PROTEOLYSIS OF PORCINE MUSCLE BY CLOSTRIDIUM PERFRINGENS

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ABSTRACT

PROTEOLYSIS OF PORCINE MUSCLE

BY CLOSTRIDIUM PERFRINGENS

By

Lynn Tilley Hapchuk

The objective of this study was to determine the mechanisms by which muscle proteins break down during growth of C. perfringens. An enzyme from a culture filtrate of this organism was isolated and its action upon muscle was compared with that of the intact organism.

C. perfringens (ATCC 12915 and 13124) were grown in amino acid and peptone media. Since only C. perfringens ATCC 13124 produced measurable quantities of proteolytic enzyme(s) in these media, it was used in the majority of these studies.

Crude enzyme was partially purified from a culture filtrate of C. perfringens ATCC 13124 by $ZnCl_2$ precipitation, followed by disodium phosphate extraction and ammonium sulfate precipitation. The crude enzyme solution was further purified by gel filtration on Bio-Gel P-100, followed by ion exchange chromatography on DEAE-cellulose and gel filtration using Bio-Gel P-200. Enzyme recovery was 11% with 158-fold purification and a final specific activity of 79 units/mg protein, with a unit being arbitrarily defined as the amount of

enzyme required to raise the absorbance of azocoll supernatant by 0.1 unit in 15 min. When subjected to disc gel electrophoresis, the purified enzyme solution contained five measurable protein peaks, indicating a lack of homogeneity.

Aseptic muscle samples were inoculated either with C. perfringens or with enzyme solution and incubated at 30 or 37°C. After incubation, the samples were analyzed for sarcoplasmic, myofibrillar and non-protein nitrogen. The sarcoplasmic extract was subjected to disc gel electrophoresis, SDS gel electrophoresis and isoelectric focusing. The myofibrillar extract was subjected to disc gel electrophoresis in urea, SDS gel electrophoresis and isoelectric focusing in urea.

After incubation with C. perfringens, the quantity of non-protein nitrogen increased, which indicated proteolytic breakdown into small nitrogen containing compounds. Total sarcoplasmic nitrogen decreased after incubation with C. perfringens, which also suggested proteolytic breakdown. Disc gel electrophoresis of the sarcoplasmic fraction indicated that growth of C. perfringens was responsible for the production of three new protein peaks and for reduction in the concentration of several other peaks. Reduction in some protein peaks was also apparent upon electrophoresis in SDS gels.

There was a consistent reduction in the concentration of troponin in muscle samples incubated with C. perfringens. This was shown by both disc gel and SDS gel electrophoresis. A reduction in the amount of actin was noted upon disc gel electrophoresis in urea, but was not apparent with SDS gel electrophoresis. The concentration of tropomyosin declined upon incubation of muscle with C. perfringens as measured by SDS gel electrophoresis and by a disc gel electrophoresis procedure utilizing urea. Electron micrographs also indicated that C. perfringens destroyed the thin filaments, which are composed mainly of actin, tropomyosin and troponin. The appearance of new peaks in the myofibrillar fraction, presumably breakdown products, was noted with both SDS gel electrophoresis and disc gel electrophoresis in urea.

This investigation clearly indicates that C. perfringens is capable of degrading both sarcoplasmic and myofibrillar proteins from muscle. However, the isolated enzyme exerted its major action on only the sarcoplasmic proteins. Since the isolated enzyme frequently did not breakdown the same proteins as the viable organism, it is presumed that C. perfringens also produces other enzymes causing proteolysis.

PROTEOLYSIS OF PORCINE MUSCLE BY *CLOSTRIDIUM PERFRINGENS*

By

Lynn Tilley Hapchuk

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INTRODUCTION

Spoilage of meat has been an economic and public health problem since muscle was first used as a food. The role of microorganisms in muscle spoilage is well recognized, but the mechanism of spoilage is, as yet, not clearly defined. Some workers (Jay and Kontou, 1967; Lerke *et al.*, 1967) originally concluded that microorganisms degraded only small compounds, such as amino acids, peptides and nucleotides, but did not break down muscle proteins. More recent work has shown that several species of bacteria cause proteolysis of muscle proteins (Ockerman *et al.*, 1969; Hasegawa *et al.*, 1970).

Clostridium perfringens has been reported to be a common contaminant of muscle foods. In addition to its known role as a causative agent of enteritis, *C. perfringens* organisms are known to cause serious disruption of muscle, such as occurs in gas gangrene infections. Several workers have described the gross and microscopic appearance of muscle infected by *C. perfringens* (Robb-Smith, 1945; MacFarlane and MacLennan, 1945; Strunk *et al.*, 1967). Hasegawa *et al.* (1970) demonstrated that proteolysis of the sarcoplasmic and urea soluble fractions of muscle occurred when it was inoculated and incubated with *C. perfringens*.

C. perfringens is known to produce multiple extracellular enzymes (toxins), and as a result causes complex changes in infected muscle. Various workers have attempted isolation and purification of the toxins from C. perfringens in order to elucidate their role in gas gangrene infections. While much has been learned regarding the in vivo effect of growth of C. perfringens, little is known of their biochemical action upon individual muscle proteins.

It may be possible to find new methods of preventing meat spoilage by studying the nature and sequence of events leading to microbial spoilage. New techniques to block the action of microbial enzymes, which are presumably responsible for the deleterious effects of microorganisms, can only be discovered after the specific mechanism of action is determined.

This investigation was undertaken to determine the effects of C. perfringens growth upon the proteins of porcine muscle. Furthermore, a proteolytic enzyme produced by C. perfringens was isolated and its influence upon muscle proteins was studied.

REVIEW OF LITERATURE

Characterization of C. Perfringens

Clostridium perfringens, which was previously known as Clostridium welchii and Bacillus aerogenes capsulatus, is a short, plump, non-motile, anaerobic, gram-positive rod with rounded ends and well marked capsules (MacLennan, 1962). Yamamoto et al. (1961) reported that the C. perfringens organism is proteolytic (liquifying gelatin), catalase negative and reduces nitrate. They further reported that C. perfringens produces acid in sucrose, glucose, maltose and lactose media, but does not produce indole. They observed that it caused a "stormy" fermentation in iron milk.

MacLennan (1962) in a review stated that C. perfringens is essentially a saprophyte, producing twelve toxins, which are not important to its metabolism or economy. He reported that the toxins have been named α, β, γ, δ, ε, η, θ, ι, κ, λ, μ and ν. Not all strains of C. perfringens produce all toxins, but most strains produce at least one to several (Hauschild, 1971).

MacLennan (1962) reported that there are five recognized types of C. perfringens, designated A, B, C, D and E. He stated the classification is based on the nature of the

toxins produced. Hauschild (1971) reported that a sixth type, F, was actually a form of type C.

Occurrence of C. Perfringens

C. perfringens is generally considered to be an ubiquitous microorganism, and is found in many environments, such as food, soil, and in human and animal feces. Upon sampling foods in Madison, Wisconsin, Strong et al. (1963) reported that C. perfringens is present in frozen prepared foods, home prepared foods, spices, market meats, ground and spiced meats, and organ meats. Canada and Strong (1964) found C. perfringens in 26 per cent of the samples of bovine liver purchased in a commercial market, while only 12 per cent of the livers from newly slaughtered cattle were contaminated. They concluded that the contamination did not originate from the bile.

Hall and Angelotti (1965) in a survey of the Cincinnati area found C. perfringens to be present in 58 per cent of the raw unprocessed meat specimens. The meat they sampled included veal, beef, chicken, lamb, and pork. Nearly 20 per cent of the processed meat and meat products contained C. perfringens. Those products, which require little or no cooking by the consumer, were found to contain fewer C. perfringens cells.

The more a meat product is handled, the greater the possibility of contamination with C. perfringens.

Baltzer and Wilson (1965) found C. perfringens contamination of pork occurred during the butchering process, after scalding, after scraping, and after inspection. They also found contamination in the scald tank water and on skin sections. They concluded that contamination from machinery was at least partially responsible for the increase in microbial numbers.

Lepovetsky et al. (1953) isolated C. perfringens from bovine lymph nodes and bone marrow. They speculated that lymph nodes may be the source of "deep spoilage" in meat.

Yamamoto et al. (1961) found that 16.4 per cent of the feces from mature chickens and 15.5 per cent of the feces from turkeys were contaminated with C. perfringens. They also isolated C. perfringens from the feces of 9.6 per cent of frying chickens. They reported that C. perfringens were found in the livers of four fryers and one fowl.

Solberg and Elkind (1970) reported 20 to 50 per cent of C. perfringens survived heating for 30 to 48 minutes in inoculated frankfurters. They found growth occurred during storage at temperatures of 12°C or higher.

Gough and Alford (1965) determined that C. perfringens survived in cured smoked hams when the final salt concentration was 3% NaCl, 500 ppm $NaNO_3$ and 200 ppm $NaNO_2$. They further observed that C. perfringens remained viable in brine concentrations ranging from 7.5% NaCl,

3,700 ppm $NaNO_3$ and 370 ppm $NaNO_2$ up to 17% NaCl, 23,000 ppm $NaNO_3$ and 2,300 ppm $NaNO_2$.

Infections Caused by C. Perfringens

C. perfringens type A is the causative agent for two types of infections in man. One is food poisoning (enteritis) and the other gas gangrene.

Food poisoning

Several workers (Hall et al., 1963; Strong et al., 1963; and Nakamura and Schulze, 1970) have written reviews of food poisoning caused by C. perfringens. They characterized the symptoms of food poisoning as being abdominal pain, diarrhea, occasional vomiting and nausea. They stated that these symptoms generally occur 8 to 12 hours after ingestion of the contaminated food and persist for 6 to 12 hours. They stated that recovery is rapid, and no deaths have been reported as a direct result of C. perfringens type A food poisoning.

Hauschild (1971), in a review article, reported that C. perfringens type C is implicated in a necrotic enteritis of man commonly known as "pig-bel." He stated that it occurs in the highlands of New Guinea and coincides with the ritual of pig feasting, which is characterized by dietary changes and over-eating. He reported that the symptoms of "pig-bel" are abdominal cramps, diarrhea, and acute inflammation of the small intestine with areas of

necrosis and gangrene, particularly in the jejunum. He stated that "pig-bel" has a high mortality rate.

Hauschild (1971) also reported that a subspecies of C. perfringens type C, originally thought to be a new type called F, was the cause of outbreaks of a necrotic enteritis called "darmbrand." He indicated that this rare disease was found after World War II in northern Germany. He stated that the causative agent of "darmbrand" was canned meat contaminated with C. perfringens type C spores. He indicated that factors involved in the onset of "darmbrand" include a sudden engorgement with rich food, and perhaps some predisposing dysfunction of the alimentary tract. He reported the symptoms of "darmbrand" included severe abdominal pain, vomiting, diarrhea, necrotic inflammation of the small intestine, particularly in the area of the jejunum, and a high mortality rate.

The causative agent for food poisoning is not known with certainty. Hauschild and Thatcher (1968) stated that α-toxin is of little importance as a factor in food poisoning. Duncan and Strong (1969) found a toxic factor in culture filtrates and cell extracts. The toxic factor was heat-labile, non-dialyzable, inactivated by pronase, but not by trypsin, lipase, or amylase, and was inactivated at a pH lower than 5.0 or higher than 12.0. They found lecithinase (α toxin) alone had no gastrointestinal effect. However, they were able to reproduce the enteritis by having volunteers ingest large quantities of viable cells.

Hobbs (1965) determined that food poisoning strains of C. perfringens in Great Britain produce low levels of α-toxin, little or no θ-toxin, and that they form heat resistant spores. Hall et al. (1963) in the United States concluded that the ability to produce heat resistant spores is a doubtful criterion for classification of food poisoning strains because contamination can occur after cooking.

Hobbs (1965) indicated that food poisoning outbreaks caused by C. perfringens are often traced to meat with a history of storage at warm temperatures. Earlier work by Hobbs (1957) showed that long, slow cooling and a lengthy warming period for meat dishes, as well as holding cooked meat dishes at serving temperatures of 39°C to 49°C for several hours contribute to food poisoning outbreaks. She found that holding meat at high temperatures (62°C to 94°C) will kill viable cells. Therefore, she recommended that cooked meat dishes be held either at refrigerator temperatures or at temperatures above 60°C.

Gas gangrene

Gas gangrene, the other infection caused by C. perfringens, causes marked structural changes in muscle (Robb-Smith, 1945; MacFarlane and MacLennan, 1945; MacLennan, 1962). Robb-Smith (1945) reported that gas gangrene results in disruption of the sarcolemma due to karyolysis and fragmentation of the muscle fibers. He

observed that the myofibrils were preserved but there was an apparent disappearance of the Z line. He noted that the "reticulin membrane" was separated from the sarcolemma with the increased endomyseal space being occupied with edemal fluid.

MacLennan (1962) reported that in gas gangrene the "reticulin fibrils" and collagen fibers were partially or completely destroyed, but elastin was unaffected. MacFarlane and MacLennan (1945) observed the gross appearance of infected muscle ranged from a friable, pinkish-grey mass to a blackish-green deliquescent mass.

Ispolatovskaya (1971) reported that the only effective method of treatment for gas gangrene has been surgical intervention. He stated that no effective antitoxin for gas gangrene in humans is known because of the complex action of the various toxins of C. perfringens. However, Ito (1970) reported development of a purified α-toxoid, which is 80 per cent effective against experimental gas gangrene in guinea pigs.

MacLennan (1962) indicated that gas gangrene is not found in every wound contaminated with C. perfringens. He stated that the proper conditions must exist, the main requirements being a lowered oxidation reduction potential (E_h) at the site of contamination and a lowered pH.

Hauschild and Thatcher (1968) demonstrated that food poisoning strains of C. perfringens are capable of producing gas gangrene in guinea pigs. They further

reported a correlation between virulence and the synthesis of α-toxin in strains causing gas gangrene.

Toxins Produced by C. Perfringens

The study of the various toxins of C. perfringens necessitates their separation and purification. Partial to complete separation has been accomplished by several workers, among them Oakley et al. (1946), Bidwell and van Heyningen (1948), Roth and Pillemer (1953, 1955), Stephen (1961), Thomson (1963), Habeeb (1964), Ikezawa et al. (1964), Hauschild (1965), and Kameyama and Akama (1971).

Alpha toxin

MacLennan (1962) indicated that phospholipase C, or α-toxin, is produced by all types of C. perfringens. He stated that phospholipase C hydrolyses lecithin to phosphoryl choline and a diglyceride. He also reported that α-toxin is activated by Ca^{+2} and Mg^{+2} but is inhibited by phosphate, citrate, pyruvate and sodium dodecylsulfate.

Ispolatovskaya (1971) indicated α-toxin also acts as an hemolysin. He further reported that α-toxin contains cystine, but is resistant to thiol poisoning and is, therefore, not a sulfhydryl enzyme. He found zinc was bound to the enzyme and postulated that it was a metallo-enzyme. He determined that the pH optimum of the enzyme in borate

buffer was 7.4 to 7.6 and in bicarbonate-CO_2 buffer was 6.7. He further reported the molecular weight of phospholipase C was 106,000±3,000. He postulated that the enzyme may exist in multiple forms or as iso-enzymes.

Beta toxin

Although little is known about β-toxin, it causes changes in the cytoplasm and nuclei of guinea pig monocytes and is followed by lysis (Hauschild, 1971).

Akama et al. (1968) purified β-toxin from C. perfringens type C. They determined β-toxin was immunologically different from any of the toxins produced by C. perfringens type A.

Epsilon toxin

Epsilon toxin causes damage to kidney cells and increased vascular permeability (Hauschild, 1971).

Thomson (1963) purified ε-toxin from C. perfringens type D. He reported that the toxin was produced as a prototoxin, which was readily converted into the active toxin by the action of proteolytic enzymes, especially by trypsin. He found that the prototoxin was converted into the active toxin in the absence of proteolytic enzyme, but the conversion was much slower.

Thomson (1963) determined the sedimentation coefficient ($S°_{20,w}$) of the prototoxin to be 2.48 S at a concentration of 6.96 mg/ml. Using the Archibald technique

of approach to equilibrium, he calculated a weight average molecular weight of 40,500. He determined the diffusion coefficient was $D = 6.76 \times 10^{-7}$ cm^2/sec and the partial specific volume to be $\bar{V} = 0.729$ ml/g. He then concluded the molecule was asymmetrical and/or hydrated to a considerable extent. He also concluded from evidence obtained by ion exchange fractionation that the prototoxin was composed of a number of fractions differing slightly in charge. Thomson (1963) suggested that the data may be indicative of a stepwise degradation of prototoxin to active toxin. He explained that the isoelectric point decreases as the prototoxin degrades, which could be caused by stepwise removal of basic groups from the prototoxin molecule.

Hauschild (1971) proposed that removal of the basic moieties from asparagine and glutamine in the prototoxin account for the high dicarboxylic acid content of ε-toxin.

Orlans et al. (1960) isolated ε-prototoxin and ε-toxin. Using mice, they found the lethal dose was 1.13 μg/kg of body weight. They determined that the $S^\circ_{20,w}$ of ε-toxin was 2.8 S. The diffusion coefficient was $D = 7.2 \times 10^{-7}$ cm^2/sec. Using these data and a partial specific volume of $\bar{V} = 0.74$ ml/g, they calculated a molecular weight of $38,000 \pm 5,000$.

Orlans et al. (1960) determined that the ε-prototoxin and ε-toxin were antigenically identical. They postulated that the toxin and prototoxin differed only by a small peptide at the active site and suggested that the

antigenic site differs from the active site.

Habeeb (1964) isolated ε-toxin from C. perfringens type D. He found the toxin was electrophoretically homogeneous at pH 4.5, but at pH 8.6 he detected five components. He determined that these five components were antigenically identical. The toxin he isolated had an $S°_{20,w}$ of 2.85 S. He obtained a molecular weight of 23,200 using sedimentation data and 25,100 using Archibald's method of approach to equilibrium.

Hauschild (1965) prepared ε-toxin by the method of Habeeb (1964) and separated the preparation into a major non-lethal fraction and a small portion containing all the ε-toxin. He proposed that the disagreement in amino acid analyses and molecular weights between Habeeb (1964) and Thomson (1963) could be explained by the presence of a non-lethal contaminant in the former preparation.

In order to explain overlapping of toxin and prototoxin peaks and the increase of toxin during storage of prototoxin preparations, both Hauschild (1965) and Thomson (1963) proposed a gradual conversion of ε-toxin from the non-toxic precursor to the toxic form.

Theta toxin

Roth and Pillemer (1955) reported that θ-toxin is an oxygen-labile hemolysin. They stated that θ-toxin is dermonecrotizing and reported that its lethality is due to its cardiotoxic action.

Roth and Pillemer (1955) determined the $S°_{20,w}$ of θ-toxin to be 6.5 S. They found the pH optimum was 6.75 to 6.8, and the temperature maximum was 37°C.

Ispolatovskaya (1971) reported θ-toxin does not require Ca^{+2} or Mg^{+2}. Roth and Pillemer (1955) observed that the action of θ-toxin was inhibited by hydrogen peroxide, potassium ferricyanide, iodoacetic acid and parachloromercuribenzoate. They reactivated the oxidized toxin using reducing agents, such as sodium sulfite and sodium hydrosulfite. They hypothesized that θ-toxin is a protein containing disulfide linkages, which must be reduced to form the active toxin.

Hauschild (1971) reported that no specific substrate is now known for θ-toxin. He proposed that θ-toxin may have a catalytic rather than an enzymatic role.

Kappa toxin

Oakley et al. (1946) determined that κ-toxin has both collagenase and a gelatinase activity. Upon injection of κ-toxin into guinea pig muscle, Oakley et al. (1948) demonstrated that collagen was destroyed, but elastin was unaffected. They found that the κ-toxin-treated collagen no longer stained red with van Gieson's stain, while elastin fibers remained intact. Oakley et al. (1946) also observed that κ-toxin destroyed the reticulum surrounding the muscle fibers and the liver trabeculae; this was true regardless of whether α-toxin was present

or not. They noted that α-toxin alone had no effect on the reticulum.

Bidwell and van Heyningen (1948) prepared κ-toxin relatively free from other toxins, but still containing small amounts of α- and θ-toxins. They found κ-toxin had a pH optimum between 6.0 and 7.5. The κ-toxin was stable in borate buffers at neutral and alkaline pH values, but was unstable at an acid pH or in phosphate buffers. These workers inactivated the κ-toxin by heating to 80°C for 5 minutes. They found that the toxin was also inactivated by Cu^{+2}, Hg^{+2}, Mn^{+2}, Co^{+2} and $Fe^{+2,+3}$ salts, while Mg^{+2} and Zn^{+2} ions were ineffective. Later work by Bidwell (1949) showed heating to 50°C at a pH above 9.0 destroyed the activity against native collagen, while activity against "azocoll" was not lost under these conditions.

Kameyama and Akama (1971) purified κ-toxin free from neuraminidase, deoxyribonuclease, hyaluronidase and caseinolytic protease. Upon ultracentrifugation, they observed a single boundary with an $S°_{20,w}$ of 5.19 S. They noted a single precipitan line with immunoelectrophoresis and immuno double diffusion. They determined that maximum absorption occurred at 276-278 nm and minimum absorption at 252-254 nm. The molecular weight, which they calculated using Sephadex, was approximately 80,000. They determined that the pH optimum was 7.5 in borate buffer and 6.5 in phosphate buffer.

Kameyama and Akama (1971) found that κ-toxin was

most stable in borate buffer and least stable in phosphate buffer. They also found the toxin was heat stable to 40°C but unstable at 60°C. They observed that 30 µg of the purified toxin killed mice by causing extensive hemorrhaging of the lungs.

Levdikova (1966), as cited by Ispolatovskaya (1971), reported the molecular weight of collagenase isolated from C. perfringens to be 113,000±3,000. He found a high carboxylic acid content, which agrees with an isoelectric point of pH 5.0. He also reported that the enzyme had a high immunological specificity. The specific site of hydrolysis was shown to be in the non-polar regions of collagen with the general sequence $(Gly\text{-}Pro\text{-}R)_n$, where Gly is glycine, R is any amino acid and Pro can be either proline or hydroxyproline. He determined that cleavage occurred between the general amino acid and glycine, i.e. $[\text{-}Pro\text{-}R\text{-}\!\!\downarrow\!\!\text{-}Gly\text{-}Pro\text{-}R\text{-}]_n$, with the arrow indicating the site of cleavage.

Lambda toxin

Lambda toxin is a proteinase which does not attack collagen (Oakley et al., 1948). Bidwell (1950) isolated λ-toxin from an extract of C. perfringens. She demonstrated that the enzyme was inactivated by heating to 60°C for 10 minutes. She also found λ-toxin was inactivated by a pH lower than 5.0 or higher than 9.0. She determined the pH optimum to be between 6.0 and 7.5. She found the

enzyme attacked hide powder, gelatin, casein, hemoglobin and seracin, but not native collagen. The λ-toxin was inhibited by Cu^{+2}, Hg^{+2}, Fe^{+2}, cysteine and citrate. Iodoacetic acid and cyanide caused slight inhibition while Ca^{+2} and Mg^{+2} had no effect on activity.

Mu toxin

Ispolatovskaya (1971) reported in a review that μ-toxin, the so-called "spreading factor," has hyaluronidase activity, and alone is not lethal. He stated that μ-toxin has a biological effect on polymerized mucopolysaccharides, causing depolymerization of the ground substance. He further reported that hyaluronidase promotes microbial growth in gas gangrene infections by liberating fermentable sugars.

Other toxins

Delta toxin is hemolytic for sheep, goat, cattle and swine red blood cells, but not for horse, rabbit and human red blood cells (Hauschild, 1971).

Iota toxin is necrotic and lethal, acting by increasing the permeability of capillaries (Hauschild, 1971).

Upsilon toxin is a deoxyribonuclease (Oakley and Warrack, 1951).

Changes in Muscle Caused by C. Perfringens

C. perfringens toxins produce drastic changes in the structure of muscle. Using a light microscope, Robb-Smith (1945) studied the lesions in muscle at the spreading edges of a gas gangrene infection. He noted an increase in endomysial connective tissue space which became edematous. He observed that the separated fibers showed a greater affinity for eosin stain. He further noted that both the sarcoplasmic nuclei and the nuclei of connective tissues disappeared due to karyolysis. He observed that isolated collagen fibers were widely separated and the fine reticular fibers between them disappeared as did the reticular membrane. The elastic fibers appeared to be unaffected.

In more advanced lesions, Robb-Smith (1945) found ruptured, distorted muscle fibers, although the myofibrils were preserved. He observed that the reticular membrane was separated from the sarcolemma and the number of fibrils were reduced, but the elastic fibers remained intact. He reported that a culture filtrate from C. perfringens produced all the above changes in vitro with the exception of the edema.

MacFarlane and MacLennan (1945) found similar changes in addition to a loss of normal muscle tone and elasticity. They also reported that the muscle fibers, though fragmented, were still recognizable.

Using an electron microscope, Strunk et al. (1967) observed crude toxin from C. perfringens left the basement membrane intact with some loosening of its internal structure. They noted that destruction of the plasma membrane began with small discontinuities, which enlarged until the membrane disappeared. They observed that the sarcoplasmic reticulum dilated and vesiculated with formation of dense internal structures. They also observed that the mitochondria displayed enlarged prominent cristae, which later vesiculated. There was no observable change in the myofibrils initially, but further action of the toxin caused fragmentation and distortion of the I band. They found that the width of the Z line increased and became less distinct until it disappeared. They determined that the sarcomere became distorted and unrecognizable as the A band filaments became disarrayed.

Strunk et al. (1967) reported that purified α-toxin alone produced the same changes as crude toxin, except for the alteration of the basement membrane, which they attributed to collagenase. They explained that the dissolution of the myofilaments was dependent upon changes in the Ca^{+2}, HCO_3^{-1} and K^{+1} ion concentrations and pH changes. Other factors that they considered to be important were the exogenous and endogenous proteolytic enzymes inherent in muscle, which previously were excluded from the myofibrils by the cell membranes.

Grossman et al. (1967), using electron microscopy, observed that the predominant change caused by α-toxin alone was the appearance of electron-dense deposits on the inner and outer mitochondrial membranes. They found oxidative functions were depressed with a decrease in Krebs cycle intermediates.

Bullen and Chushner (1962) theorized that the invasive properties of C. perfringens were not dependent upon α-toxin. They found inoculated muscle damaged by $CaCl_2$ injection showed high levels of destruction even in the presence of large amounts of α-antitoxin.

Hasegawa et al. (1970) noted that C. perfringens caused proteolysis of urea soluble fractions of rabbit and porcine muscle. They also found C. perfringens destroyed aldolase, glyceraldehyde phosphate dehydrogenase, and lactic dehydrogenase in porcine muscle.

Kameyama and Akama (1971) reported 0.7 μg of κ-toxin produced hemorrhaging on rabbit skin. In work with guinea pigs, they suggested that the toxin attacks healthy connective tissue in the skin, where collagen fibers are the main component. This conclusion was based upon pathological changes observed in the subcutaneous tissue of κ-toxin-treated guinea pigs. They postulated that the primary site of κ-toxin attack in the muscle tissue is the connective tissue supporting the muscle fiber. Kameyama and Akama (1971) confirmed the observations of Strunk et al. (1967) using α-toxin. They noted

differences in muscle destroyed by α-toxin from that destroyed by κ-toxin. Using α-toxin, they observed less prominent hemorrhaging and some of the network of connective tissue remained visible to the naked eye, which was not the case using κ-toxin.

MATERIALS AND METHODS

Culturing and Growth of C. Perfringens

C. perfringens requires a complete medium, including most of the common amino acids and vitamins for growth. For production of extracellular enzymes, the requirements are even more rigorous. C. perfringens can be grown on a Difco thioglycollate medium, but for increased toxin production a more enriched medium is necessary.

Murata et al. (1956) developed a reproducible peptone medium, which was satisfactory for toxin production, but was not completely defined chemically. Later, Murata et al. (1968) reported development of a completely defined synthetic medium, which was capable of supporting growth and toxin production. Both the peptone medium and the synthetic amino acid medium were used in the present investigation.

Stock culture

Actively growing cultures in Difco thioglycollate broth were used as inoculum throughout this investigation. Frozen stock cultures of Clostridium perfringens (ATCC 12915 and ATCC 13124) were obtained from the culture collection at the Michigan State University Meat Laboratory. The

cultures were maintained anaerobically in Difco thioglycollate broth at 37°C and transferred daily for a maximum of 30 days, at which time a new stock culture was introduced to decrease the possibility of mutations.

Culturing for toxin production

Culturing for toxin production was carried out using either a peptone or an amino acid medium.

Peptone medium. A peptone medium supplemented with 0.247 g/l Difco Bacto beef was prepared according to the method of Murata et al. (1956). The composition of the peptone medium is shown in Appendix Table 1.

The peptone solution was adjusted to pH 7.6-7.8 and brought to 95% of the desired final volume prior to sterilization (15 min at 121°C). A fructose solution (25% w/v) and an iron solution (70 mg $FeSO_4 \cdot 7\ H_2O$/100 ml 0.1 N HCl) were autoclaved separately for 15 minutes at 121°C. The fructose solution (4% of final volume) and the iron solution (1% of final volume) were then added aseptically to the cooled peptone solution immediately prior to inoculation to give the complete peptone medium.

Amino acid medium. The synthetic amino acid medium was prepared according to the method of Murata et al. (1968). The composition of the amino acid and salt solution and the growth factor solution used in making up the medium is given in Appendix Table 2. The amino acids and salts were

dissolved, the pH was adjusted to 7.4-7.6 and the solution diluted to 85% of the final desired volume prior to autoclaving at 121°C for 15 min.

The growth factor mixture was sterilized by filtration through a UM-10 membrane in an Amicon ultra filtration cell model 402 (Amicon Corporation, Lexington, Massachusetts), which had been previously sterilized with 5% formaldehyde. The sterilized growth factor mixture was stored at -20°C.

The fructose solution (25%, w/v), the thioglycolic acid solution (0.4%, v/v) and an iron solution (70 mg $FeSO_4 \cdot 7\ H_2O$/100 ml 0.1N HCl) were autoclaved separately, cooled and then combined in a ratio of 4:2.5:1. This solution (7.5 ml) was used to dissolve crystals of $NaHCO_3$ (0.17 g), which had previously been autoclaved in a test tube. Immediately prior to inoculation, the growth factor mixture (5% of final volume) and the iron-fructose-thioglycolic acid-carbonate mixture (7.5% of final volume) were added aseptically to the sterile amino acid mixture to give the complete amino acid medium.

Growth studies

Growth studies to determine the length of time required for enzyme production were carried out using both peptone medium and amino acid medium. Peptone broth (10 ml final volume) was inoculated with 1 loop of actively growing C. perfringens ATCC 13124, and incubation was carried out at 37°C. A sample was removed at hourly intervals in

order to monitor growth and activity.

The amino acid medium (4 ml final volume) was inoculated with 0.1 ml actively growing C. perfringens (ATCC 13124 or ATCC 12915) and samples were removed at 2-hour intervals during incubation at 37°C.

Growth of the organism was determined by measuring turbidity at 660 nm in a Beckman DU-2 spectrophotometer equipped with a Gilford optical density converter. The cells were then removed by centrifugation at 3,000 rpm (2,000 x g) for 15 min in an International PR-6 refrigerated centrifuge (4°C). Activity in the supernatant was determined using a modification of the Azocoll method (Kameyama and Akama, 1970) with an incubation time of 1 hour.

The ability of C. perfringens ATCC 12915 or ATCC 13124 to produce proteolytic enzyme(s) in amino acid medium was restudied using a different sampling time. Both strains were grown in amino acid medium at 37°C for 23 hours. After centrifugation to remove the cellular material, the activity of the supernatants was measured on Azocoll using incubation times of 15, 30, 60, 120 and 180 min.

Enzyme Preparation and Purification

Enzyme solution was prepared using modifications of the technique of Kameyama and Akama (1971) as outlined in Figure 1.

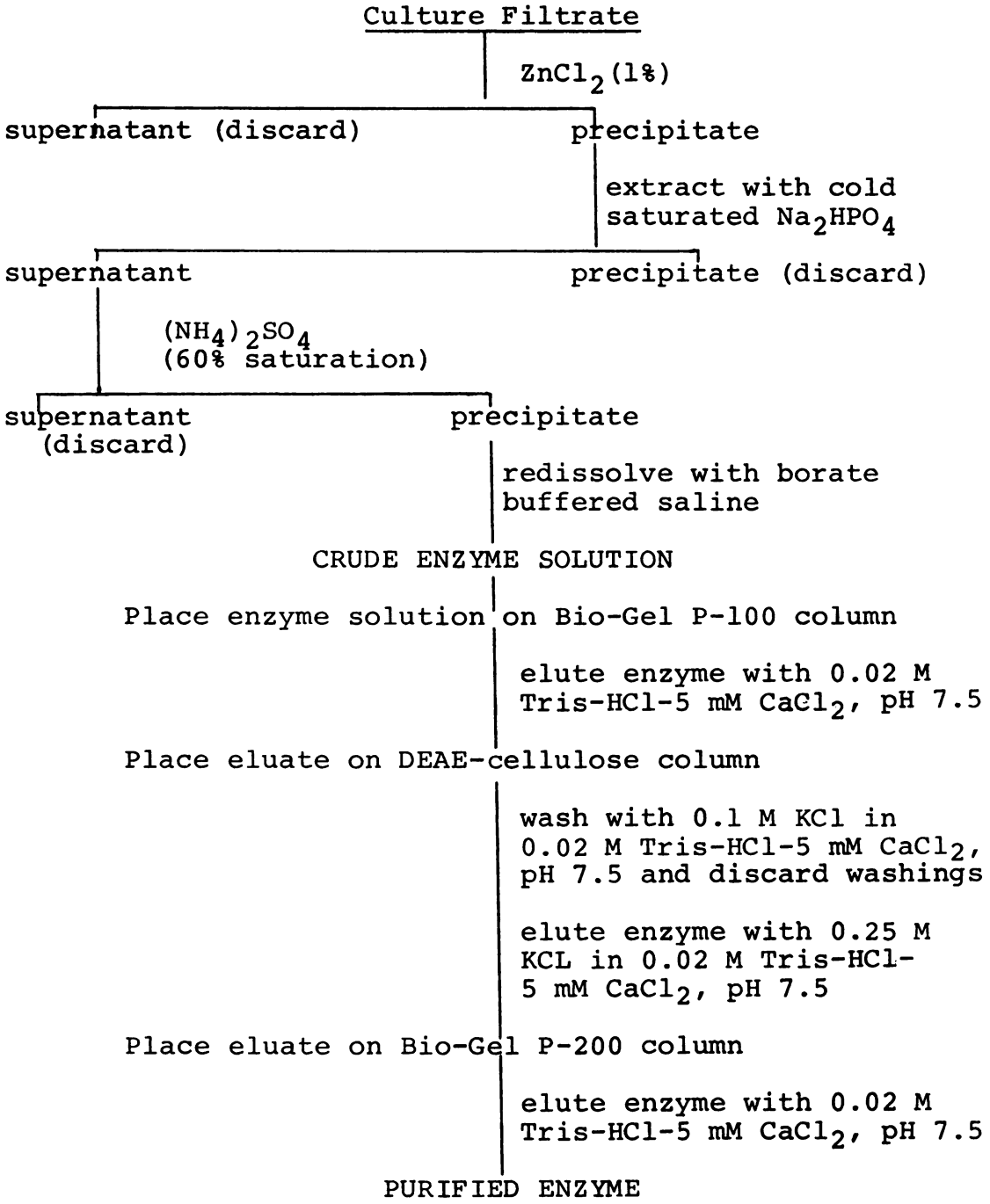

Figure 1. Enzyme purification scheme.

Preparation of culture filtrate

The peptone solution (1900 ml) was sterilized in a 2 l flask previously treated with Dow Corning Antifoam A. The flask was stoppered with a cotton plug, then autoclaved at 121°C for 15 min. Fructose solution (80 ml) and iron solution (20 ml) were added aseptically to the cooled medium.

Actively growing C. perfringens ATCC 13124 (1 ml) was used to inoculate the medium, which was then incubated for 16 to 18 hours at 37°C. The cells were removed by continuous flow centrifugation at 4°C using a Szent-Györgyi and Blum continuous flow system on a Sorvall SS-1 bench centrifuge at 11,500 rpm (≃ 16,000 x g) at a flow rate of 3-4 ml/min. Alternatively, the cells were removed by centrifuging for 30 min using the GSA rotor of a Sorvall RC2-B refrigerated centrifuge at 11,000 rpm (≃ 19,600 x g).

Zinc chloride precipitation

Precipitation of the enzymes was accomplished by adding a solution of $ZnCl_2$ (1 g/ml) slowly at 4°C with stirring, to the culture filtrate to give a final $ZnCl_2$ concentration of 1% (w/v). After stirring 1 hour at 4°C, the suspension was centrifuged at 2,400 rpm (1,500 x g) for 10 min in an International PR-6 refrigerated centrifuge at 4°C. The supernatant was discarded and the precipitate was retained for extraction with disodium phosphate.

Disodium phosphate extraction

The $ZnCl_2$ precipitate was suspended in cold saturated Na_2HPO_4 (10% of original filtrate volume), stirred 30 min at 4°C and centrifuged for 10 min at 4,000 rpm (2,500 x g) in the GSA rotor of a Sorvall super-speed RC2-B refrigerated centrifuge.

The supernatant, which contained the resolubilized toxins, was retained. The precipitate was re-extracted with cold saturated Na_2HPO_4, using 5% of the original filtrate volume, stirred for 30 min at 4°C and recentrifuged as before. The supernatants were pooled and the precipitate was re-extracted with cold saturated Na_2HPO_4 (2.5% of original filtrate volume). After stirring 30 min at 4°C, the suspension was centrifuged as before and the three supernatants pooled. The residue was discarded. If the pooled supernatant was not absolutely clear, the remaining zinc precipitate was removed by centrifugation in the GSA rotor for 20 min at 11,000 rpm (19,600 x g).

Ammonium sulfate precipitation

Solid ammonium sulfate was added slowly to the disodium phosphate extract at 4°C to precipitate the enzyme(s). The suspension was held overnight at 4°C and was then centrifuged 20 min at 8,000 rpm (7,700 x g) in the SS-34 rotor of the Sorvall RC2-B refrigerated centrifuge. The supernatant was discarded, and the precipitate was

dissolved in borate buffered saline (3% of the original culture filtrate volume). The borate buffered saline (pH 7.5) contained 0.05 M $Na_2B_4O_7 \cdot 10\ H_2O$ (1.9 g/l); 0.02 M H_3BO_4 (12.4 g/l) and 0.15 M NaCl (8.7 g/l).

The dissolved ammonium sulfate precipitate (crude enzyme) was dialyzed overnight against borate buffered saline, and then the crude enzyme was stored at -20°C until used for further purification.

Bio-Gel P-100 column

Dialyzed ammonium sulfate precipitate (25 ml) was layered on a Bio-Gel P-100 column (5.5 cm x 36 cm), which had been previously equilibrated with 0.02 M Tris-HCl-5 mM $CaCl_2$ at pH 7.5. The enzyme was eluted with 0.02 M Tris-HCl-5 mM $CaCl_2$, pH 7.5, and the active fractions were combined.

DEAE-cellulose column

The enzyme(s) from the Bio-Gel P-100 column was/were adsorbed on a DEAE-cellulose column (1 cm x 15 cm), which was equilibrated with 0.02 M Tris-HCl-5 mM $CaCl_2$, at pH 7.5. The column was then washed with 50 ml Tris-HCl buffer.

Elution was generally carried out using a stepwise increase in NaCl concentration. The column was first washed with 200 ml 0.1 M NaCl in 0.02 M Tris-HCl-5 mM $CaCl_2$, pH 7.5. These fractions were discarded. Then the

enzyme was eluted from the column using 0.25 M NaCl in 0.02 M Tris-HCl-5 mM $CaCl_2$, pH 7.5. The active fractions were combined and dialyzed against 0.02 M Tris-HCl-5 mM $CaCl_2$, pH 7.5. The dialyzed enzyme was concentrated by lyophilization and dissolved in 0.02 M Tris-HCl-5 mM $CaCl_2$, pH 7.5.

Alternatively, the enzyme solution from the Bio-Gel P-100 column was eluted from the DEAE-cellulose column by a salt gradient. The column was washed with 0.02 M Tris-HCl-5 mM $CaCl_2$, pH 7.5 as before. The enzyme was then eluted using a 0 to 0.5 M NaCl gradient in 0.02 M Tris-HCl-5 mM $CaCl_2$, pH 7.5. The salt concentration of the eluate was measured with a YSI model 31 conductivity bridge (Yellow Springs Instrument Company, Yellow Springs, Ohio).

Bio-Gel P-200 column

The dissolved enzyme from DEAE-cellulose stepwise elution was then chromatographed on a Bio-Gel P-200 column (2.7 cm x 30 cm) previously equilibrated with 0.02 M Tris-HCl-5 mM $CaCl_2$ at pH 7.5. The enzyme was eluted with 0.02 M Tris-HCl-5 mM $CaCl_2$, pH 7.5 and the active fractions were combined, lyophilized, and held at -20°C for use in muscle degradation studies.

Carboxy methyl cellulose column

In a preliminary trial, the semi-purified enzyme (after DEAE-cellulose chromatography) was further

chromatographed on a carboxy methyl (CM) cellulose column (1 cm x 15 cm), which had been previously equilibrated with 0.02 M calcium acetate-acetic acid buffer, pH 5.5. A total of 15 ml of enzyme was adsorbed on the CM-cellulose, which was then washed with 50 ml acetate buffer, pH 5.5. The enzyme was eluted with a 0 to 1.0 M NaCl gradient in 0.02 M acetate buffer, pH 5.5. The salt concentration of the eluate was measured using a conductivity bridge.

Enzyme production in amino acid medium

The synthetic amino acid medium previously described was used for preliminary enzyme purification to assess the quality and quantity of enzyme produced in this medium. The amino acid medium (1 l final volume) was inoculated with 10 ml of actively growing C. perfringens ATCC 13124 and incubated at 37°C for 14 hours. The cells were removed by centrifugation at 2,600 rpm (1,700 x g) for 30 min at 4°C in an International PR-6 centrifuge. The supernatant was passed through a XM-50 membrane, which retained the active enzyme(s). After passing the filtrate through the membrane, the fraction retained showed a two-fold increase in protein content due to concentration.

The enzymes in the retained fraction were precipitated with $ZnCl_2$ (1% final concentration). The $ZnCl_2$ slurry was held overnight at 4°C, then the precipitate was removed by centrifugation. The enzymes were extracted

from the precipitate with cold saturated disodium phosphate, and were re-precipitated by gradual addition of solid ammonium sulfate to 60% saturation. After holding overnight at 4°C, the precipitate was removed by centrifugation and redissolved in borate buffered saline.

Azocoll Method for Measuring Enzyme Activity

Enzyme activity was measured using a modification of the Azocoll method of Kameyama and Akama (1970). A 5 mg/ml suspension of Azocoll (Calbiochem, Los Angeles, California), which is an azo dye bound to hide powder, was prepared in borate buffered saline.

Azocoll (4 ml) was equilibrated to 35°C and enzyme solution (1 ml) was added. The mixture was incubated with shaking at 35°C for 15 min. After incubation, the solid material was removed by filtration through Whatman #2 filter paper and the optical density of the filtrate was measured at 520 nm using a Bechman model DU-2 spectrophotometer equipped with a Gilford optical density converter. An enzyme sample, which had been inactivated by boiling for 10 min was used as a blank. A unit of enzyme activity was arbitrarily defined as that amount of enzyme required to raise the optical density by 0.1 absorbance unit in 15 min.

Measurement of Protein

Lowry method

The protein concentration was generally measured using the method of Lowry et al. (1951). Lowry solution A (20 g Na_2CO_3, 12 g NaOH, 0.2 g $KNaC_4H_4O_6 \cdot 4\ H_2O$/l) was mixed with Lowry solution B (6 g $CuSO_4 \cdot 5\ H_2O$/l) immediately prior to use to give Lowry solution C. Phenol solution was prepared immediately prior to use by dilution (1:1) of Folin and Ciocalteu phenol reagent (Harleco, Philadelphia, Pennsylvania).

To assay for protein, 5 ml of Lowry solution C was added to 1 ml of appropriately diluted protein solution and the mixture was incubated for 20 min at room temperature. The diluted phenol solution (0.5 ml) was then added rapidly and the solution was mixed. It was allowed to stand with occasional shaking at room temperature (45 min) for color development.

The optical density was measured at 660 nm using water plus all other reagents as a blank. The protein concentration was determined by comparing with a standard curve prepared from crystalline bovine serum albumin.

Biuret method

On a few occasions, protein was measured using the biuret method (EM Reagents, Westbury, New York). In this case, protein solution (2 ml) was mixed with 0.2 ml

concentrated biuret reagent. After 30 min at room temperature, the absorbance of the mixture was read at 546 nm using 2 ml of H_2O and 0.2 ml biuret reagent as a blank. A standard curve was prepared using known concentrations of crystalline bovine serum albumin.

Kjeldahl method

Nitrogen in muscle samples was determined using a micro Kjeldahl digestion procedure followed by Nesslerization (Lang, 1958). Protein extract (4-10 ml) was transferred into a micro Kjeldahl flask with 2 glass beads and 2 ml of the digestion mixture (40 g potassium sulfate, 2 ml selenium oxychloride, water to bring the volume to 250 ml plus 250 ml of concentrated sulfuric acid). A 1,000 µg/ml lysine standard (4 ml) was added to a Kjeldahl flask and treated in the same way as above to determine the digestion efficiency.

The protein was digested using an Amicon micro Kjeldahl apparatus attached to a voltage regulator. The temperature was raised slowly to avoid loss of nitrogen and digestion was carried out until the solution became clear.

The contents of the cooled flasks were diluted appropriately and 4 ml aliquots were taken for Nesslerization. To each aliquot was added 1 ml of Nessler's solution (Koch and McMeekin, Fisher Scientific Company). After 10 min at room temperature the optical density was

read at 420 nm using a Beckman DU-2 spectrophotometer. The nitrogen content was determined by comparing to a standard curve made using solutions of ammonium sulfate containing 1 to 10 μg of nitrogen per ml.

Muscle Spoilage Studies

Preparation of C. perfringens inoculum

The C. perfringens inoculum was prepared by diluting 2 ml of an actively growing culture of C. perfringens with 100 ml of sterile 0.5% peptone-0.02% cysteine solution. The inoculum or the control (0.5% peptone-0.02% cysteine solution) was added to the muscle samples.

Preparation of enzyme solution for inoculation

The enzyme solution was prepared by redissolving lyophilized semi-purified enzyme in 0.02 M Tris-HCl-5 mM $CaCl_2$, pH 7.5 to yield a final activity of 19.2 units/ml. The enzyme solution was added to muscle samples to investigate the action of the enzyme as compared to that of the intact microorganisms. The 0.02 M Tris-HCl-5 mM $CaCl_2$, pH 7.5 solution was added to the control samples.

Muscle sample preparation

Aseptic samples of pork longissimus muscle were obtained using the method described by Hasegawa et al. (1970). A market weight pig was obtained from the

Michigan State University swine farm and slaughtered to obtain aseptic muscle samples. The neck area was scrubbed with surgical soap prior to sticking with a sterile knife. The carcass was rinsed with ethanol after dehairing and evisceration and chilled for 24 hours at 1-3°C. The middle section of the chilled carcass was rinsed with ethanol and flamed prior to removing muscle samples. The carcass was cut along the dorsal midline of the backfat-loin area with a sterilized knife. The backfat was stripped away and approximately 3 cm slices of longissimus muscle were removed and placed in sterile beakers.

The muscle was then ground twice in a sterilized grinder with C. perfringens ATCC 12915 inoculum (1 ml/100 g muscle) or a similar amount of 0.5% peptone-0.02% cysteine solution as a control. The ground meat was packed into small sterilized screw-top culture jars and incubated anaerobically at 30°C. Inoculated and uninoculated control muscle samples were removed after incubation for 0, 1, 2, 4 and 8 days and frozen for further study.

Alternatively, C. perfringens ATCC 13124 was used as inoculum. This muscle was incubated at 37°C for 0, 1, 2, 4 and 7 days.

The enzyme-treated and control samples were prepared and analyzed in the same manner as for C. perfringens ATCC 13124. The enzyme inoculum (2 ml) was added to 50 g of ground muscle and the mixture was blended for 10 sec in a sterile Virtis homogenizer (The Virtis Company, Inc.,

Gardiner, New York). An equal volume of 0.02 M Tris-HCl-5 mM $CaCl_2$, pH 7.5, was added to 50 g of muscle as a control.

After incubation the C. perfringens-treated samples (ATCC 13124) and the enzyme-treated samples were used for electrophoresis and electron microscopy.

Preparation for Kjeldahl analysis

The frozen samples, which had been inoculated and incubated with C. perfringens ATCC 12915, were thawed at room temperature and 5 g portions taken for preparation of muscle fractions as shown in Figure 2. These fractions were then used for nitrogen analysis.

The muscle sample was ground in a Waring blender for 30 sec with 50 ml of 0.03 M potassium phosphate buffer, pH 7.4. The resultant slurry was stirred for at least 3 hours at 4°C. The slurry was centrifuged at 4°C for 20 min at 1,400 x g (3,000 rpm) in a Sorvall refrigerated centrifuge, model RC2-B. The supernatant was decanted and the residue re-extracted twice. The pooled supernatant, which comprised the sarcoplasmic extract, was analyzed for non-protein nitrogen (NPN) and sarcoplasmic nitrogen.

The myofibrillar proteins were prepared by blending 5 g of muscle with 50 ml 1.1 M KCl-0.1 M potassium phosphate buffer, pH 7.4. The slurry was stirred at 4°C for 3 hours and centrifuged for 20 min at 1,400 x g. The supernatant was decanted and the residue re-extracted two more times.

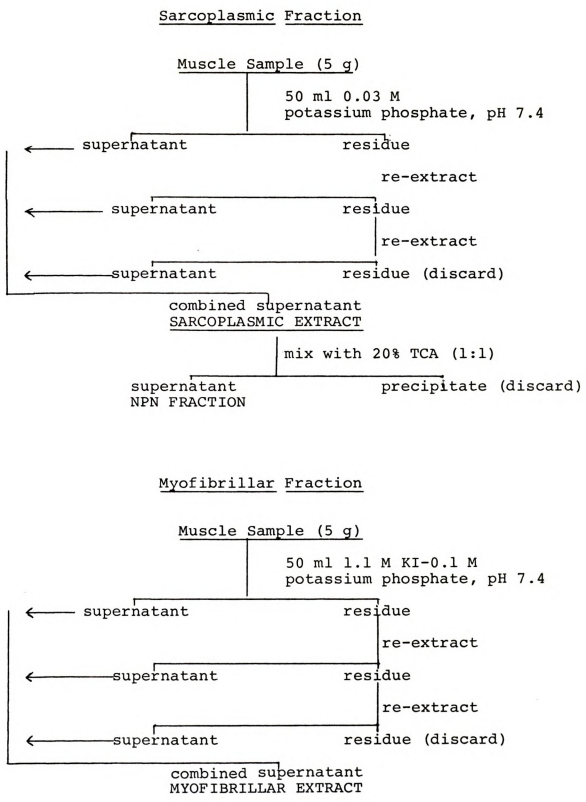

Figure 2. Preparation of muscle fractions for Kjeldahl analysis.

The supernatants were pooled to give the myofibrillar extract, which was analyzed in the same way for myofibrillar nitrogen.

The nitrogen determinations were made using the micro Kjeldahl method and the concentration of nitrogen was expressed in mg N/g muscle. To determine non-protein nitrogen, the sarcoplasmic protein was precipitated from the sarcoplasmic extract using equal volumes of 20% trichloroacetic acid (TCA). The amount of non-protein nitrogen was subtracted from the total sarcoplasmic nitrogen to give the nitrogen content of the sarcoplasmic protein. Likewise, the amount of nitrogen in the total sarcoplasmic extract was subtracted from the total myofibrillar extract to give the nitrogen content of the myofibrillar protein.

Alternatively, the protein concentration was measured using the method of Lowry et al. (1951) or the biuret method (EM Reagents Bulletin) instead of the micro Kjeldahl method.

Preparation for electrophoresis

The samples, which had been inoculated and incubated with C. perfringens ATCC 13124 or with enzyme solution or with their respective controls, were prepared for electrophoretic studies as outlined in Figure 3.

The tissue was prepared by homogenizing 5 g of muscle in a sterilized Virtis homogenizer with 50 ml of 0.03 M potassium phosphate buffer, pH 7.4. The slurry

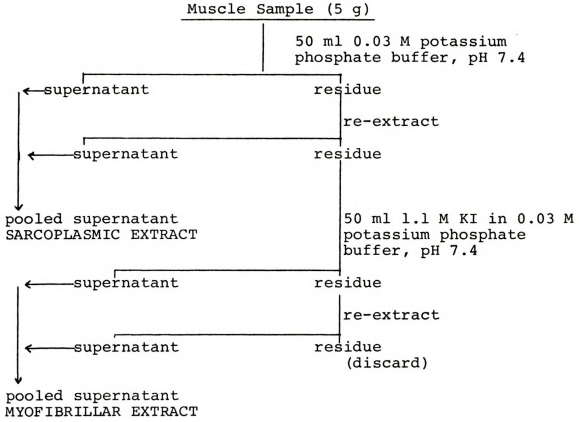

Figure 3. Preparation of muscle fractions for electrophoretic studies.

was stirred for 20 min at 4°C and centrifuged for 20 min at 10,000 x g. The residue was re-extracted with 50 ml of 0.03 M potassium phosphate buffer, pH 7.4, and the supernatants were combined to yield the sarcoplasmic extract.

The residue was extracted with 50 ml 1.1 M KI in 0.1 M potassium phosphate buffer, pH 7.4 for 1 hour at 4°C. The slurry was centrifuged for 20 min at 10,000 x g. The residue was re-extracted and the supernatants were combined to yield the myofibrillar extract. The sarcoplasmic and myofibrillar extracts were frozen for later electrophoretic analysis.

Electrophoretic Studies

All electrophoresis was done using glass tubes (15 cm x 6 mm ID) in a Polyanalyst analytical polyacrylamide vertical disc gel electrophoresis apparatus (Buchler Instruments #3-1750, Fort Lee, New Jersey) and a Heathkit Regulated High Voltage Power Supply, Model IP-17 (Heath Company, Benton Harbor, Michigan).

Disc gel electrophoresis

The sarcoplasmic proteins were separated using standard techniques of polyacrylamide disc electrophoresis (Gabriel, 1971).

Sample preparation. Aliquots of the sarcoplasmic extract were dialyzed overnight at 4°C against 20% sucrose

in 0.04 M tris-0.2 M glycine buffer, pH 8.3, prior to electrophoresis.

Gel preparation. Glass tubes were rinsed in a 2% (v/v) solution of Photo-flo 200 (Eastman Kodak Company, Rochester, New York) or a 1% (v/v) solution of column coat (Canalco, Inc., Rockville, Maryland) and oven dried. The tubes were stoppered and secured vertically in a rack.

Acrylamide gels were prepared using the formulation of Davis (1964), which is given in Appendix Table 3. The separating gel (7.5% acrylamide) was pipetted into the tubes to a height of 10-12 cm. A few drops of water were layered on top of the gel to form a flat gel surface and to protect the gel from oxygen. The tubes were refrigerated at 4°C during polymerization (20-30 min). Alternatively, the separating gel was prepared to contain 7.0% acrylamide and 0.18% BIS. All conditions were as described previously except that the separating gel stock solution (b) contained 28 g acrylamide and 0.54 g BIS in 100 ml solution.

After polymerization was completed, the water layer was removed with laboratory tissue paper. The surface was rinsed with the stacking gel solution (2.5% acrylamide), then the stacking gel solution was added on top of the polymerized separating gel to a height of 3 mm. A few drops of water were layered on the stacking gel. The stacking gel was photo-polymerized at room temperature using a fluorescent lamp a few cm from the gel. When

polymerization was completed, the stacking gel became opaque (15-20 min).

Sample application. The sample (10-100 µl) was placed on top of the stacking gel surface using a Lancer precision pipette (Sherwood Medical Industries, Inc., Bridgeton, Missouri). Buffer solution was layered on top without disturbing the sample.

Electrophoretic conditions. The glass tubes were then placed in the electrophoresis chamber and buffer was added to the upper and lower chambers. A few drops of Canalco RDS-J tracking dye or 0.05% bromophenol blue were added to the upper buffer to mark the position of maximum mobility. The electrodes were positioned with the anode in the lower chamber and the cathode in the upper.

Electrophoresis was carried out using a constant current of 2.5 ma per tube for 3-4 hours, or until the dye band had migrated 75-80% of the gel length. The gels were removed from the glass tubes using a hypodermic syringe equipped with a blunt point 22 gauge needle, 6.5 cm in length. The position of the dye band was marked by inserting a short fine wire into the gel.

Staining and destaining. The position of the protein bands was determined by staining the gels in a solution of 1% amido black 10B in 7.5% glacial acetic acid. After staining overnight, the unbound stain was removed

by diffusion in 7.5% glacial acetic acid using a BioRad model 170 gel electrophoresis diffusion destainer (BioRad Laboratories, Richmond, California).

Alternatively, the gels were placed in 12.5% TCA to denature the protein bands. After denaturation overnight, the gels were stained for 2-4 hours in coomassie blue solution (1.25 g coomassie brilliant blue-R, 46 ml glacial acetic acid, 454 ml 50% methanol). The stain was removed using 7.5% acetic acid-5% methanol.

Quantitation of protein bands. When the background stain had been removed, the position and intensity of the protein bands were recorded using a modified Kontes Chromaflex K-495000 densitometer (Kontes Glass Company, Vineland, New Jersey). The transmission of visible light from a fluorescent tube with principal line emission at 366 nm was recorded using a carriage scanning speed of 2 cm/min with an equivalent recorder speed. The migration distance of the protein bands was measured from the separating gel-stacking gel boundary to the maximum of the absorption peak.

The relative mobility (R_M) was determined by dividing the protein migration distance by the distance the marker dye migrated. The area under the peaks was determined by triangulation. The peak areas were expressed as a percentage of the total protein that entered the gel.

Disc gel electrophoresis in urea

The myofibrillar proteins were separated using disc gel electrophoresis in the presence of 7 M urea according to modifications of the method of Rampton (1969).

Sample preparation. The samples were prepared by dissolving 0.6 g solid ultrapure urea in 1 ml of myofibrillar extract. Alternatively, the samples were prepared by dialyzing aliquots of myofibrillar extract overnight against 8 M urea, which had been previously deionized by passing through a MB-3 column.

Gel preparation and sample application. The separating and stacking gels were prepared immediately prior to use using ultrapure urea as shown in the formulation given in Appendix Table 4. Each stoppered, coated glass tube contained 2 ml of separating gel solution (6.5% acrylamide), which was photopolymerized at room temperature for 20-30 min.

After polymerization, the water layer was removed and the surface rinsed with stacking gel. The stacking gel (5% acrylamide) was added to a height of 3 mm, and the gels were photopolymerized at room temperature for 20-30 min. The sample (50-100 µl) was applied as previously described.

Electrophoretic conditions. The electrophoresis was carried out at room temperature using a current of

2.0-2.5 ma per tube for 2-1/2 to 3 hours, or until the dye band had migrated 75-80% of the gel length.

Staining, destaining and quantitation of protein bands. Staining and destaining took place as previously described using both the amido black and coomassie blue stains. The position and intensity of the protein bands were recorded as described previously.

Sodium dodecyl sulfate gel electrophoresis

The proteins of the sarcoplasmic and myofibrillar extracts were separated using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) according to the method of Weber and Osborn (1969). The sarcoplasmic extract was electrophoresed using both concentrations of cross linking (0.135 or 0.270%), while the myofibrillar extract was electrophoresed using only 0.135% cross linking.

Sample preparation. Sarcoplasmic extract (0.5 ml) was incubated at 37°C for 2 hours in 2 ml of 0.01 M sodium phosphate buffer (pH 7.0)-1% SDS-1% β-mercaptoethanol solution. Myofibrillar extract (0.5 ml) was incubated in the same buffer except it also contained 8 M deionized urea. After incubation, the myofibrillar mixture was dialyzed overnight at room temperature against dialysis buffer (0.01 M sodium phosphate buffer, pH 7.0-0.1% SDS-0.1% β-mercaptoethanol solution).

Gel preparation. The gels (10% acrylamide, 0.27% or 0.135% cross linking) were prepared using 15 ml gel buffer (7.8 g $NaH_2PO_4 \cdot H_2O$, 38.6 g $Na_2HPO_4 \cdot 7\ H_2O$, 2 g SDS/1), 13.5 ml acrylamide solution (22.2 g acrylamide, 0.6 g or 0.3 g BIS/100 ml), 1.5 ml freshly made ammonium persulfate (15 mg/ml) and 0.045 ml of TEMED. After mixing, the solution was added to the glass tubes to a height of 10-12 cm and allowed to polymerize at room temperature (20-30 min).

Sample application. The sample was prepared for each gel by mixing the following in a small test tube: 3 µl tracking dye (0.05% bromophenol blue in water), 1 drop glycerol, 5 µl β-mercaptoethanol, 50 µl dialysis buffer and 50 µl of sarcoplasmic protein sample (containing approximately 0.01-0.05 mg protein). For the myofibrillar protein sample, all conditions were the same except no dialysis buffer was used and 100 µl of dialyzed sample was applied. The contents of each tube were transferred to the corresponding gel, and gel buffer, diluted 1:1 with water, was layered on top of the sample.

Electrophoretic conditions. The upper and lower chambers of the apparatus were filled with gel buffer, diluted 1:1. Electrophoresis was performed at a constant current of 8 ma per tube at room temperature for 4-5 hours or until the dye band migrated 75% of the gel length. The gels were removed from the tubes and the position of the

dye band marked with a fine wire as previously described.

Staining and destaining. The gels were stained using a solution of 1.25 g coomassie blue in 454 ml 50% methanol and 46 ml glacial acetic acid for 2-16 hours. The gels were destained by diffusion in 7.5% acetic acid-5% methanol as described earlier herein.

Quantitation and characterization of protein bands. After destaining, the position and intensity of the bands were recorded as described previously. R_M was calculated by dividing the distance the protein bands migrated from the top of the gel by the distance the marker dye migrated. The area under the peaks was determined by triangulation, and the quantity of protein was expressed as a percentage of all the protein that entered the gel.

A standard curve for determining molecular weight was prepared using the following proteins: myoglobin (17,800 daltons), chymotrypsinogen (25,700 daltons), pepsin (35,000 daltons), ovalbumin (43,000 daltons), bovine serum albumin (68,000 daltons) and phosphorylase a (94,000 daltons).

Isoelectric focusing

Isoelectric focusing in polyacrylamide gels was performed on the sarcoplasmic and myofibrillar extracts using a modification of the method described by Wrigley (1971).

Sample preparation. The sarcoplasmic extract was used directly with no further treatment. The myofibrillar extract was dialyzed overnight against 10 M deionized urea prior to use.

Gel preparation. Gel solution (7.5% acrylamide) was prepared by combining 6.5 ml H_2O, 1.5 ml sarcoplasmic extract, 3.0 ml acrylamide solution (30 g acrylamide, 1 g BIS/100 ml), 0.3 ml ampholine-pH 3.5-10.0 (40% w/v, LKB Instruments, Inc., Rockville, Maryland) and 0.7 ml of freshly prepared ammonium persulfate (1%, w/v). This amount was sufficient for 4 tubes (coated) filled to a height of 12 cm. A few drops of water were layered on the surface and the gels were allowed to polymerize at 4°C.

Gels for focusing the myofibrillar extract (5% acrylamide) were prepared using 2 ml acrylamide solution, 1 ml dialyzed myofibrillar extract, 8 ml deionized 10 M urea, 0.3 ml ampholine-pH 3.5-10.0, and 0.7 ml ammonium persulfate.

Electrofocusing conditions. The lower reservoir of the electrophoresis chamber was filled with 0.4% (v/v) ethylene diamine (cathode) and the upper reservoir was filled with 0.2% (v/v) sulfuric acid (anode). The voltage was adjusted to 400 volts, which resulted in an initial current of 75-90 ma for 12 tubes. Focusing continued 1.5 hours for the sarcoplasmic extract or 2 hours for the myofibrillar extract.

Determination of the pH gradient. One gel from each sample group was used to determine the pH gradient. The gel was divided into 11 or 12 even sections and each was allowed to sit in 3 ml of distilled, deionized water overnight. The pH of the solutions was measured using a Radiometer pH meter, PHM26 (Radiometer A/S, Copenhagen, Denmark).

Staining and destaining. The remaining gels were placed in 12.5% TCA to precipitate the protein bands, then washed in several changes of 5% TCA-5% sulfosalicylic acid. The gels were heated to 60°C in staining solution (0.1% coomassie brilliant blue-R in 150 ml methanol-372 ml H_2O-60 g TCA-18 g sulfosalicylic acid solution), and held at that temperature for 1 to 2 hours. The gels were destained by diffusion using 11% acetic acid in 36% ethanol.

Characterization of protein bands. After destaining, the position of the protein bands was recorded using the Kontes densitometer. The pH gradient was superimposed upon the densitometric tracing to determine the isoelectric point(s) of the protein band(s).

Electron Microscopy

Samples of control, C. perfringens-inoculated and enzyme-treated muscle were removed for electron microscopic examination after 1, 4 and 7 days of incubation.

Fixation and embedding

Fixation was accomplished using a modification of the procedure described by Sjöstrand (1967). Small pieces of muscle were fixed for 2 hours in a buffer solution containing 2% paraformaldehyde-1% gluteraldehyde-0.048 M sodium phosphate-0.043 M NaCl at pH 7.4. The muscle samples were then washed for 30 min in 2 changes of 0.094 M scdium phosphate-0.086 M NaCl buffer solution at pH 7.4. Then the muscle samples were fixed for 1 hour in 1% osmium tetroxide solution in veronal acetate buffer, pH 7.4, which was adjusted to 0.3 osM with NaCl, KCl and $CaCl_2$ according to the method of Sjöstrand (1967).

After fixation, the samples were dehydrated for 10 min each in 25, 50, 75 and 95% ethanol. They were then placed in 2 changes of 100% ethanol for 15 min. The dehydrated muscle samples were transferred to propylene oxide (2 changes of 30 min each) followed by 12 hours in a 1:1 mixture of propylene oxide and epon. The samples were then embedded in pure epon using flat embedding molds (LKB Instruments, Inc.). The embedded samples were placed in a dessicator under a slight vacuum for 12 hours, then put into a 60°C oven for 36 hours to allow the blocks to harden.

Sectioning and staining

Epon embedded tissue blocks were trimmed by hand with a razor blade. The muscle samples were then

sectioned on a diamond knife to a thickness of 6 to 8 μm using a LKB ultramicrotome. Sections were picked up from the knife boat on uncoated 300-mesh copper grids.

Staining of the tissue sections was accomplished by floating the grids for 30 min on a saturated solution of uranyl acetate, rinsing thoroughly with distilled water, then staining for 5 min in a solution of lead citrate (Reynolds, 1963). The sections were then washed with 0.02 M NaOH, followed by distilled water, and then 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 on Kodak Estar thick Base-70 mm film. The film was developed for 3 min in a Kodak D-19 developer, washed for 30 sec in running water, fixed in a Kodak fixer for 4 min, washed for 30 min in running water, washed in distilled water for 2 min and then dried.

The negatives were printed on Ilford Ilfoprint rapid stabilization paper using a Durst S-45-EM enlarger. Then the prints were developed in an Ilford model 1501 rapid stabilization processor. Selected prints were fixed in Kodak fixer, washed and dried on a ferrotype dryer. The photographs of the control samples and of the samples inoculated with C. perfringens or treated with the isolated enzyme(s) were examined and compared.

RESULTS AND DISCUSSION

Determination of Culturing Conditions

Selection of the organism

Both strains of C. perfringens (ATCC 13124 and ATCC 12915) exhibited considerable growth in amino acid medium. However, only C. perfringens ATCC 13124 exhibited measurable proteolytic activity (Figure 4). Further work using a single sampling time confirmed the absence of measurable proteolytic enzyme activity from cultures of C. perfringens ATCC 12915, leading to selection of C. perfringens ATCC 13124 as the test organism.

Time requirement for enzyme production

As shown in Figure 4, enzyme activity increased during the logarithmic growth phase, with a leveling off of activity followed by a slight decline during the stationary phase. In order to obtain maximum activity, it was necessary to harvest the cells in the early part of the stationary phase, before activity declined. However, practical considerations necessitated a culturing time of 16 to 18 hours, which was within the range of adequate activity, although not necessarily at the maximum.

Selection of the medium

The growth of C. perfringens ATCC 13124 and corresponding enzymatic activity in peptone medium is shown in Figure 5. Growth and activity curves are similar to those for the same strain grown on amino acid medium (Figure 4). However, enzyme activity was produced in greater quantities in the peptone medium, with the level of activity being 1.4 to 3.3 times greater than that produced in the amino acid medium.

The specific activity of the culture filtrate from amino acid medium was at least 100 fold higher than that from peptone medium, because the amino acid medium contained no protein. After storage at -20°C, however, the enzyme preparation produced in amino acid medium retained only 20% of its original activity, while that prepared from the peptone medium retained 95% of its activity. It is postulated that the presence of protein contaminants from the peptone medium resulted in a lower level of autodigestion, and thus, a higher retention of activity. The greater stability of the enzyme prepared from the peptone medium in conjunction with the relative expense and difficulty of preparation of the amino acid medium, led to the choice of the peptone medium for the majority of this study.

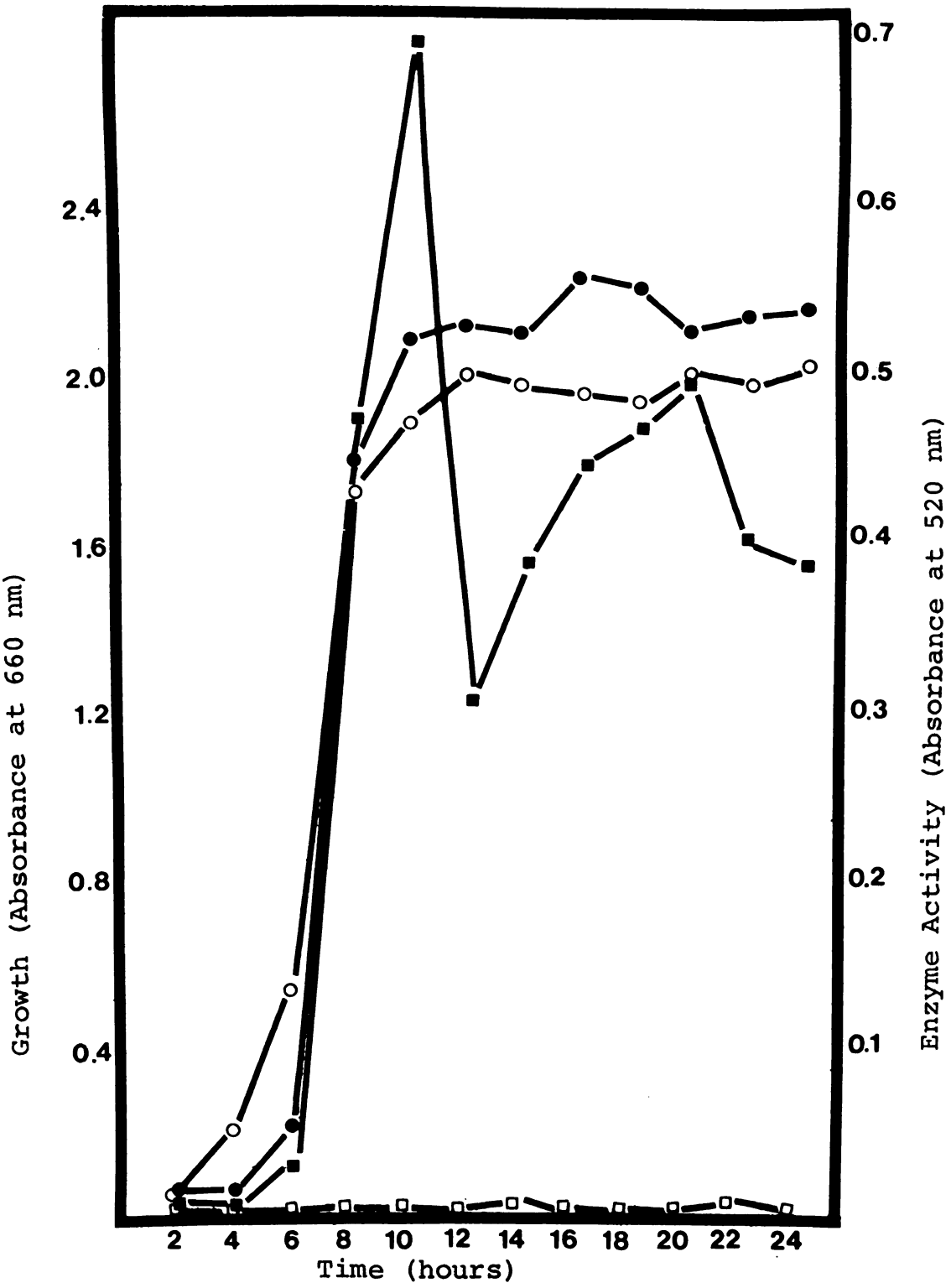

Figure 4. Growth and enzyme production by C. perfringens ATCC 13124 and 12915 in amino acid medium.

● ● ● growth of C. perfringens ATCC 13124
○ ○ ○ growth of C. perfringens ATCC 12915
■ ■ ■ enzyme activity from C. perfringens ATCC 13124
□ □ □ enzyme activity from C. perfringens ATCC 12915

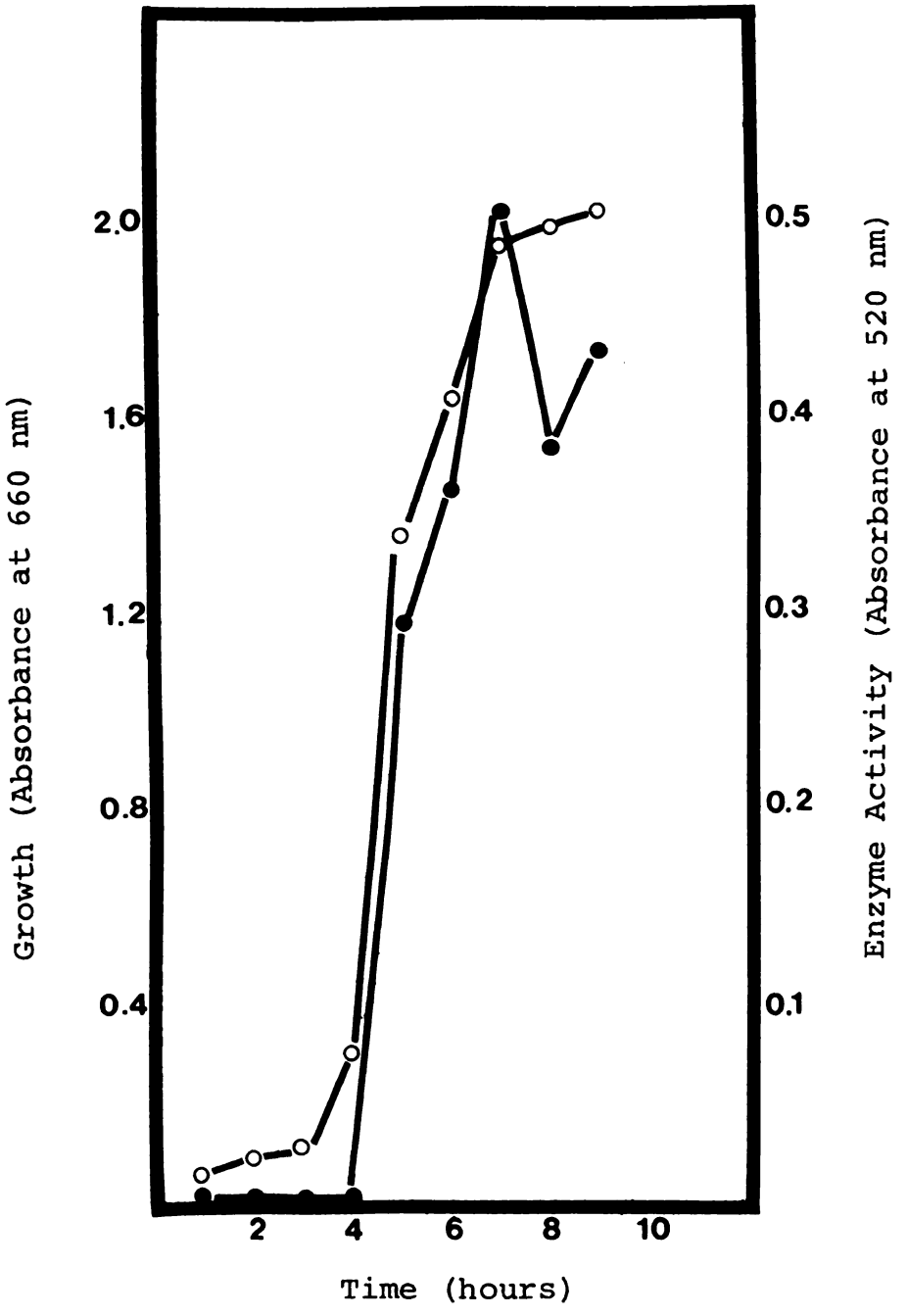

Figure 5. Growth and enzyme production by C. perfringens ATCC 13124 in peptone medium.

o o o growth of C. perfringens ATCC 13124
● ● ● enzyme activity from C. perfringens ATCC 13124

Determination of Assay Conditions

The azocoll assay was initially used with an incubation time of 1 hour. This was later reduced to 15 min after experimentation indicated the reaction rate was non-linear at longer incubation times (Figure 6).

As the enzyme concentration increased, the increase in absorbance of the solution was not linear. At absorbance values above 0.6, the reproducibility of the assay declined. Thus, the level of enzyme in the assay was adjusted to give absorbance values below 0.6.

Enzyme Purification

A typical stepwise purification is shown in Table 1. The overall recovery of enzyme was 11.2% with a 158 fold final purification. The final specific activity was 79 units/mg protein as compared to an initial specific activity of 0.498 units/mg protein. The purification in the final step (Bio-Gel P-200, Table 1) was lower than usual because of a malfunction of the fraction collector. In previous trials, the purification on Bio-Gel P-200 was as high as 3.7 fold, with a final specific activity of 224 units/mg protein. The latter preparation was used in all enzyme studies on muscle.

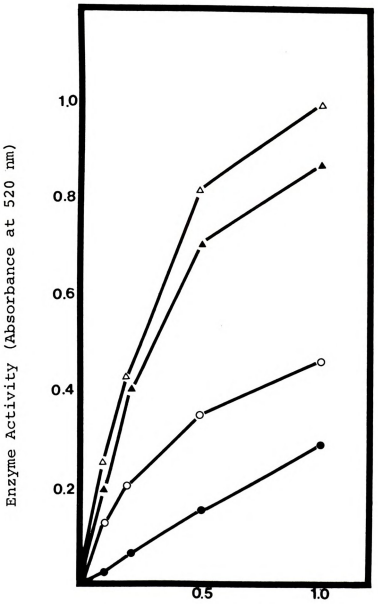

Figure 6. Effect of substrate concentration upon the azocoll reaction at different incubation times.

● ● ● 15 min incubation
○ ○ ○ 30 min incubation
▲ ▲ ▲ 45 min incubation
△ △ △ 60 min incubation

Table 1

Purification Scheme for a Protease Produced by Clostridium Perfringens ATCC 13124

PROCEDURE	FINAL VOLUME (ml)	ACTIVITY* (units/ml)	TOTAL ACTIVITY (units)	PROTEIN (mg/ml)	SPECIFIC ACTIVITY (units/mg protein)	YIELD (% protein)	ENZYME RECOVERY (% activity)	PURIFICATION (fold)
1. Culture filtrate	1815	2.42	4390	4.86	0.498	100	100	0
2. Zn Cl_2 precipitation	412	4.4	1812	1.32	3.33	6.2	41.3	6.7
3. Ammonium sulfate precipitation	59	21.9	1292	3.30	6.57	2.2	29.4	13.2
4. Bio-Gel P-100 fractionation	141	10.82	1526	0.45	24.1	0.7	34.8	48.4
5. DEAE-cellulose fractionation	135	9.75	1316	0.22	44.3	0.3	30.0	89.0
6. Bio-Gel P-200 fractionation	129	3.95	510	0.05	79.0	0.07	11.6	159

* 1 unit of enzyme activity is defined as that amount of enzyme required to raise the absorbance by 0.1 in 15 min.

Bio-Gel P-100 fractionation

A typical elution pattern from the Bio-Gel P-100 column is shown in Figure 7. The fractions of highest activity, tubes 12-16, were pooled as indicated by the shaded area and were used in subsequent steps. The pooled fraction was purified 3.66 fold with a 118% recovery of the enzyme in this step. The specific activity had increased from 6.57 to 24.1 units/mg protein.

The majority of the enzyme activity was eluted just after the major protein peak, but prior to the diffuse low molecular weight proteins. This indicated that the enzyme has a molecular weight in the general range of 80,000 to 113,000 daltons, which is in agreement with values of 80,000 and 113,000 reported by Kameyama and Akama (1971) and Levdikova (1966), respectively.

DEAE-cellulose fractionation

A typical DEAE-cellulose elution pattern using a stepwise increase in salt concentration is shown in Figure 8. Fractions exhibiting the highest levels of activity, tubes 66-69, were pooled as shown by the shaded area and used for further purification. Chromatography on DEAE-cellulose resulted in a 1.84 fold purification with a recovery in this step of 83.5%. The specific activity increased from 24.1 to 44.3 units/mg protein.

The gradient salt elution pattern from DEAE-cellulose is shown in Figure 9. The standard curve for

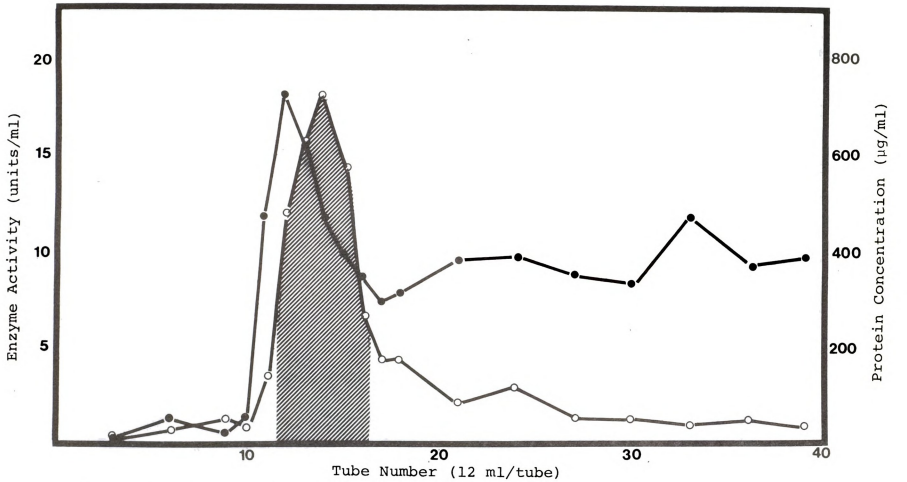

Figure 7. Bio-Gel P-100 fractionation.

● ● ● protein concentration
o o o enzyme activity
▨ pooled fractions 12-16 used in subsequent steps

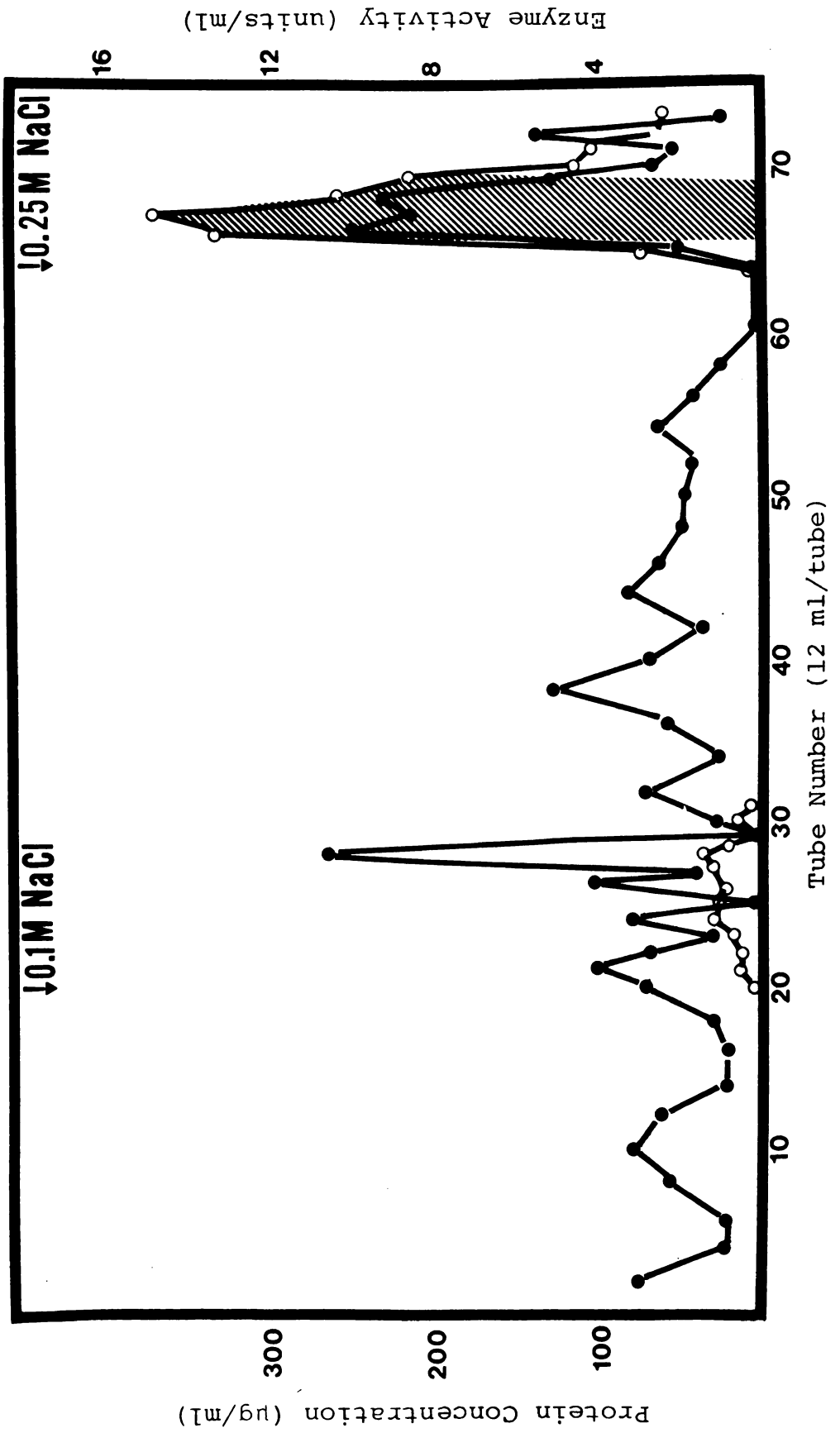

Figure 8. Stepwise DEAE-cellulose fractionation.

● ● ● protein concentration
○ ○ ○ enzyme activity
↓ change in concentration of eluent
▨ pooled fractions 66-69 used in subsequent steps

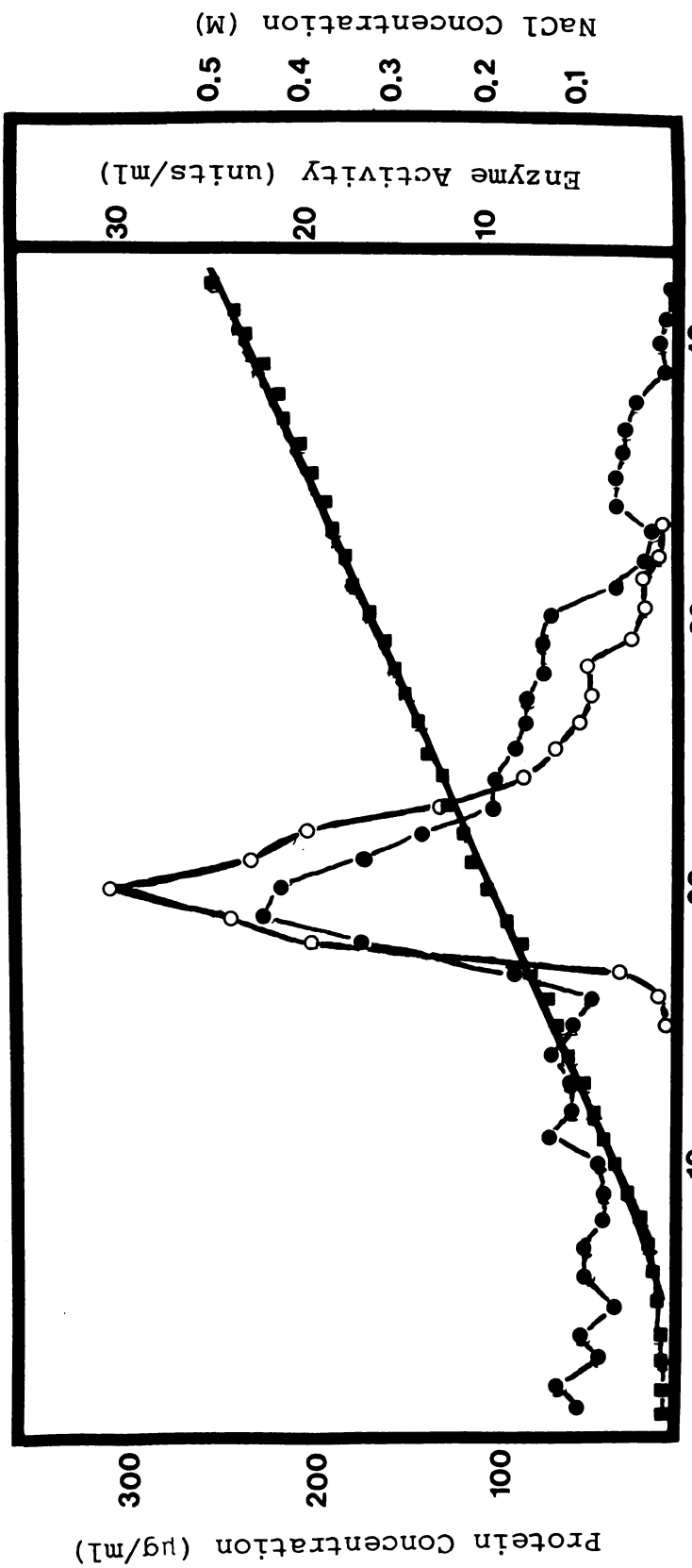

Figure 9. DEAE-cellulose gradient fractionation.

● ● ● protein concentration
○ ○ ○ enzyme activity
■ ■ ■ NaCl concentration

NaCl concentration is shown in Appendix Figure 1. The majority of the enzymatic activity was eluted between 0.15 M and 0.23 M NaCl, which corresponds closely to the major protein peak. The specific activity of the preparation adsorbed on the column was 62 units/mg protein. The specific activity of eluate fractions 17-23 had increased to 114 units/mg protein, giving a 1.8 fold purification.

Stepwise elution was used in the majority of this investigation because the abrupt change to 0.25 M NaCl concentrated most of the enzyme, which was eluted as a sharp peak.

Bio-Gel P-200 fractionation

A typical elution pattern from Bio-Gel P-200 is shown in Figure 10. While some activity was eluted with the initial protein, the majority of the activity came off the column with the second protein peak. Fractions 6, 7 and 8 were combined as indicated by the shaded area and used in the muscle incubation studies. The preparation placed on the column had a specific activity of 59.7 units/mg protein. The specific activity of the pooled eluate was 224 units/mg protein, giving a 3.7 fold purification.

CM-cellulose fractionation

Figure 11 shows the elution pattern from CM-cellulose. No protein values were reported because the

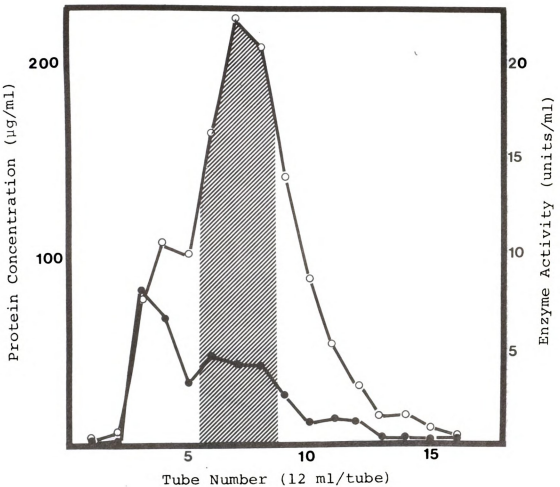

Figure 10. Bio-Gel P-200 fractionation.

● ● ● protein concentration
o o o enzyme activity
▨ pooled fractions 6-8 used in muscle studies

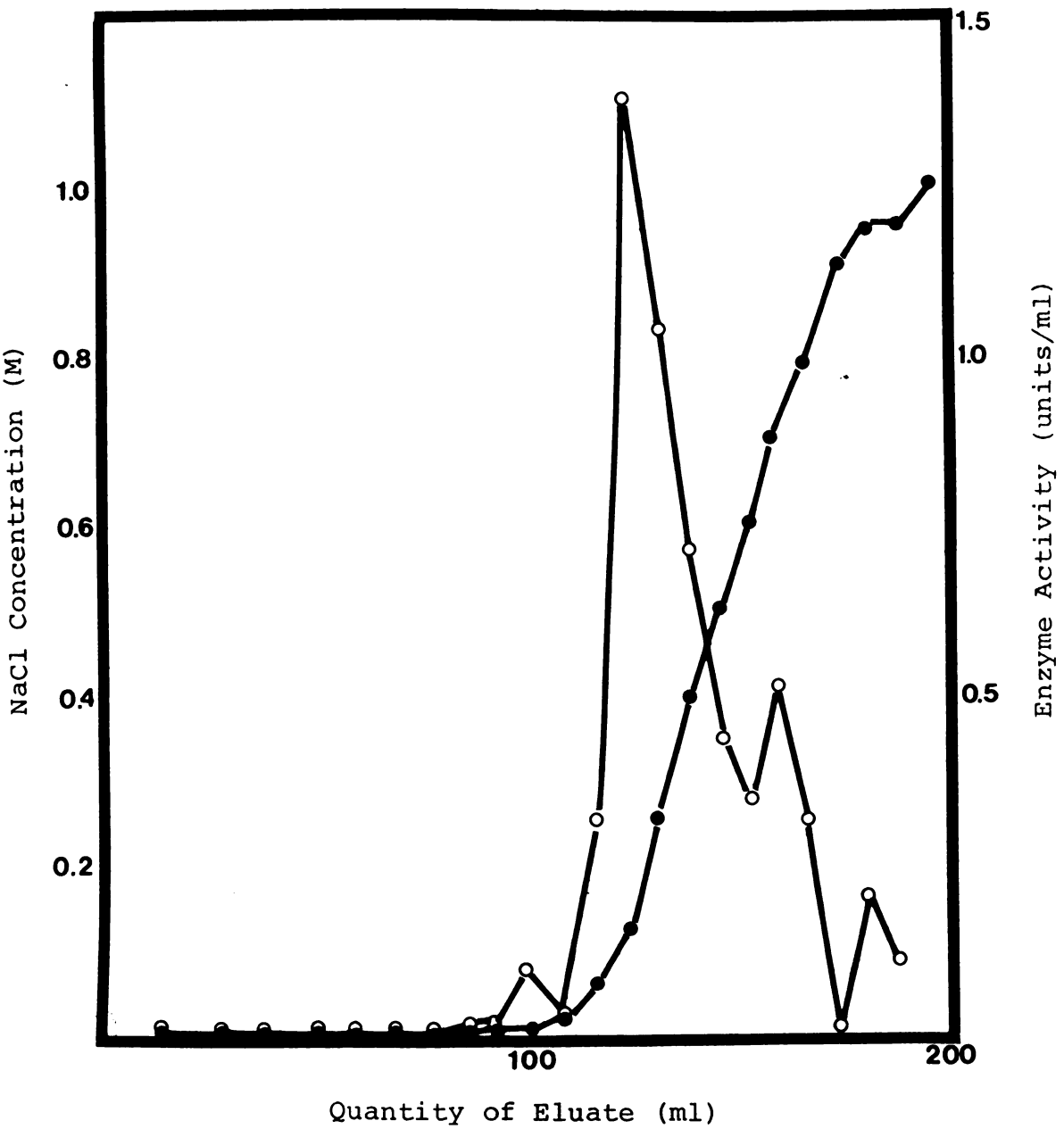

Figure 11. CM-cellulose gradient fractionation.

o o o enzyme activity
● ● ● NaCl concentration

high salt concentration of the eluent interfered with the Lowry protein determination (Lowry et al., 1951). The majority of activity was eluted between 0.05 and 0.5 M NaCl. The specific activity of the sample adsorbed on the column was 172 units/mg protein, while the specific activity of the active fractions of the eluate declined to 92 units/mg protein. Since 64.3% of the activity was lost by holding the enzyme preparation at pH 5.5, which was the pH of CM-cellulose elution, the reduction in specific activity during elution can be readily explained. Therefore, this step was not incorporated into the final enzyme purification scheme.

Disc electrophoresis of enzyme fractions

Disc electrophoresis using 7% acrylamide gels was performed on the fractions from each of the six major steps utilized in purification. Densitometric tracings of these gels are shown in Figures 12-17. Relative intensity of the bands was expressed as a percentage of the total protein applied. This method of expressing the data was used to minimize errors from applying different quantities of protein and from day to day variations in the dye binding (Fazekis de St. Groth et al., 1962).

Upon disc gel electrophoresis, the culture filtrate (Figure 12) separated into 8 peaks with the majority (73.6%) of the protein occurring in the overlapping peaks at R_M 0.33 - 0.46.

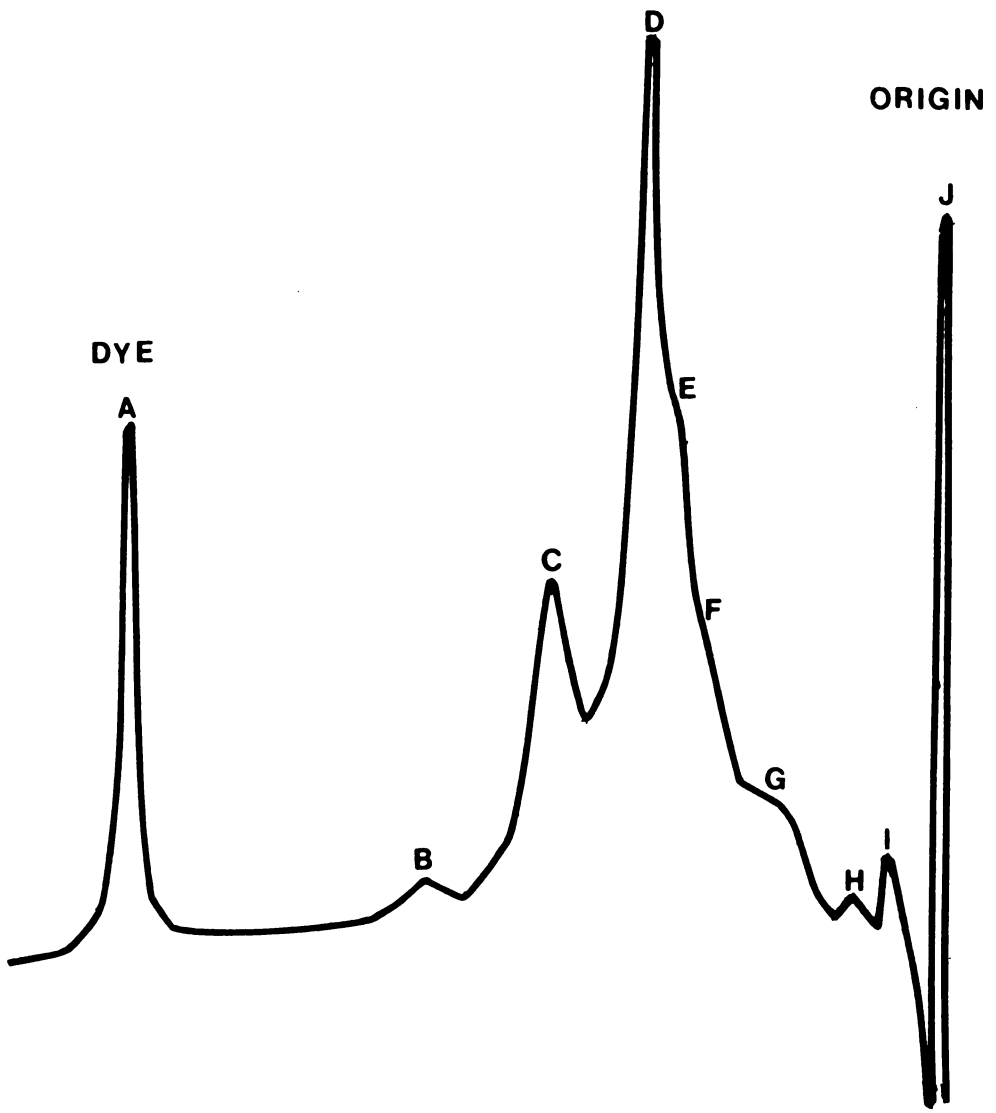

Figure 12. Disc gel electrophoresis pattern of culture filtrate.

Peak	A	B	C	D	E	F	G	H	I	J
R_M	1.0	0.62	0.46	0.36	0.33	0.29	0.20	0.09	0.05	0.0
% protein	- -	6.4	23.1	30.0	20.5	4.7	14.6	0.3	0.04	- -

Figure 13 shows the disc gel electrophoresis pattern of the resolubilized zinc chloride precipitate. The 2 major peaks (comprising 60.4% of the protein) migrated at R_M 0.38 and 0.53, with 4 other peaks comprising the remainder.

The resolubilized ammonium sulfate precipitate (Figure 14) had 2 major peaks (R_M 0.37 and 0.48) containing 68% of the total protein upon disc gel electrophoresis. The remaining protein was distributed between 5 other peaks.

Figure 15 shows the disc gel electrophoresis pattern of the pooled eluate from Bio-Gel P-100. Peaks with R_M 0.39 and 0.43 contained 52% of the protein, while 8 smaller peaks contained the remainder of the protein.

The pooled eluate from DEAE-cellulose, when subjected to disc gel electrophoresis (Figure 16), separated into 6 peaks. Two of these, R_M 0.37 and 0.47, contained 60% of the protein, while a third peak at R_M 0.34 contained 20% of the protein. The remaining protein was found in three other peaks.

Upon disc gel electrophoresis, the purified enzyme preparation separated into 5 peaks (Figure 17). The peak with R_M 0.38 contained 34% of the protein, that with R_M 0.46, 25%, and that with R_M 0.33, 20%. The two remaining peaks, R_M 0.17 and 0.56 contained 9 and 12%, respectively. The presence of multiple peaks indicated that the enzyme was not completely purified.

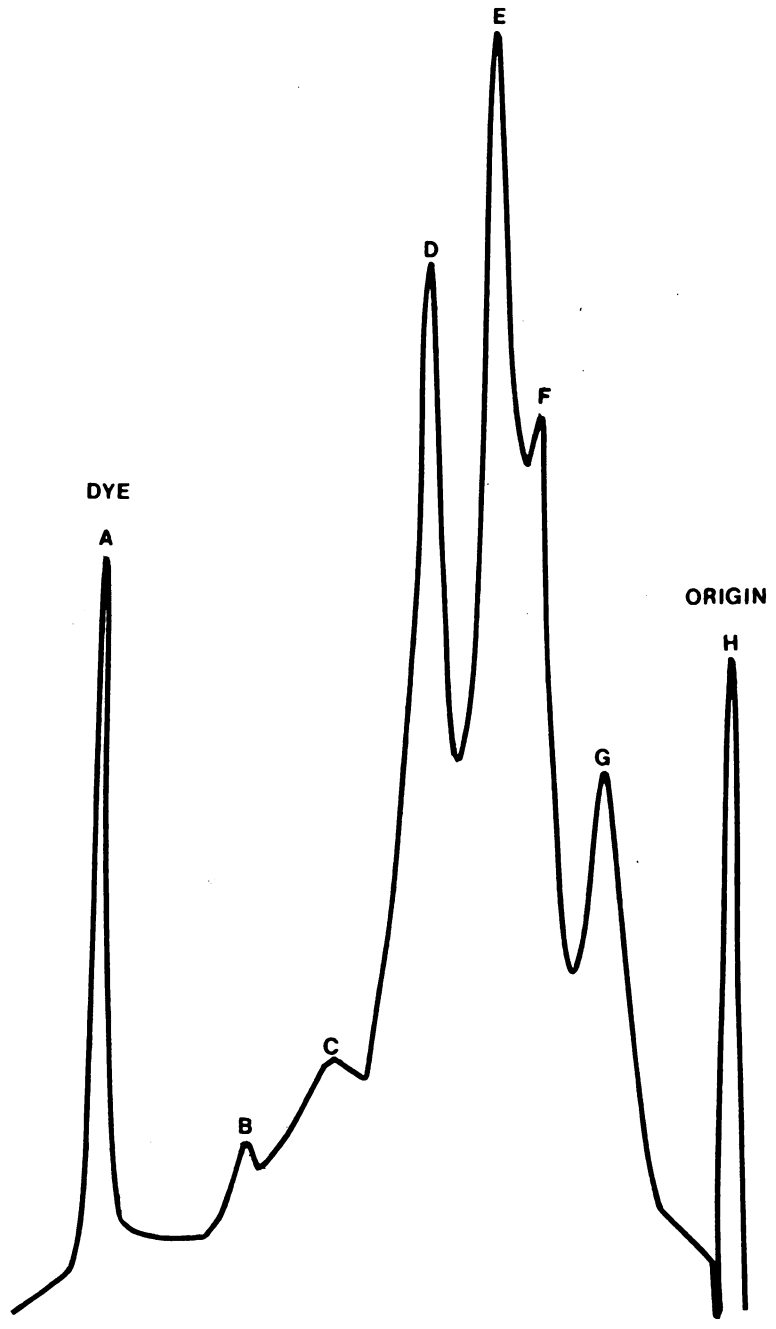

Figure 13. Disc gel electrophoresis pattern of zinc chloride precipitate.

Peak	A	B	C	D	E	F	G	H
R_M	1.0	0.76	0.63	0.53	0.38	0.30	0.18	0.0
% protein	---	0.7	7.7	27.8	32.6	16.5	14.7	---

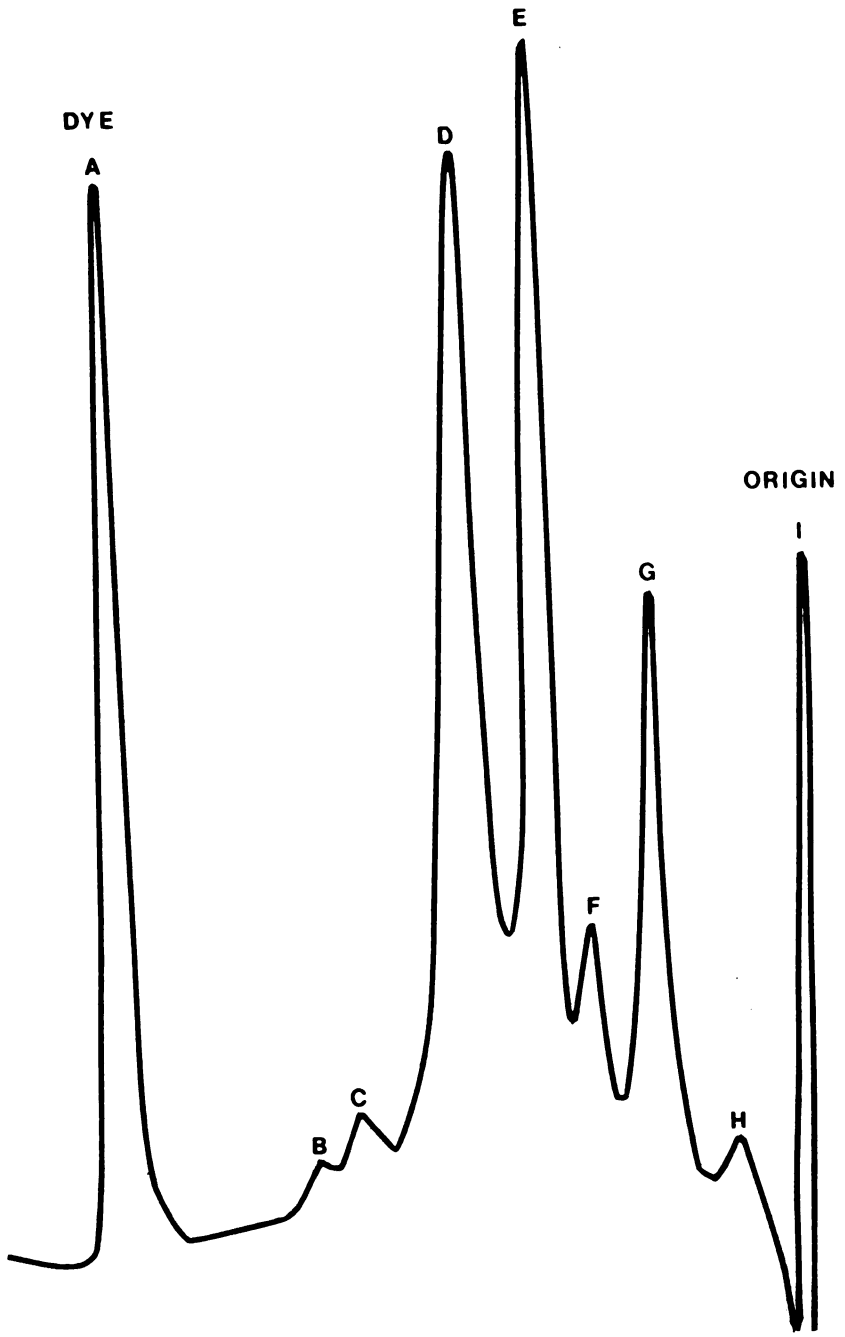

Figure 14. Disc gel electrophoresis pattern of ammonium sulfate precipitate.

Peak	A	B	C	D	E	F	G	H	I
R_M	1.0	0.70	0.64	0.48	0.37	0.29	0.18	0.06	0.0
% protein	---	1.5	6.4	36.1	31.9	8.2	13.5	2.4	---

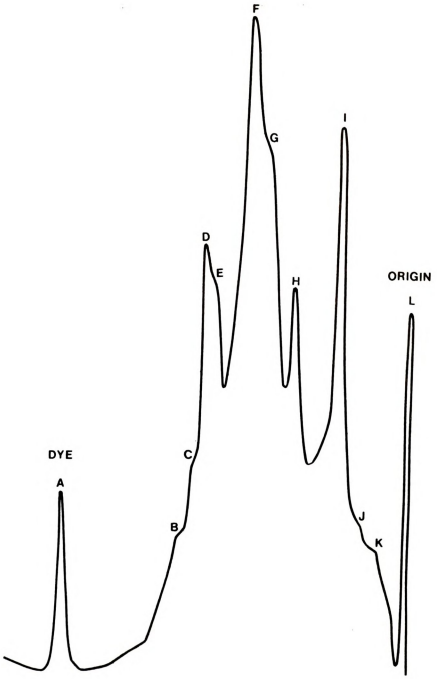

Figure 15. Disc gel electrophoresis pattern of Bio-Gel P-100 fraction.

Peak	A	B	C	D	E	F	G	H	I	J	K	L
R_M	1.0	0.66	0.62	0.58	0.56	0.43	0.39	0.30	0.19	0.13	0.07	0.0
% protein	---	3.2	3.8	7.2	9.7	31.1	20.8	10.7	9.4	0.7	3.4	---

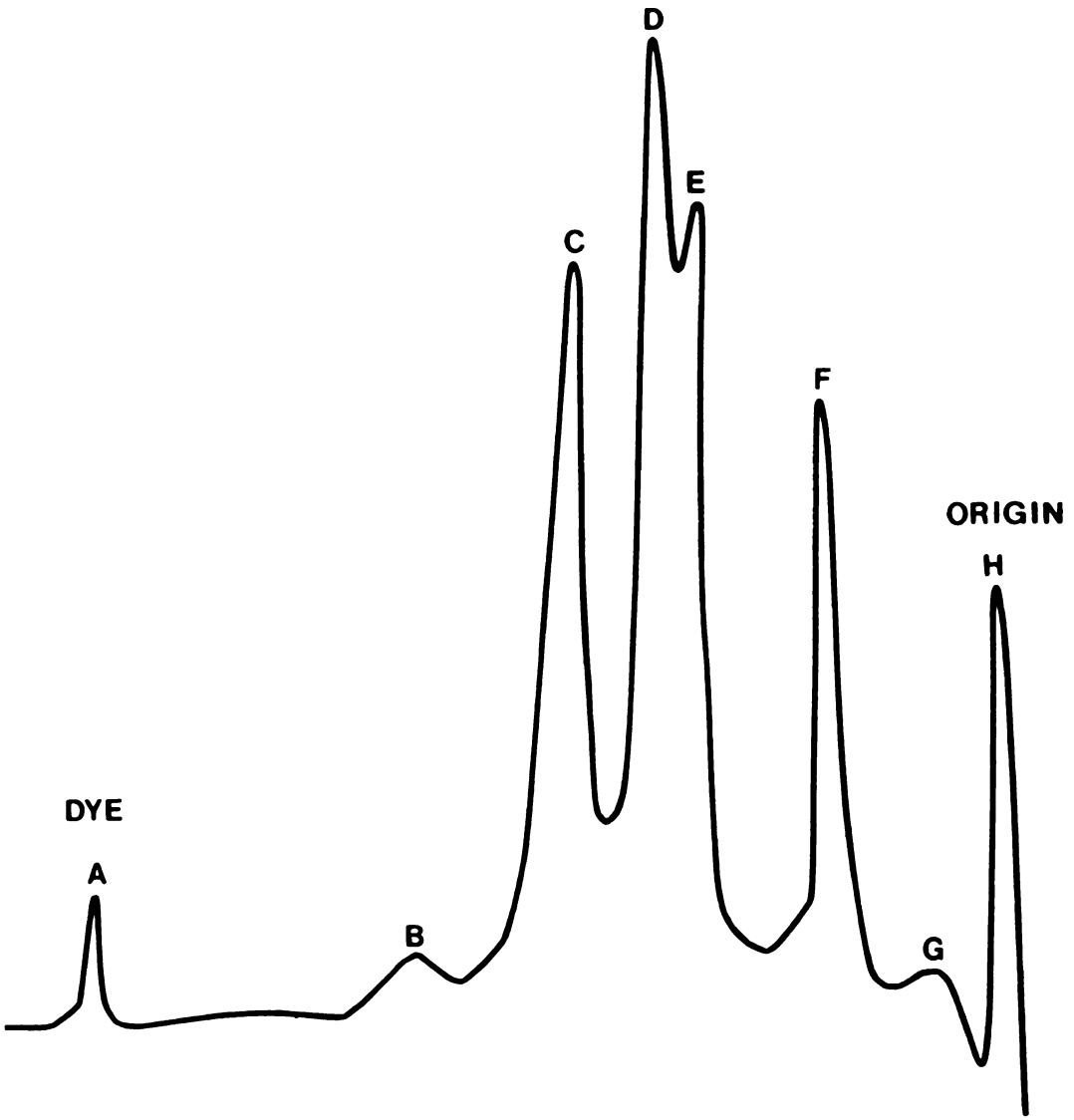

Figure 16. Disc gel electrophoresis pattern of DEAE-cellulose fraction.

Peak	A	B	C	D	E	F	G	H
R_M	1.0	0.62	0.47	0.37	0.34	0.18	0.07	0.0
% protein	---	5.2	32.9	26.9	19.6	11.3	4.2	---

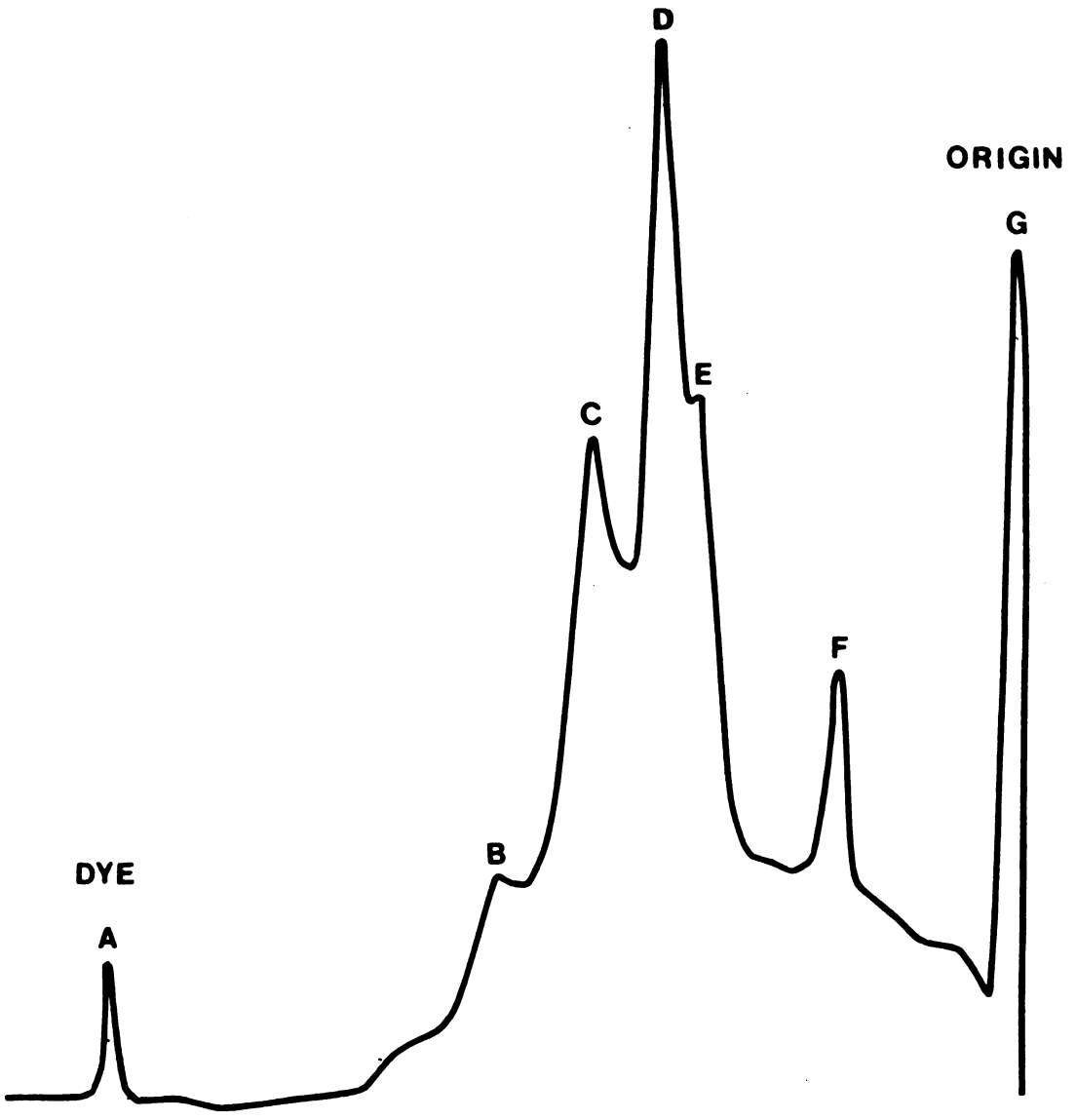

Figure 17. Disc gel electrophoresis pattern of Bio-Gel P-200 fraction.

Peak	A	B	C	D	E	F	G
R_M	1.0	0.56	0.46	0.38	0.33	0.17	0.0
% protein	---	12.0	25.3	34.2	19.8	8.7	---

Attempts to identify the protein band(s) containing the enzyme activity were unsuccessful, as activity was diffusely apparent over all the major bands, indicating either diffusion of the activity prior to or during measurement, or the presence of several active components.

Despite the obvious impurity of this enzyme preparation, it was used in the following studies upon muscle.

Changes in Nitrogen and Protein of Incubated Muscle

Muscle inoculated with C. perfringens ATCC 12915 and the uninoculated control muscle were fractionated into the sarcoplasmic and myofibrillar extracts after incubation at 30°C. The extracts were then analyzed for total nitrogen and protein content.

Measurement of nitrogen by Kjehldahl analysis

The changes seen in the nitrogen content, as measured by Kjehldahl analysis, of the sarcoplasmic, myofibrillar and NPN fractions from the incubated muscle are shown in Figure 18. The standard curve for the Nesslerization reaction is shown in Appendix Figure 2.

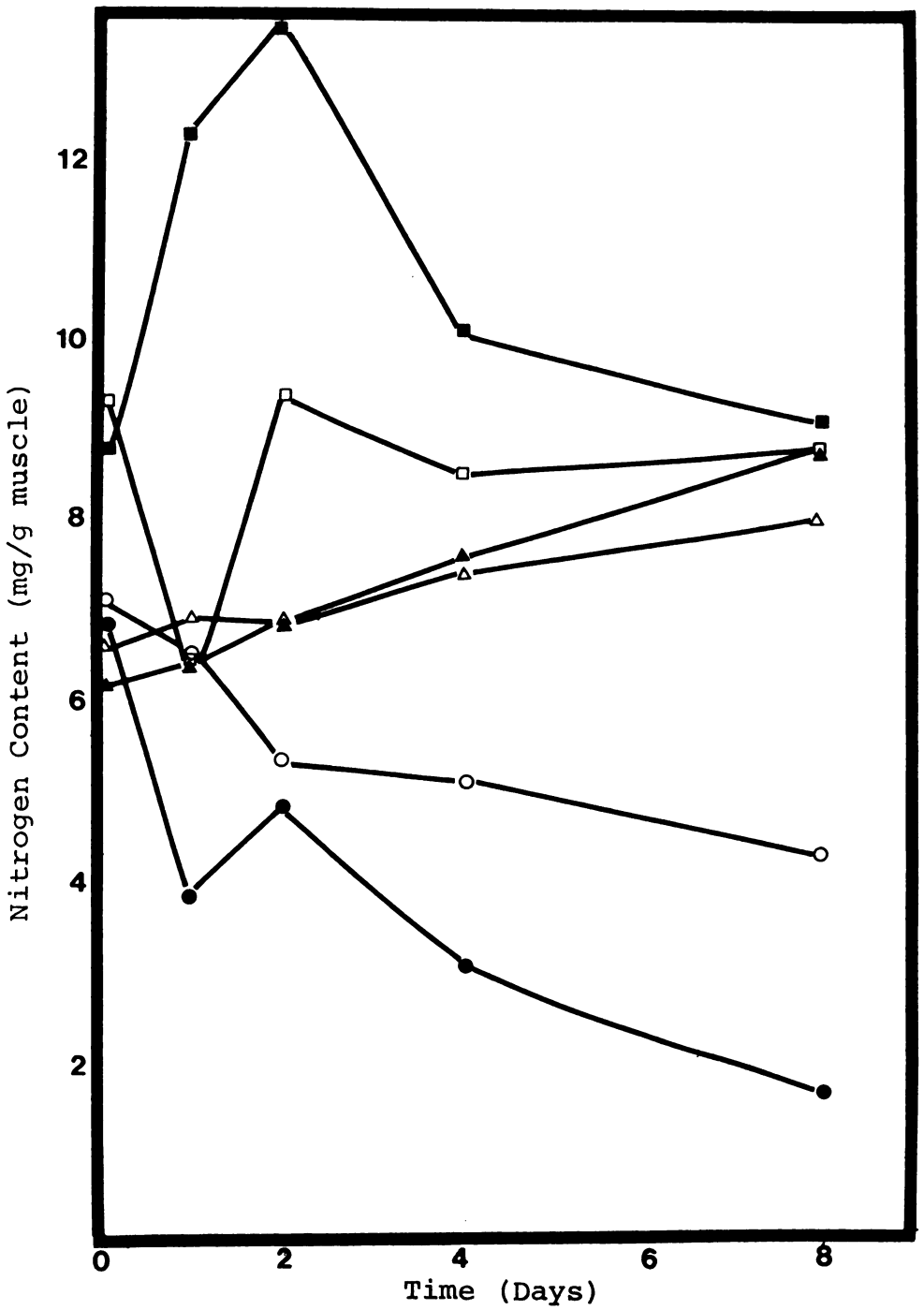

Figure 18. Nitrogen content of fractions from the incubated control and C. perfringens inoculated muscle as measured by Kjehldahl analysis.

○ ○ ○ control sarcoplasmic extract
● ● ● C. perfringens inoculated sarcoplasmic extract
□ □ □ control salt-soluble extract
■ ■ ■ C. perfringens inoculated salt-soluble extract
△ △ △ control NPN
▲ ▲ ▲ C. perfringens inoculated NPN

Changes in the sarcoplasmic extract

The quantity of sarcoplasmic protein showed a decline with incubation in both control and inoculated muscle (Figure 18). Kronman and Winterbottom (1960) reported that aging of muscle resulted in a decreased extractability of water soluble proteins. Some of the decline in nitrogen content was a result of the decrease in extractable protein, since the control sarcoplasmic protein declined from 44.7 to 27.8 mg protein/g muscle. However, the inoculated sample declined considerably more than the control, decreasing from 42.8 to 10.8 mg protein/g muscle. This is indicative of proteolysis by C. perfringens.

Changes in the myofibrillar extract

The myofibrillar (salt-soluble) proteins from inoculated and incubated muscle exhibited an increase in nitrogen content during the first 2 days of incubation and then declined to a point at or near the initial level as incubation was continued (Figure 18). Because Kjehldahl determinations measure total nitrogen only, the source of nitrogen cannot be determined by that method alone. Thus, the increase seen in salt-soluble nitrogen may have come from either the sarcoplasmic fraction or the stromal proteins.

The myofibrillar protein was obtained by subtracting the water-soluble extract from the salt-soluble

extract, while the remaining insoluble protein was assumed to be the stromal protein.

Bendall and Wismer-Pedersen (1962) reported that holding muscle at elevated temperatures resulted in an increase in the protein content of the myofilaments. They postulated that this gain in protein was caused by a layer of sarcoplasmic protein being firmly bound to the surface of the myofilaments.

McClain et al. (1965) reported the presence of both acid-soluble and salt-soluble collagen in bovine muscle. They reported an increase in the acid-soluble fraction and a decrease in the salt-soluble components during post-mortem aging. This supports the premise that not all collagen is insoluble, but varying portions may occur in the salt-soluble extract.

The changes in salt-soluble nitrogen in the inoculated, incubated samples may be indicative of proteolysis of stromal, sarcoplasmic or myofibrillar proteins, as these cannot be differentiated using simple nitrogen measurements. Another factor, which must be considered, is the microbial protein produced. This protein cannot be differentiated from muscle proteins per se, which unquestionably explains some of the discrepancies in the various protein classes.

Changes in non-protein nitrogen

During incubation there was a consistent increase in the amount of NPN in both control and inoculated samples (Figure 18). The control sample increased from 6.63 to 8.03 mg N/g muscle, while the inoculated sample increased from 6.20 to 8.77 mg N/g muscle. Sharp (1963) found a similar increase in NPN from aseptic rabbit and bovine muscle stored at 37°C, which he stated was derived from the autolysis of sarcoplasmic proteins. Tarrant et al. (1971) also observed an increase in NPN upon incubation of muscle with P. fragi. The fact that bacterial growth and the increase in NPN occurred simultaneously is contrary to the conclusions of Jay and Kontou (1967) and Lerke et al. (1967) who reported that bacteria are incapable of utilizing protein nitrogen.

Measurement of protein by Lowry and biuret methods

Although the absolute values differ, both the Lowry method and the biuret method of measuring protein content show similar trends for the sarcoplasmic and salt-soluble fractions of muscle. The standard curves used for measurement of protein by the Lowry reaction and the biuret method are shown in Appendix Figures 3 and 4, respectively.

Electrophoretic Analysis of Sarcoplasmic Proteins

After incubation at 37°C, the sarcoplasmic extracts from uninoculated muscle or from muscle inoculated with either C. perfringens ATCC 13124, with purified enzyme solution, or with tris-HCl-$CaCl_2$ buffer solution were subjected to electrophoretic analysis.

Disc gel electrophoresis

Disc gel electrophoresis was performed using two concentrations of acrylamide. The relative mobilities of the major sarcoplasmic proteins and the areas of the protein peaks are shown in Tables 2 and 3. The results shown in Table 2 were calculated following electrophoresis in 7.5% acrylamide gels and after staining with amido black. Peak areas are expressed as percentages of total protein. Expression of concentrations relative to the total protein removes a source of error caused by variation in the staining conditions and/or in the concentrations of protein applied to the gels (Fazekas de St. Groth et al., 1963). The amount of protein was determined using the method of Lowry et al. (1951). Sarcoplasmic protein was determined by subtracting NPN from the total sarcoplasmic (water-soluble) extract.

Some of the changes in electrophoretic patterns are the result of incubation of the muscle at a relatively high temperature, while other differences resulted from incubation with either the enzyme or with

Table 2

Relative Mobility and Peak Areas of Sarcoplasmic Proteins after Electrophoresis in 7.5% Acrylamide, Stained with Amido Black

TREATMENT	DAYS OF INCUBATION	PEAK AREAS (Percent of Total Protein)**								
		Relative Mobility								TOTAL PROTEIN* mg/g muscle
		0.70	0.55	0.46	0.37	0.30	0.22	0.16	0.07	
Control	0		7	5			43	26	18	92.0
	1		10	7			40	31	12	61.2
	2		15	3			27	47	7	68.8
	4		15	4			15	29	37	56.2
	7		15		26		13	31	14	60.6
Clostridium Perfringens	1		14	6	15		34	15	17	46.4
	2		14	10	16		33	21	5	44.0
	4	15	9	8	22	11	7	29		39.7
	7	15	9	7	19	10	8	31	2	34.6
Control with Ca^{+2}	1		9	5			34	35	16	55.0
	2		12	9	10		20	34	16	42.4
	4		14		8		20	49	9	38.6
Enzyme	1		12	8			32	26	23	49.4
	2		20	4			25	44	8	38.2
	4		19				44	37		34.2

*Protein concentrations were determined by the Lowry method.

**Overall standard deviation = 2.9.

the viable organism. The total protein values given in Table 2 are in general agreement with the values reported earlier (Figure 18). The quantity of extractable protein in the control declined from 92 mg/g to approximately 60 mg/g after 1 day at 37°C and then remained at about this level during further incubation. Bendall and Wismer-Pedersen (1962) reported that increasing the temperature of post-mortem muscle resulted in precipitation of an unextractable layer of sarcoplasmic protein upon the myofibrils.

In the muscle treated with C. perfringens, the quantity of extractable sarcoplasmic protein continued to decline slowly throughout incubation, reaching approximately 35 mg/g after 7 days. A continual decline in extractable sarcoplasmic protein was also evident in the muscle treated with purified enzyme or with Ca^{+2}. The levels were comparable to those found with C. perfringens treatment. The decline in total sarcoplasmic protein must be considered when interpreting changes in individual peaks after electrophoresis.

In the samples inoculated with C. perfringens, two new protein bands at R_M values of 0.30 and 0.70 (Table 2) appeared after 4 days of incubation. The band at R_M 0.70 was quite diffuse, which may be attributed to a series of low molecular weight and/or highly charged species. This band comprised 15% of the total protein. The peak at R_M 0.30 contained 10% of the total protein.

Thus, the incubation of muscle with C. perfringens was responsible for the formation of two new peaks, presumably protein breakdown products, which accounted for about one-fourth of the total extractable protein.

A third peak at R_M 0.37 appeared upon incubation (Table 2). This peak appeared in the control after 7 days of incubation, in the Ca^{+2} containing control after 2 days of incubation, but appeared after only 1 day of incubation with C. perfringens. This indicates that incubation with C. perfringens or the presence of Ca^{+2} in the control accelerated formation of the band.

The peak at R_M 0.46 (Table 2) disappeared in the control after 7 days of incubation, and in the enzyme-treated sample and in the Ca^{+2}-treated control after 4 days of incubation. On the other hand, this peak was present throughout incubation in the sample inoculated with C. perfringens.

In the control, the percentage of the band at R_M 0.22 (Table 2) decreased from 43% at 0 days to 13% after 7 days of incubation. A more rapid decrease was seen in the sample inoculated with C. perfringens, which indicated that only part of the decline can be attributed to autolysis, while the remainder was due to protein degradation by C. perfringens.

The protein with R_M 0.07 (Table 2) exhibited variable behavior. Since this peak represents material at the gel interface, slight variations in the gel

surface could alter this peak, and thus, the differences are of doubtful significance.

Protein concentrations are also reported for peaks with R_M values 0.55 and 0.16. The concentrations of these peaks varied slightly, but inconsistent and minor changes preclude any definite conclusions.

The viable microorganism appeared to have a greater effect upon the sarcoplasmic proteins than the enzyme. The changes observed in the sarcoplasmic proteins prepared from the muscle inoculated with C. perfringens cannot logically be attributed to the isolated enzyme, because the changes in the C. perfringens-treated sample did not parallel those of the enzyme-treated sample.

A factor, which may be involved in the changes occurring in the enzyme-treated sample and the corresponding control with tris-HCl-$CaCl_2$ buffer, is the presence of added Ca^{+2} ($\simeq$ 0.2 mM) in these samples. In several cases, it is apparent that addition of Ca^{+2} ions may be at least partially responsible for alteration of the bands.

The electrophoretic patterns of the sarcoplasmic proteins on 7% acrylamide gels, stained with coomassie blue are shown in Table 3. The differences between the 7% and 7.5% acrylamide gels can be attributed to differences in pore size and the affinity of the various

Table 3

Relative Mobility and Peak Areas of Sarcoplasmic Proteins after Electrophoresis in 7.0% Acrylamide, Stained with Coomassie Blue

TREATMENT	DAYS OF INCUBATION	PEAK AREAS (Percent of Total Protein)**								
		Relative Mobility								TOTAL PROTEIN* mg/g muscle
		0.64	0.50	0.39	0.31	0.28	0.21	0.10	0.06	
Control	0	7	4				41	21	28	92.0
	1	6	2			15	23	26	29	61.2
	2	8	2			30		60		68.8
	4	11	1	2		28		58		56.2
	7	11	1			34		54		60.6
Clostridium perfringens	1	6	3			26	13		53	46.4
	2	9	3			35			53	44.0
	4	8				37			55	39.7
	7	10	2	6	12				72	34.6
Control with Ca^{+2}	1	6	3			13	24	27	28	55.0
	2	5				23		41	31	42.4
	4	12		6		15		68		38.6
Enzyme	1	5	2			10	24	27	31	49.4
	2	7		4		29		35	25	38.2
	4	13		4				84		34.2

*Protein concentrations were determined by the Lowry method.

**Overall standard deviation = 3.6.

sarcoplasmic proteins for the coomassie blue or the amido black dyes.

In the 7% acrylamide gels, adjacent bands, which initially were distinguishable, overlapped and became inseparable after a few days of incubation.

Another notable difference in the 7% gels was the absence of the rapidly migrating peak in the sample inoculated with C. perfringens after 4 and 7 days of incubation. This was probably due to the staining technique, since Fazekas de St. Groth et al. (1963) reported that insulin and small peptides gave inordinately low readings upon staining with coomassie blue. They reported that acid did not fix these substrates and that the dye-substrate complex was soluble in methanol.

The protein band at R_M 0.31 (Table 3) appeared only in the muscle incubated for 7 days with C. perfringens. This peak contained about 10% of the total protein.

Although a small band at R_M 0.39 appeared intermittently in all samples after incubation (Table 3), it never exceeded 6% of the total protein. The peak at R_M 0.50 comprised 4% or less of the total protein. This band decreased or disappeared in all treatments upon subsequent incubation.

Prior to incubation, a band appeared in the control at R_M 0.21 (Table 3), which comprised about 40% of the total protein. Densitometer tracings for all the

samples indicated that incubation for 1 day resulted in splitting of this band into two peaks with R_M values of 0.21 and 0.28. The splitting of the single band into two peaks is borne out by the fact that their combined areas comprised 35-40% of the total protein. After 2 days incubation, these two bands reformed a single diffuse band in all treatments. This diffuse peak disappeared from samples incubated for 7 days with C. perfringens or for 4 days with the enzyme. Disappearance of this protein indicated proteolytic action by both C. perfringens and the isolated enzyme. The fact that the isolated enzyme caused disappearance more rapidly than the organism suggests that the enzyme alone is responsible for breakdown of this protein.

The peaks at R_M 0.10 and R_M 0.06 (Table 3) were differentiable prior to incubation, but they merged after incubation at various time periods. Merger occurred in the control at 2 days, in the enzyme-treated sample and Ca^{+2} containing control at 4 days. However, in the C. perfringens-inoculated sample, the peaks had already combined after only 1 day of incubation.

In the control sample, the relative concentration at different time periods of the peak(s) with R_M values between 0.10 and 0.06 varied from 50 to 60% of the total protein. In the C. perfringens-treated sample, the combined peak reached a maximum of about 70% of the total

protein at 7 days incubation. This peak increased even more reaching 84% in the sample incubated with enzyme for 4 days. The increased proportion of these protein peaks in the samples treated with C. perfringens and the isolated enzyme appear to be due to the action of the enzyme in both samples. This premise is supported by the greater and more rapid increase in this peak in the sample incubated with enzyme than in the sample incubated with C. perfringens.

The peak at R_M 0.64 (Table 3) did not change significantly with any of the treatments. This peak contained 5-13% of the total protein.

Using this method of separation, there was some indication that a portion of the changes occurring as a result of incubation with C. perfringens were caused by the action of the isolated enzyme. In two cases, the differences in densitometric tracings were similar for both the enzyme-treated and C. perfringens-treated samples.

SDS gel electrophoresis

The average molecular weights and the relative concentrations of sarcoplasmic proteins as determined by SDS gel electrophoresis are shown in Tables 4 and 5. Table 4 depicts the separation on gels with 0.27% cross linking while Table 5 shows the separation with 0.135% cross links.

Table 4

Molecular Weight and Peak Areas of Sarcoplasmic Proteins after SDS Gel Electrophoresis with 0.27% Cross Linking

TREATMENT	DAYS OF INCUBATION	PEAK AREAS (Percent of Total Protein)**							TOTAL PROTEIN*
		Average Molecular Weights							
		15,000	31,000	39,000	47,000	57,000	70,000	87,000+	mg/g muscle
Control	0		16	18	32	15	13	7	92.0
	1	5	17	19	25	10	20	4	61.2
	2	6	17	18	21	13	22	3	68.8
	4	6	19	22	21	12	21		56.2
	7	13	17	20	19	11	20		60.6
Clostridium perfringens	1		12	22	30	12	24		46.4
	2		8	37	27	3	25		44.0
	4	8	7	26	38		21		39.7
	7	13	7	30	26		25		34.6
Control with Ca^{+2}	1		17	22	30	13	16	3	55.0
	2	6	9	23	28	14	20		42.4
	4	16	3	26	30		25		38.6
Enzyme	1	4	12	22	28	13	18	3	49.4
	2	13	9	23	24	12	19		38.2
	4	10	5	28	32		25		34.2

*Protein concentrations were determined by the Lowry method.

**Overall standard deviation = 1.9.

With the higher level of cross linking (Table 4) major bands appeared at R_M values corresponding to molecular weights of 15,000, 31,000, 39,000, 47,000, 57,000, 70,000 and 87,000. These points are shown with the standard marker proteins in Figure 19.

The 31,000 dalton peak (Table 4) may correspond to the sarcoplasmic F-protein, identified by Scopes and Penny (1971) as having a molecular weight of 30,500. Porcine sarcoplasmic proteins, which have subunits in the molecular weight range 35,000 to 41,000, may contribute to the peak at molecular weight 39,000 (Table 4). Scopes and Penny (1971) reported that porcine sarcoplasmic proteins with subunit sizes in this weight range were glyceraldehyde phosphate dehydrogenase at 36,000, lactate dehydrogenase at 35,000 and creatine kinase at 41,000.

Scopes and Penny (1971) found phosphoglycerate kinase had a molecular weight of 48,500, which may correspond to the peak with a molecular weight of 47,000 (Table 4). They also reported that porcine phosphoglucose isomerase had a subunit size of 54,000, pyruvate kinase had a subunit size of 57,000 and phosphoglucomutase as having a molecular weight of 63,000. The protein peak with a molecular weight of 57,000 (Table 4) may correspond to one or more of these proteins. Other peaks shown in Table 4 were not identified.

In the control sample, the 31,000 molecular weight peak showed little change during incubation, while the

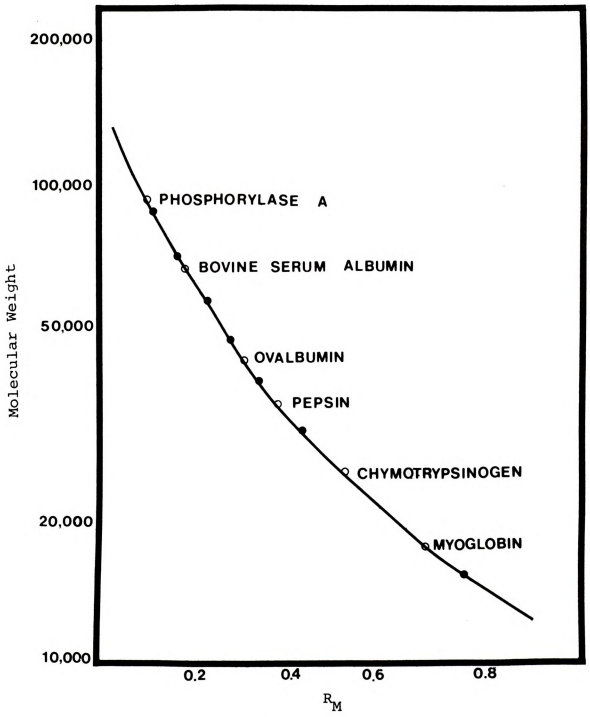

Figure 19. Molecular weights of sarcoplasmic proteins determined by comparison with standard proteins using 0.27% cross-linked SDS gels.

o o o marker proteins
● ● ● sarcoplasmic proteins

peak from the sample incubated with C. perfringens diminished. The relative concentration of this protein was also measurably reduced in both the enzyme-treated sample and the Ca^{+2}-containing control during incubation.

The relative concentration of the 57,000 molecular weight peak in the control sample (Table 4) remained fairly constant, while the sample treated with C. perfringens exhibited a sharp decline, which resulted in complete loss of the peak after 4 days of incubation. This is indicative of breakdown by C. perfringens. The enzyme-treated sample and the control with Ca^{+2} appeared to be quite similar in that the 57,000 dalton peak disappeared from both samples after the fourth day of incubation. It should be reemphasized that added Ca^{+2} ions may account for the difference between the control and the Ca^{+2}-treated control.

The peak with a molecular weight of 87,000 diminished in the control, until the band completely disappeared after 4 days incubation. This peak was not evident in the sample treated with C. perfringens at any time. This suggests that C. perfringens destroyed or altered this protein during the first 24 hours period. In both the control with Ca^{+2} and in the enzyme-treated

sample, this peak was discernable after incubation for 1 day, but disappeared from both samples incubated for longer periods of time.

The peak at a molecular weight of 15,000 was absent in the initial sample, but appeared in all samples after incubation for periods of 1 to 4 days. The levels increased in all treatments until the peak comprised 11 to 16% of the total protein.

Minor treatment differences occurred in the peaks with molecular weights of 39,000, 47,000, and 70,000. Although changes in concentration are apparent, the trends appear to be similar in all treatments for each protein.

In SDS gel electrophoresis with 0.135% cross linking (Table 5), peaks were apparent at R_M values corresponding to molecular weights of 20,000, 29,000, 36,000, 44,000, 48,000, 63,000, 88,000, 106,000 and 170,000. These points are plotted in relation to known marker proteins as shown in Figure 20.

The peak with a molecular weight of 29,000 contained from 8 to 17% of the total protein. Complete disappearance of this peak occurred only in the control treated with Ca^{+2} ions. The peak with a molecular weight of 48,000 also disappeared in the control containing Ca^{+2}, but remained present in relatively constant amounts in all other treatments.

Table 5

Molecular Weight and Peak Areas of Sarcoplasmic Proteins after SDS Gel Electrophoresis with 0.135% Cross Linking

TREATMENT	DAYS OF INCUBATION	PEAK AREAS (Percent of Total Protein)**									
		Average Molecular Weights									TOTAL PROTEIN*
		20,000	29,000	36,000	44,000	48,000	63,000	88,000	106,000	170,000	mg/g muscle
Control	0	3	17	18	21	13	16	5	6	1	92.0
	1	3	14	20	26	14	20		4		61.2
	2	5	11	19	25	16	20				68.8
	4	8	11	21	27	12	23				56.4
	7		15	23	36	7	20				60.6
Clostridium perfringens	1	17	12	17	23	11	20				46.4
	2	7	15	21	28	5	24				44.0
	4	8	11	22	25	11	22				39.7
	7	7	9	22	27	6	29				34.6
Control with Ca^{+2}	1	11	17	19	28	11	14		1		55.0
	2	9	10	20	28	13	21				42.4
	4	6		29	42		23				38.6
Enzyme	1	9	9	23	23	14	23				49.4
	2		10	21	31	15	21		2		38.2
	4		8	26	34	8	23		1		34.2

*Protein concentrations were determined using the Lowry method.

**Overall standard deviation = 2.3.

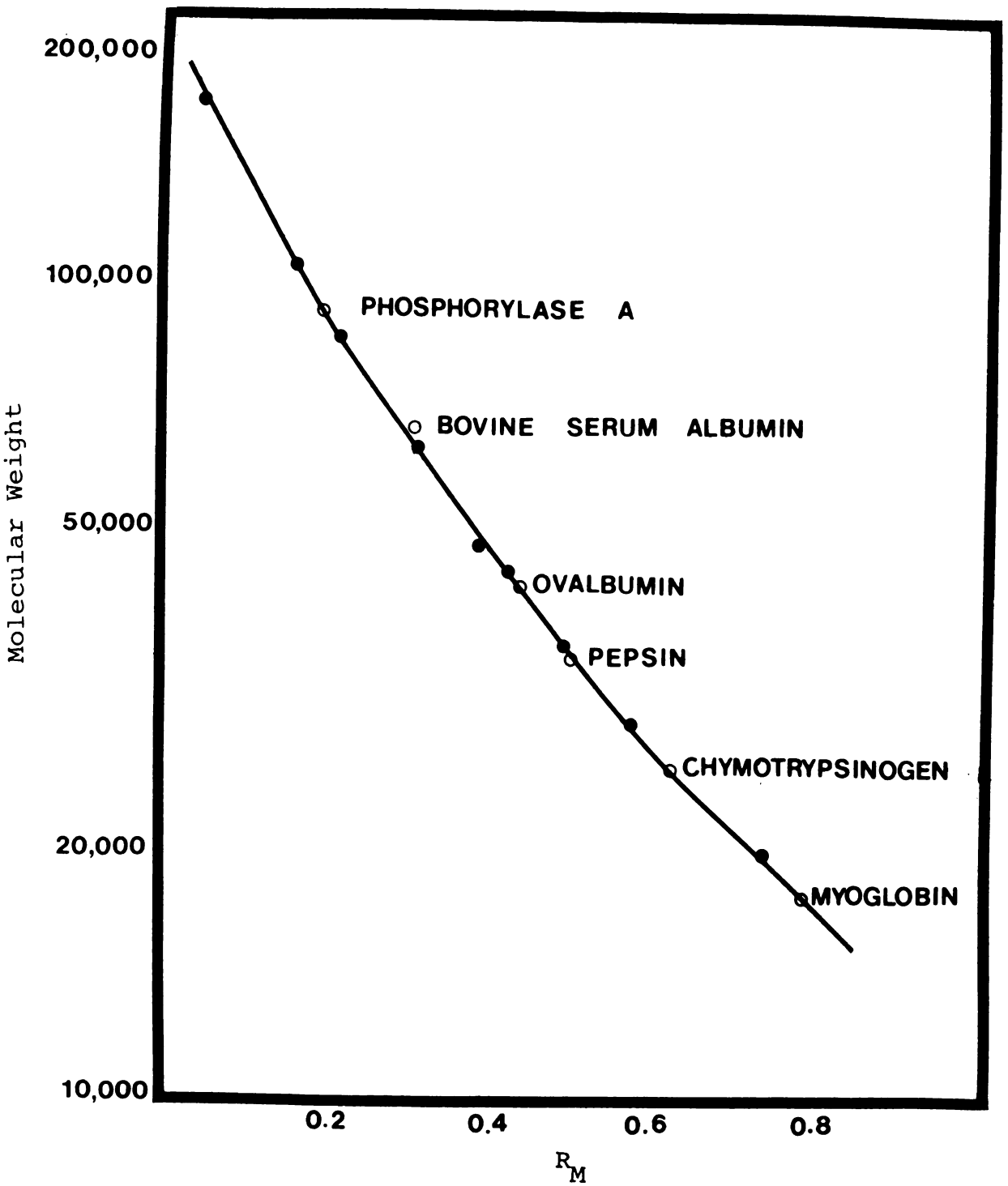

Figure 20. Molecular weights of sarcoplasmic proteins determined by comparison with standard proteins using 0.135% cross-linked SDS gels.

o o o marker proteins
● ● ● sarcoplasmic proteins

Although peaks were apparent at 88,000 and 170,000 daltons in the unincubated (0 day) control (Table 5), they disappeared in all samples following 1 day of incubation. The disappearance of these proteins indicates that they were subject either to autolysis or else precipitated upon the myofibrils.

The concentration of the protein with a molecular weight of 106,000 (Table 5) declined in the control sample, completely disappearing after 2 days of incubation. This protein was not present in the muscle incubated with C. perfringens, which suggests that the microorganism hastened its disappearance. In the enzyme-treated and the control samples with Ca^{+2}, the 106,000 molecular weight protein was present at very low levels (2% or less) at various times after incubation.

The protein with a molecular weight of 20,000 was apparent at fairly constant levels in all cases, except it was absent from the control after 7 days of incubation and from the muscle incubated with enzyme after 2 days. The relatively low concentration of this band and its disappearance upon incubation of both the control and enzyme-treated sample makes explanation difficult.

As seen previously with SDS gel electrophoresis using 0.27% cross linking (Table 4), there were no major treatment differences noted in the proteins at molecular weights of 36,000, 44,000 and 63,000.

Isoelectric focusing

Few changes were noted with isoelectric focusing, since the isoelectric points appeared over the entire range of pH values used, making the distinction of individual proteins nearly impossible. In the C. perfringens inoculated samples there appeared to be a decline in the proteins with isoelectric points in the pH range of 6.9-7.3 following 4 and 7 days of incubation. A slight decrease was also noted in the quantity of protein focused at alkaline pH values. Little difference could be detected between the enzyme and the Ca^{+2} containing control samples.

The complexity of the crude muscle extracts was such that separation of the proteins was difficult to achieve. Therefore, isoelectric focusing could be of greater value in assessing changes in simpler mixtures or for purified protein fractions.

Electrophoretic Analysis of Myofibrillar Proteins

The myofibrillar extracts from uninoculated control muscle, from muscle inoculated and incubated at 37°C with either C. perfringens (ATCC 13124), with enzyme solution, or with tris-HCl-$CaCl_2$ solution were subjected to electrophoretic analysis.

Disc gel electrophoresis in urea

Electrophoresis in urea took place using two methods of urea addition and two different protein stains. The proteins electrophoresed in the gels stained with amido black were dialyzed against 8 M urea, while those electrophoresed in gels stained with coomassie blue were brought to 8 M by the addition of solid ultrapure urea immediately prior to electrophoresis.

During overnight dialysis, cyanate ion was reformed in dialyzed urea (Bechtel, 1971), which could lead to carbamylation of free amino groups, labile sulfhydryl groups, and disulfide bonds (Stark et al., 1960). When solid urea was added directly to the salt extract, the sample contained a high salt concentration, which may interfere with electrophoresis.

The relative mobilities and relative concentrations of the myofibrillar proteins electrophoresed in the presence of urea are shown in Tables 6 and 7. Table 6 gives the analyses of the gels stained with amido black, while Table 7 shows the gels stained with coomassie blue. The relative mobilities determined using the two urea systems differed slightly, which can probably be ascribed to differences in handling and gel preparation, as well as to differences in the affinity for the dyes.

Use of the relative mobilities determined by Rampton (1969) has allowed tentative identification of

some of the myofibrillar proteins. He identified the proteins and their respective mobilities as follows: myosin (0.00-0.15), actin (0.39-0.37), monomeric (oxidized) tropomyosin (0.34), reduced tropomyosin (0.51) and extra protein, fraction IA (0.90).

The protein at R_M 0.83-0.85 (Tables 6 and 7) corresponds to extra protein, fraction IA, which Rampton (1969) tentatively identified as troponin. The protein with R_M 0.50-0.55 (Tables 6 and 7) appeared to be reduced tropomyosin, composed of two separate polypeptide subunits migrating together (Rampton, 1969), while oxidized (monomeric) tropomyosin migrated with R_M 0.34-0.35 (Tables 6 and 7). The protein with R_M 0.42-0.46 (Tables 6 and 7) was assumed to be actin. Although myosin is found in the peak at the gel boundary (R_M 0.04, Tables 6 and 7), much of the myosin in the myofibrillar fraction probably does not enter the gel structure and, therefore, cannot be analyzed.

The concentration of extractable myofibrillar protein is shown in Table 6. These values differ slightly from those reported previously (Figure 18), which can be attributed to the higher incubation temperature, as well as to the different extraction procedure for myofibrillar proteins used in electrophoresis. As already indicated, the myofibrillar fraction was extracted following the removal of the water-soluble protein.

Table 6

Relative Mobilities and Peak Areas of Myofibrillar Proteins after Electrophoresis and Staining with Amido Black

TREATMENT	DAYS OF INCUBATION	PEAK AREAS (Percent of Total Protein)**									TOTAL PROTEIN* mg/g muscle
		Relative Mobility									
		0.83	0.50	0.42	0.34	0.29	0.24	0.14	0.10	0.04	
Control	0	5	9	36	6	15	13		6	9	82.4
	1	7	4	29			18		18	24	36.0
	2	5	5	26			19		27	19	31.6
	4	3	9	19			18		22	29	23.0
	7	3	9	23			14	14	18	19	25.6
Clostridium perfringens	1	6	10	27			15		18	25	32.8
	2	2	9	15			14	17	14	29	19.0
	4	2	9	9		9	9	32		30	23.4
	7		7	6		11	7	24		45	30.6
Control with Ca^{+2}	1	3	6	15			12	25		40	42.0
	2	4	4	22			20	28		21	28.4
	4	2	8	23			13	19	20	16	19.6
Enzyme	1	4	5	17			19	24	16	15	29.4
	2	5	8	22			17	13	18	17	28.4
	4	6	8	24			16	10	22	15	31.4

*Protein determined using Lowry method.

**Overall standard deviation = 2.6.

In the control, the quantity of extractable myofibrillar protein declined from approximately 80 mg/g prior to incubation to 36 mg/g after 1 day at 37°C. The amount then slowly decreased during further incubation to about 25 mg/g. In the case of muscle incubated with C. perfringens, the extractable protein declined to 19 mg/g after 2 days with a subsequent increase to about 30 mg/g by 7 days. In the control containing Ca^{+2}, the decline in extractable myofibrillar protein continued during incubation, reaching about 20 mg/g after 4 days (Table 6). In the muscle inoculated with enzyme, the extractable protein remained relatively constant during subsequent incubation with values near 30 mg/g.

Upon incubation with C. perfringens, the percentage of troponin (R_M 0.83, Table 6) declined, disappearing by 7 days. In all the other muscle samples, troponin remained at fairly constant levels throughout incubation.

The concentration of reduced tropomyosin (R_M 0.50, Table 6) remained relatively constant throughout incubation in all samples. On the other hand, oxidized tropomyosin (R_M 0.34, Table 6) was present in the 0 time sample, but was not evident in any of the incubated samples.

The actin peak (R_M 0.42, Table 6) decreased during incubation of the control. Values declined from 36 to about 20% of the total protein. In the muscle incubated with C. perfringens, the actin peak diminished

steadily until it comprised only 6% after 7 days. In the enzyme-treated sample and the control with Ca^{+2}, the concentration of actin comprised approximately 20% of the total protein content at all incubation times.

The protein migrating at R_M 0.29 (Table 6) was evident in only two of the samples incubated at 37°C, namely, in the C. perfringens-treated samples incubated for either 4 or 7 days. It seems likely that this peak is indicative of proteolytic breakdown, being composed of products of proteolysis different from those appearing in the unincubated control. The intact organism appears to be responsible for the presence of this band, whereas, the isolated enzyme does not produce this protein peak. Thus, the organism and the isolated enzyme appear to differ in regard to formation of this protein.

The unidentified peak with R_M 0.14 (Table 6), was not evident in the unincubated sample. In the incubated control, this peak became apparent after 7 days, while for the sample incubated with C. perfringens, it appeared after 2 days. The peak was evident throughout incubation in the control with Ca^{+2} and in the muscle inoculated with enzyme.

In the control, the relative concentration of the unidentified peak at R_M 0.10 (Table 6) increased upon incubation from 5% to about 20% of the total protein. Upon incubation with C. perfringens, the peak

at R_M 0.10 increased similarly to the control at the end of 1 day, but it disappeared following 4 days of incubation. The peak was evident at levels of about 20% throughout incubation in the muscle treated with enzyme. It was absent in the control with Ca^{+2} ions at 1 and 2 days of incubation, but appeared at the 20% level after 4 days of incubation.

The large peaks at R_M 0.14 and 0.04 may mask a small peak at R_M 0.10 (Table 6) since this small peak disappeared after 4 and 7 days of incubation with C. perfringens, or after 1 and 2 days in the Ca^{+2}-treated control.

The changes in the peaks with R_M values of 0.04 (myosin) and 0.24 (unidentified) are of doubtful significance. The large increase in the R_M 0.04 peak upon incubation with C. perfringens does suggest that this organism may solubilize the high molecular weight proteins which do not normally migrate into the gel.

The gels stained with coomassie blue, shown in Table 7, were less definitive than the gels stained with amido black. The gels did not destain as readily as those stained with amido black or non-urea containing gels stained with coomassie blue. It is postulated that urea in the gels in some manner affects the staining-destaining process.

The protein migrating at R_M 0.85 in the coomassie blue stained gels (Table 7) was assumed to be identical

Table 7

Relative Mobilities and Peak Areas of Myofibrillar Proteins after Electrophoresis and Staining with Coomassie Blue

TREATMENT	DAYS OF INCUBATION	PEAK AREA (Percent of Total Protein)								
		Relative Mobility								TOTAL PROTEIN* mg/g muscle
		0.85	0.63	0.55	0.46	0.35	0.25	0.10	0.04	
Control	0	16	5	6	34	15		24		82.4
	1	7	5	7	22	14		32	16	36.0
	2	9	11	3	12	15	9	25	15	31.6
	4	8	10		22	32			28	23.0
	7	9	9	5	36	13			28	25.6
Clostridium perfringens	1	8	15	4	50	19		4		32.8
	2	6	13		39	13		6	23	19.0
	4	4	16		14	6	17	29	15	23.4
	7	2	11		11	6	19	35	17	30.6
Control with Ca^{+2}	1	14	10	8	45	16			6	42.0
	2	9	8		32	14		16	22	28.4
	4	4	11		27	15			42	19.6
Enzyme	1	10	11		39	24			17	29.4
	2	10	10		32	23			25	28.4
	4	6	10		34	16			23	31.4

*Protein determined using Lowry method.

**Overall standard deviation = 4.2.

to the peak at R_M 0.83 in the amido black stained gels (Table 6). This was tentatively identified as troponin (Rampton, 1969). The relative concentration of this protein is greater in the samples stained with coomassie blue than in the samples stained with amido black. In the control, this peak declined after 1 day of incubation, and then remained relatively constant. In the sample treated with C. perfringens, the R_M 0.85 peak continued to decline during incubation, but the difference was not significant. The enzyme-treated sample and the control with Ca^{+2} declined only slightly upon incubation.

The decline in troponin upon incubation with C. perfringens was also seen in the gels stained with amido black. This strongly suggests that the organism per se is affecting the troponin peak.

The concentration of the control peak which migrated with R_M 0.63 (Table 7) did not change significantly during incubation. The concentration of this peak was higher in the C. perfringens-treated sample, ranging from 11% to 16%. The relative concentration of this peak in the Ca^{+2}-containing control and enzyme-treated samples remained rather constant during incubation. Identification of this peak was not definite.

The reduced tropolyosin peak stained with coomassie blue (R_M 0.55, Table 7) was apparent at low

levels in the control, except after 4 days of incubation. This peak was present at quite low levels in the sample incubated for 1 day with C. perfringens, but was absent when incubated for longer periods. This peak was only apparent in the Ca^{+2}-containing control after 1 day of incubation and was not present in the enzyme treated samples at any time.

The protein migrating at R_M 0.35 (Table 7), which was tentatively identified as oxidized tropomyosin, was apparent throughout incubation on gels stained with coomassie blue, which was not the case for gels stained with amido black (Table 6). The concentration of this protein in the control sample remained relatively constant except for a sharp increase at 4 days, which corresponded to the absence of reduced tropomyosin at this time. The concentration of oxidized tropomyosin in the muscle treated with C. perfringens declined during incubation. The concentration of oxidized tropomyosin in the Ca^{+2} containing control and the enzyme-treated muscle remained relatively constant during incubation.

Oxidized tropomyosin was consistently present in the samples treated with ultra pure urea, which were stained with coomassie blue (Table 7). The presence of small amounts of reduced tropomyosin in a few samples was probably caused by the formation of some cyanate

prior to electrophoresis, which would cause disruption of disulfide bonds (Stark et al., 1960).

The protein (actin) with R_M 0.46 (Table 7) declined in the control during the first 2 days of incubation with an increase to original levels after 7 days. This peak declined considerably in the muscle inoculated and incubated with C. perfringens, decreasing from 50% to 11% of the total protein. A decline was also noted in the Ca^{+2}-containing control, while the muscle treated with enzyme did not change during incubation. Proteolysis of actin by C. perfringens was indicated by these data.

The unidentified protein with R_M 0.25 (Table 7) was apparent in the control only at 2 days of incubation. This protein appeared in the muscle treated with C. perfringens after 4 and 7 days of incubation. This suggests that C. perfringens produced this protein by breaking down some other peak.

In the control, the unidentified protein with R_M 0.10 (Table 7) was found in relatively constant amounts through the second day of incubation, after which time it was absent. This peak decreased after 1 or 2 days incubation with C. perfringens, but further incubation caused an increase in concentration. The peak was present only at 2 days of incubation in the Ca^{+2}-containing control and was absent at all times upon incubation with enzyme.

The low mobility peak (myosin, R_M 0.04) was not apparent in the unincubated control (Table 7). This may indicate that myosin was too large to enter the gel pores (Florini and Brivio, 1969). The peak appeared upon incubation for 1 day, with the concentration increasing as incubation was continued. In the C. perfringens-treated sample, myosin did not appear until the second day of incubation, while further incubation did not change the quantity greatly. In the control with Ca^{+2}, the concentration of this peak increased throughout incubation, while in the sample treated with enzyme the peak did not change significantly during incubation.

In the samples incubated for 4 or 7 days with C. perfringens, there was an increase in the concentration of the three slowest migrating peaks, R_M 0.25 (unidentified), R_M 0.10 (unidentified) and R_M 0.04 (myosin). This increase implies proteolysis of large molecules which previously did not enter the gel. Results show there was an increased quantity of salt-soluble proteins after incubation with C. perfringens for 4 days. It is assumed that the proteolysis of collagenous material is the source of the increase, since the action of C. perfringens against collagen is well known (Oakley et al., 1946; Bidwell and van Heyningen, 1948; Kameyama and Akama, 1971; Hasegawa et al., 1971).

Since the myofibrillar proteins are not completely characterized and identified upon disc gel electrophoresis with urea, the changes in individual proteins cannot always be reported with certainty. As proteolytic breakdown products are introduced into the system, the changes in myofibrillar proteins may be masked.

SDS gel electrophoresis

The average molecular weights of the myofibrillar proteins and the salt soluble proteolytic breakdown products as determined by SDS gel electrophoresis are shown in Figure 21. Major protein peaks were observed with molecular weights of 17,000, 21,000, 25,000, 31,000, 35,000, 46,000, 70,000, 92,000, 110,000, 140,000 and 200,000. The latter 3 molecular weights are extrapolated values, based upon an assumed molecular weight of 200,000 for the experimental myosin peak (Hay et al., 1973).

Tentative identification of some of the proteins was made on the basis of reported molecular weight values. Figure 22 shows the densitometric tracing of the unincubated myofibrillar fraction with tentative peak identification. The DTNB light chain of myosin, troponin C and another light myosin chain appear to have migrated together at an average molecular weight of 19,000. Reported molecular weights for the myosin light

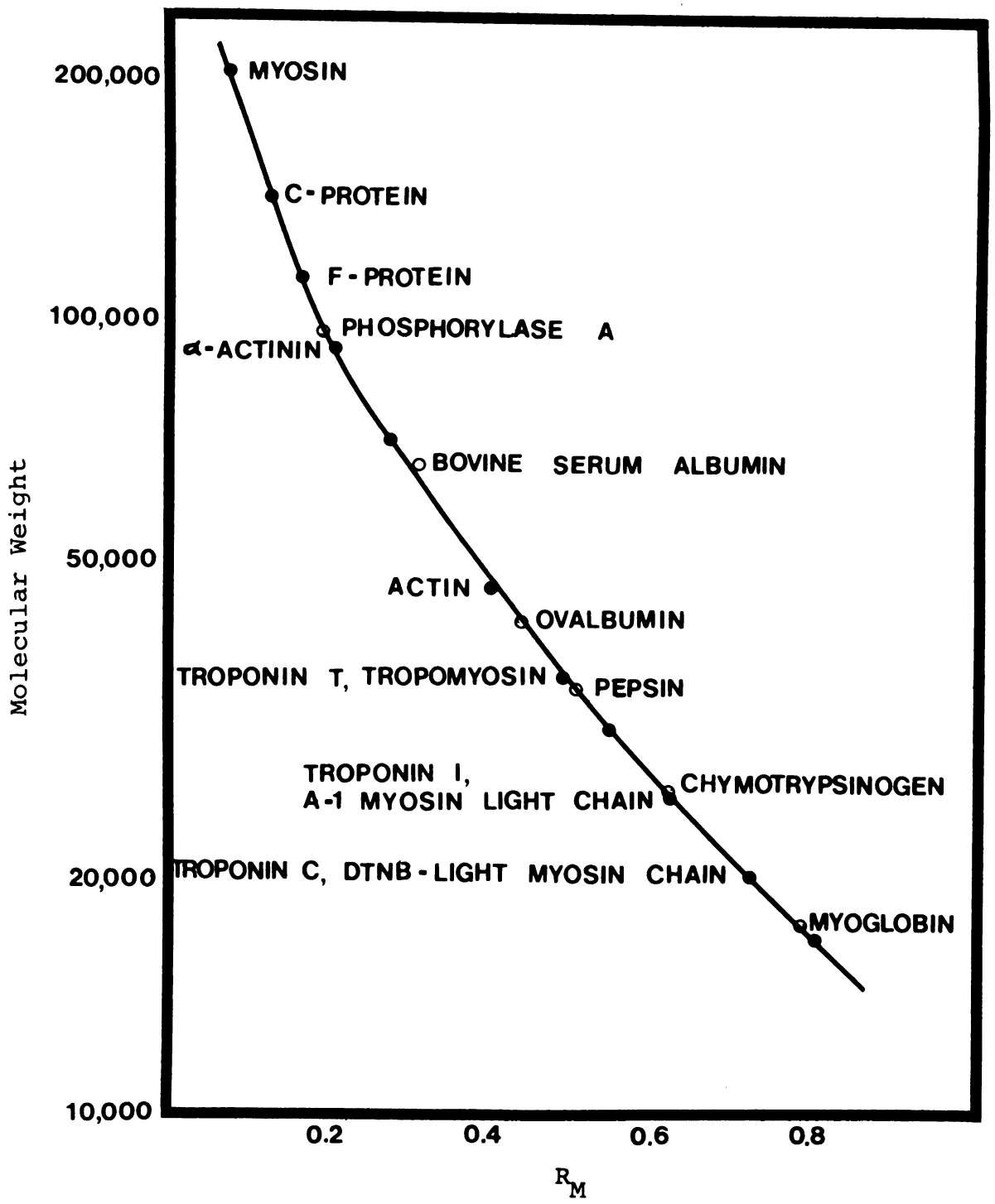

Figure 21. Molecular weights of myofibrillar proteins determined by comparison with standard proteins using 0.135% cross-linked SDS gels.

o o o marker proteins
● ● ● myofibrillar proteins

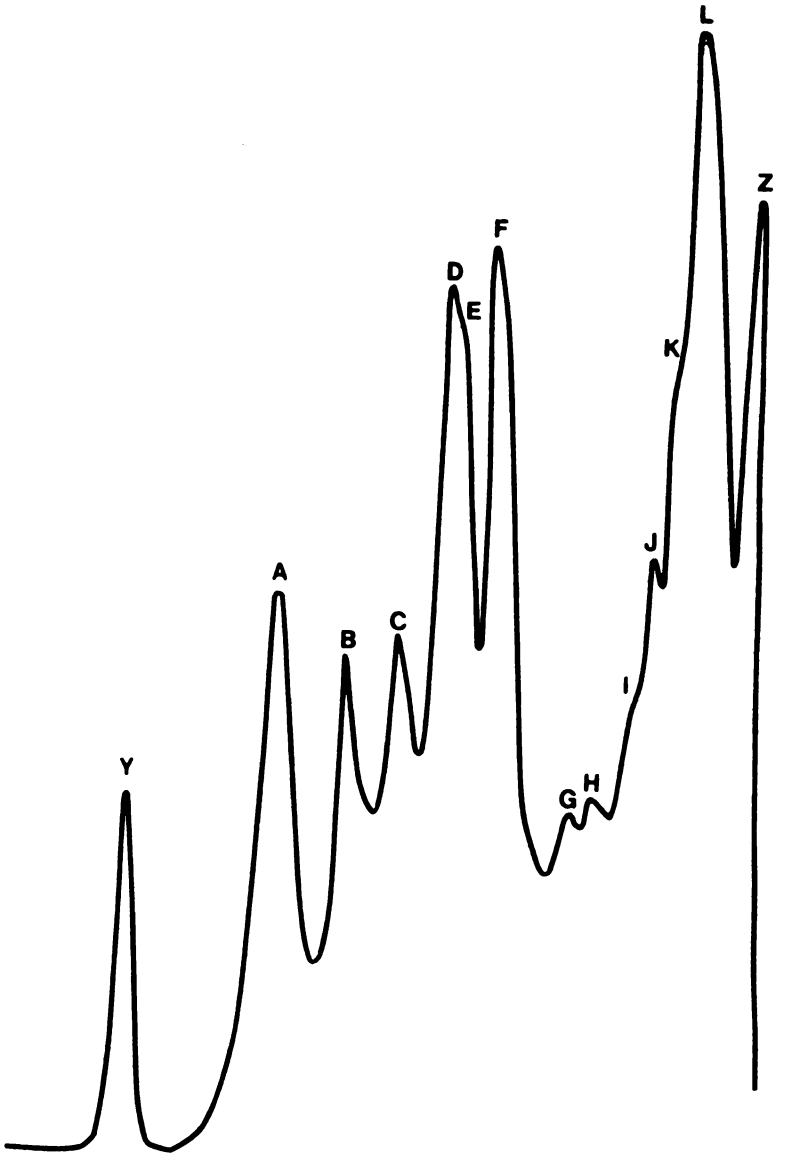

Figure 22. Densitometric tracing of the unincubated myofibrillar fraction electrophoresed upon a 0.135% cross-linked SDS gel with tentative peak identification.
(A) DTNB light chain of myosin and troponin C,
(B) A-1 light chain of myosin and troponin I,
(C) unidentified, (D) tropomyosin,
(E) troponin T, (F) actin and M-protein
(G) unidentified, (H) unidentified,
(I) α-actinin and M-protein, (J) F-protein,
(K) C-protein and M-protein, (L) myosin,
(Y) marker dye band (Z) gel origin

chains are 16,000 and 14,000 (Starr and Offer, 1971), 18,000 (Sender, 1971), 18,000 and 16,000 (Offer et al., 1973) and 16,000, 17,500 and 22,000 (Scopes and Penny, 1971). Wilkinson et al. (1972) reported troponin C had a molecular weight of 18,000.

Troponin I and the A-1 light chain of myosin are probably inseparable upon SDS gel electrophoresis, both migrating at an average molecular weight of 24,500 (Figure 22). The A-1 light chain of myosin has been reported to have a molecular weight of 24,000 by Sender (1971), 27,000 by Starr and Offer (1971), or 25,000 by Offer et al. (1973). Wilkinson et al. (1972) reported troponin I had a molecular weight of 23,000 while Offer et al. (1973) reported a value of 24,000.

The protein migrating at 31,000 daltons (Figure 22) is probably identical to an unidentified peak observed in chicken (Hay et al., 1973) and rabbit muscle (Offer et al., 1973). No other 30,000 molecular weight peaks have been observed in the myofibrillar fraction of muscle.

Tropomyosin migrated with an average molecular weight of 35,000 (Figure 22). Tropomyosin has been reported to have a molecular weight of 34,000-35,000 by Scopes and Penny (1971), 36,000 by Hay et al. (1973) and 32,000 by Offer et al. (1973).

The peak at 37,000 daltons (Figure 22) was tentatively identified as troponin T. Offer et al.

(1973) reported a molecular weight of 35,000 for troponin T, while Sender (1971) reported 38,000 and Wilkinson et al. (1972) found a value of 37,000.

The peak with an average molecular weight of 44,000 was tentatively identified as actin (Figure 22). Scopes and Penny (1971) have reported a molecular weight of 41,500 for actin, Hay et al. (1973) recorded a molecular weight of 49,000, Sender (1971) reported 44,000 and Offer et al. (1973) 41,700. Offer et al. (1973) also have reported that an M-protein component may comigrate with actin. Hay et al. (1973) speculated that the 44,000 dalton component, which was lost upon aging of chicken muscle may be M-protein.

Two small peaks with molecular weights 67,000 and 75,000 were unidentified. Scopes and Penny (1971) report unidentified bands at 55,000 and 75,000 daltons.

The protein with a molecular weight of 95,000 has been tentatively identified as α-actinin (Figure 22). Molecular weight values for α-actinin have been reported as 90,000 (Scopes and Penny, 1971; Offer et al., 1973), 115,000 (Hay et al., 1973) and 102,000 (Sender, 1971). Offer et al. (1973) also reported that a component of M-line protein comigrates with α-actinin.

An unidentified peak, possibly F-protein, was seen at a molecular weight of 120,000 (Figure 22). Scopes and Penny (1971) reported an unidentified peak at

105,000 daltons. Starr and Offer (1971) reported F-protein to have a molecular weight of 110,000.

C-protein was tentatively identified at an average molecular weight of 145,000 (Figure 22). Offer et al. (1973) noted the molecular weight of C-protein was 140,000. They also reported that a component of M-line protein was found at 140,000 daltons.

Myosin heavy chain protein was tentatively identified at approximately 200,000 daltons. The molecular weight of the heavy chain has been reported to be 200,000 by Offer et al. (1973), 207,000 by Sender (1971) and 210,000 by Hay et al. (1973). In the present study, some non-migrating proteins were noted at the gel origin, which is in agreement with the results of Sender (1971).

The relative peak areas of the salt-soluble proteins upon SDS gel electrophoresis are shown in Table 8. Some proteins are reported together, such as tropomyosin and troponin T, because they were not clearly separable on the densitometric tracings.

A low molecular weight component at approximately 15,000 appeared at all time periods in the samples incubated with C. perfringens or with enzyme (Table 8). This peak also appeared in the control with Ca^{+2} after 1 day of incubation, but at lower levels than for the corresponding enzyme-treated or the C. perfringens-treated samples. This peak is probably a proteolytic breakdown product.

Table 8

Average Molecular Weights and Peak Areas of Myofibrillar Proteins
Separated by SDS Gel Electrophoresis

TREATMENT	DAYS OF INCUBATION	PEAK AREA (Percent of Total Protein)** Average Molecular Weight										TOTAL PROTEIN* mg/g muscle
		15,000	19,000	24,500	31,000	35,000	44,000	70,000	95,000	120,000	145,000	200,000	
Control	0		9	6	6	17	14	5	5	5	6	27	82.4
	1		7	13	8	33	15	2			9	12	36.0
	2		8	11	6	39	17	3			9	7	31.6
	4		7	9	9	37	21	3	3		8	5	23.0
	7		6	6	5	32	17	8		5	14	8	25.6
Clostridium perfringens	1	9	9	3	9	25	16	5		7	10	11	32.8
	2	3	6	2	9	27	20	8	9	8	9	2	19.0
	4	5	3	3	16	16	24	28		6	4	2	23.4
	7	7		3	7	14	26	30		5	6	3	30.6
Control with Ca^{+2}	1	4	7	11	16	26	18	8	4		7	1	42.0
	2		7	11	10	34	18	8			7	4	28.4
	4		6	4	9	37	24	12			8	1	19.6
Enzyme	1	7	10	13	18	26	14	8			5	1	29.4
	2	6	8	11	13	27	17	9			7	3	28.4
	4	6	8	7	25	28	12	12			3		31.4

*Protein determined using Lowry method.

**Overall standard deviation = 1.1.

The band at 19,000 daltons containing troponin C and the DTNB-light chain of myosin (Table 8) was completely removed from the C. perfringens-treated sample after 7 days of incubation, but remained in fairly high concentrations in all other samples, indicating proteolysis by C. perfringens.

The relative concentration of the band at 24,500 daltons, which tentatively was identified as containing troponin I and the A-1 light chain of myosin, was reduced in the sample incubated for 1 day with C. perfringens and then remained at rather low levels during further incubation. The control sample had a maximum level of this protein at 1 day of incubation, after which time it gradually declined to pre-incubation levels. The enzyme-treated sample and the control with Ca^{+2} ions also had maximum levels of this band after 1 day incubation, which then gradually declined.

The reduction upon incubation with C. perfringens in the peaks containing troponin (19,000 and 24,500 daltons) is consistent with the reduction in the concentration of extra protein fraction IA (troponin) as measured by disc gel electrophoresis in urea (Tables 6 and 7).

The unidentified 31,000 molecular weight component remained fairly constant in all samples throughout incubation (Table 8) except for an increase on the fourth day of incubation with enzyme.

The tropomyosin-troponin T band (35,000) decreased in the sample incubated with C. perfringens (Table 8). The relative concentration of this peak in the control sample increased during the first day of incubation, and then remained rather constant. The control containing Ca^{+2} increased throughout incubation, while the enzyme-treated sample remained relatively constant. The decrease seen upon growth of C. perfringens is significant in light of previous reductions noted in troponin containing peaks (Tables 6, 7 and 8).

When comparisons are made between the tropomyosin peaks obtained upon SDS gel electrophoresis and disc gel electrophoresis with urea (Tables 6 and 7), some differences were noted. In the gels stained with amido black (Table 6), tropomyosin migrated in the reduced form (R_M 0.50). In the coomassie blue stained gels (Table 7), tropomyosin was seen at R_M 0.55 (reduced) as well as R_M 0.35 (oxidized). If the reduced and oxidized tropomyosin were combined, the relative amount in the C. perfringens treated sample was less than in the control sample, indicating proteolytic breakdown of tropomyosin.

Another interesting feature was the presence of an unidentified peak at R_M 0.63 (Table 7) in the coomassie blue-stained gels, which was not apparent in the amido black-stained gels (Table 6). It may be that this peak (R_M 0.63, Table 7) migrated with or

overlapped tropomyosin (R_M 0.50) in the amido black-stained gels (Table 6) and thereby concealed changes in the tropomyosin peak. If this were indeed the case, any breakdown of tropomyosin would be hidden. Never-the-less, both the SDS gels (Table 8) and the coomassie blue-stained urea gels (Table 7) suggested that tropomyosin was degraded by C. perfringens.

Upon SDS gel electrophoresis, the actin peak (44,000 daltons, Table 8) remained relatively constant in the control sample throughout incubation. The muscle incubated with C. perfringens showed a consistent but slight increase in this peak as incubation proceeded. The Ca^{+2}-containing control also exhibited a slight increase, while the muscle treated with enzyme remained relatively constant. This is contrary to evidence obtained upon electrophoresis in urea (Tables 6 and 7) which indicated a decline in the percentage of actin in samples treated with C. perfringens.

The small unidentified peaks with average weights of 70,000 upon SDS gel electrophoresis (Table 8) decreased in the control sample during the first 4 days incubation, but increased at 7 days incubation. The relative concentration of this peak in the muscle incubated with C. perfringens increased throughout incubation, probably because of the formation of proteolytic breakdown products. Both the control with Ca^{+2} and the sample treated with enzyme had similar

concentrations of this peak, and both exhibited a slight increase at 4 days of incubation.

Upon SDS gel electrophoresis, the α-actinin peak (95,000, Table 8) was discernible in only a few samples after incubation. The fact that α-actinin was not present in the control after incubation is contrary to the results of Hay et al. (1973), who reported that α-actinin was unchanged in chicken muscle up to 168 hours post-mortem.

The 120,000 molecular weight peak (Table 8) was seen upon SDS gel electrophoresis of the incubated control only after 7 days incubation, which probably indicates autolysis. This peak was seen in the sample incubated with C. perfringens in steadily diminishing amounts as incubation continued.

The concentration of the peak tentatively identified as C-protein (145,000, Table 8) remained relatively constant in the control, with a slight rise at 7 days incubation. The relative concentration of C-protein decreased slowly in muscle incubated with C. perfringens. In the enzyme-treated and Ca^{+2}-containing control samples, this protein remained at a relatively constant concentration, with the enzyme-treated sample showing only a slight decline upon 4 days incubation.

The myosin peak (200,000) is the most difficult to analyze. The concentration of this peak (Table 8)

diminished greatly during incubation. Because of the large size of myosin and the difficulty in subjecting it to electrophoresis, the fate of myosin cannot be completely determined. It may be rapidly degraded by the high temperature of incubation, rendered unextractable, or aggregated to such an extent that it does not enter the gels.

Isoelectric focusing in urea

As found with the sarcoplasmic proteins, changes in the patterns of the myofibrillar proteins seen upon isoelectric focusing are difficult to interpret. In general, incubation appeared to decrease the proportion of proteins with isoelectric points above 6.5. This trend was observed in the C. perfringens-treated sample at 1 day of incubation. At 2 days incubation, the majority of the proteins from the sample incubated with C. perfringens focused in the pH range 4.5-6.2. After 7 days incubation, however, the control sample retained some proteins having isoelectric points above pH 6.5. After 7 days incubation with C. perfringens, two peaks reappeared at pH values 8.7 and 6.6. Other peaks with isoelectric points of 6.2 and 5.3-5.8 were observed after 7 days incubation in both the C. perfringens-treated sample and the control. Both the enzyme-treated sample and the Ca^{+2}-containing control had isoelectric focusing patterns similar to the control sample.

General Observations on Electrophoresis

While many differences can be seen between one particular electrophoresis pattern and another, the changes attributable to the treatments are difficult to discern because of the complex nature of the protein system. During incubation many of the proteins changed concurrently, and the net effect was not detectable using these relatively crude separation techniques. However, it is possible that these changes are dependent upon each other and that they might not occur in more simplified systems. Until more definitive methods of separating and identifying the proteins in the inoculated and incubated samples are developed, the effect of these treatments upon individual proteins will remain difficult to assess.

Electron Microscopy

Electron micrographs of samples from control and C. perfringens-inoculated muscle were examined after incubation for both 1 and 4 days. After 7 days incubation, the muscle was degraded to such an extent that it was impossible to prepare satisfactory electron micrographs.

The myofibrils were classified on the basis of Z line width as either red fibers or white and intermediate fibers (Gauthier, 1970). Dutson (1971)

determined that the width of the Z line in porcine red fibers was about 12.0 μm, in intermediate fibers about 7.75 μm and in white fibers approximately 6.25 μm. Since the difference in the width of the Z lines for intermediate and white fibers is relatively small and the amount of disruption was relatively large in this study, no attempt to differentiate between the two was made.

Changes in the ultrastructure of incubated muscle

A typical electron micrograph of a white or intermediate fiber from control muscle incubated for 1 day is shown in Figure 23. The width of the Z lines in this fiber was about 7.5 μm. The ultrastructure remained relatively intact, with no noticeable disruption. The Z and M lines are intact and the thin filaments (I band) are clearly visible and are attached to the Z line.

A typical electron micrograph of red fibers from the control muscle incubated for 1 day is shown in Figure 24. The Z lines in this micrograph were from 10.0 to 12.5 μm in width. The red fibers appeared to be intact, with the primary features of the sarcomere being clearly evident. The intact structures seen in both the red and white fibers are in contrast to the report of Henderson et al. (1970), who reported that Z lines in prerigor porcine muscle became amorphous and disrupted within 4 hours at 37°C.

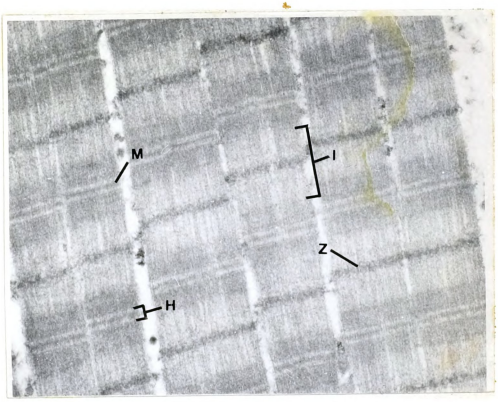

Figure 23. Electron micrograph of white or intermediate fiber from control muscle incubated for 1 day at 37°C. M = M line, Z = Z line, I = I band, H = H zone. 26,563 X.

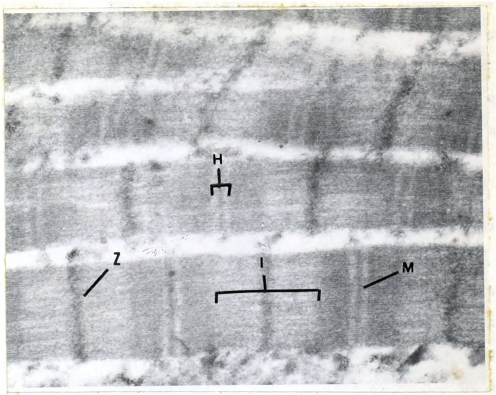

Figure 24. Electron micrograph showing red fibers from control muscle after incubation for 1 day at 37°C. M = M line, Z = Z line, I = I band, H = H zone. 36,000 X.

Figure 25 shows white or intermediate fibers after incubation for 1 day with C. perfringens. The width of the Z lines was about 6.4 μm. The electron micrograph shows some disruption of the myofibrillar structure. The thin filaments had broken loose from the Z line in several places, although the Z line itself, while diffuse and amorphous, was still recognizable. The characteristic structure of the M line had disappeared in some sarcomeres. Henderson et al. (1970) reported the loss of 60% of the M lines in prerigor porcine muscle stored for 24 hours at 37°C.

An electron micrograph of red fibers from muscle incubated with C. perfringens for 1 day is shown in Figure 26. The Z line showed some disruption, while its width varied from 12 to 13.5 μm. The attachment between the thin filaments and the Z line was severed in several places. The M-line had become quite amorphous, with almost complete disappearance of the H zone.

After 4 days incubation, the red fibers in the control muscle remained relatively intact (Figure 27). The Z line was 13.6 μm wide and was quite distinct. The thin filaments remained connected to the Z line in the majority of the sarcomeres. The M line was amorphous and had lost its distinct structure. There appeared to be some overlapping of the thin filaments, which resulted in a slightly darkened area in the region of the H zone, while the characteristic H zone had completely disappeared.

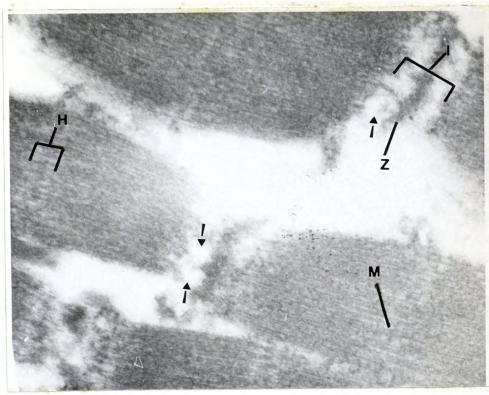

Figure 25. Electron micrograph of white or intermediate fibers after incubation for 1 day with C. perfringens at 37°C. M = M line, Z = Z line, I = I band, H = H zone, ↓↓ indicate disruption in thin filaments. 56,250 X.

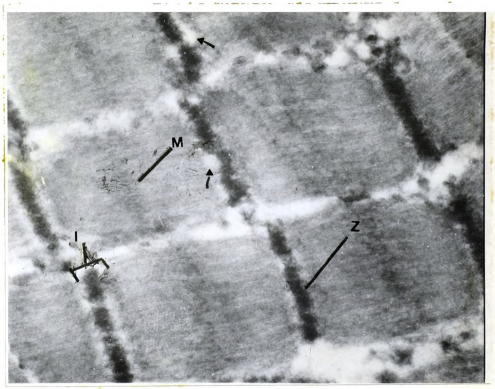

Figure 26. Electron micrograph showing red fibers from muscle incubated for 1 day at 37°C with C. perfringens. M = M line, Z = Z line, I = I band, ↓↓ indicate disruption in thin filaments. 34,230 X.

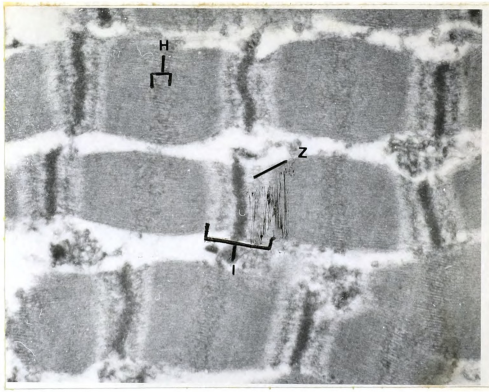

Figure 27. Electron micrograph of red fiber from muscle incubated for 4 days at 37°C. Z = Z line, H = H zone, I = I band. 22,770 X.

In the electron micrograph (Figure 28) of red fibers (Z line = 13.0 μm) from muscle incubated for 4 days with C. perfringens, the characteristic M line structure and the M line material was definitely absent. The Z line was recognizable, but showed some disruption and breakage in several spots. The thin filaments appeared to be quite degraded and were no longer attached to the Z line. This is in agreement with the observations of Strunk et al. (1967), who determined that one of the first effects of C. perfringens toxins upon the myofibrils was the fragmentation and distortion of the I band. In contrast, Dutson et al. (1971) showed that P. fragi disrupted the thick filaments (myosin) in the A band. They noted that the material in the H zone and the Z line had disappeared, but observed that the thin filaments (actin) were still distinct.

Thus, C. perfringens appears to have distinctly different effects than P. fragi upon the ultrastructure of muscle. C. perfringens causes degradation of the thin filaments, whereas, the thin filaments are not greatly affected by P. fragi. The disruption of the thin filaments is in agreement with the electrophoretic evidence (Tables 6, 7 and 8) that troponin is destroyed or altered by C. perfringens, since troponin is a constituent of the thin filament (Maruyama and Ebashi, 1970).

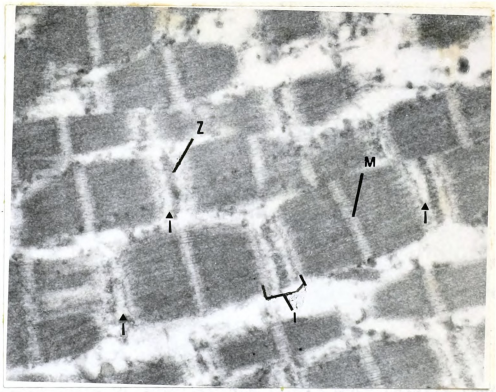

Figure 28. Electron micrograph showing red muscle fibers incubated at 37°C for 4 days with C. perfringens. Z = Z line, M = M line, I = I band, ↓↓ indicate areas of disruption in thin filaments. 23,040 X.

C. perfringens also completely removed the M line from red fibers after 4 days incubation (Figure 28). This was in contrast to the sample incubated for 1 day with C. perfringens, in which the M line from red fibers was disrupted, but could still be seen.

It was noted that treatment with enzyme or Ca^{+2} ions increased the disruption of the Z lines, but it was difficult to assess the changes because of mechanical disruption caused by incorporation of the enzyme or the Ca^{+2} solution into the muscle sample. Disruption of the Z lines has also been reported upon addition of 1 m M Ca^{+2} to intact muscle by Busch et al. (1972). Tarrant et al. (1973) also noted a loss of the Z line after incubation of muscle with an enzyme isolated from P. fragi.

There was some indication that C. perfringens degraded white or intermediate fibers more readily than red fibers. After 4 days incubation it was not possible to definitely identify white or intermediate fibers in the incubated muscle, but the red fibers were still readily discernible. A comparison of red and white fibers from inoculated muscle after 1 day of incubation with C. perfringens shows that the thin filaments from white fibers underwent a greater amount of disruption.

After 7 days incubation, samples inoculated with enzyme or with C. perfringens had an acrid, lachrymatory odor. When these samples were embedded prior to electron

microscopy, the epon blocks did not polymerize properly, making it impossible to obtain good sections.

Appearance of C. perfringens

An electron micrograph showing a longitudinal section of C. perfringens growing in muscle is shown in Figure 29. The organism measured about 220 μm in length and approximately 95 μm in width and was quite typical of other organisms observed. The outer membrane appeared to be smooth with no indication of bleb-like evaginations. This is in contrast to P. fragi, which was reported to develop surface blebs upon growth in muscle (Dutson, 1971).

Importance of Spoilage Studies

Some effects of growth of C. perfringens upon porcine muscle were determined in this study. Results indicated that proteolysis of muscle does indeed occur during microbial growth, but did not indicate whether proteolysis is the cause of spoilage or merely occurs during the spoilage process. Never-the-less, protein degradation does occur simultaneously with microbial spoilage and likely contributes to the development of the off-odors and flavors characteristic of spoiled meat.

Prevention of spoilage is of major economic importance to both the meat industry and the consumer. When the mechanism by which spoilage occurs can be

determined, it will be possible to use this knowledge in blocking spoilage. The development of new preservation techniques which interfere with biochemical deterioration would be of great benefit in increasing the shelf life of meat and meat products, thereby improving meat quality.

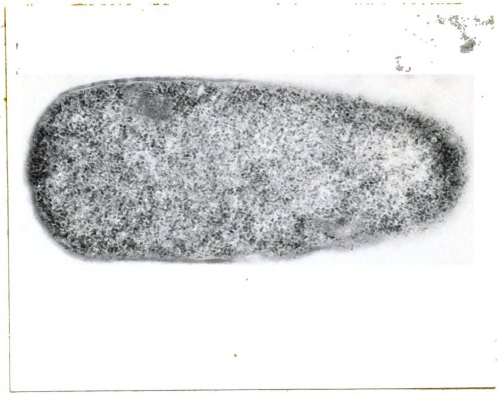

Figure 29. Electron micrograph showing a longitudinal section of C. perfringens grown in muscle for 1 day at 37°C. 53,760 X.

SUMMARY

Aseptic porcine muscle samples were inoculated either with viable cultures of C. perfringens (ATCC 13124 or 12915) or with an enzyme isolated from the culture filtrate of the same organism grown in peptone medium. The enzyme was prepared by Zn Cl_2 precipitation, disodium phosphate extraction and ammonium sulfate precipitation, followed by gel filtration and ion exchange chromatography.

After incubation at 30 or 37°C, the muscle samples were separated into sarcoplasmic, myofibrillar or non-protein nitrogen fractions. All fractions were analyzed for nitrogen. The sarcoplasmic and myofibrillar extracts were subjected to disc gel electrophoresis, SDS gel electrophoresis and isoelectric focusing.

Incubation with C. perfringens resulted in an increase in non-protein nitrogen, which implied that proteolysis had occurred with formation of small decomposition products. Proteolytic breakdown of the sarcoplasmic protein was also indicated by a decrease in sarcoplasmic nitrogen upon incubation with C. perfringens and by the reduction in size of certain protein peaks upon disc gel or SDS gel electrophoresis. Growth of

C. perfringens also resulted in the formation of new peaks in the gels of the water-soluble fraction.

New salt-soluble proteins also appeared in the gels of the myofibrillar extract after incubation with C. perfringens, indicating formation of other proteolytic breakdown products. In the myofibrillar extract, growth of C. perfringens caused a consistent reduction in the level of troponin as well as some evidence for degradation of actin and tropomyosin. Electron microscopy revealed destruction of the thin filaments in muscle during incubation with C. perfringens, which provides supporting evidence for the breakdown of troponin.

In this study, the intact organism was shown to be capable of degrading both sarcoplasmic and myofibrillar proteins from porcine muscle. Although the enzyme isolated from cultures of C. perfringens caused major breakdown in the sarcoplasmic fraction, it caused little proteolysis of the myofibrillar fraction. Since the enzyme and the intact organism did not always cause proteolysis of the same peaks, it is suggested that C. perfringens produces more than one enzyme capable of degrading muscle.

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APPENDIX

APPENDIX TABLE 1

Composition of Peptone Medium

Salt Solution I

	g/100 ml
$Na_2HPO_4 \cdot 7\ H_2O$	11.4
KH_2PO_4	2.8
$MgSO_4 \cdot 7\ H_2O$	0.4

Salt Solution II

	g/l
$ZnSO_4 \cdot 7\ H_2O$	0.6
$MnCl_2 \cdot 4\ H_2O$	0.4

Vitamin Mixture

	mg/l
Ca-d-pantothenate	20
Nicotinic acid	20
Thiamine hydrochloride	20
Pyradoxamine hydrochloride	14.2
Riboflavin	10
Biotin	0.1
Adenine sulfate	340
Uracil	250

Peptone Medium

	amount/1,900 ml
Difco proteose peptone	100 g
Difco Bacto beef	494 mg
Salt Solution I	100 ml
Salt Solution II	20 ml
Vitamin Mixture	100 ml
Thioglycolic acid	0.2 ml

APPENDIX TABLE 2

Composition of Amino Acid Medium

Amino Acids and Salts	mg/850 ml
DL-Alanine	500
L-Arginine	10,000
L-Asparagine	500
L-Cystine	50
L-Glutamic acid	750
Glycine	500
L-Histidine	250
DL-Isoleucine	500
L-Leucine	350
L-Lysine	250
DL-Methionine	500
L-Proline	150
DL-Phenylalanine	1,000
DL-Serine	500
DL-Threonine	500
L-Tryptophan	250
L-Tyrosine	100
DL-Valine	250
Na_2HPO_4	2,300
NaCl	3,000
KCl	800
$MgSO_4 \cdot 7\ H_2O$	200
$CaCl_2 \cdot 2\ H_2O$	74
$ZnSO_4 \cdot 7\ H_2O$	14
$MnCl_2 \cdot 4\ H_2O$	5

Growth Factor Mixture	mg/l
Glutamine	5,000
Ca-d-pantothenate	20
Nicotinic acid	20
Thiamine hydrochloride	20
Pyradoxamine hydrochloride	14.2
Riboflavin	10
Biotin	0.1
Adenine sulfate	340
Uracil	250

APPENDIX TABLE 3

Formulation of 7.5% Polyacrylamide
Gel Electrophoresis System

Separating Gel (7.5% Acrylamide, 0.18% BIS, pH 8.9)

Stock solutions	amount/100 ml
a) Tris	36.3 g
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	0.23 ml
1 N HCl to yield pH 8.8-9.0	
b) Acrylamide	30 g
N,N'-Methylenebisacrylamide (BIS)	735 mg
c) Ammonium persulfate	140 mg

Working solution - 1 part (a):1 part (b):2 parts (c)

Stacking Gel (2.5% Acrylamide, 0.625% BIS)

Stock solutions	amount/100 ml
a) Tris	5.98 g
TEMED	0.46 ml
1 N HCl to yield pH 6.7	
b) Acrylamide	10 g
BIS	2.5 g
c) Riboflavin	4.0 mg
d) Sucrose	40 g

Working solution - 1 part (a):2 parts (b):1 part (c):
4 parts (d)

Buffer Solution (0.04 M Tris, 0.2 M Glycine, pH 8.3)

	g/1
Tris	3.0
Glycine	14.4

APPENDIX TABLE 4

Formulation of 6.25% Polyacrylamide Gel Electrophoresis System with Urea

Separating Gel (6.25% Acrylamide, 0.1% BIS)

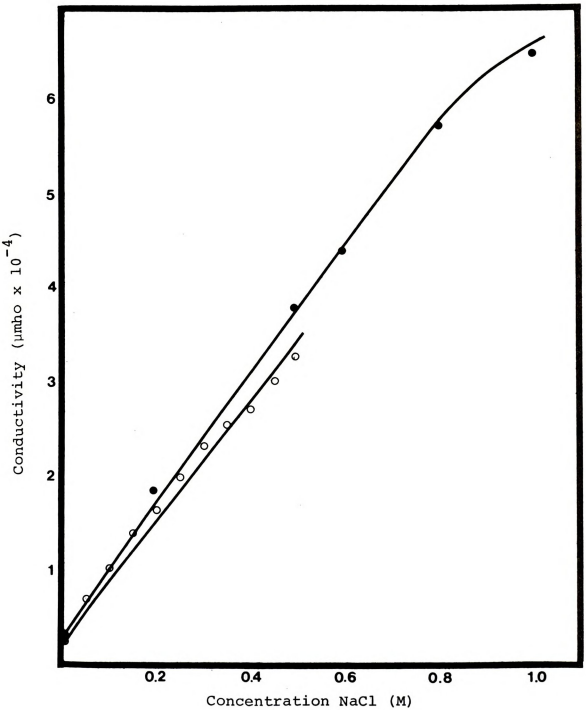
	amount/25 ml
Acrylamide	1.625 g
BIS	0.025 g
TEMED	0.01 ml
Riboflavin (4 mg/100 ml)	2.5 ml
Tris	1.21 g
Urea	10.5 g
HCl (2 N)	0.8 ml

Stacking Gel (5% Acrylamide, 0.1% BIS)

	amount/10 ml
Acrylamide	0.5 g
BIS	0.01 g
TEMED	0.004 ml
Riboflavin (4 mg/100 ml	1.0 ml
Tris	0.075 g
Urea	4.2 g
HCl (2 N)	0.3 ml

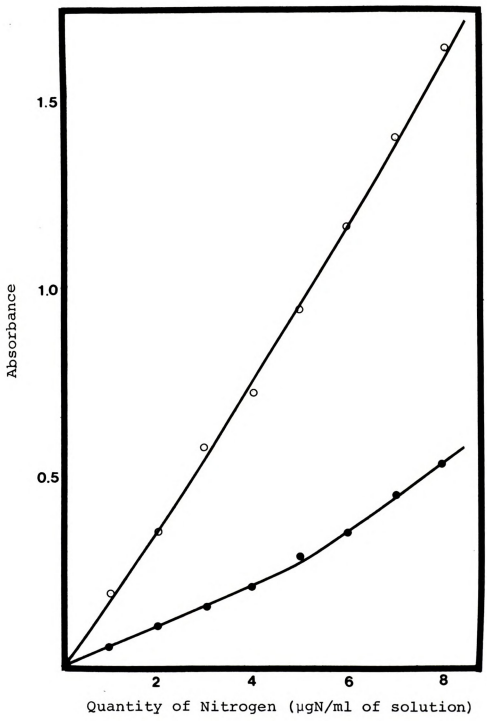
Buffer Solution (0.005 M Tris, 0.04 M Glycine, pH 8.3)

	g/l
Tris	0.6
Glycine	2.88



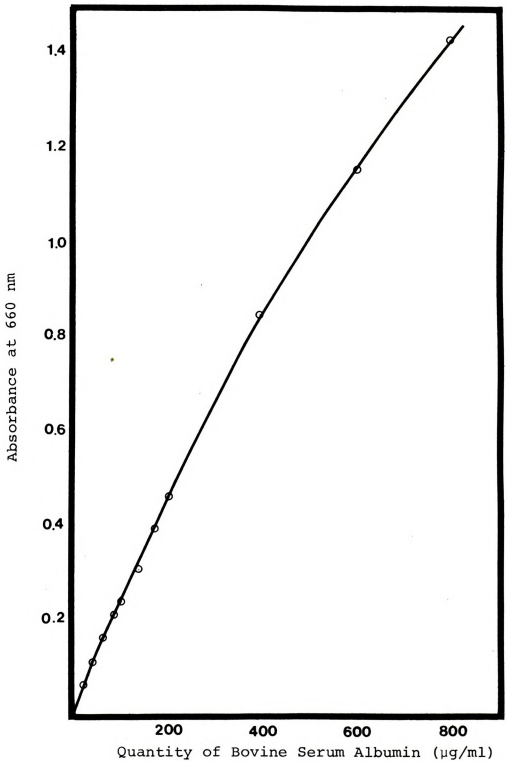
Appendix Figure 1. Conductivity of NaCl solutions.

○ ○ ○ NaCl in 0.02 M Tris-HCl
● ● ● NaCl in 0.02 M Acetate

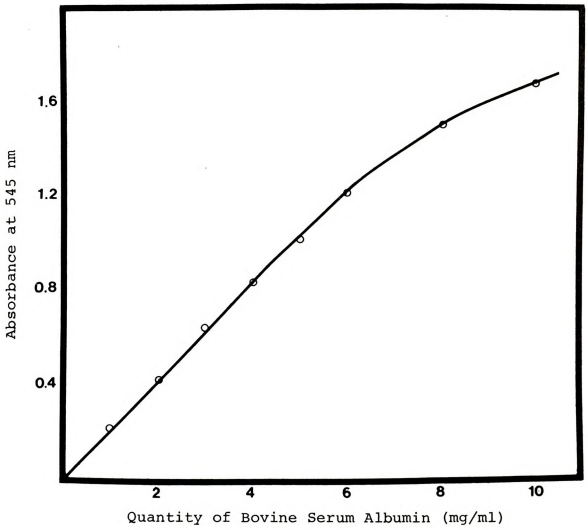


Appendix Figure 2. Standard curve for determining nitrogen using Nessler's reagent.

○ ○ ○ Absorbance at 420 nm
● ● ● Absorbance at 500 nm



Appendix Figure 3. Standard curve for determining protein using Lowry's method.



Appendix Figure 4. Standard curve for determining protein using the biuret method.