March 1, 1965

To the Graduate Council:

I am submitting herewith a dissertation written by Ted Painter McDonald entitled "An Investigation of the Effects of Vitamin A on Mucopolysaccharides of Rat Blood Platelets." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Charles S. Hobbs
Major Professor

We have read this dissertation
and recommend its acceptance:

O. Glen Hall

J. T. Miles

Theodore T. Odell, Jr.

M. C. Bell

Accepted for the Council:

Hilton A. Smith
Dean of the Graduate School

AN INVESTIGATION OF THE EFFECTS OF VITAMIN A ON MUCOPOLYSACCHARIDES OF RAT BLOOD PLATELETS

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Ted Painter McDonald

March 1965

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. T. T. Odell, Jr. of the Biology Division, Oak Ridge National Laboratory, for guidance throughout the course of this investigation and careful reading of the manuscript.

Appreciation is expressed to Dr. C. S. Hobbs, Major Professor, for his guidance and encouragement not only during the course of this investigation, but throughout the author's graduate program.

The author is indebted to Drs. M. C. Bell and N. S. Hall for their cooperation and encouragement and generous supply of materials and laboratory facilities at UT-AEC.

The encouragement and suggestions of Drs. O. G. Hall and J. T. Miles are deeply appreciated.

Most deeply and most directly, the author is indebted to his wife, Opal Mink McDonald, whose encouragement, understanding, and support have contributed so greatly to the present work.

618450

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION.	1
II.	REVIEW OF LITERATURE.	5
	Vitamin A Deficiency.	5
	Eyes.	5
	Respiratory Tract	5
	Intestinal Tract.	6
	Salivary Glands	6
	Urogenital Tract.	6
	Liver	7
	Skin.	7
	Teeth	7
	Nerves and Bones.	8
	Reproduction.	9
	Reticuloendothelial System.	9
	Blood	10
	Sulfur-35	12
	Hypervitaminosis A.	13
	Hemorrhages	13
	Reproductive Organs	15
	Bones	16
	Skin.	17

CHAPTER PAGE

Hypervitaminosis Compared with Avitaminosis A 17
Chemistry and Biochemistry of Mucopolysaccharides 18
Biochemical Effects of Vitamin A. 20
Epithelial Cells. 20
Release of Proteolytic Enzymes. 21
Glycogen Synthesis. 23
Adrenal Cortex Functions. 24
Mucopolysaccharide Production 24
Sulfate-35 Labeled Amino Acids. 28
Platelet MPS. 29
III. MATERIALS AND METHODS 31
Animals 31
Hypervitaminosis A Animals. 31
Hypovitaminosis A Animals 32
Peripheral Blood. 32
Preparation and Injection of Isotope. 33
Uptake of ^{35}S by Blood Platelets and Plasma 35
Platelet Size 36
Determination of Hexosamines. 39
Determination of Protein. 40
Identification of MPS 42
Platelet Adhesiveness 46
Statistical Analysis. 47
IV. RESULTS AND DISCUSSION. 48

CHAPTER PAGE

General Description 48
Hypervitaminosis A. 48
Hypovitaminosis A 50
Peripheral Blood Determinations in Hypervitaminosis A . . . 52
Platelets 52
Hematocrit. 52
White Blood Cells 55
Red Blood Cells 55
Peripheral Blood Determinations in Hypovitaminosis A. . . . 58
Platelets 58
Hematocrit. 60
White Blood Cells 61
Red Blood Cells 61
Platelet Size Measurements. 63
Uptake of ^{35}S by Blood Platelets and Plasma 67
Vitamin A-Treated Rats. 67
Vitamin A-Deficient Rats. 75
Hexosamine and Protein Content of Blood Platelets 78
Identification of MPS 83
Blood Platelet Adhesiveness 90
General Conclusions 92
V. SUMMARY 94
LITERATURE CITED. 98

LIST OF TABLES

TABLE PAGE

I. A Comparison of the Chemical Components of Several Mucopolysaccharides Commonly Occurring in Mammalian Tissues. 19

II. Comparison of Platelet Size (Mean Window) from Vitamin A-Treated Rats or Rats on Vitamin A-Deficient and Normal Diets. 64

III. Comparison of Platelet Size (Cells per Packed Volume) from Vitamin A-Treated Rats or Rats on Vitamin A-Deficient and Normal Diets 65

IV. Radioactivity of Platelets, Plasma-Saline, and Platelet Washes from Normal and Vitamin A-Treated Rats after Daily Intraperitoneal Injections of $Na_2{}^{35}SO_4$ 69

V. Radioactivity of Platelets, Plasma-Saline, and Platelet Washes from Normal and Vitamin A-Treated Rats after Single Intraperitoneal Injections of $Na_2{}^{35}SO_4$ 73

VI. Radioactivity of Platelets, Plasma-Saline, and Platelet Washes from Normal and Vitamin A-Treated Rats after Single Intraperitoneal Injections of $Na_2{}^{35}SO_4$ 74

VII. Radioactivity of Platelets, Plasma-Saline, and Platelet Washes from Vitamin A-Deficient and Normal Rats after Daily Intraperitoneal Injections of $Na_2{}^{35}SO_4$ 77

TABLE PAGE

VIII. Radioactivity of Platelets, Plasma-Saline, and Platelet Washes from Normal and Vitamin A-Deficient Rats after Single Intraperitoneal Injections of $Na_2{}^{35}SO_4$. 79

IX. Hexosamine and Protein Content of Platelets from Vitamin A-Treated, Normal or Vitamin A-Deficient Rats. 80

X. Relative Amounts of Several ^{35}S-Labeled Mucopolysaccharides in Blood Platelets of Rats ($Na_2{}^{35}SO_4$ Injected 5 Days before Killing). 87

XI. Adhesiveness of Blood Platelets from Vitamin A-Treated and Normal Rats. 91

LIST OF FIGURES

FIGURE		PAGE
1.	Effect of Threshold on Cells/mm^3 Using the Fisher Auto-cytometer.	34
2.	Plot Taken from Plotter of Coulter Counter Showing the Size Distribution of Normal Platelets	38
3.	Optical Density of Varying Concentrations of Hexosamine. . . .	41
4.	Optical Density of Varying Concentrations of Protein (Bovine Serum Albumin)	43
5.	Body Weights of Rats Dosed with Vitamin A or Water	49
6.	Change in Body Weight of Rats Fed Vitamin A-Deficient and Normal Diets	51
7.	Peripheral Platelet Counts of Rats Dosed with Vitamin A or Water.	53
8.	Hematocrits of Rats Dosed with Vitamin A or Water.	54
9.	Peripheral WBC Counts of Rats Dosed with Vitamin A or Water. .	56
10.	Peripheral RBC Counts of Rats Dosed with Vitamin A or Water. .	57
11.	Peripheral Platelet Counts and Hematocrits of Rats Fed Vitamin A-Deficient and Standard Laboratory Diets.	59
12.	Peripheral RBC and WBC Counts of Rats Fed Vitamin A-Deficient and Standard Laboratory Diets.	62
13.	Radioactivity of Platelets from Normal or Vitamin A-Treated Rats after Daily Intraperitoneal Injections of $Na_2{}^{35}SO_4$. . .	68

FIGURE PAGE

14. Radioactivity of Platelets from Vitamin A-Treated and Normal Rats after Single Intraperitoneal Injections of $Na_2{}^{35}SO_4$ . . 72

15. Radioactivity of Platelets from Normal Rats and Rats Fed a Vitamin A-Deficient Diet after Daily Intraperitoneal Injections of $Na_2{}^{35}SO_4$ 76

16. Chromatographic Migration on Paper Strips of 20 µl of Platelet Extract (PE), 20 µg of Chondroitin Sulfate (ChS), and 20 µg of Heparin (Hep); Developed in 0.5 M $MgCl_2$, 1.0 M $MgCl_2$, 0.4 M $AlCl_3$ or 0.5 M $FeCl_3$. 84

CHAPTER I

INTRODUCTION

Vitamin A is one of the earliest essential vitamins discovered, and so far attempts have failed to uncover its specific mode of action within the living animal. Its role in vision is best known and has been elucidated by Wald (1955), but in addition, vitamin A has other important metabolic functions that are less well understood. For example, an animal may die from vitamin A deficiency in the absence of any apparent eye damage. Morphological changes caused by a deficiency of this vitamin, such as epithelial keratinization (Wolbach and Howe, 1925), have been studied extensively. There has been a considerable amount of investigation in recent years into the action of vitamin A at the molecular level, but much remains to be deciphered. Nevertheless, it is apparent that vitamin A has an important influence on mucopolysaccharide (MPS) biosynthesis (Fell, Mellanby and Pelc, 1954; Dziewiatkowski, 1954; Wolf and Varandani, 1960; Varandani, Wolf and Johnson, 1960; Wolf, Bergan and Sundaresan, 1962; Wolf, Bergan and Sundaresan, 1963).

Deficiency of vitamin A results in a decrease in the number and activity of cells in all epithelial tissues that secrete mucus--cells in the respiratory (Wolbach and Howe, 1925), intestinal (Gross, 1924; Richards, 1935), and urinary tracts and the genital system (Moore, 1957)--and of cells in other tissues where MPS is synthesized, especially in cartilage. It has been shown that uptake of $^{35}SO_4$ into chondroitin

sulfates of cartilage is reduced in the vitamin A-deficient mammal, a result suggesting a decreased synthesis of MPS (Dziewiatkowski, 1954). Excess vitamin A, on the other hand, results in accelerated maturation of cartilage cells (Fell and Mellanby, 1953) and the mucoprotein (a mucopolysaccharide-protein complex) levels of the blood are greatly increased (Cohen, Maddock and Wolbach, 1955; Thomas et al., 1960). Toxic levels of vitamin A adversely affect the same tissues--reproductive organs (Maddock, Cohen and Wolbach, 1953; bones (Clark and Smith, 1964); skin (Chung and Houck, 1964; Barker, Cruickshank and Webb, 1964); and articular and epiphyseal cartilage matrix (McElligott, 1962; Clark and Smith, 1964)--apparently to some extent by interfering with normal MPS biosynthesis. Vitamin A has a direct action, possibly coenzymatic, on the polymerization of uridine nucleotides to form MPS or on the sulfonation of MPS (Wolf and Varandani, 1960).

In addition to altered MPS biosynthesis, hemorrhages are commonly associated both with deficiency and excess of vitamin A (Frank, 1934; Rodahl and Moore, 1943; Rodahl, 1950; Richardson, 1961), and are believed to be a cause of death associated with this vitamin (Maddock, Wolbach and Jensen, 1948). Blood platelets are involved in prevention of hemorrhages and have MPS as one of their components (Odell and Anderson, 1957; Anderson and Odell, 1958; Kerby and Taylor, 1959; Odell and McDonald, 1964a).

Of the three hemostasis-producing roles of blood platelets commonly listed [(1) clumping and fusing of the platelets at the site of blood vessel injury, (2) clotting, and (3) maintaining the resistance of

the capillaries to red cell extravasation], the least understood is its role in maintaining the integrity of the capillaries (Zucker and Borrelli, 1958). Vitamin A has also long been known to be involved in maintaining the integrity of certain specialized epithelial tissues. Of these tissues, the capillary endothelium (Rodahl, 1950) is the one most commonly associated with vitamin A deficiency, a condition leading to capillary fragility and hemorrhages.

Since blood platelets contain MPS and vitamin A has already been demonstrated to control MPS biosynthesis, it seems possible that an excess or deficiency of vitamin A may affect the synthesis of mucopolysaccharides within blood platelets. MPS compounds of various kinds have been linked both to the coagulation process and to anti-coagulation and may be among the constituents of platelets responsible for their hemostatic effect. Therefore, since both blood platelets and vitamin A are known to be involved in maintenance of the integrity of the capillary endothelium, the influence of vitamin A on capillary endothelium may be indirect by way of its effect on MPS synthesis in blood platelets.

The objective of this research problem was to study the effects of vitamin A on peripheral blood platelets, platelet MPS, and other hematologic factors. Blood platelets were compared from vitamin A-treated rats, rats fed a normal diet, a vitamin A-deficient diet, or a vitamin A-deficient diet with vitamin A added. The specific objectives of this investigation were to determine in these groups of rats:

1. Peripheral cell numbers of blood platelets, white blood cells, red blood cells as well as hematocrits and body weights.

2. Relative sizes of blood platelets.
3. Incorporation of ^{35}S-Sulfate into blood platelets and plasma.
4. Hexosamine and protein content of blood platelets.
5. Relative amounts of several mucopolysaccharides in blood platelets (chondroitin sulfates A, B, and C, and heparin).
6. Adhesiveness of blood platelets.

CHAPTER II

REVIEW OF LITERATURE

I. VITAMIN A DEFICIENCY

Eyes

In perhaps the first serious attempt to describe the underlying tissue changes in vitamin A deficiency, Mori (1922) presented evidence that a loss of secretory power occurred in the cells of the lacrimal glands and that keratinization of the cornea and conjunctiva resulted from the dryness. The stages of xerophthalmia and associated eye lesions reported by Moore (1957) are: (1) narrowing of the space between the eyelids, (2) appearance of a bloodstained exudate around the edges of lids, and (3) development of a rough, irregular surface of the eye and prominent, dull cornea.

Respiratory Tract

As mentioned before, vitamin A deficiency initially disrupts and causes atrophy of all those epithelial tissues that function in mucus secretion in the respiratory, intestinal, and urogenital tracts (Wolbach and Howe, 1925). In the respiratory tract, keratinized cells replace the original epithelium, which is probably cast off in sheets. Pneumonia often develops and, in the acute stages of the deficiency, the lungs appear dark red through congestion with blood.

Intestinal Tract

Richards (1935) reported intestinal abnormalities in rats deprived of vitamin A. These injuries included pittings, points of hemorrhage and sometimes ulceration in the glandular part of the stomach. She thought that these injuries might be expected to affect the secretion of mucus. Diarrhea is one of the common symptoms reported. Mayer and Krehl (1948), commenting on the frequency of a bloated intestinal tract in deficient rats, attributed this symptom to the blocking of the esophagus with cellular debris; blood and debris appeared in the intestine.

Salivary Glands

The salivary glands were affected adversely in the vitamin A-depleted rat (Bernard, Halpern and Kare, 1961). These animals showed a lack of sensitivity in taste responses as evidenced by a marked decrease in rejection of quinine. Vitamin A, therefore, may have a direct effect on the functioning of the taste buds.

Urogenital Tract

The urinary tract is affected adversely in vitamin A deficiency. Symptoms range from appearance of keratinizing cells to blockage of the urinary tract with debris. Blockage of the urethra may cause the bladder to distend almost to the diaphragm and be accompanied by blood-stained urine (Moore, 1957).

Very early mention (Osborne and Mendel, 1917) was made of urinary calculi associated with vitamin A deficiency. Bliss, Livermore and

Prather (1933) showed that stones are formed in vitamin A deficiency even when vitamin D is supplied, demonstrating the specificity of vitamin A.

The epithelium of both testes and uterus are highly vulnerable to vitamin A deficiency. Keratinization of the vaginal epithelium and degeneration of the testes regularly occurs in deficient animals (Moore, 1960).

Liver

The liver, which is the main storage depot of vitamin A is reduced in size as a result of a deficiency. Wolbach and Howe (1928) attributed this change to the loss of glycogen and fat. Gross (1924) noted congestion and Moore (1957) reported that the epithelial cells of the liver were affected.

Skin

The skin becomes thickened, dry, and scaly. In some parts of the body the hair follicles become enlarged and blocked with horny plugs (Moore, 1957). Dziewiatkowski (1954) reported that in the skin of vitamin A-deficient rats, the specific activity of the sulfate-^{35}S components of sulfomucopolysaccharides increased upon administration of vitamin A.

Teeth

The injuries to the teeth are not confined to the epithelial cells, but also affect the pulp and the odontoblasts (Wolbach and

Howe, 1925). The degree of specificity of vitamin A for dental abnormalities resembles that of fluorosis (McCollum et al., 1925).

Nerves and Bones

Injuries to the nerves and bones may accompany the epithelial lesions, and even surpass them in importance depending upon the species. In swine the most characteristic lesion is paralysis of the hind legs instead of xerophthalmia, which is seen in the bovine animal as a primary symptom (Moore, 1957). As a result of vitamin A deficiency, a number of abnormalities occurred in swine: marked nervous disorders, characteristic blindness, lack of coordination and spasms, and finally death (Hughes, Lienhardt and Aubel, 1929). Reports of similar symptoms in rats have been published (Aberle, 1934; and Roa, 1936). Zimmerman and Cowgill (1936), however, found that linoleic acid added to the basal diet of vitamin A-deficient rats partially reduced the incidence of paralysis. The above nervous lesions may or may not be related to deficiency of essential fatty acids, but histological studies on these vitamin A-deficient animals showed that nervous degeneration was less severe in animals treated with linoleic acid.

In vitamin A-deficient calves, Moore (1957) concluded that blindness resulted from constriction of the optic nerve by pressure from an inadequate-sized foramen caused by overdevelopment of bone due to a deficiency of vitamin A.

Mellanby (1937) found a correlation between diets high in cereals (vitamin A-deficient) and complete deafness in dogs caused by an overgrowth of bone in the labyrinthine capsule as well as other changes that

caused nervous degeneration from stretching of the auditory nerve. He also found a relationship between blindness and overgrowth of bones in the developing dog. Moore (1939) confirmed that these nervous injuries were due to vitamin A deficiency. As a simple explanation of these lesions it was inferred that vitamin A deficiency increased the growth of bone and resulted in arrest of the growth of nerves. Other theories on the production or the significance of nerve lesions in vitamin A deficiency have been advanced: Moore and Sykes (1941) found in calves that a deficiency of vitamin A caused increased pressure in the cerebrospinal fluid and Mellanby (1943) observed disorganization in the interplay between osteoblasts and osteoclasts in dogs.

Wolbach and Bessey (1941) observed in rats with vitamin A deficiency that nervous tissue grew more rapidly than bone, resulting in compression of the brain. Rigdon (1952) reported that nervous lesions in ducks suffering from avitaminosis A caused ataxia that could not be related to injuries of the bone.

Reproduction

Vitamin A can be an important factor in the production of congenital abnormalities. It is not clear, however, to what extent the fetal abnormalities found in avitaminosis A are specific to deficiency of this vitamin. This subject has been reviewed by Giroud (1955).

Reticuloendothelial System

Several early workers reported that vitamin A deficiency caused abnormalities in the reticuloendothelial system. DeRuyter (1934) first

noticed changes in the spleen and the Kupffer cells of the liver in deficient rats. Frank (1935) confirmed the abnormality of the Kupffer cells and presented data suggesting that degeneration of Kupffer cells might explain the reduced resistance to infections that is typical in vitamin A deficiency.

Uotila and Simola (1938) observed hyperplasia of the reticulo-endothelial system in deficient rats and guinea pigs. Kupffer cells increased both in size and number. In the spleen the red pulp hypertrophied and the lymphoid tissue atrophied.

In addition to liver damage, vitamin A deficiency causes damage to the spleen and lymphatic system. In the Malpighian corpuscles of the spleen and in the lymph glands, free histiocytic elements and plasma cells replaced small lymphocytes, which were greatly reduced in number.

Blood

Information on hematological abnormalities is both inadequate and inconsistent. No recent information was found concerning hematological abnormalities associated with vitamin A deficiencies. However, Sure, Kik and Walker (1929) reported that in rats suffering from the early stages of deficiency there was a suggestion of anemia, which was characterized by a reduction of either hemoglobin or erythrocytes. In the later stages, after xerophthalmia had developed, the values for both hemoglobin and erythrocytes were raised, suggesting anhydremia. These findings in the early stages of the deficiency were confirmed by Frank (1934) who also found reduced values for hemoglobin and erythrocytes.

The white blood cells have been more extensively examined. Frank (1934) found that as a result of the deficiency the total white blood cell count was reduced, with an increase in the ratio between neutrophils and lymphocytes. In the next year, Crimm and Short (1935a) reported a delay in the rate of maturation of the granulocytes with increased numbers of senile neutrophils in rats suffering from the early stages of vitamin A deficiency, although the total number of neutrophils did not change. They also observed in animals injected with foreign protein a rise for longer periods in leukocyte counts in deficient animals than in normal animals. This suggests that the system for the destruction of leukocytes (reticuloendothelial system) was working inefficiently.

Abbott and Ahmann (1938), who studied rats with prolonged vitamin A deficiency, concluded that the deficiency caused a decrease in the neutrophil count with an increase in the number of young cells and an increase in the ratio of large to small lymphocytes. The first effect of the deficiency, however, was a marked rise in neutrophils with an equally marked decrease in lymphocytes, a result that agreed with that of Frank (1934). The administration of carotene completely reversed these findings in rats as well as in humans suffering from prolonged deficiency. Crimm and Short (1935b) and Abbott, Ahmann and Overstreet (1939) produced a preponderance of young neutrophils by the prolonged and heavy dosing of normal subjects with vitamin A.

The blood clotting mechanism has been found to be affected in both rats and human infants in vitamin A deficiency with increases in fibrinogen and blood coagulation time (Frank, 1934). Anagnostu (1939)

reported a platelet decrease of 31-69 per cent in rats that had been fed a deficient diet for 5 to 7 weeks. He also observed a decrease in the cellular content of the bone marrow which affected chiefly the eosinophilic cells and decreased the development of the megakaryocytes.

From the above evidence it may be concluded that changes in the blood picture, and particularly in the number and distribution of the white cells, occurs in animals deprived of vitamin A. It is not clear to what extent these changes are attributable to deficiency directly and indirectly to secondary infection. These findings must be interpreted with caution because of the inconsistencies between reports from different workers and even from the same worker regarding different stages of his experiments.

Sulfur-35

Decreased uptake of ^{35}S was found in the femurs and tibias of vitamin A-deficient rats (Dziewiatkowski, 1954). Sulfur-35 activity in chondroitin samples isolated from the skeletons of vitamin A-deficient rats fell progressively as deficiency continued. Administration of vitamin A increased ^{35}S activity, and, in fact, the amount of ^{35}S even exceeded the value obtained from samples of the skeletons and epiphyseal cartilages of normal rats of the same age. Dziewiatkowski (1954) further reported that administration of vitamin A to vitamin A-deficient rats resulted in decreased concentration of inorganic sulfate-sulfur from 2.5 to 1.8 mg per 100 ml of serum.

II. HYPERVITAMINOSIS A

Vitamin A characteristically causes injuries when it is given in large amounts. The nature of these lesions may provide a clue as to the mode of action of the vitamin. One other outstanding characteristic of hypervitaminosis A is an accelerated maturation of cartilage cells. These cells and bones are rapidly absorbed resulting in spontaneous fractures (Wolbach, 1947).

Hemorrhages

Takahashi *et al.* (1925) showed that excess vitamin A caused hemorrhages. Other symptoms observed were: alopecia of the head, paralysis of the hind legs, and progressive emaciation. Autopsy revealed fatty degeneration of the liver, kidneys, and heart. The digestive tract showed hyperanemia accompanied by hemorrhage. Paralysis was sometimes seen within 15 minutes after concentrates had been injected; cramps and death followed, sometimes within an hour. Harris and Moore (1928) confirmed the loss of weight and skin lesions.

After vitamin A had reached a high degree of purity, Collazo and Sánchez Rodriguez (1933) noticed another striking lesion--the extreme fragility of the bones of rats given excessive doses of concentrates. This observation has been confirmed by other workers (Vedder and Rosenberg, 1938; and Weslaw *et al.*, 1938).

Rodahl and Moore (1943) drew attention to massive internal hemorrhage as a cause of sudden death in rats given excessive amounts of materials rich in vitamin A. These hemorrhages often occurred under the

skin, but were also found in various other sites including the pericardium. These reports left no doubt that excessive amounts of rich sources of vitamin A were toxic, but some of the evidence suggested that associated substances, rather than the vitamin itself were the cause of toxicity.

Moore and Wang (1945) found that young rats given large doses of crystalline vitamin A acetate (25,000 or 40,000 IU daily) developed a limping gait, sometimes within a week, and by three weeks they were dead. Autopsy revealed fractures of the bones usually accompanied by profuse subcutaneous and intramuscular hemorrhages. Bone damage seemed to result from inadequate formation of or destruction of the matrix. It appeared, therefore, that bone lesions and hemorrhages were specific effects of too much vitamin A. The occurrence of these lesions was confirmed by Rodahl (1950).

The immediate cause of these sudden hemorrhages was investigated by Light, Alscher and Frey (1944) who advanced the idea that delayed clotting time of blood plasma indicated a marked hypoprothrombinemia.

Maddock, Wolbach and Jensen (1948) found that excessive doses of vitamin A given to normal weanling rats produced multiple massive hemorrhages, most commonly found in the subdural space, epididymis, skeletal muscles, and body cavities. Lymph nodes were hemorrhagic in appearance and hematocrit values fell as low as 13 per cent. Prothrombin times were prolonged from 25 seconds or less in control animals to a range of 70-183 seconds in the excess vitamin A-treated group.

Matschiner and Doisy (1960) reported marked hypoprothrombinemia in rats receiving 500 IU per day of vitamin A for eight weeks but not in control animals or rats receiving 5 IU per day. Severe hypoprothrombinemia occurred at the 5000 IU per day level and 60 per cent of the mortality due to hemorrhage occurred at the 15,000 IU per day level. Richardson (1961) also observed that the incidence of hemorrhages was higher in rats receiving 60,000 IU of vitamin A per kilogram of diet than those receiving 30,000 IU. Doisy and Matschiner (1961) found that vitamin A acid was ten to fifteen times more detrimental in producing hemorrhages and in lowering prothrombin than vitamin A acetate.

Walker, Eylenburg and Moore (1947) noticed that the blood of hypervitaminotic A rats was thin and watery. A decreased hematocrit was observed even in the blood of rats devoid of hemorrhages. Glauert et al. (1963) have shown that rabbit erythrocytes are hemolysed in vitro by treatment with vitamin A alcohol. These findings may be related to a claim by Poumeau-Delille (1943) that toxic doses of vitamin A induce severe erythroblastic anemia in rats.

Reproductive Organs

Maddock, Cohen and Wolbach (1953) found that long-continued ingestion of vitamin A gives rise to degenerative lesions of the testes in weanling rats, but does not affect the testes of mature rats. Although reproductive lesions are important, the most prominent lesion associated with hypervitaminosis A was hemorrhage.

Bones

Clark and Smith (1964) observed increased calcium and phosphorus in the presence of large amounts of vitamin A and suggested an increased cycling of mucopolysaccharide and collagen. Toxic amounts of the vitamin, on the other hand, decreased mucopolysaccharides and collagen content of bone with no demonstrable changes in ash content.

Wolbach (1947) in comparing the effects of deficiency and excess of the vitamin on bone growth in rats reported that excessive doses caused accelerated growth of the epiphyseal cartilage cells. The processes of maturation and remodeling accompanying growth also were accelerated. From this observation Wolbach and Maddock (1952) postulated that excess vitamin A might exert its action by stimulating the anterior pituitary gland. They found, however, that the bones of hypophysectomized rats were affected even more rapidly than those of intact animals given excess vitamin A.

Wolbach, Maddock and Cohen (1955) reported that the effects of hypervitaminosis A on the skeleton of weanling rats were accelerated by adrenalectomy, but administration of cortisone did not have a significant influence on the process.

Large doses of vitamin A given to young rabbits led to a depletion of articular and epiphyseal cartilage matrix, thinning of this cartilage, and vascularization of the metaphyseal border of the plate. The costal cartilage, on the other hand, showed no detectable histological or histochemical change (McElligott, 1962). The vitamin caused a loss of chondroitin sulfate from cartilage. This loss may have been due to the degradation of a protein-chondroitin sulfate complex.

Wolbach and Maddock (1952) gave vitamin A to rats orally at the rate of 1,000 IU per gram of body weight. From post-mortem studies they concluded that skeletal responses to excessive vitamin A were not mediated through the pituitary gland. In another endocrine study, parathyroidectomy did not change the evolution of hypervitaminosis A in weanling rats (Cohen, Maddock and Wolbach, 1955).

Skin

The initial effect of vitamin A palmitate administered intraperitoneally to rats at the rate of 150,000 IU per day upon the chemistry of connective tissue was a marked loss of hyaluronic acid and chondroitin sulfate from the saline soluble components of skin (Chung and Houch, 1964). Additional doses of vitamin A resulted in a significant decrease in the glycoprotein content of these fractions. This work has been confirmed, in part, by Barker, Cruickshank and Webb (1964), who found that over 60 per cent of ^{35}S--administered as $Na_2{}^{35}SO_4$--taken up by the skin was in a mucopolysaccharide fraction. Also, excess vitamin A caused the specific activity of the MPS fraction to be nearly doubled when skin slices were incubated with $Na_2{}^{35}SO_4$.

III. HYPERVITAMINOSIS COMPARED WITH AVITAMINOSIS A

In the intact rabbit, hypervitaminosis A results in a depletion of cartilage matrix, due to loss of cartilage chondromucoprotein, followed by a rise in serum chondroitin sulfate (Thomas et al., 1960). This finding is the reverse of the effect of vitamin A excess on mucous

tissue, which indicates a difference in mode of action of vitamin A on MPS for connective and mucoid tissue.

In avitaminosis the columnar and secretory cells or membranes tend to be replaced by keratinized, squamous cells. In hypervitaminosis, membranes that are normally keratinized are changed in the reverse direction by the formation of mucus-secreting cells. In both deficiency and excess, bone formation is abnormal, the lesions differing considerably. In both conditions the reticuloendothelial system is affected--swelling of the Kupffer cells in avitaminosis and fat deposition in hypervitaminosis.

IV. CHEMISTRY AND BIOCHEMISTRY OF MUCOPOLYSACCHARIDES

Sulfated polysaccharides are found throughout the tissues of animals. Materials so widely distributed must surely have important functions to perform. The heparins at least are known to aid somehow in the maintenance of the blood's fluidity. The chondroitin sulfates (ChS) are involved in the formation of bone and in the hydration of tissues, and the mucoitin sulfates lubricate and protect the gastrointestinal tract. Our knowledge of the functions of these substances is scanty.

The pathway of biosynthesis of acid MPS has recently been advanced in a review by Dorfman, Markovitz and Cifonelli (1958).

Table I shows a comparison of the components of several MPS that are found in mammalian tissues.

TABLE I

A COMPARISON OF THE CHEMICAL COMPONENTS OF SEVERAL MUCOPOLYSACCHARIDES COMMONLY OCCURRING IN MAMMALIAN TISSUES

Mucopolysaccharide	Hexosamine	Uronic Acid	SO_4--on Hexosamine	Hydrolysis with Hyaluronidase
ChS A	D-galactosamine	D-glucuronic	C-4	Yes
B	D-galactosamine	L-iduronic	C-4	No
C	D-galactosamine	D-glucuronic	C-6	Yes
α Heparin	D-glucosamine	D-glucuronic	C-2	--
Hep Sulfate	D-glucosamine	Uronic acid?	?	--
Kerato-Sulfate	D-glucosamine	None	?	--

V. BIOCHEMICAL EFFECTS OF VITAMIN A

Epithelial Cells

Fell and Mellanby, using tissue culture techniques (reviewed by Fell in 1953) attempted to determine whether the effects of vitamin A deficiency and hypervitaminosis A were expressed indirectly and mediated through an endocrine organ or expressed directly on the tissue. In their first experiments (1952) with explants of undifferentiated chick embryo bones cultured in a medium with excess vitamin A, they found that the bone turns soft and gelatinous, growth diminishes, and fractures often occur. It is interesting to note that the cartilage matrix ceased to stain with basophilic dyes and metachromasia disappeared (the reaction of the stain toluidine blue to the acid groups of chondroitin sulfate). They later thought that dissolution of the chondroitin sulfate occurred while the collagen part of the matrix remained intact (Fell, Mellanby and Pelc, 1956). Similarly, mouse bone-cartilage matrix and bone was absorbed and disappeared from fully developed fetus. Apparently the concentration of the vitamin was similar to that found in the blood. Although this action occurs only on growing cells, it leaves little doubt that the action of vitamin A was direct.

Fell and Mellanby (1953) later found that when embryonic chick ectoderm was cultured in the presence of excess vitamin A, it formed ciliated columnar cells having secretory vacuoles that actively secreted mucus instead of developing into normal ectoderm (a keratinized layer over squamous epithelium). After removal from the high vitamin A medium the tissue reverted to normal epithelium.

These results demonstrate that vitamin A (1) disrupts chondroitin sulfate of cartilage matrix and (2) causes epithelial basal cells to differentiate into mucus-secreting cells instead of forming keratinizing cells. Furthermore, under the influence of excess vitamin A, related mucoid substances of mucous epithelium showed an increased uptake of sulfate and synthesis of sulfate-containing compounds (Fell, Mellanby and Pelc, 1954).

Wolf, Wagle and Johnson (1957) postulated that excess vitamin A increases the mucous type and decreases the connective tissue type of MPS. The reverse apparently occurs in deficiency.

Under the influence of vitamin A, the cartilage cells may produce an enzyme which converts chondroitin sulfate to a more soluble form. This would result in a soluble sulfated MPS, instead of the normal chondroitin sulfate, with the dissolution of existing matrix (Fell, Mellanby and Pelc, 1956).

In more recent studies, Fell (1960) found that vitamin A enhanced the incorporation of sulfate by explants of chicken skin. She suggests that normal keratinization of skin apparently results in a slowed biosynthesis of MPS, and vitamin A administration promotes mucous secretion replacing keratin formation.

Release of Proteolytic Enzymes

The results of studies by Fell, Dingle and Webb (1962) suggest that vitamin A enhances the release of proteolytic enzymes from cartilage by catalyzing the hydrolysis of the protein moiety of the protein-MPS

matrix to yield nonprotein-bound MPS. Accordingly, these results indicate that perhaps excessive amounts of vitamin A increase the secretion of MPS by promoting a more rapid breakdown of the MPS-protein matrix rather than by excessive MPS formation or synthesis rates (Lucy, Dingle and Fell, 1961; and Thomson and Pitt, 1960).

It was also demonstrated (Fell, Dingle and Webb, 1962) that a specific interaction of vitamin A and protein is required to obtain an effect from proteolytic enzymes. Liver lysosomes release proteolytic activity when treated with the vitamin, although the lipoprotein membrane of the lysosome remains unaltered. After studying cold lysis of erythrocytes by vitamin A as a model system of the vitamin-lipoprotein membrane interaction (Lucy and Dingle, 1962a; and Fell and Dingle, 1963) suggested that a protein-vitamin A interaction may be required for the lytic activity. In addition, vitamin A released a protease from a particulate preparation obtained from bovine and chick cartilage (Fell and Dingle, 1963).

Glauert et al. (1963) have investigated the sequence of changes in the fine structure of erythrocytes during lysis as a result of treatment (in vitro) by vitamin A alcohol. Within one minute after treatment with vitamin A, the surface area of the cells was greatly increased. Large indentations appeared in the surfaces of the cells and vacuoles were formed from the indentations by a process that resembled micropinocytosis. The cells rounded and hemoglobin was lost as breaks appeared in their membranes. Finally, ghosts were produced that were no longer spherical but contained numerous vacuoles. These observations

support the idea that one site of action of vitamin A is the lipoprotein membrane.

In further work, Lucy, Luscombe and Dingle (1963) showed that vitamin A alcohol causes swelling of rat liver mitochondria *in vivo* presumably through its action on the lipoprotein membrane, which suggested that vitamin A in optimum quantities may be required for the maintenance of the normal structure and function of mitochondria.

Many agents active at the surface have analogous effects on the cell membrane. Therefore, it remains to be seen whether a major function of vitamin A in normal metabolism is in insuring the structural integrity as well as the normal permeability of the cell membrane and of the membranes of subcellular particles.

DeLuca *et al.* (1963), who determined catabolic products in liver homogenates, found that vitamin A deficiency in young male albino rats resulted in a markedly elevated rate of oxidation of pyruvate, citrate, alpha-ketoglutarate, succinate, glutamate, and fumarate, but not beta-hydroxybutyrate and caprylate. Vitamin A given to deficient animals reduced the oxidation rates to normal values within 48 hours. Vitamin A deficiency may be related to component(s) common to all these systems, possibly to membrane structure and stability (Dingle, 1961; Lucy and Dingle, 1962b; and Dingle and Lucy, 1963) or to changes in the structure of mitochondria.

Glycogen Synthesis

The first indication of an action of vitamin A on glycogen biosynthesis was pointed out by Wolf, Lane and Johnson (1957), who presented

evidence that the metabolic effects of vitamin A were directly or indirectly involved in the reversal of glycolysis between the triose and glucose stage in the intact animal. This was shown by a reduction in the incorporation of labeled acetate, lactate, and glycerol into liver glycogen of the vitamin A-deficient rat. Reduced glyconeogenesis was later observed by Wolf and Phil (1961) although this had not been found earlier in liver homogenates from deficient rats, and results had indicated that oxidative phosphorylation was not affected (Wolf et al., 1958).

Adrenal Cortex Functions

Reduced glyconeogenesis was believed to be related to a malfunction of the adrenal cortex (Wolf et al., 1958) and, ultimately, to the interruption of adrenal glucocorticoid hormone (corticosterone) biosynthesis in the vitamin A-deficient rat. In adrenal cortex homogenates from vitamin A-deficient animals, the reduced corticosteroid biosynthesis could be restored to normal by adding the vitamin or its acid in vitro.

Mucopolysaccharide Production

One of the earliest known manifestations of vitamin A deficiency is the drying and hardening of mucous-secreting tissue. Excess of the vitamin has been shown by Fell, Mellanby and Pelc (1954) to increase the activity of the mucous-secreting tissues. These observations suggested that the vitamin would lead to an increase in the biosynthesis of the mucous type of MPS. In vitro studies using rat colon segments and colon homogenates clearly demonstrated a direct role for vitamin A in the

biosynthesis of colon MPS (Wolf and Varandani, 1960; and Varandani, Wolf and Johnson, 1960). Vitamin A deficiency decreased the incorporation of ^{35}S-sulfate into the MPS of the rat colon whereas the addition of retinol to the in vitro system restored incorporation to normal.

Pig colon mucosa has been studied by Wolf and Varandani (1960), and MPS was identified by methods similar to those for determining rat colon MPS. The synthesizing activity of MPS was located in the supernatant solution of pig colon mucosa, and evidence indicated that vitamin A was involved in MPS biosynthesis at the sulfate activation step.

Wolf, Varandani and Johnson (1961) reported that an enzyme fraction (precipitated at pH 5.2) was obtained from pig colon mucosal homogenates, which upon incubation with labeled sulfate or labeled glucose, produced labeled MPS. The optimum pH of this enzyme fraction was 6.8. The ^{35}S-MPS produced by the pH 5 enzymes could be precipitated with carrier chondroitin sulfate by cetyl-pyridinium bromide. Treatment with lipoxidase (an enzyme which destroys vitamin A) lowered incorporation, which could be restored by adding vitamin A. The activity of pH 5 enzymes from vitamin A-deficient pigs was reduced but again could be restored by adding vitamin A. The authors concluded that vitamin A exerted its influence on MPS synthesis through its effects on, or participation in, the sulfate activation reactions.

Since esterfied sulfate represents an important part of the mucopolysaccharide molecule, a number of investigations have dealt with the effect of vitamin A deficiency on sulfate incorporation into cartilage. Dziewiatkowski (1954 and 1956), for example, isolated chondroitin

sulfate after injection of labeled sulfate into vitamin A-deficient, normal, and vitamin A-treated deficient rats. The level of labeled sulfate was higher in the normal and vitamin A-treated deficient rats than in the untreated deficient rats. He also found increased breakdown as well as increased synthesis of chondroitin sulfate in the vitamin A-treated animals.

Somewhat different results were obtained by Frape et al. (1959) in vitamin A-deficient pigs. These workers found that vitamin A treatment (790 IU per pound of feed) caused decreased sulfate uptake into cartilage. Higher levels of vitamin A (up to 11,393 IU per pound of feed) raised specific activities in the blood, lung, and cartilage.

Wolf, Wagle and Johnson (1957), Wolf et al. (1958) and Wolf and Phil (1961) observed that vitamin A deficiency decreased the incorporation of carbohydrates, ammonia, acetate, and sulfate into MPS by enzymes from cell-free preparations of rat colon. Rate of synthesis was restored to normal by the addition of vitamin A to the preparation. Further study demonstrated that the metabolic defect in the absence of vitamin A was associated with the incorporation of sulfate into MPS. The normal, activated donor of sulfate residues to form MPS was 3'phosphoadenosine-5'-phosphosulphate (active sulfate). The rate of formation of active sulfate by cell-free preparations of colon from vitamin A-deficient rats was substantially slower than the rate of formation of active sulfate by tissue from normal rats or by tissue from deficient rats to which vitamin A was added during incubation (Varandani, Wolf and Johnson, 1960). Vitamin A thus may be a metabolic requirement

in obtaining a normal rate of biosynthesis of active sulfate. A decreased rate of active sulfate formation may limit the subsequent rate of formation of MPS. This result does not suggest that the vitamin may affect the biosynthesis of active sulfate in other tissues. Pasternak, Humphries and Pirie (1963) checked this point in studies on the formation of active sulfate by cell-free preparations of corneal tissue from vitamin A-deficient rats and rabbits. Their results unequivocally showed that (1) the deficiency did not reduce the rate of formation of active sulfate and (2) the addition of vitamin A to the incubation mixture did not alter the formation rate.

Vitamin A also affects net synthesis of MPS. Moretti and Wolf (1961b) determined total hexosamine content of rat colon from vitamin A-deficient rats and normal rats. The hexosamines were separated from low-molecular weight, hexosamine-containing intermediates by precipitation and dialysis. They reported that the total MPS hexosamine was lowered by about 60 per cent from deficiency of the vitamin. The glucosamine/galactosamine ratio was changed also, and vitamin A deficiency caused a decrease mainly in the content of galactosamine-containing MPS in rat colons (Moretti and Wolf, 1961a). These results, therefore, establish that vitamin A has an effect on the content and net synthesis of MPS in rat colon.

Ushimi, Sasamoto and Homma (1963) determined total hexosamine content of organs and vitamin A content in the liver of deficient and normal rats. They found a decrease in total hexosamine in the vagina,

uterus, and colon of rats fed the vitamin A-deficient diet but no decrease in the amount of vitamin A in the liver.

Wolf, Bergan and Sundaresan (1962 and 1963) developed a system utilizing particles from colon homogenates sedimenting at 20,000 x gravity, which could produce net synthesis of MPS-bound hexosamines from glucose-6-phosphate, glutamine, ATP, UTP, and DPN and Mg^{++}. Histological examination showed the suspensions to be cell-free, and bacteriological tests revealed no bacterial contamination. The level of MPS-bound hexosamines formed by whole colon homogenates of vitamin A-deficient rats was reduced as compared to controls. Addition of vitamin A to vitamin A-deficient particles did not completely restore their ability to synthesize MPS but by adding a metabolite derived from vitamin A acid it could be restored. The role of vitamin A in the biosynthesis of MPS thus may not be a direct one but may be mediated through a metabolite. The crude homogenate used previously by Wolf and coworkers may have contained this metabolite.

Evidence so far indicates that vitamin A may be involved in a controlling step in MPS biosynthesis, particularly in stimulation of such synthesis in the mucus-forming system.

Sulfate-35 Labeled Amino Acids

Smudski and Myers (1963) found a decrease in total sulfur in urine in vitamin A-deficient animals after an intraperitoneal injection of ^{35}S-labeled cystine. The sulfur was mostly in the form of sulfate.

No renal-function tests were performed in this study, however, and the possibility remains that vitamin A deficiency may have had an effect on the kidney.

Platelet MPS

The presence of mucopolysaccharides in blood platelets was strongly suggested by Gude, Upton and Odell (1956). These investigators observed that the action of hyaluronidase inhibited subsequent staining with PAS, silver methenamine, by Hale procedures, by the intense reactions with muchematein and mucicarmine, and by the red (gamma) metachromasia with toluidine blue. Following this cytochemical evidence, Odell and Anderson (1957) undertook a study to determine if the ^{35}S-sulfate in rat platelets (Odell, Tausche and Gude, 1955) is present in a mucopolysaccharide. They found that most of the radiosulfate in ^{35}S-labeled rat blood platelets was present in a sulfated mucopolysaccharide, and furthermore that it behaved similar to chondroitin sulfate when subjected to paper electrophoresis and paper chromatography. In later experiments, Anderson and Odell (1958) extracted two mucopolysaccharide fractions from rat blood platelets, one with properties similar to chondroitin sulfate and the other similar to a uronic acid-free MPS. Kerby and Taylor (1959) found that human thrombocytes contained a mixture of MPS and possibly a mixture of ChS B and C.

Odell and McDonald (1964a) concluded that within 4 to 6 hours after sulfate injection, platelets that have been labeled in the marrow--while still a part of the cytoplasm of megakaryocytes--begin to enter

the blood stream. Sulfur-35 was present in these platelets largely in a sulfated MPS and appeared to undergo little turnover in circulating platelets.

CHAPTER III

MATERIALS AND METHODS

I. ANIMALS

Male Sprague-Dawley rats were used in all experiments. These rats were obtained directly from Sprague-Dawley, Inc., Madison, Wisconsin or they were reared at University of Tennessee-Atomic Energy Commission, Agricultural Research Laboratory, using Sprague-Dawley stock. The rats were kept in a room automatically maintained at a temperature of 76 degrees F. and 55 per cent relative humidity. The rats were kept in wire cages and water bottles changed two or three times per week.

Hypervitaminosis A Animals

Rats weighing approximately 350 grams were given orally by a stomach needle 50, 100, 500, or 1000 International Units (IU) of vitamin A per gram of body weight[1] in addition to vitamin A in the normal diet.[2] Control rats were given 1 ml of distilled water daily in the same manner as the water-solubilized vitamin A. At the 50 and 100 IU levels, rats were dosed daily, while at the 500 and 1000 IU levels rats were given vitamin A only at the beginning of each experiment. Body weights were determined daily, and doses were based on these weights.

---

[1]Water-solubilized natural vitamin A (Aquasol), US Vitamin and Pharmaceutical Corp., Arlington-Funk Labs, New York, N. Y.

[2]Purina Laboratory Chow which contained approximately 21.44 IU of vitamin A per gram of feed.

Hypovitaminosis A Animals

Rats approximately 4 weeks of age and weighing 51 to 92 grams were fed a vitamin A-deficient diet[3] containing a salt mixture, vitamin D, starch, vegetable oil and casein. A second group of rats was given the above ration with vitamin A added[4] at the rate of 21.44 IU per gram of feed, and a third group was fed the standard laboratory diet. Body weights were determined weekly and when rats failed to gain weight they were considered vitamin A-deficient.

II. PERIPHERAL BLOOD

Counts of platelets, white blood cells (WBC), and red blood cells (RBC), and determinations of hematocrit and body weight were made at weekly intervals on the vitamin A-deficient groups and at daily intervals on normal and vitamin A-excess animals. Blood for platelet counts was taken into a red cell counting pipette directly from a puncture in the saphenous lateral vein of a hind limb that had been shaved and smeared with silicone stopcock grease. For platelet counts the blood was drawn to the 1.0 mark and then diluted with 1 per cent ammonium oxalate. All of the platelets in five blocks of the small square of the hemacytometer chamber (as for RBC) were counted after 20 minutes of settling, using a phase microscope, dark contrast, 43 x objective, long-working phase condenser, and a counting chamber having an optically flat bottom.

---

[3]Purchased from Nutritional Biochemicals Corp., Cleveland 28, Ohio.

[4]Pamilets, type M 250A, containing 250,000 IU of vitamin A per gram.

In most cases, both sides of the chamber were counted, and the total number counted was multiplied by 2500 to give the number of platelets per cubic millimeter. Number 1-1/2 coverslips were used in place of the thick standard hemacytometer coverslips according to the method of Brecher and Cronkite (1950).

Hematocrits were taken in quadruplicate from the same puncture, using double-oxalated microhematocrit capillary tubes (32 x 0.8 mm), and sealed with a vinyl plastic putty (Critoseal); in later experiments only one sample was taken in the larger heparinized tube (1.3 to 1.5 x 75 mm). The sealed capillary tubes were spun for 6 minutes in an International micro-hematocrit centrifuge. The percentage of cells was obtained with a micro-capillary reader.

Total WBC and RBC counts were determined (using a Fisher Autocytometer, Model 6-246V1) on blood drawn from the hind limb. A previously-determined threshold setting of 61 was used for both WBC and RBC (Figure 1). Twenty lambda blood samples were diluted in reservoirs containing 5 ml of filtered diluent (unopettes; Becton, Dickenson and Company). The dilutions were 1:250 in 3 per cent acetic acid for WBC and for RBC 20 lambda of the first dilution were added to 5 ml of normal saline which gave a final dilution of 1:62,500.

III. PREPARATION AND INJECTION OF ISOTOPE

Sodium sulfate-^{35}S in dilute HCl was purchased from Oak Ridge National Laboratory. Its pH was adjusted to 6.3 to 6.9 with approximately 4 N NaOH using a small amount of Dibromothymolsulfonphthalein

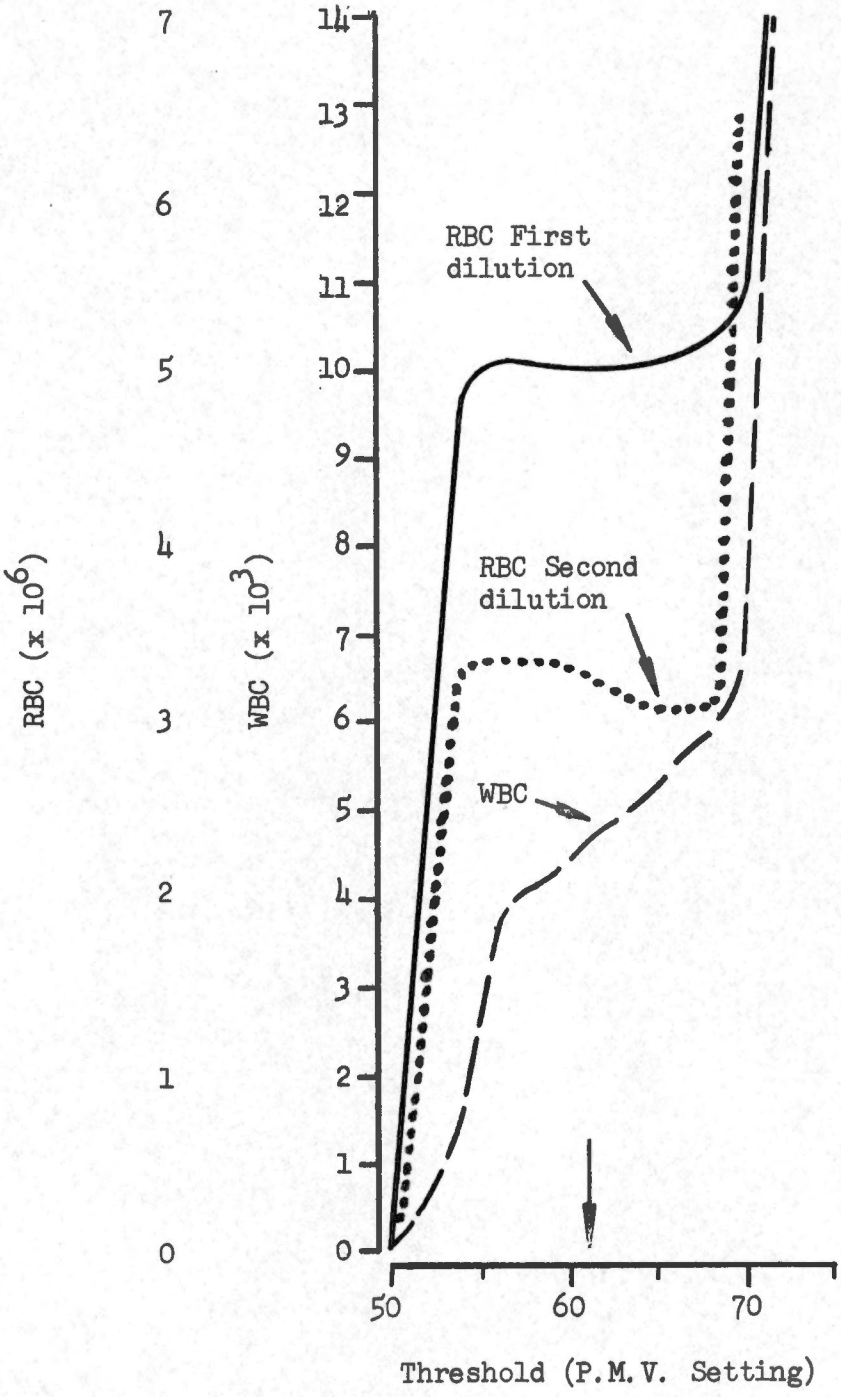


Figure 1. Effect of threshold on cells/mm^3 using the Fisher Autocytometer.

as an indicator. The final dilution with distilled water was made so that the concentration[5] was approximately 1 mc/ml. Animals were injected intraperitoneally with 1 μc of $Na_2{}^{35}SO_4$, per gram body weight.

IV. UPTAKE OF ^{35}S BY BLOOD PLATELETS AND PLASMA

Rats were killed 24 hours after the last of two to ten daily-isotope injections or 48 hours after single-isotope injections (Odell and McDonald, 1964b). Ten minutes before killing, 2 ml of 1:10 dilution of heparin (heparin sodium, 1000 IU per ml, Drake Laboratories, Chicago) was injected intraperitoneally. The animals were anesthetized with ether, and blood was drawn from the abdominal aorta into cold, siliconized, 20-ml syringes containing 1 ml of a 1 per cent solution of ethylenediaminetetraacetic acid (EDTA) in 0.538 per cent saline. In most cases, a standard volume of 9 ml of blood was collected from each rat and was diluted with 6.25 ml of saline to obtain a better yield of platelets. The blood-saline mixture from each rat was equally divided into two 15-ml conical centrifuge tubes, and the platelets were separated by differential centrifugation in a refrigerated centrifuge (5°C). After a slow centrifugation of the blood (107 x g for 40 minutes), the platelet-rich plasma-saline was removed and spun more rapidly (760 x g for 30 minutes) to obtain a platelet button. The platelets were then washed twice with 4 ml of saline and resuspended in 0.5 ml of saline.

---

[5]The amount of radioactivity per volume of isotope was given by supplier with a range of accuracy of ± 20 per cent. The amounts injected are, therefore, not directly comparable among experiments.

WBC and platelets were counted on each platelet suspension. Two 200-µl samples of platelet suspensions (containing 3.240 to 12.660 x 10^6 platelets per mm^3) were placed on planchets and dried for radioactivity counting in a windowless gas-flow proportional counter in some experiments. In other experiments, the platelet suspensions were added to polyethylene counting vials containing 5 ml of scintillation mixture of the following composition: 1 liter of 1,4-dioxane; 175 grams of napthalene; 7 grams of 2,5-Diphenyloxazole; and 0.375 grams 1,4-bis-2-(4-Methyl-5-Phenyloxazolyl)-benzene. This scintillation mixture was shown in preliminary experiments to correct for 200 µl of water with 92-93 per cent efficiency. The vials containing the activity were counted in a liquid scintillation spectrometer (Model 3214, Packard Instrument Company). The radioactivity of two 100 or 200 µl samples of platelet-free plasma-saline and one 200 µl sample of each wash was also determined. Each sample was counted three times for a 5-minute interval, and the background determined from a scintillation mixture-blank was subtracted from the final count before correcting for decay.

V. PLATELET SIZE

Platelet size was determined by: (1) measuring the volume of packed platelets and (2) estimating the platelet size distribution with a Coulter Electronic Particle Counter Model B, coupled with a particle-size distribution plotter. Washed platelets from the ^{35}S uptake experiments were used for both size determinations.

The volumes of packed platelets in the suspensions were measured in quadruplicate in microhematocrit tubes according to the method of Detwiler, Odell and McDonald (1962). The sealed capillary tubes containing the platelet suspensions were centrifuged for 6 minutes in an International Microhematocrit Centrifuge and the column lengths were measured with a cathetometer. The average platelet volumes were expressed as:

$$\frac{\text{(platelets/ml of suspension)}}{\text{(length of platelet column)/(length of total column)}} = \text{platelets per ml of packed volume}$$

Platelet sizes were measured using a Coulter Counter according to the method of McDonald, Odell and Gosslee (1964). A 50 μ aperture was used and the amplification and aperture current were set so that the mode (peak window) was near the center of the plot (see Figure 2). The same settings were used throughout the experiments so that results between experiments could be compared. The suspensions for counting were made by placing 10 to 20 μl of platelet suspensions into 100 ml of filtered saline solution (filtered through a 0.45 μ pore size filter) to give concentrations of 9 to 25 x 10^3 platelets per 50 μl. Silicone treated glassware was used throughout.

After sizing was accomplished, the plotter was disengaged and the number of particles per 50 μl determined. The lower threshold was set at 8 and the upper threshold at its maximum of 100 in counting the platelets without the plotter.

Windows 1, 2, and 3 were omitted from the size distribution since they contained counts due mainly to background noise (see Figure 2).

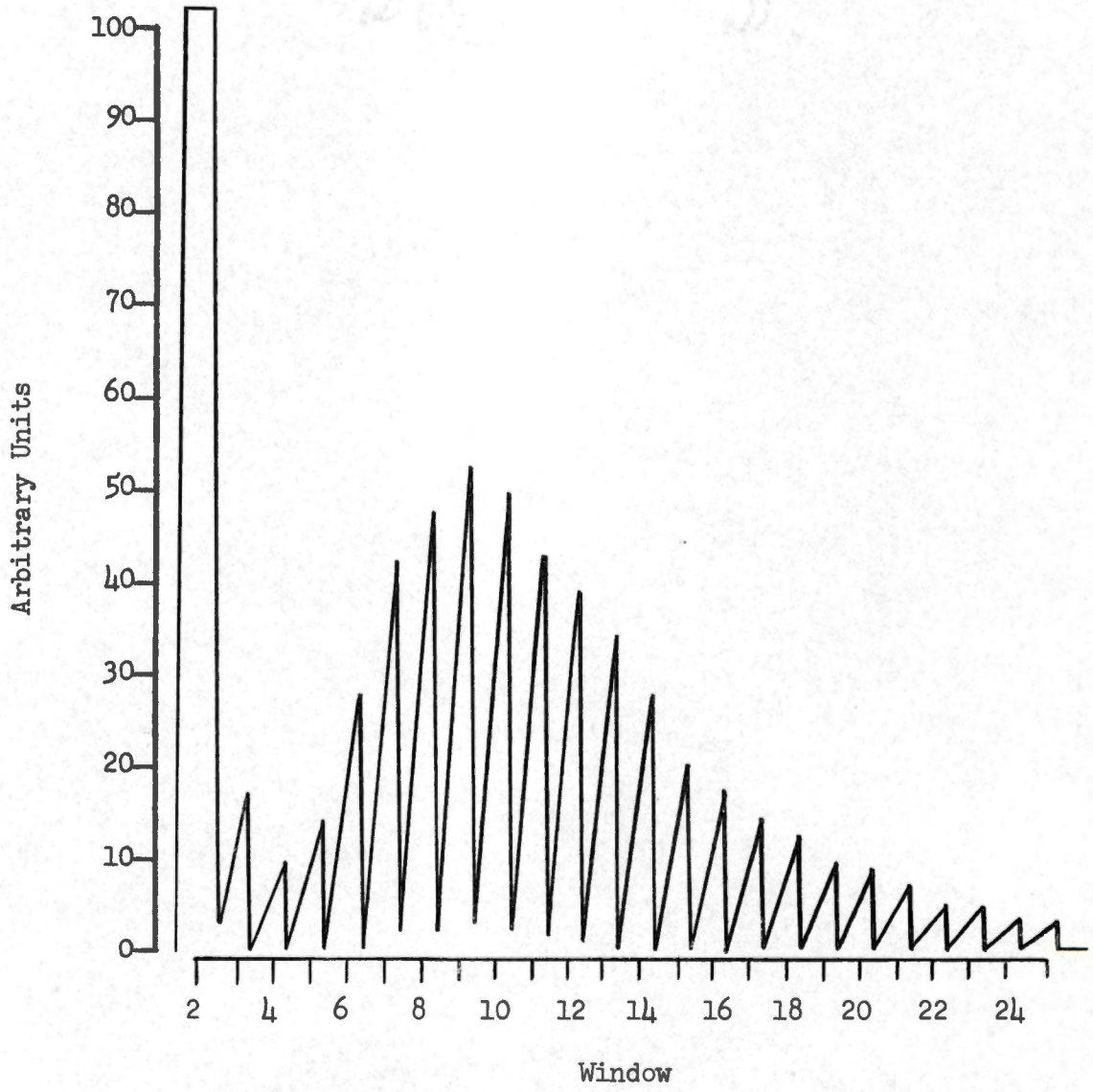


Figure 2. Plot taken from plotter of Coulter Counter showing the size distribution of normal platelets. The amplification was set at 1/2; the fine adjustment of amplification at 50 per cent; and the aperture current at .354. The plotter scale was adjusted to 4 with 4 second cycle time. This suspension contained 24,960 platelets/50 µl.

Also, counts were not possible on sizes corresponding to window numbers greater than 25, and a statistical method for truncation was used (Cohen, 1957) to obtain unbiased estimates of the mean and variance of the size distributions which are expressed here as mean windows.

VI. DETERMINATION OF HEXOSAMINES

Quantitative measurements of hexosamines were made on blood platelets of one group of rats that received 100 IU of vitamin A per gram body weight daily, a second group that received 500 IU per gram body weight at day 0, a third that was fed a vitamin A-deficient diet, and a fourth group was fed the standard laboratory diet. Hypervitaminotic rats were killed on days 4, 5, 6, and 7. Blood platelets of each rat were collected as previously described, and resuspended in approximately 1.2 ml of physiological saline solution. Platelet numbers were determined by the phase microscope method. One ml of the platelet suspension was added to 1 ml of 2 M HCl and immersed in a hot water bath (100°C) for 4 hours. To prevent evaporation in the water bath, the tubes were capped with marbles. A modification of Belcher, Nutten and Sambrook's (1954) technique for determining glucosamine was used in further treatment. After heating, the samples were cooled and 1 ml of water was added. One ml of freshly prepared acetylacetone (2,4-Pentanedione) mixture (1 ml of acetylacetone in 50 ml of 1 M Na_2CO_3) was added to the platelet samples and 0.1 to 1.0 µmoles of glucosamine or galactosamine. The samples were re-immersed in the hot water bath for 10 minutes, cooled, and 5 ml of 95 per cent ethanol was added to them. The tubes

were placed in a thermostatically controlled water bath (75°C) for 5 minutes. Then, 1 ml of p-Dimethylaminobenzaldehyde solution (made by dissolving 0.8 grams of p-Dimethylaminobenzaldehyde in 60 ml of concentrated hydrochloric acid) was added and mixed thoroughly with a vortex mixer. The tubes were re-immersed in a water bath (75°C) so that the water reached just above the level of the liquid in the tube and were held there for another 30 minutes with occasional shaking. After removal from the bath the tubes were allowed to cool to room temperature. The optical density (OD) of the samples was then determined with a Bausch & Lomb Spectronic 20 spectrometer set at 520 mμ. The OD readings were compared to readings of known amounts of glucosamine and galactosamine, and the amounts of hexosamine were determined from a standard graph prepared using the above technique (Figure 3). The visual platelet counts were then used to determine the hexosamine content per platelet.

VII. DETERMINATION OF PROTEIN

Other platelets from the platelet suspensions used in hexosamine determinations were used for protein determinations: 100 μl of platelet suspensions were added to 3.9 ml of water, mixed, and 0.5 ml of this suspension was pipetted into a test tube. To each tube 5.0 ml of an alkaline solution (4 per cent filtered Na_2CO_3; 2 per cent $CuSO_4$; 4 per cent sodium potassium tartrate, 100:1:1 by volume) was added, mixed well, and the tubes placed in a 40°C water bath for 15 minutes. One half ml of Folin-Ciocalteu reagent (FCR),[6] diluted 1:2 with water immediately before

[6]Folin-Ciocalteu reagent (Phenol reagent), Harshaw Scientific.

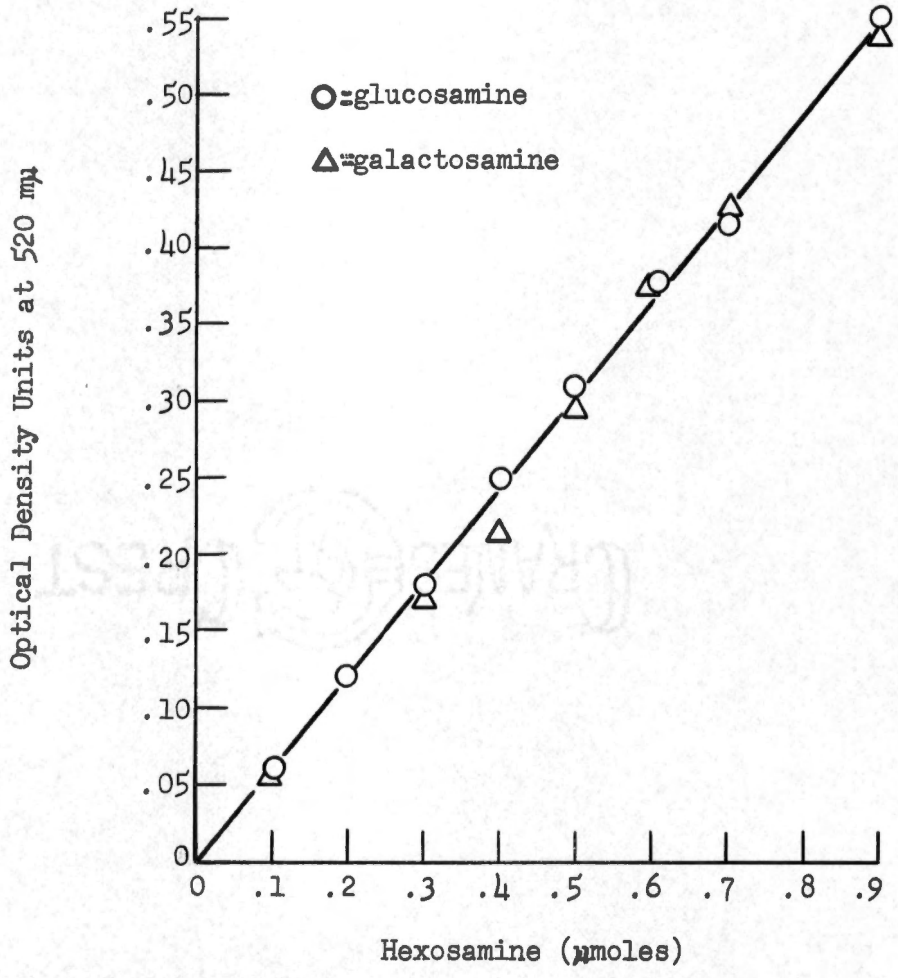


Figure 3. Optical density of varying concentrations of hexosamine.

use, was added to each tube and mixed well. After 30 minutes of color development, the samples were read at 660 mμ with a spectrometer. OD readings were compared to readings of known amounts of Bovine Serum Albumin (Figure 4) and amounts of protein were determined per platelet.

VIII. IDENTIFICATION OF MPS

Platelets from rats for identification of MPS experiments were from the same treatment groups as for hexosamine determinations. The platelets were labeled in vivo by daily intraperitoneal injections of 1 μc of $Na_2{}^{35}SO_4$ per gram of body weight for 5 days before killing. Rats killed at 4th day after vitamin A treatment, for example, were given the isotope on days minus 1, 0, 1, 2, and 3. Rats were killed at 4, 5, 6, and 7 days after treatment with vitamin A or in another group when they were vitamin A-deficient. The platelets were harvested as described above. The material was kept cold (0-5°C) throughout the experiment. At each time period the platelets of three rats were pooled and resuspended in saline to a final volume of 2.5 ml. These suspensions contained a concentration of 4.070 to 8.170 x 10^6 platelets per mm^3. The radioactivity of two 200 μl samples of the platelet suspensions and two 200 μl samples of plasma-saline, and one 200 μl sample of each wash was determined using the liquid scintillation technique discussed above.

To extract mucopolysaccharides from platelets, duplicate 1-ml samples of ^{35}S-labeled platelets were added to 4 ml of 1 M NaOH and hydrolyzed for 7 hours at 5°C with constant mixing. The samples were

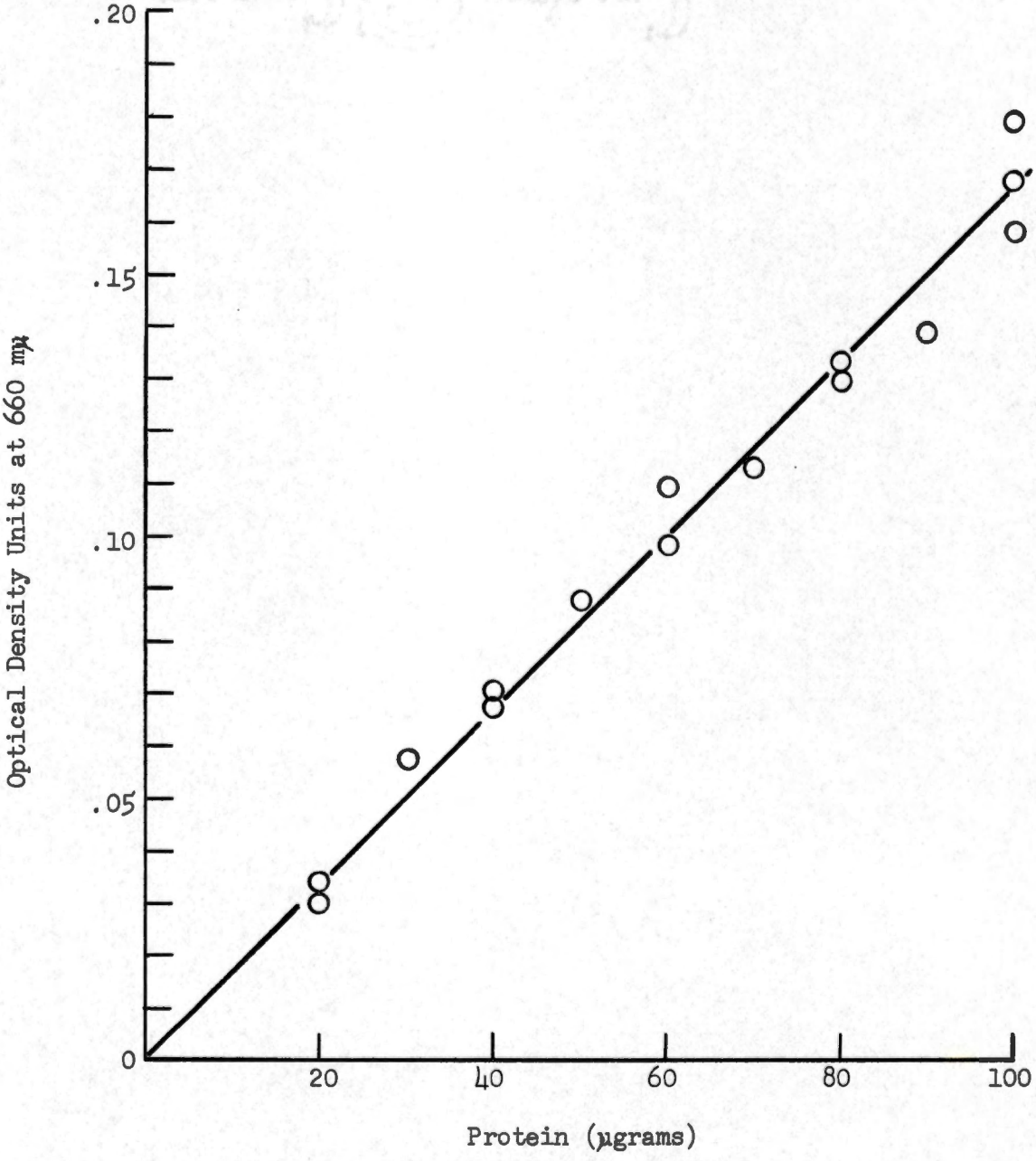


Figure 4. Optical density of varying concentrations of protein (bovine serum albumin).

then neutralized with 1 ml of 4 M acetic acid. (One sample was titrated with the 4 M acetic acid, using bromthymol blue as the indicator, to determine the 1 ml volume.) The samples were then treated 4 times with chloroform-amyl alcohol reagent (0.67 ml of amyl alcohol and 2.0 ml of chloroform per 10 ml of sample) to remove protein. No visible precipitate was seen after the third treatment. The first treatment was carried out overnight with continuous agitation, while subsequent treatments lasted for about 10 minutes with agitation. After each treatment, the samples were centrifuged for 10 minutes at 760 x g and the upper layer containing the MPS was transferred to another tube. The final supernatant fluid was placed in a dialysis bag and dialyzed (dialysis tubing No 404, Visking Co., Chicago) for 40 hours against four 1000-ml portions of distilled water at 5°C. After lypholization of the material that remained in the bag, it was dissolved in 500 µl of water.

Samples of the extracted ^{35}S-MPS were subjected to ascending chromatography (Castor and Dorstewitz, 1964). Twenty µl of the sample were spotted 1 inch from the bottom of a 3/4 x 5-1/2 inch paper strip (Blue Ribbon No. 589, Schleicher and Schuell Co.) and air-dried. To fix the MPS on the paper, the paper was immersed for 3 minutes in 0.1 per cent cetylpyridinium chloride (CPC), in 0.004 M phosphate buffer, pH 7.0 (this was made by combining 0.002 M KH_2PO_4 and 0.002 M K_2HPO_4) and then allowed to dry overnight at room temperature. The paper strips with the MPS spots were then suspended in 8 x 1.2 inch tubes with 0.5 M $MgCl_2$, 1.0 M $MgCl_2$, 0.4 M $AlCl_3$, or 0.5 M $FeCl_3$ as solvents. [MPS and their

relative solubilities are: (1) chondroitin sulfate (ChS) is soluble in 0.5 M $MgCl_2$, but heparin is not; therefore, ChS moves with the solvent front and heparin remains at the origin; (2) 1.0 M $MgCl_2$ moves ChS and heparin with the solvent front; (3) 0.4 M $AlCl_3$ leaves ChS B and heparin at the origin and moves ChS A and C with the solvent front; and (4) 0.5 M $FeCl_3$ moves only ChS A with the front.] After the solvent was allowed to migrate approximately 2 and 5/8 inches, the paper strips were removed and air-dried, placed in the 0.1 per cent CPC solution again for 3 minutes, followed by washings with 95 per cent ethanol for 3 minutes to free the solvents from the paper; absolute alcohol, 3 minutes; acetone, 2 minutes and then air-dried. After the samples were dried, pieces were cut from the strips as follows: (a) 1/2 inch on either side of the origin; (b) 1/4 inch above the solvent front and 3/4 inch behind the front. A third piece consisted of the paper between cuts. Each of the samples was placed in a polyethylene scintillation vial that contained 10 ml of the previously described scintillation fluid and counted as in the 35S-uptake experiments. After developing in solvents and clearing, other strips of paper containing MPS were stained for 5 minutes in 1 per cent Alcian Blue 8G x 300 (Arnold, Hoffman & Co., Providence, R. I.) made up in a 9:1 acetic acid and water solution, and washed well with tap water and, then, with four alternate concentrated acetic acid and water washings.

IX. PLATELET ADHESIVENESS

Five ml of whole blood, drawn from the abdominal aorta without an anticoagulant, were used for platelet adhesiveness studies (Maschauf, Robinson and LeBeau, 1964). A 20.3 cm x 7 mm (inside diameter) glass tube was filled with 8 grams of 3 mm glass beads and stoppered at each end with rubber stoppers that contained 20-gauge needles cut flush with the stoppers. The stoppers were covered heavily with silicone stopcock grease and all components of the column were siliconed except the beads. For each animal, two siliconed test tubes (13 x 100 mm) containing 1 mg of EDTA (made by evaporating 0.5 ml of a 0.2 per cent solution) were used. One-half milliliter of blood was placed in one of the tubes before the column was filled with the remaining 4.5 ml. The syringe containing the blood was then connected to the needle in the rubber stopper. The column was then inverted so that the blood was forced from the bottom to the top; when blood was seen at the top of the column a stop watch was started and the blood was allowed to remain in the column 35 seconds. One-half milliliter of blood from the column was then placed in another tube containing EDTA. The two tubes were covered with Parafilm.[7] The platelets were mixed gently and, then, immediately diluted and counted as before. The index of adhesiveness is expressed as per cent of platelets lost after filtration.

---

[7]Parafilm (Marathon, A Division of American Can Company, Menasha, Wisconsin).

X. STATISTICAL ANALYSIS

The data were always analyzed by the one-way classification analysis of variance method (Steele and Torrie, 1960). In addition, in the experiments in which treatment was the same with time, the data were subjected to Duncan's Multiple Range test (Duncan, 1955; and LeClerg, 1957).

CHAPTER IV

RESULTS AND DISCUSSION

I. GENERAL DESCRIPTION

Hypervitaminosis A

In agreement with previous findings (Rodahl, 1950), the present work shows that the symptoms following ingestion of toxic doses of vitamin A in rats may be divided into symptoms of acute intoxication (500 and 1000 IU per gram body weight given on day 0) and symptoms of chronic intoxication (50 and 100 IU per gram body weight daily for 10 days).

In the present experiment, symptoms of acute intoxication such as general muscular weakness, decreased activity, changes in the pelts, and drowsiness occurred within a few hours after single toxic doses were given orally by stomach needle. At the end of 48 hours these animals usually appeared to have recovered. It is thus probable that the symptoms of acute intoxication are related to the sudden large increase of the vitamin A level of the blood noted by Rodahl.

Continued administration of toxic doses over a period of several days resulted in symptoms of chronic intoxication such as lack of appetite, marked reduction in feed intake, loss of weight (Figure 5), muscular weakness, general ruffling of the fur, and internal hemorrhages, in agreement with Rodahl (1950). However, spontaneous fractures

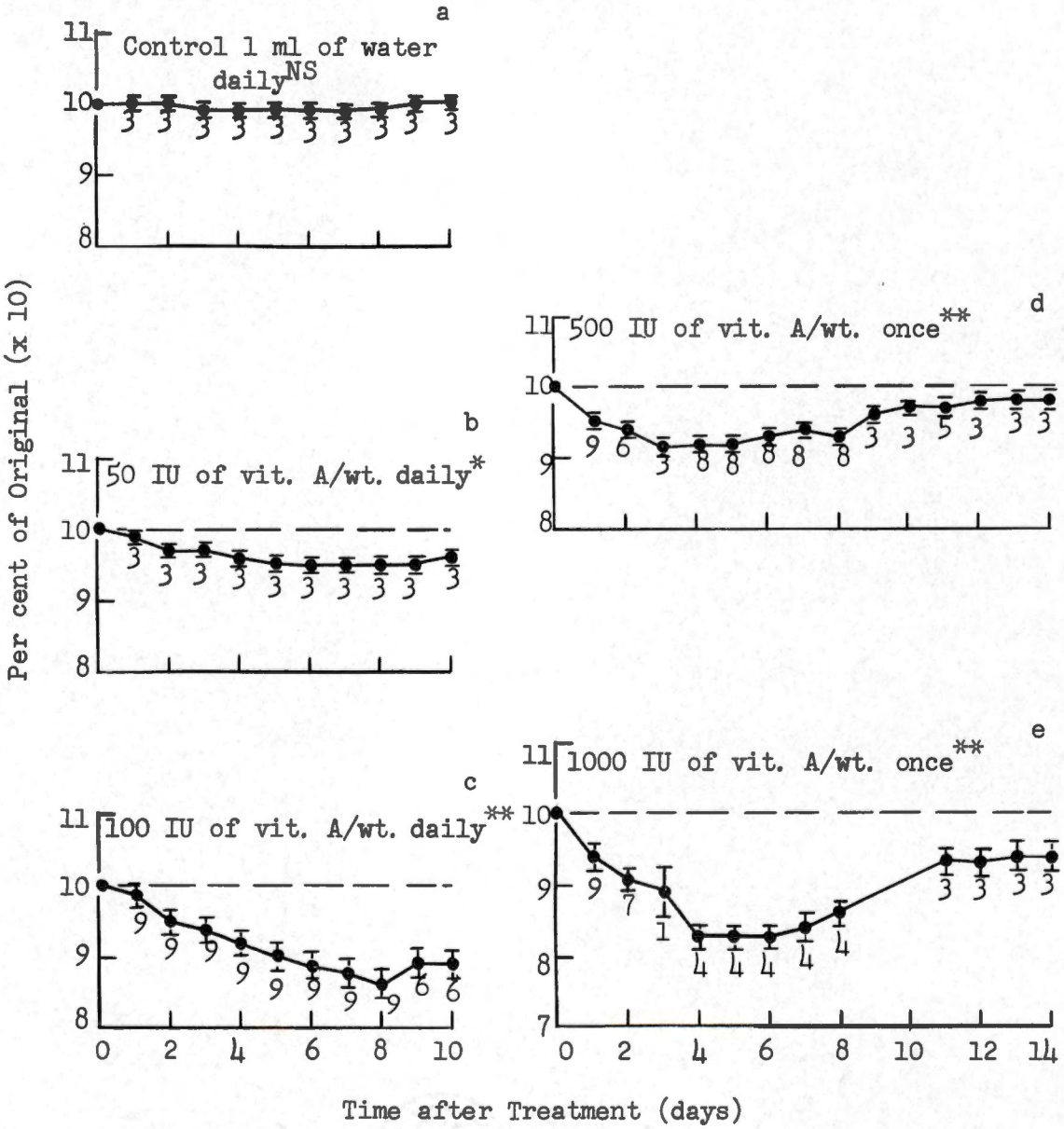


Figure 5. Body weights of rats dosed with vitamin A or water. Vertical bars indicate standard error of mean and numbers at points indicate the number of rats. NS Not significantly different; *P < 0.05; **P < 0.01.

were not observed in these rats as reported by Rodahl (1950) and Moore and Wang (1945). This difference may be due to the fact that their rats were dosed for considerably longer times than in the present experiment, since the size of daily doses was essentially the same. Rodahl (1950) found that 47 to 625 IU of vitamin A per gram body weight daily was sufficient to cause symptoms of hypervitaminosis A.

In the present experiments the amount of weight lost by vitamin A-treated rats was directly proportional to dose (Figure 5). At the highest single dose, 1000 IU per gram body weight, animals showed drastic weight loss ($P < 0.01$), weighing only 83 per cent of their initial weight at day 4. Many of these rats died within 2 days (9/12) but those surviving returned to near normal weight by day 11.

Hypovitaminosis A

Rats fed a vitamin A-deficient diet gained weight at a slower rate than did rats fed a standard laboratory diet or rats fed the vitamin A-deficient diet with vitamin A added at the rate of 21.44 IU per gram of feed (Figure 6). The vitamin added to the deficient diet did not, however, completely overcome the effects of the deficient diet.

In some of the deficient rats, loss of appetite, decreased blood pressure, roughness of coat, and typical eye lesions (xerophthalmia) were seen after about 9 weeks on the diet, while other rats did not have these symptoms until about 12 weeks. Within 2 to 3 weeks after symptoms appeared, the animals usually died. The rats fed the standard laboratory diet and rats given vitamin A in the deficient diet appeared normal.

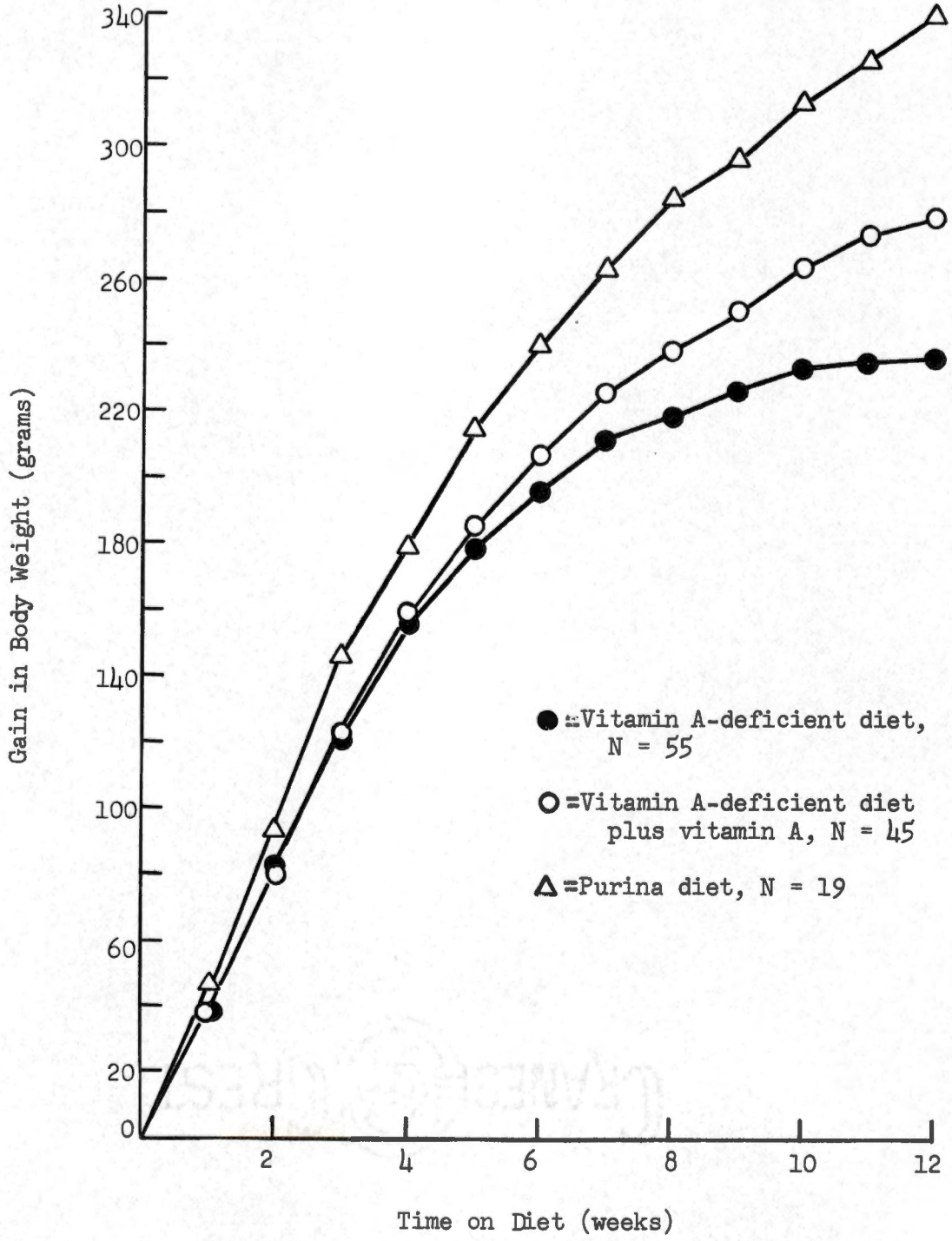


Figure 6. Change in body weight of rats fed vitamin A-deficient and normal diets.

II. PERIPHERAL BLOOD DETERMINATIONS IN HYPERVITAMINOSIS A

Platelets

Vitamin A given orally caused increases in peripheral platelet counts as high as 157 per cent of initial counts (Figure 7b) in the group receiving 50 IU of vitamin A per gram of body weight daily. This large increase in platelet counts was not observed, however, in the rats receiving 100 IU per gram of body weight daily (Figure 7c). At the higher doses, 500 and 1000 IU per gram of body weight given only at day 0 (Figures 7d and 7e), the number of platelets increased for about 48 hours and then decreased rapidly by 3 to 4 days to about 85 per cent of the initial values, then increased again. Counts approached near-normal values on the 14th day although they may have continued to oscillate. One ml of water given daily did not produce such an effect (Figure 7a). Platelet counts from control rats and the rats receiving 100 IU of the vitamin daily did not vary significantly, although the trend in the 100 IU group was similar to that in the 50 IU group.

Hematocrit

Blood from rats treated with vitamin A showed an alteration in hematocrit (Figure 8), except at the 50 IU level (Figure 8b). At 500 IU and 1000 IU levels (Figures 8d and 8e) hematocrits increased for 1 to 2 days and then fell greatly reaching by day 3 as low as 89 per cent of initial values. At times when the hematocrit was highest, difficulty was experienced in obtaining blood samples because these rats did not bleed freely, suggesting that anhydremia occurred very quickly following

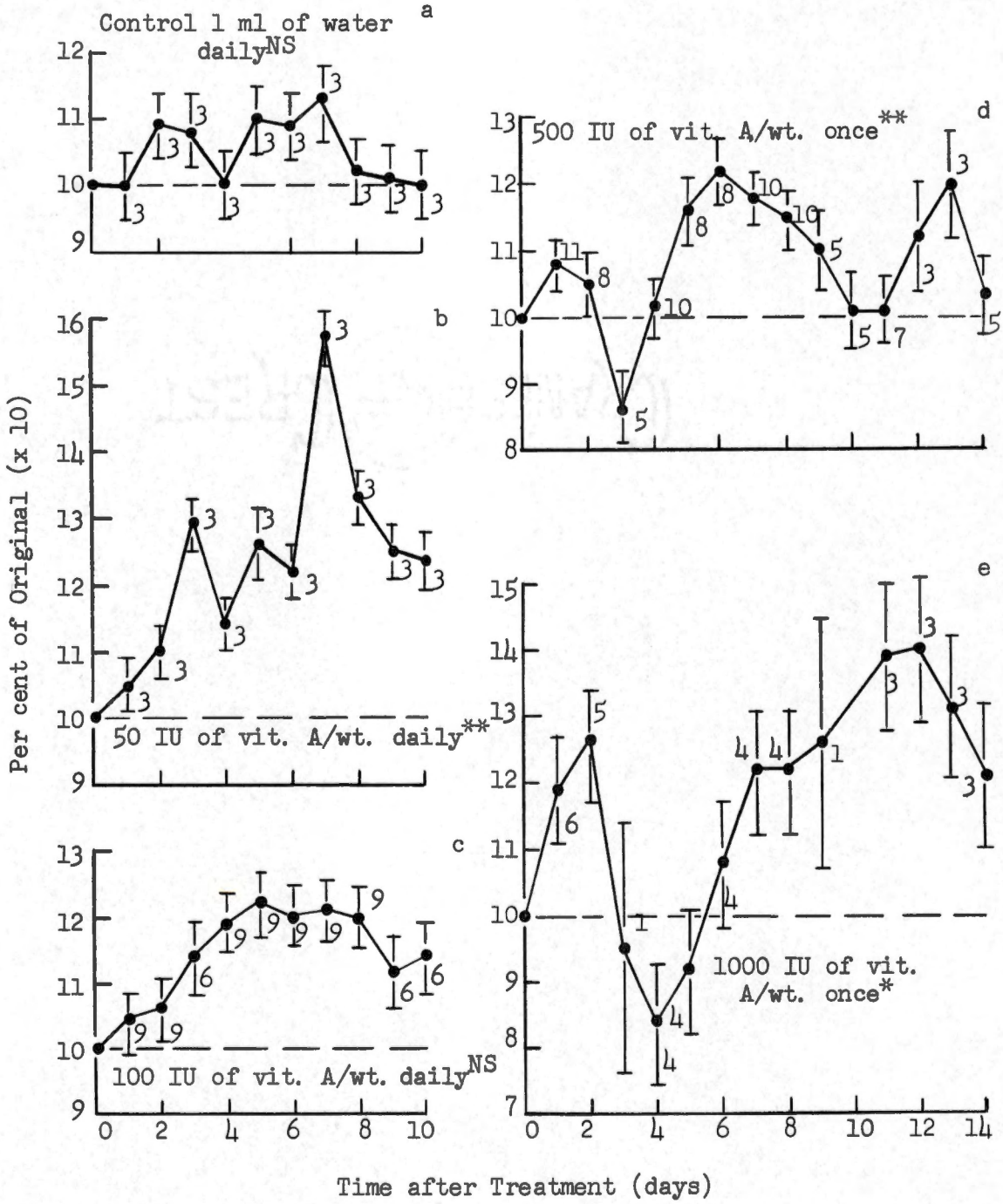


Figure 7. Peripheral platelet counts of rats dosed with vitamin A or water. Vertical bars indicate standard error of mean and numbers at points indicate the number of rats. NSNot significantly different; *P < 0.05; **P < 0.01.

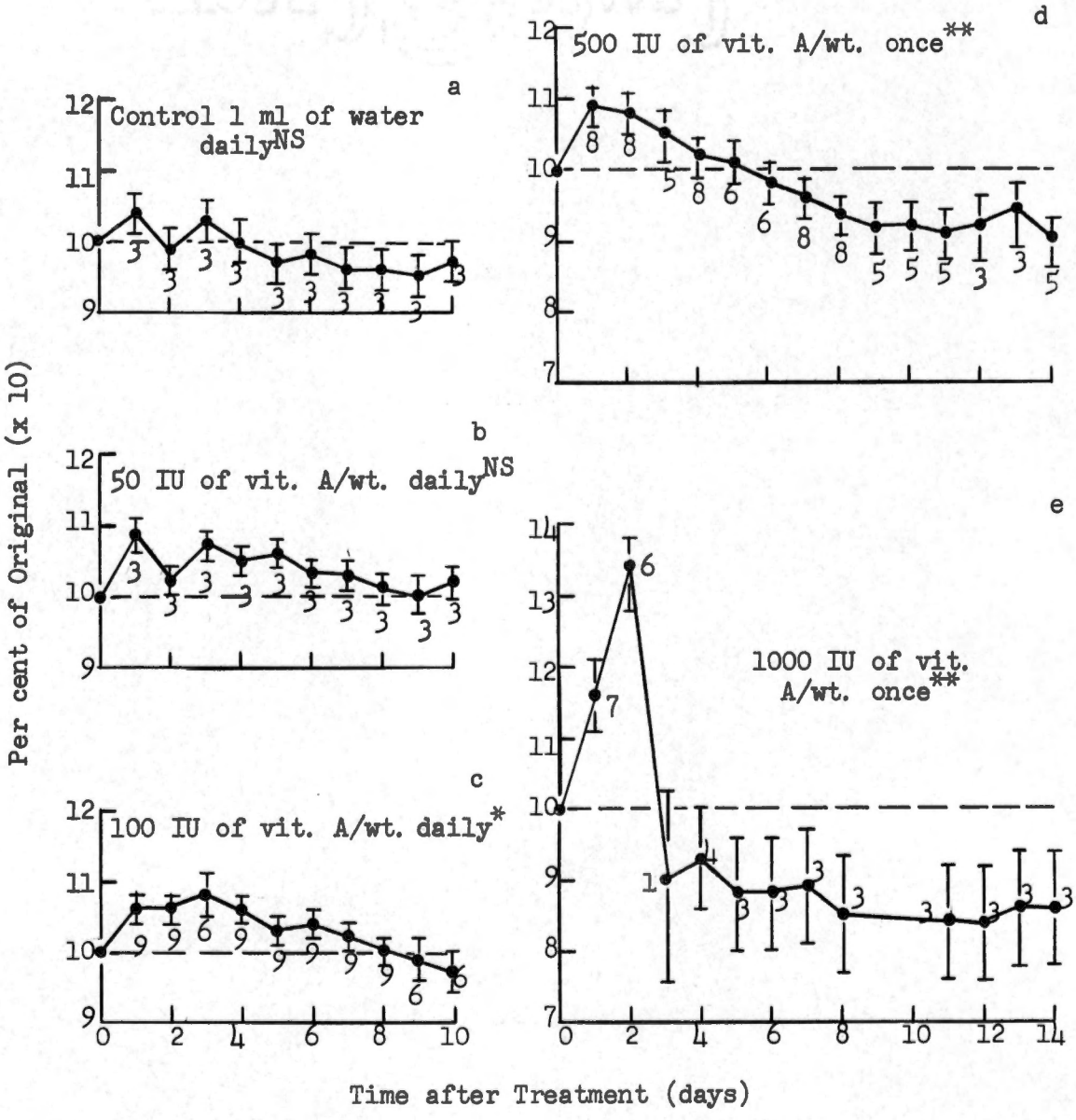


Figure 8. Hematocrits of rats dosed with vitamin A or water. Vertical bars indicate standard error of mean and numbers at points indicate the number of rats. NSNot significantly different; *P < 0.05; **P < 0.01.

administration of the vitamin. Water or 50 IU of vitamin A per gram of body weight did not produce this effect.

White Blood Cells

Total WBC counts in rats dosed with vitamin A were not significantly altered at 50 IU (Figure 9b) or 100 IU (Figure 9c) daily or 1000 IU at day 0 (Figure 9e). Surprisingly, the WBC counts of both the control (Figure 9a) and the 500 IU group were significantly altered ($P < 0.01$ and < 0.05, respectively, see Figure 9d). These changes are attributed to daily anesthesia with ether; however, the vitamin A-treated rats were similarly anesthetized for blood samples. Counts at all dose levels were highly variable and sample sizes were too small to show significant results or to draw definite conclusions on this point.

Red Blood Cells

Red blood cell counts were about the same in control and 50 IU and 100 IU animals (Figures 10a-c). Very marked changes occurred ($P < 0.01$) at the 500 and 1000 IU levels (Figures 10d, e), where the count rose as high as 142 per cent of initial value in 24 hours. The count sharply decreased to as low as 50 per cent on day 5, then increased again to 146 per cent by day 11. The effects of the vitamin at the 500 IU level were not as marked (compare Figures 10d, e) as those at 1000 IU. The sudden increase in RBC correlates well with the hematocrit results at the early time periods (Figures 8d, e), but during the next few days, hematocrits remained higher than RBC counts. This finding suggests that some red blood cells leave the peripheral circulation and those remaining

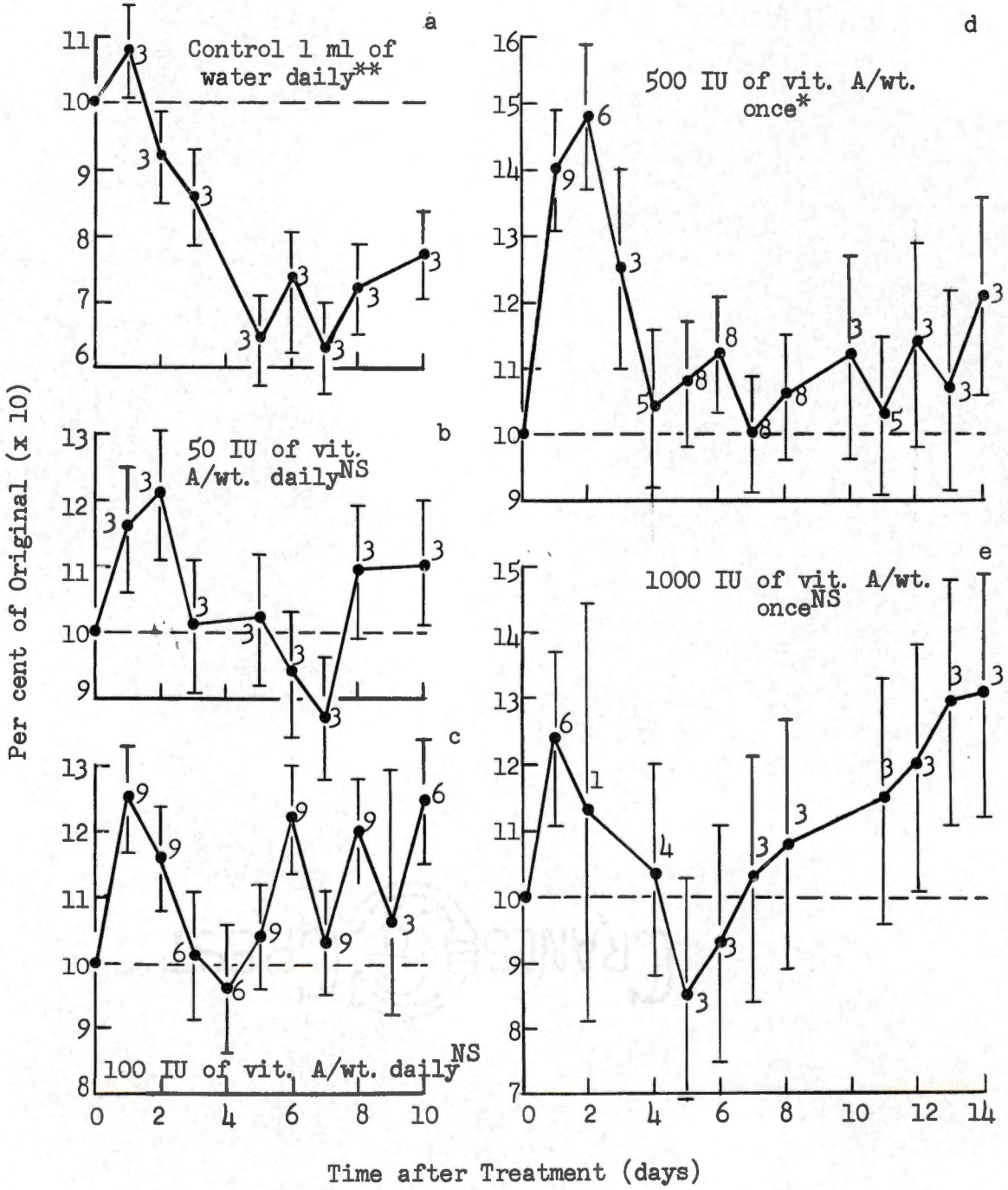


Figure 9. Peripheral WBC counts of rats dosed with vitamin A or water. Vertical bars indicate standard error of mean and numbers at points indicate the number of rats. NSNot significantly different; *P < 0.05; **P < 0.01.

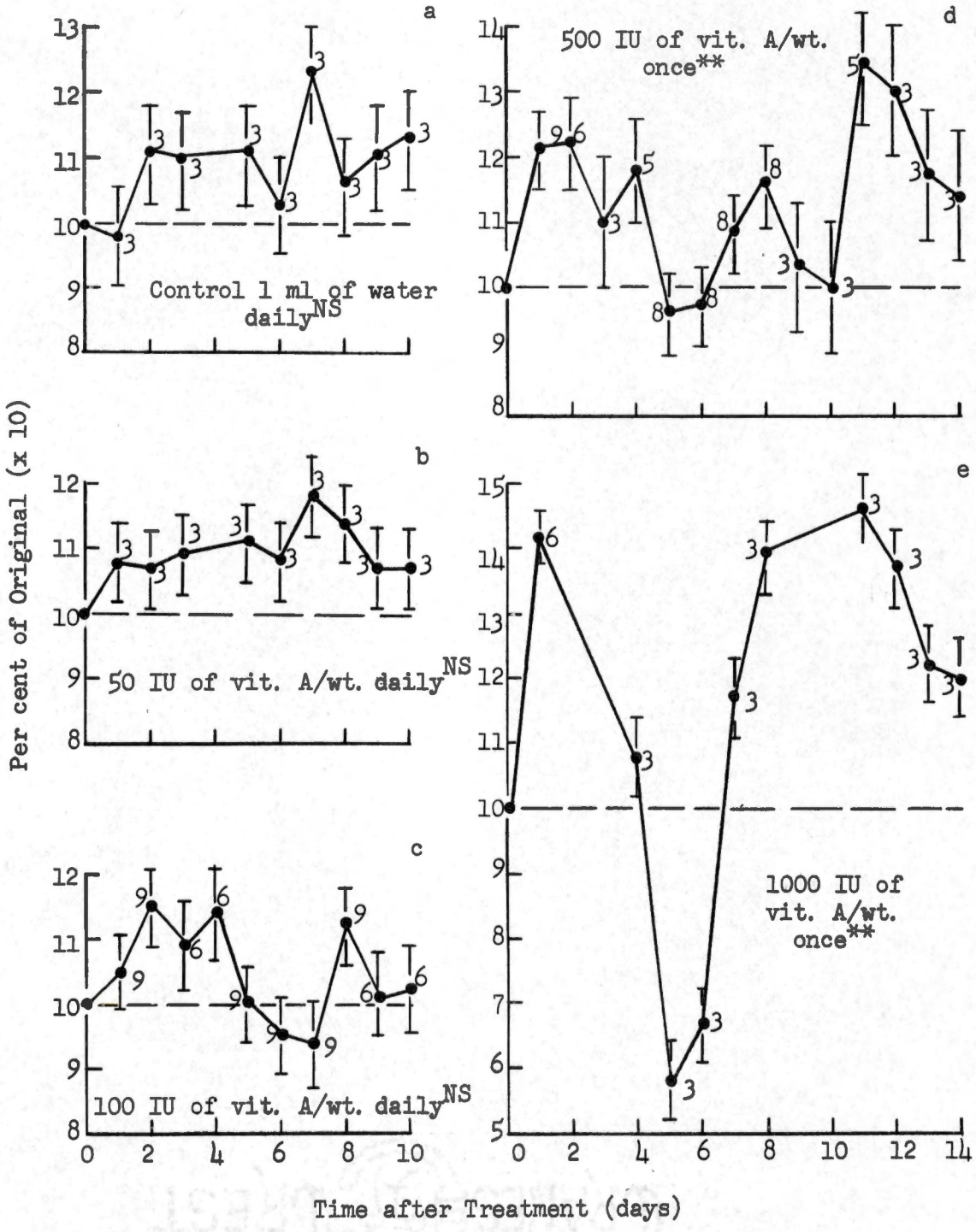


Figure 10. Peripheral RBC counts of rats dosed with vitamin A or water. Vertical bars indicate standard error of mean and numbers at points indicate the number of rats. NS Not significantly different; **P < 0.01.

become larger than normal. The presence of larger cells would account for higher hematocrit values, and the expected hematocrit decrease paralleling RBC depression would thus be offset. This phenomenon was observed by Glauert et al. (1963) who observed that within 1 minute after administration of vitamin A to rabbit erythrocytes in vitro the cells undergo changes which result in a greatly increased surface area relative to untreated cells. This was followed by loss of hemoglobin, with breaks appearing in the membranes of the cells. Ghosts finally appeared, indicating that the cells were hemolyzed.

The possibility remains, however, that part of the changes in RBC count may be derived from difference in production rates and consequent differences in sizes of peripheral cells.

III. PERIPHERAL BLOOD DETERMINATIONS IN HYPOVITAMINOSIS A

Platelets

Figure 11 shows that there was little difference in the platelet counts between control animals and those fed a vitamin A-deficient diet. In the first experiment the platelet values after 2 weeks on the diet were higher in the deficient group than in the other groups (including controls) and decreased 32 per cent ($P < 0.01$) between the second and twelfth weeks. The decrease in the control value (rats fed a standard laboratory diet) was 29 per cent which was a statistically significant change.

In the second experiment, less variation was noted, although the same general decline in platelet values with time was observed.

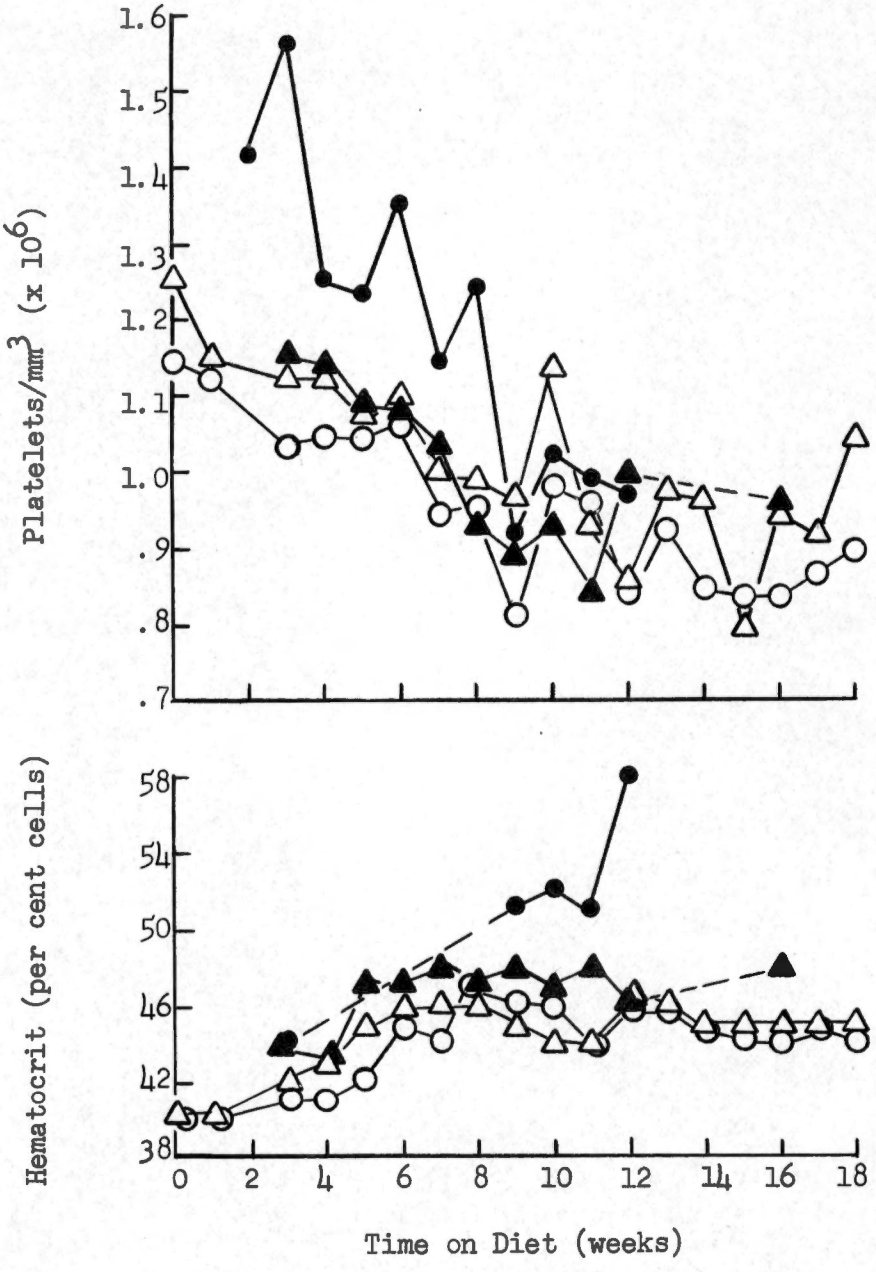


Figure 11. Peripheral platelet counts and hematocrits of rats fed vitamin A-deficient and standard laboratory diets.

●=Experiment No. 1, vitamin A-deficient diet; N = 5; platelets ($P < 0.01$); hematocrits ($P < 0.01$).
○=Experiment No. 2, vitamin A-deficient diet; N = 5.
△=Vitamin A-deficient diet plus vitamin A; N = 3.
▲=Standard laboratory diet; N = 5; platelets ($P < 0.01$); hematocrits, not significantly different.

The reason for this decrease in platelet count for all groups is not known. No reports were found of a decrease of peripheral platelet counts of laboratory rats with age; however, Anagnostu (1939) observed a much greater platelet decrease of 31 to 69 per cent in rats that had been fed a vitamin A-deficient diet for 5 to 7 weeks.

The effects of the vitamin A-deficient diet in the first and second experiments differed markedly. In the first experiment all the rats were dead after 13 weeks on the diet, whereas in the second all were alive after 18 weeks. Rats used in the first experiment were purchased from Sprague-Dawley and placed on the diet within 2 days while the rats in the second experiment were bred and reared at UT-AEC. Perhaps the stress in shipment added to the stress of the deficient diet caused trauma that increased platelet counts in the early part of the experiment and also resulted in early death. Different batches of feed also were used in the two experiments.

Hematocrit

The hematocrits (Figure 11) of control and experimental rats varied more widely than did the platelet counts. The control hematocrit values were not significantly different and remained almost constant throughout the third to sixteenth week period (five-rat average of 45 to 48 per cent). The hematocrit values of rats receiving the vitamin A-deficient diet in the first experiment, however, increased greatly before death. The five-rat average value was 43 per cent on the third week and 58 per cent on the twelfth week. More data are needed before

definite conclusions can be drawn concerning the change in hematocrits owing to hypovitaminosis A. It can be tentatively concluded that this change is related to decrease in blood pressure and/or anhydremia. Since difficulty was experienced in obtaining blood samples due to the deficient rats not bleeding freely, these findings are in agreement with those of Frank (1934), who also observed increased values for the number of erythrocytes--a condition that would increase hematocrit levels in the later stages of hypovitaminosis A.

White Blood Cells

Total WBC counts in experimental and control rats are shown in Figure 12. At first an increase in WBC counts is seen, but by the eighth to ninth week the WBC values return to normal and remain constant thereafter. These results neither confirm nor contradict those of Frank (1934) or Crimm and Short (1935a) who showed that WBC counts were reduced from deficiency of the vitamin.

Red Blood Cells

About the same RBC counts (Figure 12) were noted between control and experimental animals. Counts in all groups increased from the start of the experiment to about the sixth week, then leveled off. These data on RBC counts compare favorably with the values obtained for hematocrits (Figure 11), indicating that red blood cell sizes are probably not altered because of the deficiency.

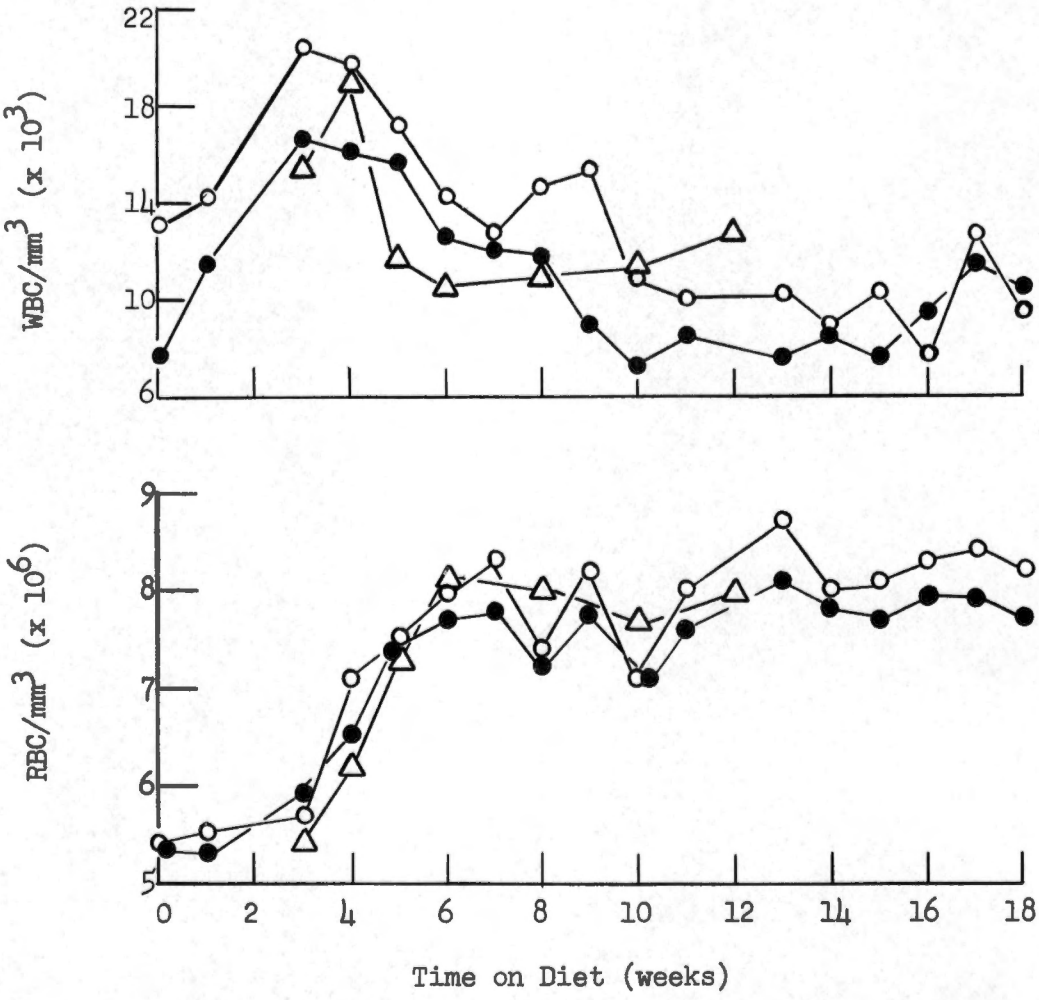


Figure 12. Peripheral RBC and WBC counts of rats fed vitamin A-deficient and standard laboratory diets.

●=Vitamin A-deficient diet, N = 5.

○=Vitamin A-deficient diet plus vitamin A, N = 3.

△=Standard laboratory diet, N = 3.

IV. PLATELET SIZE MEASUREMENTS

The results of platelet size determinations are shown in Tables II and III. Using the Coulter counter method (McDonald, Odell and Gosslee, 1964), size distribution curves of platelets from vitamin A-deficient rats were shifted to the right, indicating larger platelets. In some of the platelet curves from the vitamin A-treated rats the shift was to the left, indicating smaller platelets than that of a normal population. The window number values (obtained directly from the Coulter counter plot)were converted to logarithms, thereby transforming the skewed distributions to distributions that were approximately Gaussian (Brecher et al., 1962). Statistical analysis by Duncan's Multiple Range Test (Duncan, 1955; LeClerg, 1957) revealed that platelets from vitamin A-deficient rats were significantly larger ($P < 0.05$), than platelets from normal or the orally treated vitamin A rats. In addition, platelets collected on days 3, 4, 7, and 10 from rats receiving 100 IU per gram of body weight daily of the vitamin were smaller than platelets from untreated rats ($P < 0.05$), and platelets collected from rats 6 days after 500 IU of vitamin A per gram of body weight were smaller ($P < 0.05$) than normal platelets. Other platelet values from rats given the vitamin at the times investigated were not statistically different from control platelets.

The vitamin A-deficient diet caused rat blood platelets to increase in size; when vitamin A was added to the diet at the rate of 21.44 IU per gram of feed the change was not corrected. It appears that

TABLE II

COMPARISON OF PLATELET SIZE (MEAN WINDOW) FROM VITAMIN A-TREATED RATS OR RATS ON VITAMIN A-DEFICIENT AND NORMAL DIETS

	Time after Treatment (days)	No. Rats	$\bar{X}$ Window	$s_{\bar{X}}$
Diet[1]		17	11.239[a]	0.134
Diet[2]		10	11.486[a]	0.176
Normal Diet		34	9.895[b]	0.095
	100 IU/Gm Body Wt. (daily)			
Vitamin A Given Orally	1	3	9.421[bc]	0.323
	2	6	9.669[bc]	0.228
	3	5	9.045[c]	0.249
	4	6	9.272[c]	0.228
	5	6	9.453[bc]	0.228
	6	6	9.403[bc]	0.228
	7	6	9.214[c]	0.228
	10	6	9.208[c]	0.228
	500 IU/Gm Body Wt. (at day 0)			
	1	--	--	--
	2	2	9.582[bc]	0.395
	3	3	9.197[bc]	0.323
	4	3	9.411[bc]	0.323
	5	3	9.261[bc]	0.323
	6	3	9.004[c]	0.323
	7	3	9.270[bc]	0.323
	10	3	9.126[bc]	0.323

[1]Vitamin A-deficient diet.

[2]Vitamin A-deficient diet plus vitamin A at the rate of 21.44 IU per gram of feed.

[a]Means with superscript "a" are significantly different from means without superscript "a", according to Duncan's Multiple Range Test at the 5 per cent level of significance.

[bc]Means with superscript "b" are significantly different from means without superscript "b", and means with superscript "c" are different from those without superscript "c".

TABLE III

COMPARISON OF PLATELET SIZE (CELLS PER PACKED VOLUME) FROM VITAMIN A-TREATED RATS OR RATS ON VITAMIN A-DEFICIENT AND NORMAL DIETS

	Time after Treatment (days)	No. Rats	Platelets per ml x 10^{10}	$s_{\bar{x}}$
Diet[1]		12	10.759[a]	0.351
Normal Diet		9	13.015[b]	0.405
Vitamin A Given Orally[2]	1	3	12.500[ab]	0.705
	2	2	12.302[ab]	0.861
	4	3	12.988[b]	0.705
	5	3	12.871[b]	0.705
	6	3	11.967[ab]	0.705
	7	3	11.575[ab]	0.705
	10	3	11.135[ab]	0.705

[1]Vitamin A-deficient diet.

[2]500 IU of vitamin A per gram of body weight at day 0.

[a]Means with superscript "a" are significantly different from means without superscript "a", according to Duncan's Multiple Range Test at the 5 per cent level of significance.

[b]Means with superscript "b" are significantly different from means without superscript "b".

the amount of vitamin added to the deficient diet was not sufficient to correct platelet sizes to normal.

Similar results were obtained with the cells-per-packed-volume technique and the Coulter counter method (Tables II and III). Analysis of the data demonstrated significant differences between platelets from vitamin A-deficient rats and normal rats. Platelet sizes from the vitamin A-treated rats were not different, however, from those of normal rats, and at 5 of the 7 days investigated the platelets per packed volume were not different from the deficient group. The cells-per-ml values obtained in these experiments are larger (indicating smaller cells) than those reported by Detwiler, Odell and McDonald (1962). A possible explanation may be that in our earlier report polyvinylpyrrolidone (PVP) was added to the blood in equal volume to improve platelet yield, whereas in the present work only saline solution was used. The PVP, a plasma expander, is more viscous than saline solution and may have prevented complete settling in the microhematocrit tubes.

The alterations in platelets that are responsible for the increase or decrease in size with vitamin A deficiency or excess remain to be determined. Results by McDonald, Odell and Gosslee (1964) demonstrated a shift toward larger-than-normal size in young platelets and smaller size for older platelets. Detwiler, Odell and McDonald (1962) suggested that the content of ATP decreases with age, which may imply a gradual release of functional factors with age, in contrast to a complete utilization or destruction of the platelet components only at the time the platelet is lost from the circulatory system. The loss of ATP was

correlated with a decrease in size of blood platelets. It remains to be determined, however, if vitamin A-treated platelets are affected in the same way.

The size difference may be related, however, to action on platelet membranes by the vitamin, since evidence has been presented that one site of action of vitamin A is at lipoprotein membranes (Lucy, Luscombe and Dingle, 1963). Bell (1962) has suggested that a sulfated polysaccharide is necessary for membrane structure and hence some of its properties. He visualizes the polysaccharide as the outer layer with the sulfate groups a possible reactive site. Bell (1962) suggests that, if this is the case, changes in the amounts of MPS may cause changes in permeability. It seems possible that blood platelets could be affected in the same way and if so, changes in permeability might lead to changes in sizes of blood platelets.

V. UPTAKE OF ^{35}S BY BLOOD PLATELETS AND PLASMA

Vitamin A-Treated Rats

In normal rats or rats given vitamin A and injected intraperitoneally daily with 1 μc of $Na_2{}^{35}SO_4$ per gram of body weight, the radioactivity of whole, washed platelets increased rapidly during the first 4 days and was widely variable thereafter, without definite upward or downward trends. Mean platelet activity of the rats receiving vitamin A is presented in Figure 13 and Table IV. In Table IV radioactivity of plasma and washes of the platelets is also given. The rising lines (day 2 to day 5) were visually fitted through the means, and the

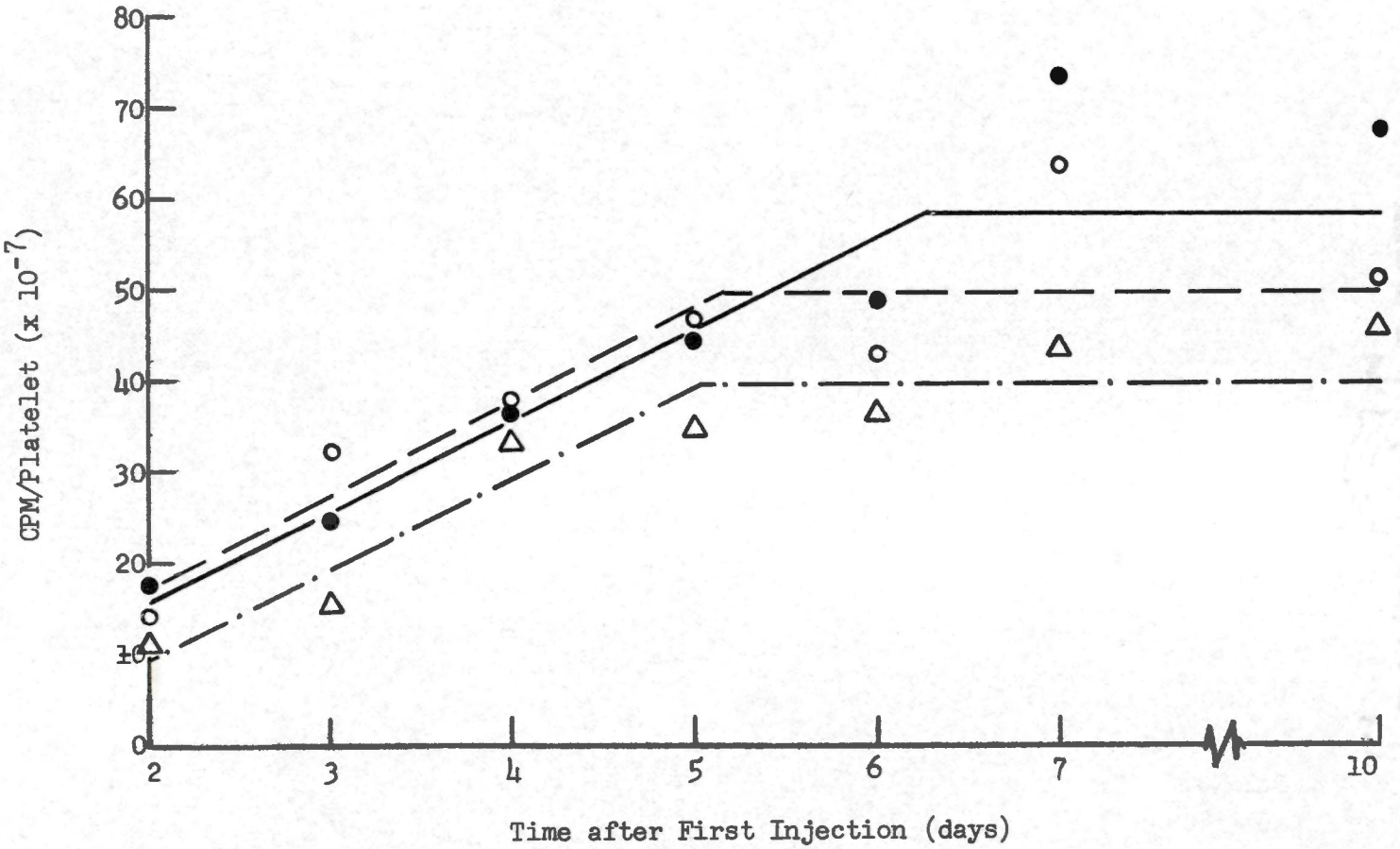


Figure 13. Radioactivity of platelets from normal or vitamin A-treated rats after daily intraperitoneal injections of $Na_2{}^{35}SO_4$.

●——●=100 IU per gram body weight daily.

○— —○=500 IU per gram body weight at day 0.

△—·—△=Normal platelets.

TABLE IV

RADIOACTIVITY OF PLATELETS, PLASMA-SALINE, AND PLATELET WASHES FROM NORMAL AND VITAMIN A-TREATED RATS AFTER[1] DAILY INTRAPERITONEAL INJECTIONS OF $Na_2{}^{35}SO_4$

Day	Number of Rats	Platelet Activity CPM/Plt. x 10^{-7}	$s_{\bar{x}}$	CPM/200 µl Plasma	Wash 1	Wash 2
		Normal Rats				
2	2	10.88	1.92	2648	52	2
3	2	15.34	1.92	2984	128	11
4	2	33.42	1.92	3650	75	4
5	2	34.64	1.92	4966	152	9
6	2	36.74	1.92	3418	86	5
7	2	43.55	1.92	4549	101	7
10	2	45.04	1.92	4582	189	5
		100 IU/Gram Body Wt. (daily)				
2	3	17.13	3.32	3420	80	11
3	3	24.63	3.32	5308	230	13
4	3	36.09	3.32	5370	173	26
5	3	44.05	3.32	4640	149	23
6	3	48.57	3.32	4937	155	14
7	3	73.09	3.32	7263	200	20
10	3	67.47	3.32	9317	323	19
		500 IU/Gram Body Wt. (at day 0)				
2	2	14.41	3.35	5625	162	4
3	3	31.88	2.73	5049	160	12
4	3	37.61	2.73	3603	110	24
5	3	41.68	2.73	6665	272	32
6	3	42.60	2.73	3944	141	5
7	3	63.23	2.73	6702	252	13
10	3	51.08	2.73	4785	216	7

[1]One µc/gram body weight daily.

horizontal lines were determined by averaging platelet activities of all rats on days 5 to 10. The activity of platelets from normal rats leveled off at 39.99 x 10^{-7} CPM per platelet (Figure 13), in agreement with earlier work (Odell and Anderson, 1959). The activity of the vitamin A-treated group, however, tended to level off higher, which is suggestive of greater incorporation of ^{35}S. Platelets from 100 IU rats reached a plateau at about 58 x 10^{-7} CPM per platelet and platelets from rats treated with 500 IU leveled off at about 49 x 10^{-7} CPM per platelet, results that lead one to believe that the greater ^{35}S incorporation indicates a greater amount of sulfated MPS per platelet. Greater ^{35}S incorporation has been observed in chondroitin samples isolated from epiphyseal cartilage after administration of vitamin A (Dziewiatkowski, 1954). The activity per platelet increased in the 100 IU group until about day 6 compared to day 5 for the control animals. This raises the question whether the life span of vitamin A-treated platelets is lengthened. Crimm and Short (1935a) observed that vitamin A caused an increase in the number of WBC senile forms. These authors suggested that the system for destruction of leukocytes was working inefficiently because of the effects of excess vitamin A. Perhaps this same explanation holds for platelets or perhaps excess vitamin A lengthens platelet life spans in another way. Increased MPS per platelet may have significant effects on the life span of platelets. The data presented here are not conclusive, and this question awaits further experimentation.

Blood platelets from rats given 500 IU per gram of body weight of vitamin A at day 0 and single ^{35}S injections 48 hours before killing

(Figure 14 and Table V) contained significantly more label than control platelets on days 4, 5, and 6. Since only one injection of ^{35}S was given 48 hours before killing, the increased platelet activity may have been caused by different rates of production (see Figure 7d). At this dose level of the vitamin, peripheral blood platelet numbers were shown to be increased by 120 per cent of original at day 6 after treatment. A more plausible explanation is that increased platelet ^{35}S activity, approximately 87 per cent greater, was due to increased MPS content per platelet. Also noteworthy is the fact that plasma-saline samples from vitamin A-treated rats were not statistically different from samples from control animals, and plasma-saline activity from vitamin A-treated rats was about the same from day to day.

Platelets from rats given vitamin A at the rate of 100 IU per gram of body weight daily and single intraperitoneal injections of 1 μc per gram of body weight of the isotope 48 hours before sacrifice took up more ^{35}S than those from rats not receiving the vitamin (Table VI). Grouping days 5, 6, 7, and 10 (although day 6 was not statistically different from control), the average activity was 28.33×10^{-7} CPM per platelet, and platelets incorporated significantly more ^{35}S than did platelets from control rats, which had 14.11×10^{-7} CPM per platelet (100 per cent more activity per platelet). Again, the plasma-saline values were not statistically different between control rats and vitamin A-treated rats. In contrast to previous reports, Thomas et al. (1960) observed that in rabbits given one million units per day of vitamin A, a rise resulted in serum ChS by 72 hours after treatment.

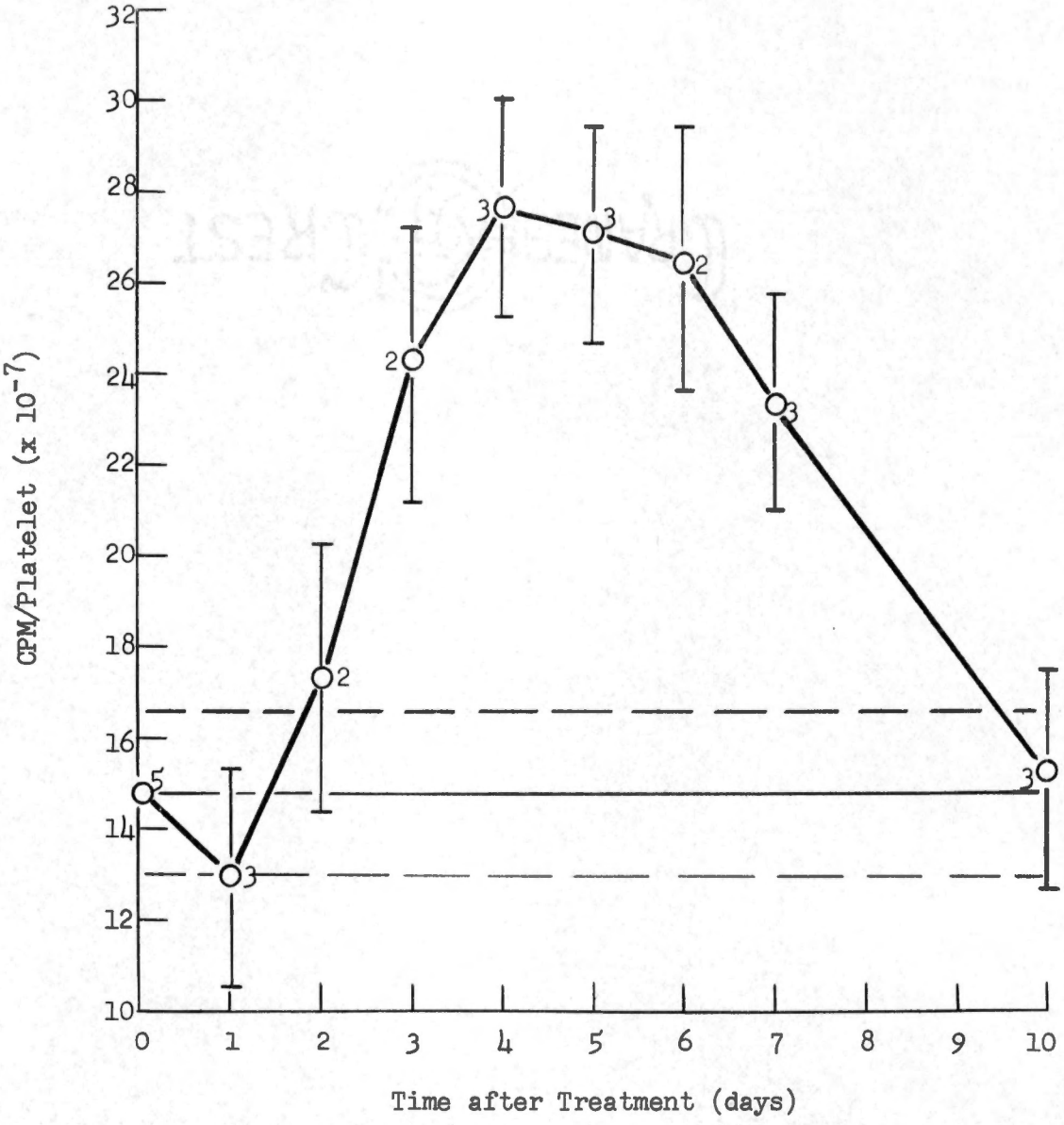


Figure 14. Radioactivity of platelets from vitamin A-treated and normal rats after single intraperitoneal injections of $Na_2{}^{35}SO_4$. Vertical bars indicate standard error of mean and numbers at points indicate number of rats. Rats were given 1 μc of ^{35}S per gram body weight 48 hours before killing and vitamin A-treated rats were given 500 IU per gram of body weight at time 0. Horizontal solid line indicates control mean and broken lines indicate standard error of mean.

TABLE V

RADIOACTIVITY OF PLATELETS, PLASMA-SALINE, AND PLATELET WASHES FROM NORMAL AND VITAMIN A-TREATED[1] RATS AFTER[2] SINGLE INTRAPERITONEAL INJECTIONS OF $Na_2{}^{35}SO_4$

Day	No. of Rats	Platelet Activity CPM/Plt. x 10^{-7}	$s_{\bar{x}}$	CPM/100 µl Plasma[3]	CPM/200 µl Wash 1	CPM/200 µl Wash 2
Control	5	14.72[d]	1.85	783	72	18
1	3	12.94[d]	2.38	851	79	3
2	2	17.36[abcd]	2.92	1293	73	11
3	2	24.28[abcd]	2.92	849	60	8
4	3	27.66[a]	2.38	1069	106	19
5	3	27.09[ab]	2.38	1073	105	16
6	2	26.38[abc]	2.92	1039	107	14
7	3	23.35[abcd]	2.38	939	86	19
10	3	15.13[cd]	2.38	874	96	12

[1] 500 IU of vitamin A per gram of body weight at day 0.

[2] 1 µc per gram of body weight 48 hours before killing.

[3] Not significantly different.

[a] Means with superscript "a" are significantly different from means without superscript "a", according to Duncan's Multiple Range Test at the 1 per cent level of significance.

[bcd] Means with superscript "b" are significantly different from means without superscript "b", means with superscript "c" are different from those without superscript "c", and means with superscript "d" are different from those without superscript "d".

TABLE VI

RADIOACTIVITY OF PLATELETS, PLASMA-SALINE, AND PLATELET WASHES FROM NORMAL AND VITAMIN A-TREATED[1] RATS AFTER SINGLE INTRAPERITONEAL INJECTIONS OF $Na_2{}^{35}SO_4$[2]

Day	No. of Rats	Platelet Activity CPM/Plt. x 10^{-7}	$s_{\bar{x}}$	CPM per 200 µl Plasma[3]	Wash 1	Wash 2
Control	6	14.11[d]	1.53	1337	21	2
1	2	13.22[cd]	2.66	1128	28	2
2	2	20.85[bcd]	2.66	1130	26	0
3	2	22.70[bcd]	2.66	1512	19	1
4	3	17.48[cd]	2.17	953	20	1
5	2	37.45[a]	2.66	1580	32	6
6	2	22.57[bcd]	2.66	1613	44	2
7	2	24.86[bc]	2.66	1219	35	1
10	3	28.44[ab]	2.17	842	30	4

[1]100 IU of vitamin A per gram body weight daily.

[2]1 µc per gram body weight 48 hours before killing.

[3]Not significantly different.

[a]Means with superscript "a" are significantly different from means without superscript "a", according to Duncan's Multiple Range Test at the 5 per cent level of significance.

[bcd]Means with superscript "b" are significantly different from means without superscript "b", means with superscript "c" are different from those without superscript "c", and means with superscript "d" are different from those without superscript "d".

Cohen, Maddock and Wolbach (1955) found greatly increased levels of mucoprotein in blood of rats after administration of 1000 IU per day of the vitamin. This difference in MPS levels of the plasma in the present experiment and those previously reported may have been due to differences in doses.

Vitamin A-Deficient Rats

Uptake of ^{35}S by platelets from control, vitamin A-deficient and -excess groups was generally the same (Figure 15 and Table VII). Activity of platelets from vitamin A-deficient rats reached a plateau at about 65 x 10^{-7} CPM per platelet, whereas the normal controls reached a plateau at about 57 x 10^{-7} CPM. Platelet activity from rats fed a vitamin A-deficient diet with vitamin A added at the rate of 21.44 IU per gram of feed was even higher (about 67 x 10^{-7} CPM). This finding may differ from that of Wolf and Varandani (1960) and Varandani, Wolf and Johnson (1960), who observed that vitamin A deficiency decreased the incorporation of ^{35}S sulfate into the MPS of the rat colon. Platelet sizes probably have an important influence on this apparent difference since the platelets with the highest activity were shown to be significantly largest among the three groups. Thus the increased ^{35}S uptake of vitamin A-deficient platelets may be related simply to the size and not to the seeming increase in total MPS per volume of mass. The fact that the activity of platelets from the control group was higher in this experiment than in that for the ^{35}S multidosed vitamin A-treated one (Figure 13 and Table IV) is probably due to the use of different batches of isotopes.

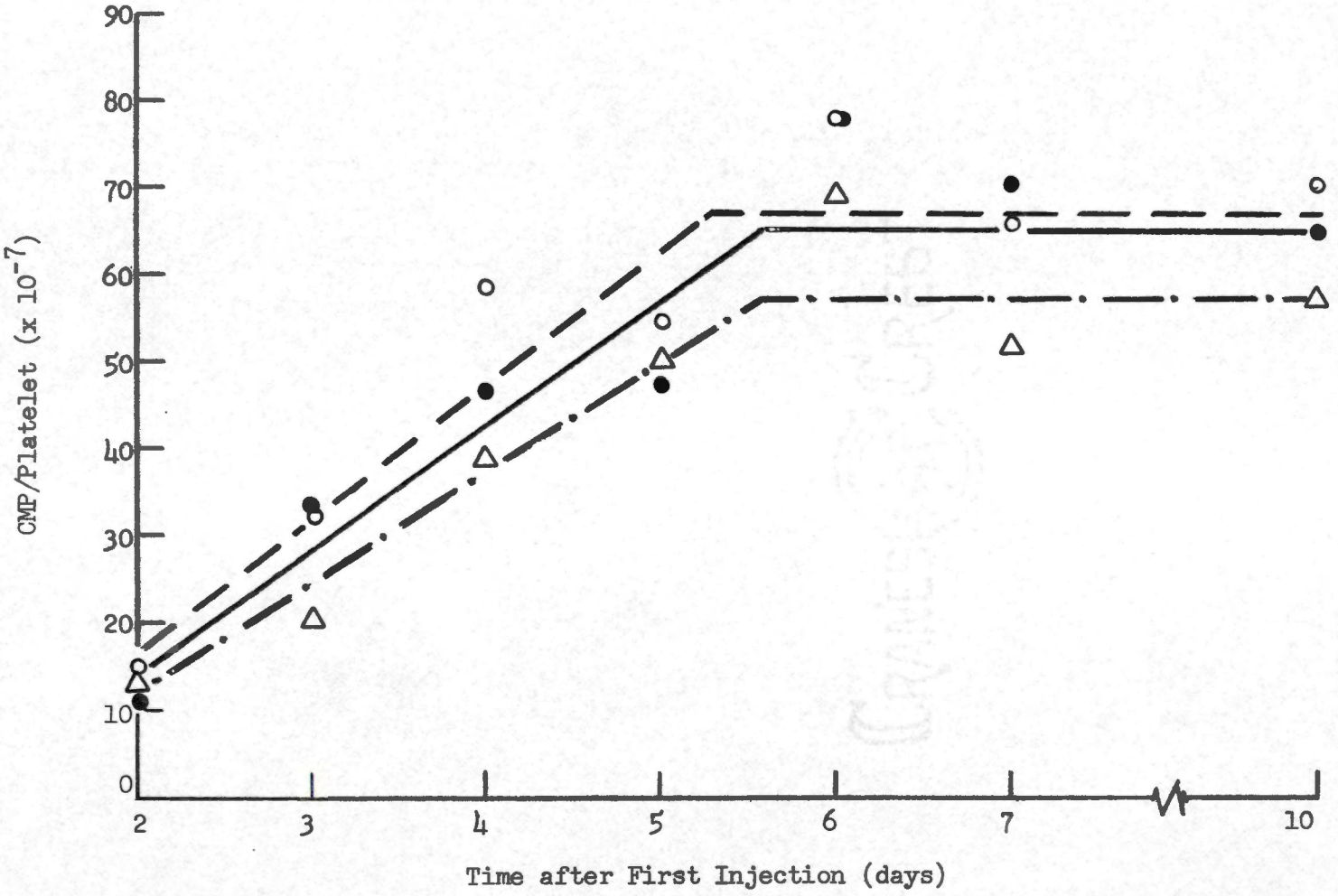


Figure 15. Radioactivity of platelets from normal rats and rats fed a vitamin A-deficient diet after daily intraperitoneal injections of $Na_2{}^{35}SO_4$.

●——●=Vitamin A-deficient diet; ○- - -○=vitamin A-deficient diet plus vitamin A; △—·—△=standard laboratory diet.

TABLE VII

RADIOACTIVITY OF PLATELETS, PLASMA-SALINE, AND PLATELET WASHES FROM VITAMIN A-DEFICIENT AND NORMAL RATS AFTER DAILY INTRAPERITONEAL INJECTIONS OF $Na_2{}^{35}SO_4$ [1]

Day	Number of Rats	Platelet Activity CPM/Plt. x 10^{-7}	$s_{\bar{x}}$	CPM/200 µl Plasma	Wash 1	Wash 2
		Normal Rats				
2	2	13.49	5.99	5422	135	6
3	2	20.32	5.99	5189	156	17
4	2	38.94	5.99	3350	126	2
5	2	50.14	5.99	3321	116	5
6	2	69.46	5.99	5176	127	17
7	2	51.98	5.99	5956	184	6
10	2	57.26	5.99	5828	203	30
		Rats Fed Vitamin A-Deficient Diet (12 weeks)				
2	2	11.02	6.50	3219	190	14
3	3	32.84	5.36	4194	224	37
4	3	46.60	5.36	4181	147	5
5	3	47.51	5.36	5115	216	15
6	3	78.60	5.36	5991	244	8
7	3	70.02	5.36	6416	248	16
10	3	65.41	5.36	5607	409	19
		Rats Fed Vitamin A-Deficient Diet with Vitamin A Added (12 weeks)				
2	1	14.76	8.11	5913	295	5
3	1	32.12	8.11	4824	168	3
4	2	58.72	5.73	5500	166	6
5	2	54.76	5.73	7480	210	12
6	2	78.39	5.73	8355	231	5
7	2	66.00	5.73	7911	279	8
10	2	70.44	5.73	6654	130	38

[1] One µc/gram body weight daily.

No statistical difference was seen in platelets or plasma-saline from rats fed a vitamin A-deficient diet for 11 weeks (Table VIII). In this experiment the isotope was given only once--48 hours before kill--and although the control values were lower, no statistical difference was noted.

VI. HEXOSAMINE AND PROTEIN CONTENT OF BLOOD PLATELETS

The influence of vitamin A on total hexosamines of platelets is illustrated by the data in Table IX. In blood platelets, "total hexosamines" are primarily MPS-bound hexosamines since small molecular weight intermediates containing hexosamines are probably nonexistent or are found in very small quantities. It has been shown that the label undergoes little turnover in normal circulating platelets (Odell and McDonald, 1964a), which means that MPS is probably formed while the platelet is still part of the cytoplasm of the megakaryocyte. One could infer that since MPS is not formed in the platelet but in its precursor, there are no intermediates of small molecular weight present in platelets.

The concentration of hexosamine or amount of protein per platelet, although quite variable (possibly because of the difficulty in determining the number of platelets in suspension), appear to be larger for the deficient platelets or vitamin A-treated platelets than for controls. The larger values for deficient platelets is perhaps related to platelet sizes, since platelets from deficient animals were shown to be larger than normal ones.

TABLE VIII

RADIOACTIVITY OF PLATELETS, PLASMA-SALINE, AND PLATELET WASHES FROM NORMAL AND VITAMIN A-DEFICIENT RATS AFTER SINGLE INTRAPERITONEAL INJECTIONS OF $Na_2{}^{35}SO_4$[1]

Weeks on Deficient Diet	No. of Rats	Platelet Activity[NS] CPM/Plt. x 10^{-7}	$s_{\bar{x}}$	CPM/100 µl Plasma[NS]	CPM/200 µl Wash 1	CPM/200 µl Wash 2
Control	4	20.85	5.04	840	61	13
6	3	32.10	5.83	1680	93	9
9	3	33.82	5.83	2117	140	10
10	3	29.85	5.83	1826	121	11
11	3	34.07	5.83	3732	194	27

[1]One µc/gram body weight 48 hours before killing.

[NS]Not statistically different.

TABLE IX

HEXOSAMINE AND PROTEIN CONTENT OF PLATELETS FROM VITAMIN A-TREATED, NORMAL OR VITAMIN A-DEFICIENT RATS

Treatment	Time After Treat. (days)	N[3]	µmoles Hexosamine per Platelet (x 10^{-11})	N	µg Protein per Platelet (x 10^{-6})	N	µmoles Hexosamine per mg Protein (x 10^{-3})	$s_{\bar{x}}$
Diet[1]		6	10.08	6	18.01	6	5.55[a]	.30
Diet[2]		6	9.94	6	16.23	6	6.19[ab]	.30
Normal Platelets		20	9.57	13	15.21	12	6.97[bcd]	.22
100 IU/Wt. of Vitamin A (daily)	4	6	11.08	3	17.83	3	7.56[d]	.43
	5	6	10.04	3	16.06	3	6.61[abcd]	.43
	6	6	8.76	3	13.61	3	6.53[abc]	.43
	7	6	9.98	5	14.87	5	7.01[bcd]	.33

TABLE IX (CONTINUED)

Treatment	Time After Treat. (days)	N[3]	µmoles Hexosamine per Platelet (x 10^{-11})	N	µg Protein per Platelet (x 10^{-6})	N	µmoles Hexosamine per mg Protein (x 10^{-3})	$s_{\bar{x}}$
500 IU/Wt. of Vitamin A (once)	4	6	9.28	3	15.32	3	6.87^{bcd}	.43
	5	5	9.99	3	11.91	3	8.83^{e}	.43
	6	6	9.86	3	15.19	3	7.72^{cd}	.43
	7	6	10.64	6	13.76	5	7.38^{cd}	.33

[1]Platelets from rats fed a vitamin A-deficient diet for 12 weeks.

[2]Platelets from rats fed a vitamin A-deficient diet plus vitamin A at the rate of 21.44 IU/gram of feed.

[3]N equals the number of rats.

^{abcde}Treatment means having different superscript letters are significantly ($P < 0.05$) different.

As can be seen in Table IX, hexosamine per platelet was unchanged in platelets of vitamin A-deficient rats. However, protein content was increased probably due to the increased size of these platelets. Protein content per volume of mass was not changed in rat colons from effects of the vitamin (Moretti and Wolf, 1961a). Therefore, the hexosamines are expressed in Table IX as μmoles of hexosamine per mg of protein.

Vitamin A deficiency results in a decrease of total hexosamine content by about 27 per cent in blood platelets as compared to controls. Five days after vitamin A was given to rats at the rate of 500 IU per gram of body weight, hexosamine values were about 26 per cent higher than controls. At other time periods, no difference was noted between platelets from control and vitamin A-treated rats. Again, the vitamin added to the deficient diet failed to restore the normal situation, the amount of hexosamine in platelets from deficient animals and from rats fed the deficient diet with vitamin A added were not significantly different (5.547 and 6.192×10^{-3} μmoles of hexosamine per milligram of protein).

Since hexosamine (glucosamine or galactosamine) is ordinarily present as a component of MPS, it is reasonable to assume that the decrease of hexosamine content in the platelets of vitamin A-deficient rats is directly proportional to a decrease in acid mucopolysaccharides. Conversely, an increase in hexosamine is interpreted as an increase in MPS.

These results are in agreement with those of other workers. For example, it has been established that decreased hexosamine values are

found in colon homogenates (Moretti and Wolf, 1961a), vagina, uterus, and colon (Ushimi, Sasamoto and Homma, 1963) as a result of vitamin A deficiency. Moretti and Wolf (1961a) reported that the colon MPS-hexosamines of vitamin A-deficient rats were lower by about 60 per cent in one experiment, and 37 to 38 per cent in another. In the present study only about 27 per cent reduction was attributed to the deficiency of the vitamin.

VII. IDENTIFICATION OF MPS

On the basis of the chromatographic methods described by Castor and Dorstewitz (1964) it appeared feasible to identify heparin and chondroitin sulfates (ChS) A, B, and C with some degree of certainty. In their study, they found that ChS C isolated from human umbilical cord or shark cartilage was soluble in 0.5 M $MgCl_2$, 1.0 M $MgCl_2$, and 0.4 M $AlCl_3$ after precipitation with cetylpyridinium chloride. ChS A (from human skin) was shown to be soluble in the same solvents plus 0.5 M $FeCl_3$ and ChS B (also isolated from human skin) was shown to be soluble only in 0.5 M $MgCl_2$ and 1.0 M $MgCl_2$. Commercially available heparin was dissolved only by 1.0 M $MgCl_2$. Figure 16 shows the results of chromatographic migration on paper strips of the control platelet extracts, commercially available ChS and heparin. It is shown here that heparin moves from the origin with the solvent front only when developed by 1.0 M $MgCl_2$. This is in complete agreement with the findings of Castor and Dorstewitz (1964). The ChS used in this study probably contained a mixture of ChS A, B, and C, since in Figure 16 it appears that the 0.5 M

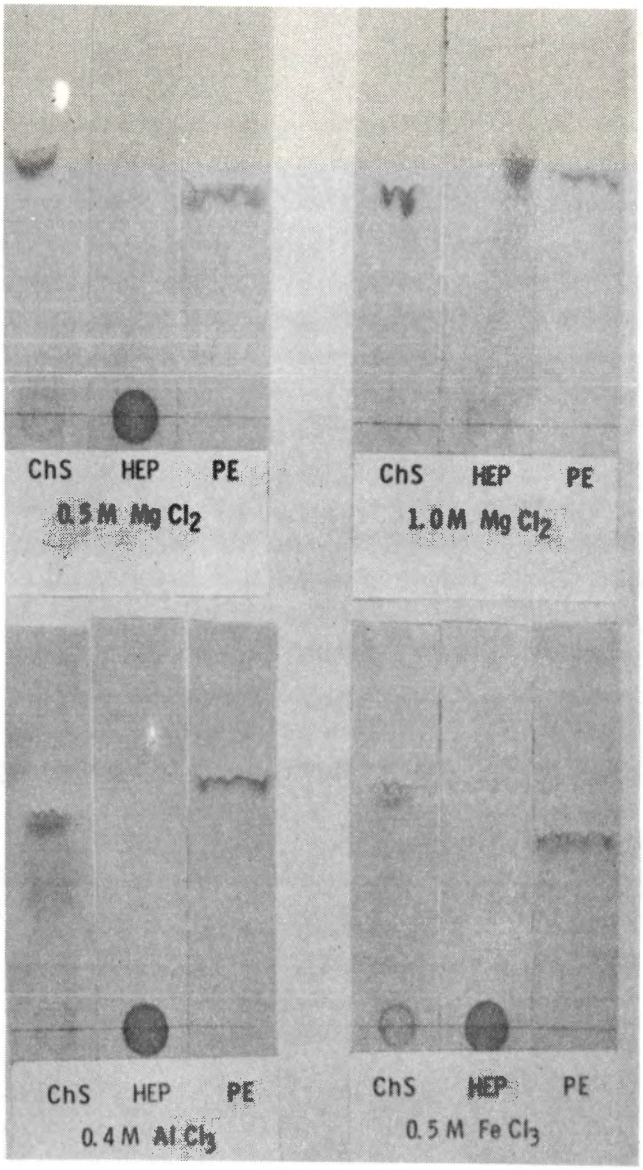


Figure 16. Chromatographic migration on paper strips of 20 µl of platelet extract (PE), 20 µg of chondroitin sulfate (ChS), and 20 µg of heparin (Hep); developed in 0.5 M $MgCl_2$, 1.0 M $MgCl_2$, 0.4 M $AlCl_3$ or 0.5 M $FeCl_3$. The material was located on the paper strip by staining with Alcian Blue 8G x 300.

$FeCl_3$ solvent moved only a part of the ChS with the solvent front and a greater amount moved with the 0.4 M $AlCl_3$ solvent. The platelet extract (PE) appears to be composed largely of compounds that are soluble in all the solvents used. By Castor and Dorstewitz's (1964) results this compound has solubilities characteristic of ChS A; therefore it is interpreted to be ChS A.

Based on the above data a system was developed to ascertain the relative amounts of ^{35}S-MPS in blood platelets by counting prescribed portions of paper strip. Other paper strips were treated as described, except not stained, and were subjected to radioactivity determinations. Each strip was cut into three pieces, which were counted individually for radioactivity, and the per cent of the total activity found in the solvent front was calculated. The relative per cent composition of blood platelet MPS was derived by the following equations:

(1) Per cent of the activity present in the solvent front of the strips chromatographed in 0.5 M $FeCl_3$ = ChS A

(2) Per cent of the activity present in the solvent front of the strips chromatographed in 0.4 M $AlCl_3$ - ChS A = ChS C

(3) Per cent of the activity present in the solvent front of the strips chromatographed in 0.5 M $MgCl_2$ - (ChS A + ChS C) = ChS B

(4) Per cent of the activity present in the solvent front of the strips chromatographed in 1.0 M $MgCl_2$ - (ChS A + ChS B + ChS C) = heparin

This procedure yields a workable method for the estimation of the ^{35}S-MPS components of blood platelets. Table X shows the results of these calculations.

Table X indicates that blood platelets contain largely a labeled MPS component that behaves like ChS A. Staining methods also indicated that the PE was largely ChS A and determinations of radioactivities of various parts of the paper chromatograph strips suggested the presence of additional MPS. The values for ChS A of extracts of control platelets range from 54 to 63 per cent of the labeled sulfated MPS. Materials designated ChS B and C are found in lesser amounts, with extracts from control platelets averaging about 6 per cent ChS B and about 17 per cent ChS C. Heparin-like materials make up about 13.5 per cent of the fractions obtained. In this system about 94 per cent of the radioactivity (MPS-^{35}S) moved with the 1.0 M $MgCl_2$ solvent. The 6 per cent of activity that remained at the origin may be MPS-bound protein that was not soluble with these solvents.

It is not clear from Table X whether differences in quality (per cent makeup) of MPS from vitamin A-treated, normal, or vitamin A-deficient platelets exist. The values obtained are not greatly different with treatment within experiments. Heparin may be reduced (percentage-wise) in the vitamin A-treated and the vitamin A-deficient groups compared to the control values (9-10 per cent compared to about 13.5 per cent). It is concluded, therefore, that vitamin A excess or deficiency did not result in noticeable alterations to platelet MPS makeup.

TABLE X

RELATIVE AMOUNTS OF SEVERAL ^{35}S-LABELED MUCOPOLYSACCHARIDES[1] IN BLOOD PLATELETS OF RATS ($Na_2{}^{35}SO_4$ INJECTED 5 DAYS BEFORE KILLING)

Treatment	Time After Treat. (days)	Plt. Cnt./ ml (x 10^9)	Platelet Activity CPM/Plt. (x 10^{-7})	CPM/200 µl Plasma	Relative Per Cent of MPS				Recovery[2]
					ChS A	ChS B	ChS C	Hep	
			First Experiment						
Normal Platelets		5.66	36.12	4930	54	10	13	17	94
		6.11	38.37	6553	61	0	20	13	94
		5.72	26.39	4860	59	4	19	12	94
		6.48	25.80	3271	63	8	11	11	93
	Mean		31.67	4904	59	6	16	13	94
Vitamin A Given Orally									
100 IU/Gram Body Weight	4	4.70	41.35	7067	57	4	20	10	91
	5	5.23	46.78	6791	61	11	13	9	94
	6	4.68	46.52	7500	69	9	7	10	95
	7	6.77	44.81	6662	62	9	12	11	94
500 IU/Gram Body Weight	4	5.60	27.53	7156	62	10	10	10	92
	5	6.11	48.31	5453	60	5	19	10	94
	6	6.14	38.08	6600	66	6	13	9	94
	7	8.17	31.47	4101	65	8	10	11	94

TABLE X (CONTINUED)

Treatment	Time After Treat. (days)	Plt. Cnt./ ml (x 10^9)	Platelet Activity CPM/Plt. (x 10^{-7})	CPM/200 µl Plasma	Relative Per Cent of MPS ChS A	ChS B	ChS C	Hep	Recovery[2]
			Second Experiment						
Normal		5.59	70.93	7190	54	2	24	14	94
Platelets		4.38	73.61	8365	58	14	13	14	94
	Mean		72.27	7778	56	8	18	14	94
Diet[3]		4.72	92.78	9670	62	13	9	10	94
		4.01	102.63	9500	64	13	6	8	91
		4.25	90.16	10729	62	16	7	9	94
	Mean		95.19	9966	63	14	7	9	93
Diet[4]		4.93	134.25	12097	64	8	10	13	95
		4.38	123.16	14509	51	2	17	14	94
		5.28	106.01	15611	62	15	8	8	93
	Mean		121.14	14072	59	8	12	12	94

[1]Mucopolysaccharide chromatograms were performed on pooled samples from 3 rats in quadruplicate.

[2]Per cent of total activity on paper strip that was present in solvent front of strips developed in 1.0 M $MgCl_2$.

[3]Platelets from rats fed a vitamin A-deficient diet.

[4]Same as above, except vitamin A was added to the diet.

The radioactivity of platelets subjected to these analyses are also shown in Table X. Although platelet activity differs considerably between experiments (probably due to different batches of isotopes), the trends are identical to those discussed previously (see section on uptake of ^{35}S by blood platelets and plasma). For example, in experiment 2 platelets from rats fed a vitamin A-deficient diet with vitamin A added showed the greatest uptake of ^{35}S (121 x 10^{-7} CPM per platelet) and platelets from vitamin A-deficient rats showed greater ^{35}S uptake (95 x 10^{-7} CPM per platelet) than did the controls (72 x 10^{-7} CPM per platelet). Also the platelets from the vitamin A-treated rats, in the first experiment, showed generally more ^{35}S-uptake than did control platelets.

Unlike previous finding, the plasma-saline ^{35}S activities appear to be different in the second experiment after treatment (see Table X).

Hexosamine studies of rat colons by Moretti and Wolf (1961a) showed a decrease in the content of galactosamine-containing MPS with a shift in the glucosamine-to-galactosamine ratio in addition to about a 60 per cent reduction of total hexosamines due to the deficiency. MPS from rat blood platelets in these experiments was not so altered. In fact the data are suggestive of a decrease in heparin (glucosamine-containing MPS) in the deficiency or excess and an increase in galactosamine-containing MPS (ChS).

Qualitative measurements of the kinds of MPS in human platelets suggested that they are composed of a mixture of chondroitin sulfates B and C (Kerby and Taylor, 1959). Odell and Anderson (1957) and

Anderson and Odell (1958) have shown that rat blood platelets contain two MPS fractions. Paper electrophoresis and chromatography of the MPS demonstrated that the first fraction was similar to chondroitin sulfate. Fraction 2 was shown to be uronic acid-free. The present results agree with these findings in that the chief component was chondroitin sulfate. More specifically it was ChS A. ChS B, ChS C, and heparin were present, but they were found in lesser quantities. According to Kerby and Taylor's (1959) results on human blood platelets, it seems possible that these platelets may differ from rat blood platelets in relative per cent composition of MPS due to species differences. Before conclusive statements can be made regarding MPS quality of blood platelets further research is needed in this very complex field.

VIII. BLOOD PLATELET ADHESIVENESS

The data on adhesiveness of blood platelets obtained from 21 rats treated with vitamin A and 5 control rats are shown in Table XI. Although the number of animals in each time period is small, significant increases in adhesiveness of blood platelets from control rats were seen at day 4 (after 100 IU per gram body weight daily) and at day 5 (after a single treatment with 500 IU per gram body weight). In the 500 IU-group the greatest adhesiveness occurred at the same time (day 5) that a significant increase in hexosamines was also observed (Table IX). No evidence was seen of a decrease in platelet numbers of control or treated animals (from blood that had not been passed through the column), since the number of platelets counted was in the normal range (about 0.9

TABLE XI

ADHESIVENESS OF BLOOD PLATELETS FROM VITAMIN A-TREATED AND NORMAL RATS

Time after Vitamin A (days)	No. of Rats	$\bar{X}$ Adhesiveness Index (Per cent)	$s_{\bar{x}}$
	Control		
	5	96^{bc}	2.60
	100 IU/Gram Body Wt.		
4	3	78^{a}	3.36
5	3	97^{c}	3.36
6	1	96^{bc}	5.81
7	3	93^{bc}	3.36
	500 IU/Gram Body Wt.		
4	2	96^{bc}	4.11
5	3	77^{a}	3.36
6	3	84^{ab}	3.36
7	3	98^{c}	3.36

[a]Means with superscript "a" are significantly different from means without superscript "a", according to Duncan's Multiple Range Test at the 1 per cent level of significance.

[bc]Means with superscript "b" are significantly different from means without superscript "b", and means with superscript "c" are different from those without superscript "c".

to 1.1 x 10^6 platelets per mm^3). It appears, therefore, that platelets from vitamin A-treated rats have greater adhesiveness than normal platelets, since a greater amount of platelets was removed by the glass beads in the vitamin A-treated rats. Whether or not the added adhesive quality of these platelets is due to increased MPS remains to be shown, but the data indicate a causal relationship. It has not been established conclusively if adhesiveness of blood platelets affects platelet role(s) in the body, but a relationship is strongly suggested. Weiner et al. (1948) reported that patients with thrombocytopenic purpura exhibited deficient adhesiveness of the thrombocytes and this was the only demonstrable defect noted in the coagulation mechanism.

IX. GENERAL CONCLUSIONS

These data have established that vitamin A influences the metabolism of MPS in blood platelets. Although the relative amounts of several MPS within blood platelets were not altered significantly, the total amount of MPS was altered by vitamin A deficiency or excess. Blood platelets of vitamin A-treated rats, which contained significantly more MPS, showed increased adhesive qualities. These results suggest that the quantity of MPS in blood platelets controls the platelet's adhesiveness.

Both blood platelets and vitamin A are known to be involved in maintenance of the integrity of the capillary endothelium. Zucker and Borrelli (1958) suggested that blood platelets contribute some chemical component to capillaries that in some way improves their resistance to

red blood cell extravasation. This study suggests that this chemical component may be a sulfated mucopolysaccharide.

Mucopolysaccharides of blood platelets have also been implicated in thrombotic thrombocytopenic purpura (Moore and Schoenberg, 1960). The lesions, which were found in the capillaries and terminal arterioles, consistently contained an accumulation of acid MPS similar to chondroitin sulfate or hyaluronic acid. The authors suggested that these lesions could be caused by a diffuse platelet thrombosis. It may be that capillary endothelium loses its integrity during vitamin A deficiency not entirely by a direct action on the endothelium, as has been previously proposed, but indirectly through the blood platelets. More data concerning this problem are needed in order to clarify these functions.

CHAPTER V

SUMMARY

Male Sprague-Dawley rats were given vitamin A orally at the rate of 50 or 100 IU per gram body weight daily or single doses of 500 or 1000 IU per gram body weight. Other rats were fed a vitamin A-deficient diet, a standard laboratory diet, and a vitamin A-deficient diet with vitamin A added at the rate of 21.44 IU per gram of feed. Rats suffering from hypovitaminosis A or hypervitaminosis A were weighed at given intervals and their peripheral white blood cells, red blood cells, platelets, and hematocrits were determined.

The following results were observed:

1. The symptoms following ingestion of excess vitamin A were: changes in the fur, lack of appetite, loss of weight, muscular weakness, and internal hemorrhages.
2. Vitamin A-deficient rats showed the following symptoms: loss of appetite, decreased gain in body weight, decreased blood pressure, roughness of fur, and eye lesions (xerophthalmia).
3. Peripheral platelet counts were increased ($P < 0.01$) when 50 IU per gram body weight of the vitamin was given daily. When 500 IU or 1000 IU per gram body weight was given at day 0, the peripheral platelet counts were variable. The platelet count of vitamin A-deficient rats was not different from that of controls, although both the experimental and control rats

showed a general decline in platelet values with time during the 18-week period.

4. Hematocrits from hypervitaminosis A rats appeared to be increased in the early part of the experiment and then decreased thereafter when 500 and 1000 IU of the vitamin per gram of body weight were given at day 0 ($P < 0.01$). In the deficiency, hematocrits were increased for about 3 weeks before death.

5. Peripheral RBC counts in rats suffering from the deficiency did not appear to be altered; however, vitamin A-treated rats showed decreased ($P < 0.01$) values as low as 50 per cent of initial at the level of 1000 IU per gram of body weight at day 0. Evidence is presented which suggests that excess vitamin A caused lysis of RBC in vivo.

Blood platelet size determinations using the Coulter counter method showed that: (1) curves of platelets from vitamin A-deficient rats were shifted to the right, indicating larger ($P < 0.05$) platelets and (2) in some of the platelet determinations from the vitamin A-treated rats the shift was to the left ($P < 0.05$) indicating smaller platelets than those of a normal population. A second technique, based on determinations of packed-cells-per-volume, substantiated these findings.

Blood platelets and plasma from the above treatment groups were analyzed for $Na_2{}^{35}SO_4$ uptake. Rats were killed 24 hours after one to ten daily intraperitoneal injections of 1 μc per gram of body weight of isotope or 48 hours after single injections. The results show the following:

1. Platelets from vitamin A-treated rats incorporated from 40 to 100 per cent more ^{35}S, depending on the dose of vitamin A, than did control platelets.
2. On a per-platelet basis, the larger platelets from the vitamin A-deficient rats showed an increased ^{35}S uptake (about 18 per cent) as compared to the smaller control platelets.

Vitamin A deficiency resulted in a decrease of total hexosamine content of blood platelets (μmoles of hexosamine per mg of protein) by 27 per cent ($P < 0.05$) as compared to controls. Five days after an excess of vitamin A was given to rats (500 IU per gram body weight), hexosamine values for platelets were 26 per cent higher ($P < 0.05$) than controls.

The relative per cent composition of ^{35}S-labeled MPS from blood platelets by a chromatographic technique demonstrated the following:

1. Of the total ^{35}S-labeled MPS found in normal blood platelets, about 58 per cent of these materials behaved like ChS A; 6 per cent, ChS B; 17 per cent, ChS C; and 13.5 per cent, heparin.
2. The results suggest that in blood platelets there may be a slight reduction in the relative amount of heparin due to vitamin A excess or deficiency.
3. A technique was developed for measuring the relative per cent composition of sulfated MPS.

Using silicone-treated glass columns filled with non-silicone glass beads, it was found that platelets from vitamin A-treated rats

have greater adhesive qualities than normal platelets ($P < 0.01$), as shown by their adhesiveness indexes (per cent cells removed by the glass beads).

These data indicate that rat blood platelets contain significantly more MPS as a result of vitamin A treatment and less MPS from deficiency. In spite of these facts, no noticeable change was found in the relative per cent composition of MPS.

LITERATURE CITED

LITERATURE CITED

Abbott, O. D. and C. F. Ahmann. 1938. Effect of avitaminosis A on the blood picture of albino rats. Am. J. Physiol. 122:589.

Abbott, O. D., C. F. Ahmann and M. R. Overstreet. 1939. Effect of avitaminosis on the human blood picture. Am. J. Physiol. 126:254.

Aberle, S. B. D. 1934. Neurological disturbances in rats reared on diets deficient in vitamin A. J. Nutr. 7:445.

Anagnostu, J. 1939. Experimentelle untersuchungen über den Einfluss von vitamin A auf die Thrombocyten und das Knochenmark. Klin. Wschr. 18:1277.

Anderson, B. and T. T. Odell, Jr. 1958. Chemical studies of mucopolysaccharides of rat blood platelets. Proc. Soc. Exp. Biol. and Med. 99:765.

Barker, S. A., C. N. D. Cruickshank and T. A. Webb. 1964. The effect of vitamin A, hydrocortisone and citral upon sulphate metabolism in skin. Exp. Cell Res. 35:255.

Belcher, R., A. J. Nutten and C. M. Sambrook. 1954. The determination of glucosomine. Analyst 79:201.

Bell, L. G. E. 1962. Polysaccharide and cell membranes. J. Theoret. Biol. 3:132.

Bernard, R. A., B. P. Halpern and M. R. Kare. 1961. Effect of vitamin A deficiency on taste. Proc. Soc. Exp. Biol. Med. 108:784.

Bliss, A. R., Jr., G. R. Livermore and E. O. Prather, Jr. 1933. Relation of vitamin A and vitamin D to urinary calculus formation. J. Urology 30:639.

Brecher, G. and E. P. Cronkite. 1950. Morphology and enumeration of human blood platelets. J. Appl. Physiol. 3:365.

Brecher, G., E. F. Jakobek, M. A. Schneiderman, G. Z. Williams and P. J. Schmidt. 1962. Size distribution of erythrocytes. Ann. N. Y. Acad. Sci. 99:242.

Castor, C. W. and E. L. Dorstewitz. 1964. Identification of acid mucopolysaccharides by paper chromatography. J. Cromatog. 13:157.

Chung, A. C. and J. C. Houck. 1964. Connective Tissue IX: Effects of hypervitaminosis A upon connective tissue chemistry. Proc. Soc. Exp. Biol. Med. 115:631.

Clark, I. and M. R. Smith. 1964. Effects of hypervitaminosis A and D on skeletal metabolism. J. Biol. Chem. 239:1266.

Cohen, A. C. 1957. On the solution of estimating equations for truncated and censored samples from normal populations. Biometrika. 44:225.

Cohen, J., C. L. Maddock and S. B. Wolbach. 1955. The effect of parathyroidectomy on the evolution of hypervitaminosis A in rats. Am. Med. Assoc. Arch. Pathol. 59:723.

Collazo, J. A. and J. Sánchez Rodriguez. 1933. Hypervitaminose A. Die Symptomatologie der durch Fütterung Von reinem A-Vitamin an jungen Ratten hervorger fenen Hypervitaminose A. Klin. Wschr. 12:1732.

Crimm, P. D. and D. M. Short. 1935a. Qualitative blood cell changes in the rat due to vitamin A. Am. J. Physiol. 111:397.

Crimm, P. D. and D. M. Short. 1935b. Qualitative blood cell changes in the human due to vitamin A. J. Indiana State Med. Ass. 28:175.

DeLuca, H. F., M. R. Manatt, N. Madsen and E. B. Olson. 1963. Action of vitamin A on liver homogenate oxidation of tricarboxylic acid cycle intermediates. J. Nutr. 81:383.

DeRuyter, T. H. 1934. The histology of avitaminosis A. I. Alterations in the organ and tissue structure. II. Vitamin A deficiency and susceptibility to infection. Acta brev. neerl. Physiol. 4:122.

Detwiler, T. W., T. T. Odell, Jr. and T. P. McDonald. 1962. Platelet size, ATP content, and clot retraction in relation to platelet age. Am. J. Physiol. 203:107.

Dingle, J. T. 1961. Studies on the mode of action of excess of vitamin A. 3. Release of a bound protease by the action of vitamin A. Biochem. J. 79:509.

Dingle, J. T. and J. A. Lucy. 1963. Vitamin A and membrane systems: Interactions of vitamin A and vitamin E. Biochem. J. 86:15.

Doisy, E. A. and J. T. Matschiner. 1961. Hemorrhagic syndrome in rats fed irradiated beef. Final report, Contract No. DA-49-007-MD-996, Research and Development Division, Office of the Surgeon General, Dept. of Army, Washington 25, D. C.

Dorfman, A., A. Markovitz and J. A. Cifonelli. 1958. Metabolism of hyaluronic acid and chondroitinsulfuric acids. Fed. Proc. 17:1093.

Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics. 11:1.

Dziewiatkowski, D. D. 1954. Vitamin A and endochondral ossification in the rat as indicated by the use of sulfur-35 and phosphorus-32. J. Exp. Med. 100:11.

Dziewiatkowski, D. D. 1956. Some aspects of the metabolism of chondroitin sulfate-S^{35} in the rat. J. Biol. Chem. 223:239.

Fell, H. B. 1953. The effect of vitamin A on organ cultures of skeletal and other tissues. In "Connective Tissues," Transaction of the Fourth Conference (C. Ragan, ed.), 142. Josah Macy, Jr. Foundation, New York.

Fell, H. B. 1960. The effect of vitamin A on tissue structure. Nutr. Soc. Proc. 19:50.

Fell, H. B. and J. T. Dingle. 1963. Studies on the mode of action of excess of vitamin A. No. 6. Lysosomal protease and the degradation of cartilage matrix. Biochem. J. 87:403.

Fell, H. B., J. T. Dingle and M. Webb. 1962. Studies on the mode of action of excess of vitamin A. No. 4. The specificity of the effect on embryonic chick-limb cartilage in culture and on isolated rat-liver lysosomes. Biochem. J. 83:63.

Fell, H. B. and E. Mellanby. 1952. The effect of hypervitaminosis A on embryonic limb-bones cultured in vitro. J. Physiol. (London) 116:320.

Fell, H. B. and E. Mellanby. 1953. Metaplasia produced in cultures of check ectoderm by high vitamin A. J. Physiol. (London) 119:470.

Fell, H. B., E. Mellanby and S. R. Pelc. 1954. Influence of excess Vitamin A on the sulphate metabolism of chick ectoderm grown in vitro. British Med. J. 2:611.

Fell, H. B., E. Mellanby and S. R. Pelc. 1956. Influence of excess vitamin A on the sulphate metabolism of bone rudiments grown in vitro. J. Physiol. 134:179.

Frank, M. 1934. Beitrag zur Hämatologie der A-Avitaminose. Mschr. Kinderheilk. 60:350.

Frank, M. 1935. Zur Frage der Resistenzverminderung gegen Infection bei der A-Avitaminose. Med. Klinik. 31:486.

Frape, D. L., R. S. Allen, V. C. Speer, V. W. Hays and D. V. Catron. 1959. Relationship of vitamin A to S^{35} metabolism in the baby pig. J. Nutr. 68:189.

Giroud, A. 1955. Les Malformations Congenitales et Leurs Causes. Biol. Med. Paris, 44:number 5.

Glauert, A. M., M. R. Daniel, J. A. Lucy and J. T. Dingle. 1963. Studies on the mode of action of excess of vitamin A. VII. Changes in the fine structure of erythrocytes during haemolysis of vitamin A. J. Cell Biol. 17:111.

Gross, L. 1924. The effects of vitamin deficient diets on rats, with special reference to the motor functions of the intestinal tract in vivo and in vitro. J. Path. Bact. 27:27.

Gude, W. D., A. C. Upton and T. T. Odell, Jr. 1956. Blood platelets of human and rat. Lab. Inves. 5:348.

Harris, L. J. and T. Moore. 1928. Hypervitaminosis and vitamin balance. Biochem. J. 22:1461.

Hughes, J. S., H. F. Lienhardt and C. E. Aubel. 1929. Nerve degeneration resulting from avitaminosis A. J. Nutr. 2:183.

Kerby, Grace P. and S. M. Taylor. 1959. The acid mucopolysaccharide and 5-hydroxytryptamine content of human thrombocytes in rheumatoid arthritic and nonarthritic individuals. J. Clin. Inves. 38:1059.

LeClerg, E. L. 1957. Mean separation by the functional analysis of variance and multiple comparisons. Agri. Res. Serv., U. S. D. A. A. R. S. 20-3.

Light, R. F., R. P. Alscher and C. N. Frey. 1944. Vitamin A toxicity and hypoprothrombinemia. Science 100:225.

Lucy, J. A. and J. T. Dingle. 1962a. Vitamin A membrane systems. 2. Membrane stability and protein-vitamin A-lipid interactions. Biochem. J. 84:76.

Lucy, J. A. and J. T. Dingle. 1962b. The effect of vitamin A on the stability of the erythrocyte membrane. Biochem. J. 82:31.

Lucy, J. A., J. T. Dingle and H. B. Fell. 1961. Studies on the mode of action of excess of vitamin A. 2. A possible role of intracellular proteases in the degradation of cartilage matrix. Biochem. J. 79:500.

Lucy, J. A., M. Luscombe and J. T. Dingle. 1963. Studies on the mode of action of excess of vitamin A. No. 8. Mitochondrial swelling. Biochem. J. 89:419.

Maddock, Charlotte L., J. Cohen and S. B. Wolbach. 1953. Effect of hypervitaminosis A on the testes of the rat. A.M.A. Arch. of Path. 56:333.

Maddock, Charlotte L., S. B. Wolbach and Dorothy Jensen. 1948. Hypoprothrombinemia with hemorrhage as a cause of death in the rat in hypervitaminosis A. Fed. Proc. 7:275.

Maschauf, C., R. W. Robinson and R. J. Le Beau. 1964. Evaluation of nialamide on the coagulation of blood. A new technic for measuring adhesiveness of the platelets. Blood 24:289.

Matschiner, J. T. and E. A. Doisy, Jr. 1960. The role of vitamin A in nutritional hypoprothrombinemia in rats. Fed. Proc. 19:421.

Mayer, J. and W. A. Krehl. 1948. The relation of diet composition and vitamin C to vitamin A deficiency. J. Nutr. 35:523.

McCollum, E. V., N. Simmonds, J. E. Becker and R. W. Bunting. 1925. The effect of additions of fluorine to the diet of the rat on the quality of the teeth. J. Biol. Chem. 63:553.

McDonald, T. P., T. T. Odell, Jr. and D. G. Gosslee. 1964. Platelet size in relation to platelet age. Proc. Soc. Exp. Biol. Med. 115: 684.

McElligott, T. F. 1962. Decreased fixation of sulphate by chondrocytes in hypervitaminosis A. J. Path. and Bact. 83:347.

Mellanby, E. 1937. Vitamin A deficiency and deafness. Chem. and Ind. 15:1054.

Mellanby, E. 1943. The effect of bone dysplasia (overgrowth) on cranial nerves in vitamin A-deficient animals. J. Physiol. 101:408.

Moore, L. A. 1939. Relationship between carotene blindness due to constriction of the optic nerve, papillary edema and nyctalopia in calves. J. Nutr. 17:443.

Moore, T. 1957. Vitamin A. Elsevier Publishing Co., New York.

Moore, T. 1960. The pathology of vitamin A deficiency. Vitamins and Hormones 18:499.

Moore, R. D. and M. D. Schoenberg. 1960. A polysaccharide component in the vascular lesions of thrombotic thrombocytopenic purpura. Blood 15: 511.

Moore, L. A. and J. F. Sykes. 1941. Terminal cerebrospinal fluid pressure values in vitamin A deficiency. Am. J. Physiol. 134:436.

Moore, T. and Y. L. Wang. 1945. Hypervitaminosis A. Biochem. J. 39: 222.

Moretti, A. and G. Wolf. 1961a. Decrease in mucopolysaccharide-bound hexosamine of rat colon in vitamin A deficiency. Biochim. Biophys. Acta 46:392.

Moretti, A. and G. Wolf. 1961b. Vitamin A and mucopolysaccharide biosynthesis in colon homogenates. Biochim. Biophys. Acta 53:263.

Mori, S. 1922. The changes in the para-ocular glands which follow the administration of diets low in fat-soluble A; with note of the effect of the same diets on the salivary glands and the mucosa of the larnyx and trachea. Bull. Johns Hop. Hosp. 32:357.

Odell, T. T., Jr. and Bonnie Anderson. 1957. Isolation of a sulfated mucopolysaccharide from blood platelets of rats. Proc. Soc. Exp. Biol. Med. 94:151.

Odell, T. T., Jr. and Bonnie Anderson. 1959. Production and life span of platelets. In the Kinetics of Cellular Proliferation. Grune and Stratton, Inc., New York.

Odell, T. T., Jr. and T. P. McDonald. 1964a. Two mechanisms of sulfate-S^{35} uptake by blood platelets of rats. Am. J. Physiol. 206:580.

Odell, T. T., Jr. and T. P. McDonald. 1964b. Methods for the estimation of the life span of platelets. In Blood Coagulation, Hemorrhage and Thrombosis (Eds., L. M. Tocantins and L. A. Kazal), pp. 68-73. Grune and Stratton, Inc., New York.

Odell, T. T., Jr., F. G. Tausche and W. D. Gude. 1955. Uptake of radioactive sulfate by elements of the blood and the bone marrow of rats. Am. J. Physiol. 180:491.

Osborne, T. B. and L. B. Mendel. 1917. The incidence of phosphatic urinary calculi in rats fed on experimental rations. J. Am. Med. Ass. 69:32.

Pasternak, C. A., S. K. Humphries and A. Pirie. 1963. The activation of sulphate by extracts of cornea and colonic mucosa from normal and vitamin A-deficient animals. Biochem. J. 86:382.

Poumeau-Delille, G. 1943. Erythroblastic anemia in rats during hypervitaminosis A. C. R. Soc. Biol. (Paris) 137:604.

Rao, M. V. Radhakrisna. 1936. Studies on vitamin A deficiency; xeropthalmia and trigeminal nerve degeneration. Ind. J. Med. Res. 24:439.

Richards, M. B. 1935. Role of vitamin A in nutrition. Brit. Med. J. 1:99.

Richardson, L. R. 1961. Investigation to determine the mechanism for production of the hemorrhagic syndrome which develops in male rats that receive diets containing irradiated beef. Carried out under Contract No. DA-49-007-MD-582 with the Office of the Surgeon General.

Rigdon, R. H. 1952. Pathologic lesions in the nervous system of a duck fed a ration deficient in vitamin A. Arch. Path. 53:239.

Rodahl, K. 1950. Hypervitaminosis A in the rat. J. Nutr. 41:399.

Rodahl, K. and T. Moore. 1943. The vitamin A content and toxicity of bear and seal liver. Biochem. J. 37:166.

Smudski, J. W. and H. M. Myers. 1963. Vitamin A and sulfur metabolism. J. Dental Res. 42:959.

Steele, R. G. D. and J. H. Torrie. 1960. Principles and Procedures of Statistics. McGraw-Hill Book Co., Inc., New York.

Sure, B., M. C. Kik and D. J. Walker. 1929. The effect of Avitaminosis on hematopoietic function. I. Vitamin A deficiency. J. Biol. Chem. 83:375.

Takahashi, K., Z. Nakamiya, M. Kawakimi and T. Kitasato. 1925. Physical and chemical properties of biosterin (a name given to fat-soluble A) and its physiological significance. Sci. Papers Inst. Phys. Chem. Res., Tokyo 3:81.

Thomas, L., R. T. McCluskey, J. L. Potter and G. Weissmann. 1960. Comparison of the effects of papain and vitamin A on cartilage. I. The effects in rabbits. J. Exp. Med. 111:705.

Thomson, J. N. and G. A. J. Pitt. 1960. Vitamin A acid and hypervitaminosis A. Nature 188:672.

Uotila, U. and P. E. Simola. 1938. Über die Beziehungen Zwischen den Vitaminen und dem reticuloendothelialen system. Virchow's Arch. Path. Anat. 301:523.

Ushimi, C., S. Sasamoto and S. Homma. 1963. Hexosamine contents in various tissues of rats fed on vitamin A deficient diet. Nat. Inst. Animal Health 3:150.

Varandani, P. T., G. Wolf and B. C. Johnson. 1960. Function of vitamin A in the synthesis of 3'-phosphoadenosine-5'-phosphosulfate. Biochem. Biophys. Res. Commun. 3:97.

Vedder, E. B. and C. Rosenberg. 1938. Concerning the toxicity of vitamin A. J. Nutr. 16:57.

Wald, G. 1955. The photoreceptor process in vision. Am. J. Ophthalmol. 40:18.

Walker, S. E., E. Eylenburg and T. Moore. 1947. The action of vitamin K in hypervitaminosis A. Biochem. J. 41:575.

Weiner, M., K. Seltmacher, C. Reich and S. Shapiro. 1948. Platelet adhesiveness. Blood 3:1275.

Weslaw, W., B. Wronski, A. Wroblewski and B. Wroblewski. 1938. Symptomatologie und Verlauf der A-Hypervitaminose bei Ratten infolge enteraler, subcutaner und percutaner Darreichung von Vitamin A-Konzentraten; Peroralė und Subcutane Verabreichung der Vitamin A. Klin. Wschr. 17:777.

Wolbach, S. B. 1947. Vitamin-A deficiency and excess in relation to skeletal growth. J. Bone and Joint Surg. 29:171.

Wolbach, S. B. and O. A. Bessey. 1941. Vitamin A deficiency and the nervous system. Arch. Path. 32:689.

Wolbach, S. B. and P. R. Howe. 1925. Tissue changes following deprivation of fat-soluble A vitamin. J. Exp. Med. 42:753.

Wolbach, S. B. and P. R. Howe. 1928. Vitamin A deficiency in the guinea pig. Arch. Path. Lab. Med. 5:239.

Wolbach, S. B. and C. L. Maddock. 1952. Vitamin-A acceleration of bone growth sequences in hypophysectomized rats. A.M.A. Arch. Path. 53:273.

Wolbach, S. B., C. L. Maddock and J. Cohen. 1955. The hypervitaminosis-A syndrome in adrenalectomized rats. Skeletal changes in weanling rats with and without cortisone. A.M.A. Arch Path. 59:130.

Wolf, G., J. G. Bergan and P. R. Sundaresan. 1962. Vitamin A and mucopolysaccharide biosynthesis by cell-free particle suspensions. Fed. Proc. 21:171.

Wolf, G., J. G. Bergan and P. R. Sundaresan. 1963. Vitamin A and mucopolysaccharide biosynthesis by cell-free particle suspensions. Biochim. Biophys. Acta 69:524.

Wolf, G., M. D. Lane and B. C. Johnson. 1957. Studies on the function of vitamin A in metabolism. J. Biol. Chem. 225:995.

Wolf, G. and D. Phil. 1961. Vitamin A in adrenal hormone and mucopolysaccharide biosynthesis. Am. J. Clin. Nutr. 9:36.

Wolf, G. and P. T. Varandani. 1960. Studies on the function of vitamin A in mucopolysaccharide biosynthesis. Biochim. Biophys. Acta 43:501.

Wolf, G., P. T. Varandani and B. C. Johnson. 1961. Vitamin A and mucopolysaccharide synthesizing enzymes. Biochim. Biophys. Acta 46:59.

Wolf, G., S. R. Wagle and B. C. Johnson. 1957. Function of vitamin A. Fed. Proc. 16:272.

Wolf, G., S. R. Wagle, R. A. Van Dyke and B. C. Johnson. 1958. The function of vitamin A in metabolism. II. Vitamin A and adrenocortical hormone. J. Biol. Chem. 230:979.

Zimmerman, H. M. and G. R. Cowgill. 1936. Lesions of the nervous system in vitamin deficiency. J. Nutr. 11:411.

Zucker, M. B. and J. Borrelli. 1958. A survey of some platelet enzymes and functions: The platelets as the source of normal serum acid glycerophosphatase. Ann. N. Y. Acad. Sci. 75:203.