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Effect of vitamin C and vitamin E deficiencies upon nitrogen constituents of muscle tissue

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NOTE TO USERS

EFFECT OF VITAMIN C AND VITAMIN E DEFICIENCIES UPON NITROGEN CONSTITUENTS OF MUSCLE TISSUE

by

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I. INTRODUCTION

The biochemistry and physiology of the nitrogen-containing compounds found in muscle tissues have been studied with increasing intensity since the early 18th century. These studies have evolved into our present-day knowledge which includes the answers, wholly or partially, to many previous mysteries, such as: the isolation and characterization of many of these nitrogen-containing compounds; the mechanochemistry of contraction; the effects of the balance or imbalance of non-nitrogen constituents; and the great importance of metabolism and the dynamic state of all components. In addition, the effects of environmental stresses have shown that the chemistry of muscle tissue is well balanced, a condition that it is imperative to maintain.

The effects of some of these outside influences must of necessity be studied with dead muscle, as compared with physiological muscle. When such factors as high and low temperature effects are studied, isolated muscle tissue is more practical. Controlled autolysis studies more closely approximate the physiological situation. In feeding experiments the effects of such environmental factors as dietary deficiencies proceed in the living tissue. In the interpretation and correlation of the data obtained with living and non-living tissues, the state of the muscle must be kept constantly in mind.

Vertebrate striated skeletal musculature is sufficiently similar, for example, in load-work, resting tension, contraction

and chemistry, from species to species, to warrant comparisons of data obtained from various animal sources. Therefore, this study has drawn upon several vertebrate species to provide a variety of muscle tissues, for example, fresh and frozen beef and chicken; fresh guinea pig and rabbit; and canned meats of different kinds, including fish. This procedure has afforded not only a wider view of the muscle chemistry of vertebrates but has prepared the way for a more careful study of some species which are subject to some of the environmental influences to which man is subject.

A deficiency of either vitamin C or vitamin E may constitute an environmental stress which is known to affect skeletal muscle, in scurvy and dystrophy, respectively. Because proteins are highly significant in muscle tissue, these nitrogen compounds, their metabolic enzymatic degration, and their fractionation and identification have been studied, in relation to certain environmental factors, in the investigations which are herewith described.

II. HISTORICAL

The following review of pertinent literature will be concerned only briefly with the physical structure of muscle, and will deal mainly with the chemical makeup of muscle tissue and the means by which these constituents may be altered or affected in certain vitamin-deficient states, and by various treatments such as heat, storage, etc. Wherever possible, citations presenting information concerning the physiology of skeletal muscle, under some of the above conditions, are included.

A. The Structure and Chemistry of Skeletal Muscle

1. Structure of skeletal muscle

Bailey (1,p.18) has stated most aptly that

> From the modern standpoint, the chemistry of contraction is the chemistry of the proteins composing muscle, and it is not possible to consider the proteins of muscle or of the fibril without relation to the microscopic or submicroscopic structures found in contractile cells.

The proteins of muscle are influenced by environmental factors. In the living muscle the proteins are in great flux and will be affected by stresses of many types. It is strongly suspected that the enzyme systems in muscle are most vulnerable under stresses such as invading organisms, excess activity and fatigue, and the adequacy of the supply of chemical compounds which the muscle requires for normal functioning. These enzyme

systems are believed to affect the proteins of muscle even after the animal is no longer alive, as demonstrated by such phenomena as autolytic changes and rigor mortis (3,8,21,128). Therefore, it is quite reasonable to study muscle protein in living and non-living preparations held under controlled conditions; and quite reasonable to suspect that some correlations may exist between the chemistry of muscle in a living animal exposed to environmental stresses such as deficiency diseases, in which the chemical balance of the tissue is upset and diminished contractile power results, and the chemistry of muscle from that animal after death.

Before considering the effect of these stresses in relation to the metabolism of muscle protein, it is desirable to present a brief survey (2,3) of the structure of skeletal muscle and to characterize the proteins which have been found. A muscle studied microscopically in cross section will be seen to consist of bundles of muscle fibers or fasciculi. About each fasciculus is a sheath of connective tissue, the perimysium. The entire mass of muscle, made up of all the fasciculi, is surrounded by a strong connective tissue sheath, the epimysium. The individual fasciculi may be irregular and many-sided when viewed in cross section and may vary quite markedly in size. Within each fasciculus are the muscle fibers, as illustrated in Figure 1, which shows two muscle fibers from a rabbit. In A, the fibrils are uniformly distributed; in B, they are aggregated into groups. In microscopic section, at

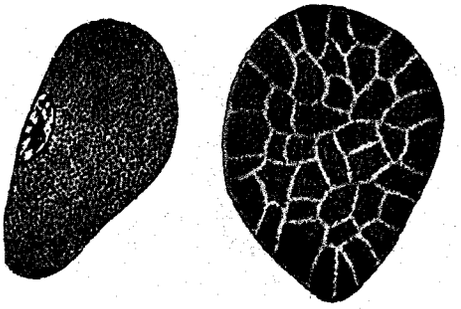


Figure 1. A B

Figure 2.

Figure 1. Cross section of two striated muscle fibers of a rabbit. In A, the fibrils are uniformly distributed; in B, they are aggregated into groups (Cohnheim's fields). (Maximow-Bloom).

Figure 2. The separation of a muscle fiber of a rabbit into fibrils after treatment with nitric acid. (Maximow-Bloom).

least, these fibrils appear to be irregular rather than circular; however, in unfixed material this may not be true (2,3). Within the fibers occasional nuclei are visible; in mammalian muscle these nuclei are close to the periphery of the fiber.

Each muscle fiber is enclosed in a tubular sheath, the sarcolemma, which some authors regard as a product of the cytoplasm of the muscle cell, while others believe it to be a product of the connective tissue surrounding the muscle fibers. However, its chemical reactions are not the same as those of collagen and elastin. The fibers may vary in width from 10 to 100 u. Within the sarcolemma can be seen the fibrils as shown in Figure 1. The protoplasm which separates these fibrils is relatively undifferentiated, and is termed the sarcoplasm. Some histologists group the fibrils into sarcostyles while others consider the "sarcostyle" to be synonymous with a fibril.

A muscle is essentially a fibrillar structure. When the muscle is separated carefully it can be dissected progressively into fasciculi, fibers, then into sarcostyles or fibrils as shown in Figure 2, which illustrates the separation of a rabbit muscle fiber into fibrils. It is believed by some that the fibrils which appear in fixed preparations but are not always visible in living uninjured fibers may be artefacts (1,2,3).

The longitudinal sections of skeletal muscle are more revealing. Viewed in this fashion many nuclei are visible and the cross striations appear very plainly, both in sections of

whole muscle or in isolated muscle fibers. When the muscle is not contracted, the dark and light bands may be of equal width. These dark and light bands are termed the A and I bands, respectively. When muscle fibers are examined under polarized light, the A bands appear doubly refracting, or anisotropic. This is believed to be the result of the presence of doubly refracting filaments aligned in a parallel fashion. The I bands are isotropic and appear dark. Figure 3 illustrates these points.

Biochemists and physiologists have employed some of the physical properties of protoplasm in elucidating the structure and composition of muscle protein. Of primary importance is double refraction or birefringence.

This physical property may be described briefly as follows: When, upon passing light through a substance, the index of refraction is different in two directions, the substance is said to be anisotropic. When the index of refraction is the same in all directions, the substance is isotropic.

When a light, either ordinary or plane polarized, is passed through an anisotropic substance, the beam is broken into two rays, or doubly refracted. The two beams emerging from such a substance are plane polarized and the planes of vibration are at right angles to each other. Light whose plane of vibration is parallel to the optic axis of the substance is called the extraordinary ray; the light whose plane of vibration is perpendicular to the optic axis is known as the ordinary ray.

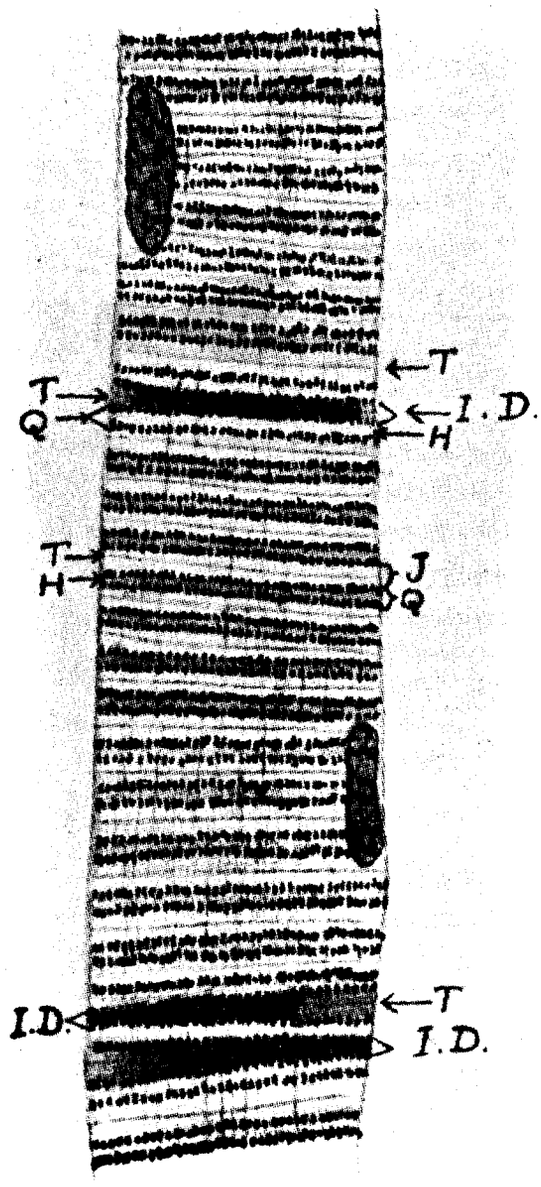


Figure 3. Stained longitudinal sections of portions of muscle fiber of man. T., telophragma; I.D., intercalated disks. (Jordan: Anat. Rec., 16:203. 1919) (Heilbrunn).

If the index of refraction of the extraordinary ray is greater than the index of refraction of the ordinary ray, the double refraction is positive; if the reverse is true, the double refraction is negative (3,4).

The muscle fibers are often free or they may be joined to the collagenous network uniting ultimately with the tendon. The length of the fiber may vary from 1 to 40 mm. Of the total muscle, the fibers comprise about 85 per cent.

According to Szent-Györgyi (5,6) the diameter of cross-striated mammalian muscle fibers is about 0.1 mm. and each fiber consists of many fibrils of about 0.001 mm. in diameter. Electron microscope studies have shown that each fibril is composed of still smaller filaments. Figure 4 illustrates a rabbit fibril (6,p.28)

> loosened by supersonic vibration into a very great number of smaller filaments, many microns long, which were thus running continuously through a number of cross-striations . . . . These filaments are believed to be composed of a positively doubly-refracting protein.

One of these isolated filaments is shown in Figure 5.

2. Mechanochemistry of muscle

As stated above (1), the chemistry of contraction is possibly the chemistry of the proteins found in the muscle. It is believed that the proteins functioning in contraction are found in the fibril, with the A bands containing the actively contracting agent and the I bands withstanding the

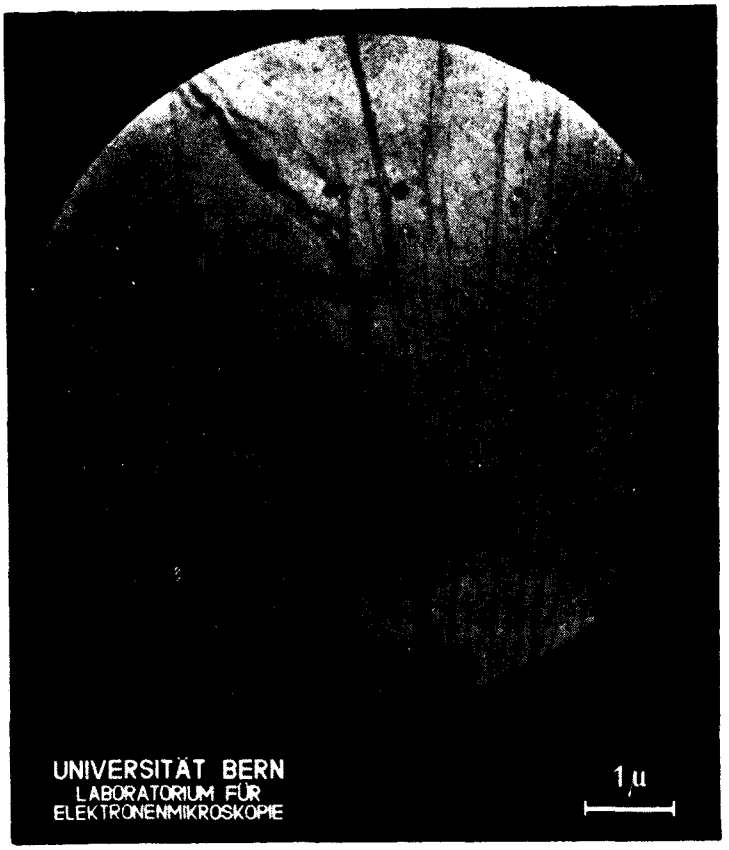


Figure 4.

Figure 5.

Figure 4. Electron microscope photography of a fibril from a cross-striated muscle of the „rabbit, loosened by supersonic radiation. (Szent-Gyorgyi, A. Nature of Life; A Study on Muscle. 1948.)

Figure 5. Electron microscope picture of a filament isolated by means of supersonic radiation. Gold shadowed. 1:30,000 (1 mm. = 330 A). (Szent-Gyorgyi, A. Nature of Life; A Study on Muscle. 1948.)

tension. Electron microscope studies have shown that the submicroscopic elements of the A bands are aligned parallel to the axis of the fibril. The optical anisotropy of the A bands also indicates this (1).

Proteins play a dominant role in the various theories of the mechanochemistry of muscle. Engelhardt (7) believes that certain physical changes in the contractile substance are induced by chemical agents which are produced during the functional metabolism of muscle tissue. He emphasized the combination of adenosinetriphosphate with myosin to form a complex of the enzyme-substrate type. He believed that myosin was the principal compound (or compounds) involved in the mechanical reactions of the muscle fiber. The interaction of these two substances is manifested in muscular work.

Sandow, in 1949, (8) expressed rather close agreement with Engelhardt's views, with the exception that he placed more emphasis upon actomyosin, rather than myosin, as the principal compound involved in the mechanochemistry of muscle. In any event, it appears from the evidence, as presented recently, that the proteins in the muscle are vitally important in muscle contraction.

In addition to the influence of the proteins of muscle upon contraction, it may be mentioned briefly that other factors are concerned. For example, certain more or less physical forces may be involved. Hill (9) has formulated a theory uniting the mechanical events and energy changes of the active

muscle. Furthermore, myo-neural transmission and electrolyte distribution are important factors involved in the physiology and chemistry of muscle contraction (10).

3. The proteins of muscle

Sandow (8,p.313) has stated that

> Although there is no doubt that both myosin and actin are present in muscle, there has been considerable uncertainty as to whether these proteins exist uncombined, in the resting fiber, or in the form of the acto-myosin complex, and whether the actin is in the globular or fibrous state.

For the sake of simplification these protein fractions will be considered separately.

a. The myosin fraction. When fresh muscle is minced and the juice expressed, numerous proteins may be obtained. According to Bailey (1), these consist mainly of enzymes and are derived from the sarcoplasm. When the fresh mince is immediately extracted with salt solutions, a viscous extract exhibiting flow-birefringence is obtained. Von Muralt and Edsall (11) gave the name myosin to the fibrous protein responsible for the high viscosity of the extract.

Myosin is a globulin which can be extracted by cold alkaline salt solutions such as potassium chloride and potassium phosphate, and it has been crystallized as a potassium complex from solutions containing a low concentration of potassium chloride (12). The concentration of myosin in muscle has been estimated at about 8 per cent. Some of the properties of crys-

talline myosin include solubility in water, a strong birefringence of flow, and a large acid-base binding capacity between the physiological limits of pH 6.4 and 7.3 (13). Molecular weight determinations on myosin have yielded varying results in the laboratories of several groups of workers. In 1933 Weber and Stover (14) reported 10^6 as the approximate molecular weight; this could be decreased to 10^5 by treating myosin with strong urea. Guba (29) found that the urea-treated myosin had a viscosity sufficiently low to indicate a globular shape, suggesting that the whole is composed of smaller globular units.

It has been accepted generally that myosin is the main protein component of the fibril and is directly responsible for the contractile property of muscle. In 1939 Engelhardt and Lyubimova reported (15) that the adenosinetriphosphatase activity of muscle was to be found in the myosin fraction and could not be separated from it. Although much subsequent work has been done, this view is still valid and is of importance for two reasons. Firstly, it was known that the breakdown of adenosinetriphosphate was practically coincidental with contraction (16); and, secondly, its cleavage either to adenosinediphosphate or adenylic acid involved a rupture of energy-rich bonds. Lipmann (17) believes that the energy may be transferred to the myosin chains by a phosphorylation of the protein itself. Bailey (1) has listed two possible reactions:

(1) ATP + myosin-ATP-ase $\longrightarrow$ ADP + myosin-phosphate

$\longrightarrow$ myosin + phosphate

(2) ADP + creatine-phosphate $\longrightarrow$ ATP + creatine

Bailey (1,p.19) also writes "By the reaction of creatine phosphate with adenosinediphosphate, the supply of adenosinetriphosphate is maintained at a high level".

In 1942, Bailey (18) reported a study of the activation of myosin adenosinetriphosphate by the divalent metals, calcium, magnesium and manganese. When myosin is in an isomolar solution of these three metals, calcium will be adsorbed predominantly; when only magnesium and manganese are present the magnesium is preferentially adsorbed. The significance of the role of calcium, in connection with myosin in particular, and muscle in general, is increased by the following information. It is believed that stimulation of the muscle is connected in some way with the availability of calcium ions to the myosin-adenosinetriphosphate fibrillar surface (18). In connection with this effect of calcium, it has been demonstrated (3) that if a muscle fiber with cut ends is placed into a solution containing calcium, the ion enters rapidly through the cut ends and a vigorous contraction occurs. The addition of excess calcium, in amounts above physiological quantities, will slow or stop the respiration of muscle minces (3).

Excerpts from Bailey's 1937 report (19), which summarizes some of the previous findings of other workers on the composition of myosin and myogen of skeletal muscle are shown in Tables

I and II. Only the data pertaining to rabbit muscle have been included in these tables.

Table I

Analysis of Pure Myosin and Myogen[1]

Protein	Author	N	S	P	Tyrosine	Tryptophane	Cystine
Rabbit myosin	Weber '33	----	1.0	0.09	5.0	1.3	----
	Todrick and Walker '37	15.3	0.94	---	---	---	0.27[2]
	Mirsky '36	----	----	---	---	---	0.67
Rabbit myogen	Weber '33	----	0.77	0.028	6.0	1.5	----

[1]Expressed as per cent of protein; dry weight

[2]As cysteine

b. The actin fraction. In 1942, Straub (20) announced the isolation from muscle of another major protein to which he gave the name actin. Straub (21) has elucidated many of the properties of this protein, but the entire picture is still incomplete. Szent-Györgyi (5,6) believes its most striking property is its ability to exist in globular as well as fibrous form. The two forms have different properties, so that the characteristics of the myosin-actin compounds are different also; only the complex with the fibrous actin has contractile power. The

two forms are reversibly transformed, a phenomenon which Szent-Györgyi thinks occurs in every contraction cycle. Actin is probably present in resting muscle in the fibrous form, but it can be extracted from the tissue only in its globular form.

Actin is not precipitated by alkali salts and is more stable than myosin; however, it is readily denatured by salts in media below its isoelectric point (pH 4.7).

If a dilute solution of potassium chloride is added to a neutral solution of actin, turbidity develops, the fibrous form is produced, the solution becomes viscous, and double refraction is exhibited (5).

Actomyosin, as the complex is termed, is viscous and fibrous; however, the properties of actomyosin vary with the ratios of actin and myosin combined. An outstanding property of the complex is its contraction and dehydration in the presence of certain salts (potassium chloride, for instance) by adenosinepyrophosphate. If a physiological salt solution in which myosin is soluble is employed, the actomyosin forms a thixotropic gel. If 0.00003 M adenosinetriphosphate is added, the viscosity falls rapidly, but as the adenosinetriphosphate is broken down by the adenosinetriphosphatase the viscosity increases. If a dilute salt solution is added, such as 0.05 M potassium chloride, adenosinetriphosphate causes the actomyosin gel to shrink with a decrease in water content from about 96 per cent to 50 per cent (1,21). Bailey and Perry (22)

have shown recently that the sulfhydryl groups in the myosin determine the ability of myosin and actin to combine and to split adenosinetriphosphate.

Bate Smith (23) has estimated that the actomyosin complex comprises 10.7 g. and the sarcoplasmic proteins 5.1 g. per 100 ml. of whole muscle mince. Bailey (1,p.21) states that "the accepted volume of extracellular space is about 15 ml., so that the corresponding fiber concentrations are about 12.6 g. for actomyosin and 6.0 g. for the sarcoplasmic proteins." These figures represent merely a uniform distribution of protein throughout the fiber, but accepting a division into sarcoplasmic and fibrillar space, it is unlikely that the concentration of protein in the fibril is less than about 20 g. per 100 ml. of the whole muscle.

c. Myogens. Some recent writings (24,25) include myogens in the discussion of the proteins found in muscle, while others do not (8). For the sake of completeness, the myogens will be included here. These components, generally considered as myogens A and B, have the general properties of an albumin; are water soluble; and can be extracted in amounts of 25 to 30 per cent. They are believed to be located largely in the sarcoplasm. The molecular weights of these components are in the range of 80,000 to 90,000. These fractions have been obtained in crystalline form; enzymatic properties (glycerophosphate dehydrogenase activity) are found in crystals of myogen A obtained

from rabbit muscle (25). These muscle proteins do not show double refraction of flow (26).

Table II presents data from Bailey (19) on certain nitrogen constituents of rabbit myosin and myogen.

Table II

Analysis of Pure Myosin and Myogen by Bailey (19)

Component	Rabbit[1] Myogen	Rabbit[1] Myosin
Nitrogen	16.60	16.70
Sulfur	1.29	1.10
Amide N as per cent of the total nitrogen	5.53	7.20
Cystine		
(Folin-Marenzi-Lugg)(a)	1.96	0.77
(Sullivan-Lugg)	1.86	0.62
Methionine (b)	2.80	3.40
Tyrosine (c)	4.21	3.38
Tryptophane (d)	1.51	0.82
Sum (a,b,c,d,)	10.48	8.37

[1]These components expressed as per cent of protein; dry weight

Table reproduced from Bailey, K., Biochem. J. 31, 1406-13 (1937)

d. Tropomyosin. In 1946, Bailey (27) announced the presence of an additional protein, tropomyosin, in skeletal muscle. It is a protein of relatively low molecular

weight, 90,000, and it appears to be bound in some special way to the fibril proteins since it is not present in muscle press-juice. It is quite soluble in water at physiological pH, but at pH 6, its solubility characteristics, in the presence or absence of salts, suggest that it is a globulin. When salts are absent completely, tropomyosin aggregates into long fibers of quite regular width, and the solutions then are very viscous. It can be obtained in crystalline form if quite exacting conditions are used.

Tropomyosin is similar in amino acid composition to myosin. (See Table II) It is similar to myosin in physico-chemical properties and is built according to the same molecular plan, giving an X-ray diffraction pattern of the α-keratin type. Bailey believes that "it seems reasonable to suppose that tropomyosin might be a unit utilized in the elaboration of myosin itself" (27,28).

e. "Protins". In addition to the major proteins of muscle listed above, Banga et al, and Szent-Györgyi (6,29) have announced the discovery of 6 new compounds which they term "protins" and which are believed by these workers to be adsorbed onto a myosin skeleton, forming a "myosin system". They claim that only this "system" possesses the enzymatic and contractile power attributed to the proteins themselves. These compounds are all acid- and heat-stable.

Szent-Györgyi has extracted another protein from rabbit muscle, the N-protein. It is a negatively doubly-refracting

protein, localized in the I-bands (6).

f. The enzyme proteins. In addition to the above proteins, there are many enzymes in muscle. Sandow (8) has emphasized the adenosinetriphosphatase activity of muscle and has pointed to the fact that the questions involved in relating this enzyme to myosin, other muscle proteins, and contraction are not definitely settled. At present this enzyme is considered of great importance in the chemistry and physiology of muscle; however, it must be recalled that Bailey emphasized the preponderance of enzymes in muscle minces and stated that they were "for the most part concerned with the glycolytic cycle" (1,p.18). Such compounds as the carbohydrate enzymes, the dehydrogenases, etc., are included in tissue extracts.

In their studies reported in 1939, Lyubimova and Engelhardt found that their myosin fraction could catalyze the dephosphorylation of adenosinetriphosphate to the di-phosphate and inorganic phosphate (30,31). The enzyme, as they considered their compound, was activated by calcium and inhibited by magnesium, had an optimum activity at about pH 9.0, and was identified with myosin.

Kielley and Meyerhof announced a new, powerful adenosinetriphosphatase in 1948 (32). This enzyme is activated by magnesium and inhibited by calcium, has a pH optimum around 7.4, and is completely separable from myosin. The activity of this adenosinetriphosphatase is closely linked with the actomyosin system in its behavior and, of course, also with the contractile mechanism.

4. The non-protein nitrogen constituents of skeletal muscle

For many years studies of the body processes, such as digestion, have attracted a large share of investigators' attention. During the course of such studies, initiated in 1783 by Spallanzani, our present concepts of enzyme chemistry have been evolved, along with basic information concerning muscle protein extracts. Liebig's meat extract, employed so extensively from the 1830's to the 1870's, was used not only for enzyme studies but to reveal many of the water-soluble proteins, non-protein nitrogen constituents, mono- and di-carboxylic acids, carbohydrates, and mineral elements.

In comparatively recent times, it has been observed that some abnormal conditions of muscle tissue are characterized by either a diminution or increase of one or more of these components. When quantitative methods for their determinations became available, more definite information was gained from a knowledge of these compounds. The water-soluble proteins and their breakdown products in particular have been of especial importance because of the quantities of these in muscle. Unfortunately, there is still a dirth of reliable quantitative methods for the protein intermediary breakdown products, but it is possible to partition them and to study their pattern of distribution by suitable methods of fractional precipitation (33).

Water extracts from muscle have been employed widely in the studies concerned with meats and meat products, with the effects of numerous environmental factors upon these; and in studies on

normal and pathological muscle. From 1900 on, the extractives of muscle have been the subject of such reports as that by von Furth and Schwartz (34) which showed the distribution of nitrogen in the water extract from the flesh of dogs and horses, as given in Table III.

Table III

Nitrogen Constituents of Muscle Extracts of Dogs and Horses[1]

Nitrogen Constituent	Per Cent of Tissue
Ammonia	4.5 to 7.0
Purines	6.1 to 11.0
Carnosine	30.3 to 36.3
Carnitine, methyl-guanidine, etc.	8.2 to 15.2
Creatine and creatinine	26.5 to 37.1
Urea, polypeptides, amino acids	6.3 to 16.0

[1]Table reproduced from von Furth, O. and C. Schwartz, Biochem. Z. 30, 413-32 (1911)

In 1912, Bennett (35) studied the purines of muscle and found adenine and guanine present. In striated muscle he reported inosinic acid as a fraction of the total hypoxanthine present.

In the same period, there was also a concentrated interest

in creatine and creatinine. Meyers and Fine (36) reported on the content of these in normal muscle, and their relation to urinary creatine; furthermore, they found that the creatinine coefficient seemed to parallel the creatine content of the muscle. The discovery of the decreased muscle creatine during experimental nutritional muscular dystrophy by Goettsch and Brown in 1931 (37) started a series of experiments which attempted to clarify the dystrophy problem.

In the meantime, Gulewitsch (38) published his isolation of carnosine nitrate from striated muscle. This was followed by numerous studies from Hunter's laboratory on the carnosine content of muscle from various species (39,40), and Clifford (41) studied the effects of storage upon the quantity of carnosine in muscle meats. Studies on carnosine are being continued at the present time since neither a wholly acceptable analytical method for its determination nor an explanation of its presence in muscle tissue has been found.

The effects of autolysis and rigor have been the subject of a great number of publications. Many of these were related not only to storage conditions but to muscular atrophy, dystrophy, excessive exercise, fatigue, etc. It was believed that autolysis was more rapid in the presence of acid, and Bradley (42, 43) carried out extensive studies on this phase of muscle chemistry and physiology. In the case of rigor, an explanation for this phenomenon was sought in studying the non-protein nitrogen components; this problem is still unsolved.

In 1929 Ackermann, Timpe and Poller (44) announced the presence of anserine in avian muscle, and this gave impetus to many studies on the isolation and quantitative determination of this compound. Because of anserine's chemical relation to histidine and carnosine, these compounds were often studied together. The di-peptides appear in quite significant quantities in many sources of muscle and this has prompted a search for some physiological function commensurate with their quantitative presence. At the time of writing, this function has not been discovered in its entirety; however, Bate Smith (45) has proposed that carnosine and anserine perform a role in the buffering mechanism of muscle.

Since isotopes have been available, many studies have appeared which show that the nitrogenous compounds found in muscle extracts are in the same dynamic flux as are other body constituents. For example, du Vigneaud and co-workers (46) in 1943 demonstrated that when deuterio-methionine was fed to a rabbit, the anserine isolated from the tissues of that rabbit contained the deuterium. They were able to postulate that the various N-methyl compounds synthesized by the animal derive their methyl groups from "labile" methyl compounds in the diet.

A study of the partition of intravenously administered amino acids was carried out by Friedberg and Greenberg (47), who found that skeletal muscle concentrated only a small quantity of the amino acids. However, Fuller, Neuberger and Webster (48) found that histidine deficiency in the rat reduced

the carnosine content of the muscle appreciably, while the anserine content was affected only slightly.

The following outline will show some of the compounds which have been reported in muscle extracts, and will also suggest the complexity of working with muscle extracts:

Non-nitrogenous extractives

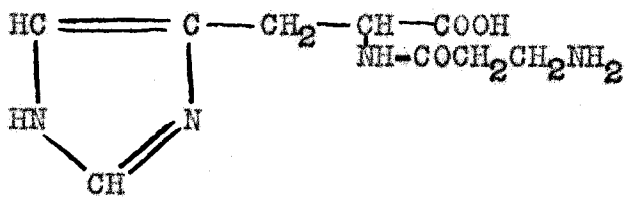
glycogen
hexosephosphate
lactic acid

Nitrogenous extractives

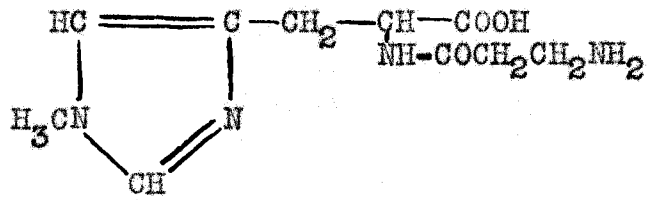
creatine
creatine phosphate
adenine
guanine
xanthine
hypoxanthine
uric acid
adenylic acid
inosinic acid
adenylic acid pyrophosphate
carnosine
anserine
carnitine

The structure of the following three compounds is given because carnosine and anserine were studied in this work and because carnitine might be confused with carnosine:

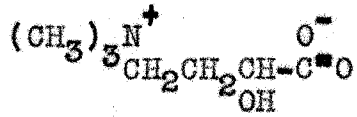
Carnosine has the structure



Anserine is N-methyl carnosine



Carnitine is a betaine of unknown function, having the structure,



The following water-soluble vitamins have been reported (49) present in muscle tissue, and, presumably, also in the water extracts of such tissue: thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, inositol, biotin, and folic acid.

B. Vitamin C Deficiency and Muscle Protein Metabolism

Vitamin C deficiency was at one time a real scourge of mankind. In comparatively recent times the etiology of the disease has been discovered, and at present the role of ascorbic acid in preventing scurvy is quite definite. Cases of scurvy among the human population are by no means unknown at present, and there remain some gaps in our understanding of the changes associated with a deficiency state. One of the gaps is the effect of the vitamin C deficiency upon the muscles of the affected animal, and more especially upon the proteins and other nitrogenous compounds found to such a large extent in muscle.

1. Qualitative description of scurvy

The general description of scurvy is somewhat colored by the extent of the deficiency of vitamin C. Guinea pigs, which are particularly susceptible to scurvy, develop gross symptoms in proportion to the quantities of the vitamin supplied. In cases where there is practically a complete lack of the vitamin, the animals develop capillary fragility and then microscopic lesions in the teeth. About the second week on the diet, activity is somewhat curtailed and the animals lose weight rapidly, with a significant wasting of tissue. Most animals die at the end of the third week. When the guinea pigs receive small quantities of the vitamin, they may survive for some time and develop a chronic condition with rough hair and rapid breathing. Upon autopsy lesions typical of scurvy may be evident.

The teeth and long bones are greatly affected. Firm, sturdy tissue is not formed, the result being teeth with a minimum of dentine present and bones made fragile by a lack of normal osseous tissue. The degree of this degradation of tissue depends upon the extent of the deficiency. The fascia and ligaments are weakened; skeletal lesions are quite evident due to the malformed bones; the gums about the teeth are swollen, spongy and hemorrhagic; and, because of the development of an inferior intercellular material in the walls of blood vessels, hemorrhages are quite extensive. The scorbutic guinea pig sits quietly most of the time in a hunched position

with his head in the characteristic "tooth-ache, or face-ache position". His joints are swollen and tender so that it is too painful for him to move about and he cries out if not handled very carefully. Toward the end of the disease the animal is usually quite emaciated; the tissues upon autopsy show a poor, unhealthy color and texture. There is usually an abnormal odor from the contents of the gastrointestinal region with apparent stasis of the whole tract (49).

2. Biochemical inferences

After scurvy became more clearly defined and definitely linked with vitamin C, attempts to learn the underlying biochemical effects of the vitamin deficiency were forthcoming. The guinea pig has been employed quite extensively for studies of this kind. One of the outstanding effects of vitamin C deficiency is upon the metabolism of certain aromatic amino acids. Sealock and Silberstein (50) demonstrated the importance of ascorbic acid in the metabolism of tyrosine, and subsequent experiments by Sealock and his co-workers have demonstrated a similar dependence of phenylalanine and 3,4-dihydroxyphenylalanine metabolism upon an adequate supply of the vitamin (51,52,53). Levine, Marples and Gordon (54,55,56) have shown that in the premature infant, a similar derangement of the metabolism of these amino acids may occur when vitamin C is not supplied.

The above authors as well as Painter and Zilva (57) have

shown that the levels of intake of the aromatic amino acids and the quantities of the vitamin available to the tissues will influence the biochemical reactions which occur. Painter and Zilva (57) have shown also that disturbances in tyrosine metabolism may occur while the tissues contain large amounts of ascorbic acid.

Further experiments of biochemical interest may be cited. Fishberg (58) has found benzoquinone acetic acid in the urine of patients showing a decreased excretion of ascorbic acid and also in patients with cyanosis. Benzoquinone is capable of producing methemoglobin in vitro. When the urinary excretion of ascorbic acid is increased by the administration of the vitamin, the excretion of benzoquinone acetic acid is reduced and the amount of methemoglobin in the blood is decreased.

The administration to rats of adrenocorticotropic hormone is followed by a temporary decrease in the amount of ascorbic acid present in the adrenals (59). The connections among vitamin C, melanin formation, and adrenal insufficiency, resulting in Addison's disease, have been studied extensively, but the link, if one exists, between these factors has not been found.

Recently more and more evidence has become available to show that there may be interrelationships between the individual vitamins in their biochemical functions. Woodruff and Darby (60) found that scorbutic guinea pigs fed either pteroylglutamic acid or ascorbic acid along with 5 per cent tyrosine in the diet,

excreted less tyrosyl derivatives and keto acids. Mayer and Krehl (61,62) have reported a connection between ascorbic acid and vitamin A in the rat; they found a 50 per cent reduction of ascorbic acid in the adrenals and blood in rats on a vitamin A-deficient diet. The animals exhibited symptoms resembling scurvy.

3. Muscle

a. Gross description of the muscle in scurvy. In severe scurvy, lesions occur in the muscles. A general weakness is characteristic. As the disease progresses there is a gradual disuse of the muscles, particularly those of the thighs. Rather than walking in the normal way, the scorbutic guinea pig hops painfully with the hind legs, and a sitting position tends to relieve the weight of his body from the sore muscles.

Goettsch and Pappenheimer (63) describe the gross appearance of the guinea pig thigh muscles as atrophied, pale, and with a yellow-brown color, instead of the light, more translucent appearance of the control animal. The tissue may be calcified, gritty when incisions are made, and has lost a large measure of its irritability and contractility.

b. Histology. Histologically, fragmentation of the striated fibers with multiplication of the sarcolemma may be evident. The primary lesion is a coagulative necrosis of the muscle fiber and, as the condition progresses, many of the necrotic fibers become resorbed and their place taken by fat

and/or connective tissue. One of the earliest changes in the fiber is shown in the appearance of transverse ridges or contraction bands, in which the striations are brought more closely together. The discs become disarranged and lose their identity by fusing into a swollen mass. A fiber cut in the long axis will show a succession of globular masses between which the sarcolemma is collapsed. The sarcolemma nuclei are displaced and distorted, and tend to become oriented at right angles to the long axis of the fiber; they may subsequently disappear completely.

The extensive hemorrhages in the muscle tissue complicate both the gross and histological lesions and the poor circulation resulting from these hemorrhages is quite likely to affect the biochemical processes going on in the muscle cells.

c. Chemistry of nitrogen compounds in scorbutic muscle.

The effects of vitamin C deficiency upon the chemistry of the constituents of muscle tissue have not been studied extensively. The studies of Wolbach and his associates (64,65) have demonstrated the effect of a vitamin C deficiency upon the formation of intercellular substances, particularly collagen. There may be some connection between the failure of this substance to be formed and the breakdown of the muscle tissue, but there is no direct evidence for it.

Quite recently Christensen and Lynch (66) have reported studies on the glycine and glutamine contents of skeletal muscle from vitamin C-deficient guinea pigs. They found that

in the deficient animal there was a large decrease of these compounds in the muscle, the values falling to 7 and 13 per cent, respectively, of the normal values. When they sacrificed their animals on the tenth day of the experiment, they found no hemorrhage in the muscles, and the analysis of such tissue was comparable to the normal. However, an animal that showed a mild spontaneous scurvy after several months upon guinea pig ration alone, gave data similar to the acutely scorbutic animals.

The authors discuss the large percentage of glycine found in the normal muscles and the diminution which they found in vitamin C-deficient animals in relation to the high glycine content of collagen.

C. Vitamin E Deficiency and Muscle Protein Metabolism

Vitamin E deficiency has numerous manifestations depending upon the species of animal, previous diet, age, sex, and level of the vitamin in the diet. What the fundamental disorder might be is still speculative. Mason (67) believes that because the damage is largely irreversible, the nucleus of the cell is affected. He gives two reasons for this: one, that the first visual evidence of the deficiency is in the structure of the nucleus; and two, that if the cytoplasm of the cell alone were affected, the damage would not be so central and fundamental. This theory seems to explain the major effects of vitamin E deficiency.

1. Qualitative description

In general terms, vitamin E deficiency results in sterility of an irreparable nature in male rats; resorption and/or abortion of the fetus in the female rat; and also nervous lesions, including involvement of the hypophysis, hemorrhagic lesions, etc., in the rat. The living young from female rats maintained on an E-low ration will develop a muscular involvement at about three weeks post-partum. The paralysis which develops resembles an upper motor neurone lesion. There is some question about the relationship between vitamin E deficiency and nervous tissue lesions (67). It is possible to develop these symptoms of weakness and dystrophy in adult rats but about five months or longer on an E-deficient diet are required. Although the rats are unable to use the hind legs, have slight incoordination and some thinning of the hair, they do not present the same symptoms of extreme weakness that are seen in herbivora.

2. Biochemical inferences

The fundamental over-all biochemical role of vitamin E may be stated according to Hickman (68) as follows: "We believe that vitamin E when synergized to the optimum in the body, is an important biologic in transitu preservative agent both to the metabolites and to the structural parts". Because the term "in transitu" seems to be a new thought as applied to living

matter, it might be explained briefly. Molecules on their way to the site of biological action, or their metabolized parts diffusing away, obviously greatly outnumber the few molecules undergoing reaction at any one time. These traveling molecules may be said to be in transitu. The importance of the in transitu history of a biological compound upon its ultimate in vivo action is obvious. The above quotation may be interpreted to include Mason's belief that vitamin E is necessary for the integrity of the nucleus of the cell. Numerous examples of its reaction in oxidation-reduction systems are available; it has been shown to be essential for the normal oxygen consumption of many tissues. Hickman has suggested that the abnormal pigmentation found in adipose tissue of the vitamin E-deficient rat and chick is the result of inefficient chemical management of organic residues arising during the reactions within the cells.

The experimental work on vitamin E, designed to determine its biochemical and biological functions, has been centered about three of the more obvious symptoms: the effect upon sterility and uterine pigmentation in rats; nutritional encephalomalacia and exudative diathesis in chicks; and muscle dystrophy in rabbits and in adult rats, or early paralysis of the muscle in weanling rats. Granted, these syndromes are not very closely related; this circumstance has complicated the experimental conclusions as to the fundamental reactions of vitamin E in the animal body. It is quite conceivable that the picture is still nebulous. In recent years, the emphasis

has been on muscle tissue and the present research has centered about this phase.

3. Muscle

a. Gross description. When certain species are deprived of vitamin E, a muscular involvement is evident. This has been termed "muscle dystrophy", although the finer characteristics of the involvement may vary intra- and inter-specifically. One of the earliest notations that muscles were involved was made by Evans and Burr (69) who noted the presence of a paralysis of the spastic type in the hind quarters of suckling rats from depleted mothers. This condition usually progressed to a muscular dystrophy. In the adult rat placed upon an E-deficient diet similar lesions will occur in the hind legs. At first there is a mere dragging of the legs with slight incoordination, but this condition will progress to complete loss of function. The adductor muscles are involved, particularly.

Goettsch and Pappenheimer (63) were the first to describe, in any detail, the muscle lesions of the depleted herbivorous animal. Their description includes a progressive weakness to complete helplessness, and finally death. This condition comes about much more rapidly with this type of animal than with the rat. Mason (67) points out that the nature of the lesions in nutritional muscular dystrophy is limited almost entirely to a necrosis of the striated musculature. Associated with the muscle lesions is a significant loss of weight and cessation

of growth, a decreased muscle creatine, creatinuria and an altered ratio between the creatine and creatinine in the urine.

Originally muscle lesions were observed only in young animals, but Mackenzie, Mackenzie and McCollum (70) succeeded in producing dystrophy in adult rats. It required eight to ten months, but the ultimate muscle degeneration was the same.

b. Histology. An adequate histopathological description of the affected muscle has been published by Goettsch and Pappenheimer (63). The changes which occur have been described by such terms as hyaline, waxy, or Zenker's degeneration. These terms may be further characterized as descriptions of a muscle in which there has been a loss of striations, a multiplication and irregular arrangement of the sarcolemma nuclei with a concurrent swelling of the sarcoplasm. There is often edema in the interstitial connective tissues and some calcification of the necrotic muscle fibers. The interpretation of the histological picture is usually complicated by the reparative changes which may be going on at the same time as the degenerative changes. This is particularly true in species that develop this syndrome under chronic conditions. The dystrophy in guinea pigs and rabbits is of this type, according to Mason (67).

c. Chemistry. The first nitrogen-containing component of muscle noted to be affected by the condition of muscular dystrophy was creatine. Goettsch and Brown (37) reported in 1932 that the concentration of this compound was lowered in skeletal

muscle in the last stages of dystrophy, and Ni (71) observed in 1936 that creatine excretion rose during the last stages. Morgulis and Spencer (72), in the same year, found that rabbit muscles, which showed marked degeneration on microscopic examination, had a total creatine content lower than that of normal muscle tissue. In a subsequent report, Morgulis and Spencer (73,p.204) stated as follows:

> During the period of progressive dystrophy, i.e., from the "critical point" to the time of death, the weight continues to fall rapidly. The phosphorus and chloride excretion gradually decrease. The creatinine and total nitrogen decrease from the high level reached at the "critical point", while the creatine excretion continues to rise until the time of death. The urine volume decreases following the brief strong diuresis at the critical point, but just before death a marked diuresis occurs again.

When vitamin E became available as the pure chemical, α-tocopherol, in 1938 (74,75), it was demonstrated by Barrie (76) and Goettsch and Ritzmann (77) that the nutritional muscular dystrophy in suckling rats could be prevented by this compound. When α-tocopherol was fed to dystrophic rabbits, Mackenzie and McCollum (78) and Mackenzie (79) demonstrated that there was a sharp drop in urinary creatine excretion; Shimotori *et al.* (80) announced that α-tocopherol prevented muscular dystrophy in guinea pigs reared on a synthetic diet; and Morris (81) confirmed Mackenzie and McCollum's observation that α-tocopherol cured dystrophy in

rabbits.

At this point four facts had been made evident: a muscular dystrophy occurred in a number of species as a result of a vitamin E deficiency; it was characterized by a lowering of the muscle creatine with an attendant increase in creatinuria; the creatinuria was decreased when dystrophic animals were given α-tocopherol; and the muscular dystrophy disappeared if the case were not too far advanced.

After it was determined that α-tocopherol could be employed in curing muscular dystrophy, experiments were performed to determine the dosage level required. Morris stated in 1939 (81) that the lower limit for a single curative dose was 20 mg. Verzar (82) reported that 100 mg. of the dl-α-tocopherol restored the metabolism of striated muscle and the urinary creatine to normal in a few days. Pappenheimer (83) found that the daily administration of 4 mg. of the synthetic α-tocopherol completely prevented nutritional myopathy of ducklings, while a 1 mg. dose was ineffective. Mackenzie, Levine and McCollum (84) placed the average daily anti-dystrophy requirement of the rabbit for α-tocopherol between 0.6 and 1.0 mg. per kg. of body weight. Eppstein and Morgulis (85) published that the minimum requirement of the rabbit for dl-α-tocopherol, calculated as the free alcohol, as determined by the cure of dystrophy, is probably about 0.32 mg. per kg. of body weight per day.

Mattill and his co-workers (86) and Pappenheimer et al (87) have published numerous valuable contributions to the vitamin E-dystrophy problem in their studies of oxygen consumption under conditions of vitamin E deficiency. This work has led directly into the recent studies on the effect of the tocopherols and their esters in enzyme and tissue functions. Mattill stated in 1949 (88) that an early guide-post in this search (for the mechanism of the action of tocopherol in tissues) was the ability of the tocopherols to delay the auto-oxidation of unsaturated fats. For those who followed this clue, antioxidants, synergists, and co-vitamins became words of significance. Some interesting matters relating to peroxides and abnormal pigments have come to light. Another guide-post was the increased consumption of oxygen by the muscles of certain species when they lack vitamin E.

These references to muscle cell respiration are included in the present discussion of the effects of vitamin E deficiency upon the nitrogen-containing constituents of the striated muscle tissue because of the great likelihood that explanations for the breakdown of muscle tissue in dystrophy may be wholly or partially found in experiments of this type.

Two of the earliest reports upon the oxygen consumption of dystrophic rabbit muscle were those of Victor (89) and Madsen (90) who found that the oxygen uptake of dystrophic muscle specimens was higher than it had been when biopsy material from the same source had been tested before death. In 1941,

Friedman and Mattill (91) confirmed these data. The increase was significant, though at times quite variable. It was also noteworthy that the muscles of young rats (92), old rats (93), and chicks (92) showed this increased oxygen uptake when deficient in vitamin E, even though the muscles showed little or no evidence of any anatomical changes. Kehler (92) believed, therefore, that the increased oxygen consumption could be attributed to changes in the metabolic activity of the muscle fibers themselves, and not to proliferative activities of connective tissue cells. It has been seen that these connective tissue cells invade and finally replace the damaged muscle tissue, especially in advanced stages. Further, it has been observed that the oxidative changes are highest during the more acute stage of the disease when there is a minimum of connective tissue invasion and cures are possible (67).

Houchin and Mattill (93,94) announced that the oxidative changes are not of the same order among species, since the oxygen uptake of normal muscle is greatest in rats and lowest in rabbits. Houchin and Mattill (93,94) reported these data from their experimental animals: The Q_{O_2}, expressed as c.mm.O_2 and based upon the fresh tissue samples employed, was 1.27-1.57 from normal rabbits; 2.22-3.88 from vitamin E-deficient rabbits; 1.88-2.08 from normal rats; 1.62-3.73 from vitamin E-deficient rats; 1.05-2.07 from normal hansters; and 3.90-4.67 from vitamin E-deficient hamsters on a low-fat diet. The normal animals were stated to have been on a stock diet.

These authors demonstrated the effect of α-tocopheryl phosphate on the oxygen uptake of hamster muscle; the hamster has been shown to be quite susceptible to muscular dystrophy. They found that muscle slices from the E-deficient animals had an oxygen consumption much above normal, with values ranging from 240 to 250 per cent of the normal. The administration of 5 mg. per cent α-tocopheryl phosphate in Ringer solution decreased this high oxygen consumption to nearly normal, but did not affect the oxygen consumption of control muscle slices from normal animals, nor of the muscle from vitamin E-deficient animals which had been given a therapeutic dose of the vitamin 48 hours previously (94). After the injection of the slightly water-soluble ester, the phosphate, Houchin and Mattill (95) found that the high oxygen consumption is reduced to normal in about 4 hours; creatine restoration after 10 or more hours, and chloride even more delayed. Houchin (96) has reported that the oxygen uptake of muscles which already contain optimal amounts of tocopherol is not lowered by the administration of additional amounts of this compound.

Houchin's paper in 1942 (96) brought out a number of interesting points, and not a few equally interesting suggestions which link vitamin E-deficient dystrophic muscle more closely with protein metabolism, oxidation-reduction systems, and enzymes. He observed that mincing or homogenizing the muscle resulted in a loss of the increased oxygen uptake which is

characteristic of the intact dystrophic muscle. Spurred on by this finding and the implications of enzymic changes, (an enzyme system dispersed and diluted with a loss of one or more water-soluble components), he investigated the succinic oxidase system of dystrophic muscle. Working with hamsters, he found that the activity of this enzyme system was proportional to the degree of dystrophy. It rose to as high as 162 per cent above normal. α-Tocopheryl phosphate was found to lower this activity; while α-tocopherol, either alone or in combination with desoxycholic acid, did not. He suggested that the tocopherols required phosphorylation in the body before they could become active in this mechanism. He postulated, too, that phosphorylated tocopherols may serve to inhibit or regulate oxidative processes of skeletal muscle; when these compounds are absent, oxidation increases, abnormal proliferation of other cells begins with connective tissue infiltration and the muscle ceases to function normally.

The entrance into the current phase of the relationship between the tocopherols and their esters with enzyme systems might well be forwarded by considering several provocative facts. An animal in dystrophy from vitamin E deficiency has an increased respiration throughout the system as well as an increased respiration and oxygen uptake in the effected muscle (97). The chemical composition of the muscle is altered with a decrease in creatine; simultaneous creatinuria occurs (37,71, 72,73). Bloch (98) had noted that in vitamin E deficiency there

was a decrease in cholinesterase content of the tissues, implying an association between this vitamin and the synthesis of acetyl choline.

In addition to the changes in creatine content, the concentrations of certain electrolytes in muscle were altered when that muscle became dystrophic. Fenn and Goettsch reported in 1937 (99) an increase in calcium and total phosphorus when they analyzed rabbit muscles which showed histological evidence of calcification. Morgulis and Osheroff (100), too, had noted an increased concentration of calcium in dystrophic muscle. In 1941, Axelrod, et al (101), in studying the succinic dehydrogenase system in tissue homogenates, discovered that it was necessary to add calcium ion in order to obtain optimum activity in aerobic work. This same group also showed that the calcium influence was probably an indirect one, that is, through the activation of diphosphopyridine nucleotidase and the subsequent destruction of diphosphopyridine nucleotide (coenzyme I, cozymase). At the present time this theory is accepted quite generally. The importance of these chemical changes and implications will become more apparent in future work with the enzyme systems operating in muscle tissue.

The impetus of Houchin's suggestions (96) has continued into the most recent publications, because of the possibilities involved. The role of phosphate was studied when he found that this particular ester would lower the succinoxidase activity of dystrophic tissue while α-tocopherol would not. This brought

forth to light once more the earlier finding of Morgulis and Spencer (72) that α-tocopherol when administered to normal animals would alter the metabolism of phospholipids. Weissberger and Harris found that phosphorylations were improved by the presence of this vitamin (102). This work has led into the present interest in α-tocopheryl phosphate as related to enzyme systems. This phosphate compound offers the advantage of being more readily soluble in aqueous solutions; when orally administered it is readily hydrolyzed in the animal body (103), and many of its reactions *in vivo* are similar to those resulting from the administration of the unesterified tocopherols.

Hummell (104) had indicated that homogenates of the muscles of some species deprived of vitamin E show a lowered rate of phosphorylation of creatine. The addition of the ester to the homogenate did not restore normal phosphorylation, nor have an effect upon phosphorylations when they were coupled reactions. It should be remembered that these are *in vitro* experiments, and that it is conceivable that the addition of vitamin E to an intact, physiological, living tissue might present a different picture. Certainly the cures noted with vitamin E therapy are of some import, but here again, when the dystrophy has progressed beyond a critical point, recovery is not attained.

One of the most interesting and fruitful lines of attack on the problem of discovering the function(s) of vitamin E in

muscle metabolism has been the study of specific enzymes. The contributions to follow are of especial interest in a study of dystrophic muscle.

Basinski and Hummel (105) have published observations on the succinic dehydrogenase system and the effects of tocopherol esters upon it. They worked with normal and dystrophic hamster muscle which, it will be recalled, shows an unusually high sensitivity to vitamin E deficiency. It will be recalled, also, that Houchin (96) had investigated previously the succinoxidase system of dystrophic muscle and found that the activity of this enzyme was proportional to the degree of dystrophy and that α-tocopherol phosphate in vitro lowered this activity. These authors reported that they found no difference in the oxygen uptake quotient in the succinic dehydrogenase system between homogenized dystrophic hamster muscle (Q_{O_2}= 38.2) and the muscle from hamsters fed the same deficient diet with a supplement of α-tocopheryl acetate (Q_{O_2}= 38.5). However, both were slightly lower than the value obtained when the muscle from hamsters fed the stock diet was studied (Q_{O_2}= 46.3).

The findings of Basinski and Hummel are at variance with those of Houchin (96) in some respects. For one thing, while Houchin found a difference in oxygen uptake between normal and dystrophic hamster muscle, Basinski and Hummel did not. The latter authors believe that one reason for this difference is to be found in the ages of the animals used. They publish a

graph which illustrates that, as age increases, the oxygen uptake tends to decline. If Houchin's control animals were older than the experimental animals the results from this group would show a lower oxygen uptake.

It has been found that a definite concentration of calcium is required by the succinic dehydrogenase enzyme. Schneider and Potter had recommended 20 mg. per reaction flask in their work with rat muscle (106). Basinski and Hummel believed that Houchin had used 20 mg. per cent calcium concentration in his work (actually, he used 20 γ per cent in the buffer or 0.6 γ per flask), and, therefore, may not have had complete activation of the succinic dehydrogenase. His normal samples gave values which were too low for this enzyme because the concentration of calcium was too low, and thus succinic dehydrogenase was inactivated. However, Houchin's values for dystrophic muscle were true since the considerable calcium in dystrophic muscle would raise the final concentration to the desired level.

Basinski and Hummel reported that the succinic dehydrogenase system was inhibited in vitro equally well by both d-α-tocopheryl phosphate and d-α-tocopheryl succinate; actually the succinate was slightly less effective in this regard than the phosphate. They tested, also, the insoluble calcium salt of dl-α-tocopheryl succinate and found that it had no inhibiting effect. According to these authors the inhibition of the succinic dehydrogenase system by α-tocopheryl phosphate is not

apparent when the concentration of the substrate is insufficient to saturate the enzyme. The inhibition becomes greater as the substrate excess is increased. They believe that since cytochrome reductase, for example, is an associated system which is normally present in excess, it may be the site of the inhibition. Basinski and Hummel state "there is probably no direct involvement of the succinic dehydrogenase system in nutritional muscular dystrophy" (105).

Hummel and Basinski (107) have furthered this work with normal and dystrophic rabbit muscle. They compared the oxygen consumption of skeletal muscle strips from normal rabbits, which had received 15 mg. α-tocopheryl acetate in olive oil every four days, with the oxygen consumption of strips from the same muscle of rabbits receiving no supplement. They found that the respiration of the dystrophic muscle strips was approximately double that of the normal strips. Muscle slices did not show this difference. They could not establish an *in vitro* influence of α-tocopherol upon cellular respiration.

Of particular interest among the nitrogen-containing components of muscle is creatine. The following enzymatic study by Hummel (108) on the formation of phosphocreatine elucidates its importance. The work was *in vitro*, with glycerophosphate or fructose-1-6-diphosphate serving as substrates, and either normal or dystrophic hamster muscle, or normal or dystrophic guinea pig muscle homogenates, present in the reaction mixture. The other constituents of the reaction mixture are significant enough to be mentioned. In addition to 100 mg. freshly homogen-

ized muscle (10 per cent suspension), it contained in micromols: creatine 230, potassium chloride 400, magnesium chloride 20, nicotinamide 20, coenzyme I 0.75, adenosine triphosphate 1.33, and sodium phosphate buffer (pH 7.4) 10. In the aerobic experiments, each flask also contained 3 X 10^{-8} moles of cytochrome c in an air atmosphere; in anaerobic: 10 micromoles of lithium pyruvate under nitrogen. When glycerophosphate was employed as the substrate, the aerobic formation of lactic acid and phosphocreatine was very small in the dystrophic muscle. However, the oxygen uptake was equal to that of the normal sample. Under anaerobiosis, lactic acid production in dystrophic muscle was smaller than that in normal muscle, but the efficiency of oxidative phosphate transfer was greatly diminished. When fructose-1-6-diphosphate was the substrate, the effects of dystrophy were less marked; nevertheless, they substantiate the experiments with glycerophosphate.

The adenosinetriphosphatase activity was appreciably lowered in the dystrophic muscle from both species. Hummel believes these are indirect effects of the vitamin E deficiency. He has taken into consideration, in his discussion (108), the possibility of the smaller mass of active muscle tissue in dystrophic animals. In order to test the influence of cellular destruction, he measured the adenosine triphosphatase activity and found that in dystrophic muscle a considerable destruction of this enzyme had taken place (108). Lyubimova and Engelhardt (30,31) had shown previously that this

enzyme is closely associated with myosin; therefore, dystrophic muscle changes may entail an alteration in the contractile structure of the muscle as well as "an impairment in the utilization of energy for contraction".

Heinrich and Mattill (109) have measured the creatine and creatinine contents of normal and dystrophic rabbit muscle. The animals were kept for approximately one month on a vitamin E-deficient diet. The controls were fed a supplement of 10 to 15 mg. per week of dl-α-tocopheryl acetate in olive oil. Portions of the gastrocnemius muscle were analyzed by an enzymic method. The muscles from the deficient animals were found to contain much less creatine than the control muscles. They analyzed the liver tissue from these animals and found that, while the creatine content of dystrophic muscle was low, in the dystrophic animal's liver it was 2 to 6 times that found in the control animals. These authors suggest that when the rabbit is dystrophic from a lack of vitamin E there is an increased rate of creatine synthesis by the liver and a decreased phosphorylation rate of creatine in muscle.

Further studies with adenosinetriphosphate and vitamin E-deficient rabbit muscle have been published by Carey and Dziewiatkowski (110). They included acid and alkaline phosphatase activity as well, and reported the following results. The adenosinetriphosphate and alkaline phosphatase activities were not altered in muscle homogenates from dystrophic rabbits; however, there was a two-fold increase in the acid phosphatase

activity. This first result does not agree with the findings of Hummel (108). Heinrich and Mattill (109) reported that α-tocopheryl phosphate inhibited the adenosinetriphosphate system; they attributed this to the precipitation of its activator, calcium ion. Although the acid phosphatase does not require calcium ion, it, too, was inhibited by α-tocopheryl phosphate.

Roderuck (111) has studied the level of free amino acids in dystrophic rabbit and guinea pig muscle. Since glutamine is known to be a neutral compound in which skeletal muscle stores its labile amino groups, this compound was studied as a possible indicator of the condition of the amino acids in normal and dystrophic muscle. The glutamine content of the skeletal muscle of dystrophic guinea pigs was strikingly decreased. In rabbits, the change from normal was less marked, perhaps not significant. This species difference may be the result of the fact that on a vitamin E-deficient diet, rabbits become dystrophic within 2 to 4 weeks, whereas guinea pigs require 6 to 7 weeks. The total non-glutamine amino acid content of the muscles was influenced little, if at all, by vitamin E deficiency. A study of the distribution of other amino acids in this condition might be revealing.

Roderuck (111) postulates that the observed reduction in glutamine in dystrophic muscle may be the direct result of the loss, "through combustion, of energy that is normally stored in high energy phosphate bonds." These data of Roderuck

are quite interesting in the light of a previous report by Christensen and Lynch (66), in their work with scorbutic guinea pigs, in which they found an appreciable decrease in glutamine in the muscle tissue.

Barber, Basinski and Mattill (112) have measured the transaminase activity between aspartic and α-ketoglutaric acids of skeletal muscle homogenates and found the activity of dystrophic guinea pig muscle to be less than half that of normal muscle. Dystrophic rabbit showed a less striking diminution. The results were uniform whether expressed on the basis of wet weight, dry weight or total nitrogen of the tissues. The decreased activity does not appear to be a result of changes in tissue structure, nor was age a variable.

Barber, Basinski and Mattill (112) added 0.05 mg. of pyridoxal phosphate to the reaction mixtures. This amount, equilibrated to allow enzyme-coenzyme complex formation, was believed to be sufficient to restore the enzyme activity of the reaction mixture if the coenzyme concentration were the limiting factor. The decrease in transamination was not the result of an increased removal of oxalacetate, as indicated by recovery experiment. In fact, with dystrophic rabbit muscle of high concentrations, the recovery of oxalacetic acid was greater than with normal muscle. This actually indicated that normal muscle possessed a better mechanism for the removal of excess oxalacetate than dystrophic muscle.

D. *In vitro* Studies of Changes in Nitrogen Compounds of Skeletal Muscle

The muscles of many animals have probably always constituted a major portion of the diet of man. At first, he may have eaten them in their raw state, but as time went on he applied heat to them before eating. He may have discovered concurrently that it took less effort to masticate such treated muscles and that the flavor was different. He discovered, too, that by drying the muscles in the sun and air, they would be preserved for future consumption. In comparatively recent times man has discovered other means of preserving his muscle-meat foods: canning, powdering and drying, freezing, etc. He has become interested, also, in delving into the reasons why the muscle-meat is still "good" after a lapse of time, and also into what happens to the tissue during such a treatment.

From a more academic point of view, it has been desirable to study the effects of these imposed processings on the constituents of muscle tissues, so that there could be gained some information which might be correlated with biological stresses that are continually impinging upon muscle tissue *in vivo*. Of special importance is the relation of nitrogen compounds to muscle tissue under some of these stresses.

One of the difficulties encountered in studying the

older literature was the interpretation of the classifications of the nitrogen compounds and the adaptation of the data to more recent studies. For example, such terms as "coagulable nitrogen" and "non-coagulable nitrogen" are difficult to evaluate. For such reasons, few of the older reports have been included, but this must not be interpreted to imply that these older studies were, and are, not important; in some instances these earlier data have not been changed.

1. The effects of prolonged temperatures below 36°F. on nitrogen constituents of muscle

One of the first studies was reported by Hoagland, McBride and Powick in 1917 (113). They had stored beef muscle just above freezing for long periods of time, and found these changes: an increase in non-coagulable nitrogen, amino nitrogen and ammonia nitrogen; a decrease in coagulable nitrogen. An interesting finding was that as the ammonia nitrogen accumulated, the rate at which it was formed decreased.

Two studies on soluble-nitrogen compounds are of interest. Foster (114) compared, over a 10-day period, beef frozen at -8°C. with unfrozen beef muscle which he considered to have autolyzed. In the frozen sample the soluble nitrogen rose from 10.5 to 16 per cent of the total nitrogen in 2 days, while the same fraction increased from 10.5 to 13 per cent of the total nitrogen in 10 days in the unfrozen

state. When McCarthy and King (115) stored muscle under the usual ripening conditions, 30 days at 35°F., they found a more rapid rise in sulfhydryl content and in soluble nitrogen compounds than in muscle stored for only 2 days at 60°F. Results found by Clifford (116) when he analyzed muscle held at 35°F. for 3 days, or 25°F. for 13 days, included no change in total nitrogen, soluble nitrogen, amino nitrogen, carnosine and creatine.

A study on denaturation of the muscle juice proteins by freezing was carried out by Finn in 1932 (117). After the onset of rigor he expressed the muscle juice and froze it. He found that at -2°C. to -3°C. denaturation is most rapid, resulting in the denaturation of 25 to 30 per cent of the total protein in about 40 days time. The additional time of 80 days did not result in further denaturation; however, when the pH was lowered from 6 there was a rapid increase in denaturation. Finn postulated that the myogen fraction was most readily denatured as a result of the influences of changing pH and concentration of the salts in the muscle juice. This brings up the question of loss of water when muscle tissues are frozen, especially at very low temperatures and are held there for any length of time. Reay (118) had reported previously that the important change during storage in the frozen state was colloidal in nature. He concluded that the muscle globulins lost their power to hold water because they were denatured as a result of the action of the concentration of salts in the frozen

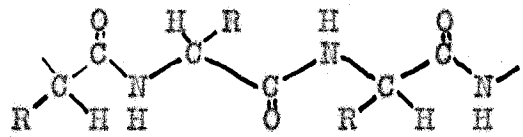
tissue.

Further considerations of the pH effects and loss of moisture from freezing are found in the reports of Drozdov and Drozdov (119) and of Johnson (120). The former workers reported that when beef muscle was frozen at -23°F., there was a shift in pH to the acid side; there was also an increase, though slight, in protein nitrogen. No changes were observed in residual nitrogen nor in amino nitrogen at this temperature. Johnson studied "bound water" in chicken muscle stored at -12.2°C. and -23.3°C. for 6 to 9 months. Less water was retained at the lower temperatures.

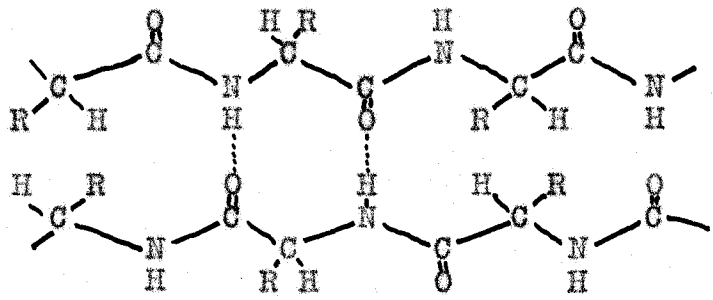
2. The effects of prolonged temperatures above 36°F. upon the nitrogen constituents of muscle

It is generally believed that high temperatures have special effects, such as coagulation, upon the proteins in muscle tissue. Of these proteins, collagen and elastin have been studied extensively because of their importance in industry as well as in the preparation of muscle-meats for food.

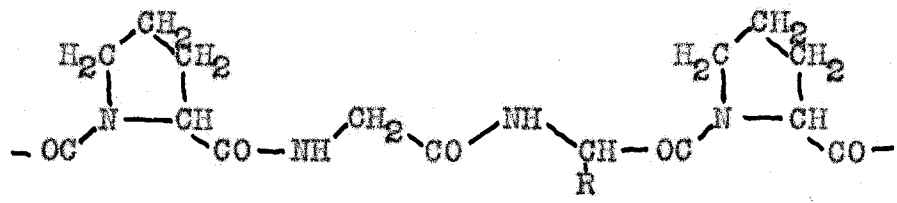
Because of the peculiar effect of heat on the protein molecule some conception of its structure is advisable for a better understanding of this phenomenon. Proteins are composed of polypeptide chains which may be pictured in either of 2 ways depending upon the length of the R-groups:



When the R-groups are short (hydrogen or methyl groups) these chains are linked through the hydrogen bonds:



Astbury (121) believes that a large protein such as collagen would be composed thusly, with the sequence of amino acids parallel to the axis of the muscle fibers:



Chains containing less constricted molecules than proline (above) would have a longer extension. Huggins (122) believes that the main protein chains are spiral with NHO bridges.

When these long chains are treated by heat (90-100°C.), with either acid or alkali present (10-15 N), they break, yielding the constituent amino acids. At lower temperatures and less drastic conditions, such as are encountered in autolysis studies

to be considered in more detail below, the breakage is more selective.

One of the most widely studied effects of high temperatures upon the muscle proteins has centered about the conversion of collagen to gelatin. Bendall (123) has recently described the process in a stepwise fashion:

(1) Collagen A is converted, at 56°C.-60°C., to Collagen B. This results in a shortening of the collagen fiber.

(2) Collagen B takes up water with consequent swelling and softening of the connective tissue.

(3) Dissolution of Collagen B to form a gelatin sol.

The last step occurs during prolonged cooking at 100°C., or during pressure cooking at 115°C. to 126°C. for a short time, or by autoclaving at 20 pounds pressure for about 6 hours.

Bendall investigated also the effect on the creatine, creatinine, nitrogen and pH values of cooking raw, lean beef muscle. He found a rise in the ratio of free to total creatine, and a rise in pH due to hydrolyzed linkages in the protein molecule. There was little effect on the non-protein nitrogen. The shift in pH to the alkaline side accompanied the coagulation of the proteins upon cooking. He also found that hydrogen ions and phosphate ions have a marked accelerating effect on the rate of

conversion of collagen to gelatin.

Prudent (124) found that when beef muscles were cooked in fat to an internal temperature of 70°C., the changes in collagen and elastin, as measured by her methods, were insignificant. The total nitrogen values showed no trend in spite of some moisture loss with time and a difference in lipid content.

Harrison (125), studying the same beef muscles as above, found that heating decreased the length and width of portions of the whole muscle and increased the thickness. These same muscles had been stored at 34°F. to 36°F. and lost significant amounts of moisture during periods ranging from 1 to 30 days. During this storage period the acid content of the muscle increased gradually. The cooked muscles were slightly more alkaline than the uncooked.

Harrison (125) found that all muscles followed much the same pattern throughout the storage periods. In general, as aging progressed beyond two days there was a tendency for the fibers to become "straighter, with fewer waves, z-z contractions, twists and kinks".

Disintegration was evident by the increasing fragility of the muscle fiber and by a breakdown of the muscle fibers in certain areas. The disintegration might extend over only a few, or over many, striae. Sometimes the sarcolemma remained intact; sometimes it broke. Areas with protoplasmic disintegration had lost all evidence of either longitudinal or cross striae, and the material within the sarcolemma had a granular

appearance. In some sections disintegration was as evident at 10 days as it had been at 30 days, with all of them showing disintegration after 10 days. Some disintegration increased after 20 and 30 days.

Harrison found that cooking had the following histological effects: connective tissue between the fibers appeared granular, and, in muscles in which there were large amounts of collagen, a film of granular tissue often covered the entire section. The fibers were usually straightened, with less kinks, twists, and waves than the raw fibers. Cooking did not intensify the microscopic characteristics of the fibers.

3. Autolysis studies on muscle

When the long protein chains referred to above are treated under less drastic conditions; when the temperature, pH, and time are controlled, the enzyme systems in muscle tissue will function and break down these long chains. However, the breaking will be much more selective than in hydrolysis to amino acids. Bergmann (126,127) has shown that it is possible to predict the breakdown scheme according to the structure of the protein skeleton, the enzymes present, etc.

Baldwin (128) has these ideas about such breakdowns:

> Animal tissues . . . as a whole are known to contain intracellular enzymes which can be extracted and shown to catalyze the breakdown of proteins and other high-molecular materials into simpler units. It seems improbable that this is their sole function in the cell. After death, these

> enzymes do in fact lead to the digestion of much of the tissue substance, a process known as autolysis, and this is why game and certain kinds of meat are allowed to "hang" before being cooked.

These intracellular enzymes of animal tissues, known collectively as "kathepsin", have been divided according to their activity. Kathepsin I is homospecific with pepsin which Bergmann (126,127) has shown can act only on certain peptide bonds; for example, a peptide link lying between an l-dicarboxylic and an l-aromatic amino acid can be broken if the conditions are right. These conditions are that the second carboxyl radical of the dicarboxylic acid residue must be free, and there must not be a free amino-group near the peptide linkage.

Kathepsin II is homospecific with trypsin which breaks only linkages adjacent to basic amino acid residues (arginine or lysine; if these are substituted the product resists trypsin), and the second amino-group of the basic amino acid unit must not be substituted.

Kathepsin III is homospecific with aminopeptidase which will remove a terminal group if the terminal unit has a free amino-group.

Kathepsin IV is homospecific with carboxypeptidase which will remove the terminal unit if the carboxyl radical is free.

In autolysis studies interest is focused primarily on the effects of these enzymes in the breakdown of tissue, while in in vivo studies, these enzymes are concerned in the synthesis of proteins and their fragments as well as their breakdown.

Baldwin (128) believes that these intracellular peptidases must have a role in maintaining the dynamic equilibrium or balance, as proposed by Schoenheimer (129), between the cell proteins and their smaller molecules either derived from them or used to synthesize them.

The conditions of autolysis, such as time, temperature, pH, and non-living tissue, will influence the results from such studies. The following more recent reports are included to indicate the trend.

Most of the early studies on autolysis have involved other types of tissues, such as liver and spleen, and have measured the effects of autolyzing enzymes upon the formation of lactic acid, pyruvic acid, etc. Some early studies, including those from the laboratories of Bradley (130), Hopkins (131), Hammett (132), Parnas (133), and Needham (134), have shown that the muscle enzymes are quite active under autolytic conditions. Chen and Bradley (42,43) found that autolysis was more rapid in the presence of acids of the approximate concentration of 0.04 to 0.02 N or pH 4.5 to 5.0, under which conditions they reported that less than 15 per cent of the protein was digested to amino acids. In subsequent studies (43) these authors related the effects and conditions of autolysis to atrophic and dystrophic conditions in muscles. They believe the changes in pH to be highly significant in the protein breakdown both in autolysis and in abnormal muscle.

Parshin (135) has investigated the changes in the nitrogen

bases of muscle tissue upon autolysis. From 1 kg. of fresh tissue he isolated 1.8 g. of carnosine, but after 1 kg. of similar tissue was autolyzed for one month, 1.33 g. was isolated. He did not observe carnosine decomposition products, but during the month's autolysis, all the creatine was transformed into creatinine. On the other hand, ammonia, residual nitrogen and amino nitrogen increased.

In the following section additional autolysis experiments will be cited.

III. EXPERIMENTAL

The following experiments on skeletal muscle were performed in an attempt to gain information concerning the functions and actions of the proteolytic enzymes, the kathepsins, described in the preceding section. The muscle tissues upon which the work was done included the psoas major, the longissimus dorsi, the semitendinosus, and the biceps femoris (semimembranosus) of beef; the pectoralis major of the fowl; and the thigh muscles of both the guinea pig and the rabbit.

Firstly, a partitioning of the nitrogen compounds into total nitrogen, total non-protein nitrogen, amino acid nitrogen, and ammonia nitrogen fractions was performed. This was followed by a further division of the amino acid nitrogen by analyses for two amino acids, tyrosine and histidine, and for two di-peptides, carnosine and anserine.

Total non-protein nitrogen determinations were done on two different types of supposedly protein-free filtrates: trichloroacetic acid filtrates and tungstic acid filtrates. The reasoning behind this was based on the observations made by Miller and Van Slyke (33) that trichloroacetic acid did not precipitate proteins quantitatively, and so it is reasonable to suppose that intermediate breakdown products from the enzymatic degradation of protein, such as proteoses, peptones, and polypeptides, would also be present in such filtrates (33). On the other hand, it has been shown, also, (33) that tungstic acid filtrates contain only amino acids, di-peptides, and other

low-molecular weight compounds. Therefore, it was believed that information concerning the extent and type of enzyme action on muscle tissue could be obtained by a comparison of these two types of non-protein nitrogen filtrates.

The employment of these methods upon the types of skeletal muscle described made it possible to study the effects of temperature, time, and dietary influences upon some of the enzyme systems in muscle tissue. It was hoped that the results might lead to some deductions concerning the relation of vitamin deficiencies, nitrogen constituents, and possibly, enzyme actions in the tissues affected by these deficiencies.

A. Effects of Prolonged Temperatures Below 36°F. on the Nitrogen Constituents of Muscle

1. Experiments with beef muscle

a. Preparation of the sample. The beef muscle samples used in this experiment had been selected from a yearling steer of "good" classification. Each muscle was analyzed individually. After the animal was sacrificed, the dressed sides were hung in the cooler at 34°F. to 36°F. for 24 hours. The muscles were then dissected from the carcass, cut into portions in anticipation of all the studies which were planned for them, and then placed unwrapped onto enamel trays with about one inch between the portions. They were stored on open shelves in the cooler at 34°F. to 36°F. for periods ranging from 1 day to 30

days. The method of sampling for chemical analysis was as follows: after the first day of storage, an aliquot was removed from each muscle and placed into a 2 to 8 oz. cork-stoppered or screw-capped glass jar. The jars were placed in the -30°F. compartment until the chemical analyses were made. This resulted in 5 samples (the longissimus dorsi were sectioned into "rib" and "loin") at each day of storage, a total of 30 samples for the entire experiment.

The conditions of the storage at 34°F. to 36°F. (cut muscles, uncovered and in the air) resulted in some surface dehydration which would increase with time of storage. There was loss of moisture after the aliquots were placed in the closed sample bottles as evidenced by the quantity of frost lining these bottles when they were removed from the -30°F. compartment.

The quantity of beef muscle in the sample jars varied from sample to sample and was, in most cases, somewhat less than the more desirable 75 to 100 g. of tissue available from the fowl or rabbit. The beef tissue was allowed to thaw, freed of as much fat as possible, weighed, and transferred quantitatively with approximately 2 volumes of distilled water to a chilled Waring Blendor jar.

The blending was carried out for 2-minute periods with intervening 2-minute chilling in an ice-salt bath. This prevented the tissue from getting warm. After the first 3 periods the sample was washed down to the bottom of the jar with dis-

tilled water. It was advisable to keep the volume of water as low as possible. The level of the water was determined by the weight of the sample. An average of 8 to 10 2-minute periods was required for most samples so that the final suspension would be sufficiently fine to pass through the tip of a 5 ml. volumetric pipette.

After sufficient blending, the sample was transferred quantitatively to a volumetric flask of a size which would result in a final suspension of 5 per cent concentration. In the experiments in which the pH of the suspension was measured with the Beckman pH meter, such measurement was made immediately after bringing the suspensions to the required volume. From this suspension or homogenate, aliquots were removed for all the determinations.

The above procedure was followed throughout the experiments in which a limited quantity of tissue could be used, as with the first beef samples and the guinea pig muscle. It was necessary to thaw only the beef samples because all subsequent material was not frozen previously. When at least 75 g. of fresh tissue could be had, duplicate samples of 25 g. each were used. In these cases the dissection was carried out and the muscle tissue was ground rapidly through the fine blades of a meat grinder, thoroughly mixed, and then the 2 25-g. aliquots weighed as quickly as possible. The greater quantity of muscle, then, made it possible to analyze the tissue in duplicate from each animal.

b. Analytical methods. Total nitrogen was determined by the usual micro-Kjeldahl procedure. The selection of aliquots for this determination may be done in two ways: one, 5 ml. of the suspension is pipetted into a 25-ml. volumetric, diluted to volume with distilled water, thoroughly shaken, and 2 ml. of this diluted suspension pipetted directly into the micro-Kjeldahl flasks; or two, 5 ml. of the undiluted suspension may be pipetted directly into the micro-Kjeldahl flasks. When the first method was employed, as with the first beef experiments, two aliquots were diluted, and duplicate determinations made from the diluted suspensions. These will digest more rapidly than the undiluted samples described in the second procedure, but will involve more error as a result of the additional manipulations. Undiluted suspension was tested, 5 ml. in triplicate, on the majority of the muscle samples.

The formol titration with the Beckman pH meter was used to determine amino acid nitrogen plus ammonia nitrogen. The method used was a modification by Northrop (136,137) of the original Henriques and Sørensen titration (138), and the use of the Beckman pH meter made possible a higher degree of accuracy than can be obtained with the visual observation of the indicator change alone. The analyses were made on 5 ml. aliquots of the tissue suspensions, usually in triplicate or more until good checks were obtained.

The details of the formol titration method employed are as follows:

Five-ml. aliquots of the suspension are pipetted into 400-ml. beakers. Into each are pipetted 50 ml. of redistilled water. It is preferable to use redistilled water whenever it is available; otherwise distilled water may be used. The electrodes of the Beckman pH meter are placed into this suspension and the beaker rotated so that the suspension is thoroughly mixed with the solutions to be added. Titration is carried out with 0.01 N NaOH to pH 7.00. It is necessary to allow time for this mixture of suspension and the alkali to come to equilibrium after each addition of alkali. At pH 7.00, value "A" is read from the burette, and recorded.

Twenty ml. of the recommended formaldehyde solution are then added and thoroughly mixed. The volume of the formaldehyde solution used will vary with the volume of the suspension to be analyzed; approximately 10 ml. of the formaldehyde solution are added for each 20 ml. of the unknown (suspension).

The mixture of suspension and alkali is titrated again with 0.01 N NaOH to pH 9.00. It is important at this point to allow adequate time for complete mixing and equilibration. If this precaution is not observed, accuracy will be sacrificed and acceptable duplication of results will not be obtained. The reading on the burette when pH 9.00 is reached is "B".

A blank or control must be determined with each preparation of formaldehyde solution which must be prepared freshly for each group of determinations. Fifty ml. of water are titrated with 0.01 N NaOH to pH 7.00; 20 ml. of formaldehyde

solution are added with thorough mixing and titrated with 0.01 N NaOH to pH 9.00. The value obtained at pH 9.00 is "C".

(B - A) - C equals the "Formol Value". This includes both amino acid nitrogen and ammonia nitrogen. The formula for the calculation of the results based upon the original suspension is as follows:

ml. 0.01 N NaOH ("Formol Value") X 0.00014 (Nitrogen Factor) X $\frac{500}{5}$ X $\frac{100}{25}$ = gm. per cent amino acid nitrogen and ammonia nitrogen in 25 g. of tissue which has been diluted to 500 ml. and of which 5-ml. aliquots have been used.

For deproteinization, a 50-ml. aliquot of the suspension was pipetted into an 125-ml. erlenmeyer flask. The tungstic acid method as described by Hiller and Van Slyke (33) was used with a proportion of 1 volume of both 10 per cent sodium tungstate[1] and 2/3 N sulfuric acid[2] to 5 volumes of the 5 per cent tissue suspension. Gradual addition of these reagents with very thorough mixing was the rule. They were allowed to stand for 30 minutes after which they were centrifuged and filtered.

[1]The sodium tungstate must contain a minimum of sodium carbonate and/or of molybdate for accurate results. The c.p. grade from either the J.T.Baker Chemical Co., Phillipsburg, N.J., or from Mallinckrodt Chemical Co., St. Louis, Missouri, is satisfactory.

[2]Both these solutions must be very accurately prepared. It is advisable to measure the quantity of sulfuric acid with a burette and the 2/3 N solution should be titrated against a standard base to confirm that it is actually 2/3 normal.

If carnosine was to be determined, the solution was not filtered through paper which adsorbs this di-peptide (138). When larger volumes of the suspension were employed, it was helpful to pipette the samples of tissue into 100-ml. centrifuge bottles and carry out the deproteinization in them. They were centrifuged for 10 to 15 minutes at 2000 to 2500 r.p.m., and the resulting supernatant which was passed through the filter became a clear and colorless filtrate. The filtrates gave negative results when the biuret test was used.

The tungstic acid method, when accurately and carefully used, is known to result in complete precipitation of all protein and protein intermediary products, leaving in the filtrate only the lower molecular weight compounds, or in other words, the non-protein compounds. These will include many nitrogen-containing molecules especially important in this study, such as the amino acids, peptides, ammonia, di-peptides, etc. Hiller and Van Slyke (33) report that the tungstic acid method, with a pH of 2.8 to 3.00 at the final concentration of reagents and suspension, results in practically complete precipitation of protein molecules, as well as intermediary protein split-products.

The total non-protein nitrogen in these filtrates was determined by analyzing 2-ml. aliquots, in triplicate, with the usual micro-Kjeldahl procedure for digestion and distillation.

The ammonia nitrogen was determined by the Parnas distillation procedure (139). Five-or 10-ml. aliquots of the clear

filtrate were taken in triplicate.

c. Results from the beef muscle experiments. The results obtained with the various beef muscles are summarized in Table IV. The values as given are the averages of all determinations for all five muscle samples. The row of figures opposite the first day of storage represents the averages from all the aliquots of all the muscles tested for each nitrogen-containing group. For example, the figure 3.31 g. per cent total nitrogen at the end of the first day's storage represents an average from 20 samples consisting of 4 analyses for each of the 5 different muscles.

The portion of each beef muscle selected for chemical analysis was chosen according to a statistical pattern described by both Prudent (124) and Harrison (125), so the averages ultimately obtained are unbiased so far as the muscle site is concerned.

The data as presented are calculated on the basis of per cent of the tissue as removed from the sample bottle after storage at -30°F. for the intervals indicated.

The data in Table IV show that there is a difference in the absolute quantities of the types of nitrogen compounds determined on these muscles and that these compounds vary in their percentage relationship to the total nitrogen in the tissue, when treated as described. However, as time of storage increases from 1 to 30 days, there is no appreciable increase or decrease in the absolute or relative quantities of either the

total nitrogen or the 3 divisions of nitrogen compounds studied.

Table IV

Effect of Prolonged Low Temperatures (35°F. and -30°F.) on the Nitrogen Constituents of Beef Muscles

Time	Total Nitrogen	Amino Acid Nitrogen		Total Non-Protein Nitrogen		Ammonia Nitrogen	
Days	Gms.pc	Gms.pc	pc of T.N.	Gms.pc	pc of T.N.	Gms.pc	pcT.N.
1	3.31	0.3355	10.12	0.4320	13.05	0.0131	0.396
2	3.93	0.3302	8.40	0.3590	9.15	0.0117	0.298
5	2.59	0.2458	9.49	0.3899	15.04	0.0129	0.498
10	3.70	0.3256	8.78	0.3724	10.05	0.0124	0.336
20	4.32	0.3633	8.40	0.5216	12.06	0.0129	0.298
30	3.64	0.3171	8.72	0.4569	12.56	0.0157	0.431

There is a considerable range in all the values; for example, the total nitrogen extends from 2.59 g. per cent to 4.32 g. per cent. Such differences may be explained, not only on the basis of the moisture variable, but on the variation in lipid content as well. Prudent (124) reported both these variables to be influential in her work with aliquots from the same frozen sample. Accepting this explanation for the fluctuations, it can be stated that there is no appreciable change in the nitrogen constituents studied, over periods of holding at 35°F. and -30°F. from 1 day to 30 days, under the conditions cited.

2. Experiments with chicken pectoralis major muscle

a. Preparation of the sample. As a supplement and enlargement to the above, further studies were conducted to test the effects upon the nitrogen constituents of holding chicken breast muscle at 32°F. for increasing lengths of time. Breast muscle (pectoralis major) was selected for this work because it could be left intact in the animal during the time at the lower temperature, and because complete muscles would be available for analyses, thus eliminating major sampling problems. The birds were sacrificed by severing the jugular veins, shocked for 10 seconds and bled for one minute. After a semi-scald in water at 130°F. for 90 seconds, the feathers were removed and the birds eviscerated. The birds were stored at 32°F. for 0, 12, 24, 36, and 48 hours.

The two pectoralis major muscles, right and left, were dissected from the bird and ground together with thorough mixing so that the final ground muscle was as homogenous as possible. The two aliquots of 25 g. each were placed into chilled Waring Blendor jars and the procedure as stated under part 1 was followed.

b. Analytical methods. The 5 per cent suspension was analyzed for total nitrogen and total non-protein nitrogen, and for combined amino acid nitrogen plus ammonia nitrogen by the formol titration.

Two precipitation procedures were carried out in order to

fractionate the nitrogen compounds in the tissue suspension into both low molecular weight fractions, as found in tungstic acid filtrates, and into high molecular weight protein breakdown products, plus the low molecular weight compounds, as found in filtrates prepared with trichloroacetic acid as the precipitant.

The tungstic acid filtrates were analyzed for total non-protein nitrogen, ammonia, amino acid nitrogen,[1] histidine, tyrosine, carnosine, and anserine.

The trichloroacetic acid precipitation was carried out according to Hiller and Van Slyke (33) who reported that if a protein digest or similar preparation was treated with this acid in such a way that the final volume contained not more than 5 per cent of the acid, only the larger protein molecules would be precipitated. From the resulting filtrate they were able to obtain such protein breakdown products as were commonly termed "proteoses", "peptides", or "polypeptides", "peptones", etc. When this procedure was used in conjunction with a filtrate prepared with tungstic acid, it was possible to obtain information about the quantities of these larger compounds which were precipitated by one reagent but not by the other.

The precipitation was carried out as follows: 25 ml. of the suspension were pipetted into an 125-ml. erlenmeyer flask, an equal volume of freshly prepared 10 per cent trichloroacetic acid was added with thorough shaking, and the mixture filtered after 30 minutes. The freshly prepared 10 per cent trichloro-

[1]Also determined on undiluted suspension.

acetic acid was made by dissolving 10 g. of the crystalline acid in distilled water with 6 ml. of concentrated HCl and brought to a final volume of 100 ml. in a volumetric flask. The filtrates were clear and colorless, the pH in the range of 1.00.

As mentioned above, the tungstic acid filtrates were used for the determinations of histidine, tyrosine, carnosine and anserine. Experiments showed that the trichloroacetic acid filtrates were not suitable because of interference with the diazo reaction. Precautions must be taken to filter the tungstic acid filtrates not through filter paper but through a closely woven fabric, glass wool, or some substance which will not adsorb carnosine (140).

The methods for carnosine and anserine were devised in this laboratory by modifying the methods available in the literature. Because of the structural similarity of histidine, carnosine and anserine, the analytical methods for them are involved. It is essential to determine tyrosine, for which a good quantitative method (Folin-Ciocalteu)(141) is available; histidine and carnosine by the diazo method (142,143); histidine alone by bromination; and anserine as methyl histidine by the bromination method after hydrolysis. The carnosine, then, is determined by difference. All the work was done colorimetrically with the Klett-Summerson photoelectric colorimeter, and the methods are given in detail below.

It is essential to be able to determine histidine in order

to obtain values for the di-peptides. The method used was a modification of the original bromine reaction published by Knoop (144). In this modification the pH of the reaction mixture was carefully controlled with a sodium acetate buffer. It had been observed by Kapeller-Adler (145) and noticed in more detail in the course of the present research, that a variety of colors could be obtained with brominated histidine solutions. The color was dependant upon the pH, so that by controlling and standardizing the pH, the same color could be obtained each time. The modification is quite specific for histidine; the only compounds known at present to give color with this test are methyl histidine and histamine. The latter is rarely found in muscle tissue in any significant quantity and the former is not found to any extent in unhydrolyzed samples, but it may be found in hydrolyzed samples if anserine had been present. However, the method is not so sensitive as might be desired; 0.2 mg. histidine or less gives an insignificantly low, if any, reading on the colorimeter.

The details of the method are:

Reagent blank:

5 ml. distilled water, or preferably, redistilled water, into 22 X 175 mm. test tube

1 per cent bromine in 33 per cent glacial acetic acid, added dropwise from a small-bore burette Number of drops is determined by the number of drops needed for the unknown; the maximum number

is used in the reagent blank

Allow to stand 10 minutes in the dark

1 ml. 0.5 per cent phenol, to decolorize the unreacted bromine is added

15 ml. 5.0 N sodium acetate buffer, pH 5.00 is added

Allow to stand 40 minutes in the dark

Adjust the Klett-Summerson photoelectric colorimeter to 0 with this blank, using filter KS 54

Unknown blank:

The following solutions are, or are not, added to:

5 ml. unknown in 22 X 175 mm. test tube

No bromine solution is added, but an equivalent number of drops of redistilled water is added from a burette with a bore similar to the one used above

Allow to stand 10 minutes in the dark

1 ml. 0.5 per cent phenol

15 ml. 5.0 N sodium acetate buffer, pH 5.00

Allow to stand in the dark for 40 minutes

Any reading with KS 54 is then deducted from the unknown only.

Unknown:

5 ml. of most filtrates are sufficient to give a significant reading. If the concentration of histidine in a 5 per cent suspension is

too low it may be advisable to increase the volume. Preliminary experiments are advisable. Into 5 ml. of the unknown in a 22 X 175 mm. test tube, the following are measured:

1 per cent bromine solution, added dropwise in a quantity which will give a faint yellow color that will be retained after standing for 10 minutes in the dark. If, upon examination, the color has faded entirely, additional drops should be added. The same number of additional drops will have to be added, either bromine or water, to the above blanks.

1 ml. 0.5 per cent phenol to decolorize all unreacted bromine

15 ml. 5.0 N sodium acetate buffer, pH 5.0

Allow to stand in the dark for 40 minutes

Read in the Klett-Summerson photoelectric colorimeter with KS 54 and make deduction of unknown blank.

Standard histidine:

Histidine monohydrochloride is used, calculated as histidine; 123.6 mg. plus 10 ml. 0.1 N H_2SO_4 are diluted to 100 ml. with redistilled water; 1.0 mg./ml. of 0.01 N H_2SO_4 is the final concentration of histidine. This standard should be made about every 10 days and preserved in

the refrigerator between determinations.

For the working standard, 5 ml. of the stock standard described above are diluted to 50 ml. with redistilled water; this is carried out each time a determination is made. The concentration of histidine in the working standard is 0.1 mg. per ml.

5 ml. of the working standard are used and treated in the same way as the unknown above. The standard will contain 0.5 mg. histidine.

The calculation for the results obtained with the above method is as follows:

$$\frac{U\text{-}Bl_u}{S} \times 0.5 \text{ mg.H.} \times \frac{240}{5} \times \frac{500}{200} \times \frac{100}{25} \times \frac{1.0}{1000} = \text{gms. per cent histidine.}$$

In this formula:

$\frac{100}{25}$, represents the weight of tissue, 25 g., converted to a percentage basis.

$\frac{500}{200}$, represents the total volume of the suspension, 500ml. out of which, in this case, 200 ml. were removed for deproteinization.

$\frac{240}{5}$, represents the total volume of filtrate, 200 plus 40 ml. of combined sodium tungstate and H_2SO_4, out of which were removed 5 ml. for this determination.

$\frac{1}{1000}$, represents the conversion of mg. (0.5 in standard) to g.

Carnosine, histidine and tyrosine will react with diazotized sulfanilic acid to produce colored compounds. This reaction was described originally by Pauly (142) and is known as the "Pauly reaction". For the determinations in this work, the Jorpes modification (143) was used. Because tyrosine produces a color, a known quantity of this amino acid was carried through the reaction so that the color from this compound might be determined.

This procedure was followed:

A quantity of unknown containing 0.05-0.005 mg. per ml. of histidine is taken.

To each aliquot (1 ml.) of unknown pipetted into 22 X 175 mm. test tubes are added 9 ml. of redistilled water or an amount to total 10 ml.

To each aliquot (4 ml.) of histidine standard are added 6 ml. of water. For this standard, 1 ml. of the stock histidine standard is diluted to 100 ml. and 4 ml. used. This represents 0.04 mg. histidine.

1 ml. of tyrosine standard (0.0905 mg. tyrosine) is pipetted into a tube and 9 ml. of water are added. It is necessary to run this tyrosine standard concurrently each time.

To each tube above are added:

2 ml. H anke-Koessler diazonium solution (146)

Allow to stand for 1 hour

5 ml. 1.1 per cent Na_2CO_3 with thorough mixing

Read against filter KS 54 within 5 minutes of adding the Na_2CO_3

The calculation for the results obtained with the above method is as follows, and is known as the "diazo value" which includes histidine, carnosine, and tyrosine:

$$\frac{U}{S} \times 0.04 \text{ mg.H.} \times \frac{10}{1} \times \frac{240}{1} \times \frac{500}{200} \times \frac{100}{25} \times \frac{1.0}{1000} = \text{gms. per cent.}$$

In this formula:

$\frac{10}{1} \times \frac{240}{1}$, represents a dilution of 1 ml. of the filtrate. The other fractions have been described.

To calculate the diazo equivalent of tyrosine:

$$\frac{T}{S} \times 0.04 \text{ mg. H.} \times \frac{1.0}{0.0905 \text{ mg.Tyr.}} \quad \text{or} \quad \frac{T}{S} \times 0.442 = \text{Tyrosine}$$

factor. This conversion factor, when multiplied by the tyrosine value obtained as grams per cent with the method of Folin and Ciocalteu (141), gives tyrosine as histidine, in grams per cent. No modifications were made in the Folin-Ciocalteu procedure; the calculation as used in these experiments is as follows:

$$\frac{U\text{-}Bl_r}{S\text{-}Bl_r} \times 0.0905 \text{ mg.T.} \times \frac{240}{1} \times \frac{500}{200} \times \frac{100}{25} \times \frac{1.0}{1000} = \text{gms. per cent.}$$

Anserine is determined as methyl histidine and is recalculated to anserine. In order to obtain methyl histidine, 25-ml.

aliquots of the filtrate are refluxed (in 22 X 175 mm. test tubes with a large marble on top) with 15 ml. of 10 N HCl for 1 hour in a constantly boiling water bath. The volume of water in the bath must be above the liquid surface in the test tubes at all times. At the end of one hour, the test tubes are removed, allowed to cool, and the solution neutralized with 15 ml. 10 N NaOH. These reagents should be prepared carefully and measured accurately with a pipette; the resulting neutralized solution should have a pH between 6.00 and 7.00. The cooled solutions are transferred quantitatively to 100 ml. volumetric flasks and 5 ml. aliquots of these unknowns carried through the bromination reaction as described above.

The duration of hydrolysis is critical; it was discovered in recovery experiments, in which the compound itself was employed, that a shorter or longer period did not prove satisfactory.

In this hydrolyzed filtrate these compounds will be present: anserine as methyl histidine, the histidine from carnosine, and the histidine originally present. The following calculation is for anserine as methyl histidine, with a subsequent conversion to anserine:

$$\frac{U\text{-}Bl_u}{S} \times 0.5 \text{ mg.H.} \times \frac{100}{5} \times \frac{240}{25} \times \frac{500}{200} \times \frac{100}{25} \times \frac{1.0}{1000} =$$

grams per cent anserine as methyl histidine.

After obtaining the above values, these further calculations are essential:

For carnosine:

(Diazo value) - (Tyr. as H_2) - (H_{Br}) = C. calculated as H_2, in grams per cent.

$C_{(as\ H_2)} \times \frac{0.0847}{0.0777}$ or $(C_{(as\ H_2)} \times 1.09)$ = C. gm_s per cent.

For anserine:

(Hydrolysis value) - (H_{Br}) - ($C_{as\ H_2}$) = $A_{*(as\ H_2)}$

$A_{*(as\ H_2)} \times 5.0 = A_{(as\ MeH_2)}$ gms. per cent.

$A_{*(as\ MeH_2)} \times \frac{240}{169}$ or $(A_{*(as\ H_2)} \times 1.42)$ = A. gms. per cent.

A convenient way to set up the calculations and data for these amino acids and di-peptides is as follows: if weights of tissue and all volumes are kept constant, the equations may be solved and factors obtained. Examples of these are 0.24 for histidine, 0.96 for the diazo value, 0.442 for the tyrosine factor, 0.217 for tyrosine by the Folin and Ciocalteu method, and 0.96 for the histidine method after hydrolysis. The colorimeter readings are recorded under such headings as "H_{Br}", "Diazo Value", "Tyr.F.C.", and "Hyd.Fil.". Under each of the 19 headings illustrated below, the necessary data and calculations may be recorded, and the required information obtained in a relatively simple way.

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Sample	H_{Br}	(2)X 0.24 = H_2 gms. per cent	Diazo Value	(4)X 0.96 = gms. per cent	Tyr. Equiv.	(6)X 0.442 = Tyr. factor

(8)	(9)	(10)	(11)	(12)	(13)
Tyr. F-C	(8)X 0.217 = gms. per cent	(7)X(9) Tyr. as H.	Hydr.Fil. A. as MeH.	(11)X 0.96 = gms. per cent	(5) - (10)

(14)	(15)	(16)	(17)	(18)	(19)
(13)-(3) C. as H. gms. per cent	(14)X 1.09 = C. gms. per cent	(12)-(3)	(16)-(14)	(17)X 5.00 = A. as MeH.	(18)X 1.42 = A. gms. per cent

If it is desirable to report these data as per cent of the total nitrogen, the following calculations are made:

Histidine: $\frac{0.271 \times \text{gms. pc H.}}{\text{Total Nitrogen}} \times 100$ = Histidine-nitrogen as per cent of the total nitrogen.

The number, 0.271, is the factor to convert histidine in grams per cent to histidine-nitrogen. The factors for the other compounds follow: Tyrosine, 0.0773; carnosine, 0.252; anserine, 0.233.

This is a sample calculation for a factor:

$$\frac{\text{(Quantity of nitrogen in the molecule)}}{\text{(Molecular weight of molecule)}}$$

The methods as described above were used to determine the values presented in Table VI.

c. Results from chicken muscle experiments. As with the beef muscle experiments, the chicken muscles were held at a low temperature (32°F.) for varying lengths of time, but were not held at -30°F. Two separate experiments were conducted; in the first, only the tungstic acid filtrate was used and the amino acid,[1] total non-protein, and ammonia nitrogens determined

[1]Also determined on undiluted suspensions.

on it. Total nitrogen data were obtained on the undiluted suspensions. In the second experiment, total nitrogen was determined on the undiluted suspension, and filtrates were prepared by both the precipitation methods described. On the tungstic acid filtrates, the amino acid and di-peptide analysis, discussed in detail above, were used.

In Table V are shown the results of the first low-temperature-time study described.

Table V

Effect of Low Temperature (32°F.) Periods on Nitrogen Constituents of Chicken Breast Muscle[1]

Time	Total Nitrogen[2]	Amino Acid Nitrogen[2]		Total Non-Protein Nitrogen[3]		Ammonia Nitrogen[3]	
Hrs.	Gms. pc	Gms. pc	pc of T.N.	Gms. pc	pc of T.N.	Gms. pc	pc of T.N.
0	3.92	0.4266	10.89	0.5056	12.90	0.0162	0.414
12	3.70	0.3405	9.20	0.5612	15.18	0.0159	0.430
24	3.99	0.4190	10.51	0.5444	13.62	0.0194	0.486
36	4.03	0.5575	13.82	0.5826	14.47	0.0163	0.404
48	3.88	0.4259	11.00	0.5469	14.10	0.0174	0.449

[1]Eight birds at 0-hour; 6 birds at other periods

[2]Determined on undiluted suspension

[3]Determined on tungstic acid filtrates

The figures given are the averages from a number of birds.

Duplicate aliquots were removed from the ground, homogenous muscle. From the prepared suspensions and filtrates triplicate aliquots were used for the individual determinations.

The total nitrogen data are in excellent agreement from 0-hour to 48-hour samples, but do not change over the period of storage at 32°F. No trend, either increase or decrease, is indicated. The excellent agreement found among these chicken muscle samples may be the result of the facts that the muscles were protected by the birds' skins; that there was more homogeneity from bird to bird with only the one kind of muscle being analyzed; and that the muscles were intact until dissection. Therefore, the moisture content and the fat, which was not noted in excess in any of the bird muscles, were more likely to remain fairly constant.

Although there is some fluctuation, the absolute and relative (per cent of the total nitrogen) values for the amino acid, ammonia, and non-protein nitrogens show a tendency to increase with time at 32°F. In all cases the 48-hour data are higher than the 0-hour data, and the 36-hour data are higher than the 0-hour data.

The absolute and relative quantities of the constituents determined are, as with the beef samples, in regular relationship to each other, for example, the values for total non-protein nitrogen are in every instance higher than the values for amino acid nitrogen which, in turn, are greater than those for ammonia nitrogen. The total non-protein nitrogen increased

more than either the amino acid or ammonia nitrogens.

The results from the second low-temperature-time experiment with chicken muscle are shown in Tables VI and VII. The 12-hour period was omitted and only two birds were studied at each period. Since information of a more general nature had been obtained with the first experiment, whose results are in Table V, the second experiment was designed to give more specific information. The tungstic acid filtrate was fractionated further into histidine, tyrosine, carnosine, and anserine. On this filtrate, too, was determined the total non-protein nitrogen for comparison with the nitrogen to be found in a trichloroacetic acid filtrate. The possibilities in such a comparison have been elucidated above.

Table VI

Effect of Storage at 32°F. on Some Constituents of the Amino Acid Nitrogen Fraction of Chicken Breast Muscle

Time	Total Nitrogen[1]	Histidine[2]		Tyrosine[2]		Carnosine[2]		Anserine[2]	
Hrs.	Gms. pc	Gms. pc	N-pc of T.N.	Gms. pc	N-pc of T.N.	Gms. pc	N-pc of T.N.	Gms. pc	N-pc of T.N.
0	4.02	0.0038	0.0249	0.0123	0.0236	0.2990	1.87	0.0000	0.00
24	3.88	0.0038	0.0259	0.0149	0.0304	0.4908	3.21	0.3497	2.11
36	3.70	0.0038	0.0271	0.0145	0.0303	0.3255	2.19	0.0604	0.40
48	3.79	0.0074	0.0521	0.0175	0.0354	0.3829	2.53	0.4235	2.58

[1]Determined on undiluted suspension

[2]Determined on tungstic acid filtrates

According to the figures presented in Table VI, both histidine and tyrosine increase when chicken breast muscle is stored at 32°F. for 48 hours. The histidine remains constant until 48 hours when it increases appreciably; the increase in the tyrosine is more gradual. Although carnosine increases, and is in greater quantity at each period than at the 0-hour, there is some fluctuation. The anserine not only fluctuates but the values are in such poor agreement compared with the other compounds studied as to merit no concluding remarks other than that they are undoubtedly of little significance.

Of more significance are the data expressed as "per cent of total nitrogen" because they show the proportioning of the constituents when compared to the whole. When these values are compared with the amino acid nitrogen and non-protein nitrogen increases in Table V, or with the increases shown in Table VII, they show a consistency which increases the reality and validity of all of them. Of particular note are the values for the total nitrogen in both experiments. There is excellent agreement among the values shown in Table V; there is not only equally excellent agreement among the values in Table VI, but also excellent agreement between the total nitrogen values from the two separate experiments. Because of these consistencies, data calculated as "per cent of the total nitrogen", can be compared more accurately and with more assurance.

In addition to the compounds noted in Table VI, the total non-protein nitrogen content of both the tungstic acid and tri-

chloroacetic acid filtrates was studied. These determinations were carried out on the same muscle tissue suspensions used for the data in Table VI, so the total nitrogen values are identical.

Table VII

Effect of Storage at 32°F. on Some Constituents of the Total Non-Protein Nitrogen Fraction of Chicken Breast Muscle

Time	Total Nitrogen[1]	Non-Protein Nitrogen Filtrates			
		Trichloroacetic Acid		Tungstic Acid	
Hours	Gms. pc	Gms. pc	pc of T. N.	Gms. pc	pc of T.N.
0	4.02	0.61	15.28	0.58	14.44
24	3.88	0.59	15.35	0.57	14.71
36	3.70	0.58	15.68	0.51	13.68
48	3.79	0.61	16.25	0.57	14.78

[1]Determined on undiluted suspension

Several outstanding points may be observed in Table VII. Firstly, both the grams per cent values for total non-protein nitrogen and their per cents of the total nitrogen are greater in the trichloroacetic acid filtrates than in the tungstic acid filtrates. Based upon these facts, it may be reasoned that at all the time periods after the sacrifice of the birds there was a greater quantity of the high molecular weight protein split-products than of the lower ones present in the tissue. Secondly, it may be generalized that there is a steady increase in these high molecular weight compounds with the additional hours

of holding at 32°F. Although there is some increase in the lower molecular weight compounds, it is, for all practical purposes, not appreciable.

An additional point, to be enlarged upon when the autolysis experiments are evaluated, is that, although there are increases with time, in the higher molecular weight compounds, these increases are not large, to wit: 15.28, 15.35, 15.68, to 16.25 from 0-hour to 48-hours. These small increases may be an effect of lowered temperature, especially when compared with incubation studies. It is interesting to note that a steady increase in degradation products was found even at 32°F., and that it probably indicates a breakdown of the larger protein molecules into intermediate fragments rather than an increase in the total degradation of these intermediate fragments. This does not imply that there was no increase in these smaller end-products, only that there was a difference in degree and/or rate of breakdown.

B. Effect of High Temperatures Upon the Nitrogen Constituents of Muscle

1. Preparation of the samples and analytical methods

In these experiments the muscle tissues were subjected to high temperatures, under pressure. In the first group, samples were prepared by placing about 100 g. of raw beef muscle into thick Pyrex test tubes, sealing the opening with heavy metal

foil, and processing in an autoclave for 2 hours at a pressure of 15 pounds. A control sample of beef muscle was not heated. Runs at two different temperatures were made; one at 40°C. and one at 100°C. After the samples were cool they were taken from the test tubes, as much fat as possible removed, and prepared for the analytical procedures in the usual way. These beef samples contained large quantities of fat which probably influenced the results, by being, of necessity, included in the starting weights of the samples.

The second group of samples consisted of commercially canned meat products obtainable in local markets. Only the products which were stated to contain unmixed tissue were selected; for instance, a type of beef product, described as containing gravies, vegetables, etc., was not taken. This second group consisted of a variety of beef, pork, lamb, and veal products, as well as salmon and sardines. The exact conditions employed in processing these samples are not known; however, it is known that high temperatures are applied. Obvious differences in these products will be described in the listing of them given below.

After the suspensions were prepared, three 5-ml. aliquots were removed for total nitrogen, and duplicates for each of both the trichloroacetic acid and the tungstic acid filtrates. This plan was followed for the samples in these two groups.

The following tabulation will describe the commercially canned products. The sample numbers correspond to those listed

in Tables IX and X.

Sample 1. A pressed beef product, known as "Mor" prepared by Wilson and Company, Chicago, Illinois. In addition to beef, the sample contained sugar, $NaNO_3$, $NaNO_2$, and NaCl. The sample contained a large quantity of fat, most of which could not be removed.

Sample 2. "Swift's Meat for Babies", composed of strained beef and broth with NaCl. The product was very finely divided and contained appreciable quantities of fat.

Sample 3. "Swift's Meats for Juniors"; diced beef with natural juices and NaCl added. The beef was in solid pieces and could be separated from the juices and particles of fat. The analyses were of practically pure muscle tissue.

Sample 4. "Libby's Roast Beef; Parboiled and Steam Roasted"; an Argentinian import containing only beef and NaCl. The tissue was very fibrous and the aliquots could be removed from the liquid and fat, so that the samples were almost all muscle tissue only.

Sample 5. "Gerber's Strained Meats"; processed by Armour and Company, Chicago, Ill. The product contained only beef, liquid, and NaCl. The beef was very finely ground and quite liquid; the fat

could not be removed from the samples.

Sample 6. "Gerber's Chopped Beef"; processed by Armour and Company, Chicago, Ill. The product contained beef, broth and NaCl. Although a chopped product, it was not as solid as Sample 3; therefore, some juice and fat were taken in the aliquots.

Sample 7. "Swift's Meats for Babies"; strained pork and broth with NaCl added. Appreciable liquid and fat which could not be removed.

Sample 8. Diced pork; processed by Swift and Company. Some liquid and NaCl were present; the meat samples could be removed from the water and fat.

Sample 9. Strained lamb; processed by Swift and Company, and containing water and NaCl. There was a very considerable quantity of fat in this sample, and it could not be removed from the aliquots.

Sample 10. Diced veal; processed by Swift and Company, and containing water and NaCl. There was some fat, but most of it could be removed before sampling.

Sample 11. Sardines; "Nor. sild". Packed in Norway. These had been packed in oil, not all of which could be removed before sampling. An attempt was made to remove the bones, skin, etc., but small portions may have been included.

Sample 12. Pink salmon; Alaska; containing little water, NaCl and little fat. Bones were not included in the samples selected.

Sample 13. Red salmon; Alaska Sockeye; only NaCl added and less liquid present than in Sample 12. Bones were not included in the samples selected.

2. Results

In the following three Tables the results of the analyses of the above products are recorded. The values include those for the pH of the suspensions, the total nitrogen, and the total non-protein nitrogen obtained in both kinds of filtrates. In Table VIII, the results from the control beef sample and the two autoclaved beef samples are given. The second group of samples is divided into two parts: the first shown in Table IX contains only the beef muscle products studied; while the second part, in Table X, includes the miscellaneous meat and fish preparations, Samples 7 to 13 in the above tabulation.

The total nitrogen data from this experiment on heat-processed beef samples are more variable than the total nitrogens in the previous experiments. The unusual quantities of fat in these samples might well have accounted for the variation in the total nitrogen results. It is quite likely that each 25-g. sample contained a different weight of fat and since there was an appreciable quantity of fat which it was not practicable to try to separate, a goodly part of the 25-g. weight could

have been fat. On a percentage basis, the values obtained for total nitrogen would be quite variable. However, in spite of the variation, the values are not out of the range of the total nitrogen figures obtained with beef and chicken muscle tissues.

Table VIII

Effect of Autoclaving for Two Hours on Some Constituents of Non-Protein Nitrogen Fractions of Beef Muscle

Treatment	pH[1]	Total Nitrogen[1]	Non-Protein Nitrogen Fractions			
			Trichloroacetic Acid		Tungstic Acid	
		Gms. pc	Gms. pc	pc of T. N.	Gms. pc	pc of T. N.
Control (raw)	5.57	3.45	0.370	8.97	0.340	9.93
40°C. 2 hr	5.70	3.69	0.370	10.06	0.345	9.25
100°C. 2 hr	6.00	4.82	0.405	7.55	0.300	6.66

[1]Determined on undiluted suspension

The comparative determinations of total non-protein nitrogen, as characterized by the 2 precipitating reagents, show several interesting facts which will be further substantiated with the autolysis experiments cited below. One of these facts is that when fresh tissue is analyzed, the 2 methods give total non-protein nitrogen values which are in fairly close agreement.

If the effect of temperature upon each filtrate is studied,

it will be seen that with the trichloroacetic acid filtrate the value for total non-protein nitrogen as per cent of total nitrogen, obtained at 40°C. is 1.09 per cent greater than at the control level, and lower by 1.42 per cent at 100°C. than at the control level. It would not be accurate to state that these values rise and fall under the influence of temperature, since these are individual samples. However, at these temperatures, compared with the control sample which was not subjected to processing, there is a definite increase in the production of the higher molecular weight breakdown products at 40°C. and no increase in these products at 100°C. over the control level.

With the tungstic acid filtrate the value for total non-protein nitrogen as per cent of total nitrogen, obtained at 40°C. is 0.68 per cent lower than at the control level, and at 100°C. this same value is 3.27 per cent lower than at the control level. Again, it would not be accurate to state that there is a steady fall in these values under the influence of temperature, but only that when compared with unprocessed samples the production of the lower molecular weight breakdown products is not increased at 40°C., and is certainly inhibited at 100°C. The difference between the control values obtained with the 2 types of filtrates plus the small (0.68 per cent) decrease at 40°C. could be interpreted to indicate that further experiments of this type might show a greater production of low molecular weight products than this particular experiment shows under the influence of an optimum temperature at 15 pounds pressure for 2 hours.

There is agreement between both types of filtrates with respect to the lower values for total non-protein nitrogen as per cent of total nitrogen when the samples are held at 100°C., 15 pounds pressure for 2 hours. It seems evident that under these conditions there is an inhibition of the proteolytic enzymes involved in the degradation of muscle meat proteins. That any values were obtained may be explained, possibly, by assuming that some enzyme action had occurred in the sample prior to treatment and, that immediately after processing began there was an initial, rapid, enzyme activity. Some products would be formed in either or both cases and these products would remain and be measurable after enzyme activity was inhibited by the processing conditions.

In Table IX, the sample numbers correspond to those previously tabulated and described. When the nature of the samples is considered in conjunction with the data obtained upon analysis of them, apparent discrepancies are explainable. For example, fluctuations in the total nitrogen results found in the different samples may be correlated with the amounts of liquid and fat which were impractical to separate and so were weighed into the aliquots. Samples number 2 and 5 had large quantities of both liquid and fat. The more fibrous muscle samples, such as samples 3, 4, and 6, gave higher values than usual, but they were comparatively dry, and parts of muscles could be weighed without liquid or fat.

The total non-protein nitrogen values from this study are

particularly interesting. In spite of the variation in the total nitrogen values from sample to sample, the total non-protein nitrogen results from the trichloroacetic acid filtrates, without exception, are higher than those from the tungstic acid filtrates. This is in agreement with the data in Table VIII in which the processed samples gave the same relationship. Evidently the proteolytic enzymes which split the

Table IX

Effect of Commercial Processing Upon Three Nitrogen Fractions of Six Different Canned Beef Products

Sample	pH[1]	Total Nitrogen[1]	Non-Protein Nitrogen Filtrates			
			Trichloroacetic Acid		Tungstic Acid	
		Gms. pc	Gms. pc	pc of T. N.	Gms.pc	pc of T. N.
1	6.29	3.06	0.408	13.34	0.281	9.18
2	6.17	2.48	0.229	9.22	0.210	8.46
3	6.00	5.63	0.432	8.56	0.376	6.66
4	6.20	5.02	0.437	8.71	0.340	6.76
5	5.95	2.93	0.477	16.30	0.318	10.87
6	6.00	5.54	0.614	11.08	0.463	8.36

[1]Determined on undiluted suspension

large protein molecules to intermediates are very active in the initial stages, at least, of the processing methods used to prepare muscle meat foods in canning. There is a lesser activity

of the enzymes which produce the ultimate products of protein degradation.

When one compares these samples, and is aware of the differences in them, so far as physical state, source of material, and so forth are concerned, the consistency found with the total non-protein nitrogen relationship is more striking. This is demonstrated even more conclusively in Table X in which the variety of the miscellaneous meat products is much greater.

Table X

Effect of Commercial Processing upon Three Nitrogen Fractions of a Variety of Muscle Tissues Canned for Food

Sample Number and Name	Total Nitrogen[1]	Non-Protein Nitrogen Filtrates Trichloroacetic Acid		Tungstic Acid	
	Gms. pc	Gms. pc	pc of T. N.	Gms. pc	pc of T.N.
7 Pork	2.70	0.245	9.07	0.166	6.16
8 Pork	4.25	0.498	11.71	0.382	8.97
9 Lamb	2.53	0.510	20.00	0.228	8.97
10 Veal	3.87	0.552	14.27	0.357	9.22
11 Sardines	3.28	0.664	20.25	0.432	13.16
12 Pink Salmon	3.89	0.690	17.72	0.470	12.07
13 Red Salmon	4.13	0.682	16.53	0.473	11.46

[1]Determined on undiluted suspension

The data presented in Table X show essentially the same

characteristics as those in Table IX. It should be emphasized, however, that the comparison between the total non-protein nitrogen values from the two precipitation methods is even more outstanding in this instance because of the greater variation in the substances analyzed. As a matter of fact, the differences between the values are greater, in most cases.

C. Effect of Autolyzing Enzymes Upon Muscle Constituents[1]

1. Preparation of the sample and analytical methods

A 5 per cent suspension of fresh chicken breast muscle was used in these experiments; it was prepared as described in previous sections. After 0-hour aliquots were removed for total nitrogen and the two precipitation methods, incubation was carried out at some defined pH and temperature over a definite period of time. A few drops of a 1:1000 aqueous solution of merthiolate were added to retard bacterial action. To follow the progress of the autolysis, aliquots were removed at intervals, deproteinized with either 10 per cent trichloroacetic acid, tungstic acid, or both, and analyzed for non-protein nitrogen and the preformed non-protein nitrogen constituents, histidine, tyrosine, carnosine and anserine. The incubation was carried out in a constant-temperature water bath and the pH adjusted every 30 minutes with a drop or two of either 5 N NaOH or 5 N HCl.

[1] All the autolysis experiments and the experiments on the autoclaved beef muscle were performed in cooperation with Lieutenant Robert S. Yare.

2. Results

a. Temperature series. Preliminary experiments had shown that pH 4.00 was optimum under the conditions selected. The following data illustrate the results obtained when a series of temperature studies was performed. Table XI presents the actual total non-protein nitrogen values, expressed both as grams per cent and as per cent of total nitrogen, obtained at 40°C., which proved to be the optimum.

Table XI is included to show a typical autolysis experiment at pH 4.00 and 40°C. The data show a steady increase in the total non-protein nitrogen, expressed both as grams per cent and the more preferable, per cent of total nitrogen, from 0-hour to 6 hours. The increase occurs in both the trichloroacetic acid and tungstic acid filtrates. The total non-protein nitrogen as per cent of the total nitrogen increases to a greater degree in the trichloroacetic acid filtrates. There is an increase also in the values which represent the per cent increase of the total non-protein nitrogen at 6 hours autolysis over the 0-hour values. These per cent increase values are calculated from the actual grams per cent of the total non-protein nitrogen found.

Figure 6 shows the average per cent increases from 0-hour to 6 hours in the total non-protein nitrogen when muscle suspensions were held at pH 4.00 at the temperatures, 25°, 35°, 40°, 45°, and 55°C. Figure 6 is not based solely on the data in Table XI. The values in the graph were taken from all

the autolytic work conducted. An attempt was made to select only those values which were in fair agreement. When no agreement existed, all the values available were averaged. The dip in the trichloroacetic acid curve may be explained by the necessary inclusion of a value (15.27 per cent increase in 6 hours) which lowered the average to 15.62 per cent increase instead of the more typical increase, 15.79, as illustrated in Table XI.

Table XI

Effect of Autolysis on the Non-Protein Nitrogen Fraction in Trichloroacetic Acid and Tungstic Acid Filtrates from Chicken Breast Muscle. Autolysis at pH 4.00; 40°C.

Time	Total Nitrogen[1]	Non-Protein Nitrogen Filtrates					
		Trichloroacetic Acid			Tungstic Acid		
Hrs.	Gms. pc	Gms. pc	pc of T.N.	pc Increase	Gms. pc	pc of T.N.	pc Increase
0	3.80	0.57	14.97		0.52	13.58	
	3.86	0.57	14.73		0.55	14.33	
2		0.59	15.52	3.51	0.56	14.70	7.69
		0.59	15.14	3.51	0.57	14.71	3.63
4		0.61	15.99	7.02	0.61	15.45	17.31
		0.61	15.71	7.02	0.61	15.80	10.91
6		0.66	17.43	15.79	0.62	16.41	19.23
		0.66	17.03	15.79	0.64	16.50	16.37

[1]Determined on undiluted suspension

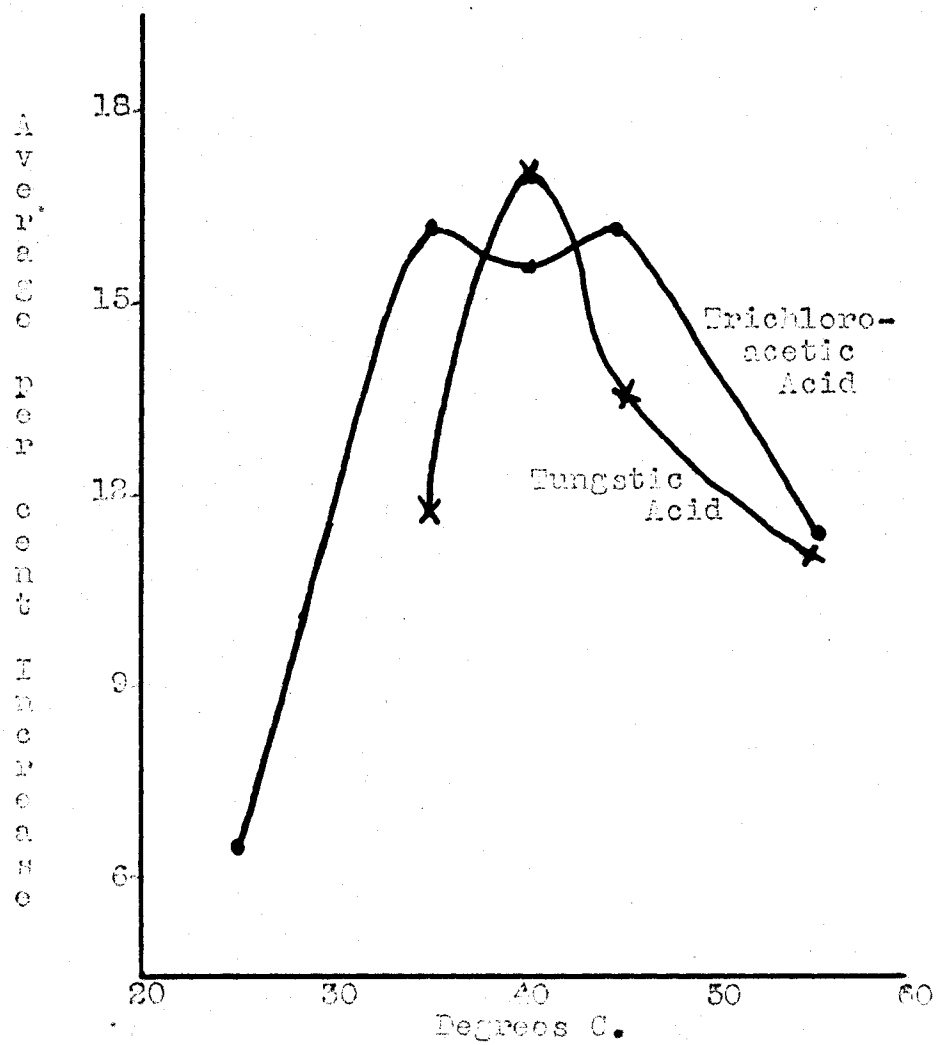


Figure 6. Average Per Cent Increase in Non-Protein Nitrogen after 6 Hours Incubation at pH 4.00 and the Temperatures Stated. Curves represent data from precipitation methods cited.

With the possible explanation for the dip in the trichloroacetic acid curve in mind, a further study of the graph will indicate at least two points: firstly, 40°C. at pH 4.00 seems to be the optimum temperature for the activity of the enzymes involved; and secondly, the broad, steady, more gradual rise and fall of the trichloroacetic acid filtrate curve indicates a greater change from protein to molecules of intermediate size than from protein fragments to the end products of protein decomposition.

Figure 7 shows the non-protein nitrogen constituents, tyrosine, histidine, carnosine and anserine, expressed as per cent increase from 0-hour to 6 hours for the temperatures, 25°C., 35°C., 45°C., and 55°C. These constituents were determined on the same tungstic acid filtrates from which the non-protein nitrogen data graphed in Figure 6 were derived. These components are representative not only of the total non-protein nitrogen but of the amino acid fraction of that general group.

These values, as well as those in Figure 6, are expressed as per cent increases, 0-hour to 6 hours, and because they were determined on the tungstic acid filtrates, may be compared with the inclusive total non-protein nitrogen curve. The anserine curve most closely resembles the total non-protein nitrogen curve, except for the rise at 55°C. The peaks for histidine and tyrosine come at 45°C., but otherwise the general trend of these curves is similar to the total non-protein nitrogen. The carnosine rises only slightly at 35°C. and then tends to re-

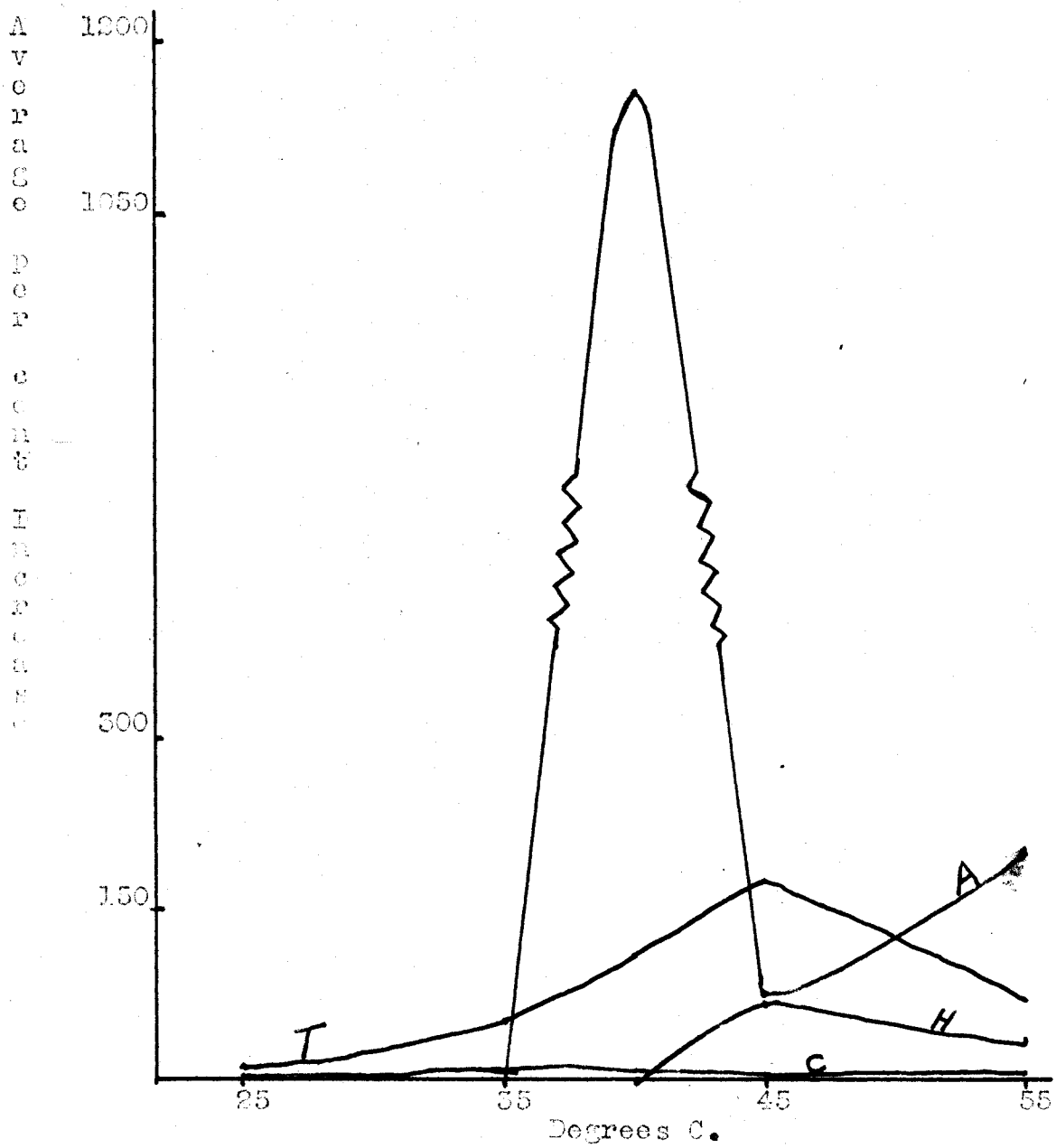


Figure 7. Average Per Cent Increase in the Compounds Cited after 6 Hours Incubation at pH 4.00 and the Temperatures Stated.

main fairly stationary after a short drop. The values for carnosine have been somewhat at variance in all the instances in which these components have been determined.

Table XII presents the total nitrogen values from the above experiments. It should be emphasized that the values summarized and graphed are the means of data from 28 birds used in 7 experiments in each of which 4 birds were analyzed. From each bird duplicate aliquots of breast muscle were analyzed.

Table XII

Total Nitrogen[1] on Chicken Muscle Autolyzed at pH 4.00 at the Temperatures Indicated

Sample	Temperatures, degrees C.				
	25°	35°	40°	45°	55°
A	3.93	3.96	3.92	3.96	4.06
B	4.04	4.01	3.97	3.96	3.90

[1]All values are expressed as grams per 100 grams fresh muscle tissue, and were determined on the undiluted suspensions.

D. Effect of Diet Upon the Nitrogen Constituents of Muscle Tissue

1. Vitamin C deficiency

a. Animals and diet. The male guinea pigs used in the

following experiments weighed from 200 to 300 gms. at the start of the experiments and were in good health. They were paired according to the weight of each animal at the beginning of the experiment by employing a random system which resulted in the two groups of each experiment having approximately the same average weight. In some experiments twelve animals were started at the same time: six in the experimental and six in the control groups. In other experiments six animals were started: three experimentals; three controls. The animals were housed in roomy, clean, wire-bottomed cages with not more than three animals in one cage. The food and water dishes were kept clean and adequate food and water supplied ad libitum. The dishes were low, heavy crockery containers which could not be tipped over and which allowed the animals free access to the diet and water at all times. This is especially essential toward the end of the experiment when the scorbutic animals find it difficult to raise themselves to eat. If the food were not accessible, they might fail to continue eating. Close observation of the animals showed that they did continue eating so that inanition was not a factor in determining the condition of the experimental animals.

The experimental animals were weighed every other day for the first week, then every day until the termination of the experiment. The controls were weighed every other day.

The diet used was commercial Purina Rabbit Chow, a complete ration which was ground in a Wiley mill and spread out in thin

layers to dry and aerate thoroughly. The ground food was fed *ad libitum*, care being taken to keep food before the animals at all times. All the animals, both control and experimental, received about 0.9 g. of brewer's yeast daily, spread on top of the food and 3 drops of a vitamin A and D concentrate (oleum percomorphum, 50 per cent, Meads) per week. The vitamin A and D concentrate was administered *per os* and provided 3,750 and 540 International Units per week of the vitamins A and D, respectively. Only the control animals received 5 mg. of vitamin C daily, administered *per os* in 1 ml. of water from a 1 ml. volumetric pipette. This regimen provided a complete diet for all the animals with the exception of the vitamin C in the case of the experimental animals.

In the first experiment to be reported, the animals received supplemental tyrosine. This compound, as the L-tyrosine, was administered as a 7.5 per cent mixture in the basal diet, layered over additional plain basal diet so that each day's supplement would be consumed entirely. Five gms. of this supplemental mixture, equivalent to 0.375 g. of tyrosine, were given each day.

Subsequent to the tyrosine supplement experiment, a series of experiments was conducted in which the guinea pigs were given the usual "scurvy-producing" diet described above. In the first three experiments of this series, the animals developed an acute form of scurvy in the expected three to four weeks period on the experimental vitamin C-free diet. In the

fourth experiment of the series, the animals developed a "chronic scurvy" on the same type of diet, to which the manufacturer of the Purina Chow had added an undisclosed quantity of animal protein factor.

b. Preparation of samples and analytical methods. Typical weight curves for normal and scorbutic guinea pigs are graphed in Figure 8. After an initial drop at the outset, when the animals are adjusting to the diet, both the normal and scorbutic individuals tend to gain weight, but, while the normal continues to rise, the scorbutic drops steadily until the termination of the experiment. When the experimental guinea pigs had dropped in weight to about 250 gms., after having risen somewhat higher during the course of the experiment, they were sacrificed. The control was sacrificed at the same time. This was accomplished by a blow on the back of the skull to stun the animal completely, and by subsequent exsanguination by severing both jugulars. The animal was suspended by the hind legs, pressure applied to the abdominal cavity until bleeding was as complete as possible. The skin was removed from the hind legs and the muscles dissected out as quickly as possible. The muscle tissue was freed of fascia and bone. The bones were tested for fragility by applying pressure with a certain pair of surgical scissors kept for this purpose. The further preparation of the muscle sample has been described in the previous sections.

Because the total muscle weight was relatively low in

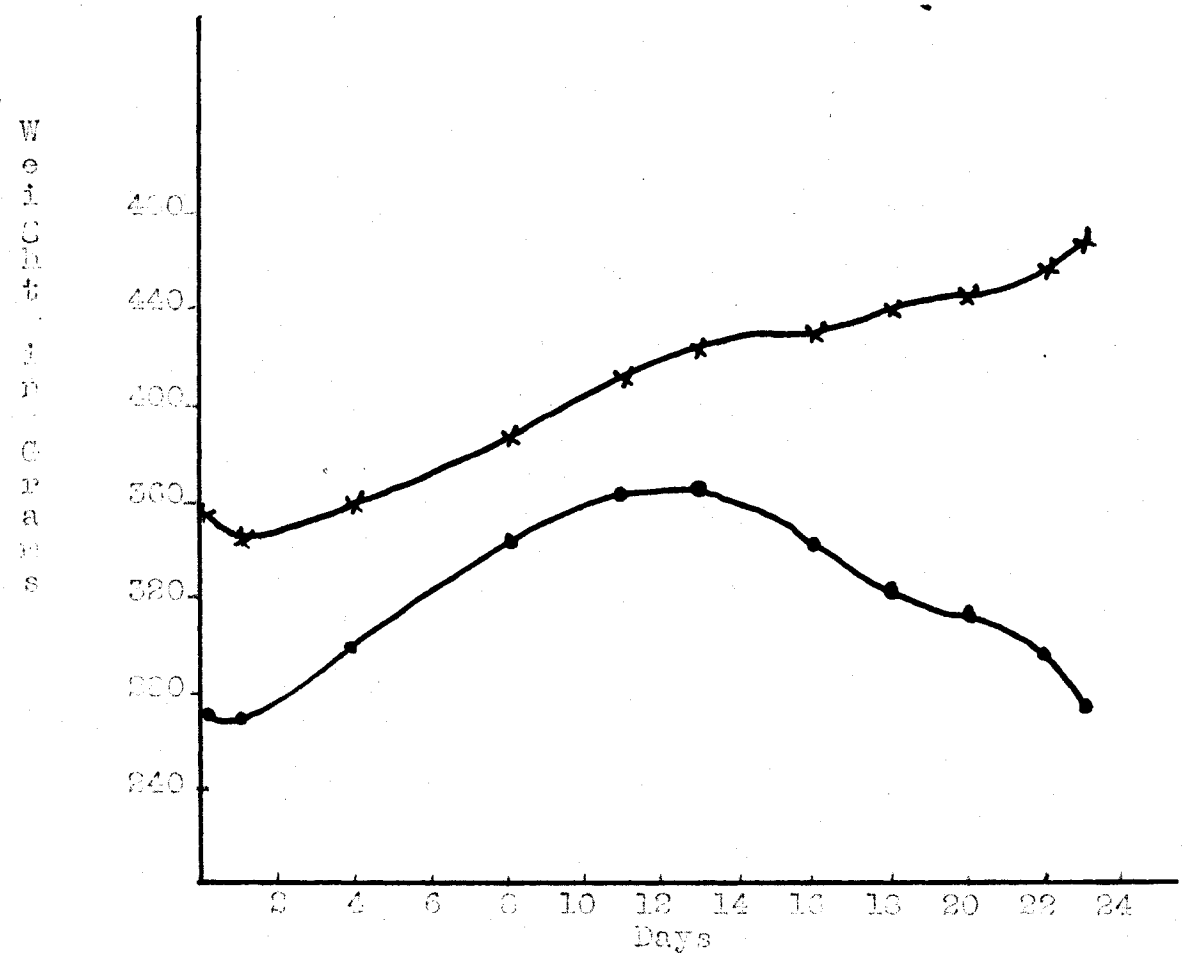


Figure 8. Typical Weight Curves of Normal (x——x) and Scorbutic (●——●) Guinea Pigs from Series 3.

guinea pigs, and especially in the experimental animals, as compared to the beef and chicken samples, it was not possible to take duplicate muscle samples from each guinea pig. However, small portions were taken for histological sections and total moisture in the last experiments.

The analytical methods used have been described. Preliminary experiments had shown that it was impractical to try to determine the amino acids and di-peptides on all the guinea pig samples, because the small amount of sample did not allow accurate histidine determinations, but one set of this kind of experiment is included.

c. Results. In the following experiments, time and temperature of storage and treatment were not matters of concern. Rather, the effects of dietary deficiencies upon the enzyme systems in living tissue were studied through the medium of measuring the metabolic reaction products.

In Tables XIII and XIV will be found the results from 2 experiments with normal and vitamin C-deficient guinea pigs. In both studies total nitrogen, total non-protein nitrogen, and amino acid and ammonia nitrogens were determined.

Table XIII shows the data obtained with the experiment in which the guinea pigs were fed the L-tyrosine supplement, amounting to 0.375 g. each day. The animals which received no vitamin C developed a very acute type of scurvy in a week. In fact, although 6 animals had been placed in each group originally, 3 of the vitamin C-deficient died during the nights before the

week had passed. The deficient animals showed all the characteristics of scurvy, including very fragile bones, extensive hemorrhages, and dark, yellow-brown muscle tissue. Their weights had dropped from over 300 gms. to 220, 172, and 216 gms. for scorbutic animals 1, 2, and 3, respectively.

Table XIII

Effect of Acute Vitamin C Deficiency, with Addition of L-Tyrosine, on Nitrogen Constituents of Guinea Pig Muscle

Sample		Total Nitrogen[1]	Amino Acid Nitrogen[2]		Non-Protein Nitrogen[3]		Ammonia Nitrogen	
		Gms. pc	Gms. pc	pc of T.N.	Gms. pc	pc of T.N.	Gms. pc	pc of T.N.
Scorbutic	1	3.01	0.2048	6.81	0.1890	6.28	0.0152	0.505
	2	2.90	0.6039	20.80	0.3587	12.38	0.0231	0.796
	3	3.00	0.2347	7.82	0.3940	13.12	0.0173	0.576
M=		2.97	0.3478	11.81	0.3139	10.59	0.0185	0.626
Normal	1	3.28	0.6078	18.50	0.3370	10.29	0.0142	0.433
	2	2.72	0.3254	11.93	0.2790	10.26	0.0176	0.648
	3	2.88	0.5470	19.00	0.4650	16.14	0.0190	0.660
M=		2.96	0.4934	16.48	0.3603	12.23	0.0169	0.580
pc dev. fm. Norm.			-29.51		-12.85		+9.47	

[1]Determined on undiluted suspension

[2]Determined, with ammonia nitrogen, on undiluted suspension

[3]Determined on tungstic acid filtrates

[4]Determined on tungstic acid filtrates and subtracted from value for amino acid nitrogen plus ammonia nitrogen.

Examination of Table XIII will show that the values for total nitrogen have the same averages. Of the total non-protein nitrogen fraction (tungstic acid filtrate) only the ammonia nitrogen was higher in the scorbutic animals, in contrast to the amino acid nitrogen and total non-protein nitrogen which were significantly lower in the scorbutic samples.

The addition of large quantities of L-tyrosine to the basal diet of the guinea pig has been shown to increase the vitamin C requirement of the animal (147). The values obtained from the guinea pigs which received the 0.375 g. daily of L-tyrosine show a wide range for both normal and scorbutic animals in the total non-protein nitrogen, amino acid nitrogen and ammonia nitrogen. There is also an overlapping of the values between the normal and scorbutic groups. The range and overlap are greater with these values than with those from less acute scurvy, as shown in subsequent experiments.

After the above experiment was performed, a second group of animals, Series 1, was started on the usual vitamin C-deficient regimen. These animals did not receive the L-tyrosine supplement, but did receive supplements of vitamins A and D and brewer's yeast as described. On this type of diet, the guinea pigs survived for three or four weeks. At the end of this time, however, they had developed the typical symptoms and their weights had fallen to the level of about 250 g. This is considered the typical course of an acute scurvy developed because of a vitamin C deficiency alone. In subsequent writings

when an "acute" scurvy is described, it was developed in this way.

The data from the Series 1 experiment are presented in Table XIV, which follows:

Table XIV

Effect of Acute Vitamin C Deficiency on the Nitrogen Constituents of Guinea Pig Muscle; Series 1

Sample		Total Nitrogen[1]	Amino Acid Nitrogen[2]		Non-Protein Nitrogen[3]		Ammonia Nitrogen[4]	
		Gms. pc	Gms. pc	pcof T.N.	Gms. pc	pcof T.N.	Gms. pc	pcof T.N.
Scorbutic	1	3.12	0.3859	12.37	0.3602	11.53	0.0179	0.574
	2	3.17	0.4218	13.31	0.3416	10.78	0.0171	0.539
	3	3.66	0.4093	11.18	0.4125	11.28	0.0139	0.380
M=		3.32	0.4057	12.29	0.3714	11.19	0.0163	0.498
Normal	1	3.26	0.1962	6.02	0.3410	10.46	0.0189	0.580
	2	3.43	0.3856	11.22	0.3573	10.41	0.0204	0.595
	3	3.43	0.4040	11.78	0.3597	10.49	0.0230	0.677
M=		3.37	0.3286	9.67	0.3527	10.45	0.0208	0.617
pc dev. fm. Norm.		-1.48	+23.45		+5.30		-21.60	

[1] Determined on undiluted suspension

[2] Determined, with ammonia nitrogen, on undiluted suspension

[3] Determined on tungstic acid filtrates

[4] Determined on tungstic acid filtrates and subtracted from value for amino acid nitrogen plus ammonia nitrogen.

A third experiment, Series 2, in which guinea pigs were exposed to the typical scorbutogenic regimen described just above, was carried out. These data are reported in Table XV, and because the conditions of the experiment were the same as those reported in Table XIV, the data from both experiments will be discussed together. In consideration of these data, however, it will be noticed that the results are not identical. The values for total nitrogen in the normal animals in Table XV are much lower than values of this kind usually are (the average is about 3.00 gms. for normal animals). Reasons for this discrepancy are not apparent.

The values for amino acid nitrogen, total non-protein nitrogen and ammonia are in the same direction although the degrees of increase and decrease vary somewhat. With the exception of the unusual total nitrogen value for the normal animals in Table XV, the results from both the experiments in which the L-tyrosine was omitted are in direct contrast to those from the experiment in which the guinea pigs received 0.375 g. L-tyrosine daily. In the latter, the amino acid nitrogen and total non-protein nitrogen were significantly lower in the scorbutic animals while in the former they were higher in the scorbutic. With 0.375 g. L-tyrosine added daily, the scorbutic animal tissue gave almost 10 per cent more ammonia nitrogen compared with values obtained when no extra tyrosine was added. In the experiments in which no tyrosine was supplemented the normal

tissue was found to have 21.60 per cent and 61.50 per cent more ammonia nitrogen than the scorbutic tissue.

Table XV

Effect of Acute Vitamin C Deficiency on the Nitrogen Constituents of Guinea Pig Muscle; Series 2

Sample		Total Nitrogen[1]	Amino Acid Nitrogen[2]		Non-Protein Nitrogen[3]		Ammonia Nitrogen[4]	
		Gms. pc	Gms. pc	pc of T.N.	Gms. pc	pc of T.N.	Gms. pc	pc of T.N.
Scorbutic	1	3.14	0.2711	8.64	0.4118	13.10	0.0102	0.325
	2	2.91	0.2442	8.39	0.3972	13.63	0.0105	0.361
M		3.03	0.2577	8.51	0.4045	13.37	0.0104	0.343
Normal	1	2.75	0.2327	8.47	0.3096	11.27	0.0266	0.968
	2	2.10	0.2673	12.71	0.3361	16.00	0.0273	1.300
M		2.43	0.2500	10.59	0.3229	13.63	0.0270	1.13
pc dev. fm. Norm.		+24.65	+3.08		+25.25		-61.50	

[1]Determined on undiluted suspension

[2]Determined, with ammonia nitrogen, on undiluted suspension

[3]Determined on tungstic acid filtrates

[4]Determined on tungstic acid filtrates and subtracted from value for amino acid nitrogen plus ammonia nitrogen

It is interesting to compare the results from these three experiments by observing the degrees of range and overlap of the values. The very acutely scorbutic (L-tyrosine-supplemented)

animals in Table XIII show a wider range within each group and there is overlap in each determination. This is not so with the data from the animals which did not receive added tyrosine. Here the ranges were narrower. There was no overlap in the ammonia values in Table XIV, nor in the total nitrogen, total non-protein nitrogen and ammonia in Table XV.

The data from the tyrosine-supplemented guinea pigs, both normal and scorbutic, show a much higher degree of variation than the data from the guinea pigs in the other experiments. The more gradual onset of the deficiency is likely to be the typical scurvy; it is interesting to observe the effects of the added tyrosine, however, not only upon the rapidity with which the animals develop the symptoms and the severity of them, but upon some of the nitrogen constituents as well.

Values for histidine, tyrosine, carnosine and anserine were obtained from the same tungstic acid filtrates used for the amino acid nitrogen values shown in Table XV. When a comparison is made between these values and the amino acid nitrogen from experiments summarized in Tables XIV and XV, it can be seen that both histidine and tyrosine are found in greater quantity in the scorbutic animal tissue. The carnosine is lower, and the anserine tests were negative. The data for the two amino acids and two di-peptides are listed in Table XVI.

Table XVI

Effect of Acute Vitamin C Deficiency
on Some Constituents of the Amino Acid Nitrogen Fraction
of Guinea Pig Muscle; Series 2

Sample		Histidine		Tyrosine		Carnosine		Anserine	
		Gms. pc	N-pc of T.N.	Gms. pc	N-pc of T.N.	Gms. pc	N-pc of T.N.	Gms. pc	N-pc of T.N.
Scor-butic	1	0.0165	0.143	0.0407	0.100	0.0000	0.000	0.00	0.00
	2	0.0083	0.077	0.0353	0.094	0.0023	0.019	0.00	0.00
M		0.0124	0.110	0.0380	0.097	0.0023	0.019	0.00	0.00
Nor-mal	1	0.0021	0.021	0.0190	0.053	0.0152	0.139	0.00	0.00
	2	0.0029	0.037	0.0187	0.069	0.0104	0.125	0.00	0.00
M		0.0025	0.029	0.0189	0.061	0.0128	0.132	0.00	0.00
pc dev. fm.Norm.		+396.00		+101.00		-82.00			

Since histidine and tyrosine nitrogen are included in the amino acid nitrogen fraction, it is reasonable to expect that the values for them would follow the same trend. However, the value for amino acid nitrogen includes all the amino acids present, so the simultaneous increase of the two amino acids studied and the amino acid nitrogen in the same filtrate might be coincidental.

When carnosine was determined on chicken muscle in previous

experiments it did not follow the same trend as the other amino acids or anserine. In this instance it is at variance also. Although no anserine was found in the guinea pig muscles, it has been reported to be present in another herbivorous rodent, the rabbit (148,149,150).

As can be seen upon inspection of the data, the ranges of the values for each constituent within each group are quite narrow, with no overlapping of values from the scorbutic to the normal.

Following the three cited experiments in which results were obtained from a relatively few animals, a more extensive experiment, Series 3, was conducted. Not only were more animals utilized, but the emphasis was shifted toward a study of the products formed when protein molecules in the muscle tissue are acted upon by the enzymes present. This was carried out by means of the two precipitation methods described in previous sections.

Table XVII presents the data from this experiment. In the table will be found all the values obtained for a total of 16 guinea pigs; 8 of which developed the typical acute scurvy characterized above. This was a particularly gratifying experiment since all the animals developed the acute symptoms in about the same length of time, and their weight curves followed the general pattern illustrated in Figure 8 on which the weight changes of two typical members of this group are plotted.

Table XVII

Effect of Vitamin C Deficiency on Nitrogen Constituents of Guinea Pig Muscle; Series 3

Sample	T. N. Gms.pc	Non-Protein Nitrogen As Gms. pc		Non-Protein Nitrogen As pc of T. N.		Total Moisture pc	pH of Susp.
		Trichl.	Tungs.	Trichl.	Tungs.		
Scorb.							
1	2.404	0.2583	0.2417	10.74	10.05	84.32	6.68
6	2.309	0.2014	0.2346	8.72	10.16	74.94	6.94
2	2.467	0.2782	0.2287	11.28	9.27	87.29	6.75
5	2.324	0.2609	0.2151	11.22	9.25	65.40	6.80
3	2.249	0.1695	0.2192	7.54	9.75	84.94	6.74
4	2.281	0.1927	0.1939	8.45	8.50	79.15	6.75
11	2.421	0.1771	0.1429	7.32	5.90	79.32	6.96
9	2.249	0.1350	0.1813	6.00	8.06	79.42	7.06
M	2.338	0.2091	0.2071	8.95	8.87	77.39	6.84
Av.dev. fm. M%	2.970	20.32	12.51	18.32	11.66	7.19	1.68
Norm.							
3	2.953	0.3191	0.2971	10.80	10.06	75.65	6.30
5	3.055	0.3043	0.3008	9.96	9.85	77.57	6.20
2	3.100	0.3393	0.3103	10.94	10.01	77.68	6.21
4	2.943	0.3129	0.2514	10.63	8.54	81.24	6.21
1	3.009	0.3195	0.2549	10.62	8.47	80.00	6.54
6	2.980	0.2483	0.2900	8.33	9.73	77.16	6.69
12	2.982	0.3451	0.3012	11.57	10.10	78.14	6.60
7	3.136	0.3348	0.2934	10.67	9.36	76.20	6.51
M	3.019	0.3154	0.2873	10.43	9.51	77.96	6.41
Av.dev. fm. M%	1.920	6.40	5.97	6.27	5.73	1.77	2.77
Av.dev. fm.Norm. %	-22.55	-33.70	-27.91				

A study of these tabulated data for the nitrogen constituents shows the following outstanding facts: in all the scorbutic guinea pig muscle there was a lower value for total nitrogen than in the normal animals. In both the normal and scorbutic samples, the range of values of the total nitrogen within each group is quite narrow, and there is no overlapping of values between the groups. The per cents deviation from the means of the total nitrogen values are low enough to warrant belief that these figures represent real differences. The deviation is higher with the scorbutic samples than it is with the normal samples.

When considered as per cent of total nitrogen, the total non-protein nitrogen values show some variability within each group and the values overlap between the groups. However, if the absolute values are taken (as grams per cent of the fresh tissue) the range of variation within the normal groups is rather small and there is no overlapping of values between the normal and scorbutic. The extent of the variability, especially in the scorbutic samples, is shown further in the per cent of deviation from the mean. Whereas the normal samples are well within experimental error, the scorbutic samples have a much greater deviation.

On the whole, both the relative values (per cent of the total nitrogen) and the absolute values of total non-protein nitrogen are higher in the trichloroacetic acid filtrates than in the tungstic acid filtrates. This is true of both conditions.

For both types of filtrates the total non-protein nitrogen is appreciably lower in the scorbutic tissue than in the normal tissue. There is a greater decrease from the normal in total non-protein nitrogen from the trichloroacetic acid filtrates than in the tungstic acid filtrates.

In both normal and scorbutic tissue there is apparently a larger proportion of intermediary breakdown products than of the low molecular weight products formed. However, when the animal has a vitamin C deficiency, the production of the intermediate compounds is decreased to a greater extent than is the production of the final low molecular weight substances, as evidenced by the per cents deviation from the normal.

Although the per cent moisture was almost the same in both groups, the pH of the suspension from the scorbutic animals was significantly higher.

In the fourth series of tests, six guinea pigs were maintained on a diet which contained insufficient vitamin C for optimum health, but contained a quantity which prevented the animals from developing the symptoms which occur when the vitamin is absent entirely. These animals had unkempt fur, rapid breathing, and their growth curves fluctuated from time to time; however, the general trend was an increase in weight. They were on this type of diet for four months. It was discovered upon examination of the composition of the Purina Rabbit Chow which these particular animals received, that an unstated quantity of animal protein factor had been added to the

ration. These animals had some of the symptoms of a chronic vitamin C deficiency, with evidences of fragile bones in addition to the above characteristics. The animals with the true, acute scurvy had much more fragile bones, considerable hemorrhage, and the other symptoms described in the literature. The chronic animals described above are included in Table XVIII. Based on the grams per cent total non-protein nitrogen, the per cent increase or decrease compared with the normal are also listed, as well as the per cent deviations from the means.

The total nitrogen, and both types of total non-protein nitrogens are presented on the histograms, Figures 9 and 10. Figure 9 shows the acutely scorbutic animals, Series 3; while Figure 10 shows the chronic animals, Series 4.

The chronic scorbutic guinea pig muscle tissue was very slightly higher in total nitrogen than the normal, but it was nearly 60 per cent higher in total non-protein nitrogen obtained with the trichloroacetic acid procedure. The values for total nitrogen in both normal and scorbutic tissue were in a narrow range with a high degree of overlapping.

In contrast to the trichloroacetic acid filtrates, the total non-protein nitrogen average values from the tungstic acid filtrates were only 5 per cent lower in the scorbutic tissue. This is different from the values expressed similarly in Table XVII, Series 3, for the acutely scorbutic guinea pig muscle, in which there were decidedly lower quantities of non-protein nitrogen in both types of filtrates, as well as a very

Table XVIII

Effect of Vitamin C Deficiency on Nitrogen Constituents of Guinea Pig Muscle; Series 4

Sample	T. N. Gms.pc	Non-Protein Nitrogen As Gms. pc		Non-Protein Nitrogen As pc of T. N.		Total Moisture pc	pH of Susp.
		Trichl.	Tungs.	Trichl.	Tungs.		
Scorb.							
8	3.008	0.2926	0.2983	9.73	9.92	73.69	6.40
7	3.022	0.2354	0.3150	7.79	10.42	77.12	6.62
10	3.092	0.3252	0.2786	10.52	9.01	65.75	6.39
M	3.041	0.2844	0.2973	9.36	9.77	72.19	6.56
Av.dev. fm.M %	1.120	11.49	4.20	11.00	3.27	5.94	1.98
Norm.							
8	3.024	0.0863	0.3274	2.85	10.83	77.03	6.67
9	2.772	0.0880	0.2878	3.17	10.48	76.55	6.60
11	3.171	0.3604	0.3240	11.37	10.22	77.03	6.40
M	2.989	0.1782	0.3131	5.96	10.48	76.87	6.47
Av.dev. fm.M %	4.850	68.18	5.36	63.25	1.93	0.28	2.05
Av.dev. fm.Norm. %	+1.74	+59.60	-5.05				

much lower quantity of total nitrogen. The values in the trichloroacetic acid filtrates extend over an abnormally wide range, especially in the normal samples. This fact produces complete overlapping of the scorbutic values by the normal values. In contrast, the tungstic acid values fall in a rather narrow range, although there is a degree of overlapping.

These data from a chronic vitamin C-deficiency are more variable in most of the normal samples than in the scorbutic samples. This is the reverse of the situation found with the acute vitamin C-deficiency. However, except for the trichloroacetic acid values, which may be disregarded because of the very obviously unusual figures for samples 8 and 9, the per cents deviation from the means are within a reliable range.

On the following pages the histograms for both the acute and chronic experiments show the differences more graphically. Figure 9 shows the difference between the total nitrogens of the normal and acutely scorbutic animals, and the uniformity within each group is emphasized. It is possible also to observe the differences between the total non-protein nitrogens from the two types of filtrates.

Figure 10, the chronic group, does not illustrate the same degree of uniformity in the total nitrogen values, but the total non-protein nitrogen values are not too divergent when those for the trichloroacetic acid filtrates of samples 8 and 9 are disregarded.

In consideration of the chronic scorbutic tissue, the

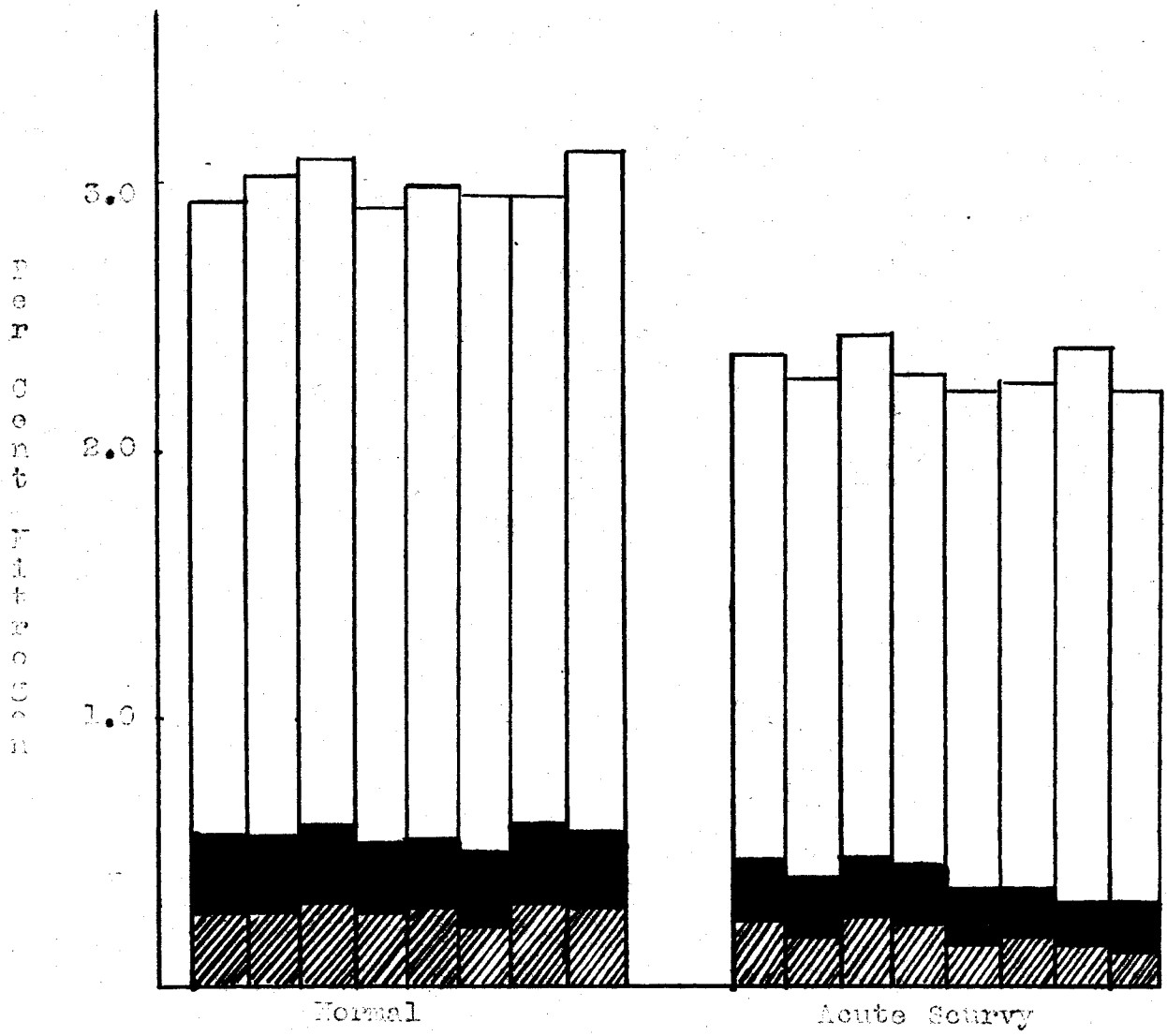


Figure 9. Total Nitrogen (clear columns), Total Non-Protein Nitrogen from Tungstic Acid Filtrates (solid columns) and Trichloroacetic Acid Filtrates (cross-hatched columns) from Normal and Scorbutic Guinea Pig Muscle; Series 3.

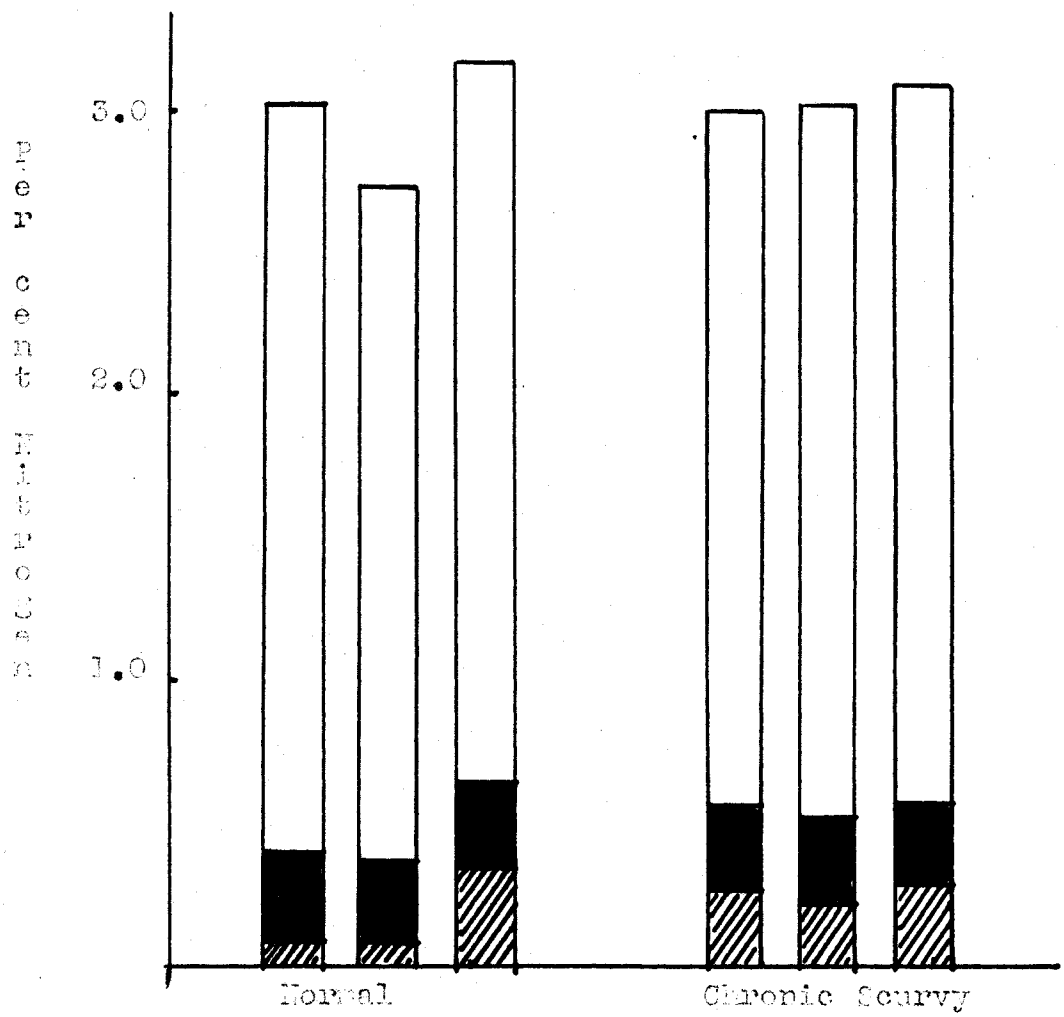


Figure 10. Total Nitrogen (clear columns) Total Non-Protein Nitrogen from Tungstic Acid Filtrates (solid column), and Trichloroacetic Acid Filtrates (cross-hatched column) from Normal and Scorbutic Guinea Pig Muscle; Series 4.

lesser number of cases must be borne in mind. The scorbutic tissues, though averaging slightly lower than the normal in total nitrogen, might not have done so if there had been more cases. The consistency within the three scorbutic cases, however, is striking, not only in the total nitrogen, but in the two fractions of it. Whereas there is little variation in the tungstic acid filtrates, either inter- or intra-groups, the trichloroacetic acid filtrates (containing the intermediates) is much lower in two out of three cases of the normal animals.

It should be emphasized that because these animals were on the experiment so much longer than the acutely scorbutic guinea pigs, the factor of age might conceivably play a part in these findings, as it has been known to do in other instances (105).

Figure 11 is a photomicrograph of a section[1] of vitamin C-deficient guinea pig thigh muscle. Some fragmentation of the striated muscle fibers is evident. The individual fibers appear to have become swollen and massed together. There is the appearance of the infiltration of fatty tissue into the areas where the muscle fiber has become fragmented.

[1]The author is indebted to Dr. Robert Getty of the Department of Anatomy, Veterinary Medicine Division of Iowa State College, Ames, Iowa, for the preparation of the histological sections and the criticism thereof.

Figure 11. Photomicrograph of longitudinal section of scorbutic guinea pig thigh muscle. 600 X

2. Vitamin E deficiency

a. Animals and diet. Male albino New Zealand rabbits weighing about 1 kg. were available for these experiments. They were paired according to starting weights by employing a random system which resulted in each group having an approximately equal average weight. Each rabbit was housed in an individual cage which allowed him ample room to move about. The cages were stainless steel with wire bottoms; low, crockery food and water dishes were supplied. The rabbits were weighed every other day until the experimental animals began markedly to lose weight, after which they were weighed each day. The weights of the two rabbits graphed in Figure 12 show relatively more gains and losses than the normal and scorbutic guinea pigs. However, the normal rabbit tends to gain more and the dystrophic one drops rather suddenly. It is this sudden drop which serves as one valuable criterion for the onset of the dystrophic condition.

Preliminary experiments had led to the selection of a diet that was superior for the purpose. The rabbits ate it reasonably well, although there was considerable wastage of the food. This necessitated putting small quantities of the food in the dish several times each day. The food and water were supplied ad libitum. The constituents of the diet, with one exception, were those published by Mattill and co-workers (93), with a special salt mixture, high calcium and low phosphorus, as recommended by Mattill and originally published by Hubbell,

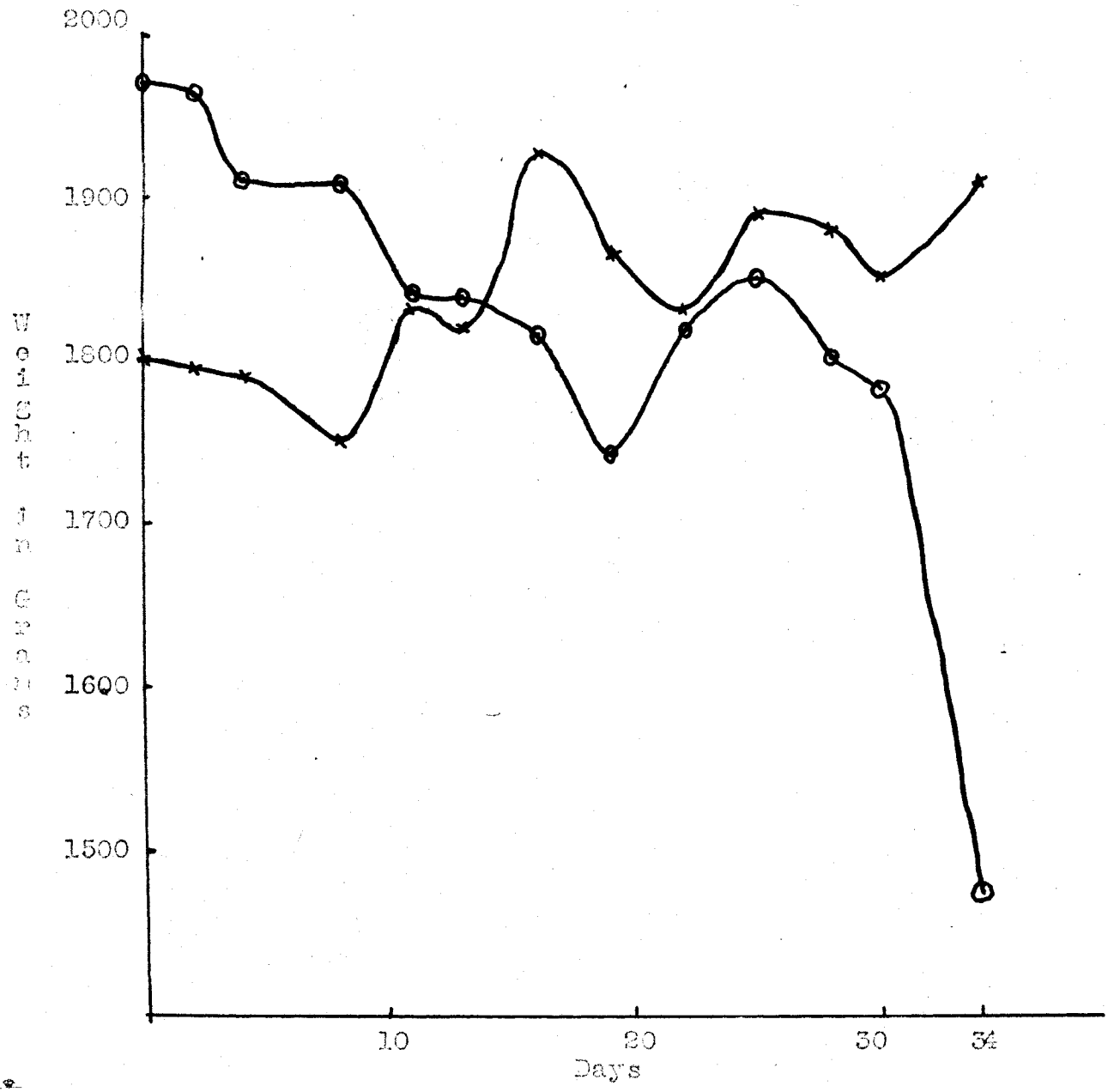


Figure 12. Typical Weight Curves of Normal (x——x) and Dystrophic (o——o) Rabbits.

Mendel and Wakeman (151). The diet constituents were:

Cellophane[1]	20 parts	Lard (E-free)	3 parts
Casein	15	Cod liver oil	3
Sucrose	10	Brewer's yeast	10
Dextrin[2]	36	Salt mixture	3

This diet was prepared in approximately 4 kg. quantities every four or five days. It was stored in a clean, dry container with a tight cover to prevent any contamination from other vitamin E-containing food. It was not kept in the room with other feed or with the animals.

In the administration of this diet it was advisable to transfer the rabbits from the usual stock diet to the synthetic mixture by following this procedure (93):

For three days they received 3/4 ground Purina Rabbit Chow mixed with 1/4 synthetic diet; the next three days they received 1/2 ground Chow and 1/2 synthetic diet; and the final three days, 3/4 synthetic diet and 1/4 ground Purina Rabbit Chow. By this procedure they became accustomed to the diet both through appetite and gastrointestinal acceptance.

Previous experiments with other synthetic diets administered to rabbits had shown that these animals develop diarrhea very

[1]The cellophane was purchased in 10-pound lots from the Traver Corporation, Chicago, Illinois. It was received as long narrow strips commonly termed "excelsior". This had to be cut into short strips on a cutting board and then ground in a Wiley mill to a small wafer about 1/16th inch in diameter. These wafers were incorporated into the diet.

[2]The dextrin was substituted for the starch stipulated in the original diet.

readily on unnatural food, and soon refuse to eat any of it. This diet caused a minimum of diarrhea and, although the rabbits did not relish it too much, they did eat it and no cases of complete refusal were observed.

To this basal diet was supplemented 5 drops of a vitamin A and D concentrate (oleum percomorphum, 50 per cent, Meads) each week. This provided the animals with 6,250 International Units of vitamin A, and 900 International Units of vitamin D, per week. The control rabbits were given 0.2 ml. of α-tocopherol in ethyl laurate *per os* with a syringe and blunt needle each day. This supplied 3.6 mg. of α-tocopherol daily, an amount sufficient to provide adequately for the animal's requirements under the conditions of this experiment. The food dishes were removed from the control animals at least two hours before the supplement was administered and not returned until at least two hours afterward. This eliminated the danger of the α-tocopherol becoming mixed with the contents of the cecum, and thus being acted upon by the rancid fats of the diet (152,153).

Since these animals were fairly large at the outset of the experiment, it took four to five weeks to bring on the complete dystrophic condition. When the animals start to lose weight they must be watched carefully for other symptoms. It was noted that the animals ate their food until they were no longer able to move, a matter of hours before being sacrificed, so the factor of inanition can be ruled out in these experiments. Before the final stages of the dystrophy develop it can be noted that

the animal's eyes develop a strange, red-violet color instead of the natural bright pink. The animal sits in a hunched position and does not jump about readily. In these experiments the alacrity and ease with which the rabbit jumped from the weighing can into the cage were unfailing criteria of the condition of its muscles. As the deficiency progressed, the rabbit either made no attempt to jump out or hung helplessly over the door. The symptoms of marked diuresis noted by Morgulis and Spencer (73) were evident with these animals.

When the animal could no longer rise to a sitting position after being placed on its side it was considered to be dystrophic. This criteria was used with success by Mattill and his co-workers in their experiments with dystrophic rabbits (109, 111). In fact, it was imperative to sacrifice them at that time, because death would come suddenly at any time after this advanced stage was reached.

The animals were sacrificed, usually a control and an experimental together, as described for the guinea pigs. Because of the large quantity of tissue, samples were reserved for histological section and total moisture; the remaining fat-free tissue was put through the meat grinder and duplicate samples weighed from the resulting homogeneous mixture.

All the animals were autopsied after the muscle tissue was prepared. Except for the color of the blood, which was very dark and venous-like in the experimental rabbits, the gross appearance of the animal was normal. There was the usual quan-

tity of fatty tissue about the organs and dispersed throughout the muscle. The experimental rabbits were not emaciated as would have been the situation in starvation or other wasting disorders. Figure 14 illustrates the histological picture of a typical dystrophic muscle sample.

The analytical methods have been described.

b. Results. In Table XIX are presented all the data from the vitamin E-deficient and normal rabbits.

In vitamin E deficiency of rabbits it was found that there was little difference in the moisture content of the muscles from the different animals, and that the pH of the dystrophic samples was higher than the normal samples.

Of the nitrogen constituents, there were found to be, firstly, a slight lowering of the total nitrogen in the dystrophic samples; secondly, a 46.24 per cent deviation from the normal in the values for the total non-protein nitrogen determined with the trichloroacetic acid method; and, thirdly, a -10.81 deviation in the same constituent with tungstic acid precipitation. These deviations from the normal should be evaluated in the light of the average deviations from the means of these components. Inspection of Table XIX will show that these deviations are not unacceptably wide. In fact, with the normal rabbits the values are well under 10 per cent.

Since the average deviations from the means are not too great, it is reasonable to suspect that the differences found between the normal and dystrophic muscle tissue for total

Table XIX

Effect of Vitamin E Deficiency on Nitrogen Constituents of Rabbit Muscle

Sample	T. N. Gms.pc	Non-Protein Nitrogen As Gms. pc		Non-Protein Nitrogen As pc of T. N.		Total Moisture pc	pH of Susp.
		Trichl.	Tungs.	Trichl.	Tungs.		
Dystr.							
12	2.840	0.3645	0.2851	12.84	10.03	64.71	6.70
10	2.915	0.3614	0.3121	12.40	10.70	76.73	6.76
4	3.156	0.5036	0.3551	15.95	11.26	74.75	6.60
2	3.384	0.4747	0.4104	14.02	12.15	76.14	6.40
M	3.076	0.4260	0.3407	13.80	11.04	73.08	6.62
Av.dev. fm.M %	6.37	14.81	12.36	8.55	6.07	5.73	1.73
Norm.							
11	3.179	0.2606	0.3869	8.19	12.18	75.39	6.40
3	3.396	0.3306	0.4018	9.75	11.83	76.26	6.50
1	3.043	0.2828	0.3572	9.31	11.74	80.82	6.35
M	3.206	0.2913	0.3820	9.08	11.92	77.49	6.42
Av.dev. fm.M %	3.96	8.99	4.32	6.61	1.51	2.86	0.93
Av.dev. fm.Norm. %	-4.12	+46.24	-10.81				

nitrogen and total non-protein nitrogen by both precipitation methods are fairly reliable.

In the histogram, Figure 13, the individual rabbits are compared. There appears to be some variation in total nitrogen, more within the groups than between the groups. A contributing cause may be the fatty tissue found in the rabbit muscle.

There is less variation in the total non-protein nitrogen as determined on tungstic acid filtrates within the groups, but there is overlapping of values among the normal and dystrophic samples; in fact, the solid columns are somewhat uniform.

The trichloroacetic acid filtrates contained a greater quantity of total non-protein nitrogen in the dystrophic samples. This can be seen readily when the cross-hatched columns of the histogram are compared. None of the values for the normal samples is so great as the dystrophic samples; the highest grams per cent value for the normal is 0.3306 while the lowest for the dystrophic is 0.3614.

According to our knowledge of the types of compounds to be found in these two filtrates, the data indicate that the proteolytic enzymes in dystrophic muscle are more active in cleaving large protein molecules to form intermediate products. The production of the low molecular weight compounds is of approximately the same degree in both normal and dystrophic muscle, and in normal muscle there seem to be fewer of the large-type molecules produced.

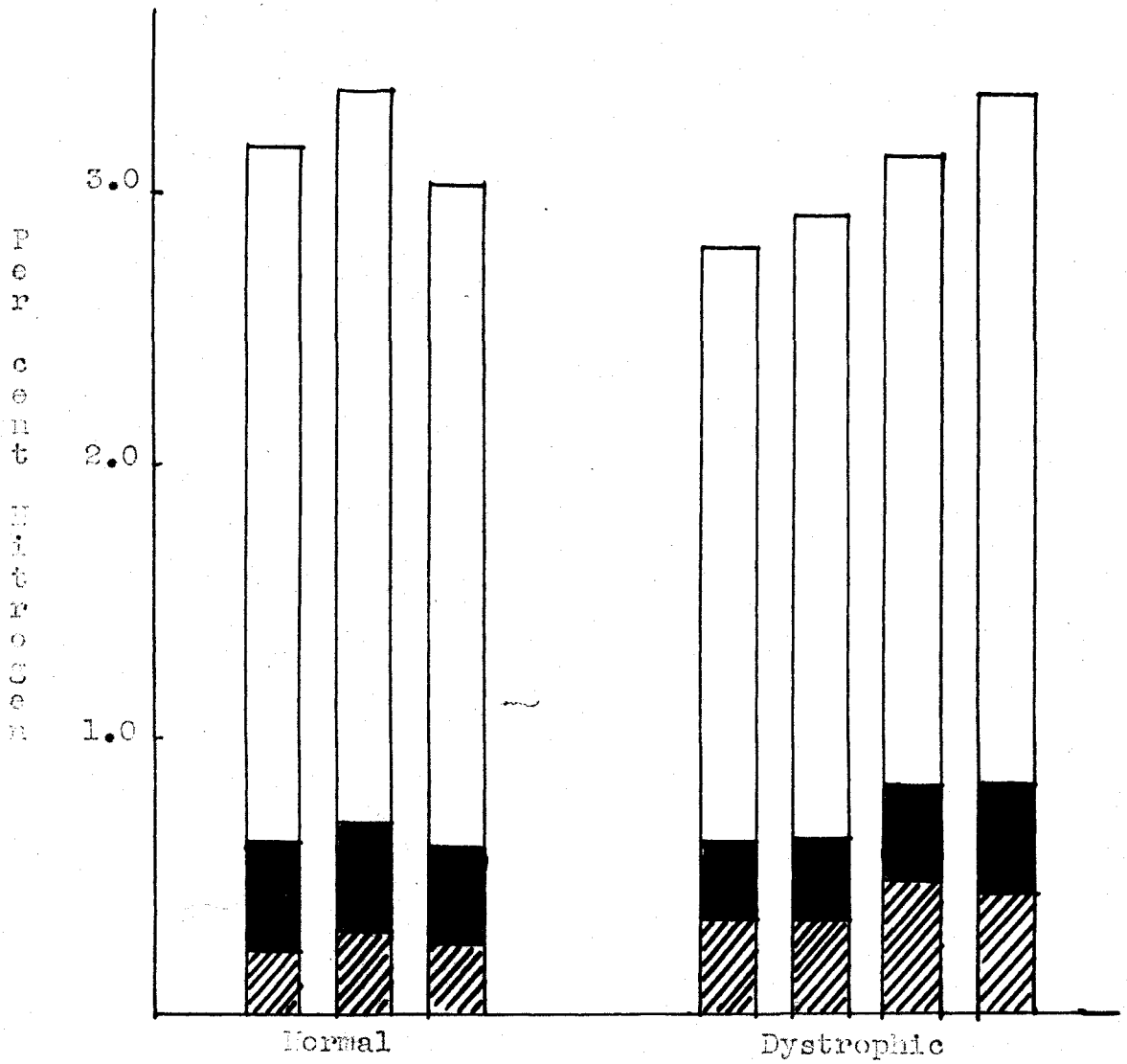


Figure 13. Total Nitrogen (clear columns), Total Non-Protein Nitrogen from Tungstic Acid Filtrates (solid columns) and Trichloroacetic Acid Filtrates (cross-hatched columns) from Normal and Dystrophic Rabbit Muscle.

Histological preparations of muscles from vitamin C- and vitamin E-deficient animals were made because in each case the striated muscle of the hind legs is affected in some way (63). In the living animal an acute deficiency of either vitamin C or vitamin E results in structural changes in the thigh muscles of such a nature that the animal cannot use them normally.

Figure 14 is a photomicrograph[1] of a section of dystrophic rabbit muscle. Some degeneration and fragmentation of the fibers is evident. There is a multiplication and irregular arrangement of the sarcolemma nuclei. The darkened areas in the fibers are believed to be invasion or replacement of the normal muscle tissue by connective tissue.

[1]The author is indebted to Dr. Robert Getty of the Department of Anatomy, Veterinary Medicine Division, Iowa State College, Ames, Iowa, for the preparation of the histological sections and the criticism thereof.

Figure 14. Photomicrograph of longitudinal section of dystrophic rabbit thigh muscle. 600 X

A comparison of the nitrogen constituents analyzed in this work indicates that there may be a difference in nitrogen metabolism in vitamin C and E deficiencies. For example, scorbutic muscle tissue contains less of the nitrogen compounds studied than the normal muscle does, and there is more variability in samples removed from vitamin C-deficient animals than from the normal ones. The nitrogen constituents in dystrophic rabbit muscle did not follow this pattern; the total non-protein nitrogen from the trichloroacetic acid filtrates of the dystrophic muscle was greater than the normal while the total nitrogen and tungstic acid total non-protein nitrogen followed the same trend as the vitamin C-deficient muscle, but not to the same extent.

When the average deviations from the means are calculated to facilitate a more reliable evaluation of the data, the greater variability in the guinea pigs can be seen, especially the fact that this variability is greater with the deficient samples. Although there is evidence of variability with the dystrophic (vitamin E-deficient) samples, it is not so great as in the vitamin C-deficient samples. It is interesting to note, however, that in the vitamin E-deficient samples there is more variability than in the normal samples from the same experiment. This is similar to the results obtained with the vitamin C experiment.

IV. DISCUSSION

Analyses have been made to determine what changes occur in the percentage composition of some of the nitrogen-containing compounds of skeletal muscle tissues subjected to various physical and physiological stresses. Because the proteolytic enzymes found in muscle tissue are in large measure responsible for these changes, the study resolved itself into one concerning the activities of these enzymes.

Certain physical influences have been studied in some detail. It is evident that when fresh muscle tissue is subjected to long periods of intense cold there is practically no enzyme action. The proteolytic enzymes seem to be moderately inhibited at temperatures below freezing; the chicken muscle experiments presented some evidence of a slow but gradually increasing activity. The enzymes which react with the large protein molecules to form intermediate-sized compounds in the tissue are more active at the lower temperatures as time increases, than were those enzymes which form smaller compounds. The condition of the muscle during cold storage, such as protection from dehydration and the intactness of the sample, may have some indirect effect upon the rate of activity of these enzymes.

When muscle tissue is subjected to temperatures which increase from below optimum to inactivating levels for the enzyme systems, it has been shown that there is the expected increase in enzyme activity, with the increase in temperature,

until the temperature reaches such a degree as to inactivate the systems (128). From a measure of the products formed under these conditions, it has become apparent that while all the proteolytic enzymes may be functioning at comparable rates in fresh untreated muscle, as the temperature is increased to a certain level these same systems increase in activity. However, the systems which split the large protein molecules directly are apparently more resistant to heat, and continue to operate after those which split off the lower molecular weight compounds have ceased to function. It is possible that these more resistant enzymes are also more general, and the less resistant enzymes more specific, in function.

When enzyme reactions in the muscle are controlled as carefully as possible through the means of incubation studies, it is not unreasonable to hope that the conditions nearly approximate those which occur in intact living muscle systems. The results of incubating samples at different temperatures have demonstrated that the enzyme systems responsible for the primary breakdown of the protein molecules have a wider temperature range of activity, produce a greater quantity of products, and are more resistant to high-temperature inactivation than are the enzymes which react upon the intermediate degradation products. On the other hand, those enzymes which split off compounds as the process of degradation is nearly at an end are more active over a much narrower range, but reach their peak of activity at about the same temperature as the less

specific group.

A further study of stresses which can be imposed upon muscle tissue involved those of a physiological nature. An insufficient intake of any of the dietary essentials for a particular species imposes a stress upon the metabolic processes of that species. "Insufficient" must be further qualified; if the animal should require a definite quantity of a dietary constituent, say vitamin C, for its usual metabolic functions, it is conceivable that it would require an additional quantity to cope with an unusual metabolic function in which vitamin C was concerned, directly or indirectly. Such an unusual metabolic situation is imposed upon the guinea pig when large quantities of L-tyrosine are fed. The inter-relationship of vitamin C and the aromatic amino acids has been demonstrated adequately (50,51,54,147), and there is some evidence that the level of vitamin C in the tissues will influence this inter-relationship (57). Data have been presented in the experiments at hand which point toward a further effect of different "tissue saturation levels" of vitamin C upon the metabolism of proteins and the component parts of proteins.

It must be emphasized that these data refer to skeletal muscle metabolism which is likely to present a different picture from such extremely active tissues as liver and kidney. However, muscle is a fairly active tissue, metabolically, and its biochemistry may correlate in some ways with similar activation studies in more active tissues. It has been demonstra-

ted that the proteolytic enzymes of muscle tissue are affected by the degree and severity of the vitamin C deficiency, ranging from an extremely acute situation such as was found under stress of added L-tyrosine, to the less acute picture produced by a longer period of insufficient intake of ascorbic acid.

One of the effects of added L-tyrosine upon the proteolytic enzyme systems is a greater production of ammonia in this type of tissue. It is possible that the added L-tyrosine necessitates an increased activity of the amino acid oxidases thus producing more ammonia. The decrease in amino acid nitrogen found may be also the result, in part, of the increased activity of these amino acid oxidases. In addition, the decrease in both amino acid nitrogen and total non-protein nitrogen might be the result of lowered activity of the enzymes which split large protein molecules directly into intermediates.

The 10 per cent greater quantity of total nitrogen in the acutely scorbutic guinea pig muscle tissue may be an indication of a retention of nitrogen by a tissue, overloaded with tyrosine, which is not supplied with sufficient vitamin C to metabolize the amino acid at the normal rate. It has been demonstrated (129) that the dynamic state of the constituents of the animal body would permit the hypothesis that large quantities of amino acids administered daily might be deposited in the muscle tissue, as well as in other tissues (47), and with an insufficient quantity of vitamin C, would tend to remain longer than normally.

In the less acute vitamin C deficiency, the analyses for nitrogen compounds present rather different results from the very acute condition discussed above. In this less acute situation, the animal not only requires a longer time to develop the same symptoms, but its enzyme systems apparently behave differently as well. There is a considerable diminution of ammonia production in the vitamin C-deficient tissue, with a concurrently greater production of such nitrogen compounds as the low molecular weight amino acids and similar cleavage products from protein. This is in direct apposition to hwat is found with very acute scurvy induced by the added L-tyrosine. It is quite possible that the time element plays a role in comparing these two degrees of vitamin C deficiency, since time has been demonstrated to influence the proteolytic enzyme activity in muscle tissue suspensions. It is not to be overlooked, however, that one cannot transfer too rigorously the data from living to those from dead tissues, although in supposedly "dead" tissue suspensions it was demonstrated that the autolytic enzymes were functioning. It is an interesting suposition that, with added time, the enzymes which produce these non-protein nitrogen compounds, just above ammonia in the catabolic scheme, are activated to a much greater extent than those which deaminate the amino acids.

When further attempts were made to characterize the enzyme systems affected by vitamin C deficiency, the differences in the degrees of the deficiency became even more apparent. Basing

conclusions upon the belief that by means of a careful selection of precipitating reagents the products and the enzymes producing them could be further elucidated, it is apparent from studies of acute vitamin C deficiency, Series 3, that many of the important proteolytic enzyme systems are inhibited.

This inhibition has manifested itself in several ways; one, there seems to be a very pronounced inhibition upon those systems which function in the whole catabolic scheme of protein metabolism; two, there is an even more pronounced inhibition of those enzymes which are believed to act directly upon the intact protein molecules; and, three, there is an indication that certain anabolic processes, too, are upset as evidenced by the decrease in muscle size and the chemical finding that vitamin C-deficient muscle contains 22.55 per cent less nitrogen than the normal.

The decrease in the production of catabolic products found when only the total non-protein nitrogen values are considered is not at variance with the values found when more specific groupings of the total non-protein nitrogen were analyzed in the acute vitamin C deficiencies presented in Series 1 and 2. Considering the specific groupings together as components of the total non-protein nitrogen, the over-all decrease is the same as that found with the trichloroacetic acid filtrates which include all protein degradation products. Therefore, it is possible to postulate that an acute vitamin C deficiency will result in an inhibition of many of the proteo-

lytic enzymes operating in muscle tissue, with perhaps a greater inhibition in those acting directly upon protein, either for anabolic or catabolic functions.

Certainly the decrease in non-protein nitrogen constituents themselves is substantiated by the findings of Christensen and Lynch (66) who describe a 7 to 13 per cent decrease in the glycine and glutamine contents of vitamin C-deficient guinea pig muscle. Since glutamine is known to serve as a neutral storage form for labile ammonia groups, these findings also substantiate the large decrease found in ammonia nitrogen in the vitamin C-deficient guinea pigs studied in Series 1 and 2.

It has been known for some time, certainly since the series of publications by Wolbach and his co-workers (64,65), that vitamin C-deficient guinea pigs were unable to synthesize such protein molecules as collagen. The appreciably lower quantity of total nitrogen in the scorbutic muscle tissue may in some way correlate with this failure to synthesize intercellular substances.

When nutritional muscular dystrophy, another type of physiological stress, was produced in rabbits some further enzymic implications were made possible. In the first place, that there was a very definite muscular involvement and a true muscular dystrophy was verified by the histological study which shows the infiltration of necrotic tissue so typical of this syndrome. From the chemical analyses of this vitamin E-deficient tissue have arisen some data which may aid in pointing

the way toward characterizing the effects of this deficiency upon the protein moiety of the tissue.

Dystrophic tissue was found to have less total nitrogen than normal tissue. It has been reported by Morgulis and Spencer (72,73), that the total nitrogen of dystrophic muscle was lower than normal. These authors and others (71,93) have found that dystrophic muscle contains, concurrently, a diminished quantity of creatine with a marked muscle degeneration observable on microscopic examination, and an increased urinary creatine excretion. These adequately-proven characteristics of vitamin E-deficient dystrophic muscle tissue should substantiate the present finding of the reduced quantity of total nitrogen in the muscle. It is further indicated by the lower quantity of the total non-protein nitrogen fraction which would contain such compounds. If it may be postulated that the lesser quantity of compounds of low molecular weight found in nutritional muscular dystrophy indicates an inhibition of the proteolytic enzymes, the activity of which results in the production of these compounds, some clue may be forthcoming to explain partially this well-known decrease in creatine.

The decreases in the low-molecular weight fractions found in both guinea pigs and rabbits with muscle degenerative nutritional deficiencies are not of the same order. It is quite true that they were not induced by the deficiency of the same vitamin, and this must be remembered. However, it is interesting to speculate upon some possible relation through the effects

upon the enzyme systems. Whereas it was found that the vitamin C-deficient muscle indicated considerable inhibition of the enzymes producing low-molecular weight fragments from protein, the vitamin E-deficient muscle indicated some inhibition, though not so great. In her study with nutritional muscular dystrophy induced by vitamin E deficiency in both guinea pigs and rabbits, Roderuck (111) found the same relationship.

An anabolic implication from the decreased quantity of low-molecular weight nitrogen-containing fragments is possible when one considers the finding of Barber, Basinski, and Mattill (112), who reported the activity of transaminase to be less than half the normal in dystrophic guinea pigs, and somewhat less in dystrophic rabbits. An inhibition of this important enzyme should result in a decreased production of amino acids from α-keto acids. This again substantiates the greater decrease found in the total non-protein nitrogen fraction in scorbutic guinea pig muscle than in dystrophic rabbit muscle, although the species and vitamin differences must be emphasized.

In complete contrast to the vitamin C-deficient guinea pig muscle, the vitamin E-deficient rabbit muscle indicated an increased activity of those proteolytic enzymes which react directly with protein molecules. It has been demonstrated many times (91,93) that vitamin E-deficient dystrophic muscle tissue has an increased oxygen consumption and a higher rate of respiration than normal tissue. These conditions are believed to result in a greater evolution of energy as heat. Since heat

has been shown, in the incubation studies, to increase the activity of proteolytic enzymes, it is possible that the greater production of heat in dystrophic tissue may account for the increased production of protein intermediates.

It is true that vitamin E-deficient muscle tissue from other species, including the guinea pig and hamster (93,94,96) also has a high oxygen consumption, so that it might be expected that the above effects upon the muscle proteins and enzymes would occur in conjunction with the increased Q_{O_2}. This may comprise one of the differences in the muscle involvements induced by the deficiencies of two different vitamins in two different species.

It has been shown in this work that the pH of the vitamin-deficient muscle is higher than the normal. Since the pH optima of the proteolytic enzymes under major consideration here are not known definitely, it is not possible to speculate upon the effects of these pH changes upon their activities. Nevertheless, it is interesting to try to explain these consistent increases in pH in the affected tissue. It is known (99,100) that there is an increase in calcium in dystrophic rabbit muscle, and this offers one possibility for the increase in pH. On the other hand, it has been demonstrated, too, that dystrophic muscle is not so efficient in removing such compounds as oxalacetic acid from the system (112), and an increase in alkaline products may be necessitated to neutralize an increase in acid products, either formed or accumulated.

Unfortunately, these proteolytic enzymes and their activities as speculated upon cannot be characterized completely at the present time. They may be related to the kathepsins I, II, III, and IV upon which Bergmann (126,127) has worked so extensively. At least these enzymes are known to have rather definite functions, as demonstrated by their behavior in the presence of Bergmann's synthesized molecules (126). The kathepsins I and II are believed to be homospecific with pepsin and trypsin; while components III and IV are homospecific with aminopeptidase and carboxypeptidase, respectively. This does not imply that these enzymes are identical, only that their specificity requirements are similar. From the measurements made of the quantities and relationships of the types of protein degradation products likely to be formed by these enzymes, it has been possible to make tentative deductions about the effects of various environmental influences upon these enzymes and their activities in skeletal muscle tissue.

V. SUMMARY

A comprehensive survey of the literature concerned with the effects of various physical and physiological stresses upon the nitrogen constituents of striated muscle has been presented. An effort has been made to increase the knowledge of this subject by performing experiments designed to show the influence of time in cold storage, heat under pressure, and autolysis on muscle tissue. Lastly, and of prime import, the effects of such physiological stresses as deficiencies of both vitamins C and E upon the nitrogen-containing components of striated muscle tissue have been studied.

When samples from five different beef muscles were excised and stored at 34°F. for periods ranging from 1 day to 30 days, no changes were found in the total nitrogen of the homogenized tissue, in the total non-protein nitrogen, in the amino acid nitrogen, nor in the ammonia nitrogen as determined upon protein-free filtrates.

Pectoralis major muscles from chickens were analyzed after storage in the intact, but eviscerated, bird at 32°F. for 0 to 48 hours. The total nitrogen data are in excellent agreement from 0-hour to 48-hour samples and the values show no change over the period of time studied. The non-protein nitrogen fractions determined on tungstic acid filtrates increased with time of storage, as illustrated below, as per cent of the total nitrogen:

Amino acid nitrogen	10.89	to	11.00
Ammonia nitrogen	0.414		0.449
Total non-protein nitrogen	12.90		14.10
Histidine nitrogen	0.0249		0.0521
Tyrosine nitrogen	0.0236		0.0354
Carnosine nitrogen	1.87		2.53
Anserine nitrogen	0.00		2.58

The poor agreement and fluctuating values obtained for the anserine nitrogen nullify the apparent increase.

A comparison between the total non-protein nitrogen in filtrates prepared with either tungstic acid or trichloroacetic acid showed that the trichloroacetic acid filtrates contained the greater quantity of total non-protein nitrogen at all times, from 0-hour to 48-hours. There was a greater increase in the total non-protein nitrogen from the 0-hour to the 48-hour period in the trichloroacetic acid filtrates than in the tungstic acid filtrates.

Commercially processed samples of muscle meats obtained at the local markets were analyzed for total nitrogen and total non-protein nitrogen in both trichloroacetic acid and tungstic acid filtrates. This study involved the effect of processing temperatures upon these constituents and showed the variation that can occur in the total nitrogen in such materials. It showed, also, that the total non-protein nitrogen values from the trichloroacetic acid filtrates were higher than the total non-protein nitrogen values from tungstic acid filtrates.

A 5 per cent suspension of the pectoralis major muscles of the chicken was held under carefully controlled conditions of pH and temperature. After 0-hour, aliquots were removed for total nitrogen and for preparing the trichloroacetic acid and tungstic acid filtrates, and the suspension was incubated in order to study the enzymatic changes which would occur. Experiments showed that pH 4.00 and a temperature of 40°C. gave optimum activity as measured by an increase in the production of total non-protein nitrogen. Aliquots were removed after 2 hours, 4 hours and 6 hours incubation.

The total non-protein nitrogen values obtained at 0-hour from the 2 filtrates are in close agreement. As time increases, the values become increasingly divergent, with the total non-protein nitrogen from the trichloroacetic acid filtrates increasing to the larger values. For example, the total non-protein nitrogen, as per cent of total nitrogen in the trichloroacetic acid filtrates at 0-hour averaged 14.85; at 6 hours, the average was 17.23. The total non-protein nitrogen, as per cent of total nitrogen, in the tungstic acid filtrates at 0-hour averaged 13.96; at 6 hours the average was 16.46.

The probable significance of these two filtrates is discussed and may be summarized briefly as follows: the tungstic acid filtrates are believed to contain only the low-molecular weight breakdown products of protein; the trichloroacetic acid filtrates contain, in addition, the intermediary products of protein breakdown. Hence, a greater increase in the total non-

protein nitrogen from the latter filtrate indicates a greater increase in the production of the intermediary products of protein breakdown under the conditions employed and described.

Guinea pig experiments were conducted to determine the effects of a dietary vitamin C deficiency upon the nitrogen constituents of muscle. A single experiment, in which L-tyrosine was supplemented, was followed by a series of experiments in which the animals were placed on a typical vitamin C-free regimen.

The first group contained guinea pigs which had been brought to a state of very acute vitamin C deficiency by incorporating 0.375 g. of L-tyrosine into the diet daily. They developed symptoms of a very severe scurvy in approximately one week. Examples of the results are: total nitrogen in the scorbutic muscle, 2.97 g. per cent, normal muscle, 2.96 g. per cent; total non-protein nitrogen from tungstic acid filtrates (expressed as per cent of total nitrogen), scorbutic, 10.59, normal, 12.23.

The first experiment of the series contained guinea pigs which had been brought to a state of typical acute scurvy by feeding ground, aerated Purina Rabbit Chow without added vitamin C. Examples of the results are: total nitrogen in the scorbutic muscle, 3.32 g. per cent, normal muscle, 3.37 g. per cent; total non-protein nitrogen from tungstic acid filtrates (expressed as per cent of total nitrogen), scorbutic, 11.19, normal, 10.45.

Series 2 contained guinea pigs brought to the typical deficiency state. Examples of the results are: total nitrogen in the scorbutic muscle, 3.03 g. per cent, normal muscle, 2.43 g. per cent; total non-protein nitrogen from tungstic acid filtrates (expressed as g. per cent of total nitrogen), scorbutic, 13.37, normal, 13.63. Also, on series 2 the following compounds were determined on the tungstic acid filtrates (expressed as nitrogen as per cent of total nitrogen): histidine, scorbutic muscle, 0.110, normal muscle, 0.029; tyrosine, scorbutic, 0.097, normal, 0.061; carnosine, scorbutic, 0.019, normal, 0.132; anserine, negative results on both normal and scorbutic muscle.

A more extensive experiment, series 3, contained guinea pigs brought to the typical deficiency state. The muscle tissue from both the normal and scorbutic animals was employed to prepare non-protein nitrogen filtrates by both trichloroacetic acid and tungstic acid. The total nitrogen in the scorbutic muscle averaged 2.338 g. per cent, normal muscle, 3.019 g. per cent. The total non-protein nitrogen values found were as follows (as per cent of total nitrogen): trichloroacetic acid filtrates, scorbutic, 8.95, normal, 10.43; tungstic acid filtrates, scorbutic, 8.87, normal, 9.51.

Series 4 was composed of guinea pigs which developed a state of chronic scurvy, and upon which a comparison of the total non-protein nitrogen found in trichloroacetic acid filtrates and tungstic acid filtrates was made. The values for total nitrogen in the scorbutic muscle were 3.041 g. per cent

normal muscle, 2.989 g. per cent. The values obtained from the filtrates are as follows (as per cent of total nitrogen): trichloroacetic acid filtrates, scorbutic, 9.36, normal, 5.96; tungstic acid filtrates, scorbutic, 9.77, normal, 10.48.

The data from Series 3, Table XVII, indicate that in the scorbutic guinea pig muscle there are no protein intermediates produced, while in the normal samples these compounds of relatively high molecular weight were present.

Vitamin E deficiency was produced in rabbits by means of a synthetic diet containing no vitamin E. Typical dystrophy of muscle was attained as demonstrated by gross symptoms and histological examination. A comparison was made between the normal and dystrophic muscle of the total nitrogen in the muscle and of the total non-protein nitrogen found in both the trichloroacetic acid filtrates and tungstic acid filtrates. Examples of the data found are as follows: total nitrogen, dystrophic, 3.076 g. per cent; normal, 3.206 g. per cent. In the filtrates, these data may be cited: trichloroacetic acid filtrates (as per cent of total nitrogen): dystrophic, 13.80, normal, 9.08; tungstic acid filtrates (as per cent of total nitrogen): dystrophic, 11.04, normal, 11.92.

The results presented in Table XIX show that, in dystrophic rabbit muscle, there is a greater quantity of protein intermediates of high molecular weight than in the normal muscle, in which a larger quantity of the protein end products of smaller molecular size is found.

The pH of the guinea pig muscle and rabbit muscle homogenates was determined and showed that in every case the pH of the affected muscles was higher than the normal muscles as illustrated by the following averages: samples taken from the acutely scorbutic guinea pig muscle gave a pH of 6.84; normal, 6.41; from the dystrophic rabbit muscle, pH 6.61, normal 6.42. In every case the pH of the normal sample was lower than the pH of the deficient sample.

The common denominator linking vitamin C deficiency in the guinea pig and vitamin E deficiency in the rabbit in these experiments was the muscle degeneration. When total nitrogen values were compared, it was found that in Series 3 of the guinea pig experiments, the most significant and extensive experiment, the deficient muscle samples were lower than the normal muscle samples. The deficient muscle samples from the dystrophic rabbits gave lower values for total nitrogen as well. In scorbutic guinea pig muscle no protein intermediates were found, while in the dystrophic rabbit muscle there is a greater quantity of these compounds present. In normal guinea pig muscle the protein intermediates were present, but in normal rabbit muscle there is a larger quantity of small molecular size end products. For example, the values from scorbutic muscle were 0.2091 g. per cent for both the high and low molecular weight compounds; and 0.2071 g. per cent for only the low molecular weight compounds. The values for the normal muscle were 0.3154 g. per cent for both the high and low

molecular weight compounds and 0.2873 g. per cent for only the low molecular weight compounds.

The values obtained from the dystrophic muscle were 0.4260 g. per cent for both the high and low molecular weight compounds and 0.3407 for the compounds of smaller molecular size. Normal rabbit muscle contained 0.2913 g. per cent of both the high and low molecular weight compounds while 0.3820 g. per cent of the low molecular weight compounds was found.

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