Fractionation of Constituents of Ribonucleoproteins Containing Heterogeneous Nuclear Ribonucleic Acid†

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ABSTRACT: A method of fractionation of hnRNP constituents adaptable to large-scale preparation is presented. It is based on differential resistance to salt dissociation of the two classes of units of hnRNP, the 30–50S monoparticles and the heterogeneous complexes. The monoparticle proteins were released from hnRNP by 0.4 M NaCl. They were separated from the salt-resistant RNP corresponding to the heterogeneous complexes in three steps: chromatography on DEAE-cellulose, high-speed centrifugation, and Bio-Gel chromatography. The latter chromatography permitted a first fractionation of monoparticle proteins according to molecular weight. Such fractions may serve for purification of individual proteins of molecular weight below 80000. After the two first steps, two fractions of salt-resistant RNP were obtained. In addition to heterogeneous RNA up to 30 S, small nuclear RNAs were detected which represented 6% of total RNA. The protein pattern was complex, and no clear-cut segregation of groups of proteins could be observed between the two fractions. They were both highly enriched in phosphoproteins as compared to monoparticle proteins. In another fraction corresponding to the void volume of Bio-Gel chromatography, one-third of the RNA was small nuclear RNA. It is suggested that this fraction contains snRNP in addition to free proteins of molecular weight above 80000 and to salt-resistant RNP similar to those described above but of small size.

The ribonucleoproteins containing the nuclear heterogeneous RNA (hnRNP)[1] are relatively large entities resembling the perichromatin fibrils described in situ (Faiferman & Pogo, 1975; Stêvenin et al., 1976; Devilliers et al., 1977). Electron microscopic examination showed that they were made of units of different sizes, and gradual treatment with ribonuclease demonstrated that they contained two classes of constituents, 30–50S monoparticles and 30–200S heterogeneous complexes interspersed along the fibril (Stêvenin et al., 1977).

For the comprehension of the structure and role of the hnRNP, it would be useful to study these two classes of constituents separately. The most important point to be taken into account for such fractionation is that these constituents are linked through hnRNA and that a ribonuclease treatment is necessary for separation. Even if minimal, such a treatment nicks also the RNA within the constituents as suggested by the small size of RNA extracted from monoparticles (Gattoni et al., 1978). In addition, RNA hydrolysis is accompanied by rearrangements such that structures which do not preexist may be obtained (Stêvenin et al., 1979). It is worth mentioning that uncontrolled endogenous ribonuclease hydrolysis often occurs, leading to the accumulation at 30–50 S of monoparticles and/or rearrangement products, according to the extent of hydrolysis. These are often considered as the sole hnRNP constituents.

As it did not seem possible to isolate the two classes of constituents as intact structures, another possibility of study was searched. It was suggested to us by previous work (Gallinaro-Matringe et al., 1975), showing that the monoparticle proteins were released from the hnRNP at low salt concentration whereas the heterogeneous complexes were salt resistant. Therefore, a salt treatment of hnRNP should allow the obtention of monoparticle proteins and of the hnRNA strand covered by the proteins of the salt-resistant complexes at given sites and bare at other sites (Figure 1). A method of separation of salt-released proteins and salt-resistant RNP suitable for large-scale preparation will be described. It allowed the obtention of the bulk of monoparticle proteins which may be further fractionated for isolation of individual proteins. In addition, three different RNA-containing fractions were obtained. This was the first isolation of salt-resistant RNP, and some general characteristics were established.

Methods

Unless otherwise indicated, the entire isolation procedure was carried out at 0–4 °C. All glassware and solutions were sterilized.

Preparation of a Nuclear Extract. Purified brain nuclei were prepared as previously described (Stêvenin & Jacob, 1972). The nuclei corresponding to approximately 108 g wet weight (72 rat brains) were suspended in 45 mL of medium B (10 mM Tris-HCl, pH 7.4, 25 mM KCl, and 1 mM $MgCl_2$) containing 0.1 M NaCl. The nuclear suspension was sonicated for 60 s at 1.25 A in a MSE sonicator (Stêvenin & Jacob, 1974). The lysate was immediately centrifuged for 5 min at 35000 rpm in a R 65 rotor (78000*g*). The supernatant is designated as the nuclear extract.

Fractionation of the Nuclear Extract on Diethylaminoethylcellulose. Whatman DEAE-cellulose (DE-23) was precycled according to the instructions of the manufacturer and equilibrated in 200 mM Tris-HCl, pH 7.4, 25 mM KCl, 2.5 mM $MgCl_2$, and 0.1 M NaCl. The DEAE-cellulose was packed in a glass column (26-mm diameter; 60-mm height) at 85 mL cm^{-2} h^{-1}.

The column was then reequilibrated with 20 bed volumes of medium B containing 0.1 M NaCl at 65 mL cm^{-2} h^{-1}. This flow rate was maintained during the entire chromatography procedure.

The nuclear extract was diluted twice with medium B

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[1] Abbreviations used: RNP, ribonucleoprotein(s); hnRNP, ribonucleoprotein(s) containing heterogeneous nuclear RNA; pre-mRNA, premessenger RNA; snRNA, small nuclear RNA; snRNP, ribonucleoprotein(s) containing snRNA.

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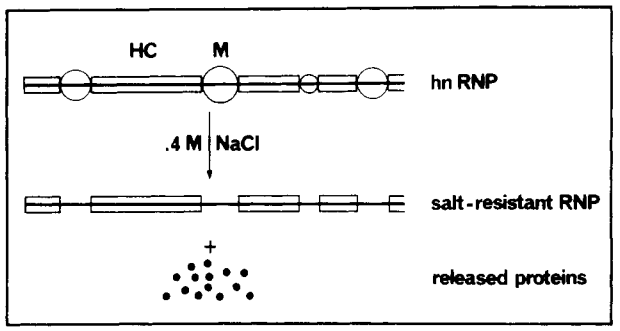

FIGURE 1: Schematic representation of the dissociation of hnRNP by NaCl. The size heterogeneity of monoparticles (M) and heterogeneous complexes (HC) was demonstrated previously (Stévenin et al., 1977; Gattoni et al., 1978).

containing 0.1 M NaCl (final volume 90 mL) and loaded on the column. The column was washed with 30 mL of the same medium, followed by 110 mL of medium B containing 0.15 M NaCl.

Elution was carried out with 140 mL of medium B containing 0.4 M NaCl (DF-0.4 M), followed by 110 mL of medium B containing 1 M NaCl and 6 M urea (DF-urea).

Treatment of DF-0.4 M for Gel Chromatography. In order to avoid aggregation of free proteins, we raised the concentration of NaCl in the DF-0.4 M fraction to 1 M by addition of solid NaCl. After 1 h, the mixture was centrifuged for 2 h at 50000 rpm in a R 60 rotor (176000*g*). The pellet is designated as P-50.

Solid $(NH_4)_2SO_4$ was added to the supernatant to bring it to 80% saturation. After 1 h, the mixture was centrifuged for 40 min at 24000 rpm in a SW 25.2 rotor (70000*g*). The pellet was dissolved in 0.4 mL of Bio-Gel medium (25 mM Tris-HCl, pH 8.0, 25 mM KCl, 1 M NaCl, and 0.1 mM dithiothreitol; Miles Laboratories, Ltd.) and dialyzed overnight against 250 mL of the same medium. This concentrated fraction is designated as S-50.

Chromatography on Bio-Gel P-150. Bio-Gel P-150, 100–200 mesh (Bio-Rad Laboratories), was swollen in Bio-Gel medium and packed according to the instructions of the manufacturer. The column diameter was 11 mm, and the packed gel bed was 950 mm high. All subsequent operations were carried out under constant pressure at 2.2 mL cm^{-2} h^{-1}.

The column was first equilibrated overnight with the Bio-Gel medium. After calibration with protein markers and overnight washing, the sample S-50 was loaded on the column, and elution was carried out in the same conditions. Fractions (0.4 mL) were collected and pooled as indicated in the text.

Polyacrylamide Gel Electrophoresis of Proteins. Samples in solution or suspension were precipitated with 10% trichloroacetic acid. The pellet obtained after high-speed centrifugation was washed with the same solution in order to remove the excess of salt. In both cases, centrifugation was for 20 min at 35000 rpm in a SW 50-L rotor (100000*g*).

The pellets were suspended in Tris–urea–DTT (10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and 6 M urea), treated with 1% sodium dodecyl sulfate, and electrophoresed on 10% acrylamide gels containing 0.1% sodium dodecyl sulfate as previously described (Gallinaro-Matringe et al., 1975).

The gels were stained with Coomassie Brillant Blue R and scanned with the aid of a Vernon recorder (Paris, France). When required, areas of selected peaks or gel regions were determined by planimetry. Under our experimental conditions, the dye intensity was proportional to the quantity of proteins.

The nomenclature of proteins was previously defined (Stévenin et al., 1978). The gel recordings were divided into four zones of molecular weight (numbered from 1 to 4) and into subzones (a, b, c, etc.) as indicated in the figures. The stained bands within each subzone were numbered.

Electrofocusing. After trichloroacetic acid precipitation, the samples were washed twice with trichloroacetic acid and twice with acetone. Reduction of S–S groups and alkylation of –SH groups were carried out as follows. The samples were incubated overnight at 4 °C in the presence of 1.5 mM dithiothreitol in phosphate buffer, pH 7.0. *N*-Ethylmaleimide was added at a final concentration of 6 mM. The mixture was incubated for 30 min at 25 °C. The proteins were precipitated and washed as above and then dissolved in a small volume of 8 M deionized urea. Electrofocusing was performed in an LKB multiphor according to the instructions of the manufacturer. All solutions were made up in 8 M deionized urea.

Determination of RNA and Phosphoproteins. Adult Wistar rats were injected intracisternally with 1 mCi of $^{32}PO_4^{3-}$ and were sacrificed 16 h after injection. It was previously shown that intracellular pools of nucleotides and RNA were equilibrated in such conditions (Stévenin et al., 1968) so that radioactivity determinations gave an index of the relative quantities of RNA in the samples. Brain DNA was not labeled, and small nuclear RNA had a low specific activity as compared to hnRNA (Weinberg & Penman, 1969).

Acid-insoluble radioactivity was determined at 0 and 90 °C. The difference corresponded to the radioactivity of RNA, the remainder being that of phosphoproteins (Stévenin et al., 1977).

For determination of ^{32}P-labeled phosphoprotein radioactivity after gel electrophoresis, the samples were first treated with ribonuclease. The gels were cut into 1-mm slices whose radioactivity was determined (Gallinaro-Matringe et al., 1975).

Slab-Gel Electrophoresis of RNA. RNA was phenol-extracted for 30 min at 38 °C in 10 mM Tris-HCl, pH 8.3, and 0.5% sodium dodecyl sulfate (Hadjivassiliou & Brawerman, 1967; Judes & Jacob, 1972). After deproteinization, RNA was precipitated with 2 volumes of ethanol in the presence of 100 mM NaCl.

Linear gradients of acrylamide (2.2–15%) were used. Buffers were made according to Loening (1967). Just before electrophoresis, the RNA samples were denatured for 10 min at 65 °C in the presence of 1% sodium dodecyl sulfate and immediately cooled in an ice bath (McKnight & Schimke, 1974). Staining was with Methylene Blue (Peacock & Dingman, 1967). The gels were recorded with a Vernon apparatus.

Results

Fractionation of a Nuclear Extract by DEAE-cellulose Chromatography. After salt treatment, the hnRNP are assumed to dissociate as illustrated in Figure 1. The free monoparticle proteins might be separated from the salt-resistant complexes by high-speed centrifugation. However, this would be a long and fastidious procedure, unsuitable for large-scale preparations as it would require two cycles of centrifugations, one for separation of hnRNP from the soluble nuclear components present in the extract (Stévenin et al., 1975) and another for separation of salt-released and salt-resistant components. A more suitable and rapid procedure was elaborated and based on the following observations: (1) the p*I* of hnRNP is acidic (pH 4–5) (Gattoni and Stévenin, unpublished experiments), and they are retained on DEAE-cellulose in neutral buffers containing 0.10–0.15 M monocationic salts; (2) the bulk of soluble nuclear proteins (mostly acidic) is eluted from DEAE-cellulose under the above conditions. It could be predicted that, after adsorption of the

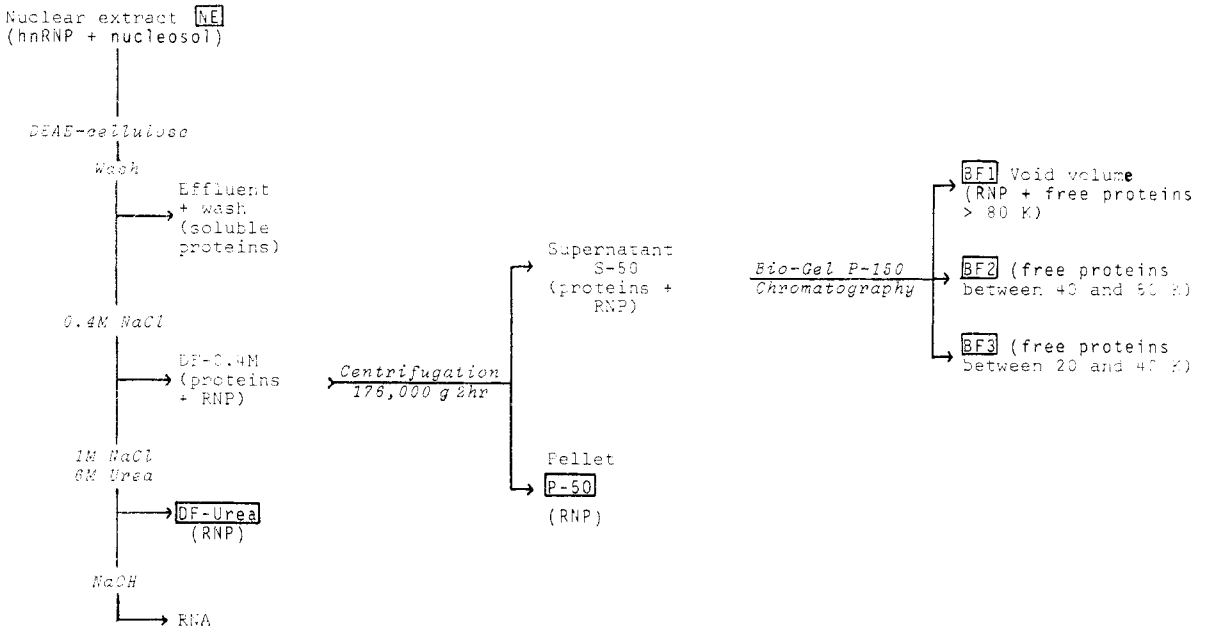

FIGURE 2: Flow diagram of the fractionation procedure.

nuclear extract and washing with 125 mM monocationic salt, pH 7.4, the soluble proteins would be found in the effluent and washing, whereas the hnRNP would remain on the column. At higher salt concentration (0.4 M NaCl), the hnRNP would dissociate into salt-resistant complexes and free monoparticle proteins. At 0.4 M NaCl, pH 7.4, the basic and acidic released proteins would be immediately eluted. The salt-resistant complexes (which are expected to be at least as acidic as untreated hnRNP since most basic proteins were eliminated and naked RNA fragments were present) would remain bound to the DEAE-cellulose. Thus, the two cycles of centrifugation might be replaced by a single chromatography on DEAE-cellulose (see flow diagram in Figure 2).

The experiments were performed with a brain nuclear extract from rat injected with $^{32}PO_4^{3-}$ in order to follow the fate of hnRNA and phosphoproteins. As expected, all the radioactivity remained bound to the DEAE-cellulose after washing, together with 80% of the proteins. The remaining 20% of the proteins were in the effluent. This amount was that previously found for nucleosol proteins (Gallinaro-Matringe et al., 1977; Stévenin et al., 1978).

NaCl (0.4 M) was then passed through the column. The eluate contained 60% of the proteins from the nuclear extract, 65% of the phosphoproteins, and 35% of the radioactive RNA. This indicated that a fraction of the salt-resistant RNP was unexpectedly eluted from the column.

The other salt-resistant RNP could not be eluted by increasing the salt concentration up to 1 M. Urea (6 M) allowed the release of 34% of the initial RNA with 18–20% of proteins and phosphoproteins. This fraction (DF-urea) may be considered as a RNP fraction, probably partly dissociated by urea. Another fraction of the RNA, without proteins, could be eluted with NaOH. The total recovery of RNA was 77%; that of proteins was 94%.

The experiments suggest the presence of at least two classes of salt-resistant RNP with different affinities for DEAE-cellulose. In the next section, we shall report our attempts to separate the free proteins from the RNP of DF-0.4 M.

Separation of Residual RNP and Free Proteins by Centrifugation and Bio-Gel Chromatography. Both high-speed centrifugation and gel chromatography could be envisaged for such fractionation. Preliminary experiments showed that some of the residual RNP were of small size and could hardly be separated from the free proteins by centrifugation adaptable to large-scale preparations. On the other hand, gel chromatography requires the application of a small volume of concentrated material. Due to the low solubility of these RNP (unpublished experiments), relatively large columns had to be used, leading to unsatisfactory recovery of the free proteins. Therefore, both methods were successively applied. High-speed centrifugation allowed the removal of the bulk of the RNP, and a sample enriched in free proteins could be submitted to gel chromatography which ensured the elimination of the remaining RNP.

Centrifugation was carried out at 50000 rpm for 2 h (Figure 2). The supernatant was concentrated by $(NH_4)_2SO_4$ precipitation, followed by dialysis of the resuspended pellet against the Bio-Gel medium (S-50).

The pellet of the high-speed centrifugation (P-50) contained 87% of the RNA from DF-0.4 M and only 13% of its proteins and constituted another RNP fraction. The supernatant (S-50) still contained RNA and was loaded on a Bio-Gel P-150 column. Three fractions were collected (Figure 2). The material of the first one (BF1) had a molecular weight above 80000, contained 90–95% of the recovered RNA (corresponding to 7% of the RNA from the nuclear extract), and was rich in proteins and phosphoproteins. Only traces of RNA radioactivity were found in the other fractions (BF2 and BF3) which may be considered as free proteins.

Distribution of RNA, Proteins, and Phosphoproteins. Our fractionation procedure yielded five different fractions (Figure 2). The distribution of RNA, proteins, and phosphoproteins relative to the nuclear extract considered as starting material is shown in Table I. Three fractions (DF-urea, P-50, and BF1) contained salt-resistant RNP as suggested by the presence of labeled RNA. The protein to RNA ratios differed according to the fractions. This was in agreement with previous data showing a large polydispersity of CsCl densities in the population of salt-resistant RNP (Stévenin & Jacob, 1974). In addition, BF1 might contain free proteins of large molecular weight, plus, as will be discussed below, ribonucleoproteins containing snRNA. The two other fractions (BF2 and BF3) primarily contained free proteins. Table I shows that the amount of phosphoproteins relative to proteins

Table I: Distribution of RNA, Proteins, and Phosphoproteins[a]

	RNA	proteins	phospho-proteins
hnRNP	100	100	100
DF-urea	34.1	18.5	20.4
P-50	31.0	8.9	8.0
BF1	6.9	24.7	19.7
BF2	0.4	4.7	1.5
BF3	0.3	8.4	1.3
recovery	72.7	65.2	50.9
recovery in salt-resistant RNP	72.0	52.1	48.1

[a] Proteins were determined by colorimetry (Lowry et al., 1951), and RNA and phosphoproteins were determined by differential thermal extraction after ^{32}P labeling (see Methods). The values for unfractionated hnRNP (nuclear extract minus effluent) were considered as 100 to allow comparisons.

was much lower in salt-released proteins than in salt-resistant RNP.

The recovery of radioactive RNA (72%) was of the order of magnitude expected from a three-step fractionation procedure. Some RNA might have been hydrolyzed and lost but such hydrolysis was probably limited as suggested by RNA size (see below). The radioactive RNA recovered in salt-resistant RNP is likely to represent that of total hnRNP (Figure 1).

The overall recovery of phosphoproteins and proteins was significantly lower than that of RNA. The losses might be of the same order of magnitude for salt-resistant and salt-solubilized proteins. However, as solubilized proteins showed a marked tendency to adsorb on various matrices (Fuchs, unpublished experiments), the losses might primarily concern the salt-solubilized proteins.

Fifty-two percent of the proteins from hnRNP were recovered in the RNA-containing fractions. Although the protein proportion might be overevaluated due to the presence of material other than salt-resistant RNP in BF1, the results indicate that salt-resistant RNP are quantitatively important hnRNP constituents.

RNA of Salt-Resistant Fractions. As small nuclear RNAs were described in hnRNP (Deimel et al., 1977; Guimont-Ducamp et al., 1977; Northemann et al., 1977) and as they were slowly labeled as compared to hnRNA, their relative proportion was determined by absorbance measurement. The RNAs of DF-urea, P-50, and BF1 were electrophoresed on polyacrylamide gels (Figure 3). The three fractions contained heterogeneous RNA plus five major low molecular weight RNAs designated as U2, U1, 5 S, 4.5 S, and 4 S (Prestayko et al., 1970). The heterogeneous RNA was of relatively large size in DF-urea and P-50 (essentially from 8 to 25 S) and smaller in BF1 (from 4 to 12 S). If we assume that the size of salt-resistant RNP is related to that of hnRNA, this may explain why the RNP of BF1 did not sediment under the conditions of obtention of P-50. The relatively large size of hnRNA in DF-urea and P-50 (part of it remaining on top of the gels) indicated that ribonuclease hydrolysis was of little importance.

The profiles of small nuclear RNA were similar in DF-urea and P-50 but there was relatively more U1 in BF1. The snRNA represented 6% of the hnRNA in DF-urea and P-50 and 50% in BF1 (Table II). In this latter fraction, only 6% of total hnRNA was detected (in agreement with results of Table I), but 35% of total snRNA was found.

Whereas the hnRNA of salt-resistant RNP is likely to represent that of total hnRNP, it is not known whether their snRNA belong to heterogeneous complexes or to monoparticles

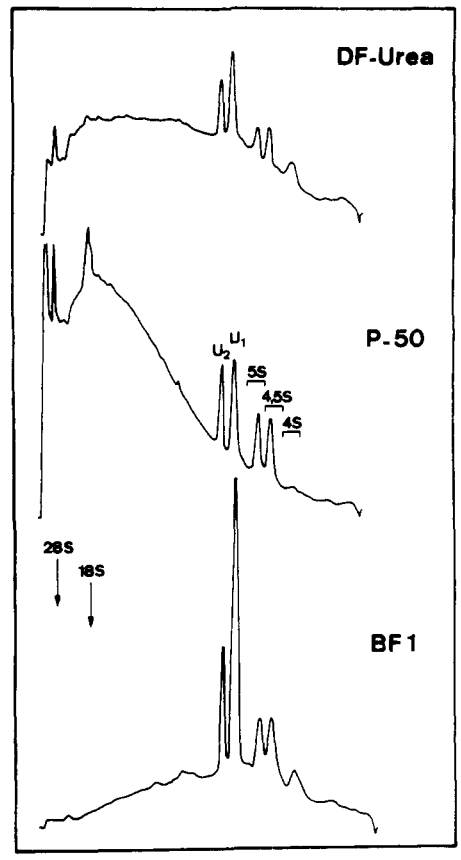

FIGURE 3: Slab-gel electrophoresis of RNA from salt-resistant RNP. The gels were recorded after staining with Methylene Blue. Five major small nuclear RNA species plus heterogeneous RNA are present. The background was flat as checked with blank gels and a mixture of ribosomal and transfer RNA.

Table II: Small Nuclear RNA and Heterogeneous RNA in Salt-Resistant Fractions[a]

	snRNA/ hnRNA × 100	distribution between fractions (%) snRNA	hnRNA
DF-urea	6.3	25	34
P-50	6.0	40	60
BF1	50.2	35	6

[a] The amount of RNA was determined by planimetry after recording. The distribution between fractions was estimated on the basis of RNA recovered from the fractions.

or to both. However, as monoparticles were dissociated, the snRNA would be that of monoparticles only if it is tightly associated with their heterogeneous RNA by hydrogen bonding (Jelinek & Leinwand, 1978; Flytzanis et al., 1978) or possibly by a few proteins resistant to salt dissociation.

BF1 contained a very high proportion of snRNA. According to their molecular weight (close to 60 000 for U2, the largest of them), free snRNA should not be found in the exclusion volume (BF1) after Bio-Gel P-150 chromatography. It is likely that a fraction of them is associated with proteins as free snRNP described by others (Raj et al., 1975). Such snRNP were found at 10–25 S (Gallinaro and Jacob, unpublished experiments) and would be present in BF1 if they are salt resistant. Therefore, BF1 would contain free snRNP in addition to the salt-resistant RNP of small size and to the free proteins initially predicted.

Protein Composition. The nuclear extract contained numerous proteins between 23 000 and 200 000 daltons (Figure 4). Their p*I* extended from 5.0 to 9.1 (Figure 5). They

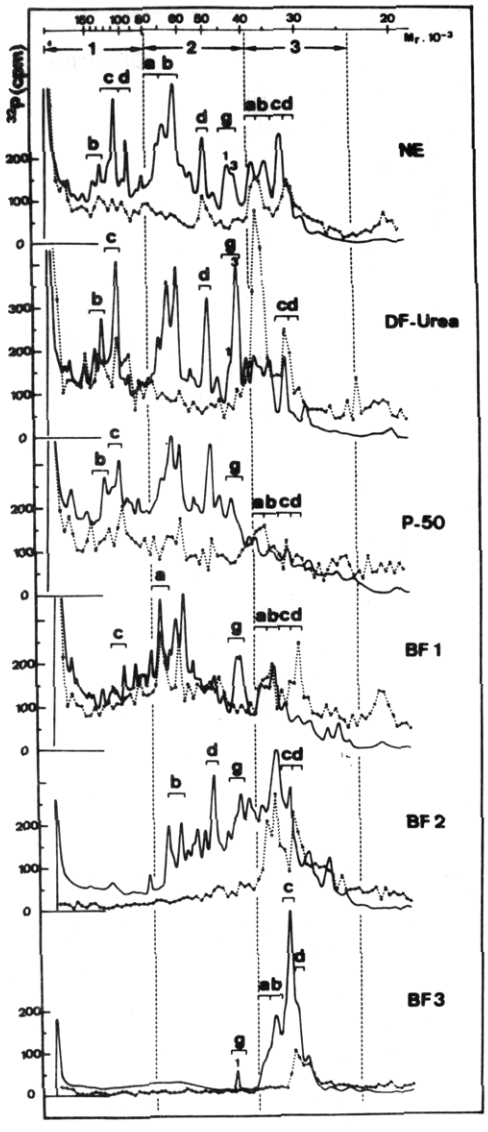

FIGURE 4: Gel electrophoresis of proteins from nuclear extract (NE), salt-resistant fractions (DF-urea, P-50, and BF1), and salt-solubilized proteins (BF2 and BF3). Absorbance was recorded after staining. The gels were then cut into slices and ^{32}P-labeled phosphoprotein radioactivity was determined. Apparent specific activities may be directly compared.

included the nucleosol proteins of molecular weight primarily above 40 000, representing 20% of the total (Stévenin et al., 1975, 1978; Gallinaro-Matringe et al., 1977). Among the hnRNP proteins, three groups could be distinguished (Stévenin et al., 1979). (1) A cluster of eight polypeptides of basic p*I* (7.5–9.1) was present between 28 000 and 38 000 daltons. They were monoparticle proteins according to our definition (Stévenin et al., 1977). (2) Another group of acidic proteins (from pH 5 to 7) including in particular the major polypeptides 1c4, 1a6, 2b5, 2c4, 2d2, 2g1, and 2h1 also belonged to monoparticles. (3) The proteins tightly bound to RNA represented those of heterogeneous complexes. Their molecular weight extended from 23 000 to 200 000 but the relative amount of proteins below 40 000 daltons was very low.

In Figures 4 and 5, we observed (1) that the basic 28 000–38 000-dalton proteins were enriched in the salt-released fractions BF2 and BF3, (2) that acidic proteins whose molecular weight is between 40 000 and 80 000 were also found among free proteins (BF2), and (3) that proteins of 28 000–38 000 daltons as well as basic proteins were minor constituents of salt-resistant RNP (DF-urea, P-50, and BF1).

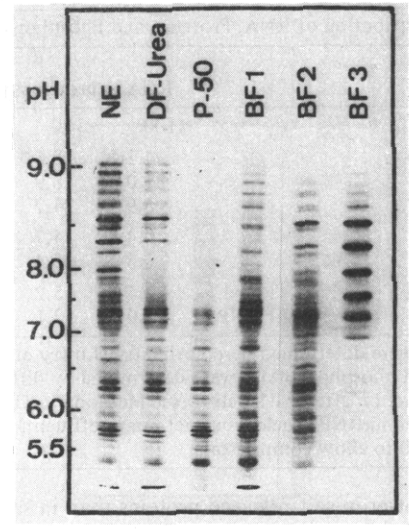

FIGURE 5: Electrofocusing of proteins from the different fractions (same fractions as described in Figure 2).

The major and most numerous proteins in these latter fractions were acidic and had molecular weights above 40 000.

We conclude that the two classes of monoparticle proteins as defined above were released by NaCl and collected in fractions BF2 and BF3. The exceptions were the monoparticle proteins of molecular weight above 80 000 expected to be present in BF1 and, possibly, a fraction of the 60 000–80 000-dalton proteins which might also be found in BF1 in consequence of overlapping.

The salt-resistant RNP proteins presented the general composition of those of heterogeneous complexes (Stévenin et al., 1977). There were few differences of composition between the three fractions. Most acidic proteins of molecular weight above 40 000 were present in all of them, though changes of distribution could be observed. An exception was a protein of 42 000 daltons (2g3), i.e., of the same molecular weight as actin previously described in hnRNP (Stévenin et al., 1977; Pagoulatos & Yaniv, 1977; Stévenin et al., 1978) and which was found in DF-urea only. A more marked difference concerned the proteins of molecular weight below 40 000. Their relative abundance as well as their distribution varied between fractions (compare DF-urea and P-50 in Figures 4 and 5, for instance). It is not known whether the relative proportions of these proteins and/or the absence of 2g3 are responsible for the difference of behavior of these two classes of salt-resistant RNP upon DEAE-cellulose chromatography.

Phosphoproteins. A heterodisperse phosphoprotein population was described in rat brain hnRNP (Gallinaro-Matringe et al., 1975; Stévenin et al., 1977) and was similar to that shown in the nuclear extract (Figure 4). Two major groups of phosphoproteins were found at the level of proteins 3a–3b and 3c–3d in the 28 000–38 000-dalton zone.

The phosphoprotein population of DF-urea, P-50, and BF1 was also heterogeneous in size. The 28 000–38 000-dalton phosphoproteins had a higher apparent specific activity in DF-urea than in P-50, in particular at the level 3a–3b. BF1 contained phosphoproteins in the 28 000–38 000-dalton range, and the apparent specific activity at the level 3c–3d was the highest in this fraction.

The salt-released proteins of 40 000–80 000 daltons were not phosphorylated, in contrast to those in the 28 000–38 000-dalton range. However, as suggested by comparison of the absorbance and radioactivity profiles of the various fractions, the apparent specific activities of the 28 000–38 000-dalton free

Table III: Relative Specific Activities of Phosphoproteins[a]

fractions	ratio of sp act. of fractions vs. BF2 (3a–3b)	ratio of sp act. of fractions vs. BF3 (3c–3d)
DF-urea	3.8	16.5
P-50	4.7	18.9
BF1	1.6	12.2

[a] The ratio of ^{32}P radioactivity to absorbance (apparent specific activity) was estimated for all fractions in zones of molecular weight 3a–3b and 3c–3d corresponding to the major peaks of phosphoprotein radioactivity in hnRNP. The apparent specific activities of phosphoproteins in salt-resistant RNP (DF-urea, P-50, and BF1) were compared to those of BF2 for zone 3a–3b and to those of BF3 for zone 3c–3d (see Figure 3).

proteins were much lower than those of the proteins of similar molecular weight in salt-resistant RNP. A tentative estimation of these differences is given in Table III.

These results are to be related with those of Table I, showing that more than 90% of the recovered phosphoproteins were present in salt-resistant RNP. It is not known whether or not phosphorylated and nonphosphorylated proteins of similar molecular weight have the same primary structure. Nevertheless, owing to the isolation of salt-resistant RNP, the results confirm and complete previous ones suggesting from indirect evidence that phosphoproteins were primarily localized in heterogeneous complexes (Gallinaro-Matringe et al., 1975; Stévenin et al., 1977).

Discussion

When devising the fractionation procedure that we propose, we had one major concern which was to achieve a clear-cut separation of salt-resistant and salt-released fractions on a large-scale basis. This was of particular importance for the study of the structure and function of the proteins as previous work had shown that proteins of close molecular weight were present in both fractions (Gallinaro-Matringe et al., 1975). The major difficulty was due to the heterogeneity of the hnRNP and of their constituents before or after dissociation by salt (Stévenin et al., 1973, 1977; Gattoni et al., 1978; Stévenin & Jacob, 1974). Methods based on sedimentation properties currently used for hnRNP preparation as well as for fraction separation were time consuming, therefore not easily adaptable to large-scale preparations, and, in addition, insufficient for total separation. As far as possible, they were replaced here by column chromatographies which allowed both a considerable reduction of the duration of manipulation and a better fractionation. The results confirmed the presence of proteins of similar molecular weight and/or p*I* in the two classes of constituents. The possibility that this might be due to aspecific adsorptions or aggregations should be evoked. These do not usually occur at the high salt concentrations used along our fractionation procedure. On the contrary, it was observed that isolated 28 000–38 000-dalton proteins (when relatively concentrated, as in our procedure) aggregated as soon as the salt concentration was lowered. Moreover, the presence of the same proteins in both fractions was also suggested by other experiments performed at low salt concentrations (Stévenin et al., 1977), and it seems unlikely that aggregation would occur similarly at low and high salt concentrations. However, the identity of primary structures should be demonstrated and its biological significance understood. This is rendered possible by the availability of a fractionation procedure.

In addition to the separation of salt-resistant RNP and salt-released proteins expected when devising our procedure, a fractionation of salt-resistant RNP and the obtention of a fraction enriched in snRNA were also achieved. We shall now discuss the advantages or limitations of the method for the study of each group of fractions.

Salt-Released Proteins. All monoparticle proteins detected by polyacrylamide gel electrophoresis up to 80 000 daltons (Stévenin et al., 1979) were recovered in fractions BF2 and BF3 after Bio-Gel chromatography. This was not the case when monoparticles were prepared prior to protein extraction (usually by endogenous ribonuclease hydrolysis). In such a case, variable amounts of polypeptides were solubilized and lost as a consequence of hydrolysis of the corresponding RNA sequences (Stévenin et al., 1977, 1979). This may explain why, in certain instances, only one or a few number of polypeptides were isolated (Krichevskaya & Georgiev, 1969; Martin et al., 1974; Christensen et al., 1977; Schweiger & Kostka, 1977; Patel et al., 1978).

The protein fractions obtained after Bio-Gel chromatography were assembled here into two large pools, but it is obvious that they might be pooled more adequately for the isolation of fractions enriched in certain polypeptides, which might then serve for further purification. Such a procedure was applied to the preparation of the basic polypeptides in the 28 000–38 000-dalton range. These are the glycin-rich polypeptides partially described by other groups (Krichevskaya & Georgiev, 1969; Martin et al., 1974; Christensen et al., 1977; Karn et al., 1977; Patel et al., 1978). Complex relationships between the various polypeptides and possible metabolic modifications were suggested by these experiments and might be of crucial importance for the comprehension of the function of that group of proteins. The detailed procedure and comparisons of the primary structures of the polypeptides will be given subsequently. In addition, 40 000–80 000-dalton proteins which were never studied could also be partially purified (Stéphan, 1978).

Salt-Resistant Ribonucleoproteins. The existence of salt-resistant RNP was shown previously (Gallinaro-Matringe et al., 1975), and comparisons of protein composition allowed their assimilation to the heterogeneous complexes defined by their relative ribonuclease resistance (Stévenin et al., 1977). However, it must be kept in mind that salt-resistant RNP are made of the heterogeneous complexes (RNA plus proteins) and of the RNA from monoparticles (see Figure 1).

A first fractionation of these ribonucleoproteins was unexpectedly achieved here but no segregation of RNP with characteristic composition could be obtained. This might be due to inadequacy of the procedure for such fractionation. On the basis of our results, and in view of the compositional heterogeneity of the hnRNA population, it seems more likely that the salt-resistant RNP represent a population of RNP with gradual changes of the protein to RNA ratio (Stévenin & Jacob, 1974) and of protein composition. The causes of the fractionation, in particular upon DEAE-cellulose chromatography, might depend on several parameters and are not clearly understood.

Among the three fractions containing salt-resistant RNP, BF1 is probably a multicomponent fraction and will be considered separately below. The most striking features of the two other fractions (DF-urea and P-50) were the presence of a majority of acidic proteins and the enrichment in phosphoproteins as compared to hnRNP. Together with resistance to salt dissociation and ribonuclease hydrolysis, these are the main characteristics of the class of ribonucleoproteins designated as heterogeneous complexes. It is likely that a large fraction, if not the totality of the snRNA detected in these

RNP, also belongs to the heterogeneous complexes, but this remains to be demonstrated unambiguously.

Our procedure is the first one allowing the obtention of heterogeneous complexes in large-scale preparations. Though some characteristics could be established, the method certainly deserves improvements for further work. In particular, as dissociation may occur, a procedure milder than elution with 6 M urea should be sought for the obtention of the RNP tightly bound to DEAE-cellulose.

Ribonucleoproteins Containing Small Nuclear RNA. After removal of the bulk of the salt-resistant RNP, the void volume of Bio-Gel chromatography was expected to contain free proteins of molecular weight above 80000 and the salt-resistant RNP too small to sediment under the conditions of obtention of P-50. The presence of high molecular weight proteins and of small-size hnRNA was in favor of this possibility.

However, the high protein to RNA ratio (Table I) and the high snRNA to hnRNA ratio (Table II) also suggested that ribonucleoproteins containing snRNA (snRNP) (Raj et al., 1975; Gallinaro and Jacob, unpublished experiments) might be additional and quantitatively important components of BF1. It should now be directly demonstrated that snRNP are present in BF1, and if so, this fraction would constitute a starting material already highly enriched for their purification and study.

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