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PROCEDURES FOR TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS OF NUCLEAR PROTEINS

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SUMMARY

A series of methods designed for electrophoresis of nuclear proteins is described. They deal with the low solubility of many nuclear proteins and with the presence of large amounts of nucleic acids. These were eliminated by enzymatic digestion, centrifugation, partition or precipitation. A combination of RNAse digestion and centrifugation is the method of choice when proteolysis is low. In the opposite case, precipitation or partition methods are preferred, at the expense of precipitation of some proteins. When the nuclear RNA content is low, centrifugation in an Airfuge is the simplest and the most efficient method, superseding the widely used S1 nuclease method.

INTRODUCTION

Nuclear proteins have been studied extensively because of their relevance to genetic regulation. One-dimensional electrophoretic techniques were first used in numerous studies on the variations of these proteins related to different genetic expression schemes[1,2]. An increase in resolution seemed necessary and two-dimensional separations were introduced by Busch and co-workers[3]. These systems used electrophoresis under two sets of conditions, but the separation criteria were linked, so that the proteins were displayed principally on the diagonal, providing therefore a small increase in resolution. Systems using two independent separation criteria (p*I* and molecular mass) were introduced by Barret and Gould[4], and their use became widespread after the pioneering work of O'Farrell[5]. The first application of this system to nuclear protein was reported by Peterson and Mc Conkey[6], and numerous other studies have followed[7–10]. However, good quality and reproducible gels have been obtained in only a few cases[6,10], most of the gels published exhibiting a marked background and considerable streaking[8]. Moreover, reproducibility is generally poor[11]. These phenomena are caused by the low solubility of most nuclear proteins and by high levels of nucleic acids, which induce numerous artefacts in two-dimensional electrophoresis[12].

Our aim in this work is to describe techniques giving a significant increase in resolution and in the reproducibility of the patterns obtained.

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MATERIALS AND METHODS

Cell culture

This work was carried out on *Drosophila* Kc cells[13]. A diploid subclone, 8-9K, was kindly provided by G. Echalier and M. Bestbelpomme, and was used throughout this study. The cells were grown on Echalier's D22 medium and plated every 5 days. The lag phase lasts approximately 1 day and the growth phase 3 days. Cells remain wholly viable for over a week after the plating (*ca.* 12 days). The radiolabelling of cells was carried out as described[14] or by labelling for 48 h in complete medium with 100 μCi/ml [^{35}S]methionine (> 800 Ci/mmol, Amersham).

Cell fractionation

The nuclei were prepared by a modification of previously described methods[15]. Briefly, cells were disrupted in hypotonic medium (0.1 *M* sucrose; 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)–sodium hydroxide; 10 m*M* $MgCl_2$) containing 0.5% Nonidet P-40. The homogenate was centrifuged for 25 min at 30 000 *g* over a 2-ml cushion of 1.8 *M* sucrose in the same buffer. The resulting pellet of nuclei contained almost no cytoplasmic tags when examined by phase-contrast microscopy.

Protein radiolabelling

The proteins were labelled either *in vivo* by [^{35}S]methionine incorporation or *in vitro* by reductive methylation using formaldehyde and tritiated sodium tetrahydroborate as described for example by Kuhn and Wilt[16]. In some experiments reductive methylation was performed as described by Jentoft and Dearborn[17] but using cold formaldehyde and tritiated sodium cyanoborohydride (30–150 Ci/g, Amersham).

Sample preparation

The samples were freed from nucleic acids and prepared for two-dimensional electrophoresis by different methods, which can be classified into four types.

Enzymatic methods. These methods use either S1 nuclease, as described by Zechel and Weber[18], or a cocktail of DNAse and RNAse. For maximum enzymatic activity, digestion was performed at 25°C for 30 min in a non-denaturing medium. This medium contains 2 *M* sodium chloride for dissociating the chromatin, 2 m*M* magnesium chloride and 25 m*M* HEPES buffer pH 7.5. DNAse and RNAse are each added at 5 μg per 100 μg DNA. In some experiments, RNAse alone was used, DNA being eliminated by another method. The digestion buffer comprised 25 m*M* HEPES buffer, 5 m*M* EDTA and 0.35 *M* sodium chloride. In all the cases, 1 m*M* phenylmethanesulphonyl fluoride (PMSF) was used as a protease inhibitor. The digestion was stopped and the proteins separated by precipitation with trichloroacetic acid (TCA). The sample was resuspended in 100 μl of modified O'Farrell lysis buffer and loaded onto the first dimension gel.

Centrifugation methods. In these methods, the nuclear pellet was resuspended in modified O'Farrell buffer containing 3-(3-cholamidopropyl)dimethylammonio-1-propanesulphonate (CHAPS), lysolecithin or lecithin as detergent. The solution was centrifuged to yield pellet particles with a sedimentation coefficient greater than 7S. The supernatant was carefully collected and loaded onto the first dimension gel.

Partition methods. This method is an adaptation of LeStourgeon's method[19] for two-dimensional electrophoresis. Briefly, the nuclear pellet was dissolved in HEPES buffer (100 m*M* pH 7.5) containing 35 m*M* EDTA, 2.5% sodium dodecyl sulphate (SDS) and 5% mercaptoethanol. The viscosity was reduced by pipetting and the solution heated at 100°C for 2 min. The insoluble material was then pelleted at 10 000 *g* for 10 min. The supernatant was collected and one volume of water-saturated phenol was added. The extraction was carried out at room temperature for 15 min and the phases were separated by centrifugation (10 000 *g* for 10 min). The phenol phase was collected and ten volumes of SDS-extracting solution, acetone–tributylamine–acetic acid–water (89:5:5:1, v/v/v/v), were added[20]. After 1 h at −20°C, the protein precipitate was collected by centrifugation, washed once with diethyl ether and thoroughly dried. The pellet was resuspended in lysis buffer and used in two-dimensional electrophoresis.

Precipitation methods. The nuclear pellet was redissolved in 9.5 *M* urea containing 2% Nonidet P-40, 50 m*M* Tris–HCl pH 7.8 and 5% mercaptoethanol. A 0.1 volume of 0.2 *M* lanthanum trichloride in water was added, and the solution left at room temperature for 1 h. It was then centrifuged at 10 000 *g* for 30 min. The supernatant was collected and 0.1 volume of ampholines was added. This mixture was used for two-dimensional electrophoresis.

In another method, the nuclear pellet was dissolved in 100 μl of HUC buffer (25 m*M* HEPES buffer pH 7.5, 8 *M* urea, 1 *M* calcium chloride). The mixture was centrifuged for 1 h at 30 p.s.i. in an Airfuge ultracentrifuge. The supernatant was collected, diluted with water to 0.7 *M* urea concentration and the proteins were precipitated by 20% TCA. The precipitate was collected by centrifugation, rinsed with diethyl ether and redissolved in modified O'Farrell lysis buffer for loading.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed essentially as described by O'Farrell, with the following alterations. For isoelectric focusing, the electrolyte molarity was raised to 50 m*M* and the voltage applied to 9500 V h, as described by Duncan and Hershey[21]. The sample was always applied at the basic end, except in a few control experiments. In some experiments, Nonidet P-40 was replaced by CHAPS in the lysis buffer and in the gel mixture, as suggested by Perdew *et al.*[22]. The second dimension gel was a linear 7–14% acrylamide gradient gel. As the proteins were radiolabelled, detection was performed by fluorography[23] using a dimethyl sulphoxide–2,5-diphenyloxazole (DMSO–PPO) procedure[23]. The impregnated gels were generally exposed for 4 days at −80°C on Kodak X-Omat S film.

RESULTS AND DISCUSSION

Two-dimensional electrophoretic analysis of nuclear proteins is very difficult. The poor solubility of many of these proteins leads to precipitation artefacts as shown in Fig. 1. Moreover, the great amount of material precipitated at the sample application point precludes all quantitative analysis of the gels. To solve this problem, we tried to increase the solubilizing power of the medium and to lower the amount of material necessary to obtain a good pattern, *i.e.*, to use a detection method with the highest sensitivity. Silver staining was not sensitive enough and detected the nucleic

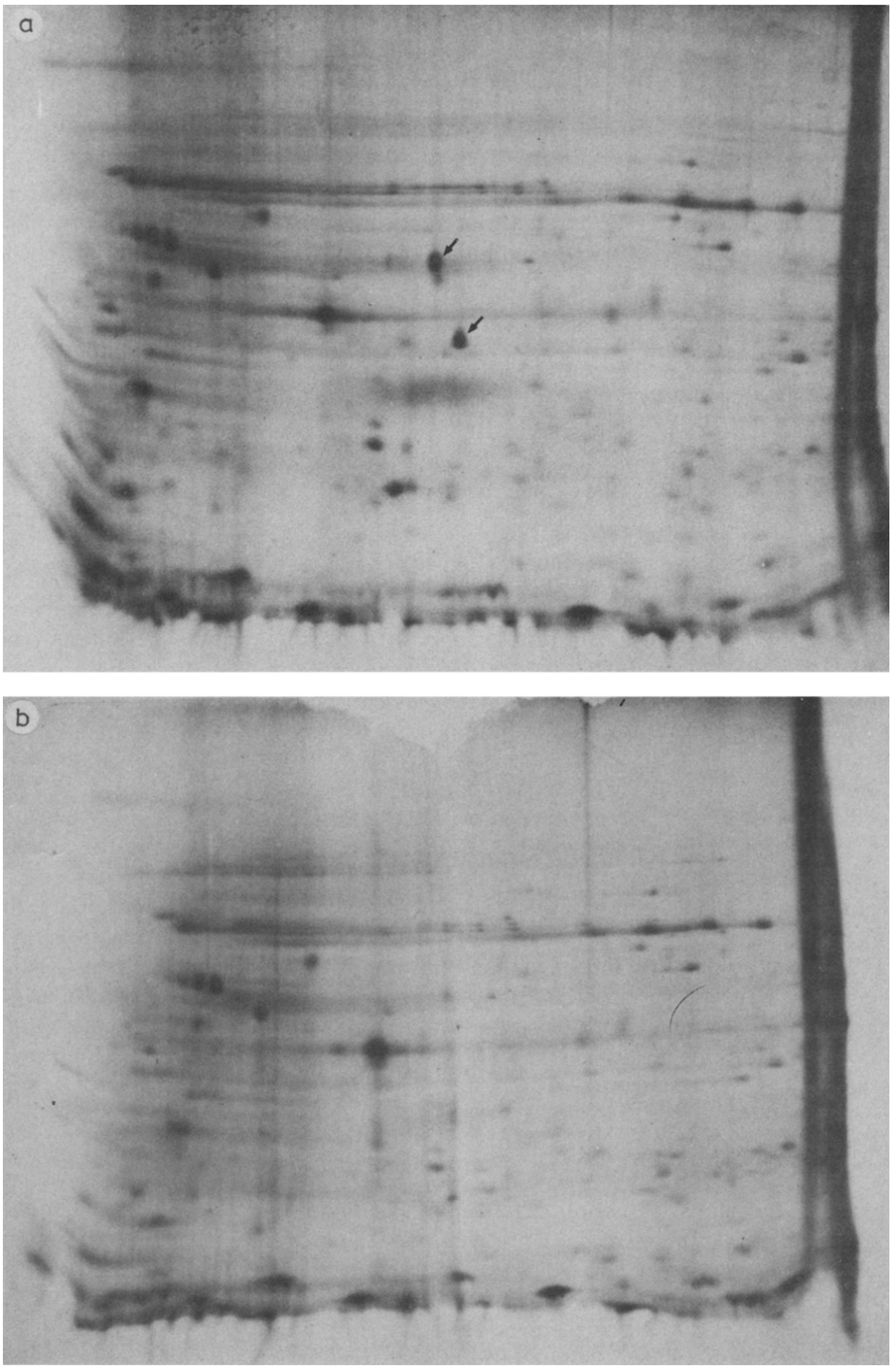

Fig. 1. Two-dimensional electrophoresis of nuclear proteins. After *in vivo* labelling with radioactive methionine, the nuclear proteins were freed from DNA and RNA by lanthanum precipitation. 150 (a) and 300 μg (b) of proteins were loaded onto the first dimension gel. The proteins which have precipitated in the most heavily loaded gel are indicated by arrows. All the gels are displayed with the acidic end on the left, and the high molecular weights at the top.

acids, causing an increased background (data not shown). We therefore chose to radiolabel the proteins. Radiolabelling by incorporation of a radioactive amino acid was not convenient for several reasons. First, labelling with a single amino acid such as [^{35}S]methionine does not allow the detection of all the proteins. Secondly, high efficiency labelling methods require depletion of the culture medium in the labelling amino acid. This may cause metabolic perturbation to the cells and therefore artefactual changes of the nuclear protein patterns. Thirdly, the incorporated radioactivity may cause irradiation damage to the cells, and changes in the protein patterns have already been observed[24]. This is especially true of long-term labelling, which is required for maximum specific activity[25].

We therefore chose an *in vitro* labelling method. Only a few methods can be applied to two-dimensional electrophoretic analysis. Among them, we chose the method described by Kuhn and Wilt[16], but we used a tritiated borohydride with a higher specific activity (80 instead of 20 Ci/mmol). Specific activities as high as 10^6 cpm per μg protein can be reached. This allows the detection of over 1000 protein spots with a 4-days exposure and a 25-μg protein load (see Fig. 4a for an example). An additional advantage of this method lies in its general use even when radioactive amino acid incorporation cannot be used, as with whole organs.

We then searched for a dissociating medium which would allow maximum solubilization of the nuclear proteins. A variety of detergents and chaotropics were tested, since O'Farrell's medium (Nonidet P-40 and urea) is known to be insufficient for hydrophobic[22] and nuclear proteins[26]. As detergents, we tried lecithins, lysolecithins[26] and sulphobetaines[27], but their low solubility in urea forced us to use Nonidet P-40 as additional detergent, which decreased the solubilization power. On the other hand, the use of CHAPS, which is a peculiar sulphobetaine, led to considerable improvement in the solubilization step[22]. In some non-equilibrium pH gradient gel electrophoresis (NEPHGE) experiments (Fig. 2), histones are clearly shown as streaks at the basic end of the gels (as in ref. 26) and no precipitate can be seen at the sample application point on the acidic side of the gel, indicating a total solubilization of the proteins. However, the chromatin is not fully dissociated under these conditions, and some proteins are eliminated with the nucleic acids. It is our feeling that additional research in the field of zwitterionic detergents will lead to media having a still higher solubilizing power, leading to full chromatin dissociation.

We also tested chaotropics other than urea. Preliminary experiments showed that tetramethylurea was a quite promising agent, and the dissociating power of a mixture of sulphobetaines (3-N-tetradecyl-N,N-dimethylammonio-1-propanesulphonate, SB 3-14) and tetramethylurea is comparable to that of boiling SDS (Table I). However, tetramethylurea is not compatible with acrylamide polymerization. While this work was in progress, the synthesis of modified acrylic derivatives compatible with organic solvents was reported[28]. This also should lead to new isoelectric focusing media with much higher performances than the current ones.

The other major problem in nuclear protein analysis lies in the high level of nucleic acids which strongly interfere with the isoelectric focusing dimension. Their level must thus be reduced, but the strong affinity of many nuclear proteins for the nucleic acids makes this reduction difficult if minimum losses in protein are expected. The first method used for the elimination of nucleic acids was devised by Peterson and Mc Conkey[6] and used digestion by S1 nuclease in the presence of SDS and urea.

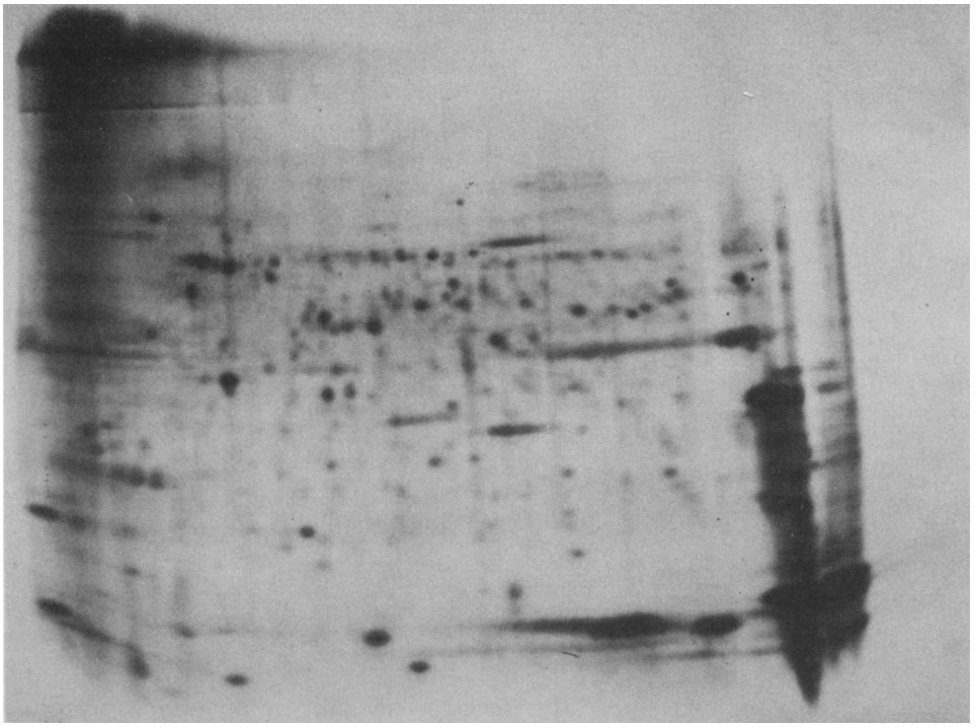

Fig. 2. NEPHGE analysis of nuclear proteins labelled *in vitro* by reductive methylation, 6000 V h were used for the electrofocusing. Histones migrate as streaks at the basic end of the gel.

TABLE I

COMPARISON OF THE EFFICIENCIES OF DIFFERENT AGENTS AS DISSOCIATING MEDIA FOR CHROMATIN

After radioactive labelling as described under Materials and methods, the nuclei were suspended at a DNA concentration of 0.5 mg/ml in the different media. The suspension was centrifuged to pellet all the molecules having a sedimentation coefficient higher than 7S. The supernatant was carefully decanted and the percentage of cpm recovered in the supernatant was taken as the yield. 2-ME = 2-Mercaptoethanol.

Solubilization medium	*Recovery of protein (%)*
2.5% SDS, 5% 2-ME, 100°C, 3 min	>97
9.5 *M* Urea, 2% Nonidet P-40, 2% ampholines	50
9.5 *M* Urea, 2% CHAPS, 2% ampholines	80
2.5% SB 3-14, 5% 2-ME, 100°C, 3 min	25
50% (v/v) Tetramethylurea, 5% 2-ME, 2% ampholines	50
2% SB 3-14, 50% (v/v) tetramethylurea, 5% 2-ME, 2% ampholines	95

However, nuclear RNA is badly eliminated by this method[18]. Moreover, the digestion is performed at high temperature, and the risk of carbamylation is greatly increased[7]. These drawbacks lead to variable results which are principally linked to the nuclear RNA content. In fact, little emphasis has previously been placed on the importance of RNA in the streaking and background problems encountered when dealing with nuclear proteins. Our *Drosophila* cells have a high nuclear RNA content and were therefore an ideal tool for such an investigation. We observed (Fig. 3) that RNA induces severe streaking and background, comparable with those previously obtained with low mobility group (LMG) protein preparations[8], which are devoid of DNA, but not of RNA. We therefore investigated several methods which can deal with large amounts of nuclear RNA and which are not subject to carbamylation artefacts. We first tried to get rid of both types of nucleic acids by ultracentrifugation[26]. This method completely eliminates DNA, but cannot reduce the RNA level to a reasonable extent (Fig. 4a, b). When using the urea–CHAPS medium already described, the yields are fair (*ca.* 80%) as shown in Table II. The fact that some RNA remains in the supernatant with the proteins may be correlated with the presence of small RNAs (snRNA) and with the presence of products of degradation of larger RNAs by the endogenous RNAses which remain active during the isolation of the

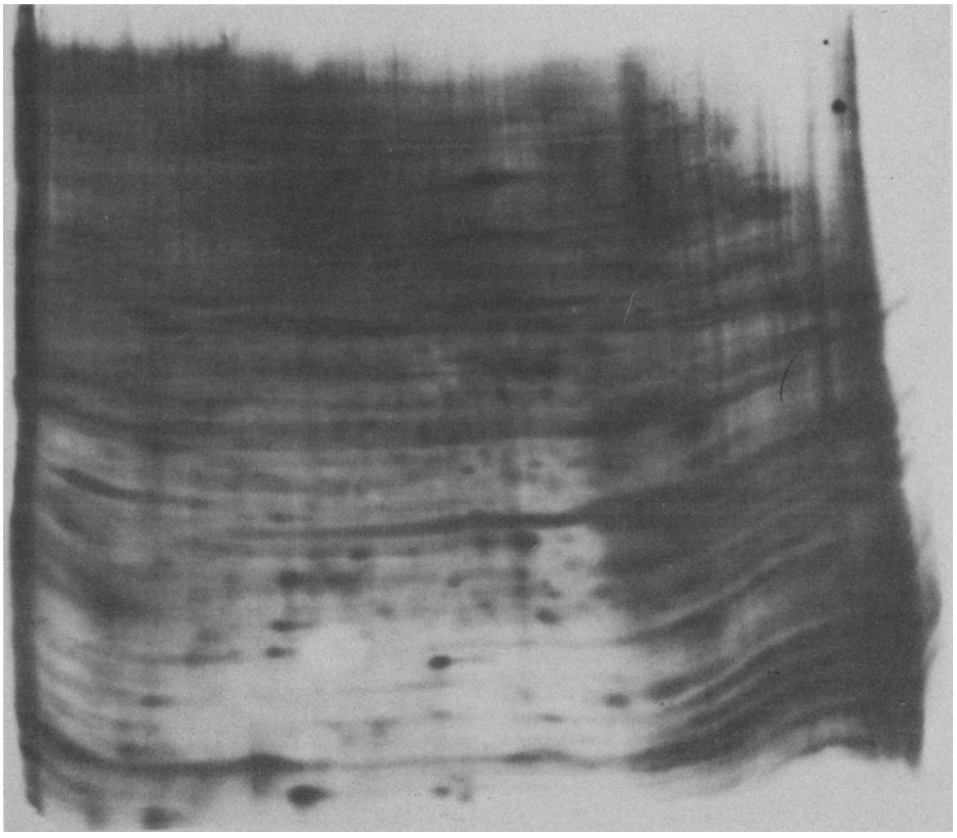

Fig. 3. Background induced by RNA in two-dimensional electrophoresis.

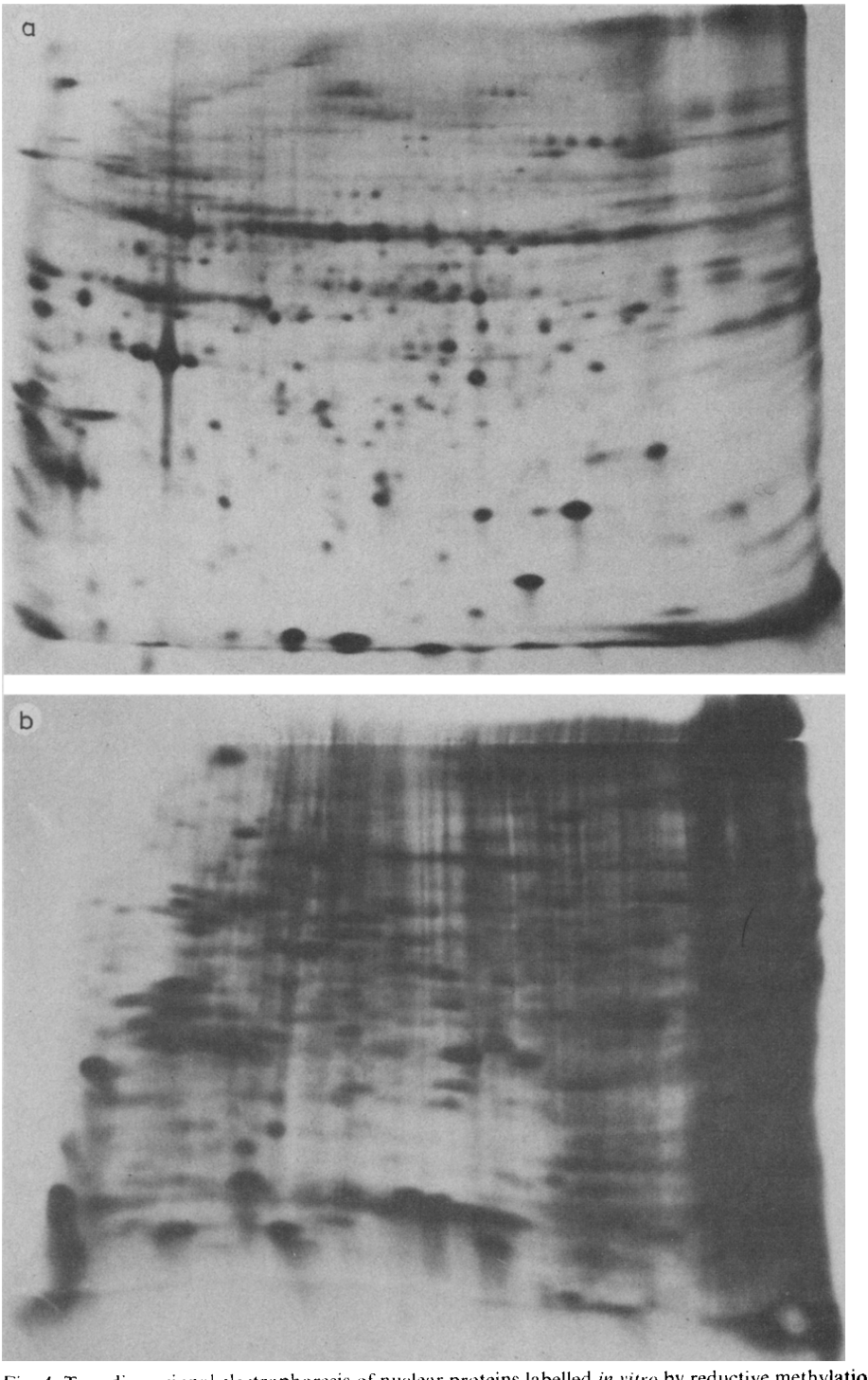

Fig. 4. Two-dimensional electrophoresis of nuclear proteins labelled *in vitro* by reductive methylation and freed from nucleic acids by centrifugation. (a) Pattern obtained from cells having a low RNA content; (b) pattern obtained from exponentially growing cells (high RNA content).

TABLE II

MAIN FEATURES OF THE DIFFERENT METHODS OF ELIMINATION OF NUCLEIC ACIDS

The yield was determined as the percentage of cpm remaining just before the gel loading compared to the cpm obtained just after labelling. The quality index was subjective and determined on exponentially growing cultures (high RNA) and confluent ones (low RNA). The RNA levels were determined as described[29].

Method	*Sensitivity to RNA*	*Sensitivity to proteolysis*	*Precipitation of protein*	*Protein recovery (%)*	*Overall quality*
S1 nuclease	+	–	–	80	Variable
DNAse–RNAse	–	+	–	80	Poor
Centrifugation	+	–	–	85	Variable
RNAse centrifugation	–	±	–	85	Good
Phenol extraction	±	–	±	70	Variable
Lanthanum precipitation	–	–	+ +	30–75	Good
Calcium precipitation	–	–	±	50–70	Rather good

nuclei. We therefore decided to digest the RNA with RNAse. The first attempt at digestion in multimolar urea was completely unsuccessful, but digestion in non-denaturing media proved far more efficient. We determined that maximum efficiency was reached when sodium chloride and EDTA were included in the digestion medium in order to dissociate the RNA–protein complexes. Elimination of the RNA by diges-

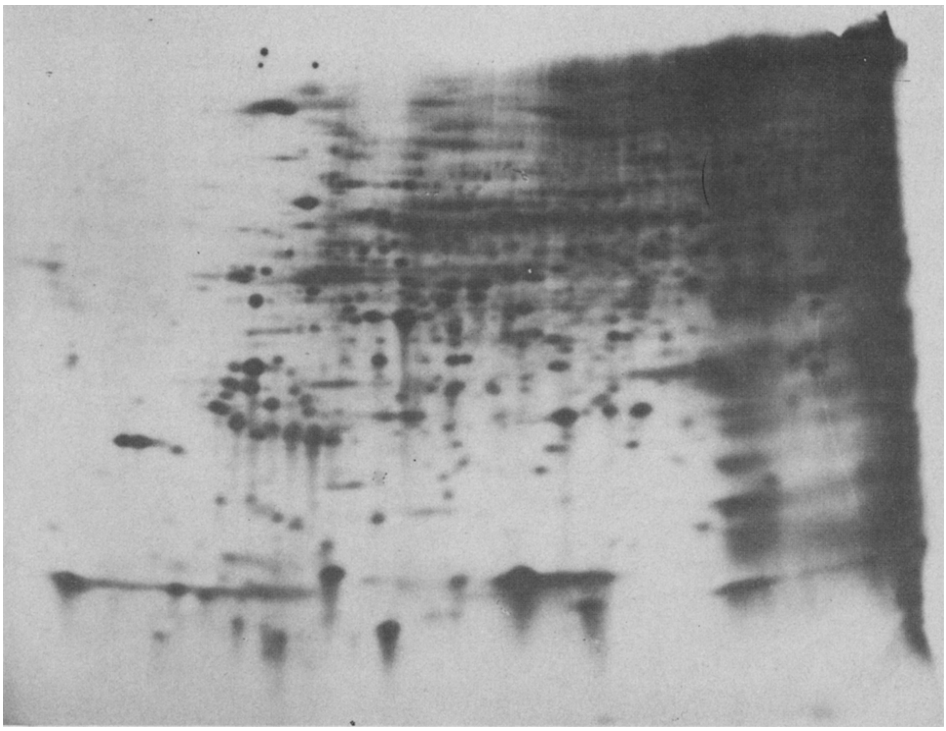

Fig. 5. Two-dimensional electrophoresis of *in vitro* labelled nuclear proteins freed from RNA by RNAse digestion and from DNA by centrifugation.

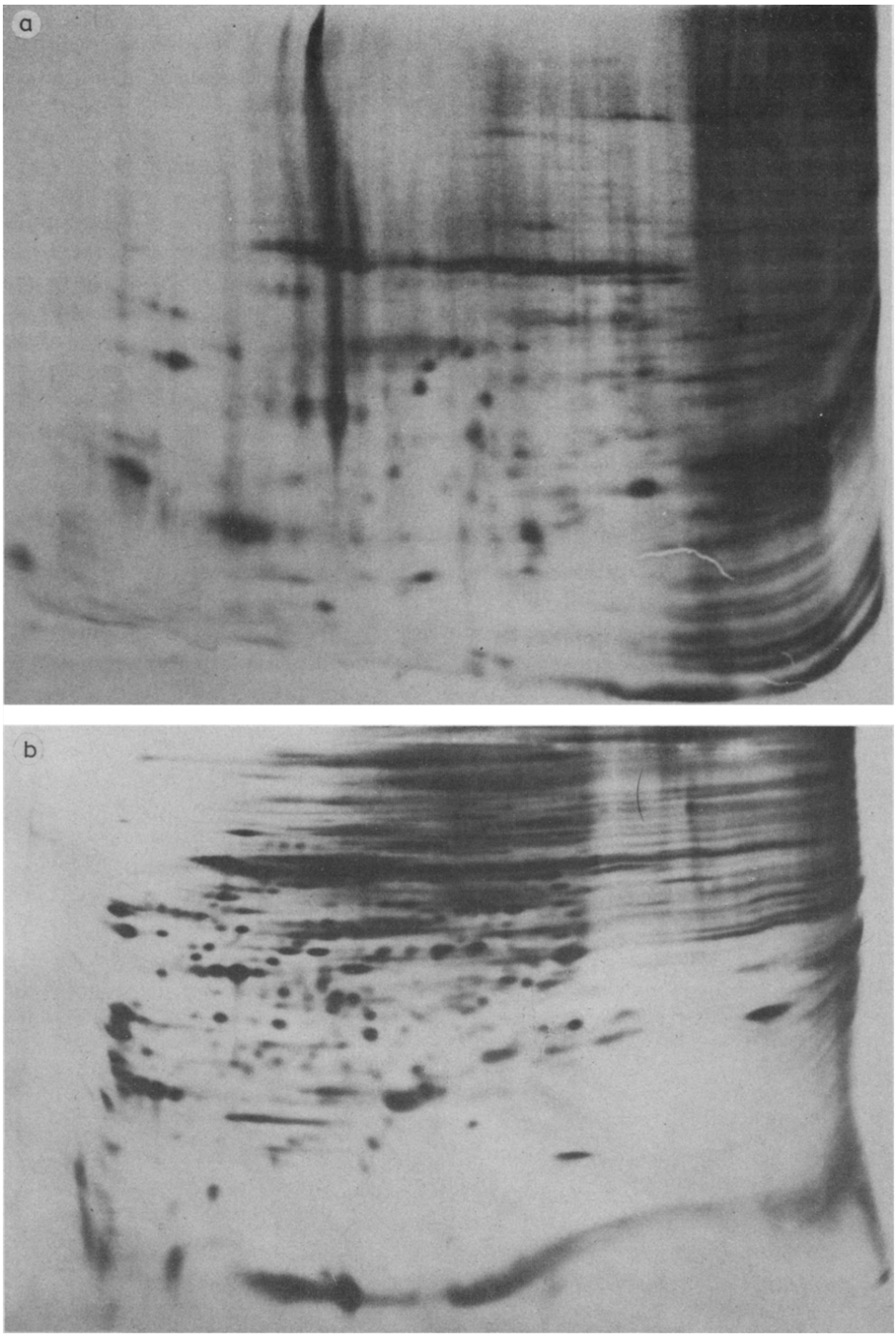

Fig. 6. Two-dimensional electrophoretic patterns of nuclear proteins freed from nucleic acids by phenol partition. (a) Without removal of SDS before the electrofocusing; (b) partition at 65°C and SDS removed by ion-pair extraction.

tion, followed by TCA precipitation to eliminate the oligonucleotides and the salts, then radioactive labelling and finally elimination of the DNA by centrifugation proved to be the method of choice in our system, leading to high quality gels (Fig. 5). On the other hand, the use of a DNAse–RNAse cocktail failed to digest DNA even in the presence of 2 *M* sodium chloride to dissociate the histones.

The major drawback of this method is its sensitivity to endogenous proteases, RNAse being heat-treated to inactivate contaminating proteases[29]. *Drosophila* cells have a low protease content, but many types of cells have high levels of nuclear proteases[30], which are not always completely inactivated by protease inhibitors. We therefore developed another set of methods in which the sample is always in highly denaturing conditions.

One method is the phenol–SDS method in which chromatin is dissociated by boiling in SDS and the proteins are partitioned into phenol, nucleic acids being excluded in the aqueous phase[19]. Some points must be kept in mind when using such a method. First, the SDS must be carefully eliminated, because CHAPS is far less efficient than Nonidet P-40 in removing SDS from proteins. Otherwise, precipitation and streaking occur at the sample application point, as shown in Fig. 6a. Secondly, the temperature of phenol extraction is of great importance. At low temperature, DNA is eliminated very well, but a lot of RNA, especially poly A + RNA, is trapped

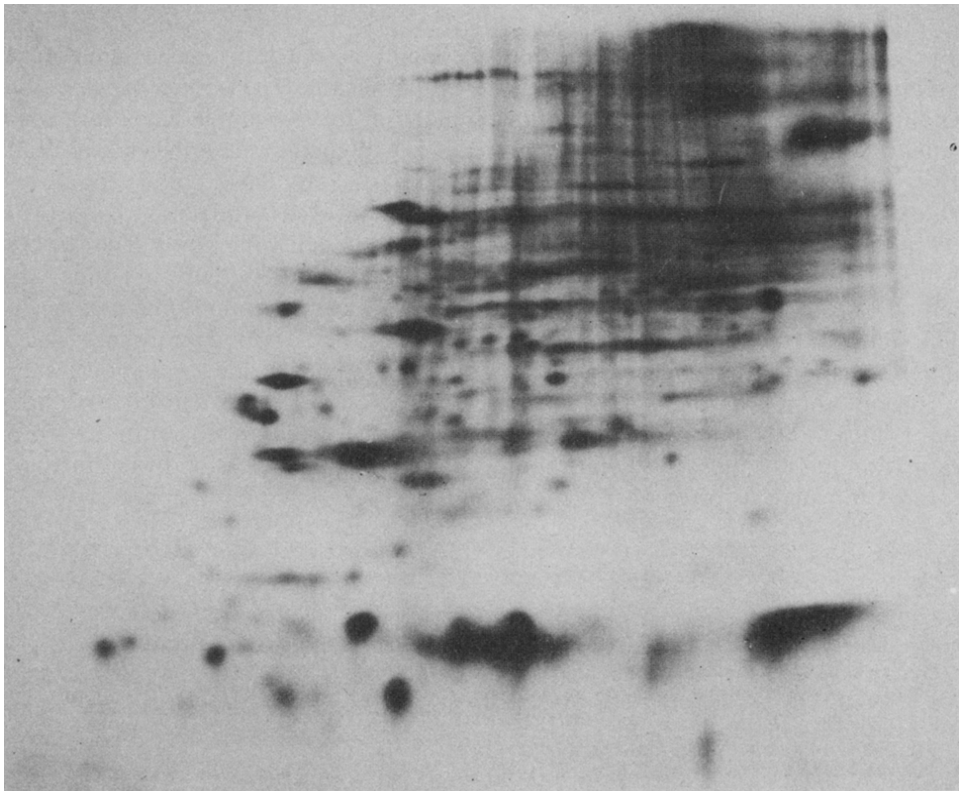

Fig. 7. Two-dimensional electrophoresis of nuclear proteins freed from nucleic acids by calcium precipitation.

with the proteins, blurring the patterns. At elevated temperature, much more RNA is eliminated, but DNA is pelleted in the phase separation, and a clean separation of the phenol phase is therefore difficult. However, good quality patterns can be obtained by this method (Fig. 6b). Moreover, this method can be applied with great success to the analysis of whole cells having both a high protease activity and a high level of RNA (data not shown).

However, this method cannot be used with cell lines which have a high nuclear poly A + RNA content. For this case of cells having both a high protease activity and a high poly A + RNA content, we developed precipitation methods. Among many precipitants tested, such as spermine, protamine, laurylamine acetate, manganese, calcium and lanthanum, only the last two gave satisfactory results. The lanthanum method is efficient but very difficult to control, and the proteins frequently precipitate along with the nucleic acids, leading to very variable yields (Table II) and considerable pattern variation from one experiment to another. Note that, for the experiment described in Fig. 1, the sample applied on both gels arose from a single precipitation. The calcium method is far safer and gives high quality patterns (Fig. 7), but the protein yield is rather low (*ca.* 60%) and selective precipitation of proteins may occur.

CONCLUSIONS

In conclusion, the main features of the methods tested have been summarized in Table II. It appears that four methods are applicable, the choice of which depending on factors linked to the biological material. If the sample has a low RNA content, a simple ultracentrifugation in urea–CHAPS medium is sufficient and leads to very high quality patterns. When the sample has a high RNA content but a low protease level, prior digestion of RNA by pancreatic RNAse in the presence of EDTA and sodium chloride followed by ultracentrifugation to pellet the DNA gives the best results. When both the RNA content and the proteolytic activity are high, this method cannot be used. In most of these cases, a phenol partition in the presence of SDS, followed by elimination of the latter, gives the best patterns. In rare cases where all these methods are inefficient, precipitation by calcium chloride gives good results, but precipitation of proteins is likely to occur. Progress in protein detection methods (more efficient labelling methods) and in dissociation media are currently under investigation in our laboratory, and should soon yield a simplification of the methods required for analysis of nuclear proteins by two-dimensional electrophoresis.

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