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Muscle Protein Analysis. I. High-Resolution Two-Dimensional Electrophoresis of Skeletal Muscle Proteins for Analysis of Small Biopsy Samples

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We have been developing a clinically useful method for high-resolution two-dimensional electrophoretic analysis of small (5–10 mg) human muscle biopsy samples with sufficient resolution to resolve the major contractile proteins and enzymes. Using rabbit psoas muscle as a model, we describe methods for sample preparation and two-dimensional electrophoresis. Basic proteins, which appear as streaks when conventional isoelectric focusing is used in the first dimension, are resolved through a modification of the nonequilibrium pH gradient electrophoresis method [*Cell* **12,** 1133 (1977)]. In the two-dimensional patterns obtained from rabbit muscle, we identify the components of 10 enzymes and of myosin, actin, tropomyosin, and troponin. These patterns indicate charge heterogeneity in a large fraction of the proteins. Comparison of rabbit and normal human muscle patterns shows many similarities, but much additional work is required to confirm identifications. We conclude that analysis of small biopsy samples is feasible, but that all aspects of human sample acquisition, storage (when necessary), and preparation require thorough study before the method becomes routine in human muscle research and, ultimately, in the diagnosis of some muscle diseases.

Additional Keyphrases: *muscle enzymes · molecular biology · human and rabbit muscle-protein patterns compared · electrophoretic "mapping" of muscle proteins and protein subunits*

Molecular biology provides increasing insight into the molecular basis of disease. To translate this work into clinical usefulness requires the analysis of small, fresh tissue samples for a large number of constituents in ways that allow subtle changes in molecular composition or amounts to be detected. There has been great progress in the analysis of constituents of low and intermediate molecular weight, but such constituents, although they may be indirectly altered in disease, have rarely been found to be the primary cause of specific diseases. Screening for alterations in the thousands of protein gene products or for sequence changes in nucleic acids, when searching for the primary cause of many diseases, presents a more difficult challenge, especially when only very small samples are available for analysis.

In this series of papers, we are concerned with the development of methods for analyzing small biopsy samples (5–10 mg) for the major enzymes and contractile proteins of muscle. The initial studies, done with rabbit muscle and to a lesser extent normal human muscle, lay the groundwork for subsequent studies on genetic polymorphisms in normal muscle proteins, on genetic variants causally related to muscle diseases, and on the molecular differences between different muscle types. These studies are part of a program to systematically index human protein gene products by using high-resolution two-dimensional electrophoresis (*1–7*) for the mapping of known proteins or protein subunits. The positions of major human plasma proteins (*8*) and human erythrocyte lysate proteins (*9*) have been previously presented.

Some of the major contractile proteins have been previously identified in maps of muscle cells derived from tissue culture (*10, 11*), but little work has been done with fresh tissue. A major problem in the two-dimensional electrophoretic mapping of muscle proteins has been the poor resolution or "streaking" of basic proteins. We have solved this problem by modifying the nonequilibrium pH gradient electrophoresis (NEPHGE)[1] technique described by O'Farrell and coworkers (*1*) and have clearly resolved the two basic subunits of troponin (troponin T and I) and enzymes with basic isoelectric points. The positions of the components of 10 enzymes and of myosin, actin, tropomyosin, and troponin are presented. The individual samples analyzed contain less than 1 mg of protein; hence, the methods are applicable to very small needle-biopsy samples.

Materials and Methods

Purified Proteins

Rabbit skeletal muscle enzymes were purchased from Worthington Biochemicals, Freehold, NJ 07728 (aldolase, EC 4.1.2.13; and phosphorylase *a*, EC 2.4.1.1) or Sigma Chemical Co., St. Louis, MO 63178 (pyruvate kinase, EC 2.7.1.40; creatine kinase, EC 2.7.3.2; enolase, EC 4.2.1.11; glyceraldehyde-3-phosphate dehydrogenase, EC 1.2.1.12; phosphoglucomutase, EC 2.7.5.1; α-glycerophosphate dehydrogenase, EC 1.1.1.8; triosephosphate isomerase, EC 5.3.1.1; and myokinase, EC 2.7.4.3). Rabbit skeletal muscle actin was obtained from Worthington Biochemicals. Purified rabbit muscle myosin and frog muscle tropomyosin were gifts of Dr. Michael Barany of the University of Illinois Medical Center, Chicago, IL; Dr. Sarah Hitchcock, Carnegie-Mellon University, Pittsburgh, PA, kindly provided purified rabbit muscle troponin.

Tissue Preparation

Prepare a homogenate of fresh rabbit psoas muscle (0.5–1 g) by homogenizing minced tissue in 10 volumes of pyro-

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[1] Nonstandard abbreviations used: NEPHGE, nonequilibrium pH gradient electrophoresis; NP 40, Nonidet P40; CHES, cyclohexylaminoethane sulfonic acid; SDS, sodium dodecyl sulfate; TnI, TnT, TnC, troponin subunits.

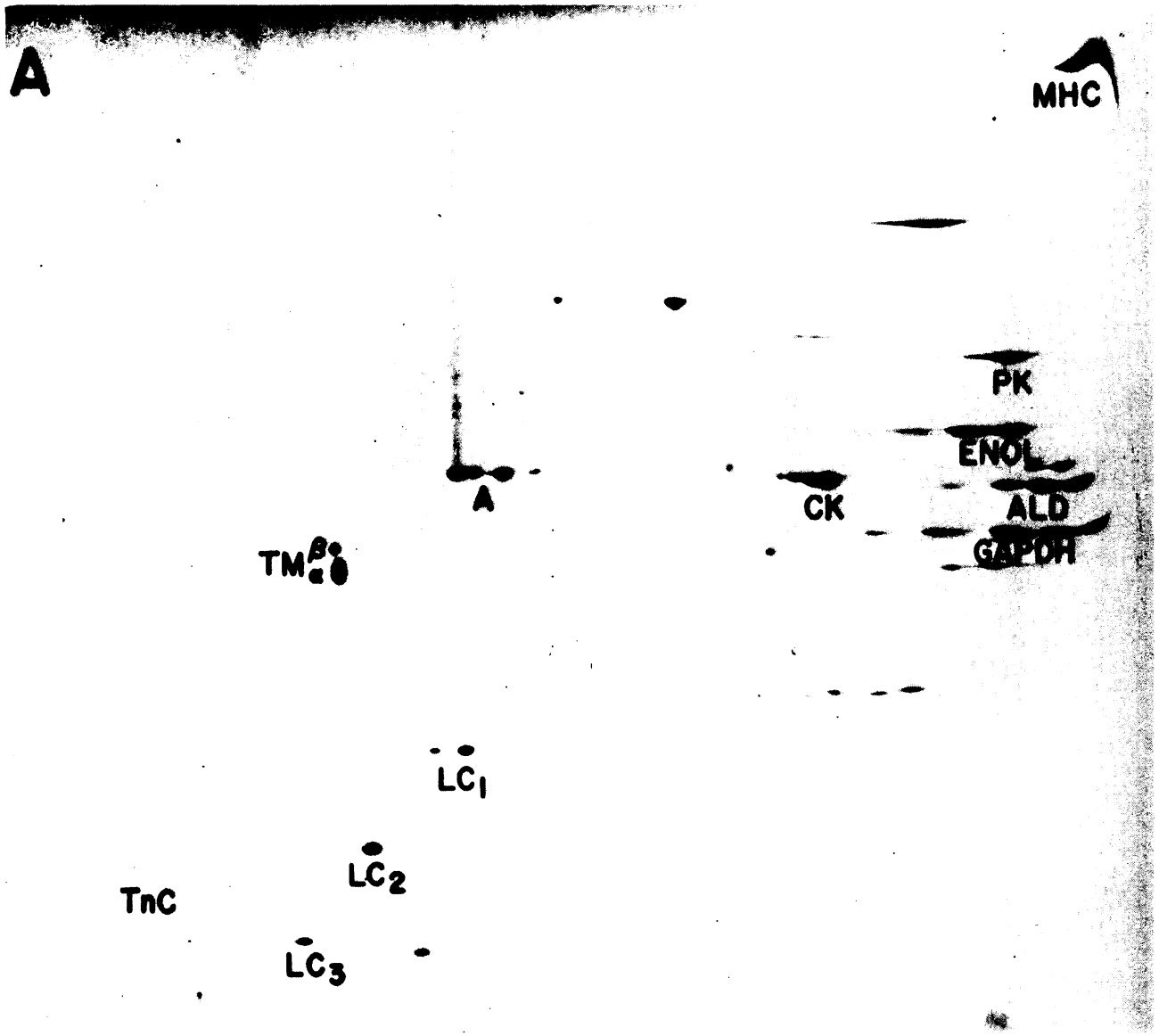


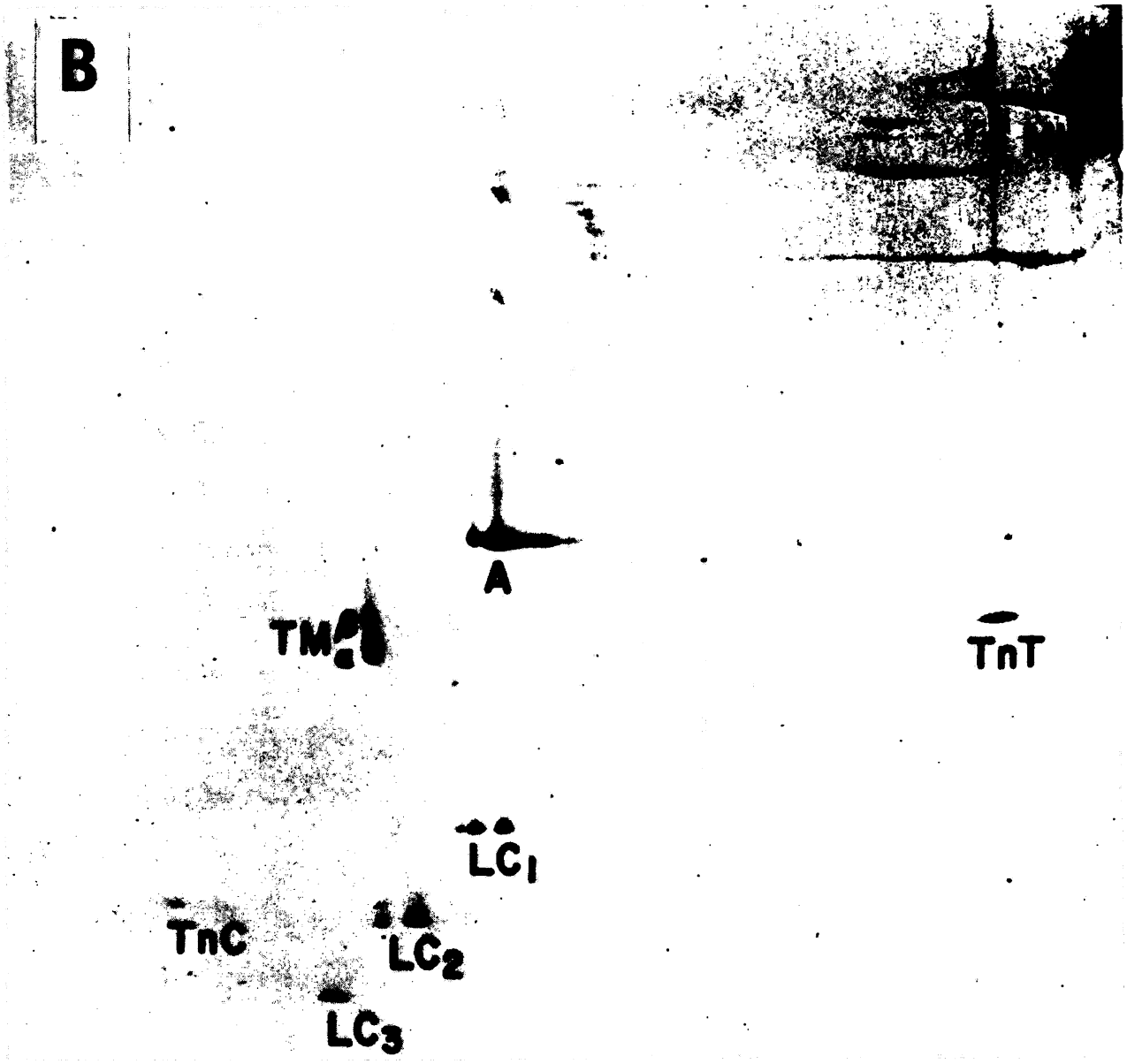
Fig. 1. Two-dimensional patterns of rabbit psoas muscle whole homogenate and myofibrils, with isoelectric focusing in the first dimension

Electrophoresis was as described in *Materials and Methods*. The gels are oriented with the acidic side to the left and the basic side to the right. (*A*) Muscle whole homogenate, 200 μg of protein; (*B*) myofibrils, 15 μg of protein. A, actin; αTM, βTM, tropomyosin; MHC, myosin heavy chain; LC_1, LC_2, LC_3, myosin light chains; TnC, calcium-binding troponin subunit; TnT, tropomyosin-binding troponin subunit; PK, pyruvate kinase; CK, creatine kinase; ENOL, enolase; ALD, aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Bottom inch of Figures (blank) cropped for page space considerations

phosphate relaxing buffer (*12*) in a Waring Blendor (Model 700A) for 30 s. Filter the homogenate through two layers of cheesecloth to remove connective tissue. Prepare myofibrils by centrifuging homogenates 15 min at 600 × *g* and washing the myofibril pellet four times in the original sample volume of relaxing buffer without pyrophosphate but with Triton X-100 (0.2 g/L) (Eastman Kodak Co., Rochester, NY 14650). Add doubly-distilled water to the final pellet to make up to the original volume.

To prepare the whole homogenate and myofibril samples for isoelectric focusing, mix each sample with one volume of 0.05 mol/L cyclohexylaminoethane sulfonic acid (CHES; Calbiochem-Behring Corp., La Jolla, CA 92112), pH 9.5 containing, per liter, 20 g of sodium dodecyl sulfate (SDS; Accurate Chemical and Scientific Corp., Hicksville, NY 11801), 10 g of dithiothreitol, and 100 mL of glycerol (this solution is henceforth referred to as SDS-CHES mix). We prepared very small (5–10 mg) rabbit psoas muscle samples by first pounding frozen tissue into a powder, using a stainless-steel mortar and pestle kept chilled on solid CO_2. The powder was then weighed and dissolved in 10 volumes of SDS-CHES mix. We prepared normal human muscle biopsy samples (kindly provided by Dr. Paul O'Brien, Medical University of South Carolina) in a similar fashion. The combination of SDS and dithiothreitol in this mix solubilizes proteins and dissociates them into subunits. The alkaline pK of CHES (9.5) provides optimum buffering action at pH 9.5 and protects the sample proteins from exposure to the high pH of the NaOH used in the upper reservoir for isoelectric focusing. To enhance solubility in the SDS-CHES mix, we heated the sample for 5 min in a 95 °C heating block after adding the mix.

We mixed muscle preparations or purified proteins to be analyzed with the modified NEPHGE (described below) for first-dimension separation with one volume of a solution containing, per liter, 20 g of Nonidet P40 (NP 40 Particle Data Laboratory, Ltd., Elmhurst, IL 60126), 10 g of β-mercaptoethanol, and 8 mol of urea (NP 40-urea mix). Very small biopsy samples (5–10 mg) were homogenized and dissolved



directly in the NP 40–urea mix. These samples were not heated.

Protein Determination

The protein concentration of muscle whole homogenates and myofibril preparations was determined by the Coomassie Blue method (*13*), with γ-globulin as the standard.

Two-Dimensional Electrophoresis

We used the Iso-Dalt system (*3, 4*) for two-dimensional electrophoresis. For analyses in which isoelectric focusing was used for the first-dimension separation, samples in SDS-CHES mix were applied to the top of isoelectric focusing gels (so-called Iso gels) containing pH 2–11 ampholines (Brinkman Instruments, Westbury, NY 11590) (*3*). The second-dimension separation was done on slab gels with linear polyacrylamide gradients (100–200 g/L), as described elsewhere (*4*). For high resolution of basic proteins in the first dimension, we used a modification of the NEPHGE described by O'Farrell and coworkers (*1*). Gels were cast in the Iso apparatus (*3*) with pH 2–11 ampholines. The lower reservoir was filled with NaOH (20 mmol/L) and the upper reservoir with H_3PO_4 (10 mmol/L). Samples dissolved in NP 40-urea mix were applied to the top of the gels with 25 μL of 1 mol/L urea layered above the sample to protect the proteins from the acid. These so-called Baso gels (by analogy with the Iso gels) were run for 8 h at 500 V. After removal from the tubes, the gels were equilibrated with SDS as usual (*3*), and layered on the SDS slab gels for the second-dimension (Dalt) separation. Less than 1 mg of protein was used per gel in each analysis.

Gels were fixed, stained, and photographed as described (*4, 8*).

Results

Rabbit Muscle Protein Analysis by Two-Dimensional Electrophoresis

Figure 1 shows the patterns obtained when rabbit psoas muscle whole homogenate and myofibrils were analyzed with two-dimensional electrophoresis in which isoelectric focusing was the first dimension. We identified the protein subunits in these patterns by electrophoresis of purified muscle proteins alone and in combination with muscle whole homogenate or myofibrils (for further discussion of this approach to spot identification, see ref. *8*). The whole-homogenate pattern is similar, but not identical, to that observed by others working with cultured muscle fibers (*10, 11*). The broad-range ampholines used here (pH 2–11) permit inclusion of many basic

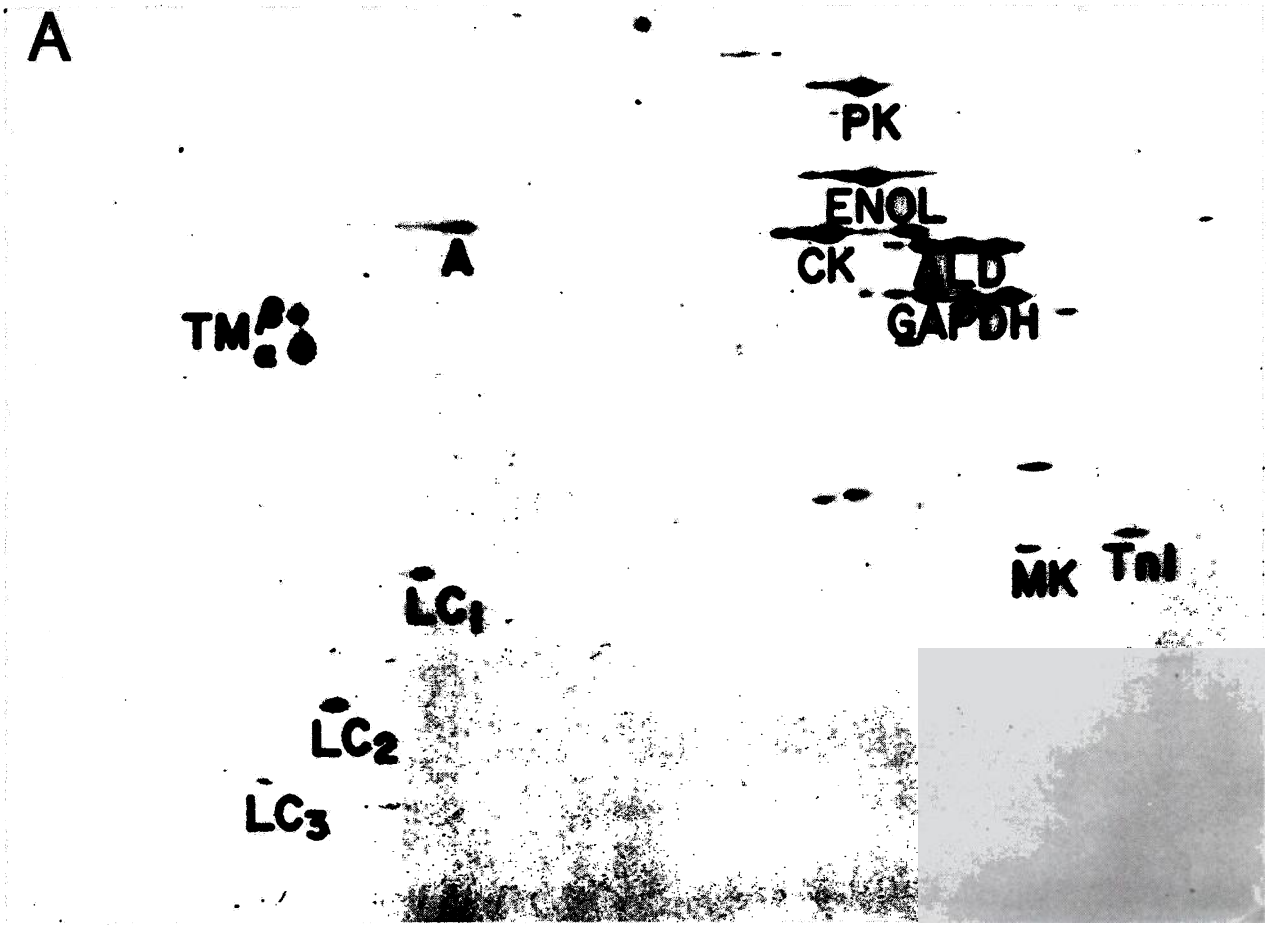


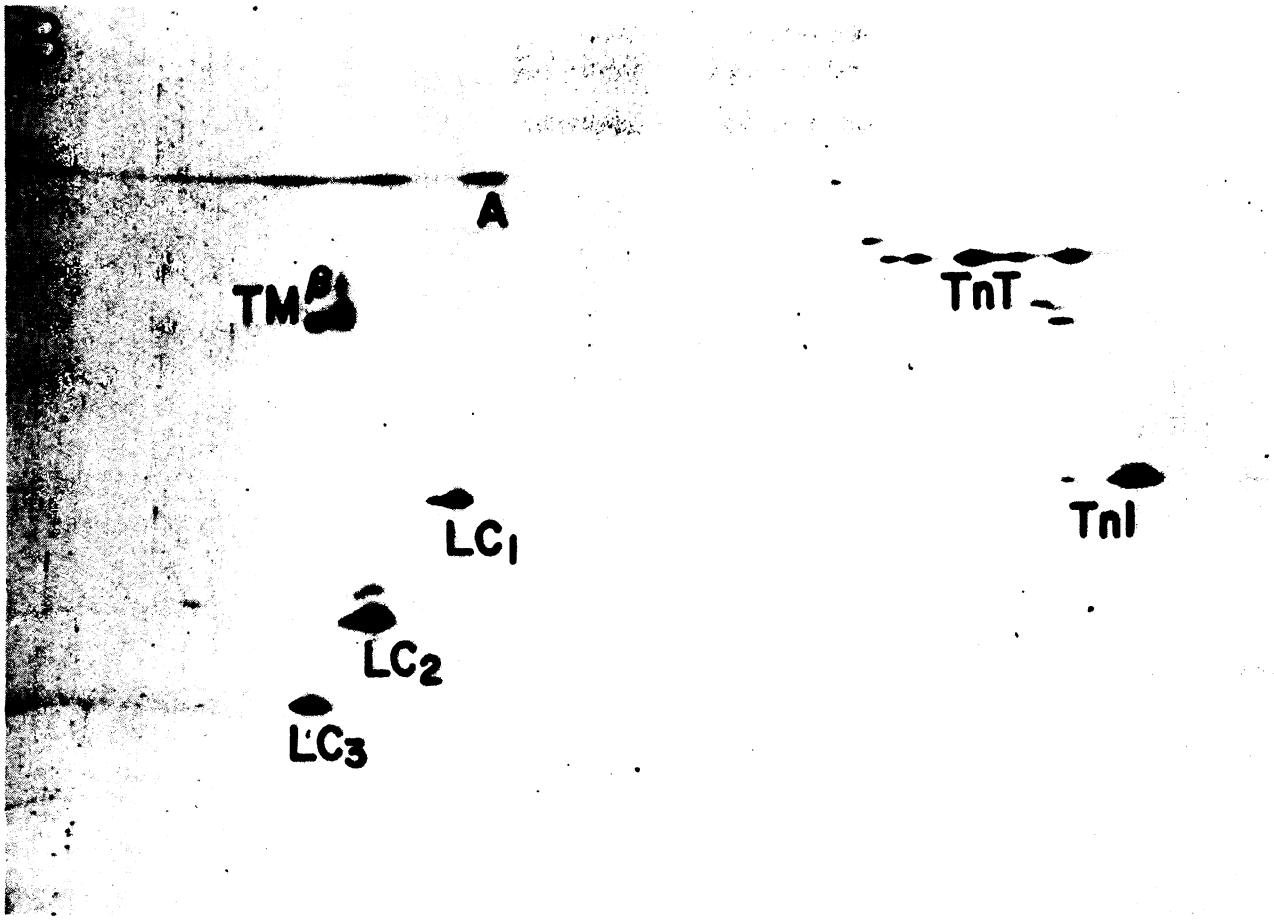
Fig. 2. Two-dimensional patterns of rabbit psoas muscle whole homogenate and myofibrils, with nonequilibrium pH gradient electrophoresis in the first dimension

Samples were prepared and separated as described in *Materials and Methods*. After electrophoresis for 4000 V-h, proteins were separated in the second dimension as previously described (*4*). Gels oriented as in Fig. 1. (*A*), muscle whole homogenate, 200 μg of protein; (*B*), myofibrils, 50 μg protein. Abbreviations as in Fig. 1, plus TnI, inhibitory troponin subunit; MK, myokinase. Top third of Figures (blank) cropped

proteins previously not seen (*11*). The myofibril pattern (Figure 1*B*) is less complex than that of the whole homogenate (Figure 1*A*), and clearly shows components of the four major muscle proteins—myosin, actin, tropomyosin, and troponin. The "stutter" pattern of spots in the center of the upper half of Figure 1*B* has also been observed in patterns from rat and rabbit heart and from rat, human, and chicken skeletal muscle (*7, 14*). This pattern demonstrates the extremely good resolution of this system: a large number of distinctive spots of differing charge and molecular weight are visible in a very limited area of the pattern. None of the proteins studied thus far gives any clue to the identity of the peptide group giving the stutter pattern, though its molecular weight heterogeneity is reminiscent of invertebrate paramyosin (*7*).

Streaking of proteins at the basic side of the two-dimensional electrophoresis patterns has been attributed to a collapse of the pH gradient in the alkaline end of the first-dimension isoelectric focusing gels (*1, 2, 15*). Rabbit muscle enzymes having isoelectric points reported to range from 7.8 (pyruvate kinase) to 8.6 (aldolase) (*16*) appear as streaks as shown in Figure 1. Although the ampholines used are presumably intended to yield a pH gradient from 2–11, our observations show that the actual gradient in a gel after focusing is from pH 3.0 to 8.5 (unpublished work). With an actual gradient of pH 3–8.5, proteins 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. (*1*) for the resolution of basic proteins, the sample is applied at the acidic rather than the basic end of the gel and submitted to electrophoresis for a shorter period than that used for isoelectric focusing (1600 V-h instead of 10 000 V-h). Although the majority of proteins do not reach their equilibrium positions in the short time used for electrophoresis, they are separated by charge.

Figure 2 shows the two-dimensional patterns obtained when rabbit psoas whole homogenate and myofibrils were analyzed with a modification of the NEPHGE technique for the first-dimension separation. Electrophoresis was carried out for a minimum of 4000 V-h to obtain the protein separation shown. We obtained identical results whether the samples were solubilized in the NP 40–urea mix described here or in the lysis buffer (which contains SDS and ampholines in addition to NP 40, urea, and β-mercaptoethanol) described by O'Farrell and coworkers (*1*). Comparison of Figure 2 (*A* and *B*) with Figure 1 (*A* and *B*) shows that the patterns with the modified NEPHGE for first-dimension separation are very similar to those with isoelectric focusing for the first dimension. Although some resolution of acidic proteins is lost, the resolution of the basic proteins (e.g., aldolase, troponin T, troponin I), is much improved when the NEPHGE technique is used. Proteins such as myokinase and the inhibitory subunit of troponin (TnI), which were completely excluded from the two-dimensional patterns in Figure 1, are resolved in the patterns in Figure 2.



Muscle Protein Identification

Figure 3 is a diagrammatic composite of the two-dimensional patterns of rabbit psoas muscle whole homogenate shown in Figures 1*A* and 2*A*. The protein identifications made thus far are indicated.

Myosin. The myosin heavy chains appear as a dark streak at the top of the two-dimensional gel patterns. The failure of these peptides to penetrate the polyacrylamide isoelectric focusing and NEPHGE gels may be related to their large size (M_r 200 000) and rod-like structure (*17, 18*). The myosin light chains display microheterogeneity in the isoelectric focusing dimension, indicating the presence of isoelectric variants. Similar microheterogeneity has also been observed in the myosin light chains of canine cardiac myosin (*19*). The different forms of light chain 2 may reflect the presence of phosphorylated and non-phosphorylated peptides (*20*).

Tropomyosin. The α- and β-subunits of tropomyosin are well resolved in the two-dimensional pattern. The presence of more α- than β-form is consistent with other reports on skeletal muscle tropomyosin (*21, 22*).

Troponin. Detection of the three components of troponin, TnC (calcium-binding subunit), TnT (tropomyosin-binding subunit), and TnI (inhibitory subunit), requires the use of both the isoelectric focusing and NEPHGE techniques for first-dimension separation. TnC, the most acidic of the three components, is only resolved when isoelectric focusing is used for first-dimension separation. TnT and TnI, on the other hand, are best resolved when the NEPHGE is used. Both TnT and TnI display charge heterogenity. Because these proteins are known to exist in vivo in phosphorylated and nonphosphorylated forms (*23*), the charge differences seen may reflect various degrees of phosphorylation.

Actin. Actin appears as a smear in the two-dimensional gel patterns, the result of the overloading required if other, more minor, components are to be seen. When lower concentrations of whole homogenate or myofibrils than those used for the patterns shown in this paper are analyzed, one major and one more acidic minor spot are seen that co-migrate with rabbit muscle actin (unpublished work). The charge heterogeneity observed requires further study before an explanation can be given. Other investigators report only one form of actin in adult rat skeletal muscle (*24*).

Enzymes. We can identify 10 enzymes in the rabbit psoas muscle whole homogenate two-dimensional pattern. Charge heterogeneity is clearly seen in all but two of these enzymes. One of the exceptions, myokinase, appears as just one spot in the pattern when the NEPHGE technique is used (the absence of myokinase from the pattern when isoelectric focusing is used is due to the failure of this very basic protein to enter the focusing gel). The second exception, phosphorylase *a*, appears as a streak in the patterns, thus masking any possible charge heterogeneity. Microheterogeneity of rabbit muscle aldolase has been observed when isoelectric focusing was done in gels containing urea (*25*). The heterogeneity displayed by the other seven enzymes in this system may reflect similar variants that have gone unrecognized previously.

Biopsy Sample Preparation and Analysis

The preparation of very small muscle-biopsy samples for analysis by two-dimensional electrophoresis must be approached differently from the preparation of 1 g quantities of muscle. To obtain the patterns shown thus far in this paper, we analyzed samples with a protein concentration of 5–10 g/L. To obtain a comparable protein concentration from pieces of

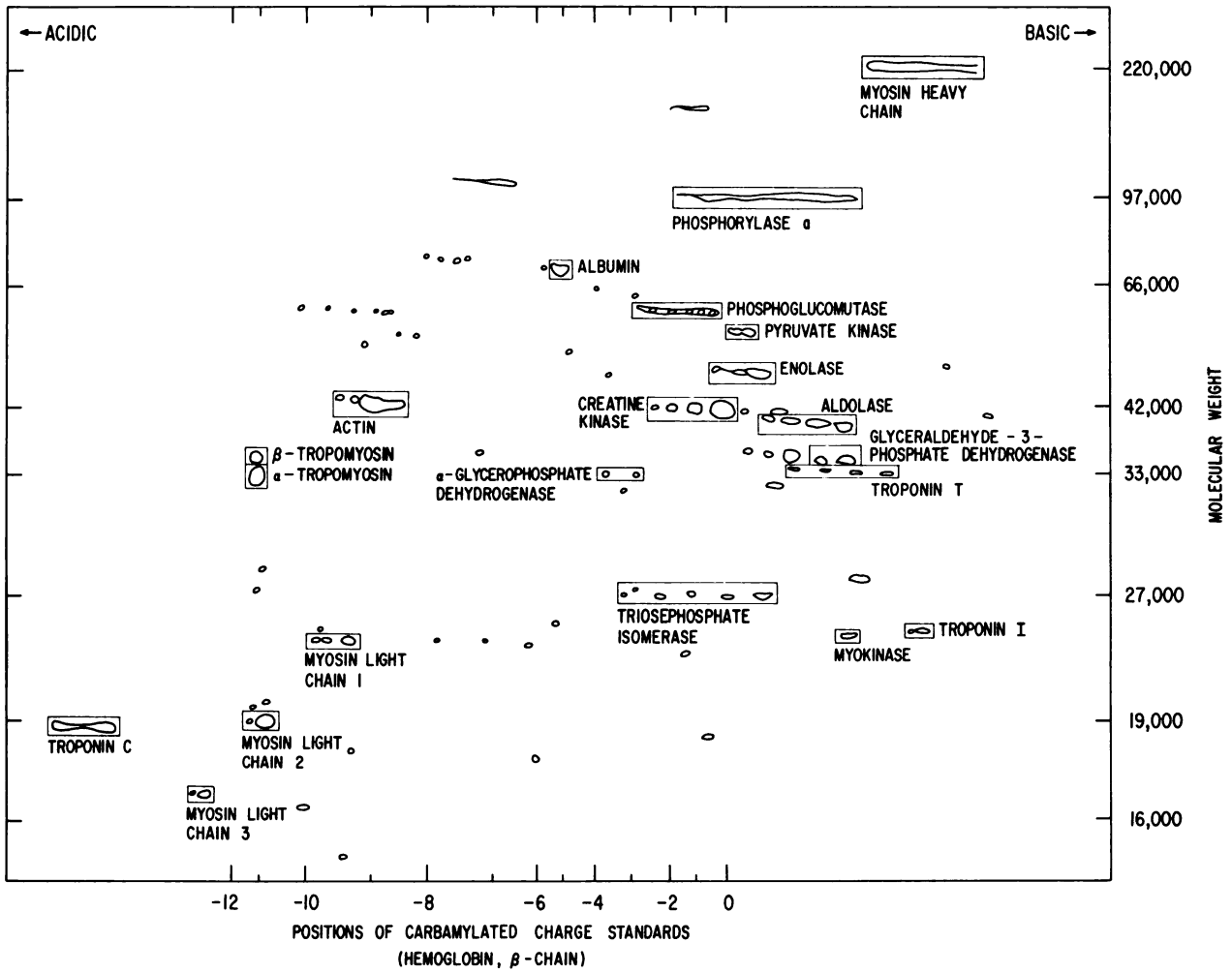


Fig. 3. Diagram of the two-dimensional pattern of rabbit muscle whole homogenate (composite of the patterns in Figs. 1*A* and 2*A*)

Protein identifications were made by co-electrophoresis of purified muscle proteins. Molecular weight calibrations (*ordinate*) obtained with rat heart streak standards (*7*); positions of carbamylated charge standards (*abscissa*) determined with carbamylated hemoglobin (*6*)

rabbit psoas muscle weighing only 5–10 mg (representative of the amount of tissue expected from a needle biopsy), the tissue can be solubilized directly in either the SDS-CHES or NP 40-urea mix. Homogenization before addition of these detergent solutions optimizes protein recovery. Tissue frozen in liquid nitrogen is pounded into a powder with a stainless-steel mortar and pestle and kept chilled on solid CO_2. The powder is then weighed and transferred to a vial containing 10 volumes of the appropriate detergent solution.

Connective tissue, which is not completely dissolved under the conditions described, is removed from large-scale muscle preparations by filtering the homogenate through cheesecloth. Such filtering is not feasible with small-scale samples, but insoluble material can be removed from those samples by centrifuging for 5 min in a Microfuge (Beckman Instruments, Fullerton, CA 92634). The patterns obtained when the resulting supernate is analyzed by two-dimensional electrophoresis are identical to those shown in Figures 1*A* and 2*A*. Our studies have shown that freezing the tissue does not alter the protein pattern from that obtained when fresh tissue is homogenized and analyzed. Evidence for the reliability of this technique will be presented in a subsequent paper.

Human Muscle Protein Analysis

A normal human muscle biopsy sample was prepared in SDS-CHES mix as described and analyzed with the Iso-Dalt system. Although not identical to the rabbit psoas muscle pattern, the human muscle pattern (Figure 4) shows some definite similarities to it. We can identify spots corresponding to myosin heavy chains, actin, tropomyosin, and creatine kinase by analogy with their positions in the rabbit muscle patterns. The verification of these identifications and the identification of other components are in progress.

Discussion

High-resolution two-dimensional electrophoresis separates proteins by differences in both charge and size. Therefore, genetic alterations in a single amino acid that alter the charge of a peptide molecule are detectable in a two-dimensional electrophoresis pattern. The clinical use of two-dimensional electrophoresis has been made feasible by the development of equipment that allows multiple analyses (*3*, *4*). The preparation and analysis of serum and urine with this equipment have been described previously (*8*, *26*, *27*). Although protein changes in these body fluids are already frequently monitored for diagnostic purposes, a look at the protein composition of tissues directly affected by certain disease processes could also provide valuable clinical information. Thus the development of methods for the two-dimensional electrophoretic analysis of tissue biopsy material has become very important.

The two-dimensional electrophoretic mapping of rabbit muscle proteins has laid the foundation for the analysis of human muscle samples. The sample preparation methods have been shown to yield comparable results when large (1 g)

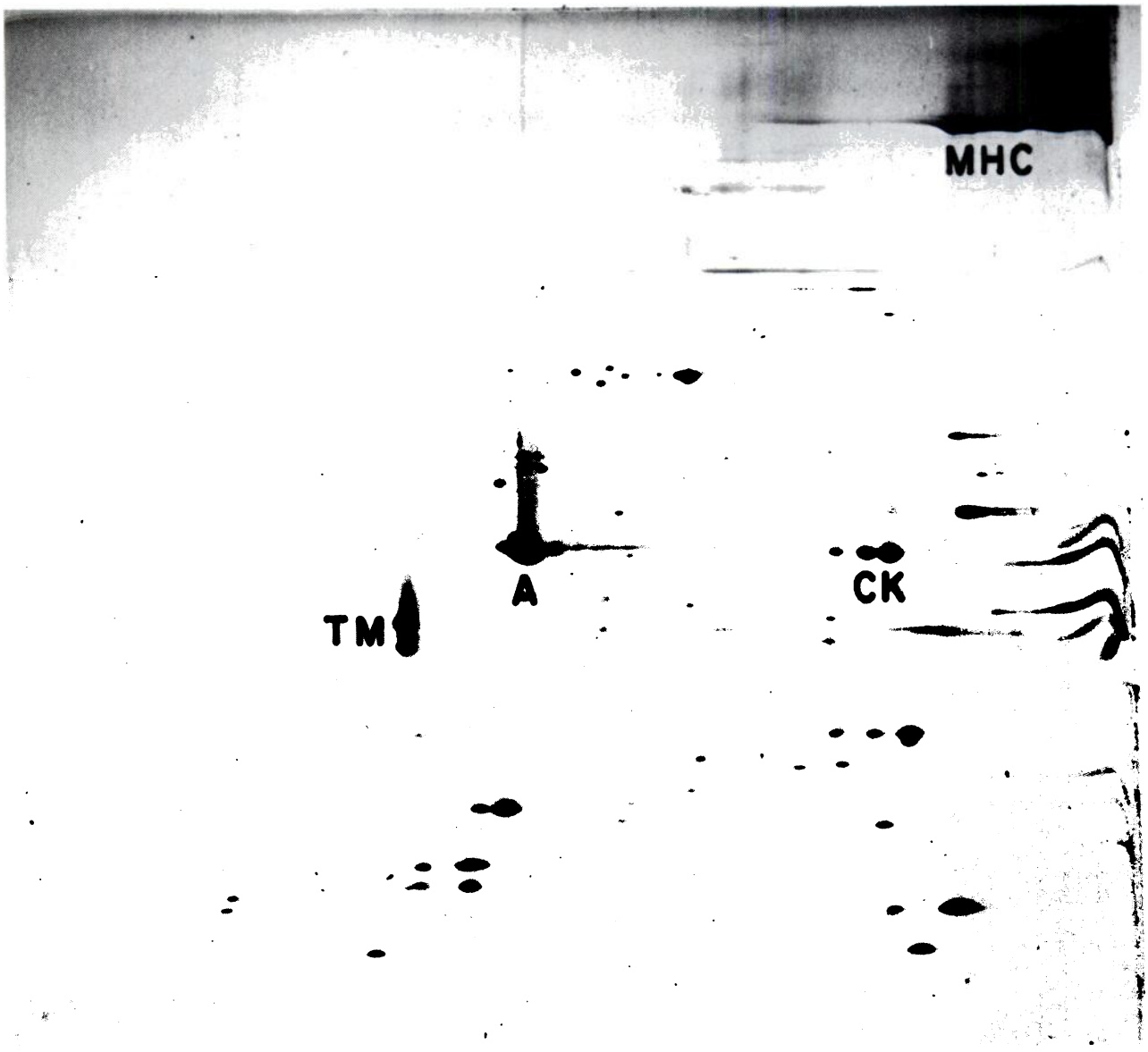


Fig. 4. Two-dimensional electrophoresis pattern of whole homogenate of normal human skeletal muscle

A human muscle biopsy sample was prepared as described in *Materials and Methods* and analyzed with 200 μg of protein applied to isoelectric focusing gel for the first dimension. TM, tropomyosin; other abbreviations, gel orientation, and cropping as in Fig. 1

or small (5–10 mg) tissue samples are used. Previous reports on the two-dimensional electrophoresis of muscle proteins have dealt primarily with cells grown in culture and have concentrated on characterization of the contractile proteins (*10, 11, 24*). The charge heterogeneity in a majority of the proteins identified in the patterns from fresh rabbit muscle may be unique to fresh tissue or may have gone undetected in those cultured cell patterns. Although the source of the heterogeneity remains to be explained, the different spots may reflect post-translational modification of proteins, which may be seen to a lesser extent in cultured cells because of their rapid protein turnover rate. The charge heterogeneity seen in the rabbit muscle proteins could also be due to the mixed fiber content of psoas muscle. To determine the contribution of fiber type to the protein composition of the total muscle, separate analysis of Type I and Type II muscle fibers will be necessary.

A central problem in work of this type is to distinguish artifacts from true post-translationally induced in vivo charge modifications. Two-dimensional electrophoretic studies on human erythrocyte pyruvate kinase have shown microheterogeneity in that protein (*9*). When these cells are separated into young and old populations, the more alkaline forms are found in young cells and the acidic forms in the old cells (J. J. Edwards, unpublished work). The chemical modification of rabbit skeletal muscle aldolase as a function of the aging process and protein turnover has also been reported (*28, 29*). Thus the detection of protein charge heterogeniety may be diagnostically useful as a measure of the rate of protein turnover. To distinguish between true charge heterogeneity and that caused during sample preparation, patterns must be studied as a function of sample preparation, temperature, pH, or other variables. If little or no effect is found, the observed heterogeneity may be considered to exist in vivo.

Subsequent papers in this series will deal with the problems of human biopsy sample acquisition, storage, and preparation, with emphasis on the minimization of autolytic breakdown. Differences in the two-dimensional protein patterns as related to muscle type, genetic variants, and pathology will also be considered as the normal human muscle pattern becomes more completely understood. This paper shows that two-dimensional electrophoresis can feasibly be used for biopsy

analysis. The way will soon be open to utilize the full clinical potential of this method.

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