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A rapid method for the fractionation of avian blood cell nuclei[1]

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Summary. Red blood cell nuclei from avian anaemic blood have been fractionated by rate sedimentation on discontinuous sucrose gradients into fractions which can be distinguished both by their RNA synthesizing ability and by their morphology as revealed by electron microscopy.

Anaemic avian blood contains both mature erythrocytes and a variable fraction of predominantly late polychromatic erythrocytes[2,3]. Avian red blood cell fractions, corresponding to different stages of development have been isolated by several workers[2-5] making use either of sedimentation in a density gradient or by separating layers from a packed cell mass after centrifugation. For subsequent preparation of nuclei a lengthy cell fractionation procedure may give rise to artefacts. A different approach is described here viz. nuclear isolation and subsequent fractionation which leads to a rapid method to prepare nuclei of distinct activity and morphology.

Materials and methods. Anaemic blood was obtained from White Leghorn cocks after phenylhydrazine injection as described before[6]. All preparations were carried out at 0-4 °C unless otherwise stated. Blood cells were washed 3 times by centrifugation at 10,000 × g for 2 min in 10 vol. 0.12 M NaCl containing 5 mM KCl and 5 mM $MgCl_2$. The buffy coat was removed taking care to remove as few red cells as possible. Cells were lysed at 0 °C for 30 min in 10 vol. 0.05% (w/v) Saponin (Merck, white pure) made up in a 0.25 M sucrose, 0.12 M NaCl, 1.5 mM $MgCl_2$, 15 mM Tris-HCl, pH 7.4 buffer (buffer A). A nuclear pellet was obtained from this suspension by centrifugation at 3000 × g for 5 min. The nuclei were washed with buffer A by centrifugation as above. 2 types of discontinuous sucrose gradients were prepared as follows:

Gradient A: Equal quantities of sucrose solutions (2.0/1.9/1.85/1.8/1.75/1.7 M sucrose in buffer A) were layered into the 34-ml-capacity tube of the Beckman SW 25.1 rotor. Nuclei ($2-5 \times 10^8$) were layered carefully onto this gradient and spun at 2500 × g for 30 min. 4 fractions, each comprising nuclei at an interface and the sucrose layer above it, were collected with a total recovery of nuclei of 91%. No nuclei were recovered from the 1.70/1.75 M interface.

Gradient B: Equal quantities of 2.0/1.75/1.5 M sucrose solutions were layered into the 250-ml-capacity tubes of the Sorvall HS-4 rotor, $2-5 \times 10^9$ nuclei were layered onto the gradient and spun at 2500 × g for 15 min. 2 fractions comprising 68% of the total nuclei, were collected from the 2 sucrose interfaces. The remaining nuclei could be recovered from the 1.75 M sucrose layer (19%), sample layer (6%) and walls of the tube (7%). The above fractions were collected by syringe or Pasteur pipette, suspended in 5 vol. buffer A, spun at 8000 × g for 15 min and the pellet finally resuspended in buffer A. Nuclei were counted in the improved Neubauer haemocytometer. RNA synthesizing activity of nuclei was determined essentially as described before[6] (incubation with 5 μCi [^{3}H]-UTP using $40-60 \times 10^6$ nuclei and 0.05 M ammonium sulphate and 0.05 M Tris-HCl pH 7.4). The reaction was terminated by the addition of an equal volume of 10% (w/v) trichloroacetic acid and the sample prepared for counting as described before[6].

Results and discussion. An initial study of the size of nuclei in stained whole cell preparations of anaemic blood showed appreciable differences between mature erythrocytes and cells with basophilic granules typical of reticulocytes. Using the formula given by Austoker et al.[7] the mean nuclear volume of stained mature erythrocyte nuclei and

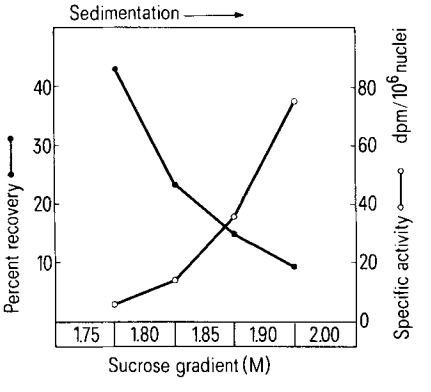

Fig. 1. Fractionation of avian anaemic red blood cell nuclei on a discontinuous sucrose gradient. RNA synthesizing activity and recovery in 4 fractions separated on gradient A are shown. The major part of each fraction was recovered from the interfaces as indicated. Recovery is expressed as percentage of nuclei applied to gradient. Specific activity is expressed as incorporation of [^{3}H]-UTP into RNA per 10^6 nuclei. Incubation conditions were as given in text except that ammonium sulphate concentration was 0.4 M.

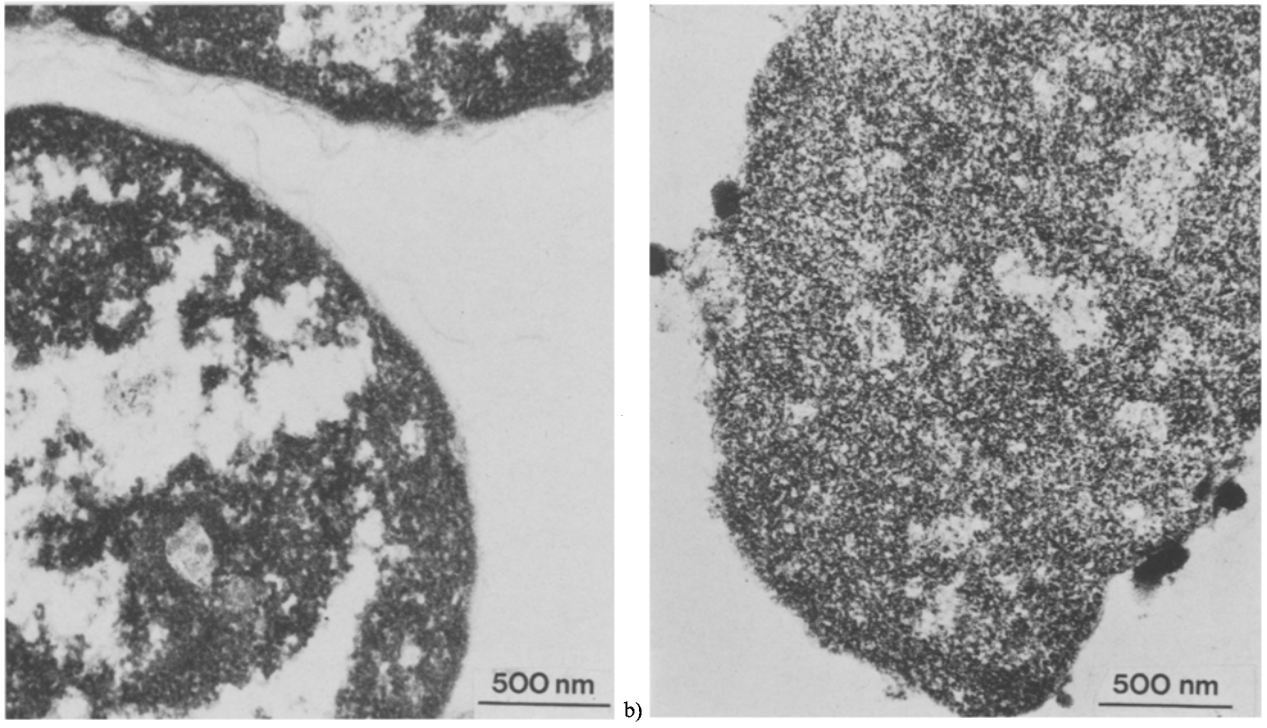

Fig. 2. Electron micrographs of nuclei fractionated on a discontinuous sucrose gradient (gradient B) illustrate the 2 classes to which nuclei were assigned. *a* 1.5/1.75 M interface: condensed chromatin. *b* 1.75/2.0 M interface: diffuse chromatin. Thin sections of nuclei embedded in Lufts Epon 812 were stained with uranyl acetate and lead citrate.

stained reticulocyte nuclei was found to be $42.5 \pm 3.7\ \mu m^3$ and $65.8 \pm 2.9\ \mu m^3$ respectively.

A comparison of RNA synthesizing activity of nuclei from anaemic blood separated by gradient A is shown in figure 1. For each fraction specific activity and percent recovery are indicated. The most active nuclei with a 13.1-×-increase in specific activity were found in the most rapidly sedimenting and smallest (9.8%) fraction. Different preparations of nuclei showed a variation in proportions and specific activities of fractions, but in each case the above trend was observed. Mathias et al.[5] have found significantly different values for nuclear volume and sedimentation rate of pigeon erythrocyte and reticulocyte nuclei. The long times of preparation and centrifugation required by these authors may explain these differences as it has been shown that the density of the nucleus can vary with the density of the medium after long centrifugation runs[8].

A simplified fractionation procedure (gradient B) demonstrated the correlation between nuclear morphology and RNA synthesizing activity in 2 fractions of nuclei. The ratio of specific activity (RNA synthesis) of the 2 fractions was 3.4 (1.75/2.0 M fraction: 1.5/1.75 M fraction). An examination of nuclei in these 2 fractions by electron microscopy showed a clear enrichment of nuclei with diffuse chromatin (60%) in the faster sedimenting and more active fraction. In contrast, the slower sedimenting less active fraction was composed of 79% nuclei with condensed chromatin (figure 2). Although it is known that the morphological appearance of chicken erythrocyte nuclei is reversibly changed by different ionic conditions and pH[9-11], the comparison here is considered valid as both fractions were isolated and examined under identical conditions. Nuclear RNA synthesis appears similar when either fractionated nuclei are tested for RNA synthesizing capacity or when cells are incubated with subsequent fractionation of nuclei. The ratios of specific activities (RNA synthesis) of nuclei in each fraction after incubation of either nuclei or cells with radioactive precursors were determined to be 3.4 and 3.1 respectively (means of 3 different preparations). These results indicate that differences in nuclear activity in intact cells are retained by nuclei after fractionation. The recovery of nuclei in the 1.75/2.0 M fraction reflected the anaemia of the blood: blood from control animals gave a reticulocyte count of 0–1% and no nuclei were recovered in this fraction; from anaemic blood (reticulocyte count: 19%) 24% of the total nuclei were recovered in this fraction.

These studies show that a rapid fractionation of nuclei with retention of differences in biosynthetic activity and morphology can be achieved. Such a method should allow a more defined study of factors affecting the activity of these nuclei.

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