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Review

Polyacrylamide gel electrophoretic methods in the separation of structural muscle proteins

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Abstract

Polyacrylamide gel electrophoresis plays a major role in analyzing the function of muscle structural proteins. This review describes one- and two-dimensional gel electrophoretic methods for qualitative and quantitative investigation of the muscle proteins, with special emphasis on determination of protein phosphorylation. The electrophoretic studies established the subunit structures of the muscle proteins, characterized their multiple forms, revealed changes in subunit composition or shifts in isoform distribution of specific proteins during development, upon stimulation or denervation of the muscle. Protein phosphorylation during muscle contraction is preferentially studied by two-dimensional gel electrophoresis. The same method demonstrated protein alterations in human neuromuscular diseases.

Contents

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1. Introduction

Polyacrylamide gel electrophoresis in the presence of SDS, described by Weber and Osborne [1] and Laemmli [2], is widely used for the separation of proteins by differences in molecular mass. To separate proteins on the basis of differences in net charge due to differences in amino acid composition or post-translational modifications such as phosphorylation, IEF is done in polyacrylamide gels containing either carrier ampholytes [3] or immobilized ampholytes [4] to generate a pH gradient. SDS-PAGE gels are made with relatively high concentrations of acrylamide (5–20%) to restrict migration distance on the basis of size; in contrast, IEF gels have low concentrations of acrylamide (3–4%) so that separation is strictly by differences in net charge, regardless of protein size.

These two procedures, IEF and SDS-PAGE, are combined in 2DE, resulting in a powerful method that can separate a large number of proteins. O'Farrell [5] first demonstrated the resolution of 2DE by separating proteins from *Escherichia coli* lysate. The technique has since been adapted for the analysis of protein structure and protein expression in a variety of biological systems [6–8]. The 2DE approach is especially advantageous in studies of whole cell or tissue homogenates, which contain many proteins with similar isoelectric points or molecular masses, allowing separation of proteins that would migrate as a single band using either IEF or SDS-PAGE alone.

Studies of muscle cell structure and function have centered on the characterization of specialized proteins such as myosin and actin, which are involved in the contractile activity, and tropomyosin, troponin, calponin and caldesmon, which are thought to regulate the contraction. In addition, there are proteins in the muscle structure, such as desmin, vimentin, α-actinin, filamin, titin and nebulin, whose roles are under investigation. All these proteins ranging from M_r 16 000 (myosin light chain) to greater than 3 000 000 (titin), are major components of muscle. Most of these proteins have been shown to exist in multiple forms, differing in isoelectric point and/or molecular mass. The subunit structure of the muscle proteins as well as changes in the subunit composition occurring during embryogenesis, development, upon a variety of stimulation, or denervation are often followed by PAGE [9,10]. Many of these proteins are expressed in different forms in different types of muscle or in different species [11]. Several proteins are phosphorylated to varying degrees in different functional states of muscle [12]. Separation of the multiple forms of these muscle

proteins has been done primarily by 2DE [13,14]. In addition to the information on the structural proteins of normal muscle, abnormal expression of these proteins related to pathological processes has also been described [15].

The purpose of this review is to summarize the literature on the application of gel electrophoresis to studies of muscle structural proteins designed to characterize muscle function. Included are methods of preparing muscle tissue for analysis by gel electrophoresis, preparing polyacrylamide gels suitable for the separation of the structural proteins of muscle, and detecting and quantitating proteins after electrophoretic separation. Special emphasis is given to the phosphorylatable myosin light chain, because its involvement in smooth muscle contraction is now widely studied.

2. Methods

2.1. Preparation of muscle extracts

To correlate the physiological state of muscle with protein phosphorylation, muscles are labeled with ^{32}P, the physiological experiments are performed, and the muscles are frozen in liquid nitrogen [16]. The frozen muscles are pulverized at 4°C by percussion using liquid nitrogen-chilled stainless-steel mortars and pestles. The frozen powders are immediately homogenized in 3% PCA and centrifuged at 27 000 *g* for 15 min; the supernatants are collected for the determination of the specific radioactivity of [^{32}P]PCr of each muscle which is equal to that of the [γ-^{32}P]ATP of the muscle [17]. The PCA-treated muscle residues are washed with a solution containing 2% TCA and 5 m*M* NaH_2PO_4 several times to remove unbound [^{32}P]phosphates and then dissolved in 1.2% SDS, 125 m*M* Na_2HPO_4, pH 8.5. After overnight dialysis against large volumes of 0.02% SDS, 2 m*M* $(NH_4)HCO_3$, insoluble connective tissue is removed by centrifugation at 100 000 *g*, and the protein content of the supernatants is determined with the biuret method [18] by measuring the optical density at 320 nm [19]. Aliquots of the supernatants are freeze-dried and subsequently subjected to 2DE (see Section 2.3 and [20]). The extensive PCA and TCA treatments in this procedure denature (and thereby inactivate) proteolytic enzymes and the enzymes involved in protein phosphorylation, therefore, the results reflect the physiological state of the proteins in the muscle. Furthermore, virtually all proteins are extracted from the muscle, and thus the information gained refers to the entire protein pattern of the cell.

In another procedure [21], muscle strips are frozen in dry ice-cooled acetone containing 10% TCA, washed with acetone, and a weighed amount of muscle powder is extracted with a solution containing 8 *M* urea, 10 m*M* DTT, 20 m*M* Tris and 22 m*M* glycine, pH 8.6, at room temperature. After centrifugation, the proteins in the extract are separated by glycerol–urea-PAGE. Alternatively, the acetone-dried powder is extracted with a solution containing 1% SDS, 10% glycerol and 20 m*M* DTT, and the proteins in the extract are separated by 2DE [22,23]. Moore and Stull [24] clamp the muscle with tongs prechilled in liquid nitrogen, pulverize at −180°C, and homogenize in a medium containing 100 m*M* $Na_4P_2O_7$, 100 m*M* NaF, 5 m*M* EGTA, 500 m*M* KCl, 40% glycerol, 15 m*M* 2-mercaptoethanol, 1 m*M* phenyl-methylsulfonyl fluoride, 0.1 m*M* leupeptin and 100 U/ml aprotinin, pH 8.8, at −30°C. The homogenate is diluted threefold in homogenizing buffer without KCl and centrifuged at 10 000 *g*. Native myosin is isolated from the supernatant on pyrophosphate-PAGE [25], and subsequently subjected to IEF for separation of the phosphorylated and non-phosphorylated forms of P-LC.

2.2. One-dimensional gel electrophoresis

The PAGE method is frequently used in the analysis of structural muscle proteins. Its resolution is high enough to compare proteins from various muscles [26,27], to follow proteolysis [28] or protein–protein interactions through cross-linking [29], or to identify myofibrillar proteins by immunoblotting [30].

In the 1970s and early 1980s, cylindrical gels in

glass tubes were generally used for PAGE. Perrie and Perry [9] separated the components of myosin light chain (LC) on 10% gels containing 40% (v/v) glycerol or 8 *M* urea in 20 m*M* Tris–glycine buffer, pH 8.6. The samples were dissolved in 8 *M* urea. For urea gels, the Tris–glycine running buffer contained 6 *M* urea. These gels also distinguished between the phosphorylated and non-phosphorylated forms of one LC component [31]. Cylindrical 10% gels in 400 m*M* Tris–glycine, pH 8.8, 5% glycerol and 0.1% SDS resolved the proteins of rabbit skeletal muscle myofibrils, as shown in Fig. 1 [32]. Similarly, 10 and 5% cylindrical gels containing

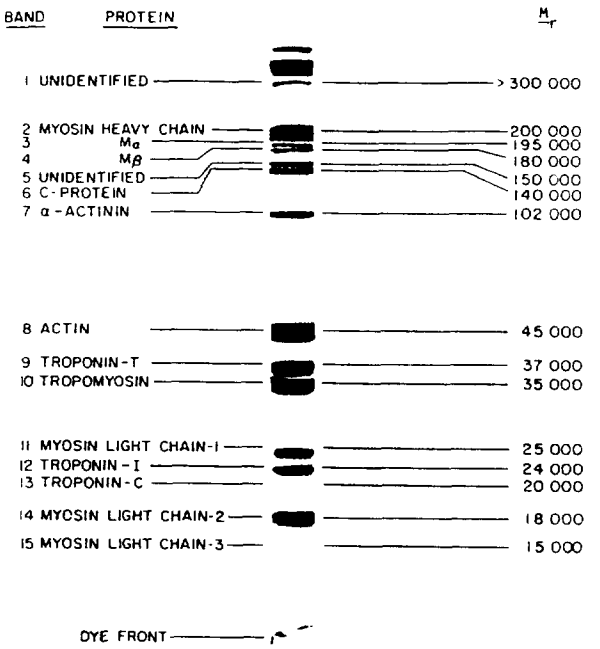


Fig. 1. Analysis by SDS-PAGE of purified rabbit skeletal muscle myofibrils using 10% acrylamide gel with 0.10% bisacrylamide (100:1) at 400 m*M* Tris–glycine (pH 8.80), 5% glycerol and 0.10% SDS. A myofibril sample of 100 μg in 20 μl was run at 1.0 mA/tube with Pyronin Y tracking dye. The gel was fixed with 25% isopropanol–10% acetic acid for 3 h and then stained overnight with 0.01% Coomassie Brilliant Blue R in 50% methanol–10% acetic acid. The gel was diffusion-destained in 5% methanol–10% acetic acid until the background was clear. Mα = M-line α; Mβ = M-line β. From Ref. [32].

0.1–1% SDS, 8.0 *M* urea and 0.1 *M* sodium phosphate, pH 7.0, resolved the proteins of myofibrils from chicken posterior and anterior latissimus dorsi muscles [27] or rat heart [17].

Recently, flat slab gels, 0.75–1.5 mm thick, have become the format for PAGE. An important advantage of slab gels is that many samples, including molecular mass marker proteins, can be electrophoresed under identical conditions in a single gel, allowing direct comparison of the band patterns of different samples. The approximate relative molecular masses of skeletal, cardiac, and smooth muscle myofibrillar proteins, obtained by comigration with proteins of known relative molecular mass on SDS-PAGE, are listed in Table 1. Fig. 2 illustrates the use of SDS-PAGE to follow a switch in the isoform population of TNI and TNT during neonatal development of rat heart myofibrils [30].

The myosin light chains, the components of troponin, calponin, tropomyosin and actin are resolved on SDS-PAGE (15%), whereas the myosin heavy chains, caldesmon, desmin and vimentin are resolved on SDS-PAGE (10%). The dimensions of the gels are $0.18 \times 14 \times 10$ cm (resolving) and $0.18 \times 14 \times 5$ cm (stacking). For preparative gel electrophoresis, required when protein is to be extracted from the polyacrylamide for further analysis [35], the gels are 0.30 cm thick instead of 0.18 cm.

The proteolysis of muscle proteins in several studies of protein structure was followed by PAGE. Examples are the digestion of rabbit skeletal or chicken gizzard myosin with papain or trypsin [28], and further digestion of the S-1 by *Staphylococcus aureus* V8 protease or chymotrypsin [36]. The kinetics of calponin and caldesmon hydrolysis by calpain I was measured using PAGE to separate the hydrolysis products [37]. Semi-logarithmic plots of the densitometric intensity of a band that retained the same mobility as the starting material plotted as a function of incubation time gave an almost linear decrease in the early phase of the reaction, indicating that the proteolytic reaction followed apparent first-order kinetics. Digestion of ^{32}P-labeled P-LC

Table 1
Relative molecular masses of skeletal, cardiac and smooth muscle myofibrillar proteins separated by SDS-PAGE

Protein	Relative molecular mass		
	Skeletal	Cardiac	Smooth
Myosin heavy chain	200 000	200 000	200 000
M-line α	195 000		
M-line β	180 000		
C-protein	140 000	140 000	
Caldesmon *h*			140 000
α-actinin	102 000	102 000	102 000
Caldesmon *l*			92 000
Vimentin			54 000
Desmin			53 000
Actin	43 000	43 000	43 000
Troponin-T	39 000	41 000	
Tropomyosin γ			41 000
Tropomyosin β	36 000		36 000
Tropomyosin α	34 000	35 000	
Calponin			34 000
Myosin light chain 1, slow	28 000	27 000	
Myosin light chain 1, fast	25 000		
Troponin-I, slow	27 000	28 000	
Troponin-I, fast	24 000		
Troponin-C	18 000	18 000	
Myosin light chain 2, slow	19 000	19 000	20 000
Myosin light chain 2, fast	18 000		
Myosin light chain 3	16 000		17 000

Source of the data: Porzio and Pearson [32]; Baumann et al. [33]; Murakami and Uchida [34]; and determinations in our laboratories. *h*, *l* = High- and low-molecular-mass form of caldesmon, respectively.

within SDS-PAGE gels by *S. aureus* (according to the procedure of Cleveland et al. [38]), was used to detect differences in the phosphopeptide pattern among the P-LC isoforms [39].

SDS-PAGE and Western blot were combined to study the structure and conformation of the interface between actin and the myosin S-1 head [40]. Hydrophobic or carboxyl group-directed zero-length protein cross-linkers were used for chemically cross-linking the rigor complex between F-actin and S-1. Comparative cross-linking patterns were visualized on SDS-PAGE using either fluorescent F-actin or fluorescent S-1. Proteolytic digestion of the fluorescent covalent complexes revealed that the carboxyl-terminal (M_r 20 000) and the central (M_r 50 000) heavy chain fragments of S-1 are involved in the cross-linking reaction, whereas peptides containing amino acids 1–28 and 40–113 in the sequence of actin were cross-linked, as identified by immunostaining with specific antibodies. The same laboratory also showed that F-actin promoted internal cross-linking of the amino-terminal (M_r 25 000) and the carboxyl-terminal (M_r 20 000) fragments of S-1 [29]. Electrophoretic analysis of photochemically cross-linked S-1 to actin subdomain-1 at Arg95 and Arg28 showed the progressive formation of five readily distinguishable new species with relative molecular masses of 165 000, 175 000, 200 000, 240 000 and 280 000 [41]. Characterization of these complexes with antibodies specific to actin or S-1 fragments

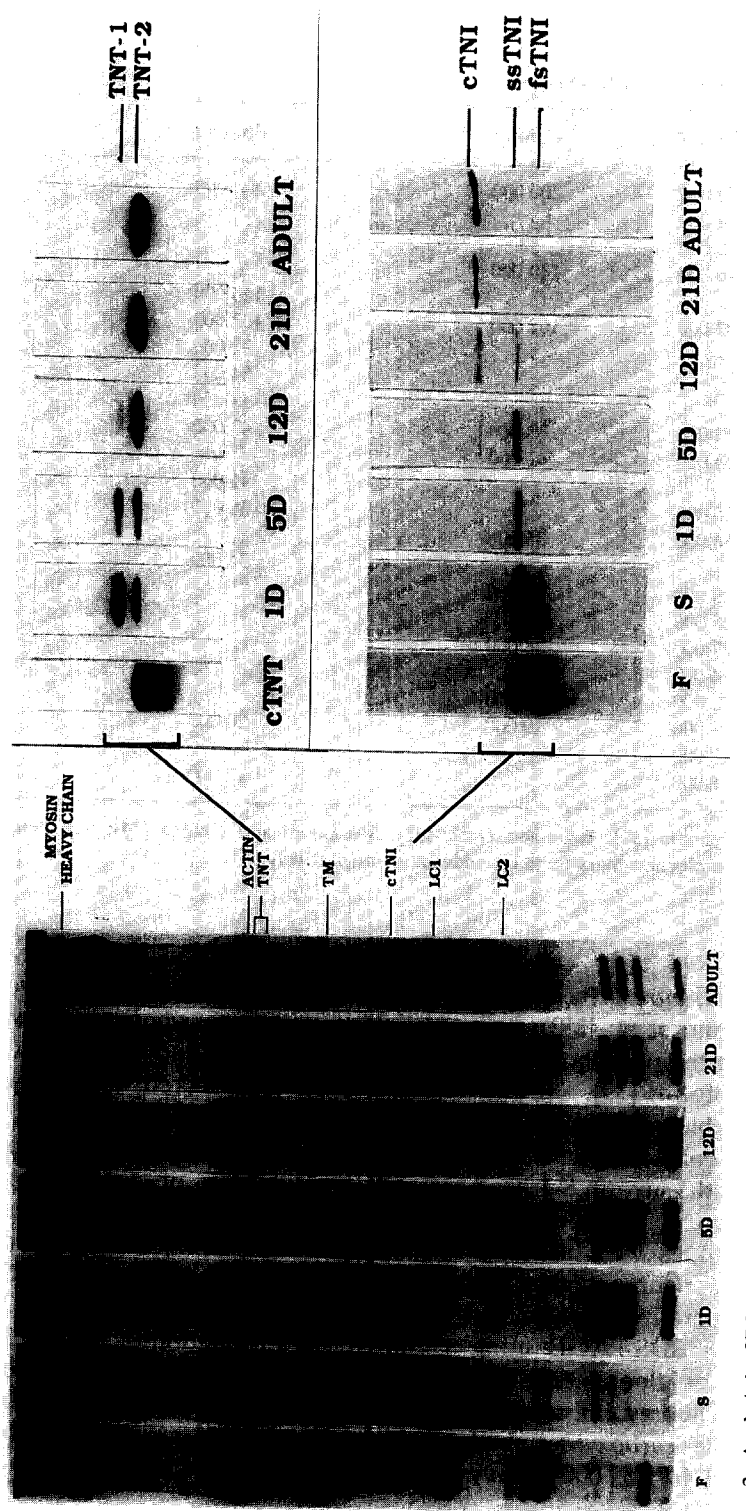


Fig. 2. Analysis by SDS-PAGE (12.5%) and Western blot of myofibrillar proteins from rat cardiac muscle during development from neonatal day 1 to adult and from tibialis anterior fast (F) and soleus slow (S) rat skeletal muscle. From each myofibrillar sample 100 μg protein was applied onto the gels. TM = Tropomyosin; cTNI = cardiac troponin I; LC1 = myosin light chain 1; LC2 = myosin light chain 2; ssTNI = slow skeletal troponin I; fsTNI = fast skeletal troponin I; TNT-1 = slow mobility isoform of TNT; TNT-2 = faster migrating isoform of TNT; D = days after birth. Left: Coomassie Blue-stained protein profile. Right: Western blot analysis of TNI and TNT isoforms. From Ref. [30].

provided an insight into the structural dynamics of the interaction of S-1 with subdomain-1 of actin during the ATPase cycle.

2.3. Two-dimensional gel electrophoresis

High-resolution 2DE of muscle tissue was first reported in 1979 [13] and was applied to structural muscle proteins in 1980 [12]. It became the preferred method for studying the phosphorylation of P-LC during smooth muscle contraction, because the phosphorylated and unphosphorylated P-LC isoforms are well separated from each other, and the P-LC isoforms are not contaminated with comigrating polypeptides of similar molecular mass (as in the case of PAGE).

Many methods for 2DE have been published, differing primarily in the apparatus used. Most of the techniques are based on that of O'Farrell [5]. In our laboratories, sixteen two-dimensional gels are run simultaneously, electrophoresing in duplicate the total protein extracts of eight ^{32}P-labeled smooth muscles frozen at various phases of the contraction–relaxation cycle. All electrophoretic boxes are laboratory-made. The procedure is inexpensive, requires moderate amount of space, and has the advantage that the migration of the tracking dye can be monitored by eye. The freeze-dried protein samples, 700 μg, (prepared as described in Section 2.1) are dissolved in 70 μl of sample buffer containing 9.5 *M* urea, 2% Nonidet-P40, 0.2 *M* DTT and 2% ampholytes at pH 4–6 or 4–6.5. Dissolution takes 2–3 h at room temperature with occasional mixing. Protein samples, 36–40 μl, are applied to IEF cylindrical gels (0.22 × 11.4 cm) containing 4% acrylamide, 8.5 *M* urea, 2% ampholytes, pH 4–6 or 4–6.5, and 2% Nonidet-P40; IEF is carried out for 8000 V h. Subsequently, the gels are equilibrated with a solution containing 2.3% SDS, 0.2 *M* DTT, 0.063 *M* Tris buffer, pH 6.8, and 10% glycerol for 15 min. The gels are transferred to slabs consisting of 3% acrylamide stacking gels (0.16 × 14.0 × 2.5 cm) in 0.1% SDS, 2 *M* urea, 0.125 *M* Tris, pH 6.8, and 15% acrylamide resolving gels (0.16 × 14.0 × 10.0 cm) in 0.1% SDS, 0.37 *M* Tris, pH 8.8. Electrophoresis is carried out in the second dimension with a buffer containing 0.1% SDS, 0.19 *M* glycine and 0.025 *M* Tris, pH 8.8, for about 3 h, so that the tracking dye is 2 cm from the bottom. The gels are stained with 0.2% Coomassie Blue R-250 in 40% methanol and 5% acetic acid for 90 min and destained in 40% methanol and 5% acetic acid overnight; a clear background is obtained.

The gels of pH 4–6 in the first dimension and 15% acrylamide in the second dimension resolve the myosin light chains, tropomyosin and actin; at pH 4–6.5 in the first dimension, the isoforms of an unidentified protein, 28K (M_r 28 000) are also resolved. Desmin, vimentin and two forms of caldesmon (M_r 92 000 and 140 000) are separated on gels of pH 4–6 in the first dimension and 10% acrylamide in the second dimension (see Fig. 10).

Proteins with isoelectric points above pH 8 cannot be resolved using the classical IEF for the first dimension of 2DE. O'Farrell et al. [42], however, substituted IEF with non-equilibrium pH gradient electrophoresis by switching the polarities and electrophoresing for much shorter V h. In such a system the basic proteins lead in the separation and are well resolved. This method, called NEPHGE, was used to separate the basic calponin isoforms (see Fig. 9) [43].

2.4. Staining

The most common method for fixing and staining muscle proteins in a polyacrylamide gel is incubating the gel with Coomassie Blue R-250 (0.2%) in a fixing solution containing alcohol and acid (e.g., methanol–water–glacial acetic acid or ethanol–water–phosphoric acid). The gel is then destained by several incubations in the fixing solution without the dye. Various modifications of the methods have been described [8]. Recently, the procedure was simplified by using very low concentration (0.003%) of Coomassie Blue in the fixing solution, which eliminates the need to destain the gel, while a clear background is obtained [44]. The use of Coomassie Blue G-250 also eliminates the need for destaining, and it is more sensitive than the R-250 derivative [45].

Silver staining is considerably more sensitive than Coomassie Blue R-250 and is the stain of choice in situations where protein load is limited (e.g., less than 5 μg of pure protein or less than 100 μg of protein in a whole homogenate, such as in studies of single muscle fibers). Numerous silver staining techniques are in use, some using silver diamine and others using silver nitrate [46]. Each technique has its own advantages and disadvantages, including background staining and protein-specific color development. Some silver stains also detect DNA, lipopolysaccharides and polysaccharides in addition to protein. With adequate understanding of the silver stain reaction used, however, this detection method can be used not only to detect proteins in polyacrylamide gels, but also to quantitate the relative abundance of proteins in those gels [47]. Giulan et al. [48], for example, quantified proteins, with relative molecular masses from 10 000 to 75 000, including actin, the troponin subunits (TNI, TNC and TNT), and the myosin light chains (LC1, LC2 and LC3) from single rabbit psoas and soleus muscle fibers on silver-stained PAGE. This work demonstrated a linear response between approximately 2 and 70 ng of protein per band. Wallace and Saluz [49] described an ultramicrodetection of proteins on SDS-PAGE, based on photochemical reactions in which very low amounts of silver are deposited around proteins and are converted in a series of steps to silver sulfide. When this conversion is carried out in the presence of [^{35}S]thiourea, the resulting radioactive silver sulfide allows detection down to femtogram quantities of protein.

With colloidal gold stain, 1–5 ng of proteins can be detected on nitrocellulose blots [8]. Gaylinn and Murphy [50] and Kitazawa et al. [51] have used this method for quantitation of P-LC phosphorylation in small (10 μg wet mass) smooth muscle tissue samples. A rapid, sensitive color–protein stain was developed for visualizing proteins on SDS-PAGE [52]. This stain detects 1 μg of protein in various colors. Eosin Y staining of proteins on SDS-PAGE has a sensitivity of 10 ng, comparable to that of silver staining [53]. It also stains sialoglycoproteins, which are not detectable by Coomassie Blue.

Muscle proteins on two-dimensional gels can also be visualized without staining, e.g., by "fixing" with potassium acetate [54]. This is based on the low solubility of the potassium–dodecyl sulfate–protein complex, making the protein spots visible against a dark background. The protein spots were excised from the gels and eluted by homogenization in distilled water. By this procedure, homogeneous tropomyosin and LC fractions were obtained from human heart [54].

2.5. *Quantification of stained proteins*

Scanning densitometers supplied with computer programs and raw data disks are available commercially. These instruments readily integrate the stained band or spot area on wet or dried gels and print out the percentage staining intensity of an individual band or spot relative to the total staining intensity of the entire gel (or only a part of it). Furthermore, the data may be displayed on computer screens, amplified, and printed out in a pictorial form. Quantitative densitometry of silver- or Coomassie Blue-stained proteins was described in a recent paper [55].

Advances in computer analysis of 2DE gel patterns allow quantitation of a large number of individual protein spots. Thus, of the 676 proteins detected in human leukocytes after silver staining, over 200 had R.S.D.s of 15% or less when data from replicate patterns were analyzed [47]. With the use of nitrocellulose membranes for immobilizing macromolecules, a new avenue opened for silver staining. The protein concentration in SDS–sample buffer could be quantified by spotting the samples onto nitrocellulose membranes and staining the dots with colloidal silver [56]. The quantification was in the range of 5-100 ng of protein.

Alternatively, well-resolved Coomassie Blue-stained protein bands or spots may be cut out of the gels, the dye eluted with 25% (v/v) pyridine in water [57,58], and the absorbance measured at 605 nm. This method allows comparison of relative protein concentrations within the same gel. If the absolute concentration of a protein is

known, the concentration of another protein can be estimated [43].

2.6. Quantification of radioactive proteins

The ^{32}P-labeled protein spots or bands are cut from the stained wet gels, digested at 105°C with 30% H_2O_2 for 30 min, and the radioactivity determined by liquid scintillation counting. In studies of muscle structural protein phosphorylation, incorporation of [^{32}P]phosphate into the proteins is quantified from the counts in the H_2O_2-digested protein spots, the specific radioactivity of [^{32}P]PCr in the muscle, the known amount of total protein applied to the gels, and the content of the specific protein in the total protein. When the content of a specific protein in a muscle is unknown, it has to be determined. PAGE (5–7.5%) followed by measuring the percentage staining intensity of the specific protein band, relative to the 100% staining intensity of all protein bands, is an appropriate procedure. The incorporation is expressed in terms of mol [^{32}P]phosphate/mol protein and computed as:

$$\frac{\text{mol [}^{32}\text{P]phosphate}}{\text{mol protein}} = \frac{\text{cpm in the protein spots}}{\text{cpm/mol [}^{32}\text{P]PCr} \cdot \text{mol protein}}$$

^{14}C-Labeled protein spots or bands can be digested with 30% H_2O_2 at 60°C for 24 h and then counted. The counts are also expressed in terms of mol ^{14}C-label per mol of protein using the specific radioactivity of the ^{14}C-containing reagent as a reference [59].

^{3}H- or ^{14}C-labeled proteins, separated on PAGE, prepared with N,N^{1}-dialyltartardiamide instead of N,N^{1}-methylenebis(acrylamide), and stained with silver, can be quantified by slicing the protein bands, placing them into glass scintillation vials with 0.5 ml of 2% sodium metaperiodate, and shaking for 30 min to dissolve the gel [60]. Liquid scintillation fluid is added after cooling the vials to prevent chemiluminescence, and the radioactivity is counted. The same procedure can be used for gels stained with Coomassie Blue or another dye.

Without counting, radioactive protein spots can also be quantified by densitometry of the autoradiograms using many of the same computer algorithms cited in the discussion of quantification of stained proteins. A specific example is given in Ref. [61].

2.7. Quantification of myosin light chain phosphorylation

^{32}P-Labeled P-LC, separated by 2DE, is quantified as described in the previous section. For non-radioactive quantification of P-LC, the stained gels are subjected to densitometry [22,23]. The phosphorylation of P-LC is calculated by taking the ratio of monophosphorylated P-LC to the sum of the non-phosphorylated and monophosphorylated P-LC. This procedure does not take into account the phosphorylation of the minor P-LC isoforms and the diphosphorylation of the major P-LC isoform, and therefore underestimates the actual phosphoryl content of P-LC.

Silver and Stull [25] quantified P-LC in muscle samples by isolating first the myosin from the crude muscle homogenate by pyrophosphate-PAGE and then subjecting the myosin to IEF in order to separate the non-phosphorylated and the phosphorylated forms of P-LC. Densitometric scanning of the IEF gels was used to quantify the phosphorylation of P-LC. Inhibitors of MLCK and MLCP were included throughout the procedure to prevent changes in the phosphate content of P-LC; however these inhibitors are less efficient than treating the muscle with PCA or TCA.

Hathaway and Haeberle [21] developed a radioimmunoblotting method for measuring P-LC phosphorylation levels in smooth muscle. The phosphorylated and non-phosphorylated forms of P-LC were separated by glycerol–urea PAGE, then transferred by electroblotting from polyacrylamide gels to nitrocellulose paper, and incubated with antiserum for P-LC; the P-LC–antibody complex was labeled with [^{125}I]protein A and the radioactivity was determined. This method of Hathaway and Haeberle was modified by Persechini et al. [62] who incubated the P-LC on the nitrocellulose paper with peroxidase-

linked P-LC-antibody, visualized the complex as a dark blue band after treatment with 4-chloro-1-naphthol, and determined the extent of P-LC phosphorylation by densitometry.

2.8. Immunoblotting

Immunoblotting (or Western blotting) is the most common procedure for identification of muscle proteins from polyacrylamide gels [8]. Fig. 3 illustrates immunoblotting of the major and minor P-LC isoforms from uterine smooth muscle [14]. The upper panel shows four Coomassie Blue-stained spots on regular two-dimensional electropherograms, while the middle panel exhibits four spots on the immunoblot stained with 4-chloro-1-naphthol as a peroxidase substrate. The relative staining intensities of the four spots in the two systems are the same indicating that all four spots contain light chain. The bottom panel shows that upon IEF in both the first and second dimensions, the spots migrate along a diagonal; thus, the spots are not produced by artifactual charge modification. Similar electrophoretic and immunoblot analysis was performed on the P-LC from arterial smooth muscle [39]. Immunoblotting has been applied for quantification of P-LC phosphorylation in smooth muscle [21,62]. Immunoblotting identified calponin in platelets [63], and the low- and high-molecular-mass forms of caldesmon on 2DE of arterial muscle extract [64].

2.9. Overlay assay

Protein interactions may be detected on SDS slab gels by overlaying the fixed gel with a radioactive protein and localizing the label by autoradiography. For example, Sellers and Harvey [65] electrophoresed smooth muscle myosin on SDS-PAGE (7.5%) and fixed the protein with methanol and acetic acid followed by extensive washes. The gel was incubated overnight with ^{32}P-labeled regulatory LC (M_r 20 000) and ^{14}C-labeled essential LC (M_r 17 000) from smooth muscle in the presence of serum albumin and then washed extensively to remove unbound protein. Following staining and destaining, the

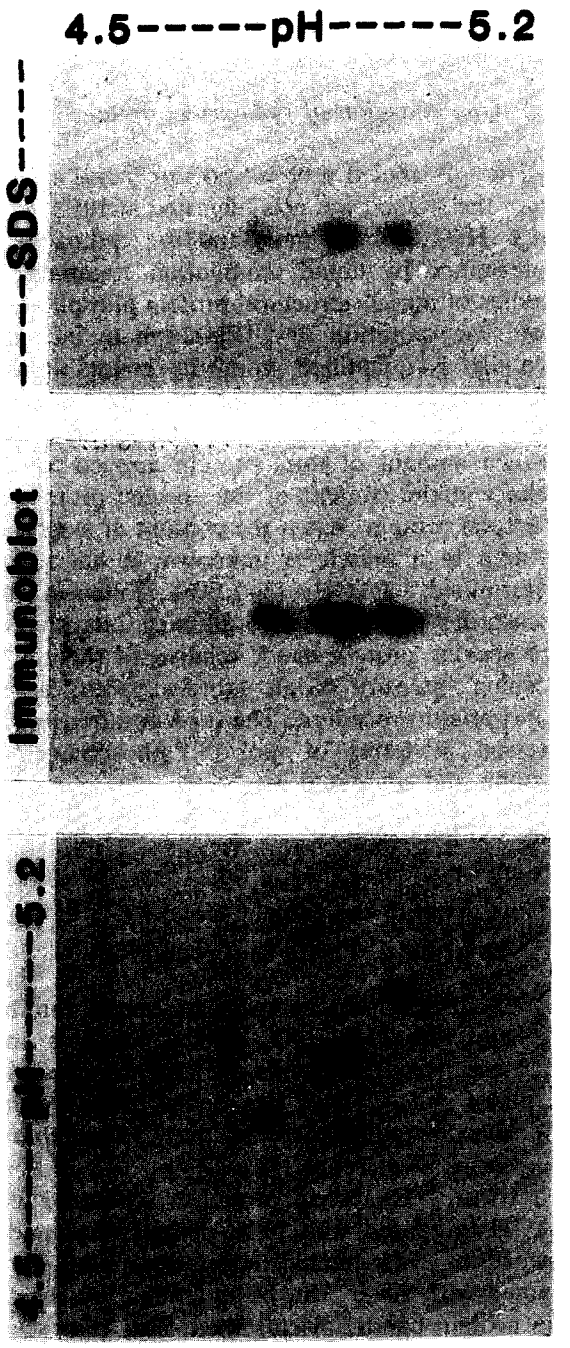


Fig. 3. Electrophoretic and immunological analysis of purified P-LC from rat uterus. Top panel: Coomassie Blue-stained electrophoretogram of 2DE. Middle panel: immunoblot of the spots from the electrophoretogram. Bottom panel: Coomassie Blue-stained two-dimensional electrophoretogram, isoelectric focusing in both first and second dimensions. From Ref. [14].

gel was autoradiographed and the HC was found to rebind the labeled regulatory and essential LCs. By fragmenting the HC proteolytically, they were able to determine the binding site for both types of LC to be within the COOH-terminal (M_r 26 000) segment of smooth muscle S-1. Using the same technique, Margossian et al. [66] have found that ^{125}I-labeled LC1 and LC2 were bound to intact cardiac S-1 or to its carboxyl-terminal proteolytic fragment (M_r 20 000). ^{125}I-Labeled TNC was used to measure TNC binding to cardiac myofibrillar proteins [30].

2.10. Combination of two-dimensional gel electrophoresis with peptide mapping

Individual protein spots, resolved by 2DE, may be further analyzed by eluting the proteins from the gels and digesting them with proteolytic enzymes such as trypsin. This approach is illustrated in Figs. 4 and 5 [67]. Fig. 4 shows the gel pattern; there are four stained P-LC spots, called spots 1, 2, 3 and 4 with increasing isoelectric points. The autoradiogram of P-LC from the K^+- and PDBu-stimulated muscle shows four spots which correspond to staining spots 1, 2, 3 and to a spot not observable by staining but only by radioactivity, called spot 0. This radioactive spot 0 could also be detected in the gel of the K^+-stimulated muscle when the corresponding area was excised and counted. Fig. 5 compares the two-dimensional tryptic phosphopeptide maps of P-LC isoforms eluted from spots 0, 1, 2 and 3 from muscles stimulated either with K^+ alone (upper row) or with K^+ and PDBu (lower row). In the maps from the muscle stimulated with K^+ alone, A and B are the predominant peptides, called MLCK/Ser, because these peptides contain phosphoserine generated by MLCK [68]. These maps also contain small quantities of peptides C and D (MLCK/Thr) and the PKC-generated peptides E (PKC/Ser) and F (PKC/

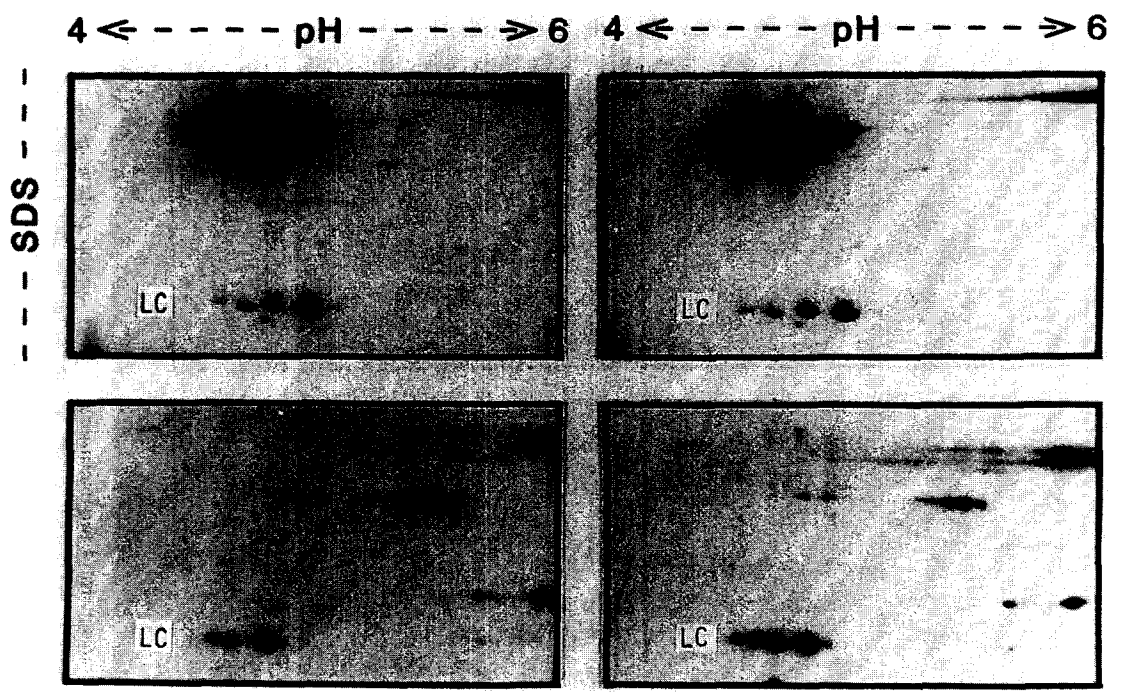


Fig. 4. Two-dimensional electrophoretograms of ^{32}P-labeled arterial muscle proteins. Left: muscle stimulated with K^+; right: muscle stimulated with K^+ and PDBu. Top: staining patterns; bottom: corresponding autoradiograms. LC = Phosphorylatable myosin light chain. From Ref. [67].

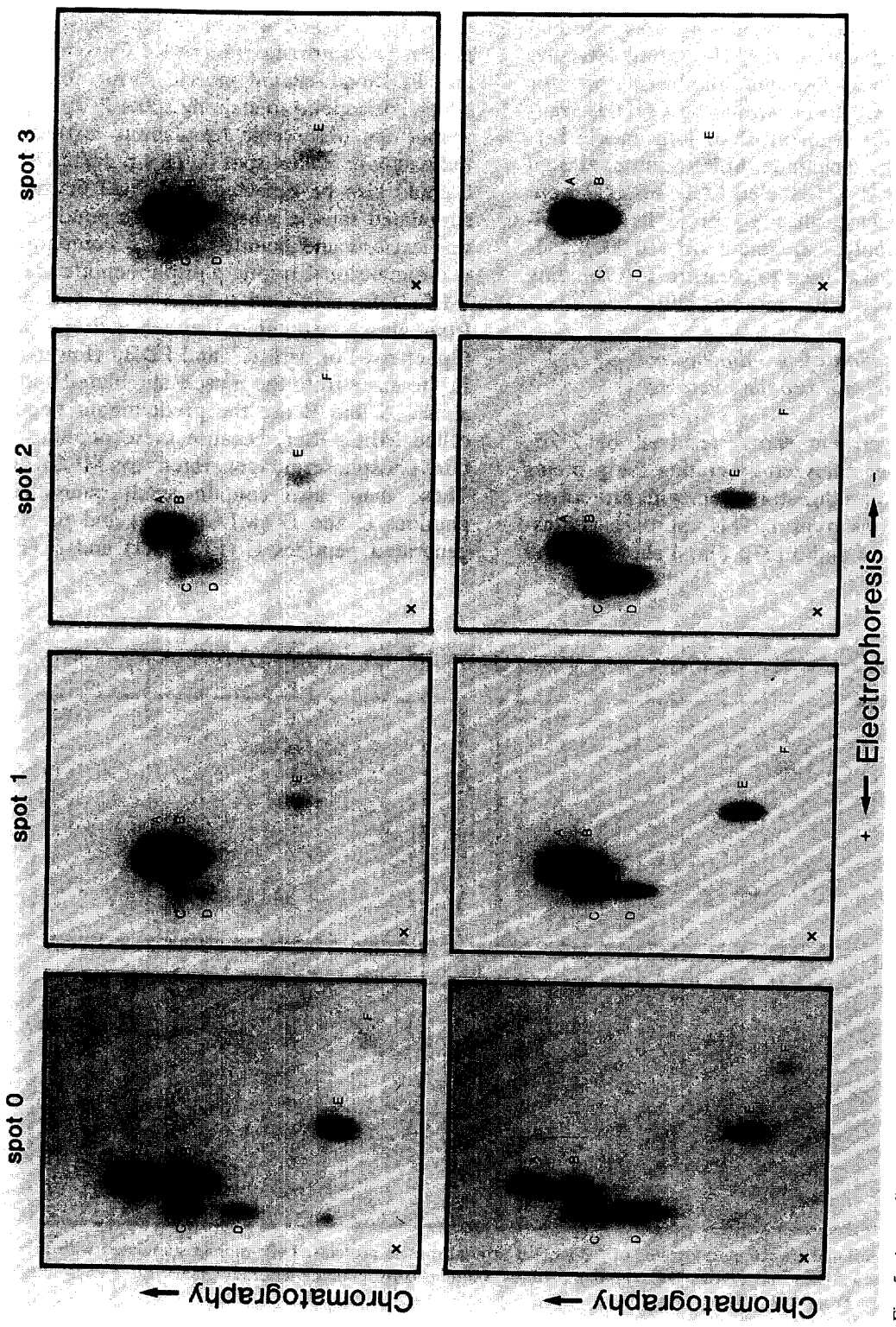


Fig. 5. Autoradiograms of two-dimensional tryptic phosphopeptide maps of individual P-LC spots isolated by 2DE, shown in Fig. 4. The upper row shows the peptides isolated from the K^+-stimulated muscle, and the lower row shows the peptides from the K^+- and PDBu-stimulated muscle. From Ref. [67].

Thr). On the other hand, in the maps from the K^+- and PDBu-stimulated muscles, peptides A and B are predominant only in spots 1 and 3, whereas in spots 0 and 2, peptides C and D appear in larger quantities than A and B. Furthermore, spots 0, 1 and 2 contain a significant amount of peptide E and traces of peptide F. Thus, one can conclude that PDBu changes the phosphorylation pattern of the P-LC isoforms, as evidenced by the difference in the phosphopeptides between muscles stimulated with K^+ alone or K^+ and PDBu.

The combination of 2DE and phosphopeptide mapping was also used to show that stretching activates MLCK in arterial smooth muscle [69].

2.11. Staining of ATPase or phosphatase activity and of alkali-labile phosphoproteins

The contractile protein myosin and its proteolytic fragments possess ATPase activity that can be used for their identification during purification procedures and subsequent PAGE. The gels can not contain SDS, so the proteins are separated in a Tris–glycine buffer system according to the method of Davis [70] or Hoh [71]. Following electrophoresis, the gels are incubated at 25°C for 20 min in a solution containing 0.02 *M* ATP, 0.01 *M* $CaCl_2$ and 0.03 *M* KCl, pH 7.0 [28]. After washing the gels with distilled water, they are placed in a solution containing 0.08 *M* Tris–maleate and 3 m*M* $Pb(NO_3)_2$, pH 7.0, for 1 h. The gels are washed exhaustively with water followed by treatment in a 2.5% $(NH_4)_2S$ solution for 5 min. After further washing of the gels, the ATPase band is visualized. Alternatively, the lead treatment is omitted and the white calcium phosphate precipitate, formed as a result of the ATPase activity, is detected by scanning the gels at 550 nm [71].

Alkaline phosphatases may be stained on polyacrylamide gels after incubation with 5 m*M* phenylphosphate at pH 10.0; the released phosphate is precipitated as lead phosphate and subsequently stained with rhodamine B [72]. Phosphatases may also be detected on gels with malachite green staining; the sensitivity is 0.2 nmol P_i [73].

Alkali-labile phosphoproteins are visualized on PAGE after alkaline hydrolysis, phosphate capture, and the formation of an insoluble rhodamine B–phosphomolybdate complex [72].

3. Proteins

3.1. Myosin

3.1.1. Native myosin

Hoh [71] was the first to electrophorese native skeletal muscle myosin on polyacrylamide gels. His method took advantage of the solubilizing effect of ATP on myosin at low ionic strength. Electrophoresis was carried out on 3.36% gels at 4°C in a continuous buffer system containing 0.025 *M* Tris, 0.19 *M* glycine, 5 m*M* ATP and 10% glycerol, pH 8.3. The Ca^{2+}-activated ATPase activity of myosin could be demonstrated on the unstained gels, providing evidence that the myosin remained in the native state. In this system, fast muscle (rat extensor digitorum longus) myosin had a higher electrophoretic mobility than slow muscle (rat soleus) myosin. Soleus myosin exhibited a major slow and a minor fast component due to two populations of muscle fibres. The same muscle cross-reinnervated by a fast muscle nerve showed only the fast component. The homogeneous extensor digitorum longus muscle exhibited only the fast component, but following cross-reinnervation it showed both fast and slow components.

Subsequently, ATP was replaced by 0.02 *M* pyrophosphate in the buffer system [74], because that gave better resolution. Chicken skeletal myosin was resolved into five components, three from the fast-twitch posterior latissimus dorsi muscle and two slower-migrating components from the slow-twitch anterior latissimus dorsi muscle. These data suggested that the components of fast- and slow-twitch muscle are isozymes of myosin. In Hoh's laboratory, pyrophosphate PAGE revealed developmental changes in chicken skeletal muscle myosin isozymes [10], and dystrophy caused changes in the

distribution of the isozymes in mouse muscles [75]. Termin and Pette [76] modified the method and thereby observed subsets of developmental, fast, and slow isomyosins of rat muscles. Neonatal isomyosins (*n*M1, *n*M2, *n*M3) migrated the fastest, the embryonic isomyosins (*e*M1, *e*M2, *e*M3, *e*M4) migrated somewhat slower. Of the nine adult fast isomyosins, the HCIIb-based isomyosins (*f*M1b, *f*M2b, *f*M3b) were the fastest, followed by the HCIId-based isomyosins (*f*M1d, *f*M2d, *f*M3d), followed by the HCIIa-based isomyosins (*f*M1a, *f*M2a, *f*M3a); the slow isomyosins (*s*M1, *s*M2, *s*M3) migrated far behind the fast isomyosins.

Pyrophosphate-PAGE of adult rat myocardium revealed the presence of five distinct components [77]: two in atrial myosin (A1, A2) and three in ventricular myosin (V1, V2, V3). Analysis of Ca^{2+}-activated myosin ATPase activity in the gels revealed that A1, A2, and V1 had about the same specific activity; V3 had the lowest activity, while that of V2 was intermediate. During the late embryonic period, V3 is predominant. After birth, there is a shift in the distribution of isomyosins, so that at three weeks of age, ventricular myosin becomes exclusively V1. The distribution of isomyosins is related to the thyroid status [78]; with a higher hormone level, V3 increases and V1 decreases.

3.1.2. Heavy chains

In SDS-containing medium, the myosin molecule dissociates into two heavy chains and the associated light chains. The HC subunits (M_r around 200 000) can be separated on 5–8% polyacrylamide gradient [79] or simply on 5% polyacrylamide gels [80]. Structural analysis of myosin through separation of the HC isoforms on SDS-PAGE takes only hours, whereas separation of the native myosin isozymes on pyrophosphate-PAGE takes days. The mobility of HCs from fast-twitch rat muscles follows the same order, HCIIb > HCIId > HCIIa [81], as that of the fast isomyosins from the same muscle [76]. Furthermore, HC electrophoresis resolved six isoforms during chick breast muscle development [79]. It was shown that the HC composition of single cells from avian slow skeletal muscle is correlated with the velocity of shortening during development [82]. Vertebrate smooth muscle HCs exist as two isoforms with relative molecular masses of 204 000 and 200 000 [80]. The ratio of HCs (204 000:200 000) was approximately 1:0.75 for myosin from the iliac-femoral artery of normotensive dogs [83]. This was not altered significantly in response to hypertension.

3.1.3. Light chains

The myosin molecule contains four light chains (M_r ranges from 16 000 to 27 000). Conventionally, the light chains are divided into two subgroups: LC1 and LC3 are called essential light chains, and LC2 is called the phosphorylatable or regulatory light chain.

The 2DE analysis of LC pattern in chronically stimulated fast-twitch muscle of the rat showed an increase in LC1*f*, with a concomitant decrease in LC3*f* [58]. The 2DE analysis of adductor longus muscle from rats subjected to space flight at zero gravity or to tail-suspension hindlimb unloading revealed an increase in the abundance of fast myosin light chains LC1*f*, LC2*f*, and LC3*f* relative to the slow myosin light chains LC1*s* and LC2*s* [84]. The 2DE analysis of diaphragm and gastrocnemius muscles of rat upon denervation demonstrated the conversion of the five-spot myosin light chain pattern from the mixed population of fast and slow fibers to a selective fast myosin light chain pattern [85]. Based on 2DE, the light chains of *Drosophila* were classified into two categories, fibrillar (flight) muscle consisting of four variants, LC1*f*, LC2*f*, LC2′*f* and LC3*f*, and tubular (leg) muscle consisting also of four variants, LC1*t*, LC2*t*, LC2′*t* and LC3*t* [86]. Human skeletal muscle LCs were analyzed during ontogenesis by 2DE using immobilized pH gradients in the first dimension [87]. The high resolution of these gels, resolving components which in the isoelectric points are < 0.01 pH apart, has allowed the identification of four phosphorylatable LCs: two slow myosin light chains (LC2*s*a and b), two fast myosin light chains (LC2*f*a and b), and their phosphorylated forms (LC2*s*aP and bP, and LC2*f*aP and bP). The polymorphism of LC was already evident at 10 weeks of development but only the non-

phosphorylated forms were present; the phosphorylated forms could be detected at 26 weeks. An embryonic LC (M_r 29 500 for $LC1_{emb}$) persisted for up to 26 weeks of fetal life.

The SDS-PAGE analysis of purified cardiac atrial (A) and ventricular (V) myosins revealed the following light chains: A-LC1 (M_r 27 000, not resolved from V-LC1), A-LC2 (22 000) and V-LC2 (19 000) [77].

IEF of purified vascular smooth muscle myosin revealed two variants of the essential LC (M_r 17 000) p*I* 4.13 (LC_{17a}) and 4.19 (LC_{17b}) [88]. Aorta, carotid and pulmonary artery muscles and tracheal smooth muscle contained both LC_{17} variants, while smooth muscle of the gastrointestinal tract (stomach and jejunum) contained LC_{17a} only. Gizzard myosin contained only the more acidic isoform [89].

3.1.4. Phosphorylatable light chain

Perrie et al. [90] were the first to show that the regulatory LC (M_r 18 500) of skeletal muscle occurs in phospho and dephospho forms. Subsequently, phosphorylation of regulatory light chains with relative molecular masses of 18 000–20 000 in various myosins was described, and following the suggestion of Frearson and Perry [31], these phosphorylatable light chains were called P-LC. Further studies with 2DE revealed the existence of P-LC variants in different muscles. The 2DE method played a major role in characterization of the multiple forms of P-LC and establishing their function in smooth muscle contraction.

Diphosphorylation of P-LC was first demonstrated in frog heart by 2DE [91]. On stained electropherograms, P-LC exhibited three spots and on the corresponding autoradiograms, two spots (Fig. 6). The specific radioactivity of [^{32}P]phosphate of the most acidic spot was two times higher than that of the middle spot, indicating the existence of diphosphorylated and monophosphorylated forms of P-LC. Both forms could be converted into their non-phosphorylated form when protein phosphatase inhibitors were omitted from the extraction medium.

2DE of P-LC of dog, cat, and adult chicken hearts [92], rat uterus [14] or porcine carotid arteries [20,93] displayed four spots on stained electropherograms. Fig. 7 illustrates this for the arteries: four spots of P-LC are resolved by staining (top panel); the percentage of stain distribution of the four spots as determined by densitometry (bottom panel), from the lower to the higher pH value (referred to as spots 1, 2, 3 and 4) is 4, 11, 22 and 63% in the resting muscle (left) and 5, 12, 48 and 35% in the contracting muscle (right). These data show an increase in staining intensity of spot 3 and a decrease in staining intensity of spot 4 in the contracting muscle relative to resting muscle. This is caused by the partial phosphorylation of spot 4, converting it to spot 3. The autoradiogram reveals that out of the four stained spots, three contain phosphorylated LC; these are spots 1, 2 and 3; the major radioactivity is in spot 3. The isoelectric points of spots 1, 2, 3 and 4 were estimated to be 4.60, 4.75, 4.90 and 5.05, respectively [14].

The nature of the multiple P-LC spots has been debated, with the possibility that they originate from the phosphorylated and non-phosphorylated proteins as a result of artifactual charge modification [22,94]. The following experiments indicated that the four spots of arterial P-LC were not artifacts: (1) pre-electrophoresis of the gels in the presence of 1 m*M* sodium thioglycolate, which has been reported to eliminate charge modification [94], had no effect on the appearance of the P-LC spots [95]; (2) two-dimensional IEF showed all P-LC spots arranged in a diagonal pattern and no off-diagonal spots were generated below any original spot ([96] and Fig. 3); (3) the same gel electrophoretic procedure which gave four P-LC spots for arterial and uterine smooth muscle showed only two spots of P-LC for skeletal muscle [14,95]. The experiments of Mougios and Bárány [95] supported the idea that the multiple P-LC spots originate from different isoforms. They showed that in fully dephosphorylated porcine arterial muscle the presence of two P-LC spots (identified with spots 2 and 4) persists at proportions of 15 and 85%. The P-LC isoforms had distinct tryptic peptide maps. The isoform concept is further supported by genetic evidence. Inoue et

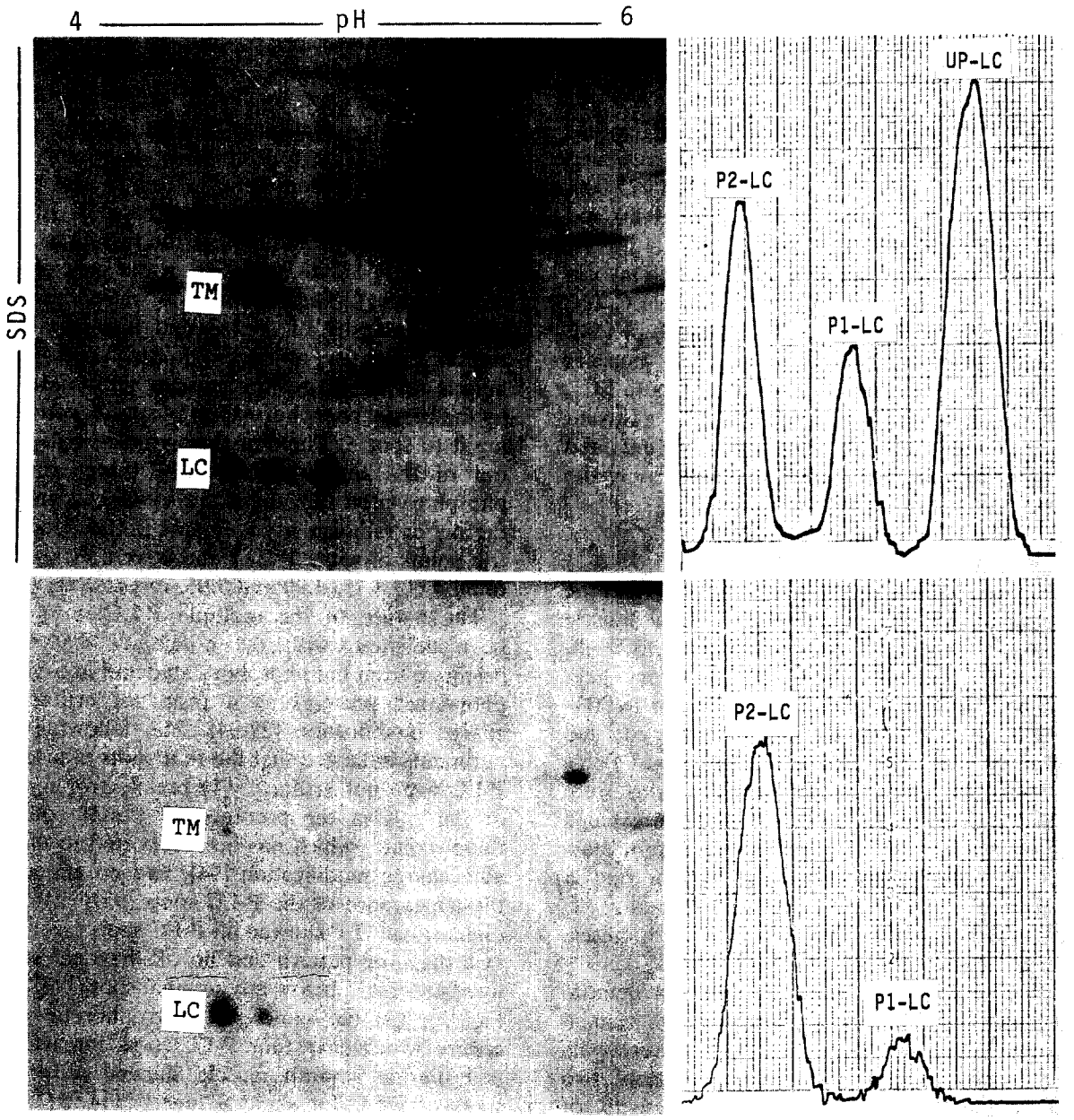


Fig. 6. Analysis by 2DE of frog heart myofibrils isolated from frogs injected with carrier-free [^{32}P]orthophosphate. Upper row: staining profile (left), scans of staining intensities of P-LC (right). Bottom row: radioactivity profile (left), scans of autoradiogram of P-LC (right). TM = Tropomyosin; P2-LC, P1-LC, UP-LC = di-, mono- and unphosphorylated forms of the phosphorylatable myosin light chain, respectively. From Ref. [91].

al. [97] isolated a cDNA clone for the minor P-LC isoform of chicken gizzard myosin with a deduced amino acid sequence that is different in ten amino acid residues from that of the major P-LC isoform of gizzard myosin. The in vitro transcription/translation product from the cDNA comigrated with the minor isoform of chicken gizzard P-LC in 2DE, it could be associ-

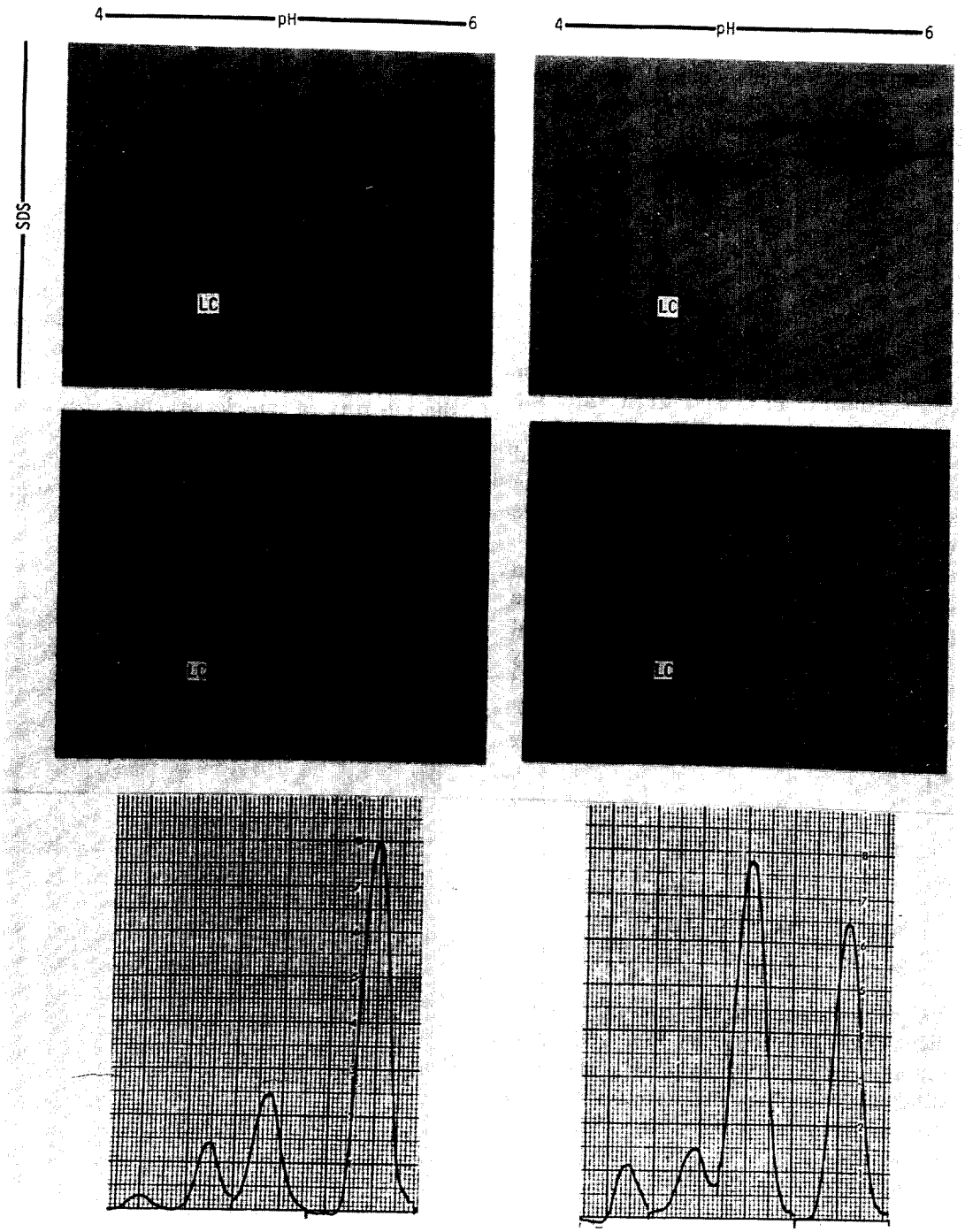
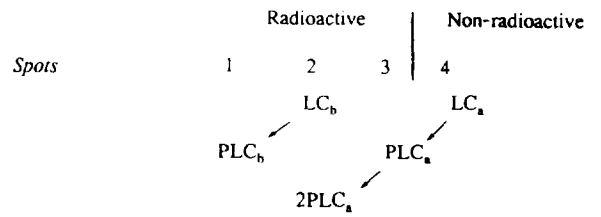


Fig. 7. The multiple forms of porcine carotid arterial P-LC, separated by 2DE. Upper panels show the Coomassie Blue staining patterns, middle panels show the autoradiograms of the arterial proteins (TCA-insoluble residues) and bottom panels show the densitometric scans of P-LC. Left: arterial muscle was frozen at rest; right: muscle was frozen 30 s after 100 m*M* K^+ challenge. LC = Phosphorylatable myosin light chain. From Ref. [93].

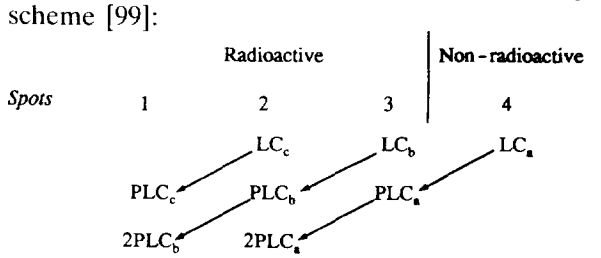
ated with native chicken gizzard myosin, and it was also rapidly phosphorylated by MLCK.

Accordingly, the four stained and three radioactive spots of Fig. 7 may be explained by two P-LC isoforms:



where LC_a is the major and LC_b is the minor light chain isoform, PLC and 2PLC are the mono- and diphosphorylated light chains. As it appears, spot 2 contains both diphosphorylated and non-phosphorylated isoforms, this is the explanation for the four stained and three radioactive spots.

The P-LC of ^{32}P-labeled rat uterus exhibited four spots on the 2DE electropherograms; the corresponding autoradiograms revealed that three spots were radioactive [98]. Completely dephosphorylated P-LC showed three stained spots, indicating the presence of three isoforms. These findings are compatible with the following scheme [99]:



where LC_a is the major and LC_b and LC_c are the minor light chain isoforms, and PLC and 2PLC are the mono- and diphosphorylated light chains.

A mathematical analysis [100] led to a function correlating apparent phosphorylation (S), determined by densitometry, with actual phosphorylation (P), determined by ^{32}P incorporation:

$$S = \left(\frac{1}{1+d} - i\right)P + i$$

where i is the fraction of non-phosphorylated LC existing as isomer and d is the fraction of all phosphorylated LC that is diphosphorylated. This function gives straight lines. From the intercepts and slopes, it was calculated that uterine P-LC contains 29% minor isoforms and arterial P-LC contains 20%. Furthermore, in uterus, 23% of the phosphorylated P-LC is diphosphorylated and in artery, 9%.

The 2DE method resolves as many as six spots in the phosphorylatable light chain component of purified and phosphorylated actomyosin (Fig. 8). The major radioactive spot contains 3 mol phosphate per mol light chain [68,95]. It is of interest to note that the difference in the phosphorylation pattern between intact muscle and actomyosin (cf. Fig. 7 with Fig. 8) is not based on the integrity of muscle structure, because aorta homogenates phosphorylated with ATP under the same conditions as actomyosin displayed a P-LC phosphorylation pattern very similar to

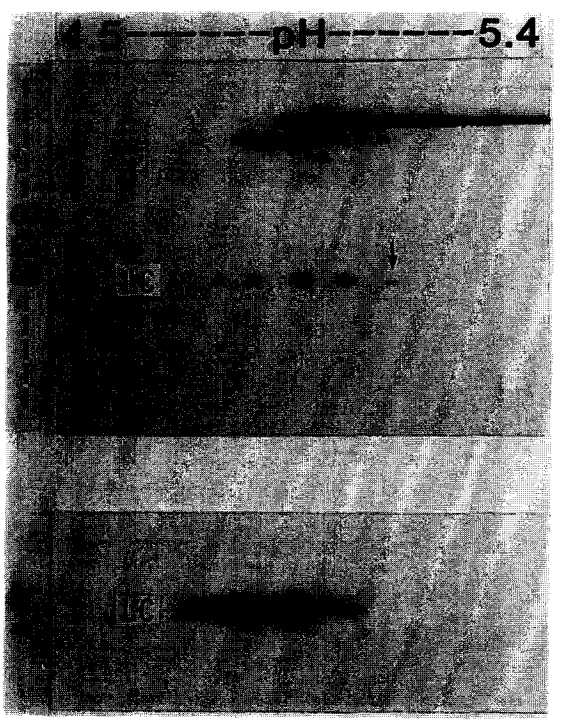


Fig. 8. Analysis by 2DE of maximally phosphorylated light chain (LC) in actomyosin from porcine carotid aorta. Top: staining pattern. The arrow points to spot 4 , containing the non-phosphorylated major isoform. Bottom: corresponding autoradiogram. From Ref. [68].

that of stimulated intact arteries [95]. Therefore, the change observed in actomyosin must be attributed to the removal of factors, presumably phosphatases, which are present in both intact and homogenized muscle.

3.2. Actin

Actin is a ubiquitous cytoskeletal protein which exists in two forms, globular (G-actin) and filamentous (F-actin). The different isoforms of actin include two striated muscle isoforms (α skeletal and α cardiac), two smooth muscle isoforms (α vascular and γ enteric), and two cytoplasmic isoforms (β and γ cytoplasmic) [101,102]. "It is extraordinary that all actin variants have the same number of residues, having 95 per cent homology from amoeba to man" [103]. Although the high concentration of actin in muscle causes the protein to appear as a long band in 2DE patterns of whole tissue extracts (see Fig. 10), resolution of the isoforms is possible by separating only small quantities of actin. When 2DE was used to separate α, β and γ actin from embryonic chicken skeletal muscle [104] and stomach smooth muscle [105], approximate p*I* values of 5.40, 5.42 and 5.44, respectively, were obtained. The relative molecular mass of all actin isoforms using SDS-PAGE is 43 000 [104].

Subtilisin, α-chymotrypsin, or trypsin digestion of G-actin yields fragments with relative molecular masses from 33 000 to 35 000 that are readily separated from the parent actin molecule by SDS-PAGE [106]. The sites available for proteolytic enzymes in G-actin are protected in F-actin or in the G-actin–subfragment-1 complex, leading to the conclusion that upon polymerization or binding to myosin subfragment-1, actin undergoes a conformational change. Proteolysis as a probe was also used for studying the actin–myosin head interactions when actin was internally cross-linked with (*m*-maleimidobenzoyl)-N-hydroxysuccinimide ester [107].

The SDS-PAGE method has the potential to identify dimers, trimers, tetramers and higher oligomers in cross-linked actin [108,109] or during lithium ion-induced actin polymerization [110]. The complex between rabbit skeletal muscle F-actin and S-1 was characterized by SDS-PAGE [111]; the actin-(11–28)-peptide was identified forming the linkage. The same part of yeast (*Saccharomyces cerevisiae*) actin was linked to rabbit skeletal muscle S-1 [112]. Actin expressed in *E. coli* [113] was also characterized by SDS-PAGE.

The electrophoretic mobility of fluorescently labeled G-actin in polyacrylamide gels under non-denaturing conditions is altered by the formation of complexes with actin-binding proteins [114]. This effect offers a convenient method for detecting and quantitating such proteins in tissue fractions and for monitoring their purification. Actin-binding proteins may also be identified by combining G-actin affinity chromatography with SDS-PAGE [115]. The interaction between talin and actin was determined by sedimentation at 100 000 *g* and analyzing both the pellet and supernatant for talin and actin by SDS-PAGE [116].

3.3. Tropomyosin

Tropomyosin, a rod-like protein, composed of two coiled-coil polypeptide chains with close to 100% α-helical content, is localized in the thin filament of muscle. It is involved in the regulation of contraction of skeletal, cardiac and smooth muscles. Skeletal muscle tropomyosin consists of two subunits, α (fast) and β (slow), based on electrophoretic mobility on SDS-PAGE [117]. The ratio of the amounts of the α and β subunits varies with muscle type. Slow (red) skeletal muscle [118] and fetal muscle [119] contain a larger proportion of β subunit than fast (white) skeletal muscle, whereas rabbit and avian cardiac muscle contain only the α subunit [118,120,121]. Despite the difference in the electrophoretic mobility of the α and β subunits, both contain the same number of amino acids, 284, and thus have a similar relative molecular mass of approximately 33 000 [121]. Tropomyosin from gizzard smooth muscle shows two subunits on SDS-PAGE [118], the β (has a mobility similar to that of skeletal muscle β-

tropomyosin) and the still slower γ subunit. Cummins and Perry [117] were the first to show that tropomyosin migrates on SDS-PAGE containing urea with a relative molecular mass of 50 000, thereby enabling the separation of tropomyosin from TNT (see also [17]).

The 2DE method resolved several forms of tropomyosin: bovine aortic tropomyosin, homogeneous on SDS-PAGE, showed the presence of two isoforms with a p*I* of 5.30 for the major spot and a p*I* of 5.25 for the minor spot [122]. In human skeletal muscle, six tropomyosin variants were identified by electrophoretic and immunochemical methods: α-tropomyosin specific to fast fibers, α′-tropomyosin specific to slow fibers, β-tropomyosin common to both fast and slow fibers, and the phosphorylated form of each [15]. In human cardiac muscle, two tropomyosin isoforms were found with the same molecular mass but different p*I* values [11]. In crayfish (*Cambarus clarki*) muscle, three tropomyosin isoforms were found by 2DE, one of them specific to cardiac muscle and the other two in skeletal and visceral muscles [123].

Changes in tropomyosin during the development of chick embryonic skeletal muscles were detected by 2DE [124]. In 10-day-old embryos, tropomyosin consists of 25% α and 75% β subunits. The entire α and 60% of the β subunit is phosphorylated. During subsequent development, the amount of α-tropomyosin increases so that by hatching it represents 50% of the total subunits. During the same time, tropomyosin phosphorylation decreases, so that after hatching 20% of each of the α and β subunits remain phosphorylated. Cardiac tropomyosin from 20-day-old chick embryos is composed of three different polypeptides having the same molecular mass but different p*I* values; one polypeptide is phosphorylated [125].

3.4. Troponin

Troponin, the regulatory protein of skeletal and heart muscle, is composed of three subunits: TNC, TNI and TNT. These subunits from vertebrate muscle migrate on SDS-PAGE with relative molecular masses of 18 000, 24 000 and 37 000, respectively [126]. The troponin found in arthropod (*Limulus*) muscles is also composed of analogous subunits [127]. An important characteristic of TNC is its ability to form a complex with TNI and TNT, this complex formation was followed on urea-PAGE in the presence of Ca^{2+} [128]. Inter- and intramolecular disulfide formation in TNC was followed by SDS-PAGE [129].

In Solaro's laboratory, extensive studies were undertaken on alterations in TNI and TNT in rat heart: SDS-PAGE of myofibrils from hearts of 7-, 14-, 21-, and 28-day-old animals indicated that both euthyroid and hypothyroid rats display a developmental shift toward the adult form of TNI [130]. However, hypothyroid rats displayed a lower percentage of adult TNI at each age studied. When adult rats were made hypothyroid, the proportion of adult TNI decreased slightly. Immunoblotting analysis showed that the development of the rat heart myofibrils is associated with isoform switching from slow skeletal TNI to cardiac TNI and from a slow-mobility isoform of TNT (TNT_1) to a faster isoform (TNT_2) [30]. Alterations in protein profiles after complete global ischemia in rat hearts were also discovered [131]. Densitometric analysis of myofibrillar proteins separated by SDS-PAGE indicated that TNI and TNT were degraded during 60 min of global ischemia. Two new protein bands appearing in ischemia-treated myofibrils were identified with Western blots as partially degraded TNI and TNT.

Basic proteins, such as TNI and TNT, present a problem when subjected to conventional 2DE. Although the ampholines used are intended to yield a pH gradient from 2–11, Giometti and Anderson [13] observed that the actual gradient in a gel after focusing was only from pH 3.0 to 8.5. With a gradient of pH 3–8.5, TNI and TNT (with alkaline isoelectric points) might not enter the focusing gel, or if they do, they could appear as streaks. In the NEPHGE system of O'Farrell et al. [42] for the resolution of basic proteins, the sample is applied at the acidic instead of the basic end of the gel with the pH gradient and is electrophoresed in the first dimension for a shorter period than for IEF (1600 V h instead of 10 000 V h). Although the majority of proteins

do not reach their equilibrium positions in that shorter time, they are separated by charge. Giometti and Anderson [13] modified the NEPHGE system of O'Farrell et al. [42] by layering urea above the sample to protect the proteins from the acid and ran the electrophoresis in the first dimension for 8 h at 500 V. Under these conditions both TNI and TNT in rabbit psoas muscle were well resolved. The heterogeneity of the TNI and TNT spots was explained by the known phosphorylation of these proteins. TNC was practically lost in this modified NEPHGE system and its detection required regular 2DE [13]. Murakami and Uchida [132] supplemented the NEPHGE system with 50 m*M* each of glutamic and aspartic acid in the first dimension and ran the electrophoresis at 400 V for 2–3 h. Following with the usual electrophoresis in the second dimension, TNI, TNT and TNC of dog cardiac troponin–tropomyosin complex and myofibrils were well separated and could be quantified on the same slab gel simultaneously.

O'Brien et al. [133] analyzed the isoforms of TNT by using immobilized pH gradients, 6–10, in the first dimension and SDS-PAGE (10%) in the second dimension. Sheep latissimus dorsi muscle showed 5 fast and 2 slow TNT isoforms. After 5 months of electrical stimulation, the slow isoforms became more dominant compared to the fast isoforms.

3.5. Calponin

Calponin is an actin-, tropomyosin- and Ca^{2+}/calmodulin-binding protein which is present in high concentration in smooth muscle [43,134]. On SDS-PAGE it migrates with a relative molecular mass of 34 000 in front of tropomyosin [134]. Its interaction with actin or actin-tropomyosin in the presence and absence of Ca^{2+} was studied by centrifugation at 120 000 *g*; the pellets containing actin and any bound calponin were analyzed by SDS-PAGE [134]; the gels contained 2 m*M* $CaCl_2$ for the separation of β-tropomyosin from actin [135].

IEF of purified aorta calponin revealed several isoforms in the pH range of 8.4 to 9.1 [136]. The NEPHGE method was used to resolve the isoforms of calponin in porcine carotid arterial smooth muscles. On the staining pattern (Fig. 9, top), six calponin spots can be recognized with staining intensities (from the higher to lower pH) 5, 15, 33, 38, 7 and 2% in the resting muscle and 6, 17, 32, 38, 6 and 1% in the contracting muscle. The identity of the calponin spots was confirmed with pure calponin from porcine aorta smooth muscle [35], which was also resolved into six spots by NEPHGE under the same conditions. Similarly, six calponin spots were resolved from resting and contracting uterine, tracheal, stomach, and bladder smooth muscles. No difference was found in the staining distribution of these spots between resting and contracting muscles. Since changes in staining intensities among multiple spots of a protein separated by 2DE is an accepted criterion for protein phosphorylation [137], the data do not indicate calponin phosphorylation during smooth muscle contraction. In contrast, there are observable changes in staining intensities of P-LC spots, due to phosphorylation during contraction. The bottom part of Fig. 9 shows the radioactivity in the calponin spots; it is localized in the most acidic spots but it is very weak. Six days were required for autoradiography to reveal the calponin spots on the autoradiograms. With such a long exposure time, other spots undetectable by stain exhibit strong radioactivity. No difference is seen in calponin radioactivity between contracting and resting muscle, in contrast to the radioactivity of P-LC from contracting muscle, which is several times higher than that in resting muscle. Based on gel electrophoretic separation of several smooth muscle proteins, therefore, one can conclude that calponin phosphorylation is not involved in smooth muscle contraction. Previous studies [35,138] also suggested that calponin phosphorylation does not accompany contraction of various smooth muscles.

3.6. Caldesmon

Caldesmon is an actin-, tropomyosin-, myosin- and Ca^{2+}/calmodulin-binding protein of smooth muscle [139]. It is a large protein with many

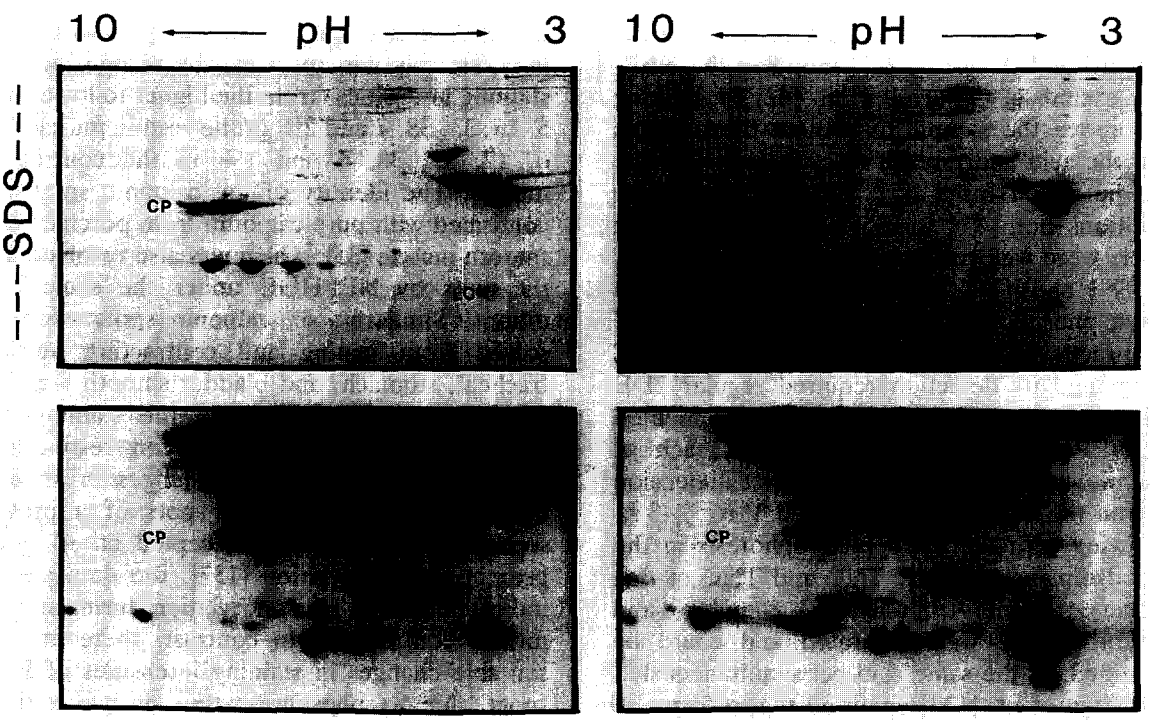


Fig. 9. Analysis by NEPHGE of arterial smooth muscle proteins. The freeze-dried samples were dissolved in a solution containing 9 *M* urea, 2% NP-40, 0.2 *M* DTT and 2% Biolyte, pH 3–10. The non-equilibrium pH gradient electrophoresis was carried out on 4% acrylamide gels containing 8.5 *M* urea, 2% NP-40 and 2% Biolyte, pH 3–10, for 2200 V h. Top: Coomassie Blue staining profiles; bottom: corresponding autoradiograms. Left: resting muscle; right: contracting muscle. Equal amounts of protein, 220 μg from either resting or contracting muscle, were applied on the gels. The specific radioactivity of [^{32}P]PCr in the muscles was the same. The dried gels were autoradiographed on Kodak X-OMAT AR film with DuPont lighting-plus intensifying screens for six days. CP = Calponin; LC = phosphorylatable myosin light chain. From Ref. [43].

isoforms, in which the relative molecular mass ranges from 87 000 to 165 000. These can be separated either by SDS-PAGE [59,140] or by 2DE [16,64]. Proteolytic digestion of caldesmon yields fragments that retain the actin- or myosin-binding abilities of the parent molecule [141,142]. Disulfide cross-linking of caldesmon to actin was localized to the C-terminus of actin [143].

The 2DE method is often used for studying the regulation of smooth muscle contraction by caldesmon through its phosphorylation [16,144] or its conformational change [59].

3.7. Desmin and vimentin

Muscle, especially smooth muscle, is rich in a three-dimensional cytoskeletal network, built of 10-nm filaments [145], the diameter of which is intermediate between the diameters of actin (thin, 6 nm) and myosin (thick, 15 nm) filaments; these are therefore called intermediate filaments. They contain a major protein, desmin (M_r 53 000) and a minor protein, vimentin (M_r 54 000). By 2DE, purified desmin shows two isoelectric variants, α-desmin (more acidic, p*I* 5.4) and β-desmin (p*I* 5.5). Recent evidence suggests that cytoplasmic intermediate filament assembly and disassembly is regulated by protein phosphorylation. In vitro experiments have shown that both cAMP-dependent protein kinase and PKC can phosphorylate vimentin [146] and desmin [147], and the resulting increase in the phosphorylation level is correlated with their disassembly. Moreover, dephosphorylation by phosphoprotein phosphatase induces reassembly

of soluble vimentin and desmin into filaments. Park and Rasmussen [148] were the first to demonstrate the phosphorylation of desmin in intact smooth muscle by treating bovine trachea with carbachol for 1 h. Both the α and β isoforms of desmin were phosphorylated. Reversible phosphorylation of desmin during a long-duration contraction–relaxation–contraction cycle of porcine carotid arteries has been demonstrated [16].

Desmin and vimentin can be separated from each other by prolonged 2DE (10% gels in the second dimension). The separation may be further improved by increasing the pH of the resolving gel buffer from 8.8 to 9.2 [149].

3.8. α-Actinin

α-Actinin is an actin-binding myofibrillar protein found in skeletal, cardiac and smooth muscle [34], including human muscle [33]. In striated muscle it is located in the Z-line; in smooth muscle, in the dense bodies and dense plaques. It is a dimer, each subunit having a relative molecular mass of 94 000-103 000; it is usually separated on SDS-PAGE, but it was also identified by 2DE of whole muscle tissues [34]. The substructure of chicken smooth muscle α-actinin molecule was deduced from chymotryptic digestion, cross-linking, and immunoblotting analyses [150]. α-Actinin is an antiparallel dimeric molecule in which two fragments with relative molecular mass of 36 000, that contain the actin-binding sites are connected by fragments with relative molecular mass of 55 000. Further characterization of the α-actinin–actin interface and comparison with filamin-binding sites on actin revealed that the two actin-binding proteins interact with closely spaced or overlapping (but not identical) sequences of the actin subdomain 1 [151]. This conclusion does not fit into results of earlier studies on the interaction of α-actinin, filamin, and tropomyosin with actin [152], which indicated that smooth muscle α-actinin and filamin can bind to actin filaments simultaneously, with little evidence of competition. In contrast, tropomyosin exhibited marked competition with either filamin or α-actinin for sites on actin filaments.

3.9. Filamin

Filamin, a major protein of various smooth muscles [153,154], is a homodimer with an estimated relative molecular mass of 500 000. The subunits in whole smooth muscle homogenates can be readily separated on SDS-PAGE (4–7%). Mild tryptic digests of human and avian filamin were studied with SDS-PAGE (12%) and 2DE [155]. The alignment of the peptides elucidated the structure–function relationship in filamin, i.e., localization of the self-association and actin-binding sites. Self-association of two monomers in a tail-to-tail orientation involves a small protease-sensitive region near the carboxyl terminal of the intact polypeptide chain. The actin-binding site is located near the blocked amino terminal of the filamin molecule. Filamin can be phosphorylated in intact mammalian smooth muscle, as detected by SDS-PAGE [154]. The same procedure was used to demonstrate that inositol phospholipids bound to filamin inhibited its ability to combine with actin [156].

3.10. Giant proteins

Giant proteins [55], having relative molecular masses over 500 000 are found in various muscle tissues. The relative molecular mass of titin, originally called connectin [157], ranges from 2 100 000 to 3 500 000; that of nebulin, another protein, varies from 700 000 to 900 000. Gel electrophoresis and immunoblots have been used extensively to determine the size, quantity, integrity, and immunoreactivity of these giant proteins in normal, pathological, and developing muscle tissues.

Gel electrophoresis of the giant proteins is carried out under a variety of conditions: SDS-PAGE (1.8%) in the presence of 6 *M* urea [158]; or SDS-PAGE, with a linear gradient of 3–10% [159], 2–12% [160], or 3.3–12% [55]. Usually, the gels are stained with Coomassie Blue, but silver staining has also been applied recently [55]. Titin tends to aggregate during electrophoresis, especially in gel systems that utilize stacking gels or discontinuous buffers to concentrate and sharpen protein bands. Granzier and Wang [55] avoid such aggregation by the use of a

continuous buffering system and a gradient gel with a low polyacrylamide concentration at the sample well. This allows titin to enter the gel without concentration-induced aggregation near the gel interface. Their gels resolve protein bands with relative molecular mass from 140 000 to 280 000 [55].

Mild proteolysis of titin with trypsin [161] or calpain [162] converts it to a protein with a relative molecular mass of 2 000 000 (titin 2 or β-connectin) that readily separates on the gels from titin (titin 1 or α-connectin). A monoclonal antibody against chicken breast muscle β-connectin reacted with the α-connectin isolated from rabbit back muscle [158]. Prolonged proteolysis of titin with trypsin yielded fragments which were separated by SDS-PAGE (12%) and transferred to nitrocellulose membranes [163]; then the C-terminal residues of the nitrocellulose-bound polypeptides were removed by carboxypeptidase Y. The results of using two distinct monoclonal anti-titin antibodies suggested that the C-terminal end of titin is located near the Z-line region and the N-terminal end is at the M-line region in the sarcomere.

Isaacs et al. [164] have used 3.2% acrylamide/1% agarose gels for studying the association of titin and HC in developing chicken leg muscle. Pulse labeling, immunoprecipitation, and cross-linking experiments indicated that within minutes of protein synthesis titin and HC could be cross-linked into very large complexes retaining many features of intact myotubes. The data suggested that the synthesis and assembly of titin and myosin are temporally and spatially coordinated in nascent myofibrils, supporting the hypothesis that titin molecules are involved in sarcomere formation.

Analyses by SDS-PAGE and Western blot of nebulin from rabbit, chicken, and beef skeletal muscle revealed that the size of the nebulin molecule varies within the skeletal muscle domain [165]. The mobilities of the nebulin bands were proportional to the length of the thin filaments in the muscles, supporting the idea that nebulin regulates thin filament assembly. In agreement with this hypothesis, SDS-PAGE studies showed that nebulin and its subfragments were bound to actin [160,166].

4. Muscle

Two-dimensional gel electrophoresis is the general method for following changes in protein phosphorylation during smooth muscle activity. Fig. 10 illustrates the separation of four major ^{32}P-labeled phosphoproteins of arterial muscle by 2DE: P-LC, 28K, desmin and caldesmon [16]. Changes in the [^{32}P]phosphate content of these proteins were measured during a short or long contraction–relaxation–contraction cycle of the muscles. It was concluded that under physiological conditions, P-LC phosphorylation initiates both short and sustained arterial contraction; desmin phosphorylation is likely to be involved in force maintenance during sustained contraction.

Fig. 11 compares the 2DE gel pattern of P-LC in resting, stretched, and K^+-stimulated arterial muscle [20]. Major changes are seen in staining intensity of spot 4, which decreases (relative to the resting state) upon stretching, stimulation, or both stretching and stimulation, and of spot 3, which increases simultaneously under these conditions. The autoradiograms show the corresponding changes. Furthermore, upon stretching, changes in densitometry and radioactivity of spot 2 can be detected. Fig. 12 illustrates that the stretch-induced P-LC phosphorylation is prevented by removal of Ca^{2+} from the muscle by EGTA.

A wealth of literature exists for the use of 2DE for the measurement of P-LC phosphorylation during muscle contraction. A few examples follow. Phosphorylation was shown to be associated with K^+-induced contraction of arterial muscle but declined before steady-state force was attained [22]. G-Protein-mediated Ca^{2+} sensitization during contraction of tonic (pulmonary and femoral artery) and phasic (portal vein and ileum) smooth muscles was shown through P-LC phosphorylation [51]. P-LC phosphorylation was completely dissociated from contraction when tracheal muscles were stimulated with carbachol in Ca^{2+}-free physiological salt solution and contracted by readmission of $CaCl_2$ [167]. Dissociation of relaxation and P-LC dephosphorylation in uterine muscle was also observed [168]. Cooperative activation of myosin by P-LC phos-

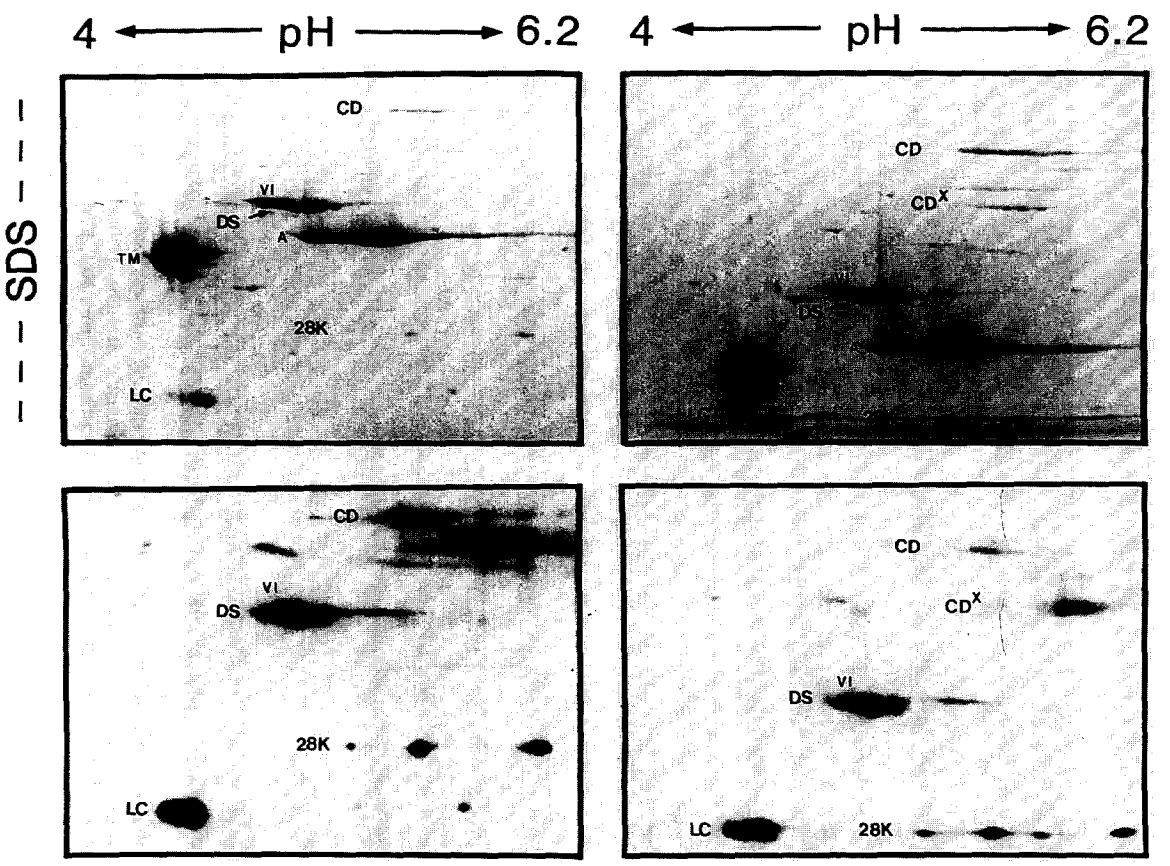


Fig. 10. Analysis by 2DE of arterial muscle proteins. In the second dimension: left: 15% gels; right: 10% gels. LC = Phosphorylatable myosin light chain; 28K = unidentified protein (M_r 28 000); TM = tropomyosin; A = actin; DS = desmin; VI = vimentin; CD = caldesmon, 140 000; CD^x = caldesmon, 92 000. From Ref. [16].

phorylation in permeabilized smooth muscle was reported [169]. The phosphorylation of P-LC during contraction of chicken fast and slow skeletal muscles was related to the rate of tension development [170]. P-LC phosphorylation in beating turtle heart has been demonstrated [171].

Methods other than 2DE also have been applied to correlate P-LC phosphorylation with muscle function. Moore and Stull [24] used the combination of pyrophosphate-PAGE isolation of myosin followed by IEF to measure P-LC phosphorylation and dephosphorylation in fast and slow rat skeletal muscles in situ. Phosphorylation depended on the frequency of muscle stimulation; higher frequency was required for the soleus than for the white portion of the gastrocnemius. The rate of dephosphorylation was four times faster in the slow soleus muscle than in the fast gastrocnemius. Adam et al. [172] determined P-LC phosphorylation by glycerol–urea-PAGE followed by radioimmunoblotting in arterial muscle stimulated with endothelin-1. Contraction was associated with phosphorylation.

5. Biomedical applications

The 2DE pattern of desmin was analyzed in a familial skeletal muscle disorder, characterized by the intra-sarcoplasmic accumulation of an electron-dense granulo-filamentous material that strongly reacted with polyclonal anti-desmin

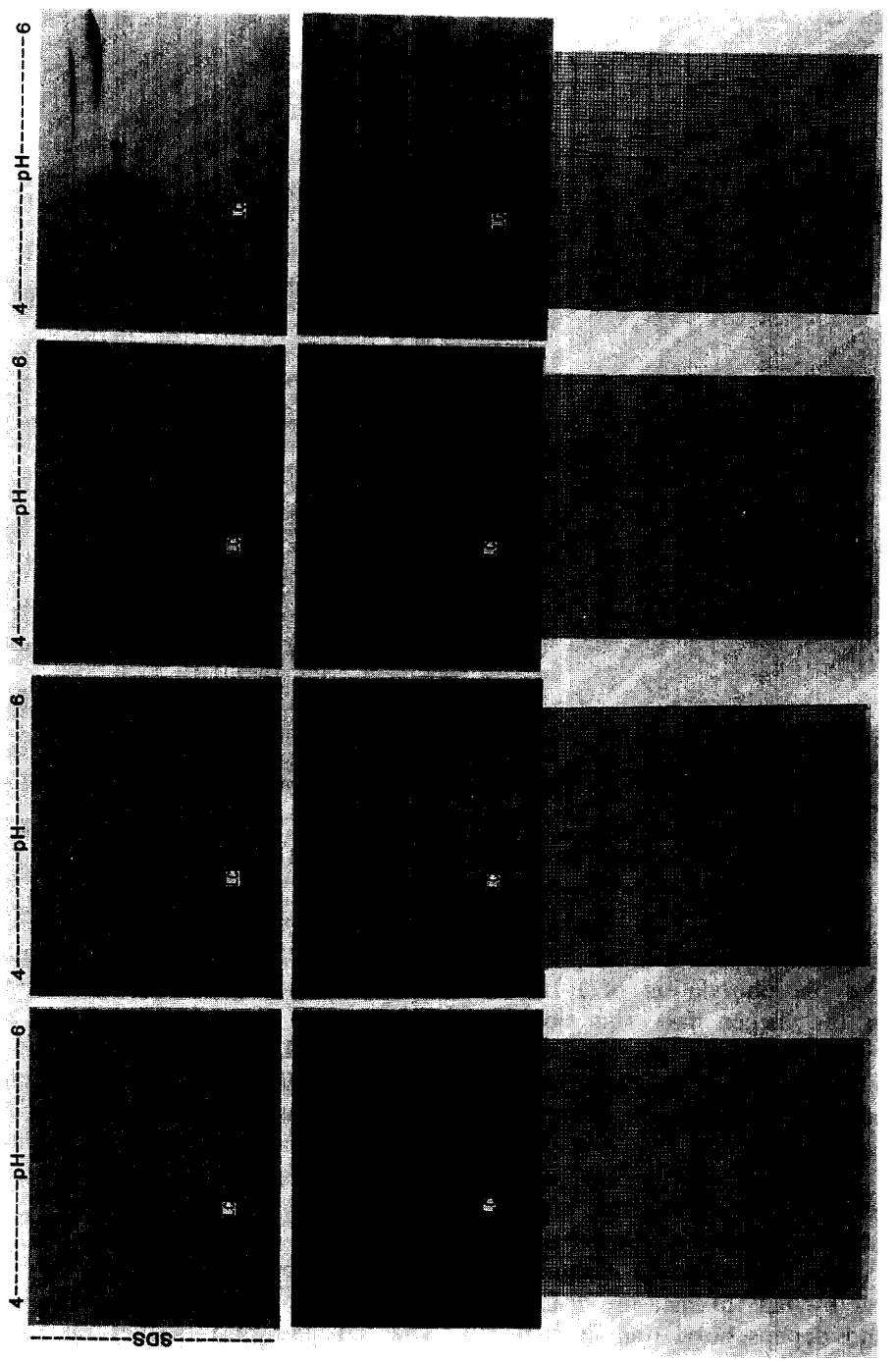


Fig. 11. Analysis by 2DE of the stimulation of P-LC phosphorylation in porcine carotid arteries. Top: staining profiles; middle: corresponding autoradiograms; bottom: densitometric tracings of P-LC. First frame: muscle at rest; second frame: muscle stretched; third frame: muscle K^+-stimulated; fourth frame: muscle stretched and then K^+-stimulated. LC = Phosphorylatable myosin light chain. From Ref. [20].

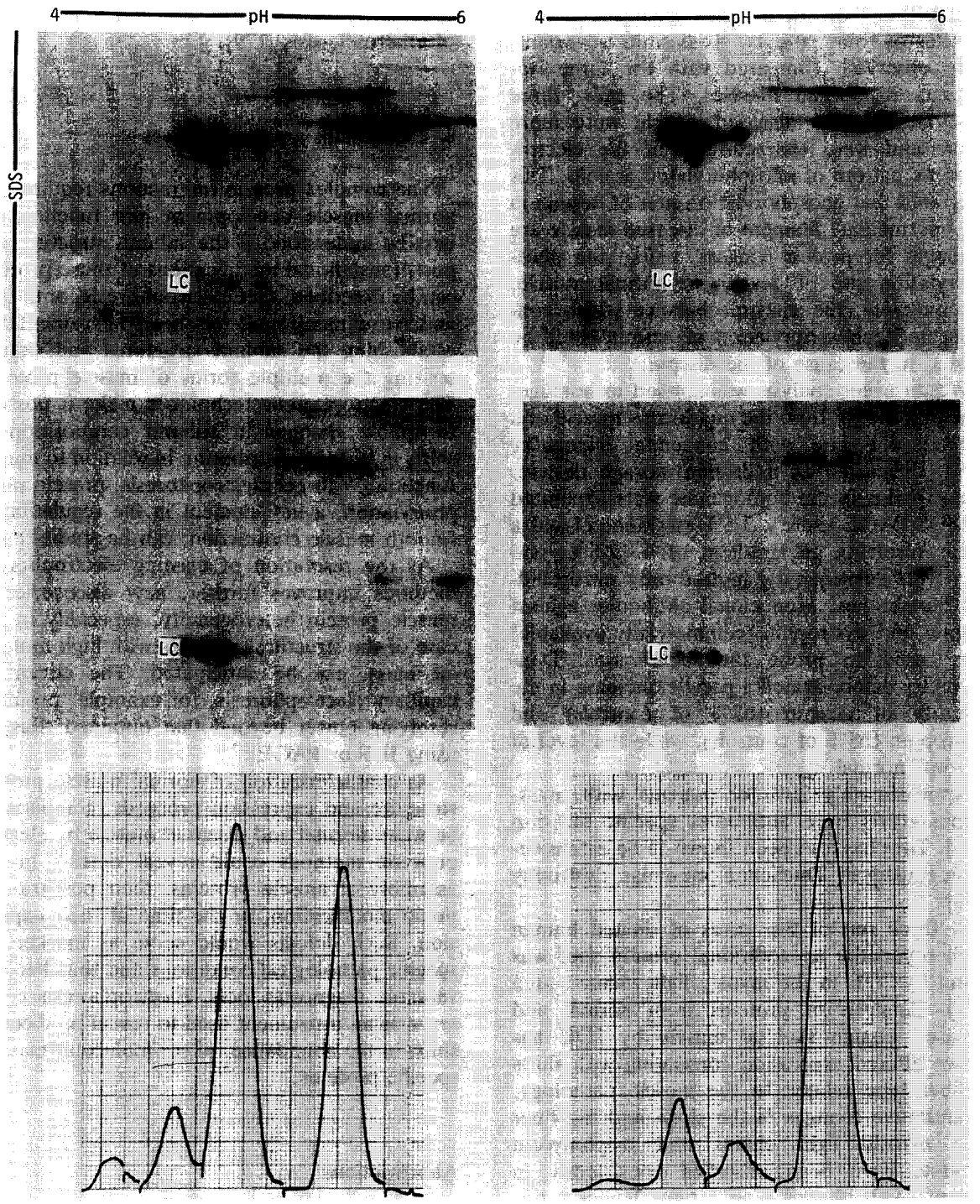


Fig. 12. Staining, radioactivity, and densitometry of P-LC, separated by 2DE, from stretched arterial muscles in the absence (left) and in the presence of EGTA (right). From Ref. [93].

antibodies [173]. In biopsies from the deltoid muscles of four patients, six desmin isovariants were observed, compared with the three isovariants in normal muscles. The extra three isovariants in the diseased muscle were more acidic and were characteristic of the electrophoretic pattern of phosphorylated desmin. This suggested that phosphorylation sites of desmin in the intermediate filament of diseased muscle are available for protein kinases, unlike the phosphorylation site of desmin in normal muscle. Alternatively, the enzymic balance which controls the dephosphorylation of desmin could be related to the cause of the disease.

Western blot analysis was applied to homogenate of a biopsy from the quadriceps muscle of a five-year-old boy with congenital myopathy [174], diagnosed as a desmin storage disease. The proteins in the homogenate were separated on SDS-PAGE using a 3.5% stacking gel and a linear resolving gel gradient of 4–12.5%, and they were subsequently blotted onto nitrocellulose membranes. Monoclonal antibodies against desmin and dystrophin (commercially available) were used to probe the membranes. Densitometry demonstrated a parallel increase in the quantity of desmin (63% of controls) and dystrophin (68% of controls), while the level of HC was normal.

Approximately 20% of patients with myasthenia gravis have antibodies specific for titin [175]. Titin has also been shown to be extensively degraded in Duchenne muscular dystrophy [176].

In three out of five cases of dilated human cardiomyopathy an additional protein spot was found by 2DE in the myosin light chain 1 area [177]. Analysis of proteins from normal and diseased human skeletal muscle by 2DE has shown altered expression correlating with shifts in fiber type related to the muscle pathology. Quantitative changes in the slow- and fast-fiber type myosin light chains and tropomyosin subunits have been observed consistently in patients with altered type 1/type 2 fiber ratios [15,178]. Human smooth muscle myosin heavy chain isoforms are important molecular markers for studying human vascular smooth muscle cell differentiation as well as the cellular mechanism of atherosclerosis [179].

6. Conclusions and perspectives

The complex protein interactions required for normal muscle development and function can best be understood if the subunit structure and post-translational modifications of those proteins can be described. Gel electrophoresis, both one- and two-dimensional, has been instrumental in establishing the subunit structures and characterizing the multiple forms of muscle proteins. The electrophoretic techniques make it possible to follow changes in subunit composition or shifts in isoform distribution in relation to muscle function. With gel electrophoresis, protein phosphorylation, a key element in the regulation of smooth muscle contraction, can be studied.

As the resolution of existing electrophoretic methods improves further, new discoveries of muscle protein heterogeneity, especially in the case of the structural proteins with high molecular mass, can be anticipated. The advent of capillary electrophoresis, for example, promises resolving power beyond that obtained thus far using IEF or PAGE.

As characterization of normal muscle protein structure and expression proceeds, comparisons between normal and diseased muscle by electrophoretic methods could reveal defects in the assembly of muscle proteins, their post-translational modification, or the time of their expression. Such defects would serve as markers of specific pathological conditions and thus become valuable diagnostic tools. Electrophoresis could serve as an instrument used to reveal predictable shifts in the abundance, p*I*, or molecular mass of specific proteins.

Abbreviations

2DE	Two-dimensional electrophoresis, comprising isoelectric focusing in the first dimension and sodium

	dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension
DTT	Dithiothreitol
EGTA	Ethylenebis(oxyethylenenitrilo)tetraacetic acid
IEF	Isoelectric focusing
HC	Myosin heavy chain
LC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NEPHGE	Two-dimensional electrophoresis, comprising non-equilibrium pH gradient electrophoresis in the first dimension and sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the second dimension
PAGE	One-dimensional polyacrylamide gel electrophoresis
PCr	Phosphocreatine
PCA	Perchloric acid
PDBu	Phorboldibutyrate
p*I*	Isoelectric point
P_i	Inorganic phosphate
PKC	Protein kinase C
P-LC	Phosphorylatable myosin light chain
S-1	Myosin subfragment 1
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TNC	Calcium-binding subunit of troponin
TNI	Inhibitory subunit of troponin
TNT	Tropomyosin-binding subunit of troponin
28K	Unidentified muscle protein, M_r 28 000

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