A Study of Nucleated Erythrocytes by Density-Gradient Centrifugation in a Zonal Rotor

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1. Several substances of high molecular weight were examined for their suitability as suspension media in the formation of density gradients for the zonal centrifugation of avian erythrocytes. None proved satisfactory. 2. The behaviour of pigeon erythrocytes in rate-sedimentation experiments in a type A zonal rotor with density gradients of sucrose was examined. The mature cells sediment more rapidly than the younger cells and have a lower RNA/DNA ratio. Maturation is accompanied by a greater loss of RNA from the nucleus than from the cytoplasm. 3. The base composition of the nuclear RNA and of the two species of cytoplasmic ribosomal RNA is reported. 4. The RNA of erythrocytes may be labelled *in vivo* by injection of inorganic [^{32}P]phosphate. The cells most active in the synthesis of RNA sediment less rapidly than the bulk of the cells. 5. Reticulocyte nuclei sediment more slowly than those from erythrocytes. Reticulocyte nuclei have a mean volume of $35\mu^3$ and are isopycnic with sucrose of density 1.2871 (measured at 20°). Maturation of the nuclei causes them to shrink to a volume of $25\mu^3$ and the density to increase to 1.2944.

The mature erythrocyte is devoid of ribosomes and incapable of protein synthesis. Immediately before this, in the reticulocyte stage, 90% of the proteins synthesized by the cells of the erythrocyte series are the globin chains of haemoglobin (Kruh & Boorsook, 1956). During the development of the reticulocytes from the comparatively unspecialized stem cells or haemocytoblasts, those parts of the genome required for haemoglobin production become selected for function whereas the remainder becomes inactivated or repressed. For these reasons the reticulocyte has attracted considerable attention. In mammals synthesis of RNA ceases with the extrusion of the nucleus after the normoblastic stage is reached. Consequently mammalian reticulocytes cannot be used for studies of its synthesis. Avian reticulocytes retain their nuclei and their RNA may be labelled *in vitro*. Bruns, Fisher & Lowy (1965) demonstrated the incorporation of precursors into both nuclear RNA and cytoplasmic RNA of duck reticulocytes. It appeared that most of the newly synthesized material remained in the nucleus. Scherrer, Marcaud, Zajdela, Breckenbridge & Gros (1966) demonstrated that some renewal of messenger RNA does occur in these immature cells incubated *in vitro*. These authors also found the predominant labelling to be in the nucleus. Attardi, Parnas, Hwang & Attardi (1966) reached similar conclusions. The nuclear RNA from reticulocytes

is the first to be labelled when [^{32}P]phosphate is injected into pigeons made anaemic by administration of phenylhydrazine (Barlow & Mathias, 1968). Label subsequently appears in the cytoplasm.

The analysis, on the one hand, of the synthesis of protein and RNA in avian reticulocytes, and, on the other, of the processes associated with maturation, which involves closure of the genome and the disappearance of the machinery for protein synthesis, is complicated by the fact that a blood sample from an anaemic pigeon will contain, besides young reticulocytes, senescent erythrocytes and cells of all ages between these extremes. Ideally a method is required that will provide a separation of cells on the basis of their age with a continuous gradation from young to old. Glowacki & Millette (1965) demonstrated that rabbit reticulocytes move less rapidly than mature erythrocytes in density gradients of bovine serum albumin. They showed that, as the cells aged, their capacity for protein synthesis and their content of ribosomes declined. Rowley & Morris (1967) used similar methods to fractionate erythrocytes. They noted that the Mg^{2+} concentration in the soluble fraction of the cell fell with maturation. Schulman (1967) achieved a partial separation of rabbit reticulocytes with gradients of Dextran C-60. He found polyvinylpyrrolidone and Ficoll unsatisfactory. Kabat & Attardi (1967) fractionated nucleated duck and chicken erythrocytes according to maturity on

linear density gradients of bovine serum albumin. The cell population from normal birds banded at a higher buoyant density than the population from birds treated with phenylhydrazine.

These experiments, both with the rabbit and with the avian erythrocytes, were conducted in swinging-bucket rotors. Consequently only very small quantities of cells could be analysed. We have sought methods for the fractionation of avian erythrocytes in zonal rotors, which offer higher capacity and better resolution than swinging-bucket rotors.

Other methods that have been employed for the fractionation of erythrocytes include electrophoresis (Stephen, 1940; Danon & Marikovsky, 1961), countercurrent distribution (Walter, Selby & Brake, 1964; Walter, Winge & Selby, 1965; Walter, Selby & Garza, 1967) and stratification of packed cells in a centrifugal field (Rigas & Kohler, 1961).

MATERIALS AND METHODS

Erythrocytes. Adult pigeons weighing 250–350g. were used. The yield of blood is about 10ml./bird, several of which were used in each experiment. The pigeons were killed by decapitation and the blood was collected in 3 vol. of ice-cold 0.15M-NaCl containing 1000 units of heparin/ml. The diluted blood was centrifuged for 5 min. at 1000 rev./min. in the 250ml. head of the MSE Major centrifuge (Measuring and Scientific Equipment Co. Ltd., London, S.W. 1). The supernatant containing the leucocytes and plasma was removed by aspiration and the procedure repeated three times. No leucocytes could be detected by microscopic examination. Pigeon erythrocytes are believed to have a life span of about 12 days (Marvin, 1954).

Reticulocytosis was induced by daily oral administration of neutralized phenylhydrazine hydrochloride [0.1 ml. of a 5% (w/v) solution (adjusted to pH 7)/bird]. For maximum reticulocytosis the administration was continued for 5 days and the birds were killed on the seventh day. The reticulocyte count for normal pigeons varies from 8 to 22% (Willis, 1932).

Media for zonal centrifugation of erythrocytes. Apart from sucrose the substances investigated included partially degraded gelatin (The Gelatine and Glue Research Association, Birmingham, 12), Ficoll (lot no. 8150; Pharmacia, Uppsala, Sweden), Dextran T-40 (lot no. 8687; Pharmacia, Uppsala, Sweden) and egg-white albumin. The latter was obtained by dialysing egg white against several changes of water at 4°, followed by freeze-drying. Experiments were performed with cells from normal birds, because these should define the maximum density to be employed in the gradient. Under iso-osmotic conditions, normal chicken erythrocytes band at a density of 1.090 (Kabat & Attardi, 1967). A density range 1.06–1.12 was used. The materials were dissolved normally in 0.15M-NaCl, although salt solutions above and below this concentration were investigated.

Fractionation of pigeon erythrocytes by zonal centrifugation. Centrifugation was carried out at 5° in a type A zonal rotor run in a Mistral centrifuge (Measuring and Scientific Equipment Co. Ltd.). The density gradient (1000 ml.) was 20–50% (w/w) A.R. sucrose in 1 mM-tris-HCl buffer, pH 7.2,

and linear with respect to volume. It was pumped into the rotor while it was spinning at 600 rev./min. at a rate of 50 ml./min. It was followed by an underlay of 55% (w/w) sucrose. Washed erythrocytes (1–1.5 ml.) were suspended in 30 ml. of 15% (w/w) sucrose and layered on to the gradient, followed by an overlay of 10% (w/w) sucrose, which was pumped in until the centre of the zone of cells had reached a distance of 6 cm. from the axis of rotation. Centrifugation was carried out for 1 hr. at 600 rev./min. The rotor contents were displaced at a flow rate of 50 ml./min. and the turbidity was monitored at 600 m μ in a flow cell (path length 1 cm.) in a Gilford 2000 extinction meter. The turbidity is a linear function of the concentration of cells up to E_{600} 0.5. Readings greater than this may be corrected from a calibration curve. The effluent was collected in fractions (normally 40 ml.). After this treatment the cells appear somewhat distorted, but are intact. The cells were recovered by dilution of the fractions either singly or in suitable groups with an equal volume of 0.25M-sucrose followed by centrifugation at 700g for 30 min. The recovered cells tend to lyse when resuspended in saline.

Labelling of pigeon erythrocytes in vivo. Carrier-free [^{32} P]orthophosphate in dil. HCl (20–60 c/mg. of P; The Radiochemical Centre, Amersham, Bucks.) was neutralized by the addition of a slight excess of NaHCO₃ and made 0.2M with respect to NaCl. Two pigeons each received 500 μ Ci (in a volume of 0.5 ml.) by direct injection into the heart. The birds were lightly anaesthetized with ether. They were killed 10 hr. after injection. The erythrocytes were collected in the usual manner and examined by zonal centrifugation. The radioactivity in the fractions recovered from the gradients was determined by adding to each fraction 0.5 vol. of 0.6N-HClO₄. After 15 min. at 0° the precipitate was collected on disks of Whatman 3MM paper by filtration. The disks were washed with 0.2N-HClO₄ and dried. They were placed in vials containing 15 ml. of 4% 2,5-bis-(5-tert.-butylbenzoxazol-2-yl)thiophen (Ciba Ltd., Basle, Switzerland) and counted in a Nuclear-Chicago scintillation counter. Less than 3% of the radioactivity could be removed from the paper disks by extraction with methanol-chloroform (2:1, v/v), showing that very little incorporation had occurred into phospholipid. Approx. 90% of the radioactivity was rendered acid-soluble by treating the disks with 0.3N-KOH (5 ml.) at 37° for 16 hr. After neutralization with 1N-HCl the precipitate was removed by filtration. The radioactivity in the filtrate, which contains hydrolysed RNA, was determined by drying suitable samples on to fresh paper disks. The concentration of RNA was determined spectrophotometrically, allowance being made for contamination by protein.

Preparation of nuclei. Washed packed erythrocytes were chilled to –30° for 1–2 hr. and then thawed in 2 vol. of 0.32M-sucrose containing MgCl₂ (3 mM), adjusted to pH 7.5 with NaHCO₃ (medium A). The lysate was centrifuged at 700g for 10 min. The pellet was resuspended in 2 vol. of lysing medium and the centrifugation repeated. The combined supernatants formed the cytoplasmic fraction. The nuclear pellet was purified by resuspension in 4 vol. of 2.4M-sucrose containing MgCl₂ (1 mM), pH 7.5, and centrifuged for 90 min. at 25000 rev./min. at 4° in the Spinco SW 25.1 rotor.

Examination of nuclei by zonal centrifugation. The pigeon erythrocyte nuclei (from about 5 ml. of packed cells) were resuspended in 20 ml. of 15% (w/w) sucrose, containing

MgCl₂ (1 mM), pH 7.5. The suspension of nuclei was examined by the method of Johnston, Mathias, Pennington & Ridge (1968) in a type A zonal rotor at 5°. The duration of centrifugation was extended to 4 hr. at 600 rev./min. The contents of the rotor were then displaced, monitored at 600 mμ and collected in 50 ml. fractions. Nuclei were recovered from the fractions after dilution with an equal volume of medium A by centrifugation at 700 g for 15 min. The nuclei were resuspended in medium A. The major and minor axes of 50 nuclei from each fraction were measured with a Wild (Heerbrug, Switzerland) M20 phase-contrast microscope fitted with a micrometer eyepiece (Baker Ltd., London) at an overall magnification of 1000. The volumes of the nuclei were calculated from the formula for a prolate spheroid $v = ab^2/6$, where a and b are the lengths of the major and minor axes.

Isopycnic centrifugation of nuclei. Washed purified nuclei (0.3–0.5 ml.) from erythrocytes or reticulocytes were suspended in 10 ml. of medium A and the sample was loaded on the type A rotor at a speed of 1100 rev./min. at 5°. The gradient (1000 ml.) of sucrose was linear with volume and from 53 to 63% (w/w), containing MgCl₂ (1 mM) and adjusted to pH 7.2. The nuclei were centrifuged at 3200 rev./min. for 10 hr., by which time they had formed sharp immobile zones. The rotor was decelerated to 1500 rev./min. and the contents were displaced and collected in 40 ml. fractions at a flow rate of 14 ml./min. The refractive index of the collected fractions was determined in an Abbe refractometer with an expanded scale (Bellingham and Stanley Ltd., London, N. 15) and the densities at 20° were determined from Tables of physical constants. Microscopic examination of the nuclei in the equilibrium position showed that most of them were aggregated.

Extraction of RNA. Nuclear RNA was prepared by the method of Barlow & Mathias (1968), with three successive extractions at 60° with phenol.

Cytoplasmic RNA was prepared from ribosomes. These were obtained by centrifugation of the cytoplasmic fraction from the preparation of nuclei at 100 000 g for 1 hr., or by centrifugation at 10 000 g for 10 min. followed by acidification to pH 6.0 with acetic acid and sedimentation of the precipitate at 1800 g for 20 min. (Arnstein, Cox, Gould & Potter, 1963). The ribosomes were purified by resuspension in 10% (w/w) sucrose containing MgCl₂ (1.5 mM) and KCl (50 mM) in 1 mM-tris-HCl buffer, pH 7.6, and centrifugation through an equal volume of 20% (w/w) sucrose containing the same salts for 5 hr. at 70 000 g. The RNA was extracted with guanidinium chloride (Cox, 1968).

Ribosomal sub-units were prepared by suspending ribosomes in 5% (w/w) sucrose at a concentration of 4 mg./ml. Immediately before centrifugation they were made 0.1 M with respect to EDTA, pH 7.2. The sub-units were separated on a small scale (2 mg.) by centrifugation in the SW 25.1 rotor for 11 hr. at 24 000 rev./min. (60 000 g) in a density gradient of 10–25% (w/v) sucrose in 0.1 M-tris-HCl buffer, pH 7.2. Large-scale separation was performed in a B4 zonal rotor by Mr E. S. Klucis. The sub-units were recovered by making appropriate fractions 10 mm with respect to MgCl₂ and 0.1 M with respect to sodium acetate, followed by centrifugation at 10 000 g for 10 min.

Determination of nucleic acids. The cells were lysed by the addition of 20 vol. of ice-cold water, and RNA and DNA were determined by the procedure of Munro & Fleck (1966) including the acid hydrolysis for the extraction of DNA.

RNA was determined from E_{260} assuming $E_{1\text{cm}}^{1\%}$, 300 or by the orcinol reaction. DNA was determined by the method of Burton (1956). For the determination of nuclear and cytoplasmic nucleic acids in fractions recovered from zonal centrifugation the cells were frozen and then lysed in 3 ml. of 0.25 M-sucrose containing MgCl₂ (3 mM) and KCl (25 mM) in 10 mM-tris-HCl buffer, pH 7.5. After centrifugation at 700 g for 10 min., the supernatant was separated. The nuclei were resuspended in 2 ml. of the sucrose solution and the centrifugation was repeated. The combined supernatants formed the cytoplasmic fraction.

Determination of protein. This was carried out by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard.

Determination of base composition. Method 1 was based on that of Katz & Comb (1963). Several batches of Dowex 50W and Zeo-Karb 225 were found to give high background absorption that rendered them unsuitable for automated systems. Accordingly a procedure was developed in which the polystyrene-based ion-exchange resin was replaced by SE-Sephadex (Pharmacia, Uppsala, Sweden). The column was 0.9 cm. × 100 cm. Elution was achieved by pumping 3 mM-HCl through the column at a flow rate of 0.25 ml./min. The effluent was monitored at 254 mμ in the flow cell of an LKB Uvicord II spectrophotometer and collected in fractions. Appropriate fractions were combined and their volumes and extinctions measured. No significant differences in base composition were observed if the extinction coefficients given by Beavan, Holiday & Johnson (1955) were employed in place of those given by Katz & Comb (1963). Method 2 was based on that of Blattner & Erickson (1967). The column was 0.9 cm. × 95 cm. Elution was achieved with 0.25 M-ammonium formate, pH 4.15, at a flow rate of 0.5 ml./min. Extinction coefficients were determined for the mixed 2'- and 3'-phosphates (all from Sigma Chemical Co., St Louis, Mo., U.S.A.) in 0.25 M-ammonium formate by recording spectra on a Cary model 14 recording spectrophotometer. The E_{260} values were: uridine phosphate, 10 000; guanosine phosphate, 11 800; adenosine phosphate, 14 700; cytidine phosphate, 7100.

The samples of RNA were hydrolysed by incubating a solution in 0.3 N-KOH (approx. 5 mg./ml.) for 18 hr. at 37°. The KOH was neutralized with HClO₄ and the precipitated KClO₄ removed by centrifugation.

RESULTS AND DISCUSSION

Zonal fractionation of erythrocytes. The first problem to be solved in attempts to fractionate cells by buoyant-density centrifugation is the choice of material for formation of the density gradient. The medium must have a range of density encompassing that of the cells. Because of the sensitivity of most cells to osmotic effects polymers of high molecular weight have frequently been used, and in particular bovine serum albumin (Lief & Vinograd, 1964). Benedict, Schumaker & Davies (1967) studied the behaviour of bovine and rabbit spermatozoa in Ficoll, Ludox (a colloidal silica solution) and umbradilic acid, and in gradients of a number of substances of low molecular weight. The large volume of type A zonal rotor (1300 ml.) rules out

the use of expensive materials such as bovine serum albumin. We explored a number of other possibilities, including partially degraded gelatin, Ficoll, Dextran T-40 and egg-white albumin.

Gelatin in the concentration range 5–30% (w/v) had a deleterious effect on the cells, causing them to become irregular in outline and to aggregate extensively. Ficoll in the concentration range 20–30% (w/w) (density 1.07–1.11) caused distortion and aggregation of the cells. The degree of aggregation could be decreased but not eliminated by lowering the concentration of sodium chloride. Attempts to mitigate the aggregation by adding citrate, sucrose or bovine serum albumin to the medium were unsuccessful. The effect of dextrans of various molecular weights on the aggregation of washed human erythrocytes was studied by Thoresen & Hint (1950), who concluded that the effectiveness of these colloids in producing aggregation was related to their molecular weight and degree of asymmetry. Aggregation appears to result from the tendency of the colloid to form surface films on the cells (Bloom, Harmer, Bryant & Brewer, 1964) with a consequent decrease in the charge on the cell (Eliason & Samelius-Broburg, 1965). These latter authors noted that the stability of suspensions of erythrocytes in the presence of dextrans of different molecular weights varied from species to species. Accordingly we examined a dextran polymer, Dextran T-40. Although the effect was much less marked than with Ficoll, aggregation was visible under the light-microscope and in small-scale experiments erythrocytes sedi-

mented rapidly through a density gradient. Egg albumin proved unsuitable for the same reasons.

As an alternative to the fractionation of avian erythrocytes by buoyant-density centrifugation, for which there is a dearth of materials suitable for zonal rotors, there is the possibility of separation by rate sedimentation. Polymeric materials, in general, appear to cause aggregation. Sucrose has the advantage that it is physiologically inert, although it has adverse osmotic effects. Preliminary experiments with erythrocytes from untreated pigeons established the conditions required to spread the cells as a band occupying about 250 ml. and centred about 13 cm. from the axis of rotation. This represents a movement of 7 cm. from the starting position (Fig. 1, curve *A*).

We then turned our attention to cells from birds in which the proportion of reticulocytes had been increased above the normal value by treatment with phenylhydrazine. The effects of different periods of exposure to this chemical on the RNA/DNA ratio are shown in Fig. 2. The population, rich in reticulocytes, that results from 5 days of treatment followed by 2 days of recovery has an RNA/DNA ratio six times that found in normal blood. The effects of an increasing degree of reticulocytosis on the profiles of the cell distribution obtained by zonal centrifugation are shown in Fig. 1 (curves *B–E*). The longer the period of exposure to phenylhydrazine the slower the movement of the cells. It

Fig. 1. Zonal profiles of pigeon erythrocytes from birds with different proportions of reticulocytes. Curve *A* (---), blood from normal birds; curve *B* (—), 2 days of phenylhydrazine treatment, killed on third day; curve *C* (-·-·-), 4 days of phenylhydrazine treatment, killed on fifth day; curve *D* (----), 5 days of phenylhydrazine treatment, killed on seventh day; curve *E* (·····), 5 days of phenylhydrazine treatment, killed on tenth day. In this and all other Figures 0 ml. is taken as the start of the gradient.

Fig. 2. Effect of treatment with phenylhydrazine on the RNA/DNA ratio in pigeon erythrocytes. The points indicated by *B*, *C*, *D* and *E* correspond to the treatments described in the legend to Fig. 1.

Fig. 3. RNA/DNA ratio within zonal profiles of pigeon erythrocyte populations with different proportions of reticulocytes. The effluent from each experiment was collected in fractions indicated by the broken vertical lines. (a), (b), (c) and (d) correspond respectively to treatments indicated in Fig. 1 curves B, C, D and E. —, Turbidity; ■—■, RNA/DNA ratio.

may be concluded that the young cells sediment less rapidly than the old cells, and that in each profile there is a gradient of maturity across the zone.

This interpretation is supported by measurements of the RNA/DNA ratio in fractions recovered from different parts of the gradient. The results are summarized in Fig. 3. In each population of cells that we examined the RNA/DNA ratio is highest in the more slowly sedimenting cells. The ratio decreases across each distribution except for cells from birds that had recovered for 5 days after 5 days of treatment (Fig. 3d). The values in this experiment should be compared with those of Fig. 3(c). The peak of the RNA/DNA ratio shifts nearer to the centre of the cell distribution after the 3 additional days of recovery. This may reflect the fact that fewer very young cells are being liberated into the circulation. The total RNA/DNA ratio for each cell distribution agrees well with the values shown in Fig. 2 for unfractionated cells. This demonstrates that there is no substantial loss of RNA from the cells in the dense sucrose solutions.

Distribution of RNA between nucleus and cytoplasm in erythrocyte and reticulocyte populations. It is known that some of the RNA synthesized in the

Fig. 4. Distribution of RNA between the nucleus and cytoplasm in the zonal profile of erythrocytes from normal pigeons. The effluent from the experiment was collected in fractions indicated by the broken vertical lines. —, Turbidity; ■—■, nuclear RNA/cytoplasmic RNA ratio.

nucleus of the avian reticulocyte is never transported to the cytoplasm (Attardi *et al.* 1966; Barlow & Mathias, 1968). Presumably it turns over within the nucleus, a process that might be related to the regulation of gene action (Soeiro, Birnboim & Darnell, 1966). The synthesis of RNA declines as the reticulocytes mature and it was important to ascertain if this was accompanied by a redistribution

Fig. 5. Distribution of RNA between the nucleus and cytoplasm in the zonal profile of erythrocytes from pigeons that had been treated with phenylhydrazine for 5 days followed by 2 days of recovery. The effluent from the experiment was collected in fractions indicated by the broken vertical lines. —, Turbidity; ■—■, nuclear RNA/cytoplasmic RNA ratio.

of RNA between the nucleus and the cytoplasm. A comparison of Figs. 4 and 5 reveals that, whereas the nuclear RNA/cytoplasmic RNA ratio has a more or less constant value of about 1 across the zone of erythrocyte cells, the ratio changes from 2 at the trailing edge of the reticulocyte zone to 1 at the leading edge. This confirms the hypothesis that the older cells sediment more rapidly. Further, as RNA synthesis is progressively switched off, the absolute amounts of RNA fall, and this loss is most marked from the nucleus.

Characterization of nuclear RNA and cytoplasmic RNA. The RNA prepared by three successive phenol extractions of the nuclei from reticulocytes was examined by sucrose-density-gradient centrifugation. The profile shown in Fig. 6 is typical. Analysis of the curve by a Du Pont 310 Curve Resolver (Du Pont Instrument Products Division, London, E.C. 4) indicates that, in addition to peaks sedimenting as the two ribosomal RNA species, there are at least two other components, one of which is polydisperse rapidly moving material. The proportion of this material is increased as the number of phenol extractions is increased. The base composition (Table 1) of the nuclear RNA is intermediate between that of the two ribosomal species, except for the tendency to a high value for

Fig. 6. Sedimentation of nuclear RNA from pigeon reticulocytes in a linear 5–20% (w/w) sucrose gradient in 0.05M-sodium acetate in a Spinco SW 25.1 rotor. Centrifugation was for 16.5 hr. at 24000 rev./min. at 5°. The contents of the tube were monitored at 260 m μ (—) in the flow cell of a Cary model 14 spectrophotometer (Edwards & Mathias, 1963). The broken lines indicate the components resolved by the Du Pont Curve Resolver.

adenine. About 70% of the extractable RNA is recovered in the first extraction. The second extract contains 17%. The fact that it contains a higher uracil content may be significant because it is known that it contains a higher proportion of material of high molecular weight (Barlow & Mathias, 1968). The cytoplasmic RNA when examined by sucrose-density-gradient centrifugation is found to contain the two expected ribosomal RNA species. (The 5s RNA is lost in the procedure using guanidinium chloride.) The base composition of the two species is given in Table 2.

Labelling of RNA. The sedimentation pattern of cells containing newly synthesized RNA after labelling *in vivo* was examined for both erythrocytes and a preparation rich in reticulocytes. The profile for normal blood is shown in Fig. 7 and that for reticulocytes in Fig. 8. The reticulocytes incorporate nine times as much radioactivity per unit wet weight of cells as the erythrocytes. For the erythrocytes, the radioactivity profile approximates to the corrected turbidity trace, and the specific radioactivity of the RNA is more or less constant across the gradient, with a tendency for the slower-moving cells to be slightly more radioactive. From this

Table 1. Base composition of nuclear RNA from pigeon reticulocytes

No. of extractions	Method of analysis	No. of analyses	Base composition (moles/100 moles)			
			U	G	A	C
Three extractions combined	1	3	19.5 $\pm$ 0.68	34.6 $\pm$ 0.7	19.2 $\pm$ 0.7	26.4 $\pm$ 0.61
	2	9	19.6 $\pm$ 0.87	34.3 $\pm$ 0.49	19.4 $\pm$ 0.68	26.2 $\pm$ 0.85
First extraction only	2	6	19.5 $\pm$ 1.07	35.9 $\pm$ 3.14	19.4 $\pm$ 1.43	25.18 $\pm$ 1.22
Second extraction only	2	3	20.6 $\pm$ 1.54	34.4 $\pm$ 1.62	18.8 $\pm$ 0	26.3 $\pm$ 2.74

Table 2. Base composition of cytoplasmic ribosomal RNA of pigeon erythrocytes

Sub-unit	Method of analysis	No. of analyses	Extinction coefficients used	Base composition (moles/100 moles)			
				U	G	A	C
Large	1	6	Katz & Comb (1963)	16.4 ± 0.96	38.3 ± 0.6	14.7 ± 0.33	30.5 ± 0.92
			Beavan <i>et al.</i> (1955)	16.3 ± 0.8	37.8 ± 0.87	15.1 ± 1.2	30.8 ± 0.7
	2	2	Beavan <i>et al.</i> (1955)	17.1 ± 0.2	35.7 ± 0.4	18.2 ± 0.25	29.2 ± 0.45
Small	1	3	Katz & Comb (1963)	26.1 ± 0.75	30.5 ± 1.5	18.6 ± 1.2	24.7 ± 0.7

Fig. 7. Zonal profile of normal pigeon erythrocytes labelled with [³²P]phosphate *in vivo*. —, Turbidity; ----, radioactivity.

experiment it may be concluded that some synthesis of RNA can occur in mature erythrocytes, although the possibility that the apparent RNA synthesis may be due to the presence of immature cells released from the bone marrow during the 10hr. period of labelling cannot be ruled out. In contrast, the peak of radioactivity does not coincide with the peak of turbidity for the labelled reticulocytes. The specific radioactivity of the labelled RNA is highest in the trailing edge of the cell zone and then decreases sharply. The region of high specific radioactivity represents cells released into the circulation before the administration of [³²P]-phosphate and still active in synthesis of RNA and cells released from the marrow during the 10hr. period of labelling. Evidently cells in the slower-moving or 'younger' region of the gradient contain

Fig. 8. Zonal profile of pigeon reticulocytes labelled with [³²P]phosphate *in vivo*. —, Turbidity; ----, radioactivity; ······, specific radioactivity.

more RNA than older cells and RNA synthesis declines progressively with aging.

Characterization of nuclei from erythrocytes. The process of aging of the erythrocytes is reflected in a change in their sedimentation properties. To some extent this must be dependent on changes in the cytoplasm, e.g. the inexorable changes in concentration of ribosomes. But it is known that the morphology of the nucleus alters as the synthesis of RNA declines. The chromatin becomes more condensed and the nucleus shrinks (Lucas & Jamroz, 1961). Therefore it was decided to compare the behaviour of nuclei from erythrocytes with those from reticulocytes. The results are shown in Fig. 9. The reticulocyte nuclei sediment more slowly than those from erythrocytes. In both populations the larger nuclei tend to move more rapidly than the smaller (Fig. 10). The reticulocyte nuclei present a wider range of sizes than nuclei from normal erythrocytes. However, it is notable that the reticulocyte nuclei, with a mean volume of 35 μ³, sediment at a lower rate than the erythrocyte

Fig. 9. Zonal profile of nuclei from pigeon erythrocytes (—) and reticulocytes (----).

Fig. 10. Histograms of the nuclear volumes of the nuclei recovered from the experiments shown in Fig. 9. (a) Reticulocyte nuclei. The nuclei examined were: for (a1) in effluent volume 220–270 ml.; for (a2) in effluent volume 320–370 ml.; for (a3) in effluent volume 420–470 ml. The histogram on the right is the summation of (a1), (a2) and (a3). (b) Erythrocyte nuclei. The nuclei examined were: for (b1) in effluent volume 220–270 ml.; for (b2) in effluent volume 320–370 ml.; for (b3) in effluent volume 420–470 ml.; for (b4) in effluent volume 520–570 ml.; for (b5) in effluent volume 620–670 ml. The ordinates represent numbers of nuclei.

nuclei, which have an average volume of $25\mu^3$. Since both types of nuclei contain the same amount of DNA it may be concluded that the reticulocyte nuclei are less dense than those from erythrocytes.

Support for this hypothesis was obtained by

Fig. 11. Isopycnic zonal centrifugation of nuclei from pigeon reticulocytes (—) and erythrocytes (----).

determination of the buoyant density of both classes of nuclei. This was achieved by centrifuging the nuclei to equilibrium in a 53–63% (w/w) sucrose gradient, which corresponds to a range of densities (measured at 20°) of 1.2462–1.3043. The reticulocyte nuclei banded at a position corresponding to a density of 1.2871, and those from erythrocytes formed a narrow zone at a density of 1.2944 (Fig. 11). These densities correspond to 60.11% and 61.35% (w/w) sucrose respectively. Measurements of the rate of sedimentation of the two classes of nuclei at different points in a 20–50% (w/w) sucrose gradient confirmed the difference in density. However, insufficient points were available to permit accurate extrapolations to buoyant density.

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