INFLUENCE OF BACTERIAL GROWTH ON PORCINE MUSCLE ULTRASTRUCTURE AND HEADSPACE VOLATILES

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ABSTRACT

INFLUENCE OF BACTERIAL GROWTH ON
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By

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The present investigation was undertaken to provide information concerning changes in muscle ultrastructure and headspace volatiles during spoilage of meat by psychrotolerant and food poisoning microorganisms. In addition, the influence of muscle fiber type on bacterial growth and muscle degradation was studied.

The red and white portions of aseptic porcine semitendinosus muscle were sliced to a thickness of approximately 3 mm, dip inoculated, and incubated with pure cultures of either Pseudomonas fragi (10°C), Bacillus pumilus (10°C), Staphylococcus aureus (15°C), or Clostridium perfringens (30°C). Samples were taken after 0, 24, 48, 96, and 168 hrs incubation for pH measurements, total bacterial counts, and electron microscopic examination. Aseptic samples of porcine longissimus dorsi muscle were ground, inoculated, and incubated with either Pseudomonas fragi or a mixed culture isolated from commercial hamburger. Samples of ground, inoculated and uninoculated aseptic tissue were analyzed by sensory and gas-chromatographic headspace

analysis after incubation for 0, 24, 48, 72 and 96 hrs.

Bacterial growth and ultrastructural degradation were not influenced by fiber type. Changes in tissue pH were related to the amount of bacterial growth which had occurred in the tissue and were not influenced by fiber type.

Extensive ultrastructural degradation was observed in tissues inoculated with Pseudomonas fragi, Staphylococcus aureus, and Clostridium perfringens. Growth of Bacillus pumilus caused no detectable ultrastructural change. Myofibrillar degradation followed the same pattern for all three organisms. Degradation appeared to start with I-band breakage, after which the I-band-Z-line material became diffuse and finally indistinguishable. These results indicate that the A-band region of the myofibril is the most resistant to microbial breakdown.

Nuclei were degraded by Clostridium perfringens and Pseudomonas fragi but were not effected by growth of either Bacillus pumilus or Staphylococcus aureus. Mitochondria appeared to be degraded mainly by autolysis, except in the case of Clostridium perfringens, which caused disappearance of the cristae. Ultrastructural observations and pH values combined with the published pH optima of catheptic enzymes suggest that pH levels near neutrality resulting from growth of either Pseudomonas fragi or Staphylococcus aureus caused greater mitochondrial resistance to degradation.

Ultrastructural observations of aseptic control tissues

showed the presence of vesicular structures in the degraded areas of the myofibril. The vesicles ranged in size from 0.01 to 1.44μ, with the larger vesicles being confined to the red fibers. The proximity of the vesicles to the degraded areas suggests that they may be related to autolytic degradation. It was also noted that intermyofibrillar mitochondria were more stable to autolysis than intramyofibrillar mitochondria. This led to greater disruption of the red fibers due to their high mitochondrial content, although fiber type did not appear to influence the rate of degradation of individual mitochondria.

Analysis of the headspace volatiles from aseptic control tissue resulted in tentative identification of acetone, lactic acid, and acetaldehyde. Tissue inoculated with Pseudomonas fragi produced chromatograms similar to the controls except for the presence of a small ethyl acetate peak. However, tissue inoculated with a mixed culture isolated from commercial hamburger produced peaks tentatively identified as lactic acid, acetaldehyde, ethyl acetate, ethanol, and propionic acid. With the exception of ethanol, the appearance of chromatographic peaks did not correlate with off-odor development. These results suggest that gas-chromatography may prove useful in detecting the onset of meat spoilage, but further work with more sophisticated systems will be required to make the data useful.

INFLUENCE OF BACTERIAL GROWTH ON PORCINE MUSCLE ULTRASTRUCTURE AND HEADSPACE VOLATILES

BY

Maxwell Todd Abbott

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INTRODUCTION

Spoilage of meat has been a public health problem since muscle was first used as a food. While the role of bacteria in meat spoilage is well recognized, as yet the mechanism of spoilage is not fully understood. Likewise, an adequate method of monitoring meat spoilage has not been developed.

The Pseudomonas and Achromobacter groups have been shown to be the dominant flora of refrigerated meat and are also responsible for producing most of the offensive aesthetic changes in meat during spoilage. Recent work (Hasegawa et al., 1970a, b; Dutson et al., 1971) has shown that several psychrophilic species of bacteria are capable of degrading meat proteins. Clostridium perfringens, a food poisoning organism, has also been shown to be capable of meat proteolysis (Hapchuk, 1974). It, therefore, seems likely that other food poisoning organisms may be able to utilize muscle proteins for growth.

Although several workers have studied bacterial proteolysis of meat using electrophoretic techniques, only a few reports employing electron microscopy are found in the literature. Almost all of these studies

used ground muscle tissue, and consequently, were unable to detect changes in nuclei, mitochondria, or membranes, which are disrupted during the grinding process.

Gauthier (1970) pointed out that failure to recognize differences in muscle fiber type has often resulted in incorrect interpretation of experimental data. It has also been noted (Dutson et al., 1971; Gann, 1974) that ultrastructural changes due to aging may be related to fiber types. This suggests that the rate and degree of bacterial spoilage may also be associated with fiber types.

Guarino and Kramer (1969) theorized that food headspace vapors could be used to identify the bacteriological flora during spoilage. However, on using meat products they were unsuccessful due to interference from the vapors normally present in the tissues. Other workers (Miller et al., 1973a, b) have identified some of the volatiles produced by the growth of Pseudomonas species on fish muscle. As yet, methods have not been developed to correlate aesthetic changes in meat with changes in the headspace volatiles occurring during the onset of spoilage.

This study was undertaken to provide information concerning changes in ultrastructure and headspace vapors occurring in meat during the growth of known spoilage and food poisoning organisms. The major objectives of the study were: (1) to identify and compare the sequence of ultrastructural changes in meat inoculated and incubated with various meat spoilage and food

poisoning microorganisms; (2) to determine the relative susceptibility of red and white muscle fibers to bacterial degradation; (3) to determine the relationship of pH and bacterial counts to the ultrastructural changes occurring during spoilage; and (4) to monitor the volatile components of meat as bacterial spoilage ensues.

LITERATURE REVIEW

Microbiology of Meats

Classification of Spoilage Bacteria

Glage (1901) first reported that the moist surfaces of meat stored at low temperature and high humidity became covered with bacterial colonies. He found these organisms to be oval to rod shaped with rounded ends and occasionally in chains. They were motile aerobes that slowly liquified gelatin and turned litmus milk alkaline. Although the indigenous bacteria grew well at 2°C, their optimum temperature for growth was 10 to 12°C.

Haines (1933) observed that with the exception of a few *Pseudomonas* and *Proteus* strains almost all bacteria growing on meat in cold storage belonged to the *Achromobacter* group. Independently, Empey and Vickery (1933) found that 95% of the initial flora of beef capable of growth at 1°C were members of the *Achromobacter* genus, with the other 5% being various species of *Pseudomonas* and *Micrococcus*. Later, Empey and Scott (1939) isolated bacteria growing at low temperatures on fresh meat and found *Achromobacter* to comprise 90%, *Micrococcus* 7%, *Flavobacterium* 3%, and *Pseudomonas* 1% of the total flora.

Ayres et al. (1950), Kirsch et al. (1952), and Wolin et al. (1957) reported that Pseudomonads are the dominant bacteria found on meat stored at low temperatures. These workers recognized that the discrepancy between their results and those of earlier investigators were due to changes in the nomenclature system, mainly as a consequence of the priority accorded the position of flagella. Due to changes in nomenclature, Brown and Weideman (1958) re-evaluated a large number of the Achromobacter isolated by Empey and Scott (1939) and found most to be polarly flagellate species of Pseudomonas. Subsequent studies (Thornley, 1967; Ayres, 1960; and Jay, 1967) have shown that the predominant meat spoilage bacteria at low temperatures are of the Pseudomonas genus. Due to a proposal to do away with the Achromobacter classification by Hendrie et al. (1974), all members of the Achromobacter genus are currently undergoing characterization studies with the possibility of reclassification.

Regardless of the animal specie or muscle, the predominant bacterial flora remains relatively the same. Ayres (1960) reported that freshly slaughtered meat contained an approximately equal number from the genus Micrococcus and Pseudomonas, with lesser populations of Achromobacter, Flavobacterium, Microbacterium, Alcaligenes, Aeromones, Bacillus, Clostridium, and Streptococcus. Organisms commonly found on poultry

carcasses include both pigmented and nonpigmented strains of Pseudomonas and strains of Acinetobacter (Barnes and Impey, 1968). Ostovar et al. (1971) found Pseudomonas, Achromobacter, and Flavobacterium to be the dominant psychrotolerant genera isolated from deboned poultry meat. They also found only 4 of 54 samples were contaminated with Clostridium perfringens. Halleck et al. (1958) reported that Achromobacter and Pseudomonas species together represented 60 and 51% of the flora for prepackaged fresh lamb and beef, respectively.

Bacterial Growth Patterns on Meat

While enormous and varied microbial populations are associated with living meat animals (Lechowich, 1971), the bacterial species responsible for the spoilage of refrigerated meat seem to be quite restricted. Although Ayres (1960) found that more than 80% of the total microbial population on fresh ground beef consisted of chromogenic bacteria, molds, yeasts, and sporeforming microorganisms, examination of spoiled meat revealed a microflora consisting primarily of single, paired, or short-chained, motile, Gram-negative, nonsporeforming rods.

One of the first reports on changes in the relative numbers of bacterial species during cold storage was by Empey and Vickery (1933), who noted an increase in the relative numbers of Achromobacter and Pseudomonas, while the proportion of Micrococcus decreased. Kirsch et al.

(1952) reported a significant population of Lactobacillus and Micrococcus on meat at the beginning of storage, but found that Pseudomonas species predominated after spoilage. Wolin et al. (1957) reported that spoiled meat contained nonpigmented, Gram-negative, aerobic, polarly flagellate rods, with half of the isolated strains being capable of liquifying gelatin.

Halleck et al. (1958) examined prepackaged meat and found that during the first 2 weeks of storage the predominant organisms were of the nonpigmented Achromobacter-Pseudomonas type, but that pigmented types became predominant during subsequent storage. Adams et al. (1964) reported that Pseudomonas and Achromobacter were the only groups in which the percentage of the total population increased during spoilage. Stringer et al. (1969) reported that Pseudomonas fragi, Pseudomonas geniculata, and Micrococcus luteus were the predominant organisms on beef carcasses, but only the Pseudomonas strains were found on meat at the retail level.

Using pure cultures, Barnes and Impey (1968) found that chicken breast muscle and leg muscle differed in their ability to support bacterial growth. They reported good growth of Pseudomonas cultures on both muscle types, but Acinetobacter grew more readily on the leg muscle. On the other hand, they reported that Pseudomonas putrefaciens grew much faster on leg than on breast muscle.

Examination of the ecological parameters effecting microbial growth in or on refrigerated meat revealed that the incubation temperature is the most important single factor influencing growth, preventing growth of all but a few genera (Jay, 1972). Green and Jezeski (1954) found that when the incubation temperature was raised, the proteolytic activity of Pseudomonads decreased. Alford and Elliott (1960) showed that elevated temperatures inhibited lipase production by Pseudomonas fluorescens, but not the activity of the lipolytic enzymes. Alford (1960) found greater lipolytic and proteolytic activity when various Pseudomonas and Achromobacter strains were incubated at temperatures lower than that for their optimal growth. Ingram (1962) found that temperature, humidity, carbon dioxide, and oxygen levels all influence the bacterial flora of meat. Jay (1972) noted that the relatively low pH of fresh meat completely prevents growth of some bacteria, but only slows the growth rate of others.

Aesthetic Changes in Meat Caused by Bacterial Growth

One of the first reports on aesthetic changes in meat due to bacterial growth was that of Mace (Circa 1900, cited by Tissier and Martelly, 1902), who observed that meat spoils in essentially two phases; first, by proliferation of aerobic sporeformers, which produce a flat odor, and secondly, by growth of Gram-negative

bacteria, which cause development of a clearly putrid odor. Tissier and Martelly (1902) subsequently confirmed these observations. Ayres et al. (1950) identified a characteristic rancid, sweetly aromatic, ester-like odor, which developed in poultry meat, and noted that production of this off-odor preceded slime formation. They also reported a pungent ammonia-like odor during the later stages of spoilage.

Ayres (1960) isolated microorganisms from refrigerated beef and found that at 10°C or lower, the bacteria responsible for slime production were almost without exception pseudomonads. Various workers (Schmid, 1931; Empey and Vickery, 1933; Haines, 1937; Kraft and Ayres, 1952; Ayres, 1959, 1960) have attempted to relate bacterial numbers to slime formation, and hence, meat salability. Their results have been somewhat variable, with slime first appearing at bacterial counts ranging from $1 \times 10^6/cm^2$ (Kraft and Ayres, 1952) to $5 \times 10^8/cm^2$ (Schmid, 1931) of the meat surface area.

Ogilvy (1950) obtained good agreement in comparing off-odor development or slime production with either bacterial load or an increase in carbon dioxide production. Ayres (1959, 1960) concluded that off-odor development in eviscerated poultry and packaged beef occurs whenever microbial populations exceeded 10^7 cells/cm^2 of surface area.

Ayres (1960) pointed out that many of the meat

contaminating bacteria are mesophiles and are not responsible for the development of off-odors, off-colors, or slime formation. Shewan et al. (1960) reported that Pseudomonas species are largely responsible for spoilage odors in fish; whereas, Achromobacter strains have little effect.

Adams et al. (1964) suggested that even though the extent of participation of individual members of a bacterial population to spoilage has commonly been attributed to the preponderance of certain genera at the time of spoilage this is not always the case. They then theorized that all bacteria or bacterial groups are not equally active in spoilage. They concluded that most of the bacteria capable of causing spoilage characteristics in fish were confined to the Pseudomonas-Achromobacter groups, but that only a small portion of those two groups were actually "spoilers". There was no selective increase for "spoilers" as storage progressed. Upon inoculation of sterile raw fish press juice with cultures from spoiled fish, they found that the initial load of "spoilers" was consistently below 10% of the total bacterial population. They also noted that even though Pseudomonas and Achromobacter strains accounted for almost 100% of the bacterial population by the end of spoilage, the percentage of "spoilers" had not increased. Subsequently, Herbert et al. (1971) reported similar results for marine fish.

McMeekin and Patterson (1975) found that only a

restricted number of bacteria are capable of producing detectable amounts of hydrogen sulfide from meat. McMeekin (1975) tested the ability of pure cultures to produce off-odors in poultry meat and found strains of both pigmented and nonpigmented Pseudomonas organisms to be capable of off-odor production. His data showed that 73% of nonpigmented Pseudomonas produced off-odors compared to only 22% for the pigmented Pseudomonas strains.

Bacterial growth on meats has been observed to cause changes in the pH of the tissue. Ockerman et al. (1969) and Adamcic and Clark (1970) reported an increase in the pH values of beef and poultry tissue upon being inoculated and incubated with Achromobacter and Pseudomonas cultures. Lobben and Lee (1968) obtained similar results with fish muscle. Several authors (Hasegawa et al., 1970a, b; Borton et al., 1970a; Tarrant et al., 1973) have reported that growth of Pseudomonas fragi on porcine muscle may raise the pH of the tissue to alkaline levels. Hasegawa et al. (1970b) reported that growth of Clostridium perfringens caused an increase in the pH of porcine tissue, but noted no change in pH due to growth of Achromobacter liquefaciens.

Evaluation of Bacterial Spoilage

Recognition of the end point of meat salability as spoilage proceeds has been a continual problem of the meat industry. Eber (1892, cited by Turner, 1960) first proposed ammonia detection

as a means of assessing meat spoilage. Shortly thereafter, several workers (Ottolenghi, 1913; Falk et al., 1919; Tillmans et al., 1921; Schmidt, 1928) attempted to correlate aesthetic changes in stored meat with bacterial loads, but the relationships were low. As microbiological techniques for sampling and enumeration improved, the relationship between bacterial counts and off-odor production and/or slime formation gave a more accurate assessment of spoilage (Schmid, 1931; Empey and Vickery, 1933; Haines, 1937; Kraft and Ayres, 1952; Ayres, 1959, 1960).

A number of workers (Proctor and Greenlie, 1939; Johns, 1944; Straka and Stokes, 1957; Ferguson, 1958; Wells, 1959; and Walker et al., 1959) have correlated bacterial numbers with resazurin dye reduction time. Kurtzman and Snyder (1960) developed a freshness test for iced shrimp based on an increase in the turbidity of a picric acid extract, which they reported to be associated with a decrease in organoleptic scores and an increase in total bacterial counts. Saffle et al. (1961) compared the resazurin dye reduction and picric acid methods, as well as a ninhydrin method, in an attempt to determine the shelf-life of meat. They concluded that the resazurin reduction method gave the best correlation between odor scores and bacterial counts.

Kraft et al. (1956) employed lead acetate impregnated filter paper strips to estimate the extent of

hydrogen sulfide production as an in-package indicator for growth of meat spoilage bacteria. Jay (1972) proposed that meat spoilage organisms alter the hydration capacity of muscle proteins, and subsequently developed procedures for measuring muscle hydration, which he claimed could be used to estimate the extent of spoilage. The extract-release volume (Jay, 1964a, b), water holding capacity (Jay, 1965) and meat swelling and viscosity (Jay, 1969) were all reported to correlate with organoleptic and bacterial population data. On the other hand, Miller and Price (1971) found that the correlation between extract-release volume and bacterial numbers did not reliably predict the bacteriological soundness of pork. Shelef and Jay (1970) also developed a titrimetric technique for estimating spoilage, which is based upon the alkalinization of meat during bacterial growth.

Fleming *et al.* (1969) noted that the volatiles from a food may include the metabolic products of the various microorganisms growing within the food. Previous workers (O'Brien, 1966; Bassette *et al.*, 1967) have shown that some bacterial species can be identified by analysis of the vapors produced when grown on culture media. Specifically, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa* (O'Brien, 1966), *Streptococcus faecalis*, *Streptococcus lactis*, *Streptococcus diacetilactis*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Achromobacter lypolyticum*, and *Pseudomonas fragi* (Bassette *et al.*,

1967) were reported to produce gas chromatograms varied enough to allow identification of the bacterial species. Guarino and Kramer (1969) speculated that analysis of headspace vapors for a food could be used as a rapid method for identifying the microbiological flora. Although they were able to identify members of the Enterobacteriaceae family, difficulty was encountered in differentiating bacterial species on hamburger due to the interference of the vapors normally present.

While studying bacterial metabolism, Keenan et al. (1967) observed that pseudomonads have the ability to reduce acetaldehyde to ethyl alcohol. Subsequently, Reddy et al. (1969) noted that Pseudomonas fragi grown in milk converted ethyl alcohol to ethyl butyrate and ethyl hexanoate. These findings prompted Miller et al. (1973a, b) to study the volatiles produced by Pseudomonas species grown on fish muscle. Miller et al. (1973a) first reported that Pseudomonas perolens produced methyl mercaptan, dimethyl disulfide, dimethyl trisulfide, 3-methyl-1-butanol, butanone, and 2-methoxy-3-isopropylpyrazine. Later, Miller et al. (1973b) reported that Pseudomonas fragi produced dimethyl sulfide, acetaldehyde, ethyl acetate, ethyl alcohol, and dimethyl disulfide.

Chemical Changes in Meat Due to Bacterial Growth

Jay and Kontou (1967) reported that fresh beef allowed to undergo microbial spoilage at 7°C showed decreases in

the quantity and types of amino acids as well as decreases in nucleotide levels. Based on the decreases in the simple nitrogenous components, these authors concluded that beef spoilage bacteria do not attack the primary muscle proteins. However, careful examination of the chromatograms presented by Jay and Kontou (1967) suggest that they had incorrectly interpreted the data. The chromatogram of tissue inoculated with a mixed culture of meat spoilage organisms showed large increases in several of the amino acids while the chromatogram from the sterile control revealed no such increases. Jay (1967) noted that not all bacteria capable of degrading gelatin were able to break down muscle proteins and concluded that any degradation of muscle proteins was probably due to the action of catheptic enzymes. He further stated that low-molecular weight compounds support the growth of spoilage bacteria and that the salt-soluble proteins are essentially untouched by bacterial proteolysis.

Rampton et al. (1970) reported that porcine myofibrillar proteins were not degraded by Achromobacter liquefaciens, Micrococcus luteus, Pediococcus cerevisiae, Pseudomonas fluorscens, Streptococcus faecalis, or a mixed culture from spoiled meat. It should be noted, however, that these authors used sucrose density centrifugation, which has since been found to be insensitive to the protein changes occurring during meat spoilage. However, the authors reported poor growth of all cultures except Achromobacter liquefaciens, which may account for their failure to find

degradation of myofibrillar proteins.

In contrast, Ockerman *et al.* (1969) found that beef muscle inoculated and incubated with *Pseudomonas* and *Achromobacter* cultures decreased slightly in stromal protein, increased in nonprotein nitrogen, and had a greater emulsifying capacity than control uninoculated beef muscle. Adamcic *et al.* (1970) reported that *Achromobacter* and nonpigmented *Pseudomonas* cultures reduced the amount of amino acids on inoculated chicken skin during the early log phase of growth, whereas, pigmented *Pseudomonas* cultures caused a marked increase in amino acid levels during the late log phase.

Borton *et al.* (1970a) found that growth of *Pseudomonas fragi* increased the water-soluble proteins in porcine muscle. They also noted increases in nonprotein nitrogen and salt-soluble protein levels when the tissue was inoculated with *Pseudomonas fragi*, *Pedicoccus cerevisiae*, *Micrococcus luteus*, or *Leuconostoc mesenteroides*. Subsequently, Borton *et al.* (1970b) reported changes in the electrophoretic pattern of porcine salt-soluble proteins caused by growth of the same four bacterial species. Samples inoculated with *Pseudomonas fragi* showed a marked decrease in a number of protein bands indicating that proteolysis of salt-soluble proteins had occurred. Growth of the other bacterial species caused no detectable changes in the electrophoretic patterns of the tissue extracts.

Hasegawa et al. (1970a) compared starch gel electrophoretic patterns of extracts from aseptic and inoculated porcine and rabbit muscles. Pseudomonas fragi showed the greatest amount of proteolytic activity on the sarcoplasmic fraction of both rabbit and pig muscle, with lower but significant amounts of activity on the urea-soluble proteins. Leuconostoc mesenteroides caused alteration of the sarcoplasmic proteins of both rabbit and porcine muscle, but Micrococcus luteus degraded only the rabbit muscle proteins. Pedicoccus cerevisiae was found to break down the urea-soluble proteins from both rabbit and porcine muscle. This organism also degraded the sarcoplasmic proteins from rabbit muscle, but had no effect on porcine sarcoplasmic proteins. Further analysis showed that Pseudomonas fragi decomposed aldolase, glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, creatine kinase and hemoglobin from pig muscle, and creatine kinase, phosphofructokinase, phosphoglycerate kinase, phosphopyruvate hydratase, and hemoglobin from rabbit muscle. Thus, results indicated that different microorganisms preferentially utilized specific proteins from rabbit and porcine muscle.

Hasegawa et al. (1970b) reported a similar study using Clostridium perfringens, Salmonella enteritidis, Achromobacter liquefaciens, Streptococcus faecalis, and Kurthia zopfii. They noted that Clostridium perfringens caused extensive breakdown of the sarcoplasmic and urea-soluble

proteins from pig and rabbit muscle, while Streptococcus faecalis and Salmonella enteritidis effected only myoglobin. Achromobacter liquefaciens and Kurthia zopfii had no measurable proteolytic activity on muscle tissue.

Tarrant et al. (1971) inoculated and incubated porcine muscle with Pseudomonas fragi. They noted a large decrease in the salt-soluble protein fraction with a corresponding increase in nonprotein nitrogen. Disc-gel electrophoretic patterns indicated significant proteolysis of the salt-soluble proteins by the 10th day of storage and almost complete breakdown by the 20th day. Proteolytic activity was found to increase significantly during the late log phase of growth.

Tarrant et al. (1973) isolated a proteolytic enzyme fraction from Pseudomonas fragi which rapidly degraded a myofibrillar protein preparation. This enzyme preparation also broke down G-actin and myosin more slowly and hydrolyzed the sarcoplasmic proteins at even slower rates. They noted that the enzyme preparation displayed optium activity at neutral pH and 35°C.

Porzio and Pearson (1975) purified and characterized a proteolytic enzyme isolated from Pseudomonas fragi. They found it to be Zn^{+2} activated and Ca^{+2} stabilized. Further characterization showed the enzyme preparation to have a molecular weight of 40,000-50,000 daltons.

Buckley (1972) studied a protease fraction from Pseudomonas perolens and found that it degraded collagen

and myofibrillar proteins, but had little effect on the sarcoplasmic proteins. This enzyme preparation was reported to have an optimum pH range of 6.5 to 9.0 and an optimum temperature of 35°C. He found that the enzyme(s) were inactivated by EDTA and theorized that they were Ca^{+2} activated.

Hapchuk (1974) studied the degradation of meat by the food poisoning organism, Clostridium perfringens. She observed that Clostridium perfringens caused an increase in nonprotein nitrogen and a decrease in total sarcoplasmic nitrogen, troponin, actin, and tropomyosin. She concluded that Clostridium perfringens was capable of degrading both the sarcoplasmic and myofibrillar proteins. However, a proteolytic enzyme fraction isolated from cultures of Clostridium perfringens exerted its major action on the sarcoplasmic proteins, leading to the conclusion that Clostridium perfringens presumably elaborates more than one protease.

Ultrastructural Changes Caused by Bacterial Growth

Walker (1969) reported that the structural proteins of beef remained essentially intact for several weeks after incipient spoilage. On the other hand, Dutson et al. (1971) reported that porcine tissue inoculated with Pseudomonas fragi was extremely disrupted after 8 days incubation at 10°C. These workers observed an almost complete absence of material in the H-zone, marked

disruption of the A-band, and some loss of dense material from the Z-line. They noted that myosin appeared to be the most susceptible protein to degradation. The ultrastructure of Pseudomonas fragi organisms observed in degraded muscle tissue showed formation of bleb-like evaginations on the outer cell wall. This led the authors to theorize that the blebs may be the mechanism for protease transport from the bacterial cell into the muscle tissue where a source of nutrients is available.

Tarrant et al. (1973) inoculated porcine muscle with an enzyme preparation from Pseudomonas fragi. They reported evidence of some dissolution of the Z-line after 6 hours incubation, and almost complete loss of the dense material from the Z-line after 72 hours incubation. The other portions of the myofibril did not seem to be affected by the enzyme(s).

Buckley et al. (1974) reported that porcine muscle inoculated and incubated with Pseudomonas perolens showed little ultrastructural change until after 8 days of storage, at which time marked Z-line fragmentation and myofibril separation were noted. On incubating the muscle with a proteolytic enzyme preparation from Pseudomonas perolens, the M-line and Z-line were completely removed by day 4, and by day 8 disruption of the actin-myosin complex became evident. Thus, the effects of the enzyme(s) and the organism per se were not identical.

Hapchuk (1974) reported that Clostridium perfringens

caused degradation of the thin filaments in the I-band region. She also noted a loss of material from the M-line which could be attributed to either proteolysis by the bacteria or the relatively high (37°C) incubation temperature. She also observed that Clostridium perfringens degraded the white fibers more readily than the red fibers, especially in being more active in degrading the thin filaments. The degradation of the thin filaments observed by electron microscopy confirmed the electrophoretic evidence for the breakdown of troponin, which is a component of the thin filament (Maruyama and Ebashi, 1970).

Red and White Muscle Fibers

Characterization of Red and White Muscle Fibers

It has long been known that mammalian skeletal muscles differ in color (Ciaccio, 1898), and that the fibers composing skeletal muscles differ in their microscopic appearance (Ranvier, 1874). Even within a given muscle the fibers are known to differ from one another (Grutzner, 1884; Knoll, 1891), and as many as three types of fibers were described over 55 years ago by Bullard (1919). Histochemical procedures for localizing enzymic activity have confirmed these early observations and extended them by revealing additional differences (Padykula, 1952; Ogata, 1958; Nachmias and Padykula, 1958; Dubowitz and Pearse, 1960).

Red fibers have been characterized as those having

a highly oxidative metabolism, large mitochondrial numbers (Dubowitz and Pearse, 1960), high succinic dehydrogenase activity (Stein and Padykula, 1962), and elevated myoglobin levels (Chinoy, 1963; James, 1968). They have also been reported to be more resistant to fatigue than white fibers (Burke et al., 1971 and 1973), and have higher levels of triglycerides (Adams et al., 1962) and lipase activity (Piantelli and Rebello, 1967).

Conversely, white fibers predominate in glycolytic metabolism with high levels of phosphorylase and myofibrillar ATPase activity (Dubowitz and Pearse, 1960; Engel, 1962). They also are characterized by having faster contraction rates and motor unit response times than red fibers (Barnard et al., 1971; Peter et al., 1972).

The original observations on muscle color by Ranvier (1874) and Grutzner (1884) led to the use of nomenclature of "red" or "white" to describe the fibers. Subsequent findings have shown fiber variations running the gamut between the classical definitions of red and white fibers. The diversity has led to the development of numerous classification schemes (Engel, 1974) for determining muscle fiber types. To avoid confusion the classical definitions of red and white fibers will be used throughout this work and intermediate fibers will not be considered.

Protein Differences Between Red and White Fibers

Seidel et al. (1964) discovered that myofibrils prepared from red muscle had a lower ATPase activity than those prepared from white muscle. Subsequently, several groups (Barany et al., 1965; Streter et al., 1966; Seidel, 1967) reported a lower Ca^{+2}-modified and EDTA-modified ATPase activity for red than for white muscle myosin. Streter et al. (1966) found that at low ionic strength the ATPase activity of red muscle myosin was activated by N-ethylamaleidmide, while white muscle myosin was unaffected. Several workers (Seidel, 1967; Guth and Samaha, 1969; Samaha et al., 1970a) have reported that the Ca^{+2}-modified ATPase activity of red muscle myosin is very labile at alkaline pH (10.5), but relatively stable at acid pH (4.35); conversely, the Ca^{+2}-modified ATPase activity of white muscle myosin is relatively stable at alkaline pH and labile at acid pH.

Kuehl and Adelstein (1970) found that red muscle myosin had no 3-methyl-histidine, but white muscle myosin contained 2 residues of 3-methyl-histidine per molecule. Locker and Hagyard (1968) and Samaha et al. (1970a, b) reported that upon treatment of red and white muscle myosin with p-chloromercuriphenylsulfonate, the red muscle myosin released two electrophoretically distinct proteins not found in white muscle myosin. Seidel (1967) and Gergely et al. (1965) reported that red muscle myosin was more resistant to tryptic digestion than white muscle

myosin.

Suzuki et al. (1973) studied α-actinin from porcine red and white muscles and found no differences in sedimentation patterns, circular dichroic spectra, rate of trypsin digestion, or in the ability to increase the Mg^{+2}-modified ATPase activity or rate of turbidity formation in suspensions of either red or white reconstituted actomyosin. However, they did find that α-actinin from red muscle contained more aspartic acid than α-actinin from white muscle; this resulted in α-actinin from red muscle having 17 more negatively charged amino acids per 100 residues than white α-actinin.

Ultrastructural Differences Between Red and White Muscle Fibers

Using the system of Gauthier (1969, 1970), Dutson et al. (1974), and Suzuki et al. (1973) classified porcine muscle fibers as either red or white. They reported that red fibers had wide, dense Z-lines approximately 120 nm in width, while white muscle fibers were characterized by narrow, less dense Z-lines approximately 6.25 nm in width. They also noted that mitochondria from red fibers were very large, showed closely packed cristae, were dense in appearance, and were arranged in groups just beneath the sarcolemma, in intrafibrillar rows, and/or in pairs at the Z-line. The mitochondria from white fibers were observed to be smaller, less dense, and contained fewer cristae. White fiber mitochondria were observed mainly

between myofibrils at the level of the Z-line, with a limited number located near the sarcolemma.

Dutson et al. (1974) reported the sarcoplasmic reticulum of white fibers to have large, open longitudinal tubules; and in the region of the H-zone, they contained open sac-like structures with some fenestrations. Red fiber longitudinal tubules were found to be narrower, more tortuous and have fewer fenestrations. Terminal cisternae of red fibers appeared dense throughout, whereas, in white fibers the dense areas were primarily next to the transverse tubule.

Autolysis of Muscle Tissue

Protein and Nonprotein Nitrogen Changes

Hoagland et al. (1917) noted an increase in noncoagulable nitrogen during low temperature storage of meat and attributed it to autolysis. McCarthy and King (1942) observed that meat aged at either cooler or high temperatures (35°C) showed an increase in soluble nitrogen values. Radouco-Thomas et al. (1959) found that the release of amino acids in postmortem muscle was inhibited by the injection of epinephrine and suggested its use as an anti-autolytic agent. Numerous other workers subsequently have observed increases in the amounts of either nonprotein nitrogen or amino acids during meat aging (Locker, 1960; Ma et al., 1961; Sharp, 1963; Davey and Gilbert, 1966;

Suzuki et al., 1967; Field and Chang, 1969; Parrish et al., 1969a).

Several authors (Kronman and Winterbottom, 1960; McLoughlin, 1963; Sayre and Briskey, 1963; Goll et al., 1964; Aberle and Merkel, 1966) have reported the sarcoplasmic proteins to be most soluble immediately after death, following which the solubility decreases with increased storage times. The effect of aging upon the myofibrillar proteins is less clear. Some workers (Zender et al., 1958; McLoughlin, 1963; Sayre and Briskey, 1963) have reported a decrease in myofibrillar solubility with aging, while Goll et al. (1964) found no change. Other authors (Hegarty, 1963; Aberle and Merkel, 1966; McIntosh, 1967; Penny, 1968; Davey and Gilbert, 1968a, b) have found an increase in the solubility of the myofibrillar proteins as postmortem time increased. It has been suggested by several groups (Sayre and Briskey, 1963; McLoughlin, 1963; Davey and Gilbert, 1968a) that pH and temperature may determine protein solubility during aging.

Structural Changes

Henderson et al. (1970) found that unrestrained porcine muscles underwent minimal shortening at 25°C and only slightly more contraction at 2 and 16°C. Gann (1974) working with bovine muscle reported variable degrees of contraction at various intervals throughout 216 hrs of cooler storage.

Davey and Gilbert (1969) observed a loss of adhesion in aged bovine myofibrils and alterations of the Z-line, which sometimes led to complete dissolution of the Z-line. Henderson et al. (1970) reported that Z-line degradation occurs more quickly and to a greater extent at storage temperatures of 25°C or above than at temperatures of 16°C or below.

Cassens et al. (1963) reported mitochondrial disruption in porcine muscle at 24 hrs postmortem. Dutson et al. (1974) observed that mitochondrial disruption or loss of cristae density occurred in all samples by 24 hrs postmortem, but was particularly obvious in white fibers. Gann (1974) noted considerable morphological variation in mitochondria by 48 hrs postmortem. Intermyofibrillar mitochondria appeared more stable than those encircling the I-band-Z-line area and suggested a fiber type degradation relationship, with red fibers being more stable than white fibers.

Dutson et al. (1974) working with porcine muscle reported that no triads or transverse tubules were apparent in either fiber type by 24 hrs postmortem. Furthermore, by 24 hrs postmortem the sarcoplasmic reticulum was no longer recognizable.

Gann (1974) observed that even though both red and white fibers showed disruption of the connection between the thin filaments and the Z-line, white fibers were easily distinguishable from red because of a greater number of

breaks.

Lehninger (1970) reported an abundant supply of 15 to 30 nm diameter glycogen granules in the intermyofibrillar cytoplasm of muscle at death. However, Gann (1974) noted that glycogen granules were completely absent in bovine muscle by 48 hrs postmortem.

Henderson et al. (1970) using porcine muscle found that storage at 37°C had caused the complete disappearance of the M-line structure. However, Gann (1974) utilizing bovine muscle reported that at cooler temperatures the M-line was unaltered at 48 hrs postmortem, but that by 216 hrs the M-line although still apparent was not discrete or prominent.

Catheptic Enzyme and Lysosomal Activity

Much attention has been devoted to isolating a component or components which would promote protein degradation in postmortem muscle. Balls (1938) isolated and partially purified a cathepsin from muscle which had a pH optimum of 4.1. Snoke and Neurath (1950) reported the isolation and partial purification of a proteolytic agent from rabbit skeletal muscle with a pH optimum of 4.0 and activity against crude muscle extracts. Sliwinski et al. (1959) purified an enzyme from beef muscle and determined that optimum activity occurred at pH 4.4 and 37°C.

Doty (1950) suggested that many of the changes observed in postmortem aging of meat could be attributed to

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proteolytic enzymes of the catheptic group. Balls (1960) in reviewing catheptic enzymes in muscle stated that the quantity of these components was very low and that their optimum activity occurs at a more acidic pH than is attained by postmortem muscle. Subsequently, Koszalka and Miller (1960a, b) purified an enzyme from rat skeletal muscle with optimum activity at pH 8.5-9.0 on both synthetic and homogenized muscle substrates. Sliwinski et al. (1961) reported on a crude bovine enzyme preparation, which was isolated at pH 5.6 and contained three different proteolytic enzymes. Landman (1963) observed that autolytic activity in beef muscle had a dual optima at pH 5.0 and 8.5-9.0. He concluded that cathepsins B and C were responsible for the dual optima.

Although proteases have been demonstrated in muscle, the most important aspect of this subject is their activity against muscle substrates. Sharp (1963) studied autolysis of bovine and rabbit muscle and observed an increase in nonprotein nitrogen but no change in the fine structure of the tissue. They concluded that the increased quantity of nonprotein nitrogen was due to the degradation of sarcoplasmic proteins.

Bodwell and Pearson (1964), using a partially purified bovine catheptic fraction, studied catheptic activity on peptides, synthetic substrates, and four natural substrates isolated from muscle. The fraction was found to have no detectable enzymatic action on crude preparations

of actin, myosin, or actomyosin, but the sarcoplasmic proteins appeared to be readily hydrolyzed. Martins and Whitaker (1968) prepared cathepsin D and found no detectable activity on actomyosin.

In related papers, Suzuki and Fujumaki (1968) and Suzuki et al. (1969a, b) reported on the isolation and purification of cathepsin D from rabbit muscle. They found cathepsin D to be most active against the sarcoplasmic proteins, followed in order by myosin, actin, and actomyosin.

Several investigators (Balls, 1960; Sharp, 1963; Bodwell and Pearson, 1964; Martins and Whitaker, 1968) have raised doubts about the significance of aseptic autolysis of primary meat proteins during aging. Martins and Whitaker (1968) and Caldwell (1970) have suggested that a combination of the various cathepsins (A, B, C, D) may be necessary for the degradation of the salt-soluble proteins of meat.

Smith (1964) reported that histochemical enzyme markers showed lysosomal activity to be confined to the vascular system of normal muscle. Parrish and Baily (1966, 1967) found that bovine cathepsins were particulate and suggested that at least a portion of the enzymes found in muscle were membrane bound. Recently, other workers (Moore et al., 1970; Bernacki and Bosmann, 1972; Tokes and Chambers, 1975) have found membrane associated proteases. Bernacki and Bosmann (1972) found cathepsin D-like activity to be associated with the membrane of human erythrocytes. These

results suggest a possible role for membrane associated proteases in the autolysis of muscle tissue.

METHODS AND MATERIALS

Preparation of Stock Cultures

Pure cultures of Pseudomonas fragi, Bacillus pumilus (formerly classified as Achromobacter liquefaciens), Staphylococcus aureus, and Clostridium perfringens were obtained from the American Type Culture Collection (Rockville, Maryland). The specific culture numbers and their incubation conditions are shown in Table 1. The Staphylococcus aureus culture was initially incubated at room temperature but was gradually acclimated to 15°C. The Clostridium perfringens culture was started at 35°C and gradually reduced to 30°C.

A mixed culture from commercial hamburger was prepared by blending the tissue with distilled water and inoculating the slurry into APT broth.

Slaughter and Tissue Preparation

Slaughtering Procedures

Seven market weight pigs (81 to 104 Kg) produced at the Michigan State University Swine Farm were slaughtered individually over a 6 month period. A modification of the method of Hasegawa et al. (1970) was employed to obtain aseptic muscle samples.

The unstunned pigs were suspended by the hind leg and the area of the neck utilized for sticking was scrubbed thoroughly with a bactericidal soap solution. Sticking was carried out with a sterile knife. After conventional dehairing and evisceration, the unsplit carcass was rinsed twice with ethanol and flamed with a dehairing torch. The carcass was then placed in a 1 to 3°C cooler for approximately 24 hrs. After chilling, the carcass was laid on a steam sterilized stainless steel table in a clean room free from excessive air currents and the ethanol rinse-flame procedure was repeated twice. The carcass was then ready for muscle excision.

Preparation of Muscle for Ultrastructural Analysis

The carcass was positioned on the sterile table so that the hams were exposed. An incision was then made starting at the lower midline, curving inward and finally outward to the hock. The skin and subcutaneous fat were stripped off exposing the upper portion of the semitendinosus muscle. The semitendinosus muscle was then dissected out from origin to incision and placed in sterile stainless steel containers.

The excised muscle was then taken to a filtered-air inoculation room. The predominantly red and white areas of the muscle were then separated and the remainder discarded. A sample was taken from both the red and white areas and frozen in liquid nitrogen for subsequent pH

and histochemical analysis. The remaining tissue was prepared for inoculation by cutting it into 1-3 mm thick slices with a sterilized Sears Model 490 electric knife. During all muscle preparation procedures only sterilized equipment was used and all personnel wore sterile disposable gloves.

Preparation of Muscle for Headspace Analysis

The pig carcasses used to study meat spoilage volatiles were prepared in the same way as the ones used for ultrastructural analysis, except that the longissimus dorsi was used instead of the semitendinosus muscle. This was done by making an incision through the backfat along the dorsal midline, followed by two incisions perpendicular to the midline. The backfat was stripped back from the midline and sections of the longissimus dorsi muscle were excised and placed in a sterile container. The precautions previously described were used in order to avoid contamination of the tissue.

Tissue Inoculation and Incubation

Tissue for Ultrastructural Analysis

Stock cultures of either Pseudomonas fragi, Bacillus pumilus, Staphylococcus aureus, or Clostridium perfringens were used to inoculate the tissue. This was done by dipping the sliced tissue into a magnetically stirred inoculum for 5 seconds and draining by suspension for 10

seconds. The inoculated tissue was then spread across the bottom of disposable petri dishes, covered immediately and placed at the proper incubation temperature (see Table I).

Clostridium perfringens samples were maintained in anaerobic conditions by placing in a National Appliance (Portland, Oregon) incubator, which was then vacuumized and nitrogen flushed three times.

A preliminary study showed that the dipped-slice inoculation technique yielded uniform bacterial loads per gram of tissue (see Appendix I).

Tissue for Headspace Analysis

Sections of the longissimus dorsi muscle were ground through a sterilized prechilled grinder, placed in sterile containers, and covered with a loose fitting sterile lid. Inoculum was prepared by diluting 100-fold a 48 hr culture of Pseudomonas fragi or a mixed culture prepared from commerical hamburger. During grinding, 50 ml of the inoculum were added to approximately 1000 gm of muscle. The ground tissue was then spread evenly across the bottom of petri dishes and incubated at 10°C.

Samples were removed daily for bacterial counting, sensory evaluation, pH measurement, and headspace analysis. A three member sensory panel was used to detect the onset of spoilage. They were presented with samples of inoculated and uninoculated control tissue and asked to indicate the absence or presence of off-odors.

Table I. Preinoculation, Experimental, and Enumeration Conditions for Bacterial Cultures.

ORGANISM	ATCC NUMBER	PREINOCULATION CONDITIONS		INCUBATION TEMPERATURE °C	ENUMERATION CONDITIONS	
		MEDIA	TEMPERATURE °C		MEDIA	TEMPERATURE °C
Pseudomonas fragi	4973	APT broth	10	10	APT agar	25
Bacillus pumilus[a]	15716	APT broth	10	10	APT agar	25
Staphylococcus aureus	14458	Brain-heart infusion broth	15	15	APT agar Mannitol-salt agar	25
Clostridium perfringens	13124	Thioglycolate broth	30	30	APT agar SPS/with D-cyco-serine	30 30
Mixed Culture[b]	----	APT broth	10	10	APT agar	25

[a] Formerly classified as *Achromobacter liquefaciens*.
[b] Culture from commercial hamburger.

Preparation of Control Tissue for Ultrastructural and Headspace Analysis

Uninoculated control samples were handled in the same way as the inoculated tissue except that sterile media was added to the aseptic tissue instead of a bacterial inoculum. Thus, uninoculated control samples were available for analysis at the same time periods as similar inoculated samples. By comparison of the results of the control and inoculated samples at the end of each storage period, it was possible to differentiate between the changes resulting from microbial action and those resulting from autolysis at the incubation temperature.

Bacterial Counting Procedures

Plate counts were performed on inoculated and uninoculated control tissues prepared for ultrastructural analysis at 24, 48, 72, 120, and 168 hrs postmortem. In the case of samples for headspace analysis, plate counts were performed daily starting at 24 hrs postmortem. Bacterial numbers were assessed by the methods described by the American Public Health Association (1966), except in the case of Clostridium perfringens, which was incubated at 30°C. The plating agars, temperatures, and other variables are shown in Table 1. All plates were incubated for 48-72 hrs before counting.

Clostridium perfringens samples were enumerated anaerobically on trptose-sulfite-cycloserine agar.

Anaerobic conditions were obtained by evacuating and nitrogen flushing a sealed incubator cabinet (National Appliance Co., Portland, Oregon) three times.

Freedom from contamination of the Staphylococcus aureus cultures was confirmed using Mannitol-salt agar. On this agar, colonies of Staphylococcus aureus are surrounded by a yellow zone.

pH Measurement

Measurement of tissue pH was done at 24, 48, 72, 120, and 168 hrs postmortem for all samples prepared for ultrastructural observations. Samples prepared for headspace analysis were monitored daily.

The pH of the muscle tissue was determined by placing 2 gm of tissue and 10 ml of 0.005 M sodium iodoacetate (pH 7.0) into a microblender jar and homogenizing for 1 minute. The pH of the resultant slurry was then measured to the nearest 0.01 unit with a Radiometer Model 26 expanded scale pH meter.

Histochemical Analysis

Tissue Preparation

Previously frozen red and white tissue samples were mounted on a microtome chuck chilled to -27°C. The chuck and tissue were then placed on a Slee-Pearse crystat and

sectioned approximately 12μ in thickness. The sections were then placed on coverslips and allowed to sit at room temperature for 30 minutes to facilitate fixing the sections to the coverslips.

Determination of Fiber Type

Fiber type was determined by the reduced diphosphopyridine nucleotide-tetrazolium reductase method of Engel and Brooke (1965). The tissue sections were allowed to incubate in a solution (see Appendix II) of 0.2 M tris buffer (pH 7.4), nitroblue tetrazolium and reduced diphosphopyridine nucleotide (NADH) for 30 minutes at 36°C. The sections were then fixed by immersion for 2 minutes each in a series of acetone-water mixtures. The mixtures (acetone to water) in order were: 30/70, 60/40, 90/10, 60/40, and 30/70. The tissue was then rinsed in distilled water and mounted on glass slides with glycerogel. This method stains red or oxidative fibers blue, while white or glycolytic fibers remain unstained.

Stained sections were observed on a Ziess Photomicroscope III. Fiber types were determined and enumerated in ten fasciculi from both red and white tissues for each ultrastructural experiment.

Electron Microscopy

Fixation and Embedding

Samples of control and inoculated tissue were fixed and embedded at 24, 48, 72, 120, and 168 hrs postmortem. Fixation was accomplished using a modification of the procedure described by Sjostrand (1967). Small pieces of muscle tissue were fixed for 2 hrs in a buffer solution containing 1.25% glutaraldehyde-0.048 M sodium phosphate-0.043 M NaCl at pH 7.4 (approximately 415 miliosmolar). The tissue samples were then washed for 1 hr in 2 changes of 0.094 M sodium phosphate-0.043 M NaCl buffer solution at pH 7.4. The samples were then postfixed for 1 hr in 1% osmium tetroxide solution in veronal acetate buffer (pH 7.4), which was adjusted to 300 miliosmolar with NaCl, KCl, and $CaCl_2$. Fixative and buffer solution preparation schedules are shown in Appendix II.

After fixation, the samples were dehydrated for 15 minutes each in 25, 50, 75, and 95% ethanol. They were then placed in 2 changes of 100% ethanol for 30 minutes each. The dehydrated muscle samples were then transferred through 2 changes of propylene oxide for 30 minutes in each, followed by 12 hrs in a 1:1 mixture of propylene oxide and epon-araldite resin. The samples were then embedded in pure epon-araldite resin using flat embedding molds (LKB Instruments, Inc.). The embedded samples were placed in a desiccator under slight vacuum for 12 hrs, then

placed in an 80°C oven for 36 hrs to allow the blocks to harden.

Sectioning and Staining

Epon-araldite embedded tissue blocks were trimmed by hand with a razor blade. The muscle samples were then sectioned with a diamond knife to a thickness of 60 to 100 nm using an LKB 4801A ultramicrotome. Sections were picked up from the knife boat on uncoated 300-mesh copper grids.

Staining of the tissue was accomplished by floating the grids for 30 minutes on a saturated solution of uranyl acetate, rinsing thoroughly with distilled water, and then staining for 5 minutes in a solution of lead citrate (Reynolds, 1963). The sections were then washed with 0.02 M NaOH, followed by distilled water, and air dried.

Observation and Photography of Muscle Sections

A Philips EM-300 Electron Microscope was used for observing the stained sections at an accelerating voltage of 60 KV. Representative photographs of each sample were taken using Kodak 8.25 x 10.16 cm sheet film. The film was developed for 4 minutes in Kodak D-19 developer, washed for 1-1/2 minutes in running water, fixed 8 to 10 minutes in Kodak fixer, washed in running water for 1 minute, rinsed in Kodak Hypo-clearing Agent, and washed for 10 minutes in running water. The washed negatives were dipped in Kodak Photo-Flo solution and dried for 45 minutes with warmed air. All processing from the latent image to the final negative was

performed on an Arkay nitrogen burst machine.

Ilford Ilfoprint rapid stabilization paper was exposed from the negatives using a Durst S-45-EM enlarger. The Ilfoprint paper was developed in an Ilford model 1501 rapid stabilization processor using Ilford activator and stabilizer chemicals. Selected prints were fixed in Kodak fixer, washed in Orbit bath, flattened with Pakosol, washed in running water, and dried on a ferrotype dryer.

Gas-Liquid Chromatography of Headspace Vapors

Sampling of Headspace Vapors

Duplicate one gram samples were taken each day of storage from the inoculated and uninoculated control samples for analysis of tissue volatiles by gas-liquid chromatography. Each sample along with 1 gm of anhydrous sodium sulfate was placed in a screw-capped vial which had a teflon-faced rubber septum. The vial was tightly sealed and placed in a 50°C water bath for 15 minutes.

A sample of the headspace vapors was obtained from the vials with a Hamilton 1002-LTN gas-tight syringe. The vapors where drawn into the barrel of the syringe, evacuated into the vial, and refilled three times before a 1.5 ml sample was removed. The syringe was immediately injected into the gas chromatograph. The syringe was cleaned between injections by means of a Hamilton syringe cleaner (Hamilton, Inc., Wittier, California).

Gas-Liquid Chromatography

Gas chromatographic analysis was performed on a Hewlett-Packard Series 5750B Research Gas Chromatograph (F-M Scientific Division, Avondale, Pennsylvania) equipped with a hydrogen flame detector. Two types of stainless steel columns were used. One column contained 80/100 mesh acid-washed Chromosorb W coated with 10% Carbowax 10M, while the other column was packed with 80/100 mesh Gas Chrom Z coated with 4% Apiezon L. Both columns were 9 feet long and had an outside diameter of 1/8 inch.

Gas Chromatography-Mass Spectrometry (GC-MS) of Headspace Vapors

Duplicate samples of inoculated and uninoculated control samples were prepared for analysis 96 hrs after inoculation. The bacterial cultures and preparation methods were the same as those used for gas chromatographic analysis.

Combined GC-MS was carried out on an LKB-9000 Mass Spectrometer, interfaced to a dedicated minicomputer (PDP-8/1, Digital Equipment Company) for data acquisition and reduction (Sweeley *et al.*, 1970). Data was displayed on a Tektronic Model 4002A storage scope with keyboard terminal and a Tektronic Model 4601 hard copy unit.

A coiled glass GC column, 6 feet in length by 2 mm (i.d.), was employed. The column was packed with 3% OE-30 on 100/120 mesh Supelcoport. Mass spectral measurements were recorded at 70 eV ionizing energy with a full

accelerating voltage of 3.5 kV and a 60μ A trap current.

accelerating voltage of 3.5 kV and a 60μ A trap current.

RESULTS AND DISCUSSION

Ultrastructural Changes Due to Microbial Growth

Histochemical Analysis of Semitendinosus Muscle

Red and white muscle fibers were differentiated by the method of Engel and Brooke (1965) which stains red fibers blue while white fibers remain unstained. The proportion of red, white, and intermediate fibers found in the red and white portions of porcine semitendinosus muscle are shown in Appendix III. The percentage of red fibers in the red portion of the semitendinosus muscle varied from 52.6 to 76.2% of the total fibers, with an average of 68.2%. White fibers comprised 75.9% of all fibers in the white portion of the semitendinosus muscle, with a range from 74.3 to 78.1%. The percentage of white fibers in the red portion was always less than 10.1% of the total fibers, whereas, the percentage of red fibers in the white portion never exceeded 2.8%. These results combined with the known ultrastructural differences of the different fiber types makes the possibility of mistaking red and white fibers quite remote.

Changes in Aseptic Control Tissue

Although separate uninoculated control samples incubated at 10-15°C for up to 168 hrs were run in the Pseudomonas fragi, Bacillus pumilus, and Staphylococcus aureus experiments, the control tissues for these organisms were found to be similar throughout incubation and will be discussed together. Because the Clostridium perfringens experiment was carried out at a higher temperature (30° vs 10-15°C) and under anaerobic conditions, tissue autolysis was found to be accelerated and will be discussed separately.

Bacterial Growth and pH Changes. Neither bacterial growth nor significant pH changes were detected in any of the uninoculated control tissues (Appendix IV). Thus, any ultrastructural changes observed during the incubation period could be attributed to autolysis or to alterations induced during preparation of the tissues.

Changes in the Ultrastructure of Aerobic Control Tissues. The myofibrils were identified based on Z-line width, mitochondrial content, and color of the original muscle. Using the system of Dutson et al. (1974), myofibrils having very wide Z-lines (approximately 120 nm) and a large number of mitochondria were classified as red fibers, whereas, those with narrow Z-lines (approximately 65 nm) and very few mitochondria were classified as white fibers. Intermediate type fibers

were also observed, but were not followed due to the difficulty encountered in characterizing them.

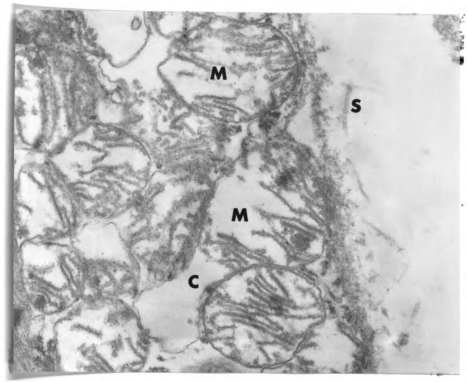
The initial uninoculated samples were removed from the carcass 24 hrs postmortem, and hereafter are referred to as the 0 hr incubation samples. At this time, most mitochondria (Figure 1) and nuclei (Figure 2) were relatively intact. As storage proceeded, the nuclei were observed to shrink in size causing an increase in the concentration of nuclear material (Figure 3).

The mitochondria (Figure 1) appeared to be intact at 0 hr incubation, although some aggregation of the cristae had occurred in all samples. After 24 hrs of incubation at 10-15°C, most of the mitochondria encircling the I-band-Z-line region appeared as large empty vesicles (Figure 4). Intermyofibrillar mitochondria appeared to be more stable than intramyofibrillar mitochondria and were found to be in relatively good condition after 168 hrs of incubation. Similar findings were reported in beef muscle by Gann (1975), who suggested that stability was related to fiber type, with mitochondria from red fibers being more stable than those from white fibers. Although intermyofibrillar mitochondria were more stable than intramyofibrillar mitochondria in the present study, differences between fiber types were not apparent.

At 0 hr the sarcolemma appeared to be quite diffuse (Figures 1 and 2). Separation of the sarcolemma from the muscle fibers was quite common after 24 hrs

Figure 1. Aseptic control mitochondria from the red portion of porcine semitendinosus muscle at 0 hr incubation (24 hrs postmortem). M = mitochondria, C = cristae, S = sarcolemma. 25,600 X.

Figure 2. Aseptic control nucleus from the white portion of porcine semitendinosus muscle at 0 hr incubation. N = nucleus, C = chromatin, S = sarcolemma, My = myofibril. 13,455 X.


M
S
M
C

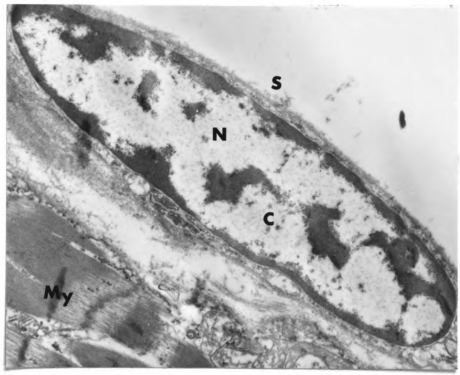
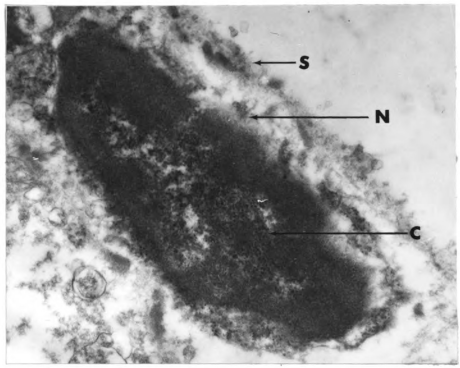
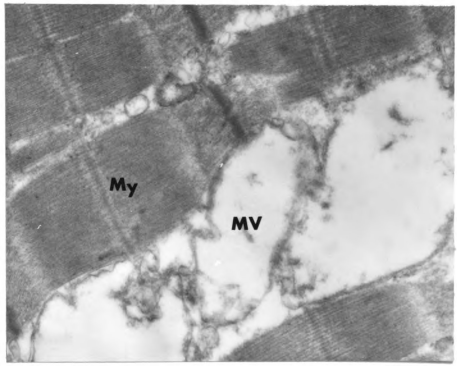

S
N
C
My

Figure 3. Aseptic control nucleus from the white portion of porcine semitendinosus muscle after 168 hrs of incubation at 10°C. N = nucleus, C = chromatin, S = sarcolemna. 32,000 X.

Figure 4. Aseptic control red fiber and intramyofibrillar mitochondrial vesicles after 24 hrs incubation at 10°C. My = myofibril, MV = mitochondrial vesicle. 34,650 X.


S
N
C


My
MV

incubation at 10-15°C. After 168 hrs incubation, the sarcolemma appeared to lose all structural integrity.

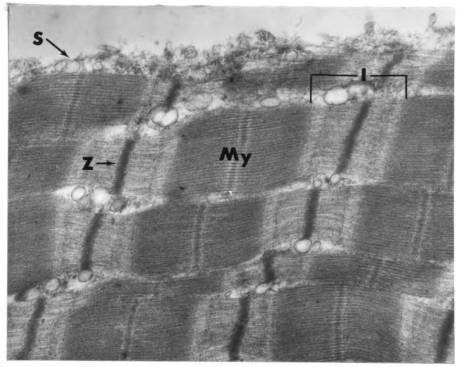
Areas of myofibrillar degradation were uncommon in 0 hr samples of both red and white fibers (Figures 5 and 6). The number of degraded areas increased with storage time, but even after 168 hrs of incubation the large majority (approximately 80%) of the myofibrils were still intact. Observation of degraded areas throughout storage showed that white muscle fibers tended to split lengthwise and appeared to have undergone more loss of Z-line material than red fibers (Figure 7).

Numerous vesicular structures were always present in the degraded areas of the tissue. Figure 8 shows an atypical 0 hr white fiber with some autolytic degradation and several vesicular structures. The vesicular structures ranged in size from approximately 0.01 μ to 1.44 μm. White muscle fibers contained only small vesicles (0.01 to 0.05 μm) whereas, red muscle fibers contained vesicles of all sizes. The size and location of the large vesicles indicate that they are probably remnants of degraded mitochondria, which would account for the larger vesicles being observed in red fibers.

Although the source of the smaller vesicles is unknown, their most likely origin would be from the existing membranes of the muscle, such as those from mitochondria, sarcoplasmic reticulum, and the T-tubule system. Some authors (Rash et al., 1968; Harsanyi and

Figure 5. Aseptic control red fiber at 0 hr. My = myofibril, Z = Z-line, I = I-band, S = sarcolemna. 27,200 X.

Figure 6. Aseptic control white fiber at 0 hr incubation. My = myofibril, Z = Z-line, I = I-band. 30,000 X.


S
I
Z
My

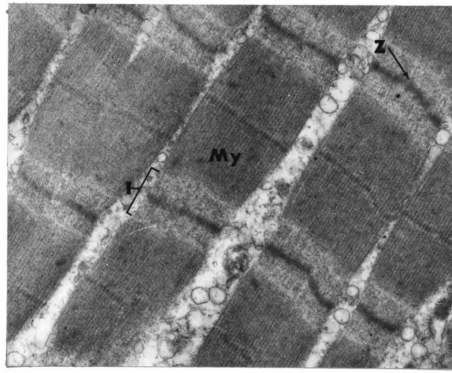
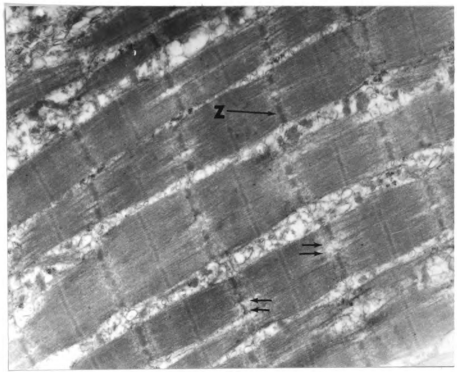
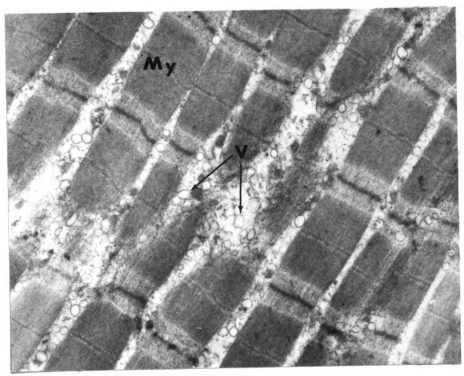

Z
My
I

Figure 7. Aseptic control white fiber after 168 hrs of incubation at 10°C showing typical type of white fiber degradation. Z = Z-line, double arrows = splits in the myofibril. 18,750 X.

Figure 8. An atypical aseptic control white fiber at 0 time. My = myofibril, V = vesicles. 17,000 X.


Z


My
V

Garamvoglyi, 1969; Walcott and Ridgway, 1967) have proposed that the Z-line contains a lipid component, and if this component does in fact exist, it could also be a source of the vesicles.

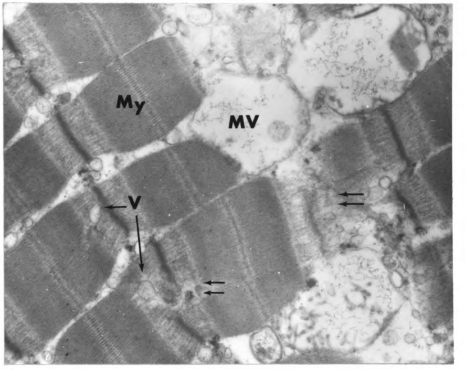
Even though the myofibrils were relatively stable during incubation periods up to 168 hrs, the I-band was the structure most susceptible to degradation during autolysis of both red and white fibers. Frequently, the mitochondrial membranes and/or small vesicles were found in the I-band-Z-line area of degrading myofibrils. Figure 9 shows a typical red fiber after 168 hrs of incubation and illustrates the large vesicular structures commonly observed during degradation of red fibers.

Figure 10 shows a ruptured mitochondrian with the area immediately surrounding the rupture undergoing autolysis. These results suggest that proteolytic enzymes located in the membranes may be active in muscle autolysis. Proteolytic activity has been reported to be associated with the membranes in muscle by Parrish and Baily (1966), and several workers (Moore et al., 1970; Bernacki and Bosmann, 1972; Tokes and Chambers, 1975) have observed proteolytic activity in other membrane systems.

Changes in the Ultrastructure of Anaerobic Control Tissue. Aseptic control tissue from the Clostridium perfringens experiment was stored under nitrogen and at a higher temperature (30°C) than the control tissue previously described, and therefore, is discussed separately.

Figure 9. Aseptic control red fiber after 168 hrs of incubation at 10°C. My = myofibril, MV = mitochondrial vesicle, V = vesicle, double arrows = degraded area. 19,500 X.

Figure 10. Aseptic control red fiber after 96 hrs of incubation at 10°C. My = myofibril, MV = mitochondrial vesicle, M = degrading mitochondrian, double arrows = degraded area. 26,875 X.


My
MV
V

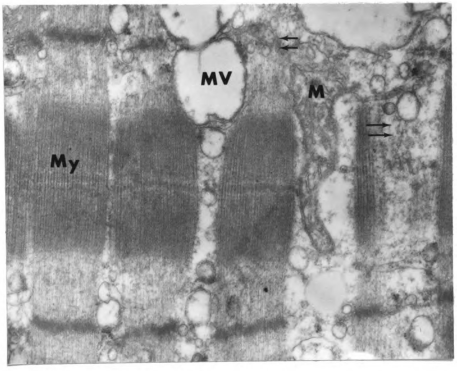

MV
M
My

Figure 11 shows a typical red fiber after 24 hrs of incubation at 30°C. Autolysis of the nuclei, myofibril, and sarcolemma appeared to proceed by the same mechanism(s) as for aerobically stored tissue but at a faster rate. After 96 hrs of incubation, approximately one-third of all myofibrils examined showed breaks in the Z-line-I-band region (Figure 12). Some loss of M-line material was also observed after 96 hrs of incubation at 30°C (Figure 12). After 168 hrs of incubation, the M-line had almost completely disappeared.

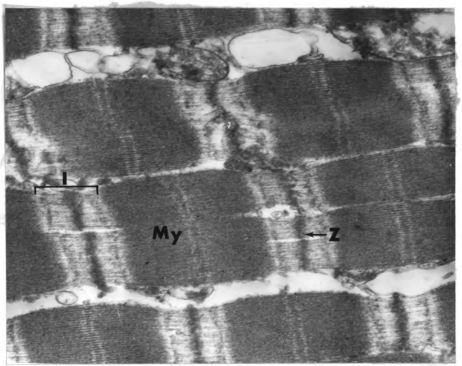
The appearance of most mitochondria undergoing anaerobic autolysis differed from that of mitochondria in the aerobic controls. Under aerobic conditions, mitochondrial cristae were found to aggregate together (Figure 1), but under anaerobic conditions most cristae formed small vesicles within the mitochondrial membrane (Figure 13). Although the exact cause of the ultrastructural change in the cristae is not clear, it is possible that different autolytic enzyme systems predominate under aerobic and anaerobic conditions. Mechanical disruption during vacuumization or tissue damage during fixation is also a possibility.

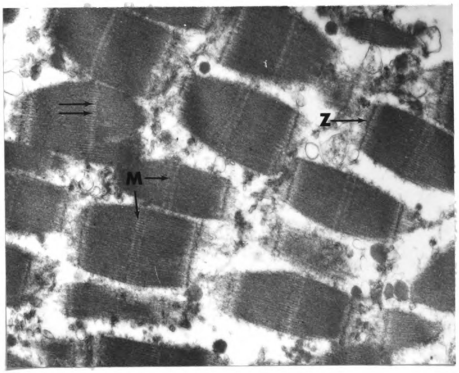
Changes in Tissue Inoculated With Pseudomonas fragi

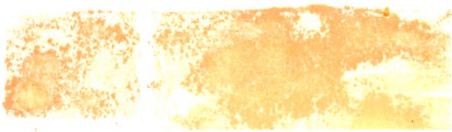
Bacterial Growth and pH Changes. Figure 14 shows the growth of Pseudomonas fragi on both the red and white portions of porcine semitendinosus muscle. As

Figure 11. An anaerobically stored aseptic control red fiber after 24 hrs incubation at 30°C. My = myofibril, Z = Z-line, I = I-band. 27,200 X.

Figure 12. An anaerobically stored aseptic control white fiber after 96 hrs incubation at 30°C. Note I-band breaks and absence of some M-lines. M = M-line, Z = Z material, double arrows = absence of M-line. 23,750 X.


I
My
Z


Z
M



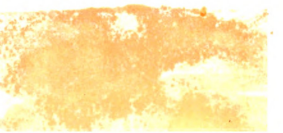


Figure 13. Anaerobically stored aseptic control inter-myofibrillar mitochondria from the red portion of the semitendinosus muscle after 24 hrs incubation at 30°C. M = mitochondria, V = vesicles, S = sarcolemma. 23,750 X.

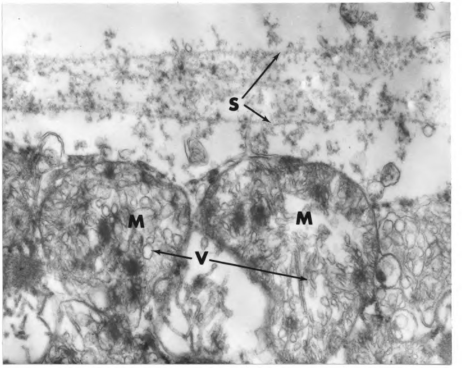
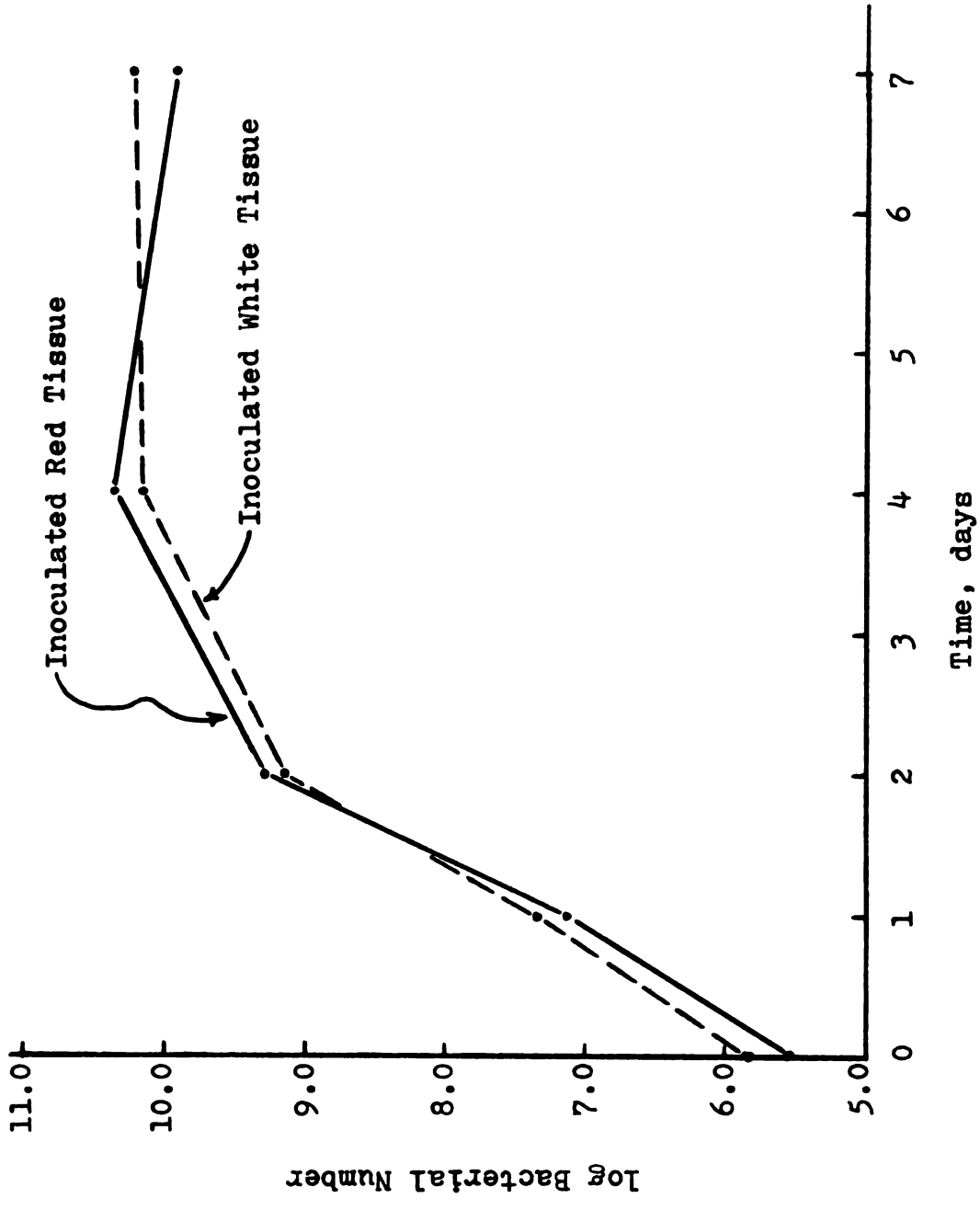

S
M
M
V

Figure 14. Bacterial growth of Pseudomonas fragi on both the red and white portions of porcine semitendinosus muscle.


Inoculated Red Tissue
Inoculated White Tissue
log Bacterial Number
11.0
10.0
9.0
8.0
7.0
6.0
5.0
0
1
2
3
4
5
6
7
Time, days

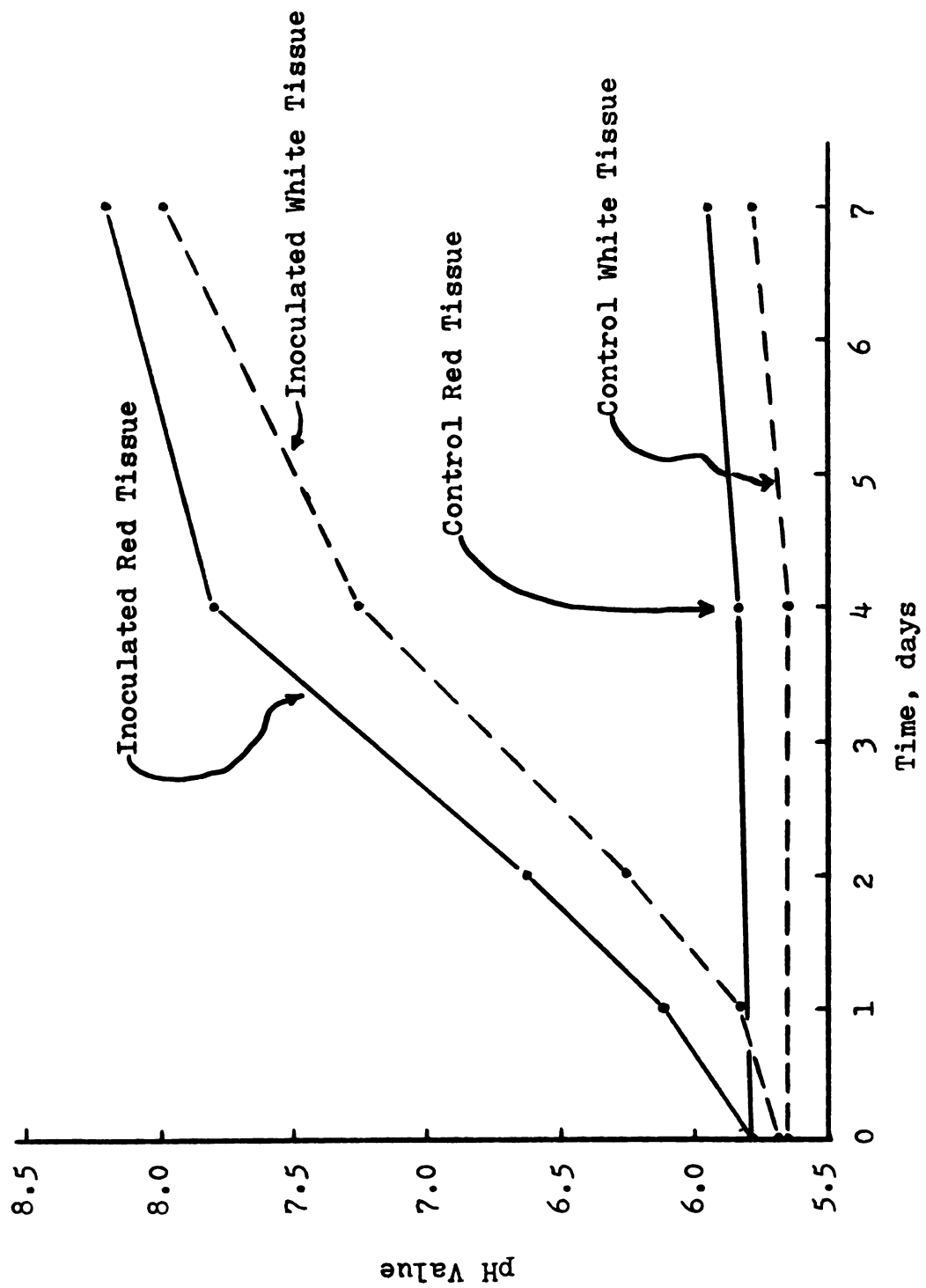
illustrated by the graph, there was no significant difference in the growth rate of Pseudomonas fragi on either red or white muscle. The absence of a lag phase during the initial stages of the growth curve may be due to a combination of factors, including the preconditioning of inoculum cultures at the experimental temperature, high inoculum numbers, and a vigorously growing culture.

Figure 15 illustrates the increase in tissue pH values due to the growth of Pseudomonas fragi on the red and white portions of porcine semitendinosus muscle. Although the white portion had a lower initial pH value than the red portion, the rate of pH increase did not vary significantly between the red and white muscle types. Furthermore, the pH of control tissues did not change significantly in either red or white muscle during incubation.

These results indicate that the growth rate of Pseudomonas fragi was not effected by different pH values for post-rigor red (pH 5.79) and white (pH 5.68) muscle. It was also noted that Pseudomonas fragi did not show a growth preference for either red or white muscle.

Changes in the Ultrastructure of Inoculated Tissue. After 24 hrs incubation with Pseudomonas fragi only localized ultrastructural changes were observed. These changes appeared to be related to the proximity of the bacteria to the tissues. After 48 hrs of incubation,

Figure 15. Changes in pH during the incubation of the red and white portions of porcine semitendinosus muscle inoculated with Pseudomonas fragi.


Inoculated Red Tissue
Inoculated White Tissue
Control Red Tissue
Control White Tissue
pH Value
8.5
8.0
7.5
7.0
6.5
6.0
5.5
0
1
2
3
4
5
6
7
Time, days

degradation became apparent throughout the tissues.

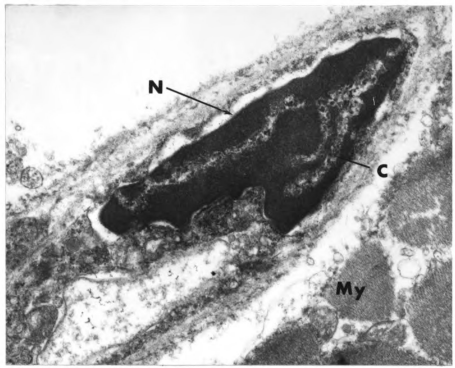
After 24 hrs of incubation, nuclei near the bacteria (Figure 16) were found to have undergone a much greater degree of shrinkage than control nuclei (Figure 2). Even though the rate of nuclei shrinkage was accelerated during incubation, most nuclei were still intact after 96 hrs of bacterial growth. However, by 168 hrs of bacterial growth the nuclei were absent.

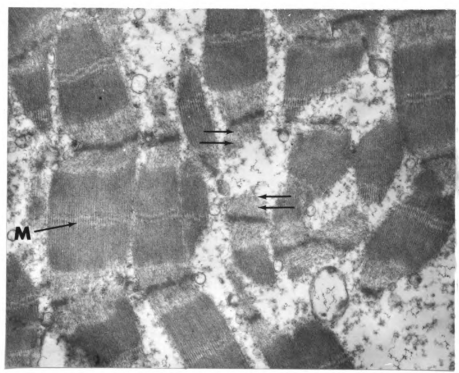
Initial mitochondrial degradation was similar to that of uninoculated control tissue, with the intramyofibrillar mitochondria being the most susceptible to degradation. After 96 hrs of incubation with Pseudomonas fragi, the remaining mitochondria appeared to be quite stable in contrast to the control, which continued to degrade up to 168 hrs. This was unexpected since Hasegawa et al. (1970a) had reported extensive breakdown of many mitochondrial enzymes during incubation with Pseudomonas fragi.

A marked increase in the pH (3.5 pH units) of the inoculated tissue was observed between 48 and 96 hrs of incubation and is probably responsible for the greater stability of the mitochondria during incubation with Pseudomonas fragi. A possible explanation for this may be found in the work of Landmann (1963) who noted that catheptic enzymes from muscle had a dual pH optima at 5.0 and 8.5 to 9.0. Thus, growth of Pseudomonas fragi which increased tissue pH values to near or slightly above

Figure 16. Typical nucleus from the red portion of porcine semitendinosus muscle incubated for 24 hrs at 10°C with Pseudomonas fragi. Note the nuclear density. N = nucleus, C = chromatin, My = myofibril. 23,000 X.

Figure 17. Typical red fiber incubated for 48 hrs at 10°C with Pseudomonas fragi. M = M-line, double arrows = breaks in I-band. 19,500 X.


N
C
My


M

neutrality may have resulted in decreased autolytic activity and hence the decreased rate of mitochondrial degradation. These results suggest that autolysis normally occurs at the usual pH of post-rigor muscle and may be the major reason for mitochondrial breakdown.

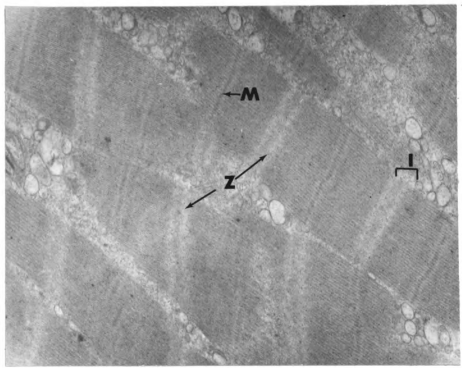
After 48 hrs of bacterial growth the myofibrillar ultrastructure varied significantly from that of aseptic control tissue. By that time, the degree of breakage in the I-band-Z-line region of the myofibril was much more extensive than in uninoculated control tissues (Figure 17). Approximately one-half of the myofibrils observed showed this type of damage after 48 hrs of bacterial growth.

After 96 hrs of incubation with Pseudomonas fragi, the I-band-Z-line region of all myofibrils had either disappeared (Figure 18) or were very diffuse and indistinct (Figure 19). The A-band region appeared to be the most stable structural segment of the myofibril to the action of Pseudomonas fragi. In red fibers no recognizable myofibrillar structures were observed after 168 hrs of bacterial growth (Figure 20), while A-bands and M-lines were still evident in a limited number of fields from the white portion of the muscle (Figure 21) at the same time interval.

Results show that Pseudomonas fragi caused extensive breakdown of both red and white muscle fibers. The results also suggest that there is very little difference, if any, in the degree or mechanism of degradation in red

Figure 18. White fiber incubated for 96 hrs at 10°C with Pseudomonas fragi. M = M-line, I = I-band area, Z = Z-line area. 29,600 X.

Figure 19. White fiber incubated for 96 hrs at 10°C with Pseudomonas fragi. M = M-line, I = I-band, Z = Z-line. 26,400 X.


M
Z
I

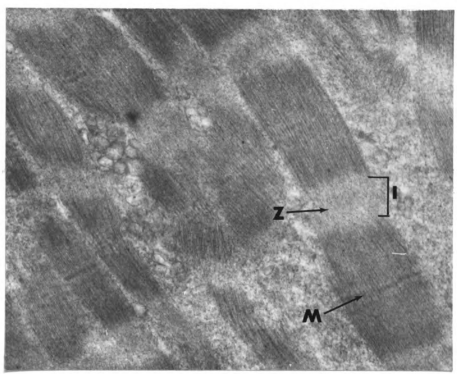
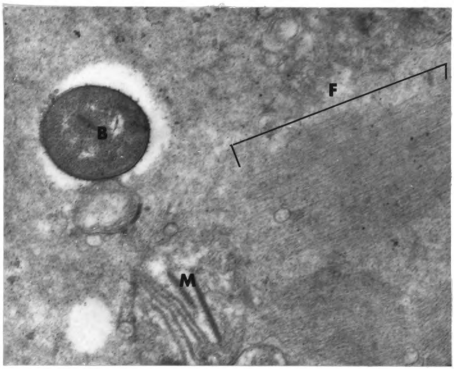
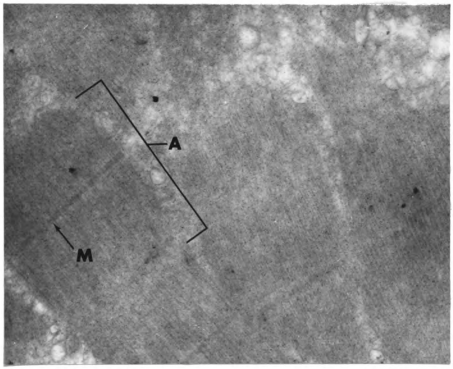

Z
I
M

Figure 20. Typical field from the red portion of porcine semitendinosus muscle after 168 hrs of incubation at 10°C with Pseudomonas fragi. Note mitochondrial cristae and areas of oriented filaments. B = bacteria, M = mitochondrian, F = filaments. 42,000 X.

Figure 21. Typical field from the white portion of porcine semitendinosus muscle after 168 hrs incubation at 10°C with Pseudomonas fragi. Note the absence of Z-lines. M = M-line, A = A-band. 39,900 X.


B
F
M


A
M

and white muscle fibers by Pseudomonas fragi. In the present study the A-band region of the myofibril, which contains mainly myosin, was found to be quite resistant to the action of Pseudomonas fragi when compared to other areas of the myofibril. These results differ from those of Dutson et al. (1971), who suggested that myosin was the most susceptible protein to degradation by Pseudomonas fragi. The difference may be explained by the fact that Dutson et al. (1971) based their conclusions on micrographs of ground muscle tissue that had undergone bacterial growth for from 8 to 20 days. Thus, it is possible that changes intermediate to total disruption of the myofibril were obscured. Another possibility is that grinding the tissue made the myosin more accessible to proteases from Pseudomonas fragi.

Thus, Pseudomonas fragi was found to cause extensive changes in the ultrastructure of tissue nuclei and myofibrils, while the mitochondria were degraded in a manner similar to that of uninoculated controls until the tissue pH approached neutrality. Nuclei were observed to shrink as in autolysis but at a much faster rate and to finally disappear. Degradation of the myofibrils appeared to start with I-band breakage, after which the I-band-Z-line material became very diffuse and finally indistinguishable. The most stable area of the myofibril, the A-band-M-line region, was also found to undergo extensive breakdown during the latter stages of the incubation period.

Changes in Tissue Inoculated With Bacillus pumilus

Bacterial Growth and pH Changes. Figure 22 shows the growth of Bacillus pumilus on both the red and white portions of porcine semitendinosus muscle. Analysis of the data shows that the population of Bacillus pumilus increased only slightly during the incubation period. Initially Bacillus pumilus (ATCC 15716) grew slightly better on the red portion of the semitendinosus muscle but by the end of the storage period the bacterial population was essentially the same on both types of tissue.

No significant increase in tissue pH was observed for either the red or white portions of porcine semitendinosus muscle during the entire incubation period. As shown in Figure 23, the inoculated tissue pH values did not vary significantly from those of uninoculated control tissue.

These results are in agreement with those of Hasegawa et al. (1970b) who reported that the population of Bacillus pumilus (ATCC 15716) (formerly Achromobacter liquefaciens) did not increase significantly or cause any pH change when grown on porcine muscle. On the other hand, Rampton et al. (1970) reported good growth of Bacillus pumilus (ATCC 15716) on porcine muscle but showed no bacterial counts or pH data.

The culture used in the present experiment grew well in the pre-inoculation medium and had to be transferred about every 3 days to avoid overgrowth of the

Figure 22. Bacterial growth of Bacillus pumilus on both the red and white portions of porcine semitendinosus muscle at 10°C.

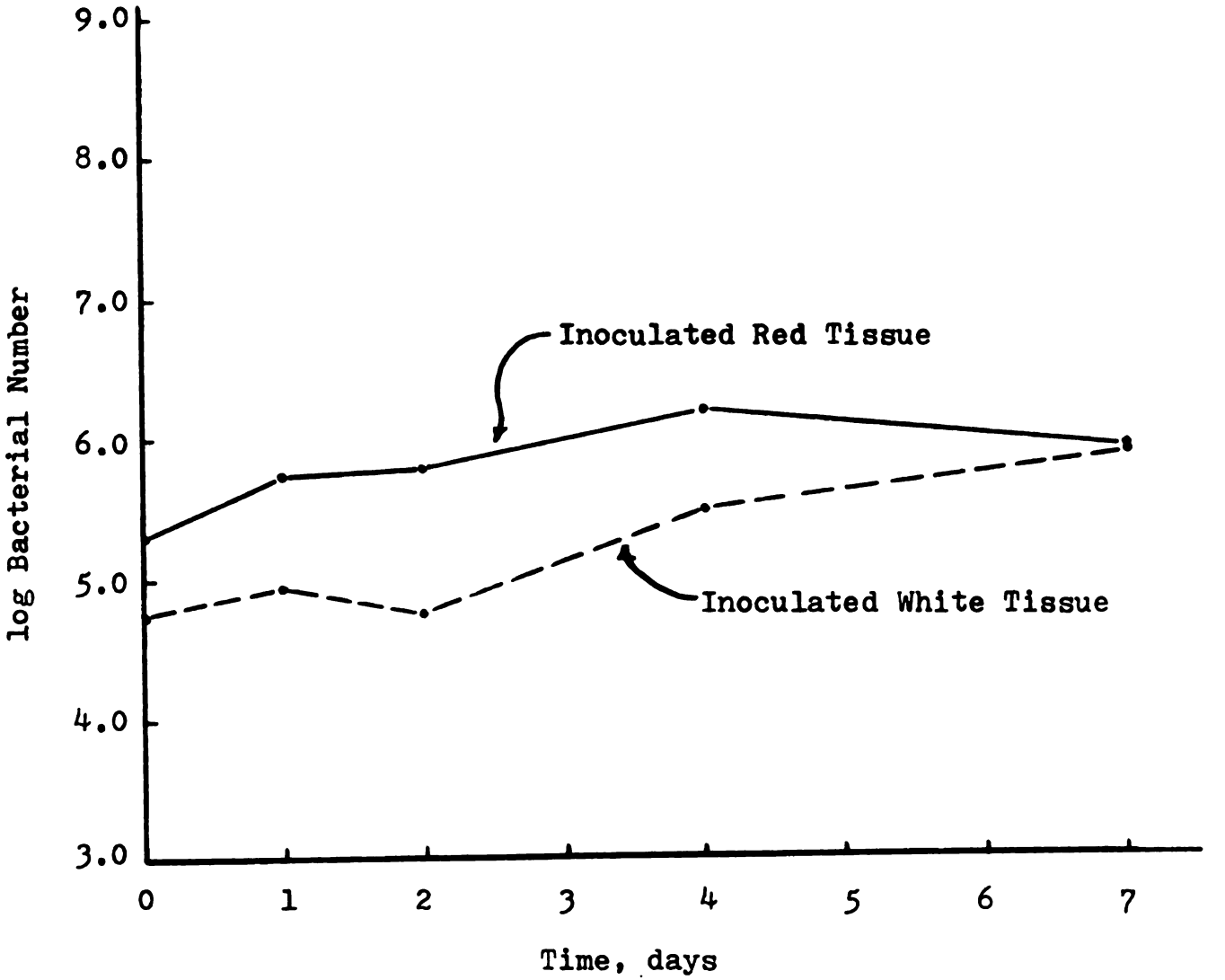
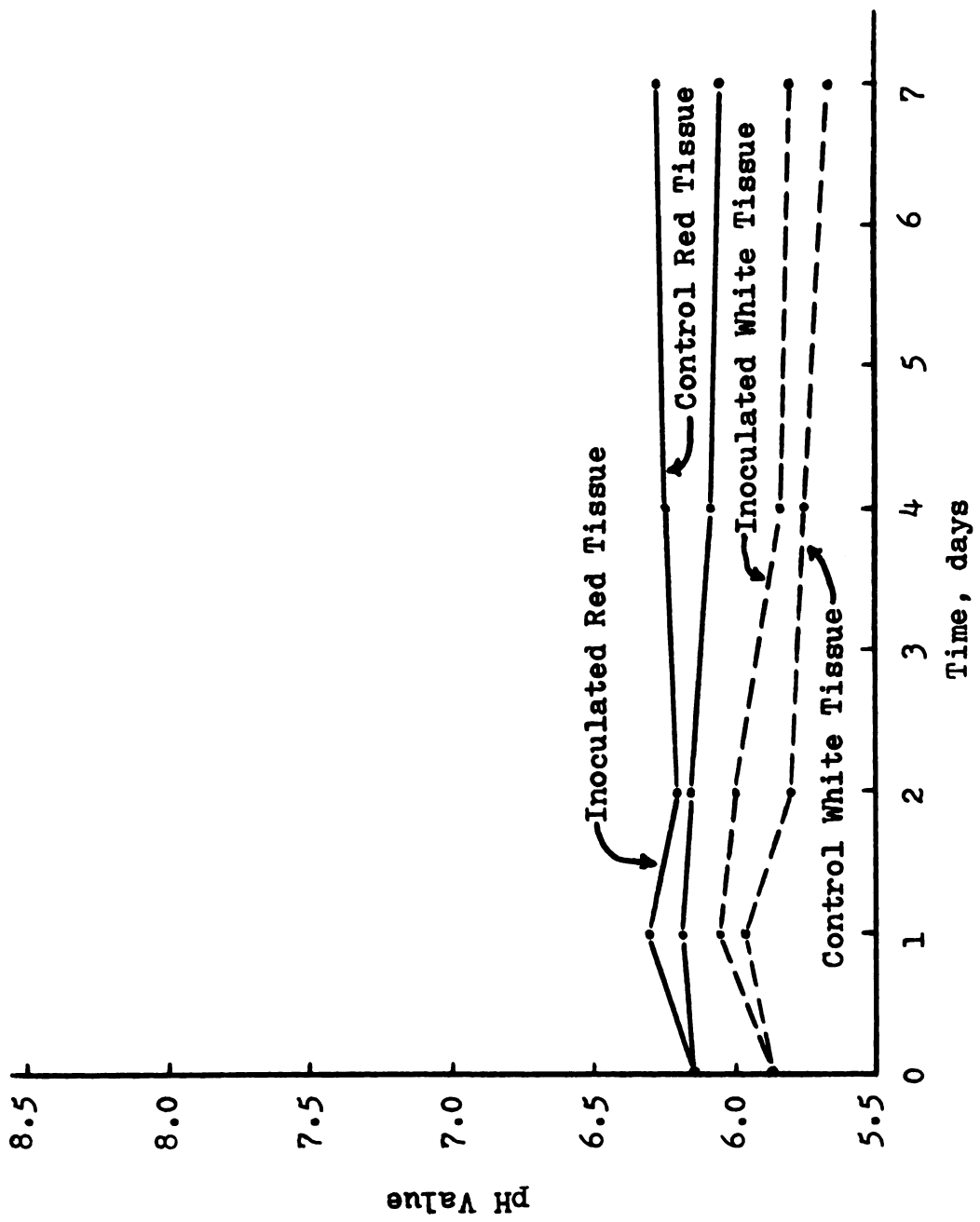

9.0
8.0
7.0
6.0
5.0
4.0
3.0
log Bacterial Number
Inoculated Red Tissue
Inoculated White Tissue
0
1
2
3
4
5
6
7
Time, days

Figure 23. Changes in pH during the incubation of the red and white portions of porcine semitendinosus muscle inoculated with Bacillus pumilus.


pH Value
8.5
8.0
7.5
7.0
6.5
6.0
5.5
0
1
2
3
4
5
6
7
Time, days
Inoculated Red Tissue
Control Red Tissue
Inoculated White Tissue
Control White Tissue

culture tubes. Therefore, even though the organism appeared to be viable, Bacillus pumilus (ATCC 15716) does not appear to grow well on porcine muscle, which is in agreement with the results of Hasegawa et al. (1970b).

Changes in the Ultrastructure of Inoculated Tissue. Bacillus pumilus had no detectable effect on the ultrastructure of either the red or white portions of porcine semitendinosus muscle. Figure 24 shows a typical red fiber 96 hrs after inoculation with Bacillus pumilus. Figure 25 shows a typical white fiber incubated with Bacillus pumilus for 168 hrs. Both micrographs show little, if any, ultrastructural variation from micrographs of aseptic control tissues. Thus, it was concluded that Bacillus pumilus causes no discernible ultrastructural damage to porcine muscle tissue.

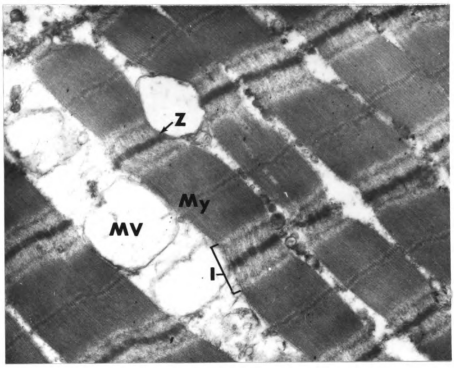
These results are in agreement with those of Rampton et al. (1970) and Hasegawa et al. (1970b) who concluded that Bacillus pumilus caused no measurable breakdown in the muscle proteins during incubation.

Changes in Tissue Inoculated With Staphylococcus aureus

Bacterial Growth and pH Changes. Staphylococcus aureus was found to grow well on both the red and white portions of porcine semitendinosus muscle. Figure 26 shows a growth curve for Staphylococcus aureus on both types of muscle fibers. As in the Pseudomonas fragi experiment, no lag phase was observed for Staphylococcus

Figure 24. Typical red fiber after 96 hrs incubation with Bacillus pumilus at 10°C. My = myofibril, MV = mitochondrial vesicle, I = I-band, Z = Z-line. 20,000 X.

Figure 25. Typical white fiber after a 168 hr incubation at 10°C with Bacillus pumilus. My = myofibril, I = I-band, Z = Z-line. 20,000 X.


Z
My
MV
I

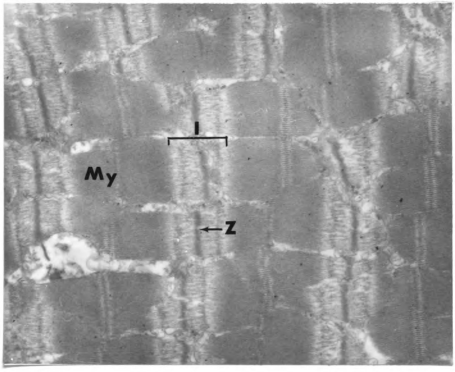
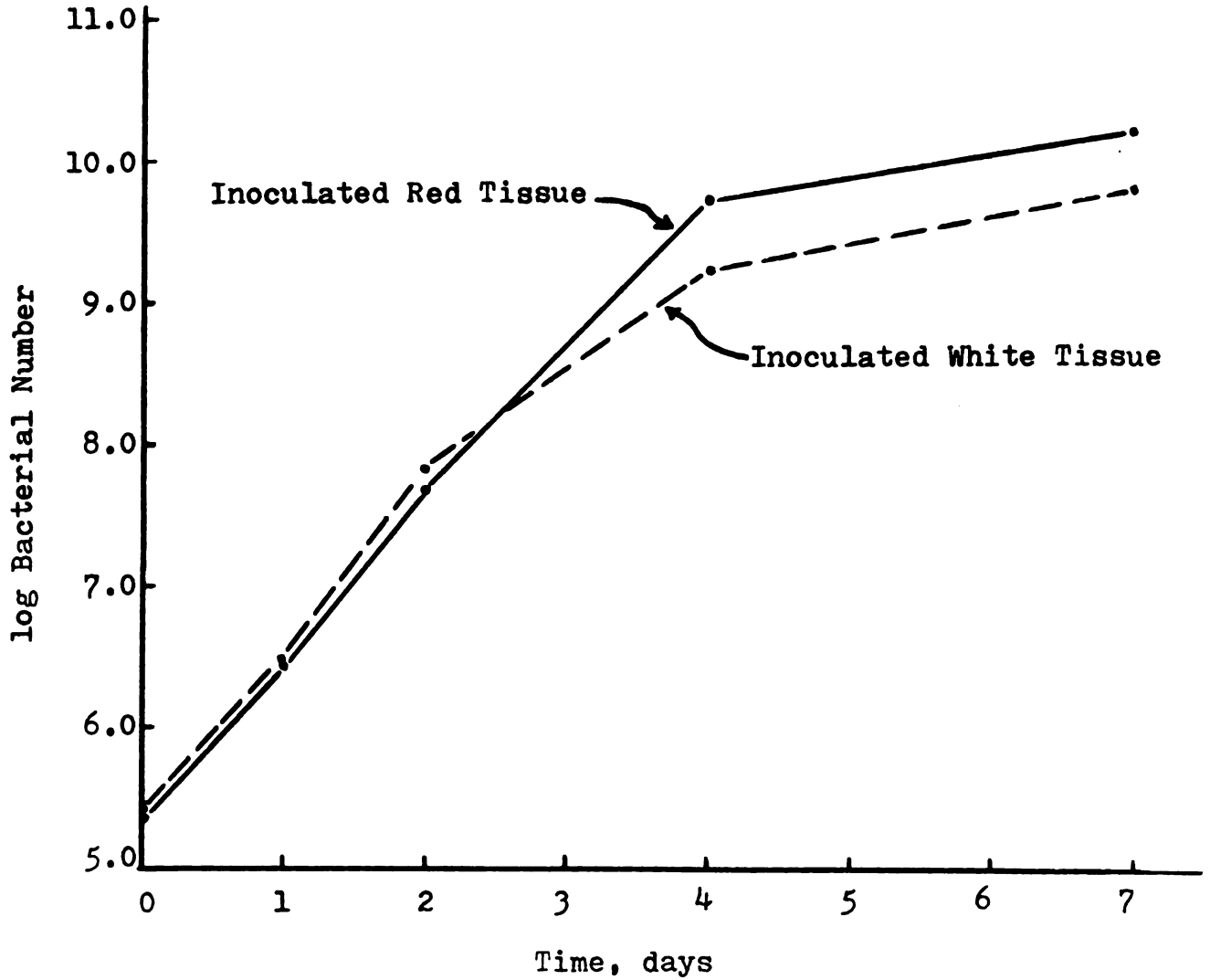

I
My
Z

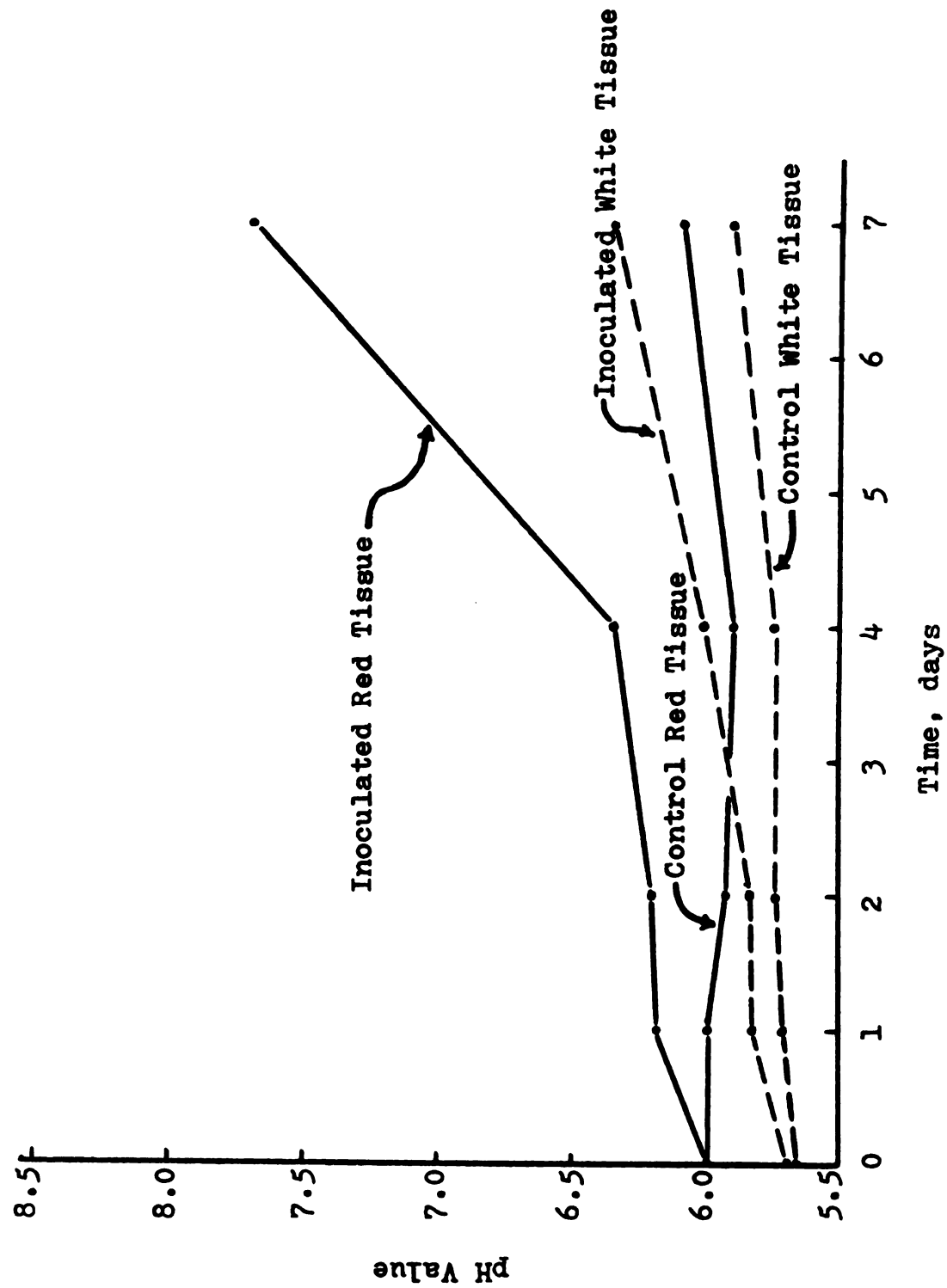
Figure 26. Bacterial growth of Staphylococcus aureus on the red and white portions of porcine semitendinosus muscle at 100°C.


11.0
10.0
9.0
8.0
7.0
6.0
5.0
log Bacterial Number
Inoculated Red Tissue
Inoculated White Tissue
0
1
2
3
4
5
6
7
Time, days

aureus on porcine tissue. Staphylococcus aureus grew at equal rates on both red and white muscle until 48 hrs after inoculation, after which time a slightly higher population was found on red tissue. However, the final bacterial populations differed by less than one-half of a log number and were, therefore, not significantly different.

Figure 27 shows the change in pH caused by growth of Staphylococcus aureus on both muscle types. There was a considerable increase in the pH of inoculated and incubated red tissue, whereas, the pH of the white tissue increased only moderately. Although the cause of the pH difference has not been elucidated, changes appear to be directly related to the amount of growth occurring in the tissues. Also, red fibers contain higher lipid levels and more triglycerides (Adams et al., 1962), and more mitochondrial enzymes (Dubowitz and Pearse, 1960; Stein and Padykula, 1962) than white fibers. On the other hand, white fibers are known to contain higher levels of glycogen, which may be a source of organic acids, and thus could contribute to a lower pH value for white tissue. Therefore, a difference in the metabolic end product levels may account for the pH difference. In addition, red fiber α-actinin contains 17 more negatively charged amino acids per 100 residues than α-actinin from white fibers (Suzuki et al., 1973). These negatively charged amino acids could be released during degradation of the Z-line by the proteases of Staphylococcus

Figure 27. Changes in pH during the incubation of the red and white portions of porcine semitendinosus muscle inoculated with Staphlococcus aureus.


Inoculated White Tissue
Inoculated Red Tissue
Control Red Tissue
Control White Tissue
pH Value
Time, days
8.5
8.0
7.5
7.0
6.5
6.0
5.5
0
1
2
3
4
5
6
7

aureus, and thus contribute to alkalinization of the tissue.

Changes in the Ultrastructure of Inoculated Tissue. No observable differences were found between the degradation of red and white muscle fibers when inoculated and incubated with Staphylococcus aureus. Both fiber types appeared to degrade in the same way and at approximately the same rate.

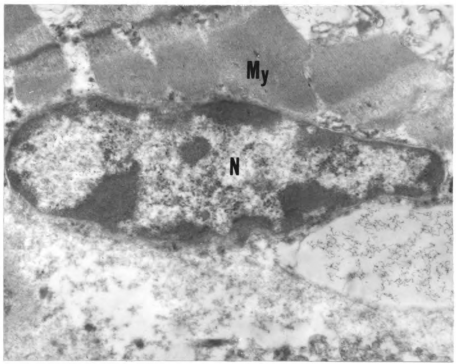
Staphylococcus aureus seemed to have little effect on the ultrastructure of either nuclei or mitochondria. Nuclei appeared to undergo autolytic-like degradation throughout the 168 hr incubation period (Figures 28 and 29).

Figure 30 shows the relatively intact condition of some typical intermyofibrillar mitochondria after 168 hrs of growth by Staphylococcus aureus. Many sarcoplasmic reticulum remnants are evident which appear to be aggregated but intact.

Some localized myofibrillar degradation was observed as early as 48 hrs after inoculation, but degradation was not widespread until 168 hrs after inoculation. By 168 hrs, almost total disruption of the tissue had occurred. The degradation pattern of myofibrils inoculated with Staphylococcus aureus was similar to that of Pseudomonas fragi. Myofibrils were observed to break mainly in the I-band region. I-band damage was followed by dissolution of the Z-line, and finally degradation of the

Figure 28. Nucleus from the white portion of porcine semitendinosus muscle after a 48 hr incubation with Staphylococcus aureus at 15°C. N = nucleus, My = myofibril. 18,225 X.

Figure 29. Nucleus from the white portion of porcine semitendinosus muscle after a 96 hr incubation at 15°C with Staphylococcus aureus. N = nucleus, My = myofibril. 36,250 X.


My
N

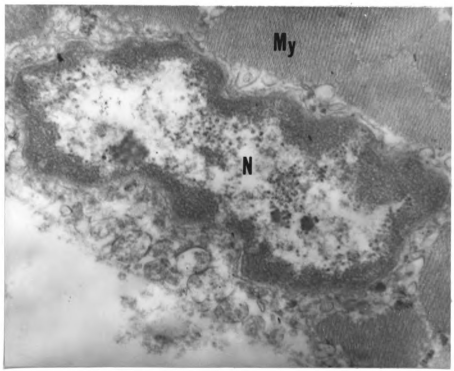
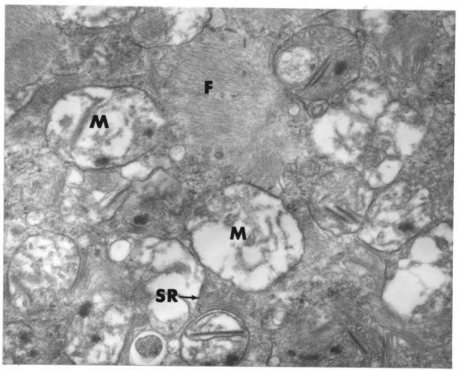

My
N

Figure 30. Intermyofibrillar mitochondria from the red portion of porcine semitendinosus muscle after a 168 hr incubation with Staphylococcus aureus. M = mitochondria, SR = sarcoplasmic reticulum remnants, F = filaments. 22,500 X.


F
M
M
SR

A-band-M-line region of the myofibril.

Figure 31 shows a red fiber surrounded by Staphylococcus aureus 96 hrs after inoculation. This micrograph shows some dissolution of the I-band, but most of the Z-lines and A-bands, although diffuse, are still intact. After 168 hrs of incubation (Figure 32) only the M-line and parts of the A-band were intact. Remnants of the I-band-Z-line structure were very obscure.

The observed ultrastructural changes indicate that the nuclei and mitochondria are at most only slightly effected by growth of Staphylococcus aureus. On the other hand, Staphylococcus aureus caused extensive degradation of the myofibril. As was the case with Pseudomonas fragi, the I-band area was found to be most susceptible to protease attack, whereas, the A-band area was the most stable.

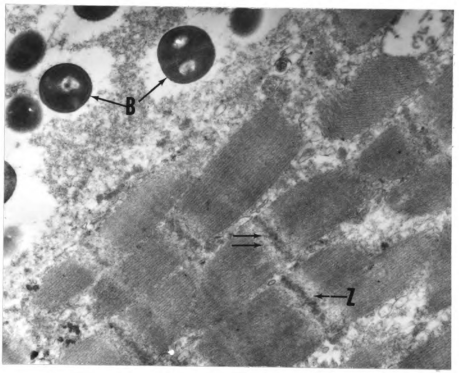
Changes in Tissue Inoculated With Clostridium perfringens

Bacterial Growth and pH Changes. Growth of Clostridium perfringens on both the red and white portions of porcine semitendinosus muscle was extremely rapid. The bacterial population approached maximum growth after only 24 hrs incubation (Figure 33). The extremely fast growth rate was probably due to the high incubation temperature (30°C).

As with Pseudomonas fragi, the initial population of Clostridium perfringens was higher on red tissue than on white. But by the end of the experimental period

Figure 31. Typical red fiber after a 96 hr incubation with Staphylococcus aureus at 15°C. B = bacteria, Z = Z-line, double arrows = degraded I-band area. 23,125 X.

Figure 32. Typical red fiber after a 168 hr incubation with Staphylococcus aureus at 15°C. B = bacteria, M = M-line, Z = diffuse Z-line. 23,125 X.


B
Z

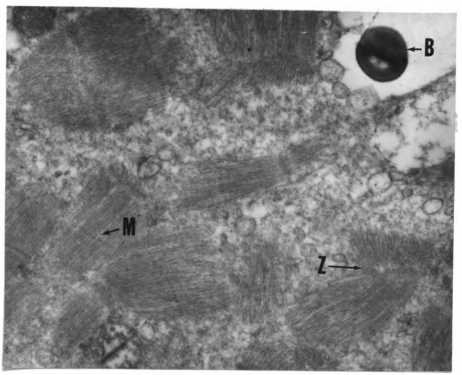

B
M
Z

Figure 33. Bacterial growth of Clostridium perfringens on the red and white portions of porcine semitendinosus muscle.

Inoculated Red Tissue
Inoculated White Tissue
log Bacterial Number
11.0
10.0
9.0
8.0
7.0
6.0
5.0
0
1
2
3
4
5
6
7
Time, days

the bacterial populations were essentially the same on both types of muscle.

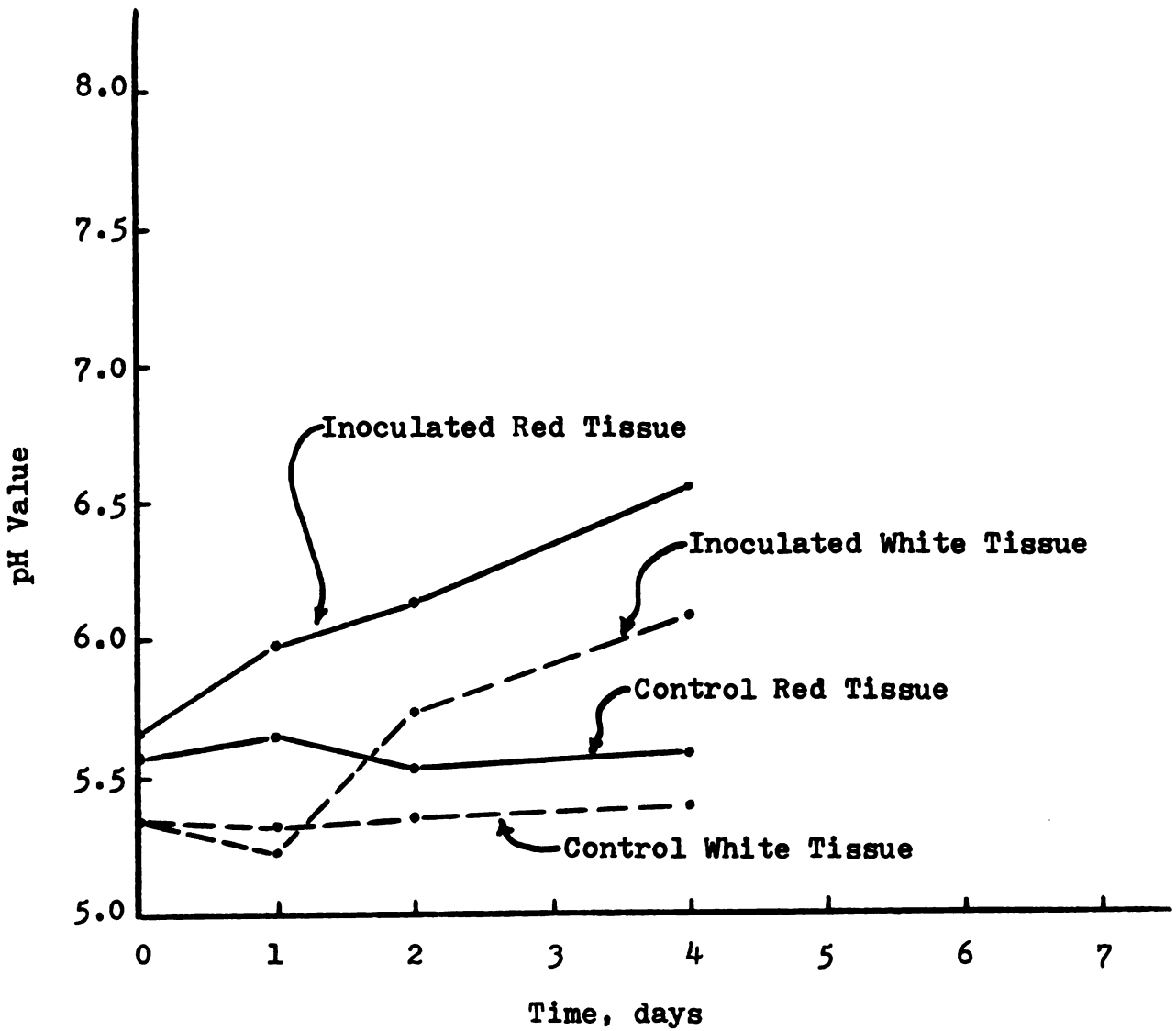
Growth of Clostridium perfringens caused an increase in the pH of both red and white muscle (Figure 34). The overall rate of pH change appeared to be equal for both red and white tissues. These results are similar to those of Hasegawa et al. (1970b), who reported a slight increase in tissue pH due to the growth of Clostridium perfringens on porcine muscle. However, it should be noted that the increase in pH of tissue inoculated with Clostridium perfringens (Figure 34) was not as great as that of tissues inoculated with either Pseudomonas fragi (Figure 15) or Staphylococcus aureus (Figure 27).

As illustrated in Figure 33, Clostridium perfringens grew equally well on red and white muscle. These results were similar to those obtained with Pseudomonas fragi, Bacillus pumilus, and Staphylococcus aureus.

Changes in the Ultrasturcture of Inoculated Tissue. There was no apparent difference in the ultrastructural degradation of red and white fibers by Clostridium perfringens. Thus, none of the organisms appeared to differentiate between the red and white portions of the semitendinosus muscle.

Considerable ultrastructural damage was noted after 24 hrs of growth with Clostridium perfringens. Nuclei in particular suffered extensive damage (Figure 35). The membranes of such nuclei were observed to be diffuse

Figure 34. Changes in pH during the incubation of the red and white portions of porcine semitendinosus muscle inoculated with Clostridium perfringens.


8.0
7.5
7.0
6.5
6.0
5.5
5.0
pH Value
Inoculated Red Tissue
Inoculated White Tissue
Control Red Tissue
Control White Tissue
0
1
2
3
4
5
6
7
Time, days

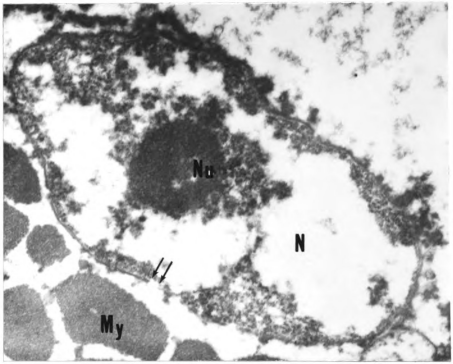
and discontinuous. The nuclei were also found to be smaller and to contain much less chromatin material than controls (Figures 2 and 35). Thus, Clostridium perfringens was capable of degrading nuclei and nuclear material. These results are in agreement with Hapchuk (1974), who isolated a proteolytic fraction from Clostridium perfringens that exerted its major action on the sarcoplasmic proteins.

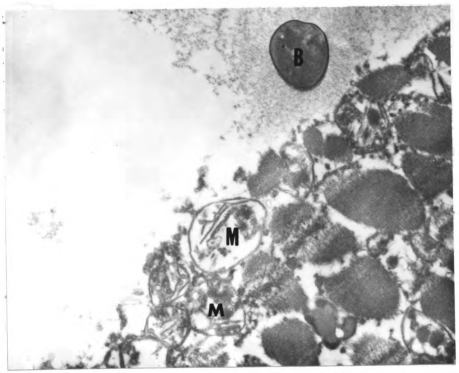
Degradation of the mitochondria by Clostridium perfringens was not as rapid as with the nuclei. Figure 36 shows some typical intermyofibrillar mitochondria which were in close proximity to several Clostridium perfringens organisms. Although the mitochondria appear to be intact, some degradation of the cristae on the interior of the mitochondria was evident. Similar mitochondria were found up to 48 hrs after inoculation of the tissue. Subsequent observations showed that by 96 hrs incubation only empty mitochondrial vesicles remained.

Myofibrillar disruption was observed to follow the same pattern as previously described for Pseudomonas fragi and Staphylococcus aureus, with initial breaks occurring in the I-band region of the myofibril. Figure 37 illustrates typical I-band damage and considerable loss of Z-line material after only 24 hrs of bacterial growth. Some disruption of the A-band was also noted but was much less prevelant than I-band breakage or Z-line disruption. A-band damage was more prevelant

Figure 35. Typical nucleus from the red portion of porcine semitendinosus muscle after a 24 hr incubation at 30°C with Clostridium perfringens. N = nucleus, My = myofibril, Nu = nucleolus, double arrows = breaks in nuclear membrane. 27,200 X.

Figure 36. Typical area from the red portion of porcine semitendinosus muscle after a 24 hr incubation at 30°C with Clostridium perfringens. B = bacteria, M = mitochondria. 18,000 X.


Na
N
My


B
M
M

in areas where I-band dissolution had occurred. This would indicate that A-band degradation probably occurs following the original I-band damage.

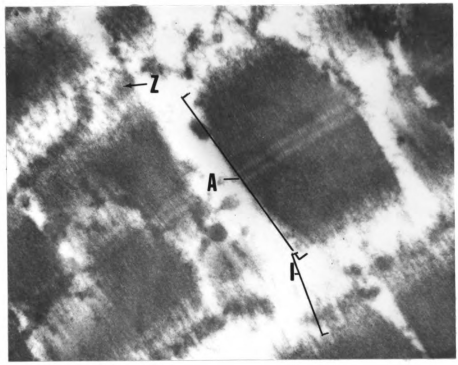
In several instances, clumps of myofibrillar material, evidently fragmented by proteases from Clostridium perfringens, were observed (Figure 38). Clostridium perfringens was the only organisms showing clumping of the myofibrillar material.

Pseudomonas fragi (Figure 17) and Staphylococcus aureus (Figures 24 and 25) bacterial cells were always surrounded by clear zones, whereas Clostridium perfringens cells were surrounded by zones filled with debris (Figures 36, 39, and 40). The reason for the difference between species is not clear but may be related to capsule formation or variations in the cell wall composition. Close examination of Figure 40 shows some apparent radial debris orientation. It is possible that some of the proteolytic activity of Clostridium perfringens may be localized within the cell wall, and thus could be responsible for the affinity between the bacterial cell and myofibrillar debris.

After 96 hrs incubation with Clostridium perfringens most of the tissue had undergone extensive proteolysis, with only the A-band remaining as a recognizable structure (Figure 41). These results are similar to those for Pseudomonas fragi and Staphylococcus aureus and suggest that the A-band is the most resistant structure to microbial attack.

Figure 37. Typical break in the I-band region of a white fiber after a 24 hr incubation at 30°C with Clostridium perfringens. A = A-band, I = I-band region, Z = Z-line remnant. 37,500 X.

Figure 38. Degraded area from a white fiber after a 24 hr incubation at 30°C with Clostridium perfringens. A = A-band, C = clumps of debris. 22,000 X.


Z
A
I

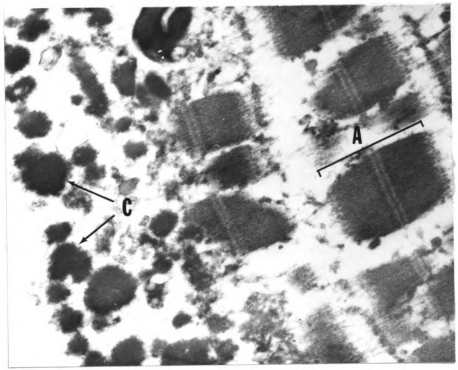
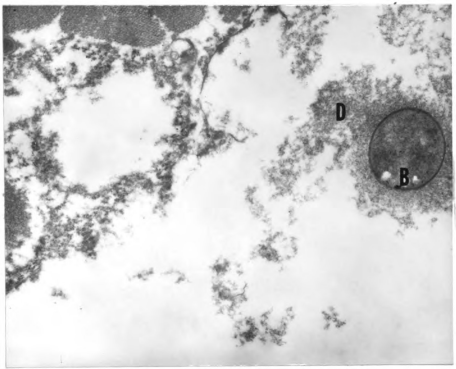

A
C

Figure 39. Typical Clostridium perfringens cell after a 24 hr incubation at 30°C in the red portion of porcine semitendinosus muscle. B = bacteria, D = debris. 22,500 X.

Figure 40. Clostridium perfringens cell with surrounding debris after a 24 hr incubation at 30°C in the red portion of porcine semitendinosus muscle. Note the radiating orientation of the debris immediately surrounding the bacterial cell. 91,000 X.


D
B

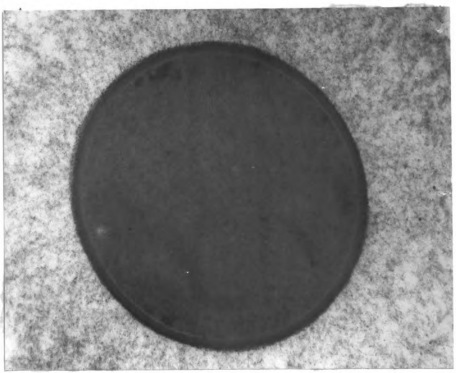
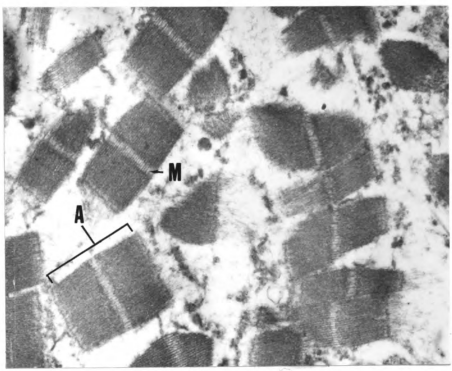


Figure 41. Typical field from the white portion of porcine semitendinosus muscle after a 96 hr incubation at 30°C with Clostridium perfringens. A = A-band, M = M-line area. 21,500 X.


M
A

Changes in Meat Headspace Volatiles Due to Microbial Growth

Bacterial Growth, pH Changes, and Off-Odor Development

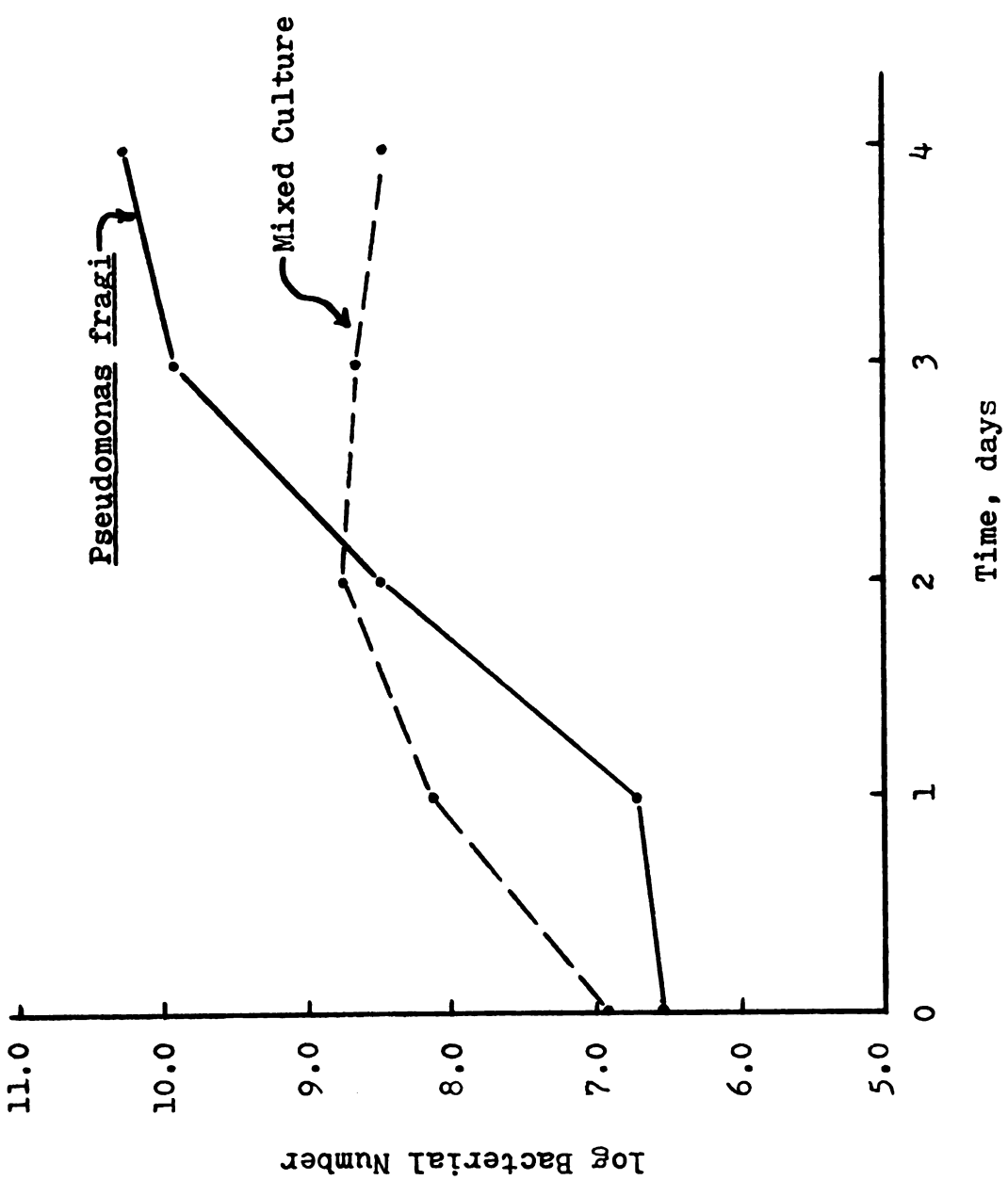
Bacterial growth was not detected in any of the uninoculated control samples. Both Pseudomonas fragi and the mixed culture from hamburger grew well on porcine longissimus dorsi muscle. The bacterial population of tissue inoculated with the mixed culture approached its maximum after only 48 hrs incubation at 10°C, whereas, bacterial counts for the tissue inoculated with Pseudomonas fragi increased throughout the 96 hr incubation period (Figure 42). Off-odor development was noted when the mixed culture and Pseudomonas fragi populations reached log numbers of 8.78 and 10.26 organisms per gm of tissue, respectively (Table 2 and Figure 42).

Table 2. Odor Evaluation of Porcine Longissimus Dorsi Muscle Inoculated with Pseudomonas fragi or a Mixed Culture and Incubated at 10°C

	Odor Evaluation[1]		
Incubation Time, hrs.	Pseudomonas fragi	Mixed Culture	Control
0	N	N	N
24	N	SO	N
48	N	DO	N
72	DO	IO	N
96	DO	IO	SO

[1]N=normal odor, SO=slight off-odor, DO=distinct off-odor, IO=intense off-odor

Figure 42. Bacterial growth of Pseudomonas fragi and of a mixed culture on porcine longissimus dorsi muscle stored at 10°C. Double arrows= detection of off odor.

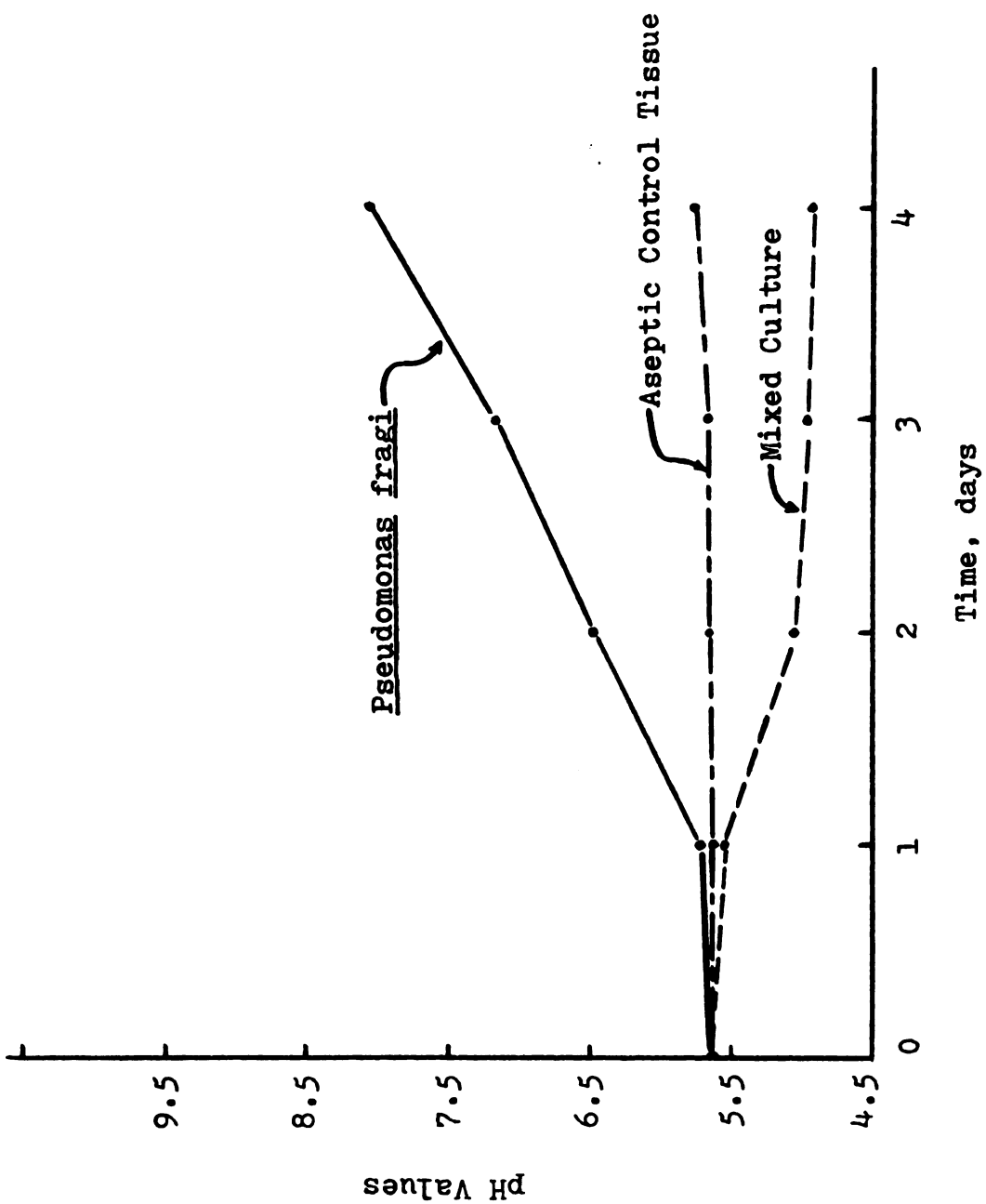

Pseudomonas fragi
Mixed Culture
log Bacterial Number
11.0
10.0
9.0
8.0
7.0
6.0
5.0
0
1
2
3
4
Time, days

Growth of Pseudomonas fragi caused a large increase in the pH of inoculated tissue, whereas, the mixed culture was found to cause a decrease in tissue pH (Figure 43). The pH of control tissues remained essentially unchanged throughout the incubation period. These results indicated that the predominant species of the mixed culture from hamburger are acid producers, probably Lactobacillus. Comparison of Figures 42 and 43 shows that the bacterial population of the tissue inoculated with the mixed culture reached a peak and then decreased as pH values dropped below 5.0. Thus, it seems likely that at least some of the bacterial species of the mixed culture were inhibited by the acidic conditions.

Gas-Chromatographic Analysis of Headspace Vapors

Standard Compounds. Several compounds reported to be either products of Pseudomonas fragi metabolism (Miller et al., 1973a, b; Reddy et al., 1969) or normal meat constituents were selected as standard compounds for gas-chromatographic identification. Standard compound retention times were established on both Apiezon L and Carbowax 20M columns by heating aqueous solutions of the compounds with sodium sulfate at 50°C for 15 minutes and injecting the resultant headspace samples directly into the chromatograph. In order to eliminate fluctuations in retention times due to experimental or instrumental variation, adjusted retention times were used to describe and compare standards with unknown peaks. The adjusted retention time is the elapsed time that each

Figure 43. Changes in the pH of longissimus dorsi muscle inoculated with *Pseudomonas fragi* or a mixed culture and incubated at 10°C.


pH Values
9.5
8.5
7.5
6.5
5.5
4.5
0
1
2
3
4
Time, days
Pseudomonas fragi
Aseptic Control Tissue
Mixed Culture

volatile is retained by the stationary phase of the column after appearance of the air peak (MacLeod, 1973). The selected standard compounds and their adjusted retention times on both columns are shown in Table 3.

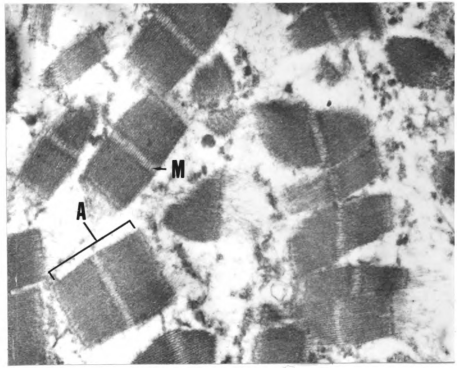
Table 3. Standard Compound Adjusted Retention Times on 3% Apiezon L and 10% Carbowax 20M Columns.

	Adjusted Retention Time[1], sec.	
Standard Compounds	Apiezon L	Carbowax 20M
Acetaldehyde	3	26
Acetone	9	45
Ethyl Acetate	11	69
Ethanol	69	99
Lactic Acid	81	51
Propionic Acid	102	49

[1]Adjusted by subtracting the air peak retention time from those of the standard compounds.

Analysis of Headspace Vapors From Uninoculated Control Tissue. Headspace vapors from ground aseptic longissimus dorsi muscle were analyzed by the same procedure as outlined for the standard compounds. Uninoculated controls at 0 time gave only one peak on both Carbowax 20M (Figure 44) and Apiezon L (Figure 45) columns. Comparison of the retention time of the unknown on both columns with those of the standards suggest that the peak was produced by acetone.

Figure 41. Typical field from the white portion of porcine semitendinosus muscle after a 96 hr incubation at 30°C with Clostridium perfringens. A = A-band, M = M-line area. 21,500 X.


M
A

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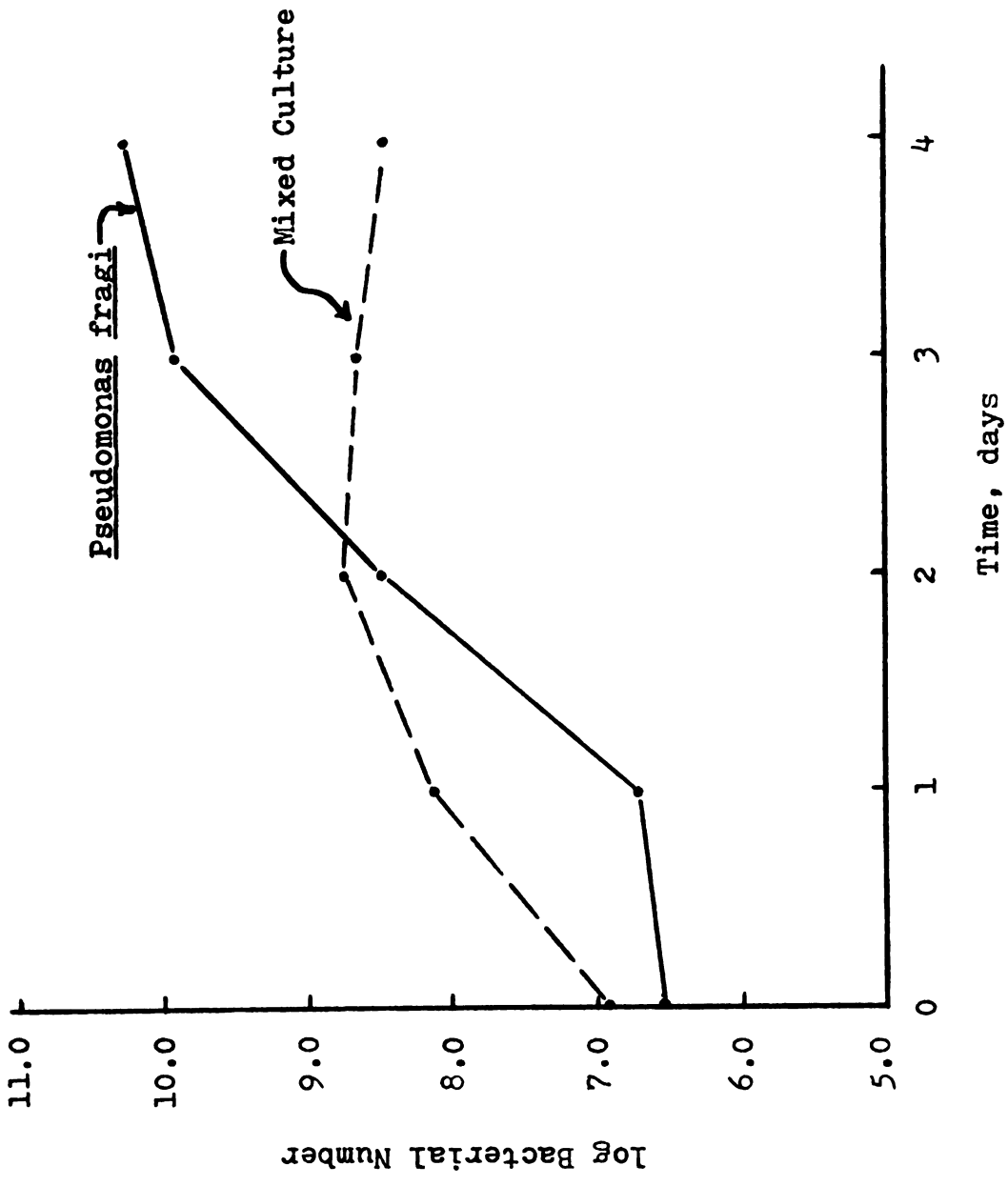
Pseudomonas fragi
Mixed Culture
11.0
10.0
9.0
8.0
7.0
6.0
5.0
0
1
2
3
4
Time, days
log Bacterial Number

Growth of Pseudomonas fragi caused a large increase in the pH of inoculated tissue, whereas, the mixed culture was found to cause a decrease in tissue pH (Figure 43). The pH of control tissues remained essentially unchanged throughout the incubation period. These results indicated that the predominant species of the mixed culture from hamburger are acid producers, probably Lactobacillus. Comparison of Figures 42 and 43 shows that the bacterial population of the tissue inoculated with the mixed culture reached a peak and then decreased as pH values dropped below 5.0. Thus, it seems likely that at least some of the bacterial species of the mixed culture were inhibited by the acidic conditions.

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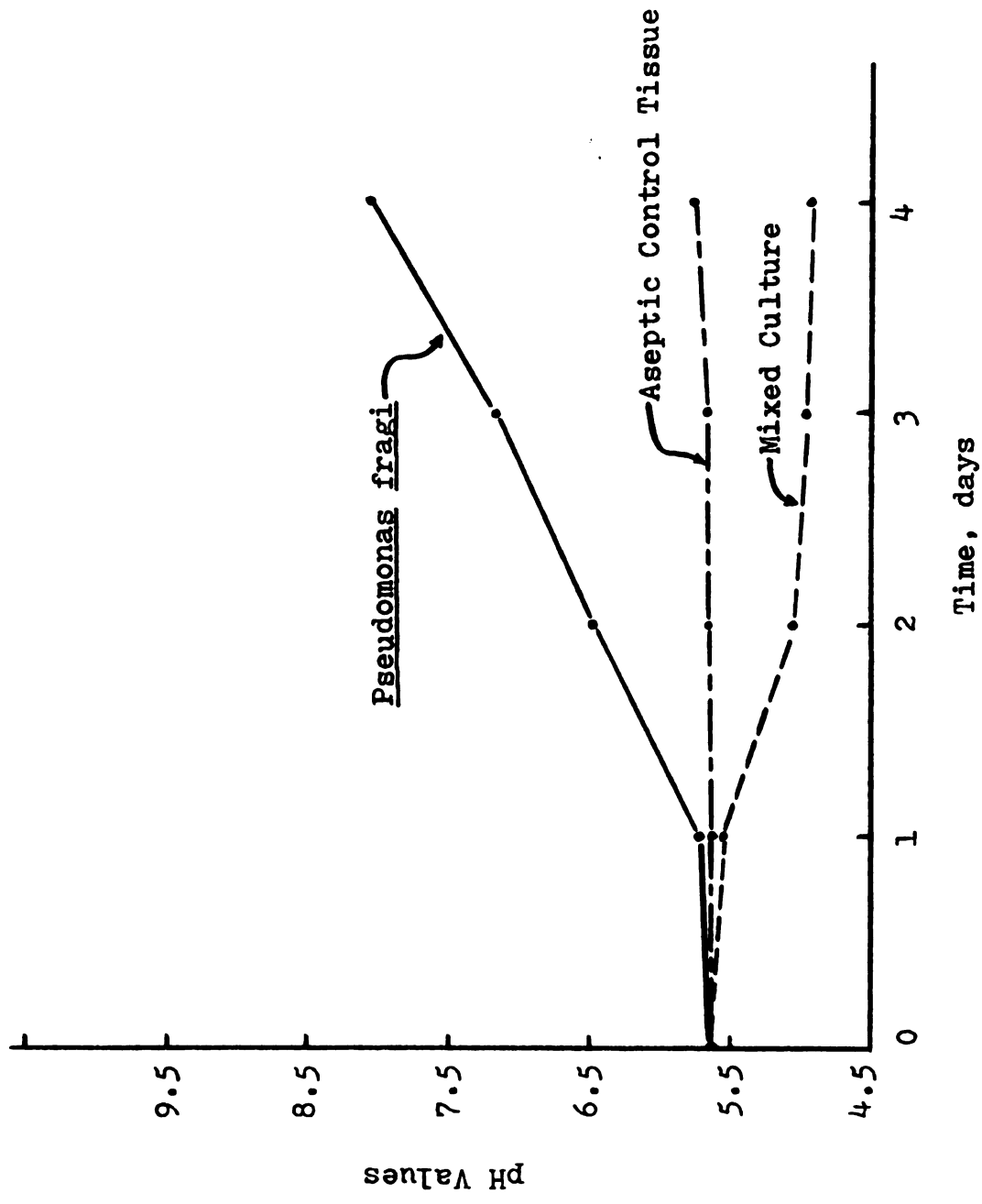
Standard Compounds. Several compounds reported to be either products of Pseudomonas fragi metabolism (Miller et al., 1973a, b; Reddy et al., 1969) or normal meat constituents were selected as standard compounds for gas-chromatographic identification. Standard compound retention times were established on both Apiezon L and Carbowax 20M columns by heating aqueous solutions of the compounds with sodium sulfate at 50°C for 15 minutes and injecting the resultant headspace samples directly into the chromatograph. In order to eliminate fluctuations in retention times due to experimental or instrumental variation, adjusted retention times were used to describe and compare standards with unknown peaks. The adjusted retention time is the elapsed time that each

Figure 43. Changes in the pH of longissimus dorsi muscle inoculated with Pseudomonas fragi or a mixed culture and incubated at 10°C.


pH Values
9.5
8.5
7.5
6.5
5.5
4.5
0
1
2
3
4
Time, days
Pseudomonas fragi
Aseptic Control Tissue
Mixed Culture

volatile is retained by the stationary phase of the column after appearance of the air peak (MacLeod, 1973). The selected standard compounds and their adjusted retention times on both columns are shown in Table 3.

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Standard Compounds	Apiezon L	Carbowax 20M
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Acetone	9	45
Ethyl Acetate	11	69
Ethanol	69	99
Lactic Acid	81	51
Propionic Acid	102	49

[1]Adjusted by subtracting the air peak retention time from those of the standard compounds.

Analysis of Headspace Vapors From Uninoculated Control Tissue. Headspace vapors from ground aseptic longissimus dorsi muscle were analyzed by the same procedure as outlined for the standard compounds. Uninoculated controls at 0 time gave only one peak on both Carbowax 20M (Figure 44) and Apiezon L (Figure 45) columns. Comparison of the retention time of the unknown on both columns with those of the standards suggest that the peak was produced by acetone.

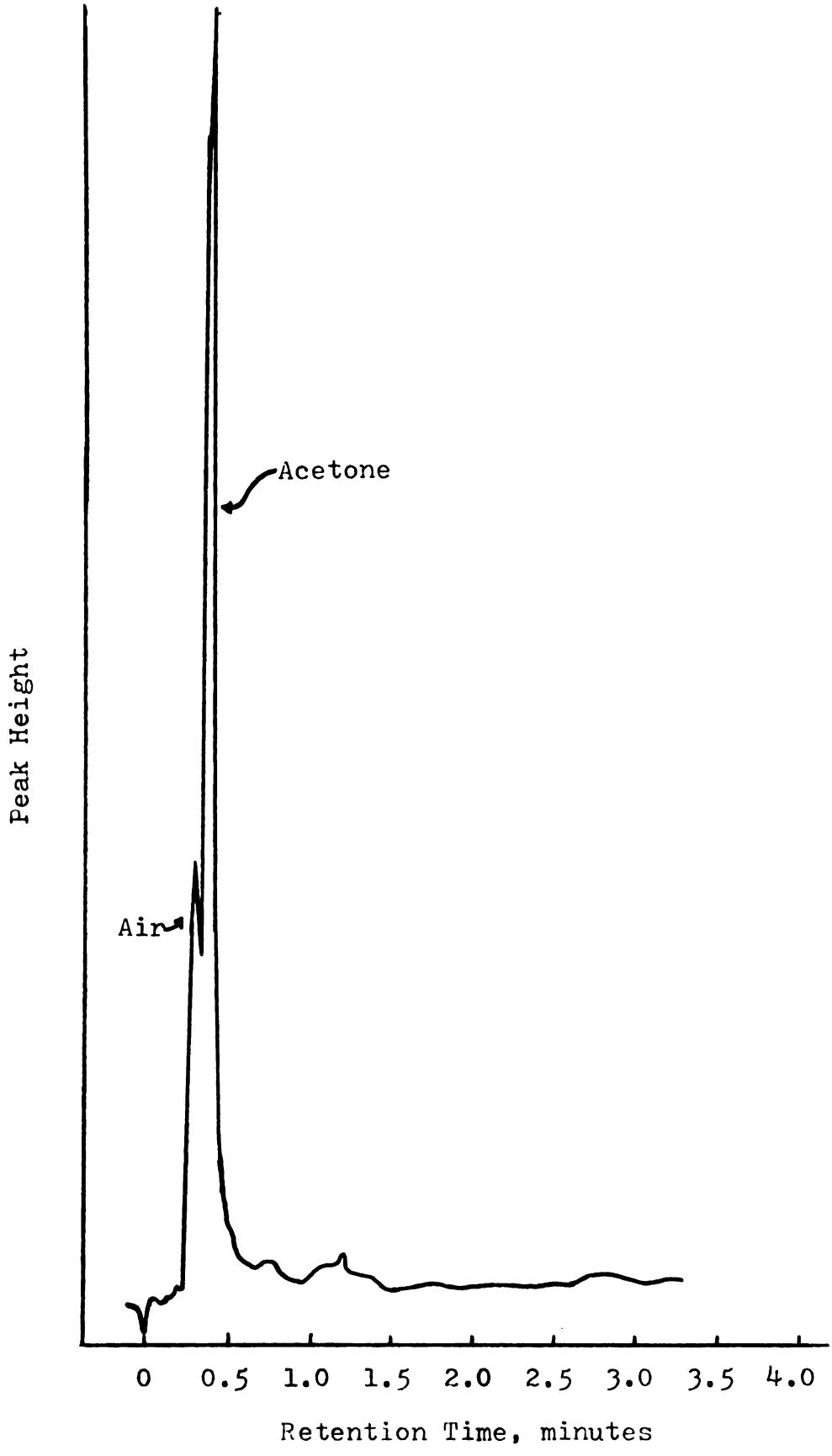
Oven Temperature - 60°C
Detector Temperature - 190°C
Injection Port Temperature - 190°C
Helium Flow Rate (carrier) - 40 ml/min
Hydrogen Flow Rate - 30 ml/min
Air Flow Rate - 500 ml/min
Sensitivity - 10

Figure 44. Chromatogram of the headspace vapors for uninoculated control porcine longissimus dorsi muscle at 0 time on a 10% Carbowax 20M column.


Peak Height
Air
Acetone
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Retention Time, minutes

Oven Temperature - 60°C
Detector Temperature - 190°C
Injection Port Temperature - 190°C
Helium Flow Rate (carrier) - 40 ml/min
Hydrogen Flow Rate - 30 ml/min
Air Flow Rate - 500 ml/min
Sensitivity - 10

Figure 45. Chromatogram of the headspace vapors of uninoculated porcine longissimus dorsi muscle at 0 incubation time on a 3% Apiezon L column.


Acetone
Air
Peak Height
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Retention Time, minutes

Although both aseptic control and inoculated samples were analyzed after 24 hrs of incubation, the results were inconclusive due to syringe leakage. Thus, results from the 24 hr incubation period indicated the presence of the major peaks, whereas, minor peaks were obscured.

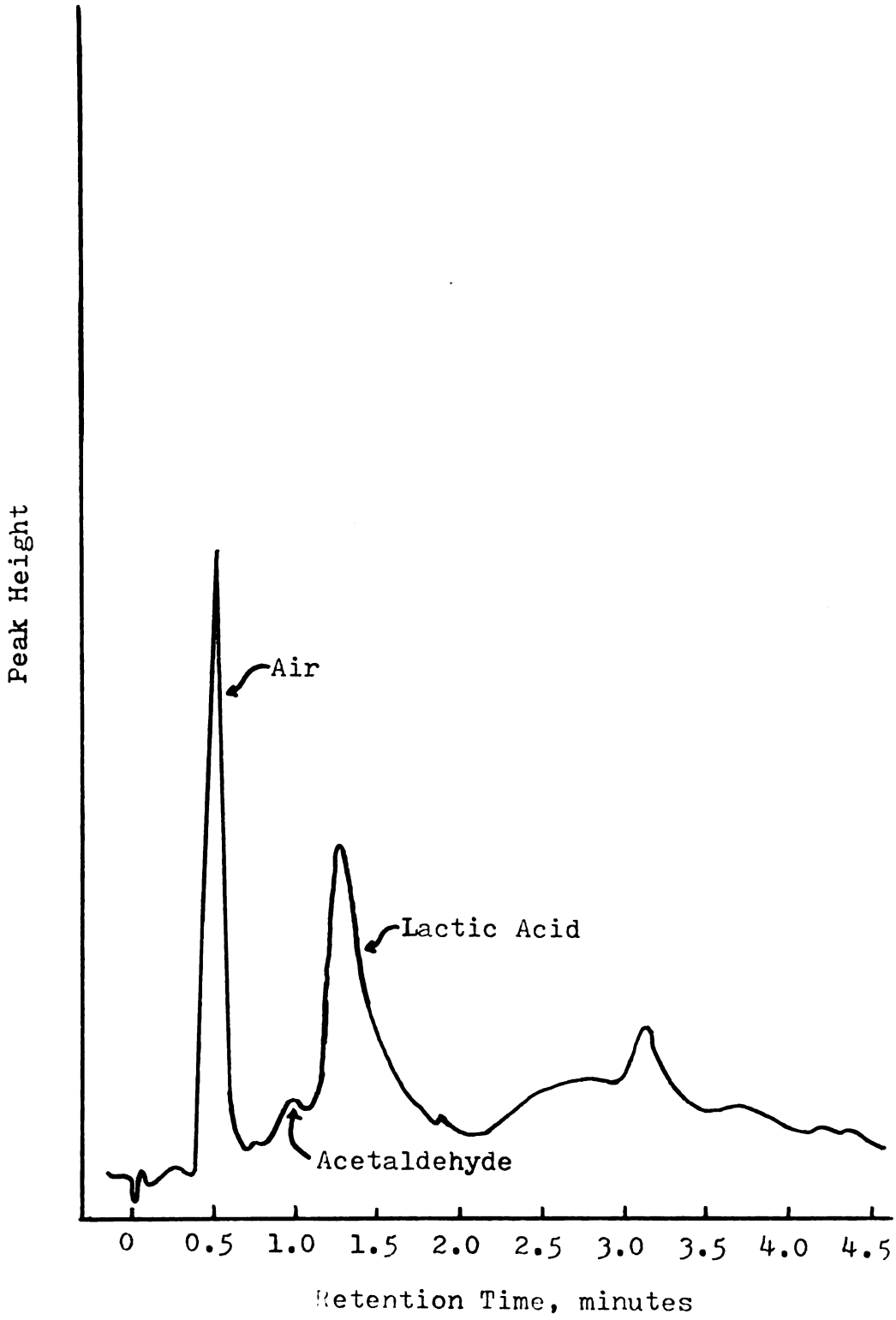
After 48 hrs incubation the uninoculated control meat sample produced three distinct peaks on the Carbowax 20M column (Figure 46). Analysis of the same sample on the Apiezon L column also yielded three peaks, the largest of which had two small shoulders. Further incubation caused no significant change in the chromatographic patterns produced on either type of column.

Analysis of retention time data from both columns (Appendix V) resulted in two of the peaks being tentatively identified as acetaldehyde and lactic acid. When data from both columns were analyzed the retention times of the other peaks did not correspond to those of the standards, and thus, were not identified.

Acetone, acetaldehyde, and lactic acid are commonly found as metabolic intermediates in muscle (Lehninger, 1970), and therefore, their presence in the samples was not unexpected. However, the low post-rigor pH of the uninoculated control sample and its consistency throughout incubation indicates that lactic acid was probably present in the 0 hr tissue. The absence of lactic acid peaks in this sample is puzzling, but the use of new columns combined with low lactic acid levels in the headspace may have led to the compound being

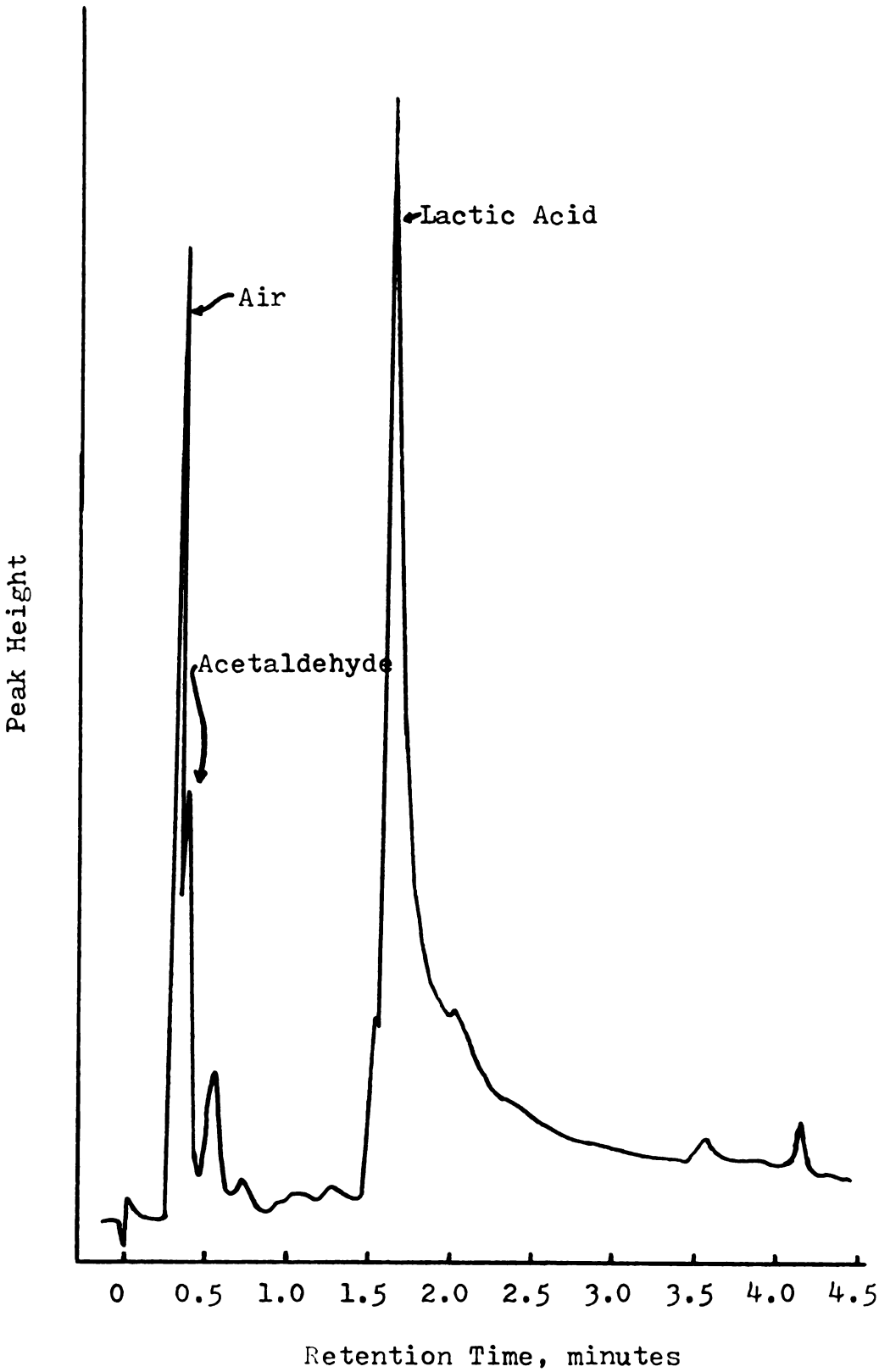
Oven Temperature - 60°C
Detector Temperature - 190°C
Injection Port Temperature - 190°C
Helium Flow Rate (carrier) - 40 ml/min
Hydrogen Flow Rate - 30 ml/min
Air Flow Rate - 500 ml/min
Sensitivity - 10

Figure 46. Chromatogram of the headspace vapors of uninoculated control porcine longissimus dorsi muscle incubated for 48 hrs at 10°C. Run on a 10% Carbowax 20M column.


Peak Height
Air
Lactic Acid
Acetaldehyde
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Retention Time, minutes

Oven Temperature - 60°C
Detector Temperature - 190°C
Injection Port Temperature - 190°C
Helium Flow Rate (carrier) - 40 ml/min
Hydrogen Flow Rate - 30 ml/min
Air Flow Rate - 500 ml/min
Sensitivity - 10

Figure 47. Chromatogram of the headspace vapors of uninoculated porcine longissimus dorsi muscle incubated for 48 hrs at 10°C. Run on a 3% Apiezon column.


Lactic Acid
Air
Acetaldehyde
Peak Height
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Retention Time, minutes

adsorbed onto the column, and thus, a failure to identify it in the headspace volatiles at 0 time.

Analysis of Headspace Vapors From Tissue Inoculated With a Mixed Culture. As expected, samples from mixed cultures of hamburger analyzed at 0 hrs incubation were similar to aseptic controls. Chromatograms from samples incubated with the mixed culture for 48 hrs or more were similar to each other but were substantially different than those of aseptic controls. Tissue incubated with the mixed culture not only contained acetaldehyde and lactic acid, but also gave peaks tentatively identified as ethyl acetate, ethanol, and propionic acid (Figures 48 and 49).

Some difficulty was encountered in identifying ethanol and lactic acid in the volatiles from the mixed culture inoculated tissue after incubation for 48 hrs. This was due to the similarity of their retention times on the Apiezon L column and the relatively large amount of ethanol in the sample. However, clear separation of these compounds was achieved on the Carbowax 20M column, thus leading to their tentative identification. Using an Apiezon L column, a small peak with a retention time similar to that of propionic acid was observed as a shoulder on the ethanol peak. However, propionic acid could not be identified on the Carbowax 20M column since its retention time was approximately the same as lactic acid.

Thus, the major differences between the headspace vapors of the aseptic control and tissue inoculated with a mixed

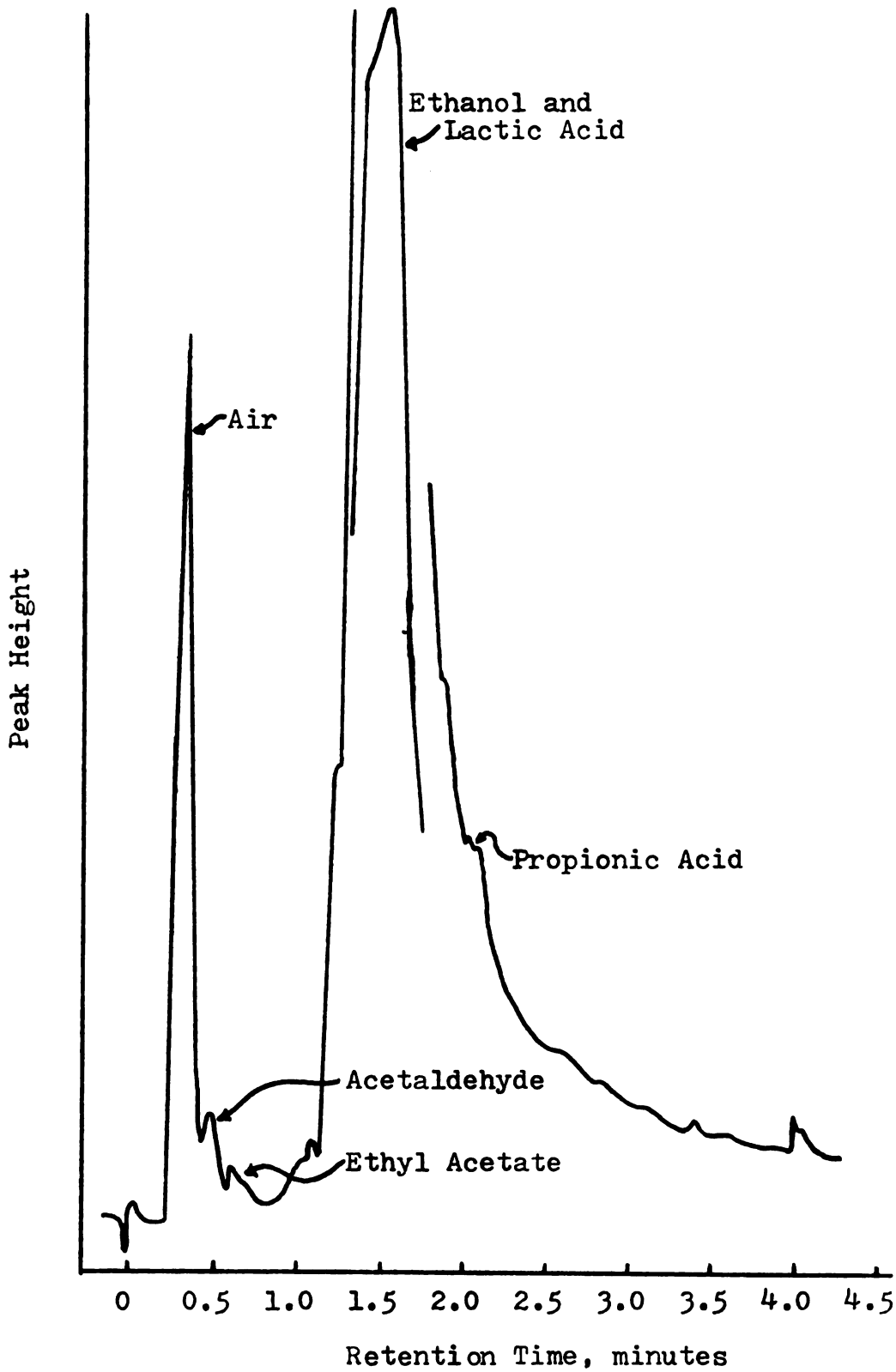
Oven Temperature - 60°C
Detector Temperature - 190°C
Injection Port Temperature - 190°C
Helium Flow Rate (carrier) - 40 ml/min
Hydrogen Flow Rate - 30 ml/min
Air Flow Rate - 500 ml/min
Sensitivity - 10

Figure 48. Chromatogram of the headspace vapors of porcine longissimus dorsi muscle incubated for 48 hrs at 10°C with a mixed culture. Run on a 10% Carbowax 20M column.

Peak Height
Air
Ethanol
Lactic Acid
Ethyl Acetate
Acetaldehyde
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
Retention Time, minutes

Oven Temperature - 60°C
Detector Temperature - 190°C
Injection Port Temperature - 190°C
Helium Flow Rate (carrier) - 40 ml/min
Hydrogen Flow Rate - 30 ml/min
Air Flow Rate - 500 ml/min
Sensitivity - 10

Figure 49. Chromatogram of headspace vapors of porcine longissimus dorsi muscle incubated for 48 hrs at 10°C with a mixed culture. Run on a 3% Apiezon column.


Ethanol and
Lactic Acid
Air
Propionic Acid
Acetaldehyde
Ethyl Acetate
Peak Height
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Retention Time, minutes

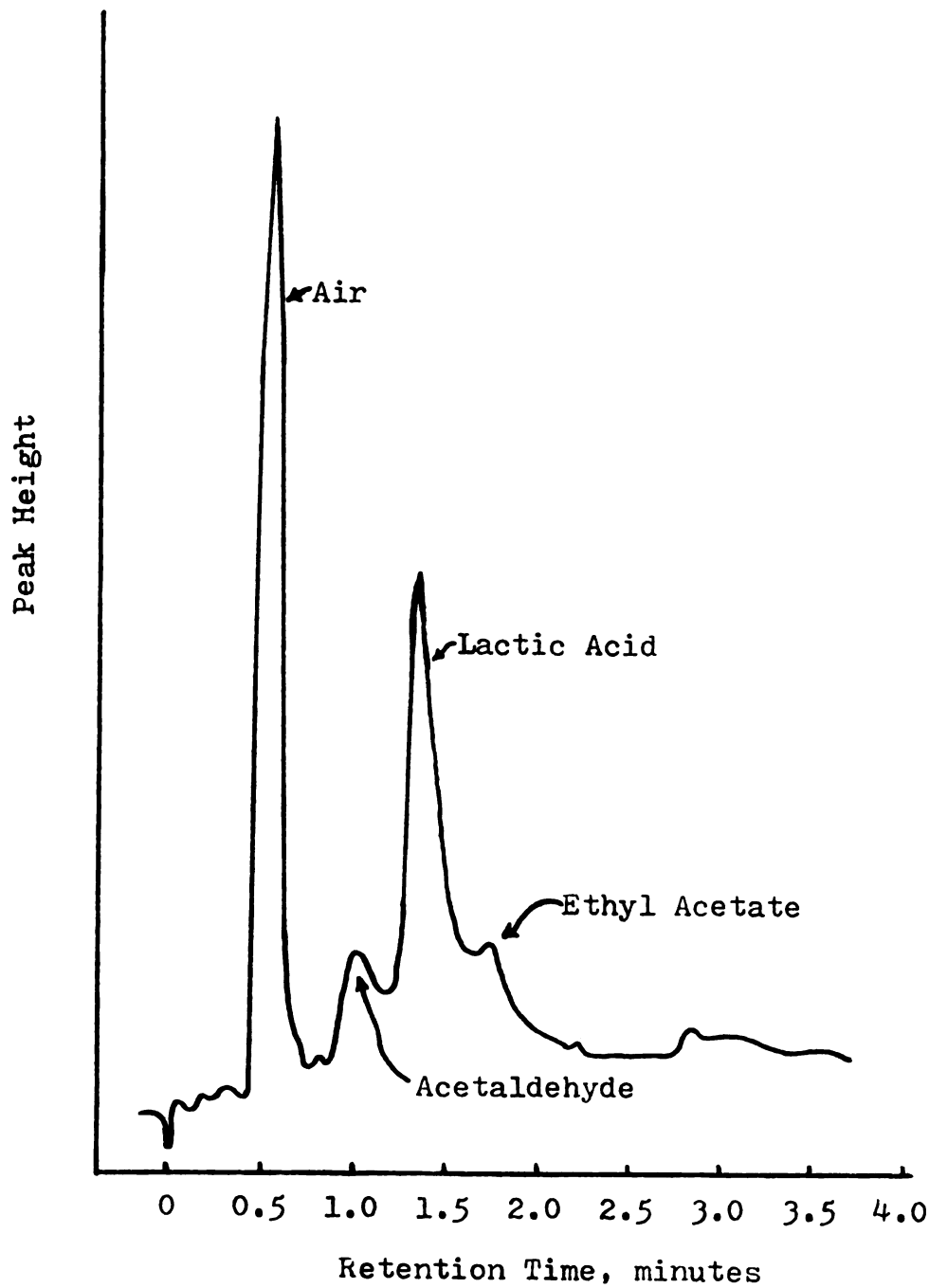
culture from hamburger were the presence and size of the ethanol peak. Analysis at 24 hrs showed that substantial amounts of ethanol were produced before off-odor production. This suggests that ethanol may be an indicator of bacterial growth when acid producers are the dominant species.

Analysis of Headspace Vapors from Tissue Inoculated With Pseudomonas fragi. At 0 hrs incubation, there was no difference in the headspace volatiles for tissue inoculated with Pseudomonas fragi and those of controls. However, after 48 hrs of incubation three peaks were produced on the Carbowax 20M column. When the same sample was analyzed on the Apiezon L column four distinct peaks were observed. Further incubation caused no changes in the chromatographic pattern produced by either type of column. Upon comparison with the controls at 48 hrs incubation, chromatograms from the Pseudomonas fragi inoculated tissue (Figures 50 and 51) and those from the aseptic controls (Figures 46 and 47) were similar. The only major difference was an additional peak on both Apiezon L and Carbowax 20M columns. Retention times for the unidentified peak on both columns were identical to the ethyl acetate standards. Thus, the headspace vapors showed that ethyl acetate was produced by Pseudomonas fragi. However, the use of ethyl acetate as an indicator of bacterial spoilage is questionable due to the small size of the peaks, their incomplete separation, and because their appearance does not coincide with off-odor production.

Analysis of Headspace Vapors Using GC-MS. Attempts to

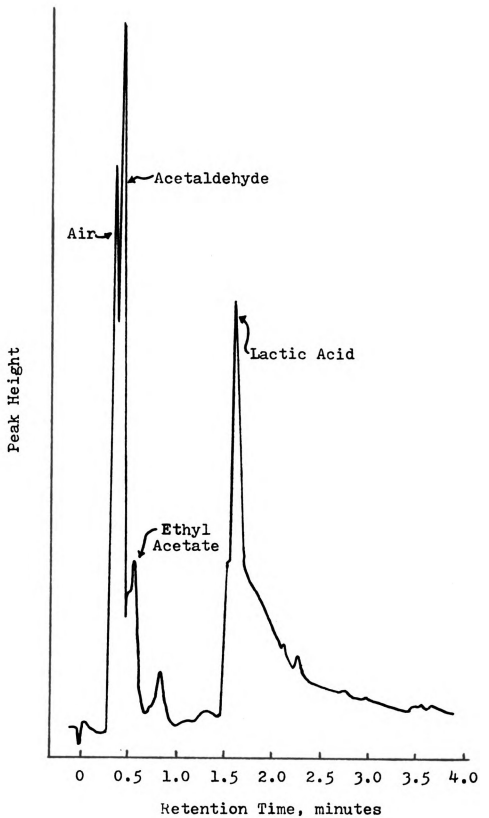
Oven Temperature - 60°C
Detector Temperature - 190°C
Injection Port Temperature - 190°C
Helium Flow Rate (carrier) - 40 ml/min
Hydrogen Flow Rate - 30 ml/min
Air Flow Rate - 500 ml/min
Sensitivity - 10

Figure 50. Chromatogram of the headspace vapors of porcine longissimus dorsi muscle incubated for 48 hrs at 10°C with Pseudomonas fragi. Run on a 10% Carbowax column.


Peak Height
Air
Lactic Acid
Ethyl Acetate
Acetaldehyde
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Retention Time, minutes

Oven Temperature - 60°C
Detector Temperature - 190°C
Injection Port Temperature - 190°C
Helium Flow Rate (carrier) - 40 ml/min
Hydrogen Flow Rate - 30 ml/min
Air Flow Rate - 50 ml/min
Sensitivity - 10

Figure 51. Chromatogram of the headspace vapors of porcine longissimus dorsi muscle incubated for 48 hrs at 10°C with Pseudomonas fragi. Run on a 3% Apiezon column.


Acetaldehyde
Air
Lactic Acid
Ethyl
Acetate
Peak Height
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
Retention Time, minutes

conclusively identify the headspace volatiles of either inoculated or uninoculated tissues by mass-spectrometry were unsuccessful. Since the mass spectrometer requires a very high vacuum to operate, the air injected as a component of the gas sample must be pumped out before any sample can be ionized. The relatively short retention times encountered with meat headspace volatiles did not allow enough time for the mass spectrometer to regain sufficient vacuum before the sample came off the column. Thus, the loss of vacuum created by injecting an air sample and the short retention times of the components to be analyzed did not permit mass-spectral analysis.

The results of these experiments, although not conclusive, indicated that there are differences in the headspace volatiles from fresh and spoiled meat. Although the techniques employed were not sensitive enough for the identification of some of the off-odor volatiles, modification of the procedures may yield more useful results.

Although sensory evaluation showed off-odor development, chromatograms at the same time interval showed no corresponding changes except for the production of ehtanol in mixed culture inoculated tissues. This was probably due to the insensitivity of the flame-ionization detector to some of the known off-odor compounds in meat, i.e., hydrogen sulfide, dimethyl disulfide, and dimethyl disulfide. Therefore, a chromatograph with a detector system suitable for identifying sulfur compounds, such as a flame photometric

detector, might show more conclusive differences as spoilage progresses. Vacuum distillation could also be expected to improve the sensitivity of the technique due to a more complete extraction and concentration of the tissue volatiles and the resultant flexibility of using various solvent systems. Thus, this type of modification would probably result in a sample more compatible with the GC-MS system.

SUMMARY

The red and white portions of aseptic porcine semitendinosus muscle were inoculated and incubated with selected bacterial cultures to determine the influence of bacterial growth upon different fiber types, pH levels, and tissue ultrastructure. In addition, the effects of growth by meat spoilage microorganisms (Pseudomonas fragi and a mixed culture from commercial hamburger) were monitored by gas-chromatographic analysis of headspace vapors from the meat at different time intervals.

Pure cultures of Pseudomonas fragi, Bacillus pumilus, Staphylococcus aureus, and Clostridium perfringens grew equally well on both the red and white portions of porcine semitendinosus muscle. Thus, fiber type did not appear to influence bacterial growth. Tissue pH increased during growth of Pseudomonas fragi, Staphylococcus aureus, and Clostridium perfringens, however, growth of Bacillus pumilus did not alter tissue pH. Increases in pH values of the inoculated tissue were not influenced by fiber type but appeared to be related to the amount of bacterial growth which had occurred in the tissue.

Ultrastructural damage was extensive in tissues

inoculated with Pseudomonas fragi, Staphylococcus aureus, and Clostridium perfringens, whereas Bacillus pumilus caused no detectable ultrastructural change. Fiber type did not appear to influence the mechanism or rate of ultrastructural damage caused by bacterial growth.

Incubation with Pseudomonas fragi, Staphylococcus aureus, and Clostridium perfringens resulted in complete disruption of the myofibrillar structure. In each case, degradation appeared to start with I-band breakage, after which the I-band-Z-line material became diffuse and finally indistinguishable. Thus, the A-band region of the myofibril was the most resistant area to microbial attack.

Growth of Clostridium perfringens and Pseudomonas fragi resulted in degradation of tissue nuclei and nuclear material, whereas, growth of Staphylococcus aureus and Bacillus pumilus did not appear to alter the nuclei.

Mitochondrial cristae disappeared due to growth of Clostridium perfringens, whereas, Bacillus pumilus, Pseudomonas fragi, and Staphylococcus aureus did not effect the mitochondria during the first 96 hrs of incubation. After 96 hrs of incubation, mitochondria in tissue inoculated with Pseudomonas fragi and Staphylococcus aureus appeared to be more stable than in aseptic controls or in tissue inoculated with Bacillus pumilus. Comparison of pH changes during growth with the published pH optima of catheptic enzymes suggested that mitochondrial degradation during incubation was probably the result of autolysis. Thus, pH increases caused by the

growth of Pseudomonas fragi and Staphylococcus aureus caused inhibition of the catheptic enzymes, resulting in greater mitochondrial stability.

Ultrastructural observations during autolysis of control samples revealed the presence of vesicular structures in the degraded areas of the myofibril. These vesicles ranged in size from 0.01 to 1.45μm and appeared to be associated with autolytic degradation. It was also noted that intermyofibrillar mitochondria were more stable to autolysis than intramyofibrillar mitochondria. This resulted in greater disruption of red fibers because of their higher mitochondrial content. However, fiber type per se did not influence the rate of degradation of individual mitochondria.

Headspace analysis of uninoculated aseptic control tissue resulted in the tentative identification of acetone, lactic acid and acetaldehyde. Chromatograms from tissue inoculated with an acid producing mixed culture showed peaks for lactic acid, acetaldehyde, ethyl acetate, ethanol, and propionic acid, whereas, Pseudomonas fragi produced chromatograms similar to the controls except for the presence of a small ethyl acetate peak. With the exception of ethanol, the appearance of chromatographic peaks did not correspond to development of off-odors. These results suggest that chromatographic profiles of headspace volatiles may prove useful for detecting the onset of meat spoilage, but further work with more refined systems will be needed to make the data useful.

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APPENDICES

Appendix I

Bacterial Numbers and Growth Using a Dip Inoculation Technique

Appendix Table 1

Bacterial Counts on Meat Inoculated with *Pseudomonas fragi* Using a Dip Technique

	Sample Number*			
Dilution	1	2	3	4
10^{-1}	TMTC	TMTC	TMTC	TMTC
10^{-2}	TMTC	TMTC	TMTC	TMTC
10^{-3}	TMTC	TMTC	TMTC	TMTC
10^{-4}	33	35	41	39
10^{-5}	0	0	1	0
10^{-6}	0	0	0	0

*TMTC = too many to count

Appendix Table 2

Effect of Either Random or Spread Packing Technique on the Growth of *Pseudomonas fragi* Dip Inoculated on Porcine Muscle

	log Bacterial Number	
Sample Number	Spread Pack	Random Pack
1	10.18	10.23
2	10.36	10.26
3	10.36	10.33
4	10.42	10.26
Average	10.33	10.27

Appendix II

Schedules for the Preparation of Histochemical and Electron Microscopy Solutions

Appendix Table 3

Schedule for the
Preparation of the Histochemical Incubating Medium

Reagents	Amount
0.2M Tris buffer (pH 7.4)	10 ml
nitro blue tetrazolium	10 mg
NADH	8 mg

Appendix Table 4

Schedule for the
Preparation of 1.25% Glutaraldehyde Fixative Solution

Reagents	Amount	Final Molarity
$NaH_2PO_4 \cdot H_2O$	1.80 gm	0.007
$Na_2HPO_4 \cdot 7H_2O$	23.25 gm	0.041
NaCl	5.00 gm	0.043
50% Glutaraldehyde	50.00 ml	
H_2O	1975.00 ml	

Appendix Table 5

Schedule for the
Preparation of Glutaraldehyde Wash Buffer

Reagents	Amount	Final Molarity
$NaH_2PO_4 \cdot H_2O$	1.80 gm	0.013
$Na_2HPO_4 \cdot 7H_2O$	23.25 gm	0.081
NaCl	5.00 gm	0.086
H_2O	925.00 ml	

Appendix Table 6

Schedule for the
Preparation of a 1% Osmium Tetroxide Fixative Solution

A. Stock solution A.

Reagents	Amount
Sodium acetate	9.714 gm
Veronal-sodium	14.714 gm
Make to 500 ml final volume with H_2O.	

B. Stock solution B.

Reagents	Amount
Sodium chloride	40.25 gm
Potassium chloride	2.10 gm
Calcium chloride	0.90 gm
Make to 500 ml final volume with H_2O	

The solutions are mixed according to the following scheme:

Solution A	10.0	ml
Solution B	3.4	ml
Dilute to 50 ml with H_2O		
0.1N HCl	11	ml (approx.)

Solutions A and B are measured out and made to a 50 ml volume with distilled water. The pH is then adjusted to 7.2-7.4 with 0.1N HCl. To this mixture 0.5 gm of osmium tetroxide is added and stored in a brown glass stoppered bottle.

Appendix Table 7

Schedule for the
Preparation Epon-Araldite Resin

Reagents	Amount
Epon 812	62 ml
Araldite 506	81 ml
DDSA (hardner)	100 ml
dibutyl-phthalate	4-7 ml
DMP-30	1.5-3.0%

Mixing solution:

Epon 812, Araldite 506, and DDSA are thoroughly mixed after which the dibutyl-phthalate and the DMP-30 are added. After mixing the resin is ready for embedding the tissue.

Appendix Table 8

Schedule for the
Preparation of Reynolds Lead Citrate Stain

Reagents	Amount
Lead nitrate	1.33 gm
Sodium citrate	1.76 gm
1N NaOH	8 ml
H_2O (freshly boiled)	make to 50 ml

Appendix Table 9

Schedule for the
Preparation of Uranyl Acetate Stain

Reagents	Amount
Uranyl acetate	8 gm
H_2O (glass distilled)	100 ml

Appendix III

Raw Data for the Histochemical Determination of Fiber Type

Appendix Table 10

Proportion of Red, White, and Intermediate Fibers in the Red Portion of Porcine Semitendinosus Muscle Inoculated with *Pseudomonas fragi*

	Red Fibers		White Fibers		Intermediate Fibers	
Fasciculi #	Number	%	Number	%	Number	%
1	20	71.4	--	---	8	28.6
2	26	70.3	1	2.7	10	27.0
3	25	71.4	1	2.9	9	25.7
4	20	76.9	--	---	6	23.1
5	30	65.2	3	6.5	13	28.3
6	16	69.6	--	---	7	30.4
7	17	68.0	--	---	8	32.0
8	24	72.7	1	3.0	8	24.2
9	17	63.0	1	3.7	9	33.3
10	17	70.8	--	---	7	29.2
Avg.	21.2	69.9	.7	1.9	8.5	28.2

Appendix Table 11

Proportion of Red, White, and Intermediate Fibers in the White Portion of Porcine Semitendinosus Muscle Inoculated with *Pseudomonas fragi*

	Red Fibers		White Fibers		Intermediate Fibers	
Fasciculi #	Number	%	Number	%	Number	%
1	3	3.8	60	75.9	16	20.3
2	2	2.7	58	79.5	13	17.8
3	4	4.3	70	76.1	18	19.6
4	---	---	17	81.0	4	19.0
5	1	1.3	62	79.5	15	19.2
6	2	2.7	58	77.3	15	20.0
7	---	---	28	82.4	6	17.6
8	---	---	20	80.0	5	20.0
9	1	3.3	22	73.3	7	23.3
10	1	2.6	29	76.3	8	21.1
Avg.	1.4	2.1	42.4	78.1	10.7	19.8

Appendix Table 12

Proportion of Red, White, and Intermediate Fibers in the Red Portion of Porcine Semitendinosus Muscle Inoculated with *Bacillus pumilus*

	Red Fibers		White Fibers		Intermediate Fibers	
Fasciculi #	Number	%	Number	%	Number	%
1	9	52.9	2	11.8	6	35.3
2	6	50.0	1	8.3	5	41.7
3	5	45.5	1	9.1	5	45.5
4	10	66.7	---	----	5	33.3
5	10	47.6	2	9.5	9	42.9
6	11	64.7	2	11.8	4	23.5
7	6	46.2	1	7.7	6	46.2
8	5	50.0	1	10.0	4	40.0
9	10	50.0	4	20.0	6	30.0
10	12	52.2	3	13.0	8	34.8
Avg.	8.4	52.6	1.7	10.1	5.8	37.3

Appendix Table 13

Proportion of Red, White, and Intermediate Fibers in the White Portion of Porcine Semitendinosus Muscle Inoculated with *Bacillus pumilus*

	Red Fibers		White Fibers		Intermediate Fibers	
Fasciculi #	Number	%	Number	%	Number	%
1	1	2.6	27	71.1	10	26.3
2	3	10.3	19	65.5	7	24.1
3	---	----	19	67.9	9	32.1
4	---	----	15	71.4	6	28.6
5	---	----	21	77.8	6	22.2
6	2	4.9	30	73.2	9	21.9
7	1	3.3	23	76.7	6	20.0
8	1	4.2	19	79.2	4	16.7
9	---	----	20	83.3	4	16.7
10	1	2.9	26	76.5	7	20.6
Avg.	.9	2.8	21.9	74.3	6.8	22.9

Appendix Table 14

Proportion of Red, White, and Intermediate Fibers in the Red Portion of Porcine Semitendinosus Muscle Inoculated with Staphylococcus aureus

	Red Fibers		White Fibers		Intermediate Fibers	
Fasciculi #	Number	%	Number	%	Number	%
1	35	81.4	----	----	8	18.6
2	32	72.7	----	----	12	27.3
3	28	71.8	1	2.6	10	25.6
4	31	72.1	----	----	12	27.9
5	29	76.3	1	2.6	8	21.1
6	39	69.6	2	3.6	15	26.8
7	31	75.6	1	2.4	9	22.0
8	28	70.0	1	2.5	11	27.5
9	21	75.0	----	----	7	25.0
10	36	75.0	1	2.1	11	22.9
Avg.	31	74.0	.7	1.6	10.3	24.5

Appendix Table 15

Proportion of Red, White, and Intermediate Fibers in the White Portion of Porcine Semitendinosus Muscle Inoculated with Staphylococcus aureus

	Red Fibers		White Fibers		Intermediate Fibers	
Fasciculi #	Number	%	Number	%	Number	%
1	----	---	20	80.0	5	20.0
2	2	6.3	25	78.1	5	15.6
3	1	7.7	9	69.2	3	23.1
4	----	---	17	81.0	4	19.0
5	----	---	20	69.0	9	31.0
6	----	---	20	71.4	8	28.6
7	1	2.9	24	68.6	10	28.6
8	----	---	18	75.0	6	25.0
9	----	---	23	76.7	7	23.3
10	1	5.3	14	73.7	4	21.1
Avg.	.5	2.2	19	74.3	6.1	23.5

Appendix Table 16

Proportion of Red, White, and Intermediate Fibers in the Red Portion of Porcine Semitendinosus Muscle Inoculated with *Clostridium perfringens*

	Red Fibers		White Fibers		Intermediate Fibers	
Fasciculi #	Number	%	Number	%	Number	%
1	35	77.8	1	2.2	9	20.0
2	20	71.4	---	---	8	28.6
3	17	77.3	---	---	5	22.7
4	31	75.6	1	2.4	9	22.0
5	28	73.7	2	5.3	8	21.1
6	21	75.0	---	---	7	25.0
7	29	80.6	1	2.8	6	16.7
8	37	72.5	2	3.9	12	23.5
9	19	76.0	---	---	6	24.0
10	23	82.1	---	---	5	17.9
Avg.	26.0	76.2	.7	1.7	7.5	22.2

Appendix Table 17

Proportion of Red, White, and Intermediate Fibers in the White Portion of Porcine Semitendinosus Muscle Inoculated with *Clostridium perfringens*

	Red Fibers		White Fibers		Intermediate Fibers	
Fasciculi #	Number	%	Number	%	Number	%
1	1	2.0	37	75.5	11	22.4
2	1	2.6	30	76.9	8	20.5
3	2	4.3	35	76.1	9	19.6
4	---	---	20	76.9	6	23.1
5	---	---	27	79.4	7	20.6
6	1	2.6	31	79.5	7	17.9
7	---	---	20	80.0	5	20.0
8	1	2.3	34	79.1	8	18.6
9	---	---	21	75.0	7	25.0
10	---	---	19	70.4	8	29.6
Avg.	.6	1.4	27.4	76.9	7.5	21.7

Appendix IV

Raw Data on Bacterial Growth and pH Changes

Appendix Table 18

Summary of the Growth of *Pseudomonas fragi* on Porcine Semitendinosus Muscle Stored at 10°C

	log Bacterial Number			
	Inoculated Tissue		Control Tissue	
Incubation Time	Red	White	Red	White
0	5.43	5.85	0.0	0.0
0	5.60	5.82	0.0	0.0
Average	5.51	5.83	0.0	0.0
24	7.18	7.38	0.0	0.0
24	7.03	7.29	0.0	0.0
Average	7.11	7.34	0.0	0.0
48	9.31	9.18	0.0	0.0
48	9.27	9.12	0.0	0.0
Average	9.29	9.15	0.0	0.0
96	10.33	10.14	0.0	0.0
96	10.38	10.18	0.0	0.0
Average	10.36	10.16	0.0	0.0
168	9.89	10.27	0.0	0.0
168	9.96	10.19	0.0	0.0
Average	9.93	10.23	0.0	0.0

Appendix Table 19

Summary of the Change in pH During the Incubation of Porcine Semitendinosus Muscle Inoculated with *Pseudomonas fragi* and Stored at 10°C

	pH Value			
	Inoculated Tissue		Control Tissue	
Incubation Time	Red	White	Red	White
0	5.75	5.60	5.80	5.63
0	5.83	5.75	5.81	5.69
0	5.80	5.70	5.74	5.64
Average	5.79	5.68	5.78	5.65
24	6.10	5.81	----	----
24	6.17	5.83	----	----
24	6.10	5.85	----	----
Average	6.12	5.83	----	----
48	6.68	6.40	----	----
48	6.57	6.21	----	----
48	6.63	6.15	----	----
Average	6.63	6.25	----	----
96	7.84	7.36	5.75	5.70
96	7.90	7.32	5.81	5.62
96	7.65	7.10	5.83	5.64
Average	7.80	7.26	5.79	5.65
168	8.17	8.04	5.93	5.77
168	8.24	7.94	5.97	5.80
168	8.21	7.98	5.96	5.81
Average	8.21	7.99	5.95	5.79

Appendix Table 20

Summary of the Growth of
Bacillus pumilus on Porcine Semitendinosus Muscle
Stored at 10°C

	log Bacterial Number			
	Inoculated Tissue		Control Tissue	
Incubation Time	Red	White	Red	White
0	5.31	4.80	0.0	0.0
0	5.28	4.65	0.0	0.0
Average	5.30	4.73	0.0	0.0
24	5.69	5.04	0.0	0.0
24	5.79	4.84	0.0	0.0
Average	5.74	4.94	0.0	0.0
48	5.79	4.71	0.0	0.0
48	5.79	4.81	0.0	0.0
Average	5.79	4.76	0.0	0.0
96	6.18	5.48	0.0	0.0
96	6.21	5.52	0.0	0.0
Average	6.20	5.50	0.0	0.0
168	6.00	5.85	0.0	0.0
168	5.85	6.00	0.0	0.0
Average	5.93	5.93	0.0	0.0

Appendix Table 21

Summary of the Change in pH During the Incubation of Porcine Semitendinosus Muscle Inoculated with *Bacillus pumilus* and Stored at 10°C

	pH Value			
	Inoculated Tissue		Control Tissue	
Incubation Time	Red	White	Red	White
0	6.02	5.83	6.15	5.81
0	6.21	5.87	6.18	5.87
0	6.19	5.89	6.10	5.88
Average	6.14	5.86	6.14	5.85
24	6.31	6.07	6.14	5.99
24	6.30	6.06	6.27	5.90
24	6.33	6.01	6.16	6.01
Average	6.31	6.05	6.19	5.97
48	6.20	5.96	6.12	5.75
48	6.21	6.99	6.21	5.82
48	6.23	6.01	6.14	5.83
Average	6.21	5.99	6.16	5.80
96	6.25	5.80	6.02	5.72
96	6.27	5.88	6.10	5.71
96	6.23	5.85	6.16	5.84
Average	6.25	5.84	6.09	5.76
168	6.30	5.83	6.01	5.66
168	6.28	5.80	6.05	5.64
168	6.27	5.79	6.11	5.70
Average	6.28	5.81	6.06	5.67

Appendix Table 22

Summary of the Growth of Staphylococcus aureus on Porcine Semitendinosus Muscle Stored at 15°C

	log Bacterial Number			
	Inoculated Tissue		Control Tissue	
Incubation Time	Red	White	Red	White
0	5.36	5.38	0.0	0.0
0	5.32	5.37	0.0	0.0
Average	5.34	5.38	0.0	0.0
24	6.44	6.41	0.0	0.0
24	6.41	6.56	0.0	0.0
Average	6.43	6.44	0.0	0.0
48	7.67	7.87	0.0	0.0
48	7.72	7.83	0.0	0.0
Average	7.70	7.85	0.0	0.0
96	9.73	9.15	0.0	0.0
96	9.79	9.36	0.0	0.0
Average	9.76	9.26	0.0	0.0
168	10.31	9.88	0.0	0.0
168	10.27	9.77	0.0	0.0
Average	10.24	9.83	0.0	0.0

Appendix Table 22

Summary of the Growth of Staphylococcus aureus on Porcine Semitendinosus Muscle Stored at 15°C

	log Bacterial Number			
	Inoculated Tissue		Control Tissue	
Incubation Time	Red	White	Red	White
0	5.36	5.38	0.0	0.0
0	5.32	5.37	0.0	0.0
Average	5.34	5.38	0.0	0.0
24	6.44	6.41	0.0	0.0
24	6.41	6.56	0.0	0.0
Average	6.43	6.44	0.0	0.0
48	7.67	7.87	0.0	0.0
48	7.72	7.83	0.0	0.0
Average	7.70	7.85	0.0	0.0
96	9.73	9.15	0.0	0.0
96	9.79	9.36	0.0	0.0
Average	9.76	9.26	0.0	0.0
168	10.31	9.88	0.0	0.0
168	10.27	9.77	0.0	0.0
Average	10.24	9.83	0.0	0.0

Appendix Table 23

Summary of the Change in pH During the Incubation of Porcine Semitendinosus Muscle Inoculated with *Staphylococcus aureus* and Stored at 15°C

	pH Value			
	Inoculated Tissue		Control Tissue	
Incubation Time	Red	White	Red	White
0	5.97	5.67	5.98	5.68
0	6.00	5.71	5.96	5.64
0	5.99	5.69	6.01	5.70
Average	5.99	5.69	5.98	5.67
24	6.07	5.86	5.97	5.73
24	6.23	5.87	6.01	5.70
24	6.26	5.75	6.00	5.72
Average	6.18	5.83	5.99	5.72
48	6.22	5.88	5.92	5.74
48	6.24	5.79	5.92	5.78
48	6.15	5.85	5.93	5.70
Average	6.20	5.84	5.92	5.74
96	6.31	6.08	5.92	5.71
96	6.29	5.97	5.91	5.78
96	6.44	6.02	5.88	5.76
Average	6.35	6.02	5.90	5.75
168	7.74	6.45	6.08	5.91
168	7.70	6.38	6.11	5.87
168	7.69	6.24	6.10	5.96
Average	7.71	6.36	6.10	5.91

Appendix Table 24

Summary of the Growth of *Clostridium perfringens* on Porcine Semitendinosus Muscle Stored at 30°C

	log Bacterial Number			
	Inoculated Tissue		Control Tissue	
Incubation Time	Red	White	Red	White
0	5.77	5.41	0	0
0	5.74	5.51	0	0
Average	5.76	5.46	0	0
24	8.66	7.71	0	0
24	8.64	7.81	0	0
Average	8.65	7.76	0	0
48	8.65	8.32	0	0
48	8.81	8.38	0	0
Average	8.73	8.35	0	0
96	8.57	8.42	0	0
96	8.59	8.45	0	0
Average	8.58	8.44	0	0

Appendix Table 25

Summary of the Change in pH During the Incubation of Porcine Semitendinosus Muscle Inoculated with Clostridium perfringens and and Stored at 30°C

	pH Value			
	Inoculated Tissue		Control Tissue	
Incubation Time	Red	White	Red	White
0	5.68	5.31	5.53	5.34
0	5.67	5.38	5.62	5.36
0	5.64	5.34	5.58	5.36
Average	5.66	5.34	5.58	5.35
24	5.98	5.17	5.62	5.39
24	6.00	5.27	5.64	5.32
24	5.95	5.26	5.68	5.25
Average	5.98	5.23	5.65	5.32
48	6.12	5.71	5.39	5.33
48	6.14	5.73	5.55	5.37
48	6.15	5.78	5.62	5.35
Average	6.14	5.74	5.52	5.35
96	6.62	6.11	5.65	5.40
96	6.50	6.08	5.54	5.35
96	6.55	6.09	5.57	5.41
Average	6.56	6.09	5.59	5.39

Appendix V

Raw Data From Headspace Analysis Experiments

Appendix Table 26

Bacterial Growth Data
for *Pseudomonas fragi* or a Mixed Culture
on Porcine Longissimus Dorsi Muscle Incubated at 10°C

Incubation Time, hrs.	log Bacterial Numbers		
	Pseudomonas fragi	Mixed Culture	Control
0	6.54	6.96	0
0	6.51	6.90	0
Average	6.53	6.93	0
24	6.72	8.14	0
24	6.69	8.15	0
Average	6.71	8.15	0
48	8.49	8.83	0
48	8.48	8.72	0
Average	8.49	8.78	0
72	9.88	8.69	0
72	9.96	8.64	0
Average	9.92	8.67	0
96	10.26	8.45	0
96	10.25	8.49	0
Average	10.26	8.47	0

Appendix Table 27

Changes in the pH Values of Longissimus Dorsi Muscle Inoculated with *Pseudomonas fragi* or a Mixed Culture from Hamburger and Incubated at 10°C

Incubation Time, hrs.	pH Value		
	Pseudomonas fragi	Mixed Culture	Control
0	5.61	5.63	5.62
0	5.64	5.63	5.65
0	5.62	5.64	5.63
Average	5.62	5.63	5.63
24	5.73	5.51	5.63
24	5.71	5.55	5.65
24	5.70	5.54	5.65
Average	5.71	5.53	5.64
48	6.47	5.09	5.66
48	6.47	5.06	5.64
48	6.49	5.06	5.69
Average	6.48	5.07	5.66
72	7.12	4.95	5.70
72	7.18	4.97	5.65
72	7.17	4.95	5.68
Average	7.16	4.96	5.68
96	8.06	4.91	5.71
96	8.01	4.95	5.79
96	8.10	4.94	5.77
Average	8.06	4.93	5.76

Appendix Table 28

Retention Time of Chromatographic Peaks of the Headspace Vapors of Uninoculated Porcine Longissimus Dorsi Muscle after Incubation at 10°C. Run on a 3% Apiezon Column.

	Retention Time, sec.[1]					
Incubation Time, hrs.	1	2	3	4	5	6
0		9				
24[2]	-	-	--	--	--	---
48	3		11	71	78	102
72	3		11	68	76	100
96	3		13	71	79	103

[1]Corrected by subtraction of the air peak retention time.

[2]Samples lost due to equipment malfunction.

Appendix Table 29

Retention Time of Chromatographic Peaks from the Headspace Vapors of Uninoculated Porcine Longissimus Dorsi Muscle after Incubation at 10°C. Run on a 10% Carbowax Column.

	Retention Time, sec.[1]				
Incubation Time, hrs.	1	2	3	4	5
0		44			
24[2]	--	--	--	---	---
48	29		49	125	155
72	27		51	129	160
96	27		50	125	157

[1]Corrected by subtraction of the air peak retention time.

[2]Samples lost due to equipment malfunction.

Appendix Table 30

Retention Time of Chromatographic Peaks from the Headspace Vapors of Porcine Longissimus Dorsi Muscle Incubated with a Mixed Culture from Hamburger at 10°C. Run on a 3% Apiezon Column.

	Retention Time, sec.[1]					
Incubation Time, hrs.	1	2	3	4	5	6
0	9					
24[2]	-	--	--	--	--	---
48	9	12	42	57	72	102
72	8	11	41	57	73	103
96	9	11	43	58	75	106

[1]Corrected by subtraction of the air peak retention time.

[2]Samples lost due to equipment malfunction.

Appendix Table 31

Retention Time of Chromatographic Peaks from the Headspace Vapors of Porcine Longissimus Dorsi Muscle Incubated with a Mixed Culture from Hamburger at 10°C. Run on a 10% Carbowax Column.

	Retention Time, sec.[1]							
Incubation Time, hrs.	1	2	3	4	5	6	7	8
0		43						
24[2]	--	--	--	--	--	---	---	---
48	30		50	69	90	117	309	339
72	29		49	69	91	110	306	338
96	30		50	71	90	107	307	342

[1]Corrected by subtraction of the air peak retention time.

[2]Samples lost due to equipment malfunction.

Appendix Table 32

Retention Time of Chromatographic Peaks from the Headspace Vapors of Porcine Longissimus Dorsi Muscle Incubated with Pseudomonas fragi at 10°C. Run on a 3% Apiezon Column.

	Retention Time, sec.[1]					
Incubation Time, hrs.	1	2	3	4	5	6
0		9				
24[2]	--	--	--	--	--	--
48	4		12	31	70	74
72	3		11	29	68	77
96	3		11	28	71	75

[1]Corrected by subtraction of the air peak retention time.

[2]Samples lost due to equipment malfunction.

Appendix Table 33

Retention Time of Chromatographic Peaks from the Headspace Vapors of Porcine Longissimus Dorsi Muscle Incubated with Pseudomonas fragi at 10°C. Run on a 10% Carbowax Column.

	Retention Time, sec.[1]			
Incubation Time, hrs.	1	2	3	4
0		43		
24[2]	--	--	--	--
48	29		50	68
72	29		51	70
96	28		49	67

[1]Corrected by subtraction of the air peak retention time.

[2]Samples lost due to equipment malfunction.