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Very alkaline immobilized pH gradients for two-dimensional electrophoresis of ribosomal and nuclear proteins

Basic proteins normally lost by the cathodic drift of carrier ampholyte focusing, or separated by NEPHGE with limited reproducibility, could be well separated by two-dimensional (2-D) electrophoresis under equilibrium conditions using immobilized pH gradients (IPGs) 4–10 and 6–10 using a previously published protocol (Görg *et al., Electrophoresis* 1988, 9, 531–546). In the present study we have extended the pH gradient to pH 12 with IPGs 8–12, 9–12 and 10–12 for the analysis of very basic proteins. Different optimization steps with respect to pH engineering, gel composition and running conditions, such as substitution of acrylamide by dimethylacrylamide and addition of isopropanol with and without methylcellulose to the IPG rehydration solution (in order to suppress the reverse electroosmotic flow) were necessary to obtain highly reproducible 2-D patterns of ribosomal proteins from HeLa cells and mouse liver. Histones from chicken erythrocyte nuclei as well as total cell extracts of erythrocytes were also successfully separated under steady-state conditions. Due to the selectivity of isoelectric focusing in IPG 9–12, where the more acidic proteins abandon the gel, the tedious procedure of nuclei preparation prior to histone extraction can be omitted.

1 Introduction

Compared to classical two-dimensional (2-D) electrophoresis with carrier ampholytes [1, 2], 2-D electrophoresis with immobilized pH gradients (IPG-Dalt) [3] has produced significant improvements in 2-D electrophoretic separation with respect to (i) higher resolution, by using well-defined narrow pH gradients [3], (ii) improved reproducibility, as demonstrated by interlaboratory comparisons [4, 5], and (iii) higher loading capacity for micropreparative runs [6–8], which has accelerated spot identification by microsequencing and mass spectrometry [9]. Basic proteins, normally lost by the cathodic drift of carrier ampholyte focusing or separated by nonequilibrium pH gradient electrophoresis (NEPHGE, [10]) with limited reproducibility, were also separated well under equilibrium conditions, especially, when IPGs 6–10 were used for the first dimension [11]. Strongly basic proteins ($pI > 10$), such as ribosomal and nuclear proteins are usually not amenable to 2-D electrophoresis with isoelectric focusing in the first dimension. NEPHGE was used for the separation of ribosomal proteins [12] and Baso-Dalt for the 2-D analysis of histones [13]. However, with carrier ampholyte IEF, only a transient state of focusing pattern is obtained, with poor resolution and limited reproducibility. Nonequilibrium focusing is difficult to control with respect to reproducibility and highly sensitive to experimental conditions, batches of carrier ampholytes, run time, gel length, and sample composition [9]. Furthermore, proteins with similar isoelectric points are fastidious to separate by NEPHGE. Therefore, several methods based on the combination of acidic-urea gels with SDS-PAGE have been described for histones [14] and the "four-corner system" for the separation of ribosomal proteins [15, 16], where four different gel systems were used, but none of them was based on isoelectric focusing in the first dimension.

In contrast to former results with carrier ampholyte IEF, the use of narrow IPGs at the basic extreme should provide reproducible and highly resolved 2-D patterns of strongly basic proteins separated under steady-state conditions. However, when trying to formulate IPGs outside the pH 4–10 range, *e.g.* a more acidic (pH 3–4) or more alkaline (pH 10–11) IPG interval, one is faced with severe problems as the "buffering power" of water becomes nonnegligible, while outside these limits it rises so sharply as to nullify any attempt at using IPGs in these regions [17]. More recently, by using Immobiline $pK > 13$, a nonlinear IPG spanning a 10–12 interval was generated and optimized for IEF [18, 19]. First results of 2-D electrophoresis using IPG 10–12 were obtained for the separation of commercial histone preparations [20]. However, by applying those experimental conditions to other strongly basic proteins such as ribosomal proteins consisting of more than 80 individuals with closely related p*I*s (10.5–11.5, [21]) and low solubility at their isoelectric points, highly streaky 2-D patterns were obtained. Attempts to improve resolution by using narrow IPGs and longer separation distances failed, the horizontal streaks being even more pronounced. A series of optimization steps with respect to pH gradient engineering, gel composition, additives and running conditions were necessary in order to obtain highly resolved 2-D patterns separated under steady-state conditions.

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Nonstandard abbreviations: **DMAA,** dimethylacrylamide; **IPG-Dalt,** two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradient

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Table 1. Casting of alkaline IPG gels

Linear pH gradient	pH 8–12		pH 9–12		pH 10–12[b]	
	Heavy solution pH 8	Light solution pH 12	Heavy solution pH 9	Light solution pH 12	Heavy solution pH 10	Light solution pH 12
Immobiline p*K* 3.6	561 µL	40 µL	–	–	–	–
Immobiline p*K* 4.6	–	–	687 µL	–	375 µL	–
Immobiline p*K* 6.2	–	–	–	–	–	–
Immobiline p*K* 7.0	–	–	–	–	–	–
Immobiline p*K* 8.5	280 µL	173 µL	–	–	–	–
Immobiline p*K* 9.3	71 µL	71 µL	300 µL	–	–	–
Immobiline p*K* 10.3	213 µL	230 µL	452 µL	253 µL	540 µL	–
Immobiline p*K* > 13	61 µL	267 µL	59 µL	393 µL	–	500 µL
Acrylamide/Bis (28.8/1.2)	1.65 mL	1.65 mL	–	–	–	–
DMAA/Bis	–	–	630 µL/20 mg	630 µL/20 mg	630 µL/20 mg	630 µL/20 mg
Deionized water	5.15 mL	7.55 mL	5.9 mL	8.72 mL	6.5 mL	8.9 mL
Glycerol (100%)	2.50 g	–	2.50 g	–	2.50 g	–
TEMED (100%)	6.0 µL	6.0 µL	6.0 µL	6.0 µL	6.0 µL	6.0 µL
Persulfate (40%)	10.0 µL	10.0 µL	10.0 µL	10.0 µL	10.0 µL	10.0 µL
Final volume	10.0 mL	10.0 mL	10.0 mL	10.0 mL	10.0 mL	10.0 mL

a) For effective polymerization, the solutions are adjusted to pH 7 with 3 N acetic acid before adding the polymerization catalysts.
b) According to Righetti *et al.* [20], modified

2 Materials and methods

2.1 Apparatus and chemicals

All equipment for IEF and horizontal electrophoresis (Multitemp II thermostatic circulator, Multiphor II electrophoresis unit, Macrodrive 5 power supply, gradient mixer, DryStrip kit) were from Pharmacia Biotech (Uppsala, Sweden). The IsoDalt System was obtained from Pharmacia-Hoefer (San Francisco, CA, USA). Immobiline II, Pharmalyte 3–10, TEMED and ammonium persulfate were from Pharmacia Biotech. The acrylamido buffer solutions p*K* 10.3 and p*K* > 13 were a gift from Bengt Bjellqvist (Pharmacia, Uppsala, Sweden). GelBond PAG film was obtained from FMC (Rockland, ME). Acrylamide (2 × crystallized), *N,N'*-methylenebisacrylamide, SDS and silicone oil DC200 were purchased from Boehringer Ingelheim (Ingelheim, Germany). *N,N*-Dimethylacrylamide (DMAA) was from Fluka (Buchs, Switzerland). Argon was from Messer-Griesheim (München, Germany). The histones H2A, H2B and H3 were purchased from Boehringer (Mannheim, Germany). Cytochrome c (bovine heart), urea, glycerol, Pefabloc and all other chemicals for electrophoresis and staining were obtained from Merck (Darmstadt, Germany). DTT, iodoacetamide, Trizma base, CHAPS, agarose (Type I-a), *N,N'*-bis[3-aminopropyl]-1,4-butanediamine (spermine) and lysozyme (chicken egg white) were from Sigma (St. Louis, MO, USA).

2.2 Sample preparation

2.2.1 Ribosomal proteins

In order to remove contaminating nonribosomal proteins and virtually all RNA molecules, preparation of ribosomes and total ribosomal proteins (Tp80S) from rat liver and HeLa cells was according to Madjar [22], comprising cell fractionation, successive removal of nuclei and mitochondria, isolation of ribosomes followed by protein extraction, and selective RNA precipitation.

Table 2. Vh needed for steady-state IEF

	Separation distance	
	11 cm	18 cm
IPG 8–12	15000	35000
IPG 9–12	30000	45000
IPG 10–12	35000	50000

Finally, the isolated ribosomal proteins (Tp80S) were reduced, alkylated, dialyzed, and lyophilized. For IPG-Dalt, the pellet was dissolved in standard lysis buffer (9 M urea, 2% CHAPS, 1% DTT, 0.8% Pharmalyte 3–10 and 0.04% Pefabloc). Twenty µL of sample solution containing 40 µg purified total ribosomal proteins were applied per IPG strip.

2.2.2 Histones

All histone preparations, including the commercially available ones, were dissolved in a lysis solution (2 mg/mL) containing 9 M urea and 1% DTT, 0.8% Pharmalyte 3–10 and 0.04% Pefabloc. Preparation of histones from chicken erythrocytes was according to Csordas [23]. Whole erythrocytes as well as isolated nuclei were extracted with 0.3 M HCl. Proteins were precipitated by adding the appropriate amount of trichloroacetic acid to the acid extract to give the final concentration of 25%. The protein pellets were washed with acetone and dried *in vacuo*. Samples were applied at the anode.

2.2.3 Mouse liver extracts

Mouse liver was ground in a liquid nitrogen-cooled mortar, and the powder obtained was immediately suspended in 10% TCA in acetone (–18°C) containing 0.12% DTT and kept at –18°C overnight. Following centrifugation (35000 *g*, 30 min at –10°C), the supernatant was discarded and the pellet resuspended in acetone containing 0.2% DTT. After 1 h at –18°C the sample was spun again with 35000 *g* for 30 min. The supernatant was discarded and the pellet dried under vacuum. Fifteen mg

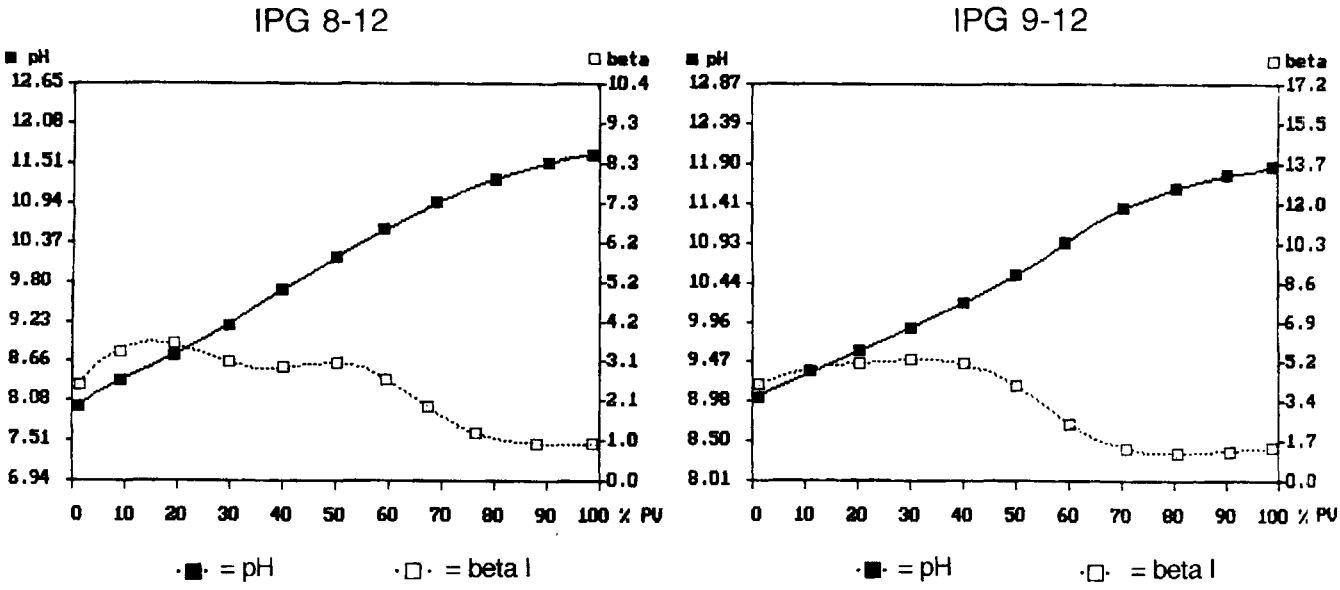

Figure 1. Profiles of pH 8–12 and pH 9–12 immobilized pH gradients corresponding to the protocols given in Table 1. The gradients were calculated by using Altland's IPGMAKER program [25]. The total amount of Immobiline species was limited to 20 mM and the β-power to 5 mequiv. L^{-1} pH^{-1}. ■, pH profiles; □, β-power.

of the pellet were suspended in 1 mL of a solution containing 9 M urea, 2% CHAPS, 1% DTT, 0.8% Pharmalyte 3–10 and 0.04% Pefabloc. After centrifugation, 20 µL per IPG strip were applied at the anode.

2.3 IPG-Dalt

IPG-Dalt was performed according to Görg *et al.* [3, 11, 24] with the modifications stated below.

2.3.1 First dimension: IEF in very alkaline IPG ranges

IPG gels with pH gradients 8–12, 9–12 and 10–12 were used. The IPG gel with a nonlinear gradient from pH 10–12 with 5%T and 4%C was cast according to the procedure optimized by Bossi *et al.* [19] using Immobilines with p*K* 4.6, p*K* 10.3 and p*K* > 13. For generation and optimization of IPGs pH 9–12 and pH 8–12, the computer program of Altland [25] was used. The optimized procedures are given in Table 2. For casting IPG gels pH 10–12 and pH 9–12, acrylamide was substituted by DMAA (not tested for IPG 8–12). When DMAA was used as monomer, the 5%T was increased to 6% DMAA (see Table 1). The washed and dried IPG gels were cut into 4 mm wide strips and rehydrated overnight. Rehydration deviated from the usual procedure in the following way: For ribosomal proteins, the reswelling solution contained 8 M urea, 10% isopropanol, 10% glycerol, 1% CHAPS, 20 mM DTT, 0.2% Pharmalyte 3–10 and 0.2% methylcellulose, or 8 M urea, 16% isopropanol, 10% glycerol, 1% CHAPS, 20 mM DTT and 0.2% Pharmalyte 3–10. For the focusing of histones the reswelling solution contained 8 M urea, 10% isopropanol, 10% glycerol, 20 mM DTT and 0.2% Pharmalyte 3–10; no CHAPS was added. Samples were applied at the anode. Twenty µL of sample solution were pipetted into sample cups using Pharmacia's dry strip system. IEF was performed under silicone oil. The silicone oil was degassed and flushed

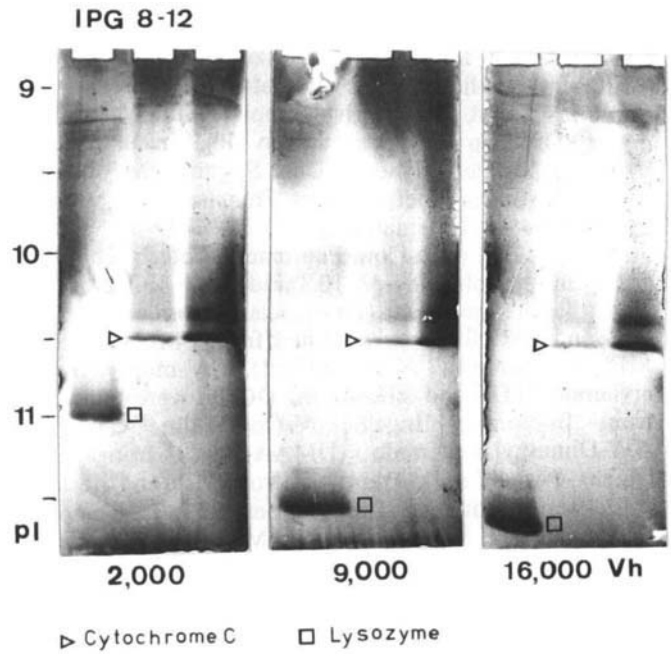

Figure 2. Native IEF with IPG 8–12 of cytochrome c (bovine heart) and lysozyme (chicken egg white). The given p*I*s of 10.6 ± 0.2 and p*I* 11.7, respectively, correspond to the calculated IPG 8–12. Steady state IEF patterns were obtained after ≈ 15000 Vh.

with argon prior to use. Additionally, the atmosphere in the chamber was replaced by argon. Electrode paper wicks were soaked with water and blotted against filter paper. The running conditions depended on the pH gradient and length of IPG gel strip used. An approximate time schedule for orientation is given in Table 2. In general, for improved sample entry, voltage was limited to 150 V (60 min) and 300 V (60 min) at the beginning. IEF, continued with a maximum of 3500 V or 5000 V to the steady state, was performed at 20°C. Current was limited to 0.05 mA per IPG gel strip. After termination

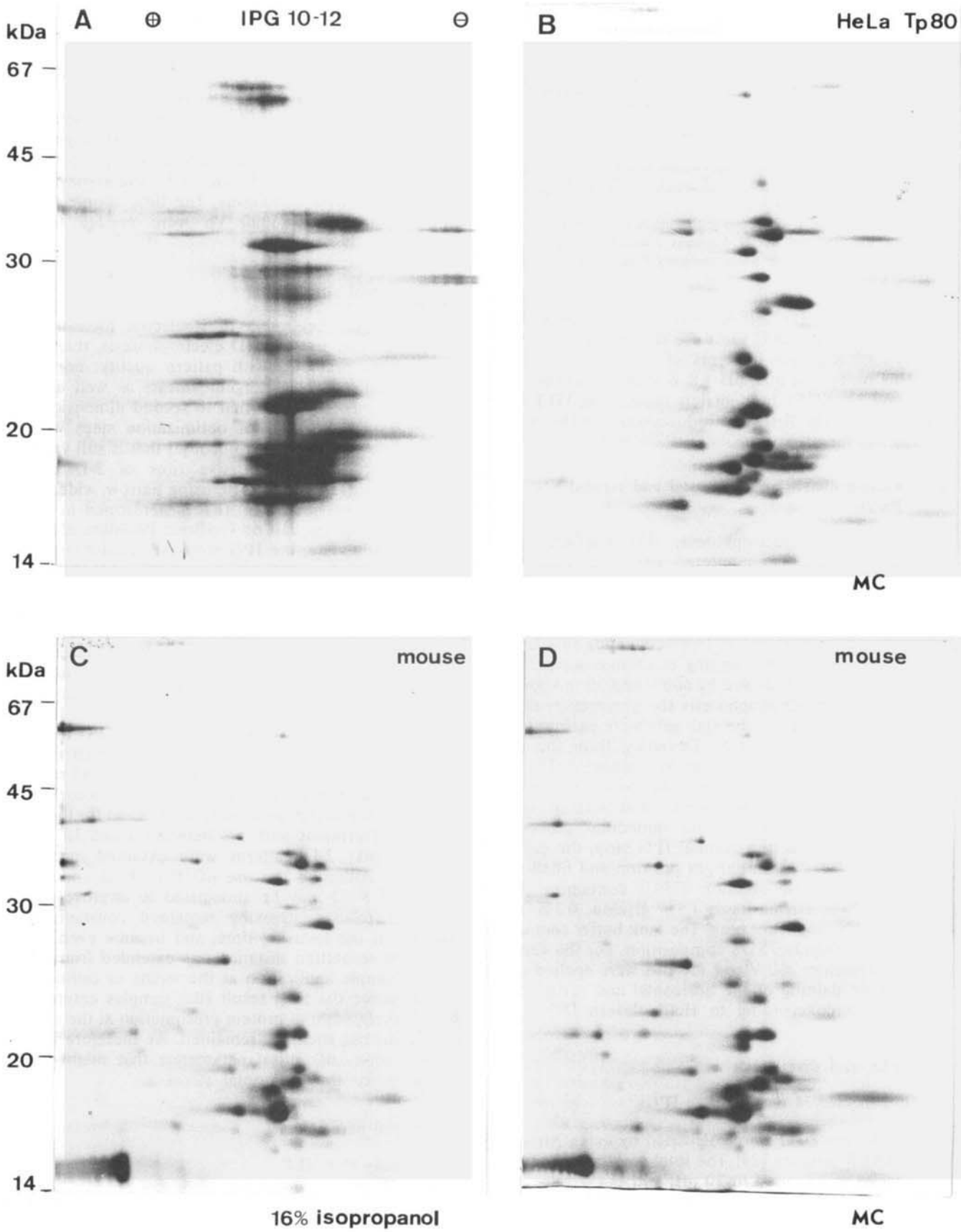

Figure 3. IPG-Dalt of ribosomal proteins using an IPG 10–12. (A) Standard protocol. Optimized protocol for very alkaline proteins: (B) highly purified ribosomal proteins Tp80S from HeLa cells and (C), (D) TCA/acetone extracts from mouse liver. For optimization, IPG gels were cast with DMAA and the strips were rehydrated (B), (D) in 8 M urea, 10% isopropanol, 10% glycerol, 1% CHAPS, 20 mM DTT, 0.2% Pharmalyte 3–10 and 0.2% methylcellulose, or (c) in 8 M urea, 16% isopropanol, 1% CHAPS, 20 mM DTT, and 0.2% Pharmalyte 3–10. See also Table 3. First dimension: IPG 10–12. Second dimension: vertical SDS-PAGE (13%T const.)

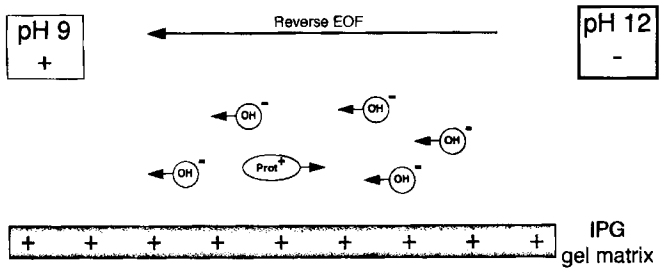

Figure 4. By using very basic Immobiline species such as p*K* 10.3 and p*K* > 13, the IPG gel matrix becomes positively charged at the cathodic end and there is water transport from cathode to anode (reverse EOF).

of the IEF, the IPG strips were stored (between two plastic sheets) at −78°C until use or equilibrated immediately for 2 × 15 min in 10 mL of a solution containing Tris-HCl buffer (50 mM, pH 8.8, 6 M urea, 30% w/v glycerol and 2% SDS). Immediately before use, DTT (1%) was added to the first, and iodoacetamide (4%) to the second equilibration step.

2.3.2 Second dimension: horizontal and vertical SDS-PAGE

Horizontal SDS electrophoresis [11] was performed on GelBond PAGfilm-supported gels (250 × 200 × 0.5 mm^3) containing 6% acrylamide in the stacking gel (4% C) and a homogeneous resolving gel containing 13% acrylamide (4%C) for the separation of ribosomal and mouse liver proteins or 15% acrylamide (4% C) for histone separation. The running conditions were 200 V and 30 mA for 1 h, followed by 600 V and 30 mA for 4 h. For vertical SDS electrophoresis the preparation of the chamber and casting of the slab gels were performed as described by Anderson [26]. Deviating from the horizontal procedure, no stacking gel was required. The gels were 1 mm thick and the cross-linker concentration was reduced to 2.7%. Total acrylamide concentration corresponded to that used for the horizontal procedure. For loading the first-dimensional IPG strip, the gel cassettes were placed in an upright position and filled with 2–3 mL of agarose solution (75°C) containing 0.5% agarose, 0.29% Trizma-Base, 1.5% glycine, 0.1% SDS and 0.03% bromophenol blue. The tank buffer contained the same Tris/glycine/SDS composition. For the vertical second dimension 200 V and 150 mA were applied overnight. Silver staining of the horizontal and vertical gels was performed according to Heukeshoven [27].

3 Results and discussion

3.1 Optimization of very alkaline IPGs

IPGs 9–12 and 8–12 were calculated by using Altland's IPGMAKER program [25]. The total amount of Immobiline species was limited to 20 mM and the β value to 5 mequiv. $L^{-1}pH^{-1}$. Our best recipes are listed in Table 1. The pH range and slope of the calculated IPGs with the corresponding profiles of β power are shown in Fig. 1. The calculation and optimization of the nonlinear IPG 10–12 was described more recently [17–19]. According to Righetti *et al.* [20] it was possible to improve and to generate more linear, very alkaline IPGs, but at a large expense in consumption of Immobiline species. For example, by increasing the amount of total Immobiline species to 50 mM, the IPG 10–12 was more linear; however, enormous swelling of the IPG gel matrix during the washing process and matrix-protein interactions during IEF were observed. In Fig. 2, the pH range of the calculated IPG 8–12 was tested by focusing cytochrome c (given p*I*s of 10.4, 10.6, and 10.8) and lysozyme (p*I* 11.7) under native conditions. The time course demonstrates that about 15000 Vh were needed for the focusing of lysozyme.

3.2 Horizontal streaking

From the very beginning of isoelectric focusing with IPGs and their use for 2-D electrophoresis, there have been severe problems with pattern quality: horizontal and vertical streaks, detergent smears as well as poor protein transfer from the first to second dimension [28]. However, after a series of optimization steps we succeeded in establishing a protocol [3] that is still valid for analytical or micro-preparative runs of 2-D electrophoresis with IPGs, pH 4–10, using narrow, wide, linear or nonlinear pH gradients. IEF is performed in individual IPG gel strips, cast on GelBond PAGfilm; after IEF to the steady state, the IPG strips are equilibrated and applied to a horizontal or vertical SDS gel [11]. To date, highly diverse samples have been separated successfully. Because of the excellent resolution and reproducibility the 2-D patterns were highly suited for interlaboratory comparison [5] and the establishment of image-based databases [29]. Moreover, basic proteins (*e.g.* mouse liver, yeast cell) were separated well under equilibrium conditions, especially when IPGs 6–10 were used for the first dimension [11]. However, by extending the IPG to pH 12 for the analysis of very alkaline proteins such as ribosomal proteins, a revival of poorly resolved 2-D patterns was observed: despite all optimization steps according to the standard protocol previously established for the great majority of proteins with p*I*s between 4 and 10 [3, 11], highly streaky 2-D patterns were obtained repeatedly (Fig. 3A). Although the use of IPGs 9–12 and 10–12 instead of 8–12 may be anticipated to improve resolution, the relative streaking remained constant, independent of the focusing time, and became even worse when the separation distance was extended from 11 to 18 cm. Sample application at the anode or cathode did not influence the final result (the samples entered the gels properly, without protein precipitation at the application point) but streaking remained. We therefore investigated a series of critical parameters that might be responsible for the horizontal streaks.

3.3 Critical parameters

3.3.1 Steady state IEF

The influence of focusing time on pattern quality was demonstrated with basic yeast cell proteins using IPG 7–10 for the first dimension [30]: horizontal streaking resulted from insufficient or excessive focusing times, whereas optimum resolution with distinct protein spots was obtained when proteins focused at equilibrium.

Table 3. Summary of the major changes of the IPG-Dalt protocol for very alkaline proteins (pH < 12) compared to the standard protocol [3] described for proteins with p*I*s up to pH 10.

Standard (pH < 10)	IPG – DALT	Very alkaline (pH < 12)
	IPG gel strip	
Acrylamide	Gel matrix	DMAA
8 M urea, 0.5% CHAPS, 0.2% CA 10 mM DTT	Rehydration	(i) 8M urea, 0.5% CHAPS, 0.2% CA, 20 mM DTT, 10% glycerol, 10% isopropanol, 0.2% methylcellulose (ii) 8 M urea, 0.5% CHAPS, 0.2% CA, 20 mM DTT, 16% isopropanol
Steady state, 20°C, no oil	IEF	Steady state, 20°C, under silicone oil, argon
	Storage	
50 mM Tris-HCl, pH 8.8, 2% SDS, 1% DTT, 6 M urea, 30% glycerol, 4% iodoacetamide	Equilibration	50 mM Tris-HCl, pH 8.8, 2% SDS, 1% DTT, 6 M urea, 30% glycerol, 4% iodoacetamide
Horizontal/vertical	SDS-PAGE	Horizontal/vertical

Nonstandard abbreviation: CA, carrier ampholytes

However, by prolonging the focusing time from 20000 to 35000 Vh, horizontal streaking remained in the 2-D patterns of ribosomal proteins focused within an IPG 9–12, indicating that a steady state IEF pattern was already obtained after 20000 Vh (not shown).

3.3.2 Nucleic acid-protein interactions

Although nucleic acids migrate outside the range of IEF gels, they can interact ionically with carrier ampholytes and polypeptides. Severe streaking, especially in the IEF dimension, was observed when samples were prepared from nuclear protein complexes. Contamination by nucleic acids can adversely affect the resolution obtained on gels since many proteins will bind nucleic acids non-specifically. This means that a protein species, instead of being a homogeneous population, can become a heterogeneous one, consisting of individual molecules carrying widely varying charges of nucleic acids bound to them [31]. To ensure that the horizontal streaks in the 2-D patterns of ribosomal proteins (Fig. 3A) were not due to the nucleic acid contamination deriving from the disruption of nuclei or incomplete RNA precipitation during sample preparation, sample solution was treated with specific nucleases for enzymatic digestion [5]. Alternatively, a basic polyamine, *e.g.* spermine, was added to the sample solution in order to precipitate remaining traces of nucleic acids [32]. However, neither nuclease treatment nor spermine addition improved the quality of the pattern (not shown), indicating that horizontal streaking was not a result of the presence of contaminating nucleic acids.

3.3.3 Carbamylation

Since cyanate ions can react with some proteins and give rise to spurious bands, the probability of artifactual charge heterogeneity due to carbamylation of polypeptides in the presence of urea (> 8 M) at alkaline pH (pH > 10) was also investigated. Formation of cyanate ions was avoided by routine precautions, *e.g.*, use of pure-grade urea, use of freshly made urea solutions, no heating above 37°C and filtration over mixed-bed ion-exchange resins before use, and addition of carrier ampholytes to the sample solution as cyanate scavenger. However, the formation of cyanate ions during IEF in IPGs up to pH 12 could not be excluded, due to the long focusing times, particularly at alkaline pH, and the lack of carrier ampholyte species which do not extend above pH 11. Therefore, spermine as primary amine and cyanate scavenger [32] was added to the sample solution (9 M urea, 1% CHAPS, 40 mM DTT and 20 mM spermine) and/or to the reswelling solution. However, there was no visible difference with respect to horizontal streaks in the 2-D patterns of ribosomal proteins.

3.3.4 Hydrophobic interactions and protein precipitation at the isoelectric point

Ribosomal proteins are dissolved well in the presence of 8 M urea, even at a basic pH (8.6), at which most of the proteins still exist as highly charged cations [22]. However, reaggregation of the ribosomal proteins during IEF at the basic extreme (IPG 10–12) could not be excluded. If so, severe streaking could occur, resulting from the slow dissolution of the aggregates during the separation process. In order to diminish possible protein precipitation at pH values close to their isoelectric points and/or to prevent protein binding to the IPG gel matrix due to hydrophobic interactions, different detergents at different concentrations were tested. Neither the addition of nonionic detergents such as Nonidet P-40 nor increasing the amount of amphoteric detergents (such as CHAPS) from 1% to 4% was successful.

3.3.5 Electroendosmotic flow

Charged groups present on the gel matrix for IEF can also be responsible for pattern disturbances. First, this can result in an ion-exchange effect of the matrix on the proteins. More importantly, those fixed charged groups lead to electroendosmosis. Usually the negatively charged groups on the matrix result in electroendosmotic migration of H^+ ions, as hydrated protons (H_3O^+),

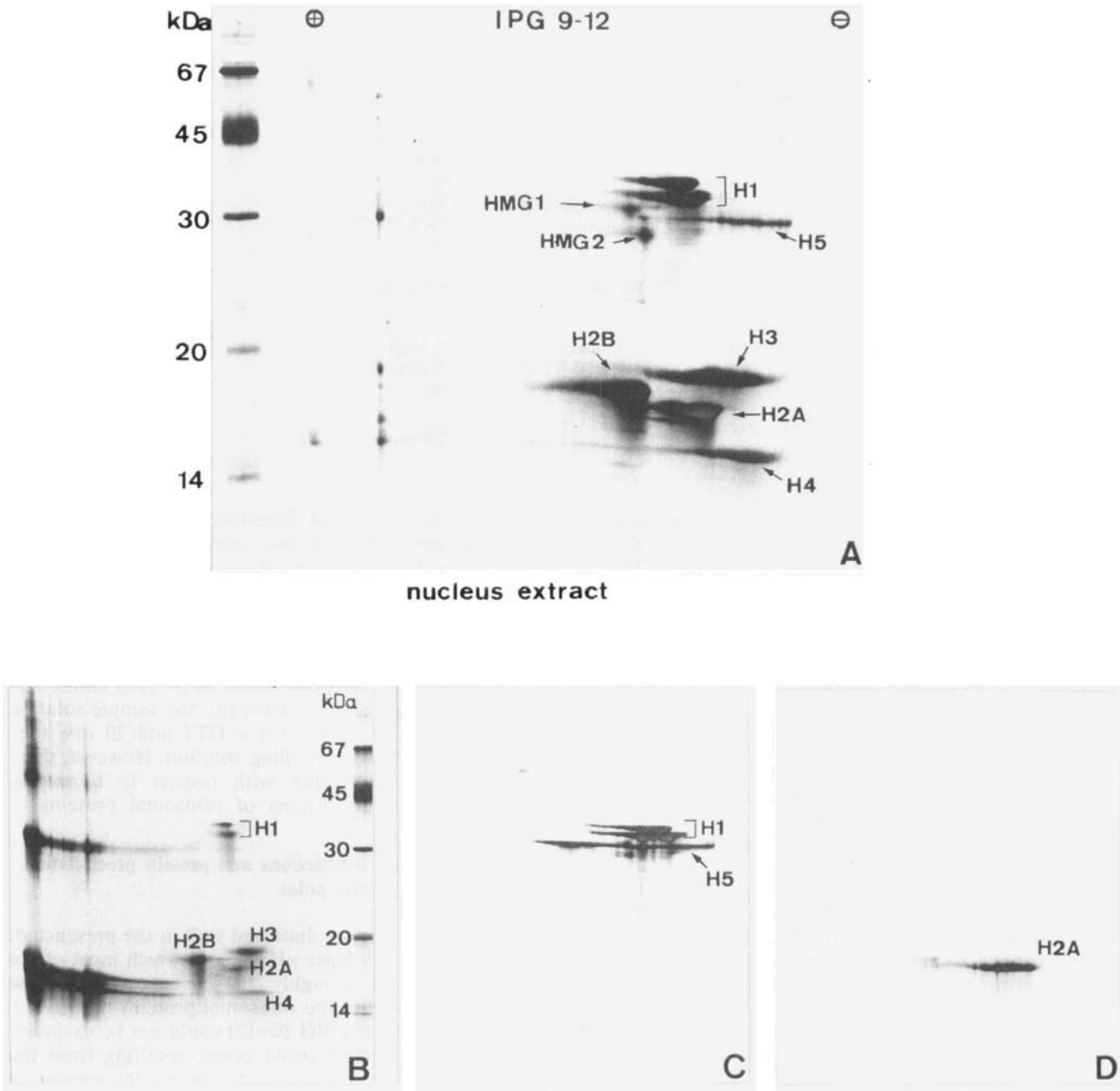

Figure 5. IPG-Dalt of histone preparations using IPG 9–12 as the first dimension. Second dimension: horizontal SDS-PAGE (15%T const.). (A) Nucleus extract from chicken erythrocytes. (B) Total cell extract from chicken erythrocytes (without preceding nuclear preparation). Heterogeneity of (C) H1 and H5 fractions and (D) H2A. Linker histones: H1 and H5. Core histones: H2A, H2B, H3 and H4. Nonhistone high mobility group proteins: HMG1 and HMG2.

towards the cathode. This effect is normally small in the case of polyacrylamide gels [9]. However, when alkaline IPGs up to pH 12 are generated with the help of very basic Immobilines with pK 10.3 (tertiary amino groups) and pK > 13 (quaternary base), the polyacrylamide matrix becomes positively charged at the cathodic gel end with a resultant active water transport from the cathode to the anode. This reverse electroendosmotic flow (EOF) is decidedly stronger when the IPG exceeds pH 11 and approaches pH 12. The strong water transport from the cathode to the anode is easy to observe because during IEF the paper wick of the cathode dries out and sticks to the gel whereas the paper strip at the anode is waterlogged (Fig. 4). During the IEF run the cathodic wick has to be replaced with new strips soaked with water, otherwise IEF will stop and overheating at the cathodic end cannot be avoided.

In order to reduce the anode-directed water flow, Bossi *et al.* [19] proposed a combination of 8 M urea and a sorbitol gradient.

3.3.5.1 The 0–10% sorbitol gradient

Adopting the procedure of Bossi *et al.* [19] the IPG gel strips were rehydrated in a cassette containing a 0–10%

sorbitol gradient (anode to cathode) in order to reduce the reverse EOF and to quench the high conductivity of the gel at the cathodic end. By applying this reswelling procedure, as well as the running conditions that were used for IEF of histones in an IPG 10–12 for IPG-Dalt of some commercial histone preparations, quite promising results were obtained [20]. However, this procedure did not apply for the 2-D analysis of ribosomal proteins (Fig. 3A) where more than 5000 Vh were necessary to obtain not only a transient but a steady-state 2-D pattern.

3.3.5.2 Addition of isopropanol

Confronted with the strong electroendosmotic flow towards the anode that inhibited all attempts to focus the alkaline proteins to equilibrium, new efforts were made to reduce the reverse EOF. Isopropanol was successfully used in capillary electrophoresis for minimizing the EOF: a reduction of 40% of the EOF was observed when 10% isopropanol was used [34]. The addition of 10% isopropanol to the reswelling solution of the very alkaline IPG gel strips improved the quality of the 2-D pattern of ribosomal proteins significantly. Moreover, a steady state was apparently reached after 20000 Vh (IPG 10–12, 110 mm): Almost identical 2-D patterns were obtained (not shown) by prolonging the focusing time to 34000 Vh. Finally, by increasing the isopropanol concentration from 10% to 16%, for the first time the majority of ribosomal proteins appeared as round spots.

3.3.5.3 Addition of methyl cellulose

Alternatively, methylcellulose was added to the reswelling solution in order to minimize the EOF. By analogy to capillary electrophoresis (M. Chiari, personal communication), 0.2% of methylcellulose proved to be most successful.

3.3.5.4 DMAA gels

In contrast to all predictions, IPG gels proved to be most stable, even at the alkaline extreme, and steady-state IEF patterns were obtained. However, a nonvisible hydrolysis of the alkaline IPG gels could not be excluded. The formation of charged breakdown products in acrylamide gels, in particular acrylic acid, might enhance reverse EOF. For this reason, IPG gels (9–12, 10–12) with DMAA instead of acrylamide gels were prepared and tested. DMAA as monomer is supposed to form the most stable gels even under extreme conditions, such as NaOH treatment over a long period of time [35]. The stability of DMAA gels was described long ago, but in view of their hydrophobic properties, they were not considered favorable for IEF, especially under native conditions [35]. However, according to our experience, the use of such gels for IEF under denaturing conditions and in the presence of isopropanol did not result in adverse effects on pattern quality. Furthermore, an immediate reduction of the dehydration at the cathodic end (gel and paper wick) was observed, indicating a reduced water transport. Optimum results for very alkaline proteins were obtained by using IPG gels cast with DMAA rehydrated in presence of 16% isopropanol (Fig. 3C) or in 10% isopropanol in combination with 0.2% methylcellulose (Fig. 3B and 3D), so that the reswelling solution contained either 8 M urea, 10% isopropanol, 10% glycerol, 1% CHAPS, 20 mM DTT, 0.2% Pharmalyte 3–10 and 0.2% methylcellulose, or 8 M urea, 16% isopropanol, 1% CHAPS, 20 mM DTT, and 0.2% Pharmalyte 3–10.

3.4 The optimized protocol

The actual protocol for 2-D analysis of very alkaline proteins is summarized in Table 3. Compared to the standard protocol [3] for proteins with p*I*s up to pH 10, the major differences are (i) the gel composition: acrylamide was substituted by dimethylacrylamide, (ii) the rehydration solution contains isopropanol with and without methylcellulose in order to reduce the reverse EOF, and (iii) IEF is performed under silicone oil flushed with argon. IPG 8–12, 9–12, and 10–12 gels, 11 or 18 cm long, were employed. For gel casting, acrylamide as monomer was replaced by DMAA. After polymerization the gels were washed, dried and cut into 4 mm strips as described before. In contrast to the standard protocol, the rehydration solution contained either 8 M urea, 10% isopropanol, 10% glycerol, 1% CHAPS, 20 mM DTT, 0.2% Pharmalyte 3–10 and 0.2% methylcellulose, or 8 M urea, 16% isopropanol, 1% CHAPS, 20 mM DTT, and 0.2% Pharmalyte 3–10. Rehydration of the gel strips in the reswelling cassette to the original gel thickness (0.5 mm) was overnight at room temperature. Before IEF the rehydrated gel strips were rinsed with water for several seconds in order to remove excess reswelling solution and in order to avoid crystallization of urea on the surface of the gels during IEF. The IPG strips were blotted against moist filter paper and placed into the tray for focusing. The electrode paper strips were soaked with water and, after blotting, placed at the anodic and cathodic end. The strips were covered with silicone oil that had been degassed and flushed with argon. Preferably, the samples were applied at the anode.

3.5 IPG-Dalt of ribosomal proteins

Figure 3 shows the 2-D pattern of total ribosomal proteins Tp80S from HeLa cells separated under standard conditions (Fig. 3A) and according to the optimized protocol (Fig. 3B). There is a significant improvement in pattern quality. The majority of ribosomal proteins appears as clear, distinct, round spots and a true steady-state 2-D pattern is obtained. For the first time, ribosomal proteins, normally not amenable to 2-D electrophoresis with IEF in the first dimension [9] could be successfully separated under steady state conditions, revealing highly reproducible 2-D patterns. Figure 3B represents highly purified ribosomal proteins Tp80S from cultured HeLa cells [22].

3.6 IPG-Dalt of very alkaline mouse liver proteins

The proper isolation of ribosomal proteins [22] is an efficient but also time-consuming procedure, comprising

cell fractionation, successive removal of nuclei and mitochondria, isolation of ribosomes, followed by protein extraction and selective r-RNA precipitation, reduction, alkylation and dialysis of the sample; therefore, a simplified extraction procedure of alkaline proteins from total cell extracts was also tested. Extraction of mouse liver tissue with lysis buffer (pH 6.5) was not successful because the ribosomal proteins appeared only as faint protein spots, showing that ribosomal proteins represent only a small proportion of total cell protein. However, TCA-acetone extraction was successfully used for sample preparation of very alkaline proteins. In this case, mouse liver was ground in a liquid nitrogen cooled mortar, and the powder was suspended immediately in a solution of 10% TCA in acetone with 0.12% DTT at −18°C. For protein precipitation, the TCA-acetone extract was kept at −78°C overnight. After centrifugation (35000 *g*, 30 min), the pellet was washed with acetone containing 0.12% DTT at −18°C. The supernatant was discarded and the pellet dried under vacuum before solubilization in a solution of 9 M urea, 2% CHAPS, 1% DTT, 0.8% w/v Pharmalytes 3–10 and 8 mM Pefabloc. Under these conditions, at acidic pH (10% TCA in acetone) alkaline proteins such as ribosomal and nonribosomal proteins are perfectly solubilized as cations and dissociated from r-RNA, and finally precipitated.

Figures 3C and 3D depict the 2-D patterns of TCA/acetone extracts from mouse liver. In Fig. 3C 16% propanol-2 was used for rehydration of the IPG gel strips, while in Fig. 3D 10% propanol-2 in combination with 0.2% methylcellulose was used. There is no significant difference between the 2-D patterns. Both rehydration solutions (see Sections 3.3.5.2 and 3.3.5.3, respectively) may be used. Presently, methylcellulose is preferred in our laboratory. By comparing the 2-D patterns of highly purified ribosomal proteins from HeLa cells (Fig. 1B) and the crude tissue extracts from mouse liver, a striking similarity is observed. This indicates that the majority of the alkaline mouse liver proteins extracted by TCA/acetone, and focused selectively in IPG 10–12, represent ribosomal proteins.

3.7 IPG-Dalt of histones

The difficulties which arise in the electrophoretic analysis of histones are due to their high positive net charge, their tendency to aggregate and their similar molecular masses. Several methods based on combinations of acidic-urea gels with SDS-electrophoresis have been described [14]. Using NEPHGE for histone protein separation [13], there are some problems in the transient state of the 2-D patterns and poor resolution exists at the basic extreme, so that the small charge differences cannot be distinguished. By applying the histone preparations from chicken erythrocyte nuclei to an IPG 9–12, histone typing in the resultant 2-D pattern was successful (Fig. 5). Clearly visible were the core histones H3, H4, H2B and H2A with the calculated p*I*s of 11.9, 11.9, 11.5, and 11.7. The linker histone H1 splits into two major bands. H5, which is typical for nucleated erythrocytes (chicken, fish, *etc.*), is subject to multiple phosphorylations. The nonhistone high mobility group proteins HMG 1 and HMG 2 are also well separated from H1/H5 and from each other. Furthermore, IPG-Dalt with highly basic pH gradients has the potential for analyzing histones and HMG proteins after their direct acidic extraction from whole cells without need for a preceding nuclear preparation (Fig. 5B). The heterogeneity of H1 and H5 fractions, probably due to multiple phosphorylations, can be demonstrated (Fig. 5C). Also, the heterogeneity of the H2A preparation of calf thymus, most likely due to modifications such as multiple acetylations, becomes visible by using IPG-Dalt 9–12 (Fig. 5D). IPG-Dalt of histones is a most promising approach compared to NEPHGE or BASO-Dalt, where only a transient state of separation with poor resolution and reproducibility is obtained.

4 Concluding remarks

In contrast to former results with carrier ampholyte IEF, the use of very alkaline IPGs such as IPG 8–12, IPG 9–12, as well as IPG 10–12 provides highly reproducible and highly resolved 2-D patterns of strongly basic proteins (*e.g.* histones and ribosomal proteins) that are focused under steady-state conditions. As shown by the TCA/acetone extracts from mouse liver, there are many more strongly basic proteins to investigate than expected.

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