THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

BIOCHEMICAL STUDIES OF LYSOSOMAL ENZYMES IN EXPERIMENTAL MUSCULAR DYSTROPHY

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSPHY

BY

ABBAS OTHMAN JIBRIL

Oklahoma City, Oklahoma

1964

BIOCHEMICAL STUDIES OF LYSOSOMAL ENZYMES

IN EXPERIMENTAL MUSCULAR DYSTROPHY

APPROVED BY

Paul B. McCay

W. R. Sheldon

Earl G. Jensen

Kenneth M. Richter

John W. Kelly

DISSERTATION COMMITTEE

ACKNOWLEDGMENT

I would like to express my sincere appreciation and thanks to Dr. Paul B. McCay for his guidance and advice during the course of this work and to Dr. Ranwel Caputto for his continued interest in the progress of this research. My sincere thanks to Dr. Mary B. Carpenter, Dr. Abbas E. Kitabchi, Dr. Jordan Tang and Dr. Raul Carubelli for their constructive ideas and criticism. I am grateful to Dr. Kenneth Richter and Dr. John Kelly for examining skeletal muscle sections histologically and histochemically and also to Dr. Richard Bottomley for his assistance in hematological procedures. Discussions with Dr. Marvin Shetlar and Dr. Earl Larsen have been very helpful.

I wish to express my gratitude to the Oklahoma Medical Research Institute for providing the facilities and materials to support this work and for the financial support as a predoctoral fellow from September 1962 to May 1964. I would like to thank the American Cancer Society for providing an institutional grant to support this work.

My sincere thanks to all my colleagues in the Biochemistry Section and the Psychosomatic Section for their cooperation and assistance.

TABLE OF CONTENTS

		Page
LIST OF TABLES		v
LIST OF ILLUSTRATIONS		viii
Chapter		
I.	INTRODUCTION	1
II.	MATERIALS AND METHOD	26
III.	RESULTS	64
IV.	DISCUSSION	149
V.	SUMMARY	180
REFERENCES		185

LIST OF TABLES

Table		Page
1.	Phagocytic Index (K) Values of Rabbits	69
2.	*In Vivo* Uptake of Carbon C-14 by Rabbit Livers	72
3.	*In Vivo* Uptake of Carbon C-14 by Rabbit Spleens	74
4.	*In Vivo* Uptake of Carbon C-14 by Rabbit Livers Expressed as Percent of Total Injected Dose	76
5.	*In Vivo* Uptake of Carbon C-14 by Rabbit Spleens Expressed as Percent of Total Injected Dose	77
6.	*In Vivo* Uptake of Carbon C-14 by Rabbit Lungs Expressed as Percent of Total Injected Dose	78
7.	*In Vivo* Uptake of Carbon C-14 by Rabbit Skeletal Muscle	80
8.	Effect of Pair Feeding and Diet Reversal on the *In Vivo* Uptake of Carbon C-14 by Rabbit Muscle	82
9.	Total Activity of Rabbit Muscle Acid Phosphatase	84
10.	Total Activity of Rabbit Muscle Cathepsin	86
11.	Total Activity of Rabbit Muscle β-Glucuronidase	88
12.	Total Activity of Rabbit Muscle Neuraminidase .	90
13.	Total Activity of Rabbit Muscle Lyso-phosphatidase	92

14. Intracellular Distribution of Neuraminidase in Skeletal Muscle of Dystrophic and Non-dystrophic Rabbits 96

15. Intracellular Distribution of Cathepsin (pH 3.8) in Skeletal Muscle of Dystrophic and Non-dystrophic Rabbits 98

16. Extracted Lipophilic Sialic Acid Compounds from Rabbit Skeletal Muscle 101

17. Total Activities of Acid Phosphatase in Liver . 103

18. Catheptic Activity (pH 3.8) in Rabbit Liver ... 104

19. Total Activities of β-Glucuronidase in Liver .. 105

20. Neuraminidase Activity in Rabbit Liver 106

21. Lysophosphatidase Activity in Rabbit Liver 107

22. Acid Phosphatase Activity in Spleen 110

23. β-Glucuronidase in Spleen 111

24. Lysophosphatidase Activity in Spleen 112

25. Distribution of Protein in Liver Subcellular Fractions 114

26. Distribution of Acid Phosphatase in Liver Subcellular Fractions from Dystrophic and Control Rabbits 115

27. Distribution of Cathepsin (pH 3.8) in Liver Subcellular Fractions from Dystrophic and Control Rabbits 117

28. Distribution of β-Glucuronidase in Liver Subcellular Fractions from Dystrophic and Control Rabbits 119

29. Distribution of Neuraminidase in Liver Subcellular Fractions from Dystrophic and Control Rabbits 121

30. Distribution of Lysophosphatidase in Liver Subcellular Fractions from Dystrophic and Control Rabbits 123

31. *In Vitro* Hemolysis of Rabbit Erythrocytes by Hydrogen Peroxide and the Inhibition of Hemolysis by Added Tocopherol 126

32. Protein, Nitrogen and Non-Collagen Nitrogen of Rabbit Skeletal Muscle 128

33. Intracellular Distribution of Neuraminidase in the Cerebrums of Encephalomalacic and Control Chicks 130

34. Intracellular Distribution of Acid Phosphatase in the Cerebrums of Encephalomalacic and Control Chicks 131

35. Intracellular Distribution of Sialic Acid Compounds in Chicken Cerebrums 132

36. Distribution of Lipophilic Sialic Acid Compounds in Liver Subcellular Fractions from Rats 134

37. Inhibition of Lysophosphatide-induced Hemolysis of Rabbit Erythrocytes by Plasma 137

38. Lysophosphatidase Activity in Rabbit Leucocytes 141

39. Leucocytes in Vitamin E-Deficient and Control Rabbits 142

40. β-Galactosidase Activity in Leucocytes of Vitamin E-Deficient and Control Rabbits 146

41. β-Galactosidase in Skeletal Muscle of Vitamin E-Deficient and Control Rabbits 147

42. Acid Hydrolases in Alveolar Macrophages of Rabbits 148

LIST OF ILLUSTRATIONS

Figure		Page
1.	Rate of Disappearance of Carbon C-14 from the Blood of Injected Rabbits	67
2.	Plot for the Determination of the Phagocytic Index (K)	68
3.	Phagocytic Index (K) Values as a Function of Days on Diet	70
4.	In Vivo Uptake of Carbon C-14 by Skeletal Muscle of Rabbits as a Function of Days on Diet	81
5.	Acid Phosphatase Activity of Rabbit Skeletal Muscle as a Function of Days on Diet	85
6.	Cathepsin Activity at (pH 3.8) of Rabbit Skeletal Muscle as a Function of Days on Diet	87
7.	β-Glucuronidase Activity of Rabbit Skeletal Muscle as a Function of Days on Diet	89
8.	Neuraminidase Activity of Rabbit Skeletal Muscle as a Function of Days on Diet	91
9.	Lysophosphatidase Activity of Rabbit Skeletal Muscle as a Function of Days on Diet	93
10.	Distribution Patterns of Neuraminidase in Skeletal Muscle of Rabbits	97
11.	Distribution Patterns of Cathepsin at (pH 3.8) in Skeletal Muscle of Rabbits	99
12.	Acid Hydrolases in Liver as a Function of Days on Diet	108
13.	Total Activities of Acid Hydrolases in Livers as a Function of Days on Diet	109

14. Acid Hydrolases in Spleen as a Function of Days on Diet 113

15. Distribution Patterns of Acid Phosphatase in Rabbit Livers 116

16. Distribution Patterns of Cathepsin (pH 3.8) in Rabbit Liver 118

17. Distribution Patterns of β-Glucuronidase in Rabbit Liver 120

18. Distribution Patterns of Neuraminidase in Rabbit Liver 122

19. Distribution Patterns of Lysophosphatidase in Rabbit Liver 124

20. Effect of Leucocytes on the Lysophosphatide-induced Hemolysis of Rabbit Erythrocytes 127

21. Leucocytes as a Function of Days on Diet 143

BIOCHEMICAL STUDIES OF LYSOSOMAL ENZYMES IN *EXPERIMENTAL MUSCULAR DYSTROPHY*

CHAPTER I

INTRODUCTION

A clear knowledge of cellular organization from electron microscopic studies correlated with molecular structure of cells is of primary importance to the understanding of the functioning of the cell. The intracellular components are considered to be in a steady state with continuous degradation and resynthesis of molecules requiring highly efficient catalysts, the enzymes. The enzymic patterns of individual mammalian tissues are varied within limits established by heredity where the adaptive enzyme systems are maintained at characteristic levels in vivo by the positive and negative feed back mechanisms. If the energy supply is cut off, the whole system runs down by loss of the unstable essential substances and by the predominance of catabolic enzyme processes which leads to autolysis of the cell. When these processes have gone sufficiently far enough, they become irreversible because the synthetic reactions cannot be resumed in the absence of the essential catalysts. By a series of enzymic transformations, the substances which serve as the initial source of energy are converted to a form which can undergo certain reactions with the formation of energy-rich bonds. The chemical energy of these bonds

may be temporarily stored as in creatine phosphate of muscle where large sudden demands for mechanical work may be made at any moment. The energy may be used immediately for biosynthesis. The two main forms in which the energy is stored are as pyrophosphate bonds in nucleoside triphosphates, especially ATP, or as phosphoamide bonds in phosphocreatine or phosphoarginine. The content of phosphate acceptors which can act as energy reservoirs varies considerably in different tissues. Skeletal muscle differs from most other tissues in having a fairly large content of adenosine phosphates and creatine. Many of the energy-requiring reactions are driven by the breakdown of ATP. Muscular contraction depends on the breakdown of ATP which is believed to be due to the action of calcium-activated adenosine triphosphatase (ATP-ase) of the myofibrils. The details of this mechanism have not been fully elucidated. The enzymes which catalyze all these reactions must be produced by biosynthesis following the patterns of other proteins. Although systems of many enzymes work well in simple solutions, a living cell cannot be considered as a bag of enzymes in a homogenous solution. In fact, the living cell is an elaborate structure and the enzymes are specifically situated in particular intracellular particles. The study of the relationship between the intracellular structures and enzyme systems have been very helpful in the elucidation of several biochemical mechanisms.

A typical cell is surrounded by a membrane which is permeable to certain ions and molecules. The membrane appears as two layers with pores in the outer layer open to the cytoplasm whereby molecules can move in either direction. The contents of a cell may be divided into two main compartments, the nucleus and the cytoplasm which contains various partic-

ulate structures among which the following are recognized: mitochondria, lysosomes (identified in liver, brain and kidney cells), microsomes, Golgi apparatus, fat droplets, granules of metabolic products, phagocytic inclusions and the endoplasmic reticulum. Some of these cytoplasmic components have been recognized as highly organized structures, for example, the mitochondria and the endoplasmic reticulum.

Biochemical studies of the intracellular components have been achieved by separating particulate fractions from tissue homogenates or disrupted cells followed by differential centrifuging. Three successive steps are involved for the performance of a fractionating experiment. The first step is to convert a tissue or cell suspension into a homogenate. The second step is to introduce a new order into the system by grouping together in separate fractions those components of the homogenate having certain physical properties falling between specific limits set by the investigator such as density or sedimentation coefficient. The final step is the biochemical analysis of the isolated fractions. In performing these experiments it is often assumed that enzymes are localized in a single intracellular site and that the population of subcellular organelles are enzymically homogeneous. These assumptions may not be valid although they helped in elucidating many enzymic reactions. It should be emphasized that most animal tissues include more than one type of cells which may differ in their enzymic contents. Experiments with tissue homogenates will merely give an average of all the cell types present. This is particularly true of liver, in which the parenchymatous and the reticuloendothelial cells are present throughtout the tissue. Similarly, cells from different tissues do not necessarily have the same enzyme systems, enzymes

which are present in a certain tissue may be absent in another. For example, D-amino acid oxidase, xanthine oxidase, and catalase are present in liver but they are practically absent in muscle. Likewise, aldolase and citrate condensing enzyme (citrate oxalacetate-lyase) are predominantly found in muscle but have low activities in liver. Different muscles also have differences in their enzymic content, for example, aldolase is more active in skeletal muscle than in cardiac muscle while citrate condensing enzyme is more active in cardiac muscle than in skeletal muscle.

The aim of this project is to study the levels of certain lysosomal hydrolases which have been associated with the muscular dystrophy. Detailed studies of the intracellular distribution of these enzymes and evidences to explain the reasons for the enzymic changes in dystrophic muscle will be presented. In these studies, the cellular heterogeneity of muscle tissues have been considered. Muscles appear surrounded by a sheath of connective tissue called the epimysium surrounding the larger bundles, the perimysium surrounding the smaller bundles and the endomysium surrounding the individual muscle fibers. The amount of connective tissue relative to muscle fibers is variable in different muscles. The elements composing the connective tissue of muscle are collagen fibers, elastic fibers, and several varieties of cells such as fibroblasts, macrophages, fat cells and a few leucocytes. Healthy muscle connective tissue is essential for muscle regeneration. According to Mason et al. (1), the regenerative process in vitamin E-deficiency state is dependent on the survival of muscle nuclei and the investing sarcoplasm together with a healthy endomysial connective tissue and a functional circulation in the area of regeneration. If any of these requirements become defective, then

the affected area becomes ischemic and whatever muscle nuclei that may have escaped the initial damage will die.

Muscle fibers are comparable to cells from other tissues. Each muscle fiber is surrounded by a thin membrane, 0.1 u thick, which is called the sarcolemma. A number of agents can cause swelling of the sarcolemma with its subsequent rupture but it is resistant to trypsin digestion. The function of this membrane has been implicated in transmitting the contractile force of muscle fibers to the connective tissue and tendons. The contents of the muscle fiber may be visualized as composed of two main compartments: the nuclei and the sarcoplasm (the cytoplasm of muscle fibers). Muscle fibers are multinucleated. Embedded in the sarcoplasm are the myofibrils which are essential in muscle contraction. There are other sarcoplasmic organelles and inclusions among which the following are recognized; mitochondria or sarcosomes, glycogen granules and fat droplets. The presence of particles analogous to lysosomes of liver cells have not been demonstrated. Granules similar to lysosomes have been demonstrated in macrophages and leucocytes (2,3). These cells are present in the connective tissue surrounding muscle fibers. The fibroblasts which are the predominant cells in connective tissue have been shown to contain most of the acid hydrolases associated with lysosomes in a high concentration along with other non-hydrolytic enzymes (4). The following enzymes have been demonstrated in fibroblasts: β-glucuronidase, acid phosphatase, aryl sulfatase, proteolytic prolidase, prolinase, catalase, peroxidase, cytochrome c reductase, lactic dehydrogenase, malic dehydrogenase, glutamic-oxalacetic transaminase and alkaline phosphatase. According to Berliner and Dougherty (4), the fibroblasts are destroyed after the administration of

a mild inflammatory stimulous and that a variable number of leucocytes and macrophages enter the inflamed area. This observation will be discussed in detail in a later section.

Lysosomes as a Biochemical Concept

As a result of the work of Schneider (5), Hogeboom (6), Claude (7), Dounce (8), Palade (9) and others, a scheme of cell fractionation has been adopted giving four fractions: nuclear and debris, mitochondrial, microsomal, and the final supernatant. This scheme is based on the assumption that the particulate components of tissue homogenates have different sedimentation coefficients and densities. De Duve et al. (10) pointed out that since the sedimentation coefficient is in itself a function of variables (size, shape, density of the particles, density and viscosity of the suspending medium) the separability of the particles is dependent on their physical properties as well as the composition of the surrounding medium. A relationship between these variables and the sedimentation of particles can be illustrated by the Stokes equation for sedimentation of a sphere (11).

$$dR/dt = 2/9\ r^2\ (d_p - d_m)\ G/N$$

dR/dt is the distance traveled in the direction of the centrifugal field in a unit time (cm/sec.), d_p is the density of the particles, d_m is the density of the medium (both densities are in grams/cc), r is the radius in cm., N is the viscosity of the medium in poises, G is the gravitational field = w^2r (w is the angular velocity), and the constant 2/9 is the shape factor for a sphere.

From the integrated Stoke equation, it can be recognized that the time required for a particle at the top of the tube to reach the bottom

is directly proportional to the viscosity of the medium.

$$t = 9/2\ n/\ w^2r^2(d_p - d_m) \cdot \ln R_{max}/R_{min}$$

Where R_{max} is the radial distance to the bottom of the tube, R_{min} is the radial distance to the miniscus. It has been pointed out by De Duve et al. (10) that the efficiency of separating two classes of particles by a single sedimentation is usually low. In the time required for complete sedimentation of the heavier particles, some of the lighter particles will also have reached the bottom and will contaminate the sediment. There are other variables which are not included in the Stoke equation but necessary for the sedimentation of mixed classes of particles such as the concentration of the suspension. Density gradient centrifuging method (12, 13) has been used by several workers to separate closely related mixed classes of particles. The particles are centrifuged in a density gradient until they reach a position where their own density approaches that of the medium and their rate of movement has become infinitely low. A detailed mathematical treatment on density gradient centrifugation has been reviewed by De Duve et al. (10). This critical evaluation of differential centrifugation techniques has led De Duve and coworkers to the discovery of new particles in the cytoplasm which have been termed lysosome (14). Studies on the intracellular location of acid phosphatase by using the classic differential centrifugation technique of Schneider and Hogeboom (15) and Palade (16), have shown that the enzyme is localized in high concentration in the mitochondrial fraction. By using a modified technique, Novikoff et al. (17) and Tsuboi (18) have shown that the enzyme is concentrated in the microsomal fraction. To clarify this discrepancy, De Duve et al. (14) repeated the experiments on the localization of acid phosphatase while recog-

nizing the many variables in sedimentation and obtained a light mitochondrial and microsomal fractions. This designation was thought to be adequate to explain the heterogeneity of the mitochondrial fraction. However, it was observed that other hydrolytic enzymes are present in high concentration and that no cytochrome oxidase can be demonstrated in the light mitochondrial fraction. Because of the special nature of this fraction, De Duve (23) suggested the name lysosome, a biochemical designation referring to the fraction's high concentration of hydrolytic enzymes. The following enzymes were reported to be concentrated in the light mitochondrial (lysosomal) fraction: acid phosphatase, acid deoxyribonuclease, acid ribonuclease, β-glucuronidase and cathepsin. Following the work of De Duve et al. (14) other hydrolytic enzymes have been observed to be localized in the lysosomal fraction among which; aryl sulfatases A and B (19), β-galactosidase (20), α-mannosidase (20), lysozyme or muramidase (22), beta N-acetyl glucosaminidase (20) and phosphoprotein phosphatase (21). In this report Neuraminidase and a lysophospholipid destroying enzyme will be shown to be associated with the lysosomal fraction.

All the identified enzymes in the lysosomal fraction favor an acidic pH optimum. They are able to hydrolyse nucleic acids, proteins and carbohydrates. The identification of a lipid-hydrolyzing enzyme will be of significant interest. A common property to all lysosomal enzymes is that they exhibit no activity towards external substrates as long as they remain associated with the intact particle. This property of lysosomal enzymes was attributed to the structure of the lysosomal particle or to the nature of the lysosomal matrix or membrane. If hydrolytic enzymes are free to act within the living cell, then they would lower the efficiency

of the synthetic systems and might interfere with the structural integrity of the cell as a whole. Intracellular segregation of hydrolases constitutes one of the means which controls and localizes the hydrolytic activity at specific sites of the cell.

A schematic representation of De Duves (23) lysosomal concept is shown below. The author supports a membrane-like barrier hypothesis to explain the structure-linked latency and activation of lysosomal enzymes.

Acid hydrolases are surrounded by a lipoprotein membrane restricting the physical freedom of the enzymes and substrate accessibility.	----------→ (Disrupting Agents)	Ruptured membrane. Enzymes released.
(Intact lysosome)		(Ruptured lysosome)

It has been reported that there are several means which contribute to the release of lysosomal enzymes; these being mechanical breakage in a Waring blender, osmotic rupture in media of low tonicity, physicochemical disruption by repeated freezing and thawing, exposure to surface active agents, such as Triton X-100, saponin, or deoxycholate; thermal activation which occurs at 37°C., lowering of the pH, sonic vibrations, enzymic actions of proteases and the lecithinase phospholipase C from C. Welchii (24). It is assumed that all these treatments act by causing a primary injury to the particle membrane which allows either the substrates to be accessible to the internal enzymes or the internal enzymes to be released to act on the substrates. It was further suggested that these phenomena may account for the simultaneous activation and solubilization of bound hydrolases. An alternative hypothesis has been proposed by Koenig (134). This proposal suggests that lysosomes are composed of macromolecules of enzyme and

non-enzymic protein conjugated to acidic glycolipids by ionic, covalent, and/or other bonds. The essential inertness of acid hydrolases *in situ* has been viewed by the author (134) as being derived from their sequestration within 'solid' glycoprotein complexes. Release or activation of the hydrolases is visualized as an attenuation or cleavage of glycoprotein-enzyme bonds, rather than a disruption of a membranous envelop. Hypotonic media, lipid solvents, and surface active agents dissolve the glycolipid or protein moieties or both. Freezing and thawing, blenderizing and ultrasonic irradiation are thought to bring about a physical fragmentation of lysosomes. The releasing action of thermal activation and proteases suggests that non-enzyme protein is requisite to the physical integrity of lysosomes and their binding of hydrolases. It was found by Koenig and Jibril (135) that cationic molecules as diverse as a basic dye, methylene blue, a polyamine, an amidine, basic proteins and phenothiazines were effective in releasing bound lysosomal hydrolases *in vitro* in brain, liver and kidney preparations. Neuraminidase from *Vibrio cholerae* was also found to activate 30-70% of the latent acid phosphatase in brain mitochondrial-lysosomal preparations, but only 3-5% of that in liver and kidney preparations. Furthermore, neuraminidase activated latent β-glucuronidase and acid deoxyribonuclease to a similar extent as acid phosphatase with a concurrent increase in the unsedimentable (soluble) activity. These findings support the concept that brain lysosomes consist of a structural matrix of glycolipoproteins in which the hydrolytic enzymes are retained in a latent state by ionic bonds. N-actylneuraminic acid appears to be the anionic site for the binding of cationic groups of the enzymes and other substances. The ionogenic molecules in non-neural lyso-

somes may include cerebroside sulfate.

The hypothesis that lysosomes belong to a distinct class of particles has been supported by the biochemical studies on the isolated lysosomal fraction. An undisputed morphological identification of lysosomes has not yet been achieved because of the apparent heterogeneity of these particles. It has been shown that within each group of lysosomes there is some degree of heterogeneity in their enzymic content, and that enzymically different lysosomes may be expected among different tissues (23). Different cytological bodies described by electron microscopic studies, such as microbodies, cytosomes, peribiliary bodies, lipofuscin granules, phagocytic vacuoles, microkinetosphares, ferritin-rich granules, specific granules of polymorphonuclear leucocytes, zymogen-like granules containing newly synthesized enzymes, pinocytic or phagocytic vacuoles at various stages of their evolution and the residual bodies that contain the remnants of the digested material, may be identical with lysosomes. Novikoff, Beaufay, and De Duve (25) examined the lysosome-rich fraction by the electron microscope and reported the presence of a large number of dense bodies with a single outer membrane. It was also observed that these electron opaque granules were absent in fractions with a low content of lysosomes and that the presence of ferritin was the cause of electron opacity. Beaufay et al. (26) demonstrated the presence of ferritin in the lysosome-rich fraction in the form of an easily detachable iron. About ten percent of the total cell ferritin was reported to be associated with lysosomes or related particles. Palade (27) suggested that ferritin molecules are picked up by the glomerular epithelial cells in pinocytic vacuoles and transported to larger vacuoles which are subsequently transformed into dense

bodies by progressive condensation. Likewise, Bennedetti et al. (28) reported that ferritin accumulates in vacuoles or granules that resemble lysosomes. It is also reported by many workers that dense bodies lacking the ferritin-like granules are present in the lysosomal fraction. It is possible, however, that several morphologically distinct entities may have been included within the biochemical concept of lysosomes. This may explain the heterogeneity of lysosomes, the presence or absence of iron particles, the difference in the enzymic content and the differences in their sensitivity to osmotic disruption. One approach to the elucidation of these phenomena was attempted by De Duve et al. (13) by analyses of mitochondrial fractions from rat liver by density gradient centrifugation. They reported that the five lysosomal hydrolases; acid phosphatase, deoxynuclease, acid ribonuclease, beta glucuronidase, and cathepsin, show typical differences from the mitochondrial group. The distribution curves of these lysosomal hydrolases were similar but not identical, indicating that if lysosomes form a single species, they are enzymically heterogeneous. It is possible that such differences reflect the existence of distinct classes of lysosomes, perhaps as many as there are lysosomal enzymes. It is equally possible that these particles are intrinsically heterogeneous with respect to their enzymic content, mode of formation, and breakdown within the cell.

The intracellular distribution of uricase is similar to the distribution of acid phosphatase. However, uricase is fully active in preparations of intact particles and it is not solubilized by most of the treatments which release the lysosomal hydrolases (29). It is assumed that uricase is either attached to the insoluble framework of lysosomes or it

may belong to a fourth group of granules with the properties of large microsomes. On the basis of density gradient centrifugation experiments, uricase appears to have a different distribution pattern.

Another type of granule, the microbodies, were reported to be rich in D-amino acid oxidase and catalase (30). The sedimentation behavior in 0.25 M sucrose was found to be similar to that of acid phosphatase. For this reason, it has been clained that these enzymes are associated with the lysosomes. Beaufay et al. (31), however, have shown from density gradient centrifuging experiments that uricase, D-amino acid oxidase, and catalase have different distribution patterns than acid phosphatase. The authors suggest that these enzymes are contained in sublysosomal particles and they prefer the terms uricase-containing particles, catalase-containing particles, and D-amino acid oxidase-containing particles rather than lysosome. They also report that the isolated lysosomal preparation contains a mixture of lysosomes and of particles containing uricase, D-amino acid oxidase, and catalase but was practically free of mitochondria. According to De Duve (23), these sublysosomal particles have a major role in the protective mechanism against radiation damage.

Lysosomes in Macrophages and Leucocytes

A new problem was brought to light from the work of Strause (32) where he injected peroxidase and found it to be concentrated in subcellular organelles similar to lysosomes. Strause named these new organelles phagosomes. Since their behavior was found to be very much like lysosomes, it was suggested that phagosomes may be a result of fusion of lysosomes with phagocytized particles. The procedures that cause the release of lysosomal hydrolases were also found to release the phagocytized per-

oxidase. There are several reports based on histochemical observations that phagocytic vacuoles possess a high acid phosphatase activity as well as other lysosomal hydrolytic enzymes. Phagocytic vacuoles are present in rat erythrophagocytes, macrophages of liver, spleen, lung, uterus and other tissues. Cells that contain phagocytic vacuoles are generally accepted as members of the reticulo-endothelial system. According to Doyle (33), most of the acid phosphatase activity in spleen is found in the macrophages. De Duve et al. (34) recognized the cellular heterogeneity of liver and performed experiments to isolate the reticuoendothelial cells from the parenchymatous cells by a technique based on the ability of macrophages to engulf colloidal iron (35, 36) and separated them from the liver suspension by a powerful magnet. It has been shown that macrophages contain all the (lysosomal) hydrolases investigated and that the intracellular distribution of these hydrolases was similar to those found in parenchymatous cells indicating that lysosomes are present in macrophages (34). These observations and others prompted the speculation that lysosomes are involved in the intracellular digestion and the engulfing process (37, 22).

The processes involved in tissue regression are assumed to be related to the phenomenon of intracellular digestion. The histochemical studies of these tissues have demonstrated that the acid phosphatase granules were either ruptured or enlarged. These observations were reported in the regressing tissues of the tail of the metamorphosing tadpole and in the keratinizing cells of the rat esophagus. The thyroid hormone has been implicated in initiating these changes which are associated with large increases in total and free activities of cathepsins (38).

The relationship between the engulfing processes such as phagocyto-

sis or pinocytosis and lysosomes have been reported by experiments on the polymorphonuclear leucocytes in peritoneal exudates of rabbits. Cohn and Hirsch (22) demonstrated that these cells possess few mitochondria and a large number of specific granules. It was suggested that these granules are lysosomes because they possess a high acid phosphatase activity as well as other hydrolytic enzymes characteristic of lysosomes. Furthermore, the lysosomal nature of these granules is indicated by the effect of low pH, freezing and thawing, and the action of saponin which releases the granule-bound enzymes.

One of the functions of phagocytic leucocytes is their ability to ingest invading microorganisms and digest them. Cohn and Hirsch (39) reported that following phagocytosis of microorganisms, the polymorphonuclear leucocytes become degranulated and their hydrolytic enzymes are released. It is proposed that a bactericidal agent, phagocytin, and the released enzymes interact with the ingested bacteria either in the cytoplasm or within the phagocytic vacuole where digestion of the bacteria is accomplished. In vitro experiments indicated that the proportion of acid phosphatase and β-glucuronidase bound to the granules is decreased while the proportion of these enzymes increases in the supernatant. No change in the total enzymic activity was observed. However, the experiments indicate that during active phagocytosis a mechanism is initiated to rupture the granules and cause the release of their hydrolytic enzymes. Active phagocytosis requires expenditure of energy derived entirely from glycolysis (40). It has been suggested that after fragmentation of the ingested bacteria by the lysosomal enzymes, the fragments may be disposed of by oxidative processes. These processes may result in an accumulation

of NADH. It has been demonstrated by Karnovsky (41) that the specific granules of the polymorphonuclear leucocytes contain a non-cytochrome-linked NADH oxidase. This enzyme was found to be released in the same manner as the hydrolytic enzymes characteristic of lysosomes. The solubilized form of the NADH oxidase was found to have a broad pH optimum at about 4.8 and exhibited a ten-fold preference for NADH over NADPH. It has been suggested that the metabolic changes occurring during active phagocytosis are attributed to the release of NADH oxidase. The granules rupture as a result of a decrease in intracellular pH when glycolysis is stimulated. Pyruvate could then become available to reoxidize NADPH through the mediation of NADPH lactate dehydrogenase (42) and thus stimulating the hexose monophosphate shunt due to increased availability of NADP. The possibility exists, however, that NADH oxidase is contained in different granules than lysosomes similar to the intracellular localization of catalase and uricase. This enzyme may be considered as a disposal agent in the same sense as the hydrolytic enzymes. This is true if its involvement in intracellular digestion is confirmed by taking part in the oxidative processes for the disposal of molecular fragments resulted from the action of the hydrolytic enzymes. An active NADPH-generating system in polymorphonuclear leucocytes have been observed (43) and that NADH oxidase is involved in lipid synthesis. Actively phagocytizing cells were found to incorporate P-32 rapidly into their phospholipids under aerobic conditions. The dramatic increases of P-32 incorporation was found in phosphatidic acid, phosphatidyl inositol and phosphatidyl serine but no significant increases were observed in phosphatidyl ethanolamine, lecithin, or sphingomyelin.

Vannotti et al. (44) studied the metabolic pattern of phagocytizing leucocytes and observed that the mature cell is especially sensitive from the enzymic point of view to the various influences of its medium. Leucocytes can modify their enzymic reactions under the influence of the endocrine system. Changes in their metabolic patterns have been observed in reponse to estrogens, cortisone and thyroxine. In addition to the phagocytic function of leucocytes, they are believed to function as carriers of enzymes which would be liberated in tissues at the moment of their destruction. The released enzymes are assumed to provoke inflammatory reactions in situ which will stimulate proliferation of other cells that take part in the inflammatory processes. It has been postulated that the released enzymes are lysosomal in nature thereby linking the inflammatory processes with lysosomes. If this is true, then it should be expected that anti-inflammatory agents, for example, cortisone, must demonstrate inhibitory effects on the release of lysosomal enzymes. An indirect evidence has been presented by Benacerraff (45) where it was demonstrated that large doses of cortisone depressed the intracellular digestion of liver Kupffer cells.

Role of Lysosomes in Pathological Autolysis

If the hypothesis that the isolation of intracellular hydrolases within lysosomes is correct, then it will provide an explanation for the fact that living cells are not attacked by their own hydrolytic enzymes. Rupture of lysosomes should be expected in massive cellular autolysis. Several authors attempted to demonstrate this hypothesis. De Duve et al. (46) demonstrated a rapid release of five lysosomal enzymes in liver tissues rendered completely ischemic by ligation of the blood vessels. Simi-

lar results have been found with respect to acid phosphatase and β-glucuronidase in autolysing mouse liver fragments according to Van Lancker et al. (47). Histochemical studies of regressing tissues demonstrated that acid phosphatase-containing particles (lysosomes?) are either enlarged or ruptured (48). Release of acid phosphatase and cathepsin after carbon tetrachloride poisoning have been reported by Martini and Dianzani (49). Beaufay et al. (50) reported that an extensive lysosomal damage was present in livers of rats rendered comatose by starvation, with or without carbon tetrachloride poisoning. Release of lysosomal enzymes was also observed by the administration of necrotizing diets. It should be mentioned that these experiments did not prove that lysosomal rupture actually preceded the cellular death and did not exclude the possibility that lysosomal rupture occurred as a consequence of cellular death. In other words, we do not know whether lysosomes can be both a cause and/or an effect of cell disintegration.

An interesting observation has been reported by Thomas et al. (51). These authors found that a metachromatic material disappeared from the cartilage of young rabbits fed excessive amounts of vitamin A. It was suggested that the degradation of the cartilage matrix under the influence of hypervitaminosis A might be due to an enhanced proteolytic activity of the chondroblasts. Dingle et al. (52) concluded that this was true and indicated that the degradation is associated with the release of a protease from lysosomes. This conclusion, however, was based on the observation that excess vitamin A alcohol caused the rupture of lysosomes from rat livers in vitro.

Bacterial endotoxins have also been shown to cause a release of lyso-

somal enzymes *in vitro* and the pathogenic alterations causing the death of injected animals were attributed to the uncontrolled release of lysosomal enzymes *in vivo* (53). The authors, however, could not rule out the possibility of other factors not related to lysosomal enzymes.

In a report by Gallagher (54) protection against carbon tetrachloride poisoning by vitamin E has been shown by histological means in rat liver. Because of the known antioxidant property of vitamin E, it was suggested that the vitamin functioned in this form of poisoning to protect the cellular lipids from oxidation (hypothetically called for) which might have resulted from carbon tetrachloride or its metabolite. However, it has been shown by Smuckler *et al.* (55) that in early carbon tetrachloride poisoning there was a dislocation of ribosomes from the lipoprotein membranes which caused the impairment of protein biosynthesis in liver. These workers suspected that the observed phenomenon was due to lipid peroxide formation in the lipoprotein membranes. After testing this possibility by the thiobarbituric acid reaction, it was concluded that lipid peroxides do not play a role in the early phase of carbon tetrachloride poisoning and therefore the protective action of vitamin E in this form of poisoning could not be attributed to its antioxidant property.

In evaluating the results of the various observations on the role of lysosomes in pathological autolysis of tissues, it must be emphasized that only indirect evidence has been presented. The role of other subcellular organelles in pathological autolysis must also be considered. The following conditions may be helpful in evaluating the *in vivo* role of lysosomes in cellular death: 1. The cells of tissues under investigation must contain lysosomes. 2. The intracellular release of the lysosomal hydrolases

must be the primary cause of cellular death. 3. The disrupting agents of lysosomes must be reasonably specific for these subcellular organelles. 4. The disrupting property of certain agents *in vitro* must be correlated with *in vivo* studies under physiological conditions. The importance of these conditions will be emphasized when the relationship between lysosomal hydrolases and muscular dystrophy is evaluated.

Role of Acid Hydrolases in Muscular Dystrophy

Several investigators observed a significant elevation in the activities of acid hydrolases (lysosomal?) in skeletal muscles of dystrophic animals regardless of the cause of dystrophy. Increased cathepsin activity in skeletal muscle of dystrophic rabbits suffering from vitamin E-deficiency has been observed by Weinstock *et al.* (56). Increased proteolytic and autolytic activities in skeletal muscle of dystrophic hamsters maintained on vitamin E-deficient diet has been reported by Mason *et al.* (57). Tappel *et al.* (58) reported increases in the activities of some acid hydrolases in muscles of genetically dystrophic chicken and later (59) they have shown that β-galactosidase, cathepsin, aryl sulfatase, and acid ribonuclease activities increase several fold in leg muscles of dystrophic rabbits suffering from vitamin E-deficiency. McCaman (60) reported significant increases in β-glucuronidase and peptidase activities and a slight increase in acid phosphatase activity in dystrophic skeletal muscle from mice with an inherited muscular dystrophy. It was further observed that the pattern of enzyme activities in normal muscle denervated by nerve section was similar to that in dystrophic muscle. Jasmin *et al.* (61) demonstrated by histochemical means a high activity of leucine amino peptidase and a cathepsin in dystrophic skeletal muscle of mice suffering

from dystrophia muscularis. This activity could not be demonstrated in normal muscle. According to these authors, the enzymic activity appeared to be confined in the interstitial connective tissue surrounding the muscle fibers and no activity could be demonstrated inside the muscle fibers. Golarz and Bourne (62) reported that various phosphatases, including acid phosphatase, were increased moderately in human dystrophic muscle using histochemical techniques. In later reports, Bourne et al. (63) demonstrated high activities of heat stable adenosine triphosphatase and adenylic acid phosphatase in the connective tissue (endomysium) proliferating between muscle fibers of human dystrophic muscle. The authors could not demonstrate these enzymic activities in normal human muscle.

Most of the acid hydrolases mentioned above may be considered lysosomal enzymes on the basis of their intracellular distribution in liver cells according to De Duve et al. (23). Demonstration of acid hydrolases in muscle tissue is not a sufficient evidence that lysosomes are present in muscle fibers since muscle tissue is a composite of several types of cells. However, Tappel et al. (59) presented a hypothesis attributing the primary cause of muscular degeneration (dystrophy) to the lability of the membranes of lysosomes (of muscle fibers?) and leakage of the bound enzymes into the muscle cytoplasm. It was further suggested that the first consequence of vitamin E-deficiency may be a free radical damage to the lipoprotein membrane of the cell and its subcellular organelles. Free radicals are thought to be produced in the preliminary phases of the peroxidation of unsaturated lipids and that a primary function of vitamin E may be to inhibit such lipid peroxidation in the tissues of animals, applying particularly to the lipoprotein membranes which contain highly unsatu-

rated fatty acids. This hypothesis is dependent on an increased lipid peroxide formation *in vivo*. The authors presented an evidence of *in vivo* lipid peroxidation based on the thiobarbituric acid reaction for the detection of malonaldehyde, a secondary product of lipid peroxidation. This method, however, has been criticized by several workers (64). A number of compounds present in animal tissues can react with thiobarbituric acid (65) producing color with the same spectra as malonaldehyde. Caputto *et al.* (66) tried a number of different chemical methods to detect lipid hydroperoxides and could not demonstrate an accumulation of such lipid hydroperoxides in tissues of vitamin E-deficient rats and rabbits.

The Vitamin E Deficiency Syndromes

Excellent reviews on the chemistry and biochemistry of vitamin E are available (64, 67) discussing the possible function or functions of the vitamin. There are several hypothesis on the mode of action of vitamin E in the living cell but as yet the mechanism of its action remains unknown. One approach to the study of its action has been the examination of the effect or effects of vitamin E deficiency state on the whole animal. The pathological disorders produced by vitamin E deficiency are varied and represent morphological alterations in a number of unrelated tissues. Development of these lesions seem to be dependent on the fat content in the experimental diet and their onset and severity seems to be accentuated in proportion to the amount and degree of unsaturation of fats used. Different species of animals seem to respond differently to vitamin E deficiency.

The classical symptom of vitamin E deficiency is considered to be the reproduction failure in rats. This disorder is not prevented by omission

of easily autoxidizable fat in the diet (68). Nutritional muscular dystrophy constitutes the most universal manifestation of vitamin E-deficiency in various species and signifies the vitally important, but as yet unknown, function of tocopherol in the metabolism of skeletal muscle. Muscular degeneration can be produced in rabbits, guinea pigs, hamsters, young and adult rats, cattle, monkeys, mink, cats, mice, and in chicks. Blaxter et al. (69) produced muscular dystrophy in calves by feeding diets rich in cod liver oil and deficient in vitamin E. These symptoms could not be prevented by ascorbic acid, ethyl gallate or biotin. Creatinurea is considered one of the earliest detectable signs of nutritional muscular dystrophy and precedes the gross physical and histopathological signs of muscle damage. Mackenzie et al. (70) found that creatinurea in rabbits can be counteracted by feeding tocopherylquinone and the corresponding hydroquinone. The authors discussed the activity of these substances in relation to their participation in an oxidation-reduction system in the body. It was further suggested that α-tocopherol may be converted into the hydroquinone form in vivo and therefore α-tocopherol functions as a provitamin for the hydroquinone. However, hydroquinone could not be detected in animal tissues (70). In chicken, the deficiency state is manifested in encephalomalacia and exudative diathesis and some protection against encephalomalacia. These symptoms are also counteracted by antioxidants such as antabuse and sulfaguanidine (71). According to Dam (72) exudative diathesis does not occur when fat is not present in the diet. Both cod liver oil and lard may provoke the subcutaneous edema called exudative diathesis. The affected tissues show edema, hyperemia, and increased permeability of the capillaries as indicated by increased absorp-

tion of intravenously injected Trypan blue (73). Nutritional encephalomalacia has been described by Peppenheimer et al. (74). This disorder is characterized by motor incoordination, ataxia, head retraction, tremors, prostration with legs spastic and claws strongly flexed. The cerebellum is more severely affected than other parts of the brain.

There are other symptoms or disorders which result from vitamin E deficiency. For example, massive liver necrosis, hemolysis of erythrocytes, lung hemorrhage, degeneration of convoluted tubules of kidneys and depigmentation or rat incisors. The fact that a vitamin E deficiency influences such a wide variety of animal tissues has tended to implicate its function to some basic mechanism common to most cells. The most prominent possibilities for the mechanism of action of vitamin E in the living organism are the following: 1. It functions as an intracellular antioxidant by preventing the oxidation of unsaturated fats and other oxygen-sensitive substances such as vitamin A and ascorbic acid. 2. It functions as a component in the cytochrome c reductase portion of the terminal respiratory chain. 3. It may function in a primary role in nucleic acid metabolism and possibly in phosphorylation reactions.

In support of the antioxidant property of vitamin E, it was reported by Draper et al. (75) that some synthetic antioxidants and other natural antioxidants than tocopherols can give partial protection against certain symptoms of vitamin E deficiency. Examples of these antioxidants are: methylene blue, N,N'-diphenyl-p-phenylenediamine (DPPD), antabuse, ascorbic acid, cystine and others. However, Green et al. (76) attributed this partial protection of these antioxidants to the sparing action of residual tocopherol levels which are otherwise insufficient for protection. These

workers demonstrated that the tocopherol-deficient diet which have been used in the feeding tests still contain small amounts which were considered to be physiologically active under the sparing influence of the antioxidants added to the diet.

CHAPTER II

MATERIALS AND METHODS

Materials

Amorphous Carbon C-14

Amorphous carbon C-14 in a powder form was purchased from New England Nuclear Corporation, Boston, Massachusetts. The specific activity of the charcoal preparation was 7.7 millicuries per gram.

Sodium β-Glycerophosphate

Crystalline sodium β-glycerophosphate was a Fisher certified reagent grade containing less than 0.1% of the alpha-isomer obtained from Fisher Scientific Company, St. Louis, Missouri.

Neuramin-Lactose

Chromatographically purified neuramin-lactose prepared from rat mammary glands was a gift from Dr. R. Carubelli (77). This material was used as the substrate for neuraminidase assays.

Phenolphthalein Mono-β-Glucuronide

This substrate of β-glucuronidase was obtained in crystalline form from Sigma Chemical Company, St. Louis, Missouri. M. Wt. = 494.4

Bovine Hemoglobin

Bovine hemoglobin enzyme substrate powder was obtained from Pentex Incorporated, Kankakee, Illinois and from Nutritional Biochemicals Corporation, Cleveland, Ohio. Acid denatured hemoglobin was prepared from this material for the measurement of cathepsin pH 3.8 activity.

O-Nitrophenyl β-Galactopyranoside

The crystalline substrate for the assays of β-galactosidase was obtained from Sigma Chemical Company, St. Louis, Missouri.

Naja Naja Venom Phospholipase A

Dried powder cobra venom (Naja naja) was used as the source of phospholipase A (a phosphatide β-acyl-hydrolase). This material was obtained from Ross Allen's Reptile Institute, Silver Springs, Florida.

Lysophosphatides

Lysophosphatides were prepared according to the method of Long and Penny (78). Phospholipids which have been isolated from the microsomal fraction of rabbit livers were dissolved in peroxide and ester free ether (1.5 to 2.0 micromoles of phosphatides per ml. ether) and were treated with snake venom phospholipase A (1 mg. venom/ml. of 0.005 M $CaCl_2$). After incubation for 2 hours, the insoluble lysophosphatides were separated by centrifuging then reprecipitated from ether without washing and the dried lysophosphatides were dissolved in chloroform. The concentration of lysophosphatides by lipid phosphorus measurements. One mole phosphorus was considered to be equivalent to one mole of lysophosphatides. Chromatography on thin layer silica Gel G demonstrated the presence of lysolecithin and lysoethanolamine.

Thiobarbituric Acid

This material was obtained from Eastman Kodak Company and was recrystallized from hot water (25 grams in 600 ml.). A 0.6 percent solution in 0.5 M sodium sulfate was prepared for the colorimetric determination of free sialic acid according to the method of Warren (79).

Resorcinol

Crystalline resorcinol Analytical Reagent grade was obtained from Mallinkrodt Chemical Works, St. Louis, Missouri. This material was used to prepare Resorcinol-HCl reagent for spraying thin layer silicic acid chromatography plates to identify sialic acid containing compounds.

Silica Gel G

Binder grade silica gel G was purchased from Research Specialties Company, Richmond, California. This material was used to prepare thin layer chromatography plates.

Triton X-100

This material is a liquid polymeric non-ionic surface active agent (based upon alkyl phenol polymers made water soluble by interaction with alkylene oxide). Triton X-100 was shown to possess haemolytic properties according to Glassman (80). It was obtained from the Rohm and Haas Company, Philadelphia.

α-Tocopherol (Vitamin E)

α-Tocopherol and α-tocopheryl acetate were obtained from Distillation Products Industries, Rochester, New York.

Materials for Diets

Casein, vitamins (except α-tocopheryl acetate), cod liver oil, and Alphacel (a pure, powdered non-nutritive cellulose added for bulk) were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Molecularly-distilled lard was obtained from Distillation Products Industries, Rochester, New York.

Vitamin E-Deficient Diets for Rabbits

The diet mixture was prepared according to Caputto et al. (81), a modification of a diet developed by Young and Dinning (82).

Basal diet	90%
Alphacel	10%

Composition of the Modified Basal Diet

Casein, vitamin free	17%
Sucrose	37.3%
Corn Starch	36%
Lard (D.P.I.)	3%
Cod liver oil	3%
Salt mixture (A)	3%
Vitamin mixture (B)	0.7%

The salt mixture (A) was prepared according to Hubbel et al. (83). The vitamin mixture (B) was prepared according to Young and Dinning (82).

Composition of Salt Mixture (A)

$CaCO_3$	54.300%
KH_2PO_4	21.200%
KCl	11.200%

Composition of Salt Mixture (A) continued

NaCl	6.900%
$MgCO_3$	2.500%
$MgSO_4$	1.600%
$FeSO_4 \cdot 7H_2O$	2.050%
NaF	0.100%
$CuSO_4 \cdot 5H_2O$	0.090%
$MnSO_4 \cdot H_2O$	0.035%
$Al(SO_4)_2KSO_4$	0.017%
KI	0.008%

Composition of Vitamin Mixture (B)
(amounts per 100 grams of basal diet)

Vitamin B-12	4.5 gamma
2-Methylnaphthoquinone, vitamin K	0.025 mg.
Biotin	0.005 mg.
Folic acid	0.50 mg.
Calcium pantothenate	1.00 mg.
Thiamine chloride	0.50 mg.
Pyridoxine HCl	0.50 mg.
Nicotinamide	20.00 mg.
Choline chloride	100.00 mg.
Riboflavin	0.50 mg.
Inositol	100.00 mg.

Vitamin E-Sufficient Diet for Rabbits

The composition of the vitamin E-sufficient diet is identical to the deficient diet with the exception that 10 milligrams of α-tocopheryl ace-

tate is added per 100 grams of diet.

Basal diet	90 grams
Alphacel	10 grams
α-Tocopheryl acetate	10 milligrams

Composition of Vitamin E-Supplemented Diet for Chickens

Diet mixture, see below	100 grams
Alphacel	10 grams
α-Tocopheryl acetate	10 milligrams

Diet Mixture

ADM Protein	35.3%
Sucrose	48.18%
Corn Oil	10.00%
DL Methionine	0.75%
Glycine	0.30%
Choline Chloride	0.20%
Salt mixture (C), see below	5.27%
Vitamin mixture (D), see below	

Salt Mixture (C)/100 Grams Diet

$CaCO_3$	2.166 gm.
KH_2PO_4	1.050 gm.
$CaHPO_4 \cdot 2H_2O$	0.940 gm.
NaCl	0.800 gm.
$MgSO_4$	0.250 gm.
$FeSO_4 \cdot 7H_2O$	0.030 gm.
$MnSO_4 \cdot H_2O$	0.020 gm.

Salt Mixture (C) continued

$ZnCO_3$	0.010 gm.
$CuSO_4 \cdot 5H_2O$	0.002 gm.
KI	0.001 gm.
$Na_2MoO_4 \cdot 2H_2O$	0.001 gm.

Vitamin Mixture (D)/100 Grams Diet

Vitamin A acetate	1000 I.U.
Vitamin D3	60 I.U.
Vitamin B-12	2.0 ug
Biotin	60.0 ug
Thiamine	10.0 mg.
Niacin	10.0 mg.
Riboflavine	1.6 mg.
Calcium pantothenate	2.0 mg.
Pyridoxine	0.6 mg.
Folic acid	0.4 mg.
Menadione	0.5 mg.

Stock Diet for Rats and Rabbits

Rats were fed a commercial diet obtained from Rockland Laboratories, Tekland Incorporated, Monmouth, Illinois. This diet had the following ingredients: soybean meal, ground yellow corn, fish meal, pulverized barley, wheat midlings, ground wheat, dehydrated alfalfa meal, pulverized oats, feeding oat meal, dried skim milk, 1% animal fat (preserved with propylene glycol, butylated hydroxytoluene and citric acid), vitamin A palmitate, irradiated dried yeast, niacin, calcium pantothenate, riboflavin supple-

ment, menadione, vitamin B-12, 1% calcium carbonate, 0.5% dicalcium phosphate, 1% sodium chloride, and traces of manganese oxide, copper oxide, cobalt carbonate, iron carbonate, zinc oxide and calcium iodate. The manufacturers guaranteed the following analysis: Crude protein 24.0%., crude fat 4.0%, and crude fiber 6.0%.

Rabbits which were not maintained on the experimental diet were fed a commercial diet obtained from Superior Feed Mills, Oklahoma City, Oklahoma. This diet has the following ingredients: Dehydrated alfalfa meal, whole ground barley, ground oats, soybean meal, wheat bran, linseed meal, dried beet pulp, wheat shorts, 1% ground limestone, 0.5% sodium chloride, meat and scraps, ground corn meal, dried whole whey, activated plant sterol (D-2), riboflavin supplement, calcium pantothenate, α-tocopheryl acetate (Vitamin E), vitamin B-12, niacin, and traces of iron sulfate, manganese sulfate, copper sulfate, cobalt sulfate, potassium iodide, and zinc sulfate. The manufacturers guaranteed the following analysis: Crude protein 15.5%, crude fat 2.5%, crude fiber 15.0%, and nitrogen free extract 48%.

Rabbits

Young male New Zealand white rabbits weighing 700 to 900 grams were purchased from National Animals Laboratories, Creve Coeur, Missouri.

Rats

White rats weighing 100 to 150 grams were obtained from the colony established in the laboratories of the Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma. The colony is highly inbred and was originally derived from the Sprague-Dawley strain of the Holtzman Company,

Madison, Wisconsin.

Chicken

One day old white Leghorn cockerels were obtained from the Capitol Hill Hatchery, Oklahoma City, Oklahoma.

Methods

Preparation of Carbon C-14 Suspension

The radioactive amorphous carbon used in these experiments was diluted with non-radioactive powdered carbon suspension which was prepared as follows: Pulverized carbon black was mixed in a solution of 1% gum arabic and 1% soluble starch (1:1, v/v). The mixture was prepared to contain 40 mg. of carbon black per ml. of suspending medium. It was then ground in a ball jar mill (Fisher Scientific Company). After one week of continuous grinding, the suspension was centrifuged at 1000 x g. for 20 minutes. The supernatant which contained the smaller carbon particles, was removed by aspiration and the amount of carbon solids in a known volume was determined by drying, then weighing the residual solids. The charcoal suspension was diluted to contain 25 mg. of carbon per ml., then 40 ml. were triturated with 500 microcuries of amorphous carbon C-14 (specific activity = 7.7 millicuries per gram). The radioactive mixture was then ground in a ball jar mill for one week and centrifuged at 1000 x g for 20 minutes. The supernatant was removed by aspiration and the sediment was resuspended in 10 ml. of the resulting supernatant and ground for another week. The resulting suspension was recentrifuged as before and the supernatants were combined. Twenty-four hours before injection, the carbon suspension was ground again, centrifuged at 1000 x g for 20

minutes and the supernatant layer was used for injection after determining its radioactivity as counts per minute per ml.

Injection Technique of Carbon C-14 Suspension

Injection was performed by a needle attached with a length of polyethylene tubing to another needle connected to a syringe. The needle portion of a 25 G hypodermic needle was cut off and attached to a polyethylene tubing 1 mm. in diameter. The tip of an intact 24 G hypodermic needle was attached to the other end of the tubing, the socket of which connected to a 2 ml. graduated syringe. After filling, the syringe was attached firmly to a metal stand. Fur in the marginal area of the rabbit's ear was removed and the veins were dilated by the application of hot moist compresses. The needle was inserted through the dorsal skin into the lumen of the marginal right ear vein at a site midway between the tip and base of the ear. The needle tip was directed downstream. One ml. of carbon C-14 suspension with a specific activity of 9.4×10^6 to 7.8×10^6 counts per minute per ml. was injected per kilogram rabbit.

Measurement of the Rate of Disappearance of Carbon C-14 from the Blood Stream of Injected Rabbits

If the right ear marginal vein was used for injection, then the left ear marginal vein was used to collect blood. Blood samples were collected into heparinized graduated capillary tubes. The volumes of blood samples collected ranged between 0.016 ml. to 0.048 ml. The volume between two marks on the graduated capillar tube was standardized to contain 0.016 ml. This was achieved by allowing distilled water to ascend by capillary action to a certain mark on the capillary tube, then weighing this volume and dividing the length to the mark into equal segments. One gram of dis-

tilled water was considered to be contained in one ml. The volume between two marks was also checked by hemoglobin measurements where a known volume of oxalated blood (0.1 ml.) was diluted with 0.1% sodium carbonate solution to obtain a measurable optical density at 540 mu and was compared with measurements obtained from the unknown volume (the volume between two marks). The two methods gave comparable results.

The collected blood samples of known volumes were transferred to small tubes containing 0.5 mll of 10% sodium oxalate solution. The tubes were stoppered and stored until assayed for counting radioactivity. The exact time of injection was noted and the duration of injection was adjusted to 3 to 4 minutes. Two samples were taken during this period at one minute intervals. The exact time of the completion of injection was noted and a blood sample was collected which was considered as the zero time sample. Subsequent samples were collected at one minute intervals for a period of 10 to 15 minutes, then at intervals of 2 to 3 minutes for a period of 15 minutes. Three or four samples were usually collected during the last 30 minutes. Known volumes of the diluted blood samples were placed on counting planchets and the radioactivity was measured by a gas flow Gieger Muller counter (Nuclear Chicago). All samples were counted twice. The background counts per minute ranged between 14 to 16 cpm. Samples with low radioactivity were counted for longer periods of time to obtain 1600 counts. The initial samples, which contained high radioactivity, were counted 5 to 6 minutes to obtain 12,800 counts. The amount of radioactivity per ml. of blood was then determined from the average values obtained for each blood sample.

Two hours after completing the injection, the rabbits were killed by

a blow on the head, then bled and the final blood sample was collected. The amount of radioactivity in the final blood sample was very small and was not included in the plot log counts per minute per ml. blood vs. time. The rate of disappearance of radioactivity from the blood stream followed an exponential function of time according to the equation:

$$(\log C_1 - \log C_2)/(t_2 - t_1) = K$$

C_1 = counts per minute per ml. blood at time (t_1) and C_2 = counts per minute per ml. blood at time (t_2). The rate constant (K) was called the phagocytic index according to Biozzi et al. (84).

Determination of Radioactivity in Organs and Muscle

The entire liver, spleen, and lungs were removed and weighed. Muscle tissue was obtained from the hind limb. All muscles from around the femur were taken, dissecting away connective tissue and tendons as much as possible, then mincing with a sharp razor blade. Samples of the minced tissues were homogenized in distilled water in all glass homogenizer equipped with a motor driven pestle. The resulting homogenates contained 500 mg. muscle tissue per ml. Preliminary experiments demonstrated that the amounts of radioactivity present in muscle homogenates were very low which made it necessary to count for longer periods of time to obtain 1600 counts. Samples of muscle homogenates were placed on planchets and dried in an oven at about 80°C. The radioactivity present was measured by a gas flow Geiger Muller counter (Nuclear Chicago). The background counts per minute ranged between 14 to 16 counts when 1600 counts were obtained. After subtracting the background counts, the values were ex-

pressed as counts per minute per gram muscle. Duplicate samples were counted twice and the average values were considered for calculation.

A technique was developed to determine the amount of radioactivity present in larger quantities of muscle tissue. Samples of muscle homogenates were mixed with equal volumes of 10 N NaOH solution and the resulting mixture was allowed to stand at room temperature overnight. About 14% of muscle tissue fragments remained undigested and were sedimented by centrifuging at 40,000 x g for 20 minutes. The supernatant did not show any radioactivity and the sediments contained all the radioactivity. The sediments were resuspended in distilled water to obtain the equivalent of two grams of muscle tissue per ml. of suspension. Samples (0.5 ml.) of this suspension, which contained the equivalent of one gram muscle tissue, were taken to measure the amounts of radioactivity present. The time required to obtain 3200 counts was determined, then the counts per minute per gram muscle were obtained by calculation. This technique, however, did not show a definite advantage over the former procedure where the amount of radioactivity was determined directly from muscle homogenates.

Deposits of carbon were visible in livers, spleens and lungs of injected rabbits. The amounts of radioactivity present in these organs were high and only small quantities of homogenized tissues were needed for counting. Samples of tissue homogenates containing 5 to 20 mg. of the original tissues were counted 7 to 9 minutes depending on the amounts of radioactivity present.

Isolation of Rabbit Leucocytes

The method of Chen and Palmer (85) was used to obtain leucocytes reasonably free from erythrocytes. To 10 ml. of oxalated rabbit blood, 0.2 ml. of phytohemoaglutinin M (Difco) was added, followed by the addition of 2.0 ml. of 0.6% dextran solution (Abbott). After shaking, the samples were allowed to stand in an ice bath for 30 minutes then centrifuged five minutes at 500 r.p.m. (International Centrifuge, Head number 242). The supernatant which contained the leucocytes, were removed by aspiration and transferred to graduated centrifuge tubes then centrifuged at 2000 r.p.m. for 10 minutes. The clear supernatants were removed by aspiration and discarded. The pellets, containing the leucocytes and a few erythrocytes were treated with five volumes of distilled water for 30 seconds to hemolyze the erythrocytes and then immediately five volumes of 1.8% NaCl solution were added to obtain a final concentration of 0.9% NaCl. This suspension was centrifuged at 2000 r.p.m. for 10 minutes and the supernatant was removed by aspiration and discarded. The pellet volume was made up to 10 volumes with distilled water to obtain a 10% cell suspension. The leucocytes were disrupted by freezing and thawing 10 times and then homogenizing in a micro-homogenizer. The resulting homogenates were used for analysis.

Isolation of Rabbit Alveolar Macrophages

The method of Myrvik et al. (86) was used to harvest macrophage cells from rabbit lungs. After killing and bleeding the animal, the thoracic cavity was opened and the upper part of the trachea was clamped

shut by a hemostat to avoid entrance of blood into the lungs. The lungs, and the trachea were dissected out as a single unit. The exterior was washed with warm water, about 37°C. and the heart was dissected free, carefully avoiding any injury to the lungs or bronchi. The lungs were sponged free of excess fluid and suspended by attaching a hemostat to the wall of the trachea, leaving the lumen open. 0.9% NaCl solution was piped into the trachea until the lungs were distended. This process required approximately 30 ml. The trachea was then clamped off and the lungs were massaged gently and the fluid was emptied into a 50 ml. beaker. The fluid was then centrifuged at 600 x g. for 20 minutes. The supernatant was removed by aspiration and discarded. The pellet, containing the macrophages was washed with five volumes of 0.9% NaCl solution and transferred into a graduated centrifuge tube. The cell suspension was then centrifuged at 1800 r.p.m. for 20 minutes. The supernatant was removed by aspiration and discarded. The fluffy layer was removed by a capillary tube. The volume of the pellet was noted and the cells were then suspended in distilled water to obtain a 10% cell suspension. Macrophages were disrupted by freezing and thawing 10 times, using a dry ice-alcohol bath as a freezing mixture. This treatment was then followed by homogenization in a micro-homogenizer. Protein determinations and enzyme assays were done on aliquots from this homogenate.

Isolation of Rabbit Erythrocytes and Plasma

Rabbits were killed by a blow on the head followed by partial decapitation and the blood was collected in oxalated beakers to prevent clot-

ting. A known volume of blood was pipetted into graduated centrifuge tubes and centrifuged at 1800 r.p.m. for 20 minutes. The volume of packed erythrocytes was noted and the plasma was removed by aspiration. The erythrocytes were shaken gently in small volumes of 0.9% NaCl solution and transferred to tubes large enough to hold 20 times the volume of packed cells. Washing with isotonic NaCl solution was done twice and the supernatant was discarded. Removal of leucocytes was done by aspirating the fluffy layer above the erythrocytes. In some experiments, the leucocytes were not removed. Washed erythrocytes were then suspended in 0.9% saline solution to obtain a 2.5% cell suspension which was used for hemolysis studies and assays of lysophosphatidase.

Assay of the Vitamin E-Deficient State by Measurement of Hemolysis

The method of Gordon et al. (87) was used for testing the effect of H_2O_2 on erythrocytes of rabbits maintained on a vitamin E-deficient diet or vitamin E-sufficient diet. Five tubes were set up with 1 ml. of 2.5% erythrocyte suspension in 0.9% NaCl solution. To four tubes, 1 ml. of 2.4% H_2O_2 solution was added. To the fifth tube, 1 ml. of 0.2 M phosphate buffer pH 7.4 was added. The five tubes were incubated at 37°C. for 15 minutes and then allowed to stand at room temperature for 4 hours with shaking. The first three tubes serve as the test in triplicate, the fourth tube serves as the index of complete hemolysis, and the fifth tube serves as the control. One ml. of the first three tubes and the fifth tube was taken and added to four new tubes containing 4 ml. of buffered saline (1.8% NaCl solution + 0.2 M phosphate buffer pH 7.4). One

ml. of the fourth tube was taken and added to 4 ml. of distilled water. The contents of each tube were mixed by shaking then centrifuged at 1800 r.p.m. for 10 minutes. The optical density of the supernatants were determined at 540 mu using buffered saline as blank.

$$\text{Percent hemolysis} = \frac{(\text{Optical Density})_{\text{test}} - (\text{Optical Density})_{\text{control}}}{(\text{Optical Density})_{\text{fourth tube containing distilled water} + H_2O_2}} \times 100$$

Determination of Protein Concentration

Spectrophotometric method. The amount of protein present in dilute solutions were determined according to the equation used by Kalckar (88) which was devised to fit the data of Warburg and Christian (89).

$$\text{Mg. protein per ml.} = 1.45\,(\text{Optical Density at 280 mu}) - 0.74\,(\text{Optical Density at 260 mu})$$

This method is based on the observation that most organic substances absorb light at wave lengths below 250 mu and the absorption of light of longer wave lengths is usually associated with the presence of unsaturated bonds in the molecule. An increase in the number of unsaturated bonds and their presence in conjugated systems contribute to light absorption at longer wave lengths. Of the protein amino acids, only phenylalanine, tyrosine, and tryptophane exhibit light absorption at wave lengths longer than 250 mu. This property may be attributed to their aromatic nature. Phenylalanine has a maximal absorption at 260 mu, tyrosine at 275, and tryptophane at 280 mu. Nucleic acids absorb strongly at 260 mu and their interference was corrected for by the empirical equation above. This method is less reliable than the Kjeldahl or biuret methods but was found

convenient in cases where the amount of material was limited such as protein determinations of leucocytes.

Biuret method. The method of Gornall et al. (90) was used. It is based on the principle that substances containing peptide bonds give a characteristic purple color when treated in a alkaline solution with copper sulfate. The color deepens as the number of peptide bonds in a series of synthetic peptides is increased. Proteins produce a deep blue violet color. Two or more peptide bonds are required to give a light pink color. Dipeptides do not give the test. The color of the biuret test is apparently due to coordination of cupric ions with the unshared electron pairs of peptide nitrogen and the oxygen of water to form a colored coordination complex. The biuret reagent was prepared by dissolving 1.5 grams of cupric sulfate ($CuSO_4 \cdot 5H_2O$) and 6 grams of sodium potassium tartarate ($NaKC_4H_4O_6 \cdot 4H_2O$) in 500 ml. of water. 300 ml. of 10% NaOH was added and then the solution was diluted to 1 liter with water. For the test, 4 ml. of biuret reagent were added to 1 ml. of a protein solution containing 1 to 5 mg. of protein. The tubes were mixed and allowed to stand at room temperature for 30 minutes. The optical density was measured in a Beckman Model DU Spectrophotometer at 540 mu. The concentration of protein in the samples tested was obtained by reference to a standard protein solution. When cloudy reaction mixtures were obtained due to presence of lipids, the reaction mixtures were shaken with 1.5 ml. of petroleum ether and centrifuged. The ether layer was removed by aspiration and the aqueous phase was read as above.

Determination of Total Nitrogen and Non-Collagen Nitrogen

Since the quantity of connective tissue present in muscle undergoing dystrophic changes generally increases, it was considered more informative to relate the enzymic activity in terms on non-collagen nitrogen. Non-collagen nitrogen was determined according to the method of Pennington (91) which was based on the method described by Lilienthal et al. (137). Samples of muscle homogenates containing 100 mg. muscle tissue fragments per ml. were digested overnight with 0.1 N NaOH solution. One volume of muscle homogenate was mixed with 9 volumes of 0.1 N NaOH and allowed to stand at room temperature overnight then centrifuged at 1800 r.p.m. (International Centrifuge, Head number 242) for 20 minutes and the supernatant was collected. The supernatant (the digest) contains the solubilized nitrogenous materials (amino acids, creatine, nucleic acids and phosphatides) and muscle fiber protein. Collagen and elastin are insoluble in a weak solution of sodium hydroxide in which other cellular protein are soluble. The nitrogen contents of the digests were determined by a micro-Kjeldahl procedure. Samples of the clear supernatants (alkaline digests) were digested with concentrated sulfuric acid in pyrex tubes and at the end of digestion, two drops of hydrogen peroxide were added. When the digestion was completed and tubes were cooled, 1 ml. of water was then added followed by 1 ml. of 10 N NaOH solution and 3 ml. of Nessler's reagent. The tubes were shaken and let stand at room temperature for 20 minutes. The color intensity was determined in a Beckman Model DU Spectrophotometer at 425 mu. For total protein determinations, aliquots of muscle homogenates containing 100 mg.

muscle tissue fragments per ml., were digested with concentrated sulfuric acid directly without pretreatment with 0.1 N NaOH solution. The nitrogen content was then determined as above.

Estimation of Organic and Inorganic Phosphates

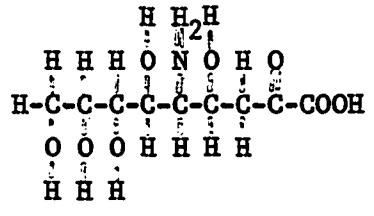
The method of Fiske and SubbaRow (92) was used. Estimation of organic phosphate compounds required digestion in sulfuric acid. This method was used to measure the phosphate content of extracted phospholipids and prepared lysophosphatides. The phospholipid preparations did not contain proteins and therefore deproteinization was not required. One ml. of 5 N sulfuric acid solution was added to samples of phospholipids. The phospholipids were dissolved in chloroform and aliquots were transferred to pyrex tubes and the chloroform was evaporated before adding the sulfuric acid solution. The phospholipid sulfuric acid mixture was heated and when the contents became charred, two drops of concentrated nitric acid were added and heating was continued until the solution was clear and colorless. After cooling, 1 ml. of water was added and the samples were heated again for about 2 minutes then cooled. The contents were transferred to a 10 ml. volumetric flask and the digesting tubes were washed three times with water, using 2 ml. each time. One ml. of molybdate reagent was added and after mixing, 0.1 ml. of the reducing reagent was added. The volume was made up to 10 ml., mixed again and allowed to stand about 20 minutes. The optical density was measured at 660 mu in a Beckman Model DU Spectrophotometer. A standard phosphate solution containing one micromole per 0.1 ml. was carried through the entire procedure in each assay.

For the inorganic phosphate determinations, 1 ml. of deproteinized samples containing 0.1 to 1 micromoles phosphate were mixed with 1 ml. of 5 N sulfuric acid followed by 1 ml. of molybdate reagent, then 0.1 ml. reducing reagent. After mixing and diluting with water to a final volume of 10 ml., the optical densities were determined as above.

The molybdate reagent is composed of 2.5% ammonium molybdate. The reducing reagent was prepared in powder form and dissolved before use. It consisted of 0.2 grams of 1-amino-2-naphthol-4-sulfonic acid, 1.2 grams of sodium bisulfite and 1.2 grams sodium sulfite. For use, about 0.25 g. were dissolved in 10 ml. of distilled water. The standard phosphate solution was prepared by dissolving K_2HPO_4 (reagent grade which had been thoroughly dried) in distilled water (136.1 mg. in 100 ml. distilled water).

Estimation of Free Sialic Acids

The naturally occurring sialic acids are substituted neuraminic acid derivatives which are collectively termed sialic acids, N-acetyl, N-glycolyl, N,O-diacetylneuraminic acid. The unsubstituted 9 carbon chain is called Neuraminic acid:



The method of Warren (79) was used to detect free sialic acids. To a sample containing up to 0.05 micromoles of sialic acid in a volume of

0.2 ml., 0.1 ml. of periodate solution was added and the tubes were shaken and allowed to stand at room temperature for 20 minutes. Arsenite solution, 1 ml., was added and the tubes were shaken until a yellow-brown color disappeared. 3 ml. of thiobarbituric acid solution were added, the tubes were shaken, capped with glass marbles, and then heated in a boiling water bath for 15 minutes. The tubes were then removed and placed in a cold water bath for 5 minutes. During the cooling, the reddish color faded and the solution became cloudy. 1 ml. of this solution was transferred to another tube containing 1 ml. of cyclohexanone or the entire 4.3 ml. were extracted with 4.3 ml. of cyclohexanone. The extraction was facilitated by agitation using a Vortex Jr. apparatus for a few minutes, after which the tubes were centrifuged. The colored, clear upper cyclohexanone phase was removed by aspiration and the optical densities were determined at 549 mu in a Beckman Model DU Spectrophotometer, using 1 cm. light path cuvettes. The amount of N-acetylneuraminic acid present in the samples was calculated according to the equation:

$$\text{Micromoles} = \frac{V \times \text{O.D. } 549 \text{ mu}}{57}$$

where V is the final volume of the test solution = 4.3

When tissues are subjected to the thiobarbituric acid assay for sialic acids, there may be a second absorption maximum at 532 mu due to 2-deoxyribose which interferes with materials absorbing at 549 mu. Other substances such as unsaturated lipids may yield malonaldehyde upon periodate oxidation and contribute to the optical densities at 549 mu and 532 mu. The following equation was used to correct for these interfer-

ences:

$$\text{Micromoles} = 0.090 \times \text{O.D.}_{549} - 0.033 \times \text{O.D.}_{532}$$

The reagents used for the assay were: 0.2 M sodium periodate (meta) in 9 M phosphoric acid; 10% sodium arsenite in a solution containing 0.5 M sodium sulfate and 0.1 N H_2SO_4, and 0.6% thiobarbituric acid in 0.5 M sodium sulfate.

Isolation and Determination of Gangliosides

The isolation of gangliosides from tissues was achieved according to the procedures of Folch et al. (93), Booth (94) and Burton et al. (95). The tissues were weighed and then cut into very small pieces. The mince was extracted with chloroform-methanol (2:1, v/v) in a blender or in an all-glass homogenizer. Usually 20 ml. of solvent were used per gram of tissue. In determining the subcellular distribution of gangliosides, the pellets (derived from differential centrifugation of the tissue homogenized in sucrose) were first removed by a minimum quantitiy of water followed by extraction with 19 volumes of chloroform methanol solvent. After extraction, the suspension was filtered and the clear filtrate was washed with 1/5th its volume of 0.5% saline and shaking. The mixture was centrifuged to separate the chloroform phase (lower phase) from water-methanol phase. The chloroform layer was aspirated and the water methanol portion, which contains the gangliosides, was dialyzed overnight in running tap water and then lyophilized. Samples were taken and hydrolyzed in 0.1 N H_2SO_4, final concentration, for 1 hour at 80°C. according to the directions of

Balakrishan *et al*. (96). After hydrolysis, free sialic acids were determined according to Warren's method (79).

Detection of Gangliosides in Lipid Extracts by Thin Layer Chromatography

The method of Wherrett and Cumings (97) was used for the detection of gangliosides from crude lipid extracts of tissues. Tissue samples were extracted with chloroform-methanol (2:1, v/v) according to Folch *et al*. (93). The crude extract which was not washed with saline was evaporated to dryness at 40-50° with an air-leak system and then redissolved in chloroform-methanol (2:1, v/v) to give a final concentration equivalent to 500 mg. of original tissue per ml. Chromatography plates were prepared by Stahl's apparatus. Glass plates, 20 cm. by 20 cm., were spread with a thin layer, 0.25-0.5 mm thick, of silica gel G and dried 4 hours before use. Samples were spotted on the base line with a micropipet. Plates were run at room temperature in paper-lined tanks. The solvent front was permitted to reach within 4-5 cm. of the top of the plate. The solvent system consisted of chloroform-methanol-H_2O (60:35:8 by volume). Detection of ganglioside spots was achieved by first drying the plates in air, then spraying lightly with resorcinol-HCl prepared according to Svennerholm (98). The test plate was covered with a second clean plate and heated in an oven at about 150°C for 8-12 minutes. Neuraminic acid-containing substances appeared as purple spots against a white background.

Isolation and Chromatography of Lysosomal Lipids

The lysosomal fraction was prepared from livers by differential cen-

trifuging as described. The pellet was first suspended in a minimum amount of water and transferred to a larger vessel. Nineteen milliliters of chloroform-methanol (2:1, v/v) were added per ml. of lysosomal suspension. Extraction was done by rapid stirring for 5-10 minutes then filtering the mixture to obtain a clear solution. The filtrate was washed by stirring with 1/5 its volume of 0.5% saline and then centrifuging to obtain two phases. The lower phase (the chloroform layer) was aspirated by a syringe leaving behind the aqueous phase which contains gangliosides. The chloroform layer, which contains phospholipids and neutral lipids was evaporated to dryness at 40°C with a vacuum rotary evaporator. Few drops of absolute ethanol were added at the end of evaporation to facilitate removal of water. The lipid material was dissolved in chloroform and stored in the cold under nitrogen. It was also observed that a white material insoluble in chloroform, methanol or water remained in the evaporating flask. Neutral lipids were separated from phospholipids by the aid of silicic acid column chromatography according to the method of Macfarlane et al. (99). One gram of silicic acid per milligram lipid phosphorous in the total extract was used in preparing columns. The lipid extract was placed on the columns in chloroform, and the neutral lipids were eluted with additional chloroform until no further material could be brought off the column. The total chloroform effluent was evaporated to dryness in a tared flask at 40° using a rotary evaporator. When the solvent evaporation was completed, the flask was weighed and the weight of lipids present was calculated. The adsorbed phospholipids were eluted with methanol without further fractionation.

The total methanol eluate was evaporated in the same manner as the chloroform effluent and the weight of phospholipids present was determined. The phospholipids were redissolved in pure chloroform and an aliquot was taken for phosphorous determination according to the method described. One mole of phosphorous was considered to be equivalent to one mole of phospholipids. Previously prepared thin layer chromatography plates were heated at 100-110°C for one hour before use. Neutral lipids were spotted on the base line. A spot composed of purified mono- and triglycerides was applied on the base line to serve as a marker for separating mixed glycerides. The plates were run at room temperature in filter paper-lined tanks for one hour, permitting the solvent front to reach within 5-6 cm. of the top of the plate. The solvent system consisted of 30% ethyl ether and 70% petroleum ether. Phospholipids were treated in a similar manner. Separate spots of purified lecithin and cephalin were included on the plate to serve as markers. An amount equivalent to 10 micrograms of phosphorus was used for each spot. The solvent system for chromatography of phospholipids consisted of chloroform-methanol-water (140:40:4). Detection of spots was achieved by drying the plates in air, then spraying lightly with ninhydrin reagent, followed by heating at 100° for a few minutes until a pink color appeared, indicating the presence of amino nitrogen containing phospholipids. After marking the positions of ninhydrin positive spots, the plates were sprayed lightly with Haynes-Ischerwood reagent (100), followed by heating until charred. Neutral lipid plates were not sprayed with ninhydrin but sprayed directly with the Haynes-Ischerwood reagent and heated until charring occurred.

Methods for the Estimation of Choline

There are several procedures for the determination of free choline based on the precipitation of choline complexes by the addition of triiodide, phosphotungtic acid, phosphomolybdic acid or reinecke salt. Application of these procedures to biological materials produces unreliable results due to the presence of interfering substances. Two methods were used: 1. By the method in which choline was precipitated as the triiodide and extracted with ethylene chloride according to Appleton *et al.* (101). This method was found unsatisfactory when tissue homogenates were used as a source of enzymes. However, when pure choline solutions were used, the procedure was satisfactory and reproducible results could be obtained. 2. By the estimation of choline by the phosphomolybdic precipitation according to the method of Wheeldon and Collins (102). This method was found to be less sensitive than the triiodide method.

Estimation of Fatty Acid Ester Groups

The method of Shapiro *et al.* (103), which is based on the reaction of hydroxylamine with esters, was used.

$$\text{R-COOR}' + \text{H}_2\text{NOH} + \text{OH}^- = \text{R-CONHOH} + \text{R}'\text{OH}$$

$$\text{RCOCl} + \text{NH}_2\text{OH} + \text{OH}^- = \text{RCONHOH} + \text{HCl}$$

$$\text{RCOOCOR}' + \text{NH}_2\text{OH} + \text{OH}^- = \text{RCONHOH} + \text{R}'\text{COOH}$$

$$\text{RCONHOH} + \text{FE}^{+++} = \text{R-C(=O)-NH-Fe(=O)} + \text{H}^+$$

The sample, containing 2-5 microequivalents of fatty acid esters, was extracted with 10 ml. of alcohol-ether mixture (3:1) and centrifuged. To 3 ml. of the clear extract, 0.5 ml. of 2 M hydroxylamine was added followed by 0.5 ml. of 3.5 N NaOH. After 20 minutes, 0.6 ml. of 30% HCl was added followed by 0.5 ml. of 0.37 M ferric chloride prepared in 0.1 N HCl. The blank consisted of 3 ml. of alcohol-ether mixture (3:1) and the reagents. After mixing, the optical density was determined at 525 mu in Beckman Model DU Spectrophotometer.

Assay of Lysophosphatides by Erythrocyte Hemolysis Test

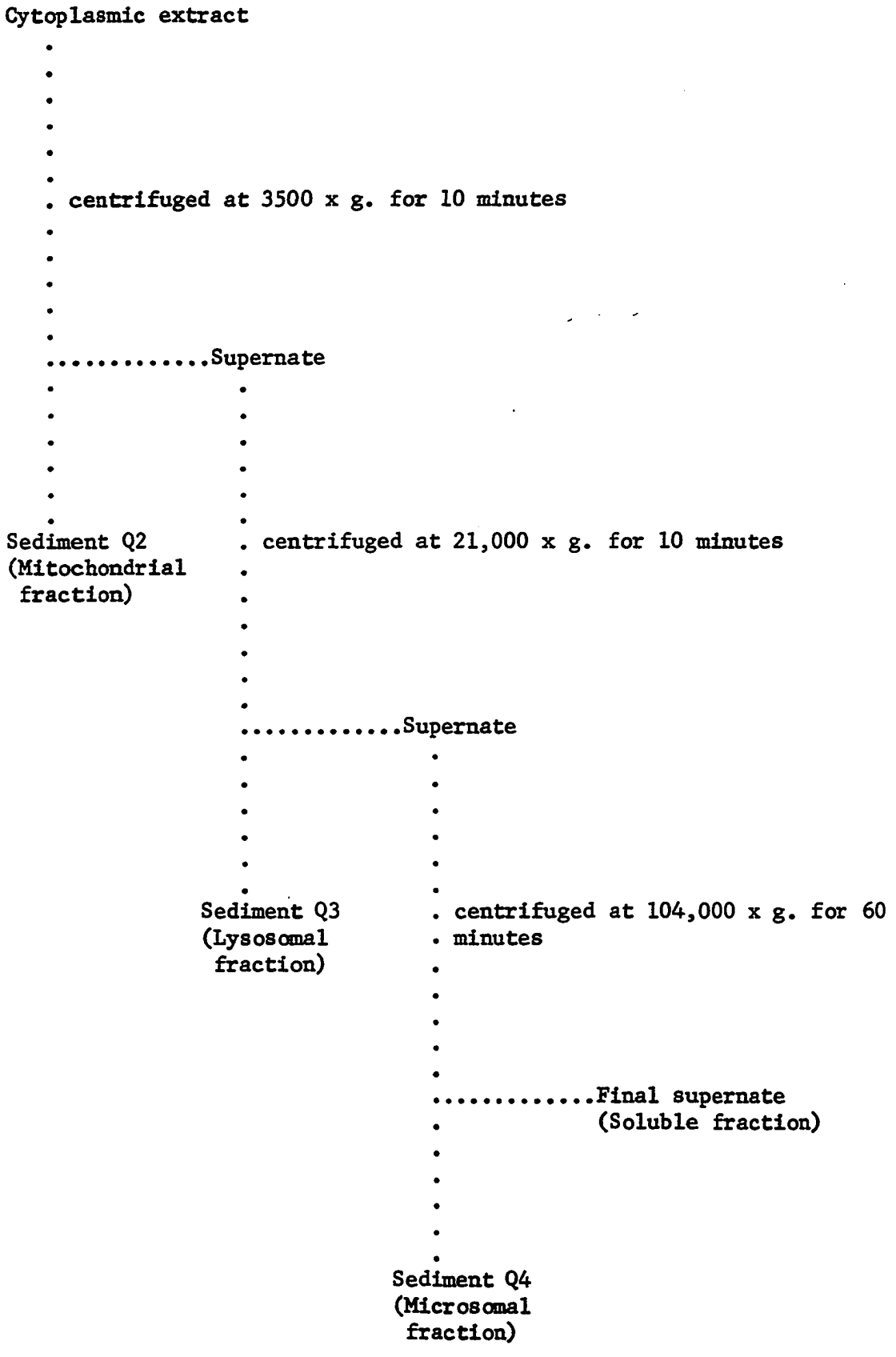
The hemolysis test of Bernheimer (104) was used to measure the concentration of lysophosphatides based on their hemolytic activity. Quantitative results were obtained when the amount of lysophosphatides was adjusted to a narrow range of 0.05 to 0.10 micromoles per ml. by dilution. Lysophosphatides were prepared according to the method described before and the minimum amount required to produce complete hemolysis was determined. An (S) shaped graph was obtained when the percent of hemolysis was plotted against the amount lysophosphatides added.

Tissue Fractionation

Intracellular particulates were prepared by differential centrifuging of tissue homogenates. The technique used was based on the procedures of De Duve *et al.* (14). The homogenizing medium was 0.25 M sucrose containing 0.001 M disodium ethylenediaminetetraacetate. The animals were killed by a blow on the head and bled. The liver, brain and all the hind

leg muscles were taken out quickly and immersed in beakers containing ice-cold suspension medium. After allowing a few minutes for chilling, samples of tissues were blotted and weighed, then cut into small pieces and transferred to a homogenizer of the Potter and Elvehjem type. The homogenizer was a small-walled glass tube fitted with a Teflon pestle manufactured by Arthur H. Thomas Company, Philadelphia. The minced tissue was dispersed in cold suspension medium, 3 ml./gram tissue, and the homogenizing tube was kept in cracked ice. Homogenization was completed by a single run where the tube was pushed upwards then downwards against the rapidly rotating pestle (about 1000 rev./min.), until all the material has been forced above the Teflon part of the pestle. The resulting slurry was centrifuged in the cold at 1000 x g. for 10 minutes. The sediment, which still contained a large number of unbroken cells in addition to the nuclei, was rehomogenized in the same quantity of medium used in the first homogenization and centrifuged at 800 x g. for 10 minutes. The sediment constituted the nuclear and the debris fraction (Q1). The supernatants were combined and made up to volume to form 1:10 cytoplasmic extract (100 mg. of original tissue per ml.).

The cytoplasmic extract was further fractionated according to the scheme outlined below using the Spinco Model L preparative ultracentrifuge with rotor No. 40 (R max. = 8.1 cm., R min. = 4.8 cm.). Three particulate fractions were obtained and the final supernatant.


Cytoplasmic extract
centrifuged at 3500 x g. for 10 minutes
Supernate
Sediment Q2
(Mitochondrial
fraction)
centrifuged at 21,000 x g. for 10 minutes
Supernate
Sediment Q3
(Lysosomal
fraction)
centrifuged at 104,000 x g. for 60
minutes
Final supernate
(Soluble fraction)
Sediment Q4
(Microsomal
fraction)

The supernates were always removed by aspiration employing either a pipette operated by a rubber bulb or a 10 ml. syringe with a long 20 G. needle. The fluffy layer on top of the lysosomal fraction was removed with the supernatant to be recovered with the microsomal fraction. The sediments were resuspended in appropriate volumes of medium using a microhomogenizer with a loose-fitting pestle. The mitochondrial and lysosomes were resuspended in amounts equivalent to 500 mg. of original tissue per ml. and equivalent to 250 mg. tissue per ml. for the microsomes.

Free and Total Activities of Lysosomal Enzymes

There are three forms of enzymic activities which should be considered when lysosomal hydrolases are assayed: The free activity, representing the small amount of enzymic activity in solution, the bound activity, representing the enzymic activity which is not accessible to the substrate, and the total activity, which equals the free plus the bound activities. For free activity determinations, the lysosomal fraction was immediately assayed in the presence of a substrate mixture containing 0.25 M sucrose for 10 minutes incubation period. Total activities were estimated after the enzymes had been completely released by suspending the lysosomal fraction in distilled water and subsequently exposing it to repeated freezing and thawing, or by preincubation at 37°C for 2 hours at pH 5, or by running the assays in presence of 1% (v/v) Triton X-100. The assays for the various lysosomal enzymes were done as follows:

Acid phosphatase. This lysosomal enzyme is an orthophosphoric mono-

ester phosphohydrolase which has a wide specificity and an acidic pH optimum. The method adopted for the estimation of acid phosphatase was primarily that of De Duve *et al.* (14) where β-glycerophosphate was used as the substrate. The α-isomer could not be used because the microsomal enzyme glucose 6-phosphatase hydrolyses this substrate. Similarly, nitrophenyl phosphate could not be used as the substrate for the lysosomal enzyme. The incubation mixture consisted of the following: 1 ml. buffered substrate solution composed of 0.1 M β-glycerophosphate and 0.1 M acetate buffer, pH 5, 0.02 ml. of 1% (v/v) Triton X-100, 0.2 ml. of enzyme solution, and 0.6 ml. of distilled water. The control system contained all components except the substrate. After incubation at 37°C for 10 minutes, the reaction was stopped by adding 1 ml. of 10% trichloroacetic acid (TCA). Substrate was added to the control tube after it had been inactivated with TCA. The tubes were then centrifuged and samples of the clear supernate were taken for the estimation of liberated inorganic phosphate according to the method described earlier. One unit of acid phosphatase activity was established to be one micromole inorganic phosphate liberated per hour. The activity was referred either to one gram of tissue or to one milligram of protein.

Cathepsin. The proteolitic activity of cathepsin was measured at pH 3.8 with acid-denatured bovine hemoglobin as the substrate. The method of Mason *et al.* (105) which was based on the original method of Anson (106) was adopted. Fresh substrate preparations were made by mixing 1 gram of bovine hemoglobin in 20 ml. of 0.1 M acetate buffer, pH 3.8, with continuous stirring until all the material was dispersed. The pH was ad-

justed to 3.8 by adding slowly 4 N HCl. The volume was brought to 25 ml. with 0.1 M acetate buffer, pH 3.8. The preparation was then centrifuged and the precipitate was discarded. The resulting solution was approximately 4% hemoglobin. There are many substances in tissue homogenates and subcellular fractions which absorb light at 280 mu, therefore, those preparations were dialyzed first before assay. Dialysis was run against 50 to 80 volumes of 0.9% saline in the cold for about 18 hours. After dialysis, the final volume was adjusted to obtain 100 mg. tissue per ml. and rehomogenized. The resulting suspension was used as the source of enzymic activity. For each assay, which was done in duplicate, the complete incubation mixture consisted of 1 ml. of 0.2 M acetate buffer, pH 3.8; 1 ml. of the freshly prepared substrate, and 1 ml. of the dialyzed enzyme source. In making up the system, the buffer was added first, then the substrate. At this point, the mixture was preincubated at 37°C for 5 minutes to equilibrate the temperature, after which the addition of enzyme source (the dialyzed homogenates) was made. The control system contained all the components except the substrate. Incubation was carried out at 37°C for 30 minutes and the reaction was stopped by the addition of 5 ml. of 5% trichloroacetic acid (TCA). After the addition of TCA to the control tube, 1 ml. of the substrate which had been incubated simultaneously in a separate tube was added. After inactivation the tubes were left at room temperature for 1 hour, and then centrifuged. The optical density of the clear supernatant was determined at 280 mu in a Beckman Model DU Spectrophotometer. The proteolytic activity was based on the difference: Optical Density (test)--Optical Density (control). One unit of activity

= optical density change of 0.100 per hour. Reference was either to one gram wet weight of tissue or one mg. of protein.

β-Glucuronidase. This lysosomal enzyme is a β-D-glucuronide glucuronohydrolase which hydrolyses conjugated glucuronides to yield glucuronic acid and the aglucurone (the conjugated group). The activity of the enzyme was determined according to De Duve's modification (14) of a method of Fishman et al. (107) which permits measurements of free and total activities as described earlier. Phenolphthalein glucuronide was the substrate in an incubation system containing 0.075 M of acetate buffer, pH 5.2, 0.00125 moles of substrate, the enzyme source, and 0.1% Triton X-100 in a total volume of 2 ml. Two controls were included in the assays. One consisted of all the materials added except the substrate and another contained all the materials added except the course of enzyme. After incubating at 37°C for 15 minutes, the reaction was stopped by the addition of 5 ml. of glycine buffer, pH 10.4 to 10.6. After mixing and centrifuging, the pink color of the alkalinized free phenophthalein was read at 540 mu in a Beckman Model DU Spectrophotometer against a blank consisted of Triton X-100, acetate buffer, glycine buffer and water to make a total volume of 7 ml. Freshly prepared substrate contributed no color in the second control mentioned above. When cloudiness persisted, the contents were centrifuged again at about 20,000 x g. for 10 minutes. The optical density measurements were then converted to micrograms of phenolphthalein from a standard curve of alkalinized phenolphthalein.

β-Galactosidase. The method of Sellinger et al. (20) was used for the determination of β-D-galactoside galactohydrolase (β-galactosidase) activity. This lysosomal enzyme hydrolyses β-D-galactosides to yield D-galactose and an alcohol. Total activities were assayed by incubating the enzyme preparation for two hours at 37°C in a final volume of 2 ml. containing 0.05 M of pyridine-HCl buffer, pH 5.0, 2.5 mM of ortho-nitrophenyl-β-D-galactoside as substrate, and 0.1% Triton X-100. The reaction was stopped by the addition of 3 ml. of 3% trichloroacetic acid, mixing, and centrifuged at 1800 r.p.m. for 10 minutes. To 2 ml. of the supernatant were added: 0.75 ml. of 0.5 N NaOh and 1.25 ml. of 0.25 M glycine-Na_2CO_3 buffer, pH 10.0. The ortho-nitrophenol liberated was measured colorimetrically at 420 mu in a Beckman Model DU Spectrophotometer. The enzyme preparation control and the substrate blank were run concurrently with the test.

Neuraminidase. This enzyme which has been shown to be present in animal tissue is an N-acetylneuraminyl glycohydrolase which hydrolyses the a-2,6 links between N-acetylneuraminic acid and 2-acetylamino 2-deoxy D-galactose residues in mucopolysaccharides. The method was used as described by Carubelli et al. (108) where neuramin-lactose served as the substrate. The incubation system consisted of 0.3 ml. of the enzyme preparation suspended in 0.1 M acetate buffer, pH 5.0; 0.1% Triton X-100 and 0.0001 M calcium chloride, and 0.06 micromoles of neuramin-lactose in a final volume of 0.4 ml. After incubation at 37°C for 3 hours, the reaction was stopped by the addition of 0.2 ml. of periodate reagent and the free sialic acid was determined by the thiobarbituric

acid reaction of Warren as described earlier. Controls were run in which neuramin-lactose was added following the periodate reagent and their values were subtracted. In some experiments, α-tocopherol was homogenized with tissues (2 mg. per gram) to reduce the interfering background color.

Lysophosphatidase (lysophosphatide destroying enzyme). A method was developed which was based on the property of lysophosphatides to hemolyze erythrocytes and assuming that the alteration of these compounds by any reaction would result in a loss of their hemolytic property. The consumption of lysophosphatides by an enzymic reaction could then be determined by measurement of the hemolytic activity remaining after incubation. The enzyme preparation was suspended in 0.9% NaOl and incubated for one hour at 37°C. 0.2 ml. aliquots were then transferred to a series of tubes which contained 0.5 ml. buffered isotonic saline, pH 6.0, and 0.1 ml. of lysophosphatides in isotonic saline solution in quantities ranging from 0.1 to 0.4 micromoles. 0.2 ml. isotonic saline was added to the incubation mixture to obtain a total volume of 1 ml. The control contained all the materials in the incubation mixture except the lysophosphatides. After incubating at 37°C. for 15 minutes, the tubes were immersed in an ice bath and immediately 1 ml. of a cold 2.5% erythrocyte suspension was added to each tube, mixed gently by whirling, allowed to stand at room temperature for 5 to 7 minutes, then centrifuged at 1800 r.p.m. for 5 minutes. The supernatants were removed by gentle aspiration, and diluted, then the optical densities were determined at 550 mu in a Beckman Model DU Spectrophotometer. The reference mixture which produced hemolysis was the same as the incubation mixture excepting that it was kept

in an ice bath and the erythrocyte suspension was added to it without delay. In other words, the hemolysis after 15 minutes incubation was compared to the hemolysis at zero time. The amount of lysophosphatides consumed during a 15 minute incubation was determined as follows: If the tubes containing 0.1 and 0.2 micromoles of lysophosphatides showed no hemolysis, while the tube containing 0.3 micromoles showed a 50% hemolysis, and the tube containing 0.4 micromoles showed a 100% hemolysis, then the amount consumed must be between 0.2 and 0.3 micromoles. From a previously prepared graph, 50% hemolysis was equivalent to the presence of 0.05 micromoles. Therefore, from 0.3 micromoles there remained 0.05 micromoles and the amount consumed was 0.25 micromoles.

Phosphatidylcholine Cholinephosphohydrolase (Phospholipase C)

This hydrolytic enzyme acts on lecithin to produce α,β-diglyceride and phosphorylcholine. The method of Macfarlane *et al.* (151) was used to detect phospholipase C activity in rabbit tissue homogenates. The assay mixture consisted of 0.2 ml. of 0.3 M $CaCl_2$; 1 ml. of 0.1 M borate buffer (pH 7.1); 1 ml. of 2.5% aqueous lecithin solution; tissue homogenate (as source of enzyme) and water to make a total volume of 6 ml. The mixture was incubated 30 minutes at 37°C and the reaction was stopped by the addition of 1 ml. of 20% trichloroacetic acid. The total phosphate in the filtrate was determined according to the method described before. A unit of enzymic activity was arbitrarily defined as the quantity of enzyme which under the conditions described above produced 0.1 mg. acid-soluble phosphorus from lecithin in 1 hour at 37°C.

Phosphatidylcholine Phosphatidohydrolase (Phospholipase D)

This hydrolytic enzyme acts on lecithin, phosphatidylethanolamine and phosphatidylserine and yields phosphatidic acid and free base (choline, ethanolamine or serine). Phospholipase D activity was measured according to the method of Davidson and Long (152). The enzymic degradation was carried out in 15 ml. glass-stoppered test tubes. To 1.25 ml. of an emulsion of lecithin (16 micromoles) in 0.1 M acetate buffer, (pH 5.6), 0.25 ml. of 1 M $CaCl_2$ and 1 ml. of enzyme preparation (tissue homogenate) were added. At zero time, 1 ml. of ether was added, the contents of the tubes were shaken vigorously and the reaction mixture was incubated at room temperature for 15 minutes. The reaction was stopped by the addition of 1 ml. of 30% trichloroacetic acid. Phosphatidic acid and unchanged lecithin were extracted with 1.5 ml. ether and the amount of choline liberated was determined on aliquots of ether-free filtrate.

CHAPTER III

RESULTS

Development of Muscular Dystrophy in Rabbits

Experimental muscular dystrophy was produced in rabbits when they were fed ad libitum a diet deficient in tocopherol (vitamin E). The composition of the experimental diet has been described earlier. The food consumption of the vitamin E-deficient animals was approximately the same as the control group which received vitamin E in their diet. To prevent the effects of a sudden change in diet, the experimental diet was mixed in varying amounts with the commercial rabbit pellet diet and given to the animals. After arrival in the laboratory, the rabbits were fed the commercial food for 2 days. Then there was a 10% reduction of that food each day and substitution of the same amount with the experimental diet until all the commercial diet was substituted in a period of 10 days. The day when the rabbits received a 100% experimental was considered the first day on full diet. In presentation of results that follows where the number of days on diet is mentioned, the reference is to the number of days during which the rabbits were fed the 100% experimental diet. The group of rabbits that were maintained on an all vitamin E-deficient diet for a period of 19 to 25 days showed signs of muscular dystrophy. The onset of the disease was sudden and was demonstrated by the inability of rabbits to right themselves when placed on their

backs. The disease caused death of rabbits when they were kept 24 to 72 hours after the gross symptoms of muscular dystrophy appeared. When the animals were fed vitamin E-deficient diet for 15 days, then vitamin E was supplemented, symptoms of muscular dystrophy were prevented.

Clearance of Carbon C-14 from the Blood of Rabbits

Cells of the reticuloendothelial system (RES) have the ability to engulf (phagocytize) particulate matter such as finely divided carbon, colloidal dyes or bacteria. Removal of injected carbon C-14 from the blood is primarily dependent on the RES cells that are in contact with the blood stream, such as the Kupfer cells, RES cells of spleen and bone marrow. Phagocytes present in other tissues and organs of the body will also engulf the carbon particles.

Rabbits which were maintained on vitamin E-deficient diet or vitamin E-supplemented diet were injected intravenously with radioactive carbon suspension and samples of blood were taken at various intervals of time according to the method described earlier. The concentration of carbon C-14 per ml. of blood was determined as (counts per minute per ml. blood) which was plotted against time (in minutes after completion of the injection). The rate of disappearance of C-14 from blood of dystrophic and non-dystrophic rabbits followed an exponential disappearance curve as illustrated in (Figure 1).

The exponential disappearance curve was represented by the following differential equation: $- dC/dt = k C$

where C = concentration of carbon in blood as (cpm/ml.)

t = time in minutes

k = proportionality constant

Integrating the equation between limits, C_1 at t_1 and C_2 at t_2, the following equation is obtained:

$$\ln (C_1/C_2) = k (t_2-t_1)$$

$$\log (C_1/C_2) = k (t_2-t_1)/2.303$$

Therefore:

$$k/2.303 = (\log C_1 - \log C_2)/(t_2 - t_1)$$

By definition, the Phagocytic Index, (K) = k/2.303

Therefore: $K = (\log C_1 - \log C_2)/(t_2 - t_1)$

The (K) values were determined experimentally by plotting log concentration of carbon = log (cpm/ml. blood) vs. t = time in minutes. This procedure is illustrated in (Figure 2). The calculated values of (K) for rabbits maintained on vitamin E-supplemented and vitamin E-deficient diet are presented in (Table 1). Variations of the (K) values for each group of rabbits classified according to number of days on diet are illustrated graphically in (Figure 3). Group A, the rabbits were maintained on the diet 7 to 8 days. Group B, the rabbits were maintained on the diet 14-16 days. Group C, 19 to 25 days. Vitamin E-deficient rabbits of Group C were dystrophic.

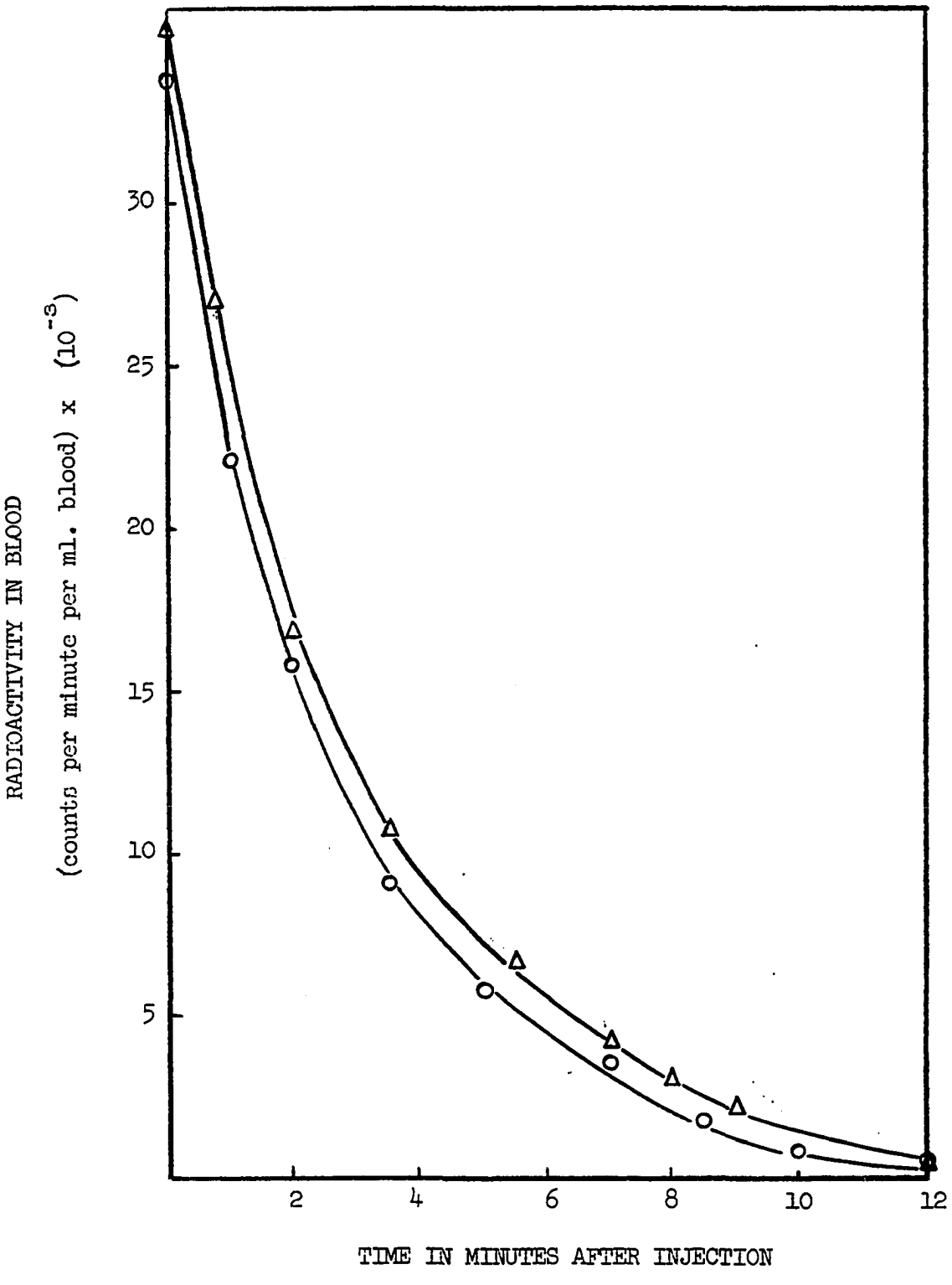


Figure 1.-Rate of disappearance of injected carbon C-14 from the blood of rabbits. o——o, dystrophic vitamin E-deficient rabbits. Δ——Δ , control rabbits.

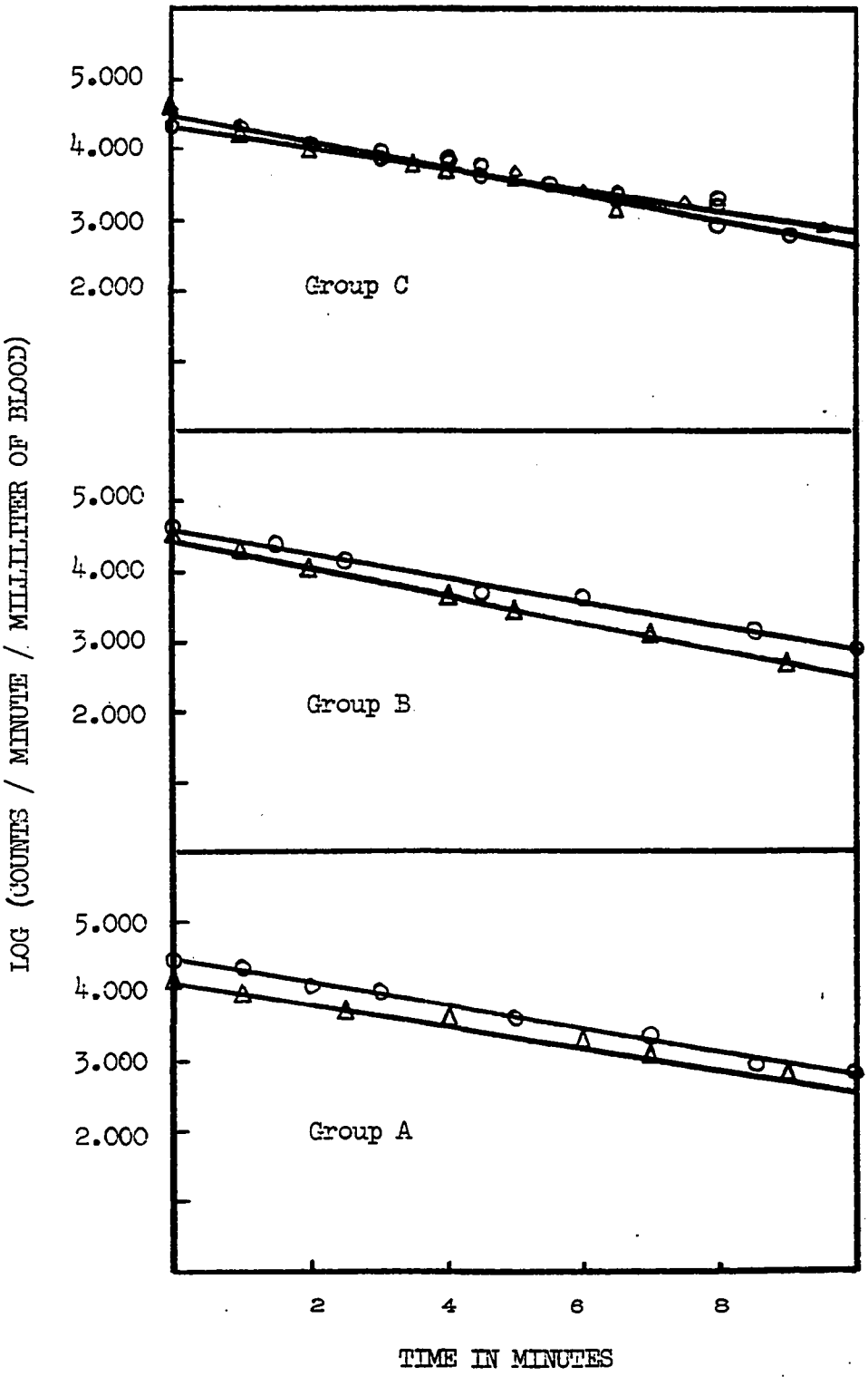


Figure 2.-Plot for the determination of the phagocytic index (K) in rabbits. o——o, vitamin E-deficient. Δ——Δ, vitamin E-supplemented. Groups A, B, and C are explained in text.

TABLE 1

PHAGOCYTIC INDEX (K) VALUES OF RABBITS

Group	Days on Diet (+)	Days on Diet (-)	(+) E (K)	(-) E (K)
A	7	7	0.143	0.145
A	7	8	0.137	0.142
A	7	8	0.141	0.140
B	14	14	0.148	0.138
B	14	15	0.144	0.145
B	14	15	0.138	0.138
B	15	16	0.146	0.145
C	26*		0.149*	
C	20	19	0.142	0.151#
C	20	20	0.139	0.145#
C	21	20	0.145	0.148#
C	22	21	0.138	0.140#
C	25	25	0.142	0.150#

Group A, rabbits were maintained 7-8 days on diet.
Group B, 14 to 16 days. Group C, 19 to 25 days.
(+) E, vitamin E-supplemented diet.
(-) E, vitamin E-deficient diet.
*Rabbit fed commercial pellets.
#Rabbits were dystrophic.

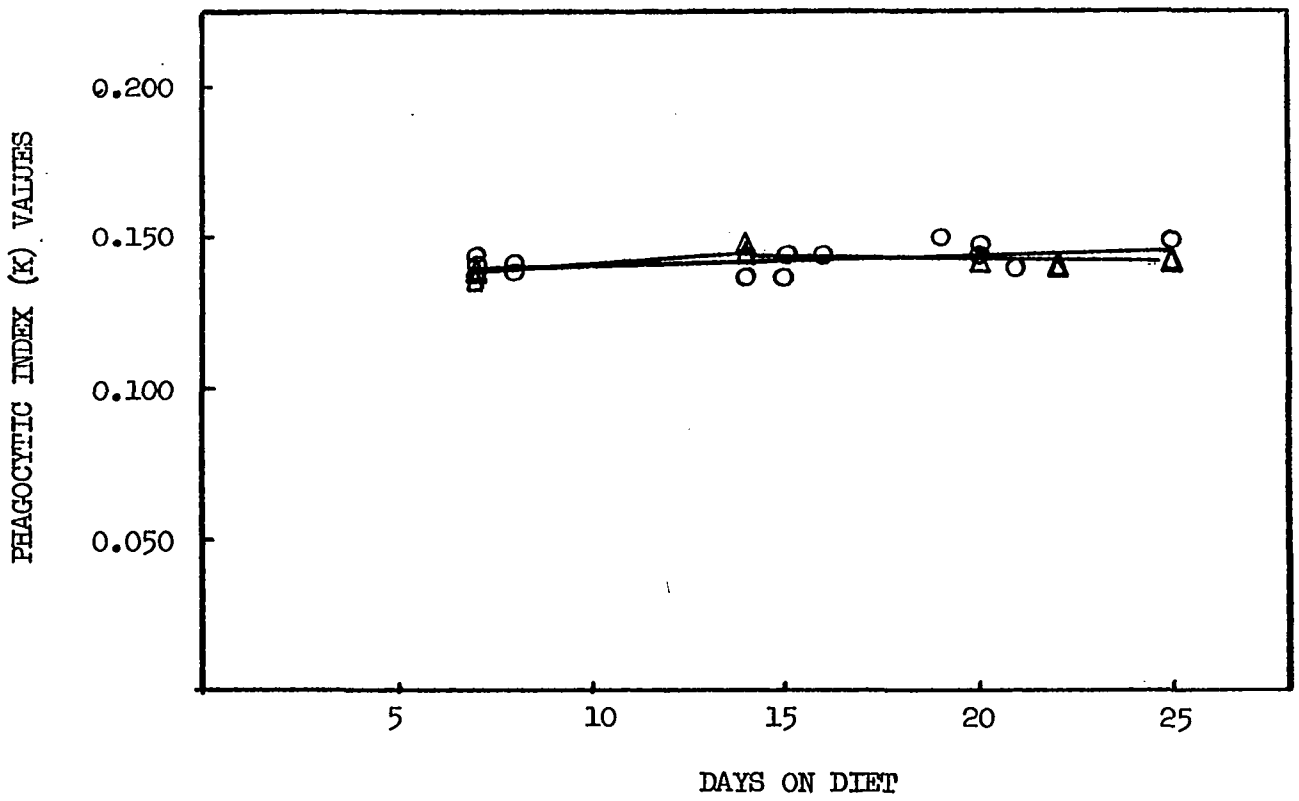


Figure 3.-Phagocytic index (K) values as a function of days on diet. o——o, rabbits fed vitamin E-deficient diet. Δ——Δ, rabbits fed vitamin E-supplemented diet.

In Vivo Uptake of Carbon C-14 by Livers, Spleens and Lungs of Rabbits

Two hours after the injection of carbon C-14 was completed, the rabbits were killed and bled then the organs were removed and their total weight was determined. The amount of radioactivity was determined from the tissue homogenates and was represented as counts/minute/gram tissue. Values obtained from liver, spleen and lungs are shown in Tables 2, 3 and 6. Because of the variations in the total weights of organs from different rabbits, therefore, a reference, grams organ percent rabbit was introduced. This reference is based on the assumption that the weight of a rabbit is directly proportional to the weights of its organs. By definition, grams organ percent rabbit = the weight in grams of an organ present in a theoretical rabbit weighing 100 grams. The amount of radioactivity present in organs obtained from rabbits of different weights can then be standardized. The calculations to obtain these values are as follows:

If (WL) = total weight of liver in grams
and (WR) = weight of the rabbit in grams
and (Q) = counts per minute per gram of liver
and (LG) = grams liver percent rabbit
then (LG) = 100 (WL)/(WR) and
(Q)·(LG) = the amount of radioactivity present in the liver of a rabbit weighing 100 grams.

Results of these calculations for liver and spleen are shown in Tables 2 and 3. (SG) represented grams spleen percent rabbit.

TABLE 2

IN VIVO UPTAKE OF CARBON C-14 BY RABBIT LIVERS

Group and Experiment Number		(+) E Rabbits		(-) E Rabbits	
		Weight of Liver in Grams	cpm/gm.	Weight of Liver in Grams	cpm/gm.
A	1	30.5	116.1 M	40.7	113.2 M
A	2	82	136.4 M	35	137.8 M
A	3	41.3	108.5 M	45.5	109.5 M
A	4	46.6	104.2 M	36.8	120.0 M
B	1	48	99.5 M	47.5	93.8 M
B	2	79	74.0 M	38.9	107.5 M
B	3	69	87.3 M	45.3	117.5 M
B	4	36	136.8 M	46.5	79.2 M
C*	1*	72.3*	135.5 M	--	--
C	2	48.2	156.2 M	45	114.0 M #
C	3	45	99.5 M	31	119.2 M #
C	4	65.7	156.0 M	43	116.0 M #
C	5	30.2	129.5 M	32	98.2 M #
C	6	67	70.1 M	41.5	115.6 M #
C	7	41	89.4 M	43.9	104.0 M #
C	8	--	--	34	97.2 M #

*Rabbit fed commercial diet. M, values multiplied by 1000.
cpm = counts per minute. Group A, 7 to 8 days on diet,
Group B, 14 to 16 days, Group C, 19 to 25 days.
Rabbits were dystrophic.

TABLE 2 (Continued)

Group and Experiment Number		(+) E Rabbits		(-) E Rabbits	
		(LG)	(Q)·(LG)	(LG)	(Q)·(LG)
A	1	3.6	418 M	4.0	453 M
A	2	3.1	424 M	3.2	438 M
A	3	3.9	422 M	4.2	462 M
A	4	4.1	438 M	3.6	434 M
B	1	4.5	448 M	5.9	436 M
B	2	6.0	443 M	4.2	452 M
B	3	5.0	437 M	3.7	433 M
B	4	3.3	452 M	5.5	435 M
C*	1*	3.4*	462 M	--	--
C	2	3.0	468 M	4.1	467 M #
C	3	4.7	466 M	3.8	454 M #
C	4	2.9	452 M	3.8	452 M #
C	5	3.3	427 M	4.6	452 M #
C	6	6.7	470 M	4.2	486 M #
C	7	5.2	468 M	4.4	456 M #
C	8	--	--	4.7	457 M #

(LG) = calculated weight of liver in grams per 100 grams rabbit.
(Q) = counts per minute per gram liver.
All other symbols are as before.

TABLE 3

IN VIVO UPTAKE OF CARBON C-14 BY RABBIT SPLEENS

Group and Experiment Number		(+) E Rabbits		(-) E Rabbits	
		Weight of Spleens in Grams	cpm/gm.	Weight of Spleens in Grams	cpm/gm.
A	1	0.300	630 M	0.470	535 M
A	2	1.150	532 M	0.340	810 M
A	3	0.335	726 M	0.335	805 M
A	4	0.480	577 M	0.315	794 M
B	1	0.570	416 M	0.410	493 M
B	2	0.550	509 M	0.330	688 M
B	3	0.680	488 M	0.680	443 M
B	4	0.480	515 M	0.980	201 M
C*	1*	1.470*	351 M	--	--
C	2	0.770	513 M	0.450	654 M
C	3	0.620	413 M	0.380	573 M
C	4	0.830	642 M	0.650	460 M
C	5	0.540	434 M	0.505	404 M
C	6	1.210	193 M	0.840	330 M
C	7	0.840	241 M	0.420	682 M
C	8	--	--	0.760	266 M

M, values are multiplied by 1000.
All other symbols are as before.

TABLE 3 (Continued)

Group and Experiment Number	(+) E Rabbits		(-) E Rabbits	
	(SG) Grams	(Q)·(SG)	Grams	(Q)·(SG)
A 1	0.035	22.2 M	0.047	25.2 M
A 2	0.044	23.4 M	0.031	25.1 M
A 3	0.031	22.5 M	0.031	24.9 M
A 4	0.044	25.3 M	0.031	24.6 M
B 1	0.053	22.0 M	0.051	25.2 M
B 2	0.042	21.4 M	0.036	24.7 M
B 3	0.049	23.8 M	0.057	25.3 M
B 4	0.044	22.7 M	0.115	23.2 M
C* 1*	0.068	23.7 M	--	--
C 2	0.048	24.6 M	0.042	27.5 M
C 3	0.065	26.8 M	0.047	26.9 M
C 4	0.036	23.2 M	0.059	27.1 M
C 5	0.060	26.0 M	0.072	29.1 M
C 6	0.120	23.3 M	0.085	28.1 M
C 7	0.107	25.6 M	0.042	28.7 M
C 8	--	--	0.105	28.1 M

(SG) = calculated weight of spleen per 100 grams rabbit.
M = values are multiplied by thousands.
(Q) = counts/minutes/gram spleen.

TABLE 4

IN VIVO UPTAKE OF CARBON C-14 BY RABBIT LIVERS
EXPRESSED AS PERCENT OF TOTAL INJECTED DOSE

Group		(+) E Rabbits	(-) E Rabbits
A	1	61.2	63.9
A	2	63.7	61.6
A	3	64.2	63.2
A	4	61.8	63.8
B	1	63.9	60.7
B	2	62.8	64.2
B	3	60.9	61.6
B	4	62.3	63.8
C*	1*	63.8*	--
C	2	64.5	65.6 #
C	3	62.2	64.8 #
C	4	65.2	63.5 #
C	5	60.4	62.2 #
C	6	65.2	67.8 #
C	7	64.2	63.2 #
C	8	--	65.3 #

Group symbols were explained previously.
= rabbits were dystrophic.
* = rabbit fed commercial pellet diet.

TABLE 5

IN VIVO UPTAKE OF CARBON C-14 BY RABBIT SPLEENS EXPRESSED AS PERCENT OF TOTAL INJECTED DOSE

Group		(+) E Rabbits	(-) E Rabbits
A	1	3.32	3.50
A	2	3.21	3.48
A	3	2.92	3.30
A	4	3.50	3.46
B	1	3.16	3.51
B	2	3.12	3.51
B	3	3.35	3.49
B	4	3.16	3.41
C*	1*	3.41*	--
C	2	3.46	3.72 #
C	3	3.56	3.82 #
C	4	3.43	3.82 #
C	5	3.60	4.10 #
C	6	3.24	3.85 #
C	7	3.48	3.98 #
C	8	--	4.05 #

All symbols are the same as before.

TABLE 6

IN VIVO UPTAKE OF CARBON C-14 BY RABBIT LUNGS EXPRESSED AS PERCENT OF TOTAL INJECTED DOSE

Group		(+) E Rabbits	(-) E Rabbits
A	1	1.23	1.7
A	2	1.12	1.17
A	3	1.52	1.35
B	1	1.63	1.38
B	2	1.26	1.28
B	3	1.31	1.6
C*	1	1.53*	--
C	2	1.16	1.35 #
C	3	1.14	1.42 #
C	4	1.71	1.48 #
C	5	1.23	1.1 #
C	6	--	1.4 #

All symbols were explained previously.

In Vivo Uptake of Carbon C-14 by Rabbit Skeletal Muscle

Muscle tissues were collected and homogenized according to procedures described before in Chapter II. The amount of radioactivity present in muscle homogenates were determined and expressed as counts per minute per gram. Since the total weight of muscle was not known, therefore, calculations to obtain values of grams muscle percent rabbit and the percentage uptake of radioactivity in muscle could not be possible.

Table 7 shows the results obtained from muscles of rabbits fed vitamin E-deficient and vitamin E-supplemented diet. These were designated as (-) E and (+) E, respectively. Rabbits were fed ad libitum except those designated by (P) which were pair fed. Pair feeding indicates that (+) E rabbits were fed the same amount of food which was consumed by the (-) E rabbits. To observe the effect of reversal in diet, a group was maintained on vitamin E-deficient diet for 15 days then the diet was changed before appearance of muscular dystrophy symptoms to a vitamin E-supplemented diet for 7 days. Rabbits in this group did not show signs of muscular dystrophy, indicating that vitamin E prevented the occurrence of the disease (Table 8).

The dystrophic rabbits were designated by the symbol (#) and the extent of muscular weakness was indicated by an arbitrary index based on the number of times after which they were unable to right themselves when placed on their backs.

Graphical representation of these data is shown in Figure 4. The amount of radioactivity present expressed in counts per minute per gram muscle was plotted as a function of days on diet.

TABLE 7

IN VIVO UPTAKE OF CARBON C-14 BY RABBIT MUSCLE

Group	Days on Diet		Counts Per Minute Per Gram	
	(+) E	(-) E	(+) E	(-) E
A	7	7	48	80
A	7	8	32	50
A	7	8	30	40
A	8	8	42	48
A	7 P	7	63 P	92
A	7 P	7	40 P	50
A	7 P	7	52 P	42
B	14	14	38	136
B	14	15	48	96
B	14	15	36	112
B	15	16	40	80
C	20	19	32	192 #
C	20	20	40	172 #
C	21	20	42	200 #
C	22	21	36	164 #
C	23	22	60	320 #
C	-	25	-	250 #
C	26*		52*	

(*) = rabbit fed commercial pellets. (P) = rabbits were pair fed. (#) = rabbits were dystrophic.

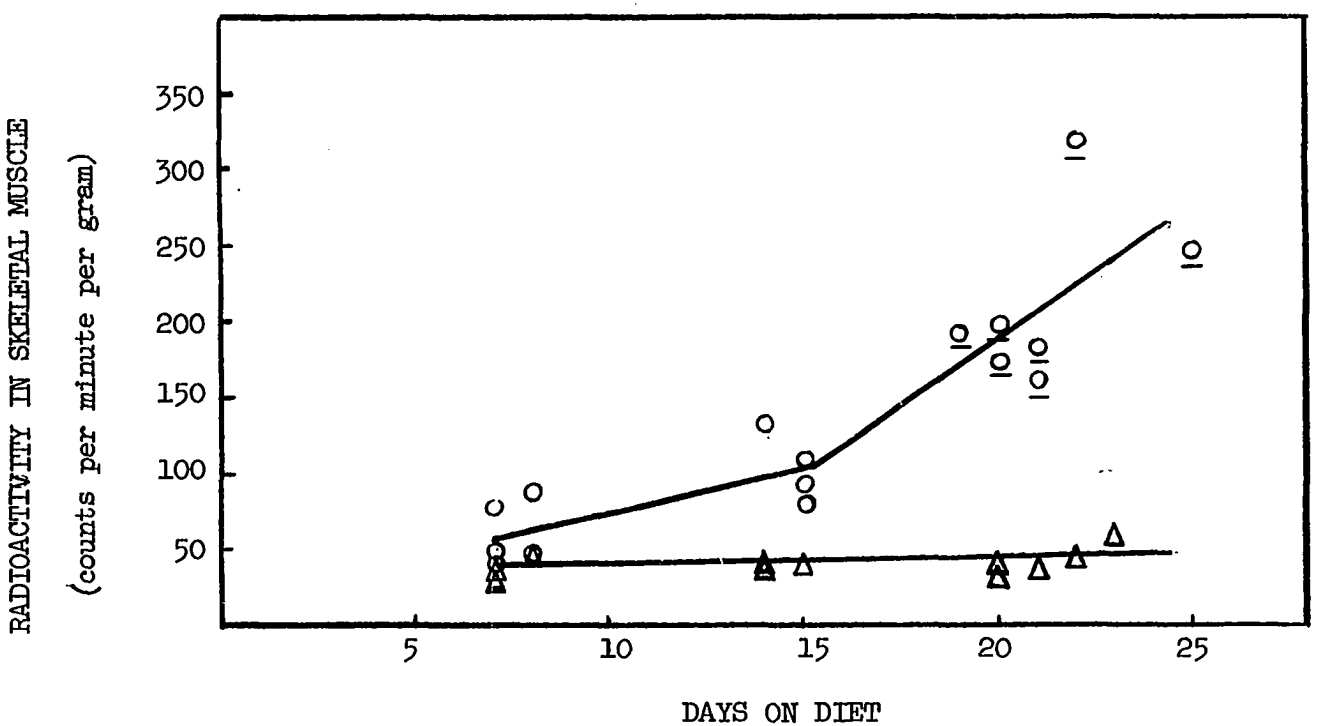


Figure 4.-In vivo uptake of injected amorphous carbon C-14 by rabbit skeletal muscle as a function of days on diet. o——o, vitamin E-deficient rabbits. Underlined circles indicate the rabbits were dystrophic. Δ——Δ, control rabbits fed vitamin E-supplemented diet.

TABLE 8

EFFECT OF PAIR FEEDING AND DIET REVERSAL ON THE IN VIVO UPTAKE OF CARBON C-14 BY RABBIT MUSCLE

Days on Diet		Counts Per Minute Per Gram	
Pair Fed (+) E Diet	Fed *ad libitum* (+) E Diet	Pair Fed	*Ad libitum*
7	7	63	48
7	8	40	32
7	8	52	30
22	22	60	42
22	22	40	36

DIET REVERSAL	
	Counts Per Minute Per Gram
Two Rabbits fed *ad libitum* (-) E diet for 15 days then the diet was changed to (+) E diet and fed *ad libitum* for 7 days	100 80

Lysosomal Enzymes in Rabbit Skeletal Muscle

In this report, lysosomal enzymes are defined as those hydrolases which favor acid pH media and which seem to be associated with special cytoplasmic particles, the lysosomes. This definition is based on the intracellular distribution of these enzymes in liver cells. Presence of lysosomes in muscle cells have not been demonstrated. An essential property of lysosomal hydrolases is that they exhibit structure-linked latency of activation and solubilization. To obtain total activities (free + bound) of lysosomal hydrolases, special treatments were required as has been described earlier in Chapter II. It should be pointed out that crushed muscle tissues are composite of various types of cells. In addition to muscle cells (fibers and myoblasts) there are fibroblasts fixed macrophages and leucocytes.

Skeletal muscles were obtained from rabbits maintained on vitamin E-supplemented or vitamin E-deficient diet and the enzymic activities were measured as a function of the number of days on diet. Total activities of the following enzymes were determined from muscle homogenates: acid phosphatase, β-glucuronidase, cathepsin, neuraminidase and lysophosphatidase. Acid phosphatase, cathepsin and β-glucuronidase are among the original lysosomal enzymes described by De Duve et al.(14). Neuraminidase and lysophosphatidase were investigated in this laboratory and found to be related to lysosomes as will be shown in a later section. The results are shown in (Tables 9, 10, 11, 12 and 13) and were illustrated graphically in (Figures 5, 6, 7, 8 and 9) to demonstrate the enzymic changes as function of days on diet.

TABLE 9

TOTAL ACTIVITY OF RABBIT MUSCLE ACID PHOSPHATASE

Group	Days on Diet (+)	Days on Diet (-)	Units*/gram/hour (+) E	Units*/gram/hour (-) E	Specific Activity (+) E	Specific Activity (-) E
A	7	7	12.2	13.2	0.090	0.100
A	7	8	11.0	10.2	0.085	0.125
A	7	8	10.8	14.0	0.082	0.106
A	8	8	13.4	12.8	0.102	0.097
B	14	14	12.0	19.5	0.110	0.150
B	14	15	13.2	17.8	0.120	0.140
B	15	15	12.8	18.5	0.098	0.147
C	20	19	13.5	36.0	0.104	0.300 #
C	20	20	13.8	28.5	0.104	0.240 #
C	21	20	12.2	39.0	0.093	0.320 #
C	22	21	12.0	32.0	0.091	0.280 #
C	22	22	14.2	35.0	0.107	0.300 #
C	25	25	13.0	38.0	0.098	0.330 #

*One unit of acid phosphatase activity = 10^{-6} M phosphate liberated. Rabbit groups are classified according to number of days on diet, A, 7 to 8 days, B, 14 to 16 days, C, 19 to 25 days. #, rabbits were dystrophic.
Specific activity = units/mg. protein/hour.

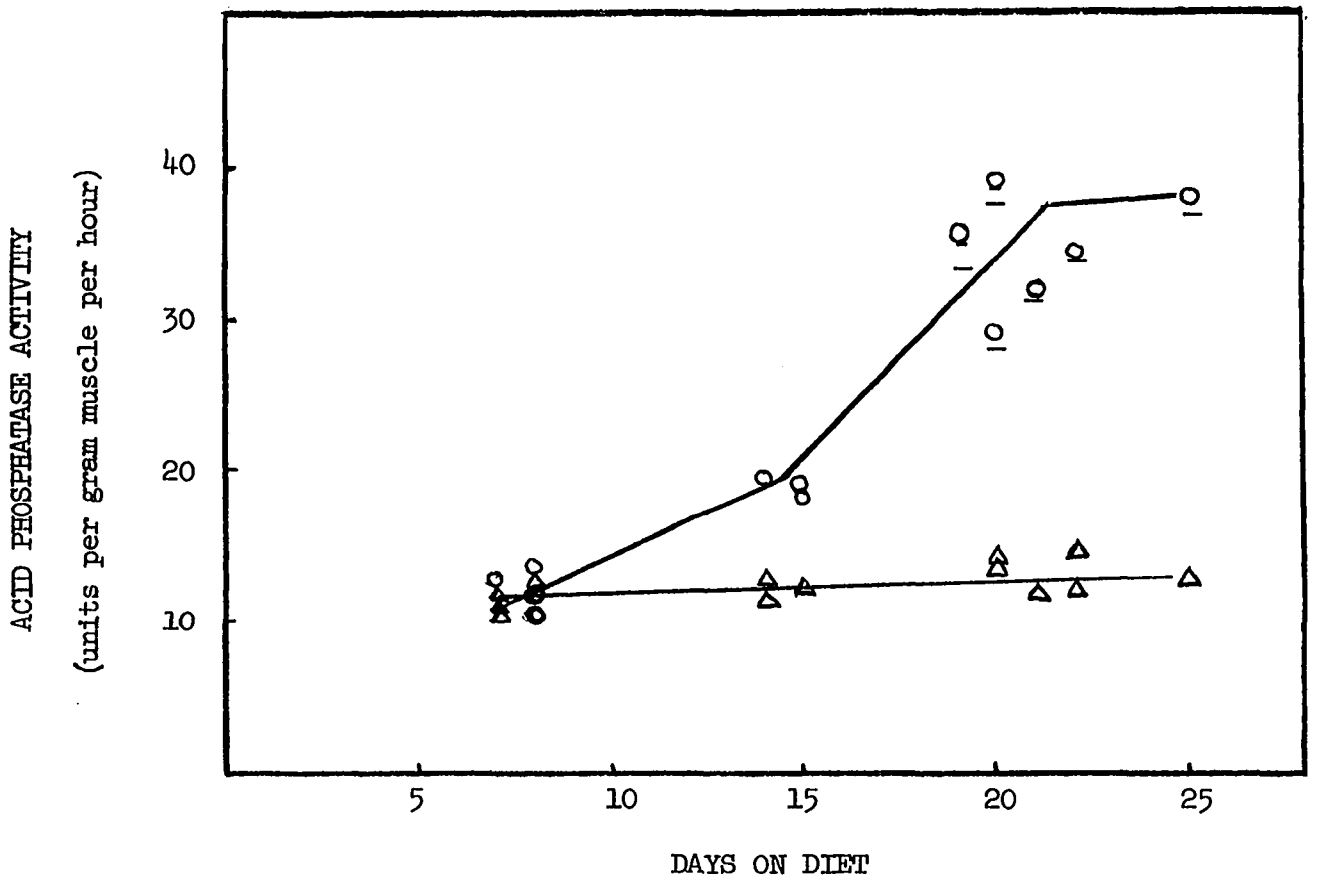


Figure 5.-Acid phosphatase activity of rabbit skeletal muscle as a function of days on diet. o——o, vitamin E-deficient rabbits. Δ —— Δ, control rabbits fed vitamin E-supplemented diet. Unit of activity = one micromole inorganic phosphate liberated. Underlined circles indicate the rabbits were dystrophic.

TABLE 10

TOTAL ACTIVITY OF RABBIT MUSCLE CATHEPSIN
(PROTEOLYTIC ACTIVITY AT pH 3.8)

Group	Days on Diet		O.D. units/gram/hour		
	(+)	(-)	(+) E	(-) E	
A	7	7	11.70	12.50	
A	7	8	11.50	12.00	
A	8	8	12.00	12.20	
A-P	7	7	12.30 P	11.70	
A-P	7	7	13.20 P	12.60	
A-P	7	7	11.80 P	12.50	
B	14	14	12.20	21.00	
B	14	15	11.50	21.50	
B	15	15	13.00	19.80	
C	20	19	14.50	46.50 #	
C	20	20	12.70	42.20 #	
C	21	20	12.00	48.00 #	
C	22	21	13.80	50.50 #	
C	22	22	12.80	53.20 #	
C	25	25	12.40	47.50 #	
Diet change			15.50 P		26.5 DC
Diet change			13.50 P		24.0 DC

One O.D. unit = change in optical density of 0.100.
DC = Diet change, P = Pair fed.

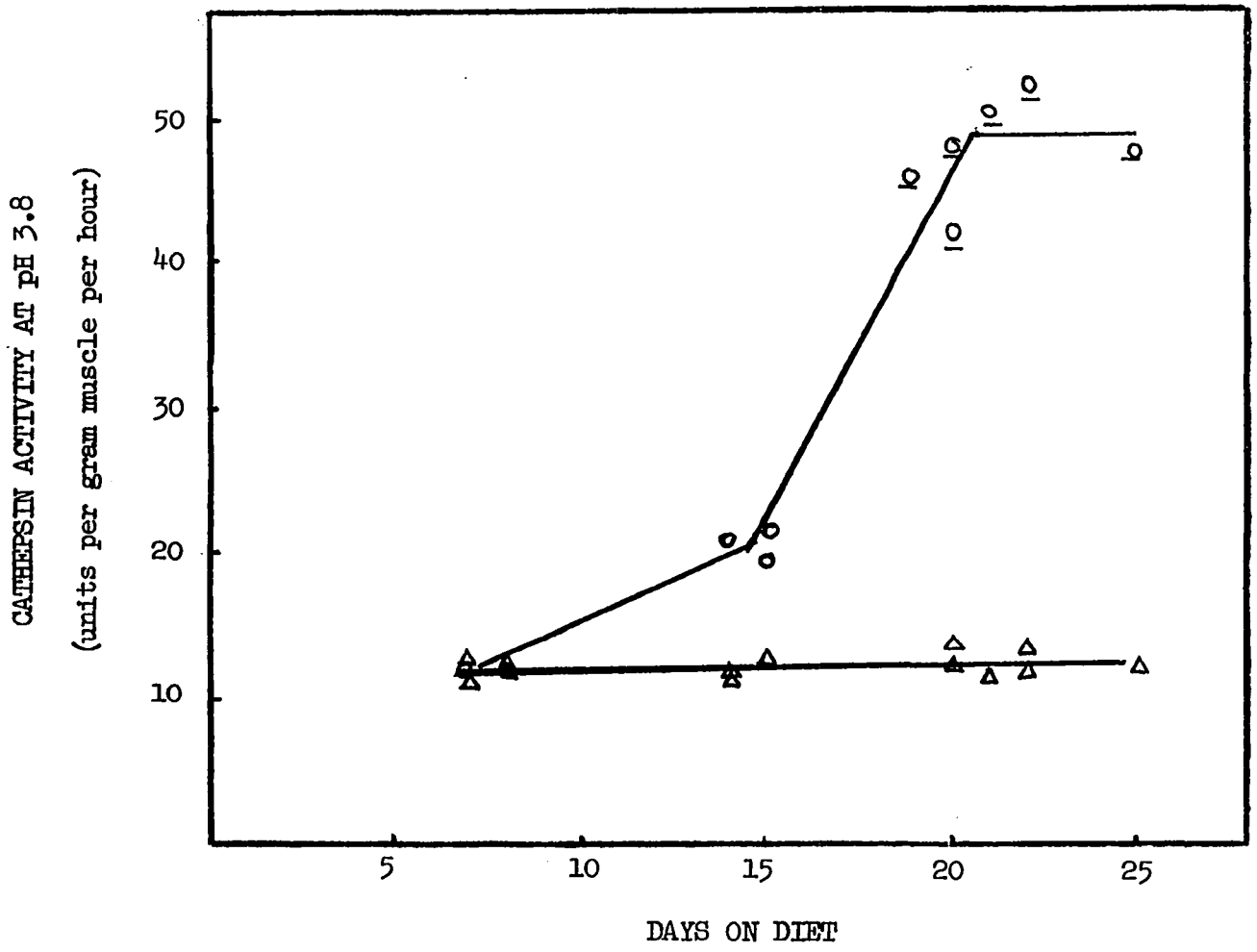


Figure 6.-Cathepsin activity at pH 3.8 of rabbit skeletal muscle as a function of days on diet. o——o, vitamin E-deficient rabbits. △——△, control rabbits fed vitamin E-supplemented diet. Unit of activity = change of optical density at 280 mμ of 0.100
Underlined circles indicate the rabbits were dystrophic.

TABLE 11

TOTAL ACTIVITIES OF RABBIT SKELETAL MUSCLE
β-GLUCURONIDASE

Group	Days on Diet (+)	Days on Diet (-)	Units*/gram/hour (+) E	Units*/gram/hour (-) E	Specific Activity (+) E	Specific Activity (-) E
A	7	7	1.25	2.10	0.0096	0.0160
A	7	8	1.60	1.80	0.0124	0.0139
A	8	8	1.50	2.00	0.0113	0.0153
A	7	7	1.75	1.70	0.0133	0.0129
A	7	7	1.65 P	1.50	0.0127	0.0125
A	7	7	1.80 P	1.80	0.0138	0.0139
A	7	7	1.35 P	1.65	0.0107	0.0130
B	14	14	1.45	4.10	0.0110	0.0319
B	14	15	1.80	3.60	0.0138	0.0283
B	15	15	1.70	5.00	0.0129	0.0396
C	20	19	1.65	12.68 #	0.0127	0.1050 #
C	20	20	1.70	11.50 #	0.0127	0.0960 #
C	21	20	1.55	14.25 #	0.0117	0.1190 #
C	22	21	1.55	12.50 #	0.0117	0.1080 #
C	22	22	1.85	14.00 #	0.0140	0.1190 #
C	25	25	1.80	14.80 #	0.0135	0.1280 #
Diet change			1.60 P	6.25 DC	0.0126 P	0.0495 DC
			1.60 P	5.70 DC	0.0126 P	0.0440 DC

*One unit of activity = one micromole phenolphthalien liberated.

All other symbols were explained before. DC = Diet change, explained in text. P = Pair-fed.

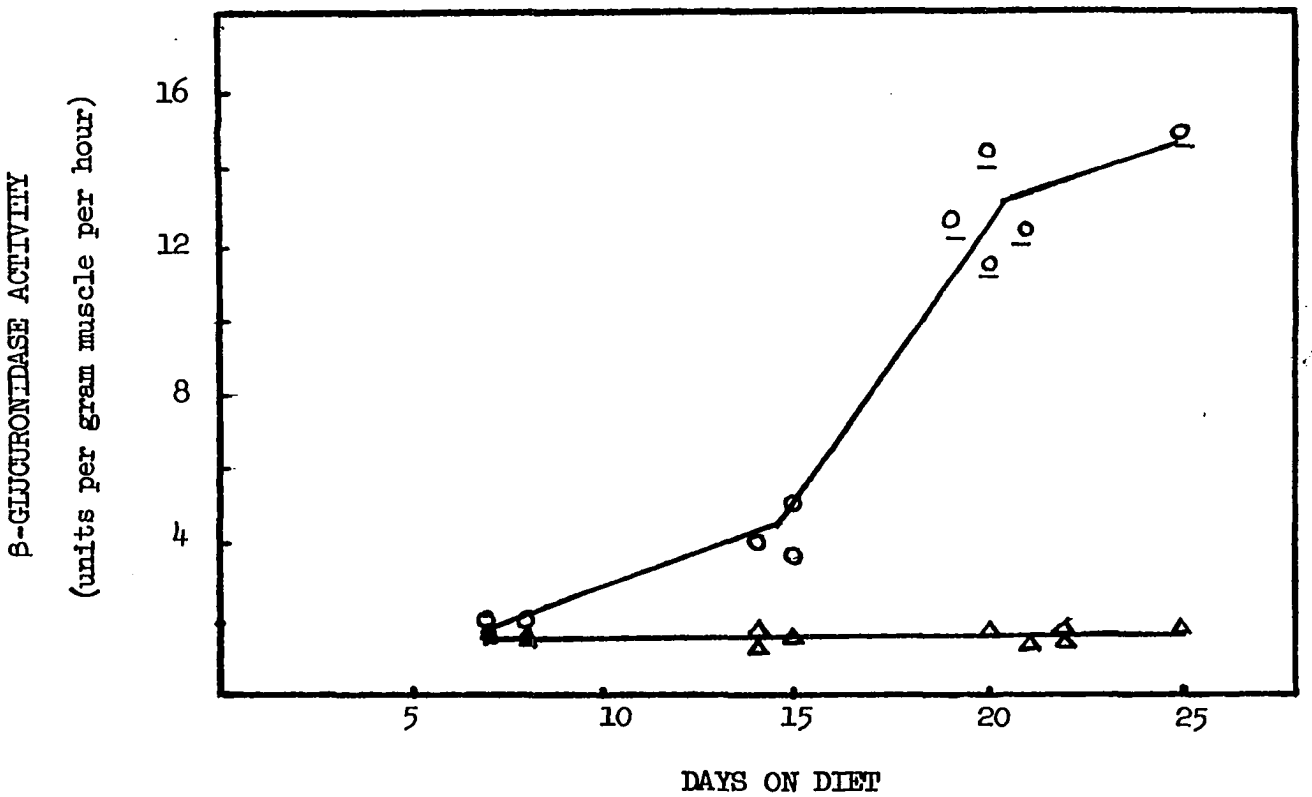


Figure 7.- β-Glucuronidase activity of rabbit skeletal muscle as a function of days on diet. o——o, vitamin E-deficient rabbits. Δ——Δ, control rabbits fed vitamin E-supplemanted diet. Unit of activity = one micromole phenolphthalein liberated. Underlined circles indicate the rabbits were dystrophic.

TABLE 12

TOTAL ACTIVITIES OF RABBIT SKELETAL MUSCLE NEURAMINIDASE

Group	Days on Diet		Units*/gram/3 hours	
	(+)	(-)	(+) E	(-) E
A	7	7	102	102
A	7	8	96	110
A	8	8	84	86
B	14	14	112	168
B	14	15	104	176
B	15	15	112	184
C	20	19	103	342 #
C	20	20	110	344 #
C	21	20	98	320 #
C	22	21	94	308 #
C	22	22	118	278 #

*One unit of activity = one millimicromole (10^{-9}) M neuraminic acid released.
Three hours of incubation at 37°C were required to obtain measurable values.
= Rabbits were dystrophic.

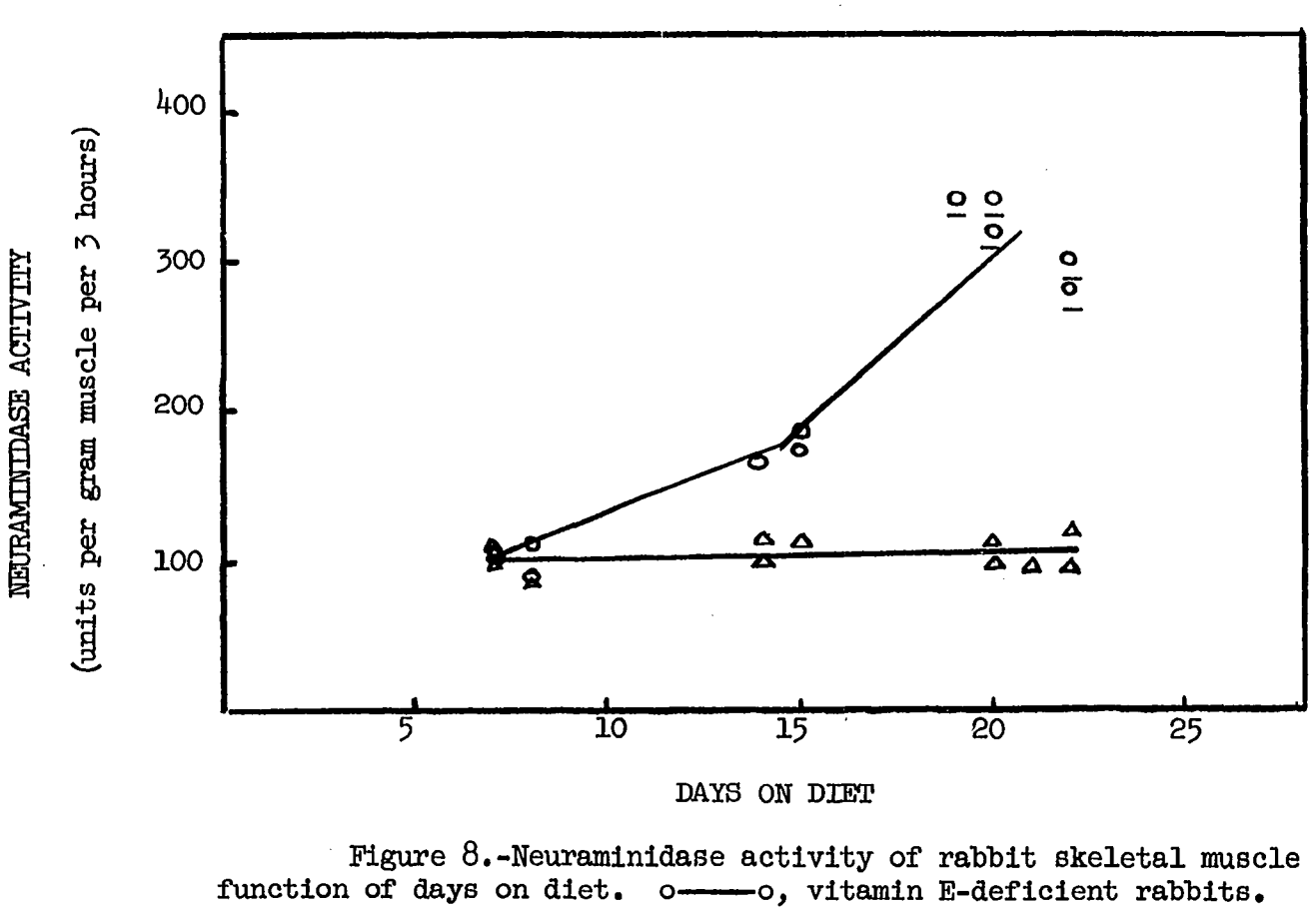


Figure 8.-Neuraminidase activity of rabbit skeletal muscle as a function of days on diet. o——o, vitamin E-deficient rabbits. Δ——Δ, vitamin E-sufficient rabbits. Unit of activity = one millimicromole of neuraminic acid released. Underlined circles indicate the rabbits were dystrophic.

TABLE 13

TOTAL ACTIVITY OF RABBIT SKELETAL MUSCLE LYSOPHOSPHATIDASE (LYSOPHOSPHATIDE DESTROYING ENZYME)

Group	Days on Diet		Units*/gram/hour	
	(+)	(-)	(+) E	(-) E
A	7	-	12	-
A	7	8	7	7
A	7	7	7 P	8
A	7	7	8 P	7
A	7	7	12 P	8
B	14	14	-	60
B	14	15	12	48
C	20	20	12	72 #
C	22	21	8	60 #
C	23	22	8	72 #
C	24	23	14	72 #
C	26	25	12	86 #
C	22	22	12 P	48 DC
C	22	22	14 P	52 DC

*One unit of activity = one micromole lysophosphatides consumed.

All other symbols were explained previously.

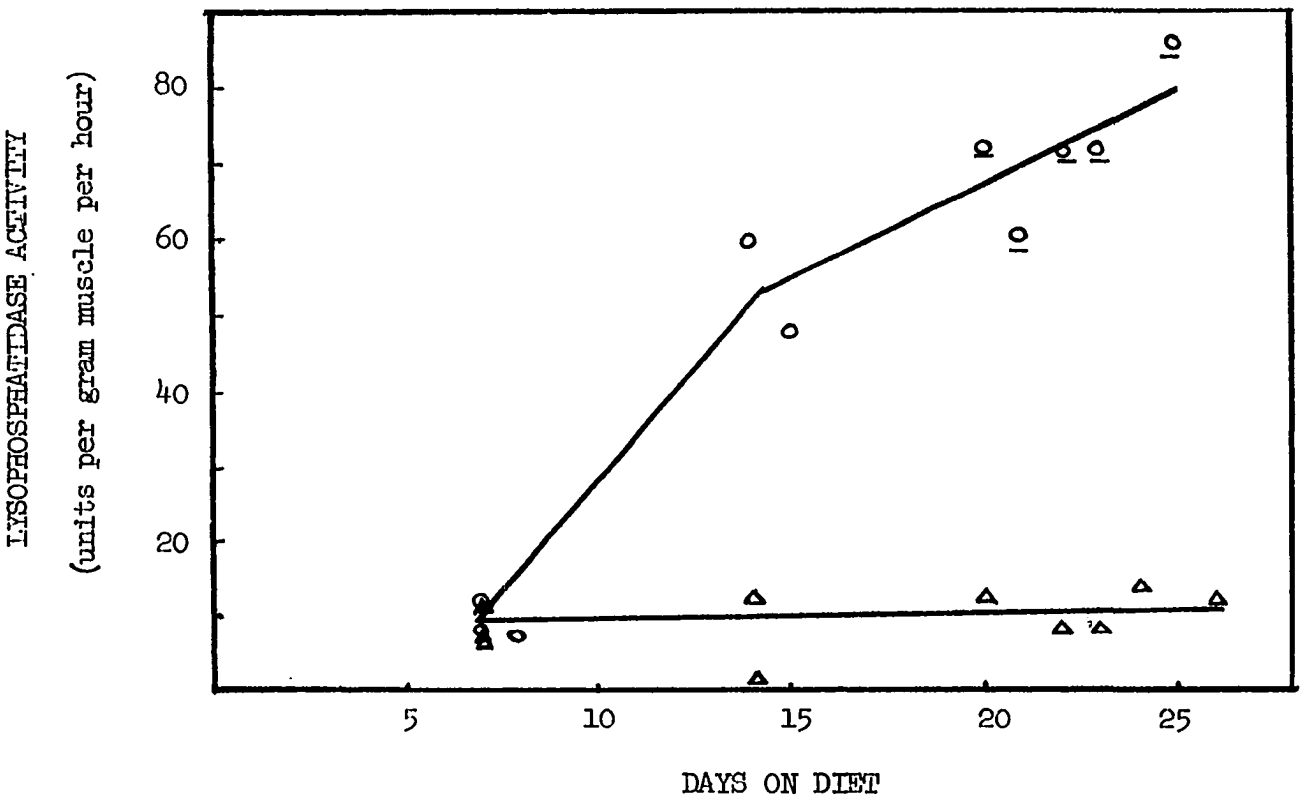


Figure 9.-Lysophosphatidase activity of rabbit skeletal muscle as a function of days on diet. o——o, vitamin E-deficient rabbits. Δ ——Δ, control rabbits fed vitamin E-supplemented diet. Unit of activity = one micromole of lysophosphatides consumed. Underlined circles indicate the rabbits were dystrophic.

Intracellular Distribution of Neuraminidase and Cathepsin in Rabbit Skeletal Muscle

Skeletal muscle was obtained from dystrophic and non-dystrophic rabbits which were maintained on vitamin E-deficient and vitamin E-supplemented diet. Muscle tissues were homogenized in 0.25 M sucrose and the slurry of the crushed tissues were fractionated by differential centrifugation as described earlier in Chapter II. Fraction A, which constituted the nuclei, myofibrils, connective tissue fragments, erythrocytes and unbroken cells, was sedimented at 1000 x g x 10 minutes. Fraction B, which was considered to contain the sarcosomes (muscle mitochondria), was centrifuged at 3500 x g x 10 minutes. Fraction C, which was assumed to contain the lysosomes was sedimented at 20,000 x g x 10 minutes. Fraction D, which was assumed to contain muscle microsomes was sedimented at 104,000 x g x 60 minutes. Fraction E, represented the soluble fraction. Only the cytoplasmic extract and its constituents were analyzed. The cytoplasmic extract is defined here as that part of the original muscle homogenate which was not sedimented at 1000 x g x 10 minutes and which is composed of Fractions B, C, D, and E. In each fractionation experiment, it was repeatedly observed that if equivalent amounts of dystrophic and non-dystrophic muscle tissues were fractionated, the amount of sediment in Fraction C obtained from the dystrophic muscle was about three times more than that obtained from the non-dystrophic muscle. This ratio, 3:1, (dystrophic:non-dystrophic) was based on turbidity measurements of the diluted sediments.

Total activities of neuraminidase and cathepsin in the cytoplasmic

extract and the subcellular fractions were determined according to the methods presented earlier.

Tables 14 and 15 show the relative specific activities of neuraminidase and cathepsin in the subcellular fraction. The relative specific activity is defined as the ratio of the specific activity of an enzyme in a subcellular fraction to the specific activity of the enzyme in the extract before fractionation. Values of the relative specific activity can be calculated from individual specific activities in the fractions according to the definition above or can be calculated as follows:

$$\text{Relative specific activity} = \frac{\% \text{ activity in the fraction}}{\% \text{ protein in the fraction}}$$

This method of calculation was found convenient to illustrate the results graphically as shown in (Figures 10 and 11) where the relative specific activity was plotted against percent protein in the fractions. The relative specific activity of the cytoplasmic extract before fractionation = 1 and the percent protein = 100.

TABLE 14

INTRACELLULAR DISTRIBUTION OF NEURAMINIDASE IN SKELETAL MUSCLE OF DYSTROPHIC AND NON-DYSTROPHIC RABBITS

Subcellular Fractions	% Activity		% Protein		Relative Specific Activity	
	(+) E	(-) E	(+) E	(-) E	(+) E	(-) E
B (4,4)	16.1	8.0	7.8	11.4	2.1	0.7
C (4,4)	34.0	20.6	5.3	9.5	6.6	2.2
D (4,4)	27.0	20.2	18.7	16.5	1.5	1.2
E (4,4)	22.3	51.2	68.2	62.6	0.35	0.82

Subcellular fractions are referred to fractions obtained by fractionating the cytoplasmic extract as described in the text. Fraction B, was sedimented at 3500 x g x 10 minutes. Fraction C, sedimented at 20,000 x g x 10 minutes. Fraction D, sedimented at 104,000 x g x 60 minutes. Fraction E, was the final supernate.
(+) E = rabbits were non-dystrophic and fed vitamin E-sufficient diet.
(-) E = rabbits were dystrophic fed vitamin E-deficient diet.
Relative specific activity = percentage of total activity/percentage of total protein. The values are mean averages of 4 experiments (+) E and (-) E shown as (4,4).

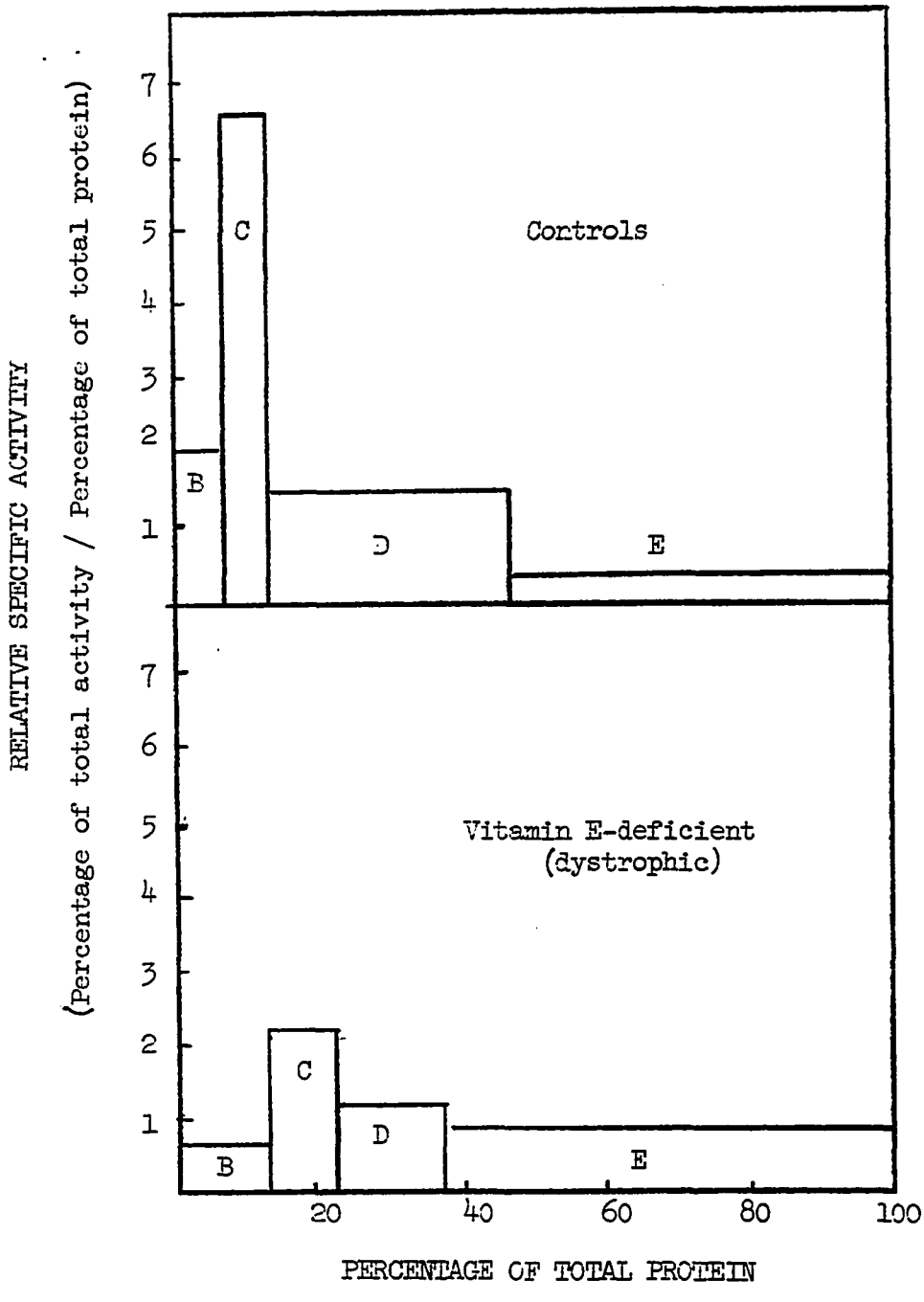


Figure 10.-Distribution patterns of neuraminidase in rabbit skeletal muscle. Upper graph, vitamin E-sufficient rabbits. Lower graph, vitamin E-deficient rabbits (dystrophic). B, C, D, and E, represent the intracellular fractions explained in text.

TABLE 15

INTRACELLULAR DISTRIBUTION OF CATHEPSIN (pH 3.8) IN SKELETAL MUSCLE OF DYSTROPHIC AND NON-DYSTROPHIC RABBITS

Subcellular Fractions	% Activity		% Protein		Relative Specific Activity	
	(+) E	(-) E	(+) E	(-) E	(+) E	(-) E
B (4,4)	23.1	18.8	7.8	11.4	2.9	1.65
C (4,4)	22.8	11.9	5.3	9.5	4.3	1.25
D (4,4)	19.9	14.8	18.7	16.5	1.1	0.9
E (4,4)	34.2	54.5	68.2	62.6	0.5	0.9

Subcellular fractions are referred to fractions obtained from the cytoplasmic extract. Fraction B, was sedimented at 3500 x g x 10 minutes. Fraction C, at 20,000 x g x 10 minutes. Fraction D, at 104,000 x g x 60 minutes. Fraction E is the final supernate.
(+) E, indicates that the rabbits were non-dystrophic and were fed vitamin E-sufficient diet. (-) E, the rabbits were dystrophic and fed vitamin E-deficient diet.
% Activity and % Protein are referred to the cytoplasmic extract as 100.
Relative Specific Activity = % Activity/% Protein.
Values are averages of 4 experiments (+) E and 4 experiments (-) E shown as (4,4).

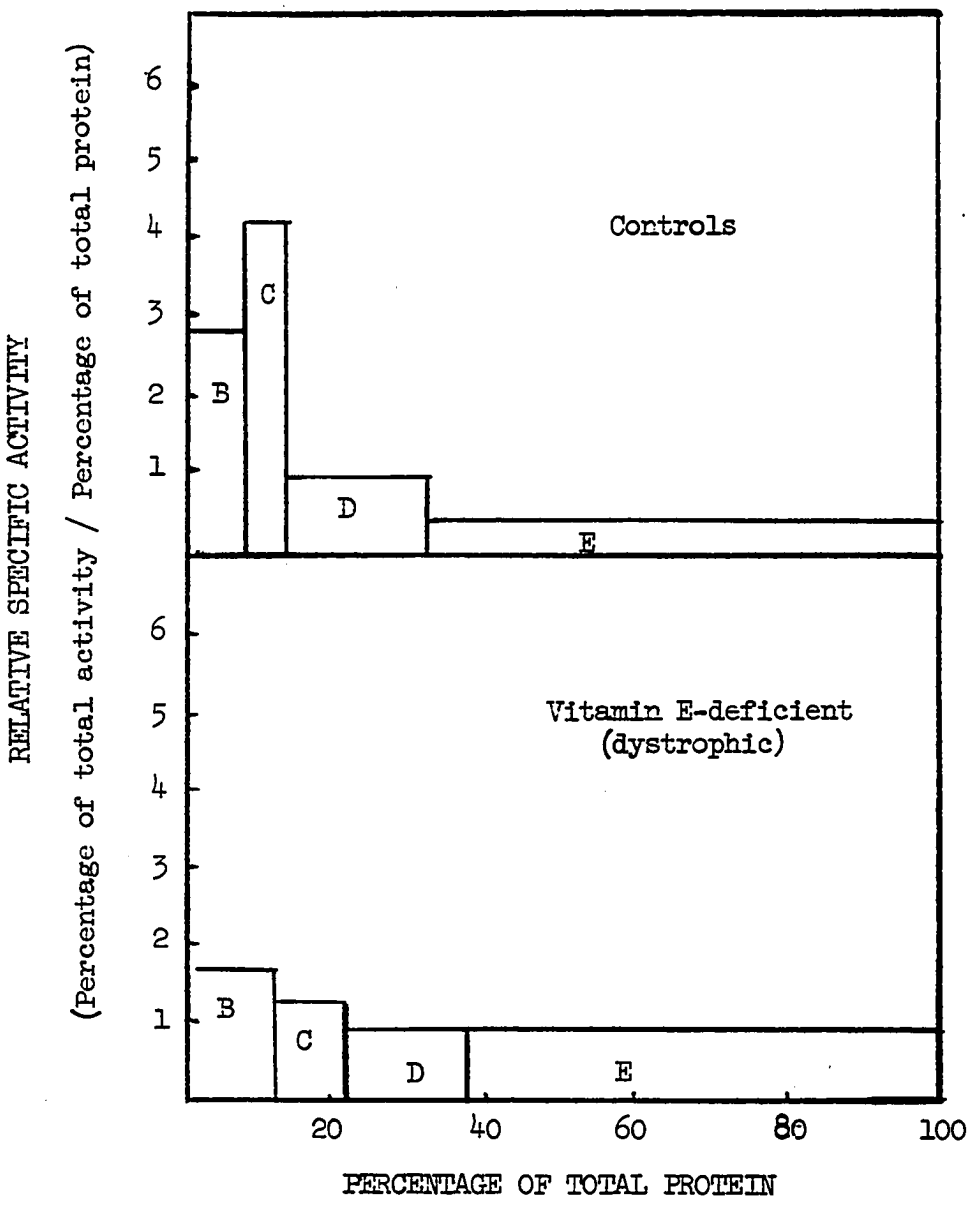


Figure 11.-Distribution patterns of cathepsin in rabbit skeletal muscle. Upper graph, vitamin E-sufficient rabbits. Lower graph, vitamin E-deficient rabbits (dystrophic). B, C, D, and E, represent the intracellular fractions explained in text.

Lipophilic Sialic Acid Containing Compounds in Rabbit Skeletal Muscle

Muscle tissues were obtained from rabbits that were maintained on vitamin E-deficient or vitamin E-supplemented diet. Chloroform-methanol (2:1) extracts from the tissues have been prepared by the method described before and the sialic acid containing compounds were detected by chromatography on thin layer of silica gel G according to the procedure outlined in Chapter II. The water methanol phase obtained from washing the crude lipid extract was dialyzed and concentrated. Samples were then taken to measure free sialic acid after acid hydrolysis for 1 hour at 80°C by the thiobarbituric acid reaction of Warren (79). Free sialic acid was not detected before acid hydrolysis. The color produced by the thiobarbituric acid reaction of the hydrolysed samples was extracted with cyclohexanon and the spectrum was determined in a Cary recording spectrophotometer between 450 mu and 650 mu. The obtained spectrum was identical with authentic N-acetylneuraminic acid with a maximum absorption at 551 mu and a shoulder at 520 mu. The optical density ratio (549 mu/532 mu) was 2.2. The amount of sialic acid (free) was calculated according to the equation suggested by Warren (79).

Micromoles sialic acid = (optical density at 549 mu) x 0.09 - (optical density at 532 mu) x 0.033.

Table 16, shows the results of sialic acid compounds expressed as micromoles sialic acid per gram muscle. Rabbits that were fed seven days on vitamin E-deficient diet (non-dystrophic) were young rabbits. The second group, 21 to 23 days on diet were older and bigger rabbits.

TABLE 16

EXTRACTED LIPOPHILIC SIALIC ACID COMPOUNDS
FROM RABBIT SKELETAL MUSCLE

Days on Diet		Weight of Rabbits (gm)		Micromoles Sialic Acid Per Gram Muscle Wet Weight	
(+) E	(-) E	(+) E	(-) E	(+) E	(-) E
7	7	870	895	0.0096	0.0088
7	7	890	910	0.0064	0.0078
7	7	930	920	0.0076	0.0058
17	17	1855	1878	0.0186	0.1928 (dystrophic)
24	24	1510	1560	0.0136	0.2146 (dystrophic)
35	35	1644	2085	0.0164	0.1970 (dystrophic)

(+) E and (-) E indicate vitamin E-supplemented and vitamin E-deficient rabbits.

Lysosomal Enzymes in Rabbit Livers and Spleens

Definition of lysosomal enzymes has been given before in the section on muscle lysosomes. Livers from rabbits fed the experimental diets were assayed for acid phosphatase, cathepsin at pH 3.8, β-glucuronidase, neuraminidase and lysophosphatidase, to observe if there are any changes occurring in the activities of lysosomal enzymes when the rabbits become dystrophic. The assay procedures of these enzymes were described in Chapter II. Total activities are reported and the data for livers are shown in Tables 17, 18, 19, 20 and 21, and illustrated in Figures 12 and 13. Data for spleens are shown in Tables 22, 23 and 24, and illustrated in Figure 14.

Intracellular Distribution of Acid Hydrolase in Livers of Dystrophic and Non-dystrophic Rabbits

Liver tissues were homogenized in 0.25 M sucrose and fractionated by centrifuging according to the procedures described in Chapter II. The total activity of each fraction except the first fraction (nuclear and debris) was assayed and the results were expressed as units per gram equivalent (the units of activity that would have been obtained from fractionating one gram of liver). The protein content of the fractions was expressed as milligrams protein per gram liver (equivalent). The results are shown in Tables 25, 26, 27, 28, 29 and 30, and illustrated in Figures 15, 16, 17, 18 and 19. The values are reported as mean averages of four pairs of fractionation experiments. The relative specific activity was chosen as the index to show the relationship between the subcellular fractions and the original homogenates as has been described before in muscle fractionation.

TABLE 17

TOTAL ACTIVITIES OF ACID PHOSPHATASE IN LIVER

Days on Diet		Total Activity		Specific Activity	
(+) E	(-) E	(+) E	(-) E	(+) E	(-) E
7	7	170	178	0.86	1.00
7	8	188	194	0.93	0.95
8	8	167	182	0.89	0.96
14	14	184	176	0.96	0.94
14	15	192	208	0.94	1.08
15	15	175	212	0.92	1.16
20	19	196	222	1.00	1.24 #
20	20	201	246	0.98	1.21 #
20	21	173	208	0.93	1.08 #
21	21	185	256	1.02	1.46 #
22	22	177	263	0.86	1.47 #

Total activity = micromoles inorganic phosphate liberated per gram liver per hour.
Specific activity = micromoles phosphate liberated per milligram protein per hour.
(#) = rabbits were dystrophic.
(+) E = vitamin E-sufficient diet.
(-) E = vitamin E-deficient diet.

TABLE 18

CATHEPTIC ACTIVITY (pH 3.8) IN RABBIT LIVERS

Days on Diet		Total Activity (Units/gram liver/hour)	
(+) E	(-) E	(+) E	(-) E)
7	7	91	99
7	8	94	95
14	14	96	98
14	15	112	127
20	19	97	124 #
20	20	95	136 #
21	20	89	118 #
21	21	106	142 #
22	22	98	128 #

Units of activity are expressed in optical density units at 280 mu = change in optical density at 280 mu of 0.100 using acid denatured hemoglobin as the substrate.
(#) = rabbits were dystrophic

TABLE 19

TOTAL ACTIVITIES OF β-GLUCURONIDASE IN LIVER

Days on Diet		Total Activity		Specific Activity	
(+) E	(-) E	(+) E	(-) E	(+) E	(-) E
7	7	35.6	37.2	0.18	0.21
7	8	28.4	32.5	0.14	0.16
8	8	38.1	27.8	0.20	0.15
14	14	29.5	31	0.18	0.18
14	15	37.2	35.3	0.19	0.19
15	15	36	33.5	0.18	0.18
20	19	33.5	39	0.17	0.22 #
20	20	36	41	0.18	0.20 #
21	20	32	38.5	0.17	0.20 #
21	21	30	42	0.15	0.24 #
22	22	37	47	0.18	0.25 #

Total activity = micromoles phenolphthalein released per gram liver per hour.
Specific activity = micromoles phenolphthalein released per milligram protein per hour.
(#) = rabbits were dystrophic.

TABLE 20

NEURAMINIDASE ACTIVITY IN RABBIT LIVERS

Days on Diet		Total Activity (Units/gram liver/3 hours)	
(+) E	(-) E	(+) E	(-) E
7	7	98	86
7	8	78	106
14	14	108	145
14	15	112	172
20	19	103	224 #
20	20	96	288 #
21	20	116	314 #
21	21	106	268 #
22	22	87	256 #

Unit of activity = millimicromoles neuraminic acid released.
Substrate used = N-acetylneuraminyllactose.
Three hours incubation at 37°C was required.
(#) = rabbits were dystrophic.

TABLE 21

LYSOPHOSPHATIDASE ACTIVITY IN RABBIT LIVERS

Days on Diet		Total Activity		Specific Activity	
(+) E	(-) E	(+) E	(-) E	(+) E	(-) E
7	8	48	60	0.24	0.32
8	8	60	72	0.32	0.40
14	15	60	72	0.31	0.39
15	16	48	84	0.26	0.45
21	21	54	156	0.26	0.84 #
22	21	60	113	0.31	0.59 #
22	22	70	124	0.37	0.66 #
24	24	60	124	0.30	0.70 #

Total activity = micromoles lysophosphatides consumed per gram liver per hour based on the disappearance of their hemolytic activity.
Specific activity = micromoles lysophosphatides consumed per milligram protein per hour.
(#) = rabbits were dystrophic.

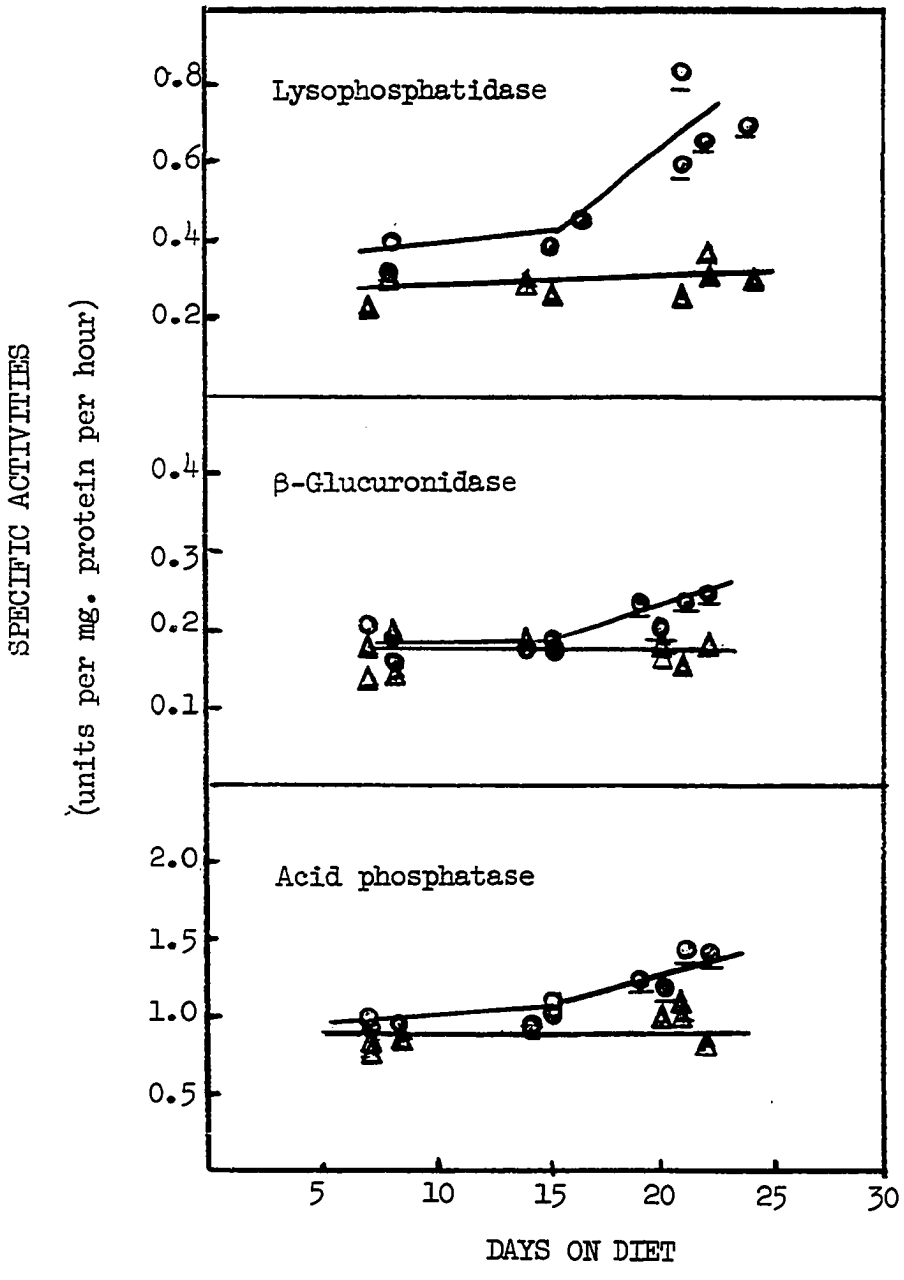


Figure 12.-Acid hydrolases in rabbit livers as a function of days on diet. o——o, vitamin E-deficient rabbits. Underlined circles indicate the rabbits were dystrophic. Δ——Δ , vitamin E-supplemented rabbits. Units of activity are explained in text.

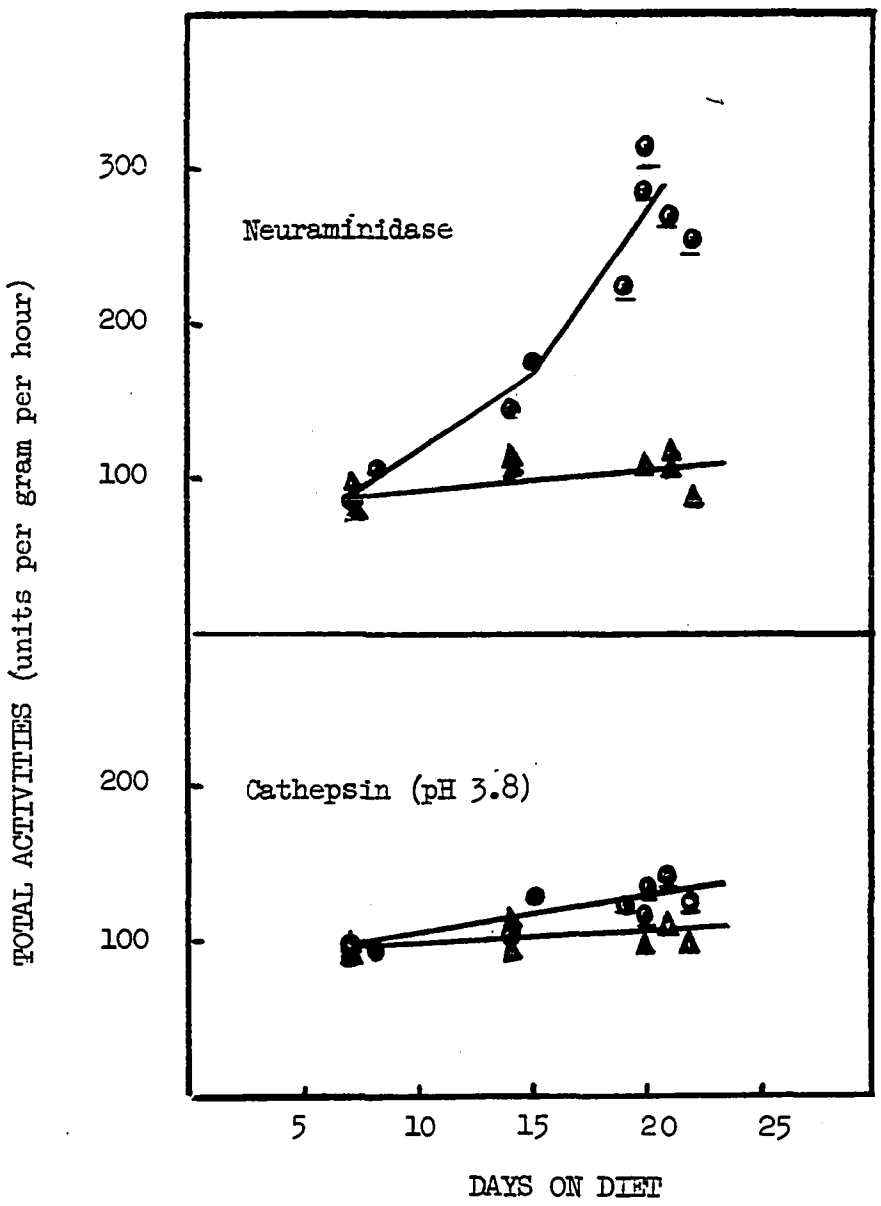


Figure 13.-Total activities of cathepsin and neuraminidase in rabbit livers as a function of days on diet. o——o, vitamin E-deficient rabbits. Underlined circles indicate the rabbits were dystrophic. Δ——Δ , vitamin E-sufficient rabbits. Activity units are explained in text.

TABLE 22

ACID PHOSPHATASE ACTIVITY IN SPLEENS

Days on Diet		Specific Activity (Units/mg. protein/hour)	
(+) E	(-) E	(+) E	(-) E
7	7	0.26	0.32
7	8	0.30	0.31
14	14	0.30	0.32
14	15	0.28	0.30
20	19	0.29	0.43 #
20	20	0.30	0.53 #
21	20	0.29	0.46 #
21	21	0.29	0.43 #

Unit of activity = micromoles phosphate released.
(#) = rabbits were dystrophic.

TABLE 23

β-GLUCURONIDASE ACTIVITY IN SPLEENS

Days on Diet		Specific Activity (Units/mg. protein/hour)	
(+) E	(-) E	(+) E	(-) E
7	7	0.100	0.104
7	8	0.106	0.102
14	14	0.102	0.103
14	15	0.103	0.105
20	19	0.106	0.117 #
20	20	0.105	0.121 #
21	20	0.102	0.121 #
22	22	0.105	0.126 #

Unit of activity = micromoles phenolphthalein released.
(#) = rabbits were dystrophic.

TABLE 24

LYSOPHOSPHATIDASE ACTIVITY IN SPLEENS

Days on Diet		Specific Activity (Units/mg. protein/hour)	
(+) E	(-) E	(+) E	(-) E
8	8	0.19	0.20
14	15	0.20	0.23
15	16	0.22	0.26
21	21	0.22	0.52 #
22	21	0.26	0.45 #
22	22	0.22	0.61 #
24	24	0.23	0.47 #

Unit of activity = micromoles lysophosphatides consumed based on the loss of their hemolytic activity.

(#) = rabbits were dystrophic.

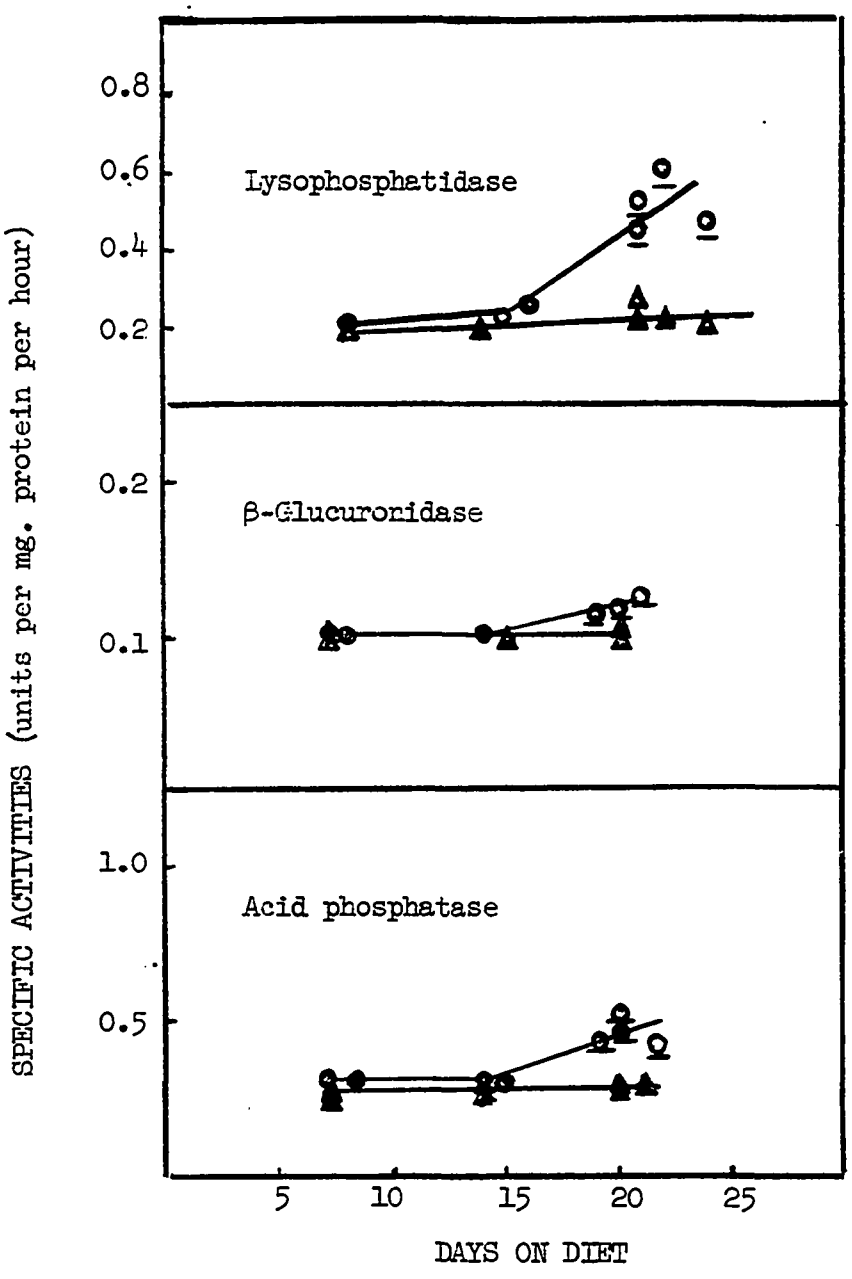


Figure 14.-Acid hydrolases in spleen as a function of days on diet. o——o, vitamin E-deficient rabbits. Underlined circles indicate the rabbits were dystrophic. Δ——Δ , vitamin E-sufficient rabbits.

TABLE 25

DISTRIBUTION OF PROTEIN IN LIVER SUBCELLULAR FRACTIONS

Number of Experiments (4,4)	(+) E	(-) E
Total protein in homogenates. (mg. per gram liver)	197 (±2.5)	193 (±3.1)
Percentage of total protein in subcellular fractions	%	%
Q1, nuclear and debris**	17.8	18.5
Q2, mitochondrial	23.1	21.5
Q3, lysosomal	9.2	8.3
Q4, microsomal	16.5	14.2
Q5, soluble fraction	33.4	37.5

(±) = standard error. Protein content was based on the wet weight of livers. (4,4) = four vitamin E-sufficient (controls) and four vitamin E-deficient (dystrophic) rabbits.
(**) = values obtained by subtraction, considering 100% recovery of protein in fractions.

TABLE 26

DISTRIBUTION OF ACID PHOSPHATASE IN LIVER SUBCELLULAR FRACTIONS FROM DYSTROPHIC AND CONTROL RABBITS

Subcellular Fractions	Percentage of Total Activity		Relative Specific Activity	
	(+) E	(-) E	(+) E	(-) E
Q1, nuclear and debris**	12.2	9.2	0.66	0.57
Q2, mitochondrial	35.5	28.2	1.52	1.30
Q3, lysosomal	29.7	20.5	3.14	2.16
Q4, microsomal	7.7	10.2	0.44	0.55
Q5, soluble fraction	14.9	31.9	0.46	0.89

(+) E = rabbits fed vitamin E-supplemented diet.
(-) E = rabbits fed vitamin E-deficient diet and were dystrophic.
(**) = values obtained by subtraction.
Relative specific activity = percentage of total activity/percentage of total protein.
All values are mean averages of four experiments.

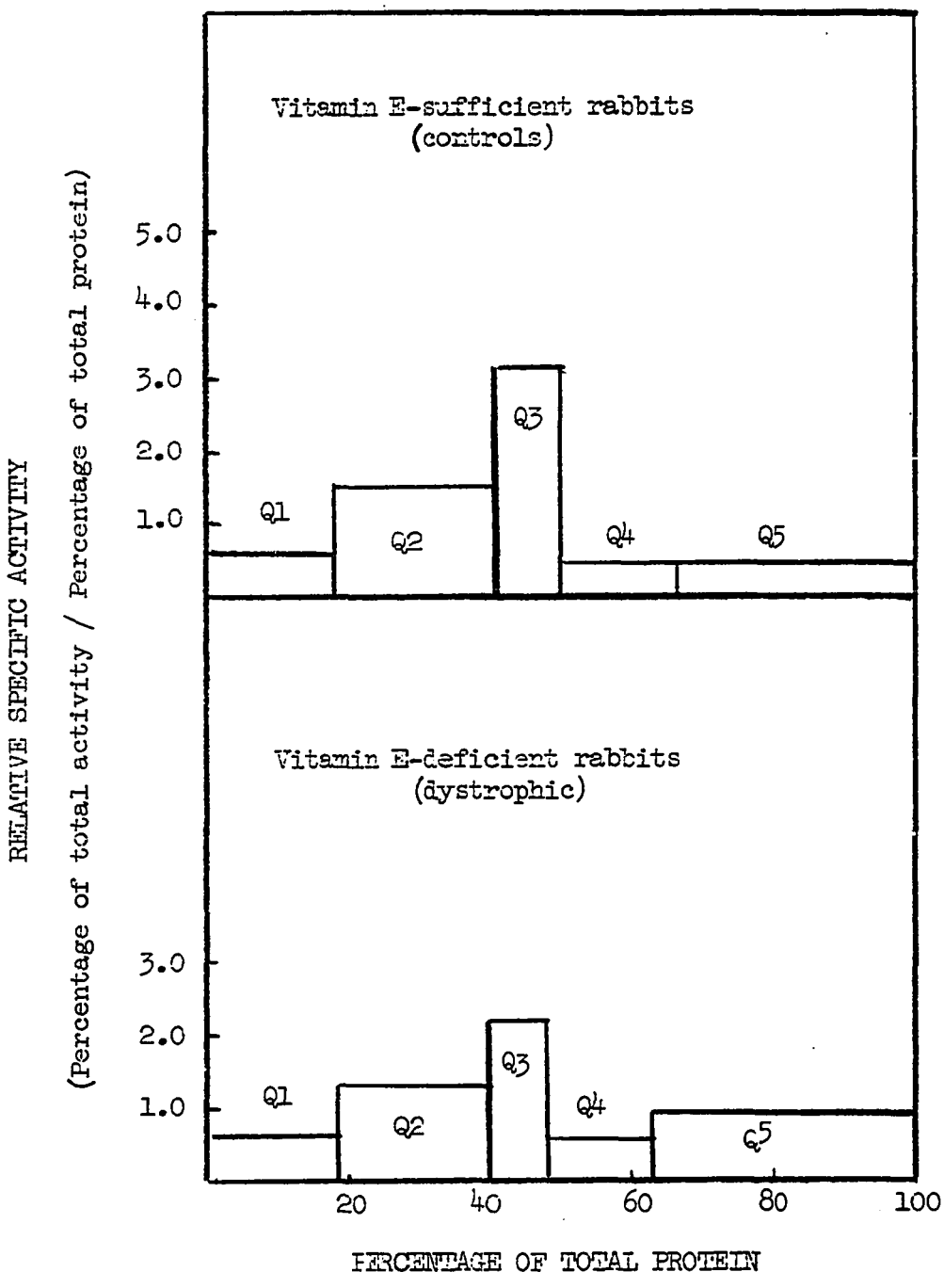


Figure 15.-Distribution patterns of acid phosphatase in rabbit liver. Q1, Q2, Q3, Q4, and Q5 , represent the intracellular fractions explained in text.

TABLE 27

DISTRIBUTION OF CATHEPSIN (pH 3.8) IN LIVER SUBCELLULAR FRACTIONS FROM DYSTROPHIC AND CONTROL RABBITS

Subcellular Fractions	Percentage of Total Activity		Relative Specific Activity	
	(+) E	(-) E	(+) E	(-) E
Q1, nuclear and debris**	6.6	6.0	0.35	0.39
Q2, mitochondrial	33.1	24.6	1.43	1.27
Q3, lysosomal	37.1	20.3	4.76	2.0
Q4, microsomal	7.1	11.38	0.37	0.67
Q5, soluble fraction	16.1	37.7	0.50	0.99

(+) E = rabbits fed vitamin E-supplemented diet.
(-) E = rabbits fed vitamin E-deficient diet and were dystrophic.
(**) = values obtained by subtraction.
Relative specific activity = percentage of total activity/percentage of total protein.
All values are mean averages of four experiments.

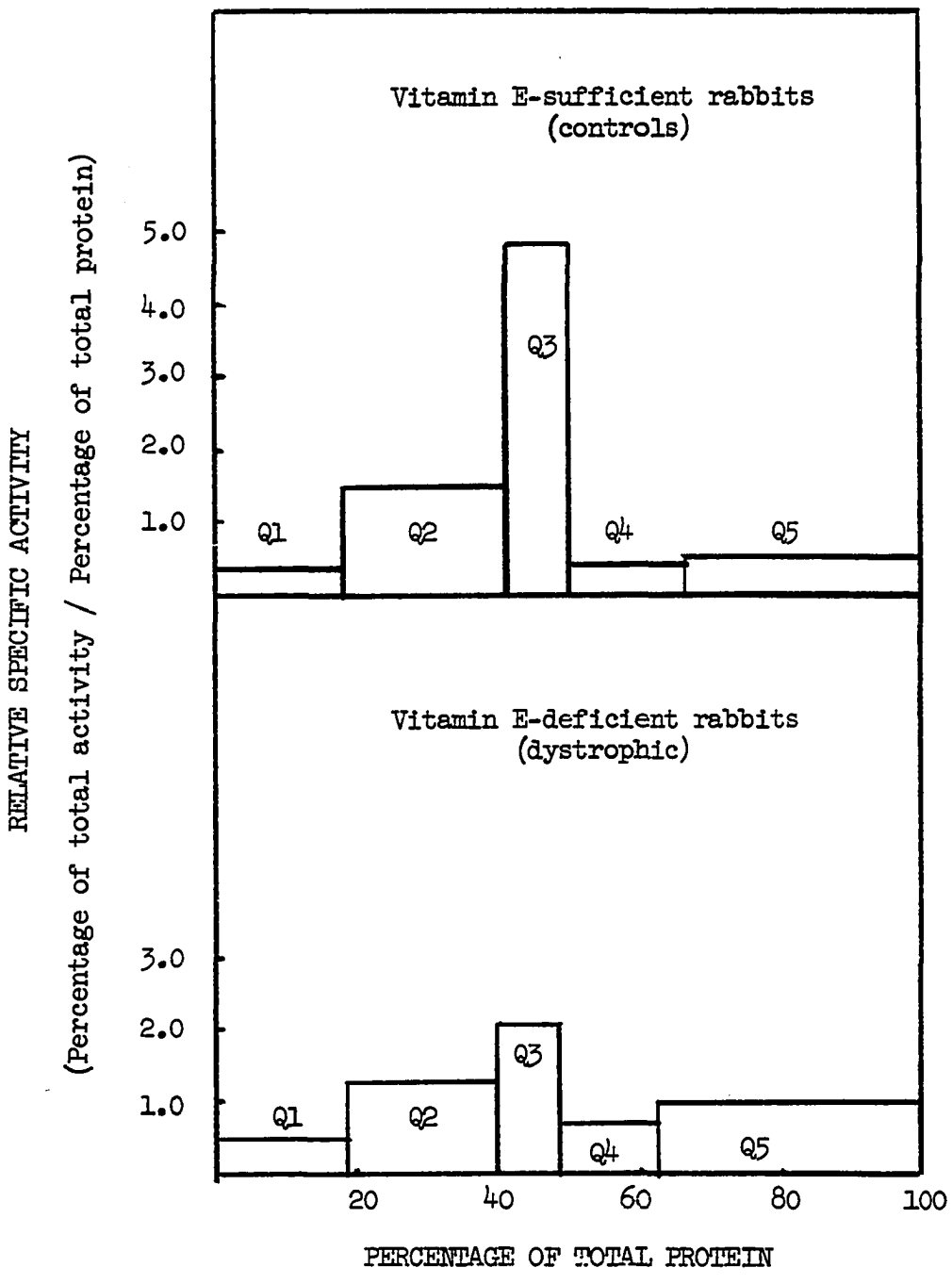


Figure 16.-Distribution patterns of cathepsin (pH 3.8) in rabbit liver. Q1, Q2, Q3, Q4, and Q5, represent the intracellular fractions.

TABLE 28

DISTRIBUTION OF β-GLUCURONIDASE IN LIVER SUBCELLULAR FRACTIONS FROM DYSTROPHIC AND CONTROL RABBITS

Subcellular Fractions	Percentage of Total Activity		Relative Specific Activity	
	(+) E	(-) E	(+) E	(-) E
Q1, nuclear and debris**	6.6	10.3	0.37	0.56
Q2, mitochondrial	30.6	18.8	1.32	0.84
Q3, lysosomal	18.8	14.6	2.04	1.75
Q4, microsomal	29.0	17.5	1.76	1.23
Q5, soluble fraction	15.0	38.8	0.45	1.03

(+) E = rabbits fed vitamin E-supplemented diet.
(-) E = rabbits fed vitamin E-deficient diet and were dystrophic.
(**) = values obtained by subtraction.
Relative specific activity = percentage of total activity/percentage of total protein.
All values are mean averages of four experiments.

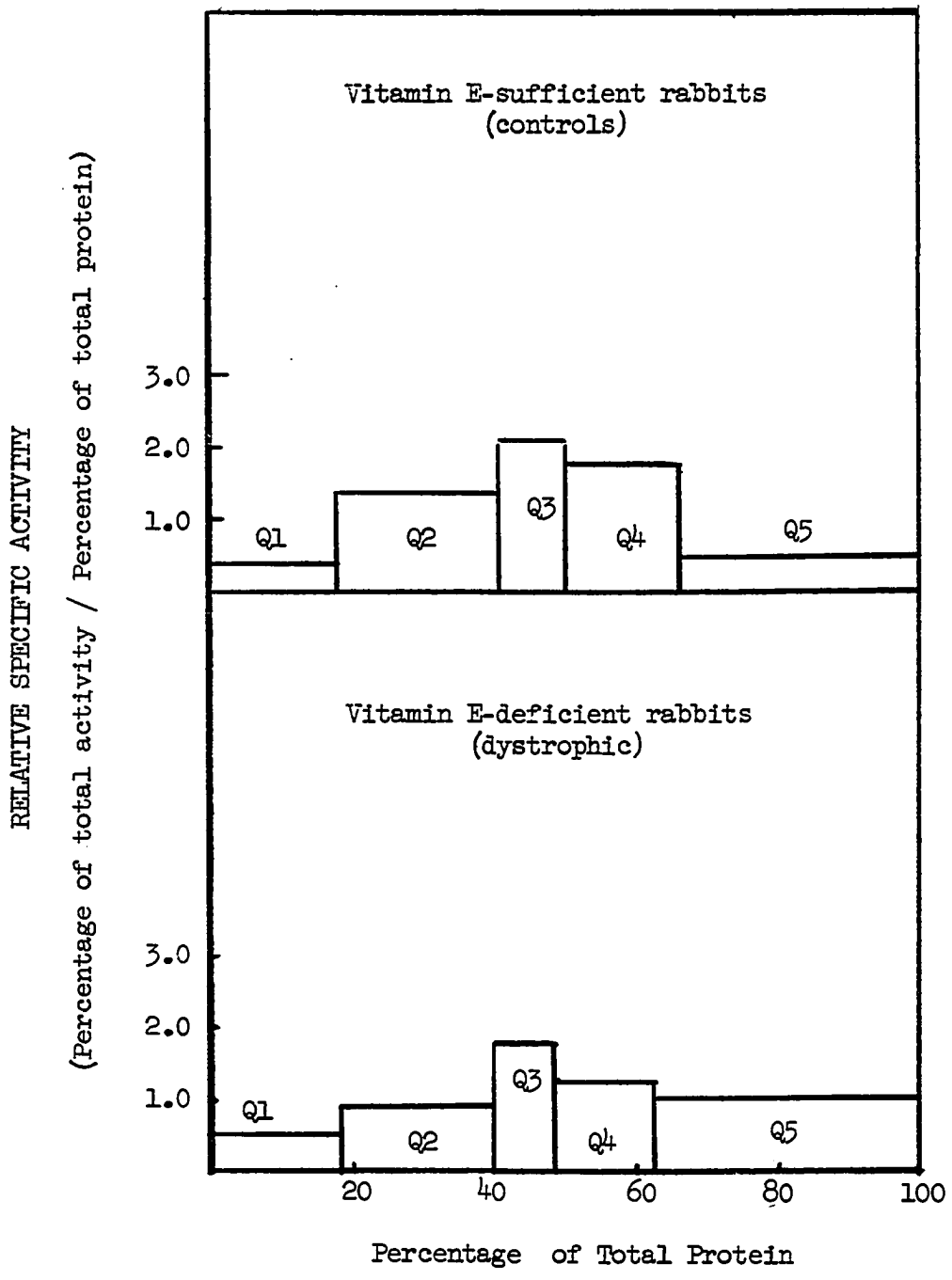


Figure 17.-Distribution patterns of β-glucuronidase in rabbit liver. Q1, Q2, Q3, Q4, and Q5 represent the intracellular fractions.

TABLE 29

DISTRIBUTION OF NEURAMINIDASE IN LIVER SUBCELLULAR FRACTIONS FROM DYSTROPHIC AND CONTROL RABBITS

Subcellular Fractions	Percentage of Total Activity		Relative Specific Activity	
	(+) E	(-) E	(+) E	(-) E
Q1, nuclear and debris**	6.9	5.1	0.41	0.4
Q2, mitochondrial	13.6	10.2	0.65	0.54
Q3, lysosomal	28.3	15.8	2.97	1.9
Q4, microsomal	9.6	11.7	0.42	0.82
Q5, soluble fraction	41.6	57.2	1.25	1.53

(+) E = rabbits fed vitamin E-supplemented diet.
(-) E = rabbits fed vitamin E-deficient diet and were dystrophic.
(**) = values obtained by subtraction.
Relative specific activity = percentage of total activity/percentage of total protein.
All values are mean averages of four experiments.

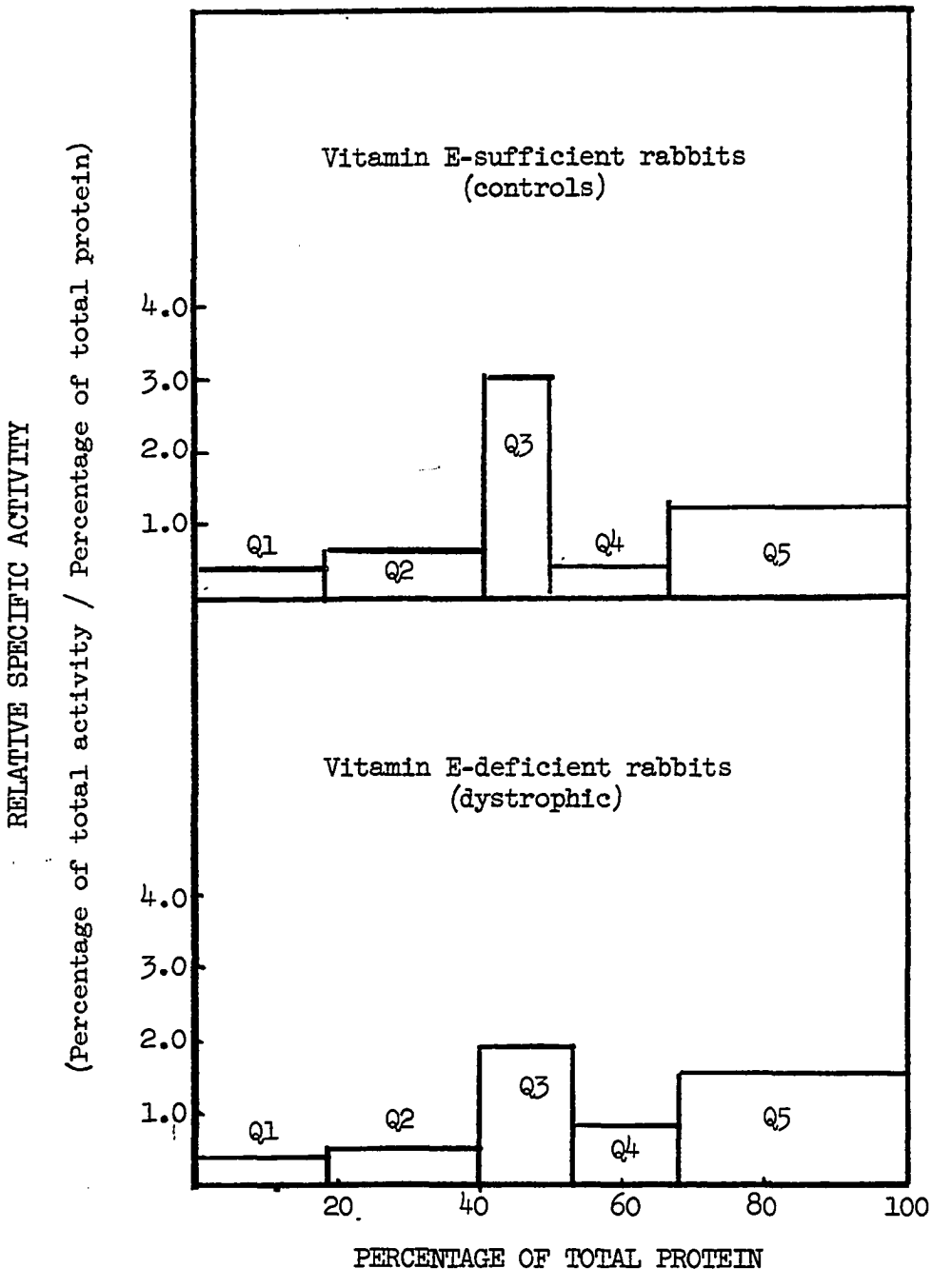


Figure 18.-Distribution patterns of neuraminidase in rabbit liver. Q1, Q2, Q3, Q4, and Q5, represent the intracellular fractions.

TABLE 30

DISTRIBUTION OF LYSOPHOSPHATIDASE IN LIVER SUBCELLULAR FRACTIONS FROM DYSTROPHIC AND CONTROL RABBITS

Subcellular Fractions	Percentage of Total Activity		Relative Specific Activity	
	(+) E	(-) E	(+) E	(-) E
Q1, nuclear and debris**	4.2	3.1	0.23	0.18
Q2, mitochondrial	9.5	10	0.48	0.48
Q3, lysosomal	33.0	10	3.7	1.3
Q4, microsomal	11.5	14.5	0.67	0.87
Q5, soluble fraction	41.8	62.4	1.16	1.65

(+) E = rabbits fed vitamin E-supplemented diet.
(-) E = rabbits fed vitamin E-deficient diet and were dystrophic.
(**) = values obtained by subtraction.
Relative specific activity = percentage of total activity/percentage of total protein.
All values are mean averages of four experiments.

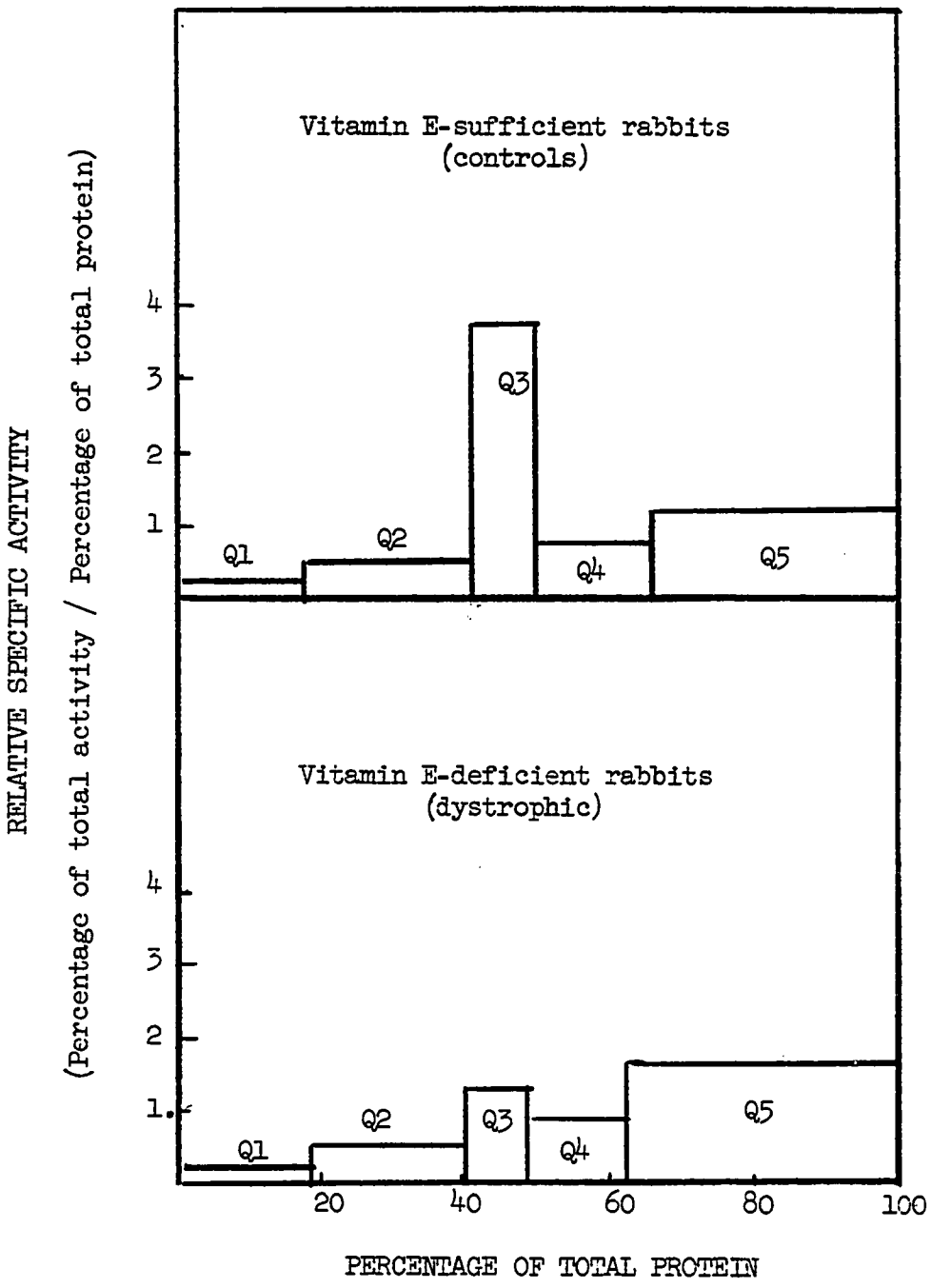


Figure 19.-Distribution patterns of lysophosphatidase in rabbit liver. Q1, Q2, Q3, Q4, and Q5, represent the intracellular fractions.

Demonstration of the Vitamin E-deficient State by the in vitro Hemolysis of Rabbit Erythrocytes with Hydrogen Peroxide

It has been shown by Gyorgy and Rose (109) that hemoglobinuria, intravascular hemolysis, and death which occur in alloxan-treated rats are prevented by dietary tocopherols (vitamin E) but the diabetic phenomena were not affected. It has been found later that dialuric acid which is a decomposition product of alloxan, produce hemolysis of erythrocytes of vitamin E-deficient animals. Other autoxidizable substances, ascorbic acid and sulfhydril compounds such as cysteine and glutathione, were found to cause hemolysis, but the reaction was slower and required much larger amounts than with dialuric acid or hydrogen peroxide. It has been postulated that vitamin E protects the erythrocytes by counteracting free radicals or peroxides formed as intermediates of the oxidation-reduction systems of dialuric acid and alloxan. Inhibition of hemolysis by these agents was obtained by treatments of the erythrocytes in vitro with tocopherols and a number of fat soluble antioxidants. On the basis of these reactions, hemolysis tests were developed to demonstrate the vitamin E-deficient state in animals. Hydrogen peroxide was used in place of dialuric acid where its hemolytic activity was found to be related to the vitamin E content of the erythrocytes. It was demonstrated by Gyorgy and Rose (109) that catalase decreased the effectiveness of dialuric acid as a hemolytic agent and therefore linking the formation of hydrogen peroxide during autoxidation. The results are shown in Table 31. This test demonstrated a vitamin E-deficiency state after 7 to 8 days on diet.

TABLE 31

IN VITRO HEMOLYSIS OF RABBIT ERYTHROCYTES BY HYDROGEN PEROXIDE AND THE INHIBITION OF HEMOLYSIS BY ADDED TOCOPHEROL *IN VITRO*

Days on Diet	Number of Rabbits		No Addition		Tocopherol Added to Cells *in vitro*	
			% Hemolysis		% Hemolysis	
	(+)	(-)	(+)	(-)	(+)	(-)
7 to 8	7	7	4.2	78	4	13
14 to 15	3	3	4.5	82	5.2	17
19 to 26	7	8	4.3	89.5	0	19.5

(+) = rabbits fed vitamin E-supplemented diet. (-) = rabbits fed vitamin E-deficient diet. Excess tocopherol was added to packed erythrocytes. The values are mean averages.

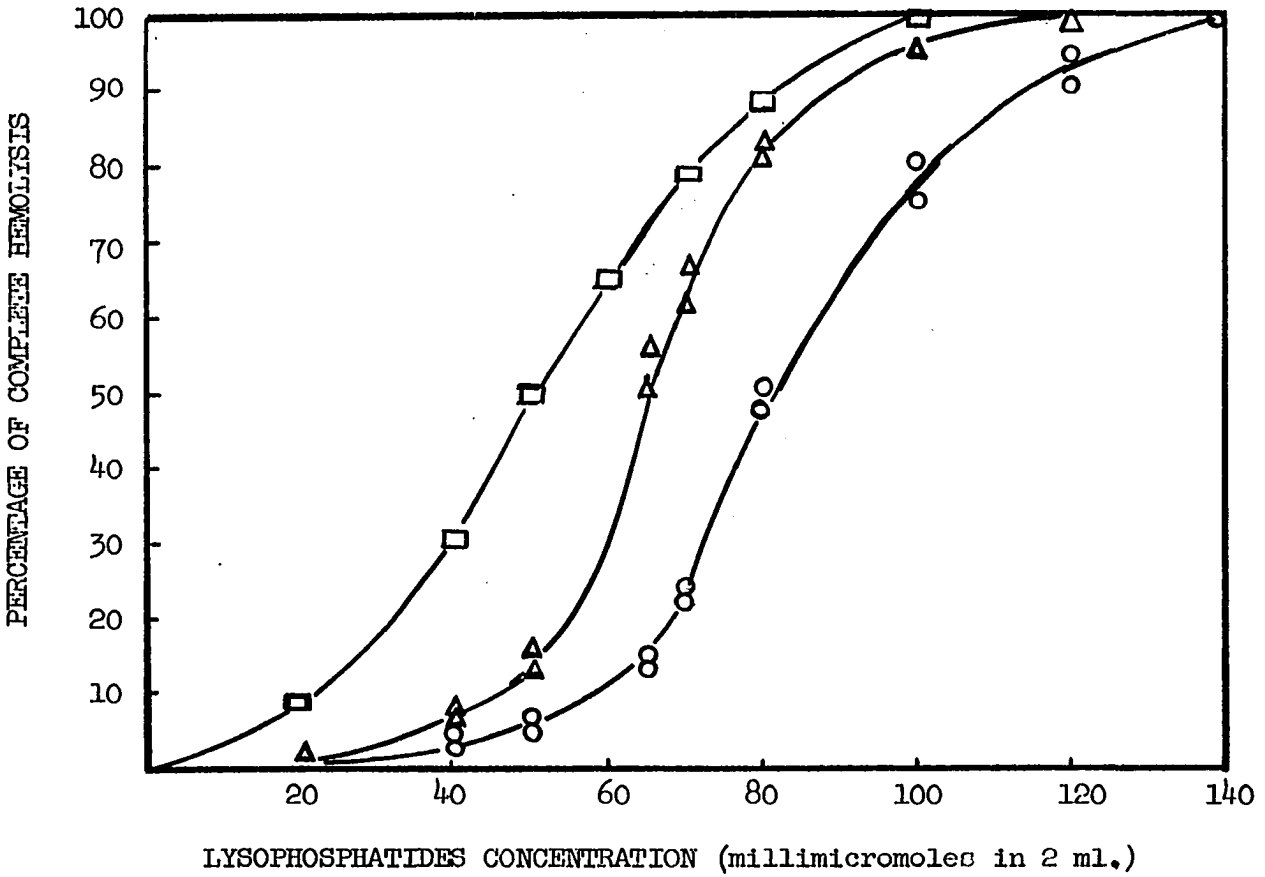


Figure 20.-Effect of leucocytes on lysophosphatide-induced hemolysis of rabbit erythrocytes. o——o, (erythrocytes + leucocytes) from dystrophic vitamin E-deficient rabbits. Δ——Δ, (erythrocytes + leucocytes) from vitamin E-sufficient rabbits. □——□, (erythrocytes alone) from either vitamin E-deficient or vitamin E-sufficient rabbits.

TABLE 32

PROTEIN, NITROGEN, AND NON-COLLEGEN NITROGEN OF RABBIT SKELETAL MUSCLE

Days on Diet	Number of Rabbits	Mg. per Gram Muscle Wet Weight					
	(+),(-)	Total Protein		Total Nitrogen		Non-collegen Nitrogen	
		(+) E	(-) E	(+) E	(-) E	(+) E	(-) E
7 to 8	(7,7)	129.8	128.4	20.8	20.5	19.5	19.4
14 to 15	(3,3)	131.2	125.3	21.2	19.2	19.1	18.1
19 to 25	(7,8)	131	116.8 #	21.5	17.6 #	18.8	16.4 #

All values are mean averages of the number of rabbits shown in parenthesis. (+) E = rabbits were fed vitamin E-supplemented diet. (-) E = rabbits were fed vitamin E-deficient diet. (#) = rabbits were dystrophic.

Distribution of Neuraminidase, Acid Phosphatase and Lypophilic Sialic Acid Compounds in Brain Subcellular Fractions from Chicken

Brain tissues were obtained from chicken which were maintained on vitamin E-supplemented or vitamin E-deficient diet expecially formulated to develop encephalomalacia in chicken. Muscular weakness was also observed in the chickens fed vitamin E-deficient diet. It was of interest to observe any enzymic changes in chick brains that developed encephalomalacia, particularly the lysosomal enzymes which were studied in dystrophic rabbit muscles. Neuraminidase was found to be associated with the lysosomal fraction as shown in Table 29. It is apparent, however, that the soluble fraction has a higher relative specific activity than the classical lysosomal enzymes such as acid phosphatase. The distribution of acid phosphatase and neuraminidase were studied in chicken brains and their assays were carried concurrently from the same materials. Acid phosphatase in chicken brains served as a marker of the well established lysosomal enzymes. The cerebrum was studied in these experiments. Table 33 shows the distribution of neuraminidase and Table 34 shows the distribution of acid phosphatase. Lipophilic sialic acid compounds, presumably gangliosides, were isolated from the materials used for the enzymatic assays. Table 35, shows the subcellular distribution of lipophilic sialic acid compounds (gangliosides). The results are expressed as percentages of the total lipophilic sialic acid compounds in the homogenates before fractionation. The nuclear and debris fraction (Q1), was not assayed. Recovery in the subcellular fractions was considered as 100% and the values for (Q1) were obtained by subtraction.

TABLE 33

INTRACELLULAR DISTRIBUTION OF NEURAMINIDASE IN THE CEREBRUMS OF ENCEPHALOMALACIC AND CONTROL CHICKENS

Subcellular Fractions	Percentage of Total Activity		Percentage of Total Protein		Relative Specific Activity	
	(+)	(-)	(+)	(-)	(+)	(-)
Nuclear and debris	14.8	12.7	17.1	16.5	0.87	0.77
Mitochondrial	18.1	13.0	28.3	26.6	0.71	0.49
Lysosomal	31.4	13.2	9.2	8.9	3.4	1.47
Microsomal	10.2	16.4	15.3	14.4	0.67	1.14
Soluble	25.5	44.7	30.1	33.6	0.85	1.33

Total activity in the homogenate before fractionation = 31.4 units/gram/3 hours for the control chicken, and 54.7 units/gram/3 hours for the encephalomalacic chicken. Total protein = 87.3 mg./gram for the controls, and 85.9 mg./gram for the sick chicken. One unit activity = millimicromoles sialic acid released. (+) = control chicken, fed vitamin E-supplemented diet. (-) = encephalomalacic chicken, fed vitamin E-deficient diet. Relative specific activity = (percentage of total activity)/(percentage of total protein). The values are averages of three experiments.

TABLE 34

INTRACELLULAR DISTRIBUTION OF ACID PHOSPHATASE IN THE CEREBRUMS OF ENCEPHALOMALACIC AND CONTROL CHICKENS

Subcellular Fractions	Percentage of Total Activity		Relative Specific Activity	
	(+)	(-)	(+)	(-)
Nuclear and debris	14.1	9.1	0.82	0.55
Mitochondrial	37.3	31.8	1.33	1.2
Lysosomal	28.1	18.7	3.55	2.1
Microsomal	6.9	11.2	0.45	0.78
Soluble	13.6	30.2	0.45	0.90

Total activity in the homogenate = 89.7 units/gram/hour for the (+) chicken, 144.3 units/gram/hour for the (-) chicken. Total protein was the same as shown in Table 35. (+) = control chicken, fed vitamin E-supplemented diet. (-) = encephalomalacic chicken, fed vitamin E-deficient diet. One unit of activity = micromoles inorganic phosphate released. The values are averages of three experiments.

TABLE 35

INTRACELLULAR DISTRIBUTION OF LIPOPHILIC SIALIC COMPOUNDS IN CHICKEN CEREBRUMS

Subcellular Fractions	Percentage of Total Sialic in Homogenates (+)
Nuclear and debris	11.6**
Mitochondrial	33.8
Lysosomal	23.7
Microsomal	18.3
Soluble	12.5

Total sialic acid in homogenates = 3.85 micromoles per gram wet weight. The values are averages of three experiments.
(**) = values obtained by subtraction, this fraction was not assayed.
(+) = values from control chicken, non-encephalomalacic, fed vitamin E-supplemented diet.

Distribution of Lypophilic Sialic Acid Compounds in Liver Subcellular Fractions from Rats

Liver tissues were obtained from rats which were fed commercial rat pellet diet. The tissues were homogenized and fractionated by centrifuging according to the methods described in Chapter II. The primary interest was to study the intracellular distribution of neuraminidase in rat liver fractions. The distribution of this enzyme was found to be similar to its distribution in rabbit livers. It was of interest to find the distribution of sialic acid compounds in liver that would serve as the natural substrate of neuraminidase *in vivo*. Isolation of the sialic acid compounds was done according to the procedure described before. The subcellular fractions were suspended in a minimum amount of water and extracted with chloroform-methanol (2:1). The crude lipid extract was washed with 0.5% NaCl and the water methanol phase was separated, dialyzed, then concentrated. After acid hydrolysis, free sialic acids were assayed by the thiobarbituric acid reaction of Warren (79) and the amount was then determined according to the methods described before in Chapter II. The first fraction, Q1, the nuclear and debris fraction, was not analyzed because of its low content and the presence of interfering substances. Recovery in the fractions was considered as 100% and the values of the nuclear and debris fraction was obtained by subtracting the sum recovered in the remaining fractions from the amount present in the homogenate before fractionation. Table 36 shows the results expressed as percentage of total sialic acids in the homogenates.

TABLE 36

DISTRIBUTION OF LIPOPHILIC SIALIC ACID COMPOUNDS IN LIVER SUBCELLULAR FRACTIONS FROM RATS

	Micromoles Sialic Acid per Gram Liver Wet Weight
Homogenate	2.332 ± 0.117
Subcellular Fractions	Percentage of homogenate
Q1, nuclear and debris	11.8**
Q2, mitochondrial	36.9
Q3, lysosomal	28.2
Q4, microsomal	8.3
Q5, soluble fraction	14.8

(**) = values obtained by subtraction. All values are mean averages of three experiments. (±) = standard error.

Effect of Leucocytes on the Lysophosphatide-induced Hemolysis of Rabbit Erythrocytes

Fresh whole blood was obtained from vitamin-deficient or vitamin E-sufficient rabbits and the cells were washed with isotonic sodium chloride solution as described in Chapter II. In preparing the erythrocytes suspensions, the leucocytes were either removed by aspiration or they were allowed to remain. Hemolysis of erythrocyte in these preparations by lysophosphatides was then determined by measuring the released hemoglobin. The effect of leucocytes on the hemolysis of erythrocytes is shown in (Figure 20) where the percentages of complete hemolysis were plotted against the amount of lysophosphatides present in a total volume of 2 ml. In the experiments where erythrocytes and leucocytes were present, it was found that more lysophosphatides were required to obtain complete hemolysis than in the experiments where only erythrocytes were present. In the absence of leucocytes, it was found that the same amounts of lysophosphatides can cause complete hemolysis of erythrocytes of vitamin E-deficient or vitamin E-sufficient rabbits. Complete hemolysis is defined here as the amount of hemoglobin released from erythrocytes in presence of excess lysophosphatides in a hypotonic saline solution. Lysophosphatides caused a rapid release of hemoglobin at room temperature and the lysis at 37°C for 15 minutes was not greater than at room temperature for 5 minutes. It was assumed, therefore, that the hemolysis of rabbit erythrocytes by lysophosphatides is independent of temperature.

Inhibition of Lysophosphatide-induced Hemolysis of Rabbit Erythrocytes by Plasma

Plasma was collected from oxalated fresh whole blood obtained from vitamin E-deficient or vitamin E-sufficient rabbits and the plasma proteins were determined according to the procedures described in Chapter II. Preliminary experiments indicated that large amounts of lysophosphatides were required to hemolyse 1 ml. of a 2.5% suspension of whole blood in a final volume of 2 ml. In subsequent experiments, 1 ml. of a 2.5% susepnsion of washed erythrocytes freed from leucocytes required a relatively small amount of lysophosphatides (100 millimicromoles) in a final volume of 2 ml. to produce complete hemolysis.

The effect of plasma on the induction of hemolysis by lysophosphatides was studied by mixing varied amounts of plasma with a constant amount of lysophosphatides (200 millimicromoles) then testing the hemolytic activities of the resulting mixtures. In all of these experiments, 1 ml. of 2.5% suspension of washed erythrocytes (freed from leucocytes) in 0.9% saline was added to 1 ml. of an isotonic solution containing 200 millimicromoles lysophosphatides and an amount of plasma in a final volume of 2 ml. Table 37 shows the results of these experiments. Incubation of the lysophosphatide-plasma mixture at 37°C for one hour did not result in greater inhibition of hemolysis than at 5°C or room temperature for 5 minutes. It was assumed, therefore, that the loss of the hemolytic activity of lysophosphatides by plasma is independent of temperature. The ratio (lysophosphatides)/(mg. protein) appears to be constant and equals 57 millimicromoles per mg. plasma protein.

TABLE 37

INHIBITION OF LYSOPHOSPHATIDE-INDUCED HEMOLYSIS OF RABBIT ERYTHROCYTES BY PLASMA

200×10^{-9} Moles Lysophosphatides Were Mixed with the Following Amounts of Plasma:	Percentage of Complete Hemolysis of Erythrocytes from:	
	(+) E Rabbits	(-) E Rabbits
No plasma	100	100
Plasma from (+) E rabbits		
0.100 ml.	000	000
0.075 ml.	000	000
0.050 ml.	000	000
0.025 ml.	73	79
0.0125 ml.	100	100
Plasma from (-) E rabbits		
0.100 ml.	000	000
0.075 ml.	000	000
0.050 ml.	000	000
0.025 ml.	60	62
0.0125 ml.	100	100
Mg. protein/ml. plasma = 70 from (+) E rabbits = 68.4 from (-) E rabbits		

(+) E = rabbits were fed vitamin E-supplemented diet.
(-) E = rabbits were fed vitamin E-deficient rabbits, and were dystrophic.
The values are averages of two experiments.
The test mixture consisted of 1 ml. isotonic solution composed of 200 millimicromoles lysophosphatides and plasma, and 1 ml. of 2.5% erythrocyte suspension in 0.9% sodium chloride solution.

Lysophosphatidase Activity in Rabbit Leucocytes

The effect of leucocytes on lysophosphatide-induced hemolysis was demonstrated in (Figure 20). The results indicated that elucocytes appear to possess certain properties which enabled them to destroy the hemolytic activity of lysophosphatides. It was of interest to investigate these properties in isolated leucocytes. Leucocytes were isolated from fresh whole blood of vitamin E-deficient or vitamin E-sufficient rabbits according to the procedure described in Chapter II. The cells were ruptured by repeated freezing and thawing and microhomogenization. Identification of an enzymic activity which may be responsible for the disappearance of the hemolytic activity of lysophosphatides was attempted by performing preliminary experiments on the disrupted leucocyte preparations. The results of these experiments were as follows: there was no destruction of the hemolytic activity of lysophosphatides when incubated with ruptured leucocytes at 5°C for one hour; heated leucocyte preparations did not possess the ability to destroy the hemolytic activity of lysophosphatides; 18 to 26 per cent of total lysophosphatides were destroyed when incubated at room temperature for 30 minutes while the same amount was completely destroyed when incubated at 37°C for 10 minutes; the hemolytic activity of lysophosphatides decreased exponentially with time when the reaction mixture was incubated at 37°C, and the optimum pH was 6.0. These criteria strongly suggest that enzymic mechanisms are involved in the destruction of the hemolytic activity of lysophosphatides by disrupted leucocyte preparations. When these observations were compared with the

inhibition of lysophosphatide-induced hemolysis by plasma, it was found that the inhibition was dependent on a constant ratio of lysophosphatides to plasma protein. Deviation from the established ratio, 60 ± 3 millimicromoles lysophosphatides per milligram plasma protein, will result an incomplete inhibition. In the case of leucocytes, Table shows that 620 to 720 millimicromoles lysophosphatides were destroyed per milligram protein from leucocytes of non-dystrophic rabbits and up to 2540 millimicromoles per milligram protein were destroyed by the leucocytes of dystrophic rabbits. Plasma inhibition was independent of temperature while the action of leucocytes was temperature dependent. Longer incubation with plasma did not result in greater inhibition, while the destruction of lysophosphatides by leucocytes increased with time. It may be assumed, therefore, that the inhibition by plasma is non-enzymic and the action of leucocytes is enzymic and termed as lysophosphatidase.

Leucocytosis in Vitamin E-deficient and Vitamin E-sufficient Rabbits

The results in Table 40 indicated that the leucocytes of vitamin E-deficient rabbits which developed muscular dystrophy have a higher activity for the destruction of lysophosphatides (lysophosphatidase). It was of interest to know the reasons for this elevated activity. One possibility is that the leucocytes in the dystrophic rabbits have increased in number. Table 39 shows the leucocyte counts per microliters from vitamin E-deficient or vitamin E-sufficient rabbits as a function of days on diet. The dystrophic rabbits have a slightly higher count

than the control rabbits but that difference is not sufficiently higher to account for an approximately three-fold increase in lysophosphatidase activity. It was observed, however, that the percentage of granulocytes is greatly elevated in the dystrophic rabbits and the percentage of lymphocytes was high in the control rabbits (Figure 21 and Table 39).

TABLE 38

(LYSOPHOSPHATIDASE) ACTIVITY IN RABBIT LEUCOCYTES

Days on Diet		Specific Activity (Micromoles Lysophosphatides Consumed/mg. Protein/Hour)	
(+) E	(-) E	(+) E	(-) E
7P	7	0.74	0.89
7P	7	0.68	0.72
7P	7	0.72	0.76
20	20	0.62	2.13 #
21	20	0.67	2.54 #
24	24	0.62	2.28 #

(+) E = vitamin E-sufficient rabbits.
(-) E = vitamin E-deficient rabbits.
(#) = rabbits were dystrophic.
(P) = vitamin E-sufficient rabbits were pair fed.

TABLE 39

LEUCOCYTES OF VITAMIN E-DEFICIENT AND CONTROL RABBITS

Days on Deit		Total Leucocytes (thousands/0.001 ml.)		Percentage of Total Leucocytes			
				Lymphocytes		Granulocytes	
(+) E	(-) E	(+) E	(-) E	(+) E	(-) E	(+) E	(-) E
7P	7	5.2	11.4	74	63	25	35
7P	7	6.4	7.4	80	70	18	26
7P	7	6.5	16.0	65	46	31	51
14P	14	9.8	14.4	76	31	22	68
14P	14	11.6	13.8	75	41	24	57
14P	14	12.4	19.6	62	25	35	72
20	20 #	14.6	24.1 #	81	19 #	17	79 #
21	20 #	18.2	25.2 #	76	14 #	14	83 #
24	24 #	17.8	26.8 #	65	22	34	75 #

(+) E = vitamin E-sufficient rabbits (controls). (-) E = vitamin E-deficient rabbits. (#) = rabbits were dystrophic. Percentages of granulocytes include neutrophils, eosinophils and basophils. Percentages of monocytes are not shown. P = indicates that rabbits were pair fed. The groups of rabbits (7P,7) and (14P,14) days on diet were the same animals.

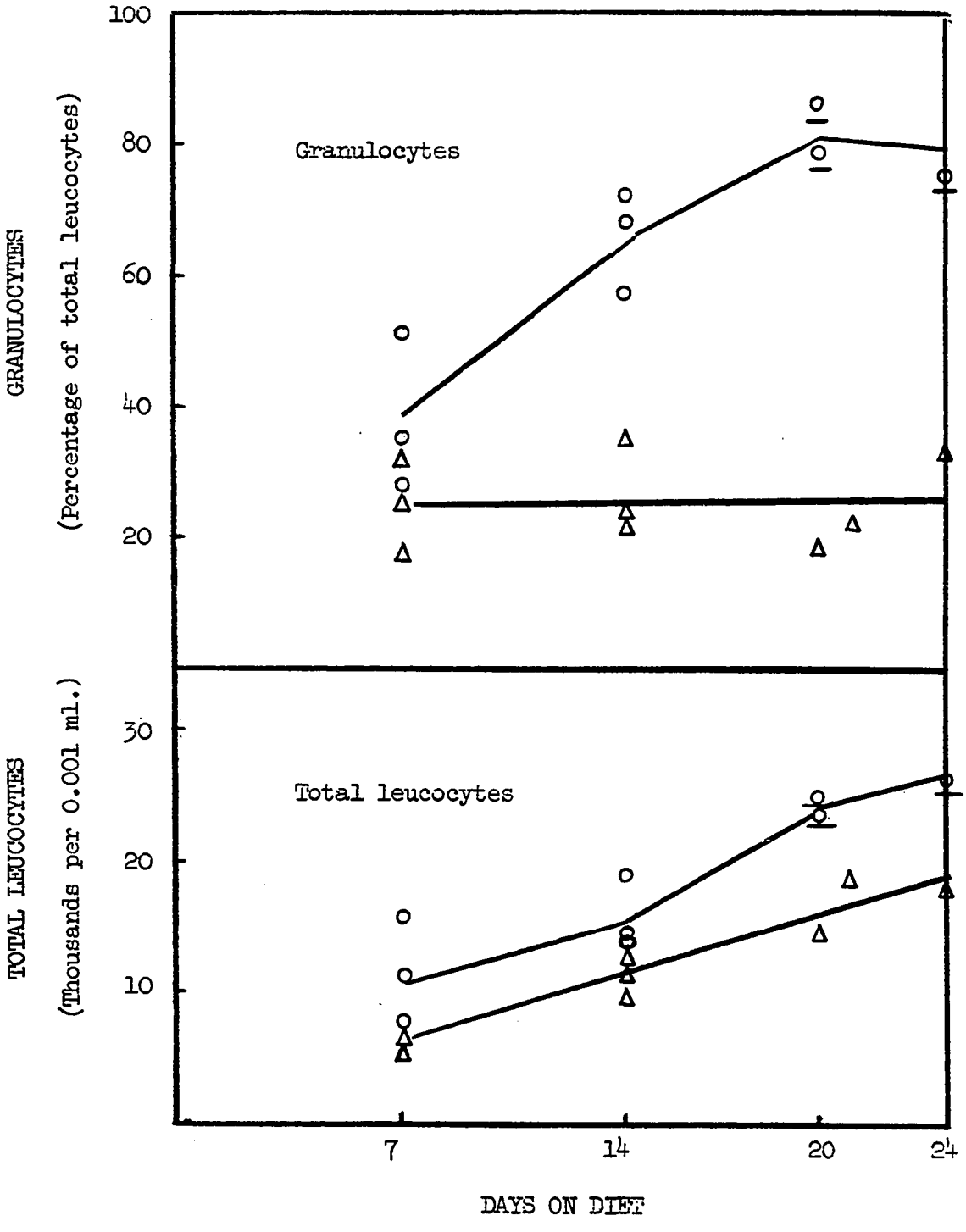


Figure 21.-Leucocytes as a function of days on diet. Upper graph, granulocytes percentages of total leucocytes. Lower graph, total leucocytes. o——o, vitamin E-deficient rabbits. Underlined circles indicate the rabbits were dystrophic. Δ——Δ , vitamin E-sufficient rabbits.

β-Galactosidase Activity in Leucocytes of Vitamin E-Deficient and Control Rabbits

β-Galactosidase has been identified by De Duve et al (20) as a lysosomal enzyme in hepatic cells. Tappel et al (59) have shown that this enzyme increases in dystrophic rabbit muscle. The results shown in Table 39 indicate that rabbits suffering from nutritional muscular dystrophy have exhibited leucocytosis and granulocytosis. Although these experiments do not prove that leucocytosis and granulocytosis are a direct result of vitamin E-deficicney, these phenomena may reflect a leucocytic infiltration of the dystrophic muscle and need not be specific for vitamin E-deficiency. It became of interest, therefore, to identify β-galactosidase in leucocytes as an attempt to find explanations for the observed elevation of this enzyme and other lysosomal enzymes in dystrophic muscle.

The disrupted leucocyte preparations used for the detection of lysophosphatidase activity were also assayed for β-galactosidase activity. β-Galactosidase was assayed according to the method of De Duve as described in Chapter II. Table 40 shows the results of these experiments. Leucocytes of the dystrophic rabbits appeared to possess a higher activity of β-galactosidase than leucocytes of the control rabbits. Skeletal muscle samples from the same rabbits were assayed for β-galactosidase as shown in Table 41. An experiment to show the effect of reversal in diet was done. Three rabbits were fed vitamin E-deficient diet for 14 days and three control rabbits were pair fed vitamin E-supplemented for the same number of days (given the same amount of diet which was consumed by the vitamin E-deficient rabbits). After this period,

the rabbits which received vitamin E-deficient diet, were given vitamin E-supplemented diet for 7 days and the control rabbits were paid fed the same diet. Two rabbits survived, their leucocyte counts were similar to those rabbits which were maintained 14 days on diet as shown in Table 39. Leucocytes of these rabbits did not show elevation in lysophosphatidase or β-galactosidase. Lysosomal enzymes were tested in the skeletal muscle of these rabbits and the results were shown before.

Acid Hydrolases in Alveolar Macrophages from Vitamin E-deficient and Control Rabbits

Because of the ease of obtaining macrophages from lungs of rabbits, it was of interest to identify lysophosphatidase, representing a lipid enzyme, and β-galactosidase, representing the lysosomal enzymes. Only three experiments were done from dystrophic and control rabbits. The macrophages were isolated according to the method described earlier in Chapter II and were disrupted by repeated freezing and thawing and microhemogenization. The specific activities of β-galactosidase and lysophosphatidase are shown in Table 42.

TABLE 40

β-GALACTOSIDASE IN LEUCOCYTES OF VITAMIN E-DEFICIENT AND CONTROL RABBITS

Days on Diet		Specific Activity	
(+) E	(-) E	(+) E	(-) E
7P	7	21.8	21.2
7P	7	19.6	18.4
7P	7	18.8	20.7
20	20 #	16.4	47.2 #
21	20 #	28.2	43.6 #
24	24 #	21.6	48.4 #

(+) E = vitamin E-sufficient rabbits.
(-) E = vitamin E-deficient rabbits.
(#) = rabbits were dystrophic. (P) = pair fed rabbits. Specific activity = millimicromoles O-nitrophenol liberated per mg. protein per hour.

TABLE 41

β-GALACTOSIDASE IN SKELETAL MUSCLE OF VITAMIN E-DEFICIENT AND CONTROL RABBITS

Days on Diet		Specific Activity	
(+) E	(-) E	(+) E	(-) E
7P	7	0.61	0.68
7P	7	0.52	0.61
7P	7	0.66	0.58
20	20 #	0.59	2.22 #
21	20 #	0.73	2.15 #
24	24 #	0.68	1.89 #

The rabbits were the same ones of Table 42.
(+) E = vitamin E-sufficient rabbits.
(-) E = vitamin E-deficient rabbits.
(#) = rabbits were dystrophic.
(P) = pair fed rabbits.
Specific activity = millimicromoles o-nitrophenol liberated per mg. protein per hour.

TABLE 42

ACID HYDROLASE IN ALVEOLAR MACROPHAGES OF RABBITS

Experiment No.	Specific Activity			
	Lysophosphatidase		β-Galactosidase	
	(+) E	(-) E	(+) E	(-) E
1	2.3	2.8 #	54.4	50.6 #
2	2.4	2.2 #	45.2	53.4 #
3	2.5	2.2 #	52.6	48.4 #

Specific activity of lysophosphatidase = micromoles lysophosphatides consumed per mg. protein per hour.
Specific activity of β-galactosidase = millimicromoles o-nitrophenol liberated per mg. protein per hour.
= rabbits were dystrophic.
(+) E = vitamin E-sufficient rabbits.
(-) E = vitamin E-deficient rabbits.

CHAPTER IV

DISCUSSION

Experimental Muscular Dystrophy in Rabbits

The gross symptoms of muscular weakness in the rabbits suffering from vitamin E-deficiency were apparent after 19 to 25 days on diet as defined in this report. The dystrophic rabbits were unable to right themselves when placed on their sides or backs. There was also clinching in the forepaws, dragging and weakness of the extremities, diminution of respiration and listlessness. The vitamin E-deficient state (assayed by measurement of hemolysis of erythrocytes sensitive to the action of hydrogen peroxide) occurred after 7 days on diet as shown in Table 31. The in vitro addition of α-tocopherol to packed erythrocytes of vitamin E-deficient rabbits prevented the lability to hemolysis by hydrogen peroxide. These experiments indicate that there was an early depletion of vitamin E from the erythrocytes of vitamin E-deficient rabbits. The sequence of events leading to muscular dystrophy may have already started at this early phase of vitamin E-deficiency. The in vivo uptake of carbon C-14 by skeletal muscle of vitamin E-deficient rabbits (measured by determining the amount of radioactivity present in muscle homogenates) appeared to have increased slightly after 7 days on diet as illustrated in Figure 4. After this period on diet, there was no apparent elevation in the activities of the acid hydrolases studied in

muscle homogenates of vitamin E-deficient rabbits as shown in Figures 5, 6, 7, 8 and 9. Significant increases in the activities of enzymes studied were apparent after 14 days on diet. At this time, there were no apparent signs of muscular dystrophy but the in vivo uptake of carbon C-14 by skeletal muscle of vitamin E-deficient rabbits was significantly higher than that of control rabbits (Table 7).

The skeletal muscles of the hind limbs of the dystrophic rabbits appeared pale, moist and sometimes gritty. Samples of muscle tissue from dystrophic and control rabbits were fixed in 10% formalin solution for several days, then embedded in paraffin wax and sectioned at 10 micra. The sections were stained with hematoxylin and eosin by the conventional histological procedures. Microscopic examination of these sections indicated an extensive deterioration in the dystrophic muscle and a loss of the connective tissue surrounding muscle bundles and fibers. There was also a significant shrinkage of cross sections of the muscle fibers. It could not be ascertained, however, if the observed shrinkage was due to the method of preparing the muscle sections or it was a true picture of dystrophic muscle. These muscle samples were taken from rabbits that were injected with carbon C-14 suspensions. Very few carbon particles could be seen in both dystrophic and control muscle sections. However, the probability of finding carbon particles was higher in sections of dystrophic muscle. The carbon particles, when observed, were found outside the muscle fibers in the area normally occupied by the connective tissue. A low in vivo uptake of carbon C-14 by skeletal muscle was found as shown in Table 7. The observed changes in connective tissue are in agreement with the results obtained from nitrogen determina-

tions as shown in Table 32. There seems to be a reduction in the amount of collagen nitrogen. Menschik (139) studied the dietary factors influencing the collagen-producing activity of the fibroblasts by histochemical and cytological procedures of connective tissue of mice, guinea pigs and rabbits. It was found that proper differentiation of mesenchymal cells into fibroblasts was retarded by vitamin E deficiency. It was further observed that collagen-producing activity of the fibroblasts can be regulated by dietary factors, namely vitamin E, saturated triglycerides, and unsaturated fatty acids. The administration of large doses of unsaturated fatty acids superimposed on vitamin E deficiency was shown to cause metabolic disturbances of collagen leading to its fibrinoid degeneration. Such changes were observed in the connective tissue of skeletal muscle and cardiac muscle. It was mentioned earlier in Chapter I that the regenerative process of muscle is dependent upon the survival of muscle nuclei and the investing sarcoplasm together with a healthy endomysial connective tissue and functional circulation in the same area. If any of these requirements becomes defective, the affected area becomes ischemic and whatever muscle nuclei that may have escaped the initial damage will die. It is generally accepted that regeneration of muscle occurs only by budding or sprouting from the undamaged portion of fibers. The endomysial connective tissue and the sarcolemma are essential for muscle stimulation to contraction and muscle innervation. Defective sarcolemma and endomysial connective tissue result in degenerative processes which are not accompanied by regeneration. Denervation of muscle cause degeneration of the tissue, invasion by macrophages and polymorphonuclear leucocytes, and elevations in the activities of acid

hydrolases and TPN-dependent dehydrogenases. According to McCaman (60) the pattern of enzyme activities found in normal muscle denervated by nerve section was similar to that in dystrophic muscle of mice suffering from hereditary muscular dystrophy. Pearse et al. (111) demonstrated a dispersion of hydrolytic enzymes from the interstitial connective tissue to muscle fibers in muscles of dystrophic human patients. Similar results were observed by Jasmin et al. (114) and it was suggested that the observed changes in muscle fibers are secondary to the carcolemmal and endomysial damage. Studies by Bourne et al. (63) concerning the determination of some acid hydrolases in human muscular dystrophy indicated that the pathological process primarily involves the interstitial connective tissue.

There are several reports on the histopathology of muscular dystrophy produced by various causes. There are several forms of muscular dystrophy, for example: Nutritional muscular dystrophy in rabbits, hamsters, rats, guinea pigs and other animals, developed by vitamin E deficiency. Genetic muscular dystrophy in humans, rats and mice. Developed muscular dystrophy by various means; ischemia, trauma, application of solid carbon dioxide, dry ice (140), injection of dihydrotachysterol (DHT) plus 5-hydroxytryptamine (serotonin) in rats (113), injection of p-phenylenediamine (PPD) (114) and muscle denervation by nerve section of normal muscle (60).

It is generally accepted that muscle undergoes certain reactions in response to injury before necrosis and after which, the sequence of cellular changes are the same regardless of the initiating cause. The following descriptions are based on the reported observations by West and Mason

(1), Mackenzie et al. (110), Pearse et al. (111) and Bourne et al. (62). Microscopic examination of dystrophic muscle indicated the presence of a widespread interstitial edema, loss of cross straition, proliferation of nuclei, nuclear fragmentation, rupture of the sarcolemmal sheath, hyaline necrosis of the fibrillar substances, nuclear rowing, reaction clotting, coagulation necrosis, formation of granular masses within the fibers, extensive polymorphonuclear and mononuclear leucocytic infiltrations, giant cell formation, histiocytic infiltration and proliferation of macrophages. It should be emphasized that the above features are common to all forms of muscular dystrophy. The name muscular dystrophy may be misleading. According to Innes et al. (115) the pathological process of muscular dystrophy is essentially that of degeneration, necrosis, and inflammation. The lesions in paralysis are characterized by the degenerative and necrotizing changes which are the counterparts of the so-called experimental muscular dystrophy.

Role of the Reticuloendothelial System in Vitamin E Deficiency

Histopathological studies of dystrophic muscle tissues, developed by lack of dietary vitamin E, have indicated an increased histiocytic activity (polymorphonuclear and mononuclear leucocytic infiltration, giant cell formation, histiocytic infiltration and proliferation of macrophages). These responses of reticuloendothelial cells are nonspecific to vitamin E deficiency but rather a manifestation of inflammatory reactions. According to Harris (141) leucocytes and macrophages respond to certain stimuli chemotactically and migrate towards the injured or in-

flammed areas. It was demonstrated by Menkin (142) that inflammation is accompanied by an increased capillary permeability induced by a chemotactic agent (leucotaxine). As a result of the primary injury to muscle due to vitamin E deficiency, it is possible that some inflammatory stimuli have been produced, perhaps materials similar to leucotaxine or leucocytosis producing factors. In this respect, the primary cause of muscular degeneration as a result of vitamin E deficiency remains unknown. Whereas the vitamin E deficiency state occurred after 7 days on diet (Table 31), muscular weakness and degeneration was apparent after 19 to 25 days on diet. However, significant increases in the activities of acid hydrolases appeared after 14 days on diet (Figures 5, 6, 7, 8 and 9), and it can be seen that the in vivo uptake of carbon C-14 by skeletal muscle of vitamin E-deficient rabbits was increased slightly after 7 days on diet (Table 7 and Figure 4). These results may indicate that the inflammatory stimuli were produced at an early phase of the vitamin E-deficiency state. Mild inflammatory stimuli are known to cause destruction of connective tissue fibroblasts and the subsequent entrance of leucocytes and macrophages to the inflamed area (4).

Studies on several mesenchymal cell types bearing on the problem of the reticuloendothelial system have been reported by Richter (116). It was indicated that the reticuloendothelial system is dependent on how it is defined. To some, the histiocyte is of prime concern, as phagocytosis of some kind is the only clearly established functional structure attribute of this system. To others, the reticuloendothelial system is a particular physiological or functional cell state, which is completely independent of genetic limitations and can be achieved by many diverse

types of cells. It was further stated that the acquisition by specific cells of morphologic and phagocytic functional characteristic of the histiocyte has been viewed as a cellular modulation. There are several types of cells which have been shown to have the capacity to segregate within their cytoplasm colloidal particles and/or cell fragments or to have the capacity to transform into or give rise to histiocytic type cells. For example, in addition to fixed and free macrophages (histiocytes) there are the reticulum cells, the lining cells of lymph and certain vascular sinuses, the monocytes, neurolemmal cells, epididymal epithelial cells, germinal cells, lymph and blood endothelial cells, ordinary connective tissue fibroblasts, and fat cells.

It was discussed earlier that numerous factors must be considered when experimental lesions are produced in skeletal muscle. The histopathological descriptions of the injured muscle are similar regardless of the initiating cause and indicated the involvement of various reticuloendothelial system cells and increased levels of some acid hydrolases and TPN-dependent dehydrogenases.

The use of radioactive carbon particles has made it possible to obtain quantitative measurements of the phagocytic capacity of the reticuloendothelial system cells <u>in vivo</u>. This is especially important in determining the <u>in vivo</u> uptake of particulate matter by phagocytes present in muscle tissue. The size of carbon particles was reduced by grinding which should be less than 0.1 micron. Carbon black is not a metabolite and its removal from the blood stream is dependent on reticuloendothelial cells (RES) such as macrophages which are able to engulf (phagocytize) particulate matter. Several materials have been used to study the phago-

cytic capacity of the reticuloendothelial system cells among which are the following: Trypan blue, thorostat, carcinogenic hydrocarbons, fats, bacteria, proteins, dust, colloidal iron, iron dextran, cholesterol and cholesterol esters, proteins and others. The functioning of the reticuloendothelial system can be conditioned by certain stimulatory or inhibitory factors. It was demonstrated by Stuart et al. (117) that the injection of glyceryl trioleate had a stimulatory effect on the reticuloendothelial system function whereas ethyl oleate caused a depressant action. Relatively high dosages of cholesterol esters, when injected intravenously, can cause an inhibition of the phagocytic function, whereas relatively low dosages produce no effect. Berliner et al. (4) have shown that reticuloendothelial cells retain cholesterol in vivo. Cholesterol and cholesterol esters were found to increase in plasma of (dystrophic) vitamin E-deficient rabbits and guinea pigs, but in their skeletal muscles, free cholesterol showed the principal increase (143). The increased cholesterol levels in muscle of dystrophic rabbits may be a reflection of the increased number of the reticuloendothelial cells in the areas of muscular degeneration.

It has been demonstrated by Biozzi et al. (84) that the Kupffer cells of liver and macrophages of spleen are the most important cells of the reticuloendothelial system to remove particulate matter from the blood stream. The results of this study are in agreement with this view as shown in Tables 5 and 6. The rate of phagocytosis is therefore dependent on the phagocytic activity of liver and spleen macrophages and the weight of these organs. The rate of phagocytosis is a reflection of the rate of disappearance of injected carbon C-14. The rate constant (K) has

been named the phagocytic index. The phagocytic index values of rabbits fed vitamin E-deficient or vitamin E-supplemented diet are shown in Table 1. The results indicate that there was no significant difference between the phagocytic capacity of vitamin E-deficient and control rabbits. The phagocytic index values reamined virtually constant when plotted as a function of days on diet as shown in Figure 3.

It was interesting to note that the weights of livers and spleens per 100 grams rabbit were within the same range. The in vivo uptake of carbon C-14 by these organs based on grams liver percent rabbit were similar in vitamin E-deficient and control rabbits, shown in Tables 2 and 3. There was, however, a modest increase in the uptake of carbon by spleens of dystrophic rabbits but such increases could not be ascertained when the results are expressed as percentages of total injected dose, shown in Tables 4 and 5. The results seem to indicate that there was no significant difference between the phagocytic capacity of liver and spleens of vitamin E-deficient and control rabbits. Similarly, no correlation could be established between vitamin E-deficiency and the phagocytic capacity of lungs (Table 6). These findings are in contrast to the observed stimulation of the phagocytic activity by feeding carcinogenic diets (117).

The most prominent difference was the capacity of skeletal muscle of dystrophic rabbits to accumulate carbon C-14 particles. Table 7 shows the in vivo uptake of carbon C-14 by skeletal muscle as a function of days on diet and the values are represented graphically in Figure 4. After 7 days on diet, there was a noticeable increase in the in vivo uptake of carbon by skeletal muscle of rabbits maintained on vitamin E-de-

ficient diet. Such increases could not be ascertained because there was an overlapping with control rabbits. After 14 days, however, there was a 2- to 3-fold increase in the *in vivo* uptake of carbon C-14 by muscle of vitamin E-deficient but non-dystrophic rabbits when compared with control rabbits. The *in vivo* uptake of carbon C-14 by skeletal muscle of dystrophic rabbits suffering from vitamin E-deficiency was 4 to 5 times more than the *in vivo* uptake by control rabbits. The *in vivo* uptake by skeletal muscle of control rabbits fed vitamin E-supplemented diet remained constant when measured after various days on diet as shown in Figure 4. The results of Table 7 are expressed in counts per minute per gram muscle. If the results are expressed as counts per minute per gram protein, then the differences between the uptake of vitamin E-deficient and control rabbits would be slightly higher because one gram of dystrophic muscle contains about 0.9 of the amount of protein found in one gram of non-dystrophic muscle. Furthermore, the amount of injected carbon C-14 was proportional to the weight of the whole animal where the loss of weight was counteracted by injecting less material. Since the rabbits were fed *ad libitum*, it was of interest to see how pair-feeding influences the *in vivo* uptake of carbon C-14. The results indicated that there was no significant change resulted from the two forms of feeding as shown in Table 8. Feeding a vitamin E-supplemented diet to rabbits which were maintained 14 days on diet (before appearance of gross symptoms of muscular dystrophy) prevented the appearance of the gross symptoms of muscular weakness. The effect of the diet reversal on the *in vivo* uptake of carbon C-14 is shown in Table 8 where it is apparent that although the rabbits did not develop muscular dystrophy, the carbon C-14 content did not

drop to the low levels of control rabbits and there was still a noticeable increase but very much lower than in the dystrophic state.

Acid Hydrolases (Lysosomal Enzymes) in Skeletal Muscle of Vitamin E-Deficient and Control Rabbits

The total activities (bound + free) of acid phosphatase, cathepsin (pH 3.8), β-glucuronidase, neuraminidase, lysophosphatidase, and β-galactosidase were determined in preparation of skeletal muscle of rabbits maintained on vitamin E-deficient or vitamin E-sufficient diets. The results of these determinations are shown in Tables 9, 10, 11, 12, 13, and 43. The changes in the levels of these enzymes as a function of days on diet can be observed in Figures 5, 6, 7, 8, and 9. After 14 days on diet, the activities of the enzymes mentioned above, except lysophosphatidase, appear to have increased slightly in the homogenates of skeletal muscle of vitamin E-deficient rabbits. However, the activities of all the enzymes increased several fold when the rabbits became dystrophic after 19 to 25 days. Acid phosphatase increased 2- to 3-fold, β-glucuronidase increased 7- to 9-fold, cathepsin increased about 4-fold, neuraminidase increased 2- to 3-fold, β-galactosidase increased 3- to 4-fold, and lysophosphatidase increased 6- to 7-fold. The increases in the levels of these enzymes in muscle homogenates of vitamin E-deficient rabbits seem to parallel the observed increases in the *in vivo* uptake of carbon C-14 by skeletal muscle of the same rabbits. Compare Figures 5, 6, 7, 8 and 9 with Figure 4. These results indicate that there is a relationship between the increased number of phagocytes and the increased levels of acid hydrolases (lysosomal enzymes) in skeletal muscle of dystrophic rab-

bits due to vitamin E-deficiency. Since the vitamin E-deficiency state did not affect the function of the reticuloendothelial system (Figure 3) the increased phagocytic activity by phagocytes of skeletal muscle (Figure 4), can be considered proportional to the increased number of these cells.

Studies on the intracellular distribution of two acid hydrolases (neuraminidase and cathepsin) in skeletal muscle of vitamin E-supplemented rabbits (controls) indicate that the two enzymes seem to be concentrated in Fraction (C) which is the counterpart of the lysosomal fraction obtained from liver subcellular fractionation experiments. The lysosomal fraction of liver homogenates and Fraction (C) of skeletal muscle preparations are defined as those fractions obtained by centrifuging the homogenates between 3500 x g x 10 minutes and 20,000 x g x 10 minutes. The results of tissue fractionation experiments are shown in Tables 14 and 15 for skeletal muscle and Tables 26, 27, 28, 29 and 30 for liver. The graphical illustrations of the results are shown in Figures 10 and 11 for skeletal muscle experiments and Figures 15, 16, 17, 18 and 19 for liver studies.

The activities of neuraminidase and cathepsin in Fraction (C) which were obtained from skeletal muscle preparations of dystrophic (vitamin E-deficient) rabbits, dropped to a lower level. There was also an accompanying increase of both activities in the soluble fraction which is an indication of released enzymes from the bound form in the particulate fraction (Fraction C). The results of Tables 14 and 15 may suggest that neuraminidase and cathepsin are present or associated with certain intracellular organelles (lysosomes?) which may have ruptured <u>in vivo</u> in the

degenerating muscle tissue or they may have become more labile to disruption by homogenization due to certain changes in their structural organization. The interpretation of the results of these experiments is complicated by two considerations: 1. The heterogeneity of cellular populations in skeletal muscle tissues. In addition to muscle fibers, there are macrophages, fibroblasts and leucocytes which are present in the interstitial connective tissue. 2. The cellular populations of skeletal muscle of vitamin E-deficient (dystrophic) rabbits and controls (vitamin E-sufficient) are not comparable since the skeletal muscle of dystrophic animals is characterized by the presence of degenerative lesions infiltrated by various types of inflammatory cells. What cells of muscle tissue are the primary source of acid hydrolases? Are the sedimented granules or organelles in Fraction (C) comparable to lysosomes? Tissue fractionation experiments do not give information on the cellular source of the sedimented intracellular organelles. This is especially true if the tissue under study is a composite of various types of cells, as in the case of skeletal muscle or liver. Because of these difficulties, the two enzymes studied in muscle fractionation experiments, neuraminidase and cathepsin, may have originated from certain granules present in muscle fibers, macrophages, fibroblasts or leucocytes. There is no good evidence that muscle fibers contain particles which serve as reservoirs of hydrolytic enzymes, corresponding to the lysosomes of liver. Histochemical means which are used to identify lysosomes in liver, brain and kidney have failed to demonstrate lysosomes in skeletal muscle fibers (138). However, evidence has accumulated which support the view that granules similar to lysosomes are present in macrophages (34), leucocytes

(2), and fibroblasts (4). These cells are rich sources of acid hydrolases and the specific activities of the enzymes studied are several times higher than those observed in dystrophic skeletal muscle. Leucocytes appear to play an important role in the increased activities of enzymes in degenerative muscle. Lysophosphatidase activity which is present in very small amounts in normal muscle was found to increase 6-fold in skeletal muscle of dystrophic rabbits (Table 13). Leucocytes of dystrophic (vitamin E-deficient) rabbits exhibited increased lysophosphatidase activity (Table 38). These observations and the results of the in vivo uptake of carbon C-14 by skeletal muscle of vitamin E-deficient rabbits (Figure 4) which parallel the observed increases in the levels of the acid hydrolases studied (Figures 5, 6, 7, 8 and 9) appear to support the hypothesis that the observed increases in the levels of some enzymes in skeletal muscle are primarily due to an elevated number of reticuloendothelial system (RES) cells which are rich sources of the same enzymes. There are several known factors which influence cells of the reticuloendothelial system, among which are the following: Histamine, serotonin, bradykinin, leucotaxin, leucocytosis factor and necrosin (142). These factors promote the appearance of young macrophages, cause an increased migration of RES cells, and can stimulate phagocytosis. Goldberg et al. (144) demonstrated the effect of iron dextran on macrophages in vivo. It was observed that repeated injections of iron dextran into skeletal muscle of animals can cause an influx of macrophages at the site of injection accompanied by an elevation in the activities of acid hydrolases at the same injection site. It was proposed that the increased levels in acid hydrolases at the injection site were due to the increased number of

macrophages. Leucocytic infiltration and macrophage proliferation in the areas of degenerative lesions of skeletal muscle of animals have been observed by several workers. Degenerative lesions can be produced by various means other than lack of dietary vitamin E. The observed elevations in DNA synthesis in skeletal muscle of vitamin E-deficient hamsters were attributed to the presence of the large number of inflammatory cells in the degenerative lesions (145). The autoradiographic studies of Gerber et al.(145) indicated that DNA labeling in muscles of vitamin E-deficient animals was confined to the areas of degenerative lesions, and the labeled cells were mainly macrophages, fibroblasts and leucocytes. H^3-labeled thymidine was injected intraperitoneally to Syrian golden hamsters and DNA labeling was studied in skeletal muscle and in various tissues and organs. It was found that DNA synthesis and turnover were increased in skeletal muscle and bone marrow of vitamin E-deficient hamsters when compared with vitamin E-supplemented animals.

Macrophages and polymorphonuclear leucocytes have been shown to be rich sources of TPN-requiring dehydrogenases (140). Examples of these enzymes are: Glucose 6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase, isocitric dehydrogenase, α-glycerophosphate dehydrogenase and TPN-linked lactic dehydrogenase. The last enzyme has been identified by Navazio et al. (147). It was demonstrated by Karnovsky (40) that under aerobic conditions, polymorphonuclear leucocytes exhibit a very considerable increase in oxygen uptake during active phagocytosis.

The above listed TPN-requiring enzymes were found to increase significantly in degenerative lesions of skeletal muscle (60, 140) and several workers demonstrated an increased oxygen consumption of skeletal muscle

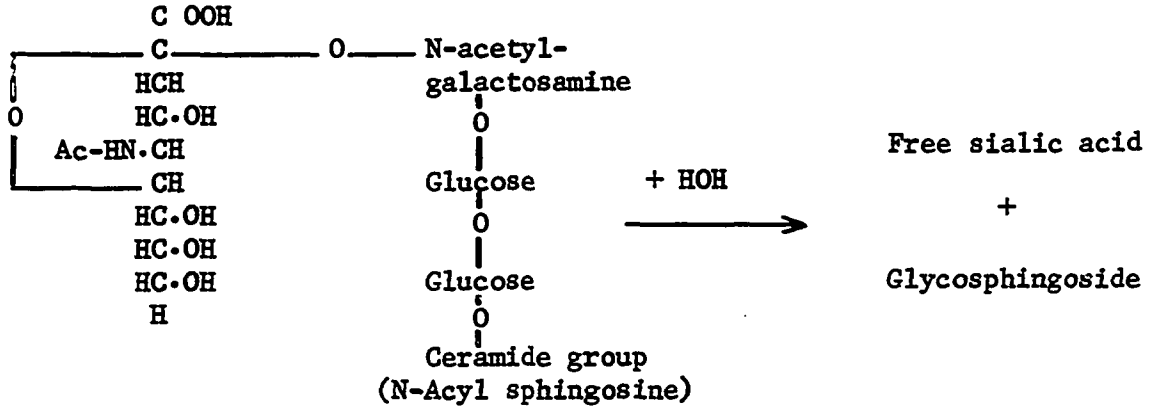
strips and homogenates from vitamin E-deficient (dystrophic) animals (148, 149, 150). These workers suggested that vitamin E-deficiency caused an uncoupling effect of oxidative phosphorylation.

The results shown in Table 7 and Figure 4 indicated that the phagocytic activity of reticuloendothelial cells of skeletal muscle of vitamin E-deficient rabbits was increased. The results also indicate that there was an increased number of phagocytes in the degenerative lesions of skeletal muscle of the dystrophic rabbits. Increased leucocytic infiltration to the degenerative areas of skeletal muscle of dystrophic animals were observed by several workers (1, 57, 113, 145). It is suggested, therefore, that the observed increases in the activities of TPN-requiring dehydrogenases, in the oxygen consumption, in the activities of some acid hydrolases, and DNA synthesis, in degenerative lesions of skeletal muscle produced by vitamin E-deficiency are due to the increased numbers of reticuloendothelial system cells in response to certain inflammatory stimuli.

Lipophilic Sialic Acid Compounds in Skeletal Muscle of Vitamin E-Deficient and Control Rabbits

In the present studies it was observed that neuraminidase activity has increased several fold in skeletal muscle of dystrophic rabbits suffering from vitamin E-deficiency (Table 12). It became of interest to investigate the significance of these elevated activities. Neuraminidase hydrolyses the terminal α-2-ketosidic bond between N-acetylneuraminic acid and 2-acetylamino-2-deoxy-D-galactose residues in various mucopolysaccharides and glycolipids to produce free neuraminic acid (sialic acid). For example, neuraminidase hydrolyses gangliosides as

follows:



Folch et al. (119) identified a lipophilic sialic acid substance in skeletal muscle of animals which they called "strandin" which has a high molecular weight (250,000) and forms well-oriented strands when its aqueous solutions are evaporated. The authors indicated that the amounts of strandin in skeletal muscle are very small (less than 0.01 percent). The amounts of sialic acid present in the lipid extracts of rabbit skeletal muscle are shown in Table 16. These results indicate that the levels of extracted sialic acid compounds by lipid solvents from skeletal muscle of dystrophic rabbits suffering from vitamin E-deficiency have increased significantly (10- to 12-fold). These results, however, may not indicate that elevated amounts of sialic acid compounds are present in the dystrophic skeletal muscle in vivo. They may merely indicate that as a result of muscle degeneration, these lipophilic sialic acid-containing compounds were rendered more extractable by lipid solvents namely chloroform-methanol (2:1, v/v). In a report by Booth (94) it was indicated that protamine and other highly basic proteins can interact with N-acetylneuraminic acid of gangliosides and renders half of this lipophilic sialic

acid-containing material inextractable into chloroform-methanol (2:1, v/v). It was further observed that the amount of gangliosides extractable from a protamine-ganglioside mixture can be increased by the addition of polysulfonic acids such as suramin. It was reported by Oppenheimer et al. (120) that sialic acid levels have increased significantly in α_2-globulin in serums of children with pseudohypertrophic muscular dystrophy. Sialic acid levels in serums of rabbits suffering from vitamin E-deficiency was shown to increase only when the rabbits became definitely dystrophic. These observations and the reported results in Table 16 seem to indicate the involvement of sialic acid-containing compounds in muscular dystrophy. There are recent reports by Castellani et al. (121) which suggest the presence of sialic acid in connective tissue. Reynolds et al. (122) reported that the concentration of sialic acid in wound tissues rose to fairly high levels from the usual concentration in skin. These high levels were found to decline during the period of wound healing. These authors support the view that sialic acid levels are an excellent index of intermediary connective tissue metabolism. They believe that an enzyme similar to the receptor destroying enzyme (neuraminidase) causes the loss of fibroblasts polarity in the regeneration process. There are no reports, however, to indicate that fibroblasts or mast cells are responsible for the synthesis of sialic acid compounds. Furthermore, there are no reports to indicate sialic acid compounds are actually present in the connective tissue of skeletal muscle. The results in Table 16 also show that control rabbits which were fed vitamin E-supplemented diet and weighing about 900 grams seem to have less sialic acid in their skeletal muscle than the rabbits weighing 1500 to 2000 grams. This ob-

servation may suggest that sialic acid is somehow involved in the processes of growth and aging.

Acid Hydrolases (Lysosomal Enzymes) in Livers and Spleens of Vitamin E-Deficient and Control Rabbits

Total activities (free + bound) of acid phosphatase, cathepsin at pH 3.8, β-glucuronidase, neuraminidase and lysophosphatidase were determined in liver homogenates of rabbits maintained on vitamin E-deficient or vitamin E-supplemented diets. The results are shown in Tables 17, 18, 19, 20 and 21. The changes in the levels of these enzymes at various number of days on diet are shown in Figures 12 and 13. These results indicate that the levels of acid phosphatase, cathepsin, β-glucuronidase, neuraminidase and lysophosphatidase did not change in liver preparations of vitamin E-deficient rabbits before the appearance of muscular dystrophy symptoms. When the vitamin E-deficient rabbits became dystrophic, the levels of two enzymes increased significantly, neuraminidase and lysophosphatidase, but the other hydrolases which have been identified as lysosomal enzymes according to De Duve *et al.* (14), namely, acid phosphatase, cathepsin, and β-glucuronidase, increased only slightly. Therefore, it became important to see if neuraminidase and lysophosphatidase are lysosomal enzymes.

Intracellular Distribution of Acid Phosphatase, Cathepsin, β-Glucuronidase, Neuraminidase and Lysophosphatidase

Subcellular fractions were obtained by differential centrifugation of homogenates of livers of rabbits which were dystrophic due to vitamin

E-deficiency and of control rabbits which were fed vitamin E-supplemented diet. The results of these fractionation experiments are shown in Tables 26, 27, 28, 29 and 30. The distribution patterns of these enzymes are illustrated in Figures 15, 16, 17, 18 and 19. It can be seen that in liver preparations of nondystrophic rabbit (vitamin E-sufficient), these enzymes exhibit their highest specific activity in the lysosomal fraction, the fraction intermediate between the mitochondrial and microsomal fractions. Neuraminidase and lysophosphatidase seem to be concentrated in the lysosomal and soluble fractions (Tables 29 and 30) whereas the classical lysosomal enzymes according to De Duve et al. (14) appear to concentrate their activities in the particulate fractions. For example, acid phosphatase and cathepsin are concentrated in the lysosomal and mitochondrial fractions and exhibit a small activity in the soluble fraction. β-Glucuronidase appears to be concentrated in the lysosomal and microsomal fractions and exhibits a small activity in the soluble fraction. It appears, therefore, that there are significant differences between the intracellular distribution of the classical lysosomal enzymes and the two enzymes, neuraminidase and lysophosphatidase. Furthermore, the lysosomal concept advanced by De Duve et al. (23) cannot include enzymes which exhibit a high specific activity in the soluble fraction under normal conditions. The problem of lysophosphatidase will be discussed in a separate section. It will be shown that the observed activity for the destruction of lysophosphatides may be a result of two enzymic reactions, one enzyme is localized in the particulate fractions (lysosomal) and another enzyme is localized in the soluble fractions. The problem of neuraminidase is more difficult to explain and its existence in the lysosomal

and soluble fractions may suggest the presence of two enzymic activities which are specific for different sialic acid-containing compounds. Raffelson et al. (123) demonstrated a number of differences between two neuraminidases obtained from two strains of virus, the Asian virus and the PR 8 virus. These enzymes were shown to act preferentially on different substrates. The intracellular localization of sialic acid-containing compounds in rat liver can be seen in Table 36. These compounds seem to be concentrated in the particulate fractions. However, the chemical composition of these compounds has not been determined. On the basis of thin-layer chromatography of the crude extracts of liver by chloroform-methanol (2:1), there were three bands which showed the presence of sialic acid. Therefore, it is possible that there are several types of sialic acid compounds within the cell.

The specific activities of all the enzymes studied were reduced significantly in the lysosomal fractions obtained from the subcellular fractionation experiments of livers of rabbits with muscular dystrophy as shown in Figures 15 to 19. There was a concurrent increase in the specific activities of these enzymes in the soluble fractions. These phenomena, if actually occurred in vivo, may reflect an induction of liver necrosis or degeneration in the vitamin E-deficient rabbits which became dystrophic. Dianzani et al. (49) described the release of acid phosphatase and cathepsin in liver homogenates from animals treated with carbon tetrachloride. Beaufay et al. (50) found an increase of the lysosomal enzymes present in the soluble fraction by feeding necrotizing diets. De Duve et al. (46) reported that a rapid release of five lysosomal enzymes has been observed in liver tissues rendered completely ischemic by

ligation of the blood vessels. Similar results have been obtained with respect to acid phosphatase and β-glucuronidase by Van Lancker et al. (47) on autolysing mouse liver fragments. All these workers indicated that there was a concurrent increase in the total activities of the enzymes studied. De Duve et al. (46) could not establish a correlation between the appearance of microscopic lesions in liver and the observed enzymic changes. These workers suggested that the lack of correlation is an indication that the enzymic changes precede the appearance of lesions. It was further suggested that liver degeneration is a result of the damaging action of the released acid hydrolases in vivo due to rupture of lysosomes.

The gross examination of the livers of the dystrophic rabbits studied did not show signs of necrosis or deterioration. Since the Kupffer cells seem to be involved in dietary liver necrotic degeneration, then it should be expected that the phagocytic index and the uptake of carbon C-14 by liver should be altered in liver necrosis. The results shown in Tables 1, 2 and 4 indicated that there was no significant difference between the values obtained from the dystrophic rabbits and controls.

There is a vast and confusing literature on the subject of liver degeneration due to vitamin E-deficiency. According to Schwarz (124) liver degeneration can be produced in rats by a simultaneous lack of three factors: cystine, vitamin E and Factor 3.

The effect of vitamin E-deficiency on the enzymic levels of spleens was also studied. The following enzymes were investigated: acid phosphatase and β-glucuronidase as markers for lysosomal enzymes, and lysophosphatidase. The results of these studies are shown in Tables 22, 23

and 24. The changes in the levels of the enzymes as a function of the number of days on diet are represented graphically in Figure 14. Similar to the observed increases in liver, only lysophosphatidase showed a significant increase when the rabbits became definitely dystrophic.

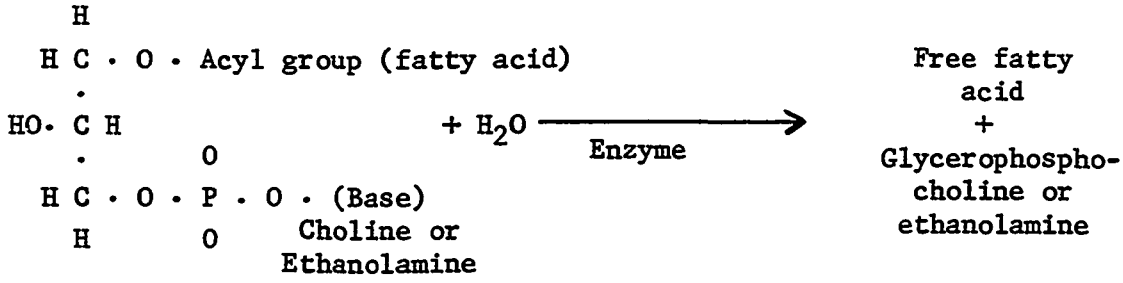
Because of the observed elevation in the activities of lysophosphatidase in liver, spleen and muscle of dystrophic rabbits, it became of interest to see if other phospholipases show similar increases. Attempts were made to identify phospholipase A (a phosphatide β-acylhydrolase), phospholipase C (a phosphatidylcholine cholinephosphohydrolase) and phospholipase D (phosphotidylcholine phosphatidohydrolase). The tissues examined were liver, spleen and muscle. No activity of any of these enzymes listed above could be observed in fresh tissue homogenates. After aging for two to three weeks, a small activity of these enzymes was detected. However, due to the difficulties in the assay methods used, it could not be ascertained whether such small activities are significant. Furthermore, storing in the cold room could have resulted a contamination with microorganisms. It was reported by Shapiro et al. (125) that phospholipase A activity was measurable in aged pancreatic tissues and that no phospholipase A activity could be demonstrated in fresh tissue.

Lysophosphatidase Activity (Lysophosphatide Destroying Enzyme)

The loss of the hemolytic activity of lysophosphatides by the action of tissue homogenates has been attributed to some enzymic reactions which caused alteration of these compounds. The criteria which indicated that the destruction of the hemolytic activity of lysophosphatides are

the following: there was no destruction of the hemolytic activity of lysophosphatides when incubated at 5°C for 1 hour with tissue preparations, heated tissue preparations at 100°C for 10 minutes did not affect the hemolytic activity of lysophosphatides, the hemolytic activity of lysophosphatides decreased exponentially with time when the reaction mixture was incubated at 37°C, there was a pH optimum about 6.0, and the reaction was temperature dependent.

There are two known enzymic reactions in animal tissues which catalyse the hydrolysis of lysophosphatides: 1. By the action of a lysophosphatide acylhydrolase (phospholipase B) (126). 2. By the action of a β-acylase (127). Phospholipase B catalyzes the following reaction:

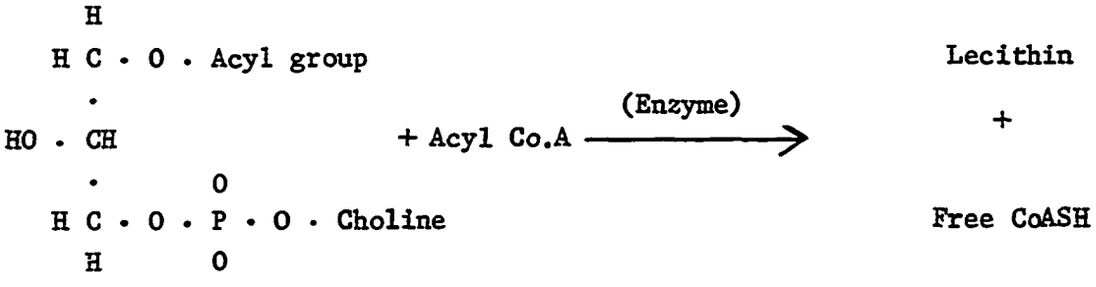


Glycerophospho-choline or -ethanolamine can be hydrolyzed to α-glycerophosphate + free base (choline or ethanolamine). The assay of this reaction depends on the measurements of free choline or ethanolamine.

Phospholipase B activity can be measured by two methods: 1. By measuring the decrease in the number of acyl groups or by estimating the increase in free fatty acids. 2. By the measurement of glycerophosphorylcholine or glycerophosphorylethanolamine. This latter method is dependent on free choline measurements after acid hydrolysis of glycerophosphorylcholine or by the action of glycerophosphorylcholine diesterase. Both of these methods were found unsatisfactory as mentioned earlier

in Chapter II.

β-Acylase activity, which introduces an acyl group on the β-position of glycerol to form a phosphatide, has been studied by Lands (127). The reactions involved in converting lysolecithin to lecithin were suggested to be as follows:



The author determined β-acylase activity by incubating radioactive lysolecithin with oleic acid, ATP, CoA, and $MgCl_2$ in the presence of rat liver homogenates. It was shown that the conversion of lysolecithin into lecithin occurred predominantly in the heavy and light microsomal fractions. The heavy microsomal fraction according to the terminology of Lands (127) is equivalent to the lysosomal fraction according to the terminology of liver subcellular fractions described in this report. The hydrolysis of lysolecithin to give free fatty acids was shown to be carried out chiefly in the soluble fraction. These observations seem to be in agreement with the observed lysophosphatidase activity in liver subcellular fractionation experiments (Table 30 and Figure 19). The method used for the determination of lysophosphatidase activity in these experiments was based on the disappearance of the hemolytic activity of lysophosphatides. Therefore, the observed activity for the destruction of lysophosphatides is possibly a result of the action of two enzymes,

phospholiOase B and β-acylase. The presence of two enzymes can then explain why lysophosphatidase activity is distributed in the lysosomal fraction and the soluble fraction, whereas the classical lysosomal enzymes are predominantly present in the particulate fractions with a small activity in the soluble fractions. In this connection, it should be mentioned that neuraminidase seems to present a different problem which cannot be solved on the basis of the available information on the properties of this enzyme.

Leucocytes and alveolar macrophages appear to be rich in lysophosphatidase activity (Tables 38 and 42). A recent report by Rizack et al. (128) described the presence of a lysophospholipase in rabbit polymorphonuclear leucocytes from peritoneal exudates. These authors determined the enzymic activity by measuring the liberated free fatty acids.

The significance of increased lysophosphatidase activities in the various rabbit tissues when the animals became dystrophic cannot be explained at the present time because of the lack in the formation concerning the reactions that can lead to the synthesis of lysophosphatides in vivo. There is no conclusive evidence to prove the presence of phospholipase A (phosphatide β-acylhydrolase) in animal tissues. The biosynthetic reactions described by Kennedy et al. (129) for lecithin and cephalin synthesis do not clearly establish the possibility of a similar biosynthetic pathway for the production of lysophospholipids.

Lysophosphatides were shown to cause a rapid hemolysis of rabbit erythrocytes (Figure 20). Similarly, they caused a rapid rupture of isolated lysosomes which were obtained from rat and rabbit liver subcellular fractionation experiments, causing the release of acid hydrolases,

β-glucuronidase and cathepsin.

Experiments on Encephalomalacic Chickens Due to Vitamin E-Deficiency

If one-day-old chicks are fed a vitamin E-deficient diet, they usually will develop a state of nutritional encephalomalacia or exudative diathesis, or sometimes both, during the first two months of life. The two manifestations seem to be secondary to certain dysfunctions of the capillary bed and are influenced considerably by variations in fats and other dietary components.

Pappenheimer et al. (130) described the symptoms of nutritional encephalomalacia in chicks. The cerebrum is also affected although the cerebellum is the usual tissue where necrosis occurs. These workers indicated that although capillary thrombosis seems to be the primary cause of ischemic necrosis, it is possible that some of the symptoms may be secondary to prolonged vasoconstriction or vasomotor paralysis of larger blood vessels. It was observed also that there was an involvement of phagocytes during the spontaneous or induced recovery. Nutritional encephalomalacia in chicks may be considered the counterpart of nutritional muscular dystrophy in rabbits. Both diseases are caused by the lack of dietary vitamin E. It became of interest to see if acid hydrolases exhibit an elevated activity in encephalomalacia as it has been observed in the muscle of dystrophic rabbits.

Two enzymes were examined in the cerebra of encephalomalacic and control chicks, acid phosphatase as a representative of lysosomal enzymes and neuraminidase. There was a significant increase in the activities of

both enzymes but to a much lesser extent than the increases in muscle of dystrophic rabbits (Tables 33 and 34). The intracellular localization of these two enzymes appears to correspond fairly well with their distribution in rabbit liver subcellular fractions. Similar to liver subcellular fractionation studies, there was a significant reduction of the enzymic activities in the lysosomal fraction with a concurrent increase in the soluble fractions. There was a noticeable increase in the specific activity of neuraminidase in the microsomal fraction when the chicks are encephalomalacic, the relative specific activity of the control was 0.67 and increased to 1.14. This observation may indicate an accelerated synthesis. It is not known whether phagocytes play an important role in necrotic chick brain. The mechanism by which acid phosphatase has been released cannot be established on the basis of these experiments. This enzyme may have been released in vivo and caused an extensive damage to brain cells or that the granules containing it (lysosomes) became more labile to homogenization than in control chicks. There are no histochemical reports to indicate that there was a decrease in the acid phosphatase containing granules, at least as an indirect evidence that the enzymic activity has been released.

Neuraminidase appears to be concentrated in the lysosomal and soluble fraction similar to the observed distribution in liver cells. The hypothesis that there are two enzymes which can catalyse the hydrolytic cleavage of the glycosidic bond joining the keto group of neuraminic acid to D-galactose of glycopolysaccharides may be supported by the finding of Morgan et al. (131). These workers reported the presence of an enzyme (neuraminidase) in mammalian brain which releases sialic acid from

the endogenous sialic acid-containing compounds. This enzyme was shown to have an optimum pH of (3.5-4.0). In this report, the neuraminidase activity was tested at (pH 5.0).

The intracellular distribution of lipophilic sialic acid-containing compounds was studied in chick cerebra and was found to be largely concentrated in the mitochondrial and lysosomal fractions (Table 35). Wherrett and McIlwain (132) studied the intracellular distribution of gangliosides (sialic acid-containing compounds) in the gray matter from the cerebral cortex of guinea pigs. It was found that these compounds were localized primarily in the microsomal fraction. The results in Table 35 were obtained by a different subcellular fractionation procedure than that used by the above authors (132). The following differences were noted which may have contributed to the dislocation of sedimented materials in different fractions. The authors used 0.32 M sucrose as the suspending medium, the starting material for the isolation of gangliosides was the gray matter of the cerebral cortex of guinea pigs, and a basically different centrifuging procedure. The procedures used in this project for the isolation of sialic acid-containing compounds were presented in Chapter II. The suspending medium was 0.25 M sucrose, the starting material was the cerebra of chicks, and a centrifuging technique was adopted to obtain five fractions.

Experiments on Isolated Leucocytes from the Blood of Vitamin E-Deficient and Vitamin E-Sufficient Rabbits

The results shown in Table 39 indicate that vitamin E-deficient rabbits display an increased leucocytosis accompanied by granulocytosis

(Figure 21). The leucocytes of vitamin E-sufficient rabbits (controls) were also increased after 20 to 24 days on diet. The differences in the enzymic contents of leucocytes isolated from vitamin E-deficient and vitamin E-sufficient rabbits appear to be dependent on the differences in cell types. The leucocytes of dystrophic rabbits (vitamin E-deficient) were predominantly granulocytes (neutrophils + eosinophils + basophils) and exhibited significantly higher specific activities of lysophosphatidase and β-galactosidase. These points are illustrated in Tables 38, 39 and 40. The results indicate that granulocytes are rich in these acid hydrolases. The work of Cohn and Hirsh (22) indicated that polymorphonuclear leucocytes from peritoneal exudates of rabbits and guinea pigs are rich sources of acid phosphatase, β-glucuronidase, and cathepsin. The specific activities of these enzymes in leucocytes (from blood and peritoneal exudates) are several times higher than the specific activities of the same enzymes in skeletal muscle of dystrophic rabbits. Leucocytic infiltration to injured skeletal muscle areas developed by vitamin E-deficiency or by other means have been observed by several workers as discussed before. Similarly, the observed leucocytosis and granulocytosis are not specific for vitamin E deficiency. It is possible, however, that granulocytosis is produced in response to certain stimulatory effects (on the bone marrow) caused by agents liberated from the lesions developed in skeletal muscle of vitamin E-deficient animals. The mechanism responsible for the increased replacement of granulocytes (145) is not clear. DNA synthesis and cell renewal were found to be accelerated in the bone marrow of vitamin E-deficient animals (146). The observed leucocytosis and

granulocytosis reported in Table 39 are in agreement with the observation of Dinning (133).

Lysophosphatidase and β-galactosidase activities in lung macrophages of control rabbits (vitamin E-supplemented) were significantly higher than in leucocytes of the same rabbits. The activities of these two enzymes were similar in lung macrophages of vitamin E-deficient (dystrophic) rabbits and controls (Table 42). These results indicate that alveolar macrophages were not affected by lack of dietary vitamin E. The phagocytic function of alveolar macrophages was not altered as shown in Table 6. These experiments support the view that there is a close correlation between the changes in the enzymic activities in macrophages and the changes in the phagocytic capacity of these cells.

Granulocytes may act as carriers of enzymes which can be liberated into the tissues at the moment of their destruction, helping to provoke inflammatory reaction in situ and eventually to stimulate the activity of other cells which take part in the inflammatory process.

CHAPTER V

SUMMARY

Studies were made to correlate the proliferation of phagocytic cells in various tissues with the levels of acid hydrolases (lysosomal enzymes). Significant increases in both the enzymic levels and the *in vivo* uptake of carbon C-14 particles in skeletal muscle were observed before the appearance of the earliest symptoms of muscular dystrophy due to lack of dietary vitamin E.

The role of the reticuloendothelial system was investigated by measuring the rate of disappearance of radioactive carbon particles from the blood stream of injected rabbits which were fed vitamin E-deficient or vitamin E-supplemented diets. The calculated values of the phagocytic index (K) obtained at various lengths of time on the experimental diet indicated that there was no significant difference between those obtained from vitamin E-deficient and control rabbits. There were no significant differences between the *in vivo* uptake of carbon particles by spleens, livers and lungs of vitamin E-deficient (including those which became dystrophic) and vitamin E-supplemented rabbits. Most of the injected carbon particles were retained by liver and spleen.

The *in vivo* uptake of carbon C-14 particles by skeletal muscle was found to be significantly higher in vitamin E-deficient animals than

controls which were fed vitamin E-supplemented diet. The increase apparently begins at the seventh day on the experimental diet. After the second week on diet and before the appearance of the earliest symptoms of muscular dystrophy, there was a 2- to 3-fold increase in the in vivo uptake of carbon C-14 particles by skeletal muscle of vitamin E-deficient rabbits. There was a 4- to 6-fold increase when the rabbits became dystrophic. These results indicate that there was an early proliferation of phagocytic cells.

Significant elevations in the activities of acid hydrolases in skeletal muscle of vitamin E-deficient rabbits were apparent by the second week on diet. A similar increase in the in vivo uptake of carbon C-14 particles in the same muscles was observed. Although the carbon uptake by skeletal muscle of vitamin E-deficient animals indicated that the phagocytic activity was increasing after only one week on diet, a similar elevation of hydrolytic enzymes at one week was not observed. It is suggested that an initial activation of macrophages may have occurred before proliferation or before the invasion of blood-born leucocytes to the affected areas of skeletal muscle. Of the enzymes studied, the increases in acid phosphatase, cathepsin at (pH 3.8), β-glucuronidase, β-galactosidase and neuraminidase followed a similar pattern while lysophosphatidase activity showed proportionately greater elevation during the course of the experiment.

The observed elevations in the activities of neuraminidase and lysophosphatidase in skeletal muscle of dystrophic rabbits suffering from vitamin E deficiency were demonstrated for the first time in this laboratory. On the basis of liver tissue fractionation experiments,

all the above acid hydrolases can be considered lysosomal enzymes, Neuraminidase and lysophosphatidase appear to have different intracellular distribution patterns than the classical lysosomal enzymes studied (acid phosphatase, β-glucuronidase, and cathepsin).

The presence of lysosomes in muscle fibers per se could not be established unequivocally on the basis of muscle tissue fractionation experiments because of the heterogeneity of cells present in skeletal muscle. These fractionation experiments cannot exclude the possibility that certain cytoplasmic granules or organelles similar to lysosomes are present in cells located in the connective tissue surrounding muscle bundles (fibroblasts, macrophages and leucocytes). The observed decrease in the activities of cathepsin and neuraminidase in fractions sedimented between (3500 x g x 10 minutes) and (20,000 x g x 10 minutes) obtained from muscle tissue fractionation experiments of vitamin E-deficient animals can be attributed to their release from granules (lysosomes?) of fibroblasts, macrophages and leucocytes.

Lysophosphatidase activity was newly discovered in this laboratory to be present in leucocytes of peripheral blood of rabbits. This enzymic activity was found to be several fold higher in the leucocytes of rabbits suffering from lack of dietary vitamin E than in the leucocytes of control rabbits which were fed vitamin E-supplemented diet. This increase was found to be in correlation with the observed granulocytosis in the peripheral blood of the same animals.

Vitamin E deficiency in rabbits resulted in leucocytosis and granulocytosis. It is suggested that the infiltration of leucocytes to the injured muscle areas contributed significantly to the observed eleva-

tions in the activities of acid hydrolases.

In liver and spleen, the classical lysosomal enzymes (acid phosphatase, cathepsin (pH 3.8) and β-glucuronidase) increased only slightly when the rabbits became definitely dystrophic due to lack of dietary vitamin E, whereas neuraminidase and lysophosphatidase were increased significantly.

The intracellular distribution studies of lysophosphatidase in liver tissue fractionation experiments indicated that the activity is concentrated in both the lysosomal and soluble fractions. It is suggested that the observed lysophosphatidase activity in the two fractions is represented by two enzymes, one being localized in the particulate fraction (lysosomal), probably an acylase, and the other in the soluble fraction, probably an acyl hydrolase.

Neuraminidase in liver fractions was also shown to be concentrated in both the lysosomal and the soluble fractions. The presence of high neuraminidase activity in the soluble fraction is contradictory to the lysosomal concept and again suggests the presence of two neuraminidases.

The intracellular distribution of sialic acid compounds in liver tissue fractionation experiments indicated that these compounds (which may be different in their chemical composition) are concentrated in the particulate fractions. The mitochondrial and lysosomal fractions contained the highest percentage of total sialic acid compounds extracted from tissue homogenates. The presence of neuraminidase in the soluble fraction would make the sialic acid containing substances vulnerable to the hydrolytic action of the enzyme unless the compounds are inactive

as substrates. It is suggested that if there are two neuraminidases in liver, they would have different substrate specificities.

The results support the concept that changes in muscle connective tissue precedes any measurable alterations of muscle fibers in vitamin E deficiency and that the source of the increased enzymic activities in skeletal muscle of dystrophic rabbits suffering from vitamin E deficiency are the increased cells of the reticuloendothelial system, fibroblasts, macrophages and leucocytes.

REFERENCES

1. West, W.T., and Mason, K.E., Am. J. Anat., 102, 323 (1958)

2. Cohn, Z.A., and Hirsch, J.G., J. Exptl. Med., 112, 983 (1960)

3. Wattiaux, R., Baudhuin, P., Berleur, A.M., and De Duve, C., Biochem. J., 63, 608 (1956)

4. Berliner, D.L., and Dougherty, T.F., Ann. N.Y. Acad. Sci., 88, 14 (1960)

5. Schneider, W.C., J. Biol. Chem., 161, 293 (1945)

6. Hogeboom, G.H., in Methods of Enzymology, S.P. Colowick and N.O. Kaplan, edit., Vol. 1, 16, Acad. Press, New York (1955)

7. Claude, A., J. Exptl. Med., 84, 51 (1946)

8. Dounce, A.L., Ann. N.Y. Acad. Sci., 50, 982 (1950)

9. Palade, G.E., J. Exptl. Med., 95, 285 (1952)

10. De Duve, C., Berthet, J., and Beaufay, H., Prog. Biophys., 9, 325 (1959)

11. Svedburg, T., and Nichols, J., J. Am. Chem. Soc., 45, 2910 (1923)

12. Anderson, N.G., Exptl. Cell Res., 9, 446 (1955)

13. Beaufay, H., Bendal, D., Baudhuin, P., Wattiaux, R., and De Duve, C., Biochem. J., 73, 628 (1959)

14. De Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R., and Applemans, F., Biochem. J., 60, 604 (1955)

15. Schneider, W., and Hogeboom, G., J. Biol. Chem., 195, 161 (1952)

16. Palade, G.E., Arch. Biochem., 30, 144 (1951)

17. Novikoff, A.B., Podler, E., Ryan, J., and Noe, E., J. Histochem. and Cytochem., 1, 27 (1953)

18. Tsoboi, K.K., Biochim. Biophys. Acta, 8, 173 (1952)

19. Roye, A.B., Biochem. J., 68, 519 (1958)

20. Sellinger, O.Z., Beaufay, H., Jacques, P., Doyen, A., and De Duve, C., Biochem. J., 74, 450 (1960)

21. Paigen, K., and Griffiths, S.K., J. Biol. Chem., 234, 299 (1959)

22. Hirsch, J.G., and Cohn, Z.A., J. Exptl. Med., 112, 1005 (1960)

23. De Duve, C., in Subcellular Particles, T. Hayashi, edit., pp. 128, Ronald Press Co., New York (1959)

24. Beaufay, H., and De Duve, C., Biochem. J., 73, 604 (1959)

25. Novikoff, A.B., Beaufay, H., and De Duve, C., J. Biophys. Biochem. Cytol., 2, Suppl. 179 (1956)

26. Beaufay, H., Bendal, D.S., Baudhuin, P., and De Duve, C., Biochem. J., 73, 623 (1959)

27. Palade, G.E., J. Biophys. Biophys. Biochem. Cytol., 2, Suppl. 85 (1956)

28. Bennedetti, E.L., and Bernhard, W., J. Ultrastructure Research, 1, 309 (1958)

29. De Duve, C., Beaufay, H., Jacques, P., Rahman-Li, Y., Sellinger, O.Z., Wattiaux, R., and De Coninck, S., Biochim. Biophys. Acta, 40, 186 (1960)

30. Gansler, H., and Rouiller, C., Schweiz. Z. Path. u. Bakteriol., 19, 217 (1956)

31. Beaufay, H., and De Duve, C., Arch. Intrn. Physiol. et Biochem., 65, 156 (1957)

32. Strause, W., J. Biophys. Biochem. Cytol., 4, 541 (1958)

33. Doyle, W.L., J. Biophys. Biochem. Cytol., 1, 221 (1955)

34. Wattiaux, R., Baudhuin, P., Berleur, A., and De Duve, C., Biochem. J., 63, 608 (1956)

35. Rous, P., and Beard, J.W., J. Exptl. Med., 59, 577 (1934)

36. St. George, S.S., Friedman, M., and Byers, S.O., Science, 120, 463 (1954)

37. Bennett, H.S., J. Biophys. Biochem. Cytol., 2, Suppl., 185 (1956)

38. Weber, R., Experientia, 13, 154 (1957)

39. Cohn, A.Z., and Hirsch, J.G., J. Exptl. Med., 112, 1015 (1960)

40. Karnovsky, M.L., in Biological Activity of the Leucocyte, Ciba Foundation Study Group, No. 10, 10 (1960)

41. Sbarra, A., and Karnovsky, M.L., J. Biol. Chem., 235, 2224 (1960)

42. Evans, W.H., and Karnovsky, M.L., Biochemistry, 1, 159 (1962)

43. Evans, W.H., and Karnovsky, M.L., J. Biol. Chem., 236, PC 30 (1960)

44. Borel, C., Frei, J., Montri, S., and Vannotti, A., Helv. Med. Acta, 26, 785 (1959)

45. Benacerraff, B., Biozzi, G., Halpern, B.N., and Stiffel, C., Res. Bull., 2, 19 (1956)

46. De Duve, C., and Beaufay, H., Biochem. J., 73, 610 (1959)

47. Van Lancker, J.L., and Holtzer, R.L., Am. J. Path., 35, 563 (1959)

48. Weber, R., Rev. Suisse Zool., 64, (1957)

49. Martini, E., and Dianzani, M.U., Experientia, 14, 285 (1959)

50. Beaufay, H., Van Campenhout, E., and De Duve, C., Biochem. J., 73, 617 (1959)

51. Thomas, L., Mclusky, R.T., Potter, J.L., and Weissman, J., J. Exptl. Med., 111, 705 (1960)

52. Bell, H.B., Dingle, J.T., and Webb, M., Biochem. J., 83, 63 (1962)

53. Weissman, G., and Dingle, J.T., Exptl. Cell Research, 25, 207 (1961)

54. Gallagher, C.H., Nature, 192, 881 (1961)

55. Priest, R.E., Smuckler, E.A., Iseri, O.A., and Beneditt, E.P., Proc. Soc. Exptl. Biol. Med., 111, 50 (1962)

56. Weinstock, I.M., Goldrich, A.D., and Milhorat, A.T., Proc. Soc. Exptl. Biol. Med., 88, 257 (1955)

57. Kozalka, T.R., Mason, K.E., and Krol, G., J. Nutrition, 73, 78 (1961)

58. Tappel, A.L., Zalkin, H., Caldwell, K.A., Desai, F.D., and Shibko, S., Arch. Biochem. Biophys., 96, 340 (1962)

59. Zalkin, H., Tappel, A.L., Caldwell, K.A., Shibko, S., Desai, F.D., and Holliday, T.A., J. Biol. Chem., 237, 2678 (1962)

60. McCaman, M.W., Am. J. Physiol., 205, 897 (1963)

61. Bajusz, E., and Jasmin, G., Rev. Canad. Biol., 21, 409 (1962)

62. Bourne, G.H., and Golarz, M.N., Rev. Canad. Biol., 21, 383 (1962)

63. Bourne, G.H., and Golarz, M.N., Nature, 183, 1741 (1959)

64. Nason, A., Biochemistry of Vitamin E, in Vitamins and Hormones, Vol. 18, Academic Press, Inc., New York (1960)

65. Kohn, H.I., and Liversedge, M., J. Pharmacol. Exptl. Therap., 82, 292 (1944)

66. Chenau, U., Trucco, R., El-Khatib, S., Carpenter, M., and Caputto, R., Fed. Proc., 21, 473 (1962)

67. Harris, R.S., Mattill, H.A., and Mason, K.E., The Tocopherols, in The Vitamins, Vol. III, W.H. Serbell, Jr., and R.S. Harris, ed., Academic Press, Inc., New York (1954)

68. Evans, H.M., and Burr, G.O., J. Biol. Chem., 76, 273 (1928)

69. Blaxter, K.L., Brown, F., Wood, W.A., and McDonald, A.M., Brit. J. Nutrition, 7, 337 (1953)

70. Mackenzie, J.B., and Mackenzie, C.B., Proc. Soc. Exptl. Biol. Med., 84, 388 (1953)

71. Dam, H., Kruse, I., Prange, I., and Sondergaard, E., Acta Physiol. Scand., 22, 299 (1951)

72. Dam, H., and Glavend, J., Nature, 143, 810 (1939)

73. Dam, H., J. Nutrition, 27, 193 (1943)

74. Pappenheimer, A.M., Physiol. Rev., 23, 37 (1943)

75. Draper, H.H., Goodyear, S., Barbee, K.D., and Johnson, B.C., Brit. J. Nutrition, 12, 89 (1958)

76. Green, J., Diplock, A.T., Bunyon, J., and Edwin, E.E., Biochem. J., 79, 108 (1961)

77. Carubelli, R., Ryan, L.C., Trucco, R.E., and Caputto, R., J. Biol. Chem., 236, 2381 (1961)

78. Long, C., and Penny, I.F., Biochem. J., 65, 382 (1957)

79. Warren, L., J. Biol. Chem., 234, 1971 (1959)

80. Glassman, H.N., Science, 111, 688 (1950)

81. Caputto, R., McCay, P., and Carpenter, M., J. Biol. Chem., 233, 1025 (1958)

82. Young, J.M., and Dinning, J.S., J. Biol. Chem., 193, 743 (1951)

83. Hubbell, R.B., Mendel, L.B., and Wakeman, A.J., J. Nutrition, 14, 273 (1937)

84. Biozzi, G., Benacerraff, B., and Halpern, B.N., Brit. J. Exptl. Path., 34, 441 (1953)

85. Chen, H.P., and Palmer, G.K., Am. J. Clin. Path., 30, 567 (1958)

86. Myrvik, Q.N., Leake, E.S., and Fariss, B., J. Immunol., 86, 128 (1961)

87. Gordon, H.H., Nitowsky, H.M., and Cornblath, M., A. M. A. Am. J. Dis. of Children, 90, 669 (1955)

88. Kalckar, H.M., J. Biol. Chem., 167, 461 (1947)

89. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1941)

90. Gornall, A.G., Bardawil, C.S., and David, M.H., J. Biol. Chem., 177, 75 (1949)

91. Pennington, R.J., Biochem. J., 88, 64 (1963)

92. Fiske, C.H., and SubbaRow, Y., J. Biol. Chem., 66, 375 (1925)

93. Folch, J., Ascoli, I., Lees, M., Meath, J.A., and La Baron, F.N., J. Biol. Chem., 191, 833 (1951)

94. Booth, D.A., J. Neurochem., 9, 265 (1962)

95. Burton, R.M., Garcia-Bunuel, L., and Golden, M., Biochem., 2, 580 (1963)

96. Balakrishnan, S., and McIlwain, H., Biochem. J., 81, 76 (1961)

97. Wherrett, J.R., and Cumings, J.N., Biochem. J., 86, 378 (1963)

98. Svennerholm, L., Biochim. Biophys. Acta, 24, 604 (1957)

99. Macfarlane, M.G., Gray, C.M., and Wheeldon, L.W., Biochem. J., 77, 626 (1960)

100. Isherwood, F.A., and Hanes, C.S., Biochem. J., 55, 824 (1953)

101. Appleton, H.D., La Du, B.N., Levy, B.B., Steel, J.M., and Brodie, B.D., J. Biol. Chem., 205, 803 (1953)

102. Wheeldon, L.W., and Collins, F.D., Biochem. J., 70, 43 (1958)

103. Stern, I., and Shapiro, B., Brit. J. Clin. Path., 6, 158 (1953)

104. Bernheimer, A.W., J. Gen. Physiol., 30, 337 (1947)

105. Kozalka, T.S., Mason, K.E., and Krol, G., J. Nutrition, 73, 78 (1961)

106. Anson, M.L., J. Gen. Physiol., 20, 565 (1936)

107. Fishman, W.H., Springer, B., and Bruntti, R., J. Biol. Chem., 173, 449 (1948)

108. Carubelli, R., Trucco, R., and Caputto, R., Biochim. Biophys. Acta, 60, 196 (1962)

109. Gyorgy, P., and Rose, C.S., Science, 108, 716 (1948)

110. Mackenzie, C.M., Proc. Soc. Exptl. Biol. Med., 49, 313 (1942)

111. Pearse, A.G.C., and Dubowitz, V., Nature, 185, 701 (1960)

112. Bourne, G.H., and Golarz, M.N., Rev. Canad. Biol., 20, 383 (1962)

113. Selye, H., Dieudonne, J.M., and Veilleux, R., Proc. Soc. Exptl. Biol. Med., 109, 232 (1962)

114. Jasmin, G., and Gareau, R., Brit. J. Exptl. Path., 62, 592 (1962)

115. Innes, J.R.M., and Yevich, P.P., Am. J. Path., 30, 555 (1954)

116. Richter, K.M., Ann. N. Y. Acad. Sci., 73, 139 (1958)

117. Stuart, A.E., Nature, 196, 78 (1962)

118. Kampschmidt, R.E., Clabaugh, W.A., and Arrendondo, M.I., Proc. Soc. Exptl. Biol. Med., 108, 216 (1961)

119. Folch, P., Arsove, S., and Meath, J.A., J. Biol. Chem., 191, 819 (1951)

120. Oppenheimer, H., Shulman, S., Roberts, S., and Milhorat, A.T., Proc. Soc. Exptl. Biol. Med., 97, 882 (1958)

121. Castellani, A.A., Ferri, G., Bolognani, L., and Graziano, V., Nature, 185, 37 (1960)

122. Reynolds, B.L., Leveque, T.F., and Buxton, R.W., Am. Surgeon, 29, 325 (1963)

123. Rafelson, M.E., Jr., Schneir, M., and Wilson, V.W., Jr., Arch. Biochem. Biophys., 103, 424 (1963)

124. Schwarz, K., Proc. Soc. Exptl. Biol. Med., 78, 852 (1951) and 80, 319 (1952)

125. Epstein, B., and Shapiro, B., Biochem. J., 71, 615 (1959)

126. Dawson, R.M.C., Biochem. J., 64, 192 (1956)

127. Lands, W.E.M., J. Biol. Chem., 235, 2233 (1960)

128. Elsbach, P., and Rizack, M.A., Am. J. Physiol., 205, 1154 (1963)

129. Rossiter, R.J., and Strickland, K.P., in The Metabolism and Function of Phosphatides, Lipid Metabolism, p. 69, K. Bloch, ed., John Wiley and Sons, Inc., New York (1960)

130. Pappenheimer, A.M., Physiol. Rev., 23, 37 (1943)

131. Morgan, E.H., and Laurel, C.B., Nature, 197, 921 (1963)

132. Wherrett, J.R., and McIlwain, H., Biochem. J., 84, 232 (1962)

133. Dinning, J.S., Proc. Soc. Exptl. Biol. Med., 79, 231 (1952)

134. Koenig, H., Nature, 195, 782 (1962)

135. Koenig, H., and Jibril, A., Biochim. Biophys. Acta, 65, 543 (1962)

136. Faillard, H., Z. Physiol. Chem., 307, 62 (1957)

137. Lilienthan, J.L., Zierler, K.L., Folk, B.P., Buka, R., and Riley, M.J., J. Biol. Chem., 182, 501 (1950)

138. De Duve, C., Lysosomes and Chermotherapy, in Biological Approaches to Cancer Chemotherapy, R.J.C. Harris, ed., Academic Press, New York (1961)

139. Menschik, Z., Internat. Physiol. Congress, Montreal, Canada, 612 (1953)

140. Smith, B., and Rubenstein, L.J., J. Path. Bact., 83, 572 (1962)

141. Harris, H., Brit. J. Exptl. Path., 34, 276 (1953)

142. Menkin, V., Brit. J. Exptl. Path., 34, 412 (1953)

143. Deuel, H.J., Cox, R., Alfin-Slater, R.B., and Ershoff, B.H., Biochem. J., 61, xiv (1955)

144. Golberg, L., Martin, L.E., and Batchelor, A., Biochem. J., 77, (1960)

145. Gerber, G.B., Aldridge, W.G., Kozalka, T.R., and Gerber, G., J. Nutrition, 78, 307 (1962)

146. Dinning, J.S., and Day, P.L., J. Biol. Chem., 233, 240 (1958)

147. Navazio, F., Ernster, B.B., and Ernster, L., Biochim. Biophys. Acta, 26, 416 (1957)

148. Houchin, O.B., J. Biol. Chem., 146, 313 (1942)

149. Rosenkrantz, H., J. Biol. Chem., 214, 789 (1955)

150. Hummel, J.P., and Melville, R.S., J. Biol. Chem., 191, 391 (1951)

151. Macfarlane, M.G., and Knight, B.C.J.G., Biochem. J., 35, 884 (1941)

152. Davidson, F.M., and Long, C., Biochem. J., 69, 458 (1958)