

Lactate dehydrogenase isoenzymes in foetal and neonatal tissues

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WITH THREE PLATES

THE EXISTENCE of isoenzymes, one class of the multiple molecular forms of enzymes, is now well recognized. Isoenzyme distribution patterns, particularly those of lactate dehydrogenase (LDH), have been extensively studied and are often tissue and species specific (Markert & Møller, 1959; Wieland, Pfeleiderer, Haupt & Wörner, 1959; Plagemann, Gregory & Wróblewski, 1960; Tsao, 1960). Various techniques, such as zone electrophoresis and column chromatography, have been used to separate isoenzymes of lactate dehydrogenase. Using starch-gel electrophoresis combined with visual demonstration of the isoenzyme patterns, we have detected the usual five zones of activity in human tissues (Latner & Skillen, 1961). They are termed LD₁, LD₂, LD₃, LD₄ and LD₅ where, according to the terminology we use, the latter is the isoenzyme which migrates furthest towards the anode and is most prominent in heart muscle.

It has been suggested that the LDH isoenzyme patterns are specific for each tissue at each stage of development (Markert & Møller, 1959). However, in the human foetus it has been shown that most tissues show very similar LDH isoenzyme distribution patterns (Pfeleiderer & Wachsmuth, 1961).

Studies on the LDH isoenzymes in the developing mouse, rat and guinea-pig brain have shown that during the first weeks of extrauterine life there is a gradual increase in the activity of those components moving nearest to the anode, i.e. a predominance of LD₄ and LD₅ (Flexner, Flexner, Roberts & De La Haba, 1960; Bonavita, Ponte & Amore, 1962).

In the foetal and neonatal development of mouse and guinea-pig liver there is a reduction in the number of LDH components until the adult pattern (mainly LD₁) is reached (Flexner *et al.*, 1960). In embryonic rabbit skeletal muscle and liver the isoenzymes LD₂, LD₃, LD₄ and LD₅ are prominent whereas the adult tissues show only LD₁ and LD₂ in large concentrations. In embryonic rabbit

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heart muscle the same four bands are present whereas the adult tissue shows only the fastest moving component (Vesell, Philip & Bearn, 1962). Similar differences between adult and foetal tissues have been described in the chicken (Philip & Vesell, 1962; Cahn, Kaplan, Levine & Zwilling, 1962). Studies of foetal mouse tissues (Markert & Ursprung, 1962) and of foetal rat tissues (Fine, Kaplan & Kufnec, 1963) have shown that in nearly all the tissues examined the principal LDH activity was in the LD₁ and LD₂ position and that during ontogenesis there was a gradual change towards the more anodically moving isoenzymes.

As the LDH isoenzymes have been shown to have different affinities for coenzyme analogues and different substrate optima (Kaplan, Ciotti, Hamolsky & Bieber, 1960; Kaplan & Ciotti, 1961) the changes in isoenzyme patterns during development may reflect changing metabolic rôles of the tissues. It has, in fact, been shown that the affinity for coenzyme analogues of foetal isoenzymes is different from that found in adult tissues (Wiggert & Vilee, 1962; Cahn *et al.*, 1962).

It has recently been suggested that the lactate dehydrogenase enzyme system is built up from two different polypeptide sub-units arranged at random in groups of four. Each group of four corresponds to a single isoenzyme (Appella & Markert, 1961; Cahn *et al.*, 1962). If the polypeptide sub-units are designated M and H respectively, random combinations in groups of four would result in the following five formations M₄, M₃H₁, M₂H₂, M₁H₃ and H₄. M₄ corresponds to the isoenzyme which moves most slowly during electrophoresis and H₄ to the fastest moving entity. The other groupings correspond to the remaining three isoenzymes and those containing more of H move correspondingly more rapidly during electrophoresis (Cahn *et al.*, 1962). It has been postulated that the sub-unit hypothesis can be used to explain the shift in tissue isoenzyme patterns during ontogeny (Markert & Ursprung, 1962; Fine *et al.*, 1963). There could well be a great predominance of either of the two basic sub-units M and H in the early stages of development and M₄ or H₄ could be the predominant isoenzyme in embryonic tissues.

This work was undertaken in order to obtain some idea of the development of LDH isoenzymes in the rat and human being and to ascertain whether the concepts for the origin of the five LDH isoenzymes could be supported.

MATERIALS AND METHODS

Rat tissues

Adult rats of the Scott-Russ strain were killed by a blow on the back of the head. At intervals from birth to 26 days old, young rats were killed by decapitation. Rat embryos were obtained at various stages of the gestation period, the approximate date of conception being determined by allowing the pairs 48 hr. to mate. The tissues required were dissected and washed in ice-cold 0.9 per cent. NaCl. Enough foetal tissue was obtained by pooling individual organs from each

litter and in the first few days of life it was also necessary to pool organs from two or three litter mates.

Human tissues

Adult human tissues were obtained from recently dead individuals and human foetal tissues from spontaneous and therapeutic abortions.

All tissues were homogenized with approximately three times their volume of ice-cold 0.9 per cent. NaCl using an all glass Potter-Elvehjem type homogenizer. The homogenates were centrifuged for 30 min. at $10^5 g$ and the supernatants removed. Wherever possible the extracts were examined for their isoenzyme pattern the same day as the extracts were prepared. In a few cases they were stored at -20°C .

The extracts were assayed for LDH activity using a standardized substrate technique (Sigma Technical Bulletin No. 500) so that approximately equivalent enzyme activities from each tissue could be compared. Usually extracts containing 1000–1500 units/ml. of LDH were used as samples for electrophoresis.

Vertical starch-gel electrophoresis, using 0.05 M Tris-HCl buffer pH 8.8, was used to separate the isoenzymes. A voltage gradient of 10 V./cm. was applied in the cold room at 4°C . for $1\frac{1}{4}$ – $1\frac{1}{2}$ hr. The gels were sliced and the isoenzymes visualized using a tetrazolium salt MTT (3-(4,5-dimethyl-thiazolyl-2)-2, 5-diphenyl tetrazolium bromide) in the presence of phenazine methosulphate (Latner & Skillen, 1961). The gel slices were incubated with the substrate for 20 min. and the stained gel slice washed for 15 min. with three changes of distilled water before immersing in 80 per cent. glycerine. The gel slice became transparent and the isoenzyme patterns were clearly seen as purple bands.

RESULTS

Adult tissues

The LDH isoenzyme patterns given by some adult human tissues are shown in Plate 1, Fig. 1. The marked variations between the adult tissues are clearly demonstrated. Liver and skeletal muscle contain large amounts of the slowest moving isoenzyme (LD_1) whereas heart and kidney contain large amounts of the fastest moving isoenzyme (LD_5). Similar patterns were obtained with adult rat tissues.

One of the difficulties with tissue extracts, using our technique, is the fact that LD_1 and LD_2 sometimes run very closely together or even as a single broad band. Usually, however, it is possible to detect a line of demarcation even though it may be somewhat narrow.

Rat foetal and neonatal tissues

In the rat foetus all the tissues examined showed great similarity in their isoenzyme patterns with a large preponderance of LD_2 and lesser amounts of LD_1 and LD_3 . Foetal tissue isoenzyme patterns also resembled that of an extract of

the corresponding placenta (Plate 1, Figs. 2 and 3). The differences between foetal and adult rat brain (Plate 1, Fig. 2) and foetal and adult rat heart (Plate 1, Fig. 3) were most noticeable, in so far as there was a total absence of the faster moving isoenzymes. Foetal and adult rat liver and muscle were found to have similar patterns (Plate 1, Fig. 3).

At birth most of the tissues examined also gave isoenzyme patterns different from those of the adult rat; all showing greatest activity in LD₂ or LD₁. LD₃ was also prominent in a number of the tissue extracts (Plate 2, Fig. 4). The most noticeable differences from the adult were apparent in heart and brain where the LD₅ band, which is characteristic of the adult tissues, was absent or barely detectable in the newborn tissues. The pattern given by newborn rat kidney more closely resembled the adult pattern than did the newborn rat heart, there being more LD₄ and LD₅, and in this context it could be said to be more developed.

In the first 2 to 3 weeks after birth the tissue isoenzyme patterns became more complex, i.e. more like the adult. There was a gradual shift from those bands moving nearest to the cathode to those moving more anodically. Up to 1 week after birth the isoenzyme patterns still showed a somewhat abnormally large proportion of LD₁, LD₂ and LD₃. These changes in pattern were most obvious in heart extracts, in which LD₄ was present 1 week after birth with the LD₂ band less concentrated than at birth. During the 2nd week of extrauterine life LD₅ became just detectable, and in the subsequent 2 weeks the pattern shown by the young rat heart became similar to that of the adult animal (Plate 2, Fig. 5A). Using the heat stable property of LD₅ (Plagemann, Gregory & Wróblewski, 1961) this gradual change into the adult pattern was clearly seen. Only the heart extracts from the 2 and 3–4-week-old rats showed any activity after heating at 60°C for 1 hr. (Plate 2, Fig. 5B).

Human foetal tissues

A range of human foetal tissues at gestation periods ranging from 10–26 weeks was examined. In a 10-week-old foetus the isoenzyme patterns of heart, brain, kidney, lungs and liver appeared very similar, contrasting with the marked differences found between adult tissues such as heart and liver.

In the 14–16-week foetus the isoenzyme patterns of most of the foetal tissues still differed from those of the normal adult (Plate 3, Fig. 6A). In the foetal tissues there was a greater concentration of the slower moving isoenzymes, especially

PLATE 1

- FIG. 1. LDH isoenzyme patterns in adult human tissues (adult rat tissues give similar patterns).
FIG. 2. LDH isoenzyme patterns in 1–2 week foetal rat tissues compared with adult rat brain which, in contrast, contains large amounts of LD₄ and LD₅.
FIG. 3. LDH isoenzyme patterns in 2–3 week foetal rat tissues. All the foetal tissues have relatively similar patterns. Foetal liver and foetal skeletal muscle are not very different from the adult tissues as they all contain large amounts of the slower moving isoenzymes. Foetal heart is very different from adult heart with no LD₄ or LD₅ in the foetal tissue.

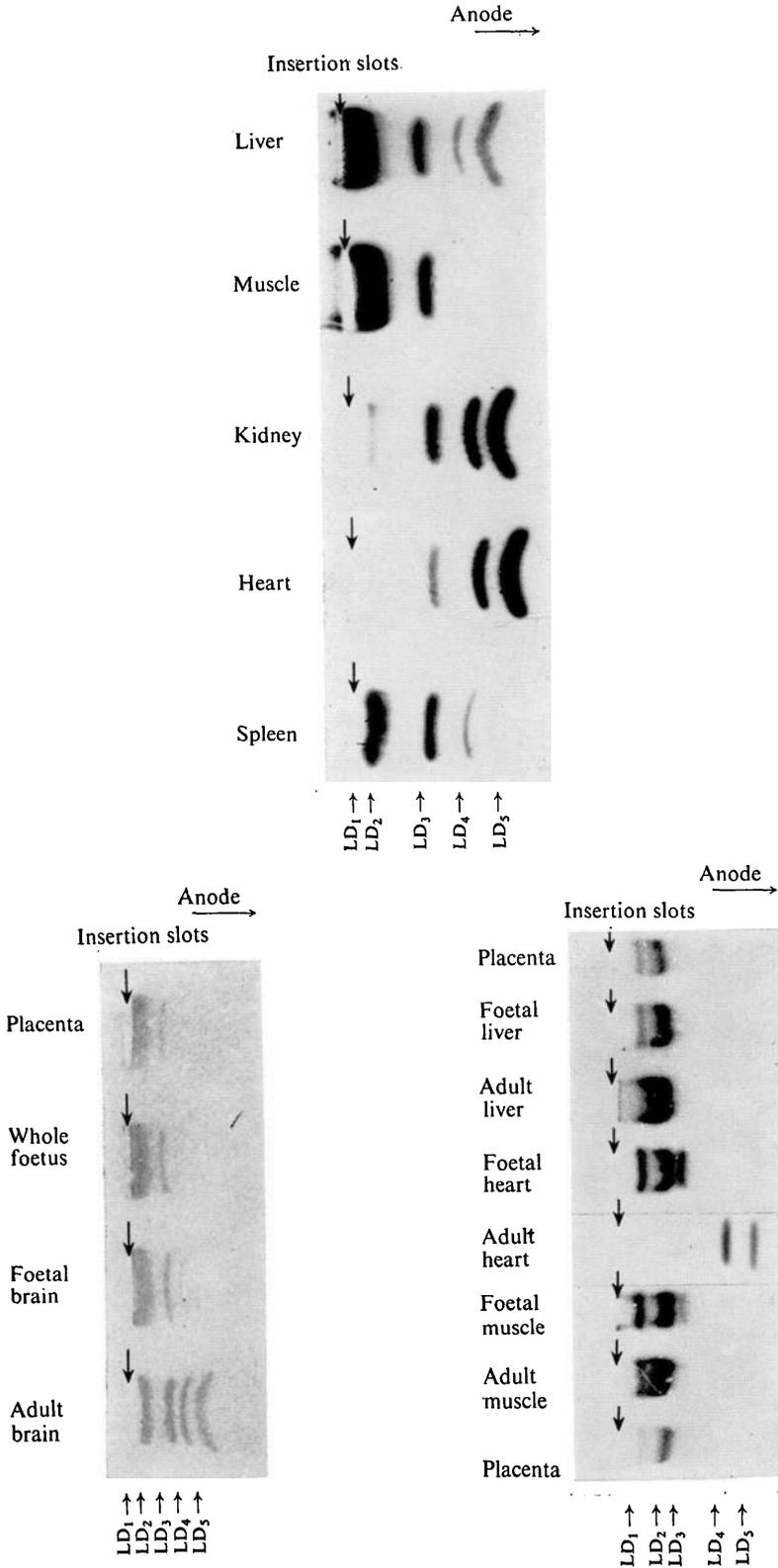


PLATE 1

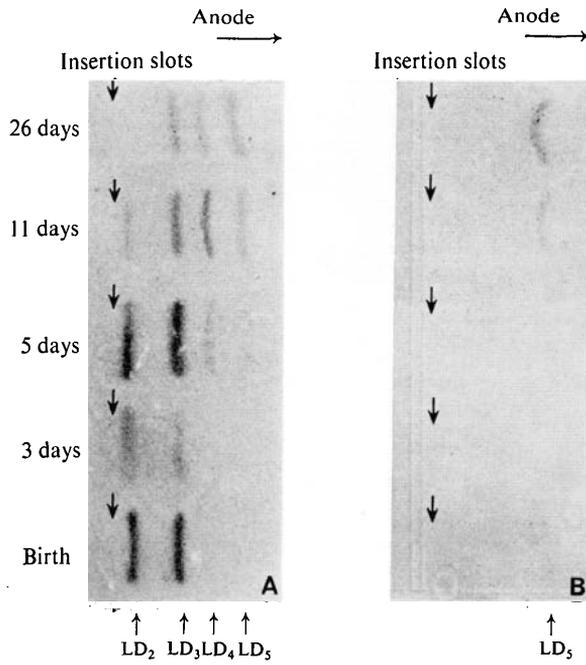
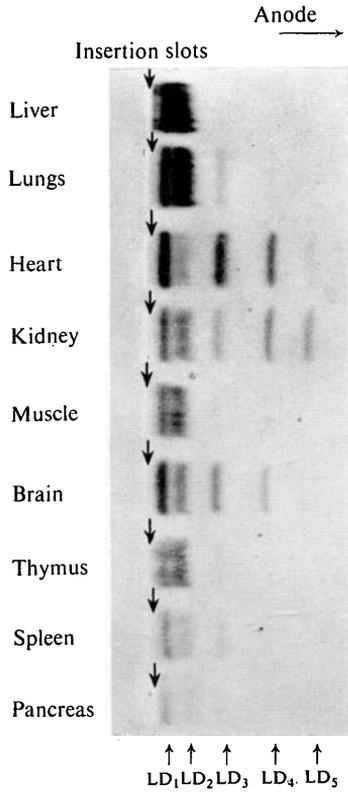


PLATE 2

LD₂; a situation similar to that found in the foetal and young rat tissues. The LD₂ and LD₃ isoenzymes were the most concentrated in all human foetal tissues and LD₅ was present in significant amounts only in brain extract. Brain and muscle at this stage showed patterns approximating to the adult. The pattern given by foetal heart was the one most distinctly different from the adult tissue and was similar to that found in foetal and neonatal rat heart with most activity in those isoenzymes migrating nearest to the point of insertion. In general, the tissue isoenzyme patterns of the human 14-week foetus resembled those of the neonatal rat.

In the 26-week foetus the tissue isoenzyme patterns were found to be considerably more developed in so far as there was a much greater proportion of LD₄ and LD₅. All the tissues at this stage had isoenzyme patterns closely approaching those of the adult except that there was still a slightly higher proportion of LD₂ (Plate 3, Fig. 6B).

In some instances abnormal isoenzyme patterns were detected in human foetal tissue extracts. These abnormal patterns took the form of what was either a new abnormally migrating isoenzyme or an abnormal position of LD₂ (Plate 3, Fig. 7).

DISCUSSION

The ontogenesis of LDH isoenzyme patterns has been studied in the rat and human being using starch-gel electrophoresis. In both human and rat foetal tissues there appears to be a preponderance of those isoenzymes with least mobility towards the anode, i.e. LD₁, LD₂ and LD₃. LD₂ has always been present in greatest amounts in tissues from the foetal and young rat.

Our findings with foetal and young rat tissues agree in general with those of Fine *et al.* (1963). The results with the rat are, however, markedly different from those obtained with the rabbit (Vesell *et al.*, 1962). In the embryonic rabbit heart, LD₂, LD₃, LD₄ and LD₅ are present whereas the adult tissue contains only LD₅. The development of the liver LDH isoenzymes in the rabbit is also strikingly different from that in the rat. In the rat and human being we found that the foetal liver pattern was not much different from the adult pattern whereas in the rabbit and guinea-pig it appears that as many as four isoenzymes are present in the foetal liver (Flexner *et al.*, 1960; Markert & Ursprung, 1962).

PLATE 2

FIG. 4. LDH isoenzyme patterns in newborn rat tissues. All tissues still contain large amounts of the slower moving isoenzymes. The heart and brain extracts do not contain the large amounts of LD₅ present in the adult tissue. The kidney extract gives a pattern more similar to the adult type.

FIG. 5. LDH isoenzyme patterns in the developing rat heart. A: Normal tissue extracts. At birth and 3 days of age LD₄ and LD₅ are absent. The normal adult pattern is developed about 4 weeks after birth. B: Heated tissue extract. Only LD₅ is active after heating at 60°C for 1 h. and cannot be detected before the rat is 11 days old.

Our results with human foetal tissues at the 10th and the 14th week are very similar to those found by Vesell *et al.* (1962) in the human foetus at the 10th week. It has been stated that all human foetal tissues show a great preponderance of LD₃ (Pfleiderer & Wachsmuth, 1961). Our results would tend to confirm this statement in so far as the remarks are applicable before the 26th week of intrauterine life.

The LDH isoenzyme patterns of most human foetal tissues appear to approach those of the adult tissues at about 26 weeks, although at this stage there has been more LD₂ present in foetal material than in the corresponding adult tissues. In most instances the appearance of significant amounts of LD₅ seems to be an index of the foetal tissue approaching the adult state. In the rat, however, some tissues do not have adult LDH isoenzyme patterns until 3–4 weeks after birth.

The comparative development of the foetal brain and the foetal heart in both the rat and human being is of great interest. Whereas in the rat the foetal heart and foetal brain advance towards the adult type at approximately the same rate, in the human being the brain pattern closely approximates that of the adult as early as the 14th week of intrauterine life. At this stage the heart pattern is still of the foetal type. However, the results for the development of LDH isoenzymes in the human foetus indicate that tissues assume their adult patterns at a much earlier stage of development than those of the rat. It is also quite obvious that within the same species tissues do not develop their adult patterns at similar rates. It has already been indicated that during development LD₄ and LD₅ appear much earlier in mouse kidney than in mouse heart (Markert & Ursprung, 1962).

The relative abundance of the different isoenzymes may correspond to the metabolic requirements of the tissue (Markert & Ursprung, 1962; Wilson, Cahn & Kaplan, 1963). Anaerobic glycolysis has been found to be very active in embryonic tissues (Needham & Nowinski, 1937) and tissues whose metabolism takes place under relatively anaerobic conditions have been shown to have isoenzyme patterns containing large amounts of LD₁, LD₂ and LD₃ (Pfleiderer & Wachsmuth, 1961). The findings we have described in relation to the preponderance of the slowest moving isoenzymes in foetal tissues could possibly explain their facility for anaerobic glycolysis. The general change in isoenzyme patterns from those involved in anaerobic glycolysis to those involved in aerobic glycolysis probably reflects some general change in metabolic environment, possibly oxygen tension. The varied metabolic environments in other mammals could possibly account for the differences in development of their isoenzyme patterns.

The theories postulating that LD₁ is the first active isoenzyme in foetal life (Cahn *et al.*, 1962; Markert & Ursprung, 1962) are to some extent supported by our findings of a greater proportion of the slower moving isoenzymes in foetal tissues than in those from adults. We must, however, emphasize that in certain foetal tissues, of which heart is a good example, LD₁, LD₂ and LD₃ are the only isoenzymes we can detect. This has an important bearing in regard to modern

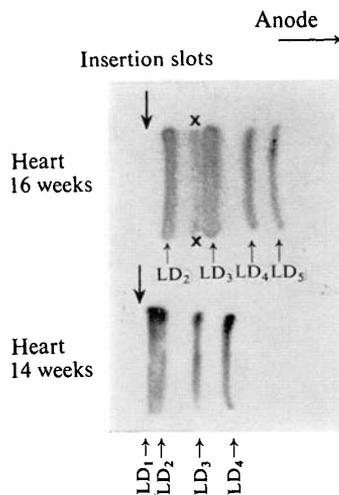
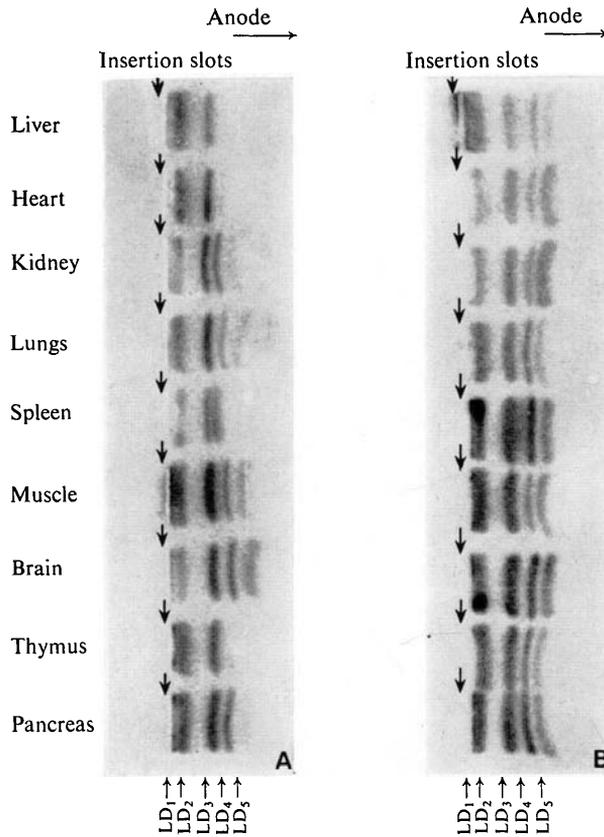


PLATE 3

FIG. 6. LDH isoenzyme patterns in human foetal tissues. A: 14th-week foetus. All extracts contain large amounts of LD₁, LD₂ and LD₃. Heart extract is the most markedly different from normal. The brain and liver patterns are similar to the adult type. B: 26th-week foetus. All extracts have patterns relatively similar to the adult type—heart, kidney and brain extracts containing significant amounts of LD₄ and LD₅.

FIG. 7. LDH isoenzyme patterns in human foetal hearts showing abnormal isoenzyme (X) in the 16-week specimen compared with the normal at 14 weeks.

concepts of the unit structure of LDH isoenzymes (Markert, 1963). If each is made up of four sub-units and there are only two kinds of sub-unit available, it is rather surprising that we can detect LD₂ and LD₃ in relative abundance but find no evidence for the existence of LD₄ or LD₅ in significant amounts. Fine *et al.* (1963) have also pointed out that in rat foetal tissues the hybrids appear before the pure H type. The apparent absence of LD₁ in some instances may be due to its low temperature lability (Zondag, 1963). There are, however, other factors which must be considered in regard to the sub-unit hypothesis. There is always a possibility that intracellular conditions are such that the sub-units are capable of combining only in the ways we have found. It is by no means unlikely, for example, that intracellular oxygen levels could play a part in this phenomenon in relation to bonding of one kind or another between the sub-units.

Other observations which could be regarded as apparent contradictions of the sub-unit theories are the presence of a sixth LDH isoenzyme in human sperm and testis (Blanco & Zinkham, 1963; Goldberg, 1963), the isoenzyme migrating between LD₃ and LD₄ in human red cell haemolysates (Vesell & Bearn, 1962) and the apparently abnormal isoenzymes which we have sometimes demonstrated in human foetal and tumour tissues (Latner, Skillen & Turner, unpublished observations). The recent findings that the sperm isoenzyme (Zinkham, Blanco & Kupchyk, 1963) and another red cell LDH isoenzyme (Boyer, Fainer & Watson-Williams, 1963; Nance, Claffin & Smithies, 1963) are probably subject to different gene control from the isoenzymes previously described, go some way to overcome these apparent contradictions. Some of the apparent anomalies may also be due to combination of the isoenzymes with different amounts of NAD (Fritz & Jacobson, 1963) or to the effect of other proteins on their electrophoretic migration. The effect of γ -globulin on the separation of LD₁ and LD₂ by starch-gel electrophoresis at pH 8.6 has already been demonstrated (Carr & Skillen, 1963). Protein binding could possibly account for the abnormal bands found in some of our foetal tissue extracts. It must be emphasized, however, that this type of binding does not explain the shift towards the slowest moving isoenzymes commonly found in foetal tissues. Those slow moving bands which we demonstrated all moved to positions corresponding to those associated with the known isoenzymes of lactate dehydrogenase and do not require any special explanation.

SUMMARY

1. Lactate dehydrogenase isoenzymes have been studied in human foetal and rat foetal and neonatal tissues using starch-gel electrophoresis.
2. Foetal and neonatal rat tissues contain greater amounts of the slower moving isoenzymes than the corresponding adult tissues. Certain rat tissues, especially cardiac muscle, did not give the normal adult isoenzyme pattern until 4 weeks after birth.
3. Human 14th-week foetal tissue isoenzyme patterns also differ markedly from the adult type. In the 26th-week foetus the tissue isoenzyme patterns closely

approach the adult form, although there is still more LD₂ in foetal than in the corresponding adult tissues.

4. The marked predominance of LD₂ and LD₃ in foetal tissues suggests that the sub-unit hypothesis does not account entirely for the development of the lactate dehydrogenase pattern, which may also be subject to influences such as protein-enzyme interactions.

RÉSUMÉ

Isoenzymes de la lactate-déshydrogénase dans les tissus de foetus et de nouvea-nés

1. On a étudié les isoenzymes de la lactate-déshydrogénase dans les tissus de foetus d'homme et de rat et dans les tissus de rats nouveau-nés, au moyen de l'électrophorèse sur gel d'amidon.

2. Les tissus de foetus et de nouveau-nés de rat contiennent des quantités plus fortes d'isoenzymes à déplacement lent que les tissus adultes correspondants. Certains tissus de rat, en particulier le muscle cardiaque, ne montrent pas une répartition normale, du type adulte, des isoenzymes jusqu'à la quatrième semaine après la naissance.

3. Les aspects isoenzymatiques des tissus foetaux humains de la 14^{ème} semaine sont également nettement différents du type adulte. Chez le foetus de 26 semaines, ces aspects se rapprochent étroitement de ceux de l'adulte bien qu'il y ait encore plus de LD₂ dans les tissus foetaux que dans les tissus adultes correspondants.

4. La prédominance marquée de la LD₂ et de la LD₃ dans les tissus foetaux suggère l'idée que l'hypothèse des sous-unités ne rend pas compte entièrement du développement du complexe lactate-deshydrogénasique, qui peut être également soumis à des influences telles que les interactions entre protéines et enzymes.

Note added in proof: The International Union of Biochemistry has indicated that the correct terminology for lactate dehydrogenase isoenzymes should be different from that used above, i.e LD₁ should read LDH-5, LD₂ should read LDH-4, LD₃ should read LDH-3, LD₄ should read LDH-2 and LD₅ should read LDH-1.

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