RAPID ISOLATION AND SEPARATION OF THE NON-HISTONE PROTEINS OF RAT LIVER NUCLEI

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1. Introduction

For many years the separation and classification of the nuclear non-histone proteins has been the subject of numerous investigations [1, 2] because of their possible importance in the control of gene expression in the eukaryote cell [e.g. 3]. The classical methods of nuclear protein extraction usually involve the extraction of isolated nuclei with isotonic and 1–2 M sodium chloride solutions, dilute acids and bases. These are often time-consuming and the relative insolubility of some fractions in normal aqueous solvents leads to analytical difficulties.

A one-step procedure has been described [4, 5] to solubilize the bulk of the non-histone proteins of rat hepatoma 223 nuclei. This extract did not contain DNA. The method was also found to be applicable to rat liver nuclei. This communication describes the separation of the components of the mixture using the technique of isoelectric focussing in polyacrylamide gel.

2. Materials and methods

2.1. *Nuclear extraction procedure*

Rat liver nuclei were prepared, free of cytoplasmic contamination, by a modification of the Chauveau procedure [6] using 2.3 M sucrose containing 3 mM Ca^{2+} at 4°. The nuclei were immediately extracted (in a hand-operated tight gap homogenizer) with 8 M urea 0.05 M sodium phosphate pH 7.6 containing a two-fold excess of *N*-ethylmaleimide (NEM) at 10°. The nuclei from the liver of a 300–350 g rat were extracted with 5 ml of the urea solution containing 5 μmoles of NEM. After 15 min the suspension was tested with the Ellman reagent (DTNB) [7] to ensure that all the –SH had reacted. When complete the suspension was centrifuged at 20,000 *g* for 15 min. The sedimented nuclei were re-extracted with an equal volume of the buffered urea solution and finally with half the initial volume of this solvent. Although the first two extracts contain the bulk of the protein material, the three extracts were combined.

The residue was extracted several times with 0.25 N HCl to remove the histones and the insoluble material left, mainly DNA, dissolved in 0.05 M tris buffer pH 8.0 containing 1% SDS at 37° overnight.

2.2. *Separation by isoelectric focussing in polyacrylamide gel*

As normal conditions were not found to be applicable, the technique used was a modification of existing procedures [see 9] using chemical polymerization [10]. A 5% (w/v) acrylamide gel containing urea (Analar grade, deionized) with 2% Ampholine carrier ampholytes (LKB Produkter AB, Stockholm, Sweden) was used.

Solutions

(1) 5.0 g of acrylamide (from Kodak, Ltd.) and 0.2 g of BIS (*N, N'*-methylenebisacrylamide, Kodak) were dissolved in 40 ml of deionized 8 M urea.

(2) 25 mg of ammonium persulphate in 10 ml of deionized 8 M urea, freshly made up.

For three gels approximately 6.5 cm long × 0.5 cm diameter, the mixture consisted of 2 ml of solu-

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tion (1), 0.25 ml of ampholine carrier (40%) pH 3–10, 2 ml containing the sample (300–500 μg of protein) preferably in 8 M urea, and finally 0.75 ml of solution (2). After de-aerating, the mixture was transferred to the running tubes, overlayered with a small quantity of distilled water and left to set for one hour. The tubes were loaded into a Shandon Disc electrophoresis apparatus and run at 150 V (constant voltage) for 5 hr at room temperature. The anode solution was 5% orthophosphoric acid and the cathode 5% 1,2-diamino-ethane (upper electrode). Gels were soaked in 12% trichloroacetic acid overnight and washed with two further changes of acid to remove excess carrier ampholytes. They were stained overnight in 0.1% Coomassie Brilliant Blue R (G.T. Gurr, Ltd., London) in ethanol-acetic acid -water (45:10:45 v/v). Destaining was achieved by soaking in several changes of ethanol–acetic acid–water (25:10:65 v/v) until the background was clear.

For analysis in this system the 8 M urea nuclear extract was usually dialysed overnight at room temperature against deionized 8 M urea but it can be used directly without further treatment.

2.3. *Electrophoresis in SDS-acrylamide gels*

The system of Shapiro et al. [11] was used with the modifications described by Elgin and Bonner [8] for nuclear protein analysis using 5% gels.

a) The 8 M urea soluble proteins were dialysed overnight at room temperature against 8 M urea, 0.1% SDS, 0.1% β-mercaptoethanol and 10% glycerol in 0.01 M sodium phosphate buffer pH 7.1 (c.f. buffer III [8]). 50–100 μg of protein was applied to 6.5 cm long gels.

b) The DNA in the 1% SDS–0.05 M tris (pH 8) extract was removed by ultracentrifugation and after dialysis of the protein solution against the modified buffer III, 50–100 μg of protein was applied to an 11.0 cm long gel.

2.4. *Electrophoresis of acid-soluble proteins*

a) Proteins extracted from the nuclei with 0.25 N HCl were subjected to electrophoresis in 15% polyacrylamide gels according to the technique of Reisfeld et al. [12] in β-alanine acetic acid buffer pH 4.4 at 4 mA/tube for 2.5 hr.

b) The 8 M urea 0.05 M phosphate extract was dialysed exhaustively against 0.25 N HCl at 4°. The precipitate formed was removed by centrifuging the mixture at 100,000 *g* for 45 min and the supernatant examined using the above electrophoresis procedure.

2.5. *General analyses*

DNA was estimated by the diphenylamine procedure [13]; protein by both the biuret method and the Folin-Lowry [14]; –SH by the Ellman reagent [7].

3. Results and discussion

The 8 M urea 0.05 sodium phosphate was shown to extract 70.3 ± 5.0% of the total nuclear protein of rat liver nuclei but none of the DNA. This procedure also removes 88.2 ± 1.4% of the nuclear –SH material at a 'specific activity' of approximately 70 nmoles of –SH/mg protein. It is therefore important to block these –SH groups prior to analysis to prevent intermolecular disulphide formation which could make subsequent analysis irreproducible.

This extraction procedure also avoids the often undersirable acid extraction of nuclei prior to extraction of acidic and residual proteins.

Isoelectric focussing of the extract in the system described is highly reproducible and gives rise to some 23 different bands using the pH 3–10 ampholine carrier. The same pattern was obtained when the proteins were fully reduced. Fig. 1 shows the patterns obtained in 4 short gels each containing 150–200 μg of the 8 M urea extracts from the liver nuclei of four different rats. The longer gel shown contains twice as much protein and was focussed for 10 hr. Shorter or longer running times for both long and short gels gave considerably poorer resolution. Obviously the blocking of protein –SH groups by NEM will alter the isoelectric point somewhat, but the time taken for focussing indicates that these proteins are not heavily charged. It has been reported [5] that a large proportion of this type of protein in rat hepatoma nuclei is not absorbed on DEAE cellulose at pH 8.4 despite the high proportion of aspartic and glutamic acids in their composition. This would seem to indicate that the carboxyl groups are blocked in some way, perhaps as amides [15].

It could be argued that the inclusion of the proteins in the gel mixture during chemical polymeriza-

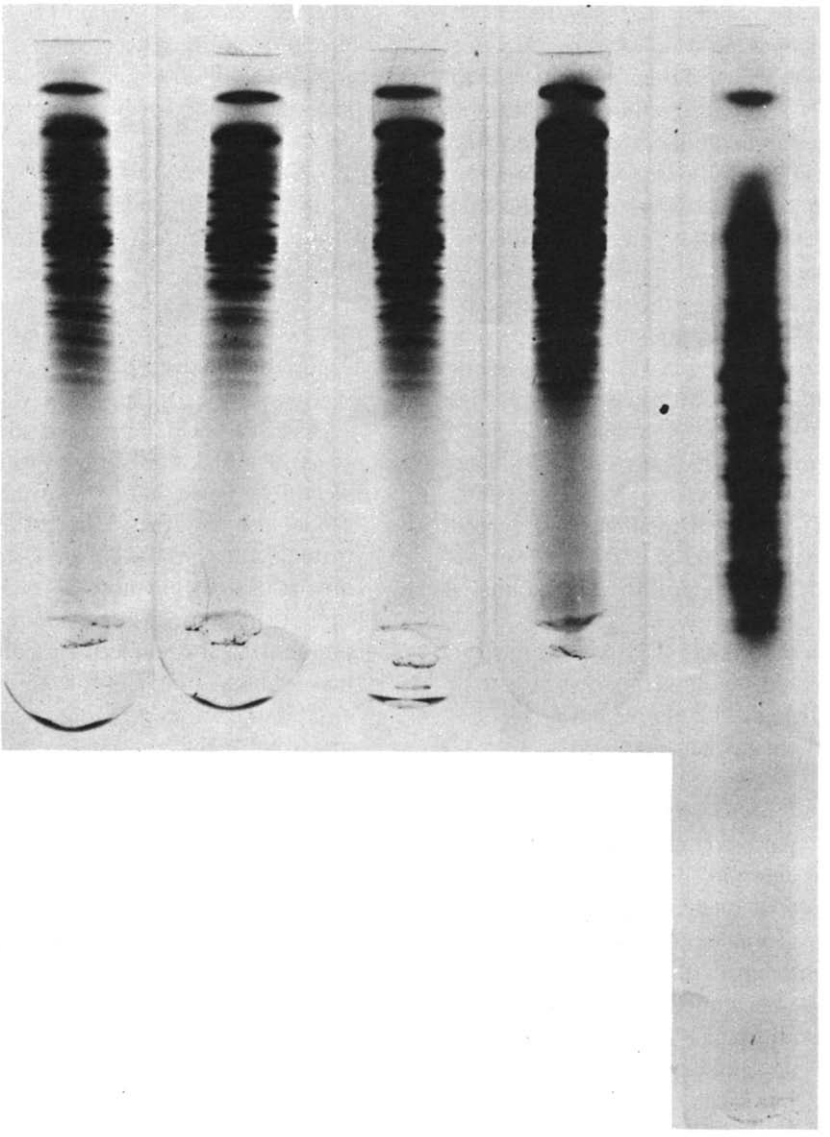

Fig. 1. Isoelectric focussing of nuclear urea extracts in 5% polyacrylamide gel. Highest pH (ca. 9) at the top of the gel. Each gel contains proteins from a different rat liver. Short gels contained approximately 130 μg of protein per gel and were run for 5 hr. The longer gel contained 250 μg of protein and was run for 10 hr. Gels stained with Coomassie Brilliant Blue, stored in 10% ethanol and photographed in test-tubes using a Kodak 23A filter.

tion might lead to artifact formation [9]. This is probably mainly due to –SH oxidation, a problem which does not arise in these studies since this reactive group has been blocked with NEM. When the persulphate concentration quoted was halved or doubled, identical gel patterns were obtained. Also, loading the protein sample onto the top of the gel [9] gave a similar result.

Fig. 2 shows SDS gels of the proteins in the 8 M urea extract and the remaining residual fraction soluble in 1% SDS-tris. Since the distance migrated by the protein is proportional to its molecular weight, reduced bovine serum albumin (Sigma) was run as a standard molecular weight marker (M.W. 67,000). The position of the bromophenol blue band at the end of the run

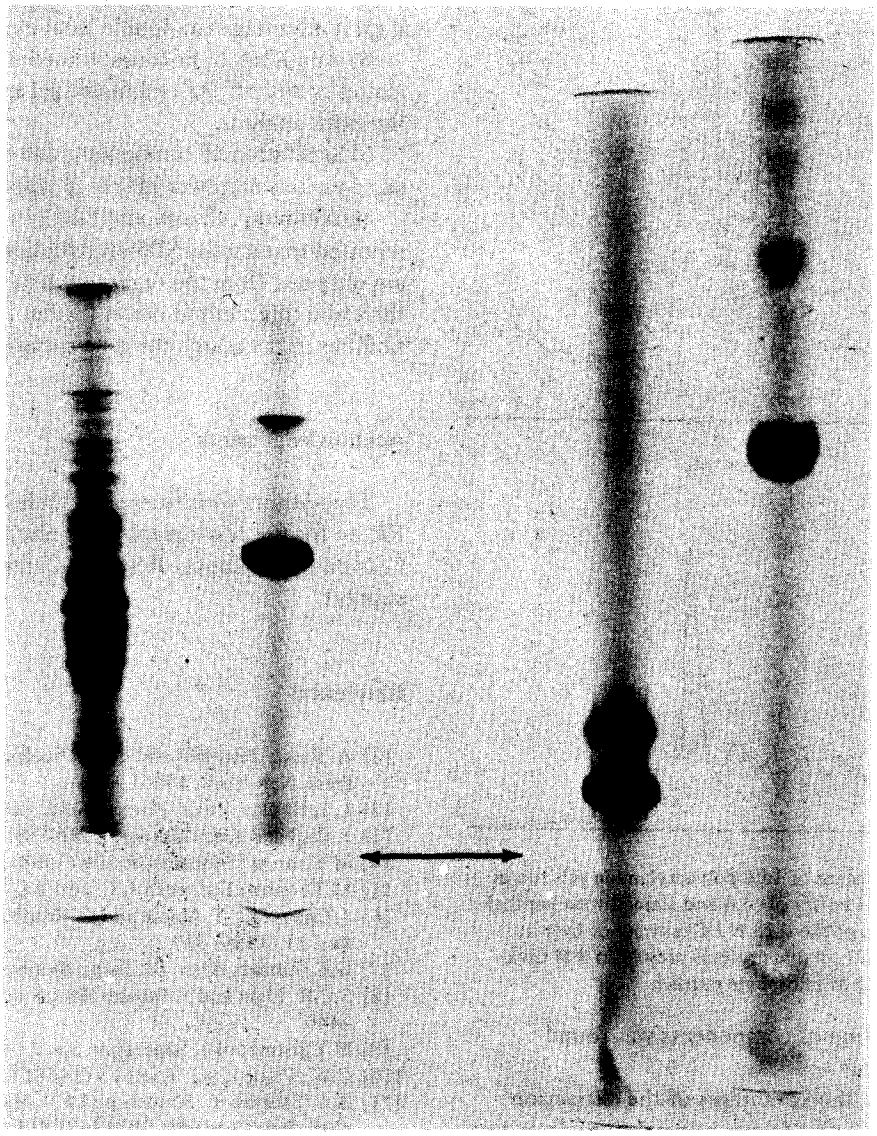

Fig. 2. SDS-polyacrylamide gel patterns. Left to right: short gels (i) urea soluble proteins; (ii) bovine serum albumin marker; long gels (iii); tris-SDS residue proteins; (iv) bovine serum albumin. The arrows indicate the position of the Bromophenol Blue marker at the end of the run (positive electrode end). Gels stained with Coomassie Brilliant Blue.

is also marked. This shows clearly that, while the 8 M urea extract contains most, if not all, of the 13 major polypeptide bands of molecular weight, ca. 5,000–100,000 shown to be present in rat liver non-histone chromosomal protein [8], the remaining SDS soluble non-histone protein fraction contains only two components of low molecular weight.

Gel electrophoresis of the 0.25 N HCl extract (fig. 3A) demonstrated all the histone species normally found in rat liver nuclei to be present. In the urea extract, however, only one fast moving band probably corresponding to the F2a1 histone was present (fig. 3B).

When nuclear fractions obtained by conventional buffer and saline extraction procedures were analysed in this system using these techniques considerable

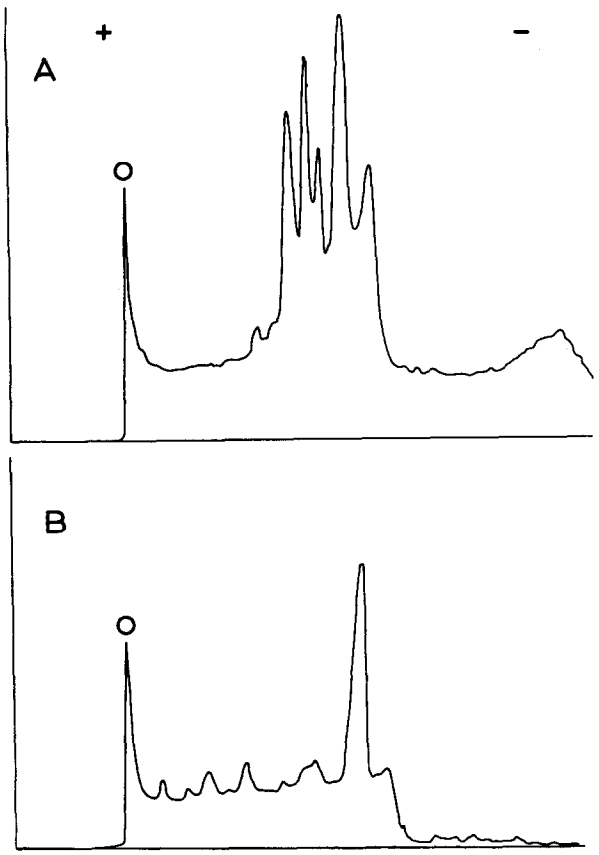

Fig. 3. Densitomer scans of 15% polyacrylamide gels run in β-alanine acetic acid buffer pH 4.4 and stained with napthalene black. (A) Scan of the 0.25 N HCl extract of liver nucleic. (B) 0.25 N HCl soluble proteins present in 8 M urea 0.05 M phosphate extract.

cross contamination of components was found (unpublished data).

To summarize the advantages of the extraction method and isoelectric focussing procedure described:

1) It is rapid and simple to perform (one-step preparation of gels);

2) Only very small amounts of liver nuclei are required;

3) The components are focussed to narrow bands instead of being spread as sometimes occurs in conventional electrophoresis;

4) Large amounts of protein ca. 300 μg in dilute solution can be loaded in a 6.5 × 0.5 cm gel. This is a great advantage for double label experiments;

5) Any RNA or histones in the mixture are automatically run off the end of the gel and do not interfere with analysis.

6) Separation of components can be improved by using various narrower pH range ampholytes.

MacGillivray, Carroll and Paul [16] have recently reported that similar SDS-electrophoretic patterns are obtained from the organs of different species and therefore this method offers further interesting possibilities in the search for gene-specific proteins.

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