Separation of haemopoietic cells for biochemical investigation

Preparation of erythroid and myeloid cells from human and laboratory-animal bone marrow and the separation of erythroblasts according to their state of maturation

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The separation of haemopoietic bone-marrow cells by centrifugation through discontinuous density gradients of Percoll is described. This method was used to prepare fractions enriched in erythroblasts, myeloid blast cells or reticulocytes from bone marrow of anaemic and non-anaemic rabbits, from the marrow of other anaemic laboratory animals and from human samples. It is a simple, rapid, reproducible and inexpensive technique that can be readily adapted to suit individual requirements. Secondly, a convenient method is presented for the separation of large quantities of bone-marrow cells into fractions enriched in erythroblasts at different stages of maturation, by velocity sedimentation through a linear gradient of 1–2% sucrose at unit gravity. *In vivo*, erythroblasts adhere together strongly via a mechanism almost certainly involving a β -galactoside-specific surface lectin termed erythroid developmental agglutinin. Since the efficiency of cell-separation techniques depends heavily on the maintenance of a single cell suspension in which each unit can move independently, the presence of an adhesive molecule at the cell surface is of considerable significance. The effect of washing the marrow with a lactose-containing medium, which has been shown to remove the agglutinin, was therefore investigated in relation to both methods. The separation on Percoll gradients is considerably enhanced by this treatment. In addition, the unit-gravity sedimentation gradient can be loaded with 5–10 times more cells after lactose extraction in comparison with intact marrow. Although enrichment is less, a useful fractionation according to maturation is still obtained.

Over the past ten years, many aspects of the biochemistry of erythropoiesis in the adult rabbit marrow have been investigated in this Department. Of particular interest have been studies on transcription (Clissold *et al.*, 1977; Hsu *et al.*, 1978), translation (Stewart *et al.*, 1976; Bonanou-Tzedaki & Arnstein, 1976), enzyme changes (Setchenska & Arnstein, 1978, 1979; Hulea & Arnstein, 1977; Denton *et al.*, 1975; Jeffery & Spencer, 1978), cell aging mechanisms (McKay *et al.*, 1980), cyclic nucleotide metabolism (Setchenska *et al.*, 1980) and cell–cell recognition and adhesion (Harrison & Chesterton, 1980). To carry out this work, methods have been devised for the fractionation of the complex marrow cell population. Since many have expressed an interest in this technology, we have set down here our current procedures.

Haematopoiesis in the adult mammalian bone marrow constitutes a developmental system readily accessible to experimental investigation by cellular physiologists, biochemists and molecular biologists. A pluripotent stem cell gives rise to the myeloid, erythrocytic and megakaryocytic cell lines, which all develop within the bone marrow. The erythroid lineage is of particular interest in this laboratory. The committed erythropoietic stem cell divides repeatedly in response to erythropoietin, to form clusters of rapidly differentiating proerythroblasts. The subsequent stages of erythroid differentiation, resulting in the release of immature reticulocytes from the bone marrow, can be observed and investigated, since cells at each stage of development are easily recognized by their characteristic morphological appearance under the light microscope and by histochemical staining.

Cellular heterogeneity and interactions between the different cell types prevent the study of erythro-

Abbreviation used: PBS, phosphate-buffered saline.

poiesis in whole bone marrow, and a variety of procedures have been developed to isolate erythroid cells and to fractionate them according to their state of maturation (Denton & Arnstein, 1973; Clissold, 1974; Meyskens *et al.*, 1979). We report here the development of a method for the preparation of fractions enriched in erythroblasts and myeloid blast cells from anaemic-rabbit bone marrow on discontinuous gradients of Percoll (polyvinylpyrrolidone-coated colloidal silica). The simplicity and speed of this technique, together with the highly reproducible results obtained, make this method particularly useful for the preparation of a large quantity of cells for subsequent physiological or biochemical investigations. These separation procedures have also been successfully applied to the preparation of erythroblast fractions from other animal species.

We describe also the use of an inexpensive, osmotically balanced, sucrose-gradient system for the large-scale separation of erythroblasts according to their stage of development. As erythroid cells differentiate, they become smaller and, although their density fluctuates, the change in size is sufficient to allow a separation by velocity sedimentation at unit gravity (Peterson & Evans, 1967; McCool *et al.*, 1970; Stephenson & Axelrod, 1971; Denton & Arnstein, 1973; Glass *et al.*, 1975). Most of the techniques described, however, utilize expensive gradient-forming materials such as serum albumin or Ficoll, and large quantities of reagents are necessary to provide an adequate number of separated cells for experimentation. To circumvent this problem, we have developed a unit-gravity sedimentation method based on the osmotically balanced 1–2% sucrose gradient recommended by Shall (1973) and using the sedimentation tank designed by Denton & Arnstein (1973).

Materials

(a) General

Anaemia was induced in New Zealand White rabbits by intraperitoneal phenylhydrazine injection [0.3 ml of 2.5% phenylhydrazine (pH 7.4)/kg body wt. daily for 5 days, followed by 2 days rest] (Denton & Arnstein, 1973), and in Schofield mice, Dunkin Hartley guinea pigs and Wistar rats, with the same daily regime at respective dosages of 0.1 ml of 0.8% solution/25 g body wt., 1.0 ml of 2.5% solution/1200 g body wt. and 0.1 ml of 2.5% solution/100 g body wt. We are grateful to Dr. Janet Young at the Hammersmith Hospital for kindly providing human bone-marrow aspirates from anaemic patients.

PBS solutions were prepared from tablets (Oxoid, Basingstoke, Hants., U.K.) routinely dissolved in 85 ml of water per tablet. All buffers used were adjusted to pH 7.2 and osmolarity of 295–310-

mosmol/l, as determined in an Advanced Osmometer (MSE), and cooled to 0°C. When the separated cells were to be cultured, all solutions used in the preparation of Percoll gradients were freshly made up from sterile stock solutions. Typhoid-paratyphoid A and B (TAB) vaccine was purchased from the Lister Institute, Elstree, Herts., U.K.

(b) Percoll gradients

Stock solutions of 10 times the standard strength of PBS (10× PBS) and of 1% (w/v) CaCl_2 /1% (w/v) MgCl_2 (Ca/Mg) were stored at -20°C. The osmolarity of the Percoll supplied was adjusted by the following procedure. For each gradient, 20 ml of Percoll + 1.5 ml of 10× PBS + 0.2 ml of Ca/Mg solution was adjusted to pH 7.2 by the dropwise addition of 0.01 M-HCl with rapid stirring. Then the osmolarity was adjusted to 295–310 mosmol/l by the dropwise addition of water or NaCl. This solution is by definition 100% Percoll and was diluted to make 10 ml gradient fractions of 40% (1.03 g/ml), 70% (1.09 g/ml) and 80% (1.10 g/ml) Percoll by using PBSS solution (10 ml of 10× PBS + 1 ml of Ca/Mg solution per 100 ml).

(c) Unit-gravity separation gradient

The following stock solutions were stored at -20°C: a solution containing 9% (w/v) NaCl, 2.25% (w/v) KCl, 13% (w/v) Na_2HPO_4 , 2.25% (w/v) KH_2PO_4 , 11.25% (w/v) glucose (stock salts solution); 0.2% (w/v) CaCl_2 /1% (w/v) MgCl_2 (modified Ca/Mg); 12.5% (w/v) streptomycin sulphate + 4.5×10^4 units of penicillin/ml (Pen/Strep). The gradient buffers were stored at 4°C overnight. The 1%-sucrose buffer contains, per 5½ litres: 56.25 g of sucrose + 35.5 g of NaCl + 1.0 g of bovine serum albumin + 56 ml of modified Ca/Mg + 50 ml of stock salts + 0.5 ml of Pen/Strep. The 2%-sucrose buffer contains, per 5½ litres: 112.5 g of sucrose + 27.5 g of NaCl + 1.0 g of albumin + 56 ml of modified Ca/Mg + 50 ml of stock salts + 0.5 ml of Pen/Strep. The 2.5%-sucrose supporting pad contains, per 10 litres: 250 g of sucrose + 47.25 g of NaCl + 100 ml of modified Ca/Mg + 100 ml of stock salts + 5 ml of Pen/Strep. After preparation the osmolarity of each should be 295–300 mosmol/l.

Methods

Separation of cell types on Percoll gradients

Bone marrow was removed from the femur, humerus and tibia bones of an anaemic rabbit immediately after death and gently disaggregated in 10 ml of LMES medium [0.3 M-lactose/14 mM-2-thioethanol/2 mM-EDTA (pH 8)/150 mM-NaCl] to form a single cell suspension, by using pipettes of 4 mm, and then of 2 mm diameter aperture sequentially. The cells were pelleted at 150g for 5 min, washed

with a further 10 ml of LMES medium and pelleted again. The cells were then resuspended in approx. 2 ml of PBSS medium and filtered through nylon bolting cloth. The cell concentration in this suspension was determined by counting a diluted sample in a haemocytometer or Coulter Counter.

A three-step Percoll gradient was produced by sequentially layering each gradient fraction into the bottom of a 50 ml centrifuge tube from a 10 ml syringe, under gravity flow. Bone-marrow cells (1×10^9) were loaded, after thorough resuspension, on to the top of the gradient in 2 ml of PBSS medium and the gradient was centrifuged at $3000g$ for 30 min. Fractions (2 ml) were collected by hand from the top of the gradient by using a fired Pasteur pipette, and the cell distribution was determined by counting each fraction in a Coulter Counter. The positions of the erythroblast and myeloid blast cells at the interfaces of the gradient could generally be readily determined visually and the appropriate fractions pooled. To remove Percoll, the combined fractions were diluted 20-fold with PBSS medium and the cells pelleted at $300g$ for 5 min. The cell fractions were then resuspended in the media required for subsequent experiments and samples were used to prepare cytocentrifuge slides for subsequent staining with benzidine and Giemsa. Identification of the different cell types present in each fraction was as described by Denton & Arnstein (1973) and Clissold *et al.* (1977).

Separation of erythroblasts by unit-gravity sedimentation

At 2 h before death, the rabbit was injected intravenously with 0.5 ml of TAB vaccine/kg body wt., as described by Denton & Arnstein (1973). The following cell-suspension medium was prepared and cooled to 0°C : 1.6 litres of PBS + 1.6 g of albumin + 16 ml of modified Ca/Mg + 6 ml of Pen/Strep (PBS/BSA medium). Immediately after death by exsanguination the marrow was removed and disaggregated gently, as described above, in 10 ml of PBS/BSA medium, pelleted at $150g$ for 5 min and washed again. After suspension in 400 ml of PBS/BSA medium and filtration through nylon bolting cloth, the cell density was estimated in the Coulter Counter.

The apparatus illustrated in Fig. 1 was assembled in the cold-room at 4°C , ensuring that the separation tank (A) is level. The tank outlet (C) was closed and 500 ml of PBS solution poured carefully into the bottom of the tank, then the cone (B) was filled to the brim with silicone-coated glass beads. These prevent disturbance of the gradient during loading. All subsequent movement of solutions into or out of the tank was achieved by raising or lowering the tank outlet (C).

A suspension of 1.2×10^9 cells in 400 ml of

Fig. 1. Apparatus for velocity-sedimentation procedure at unit gravity

A, Perspex separation tank in cross section (50 cm square in aerial view). The cone, B, is filled with glass beads (see text). C, screw-clipped tank outlet. E, 10 litre linear-gradient maker with air-bubble trap D.

PBS/BSA medium was loaded into the tank under the PBS, at a flow rate of 100 ml/min, via a funnel inserted into the outlet tubing (C), followed by 250 ml of 1% sucrose buffer. The tank was then connected to the gradient maker as indicated in Fig. 1 (D and E), and a 10-litre linear gradient of 1–2% sucrose was loaded at 500 ml/min. Finally, 10 litres of supporting pad was loaded in the same manner. The gradient and 2.5% pad were sterilized by passage through a u.v.-irradiated quartz tube during loading (Fig. 1).

The cells were allowed to sediment through the sucrose gradient for 4.5 h from initial loading. The support pad was then run out at about 250 ml/min. The gradient was collected similarly in 250 ml fractions and the cell distribution determined by counting samples in the Coulter Counter. The cells were harvested at $300g$ for 10 min and resuspended in PBS/BSA medium. Those fractions containing the erythroblast fractions required (Fig. 2) were combined and samples were taken to prepare cytocentrifuge slides for subsequent staining and cell identification.

Results

Preparation of an erythroblast-enriched fraction from anaemic-rabbit bone marrow on Percoll gradients

Initially bone-marrow cells were washed twice with PBSS before loading on to a Percoll gradient. The separation profile obtained was essentially that seen in Plate 1. The 2 ml fraction on top of the gradient was found to contain mainly damaged cells, i.e. cells permeable to Trypan Blue dye, and debris. The erythroblast fraction banded at the 40%/70% interface. The composition of this band of cells, as

determined by counting stained cytocentrifuge slides, is presented in Table 1. It was noted, however, both before application to the gradient and when collecting fractions from the gradient, that the bone-marrow cells readily formed small aggregates, which decreased the efficiency of the cell separation. Cell clumping has often proved a problem in cell-separation procedures. Consideration of the discovery of the β -galactoside-specific erythroblast lectin termed erythroid developmental agglutinin (EDA) in rabbit bone marrow (Harrison & Chesterton, 1980) suggested that washing bone-marrow cells with lactose before their application to the gradient might improve the separation. This procedure routinely yielded the cell separation profile illustrated in Plate 1 and markedly improved the relative purity and yield of the erythroblast fraction (Table 1). Loads of 0.5×10^9 , 1.0×10^9 and 1.5×10^9 cells on the Percoll gradient have all yielded comparable cell separations. Erythroblast-enriched fractions prepared in this manner contained over 98% Trypan Blue-excluding cells and appeared undamaged when viewed by phase-contrast microscopy.

Preparation of erythroblasts from other laboratory animals and from human marrow

The simplicity and efficiency of this separation prompted the application of these techniques to other systems. Results of these experiments are also shown in Table 1. Erythroblast fractions prepared from anaemic rats, guinea pigs or mice proved to be

markedly enriched for erythroblasts with respect to the whole bone marrow, although the purity of these fractions was significantly lower than those routinely prepared from anaemic-rabbit bone marrow. It is evident, however, from the overall composition of the bone marrow that anaemia in terms of the percentage content of erythroblasts was far less severe in these animals than is generally observed in rabbits.

It is evident from the data presented in Table 1 that in human bone-marrow aspirates from anaemic patients the major problem is reticulocyte and erythrocyte contamination of the erythroblast fraction. Preliminary experiments have suggested a way in which this problem might be overcome. A sample (2 ml) of lactose-washed human bone-marrow aspirate was layered on top of 10 ml of 70% Percoll and centrifuged at 3900 g for 30 min. The cell layer on top of the Percoll was enriched from approx. 0.5% erythroblasts to 30% erythroblasts. Subsequent loading of such an erythroblast-enriched fraction on to a standard Percoll gradient would be expected to improve greatly the relative purity of the erythroblast fraction, but, owing to a lack of material, we have so far been unable to investigate this possibility further.

Preparation of fractions enriched with either myeloid blast cells or marrow reticulocytes on Percoll gradients

When bone-marrow cells were applied to a three-step gradient as illustrated in Plate 1, the cells

Table 1. *Cell composition of erythroblast and myeloid-blast-cell-enriched fractions prepared on Percoll gradients*
Percentage cell distribution ($\pm$ s.d.) was determined as described in the text. Abbreviations: E, erythroblast; R, reticulocyte; M, myeloid blast cells.

		Cell composition (%)						Recovery of cell type (%)	No. of expts. performed
		Cells loaded			Cells in fraction				
	Wash	E	R	M	E	R	M		
(a) Erythroblast fraction (40/70%-Percoll interface)									
Anaemic rabbit	PBS	68 ± 5.5	14 ± 4.3	17 ± 6.2	77 ± 2.3	15 ± 1.8	8 ± 2.3	74	7
Anaemic rabbit	LMES	68 ± 5.5	14 ± 4.3	17 ± 6.2	84 ± 2.2	11 ± 2.7	4 ± 2.3	80	11
Anaemic rat	LMES	54	15	31	67	27	9	—	2
Anaemic guinea pig	LMES	55	18	27	72	5	23	—	2
Anaemic mouse	LMES	15	16	29	61	13	26	—	2
Anaemic human	LMES	—	>99	—	61	38	1	—	1
Non-anaemic rabbit	PBS	26	46	27	58	34	8	54	2
Non-anaemic rabbit	LMES	26	46	27	60	20	20	90	2
(b) Myeloid blast-cell fraction (70/80%-Percoll interface)									
Anaemic rabbit	LMES	73	10	17	10	14	76	17	2
Non-anaemic rabbit	PBS	26	46	27	0	1	99	26	1
Non-anaemic rabbit	LMES	26	46	27	3	10	87	14	1
(c) Myeloid/reticulocyte fraction (80%-Percoll)									
Anaemic rabbit	LMES	73	10	17	6	31	63	12	2
Non-anaemic rabbit	PBS	26	46	27	0	62	38	23	2
Non-anaemic rabbit	LMES	26	46	27	4	61	35	20	2

EXPLANATION OF PLATE 1

Separation of bone-marrow haemopoietic cells on a Percoll gradient

Results of a typical separation of anaemic rabbit marrow cells (1.0×10^6) washed in LMES medium, showing the appearance of a gradient after centrifugation, the cell distribution through the gradient and stained cytocentrifuge slides prepared from each band of cells (magnification $\times 175$). Abbreviations: E, erythroblasts; R, reticulocytes; W, myeloid cells.

banding at the 70%/80% interface were found to consist predominantly of myeloid blast cells (Plate 1 and Table 1b), particularly when bone marrow of non-anaemic animals was separated. Although only a relatively small proportion of the total number of myeloid blast cells in the bone marrow could be recovered from this fraction, it was often particularly useful to be able to prepare simultaneously erythroblast and myeloid-blast-cell-enriched fractions from the same bone-marrow sample by directly comparable experimental procedures. In anaemic rabbits, more white cells could be recovered from the 80%-Percoll layer, but reticulocyte contamination here could rise to 40% (Table 1c). In non-anaemic rabbits, however, the 80%-Percoll fraction consisted predominantly of reticulocytes (Table 1c) and could therefore be used as an enriched source of bone-marrow reticulocytes for further separation procedures.

Separation of erythroblasts by unit-gravity sedimentation

The separation profile obtained when bone-marrow cells were washed twice in PBS/BSA medium before unit-gravity sedimentation is shown in Fig. 2(a). As found with previous methods, the fractions obtained were not completely homogeneous for cell type (Table 2). The extent of cross-contamination was variable and probably dependent on the state of anaemia and the lipid content of each marrow. In general, however, fractions containing 40–60% of the selected erythroblast cell type could be obtained (Plate 2).

The improvement in the Percoll-separation technique achieved by washing the bone-marrow cells with LMES medium prompted the application of equivalent procedures to the unit-gravity sedimentation. However, in this case, lactose-washing of the cells did not improve the cell separation in itself, but did decrease the cell clumping and subsequent streaming phenomena previously observed at high cell concentrations (Peterson & Evans, 1967). This effect enabled the application of 5–10-fold more cells to the sucrose gradient. It is evident from the separation profile shown in Fig. 2(b) that the lactose-washed cells sedimented at a slightly faster rate than did cells prepared by washing in PBS/BSA medium, but did not aggregate during sedimen-

tation, and the identification of the cell types present in the pooled fractions revealed that an enrichment for each cell type was obtained. However, in general, the enrichment was poorer than that observed with the standard loading of cells prepared for sedimentation by washing with PBS/BSA medium, particularly among the smaller cell types (Table 2). Cells produced by either method were at least 80% viable, as judged by a Trypan Blue exclusion test.

It seemed that preparation of an erythroblast-enriched fraction for unit-gravity sedimentation by a preliminary Percoll gradient might lessen the relatively high contamination of the proerythroblast fraction with myeloid cells and the contamination of

Fig. 2. Cell distribution on a linear sucrose gradient after sedimentation at unit gravity

Experimental details were as described in the text: (a) 1.2×10^9 cells washed with PBS/BSA medium loaded on gradient; (b) 6.0×10^9 cells washed with LMES medium loaded on gradient. Fractions collected: PE, proerythroblasts; B, basophilic erythroblasts; PC, polychromatic erythroblasts; OI, orthochromatic I erythroblasts; OII, orthochromatic II erythroblasts; R, reticulocytes. The average sedimentation rate (mm/h) of cells within each fraction is indicated on the Figure.

EXPLANATION OF PLATE 2

Erythroblast fractions separated by unit gravity sedimentation

Cytocentrifuge slides (magnification $\times 625$) of pooled fractions from gradient (1.2×10^9 cells washed in PBS/BSA medium loaded) stained for cell identification as described in the text. Fractions enriched for: (a) proerythroblasts; (b) basophilic erythroblasts; (c) polychromatic erythroblasts; (d) orthochromatic I erythroblasts; (e) orthochromatic II erythroblasts.

Table 2. *Cell composition of erythroblast fractions obtained by unit-gravity sedimentation*

Percentage cell distribution ($\pm$ s.d.) was determined as described in the text and Fig. 2: (a) 1.2×10^9 anaemic marrow cells washed in PBS/BSA medium; data from five separations; (b) 6×10^9 anaemic marrow cells washed in LMES medium to remove EDA. Data are from three separations. Abbreviations for fractions; PE, proerythroblast; B, basophilic erythroblasts; PC, polychromatic erythroblasts; OI, orthochromatic I erythroblasts; OII, orthochromatic II erythroblasts (see Fig. 2).

Cell type		Fraction (%)				
		PE	B	PC	OI	OII
Immature myeloid cells	a	23 $\pm$ 7	15 $\pm$ 3	6 $\pm$ 4	3 $\pm$ 4	—
	b	20 $\pm$ 5	16 $\pm$ 4	10 $\pm$ 1	9 $\pm$ 6	5 $\pm$ 2
Proerythroblasts	a	46 $\pm$ 7	13 $\pm$ 8	3 $\pm$ 5	—	—
	b	37 $\pm$ 2	11 $\pm$ 4	4 $\pm$ 1	—	—
Basophilic erythroblasts	a	22 $\pm$ 7	53 $\pm$ 3	17 $\pm$ 9	3 $\pm$ 3	—
	b	24 $\pm$ 2	43 $\pm$ 7	22 $\pm$ 8	6 $\pm$ 1	—
Polychromatic erythroblasts	a	6 $\pm$ 2	16 $\pm$ 4	62 $\pm$ 9	17 $\pm$ 7	5 $\pm$ 4
	b	10 $\pm$ 3	18 $\pm$ 4	49 $\pm$ 11	25 $\pm$ 6	8 $\pm$ 4
Orthochromatic I erythroblasts	a	1 $\pm$ 1	1 $\pm$ 1	6 $\pm$ 2	54 $\pm$ 6	16 $\pm$ 6
	b	5 $\pm$ 2	6 $\pm$ 2	8 $\pm$ 4	36 $\pm$ 6	22 $\pm$ 7
Orthochromatic II erythroblasts	a	1 $\pm$ 1	—	5 $\pm$ 3	16 $\pm$ 7	47 $\pm$ 5
	b	2 $\pm$ 1	3 $\pm$ 1	3 $\pm$ 1	10 $\pm$ 3	27 $\pm$ 7
Reticulocytes	a	1 $\pm$ 1	1 $\pm$ 1	3 $\pm$ 1	7 $\pm$ 2	31 $\pm$ 9
	b	2 $\pm$ 0	2 $\pm$ 2	4 $\pm$ 2	11 $\pm$ 3	38 $\pm$ 12

the late orthochromatic cells with reticulocytes. However, the application to a unit-gravity separation of neither PBSS- nor LMES-washed erythroblasts from a Percoll gradient produced fractions enriched in the different cell types equivalent to those produced by standard means.

Discussion

Convenient and reproducible methods of cell isolation and separation greatly facilitate biochemical investigations, and the application of Percoll-gradient separation procedures has enabled the investigation of the activity *in vitro* of the rabbit bone-marrow agglutinin, erythroid developmental agglutinin (EDA), on erythroblast- and myeloid-blast-cell-enriched fractions from the bone marrow of human and laboratory animals (Harrison & Chesterton, 1980; F. L. Harrison & C. J. Chesterton, unpublished work). Removal of non-erythroid cells from bone-marrow samples has been attempted by various means, such as the selective adherence of myeloid cells to glass surfaces (Cutt, 1970) or packed fibrous cotton-wool (Light & Tanner, 1977). The efficiency of these methods is variable, however, and they tend to deplete the erythroblast component of the cell suspension. The induction of severe anaemia by phenylhydrazine injection and the pretreatment of laboratory animals with TAB vaccine (Denton & Arnstein, 1973) can also be used to lessen the myeloid complement of bone marrow. In our experience, however, even when these

methods are used in conjunction, it is difficult to decrease the myeloid component below 15% of the bone-marrow cell population. Consequently, such treatments have generally been used before other preparative procedures.

Density-gradient-centrifugation procedures have also been employed, notably the use of bovine serum albumin gradients by Borsook *et al.* (1969) and Clissold (1974) for non-anaemic and phenylhydrazine-induced anaemic tissues respectively. Both procedures require large quantities of albumin, and the gradients, which are time-consuming to prepare, have a high viscosity, which makes them difficult to handle. In addition, ultracentrifugation is required for the cells to reach equilibrium positions. The use of counterflow centrifugal elutriation to separate human bone-marrow cells has more recently been described (Meyskens *et al.*, 1979), but this method seems to be better suited to the preparation of subpopulations of non-erythroid cells.

The Percoll-gradient technique proved preferable to the established procedures because it is relatively cheap and is a reproducible, simple and rapid procedure requiring only brief centrifugation at a relatively low speed. Large quantities of erythroblasts can be prepared; for example, up to 1.5×10^9 cells have been successfully separated on one 30 ml gradient. The simultaneous preparation of erythroid and myeloid fractions from the same marrow is a considerable advantage for comparative experiments, and it is particularly useful that the Percoll-gradient technique is so readily applicable to the

separation of haemopoietic cells from different animal species.

Cells separated on Percoll gradients show a high degree of viability, and immature erythroblasts from a Percoll gradient have been found to respond to erythropoietin and to mature in culture *in vitro* (T. M. Beswick & S. Bonanou-Tzedaki, unpublished work).

It was found that cells separated on albumin gradients displayed altered cell-surface properties with respect to agglutination by EDA, whereas erythroblasts and myeloid cells separated on a Percoll gradient demonstrated characteristics comparable with those observed in whole bone-marrow suspensions (Harrison & Chesterton, 1980). This phenomenon suggested that either membrane components are lost on centrifugation through albumin gradients or are masked by the albumin to an extent not readily reversible by washing in PBS.

The separation of erythroblasts according to their state of maturation is difficult to achieve. The albumin-density-gradient-centrifugation procedures of Borsook *et al.* (1969) and Clissold (1974) produce only two distinct cell fractions. As reported by Mezl *et al.* (1979), the method of Borsook *et al.* (1969) yields two fractions similarly enriched for immature erythroblasts, 63% of total cells are proerythroblasts or basophils, and two fractions consisting of 40% polychromatic erythroblasts. The procedure of Clissold (1974) yields fractions consisting largely of early erythroblasts, 69% proerythroblasts and basophils and fractions of predominantly late cells, 67% polychromatic and orthochromatic erythroblasts (Hsu, 1979). When the preparation of early erythroblasts is particularly desired, anti-erythrocyte antisera can be used to remove the reticulocytes and more mature erythroblasts from the bone-marrow sample before application to the gradient (Borsook *et al.*, 1969; Clissold, 1974). These techniques therefore provide a partial separation of erythroblasts not produced by the Percoll-density-gradient separation. However, preliminary experiments (T. M. Beswick, unpublished work) have shown that two consecutive Percoll gradients can produce a fraction enriched to 60–65% with early erythroblasts.

Alternatively, the sucrose-gradient unit-gravity velocity-sedimentation technique can be employed. This method produces fractions enriched for the five recognized developmental stages from proerythroblast to late orthochromatic erythroblast, and is routinely used in this department to prepare large quantities of separated cells for biochemical investigation (see, e.g., Clissold *et al.*, 1977). However, for some experiments, such large numbers of separated cells are required (Hsu *et al.*, 1978) that until the development of the lactose-washing procedure these investigations were not practicable. The lower efficiency of the unit-gravity sedimentation

technique when 5–10 times the normal load of lactose-washed cells is employed undoubtedly stems from the fact that each developmental compartment contains a higher density of cells in the same volume of gradient buffer. The increased rate of collision will cause a widening of the peak size and enhanced contamination of neighbouring fractions. Nevertheless, washing bone-marrow cells with lactose before separation on either Percoll or sucrose gradients has proved an invaluable modification of these methods, and similar sugar washing procedures might perhaps be generally applicable in the field of cell-separation techniques. Unfortunately, attempts to lessen the myeloid and reticulocyte contamination of cell fractions prepared by unit-gravity sedimentation by prior fractionation in Percoll proved less successful. It seems likely that the relative densities of the erythroblasts at different stages of maturation are altered in some way during sedimentation through a Percoll gradient.

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