Changes in some cytoplasmic enzymes from red cells fractionated into age groups by centrifugation in Ficoll/Triosil gradients

COMPARISON OF NORMAL HUMANS AND PATIENTS WITH DUCHENNE MUSCULAR DYSTROPHY

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1. A procedure is described for the fractionation into age classes of human red cells on a Ficoll/Triosil discontinuous density gradient. The fractionated cells appear minimally affected by the procedure. 2. Individual blood samples show a normal cell distribution, but the mean cell density may vary slightly. To allow for this in comparing samples, cell ages are expressed in terms of the mean and standard deviations from the mean. 3. Reticulocyte number decays exponentially in the gradient. By back extrapolation, a standard deviation can be computed corresponding to the theoretical age at which the sample contains 100% reticulocytes. This value can be used to calculate the theoretical activity of an enzyme in the reticulocytes and the enzyme half-life. 4. Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and glutathione reductase activities in unfractionated and age-fractionated cells were investigated in adults, children and patients with Duchenne muscular dystrophy. 5. The half-life for adult glucose 6-phosphate dehydrogenase (48 days) compares with that found by other workers. In children the half-life is 24 days, although the initial activity is essentially unaltered. 6. The half-life of 6-phosphogluconate dehydrogenase was found to be 117 days; the activity of glutathione reductase did not alter throughout the life of the cell. There was no difference between adults and children for these enzymes. The same results were obtained with dystrophic patients as for control children for all three enzymes.

The life-span of a human red cell in the blood-stream is approx. 120 days. During this period, from reticulocyte to senescent erythrocyte, biophysical and biochemical properties of many of the cell components undergo progressive change (Danon, 1966; Fornaini, 1967; Edwards & Rigas, 1967; Edwards *et al.*, 1971). A number of red-cell enzymes have been found to undergo an age-dependent loss of activity, the extent of which may vary considerably for different enzymes. Hexokinase and aldolase, for example, appear to lose activity rapidly (Chapman & Schaumberg, 1967), whereas lactate dehydrogenase remained unaltered (Lohr & Waller, 1962). Most of the early studies were carried out *in vivo* with the use of difficult methods that are also prone to error. Key (1921) found that the density of erythrocytes increased with cell age. Since that time, equilibrium density-gradient centrifugation has been found to give the most satisfactory quantification of the density distribution of an erythrocyte population (Piomelli *et al.*, 1967). By this means human

erythrocytes have been shown to exhibit a normal distribution with respect to density (Danon & Marikovsky, 1964; Leif & Vinograd, 1964). The mean specific gravity of erythrocytes varies between individuals, but the variance of the distribution is always very similar (Leif & Vinograd, 1964). Hence, when several individuals are compared, the cumulative distribution function rather than the absolute specific gravity of the fractions should be used. This was done in the present work.

For the earlier density-gradient studies, bovine serum albumin was used (Piomelli *et al.*, 1968). More recently, excellent results were obtained with discontinuous gradients prepared from mixtures of Ficoll and Triosil, the latter compound being added to decrease the viscosity of the Ficoll used to generate the gradient (Turner *et al.*, 1974). The present paper describes in more detail the preparation of a discontinuous Ficoll/Triosil density gradient with a modified density range for red-cell fractionation.

A number of investigations have indicated that

erythrocytes are affected in Duchenne muscular dystrophy, possibly as a result of the hereditary defect producing widespread changes in cell membranes (see, e.g. Vickers *et al.*, 1978; Ruitenbeek *et al.*, 1979; Wakayama *et al.*, 1979). The present work was undertaken to see if there was any difference with red-cell age, as determined by density distribution, and whether there were any obvious changes in some cytoplasmic enzymes. The cytoplasmic enzymes were chosen partly so that our results could be compared with those of previous workers and partly because of previous evidence that enzymes involved in glutathione metabolism might be affected by muscular dystrophy (Hooton & Watts, 1967) in association with changes in the oxidative state of the cell membranes (Omaye & Tappel, 1974).

Results are presented for three red-cell cytoplasmic enzymes as well as for erythrocyte and reticulocyte distribution for samples from normal adults and children, and for young males suffering from Duchenne muscular dystrophy.

An important feature of any fractionation system is that it should preserve the cells in as near normal condition as possible, and evidence is presented to indicate that the method is very satisfactory in this respect.

Experimental

Anticoagulant (acid/citrate/dextrose solution; ACD) contained 75 mM-trisodium citrate, 40 mM-citric acid and 125 mM-glucose (chemicals from BDH Chemicals, Poole, Dorset, U.K.). Phosphate-buffered saline contained 0.85% (w/v) NaCl in 5 mM-sodium phosphate buffer, pH 7.0 (chemicals from Hopkin and Williams, Romford, Essex, U.K.).

Isoton was obtained from Coulter Electronics, Harpenden, Herts., U.K. Triosil 440 was obtained from Nyegaard (marketed by Vestric Ltd., Runcorn, Cheshire, U.K.). All other general chemicals were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Haemolysate preparation

Blood (10 cm³) was collected by venepuncture (with informed consent and the ethical approval of Guy's Hospital Medical School) into acid/citrate/dextrose anticoagulant (2 cm³). Plasma was removed by centrifugation at 300 g at 4°C for 5 min. The cells were then washed three times in 30 vol. of phosphate-buffered saline, pH 7.0; each time as much of the buffy coat was removed as was consistent with the minimal removal of red cells. Cells were counted with a Coulter Counter (model ZF, with a 100 µm aperture; Isoton was used for all dilutions) and then lysed by diluting them 30-fold in 5 mM-sodium phosphate buffer, pH 7.4, and shaking for 1 h at approx. 4°C. This haemolysate was centrifuged at 30 000 g for 30 min at 4°C.

The supernatant was kept for measurement of the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The whole haemolysate was used for the determination of glutathione reductase activity.

Separation of erythrocytes into groups of different mean ages

A method based on that of Turner *et al.* (1974) was used. A discontinuous density gradient was made up of layers of Ficoll/Triosil solutions (Table 1), with variations in the Ficoll 400 [Pharmacia (G.B.) Ltd., Hounslow, Middx., U.K.] concentra-

Table 1. *Composition of the gradient used for red-cell fractionation*

A 30% (w/v) stock solution of Ficoll 400 was made up in distilled water and then added in the amounts shown below to 8 cm³ of a solution obtained by diluting 20 cm³ of Triosil 440 with 24 cm³ of distilled water (final concn. 13.6%, w/v) at approx. 4°C, and then adding water as indicated. The solutions were pre-equilibrated at 4°C to minimize the mixing of layers, which were formed by layering with a syringe the volumes shown, in reverse order, into a 30 ml cellulose nitrate centrifuge tube. The washed red cells, diluted to twice the packed volume with phosphate-buffered saline, were layered on top in 1.5 cm³. Centrifugation was for 30 min at 4°C in a SW30 rotor of the Omega ultracentrifuge at 45 000 g to achieve equilibrium. Discrete layers of cells form at the interfaces of solutions 1–8 and were aspirated with a syringe.

Solution no.	Calculated density (g/cm ³)	Volume of 30% Ficoll added (cm ³)	Final concn. of Ficoll (w/v)	Volume of water (cm ³)	Volume of solution used for one 30 cm ³ gradient (cm ³)
1	1.095	1.92	2.9%	10.08	2
2	1.100	3	4.5%	9	3
3	1.105	4	6.0%	8	3.5
4	1.110	4.8	7.2%	7.2	4.5
5	1.115	5.8	8.7%	6.2	4.5
6	1.12	6.8	10.2%	5.2	3.5
7	1.13	8.68	13%	3.32	3
8	1.15	12.0	18%	0	2

tions being used to produce the appropriate density differences. The solutions were pre-equilibrated to approx. 4°C and the gradients were prepared by layering appropriate volumes (Table 1) into cellulose nitrate tubes of capacity 30 cm³. Three equivalent gradients were prepared. Each was loaded with 1.5 cm³ of packed red cells that had been diluted to twice their original packed volume with phosphate-buffered saline. The gradients were centrifuged for 30 min at 4°C at 45 000 *g* (Table 1). After centrifugation the seven discrete layers of red cells were removed from each of three gradients, and equivalent fractions were combined in order to obtain an adequate number of cells, particularly from the uppermost and lowermost layers. The fractions were washed three times in phosphate-buffered saline, pH 7.0, to remove Ficoll and Triosil.

The sample percentile of each fraction (Piomelli *et al.*, 1968) is the percentage of cells in a gradient of a lower density than the mean density of a particular fraction. It is calculated from the formula:

$$\text{Sample percentile of fraction no. } x = \frac{R_x}{2} + \sum_{y=1}^{y=x-1} R_y$$

where R_x is the percentage of cells in the fraction number being considered (x) of the total cells on the gradient, and R_y is the percentage of cells in each other fraction of a lower density than x (i.e. the fractions where $y = 1$ to $y = x - 1$). Thus the sample percentile indicates the midpoint position of each fraction in the gradient expressed as a percentage of the total number of cells. In the results this value has been converted into the corresponding standard deviation from the mean density. This calculation depends on the assumption that human erythrocytes exhibit a normal distribution with respect to density as shown by Leif & Vinograd (1964) and Danon & Marikowsky (1964). It was confirmed for each sample on which experiments were carried out in this work.

Counting of reticulocytes

Reticulocytes in whole blood were stained with Brilliant Cresyl Blue in plastic tubes. Blood smears were made after incubation with the stain for 15 min. The percentage of reticulocytes was counted with a light microscope at 400× magnification, fitted with a graticule. At least 1000 red cells were counted for each sample.

Measurement of enzyme activities

The activities of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were measured in a reaction volume of 0.5 cm³. The reaction mixture contained 20 µl of red-cell supernatant (from the first centrifugation after lysis) and (final concentrations) 66 mM-Tris/HCl buffer, pH 8.0, 1.2 mM-glucose 6-

phosphate, 1.2 mM-6-phosphogluconate, 10 mM-MgCl₂ and, added to start the reaction, 0.15 mM-NADP⁺. The reaction was followed at 340 nm with a Cary 16S recording spectrophotometer, thermostatically controlled at 30°C. The assay for each extract was then repeated by using the same reaction medium but without glucose 6-phosphate, thus enabling the measurement of 6-phosphogluconate dehydrogenase activity alone. Glucose 6-phosphate dehydrogenase activity was obtained by subtraction. The molar absorption coefficient of NADPH was taken as 6.22×10^3 at 340 nm.

The activity of glutathione reductase (EC 1.6.4.2) was measured by following the oxidation of NADPH at 30°C. Haemolysate, obtained as described above, was used for the assay. The basic method of Beutler (1969) was followed. Samples (50 µl) of haemolysate were incubated in a reaction mixture containing (final concentrations) 0.5 M-Tris/HCl buffer, pH 8.0, 2 mM-EDTA (disodium salt) and 3.3 mM-oxidized glutathione for 10 min at 30°C. The reaction was initiated by the addition of NADPH to give a final concentration of 0.1 mM in a final volume of 0.5 cm³.

Results

Erythrocyte distribution

Fig. 1 shows the results for the distribution of

Fig. 1. Distribution of erythrocytes fractionated into age classes on Ficoll/Triosil discontinuous density gradients

The histogram represents the mean percentage of total cells on the gradient ($\pm$ S.E.M.) in each fraction taken from the gradients. These results were compiled from values obtained from the blood of 12 control children (of both sexes and aged between 5 and 17 years of age), five adults (of both sexes and aged between 18 and 52 years of age), six boys with Duchenne muscular dystrophy (aged between 4 and 19 years of age) and 15 donors with other neuromuscular disorders. The broken line represents the calculated normal distribution curve for the same data.

erythrocytes through the gradient for normal children aged 5 to 17 years, for adults, and for boys with Duchenne dystrophy and other neuromuscular disorders. The broken line represents an ideal normal distribution curve based on the mean and standard deviation of the observed data and shows a good fit with the experimental results. The mean calculated density of the erythrocytes of these donors was $1.1133 \pm 0.0003 \text{ g/cm}^3$ (standard deviation, $\sigma = 0.001$). The erythrocytes of normal adults, carrier women, control children and boys with Duchenne, Becker and limb-girdle dystrophies all had mean densities within a range of $\pm 0.002 \text{ g/cm}^3$ around the mean value.

Distribution of reticulocytes

Red cells enter the bloodstream as reticulocytes, also with a normal distribution as regards their density (Leif & Vinograd, 1964). The variance of this distribution does not vary from individual to individual. Reticulocytes increase in density at the same rate, thus the variance of each age group is the same (Leif & Vinograd, 1964). In any density range there are erythrocytes of various ages, as the age ranges overlap. Thus no precise age can be given to any one fraction of cells (although a mean age may be calculated). If a sample of pure-reticulocytes could be isolated, information about the structure and enzyme activities of the red cell, on first entering the bloodstream could be obtained. Although pure reticulocytes cannot be obtained in practice, one can usefully calculate the theoretical enzyme activity corresponding to 100% reticulocytes in the gradient. To do this, reticulocytes were counted in the different fractions of a number of gradients and the number was expressed as a percentage of the number of red cells in each fraction (Fig. 2). The proportion of reticulocytes decreases exponentially with the position down the gradient. By back-extrapolation a density value, defined in terms of σ , can be found corresponding to the theoretical expectation of finding 100% reticulocytes. In the same way, changes in other cell components can now be back-extrapolated to the same standard deviation to indicate the situation in 100% reticulocytes. From Fig. 2 it is estimated that at -4.35σ from the mean position of the gradient a fraction should be obtained that contains 100% reticulocytes. In both controls and dystrophy patients, the reticulocytes distributed throughout the gradient in the same way.

Integrity of fractionated erythrocytes

It is well known that the simple washing of erythrocytes with normal saline (0.85% NaCl) is sufficient to initiate the formation of spherocytes and echinocytes in a proportion of the cells. The normal biconcave shape is restored by resuspending the cells in plasma. Essentially similar results were obtained

Fig. 2. Relationship between the percentage of reticulocytes in a series of blood samples and position in a Ficoll/Triosil gradient

The position in the gradient is expressed as the standard deviation from the mean (σ). The number of reticulocytes was counted in each fraction separated from the blood of three boys with Duchenne muscular dystrophy and three child male controls. The cells were stained and counted as described in the Experimental section. The line through the points was calculated by linear regression. The broken line indicates the standard deviation corresponding to 100% reticulocytes in the sample.

with cells that had first been fractionated on a Ficoll/Triosil gradient and then washed in phosphate-buffered saline solution. After return to plasma, the cells rapidly regained their normal shape, and no obvious differences could be seen by light, phase-contrast or scanning electron microscopy.

As a further test, fractionated cells were lysed, washed and the membrane proteins analysed by conventional sodium dodecyl sulphate/polyacrylamide-gel slab electrophoresis (Laemmli, 1970). No

difference in the protein bands could be detected visually as compared with unfractionated cells.

Finally, unfractionated cells were incubated with various concentrations of Ficoll/Triosil mixture under the conditions used for age fractionation, but without centrifugation. The cells were then washed with phosphate-buffered saline in the conventional way and the activity of acetylcholinesterase, an erythrocyte outer-membrane enzyme, was compared with that in an untreated control portion of the original washed cells. Acetylcholinesterase was assayed by the method of Ellman *et al.* (1961). No

difference was found between control and treated cells. It has been shown by Billington & Coleman (1978) that acetylcholinesterase may be selectively eluted from erythrocytes without lysis by certain bile salts.

It was concluded that cell fractionation on Ficoll/Triosil gradients was no more deleterious to erythrocyte preparations than the washing procedures in current use.

Distribution of enzyme activities

Glucose 6-phosphate dehydrogenase, 6-phospho-

Table 2. *Enzyme activities in unfractionated red cells*

Enzyme	Donors	No.	Activity range or mean activity ± S.E.M.
Glucose 6-phosphate dehydrogenase (nmol of NADP ⁺ reduced/min per 10 ⁹ red cells)	Adults	4	85–116
	Children	6	60–140
	Duchenne	3	75–106
	All	13	95 ± 13
6-Phosphogluconate dehydrogenase (nmol of NADP ⁺ reduced/min per 10 ⁹ red cells)	Adults	4	80–136
	Children	6	102–142
	Duchenne	3	85–128
	All	13	112 ± 7
Glutathione reductase (in nmol of glutathione reduced/min per 10 ⁹ red cells)	Adults	3	45–61
	Children	7	46–60
	Duchenne	2	46–57
	All	12	51 ± 2

Table 3. *Comparison of the half-life of red-cell enzymes estimated by various workers*

Enzyme	Method of separation	Half-life (days)	Estimated enzyme activity of reticulocytes	Reference
Glucose 6-phosphate dehydrogenase	Ficoll/Triosil density gradient	48 (adults)	205 nmol of NADP ⁺ reduced/min per 10 ⁹ red cells	The present work
	Ficoll/Triosil density gradient	24 (children)	240 nmol of NADP ⁺ reduced/min per 10 ⁹ red cells	The present work
	Differential agglutination <i>in vivo</i>	53	—	Lohr & Waller (1962)
	Bovine serum albumin density gradient, <i>in vitro</i>	62	—	Piomelli <i>et al.</i> (1968)
	Ficoll/Triosil density gradient	42	—	Turner <i>et al.</i> (1974)
6-Phosphogluconate dehydrogenase	Ficoll/Triosil density gradient	117	165 nmol of NADP ⁺ reduced/min per 10 ⁹ red cells	The present work
Glutathione reductase	Ficoll/Triosil density gradient	∞	50 nmol of glutathione reduced/min per 10 ⁹ red cells	The present work

Fig. 3. Distribution of glucose 6-phosphate dehydrogenase activity in red cells from normal adults and children fractionated into age classes in discontinuous Ficoll/Triosil density gradients

The enzyme activity was measured in red-cell lysates from the fractions. Samples were analysed from (a) four adults (three male and one female) and (b) children (O, four control males; ●, three males with Duchenne muscular dystrophy). Measurement of enzyme activity was made in the red-cell lysate obtained from each fraction. Activity is expressed as the percentage of the activity found with the unfractionated blood sample. This is to normalize the results with respect to the variation in enzyme activity between individual samples.

gluconate dehydrogenase and glutathione reductase activities were measured in the unfractionated cells of a number of donors (Table 2). No difference was found between activities from control adults, control children or from patients for any of the three enzymes.

A number of samples from adults were fractionated and glucose 6-phosphate dehydrogenase activity was measured in the different fractions (Fig. 3a). The results are expressed as a percentage of the activity in unfractionated red cells to allow for variation in enzyme activity between individuals. The line through the plot was calculated by linear regression and shows an exponential decrease in activity with cell density (correlation coefficient 0.96).

By extrapolating back to -4.35σ , the mean glucose 6-phosphate dehydrogenase activity of the red cells on first entering the bloodstream was estimated (Table 3). The half-life of the enzyme activity has also been calculated on the assumption that the lifespan of the red cell is 120 days. This is compared with estimates by other workers in Table 3.

Fig. 3(b) expresses similar results for four child controls and three patients of similar age with Duchenne dystrophy. The enzyme activity in children was found to be clearly proportional to the

Fig. 4. Distribution of 6-phosphogluconate dehydrogenase activity in red cells from normal adults and children fractionated into age classes in discontinuous Ficoll/Triosil density gradients

The enzyme activity was measured in red-cell lysates from the fractions obtained from (O) adults, (●) two boys with Duchenne muscular dystrophy, and (▲) three normal boys. The enzyme activity is expressed as described in Fig. 3.

Fig. 5. Distribution of glutathione reductase activity in red cells fractionated into age classes in discontinuous Ficoll/Trisil density gradients

The enzyme activity was measured in red-cell haemolysates from (O) six children (four male, two female), (●) one dystrophic boy, and (Δ) one adult. The enzyme activity is expressed as described in Fig. 3.

position of the cells in the gradient, i.e. to cell density (correlation coefficient 0.97). The calculated enzyme activity of 100% reticulocytes in the bloodstream is similar to that estimated for the adults, but the half-life of the enzyme was calculated to be only about half the adult value (Table 3).

Fig. 4 shows results for the activity of 6-phosphogluconate dehydrogenase. There was an exponential loss of activity with increasing cell density, which occurred at a slower rate than with the previous enzyme. No difference in the rate of loss of activity was found for control adults, control children and boys with Duchenne dystrophy. The estimated half-life is listed in Table 3, together with the calculated activity corresponding to 100% reticulocytes.

Similar measurements were made of glutathione reductase activity, and again no differences were found between the groups of individuals studied. The data for unfractionated cells are listed in Table 2. The cell distribution with age was determined on one normal adult, three normal children and four children with myopathies. Fig. 5 and Table 3 show that this enzyme retained its full activity throughout the lifespan of the cell in all these samples (correlation coefficient 0.12).

Discussion

The fractionation procedure used in this work

distributes the cells into seven fractions. The method as originally described by Turner *et al.* (1974) was found to give the correct density values for the fractions as derived empirically, but the practical instructions for obtaining these density values were found to be incorrect. This may be because the properties of the Nygaard Triosil 440 has been altered slightly in the intervening period since their publication. The procedure described here has been found to be reliable and reproducible for the past 4 years.

Although lacking the resolution of a continuous gradient, the method combines procedural economy with the convenience of separating fractions of a size suitable for subsequent analysis, except for some small pathological samples, where fractions from the ends of the gradient had to be combined because of the small numbers of cells obtained. The density distribution of all the samples tested was within the same range as was observed by Leif & Vinograd (1964) and Turner *et al.* (1974) for normal adults. Thus our data indicate that from childhood onwards for both sexes there is no marked difference in the age/density profile of red cells, although the possibility of differences in very young and very old people remains to be investigated. Similarly, no differences were found with Duchenne dystrophy, the more benign but also sex-linked Becker dystrophy, the autosomally inherited limb-girdle dystrophy or spinal muscular atrophy. A difference was found (results not shown) in a single sample from a 12-year-old boy with myotonic dystrophy (autosomal dominant inheritance) in which the cells had a mean density of 1.1158 g/cm^3 , which falls outside $\pm 2\sigma$ for normal cells. This merits further investigation, as membrane involvement is clearly recognized in this disease (see, e.g. Plishker *et al.*, 1978).

The normal distribution of cells as a function of density means that the cells do not change density at a steady continuous rate throughout their life, but rather that the change is most rapid in young and old cells, with a relatively quiescent period during the middle of the life-span. Hence the time scale is a non-linear function of the linear density scale, and this has to be borne in mind in the calculation of enzyme half-lives by this procedure. The procedure adopted here is in line with that used by other workers (Table 3), so that direct comparison can be made.

The proportion of reticulocytes in the gradient decreases exponentially with increasing density (Fig. 2) and showed no significant change with age, sex or in the four neuromuscular diseases with normally distributed cell populations. The theoretical position in the gradient corresponding to 100% reticulocytes was found to occur at -4.35σ , and the calculated theoretical activities of three enzymes when the red cell first enters the bloodstream are listed in Table 3.

The values for glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are markedly higher than the average values obtained from unfractionated blood samples (Table 2). The slightly higher glucose 6-phosphate dehydrogenase activity found with children than with adults is probably not significant. The activities of the three enzymes investigated showed similar ranges in unfractionated cells with all the samples tested (Table 2).

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