THE FRACTIONATION OF ACTIVE AND INACTIVE CHROMATINS FROM ERYTHROID CELLS OF CHICKEN

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1. Introduction

Biochemical studies of cell differentiation in multicellular eukaryotes are often hampered by the heterogeneity of the cell populations. Populations of varying degrees of maturation have been obtained, and the cells have been fractionated with some success according to their state of maturity. In erythroid systems the regenerating blood or marrow of birds recovering from experimental anaemia has been enriched in erythroblasts by centrifugation into gradients or barriers of Ficoll [1] or bovine serum albumin [2,3]. However, these enrichments have not produced substantial amounts of cells still capable of cell division from which reasonably pure chromatin retaining transcriptional potential could be prepared for biochemical examination. We have succeeded in approaching this goal by taking the marrow of chickens recovering from phenylhydrazine-induced anaemia, preparing nuclei relatively free of cell membranes following the technique of Zentgraf et al. [4], removing extranuclear membranes with non ionic detergents, and fractionating the unsheared chromatin by isopycnic centrifugation in density gradients of sucrose and glucose as described by Raynaud and Ohlenbusch [5].

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2. Preparation of intact chromatin

To obtain a density distribution which reflects the constitution of the chromatin it is important to remove contaminating cell membranes. This requires elimination of erythrocyte ghosts, which has been done most effectively by the method of Zentgraf et al. [4], using high-speed blending in the presence of gum arabic. Slight variants of this method have been applied to preparing mature erythrocyte nuclei for ultrastructural studies [6], and reticulocyte chromatin for studies of transcription [7] and of non-histone proteins [8]. We have taken the marrows of chickens rendered anaemic by three successive daily injections of phenylhydrazine (0.3 ml of 2.5% phenylhydrazine per kg body weight [9]), followed by sacrifice of the birds one day after the last injection. Marrows from the chilled legbones were quickly recovered and dispersed in isotonic sucrose (I.S. : 0.294 M sucrose, 1 mM $MgCl_2$, in 1 mM sodium phosphate, pH 6.8) containing 8 mg heparin/100 ml. According to Williams [10] such marrow typically contained 39% erythroblasts, 37% polychromatophilic erythrocytes, 8% reticulocytes and erythrocytes, and 16% non-erythroid cells. After extrusion through cheesecloth, the marrow cells were separated from lipid and plasma by repeated centrifugation for 10 min at 550 *g*. After washing by suspension and centrifugation in I.S., and in S.T.G. (3% gum arabic (w/v) in 0.4 M sucrose, 10 mM Tris, pH 7.0), the chilled cell suspension (in S.T.G. containing a few drops of *n*-octanol) was blended for 30 sec at full speed in a

Virtis 23 homogenizer (Gardiner, N.Y.). This treatment was repeated twice, each time removing and saving the supernatants from the intervening centrifugations and resuspending the pellets in S.T.G. The resultant homogenates, including the saved supernatants, were re-homogenized at half-speed and the crude nuclear pellets collected by centrifugation for 10 min at 2500 *g*; this operation was repeated once and the ultimate pellet was washed by manual suspension in I.S. and recentrifugation. These nuclei appeared largely free of cell ghosts, according to phase microscopy, but intracellular membranes still adhered to the nuclei [6]. These preparations were further treated for 15 min in cold with 0.3% (w/v) saponin in I.S., followed by eight phases of the tight-fitting piston of a Dounce hand-homogenizer (Kontes Glass, Vineland, N.J.); this treatment caused no apparent change in the appearance of nuclear preparations.

To denude the nuclei of cytoplasmic membranes, the chilled suspension in I.S. was rendered 1% (w/v) in Triton X-100 shortly before isopycnic centrifugation. These nuclei appeared to lose their extranuclear membranes [6], swelled slightly and were very susceptible to aggregation if completely pelleted; manipulations which might shear the intact chromatin were avoided subsequently. Prior to the treatment with non-ionic detergent the bulk of the chromatin banded at densities between 1.310 and 1.335 on sucrose–glucose gradients, with only a minor peak at 1.360. After Triton X-100 treatment, all of the chromatin banded above 1.340 with the main peak above 1.362. This is in the same range of density as calf thymus chromatin [5].

3. Density gradient fractionation

The denuded chromatin (i.e. Triton-treated nuclei) was fractionated by centrifugation in sucrose-glucose gradients made as described by Raynaud and Ohlenbusch [5]. The chromatin suspensions in Triton-containing I.S. were diluted with two parts by weight of Dense S. G. (220 g sucrose, dissolved in 112 g TMP (10 mM Tris, 1 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0) before addition of 68 g glucose, and water to maintain 400 g total). This supersaturated solution has a nominal sugar concentration of 75% (w/w), a refractive index of 1.4775 and a nominal density of 1.378 [11]. Light S.G. (nominal sugar concentrations of 65%; refractive index of 1.4531, and nominal density of 1.315) was made by dilution of Dense S. G. with T.M.P.

Each gradient was preformed by mixing 16 g of Dense S. G. and 14 g of Light S. G. at room temperature while flowing slowly into 38 ml cellulose nitrate centrifuge tubes (Beckman, Palo Alto, Cal.) containing a cushion of 7 g of Dense S. G. After cooling to less than 5°C overnight, the gradient was loaded with 12 g of chromatin suspension containing no more than 400 O.D. 260 units, and the tubes were centrifuged for 1000 min at 20 000 rev/min and 3°C in the SW-27 rotor, followed by unbraked deceleration. 1-ml fractions were recovered from the gradient by slow injection of chloral hydrate (100 g/75 ml) through a needle piercing the bottom of the tube. The density of fractions was determined by refractive index [11].

For most other analyses, the fractions were diluted with I.S. and sampled or centrifuged. For ultraviolet absorption spectra, the suspended chromatin was dissolved with prolonged agitation in 4 M sodium chloride.

A typical distribution of anaemic chicken marrow chromatin, as indicated by O.D. 260 vs. sucrose density, is shown in fig.1. Several replicate preparations consistently showed a peak between 1.362 and 1.369 with a bias to the light side. A recentrifuged fraction retained its original banding density. By comparison chromatin prepared from normal chicken blood has a density greater than 1.372.

The ultraviolet absorption spectra, corrected for turbidity, indicated that the protein/DNA ratio of these chromatin fractions decreased gradually from 3.1 at densities below 1.350 to 1.4 at densities near 1.370. That these differences are caused by non-histone chromosomal (NHC) proteins was shown by extracting total chromosomal proteins of each fraction (in 1% SDS–8 M urea–3 M NaCl–1% β-mercaptoethanol–0.01 M phosphate buffer pH 7) and subjecting the extracts to SDS-acrylamide gel electrophoresis [12]. A major band of NHC-proteins (mol. wt. 35 000) overlaps histones H_1 and H_5 (J. Burckard, personal communication). Planimetry of the peaks recorded by densitometry of stained gels (table 1) indicated a decline in the ratio of the larger proteins (NHC-proteins, H_1 and H_5) to the smaller histones

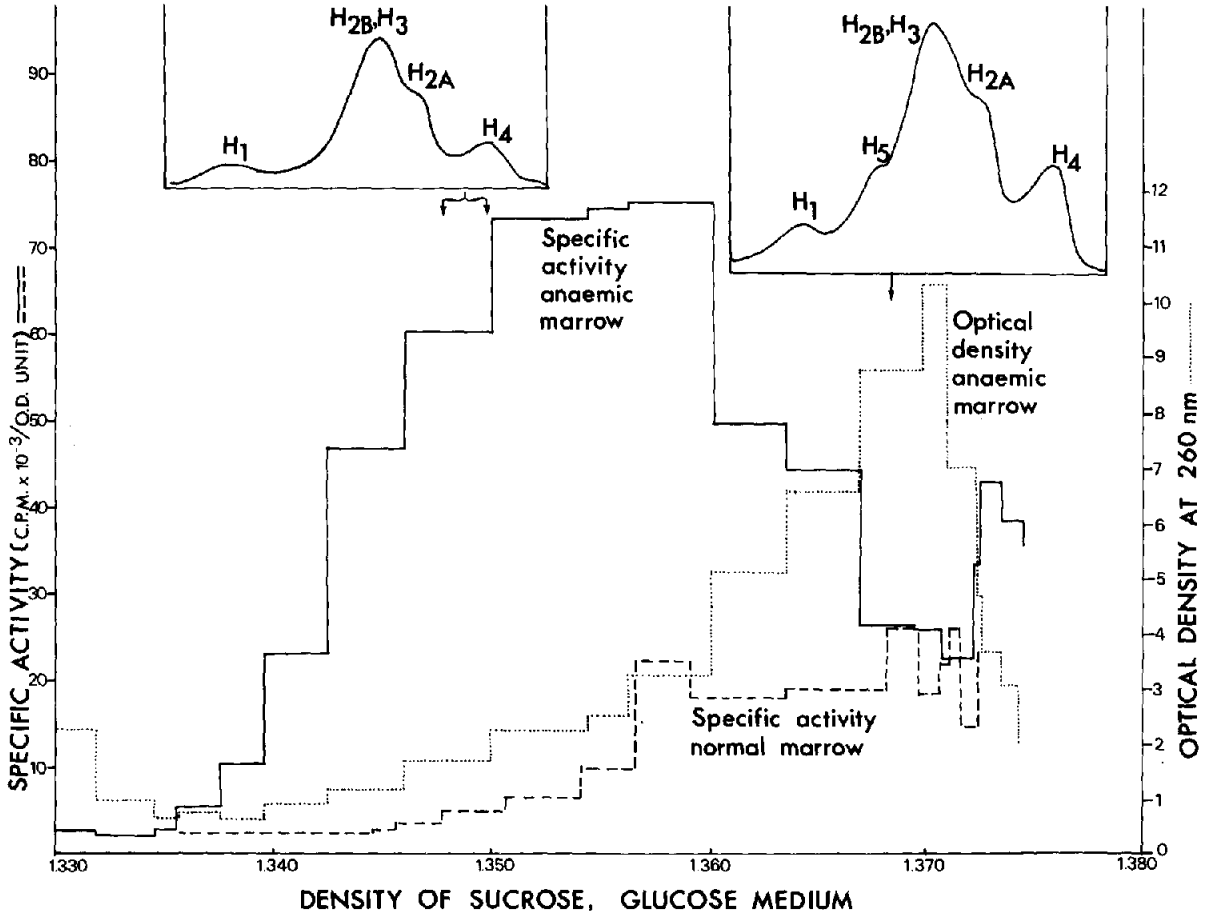

Fig.1. Distribution of unsheared, denuded chromatin from anaemic and normal chicken marrows after isopycnic centrifugation on gradients of sucrose, glucose solutions [5]. Densities of 1-ml fractions were determined by refractive index. The chromatin was located by optical density at 260 nm after dispersion in 4 M NaCl. In the experiment shown, marrow cells from 7 g of fresh marrow were washed and suspended in SWIM's 77 medium containing 10% calf serum (v/v), preincubated at 37°C for 30 min, then incubated for 30 min with 200 μCi of [^{3}H]thymidine with stirring, before recovery of the chromatin as usual. Radioactivity was determined on 0.5 ml aliquots of gradient fractions, diluted with two volumes of isotonic sucrose and added to a 14 ml cocktail containing 87% Aquasol, 5% water, 8% acetic acid, (v/v) before measurement of counts per minute in an Intertechnique SL32 liquid scintillation counter. Quenching was uniform in all fractions. The inset figures indicate the densitometric profiles of acrylamide gel electropherograms in acid-urea of total histones from two chromatin fractions corresponding to the densities indicated by the arrows. The main histone bands are labelled. Relative peak areas of these and other fractions are presented in table 1. (——) Specific activity of anaemic marrow. (- - - -) Specific activity of normal marrow. (· · · ·) Optical density of anaemic marrow.

Table 1

Relative proportions of histones[a] and non-histone chromosomal (NHC) proteins in chromatin fractions[b] from marrow of anaemic chicken

Experiment	Density of sucrose/glucose (g/cc (4°C))	Incorporated [^{3}H]thymidine (d.p.m./O.D. 260)	Ratio, areas of NHC + H_1 + H_5 to H_{2A} + H_{2B} + H_3 + H_4	Ratio, areas of H_1 to H_{2A} + H_{2B} + H_3 + H_4	Ratio, areas of H_5 to H_{2A} + H_{2B} + H_3 of H_4	Ratio of NHC to all histones
M_a-1, M_a-2	1.342 – 1.347	1150	1.3	0.12	0.0	1.0
M_a-1, M_a-2	1.347 – 1.351	1390	1.9	0.16	0.0	1.5
M_a-1, M_a-2	1.356	1150	1.5	0.10	0.12	1.1
M_a-1	1.360	–	–	0.09	0.13	–
M_a-1, M_a-2	1.362	600	1.0	0.11	0.16	0.6
M_a-1	1.364	–	–	0.09	0.14	–
M_a-1, M_a-2	1.367	400	0.9	0.10	0.15	0.5
M_a-2	1.372	400	0.6	–	–	0.3
S_n-3	1.380	0	$\leqslant$ 0.5	0.14	0.41	$\leqslant$ 0.3

[a] The nomenclature recommended at the C.I.B.A. Foundation Symposium on 'Structure and Function of Chromatin' [14] has been used.

[b] After isopycnic centrifugation in sucrose/glucose gradients, fractions of chromatin from anaemic chicken marrow (M_a) were extracted with 0.25 M HCl and the histones were electrophoresed in acid-urea [13] to determine the relative contents of histones H_1, H_5 and H_{2A} + H_{2B} + H_3 + H_4 by planimetry of densitometrically recorded, stained bands (M_a-1); alternatively fractions from anaemic marrow after [^{3}H]thymidine incorporation were extracted with SDS–urea–NaCl–mercaptoethanol and the total proteins were electrophoresed in SDS–urea to determine the relative contents of NHC-proteins + H_1 + H_5 vs. H_{2A} + H_{2B} + H_3 + H_4 (M_a-2); the relative contents of NHC-proteins vs. all histones were calculated from the two series. A control of normal chicken blood chromatin is included (Sn-3) with NHC/histone taken from the litterature [8,15].

(H_{2A}, H_{2B}, H_3 and H_4). When these ratios (which do not represent actual weights, but which should reflect relative differences) are adjusted for the relative content of H_1 and H_5, (determined by planimetry of stained bands after acrylamide gel electrophoresis of histone extracts in acid-urea [13]), two trends are evident (table 1). Firstly, the NHC-protein decreases five-fold as chromatin increases in density from 1.350 to 1.372; in mature erythrocyte chromatin, the small amount of NHC-protein [8,15] is reflected in the highest density, 1.38. Secondly, the content of the erythrocyte-specific histone H_5 is essentially nil in chromatin of density less than 1.351 (table 1 and fig.1, Inset). In denser chromatin the proportions of H_5 and H_1 remain constant, although the NHC-protein content is declining sharply. The relative level of H_5 in marrow chromatin fractions is much less than that in mature erythrocyte chromatin treated similarly (table 1); it seems improbable that this discrepancy could be entirely accounted for by the content of non-erythroid cells in the marrow, because the relative content of H_1 remains low in marrow fractions, even though $H_1/H_{2A} + H_{2B} + H_3 + H_4$ is much greater in leukocytes than in mature erythrocytes [16].

The problem of contamination by leukocytes in the original marrow cell suspension, and therefore of leukocytes chromatin in the fractions, has not been entirely resolved. However, the chromatin from cells regenerating in response to anaemia has been located on the gradients, by incubating marrow cell suspensions from anaemic chickens and from normal chickens with [^{3}H]thymidine at 37°C for 30 min. prior to preparation and fractionation of the chromatin (fig.1). The specific activity (c.p.m. per O.D. 260) of normal marrow chromatin was relatively low and broad, rising to a plateau between densities 1.356 and 1.372. The specific activity of anaemic marrow chromatin was already substantial in light fractions, formed a broad peak between densities 1.350 and 1.360, and fell gradually at higher densities. (This distribution was essentially confirmed in a separate experiment). As a control, it was shown that mature erythrocytes from normal chicken blood, incubated with an aliquot of anaemic marrow cells, incorporated negligible levels of [^{3}H]thymidine into their denser chromatin and thus sharply diluted specific activities of marrow chromatins denser than 1.36. Presumably, the NHC-protein-rich chromatins of densities less than 1.36 represent marrow cells actively responding to the destruction of erythrocytes in the anaemic chicken. The lower levels of incorporation into denser chromatins may represent normal production of non-erythroid cells in the marrow, which are little affected by anaemia.

Characterization of the chromatin fractions of various densities is continuing at the biochemical and microscopic levels to determine transcriptional capacities and compactness of chromatin fibrils. Meanwhile, it appears to us that the fractionation of essentially unsheared, denuded erythroid cell chromatin provides a source of two valuable stages in erythrocyte chromatin maturation: chromatin which contains twice as much NHC-protein as histone, and no detectable erythrocyte-specific H_5, but which responds to anaemia by active thymidine incorporation; chromatin which contains less NHC-protein and significant levels of H_5, but which still incorporates thymidine. These two fractions should be of value in assessing the role of first synthesis of H_5 and its later modification in the declining activities of maturing erythroid cells.

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