Organization in the Cell Nucleus: Divalent Cations Modulate the Distribution of Condensed and Diffuse Chromatin

ROBERT PETER AARONSON and EDWIN WOO
Department of Microbiology, The Mount Sinai School of Medicine, New York 10029

ABSTRACT The organization of rat liver nuclei in vitro depends on the ionic milieu. Turbidity measurements of nuclear suspensions in the presence of varying concentrations of divalent cations have been correlated with nuclear ultrastructure. The concentration of $MgCl_2$ (2 mM) at which turbidity of nuclear suspensions is maximal and chromatin condensation appears most extensive is the same concentration that reportedly (Gottesfeld et al., 1974, *Proc. Natl. Acad. Sci. U. S. A.* 71:2193–2197) precipitates "inactive" chromatin. Thus, a mechanism is suggested by which chromatin activity and ultrastructural organization within the nucleus may be mediated. The nuclear organizational changes attendant upon the decrease in divalent cation concentration were not entirely reversible.

Cell nuclei exhibit, in vivo, a variety of shapes and varying degrees of "condensation" or pyknosis. It is accepted that at least some of these morphological variations are associated with differing functions and/or developmental history of the nuclei (13). Whether the structural variations cause or reflect these different functions is still unresolved.

Nuclei can be isolated so that they remain virtually morphologically identical to nuclei in vivo (20). A diverse assortment of agents are known to modify nuclear structure in vivo and in vitro (24). This affords the opportunity of examining the contribution of various factors to nuclear structure. For instance, polyanions (16), polycations, and inorganic salt solutions of differing ionic strength (4, 21, 22) have been used to effect gross changes in nuclear structure. In the present report, we describe a relatively simple optical method for evaluating gross changes in nuclear structure and show that varying the divalent cation concentration over a narrow range causes gross changes in the organization of the nuclear contents and that some of these changes are apparently not fully reversible. For the present purposes, nuclear organization or structure will be broadly defined as a combination of the relative amounts and distribution of condensed and diffuse chromatin within the nucleus and overall nuclear size and shape.

MATERIALS AND METHODS

Rat liver nuclei were prepared from fasted 150 g male Sprague-Dawley rats (1, 3) in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (11), collected by low speed centrifugation, and resuspended in STKMP buffer (0.25 M sucrose, 50 mM Tris-Cl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM PMSF). In general, the stock suspensions of nuclei were diluted at least 50-fold into appropriate solutions to achieve the indicated final concentrations of ions.

Absorbance measurements were performed with 10-mm path length semimicrocuvettes in a Gilford spectrophotometer, model 2400 (Gilford Instrument Laboratories Inc., Oberlin, Ohio). Identical results were obtained with nuclei that were quick-frozen as a pellet in a dry ice-ethanol bath and stored at −80°C.

Nuclei were fixed for electron microscopy either as thin discoid pellets (10) or in suspension at 4°C with 1–2% glutaraldehyde in the presence of 50 mM triethanolamine Cl, pH 8.5, and the indicated concentrations of $MgCl_2$. Fixation was continued for 16 h at 4°C. The pellets of nuclei were postfixed at 4°C for 1 h in 1% OsO_4, 0.1 M *s*-collidine, pH 7.4, and the indicated concentration of $MgCl_2$. The pellets were then stained en bloc with uranyl acetate in acetate-veronal buffer (12), dehydrated, and embedded in Epon (18). The disk-shaped pellets were oriented so that sectioning was performed parallel to the direction of sedimentation so that both the top and bottom of a pellet were included for evaluation. Silver and gray thin sections were stained with uranyl acetate (25) and lead citrate (23) and viewed in an AEI 801 electron microscope at an accelerating voltage of 80kV.

Glutaraldehyde and osmium tetroxide were obtained from Electron Microscopy Sciences (Fort Washington, Pa.) and *s*-collidine from Polysciences Inc. (Warrington, Pa.). $MgCl_2$ was purchased as a 1-M solution (Fisher Scientific Co., Pittsburgh, Pa.). All other reagents were analytical grade.

RESULTS

Turbidity as an Indicator of Nuclear Organization

Rat liver cell nuclei isolated by sedimentation through concentrated sucrose solutions in the presence of 5-mM divalent cations and 25-mM monovalent cations at pH 8.5 are, morpho-

 · 0021-9525/81/07/0181/06 $1.00

logically, virtually identical with such nuclei in vivo (20). By phase-contrast microscopy, they generally appear roughly spherical with an uneven periphery and very granular contents and exhibit two or three prominent nucleoli. A suspension of the nuclei is turbid. If the turbidity of a suspension is estimated by measuring its absorbance at a wavelength of light at which none of the principal nuclear constituents absorb, it is found (Fig. 1) that the turbidity (absorbance at 320 nm, A_{320}) does not follow Beer's Law, i.e., it is not a linear function of the concentration of nuclei. The turbidity arises from complex light-scattering phenomena and a true extinction coefficient cannot be determined. This explanation is supported by the fact that the A_{320} of any particular nuclear suspension increases with increasing distance between the cuvette and the photodetector (21, and unpublished observations). The absorbance in the range 300–500 nm is also not a linear function of λ^{-4} (not shown) as would be expected for Rayleigh light scattering. This may not be unreasonable (8), inasmuch as the nuclei are very large relative to the wavelengths of light in this range (effective particle size = diameter of nucleus/wavelength in medium ≈10–20). Finally, the turbidity must be a measure of gross nuclear structure because in the presence of structure-perturbing reagents (SDS, urea, etc.), the turbidity of the suspension is nil (Table I).

It is worth noting that the absorbance at 260 nm is virtually linear over the same concentration range.

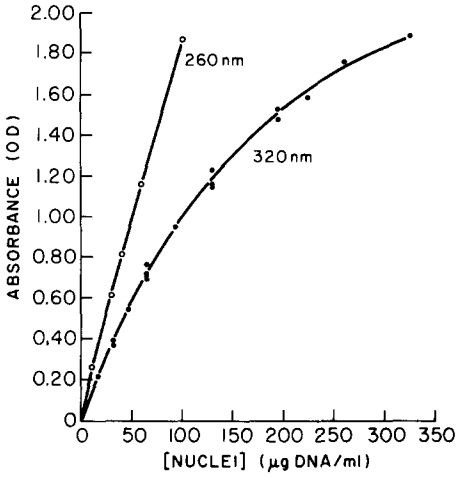

FIGURE 1 Turbidity of a nuclear suspension as a function of nuclear concentration. Fresh rat liver nuclei, isolated in 0.25 STKMP, were diluted into the same buffer and the absorbance was measured at 260 nm (O) and 320 nm (●).

TABLE I

Effect of Buffer Constituents on Nuclear Organization

	A_{320}
	OD
S buffer*	0.304
S buffer − sucrose (5 mM)‡	0.289
S buffer − Tris Cl (6 mM)‡	0.289
S buffer − KCl (0.5 mM)‡	0.300
S buffer − $MgCl_2$ (0.1 mM)‡	0.170
Minimal buffer§	0.081
Minimal buffer§ + 0.1% SDS	0.004

Fresh rat liver nuclei suspended at a DNA concentration of 1 mg/ml in STKMP buffer and diluted 1:50 in the appropriate diluent to yield the indicated final concentrations of buffer constituents.

* S buffer: 0.25 M sucrose, 50 mM Tris Cl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$.

‡ Final concentration of the constituent omitted from diluent.

§ Minimal buffer consisted of 5 mM Tris Cl, pH 7.5.

Dispersal of Nuclear Organization

The turbidity of a nuclear suspension is especially sensitive to the presence of $MgCl_2$, because its omission results in a dramatic decrease in the A_{320} (Table I), whereas omission of each of the other buffer constituents, individually, produces virtually no gross difference in turbidity. On the other hand, at low concentrations of $MgCl_2$ the other buffer constituents do contribute to nuclear structure, e.g., compare "$-MgCl_2$" with "only 5 mM Tris Cl" in Table I.

When the effects, on nuclear structure, of each of the ionic buffer constituents are determined in the absence of the others (Figs. 2–4), it can be seen that at both high and low concentrations the turbidity of the suspensions is minimal, but that at intermediate concentrations the turbidity is increased. This pattern is not simply a function of ionic strength nor of anion concentration, as the effective concentration ranges for the monovalent and divalent salts are too different.

Upon varying the concentration of monovalent ions (Figs. 2 and 3), a general increase in turbidity with increasing salt concentration is observed, until a critical concentration is achieved, at which point the turbidity decreases with increasing

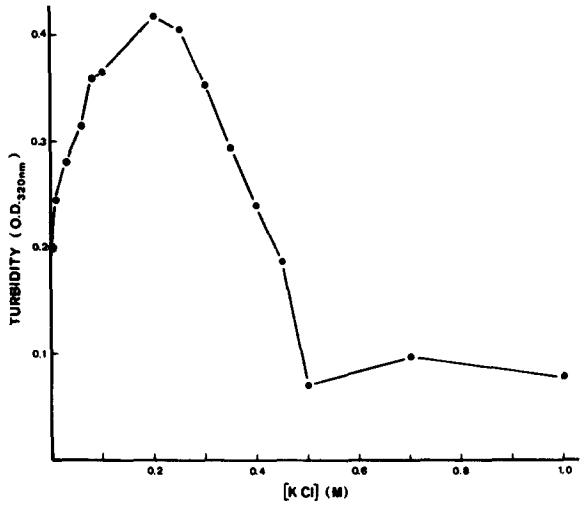

FIGURE 2 The contribution, in the absence of divalent cations of KCl to gross nuclear structure. Nuclei were diluted, as in Fig. 1, to a final DNA concentration of 90 μg/ml into a buffer containing 10 mM Tris Cl, pH 7.5 and the indicated concentration of KCl.

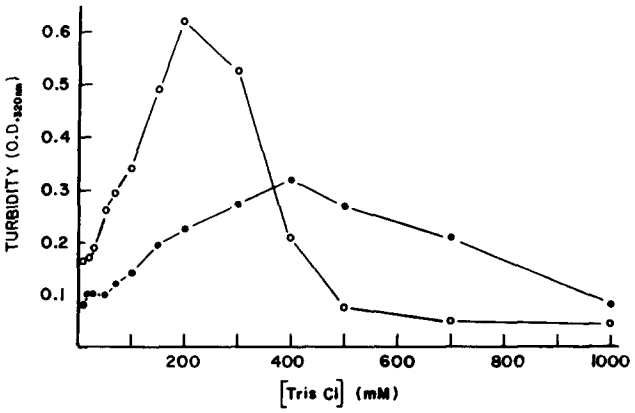

FIGURE 3 The contribution, in the absence of divalent cations, of Tris Cl to gross nuclear structure. Nuclei were diluted, as in Fig. 1, to a final DNA concentration of 85 μg/ml, into buffer containing Tris Cl at the indicated concentration and pH. O, pH 8.5; ●, pH 7.5.

salt concentration. The optimal concentration of KCl (or other monovalent cations, not shown) at which maximal turbidity is achieved is ~200 mM.

The pH of the suspension is also a factor in determining gross nuclear structure, as can be seen in Fig. 3. Even correcting for the differing ionic strengths at different pH values of equal concentrations of Tris (approx. 10-fold between pH 7.5 and 8.5), the maximum turbidity is greater at pH 7.5 than at pH 8.5. Similar results are obtained if the turbidity of the nuclear suspensions, as a function of monovalent ion concentration, is determined at pH 7.5 and 8.5 (data not shown).

For divalent cations (see Fig. 4) there is a relatively broad concentration range of $MgCl_2$ (and of $CaCl_2$, not shown) between ~2 and 30 mM in which the turbidity of the nuclear suspensions is maximal. There is a dramatic decrease in turbidity over a relatively narrow range both above 30 mM and below 2 mM $MgCl_2$. The more abrupt transition occurs in the range of 1.0–1.5 mM $MgCl_2$. A second transition of smaller magnitude occurs in the range of 0.2–0.5 mM $MgCl_2$. Nuclear suspensions in the range between these two transitions (0.5–1.0 mM $MgCl_2$) exhibit a plateau with respect to turbidity.

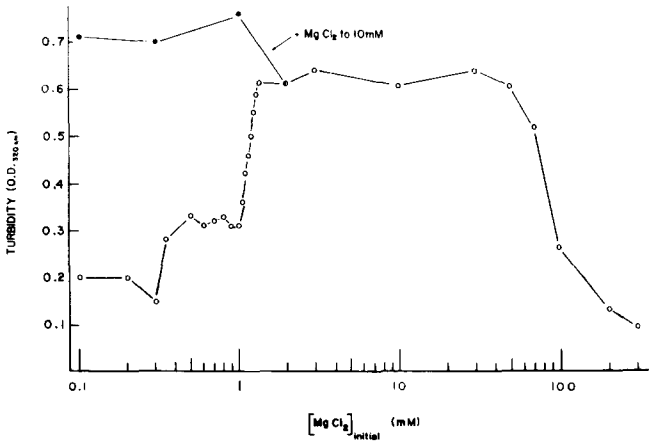

FIGURE 4 The contribution of $MgCl_2$ to gross nuclear structure. Nuclei were diluted, as in Fig. 1, to a final DNA concentration of 40 μg/ml, into buffer containing 10 mM Tris Cl, pH 7.5, and $MgCl_2$ at the indicated initial concentration. To four of the samples (filled circles) additional $MgCl_2$ was then added, in a bolus, to increase the concentration to 10 mM.

Reversal of the Dispersal of Nuclear Structure

Preliminary experiments attempting to reverse the loss of gross nuclear organization upon exposure of nuclei initially to high concentrations of salts and then dilution to the more optimal range were totally unsuccessful, i.e., large clumps of aggregated material were formed that could not be evenly suspended. On the other hand, simple readdition of $MgCl_2$, to a final concentration of 5–10 mM, to nuclei suspended in its absence allowed an even opalescence to appear in the suspension as well as the reformation, observable in the phase-contrast microscope, of smaller, granular, inhomogeneous "nuclei" similar in appearance to the originally isolated nuclei. Somewhat surprisingly, the A_{320} of this suspension consistently exceeded the A_{320} of an equal concentration of nuclei that had been maintained in 5–10 mM $MgCl_2$ (see Fig. 4).

Ultrastructural Analysis of Nuclear Organizational States

Ultrastructural analysis was performed on fresh rat liver nuclei which were fixed in buffers which allowed comparison between optically defined states. As can be seen in Fig. 5, nuclei suspended in a low concentration of $MgCl_2$, e.g., 0.2 mM $MgCl_2$ (or 0.1 mM $MgCl_2$, not shown) exhibit an expanded, relatively homogeneous, granular-fibrillar appearance. The disjointed fibrils appear to have diameters of up to 10 nm and the densely staining granules vary in size between 20 and 50 nm. Some areas (0.6–1.3 μm in diameter) appear to contain a greater numerical density of densely staining granules and may represent remnants of nucleoli. There is no apparent enrichment of granules or fibers at the periphery of the nuclei.

Nuclei suspended in the presence of 0.5 mM $MgCl_2$ (Fig. 6), i.e., in the range in which the intermediate turbidity plateau is observed, also exhibit a relatively homogeneous granular-fibrillar appearance. Nucleoli are easily identified.

Finally, nuclei suspended in a solution containing 10 mM $MgCl_2$ (Fig. 7) exhibit well-defined nucleoli and large aggregates of condensed chromatin granules. These aggregates are, for the most part, distributed at the nuclear periphery and in close apposition to the nucleoli. Nuclei suspended in solutions containing 2 and 5 mM $MgCl_2$ demonstrate (not shown) the same ultrastructure.

Ultrastructural Analysis of Nuclei Returned to 10 mM $MgCl_2$

As mentioned above, readdition of $MgCl_2$, in a bolus, to 10 mM causes a marked turbidity increase (Fig. 4). Morphological examination of this material (Fig. 8) demonstrates that nucleoli can be observed and that there is a marked rearrangement of the nuclear material uniformly but inhomogeneously throughout the nucleus. This altered distribution has been thought to reflect the presence of a nuclear matrix (2).

DISCUSSION

Previous workers (21) have examined the effect of ionic strength on nuclear superstructure by observing nuclei with phase-contrast microscopy and have reported "phase diagrams" that reflect gross changes in the nuclear superstructure. Similar experiments have been described here using turbidity as a measure of gross nuclear structure. Rat liver nuclei, diluted so that the final concentration of $MgCl_2$ is below 1.5 mM, exhibit diminished turbidity relative to "standard" conditions (5–10 mM $MgCl_2$). The decrease in turbidity largely occurs within 15 s and the absorbance is stable for at least 1 h.

The major portion of the diminution in turbidity occurs over a narrow concentration range (1–1.5 mM $MgCl_2$) and the rest of the diminution also occurs over a narrow range (~0.2–0.5 mM). The narrowness of both these ranges suggests a high level of cooperativity for two separate structural changes in the nucleus.

Upon readdition of $MgCl_2$ to a final concentration of 10 mM, the turbidity of the suspension increases (within 1–2 min) to a value greater than that observed under the standard conditions. This "overshoot" is reproducible and only appears to occur after nuclei have been exposed to a concentration of $MgCl_2$ below 2 mM.

In a similar fashion, if the nuclei are diluted into monovalent ion-containing buffers, the maximum turbidity obtained occurs at ~200 mM KCl (Fig. 2) and is only about two-thirds as great

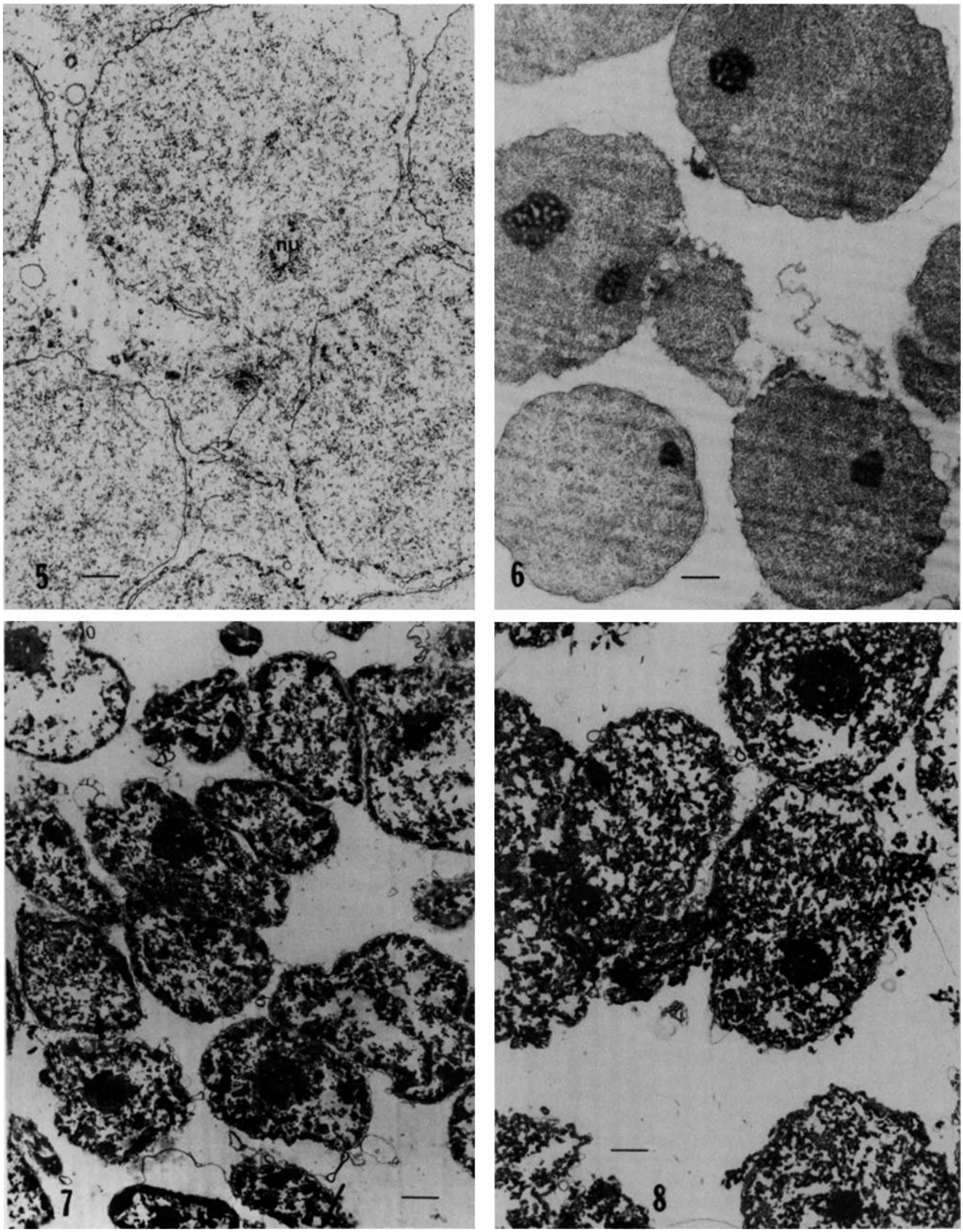

FIGURE 5 Rat liver nuclei suspended in a low concentration of $MgCl_2$. Nuclei were diluted, as in Fig. 1, to a final DNA concentration of ~100 μg/ml, into a buffer containing 10 mM triethanolamine, pH 7.5, 0.2 mM $MgCl_2$, 0.1 mM PMSF. The suspension was centrifuged and the pelleted material fixed in 0.2 mM $MgCl_2$ as described in Methods. Chromatin is distributed throughout the nucleus, although there appear to be slightly more concentrated regions. Note areas containing relatively large numbers of granules. *nu*, Remnants of nucleoli (presumptive). Bar, 2 μm. × 3,500.

as is obtained with the same concentration of nuclei in 5–10 mM $MgCl_2$.

Light scattering by large particles is a function of reflection, diffraction, refraction, interference, and absorption. For particles that are much larger than the wavelength of transmitted light, the contributions of "whole particle" scattering, and "internal" scattering, and of absorption to total light scattering have been discussed (6, 17). For very large nonabsorbing homogeneous (spherical) particles, an increase in particle volume, with no change in dry weight of the particles, would be expected to increase the whole particle scattering component, thereby increasing the turbidity of the suspension. On the other hand, if the particles were internally inhomogeneous, a decrease in internal structure (to a more homogeneous distribution of particle contents) would be expected to decrease the suspension turbidity because "internal" scattering decreases. Suspensions of nuclei in the presence of 5 mM $MgCl_2$ are more turbid than suspensions at 0.1 mM $MgCl_2$. Thus, in the transition from 5 to 0.1 mM $MgCl_2$, the contribution to the total light scattering of whole particle scattering (an anticipated increase in turbidity owing to the increase in particle volume) must be more than compensated by internal scattering (an anticipated decrease in turbidity owing to more internal homogeneity).

In principle, the actual contributions of these two components may be quantitatively calculated and compared with the measured turbidity (5, 6, 9, 15). Unfortunately, these calculations are practical only for predicting the amount of light scattered at specific angles or for predicting the absolute extinction of transmittance (= turbidity with a 0° collection angle for the photometer). Because the photomultiplier tube of the spectrophotometer used in this study ordinarily collects light from the cuvette through an angle of ~7°, it is not appropriate (7) to perform the calculations for quantitative comparison with the present data.

Ultrastructural examination of nuclei fixed under the appropriate conditions revealed a qualitative correspondence between the amount of condensed chromatin in the nuclei and the turbidity of the suspension. At present, no quantitative relationship has been demonstrated. Moreover, other organizational factors or nuclear structures (e.g., intermediate levels of chromatin organization, nucleoli, etc.) may contribute to the total turbidity. In this latter regard, it is worth noting that the redistribution of condensed chromatin after readdition of $MgCl_2$ to 10 mM appears to cause an increase in turbidity over that exhibited by nuclei maintained at the higher $MgCl_2$ concentration (the overshoot phenomenon of Fig. 4). Moreover, nucleoli were present when the turbidity of a suspension was at the "intermediate" level, although virtually none of the chromatin was condensed (Fig. 6).

Although a quantitative relationship has not been shown between turbidity (as measured here) and degree of nuclear organization, it seems reasonable to assign the loss of turbidity in nuclear suspensions which occurs between 1.5 and 1 mM $MgCl_2$ to the de-"condensation" of the chromatin and the loss of turbidity occurring between 0.4 and 0.3 mM $MgCl_2$ with de-"condensation" of the nucleoli. Both of these processes appear to be highly cooperative. Subsequent experiments will be designed to test these assignments.

Relatively inactive chromatin is precipitated in the presence of 2 mM $MgCl_2$ (14, 19). This concentration is sufficient to achieve maximal turbidity in nuclear suspensions and condensation of the chromatin. Thus, it seems likely that the condensed chromatin is not actually in solution and is, for that reason, inactive. Further examination of the factors that govern chromatin condensation, as well as the distribution of chromatin in the cell nucleus, should help illuminate mechanisms of controlling gene expression in eukaryotes.

This work was supported by grants from the National Institutes of Health (GM21950 and GM00278), and from the National Science Foundation (PCM77-26587).

Received for publication 26 September 1980, and in revised form 6 March 1981.

REFERENCES

1. Aaronson, R. P. 1977. Isolation of nuclear proteins associated with the nuclear pore complex and the nuclear peripheral lamina of rat liver. *Methods Cell Biol.* 13:337–342.
2. Berezney, R., and D. S. Coffey. 1977. Nuclear matrix. Isolation and characterization of a framework structure. *J. Cell Biol.* 73:616–637.
3. Blobel, G., and V. R. Potter. 1966. Nuclei from rat liver. Isolation method that combines purity with high yield. *Science (Wash. D. C.).* 154:1662–1665.
4. Brasch, K., V. L. Seligy, and G. Setterfield. 1971. Effects of low salt concentration on structural organization and template activity of chromatin in chicken erythrocyte nuclei. *Exp. Cell Res.* 65:61–72.
5. Bryant, F. D., and P. Latimer. 1969. Optical efficiencies of large particles of arbitrary shape and orientation. *J. Colloid Interface Sci.* 30:291–304.
6. Bryant, F. D., P. Latimer, and B. A. Seiber. 1969. Changes in total light scattering and absorption caused by changes in particle conformation. A test of theory. *Arch. Biochem. Biophys.* 135:109–117.
7. Bryant, F. D., B. A. Seiber, and P. Latimer. 1969. Absolute optical cross sections of cells and chloroplasts. *Arch. Biochem. Biophys.* 135:79–108.
8. Camerini-Otero, R. D., and L. A. Day. 1978. The wavelength dependence of the turbidity of solutions of macromolecules. *Biopolymers.* 17:2241–2249.
9. Camerini-Otero, R. D., R. M. Franklin, and L. A. Day. 1974. Molecular weights, dispersion of refractive index increments, and dimensions from transmittance spectrophotometry. Bacteriophages R17, T7, and PM2, and tobacco mosaic virus. *Biochemistry.* 13:3763–3773.
10. Cotman, C. W., and D. A. Flansburg. 1970. An analytical micro-method for electron microscopic study of the composition and sedimentation properties of subcellular fractions. *Brain Res.* 22:152–156.
11. Fahrney, D. E., and A. M. Gold. 1963. Sulfhydryl fluorides as inhibitors of esterases. I. Rates of reaction with acetylcholinesterase, a-chymotrypsin, and trypsin. *J. Am. Chem. Soc.* 85:997–1000.
12. Farquhar, M. G., and G. E. Palade. 1965. Cell functions in amphibian skin. *J. Cell Biol.* 26:263–291.
13. Frenster, J. H. 1974. Ultrastructive and function of heterochromatin and euchromatin. *In* The Cell Nucleus. H. Busch, editor. Academic Press, Inc., New York. 1:565–580.
14. Gottesfeld, J. M., W. T. Garrard, G. Bagi, R. F. Wilson, and J. Bonner. 1974. Partial purification of the template-active fraction of chromatin. A preliminary report. *Proc. Natl. Acad. Sci. U. S. A.* 71:2193–2197.

FIGURE 6 Rat liver nuclei in a suspension that exhibits intermediate turbidity. Nuclei were fixed in suspension at 4°C in 50 mM triethanolamine Cl, pH 8.5, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM PMSF, 1% glutaraldehyde at a DNA concentration of 100 μg/ml. Most nuclei contain no condensed chromatin but are, instead, composed of an almost uniform mass of fibers and granules. Nucleoli are still composed of fibrillar and granular portions and exhibit occasional lacunae. Immediately underneath the inner nuclear membrane there is often a single row of dark granules. Bar, 2 μm. × 3,500.

FIGURE 7 Rat liver nuclei exhibiting maximal nuclear organization. Nuclei were treated as in Fig. 5 except that the $MgCl_2$ concentration was maintained at 10 mM throughout. The highly condensed chromatin is distributed largely at the nuclear periphery and adjacent to nucleoli. Bar, 2 μm. × 3,500.

FIGURE 8 Rat liver nuclei after readdition of $MgCl_2$ to 10 mM. Nuclei were treated as in Fig. 5, except that $MgCl_2$ was readded, after dilution of the nuclei but before centrifugation, to a final concentration of 10 mM and maintained at that concentration thereafter. Relatively thick cords of condensed chromatin are apparently randomly distributed throughout the nuclear interior. Although some of the condensed chromatin is distributed peripherally there is virtually none in close apposition to the apparently reformed nucleoli. Bar, 2 μm. × 3,500.

15. Holzwarth, G., D. G. Gordon, J. E. McGiness, B. P. Dorman, and M. Maestre. 1974. Mie scattering contributions to the optical density and circular dichroism of T2 bacteriophage. *Biochemistry.* 13:126–132.
16. Kraemer, R. J., and D. S. Coffey. 1970. The interaction of natural and synthetic polyanions with mammalian nuclei. I. DNA synthesis. *Biochem. Biophys. Acta.* 224:553–567.
17. Latimer, P., D. D. Moore, and F. D. Bryant. 1968. Changes in total light scattering and absorption caused by changes in particle conformation. *J. Theor. Biol.* 21:348–367.
18. Luft, J. H. 1961. Improvements in epoxy embedding methods. *J. Biophys. Biochem. Cytol.* 9:409–414.
19. Marushige, K., and J. Bonner. 1971. Fractionation of liver chromatin. *Proc. Natl. Acad. Sci. U. S. A.* 68:2941–2944.
20. Monneron, A., G. Blobel, and G. E. Palade. 1972. Fractionation of the nucleus by divalent cations. Isolation of the nuclear membranes. *J. Cell Biol.* 55:104–125.
21. Olins, D. E., and A. L. Olins. 1972. Physical studies of isolated eucaryotic nuclei. *J. Cell Biol.* 53:715–736.
22. Philpot, J. St. L., and J. E. Stanier. 1956. The choice of suspension medium for rat liver cell nuclei. *Biochem. J.* 63:214–223.
23. Reynolds, E. S. 1963. The use of lead citrate at a high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17:208–215.
24. Simard, R., Y. Langelier, R., Mandeville, N., Maestracci, and A. Royal. 1974. Inhibitors as tool in elucidating the structure and function of the nucleus. *In* The Cell Nucleus. H. Busch, editor. Academic Press, Inc., New York. 3:447–487.
25. Stempak, J. G., and R. T. Ward. 1964. An improved staining method for electron microscopy. *J. Cell Biol.* 22:697–701.