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Title   FRACTIONATION OF BOVINE MUSCLE PROTEINS BY

        CELLULOSE ION EXCHANGE CHROMATOGRAPHY

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The purposes for which the fractionation of proteins are

carried out are quite varied and manyfold. However, one of the

more important reasons is that of determining the nature and the

extent of autolysis or proteolysis on the bovine muscle proteins

during post mortem aging. Hence, the development of a procedure

for the adequate fractionation of muscle proteins would greatly stimu-

late the interest and research progress in this difficult field of study.

The research reported herein pertains to a study of the

fractionation of fresh bovine muscle proteins by ion exchange chro-

matography. A KCl-phosphate buffer, pH 7.5 and an ionic strength

of 0.55, was used to extract the proteins from the muscle. This ex-

tract was then diluted to specific ionic strengths in order to separate

the gross fractions (actomyosin, myosin, sarcoplasmic + actin)

from the total KCl-phosphate soluble proteins. The major protein fractions were then separated by diethylaminoethyl-cellulose (DEAE-cellulose) ion exchange chromatographic procedure. A non-linear gradient elution schedule was used throughout the chromatographic procedure. The disc electrophoresis technique was used to determine the homogeneity of the various protein fractions and sub-fractions.

The total KCl-phosphate soluble proteins were fractionated into 9 - 12 fractions by DEAE-cellulose ion exchange chromatography. The number of fractions differed from one sample to another. The disc electrophoresis results paralleled those of the chromatographic procedure. Some of the fractions appeared to be homogeneous while others were not.

The KCl-phosphate soluble proteins minus actomyosin were fractionated by column chromatography into 9 - 10 different protein components. The disc electrophoresis results also indicated that this fraction contained 9 - 10 components.

The sarcoplasmic + actin proteins were fractionated into six fractions by the chromatographic procedure. These fractions appeared to be electrophoretically homogeneous.

Although some success was attained in the separation of the actomyosin proteins, the myosin fraction was quite resistant to chromatographic separation.

In spite of the ineffectiveness of the DEAE-cellulose column chromatographic technique to fractionate the myosin and actomyosin proteins, the procedure appears to have some value for studying the other protein fractions during autolysis. Moreover, further work on the adoption of this procedure might provide the necessary information to perfect the technique for muscle protein fractionation.

FRACTIONATION OF BOVINE MUSCLE PROTEINS
BY CELLULOSE ION EXCHANGE CHROMATOGRAPHY

by

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FRACTIONATION OF BOVINE MUSCLE PROTEINS BY CELLULOSE ION EXCHANGE CHROMATOGRAPHY

INTRODUCTION

Some of the most challenging but perplexing problems involved in the post-mortem aging of meat are related to a series of intricate chemical events responsible for the dissolution of rigor mortis. Although many research studies have been completed in an attempt to elucidate the fundamental nature of these changes, the necessary evidence to provide a logical explanation has thus far eluded the efforts of many scientists.

Autolysis, indicating the degradation of tissue proteins, is assumed to be the major factor regulating the dissolution of rigor mortis. Although such may well be the case, the synonymous terms "autolysis"and"proteolysis" generally encompass a wide and unspecified range of chemical activities with reference to the breakdown of muscle proteins.

A variety of experimental procedures have been used in numerous investigations to study the effects of proteolysis in meat. However, most of these procedures measured changes in the accumulation of the end products rather than following changes in the initial and intermediate compounds. Furthermore, many of the prior studies reported changes in the nitrogenous components of meat held appreciably longer than the normal aging period. Thus, data from these studies have provided a rather wide range of results

and conclusions. Whitaker (91) recently emphasized the fact that muscle proteins can undergo extensive degradation before many amino acids are liberated and detected. Hence, it becomes strikingly apparent that the initial and the intermediate proteolytic changes must be measured and studied in order to determine the extent and role of proteolysis in the dissolution of rigor mortis and also in the subsequent tenderization process.

The lack of a relatively inexpensive but adequate means for the separation of proteins in complex biological mixtures has greatly hindered research progress in the field of meat proteins. Many of the techniques used in the separation of protein fractions were very laborious, time-consuming and demanding. Moreover, these techniques frequently require the use of expensive instruments, such as an ultracentrifuge, to complete the separation procedure. Thus, these techniques were mainly used by the basic scientist to isolate, purify and characterize the various protein components.

Cellulose ion exchange chromatography (72, 73) has emerged recently as a powerful tool for the separation and fractionation of some rather divergent protein components. This technique has been used successfully for the separation of milk proteins (92), analysis of blood sera proteins (74), purification of various hormones and enzymes (73), and characterization of egg white proteins (65).

Moreover, the procedure appears to fulfill the necessary require-

ments of being a mild and delicate means for fractionating proteins

as well as being a relatively inexpensive acquisition obtainable by

most laboratories.

In view of the apparent advantages of this procedure, research

reported herein pertains to the study of the fractionation of fresh mus-

cle proteins by DEAE-cellulose ion exchange chromatography. In ad-

dition, disc electrophoresis analysis was used to test the homogeneity

of the various protein fractions.

LITERATURE REVIEW

Proteins commonly occur in complex mixtures. The number of proteins in some of these mixtures is relatively small with one type often predominating, such as casein in milk (53, p. 202). In others, particularly those composing the cells of most animal tissues, the number of proteins tends to be very large (1, p. 708).

Muscle Proteins

Szent-Gyorgi (81, p. 1) has divided the muscle proteins into four general classes as follows: the sarcoplasmic proteins, the granular proteins, the stroma proteins and the myofibrillar proteins. He based this classification on the differences between the protein fractions in regard to extractability, locality in the muscle cell, and physiological properties.

Sarcoplasmic Proteins

The sarcoplasmic proteins contribute about 30 to 35 percent of the total muscle protein. These proteins occupy the spaces between the myofibrils and do not appear to be involved in the structural organization of muscle that results in contraction or relaxation. Myoglobin, enzymes, and all of the components of the

glycolytic cycle are found in this group (81, p. 1-2).

Granular Proteins

These proteins are also located between the myofibrils and somewhat in the specialized Z-membranes of the muscle cells. The important components of the granules are the nuclei, mitochondria and the microsomes. Although these proteins are mainly involved in the oxidative reactions of the muscles, some of their products may indirectly influence the activities of the structural proteins during muscular activity (81, p. 2-3).

Stroma Proteins

The stroma proteins account for 15 to 20 percent of the muscle proteins. They appear to be of a collagenous nature and are involved in the make-up of various muscle membranes and connective tissues. Knowledge of the stroma proteins is very limited because research on these proteins has been grossly neglected due to their extremely poor solubility properties (81, p. 3).

Myofibrillar Proteins

This class of proteins form the filamentous organization of muscle that is directly involved in muscle contraction and relaxation.

They are commonly referred to as the "structural proteins" since they are of a fibrous nature. The myofibrillar proteins make-up over 50 percent of the total muscle protein content. Actin, myosin and tropomyosin are the major components of this group. In addition, actin and myosin form a complex, actomyosin, when muscle goes into contraction or rigor. The amount of actomyosin formed at any given time is variable with degree of contraction or phase of rigor (2, p. 75).

According to Huxley (39, p. 68), the interaction of myosin, actin and adenosine triphosphate (ATP) appear to have all the essentials of a contractile system. His studies on muscle structure with electron microscopy techniques have indicated that myosin exists as the thick filaments of the myofibrils while the thin filaments are composed of actin. The thin or actin filaments tend to oscillate or slide back and forth along the intact myosin filaments during contraction and relaxation of the muscle. The rigidity of the muscle in rigor mortis is thought to be due to the formation of permanent links between the actin and myosin filaments to produce the actomyosin complex. Huxley visualizes these links to resemble that of a motor in which the pistons have "seized" from the lack of lubrication.

Post-mortem Changes in Muscle

The muscles of an animal at the time of slaughter are soft
and pliable. Instantaneous with death, ATP is broken down and its
concentration decreases. As the concentration of ATP diminishes,
the muscle slowly hardens until it becomes very rigid -- the full
state of rigor mortis. This condition, however, is actually the net
effect of a series of changes that begins the instant the animal dies
(2, p. 76-77).

Meat in the state of rigor is very tough. If the meat is placed
in a cooler for a few days, it gradually begins to soften again. The
events leading to the softening condition are often referred to as the
dissolution of rigor mortis (91, p. 31). This term is bravely used
as an all-inclusive phrase to describe a series of ill-defined changes.

During post-mortem aging, the actomyosin complex appears
to be split into its component parts of actin and myosin (2, p. 76-77).
The data of Bailey (4, p. 1005) show that during maximum rigor the
amounts of extractable actin and myosin are at a minimum, but they
become more soluble as the aging time increases. Since actomyosin
is less soluble than either actin or myosin, Bailey's results lend
support to the theory that actomyosin is degraded during aging.

The catheptic enzymes are considered to be one of the prime

factors causing the breakdown of the actomyosin complex even though they are present in muscle in minute amounts (91, p. 35). Information on the catheptic enzymes is very meager due to the extreme difficulties encountered in their extraction, purification and characterization (28, p. 405). Thus far, only three different proteinases of muscle have been adequately characterized. It is very difficult to evaluate the role of inherent proteolytic enzymes in the autolysis of meat since so much of the necessary evidence still remains in obscurity (91, p. 37).

Extraction and Properties of Muscle Proteins

Information presented under this topic describes some of the more pertinent isolation procedures and properties of the major muscle fractions. Although a variety of extraction procedures have been used, only the details of those which were reported to give fairly pure preparations are discussed.

Sarcoplasmic and Granular Proteins

These proteins can be extracted quite easily with water or solutions of neutral salts of a low ionic strength, $\Gamma/2 < 0.2$ (81, p. 1). Robinson (67, p. 621) reported excellent extraction of the sarcoplasmic proteins of chicken muscle with a phosphate buffer

containing 0.1 M KCl, pH of 7.1 and an ionic strength of 0.2. However, at an ionic strength of 0.2, the granular proteins are also extracted with the sarcoplasmic fraction. The granules are then separated from the sarcoplasmic proteins by differential centrifugation (81, p. 2). The sarcoplasmic and granular proteins are mainly concerned with the glycolytic and oxidative activities of the muscle.

Stroma Proteins

These proteins are retained in the residue of a well-homogenized muscle sample after prolonged extraction with a strong salt solution (81, p. 3). Since they are so difficult to extract, very little knowledge has been accumulated that would be of any significance in helping to explain their role in post-mortem aging.

Myofibrillar Proteins

Since the proteins of the myofibrils form the contractile structure of muscle, considerable information is found in the literature in regard to their extraction procedures. Thus for reasons of clarity, each component of this fraction will be discussed individually.

1. Myosin. The extraction of myosin from muscle is enormously complicated by its interaction with actin, ATP and various

ions (81, p. 5). However, satisfactory preparations have been obtained by the rapid extraction of the homogenized muscle with 0. 6 M KCl solution followed by dilution to lower the salt concentration to an ionic strength less than 0. 2 which allows the myosin to precipitate (81, p. 6). Weber and Meyer (89, p. 137) extracted myosin with 0. 6 M KCl, pH 8. 9. The myosin was then precipitated from the preparation by dialysis at pH 7. 0 while the KCl concentration decreased to 0. 03 M.

During the extraction of myosin, the main impurities carried along are actin and actomyosin (81, p. 7). Several procedures based on differences in the solubility between myosin and actin in ammonium sulfate or in potassium chloride are used to separate these two components. Actomyosin can be separated from myosin by differential precipitation at slightly alkaline pH and low ionic strength (81, p. 7), at slightly alkaline pH and ionic strength of 0. 15 in the presence of ATP (62, p. 62), or at neutral pH and ionic strength of 0. 3 in the absence of ATP (76, p. 180). Since myosin precipitates at higher ammonium sulfate saturation than actomyosin, Tsao (83, p. 368) used this fact to salt-out the actomyosin in ammonium sulfate and obtained an electrophoretically homogeneous myosin preparation.

Myosin contains large amounts of aspartic and glutamic acids

and a considerable amount of the dibasic amino acids which give the protein a relatively high charge (2, p. 74). Amino acid analysis, titration and electrophoretic data indicate that myosin is a negatively charged protein at physiological pH (2, p. 74). Myosin has been reported to have an isoelectric point of about pH 5.4 (81, p. 9).

When the extraction of the structural proteins is performed under sufficiently mild conditions, such as at neutral pH and an ionic strength of about 0.5, myosin and the other myofibrillar components retain their biological properties without exhibiting the obvious signs of denaturation (4, p. 962). This characteristic is very advantageous because the solubilized fractions are suited for further study of their general properties.

Myosin can be split into two smaller fractions by such enzymes as trypsin and chymotrypsin (2, p. 74). These two components show different sediment rates in the ultracentrifuge. The fraction that sediments slowly is called light meromyosin and the other, which sediments more rapidly, is designated as heavy meromyosin. The latter retains many of the properties of the intact myosin molecule (81, p. 10).

Szent-Gyorygi (81, p. 19) reported that myosin can be depolymerized by such denaturing agents as urea, which excludes the fission of the peptide bonds. The relationship between the

components obtained after urea treatment and the meromyosins has

not yet been clarified.

2. Actin. Actin possesses the characteristic property of

aggregating in the presence of salts but dissociating into monomers

in the absence of these ions (79, p. 80). Although actin is soluble

in water or low concentrations of neutral salt solutions, these solu-

tions do not readily extract it from the muscle (81, p. 25).

The difficulties encountered in extracting actin per se from

muscle arises from the fact that it is naturally present in muscle

in the fibrous form (81, p. 29). However, Barany et al. (6, p. 819)

observed that actin is readily extracted with such agents as potas-

sium iodide which depolymerize the actin.

Szent-Gyorgyi (78, p. 361) was able to obtain a fairly pure

actin preparation by extracting it with 0. 6 M KI in the presence of

ATP followed by ethanol fractionation. Hasselbach and Schneider

(33, p. 462) developed a procedure for the direct estimation of actin

by homogenizing the muscle in 0. 6 M KCl, pH 6-6. 3, containing

traces of sodium pyrophosphate. The latter reagent tends to disso-

ciate actin while myosin diffuses to the solvent leaving the actin as-

sociated with the stroma proteins. This slurry is homogenized

again in 0. 6 M KCl which yields a turbid, viscous extract of actin.

Another procedure for obtaining actin involves the use of the

muscle residue remaining after the partial removal of myosin (81, p. 25). When this residue is dried with a nonpolar solvent, such as acetone, any remaining myosin is denatured. The actin is then extracted in the globular form (G-actin) by distilled water at a neutral or slightly alkaline pH. Upon the addition of 0.1 M KCl and traces of $MgCl_2$, the G-actin is converted into the fibrous form (F-actin). In the presence of ATP, actin can be reversibly depolymerized (81, p. 25).

Mommaerts (58, p. 545) has found that during ultracentrifugation, 100,000 G, the F-actin sediments to form a pellet which can be redissolved as F-actin in 0.1 M KCl or as G-actin in ion-free water containing a low concentration of ATP.

The F-actin isolated directly from muscle differs somewhat from the polymers of G-actin formed in vitro (2, p. 75). Since actin can exist in either of two forms, as a monomer, termed G-actin (globular) or as a polymer, called F-actin (fibrous), such differences emphasize the caution that must be considered when attempting to compare in vivo and in vitro measurements (2, p. 75). Furthermore, the thixotropic effects of actin present serious problems in studying the purity of actin by electrophoresis techniques (76, p. 180; 58, p. 546).

Actin is present in muscle with respect to myosin in a ratio

of 1:3 (2, p. 75). Its isoelectric point, about pH 4.7, is somewhat

lower than that of myosin.

3. <u>Actomyosin.</u> Actomyosin is the complex formed when

solutions of actin and myosin are brought together. The complex is

characterized by a viscosity higher than the sum or the components,

actin and myosin, by a high sedimentation constant, and by a high

molecular weight (81, p. 68).

Actomyosin precipitates at a higher KCl concentration and at

a lower ammonium sulfate saturation than myosin. Normally, neu-

tral salt solutions having an ionic strength of 0.5 or greater are

used to extract it from muscle (4, p. 986). However, this extrac-

tion condition is also suitable for extracting the other muscle pro-

teins along with the actomyosin. The latter is then separated by

lowering the ionic strength of the buffer to a point where the acto-

myosin precipitates but leaving the other extracted proteins in solu-

tion.

Khan (41, p. 430) has developed a procedure whereby the

initial extracting buffer is diluted from an ionic strength of 0.5 to

0.25 followed by differential centrifugation to obtain a fairly com-

plete separation of the actomyosin from the other components.

Due to the complexity of actomyosin, it has not been very

well characterized.

4. <u>Tropomyosin.</u> This protein was isolated and character-
ized by Bailey in 1948 (5, p. 271). It now appears to be a fibrous
constituent of the myofibril universally occurring in mammalian
muscle (81, p. 30). The relationship of tropomyosin to the activity
of the muscle cells is not known.

Tropomyosin is very resistant to alkali, acid or heat treat-
ments and can be stored as a lyophilized powder (81, p. 30). Tropo-
myosin is generally extracted from muscle which has been dried by
ethanol and ether. After the muscle has been dried, tropomyosin is
extracted with 1 M KCl at pH 7 followed by repeated ammonium sul-
fate fractionations until the protein is purified. The purity of tropo-
myosin can be followed quite easily since this protein contains no
tryptophan and only minute amounts of proline. The isoelectric
point of tropomyosin is reported to be about pH 5.1 (81, p. 31).

Hamoir (31, p. 116) has found that tropomyosin obtained by
salt extraction without dehydration is always contaminated with
nucleic acid. He has labeled this preparation as "nucleotropomyosin."
This complex may have been formed during extraction, since the
association between the tropomyosin and the nucleic acid appears to
be very weak.

Tropomyosin can be crystallized. It is the only known fibrous
protein that forms true crystals with sharp edges and faces

(81, p. 30). In addition, these crystals appear to be unique because they contain approximately 90 percent water.

Cellulose Ion Exchange Chromatography

The separation of components of complex mixtures has always been a serious problem, a problem particularly difficult when macromolecules of biological systems are to be investigated. In regard to proteins, it is necessary to disrupt the cellular organization characteristic of life and extract them with a suitable solvent. This procedure inevitably brings into solution many of the other constituents present in the cells, and the task of separating the desired protein from the unwanted materials becomes a test of the experimenter's skill and, very frequently, of his good fortune. Since proteins are fragile chemical entities, the nature of the laboratory procedures for separating them is restricted. It is general knowledge that a slight change in the structure of the protein molecule may lead to alteration of its properties. Thus, it is important to avoid or to control changes in the molecule during the isolation procedure.

General Considerations

Sober and Peterson (72, p. 1116-1126) have developed a series of cellulosic ion exchangers that possess a high capacity for

the adsorption of proteins, yet release them completely under mild

conditions. These cellulosic adsorbents are available as either

anion or cation exchangers. Diethylaminoethyl-cellulose (DEAE-

cellulose) and carboxymethyl-cellulose (CM-cellulose) are examples

of anion and cation exchangers, respectively. The acidic or basic

groups are attached to the cellulose by reacting the appropriate halo-

gen derivative with alkali-swollen alpha cellulose. The ionizing

groups are attached by ether linkages and the stability of these ad-

sorbents is assumed to be essentially that of the cellulose matrix.

They can stand short treatment with fairly strong mineral acids

while being very resistant to strong alkali (72, p. 1116).

When a mixture of proteins in an appropriate buffer is applied

to an ion exchange column, some of the proteins form many electro-

static bonds with the exchanger and thus become tightly bound to the

column. As long as the initial conditions are maintained, the pro-

teins will not move or migrate. Some of the other proteins of the

mixture move through the column without hindrance because they do

not contain sufficient charges of appropriate sign to form multiple

bonds with the adsorbent. Still a third group of proteins may have

charges in such number and sign that under the existing conditions,

the number of electrostatic bonds formed with the exchanger permits

a reasonable probability, at a given moment, for the simultaneous

dissociation of all those restraining a given molecule. At the instant

of release the protein moves, only to be held again at another site as

electrostatic bonds are momentarily re-established. These mole-

cules undergo "true chromatography" which depends upon a series

of successive adsorptive and desorptive processes for its high re-

solving potential (73, p. 65, 66).

Although proteins can sometimes be eluted from the ion ex-

change adsorbent by one eluant under specific conditions of pH and

salt concentration, the range of adsorption affinities usually encoun-

tered with proteins is too great to allow differential elution of all with

a single eluant. Hence, stepwise or gradual and continuous increase

in salt concentration and/or change in pH are most often used during

the elution procedures. Adsorption is enhanced at low salt concen-

trations while desorption is favored by increased salt and/or a

change in pH. The effect of salt appears to be predominantly one of

promoting dissociation of the electrostatic linkages formed between

the protein and adsorbent while the main effect of pH is assumed to

be that of changing the number of charges on the protein and, in some

cases, on the adsorbent (72, p. 1117).

Application

The cellulose ion exchangers have been used very successfully

for the fractionation of biological mixtures containing closely associated proteins (74, p. 763). Boardman and Partridge (16, p. 208) reported that they were able to separate neutral proteins differing in isoelectric points of only 0.6 of a pH unit on cellulose adsorbents. Sera proteins from both human and horse blood have been adequately separated on DEAE-cellulose columns, while CM-cellulose was used for the successful fractionation of the egg white proteins (72, p. 1116-1126). The enzymes of bovine pancreatic juice have been separated and isolated by DEAE-cellulose chromatography without apparent loss of their enzymatic activity (42, p. 233).

Cellulose ion exchange chromatography appears to be of special importance in certain areas of medical research. Huismann et al. (37, p. 312-327) were able to study specific changes occurring in blood proteins during certain disease conditions by this technique. Humphrey and Porter (38, p. 196-197) successfully use DEAE-cellulose for the isolation of the reagins (skin-sensitizing antibodies) directly from the blood serum of allergic individuals. Hoyer, et al. (35, p. 517; 36, p. 312) and Ornsbee and Hoyer (59, p. 529) have made considerable progress in the purification of several representative animal viruses and rickettsiae by utilizing both DEAE- and ECTEOLA-celluloses in their chromatographic procedures. The ECTEOLA-cellulose is a complex produce of

epichlorohydrin and triethanolamine.

Recovery of the ionized substances from the columns is quite good except in the case where a substance is known to be labile under the conditions existing in the column or where retention is by forces other than straight forward ion exchange (17, p. 102). In general, an excess of 90 percent recovery of the proteins has been achieved. An exception to this occurs with avidin of egg white, of which only 75 percent can be recovered (73, p. 68). Mandeles (51, p. 260) has stated that the elution patterns are reproducible to within five percent provided that the columns used in the comparison were made from the same lot of washed cellulose, and the material being chromatographed was the same for each column. Furthermore, Mandeles reported that if all experimental conditions were rigidly controlled to hold the differences to an absolute minimum, patterns obtained with egg white on comparative columns were practically indistinguishable from each other.

In the interpretation of the effluent diagrams obtained from column chromatography, it must be borne in mind that variations in the position of the peaks can be due to several factors. In most instances, the minor variations can be attributed to slight differences in flow rate, buffer concentrations and temperature (57, p. 191). If the variations in comparative effluent curves tend to be somewhat

large, then it is fairly certain that contamination or changes in the composition of the sample occurred prior to placing it on the column (57, p. 230).

In many instances, buffers of very low ionic strength are used at the beginning of the column separation, whereby the position of a given protein peak may be extremely sensitive to extraneous factors and possibly to protein-protein interaction as well. Therefore, it may not be possible to conclude that two mixtures of proteins are the same or different by examining the effluent curves (73, p. 65). Hence, it is important to check the homogeneity of the fractions corresponding to the various peaks of the chromatogram by several independent means, such as by electrophoretic behavior, biological and enzymatic activity (57, p. 231).

Trial #3: A KCl-phosphate buffer (0.4 M KCl, 0.049 M

$Na_2HPO_4 \cdot 7 H_2O$, 0.0045 M $NaH_2PO_4 \cdot H_2O$),

pH 7.5, ionic strength of 0.55 (41, p. 431).

<u>Elution schedules</u> (non-linear gradient)

Trials #1 and #2, elution scheme and eluant buffer were

constant for both trials as given below:

1st buffer ------- 0.02 M glycine

2nd buffer ------- 0.02 M glycine + 0.02 M KH_2PO_4

+ 0.02 M K_2HPO_4

3rd buffer ------- 0.02 M glycine + 0.1 M KH_2PO_4

+ 0.1 M NaCl

4th buffer ------- 0.02 M glycine + 0.1 M KH_2PO_4

+ 0.1 M NaCl + 0.03 M HCl

Trials #3

1st buffer ------- 0.005 M NaH_2PO_4, pH 7.0

2nd buffer ------- 0.02 M NaH_2PO_4, pH 6.0

3rd buffer ------- 0.05 M NaH_2PO_4, pH 5.0

4th buffer ------- 0.05 M NaH_2PO_4 + 0.1 M NaCl,

pH 4.0

5th buffer ------- 0.10 M NaH_2PO_4 + 0.5 M NaCl,

pH 3.5

The results of the above preliminary studies are briefly mentioned at this point because they served as the basis for choice of buffer in the main experiments.

The number of fractions appearing on the effluent diagrams from the DEAE-cellulose chromatographic separation of the total muscle proteins extracted by the three media were 5, 1, and 12, for the glycine-NaOH, borate and phosphate buffers, respectively. Since the phosphate buffer showed greater superiority in extracting the muscle proteins and/or greater compatibility with the DEAE-cellulose separation procedure, this buffer was used solely in the main experiments.

Main Experiments

Sample Source and Preparation

Sections of loin muscle (longissimus dorsi) obtained from three beef animals were used throughout the course of this study. Animal description and time intervals are given below:

Animal #1 - Cow, approximately 3.5 years of age, time interval between death and mincing of sample was two hours.

Animal #2 - Bull, about five years of age, three hour time interval between death and mincing of sample.

Animal #3 - Cow, about six years of age, three hour time

interval between death and mincing of sample.

After all of the visible external fat and membranes were re-
moved from the meat, a 50 gram sample of the lean muscle was cut
into as small pieces as possible. This sample was ground with one
and one-half times its weight of acid-washed sand plus eight times
its weight of Na-phosphate KCl buffer, pH 7.5 and ionic strength of
0.55. The grinding was accomplished manually with a pre-cooled
mortar and pestle at 34°F. Approximately 30 minutes were required
for complete dispersion. The slurry was then centrifuged at 750 x G
for 15 minutes at 34°F. The supernatant, containing the total Na-
phosphate KCl soluble proteins, was transferred into a volumetric
flask and adjusted to a fixed volume which was ten times the initial
weight of sample extracted. This procedure was recommended by
Weinberg and Rose (90, p. 376-379).

The scheme for the gross separation of the total Na-phosphate
KCl soluble proteins into actomyosin, myosin and sarcoplasmic +
actin fractions is shown in Figure 1. This procedure was devised
by Weinberg and Rose (90, p. 376) and has been successfully applied
by Khan (41, p. 431), for the fractionation of chicken muscle pro-
teins. Both actomyosin and myosin were initially obtained as

Total Na-phosphate KCl soluble
proteins

|

diluted to $\Gamma/2 = 0.225$ kept overnight and
centrifuged at 1500 x G for 15 minutes

Supernatant   Residue
(Myosin and sarcoplasmic + actin   (Actomyosin)
proteins)
diluted to $\Gamma/2 = 0.05$, kept over-
night and centrifuged at 1500 x G
for 15 minutes

(Sarcoplasmic + Actin)   Residue
                                                     (Myosin)
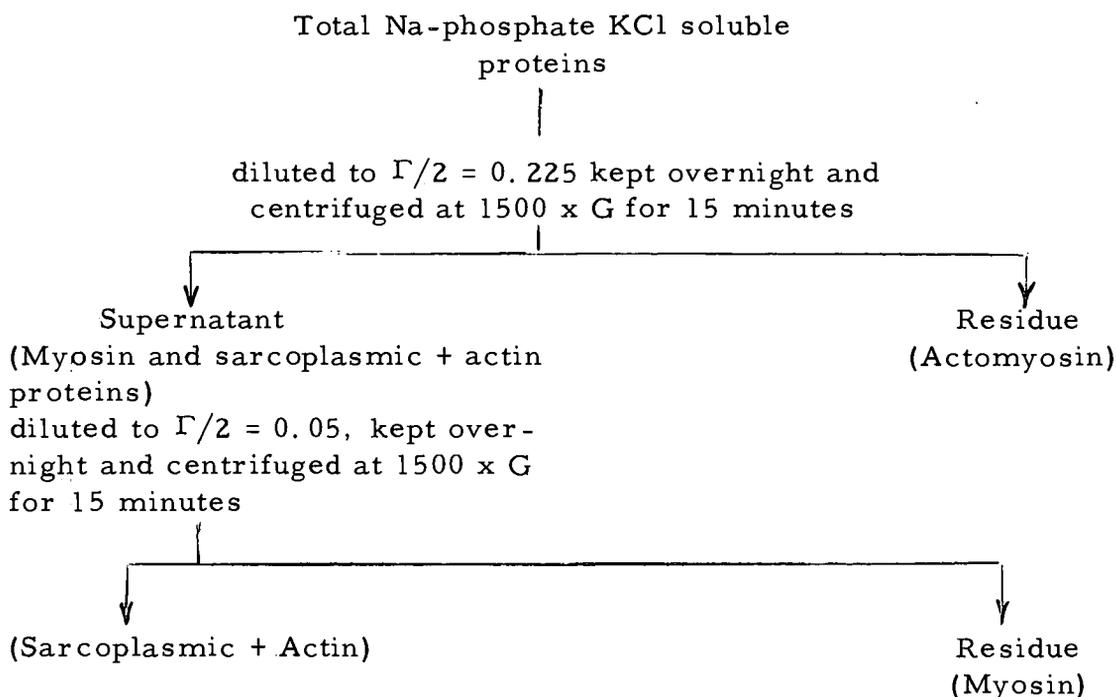
Figure 1. Scheme for the separation of bovine muscle proteins.

residues but were brought back into solution by the same initial ex-

tracting buffer (Na-phosphate KCl, pH 7.5) for further analysis.

All operations were performed at $34^{\circ}F$.

Column Chromatography Analysis

Since most of the known proteins of muscle have isoelectric

points between pH 4 and 7 (81, p. 9), the anion exchanger, DEAE-

cellulose, was used in this study.

Preparation of the cellulose column. DEAE-cellulose, type

20, 0.94 mequiv./g was used in this experiment. Before use, the

DEAE-cellulose was washed on a Büchner funnel with fritted glass disc successively with 0.1 N HCl - 1 M NaCl, water, 1 M NaHCO$_3$, water, 1 M Na$_2$CO$_3$, water, 0.1 N NaOH, water, ethanol and water. The cellulose was then suspended in water overnight to allow the trapped air bubbles to escape. After the suspension had settled, any fines that remained were decanted (92, p. 590). The washed cellulose was soaked in 0.1 N NaOH to insure complete regeneration (51, p. 256). The adsorbent was then washed with large volumes of distilled water to neutrality, followed by washing with several volumes of the starting eluting buffer to equilibrate the cellulose to pH 7.0.

The washed cellulose suspension (50 g suspended in one and one-half liters of the starting buffer) was placed in a beaker fitted with a magnetic stirrer which agitated the mixture continuously in order to maintain a homogeneous suspension throughout the course of packing the column. A glass column (70 cm long x 2.5 cm diameter) fitted at the bottom with a plug of glass wool was used as the chromatographic column. After a piece of filter paper was placed on top of the glass wool, the column was filled with the starting eluting buffer. Glass tubing (one quarter inch in diameter) was then used to siphon the cellulose into the column. The adsorbent was allowed to pack and settle in the column under gravity. After

the desired height (35 - 50 cm) was obtained, a piece of filter paper
was put on top of the packed cellulose to prevent erosion by the in-
coming protein solution. The column was held in the cold room
(34 - 38°F.) at least 12 hours before being used. All chromatogra-
phic fractionation runs were performed at the above mentioned tem-
perature range.

Elution schedules. Throughout the entire course of frac-
tionating the proteins on the DEAE-cellulose column, a non-linear
gradient elution schedule was carried out according to the procedure
of Sober, et al. (74, p. 757). Fifty ml of protein extract was enter-
ed at the top of the column and permitted to sink almost entirely
into the column before being washed with portions ranging between
20 - 100 ml of the starting buffer. Non-linear gradient elution was
then initiated, using a mixing chamber containing the starting eluting
buffer and a reservoir containing the second eluting buffer. As the
buffer in the mixing chamber flowed into the column, the next buffer
automatically flowed into the chamber because of the slight vacuum
created by the reduction in volume of the starting buffer. Thus the
amount of buffer in the mixing chamber is always nearly constant.
When the reservoir chamber became empty, it was filled with the
buffer next in series to the exhausted one, and this operation was
repeated until the last buffer was added. The schedule of eluting

solutions (500 ml/buffer) was in the following order:

1st buffer ---- 0.005 M Na phosphate, pH 7.0

2nd buffer ---- 0.02 M Na phosphate, pH 6.0

3rd buffer ---- 0.05 M NaH_2PO_4, pH 5.0

4th buffer ---- 0.05 M NaH_2PO_4 + 0.1 M NaCl, pH 4.0

5th buffer ---- 0.10 M NaH_2PO_4 + 0.5 M NaCl, pH 3.5

Flow rates in the order of 2 - 3 ml/min were used, and the

effluent was collected by LKB fraction collector, Assembly OA

equipped with a 10 ml volumetric siphon. Approximately 22 hours

were required per chromatographic separation of any one of the

gross protein fractions when the flow rate was 2 ml/min as contrast-

ed to 14 hours when the rate was 3 ml/min.

During the course of chromatographic separation, the elu-

tion of proteins was followed by measuring the absorbancy of each

of the 10 ml fractions collected at 280 mμ in a Beckman DK-1 record-

ing spectrophotometer (51, p. 257). In addition, the pH of each of

the above fractions was measured with a Beckman Zeromatic pH

meter. The abosrbancy and pH data were then used to plot the efflu-

ent diagrams to obtain a graphic record for comparative purposes.

Total Nitrogen Determinations

Nitrogen estimation was determined by the standard

micro-Kjeldahl method (A. O. A. C. , 1955). Total nitrogen was de-

termined in aliquots ranging from 2 to 50 ml of the various protein

solutions. The actual volume per aliquot depended on the relative

protein concentration of a particular solution as indicated by the ab-

sorbancy reading at 280 mμ.

Disc Electrophoresis

The disc electrophoresis technique, as described by Davis

and Ornstein (22, p. 1-21), was used to determine the homogeneity

of the various protein fractions separated and eluted by the DEAE-

cellulose column. This electrophoretic procedure involves the se-

paration of the protein components on a small column of polyacryla-

mide gel in glass tubes (6. 5 long x 0. 5 cm internal diameter) under

a constant current of 5 ma. per tube for 25 minutes. However, the

duration of some runs was reduced to 15 minutes because a longer

duration caused fading of the color of the protein bands. After com-

pletion of an electrophoretic run, the proteins were fixed with seven

percent acetic acid and the fractions stained by one percent amido

schwarz dye. These protein fractions appear as dark bands on the

gel columns. The columns were then photographed in order to obtain

permanent records.

Amino acid determination

Sample hydrolysis. Two to 50 ml of a protein solution
(volume dependent upon concentration of protein/sample) were reflux-
ed for 12 hours at 100°C with an equivalent amount of 12 N HCl (15,
p. 99). To evaporate the hydrochloric acid after hydrolysis, the
samples were placed in a glass vacuum desiccator heated between
80 - 90°C and evacuated by water aspirator until dryness. Two ml
of pH 10 buffer (0.053 M boric acid, 0.053 M KCl and 0.047 M NaOH)
containing 10 percent isopropanol were added to the residue to bring
the hydrolyzed constituents into solution (30, p. 286).

Paper chromatography analysis. The one dimensional
paper chromatography technique of Hackman (30, p. 281-292) was
used to estimate the amino acid content of the analyzed samples.

Whatman #1 filter paper was used throughout the course of
the experiment with two different solvents. One solvent was com-
posed of phenol-water (80/20, V/V) with the addition of 50 mg of
8-hydroxyquinoline per 100 ml of solvent to preserve the phenol.
This solvent was used to separate the following amino acids: cysteine,
cystine, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine
lysine, serine, threonine and valine. The other solvent used was

n-butanol-acetic acid-water (77:6:17, V:V:V). The amino acids se-
parated by this solvent were proline, hydroxyproline, isoleucine,
leucine, methionine, phenylalanine, tryptophan and tyrosine (15,
p. 113; 30, p. 286).

Preliminary runs were completed on the various protein
effluent fractions to determine the amount to be spotted for a parti-
cular fraction. The amounts spotted on the regular runs ranged
from one to 50 microliters depending on the protein concentration of
the fraction. Eight spots were spaced on any one sheet of paper by
the use of micro-pipettes. The known standard was also spotted on
the same paper. A period ranging between 18 to 24 hours was usu-
ally allowed for the paper to reach equilibrium with the vapor of the
chromatographic chamber before the paper was dipped into the sol-
vent.

Three reagent solutions were used for the color develop-
ments of the analyzed amino acids (30, p. 284):

(1) For the amino acids developed in phenol-water solvent,
a solution of one percent ninhydrin in 95 percent ethyl alcohol con-
taining two percent glacial acetic acid was used.

(2) For the amino acids developed in butanol-acetic acid-
water, excluding proline and hydroxyproline, a solution of one per-
cent ninhydrin in 95 percent ethyl alcohol containing 0.25 percent

triethanolamine was used.

(3) For proline and hydroxyproline, one percent isatin in ethanol was used.

The color developing reagent was applied by spraying, followed by drying in stream of air for eight hours when ninhydrin was used or by drying at 100°C. for ten minutes when isatin was used. A Photovolt electronic densitometer Model 525 with a #47 Wrattan filter was used to measure the density of the colored spot. The area of the spot was carefully measured and a correlation between the area and the average density (nine readings) of the colored spot was calculated, which is explained in detail in the following section. The amount of each amino acid, by means of its area and average density values, was obtained by direct readings from the standard curves (15, p. 107).

Standard curves. A standard solution for each of the 20 amino acids to be analyzed was prepared with 0.005 M of the amino acid in pH 10 buffer (0.053 M boric acid, 0.053 M KCl and 0.047 M NaOH) containing ten percent isopropanol (30, p. 287). Four different concentrations of the standard amino acid solution were spotted on the chromatography paper and developed by the same technique described previously. During the preliminary experimental paper chromatography work, when different concentrations of one amino acid

were spotted and developed, the color intensities of these spots re-
mained fairly constant while the area of the spots increased almost
proportionately with the increase in amino acid concentration. Thus
it was possible to correlate the area of the spot and the average den-
sity reading with the known concentration of the amino acid in order
to construct a standard curve for each of the twenty amino acids to
be measured (15, p. 107). The procedure for the development of
the standard curves is described in this section. After completion
of the chromatogram, the circumference of the colored spot of each
amino acid was carefully marked in pencil and the area determined.
Nine absorbency readings were taken on the spot at arbitrary select-
ed points and the average value represents the intensity of the color
throughout the entire spot. Since each reading was measured from
an area of 3.14 mm^2 on the spot, a relativity factor between the
total area of the spot and the area of each density reading was calcu-
lated. This corrected reading when plotted on the log scale of semi-
log paper versus the concentration of the tested amino acid gave a
straight line relationship, thereby producing a standard curve (15,
p. 107-108).

RESULTS AND DISCUSSION

The results of the preliminary work are discussed briefly because they had a direct bearing on the nature of the main experiments.

Preliminary results

The initial experiments were conducted to determine which of the three extracting buffers(glycine-NaOH, KCl-borate and KCl-phosphate) would provide optimum conditions for both the extraction of muscle proteins and their subsequent separation by the DEAE-cellulose ion exchange chromatography procedure.

Although Zender, et al. (93, p. 307) have indicated that the glycine-NaOH buffer extracts only about 40 percent of the myofibrillar proteins, this buffer possesses a low specific electrical conductivity which permits its direct use in electrophoretic analysis. The DEAE-cellulose column separated the glycine-NaOH soluble proteins into five components which indicates that this buffer extracts the muscle proteins rather incompletely.

Khan (41, p. 432) reported that KCl-borate buffer is an excellent medium for the extraction of chicken muscle proteins. He found that it removed about 95 percent of these proteins. However, he

was interested only in the routine estimation of total soluble proteins at any given time during processing and storage treatments and not particularly concerned about maintaining the extracted proteins in their native state.

In the present study, the data showed that the DEAE-cellulose column did little or no separating of the KCl-borate soluble proteins since only one fraction was observed to be eluted during the entire chromatographic run. It has been reported (4) that this buffer is not applicable for those compounds containing two or more hydroxyl groups because the boric acid will react with these groups to form complex acids of an appreciable strength. Thus, the pH of the borate buffer may be perceptibly altered and the substances extracted are no longer present in their original state when in contact with this buffer.

The KCl-phosphate buffer has been widely used with good results in the extraction of muscle proteins (4, 41, 81). In this experiment, KCl-phosphate buffer extracted 80.4 percent of the total muscle protein. The proteins remaining in the residue were mainly the stroma proteins and perhaps a small amount, three to five percent, of the myofibrillar fraction. When the KCl-phosphate soluble proteins were chromatographed on the DEAE-cellulose column, 12 different protein components were separated and eluted in the pH range

of 7. 0 to 3. 5. These results indicated that the KCl-phosphate was an efficient extraction buffer while being compatible with the DEAE-cellulose column in separating muscle proteins. Hence, this buffer was used in the remainder of the experimental work.

Main Experiments

In order to obtain some idea of the efficiency and reproducibility of the diethylaminoethyl cellulose (DEAE-cellulose) anion exchange chromatography procedure in the separation of bovine muscle proteins, fractionation studies on the loin muscle proteins of three different animals were completed. In effect, this more or less represented three replications.

The disc electrophoretic patterns of the different KCl-phosphate soluble proteins that contribute to the formation of the bovine loin muscle are shown in Figure 2. This figure is presented at this point to show that the major protein fractions can be further separated into smaller components. In addition, these patterns provide an indication of the heterogeneity of the different fractions normally encountered under the present muscle protein classification. The components separated for each of the major protein fractions are shown as dark bands on the gel columns and are described as follows:
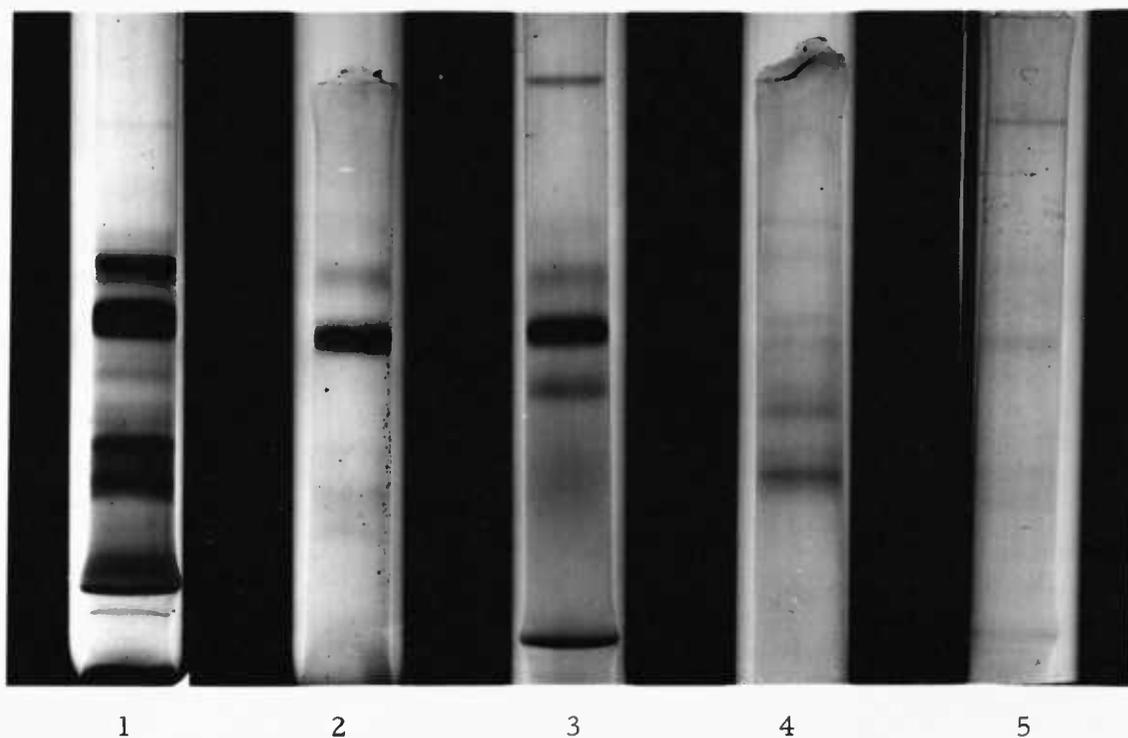
Figure 2. Disc electrophoresis patterns of the major bovine loin
muscle protein fractions of animal No. 3.
Legend:
1. Total KCl-phosphate soluble proteins, (13 bands)
2. Total KCl-phosphate soluble proteins minus the
actomyosin proteins, (9 or 10 bands)
3. Actomyosin proteins, (4 bands)
4. Myosin proteins, (8 bands)
5. Sarcoplasmic + actin proteins, (6 bands)

Fraction #1.   Total KCl-phosphate soluble proteins (13 bands).

Fraction #2.   Total KCl-phosphate soluble proteins minus actomyosin

(9 or 10 bands).

Fraction #3.   Actomyosin proteins (4 bands)

Fraction #4.   Myosin proteins (8 bands).

Fraction #5.   Sarcoplasmic + actin proteins (6 bands).

Although it is somewhat difficult to count the protein bands on

the gel column in the photogram, it was possible to count the bands

when the gels were placed between the observer and fluorescent light.

The differences in the disc electrophoretic mobilities of the

various components were probably due to a combination of factors

rather than to one specific effect.   The net charge of the protein com-

ponents undoubtedly plays a major role in determining the rate of their

migration.   However, the polyacrylamide gels also have the ability

to do a certain amount of molecular sieving whereby the molecular

weight and size of a protein exert an effect upon the electrophoretic

mobility.   When all conditions remain constant, the smaller proteins

show a greater rate of migration than the larger sized molecules

(22, p. 1-22).

Another point of interest in the photogram, Figure 2, is that

the sum of the protein bands or components of the actomyosin, myo-

sin, and sarcoplasmic + actin fractions is 18 as contrasted to a total

of 13 for the total KCl-phosphate soluble proteins. This difference

appears to be due to the overlapping of some of the bands on the gel

column of the total KCl-phosphate soluble fraction.

Protein recovery data

The term "protein recovery" refers to the amount of protein

eluted from the DEAE-cellulose column during a chromatographic

run. In practically every instance, there appears to be a certain

amount of protein firmly absorbed on the column and hence cannot be

accounted for in the effluent fractions (72, p. 1117). Sober and

Peterson (73, p. 61-65) have stated that although the binding forces

in effect during cellulose column chromatography are primarily of an

electrostatic nature, other factors may also be involved which tend

to hold some of the protein on the column.

Protein recovery was measured for each DEAE-cellulose

chromatographic run by determining the nitrogen content of the pro-

tein solution placed on the column and measuring the total amount

eluted into the various components. These data are shown in Table 1.

The results are expressed on a protein basis, and the recovered pro-

tein is given as a percentage of the protein initially applied on the

column. The amount of protein recovered per chromatographic run

appears to be quite good with the exception of the actomyosin fractions

Table 1. Protein recovery data of the various bovine loin muscle protein fractions eluted during DEAE-cellulose column chromatography.

	Amount applied	Amount recovered	Percent recovery
Total KCl-phosphate soluble proteins, animal No. 1	0. 511 g	0. 446 g	87. 3
Total KCl-phosphate soluble proteins, animal No. 2	0. 473 g	0. 408 g	86. 2
Total KCl-phosphate soluble proteins, animal No. 3	0. 951 g	0. 785 g	82. 5
Total KCl-phosphate soluble proteins minus actomyosin, animal No. 2	0. 361 g	0. 306 g	84. 7
Total KCl-phosphate soluble proteins minus actomyosin, animal No. 3	0. 334 g	0. 285 g	85. 4
Actomyosin proteins, animal No. 1	45. 47 mg	30. 60 mg	67. 3
Actomyosin proteins, animal No. 2	33. 62 mg	25. 72 mg	76. 5
Actomyosin proteins, animal No. 3	29. 87 mg	24. 94 mg	83. 5
Myosin proteins, animal No. 1	84. 73 mg	74. 56 mg	88. 0
Myosin proteins, animal No. 2	94. 17 mg	62. 71 mg	66. 6
Myosin proteins, animal No. 3	59. 22 mg	51. 29 mg	86. 6
Sarcoplasmic + actin proteins, animal No. 1	75. 13 mg	63. 49 mg	84. 5
Sarcoplasmic + actin proteins, animal No. 2	69. 66 mg	68. 42 mg	.98. 5
Sarcoplasmic + actin proteins, animal No. 3	168. 34 mg	157. 23 mg	93. 4

from animals No. 1 and 2 and the myosin fraction of animal No. 2.

The literature (72, p. 1124) indicates that generally 90 percent or

more of the protein applied to the columns was recovered in the ef-

fluents of most of the proteins thus far studied, which include the

proteins of blood sera, egg white and milk. However, it must be re-

membered that the muscle proteins are very complex macromolecules

possessing unique properties and characteristics which are quite di-

vergent from other known protein systems (81, p. 1-49). The low

protein recovery data of the three fractions mentioned above might

possibly be due to the development of strong binding between these

protein molecules and the adsorbent which hindered their release

under the buffer and eluting conditions employed. Furthermore, the

large size and peculiar configuration of the myosin and actomyosin

molecules may have somewhat retarded their passage through the

cellulose column.

Total KCl-phosphate Soluble Proteins

Column Chromatography and Disc Electrophoresis Analysis

Figures 3a, 4a and 5a show the effluent diagrams obtained

from the DEAE-cellulose ion exchange chromatographic separation

of the total KCl-phosphate soluble proteins extracted from a loin

muscle of each of three animals. Concentration of protein applied

for each run was as follows: Figure 3a, 50 ml of extract containing

0.511 g of protein; Figure 4a, 50 ml of extract having 0.473 g of

protein; and Figure 5a, 100 ml of extract with 0.951 g of protein.

The protein components were eluted from the column by the non-

linear gradient elution procedure which provides for means of a

gradual and continuous increase in the ionic strength of the eluting

buffer coupled with a concurrent decrease in the pH during the

chromatographic run. Throughout the course of this study, the

eluted protein components were given numerical designations accord-

ing to their sequence of emergence from the DEAE-cellulose columns.

Figures 3a, 4a and 5a also show the effluent pH curves which

graphically depict the changes in the pH of the effluent during the

course of each chromatographic run. The pH ranges at which the

different protein components were separated and eluted, and the re-

lative percentage of each component, calculated on the basis of total

protein recovered, are summarized in Table 2. Table 2 also gives

the number of protein bands shown on the disc electrophoresis gels

in Figures 3b, 4b and 5b. These were included in Table 2 as a mat-

ter of convenience for comparative purposes.

Figures 3b, 4b and 5b show the disc electrophoresis patterns

of the components eluted from the DEAE-cellulose column. The

fraction having the highest absorbancy value for each component was

used in disc electrophoresis analysis. This procedure was used to

test the homogeneity of components separated and eluted by the

chromatographic technique. Some of the components did not show

any sign of protein separation during disc electrophoresis and hence

they were omitted in the photograms.

Figure 3a shows that the DEAE-cellulose ion exchange chro-

matographic technique separated the total KCl-phosphate soluble

proteins of animal No. 1 into ten different components. This effluent

diagram and the protein recovery data of Table 2 show that the first

component to be separated and eluted contained 80 percent of protein

to pass through the column. The disc electrophoresis analysis,

Figure 3b, shows that this component is very heterogeneous since

six or more fractions could be separated by this technique. Protein

components 2 and 3 appear to be very closely related since both

emerge at almost the same pH range. The disc electrophoretic pat-

tern of component 3 shows two different protein bands while compon-

ent 2 shows none. Hence, there may have been a combination of

overlapping of the two components or poor column separation. Com-

ponent 9 appears to be eluted from the DEAE-cellulose column as a

single entity since the photogram, Figure 3b, indicates that it may

be electrophoretically homogeneous. Although component 10 exhi-

bited a fairly sharp peak on the effluent diagram, it was found to

Figure 3 b. Disc
electrophoresis pat-
terns of effluent
components shown
in Figure 3 a.

Figure 3 a.   Effluent diagram of total KCl-phosphate soluble
proteins extracted from the loin muscle of animal
No. 1.   Adsorbent was DEAE-cellulose (40 x 2.5
cm).   Fifty ml of extract containing 0.511 g of
protein was applied.   Starting buffer, 0.005 M Na-
phosphate, pH 7.0, non-linear gradient elution,
$36^{\circ}F$.

*   Dash lines (---) indicate components analyzed for nitrogen while
arrows indicate fractions analyzed by disc electrophoresis.

Table 2. Percentage of protein and pH range of emergence of the protein components of the total KCl-phosphate soluble loin muscle proteins separated and eluted by DEAE-cellulose ion exchange chromatography.

Sample number	Component number	pH range of emergence	Percentage of recovered protein as eluted per component	Disc bands[*]
Animal	1	8.23 - 7.28	80.31	6 or more
No. 1	2+3	7.57 - 7.73	2.04	2
	4	7.23 - 7.00	0.34	-
	5	7.00 - 6.57	1.68	-
	6	6.56 - 5.98	2.05	-
	7	5.92 - 5.67	3.64	1
	8	5.64 - 5.18	0.68	-
	9	5.14 - 4.65	6.09	1
	10	4.62 - 4.34	4.15	2
			Total 100.98	
Animal	1	8.47 - 7.62	66.23	6 or more
No. 2	2	7.60 - 8.22	0.42	-
	3	8.22 - 7.06	6.71	2
	4	7.08 - 6.45	3.03	1
	5	5.93 - 6.01	1.51	-
	6	6.00 - 5.00	6.75	1
	7	4.93 - 4.37	9.64	1
	8	4.35 - 4.18	3.49	-
	9	4.17 - 4.08	2.20	1 or 2
			Total 99.98	
Animal	1	8.70 - 8.06	67.76	6
No. 3	2	8.06 - 7.82	18.16	5
	3	7.82 - 7.83	5.04	2
	4	7.83 - 7.46	0.44	-
	5	7.46 - 6.98	0.44	-
	6	6.96 - 6.84	0.15	-
	7	6.81 - 6.32	0.96	-
	8	6.32 - 6.20	0.37	-
	9	6.18 - 5.27	1.71	1
	10	5.27 - 4.41	0.49	-
	11	4.41 - 4.30	3.23	1
	12	4.30 - 4.10	1.26	1 or 2
			Total 100.01	

[*]Number of bands appearing on the disc electrophoresis gels of the separated protein components.

contain two electrophoretically different protein compounds. When analyzed by disc electrophoresis, components 2, 4, 5, 6 and 8 did not show any stainable protein fractions. One possible explanation for the failure of these components to show protein bands is that at the time they were eluted from the column the particular conditions of pH and ionic strength of the buffer may have altered and rendered them electrophoretically inert. Another reason may be that the eluting conditions caused the components to lose their capacity to be stained by the staining dye used. In any event, the above explanations are purely speculative.

Figure 4a represents the effluent pattern of total KCl-phosphate soluble protein extracted from loin muscle of animal No. 2. This diagram shows that nine protein components were fractionated from this sample. Table 2 indicates that 60 percent of the total protein eluted from the column was contained in component No. 1.

The disc electrophoresis patterns of the protein components separated by the column for animal No. 2 are shown in Figure 4b. These results show that the first component can be further separated into at least six fractions. Component 3 contains two electrophoretically different fractions. Components 4, 6, 7 and 9 each show only one band which is an indication of homogeneity. Components 2, 5 and 8 did not show any electrophoretic fractions.
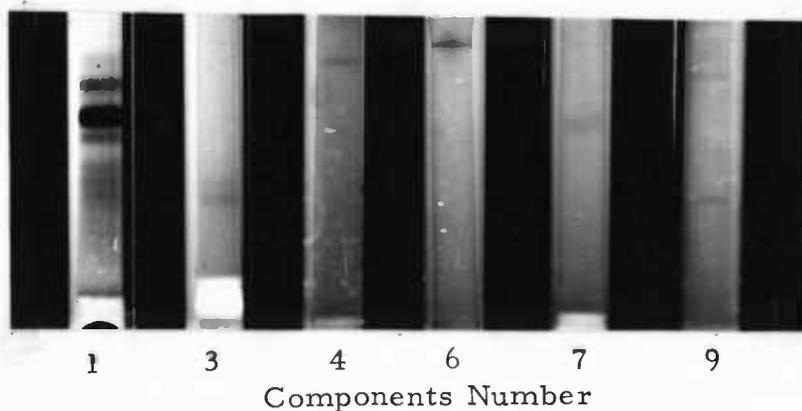
Figure 4 b.   Disc electrophoresis patterns of the
effluent components shown in Figure
4 a.

*Dash lines (---) indicate
components analyzed for
nitrogen while arrows
indicate fractions analyzed
by disc electrophoresis.

Effluent pH
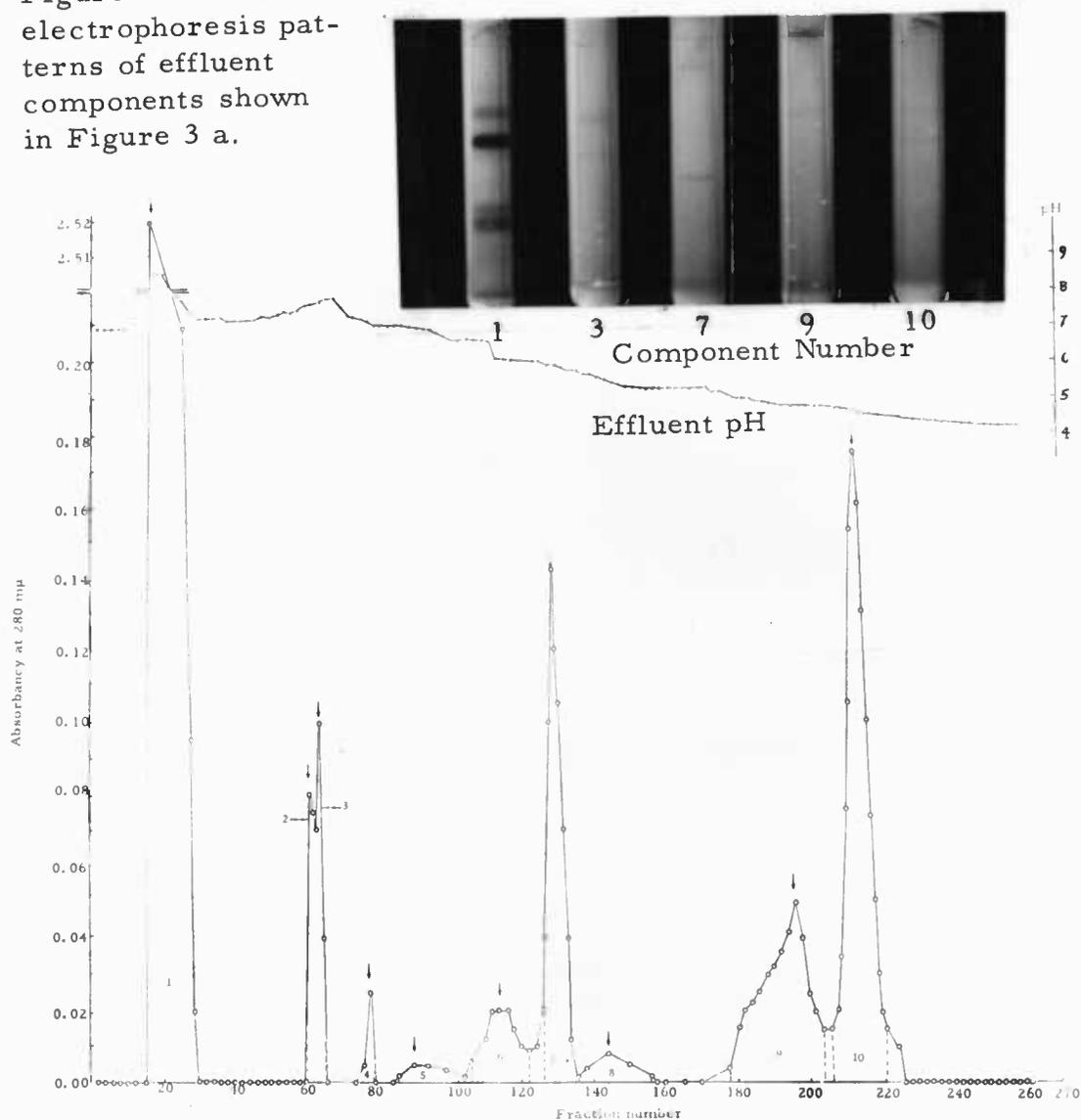
Figure 4 a.   Effluent diagram of total KCl-phosphate soluble pro-
teins extracted from loin muscle of animal No. 2.   DEAE-cellulose
column (40 x 2.5 cm).   Fifty ml of extract containing 0.473 g of
protein was applied.   Starting buffer, 0.005 M Na-phosphate, pH
7.0,   non-linear gradient elution, 36°F.

The effluent diagram of the KCl-phosphate soluble proteins of animal No. 3 is given in Figure 5a. However, the amount of protein applied to this column (0.951 g) was about twice that applied for the previous two samples (0.511 and 0.473 g). Since some of the fractions were observed to be present in minute amounts in the two prior runs, it was thought that better separation might occur by increasing the protein concentration. Figure 5a shows that 12 components were separated during this run. Table 2 indicates that over 60 percent of the protein recovered was contained in the first component to be eluted while 18 percent was in the second component.

Figure 5b shows the disc electrophoresis analysis of the eluted components. Components 1 and 2 appear to be very heterogeneous by showing at least five protein bands. Component 3 shows two protein fractions while components 9, 11 and 12 each seem to have only a single band. The remaining components gave negative electrophoretic results.

The larger quantity of protein applied on the DEAE-cellulose column for the third sample might be the reason for the emergence of more protein components in this case than in the two previous runs. Some of the protein fractions that were not separated in the first and second samples had a better chance to appear in the third run when the amount of protein applied to the column was increased.

Component Number
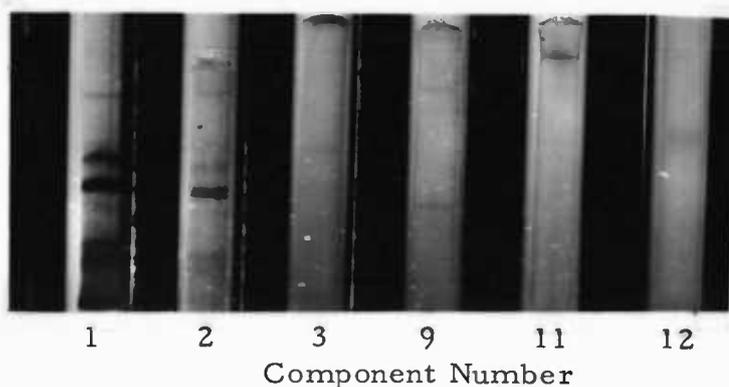
Figure 5 b. Disc electrophoresis patterns of
the effluent components shown in
Figure 5 a.

Effluent pH

*Dash lines (---) indicate
components analyzed for
nitrogen while arrows
indicate fractions ana-
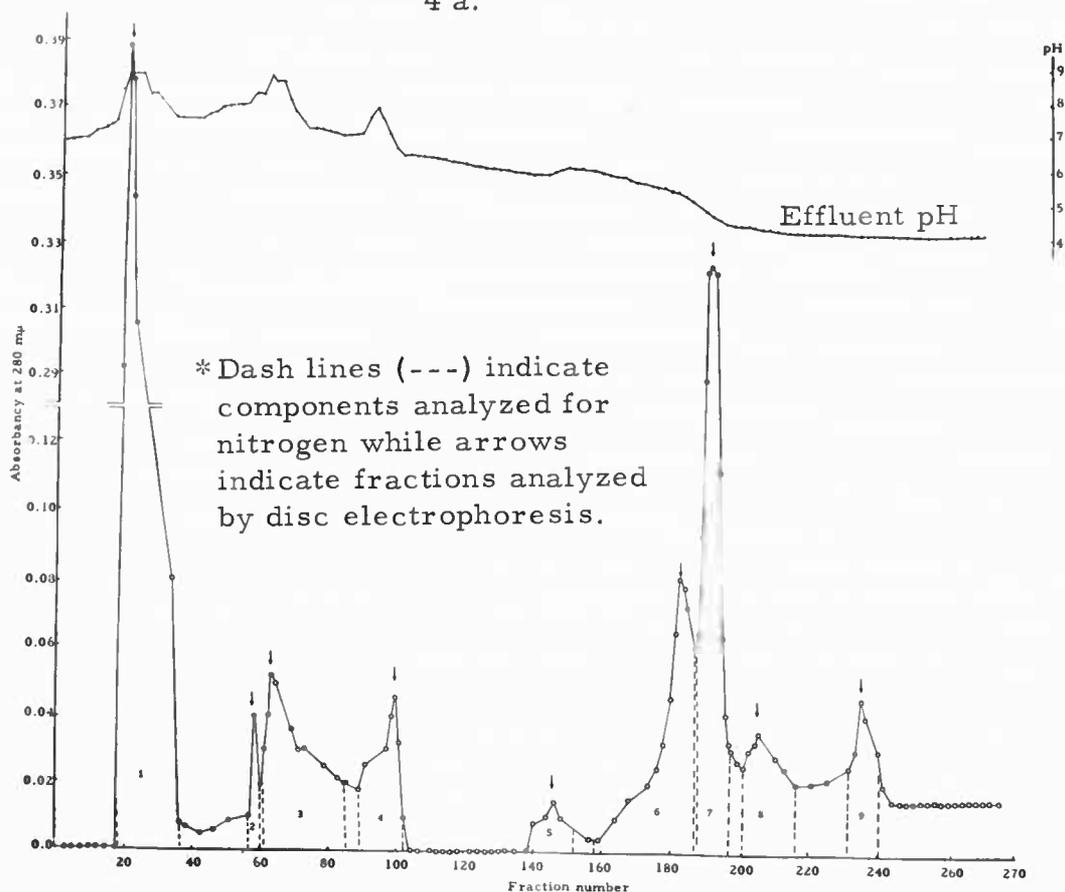lyzed by disc electro-
phoresis.
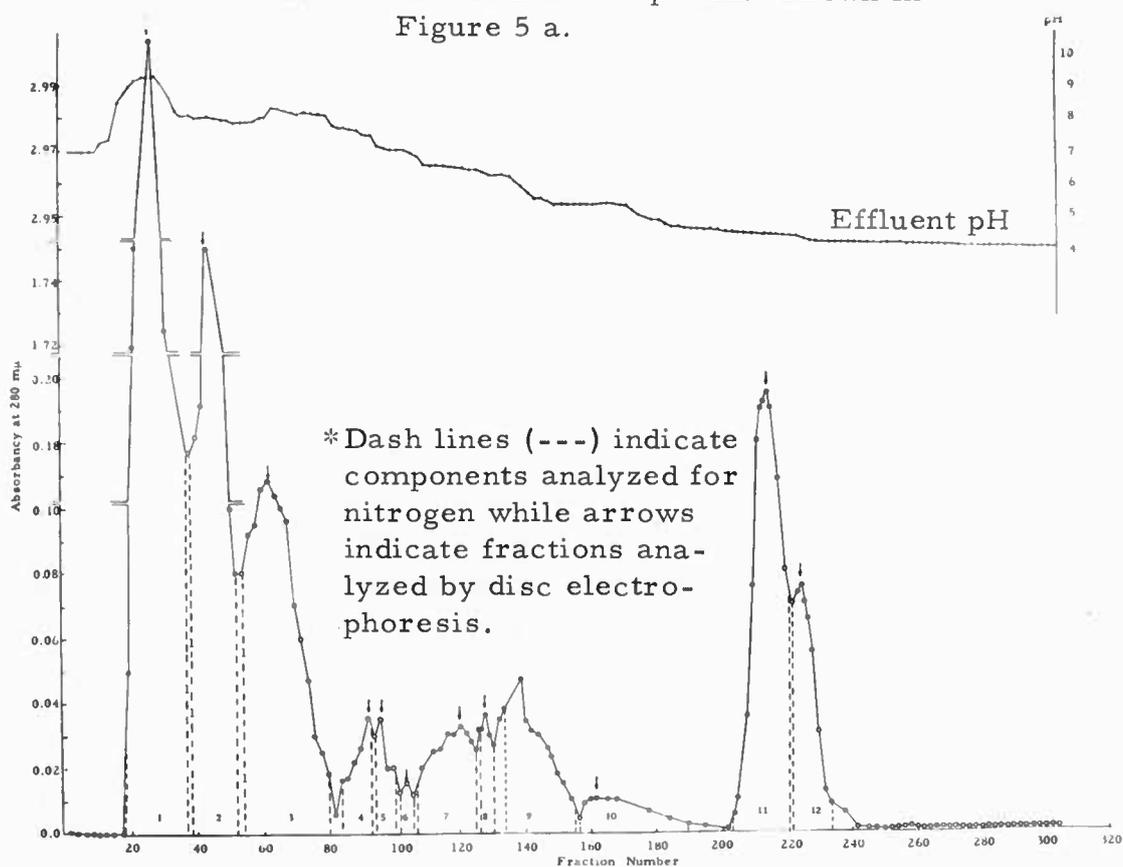
Figure 5 a.   Effluent diagram of total KCl-phosphate soluble pro-
teins extracted from loin muscle of animal No. 3.
DEAE-cellulose column (40 x 2. 5 cm).   One hundred
ml extract containing 0. 951 g of protein applied.
Starting buffer, 0. 005 M Na-phosphate, pH 7. 0, non-
linear gradient elution, 36°F.

The differences in behavior of the total KCl-phosphate soluble proteins of animals 1, 2 and 3 on the DEAE-cellulose column are somewhat similar to the results obtained from the disc electrophoresis analysis of the same initial protein extracts. Figure 6 represents the three patterns obtained by disc electrophoresis. Patterns A and B appear to be similar while pattern C is somewhat divergent. It is of interest to note that patterns A and C are from the loin muscle of cows while pattern B was from a bull. The smallest number of protein components to be separated on the DEAE-cellulose column was the sample of animal No. 2. This sample also gave the smallest number of protein zones on the disc electrophoresis patterns.

The observation that the first component in the effluent diagrams of animals No. 1 and 2 and components 1 and 2 for animal No. 3 showed at least six electrophoretically heterogeneous protein fractions, indicates that the column has a certain protein binding capacity. Overloading the column results in a condition where the unadsorbed proteins pass through the column concurrently with the fractions being eluted at the existing conditions.

The effluent diagram, Figure 5a, of the column separation of the total KCl-phosphate soluble proteins of animal No. 3 shows that too much protein was applied to the column in this run. The first three components to be eluted have relatively high absorbancy
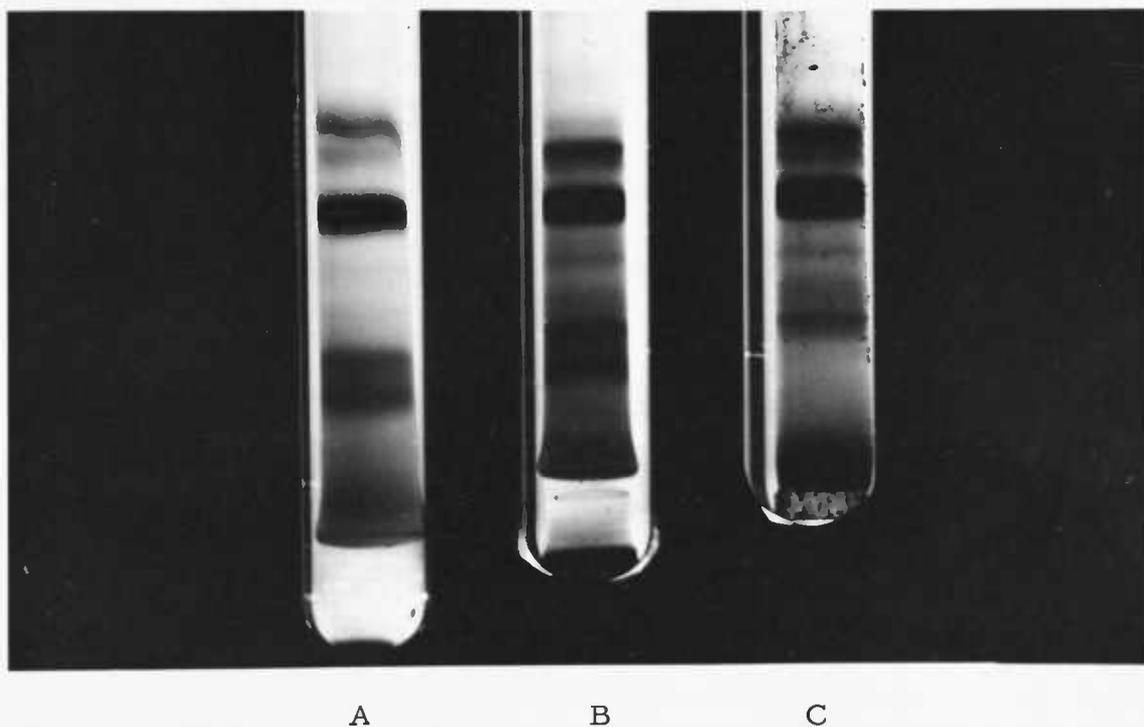
Figure 6. Disc electrophoresis patterns of total KCl-phosphate
soluble proteins extracted from the loin muscle of the
three different animals.
Legend:

A - Pattern for animal No. 1.
B - Pattern for animal No. 3.
C - Pattern for animal No. 2.

values, indicating that a considerable amount of protein had passed through the column without being adsorbed and fractionated. The disc electrophoresis patterns of the eluted components support this view because the first three components contained at least nine out of the total 12 or 13 bands shown for the whole series.

Components designated as 1 in the eluting diagrams shown on Figure 3a and 4a, both emerged at almost an identical pH region, the pH range of 8.47 - 7.62 in the case of animal No. 1 and 8.23 - 7.28 for that of animal No. 2. However, it is difficult to conclude any similarity of the other components on the basis of their pH range of emergence.

In each of Figures 3a, 4a and 5a, the effluent pH curves show an increase in pH values as the protein components emerge from the column. This effect was especially noticeable at the beginning of a run when the pH is near neutrality, but becomes less pronounced when approaching the acidic region. The initial increase in the pH value from 7 to 8.5 indicates a rapid exchange of ions between the proteins and the adsorbent.

Amino Acid Analysis

In Table 3 the amino acid analysis of the total KCl-phosphate soluble proteins of animals No. 1, 2 and 3 are tabulated. This

Table 3. Amino acid composition of total KCl-phosphate soluble
proteins expressed as percentage of total proteins.

Amino Acid	Animal No. 1	Animal No. 2	Animal No. 3
alanine	2. 42	3. 5	2. 42
arginine	5. 73	11. 3	10. 0
aspartic acid	6. 04	13. 5	14. 8
cystine	3. 65	1. 47	1. 08
glutamic acid	4. 3	10. 4	9. 58
glycine	1. 82	2. 56	3. 52
histidine	3. 69	7. 27	8. 16
hydroxyproline	- - -	- - -	- - -
isoleucine	3. 80	7. 09	6. 88
lysine	2. 55	4. 17	1. 84
methionine	4. 80	7. 99	7. 60
phenylalanine	3. 80	11. 6	7. 04
proline	3. 38	7. 05	7. 01
serine	- - -	- - -	1. 81
threonine	4. 51	6. 69	8. 26
tryptophan	- - -	- - -	- - -
tyrosine	4. 27	7. 18	7. 83
valine	2. 05	4. 17	3. 28
Total	56. 81	105. 94	101. 11

(- - -) indicates none or too minute to be detected.

table shows some resemblance as well as some variation in the

amino acid distribution. Except for the amino acids lysine and

phenylalanine, there is close similarity between the distribution of

each amino acid in the case of animals 2 and 3, while the results

for animal No. 1 differ considerably from those of the other two

animals. The very low level or non-detection of the amino acids

hydroxyproline, serine and tryptophan may be attributed to their

destruction or alteration during hydrolysis (2, p. 67 - 69) or perhaps

they existed in such a low concentration initially that the volume of

the sample applied on the paper was not sufficient for detection.

High levels of aspartic and glutamic acids and arginine are noticeable

in the three different samples.

Total KCl-phosphate Soluble Proteins Minus Actomyosin

Column Chromatography and Disc Electrophoresis Analysis

The effluent diagram shown in Figure 7a was obtained when

100 ml of extract, containing 0.361 g of protein, of the total KCl-

phosphate soluble proteins minus actomyosin from animal No. 2

were fractionated on the DEAE-cellulose column. Nine components

were separated and eluted.

Figure 7b represents the disc electrophoretic patterns of

components 1, 6, 7 and 9 when analyzed by the disc electrophoresis

56

Figure 7 b. Disc electro-
phoresis patterns of the
effluent components
shown in Figure 7 a.

Effluent pH

*Dash lines (- - -) indicate
components analyzed for
nitrogen while arrows
indicate fractions analyzed
by disc electrophoresis.

Figure 7 a. Effluent diagram of total KCl-phosphate soluble pro-
teins minus the actomyosin of loin muscle of animal
No. 2. One hundred ml extract containing 0.361 g of
protein applied to DEAE-cellulose column (40 x 2.5
cm). Starting buffer 0.005 M Na-phosphate, pH 7.0,
non-linear gradient elution, 36°F.

technique. The other components gave negative electrophoretic results. Component 1 shows a heterogeneous electrophoretic pattern while each of the patterns 6 and 9 indicate the presence of two electrophoretically different protein fractions. Component 7 appears to be homogeneous since it shows a single protein fraction.

The heterogeneity observed in the case of pattern 1 can probably be attributed to that part of the sample which was not adsorbed by the DEAE-cellulose. The electrophoretic data indicate that ten different protein fractions were present in the total sample which agrees somewhat with the results of the fractionation of these proteins on the DEAE-cellulose column.

Figure 8a shows the effluent pattern when 100 ml of the total KCl-phosphate soluble proteins minus actomyosin extract of animal No. 3. containing 0.334 g of protein, were fractionated on DEAE-cellulose column. The figure shows that eight or nine different protein components were eluted. Components 1 and 2 are probably one component since there was no sharp division between their absorbancy peaks. Table 4 gives a brief description of the pH ranges at which these protein components emerged from the column and their percentage of the total protein recovered.

Figure 8b shows the disc electrophoretic patterns of the eluted components 1, 5, 8 and 9. Negative results were obtained

Figure 8 b. Disc electro-
phoresis patterns of the
effluent components
shown in Figure 8 a.

* Dash lines (---) indicate
components analyzed for
nitrogen while arrows
indicate fractions analyzed
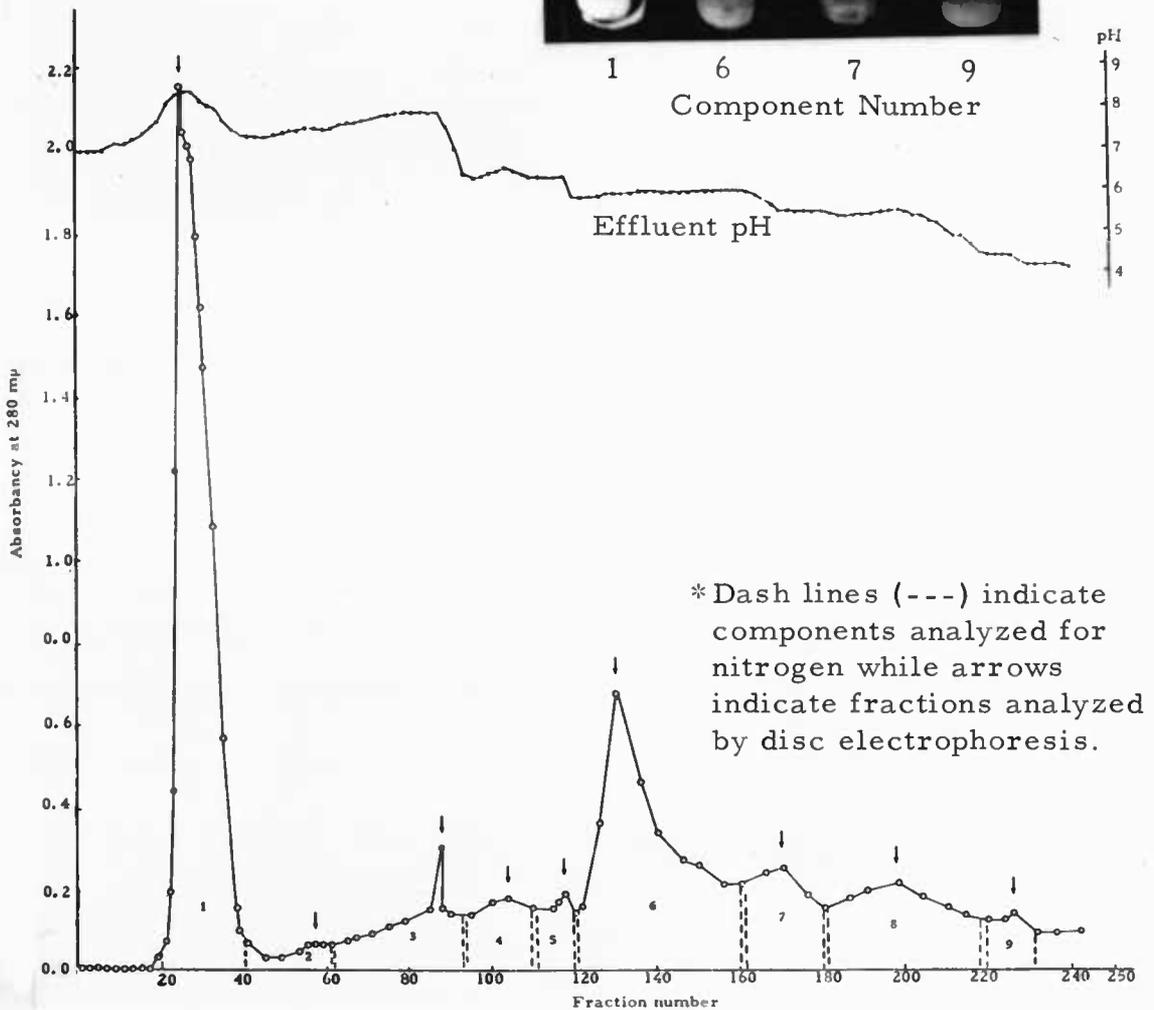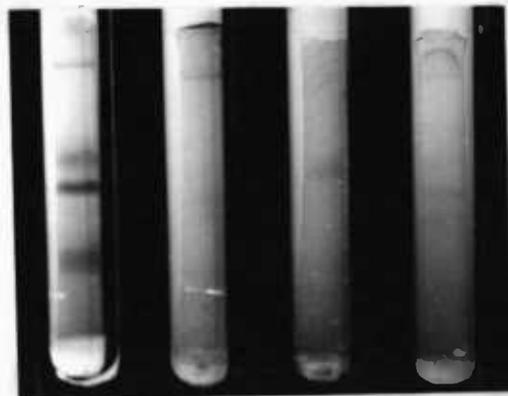by disc electrophoresis.
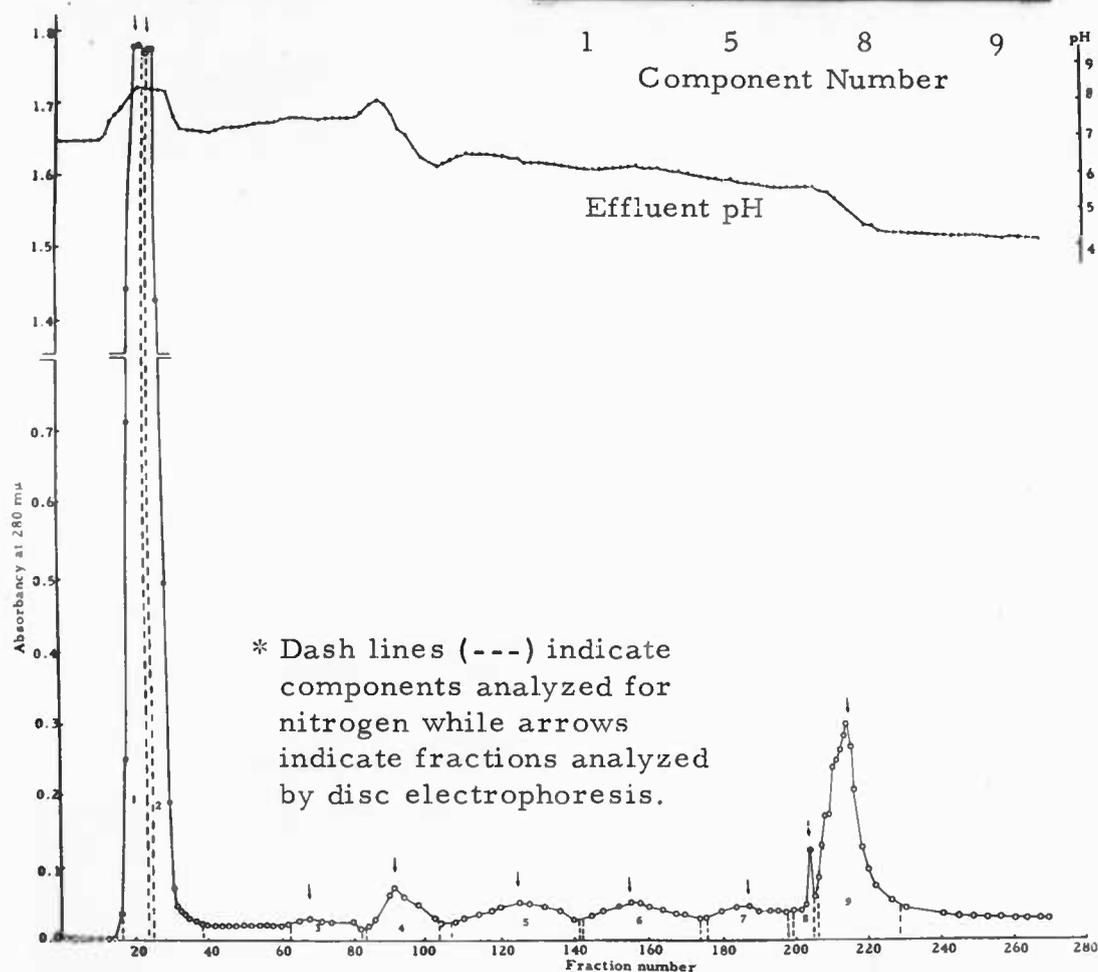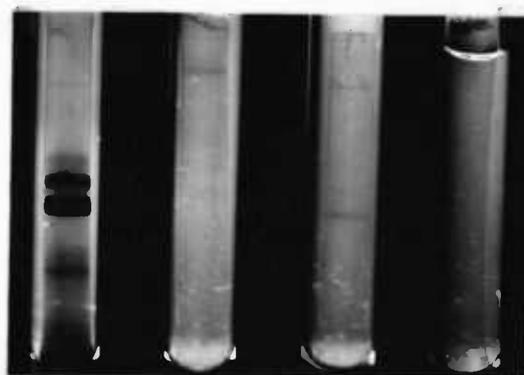
Figure 8 a. Effluent diagram of total KCl-phosphate soluble pro-
teins minus the actomyosin of loin muscle of animal
No. 3. One hundred ml extracts containing 0.334 g
of protein applied to DEAE-cellulose column (40 x
2.5 cm). Starting buffer 0.005 M Na-phosphate, pH
7.0, non-linear gradient elution, 36°F.

Table 4. Percentage of protein and pH range of emergence of the protein components of the total KCl-phosphate soluble loin muscle proteins minus actomyosin separated and eluted by DEAE-cellulose ion exchange chromatography.

Animal number	Component number	pH range of emergence	Percentage of recovered protein as eluted per component	Disc bands[*]
2	1	7. 48 - 7. 28	51. 19	5
	2	7. 28 - 7. 46	0. 68	-
	3	7. 48 - 6. 46	5. 77	-
	4	6. 34 - 6. 22	3. 97	-
	5	6. 22 - 5. 76	2. 94	-
	6	5. 76 - 5. 94	16. 94	2
	7	5. 92 - 5. 43	4. 72	1
	8	5. 40 - 4. 40	12. 63	-
	9	4. 40 - 4. 18	1. 12	2
		Total	99. 95	
3	1	7. 56 - 8. 40	18. 60	5
	2	8. 40 - 7. 20	19. 80	-
	3	7. 45 - 7. 50	1. 59	-
	4	7. 52 - 6. 20	9. 59	-
	5	6. 20 - 6. 12	11. 65	2
	6	6. 10 - 5. 90	9. 59	-
	7	5. 85 - 5. 55	5. 12	-
	8	5. 52 - 5. 54	1. 99	1
	9	5. 54 - 4. 26	21. 97	2
		Total	99. 90	

[*] Number of bands appearing on the disc electrophoresis gels of the separated protein components.

with components 3, 4, 6 and 7. Although positive results were obtained for component 2, it was inadvertently omitted from the photogram.

The electrophoretic pattern for component 1 shows that it was heterogeneous, since at least five different bands were shown on the pattern. Components 5 and 9 each contain two electrophoretically different protein fractions. Pattern 8 appears to be an electrophoretically homogeneous protein component since only one zone was shown on the pattern. The electrophoresis data and the results of the DEAE-cellulose column separation show some similarity. The effluent diagram (Figure 8a) indicates eight and possibly nine different protein components while the electrophoresis patterns show the existence of 10.

Although some variations were noted between the eluting patterns of these samples from animals 2 and 3, the differences were less pronounced than those observed between the total KCl-phosphate soluble proteins of the same animals (Figures 5a and 6a).

Comparison of the data in Table 4 shows that the pH values at which the different components emerge from the column were very similar for both samples. For example, component 1 of animal No. 2 emerged at a pH of 7.48 and terminated at pH 7.2, while components 1 and 2 of animal No. 3, which were most likely to be one

component, emerged at pH 7.56 and ended at pH 7.20. Although components 5 of both samples first appeared at a pH value of 6.22, they terminated at two different regions. Component 6 of the second extract ended at pH 5.94 while the same component of the third sample stopped at pH 5.90. In addition, there was some similarity in the pH emergence of the eighth component of both samples.

Mechanical difficulties were encountered with the fraction collector during the early stages of the chromatographic separation of the sample from animal No. 1. As a result, this sample was completely lost.

Amino Acid Analysis

The amino acid composition of the total KCl-phosphate soluble proteins minus actomyosin for animals No. 2 and 3 are given in Table 5. The data of the two samples appear to be quite comparable. For both samples, aspartic acid was the predominant amino acid followed by glutamic acid.

It is of interest to note that the good agreement between the amino acid analysis of the above two samples parallels the results obtained for the total KCl-phosphate soluble proteins of the same two animals.

Table 5.   Amino acid composition of the total KCl-phosphate soluble
           proteins minus actomyosin expressed as percentage of
           total protein.

Amino acid	Animal No. 2	Animal No. 3
alanine	2.93	3.02
arginine	7.94	7.97
aspartic acid	15.20	12.52
cystine	1.72	0.71
glutamic acid	9.15	9.46
glycine	1.79	2.92
histidine	6.35	6.68
hydroxyproline	---	---
isoleucine	6.64	8.50
lysine	5.96	1.39
methionine	1.71	5.30
phenylalanine	7.63	6.97
proline	5.31	5.42
serine	---	2.80
threonine	5.89	6.30
tryptophan	---	---
tyrosine	5.84	6.42
valine	3.92	5.09
Total	87.98	91.47

(---) indicates none or too minute to detect.

Sarcoplasmic + Actin Proteins

Column Chromatography and Disc Electrophoresis

The effluent diagram shown on Figure 9a was obtained when 100 ml of sarcoplasmic + actin proteins of animal No. 1, containing 75. 13 mg of protein, were subjected to chromatographic analysis. The volume of buffer used in this run was reduced to 100 ml for the first buffer and to 150 ml for each of the other four buffers. In all of the previous runs, each of the five eluting buffers were held at a constant volume of 500 ml. This alteration was thought necessary at the time of analysis to compensate for the small amount of protein available for the chromatographic run.

Six different protein components were fractionated as shown in the effluent diagram of Figure 9a. A summary of the pH ranges at which these components emerged and their percentage to the total protein recovered from the column are presented in Table 6. The disc electrophoresis analysis showed no protein bands on any of the six components tested.

Figure 10a was obtained when 100 ml of sarcoplasmic + actin proteins extracted from animal No. 2, containing 69. 66 mg of protein, were chromatographed on DEAE-cellulose using a volume

Figure 9. Effluent diagram of sarcoplasmic + actin proteins of loin
muscle of animal No. 1. One hundred ml extract contain-
ing 75.13 mg protein applied to DEAE-cellulose column
(35 x 2.5 cm). Starting buffer 0.005 M Na-phosphate,
pH 7.0, non-linear gradient elution, 36°F.

Table 6. Percentage of protein and pH range of emergence of the protein components of sarcoplasmic + actin proteins of loin muscle, separated and eluted by DEAE-cellulose ion exchange chromatography.

Animal number	Component number	pH range of emergence	Percentage of recovered protein as eluted per component	Disc bands[*]
1	1	7.40 - 7.60	4.04	-
	2	7.62 - 7.20	22.90	-
	3	7.20 - 6.35	16.76	-
	4	6.30 - 6.12	29.34	-
	5	6.10 - 5.07	12.57	-
	6	5.00 - 4.18	14.37	-
		Total	99.88	
2	1	7.35 - 7.50	15.55	1
	2	7.50 - 7.00	15.31	1
	3	7.00 - 6.20	20.20	1
	4	6.10 - 6.15	7.31	1
	5	6.15 - 5.70	3.38	1
	6	5.70 - 5.40	38.23	1
		Total	99.98	
3	1	7.35 - 7.75	52.17	-
	2	7.60 - 7.10	2.52	-
	3	7.00 - 6.75	5.91	-
	4	6.30 - 6.00	2.85	-
	5	6.00 - 5.50	15.93	-
	6	5.40 - 4.15	20.59	-
		Total	99.97	

[*] Number of bands appearing on the disc electrophoresis gels of the separated protein components.

of 500 ml per buffer. This diagram shows the presence of six different protein components. Their relative percentage to the total protein recovered from the column and the pH at which they emerge are presented in Table 6.

Figure 10b contains the electrophoretic patterns of the maximum tube of each peak of the six different protein components. This figure also shows a component designated as "o" which is the pattern of the extract prior to column separation. The duration of this run was cut from 25 to 15 minutes because at a longer duration these particular fractions after staining seemed to be more susceptible to fading or color loss. Each of the six components showed the presence of only one electrophoretic fraction. The other zones shown belong to the dye which was used as a marker to indicate the front of the run.

Figure 11 shows the diagram obtained when 200 ml of sarcoplasmic + actin proteins of animal No. 3, containing 168.34 mg protein, were chromatographed on DEAE-cellulose with the original elution schedule. The diagram shows the appearance of six different protein fractions. Their relative percentage to the total protein recovered from the column and the pH range at which they emerged are reported in Table 6.

A comparative study of the behavior of the three sarcoplasmic
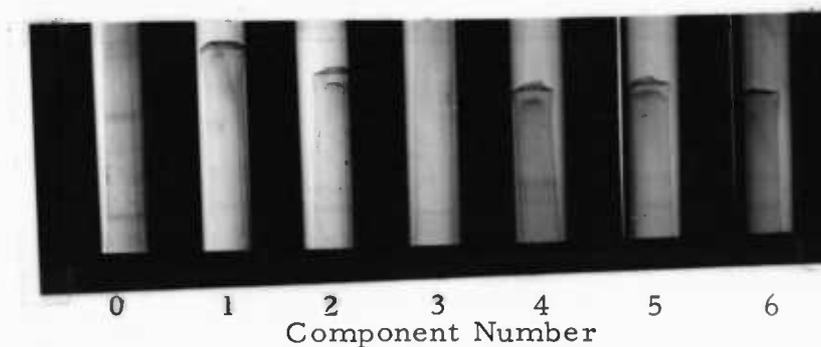
Figure 10 b.   Disc electrophoresis patterns of
effluent components shown in Figure 10 a.   Com-
ponent 0 is the sarcoplasmic + actin extract prior
to column chromatography.
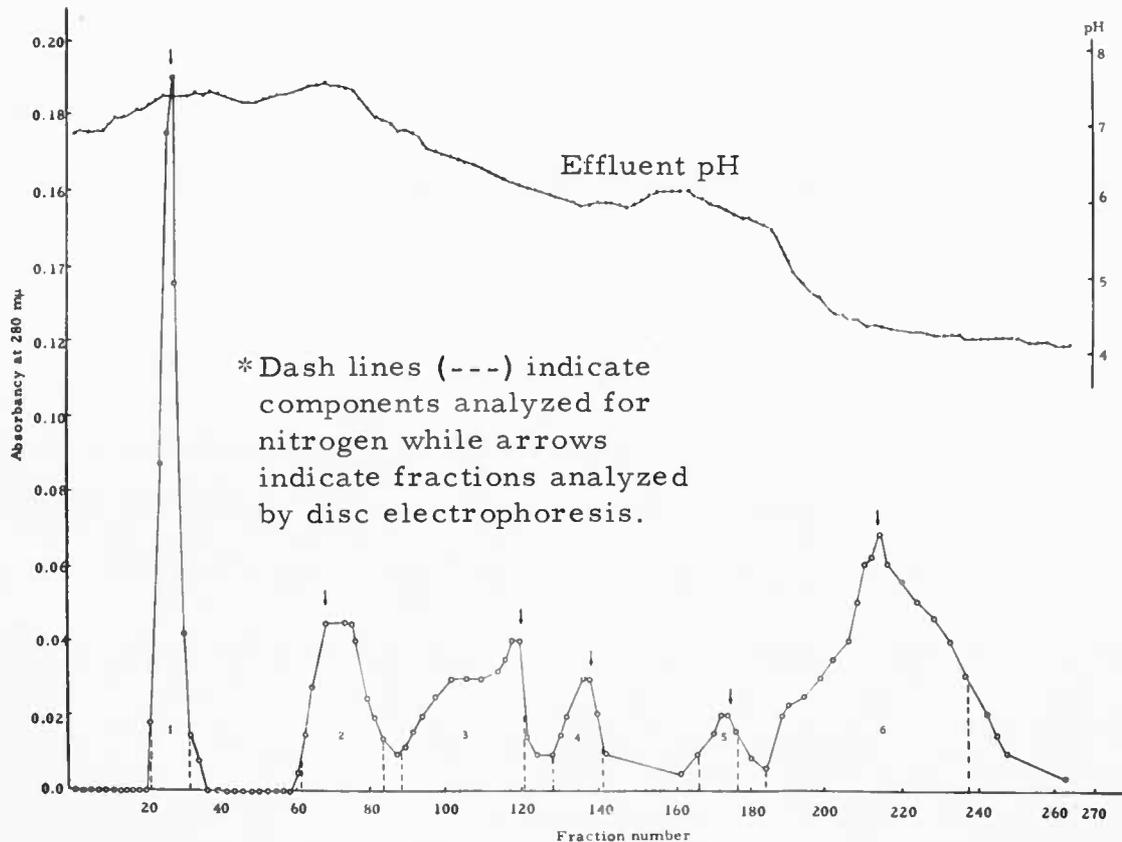
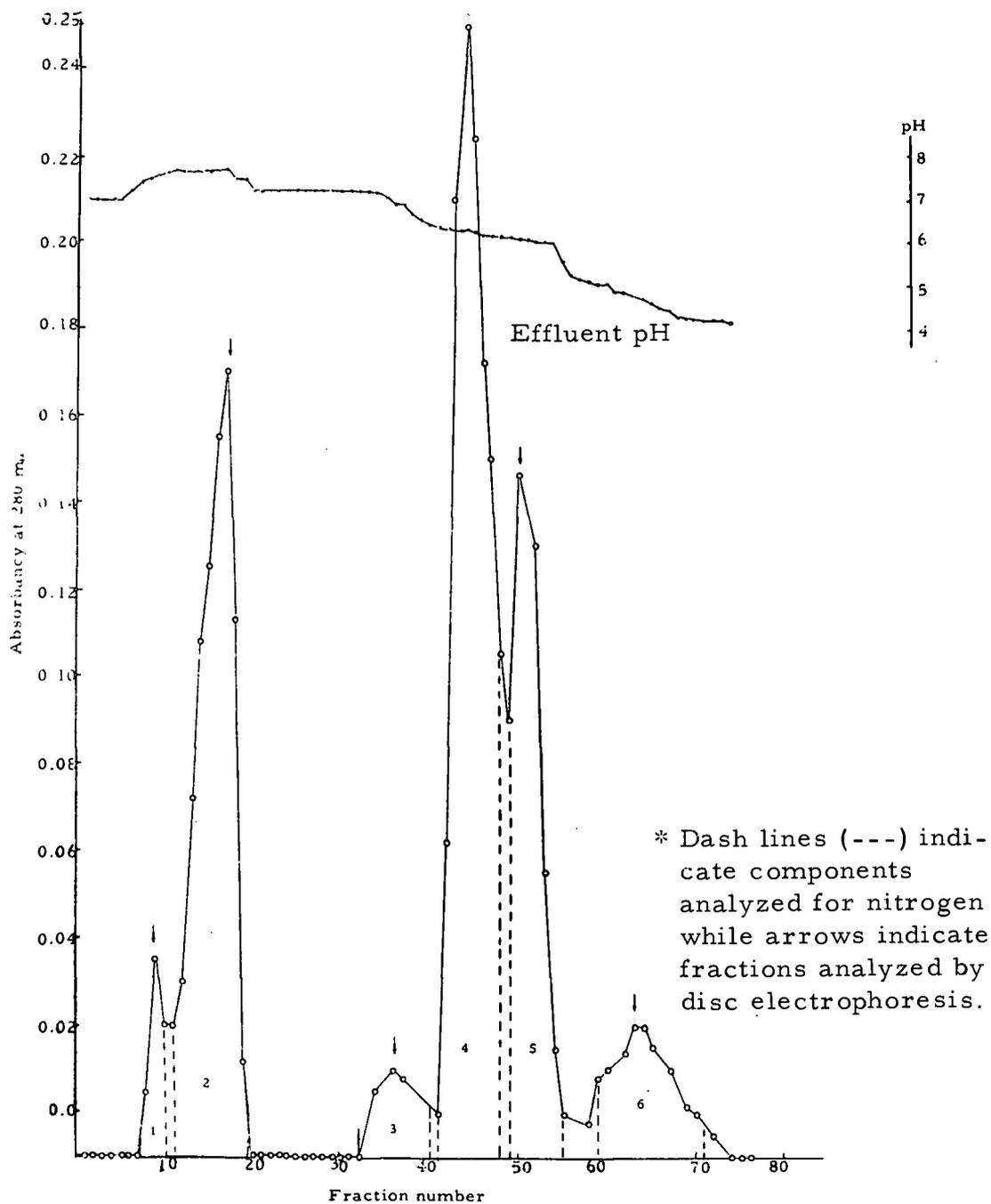Figure 10 a.   Effluent diagram of sarcoplasmic + actin proteins of
loin muscle of animal No. 2.   One hundred ml of
extract containing 69. 66 mg protein applied to DEAE-
cellulose column (40 x 2. 5 cm).   Starting buffer 0. 005
M Na-phosphate, pH 7. 0 non-linear gradient elution,
$36^{O}F$.

Figure 11. Effluent diagram of sarcoplasmic + actin proteins of
loin muscle of animal No. 3. Two hundred ml of extract
containing 168. 34 mg protein applied on DEAE-cellulose
column (40 x 2. 5 cm). Starting buffer 0. 005 M Na-
phosphate, pH 7. 0, non-linear gradient elution, 36°F.

* Dash lines (---) indicate components analyzed for nitrogen while
arrows indicate fractions analyzed by disc electrophoresis.

+ actin samples on DEAE-cellulose column indicates some similarity. Each sample contained six different protein components although the volume of the eluting buffer differed. Although twice as much protein was applied on the column for the third sample as for the other two samples, data in Table 6 indicated that the six components of each sample emerged at pH regions very similar to one another. The first component of samples 1, 2 and 3 started to emerge at pH values of 7.62, 7.50 and 7.60, respectively. The third component emerged at pH values of 7.20, 7.00 and 7.00 for samples 1, 2 and 3. Although these data might be construed to indicate similarity between the three samples, agreement of the results of the protein recovery per component between samples is much too divergent to allow for much emphasis to be placed on the data of pH emergence.

Amino Acid Analysis

The results of the amino acid analysis of the sarcoplasmic + actin proteins, Table 7, are irrelevent since only slightly over 50 percent of the total protein content is accounted for by the amino acids.

The low total levels observed in this case might be due to the insensitivity of the one dimensional paper chromatography method used for the amino acid determination in this study.

Figure 12. Effluent diagram of actomyosin protein of loin muscle
of animal No. 1. Fifty ml of extract containing 45.47
mg of protein applied on DEAE-cellulose column (40 x
2.5 cm). Starting buffer 0.005 M Na-phosphate pH 7.0
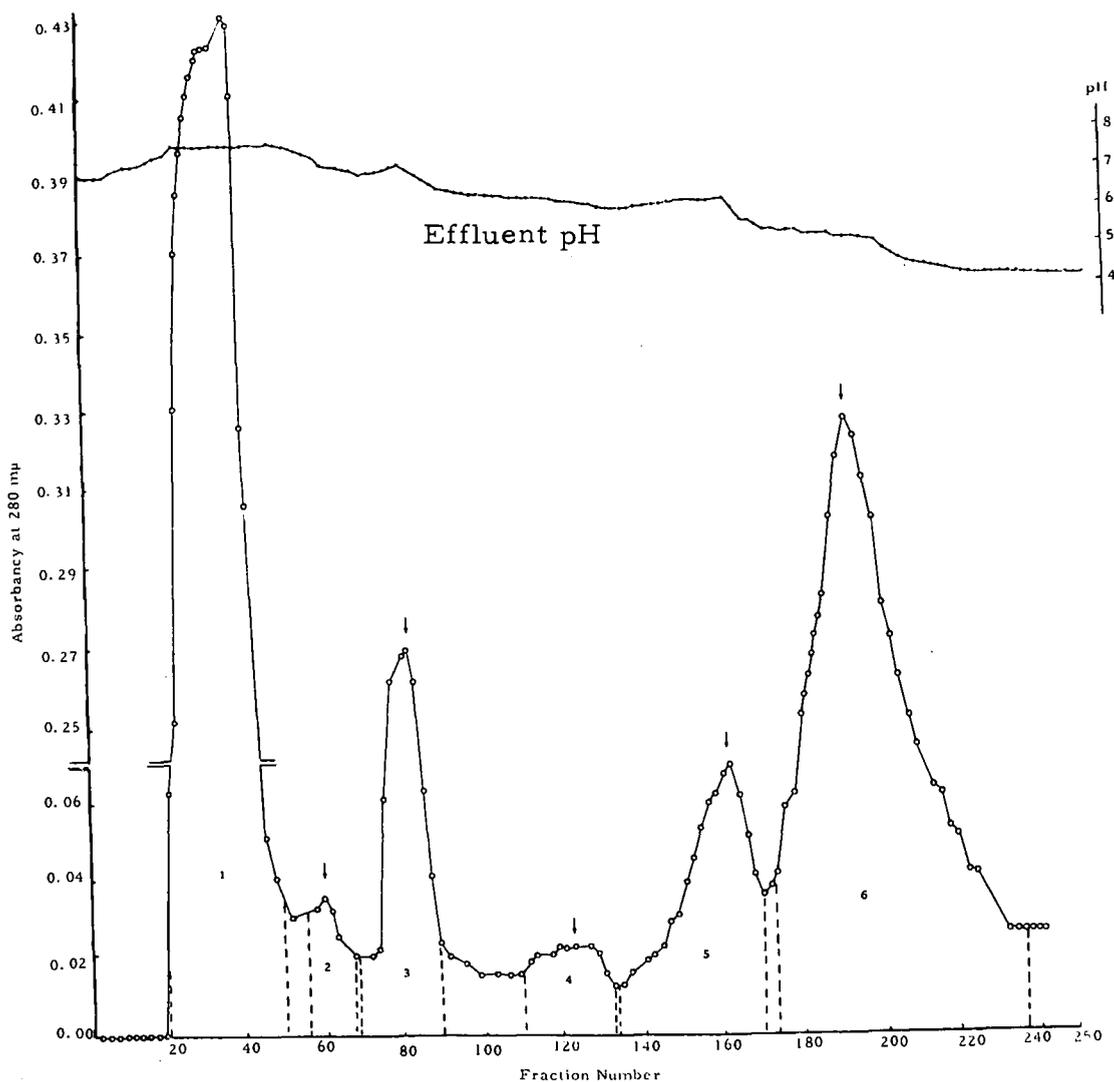non-linear gradient elution, 36°F.

* Dash lines (---) indicate components analyzed for nitrogen while
arrows indicate fractions analyzed by disc electrophoresis.
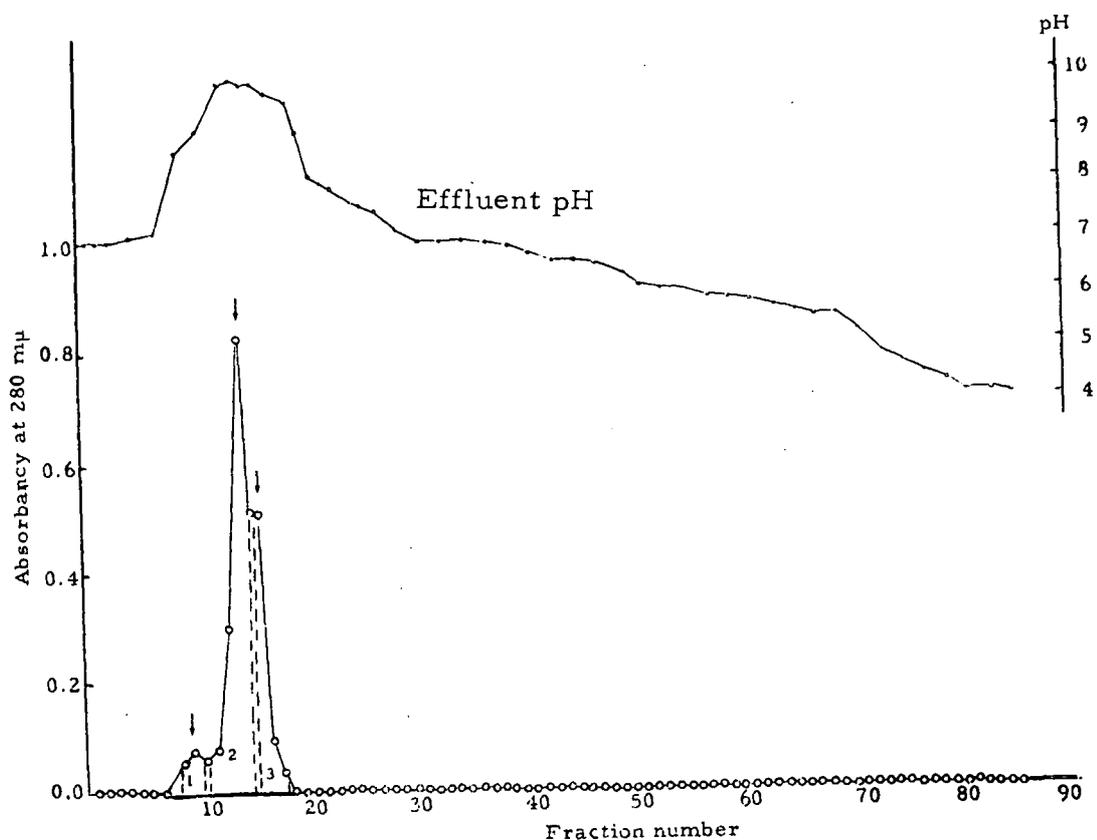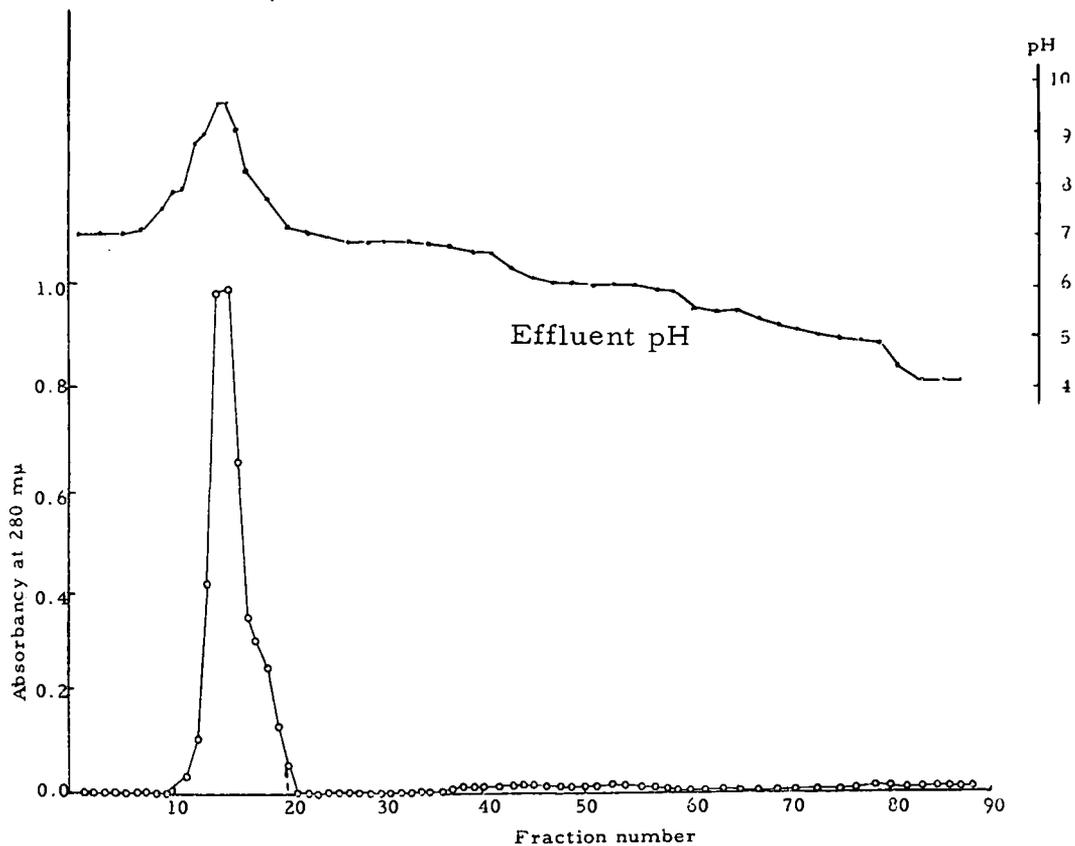
Figure 13. Effluent diagram of actomyosin proteins of loin muscle
of animal No. 2. Fifty ml of extract containing 33.62
mg protein applied to DEAE-cellulose column (40 x 2.5
cm). Starting buffer 0.005 M Na-phosphate, pH 7.0,
non-linear gradient elution, 36°F.

* Dash lines (---) indicate components analyzed for nitrogen while
  arrows indicate fractions analyzed by disc electrophoresis.

0. 672 mg/ml for the second sample. At these concentrations, both solutions appeared to be somewhat viscous. Such a condition would undoubtedly hinder the DEAE-cellulose ion exchange chromatographic separation of these solutions.

In an attempt to overcome this difficulty, the actomyosin solution of the third sample was diluted to a considerable extent. Thus, 250 ml of actomyosin having 33. 62 mg of protein or a concentration of 0. 116 mg/ml of buffer were applied on the chromatographic column. The volume of each of the five eluting buffers was 500 ml and non-linear gradient elution schedule was used as described previously. The results of this run are shown in Figure 14a.

Four different protein components were shown to be separated. Component 1 was eluted at pH value close to neutrality while components 2, 3 and 4 were eluted at an acidic pH range. Table 8 gives the pH range at which these fractions were eluted and their percentage of the total protein recovered from the column.

Figure 14b shows the electrophoretic pattern of the actomyosin protein sample of animal No. 3 prior to column separation. It was also shown previously in Figure 2 as pattern 3. This pattern shows that the disc electrophoresis technique fractionated the original extract into four components which coincides with the results of the DEAE-cellulose separation of a sample of the same extract.

Figure 14b. Disc electrophoresis pattern of the actomyosin fraction prior to DEAE-cellulose chromatographic separation.

Figure 14a. Effluent diagram of actomyosin proteins of loin muscle of animal No. 3. Two hundred fifty ml of extract containing 29.87 mg protein applied to DEAE-cellulose column (40 x 2.5 cm). Starting buffer 0.005 M Na-phosphate, pH 7.0, non-linear gradient elution, 36°F.

* Dash lines (---) indicate components analyzed for nitrogen while arrows indicate fractions analyzed by disc electrophoresis.

Table 8. Percentage of protein and pH range of emergence of the protein components of the actomyosin proteins of loin muscle of animal No. 3 separated and eluted by DEAE-cellulose ion exchange chromatography.

Animal number	Component number	pH range of emergence	Percentage of recovered protein as eluted per component
3	1	7.38 - 7.32	35.31
	2	5.36 - 5.00	38.05
	3	4.90 - 4.30	21.30
	4	4.25 - 4.20	5.32
		Total	99.98

These results indicate that the actomyosin proteins can be separated into four components.

Negative results were obtained when the maximum tube of each peak of the cellulose column eluted components of the three samples was analyzed by disc electrophoresis method.

Amino Acid Analysis

Table 9 shows the amino acid composition of the actomyosin proteins extracted from the three different animals. There is very little agreement between the data of the three samples except for the high contents of glutamic acid.

Myosin Proteins

Column Chromatography and Disc Electrophoresis Analysis

Figure 15 shows the eluent pattern when 20 ml of myosin solution, containing 84. 726 mg of protein, of animal No. 1 were subjected to DEAE-cellulose column separation. The buffer system was modified in this run as follows: 300 ml of each of the first four buffers and 400 ml of the fifth buffer. The diagram showed the appearance of one, but more likely two, protein components. Their emergence was accompanied by a rise in the pH value as shown in

Table 9. Amino acid composition of the actomyosin proteins
expressed as percentage of total protein.

Amino acid	Animal No. 1	Animal No. 2	Animal No. 3
alanine	1.75	4.46	1.35
arginine	2.24	1.34	3.73
aspartic acid	3.37	1.43	4.15
cystine	2.87	0.96	0.46
glutamic acid	11.1	11.9	12.0
glycine	6.71	5.54	0.72
histidine	3.02	9.77	2.41
hydroxyproline	---	---	---
isoleucine	2.98	6.11	2.48
lysine	4.03	1.65	3.92
methionine	3.17	10.50	2.20
phenylalanine	3.92	0.22	1.04
proline	2.84	7.68	1.36
serine	---	---	---
threonine	---	4.35	3.73
tryptophan	---	---	---
tyrosine	3.68	6.53	2.49
valine	2.00	4.68	4.68
Total	53.68	77.13	46.72

(---) indicates none or too minute to be detected.
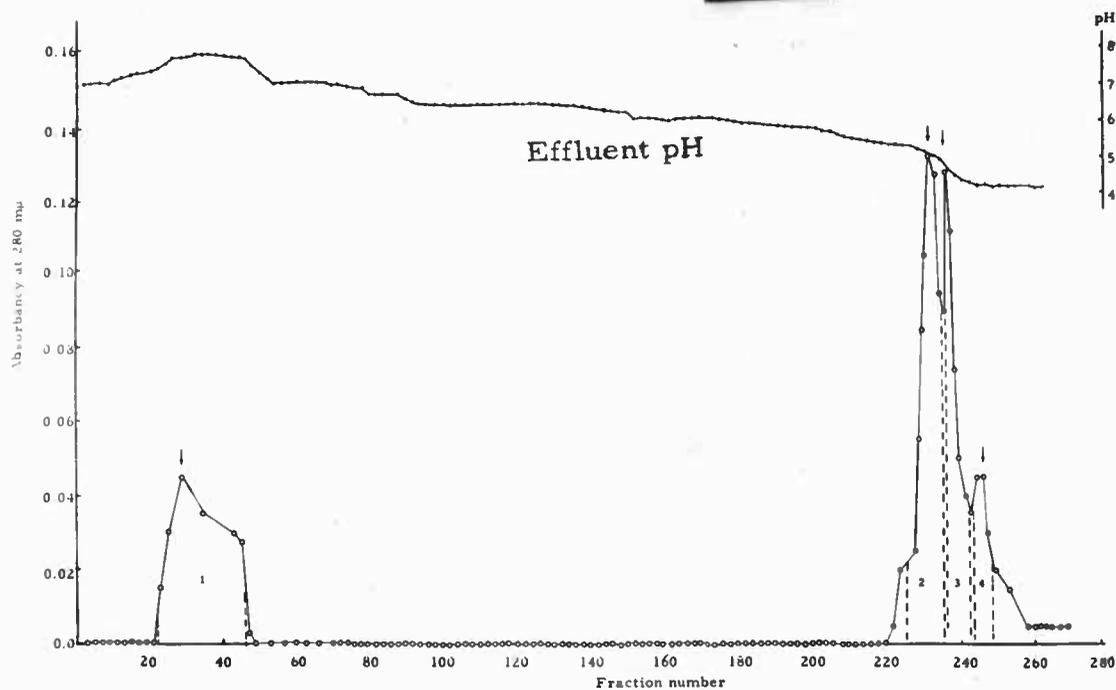
Figure 15. Effluent diagram of myosin proteins of loin muscle of animal No. 1. Twenty ml of extract containing 84.72 mg of protein applied on DEAE-cellulose column (40 x 2.5 cm) Starting buffer 0.005 M Na-phosphate, pH 7.0, non-linear gradient elution, 36°F.

* Dash lines (- - -) indicate components analyzed for nitrogen while arrows indicate fractions analyzed by disc electrophoresis.

the effluent pH curve.

The effluent pattern obtained when 94. 17 mg of myosin pro-
teins in 20 ml KCl-phosphate buffer were chromatographed on DEAE-
cellulose is shown on Figure 16.   This sample was from animal No.
2.   Only one protein component appeared to be eluted during the
course of the chromatographic run.   Although non-linear gradient
elution system was employed, the first buffer had a volume of 200
ml while the other four buffers each had a volume of 300 ml.

Figure 17 shows the effluent pattern obtained when 59. 22 mg
of myosin of animal No. 3 were dissolved in 250 ml of KCl-phosphate
buffer and chromatographed on DEAE-cellulose column.   The elu-
tion schedule used was that originally described under the experi-
mental procedure which employs a volume of 500 ml for each buffer.
Only one protein component appears to have been eluted.   The efflu-
ent pH curve shows an increase in pH during the elution of the com-
ponent.

Although the disc electrophoresis analysis was carried out
on each of the eluted components of the three myosin samples, nega-
tive results were obtained in each case.   However, Figure 2 shows
that when the original myosin extract was subjected to disc electro-
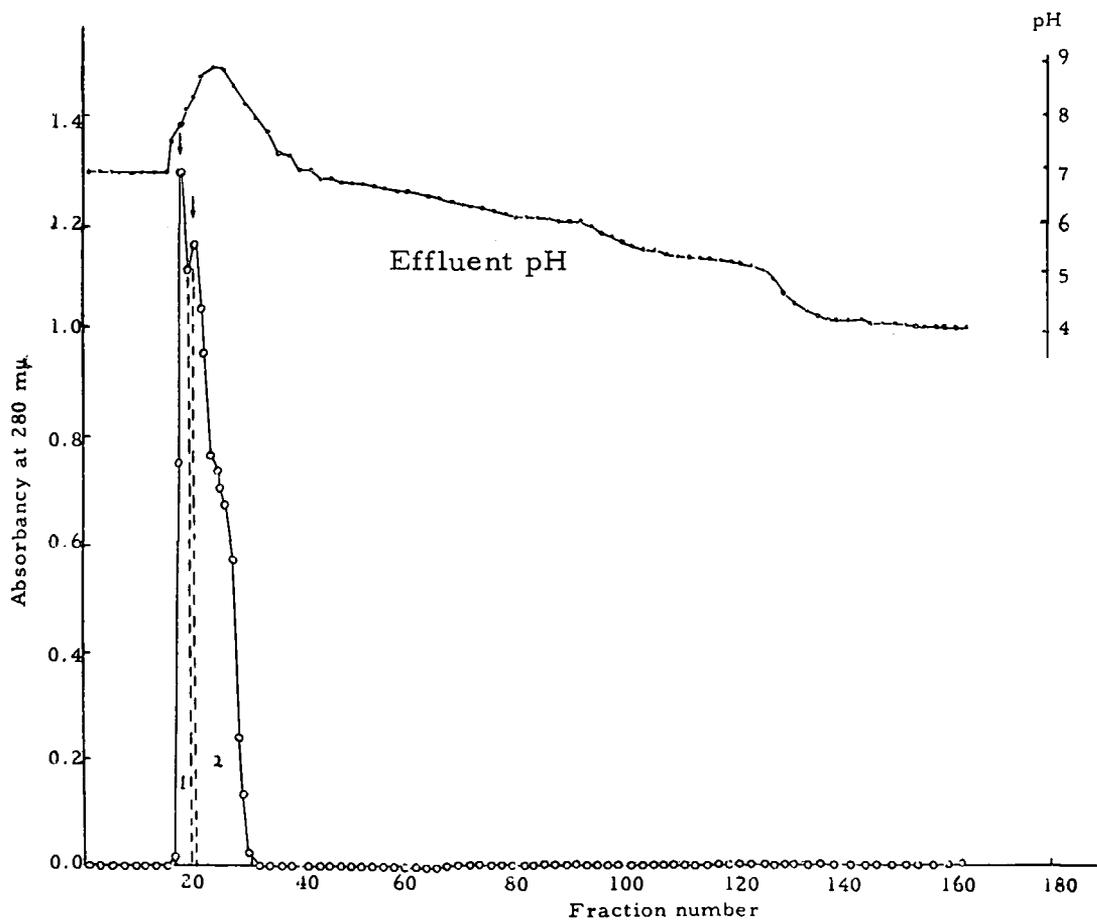phoresis, eight fractions were separated.
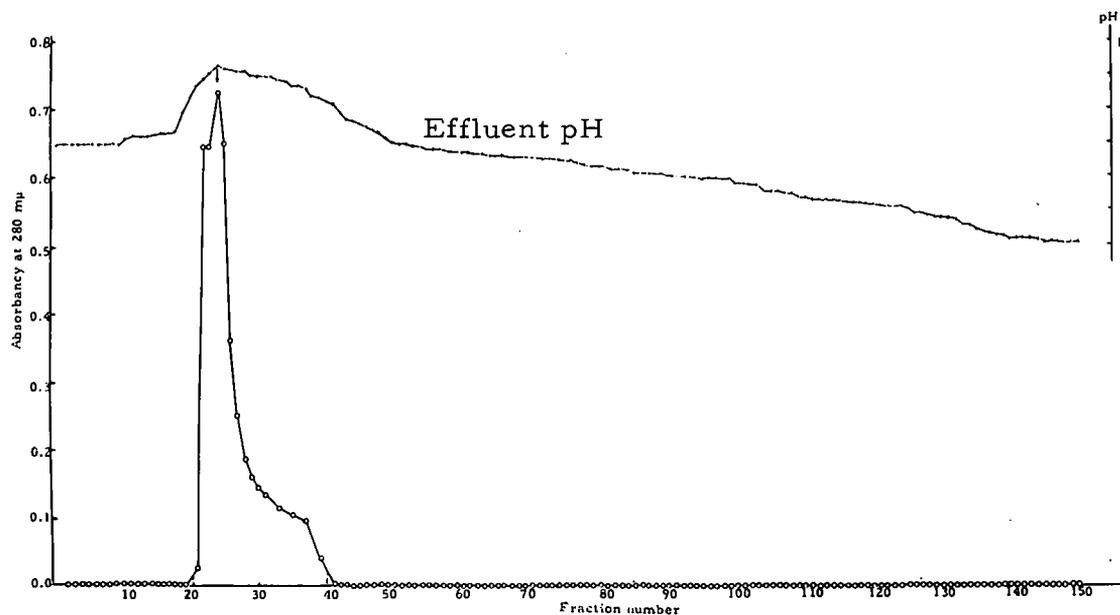
Figure 16. Effluent diagram of myosin proteins of loin muscle
animal No. 2. Twenty ml of extract containing 94.19
mg of protein applied on DEAE-cellulose column
(40 x 2.5 cm). Starting buffer 0.005 M Na-phosphate,
pH 7.0, non-linear gradient elution, 36°F.

* Dash line (---) indicate components analyzed for nitrogen while
arrows indicate fractions analyzed by disc electrophoresis.
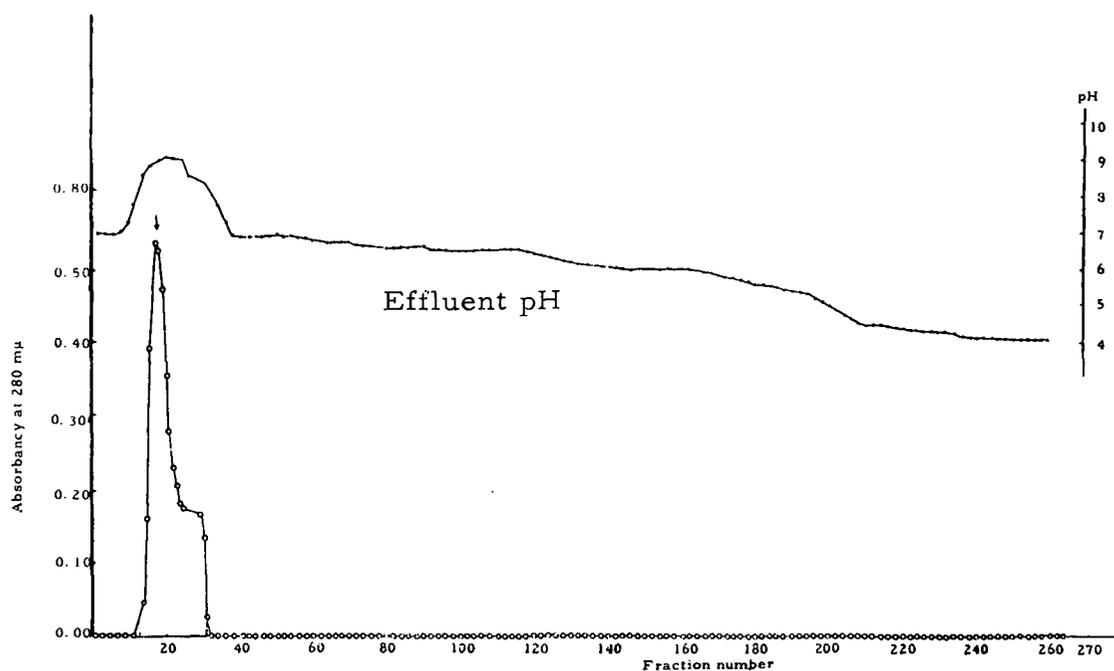
Figure 17. Effluent diagram of myosin proteins of loin muscle of
animal No. 3. Two hundred fifty ml of extract con-
taining 59. 22 mg of protein applied to DEAE-cellulose
column (40 x 2. 5 cm). Starting buffer 0. 005 M Na-
phosphate, pH 7. 0, non-linear gradient elution, 36°F.

* Dash lines (---) indicate components analyzed for nitrogen while
arrows indicate fractions analyzed by disc electrophoresis.

Amino Acid Analysis

The amino acid composition of the myosin proteins of the three samples are shown in Table 10. Although there is some similarity between the contents of certain amino acids of the three samples, the variability among the others is too great for these data to be of much value.

General discussion. Numerous problems of great difficulty are usually encountered in the study of muscle proteins. Some of the most evident ones are those related to sample variability. This lack of homogeneity is very evident when attempts are made to adapt or modify analytical procedures that have been proven to be of great value in studies of other materials to that of meat research. A certain amount of variation in the composition and properties exists within different localities of an intact muscle as well as between the same muscle of different animals (2, p. 223). In addition, such factors as age, sex, physiological conditions prior to and at time of slaughter, the time interval between death and sample analysis, phase of rigor mortis, all have an important influence on the properties and characteristics of the muscle proteins. In some instances, it is possible for the tissue sample to be frozen and maintained at sub-zero temperatures for long intervals or the sample can be dried

Table 10. Amino acid composition of the myosin proteins expressed as percentage of total protein.

Amino acid	Animal No. 1	Animal No. 2	Animal No. 3
Alanine	8.0	5.30	1.19
arginine	3.51	2.18	3.62
aspartic acid	4.25	3.41	4.00
cystine	0.28	---	0.30
glutamic acid	28.60	18.65	20.52
glycine	0.45	0.56	0.92
histidine	1.25	---	1.79
hydroxyproline	---	---	---
isoleucine	1.47	---	0.75
lysine	---	2.54	1.99
methionine	---	0.46	---
phenylalanine	14.40	14.40	9.60
proline	2.61	3.71	6.18
serine	2.00	---	---
threonine	1.48	1.94	1.20
tryptophan	---	---	---
tyrosine	1.48	1.30	1.91
valine	1.46	1.19	---
Total	71.24	55.64	53.97

(---) indicates none or too minute to be detected.

by lyophilization and stored as a powder for a considerable length of time without encountering too much deterioration. With muscle proteins, however, a certain amount of degradation or undesirable changes occur as a result of these treatments. Denaturation appears to be the most serious defect to occur when muscle proteins are stored by the above procedures.

Muscle tissue proteins remain in a very dynamic state from time of death until autolysis has been completed. In the present research, it was desired to study the muscle proteins as close to the native state as possible rather than attempt to store them by the conditions described above. Although the primary objective of the research reported herein was that of adapting the column chromatography procedure to the fractionation of muscle proteins, it was also desirable to learn more of the nature of the protein fractions as they exist in the fresh state. The latter data should prove to be quite valuable for future studies on the nature of the changes occurring in the muscle proteins during autolysis. Hence, the loin muscle of three different animals were used in order to fulfill the objectives of the study.

One of the most apparent results of the DEAE-cellulose chromatographic separation of the various muscle proteins was that the first component to be eluted, in most instances, was very

heterogeneous. Generally, the initial component contained at least five or more fractions as shown by the disc electrophoresis results. One of the reasons for such an occurrence may have been an overloading of the cellulose column with the initial protein extract. If such was the case, then it can be assumed that the ion exchange capacity of the column was exceeded and thereby resulted in poor protein separation. However, studies on the fractionation of milk proteins by the same procedure employed in this study, reported the application of a much higher concentration of skimmilk proteins and casein on the column with excellent separation and resolution being obtained (92, p. 6 - 12).

In view of these facts, it is almost certain that other factors must also be exerting an influence to cause the first eluted component of the muscle extract to be so heterogeneous. Sober and Peterson (72, p. 1117) have reported that proteins adsorbed tightly to a column contain many more charges initially than does a substance that is loosely adsorbed. Whether a particular molecule is eluted from an adsorbent depends upon the number of bonds that can be formed between them. Moreover, a molecule having the same net surface charge density as another but being sufficiently larger in size, would require much stronger eluting conditions since it has the ability of forming more bonds with the adsorbent. In addition, the

spatial arrangement of the ionizable groups on the molecule can readily influence their effectiveness in forming bonds with the adsorbent (73, p. 61-83).

Another factor that might influence the heterogeneity of the first eluted component is that the initial protein mixture may have contained numerous protein components that lacked sufficient charges of an appropriate sign to form multiple bonds with the ion exchange adsorbent and thus moved through the column without being retarded. In conjunction with this explanation, the disc electrophoresis results indicated that some of these substances showed considerable electrophoretic mobility. Although the charge of the molecule has an effect in this technique, molecules of very small size but possessing a slight charge can move rapidly through the polyacrylamide gel column on the basis of not being retarded to any extent by molecular sieving (22, p. 1-22). Hence, it seems logical to suspect that many of the substances composing the first eluted component of the muscle extract are of small size and have a slight charge.

Some of the major muscle protein fractions such as the total KCl-phosphate soluble proteins, the total KCl-phosphate minus actomyosin, and the sarcoplasmic plus actin fractions were found to be separated into several components by the DEAE-cellulose chromatographic procedure. Several of these components appeared to be

eluted as homogeneous entities according to the electrophoresis data. The myosin and actomyosin fractions resisted chromatographic separation. These two fractions are very complex systems. They are difficult to extract from muscle and several procedures must be used concurrently to obtain them in the purified form.

Myosin can be dissociated into two or more components by enzymatic attack or can be depolymerized by moderately high levels of urea plus several days of incubation. Actomyosin can be dissociated by ultracentrifugation, by adenosine triphosphate or by certain minerals under the proper conditions (81, p. 1-49). These two muscle proteins were not separated to any great extent by the chromatographic procedure and conditions employed in this study. However, it should be pointed out the actomyosin fraction of the third animal was separated into four components when the concentration of the initial extract was diluted three to four fold over that used in the first two chromatographic runs. At the higher concentrations, it was a viscous fluid resisting column separation and elution as indicated by the protein recovery data.

The pH effluent curves showed an increase in pH values as the proteins components began to emerge from the column. This was particularly noticeable when the pH was near neutrality. It became less pronounced as it approached the acidic pH region.

Sober and Peterson (73, p. 65) have found that the protein compon-
ents eluted from the column close to a neutral pH are the ones hav-
ing relatively high isoelectric points (I. P.) and they lose their nega-
tive character earlier than those with a lower I. P. Consequently,
the proteins with the lower isoelectric points are eluted when the
conditions become more acidic. Although this would suggest that
differences existed between the isoelectric points of the various
eluted protein components, it must be borne in mind that it is quite
possible for factors other than the I. P. to influence the emergence
of the components from the column. Such factors would include the
net surface charge density, molecular size, formation of protein
complexes which might be stable under certain chromatographic
conditions but unstable with pH and ionic changes, and the existence
of specific affinities of a non-electrostatic nature between the ad-
sorbent and the protein (72, p. 1120).

The mechanism of causing an increase in the pH of the initial
effluent may be partially explained by a rapid exchange of ions be-
tween the protein and the adsorbent. Since the column was originally
regenerated with NaOH, the most readily exchangeable ions on the
adsorbent are the OH⁻ group. Conversely, the cations of the pro-
teins having a high isoelectric point are the most likely ions to be
released from the protein. Thus, when the protein is being

adsorbed on the cellulose the OH⁻ ions are released from the column

and combine with the Na^+ or K^+ ions freed from the protein to form

either NaOH or KOH which in turn increases the pH of the effluent.

The reproducibility of this procedure in the separation of

similar protein fractions from the same muscle but from three dif-

ferent animals may not appear, off-hand, to be what is desired.

Nevertheless, it does provide impetus for conducting further re-

search on the muscle proteins by this technique. All in all, the

agreement between the number of components separated per fraction

between the muscle samples is quite good when fully considering the

heterogeneity found within a muscle plus the additional variables

existing between animals. In addition, there are a number of en-

vironmental factors which may also influence and possibly intensify

the variability within and between muscle samples.

Although there can be no question that further work is neces-

sary before this procedure and technique can be adapted for standard

or routine use in studies on muscle proteins, it is quite easy to

visualize the many advantageous ramifications it may offer once it

has been perfected. For example, the post-mortem changes occur-

ring in the muscle proteins can be readily followed at systematic in-

tervals. Furthermore, while following the proteolytic changes, the

various fractions can be collected and used for integrated studies to

isolate, identify and characterize the proteolytic enzymes intimately

associated with autolysis.

SUMMARY AND CONCLUSIONS

A study of the adaptability of the column chromatographic technique for the fractionation of the bovine loin muscle proteins was completed. The muscle proteins were initially extracted with a KCl-phosphate buffer and separated into major fractions by changing the ionic strength of the buffer and by centrifugation.

The major fractions were then separated by column chromatography using diethylaminoethyl cellulose (DEAE-cellulose) as the anion exchange adsorbent. As fractionation proceeded, the various components were collected and analyzed for homogeneity by the disc electrophoresis technique.

When the total KCl-phosphate soluble proteins were fractionated on the DEAE-cellulose column, 9 to 12 protein components could be separated. The number of components varied between the samples of the three animals. The first component to be eluted from each sample was very heterogeneous. Disc electrophoresis analysis showed that it generally contained at least five or six additional fractions. The remaining components appeared to be fairly homogeneous since the electrophoresis data indicated that most of them contained one fraction.

The KCl-phosphate soluble proteins minus the actomyosin

were fractionated by the column into eight or ten different protein components. The disc electrophoresis results also showed that this fraction contained nine or ten different components.

The sarcoplasmic + actin proteins were separated into six protein components by the column procedure. The results of the electrophoretic analysis indicated that each of the six components was eluted as a homogeneous entity.

The myosin and actomyosin proteins were not adequately fractionated by the DEAE-cellulose ion exchange chromatography procedure. However, the actomyosin proteins of the third animal were fractionated into four components which agrees with the electrophoresis results obtained on the extract prior to column separation.

In most instances, the protein recovery data indicated that 85 percent or more of the protein applied to the column was accounted for in the subsequent effluent. However, protein recovery for the myosin and actomyosin proteins was somewhat less, ranging from 66 to 88 percent.

Some of the difficulties encountered with chromatographic procedure in this study are discussed. In addition, the possible advantages for the use of a perfected chromatographic technique in future studies on muscle proteins are described.

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