Biochemical and Enzymic Changes during Erythrocyte Differentiation THE SIGNIFICANCE OF THE FINAL CELL DIVISION

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1. The haemoglobin content of developing erythroblasts was shown to increase rapidly when the cells completed the final cell division of erythroid development and passed from the dividing into the non-dividing cell compartment. 2. The activity of carbonic anhydrase was measured and shown to increase continually throughout erythroid differentiation. The activity increased most rapidly in the polychromatic stage. 3. Catalase activity did not increase significantly during erythroid differentiation until the reticulocyte stage. 4. The activity of four enzymes, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, adenosine deaminase and nucleoside phosphorylase, exhibited a similar pattern of change during erythroid differentiation. In the dividing cell compartment their activity was relatively high but exhibited a steep decline between the polychromatic stage and the orthochromatic stage, that is, as the cell completed its final cell division and moved from the dividing to the non-dividing compartment. After this the activity of these enzymes was stabilized at a relatively low value, and this activity persisted at such a value until the reticulocyte stage. 5. Lactate dehydrogenase activity also declined after the cell had crossed from the dividing into the non-dividing stage, but in this case the decline was less than in the case of the above four enzymes. 6. Adenylate kinase activity was relatively constant in the dividing cell compartment but exhibited a 60% increase when the cell passed from the dividing into the non-dividing compartment. 7. The cessation of cell division appears to coincide with a set of complex biochemical changes.

The mature mammalian erythrocyte is probably one of the most intensively investigated of cells. A great deal is known about its physiology, biochemistry, metabolism, genetics and structure, and the synthesis of its major protein, haemoglobin, has served as a model system for the study of mammalian protein synthesis. However, as far as its differentiation in the bone marrow is concerned, although something is known of the cytology and cell kinetics of the process, we are still to a large extent ignorant of even the most basic facts of the organization and sequence of biochemical change that must be responsible for the dramatic morphological reconstruction which, in a space of about 70h, converts the round nucleated haematopoietic stem cell into the mature anucleate plate-shaped erythrocyte.

Unfortunately, a biochemical description of the process of erythroid differentiation is dependent on the solution of the difficult technical problem common to most other developing system, that of the physical separation of cells at different stages of development, both from one another and from cells of different types and their subsequent collection in sufficient quantities to permit accurate biochemical characterization. The development of a suitable technique for this purpose is an essential prerequisite

to any advance in understanding the complex molecular events of differentiation. Until this problem is solved most models of differentiation will remain highly speculative, and at present there is still hardly any information about the detailed sequence and organization of chemical change that occurs in any case of differentiation. One exception to this is possibly the case of the cellular slime mould (Newell, 1971).

Various attempts have been made to fractionate developing erythroblasts. Fantoni *et al.* (1969) utilized the fact that the circulating erythroid cells of the foetal mouse increase in maturity day by day during certain stages of embryonic development. Others have attempted to collect synchronized fractions of developing erythroid cells on various days after actinomycin injections (Hershko *et al.*, 1969). The physical techniques of density centrifugation (Borsook *et al.*, 1969) and velocity sedimentation (McCool *et al.*, 1970) have also been used in an attempt to separate developing erythroblasts.

We have used the velocity-sedimentation technique to separate erythroblasts at different stages of development (Denton & Arnstein, 1973). The resolution obtainable by this method is especially satisfactory for cells at later stages of differentiation and

complete separation of erythroblasts before and after the final cell division was achieved. With this technique we have been able to characterize some of the enzymic and biochemical changes that occur during erythroid differentiation. The results indicate that the period of time when the erythroblast moves from the dividing into the non-dividing cell compartment is a time of complex biochemical reorganization.

Experimental

Materials

The animals were white New Zealand rabbits of 2 kg body weight. Ficoll used in the preparation of the gradient for cell separation was obtained from Pharmacia, London W.5, U.K. The enzymes and substrates used in the assay systems were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. All other chemicals were AnalaR grade or the best preparations commercially available.

Treatment of animals

The animals were made anaemic by giving them five daily injections of neutralized phenylhydrazine hydrochloride [0.3 ml of 2.5% (w/v) phenylhydrazine/kg body wt.] (Arnstein *et al.*, 1964). The animals were killed on the third day after cessation of injections. A bone-marrow-cell suspension was prepared from the animal, which contained approx. 95% erythroid cells (Denton & Arnstein, 1973).

Cell separation

The erythroid-cell suspension was subjected to fractionation by the velocity-sedimentation technique. Separation depends on the fact that during erythroid development there is a continual decrease in cell size from the large 15 μ m-diameter basophilic erythroblast to the 8 μ m-diameter reticulocyte. At unit gravity the sedimentation velocity of a cell is approximately proportional to its size. Thus the more mature the erythroblast the more slowly it will sediment at unit gravity. Complete separation between the dividing and non-dividing cells is possible because at the final cell division during erythroid differentiation the cell volume is halved and during subsequent maturation to the reticulocyte the cell size continues to decline.

The details of the technique and apparatus, including preparation of the gradient and the loading and emptying of the sedimentation chamber, were similar to those described by Denton & Arnstein (1973), but instead of a square Perspex chamber with a cross-sectional area of 2500 cm² a larger round metal chamber with a cross-sectional area of 5000 cm² was used. The larger size permitted the separation of

up to 1.5×10^9 cells (1.5 ml packed cell volume). After the cells had sedimented at unit gravity for 4.5 h 30 fractions (each 500 ml) were collected from the chamber. The first fraction contained large immature erythroblasts that had sedimented nearly to the bottom of the chamber. The following 29 fractions consisted of successively smaller and more mature erythroid cells. Fractions 1–18 contained the cells of the dividing compartment and the remaining fractions the non-dividing cells. Fractions 1–8 consisted mainly of basophilic cells, fractions 9–18 mainly of polychromatic cells, fractions 19–25 of orthochromatic cells and fractions 25–30 of reticulocytes (see Fig. 1). The orthochromatic erythroblasts (fractions 19–25, Fig. 1) comprise all non-dividing nucleated cells, including those termed 'small polychromatic cells' by Denton & Arnstein (1973). The cells in each fraction were spun down and lysed by hypo-osmotic shock by resuspending them in 4 vol. of water. The nuclei, mitochondria and membranes were sedimented by centrifuging at 10000g for 15 min and the cytoplasmic lysates examined for haemoglobin and a variety of enzymic activities.

Protein and haemoglobin content

Protein concentration in the lysate was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. Haemoglobin concentration was determined by the method of Drabkin (1945).

Enzyme assays

All spectrophotometric measurements were made with a Unicam SP.800 recording spectrophotometer in silica cuvettes of 1 cm light-path and 2 ml minimum volume unless otherwise stated.

Carbonic anhydrase activity (EC 4.2.1.1). Carbonic anhydrase activity was assayed by measuring the rate of colour change of Bromothymol Blue indicator in a CO₂-saturated buffer. The details of the assay system and the method of calculating enzyme activity are given by Rickli *et al.* (1964). The activity of carbonic anhydrase was expressed as enzyme units per mg of protein, the unit (E.U.) being derived from the equation, $E.U. = \frac{t_0 - t}{t}$ where t and t_0 are the reaction

times in the presence and absence of catalyst respectively required to decrease the pH to 6.3.

Catalase activity (EC 1.11.1.6). Catalase activity was determined by the method of Aebi & Suter (1969). The assay is based on measurement of the decrease of E_{240} during enzymic decomposition of H₂O₂. The details of the assay and the method of calculating the velocity constant of the reaction are given by Aebi & Suter (1969). The velocity constant k was related to

the protein content in the cell lysates and was expressed as velocity constant per g of protein. Before commencing the assay the concentration of protein in the cell lysates was diluted to 100 μg per ml. The measurements were carried out at 20°C.

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.43) activities. Glucose 6-phosphate dehydrogenase was assayed by a method based on that of Glock & McLean (1953). Glucose 6-phosphate dehydrogenase activity is given by the difference between combined glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities obtained in the presence of glucose 6-phosphate and 6-phosphogluconate substrates and the 6-phosphogluconate dehydrogenase activity obtained in the presence of 6-phosphogluconate alone as substrate. The details of the assay mixture are given by Harris *et al.* (1963). 6-Phosphogluconate dehydrogenase activity was determined in an identical system except for the omission of the glucose 6-phosphate. In all cases the reaction was started by the addition of NADP⁺ and the change in E_{340} was determined. Enzyme activity is expressed as nmol of NADP⁺ reduced/min per mg of protein.

Lactate dehydrogenase activity (EC 1.1.1.27). The lactate dehydrogenase activity was determined by the method of Wroblewski & Ladue (1955), which depends on measuring the change in E_{340} when pyruvate is converted into lactate in the presence of NADH. The activity is expressed as nmol of NADH converted/min per mg of protein.

Purine nucleoside phosphorylase activity (EC 2.4.2.1). The assay method is based on the determination of hypoxanthine formed during the phosphorylation of inosine by purine nucleoside phosphorylase. The hypoxanthine is oxidized to uric acid by xanthine oxidase and the formation of uric acid followed by measuring the increase in E_{293} . The details of the method were given by Price *et al.* (1955). Enzyme activity is expressed as nmol of inosine converted/min per mg of protein.

Adenosine deaminase activity (EC 3.5.4.4). This was measured by using essentially the method of Kalckar (1947) as given by Osborne & Spencer (1973). Reaction rates as measured by the decrease in E_{265} were proportional to enzyme concentration. The specific activity is expressed as the number of nmol of adenosine converted/min per mg of protein.

Adenylate kinase activity (EC 2.7.4.3). This was measured in the direction $2\text{ADP} \rightarrow \text{AMP} + \text{ATP}$ by using the coupled assay system of Oliver (1955). The details of the method are given by Brownson & Spencer (1972). The reaction was initiated by the addition of enzyme solution. Measurements were made using semi-micro cuvettes with a 1 cm light-path. Adenylate kinase activity is expressed as μmol of ATP produced/min per mg of protein.

Results

Haemoglobin content

As the erythroid cell matures from the basophilic to the polychromatic compartment the haemoglobin concentration in the cytoplasm increases from 100 to 300 $\mu\text{g}/\text{mg}$ of protein (Fig. 1). Even the largest, most immature erythroblasts in fraction 1 contain considerable quantities of haemoglobin, and this agrees with cytological observations of early erythroblasts (Grasso *et al.*, 1963) and the fact that the synthesis of haemoglobin commences shortly after commitment of the erythropoietin-sensitive cell in the marrow (Hodgson, 1970). An erythroid cell spends 50h and undergoes several cell divisions in the dividing compartment (Tarbutt, 1969) and at the end of this period it has accumulated about one-third of its final complement of haemoglobin. After the cell undergoes the final cell division, leaving the polychromatic compartment to enter the non-dividing orthochromatic compartment, the concentration of haemoglobin increases rapidly from 300 to 600 $\mu\text{g}/\text{mg}$ of protein. Thus, during the 10h after the final cell division and passage through the orthochromatic compartment the concentration increases by as much again as in the previous 50h. The period after the final cell division therefore coincides with important changes in the control of haemoglobin synthesis.

Carbonic anhydrase activity

Carbonic anhydrase activity rises continually from fraction 1 to fraction 30, although the overall increase

Fig. 1. Haemoglobin content of cytoplasmic lysates of separated developing erythroblasts

Cells were separated by velocity sedimentation at unit gravity and the cell types present in the main regions of the gradient are indicated. Experimental details are given in the text.

Fig. 2. *Carbonic anhydrase and catalase activity of cytoplasmic lysates of developing erythroblasts*

●, Carbonic anhydrase; ▲, catalase. Enzyme assays were carried out on cells fractionated by velocity sedimentation at unit gravity, details being given in the text. The cells present in different fractions of the gradient are as shown in Fig. 1.

Fig. 3. *Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity of cytoplasmic lysates of separated developing erythroblasts*

●, Glucose 6-phosphate dehydrogenase; ▲, 6-phosphogluconate dehydrogenase. Enzyme assays were carried out on cells fractionated by velocity sedimentation at unit gravity, details being given in the text. The cells present in different fractions of the gradient are as shown in Fig. 1.

is not as great as that of haemoglobin (Fig. 2). Unlike haemoglobin content, the greatest rise in carbonic anhydrase activity occurs in the polychromatic stage. The fall in activity round about fraction 20 may be due to a dilution effect caused by the enormous increase in haemoglobin concentration at this time. The blood cells of most mammals, including rabbits, contain two isoenzymes of carbonic anhydrase, isoenzymes CA1 and CA2, and in the rabbit erythroid cell the specific activity of isoenzyme CA2 is several times that of CA1 (McIntosh, 1970). This means that most of the carbonic anhydrase activity detected in rabbit erythroid cell lysates is probably due to isoenzyme CA2. The fact that the activity of carbonic anhydrase continues to rise until the reticulocyte stage suggests that at least the synthesis of isoenzyme CA2 continues until this stage of maturity. The increase in carbonic anhydrase activity observed in the polychromatic cells suggests that a burst of isoenzyme CA2 synthesis occurs at this stage of erythroid differentiation. A similar conclusion can be inferred from the results of Desimone *et al.* (1973) with the monkey.

Catalase activity

The catalase activity is almost constant from the basophilic to the orthochromatic compartment, after which an increase in activity occurs at the reticulocyte stage (Fig. 2). The physiological significance of catalase in the mature cell is controversial (Aebi & Suter, 1969). However, the rise in catalase activity towards the later stages of development suggests that it does play some adaptive role in the erythrocyte.

Activity of glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, adenosine deaminase and purine nucleoside phosphorylase

The activity of each of these four enzymes exhibits a similar pattern of change during erythroid development (Figs. 3, 4 and 5). Their activity is almost constant in the dividing cell compartment. After the cell undergoes the final cell division, leaving the polychromatic compartment to enter the orthochromatic, the activity of all four enzymes declines three- to five-fold. The decline ceases in the orthochromatic compartment and remains relatively constant throughout the non-dividing compartment as the cell matures into a reticulocyte.

The decline in activity of the above enzymes that occurs between the polychromatic and orthochromatic stage is unlikely to be due to a passive dilution effect but more probably arises from some type of actively controlled process. Assuming that the time taken for a cell to move from the polychromatic to the orthochromatic stage is approx. 10h, and assuming further that their enzymic activity only decreases marginally during the life-span of the erythrocyte, a period of approx. 100 days, then their rate of decline in activity over this short period is nearly 10^3 times faster than in peripheral blood cells. If such a decline continued for a few hours longer then the mature erythrocyte would be totally lacking in the activity of these enzymes. The decline must be tightly controlled, because in the case of glucose 6-phosphate dehydrogenase (Fig. 3), which catalyses the initial step in the pentose phosphate shunt producing NADPH, a certain activity is essential for the integrity of the mature erythrocyte (Motulsky & Yoshida, 1969).

The physiological significance of the parallel decline in activity of all the four enzymes is probably their common implication in the so-called 'salvage pathway' (Murray, 1971), which is considered to be the major source of nucleotides utilized by dividing erythroblasts in the bone marrow (Lajtha & Vane, 1958). This source of nucleotides is unnecessary after the final cell division and cessation of DNA synthesis. The molecular organization which may be responsible for coincidental changes in different enzyme systems is not difficult to envisage. Many metabolic pathways are interrelated, and shifting distributions and concentrations of substrates and cofactors, which presumably occur as an automatic outcome of the changes of differentiation, may lead to a variety of effects on a wide spectrum of different enzymes. Change in the concentration of a few key intermediaries could lead to coincidental breakdown of a number of different enzymes which function in only distantly related metabolic pathways. It is well known that the substrate concentration can alter the stability of an enzyme, and in addition enzymes may be multifunctional (Reed, 1969). Thus it is relatively easy to imagine the destabilization of only a few multienzyme systems leading to coincidental degradation of a variety of different enzymes.

The persistence of a low activity of adenosine deaminase (Fig. 5) and purine nucleoside phosphorylase (Fig. 4) in the non-dividing compartment may be because of their role in nucleotide turnover in the mature erythrocyte (Mager *et al.*, 1967), which is itself part of the design of the erythrocyte as a purine-transporting system (Henderson & Le Page, 1959).

Lactate dehydrogenase activity

The activity of lactate dehydrogenase remains almost constant throughout the dividing compartment. After the final cell division the activity continually declines (see Fig. 4). The reason for the relatively slower decline in lactate dehydrogenase activity compared with the other enzymes connected with glucose metabolism might be that whereas the phosphate shunt is no longer required as a source of ribose 5-phosphate, 95% of glucose in the mature erythrocyte is metabolized via the Embden-Meyerhof pathway (Murphy, 1960). Most of the pyruvate utilized by the early erythroblasts is probably metabolized via the tricarboxylic acid cycle, which is not available after loss of mitochondria, and therefore persistence of a relatively high lactate dehydrogenase activity may assist in pyruvate metabolism in the mature cell.

Adenylate kinase activity

The activity of adenylate kinase is almost constant in the dividing cell compartment. A considerable increase in activity occurs after the cell has undergone the final cell division and entered the orthochromatic compartment (Fig. 5). The physiological significance of this increase in the activity of adenylate kinase in the non-dividing compartment may be related to the general preponderance of adenine nucleotides in the mature cell (Hershko & Mager, 1971) and the importance of ATP regeneration from ADP during glycolysis (Brewer, 1969).

Fig. 4. Lactate dehydrogenase and purine nucleoside phosphorylase activity of cytoplasmic lysates of separated developing erythroblasts

▲, Lactate dehydrogenase; ●, nucleoside phosphorylase. Enzyme assays were carried out on cells fractionated by velocity sedimentation at unit gravity, details being given in the text. The cells present in different fractions of the gradient are as shown in Fig. 1.

Fig. 5. Adenylate kinase and adenosine deaminase activity of cytoplasmic lysates of separated developing erythroblasts

▲, Adenylate kinase; ●, adenosine deaminase. Enzyme assays were carried out on cells fractionated by velocity sedimentation at unit gravity, details being given in the text. The cells present in different fractions of the gradient are as shown in Fig. 1.

Discussion

The results demonstrate that the activity of a number of enzymes and the concentration of haemoglobin varies considerably over the period when the erythroid cell moves from the dividing into the non-dividing compartment. Only catalase and carbonic anhydrase do not exhibit any major change in their activity at this time. As well as the biochemical changes reported here we have shown that a variety of other biochemical changes occur over the same period. These include changes in the pattern of ribonuclease activity, a large-scale mobilization of iron from the stromal compartment of the cell, as well as a considerable amplification of haemoglobin synthesis (M. J. Denton, H. T. Delves & H. R. V. Arnstein, unpublished work).

These biochemical changes occur at a time when the morphology of the cell is undergoing its most rapid period of change. Temporal correlation of enzymic, functional and morphological change has been discussed by Needham (1931). Flexner *et al.* (1953) examined enzymic change in the developing rodent brain and demonstrated that the activity of certain enzymes changes rapidly over a short period of time, termed by them 'a critical period', coincident with sudden functional and morphological change interspersed with longer intervals of relative quiescence. Presumably during a quiescent period a differentiating system becomes preset in preparation for the sudden functional and morphological changes in the next crucial phase. In the developing liver, enzyme activities do not rise at an even pace over a prolonged period of weeks, but all or most of the amount present in adult tissues accumulates suddenly within hours or days (Greengard, 1971). The emergence of new enzymes or the increase in activity of enzymes already present in low concentration is not evenly distributed along the age axis but occurs in clusters, giving the impression of a succession of eventful periods followed by less eventful ones. It is of interest that in addition to increases in activity of a number of enzymes others that were originally present in high concentration decrease precipitously to low adult values, whereas still others remain unchanged.

The development of the amphibian embryo also exhibits bursts of intense biochemical activity coincident with major morphological events (Gurdon & Woodland, 1968).

The results presented here suggest that the final cell division of erythroid maturation may be considered a 'critical period' analogous to those that have been described in the developing brain, liver and amphibian embryo, which divides the process of erythroid differentiation into two quite distinct phases in terms of physiology, biochemistry and morphology.

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