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Analytical Techniques for Cell Fractions

XXVI. A Two-Dimensional Electrophoretic Analysis of Basic Proteins Using Phosphatidyl Choline/Urea Solubilization[1,2]

KAREN E. WILLARD, CAROL SMITH GIOMETTI, N. LEIGH ANDERSON, TIMOTHY E. O'CONNOR, AND NORMAN G. ANDERSON

Molecular Anatomy Program, Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

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The ISO–DALT system of two-dimensional electrophoresis allows high-resolution separations of proteins and protein subunits. However, the conventional isoelectric focusing employed in this system does not give satisfactory resolution of the more basic proteins, such as histones. The BASO–DALT system was designed to obtain improved resolution of these basic proteins in the first dimension. In this system, phosphatidyl choline is used as the solubilization agent, and allows resolution of many low molecular weight basic proteins that were not seem with more conventional detergents. Using the BASO–DALT system, Novikoff hepatoma chromosomal proteins have been analyzed, and the five histones identified.

Very high-resolution separations of proteins and protein subunits can be obtained by two-dimensional polyacrylamide gel electrophoresis. The ISO–DALT system (1,2), a modification of O'Farrell's technique (3), utilizes isoelectric focusing in urea in the first dimension coupled with sodium dodecyl sulfate (SDS)[3] slab gel electrophoresis in the second. This procedure resolves mainly those proteins with acidic or neutral isoelectric points; the few basic proteins that enter the ISO gel usually appear as streaks. O'Farrell *et al.* (4) have described an alternative first-dimension procedure to resolve basic proteins which they call nonequilibrium pH gradient electrophoresis (NEPHGE). In this procedure, improved separation of many basic proteins resulted from loading samples at the acidic end of the focusing gel and running for 1600 V-h instead of the 10,000 V-h used in isofocusing. We have been unable, however, to obtain satisfactory resolution of basic proteins from Novikoff hepatoma cells by following O'Farrell's procedure; the histones were not solubilized by O'Farrell's SDS and NP-40 lysis buffers, and a considerable proportion of the total protein applied failed to enter the gel. By utilizing a mixture of dipalmitoyl L-α-phosphatidyl choline (PC) and urea as the solubilizing agent, and then electrophoresing the sample for 3200 V-h, we obtained more complete patterns of the basic proteins (including the histones) in Novikoff hepatoma cells. Longer periods of electrophoresis (4000 V-h) focused all but the most basic proteins. This system is called the BASO–DALT system, since the second

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[3] Abbreviations used: SDS, sodium dodecyl sulfate; NEPHGE, nonequilibrium pH gradient electrophoresis; PC, L-α-phosphatidyl choline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CK, creatine phosphokinase.

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(SDS) dimension is identical with that used in the ISO–DALT system. Our initial studies were concerned with Novikoff hepatoma cells, but the BASO–DALT method to be described has also been used for studying liver, muscle (5), serum, and urinary proteins.

MATERIALS AND METHODS

Sample preparation. Urine and serum samples were lyophilized to remove excess water and cells were pelleted to allow samples to be reconstituted in the highest concentration of urea. Lyophilized samples were resuspended in 0.5% PC (Sigma), 9.5 M urea, 2% ampholytes, and 5% mercaptoethanol, with the pH adjusted to 2.8 with H_3PO_4. Samples were allowed to stand in this opalescent solution for 1 h at room temperature.

After solubilization, all samples were subjected to high-speed centrifugation to pellet high molecular weight materials such as DNA and to remove the excess PC, both of which would otherwise tend to clog the focusing gel. A method for high-speed centrifugation of small samples has been previously described (6). Briefly, samples are placed in 75-μl centrifuge tubes which are then fitted into polycarbonate adapters for the Beckman VTi 50 vertical tube rotor. These adapters are designed to hold all tubes at the same radius in the horizontal position. The samples are then centrifuged for 1 h at 20°C and 50,000 rpm ($\omega^2 t = 85 \times 10^7$) to sediment all particles with an S value greater than 12. The lipid layer on top is removed, and the aqueous supernatant removed for analysis in the BASO–DALT system.

Two-dimensional electrophoresis. First-dimension (BASO) gels were cast in the ISO apparatus using solutions and procedures previously described (1,2). Either LKB 3.5–10 and/or 9–11 Ampholines or Brinkman 2–11 ampholytes were used. The lower tank reservoir was filled with 2 liters of degassed 20 mM NaOH. BASO gels were not prefocused and samples were applied directly to the upper gel surface. To protect the proteins from the acid used in the upper reservoir, samples were overlaid with 8 M urea and 1% mercaptoethanol (filling the remainder of the focusing gel tube). The upper reservoir was then filled with 250 ml of 10 mM H_3PO_4. It should be noted that the lower reservoir now contains the cathode and the upper reservoir, the anode (the reverse of the ISO system).

After electrophoresis, the gels were extruded from the tubes and equilibrated in SDS buffer (1). They were then loaded on the SDS slab gels for the second-dimension (DALT) separation, and were subsequently fixed, stained, and photographed as described earlier (2).

Carbamylated proteins. Rabbit muscle aldolase was obtained from Worthington Biochemicals. Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (GAPDH), myokinase (adenylate kinase), and creatine phosphokinase (CK) were purchased from Sigma. Rabbit muscle soluble proteins were extracted from psoas muscle by homogenizing the tissue in four volumes of phosphate-buffered saline with a Waring Blendor. The myofibrils were removed by centrifugation of the sample for 1 h at 100,000g.

Proteins were prepared as 5-mg/ml solutions in 8 M urea. Carbamylation was performed by heating these solutions for varying time intervals at 95°C (generally 0, 4, 6, 8, 10, 15, 30, 45, 60 min) and then rapidly cooling them on ice. Each fraction was then analyzed in the BASO–DALT system, and a uniform blend of these different fractions was prepared to give the standard mix for each protein. Use of carbamylation standards for the ISO–DALT system has been previously described (7).

RESULTS

Protein solubilization. A conventional two-dimensional electrophoretic analysis of nuclear proteins (using the ISO–DALT

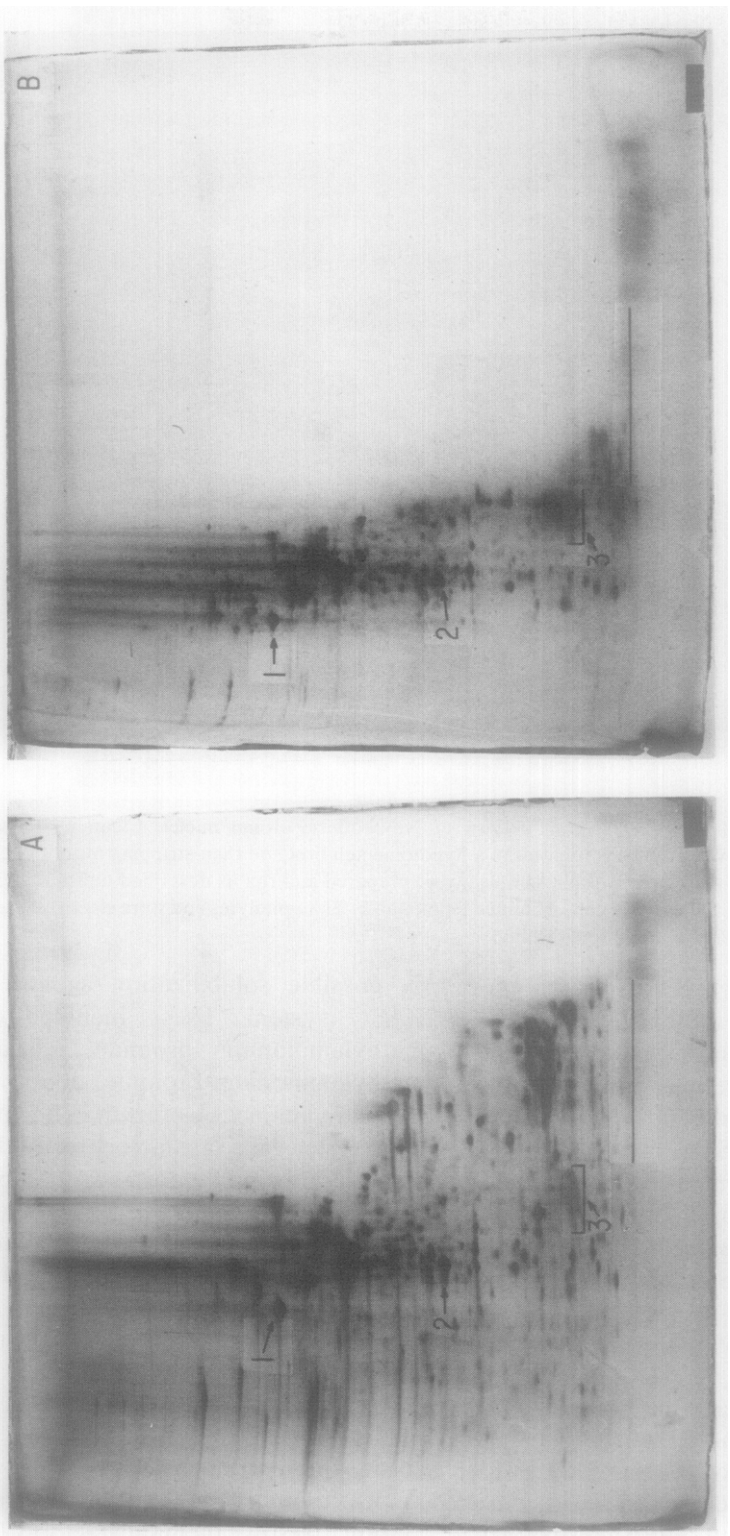


FIG. 1. BASO–DALT patterns of Novikoff hepatoma cells illustrating different methods of solubilization. Samples were electrophoresed for 1600 V-h using LKB 3.5–10 Ampholines in the ISO–DALT apparatus as described under Materials and Methods. The gels are oriented with the basic end to the right and the acidic end to the left. (A) Cells were lysed in the PC/urea mixture, allowed to stand at room temperature for 1 h, and then centrifuged for an hour at 50,000 rpm, as described under Materials and Methods. (B) Cells were mixed 1:1 with the SDS lysis buffer described by O'Farrell *et al.* (4) and allowed to stand for 10 min. This mixture was then diluted 1:1 with their NP-40 lysis buffer, and the final protein concentration equaled the samples in A. Selected proteins are labeled 1–3 for reference purposes in both A and B. The area above the bar represents the greatest difference between the gels.

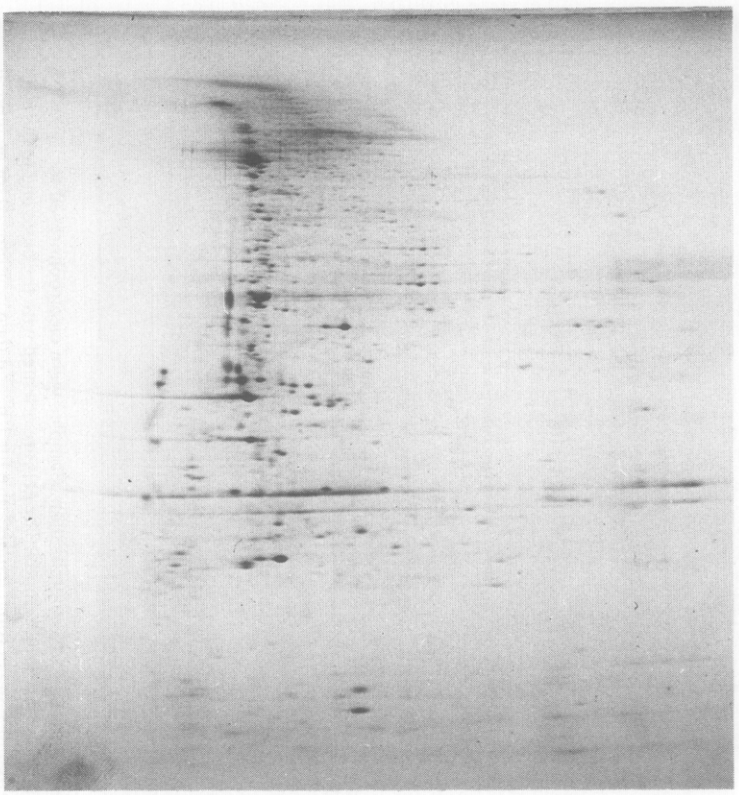


FIG. 2. Portion of a BASO–DALT pattern of Novikoff hepatoma nuclei. Clean nuclei were prepared by first allowing the cells to swell in a hypotonic solution and then stripping away the cytoplasm with 0.5% NP-40. Nucleoprotein samples were prepared and run as described under Materials and Methods. The first-dimension gels contained Brinkman 2–11 ampholytes and were electrophoresed for 4000 V-h. The gels are oriented as in Fig. 1.

system) fails to reveal those with more basic isoelectric points. O'Farrell's NEPHGE system was designed to give high resolution of basic cellular proteins, but in our experience Novikoff hepatoma cells solubilized in the SDS and NP-40 lysis buffers of this system often give rise to a precipitate at the top of the focusing gel that prevents the sample proteins from entering the gel. Samples that do not precipitate give well-resolved patterns, but a large percentage of the sample still does not enter the gel (Fig. 1B). This phenomenon could be due to inadequate solubilization of the sample by the SDS/NP-40 method and/or the inability of the sample to enter the gel because of the attached SDS. Based on these assumptions, we began to test a number of cationic detergents and amines as possible solubilization agents for the BASO system. These included cetyltrimethylammonium bromide, tetradecyltrimethylammonium bromide, dodecylamine, and amylamine. Unfortunately, none of those tested gave satisfactory results because a dense precipitate formed on top of the focusing gel as soon as the current was applied. Surprisingly, the use of phosphatidyl choline in conjunction with urea for cell lysis did not precipitate the sample and gave higher resolution and better solubilization than any other agent tested (Fig. 1A). Patterns of Novikoff hepatoma nuclei solubilized by this technique demonstrate the extreme heterogeneity of the nuclear proteins and the ability of the BASO system to resolve them satisfactorily (Fig. 2).

Duration of electrophoresis. O'Farrell

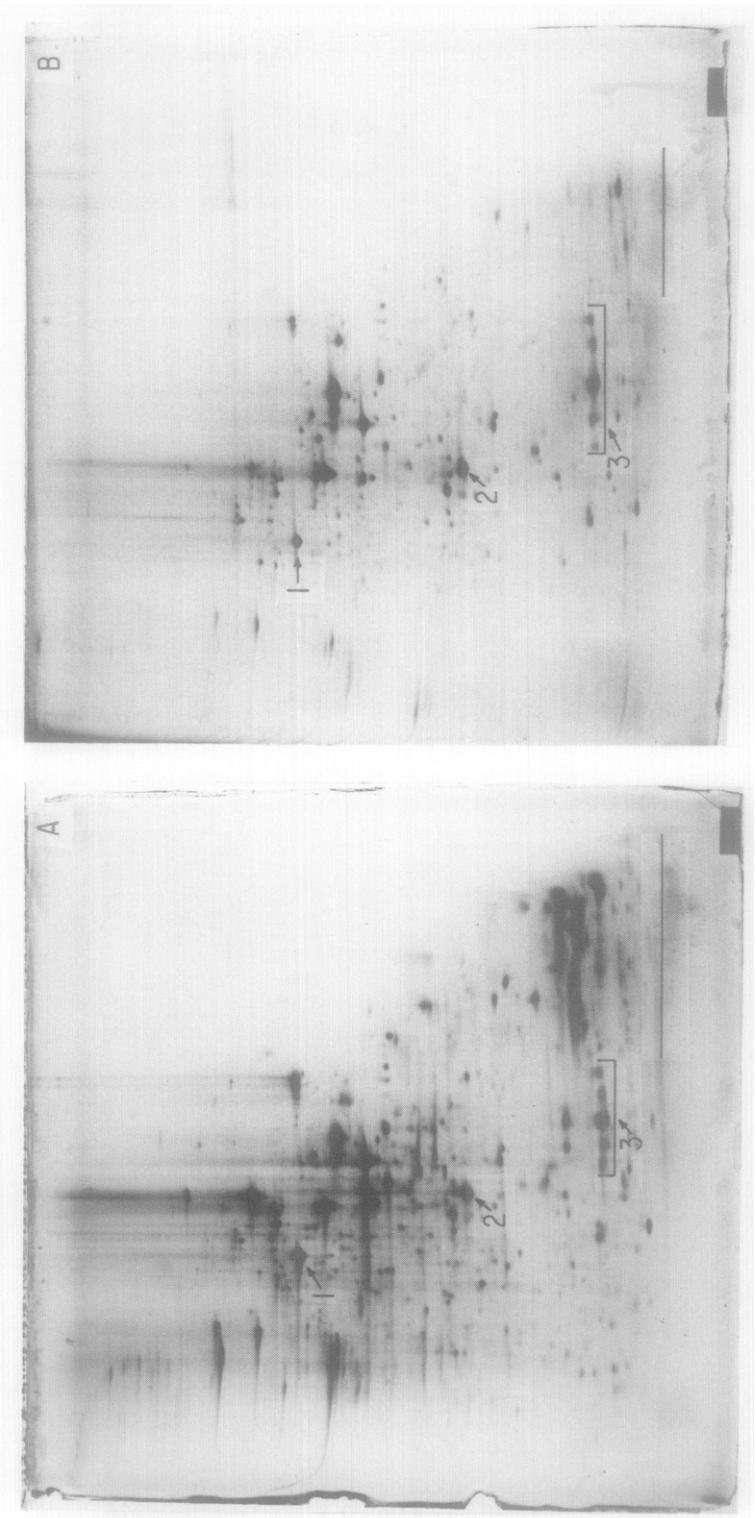
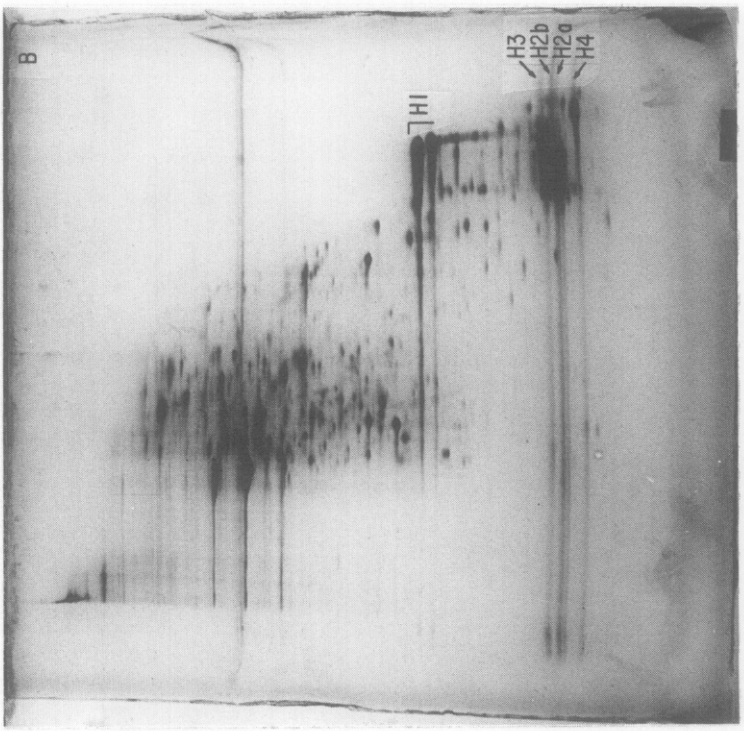
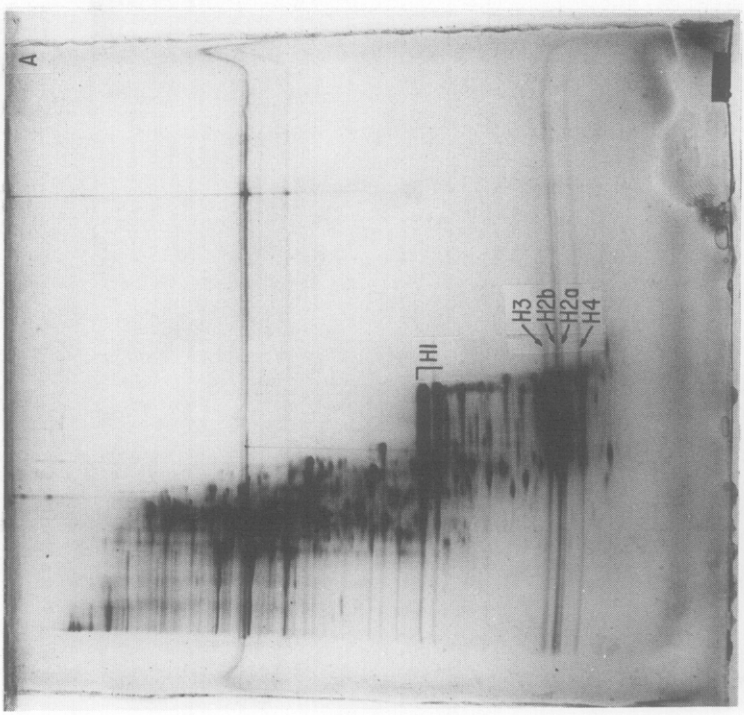


FIG. 3. BASO–DALT patterns of Novikoff hepatoma cells after 3200 V-h of electrophoresis. Samples A and B were solubilized as described in Fig. 1. Samples were electrophoresed using an I.KB 3.5–10 Ampholine gradient in the ISO–DALT apparatus as described under Materials and Methods. The gels are oriented as in Fig. 1. Reference proteins 1–3 and the area above the bar are the same as in Fig. 1.


B
H3
H2b
H2a
H4
H1


A
H3
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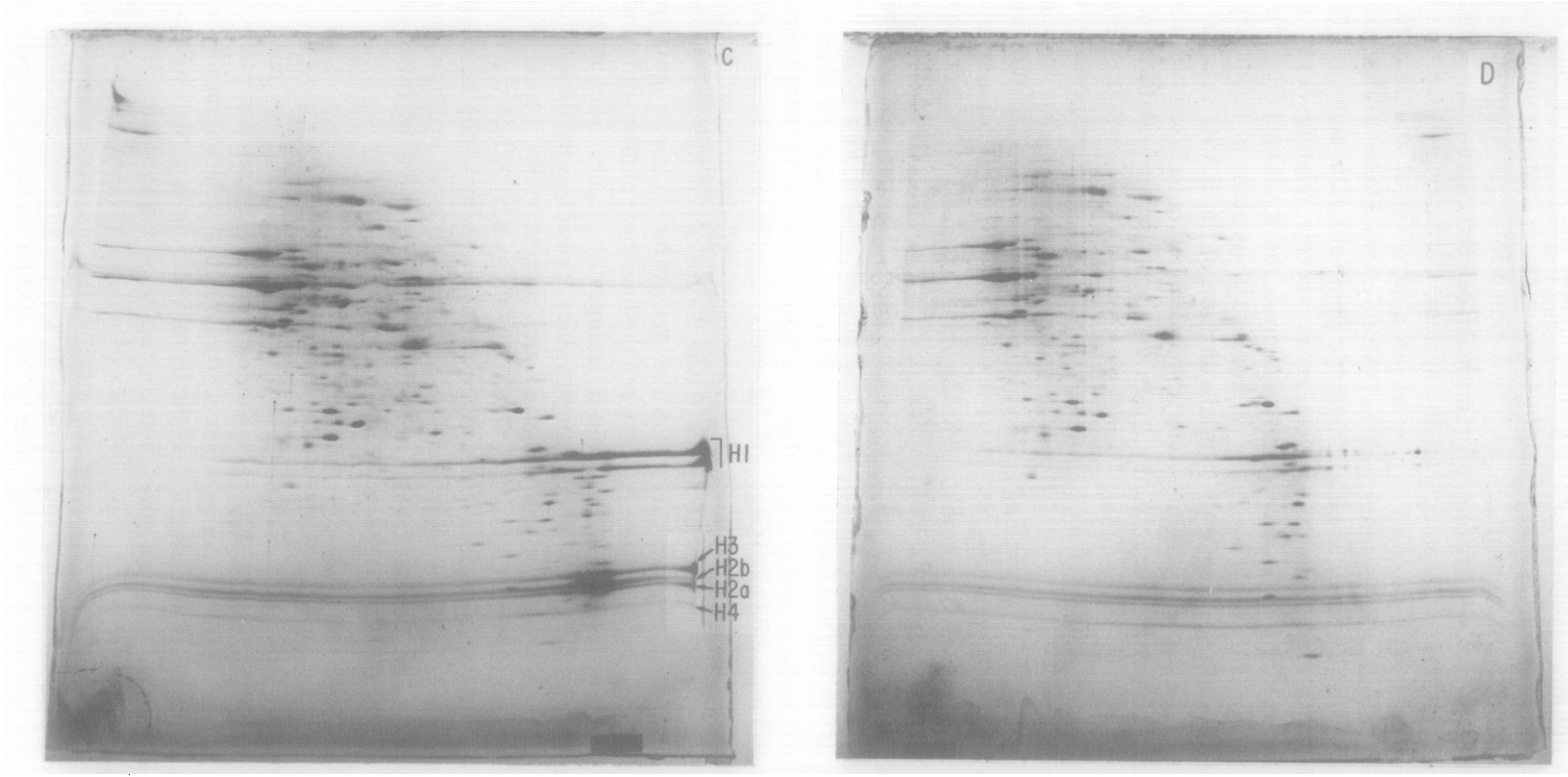

FIG. 4. Protein migration as a function of time in the BASO system. Novikoff hepatoma nuclei were solubilized in phosphatidyl choline as described under Materials and Methods. They were then subjected to electrophoresis for 2–6 h at 800 V using a 3.5–10 Ampholine gradient. The proteins were then separated in the DALT system as previously described (2). The gels are oriented as in Fig. 1: (A) 1600 V-h, (B) 3200 V-h, (C) 4000 V-h, and (D) 4800 V-h. The histones H1, H2a, H2b, H3, and H4 are identified in A, B, and C. These identifications are based on analyses of purified Novikoff histones in the BASO–DALT system.

et al. (4) described optimal resolution of *Escherichia coli* and African green monkey kidney cells in their NEPHGE system after electrophoresis for 1600 V-h. Comparisons of Novikoff hepatoma cell lysates prepared in PC versus those prepared in the SDS/NP-40 lysis buffers demonstrate that optimal resolution of these samples is not achieved until after 3200 V-h (Fig. 3A). Proteins solubilized in SDS and NP-40 appear to move much more slowly than their PC solubilized counterparts (Fig. 3B) and do not appear to reach their isoelectric points until after 4000 V-h of electrophoresis. Presumably, the slower movement is due to residual SDS attached to the proteins which reduces their net positive charge.

Further investigation into the movement of the PC solubilized nuclear proteins (Fig. 4) demonstrated that most of the proteins reached their isoelectric point after 4000 V-h of electrophoresis. The most basic proteins, such as the histones, continued to move throughout the time of electrophoresis and ultimately migrated off the basic end of the gel. The ampholyte gradient achieved using Brinkman 2–11 ampholytes actually extends from pH 3.0 to 8.5 after focusing (unpublished work) and would thus explain the inability of the histones to focus at their isoelectric point. Electrophoresis for 3200 V-h achieves good separation of all the proteins entering the gel and does not lose the histones off the basic end (Fig. 4B).

Isoelectric point standards. Isoelectric point standards for the ISO–DALT system based on carbamylation of proteins by heating at 95°C in 9.5 M urea have been described (7). Under these conditions, the cyanate formed by heating the urea will carbamylate the terminal α-amino and the lysine ϵ-amino groups of the protein. Carbamylation raises the net negative charge of the molecule because a positively charged amino group is transformed into an uncharged carbamylamino group of homocitrulline (8). Purified proteins can be carbamylated for varying lengths of time, and then mixtures can be prepared to yield equal amounts of the protein at each charge shift. These mixtures form a train of proteins on a two-dimensional gel that can be used for internal isoelectric point standardization of the system. Carbamylated CK has been chosen as a provisional standard for the ISO–DALT system. However, because the isoelectric point of CK is about 6.1 (9) the most basic species migrates only about halfway across the BASO gel (Fig. 5).

A more basic protein is necessary for standardization of the BASO system. Carbamylation of rabbit muscle soluble proteins yields a map showing several different carbamylation trains of basic proteins (Fig. 5). Using commercially purified proteins from rabbit muscle, the carbamylation trains of aldolase, GAPDH, and adenylate kinase were investigated (Fig. 5). Carbamylation of aldolase yields a train of spots that runs as a single row across the gel. However, the spots lack good definition and tend to run into one another. Adenylate kinase (myokinase) also runs as a single row of spots, but there is considerable variation in the spacing of the spots. On the other hand, GADPH yields a train of well-defined, evenly spaced spots which runs about halfway down the SDS slab gel. Although minor spots appear between the first three charge shifts and two minor trains with higher SDS molecular weight values appear toward the acid end of the gel, this protein yields a train of spots suitable for our needs. While the GADPH carbamylation train may not be useful in every situation, it will provide internal standardization for most of the spots in the BASO system.

DISCUSSION

Nonhistone chromosomal proteins have not been characterized biochemically as well as the histones because of their tremendous heterogeneity and poor solubility. Most studies of nuclear proteins have employed one-dimensional polyacrylamide gel electrophoresis in the presence of SDS. Using the BASO–DALT system, it is possible to

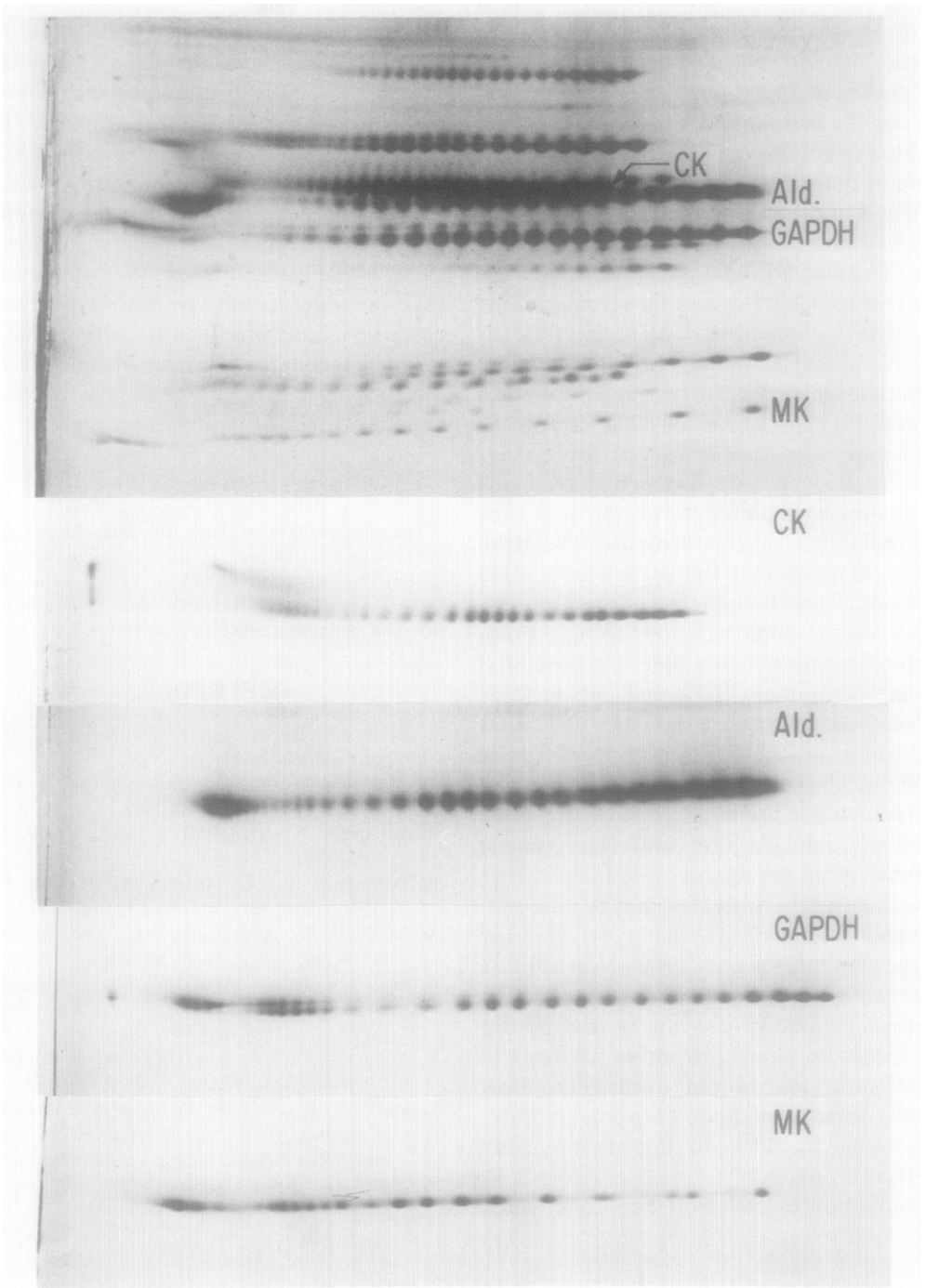

FIG. 5. Carbamylation trains of rabbit muscle soluble proteins. Procedures used to identify these muscle proteins have been described (5). First-dimension BASO gels contained Brinkman 2–11 ampholytes and were electrophoresed for 4000 V-h. Second-dimension gels were run as previously described (2). Gels are oriented as described in Fig. 1. (A) Rabbit muscle soluble proteins with creatine phosphokinase (CK), aldolase (Ald.), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and myokinase (MK) are identified. Sections of BASO–DALT gels showing the complete carbamylation trains of the purified proteins are also shown.

obtain high resolution two-dimensional polyacrylamide gel maps of these proteins. Except for the most basic cellular proteins, such as the histones, the proteins analyzed in this system appear to focus by 4000 V-h of electrophoresis. The patterns obtained at 4000 V-h are reproducible from experiment to experiment. Studies performed on histones require shortening the time of electrophoresis to 3200 V-h and these separations are also reproducible. Thus, the time of electrophoresis can be varied depending upon the sample, but we recommend elecrophoresis of the sample for 4000 V-h unless studies are being done on very basic proteins.

It has been demonstrated that changing the sample lysis buffer can greatly influence the mobilities of the proteins. O'Farrell *et al.* (4) state that the presence of SDS does not affect the mobility of the proteins. However, by comparing our sample preparation mixture containing phosphatidyl choline with their SDS and NP-40 lysis buffers, we found that the SDS does alter the electrophoretic mobility of basic proteins. Use of phosphatidyl choline and urea for sample preparation allows the most complete solubilization and the greatest mobility of all the agents tested.

Because of the variable times required for electrophoresis of BASO gels, internal charge standards are necessary to quantitate spot positions. Carbamylation trains used as internal standards provide a means for accurate comparisons from experiment to experiment, and would eventually allow comparisons to be made between laboratories, although they do not define exact isoelectric points.

In addition to Novikoff hepatoma cells, we have used the BASO–DALT system to examine the basic proteins in muscle samples (5), concentrated urinary proteins, liver proteins, and serum proteins. This technique should provide a better means for analysis of basic protein mixtures found in tissues and cells. Except for the histones, there have been relatively few studies of these basic proteins. Use of the conventional ISO procedure and the BASO technique described here provides a comprehensive mapping technique, showing histones as well as other basic proteins necessary to catalog human protein gene products.

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