THE RELATION OF FIRMNESS TO CERTAIN OTHER CHARACTERISTICS OF BEEF MUSCLE

PART I. VARIATION IN THE PHYSICAL STRUCTURE OF THE LONGISSIMUS DORSI MUSCLE AT THE TWELFTH RIB AND ITS RELATIONSHIP TO FIRMNESS AND TENDERNESS

PART II. THE RELATIONSHIP OF OBJECTIVELY MEASURED FIRMNESS AND TENDERNESS TO THE PHYSICAL STRUCTURE OF THE LONGISSIMUS DORSI

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May, 1964

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ACKNOWLEDGMENTS

The writer wishes to express appreciation to his major advisor, Dr. L. E. Walters of the Department of Animal Husbandry, for his assistance and many helpful suggestions during this investigation and in the preparation of this dissertaion. Also, the author wishes to acknowledge the guidance and encouragement given by Dr. L. M. Henderson, Head of the Biochemistry Department, during the chemical phases of this study. Furthermore, the writer wishes to acknowledge the assistance given by Dr. J. V. Whiteman of the Department of Animal Husbandry for the guidance given in the statistical analysis.

He further extends appreciation to his wife, Barbara, for her encouragement during this study and for typing this dissertation.

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INTRODUCTION

The term quality, when used to describe fresh meat, generally refers to the character of the lean and fat, as well as the maturity of the animal from which a carcass or cut is derived. For example, the "quality" of the lean in a beef carcass is determined by a subjective evaluation of the color, texture, firmness, and marbling in the longissimus dorsi (ribeye) muscle. In an effort to facilitate marketing and to minimize the variation in the palatability of cuts from beef carcasses, the United States Department of Agriculture (1956) places considerable emphasis on the "quality" attributes of the ribeye. Within a particular carcass grade, the standards for each of the quality factors differ for each degree of maturity. However, within a maturity group minimum requirements are set forth for each quality factor in such a way that one quality factor alone may be responsible for downgrading a beef carcass if this quality attribute is below the minimum requirement for the grade. An example is the minimum requirement for firmness of the longissimus dorsi from U.S. Choice carcass beef as stated by the Official United States Standards for Grades of Carcass Beef: "Regardless of the extent to which other factors may exceed the minimum requirements for the grade, carcasses whose flesh is moderately soft and slightly watery are not eligible for the Choice grade" (United States Department of Agriculture, 1956).

During recent years, there has been an increase in the per capita consumption of beef. Accompanying this increase is a demand for beef

with uniform palatability characteristics. Cover et al. (1958) have reported a wide variation in the tenderness, flavor, and juiciness of cooked meat from beef carcasses within the same grade. Consequently, many questions have arisen as to the actual influence which each "quality" factor may have on the ultimate acceptability of a cooked steak or roast.

Marbling is one of the quality factors which has been studied rather extensively. The results of such investigations indicate that marbling has the following relationships with cooked beef: 1. marbling is positively associated with tenderness, when the muscles are from the same anatomical location, and when the carcasses differ widely in grade. However, an increase in marbling may not necessarily increase the tenderness of comparable muscles (carcasses of the same grade), and 2. marbling is positively correlated with the flavor and juiciness of cooked beef.

Studies relating to the importance of texture are few in number. However, Ramsbottom et al. (1945) and Brady (1937) have reported that "finer textured" beef muscle is more tender than "coarse textured" muscle.

A bright "cherry-red" color has been associated with "high quality" beef, although the color will vary according to the chronological age and physiological condition of an animal at the time of slaughter. A common problem in this area is the abnormally dark-colored ribeyes ("dark cutters") occurring in high grading beef carcasses. Among the most recent investigations in this area are those reported by Hedrick et al. (1959). These authors found no significant difference between the eating qualities of ". . . dark and bright beef from animals of the same approximate age and degree of finish." They concluded that, "If the housewife could be assured that the undesirable appearance of dark-cutting beef was not

due to deterioration, she could purchase dark beef with the same assurance of eating satisfaction as if she purchased bright beef."

Although firmness has been associated with the "quality" of carcass beef for many years, few studies have been conducted to establish the exact nature of this relationship. Using a subjective scoring method for beef firmness evaluation, Kropf and Graf (1959) found firmness to be positively correlated with tenderness and fat content of the longissimus dorsi muscle at the twelfth rib. Objectively measured firmness has been reported to be positively associated with juiciness and with the fat content of beef rib steaks (Pilkington, 1960). This latter study suggested, however, that when the fat content was held constant, the softer steaks were more tender.

The purposes of this study were to investigate: (1) variation in the physical structure of the longissimus dorsi muscle at the twelfth rib and its relationship to firmness and tenderness, and (2) the relationship of objectively measured firmness and tenderness to the physical properties of the longissimus dorsi muscle.

REVIEW OF LITERATURE

Meat is commonly defined as the edible flesh from animals used for food. The lean portion is composed of approximately 70 percent moisture, 20 percent protein, 1.0 percent ash, and the remainder is fat. According to Neuman and Logan (1950b), and Walls (1960), connective tissue accounts for 3.0 to 30.0 percent of the total protein. This variation is due to the location of the muscle in the body, as well as the location along the muscle from which the sample is obtained. To exemplify more fully the relationship between the various parts within a muscle system, a brief description of the microanatomy of a muscle is presented.

The carcass is composed of four basic types of tissue: epithelial, muscle, connective, and nervous. These tissues are differentiated according to their morphology and specialized functions. The specialized functions ascribed to each type of tissue are as follows: 1. epithelial - protection, absorption, and secretion; 2. muscle - conduction and contraction; 3. connective - support and 4. nervous - irritability and conduction. Each tissue is composed of cells, intercellular substance (formed and amorphous), and fluid. In meat science, one of the primary areas of research is concerned with skeletal muscle. A muscle system is composed of a mixture of muscle fibers and connective tissues. (Ham, 1957). It has been estimated that skeletal muscle comprises 40.0 percent of the body weight.

Embryologically, skeletal muscle arises from a particular area in the mesoderm, the myotome. (Patton, 1948). Boyd (1960) states that,

"A study of the histogenesis of muscle leads one to consider that in the embryo it is formed in two components. There is first the cell of the mesenchymatous background which becomes the fibrous tissue of the muscle and probably gives origin to the fibroblastic cells and their connective tissue derivatives are directly continuous with the tendon, where there is one. The other element is the contractile one, and is formed by the primary myoblasts." After embryonic and fetal growth, it has generally been accepted that there is no increase in the genetically controlled number of muscle fibers. Although the diameter of these fibers increases after birth, the degree of increase depends on the function and location of the muscle system. Hiner et al. (1953) in a study with beef cattle stated that in terms of percent, the psoas major (tenderloin) muscle fibers increased in size the most from veal to cows, whereas, the serratus ventralis (neck) muscle increased the least. In general, the less active muscle fibers increased in size more than those that were more active. Even within a single muscle, the muscle fiber diameter is not uniform. Walls (1960) reviewed articles which indicated that the larger muscle fibers were generally along the periphery of the muscle. Others have found that thick and thin muscle fibers exist adjacent to one another. Presently, there is no generally accepted explanation for this phenomenon.

Upon examining a cross-section of a muscle, it can be observed that there are many separate groups of muscle fibers. These groups are referred to as muscle bundles, and may be further classified as either primary or secondary bundles. The distinction between the two types of bundles is based on the thickness of the encompassing connective tissue.

Connective tissue surrounds and holds each individual muscle fiber in place, as well as surrounding the entire muscle system and providing a mechanism for its attachment to bone. The elements constituting the

connective tissue of muscle are collagen fibers, reticular fibers, elastic fibers and several varieties of cells, such as fibroblasts, histocytes (macrophages - also called clasmatocytes or resting wandering cells), and fat cells. (Walls, 1960). The fibrous (formed) elements of connective tissue (collagen, elastin, and reticular fibers) are embedded in a soft amorphous ground substance composed primarily of mucopolysaccharides. Such a combination of formed and amorphous substance is referred to as the intercellular substance. The elastic fiber component varies the most and is found in highest concentrations in the muscles of the soft parts of the body, e.g., the muscles of the tongue and face. (Walls, 1960).

The connective tissue components associated with a muscle system are classified as endomysium, perimysium, and epimysium. The endomysium invests each muscle fiber and is attached to the sarcolemma of the muscle fiber (Wang, 1960) by reticular connective tissue. Surrounding a bundle of fibers is a heavier layer of connective tissue referred to as the perimysium. The epimysium is that layer of connective tissue surrounding the entire muscle. In reality, these different layers of connective tissue are attached so that they function as a unit during muscle contraction. In addition to participating in the contraction of muscle, each layer of connective tissue supports other tissues within the muscle. The reticular connective tissue and the endomysium supports the capillaries and smaller branches of the nerves, as well as forming sheaths for the regeneration of damaged muscle fibers. Larger blood vessels and nerves are supported throughout the muscle by the perimysium. Since muscle fibers are never attached to bone, the three types of connective tissue (endomysium, perimysium, and epimysium) blend together and form the muscle-tendon junction. (Lockhart, 1960). Thus the area of the carcass

from which a muscle sample is obtained may influence the concentration of connective tissue and the over-all histological picture of the muscle.

Research dealing with the relation of muscle microanatomy to certain qualitative characteristics of meat is reviewed according to the following areas: A. Factors associated with firmness and tenderness; B. Methods of measuring firmness and tenderness; and C. Chemistry of collagen.

Factors Associated with Firmness and Tenderness

From a brief description of the microanatomy of muscle, it can be seen that there are two major physical components in muscle which are associated with tenderness and firmness, i.e., the muscle fibers and the connective tissues. Numerous variables relating to muscle fibers have been studied: 1. muscle fiber diameter; 2. density of muscle bundles; and 3. chemical components of the muscle fiber. The connective tissue content in beef tissue has been studied quite extensively. A major part of the work in this area has attempted to quantitate the collagen fraction by chemical methods, while others have dealt with the histology of connective tissues. Since intramuscular fat (marbling) is actually the result of the deposition of fat in a connective tissue cell, research dealing with the relationship of marbling to firmness and tenderness is reviewed with that of connective tissue.

The muscle fibers have been designated as one component of the tenderness sensation when taste panel members are subjectively evaluating cooked meat. (Cover, 1959; Cover et al., 1962a,c). Cover et al. (1962, a,c) suggested that there are three different sensations which can be ascribed to the muscle fibers, "ease of fragmentation across the grain", "mealiness", and "apparent adhesion between fibers". These properties were described as follows: "Ease of fragmentation required cutting or breaking

the muscle fibers across the grain. Mealiness was a special kind of fragmentation: the fragments were tiny, dry, and hard, and clung to cheek, gums, and tongue. Scores for lack of apparent adhesion were lowest when the muscle fibers seemed almost felted together." A nine point scale was used to classify each of the three muscle fiber components. Results were presented (Cover et al., 1962c) which suggested that the ease of fragmentation and lack of adhesion between muscle fibers tended toward greater toughness in longissimus dorsi and toward greater tenderness in biceps femoris. Mealiness was not observed when the steaks were cooked to 61°C., but became apparent at higher internal temperatures. In an earlier paper, Cover et al. (1962b) reported an attempt to relate muscle fiber diameter to two other components of tenderness (1. softness to tongue and cheek which was judged by feel; and 2. softness to tooth pressure which was judged by the amount of muscular force exerted without shearing the cooked sample of meat). They noted a decrease in muscle fiber diameter and lower scores for the two softness components when comparing the results from steaks cooked to 61° and 80°C. However, these authors suggested that improved histological methods were needed before any significance could be attached to these findings.

Fiber diameter and/or muscle bundle size have been reported to be associated with the tenderness of meat. (Brady, 1937; Hammond and Appleton, 1932; Hiner et al., 1952, 1953; Lorincz and Biro, 1960; Ramsbottom et al., 1945; Strandine et al., 1949; Tuma et al., 1962). Brady (1937) found a correlation of 0.22 between muscle fiber diameter and the Warner-Bratzler shear values of aged, raw steaks. However, the correlation was 0.55 between the same two variables when the steaks were cooked. Tuma et al. (1962) reported similar correlations. Hiner et al. (1952, 1953) presented data which suggested that the relationship

between fiber diameter and tenderness was curvilinear. Their data were collected on nine muscles from 52 animals. Within each muscle, the simple correlations varied from 0.31 to 0.75. The pooled simpled correlation was 0.83. Thus, there appears to be a positive relationship between fiber diameter and tenderness, i.e., the smaller the diameter the more tender the meat. Sartorius and Child (1938) suggested that large bundles with many small fibers were indicative of tender meat, whereas the reverse was true when the bundles were small and contained large fibers. Similar conclusions have been presented by Brady (1937), Ramsbottom et al. (1945), and Strandine et al. (1949).

Detailed studies have been conducted to elucidate the factors responsible for the variation in the diameter of muscle fibers. Because of the complexity involved in many of the studies, this review will present the conclusions and details only when it is necessary for clarity.

1. Species - Joubert (1956a) conducted a study involving mature male rabbits, swine, sheep and cattle. He found no consistent relationship between body weight and mean fiber diameter or between the species at birth. However, the order of relative increase for muscle fiber diameter over the same period of time corresponded with that of body weight in the four species studied. (Joubert, 1956a). At maturity, the fiber diameter of the four species in order of decreasing magnitude was as follows: swine, rabbit, cattle, and sheep. Joubert (1956a) cited the work of Warringsholz (1903) which supported the above conclusions, as well as indicating that the mean fiber diameter of the horse was intermediate to that of cattle and sheep. Strandine et al. (1949) reported that there was a difference in the histological structure of the same muscle from the carcasses of cattle and chickens. Thus, there appears to be a difference in the structure of muscles from different species, independent

of body weight.

2. Breeds within species - Tenderness has been shown to differ between breeds. (Black et al., 1934; Hostetler et al., 1936; Hiner and Yao, 1954; Cartwright et al., 1958). Joubert (1956a), for example, found that cattle of the Friesian breed had larger muscle fibers than those of the Dairy Shorthorn cattle. However, he did not relate these findings to the palatability of the cooked product. Furthermore, there is very little if any published research findings relating the differences in the diameter of the muscle fibers of different breeds to the resulting palatability of the cooked steaks or roasts.

3. Sex within breed - Joubert (1956c) noted that male lambs consistently had larger fibers at birth than females. However, in earlier work the females were reported to have the larger muscle fibers. (Joubert, 1956a). In this same paper, it was reported that the males had larger fibers than the females at 290 days of age. This difference, however, was small and non-significant. Joubert (1956c) cited studies by others (Hammond and Appleton, 1932; Mehner, 1938; Tsuchiya and Yoshida, 1953) which have indicated that mature males generally have larger muscle fibers than females. Although Brady (1937) and Satorius and Child (1938) reported that steers had larger fibers than cows, it should be noted that these studies were conducted with the carcasses from steers of beef breeding and cows of Holstein breeding. In summary, it may be concluded that the muscles from the carcasses of mature males tend to have larger fiber diameters than the same muscles from mature females.

4. Nutrition - Regardless of species, the results of several investigations indicate that the muscle fiber diameter in animals with a high plain of nutrition is larger than that of animals on a low plane of nutrition. (Hammond, 1960; Joubert, 1956a; McMeekan, 1940b; Robertson et al.,

1933). The difference due to the level of nutrition may not be present in the immature animal. (Joubert, 1956a). McMeekan (1940a) has presented data which suggest that the increase in fiber diameter is not related to fat deposition. He observed that in four and eight week old pigs the intramuscular fat was located within the muscle fibers as well as between the fibers; however, after 16 weeks of age, the fat within the fibers could not be detected. Robertson et al. (1933) were also unable to detect fat within the fibers of mature beef. Wang et al. (1954a) and Auerbach et al. (1954) have reported correlations of approximately 0.60 between fiber diameter and moisture content of meat. Since there is a negative relationship between moisture and fat content, these data would support the conclusion that fat deposition is not associated with fiber diameter. Joubert (1956a), in summarizing the influence of nutrition on fiber diameter, stated that growth of the muscle fiber, similar to growth in general, is essentially a function of physiological or developmental age, rather than of chronological age.

5. Chronological age - With increasing chronological age, there is reported to be an increase in the diameter of the muscle fiber. (Dickerson, 1960; Doty and Pierce, 1961; Hammond, 1960; Hiner et al., 1952, 1953; Lowe and Kastelic, 1961; McMeekan, 1940a; Tuma et al., 1962). Hammond (1960) also stated that there was a corresponding increase in the size of the muscle bundles with an increase in the coarseness of the "grain" of the meat. The location of the muscles, however, influences the relative rate of growth. (Hiner et al., 1953; Joubert, 1956a; Tuma et al., 1962).

6. Individual muscles - There appears to be an inverse relationship between the pre- and post-natal development of muscle fiber diameter. In a study on the pre-natal growth of sheep, Joubert (1956b) stated that the foetal muscle fiber undergoes relatively little change in diameter from

a weight of approximately 8 g. to just under 1000 g. (approximately 45 to 103 days). However, noticeable changes were evident at 125 days and at birth. In addition, cross-striations in the muscle fibers became increasingly clearer from 85 days onward. Further observations were made in this study which indicated that the anatomical location of the muscle influenced the relative rate of maturation. For example, the longissimus dorsi muscle of the trunk matured earlier than the gastrocnemius muscle of the pelvic limb. At birth, the size of the fibers was found to be smallest for the earliest maturing muscle and largest for the latest maturing muscle. (Joubert, 1956c). The rate of post-natal hypertrophy may also be associated with the rate of pre-natal maturation. In lambs, the fibers of the longissimus dorsi grow at a slightly faster rate than those of the gastrocnemius from birth to 60 days. (Joubert, 1956a). As the lambs continued to grow, the gastrocnemius became the slowest growing muscle while the longissimus dorsi maintained its faster rate of growth. Hiner et al. (1952, 1953) reported that nine muscles from different locations in beef carcasses could be separated into four groups on the basis of their average muscle fiber diameters. The four general groups in increasing fiber diameter were as follows: 1. psoas major; 2. longissimus dorsi (rib and short loin regions) and gluteus medius; 3. semimembranosus, semitendenosus, and biceps femoris; and 4. serratus ventralis and deep digital flexor. These authors also reported that not only was tenderness related to the muscular activity but also to the increase in muscle fiber diameter from birth to maturity. In this study, the psoas major increased the most while the serratus ventralis increased the least. The greatest increase in diameter occurred from eight to fourteen months of age. A similar growth pattern for the longissimus dorsi and biceps femoris has been reported by Tuma et al. (1962). Using the rate of

increase in muscle fiber diameter as the basis for the growth rate of muscles, these studies indicated that the muscles (such as the longissimus dorsi) which decrease their rate of growth early during the pre-natal period are the muscles which mature late during post-natal growth. These same muscles are also considered to be the more tender muscles in the carcass. It is generally accepted that the meat from mature animals is less tender than that from immature animals. Part of this decrease in tenderness with increasing animal age has been associated with the increase in muscle fiber diameter.

7. Post-morten aging of meat - The aging of meat has been demonstrated to influence the structure of fresh meat and enhance its ultimate tenderness. (Doty and Pierce, 1961; DeFremery and Pool, 1960; Hanson et al., 1942; Harrison et al., 1949; Locker, 1960; Paul et al., 1944; Ramsbottom and Strandine, 1949; Tuma et al., 1962). Ramsbottom and Strandine (1949) described the structural changes in the longissimus dorsi of beef from 2 hours to 12 days post mortem. During the first several hours after slaughter, the muscle fibers appeared straight to slightly wavy (pre-rigor phase). After 8 to 24 hours post mortem, the muscle fibers were in a state of contraction and they appeared as sharply defined waves (rigor-mortis phase). This state of contraction was reported to last from 24 to 72 hours post-mortem. Following this phase, the fibers began to go into a relaxed or non-contracted state (post-rigor phase). While in this relaxed state, the muscle fibers gradually broke transversely and longitudinally as a result of enzymatic action or autolysis.

Accompanying these three phases of rigor mortis was a decrease in tenderness during rigor-mortis and then a continual increase in tenderness during post-rigor. (May et al., 1962; Ramsbottom and Strandine, 1949). Also, the muscle bundles were not hard during pre-rigor, but became

firm during rigor. When the muscle was in post-rigor, there was a gradual softening and loosening of the bundles, which was quite noticeable by the twelfth day. (Ramsbottom and Strandine, 1949).

The extensibility of the muscle fibers (resistance to breaking under pressure) has been reported to be negatively related to tenderness. (Hostetler and Cover, 1961; May et al., 1962; Wang et al., 1954a,b, 1956). May et al. (1962) noted that the percent extensibility of pork and chicken muscles decreased and the shear values increased with the stage of mortis. It has been postulated that the actomyosin complex, which forms during contraction, is responsible for the various degrees of extensibility. (Wang et al., 1958; Weir et al., 1958).

Connective tissue has long been associated with tenderness. One of the early published reports on this relationship was by Lehmann (1897). Since that time many studies have been conducted which indicate a negative relationship between tenderness and connective tissue, i.e., the more tender the cooked meat the lower the collagen content. Some of the investigators who have reported a close association between these two factors are Adams et al. (1960), Cover (1959), Doty and Pierce (1961), Hall et al. (1944), Hiner et al. (1955), Husaini et al. (1950a), Mitchell et al. (1927), Nottingham (1956), Parrish et al. (1962), Ramsbottom et al. (1945), Ritchey and Cover (1962), Ritchey et al. (1963), Satorius and Child (1938), Strandine et al. (1949), and Winegarden et al. (1952). Other workers have suggested that the role of connective tissue is not an important factor in tenderness. (Hershberger et al., 1951; Husaini et al., 1950b; Lorincz and Szeredy, 1959; Mackintosh et al., 1936; Naumann et al., 1953; Wierbicki et al., 1954; and Wilson et al., 1954). According to Doty and Pierce (1961), tenderness was only slightly correlated (negatively) with the collagen content of raw or cooked meat. However,

it is perhaps significant that, with two exceptions, all correlation coefficients between collagen content of the meat and tenderness were negative. Lorincz and Szeredy (1959) stated that it was not the quantity of connective tissue which determined the chewing (cutting) resistance of meat, but the quality of such connective tissue.

Using histological methods, Ramsbottom et al. (1945) reported data which indicated that muscles with sufficient connective tissue to result in definite muscle bundles (ex. superficial pectorial) were less tender than those muscles containing a small amount of connective tissue with correspondingly fewer muscle bundles (ex. psoas major). Further work was conducted by this group to elucidate the relationship between connective tissue distribution and palatability. (Ramsbottom and Strandine, 1948, 1949; Strandine et al., 1949). In the study by Strandine et al. (1949), the size of the perimysium was classified as large, medium, small, or indistinct. They concluded that the muscles with abundant connective tissue were much less tender. When the combined histological rating for collagen and elastin on the 50 muscles from each of four animals were analyzed, "The correlation coefficient for these data on all of the beef muscles studied was 0.70. . . showing that connective tissue is a significant factor influencing tenderness but not the only factor." (Strandine et al., 1949). Doty and Pierce (1961) reported a study involving 153 beef carcasses representative of the light and heavy weight U.S. Prime and Good carcasses and 650 lb. U.S. Commercial cow carcasses. These authors stated that, "There was no indication from observations made during this study that the distribution of collagen was consistantly different from carcasses of different grades. Thus, it would appear that the collagen content and distribution in similar muscles from carcasses of different grades are so similar that this cannot

generally be of primary importance in determining organoleptic differences between grades." However, Doty and Pierce (1961) concluded that the collagen content of the ribeye was perhaps a minor factor contributing to tenderness.

Hiner et al. (1955) observed that the amount of elastin dispersed through a cut of meat was closely associated with tenderness. In the less tender cuts, the elastic fibers were bunched together in definite areas between the larger muscle bundles. There was only a small amount of elastin in the more tender cuts. Winegarden et al. (1952) stated that since the elastin content of most muscles is small compared to the collagen content, it seems that the tenderness of cooked meat is affected to a greater extent by collagen than elastin.

When studying the relationship between the tenderness and the connective tissue content of meat, the manner in which tenderness is measured can greatly influence the resulting analyses. Nottingham (1956) found no correlation between collagen content and tenderness when tenderness was mechanically measured by shearing meat across the muscle fibers. A correlation of 0.78 was observed between the same two variables when meat samples were sheared parallel with the muscle fiber direction. This author postulated that shear force measured parallel to the fibers represents the force needed to separate adjacent fibers against the binding of the connective tissue and would be expected to be related to connective tissue content.

Ramsbottom et al. (1945) advanced the theory that differences in the amount of connective tissue associated with the intramuscular fat may explain why it was not possible to show a positive relationship between high fat content of muscles and low shear values.

Cooking results in the hydrolysis, of at least a part of the collagen, and the subsequent formation of gelatin. Factors affecting this process in meat are the internal temperature, the pH, the size of the cut, and the denseness or kind of collagen. (Lowe, 1955). According to Winegarden et al. (1952) heat influences collagenous tissues in the following manner: 1. a shortening in length, 2. a decrease in width, 3. an increase in thickness, 4. a loss in weight, and 5. a softening process. Bell et al. (1941) have reported that 22 percent of the collagen is converted to gelatin during cooking. Similar results have been reported by Irvin and Cover (1959). This conversion can be either increased or decreased, depending on the final internal temperature of the steak or roast. (Bell et al., 1951; Griswold and Keffler, 1952, Adams et al., 1960; Doty and Pierce, 1961; Ritchey et al., 1963; Ritchey and Cover, 1962). Ritchey and Cover (1962) for example have reported that, "Collagen nitrogen loss approaches 100 percent when steaks of either muscles (longissimus dorsi or biceps femoris) are cooked to 100°C. and held there for 25 minutes. . ." The effect of collagen hydrolysis on the tenderness of this fraction has been studied by taste panel methods. (Cover, 1959; Cover et al., 1962; Ritchey et al., 1963). All three studies pointed to a tenderization of the connective tissue during the cooking process. Ritchey et al. (1963) reported that a mouth sensation of tenderness or toughness of connective tissue can detect extensive chemical changes in connective tissue as measured by collagen content.

Harrison et al. (1949) reported that cooked collagenous tissue usually is not affected by van Geisen's stain (specific for collagen) due to heat degradation. Earlier workers had observed this same phenomenon. (Paul et al., 1944; Paul and McLean, 1946; Ramsbottom and Strandine, 1949).

In discussing the changes in collagen upon heating, one must be cognizant of the concomitant changes in the fat cells of connective tissue. Siemers and Hanning (1953) investigated the relationship of fat content to the juiciness of lean ground beef. They reported that, "The heat transfer studies showed that probably the major part of the juice retention caused by suet content was due to the slower rates of heat transfer in the suet and connective tissue." This association was further elucidated by Wang et al. (1954) in a histological study on the dispersion of fat within cooked muscle. They observed that if dispersion occurred within a perimysium of considerable size, and if the collagen in it were only partly degraded by hydrolysis, the fat droplets would tend to mingle only with the degraded collagen, leaving the intact collagenous fibrils relatively fat free. Sartorius and Child (1938) suggested that for meats cooked to an internal temperature of 58° to 67°C. the hydrolysis of collagen was the most important tenderizing process. At temperatures greater than 67°C., coagulation of the muscle protein began to influence tenderness of meat. This may help to explain the time-temperature curves presented by Cover (1937). Her work showed that there was a "plateau region" in the curves from approximately 65° to 75°C. during the heating phase, regardless of the oven temperature at which the meat was being cooked.

Factors which have been reported to influence the collagen content of fresh muscle are animal age, location of the muscle in the carcass, degree of marbling, management of the cattle prior to slaughter, and post-mortem aging.

Collagen content has been reported to increase with chronological age. (Shock, 1961, Strandine et al., 1949). Some workers, however, have

reported that veal contains more collagen than mature beef; (Lorincz and Szeredy, 1959; Wilson et al., 1954); while Spencer et al. (1937) indicated that the collagen content of muscle was independent of animal age. The work of Loyd and Hiner (1959) was in agreement with that of Spencer et al. (1937) when the alkali-insoluble collagen fraction was combined with the fat fraction of the Lowry et al. (1940) technique. When the fat content of the meat sample was low, such as with veal, the fat fraction did not contain an appreciable amount of collagen. However, the fat content of mature beef was so high that the fat fraction could contain as much as 71.0 percent of the collagen. Thus, their data suggested that the higher collagen content in veal than mature beef was due to the amount of collagen being retained in the discarded fat fraction of the alkaline hydrolyzed meat sample. Bray et al. (1949, 1951) have also noted that the connective tissue from mature animals contains more fat than similar connective tissue from youthful animals.

Location of the muscle in the animal body, as related to function, has been shown to influence the amount of connective tissue present in a particular muscle. (Hiner et al., 1955; Nottingham, 1956; Mitchell, et al., 1928; Parrish et al., 1962; Ramsbottom et al., 1945; Ramsbottom and Strandine, 1948; Ritchey et al., 1963; Strandine et al., 1949). Ramsbottom et al. (1945) in a study of 25 muscles from various locations in the body reported that muscles in the neck, for example, contained more connective tissue than muscles in the back region. A more comprehensive study on the influence of location on connective tissue content was undertaken by Strandine et al. (1949). These authors stated that the cross-sectional patterns in a given muscle were fairly constant from animal to animal. However, the histological patterns of different muscles were not alike, but varied in the amounts and kinds of connective tissue elements in the

perimysium.

Batterman et al. (1952) and Lorincz and Szeredy (1959) have suggested that there is a decrease in the connective tissue of muscle as an animal fattens. Other workers (Cover and Hostetler, 1960; Mitchel et al., 1928; Wilson et al., 1954) have found no relationship between fat and collagen content. Ramsbottom et al. (1945) have suggested that the type of fat laid down influences the connective tissue content of the muscle. For example, kidney fat has only a small amount of connective tissue while the fat in the brisket contains a large amount of connective tissue. (Ramsbottom et al., 1945). Using histological techniques, Hiner et al. (1955) observed that muscles with a high degree of marbling had a loose network of collagenous fibers; muscles with less intramuscular fat were found to contain clumps of collagenous fibers. Thus, there appears to be no concrete relationship between the level of marbling and the concentration of collagen.

The collagen content of muscle may be influenced by certain genetic, nutritional, and physiological conditions. Although tenderness has been shown to be an inherited characteristic (Cartwright et al., 1958; Cover et al., 1957; Hershberger et al., 1951), Husaini et al. (1950b) have reported that there was no difference in tenderness between steaks from Holstein and Hereford steer carcasses. McIntosh et al. (1961) and Wierbicki et al. (1953, 1955, 1956) indicated that the feeding of diethylstilbestrol to cattle increases the hydroxyproline content of muscle. Wierbicki et al. (1956) also indicated that carcasses from bulls and hormone implanted bulls contained more hydroxyproline than those of steers and heifers. Exercise may contribute to a decrease in the collagen content of muscle. (Mitchell and Hamilton, 1933).

Post-mortem aging of meat has been reported to improve the tenderness of the cooked product. (Ewell, 1940; Hall et al., 1944; Harrison et al., 1949; Paul et al., 1944). Ramsbottom et al. (1949) have attributed some of the increase in tenderness to collagen degradation. However, Wierbicki et al. (1954) noted no change in the alkali insoluble proteins as a result of aging. Lawrie et al. (1961) reported on the influence of ionizing radiation on meat and subsequent storage, and stated that the absence of soluble hydroxyproline and the presence of clearly marked cross-striation indicated that the autolysis must have involved sarcoplasmic and not fibrillar or connective tissue protein.

From the above review, it may be concluded that there is a negative relationship between tenderness and the collagen content of meat. Some of the factors which may influence the collagen content in meat are: 1. cooking method and final internal temperature, 2. location in the carcass, 3. degree of marbling, 4. genetic, nutritional and physiological conditions, and 5. post-mortem aging.

Objective Methods of Measuring Firmness and Tenderness

Firmness

The term "firmness", as used in this paper, refers to the resistance of a "semi-solid" object to an applied blunt force. This connotation does not mean that the object will resist a cutting force when such is applied.

Many foods are critically evaluated on the basis of the degree of firmness. Examples of these foods include vegetables, fruits, and dairy products. The industries associated with these products have studied objective methods for determining the firmness of the product. Dolby (1941) listed five different measurements used to express this factor with

butter. These methods were designated as : 1. compression - a cube or cylinder of butter is compressed between two plates and reported as the load per unit time or degree of compression; 2. penetrometer - a blunt instrument is forced into a certain sized sample with the results expressed as a variable weight, time, or depth of penetration with a constant weight and time; 3. resistance to cutting by a wire - the length of time to cut through a certain dimension of butter; 4. extrusion - the amount of weight to extrude a certain quantity of butter; and 5. rate of sag - the rate of sag by a certain size cube of butter held horizontally. Since this report, these methods or combinations of them have been modified and used as standard measures of firmness for food products.

Emmons (1959) combined the compression and the cutting wire methods into a procedure which closely related the subjective firmness of cottage cheese curds to that of organoleptic panel scores ($r \sim 0.96$).

Cox and Higby (1944) modified the Lockwood and Hayes "Ridgelimiter" to determine the jelling power of pectin. These workers reported that the percent sag of an inverted jelly from a standard mold was associated with the firmness of the jelly.

Kramer et al. (1951) developed an instrument for measuring the tenderness of fruits and vegetables. This device measures the amount of force required to plunge a series of blades through a sample confined in a specific size box. Shallenberger et al. (1963) have used this procedure to evaluate the firmness of cooked apple slices. A discussion of this instrument as used in meat research is included in the tenderness methods section.

The depth of penetration for a specified time and a constant weight load has also been used to measure the firmness of food products other than butter. In the study reported by Shallenberger et al. (1963), raw

apples were subjected to penetrometer determinations as an evaluation of their quality.

Some meat tenderness investigations have been conducted using the penetrometer as a tenderness measuring device. Tressler, Birdseye and Murray (1932) were among the first to use the penetrometer as a firmness measure. In the initial phases these workers used a cylinder of meat and forced a needle under 255 gm. of weight into the meat. Later, the cylinder was replaced by an eight hole box to permit more penetrometer readings per meat sample. (Tressler and Murray, 1932). Noble et al. (1934) and McCarthy and King (1942) also used the penetrometer type of measurement in meat research. However, the only reported meat studies in which firmness (as determined by the penetrometer) was specifically involved were those of Hiner and Hankins (1941), Gannaway (1955), Pilkington (1960), and Doty and Pierce (1961). All of these workers used a rounded object as the plunging device. When penetrometer readings were compared to panel firmness scores for pork, the correlation coefficients were 0.82 and 0.90. (Gannaway, 1955; and Hiner and Hankins, 1941; respectively). A similar study by Pilkington et al. (1960) in which the penetration objectively was modified to study beef lean, reported a similar correlation coefficient (0.93). Thus, it appears that the penetormeter may be used to objectively determine the meat quality factor, firmness. The investigation by Pilkington (1960) indicated that a ball plunging device was more desirable for measuring beef lean firmness than a "spike" shaped device.

Tenderness

Tenderness may be defined as the kinesthetic characteristic of meat. This includes all the sensations associated with the chewing and the subsequent evaluation of meat for tenderness. Cover et al. (1962a)

have concluded that there are at least three major components of tenderness: 1. two softness components, 2. three muscle fiber components, and 3. the connective tissue component. Since these factors are "quantitatively" and "qualitatively" evaluated subjectively by sensations originating in the mouth, a precise and/or accurate measurement of tenderness has been difficult to obtain.

Research leading to the development of objective methods for measuring tenderness was conducted by Lehmann as early as 1897. (Schultz, 1957). There are presently many objective methods for determining the tenderness of meat. These instruments may be classified according to their mode of operation: 1. simulated chewing of a meat sample; 2. work required when grinding a meat sample; 3. puncturing or pressing a meat sample; and 4. shearing a meat sample.

1. Simulated chewing of a meat sample - Two types of instruments have been designed using this principle. Volodkevick (1938) developed an instrument which became popular in Germany (Bate-Smith, 1948), which consisted of one stationary wedge and one movable wedge. As the meat sample was sheared and squeezed, the measurement was recorded as force per unit of time. Tenderness was estimated by measuring the "shearing" portion and "squeezing" portion of the force-time curve. Several modifications of this instrument have been made in an effort to improve the accuracy of the determinations. (Sale, 1960; Shrimpton and Miller, 1960; Winkler, 1939). In 1955, Proctor et al. designed a strain gage denture tenderometer. This instrument contained a set of dentures to simulate the chewing process. By using a photography arrangement (Proctor et al., 1956a), a force curve was obtained to evaluate the degree of tenderness. Kelly et al. (1960) developed an instrument similar to that of Proctor et al. (1955). After several modifications (Kelly et al.,

1963), the correlation between the tenderness measured by this instrument and taste panel tenderness scores still was not as high as when Warner-Bratzler shear values were related to the subjective scores, r = 0.32 and 0.61 respectively.

2. Work required when grinding a meat sample - Miyada and Tappel (1956a) wired an ammeter in series with a household type food grinder. The resulting work per unit time curve was used to compare the tenderness of meat samples. Emerson and Palmer (1958) reported that the Warner-Bratzler Shear was more closely related to organoleptic values than the food grinder method. Bockian et al. (1958) obtained correlations of -.59 and -.60 between the subjective and the grinder values when evaluating meat samples over a two year period. A study on the causes of variation in the grinder method was reported by Schoman et al, 1960). Factors that affected the results were changes in motor resistance, line voltage fluctuations, and variations in load caused by friction of the movable grinder elements. After corrective measures were taken, the coefficient of variation was less than that for the Warner-Bratzler Shear method, 5.5 and 19.4 percent respectively.

3. Puncturing or pressing meat sample - Instruments based on this principle have been designed and/or modified by several workers. (Doty and Pierce, 1961; Kulwich et al., 1963; Miyada and Tappel, 1956a; Sperring et al., 1959; Tressler et al., 1932; Tressler and Murray, 1932). The technique of Sperring et al. (1959) has been used in a tenderness study by Bratzler and Smith (1963). Results of this study indicated that the pressing method was associated with the tenderness of cooked beef and lamb (r = -.95 and -.51 for beef shortloin steaks, n = 15; and lamb loin chops, n = 129; respectively).

4. Shearing a sample of meat - The instruments based on this principle include the Warner-Bratzler Shear (Black et al., 1931; Warner, 1952) and the Kramer Shear-Press (Kramer et al., 1951). At least three other instruments, in addition to that of Lehman (1907), have been developed for estimating tenderness by shearing; Dassow et al. (1962), Satorius and Child (1938), Tressler et al. (1932), and Tressler and Murray (1933).

The Warner-Bratzler Shear has been the most widely used objective tenderness measurement in the United States because of its close relationship with organoleptic tenderness values. Some of the research groups who have reported a high correlation between these two methods ($r = -.60$ to $-.80$) are Bratzler and Smith (1963), Gainer et al. (1951), Kulwich et al. (1963), Kelly et al. (1960, 1963), Mackintosh et al. (1936), Pilkington (1960), and Sperring et al. (1959). However, low correlations ($r = -.18$ to $-.37$) have also been reported between these two methods. (Deatherage and Garnatz, 1952; Fielder et al., 1963; Mahon et al., 1956; Tressler and Murray, 1932). Schoman et al. (1960) indicated that the coefficient of variation was 19.4 percent when evaluating the tenderness of beef rounds by this method. In an effort to decrease the variation, Hurvicz and Tischer (1954), and Spencer et al. (1962) have recommended changes in the instrument. Hurvicz and Tischer (1954) noted that the tenderness measurement expressed as time vs. shear force decreased the coefficient of variation from 7.41 for maximum shear force to 4.79 percent when beeswax was used as the testing medium. Spencer et al. (1962) modified the horizontal model by using an electrical force transducer and recording system. Using the same testing medium as Hurwicz and Tischer (1954), they reported coefficients of variation from 6.6 to 10.6 percent. Klose et al. (1959), using muscles from poultry, studied the variation in the Warner-Bratzler shear values. In this study, they

assumed that the difference in shear values between duplicate samples of the same muscle within a bird was attributable to the precision of the Warner-Bratzler Shear. The results of the analysis of variance indicated that the among bird variation was at least three times greater than the within muscle variation. These workers concluded that these results are indicative of the very desirable repeatability of the Warner-Bratzler Shear for estimating the variation in tenderness among meat samples from different birds.

Although the Kramer Shear-Press was developed and modified for evaluating vegetables (Kramer et al., 1951; Kramer, 1957; Werner et al., 1963), it has been used for estimating the tenderness of meat (Batcher et al., 1962; Cameron and Ryan, 1955; Parrish et al., 1962; Shannon et al., 1957). Cameron and Ryan (1955) reported a correlation of -.97 between taste panel tenderness scores and the Kramer Shear-Press values. Batcher et al. (1962) found a correlation of -.76 between the two variables. In addition, they reported that the correlation between the Kramer and Warner-Bratzler instrument was 0.87 and 0.83 for cooked and raw lamb chops.

Tenderness of meat, when evaluated by individual members of an organoleptic panel, is the result of energy expended in cutting, squeezing and tearing a sample into a size that can be ingested. Although many objective instruments have been developed to measure this sensation, those most commonly used involve the shearing principle, such as the Warner-Bratzler Shear and the Kramer Shear-Press.

Chemistry of Collagen

Chemical and physical properties of collagen

Collagen is that portion of the skeletal muscle often referred to as "white fibrous connective tissue." On a weight basis, collagen is the most

abundant protein found in mammals. Neuberger (1955) estimated that 25 to 35 percent of the protein in the mammalian body is collagen.

Collagen, as contrasted to many other proteins, has a different amino acid spectrum. Although collagen contains traces of tyrosine and phenylalanine, there is no evidence of tryptophan, cysteine, and cystine in its structure. Among the characteristics which distinguish collagen from many other proteins are its high concentration of glycine, proline, and hydroxyproline (27, 15, and 14 percent respectively); plus the presence of hydroxylysine (1.0 percent). The three amino acids, glycine, proline, and hydroxyproline, comprise more than 50 percent of the collagen amino acid residues. Neuberger (1955) has calculated that over 70 percent of the glycine and proline in a mammalian body is in collagen. According to the results of Bowes and Kenten (1949), collagen is the only source of hydroxyproline in the mammalian body. Similarly, hydroxylysine in mammals can be found only in the collagen molecule.

Glycine, proline and hydroxyproline have a definite relationship to the structure of collagen. Vies (1962), in his review article on collagen, cited papers which indicated that the following amino acid sequence frequently appears: -proline - hydroxyproline - glycine - proline -. However, Grassman et al. (1956) have reported evidence that there are some areas along the protein chain which are low in hydroxyproline. Michales et al. (1958) have suggested that collagenase acts on collagen to release only N - terminal glycine residues. Since 60 percent of the peptide bonds involving glycine are hydrolyzed by this enzyme (and with the frequency of the three specific amino acid sequence), these workers postulated that this sequence was necessary for the enzyme specificity. Other workers (Danby, 1958; Rigby and Spikes, 1960; Gross, 1961) have shown that the shrinkage temperature of collagen (a measure of its

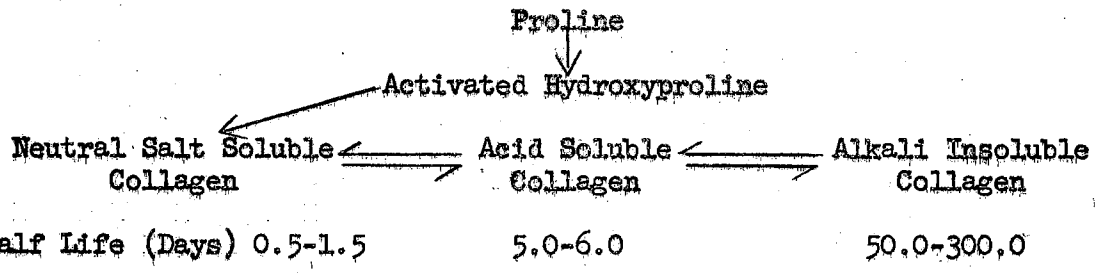
stability) is related to hydroxyproline concentration. For example, there is an abnormally low hydroxyproline content in the fibrinoid tissue from rheumatic fever patients (Rigby and Spikes, 1960). In addition, there is a decrease in the shrinkage temperature of the collagen to approximately 40°C. With a decrease in the shrinkage temperature, a structural breakdown of collagen could occur at body temperature.

With the widespread interest in hydroxyproline and its relation to collagen structure, many biochemical studies have been reported dealing with collagen formation. Recent work has been directed toward the synthesis of hydroxyproline and its incorporation into the collagen molecule.

Early work in this field indicated that proline was a precursor for hydroxyproline. Robertson et al. (1959) also showed that ascorbic acid was required for the hydroxylation of the proline. In 1961, Hausmann and Neuman injected C^{14} proline into guinea pigs and then assayed the skin for the appearance of the two labeled imino acids. These authors stated that, "The data are most consistent with the view that hydroxylation occurs in a bound form of proline, not of macromolecular dimensions, but which contains within the same molecule both proline destined for hydroxylation and proline destined for collagen proline." Peterkofsky and Udenfriend (1961) conducted a similar study using a cell-free system from 10 to 11 day old chick embryos. Their results indicated that: 1. hydroxylation was occurring, since hydroxyproline appeared immediately after injection and ATP was required as an energy source; 2. hydroxyproline was forming a peptide because the only fraction that contained hydroxyproline was the microsomal fraction where protein formation occurs; and 3. the rate of release from solution for proline, hydroxyproline and the alpha amino acids studied was the same.

Based on solubility, collagen can be characterized into three fractions: 1. neutral salt-or alkali-soluble collagen; 2. acid soluble collagen; and 3. alkali insoluble collagen. The neutral salt - or alkali-soluble fraction is extracted with phosphate buffers having a pH of 7 to 9. Neuberger (1955) has reported that this fraction constitutes approximately four percent of the total collagen. Acid soluble collagen is that portion obtained by using dilute acetic or citric acid buffers at a pH $<$ 5.0. This fraction is often referred to as tropo - or pro-collagen. (Schmitt et al., 1955; Orekhovitch and Shpikiter, 1958). Courts (1961) has reported that this fraction varies with chronological age. In the embryo, this fraction accounts for approximately 13 percent of the collagen while in the adult it accounts for only 1 percent of the collagen. (Courts, 1961; Grassmann, 1958). The alkali-insoluble portion is the major fraction of collagen and is usually prepared by using 0.05 to 0.10 N NaOH as the extracting solvent. (Mellon et al., 1960; Miller and Kastelic, 1956; Bowes et al., 1957).

Lindstedt and Prockop (1961) summarized the work on hydroxyproline metabolism and the three collagen fractions and proposed a pathway for the formation of the collagen molecule. Their schema is based on the half life (days) of C^{14} hydroxyproline in each of the three fractions. The following diagram depicts the Lindstedt and Prockop (1961) scheme:



In studying the catabolism and anabolism of hydroxyproline and its relation to collagen, it is now considered that hydroxyproline exists in

both a "bound" and "free" form. Thus, a measure of hydroxyproline in the blood and urine would be an estimate of both forms of the imino acid. Gerber et al. (1960) postulated that two metabolic pools of hydroxyproline could exist. Their data suggested that there were two metabolically distinct components. One component had a rapid turnover rate, the labile fraction, and was catabolized readily to pyrrole - 2 - carboxylic acid or free hydroxyproline. The component with the slow turnover rate was designated as the "bound" hydroxyproline. Woessner and Boucek (1961), using the implanted sponge technique, helped to confirm the theory of two metabolic pools of hydroxyproline. By recording the appearance of D.N.A., collagen, and "free" hydroxyproline, several conclusions were drawn from their work on the gross formation of collagen. D.N.A. could be detected as early as three days after the sponge had been implanted. From the sixth through the twentieth day the D.N.A. curve was a straight line. This was thought to coincide with fibroblast formation. After the twentieth day, the D.N.A. concentration was found to be constant. However, collagen formation did not follow the same curve as D.N.A. Instead, collagen formed at an appreciable rate from the seventh through the thirteenth day, leveled-off from the sixteenth through the twentieth day, and then rose again through the thirty-fifth day, which completed collagen formation. These workers concluded from these data that collagen formation must be dependent on prior proliferation of the fibroblasts and unknown factors, in addition to the gross number of cells present. Regarding "free" hydroxyproline, Woessner and Boucek (1961) stated that, "The parallelism between the "free" hydroxyproline curve and the D.N.A. curve supports the hypothesis that the free hydroxyproline is intracellular and that there may normally be a constant amount of free hydroxyproline present in the fibroblast."

Daughaday and Mariz (1962) have concluded that "free" hydroxyproline arises from an activated hydroxyproline intermediate that exists before the stage of peptide synthesis.

Gross (1961) in a review article proposed the location for collagen formation, i.e., intra- or inter-cellular. The first step in the process by which the body produces collagenous tissue is that the fibroblast evidently synthesizes complete collagen molecules. These molecules are then extruded into the space outside the cells, where they polymerize into fibrils. The fibrils then grow in size until the original supply of collagen molecules is exhausted. Apparently the change in solubility is associated with the increasing perfection of fit between the molecules as they gradually pack together in a "lock and key" type of association along their length.

Methods of measuring collagen

Solubility methods:

One of the early methods for separating collagen from other mammalian proteins was devised by Stokes (1897). In this procedure, the proteins, except collagen, were removed by precipitation with mercuric nitrate. After filtration, the gelatin (primarily collagen) was precipitated from the filtrate with picric acid. Jacobs and Jaffe (1932) modified the procedure by: 1. precipitating the various proteins with lead nitrate, 2. subjecting the resulting filtrate to activated charcoal which absorbed the "pseudogelatins" and "other proteins", and 3. removing the gelatin from the final filtrate by forming a tannic acid-gelatin complex which precipitated out of solution. Farkas in 1933 reported that the gelatin - picrate complex of Stokes (1897) was soluble at 40°C., but that the other precipitated proteins were not soluble. Thus, the 40°C. supernatant could be cooled to allow the

gelatin complex to precipitate. The precipitate could then be used as an estimate of collagen. Spencer et al. (1937) adapted the tannic acid procedure for a micro-determination of collagen. The major modification was that the collagen fraction was separated from the other proteins by first autoclaving and washing the resulting residue with hot water. All the supernatants were combined and the collagen removed as the insoluble tannic acid complex.

Mitchell et al. (1927) used a different approach to the problem of separating collagen from muscle tissue. To separate the connective tissue (collagen and elastin) from the muscle tissue, the meat sample was mixed with water and then placed in a ball mill. Following this treatment, the sample was filtered through a 40 mesh sieve and washed with cold water at least three times. The residue remaining on the sieve was considered to consist of collagen and elastin. These two proteins were then separated in the following manner. First the residue was autoclaved on the premise that collagen was converted to gelatin and became soluble upon heating. When the autoclaved material was filtered, the filtrate should have contained the collagen and the residue should have contained elastin, along with any contaminants. The remaining residue was then subjected to trypsin digestion. Since trypsin does not attack elastin, an analysis of the residue should be an estimate of elastin concentration. Mitchell et al. (1928) modified the original procedure by replacing the 40 mesh sieve with a 100 mesh sieve to decrease the loss of collagenous material going into the initial discarded filtrate. However, Bell et al. (1941) noted that there was still a loss of collagen due to filtration and replaced the sieve with a linen filter. Another modification of this procedure was made by Hartley and Hall (1949). These workers replaced the ball mill with a Waring blendor. Also, the

pH of the blending solution was adjusted to pH 5.0 (the isoelectric point of raw beef proteins). After blending the sample, the liquid was decanted into a centrifuge tube. In general, the remainder of this procedure was similar to that of Mitchell et al. (1928) except that centrifugation was used in place of filtration.

The procedure of Lowry et al. (1941) is presently the most commonly used technique for the separation of the collagen fraction from other muscle proteins. Part of this procedure was used earlier by Schepilewsky (1899). This procedure is based on the theory that collagen and elastin are insoluble in weak alkaline solutions while the other muscle proteins are soluble. The extraction phase of the technique consisted of placing the meat sample in 0.10 N NaOH for 18 hours. Lowry et al. (1941) and Ritchey et al. (1963) have stated that some collagen will solubilize during the extraction step. However, they did not take this small loss into account when reporting the analytical data. Another change in this technique, as compared to the others, involved the residue left after centrifugation of the extraction solution. Prior to autoclaving the residue, the water-residue mixture was adjusted to pH 7.0 so that the non-collagenous material would not solubilize at the same time as the collagen. Elastin was also determined in the Lowry et al. (1941) procedure. To separate the collagen and elastin, the 0.10 N NaOH extraction sample went through two, ten minute boiling water baths. This type of extraction process for elastin is based on the theory that elastin is not soluble in hot dilute alkaline solutions. The repeatability of this procedure, using gravimetric determinations, was ± 5.0 percent.

Numerous studies have been conducted in an effort to improve the precision and accuracy of the original Lowry et al. (1941) procedure.

One major change has been the adoption of the practice of expressing the collagen results in terms of Kjeldahl nitrogen or collagen on the basis of hydroxyproline. Kastelic (1955) in working with beef muscles reported that the separation of the salt or NaOH dispersed in solubilized protein fractions from the stromal proteins could not be accomplished successfully by centrifuging. To obtain a complete separation of the fractions, he trapped the connective tissues on glass wool in an Erlenmeyer flask. Data were also presented in this article which substantiated the theory of the NaOH extraction process: "One may conclude from these studies that if it is accepted that the presence of hydroxyproline specifically identifies collagen and elastin in muscle tissue the Kjeldahl nitrogen values for the autoclaved, soluble fraction are not a valid measure of collagen content in the absence of a prior NaOH treatment of the tissue." Similar results were later reported by Miller and Kastelic (1956).

Nottingham (1956) has suggested that the meat samples be sectioned at 30 microns to avoid loss of connective tissue due to wrapping around the blendor blades. Furthermore, the extraction phase should be carried out in the cold (0 - 5°C.) to avoid solubilization of some collagenous material.

A comprehensive study of factors affecting the NaOH extraction has been reported by Lorincz and Szeredy (1959). Results of this investigation were as follows:

1. The finer the meat sample is chopped, the greater the loss of collagen during extraction.
2. The higher the temperature during extraction, the greater loss of collagen.

3. The NaOH concentration can vary from 0.01 to 0.10 N without affecting the connective tissue proteins, but above 0.10 N NaOH some of the connective tissue proteins will solubilize.
4. The time of extraction had no effect when the time interval was over 24 hours.
5. The concentration of fat in the meat sample does not influence the collagen values.

To make sure that all substances other than collagen and elastin were removed from the remaining residue after NaOH extraction, Irvin and Cover (1959) extracted the water and alkali soluble material for 30 hours. "Each meat sample was subjected to two water extractions within a period of approximately four hours and then to five 0.1 N NaOH extractions within a period of approximately 26 hours." After the extraction period, the connective tissue was trapped in glass wool similar to the procedure of Kastelic (1955).

Loyd and Hiner (1959) modified the Lowry et al. (1941) procedure slightly by retaining the fat layer from the centrifuged extraction mixture. Using the presence of hydroxyproline as a test for collagen, they stated that in the fatter samples most of the hydroxyproline was recovered from the fat fraction. In the longissimus dorsi, on the average, 71 percent of the hydroxyproline was found in the fat fraction, while in the psoas major, 58 percent of the hydroxyproline was found in the fat fraction. The percent fat in the longissimus dorsi and psoas major was 11.51 to 19.20 percent and 10.10 to 13.65 percent, respectively.

Adams et al. (1960) used the enzyme Protease-15 (Rhom and Haas) to remove all the proteinaous material, except connective tissue, from meat samples. One of the objectives of this study was to compare the collagen Kjeldahl nitrogen values using the Waring blendor method (Hartley

and Hall, 1949) and the enzyme technique to study differences in collagen content of raw and cooked steaks. The experimental material consisted of top round steaks from ten U.S. Good beef carcasses. In summarizing the study, they stated that the collagen values were lower in cooked than in raw meat by both methods, but differences between raw and cooked samples were generally greater by the enzyme method. Thus, it would appear that the enzyme method offers promise for a procedure that is reproducible, rapid, and feasible. These authors did not determine the presence of hydroxyproline as evidence of collagen degradation by the Protease-15 enzyme.

From this discussion one can readily conclude that the determination of collagen by the solubility method is somewhat empirical. Although many studies have been conducted using this method to measure collagen, many modifications have been made in an effort to improve the repeatability and/or accuracy of the method. In addition, the alkali insoluble fraction which has been commonly used as an index of collagen is a measure only of "mature" collagen. Kastelic (1955) reports that the alkali insoluble fraction does not account for 8 to 12 percent of the collagen moiety. Thus, studies using this method as a quantitation for collagen are in reality only studying approximately 90 percent of the total collagen present in the material.

Hydroxyproline methods:

The hydroxyproline concentration in a sample is the most widely used method of quantitatively estimating collagen. Dakin (1920) and Bergmann (1935) were among the first to indicate that hydroxyproline could be quantitatively measured. These workers determined hydroxyproline concentration by crystallization. By using an alcohol extraction and direct crystallization technique, Dakin (1920) reported that gelatin contained

14.1 percent hydroxyproline. Bergmann (1935) found a hydroxyproline concentration of 14.4 percent in gelatin by precipitating it as the Reinecke salt.

When using hydroxyproline as a quantitative index for collagen, one must be cognizant of the fact that there are changes in the physical structure of collagen associated with chronological age. Unless the three different collagen fractions are analyzed separately, the hydroxyproline content is only an indication of total collagen irrespective of the state of polymerization. Another variable to consider is the health of the subject from which the sample was obtained. As was pointed out earlier, in people with rheumatic fever the collagen may have a low hydroxyproline content. Recently, data from three separate research groups have proved quite conclusively the existance of a structural isomer for 4-hydroxyproline, 3-hydroxyproline. (Arlinghaus, 1962; Ogle et al., 1962; Sheehan and Whitney, 1962). The work of Ogle et al. (1962) indicated that the 3-hydroxyproline was present in the commonly found tripeptide of collagen in place of the 4-hydroxyproline. According to their work, the 3-hydroxy isomer will not react with p-dimethylaminobenzaldehye (p-DMAB). It will react, however, with ninhydrin. Using the ninhydrin reaction to quantitate the new isomer, it has been reported to account for 0.26 percent of the Achilles tendon of cattle. (Ogle et al., 1962). Consequently, the reported concentration for this imino acid depends to some extent on the reagent used for the analysis.

Ninhydrin methods - A general review pertaining to the development of and the general procedures for the reaction of ninhydrin with amino acids has been prepared by Greenstein and Winitz (1961).

Ruhemann (1911) discovered that all acids containing a free alpha-amino group reacted with triketohydrindene hydrate (ninhydrin) to give an

intense blue color. He also isolated the compound responsible for the color, diketohydrindyline-diketo-hydrindamine. In 1915, Harding and MacLean reported a colorimetric ninhydrin procedure for the estimation of amino nitrogen. They found that the technique was quantitative when an excess of ninhydrin was present. Further work by Harding and Warneford (1916), indicated that the presence of reducing agents would intensify the color of the reaction mixture. However, these workers (Harding and MacLean, 1916) published data which showed that the reaction gives positive results for compounds other than amino acids. From this work, they concluded that the ninhydrin reaction was open to criticism.

Earlier, Van Slyke (1911, 1912 and 1913-14) had used the nitrous acid reaction to quantitate the amino nitrogen in a sample. However, the reaction would not determine proline or hydroxyproline since the amino group is in the pyrrole ring. Van Slyke et al. (1941) later revised the initial mannometric technique by measuring the CO_2 released from amino acids on reaction with ninhydrin. They cited the work of Grassmann and von Armin (1934) as to the manner in which the imino acids (proline and hydroxyproline) reacted with ninhydrin. Grassmann and von Armin (1934) showed that when proline and hydroxyproline reacted with ninhydrin there was a release of CO_2 but no NH_3 was released nor was an aldehyde formed as was the case with the other amino acids. These workers also reported that the decarboxylated pyrroline reacted with one molecule of reduced ninhydrin to give a yellow product. This product then condensed with another molecule of ninhydrin to give a red product. Van Slyke et al. (1941) noted that these colored compounds were formed during the heating phase of their mannometric determination. They also noted that pH influenced the color stability of the two compounds. Although the yellow color was formed at various acidic pH's, it was more stable at pH 1.0

than at a higher pH.

It was not until 1948 that Moore and Stein developed a repeatable quantitative colorimetric ninhydrin procedure for the alpha-amino acids. In addition, proline and hydroxyproline could be determined, but not as accurately as the other amino acids. These authors observed that the yellow ninhydrin product could be measured at 440 mμ. providing the reaction was continued about twice as long in the boiling water bath.

In 1953, Troll and Cannan developed a technique to quantitatively measure hydroxyproline by differential spectrophotometry. They reported that with their reaction system the red derivatives of Grassmann and von Armin (1934) were actually intermediates in the formation of the yellow compound. Furthermore, they found that the products of ninhydrin and alpha-amino acids were insoluble in benzene, as were the yellow pigments. By continuous extraction into benzene, Troll and Cannan obtained data indicating that the products of the reaction of ninhydrin with proline and hydroxyproline would have maximum absorbancy peaks at 550 and 570 mμ. respectively. However, the red derivative of proline forms more slowly and is less stable than the one formed by hydroxyproline. Therefore, readings can be made at the above two wave lengths and the corrected concentration for hydroxyproline found by solving the two algebraic equations. These workers reported recoveries of 97.8 - 103 percent for hydroxyproline from protein hydrolysates.

Parrish et al. (1962) have modified the Troll and Cannan (1953) procedure to estimate the hydroxyproline content in alkaline hydrolysates of partially defatted and dehydrated beef muscle. They found that a decrease in the temperature of the reaction improved the reproducability of the results.

However, Aronson and Elvehjen (1956) used the original Troll and Cannan method for the analysis of hydroxyproline in animal tissues. Their results indicated that the technique per se was adequate to measure small differences in hydroxyproline concentration.

Rodgers et al. (1954) also reported the procedure satisfactory for measuring small changes in hydroxyproline in blood plasma and urine. Their measurements, however, were made on hydroxyproline that had been separated from the other amino acids by ion-exchange chromatography.

Para-dimethylaminobenzaldehyde methods - The initial work conducted in this area was by Morse (1933) and Lang (1933). Morse (1933) developed a qualitative test for hydroxyproline based upon the reactivity of the secondary alcohol group. This test involved heating a mixture of methyl-hexylcarbinol, a hydroxyproline sample, and a strong alkaline oxidizing agent (example, either Na_2O_2 or NaOH and H_2O_2). After the mixture was boiled dry and cooled, 2.0 ml. of 5.0 N HCl was added and the sample placed in a boiling water bath. The appearance of an amberrose color was indicative of hydroxyproline. The author concluded that the specificity of the reaction was due to the hydroxyl group because proline failed to give the reaction.

Lang (1933) proposed a quantitative technique for hydroxyproline. This procedure was based on first oxidizing the proline and hydroxy-proline in protein hydrolysates to pyrroline and hydroxypyrroline with NaOCl and then by steam distillation separating the oxidized imino acids from the other oxidized products. Both imino acid oxidation products were reacted with p-DMAB and then the hydroxyproline oxidized product was reacted with isatin in dilute H_2SO_4. Hydroxyproline and proline concentrations were then calculated from photometric measurements. This was accomplished by substracting the concentration of the isatin reacted

products (hydroxyproline) from the concentration of the p-DMAB products. The difference was supposed to represent the concentration of proline.

Waldschmidt-Leitz and Akabori (1934) reported work that refuted the conclusion of Lang (1933) regarding the oxidized products. These authors indicated that proline does not react with NaOCl. Also, NaOCl oxidized hydroxyproline yielded 80 percent pyrrole. If the NaOCl was destroyed before the p-DMAB step, the p-DMAB gave ten times as much absorbancy for hydroxyproline as the product with isatin. They reported that gelatin contained approximately 9.0 percent hydroxyproline.

Guest and McFarlane (1939) reported that the p-DMAB ". . . is lacking in specificity, being given by substituted pyrroles, pyrrolines, pyrrolidines, and indoles. On the other hand, the isatin reaction is highly specific, being given only by pyrroles with an α- position unsubstituted and not given by reduced pyrroles or indole derivatives." Furthermore, interfering substances could be avoided in the isatin reaction mixture by extracting the pyrrole with ether. These authors reported a hydroxyproline value for gelatin of 14.1 percent based upon the removal of pyrrole from gelatin by dry distillation and then reaction with isatin. In fact, their data suggested that proline and hydroxyproline accounted for all the pyrrole derived from gelatin. Data were also presented on the effect of different oxidizing agents on the pyrrole yield. Their results showed that only Na_2O_2 increased the pyrrole concentration. Other oxidizing agents studied were NaOCl, $FeCl_3$, H_2O_2 and alkaline permanganate. When the imino acids were oxidized with Na_2O_2 in the presence of $CuSO_4$, the apparent hydroxyproline content was decreased and the proline increased.

McFarlane and Guest (1939) found that if a solution of hydroxyproline, sodium peroxide and dilute cupric sulfate is acidified with hydrochloric

acid, a red color developes when the solution is placed in boiling water. No other amino acid, including proline, gave a positive test under these conditions. These workers studied the influence of $CuSO_4$ in an effort to determine the reason for the specificity of this reaction. When different sulfates were used in place of $CuSO_4$ and the resulting products reacted with isatin, the color values were low. From these data, they concluded that the complex, ". . . may possibly be a complex copper salt of a dipyrrylmethene." A 1:1 mixture of six percent H_2O_2 and ten percent NaOH was found to minimize the variable results obtained with solid Na_2O_2. The reason for this change in the oxidizing agent was that the rate of oxidation could be somewhat controlled by forming Na_2O_2 in situ. Using this information, they developed a photometric procedure for determining hydroxyproline in protein hydrolysates. The initial step was to form the copper complex by using a solution containing 1.0 ml. each of hydroxyproline, 0.01 M $CuSO_4$, 10% NaOH, and 6% H_2O_2. After oxidizing at room temperature the samples were placed in a boiling water bath to complete the oxidation. The oxidized solution was then cooled, acidified, and then reacted with isatin in a boiling water bath. Maximum absorbancy for the stable product from isatin was 520 mμ. Results from different gelatin hydrolysates indicated that hydroxyproline accounted for approximately 14.0 percent of the amino acids present.

Neuman and Logan (1950a), along with Devine (1941), have reported that the McFarlane and Guest (1939) hydroxyproline procedure is not accurate. Both groups of workers indicated that the results from gelatin were much lower than the generally accepted value of approximately 14 percent hydroxyproline. To obtain a more accurate and precise colorimetric technique, Neuman and Logan (1950a) developed a procedure based on the work of McFarlane and Guest (1939), Guest and McFarlane (1939),

and Guest (1939). This new procedure or minor modifications thereof, has been one of the more commonly used methods for determining the concentration of hydroxyproline in tissue hydrolysates.

The basic oxidizing step of McFarlane and Guest (1939) was not changed by Neuman and Logan (1950a). However, they did change the first heating phase. Instead of placing the reaction tubes in a boiling water bath, they were placed in an 80°C. water bath for five minutes with occasional shaking. They stated that, "The heating and shaking destroys the excess of peroxide. Traces of peroxide which remain will decrease color formation and produce an orange-red hue." Neuman and Logan (1950a) completely changed the steps involved in developing the chromophoric group. One of the major modifications was the use of p-DMAB in place of isatin to form the color complex. Prior to adding the p-DMAB to the reaction mixture, Neuman and Logan (1950a) acidified the mixture with H_2SO_4 instead of HCl. To obtain maximum absorption, they used a 70°C. water bath in place of the boiling water bath used by McFarlane and Guest (1939) to develop the color complex. According to Neuman and Logan (1950a) a 540 mμ filter should be used to determine A. in place of the 520 mμ filter recommended by McFarlane and Guest (1939) or Guest (1939). Neuman and Logan (1950a) also reported that tyrosine and hydroxyproline were the only two amino acids in acid hydrolyzed proteins which reacted with p-DMAB in sufficient quantity so that their absorption values could be measured. However, tyrosine yielded only 1.5 percent as much color as did hydroxyproline. In addition, recrystallized proline gave A. values corresponding to 0.17 percent that of hydroxyproline. Although these results were in contrast to the work of Guest (1939), the data presented by Neuman and Logan indicated that reagent-grade proline contained hydroxyproline. Consequently, nonrecrystallized proline reacted with p-DMAB to such a degree

that the p-DMAB reaction appeared to not be specific for hydroxyproline.

Many modifications have been made in the Neuman and Logan (1950a) procedure. These modifications have generally been concerned with one or more of the following three areas: 1. destruction of the excess hydrogen peroxide; 2. stabilization of the p-DMAB hydroxyproline complex; and 3. correction for interfering color complexes, such as that produced by tyrosine and/or excessive concentration of the other amino acids.

To eliminate the excess H_2O_2, Leach (1960) conducted the oxidation and destruction of H_2O_2 steps together in a 40°C. water bath. By following this procedure, "The oxidation . . . is completed before the total destruction of the excess of peroxide has occurred." (Leach, 1960). However, Lollar (1957) reported that the excess hydrogen peroxide could be destroyed by vigorous shaking after oxidation was complete. Thus, the 80°C. step was deleted from the procedure. Martin and Axelrod (1953) used a combination of acidified, dilute ferric sulfate and shaking in place of the 80°C. step to destroy any unreacted peroxide.

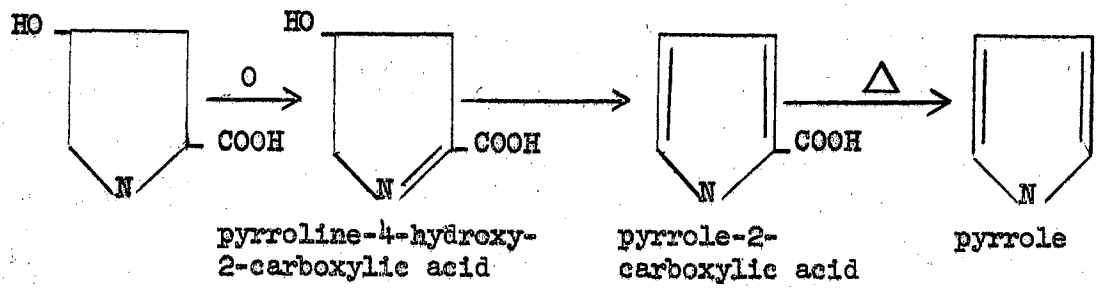
Another approach was tried successfully by Hutterer and Singer (1960). These authors decreased the temperature of the first water bath to 70°C. After five minutes in the water bath, the excess H_2O_2 was removed by pulling a vacuum on each tube for 30 seconds. Chloramine T has been used in place of H_2O_2 as the oxidative agent. (Prockop and Udenfriend, 1960). This change was made because it is available in a stable form and solutions of reproducible strength can be prepared readily. An excess of alanine was added to help control the oxidation step, as well as destroy any excess chloramine T. Wiss et al. (1949) have proposed that a mixture of sodium carbonate and hypochlorite may be used as the oxidizing agent. By forming sodium hypochlorite in situ, the reaction rate was slower and more complete. Glutamic acid was then added to

destroy excess hypochlorite.

Instability of the color complex has generally been a problem whenever the p-DMAB technique is used. To avoid variation due to fading, many workers have merely read the samples at a definite time interval after color formation. Woessner (1961) overcame this problem with samples containing a low hydroxyproline concentration by using a fading constant. This constant was then used in a mathematical formula to calculate the corrected A. absorbancy. According to Baker et al. (1953), Lollar (1957), and Hutterer and Singer (1960), a majority of the instability can be associated with impure p-DMAB. To purify p-DMAB, the method of Neuman and Logan (1950a) will often be used. Another method (Adams and Coleman, 1941) involving reprecipitation and recrystallization also can be used to purify p-DMAB. Mitoma et al. (1959) reported that, "The rapid formation and fading of the chromophore described by Wiss et al. (1949) in his method is due to the concentrated hydrochloric acid used in the procedure." These authors employed a less concentrated acid (1.0 ml. of 2.0 N H_2SO_4) and controlled the heating conditions to overcome the instability of the color complex. Miyada and Tappel (1956b) obtained similar results with the Neuman and Logan (1950a) procedure. Consequently, they decreased the concentration of the H_2SO_4 from 3.0 to 1.5 N. The alcohol used has also been shown to influence color stability (Hutterer and Singer, 1960). These authors reported that, "Both increased concentration and lengthened alkyl chain of the alcohol (lower dielectric constant) used result in enhanced intensity and stability of the color."

The problem of interfering chromogens will often cause problems with colorimetric procedures. In the p-DMAB procedure, the two most common type chromophores influencing the A.values result from tryrosine, as well

as the other amino acids. The tyrosine contribution has been quantitated and its influence removed from the observed A.values. (Wierbicki and Deatherage, 1954; Miyada and Tappel, 1956b). However, Stegemann (1958) reported that tyrosine did not interfere when chloramine T was used as the oxidizing agent. When Grunbaum and Glick (1956) modified the Neuman and Logan (1950a) technique for histochemistry studies, their data suggested that the use of 2.0 percent H_2O_2 decreased the influence of tyrosine to less than 1.0 percent of the hydroxyproline value. Baker *et al.* (1953) have defined the influence of other amino acids: "When other amino-acids (as a mixture or in the form of protein hydrolysates) were present in the ratio of amino-acid to hydroxyproline of 8 to 1, the colour density was not affected; a slight decrease in colour density was noted at a ratio of 50 to 1, which became more marked at ratios of 100 to 1 and 1200 to 1." These conclusions were confirmed by Lampitt *et al.* (1954). They avoided the influence of the other amino acids by using defatted freeze-dried meat and direct acid hydrolysis. This procedure was selected over 0.10 N NaOH extraction and autoclaving to obtain only the collagen fraction because, ". . . the dissolution of the collagen on autoclaving is not quite complete." According to Prockop and Udenfriend (1960), hydroxyproline undergoes an oxidation and decarboxylation to form pyrrole during the various steps of the procedure. They indicated that the following compounds were formed during the degradation:



pyrroline-4-hydroxy-2-carboxylic acid

pyrrole-2-carboxylic acid

pyrrole

On the basis of this principle, several procedures have been developed to extract the intermediate compounds. Wiss et al. (1949) used a distillation step after oxidation to remove the intermediate compounds and then they reacted them with p-DMAB. Mitoma et al. (1959), in referring to their modification of the Neuman and Logan (1950a) technique, stated that, "The present procedure involves extraction of pyrrole-2-carboxylic acid with ether to separate it from the degradation products formed from such compounds as tyrosine. . . thus, conferring specificity to the method." Prockop and Udenfriend (1960) used toluene in place of ether. Woessner (1961) allowed the color complex to form with all the compounds in the reaction mixture and then corrected for the interfering chromogens. Corrections were based on the theory that the color complexes formed by the other chromogens were not sensitive to oxidation by hydrogen peroxide. Thus, a reading was taken before and after a peroxide treatment. The difference between the first and second reading equalled the correct value for hydroxyproline concentration. Differential spectrophotometry has been used very effectively to correct for all interfering color complexes. (Hutterer and Singer, 1960). These workers stated that, "This interfering absorbance constitutes about 20 to 25 percent (23.7 ± 3.0) of the total absorbance at 560 mμ." By making readings at 500 and 560 mμ and using their correction formula, Hutterer and Singer (1960) reported that, "Recovery experiments yielded satisfactory results, the standard deviation from the mean being 2.8%. . ."

Although numerous problems have been encountered in using the basic Neuman and Logan (1950a) procedure, changes have been made which contribute to its increased reliability for quantitatively determining hydroxyproline. However, it appears that the ratio of amino acids to hydroxyproline must be less than 50 to 1. (Baker et al., 1953).

In order to minimize the interference from a high ratio of amino acids to hydroxyproline, there are procedures available which may be used to eliminate the excess amino acids prior to using the colorimetric procedure. One method is that of nitrosation. This reaction can be used since the amino group is split from the aliphatic chain and replaced by a hydroxyl group when the reaction is carried out at room temperature. Therefore, the only amino acids which will be available to condense with the p-DMAB are proline and hydroxyproline. Several procedures have been published on the applicability of the nitrosation principle. (Van Slyke, 1911 and 1912; Hamilton and Ortiz, 1950; Levine, 1959). Ion exchange chromatography may also be employed to quantitatively separate hydroxyproline from the other amino acids. This is in fact one of the most commonly used techniques for separating amino acids prior to quantitative and qualitative tests. (Leach, 1960; Levine, 1959; Moore et al., 1958; Moore and Stein, 1951; Piez et al., 1956; and Rodgers et al., 1954).

STUDIES OF METHODS FOR MEASURING COLLAGEN BY HYDROXYPROLINE DETERMINATIONS

Ninhydrin Methods

This investigation was undertaken to determine the cause or causes for high recovery values obtained when the Parrish modification for hydroxyproline was used to analyze similar meat samples.

Experimantal procedures

Techniques reported by Troll and Cannan (1953) and Parrish et al. (1962) were followed along with modifications of the latter procedure. Assay grade ninhydrin, redistilled methoxycellosolve and deionized distilled water were used to minimize variation in the results due to the reagents. Anhydrous sodium sulfate was used as a dessicant to obtain an optically clear solution. Prior to determining the absorbancy of the reaction mixture, the sodium sulfate was removed by filtering the solution through Whatman No. 42 filter paper.

Results and discussion

A decrease in the water bath temperature from 75° to 71°C. was the major change in the Troll and Cannan (1953) procedure made by Parrish et al. (1962). This study verified the conclusion that more repeatable results are obtained by using the lower temperature; although the lower temperature decreased slightly the maximum absorbancy values. Therefore, a water bath temperature of 71° $\pm$ .25°C. was used throughout the remainder of this study.

Table I indicates the influence of time after completion of the reaction on the variation and maximum A_{λ} values of standard hydroxyproline determinations. From this experiment and others similar to it, all subsequent samples were stored in the dark one hour prior to determining A_{λ} values.

TABLE 1

INFLUENCE OF TIME ON THE CORRECTED A_{566} VALUES FOR STANDARD HYDROXYPROLINE

Time (Hours)[1]	Replicates[2] 1	2	3	Average	Maximum Difference
0.25	0.229	0.226	0.221	0.225	0.008
0.50	0.232	0.227	0.232	0.230	0.005
1.25	0.240	0.246	0.246	0.244	0.006
2.00	0.244	0.246	0.246	0.245	0.002
2.25	0.243	0.245	0.245	0.244	0.002

[1]Stored in the dark.
[2]200 µg/ml. concentration.

Maximum absorbancies for proline and hydroxyproline, when reacted with ninhydrin, have been reported to be 550 and 570 mµ, respectively. (Troll and Cannan, 1953). However, scanning of the visible spectrum of hydroxyproline products by this method indicated that the maximum absorption was 566 mµ (Fig. 1). The maximum absorbancy for ninhydrin reacted with proline (Fig. 2) agreed with that of Troll and Cannan (1953). These above results were obtained using the Beckman Model D.U. spectrophotometer.

Recovery studies were conducted on mixtures containing assay grade proline and hydroxyproline. Absorbancy was determined at 570, 566, and

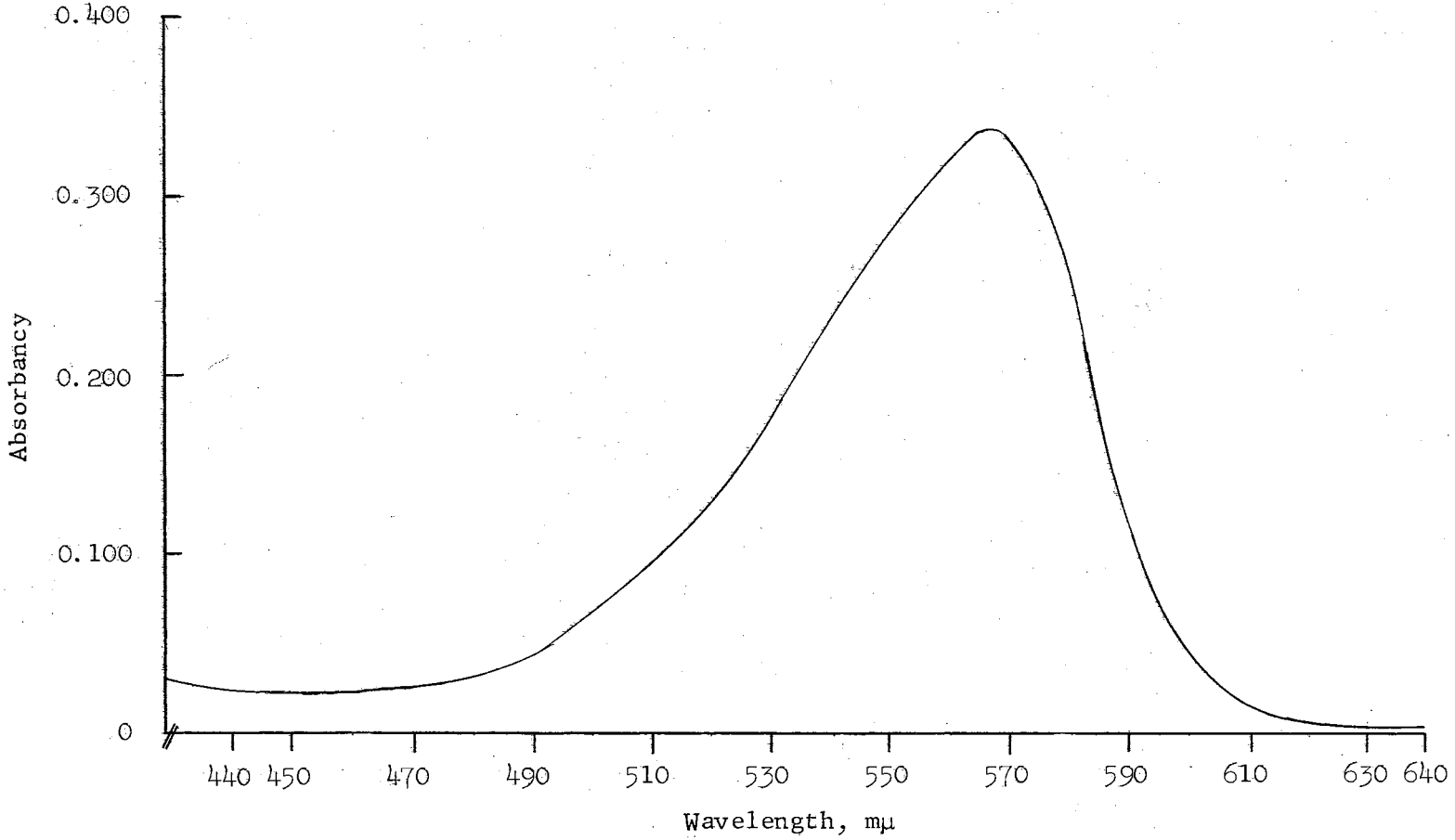


Figure 1: Visible Spectrum of the Reaction Products of Ninhydrin and Hydroxy-L-Proline Obtained with a Model 14, Carey, Recording Spectrophotometer.

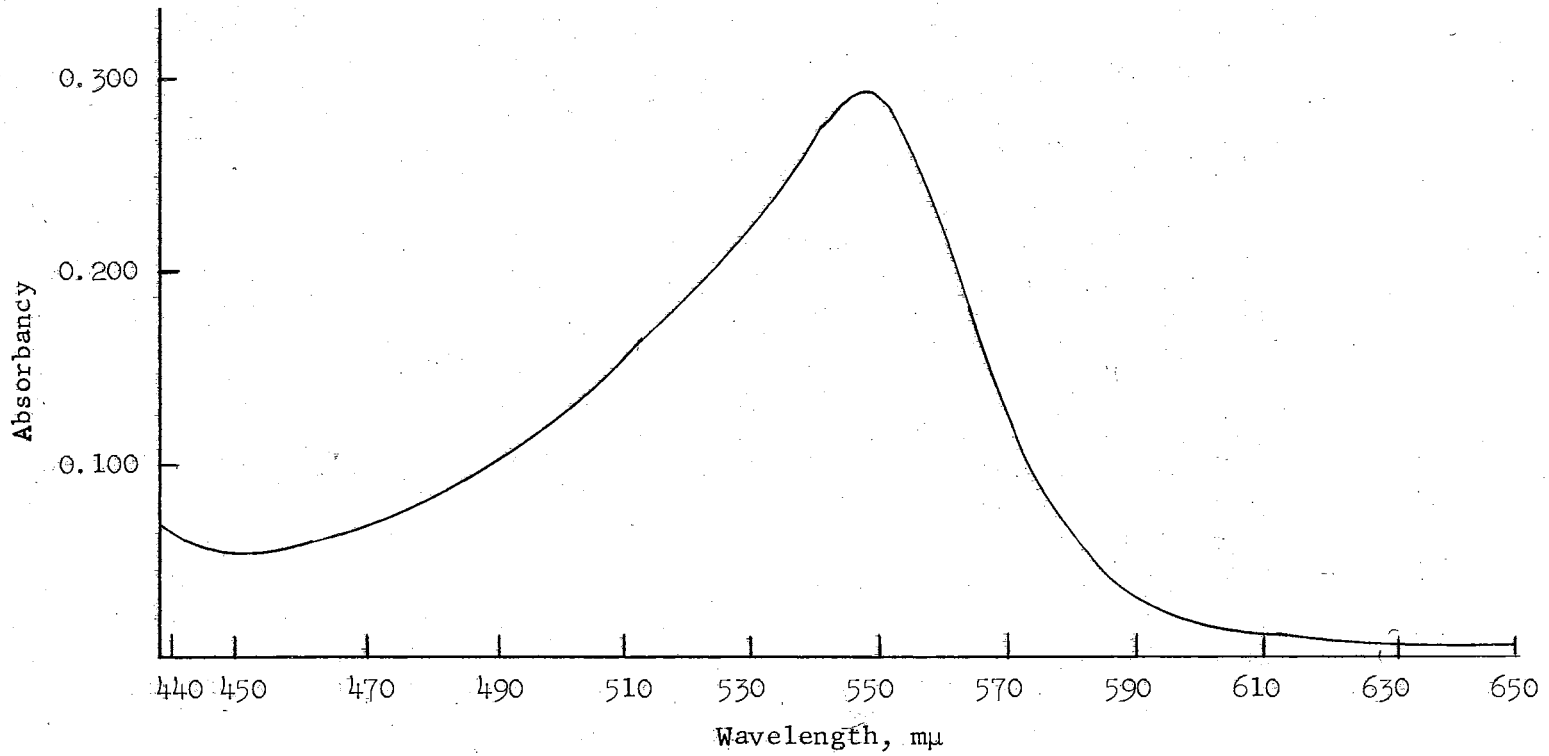


Figure 2: Visible Spectrum of the Reaction Products of Ninhydrin and Proline (1 mg/ml) Obtained with a Model 14, Carey, Recording Spectrophotometer.

550 mμ. Using the formula of Troll and Cannan (1953), Hypro A_{570} = A_{570} x 1.46 - A_{550} x 0.592, the recovery was approximately 95 percent. When the A_{570} value was replaced by the A_{566} value, the percent recovery was increased to about 98 percent. Furthermore, the corrected A_{566} value for hydroxyproline was similar to the observed A_{570} value for the reaction products. This same phenomenon did not hold true when the corrected A_{566} and the observed A_{570} were compared on the reaction mixture of meat hydrolysates.

Similar recovery studies were conducted using acid hydrolyzed meat samples. The samples were 500 mg. of partially "dried and fat-free", finely ground beef ribeye. (Parrish et al., 1962). These samples were placed in a covered beaker containing 40 ml. of 3 N HCl and autoclaved at 121°C. for ten hours. They were then neutralized according to the Swift and Company procedure for microbiological assays. (Mason, 1962). Mixtures containing 100 μg/ml. hydroxyproline and various concentrations of hydrolysate were used in this recovery study. The percent recovery of hydroxyproline varied from 128.0 to 141.0. Curves for absorbancy in the visible spectrum from this study indicated that there were two peaks of maximum absorption due to ninhydrin reaction products extracted into the benzene layer (Fig. 3).

Studies were then conducted to elucidate the reaction products responsible for these high recoveries. The factors studied were the influence of hydrolysis time and concentration of HCl. Figure 3 illustrates the visible spectrum from representative determinations. These graphs indicate that the increase in hydrolysis time and HCl concentration decreases the absorbancy at 500 mμ., although there was essentially no change in the absorbancy at 560 mμ. However, there was no change in the

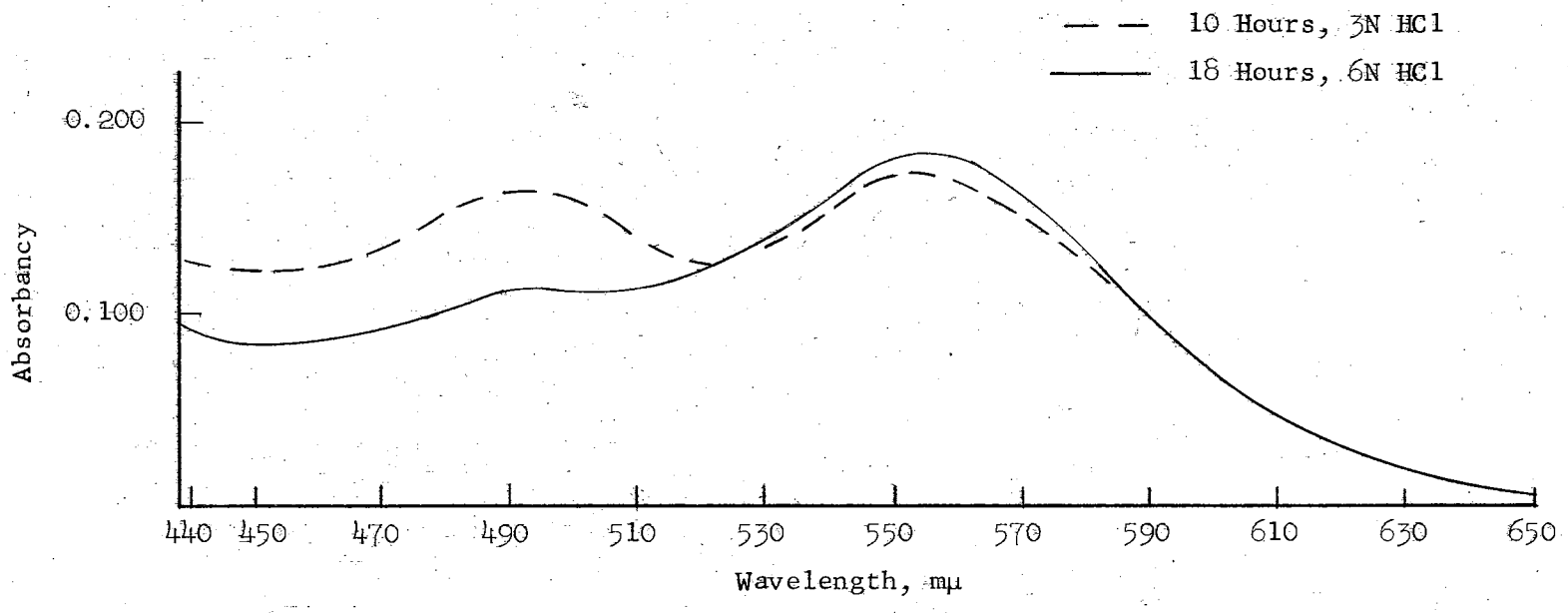


Figure 3: Visible Spectrum of the Reaction Products of Ninhydrin and Meat Samples (8 mg/ml) Hydrolyzed Under Different Conditions. Spectrum Obtained with a Model 14, Carey, Recording Spectrophotometer.

percent recoveries from the 10 hour, 3 N HCl hydrolysates and the 18 hour, 6 N HCl hydrolysates.

Studies were then initiated using synthetic amino acids in an effort to determine whether ninhydrin reacted amino acids were responsible for the absorption peaks at 500 and 560 mμ. Figure 4 illustrates the visible spectrum of the reaction products formed between ninhydrin and the 18 naturally occurring amino acids in a microbiological assay. This graph indicates that there are ninhydrin reacted amino acids absorbing at 500 and 560 mμ. Since proline was present in the above mixture, a synthetic hydrolysate was made according to Greenwood et al. (1953). Trytophan, proline, and hydroxyproline were deleted from the Greenwood mixture. To help explain these absorption peaks, the visible spectrum was scanned for each reagent used in the reaction mixture. Also, spectra were obtained for the reaction products of ninhydrin with glycine (representative of the alpha amino acids) and the aromatic amino acids; histidine, tyrosine, and phenylalanine. Results of this experiment indicated that: 1. the ninhydrin mixture was the only reagent absorbing in the visible spectrum, 2. the ninhydrin reaction products of glycine, histidine, and tyrosine had maximum absorption at 500 mμ. (Fig. 5); and 3. phenylalanine shows several absorption peaks in the visible spectrum (Fig. 5). These results indicate that the large 500 mμ. peak observed in the synthetic hydrolysate (Fig. 6) is due to the ninhydrin reaction products of amino acids, other than hydroxyproline, being somewhat soluble in benzene. Hydroxyproline was then added to the synthetic hydrolysate, holding the concentration of the hydrolysate constant to give 0.005 A. Results from several experiments indicated that the percent recovery of hydroxyproline from these mixtures was similar to those obtained with the meat hydrolysates plus known amounts of hydroxyproline. Figure 6 is

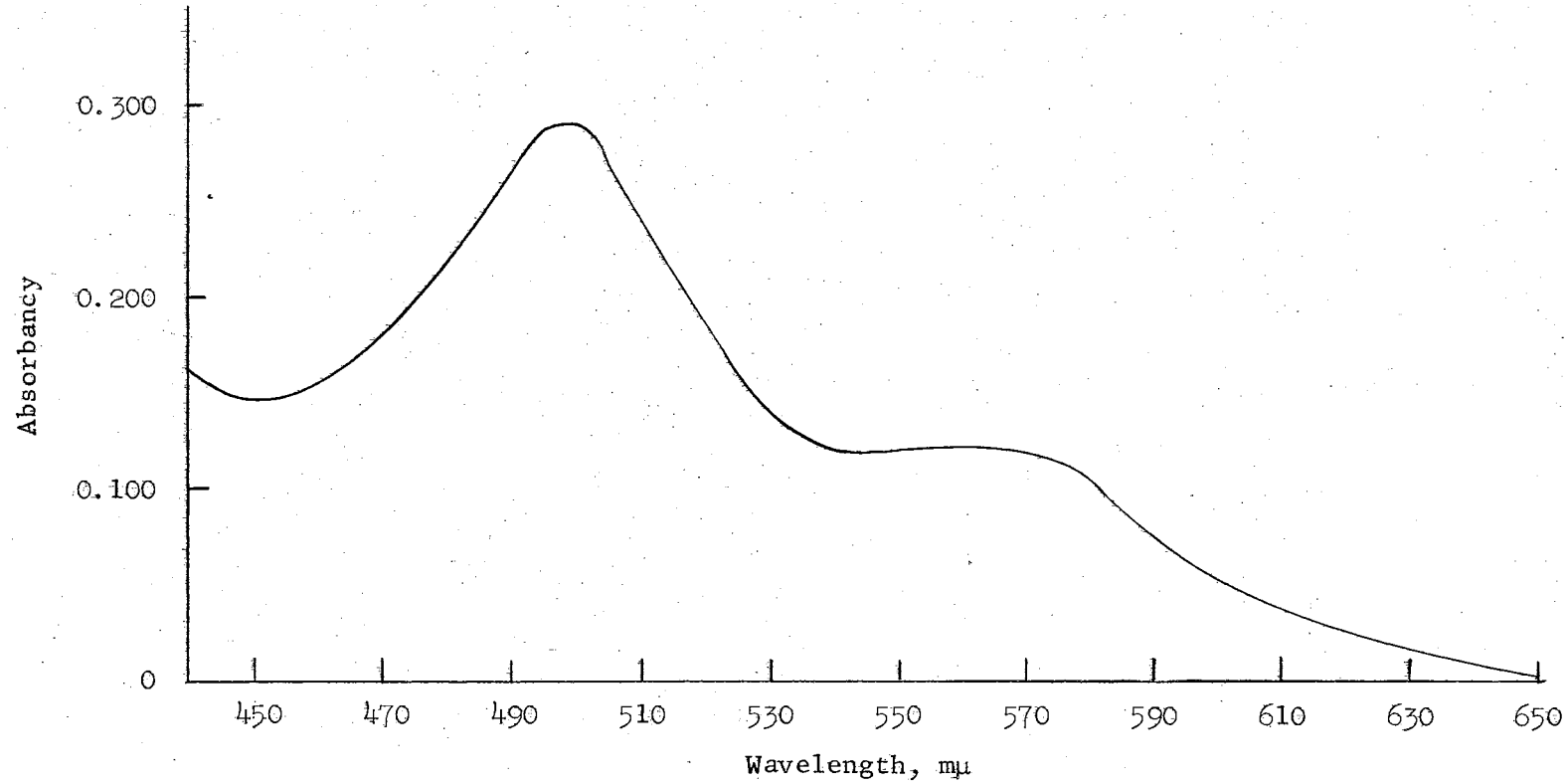


Figure 4: Visible Spectrum of the Reaction Products of Ninhydrin and the Naturally Occurring Amino Acids Obtained with a Carey, Model 14, Recording Spectrophotometer.

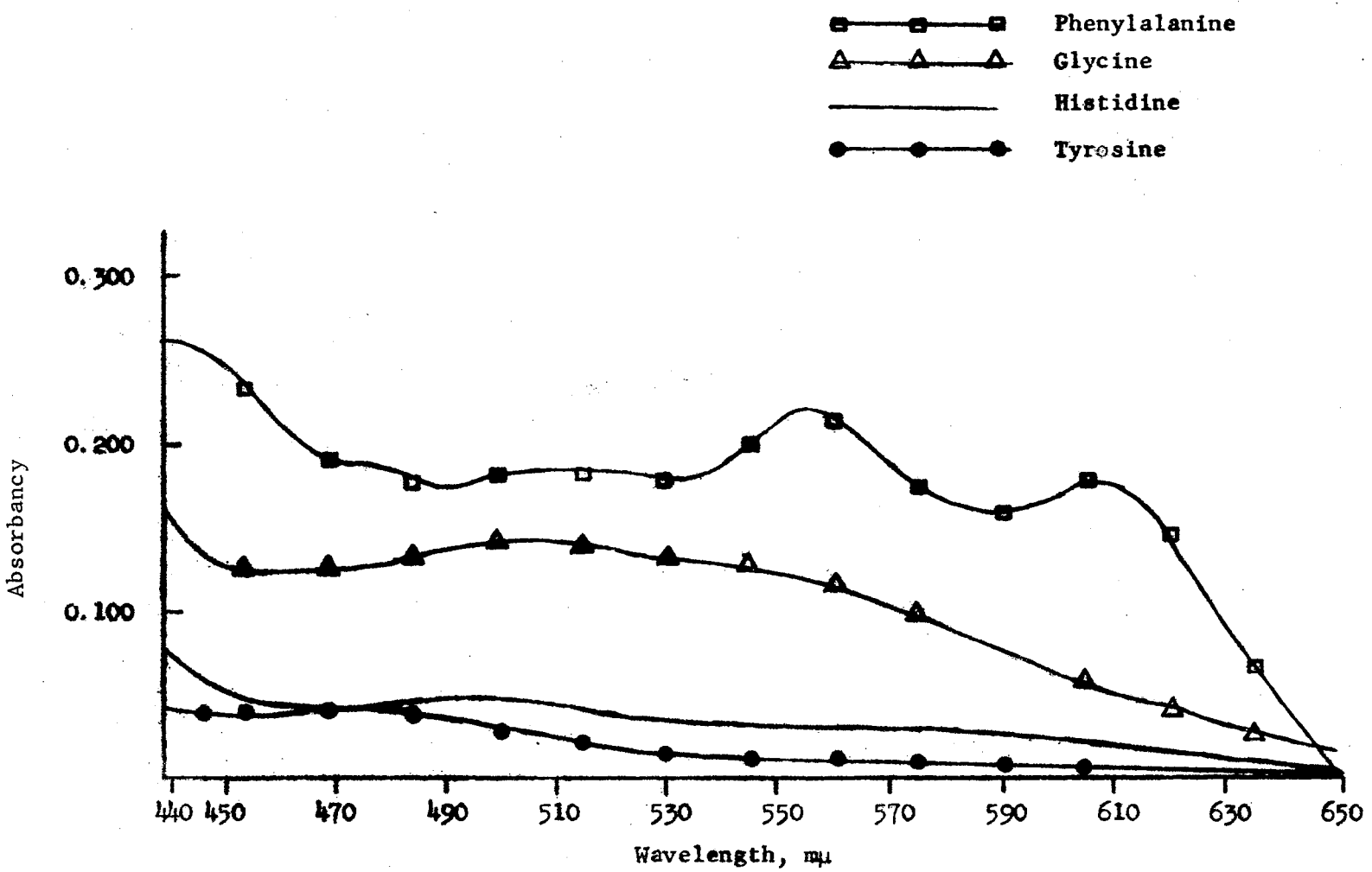


Figure 5: **Visible Spectrum of the Reaction Products of Ninhydrin and Phenylalanine, Glycine, Histidine and Tyrosine**

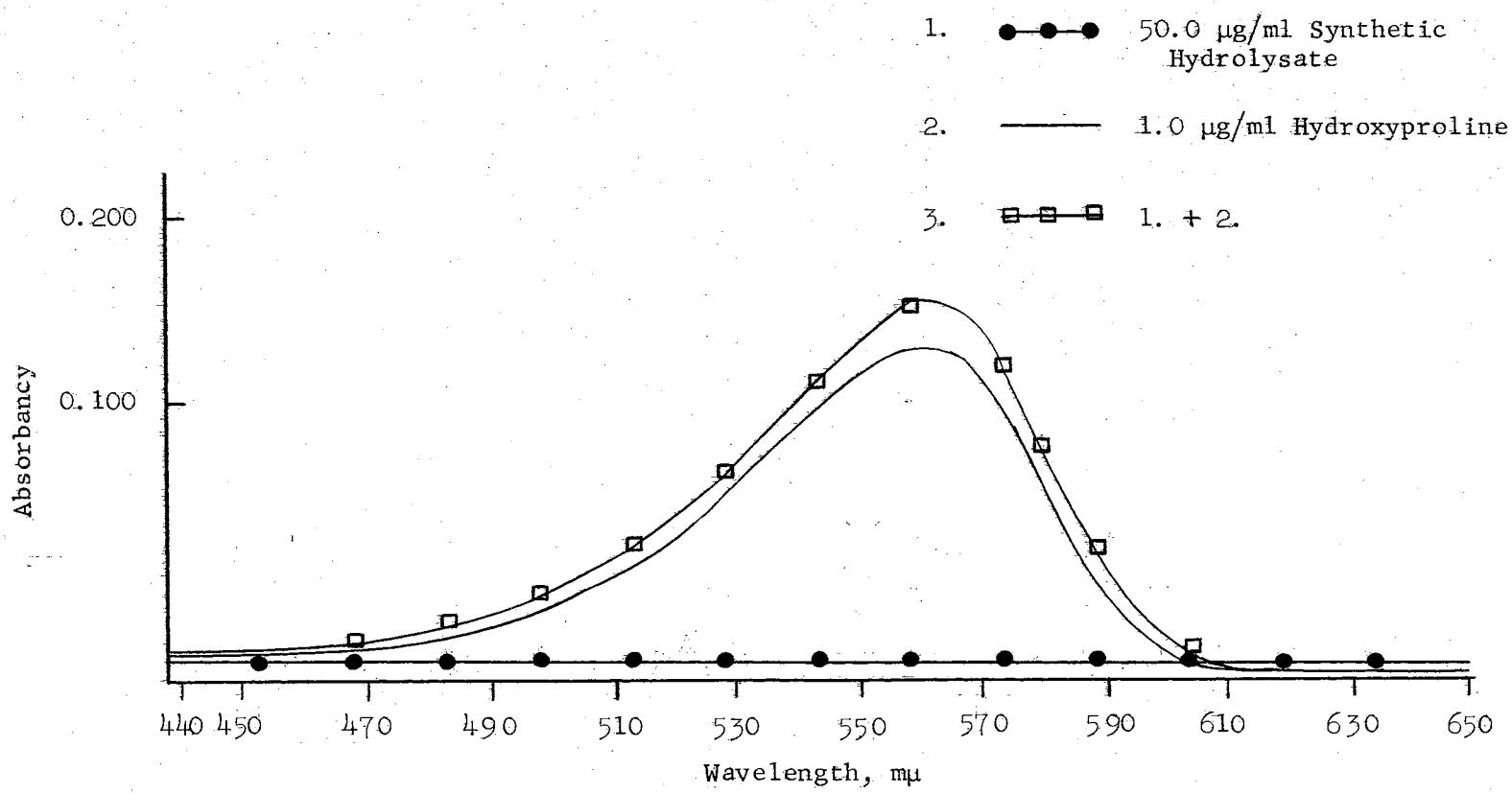


Figure 6: Visible Spectrum of the Reaction Products of Ninhydrin and a Synthetic Amino Acid Mixture and Hydroxyproline

representative of the visible spectrum obtained from these experiments.

Summary and conclusions

Based on the variable and high percentage recovery, this phase of the investigation indicates that neither the Troll and Cannan method (1953) nor the modification of Parrish et al. (1962) is a desirable quantitative technique to measure small differences in hydroxyproline in meat hydrolysates. However, Rodgers et al. (1954) have adequately shown that this method will give very desirable results (98 percent recovery) if the hydroxyproline is first separated by ion-exchange chromatography.

Para-Dimethylaminobenzaldehyde Methods

The purpose of this study was to determine the feasibility of assaying for hydroxyproline by using a p-DMAB reaction.

Experimental procedures

Two modifications of the Neuman and Logan (1950) procedure were used during the course of this investigation. The procedures were those of Ritchey et al. (1962), and Hutterer and Singer (1960). All chemicals used in this study were reagent grade. In addition, the n-propanol was redistilled and the p-DMAB was reprecipitated and then recrystallized. (Adams and Coleman, 1941). To obtain maximum yield and purity of p-DMAB, the addition of alkali was stopped just prior to the point of complete neutralization. The reprecipitated p-DMAB was air-dried on a Buchner funnel attached to a water aspirator and recrystallized three times from absolute ethanol. It was then dried and stored in a desiccator over $CaSO_4$.

At the beginning of this study all the solutions were prepared daily. During the final stages of this investigation, only the hydrogen peroxide, p-DMAB, and ferrous sulfate (Ritchey et al., 1962) were prepared daily.

The hydrogen peroxide solutions were prepared from a 30.6 percent stock solution. Acid hydrolysis of gelatin and meat sample was conducted as outlined on page 54. Absorbancy was determined with a Beckman Model D.U. spectrophotometer.

Results and discussion

The Ritchey et al. (1962) procedure was used during the initial phase of this investigation. This procedure was modified due to the poor repeatability between duplicate aliquots within a determination. This particular problem was overcome by standardizing the shaking steps conducted at room temperature. The modification consisted of shaking each tube with a Cyclo-Mixer for five seconds once every minute. Figure 7 illustrates a typical hydroxyproline standard curve from a study in which the manual shaking steps were replaced with the mechanical standardized steps. Although this procedure was repeatable within an assay, it often lacked repeatability between assays. The data in Table II indicates the variation in results from two samples (6R and 6L) when the concentration of hydroxyproline was determined at two different times (A and B). With this amount of variation between duplicate assays, the Ritchey et al. (1962) procedure may not be desirable to quantitate small differences in hydroxyproline for a large number of samples.

TABLE II

VARIATION BETWEEN AND WITHIN ASSAYS BY THE RITCHEY PROCEDURE

Assay	Sample (μg. Hypro Per mg. sample)					
	6R			6L		
	1	2	Av.	1	2	Av.
A	2.88	2.88	2.88	2.53	2.56	2.54
B	3.50	3.41	3.46	3.36	3.64	3.50

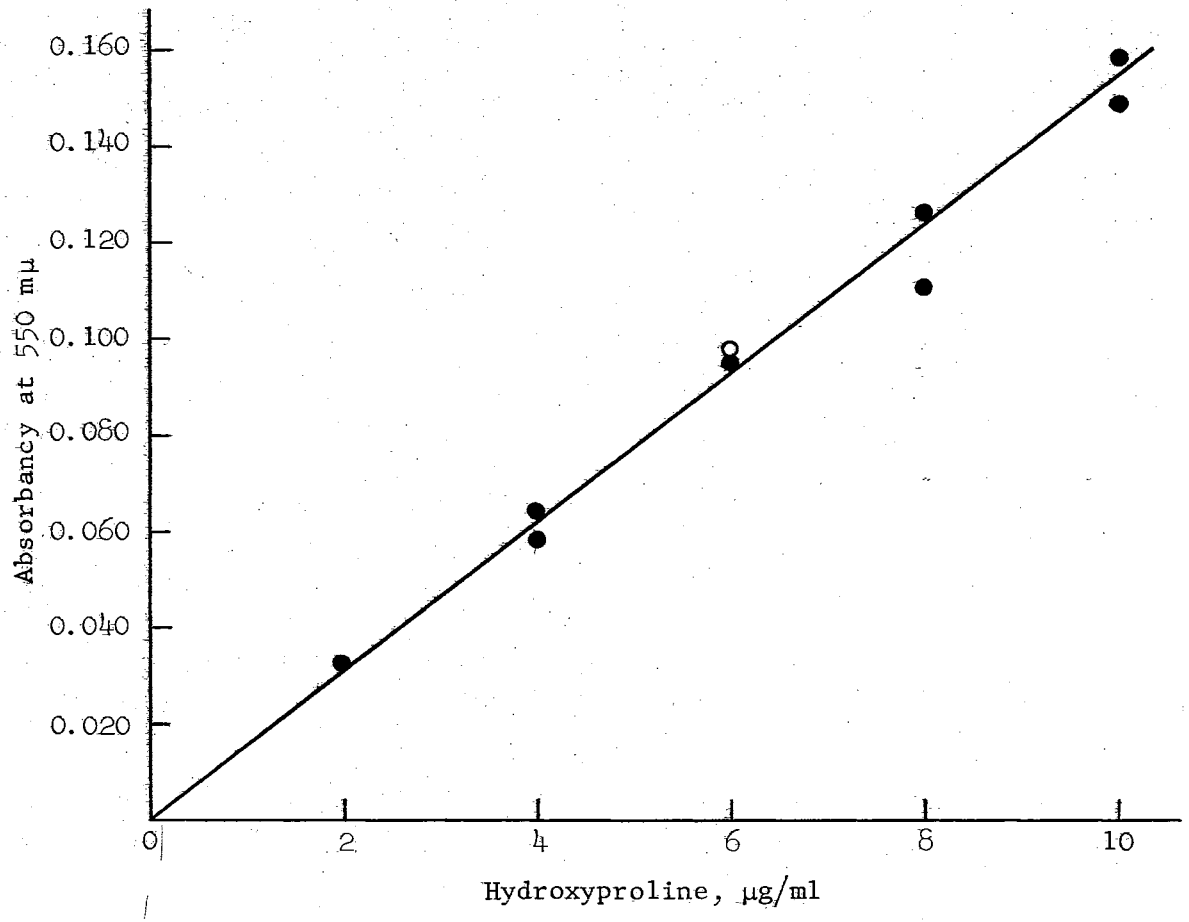


Figure 7: Hydroxy-L-Proline Standard Curve by the Ritchey Procedure

Preliminary studies indicated that the precision of the Hutterer and Singer (1960) technique was adequate for assaying a large number of samples, over a period of time, which have a small variation in hydroxyproline concentration. During these initial investigations, the vacuum stage of the procedure was conducted by two methods. In one method, the vacuum was applied by using the Buchler "Roto-Vap" and applying the vacuum for 30 seconds. The other method involved removing the test tubes from the 70°C. water bath and pulling a vacuum to 50 mm. mercury (approximately 30 seconds). Water aspirators were used to pull the vacuum for both methods. The results from these two methods were essentially the same for hydroxyproline standard curves, $K_{560} = 0.035$ A. per µg hydroxyproline.

Recovery studies were also conducted using both methods. Again, these methods gave similar results, approximately 100 percent recovery of hydroxyproline from meat hydrolysates. Consequently, the remainder of the investigations were conducted using the method by which the excess hydrogen peroxide was removed by pulling a separate vacuum on each individual sample. This method was selected because of the accessibility to all pieces of equipment involved in conducting a hydroxyproline assay.

The day to day repeatability for this method was determined by the variation of the regression coefficients at A_{560} and A_{500}. These data indicated that this technique was very precise, $K_{560} = .0351 \pm .0001$ and $K_{500} = .0116 \pm .0001$ A. per µg. hydroxyproline. Figure 8 illustrates a hydroxyproline standard curve from one of these studies.

Partially "dried and fat-free" meat samples (500 mg.) were used to determine the optimum time for acid hydrolysis. The results of this study indicated that the maximum concentration was obtained at 18 hours (Fig. 9).

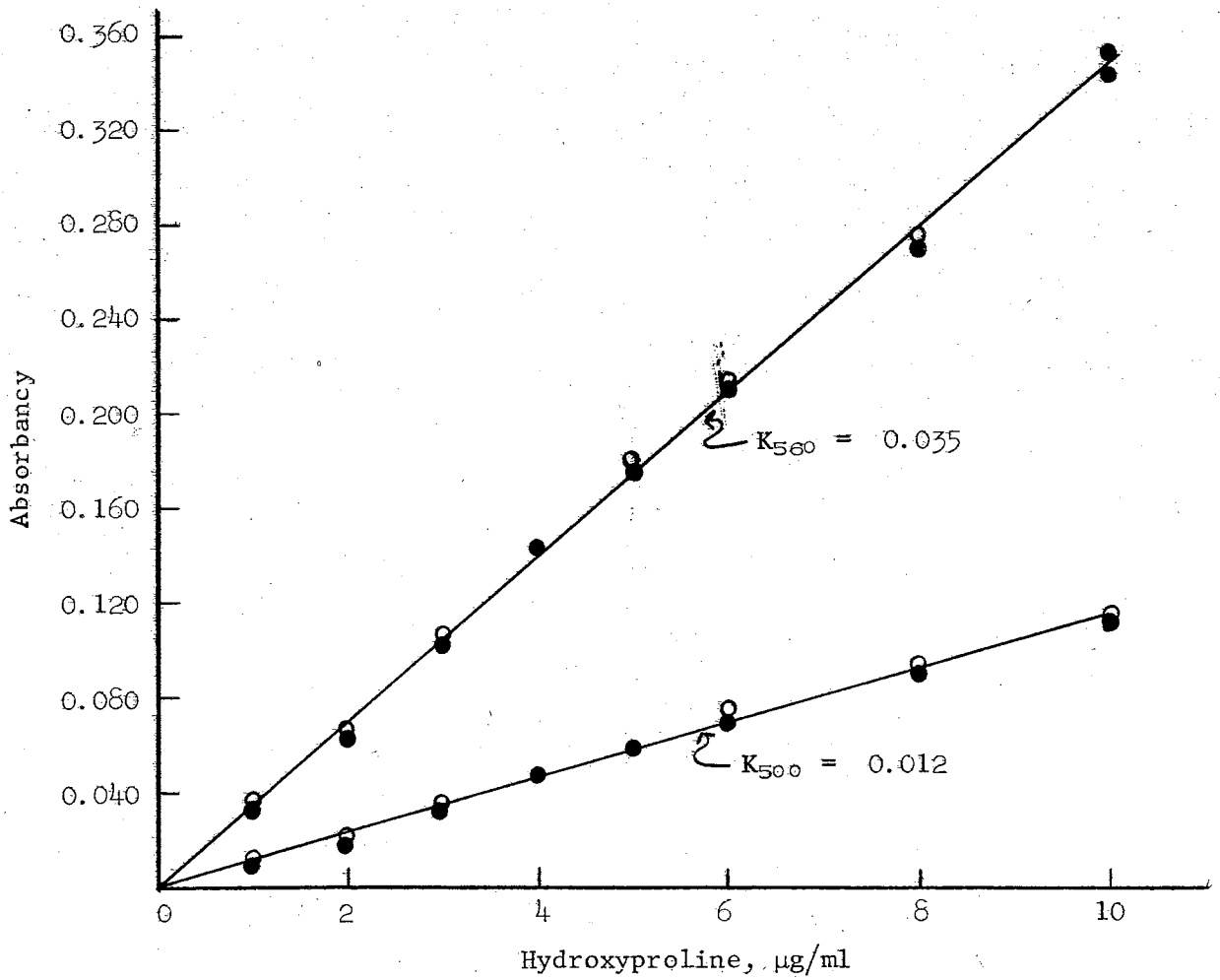


Figure 8: Hydroxy-L-proline Standard Curve by the Hutterer and Singer Procedure

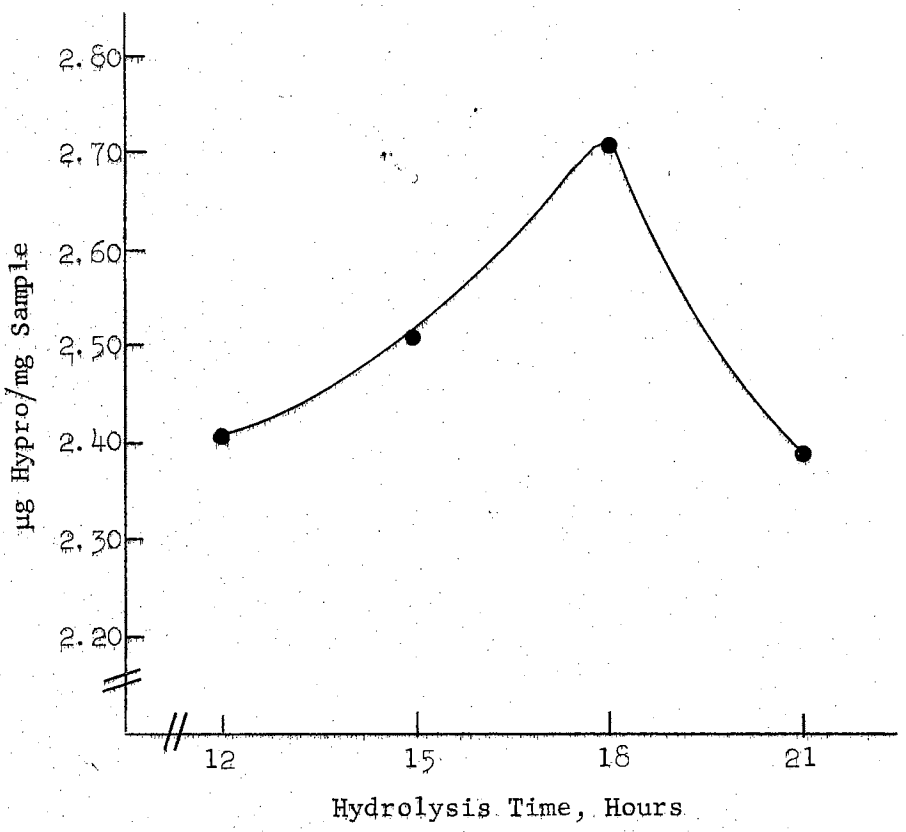


Figure 9: Optimum Hydrolysis Time

Also included in this study was the loss of hydroxyproline due to the hydrolysis and neutralization procedures. These data suggested that with this procedure there is no loss of hydroxyproline, whether it is autoclaved alone or with a meat sample.

Before further investigations were conducted, the repeatability of the hydrolysis and neutralization procedure was studied. The samples consisted of 250 mg. of Difco-Bacto gelatin which was hydrolyzed and neutralized on seven different days. Table III contains the data from these seven gelatin samples. These results indicated that the mean and standard deviation was 12.97 $\pm$ 0.31 µg./mg. Thus, the variation between samples was 2.39 percent of the mean ($\bar{x}$/SxX100).

TABLE III

VARIATION IN THE HYDROXYPROLINE CONCENTRATION OF GELATIN SAMPLES

Duplicate	Sample Number							Average
	1	2	3	4	5	6	7	
A	12.85	12.70	13.13	12.70	13.00	12.85	12.55	12.83
B	13.45	13.45	13.00	12.55	13.45	12.85	13.00	13.11
Average	13.15	13.08	13.06	12.63	13.22	12.85	12.78	12.97

Another investigation was initiated to determine the optimum concentration for quantitating the hydroxyproline content in meat samples. The results are illustrated in Figure 10. These data indicated that there were two straight line portions in the dilution curve, one from 0 to 1 and another one from 2 to 5 mg. of sample. The apparent reason for the curvilinear portion was the influence of the color from humin formation. When a gelatin sample was diluted, the dilution curve was a straight

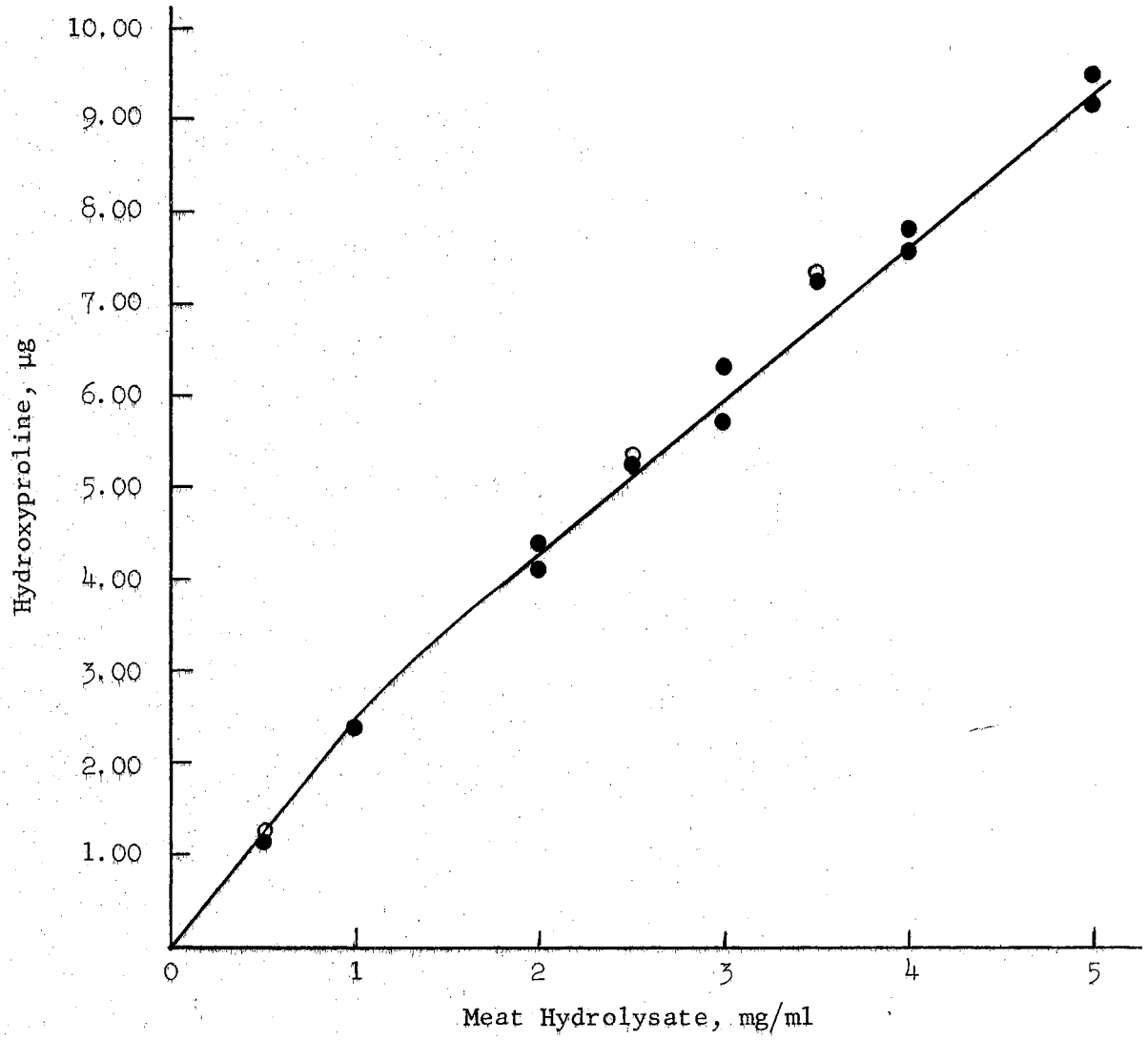


Figure 10: Dilution Curve of Meat Hydrolysate

line from 0 to 12.55 µg. hydroxyproline per mg. gelatin. An attempt was made to decolorize the meat samples with activated charcoal so that the hydroxyproline could be measured in larger than 1 mg. samples. However, the charcoal adsorbed some of the hydroxyproline (Table IV). Consequently, all the meat samples were diluted to 1 mg. per ml. concentration.

TABLE IV

LOSS OF HYDROXYPROLINE FROM MEAT SAMPLES BY CHARCOAL DECOLORIZATION

Sample	Replicates (µg. Hypro Per mg. Sample) 1	2	Average
17L	2.40	2.49	2.44
17L (decolorized)	2.20	2.08	2.14

Recovery studies were initiated using assay grade hydroxyproline and the partially "dried and fat-free" meat samples. These investigations indicated that the percent recovery of hydroxyproline was 102.48 ± 3.01. The apparent reason for the percent recovery being over 100 percent was the amount of huminous material in a sample. When the meat sample had a large amount of humin formation, the color of the resulting hydrolysate would depress the absorption values. However, the recovery studies were conducted with samples containing 0.5 mg. of sample and 1.0 µg. of hydroxyproline instead of 1.0 mg. of sample. The 50 percent dilution of the meat hydrolysate eliminated the effect of the hydrolysate color on the absorbancy values. Consequently, the concentration of hydroxyproline in the recovery samples would be more than expected due to the increased absorbancy of the hydroxyproline in the meat samples. Futhermore, the

coefficient of variation for the recovery study samples (2.94 percent) was essentially the same as the coefficient of variation for the gelatin samples (2.39 percent). This indicates that the high percent of recovery has little influence on the variation among sample means.

Summary and conclusions

The p-DMAB methods of Ritchey et al. (1962), and Hutterer and Singer (1960) were studied in an effort to quantitatively measure the hydroxyproline concentration in partially "dried and fat-free" meat samples. Due to the day to day variation in the Ritchey et al. (1962) procedure, this method was not precise enough to measure the small variation in meat samples that must be assayed over an extended period. However, the Hutterer and Singer (1960) technique appeared to be a more precise and accurate method to quantitatively measure hydroxyproline in meat samples. The day to day variation between hydroxyproline standard curves was 0.0001 A. per μg. hydroxyproline. In addition, the analytical procedures were associated with 2.39 percent of the variation (coefficient of variation) among standard gelatin samples. The percent recovery of hydroxyproline from meat samples was 102.48 ± 3.01 percent. These results indicate that the Hutterer and Singer (1960) procedure is a precise and accurate technique to assay for hydroxyproline in partially "dried and fat-free" meat samples.

EXPERIMENTAL MATERIAL

Meat used in this study was obtained from the carcasses of 13 month-old Angus steers from a beef cattle breeding study at the Oklahoma Agricultural Experiment Station. Detailed breeding, feeding, gain, and slaughter data for these cattle has been published (Chambers et al., 1962) and were designated as "1960 steers" in the publication. Table V presents the average carcass weight and grade by sire groups. Six of the sire progeny groups (those designated by three digits) were by bulls that were either one-half, three-quarter, or full sibs.

TABLE V

AVERAGE CARCASS GRADE AND WEIGHT BY SIRE GROUP

		Grade[1]		Weight[2]	
Sire	No. Per Sire	Average	Range	Average	Range
264	5	11.0	10-12	630.8	587-661
337	8	10.8	9-12	567.5	506-652
327	8	10.9	10-12	620.5	582-662
187	8	10.2	8-11	566.2	521-702
047	4	10.3	9-11	591.3	545-602
157	7	10.7	9-12	619.8	532-704
6	4	9.0	8-11	625.0	601-646
21	4	10.8	10-11	538.7	498-565
22	4	11.0	9-12	585.0	545-623
24	5	10.4	9-12	571.6	517-606

[1]Carcass grades: 8 = average good; 9 = high good; 10 = low choice; 11 = average choice; 12 = high choice.

[2]Chilled carcass weight - 48 hours post-mortem.

All of the cattle were slaughtered on the same day at a major packing plant in Oklahoma City. Carcass measurements, weights, and grades were obtained 48 hours post-mortem. Both sides of each carcass were cut into wholesale cuts either 48 or 72 hours post-mortem. At the time of cutting, a two rib section was taken from the posterior portion of each wholesale rib for this study. The cut was made perpendicular to the backbone between the tenth and the eleventh vertebra. These cuts were processed for further study at the Oklahoma State University Meat Laboratory.

Each rib section was cut into a two-inch twelfth rib and a one-inch eleventh rib steak, 96 hours post-mortem. The data obtained from the twelfth rib steak were as follows: 1. penetrometer firmness readings, 2. histological observations, 3. ether extract, and 4. chemical determination for collagen. Warner-Bratzler shear readings were taken on the cooked eleventh rib steaks.

After the penetrometer readings and histological samples were obtained, the remainder of the longissimus dorsi of the twelfth rib was cut into small pieces, put into a screw top jar, frozen at -40°C., and then stored at -30°C. for the chemical analyses. The eleventh rib steak was boned, wrapped, quick frozen, and stored at -30°C. until cooked and sheared.

EXPERIMENTAL METHODS

Penetrometer Determination

The steaks were placed in a 2.0°C. cooler for approximately 18 hours after cutting. This was done to allow the steaks to reach a uniform internal temperature. To prevent dehydration during this phase, the steaks were placed separately on cutting tables and covered with waxed wrapping paper. A damp shroud was then placed on top of the paper. Since previous work had shown a difference in penetrometer readings between anterior and posterior faces of rib steaks (Pilkington, 1960), the anterior face of the steaks was placed on the table. Thus, the posterior face of the steak remained intact for penetrometer firmness determinations.

Firmness readings were made using the single ball penetrometer described by Pilkington (1960). Readings were randomly obtained between the lateral and dorsal ends of each longissimus dorsi steak as described in the work cited above.

Warner-Bratzler Shear Determination

Prior to cooking, the eleventh rib steaks for Warner-Bratzler shear determinations were thawed at 4.4°C. for approximately 15 hours. They were then cooked in a 177°C. oven until the internal temperature of the steaks was 65.5°C. Immediately after the steaks reached the desired internal temperature, one-inch cores were removed from the dorsal and lateral locations as described by Mjoseth (1962). However, the cores

remained in the steaks until they were ready to be placed in the Warner-Bratzler Shear for the tenderness determinations. This procedure was followed so that the temperature of the cores would be uniform at the time of shearing. One shear reading was taken perpendicular to the predominating muscle fiber direction of each core. The shear determinations were made at random with respect to the location within the steak. Each sire group and each steak within a sire group were taken at random for cooking within a period of two days.

Histological Procedures

Histological samples were taken from the lateral and dorsal ends of each twelfth rib steak. These samples were from the same area of the steak as that used for the penetrometer readings. A one-inch core was removed from each location in the steak in such a manner as to cut as nearly parallel to the predominating "grain" as possible. Three 5 mm. thick cross-sectional samples were taken from the anterior side of the core. The samples were placed in individual "Tissue Tek" plastic capsules. The first and second samples were labeled for future collagen studies, while the third sample was used for fiber diameter determinations. Four capsules were placed in a four ounce, wide mouthed sample jar containing a 10 percent physiological saline formalin solution buffered with 1.0 percent sodium acetate. (Venable, 1962).

Because of the high ratio of sample to fixative, the solution was changed according to the following time schedule: 12 hours, 36 hours, 72 hours, and then the samples were placed into the final storage solution. Throughout the preparation and fixative stages, along with the subsequent storage period, the samples remained in a 4.4°C. cooler.

The following detailed histological observations were obtained only from sire groups 337, 327 and 187.

Muscle fiber diameter

Preliminary studies were conducted in an effort to determine the most desirable method for preparing the formalin fixed meat tissue for muscle fiber diameter determinations. The basic criteria for selecting a method were: 1. the degree of complete muscle fiber separation with the minimum amount of fiber damage, and 2. the most uniform method for obtaining a representative sample of muscle fibers.

Tuma et al. (1962) used a Waring Blendor with the blade reversed to separate muscle tissue into the component fibers. In this work, the fibers were placed in a coplin jar lid and then measured using a compound microscope equipped with an ocular micrometer. In general, the fibers in the bottom were those being measured. This procedure was tried and slightly modified because the speed of the blendor influenced the amount of fiber destruction. Also, the density of the fibers somewhat determines which fibers settle to the bottom of the suspending media. Consequently, such measurements may tend to bias the results since only those fibers of similar density are measured.

Preparation of the formalin fixed samples was found to greatly affect the ease (and possibly the accuracy) of the fiber diameter readings. Five preparation factors were noted to influence the condition of the separated fibers: 1. time of blending, 2. speed of blending, 3. temperature of the blending solution, 4. size of the blended sample, and 5. ratio of sample size to volume of blending solution.

A Model 700B Waring Blendor was used throughout this study. It was attached to a rheostat for regulating the speed of the blendor. A one quart blendor jar, with the blade reversed, was used for blending the

samples. The fixative solution was also the blending solution. By using the fixative solution, theoretically the amount of diffusion, which could possibly affect the fiber diameter and stability, would be at a minimum.

The initial studies on the time and speed of blending indicated that 30 seconds with a rheostat setting of 40 volts partially fulfilled the requirements for a desirable sample. Further work suggested that the samples and the blending solution should be cold at the time the tissues were prepared for fiber diameter determination. Therefore, the fixative was kept in the 4.4°C. cooler with the "fixed" samples. A one and three-fourths inch petri plate with a smooth, flat bottom was selected as the counting receptacle in an effort to minimize the stacking effect of muscle fibers in suspension. It was found that 3 ml. of liquid covered the bottom of these petri plates. Experience indicated that by just covering the bottom of these plates, the density of the fibers had little influence on which fibers were brought into the microscopic field.

The proper ratio of sample to solution was based on the dispersion and concentration of the fibers in suspension. It was found that the concentration should be great enough that at least 25 fibers could be counted from one 3 ml. pipetting. However, the concentration should not be so high that the fibers would stack on top of one another. A sample approximately 5 x 8 x 5 mm. in 70 ml. of solution was found to fulfill the above requirements.

As a result of the above mentioned study, the following procedure was used to prepare for and measure fiber diameter.

Procedure - Use sections fixed in 10 percent formalin buffered with 1.0 percent sodium acetate and 0.9 percent sodium chloride.

1. Remove a sample approximately 5 x 8 x 5 mm. from the middle of a tissue that has been in cold fixative.

2. Place 70 ml. of cold fixative in a Waring blendor jar which has the cutting blade reversed.
3. Add the sample and blend for 30 seconds with a rheostat setting of 40 volts.
4. Allow the air bubbles to clear from the blended sample.
5. Shake the sample so that the fibers are evenly dispersed. Quickly remove a 3.0 ml. sample and place it in a one and three-fourths inch petri plate. (A 10 ml. graduated pipet with the tip cut off may be used).
6. Measure the diameter of 25 fibers to the nearest 0.05 micron using 100X magnification.
7. Repeat steps 5 and 6 until a total of 50 fibers are measured.

Measurement procedure -

1. Measure only those fibers that are at least one-half the length of the field.
2. Measure only those fibers not in rigor mortis.
3. Measure only the widest part of fibers that fit the above two specifications but are non-linear in shape.
4. Cover the area of the petri plate in a systematic manner so that a fiber will not be measured more than once.

Muscle bundle density

For this phase of the study, the formalin fixed tissues were embedded and stained according to the procedures outlined by Venable (1962). This paraffin embedding technique was selected because of the quick embedding, without sacrificing complete paraffin impregnation of the muscle tissue. Venable's staining process is a modification of the Van Gieson acid fuchsin procedure for collagen. Modifications in this technique were made in order to obtain a maximum contrast between collagen

and the other tissue components present in muscle. The modifications and the purpose for each are listed below:

1. Twenty-five minutes in saturated aqueous picric acid solution. This was the first solution into which the sections were placed after they were rehydrated. The purpose of this step was to insure that all possible tissues would be masked by picric acid.
2. One hour in a 0.1 percent acid fuchsin solution of saturated aqueous picric acid. By following the above solution with this step, the equilibrium condition was maintained for the picric acid stained tissues. However, the collagen would take the acid fuchsin.
3. Ten minutes in a 0.1 percent acid fuchsin solution of 90 percent saturated aqueous picric acid. Essentially, this step starts the differentiating phase for this staining technique. By decreasing the picric acid concentration, there is a loss of picric acid from the section. Therefore, the contrast between collagen and the other components in the muscle will be increased.

Venable's procedure for washing, dehydrating, and embedding beef muscle samples in paraffin is as follows:

1. Wash in running tap water	24 hours
2. Dehydration -	
a. 30% isopropanol	2 hours
b. 50% isopropanol	1 hour
c. 70% isopropanol	1 hour
d. 80% isopropanol	1 hour
e. 90% isopropanol	1 hour

f. 99% isopropanol 2 hours

g. 99% isopropanol 16 hours

h. Acetone (Reagent Grade) 2 hours

i. Acetone (Reagent Grade) 2 hours

3. Embedding - Place the samples in 53° - 55°C. Fisher tissuemat for four hours in a 56°C. paraffin oven, under a slight vacuum with an air wash.

By using such embedding procedures, thin sections cannot be obtained when the blocks of muscle are at room temperature. Therefore, many reseachers follow the practice of soaking the muscle blocks in ice water for several hours. The purpose of the ice water bath is to rehydrate the muscle and to harden the paraffin to the same degree as some of the components in the muscle. Thus, the shattering of the tissue upon sectioning can be avoided.

Preliminary observations in this study indicated that the following soaking and hardening steps developed optimum conditions for thin sectioning:

1. Trim the blocks so that one surface is exposed.
2. Place the blocks in individual beakers of water and soak for approximately two hours at 4.4°C.
3. Transfer the beakers to a blast freezer (-40°C.). Leave them long enough to form a thin film of ice on top.
4. Remove and section the blocks. Ten blocks at a time were carried through this procedure.

Section and mounting

An American Optical Spencer rotary microtome was used to section the blocks. Prior to sectioning each group of ten blocks, the microtome blade was placed in the blast freezer for approximately 30 minutes. All

blocks of tissue were sectioned at a thickness of six microns.

Each section of muscle was floated on a 48°C. water bath. One function of the bath was to straighten out the paraffin sections. If the temperature was much higher than 48°C., the muscle and paraffin matrix would separate. However, temperatures lower than 45°C. failed to allow the sections to straighten out sufficiently. Another function of the bath was related to the floating medium. To form a slight adhesive between the section and the slide, a 0.02 percent gelatin solution was used as the floating medium. Difco Bacto Gelatin was used as the gelatin source.

After the sections were mounted, they were allowed to dry approximately 20 hours on a 46°C. slide warmer. No difficulties were encountered with loose sections during staining by following the above sectioning, mounting and drying techniques.

Venable's modification (1962) of the Van Gieson collagen stain consists of the following steps:

1. Xylene	3 minutes
2. Xylene	3 minutes
3. 98% Isopropanol	2 minutes
4. 98% Isopropanol	2 minutes
5. 80% Isopropanol	2 minutes
6. 70% Isopropanol	2 minutes
7. Distilled Water	2 minutes
8. Saturated Picric Acid	25 minutes
9. 0.1% Aqueous Acid Fuchsin Saturated with Picric Acid	1 hour
10. 0.1% Aqueous Acid Fuchsin 90% saturated with Picric Acid	10 minutes

11. 70% Isopropanol	Dip to differentiate
12. 80% Isopropanol	10 dips
13. 98% Isopropanol	10 dips
14. 98% Isopropanol	10 dips
15. Xylene	2 minutes
16. Xylene	2 minutes

The slides were cover sliped using Fisher Permount as the adhesive.

A greater part of meat histological studies dealing with muscle bundles has expressed results in terms of the number of primary bundles per secondary bundle. However, it is most difficult to define a secondary bundle in the longissimus dorsi. Consequently, in these studies a method was used in which the results may be expressed as the number of muscle bundles per unit area. The size of the primary bundles as well as the size of the interbundular space influences the density. Thus, such a measure is an indication of the area of skeletal muscle fibers per unit of muscle area (the greater the density, the smaller the amount of skeletal muscle fibers).

An American Optical Spencer Microstar microscope equipped with a calibrated stage was used for this phase of the study. A 10X objective and a 15X ocular (150X magnification) was found to give sufficient detail and an area large enough to count the number of bundles. Preliminary work suggested that at lower magnifications, which gave larger fields, poor repeatability occurred when the same field was counted by several people. With magnification greater than 150X, it was found that the number of complete bundles per area was too small. With only a few complete bundles in a given field, difficulty was encountered in obtaining a good estimate of this variable. Some samples had such large bundles that the average value was less than 1.0 when higher

magnifications were used.

In order to avoid artifacts resulting from the preparation of the slides, the measurements were made using the middle portion of each slide. This area was determined by taking one half the width and then measuring from right to left on the section. Initial work with this determination indicated that the maximum number of fields along this line was five per section. Therefore, the length of each section was divided by five to determine the point for each observation. This point was changed only when the calculated area primarily contained adipose connective tissue.

The only criterion used for determining what constituted a primary muscle bundle was that each group of fibers be a discrete entity. Although a primary bundle is normally defined as a group of muscle fibers surrounded by a thin layer of connective tissue (perimysium), it is possible that at low magnification this connective tissue may not be apparent. Consequently, a group of muscle fibers completely separated from other fibers by a distinct intercellular space was counted as one primary bundle.

Chemical Methods

All of the quantitative chemical analyses were conducted on homogenous samples of the longissimus dorsi muscle from the twelfth rib area of each carcass. Prior to the homogenization step, the samples were partially thawed in a 4.4°C. cooler. They were then homogenized in a Waring Blendor. Two aliquots from each sample were placed in separate glass jars and refrozen until analyzed for ether extract and collagen content. The ether extract was determined by the A.O.A.C. method (1945).

Two 10 gm. aliquots from the homogenous samples were subjected to acetone and ether extraction to remove a major part of the moisture and fat according to the method of Parrish et al. (1962). The two partially "dried and fat-free" samples were combined and finely ground with a mortar and pestle. Before the samples were weighed for collagen determinations, they were dried for 24 hours under vacuum in a desiccator containing $CaSO_4$. For collagen determinations, duplicate 500 mg. samples were placed in 100 ml. beakers containing 10 ml. 6 N HCl and covered with a watch glass. The samples were then autoclaved at 121°C. for 18 hours. To remove the HCl, the hydrolyzed samples were placed in round bottom flasks and taken down to dryness three times on a rotary evaporator. They were then neutralized according to the procedure outlined by Mason (1960). Duplicate aliquots from each hydrolyzed sample were used for the collagen assays. The collagen content was estimated by determining the hydroxyproline concentration by the Hutterer and Singer technique (1960). By conducting duplicate assays on each duplicate sample, the precision of the method could be estimated by statistical analysis. Duplicate nitrogen determinations were also made on each duplicate sample by the micro-kjeldahl method of Miller and Houghton (1945). In reporting the μg. hydroxyproline per mg. nitrogen, an average of the two aliquots was used. Consequently, the precision of the nitrogen determination was confounded with sampling error in the statistical analysis.

RESULTS AND DISCUSSION

Part I. Variation in the Physical Structure of the Longissimus Dorsi Muscle at the Twelfth Rib and Its Relationship to Firmness and Tenderness

The purpose of this investigation was to study the variation in the size of the muscle fibers and the muscle bundles with respect to the lateral and dorsal ends of the longissimus dorsi at the twelfth rib. In addition, the firmness and tenderness values at the dorsal and lateral locations were related to the corresponding muscle fiber diameter and muscle bundle density values. To obtain a more accurate estimate of each variable at the two locations within each steak, the total of the values from the corresponding positions on the right and left sides were combined into one observation. There were two types of correlations calculated for each comparison made in this study. "All animal" correlations refer to the degree of association for two variables among the 24 animals used in this phase of the study. "Within sire" correlations were used to study the relationship of two variables when the sire effect was statistically removed.

Table VI contains the means and standard deviations for the measurements obtained at the dorsal and lateral locations. Except for the muscle bundle density values, these data indicated that there was no significant difference ($P > .05$) in the variances of the two locations for the various measurements. However, there was a significant difference ($P < .05$) in the variances of the muscle bundle density values

at the dorsal and lateral locations. These data suggested that the dorsal position had more primary bundles per unit area and had more variation (8.22 ± 1.67) than the lateral location (5.95 ± 1.15). Thus, correlations involving the dorsal location may be larger than those involving the lateral location due to the significant difference ($P < .05$) in variation of these two positions on rib steaks.

TABLE VI

THE MEAN AND STANDARD DEVIATION OF THE VARIOUS MEASUREMENTS AT THE DORSAL AND LATERAL LOCATIONS

	Dorsal	Lateral
Penetrometer	34.75 ± 10.38 mm.	35.91 ± 8.42 mm.
Warner-Bratzler Shear	16.69 ± 4.59 lbs.	18.24 ± 5.10 lbs.
Muscle Fiber Diameter	62.73 ± 8.66 microns	61.20 ± 10.15 microns
Muscle Bundle Density	8.22 ± 1.67	5.95 ± 1.15[1]
Muscle Fiber Diameter: Muscle Bundle Density	7.98 ± 1.97	10.74 ± 2.64

[1]Significant difference in variance between the dorsal and lateral positions ($P < .05$).

Simple correlations between the dorsal and lateral measurements for both muscle fiber diameter and muscle bundle density are presented in Table VII. These correlations indicate that the values obtained at one location were associated with 30 to 35 percent of the variation at the other location ($r^2 \times 100 = 27$ to 39). Consequently, different factors may be influencing the diameter of muscle fibers, as well as the density of the muscle bundles, at the opposite ends of the rib steaks. Therefore, a particular measurement may be more closely associated with

the variation in muscle fiber diameter and/or the muscle bundle density at the dorsal location than at the lateral location.

TABLE VII

COMPARISON OF THE SIMPLE CORRELATIONS BETWEEN DORSAL AND LATERAL MEASUREMENTS FOR MUSCLE FIBER DIAMETER AND MUSCLE BUNDLE DENSITY

	All Animals (d.f. = 22)	Within Sire (d.f. = 20)
Muscle Fiber Diameter	0.52**	0.59**
Muscle Bundle Density	0.63**	0.55**

**$P < .01$

The association between muscle fiber diameter and muscle bundle density within each location was also studied. The within sire correlation between these two variables was -.46 (d.f. = 20) at the dorsal location and -.41 (d.f. = 20) at the lateral location. These results indicate that there was a tendency for large primary muscle bundles to contain large muscle fibers. The coefficients of determination from these correlations ($r^2 \times 100$) suggested that approximately 16 percent of the variation in one variable can be associated with the variation in the other variable. This would then suggest that a majority of the within location variation between these two measurements is attributable to different factors. Consequently, a particular component within the muscle tissue could be associated with either muscle fiber diameter or muscle bundle density within one of the particular cross-sectional areas of the longissimus dorsi, but it may not be associated with either variable within the other location.

A comparison of the relationship of percent ether extract to muscle fiber diameter and muscle bundle density is presented in Table VIII. These correlations point out that there was a difference in the

TABLE VIII

COMPARISON OF THE SIMPLE CORRELATIONS BETWEEN PERCENT ETHER EXTRACT AND MUSCLE FIBER DIAMETER AND MUSCLE BUNDLE DENSITY AT THE DORSAL AND LATERAL LOCATIONS

	Location					
	Dorsal		Lateral		Animal Av.	
	All Animals	Within Sire	All Animals	Within Sire	All Animals	Within Sire
Muscle Fiber Diameter	-.17	-.04	0.12	0.12	-.01	0.05
Muscle Bundle Density	0.20	0.45*	0.02	0.14	0.14	0.35

*$P < .05$.

association of fat with the above two variables, both within and between location. For example, the muscle fiber diameter of a rib steak had little relationship to percent ether extract. This low relationship would be expected since the correlations between these two variables are negative at the dorsal location and positive at the lateral location. These results would suggest that if any relationship does exist between fat content and muscle fiber diameter, it may vary according to the position within the muscle from which the samples were removed.

Muscle bundle density in the dorsal location, however, was positively related to percent ether extract (Table VIII), i.e., the higher the fat content, the smaller the muscle bundles. The all animal and within sire correlations between muscle bundle density and fat content

at the lateral location were lower than the corresponding correlations for the dorsal location. In addition, there was a difference in the within sire correlations of fat content to muscle fiber diameter and muscle bundle density for the dorsal location ($P < .10$). These results would suggest that muscle bundle density in the dorsal location is more closely related to the average fat content of the ribeye at the twelfth rib than muscle fiber diameter. Although the correlations were low and non-significant at the lateral location, there was no detectable difference in the association of muscle bundle density and muscle fiber diameter to fat content for this position within the muscle.

Firmness, as estimated by penetrometer values, was significantly related ($P < .05$) to the muscle fiber diameter, muscle bundle density, and the ratio of these two measurements at the dorsal position of the longissimus dorsi (Table IX). With the exception of the correlations

TABLE IX

COMPARISON OF THE SIMPLE CORRELATIONS BETWEEN THE PENETROMETER VALUES AND THE PHYSICAL PROPERTIES OF THE LONGISSIMUS DORSI AT THE DORSAL AND LATERAL LOCATIONS

	All Animals (d.f. = 22)		Within Sires (d.f. = 20)	
	Dorsal	Lateral	Dorsal	Lateral
Muscle Fiber Diameter	0.44*	0.32	0.52*	0.15
Muscle Bundle Density	-.42*	-.07	-.57**	0.01[1]
Muscle Fiber Diameter: Muscle Bundle Density	0.43*	0.15	0.53*	0.03

**$P < .01$.

*$P < .05$.

[1]Significant difference between dorsal and lateral ($P < .05$).

involving the muscle bundle density values, the difference in the within sire correlations between the dorsal and lateral locations for each measurement was $P < .10$. However, the difference between the two positions in the ribeye for the within sire correlations involving the muscle bundle density values was $P \leq .05$. Therefore, the results in Table IX indicated that the larger the muscle fibers in the dorsal end of rib steaks, the softer the meat. When the penetrometer values were related to the muscle bundle density, the results suggested that the steaks with the larger muscle bundles tended to be softer than the steaks with small muscle bundles in the dorsal location. The ratio of muscle fiber diameter to muscle bundle density, when compared to the firmness values, confirms the relationship of these two muscle tissue components to firmness. More specifically, the steaks which were firmest in the dorsal position were generally those containing small fibers in small primary bundles and conversely, those containing large fibers in large primary bundles were softest.

Tenderness at the dorsal and lateral locations, as estimated by the Warner-Bratzler Shear, was not closely associated with either of the corresponding location estimates for muscle fiber diameter, muscle bundle density, and the ratio of muscle fiber diameter to muscle bundle density (Table X). The significant correlation between Warner-Bratzler Shear and muscle bundle density values at the dorsal location ($P < .05$) suggested that the dorsal position increased in tenderness as the muscle bundles increased in size. However, the results of "Z" transformations (Snedecor, 1956) indicated that there were no significant differences between any of the correlations ($P > .05$), whether the comparisons were made between or within locations for the various measurements. Thus, muscle fiber diameter, muscle bundle density, or the ratio

of these two measurements does not appear to account for a significant amount of the variation in tenderness, either within a particular location or between the dorsal and lateral locations of rib steaks. However, it has been reported that muscle fiber diameter, muscle bundle density and/or the ratio between these measurements are closely associated with the variation in tenderness between animals (Brady, 1937; Hiner et al., 1952, 1953; Ramsbottom et al., 1945; Satorius and Child, 1938; Strandine et al., 1949).

TABLE X

COMPARISON OF THE SIMPLE CORRELATIONS BETWEEN THE WARNER-BRATZLER SHEAR VALUES AND THE PHYSICAL PROPERTIES OF THE LONGISSIMUS DORSI AT THE DORSAL AND LATERAL LOCATIONS

	All Animals (d.f. = 22)		Within Sire (d.f. = 20)	
	Dorsal	Lateral	Dorsal	Lateral
Muscle Fiber Diameter	-.09	-.20	-.39	-.17
Muscle Bundle Density	0.42*	0.16	0.20	-.04
Muscle Fiber Diameter: Muscle Bundle Density	-.39	-.22	-.23	-.04

*P < .05

Comparisons were made between penetrometer and Warner-Bratzler shear values at both locations within the cross-section of the longissimus dorsi from the three sire groups used for the detailed study on the physical factors. Also, the data from the additional seven sire groups were included in these comparisons in an effort to determine whether the results from a small sample (24 animals) is representative of the results from a larger population (57 animals). The results indicated that firmness and tenderness are negatively related ($P < .01$) when the measurements

are taken on the dorsal end of the rib steaks (Table XI), i.e., the softer the meat in the dorsal location, the more tender the meat. Although the relationship was in the same direction for these measurements

TABLE XI

COMPARISON OF THE SIMPLE CORRELATIONS BETWEEN THE PENETROMETER AND THE WARNER-BRATZLER SHEAR AT THE DORSAL AND LATERAL LOCATIONS

	Dorsal	Lateral
Three Sire Groups		
All Animals (d.f. = 22)	-.59**	-.21
Within Sires (d.f. = 20)	-.71**	-.17[1]
Ten Sire Groups		
All Animals (d.f. = 55)	-.36**	-.21
Within Sires (d.f. = 46)	-.54**	-.15[1]

**P < .01

[1]Significant difference between the dorsal and lateral (P < .025).

at the lateral location, the correlations were low and not significant (P > .05). Fisher's "t" test (Snedecor, 1956) suggested that there was a significant difference (P < .05) in the within sire correlations between dorsal and lateral locations, regardless of the number of observations used in the comparisons.

Part II. The Relationship of Objectively Measured Firmness and Tenderness to the Physical Structure of the Longissimus Dorsi Muscle

The analysis of variance was used to study the influence of sire, side (right versus left side of the carcass), and when applicable the location effect (dorsal versus lateral position within a cross-section of the longissimus dorsi muscle) for the variables being studied. An analysis of variance was computed for each of the following observations: penetrometer values, Warner-Bratzler shear values, muscle fiber diameter, muscle bundle density, collagen content of partially "dried and fat-free" meat samples expressed as micrograms of hydroxyproline per milligram of sample or milligram of nitrogen, and percent ether extract. Only the observations obtained from the carcasses in sire group numbers 337, 327, and 187 were used for these calculations.

The results of the analysis of variance for the penetrometer values are presented in Table XII. These results indicated that there was a statistically significant location X side interaction ($P < .01$). The data in the location X side two-way table (Table XIII) points out that the dorsal location is softer than the lateral location on the right side. However, the lateral location is softer than the dorsal location on the left side. Earlier work by Pilkington (1960) suggested that there was an interaction between the penetrometer measurements taken on the adjacent faces of rib steaks on the same side of a carcass, but there was no interaction when the measurements were made on the same face of corresponding rib steaks from both sides of beef carcasses. Although the side effect was not significant ($P > .05$), the steaks from the right side were always softer than those from the left side for each sire group (Table XIV). This effect may be associated with the significant

difference in fat content ($P < .01$) between the right and left side of these carcasses. Gannaway (1955), Kropf and Graf (1959), and Pilkington (1960) have indicated that the firmness of meat is closely related to amount of intramuscular fat.

TABLE XII

ANALYSES OF VARIANCE FOR PENETROMETER AND WARNER-BRATZLER SHEAR VALUES

Source	d.f.	Penetrometer M.S.	Warner-Bratzler Shear M.S.
Total	95		
Between Side			
Animal	23		
Sire	2	49.30	135.25*
Within Sire[1]	21	134.89	32.18
Side	1	70.04	0.63
Side X Sire	2	15.79	0.35
Discrepency[2]	21	49.02	4.89
Within Side			
Location	1	32.67	57.35**
Location X Side	1	165.38**	19.62*
Location X Sire	2	78.04	0.15
Location X Animal[3]	23	51.23	6.12
Remainder[4]	21	15.45	4.48

**$P < .01$.

*$P < .05$.

[1]Estimate of σ^2 used for testing sire.

[2]Estimate of σ^2 used for testing side and side X sire.

[3]Estimate of σ^2 used for testing location X sire.

[4]Estimate of σ^2 used for testing location and location X side.

TABLE XIII

AVERAGE PENETROMETER VALUES (LOCATION X SIDE TWO-WAY TABLE)

Location	Side [1,2]		Average
	Right	Left	
Dorsal	36.9	32.6	34.8
Lateral	35.4	36.4	35.9
Average	36.2	34.5	35.4

[1]Depth of penetration in 0.1 mm. (sum of 24 measurements).

[2]The deeper the penetration the softer the ribeye.

TABLE XIV

AVERAGE PENETROMETER VALUES (SIDE X SIRE TWO-WAY TABLE)

Side	Sire Groups [1,2]			Average
	337	327	187	
Right	35.6	37.0	36.0	36.2
Left	32.3	35.6	35.5	34.5
Average	33.9	36.3	35.8	35.4

[1]Depth of penetration in 0.1 mm. (sum of 16 measurements).

[2]The deeper the penetration the softer the ribeye.

Results from the Warner-Bratzler shear values suggested that the tenderness of meat is related to the sire and the sample location within the muscle (Table XII). Several studies have been reported which indicated that tenderness is a heritable trait (Christians, 1962; Cartwright et al., 1958; Cover et al., 1957). These data suggested that the dorsal position is more tender than the lateral position (Table XV). Similar data has

been reported by Pilkington (1960) and Tuma et al. (1962). The reason(s) for this difference in tenderness between these extreme locations within the cross-section of the longissimus dorsi are not apparent at the present time. However, the -.71 ($P < .01$) correlation between firmness and tenderness at the dorsal location suggests that some factors associated with softness are also related to tenderness. The correlations between tenderness and the physical factors of the longissimus dorsi muscle (Table X) indicated that these physical factors were not closely associated with tenderness at either location. There was an apparent location X side interaction which influenced the tenderness values. The data in Tables XV and XVI suggested that the dorsal location was the most tender position, regardless of the sire group or the side of the carcass. The reason for the statistical significance of the location X side interaction is brought out in Table XVI. In these data, the dorsal location was more tender on the left than the right side; while the lateral location was more tender on the right than the left side. Thus, the largest difference in the tenderness between the two positions within the muscle is on the left side of these carcasses.

TABLE XV

AVERAGE WARNER-BRATZLER SHEAR VALUES (LOCATION X SIRE TWO-WAY TABLE)

Location	Sire [1,2]			Average
	337	327	187	
Dorsal	17.0	14.6	18.5	16.7
Lateral	18.6	16.0	20.2	18.2
Average	17.8	15.3	19.3	17.4

[1]Pounds of force required to shear a one-inch core of meat (sum of 16 measurements.

[2]The lower the value the more tender the meat.

TABLE XVI

AVERAGE WARNER-BRATZLER SHEAR VALUES (LOCATION X SIDE TWO-WAY TABLE)

Location	Side[1,2]		Average
	Right	Left	
Dorsal	17.2	16.2	16.7
Lateral	17.9	18.6	18.2
Average	17.5	17.4	17.4

[1]Pounds of force required to shear a one-inch core of meat (sum of 24 measurements).

[2]The lower the value the more tender the meat.

Muscle fiber diameter appears to be influenced by sire and location, in addition to a location X sire interaction (Table XVII). The within location variation in this analysis is an estimate of the variance associated with the two determinations on each individual location. The standard deviation of the mean between these two estimates was 2.51 microns. Table XVIII contains the average fiber diameter as influenced by sire, side, and location. These data indicate that the muscle fibers were larger in the dorsal than the lateral location for sire groups 337 and 187. In sire group 327, the average diameter of the muscle fibers at the lateral location was larger than the diameter for those in the dorsal location. These observations would indicate that there was a true sire X location interaction when the precision of the determination is considered ($S_{\bar{x}} = 2.51$). Thus, there may be a significant difference in the diameter of fibers from the lateral and dorsal locations within a rib steak. However, the location with the larger fibers would depend on the sire of the animals involved. Joubert (1956a) has

reported that the sire does influence the diameter of muscle fibers. Therefore, histological samples from the same anatomical location and side will demonstrate the sire effect on muscle fiber diameter.

TABLE XVII

ANALYSIS OF VARIANCE FOR MUSCLE FIBER DIAMETER

Source	d.f.	M.S.
Total	191	
Between Side		
Animal	23	
Sire	2	387.11*
Within Sire[1]	21	111.08
Side	1	5.34
Side X Sire	2	17.54
Discrepency[2]	21	20.77
Within Side		
Location	1	111.01*
Location X Side	1	14.09
Location X Sire	2	182.08*
Location X Animal[3]	23	42.96
Discrepency[4]	21	14.91
Within Location	96	6.29

*$P < .05$.

[1]Estimate of σ^2 for testing sire.

[2]Estimate of σ^2 for testing side and side X sire.

[3]Estimate of σ^2 for testing location X sire.

[4]Estimate of σ^2 for testing location and location X side.

TABLE XVIII

AVERAGE MUSCLE FIBER DIAMETER (SIRE X SIDE X LOCATION THREE-WAY TABLE)

Sire Groups	Location[1]					
	Dorsal Side			Lateral Side		
	Right	Left	Average	Right	Left	Average
337	61.00	60.50	60.75	57.81	57.62	57.72
327	61.94	61.06	61.50	62.69	65.00	63.85
187	66.56	65.31	65.94	62.81	61.31	62.06
Average	63.17	62.29	62.73	61.10	61.31	61.21

[1]An average of 16 determinations in microns.

The analysis of variance for muscle bundle density (Table XIX) indicated that sources of variation in this determination were sire, side, side X sire, and location. Although the data in Table XX indicates that the right side generally contains larger bundles than the left side, the reverse situation was true for the observation from the carcasses in sire group 337. These data, when combined with the muscle fiber diameter data, suggested that the sire effect may be one of the factors influencing the relationship between these two variables at each location. The data in Table XX indicated that sire group 327 differs from the other two groups in regard to the difference in muscle fiber diameter between the lateral and dorsal positions. In the case of the muscle bundle density, the observations from the carcasses in sire group 337 differed from those for sire groups 327 and 187 with respect to the difference between sides. However, the muscle bundles were always smaller for the dorsal than the lateral on both the right and left sides

(Table XXI). The average density values for the dorsal and lateral locations was 8.22 and 5.95 respectively.

TABLE XIX

ANALYSIS OF VARIANCE FOR MUSCLE BUNDLE DENSITY

Source	d.f.	M.S.
Total	95	
Between Sides		
Animal	23	
Sire	2	613.51*
Within Sire[1]	21	119.58
Side	1	162.76*
Side X Sire	2	413.44**
Discrepency[2]	21	32.81
Within Side		
Location	1	3116.76**
Location X Side	1	10.01
Location X Sire	2	115.01
Location X Animal[3]	23	42.78
Remainder[4]	21	33.79

**P< .01.

*P < .05.

[1]Estimate of σ^2 for testing sire.

[2]Estimate of σ^2 for testing side and side X sire.

[3]Estimate of σ^2 for testing location X sire.

[4]Estimate of σ^2 for testing location and location X side.

average percent ether extract for the side and sire effects. These data suggest that the right side contains more fat than the left side. As was mentioned earlier, this variation in side may account for part of the side variation observed in firmness. (See page 92).

TABLE XXII

ANALYSIS OF VARIANCE FOR HYDROXYPROLINE
(UG PER MG. SAMPLE)

Source	d.f.	M.S.
Total	191	
Between Side		
Animal	23	
Sire	2	0.43
Within Sire[1]	21	0.28
Side	1	0.05
Side X Sire	2	1.50**
Discrepency[2]	21	0.22
Within Side	144	0.006
Sampling	48	0.0098
Aliquot	96	0.0042

**P < .01.

[1]Estimate of σ^2 for testing sire.

[2]Estimate of σ^2 for testing side and side X sire.

TABLE XXIII

ANALYSIS OF VARIANCE FOR HYDROXYPROLINE
(MG PER MG. NITROGEN)

Source	d.f.	M.S.
Total	95	
Between Side		
Animal	23	
Sire	2	7.17
Within Sire[1]	21	6.93
Side	1	0.17
Side X Sire	2	35.28**
Discrepency[2]	21	5.59
Sampling Error	48	0.28

**P < .01.

[1]Estimate of σ^2 for testing sire.

[2]Estimate of σ^2 for testing side and side X sire.

TABLE XXIV

AVERAGE MG. HYDROXYPROLINE PER MG. SAMPLE
(SIDE X SIRE TWO-WAY TABLE)

Side	Sire Groups[1]			Average
	337	327	187	
Right	2.30	2.54	2.13	2.32
Left	2.49	2.22	2.36	2.36
Average	2.40	2.38	2.25	

[1]An average of 32 determinations.

TABLE XXV

ANALYSIS OF VARIANCE FOR PERCENT ETHER EXTRACT

Source	d.f.	M.S.
Total	95	
Between Side		
Animal	23	
Sire	2	7.56
Within Sire[1]	21	7.77
Side	1	2.88*
Side X Sire	2	0.53
Discrepency[2]	21	0.52
Within Side	48	0.023

*P < .05.

[1]Estimate of σ^2 for testing sire.

[2]Estimate of σ^2 for testing side and side X sire.

TABLE XXVI

AVERAGE PERCENT ETHER EXTRACT
(SIDE X SIRE TWO-WAY TABLE)

Side	Sire Groups[1]			Average
	337	327	187	
Right	6.41	6.74	5.58	6.24
Left	5.88	6.29	5.53	5.90
Average	6.14	6.52	5.55	

[1]An average of 16 determinations.

This phase of the study was designed to investigate the association between the firmness and tenderness of each carcass to the measurements of different physical properties of the meat. Since the analyses of variance indicated that there were interactions between several of the sources of variation in this population, the animal totals were used as an estimate of each observation. In addition to the all animal correlations, within sire correlations were computed to study these relationships when the sire variation was removed.

Table XXVII contains the simple correlations between the penetrometer values (estimate of firmness) and the physical properties of the

TABLE XXVII

COMPARISON OF THE SIMPLE CORRELATIONS BETWEEN FIRMNESS[1] AND THE PHYSICAL PROPERTIES OF THE LONGISSIMUS DORSI

	All Animals (d.f. = 22)	Within Sire (d.f. = 20)
Muscle Fiber Diameter	0.47*	0.46*
Muscle Bundle Density	-.45*	-.47*
Muscle Fiber Diameter: Muscle Bundle Density	0.46*	0.46*
µg. hypro per mg. sample	0.09	0.13
µg. hypro per mg. nitrogen	0.06	0.07
Percent Ether Extract	-.38	-.41

*$P < .05$.

[1]Penetrometer values measured in 0.1 mm. (The higher the reading, the softer the meat).

ribeye. The results of " Z " transformation (Snedecor, 1956) indicated that there was no significant difference between the all animal and within sire correlations. Thus, there appears to be little or no sire influence on any of these relationships. These data also suggested that the muscle fiber diameter and muscle bundle density, as well as the ratio between these two measurements, was related to the firmness of the longissimus dorsi ($r \sim .46$, d.f. = 22). In addition, the variation in each observation was associated with approximately 21 percent of the variation ($r^2 \times 100$) in the penetrometer readings. The positive relationship between the penetrometer values and muscle fiber diameter suggested that ribeye steaks with large fibers tend to be softer than steaks with small fibers. Muscle bundle density, however, was negatively related to firmness. This indicates that a decrease in the number of primary bundles per unit area (larger bundles) is associated with an increase in the softness of the longissimus dorsi. The ratio of the muscle fiber diameter to the muscle bundle density, further substantiated the relationship of these two variables with firmness. Thus, ribeyes with large fibers and large bundles tend to be soft, while the reverse appears to be true for the ribeyes containing small muscle fibers and primary bundles. Similar results were obtained when the same observations were made on the dorsal location of the longissimus dorsi (see page 87).

Collagenous tissue, as estimated by hydroxyproline concentration, appeared to have little relationship to the firmness or softness of the steaks from the carcasses used in this study (Table XXVII). These low correlations (approximately 0.10) suggest that factors other than collagen may be associated with the variation in firmness of the longissimus dorsi; especially when the carcasses are from a restricted population

(the same breed, and similar in age and condition).

The percent ether extract was negatively correlated with the penetrometer measurements (Table XXVII). Although this correlation coefficient is lower than those reported by Gannaway (1955), Kropf and Graf (1959), and Pilkington (1960), it is in the same direction as reported in these studies, i.e., the firmer the meat, the higher the fat content.

Tenderness, as estimated by the Warner-Bratzler shear values, was not significantly ($P > .05$) related to the observations in this study (Table XXVIII). In addition, there is no statistically significant

TABLE XXVIII

COMPARISON OF THE SIMPLE CORRELATIONS BETWEEN TENDERNESS[1] AND THE PHYSICAL PROPERTIES OF THE LONGISSIMUS DORSI

	All Animals (d.f. = 22)	Within Sire (d.f. = 20)
Muscle Fiber Diameter	-.16	-.27
Muscle Bundle Density	0.38	0.40
Muscle Fiber Diameter: Muscle Bundle Density	-.39	-.20
µg. hypro per mg. sample[2]	-.25	-.13
µg. hypro per mg. nitrogen[2]	-.22	-.10
Percent Ether Extract	-.21	-.07

[1]Pounds of force required to shear a one-inch core of meat (the lower the force, the more tender the meat).

[2]Estimate of collagen.

difference between the all animals and within sire correlations. However, these correlation coefficients are of sufficient magnitude that they may be indicative of the positive or negative direction of the relationship. These data indicated that there was a tendency for the cooked steaks to be more tender when the muscle fibers were large in diameter ($r = -27$, d.f. = 20). Tuma et al. (1962) reported a correlation of 0.41 ($P < .05$) between taste panel tenderness scores and fiber diameter measurements, even though the population included carcasses from calves and mature cows. Other workers, however, have reported a negative correlation between these same two observations. (Brady, 1937; Hiner et al., 1952, 1953; Paul, 1962). These apparently contrasting results may be associated with the type of population involved in the different investigations. For example many workers have conclusively shown that the diameter of the muscle fibers increases with chronological age. (Dickerson, 1960; Doty and Pierce, 1961; Hiner et al., 1952, 1953; Joubert, 1956a,b; Lowe and Kastelic, 1961). Moreover, the tenderness of cooked steaks has been reported to decrease as animal age increases. (Hiner and Hankins, 1950; Hiner et al., 1952, 1953; Lowe and Kastelic, 1961). Consequently, the results from studies in which fiber diameter is confounded with animal maturity should show that the youthful animals with small fibers are more tender than the more mature animals with large fiber diameters. Therefore, it is possible that a small positive relationship can exist between tenderness and fiber diameter when the muscle samples are from the carcasses of animals similar in maturity.

Muscle bundle density was positively correlated with Warner-Bratzler shear values (Table XXVIII), i.e., the larger the bundles the more tender the meat. Similar conclusions have been reported by Brady (1937),

Ramsbottom et al. (1945), Satorius and Child (1938), and Strandine et al. (1949).

The ratio of muscle fiber diameter to muscle bundle density was negatively related to tenderness (Table XXVIII). Therefore, the steaks with small fibers and large bundles tend to be more tender than the steaks containing large fibers and small muscle bundles. These results are similar to those of Brady (1937), Ramsbottom et al. (1945), Satorius and Child (1938), and Strandine et al. (1949).

Collagen content and percent ether extract were negatively related to the Warner-Bratzler shear values (Table XXVIII). These data indicated that within this restricted population, fat content and collagen content are not closely associated with the tenderness of cooked rib steaks. Similar conclusions have been reported by Doty and Pierce (1961), Hershberger et al. (1951), Lorincz and Szeredy (1959), and Wilson et al. (1954). Other workers have suggested that these two factors are associated with tenderness. (Adams et al., 1960; Cover and Hostetler, 1960; Nottingham, 1956; Parrish et al., 1962). In this study, the correlation between percent ether extract and hydroxyproline content was 0.36 (d.f. = 22). Thus, carcasses with high fat content tended to have a high content of collagen. Similar results have been reported by Batterman et al. (1952), and Lorincz and Szeredy (1959). Consequently, there may be a positive correlation for tenderness with percent ether extract and collagen content when the population variation is restricted for fat content, animal age, and management conditions.

The simple correlation coefficients in Table XXIX indicated that there is a negative relationship between firmness and tenderness. Furthermore, there was no significant difference ($P \sim .40$) in the

magnitude of the correlations from the three and ten sire groups. Thus, these data substantiate the work of Pilkington (1960); which indicated that when steaks of equal fat content differed in their degree of firmness, the softer steaks tend to be more tender.

TABLE XXIX

COMPARISON OF THE SIMPLE CORRELATIONS BETWEEN PENETROMETER VALUES AND WARNER-BRATZLER SHEAR VALUES

	3 Sire Groups (n = 24)	10 Sire Groups (n = 57)
All Animals	-.52**	-.29*
Within Sire	-.58**	-.35*

**P < .01.

*P < .05.

SUMMARY AND CONCLUSIONS

The purposes of this study were to investigate: (1) variation in the physical structure of the longissimus dorsi muscle at the twelfth rib and its relationship to firmness and tenderness, and (2) the relationship of objectively measured firmness and tenderness to the physical properties of the longissimus dorsi muscle.

The experimental material consisted of the eleventh and twelfth rib steaks from the carcasses of 57, 13 month-old Angus steers. These steers were from ten sire progeny groups which had been raised under the same management regime. The carcasses ranged in grade from average Good to high Choice. Carcass weight ranged from 506 to 704 pounds. Firmness (penetrometer determinations) was obtained on the lateral and dorsal positions of the twelfth rib steaks from each side of all the carcasses. Tenderness (Warner-Bratzler shear determinations) was obtained on the lateral and dorsal positions of all of the oven cooked eleventh rib steaks. Muscle fiber diameter, muscle bundle density, and collagen concentration (estimated by hydroxyproline content) were also obtained on 24 of the carcasses (three sire groups, eight animals each). The muscle fiber diameter and muscle bundle density measurements were made on the right and left twelfth rib steaks from these carcasses. In addition, the hydroxyproline and percent ether extract determinations were made from homogenized samples of the longissimus dorsi at the twelfth rib.

These studies indicated that the lateral and dorsal ends of the longissimus dorsi differed with respect to muscle fiber diameter, muscle bundle density, and tenderness. The diameter of the muscle fibers was larger in the dorsal than the lateral position ($P < .05$), although these data suggested that there may be some sire influence on the difference between the two positions. In addition, the muscle bundles were smaller in the dorsal than the lateral locations ($P < .01$). Also, the variation in the muscle bundle density was more closely associated with the variation in fat content at the dorsal than the lateral position. The Warner-Bratzler shear values indicated that the dorsal position was more tender than the lateral ($P < .01$). Although previous studies had suggested that beef rib steaks were firmer at the dorsal than the lateral location (Pilkington, 1960), the results of the penetrometer readings in this study indicated that there was no difference in the firmness between these two locations ($P > .05$). The variances for firmness, tenderness, and muscle fiber diameter were similar ($P > .05$) at the dorsal and lateral positions. However, the muscle bundles were smaller and more variable in the dorsal than the lateral position.

Simple correlations were computed to study the relationship of penetrometer values to the measurements for muscle fiber diameter, muscle bundle density, and the ratio of muscle fiber diameter to muscle bundle density at the dorsal and lateral locations. The within sire correlations indicated that the penetrometer readings in the dorsal location were related to muscle fiber diameter ($r = 0.52$) to muscle bundle density ($r = -.57$), and to the ratio of these two muscle properties ($r = 0.53$); i.e., the dorsal location tends to be softer when the meat contains large muscle fibers incased in large muscle bundles. However, the

correlation coefficients were low ($r < .15$) and non-significant when these same factors were correlated at the lateral position. The lateral and dorsal measurements were also pooled to obtain an estimate of each variable on a carcass basis. When the firmness of each carcass was compared to the diameter of the muscle fibers and the density of the muscle bundles, the correlations were similar in magnitude to the comparable correlations on the dorsal measurements ($r = 0.46$). Therefore, firmness at the lateral location apparently has little or no relationship to muscle fiber diameter or muscle density. Consequently, part of the variation in firmness at the dorsal position of ribeye steaks is attributable to the variation in muscle fiber diameter, muscle bundle density, or the ratio of these two structural components in beef muscle.

Tenderness was not closely associated with either muscle fiber diameter or muscle bundle density ($r \sim .40$); irrespective of whether the comparison involved the location within the ribeye or the pooled carcass measurements. However, these correlations indicated that the tenderness of the cooked steaks tended to increase as the size of the muscle fibers and bundles increased.

The correlations between firmness and collagen (as estimated by hydroxyproline concentration) indicated that the variation in firmness was not associated with the variation in collagen ($r \sim 0.10$). In addition, the Warner-Bratzler shear values were not significantly correlated with the hydroxyproline values ($P > .05$). Thus, these data suggest that neither firmness nor tenderness are related to the variation in the collagen content of the longissimus dorsi muscle for this population.

Simple correlations were also computed to study the relationship of penetrometer, Warner-Bratzler shear, muscle fiber diameter, and

muscle bundle density values to the average percent ether extract in the ribeye of each carcass. The all animal correlation coefficients for these comparisons were non-significant, indicating that factors other than fat content are associated with the variation in firmness, tenderness, muscle fiber diameter, and muscle bundle density of the carcasses in this population.

A comparison of the all animal correlation coefficients for penetrometer versus Warner-Bratzler shear values indicated that these measurements were negatively associated ($r \sim -.35$, d.f. = 55). When these same measurements were compared on a within location basis, the results indicated that the dorsal measurements were negatively related ($r = -.54$, d.f. = 46), but they were not related at the lateral location ($r = -.15$, d.f. = 46). These results suggest that variations in the firmness of the longissimus dorsi are associated with the variations in tenderness primarily at the dorsal location.

In conclusion, these studies indicate that:

1. The muscle density in the dorsal location of the twelfth rib steaks is related to the percent ether extract.
2. The firmness of the dorsal location in the longissimus dorsi at the twelfth rib is associated with the size of the muscle fibers and muscle bundles.
3. The tenderness of cooked eleventh rib steaks is apparently more closely related to factors other than muscle fiber diameter, muscle bundle density, collagen, and fat content.
4. Firmness is negatively correlated with the tenderness of the dorsal location of twelfth rib beef steaks.

Further studies need to be conducted in an effort to elucidate the factors associated with the variation in firmness, particularly at the lateral location, as well as those associated with the variation in tenderness at both the lateral and dorsal locations of *longissimus dorsi* muscle at the twelfth rib for beef carcasses similar in grade and physiological maturity.

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VITA

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Doctor of Philosophy

Thesis: THE RELATION OF FIRMNESS TO CERTAIN OTHER CHARACTERISTICS OF BEEF MUSCLE

PART I. VARIATION IN THE PHYSICAL STRUCTURE OF THE LONGISSIMUS DORSI MUSCLE AT THE TWELFTH RIB AND ITS RELATIONSHIP TO FIRMNESS AND TENDERNESS

PART II. THE RELATIONSHIP OF OBJECTIVELY MEASURED FIRMNESS AND TENDERNESS TO THE PHYSICAL STRUCTURE OF THE LONGISSIMUS DORSI MUSCLE

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