# PHYSICAL STUDIES OF ISOLATED EUCARYOTIC NUCLEI

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## ABSTRACT

The degree of chromatin condensation in isolated rat liver nuclei and chicken erythrocyte nuclei was studied by phase-contrast microscopy as a function of solvent pH, $K^+$ and $Mg^{++}$ concentrations. Data were represented as "phase" maps, and standard solvent conditions selected that reproducibly yield granular, slightly granular, and homogeneous nuclei. Nuclei in these various states were examined by ultraviolet absorption and circular dichroism (CD) spectroscopy, low-angle X-ray diffraction, electron microscopy, and binding capacity for ethidium bromide. Homogeneous nuclei exhibited absorption and CD spectra resembling those of isolated nucleohistone. Suspensions of granular nuclei showed marked turbidity and absorption flattening, and a characteristic blue-shift of a crossover wavelength in the CD spectra. In all solvent conditions studied, except pH < 2.3, low-angle X-ray reflections characteristic of the native, presumably superhelical, nucleohistone were observed from pellets of intact nuclei. Threads (100–200 A diameter) were present in the condensed and dispersed phases of nuclei fixed under the standard solvent conditions, and examined in the electron microscope after thin sectioning and staining. Nuclei at neutral pH, with different degrees of chromatin condensation, exhibited similar binding capacities for ethidium bromide. These data suggest a model that views chromatin condensation as a close packing of superhelical nucleohistone threads but still permits condensed chromatin to respond rapidly to alterations in solvent environment.

## INTRODUCTION

Isolated eucaryotic nuclei undergo rapid morphological changes in response to alterations of solvent pH and electrolyte concentration (Ris and Mirsky, 1949; Anderson and Wilbur, 1952; Philpot and Stanier, 1956). As examined with the phase-light microscope, nuclei appear to swell and become optically homogeneous with removal of divalent cations at neutral pH, whereas in the presence of sufficient monovalent or divalent cations, or at acid pH, nuclei are not swollen and appear characteristically "granular," due to differential refractility of chromatin condensations. These nuclear states furnish convenient material for comparing morphologic and other physical properties, in order to understand the mechanisms of chromatin condensation. These states gain further interest by analogy with similar morphologic changes seen during nuclear "activation" (Harris, 1967; Ringertz, 1969).

In the present studies, nuclei have been isolated from two tissues, rat liver and chicken erythrocyte. Rat liver nuclei contain considerable amounts of dispersed chromatin; chicken erythrocyte nuclear chromatin is principally condensed. Employing phase-contrast microscopy, morphologic "phase" maps relating nuclear appearance to solvent composition have been constructed. Standard solvent conditions have been defined, and the properties

of nuclei in the different states have been studied by ultraviolet spectrophotometry, circular dichroism, low-angle X-ray diffraction, the binding of ethidium bromide, and electron microscopy.

## MATERIALS AND METHODS

### *Reagents*

Native calf thymus DNA was purchased from Sigma (Type I, lot 50C-4830; Sigma Chemical Co., St. Louis, Mo.). Bovine serum albumin was a lyophilized product of Sigma Chemical Co. Gum arabic (acacia) was obtained from BDH Chemicals Ltd., Poole, England. Ethidium bromide was obtained from Boots Pure Drug Co., Ltd., Nottingham, England. Other chemicals were reagent or analytical reagent grade. All buffers and solutions were made with glass-distilled water. The following buffer stock solutions were employed: pH 1.88, 2.28, and 2.88, 0.1 M glycine·HCl buffer, pH 3.90, 4.87, and 5.50, 0.1 M sodium acetate–acetic acid buffer; pH 6.95, 7.50, 8.16, and 8.50, 0.1 M tris (hydroxymethyl) aminomethane·HCl buffer, pH 10.00 and 10.90, 0.1 M sodium carbonate–sodium bicarbonate buffer

For many studies standard solvent conditions were used. The most frequently used solvents were: (*a*) 0.02 M Tris, pH 7.5, 0.02 M KCl, 0.25 M sucrose, (*b*) 0.02 M Tris, pH 7.5, 0.20 M KCl, 0.25 M sucrose, and (*c*) 0.02 M Tris, pH 7.5, 5 mM $MgCl_2$, 0.25 M sucrose.

### *Preparation of Nuclei*

Rat liver nuclei were prepared by the method of Blobel and Potter (1966). Sprague-Dawley rats (mixed sexes, approximately 250 g, fed ad libitum) were killed by decapitation and the livers immediately placed in ice-cold TKM homogenizing buffer (0.05 M Tris-HCl, pH 7.5, 0.025 M KCl, 0.005 M $MgCl_2$, 0.25 M sucrose). Homogenates, made 1.62 M sucrose and layered over 2.3 M sucrose-TKM, were centrifuged for 1 hr at 25,000 rpm in a SW 25.2 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Nuclei were washed as described by Blobel and Potter, except that in most experiments Triton X-100 was not employed, as detergent-washed nuclei exhibited considerable clumping. Rat liver nuclei were also isolated by homogenization in 1.5% citric acid, pH 2.3, as described by Busch (1967).

Chicken erythrocyte nuclei were prepared by the method of Zentgraf et al., (1969). Rhode Island red hens and Light Sussex hens (approximately 5 lb, fed ad libitum) were anesthetized with ether and exsanguinated by cutting the jugular vein. Blood (50–70 ml) was collected in an equal volume of ice-cold homogenization medium (0.01 M Tris-HCl, pH 7.0, 0.001 M $CaCl_2$, 0.004 M *n*-octanol, 0.5% bovine serum albumin, 3% gum arabic, 0.4 M sucrose). Blood was centrifuged for 10 min at top speed in a refrigerated clinical centrifuge (8000 rpm). Erythrocytes were resuspended in 50–70 ml of homogenization media, blended 3 times for 30 sec at top speed in a VirTis 45 microhomogenizer (VirTis Co., Inc., Gardiner, N.Y.), filtered through two layers of cheese cloth, and centrifuged for 10 min in a clinical centrifuge. Nuclear pellets were resuspended in 50–70 ml of homogenization medium, and the procedure was repeated twice more. The pellet from the third homogenization contained no intact erythrocytes and was suspended in 10 vol of supersaturated sucrose (made 0.01 M Tris, pH 7.0, 0.001 M $CaCl_2$, 0.004 M *n*-octanol), blended for 30 sec at half-full speed in the VirTis, layered over supersaturated sucrose buffer, and centrifuged for 90 min at 25,000 rpm in a SW 25.2 rotor. Nuclei were observed in the pellet and at the density interphase. The pellet was saved, suspended in 50 ml of TKM buffer, centrifuged, and washed several times

Washed rat liver and chick erythrocyte nuclear pellets were resuspended in several milliliters of TKM buffer, and the clumps of nuclei were broken by repeated pipetting. The concentrations of nuclei were measured by quantitative dilution of the nuclear suspension in TKM buffer and counting these dilutions with a hemocytometer. Typical nuclear concentrations of the stock solutions were: rat liver nuclei, $3 \times 10^8$ particles/ml, chick erythrocytes, $1 \times 10^9$ particles/ml. Expected DNA concentrations for these stock solutions were calculated, assuming contents of $7.5 \times 10^{-6}$ μg DNA per rat liver nucleus and $2.6 \times 10^{-6}$ μg DNA per chicken erythrocyte nucleus (Sober and Harte, editors, 1968). A single determination of the DNA content of isolated rat liver nuclei with the diphenylamine method (Giles and Myers, 1965), using calf thymus DNA as standard, gave a value of $8.09 \times 10^{-6}$ μg DNA per nucleus. Expected DNA concentrations in the nuclear suspensions were also expressed as expected $A_{260\ nm}$, assuming $E^{1\ cm}_{260\ nm}$ (1 mg DNA/ml) = 20; expected DNA phosphate concentrations from $\epsilon^{1\ cm}_{260\ nm}$ (1 M DNA phosphate) = 6500.

Sonication of isolated rat liver nuclei and chick erythrocyte nuclei was performed with a Mullard Sonicator (Mullard Ltd., London). Nuclei were diluted to approximately $2 \times 10^6$ per ml (rat liver) or $2 \times 10^7$ per ml (chicken erythrocyte) in the chosen buffer. 10- and 25-ml vol of suspension in 50-ml beakers, surrounded by an ice bath, were irradiated with a 0.9 cm diameter probe at a current of approximately 1.7 amp for periods up to 7 min. 3.0-ml portions were withdrawn at various times for spectral analysis and counts of intact nuclei.

PHASE MICROSCOPE OBSERVATIONS: All observations were made with a Zeiss WL micro-

scope (Carl Zeiss, Inc., New York) equipped with camera and exposure meter attachments. Most photographs and visual observations were made with a ×40 phase neofluar objective. Suspensions of nuclei in TKM buffer ($10^6$–$10^7$ particles per ml) were mounted under a cover slip which was secured with Vaseline. Solvent changes were accomplished by sucking buffer across the slide with Kleenex wicks.

ULTRAVIOLET SPECTROSCOPY: Spectral measurements of nuclear suspensions were performed with a Unicam SP. 800 recording spectrophotometer. 1 cm quartz cuvettes were employed, and spectra were frequently recorded at two sample positions, a standard position and close to the photomultiplier. Nuclear suspensions diluted from the stock solutions into the buffers of choice were measured over a range of concentrations (rat liver nuclei, 1–6 × $10^6$ particles/ml, chicken erythrocyte nuclei, 5–40 × $10^5$ particles/ml) and appeared to follow Beer's law throughout the spectral range employed, 210–360 nm. Spectral data are expressed in absorbance due to variations in measured absorbance between different nuclear preparations (in constant solvent and at constant nuclear concentrations). Swollen and homogeneous nuclei tend to aggregate into a gel. Even after low speed centrifugation a pellet of swollen nuclei is impossible to resuspend. Care was taken, therefore, to dilute a nuclear stock in a manner to prevent aggregation, and solutions were repeatedly gently shaken to maintain the suspension.

CIRCULAR DICHROIC SPECTRA: Measurements were made on a Cary Model 61 recording dichrograph (Cary Instruments, a Varian Subsidiary, Monrovia, Calif.). Nuclear suspensions were the same as those examined by ultraviolet spectroscopy, except that all samples had apparent $A_{260\,nm} < 1.0$ (as determined from the standard cuvette position in the Unicam spectrometer). 1.0 and 0.5 cm quartz cuvettes were employed. The variation in measured ellipticity between different nuclear preparations was not as great as that of the apparent absorbance. The data, therefore, are expressed as $\epsilon_L - \epsilon_R$, the difference between the molar extinction coefficients for left- and right-handed circularly polarized light, expressed per mole DNA phosphates.

LOW-ANGLE X-RAY DIFFRACTION: 0.5–1.0 ml quantities of the nuclear stock solutions were diluted with 10.0 ml of a chosen buffer, centrifuged for 5 min at top speed in a refrigerated clinical centrifuge, and resuspended, washed, and centrifuged twice more. For most X-ray experiments, high speed pellets were obtained by resuspending the washed nuclei in buffer and centrifuging in a type 65 or an SW 65 rotor for 1 hr at 65,000 rpm. These "high speed pellets" were carefully pushed into glass capillaries, 0.7 and 1.0 mm diameter (Pantak Ltd., Windsor, England), and the capillaries sealed by flame. A small volume of buffer was generally observed at the ends of the column of packed nuclei. For several experiments "low speed pellets," those obtained by brief centrifugation in the clinical centrifuge, were sealed in capillaries. Low-angle X-ray diffraction patterns of pelleted nuclei were obtained on flat films. Cu $K\alpha_2$ (1.541 A) radiation, from a Hilger semimicrofocus X-ray generator, was selected with a line-focusing quartz crystal monochromator attached to a specially constructed camera (Venable et al., 1970), modified to refrigerate the sample during exposure. Samples were kept at 4–7°C throughout exposure. High speed pellets were irradiated, generally, for 17 hr; low speed pellets for up to 50 hr. The positions of reflections were measured by eye and the Bragg spacings computed with reference to the 3.035 A reflection of calcite crystals mounted at the same specimen positions. Several films were analyzed with a recording microdensitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England) modified to scan with a long line (5 mm × 0.1 mm).

Sample concentrations were determined by measuring the pellet volume in a microscope with a calibrated ocular micrometer, crushing the capillary in 10% perchloric acid, and analysis by the diphenylamine reaction. Inhomogeneity within the nuclear pellets, trapped air bubbles, and errors in volume calculations compel that the concentration measurements be assigned only semiquantitative significance.

ELECTRON MICROSCOPY: Low speed pellets of rat liver nuclei and chick erythrocyte nuclei prepared as described above (for X-ray diffraction) were fixed for 2 hr in 3% glutaraldehyde, 0.25 M sucrose, the required level of salt, and 0.02 M buffers. Samples fixed at pH 7.5 were buffered with 0.02 M sodium cacodylate buffer, fixation at pH 4.87 employed 0.02 M acetate buffer containing 0.02 M KCl. After fixation the nuclear pellets were washed several times and washed overnight with buffer containing 0.54 M sucrose (extra sucrose to compensate for the osmolarity of glutaraldehyde). The pellets were then cut into small blocks, fixed in 1% $OsO_4$ buffer, 0.54 M sucrose for 1 hr, and washed. Up until this point, buffer and salt concentrations and osmolarity were kept constant. Fixed nuclear pellets were dehydrated with ethanol, embedded in Araldite, sectioned with a diamond knife on a Cambridge-Huxley microtome at a thickness of 90–150 nm (silver and gold reflections), and stained with magnesium uranyl acetate and lead citrate. Electron micrographs were taken on a Siemens Elmiskop I at 80 kv, objective aperture 50 $\mu$m, at magnifications up to × 40,000. A calibration grating (28,800 lines/inch) was photographed with each series of observations, magnification values calculated for the final photographic prints had a standard deviation of ± 7%.

Measurements of chromatin fiber widths were made on prints at × 112,000 magnification, using a trans-

parent plastic ruler calibrated in 0.5 mm spaces. 30–150 measurements of fiber widths were made for each solvent (see Table III). Attempts were made to randomize the fibers selected for measurement, except that they were classified to be within either condensed or dispersed regions. Furthermore, measurements presented in this paper are taken from lateral views of fibers, since end views are not easily distinguished from nuclear granules.

ETHIDIUM BROMIDE TITRATIONS: The binding capacity of isolated rat liver nuclei and chicken erythrocyte nuclei for ethidium bromide, over a range of pH and different concentration of $K^+$ and $Mg^{++}$, was measured as described by LePecq and Paoletti (1967). Measurements were made in a Locarte fluorimeter (The Locarte Co, London). Excitation in the region of 546 nm was accomplished with their LF4 filter; emission was recorded at 590 nm, using an LF9 filter. Reaction mixtures contained approximately 7.75 $\mu$g ethidium bromide per ml and 6.75 $\mu$g DNA (or nuclear DNA) per ml. Fluorescence was read at room temperature 30 min after mixing.

## RESULTS

### *Light Microscopy, Phase Maps, and Standard Solvents*

When isolated rat liver nuclei or chick erythrocyte nuclei are washed with buffers containing no divalent cations and very low concentrations of monovalent cations, a rapid morphologic change can be observed with phase-contrast microscopy (Ris and Mirsky, 1949; Anderson and Wilbur, 1952; Philpot and Stanier, 1956; Brasch et al., 1971). Within 1 min the nuclei, which had appeared granular in buffers containing 5 mM $MgCl_2$, swell and become optically homogeneous (Fig. 1).

This swelling and loss of granularity has been studied systematically as a function of pH and $K^+$ and $Mg^{++}$ concentrations for both rat liver nuclei and chick erythrocyte nuclei (Fig. 2). Both types of nuclei show marked granularity at pH 4.87, except at above 0.3–0.4 M KCl. At pH 7.5, isolated nuclei manifest the low cation swelling phenomena. Nuclei appear homogeneous at less than 1–2 mM $MgCl_2$, and less than 0.075–0.1 M KCl. An interesting difference appears between rat liver and chicken erythrocyte nuclei at neutral pH: at less than 1–2 mM $MgCl_2$, and between 0.1–0.3 M KCl, rat liver nuclei lose most of their granularity, whereas chicken erythrocyte nuclei remain granular. This difference is even more apparent in basic solvents (i. e., pH 8.50) where rat liver nuclei appear homogenous in solvents less than 3–4 mM $MgCl_2$ and 0.26–0.3 M KCl, although chicken erythrocyte nuclei are still quite granular in 0.20 M KCl buffers. Phase maps at pH 6.00, 6.50, and 6.95 resembled those obtained at pH 7.50.

For convenience we defined three solvent conditions that reproducibly yield nuclei of different phase states: pH 7.5, 0.02 M KCl, produces homogeneous nuclei; pH 7.5, 5 mM $MgCl_2$, granular nuclei; and pH 7.5, 0.20 M KCl, granular erythrocyte nuclei, and slightly granular rat liver nuclei.

We do not mean to imply that the granular nuclei observed at pH 4.87, or pH 7.50 and 8.50 (with sufficient cation), represent a single structural state, or that all homogenous nuclei are a single, simple phase. Subtle differentiation within a nuclear state is difficult, but can be observed with the phase-light microscope: acid-granular nuclei appear more "brilliant" than those at neutral and alkaline pH; the nucleoli of homogeneous rat liver nuclei are clearly visible at pH 7.5, but less readily observed in alkali

Estimates of volume changes accompanying swelling and loss of granularity for rat liver nuclei have been made by Anderson and Wilbur (1952). From their data, nuclei in 5 mM $MgCl_2$ would have a volume of approximately 400 $\mu^3$; in 0.20 M KCl, 450 $\mu^3$; and in 0.02 M KCl, 700 $\mu^3$. Thus, changing buffers from 5 mM $MgCl_2$ to 0.02 M KCl produces approximately a 1.75-fold increase in nuclear volume, or a 57% decrease in average nuclear DNA concentration.

We have also made measurements of the diameters (and volumes) of isolated rat liver nuclei in two solvent conditions: pH 7.5, 0.02 M KCl; and pH 7.5, 5 mM $MgCl_2$. Although our calculated average nuclear volumes are less than those of Anderson and Wilbur (5 mM $MgCl_2$, 205 $\mu^3$; 0.02 M KCl, 354 $\mu^3$), we obtain a 1.73-fold volume increase. Using our volume data, and a value of $7.5 \times 10^{-6}$ $\mu$g DNA per rat liver nucleus (Sober and Hart, editors, 1968), we obtain average nuclear DNA concentrations of 21 mg DNA/ml (in 0.02 M KCl) and 37 mg DNA/ml (in 5 mM $MgCl_2$). Further assuming that, in 5 mM $MgCl_2$, approximately 50% of the nuclear DNA is condensed in roughly one-tenth of the nuclear volume, one can calculate that local DNA concentration might be as high as 200 mg DNA/ml. This degree of condensation is based upon observations, from electron micrographs, that the peripheral chromatin seen in granular nuclei can be approximated to a spher-

ical shell with thickness about 5% that of the radius of the spherical nucleus The problem of volume shrinkage during fixation and embedding is ignored for this computation Thus, nuclear swelling from 5 mM $MgCl_2$ to 0 02 M KCl could produce a 10-fold decrease in DNA concentration for much of the rat liver nuclear chromatin

Unfortunately, volume data for chicken erythrocyte nuclei in the granular and homogeneous states are lacking. On the basis of microspectrophoto-

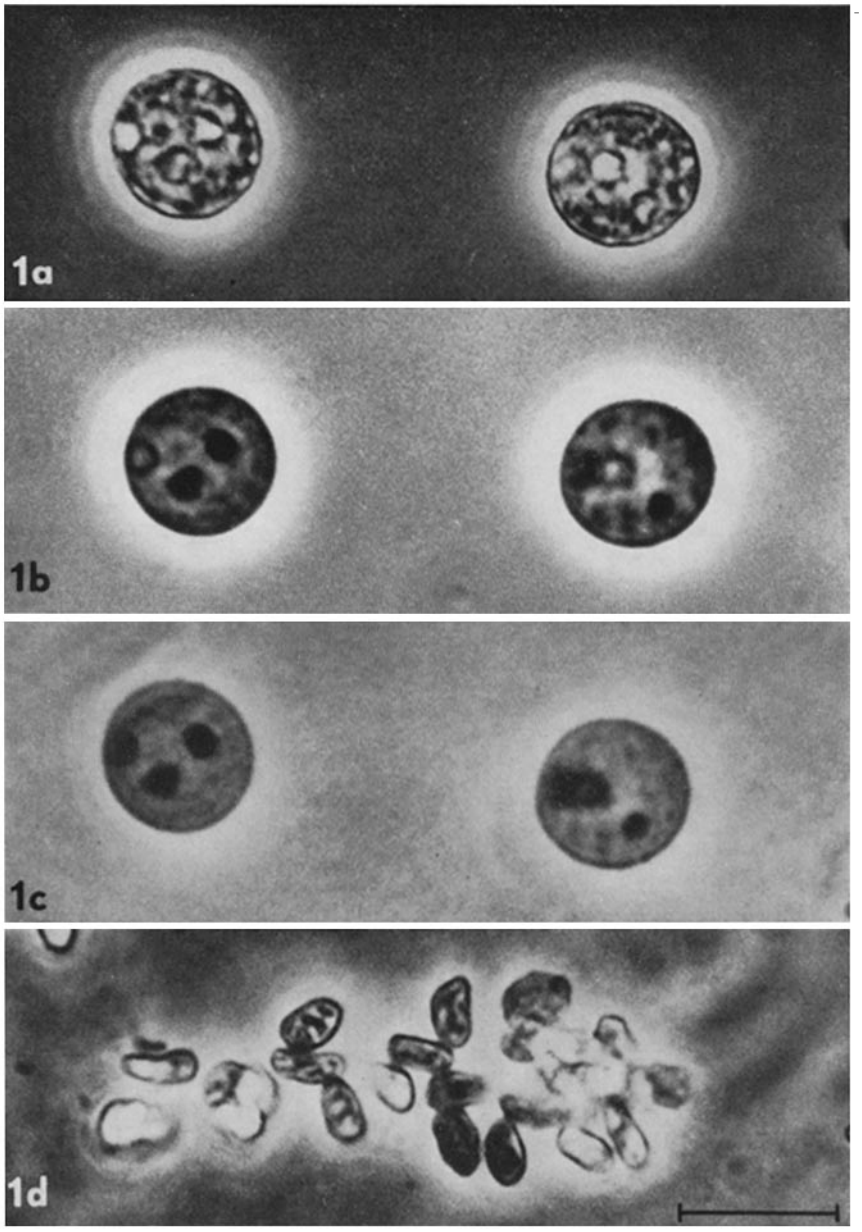


FIGURE 1 Phase-contrast microscopy of isolated nuclei in different solvents. (*a*) granular rat liver nuclei in 5 mM $MgCl_2$, pH 7 5; (*b*) slightly granular rat liver nuclei in 0 20 M KCl, pH 7.5; (*c*) homogeneous rat liver nuclei in 0.02 M KCl, pH 7.5; (*d*) granular chicken erythrocyte nuclei in 5 mM $MgCl_2$, pH 7.5. Bar denotes 10 $\mu$. × 2000.

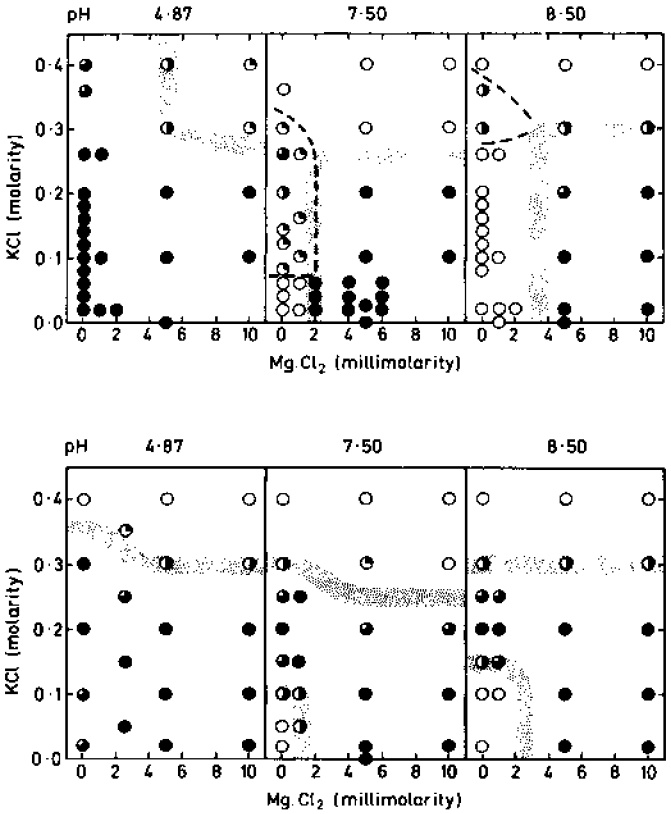


FIGURE 2 Morphologic phase maps relating nuclear chromatin condensation to solvent pH, $K^+$ and $Mg^{++}$ concentration. (A) top frames, isolated rat liver nuclei. (B) bottom frames, isolated chicken erythrocyte nuclei. Open circle, one-quarter filled circle, half-filled circle, three-quarter filled circle, solid circle: increasing levels of condensation. Phase boundaries, corresponding to approximately half-maximum granularity, are indicated by stipple; an additional boundary of lower granularity for rat liver nuclei is indicated by a dashed line. Most points represent multiple observations.

metric data for the condensed chromatin of amphibian erythrocyte nuclei (i. e., $A_{260\ nm} = 0.38$ for a 0.5 $\mu$ tissue section; Small and Davies, 1970), one can estimate that local DNA concentrations of 375 mg DNA/ml are not unreasonable.

### *Absorption Spectra*

Measurements of the absorption spectra of isolated rat liver and chicken erythrocyte nuclei reveal a close correlation with their observed morphologic states: granular nuclei exhibit a marked turbidity at $\lambda > 300$ nm, and a flattened spectrum at $\lambda < 300$ nm; homogeneous nuclei show little turbidity, and the ultraviolet spectrum is much more like that of soluble nucleohistone (Tuan and Bonner, 1969); nuclei with moderate levels of granularity display intermediate spectral properties. Fig. 3 shows the spectra obtained for isolated nuclei in the three standard solvents at pH 7.5. Spectra of rat liver nuclei in solvents of varying pH, with and without $MgCl_2$, appear in Fig. 4. Suppressed spectra are seen for nuclei at acid pH (1.88 and 4.87) with and without $MgCl_2$, and in neutral (7.50) and basic (8.50) buffers containing 5 mM $MgCl_2$, all solvents that promote nuclear granularity. At pH 7.50, 8.50, and 10.00, in the absence of $MgCl_2$, nuclei are homogeneous and spectra do not appear flattened. Similar turbidity changes, correlating with nuclear morphology, have recently been reported by Kraemer and Coffey (1970).

There are several mechanisms that could explain these spectral changes. Spectral perturbations could be due to (*a*) light-scattering effects, (*b*) shadowing of chromophores (the sieve effect), or (*c*) changes

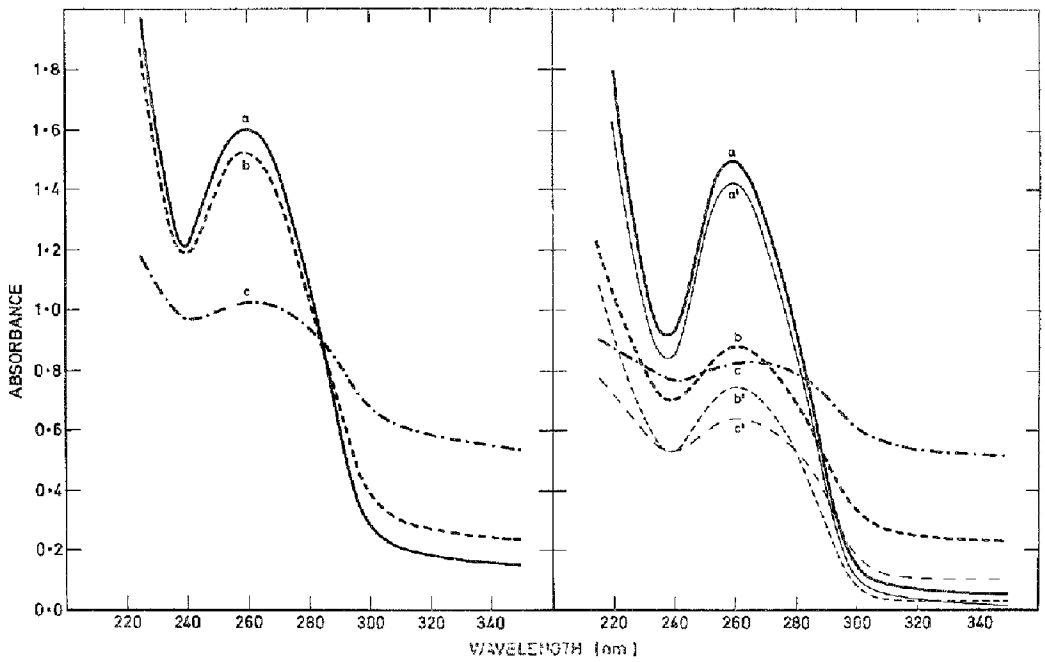


FIGURE 3 Ultraviolet absorbance spectra of isolated nuclei at neutral pH. (A) left frame, rat liver nuclei, concentration: $5.75 \times 10^6$ nuclei/ml (B) right frame, chicken erythrocyte nuclei; concentration: $3.55 \times 10^7$ nuclei/ml. Curves. *a*, 0.02 M KCl, pH 7.5, *b*, 0.20 M KCl, pH 7.5; *c*, 5 mM $MgCl_2$, pH 7.5; *a'*, *b'* and *c'* were recorded with the cuvette close to the detector.

in the chemical environment of the chromophores Although the effects of light scattering upon spectral measurements are extremely difficult to calculate for particles such as those studied here, these effects can be empirically minimized by introducing scattering elements in both the reference and sample light paths, or by moving the reference and sample cuvettes very close to the detector in order to collect the maximum amount of scattered light (Shibata, 1959) The Unicam SP 800 recording spectrophotometer has facilities for taking measurements in a "standard" cuvette position, and a position close to the detector. Fig 3 B illustrates the consequences of changing the cuvette position Apparent turbidity is markedly reduced for nuclei in 0 20 M KCl and in 5 mM $MgCl_2$, and spectral peaks and troughs are more clearly defined Altering the cuvette position has only minor influence upon the spectra of homogeneous nuclei, but markedly alters those of granular nuclei. Similar changes have been seen with erythrocyte nuclei It is clear that light scattering has a profound effect upon the observed spectra, but spectral differences between granular and homogeneous nuclei still remain Comparing nuclei in 0 02 M KCl, pH 7.5 and in 5 mM MgCl, pH 7 5, the following differences remain: (*a*) very slight red-shifts (i e , 1–2 nm) of the granular nuclear spectra were observed at the $\lambda_{min}$ and $\lambda_{max}$ (approximately 240 and 260 nm, respectively), (*b*) $A_{260\ nm}$ for nuclei in 5 mM $MgCl_2$ is considerably less than $A_{260\ nm}$ for nuclei in 0 02 M KCl (granular rat liver nuclei averaged $A_{260\ nm}$ = 50% that of homogeneous nuclei, granular chicken erythrocyte nuclei had 42% of the absorbance seen for homogeneous nuclei) These spectral differences and their magnitudes have been consistently observed despite an almost twofold variation in nuclei per ml/$A_{260\ nm}$ that we have experienced between different nuclear preparations of the same tissue type The wavelength shifts are very small and will not be considered further; the absorption "flattening" should be viewed from the point of view of the sieve effect.

Absorption flattening, as a consequence of the "shadowing" of chromophore by chromophore on different particles, while a fraction of the incident light passes, unabsorbed, to the detector (the "sieve" effect), has received recognition and theoretical treatment since the studies of Duysens (1956) and of Rabinowitch (1956). The goal of the

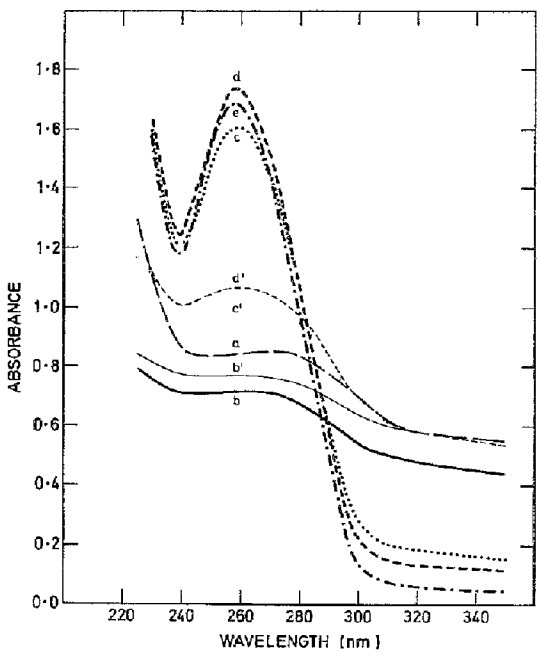


FIGURE 4 Ultraviolet absorbance spectra of isolated rat liver nuclei in various solvents. Concentration: $5.75 \times 10^6$ nuclei/ml. Solvents: *a*, *b*, *c*, *d*, and *e*, all 0.02 M KCl and pH 1.88, 4.87, 7.50, 8.50 and 10.00, respectively, *b'*, *c'*, and *d'*, all 5 mM $MgCl_2$ and pH 4.87, 7.50 and 8.50, respectively.

computations is $Q_A$, the flattening coefficient, defined as $A$ suspension/$A$ solution, the ratio of the absorbance of the particle suspension to that of a dispersed solution of the chromophores, assuming no intrinsic spectral differences between the chromophores in the two environments. $Q_A$ varies from 0 to 1, and is calculated, assuming the appropriate model of the particle, from particle dimensions and absorbance, and the volume fraction of particles in the suspension. Such a computation will be described later.

An alternative means of examining the magnitude of the sieve effect (i. e., the value of $Q_A$) is to compare the spectra (minus scattering) of intact nuclei in suspension with spectra of dispersed or fragmented nuclei, assuming no intrinsic changes in chromophore. We have employed two methods for fragmenting isolated nuclei, by sonication and by disruption in high NaCl (2 M or 4 M). Isolated nuclei were sonicated in 0.02 M KCl, pH 7.5 and in 5 mM $MgCl_2$, pH 7.5 buffers. As monitored by counts of the titer of intact nuclei as a function of sonication time, marked differences were observed in susceptibility to breakage. Homogeneous rat liver nuclei (0.02 M KCl) were completely fragmented by 15 sec, whereas granular nuclei (5 mM $MgCl_2$) required 4–5 min for complete disruption. Swollen chicken erythrocyte nuclei (0.02 M KCl) were also totally disrupted by 15 sec, but in 5 mM $MgCl_2$ erythrocyte nuclei were only 50% broken after 7 min of sonication (the maximum time allowed for sonication, in this study). Disruption of homogeneous rat liver and chicken erythrocyte nuclei in 0.02 M KCl buffers produces only minimal changes in the absorbance spectra (i. e., changes in $A_{260\ nm} \sim \pm\ 10\%$). Spectra of isolated nuclei in 2 and 4 M NaCl are also quite similar to those of the intact homogeneous nuclei. Sonication of isolated rat liver nuclei (Fig. 5) and chicken erythrocyte nuclei in 5 mM $MgCl_2$, on the other hand, produced dramatic alterations in the spectra. Measured at the standard cuvette position, turbidity increased almost 100% for rat liver nuclei, 50% for chicken erythrocyte nuclei. Measured close to the detector, $A_{260\ nm}$ for broken rat liver nuclei was almost equal to that of nuclei disrupted in 2 or 4 M NaCl, sonicated chicken erythrocyte nuclei achieved only about 66% of the absorbance observed with nuclei in 2 M NaCl. Assuming 50% breakage for chicken erythrocyte nuclei, $A_{260\ nm} = 0.43$ (intact nuclei), and $A_{260\ nm} = 1.10$ (disrupted nuclei in 2 M NaCl), additive spectra would yield $A_{260\ nm} = 0.87$, somewhat higher than actually observed. Ignoring scattering effects, it appears then that the absorption spectra of homogeneous nuclei (0.02 M KCl), sonically disrupted nuclei (0.02 M KCl, and in 5 mM $MgCl_2$), and nuclei dispersed in high NaCl (2 and 4 M NaCl) are all very similar in magnitude (i. e., $Q_A \sim 0$) whereas intact nuclei in 5 mM $MgCl_2$ reveal considerable flattening (i. e., $Q_A = 0.4$–$0.5$).

As mentioned above we have attempted approximate computations of $Q_A$ from the equations of Duysens (1956). If one assumes that isolated rat liver nuclei can be considered as tiny spheres with chromatin uniformly dispersed inside the nucleus, and assumes the following volume and DNA concentration data: (*a*) 0.02 M KCl: volume/nucleus, 354 $\mu^3$, nuclear DNA concentration, 21 mg/ml; nuclear absorbance, $A_{260\ nm,\ 1\ cm} = 420$ (*b*) 5 mM $MgCl_2$: volume/nucleus, 205 $\mu^3$; nuclear DNA concentration, 37 mg/ml.; nuclear absorbance $A_{260\ nm,\ 1\ cm} = 740$, one can calculate an average value of $\alpha_p$, the absorbance through the center of the sphere, and in turn, $Q_A$ (Duysens,

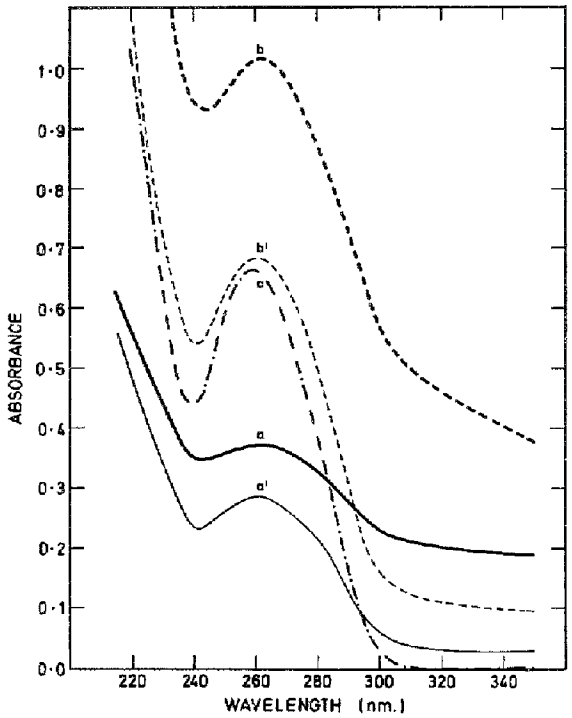


FIGURE 5 Ultraviolet absorbance spectra-sonication of rat liver nuclei. Solvent: 5 mM $MgCl_2$, pH 7.5. Concentration: $2 \times 10^6$ nuclei/ml Curves: *a* and *a'*, before sonication, standard and close cuvette positions, respectively; *b* and *b'*, after 5 min sonication, standard and close cuvette positions, respectively; *c*, in 4 M NaCl. Similar spectra were obtained for nuclei disrupted with 2 M NaCl.

1956). For rat liver nuclei in 0.02 M KCl, we calculate $Q_A = 0.87$; for 5 mM $MgCl_2$, $Q_A = 0.82$. These computations would predict that little absorbance flattening would be observed in either 0.02 M KCl or 5 mM MgCl Rather than conclude that the large discrepancy between predicted and observed $Q_A$ for nuclei in 5 mM $MgCl_2$ necessarily implies environmental or conformational changes of the DNA chromophore, we believe that more complete data combined with a consideration of other models, such as spherical shells (Gordon and Holzwarth, 1971; Papageorgiou, 1971, Glaser and Singer, 1971) and spheres with internal structure (Latimer et al., 1968), are first required

### *Circular Dichroic Spectra*

The circular dichroic (CD) spectra of isolated rat liver and chicken erythrocyte nuclei also appeared to correlate with the morphologic state of the nuclei (Fig 6 and Table I) Granular nuclei revealed flattened spectra; homogeneous nuclei exhibited sizeable ellipticities. Recent measurements have appeared of the circular dichroic spectra of isolated chromatin (Permogorov et al. 1970; Henson and Walker, 1970, Shih and Fasman, 1970; Simpson and Sober, 1970). These spectra, on isolated and extensively sheared chromatin, reveal striking similarities to spectra that we have obtained with homogeneous nuclei (especially rat liver nuclei) At $\lambda > 260$ nm, a positive Cotton effect is observed, frequently bimodal, and, in magnitude, 40–60% that of native DNA; below $\lambda = 260$ nm, a negative shoulder ($\lambda \sim 245$ nm) with magnitude comparable to native DNA, and a strongly negative Cotton effect ($\lambda \sim 222$ nm and 208 nm) ascribed to $\alpha$-helical protein Representative spectral parameters from our data are compared with data from the literature for isolated soluble nucleohistone, converted into units of $\epsilon_L - \epsilon_R$, and shown in Table I. Our data from isolated rat liver nuclei, swollen in 0 02 M KCl, strongly resemble the literature data Isolated chicken erythrocyte nuclei in 0.02 M KCl appear more suppressed at $\lambda \sim 275$–283 nm than do rat liver

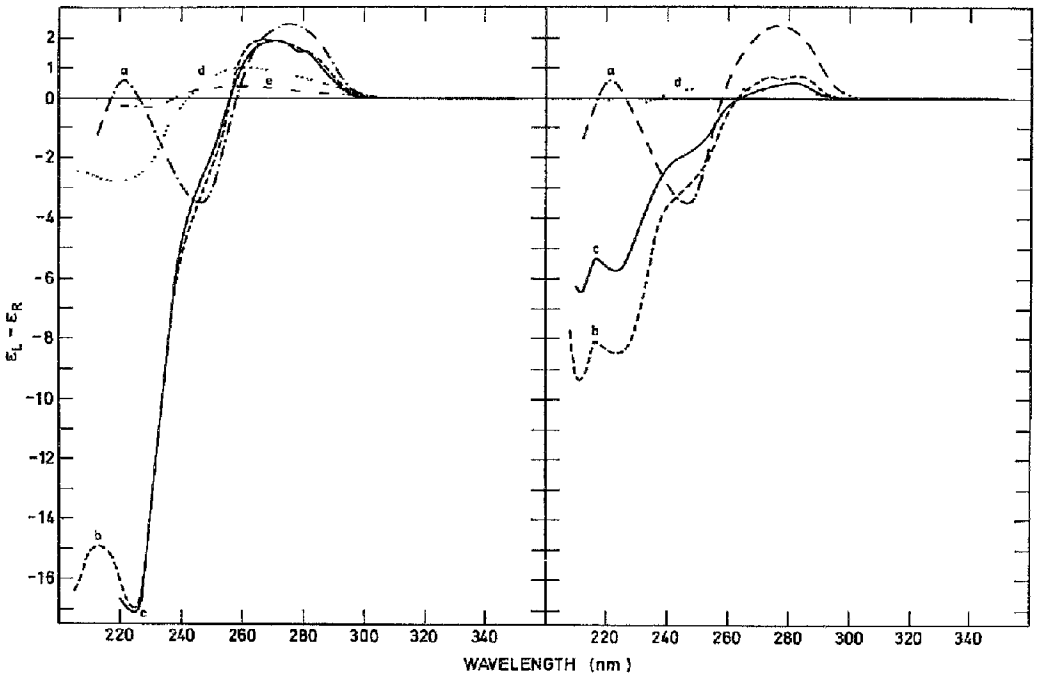


FIGURE 6 Circular dichroic spectra of intact nuclei in various solvents: (A) left frame, rat liver nuclei; (B) right frame, chicken erythrocyte nuclei. $\epsilon_L - \epsilon_R$, difference between the extinction coefficient (per mole DNA phosphate) for left- and right-handed circularly polarized light. Curves: *a*, calf thymus DNA, 0.02 M KCl, pH 7.5; *b*, nuclei, 0.02 M KCl, pH 7.5; *c*, nuclei, 0.20 M KCl, pH 7.5; *d*, nuclei, 5 mM $MgCl_2$, pH 7.5; *e*, nuclei, 0.02 M KCl, pH 4.87.

nuclei under the same conditions. Crossover points ($\lambda \sim 260$ nm) for swollen nuclei and isolated chromatin are also quite similar. We would also point out that calf thymus DNA dissolved in 2 M NaCl reveals a flattening of absorption at $\lambda > 260$ nm, comparable to that seen for swollen nuclei and for isolated chromatin. Similar observations have been made by Permogorov et al. (1970) and by Henson and Walker (1970). In 5 mM $MgCl_2$, pH 7.5, or at acid pH (4.87), the spectra of nuclei are considerably flattened, and the crossover points markedly shifted. Flattening coefficients, $Q$, comparing spectra in 5 mM $MgCl_2$ with those in 0.02 M KCl, reveal that the flattening effect is greatest at $\lambda < 230$ nm. Crossover points for nuclei in 5 mM $MgCl_2$ are considerably blue-shifted. Rat liver nuclei, pH 4.87, revealed a crossover point of $\lambda = 237$ nm.

Circular dichroic spectra of turbid particulate suspensions are also prone to a number of optical artifacts (Urry and Krivacic, 1970; Schneider et al, 1970, Gordon and Holzwarth, 1971; Glaser and Singer, 1971). (*a*) Light scattering can affect measured ellipticity if an appreciable fraction of the light that would have been absorbed is scattered from solution. This effect tends to diminish the measured absorbance, and consequently the circular dichroism ($A_L - A_R$). (*b*) Differential light scattering, due to particle asymmetry with respect to the refractive indices for left and right circularly polarized light, alters the measured absorbance, the magnitude and sign of this effect being a function of $n_L$, $n_R$, and $n_0$ (solvent refractive index). (*c*) Absorption flattening due to a sieve effect decreases the magnitudes of $A_L$, $A_R$, and $A_L - A_R$. These effects have presented considerable difficulty to workers concerned with membrane protein conformations (Schneider et al, 1970, Glaser and Singer, 1971), who have attempted to estimate their relative contributions to observed spectra by fragmentation of the membranes.

In our studies, the influences of light scattering and of differential light scattering did not appear to be great; varying the position and path length of the optical cells did not appreciably alter the

TABLE I

*Circular Dichroism Measurements*

| Nuclei or chromatin | Solvent | $\epsilon_L - \epsilon_R$ | | | | | Crossover $\lambda_{nm}$ | | Reference |
|---|---|---|---|---|---|---|---|---|---|
| | | 285 | 275 | 245 | 223 | | | | |
| Rat liver nuclei | 0.02 M KCl, pH 7.5 | 1.32 | 1.70 | −4.58 | −21.54 | 257 | | | |
| | 0.20 M KCl, pH 7.5 | 1.31 | 1.85 | −3.20 | −16.95 | 257 | | | |
| | 5 mM $MgCl_2$, pH 7.5 | 0.57 | 0.79 | +0.12 | −3.17 | 245 | | | |
| | Q (5 mM Mg/0.02 M K) | (0.43) | (0.46) | | (0.15) | | | | |
| Chicken erythrocyte nuclei | 0.02 M KCl, pH 7.5 | 0.74 | 0.64 | −3.05 | −8.88 | 263 | | | |
| | 0.20 M KCl, pH 7.5 | 0.48 | 0.32 | −2.35 | −5.77 | 263 | | | |
| | 5 mM $MgCl_2$, pH 7.5 | 0.18 | 0.24 | +0.18 | −0.15 | 237.5 | | | |
| | Q (5 mM Mg/0.02 M K) | (0.24) | (0.38) | | (0.02) | | | | |
| Calf thymus DNA | 0.02 M KCl, pH 7.5 | 2.04 | 2.39 | −3.31 | 0.49 | 258 | 226 | 217 | |
| | 2.0 M NaCl | 1.58 | 1.42 | −3.60 | 0.61 | 261 | 226.5 | 217 | |
| Calf thymus chromatin | 0.7 mM sodium phosphate, pH 6.8 | 1.3 | 1.2 | −3.5 | −13 | 260 | | | Henson and Walker (1970) |
| Calf thymus chromatin | 0.14 M NaF, pH 8.0 | | 1.21* | −3.6 | −11.1 | 259.5 | | | Shih and Fasman (1970) |
| Calf liver chromatin | 1.0 mM Tris, pH 7.8 | 1.67‡ | 1.82§ | −1.82 | | 258 | | | Simpson and Sober (1970) |

* 277 nm

‡ 280 nm

§ 272 nm

observed spectra for homogeneous or granular nuclei. Attempts were made to estimate the sieve effects by sonication of nuclei, and by disruption in 2 M NaCl. Small changes in the magnitude of absorbance were observed due to sonication. Probably the most dramatic effect, however, was the red-shift of the crossover point for nuclei fragmented in 5 mM $MgCl_2$, to $\lambda \sim 260$ nm, whereas nuclei disrupted in 0.02 M KCl revealed no shift in crossover point (Fig. 7). Equally clear is the finding that nuclei disrupted in 5 mM $MgCl_2$ reveal considerable differences compared to intact or disrupted nuclei in 0.02 M KCl, especially with respect to absorbances at $\lambda \sim 220$ nm. Recent studies of Wagner and Spelsberg (1971) reveal similar shifts of the crossover point in circular dichroic spectra of intact and disrupted calf thymus nuclei in 3.3 mM $CaCl_2$. Nuclei disrupted with 2 M NaCl strongly resemble, at $\lambda > 245$ nm, native DNA in the same solvent, and revealed no trace of the biomodal peak at $\lambda \sim 270$–280 nm. Our observations that disruption of isolated nuclei in 0.02 M KCl yields only small changes in measured ellipticities, the resemblance of these spectra to literature data on sheared chromatin, and the earlier observation of minimal ultraviolet absorbance changes upon disruption of nuclei in 0.02 M KCl, all suggest that removal of divalent cations at low ionic strength results in a state similar to that of extensively sheared and purified chromatin. We cannot state whether the crossover point shifts and apparent flattening observed for isolated nuclei in 5 mM $MgCl_2$ are due to optical effects only or to conformational effects. Tunis-Schneider and Maestre (1970) report blue-shifts in the crossover point for CD spectra of films of sodium DNA at 75% relative humidity, conditions that promote *A* form DNA. We note, also, that Simpson and Sober (1970) report that $Ca^{++}$ added to calf liver nucleohistone resulted in a 25% decrease in el-

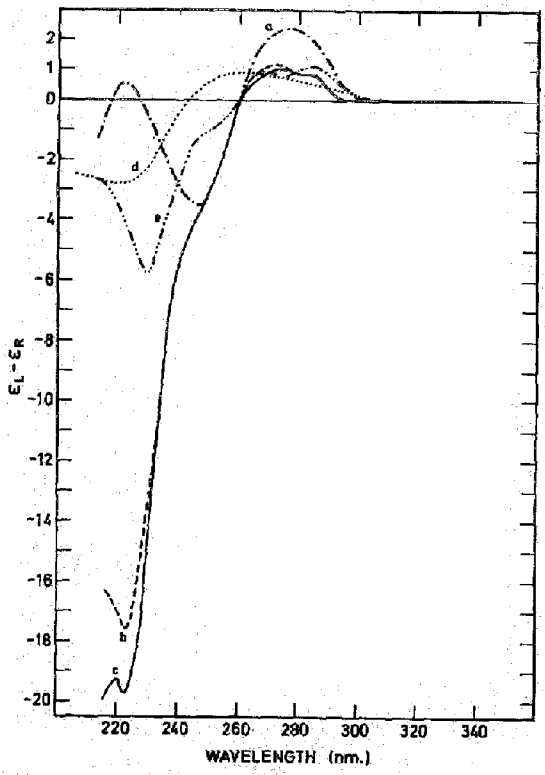


FIGURE 7 Circular dichroic spectra of sonicated rat liver nuclei: $\epsilon_L - \epsilon_R$, difference between the extinction coefficient (per mole DNA phosphate) for left- and right-handed circularly polarized light. Curves: *a*, calf thymus DNA, 0.02 M KCl, pH 7.5; *b*, nuclei before sonication, 0.02 M KCl, pH 7.5; *c*, nuclei after 15 sec sonication, 0.02 M KCl, pH 7.5; *d*, nuclei before sonication, 5 mM $MgCl_2$, pH 7.5; *e*, nuclei after 5 min sonication, 5 mM $MgCl_2$, pH 7.5.

lipticity at $\lambda \sim 276$ nm, but did not affect spectra at $\lambda \sim 250$ nm. It appears probable, therefore, that both optical and structural effects intravene.

### *Low Angle X-Ray Diffraction*

Evidence for periodic structure within chromatin has come from a variety of wide- and low-angle X-ray studies (Luzzati and Nicolaieff, 1959, 1963; Wilkins et al., 1959; Wilkins, 1964; Pardon, 1966; Pardon et al., 1967; Bram and Ris, 1971; Bram, 1971; Garrett, 1971). Luzzati and Nicolaieff (1959, 1963) studied the low-angle X-ray diffraction of nucleohistone gels, observed reflections at 110, 55, 35 A (among others), and analyzed these reflections in terms of several concentration-dependent phases. The 110 A reflection, in particular, was assumed to represent interparticle distances. Wilkins (1964), Pardon (1966), and Pardon et al., (1967) observed a series of low-angle spacings (110, 55, 37, 27, and 22 A) and their orientations with respect to fiber axis in oriented nucleohistone fibers. On the basis of these studies, they proposed that nucleohistone filaments (width, 30 A) are coiled into a superhelix (pitch 120 A, diameter 100 A). More recently, Bram and Ris (1971) have examined the low-angle diffraction intensities of nucleohistone gels. They obtained evidence of reflections at 41, 23, 12, and 9 A, but did not observe the 110- or 55-A spacings. On the basis of the central scattering intensities, and companion electron microscope studies, Bram and Ris (1971) propose that nucleohistone can be approximated to a long cylinder (diameter 80–120 A) composed of irregularly folded or supercoiled (pitch 45 A) nucleohistone filaments (width 22 A).

We have been concerned with X-ray diffraction patterns obtained from intact, unoriented, freshly isolated nuclei in a variety of solvent systems. Use of a low-angle camera in conjunction with a line-focusing quartz crystal monochromator proved advantageous in visualizing the low-angle rings. Data obtained on nuclear pellets, from low or high speed centrifugation, and in a variety of solvents, are summarized on Table II. It is clear that, given sufficient exposure time, the $\sim$ 110-, $\sim$55-, and $\sim$ 35-A reflections can be observed in homogeneous and granular nuclei in all the solvents employed, except at pH 1.88 and 2.3, where the two lowest angle reflections are replaced by a broad smear of scattering. Presumably, the apparent loss of periodic structure in acid pH is a consequence of acid denaturation of the native DNA structure (Steiner and Beers, 1961; Michelson, 1963), since at these pH's the only histone that would be completely removed, the lysine-rich (F1) histone (Murray, 1969), does not appear to be necessary for maintenance of the superhelix (Richards and Pardon, 1970; Murray et al., 1970).

The length of exposure required to visualize the X-ray reflections appeared to correlate with the degree of nuclear swelling. Comparing high speed pellets, rat liver nuclei and rat liver chromatin in 5 mM $MgCl_2$, pH 7.5 had DNA concentrations (as measured diphenylamine analyses) of 500–650 mg DNA/ml pellet. Pellets of nuclei and chromatin in 0.02 M KCl, pH 7.5, contained 200–350 mg DNA/ml. Generally, a twofold increase of exposure time was adequate to visualize the low-angle reflections

TABLE II

*Low-Angle X-Ray Reflections (A)*

| Nuclei | Solvent | | | Pellet | Reflections* | | |
|---|---|---|---|---|---|---|---|
| | pH | M KCl | mM $MgCl_2$ | | | | |
| Rat liver nuclei | 1.88 | — | 5 | H | — | — | 41 |
| | 2.30‡ | — | — | H | — | — | 36 |
| | 3.90 | — | 5 | H | 136 | 63 | 40 |
| | 4.87 | 0.02 | — | H | 121 | 68 | 39 |
| | 7.50 | 0.02 | — | H | 97 | 61 | 37 |
| | | 0.02 | — | L | 113 | 64 | 39 |
| | | 0.10 | — | H | 92 | 63 | 38 |
| | | 0.20 | — | H | 109 | 60 | 39 |
| | | 0.20 | — | L | 109 | 63 | 39 |
| | | — | 5 | H | 112 | 58 | 39 |
| | | — | 5 | L | 102 | 57 | 39 |
| | | 0.025 | 5 | H | 101 | 61 | 37 |
| | | 0.025 | 10 | H | 95 | 57 | 37 |
| | | 0.02 | 5 (EDTA)§ | H | 109 | 69 | 40 |
| | 8.50 | 0.02 | — | H | 109 | 64 | 37 |
| | | — | 5 | H | 124 | 62 | 37 |
| | 9.20 | — | 5 | H | 95 | 56 | 37 |
| | 10.00 | — | 5 | H | 99 | 55 | 37 |
| | 10.90 | — | 5 | H | 104 | 63 | 38 |
| Chicken erythrocyte nuclei | 4.87 | 0.02 | — | L | 111 | 60 | 38 |
| | 7.50 | 0.02 | — | H | 109 | 64 | 39 |
| | | 0.02 | — | L | 109 | 68 | 39 |
| | | 0.20 | — | L | 116 | 63 | 39 |
| | | — | 5 | L | 113 | 63 | 39 |
| Rat liver chromatin‖ | 7.50 | 0.02 | — | H | 133 | 64 | 35 |
| | | 0.20 | — | H | 99 | 57 | 35 |
| | | — | 5 | H | 99 | 64 | 38 |
| | | | Average | | 109 ± 11 | 62 ± 4 | 38 ± 1 |

* Wide-angle reflections (~28, 12, and 8A) were observed with many samples, but are not recorded here. Average values and their standard deviations are also shown. H and L, high and low speed pellets after centrifugation.

‡ 1.5% citric acid.

§ 5 mM Na EDTA.

‖ Prepared by repeated shearing with a Teflon-glass homogenizer, following the method of Zubay and Doty (1959).

Because of the suggestion (Luzzati and Nicolaieff, 1959, 1963) that the 110 A reflection might represent interparticle distances, and considering that high speed pellets contain DNA concentrations, possibly 10–20 times more concentrated than native nuclei, diffraction data were also obtained from low speed pellets. Greater exposure time was required to visualize the reflections (see Materials and Methods), and, when measured, the low-angle spacings showed no significant differences from those obtained on high speed pellets. Therefore, within the range of DNA concentrations studied (i. e., 35–650 mg DNA/ml), no evidence is obtained for the type of concentration-dependent interparticle distances observed with F-actin (Spencer, 1969) and ribosomes (Venable et al. 1970).

Wilkins and Zubay (1963) have demonstrated that 60-A reflections can arise from small amounts of lipid present within isolated nucleoprotein gels. In several experiments, isolated rats liver nuclei were washed with TKM buffer containing 0.5% Triton X-100, which has been shown to remove the outer nuclear membrane (Blobel and Potter,

1966), with no observable consequence to the low-angle X-ray reflections. In any case, the characteristic pattern was not considered to be present unless the series ~ 110, ~ 55, and ~ 38 A was observed.

The observed series of low-angle X-ray reflections, and the periodic structure they connote, appear, therefore, to be remarkably stable to alterations in solvent composition and attendant changes in nuclear morphology, probably a consequence of the stability of histone-DNA interaction (Richards and Pardon, 1970).

### *Electron Microscopy*

Electron microscope observations of thin sections of fixed nuclei and chromosomes have revealed an array of largely disorganized threads and granules (generally assumed to be end views of threads) ranging in widths from 20 to 500 A, and most frequently approximately 100–200 A (Davies, 1968; for reviews see: Kaye and McMaster-Kaye, 1966; Ris, 1969). In many types of nuclei, and particularly well delineated in chicken erythrocyte nuclei, unit threads (170 A width) reveal an orderly arrangement into layers within condensed chromatin adjacent to the nuclear membrane (Davies, 1968; Everid et al., 1970).

In this study isolated rat liver and chicken erythrocyte nuclei were fixed in solvents similar to the standard solvents mentioned earlier, in order to examine the relationship of the dimensions of chromatin threads to the degree of nuclear condensation. Figs. 8, 9, and 10 present low and high magnification micrographs of rat liver and chicken erythrocyte nuclei fixed in solvents containing either 0.02 M KCl, 0.20 M KCl, or 5 mM $MgCl_2$, pH 7.5. The low power micrographs reveal good correlation with phase-light microscope observations upon unfixed nuclear preparations. Homogeneous nuclei reveal a uniform distribution of chromatin threads, i. e., all of the chromatin appears dispersed. Granular and slightly granular nuclei display regions of condensed chromatin and regions of dispersed chromatin threads.

Measured average widths of chromatin threads within homogeneous and granular nuclei are presented in Table III. The data were obtained from lateral views of the nucleohistone threads as described in Materials and Methods. As can be seen, average widths range from 100 to 190 A. The measured widths were analyzed in frequency-size distribution plots. The distributions of widths for the different nuclear states were, generally, broad unimodal curves.

Several observations have resulted from a detailed examination of these electron micrographs. (*a*) Homogeneous rat liver nuclei in 0.02 M KCl display a larger proportion of thin threads (50–100 A width) than in 0.20 M KCl. This is reflected in the lower average width of rat liver nuclei in 0.02 M KCl (Table III). (*b*) In 5 mM $MgCl_2$, chicken erythrocyte nuclei contain almost no remaining dispersed chromatin. What remains of the dispersed chromatin is composed of a few fibers, less than 100 A wide, and occasional clusters of "granules," 50–300 A in diameter. (*c*) Rat liver nuclei, on the other hand, possess considerable dispersed chromatin in 5 mM $MgCl_2$, and its threads resemble those seen at 0.20 M KCl.

In summary, nuclei swollen in 0.02 M KCl, pH 7.5, or condensed to varying degrees in 0.20 M KCl or 5 mM $MgCl_2$, pH 7.5 appear to contain chromatin threads possessing approximately the same spectrum of fiber widths, with average diameters, as viewed laterally, between 100 and 200 A. Such dimensions are in agreement with most literature data obtained by study of thin-sectioned material (see Davies, 1968, and reviews cited earlier), but are larger than the average fiber widths (~ 75 A) reported by Brasch et al. (1971) for thin sections of isolated chicken erythrocyte nuclei swollen in distilled water. Bram and Ris (1971) also observe thin (80–120 A) fibers for isolated calf thymus nucleohistone in 0.8 mM phosphate buffer, pH 6.8, examined after formalin fixation, critical point drying, and uranyl acetate stain.

### *Ethidium Bromide Titrations*

Isolated nucleohistone has been shown to possess considerable binding capacity for a variety of basic dyes, actinomycin D, and polylysine (Klein and Szirmai, 1963; Miura and Ohba, 1967; Ringertz and Bolund, 1969; Bolund, 1970; Borisova and Minyat, 1970; Itzhaki, 1970, 1971; Kleiman and Huang, 1971; Clark and Felsenfeld, 1971). From these studies, the estimated binding capacity of chromatin is 30–50% that of native naked DNA.

It was of interest to examine whether the state of condensation of chromatin within isolated nuclei had any influence upon its binding capacity for small molecules. The intercalation of ethidium bromide into helical polynucleotides can be conveniently observed with fluorimetric measurements (LePecq and Paoletti, 1967). We chose to compare

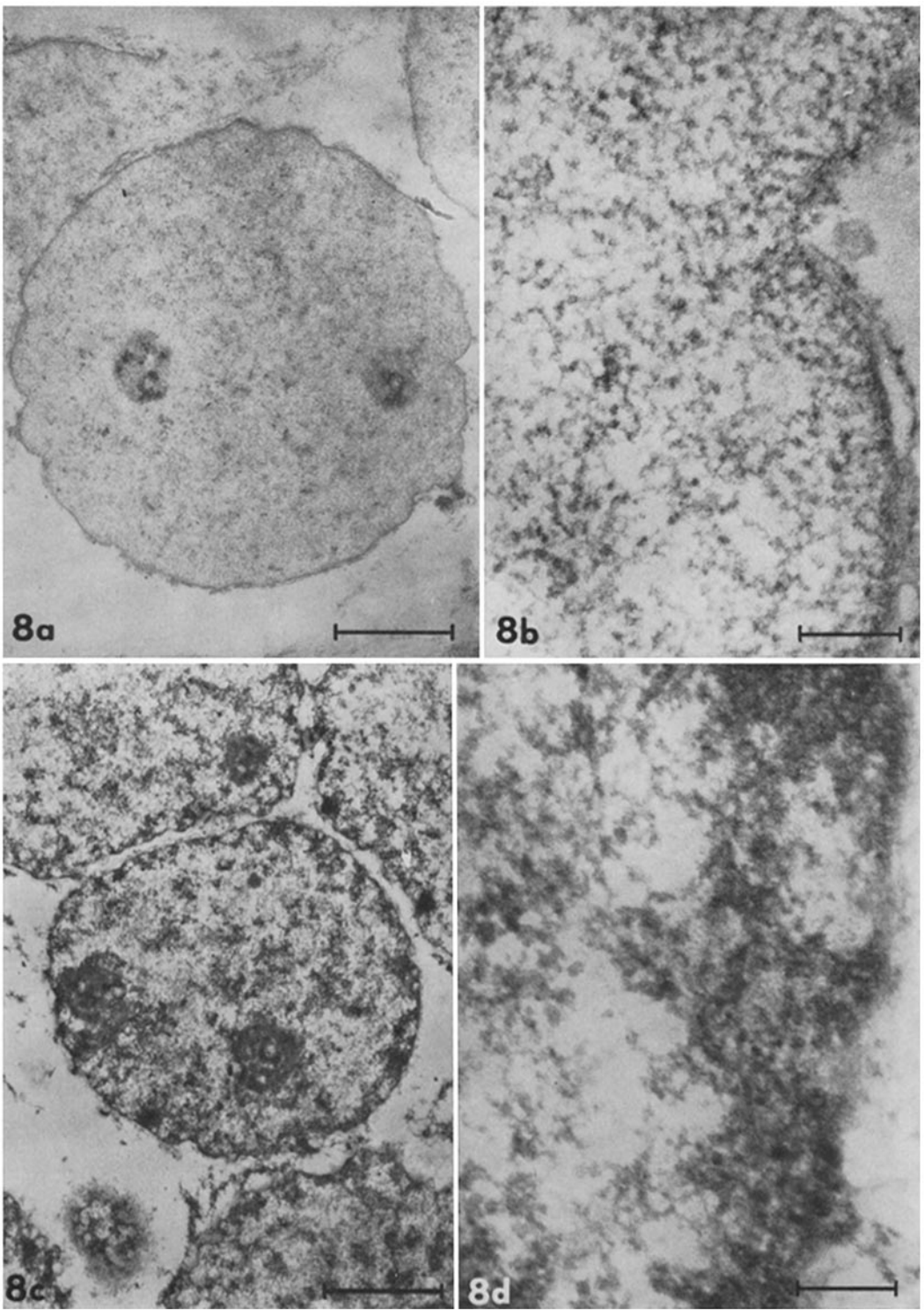


FIGURE 8 Electron micrographs of isolated rat liver nuclei. (*a*) and (*b*), fixed in 0.02 M KCl, pH 7.5; (*c*) and (*d*), fixed in 0.20 M KCl, pH 7.5. Magnification. (*a*) and (*c*), bar denotes 2 $\mu$. × 8900; (*b*) and (*d*), bar denotes 0.2 $\mu$. × 74,900.

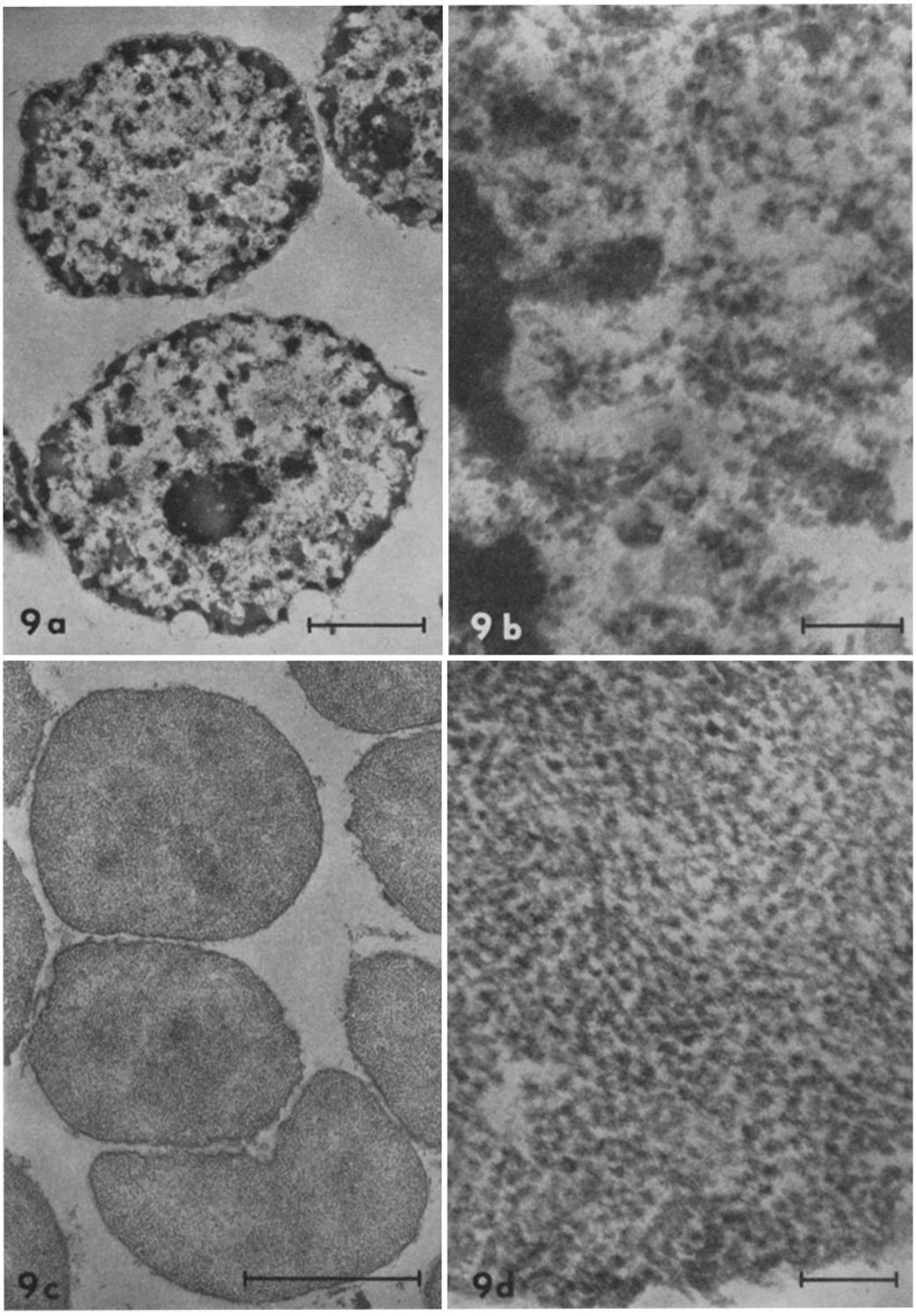


FIGURE 9 Electron micrographs of isolated nuclei. (*a*) and (*b*), rat liver nuclei, fixed in 5 mM $MgCl_2$, pH 7.5; (*c*) and (*d*), chicken erythrocyte nuclei, fixed in 0.02 M KCl, pH 7.5. Magnification: (*a*) and (*c*), bar denotes 2 $\mu$; (*a*) × 8900, (*c*) × 13,400. (*b*) and (*d*), bar denotes 0.2 $\mu$. × 74,900.

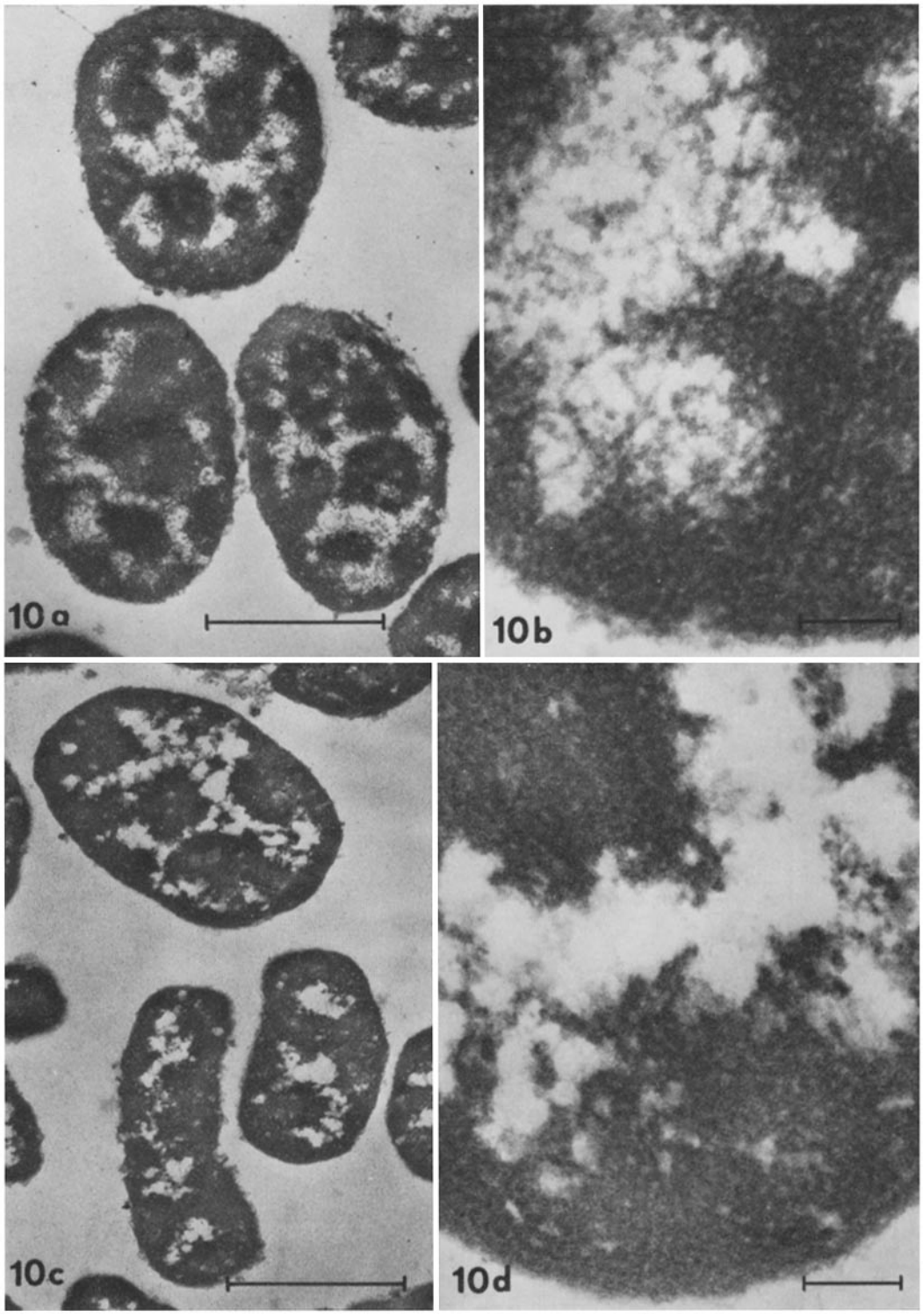


FIGURE 10 Electron micrographs of isolated chicken erythrocyte nuclei. (*a*) and (*b*), fixed in 0.20 M KCl, pH 7.5; (*c*) and (*d*), fixed in 5 mM $MgCl_2$, pH 7.5. Magnification: (*a*) and (*c*), bar denotes 2 $\mu$; × 13,400; (*b*) and (*d*), bar denotes 0.2 $\mu$; × 74,900.

TABLE III

*Widths of Chromatin Threads (A)*

| Nuclei | Solvent | Chromatin* | |
|---|---|---|---|
| | | Dispersed | Condensed |
| Rat liver | 0.02 M KCl | 100 ± 40 | — |
| | 0.20 M KCl | 125 ± 60 | 160 ± 50 |
| | 5 mM $MgCl_2$ | 130 ± 45 | ‡ |
| Chicken erythrocyte | 0.02 M KCl | 140 ± 60 | — |
| | 0.20 M KCl | 140 ± 50 | 170 ± 45 |
| | 5 mM $MgCl_2$ | ‡ | 190 ± 45 |

The data are expressed in angstroms ± one standard deviation.

* "Dispersed chromatin" describes regions of the nucleus where chromatin threads are widely separated from one another; in "condensed chromatin" the fibers are more closely packed. In homogeneous swollen nuclei (0.02 M KCl) all of the chromatin appears dispersed, whereas in granular or slightly granular nuclei (i.e., in 5 mM $MgCl_2$ and 0.20 M KCl) most of the chromatin appears condensed.

‡ Too difficult to visualize chromatin threads. Accurate measurements were not possible for these samples.

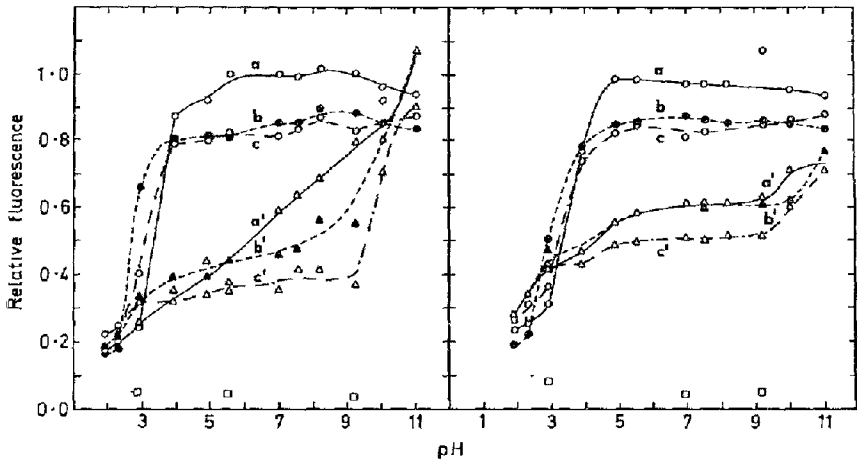


FIGURE 11 Ethidium bromide binding by DNA and nuclei: (A) left frame, rat liver nuclei; (B) right frame, chicken erythrocyte nuclei. Relative fluorescence, normalized to complex of ethidium bromide and calf thymus DNA, 0.02 M KCl, pH 7.5, assigned a value of 1.0. Curves: *a*, *b*, and *c*, calf thymus DNA in 0.02 M KCl, 0.20 M KCl and 5 mM $MgCl_2$, respectively; *a'*, *b'* and *c'*, isolated nuclei in 0.02 M KCl, 0.20 M KCl and 5 mM $MgCl_2$, respectively; dotted line, solvent levels of fluorescence.

the binding of ethidium bromide to isolated nuclei and to native calf thymus DNA as a function of pH and cation concentration (Fig. 11). Our results with native DNA closely parallel those obtained by LePecq and Paoletti (1967, see their Fig. 6). From pH 4 to 11, in 0.02 M KCl, 0.20 M KCl, or 5 mM $MgCl_2$, the relative fluorescence of bound ethidium bromide is essentially constant, although the plateau level of fluorescence is ~ 20% less for DNA in the latter two concentrations of cation. Below pH 4, relative fluorescence (and assumed binding) decreases sharply, presumably reflecting acid denaturation of native DNA. Isolated rat liver and chicken erythrocyte nuclei (with the exception of rat liver nuclei in 0.02 M KCl containing buffers) display a plateau of binding from pH ~ 4 to ~ 9, with levels of binding 30–60% that of naked native DNA. Below pH ~ 4, nuclear binding of ethidium bromide decreases, probably due to acid denaturation of DNA. Between pH ~ 9 and pH ~ 11, nuclear binding of ethidium bromide markedly increased toward the level of fluorescence of naked DNA. This increased binding at alkaline pH conceivably represents

deprotonation of histone lysyl groups, reduced neutralization of DNA phosphates, and intercalation of ethidium bromide into these "loosened" regions. Phase-contrast microscope observations of rat liver nuclei in similar buffers to those used in the dye titration revealed that: (*a*) in 5 mM $MgCl_2$, nuclei were granular up to pH 9 20, and homogeneous at pH 10 00 and 11 00; (*b*) in 0.20 M KCl, nuclei were granular up to pH 5.00, slightly granular to pH 7.50, and homogeneous at pH 8.50, 9 20, 10 00, and 11 00, (*c*) in 0 02 M KCl, nuclei were granular to pH 5 00, and homogenous at higher pH. The present results suggest that no systematically large difference in binding capacity can be observed between condensed and dispersed chromatin. The only exceptions to such a generalization are the increased binding in alkali, which is tentatively ascribed to weakened DNA-histone interaction, and the almost linear increase in fluorescence, as a function of pH, of rat liver nuclei in 0.02 M KCl.

## DISCUSSION

The present study demonstrates that isolated eucaryotic nuclei respond to changes in solvent conditions (i e , pH and cation concentrations) in a highly reproducible manner, as observed by a variety of physical methods. These changes in condensation of chromatin have been represented as phase diagrams relating solvent conditions to nuclear morphology, and used to define standard solvents. The use of a phase map representation must be considered an analogy to thermodynamic maps until detailed data on the reversibility of chromatin condensations are available. Employing standard solvents, such as 0 02 M KCl, 0 20 M KCl, or 5 mM $MgCl_2$, pH 7 5, we have observed absorption and circular dichroism differences that correlate with nuclear morphology: in addition to light-scattering effects, granular nuclei reveal considerable flattening of absorption; homogeneous nuclei exhibit virtually no flattening. On the other hand, spacings observed by low-angle X-ray diffraction, the diameters of chromatin threads revealed by electron microscopy, and capacity for the binding of ethidium bromide reveal no striking changes in different states of chromatin condensation. Thus the phase map representation correlates with several, but not all, physical parameters.

Isolated nuclei, swollen in low ionic strength (0.02 M KCl) at neutral pH, show considerable resemblance to purified, sheared, soluble nucleohistone Circular dichroic spectra are similar, particularly the 30–50% decrease in positive absorbance at $\lambda \sim 275$–285 nm, observed here for homogeneous rat liver nuclei, and for soluble nucleohistone (Permogorov et al , 1970; Shih and Fasman, 1970; Simpson and Sober, 1970, Henson and Walker, 1970) This similarity in spectral properties is even more striking when one considers the 1000-fold difference in DNA concentration between swollen nuclei ($\sim$ 30–50 mg DNA/ml) and solutions of nucleohistone ($\sim$ 0.05 mg DNA/ml) employed for spectral measurements (e. g , see Simpson and Sober, 1970). Both swollen nuclei and nucleohistone gels (Luzzati and Nicolaieff, 1959, 1963, Garrett, 1971) and fibers (Wilkins, 1964, Pardon, 1966, Pardon et al , 1967) reveal the series of low-angle X-ray reflections ( $\sim$ 110, 55, 38 A) attributed to a supercoiled nucleohistone filament (Wilkins, 1964; Pardon, 1966, Pardon et al , 1967). Similar dye-binding capacity, suggesting $\sim$ 50% unneutralized DNA phosphates, is observed for nuclei and for soluble nucleohistone (Klein and Szirmai, 1963, Miura and Ohba, 1967; Ringertz and Bolund, 1969, Bolund, 1970; Borisova and Minyat, 1970; Itzhaki, 1970, 1971; Kleiman and Huang, 1971, Clark and Felsenfeld, 1971). Thus, it appears that, excluding properties such as nucleohistone fiber length and degree of cross-linking of nucleohistone fibers, extensively sheared and purified nucleohistone (Zubay and Doty, 1959) retains a considerable degree of the local structure and, with proper solvent conditions (Garrett, 1971), a considerable degree of the long-range periodic structure observed in swollen intact nuclei.

It should be mentioned that the characteristic circular dichroic spectra of soluble nucleohistone and of swollen nuclei have considerable resemblance, at $\lambda > 245$ nm, to spectra observed for native DNA in high concentrations of ethylene glycol (Green and Mahler, 1968, 1970, 1971; Nelson and Johnson, 1970), in concentrated salt solutions (Tunis and Hearst, 1968), and on unoriented films at defined relative humidities (Tunis-Schneider and Maestre, 1970) The latter authors were able to establish a correlation between this characteristic CD spectrum (i e , a decreased Cotton effect at $\sim$275 nm) and conditions (lithium DNA at 75% relative humidity) favoring the C form of DNA (Marvin et al , 1961). Probably the principal argument favoring the B form for DNA in soluble nucleohistone, and not the A or C forms, derives

from the recent wide-angle X-ray diffraction studies of Bram (1971) Clearly, other types of absorption flattening effects must be considered before an argument based only upon circular dichroism measurements can be considered convincing.

The exact relationship between the dimensions of nucleoprotein filaments obtained by ultrastructural studies and those deduced from X-ray diffraction experiments is, unfortunately, not adequately understood. Davies (1968) has suggested that the unit threads (~170 A wide) observed in electron micrographs of thin sections of nuclei might represent views of the proposed superhelix (100 A diameter, 120 A pitch) of Pardon et al. (1967). Recently, Lampert (1971) has argued that the 250 A nucleoprotein thread, observed with spread chromosomes, is a consequence of a coiling of the superhelix, and that the threads observed with thin sections of nuclei are thinner due to shrinkage during fixation and embedding Against these views, based upon the superhelix of Pardon et al., are the propositions of Bram and Ris (1971), that nucleohistone is coiled into a nonuniform helix (60 A diameter, 45 A pitch), and that this helix corresponds to the fibrils of 100 A diameter observed in electron micrographs of nucleohistone solutions dried by the critical point method. According to Ris (1968, 1969), this 100 A fibril is additionally folded, in the presence of divalent cations, to yield the 250 A thread mentioned above. Bram and Ris (1971) further suggest that the 110 and 55 A low-angle X-ray reflections represent spacing between nucleohistone fibers, and, by this, imply that they should be concentration dependent. The fact that the 110 A reflection disappears upon drying of nucleohistone gels (Luzzati and Nicolaieff, 1959, 1963) and fibers (Pardon, 1966) is well documented. But in no study that we are aware of has a shift in the spacing of this reflection been observed, analogous to the concentration-dependent interparticle spacing observed with F-actin (Spencer, 1969) and ribosomes (Venable et al., 1970) We are, therefore, inclined to the view that the 110 and 55 A spacings represent an integral part of the coiled nucleohistone and are not interfibril distances Our data do not permit a decision on the relationship between the 100–200 A threads observed in electron micrographs of thin sections of nuclei and the postulated superhelix of Pardon et al. On occasion, lateral views of nucleohistone threads have been observed showing periodicities of ~300 A, perhaps related to the coiled superhelix model suggested by Lampert (1971).

On the basis of the present data, one can suggest a simplified model for chromatin condensation within the interphase nucleus:

$$\text{condensed} \rightarrow \text{dispersed}_1 \rightarrow \text{dispersed}_2$$

Both the condensed and dispersed$_1$ states would possess superhelical nucleohistone (100 A diameter, 120 A pitch) and 100–200 A wide nucleohistone threads. These two states represent conditions studied in the present paper (i.e., the condensed state corresponds to granular nuclei; the dispersed$_1$ state, to homogeneous nuclei) The degree of chromatin condensation is determined by the concentration of divalent and monovalent cations, and the structural change would involve alterations in the close packing of superhelical nucleoprotein fibers The postulated dispersed$_2$ state would account for the electron microscope data of Brasch et al. (1971), and could correspond to the nucleohistone coil proposed by Bram and Ris (1971). This state would describe nucleohistone fibers thinner than the superhelix of Pardon et al Dispersed$_2$ state should be present in solvents of very low divalent and monovalent cation concentration. The relative contributions of metal binding and of electrostatic interaction to stability of the superhelix have just begun to be studied. Garrett (1971) has shown that increasing the concentration of calf thymus nucleohistone gels (to ~150 mg nucleohistone/ml) or their salt concentration (to 150 mM sodium phosphate) did not generate the 110- and 55-A reflections, whereas 1 mM $CaCl_2$ or Mg acetate did promote these low-angle X-ray reflections. In our studies, we have observed these low-angle reflections with pellets of intact or broken rat liver nuclei suspended in 5mM sodium EDTA, 0 02 M KCl Therefore, chelation of divalent cations is probably not sufficient to totally disrupt the superhelical structure However, the extensive shearing of chromatin in chelating buffers followed by dispersion of the nucleohistone into distilled water, as introduced by Zubay and Doty (1959), would appear to be sufficient conditions for the complete disruption of superhelical nucleohistone. It seems likely to us, therefore, that the relative importance of divalent cation concentration, ionic strength, and nucleohistone gel concentration may differ, depending upon whether one is examining the disruption or the generation of the presumptive superhelical structure We would also mention our observations that, in the absence of added $Mg^{++}$, 0.20 M KCl is effective in the condensation of

chicken erythrocyte nuclear chromatin. The decrease in average fiber widths observed for rat liver nuclei in 0.02 M KCl, pH 7.50 suggests that these nuclei change toward the dispersed$_2$ state more readily than chicken erythrocyte nuclei, which reveal very few thin fibers in 0.02 M KCl. Chromatin condensation might then be visualized as a combination of metal binding and suppression of electrostatic repulsions, possibly strengthened by noncovalent and covalent linkages between separate nucleohistone fibers. Cantor and Hearst (1970) have demonstrated that the addition of $Mg^{++}$ to ion-free metaphase chromosomes provokes the release of 21 protons per bound $Mg^{++}$ Clearly, cooperative effects beyond simple electrostatic neutralization are indicated. Massive structural transitions, probably controlled by electrostatic interactions, have previously been observed for synthetic complexes of DNA and histone (Olins and Olins, 1971)

Due to the similar levels of binding of ethidium bromide, at neutral pH, comparing granular and homogeneous nuclei, we would suggest that unneutralized DNA phosphates are fairly uniformly distributed throughout the chromatin, and that small molecules (e.g , dyes, hormones) and simple cations can quickly equilibrate in the condensed and dispersed phases The observation that condensed chromatin of erythrocyte nuclei contains only 10% as much hemoglobin as does erythrocyte cytoplasm (Small and Davies, 1970) suggests an upper limit of molecular size for diffusion into condensed chromatin One reason for the nuclear swelling observed during activation (Ringertz, 1969) might be to allow free diffusion of macromolecules to chromatin sites

Current evidence on the intranuclear concentrations of mono- and divalent cations (Langendorf et al , 1961) suggests that their concentrations are sufficient to maintain much of the chromatin in a condensed state Nuclear swelling, under such conditions, would seem to require as yet unknown mechanisms which either reduce the level of unbound nuclear cations below the threshold necessary to maintain condensation, or add sufficient fixed negative charges or metal-ion binding sites to condensed chromatin and, thereby, raise its threshold for cation-induced condensation.

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