Salt Dissociation of Nuclear Particles Containing DNA-Like RNA. Distribution of Phosphorylated and Nonphosphorylated Species†

Hélène Gallinaro-Matringe,‡ James Stevenin,§ and Monique Jacob*,#

ABSTRACT: Electrophoresis of proteins from nuclear particles containing DNA-like RNA gave a pattern with 45 bands. The possibility that some of these proteins arose by contamination with ribosomes, chromatin, or soluble nuclear proteins was examined and eliminated. The fate of the proteins of the particles was studied after partial dissociation with 0.25 and 0.70 *M* NaCl. The individual proteins were released progressively and in different quantities. A group of easily released species (75 and 95% removed with 0.25 and 0.70 *M* NaCl) was demonstrated. This group contained 8 species between 29,000 and 39,000 daltons which represented approximately one-half of the total number of molecules. It is suggested that they are bound to repetitive sequences of the RNA. At least 30 and 60% of the other proteins were released at 0.25 and 0.70 *M* NaCl, respectively. There were no specific proteins tightly bound to the RNA, unless the nature of the remaining species is different from that of the released ones of the same molecular weight. The phosphorylated proteins were more tightly bound to the RNA than the nonphosphorylated species of similar molecular weight. In several instances, the ^{32}P radioactivity was associated with quantitatively minor bands of proteins.

The nuclear particles containing the DNA-like RNA (the putative premessenger RNA) are heterogenous in size (40–300 S). Upon mild ribonuclease treatment they were converted into 30–50S structures (Samarina et al., 1968; Stévenin et al., 1970; Pederson, 1974). This suggested a polymeric structure and, therefore, the largest particles were designated as "polyparticles" which would be made of a variable number of "monoparticles". This would not imply that all the monoparticles are identical, however. CsCl density analysis indicated that the particles contain four to five times more protein than RNA. There is, however, a large discrepancy concerning the number of protein species in the particles. This number was found to be either small (1 to 3) (Krichevskaya and Georgiev, 1969; Martin et al., 1974) or large (more than 10) (Faiferman et al., 1971; Niessing and Sekeris, 1971; Matringe and Jacob, 1972a; Albrecht and Van Zyl, 1973; Ducamp and Jeanteur, 1973; Gallinaro-Matringe and Jacob, 1973, 1974; Sommerville, 1973; Pederson, 1974). The discrepancy was only partially resolved by the demonstration that a large fraction of the proteins escaped detection when the analysis was performed by gel electrophoresis in urea at pH 4.5 (Gallinaro-Matringe and Jacob, 1974), since recently a small number of proteins was also reported after gel electrophoresis in the presence of dodecyl sulfate (Martin et al., 1974). There is no obvious explanation yet for this discrepancy but it should be noted that a large number of proteins was always found in polyparticles and that the discrepancy was observed only for the 30–50S complexes. These were the products of endogenous degradation of polyparticles and it can be assumed that such a degradation might vary in degree and lead to the release of different amounts of material. This opinion is strengthened by the fact that poly(A) was found in the 30–50S structures in some cases (Cornudella et al., 1973; Ducamp and Jeanteur, 1973), but in 15–17S complexes and not in 30S complexes in other cases (Quinlan et al., 1974). In order to avoid the source of error due to uncontrolled degradation, it seemed necessary to study the more native form of particles, namely the polyparticles. With such preparations and using improved analytical methods, more than 40 bands were resolved from rat brain nuclear polyparticles. It was then crucial to demonstrate that none of them were contaminants and several arguments against contamination will be developed.

The nuclear particles are likely to be folded ribonucleoprotein strands, but the arrangement of their proteins is unknown. It was shown previously (Stévenin and Jacob, 1972, 1974; Stévenin et al., 1973) that the proteins were progressively released by increasing the salt concentration of the medium or by adding deoxycholate. As a first attempt to unravel the structure of the particles, we tried to determine if certain proteins might be more tightly bound than others to the RNA as determined by their differential solubilization by NaCl. In addition, the possible effect of phosphorylation on the release of the proteins was studied with particles labeled with $^{32}PO_4^{3-}$ in vivo (Gallinaro-Matringe and Jacob, 1973; Stévenin and Jacob, 1974). Groups of proteins with different solubility properties were demonstrated.

Methods

Labeling of Particles. Adult Wistar rats were injected intracisternally with either 50–100 μCi of tritiated uridine or 1–2 mCi of $^{32}PO_4^{3-}$. The rats were sacrificed 4 hr ([^{3}H]uridine) or 16 hr ($^{32}PO_4^{3-}$) after injection.

Preparation of Particles. Purified brain nuclei were prepared as previously described (Stévenin and Jacob, 1972).

† From the Centre de Neurochimie du C.N.R.S. and Unité 44 de l'Inserm, Université Louis Pasteur, 67085-Strasbourg Cedex, France. *Received November 11, 1974.* This work was supported in part by Grants RCP 281 and ATP 655-4303 from the C.N.R.S. and by gifts of radioactive material from the Commissariat à l'Energie Atomique.

‡ Attachée de Recherche au C.N.R.S.

§ Chargé de Recherche à l'I.N.S.E.R.M.

Maître de Recherche au C.N.R.S. Address correspondence to Centre de Neurochimie du C.N.R.S.

They were suspended in 1 ml per brain of medium F (10 m*M* Tris-HCl (pH 8)-100 m*M* NaCl-25 m*M* KCl-1 m*M* $MgCl_2$) containing cytoplasmic ribonuclease inhibitor. The inhibitor was prepared according to Roth (1958) and dialyzed against 10 m*M* triethanolamine-HCl (pH 7.4)-25 m*M* KCl. They were rapidly lysed with 0.2% (w/v) deoxycholate and the lysate was immediately centrifuged for 5 min at 35,000 rpm (100,000*g*) in a SW50 rotor (Stévenin and Jacob, 1972). The supernatant designated as "nuclear extract" was centrifuged on a 10–25% (w/v) linear sucrose gradient in buffer B (10 m*M* triethanolamine-HCl (pH 7.4)-25 m*M* KCl-1 m*M* $MgCl_2$) for 3.5 hr at 23,000 rpm (63,800*g*) in an SW 25-2 rotor (preparative gradient). Sedimentation coefficients were determined according to Martin and Ames (1961). Fractions of sedimentation coefficients lower than 60 S, containing free or aggregated soluble proteins, were discarded (Stévenin and Jacob, 1974). CsCl buoyant densities were determined after formaldehyde fixation as previously described (Stévenin and Jacob, 1972).

Preparation of Soluble Nuclear Proteins. The purified nuclei were suspended in buffer F without cytoplasmic ribonuclease inhibitor. They were lysed by ultrasonication (45 sec, 1.25 A, MSE Sonicator) (Stévenin et al., 1973; Stévenin and Jacob, 1974). Subsequent steps were identical with those described above for particles. CsCl density was determined after labeling with ^{3}H-labeled amino acids. Proteins in different fractions of the gradient were analyzed. Free proteins were mainly present in the 0–30S and particles in the 30–200S regions. The amounts of various proteins in these regions were estimated and were considered as representative of the actual pools of soluble and particle proteins. A more detailed account of this work will be given elsewhere (manuscript in preparation).

Preparation of Chromatin. Chromatin was prepared from purified brain nuclei according to Bhorjee and Pederson (1973). An additional ribonuclease treatment was necessary in order to remove remaining particles (manuscript in preparation). For electrophoresis of proteins, chromatin was dissociated overnight with 6 *M* urea, 1% dodecyl sulfate, and 1 m*M* dithiothreitol.

Treatment of Particles with NaCl. NaCl (5 *M*) was added to selected fractions of the preparative gradients up to the chosen final concentration. For 0.25 *M* NaCl treatment the suspension was layered on a cushion of 2.5 ml of 1.2 *M* sucrose in buffer B in rotor R 65 tubes and centrifuged for 3.5 hr at 54,000 rpm (169,200*g*). For 0.70 *M* NaCl treatment a double cushion (1.5 ml of 1.2 *M* sucrose in buffer B and 1.0 ml of 1.0 *M* sucrose in buffer B containing 0.4 *M* NaCl) was used. Centrifugation was for 4 hr at 60,000 rpm (229,400*g*). In both cases the rotor reached the maximum speed 30 min after the addition of NaCl to the particles. The supernatant, sucrose cushion, and the pelleted complexes were collected.

Polyacrylamide Gel Electrophoresis of Proteins. The particles in suspension in sucrose, the NaCl supernatant, and the sucrose cushions were precipitated overnight with 10% (w/v) trichloroacetic acid and centrifuged for 20 min at 35,000 rpm (120,000*g*) in an SW 56 rotor. The trichloroacetic acid insoluble pellets and the pelleted complexes were dissolved in 10 m*M* Tris-HCl (pH 7.5)-1 m*M* dithiothreitol-6 *M* urea. The final pH was adjusted to 7.5. In ^{32}P-labeling experiments the material was treated overnight with 1 (particles) or 2 (complexes) μg/pancreatic ribonuclease at 4°.

Samples were treated with 1% (w/v) sodium dodecyl sulfate and additional 1 m*M* dithiothreitol at 60° for 10 min. Electrophoresis was carried out according to Waehneldt (1971) as previously described (Matringe and Jacob, 1972a) with the following modifications: 0.4 ml of a 12% polyacrylamide solution was polymerized at the bottom of the tubes; 1.6 ml of a 10% polyacrylamide solution was layered and polymerized on top. The stacking gel was unmodified. The tubes (6 mm i.d.) were pretreated with 1% (v/v) silicone (Siliclad, Clay Adams) to avoid the formation of gas bubbles. Electrophoresis was carried out first at 0.35 mA/gel until the tracking dye (Bromophenol Blue) reached the end of the stacking gel (approximately 2.5 hr), then at 2.5 mA/gel until the dye reached the end of the running gel (3 to 4 hr). Molecular weights were estimated according to Weber and Osborn (1969). The gels were stained with Coomassie Brilliant Blue R and scanned with the aid of a Vernon recorder (Paris, France). For ^{32}P-labeling experiments, the position of several bands along the gels was marked by the insertion of a thin wire. The gels were then frozen and cut into 1-mm slices which were counted for radioactivity.

Estimation of the Amount of Proteins in the Gels. The area of selected peaks or groups of peaks was measured on the recordings by planimetry (A. Ott, Planimeter). The intensity of the dye was proportional to the amount of proteins in the range of 0.1 to 1.5 μg as checked with markers (serum albumin, ovalbumin, chymotrypsinogen, and myoglobin). This range corresponded to our experimental conditions. The method is only valid for the comparison of the amount of a given protein since the intensity of the dye changes with the nature of the protein. The experimental error was estimated to be about 10%.

Results

Number of Proteins and Nomenclature. The proteins from nuclear particles covered a wide range of molecular weight (from 20,000 to 150,000) (Niessing and Sekeris, 1971; Albrecht and Van Zyl, 1973; Ducamp and Jeanteur, 1973; Gallinaro-Matringe and Jacob, 1973, 1974; Pederson, 1974). The modification of the electrophoresis conditions (see Methods) led to a much better resolution than obtained previously (Matringe and Jacob, 1972a; Gallinaro-Matringe and Jacob, 1973) and increased the number of visible bands by a factor of 2 or 3 (Figure 1, P). The treatment of the particles by salt (Figure 1, C) which released some quantitatively major proteins (see below) also permitted the visualization of some additional bands. For instance, with our former procedure 3a and 3b were two ill-resolved bands. With the new method, 3a was found as a relatively large band well separated from 3b in which two species were visible (Figure 1, P). After treatment with 0.25 *M* NaCl (Figure 1, C) it clearly appeared that 3a and 3b contained two and three bands, respectively. In total, 40 to 45 bands were separated in the region of molecular weight from 22,000 to 150,000.

The former nomenclature (Matringe and Jacob, 1972a) based on the division of the gels into four molecular weight zones (zones 1, 2, 3, and 4 of decreasing molecular weight) and on designation of the different bands inside each zone by lower case letters (2a, 2b, etc. . . .) was maintained. The newly resolved bands were designated as the former unique band with an additional number; for instance, 2c was separated into $2c_1$, $2c_2$, $2c_3$.

Presence of Contaminants in the Particles. Polysomes

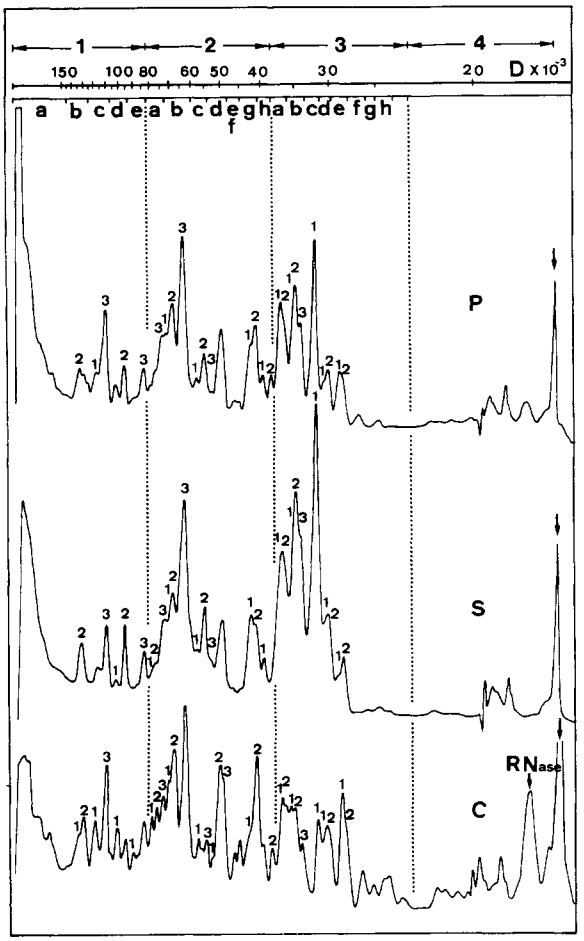

FIGURE 1: Electrophoresis of the proteins from nuclear particles (P) and comparison with the proteins released from (S) and bound to the RNA (C) at 0.25 *M* NaCl. The scale of molecular weight (second line from the top), the delimitation of zones (first line), and subzones (third line) are indicated. The numbers along the profiles point to individual protein bands. The position of the tracking dye is indicated by a vertical arrow.

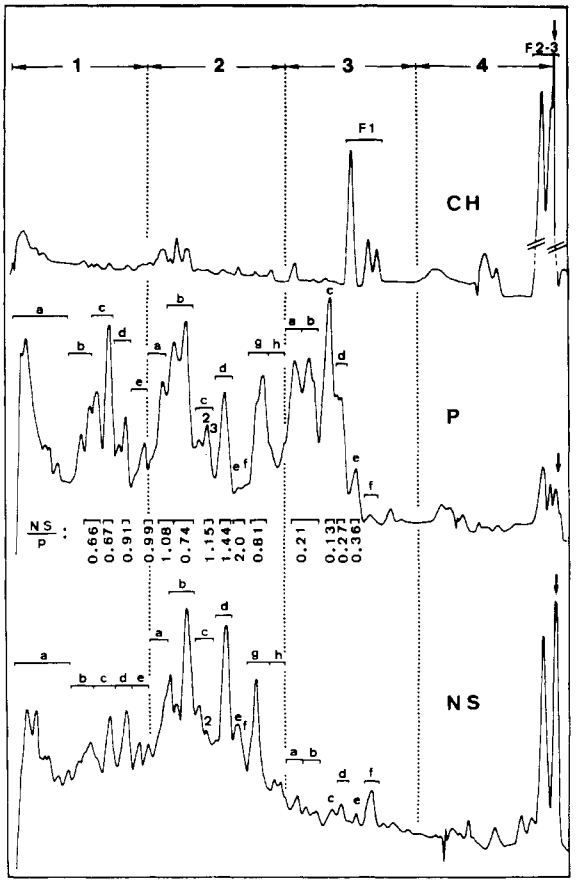

FIGURE 2: Comparison of the proteins from brain chromatin (CH), particles (P), and nuclear soluble proteins (NS). Zones, subzones, protein numbers, and arrow are as in Figure 1. The position of histones F1 and F2-3 is indicated in the upper profile (CH). The scale at the level of F2-3 is half that used for the other parts of the profile. The molecular weights corresponding to zones and subzones are identical with those of Figure 1. The vertical dotted lines indicate 82,000, 38,000, and 23,000 daltons, the limits between zones 1 and 2, 2 and 3, and 3 and 4, respectively.

and preribosomes, chromatin, and soluble proteins are the most probable contaminants in nuclear particles preparations. The presence of few polysomes in the particles preparation was shown by electron microscopic examination (unpublished observations) but various arguments mainly based on the determination of CsCl density of particles preparation and on RNA analysis demonstrated that this amount was small (Stévenin et al., 1970; Stévenin and Jacob, 1974). This was also indicated by the low amount of proteins in zone 4 of the gels (Figures 1, 2, and 3, P) where many of the structural proteins of ribosomes migrate (Matringe and Jacob, 1972a,b). In addition, the electrophoretic profile of the particles proteins was unchanged upon an EDTA treatment (results not shown) which dissociated ribosomes but not nuclear particles (Stévenin et al., 1970; Faiferman et al., 1970; Pederson, 1974). In conclusion, the contamination with polysomes was too low to reveal any additional bands.

The particles preparations contained 5-10 μg of DNA/100 μg of RNA and might therefore be contaminated with chromatin. It was estimated that for the maximal DNA/RNA ratio of 0.1, there would be 1.4 and 3% of nonhistone proteins and histones, respectively. This would make the numerous nonhistone proteins undetectable. In addition, histones (Figure 2, CH) were never detected among particles proteins (Figures 1, 2, and 3, P). Histone F1 was shown by split gel analysis to have a mobility different from that of particles proteins 3d and 3e.

The possibility of contamination with soluble proteins had to be carefully eliminated, since we intended (see next paragraph) to study proteins released by NaCl. For the isolation of soluble proteins our routine procedure of lysis of nuclei in the presence of ribonuclease inhibitor could not be used since it introduced exogenous soluble proteins. This excluded the possibility of lysis with 0.2% deoxycholate which released latent ribonucleases (Stévenin and Jacob, 1972). Therefore, the nuclei were ultrasonicated. Particles prepared from nuclei lysed by each of the two methods had the same sedimentation coefficient and CsCl density. In addition, the amount of proteins released by various concentrations of NaCl as determined by density in CsCl was similar in both cases (Stévenin et al., 1973; Stévenin and Jacob, 1974). It was also shown (manuscript in preparation) that the degradation of particles by endogenous ribonucleases was very limited under the conditions used for ultrasonication. The present work demonstrated that the electrophoretic profiles of the particles proteins prepared with the two methods were practically superimposable (compare Figure 1, P, and Figure 2, P) and that the proteins released with NaCl were the same (not shown). In conclusion, the use of

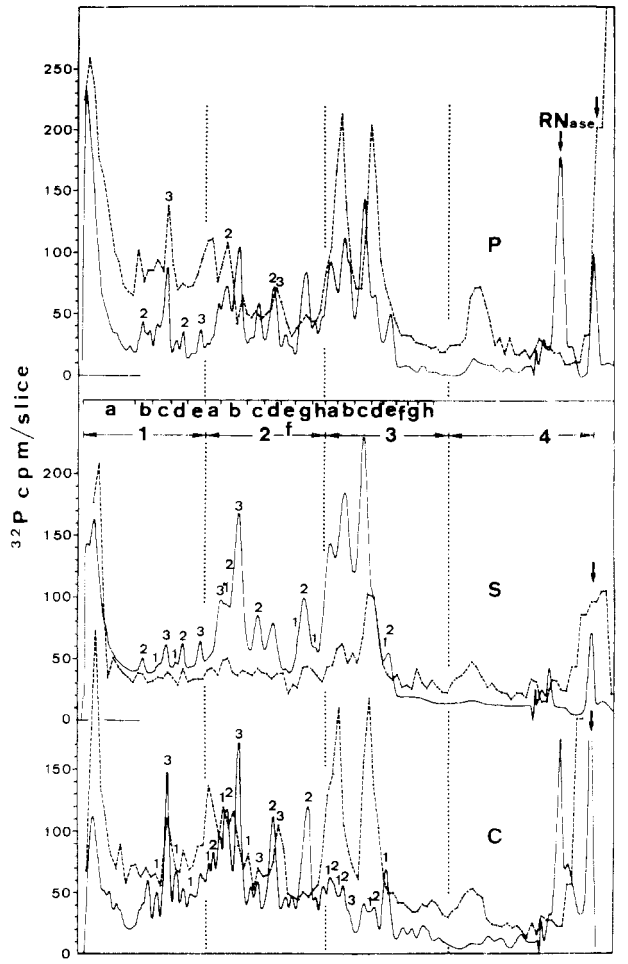

FIGURE 3: Electrophoresis of phosphorylated proteins from nuclear particles after 0.25 *M* NaCl treatment. Nomenclature and symbols as in Figure 1: (----) ^{32}P radioactivity. The molecular weights corresponding to zones and subzones are identical with those of Figure 1. The vertical dotted lines indicate 82,000, 38,000, and 23,000 daltons, the limits between zones 1 and 2, 2 and 3, and 3 and 4, respectively.

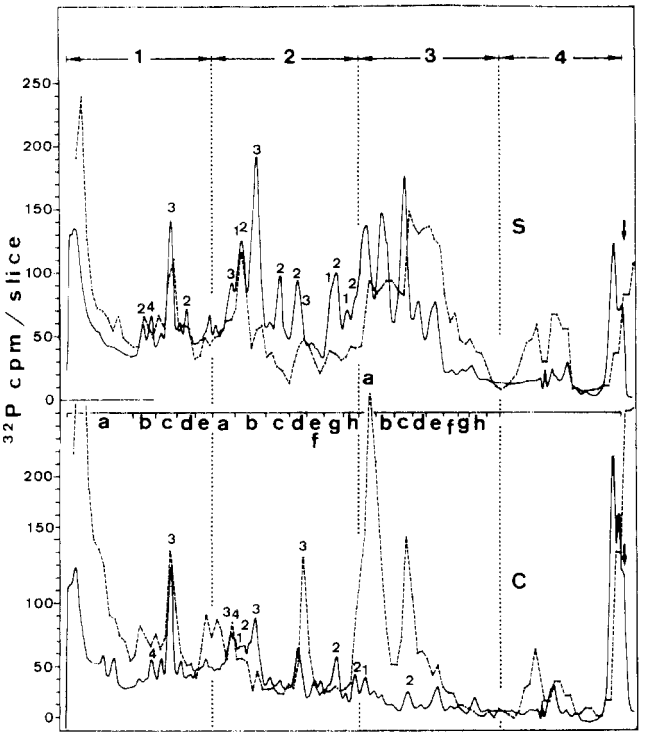

FIGURE 4: Electrophoresis of proteins from nuclear particles after 0.70 *M* NaCl treatment. Nomenclature and symbols as in Figure 1: (----) ^{32}P radioactivity. The molecular weights corresponding to zones and subzones are identical with those of Figure 1. The vertical dotted lines indicate 82,000, 38,000, and 23,000 daltons, the limits between zones 1 and 2, 2 and 3, and 3 and 4, respectively.

ultrasonication as an alternative method of lysis of nuclei is justified.

CsCl density analysis had shown that detectable amounts of free aggregated proteins were not found in regions of the gradients above 60 S (Stévenin and Jacob, 1974). However, soluble proteins might associate nonspecifically with the particles of higher sedimentation coefficient or their RNA and be mistaken for particle proteins. In order to explore this possibility we compared the proteins from particles and from a soluble fraction. A detailed account of the relationships between particles and soluble proteins will be reported elsewhere and only results pertaining to this discussion will be given here.

There were large differences between the electrophoretic profiles of soluble (0–10 S) and particles (75–85 S) proteins (Figure 2, NS and P). The relative amount of proteins of zone 3 as compared to zones 1 and 2 was much smaller in NS than in P. It was estimated that the ratio of the absolute amount of various proteins of zone 3 in the soluble pool to that in the corresponding particles was 0.1 to 0.4 (ratio NS/P, Figure 2). The proteins of larger molecular weight (zones 1 and 2) had a different distribution in NS and P and may or may not be different species. The NS/P ratio was below or close to 1 in most cases with the only exception being the region 2e–f where it reached 2. The probability of nonspecific association is likely to be dependent, at least partly, upon the NS/P ratio and thus we conclude that the probability of such an association is low for these groups of proteins, especially those of zone 3.

Distribution of the Proteins after Dissociation of the Particles with NaCl. The determination of density in CsCl of complexes obtained after NaCl or KCl treatment had shown that 30–40% and 70–80% of the proteins were released from the particles at 0.25 and 0.70 *M* NaCl, respectively (Stévenin et al., 1973). These NaCl concentrations were adopted for the demonstration of easily released and tightly bound proteins. Higher concentrations of NaCl were not used since the amount of proteins remaining bound to the RNA was prohibitively low.

Sucrose gradient fractions containing the particles were adjusted to the chosen NaCl concentration, layered on a sucrose cushion, and centrifuged (see Methods). The supernatant and the pellet contained the released proteins and the remaining complexes, respectively. Several verifications were performed. (1) When untreated particles were centrifuged under the same conditions as salt-treated particles, only traces of material were found in the supernatant and the sucrose cushion. (2) After salt treatment the decrease of sedimentation coefficient of the particles was that expected by the release of proteins (Stévenin et al., 1973). This indicated the absence of marked ribonuclease hydrolysis of the polyparticles, which was ascertained under the present experimental conditions by the use of uridine-labeled particles. Less than 5 and 10% of the radioactive RNA were found in the supernatant and sucrose cushion, respectively. (3) The sucrose cushion used to ensure a good separation between released proteins and remaining complexes con-

tained less than 5% of the total proteins. Their electrophoretic profile was that of a mixture of both fractions.

The proteins from the supernatant (profiles S) representing the released species and from the remaining complexes (profiles C) were analyzed and their electrophoretic profiles were compared to those of untreated particles (profiles P). The profiles will be commented upon zone by zone (Figures 1, 3, and 4).

Zone 1. The region 1a close to the boundary between the stacking and running gels contained irreproducible bands and will not be examined. There were 12 bands in the region from 1b to 1e (Figures 1 and 3, P). The major one was $1c_3$ followed by $1b_2$, $1d_2$, and $1e_3$. After treatment with 0.25 *M* NaCl (Figures 1 and 3), these four species were also major in S, but the relative amount of $1c_3$ was decreased. Among the minor bands, only $1c_1$ and $1d_1$ were detectable in S. In C, $1c_3$ was the major species; the relative amount of the minor bands, in particular $1c_1$, $1d_1$, and $1e_1$, increased.

After 0.70 *M* NaCl, $1c_3$ increased in S (Figure 4) as did the minor bands (such as $1b_4$). The amount of most bands relative to $1c_3$ decreased in C. $1c_3$ remained the main species in this fraction. $1d_2$ was almost totally released.

Zone 2. There were 18 bands in this zone (Figures 1 and 3, P). After 0.25 *M* NaCl, the main species found in S were $2a_3$, $2b_{1-2}$, $2b_3$, $2c_2$, 2d, $2g_1$, $2g_2$, and $2h_1$. These species were also present in C, where a relative enrichment in $2b_{1-2}$, 2d, and $2g_2$ was observed. Some bands were not detectable ($2h_2$) or were found only as traces in S ($2a_{1-2}$, $2c_1$, $2c_3$, 2e, and 2f). They were clearly enriched in C where new bands such as $2d_1$ and $2d_3$ were sometimes revealed as shoulders of the main bands. $2a_1$, $2a_2$, and $2a_3$ were seen as discrete peaks in this fraction whereas they were not separated in P.

NaCl (0.70 *M*) removed more of the same species as did 0.25 *M* NaCl (Figure 4, S). Part of $2h_2$ was released and the ratio $2g_2/2g_1$ was similar to that in P. The profile in C (Figure 4) resembled that obtained with 0.25 *M* NaCl (Figure 3) but the amount of the major bands ($2a_3$, $2b_{1-2-3}$, 2d, and $2g_2$) decreased relative to those of the minor species.

Zone 3. Large quantities of 3a, 3b, and especially 3c were found in S after 0.25 *M* NaCl (Figures 1 and 3). Consequently they decreased in C and thus permitted a better resolution and even revealed components ($3a_1$, $3a_2$, $3b_1$, $3b_2$, and $3b_3$). 3d and 3e (two bands each) were distributed between S and C. There was a relative enrichment of $3e_1$ in C. The minor bands in regions 3f, 3g, and 3h that were barely detectable in P were found almost exclusively in C. At least 15 bands were resolved in zone 3.

NaCl (0.70 *M*) (Figure 4) released most of the proteins of zone 3. Their profile in S was practically superimposable on that in P. The small amounts of these proteins remaining in C consisted mainly of four bands with mobilities corresponding to $3a_1$, 3c, 3e, and 3h. It is not known whether some of these bands represented a small amount of the main species or whether they are different species revealed by the salt treatment. For instance, the peak tentatively designated as $3c_2$ may have been masked in the profile of nondissociated particles by the high amount of the major 3c.

There were a few quantitatively minor proteins in zone 4. One of the peaks corresponded to the limit between the 10 and 12% polyacrylamide gels; another one was an artefact also found on blank gels. Ribonuclease, when added, also migrated in this zone. The proteins of zone 4 will not be discussed.

In the NaCl experiments the distribution of some proteins or groups of proteins between the supernatant (S) and the remaining complexes (C) was estimated, taking into account the peak's areas and the amount of material in the fractions. Due to the lack of precision inherent in the method of area delimitation, this estimation was obviously rough, especially for the minor species. The relative amount of release gradually varied for the individual species between 30 and 89% at 0.25 *M* NaCl; therefore, the division into groups was somewhat arbitrary (Table I). However, a group (I) of easily released proteins (3a, 3b, 3c, and 3d) could be clearly defined. Approximately 75 and 95% of these species were removed with NaCl, 0.25 and 0.70 *M*, respectively. In group IV, 0.25 *M* NaCl removed only 30–40%, i.e., half of the amount of the proteins of the first group. No more than 67% of these species were released at 0.70 *M* NaCl. The two other groups (II and III) showed an intermediate behavior.

Table I: Estimation of the Percentage of Proteins Released with NaCl.

Groups	Protein Bands	% of Proteins (Absorbance) Released at NaCl: 0.25 *M* *a*	0.25 *M* *b*	0.70 *M*	% of Proteins, ^{32}P, Released at NaCl[c]: 0.25 *M*	0.70 *M*
I	3c	78	89	95		
	3a	{66	74	92	{22	
	3b		80	95		46
	3d	71	65	96	30	76
II	$2c_2$	56	65	85		
	$2b_3$	52	58	79	30	68
III	3e	31	42	85		
	$2h_1h_2$		45	80		
	2g	44	53	78		
	$1d_2$		47	76		
	$2a_3a_4b_1b_2$	43	40	70	27	71
IV	$2a_1a_2$	40	34	67		
	2d	37	40	66	26	48
	2e, 2f	25	40	64		
	$1e_3$		42	64	36	54
	$1c_1$		37	61		
	$1c_3$		30	65	33	60
	$1d_1$		35	60		

[a] In this series of two experiments, the average dissociation of particles was slightly lower than in the following ones. [b] Average of the experiments of Figures 1 and 3. [c] These percentages correspond to regions of the gel rather than to percise bands.

It was already evident from the observation of electrophoretic profiles that no protein remained specifically bound to the RNA, since at least 60% of each band was released at 0.70 *M* NaCl. However, it cannot be excluded that some minor bands (such as, e.g., the remaining 3a and 3c) represented different species tightly and specifically bound to the RNA.

Phosphorylated Proteins. ^{32}P was incorporated into proteins from particles as demonstrated previously by sensitivity to Pronase, insensitivity to ribonuclease at low salt concentration, and insolubility in trichloroacetic acid at 90° (Gallinaro-Matringe and Jacob, 1973). The ^{32}P radioactivity insoluble in trichloroacetic acid was found exclusively associated to material of density in CsCl of 1.39–1.40, identical with that of particles.

In the presence of NaCl the phosphorylated proteins were more firmly associated to the RNA than the nonphosphorylated species (Figures 3 and 4). NaCl, 0.25 and 0.70 *M*, respectively, released 23 and 65% of the ^{32}P material

from the region 22,000–150,000 daltons vs. 55 and 87% for the bulk of the proteins (estimation of the total area under the peaks). CsCl density analysis after salt treatment confirmed that the ^{32}P material insoluble in trichloroacetic acid at 90° remained preferentially associated to the ribonucleoprotein complexes containing the RNA. As previously observed many species were phosphorylated in the particles and a high amount of radioactivity was found at the level of 3a–b and 3c–d–e. Due to overlapping it was usually difficult to attribute the radioactivity to a given band; therefore, in most cases, regions rather than bands will be considered.

At 0.25 *M* NaCl (Figure 3) only a little, randomly distributed ^{32}P radioactivity was found in S. A discrete peak was present at the level of 3d only. Most of the phosphorylated proteins remained in C.

At 0.70 *M* NaCl (Figure 4) some phosphorylated species were released (S), in particular $1c_3$, $2b_{1-2}$, and $2d_3$; ^{32}P-labeled material with the same molecular weight remained associated to the RNA (C). Some ^{32}P from the regions 3a–b and especially 3c–d–e was also released. A large amount of ^{32}P was still found in C at the level of 3a–b as well as a lower amount at the level of 3c–d–e.

In some cases (for instance, $1c_3$, 1e, $2a_{3-4}$, and b_{1-2}) the distribution of radioactivity between S and C was similar to that of absorbance in the same region (Table I). In other cases, and particularly in region 3, much more radioactivity than absorbance remained associated to the RNA; whatever the band (or bands) to which the radioactivity was associated, it was obvious (Figure 4, C) that the specific activity of the remaining proteins 3a–b and 3c was very high. This was also true for the peak $2d_3$ whose radioactivity seemed to be associated to very little absorbance.

The exact number of phosphorylated proteins could not be determined from our experiments though this number was high as shown in particular after 0.70 *M* NaCl treatment (Figure 4). The heterogeneity of phosphorylated species in zone 3 might be as marked as that observed in the absorbance studies.

Discussion

A prerequisite for a study of the proteins from the nuclear particles was the absence of detectable amounts of contaminating proteins. It was demonstrated that the contaminating polysomes and chromatin were below detection at the level of stained bands. The contamination by aggregates of soluble proteins was avoided by studying only particles with sedimentation coefficients higher than 60 S (Stévenin and Jacob, 1974). On the other hand, a large nonspecific fixation was unlikely for the following reasons. (1) Nuclear particles of various origins extracted by various methods [ultrasonication (Stévenin et al., 1973; Pederson, 1974), deoxycholate lysis (Stévenin et al., 1970; Stévenin and Jacob, 1972), diffusion (Samarina et al., 1968; Niessing and Sekeris, 1971; Martin and McCarthy, 1972; Ducamp and Jeanteur, 1973), high pressure (Faiferman et al., 1971), and incubation with ATP (Ishikawa et al., 1974)] had similar protein to RNA ratios as determined by CsCl density gradients. (2) The particles were isolated in media containing 0.125 *M* monocations which do not favor nonspecific fixation (Baltimore and Huang, 1970; Olsnes, 1971). (3) Complexes made in vitro between RNA and soluble proteins were much less stable than native particles even upon centrifugation in sucrose gradients (Spirin, 1969; Zawislak et al., 1974). (4) Exogenous free RNA, when present during the lysis of nuclei, did not bind proteins (Pederson, 1974).

Nevertheless, the possibility of a nonspecific fixation of a few proteins was not excluded. It was crucial for our study to determine whether the most easily released proteins were not such contaminants. These proteins (zone 3, Table I) were very scarce in the nucleosol (Figure 2, C) and the probability of a contamination was very low. Proteins in the regions 2d, 2e, and 2f were those where the probability of contamination, though still low, was the highest (Figure 2). However, these proteins belonged to group IV (Table I) and were among the most tightly bound to the RNA. Therefore, we assume that they were not contaminants. Our conclusions obviously do not exclude that some proteins from the particles can be found in a soluble form. This possibility will be developed elsewhere.

Taken together, the results strongly suggest that the 45 protein bands are native to the particles. This number must be taken as an underestimation since proteins of very low (zone 1a) and very high (zone 4) electrophoretic mobility were not analyzed. Moreover, even better resolution should be achieved (by the use, for instance, of two-dimensional electrophoresis) to determine the protein composition of the particles. The same protein composition was observed when nuclei were lysed with 0.2% deoxycholate (Figure 1, P) or by ultrasonication (Figure 2, P). Therefore, the procedure per se was not responsible for a variation in the number of proteins. It is worth mentioning that large-size particles were obtained with both methods. It is suggested that a partial degradation of particles might lead to structures with fewer proteins and this possibility is under current investigation.

The dissociation of the particles by NaCl permitted the separation of their proteins into four groups (Table I). Although this separation was somewhat arbitrary due to the existence of a range of proteins of gradually changing behavior, a group (I) of easily released proteins was conspicuous. These proteins had an apparent molecular weight between 29,000 and 39,000 (zone 3). The preferential release of proteins in the same range of molecular weight was also described with nuclear particles of HeLa cells treated with 0.5 or 1.0 *M* NaCl (Pederson, 1974). Their release was not an "all or nothing" or a cooperative phenomenon [like the release of histone F1 from chromatin (Ohlenbusch et al., 1967)] but was progressive. The phosphorylated proteins of this group were more tightly bound to the RNA than the nonphosphorylated species. It is not yet known whether they were different species or whether some proteins were partially phosphorylated and only the phosphorylated fraction was tightly bound to the RNA. The small amount of the protein remaining after 0.70 *M* NaCl, tentatively identified as $3c_2$ (Figure 4), could be an example of the first possibility. In the latter case it could be imagined by analogy with histone F1 and DNA (Louie and Dixon, 1973) that the phosphorylation modulates the association of the protein to the RNA in vivo and perhaps the configuration of the particle.

The dissociation of the proteins of the other groups was also progressive. After 0.70 *M* NaCl at least 60% of them were removed. They can, therefore, be considered as tightly bound only relatively to the proteins of group I. Most, though not all, of the proteins of highest molecular weight were thus relatively tightly bound (zone 1, group IV). As in group I and with the same possible interpretation, the phosphorylated species were more tightly bound than the other

proteins of similar molecular weight (with the exception of $1c_3$).

RNA–protein and protein–protein interactions probably ensure the structural organization of the particles. It might be assumed that NaCl dissociates electrostatic bonds, in all likelihood those between RNA and proteins. Moreover, deoxycholate at concentrations of 1% or higher released proteins from the particles (Faiferman et al., 1971; Stévenin and Jacob, 1972) and the most easily released species were those of group I (unpublished observations), suggesting that the same bonds were dissociated as with NaCl. The deoxycholate concentration required to provoke the same dissociation as NaCl was approximately an order of magnitude lower, as also found for the dissociation of histones from chromatin (Smart and Bonner, 1971). As suggested by these authors, this might be due to a binding of the anion deoxycholate, stronger than that of chloride to specific sites of the proteins. In addition to electrostatic bonds, the possibility of the existence of hydrophobic bonds dissociated by deoxycholate, as suggested by Faiferman et al. (1971), must also be taken into account. Moreover, the direct dissociation action of agents like NaCl or deoxycholate might be modified by such parameters as the accessibility of a given macromolecule to the agent or as the interactions with other molecules in its vicinity. The intervention of such parameters is suggested by the progressivity of the release of the proteins from the particles. Due to the complexity of the large ribonucleoprotein particles the interpretation of the experimental results must be considered as partially speculative in the actual state of our knowledge.

Even less is known about the role of the proteins from the particles. It might be expected that a different role is played by those bound to the messenger and nonmessenger sequences of the premessenger RNA. The results already obtained permit a first tentative classification that will be discussed now.

The most easily released proteins (group I) were among the most abundant species of the particles. From the absorbance profiles it was estimated that 3a, 3b, 3c, and 3d (eight species) accounted for approximately one-third of the total mass of proteins. Due to their low molecular weight relative to that of the other proteins they represented more than one-half of the total number of molecules. Insofar as a specificity of the protein–RNA interaction is assumed, this suggests that the proteins of group I are associated to repetitive sequences common to the RNA of most or all brain nuclear particles. Preliminary work (Mangenot, 1974) showed that the proteins of group I had identical electrophoretic mobilities in several tissues of the same animal species. This is an argument in favor of the association with repetitive sequences common to many if not all the nuclear RNAs of the same organism. Repetitive sequences located in the nonmessenger portion of the premessenger RNA have been demonstrated (Pagoulatos and Darnell, 1970; Greenberg and Perry, 1971; Georgiev et al., 1972) as well as a large homology between the rapidly labeled RNA of various tissues (McCarthy and Hoyer, 1964).

It is relevant to mention here that the one or two proteins obtained from the 30S particles (Georgiev and Samarina, 1971; Martin et al., 1974) are in the same molecular weight range as the proteins of group I. The RNA of these particles hybridized with moderately repetitive sequences of DNA, an observation which is also in favor of the association of these proteins with repetitive sequences of premessenger RNA (Martin and McCarthy, 1972).

On the basis of these various observations it is proposed that the proteins of group I are associated to relatively abundant nuclear-restricted sequences of premessenger RNA. Obviously other proteins might also be bound to such sequences since such association is not a priori related to the affinity of the protein to RNA in the presence of salt. This proposition as well as our initial assumption about the specificity of the RNA–protein interaction will require further experimental verification.

The proteins bound to the messenger RNA sequences and to the attached poly(A) are expected to be relatively scarce since these sequences correspond only to a small fraction of the premessenger RNA and, in addition, poly(A) is only attached to a limited number of molecules (Greenberg and Perry, 1972). If some of them are to accompany the RNA during its transport to the cytoplasm, their association with the RNA should be similar to that of proteins from cytoplasmic particles. Proteins from 49,000–52,000 and 73,000–78,000 daltons which remained bound to polysomal messenger RNA in the presence of 0.5 *M* NaCl have been described and seem to be common to several species or tissues (Blobel, 1973; Bryan and Hayashi, 1973). There were several minor species in this range of molecular weight in the nuclear particles ($2a_1$, $2a_2$, $2c_1$, $2c_2$, $2d_1$, 2e, and 2f) and they were among the relatively tightly bound species. At the moment, no argument can be presented suggesting that one or another of these species is truly bound to the messenger RNA or the poly(A) sequences in the nuclear particles.

Much work will certainly be needed to confirm these speculations and to get an insight into the complex structural organization of the nuclear particles.

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A Comparison of the Fluorescence of the Y Base of Yeast $tRNA^{Phe}$ in Solution and in Crystals†

Richard Langlois, Sung-Hou Kim, and Charles R. Cantor*

ABSTRACT: The fluorescence properties of the Y base of yeast $tRNA^{Phe}$ are known to be quite sensitive to the environment. The fluorescence lifetime of the Y base in yeast $tRNA^{Phe}$ is identical in orthorhombic crystals and in the mother liquor from which these crystals are grown. It is 10% higher than the lifetime observed in dilute solutions of tRNA. This small change is a solvent effect due to isopropyl alcohol in the crystallization medium. Isopropyl alcohol does not change the accessibility of the chromophore of the Y base as measured by iodide quenching rates in solution. The accessibility in intact $tRNA^{Phe}$ is much less than in a ribonuclease digest. Thus, within the limits of the sensitivity of the method, the Y chromophore occupies the same environment in solution and in the crystal and it must be at least partially buried.

High-resolution X-ray crystallography is the most powerful tool for the elucidation of the three-dimensional structure of proteins and nucleic acids. There are always lingering doubts, however, whether the structure determined in the crystal is the same as the structure in solution. This is especially true when one considers the relatively weak interactions that stabilize the tertiary structure of biopolymers. This uncertainty is particularly significant for tRNA. The solution structure as measured by circular dichroism (CD) is quite variable, depending on specific solvent conditions (Prinz et al., 1974), which may indicate that structural changes occur during in vivo protein synthesis. Also, at least eight different crystal forms have been observed for yeast $tRNA^{Phe}$, some containing up to 80% solvent (Cramer et al., 1974). X-Ray diffraction studies on two crystal forms have yielded the three-dimensional structure of this tRNA (Kim et al., 1974; Robertus et al., 1974). Low field nuclear magnetic resonance NMR (Kearns and Shulman, 1974) and singlet-singlet energy transfer measurements (Yang and Soll, 1974) are consistent with a tRNA solution structure similar to that found in crystals. However, these mea-

† From the Departments of Chemistry and Biological Sciences, Columbia University, New York, New York 10027 (R.L. and C.R.C.), and Department of Biochemistry, Duke University, Durham, North Carolina 27710 (S.-H.K.). *Received November 25, 1974.* This work was supported by Grants from the U.S. Public Health Service, GM 14825 and CA 15802, and from National Science Foundation (GB 40814).