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Muscle Protein Analysis. III. Analysis of Solubilized Frozen-Tissue Sections by Two-Dimensional Electrophoresis

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Proteins from frozen histological sections of human muscle were analyzed by two-dimensional gel electrophoresis. Patterns so obtained were identical to those from whole homogenates of muscle prepared from frozen tissue powders that had much higher protein concentrations. To increase the number of proteins visible on gels of samples low in protein content, the gels were silver stained, or the proteins were labeled with [^{14}C]iodoacetamide before electrophoresis and the gels were fluorographed. The latter method allows use of a single frozen-tissue section for two-dimensional electrophoretic analysis and brings the technique closer to practicable clinical use.

Additional Keyphrases: *ISO-DALT system · tissue analyses · electrophoresis, polyacrylamide gel · correlation of histological and electrophoretic observations*

Two-dimensional electrophoresis provides the highest-resolution separation of proteins and protein subunits currently available. In previous papers we have explored ways in which this technique might be used to characterize both normal and diseased human cells and tissues at the molecular level (*1–3*), and thus be used as a tool for clinical diagnosis. To be of use to pathologists, the electrophoretic mapping techniques must be successfully interfaced with existing procedures in the clinical pathology laboratory, and clinical chemistry laboratories using the technique must be able to analyze very small samples and must ultimately be able to deal with the problem of tissue sample inhomogeneity. Tumor samples, for example, may contain necrotic areas, areas of surrounding normal tissue, and areas of tumor cells admixed with other cells to varying degrees. Clearly it is desirable to use as much information as can be obtained from the pathologist in choosing the sample areas to be analyzed and to be able to make molecular maps from very small, precisely chosen samples.

Our initial approach to the problem of applying two-dimensional electrophoresis to clinical pathology was to develop methods for analyzing very small biopsy samples that had been rapidly frozen after excision (*1*). The samples were pounded into a fine powder at low temperatures, transferred to small tubes, and weighed. (In spite of every effort to keep the tissue frozen, this was difficult during transfer of small samples.) The samples were then dissolved in a denaturing solution before electrophoresis. Although this method requires only 5 mg of tissue (*1*), this is often a very substantial portion of a needle-biopsy sample. Further, although the method is useful for research purposes, it is not easily adapted to routine clinical laboratory use.

We have therefore explored the two-dimensional electrophoretic analysis of frozen tissue sections such as those routinely prepared in hospital pathology laboratories and describe here the results we obtained with sections of human muscle. We find that a single appropriately treated frozen section is adequate to show the major contractile proteins in gels stained with Coomassie Blue. Other, less-abundant proteins can be detected by using the more sensitive silver-staining technique (*4*) or by radio-labeling sample proteins before electrophoresis and using fluorography for detection.

Materials and Methods

Sample preparation. Muscle samples were provided by Dr. M. J. Danon (Department of Neurology, University of Illinois at the Medical Center, Chicago, IL). All samples were flash frozen immediately after removal from the patient and were transported and stored at −80 °C. Sectioning was done in our laboratory with a cryostat at −25 °C. The 5 × 5 mm sections were 16 μm thick. The frozen sections were placed in chilled multiwell plates (Falcon 3830) containing 100 μL of frozen solubilization mixture per well. For samples to be labeled with [^{14}C]iodoacetamide, the solubilization mix contained, per liter, 9 mol of urea, 50 mL of Nonidet P40 (a non-ionic detergent; Particle Data Laboratory, Ltd., Elmhurst, IL 60126), 20 g of ampholyte (LKB pH range 3.5–10; LKB Instruments, Inc., Rockville, MD 20852), and 50 mCi of [^{14}C]iodoacetamide (Amersham/Searle, Arlington Heights, IL 60005). All other samples were solubilized in, per liter, 9 mol of urea, 50 mL of Nonidet P40, 20 g of ampholyte (3.5–10), and 1 g of dithioerythritol (the "NP40-urea" mixture). Samples were kept frozen in the multiwell plates until 45 min before isoelectric focusing. The plates were then incubated, with rocking, at room temperature for 30 min. The solubilized samples were transferred to tubes and centrifuged for 3 min in a Beckman Microfuge B to remove all insoluble material. Aliquots of the resulting supernates were used for the analysis by two-dimensional electrophoresis.

For comparison with these tissue-section preparations, we prepared a sample of muscle by the frozen powder method described previously (*1*). Frozen tissue was pounded into a fine powder, transferred to a tared Microfuge tube, and weighed. The sample was solubilized in 10 volumes of NP40-urea mixture, allowed to stand at room temperature for 10 min, mixed well, and then centrifuged for 3 min in a Beckman Microfuge B. The supernatant fluid was then centrifuged for 1 h at 42 000 rpm in a Beckman LP 42 Ti rotor ($\omega^2 t = 6.4 \times 10^{10}$). Aliquots of the final supernatant fluid were used for two-dimensional electrophoresis. Sample protein concentrations were determined by the Coomassie Blue method (*5*).

Electrophoresis. First-dimension isoelectric focusing was performed as described previously (*6*) with use of LKB Ampholines (10% of pH range 2.5–4 with 90% of pH range 3.5–10). Isoelectric focusing was carried out for 11 000 V · h.

The second-dimension sodium dodecyl sulfate gel electrophoresis, Coomassie Blue staining, and destaining were all done as previously described (*7*). For silver staining we used a modification of the method described by Oakley et al. (*8*). Fluorography was according to Bonner and Laskey (*9*).

Results

Figure 1 compares the two-dimensional electrophoresis patterns of whole-homogenate proteins from normal human

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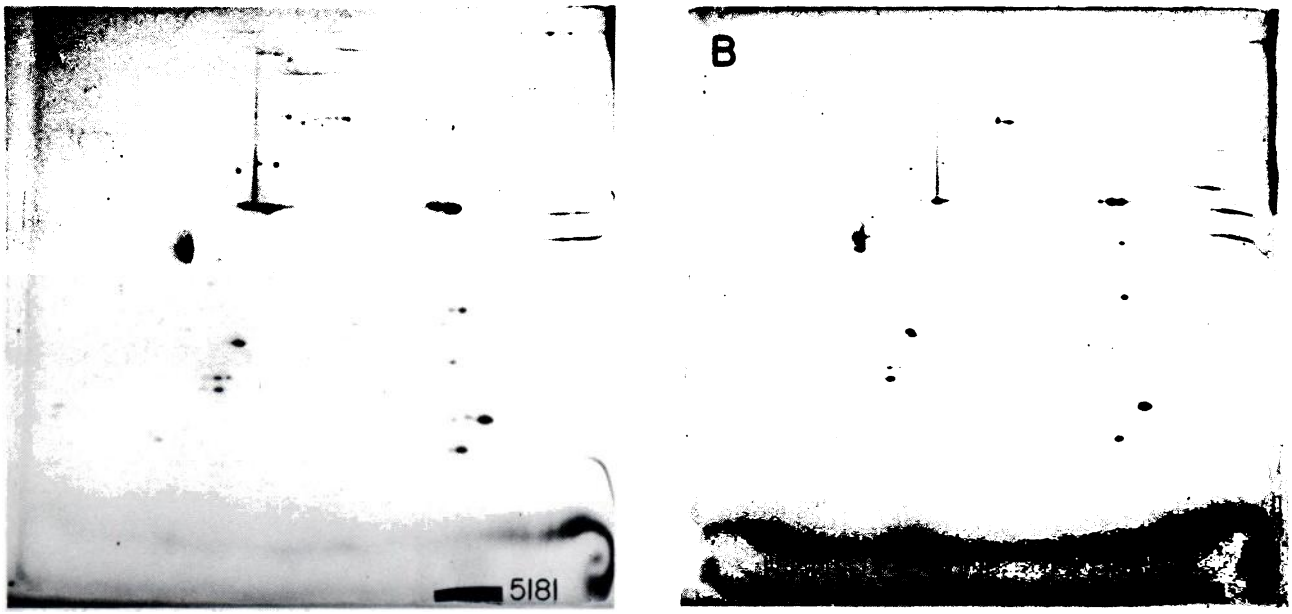

Fig. 1. Comparison of results of some tissue-biopsy preparation methods

A specimen of normal human deltoid was prepared for two-dimensional electrophoresis by two different methods: (*A*) frozen tissue-powder method and (*B*) frozen section method (tissue sections were 16 μm × 5 mm × 5.5 mm). Both gels were stained with Coomassie Blue and are oriented with the acid side to the left and basic side to the right

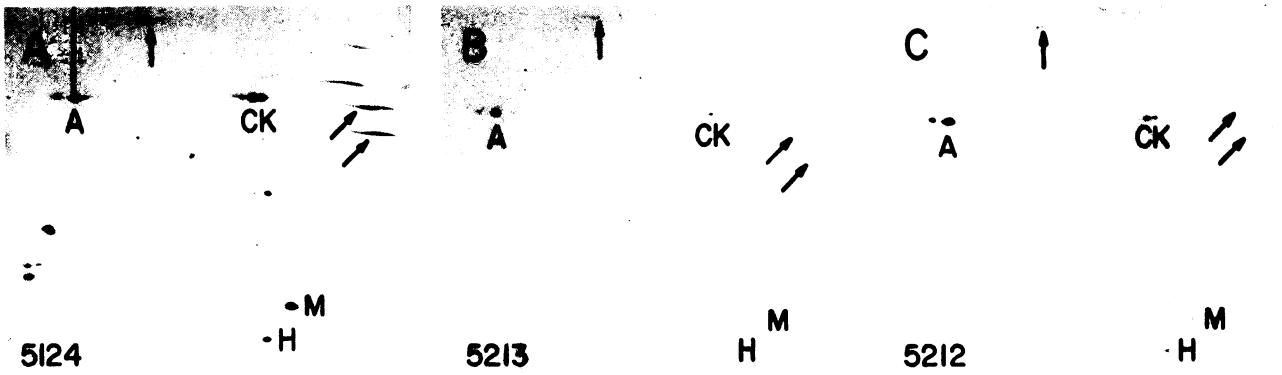

Fig. 2. Comparison of two-dimensional electrophoresis patterns of muscle after Coomassie Blue or silver staining

(*A*) Three frozen sections of human deltoid with Coomassie Blue stain. (*B*) One frozen section with Coomassie Blue Stain. (*C*) Same as *B*, but after silver stain. *A*, Actin; *CK*, creatine kinase; *M*, myoglobin; *H*, hemoglobin. *Arrows* indicate proteins that were only very faintly stained in the one-section sample when Coomassie Blue was used. Gel orientation same as in Figure 1

deltoid prepared by using 40 mg of frozen-tissue powder (*A*) or by using three frozen-tissue sections (*B*). The protein concentrations of the two samples were 25 and 2.5 g/L, respectively, and the protein load on the gels was 125 and 62 μg, respectively. The patterns are of comparable resolution and demonstrate that major muscle-homogenate proteins are equally well represented with either sample preparation technique. When the frozen-powder technique was used, samples were too viscous to pipet and had to be ultracentrifuged for an hour to remove the DNA. The small amount of material required in the tissue-section technique did not produce a viscous sample. Centrifuging in a Microfuge for just 3 min sufficed to remove debris that would interfere with isoelectric focusing.

In Figures 2*A* and 2*B* the Coomassie Blue patterns of proteins obtained from three frozen sections or only one section of normal human deltoid are compared. Protein concentrations of the samples shown were 2.5 and 0.65 g/L, respectively; protein loaded on the gels was 38 (2*A*) and 6.5 (2*B*) μg. Actin and creatine kinase are easily found in Figure 2*B* and myoglobin and hemoglobin are faintly visible, but the minor proteins are not visibly stained. When a gel comparable to the one shown in Figure 2*B* was stained with silver (Figure 2*C*), a technique that is 100 times more sensitive for protein detection than Coomassie Blue (*4*), the protein pattern more closely resembled that seen in Figure 2*A*.

Although the silver stain is more sensitive than staining with Coomassie Blue, the procedure is more tedious. Therefore, we investigated other alternatives for detection of proteins in low concentrations. When tissue sections were solubilized in 9 mol/L urea with Nonidet P40 in the presence of [^{14}C]iodoacetamide, the free -SH groups of the denatured proteins were labeled with ^{14}C. This irreversible reaction causes no charge alterations in the treated proteins (*10*), as demonstrated by comparison of the protein patterns shown in Figure 3*A* and *B*. These patterns, both stained with Coomassie Blue, are from a sample of normal human gastrocnemius that was analyzed without treatment (Figure 3*A*) or after treatment with [^{14}C]iodoacetamide (Figure 3*B*). There is no difference in the two patterns with respect to isoelectric point or molecular mass distribution of proteins. The untreated sample was prepared by using two tissue sections, whereas the treated

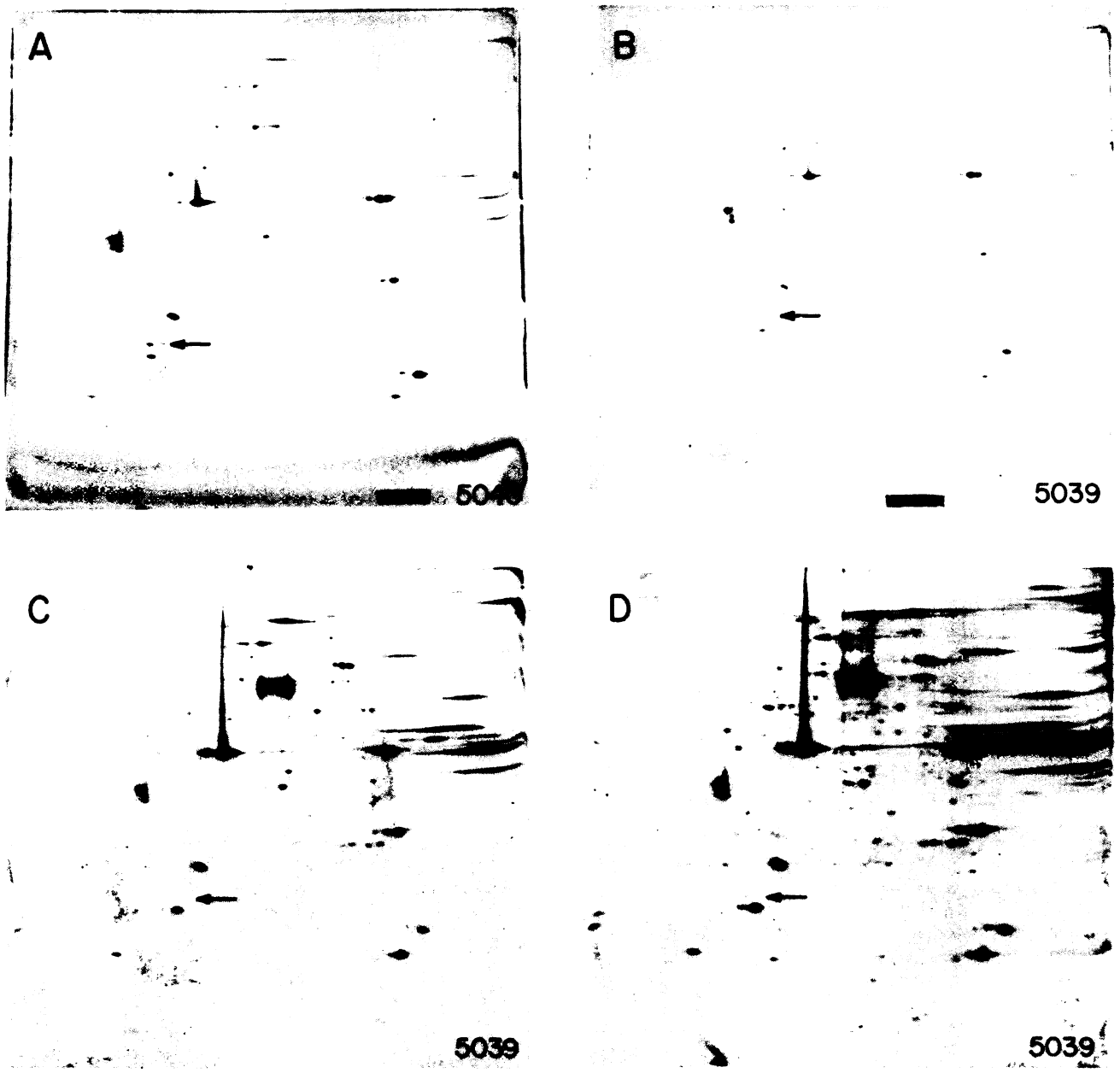

Fig. 3. Comparison of Coomassie Blue staining with ^{14}C-labeling for detection of tissue proteins on two-dimensional gels

(A) Two untreated sections of human gastrocnemius on a gel that was Coomassie Blue stained. *(B)* One section treated with [^{14}C]iodoacetamide on a gel that was Coomassie Blue stained. *(C)* A 3-day fluorogram of *B*. *(D)* A 7-day fluorogram of *B*. The *arrows* indicate position of protein designated MLC_{2S} *(2)*. Gel orientations same as in Figure 1

sample was prepared by using just one section. Fluorography of the gel shown in Figure 3*B* with use of a three-day exposure revealed the proteins labeled with ^{14}C (Figure 3*C*). By extending the exposure time of the fluorogram to seven days (Figure 3*D*), very minor proteins could be seen that were not visible by Coomassie Blue stain, even in the pattern from a sample of two tissue sections. Comparison of Figures 2 and 3 shows that the [^{14}C]iodoacetamide labeling is a more sensitive method of detecting protein than is the silver stain. However, detection of proteins by this technique depends on their cysteine content, so that some proteins with low cysteine content that are seen when Coomassie Blue staining is used may not be visible in the fluorograms. Most human muscle proteins are efficiently labeled with [^{14}C]iodoacetamide, but the myosin light chain designated MLC_{2S} *(2)* is an example of a protein that is not well labeled. The spot corresponding to this protein is only faintly visible in the fluorogram, although it is a major protein in the patterns stained with Coomassie Blue. This difference will be useful for the identification of MLC_{2S} is non-muscle tissues.

Discussion

The two-dimensional electrophoresis patterns obtained when frozen-tissue sections are solubilized are similar to those obtained for samples prepared from frozen muscle powders. In addition the lower protein load on each gel improves the separation and results in sharper, more well-defined spots. Use of the frozen-tissue-section technique decreases sample handling, because the sections of muscle can be prepared for electrophoresis at the same time tissue sections are made for clinical histology tests, which decreases the possibility of sample damage due to premature thawing. One frozen section of muscle (16 μm × 5 mm × 5 mm) contains about 65 μg of protein, the same amount of protein found in 200 μg of frozen tissue powder. Techniques used to prepare frozen powders, however, require a minimum of 1 mg of frozen tissue because

of the physical limitations of the apparatus used. The sectioning technique eliminates the waste of material caused by such physical limitations of sample-preparation techniques; only as much material is required as needed for the electrophoretic analysis. The tissue-section samples that we have shown to suffice for electrophoretic analysis thus are more compatible with the specimens obtained clinically, which must be divided for use in several tests, than the larger samples required for the frozen-powder method. Sections that are only 10-μm thick (the thickness preferred for use in some histological procedures) produce two-dimensional protein patterns comparable to those seen when 16-μm sections are used.

With Coomassie Blue staining, a single tissue section can suffice for examination of the major protein components of muscle. Additional techniques and (or) a larger sample must be used for detection of the less-abundant proteins. The silver staining technique will show the proteins detected with Coomassie Blue plus other proteins that are present in amounts that escape detection with Coomassie Blue staining. Radiolabeling cysteine residues by reacting them with [^{14}C]iodoacetamide is an even more sensitive method of protein detection than silver staining, and it has the additional advantage that it provides some information concerning the free cysteine content of the muscle proteins. For example, by this technique the myosin light chain MLC_{2S} in human muscle samples is seen to contain little cysteine.

In addition to the two human samples shown in this report (normal deltoid from a 21-year-old man and normal gastrocnemius from a 28-year-old woman), we have analyzed 19 other normal and 13 pathological muscle biopsies by two-dimensional electrophoresis after [^{14}C]iodoacetamide-labeling of single frozen sections. The reproducibility of the sample preparation technique is demonstrated by the fact that each of those 32 patterns is almost identical to the patterns shown in this report (data not shown). Variations in the protein patterns are attributable to normal individual variation among patients, differences in the tissue composition of the biopsy, and (or) effects of muscle pathology on the tissue protein components (unpublished observations).

A major advantage of the use of tissue sections as starting samples for electrophoresis instead of a powder made from an entire muscle biopsy is that variations in the protein composition of samples can be correlated with the histochemical data available from adjacent tissue sections. Just as pathologists study more than one slide of a biopsy or biopsy more than one site before reaching a diagnosis, more than one tissue section may have to be analyzed by electrophoresis before protein alterations can be accurately and confidently interpreted.

The feasibility of using two-dimensional electrophoresis for clinical analysis of human tissue samples has been previously discussed (*1*, *2*), and systems for analyzing many samples in parallel have been described (*6*, *7*). In time, automation of the methods and data analysis will be perfected. However, if these procedures are to come into general use, the problems of tissue-sample preparation for two-dimensional electrophoresis in a clinical laboratory must be solved. The use of frozen-tissue sections means a very small sample requirement, especially with silver staining and radiolabeling techniques. The tissue sections, prepared in the pathology laboratory according to existing procedures, could be put into holders containing solubilization media, transported to the laboratory involved with two-dimensional gel analysis, thawed, centrifuged, and loaded onto the gels. The results of the two-dimensional electrophoretic analysis could then be directly correlated with histological results for the same specimen. If alternate samples are analyzed microscopically and electrophoretically, individual spots in the electrophoretic pattern can be correlated with different cell types or pathological conditions seen in sections. This should prove both interesting and useful in the analysis of tissues containing layers of cells of different types, or in organs such as the pituitary that have different cell types in different regions. Ultimately, however, correlation of individual peptides with specific cell types should be confirmed by use of specific antibodies. The most obvious initial use of the results reported here will most probably be in cancer diagnosis. Our laboratory is currently investigating the use of this technique with tissues other than muscle, including biopsies of brain and prostate.

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