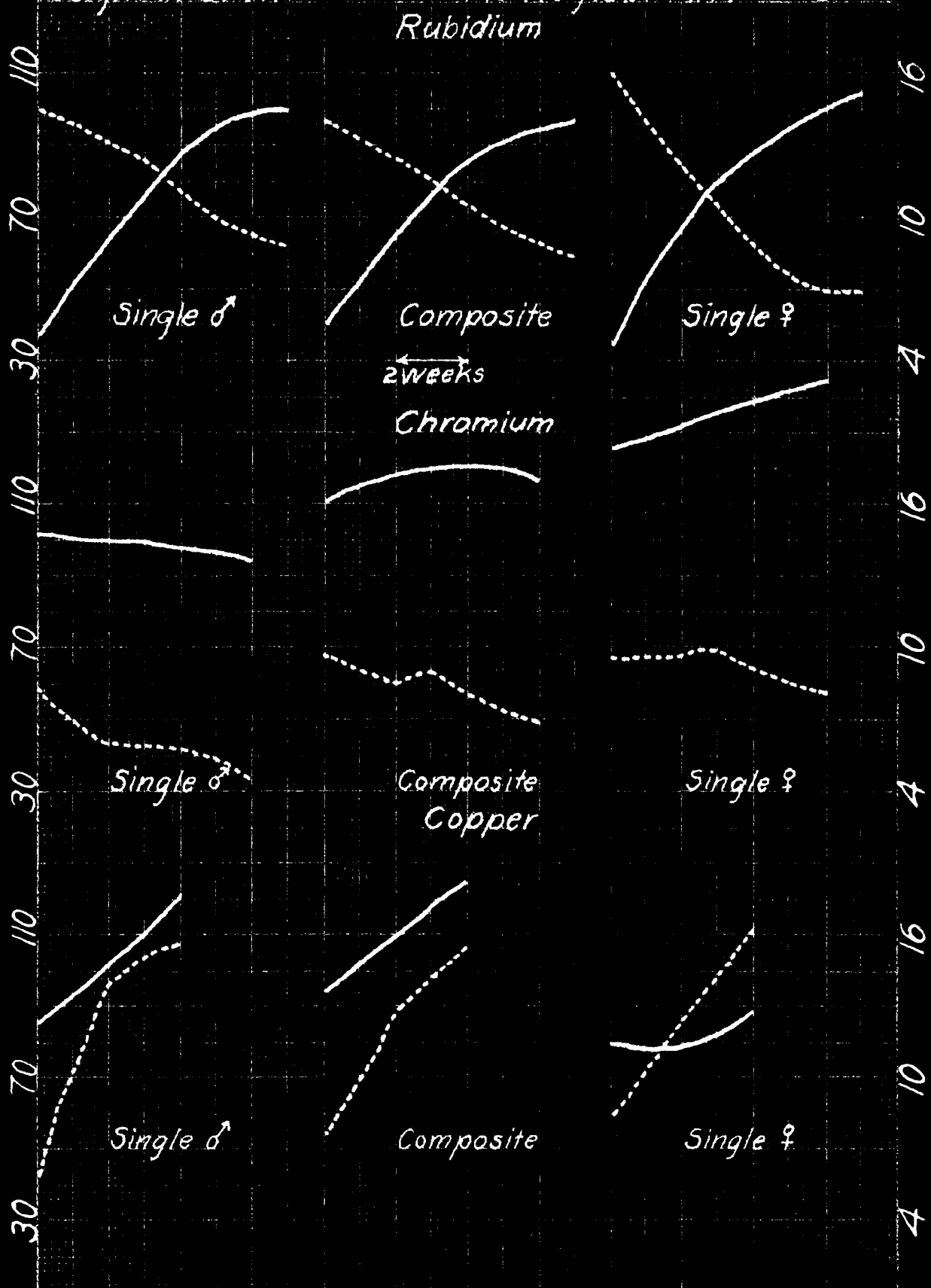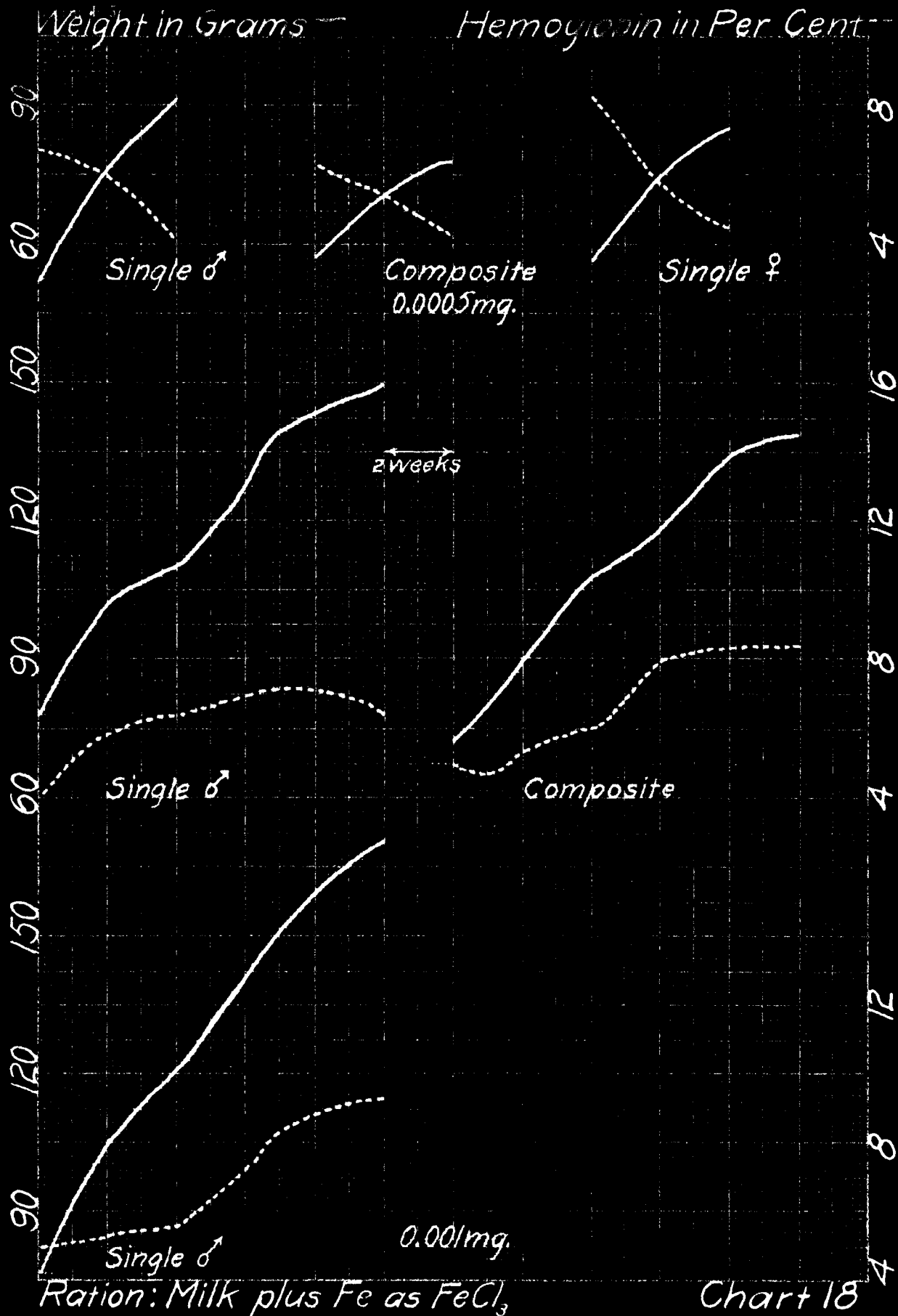
Ration: Milk plus Fe as FeCl₃   Chart 16

RESULTS OF AMINO ACID ADDITIONS

Weight in Grams — Hemoglobin in Per Cent
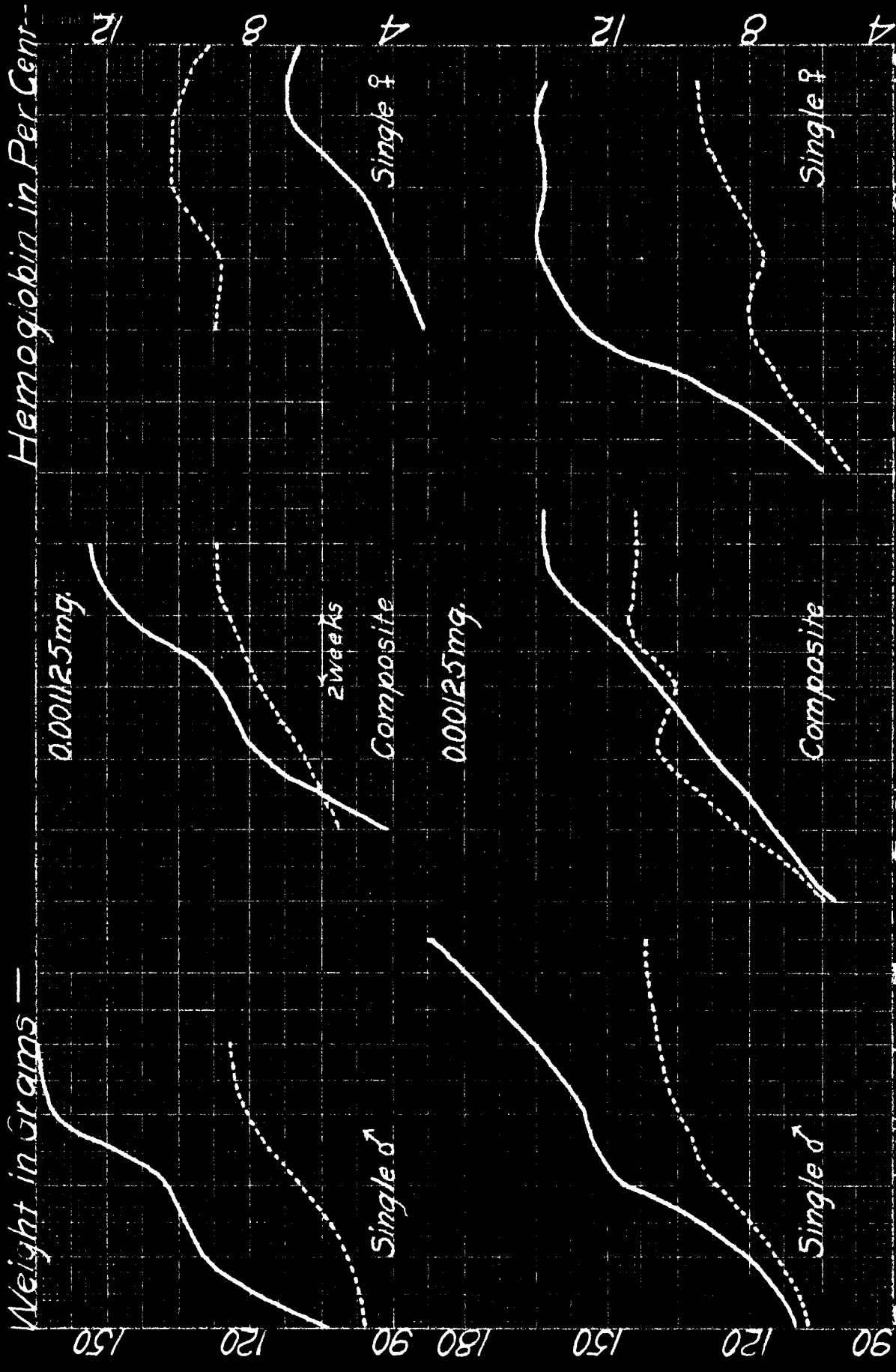
Ration: Milk plus Fe as FeCl₃   Chart 17

RESULTS OF COPPER INJECTIONS

Weight in Grams —   **Hemoglobin in Per Cent —**

90

60

Single ♂   Composite 0.0005mg.   Single ♀

150

120   ← 2weeks →

90

60   Single ♂   Composite

150

120

90   Single ♂   0.001mg.

Ration: Milk plus Fe as FeCl₃   Chart 18

RESULTS OF COPPER INJECTIONS

— Hemoglobin in Per Cent· — Weight in Grams —

Single ♀   Single ♂   2 weeks   Composite   Single ♀   Single ♂   Composite

0.0011.25mg.   0.00/.25mg.

Ration: Milk plus Fe as $FeCl_3$

Chart 19

RESULTS OF COPPER INJECTIONS

Weight in Grams —

Hemoglobin in Per Cent - - -

240

200

160

120

80

40

16

12

8

4

2 weeks

♂

♀

Single ♂

Single ♀

0.0015mg.
Changed to 0.002mg. at "X"
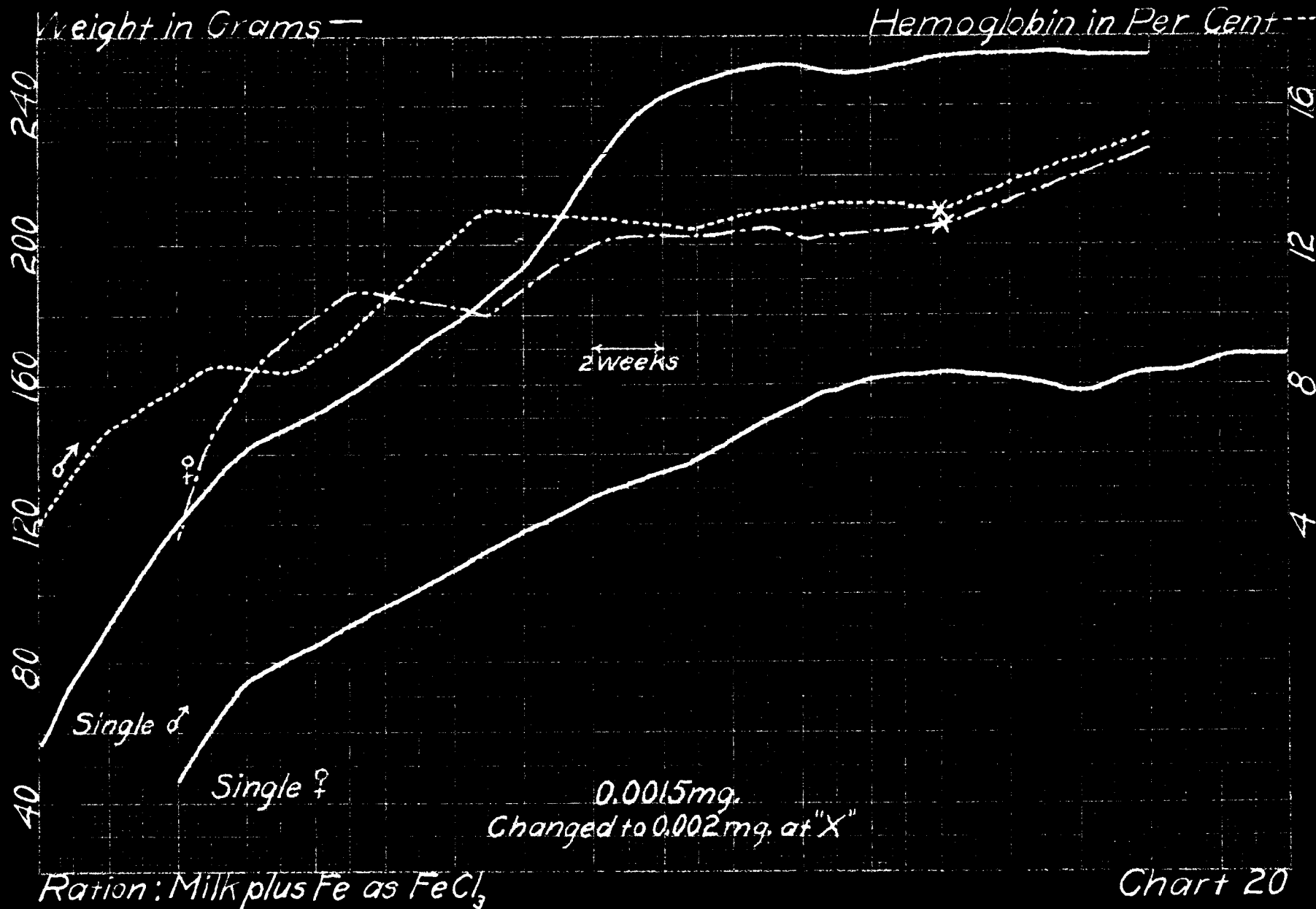
Ration: Milk plus Fe as FeCl₃

Chart 20

RESULTS OF COPPER INJECTIONS

Weight in Grams —

Hemoglobin in Per Cent ---

2 weeks

Composite

0.0015 mg.

Changed to 0.002 mg. at "X"

Ration: Milk plus Fe as FeCl₃

Chart 21

RESULTS OF COPPER INJECTIONS

Weight in Grams _____ Hemoglobin in Per Cent - - -

0.00175mg.

←2weeks→

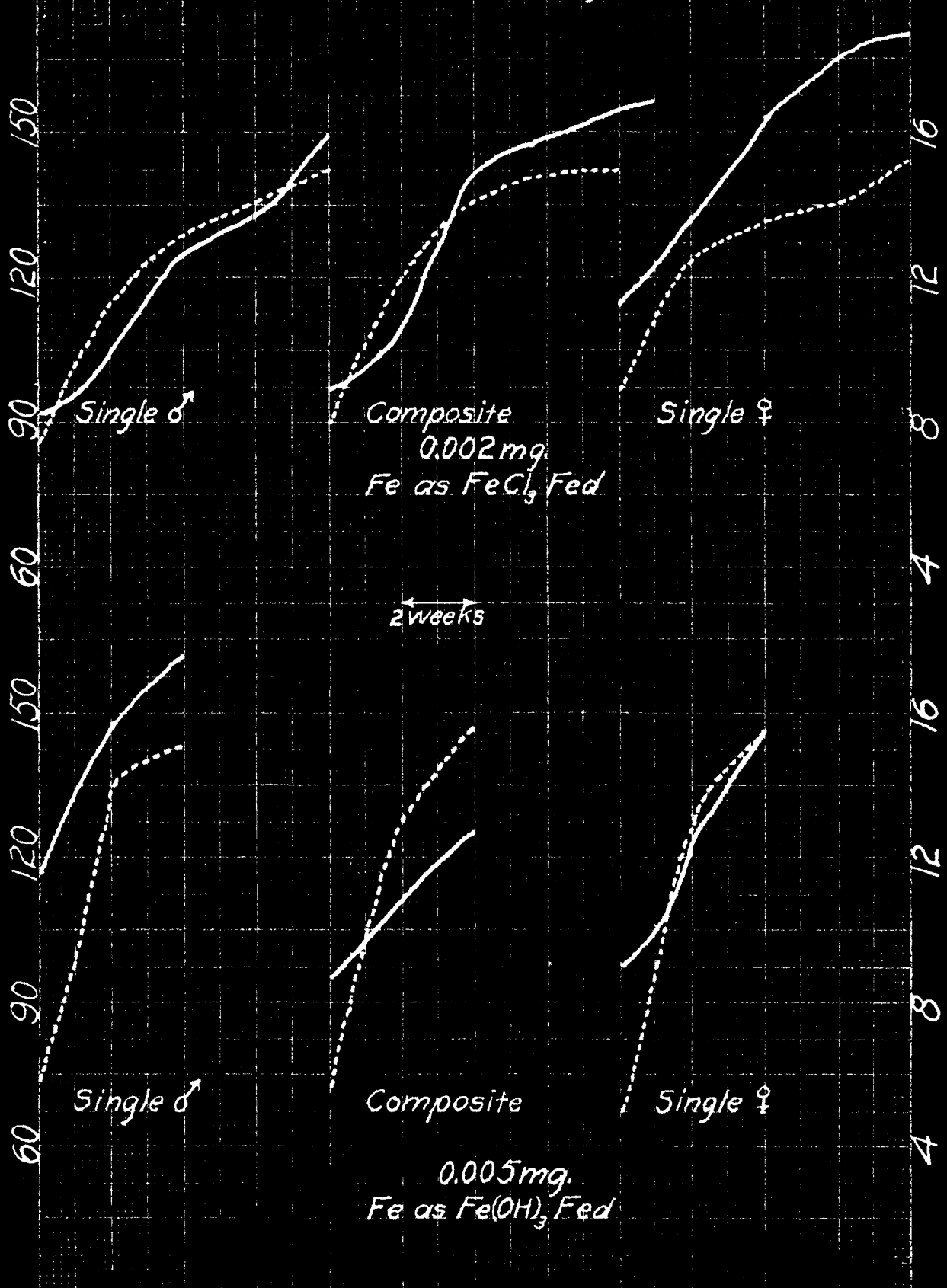Changed to 0.002 mg at "X"

Single ♀

Composite   Single ♂
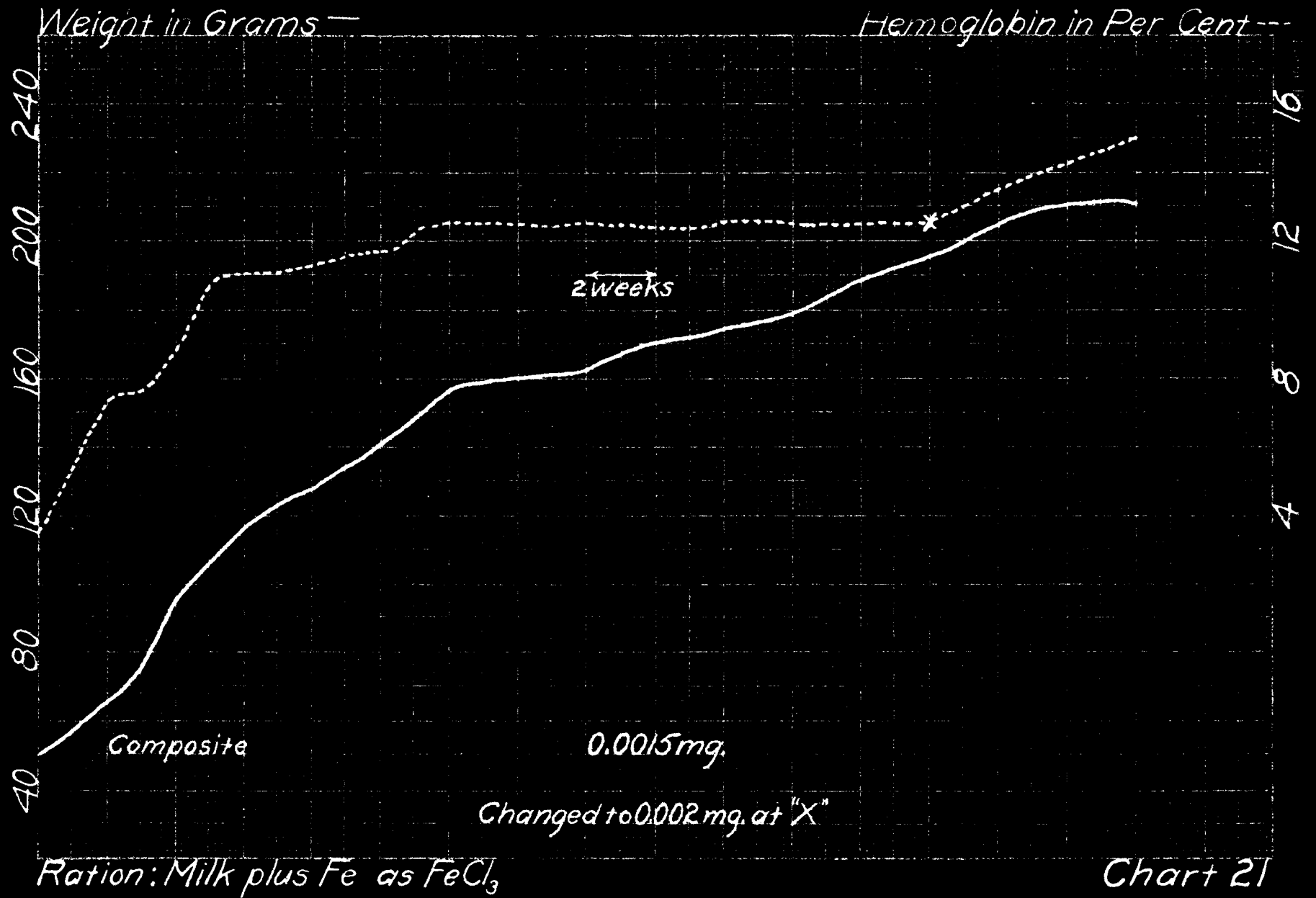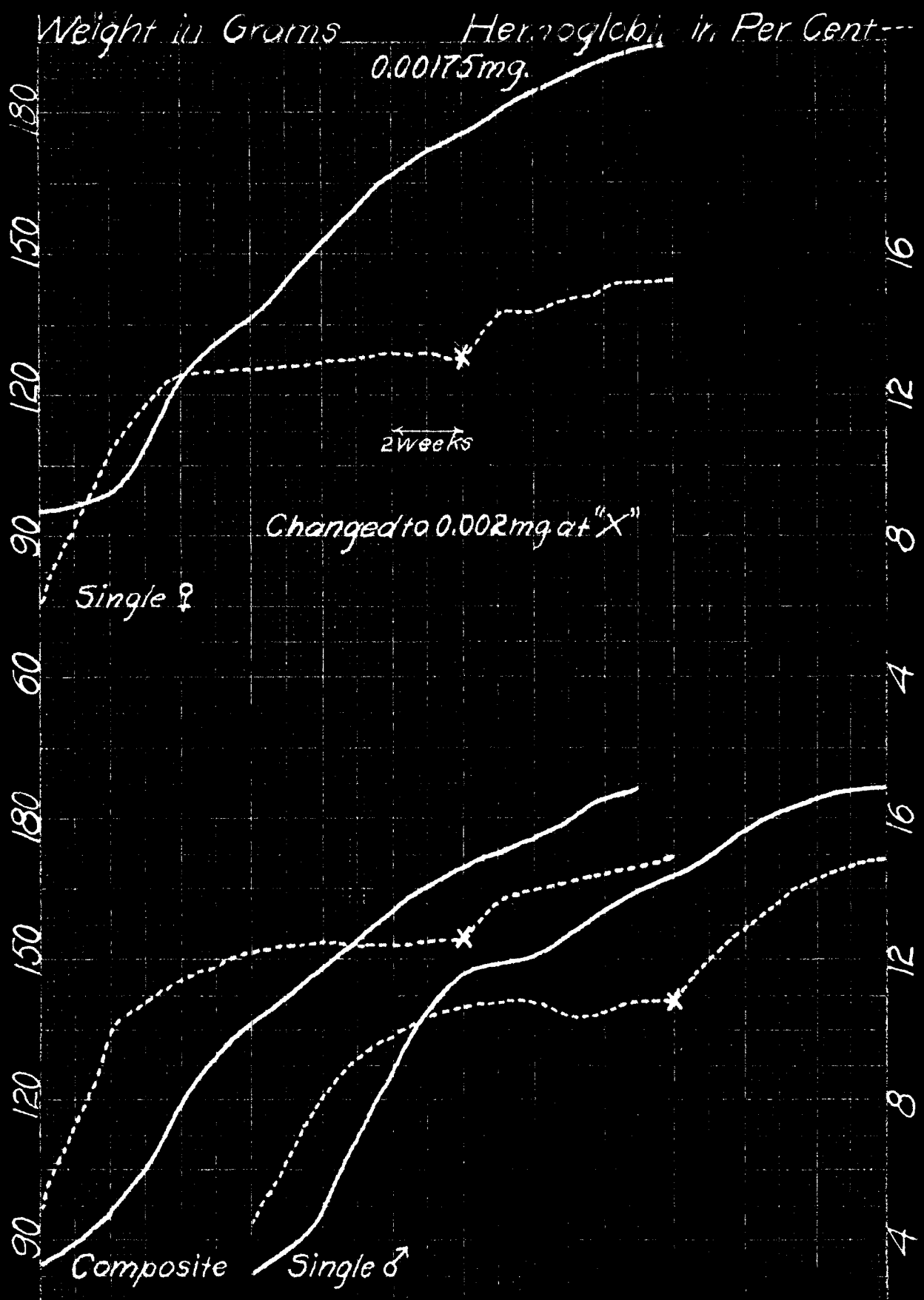
Ration: Milk plus Fe as FeCl₃   Chart 22

RESULTS OF COPPER INJECTIONS

Weight in Grams —   Hemoglobin in Per Cent --

Single ♂   Composite   Single ♀

0.002 mg.
Fe as FeCl₃ Fed

2 weeks

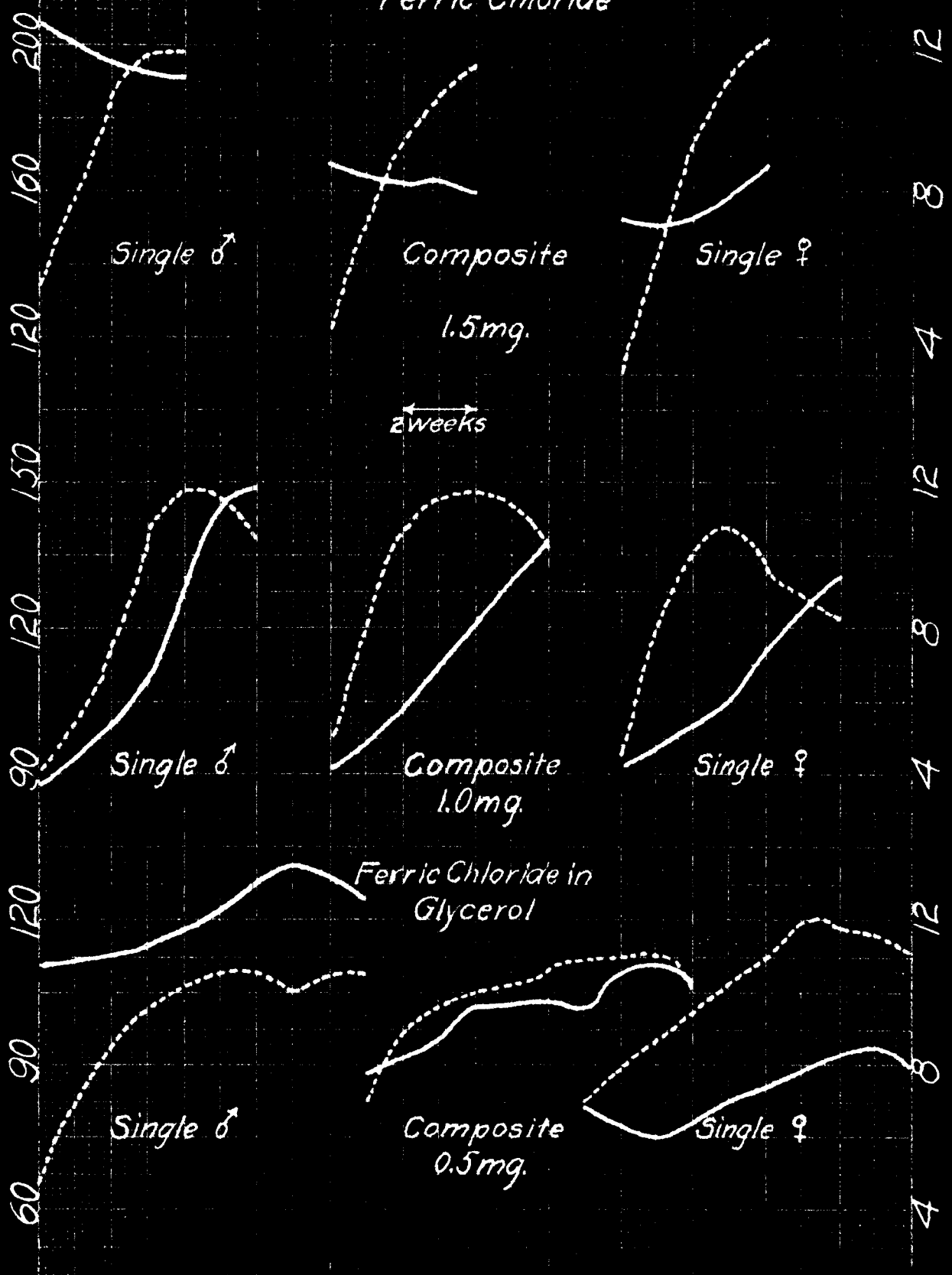Single ♂   Composite   Single ♀

0.005 mg.
Fe as Fe(OH)₃ Fed

Ration: Milk plus Fe as shown   Chart 23

RESULTS OF IRON INJECTIONS

Weight in Grams —   Hemoglobin in Per Cent ---

Ferric Chloride

Single ♂   Composite   Single ♀

1.5 mg.

←→ 2 weeks

Single ♂   Composite   Single ♀
                  1.0 mg.

Ferric Chloride in
Glycerol

Single ♂   Composite   Single ♀
                  0.5 mg.

Ration: Milk   Chart 24

RESULTS OF IRON INJECTIONS

Weight in Grams —   Hemoglobin in Per Cent ---

Ferric Citrate

120

90

Single ♂   Composite   Single ♀

0.5 mg. Fe

60

←2 weeks→

120

90

Single ♂   Composite   Single ♀

0.2 mg. Fe

60

120

90

Single ♂   Composite   Single ♀
                  0.1 mg. Fe

60

12

8

4

12

8

4

12

8

4

Ration: Milk   Chart 25
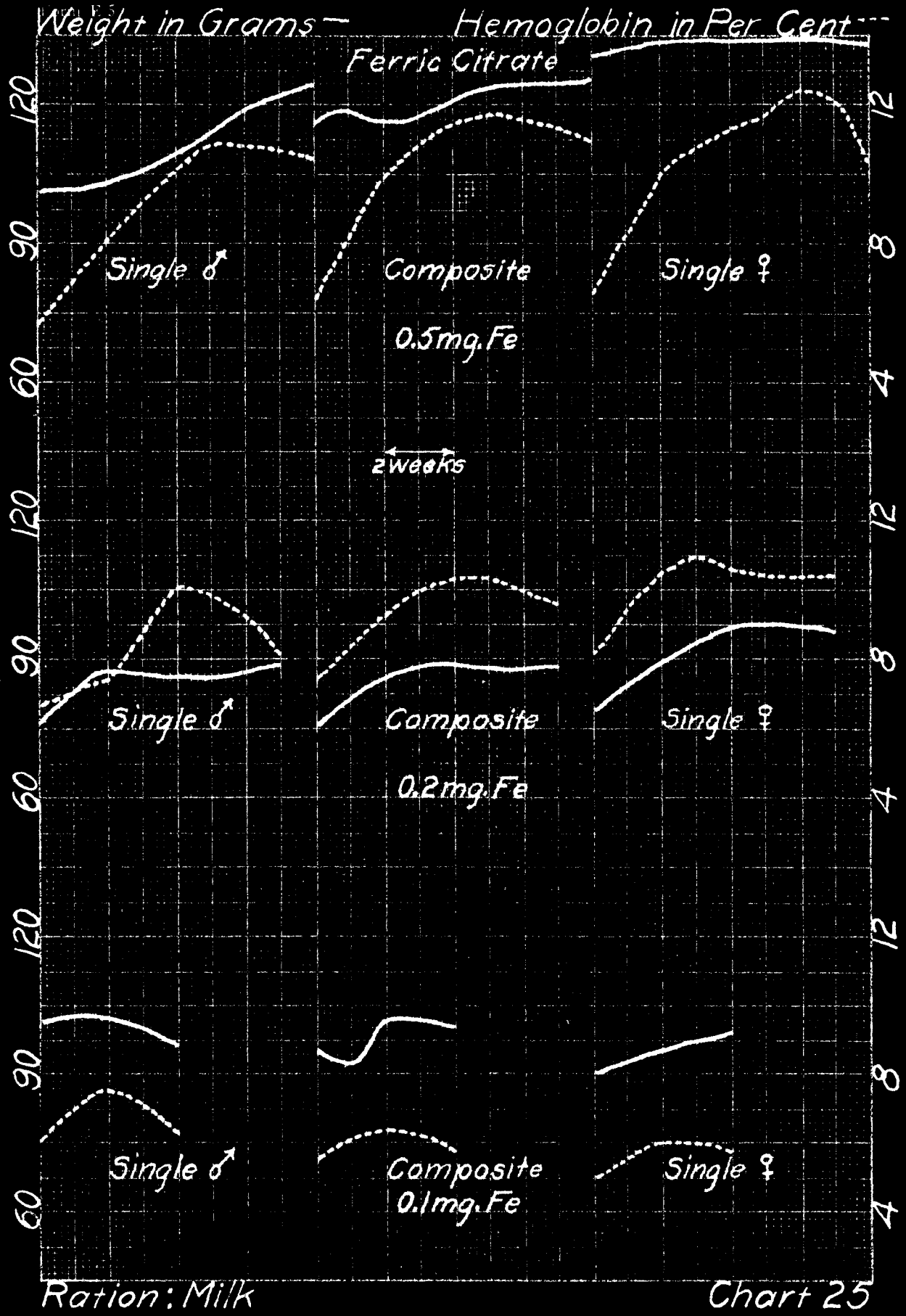
RESULTS OF ACID INJECTIONS

Weight in Grams —   Hemoglobin in Per Cent--

0.033 N HCl
Fe as FeCl₃ Fed

Single ♂   Composite   Single ♀

0.092 N HCl
Fe as Fe(OH)₃ Fed

2 weeks

Single ♂   Composite   Single ♀

0.092 N HCl
Fe as FeCl₃ Fed

Single ♂   Composite   Single ♀
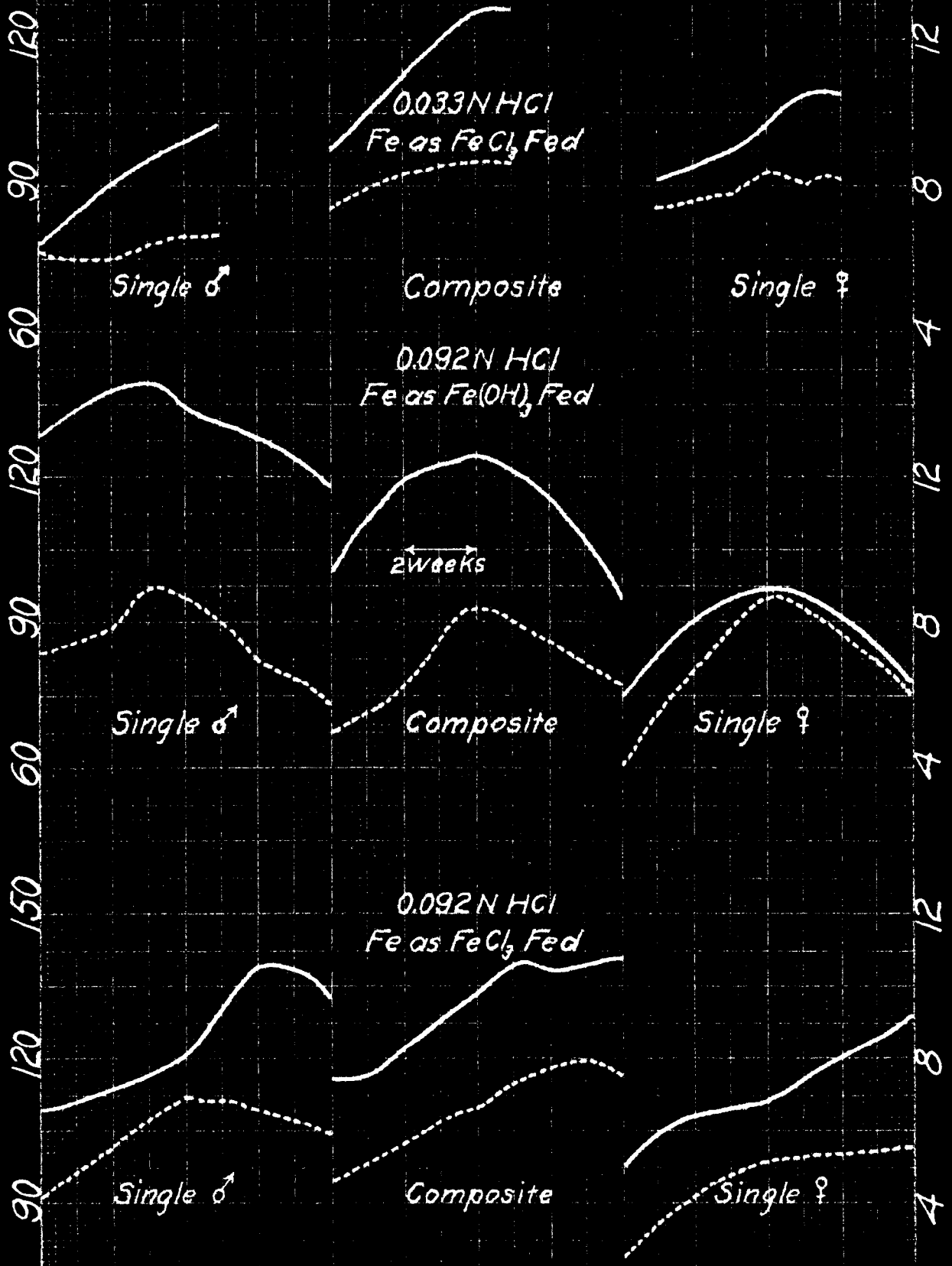
Ration: Milk   Chart 26

RESULTS OF COPPER AND IRON INJECTIONS

Weight in Grams —

Hemoglobin in Per Cent --

0.002 mg. Cu as CuSO₄
0.05 mg. Fe as FeCl₃

Single ♂

2 weeks

Composite

Single ♀

0.002 mg. Cu as CuSO₄
0.1 mg. Fe as the colloidal
hydroxide

Single ♂

Composite

Single ♀

Ration: Milk

Chart 27

RESULTS OF COPPER AND IRON INJECTIONS

Weight in Grams ——— Hemoglobin in Per Cent--

Single ♂   Composite

2weeks

0.002mg. Cu plus 0.1mg. Fe as FeCl₃
At "X" Fe raised to 0.15mg.
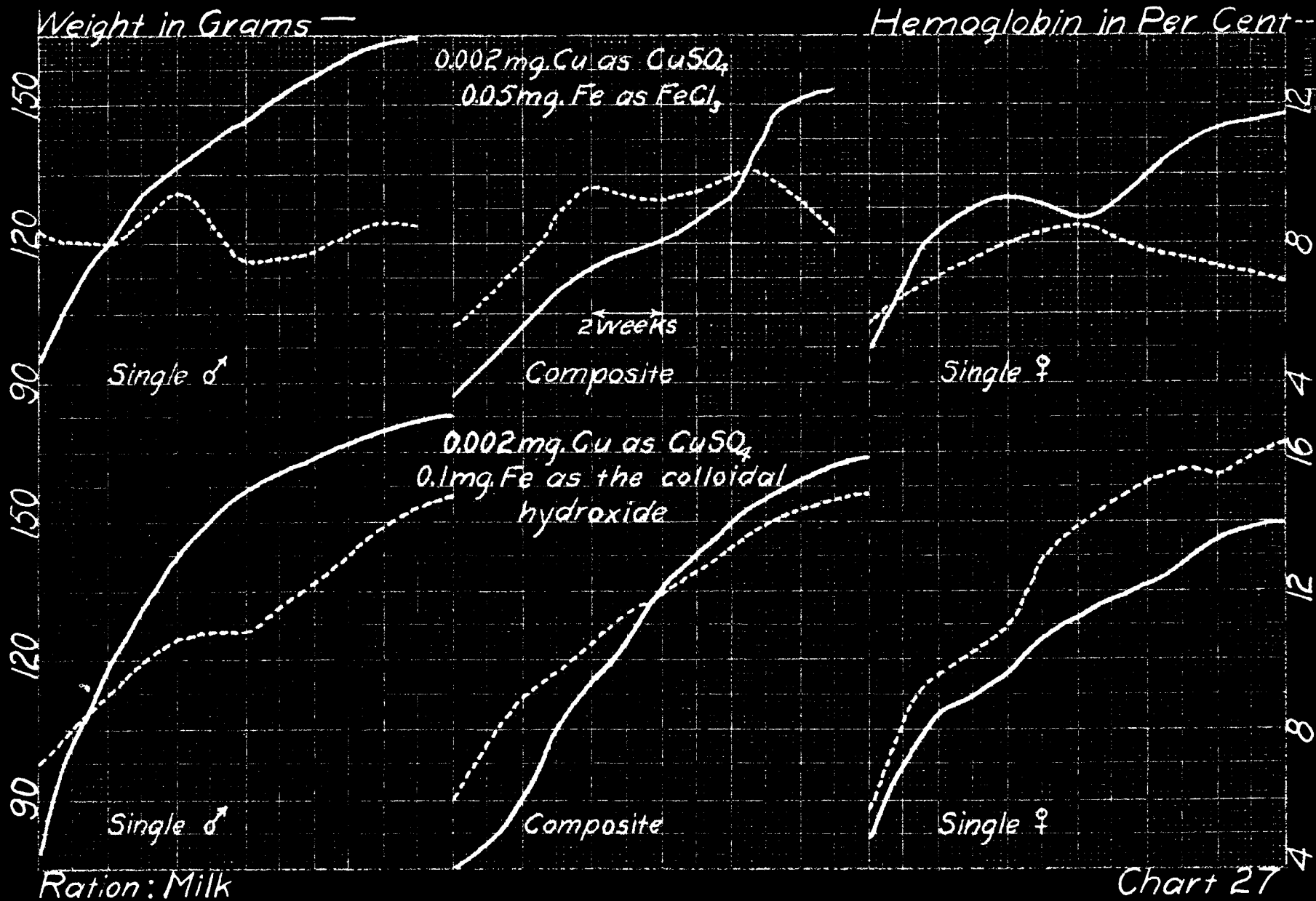
Single ♀
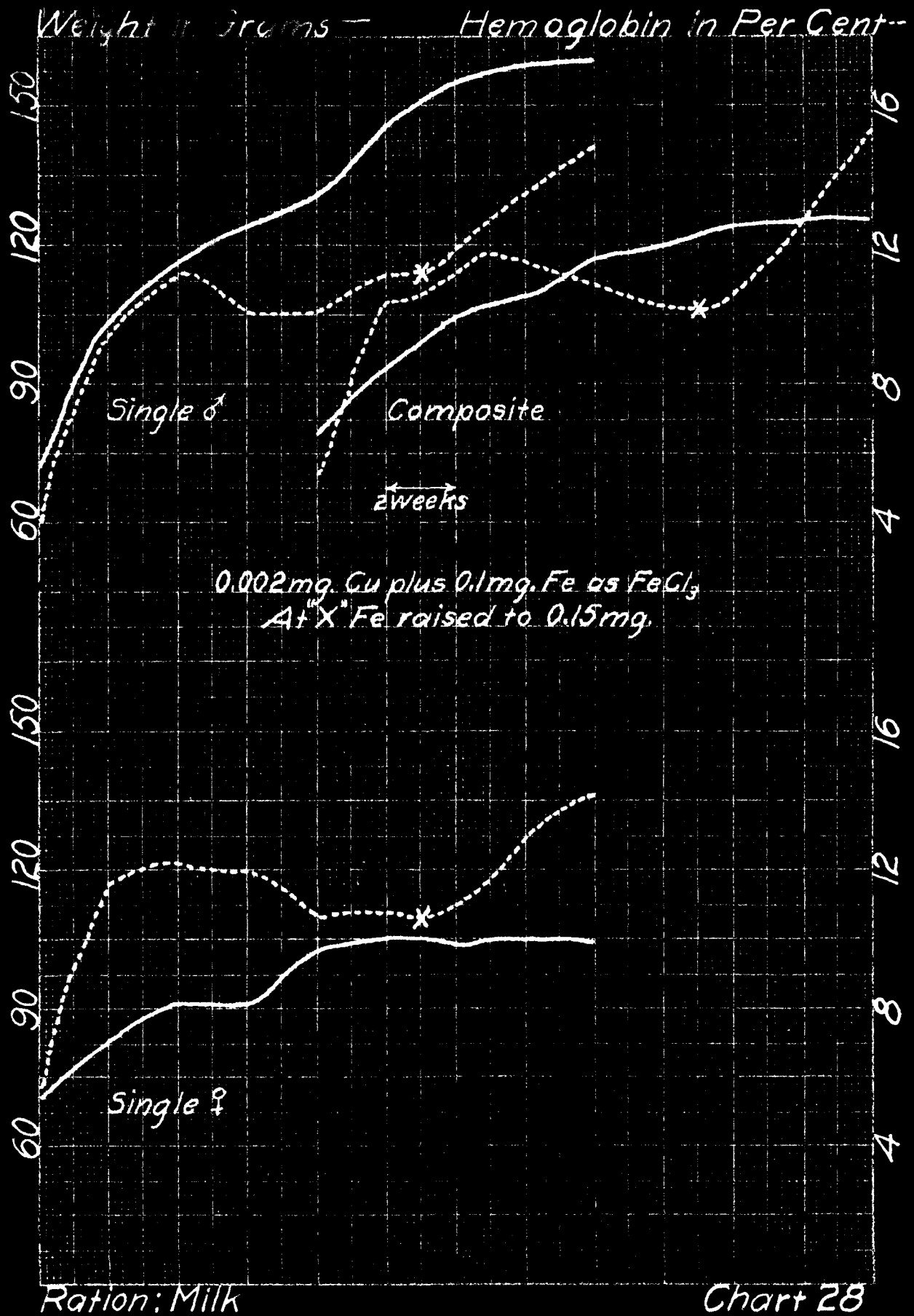
Ration: Milk   Chart 28

RESULTS OF Fe(OH)₃ ADMINISTRATION

Weight in Grams —   Hemoglobin in Per Cent--

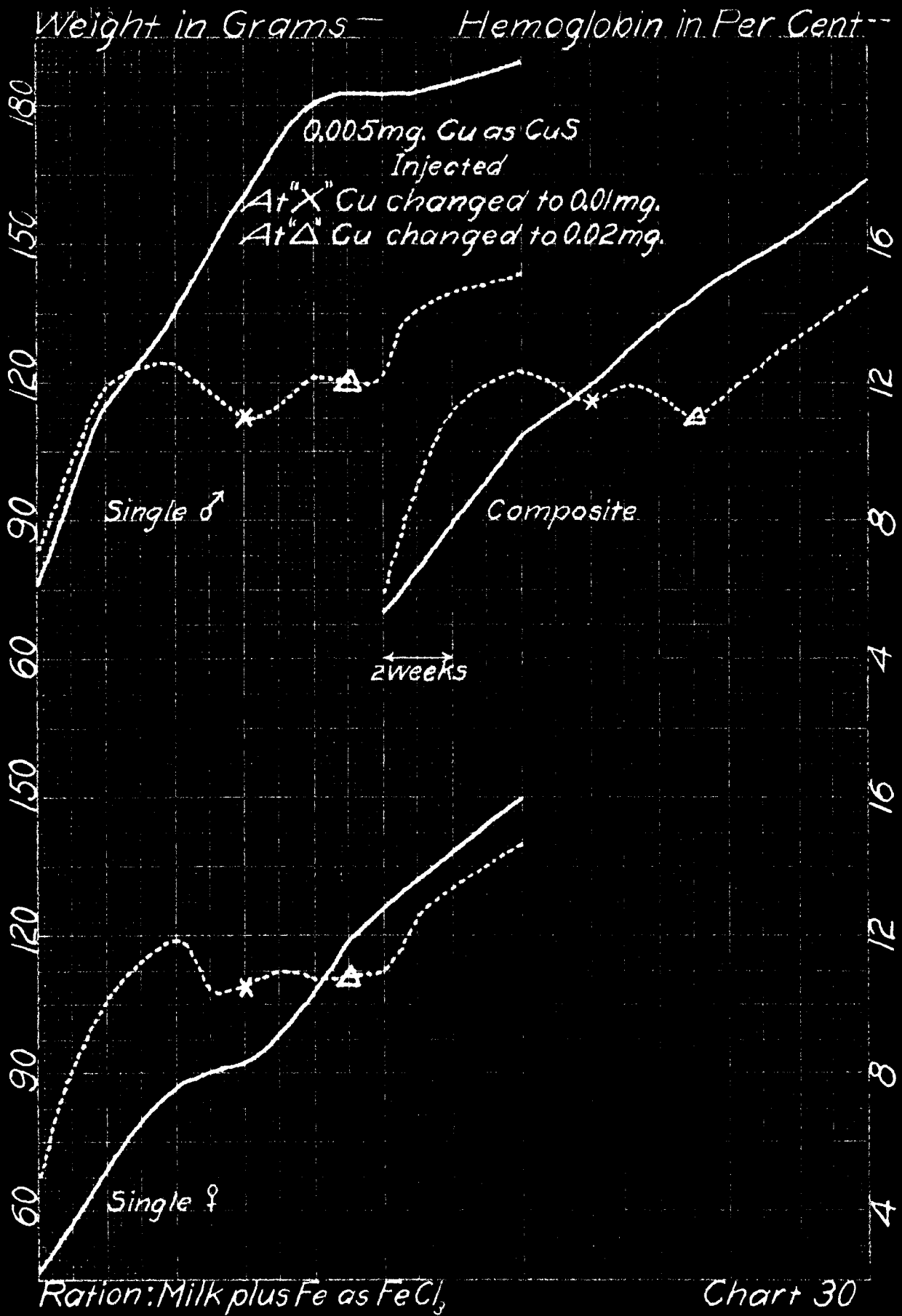### 0.5 mg. Fe Fed

Single ♂   Composite   Single ♀

2 weeks
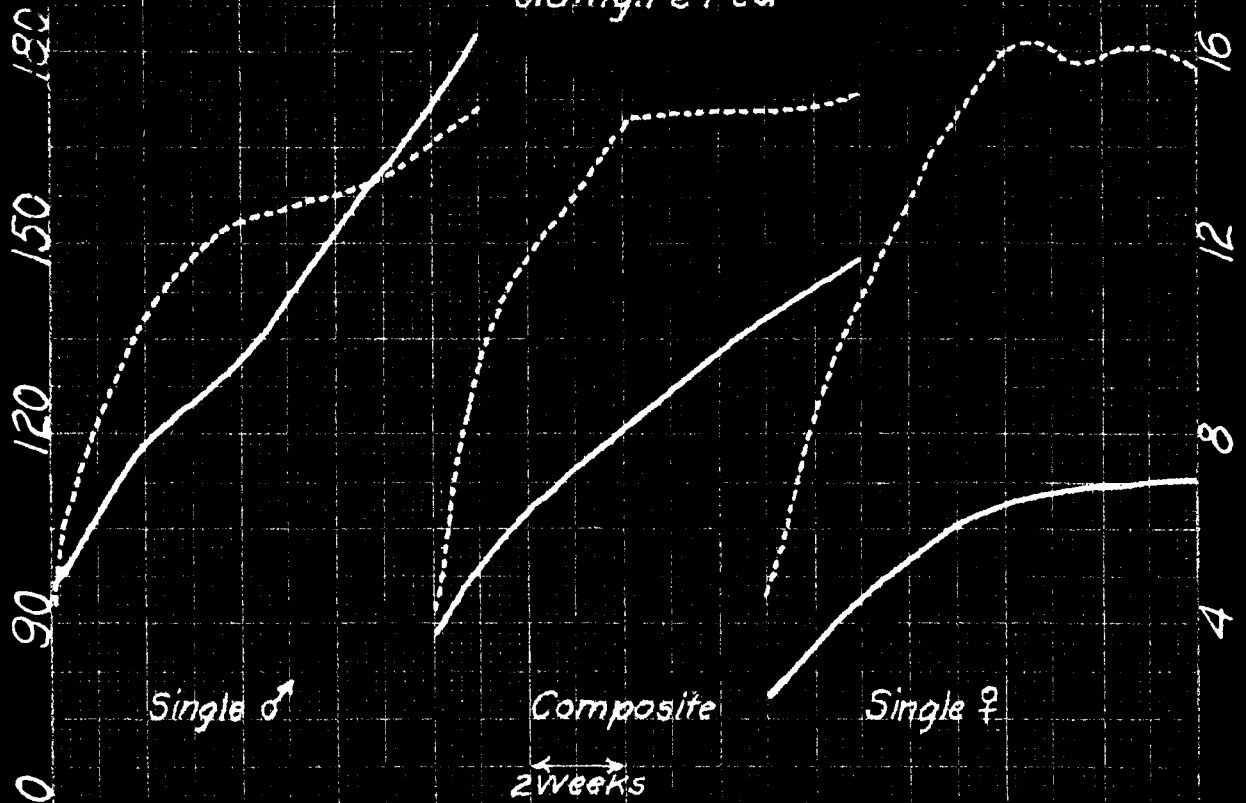
0.5 mg. Fe Injected
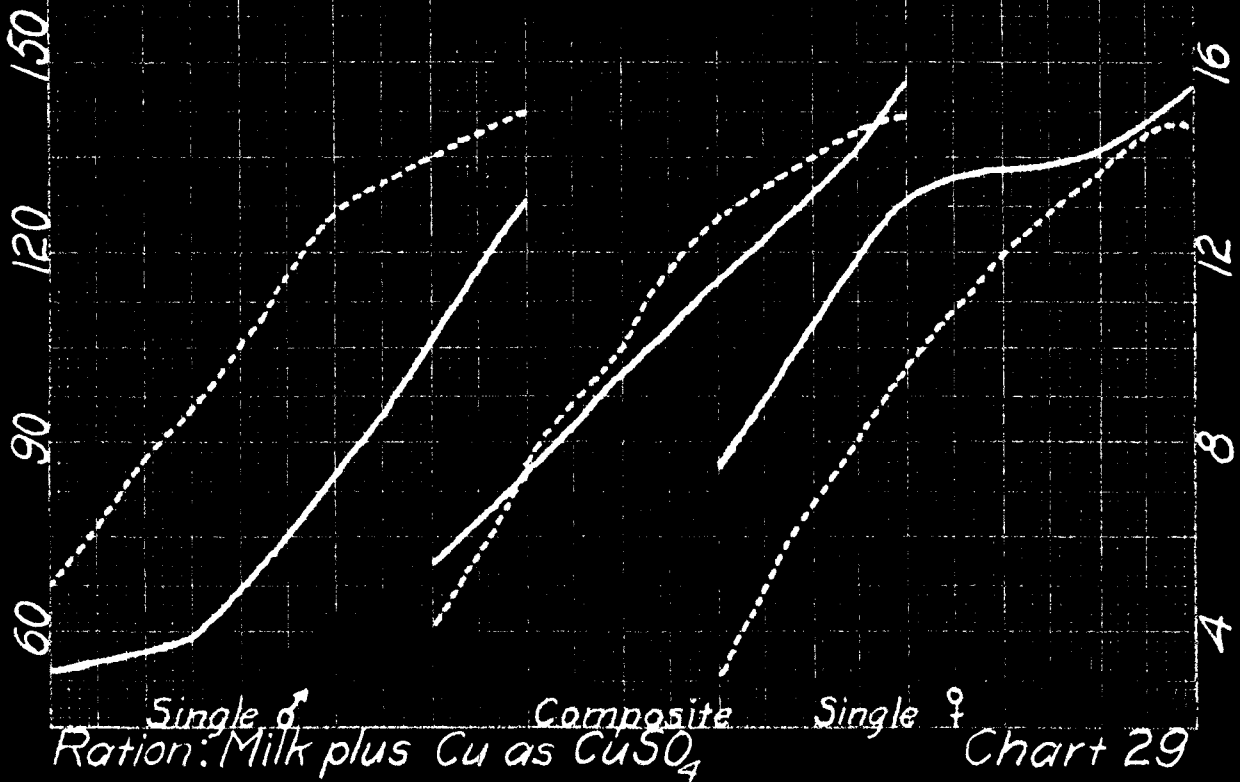
Single ♂   Composite   Single ♀

Ration: Milk plus Cu as CuSO₄   Chart 29

RESULTS ON INSOLUBLE COPPER COMPOUNDS

Weight in Grams —— Hemoglobin in Per Cent --

0.005mg. Cu as CuS
Injected
At "X" Cu changed to 0.01mg.
At "△" Cu changed to 0.02mg.

Single ♂

Composite

2 weeks

Single ♀

Ration: Milk plus Fe as FeCl₃

Chart 30

RESULTS ON INSOLUBLE COPPER COMPOUNDS

Weight in Grams ——— Hemoglobin in Per Cent ---

0.005 mg. Cu as Cu(OH)₂
Injected

Single ♂

Composite

2 weeks

Single ♀

Ration: Milk plus Fe as FeCl₃   Chart 31

INSOLUBLE COPPER COMPOUNDS

Weight in Grams —— Hemoglobin in Per Cent ---

0.01 mg. Cu as Cu$_2$O
Injected

Single ♂   Composite   Single ♀

a weeks

0.01 mg. Cu as CuI
Injected

Single ♂   Composite   Single ♀
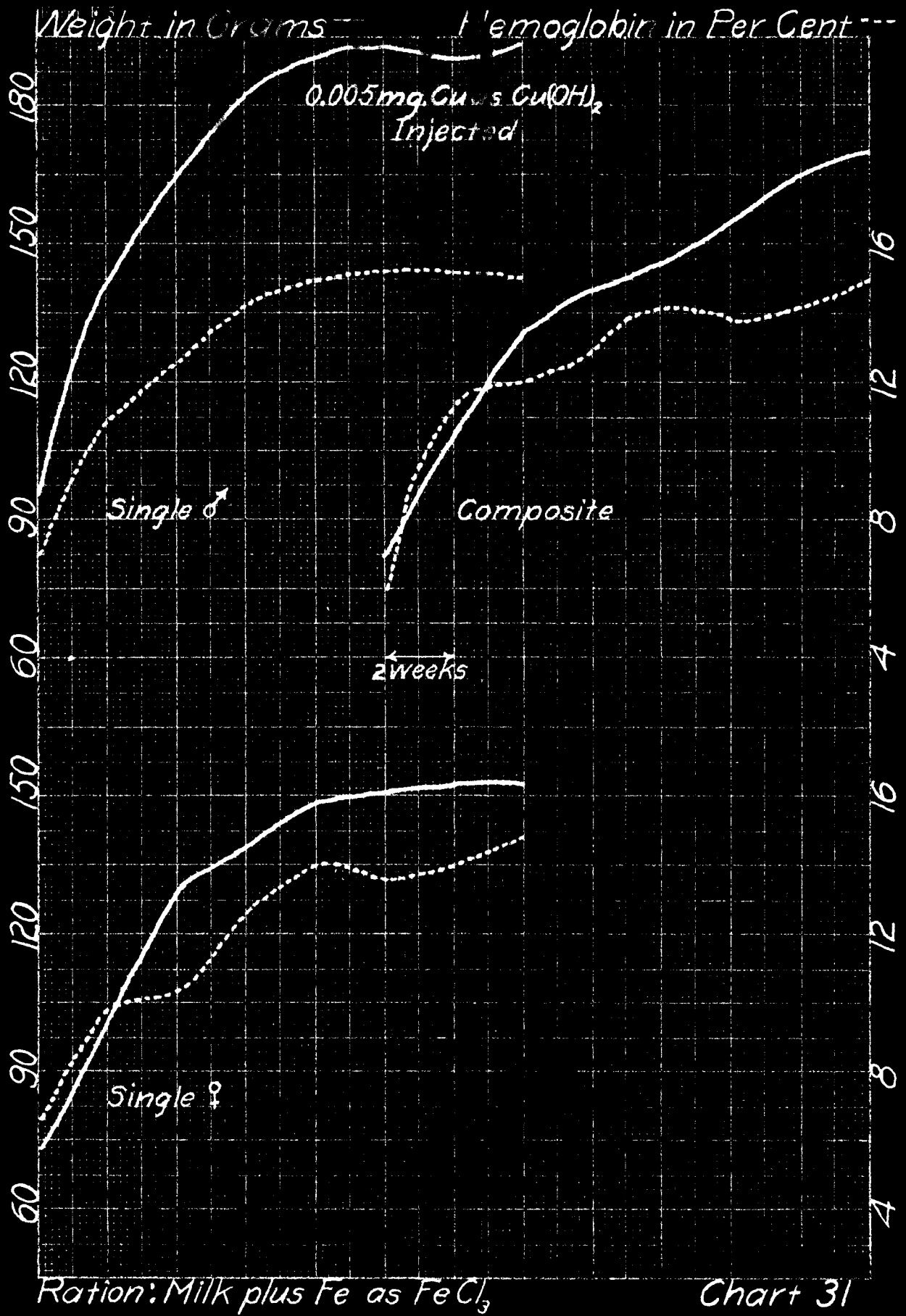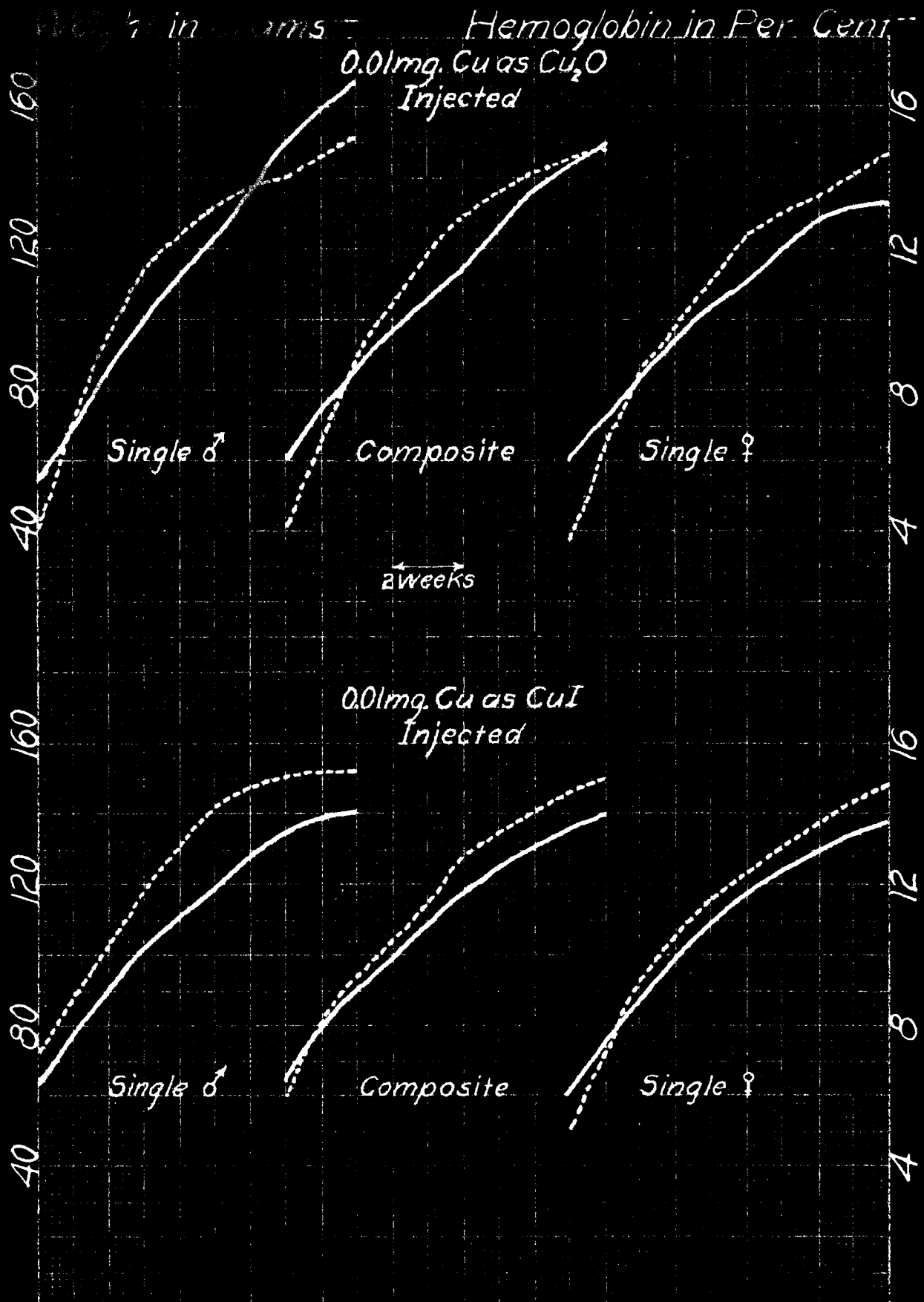
Ration: Milk plus Fe as FeCl$_3$ Chart 32

RESULTS OF FEEDING COLLOIDAL HYDROXIDES
Weight in Grams   Hemoglobin in Per Cent ---

Single ♂

Composite
0.05 mg. Cu as Cu(OH)₂
0.5 mg. Fe as Fe(OH)₃

2 weeks

Single ♀
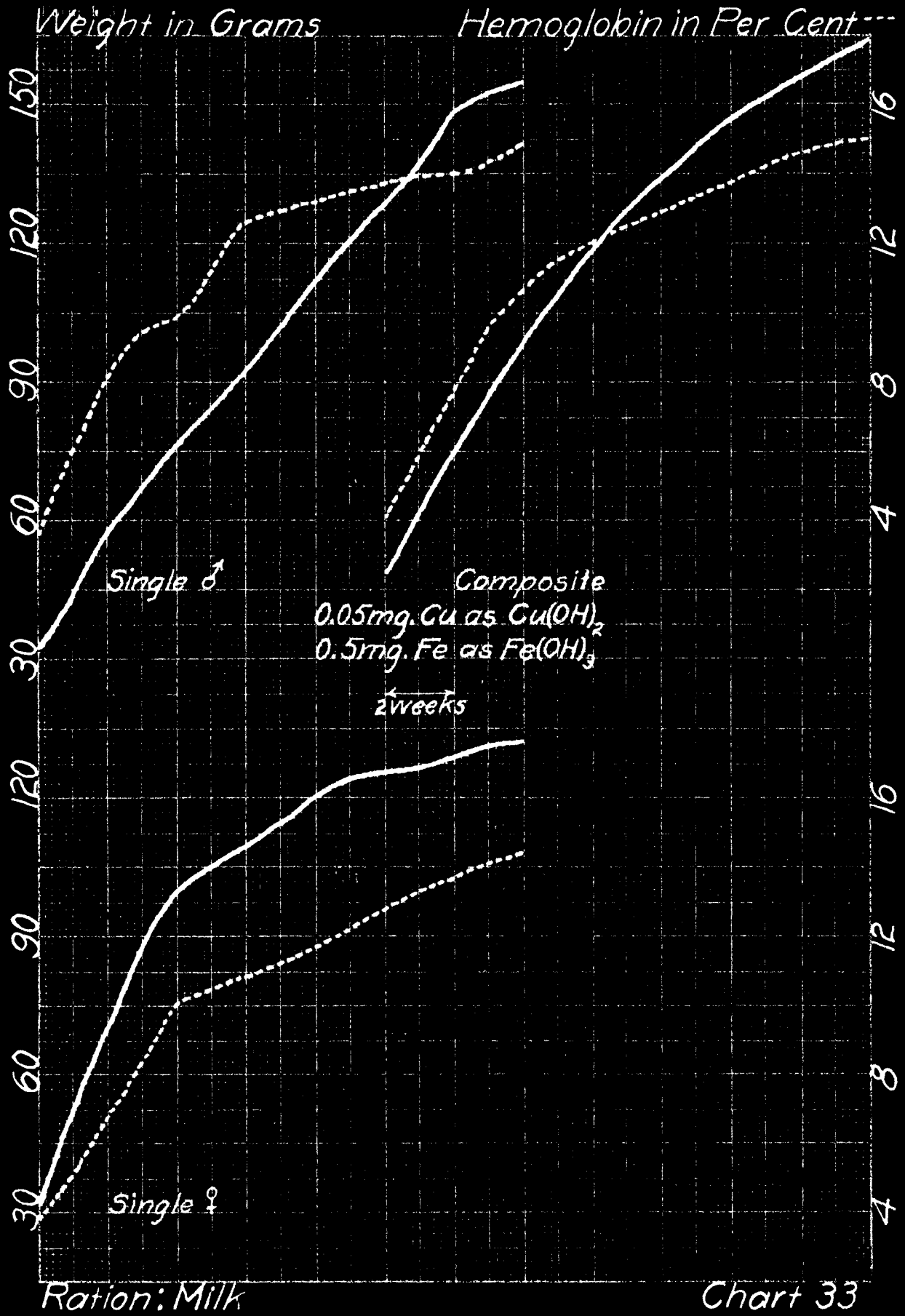
Ration: Milk   Chart 33

RESULTS OF COLLOIDAL HYDROXIDE INJECTIONS

Weight in Grams —   Hemoglobin in Per Cent

0.002 mg. Cu as Cu(OH)$_2$
0.1 mg. Fe as Fe(OH)$_3$

Single ♂   Composite

2 weeks

Single ♀

Ration: Milk   Chart 34