Metrizamide dissociates nuclear particles containing heterogeneous nuclear RNA

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ABSTRACT

Metrizamide gradients were tested for the possible fractionation of the constitutive units of nuclear particles. Material from 35-55 S monoparticles was indeed distributed along the gradient but rerun experiments, CsCl density determinations, formaldehyde fixation prior to centrifugation suggested that the separation was due to dissociation or (and) action of endogeneous ribonucleases rather than to monoparticle fractionation. That dissociation had indeed occurred was confirmed by the study of 60-110 S polyparticles. They were dissociated into ribonucleoproteins rich in phosphoproteins and into free proteins. These products were essentially similar to those obtained after NaCl treatment of the particles though the modes of action of metrizamide and NaCl are likely to be different. The loss of proteins from particles reaches 60-70 % and we conclude that metrizamide gradients are not utilisable for the fractionation of the units of nuclear particles.

INTRODUCTION

A study of the action of ribonuclease on brain and liver nuclear particles permitted the demonstration of at least 3 constitutive units, 2 types of 30-50 S monoparticles (M_{α} and M_{β}) and heterogeneous (30-200 S) ribonucleoprotein complexes (1). A physical separation of the 2 types of monoparticles was necessary for their further characterization.

Metrizamide gradients have previously been used for the fractionation of sheared chromatin (2-5) or of various ribonucleoproteins including nuclear particles (6-10). This substance is reputed to be chemically inert since it has no ionizable groups and its solution in water may reach high densities (11). Therefore, cellular constituents of different densities in solution may be separated on metrizamide gradients without prior fixation and analyzed afterwards.

The results obtained after centrifugation of brain nuclear mono- and polyparticles in metrizamide gradients are reported. They show that metrizamide dissociates the nuclear particles and cannot be used for the fractionation of their constitutive units.

METHODS

Labelling of particles. Adult Wistar rats were injected intracisternally with either 250 μCi of ^3H -uridine or with a mixture of 500 μCi ^{32}P -orthophosphate and 150 μCi each of ^3H -proline (27 Ci/mM), ^3H -phenylalanine (10 Ci/mM) and ^3H -lysine (28 Ci/mM) (12). They were sacrificed 4 h after injection.

Preparation of nuclear mono and polyparticles. Purified brain nuclei (13) were sonicated and a nuclear extract containing the particles and soluble proteins was prepared in the presence of cytoplasmic ribonuclease inhibitor (14) as previously described (12). The nuclear extract was centrifuged on a 10-25 % sucrose gradient (in buffer B : 10 mM Triethanolamine-HCl, pH 7.4, 25 mM KCl, 1 mM MgCl_2) for 4 h at 25,000 rev./min in a SW 25-2 rotor (75,500 x g). 60-110 S fractions containing polyparticles were pooled for analysis. For the obtention of a large amount of monoparticles the cytoplasmic ribonuclease inhibitor was omitted and the extract was incubated for 10 min at 20°C. Most of the polyparticles were hydrolyzed into 35-55 S monoparticles by endogeneous ribonucleases and only the 35-55 S fractions were collected.

Centrifugation on metrizamide gradients. 2-(3 acetamido-5-N-methyl-acetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose (Metrizamide, Nyegaard and Co A/S, Oslo) was dissolved in 20 mM Triethanolamine-HCl, pH 7.4, 25 mM KCl, 3 mM MgCl_2 , 10 mM mercaptoethanol. Two methods were used for making the gradients : Method I : Fractions of sucrose gradients were pooled and 1.8 ml were layered on 3.4 ml of a solution of 40 % (w/v) of metrizamide (7).

Method II : Metrizamide and MgCl_2 were added to sucrose gradient fractions (1.7 ml) up to final concentrations of 20 % (w/v) and 3 mM, respectively. The sample was deposited on 3 layers of 50, 40 and 35 % metrizamide (0.5, 1.9 and 0.5 ml, respectively).

In both cases, centrifugation was for 17 h at 50,000 rev./min (212,000 x g) in a SW 50-1 Spinco rotor at 4°C. The product of acceleration by time was similar to that used by the various groups studying RNP in metrizamide gradient (6-10). These conditions of centrifugation were such that equilibrium was reached for the high molecular weight material like ribonucleoproteins but not for free proteins or small RNA and ribonucleoproteins (15). Density was determined on aliquots according to Birnie and Rickwood (11). Fractions were pooled for subsequent analysis as indicated in figure 1 : region H (1.22-1.35 g/ml), region M (1.15-1.22 g/ml), region L (density lower than 1.15 g/ml).

The major differences between methods I and II were as follows : a) the gradient was partially preformed with Method II and not with Method I ; b) the particles were in contact with metrizamide from the start in method II whereas

they were in aqueous solutions with method I.

CsCl density determination. 0.1 or 0.2 ml fractions from metrizamide gradients were diluted to 1 ml with buffer B and fixed with 3 % formaldehyde (pH 7.4) for 6-7 h. They were layered on a preformed 1.3-1.6 g/ml CsCl gradient and analyzed as described previously (13).

Polyacrylamide gel electrophoresis of proteins. After trichloroacetic acid precipitation, aliquots from metrizamide gradients were dissociated in the presence of urea, dithiothreitol and sodium dodecylsulfate as described previously (16). They were analyzed on 10 % polyacrylamide gels in the presence of 0.1 % sodium dodecylsulfate.

RESULTS AND DISCUSSION

In the first series of experiments, uridine-labelled monoparticles, prepared from a previously incubated (10 min, 20°C) nuclear extract were studied. Upon analysis on metrizamide gradients (fig. 1), a peak of radioactivity was found at 1.22-1.35 g/ml (region H, fig. 1A and C) whatever the method used for making the gradients. A second peak was present at 1.15-1.22 g/ml (region M) and was relatively broad, apparently in relation to the slope of the gradient (steeper with method II than with method I). At the position corresponding to the input sample and to material of density lower than 1.15 g/ml, a trail of radioactivity was detected only when method II was used (fig. 1C). In general, more material was found in region M and L with method II than with method I.

When 35-55 S monoparticles were prepared from a non-incubated nuclear extract (in this case, the amount of monoparticles is low), results were qualitatively similar to those obtained with incubated particles (not shown).

When the particles were slightly prefixed with formaldehyde (1 %, 30 min) before analysis, a net decrease of the quantity of material of regions M and L was observed (fig. 1B and 1D). The peak of region H contained 81 and 71 % of the radioactivity with methods I and II, respectively, against 57 and 44 % in the absence of fixation.

The material from regions H and M of the experiment of fig. 1C was pooled and recentrifuged on a second metrizamide gradient. The radioactivity of the peak of region H was redistributed in the 3 regions of the new gradient, only 42 % of it keeping its initial density (fig. 1E), against 85 % for prefixed material. The peak of the intermediary region remained at its initial density (fig. 1F).

The results do not seem to be compatible with the separation of classes

Figure 1. Metrizamide gradients of 35-55 S monoparticles. Monoparticles were analyzed in metrizamide gradients made by method I (A, B) or II (C, D) without (A, C) or with prior formaldehyde fixation (B, D). Material from region H and M from the experiment shown in C were reanalyzed by method II (E, F).

of monoparticles. They rather suggest that the complexes of region H are transformed into apparently less dense material in the presence of metrizamide (method II versus method I, rerun) and that this change may be partially prevented by formaldehyde fixation.

In order to gain information about the causes of the phenomenon, the CsCl density of the metrizamide complexes was studied (fig. 2). Intact nuclear particles have a CsCl density of 1.38-1.40 g/ml (12) and this was also the density of the peak from the heavy region of metrizamide gradient (fig. 1D) when the material was prefixed with formaldehyde (dashed profile in fig. 2D). The peak from region H from metrizamide gradient, method I, had a CsCl density of 1.50 g/ml and trailed towards lower densities (fig. 2A), whereas the material from region M had a polydisperse density from 1.30 to 1.50 g/ml (fig. 2B). The results were different with method II. The material from region H had an

Figure 2. CsCl density determination of material fractionated on metriza-mide gradients. (A) region H and (B) region M from the experiment of figure 1A, (C) region H and (D) region M from the experiment of figure 1C. Dashed line (D) : region H from prefixed particles of fig. 1B. The densities indicated by the arrows are in g/ml.

average CsCl density of 1.39 g/ml (fig. 2C) similar to that of the native or fixed particles but the peak was much broader. The radioactivity of region L was heterogeneous with an average at 1.32 g/ml (fig. 2D).

No clear-cut conclusion could be drawn from these results except that the very reproducible CsCl density of native particles was not preserved in the presence of metriza-mide and that material from a same region of metriza-mide gradient may have variable CsCl densities. The results might be attributed to both a release of proteins which leads to an increase of density of the remaining ribonucleoproteins (12) and to an action of endogeneous ribonuclease which was shown to be present in the nuclear particles (17) and to lead to a decrease of density (12, 13). The dissociation would predominate with method I and the ribonuclease action with method II.

If dissociation of the monparticles had occurred in the presence of metriza-mide, it should also be observed with the large native polyparticles. 60-110

S particles, double-labelled with $^{32}\text{P}\text{O}_4^{3-}$ and ^3H - amino acids, were prepared in the presence of cytoplasmic ribonuclease inhibitor and the fate of their proteins, RNA and phosphoproteins, was studied. With methods I and II, approximately 90 % of the RNA radioactivity was found in region H (fig. 3A, 3C). In contrast, the proteins were found in all regions of the gradient. 55 % were present in region M and L with method I and 60 % with method II. A certain quantity of proteins was present at the level of the input material with method II but not with method I. The behavior of the phosphoproteins was not similar to that of the proteins since 80 % of their radioactivity was present in region H, with the bulk of the RNA.

In contrast, when the particles were prefixed with formaldehyde (fig. 3B,

Figure 3. Metrizamide gradients of 60-110 S polyparticles. Particles were double labelled with ^3H -amino acids and $^{32}\text{P}\text{O}_4^{3-}$. RNA and phosphoprotein radioactivities were discriminated by the 90°C trichloroacetic acid procedure (23). A. B. C. D. as in figure 1.

3D), no more than 20 % of the proteins (^3H -radioactivity) trailed in regions M and L.

The results strongly suggest that proteins were released from the particles in the presence of metrizamide. It is unlikely that the proteins of region M and L corresponded to ribonucleoproteins containing slowly-labelled RNA (18) since 1 h and 8 h labelling times (instead of 4 h) did not change the proportion of radioactive RNA at their level (not shown). Aggregates of free proteins were shown to be absent from the 60-110 S polyparticles (12) and, therefore, their presence cannot account for the results. Another argument in favor of a dissociation by metrizamide is the presence of proteins in the input layer on top of the gradient with method II only. This fraction is devoid of metrizamide in method I but contains 20 % metrizamide which may already have started the dissociation in method II (this was also the case with monoparticles as shown by comparison of fig. 1A and 1C).

The CsCl density of the fractions H and M of metrizamide gradients from polyparticles (corresponding to the experiment of fig. 3) was determined. Since results were similar with method I and II, only the results of the latter are shown in figure 4. When the polyparticles were fixed with formaldehyde prior to the metrizamide gradient, the double-labelled material of region H was found as a peak at the same CsCl density as that of native particles which were never in contact with metrizamide (1.39-1.40 g/ml, fig. 4C). When fixation was omitted, a major peak of ^3H - and ^{32}P -radioactivity was found at

Figure 4. CsCl density determination of material fractionated on metrizamide gradients. (A) region H and (B) region M from the experiment of figure 3C. (C) region H from figure 3D.

1.50 g/ml (fig. 4A). Some double-labelled material trailed up to 1.35 g/ml. In addition, ^3H -radioactivity was present at the density of free proteins (1.30-1.33 g/ml) (12). The material of region M corresponding primarily to ^3H -radioactivity was polydisperse and its CsCl density (1.24-1.34 g/ml) was centered around that of free proteins (fig. 4B).

The CsCl density determinations support the hypothesis of dissociation of particles into ribonucleoprotein complexes and free proteins in the presence of metrizamide. Quantitatively this dissociation was far from negligible. The transformation of a particle with a CsCl density of 1.39 g/ml into a 1.50 g/ml ribonucleoprotein corresponds to a loss of proteins of 70 % (13). A similar high level of dissociation (60-65 %) was also estimated by comparing the ratio of H^3 to ^{32}P radioactivities in figures 4A and 4C. Remark that, when the equilibrium of a metrizamide gradient is reached, free proteins band in region H (1.27 g/ml) (11). Under our experimental conditions, the equilibrium was reached for nucleoproteins, not for proteins. Therefore, these were found along the gradient and their position was probably related to that of the ribonucleoproteins at the moment of the dissociation. This is supported by the fact that most of the free proteins were found in region M of the gradient and that some of them remained on top with method II (fig. 3A, C).

In respect to CsCl densities, the dissociation in the presence of metrizamide resembled that obtained in the presence of NaCl or sodium deoxycholate (12, 13). In order to determine whether the same proteins were also released, fractions H, M and L from a metrizamide gradient, similar to that illustrated in figure 3C were analyzed for proteins (fig. 5). The most striking feature of the electrophoretic profiles was that the relative quantities of proteins 2b3, 3b, 3c (66,000, 36,000, 34,000 Mr) decreased in region H (fig. 5D) as compared to unfractionated particles (fig. 5A). In contrast, these same proteins were enriched in region L (fig. 5B). This is in agreement with another work (10) showing that proteins in the 30,000-40,000 Mr range predominated in the light fractions of a metrizamide gradient. The proteins that remained the more tightly bound to the RNA were those of zone 1, subzones 2a, 2b1-2, 2ef, 3a, 3e (fig. 5D). In fact, the overall electrophoretic profile of the heaviest complexes (fig. 5D) resembled that obtained after a 0.25 M NaCl treatment of native particles which also led to a preferential release of proteins of zone 3 and of 2b3 (16). These heavy complexes were also enriched in phosphoproteins (fig. 3). The material of regions M and L had the same overall protein composition as the proteins released by NaCl, with, however, quantitative differences for certain proteins.

Figure 5. Electrophoregrams of proteins from material fractionated on metrizamide gradients. Proteins from (A) control, unfractionated particles; (B) region L, (C) region M, (D) region H from a metrizamide gradient (Method II) similar to that shown in figure 3C. 60 (B), 60 (C) and 100 % (D) of the total proteins of each region were analysed.

The protein compositions of the metrizamide gradient fractions from monoparticles (corresponding to figures 1A and 1C) were similar to those of figure 5 (not shown). When polyparticle fractions obtained by method I were analysed, only traces of proteins were found in region L, as expected (not shown). The proteins of zone 3 plus 2b3 were enriched in region M, but their amount in region H was higher than with method II. The differences are proba-

bly to be related to the delayed action of metrizamide in method I as compared to method II.

In short, the primary effect of metrizamide on particles seems to be a dissociation of the particles into ribonucleoproteins and free proteins. The extent of the dissociation may be modified by the concentration of metrizamide and the time of contact with this reagent. Our observations meet those of other investigators who showed a partial dissociation of chromosomes and nucleosomes in the presence of metrizamide (19). The endogeneous ribonucleases may provoke additional variability either by rendering more proteins available for dissociation or by provoking the formation of short ribonucleoprotein fragments which would not reach their equilibrium under the conditions of centrifugation. Protease activities were shown to be very low in brain nuclei (20) and brain nuclear particles (21) and these enzymatic activities are not likely to have interfered with our results.

Apparently, metrizamide which has no ionizable groups has the same effect on particles as NaCl. This does not obligatory mean that the modes of action of these agents are similar. It was shown that metrizamide may bind proteins in certain conditions (22). Its association with certain particle proteins may modify their interaction with the RNA or with other proteins and provoke a dissociation. However, there is no reason to believe *a priori* that the same proteins are released in the presence of salt and of metrizamide, except if one assumes that the most accessible proteins are concerned in both cases.

In conclusion, metrizamide gradients are not utilizable for the fractionation of the constitutive units of brain nuclear particles. It is likely that similar results would have been reached with nuclear particles from other origins but our results do not imply that similar phenomena occur with other types of ribonucleoproteins or subcellular fractions. They merely stress the necessity of adequate controls before using this method which may be very valuable in certain instances.

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REFERENCES

1. Stévenin, J., Gallinaro-Matringe, H., Gattoni, R. and Jacob, M. (1977) *Eur. J. Biochem.* 74, 589-602.
2. Rickwood, D., Hell, A. and Birnie, G.D. (1973) *FEBS Lett.* 33, 221-224.
3. Rickwood, D., Hell, A., Malcolm, S., Birnie, G.D., Macgillivray, A.J. and Paul, J. (1974) *Biochim. Biophys. Acta* 353, 353-361.
4. Monahan, J.J. and Hall, R.H. (1974) *Nucleic Acids Res.* 1, 1359-1370.
5. Levy, A., Jakob, K.M. and Moav, B. (1975) *Nucleic Acids Res.* 2, 2299-2303.
6. Buckingham, M.E. and Gros, F. (1975) *FEBS Lett.* 53, 355-359.
7. Houssais, J-F (1975) *FEBS Lett.* 56, 341-347.
8. Dissous, C., Lempereur, C., Verwaerde, C. and Krembel, J. (1976) *Eur. J. Biochem.* 64, 361-367.
9. Auger, M-A and Longuet, M. (1976) *C. R. Acad. Sc. Paris* 282, 1889-1892.
10. Houssais, J-F (1977) *Mol. Biol. Rep.* 3, 251-261.
11. Rickwood, D. and Birnie, G.D. (1975) *FEBS Lett.* 50, 102-110.
12. Stévenin, J. and Jacob, M. (1974) *Eur. J. Biochem.* 47, 129-137.
13. Stévenin, J. and Jacob, M. (1972) *Eur. J. Biochem.* 29, 480-488.
14. Roth, J.S. (1958) *J. Biol. Chem.* 231, 1085-1095.
15. Birnie, G.D., Rickwood, D. and Hell, A. (1973) *Biochim. Biophys. Acta* 331, 283-294.
16. Gallinaro-Matringe, H., Stévenin, J. and Jacob, M. (1975) *Biochemistry U.S.A.* 14, 2547-2554.
17. Niessing, J. and Sekeris, C.E. (1970) *Biochim. Biophys. Acta* 209, 484-492.
18. Deimel, B., Louis, Ch. and Sekeris, C.E. (1977) *FEBS Lett.* 73, 80-84.
19. Pignatti, P.F., Cremisi, C., Croissant, O. and Yaniv, M. (1975) *FEBS Lett.* 60, 369-373.
20. Sellwood, S.M., Riches, P.G., Harrap, K.R., Rickwood, D., Macgillivray, A.J. and Capps, M. (1975) *Eur. J. Biochem.* 52, 561-566.
21. Stévenin, J., Gallinaro-Matringe, H. and Jacob, M. (1977) *Mol. Biol. Rep.* in press.
22. Rickwood, D., Hell, A., Birnie, G.D. and Gilhuus-Moe, C.Chr. (1974) *Biochim. Biophys. Acta* 342, 367-371.
23. Stévenin, J., Gallinaro-Matringe, H. and Jacob M. (1975) *Biochimie* 57, 1099-1102.