Fractionation of Rat Liver Chromatins: Effects of Cations, Hepatectomy and Actinomycin D[1] (35111)

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Nuclear chromatin of differentiated interphase cells is present partly in a condensed state and partly in a dispersed state. It is known that RNA transcription (1, 2) and DNA replication (3) occur in diffuse chromatin and not in dense chromatin. Frenster *et al.* (4) fractionated thymic lymphocyte nuclei by differential centrifugation and obtained a diffuse, active chromatin; a dense, inactive chromatin; and a transitional chromatin of intermediate activity; and partially characterized their chemical components.. Harbers *et al.* (5) subsequently applied this method to rat liver nuclei and recovered three similar chromatin fractions, but did not provide data on the metabolic activity or chemical composition of these fractions. This communication describes an improved method for subfractionation of rat liver nuclei which affords considerable resolution of the major chromatin fractions. We also report the metabolic activity and principal chemical components of these fractions and present the effects of physiological cations, hepatectomy, and actinomycin D on the fractionation pattern of chromatin. A brief account of some of this work has appeared (6).

Material and Methods. Liver of adult albino rats was used in these studies. The liver was perfused with 0.9% NaCl, homogenized in 10 vol of cold Solution 1 (0.32 *M* sucrose, 0.0015 *M* $MgCl_2$, and 0.004 *M* potassium phosphate buffer, pH 6.8) and filtered through 4 layers of cheese cloth. A crude nuclear pellet, obtained by spinning at 1000 *g* for 10 min, was suspended in 10 ml of Solution 1, mixed with 45 ml of 2.5 *M* sucrose and centrifuged in a Spinco Model L2 ultracentrifuge for 1 hr at 48,000*g* in an S.W. 25.2 rotor to deposit a purified nuclear pellet. The purified nuclear pellet was suspended in 20 ml of 1 *M* sucrose, allowed to stand for 10 min, centrifuged at 2500*g* for 10 min, resuspended in 10 ml of 0.20 *M* sucrose for another 10 min and then sonicated in a Raytheon Model S-102A Sonic oscillator (50 W, 9 kcps) for 6 sec with the output voltage set at 80 V. Five ml of the nuclear sonicate was layered onto 50 ml of a 0.25–2.5 *M* linear sucrose gradient and centrifuged for 45 min at 64,000*g* in the S.W. 25.2 rotor in the Model L2 ultracentrifuge. All manipulations were at 4°. Three-ml fractions were collected by means of an ISCO Model 270 fraction collector. Proteins were precipitated with 10% trichloroacetic acid (TCA) at 4°, collected on Polyvic filters (Millipore, 0.6 μ), washed with cold 10% TCA followed by ether, nucleic acids were extracted in 3 ml of 0.6 *N* perchloric acid (PCA) for 20 min at 70°, and acid-insoluble protein was removed by filtering. DNA was measured by a modified diphenylamine reaction (7) with calf thymus DNA as a standard. RNA was measured by ultraviolet spectrophotometry. The $OD_{595m\mu}$ of DNA yielded by the diphenylamine reaction was converted to $OD_{260m\mu}$ and the latter value subtracted from the total $OD_{260m\mu}$ of the PCA extract to give the $OD_{260m\mu}$ of RNA. Protein was determined (8) before and after extraction of histones with 0.2 *M* NaCl and 0.2 *N* HCl to give total protein and nonhistone protein, respectively. Histone was calculated by difference. Phospholipid was determined by phosphorus measurements of lipid extracts of TCA-precipitated fractions (9). ^{14}C-Adenine

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and ^{14}C-orotic acid (20-30 mCi/mmole, Nuclear Chicago) were used as RNA precursors. The isotope (10–30 μCi/rat) was injected intrahepatically 5–10 min before sacrifice, and aliquots of the PCA extract were radioassayed.

Results. We found that resuspending the nuclei in 1 *M* sucrose before sonication in 0.2 *M* sucrose protects nucleoli against disruption, thereby avoiding the addition of divalent cation normally used for the preservation of nucleoli which would cause aggregation of diffuse chromatin (see below). The distribution of chromatin in the gradient was markedly sensitive to sonication time. Excessive sonication dispersed dense chromatin, thereby increasing the yield of diffuse chormatin, while inadequate sonication ruptured too few nuclei. When the sonication time was extended from 5 sec to 2 min, the ratio of dense to diffuse chromatin decreased from approximately 5:1 to less than 1:1. The importance of mild conditions for sonication to preserve dense chromatin has been stressed by previous workers (4, 5).

The DNA profile of the gradient showed two broad peaks, a smaller one consisting of diffuse chromatin in the light part of the gradient, and a second larger peak of dense chromatin in the heavy part of the gradient (Fig. 1). The diffuse chromatin appeared in the first 6–8 tubes with a maximum usually in tube no. 4 ($\sim$0.4 *M* sucrose). The dense chromatin occupied the remainder of the gradient, with a well-defined maximum in tube nos. 15 or 16 (1.75–1.9 *M* sucrose). The diffuse chromatin fraction contained 20-25%, and the dense chromatin fraction 75–80% of the total DNA in the gradient. When ^{14}C-orotate or ^{14}C-adenine was used as an RNA precursor, most of the acid-insoluble radioactivity in the gradient, approximately 70% of the total, was associated with the diffuse chromatin; about 30% of the radioactivity occurred in the dense chromatin. The great bulk of this radioactivity, more than 98% of the total, resided in RNA, as judged by its susceptibility to alkaline hydrolysis. The specific activity of RNA (cpm/μg of DNA-P) in the light portion of the gradient rose to a peak

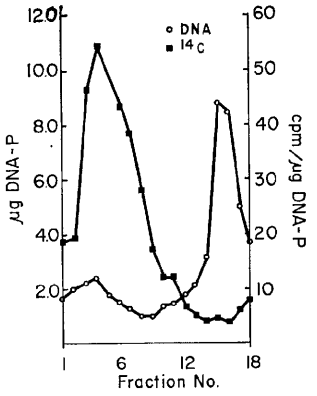

FIG. 1. Fractionation of rat liver nuclei by differential centrifugation of a nuclear sonicate over a 0.25–2.5 *M* linear sucrose gradient. Rats received ^{14}C-orotate 10 min before sacrifice. ^{14}C-Orotate uptake into acid-insoluble fraction (> 98% in RNA) is expressed as counts per minute per microgram of DNA-P. Additional details are given in Materials and Methods.

value in tube nos. 4 or 5 and then declined gradually to reach a broad trough in the gradient containing the dense chromatin (Fig. 1). Presumably the differences in specific activity of RNA in the first 6–8 fractions comprising the diffuse chromatin reflect differences in transcription rates *in situ*. The specific activity of the most active fraction of diffuse chromatin, usually tube no. 4, was about 10–15 times greater than that of the least active fraction of dense chromatin, generally tube no. 14 or 15. A region of the gradient overlapping the diffuse and dense peaks of DNA, generally fractions 7 through 11, exhibited intermediate specific activities and was designated the "transitional" chromatin fraction. The percentage distribution of DNA (mean $\pm$ standard error, $n = 4$), in these chromatin fractions was: diffuse chromatin, 22 $\pm$ 4%; transitional chromatin, 10 $\pm$ 4%; dense chromatin, 68 $\pm$ 5%.

Microscopic examination revealed some contaminating nuclei and nucleoli in the last three tubes of the gradient just above the pellet. Tube nos. 1–16 were without these structures. On electron microscopy the diffuse chromatin peak contained fibrous and granular components in a loose open arrangement; tube nos. 13–15 in the dense chromatin region showed similar material in a compact, highly condensed state (Fig. 2). Preliminary

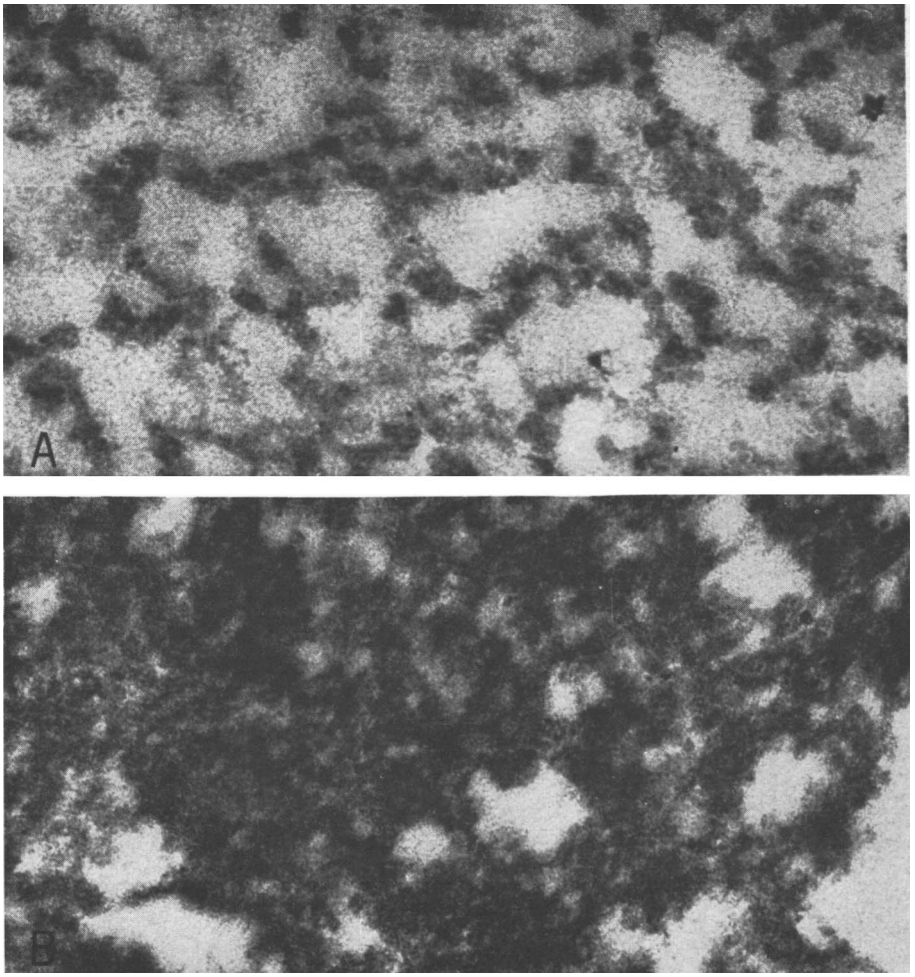

FIG. 2. Electron micrographs of chromatin fractions: (A) diffuse chromatin (B) dense chromatin; ×90,000.

chemical analysis revealed that the diffuse chromatin peak contained 2–3 times more RNA, phospholipid and nonhistone protein per unit of DNA than the dense chromatin, while the histone content was similar. In diffuse chromatin the mean concentrations (mg/mg DNA) were: RNA, 0.7; phospholipid, 0.4; histone, 1.2; and nonhistone protein, 3.4. In dense chromatin the corresponding values were 0.3, 0.2, 1.4, and 1.4, respectively. These results confirm the findings of Frenster *et al.* for calf thymic lymphocytes (4).

Addition of cations to the nuclear sonicate caused noteworthy changes in the sedimentation profile of the chomatin. The divalent cation Mg^{2+} (0.001–0.003 *M*) caused the DNA and acid-insoluble radioactivity of the diffuse chromatin fraction to sediment with the dense chromatin (Fig. 3). Ca^{2+} exerted a similar effect. The total nuclear protein, consisting of histone and nonhistone protein, underwent a similar translocation in the gradient. The altered sedimentation of diffuse chromatin seems to be due to aggregation. Coarse chromatin aggregates also appear in unbroken isolated nuclei in the presence of 3 m*M* Ca^{2+} or Mg^{2+}. These findings suggest that RNA and nonhistone protein are associated in a structural complex with nucleohistone in the diffuse chromatin, as it is unlikely that the sedimentation characteristics of these com-

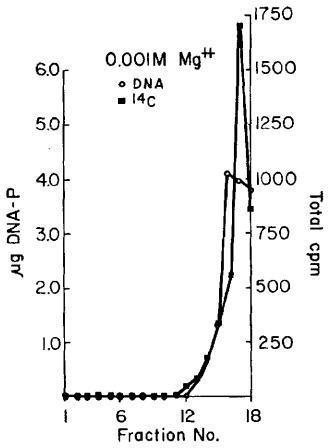

FIG. 3. Effect of Mg^{2+} on the sedimentation profile of chromatin-associated DNA and ^{14}C-labeled RNA in rat liver nuclei. Rats received ^{14}C-orotate 10 min before sacrifice. Mg^{2+} (0.001 *M*) was added to the nuclear sonicate and the latter fractionated as described in legend to Fig. 1.

ponents would be altered to a like degree by divalent cations. The monovalent cations Na^+ and K^+, added to the nuclear sonicate in a concentration of 0.05 *M*, shifted the dense chromatin into lighter regions of the gradient, possibly by causing a dispersion of the dense chromatin. K^+ was more effective in this respect than Na^+ (Fig. 4). These results suggest that the stimulatory effect of monovalent cations on the RNA polymerase reaction in intact nuclei (10) may be partly due to an

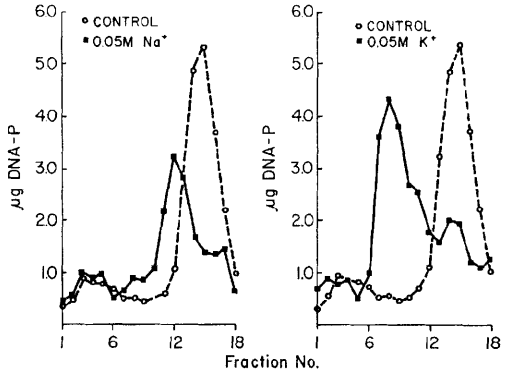

FIG. 4. Effect of Na^+ and K^+ on the sedimentation profile of chromatin-associated DNA in rat liver nuclei. Na^+ (0.05 *M*) or K^+ (0.05 *M*) was added to the nuclear sonicate and the latter fractionated as described in legend to Fig. 1.

alteration in the structure of the chromatin template.

We have applied this procedure for fractionating nuclear chromatin to two experimental treatments in which an alteration in the chromatin profile might be expected, namely hepatectomy and actinomycin D. RNA transcription is increased in regenerating liver (11), partly due to an increase in the number of DNA sites available for RNA transcription (12). We studied regenerating liver 12 hr after partial hepatectomy at a time when DNA replication and cell division have not yet begun (13). In regenerating liver the distribution of DNA in the diffuse chromatin region of the gradient was broadened and polydisperse (Fig. 5). The diffuse chro-

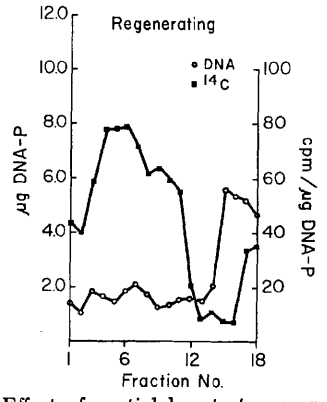

FIG. 5. Effect of partial hepatectomy on the sedimentation profile of chromatin-associated DNA and ^{14}C-labeled RNA in rat liver nuclei. Animals were killed 12 hr after subtotal hepatectomy and 10 min after ^{14}C-orotate injection. Nuclear fractionation was performed as described in legend to Fig. 1.

matin fraction comprised 36 ± 5% ($n = 3$) of the total DNA as compared with 22 ± 4% in the control; the transitional and dense chromatin fractions declined from control values of 10 ± 4% and 68 ± 5% to 4 ± 0.5 and 60 ± 5%, respectively, in regenerating liver. In addition, total incorporation of ^{14}C-orotate into the RNA in the diffuse chromatin increased about threefold. These experiments thus provide direct evidence that activation of the genome in liver regeneration entails a major alteration in the structure and properties of the chromatin involving a conversion of some dense and transitional chro-

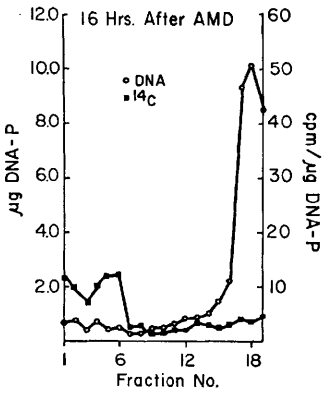

FIG. 6. Effect of actinomycin D on the sedimentation profile of chromatin-associated DNA and ^{14}C-labeled RNA in rat liver nuclei. Animals were killed 16 hr after a single dose of actinomycin D, 1 mg/kg, and 10 min after ^{14}C-orotate injection. Nuclear fractionation was carried out as indicated in legend to Fig. 1.

matin to a diffuse, highly active chromatin.

We also examined the chromatin profile in rat liver when RNA transcription is repressed by administration of actinomycin D (AMD) (14). AMD, given as a single dose of 1 mg/kg intraperitoneally 16 hr before ^{14}C-orotate, inhibited the uptake of ^{14}C-orotate into RNA about 70% from the control. The diffuse chromatin fraction comprised 26% of the total DNA in control liver, and 9% of the total DNA in AMD-treated liver, a reduction of 65% (Fig. 6). In addition, the dense chromatin peak was displaced into the denser region of the gradient. Thus the repression of RNA transcription by AMD seems to be associated with a diminution of diffuse chromatin and a further compaction of the dense chromatin. This finding is in agreement with morphological observations showing that AMD causes a nuclear heterochromatization, *i.e.*, a condensation of nuclear chromatin (15, 16), and is consistent with the hypothesis that AMD represses RNA transcription partly by converting the diffuse active chromatin to a dense inactive chromatin (16, 17).

This procedure is applicable to other tissues as well. We have found that nuclei isolated from cat cerebrum, when fractionated by this method, yield chromatin and radioactivity profiles similar to those obtained from hepatic nuclei (18). This fractionation procedure has recently enabled us to elucidate the binding sites of AMD and neutral red in neural nuclei (18, 19), and should be useful for investigating the intranuclear distribution of drugs, hormones, and other biologically active agents.

Summary. Rat liver chromatin was fractionated by differential centrifugation of nuclear sonicates in a 0.25–2.5 *M* linear sucrose gradient. Diffuse, transitional, and dense chromatin fractions were resolved which contained 22 ± 4, 10 ± 4, and 68 ± 5% of the total DNA, respectively. The diffuse chromatin incorporated RNA precursors *in vivo* 10–15 times more actively, and contained 2–3 times more RNA, phospholipid and nonhistone protein per unit DNA than dense chromatin. Mg^{2+} and Ca^{2+} (0.001–0.003 *M*) caused the diffuse chromatin to sediment with the dense chromatin apparently due to aggregation. Na^{+} and K^{+} (0.05 *M*) rendered the dense inactive chromatin more buoyant, possibly by dispersing it. During liver regeneration (12-hr posthepatectomy), the diffuse chromatin content increased 50% and incorporation of ^{14}C-orotate into its RNA trebled. Actinomycin D (a single dose of 1 mg/kg administered 16 hr before sacrifice) reduced the size of the diffuse chromatin fraction by 65% and the specific activity of its RNA by 70%.

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