Changes in the Lactate Dehydrogenase Isoenzyme Pattern during Differentiation of Rabbit Bone-Marrow Erythroid Cells

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1. Differentiation and maturation of rabbit bone-marrow erythroid cells was accompanied by a 15-fold decrease in lactate dehydrogenase activity from approx. 0.1 pmol of NADH utilized/min per cell in basophilic cells to 0.007 pmol of NADH/min per cell in reticulocytes. 2. In early cells, cell division takes place with a corresponding decrease in cell volume, but the concentration of lactate dehydrogenase remains almost constant. 3. When cell division ceases, qualitative as well as quantitative changes in the lactate dehydrogenase isoenzyme pattern become apparent and reticulocytes were found to contain almost exclusively the H₄ isoenzyme, whereas early erythroblasts contained also the M₄ and hybrid isoenzymes. 4. Extracts from a lysosome-enriched subcellular fraction of bone-marrow erythroid cells specifically degraded the M₄ isoenzyme *in vitro*, but the H₄ form was stable. It is suggested that lysosomal enzymes are involved in bringing about the observed changes in lactate dehydrogenase isoenzyme patterns *in vivo*.

After differentiation of multipotent haematopoietic stem cells into the erythroid lineage, the subsequent development and maturation of erythroid cells involves not only the initiation of haemoglobin biosynthesis, but also complex changes in several enzymes. Thus in previous papers from this laboratory it was shown that early rabbit bone-marrow erythroblasts appear to maintain almost constant enzyme activities, whereas after the final cell division marked changes occurred in the activities of a number of enzymes (Denton *et al.*, 1975; Hulea *et al.*, 1975). Some enzyme activities increased and with carbonic anhydrase this was mainly due to an increase in enzyme protein rather than to activation (Spencer & Peller, 1976). Other enzymes, however, decreased in terms of both specific activity and activity per cell. Lactate dehydrogenase activity appeared to be anomalous in that the observed changes in activity were much less marked than those of the other enzymes investigated. It was thought that differential changes in lactate dehydrogenase (EC 1.1.1.27) isoenzymes might be at least partly responsible for this result, and we have therefore investigated the isoenzyme pattern during bone-marrow erythroid-cell development using a technique developed in this laboratory for fractionating rabbit bone-marrow erythroid cells by velocity sedimentation in a Ficoll–bovine serum albumin gradient (Denton & Arnstein, 1973).

The active isoenzymes of lactate dehydrogenase are tetramers composed of various proportions of two polypeptide chains, H and M. The five isoenzymes may be distinguished by electrophoresis in the pH range 7–9, when their mobility towards the anode decreases in the order H₄, H₃M, H₂M₂, HM₃ and M₄. These isoenzymes have also been designated LDH 1, 2, 3, 4 and 5 respectively.

A number of reports deal with the distribution of lactate dehydrogenase isoenzymes in haemolysates of human erythrocytes. Here three bands have been demonstrated, namely isoenzymes LDH 1, 2 and 3, of which LDH 1 is the most prominent (Vesell, 1961). Species with nucleated erythrocytes, such as duck and chicken, contain some isoenzyme LDH 5 (Vesell & Bearn, 1962), and this band has also been demonstrated in the nucleated stages of human and guinea-pig erythroid cells (Vesell, 1964). It has been suggested therefore that isoenzyme LDH 5 is associated with the cell nucleus. Rosa & Schapira (1966) separated young and old erythrocytes from rabbits by centrifugation in the presence of bovine serum albumin, and showed that haemolysates from both these cells on starch-gel electrophoresis in borate buffer gave rise to two main anodic bands which migrated more rapidly than haemoglobin. These fractions seem to correspond to isoenzymes LDH 1 and LDH 2. In younger cells, two additional faint bands migrating more slowly than the main bands were observed. An increase in human erythrocyte isoenzyme LDH 5 has been reported to indicate active or hyperactive erythroid tissue (Starkweather

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et al., 1965). Plagemann *et al.* (1960) have shown that rabbit skeletal muscle is a tissue containing only the slowest-migrating isoenzyme M_4 , whereas rabbit heart muscle and erythrocytes contain only the fastest-migrating isoenzyme H_4 . The presence of different isoenzymes in cells and tissues is usually ascribed to differential gene expression. The results described in the present paper show, however, that the changes in the lactate dehydrogenase isoenzyme pattern during erythroid-cell development may be accounted for by differences in the degradation of the M and H forms.

Experimental

Materials

The animals were white New Zealand rabbits. Pyruvate (monosodium salt), NADH (disodium salt), NAD^+ (free acid) and pure lactate dehydrogenase isoenzymes H_4 (rabbit heart) and M_4 (rabbit muscle) were obtained from Boehringer Corp. (London) Ltd., Lewes, East Sussex BN7 1LG, U.K. L-(+)-Lactate (lithium salt) was purchased from BDH Chemicals, Poole, Dorset, U.K. Cellogel cellulose acetate gel strips were purchased from Whatman Labsales, Maidstone, Kent ME14 2LE, U.K. All other reagents were AnalaR (BDH Chemicals, Poole, Dorset, U.K.)

Preparation of the cell suspensions and lysates

Rabbits were made anaemic by five daily injections of 0.3 ml of 2.5% (w/v) phenylhydrazine/kg body wt. (Arnstein *et al.*, 1964). The animals were killed 3 days after the last injection by bleeding from the heart. Blood reticulocytes (or erythrocytes from normal rabbits) were separated from the plasma by centrifugation at 1000g for 10 min, and the 'buffy' coat of white cells at the top was removed by aspiration. The cells were washed twice with 0.145M-NaCl in 0.02M-sodium phosphate buffer, pH 7.4 (phosphate-buffered saline). Bone marrow was scraped from the femur and humerus and a cell suspension was prepared in ice-cold phosphate-buffered saline (approx. 10 vol.) by forcing the marrow clumps through a 10 ml pipette. All operations were carried out at 0°C unless otherwise stated. After filtration through a double layer of muslin, the cells were collected by centrifugation at 1000g for 10 min and washed twice with phosphate-buffered saline.

In some experiments bone-marrow erythroid cells were separated by velocity sedimentation into 30 fractions. The details of the technique and apparatus have been described elsewhere (Denton & Arnstein, 1973). Consecutive fractions were combined in pairs so that 15 fractions were obtained for analysis. Fractions 1–4 consisted mainly of basophilic erythro-

blasts, fractions 5–10 mainly of polychromatic cells and fractions 11–15 of orthochromatic cells and reticulocytes. Thus fractions 1–10 contained dividing cells, whereas the more mature non-dividing cells were present in fractions 11–15. The number of cells was determined with a Coulter counter after appropriate dilution.

Reticulocytes or erythrocytes, total bone-marrow cells and bone-marrow cell fractions were lysed at 0°C in 10 mM-Tris/HCl buffer, pH 7.4, and cell debris was removed by centrifugation at 12000g for 30 min. The supernatants were kept frozen at -70°C in small portions. All experiments were carried out after a single thawing of the sample.

To obtain a lysosome-enriched fraction, bone-marrow cells (1–2 ml packed cell volume) were homogenized in 2 vol. of buffer containing 0.25M-sucrose, 0.05M-Tris/HCl, pH 7.4, 25 mM-KCl and 5 mM-MgCl₂ with a Potter Teflon/glass homogenizer. The unbroken cells (60–70% by this procedure) and cell debris were sedimented at 1000g for 10 min. The mitochondrial and lysosomal fraction was obtained from the supernatant by centrifugation at 10000g for 30 min and then lysed in 1 ml of 10 mM-Tris/HCl buffer, pH 7.4.

Protein determination

Portions (0.2 ml) of the lysates were precipitated with 10% (w/v) trichloroacetic acid (2 ml), the precipitate was redissolved in 1M-NaOH (2 ml) and suitably diluted samples were used for the determination of protein by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. Reticulocytes and erythrocytes contained 35–45 pg of protein/cell, whereas the average protein content of total bone marrow was 16–20 pg/cell.

Enzyme assays

Lactate dehydrogenase activity (EC 1.1.1.27) of erythroid cell lysates was determined by measuring the change in A_{340} at 20°C in a cell with 1 cm light-path at 30 s intervals. The reaction mixture (final vol. 3 ml) contained 100 mM-Tris/HCl buffer, pH 7.6, 0.66 mM-sodium pyruvate, 0.13 mM-NADH and the enzyme. The activity was calculated as nmol of NADH utilized/min per 10⁶ cells. The results are expressed per 10⁶ cells, rather than per mg of protein, because of the marked increase in cellular protein resulting from haemoglobin synthesis during the final stages of cell development. The determination of the proportion of H- and M-subunits was carried out as described by Bernstein & Everse (1975). The method is based on the differential inhibition of the H-type lactate dehydrogenase by high concentrations (10 mM) of pyruvate in the presence of NAD^+ (13 μ M),

whereas the activity of the M-type isoenzyme is not significantly affected. To calculate the isoenzyme content of an unknown solution, the inhibition of the sample was compared with that of the pure H_4 and M_4 isoenzymes (from the same species) under identical conditions.

The isoenzyme pattern of lactate dehydrogenase was determined by electrophoresis in veronal buffer, pH 7.5, on Cellogel cellulose acetate strips (14 cm $\times$ 2.5 cm). The samples were applied to the centre of the absorbent face of the strips and were electrophoresed for 90 min at 200 V (20 V/cm). After electrophoresis, the strips were stained for enzyme activity with a reaction mixture containing 50 mM-Tris/HCl buffer (pH 7.6), 10 mM-MgCl₂, 1 mM-NAD⁺, 100 mM-lactate, 0.003 % phenazine methosulphate and 0.03 % 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The isoenzyme bands were left to develop for 15 min in darkness at room temperature and were fixed in 5 % (v/v) acetic acid. The bands were scanned with a microdensitometer (Joyce, Loebel and Co. Ltd., Gateshead 11, U.K.) set for reflectance with a yellow filter. In some experiments the areas under the peaks were measured with the integrator, in others the lactate dehydrogenase bands were cut out, extracted with 1 ml of methanol and the intensity of the colour was measured by the A_{570} as an index of enzyme activity.

Results

The total lactate dehydrogenase activity (Table 1) of lysates obtained from bone-marrow cells (20.7 nmol of NADH utilized/min per 10⁶ cells) was considerably greater than that in lysates from circulating reticulo-

cytes or erythrocytes (approx. 7 nmol of NADH/min per 10⁶ cells). Determination of lactate dehydrogenase in bone-marrow erythroid cells fractionated by velocity sedimentation at unit gravity (Fig. 1) showed that the enzyme activity of basophilic cells (100 nmol of NADH/min per 10⁶ cells) was consistently very much higher than that of late cells. The activity of the polychromatic cells was approx. 50 nmol of NADH/min per 10⁶ cells and after the final cell division the lactate dehydrogenase activity decreased markedly to a constant low value. These data show that the activity of the total bone-marrow lysate represents mainly the activity of the cells before the last cell division. Reconstitution of the total bone-marrow cells from the fractions obtained after cell separation gave an average value of 19 nmol of NADH/min per 10⁶ cells, which is in good agreement with the value of 20 nmol of NADH/min per 10⁶ cells for the total population of erythroid bone-marrow cells (Table 1).

There was a dramatic difference between the electrophoretic isoenzyme patterns of bone-marrow and reticulocyte lysates. A typical electrophoretogram is shown in Fig. 2. The mixed population of bone-marrow cells contains all five lactate dehydrogenase isoenzymes: H_4 is predominant, M_4 is present in the smallest amount, whereas H_2M_2 and H_1M_3 are found in almost equal proportions. In the same number of circulating reticulocytes or erythrocytes only isoenzyme H_4 is detectable, but traces of isoenzymes H_3M_1 and H_2M_2 can be seen at higher loadings. By scanning the strips and measuring the areas under the peaks (Table 2) we obtained values of approx. 37 % for the H_4 isoenzyme, 15 % for H_3M_1 , 20 % for H_2M_2 and H_1M_3 and only 10 % for M_4 . In circulating reticulocytes, almost 90 % of the total activity is represented by the H_4 isoenzyme. The activity per cell of the H_4 isoenzyme in reticulocytes is about 75 % of that in bone-marrow erythroid cells, but because of the increase in protein content the specific activity per mg of protein in reticulocytes is only 50 % of that in bone marrow. When the percentage of H and M subunits was calculated from the electrophoretic data (see Table 2) the total lactate dehydrogenase activity in the bone-marrow cells was made up of approx. 63 % H subunits and 37 % M subunits. The subunit composition of bone-marrow cells and reticulocytes was also estimated by inhibition of H subunits in the presence of high concentrations of pyruvate and NAD⁺ which gives the proportion of H and M subunits (Table 3). By this method the H subunits accounted for 73 % of the enzyme activity in the total bone-marrow cells, whereas in reticulocytes this value was 92 %. Thus the ratio of H/M activity increased greatly, from about 3 in bone-marrow cells to 11.5 in reticulocytes.

The changes in lactate dehydrogenase isoenzyme pattern during erythroid-cell development are

Table 1. *Lactate dehydrogenase activity of erythroid cells*

Erythrocytes were obtained from normal rabbits by bleeding from the ear vein. The animals were then made anaemic by injecting phenylhydrazine as described in the Experimental section. Bone-marrow cells and circulating reticulocytes were obtained after killing the animals 2 days after the last injection. Enzyme activities are given as mean values for the indicated number of experiments $\pm$ S.E.M.

Cells	No. of animals	Lactate dehydrogenase activity (nmol of NADH/min per 10 ⁶ cells)
Total bone marrow	8	20.7 $\pm$ 0.97 (range 18–25)
Circulating reticulocytes	10	6.9 $\pm$ 0.59 (range 4.6–10)
Erythrocytes	3	6.7 $\pm$ 0.01 (range 6.6–6.9)

Fig. 1. *Lactate dehydrogenase activity of separated developing erythroblasts*

Enzyme assays were carried out on cells fractionated by velocity sedimentation and lysed as described in the Experimental section. 1, Basophilic cells; 2, polychromatic cells; 3, orthochromatic cells and reticulocytes.

Fig. 2. *Lactate dehydrogenase isoenzyme pattern of rabbit bone-marrow cells and circulating reticulocytes*

The electrophoresis was carried out on cellulose acetate strips. Experimental details are given in the text. (a) Total bone-marrow lysate; (b) reticulocyte lysate. Band 1, H₄; band 2, H₃M₁; band 3, H₂M₂; band 4, H₁M₃; band 5, M₄; Hb, haemoglobin.

Table 2. *Lactate dehydrogenase isoenzymes in rabbit bone-marrow cells and circulating reticulocytes*

Bone-marrow cells and circulating reticulocytes were obtained from anaemic rabbits. The cells were lysed and the 12000g supernatant was analysed for lactate dehydrogenase isoenzymes by electrophoresis on Cellogel strips. After staining for enzyme activity the strips were scanned and the areas under the peaks were measured, with an automatic integrator.

	Bone marrow		Reticulo-cytes		Bone marrow	
	Peak area	(%)	Peak area	(%)	% of H subunits in hybrids	% of M subunits in hybrids
H ₄	203	36.6	149	90	36.6	0.0
H ₃ M ₁	84	15.1	Traces	—	11.4	3.7
H ₂ M ₂	110	19.8	Traces	—	9.9	9.9
H ₁ M ₃	105	18.9	Absent	—	4.7	14.2
M ₄	53	9.6	Absent	—	0.0	9.6
Total	555	100.0			62.6	37.4

Table 3. *Subunit composition of rabbit erythroid lactate dehydrogenase*

Bone-marrow cells and circulating reticulocytes were obtained from anaemic rabbits. Lactate dehydrogenase activity of lysates was measured in the presence of low (0.66mM) and high (10mM) pyruvate concentrations by the method of Bernstein & Everse (1975), with pure H₄ (rabbit heart) and pure M₄ (rabbit muscle) isoenzymes as standards. The concentrations of H and M subunits in the bone-marrow cells and reticulocytes are given in arbitrary units. In our experiments 1 unit represents ΔA_{340} of 0.001/min per 0.01ml of lysate, which contained 3×10^6 bone-marrow cells and 4×10^6 reticulocytes respectively. The values are representative of three different experiments, which all gave essentially similar results.

	H subunits		M subunits		Ratio [H]/[M]
	[H]	(%)	[M]	(%)	
Total bone-marrow cells	1.70	73.4	0.62	26.6	2.7
Circulating reticulocytes	0.47	92.0	0.04	8.0	11.5

presented in Fig. 3. In the basophilic cells all five bands have high activities, isoenzyme M₄ being almost as active as isoenzyme H₄, and only slight variations were observed at this stage. At the polychromatic-cell stage, the activities of all five isoenzymes decreased sharply. The most dramatic change involved isoenzymes M₄ and H₁M₃, which disappear almost simultaneously soon after the last cell

division. The activity of isoenzyme H₄ changes relatively slowly, and in the non-dividing erythroblasts this becomes the predominant lactate dehydrogenase isoenzyme. The activities of isoenzymes H₃M₁ and H₂M₂ also decline in the orthochromatic cells. In the non-dividing cells they remain constant but very low. These isoenzyme patterns in the different bone-marrow cell populations support the observation that most of the lactate dehydrogenase activity in the total bone-marrow lysate is mainly due to early dividing cells, when isoenzymes LDH 1-5 are present in about the following proportions: 33:12:25:22:10. If the activities of H₄ and M₄ isoenzymes are calculated as percentages of the total lactate dehydrogenase activity during erythrocyte development (Fig. 4), it can be seen that the activity of isoenzyme H₄ increases from approx. 20% to almost 60%, whereas the percentage of isoenzyme M₄ decreases to practically zero.

It occurred to us that the disappearance of isoenzyme M₄ during erythroid cell development might be due to its specific degradation and that lysosomal enzymes may be involved in bringing about the observed changes in lactate dehydrogenase isoenzyme patterns *in vivo*. Since hypo-osmotic lysis of bone-marrow cells would be expected to damage the lysosomes, it was assumed that lysosomal enzymes would be present in these lysates. An experiment was therefore carried out in which pure isoenzyme M₄ was incubated with bone-marrow lysates for different periods of time at 38°C. Because of heat-lability of lactate dehydrogenase isoenzyme M₄ in buffer (Plagemann *et al.*, 1961), we determined the effect of adding bovine serum albumin (final concn. 1 mg/ml) in a preliminary experiment and found that under these conditions there was no loss of either M₄ or H₄ isoenzyme activity during incubation at 38°C for up to 4 h. Under identical conditions of incubation in the presence of bovine serum albumin, the total lactate dehydrogenase activity of a bone-marrow cell lysate decreased only by less than 10% in 2 h (Table 4). When bone-marrow lysate supplemented with exogenous M₄ isoenzyme was incubated under the same conditions the total lactate dehydrogenase activity decreased by approx. 20% within 2 h. A control incubation mixture kept at 0°C for 2 h showed no change in lactate dehydrogenase activity. No decrease in enzyme activity was detected when the same experiment was carried out with bone-marrow lysate that had been boiled for 3 min. The activity of pure H₄ isoenzyme was not affected under the same conditions.

By subtracting the lactate dehydrogenase activity of the bone-marrow lysate from the total activity of lysate plus added isoenzyme before and after incubations the effect of the lysate on isoenzymes M₄ and H₄ was calculated from the results given in Table 4. Whereas the activity due to exogenous isoenzyme M₄

Fig. 3. Changes in lactate dehydrogenase isoenzymes in developing erythroblasts

Electrophoresis was carried out on lysates from cells fractionated by velocity sedimentation; details of electrophoresis conditions are given in the text. Lysates from 10^6 cells were loaded on each cellulose acetate strip. After staining for enzyme activity the bands were cut out, the colour was extracted with 1 ml of methanol and its intensity was measured at A_{570} . (a) ●, H_4 ; ○, M_4 . (b) ▲, H_3M_1 ; ■, H_2M_2 ; △, H_1M_3 . 1, Basophilic cells; 2, polychromatic cells; 3, orthochromatic cells and reticulocytes.

Fig. 4. Lactate dehydrogenase isoenzymes H_4 and M_4 in separated developing erythroblasts

The percentage of H_4 (●) and M_4 (○) isoenzymes has been calculated from the results shown in Fig. 3.

decreased from 0.048 to 0.024 $\Delta A_{340}/\text{min}$ per 0.02 ml of extract, corresponding to a 50% inactivation after incubation for 2 h, there was no significant change in the activity of isoenzyme H_4 . The inactivation of isoenzyme M_4 by the bone-marrow lysate was much

decreased by the proteinase inhibitor 7-amino-1-chloro-3-L-tosylamidoheptan-2-one at 0.5 mM concentration and completely abolished at 1 mM.

When bone-marrow cells were lysed by gentle homogenization in an iso-osmotic medium, the mitochondrial plus lysosomal fraction could be separated from the rest of the cytoplasm by differential centrifugation. When this fraction was hypo-osmotically lysed in 10 mM-Tris/HCl buffer, pH 7.4, and the same experiment as above was performed, the endogenous lactate dehydrogenase activity was localized in the cytosol, but this fraction did not degrade added pure isoenzyme M_4 . On the other hand, the mitochondrial plus lysosomal fraction contained negligible lactate dehydrogenase activity, but degraded added pure M_4 isoenzyme to the extent of 20% within 2 h (Table 5). It is likely, therefore, that the activity of the total bone-marrow lysates was due to lysis of lysosomes under hypo-osmotic conditions.

Discussion

During the development of rabbit bone-marrow erythroblasts, the period when cell division ceases appears to coincide with several complex morphological and biochemical alterations, including changes in the activities of several enzymes (Denton *et al.*,

Table 4. *Inactivation of lactate dehydrogenase isoenzyme M₄ by incubation with bone-marrow lysates*

Bone-marrow cells were lysed in 10mM-Tris/HCl buffer, pH7.4. Pure isoenzymes M₄ and H₄ were diluted with bovine serum albumin solution (1 mg/ml) in 10mM-Tris/HCl buffer, pH7.4. All incubation mixtures contained bovine serum albumin at the same concentration. Incubation was carried out for 2h at 38°C. Lactate dehydrogenase activity was measured before and after incubation of the samples, the activity being given as $\Delta A_{340}/\text{min}$ per 0.02ml of extract. The proteinase inhibitor 7-amino-1-chloro-3-L-tosylamidoheptan-2-one was added where shown. —, Not significant.

Incubation mixture	Lactate dehydrogenase activity		Inhibition of lactate dehydrogenase (%)
	Before incubation	After incubation	
Pure isoenzyme M ₄	0.054	0.056	—
Pure isoenzyme H ₄	0.052	0.055	—
Bone-marrow lysate	0.102	0.095	7
Bone-marrow lysate + isoenzyme M ₄	0.150	0.120	20
Boiled bone-marrow lysate + isoenzyme M ₄	0.053	0.055	—
Bone-marrow lysate + isoenzyme M ₄ + 1 mM-inhibitor	0.156	0.149	5
Bone-marrow lysate + isoenzyme M ₄ + 0.5 mM-inhibitor	0.153	0.139	10
Bone-marrow lysate + isoenzyme H ₄	0.156	0.154	—
Boiled bone-marrow lysate + isoenzyme H ₄	0.049	0.047	—
Bone-marrow lysate + isoenzyme H ₄ + 1 mM-inhibitor	0.157	0.160	—

Table 5. *Degradation of lactate dehydrogenase isoenzyme M₄ by extracts from a lysosome-enriched cell fraction*

Lysosomal extracts were obtained as described in the Experimental section. Incubations were carried out at 38°C for 2h in the presence of bovine serum albumin (1 mg/ml). The activity of lactate dehydrogenase is expressed as $\Delta A_{340}/\text{min}/0.05 \text{ ml}$ of extract. —, Not significant.

Expt. no.	Incubation mixture	Lactate dehydrogenase activity		Inhibition of lactate dehydrogenase (%)
		Before incubation	After incubation	
1	Isoenzyme M ₄	0.126	0.123	—
	Isoenzyme M ₄ + lysosomal extract	0.135	0.108	20
2	Isoenzyme M ₄	0.230	0.223	—
	Isoenzyme M ₄ + lysosomal extract	0.250	0.197	22

division, but after the final cell division there are more extensive quantitative as well as qualitative changes. Thus the total bone-marrow cells have all five lactate dehydrogenase isoenzymes whereas the circulating reticulocytes possess mainly isoenzyme H₄ and only traces of H₃M₁ and H₂M₂. During erythroid-cell development, isoenzyme M₄ shows a very rapid decline and after the final cell division disappears altogether. The activity of isoenzyme H₄ changes most slowly and in the non-dividing erythroblasts this isoenzyme is the predominant one. The loss of isoenzyme M₄ is accompanied by a gradual relative increase of H₄ during aging. Starkweather *et al.* (1965) have proposed that isoenzyme M₄ might be a measure of the developmental stage of the erythrocyte. Thus isoenzyme M₄ may be necessary for the dividing cell, but not important for the metabolic functioning of the mature cell.

It is known that during the development of an animal species to its adult stage the lactate dehydrogenase isoenzyme pattern of a particular tissue is significantly altered. For example, more electrophoretic bands have been demonstrated in extracts of foetal liver from the mouse and guinea pig than were found in the adult organ (Flexner *et al.*, 1960). It has also been shown that embryonic skeletal muscle and cardiac muscle of rabbit give all five bands (Vesell *et al.*, 1962), whereas adult rabbit skeletal muscle contains only isoenzyme M₄ and adult rabbit heart only isoenzyme H₄. When the changes in lactate dehydrogenase isoenzyme in rat tissues during postnatal development were studied (Kanungo & Singh, 1965), it was found that from 4 to 74 weeks of age the amount of isoenzyme H₄ in the brain increased and that of isoenzyme M₄ decreased by about 30%;

1975). The observed decreases in the specific activities of enzymes such as glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, adenosine deaminase and nucleoside phosphorylase may be accounted for largely by dilution as a result of increased haemoglobin synthesis after cessation of cell division, but there may be also some selective degradation of enzymes by intracellular proteolytic enzymes (Arnstein, 1976). The results obtained in the present work extend our earlier observations (Denton *et al.*, 1975) on the changes in lactate dehydrogenase by showing that the activity per cell is approx. 10–20 times higher in the early basophilic erythroblasts than in the late orthochromatic erythroblasts and reticulocytes. In dividing cells the decrease in activity per cell (Fig. 1) corresponds closely to the decrease in cell volume, which is approximately halved at each

in the same period rat heart isoenzyme M_4 decreased by about 70%, whereas isoenzyme H_4 increased to a similar extent as in brain.

The question arises as to the reason for the dramatic difference in lactate dehydrogenase isoenzyme pattern between the early cells and mature erythrocytes. Cahn *et al.* (1962) and Markert (1963) have suggested that lactate dehydrogenase formation is controlled by the action of two genes. Each gene is responsible for the synthesis of a unique polypeptide. The present data show that the changes in the lactate dehydrogenase isoenzyme pattern in the basophilic cells are not significant. This would probably indicate that the activities of both the genes responsible for the H and M subunits remain constant at this stage of development. The most dramatic changes occur in the polychromatic cells, when synthesis of the M subunits probably decreases sharply.

Vesell & Bearn (1962) found that closely related species have similar lactate dehydrogenase isoenzyme patterns and that young human and guinea-pig erythrocytes have all five LDH isoenzymes, but that mature erythrocytes have lost isoenzyme M_4 . They offered some evidence that this isoenzyme is associated with the cell nucleus, so that it would be absent for the mature mammalian erythrocyte, which is anucleate. Our data show that disappearance of isoenzyme M_4 results in a relative increase of isoenzyme H_4 in the later cells, which does not, however, represent increased enzyme synthesis. It is most likely that the H subunit is more stable and the relatively minor decrease in isoenzyme H_4 may be due to dilution of the existing H species by cell division. It is noteworthy that after the changes in isoenzyme pattern in the bone marrow the remaining isoenzyme H_4 in circulating erythrocytes appears to be quite stable, as shown by the identical lactate dehydrogenase activities of circulating reticulocytes and mature erythrocytes (Table 1).

It is likely that the observed decrease in lactate dehydrogenase activity on incubation of isoenzyme M_4 with bone-marrow lysates (Table 4) or extracts from a lysosome-enriched fraction (Table 5) is due to enzymic degradation, since boiled preparations were inactive and the presence of the proteinase inhibitor 7-amino-1-chloro-3-L-tosylamidoheptan-2-one prevented the loss of lactate dehydrogenase activity. It is noteworthy that this compound has been shown to be very active as an inhibitor of cathepsin L from rat liver lysosomes (Kirschke *et al.*, 1977).

In this laboratory it has been shown that early erythroid cells are rich in lysosomes, but after the last cell division lysosomes disappear and lysosomal enzymes are lost or inactivated (Hulea, 1976). *In vivo*, the maximum degradation of isoenzyme M_4 takes place in polychromatic erythroblasts, and lysosomes present in these cells may be unstable, since there are

very few left at the subsequent stages of cell development (Hulea, 1976). This observation, together with data in the present paper showing that a lysosome-enriched fraction of bone-marrow cells can inactivate lactate dehydrogenase isoenzyme M_4 *in vitro* (Table 5), supports the suggestion (Arnstein, 1976) that lysosomes may be involved in giving rise to enzyme changes *in vivo* at the stage of erythroid-cell development when the nucleus condenses.

It is known that the predominance of isoenzyme H_4 is a characteristic of aerobic tissues (cardiac muscle), whereas isoenzyme M_4 is associated with anaerobic tissues (skeletal muscle). As the mature erythrocyte is an anaerobic cell with the glycolytic pathway as a single source of ATP synthesis, the conversion of pyruvate into lactate should be the main direction in which lactate dehydrogenase would function. The disappearance of isoenzyme M_4 during erythroid-cell differentiation therefore seems anomalous from a metabolic point of view. The K_m of isoenzyme H_4 for pyruvate is higher than that of isoenzyme M_4 , and the predominant presence of lactate dehydrogenase H_4 in mature cells might be expected to result in the accumulation of NADH and in a decreased rate of glycolysis and ATP synthesis. However, this isoenzyme is inhibited by higher concentrations of pyruvate, which might be a mechanism for preventing over-production of NADH.

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