Biochemical changes during reticulocyte maturation in culture

A comparison of genetically different sheep erythrocytes

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A simple culture technique was used to follow sheep reticulocyte maturation. Cells remained in good condition without lysis for at least 7 days and maintained normal intracellular ATP concentrations. Amino acid and nucleoside transport were measured during maturation together with changes in reduced glutathione (GSH), amino acid, K^+ and Na^+ concentrations. Reticulocytes of the genetic types high- K^+ and low- K^+ ; high-GSH and low-GSH; nucleoside-permeable and nucleoside-impermeable were indistinguishable both in transport properties and cellular constituents. Cell maturation was associated with marked decreases in L-alanine transport (Na^+ -dependent and independent), uridine transport and GSH and K^+ concentrations. Typical mature-cell levels for all parameters were reached within the culture period, during which time predicted differences between cells of different genotypes became apparent. Loss of amino acid-transport activity from low-GSH cells was accompanied by an increase in intracellular ornithine and lysine concentrations. The rates at which different parameters changed during culture were compared.

In its mature state the sheep erythrocyte possesses a number of different genetically determined biochemical variants. One well-known polymorphism involves intracellular concentrations of potassium, whereby some sheep have erythrocytes with high K^+ and low Na^+ concentrations, and others have low K^+ and high Na^+ concentrations (Tucker, 1971; Ellory, 1977). A second polymorphism concerns GSH. Some sheep have lower than normal concentrations of GSH in their erythrocytes (low-GSH type) resulting from an impaired transport system for small neutral amino acids (Tucker & Kilgour, 1970; Young *et al.*, 1976; Young & Ellory, 1977*a,b*). These cells are also unusual in that they accumulate relatively high concentrations of the dibasic amino acids lysine and ornithine, in contrast with erythrocytes with normal amino acid transport (high-GSH type) (Ellory *et al.*, 1972). A third genetic polymorphism lies in the relative ability of the erythrocytes to transport nucleosides. The mature erythrocytes of most sheep are unable to transport nucleosides (nucleoside-impermeable type), but a small proportion of animals have erythrocytes that are capable of nucleoside transport (nucleoside-permeable) (Young, 1978; Jarvis & Young, 1978).

A common characteristic of the phenotypes

Abbreviations used: GSH, reduced glutathione; Hb, haemoglobin.

mentioned above is that they are not distinguishable from each other at the reticulocyte stage of development (Tucker & Ellory, 1971; Tucker & Kilgour, 1973). Reticulocytes also possess higher overall transport rates than are ever found in mature erythrocytes. Their maturation therefore presents a convenient system for studying the genetic control of biochemical changes during cell development. It is, however, difficult to monitor these changes *in vivo*, because cell populations of different ages are present in the blood during recovery from anaemia. For this reason we have devised a culture technique that enables reticulocyte maturation to be followed *in vitro*. The present paper describes the technique and presents initial findings on some of the biochemical changes occurring during the final maturation process from reticulocyte to erythrocyte in sheep of different phenotypes. A preliminary account of this study has already been published (Tucker *et al.*, 1979).

Materials and methods

Selection of sheep

Sheep of appropriate genotype were selected from the special flocks maintained at Babraham for blood-group studies (see Tucker, 1971; Tucker *et al.*, 1976; Young, 1978).

Preparation of reticulocytes

Adult sheep were made anaemic by the removal of 800–1000 ml blood per day from the jugular vein. Approx. 4 days later, when the circulating reticulocyte count was 6–8%, 300–500 ml of blood was withdrawn. By using sterile technique throughout, the blood was filtered three times through glass wool to remove platelets and leucocytes. The cells were then washed in 0.9% NaCl, resuspended in an equal volume of 0.9% NaCl and the reticulocytes were separated from the mature erythrocytes by centrifugation in polythene tubes in a bench centrifuge as described previously (Tucker & Ellory, 1971). The top quarter of each cell column was collected and pooled. These cells were resuspended in 0.9% NaCl and spun again in polythene tubes. The top quarter of each packed-cell column was again harvested and pooled. This process yielded about 3.0 ml of packed cells consisting of 80–90% reticulocytes.

Culture technique

The reticulocytes were suspended in 100 vol. of pre-warmed culture medium composed of 80% (v/v) RPMI 1640 (Flow Laboratories, Irvine, Scotland, U.K.), 20% (v/v) foetal bovine serum (mycoplasma and virus screened; Flow Laboratories), 0.66 mM-L-glutamine, Crystamycin to give a final concentration of 50 units of penicillin/ml and 50 µg of streptomycin/ml (Glaxo Laboratories, Greenford, Middx., U.K.), and Fungizone to give a final concentration of 2.5 µg/ml (E. R. Squibb and Sons, New York, U.S.A.). The cells in culture medium were dispensed in 10.0 ml portions into a series of disposable plastic culture vessels (38 ml total capacity) and incubated at $38 \pm 0.5^\circ\text{C}$ for up to 11 days. The cultures were mixed gently twice daily. One or more culture vessels were removed from the incubator when required for cell counts and biochemical determinations. The cells were washed free of culture medium before assay.

Haematology

Red cell counts were carried out microscopically with a Neubauer counting chamber. Haemoglobin was estimated as cyanmethaemoglobin. Reticulocytes were stained supravitaly with Brilliant Cresyl Blue (Archer, 1965).

K⁺, Na⁺ and GSH determinations

K⁺ and Na⁺ were measured by flame photometry after the cells had been washed thoroughly in ice-cold 150 mM-choline chloride containing 10 mM-Tris/HCl (pH 7.5 at 20°C). GSH was determined by using 5,5'-dithiobis-(2-nitrobenzoate) as chromogen (Beutler *et al.*, 1963).

L-Alanine transport

Initial L-[U-¹⁴C]alanine influx rates (extracellular

concentration 0.2 mM, 3 min incubation at 37°C) were determined in the presence and absence of Na⁺ as described previously (Young *et al.*, 1976) with choline to replace Na⁺.

Uridine transport

Initial [U-¹⁴C]uridine uptake rates (1 mM, 30 s incubation at 37°C) in Na⁺-containing medium were determined as described previously (Young, 1978).

ATP

This was estimated by a spectrophotometric NADH-enzyme assay (Boehringer Test Combination, Diagnostics).

Amino acids

Amino acid concentrations were determined in sulphosalicylic acid extracts of cells by using a Locarte amino acid analyser.

Calculation of maturation half-times

Maturation rates for different biochemical parameters were compared by graphical estimation of the time required to reach an activity of $(A_0 + A_f)/2$, where A_0 is the total activity at the beginning of the culture and A_f the final stabilized activity. In cases where a stable activity was not reached by the end of the culture period, A_f was taken as the activity normally found in mature circulating erythrocytes from animals of the appropriate genotype.

Results

Cultures were set up with cells from three sheep with different combinations of genetic types, namely (i) high-K⁺, low-GSH, nucleoside-impermeable (sheep 6X59); (ii) low-K⁺, low-GSH, nucleoside-impermeable (sheep 7X4); (iii) low-K⁺, high-GSH, nucleoside-permeable (sheep 7F207). The culture of cells from sheep 6X59 was continued for 3 days only, but samples were tested at 5, 10, 16, 21 and 28 h to study the cells over the time at which the greatest changes were taking place. Cultures from sheep 7X4 and 7F207 were studied for 7 and 11 days respectively.

Maturation of reticulocytes

All three cultures started with at least 80% reticulocytes. Reticulocyte counts in cultures 7X4 and 7F207 fell rapidly, no reticulocytes being found after 60 h incubation (Fig. 1). Total cell counts remained relatively constant, indicating that the observed loss of reticulocytes was due to cell maturation. In culture 6X59 the maturation process was slower, 24% reticulocytes still being present at day 3. For all three cultures, the reticulatin content of the reticulocytes became progressively less as the cells matured. At the time the cultures were

Fig. 1. *Reticulocyte maturation in culture*

Total cell counts (O) and reticulocyte counts (●) were measured in cultures from three different sheep [(a) 6X59, 87% reticulocytes at time zero; (b) 7X4, 80% reticulocytes; (c) 7F207, 80% reticulocytes] as described in the Materials and methods section. Changes in other parameters are given in Figs. 2–6.

Fig. 2. *Changes in Na^+ and K^+ concentrations during reticulocyte maturation*

Intracellular Na^+ (O), K^+ (●) and total ($\text{Na}^+ + \text{K}^+$) (■) concentrations of (a) 6X59 (high- K^+), (b) 7X4 (low- K^+) and (c) 7F207 (low- K^+) cultures were determined as described in the text.

terminated all were of good appearance, the cells being well oxygenated and the pH neutral. The cells also handled well during the tests. ATP concentrations in 6X59 cells measured over the first 28 h of culture were 7.06, 6.92, 4.22, 3.83, 4.24 and 3.86 $\mu\text{mol/g}$ of Hb at 0, 5, 10, 16, 21 and 28 h respectively. In 7F207 cells, even after 11 days culture, ATP concentrations were still 2.07 $\mu\text{mol/g}$ of Hb.

K^+ and Na^+ concentrations

Mean K^+ and Na^+ concentrations for the two low- K^+ cell preparations (each composed initially of 80% reticulocytes) were 0.388 and 0.123 mmol/g of Hb respectively. The K^+ and Na^+ concentrations for

the high- K^+ preparation (87% reticulocytes) were 0.430 and 0.078 mmol/g of Hb respectively. In the two low- K^+ cultures (7X4 and 7F207) loss of K^+ was accompanied by a concomitant rise in intracellular Na^+ concentration (Fig. 2). In the high- K^+ cells (6X59) both K^+ and Na^+ concentrations fell. Total K^+ and Na^+ showed an overall fall in concentration in both low- K^+ and high- K^+ cultures as the cells matured.

L-Alanine transport and GSH concentrations

Reticulocytes of both low- and high-GSH phenotypes showed a high permeability to L-alanine that was largely Na^+ -dependent (Fig. 3). The ability to transport L-alanine was lost in the two low-GSH

Fig. 3. Changes in amino acid transport during reticulocyte maturation

Initial L-alanine influx rates (extracellular concentration 0.2 mM) [(a) $-Na^+$; (b) $+Na^+$] of cells from (●) 7F207 (high-GSH), (○) 6X59 (low-GSH) and (△) 7X4 (low-GSH) cultures were measured as described in the Materials and methods section.

Fig. 4. Intracellular GSH concentrations during reticulocyte maturation

Intracellular GSH concentrations of cells from (●) 7F207 (high-GSH), (○) 6X59 (low-GSH) and (△) 7X4 (low-GSH) cultures were measured as described in the text.

cultures (6X59 and 7X4) within 45 h. Amino acid transport by the high-GSH type cells (7F207) also decreased rapidly, but stabilized at a value characteristic of mature high-GSH type erythrocytes. This residual flux was not Na^+ -dependent. GSH

Fig. 5. Uridine transport during reticulocyte maturation

Initial uridine influx rates (extracellular concentration 1 mM) of cells from (●) 7F207 (nucleoside-permeable), (○) 6X59 (nucleoside-impermeable) and (△) 7X4 (nucleoside-impermeable) cultures were determined as described in the Materials and methods section.

concentrations decreased more slowly than did L-alanine transport, reaching similar low levels in the two cell types (Fig. 4).

Uridine transport

In the two nucleoside-impermeable cell cultures (6X59 and 7X4) there was still some residual uridine transport activity present after 2 days of culture (Fig. 5). However, these fluxes were 4-fold lower than that for nucleoside-permeable cells (7F207) at the same stage of culture. Uridine transport in the nucleoside-permeable culture had stabilized by day 5. By this time, no uridine transport could be detected in the 7X4 culture.

Dibasic amino acid concentrations in low-GSH reticulocytes

Fig. 6 shows the ornithine, lysine and arginine concentrations of low-GSH type cells in culture (6X59). The hatched area indicates the amino acid contribution from mature erythrocytes already present in the culture (initial reticulocyte count 87%). For both ornithine and lysine the concentration rose within the first 30 h of culture and then slowly declined. The initial rise coincided with the loss of amino acid transport activity, and the levels reached between 20 and 60 h in culture were comparable with those normally found in fully mature low-GSH type cells. Arginine concentrations were considerably lower than those of ornithine and lysine, rose slightly within the first 5 h of culture, and then declined slowly to zero. As a control, amino

Fig. 6. Intracellular dibasic amino acid concentrations during maturation of low-GSH cells in culture. Ornithine (●), lysine (○) and arginine (▲) concentrations were determined by using a Locarte amino acid analyser. The hatched area represents the ornithine and lysine contributions (each approx. 3 μmol/g of Hb) from mature erythrocytes already present in the culture (6X59, initial reticulocyte count 87%). The L-arginine contribution of normal mature sheep erythrocytes is negligible.

acid concentrations were measured in 7F207 cells (high-GSH) both before and after 40h in culture. Ornithine, lysine and arginine concentrations were <0.5, 1.6 and 3 μmol/g of Hb respectively for reticulocytes (80%) and 1.2, 2.2 and 3.7 μmol/g of Hb respectively for cells after 40h in culture.

Relative loss of transport activity

The above results were analysed to determine the relative rates at which these parameters changed during the transition from reticulocyte to mature state (Table 1). Also included in Table 1 are data from two preliminary experiments (6F66 cells: type high-K⁺, high-GSH, nucleoside-permeable; 5X56 cells: type low-K⁺, low-GSH, nucleoside-impermeable) where only partial separation of reticulocytes from mature cells was achieved (45 and 31% reticulocytes, respectively). The half life of reticulocytes was, on average, 15 h, but by this time, most of the Na⁺-dependent and Na⁺-independent amino acid transport activity had been lost. In contrast, loss of uridine transport activity was generally slower than the reticulocyte maturation rate. The intracellular GSH concentration decreased even more slowly. The probable relative rate of loss is: L-alanine (Na⁺-dependent, Na⁺-independent) > reticulocytes > uridine > GSH. Amino acid transport activity was lost at similar rates in high- and low-GSH cells. Similarly, no obvious difference

Table 1. Relative rates of change of the parameters measured during maturation. Data for 6X59, 7X4 and 7F207 cells were calculated from Figs. 1-5.

Cells from animal	Genetic type	Reticulocytes at time zero (%)	Parameter ...	Half-time for maturation (h)						Relative rate of loss
				L-Alanine transport		Reticulocytes	Uridine transport	Concentration of		
				+Na ⁺	-Na ⁺			GSH	K ⁺	
5X56	Low-GSH, low-K ⁺ , nucleoside-impermeable	31		8	10	18	11	22	—	Alanine > uridine > reticulocytes > GSH
6F66	High-GSH, high-K ⁺ , nucleoside-permeable	45		<11	<11	12	18	27	—	Alanine > reticulocytes > uridine > GSH
6X59	Low-GSH, high-K ⁺ , nucleoside-impermeable	87		8	11	17	20	25	4	K ⁺ > alanine > reticulocytes > uridine > GSH
7X4	Low-GSH, low-K ⁺ , nucleoside-impermeable	80		<11	<11	14	—	25	36	Alanine > reticulocytes > GSH > K ⁺
7F207	High-GSH, low-K ⁺ , nucleoside-permeable	80		<23	<23	23	28	33	94	Alanine, reticulocytes > uridine > GSH > K ⁺

was found in the rate at which nucleoside transport activity was lost from nucleoside-permeable and nucleoside-impermeable cultures. The relatively high K^+ content of mature sheep erythrocytes made it difficult to estimate with any degree of accuracy the rate of K^+ loss from reticulocytes in the two preliminary experiments (5X56, 6F66). Of the remaining three cultures, K^+ was lost at the same rate as, or faster than, L-alanine transport activity in the high- K^+ culture (6X59) but slower than GSH in the two low- K^+ experiments (7X4, 7F207).

Discussion

The present series of experiments demonstrate that the process of reticulocyte maturation can be followed successfully in culture. The results also confirm that the sheep erythrocyte K^+ , amino acid and nucleoside-transport phenotypes are not distinguishable from each other at the reticulocyte stage of development. For both L-alanine and uridine, reticulocyte maturation was associated with marked and reproducible decreases in transport activity. Predicted transport differences between cells of different genotypes were clearly visible at the time the cultures were terminated. Intracellular Na^+ and K^+ concentrations also reached typical adult levels, indicating that active K^+ transport rates similarly declined over this period of time. Intracellular GSH levels also dropped, but there were no obvious differences in GSH concentration between high-GSH and low-GSH type cells after culture. This agrees with earlier studies *in vivo* that found that erythrocyte GSH concentrations in high-GSH-type sheep fell below normal levels during recovery from anaemia, whereas in low-GSH-type sheep, values were higher than normal (Tucker & Kilgour, 1973). It may therefore be concluded that reticulocytes in culture undergo changes similar to those that occur *in vivo*, at least for the parameters considered in the present study.

L-Alanine transport activity was lost more rapidly than uridine transport, suggesting that amino acid and nucleoside transport have different regression schedules. This extends an earlier study *in vivo* by Antonioli & Christensen (1969) that provided evidence that amino acid transport systems in rabbit reticulocytes were lost at different rates during cell maturation. GSH concentrations decreased more slowly than any of the above parameters. Loss of Na^+ -dependent amino acid transport activity in low- K^+ type cells preceded intracellular cation changes. Similar changes in Na^+ -dependent glycine transport and Na^+ independent accumulation of large neutral amino acids have been observed by Benderoff *et al.* (1978a,b).

The amino acid transport system (the C system) that is functionally absent from sheep low-GSH

erythrocytes, although having L-cysteine and L-alanine as its optimal substrates, is also the major route for dibasic amino acid transport in normal sheep erythrocytes (Young *et al.*, 1976; Young & Ellory, 1977a; Young *et al.*, 1980). It is therefore likely that the absence of this system from low-GSH cells is responsible for the intracellular accumulation of dibasic amino acids. However, the origin of these amino acids is unknown. One possibility we have considered is that they are derived from the substantial protein degradation that occurs during reticulocyte maturation (Rapoport *et al.*, 1974). The observation that arginine replaces ornithine in low-GSH cells that are also arginase-deficient (Tucker *et al.*, 1977) and the present finding that intracellular amino acids appear within the first 30 h of culture are both consistent with this idea. Our culture technique *in vitro* offers a suitable system to investigate this and other aspects of reticulocyte maturation.

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