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The electrophoretic behavior of sarcoplasmic proteins was investigated by the use of vertical acrylamide gel electrophoresis. The first part of the study was concerned with the separation of sarcoplasmic proteins extracted at different times of post-mortem aging. Electrophoresis of the sarcoplasmic extracts by the discontinuous technique, using ten percent acrylamide gels, resulted in the separation of 18 (possibly 20) electrophoretically different proteins. Differences between electrophoretic patterns of sarcoplasmic proteins as post-mortem aging proceeded were slight.

Heterogeneity of sarcoplasmic protein fractions obtained by DEAE-cellulose chromatography was investigated by vertical acrylamide gel electrophoresis in the second part of this study. Electrophoretic analyses of the sarcoplasmic fractions from the above separations indicated that the major peaks of the chromatographic

profiles were quite heterogeneous. Fraction Areas I and IV, which appeared as single homogeneous chromatographic peaks, showed five and six distinct bands, respectively, when subjected to gel electrophoresis. Once again changes in the electrophoretic patterns at 0 and 10 days of post-mortem aging were slight with main differences being in the density of the bands.

The versatility and high resolving power of acrylamide gel electrophoresis for separating sarcoplasmic proteins was demonstrated. Excellent reproducibility of electrophoretic patterns was noted and in most instances, the patterns were clear and showed well-defined bands.

Attempts to fractionate sarcoplasmic proteins by density gradient electrophoresis resulted in separations characterized by poor resolution.

Gel Electrophoretic Analysis of Bovine Sarcoplasmic Proteins

by

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INTRODUCTION

In recent years much of the fundamental research concerned with meat has been centered around the proteolytic changes occurring in muscle proteins during post-mortem aging. These changes are assumed to have a major influence upon the tenderness properties of meat.

Parallel to the above, considerable effort has been directed toward the development of procedures or techniques capable of detecting proteolytic changes systematically. Cellulose ion exchange chromatography has been employed in our laboratories with some degree of success in fractionating bovine sarcoplasmic proteins (Rampton, 1965; Thompson, 1967). Results of these studies, however, have indicated that several of the fractions thus obtained were heterogeneous. Thus, it became evident that additional techniques should be employed to determine whether a higher degree of protein separation could be obtained.

Zone electrophoresis, utilizing porous supporting media such as starch or acrylamide gels, has been used advantageously to separate and study muscle proteins of various species (Giles, 1962; Aberle and Merkel, 1966; Maier and Fischer, 1966).

The versatility and flexibility with which polyacrylamide gels

can be manipulated to improve resolution of protein separation (Davis, 1964; Ornstein, 1964; Raymond, 1964) prompted this study. Thus, research reported herein pertains to electrophoretic analysis of bovine sarcoplasmic proteins by various modifications of vertical gel electrophoresis using polyacrylamide gels. In addition, the heterogeneity of sarcoplasmic protein fractions obtained by diethylaminoethyl-cellulose (DEAE-cellulose) chromatography was determined.

LITERATURE REVIEW

The term "electrophoresis" is currently used to describe the migration of charged particles in solution under the influence of an electric field (Tiselius, 1959). Longsworth (1959a) and Wunderly (1959) both cited the studies of Hittorf in 1853 and Lodge in 1866, as being the earliest recorded investigations concerned with the transport of dissolved substances by electric current. The discovery by Hardy (1899) that many biocolloids showed characteristic electrophoretic mobilities that were largely dependent upon the pH of their solution greatly stimulated the application of electrophoresis in the separation and study of enzymes and proteins. An early example of such application was the determination of the isoelectric points of enzymes by migration experiments at different levels of pH (Michaelis, 1909). Since the publication of these early studies, electrophoretic migration has been investigated extensively.

Electrophoretic Mobility

Generally speaking, electrophoresis is based upon the existing differences in the electrophoretic mobility of ions suspended in a liquid (solvent) when an electric field of given strength is applied (Tiselius, 1959). This mobility depends directly on the magnitude of the net charge on the ion, and inversely upon the frictional

resistance exerted on the charged particle by the solvent (Fox and Foster, 1957). The magnitude of charge on the protein ion is influenced by the pH of the solution. For any protein there exists a pH where mobility is zero, the isoelectric point. At pH lower than the isoelectric point, the protein (positively charged) migrates as a cation, while at pH higher than the isoelectric point (negatively charged), it migrates as an anion. Electrophoretic mobility is defined as the velocity attained in a unit potential gradient since the force exerted on an ion in an electric field depends upon the potential gradient while frictional resistance is proportional to the velocity. At the velocity attained, the force of the electrostatic field and that of resistance are equal, assuming that the protein can be considered as a simple isolated ion acting independently of other ions in the system. In most instances, however, the protein ion in question is surrounded by other ions, including other protein ions and particularly small ions such as H^+ or OH^-, and various buffer ions. The protein ion generally is immersed in a cloud of opposite charge, moving slightly in the opposite direction which tends to retard its migration. Thus, the actual electrophoretic mobility decreases with increasing ionic strength while the idealized mobility can be attained only at zero ionic strength (Fox and Foster, 1957).

Types of Electrophoresis

Electrophoresis is considered as one of the most effective methods for the separation of proteins of a mixture (Tiselius, 1959). The various methods which have been used to study the electrophoretic behavior of proteins can be divided into three groups: (1) direct observation, (2) the moving boundary technique, and (3) zone electrophoresis (Longsworth, 1959a).

Direct Observation

This method is applicable when the particles are large enough so that their motion can be observed directly in the field of a microscope. Microorganisms and other living cells can be studied by this method. Conversely, many charged particles (e.g. protein ions) are not large enough to be observed microscopically (Longsworth, 1959a). Hence, this method has been supplanted in protein studies by the other methods to be described.

Moving Boundary Technique

The moving boundary method is based upon the principle that the sharp boundary which can be formed between a protein solution and a buffer solution, at pH values above or below the isoelectric point, migrates upon application of a potential gradient. Protein

molecules of the same kind move at the same rate. Thus if a protein solution contains more than one protein, the originally homogeneous boundary will separate into two or more boundaries during electrophoresis (Fox and Foster, 1957; West, 1963). Moving boundary electrophoresis is carried out in a specially designed cell developed by Tiselius (1937). After completion of an electrophoretic run, the observation and photography of boundaries (electrophoretic pattern) can be carried out by the use of appropriate optical systems. Such systems are based on the principle that the refractive index of boundaries varies from point to point in the electrophoresis tube with variations in protein concentration (refractive gradient) (Fox and Foster, 1957).

During an electrophoretic run, boundaries can be disturbed and lose their sharpness by electroosmotic streaming, thermal convection due to the heat generated by passage of electric current, and convection caused by the development of layers of solution less dense than those above or more dense than those underneath (Longsworth, 1959b; West, 1963). The characteristic feature of moving boundary electrophoresis is that complete separations are not possible. In order to increase the resolution, especially of protein ions with slightly different mobilities, countercurrent electrophoresis can be used. In this technique, the movement of boundaries against a flow of solution in the opposite direction extends the height of the

electrophoretic channel to improve the resolution (Longsworth, 1959a).

Zone Electrophoresis

In zone electrophoresis, complete separation is possible and each component of a protein solution can be resolved as a discrete zone (Fox and Foster, 1957). Although it is not possible to obtain complete separation in moving boundary electrophoresis due to convective mixing, this defect is eliminated in zone electrophoresis by the use of a suitable supporting medium. Of the two types of zone electrophoresis thus far developed, one involves the use of density gradients while the other is conducted with porous media (Longsworth, 1959a).

Zone Electrophoresis in Density Gradients. Prior to an electrophoretic run with the zonal density gradient method, a gravitationally stable density gradient must be formed in a vertical channel (column) by the appropriate mixing of buffer solutions to which a gradient forming material has been added to increase the density (Colehour, 1960; Longsworth, 1959a). The gradient forming material must be of high density and molecular weight, of low viscosity, readily soluble in the buffer solutions used, chemically inert, and non-toxic to the molecules being studied (Beckman Instruments, 1960). In this technique, the stabilizing power of the density gradient

column is utilized to insure stability of both the initial and the migrating zones. During the electrophoresis of a protein mixture, components migrate convection-free to form bands stabilized by the density gradient zones (Svensson et al., 1957). Kolin (1954, 1955) has described the use of density gradient columns with and without superimposed pH gradients. Svensson et al. (1955, 1957) also used density gradient columns without a pH gradient.

The sample mixture to be separated is placed either in a zone near the top of the column so that the proteins descend (descending technique) upon the application of current, or at the bottom of the column, whereby the proteins ascend (ascending technique) during electrophoresis (Longsworth, 1959a). The absence of both the disturbance, caused by electroosmosis, and the adsorption of the sample on the stabilizing material plus the possibility of recovering the fractions without elution, are additional advantages of this electrophoretic method. Density gradient electrophoresis is not only used for electrophoretic analysis of protein mixtures but also may be employed for large-scale preparative separations (Bier, 1959). Although preparation of the density gradient is probably the most sensitive part of this method, the use of specially designed devices simplifies the preparation of either linear or non-linear density gradients as desired (Beckman Instruments, 1960; Oster, 1965). According to Svensson (1958), other major difficulties encountered

with this procedure involve the injection of the initial zone (sample) and the subsequent problem of droplet sedimentation. Formation of protein droplets which descend to lower parts of the column distort the analysis curves. With the ascending technique, however, this difficulty can be minimized.

Zone Electrophoresis in Porous Media. Zone electrophoresis can be performed in various kinds of porous stabilizing media in columns or troughs either horizontally or vertically (Kunkel and Trautman, 1959). An early example of the use of paper as a porous supporting medium was reported by von Klobusitzky and Konig in 1939. From that date on, paper electrophoresis has found wide application. However, this method is not free of electroosmosis due to the charge which the fiber surface of the filter paper acquires with respect to buffer fluids (Wunderly, 1959).

The use of agar as supporting medium was first described by Gordon et al. (1950). In 1955, Smithies employed starch gel as a medium for electrophoresis. This method, comprised of zone electrophoresis with a starch gel, proved to have additional resolving power beyond that of the moving boundary method.

Polyacrylamide gels have been introduced as supporting media for zone electrophoresis in recent years (Raymond and Weintraub, 1959). These gels produce results similar to those obtained with starch gels although they are much easier to manipulate and can be prepared

in a wider range of concentrations (Tombs, 1965). To separate two component ions, it is necessary to permit electrophoretic migration to continue until one of the kinds of ions has travelled at least one thickness of volumes that it initially occupied (starting zone) further than the other. However, the sharpness, and therefore the resolution, of the zones occupied by each ion diminishes with time due to the spreading of zones as a result of diffusion (Ornstein, 1964). With the development of acrylamide gels, it was found that by varying the pore size of the gels, diffusion could be significantly retarded and sharper resolution of all fractions could be obtained. Acrylamide gels have additional advantages in that they can be prepared in a wide range of average pore sizes and they are thermostable, transparent, strong and relatively inert chemically. They are also non-ionic which indicates the absence of electroosmosis (Raymond and Nakamichi, 1962, 1964). This is remarkable considering the fact that even starch carries a few anionic groups which are, in part, responsible for the backwards endosmotic flow noted with its use (Ornstein, 1964).

In moving boundary electrophoresis, the mobility of charged particles (e.g. proteins) is a function primarily of particle size, shape and electric charge, the latter varying with pH (West, 1963). Acrylamide gel electrophoresis contains an additional characteristic effect of "sieving" or "molecular filtration" (Ornstein, 1964). Since

acrylamide gel electrophoresis combines the effects of both free electrophoresis and molecular filtration, this procedure offers a degree of flexibility and versatility not readily attained by other electrophoretic techniques (Davis, 1964; Raymond, 1964).

Gels are formed when acrylamide is copolymerized in an aqueous solution with methylenebisacrylamide, using an oxidation-reduction catalyst system, usually an amine, with ammonium persulfate (White, 1960). Polyacrylamide gel lattices are carbon-carbon polymers with pendant amide groups (Davis, 1964). These polymers consist of random intertwining of polyacrylamide chains that are occasionally crosslinked with the methylenebisacrylamide (White, 1960). Since water-soluble materials can diffuse into, and out of, the so-formed gel, electrophoretic measurements can be obtained. The permeability coefficient depends upon the percent acrylamide in the gel and is independent of the amount of crosslinking. This means that the pore size of a gel (e.g. from 95 percent acrylamide and five percent methylenebisacrylamide) is controlled by the concentration of polymer and is little affected by the amount of crosslinking (White, 1960). Other experiments (Raymond and Nakamichi, 1962) have demonstrated that the amount of catalyst has no significant effect on the electrophoretic properties of the gel although by increasing the concentration of catalyst, the time of polymerization is decreased.

The capacity of acrylamide gels to "sieve" high molecular

weight substances (e.g. proteins) initiated the possibility for resolving ions of similar, and even identical, free mobilities. Sieving properties of these gels are unique. Charged proteins moving through the gel experience a frictional resistance that depends on their dimensions and the average pore size of the gel. The "sieving" power of a gel is low when the diameter of the moving particle is small compared to the average pore size of the gel, and virtually infinite when the diameter is very large compared to the pore size. As the average pore size approaches the range of dimensions of proteins, various components will be differentially retarded to degrees proportional to their dimensions. Thus, during electrophoresis, protein mixtures are fractionated through dimensional as well as by charge differences (Davis, 1964). It has been computed, for example, that a 7.5 percent polyacrylamide gel has an "average pore size" of about 50 Angstroms while a 30 percent gel produces about a 20 Angstrom pore (Ornstein, 1964).

Parameters of some well-known proteins are given in Table 1. In considering the average pore size of a 7.5 percent gel in conjunction with data of Table 1, it can be predicted that β_1 lipoprotein, fibrinogen, γ globulin, and perhaps, α_2 macroglobulin, should experience higher frictional resistance than the other proteins when an electrophoretic run is carried out in such a gel. Hence, the pore size of a gel can be tailored to the dimensions of molecules

to be separated (Ornstein, 1964).

Table 1. Parameters of some proteins.[a]

Protein	Mobility, M_w[b]	Molecular weight	Length[c]	Diameter[c]
Albumin	-6.1	69,000	150	38
Transferrin	-3.3	90,000	190	37
β_1 lipoprotein	-3.0	1,300,000	185	186
γ globulin	-1.0	156,000	235	44
Fibrinogen	-2.1	400,000	700	38
a_2 macroglobulin	-4.2	850,000	---	---

[a] Data from Ornstein (1964)

[b] M_w expressed in mobility units at 0° C; one unit = 10^{-5} cm^2/volt-sec.

[c] Dimensions expressed in Angstroms.

Polyacrylamide gels have been used in the development of both disc and vertical gel electrophoresis. In disc electrophoresis, a polyacrylamide gel column is formed in a suitable container such as a cylindrical glass tube. The column is prepared in three layers: (1) a large-pore sample gel, (2) a large-pore spacer gel, and (3) a small-pore gel in which the electrophoretic separation takes place. After an electric current has been applied for a certain length of time, gels are removed from the tubes and stained. Upon completion of destaining an electrophoretic pattern of stained proteins in the form of discs is obtained (Davis, 1964).

Vertical acrylamide gel electrophoresis is carried out in a specially designed cell which is divided in two electrode chambers (Raymond, 1964). An overflow tube in the upper chamber plus a suitable pump permit, if desirable, the continuous recirculation of buffer during the run. In this method, the acrylamide gel is formed in the space between two cooling plates of the electrophoretic cell while it is in horizontal position. During electrophoresis, the cell and gel slab are maintained in a vertical position. Samples are placed in wells at the top edge and migrate downwards.

Vertical gel electrophoresis combines the advantages of the acrylamide gel with those of the flat slab. Such advantages are: (1) provides maximum surface area for cooling of the gel, which is particularly important in the electrophoresis of proteins, (2) allows the application of two-dimensional techniques, which is not possible with cylindrical gels, and (3) facilitates the direct comparison of many samples which can be processed in a single gel at the same time. In addition, a recording densitometer can be used for quantitation of results (Raymond, 1964).

Vertical gel electrophoresis can be carried out by either the continuous or discontinous techniques. The objective of the continuous technique is to maintain uniform conditions within the electrophoresis medium during a run. The continuous recirculation of buffer is the means employed to achieve uniformity of conditions

in the cell. This recirculation counteracts pH and concentration changes within the cell. In addition, a prerun, involving the passage of electric current through the buffer and gel for a certain length of time prior to electrophoresis, helps to establish the desired uniformity of conditions (Raymond, 1964).

The discontinuous technique combines pore size control and stacking of the sample into thin starting zones (Ornstein, 1964). Discontinuous gel and discontinuous buffer systems are two essential feature of this technique (E-C Apparatus Corporation, 1966). The discontinuous gel system consists of both large-pore spacer and small-pore running gels. The large-pore spacer gel is used to preconcentrate the sample into a thin layer electrophoretically prior to separation. In this case, however, the sample is prepared with acrylamide to form an anticonvection mixture. The sample is then separated by a combination of electrophoresis and molecular sieving in the small-pore running gel. The discontinuous buffer system includes a running buffer, a space buffer and an electrode buffer. The necessary ionic constituents of this system are the trailing ion, the leading ion and the buffer counter-ion (Williams and Reisfield, 1964). The leading ion (e.g. Cl^-) is present in the sample mixture and in both the spacer and running buffers. It has the highest mobility of the three types of ions and is independent of pH. The trailing ion (e.g. glycine) is found in the electrode buffer. It has lower

mobility than any of the proteins in the spacer gel but a higher one in the running gel. The pH discontinuity between the spacer and running gels increases the mobility of the trailing ion in the running gel. Although the leading ion-trailing ion boundary in the spacer gel concentrates the proteins in a thin layer, in the running gel this boundary moves fast enough to leave the proteins behind. Thereafter, proteins in the running gel are subjected to electrophoresis (Williams and Reisfield, 1964). The buffer supplies counter-ions to maintain electrical neutrality. These counter-ions are present in all sections of the cell. The conductivity jump across the moving boundary creates thin starting zones and consequently, sharper resolution of protein mixtures. However, the use of buffer systems with ions of special properties decreases the possibility of varying the pH and composition of buffer. In addition, substances from biological material are sometimes stable only in specific buffers and a multi-buffer system may result in their degradation. A less complicated method to achieve a thin starting zone is to decrease sample conductivity in order for it to be lower than that of the buffer (Hjerten et al., 1965).

The two-dimensional electrophoretic technique (orthogonal or orthacryl) is a recent innovation concerning the application of vertical gel electrophoresis. This method employs acrylamide gel in two orthogonal directions. Gel concentration and pH are the main

experimental factors which are varied to produce the desired orthogonal pattern. Alteration of buffer composition and field strength plus chemical modification of gels may also be employed as experimental variables (Raymond, 1964). An orthogonal-gel concentration pattern provides information about the molecular size of proteins whereas an orthogonal-pH pattern gives data pertaining to molecular charge. Two-dimensional electrophoresis has been used to study the nature of protein interactions occurring during gel electrophoresis (Bier, 1959). The following protein-protein interactions are those generally considered to take place in electrophoresis:

(a) the polymerization of $nB \rightleftharpoons Bn$

(b) the bimolecular association of $B + C \rightleftharpoons BC$

and

(c) the isomerization of $B \rightleftharpoons B'$

Some possible signs of such interactions occurring in zone electrophoresis are tailing of fractions, appearance of one or more new bands and changes in mobility (Ewart, 1966). However, protein-buffer interaction has also been reported to give electrophoretic patterns showing two or more new peaks (Cann, 1966).

Ammonium persulfate has been reported to cause increased electrophoretic heterogeneity during disc electrophoresis in gels containing 8 M urea. Brewer (1967) has found that under certain conditions the persulfate inactivates yeast enolase to produce

patterns containing additional protein zones.

After completing an electrophoretic run, gels are stained with an acidic dye solution to locate protein zones. Excess dye is removed from the gel after the protein has been fixed and stained. Rapid electrophoretic methods using specially designed cells have been developed for destaining the gels (Burger and Wardrip, 1965; Nagy et al., 1966). Acrylamide gels retain their original size and shape during the staining and destaining processes. These gels can be stored wet in plastic bags for as long as two years (Tombs, 1965) or they can be allowed to dry out completely. Gels that have shrunk uniformly can be rehydrated to their original dimensions by soaking in water. Although stained gels tend to fade with time, such patterns can be restored by soaking in water, followed by restaining and destaining (Raymond and Weintraub, 1959).

Preparative Electrophoresis

While full advantage has been taken of the possibilities of electrophoresis at the analytical level, progress at the preparative level has been much slower (Bier, 1959). Separation and recovery of pure proteins on a laboratory scale is required for further studies. The ordinary methods of protein separation and purification (e.g. salt precipitation, pH variation, adsorptimetric procedures, etc.) often produce small amounts of chemically altered proteins.

Recently, preparative electrophoretic techniques have been developed by Vande Woude and Davis (1963) and Raymond and Jordan (1966). The first step of these techniques is the separation of proteins from each other. Secondly, recovery of fractionated proteins in a suitable form from the gel after resolution has been achieved. Purified protein components can be recovered by elution-convection electrophoresis. The high-resolution gel slab pattern obtained with the analytical electrophoresis gel is used in the elution-convection cell for removal and recovery of separated protein components. Compared with its original orientation in the vertical gel cell, the gel slab is rotated 90 degrees in order to be placed in the elution-convection cell. Protein zones are then eluted from the gel electrophoretically. The emerging proteins are trapped in vertical channels sealed on one side while the other side contains a dialyzing membrane which bars the passage of proteins. The proteins accumulate by electro-convection and are recovered in collecting tubes in a concentration almost equal to that of the original sample (Raymond and Jordan, 1966).

EXPERIMENTAL PROCEDURE

Sample Preparation

Muscle Extract

Wholesale beef rib sections (cow, U.S.D.A. commercial grade) were obtained from a local meat processing plant and held at 4° C until used.

Fifty gm of longissimus dorsi muscle, excluding as much intermuscular fat and connective tissue as possible, were diced and homogenized with 50 ml of distilled water for 90 seconds in a water-jacketed Waring blendor cooled with flowing tap water (10-12° C). The pH of the homogenate was adjusted from about 5.5 to 7.0 with 1 N NaOH (Thompson, 1967). Homogenization was continued for an additional 30 seconds to thoroughly mix the homogenate. The homogenate was centrifuged at 30,000 × G for 25 minutes at 4° C. The supernatant was filtered through Whatman No. 12 filter paper to remove floating fat particles. A portion of the filtrate was used as undialyzed extract while another portion was dialyzed against a 200-fold volume of 0.04 M Tris (hydroxymethyl-aminoethane)-phosphate buffer, pH 9.0, for 24 hours at 4° C (Rampton, 1965). The remainder of the extract was dialyzed against a 200-fold volume of distilled water, adjusted to pH 7.0 with 1 N NaOH. In the latter

case, the precipitate formed during dialysis was removed by additional centrifugation, as described above, for 15 minutes.

To obtain the desired density, sucrose, an inert solute used in the continuous electrophoretic technique, or a "sample mixture", a solution used in the discontinuous technique, were added to the undialyzed or dialyzed muscle extracts (Raymond, 1964; E-C Apparatus Corporation, 1966). The "sample mixture" was prepared by dissolving 4 gm of acrylamide and 5 gm of sucrose in Tris-HCl buffer (spacer buffer), pH 6.7, diluting to 100 ml and adding to this solution, 0.1 ml of TMED (N, N, N', N'-tetramethyl ethylene diamine) and 0.1 gm of AP (ammonium persulfate) with vigorous stirring. After polymerization, the solution was colored with a minimum amount of bromophenol blue. Muscle extracts were mixed with appropriate volumes of "sample mixture" to obtain protein concentrations of 4 to 5 mg/ml (E-C Apparatus Corporation, 1966).

DEAE-Chromatography Fractions

In conjunction with other experimental work (Thompson, 1967), fractions of sarcoplasmic proteins obtained by DEAE (diethyl aminoethyl)-cellulose ion exchange chromatography were collected and analyzed.

After passing through UV monitors where transmittance was

measured at 280 mμ, effluents of 0- and 10-day post-mortem sampling times were collected in 10 ml fraction collecting tubes. Fractions were marked on appropriate absorption recordings, by automatic fraction markers, to facilitate collection of fractions contained in areas designated I, II, III, IV and V in Figure 1.

Although fraction areas I and III are shown as large single peaks on the chromatographic profile, the fraction areas II, IV and V each contain several small peaks. The latter two fraction areas were arbitrarily grouped as such to obtain samples containing protein levels sufficient for electrophoretic analysis. Effluents of fraction areas were freeze-dried to obtain concentrated protein samples. In the first series of experiments, effluents were not dialyzed. In the second series, however, they were dialyzed against a 150-fold volume of distilled water for 24 hours at 4° C. Dried protein samples were diluted with distilled water to obtain samples having the desired protein concentration. For the continuous electrophoretic technique, a small amount of sucrose was added to these samples. Also, a small amount of dye (bromophenol blue) was added to the colorless samples. For the discontinuous technique, samples were mixed with appropriate volumes of "sample mixture" prior to electrophoretic runs.

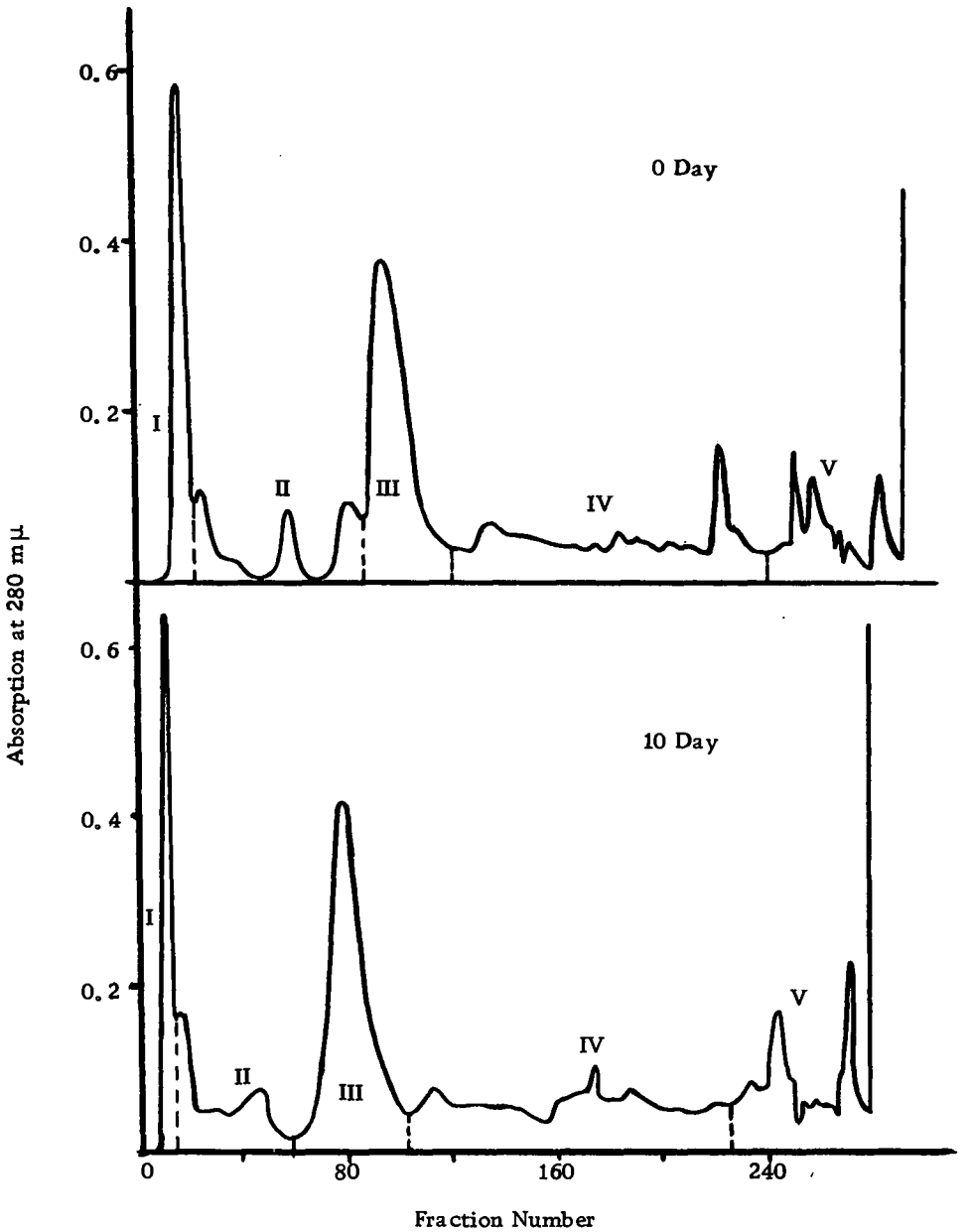

Figure 1. DEAE-cellulose chromatography of bovine sarcoplasmic proteins aged 0 and 10 days. Sample size: 9 ml of dialyzed extract (270 mg of protein). Concave gradient elution: starting buffer, 0.04 M Tris-phosphate, pH 9.0; final buffer, 0.5 M Tris-phosphate, pH 3.6. Fraction areas analyzed electrophoretically are designated by Roman numerals. Vertical lines indicate division of fraction areas.

Vertical Gel Electrophoresis

A vertical gel electrophoresis apparatus (Model EC-470, E-C Apparatus Corporation, Philadelphia, Pa.) and companion power supply (Model EC-454) were used for electrophoretic runs. Continuous, discontinuous and orthogonal procedures were the three vertical gel electrophoretic techniques employed.

Continuous Gel Electrophoresis

Tris-EDTA-boric acid (TEB) buffer was prepared by dissolving 180 gm of Tris, 19.9 gm of $Na_2EDTA \cdot 2H_2O$ (disodium ethylene diamine tetraacetic acid) and 6.8 gm of boric acid in 18 liters of distilled water (stock solution). The buffer was adjusted to pH 9.2 with boric acid. To 150 ml of this buffer, appropriate amounts of Cyanogum-41 (composed of 95 percent acrylamide and five percent N,N'-methylenebisacrylamide) were added to obtain gels of 5, 7.5, 10 and 13 percent concentrations. To catalyze gel polymerization, 0.20 ml of TMED was added first and after filtration through Whatman No. 12 filter paper, 0.2 gm of AP was dissolved in the filtrate. The gel solution was immediately poured into the space between the cooling plates of the electrophoretic cell maintained in a horizontal position. The slot form was inserted and the cell was allowed to stand undisturbed until the solution gelled (approximately 40 to 50

minutes).

Water was circulated continuously through the cooling channels of the cell during both the gelling period and the electrophoretic run. Most runs were carried out in a cold room where the temperature of the circulating water was 4° C. In remaining runs, tap water (10-12° C) was used for cooling.

Subsequent to polymerization, the exposed block of acrylamide gel above the slot form was excised, the cell turned to a vertical position, and both upper and lower chambers of the cell filled with TEB buffer. Buffer was recirculated continuously during electrophoresis (Raymond, 1964). The slot form was then removed and an electric current was applied at 300 V (volts) for 30 to 60 minutes. After this prerun, the gel was ready to receive the sample.

Samples were applied with a syringe directly into the slots at the top of the gel slab to form a layer under the buffer. Sample slots were usually completely filled. During sample application, and for a few minutes thereafter, buffer was not recirculated to facilitate "stacking" of sample. Current was maintained at 300 V until the colored band of the samples from the muscle extract had travelled about three-fourths the length of the gel. For the colorless samples from DEAE-cellulose chromatography fractionations, the optimum running times were determined by the use of dye migration guides or by adding samples at various time intervals.

Discontinuous Gel Technique

Buffers and solutions required for this procedure are as follows:

1. Running buffer, pH 9.0; 138 gm of Tris and 12 ml of concentrated HCl were used to make three liters of this buffer. The Tris was dissolved in 1500 ml of deionized water. After addition of most of the concentrated HCl, the pH was adjusted to 9.0 with dilute HCl. Final volume was made up to three liters with deionized water.

2. Spacer buffer, pH 6.7; 22.5 gm of Tris and 12 ml of concentrated HCl were used to make three liters of this buffer. Preparation and adjustment of pH were carried out as described above.

3. Electrode buffer, pH 8.3; 10.8 gm of Tris and 52.2 gm of glycine were dissolved in deionized water to make up 18 liters of buffer. Tris or glycine were added to adjust pH as required,

The above stock solutions were stored in tightly sealed bottles to avoid atmospheric contamination (E-C Apparatus Corporation, 1966).

4. Agarose solution; 100 ml of 0.5 percent agarose solution were prepared with distilled water.

5. Plug and running gel solution; Appropriate amounts of Cyanogum-41 were dissolved in running buffer (described above)

to make 200 ml of 5, 7, 10 and 13 percent solutions. Then 0.2 ml of TMED and 0.02 ml of Tween 80, a wetting agent, were added prior to filtration through Whatman No. 12 filter paper.

6. Spacer gel solution; 14 gm of Cyanogum-41 were dissolved in spacer buffer to make 100 ml of solution. One-tenth ml TMED and 0.01 ml Tween 80 were added and the solution filtered.

Due to instability, it was necessary to prepare the plug and running gel and spacer gel solutions just prior to use (Ritchie et al., 1966).

Before assembling the electrophoretic cell, heated agarose solution was placed along the outer edges of the gaskets and allowed to gel. The cell was placed at an angle after assembling. One hundred ml of running gel solution were mixed with 0.1 gm AP and poured between the cooling plates. Angle of the cell was adjusted so that the level of solution was 2.5 to 3 cm from the bottom of the inner cooling plate (see Figure 2). The surface of the solution was covered with 5 ml of distilled water to trap and remove air bubbles. After gel polymerization, the water was removed, the gel plug rinsed with running gel solution, and the cell placed in a vertical position. Then 100 ml of running gel solution were mixed with 0.1 gm AP and poured between the cooling plates to a level 2.5 cm below the top of the inner cooling plate (Figure 2). The surface of the gel solution was again covered with 5 ml of distilled water. After polymerization

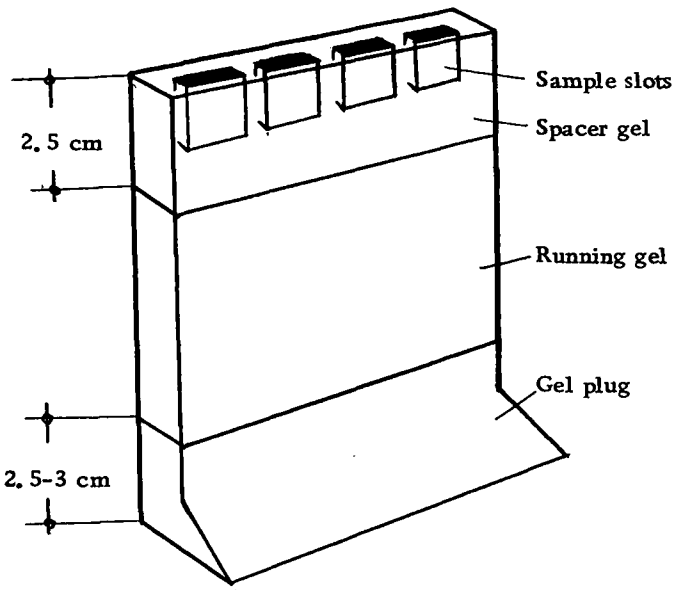

Figure 2. Schematic diagram of the discontinuous gel system used in vertical gel electrophoresis. (Adapted from E-C Apparatus Corporation, 1966).

and removal of water, the gel was rinsed with spacer gel solution and the apparatus was returned to a horizontal position. One hundred ml of spacer gel solution were mixed with 0.1 gm AP and used to fill the remaining gel space. The slot form was inserted and the cell was allowed to stand undisturbed until the solution was completely polymerized. Polymerizations generally occurred within 30-35 minutes although longer times were often required for gels of low concentrations.

Excess gel above the slot form was removed and the cell was turned to vertical position. The upper electrode chamber was filled with electrode buffer allowing buffer to overflow until the lower chamber electrode was completely covered. The slot form was removed and each sample slot was filled with the prepared sample. The electrophoretic cell was cooled by circulation of water as described previously. An electric current of 200 V was applied until the sample was completely stacked in spacer gel and the sharp sample layer had moved to the surface of the running gel (a prerun). Current was then increased to 400 V for the remainder of the electrophoretic run. Buffer was not circulated during electrophoresis (E-C Apparatus Corporation, 1966).

During all electrophoretic runs, continuous or discontinuous, the cathode and anode were oriented in the upper and lower chambers of the cell, respectively.

Orthagonal (Orthacryl) Gel Electrophoresis

The initial stage of this procedure was identical to that described previously for the continuous and discontinuous techniques. Gel patterns were prepared under aforementioned conditions in the electrophoretic cell using a four space slot form. Vertical strips of these patterns (10-12 cm long and 0.8-1 cm wide) were excised and used for second electrophoretic runs. Stained gels of identical runs or strips of the same gel pattern were stained and used for reference. Gel concentration and buffer pH were the two factors varied to produce an orthogonal pattern.

1. Orthagonal gel concentration procedure. Seven percent gels were prepared for initial electrophoretic runs while ten percent gels were used in the second runs. Other experimental factors were held constant. When the continuous technique was applied, initial runs of four hours were preceded by 30 minute preruns. Current was applied for 5.5 hours in the second runs. After completion of the first run, gel slabs were removed from the cell and strips were excised longitudinally from the patterns. These strips were cross-matched with those that had been stained in identical runs in order to locate the protein bands. The gel compartment of the cell was filled with ten percent gel solution. In place of the slot form, the excised strips of the first runs were immediately inserted into the

compartment before the solution polymerized. Following gel polymerization and embedment of sample strips, the cell was returned to a vertical position for the second run. The discontinous technique was also used in both directions to obtain orthogonal patterns. In this case, concentrations of the plug and running gels were seven percent in the first run and ten percent in the second. Spacer gel concentrations were maintained at four percent.

2. Orthogonal pH procedure. In this method, the variable experimental factor was buffer pH (Raymond, 1964). TEB buffer, pH 9.2, was used for initial runs while a Tris buffer, pH 8.6, was employed in the second runs. The latter buffer was prepared by mixing 32.2 gm of Tris, 2.78 gm of disodium-EDTA and 4.25 ml of glacial acetic acid in three liters of distilled water. Although the second run gel concentration was held at seven percent, the solution was made from the freshly prepared Tris buffer, pH 8.6.

Staining and Destaining of Gels

After electrophoresis was completed, gel slabs were stained for one to two hours in the dye solution with intermittent stirring. Dye solution was prepared by dissolving 4 gm of amido black-10B in a solvent consisting of 1000 ml of methanol, 1000 ml of distilled water plus 200 ml of acetic acid. After the proteins were fixed and stained, excess dye was removed with an electrophoretic destainer

(Model EC-479). The destaining solution consisted of eight liters of distilled water, eight liters of methanol and one liter of glacial acetic acid. When necessary, contaminated solutions were replaced with fresh solutions in order to maintain three to four amperes current during destaining. Electrophoretic destaining was generally limited to 20 to 30 minutes each time since gels were subject to damage by ohmic heating when longer times were employed. Further destaining, if necessary, was achieved by leaving gels in the destaining solution overnight. Destained gels were washed with tap water and clarified with a five percent solution of trimethylol propane. Gels were photographed by the Oregon State University Photo Service to obtain a permanent record. After photography, gels were stored wet in sealed plastic bags (Tombs, 1965) for further reference.

Density Gradient Electrophoresis

In addition to the gel electrophoretic studies, density gradient electrophoresis was also employed to investigate separation of sarcoplasmic proteins.

A column electrophoresis apparatus (Model 3340C, LKB Instruments, Inc.), requiring the following operating solutions, was used in this study.

1. Buffer, pH 8.6, 0.05 M barbiturate-acetate-HCl; prepared by adding 41.3 gm of sodium barbital, 27.2 gm of sodium acetate

trihydrate and 3 ml of concentrated HCl to make up four liters with distilled water.

2. Buffer, pH 8.6, 0.025 M barbiturate-acetate-HCl; prepared from the above solution by a 1:1 dilution with distilled water.

3. Heavy sucrose solution; prepared by adding sucrose to 0.05 M barbiturate-acetate-HCl buffer to give a final solution having a specific gravity of 1.24.

4. Light sucrose solution; prepared by adding sucrose to 0.025 M barbiturate-acetate-HCl buffer to give a final solution having a specific gravity of 1.17.

The ascending technique of Colehour (1960) was followed. The heavy sucrose solution was added from the Mariotte flask until it reached a level about 7 cm above the bottom of the inner column. The U-shaped left arm was then filled with 0.05 M buffer and the anode inserted. The pinch clamp of the anode and the opening of the center column were closed while the right arm was filled with 0.05 M buffer. Precautions were employed to avoid mixing of buffer with the heavy sucrose solution. The cathode was then inserted and the pinch clamp closed.

The stopper of the center column was removed and 4 ml of sample were added, by means of a capillary tube, to a level extending slightly above that of the heavy sucrose solution. The density of the sample was adjusted by addition of sucrose so that it floated

on the heavy sucrose solution and remained as a sharp layer after the density gradient had been added to fill the center column. Finally, pinch clamps of the electrodes were opened, the center column closed and current applied at 420 V (18-20 mA) for 48 hours. Before the addition of the sample and during the electrophoretic run, cold water (3° C) was circulated through the cooling jacket of the column. The density gradient was prepared by gradual mixing of 500 ml of the 0.025 M buffer with 140 ml of light sucrose solution which was advanced to the column by a micropump. After completion of electrophoresis, pinch clamps of electrodes were closed and the center column opened. The column was emptied through the bottom capillary when the polyethylene funnel was seated on the bottom of the inner column. The density gradient containing the separated protein fractions was passed through a Gilson UV monitor where transmittance was continuously measured at 280 mμ and recorded on a Texas Instruments "recti-riter" recorder. The flow rate of elution was 180 ml/hour while the pH of the effluent was measured at 4 ml intervals.

RESULTS AND DISCUSSION

Determination of Optimum Experimental Conditions

Trial runs were carried out prior to the main electrophoretic experiments to determine optimum experimental conditions for each category of samples. In preliminary runs, such experimental variables as gel concentration, field strength, temperature, buffer composition, pH and other factors which might affect the resolution and migration rate (mobility) of sarcoplasmic proteins were studied.

Gel Concentration

Since polyacrylamide gels can be prepared in a wide range of concentrations (3-30%), it was necessary to determine the influence of gel concentration on mobility and resolution. The influence of gel concentration on migration rate was studied with the continuous technique (Figure 3). Identical samples, sarcoplasmic extracts, were applied to 5, 7, 10 and 13 percent gels at designated time intervals. Preruns were employed before and, in some cases, after application of samples. The absence of detectable electroosmotic backflow in acrylamide gels prepared without additives (Raymond and Nakamichi, 1964; Wieme, 1964) permitted the measurement of

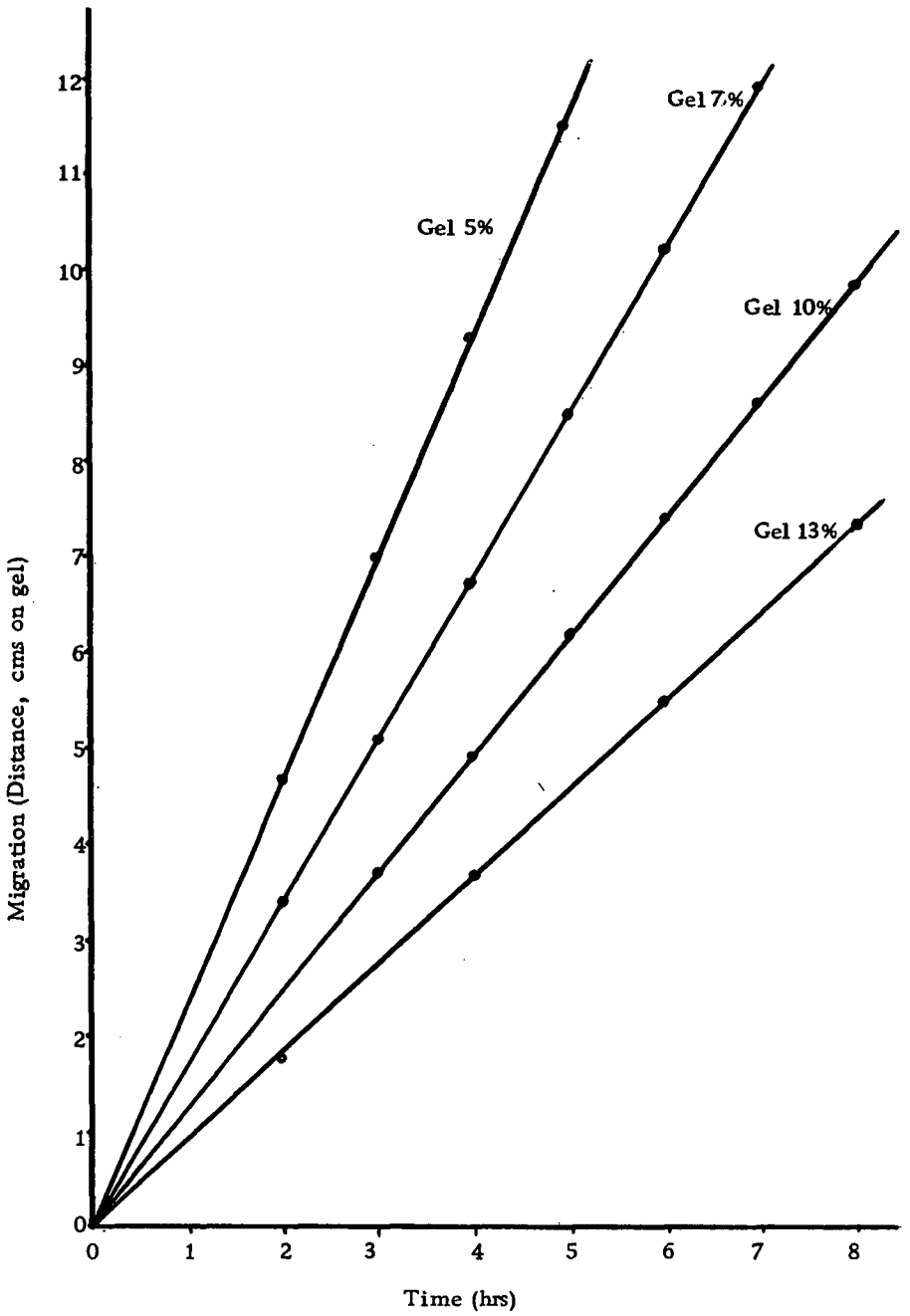

Figure 3. Effect of gel concentration upon electrophoretic migration. Electrophoretic conditions--Sample: pigment band of sarcoplasmic extract. Technique: continuous; buffer, TEB; pH 9.2; current, 300 V; temperature, 4° C.

protein migration without the use of marker proteins. From the results it was evident that migration of protein bands, while retarded during the prerun (Raymond, 1964), was thereafter linear with time. In Figure 3, for example, the migration of the pigment band in different gel concentrations was plotted against time and a linear relationship was observed. Straight lines, with different slopes, were also obtained by plotting the migration rate of other bands (data not presented). For the range of gel concentrations used and for the sarcoplasmic proteins studied, migration rate decreased as gel concentration increased (Figure 3).

Tombs (1965) noted that mobility was not proportional to the mean pore size of the gel throughout a concentration range of five to 15 percent. He noted non-linear response at both high and low gel concentrations. Similar results were found by plotting measured migration (M) of the pigment band against the reciprocal of gel concentrations (Figure 4). Similar curves were obtained by plotting the migration of other bands against the reciprocal of gel concentration.

The effect of the gel concentration on the resolution of the proteins was studied with the discontinuous technique using 5, 7, 10 and 13 percent gels. Results of these studies indicate that the most useful gels for the separation of sacroplasmic proteins approximate ten percent concentration. Seven percent gels were characterized by

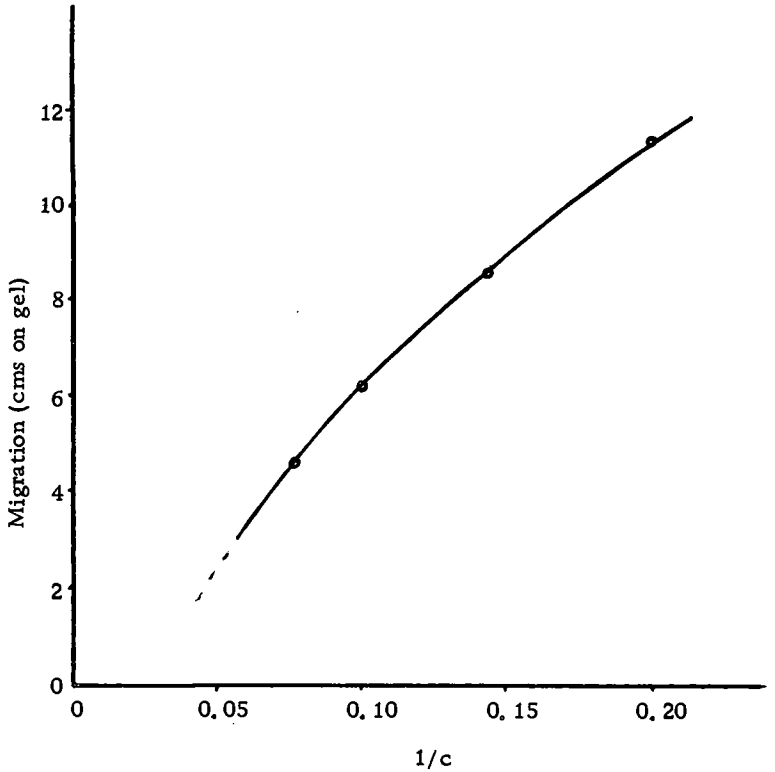

Figure 4. Plot of measured electrophoretic migration versus reciprocal of gel concentration. Electrophoretic conditions--Sample: pigment band of sarcoplasmic extract. Technique: continuous; buffer, TEB; current, 300 V; temperature, 4° C; length of run, 5 hrs.

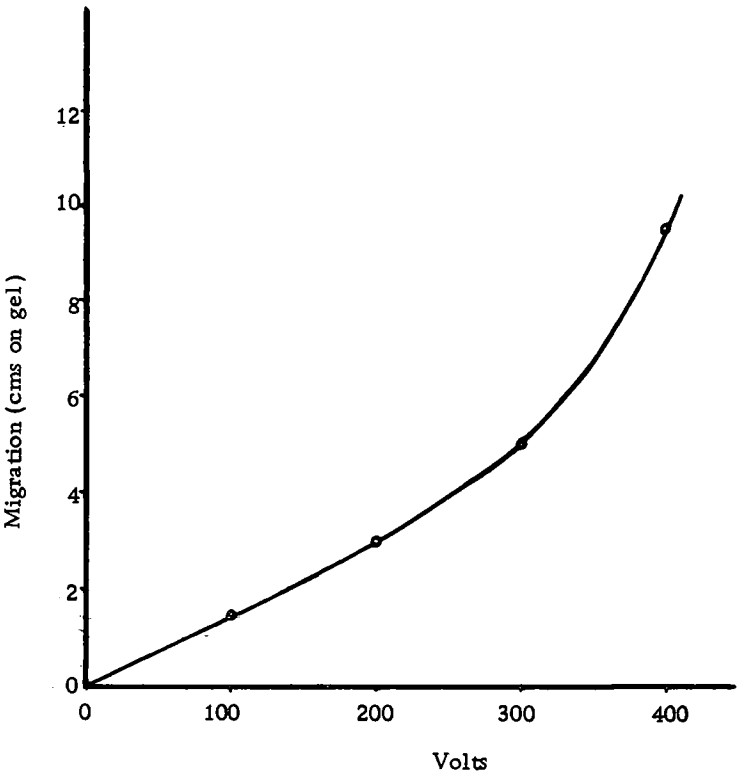

Figure 5. Effect of applied voltage upon electrophoretic migration. Electrophoretic conditions--Sample: pigment band of sarcoplasmic extract. Technique: continuous; buffer, TEB; gel concentration, ten percent; temperature, 4° C; length of run, 4 hrs.

more intense faster moving bands but inferior resolution of the slower migrating bands was evident. Although superior resolution of slower migrating bands was achieved by using 13 percent gels, faster moving bands almost disappeared.

The above observations might be explained on the basis of concentration and average pore size. The smaller pore size of the 13 percent gel, in comparison to the large pore size of the seven percent gels, would reduce proportionately the relative mobilities of all bands by providing increased resistance to migrating protein molecules. Similar results were observed using the continuous technique with 7.5, 10 and 13 percent gels. Results of a series of experimental runs with samples from DEAE-cellulose chromatography fractionation indicated that the range of optimum gel concentration was seven to ten percent.

Field Strength

Electrophoretic migration, as a function of applied voltage, was studied with the continuous technique. Potentials of 100, 200, 300 and 400 volts (V), in ten percent acrylamide gel, were applied to portions of the same sarcoplasmic extract at prescribed time intervals. Four hour migration distances of the pigment band, as a function of voltage level, are presented in Figure 5. According to Giddings and Boyack (1962), taking into account the obstructing matrix of the gel, mobility should not be a linear function of voltage.

Data shown in Figure 5 are in agreement with results of similar studies reported by Raymond (1964) concerning the electrophoretic migration of bovine serum albumin in acrylamide gel.

In this work, an electric current was passed through the buffer and gel for prescribed periods of time before introducing the sample. During this time, a prerun, there is established a steady state flux of buffer ions through the gel which remains essentially unaltered throughout the duration of the experiment (Raymond, 1964). In the continuous technique during the 30-60 min of prerun, the current at 300 V decreased from 130-140 to 90-100 mA. During runs of several hours, current continued to decline slightly to levels of 75-90 mA.

In the discontinuous technique, the application of 200 V for 60-90 min time was considered as a prerun. During this period, current decreased from 80 to 45-50 mA. During several hours of a run at 400 V, the current decreased from 75-90 to 35-50 mA.

While it is evident from data presented in Figure 5 that application of higher voltage levels decreases the duration of electrophoresis, voltage and current level are limited by possible overheating of the gels. Hjerten et al. (1965) stated that current strength should be low during the migration of the sample into the gel to minimize the risk of thermal convection. Overheating was determined visually since it caused the top edge of the gel to swell outward. In the

continuous technique with TEB buffer and using tap water (12-13° C) as the circulating coolant, the starting current was high enough (130-140 mA) to occasionally cause the gels to overheat. If this condition was corrected immediately by reducing the voltage (and current) applied, the swelling disappeared. In this case the patterns could be saved, however, interpretation of results was difficult because of the variable experimental conditions. When electrophoretic runs were carried out in the cold room (cooling water 4° C), overheating of gels was not observed up to 300 V. Higher voltage levels caused swelling of the gels. Thus, for the continuous technique, a maximum voltage of 300 V was used and the main runs were carried out in the cold room (4° C).

In the discontinuous technique, using appropriate buffers, the starting current strength was lower and overheating of the gel was not usually observed. In this case, attempts to apply higher voltage caused swelling of gels even when runs were carried out in the cold room. This was especially true for the low concentration spacer gels. In this technique, the main runs were carried out in the cold room. Optimum voltage levels for the prerun and the main run were 200 and 400 V, respectively. Increasing the current to 400 V occasionally caused the spacer gel to swell and extrude into the upper chamber of the cell. The same phenomenon was observed by Ritchie et al. (1966). This condition did not affect the electrophoretic

separation since the sample layer had already passed into the stationary running gel.

Buffers.

The use of Tris-phosphate, pH 9.0, Tris-acetic acid, pH 8.6, or barbital, pH 8.6, buffers in the continuous technique, did not provide better resolution that that obtained with TEB buffer, pH 9.2. TEB buffer permitted the use of 300 V, providing for a relatively fast migration rate without heating of the gel. Recirculation of buffer counteracted changes in pH and concentration within the cell. For this reason the TEB buffer was used for three to four runs. The use of buffer for more than three to four runs was avoided because of possible contamination from protein, residual catalyst and low-molecular weight polymers from the gels (Raymond, 1964). The pH of TEB buffer before and after the run was 9.2. At this pH nearly all proteins were negatively charged and migrated toward the anode located at the bottom of the gel.

After a run with the discontinuous technique, the pH of electrode buffer was observed to change. The extent of change was time dependent and not necessarily the same in the upper and lower chambers of the cell. Hence, the electrode buffer was not reused. The discontinuous anionic buffer system of Ornstein and Davis, which is considered to be nearly identical to that of Williams and Reisfeld

(1964), was used. Both of the above buffer systems were initially designed for disc electrophoresis. It has been reported that the actual running pH in the discontinuous anionic system is approximately one-half pH unit higher than the initial pH of the running gel (Williams and Reisfeld, 1964). Thus the pH 8.9 of the running gel in the present work corresponded to an actual running pH of about 9.4. At this pH, the proteins are soluble and negatively charged. In addition, most proteins are chemically and biologically stable at this pH. The leading ion, in the buffer system used, was the chloride ion. It has, as required, a high electrophoretic mobility that is independent of pH and also has insignificant damaging effects on proteins (Williams and Reisfeld, 1964). Glycine, with a pK_a of 9.8, fits the suggested requirements for the pK_a (8.9-9.9) of the trailing ion. The pK_a of Tris buffer is 8.1 which is within the required range (7.9-8.9). The pH of the electrode buffer at 8.3 was similar to pH 8.1 suggested by Williams and Reisfeld (1964).

Another anionic system mentioned by Williams and Reisfeld (1964), with running gel pH of 7.5 (or running pH of 8.0), was not tested. This particular system can be used advantageously for many enzymes because the stability of proteins at this pH is better than at higher pH values.

Other Experimental Factors

The approximate amount of protein in muscle extracts was determined spectrophotometrically by measuring the absorption at 280 mμ (Dixon and Webb, 1964). The optimum protein content of samples was 2-5 mg/ml. Table 2 lists the protein content of extracts calculated from corresponding absorbance values. After determination of approximate protein content, muscle extracts were mixed with the appropriate volumes of "sample mixture" or diluted as described in the experimental procedure. The amount of protein in these samples was maintained at 4-5 mg/ml. Differences in salt concentration during dialysis affected the solubility of proteins. When the extracts were dialyzed against water (pH 7.0), or occasionally with Tris-phosphate buffer (pH 9.0), a light precipitate was formed. Zender et al. (1958) reported that the precipitate might be globulin X or other insoluble proteins that had undergone partial proteolysis.

Fractions obtained from the DEAE-cellulose chromatography were freeze-dried immediately after dialysis. The total volume of DEAE-column effluents was approximately 2.5 liters. Freeze-drying was chosen since it is considered to be an excellent method for concentrating large volumes of protein solutions (Haurowitz, 1963). The use of these effluents without dialysis was avoided

Table 2. The protein content of sarcoplasmic extracts.

	Age of sample[a] and treatment	O. D. at 280 mμ	Protein content mg/ml
1.	24 hours, undialyzed	0. 860	86. 0
2.	24 hours, dialyzed with distilled water	0. 215	21. 5
3.	48 hours, undialyzed	1. 100	110. 0
4.	48 hours, dialyzed with water + 1 N NaOH, pH 7	0. 170	17. 0
5.	48 hours, dialyzed with Tris buffer, pH 9	0. 352	35. 2
6.	10 days, undialyzed	0. 730	73. 0
7.	10 days, dialyzed with Tris buffer, pH 9	0. 300	30. 0

[a] 0. 25 ml of sample was diluted to 25 ml with distilled water.

because of the possible presence of large amounts of salt in the dried material after freeze-drying. The DEAE-cellulose chromatography fractions were dialyzed against distilled water to minimize the amount of salt in the final samples. Since the molarity (M) of the elution buffer in DEAE-cellulose chromatography increases (starting buffer 0.04 M Tris; final buffer 0.5 M Tris), the fractions in Areas IV and V required more thorough dialysis. It is possible that, at the elevated pH of the elution buffer, the charged proteins interact with the salt ions and thereby decrease or limit the amount of salt that can be removed by dialysis. During the dialysis of the DEAE-cellulose chromatography fractions IV and V, a light precipitate was formed but was later removed by centrifugation.

Subsequent to freeze-drying, fraction residues were weighed. Samples could not be considered as pure protein because of the residual salt from the elution buffer. Thus, samples were prepared by gradual dilution with distilled water to a limit of 2-5 mg/ml. Hjerten et al. (1965) reported that a sharpening of the starting electrophoresis zone could be achieved by reducing conductivity of the sample below that of the electrophoretic buffer. Because of this, dialysis of fractions and the the dilution of samples were carried out with distilled water.

The use of agarose solution to form a gel to seal the acrylamide gel from the rubber gaskets of the electrophoresis cell was valuable

since rubber inhibits gel polymerization (E-C Apparatus Corporation, 1966). A faint opalescence was indicative of gel polymerization. The addition of the wetting agent, Tween 80, insured a smooth flow of the gel-buffer solutions down the plastic walls of the electrophoresis cell.

After electrophoresis, protein bands were stained with amido black and precipitated by the methanol and acetic acid of the dye solution. Electrophoretic destaining was not allowed to proceed too long in an attempt to avoid overheating of gels and complete decolorization of faintly visible bands. Better patterns were obtained by minimizing electrophoretic destaining and allowing gels to remain in the destaining solution overnight. During the electrophoretic destaining, the anode was oriented toward the overflow tube of the destainer. Thus, the anionic dye was removed following the recirculating destaining solution (Burger and Wardrip, 1965).

Gels had a tendency to shrink with the passage of time. The stained protein bands also became less distinct because of diffusion of the dye. Unfortunately, gels were not photographed immediately after destaining and clarification at which time the electrophoretic patterns were sharpest and clearest. Restoration of gels, when needed, was possible by soaking them in water (rehydration), restaining and destaining. The patterns, so obtained, although not equally sharp, were identical to initial ones.

Main Experiments

Subsequent to preliminary studies, experiments were conducted (1) to determine the electrophoretic patterns of the sarcoplasmic extracts, and (2) to investigate the possible protein heterogeneity of DEAE-cellulose ion exchange chromatography fractions.

Sarcoplasmic Extracts

Electrophoretic separation patterns of bovine sarcoplasmic proteins by continuous and discontinuous vertical acrylamide gel techniques are presented in Figures 6 and 7. The discontinous technique provided more discrete, highly resolved band patterns than the continuous technique. In both techniques, the cathode was oriented at the top of the gel and the anode at the bottom. Thus, proteins, negatively charged, migrated toward the anode. Stained bands, corresponding to electrophoretically different proteins, are designated by numbers at the right-hand margin of the patterns. In patterns obtained with the discontinuous technique, 18 different protein bands can be distinguished (Figure 7). Two very faintly visible areas might also be interpreted as possible protein bands.

Giles (1962) used starch gel electrophoresis to separate bovine sarcoplasmic proteins. With the methods and conditions described by Smithies (1959), he was able to detect seven distinct bands in the

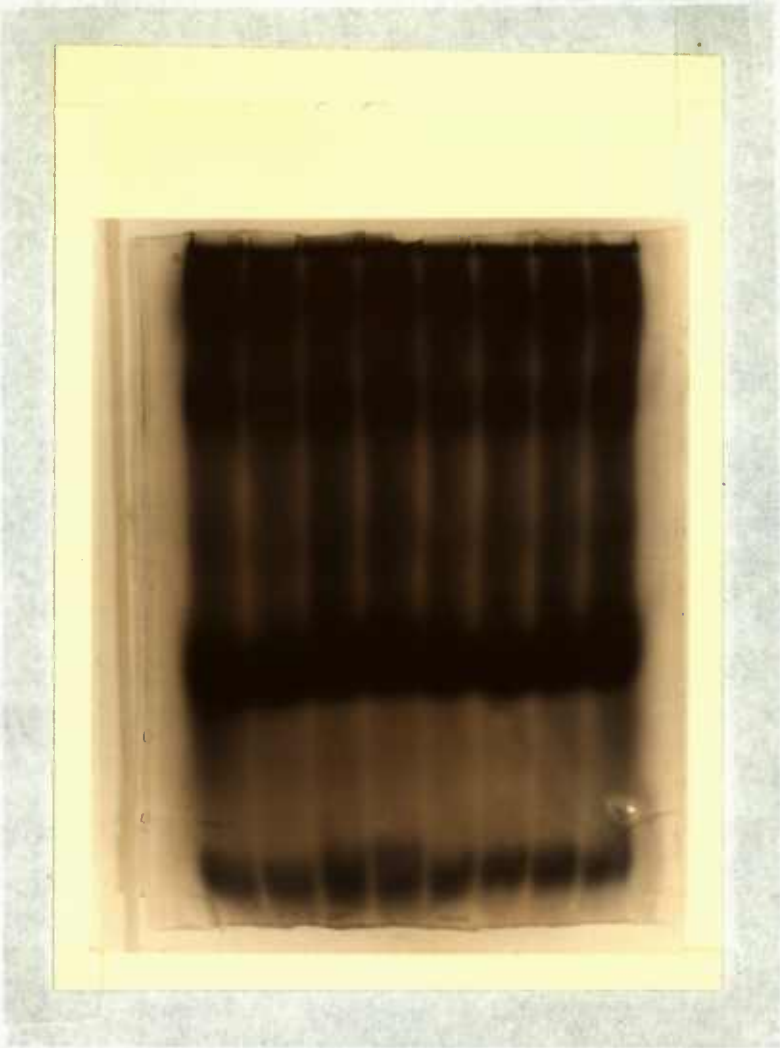

Figure 6. Electrophoretic pattern of sarcoplasmic proteins (aged 24 hours) obtained by continuous technique. Electrophoretic conditions: buffer, TEB; gel concentration, ten percent; current, 300 V; temperature, 4° C; length of run, 8 hrs.

electrophorotograms. More recently, Aberle and Merkel (1966) reported that 15 zones (bands) were definable in the starch gel electrophoretic patterns of bovine sarcoplasmic proteins. Patterns obtained in this study, with acrylamide gel discontinuous electrophoresis, appear to be quite similar to the starch gel electrophoretic patterns published by Aberle and Merkel.

As shown in Figure 7, the electrophoretic patterns of bovine sarcoplasmic proteins appear to change only slightly, if at all, with post-mortem aging of the l. dorsi muscle. Zender et al. (1958) reported that the electrophoretic pattern of proteins extracted from muscles of lamb and rabbit, stored aseptically at 25° C, was modified only after 20 days and 50-70 days of storage, respectively. On the other hand, they showed that when muscles were stored at 38° C, electrophoretic modifications were apparent at nine days post-mortem. Kronman and Winterbottom (1960) studied the alteration of the water-soluble proteins of the bovine muscle aged for seven days at 3° C. They reported that patterns obtained from moving boundary electrophoresis showed changes in the relative amounts of protein components, but these changes were not consistent.

Maier and Fischer (1966), using acrylamide gel disc electrophoresis, studied the chicken breast muscle proteins during a 24 hour post-mortem aging period in ice water. The electrophoretic patterns obtained with the water-soluble extracts, utilizing 7.5

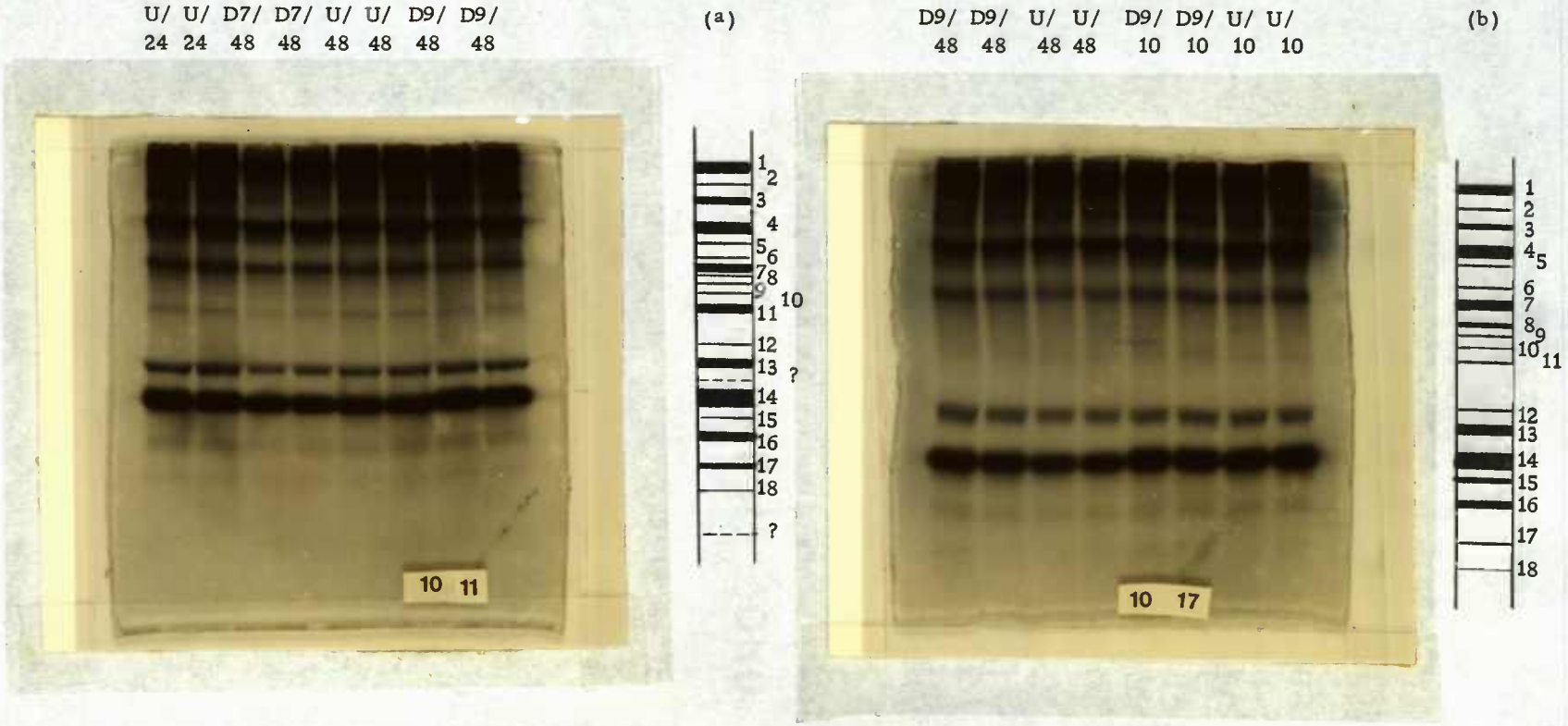

Figure 7. Electrophoretic patterns obtained by discontinuous techniques of dialyzed and non-dialyzed sarcoplasmic proteins extracted at different aging intervals. Dense band (No. 14) corresponds to pigment band. Electrophoretic conditions: buffer, discontinuous system; gel concentration, ten percent; current, 200-400 V; temperature, 4° C; length of run, 5 1/2 hrs for pattern a and 6 hrs for pattern b.
Legend: U = non-dialyzed; D7 = dialyzed at pH 7.0; D9 = dialyzed at pH 9.0; 24 hours, 48 hours and 10 days refer to aging times.

percent gels, consisted of four heavy bands and several light bands. The heavy bands showed only some minor and inconsistent changes in relative intensities with aging. Using 15 percent gel, they observed only a slight increase in the relative intensity of faint, fast-moving bands with post-mortem aging. No consistent changes with aging were noted in the electrophoretic patterns obtained with salt- and urea-soluble extracts. Aberle and Merkel (1966) reported that the electrophoretograms obtained from the starch gel electrophoresis of sarcoplasmic proteins were very similar at 1, 7 and 14 days (at 4° C) post-mortem aging. They did suggest, however, that the individual zones became more discrete. In addition, they noted a fast-moving protein zone in the 14 days electrophoretic pattern and some changes in the intensities of two other zones.

Comparison of the patterns (a) and (b) in Figure 7 of this study shows slight changes between the 1, 2 and 10 day aged samples. Some zones (8, 9, 10) are more discrete, and their boundaries more distinct, at ten days post-morten than at either one or two days. Changes in the intensity of zones can also be observed. Zone 11 is more intense in pattern (a) and the very faintly visible zone 18 is even more faint (almost disappears) in pattern (b). Comparison of the vertical strips of pattern (b) alone reveals only minor differences. Ten day sample zones appear slightly more intense. To obtain this pattern, the extract at one day of aging was held for ten days at 4° C

and the electrophoretic run was carried out by introducing this sample in the same run with the extract of muscle after ten days of aging. In this case, if it is assumed that slight changes occurred during the aging of muscle in the sarcoplasmic proteins, it is possible that such alterations could also occur when the extract was held at 4° C for ten days.

In conclusion, it is difficult to state that consistent electrophoretic pattern changes were noted with post-mortem extraction times under the conditions of the present experiments. Much more evident were pattern differences between dialyzed and undialyzed sarcoplasmic extracts of the same post-mortem extraction times. It can be observed that band 3 in the electrophoretic pattern (Figure 7a), obtained with extract dialyzed at pH 7, is less distinct than the corresponding bands obtained from the undialyzed and dialyzed samples at pH 9. In addition, zones 6 and 15 are not visible in the same pattern while other zones appear less intense (pattern a, Figure 7). The electrophoretic patterns of the undialyzed and dialyzed (at pH 9.0) sarcoplasmic extracts are very similar (patterns a and b, Figure 7).

DEAE-Cellulose Chromatography Fractions

Two sets of electrophoretic runs were completed on fractions obtained by the DEAE-cellulose chromatography of sarcoplasmic

proteins of meat samples aged for 0 and 10 days. As with the sarcoplasmic extracts, both the continuous and and discontinuous techniques were applied in these experiments. The results thus obtained are described and discussed hereafter according to fraction area.

Fraction Area I. Electrophoretic patterns of the effluent comprising Fraction Area I are presented in Figure 8. These patterns were obtained by the continuous technique using 7.5 percent acrylamide gels with Tris-EDTA-boric acid buffer, pH 9.2. Five distinct bands can be observed. Reproducible patterns were obtained when samples were introduced at different time intervals.

Although the above findings suggest heterogeneity of Fraction Area I, Parker and Bearn (1963) showed that the three zones exhibited by conalbumin in starch gel electrophoresis at pH 8.9 arose from the reversible interaction with borate buffer. Human myoglobin also was found to give three zones during starch gel electrophoresis in a Tris-borate buffer (Kossman et al., 1964). However, it was not clear to the authors whether such results were due to protein-buffer interaction. More recently, Cann (1966) reported that between pH range 6.2 to 9.2, BSA (bovine serum albumin) interacted reversibly with phosphate-borate and sodium borate buffers to give starch gel electrophoretic patterns showing two or more bands. Cann further stated that such data cannot be considered indicative of

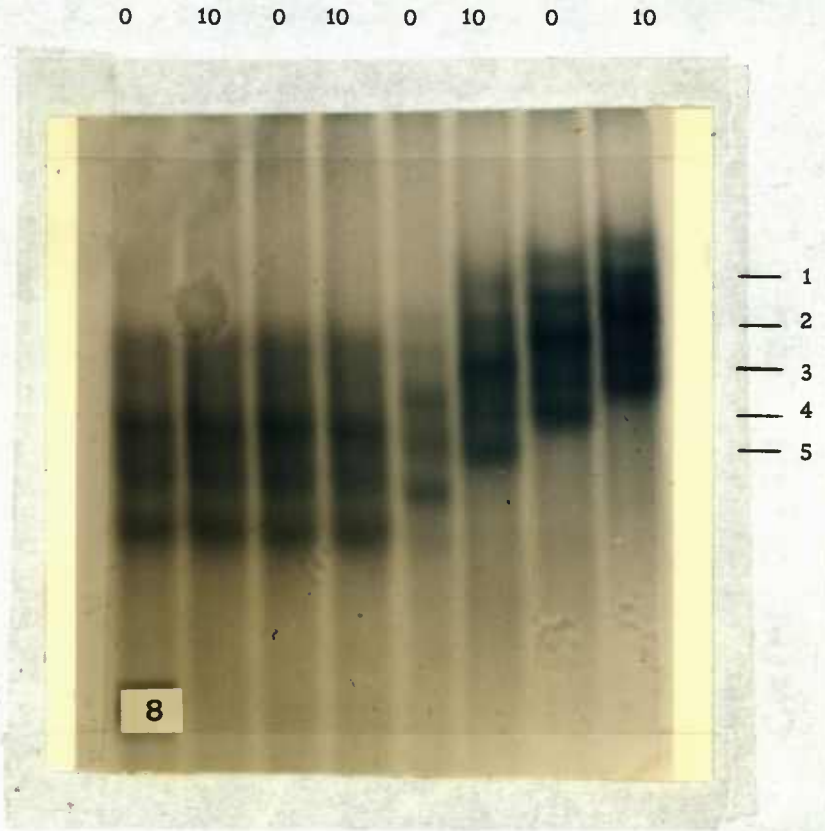

Figure 8. Electrophoretic pattern of Fraction Area I of DEAE-cellulose chromatography (Figure 1). Electrophoretic conditions: technique, continuous; gel concentration, 7.5 percent; buffer, TEB; current, 300 V; temperature, 4° C, length of run 20-14 hours. Legend: 0 = zero days of aging; 10 = 10 days of aging.

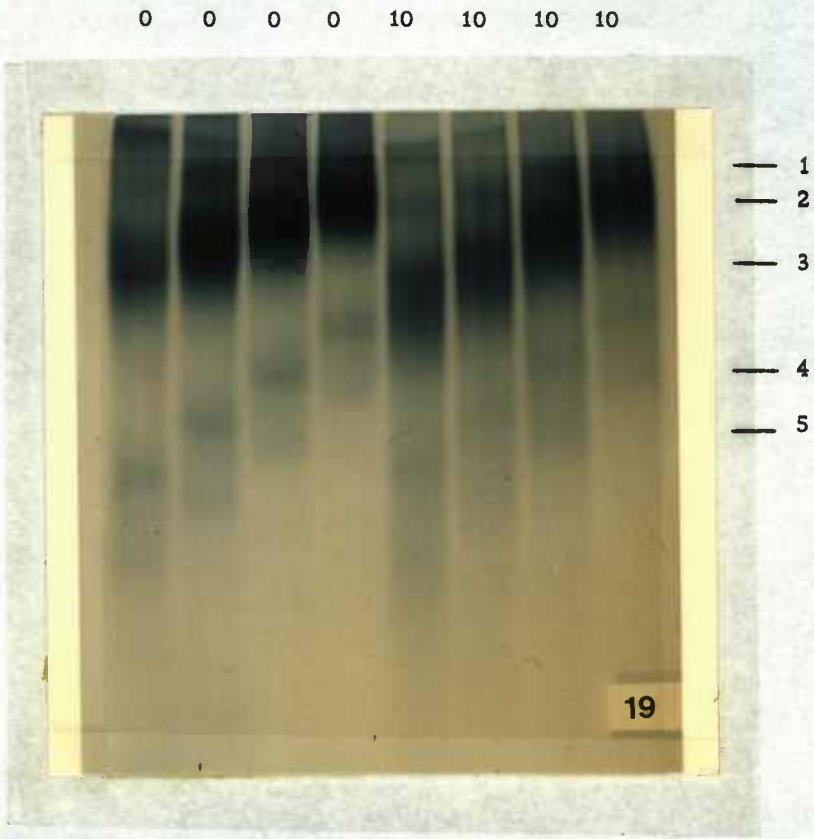

Figure 9. Electrophoretic pattern of Fraction Area II of DEAE-cellulose chromatography (Figure 1). Electrophoretic conditions: technique, continuous; gel concentration, 7.5 percent; buffer, TEB; current, 300 V; temperature, 4° C; length of run, 9-6 hours. Legend: 0 = zero days of aging; 10 = 10 days of aging.

true heterogeneity.

In spite of the above references, patterns in Figure 8, obtained with the continuous technique using Tris-EDTA-boric acid buffer, can be considered as good evidence for the heterogeneity of Fraction Area I. The presence of five distinct, clearly visible bands cannot merely be explained as buffer-protein interaction. In addition, patterns similar to those in Figure 8 were obtained with the discontinuous technique employing Tris-HCl buffers. Unfortunately, there is no record of the discontinuous technique pattern since the photonegative was inadvertently destroyed and the gel could not be restored to the original condition. Moreover, the lack of sample did not allow repetition of the experiment. Differences between patterns of samples aged 0 and 10 days were quite minor and considered to be insignificant.

The quantity of sample available for vertical gel electrophoresis from the DEAE-cellulose chromatographic separation of sarcoplasmic proteins was, at best, relatively small. The total amount, and consequently that available for each of the five fraction areas, was limited by the amount of extract (9 ml) originally run through the columns. For example, the sample from Fraction Area I, after freeze-drying and dilution with distilled water, was only 2.2 ml. The other diluted volumes were 5.5, 4.3, 4.5 and 3.2-4.5 ml for Fraction Areas II, III, IV and V, respectively. Hence, the volume

of sample from the designated areas allowed for only a few electrophoretic runs.

Fraction Area II. Electrophoretic patterns of DEAE-cellulose chromatography of Fraction Area II show five distinct bands (Figure 9). Since similar results were obtained using both the continuous and discontinuous techniques, only those patterns produced by the continuous technique in a 7.5 percent gel are presented. Although samples of 0 and 10 days of aging were introduced at various time intervals, the resulting patterns are similar. However, it is evident that there are slight differences in both the intensity and electrophoretic mobility between the patterns of the two aging treatments.

Fraction Areas I and II, eluted during DEAE-cellulose chromatography of the sarcoplasmic proteins, correspond to the slower moving bands in the electrophoretic patterns. The slow moving bands have a low level of electronegativity since the time required for electrophoresis decreased from 20 hours for Fraction Area I and ten hours for Fraction Area II, to 7, 5 and 4 hours for Fraction Areas III, IV and V, respectively. In addition, the optimum gel concentration which was 7.5 percent for Fraction Areas I and II, was ten percent for the remaining fraction areas. The more rapid electrophoretic mobility of the proteins in the latter three fraction areas, and therefore, the necessity for increasing gel concentration and reduced running time, might be explained on the basis of net electrical charge

and molecular weight. Hence, it might be concluded that the slower moving bands of Fraction Areas I and II represent proteins of higher molecular weight and/or lower net negative charge.

Fraction Area III. The sample containing DEAE-cellulose chromatographic Fraction Area III contains the pigment peak. Both continuous and discontinuous techniques were used to investigate the possible heterogeneity of this fraction. Samples were applied at staggered intervals in the continuous technique and reproducible patterns were obtained showing a linear relation of migration with time. Although patterns obtained with the discontinuous technique were in agreement with those produced by the continuous procedure, bands of the former were more discrete and their boundaries more distinct. The discontinuous technique patterns of Fraction Area III are presented in Figure 10. In this case, a ten percent gel concentration was found to be optimum for electrophoretic separation. Six bands are visible which indicate the heterogeneity of the pigment peak of the DEAE-cellulose chromatography profile. Simultaneous introduction of samples aged 0 and 10 days allowed for direct comparison of the effect of aging on this pigment fraction. In the patterns of Figure 10, the second band appears more intense in the ten-day sample.

Fraction Area IV. The electrophoretic patterns of Fraction Area IV produced by the discontinuous technique are shown in

Figure 11. A gel concentration of ten percent was found to give better separation than did gels of other concentrations. Patterns obtained with the continuous technique were in agreement with those of the discontinuous procedure although better resolution was achieved with the latter method. Eight bands are clearly visible in Figure 11 while a ninth band, slow migrating, might also be present. Zero- and 10-day aged samples were analyzed in the same electrophoretic run. No appreciable pattern changes were observed with post-mortem aging since the vertical strips showing the 0- and 10-day samples appear to be identical (Figure 11).

Fraction Area V. Continuous and discontinuous techniques were also employed for the electrophoretic analysis of the DEAE-cellulose chromatographic Fraction Area V. Although patterns of both techniques were similar, the discontinuous procedure again gave better resolution. Patterns of this fraction obtained by the discontinuous technique using a ten percent gel are presented in Figure 12. Three bands are definable. The fast moving band appears to be most intense while the other two are faintly visible. The vertical strips corresponding to 0 and 10 days of aging appear to be identical.

From the electrophoretic analysis of the DEAE-cellulose column chromatography of bovine sarcoplasmic proteins, a total of 26, possibly 27, protein bands are definable. However, caution must be exercised in stating that each of these bands represents a

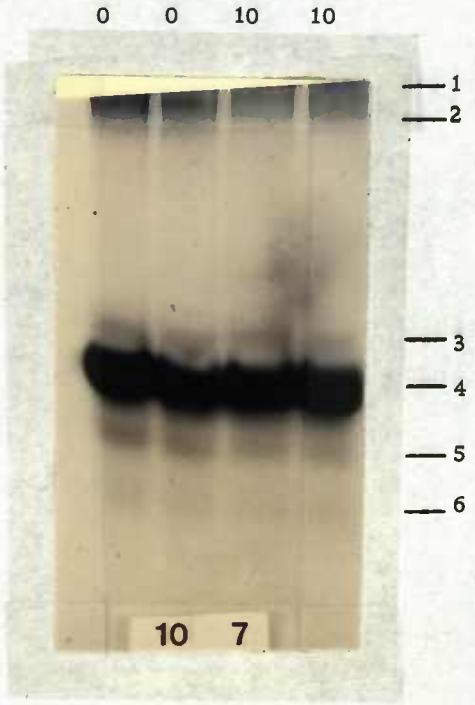

Figure 10. Electrophoretic pattern of Fraction Area III of DEAE-cellulose chromatography (Figure 1).

Figure 11. Electrophoretic pattern of Fraction Area IV of DEAE-cellulose chromatography (Figure 1).

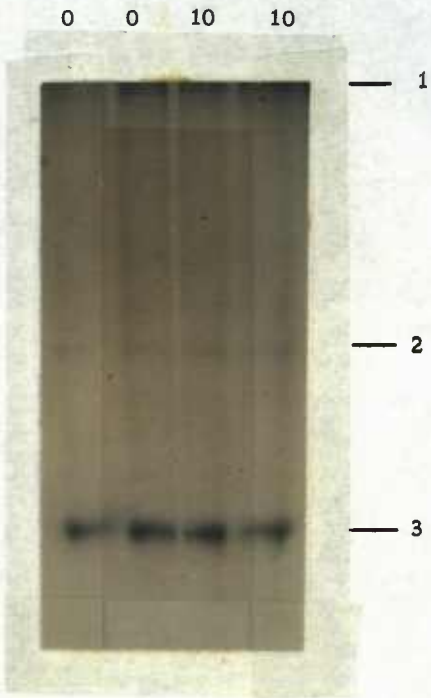

Figure 12. Electrophoretic pattern of Fraction Area V of DEAE-cellulose chromatography (Figure 1).

Electrophoretic conditions were similar for the above three figures: technique, discontinuous; gel concentration, 10 percent; buffer, discontinuous system; current, 200-400 V; temperature, 4°C; length of runs, 6, 5, 4 hours respectively.

Legend: 0 and 10 refer to zero and 10 days of aging respectively.

different protein. For example, Fujimaki and Deatherage (1964) obtained reproducible results in the ion exchange chromatographic fractionation of bovine sarcoplasmic proteins but they also reported that various enzymes tested for appeared in several of the chromatographic fractions. Thus a similar overlapping may have occurred in this study. The multiple zone (bands) electrophoretic patterns obtained are indicative of the heterogeneity for the five major fractions of the DEAE-cellulose chromatographic protein profile. Such heterogeneity is quite evident for Fraction Areas I and III which appeared as single homogeneous peaks in Figure 1, but showed five and 6 protein bands, respectively, when separated by gel electrophoresis. The observed differences in electrophoretic mobility and the changes occurring mainly in the electrophoretic patterns of Fraction Areas II and III as aging proceeded indicate that some qualitative changes do occur in the sarcoplasmic proteins during postmortem aging of beef.

Orthogonal Acrylamide Gel Electrophoresis

Two-dimensional electrophoresis techniques were employed to gain information about the applicability of such procedures for studying sarcoplasmic proteins. The orthogonal gel concentration procedure provides data concerning molecular size while the orthogonal pH technique pertains to molecular charge. Results of the use

of these two techniques are discussed below.

Orthogonal Gel Concentration Procedure

The orthogonal gel concentration pattern of the sarcoplasmic proteins is shown in Figure 13. Continuous electrophoretic runs employing acrylamide gels of different concentrations were completed in both directions. In Figure 13, a straight line through the point of origin intersects proteins of equal molecular size. The stained spots outside the straight line represent proteins of different molecular size. The spot at the far right, corresponding to the fast migrating band of the first direction pattern, demonstrates that the fast moving bands are more dense in seven percent gel than in gels with higher concentration. In the orthogonal concentration pattern (Figure 13), the pigment band (mostly myoglobin) separates into two additional faster moving bands. According to Gurd (cited by Raymond, n.d.), native myoglobin in solution undergoes successive isomerization with unit increases in migration velocity.

Orthogonal pH Procedure

The orthogonal pH pattern of the sarcoplasmic proteins is presented in Figure 14. A straight line through the point of origin intersects spots of stained proteins of equal molecular charge. In addition, passage of straight lines through the point of origin in such

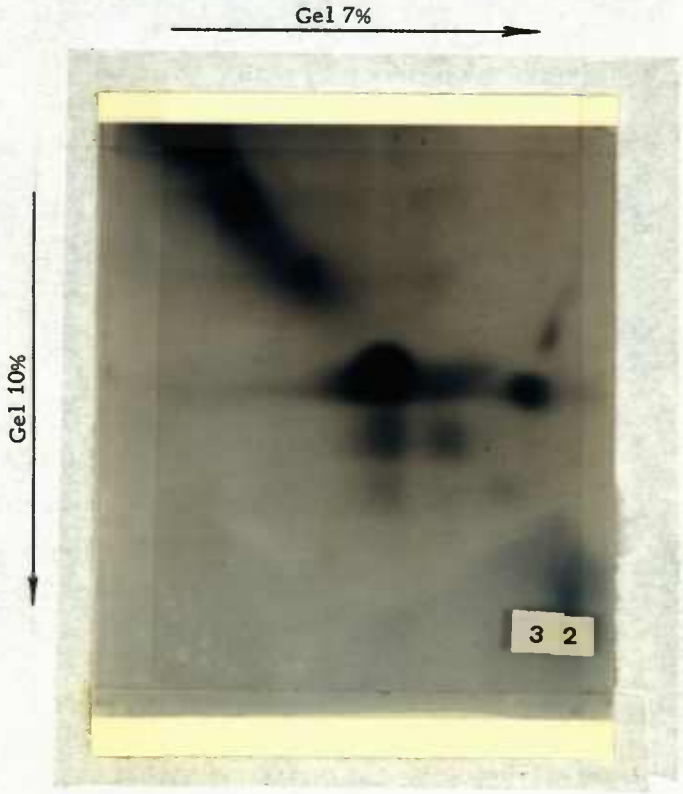

Figure 13. Orthogonal-gel concentration electrophoretic pattern of sarcoplasmic proteins aged 24 hours. Electrophoretic conditions: gel concentration seven percent in first direction and ten percent in second direction; buffer, TEB; current, 300 V; temperature, 4° C.

Figure 14. Orthogonal-pH electrophoretic pattern of sarcoplasmic proteins aged 24 hours. Electrophoretic conditions: gel concentration, seven percent; buffer system, pH of 9.2 in first direction and pH of 8.6 in second direction; current, 300 V; temperature, 4° C.

patterns is indicative of the lack of electroendoosmosis in these gels (Raymond, n. d.).

Density Gradient Electrophoresis

Density gradient electrophoresis was carried out to assess the feasibility of this technique for separating bovine sarcoplasmic proteins. Preliminary results obtained on the separation of sarcoplasmic extracts did not encourage further application of the procedure to DEAE-cellulose chromatographic fractionation heterogeneity studies.

The density gradient electrophoretic patterns of the sarcoplasmic extract from bovine l. dorsi muscle, aged 24 hours, is presented in Figure 15. These results are presented with the effluent volume on the abscissa and the absorbance (280 mμ) of the eluent on the ordinate. While the profile pattern is divided into five separate areas or peaks, no attempt was made to quantitate the results. Changes in the optical density of proteins in association with changes in pH eliminates quantitation of such results (Wetlaufer, 1962).

Interpretation of the profile in Figure 15 must be done with care due to the variability of experimental conditions. Comparison of results of density gradient electrophoresis with those obtained with either DEAE-cellulose chromatography or vertical gel electrophoresis suggest that the former technique, as conducted in this

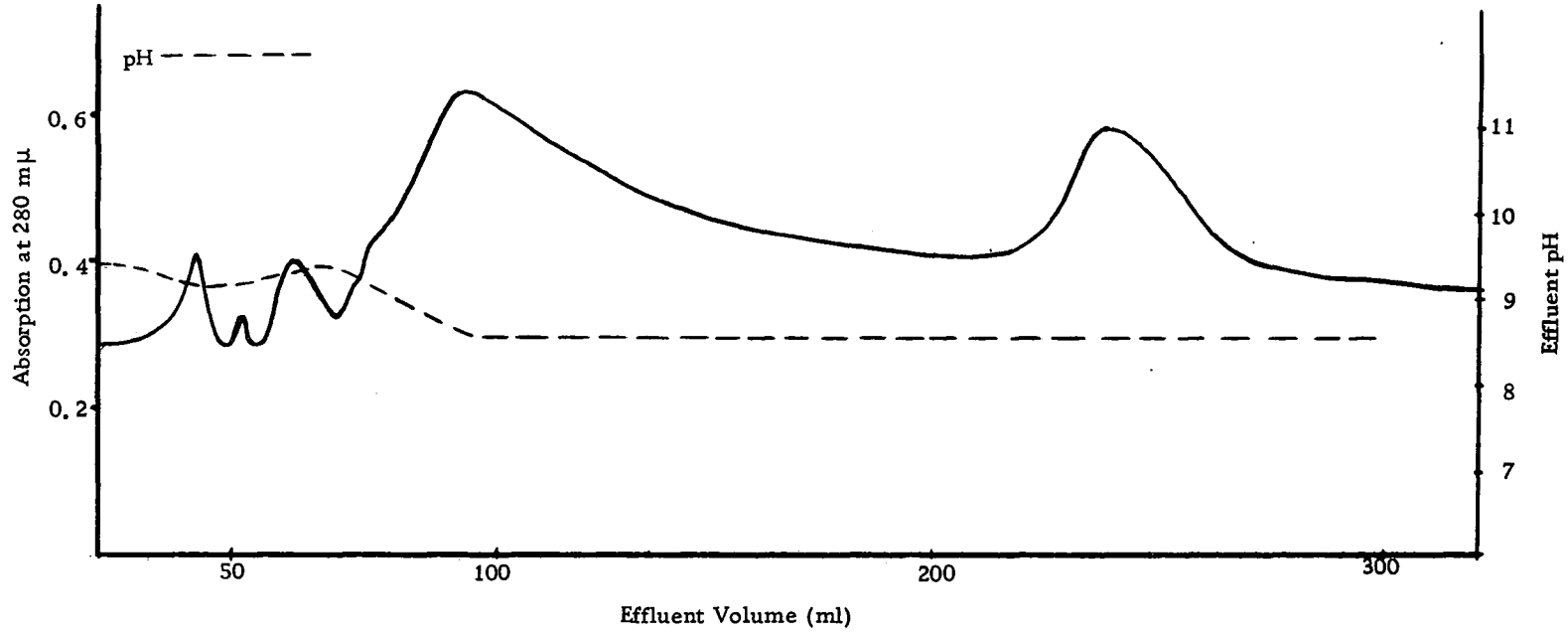

Figure 15. Density gradient electrophoretic pattern of sarcoplasmic proteins aged 24 hours. Length of run was 48 hours at 420 V, 21-18 mA and 3° C temperature.

study, does not separate the various sarcoplasmic proteins to the extent that the latter two techniques do. Many difficulties were encountered in the use of sucrose as gradient-forming material due to its high viscosity and stickiness. The use of gradient forming material (e.g. Ficoll, etc.) other than sucrose (Beckman Instruments, 1960) might facilitate the operational procedure of the apparatus. In addition, it is possible that radical changes in other operational conditions might also improve the separation of sarcoplasmic proteins.

SUMMARY AND CONCLUSIONS

Vertical acrylamide gel electrophoresis was used to study the electrophoretic behavior of bovine sarcoplasmic proteins. Part of the study was concerned with the influence of experimental variables to achieve a high degree of electrophoretic fractionation of the proteins of sarcoplasmic extracts. The remainder of the study was largely devoted to the investigation of the heterogeneity of sarcoplasmic protein fractions obtained by DEAE-cellulose chromatography.

The high resolving power and versatility of acrylamide gel electrophoresis for separating sarcoplasmic proteins were observed in these studies. In addition, excellent reproducibility of electrophoretic patterns was also noted. An overall comparison between the continuous and discontinuous techniques indicated the superiority of the latter method for separating sarcoplasmic proteins. Although the discontinuous technique is more complicated than that of the continuous procedure, electrophoretograms obtained by the former method showed greater resolution and more distinct boundaries of the protein bands.

Electrophoresis of the sarcoplasmic extracts by the discontinuous technique resulted in 18 (possibly 20) electrophoretically different proteins being separated. This degree of resolution is

higher than that obtained by other techniques reported in the literature. The electrophoretic patterns were clear and the bands were well-defined, particularly when ten percent acrylamide gels were used.

Results of the electrophoretic analysis of the sarcoplasmic fractions obtained from DEAE-cellulose chromatography indicate that the major peaks of the chromatographic profile were quite heterogeneous. Although a total of 27 bands were observed upon the above electrophoretic analysis, each band does not necessarily represent a different protein since there may have been carry-over of some proteins from one chromatographic fraction to another. Changes in the electrophoretic patterns of sarcoplasmic proteins during several days of post-mortem aging were slight. These small differences do indicate that the sarcoplasmic proteins undergo some qualitative change upon aging.

Results of attempts to separate the sarcoplasmic proteins by density gradient electrophoresis were not encouraging. Low resolution of separation was quite apparent.

So far as future studies are concerned, the possible protein-buffer and protein-protein interactions that might occur during the electrophoresis of sarcoplasmic proteins should be investigated. In addition, the extractability of sarcoplasmic proteins under different conditions must be considered. The use of elution convection

preparative electrophoresis for the collection of proteins separated by vertical gel electrophoresis should be attempted. Availability of such fractions would allow for more complete and detailed studies of the proteins fractionated electrophoretically. For better interpretation of gel electrophoretic patterns, a recording densitometer should be used.

So far as density gradient electrophoresis is concerned, this procedure might be used advantageously as preparative technique.

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