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I am submitting herewith a thesis written by Cing-ya Chiao entitled "Comparative Hematology During Deficiencies of Iron and Vitamin A in the Weanling Rats." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

John T. Smith, Major Professor

We have read this thesis and recommend its acceptance:

Jane R. Savage, Mary Rose Gram

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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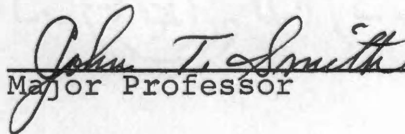
November 20, 1972

To the Graduate Council:

I am submitting herewith a thesis written by Cing-ya Chiao entitled "Comparative Hematology During Deficiencies of Iron and Vitamin A in the Weanling Rats." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.



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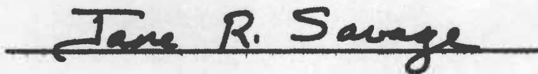

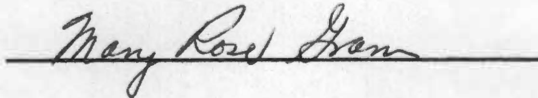

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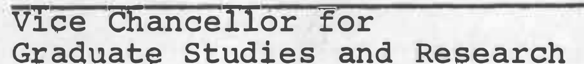

Vice Chancellor for
Graduate Studies and Research

COMPARATIVE HEMATOLOGY DURING DEFICIENCIES OF IRON AND VITAMIN A IN THE WEANLING RATS

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Cing-Ya Chiao

December 1972

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ABSTRACT

The purpose of this investigation was to determine the effect of feeding male, weanling rats diets which were deficient in both vitamin A and iron on hematologic parameters.

The hematologic parameters which were measured were hemoglobin concentration, hematocrit values, red blood cell counts and cellular morphology of the blood smears as well as the level of iron stored in the liver.

In the first experiment, it was found that rats with iron and vitamin A deficiency, not only had higher hemoglobin and hematocrit values as well as higher red blood cell counts. They also had higher levels of iron in their livers when compared with rats fed diets without iron. Rats deficient in iron developed hypochromic, microcytic anemia, whereas rats deficient in vitamin A had the highest hemoglobin and hematocrit values and red cell counts.

Growth rate was retarded in rats fed diets deficient in either iron or vitamin A. However, the greatest growth retardation was obtained in rats fed diets deficient in both iron and vitamin A.

In order to study whether iron storage of the liver is impaired in vitamin A deficient rats, the second experiment was designed using $^{59}Fe(NH_4)(SO_4)_2$ as a tracer. Rats were fed different levels of vitamin A; namely, "0", "4",

"250" IU per kilogram of body weight per day for a six week period. At the end of the dietary period no statistically significant difference in iron storage by the liver was observed in any of the three, i.e., vitamin A deficient, vitamin A free or the control groups.

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CHAPTER I

INTRODUCTION

It is generally believed that iron is one of the prerequisite elements for red blood cell formation, whereas vitamin A is a prime nutrient for visual studies. But there are several interesting observations about hemotological changes in association with vitamin A and iron deficiency.

McLaren et al. (1) in their survey of xerophthalmia in Jordan, pointed out that young children who were suffering from severe vitamin A deficiency had significantly higher hemoglobin and hematocrit values than those who were equally malnourished, but without clinical evidence of vitamin A deficiency. After reviewing the dietary picture of these children, which was undoubtedly low in protein, iron and other hematinic factors, McLaren et al. concluded that "These children were anemic but that the hemoconcentrating effect of vitamin A deficiency masked this by increasing hemoglobin and hematocrit values." However, in this study, hematological changes may have varied because of a deficiency of nutrients other than vitamin A or may have been complicated by intercurrent infections. A comparison of changes in children receiving diets low in vitamin A with those of rats fed diets restricted in vitamin A follows. Rats fed vitamin A

deficient diets gave evidence of a higher hemoconcentration than either normal animals or those fed a diet low in both vitamin A and iron (2,3).

Another interesting observation made by Pollard et al. and Kon (4,5) was that when vitamin A alcohol was given intravenously to anemic rabbits or rats, the vitamin A quickly disappeared from the circulation, and only one-fourth of the dose was found in the liver of the rats. Amine et al. (3) confirmed these results, the animals deficient in iron (with vitamin A) had serum vitamin A concentrations significantly lower than the groups fed a diet with both iron and vitamin A.

It seems that vitamin A is elegant in its photoreceptor role and yet is tantalizingly mysterious in its other biological involvements. Iron deficiency affects the metabolism of vitamin A. From the above observations it appears as if a study comparing the effect of both vitamin A and iron deficiency should be of value.

In this study, stress will be placed on the hematologic changes due to vitamin A deficiency, about which we know little, rather than hematologic changes in iron deficiency, about which we know a great deal. Finally, consideration will be given to whether iron uptake is impaired in vitamin A deficiency.

CHAPTER II

REVIEW OF THE LITERATURE

The early literature contains numerous references to the influence of vitamin A deficiency on various peripheral blood elements. Koessler et al. (6) reported that when rats are fed a vitamin A free diet, the following changes are observed. There is an initial weight gain, which is associated with a concomitant increase in hemoglobin and erythrocytes. Soon after, the weight curve began to go down, there was a drop both in erythrocyte count and hemoglobin concentration. These changes were soon followed by a rise of both erythrocyte count and hemoglobin concentration. Most reports pointed out that especially at the period of onset of xerophthalmia, the hematopoietic disturbance is characterized by changes in concentration of either hemoglobin or erythrocytes or both. The work was confirmed by Kik et al. (7) and Crimmand Short (8). However, their data may be complicated by the rather crude diets employed in the experiments and the possibility of another deficiency in addition to vitamin A. However, recent literature, using more clearly defined diets, has shown evidence for the hematologic effects in the blood of vitamin A deficient animals (2,9,10,11).

It seems no specific features have been described in man or laboratory animals in relation to the atrophy of blood-forming bone marrow and atrophy of lymphoid tissue--spleen, lymph nodes and thymus from hypovitaminosis A. There is, however, a greater deposit of hemosiderin in liver and spleen than that which accompanies a deficiency of other vitamins (12). Interestingly, the hemosiderin rapidly disappeared by resumption of vitamin A feeding. This work was linked with that of Koessler et al. (5). They demonstrated that following addition of vitamin A to the diet, the hemoglobin level as well as the erythrocyte count showed a decided decrease. After more than two weeks of vitamin A refeeding, erythrocytes and hemoglobin returned to normal. In a recent report, Amine et al. (3) found that vitamin A supplementation of a rat which was both deficient in iron and vitamin A reduced the red blood cell numbers but slightly increased hemoglobin and hematocrit values after eight days of treatment. It appears that vitamin A may exert some special effect on blood cell formation in the experimental animals.

In dealing with iron-deficient anemia in rats, numerous observations have documented that the development of the anemia was characterized either by changes in the morphology of red blood cells--microcytosis, hypochromia, poikilocytosis--or falling hemoglobin concentration, red blood cell number and volume (13-19).

Polycythemia anemia is a condition which is still not well understood. The term generally implies real increase in the number of circulating red cells with a corresponding elevation of the hemoglobin and hematocrit values (20). It has been shown that the increase in the total volume of red cells in polycythemia anemia is due to an increased rate of red blood cell production and not to an increase in the life span of the red cells (21-24).

The term "polycythemia anemia" related to the animal with increased red blood cell volume due to vitamin A deficiency was first introduced by Amine et al. (3). There are, in general, two theories of the basic abnormality in erythropoiesis in polycythemia: neoplastic and physiologic. Many patients or animals with hypoxia and an appropriate increase in erythropoiesis due to high altitude (25-27), pulmonary disease (28,29), chronic heart disease (30,31), or obesity with inadequate pulmonary ventilation (32), have been shown to have increased blood (25-28) or urinary (25,26) erythropoietin levels. Some cases are secondary to an inappropriate and autonomous secretion of hormone due to renal neoplasms, cysts or hydronephrosis, hepatomas, or other tumors (33,34). A possible model for the study of inappropriate erythropoiesis due to autonomous marrow production of red cells exists in leukemia or in rats with decreased level of androgens (35,36).

The pathophysiology, mentioned above, can be easily found in hypovitaminosis A animals or humans. One literature report showed high hematocrit values in cockerels due to a depression of androgen in vitamin A deficiency (11). The abnormal erythropoiesis may be complicated by intercurrent infections resulting from vitamin A deficiency. But the most interesting finding is that more ^{59}Fe was retained in the whole body tissue of vitamin A deficient rats than in the whole body tissue of normal rats (3). Whether the hematologic changes shown in vitamin A deficient rats are due to increased iron retention or not is unknown.

Decreased iron stores are a common occurrence in polycythemia. A recent study of polycythemia demonstrated that polycythemia is related to variations in the iron stores (37).

Liver is the main organ for vitamin A storage (38,39). Therefore, the choice of the liver for studying the relationship between iron and avitaminosis A seems reasonable.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. EXPERIMENT 1

Twenty-four weanling, male rats of the Sprague-Dawley strain were allotted to four groups according to the initial weight (50-60 gm). Group I was fed the basal diet without vitamin A and iron. Group II was offered the basal diet plus vitamin A. Group III was fed the basal diet plus iron. And Group IV, the control group, was fed the basal diet plus vitamin A or iron.

The composition of the basal diet is shown in Table 1. All animals consumed distilled water ad libitum. The rats were housed in individual cages. Feed and feed intake were recorded daily. Body weight was measured and recorded weekly. Blood samples were taken from a vein in the rat's tail every week. During week five all rats were killed by a blow on the head followed by rapid decapitation. The livers were saved for the determination of iron storage and vitamin A levels.

The detailed procedures for red blood cell counting, hematocrit measuring, hemoglobin determination, blood smear staining, and determination of iron and vitamin A concentration in the liver are delineated in the Methods section.

TABLE 1

COMPOSITION OF BASAL DIET[a]

Component	g/Kg diet
Casein (vitamin free)[b]	240
Cornstarch	660
Wesson oil	30
Iron-free-salt mixture[c]	50
Choline chloride	10
Vitamin mixture[d]	10
Vitamin D_2	0.023

[a]The compositions of the diets for different groups were: Group I--basal diet
Group II--basal diet + 4000 IU vitamin A acetate/Kg diet
Group III--basal diet + 250 mg iron as $FeSO_4 \cdot 7H_2O$/Kg diet
Group IV--basal diet + 4000 IU vitamin A + 250 mg $FeSO_4 \cdot 7H_2O$/Kg diet

[b]Nutritional Biochemicals Company, Cleveland, Ohio.

[c]See Table 2.

[d]The vitamin mixture contained in (mg) thiamin hydrochloride, 250; riboflavin, 150; calcium pantothenate, 400; nicotinic acid, 1000; pyridoxin HCl, 60; biotin, 6; folic acid, 40; menadione, 40; vitamin B_{12}, 0.5; inositol, 2000; para-aminobenzoic acid, 60; α-tocopheryl succinate, 600; with sufficient sucrose to make 100 gm.

TABLE 2

COMPOSITION OF SALT MIXTURE[a]

Component	Grams
NaCl	73.13
KH_2PO_4	204.15
$MgSO_4$	30.07
$CaCO_3$	200.20
KI	0.413
$MnSO_4 \cdot 2H_2O$	2.335
$ZnCl_2$	0.1363
$CuSO_4 \cdot 5H_2O$	0.2497
$CoCl_2 \cdot 6H_2O$	0.0119

[a]Source: Jones, J. H. and C. Foster 1945 J. Nutr. 24: 245.

II. EXPERIMENT 2

In order to determine if iron uptake was impaired in vitamin A deficient rats, fifteen weanling Sprague-Dawley rats were divided into three groups. The groups were fed diets with graded amounts of vitamin A. Group I received an adequate amount of vitamin A acetate (250 I.U.) per kilogram body weight per day.[1] Group II was fed inadequate vitamin A (4 I.U.) per kilogram body weight per day. Group III was fed a vitamin A free diet. The composition of the diet is the same as shown in Table 1, except 250 mg iron as ferrous sulfate was added per kilogram of diet. Following a six weeks dietary period 40 ug iron as $^{59}Fe(NH_4)(SO_4)_2$ labeled with 0.2 uCi ^{59}Fe was fed to each rat by stomach tube. The radioactivity was determined as described in the Methods section.

III. METHODS

Hemoglobin Concentration

Determination of iron concentration in hemoglobin solution.

1. Reagents. All of the reagents except the standard must be iron free.

   (a) Sulfuric acid, concentrated.

---

[1] Vitamin A intake, based on body weight, was approximated by supplementing 1 kg of diet with ten times the daily I.U. desired, found practical in pilot studies for these animals and conditions.

(b) Potassium persulfate saturated solution. Approximately 7 grams of reagent potassium persulfate was transferred to a glass-stoppered bottle. About 100 ml of water was added, and the bottle shaken until a solution was obtained. This solution should be prepared fresh each week.

(c) Sodium tungstate, 10% solution (weight per volume).

(d) Standard stock solution of iron. 100 mg of iron analyzed grade was weighed out and placed in a 500 ml beaker, 20 ml of concentrated HCl and 100 ml of water were added. The mixture was heated on a steam bath until solution was complete. The small carbon residue was disregarded. The solution was cooled to room temperature, transferred to a one-liter volumetric flask, and diluted to volume with distilled water. Complete mixing was obtained by repeated inversion and shaking of the flask. Each milliliter contained 0.1 mg of iron.

(e) Potassium thiocyanate, approximately 3N KSCN in the amount of 146 grams was transferred to a beaker and dissolved in about 300 ml of water; 20 ml of acetone was added as a preservative.

The solution was transferred to a 500 ml volumetric flask and diluted to the mark. Only those solutions which were cloudy were filtered.

2. Calibration curve. Five milliliters of a standard stock solution of iron were diluted to 100 ml with water in a volumetric flask and mixed well. Using a wax pencil, one of the following numbers was placed on each of eight, 25 ml volumetric flasks: 0, 1, 2, 3, 5, 8, 10, and 15. Using a buret, the number of milliliters of dilute iron standard that corresponded to the numbers on the flasks was added to each flask. Then 0.4 ml of concentrated sulfuric acid was added to each flask, and the mixture diluted to about 15 ml with distilled water. One ml of saturated potassium persulfate was added, and the solutions were mixed thoroughly.

The Beckman B spectrophotometer was adjusted to a wavelength of 540 nm. To the flask marked "0" 4 ml of 3N potassium thiocyanate solution was added. The mixture was diluted to 25 ml with distilled water, stoppered and mixed thoroughly. The solution was poured into a cuvet of appropriate size which was then placed in the spectrophotometer to serve as the "zero" blank.

To the flask marked "1" 4 ml of 3N potassium thiocyanate was added. The mixture was diluted to 25 ml with distilled water, stoppered and mixed thoroughly. The cuvet was rinsed twice with the solution from flask "1" and then filled. The cuvet was placed in the spectrophotometer, the absorbance was determined and the reading recorded. The reading must be made within three minutes, after the addition of color reagent. The addition of potassium thiocyanate to each of the remaining flasks was repeated.

A calibration curve was prepared on coordinate paper, using the following figures to correspond to the amounts of standard solution used: 0, 5, 10, 15, 25, 40, 50 and 75 mg of iron per 100 ml of blood; and plotting against the corresponding reading in the spectrophotometer.

Determination of hemoglobin concentration.

1. Reagents.

   (a) Ammonium hydroxide, 0.04%. A stock solution is prepared by diluting 2.35 ml ammonium hydroxide (17%, $^{v}/v$) to 1000 ml with distilled water.

   (b) Oxalated blood, 10 to 15 milliliters.

2. Calibration curve. Ten to 15 ml of oxalated blood was centrifuged at 2000 rpm for 15 minutes in an

International centrifuge, size 1 (Model SBV). Plasma was removed and discarded. Physiological salt solution was added to the cells in an amount two or three times the volume of the cells. The cells were carefully resuspended and centrifuged in the International Model SBV, size 1, at 2000 rpm for 15 minutes and the supernatant saline solution was removed and discarded. The cells were washed three times more. To the washed cells an equal volume of distilled water was added. The water was mixed with the cells until they hemolyzed. The solution was centrifuged as before, and the bottom layer of "cell ghosts" removed. The iron concentration was determined on duplicate samples of the resulting hemoglobin solution.

An empty 25 ml volumetric flask was marked "B" (blank); to this flask was added 0.4 ml of concentrated sulfuric acid, and approximately 15 ml of distilled water. One ml of potassium persulfate solution was then added. The spectrophotometer was adjusted to a wavelength of 540 nm. Four ml of 3N potassium thiocyanate solution was added to the flask marked "B" and the mixture diluted to the mark with distilled water. After thorough mixing the solution was transferred to a cuvet, placed in

the spectrophotometer and its absorbance determined and recorded.

Five-tenths ml of well-mixed blood was transferred to a dry 50 ml volumetric flask. Two ml of concentrated iron-free sulfuric acid was added and mixed by whirling one or two minutes. Two ml of saturated potassium persulfate solution was added, mixed and the mixture diluted to about 25 ml with distilled water. Three ml of 10% sodium tungstate solution was added and mixed. The resultant solution was cooled to room temperature and diluted to 50 ml with distilled water. After thoroughly mixing, the mixture was allowed to stand for five minutes. The contents of the flask were then filtered through a dry filter, and the filtrate collected in an Erlenmeyer flask. Ten ml of the filtrate were transferred to a 25 ml volumetric flask, marked "Hb".

Four ml of the potassium thiocyanate solution was added to the flask marked "Hb". The mixture was diluted to 25 ml with distilled water and mixed. The cuvet was rinsed twice, filled and read in the spectrophotometer within three minutes. The reading was recorded and the concentration of the unknown was determined from the calibration curve of iron concentration in hemoglobin. The results were

averaged and the hemoglobin concentration determined as follows:

$$\frac{\text{Mg of iron per 100 ml}}{3.40} = \text{Gms of hemoglobin per 100 ml of solution}$$

Three, 4, 5 and 8 ml of NH_4OH solution were transferred to cuvets. The hemoglobin solution, in the amount of 0.02 ml was transferred to each cuvet, using the calibrated Sahli pipet. The pipet was rinsed three times in each solution. The cuvets were shaken vigorously. The spectrophotometer was adjusted to a wavelength of 540 nm. A cuvet was labeled "B" (blank), filled with 0.4% NH_4OH solution and placed in the spectrophotometer. Absorbance readings were recorded. The hemoglobin concentration of each dilution was calculated by multiplying the concentration of Hb solution by the appropriate dilution factor (Table 3).

Red Blood Cell Counting

1. Reagent.

(a) Diluent: 100 ml of 3% sodium citrate, 1 only of formalin (37-40%) and 0.6 g eosin B.

In making a count, blood is drawn by suction into a Thoma red cell pipet to the 0.05 mark or, if there is marked

TABLE 3

THE APPROPRIATE DILUTION FACTOR, HEMOGLOBIN IN SOLUTION CALCULATED, HEMOGLOBIN CONCENTRATION AND SPECTROPHOTOMETER ABSORBANCE[a]

NH_4OH ml	Dilution factor	Hemoglobin in solution g/100 ml	Calculated Hb concentration g/100 ml	Spectrophotometer reading
3	1.331	4.410	5.869	0.280
4	1.000	4.410	4.410	0.210
5	0.801	4.410	3.532	0.185
8	0.501	4.410	2.209	0.130

[a]Method of Sheard and Sanford, Szigeti, Modified 1940 Biochem. 34: 1460.

anemia, to the 0.1 mark. This must be done meticulously since any error is magnified 200 times by the subsequent dilution. The blood adhering to the outside of the pipet is next wiped off and the diluent is drawn in until it fills the bulb and reaches the mark 101. After the desired quantity of solution has been drawn into the pipet, it is held horizontally and shaken in order to secure thorough mixing. Shaking constantly in any one direction should be avoided. The mixing should be repeated each time before expelling a drop for examination.

The cover-glass is next placed on the counting chamber. Several large drops of fluid are expelled from the pipet and discarded. This is done in order to remove the fluid in the capillary portion of the pipet which has not come in contact with the blood. A small quantity of diluted blood is then placed between the cover-glass and the ruled platform of the counting chamber. The fluid will run under the cover-glass by capillary attraction. The platform should be completely covered with fluid and none should run over the sides.

After a few minutes have been allowed for the blood to settle, the slide is examined to ensure even distribution of the cells in chamber. If distribution is satisfactory, the cells are counted. The high power objective (450 x) of the microscope should be used.

In the new Neubauer ruling the small squares in the central square millimeter are employed in the enumeration of red cells. The smallest squares are counted, a volume of 0.00025 × 80 or 0.02 c.mm. has been covered. In order to give the value per 1 c.mm., the number of cells counted must be multiplied by $^{1}/0.002$, or 50. However, since the dilution was 1 to 200, the multiplication factor is 50 × 200 or 10,000.

Determination of Hematocrit

Blood was drawn by capillary attraction into the heparinized microtube until it was one-half to two-thirds full. The tube was then closed with sealing compound and centrifuged at 800 xg for thirty minutes. The height of packed cells and the height of packed cells plus plasma was measured. The calculations were done by the following equation:

$$\frac{\text{Height of packed cell}}{\text{Height of packed cell + plasma}} \times 100 = \text{\% hematocrit}$$

Blood Smear Staining

The air-dried blood film was placed with smeared side upwards on a support from which it could be picked up. The blood smear was completely covered with Wright's stain, which was allowed to remain for one minute. The stain was then diluted with distilled water. This was added drop by drop, using approximately the same number of drops of water

as had been used of dye. A greenish metallic scum should appear and the margins should show a reddish tint. After three or four minutes, the stain was washed off with water until the film was yellowish or pink. After the preparation had been thoroughly washed, it was stood on edge to dry.

Photomicrographs of blood smears were taken with Miranda-Sensorex-f 1.4, with a Kodak ATB, 80-B Series 5 filter, magnification (500X).

Determination of Vitamin A Level in Liver

The livers of four groups (I, II, III, IV) were lyophilized overnight, and ground into a powder with a mortar and pestle.

Liver powder equivalent to one-half liver was shaken with 40 ml of anhydrous ether for two minutes. The ether was decanted into a 50 ml conical centrifuge tube, and the sediment was packed by centrifugation in the International Model SBV, Size 1, centrifuge for ten minutes at 2000 rpm. The ether was decanted into a 125 ml Erlenmeyer flask of actinic glass. The liver powder was extracted a second time with 20 ml of anhydrous ether, which after it was centrifuged and the supernatant fluid was added to the flask, the ether was evaporated; the last few milliliters of ether were removed under a stream of nitrogen. The residue was dissolved immediately in 10 ml of chloroform. One-half milliliter of the chloroform solution was taken for the determination. When

the samples contained higher levels of vitamin A, a further dilution with chloroform was necessary to obtain absorbance readings from 0.2 to 0.8.

The spectrophotometer was set at 620 nm. Five milliliters of antimony trichloride reagent (20 gm of antimony trichloride in sufficient reagent grade chloroform to make 100 ml) were added to the cuvet as a blank. For vitamin A measurements, one-half milliliter of the test solution was pipetted into a cuvet followed by four and one-half milliliters of the antimony trichloride reagent. The antimony trichloride was added rapidly and the cuvet was transferred immediately to the spectrophotometer. The maximum steady absorbance, which was obtained in approximately three seconds, was recorded.

<u>Determination of the Iron Level in Liver</u>

1. <u>Reagent.</u>
   - (a) Perchloric-sulfuric acid, 7:1 v/v
   - (b) Concentrated nitric acid
   - (c) Nitric acid, 50%
   - (d) Sulfuric acid, 1%
   - (e) Working solution: 100 ppm iron in aqueous solution
   - (f) Iron standards: 25 ml solution containing 5, 10, 15 and 20 ppm iron

Liver powder equivalent to one-half liver was added to a 125 ml Erlenmeyer flask.[2] Four ml of distilled water and 4 ml of concentrated nitric acid were added to the flask. The mixture was digested on an electric heater and the fluid volume was replenished with 50% nitric acid as needed. If oily drops occurred, 0.5 to 1 ml of perchloric-sulfuric reagent was added until they disappeared.

The clear solution was allowed to cool. One milliliter of demineralized water was added, mixed thoroughly and the contents of the Erlenmeyer flask were quantitatively transferred to a 25 ml volumetric flask and the flask filled up to the mark with demineralized water.

The percent absorption due to the iron was determined with a Perkin-Elmer atomic absorption spectrophotometer 303. The absorption was converted to absorbance by use of a conversion chart.

<u>Determination of ^{59}Fe Uptake by Liver</u>

Since a survey of the literature could not document the time required for the liver mechanism to complete its uptake of a test dose of ^{59}Fe, a time study was conducted. Nine groups of three rats each were sacrificed 1, 2, 3, 4, 6, 8, 12, 24, and 48 hours after feeding 40 ug of iron as ^{59}Fe $(NH_4)(SO_4)_2$ labeled with 0.2 uCi ^{59}Fe by stomach tube.

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[2] Thirty ml Kjeldahl flask is too small to digest sample. Four samples of Group I in Experiment 1 were lost.

After the appropriate time intervals the animals were sacrificed and their livers removed, weighed and homogenized. A constant volume of the homogenate (1 ml, 20% of homogenate) was transferred to a counting vial with 10 ml toluene-ppo and 8 ml 2-ethoxyethanol for counting in the scintillation counter (Picker nuclear/liquimat 220).

Based on the results of the time study, eight hours was selected as the optimum time between ^{59}Fe feeding and sacrifice of the animals.

Three groups of five rats were fed different levels of vitamin A: . "0", 4 IU/kg body weight per day, 250 IU/kg body weight per day. The same dose of ^{59}Fe as was used in the time-study was tube fed to the rats. Analyses of the liver were carried out by combusting one-third of the dried liver in pill form in a Parr sulfur bomb apparatus. After combusting, duplicate 1 ml aliquots of the sample were added to counting vials by pipet. Ten ml of toluene-ppo and 8 ml of 2-ethoxyethanol were added to the vials and their ^{59}Fe activity evaluated with a Picker nuclear/liquimat 220. The ^{59}Fe activity was expressed as CPM/g of liver.

Statistical Method

The data were analyzed by the method of Duncan's new multiple-range test. All computations were performed using the Olivetti-Underwood programma 101.

CHAPTER IV

RESULT

Growth

The mean difference in weight gain per week and mean feed intake per week from Experiment 1 are shown in Figure 1. Growth was reduced as feed intake decreased; this phenomenon began during the second week of the experiment. Moreover, statistical analysis of the mean body weight gain (Table 4) reveals that there was a highly significant difference between the deficient groups and the control group ($p < 0.05$).

A comparison of weight gain between iron and vitamin A deficient groups is presented in Table 4. Animals fed the diet without added iron and vitamin A gained less weight than those fed diets low in either iron or vitamin A.

In Experiment 2, the control group had a statistically significant higher weight gain than animals fed either vitamin A inadequate (4 IU/kg body weight per day) or vitamin A free diet ($p < 0.05$). No statistically significant difference was found between the two deficient groups (Table 4).

Hematologic Parameters

A summary of hematologic parameters for control, iron or vitamin A deficient, and iron plus vitamin A deficient rats after a four-week dietary treatment is given in Table 5.

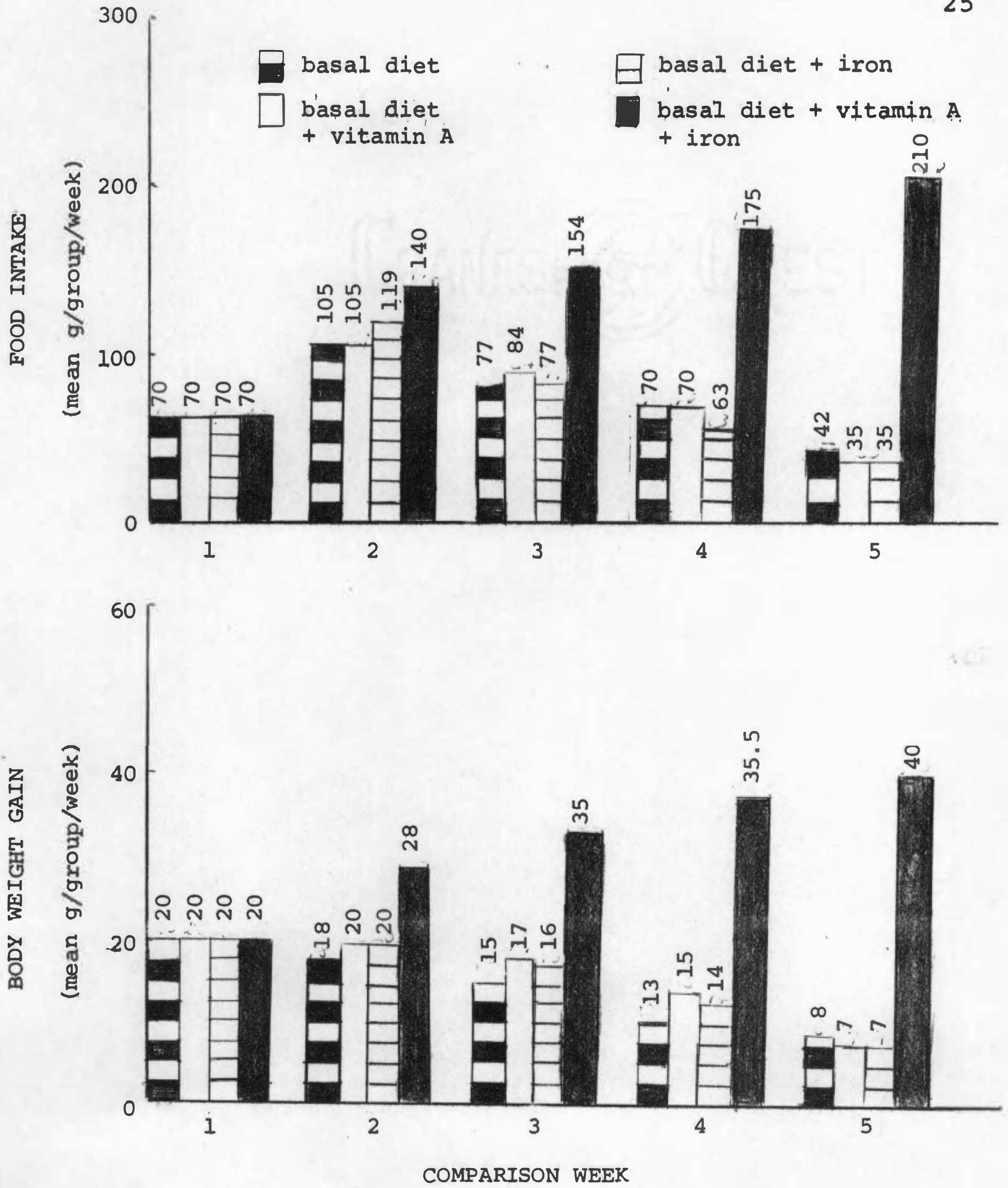


Figure 1. Comparison of food intake and body weight gain during dietary treatment in Experiment 1.

TABLE 4

MEAN BODY WEIGHT GAIN AND STANDARD DEVIATIONS FOR THE 5-WEEK AND 6-WEEK COMPARISON PERIOD FOR RATS IN EXPERIMENT 1 AND EXPERIMENT 2

Diet	Mean + Standard Deviation
EXPERIMENT 1	
Group I: basal diet	73.3 ± 6.7[a]
Group II: basal diet + vitamin A	79.0 ± 7.0[a,b]
Group III: basal diet + iron	84.3 ± 5.5[b]
Group IV: basal diet + vitamin A + iron	117.0 ± 14.2[c]
EXPERIMENT 2	
Group I: 250 IU vitamin A/Kg body weight/ day	223.0 ± 10.3
Group II: 4 IU vitamin A/Kg body weight/ day	183.6 ± 14.2[a]
Group III: 0 IU vitamin A/Kg body weight/ day	172.6 ± 12.2[a]

[a,b,c]All means without common superscripts differ significantly, $P < 0.05$.

TABLE 5

MEAN TERMINAL VALUES AND STANDARD DEVIATIONS FOR HEMATOLOGIC PARAMETERS, IRON AND VITAMIN A STORAGE IN LIVER FOR THE FOUR WEEK COMPARISON PERIOD OF RATS IN EXPERIMENT 1

Criterion	DIETS			
	basal diet	basal diet + vitamin A	basal diet + iron	basal diet + vitamin A + iron
BLOOD				
Hemoglobin, g/100 ml	8.1 ± 1.6[a]	7.4 ± 1.9[a]	16.2 ± 0.6[b]	14.1 ± 0.2[c]
RBC, millions/mm^3	4.1 ± 0.9[a]	3.8 ± 0.2[a]	8.7 ± 1.4[b]	5.2 ± 0.3[c]
Hematocrit, %	27.6 ± 5.0[a]	24.7 ± 4.3[a]	49.9 ± 2.0[b]	43.3 ± 6.3[c]
Mean cell volume, u^3	67.3 ± 1.2[a]	65.0 ± 3.2[a]	57.4 ± 0.5[b]	83.3 ± 2.1[c]
Mean corpuscular hemoglobin, rr	20.0 ± 1.8[a]	18.9 ± 2.0[b]	23.4 ± 0.6[b]	27.2 ± 1.0[c]
TISSUE STORAGE				
Iron, storage, mg/100 gm	0.57 ± 0.2[a]	0.41 ± 0.2[a]	42.2 ± 13[b]	42.1 ± 10[b]
Vitamin A storage	none	have	none	have

[a,b,c]All means without common superscripts differ significantly, $P < 0.05$.

These data include mean hemoglobin values, red blood cell counts (R.B.C.), hematocrit values and the indices calculated from the foregoing values--mean red cell volume (M.C.V.), and mean corpuscular hemoglobin (M.C.Hb). Also included are the values for iron and vitamin A storage in the liver. The statistical significance of differences in the means are recorded under the Table. It is to be noted that all hematologic parameters of those rats which were deficient only in vitamin A are significantly different from those of the other three groups ($p < 0.05$). Rats fed diets deficient in both iron and vitamin A have slightly higher mean values for hemoglobin concentration, R.B.C. count and hematocrit values than animals fed the iron deficient diet. But the values were not as high as those of rats fed the vitamin A deficient diet. The complete series of red blood cell counts and hemoglobin concentration as determined at intervals throughout the experiment are presented in Figure 2.

Photomicrographs of the blood smears of the treatment rats are shown in Figure 3. The erythrocytes from iron-free rats are smaller than average (microcytosis); the hemoglobin content of these cells was also low (hypochromia). A large number of elliptical cells (poikilocytosis) and target cells were very evident. In the doubly deficient rats, the red blood cells showed less variation in size or hemoglobin content than in the iron deficient rats. In addition, the

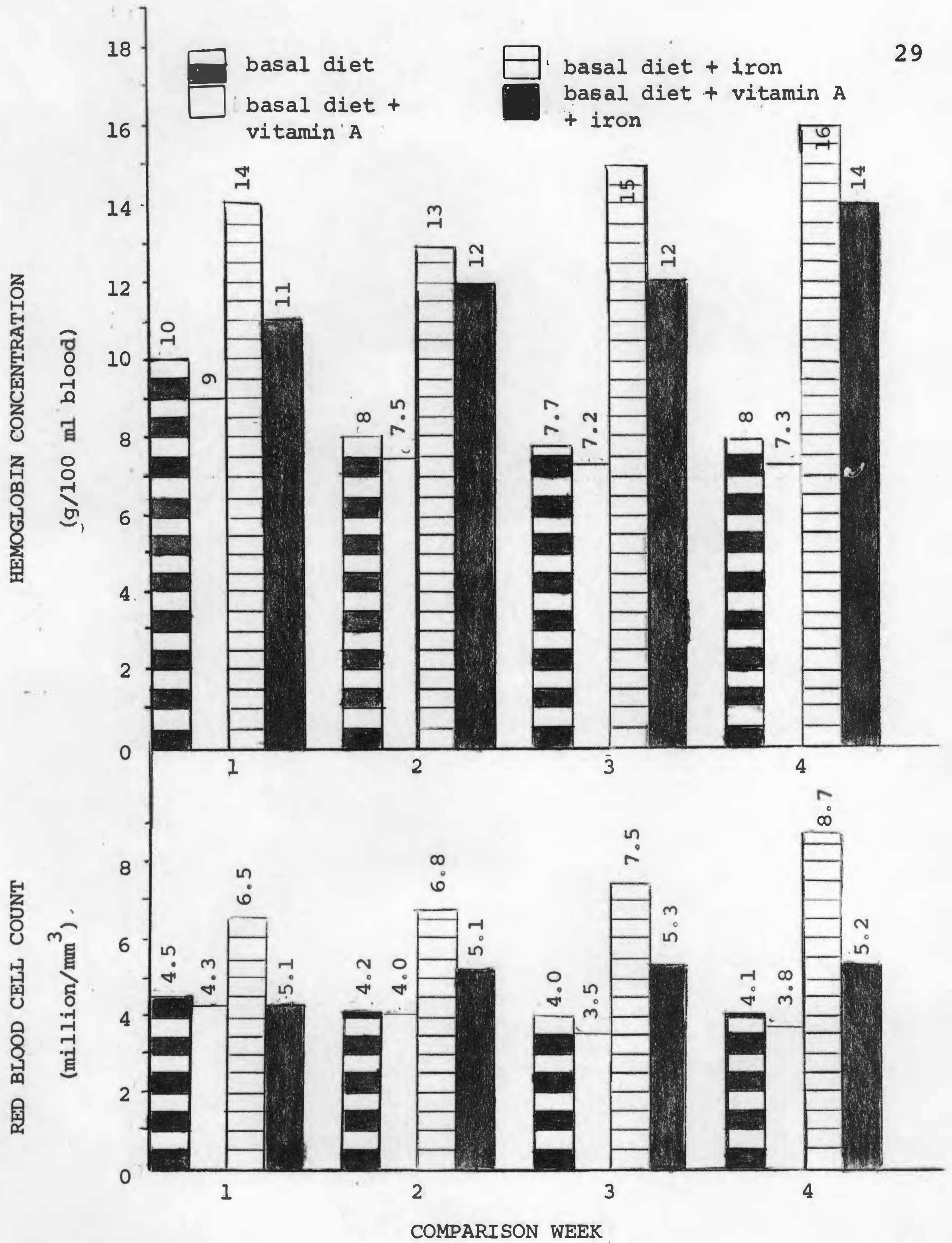
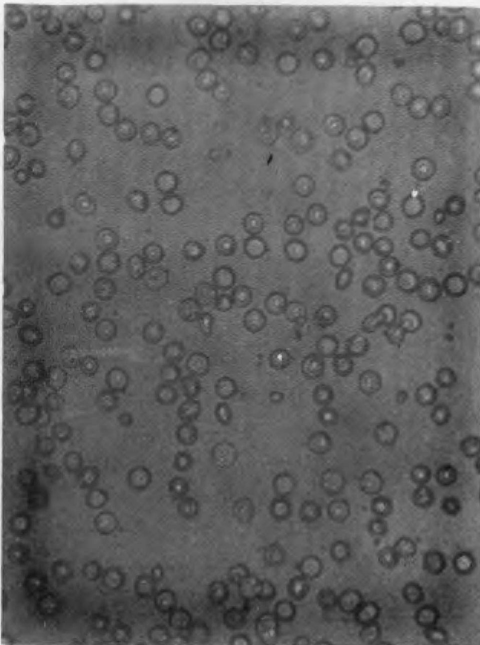
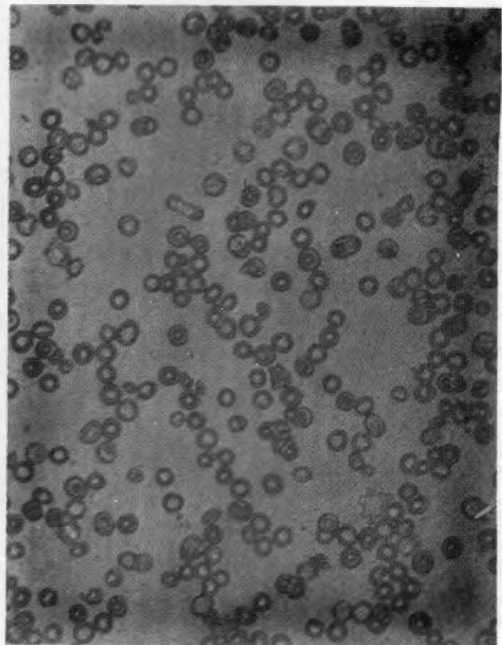


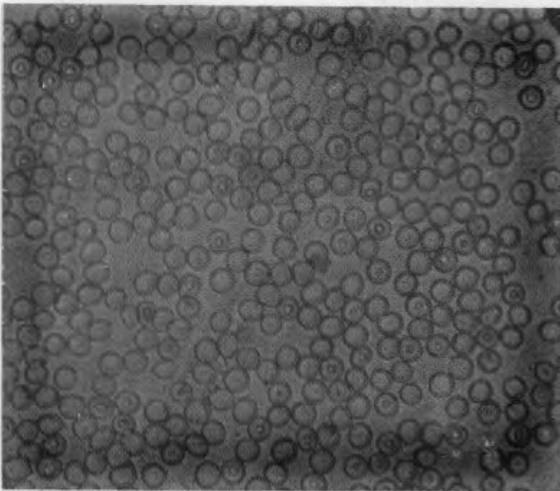
Figure 2. Comparison of hemoglobin concentration and red blood cell count between different dietary treatment in Experiment 1.



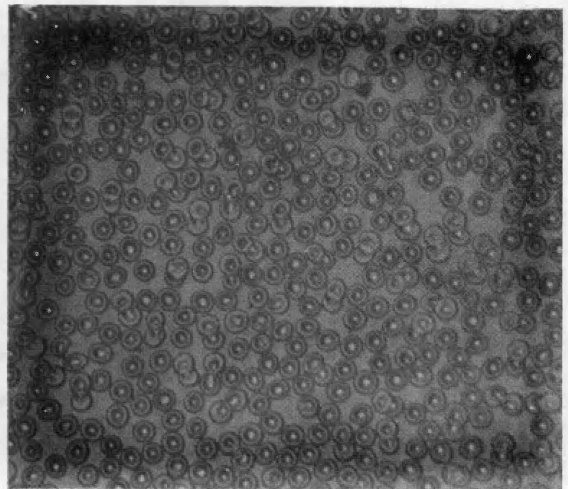
a. Vitamin A and iron deficiency.



b. Iron deficiency.



c. Vitamin A deficiency.



d. Control group.

Figure 3. Characteristic morphology of red blood cells from rats in each dietary group.

degree of poikilocytosis was relatively slight. Disk-sphere, spindle-like or tear-drop shaped erythrocytes were numerous and pronounced in the case of the vitamin A deficient rats. The erythrocytes of the control group were biconcave and normal in size.

Iron storage in the liver of rats fed the vitamin A free diet was slightly higher as compared to the control group. But the difference was not statistically significant. Similar results were obtained between the double deficient and iron deficient rats.

Because of problems encountered in the determination of vitamin A, no comparison could be made.

The ^{59}Fe Uptake by Rat's Liver

The uptake of a test dose of $^{59}Fe\ (NH_4)(SO_4)_2$ by rat's liver with time is given in Table 6. The absorption (CPM/g of liver) of the dose taken up by the rat's liver rose highly in eight hours, fell at twelve hours and then slightly rose at twenty-four and forty-eight hours.

The ^{59}Fe Uptake by Liver of Rats Fed Different Levels of Vitamin A Diet

Results of the analysis of ^{59}Fe uptake by the liver of the three groups fed various levels of vitamin A are presented in Table 7. It can be seen that there was no significant difference between the ^{59}Fe uptake of any of the groups;

TABLE 6

UPTAKE OF A TESTING DOSE OF $^{59}Fe(NH_4)(SO_4)_2$ BY RAT'S LIVER WITH TIME

Time after ^{59}Fe Force Feeding	Uptake $^{59}Fe(NH_4)(SO_4)_2$
Hours	CPM/g liver
1	512 ± 12[a]
2	505 ± 25
3	412 ± 18
4	386 ± 19
6	389 ± 5
8	1336 ± 20
12	466 ± 8
24	1196 ± 7
48	1217 ± 18

[a]Mean uptake of ^{59}Fe by three rats per time period ± standard deviation.

TABLE 7

MEAN VALUE AND STANDARD DEVIATION OF $^{59}Fe(NH_4)(SO_4)_2$ UPTAKE BY LIVER OF RATS FED DIFFERENT LEVELS OF VITAMIN A

Group	Counts/minute/g dried liver
I: 250 IU vitamin A acetate per kilogram body weight per day	153 ± 77[a]
II: 4 IU vitamin A acetate per kilogram body weight per day	115 ± 33[a]
III: 0 IU vitamin A acetate per kilogram body weight per day	111 ± 26[a]

[a]All means without common superscripts are significantly different at $P < 0.05$.

however, the mean radioactivity of ^{59}Fe in either the vitamin A inadequate or free group was less than that of the controls.

Clinical Sign of Vitamin A or Iron Deficient Animals

The signs of avitaminosis A which were present in the depleted animals were mild, in either Experiment 1 or 2. The rats were small in size as compared to the controls; some of them exhibited a thinning of the fur on the head and slight inflammation and crustiness of the eyelids. Whereas, iron deficient rats were lethargic and had rough coats and white incisor teeth.

CHAPTER V

DISCUSSION

All of the deficient groups were characterized by growth retardation as compared to the control groups. Growth depression in iron deficiency has been demonstrated in studies designed to determine the effect of iron deficiency on enzymes containing iron in heme (catalase, cytochrome, and cytochrome oxidase) and nonheme groups (succinic dehydrogenase) and iron-requiring groups (aconitase)(40). Srivastava (41) found hemoglobin concentration decreased from 15.8 to 7.6 grams per 100 ml of blood, and liver glucose-6-phosphate dehydrogenase activity was completely lost in iron deficient rats. Also inadequate oxygenation resulted from the lowered red blood cell counts or hemoglobin concentration.

Animals fed a vitamin A deficient diet lost weight. This has been attributed to diminution of appetite, resulting in a depression of the rate of protein synthesis (42), and impairment of oxidative phosphorylation in the liver as evidenced by an elevated rate of pyruvate, citrate, α-ketoglutarate, succinate, glutamate and fumarate oxidation (43).

As we mentioned before, the single deficient animals demonstrated a comparable growth picture. It thus appears as if the effect of vitamin A deficiency is independent of the effect of iron deficiency.

In the experimental group deficient in both vitamin A and iron, three phenomena were evident: (1) growth depression, (2) a slightly higher hemoglobin concentration and red cell count and, (3) a higher mean iron storage, compared to iron deficient rats. The results suggest that growth retardation is not impaired by poor iron absorption due to vitamin A deficiency (Table 7, page 33), but rather, that the growth retardation is a reflection of hypovitaminosis A. The data from Experiment 2 also suggest that vitamin A does exert a special role in hemopoiesis but indirectly functions to conserve iron for hemopoiesis. This suggestion is supported by the data that were obtained with the vitamin A deficient group; the iron storage in liver was slightly higher than in the control group. The results of other investigations should be born in mind, namely that an anemia in children or animals may be masked by hemoconcentration and give a normal biochemical test in iron.

As can be seen in Figure 3, page 30, rats that received a vitamin A free diet showed a disk-sphere, tear-drop or spindle shaped erythrocytes without bioconcavity. Little is known about the biochemical basis of the maintenance of the bioconcave shape of erythrocytes. It has been proposed that inhibition of the membrane ATPase activity, decreased binding of ATP to the membrane, or decreased cell diameter, may induce disk-sphere transformation. Early reports about the

mode of action of vitamin A in maintaining the functional integrity of subcellular membranes have been intensively investigated but are still a matter of speculation (44). In addition to lysosomes, other membranes, such as those of erythrocytes and mitochondria are sensitive to vitamin A deficiency (45). In rats, the erythrocyte membrane was distorted in hypovitaminosis A, and bound ATPase was more readily released; this effect of vitamin A was observed in vivo (46). Retinol itself in vitro can cause a loss in the smooth contour or increase the hemolytic properties of red cells as was observed by Dingle and Lucy (47).

Several questions can be raised. For example, will erythrocytes with the disk-sphere shape function in oxygen-carbon dioxide transport? Does the change in size or shape have any advantageous effect such as to lower the blood viscosity of animals with hemoconcentration due to vitamin A deficiency?

The degree to which the fluctuation in hemoglobin concentration or erythrocyte count among the vitamin A deficient rats may be attributed to decreased food consumption or to a real increase in blood mass is uncertain.

In the present study a slightly higher storage of iron in vitamin A deficient rats is shown in Table 5, page 27. In Experiment 2, iron absorption was not significantly impaired by vitamin A. Two explanations are possible.

First, Yoshikawa and Yoneyama reported the activity of the enzyme Ferrochelatase as determined by ^{59}Fe incorporation into heme, increased as the phospholipid concentration increased (48). However, phospholipid alone did not catalyse the insertion of iron into protoporphyrin. A hypothesis based on this idea is that the increase in iron retention might have been due to solubilisation of protoporphyrin in the presence of a "special substance" which might be synthesized or released under vitamin A deficiency.

Secondly, recent studied have shown that homogenates of human and rat liver, spleen and human bone marrow, are sources of an iron-incorporating enzyme system (I.I.E.). Subfractionation of these tissue components revealed that the mitochondria were the richest sources of I.I.E. Beaufay et al. (49) also found a significant peak of iron in liver light mitochondrial fraction. The light liver fraction is richest in lysosomes; and based on electron microscope observations, particles loaded with ferritin are concentrated together with lysosomes. The nature of the relationship between I.I.E. system--ferritin--lysosome--vitamin A seems to be worthy of additional study.

CHAPTER VI

SUMMARY

A study was made of the effects of vitamin A, iron and a deficiency of both vitamin A and iron on hematologic changes of fifty-six day old male rats of the Sprague-Dawley strain.

Rats, fed iron and vitamin A deficient diets had slightly higher iron storage with normocytic hypochromic anemia than iron deficient rats who developed hypochromic microcytic anemia. Hemoconcentration was pronounced in vitamin A deficient animals. Iron storage in the hypovitaminosis A animals is high when compared with the control group but was not statistically significant.

The possibility of a decrease in iron absorption as a result of avitaminosis A was investigated but no statistical significance was obtained.

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VITA

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