



Blood and enzyme changes in rats fed hypercholesterolemia-inducing diets  
by Jane Alexandra Afanasiev

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of  
Master of Science in Home Economics

Montana State University

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**Abstract:**

This study was made to investigate the interrelationships between the hematopoietic processes, serum lipids and the biological oxidation mechanism as affected by dietary factors. Groups of male rats were fed a basal hypercholesterolemia-inducing diet; a similar diet with and without a certain level of ascorbic acid; also a similar diet but without iron; and the same basal diet with additional minerals for seven weeks. Biochemical changes that occurred within the blood, serum, heart, liver and kidneys of these rats were determined chemically and the data analyzed statistically. Rats fed any of the five hypercholesterolemia diets, when compared to rats' fed the chow diet, had significantly (a) lower hemoglobin and hematocrit levels; (b) higher values for serum protein; (c) higher values of succinic dehydrogenase activity in the heart, when expressed in relationship to the rat weight; and (d) lower values of succinic dehydrogenase activity in the liver and kidney when expressed either as  $\mu\text{l O}_2/\text{hr}/\text{mg}$  dry weight or related to the weight of the rat. Although not analyzed statistically, all rats fed the hypercholesterolemia-inducing diets had hearts and livers weighing more than, and kidneys weighing less than, those rats fed the chow diet, when expressed as g/100 g of rat. No statistical differences were observed in the biochemical values obtained for rats fed either the diets with and without ascorbic acid or the diet with the additional minerals, when these values were compared to those obtained from rats fed the basal hypercholesterolemia-inducing diet. A relationship was found, however, in rats fed an iron-deficient high-fat diet between disturbances in the hematopoietic processes and the iron-transport system.

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HYPERCHOLESTEROLEMIA--INDUCING DIETS

by

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## ABSTRACT

This study was made to investigate the interrelationships between the hematopoietic processes, serum lipids and the biological oxidation mechanism as affected by dietary factors. Groups of male rats were fed a basal hypercholesterolemia-inducing diet; a similar diet with and without a certain level of ascorbic acid; also a similar diet but without iron; and the same basal diet with additional minerals for seven weeks. Biochemical changes that occurred within the blood, serum, heart, liver and kidneys of these rats were determined chemically and the data analyzed statistically. Rats fed any of the five hypercholesterolemic diets, when compared to rats fed the chow diet, had significantly (a) lower hemoglobin and hematocrit levels; (b) higher values for serum protein; (c) higher values of succinic dehydrogenase activity in the heart, when expressed in relationship to the rat weight; and (d) lower values of succinic dehydrogenase activity in the liver and kidney when expressed either as  $\mu\text{l O}_2/\text{hr}/\text{mg}$  dry weight or related to the weight of the rat. Although not analyzed statistically, all rats fed the hypercholesterolemia-inducing diets had hearts and livers weighing more than, and kidneys weighing less than, those rats fed the chow diet, when expressed as g/100 g of rat. No statistical differences were observed in the biochemical values obtained for rats fed either the diets with and without ascorbic acid or the diet with the additional minerals, when these values were compared to those obtained from rats fed the basal hypercholesterolemia-inducing diet. A relationship was found, however, in rats fed an iron-deficient high-fat diet between disturbances in the hematopoietic processes and the iron-transport system.

## INTRODUCTION

Hypercholesterolemia-inducing diets, which contain adequate amounts of iron for the hematopoietic processes under normal dietary conditions, have been shown to induce a suboptimal blood state and a condition of abnormal lipid metabolism in rats, guinea pigs and rabbits (1-6). A review of the literature (7-13) indicates a relationship between (a) the ascorbic acid intake of certain animals and the condition of hypercholesterolemia, (b) dietary ascorbic acid and the hematopoietic processes and, (c) dietary ascorbic acid and the enzymatic system (biological oxidation mechanism) of certain body organs.

However, there are no reports concerning the effect of dietary ascorbic acid on these three conditions as they might be affected by the feeding of high fat or hypercholesterolemic diets. The intent of this research is to investigate the effect of supplementary ascorbic acid on these three phenomena, serum lipids, hematopoietic processes and the biological oxidation mechanism when hypercholesterolemic diets are fed.

The rat was chosen as the experimental animal for these tests even though this animal is capable of synthesizing its own body needs for ascorbic acid under normal conditions (14). It is known that the rat responds to added ascorbic acid under certain conditions (14,15) and that it is added to the usual vitamin mixtures used in rat feeding experiments (16). Ascorbic acid is a highly reductive substance and it is suggested that the animal body makes use of additional amounts of this factor in at least one area (14), over and above that needed for normal body metabolism, because of its chemical properties.

## REVIEW OF LITERATURE

Pinter and Bailey (1) observed that rabbits fed cholesterol-containing diets, developed a hemolytic anemia. The red cell count decreased and reached the lowest level at about 8-12 weeks after the rabbits were placed on the diet. After conducting cross-transfusion studies, they concluded that the anemia developed as the consequence of the production of red cells through an alteration in the function of the erythropoietic tissue.

Roehm and Mayfield (2) using rats, fed diets containing a medium level (13% fat) and a high level (34%) of either animal or vegetable fat, with and without additional cholesterol. Both levels of fat were fed with and without an additional mineral supplement. These additional minerals did not affect the cholesterol and lipid values of the serum and liver. The rats fed butter-containing diets had higher serum cholesterol and higher liver lipids than did rats fed the vegetable-fat diets. There was no difference in liver cholesterol values due to the type of fat fed. Diets with a high level of either fat brought about higher cholesterol and liver lipid values than did similar diets containing a medium level of fat. The rats fed diets containing the high level of either vegetable or animal fat (34%) had hemoglobin levels of approximately 2 g/100 ml lower than did the rats fed similar diets with the medium level of fat (13%).

Hemolytic anemia in rats was examined by Priest and Normann (3). They observed that rats fed a diet high in butter, cholesterol, sodium cholate and thiouracil became hyperlipemic and anemic. Two of these animals that had the greatest concentration of serum cholesterol were also

the most severely anemic. The anemia was characterized by lowered hematocrit levels, reticulocytosis, and hyperbilirubinemia.

Ostwald and Shannon (4) found that guinea pigs fed a semi-synthetic diet containing 1% cholesterol developed enlarged and fatty livers, very large spleens and hemolytic anemia. Feeding the cholesterol produced a large increase in the cholesterol ester fraction of livers, plasma and spleens. It also produced an increase in the unesterified cholesterol. The authors suggested that, in the guinea pig, the rate of cholesterol esterification was insufficient to maintain the normal tissue-lipid composition when cholesterol was included in the diet.

In a study by Wohl and Merskey (5), rats were fed diets containing cholesterol (5%), thiouracil and cholic acid. Hemoglobin and hematocrit levels rose initially, then fell progressively. After 60 days, the hemoglobin levels were 1.5 g/100 ml less and hematocrit levels were 8% less than the controls. They concluded that the atherogenic diets fed to the rats brought about an abnormal development of the red cells.

Roehm and Mayfield (6) investigated the interrelationships between hemoglobin levels and serum lipids in rats fed hypercholesterolemia-inducing diets. These diets were fed with and without 1% added cholesterol and with and without sufficient iron. The control rats were fed a commercial chow. Hemoglobin, hematocrit and serum iron of the rats fed the experimental diets were lower, and serum protein higher, than those for the control rats. Serum cholesterol and serum triglyceride levels were markedly increased in the iron deficient rate.

Similar decreases in hemoglobin and hematocrit values occurred when man was given multiple infusions of a fat emulsion as reported by Mueller and Viteri (17). The anemic conditions they observed were of the normocytic and normochromic types and associated only rarely with reticulocytosis. The genesis was not established but by negative reasoning it was concluded to be "dilutional" anemia. The factors governing the phenomenon of lowered hemoglobin and hematocrit levels have not been elucidated at the present time.

According to recent evidence, the activity of some of the enzymes functioning in the electron transport-biological oxidative mechanism, particularly that of succinic dehydrogenase (SDH), has been linked to the iron content of the enzyme (18-20). Singer, et al. (18) state that the iron of the SDH molecule is bound so tightly that no method has been found for its removal without causing denaturation of the protein. More recent work by Kearney and Singer (19) as summarized by Velick (20) states that SDH contains one to two atoms of iron per molecule with the activity directly proportional to this iron. Also stated is that SDH is not affected by chelating agents with high affinities for ferrous and ferric ions.

The SDH activity in the heart, liver and kidney of rats fed iron-deficient diets was studied by Beutler and Blaisdell (21). They observed that after prolonged, moderately severe iron deficiency, no decrease in SDH activity was found in the liver. However, SDH activity was lowered in the hearts and kidneys of these iron-deficient rats.

In their investigation, Roehm and Mayfield (6) report that the liver SDH activity per gram of rat appeared related to the hypercholesterolemic effect of the diet and not to its effect of lowering the hemoglobin level. The heart SDH activity was not affected. The SDH activity of the kidney was lower and appeared to be related to the lowered hemoglobin level.

According to a recent article by Beutler (22), the symptoms of iron-deficiency anemia, commonly attributed to the lowering of blood hemoglobin levels were possibly due to disorders in tissue metabolism. He also stated that in iron deficiency, cytochrome c, cytochrome oxidase, aconitase and the SDH activity were depleted. At the present time, the exact principle by which the SDH activity affects the hematopoietic processes is unknown.

Basic investigations concerning the iron content and iron-binding capacity of the serum or plasma of rats and humans under conditions of normal nutrition have been reported (23-29). In their studies, Itzhaki (23) and Itzhaki and Belcher (29) found that rats of different strains, fed diets varying widely in their iron content, had similar plasma iron levels. There was no correlation between either body weight or age and plasma iron concentration.

Beutler (25) and Beutler and Blaisdell (21,26) using the rat, investigated the relationship of the iron enzyme system to the iron content and iron-binding capacity of the serum under hypercholesterolemic conditions as well as in the normal state. It was reported by Rechenberger and Hevelke (27) that when man was given intravenous iron (1 mg/kg) there

was a definite relationship between the rate of disappearance of the iron from the blood stream and the age of the person.

Diurnal variations in the plasma iron of man were observed by Hamilton, et al. (28). They found that the plasma iron underwent a regular variation with the highest values occurring during the early morning, decreasing during the day and reaching the lowest level during the evening.

Hemoglobin concentrations, fasting serum iron and serum iron-binding capacity of men and women given a measured amount of iron daily were studied by Verloop, et al. (29). Hemoglobin concentrations increased, and fasting serum iron and serum iron-binding capacity decreased a similar degree in both sex groups during the time of the study. The difference between the sexes was attributed to differences in endocrine systems.

Investigations into the possibility that nutritional variations may affect the hematopoietic and iron transport systems have been made recently (7-13). Takeda and Hara (7) stated that iron was part of the biological oxidation system, in which ascorbic acid has been found to play a role. It was their proposal that the primary function of ascorbic acid was to mobilize the ferrous iron.

In a study performed by Tantengco, et al. (8), ascorbic acid was given to groups of cockerels in conjunction with nicotinic acid and L-triiodothyronine and a significant lowering of the serum cholesterol level was observed. When the ascorbic acid was given alone, no hypocholesterolemic affect was found. The same conclusion was found by Zaitzen, et al. (9) in their study with rabbits on the influence of

ascorbic acid on the cholesterol distribution in experimental atherosclerosis.

Greenberg and Rinehart (10), working with monkeys in a condition of chronic ascorbic acid deficiency, observed that oral administration of iron had no influence on the accompanying state of anemia and only slightly increased the erythrocyte and hemoglobin levels. However, when ascorbic acid was added to the iron, they observed that the combined therapy markedly increased the hemoglobin and serum iron levels over what was obtained with only the iron.

Histological examinations made by Coluzzi (11) on the blood of dogs inoculated with Staphylococcus aureus showed a greater number of reticular cells in the inoculated subjects as compared with the controls. The fact that these cells were found in proximity to erythrogranules of iron supported his hypothesis that ascorbic acid facilitates the passage of iron from the reticular cells to the erythroblasts.

Rats given both ascorbic acid and alpha-tocopherol along with supplementary iron showed a much greater hemoglobin regeneration rate according to Greenberg, et al. (12), than when either vitamin was administered separately with the iron. They also observed that hemoglobin levels were better sustained after the cessation of iron supplements if the iron was given with both ascorbic acid and alpha-tocopherol.

Bencze, et al. (13) noted that rats fed protein-deficient diets had a decreased hemoglobin level, 3.4 g% as compared with 12.2 g% in rats fed normal protein diets. However, when these protein-deficient animals

received 40-60 mg of additional alpha-tocopheral daily, the hemoglobin concentration increased to 13.1 g%.

## EXPERIMENTAL PROCEDURE

The methods used by Roehm and Mayfield (6) were employed as the basis for the experimental procedure in this investigation. Sixty-five weanling male rats of the Holtzman strain, three weeks of age, were obtained at two different times during a three-month period in lots of 24 and 39 rats respectively. Each lot was used in a complete replication of the experimental design. The rats were randomly placed in individual screen-bottom cages, weighed and that weight recorded. They were maintained in a temperature-controlled room of approximately 24° C at an elevation of 1.46 km or 4800 feet. The rats were fed Purina Laboratory Chow ad libitum (ad lib.) for a three day period after which they were again weighed and that weight recorded. The average weight for both replications after the three day stabilizing period was 65 grams.

Table 1  
Distribution of Rats

Diets	Replications		Total
	1	2	
I	4	5	9
II	4	6	10
III	4	6	10
IV	4	6	10
V	4	6	10
VI	4	10	14

In the first lot, four rats were assigned to each of the five diet groups with the remainder serving as controls. Because of poor health, two of the control rats in group VI died within three weeks of arrival. Autopsies revealed that the rats died from causes unrelated to the experiment. The second lot of rats was assigned to the diet groups in a similar manner as shown in Table 1. The rats in the experimental groups I-V were then fed, ad lib., one of the diets as described in Table 2 with the number VI group continuing to receive the laboratory chow. This group served as a normal control group.

The hypercholesterolemia-inducing diets fed in this experiment were similar to those used by Okey and Lyman (30) and as modified by Roehm and Mayfield (6). Group I was chosen for the basal diet with rats in groups II and III being fed similarly except the vitamin mixture did not contain ascorbic acid. Group III rats received, orally, 50 mg ascorbic acid per day, five days per week. The diets fed to group IV differed from group I in that all the iron salts were omitted from the salt mixture. Rats in group V were fed the basal diet with additional manganese, zinc and copper. These additional minerals were added to this diet in order to check on the adequacy of these minerals as supplied by the USP XIV salt mixture. These three minerals, all of which may be involved in the hematopoietic processes, are supplied in the USP XIV salt mixture in lesser amounts than are stated in the National Research Councils recommendations for the nutrient requirements of the rat (31). Rat food intake was measured and recorded three times per week and rat weight, two times. Distilled water was provided for the rats to drink.

Table 2  
Composition of Experimental Diets

Diet Groups	I %	II %	III %	IV %	V %
Albumin, egg <sup>1</sup>	10.0	10.0	10.0	10.0	10.0
Casein <sup>2</sup>	5.0	5.0	5.0	5.0	5.0
Cottonseed oil <sup>3</sup>	10.0	10.0	10.0	10.0	10.0
Sucrose	67.8	67.8	67.8	67.8	67.8
Salt mixture <sup>4</sup>	4.0	4.0	4.0	4.0 <sup>5</sup>	4.0 <sup>6</sup>
Vitamin mixture <sup>7</sup>	2.2	2.2 <sup>8</sup>	2.2 <sup>8,9</sup>	2.2	2.2
Cholesterol <sup>10</sup>	1.0	1.0	1.0	1.0	1.0

<sup>1</sup>Nutritional Biochemicals Corporation (NBC), Cleveland.

<sup>2</sup>Vitamin-Free Casein, NBC.

<sup>3</sup>Wesson Oil, Wesson Oil Sales Company, Fullerton, California.

<sup>4</sup>Salt mixture, USP XIV, NBC, containing the following in grams/kg: cupric sulfate, 0.08; ferric ammonium citrate, 15.28; manganese sulfate, 0.02; ammonium alum, 0.09; potassium iodide, 0.04; sodium fluoride, 0.51; calcium carbonate, 68.6; calcium citrate, 308.3; calcium biphosphate, 112.8; dibasic potassium phosphate, 218.8; and sodium chloride, 77.1.

<sup>5</sup>Salt mixture, USP XIV, as above but with all iron salts omitted, NBC.

<sup>6</sup>Salt mixture, USP XIV, as above but with these additions in g/100 g: Manganese sulfate, 0.365; zinc carbonate, 0.115; and copper sulfate, 0.090.

<sup>7</sup>Vitamin Diet Fortification Mixture, NBC, containing the following in g/kg: vitamin A concentrate, 4.5 (200,000 units/gm); vitamin D concentrate, 0.25 (400,000 units/gm); alpha-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCL, 1.0; thiamine·HCL, 1.0; calcium pantothenate, 3.0; and the following in mg/kg: biotin, 20.0; folic acid, 90.0; and vitamin B<sub>12</sub>, 1.35.

<sup>8</sup>Vitamin Diet Fortification Mixture as above but without ascorbic acid and alpha-tocopherol, NBC. 5.0 g/kg alpha-tocopherol added to bring the level up to the other diets.

<sup>9</sup>Ascorbic acid, 50 mg, pipetted orally to each rat, daily, 5 days/week.

<sup>10</sup>Cholesterol USP, NBC.

Hemoglobin, hematocrit and serum protein determinations were made three times on tail blood samples from non-fasted rats after these rats were fed for 3, 5 and  $6\frac{1}{2}$  weeks on the experimental diets. These determinations were made in the morning with one exception. The first determination of the first replication was partially completed in the morning and concluded in the early afternoon. Measurement of the hemoglobin in duplicate samples was done by the cyanmethemoglobin method as described by Wintrobe (32). This method utilizes the conversion of hemoglobin to cyanmethemoglobin, then the measurement of the optical density of this solution against a known hemoglobin standard, Acuglobin<sup>1</sup>. Readings were made using a Beckman B spectrophotometer.

Standard, heparanized capillary tubes were used in the hematocrit determinations. These micro-hematocrits were first centrifuged in a hematocrit centrifuge and then read in a micro-capillary reader. The serum protein was determined directly from serum in the hematocrit capillary tube by the use of a temperature compensated Goldberg Proteinometer. At the end of the first three week period and after the hemoglobins and hematocrits were measured, a 1.5-2.0 ml representative sample of blood was withdrawn from the tail of each rat. This removal of blood was made in order to induce a slightly lowered hemoglobin level, a condition that would call for hemoglobin regeneration in all six groups of rats. It was believed that this condition would better test the adequacy of all the diets

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<sup>1</sup>Acuglobin, Hemoglobin Standard, supplied by the Ortho Pharmaceutical Corporation, Raritan, New Jersey.

for hemoglobin regeneration.

At the end of the seven-week feeding period and from 3 to 17 days after the blood determinations were made, the rats were sacrificed. Food was removed from the cages the night before, approximately 10 hours prior to decapitation, with the weight of the rat being recorded at the same time. The blood was collected and serum prepared, frozen and held at  $-23^{\circ}$  C for later analyses. Because of the length of time required for the enzyme determinations, only four rats could be sacrificed each morning. The heart, liver and kidneys were removed as rapidly as possible, wiped free of blood by pressing lightly on filter paper, placed on tared weighing papers and weighed. Homogenates were prepared at once using a Virtis "45" Homogenizer. They were placed in covered flasks and chilled in crushed ice until used for enzyme determinations. The entire heart, both kidneys and from a 1.0-2.0 gram representative sample of the liver was used in the preparation of the respective homogenates for the enzyme activity measurements. The succinic dehydrogenase (SDH) activity was determined by the procedure of Schneider and Potter (33) as given by Umbreit, et al. (34). This method measures the oxygen consumption by the SDH enzyme in a flask held in a water bath at  $37^{\circ}$  C through a pressure change as recorded on a thermobarometer. Also contained in the flask is a solution of phosphate buffer, cytochrome c, calcium chloride, aluminum chloride, and sodium succinate. A sodium hydroxide wick is used to draw up the carbon dioxide produced. One group of SDH determinations was made in the morning and one in the afternoon.

The SDH activity of these organs was measured in duplicate over a 40 minute interval using a 14-manometer Lardy Warburg apparatus. SDH activity was first calculated and expressed in the usual manner as microliters of oxygen per hour per milligram of dry tissue and then later calculated on the basis of activity per organ weight per gram of rat weight. For purposes of these calculations, the heart tissue was found to be 22% solids, the liver was 30% and the kidneys were 22% solids. These percentages were established by analyses made on composite samples and the average composition accepted by other workers in the field (6).

The serum, which had been frozen at the time of sacrifice from the fasted rats, was later chemically analyzed for iron content, total iron-binding capacity (TIBC), total cholesterol and triglyceride content. Serum iron and TIBC determinations were made on the frozen serum samples using the method of Peters, et al. (35) as modified by Mandel (36). The serum iron procedure utilizes the oxidation of ferrous iron to ferric iron and the color produced is measured using a spectrophotometer. In the TIBC determination ferric iron is added to saturate the serum transferrin, the excess iron is removed, and then colorimetric determinations are made as in the serum iron determination. A micro-adaptation of the procedure of Abel, et al. (37) was used in the total cholesterol determinations. The cholesterol determination utilizes saponification with alcoholic KOH to form glycerol, extraction with N-Hexane and color production with the Lieberman-Burchard reagent. Serum triglycerides were determined by using the method of Van Handel and Zilvermit (38) as was later modified by Van Handel (39). In this determination the phospho-

lipids are first removed from the serum. The triglycerides are then extracted with chloroform, saponified to form glycerol, the glycerol oxidized to formaldehyde and chromotropic acid added to produce a color which can be measured.

Statistical treatment of all data reported was made using the services of the Montana State University Computing Center. The treatment consisted of an analysis of variance and comparisons were made using Duncan's multiple range test (40). Only differences considered statistically significant at the 1% level have been considered.

## RESULTS AND DISCUSSION

Food and growth records of groups of rats fed the five hypercholesterolemia-inducing diets and diet VI, Purina Laboratory Chow, are presented in Table 3. Biochemical changes in the blood, serum, heart, liver and kidney of the rats fed these diets are shown in Tables 4, 6 and 9. Tables 5 and 7 show the accepted values for these biochemical measurements as given by Albritton (41) and Roehm and Mayfield (6) for rats fed normal diets. Table 8 presents the organ weights of the rats fed the experimental and the chow diets.

Comparisons of the data were made using Duncan's multiple range test (40) by which statistically significant differences between means for each of the biochemical treatments were determined, as shown in Tables 4, 6 and 9. Means with dissimilar superscripts are considered statistically different at the 1% level. The highest value(s) when the Duncan's multiple range test was used are denoted with the superscript letter a, the next highest value(s) with b and the lowest value with letter c. For simplification, the values given in the tables in this study represent the average value or mean of each treatment for each group of rats. Standard errors of the means were calculated for each treatment and are shown in Tables 4, 6, 8 and 9. Treatment values presented in this study will be compared to values obtained by Roehm and Mayfield (6) as similar diets were used in both instances. However, rats used in the former study (6) were 11 days older than those used in the present study.

Table 3

Food and Growth Records of Rats Fed the Experimental Diets

	Experimental Diets					Control
	I	II	III	IV	V	IV
Rats/group	9	10	10	10	10	12
Food consumption, g/day	13.6	13.9	12.9	11.3	14.0	18.8
Weight gain, g/49 days	229.0	218.0	201.0	185.0	228.0	232.0
Weight at end of test, g	252.0	241.0	220.0	210.0	256.0	265.0

## FOOD AND GROWTH

Food consumption, weight gain and the weight at end of the test of rats fed any of the hypercholesterolemia-inducing diets were lower than the control rats fed the chow diet, as shown in Table 3. Rats fed the basal diet, diet I; diets II and III, with and without ascorbic acid; and diet V, with additional minerals all consumed more food and gained more in weight than did rats fed diet IV which was iron-deficient. When records are compared for rats fed diets II (no ascorbic acid present) and diet III (50 mg ascorbic acid fed orally 5 days per week), it will be noted that food consumption and growth rate levels were lower for rats fed diet III. This may have been due, in part, to the added strain of orally feeding the ascorbic acid. Although food consumption and growth rates of rats fed the hypercholesterolemia-inducing diets were lower than those of the control group, they still were within the range of values given by other workers (31).

Table 4

Hemoglobin, Hematocrit and Serum Protein Levels of Rats<sup>1</sup> Fed the Experimental Diets

Rats/group	Experimental Diets					Control
	I 9	II 10	III 10	IV 10	V 10	VI 12
After 3 wks. on experimental diet:						
Hemoglobin, g/100 ml	b,2 13.2±0.22 <sup>3</sup>	b 12.6±0.21	b 12.9±0.34	c 7.8±0.50	b 13.2±0.24	a 14.1±1.66
Hematocrit, %	a 44.6±1.17	a 41.0±0.66	a 43.0±1.29	b 26.6±1.47	a 43.4±0.87	a 44.6±0.61
Serum protein, g/100 ml	a 7.0±0.66	a 6.7±0.08	a 7.0±1.18	a 6.6±0.15	a 7.0±0.14	a 6.4±0.08
After 5 wks. on experimental diet:						
Hemoglobin, g/100 ml	b 14.0±0.23	b 13.6±0.12	b 13.6±0.18	c 5.4±0.34	b 14.3±0.22	a 15.5±0.07
Hematocrit, %	b 45.2±0.37	b 44.4±0.36	b 44.8±0.64	c 22.4±0.98	b 45.9±0.46	a 48.6±0.66
Serum protein, g/100 ml	a 7.6±0.27	a 7.4±0.04	a 7.5±0.07	b 7.0±0.09	a 7.3±0.10	c 6.8±0.07
After 6½ wks. on experimental diet:						
Hemoglobin, g/100 ml	b 15.0±0.16	b 14.6±0.16	b 14.5±0.14	c 5.7±0.28	b 15.0±0.12	a 16.1±0.11
Hematocrit, %	b 46.8±0.46	b 46.3±0.36	b 46.4±0.26	c 24.2±0.89	b 47.3±0.50	a 49.3±0.36
Serum protein, g/100 ml	a 8.0±0.24	a 7.6±0.08	a 7.6±0.08	b 7.2±0.10	a 7.8±0.08	c 7.0±0.08

<sup>1</sup>Non-fasted rats.<sup>2</sup>Duncan's multiple range test (40) with comparisons made horizontally. Means with different superscripts are statistically significant at the 1% level.<sup>3</sup>Standard error of the mean.

HEMOGLOBIN, HEMATOCRIT AND SERUM PROTEIN

Mean hemoglobin and hematocrit values for rats fed the hypercholesterolemia-inducing diets, as shown in Table 4, are significantly lower than those for rats fed the chow diet. However, the removal of ascorbic acid from the basal diet, diet II, and the addition of minerals, diet V, did not significantly alter these values when compared to the values obtained when the basal hypercholesterolemia-inducing diet was fed. Neither were the hemoglobin and hematocrit values significantly altered when ascorbic acid was given orally to this group of rats, diet III. The values for diets I, II, III and V, were lower than those of rats on the control diet fed chow. However, they are within the ranges given by Albritton (41) as presented in Table 5. As a point of interest, Table 5 also includes these values for normal rats as reported by Roehm and Mayfield (6). It will be noted that these mean values (6) for normal rats are all higher than those given by Albritton (41), probably reflecting the effect of altitude.

When the iron was removed from the basal diet, these hemoglobin and hematocrit values were significantly lower than the values from rats fed either the basal hypercholesterolemia-inducing diet or the control chow diet. These hemoglobin and hematocrit values are slightly lower than those reported by other workers feeding similar diets (6). This may be due to the age (26 days) of the rats when fed the experimental diets in the present study, as compared to the age of the rats (36 days) when fed the experimental diets in a previous study (6). Although the

Table 5

Hemoglobin, Hematocrit and Serum Protein Values for Normal Rats as Reported in the Literature (41,6).

	Albritton (41)		Roehm and Mayfield (6)	
	Mean	Range	Mean	Range
Hemoglobin, g/100 ml	14.80	12.0-17.5	16.1	16.0-16.2
Hematocrit, %	46.00	39.0-53.0	49.4	49.1-49.7
Serum Protein, g/100 ml	6.04	5.5-7.9	6.5	6.4-6.6

hemoglobin values differed significantly after the rats were fed the diets for three weeks, the hematocrit values did not. After five weeks, however, the differences were highly significant. Mean hemoglobin and hematocrit levels for the control rats were slightly higher than values reported by Albritton (41). This may be due to the effect of altitude (1.48 Km or 4800 feet) on the hematopoietic processes. Similar increased hemoglobin and hematocrit values were found at this altitude by Roehm and Mayfield (6,42).

Serum protein values, as shown in Table 4, for rats fed any of the five hypercholesterolemia-inducing diets for three weeks were higher on those diets but became significantly higher after five and 6½ weeks, than those of the control group. Even though this increase is not large, the levels of serum protein are greater than those given by Albritton (41), Table 5. Rats fed the basal hypercholesterolemia-inducing diet, diet I, had slightly higher (not statistically significant) serum protein values than did the rats fed the diets with and without ascorbic acid, diets II

and III, and with additional minerals, diet V. Rats fed diets I, II, III and V had significantly higher serum protein values than did those fed the diet which contained no iron. These higher serum protein levels associated with the hypercholesterolemia-inducing diets have been related (6) to the accompanying changes in serum cholesterol and triglycerides. This theory is based on an investigation by Rodbell, et al. (43) and a discussion by Korn (44) which reports that when most of the exogenous triglyceride and cholesterol is transported from the intestinal tract to the tissues, via the lymph and blood, they are in the form of chylomicrons, a complex of triglyceride, cholesterol ester, phospholipid and protein. These workers (43,44) reported that although in some instances the entire chylomicron may leave the circulatory system intact, there was also evidence that some of the protein may be left behind when the lipid disappears. Because of these factors, Roehm and Mayfield (6) concluded that this accompanying higher serum protein level may be due to the protein being left behind in the blood stream after the partial hydrolysis of the cholesterol-triglyceride-protein-containing chylomicrons. It is believed that these higher serum protein levels suggest and support the theory that the lowered hemoglobin and hematocrit values observed in these rats were not the result of a process of dilution of the blood stream. This type of anemia was suggested by Mueller and Viteri (17) and discussed by Popjak (45) since more water than usual may be consumed by test animals fed these hypercholesterolemia-inducing diets.

### SERUM IRON AND TIBC

Rats fed the iron-deficient, hypercholesterolemia-inducing diet had significantly lower serum iron values than rats fed the other high-fat diets and the laboratory chow diet. These values are presented in Table 6. Even though rats fed diet V, the basal diet with additional minerals, had a higher mean serum iron value than did those fed the other diets, this value was not significantly higher when Duncan's multiple range test (40) was used. The serum iron value for rats fed diet III (additional ascorbic acid) was higher than diet II (no ascorbic acid) and very similar to diet I, but no significant difference occurred between any of the diets. The serum iron values of rats fed any of the diets with the exception of diet IV were lower than have been reported (6). All of the serum iron values obtained were lower than values given in Table 7 for normal rats by Albritton (41). A possible explanation for these lowered values may be the age (26 days) at which the rats were placed on the experimental diets.

TIBC values given in Table 6 for rats fed the hypercholesterolemia-inducing diets were significantly higher than for rats fed the chow diet. These values are the inverse of values found by Roehm and Mayfield (6). They found that rats fed similar high-fat diets had TIBC values significantly lower than did rats fed the chow diet. The exact reason for this phenomenon cannot be explained at this time. No TIBC values were given by Albritton (41). In the analysis of the serum iron, TIBC, cholesterol and triglyceride determination, a large standard error of the mean was

Table 6

Serum Iron, Total Iron-Binding Capacity (TIBC), Serum Cholesterol and Serum Triglyceride Levels of Rats<sup>1</sup> Fed the Experimental Diets

Rats/group	Experimental Diets					Control
	I	II	III	IV	V	VI
	9	10	10	10	10	12
Serum iron, mg/100 ml	a, <sup>2</sup> 183.2±18.9 <sup>3</sup>	a 168.4±13.2	a 184.8±17.2	b 48.4± 4.2	a 212.2±14.6	a 174.4±11.3
TIBC, mg/100 ml	a 696.2±36.8	a 680.0±29.9	a 678.0±26.5	a 693.6±12.8	a 670.8±31.2	b 526.8±21.1
Cholesterol, mg/100 ml	a 129.1±10.6	a 124.2± 9.8	a 116.2± 9.2	a 134.5±17.6	a 131.6±10.6	b 79.3± 3.5
Triglycerides, mg/100 ml	b 48.7± 7.6	a 68.6± 7.2	a 67.5± 8.8	a 83.0±10.5	a 54.2± 6.2	a 65.0± 6.2

<sup>1</sup>Fasted rats.

<sup>2</sup>Duncan's multiple range test (40) with comparisons made horizontally. Means with different superscripts are statistically significant at the 1% level.

<sup>3</sup>Standard error of the mean.

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observed. This may be due to individual differences in the rats themselves rather than to an experimental error as similar, and in some cases higher, variances were found (6) using the same type diets.

#### SERUM CHOLESTEROL

Serum cholesterol values of rats fed the hypercholesterolemia-inducing diets shown in Table 6 were significantly higher than the values of rats fed the laboratory chow. This was also reported by others (2,6) but with values that were either higher or lower than values reported in this study. Rats fed the basal high-fat diet, diet I had slightly higher (non-significant) serum cholesterol levels than did rats fed diets with and without ascorbic acid, diets II and III, yet lower (non-significant) than rats fed either diet IV, iron deficient or diet V, with additional minerals. Rats fed diet III and receiving 50 mg ascorbic acid per day had a lower, but not significantly lower serum cholesterol level than rats fed diet II which contained no ascorbic acid. Hence there is no statistically significant difference in the serum cholesterol of rats fed any of the five hypercholesterolemia-inducing diets, but all are significantly higher than the control diet values.

#### SERUM TRIGLYCERIDES

As shown in Table 6, the serum triglyceride levels of rats fed the hypercholesterolemia-inducing diets II, III, IV and V were significantly higher than those found in rats fed the basic hypercholesterolemia-inducing

Table 7

Serum Iron, Total Iron-Binding Capacity (TIBC), Cholesterol and Triglyceride Levels for Normal Rats as Reported in the Literature (41,6).

	Albritton (41)		Roehm and Mayfield (6)	
	Mean	Range	Mean	Range
Serum iron, mg/100 ml	261.0	—	254.0	234.5-273.5
TIBC, mg/100 ml	—	—	728.0	706.8-749.2
Cholesterol, mg/100 ml	52.0	28.0-76.0	76.2	74.3-78.1
Triglyceride, mg/100 ml	85.0	26.0-144.0	90.4	86.2-94.6

diet I. Rats fed the iron-deficient diet, diet IV, had the highest serum triglyceride level of any of the groups, and those fed the additional minerals was considerably lower. The omission of ascorbic acid from diet II caused a significant increase in the level of serum triglycerides (diet I versus diet II). However, the addition of orally fed ascorbic acid did not alter the triglyceride level (diet II versus diet III). Serum triglyceride values for rats as reported by Roehm and Mayfield (6) and Bizzi, et al. (45), were considerably higher than were found in this study. Tinoco, et al. (47) report a lower value for rats fed a purified diet somewhat low in methionine and vitamin B<sub>12</sub>, but otherwise complete. However, serum triglyceride values found in this study are within the range given by Albritton (41) in Table 7.

Serum cholesterol and triglyceride levels of rats fed these hypercholesterolemia-inducing diets showed a very interesting phenomenon, they did not respond to dietary variations by increasing or decreasing in a

similar manner. This inverse relationship was recognized by Albrink (48) in work on coronary artery disease. Later work by Bizzi, et al. (46) also recognized these variations and reversals in blood lipids but they were not able to establish fully all factors in the diet which bring this phenomenon about. Their work showed high levels of serum triglycerides accompanied a condition of thrombosis in the heart chambers of the rat and that elevated serum cholesterol, together with a normal triglyceride level, was associated with atherosclerosis of the aorta. This inverse relationship was also noticed by Roehm and Mayfield (6) who concluded that the degree of impairment in the iron transport system, as well as in the hematopoietic process, might be an influencing factor in the thrombogenic system. They based this conclusion on the fact that rats in the group with the highest level of serum triglycerides also had the lowest hemoglobin and hematocrit levels together with the reversed phenomenon of a low serum iron level. Similar results were obtained in this study with one additional point, the serum cholesterol level of rats fed this iron-deficient diet were also high. Since the whole heart was used in the enzyme determination, no histological examination of this organ was made.

#### ORGAN WEIGHTS

Weights of the heart, liver and kidneys of rats fed the experimental diets and the laboratory chow, expressed in g/100 g rat, are shown in Table 8. The weight of the hearts of rats fed the five hypercholesterolemia-inducing diets varied little between groups but were higher than

Table 8

Organ Weights of Rats<sup>1</sup> Fed the Experimental Diets

Rats/group	Experimental Diets					Control
	I 9	II 10	III 10	IV 10	V 10	VI 12
Heart, g/100 g rat weight	0.32±0.01 <sup>2</sup>	0.35±0.01	0.35±0.01	0.40±0.01	0.32±0.01	0.30±0.01
Liver, g/100 g rat weight	3.17±0.09	3.81±0.08	3.82±0.16	3.18±0.06	3.74±0.07	3.00±0.03
Kidney, g/100 g rat weight	0.66±0.02	0.62±0.01	0.62±0.01	0.59±0.01	0.63±0.01	0.67±0.01
<sup>1</sup> Fasted rats.						
<sup>2</sup> Standard error of the mean.						

those of rats fed the laboratory chow. The largest heart weights were observed in rats fed diet IV which was iron-deficient. Since no histological examinations were made on this organ, it is not known whether this increased weight was due to normal tissue development or to some abnormality, such as a thrombogenic condition within the heart chambers as noted by Bizzi, et al. (46). As was the case in the heart weights, the liver weights of the rats fed the high-fat diets were higher than rats fed the laboratory chow. It is known, however, that rats fed the hypercholesterolemia-inducing diets showed a deposition of fat within the structure of the liver which was quite obvious to the eye. Rats fed the laboratory chow diet had normal, non-lipogenic livers. The converse was true with the kidney weights, as rats fed the high-fat diets, diets I-IV, had slightly lower kidney weights than rats fed the laboratory chow. Duncan's multiple range test was not performed on the values for organ weights so it is not known if these values differ significantly. Rats fed the iron-deficient diet IV showed lowered hemoglobin values, a disturbance in the iron transport system and also had kidney weights less than those of the control rats. Similar conditions were reported (6,21) in which the iron-deficient diet induced an increase in the weight of the heart and a decrease in the weight of the kidneys. Similar but larger organ weights than were found in this survey were observed by Roehm and Mayfield (6) but this increase may be attributed directly to the age of the rats since rats used in their study were 11 days older.

Table 9

Succinic Dehydrogenase (SDH) Activity of Organs of Rats<sup>1</sup> Fed the Experimental Diets

Rats/group	Experimental Diets					Control
	I 9	I 10	III 10	IV 10	V 10	VI 12
SDH, Heart	a, <sup>2</sup>	a	a	b	a	a
Dry weight, $\mu$ l O <sub>2</sub> /hr/mg	248.1 $\pm$ 6.8 <sup>3</sup>	248.5 $\pm$ 7.1	244.8 $\pm$ 4.0	207.0 $\pm$ 4.5	258.6 $\pm$ 5.0	242.0 $\pm$ 5.6
Relative SDH/g rat weight <sup>4</sup>	a 176.8 $\pm$ 4.7	a 193.1 $\pm$ 5.4	a 192.0 $\pm$ 5.1	a 182.3 $\pm$ 8.3	a 180.9 $\pm$ 4.1	b 163.6 $\pm$ 4.6
SDH, Liver	b	b	b	b	b	a
Dry weight, $\mu$ l O <sub>2</sub> /hr/mg	42.4 $\pm$ 1.2	43.0 $\pm$ 2.8	42.1 $\pm$ 2.4	47.2 $\pm$ 3.7	43.1 $\pm$ 1.8	80.9 $\pm$ 1.9
Relative SDH/g rat weight	b 482.4 $\pm$ 17.2	b 504.9 $\pm$ 28.1	b 495.1 $\pm$ 43.1	b 450.3 $\pm$ 29.0	b 496.0 $\pm$ 17.6	a 733.6 $\pm$ 14.2
SDH, Kidney	b	b	b	c	b	a
Dry weight, $\mu$ l, O <sub>2</sub> /hr/mg	149.6 $\pm$ 3.0	151.6 $\pm$ 6.0	150.6 $\pm$ 2.3	108.6 $\pm$ 2.6	156.2 $\pm$ 4.4	170.6 $\pm$ 3.7
Relative SDH/g rat weight	b 221.3 $\pm$ 7.5	b 210.3 $\pm$ 8.0	b 206.9 $\pm$ 6.0	c 142.3 $\pm$ 5.4	b 220.2 $\pm$ 11.0	a 253.9 $\pm$ 4.4

<sup>1</sup>Fasted rats.<sup>2</sup>Duncan's multiple range test (40) with comparisons made horizontally. Means with different superscripts are statistically significant at the 1% level.<sup>3</sup>Standard error of the mean.<sup>4</sup>Relative SDH/g rat weight calculated at follows:

$$\frac{\mu\text{l O}_2/\text{hr/mg moist tissue} \times \text{organ weight, in mg}}{\text{rat weight in grams}}$$

### SUCCINIC DEHYDROGENASE ACTIVITY (SDH) OF THE HEART

Rats fed the hypercholesterolemia-inducing diet which was iron-deficient, showed a significant lowering in the SDH activity of the heart when expressed as microliters of oxygen per hour, per milligram of dry weight tissue, as presented in Table 9. However, when this was calculated on the basis of relative SDH activity per gram of rat weight, this lowering was no longer evident. This may be due in part to the fact that the heart weights of the iron-deficient rats were increased and therefore, when the activity was expressed on a relative SDH basis, the total amount per 100 g of rat weight for the control and iron-deficient animals was approximately equal. These results agree in general, with those reported by others (6,21). When the SDH activity of the heart of rats fed the laboratory chow was calculated on the relative SDH activity/g of rat weight basis, it was found to be significantly lower than that of rats fed any of the five hypercholesterolemia-inducing diets.

### SDH ACTIVITY OF THE LIVER

The SDH activity of the liver of rats fed the hypercholesterolemia-inducing diets was significantly lower than that of rats fed the laboratory chow diet when this activity was calculated both on the dry weight or the per rat weight basis. Liver SDH levels of rats fed diet IV, which was iron-deficient did not show any further lowering. Therefore, these significant lower liver SDH values for rats fed these high-fat diets were probably the result of the hypercholesterolemic effects of the diet and

not of its effect of lowering hemoglobin. This is in agreement with what was found by Roehm and Mayfield (6) and Beutler and Blasidell (21) who reported that moderately severe iron-deficiency caused no decrease in the SDH activity of the liver.

#### SDH ACTIVITY OF THE KIDNEY

The kidney SDH activity of rats fed the hypercholesterolemia-inducing diets was significantly lower than was observed for the control rats fed the laboratory chow as calculated on either basis. This decreased activity appeared to be related to the hemoglobin level since rats fed the iron-deficient diet IV had significantly less SDH activity than rats fed the basal high-fat diet with a normal level of iron. Other workers (6,21) have also reported similar significant lowerings of kidney SDH activity when rats were fed iron-deficient diets. As was the case in the serum analyses, the SDH levels for the heart, liver and kidney of rats fed these experimental diets, showed a large standard error of the mean. Similar but lower variances were noticed by others (6).

No significant difference in heart, liver and kidney SDH levels was observed when rats were fed the basal diet with and without ascorbic acid, diets II and III. Neither was there any significant difference in these values when additional minerals were added to the basal hypercholesterolemia-inducing diet.

## SUMMARY

Six groups of weanling male rats, averaging 10 rats per group were fed one of five experimental diets or a laboratory chow diet as follows: diet I, a basal hypercholesterolemia-inducing diet; diet II, similar to diet I but without ascorbic acid; diet III similar to diet II but rats were fed orally 50 mg of ascorbic acid five days per week; diet IV, similar to diet I but with all iron salts omitted; diet V, similar to diet I but with additional copper, zinc and manganese; and diet VI, Purina Laboratory Chow with rats serving as a control group. Biochemical changes that occurred in the blood, serum, heart, liver and kidneys of these rats were determined chemically and the data analyzed statistically.

Food consumption and weight gains were considerably lower for rats fed the hypercholesterolemia-inducing diet which was iron-deficient than for rats fed any of the other diets.

Mean hemoglobin and hematocrit values for rats fed any of the five hypercholesterolemia-inducing diets were significantly lower, and serum protein levels higher than those for the control rats fed the chow diets. The removal of ascorbic acid from the basal diet, or the addition of copper, zinc and manganese to the basal diet did not affect the hemoglobin, hematocrit or serum protein levels of the rats when compared to values for rats fed the basal hypercholesterolemic diet. Rats fed the iron-deficient hypercholesterolemic diet had significantly lower hemoglobin and hematocrit levels than did the rats fed the other four hypercholesterolemic-inducing diets. Serum protein levels of these iron-deficient rats were similar

to those of the control, chow fed rats.

Serum iron was lowest in rats fed the iron-deficient diet but was similar in all other groups.

Total iron-binding capacity (TIBC) and cholesterol values were significantly higher in rats fed the five experimental diets than in those fed the chow diet. These values were not affected by the factors under investigation, namely, ascorbic acid or the minerals, copper, zinc and manganese.

Rats fed the basal hypercholesterolemia-inducing diet had a significantly lower serum triglyceride level than did rats fed any of the other diets. These triglyceride values were unaffected by the factors under investigation.

Succinic dehydrogenase (SDH) activity of the heart and the iron-deficient rats, when expressed as  $\mu\text{l O}_2/\text{hr}/\text{mg}$  of dry weight of tissue, was significantly lower than the SDH values of rats in the other groups. This difference was not evident when expressed on a relative SDH/g rat weight basis. The SDH activity of the heart was not affected by the presence or absence of ascorbic acid or the addition of the extra minerals in the diet.

Rats fed any of the hypercholesterolemic diets had lower SDH activity in the liver than did the control rats. Likewise, these values were not affected by the factors under investigation, ascorbic acid and additional minerals.

The SDH activity of the kidney was significantly lower in the five groups of rats fed the hypercholesterolemic diets than in the control,

chow fed rats. This value was greatly lowered when the rats were fed the iron-deficient diet but was not affected by the absence or presence of ascorbic acid or the addition of minerals to the diet.

The weights of the heart, liver and kidneys, expressed as g/100 g rat weight, were affected by the five hypercholesterolemia-inducing diets. The weight of the heart and liver of rats fed any of the five hypercholesterolemic diets were greater and the weight of the kidney lower than the respective weights of the control, chow fed rats. Rats fed the iron-deficient diet had the largest heart weight and the smallest kidney weight of rats in any of the groups.











