The Foetal Development of Lactate Dehydrogenase Isoenzymes, Glucose 6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase from Human Striated Muscle

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(Received 22 May 1968)

1. Human foetal skeletal muscles involved in support and in periodic contractility were studied for their content of total extractable lactate dehydrogenase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities as well as for the relative distribution of lactate dehydrogenase isoenzymes. 2. During foetal development a linear steady increase in total lactate dehydrogenase activity as well as a linear decrease in the H/M sub-unit ratio of the isoenzymes was found. 3. No significant changes were found in the activities of the enzymes of the hexose monophosphate shunt (C-6 oxidation). 4. The changes found suggest a steady increased synthesis of lactate dehydrogenase M-sub-units in human skeletal muscles during foetal development. 5. The weekly changes in the total lactate dehydrogenase activity and in lactate dehydrogenase isoenzymes are lower in muscles involved in support than in those involved in periodic contractility. 6. These findings, together with the literature available, are consistent with the morphological fact that foetal development of skeletal muscles mostly concerns the white muscle fibres and not the red muscle fibres.

LDH* exists in five electrophoretically distinguishable isoenzyme forms that are tetramers of two different sub-units, H and M (see the survey by Wilkinson, 1965), each under the control of a specific gene (Shaw & Barto, 1963). The M-sub-unit predominates in skeletal muscle and liver, the H-sub-unit predominates in heart muscle (Cahn, Kaplan, Levine & Zwilling, 1962). Each isoenzyme has a unique amino acid composition and characteristic physical, chemical, immunological and kinetic properties (Markert & Apella, 1961). Although bound in a tetramer structure, each sub-unit can function as an individual unit independent of the other sub-units (Fine, Kaplan & Kuftinec, 1963).

Dawson, Goodfriend & Kaplan (1964) correlated the kinetics of the LDH-H₄ and LDH-M₄ isoenzymes with the aerobic and anaerobic conditions respectively of different tissues. Thus the energy requirements of tissues possessing a preponderance of LDH-H₄, e.g. cardiac tissue, agree with the kinetics of this isoenzyme, i.e. a low turnover number with pyruvate as substrate, maximal LDH activity at low pyruvate concentration, but showing inhibition at higher pyruvate concentrations. The energy available for heart tissue is required continuously; this demand is met by complete

combustion of lactate and pyruvate. Pyruvate does not accumulate in this tissue.

On the other hand, the skeletal muscles require the release of energy in discontinuous bursts under relatively anaerobic functional conditions. The energy requirements in skeletal muscles are satisfied by glycolysis and the high concentrations of pyruvate are reduced to lactate by the predominating LDH-M₄ isoenzyme, which possesses optimum kinetics for these demands.

The abrupt energy requirements of skeletal muscles are related to the neural activity. As this activity first appears in the last 3 months of the gestation period (see below) it was decided to relate changes in enzymes involved in glycolysis to the development of the neuromuscular system. The aim of the present work was to correlate these changes by relating total LDH activity and distribution of LDH isoenzymes to foetal age. As ontogenesis to some extent reflects the phylogenetic development, it was decided to relate the foetal development of metabolic pathways of different phylogenetic age. As the glycolytic pathway seems to be present even in primitive micro-organisms, and as the hexose monophosphate shunt (C-6 oxidation) occurs predominantly in aerobic phylogenically higher organisms but not in primitive anaerobic micro-organisms (see the survey by

* Abbreviation: LDH, lactate dehydrogenase (L-lactate-NAD⁺ oxidoreductase, EC 1.1.1.27).

Horecker, 1965), the present paper also relates the foetal changes in LDH to those of enzymes involved in C-6 oxidation through the hexose monophosphate shunt.

MATERIALS AND METHODS

Materials

Human material. Human foetuses ranging in age from 13 to 25 weeks gestation age were obtained from the Obstetric and Gynaecological Departments of Øresund Hospital and Gentofte Hospital and from the Anatomical Institute of the University of Copenhagen; we are grateful to the Chiefs of these Departments for the specimens obtained. All specimens were received and processed within 12 hr. after the operation and in most instances within 3 hr.

Muscles involved in locomotive function (gastrocnemius, psoas major, sartorius) and in maintaining posture (gluteus maximus, quadriceps femoris, soleus) (Johnston & Whillis, 1950) were isolated.

Reagents. All chemicals used were of highest commercial purity from British Drug Houses Ltd. (Poole, Dorset), except for the nucleotides, which were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany).

Methods

Handling of tissue. The specimens used were cut out from the parenchymatous parts of each muscle. They were immediately washed in 0.15M-NaCl and divided into two parts: one part for the determination of the total enzyme activities and the other for the determination of the LDH isoenzyme pattern.

The extract used for measuring the total enzyme activities was prepared by homogenizing the tissue sample in 5 vol. (v/w) of 0.05M-sodium glycylglycine buffer, pH 7.6, containing Triton X-100 (1%) in a Hostalen centrifuge tube by means of a rounded glass stirring rod (140 rev./min.). The sample was then centrifuged at 18000g for 30 min. at 4°. Extracts for enzyme-electrophoresis were prepared without adding Triton X-100, as this detergent gives rise to an artificially stained zone in electrophoresis. The content of

blood in the specimens was evaluated to be below 1% by spectrophotometric determination at 412nm. (standard: bovine oxyhaemoglobin).

Assay of total LDH activity. The reaction mixture contained 100 µl. of suitably diluted extract and (final concentrations) 1.0 mM-pyruvate, 0.33 mM-NADH and 50 mM-sodium phosphate buffer, pH 7.5, in a final volume of 3.0 ml. (24°). All enzyme activities were determined by measuring the change in E_{366} on an Eppendorph spectrophotometer equipped with a Siemens recorder. The total LDH activity (µmoles of NADH oxidized/min. per ml. of sample or per mg. of extractable protein) and the experimental conditions were as defined by Clausen & Øvliisen (1965).

Assay of total glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities. The reaction mixture contained 200 µl. of suitable diluted extract and (final concentrations) 2.8 mM-glucose 6-phosphate or 2.8 mM-6-phosphogluconate (sodium salts) or both, 20 mM-MgCl₂, 1.30 mM-NADP⁺, 33.5 µM-EDTA (sodium salt) and 0.1 M-sodium glycylglycine buffer, pH 7.8 or 8.3, in a total volume of 1.0 ml. (24°) for assay of total activity of both enzymes and the 6-phosphogluconate dehydrogenase activity alone respectively (Greenberg & Glick, 1960). The tris buffer recommended by Greenberg & Glick (1960) was replaced by sodium glycylglycine buffer, as preliminary studies showed that tris inhibited the enzymic activities.

Determination of protein. Total protein was determined by the Lowry method as described by Lous, Plum & Schou (1956) on the same extract as was used to measure enzyme activities.

Determination of LDH isoenzymes. Agar-gel micro-electrophoresis was performed on an extract prepared by homogenizing the other part of the tissue sample in an equal volume of 0.05M-sodium glycylglycine buffer, pH 7.5. Homogenization and centrifugation was performed in the same manner as described above. The slides were run for 30 min. at 140 v and then stained for LDH activity by the formazan method as described by Van der Helm, Zonnadag & Harlog (1962). After staining for 30 min. at 37°, the slides were fixed for 30 min. in 4% acetic acid and then dried on filter paper and scanned in a Vitatron scanning apparatus (Clausen & Øvliisen, 1965). From the percentage distribution of the LDH isoenzymes and known sub-unit structure the H/M sub-unit ratio was calculated.

Table 1. Weekly changes in total LDH activity and changes in the H/M sub-unit ratio of human foetal muscles

All changes are significant at the $P < 0.001$ level.

	Change in LDH activity/week		Change in H/M sub-unit ratio/week	
	Slope of regression line	Interval of confidence	Slope of regression line	Interval of confidence
Muscles maintaining support (postural muscles)				
Gluteus maximus	$+2.68 \times 10^{-3}$	$\pm 1.54 \times 10^{-3}$	-50.0×10^{-3}	$\pm 33.1 \times 10^{-3}$
Quadriceps femoris	$+3.62 \times 10^{-3}$	$\pm 0.38 \times 10^{-3}$	-56.0×10^{-3}	$\pm 30.8 \times 10^{-3}$
Soleus	$+3.90 \times 10^{-3}$	$\pm 0.37 \times 10^{-3}$	-52.0×10^{-3}	$\pm 21.6 \times 10^{-3}$
Muscles mostly concerned with phasic contraction				
Psoas major	$+3.62 \times 10^{-3}$	$\pm 1.46 \times 10^{-3}$	-57.0×10^{-3}	$\pm 17.7 \times 10^{-3}$
Sartorius	$+3.17 \times 10^{-3}$	$\pm 0.36 \times 10^{-3}$	-71.0×10^{-3}	$\pm 32.0 \times 10^{-3}$
Gastrocnemius	$+4.58 \times 10^{-3}$	$\pm 1.93 \times 10^{-3}$	-55.0×10^{-3}	$\pm 2.4 \times 10^{-3}$

Determination of foetal age. The crown-rump length of the fetuses was used as a measure of age and was determined as described by Shepard, Andersen & Andersen (1964).

Statistical evaluation. The results were evaluated by calculation of the regression curve (Hald, 1952) relating respective enzymic activities to foetal age. The significance of the results was evaluated by determining whether the inclination of the regression curve was different from zero (Hald, 1952).

RESULTS

In all foetal muscles studied there is a significant increase in the total LDH activity/mg. of extracted protein and a significant decrease in the H/M sub-unit ratio with increasing foetal age (Table 1 and Figs. 1 and 2). The shift in the H/M sub-unit ratio also involves a progressively significant decrease in fractions I, II and III and an increase in fractions IV and V (Figs. 3 and 4). These findings show that the enzymic activity in the young foetal muscle is confined predominantly to fractions II, III and IV.

The change in total LDH activity/week and the change in H/M sub-unit ratio/week are for most of the muscles directly related (Table 1). Maximal and minimal values of changes in LDH activity/week and H/M sub-unit ratio/week correspond, but for values between these extremes no direct relationship can be deduced between the two series of values. Further, apart from the change in total LDH activity/week for soleus muscle, the group of muscles concerned with phasic contraction exhibit values higher than those of muscles maintaining the posture of the human body.

The results for glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are presented in Table 2 and Fig. 5. There were large variations and no significant change in activity could be demonstrated, although there was a slight insignificant increase in the 6-phosphogluconate dehydrogenase activity of all muscles. There was a similar insignificant increase in glucose 6-phosphate dehydrogenase activity in the gastrocnemius, psoas major, sartorius and quadriceps femoris muscles.

DISCUSSION

The present results, demonstrating a developmental increase in total LDH activity of human striated muscles, are in agreement with the data of Dubowitz (1963), who, on the basis of studies of animal foetal material, suggested that the increase in LDH activity may correspond to the appearance of contractile activity. Thus the increase in total LDH activity may be related to the increase in

Fig. 1. Relationship between total LDH activity extractable from gluteus maximus muscle and foetal age. Experimental details are given in the text. Slope of regression line $\beta = 2.68 \times 10^{-3}$; interval of confidence $\pm 1.54 \times 10^{-3}$; $P < 0.001$.

Fig. 2. Relationship between H/M sub-unit ratio of LDH isoenzymes and foetal age. Experimental details are given in the text. Slope of regression line $\beta = -7.10 \times 10^{-2}$; interval of confidence $\pm 3.20 \times 10^{-2}$; $P < 0.001$.

oxygen consumption caused by the contractile activity appearing in the last half of the gestation period. Studies of the enzymic changes after stimulation to muscles have shown that there is indeed an increase in enzyme activities, including that of LDH, after the appearance of contractile activity (Kendrick-Jones & Perry, 1965). The diminished muscular activity in old age seems, on the other hand, to be associated with decreased total LDH activity (Emery, 1967).

Fig. 3. Relationship between proportional LDH isoenzyme activities (expressed as percentages of total LDH activity) of gluteus maximus muscle and foetal age. Experimental details are given in the text. (a) LDH-H₄; (b) LDH-H₃M; (c) LDH-H₂M₂; (d) LDH-HM₃; (e) LDH-M₄.

Fig. 4. Relationship between proportional LDH isoenzyme activities (expressed as percentages of total LDH activity) of quadriceps muscle and foetal age. Experimental details are given in the text. (a) LDH-H₄; (b) LDH-H₃M; (c) LDH-H₂M₂; (d) LDH-HM₃; (e) LDH-M₄.

Table 2. Weekly changes in total glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities of human foetal muscles

The changes indicated are not significant at the $P < 0.001$ level. G6PDH, Glucose 6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase.

	Change in G6PDH activity/week		Change in 6PGDH activity/week	
	Slope of regression line	Interval of confidence	Slope of regression line	Interval of confidence
Muscles maintaining support (postural muscles)				
Gluteus maximus	-0.10×10^{-4}	$\pm 1.03 \times 10^{-4}$	$+0.04 \times 10^{-4}$	$\pm 0.48 \times 10^{-4}$
Quadriceps femoris	$+0.04 \times 10^{-4}$	$\pm 0.82 \times 10^{-4}$	$+0.07 \times 10^{-4}$	$\pm 0.58 \times 10^{-4}$
Soleus	$+0.00 \times 10^{-4}$	$\pm 0.44 \times 10^{-4}$	$+0.37 \times 10^{-4}$	$\pm 0.44 \times 10^{-4}$
Muscles mostly concerned with phasic contraction				
Psoas major	$+0.40 \times 10^{-4}$	$\pm 0.75 \times 10^{-4}$	$+0.15 \times 10^{-4}$	$\pm 0.49 \times 10^{-4}$
Sartorius	$+0.36 \times 10^{-4}$	$\pm 0.85 \times 10^{-4}$	$+0.13 \times 10^{-4}$	$\pm 0.35 \times 10^{-4}$
Gastrocnemius	$+0.49 \times 10^{-4}$	$\pm 0.85 \times 10^{-4}$	$+0.11 \times 10^{-4}$	$\pm 0.44 \times 10^{-4}$

Fig. 5. Relationship between total glucose 6-phosphate dehydrogenase (G6PDH) (∇) and 6-phosphogluconate dehydrogenase (6PGDH) ($\circ$) activities of soleus muscle and foetal age. Experimental details are given in the text.

The increase in total LDH activity appears to be roughly proportional to the developmental shift to a higher percentage of M-sub-units. Thus the H-sub-units are being synthesized at a constant rate whereas M-sub-units are being produced at a constantly increasing rate, a change necessary to produce the adult pattern eventually. These changes are in agreement with Cahn *et al.* (1962), Philip & Vesell, 1962, Vesell & Philip (1963), Master (1964) and Latner & Skillen (1964).

The theory that the H/M sub-unit ratio is related to the oxygen concentration is supported by the demonstration that the M-sub-units can function in an environment characterized by low oxygen

concentration and high pyruvate and lactate concentrations (Pfleiderer & Wachsmuth, 1961; Regnier & Wieme, 1962). A foetal shift to a predominance of M-sub-units would then be expected in a tissue experiencing a metabolic transition necessitating an increased dependence on anaerobic energy production. Such a dependence may indeed occur in foetal muscle in the last 3 months of gestation, when contractile activity appears.

The developmental increase in muscle mass (probably white muscle fibres; see below) and amount of cells could not be related to increase in the two enzymes of the hexose monophosphate shunt forming metabolic hydrogen (NADPH) and ribose for synthetic purposes. Thus it was not possible to relate the foetal development of these enzymes to phylogenesis. Van Wijhe, Blanchaer & St George-Stubbs (1964) and Nene & George (1965) demonstrated that, among the red muscle fibres and white muscle fibres occurring in both adult and foetal muscles (Wiisen & Larsson, 1964), an uneven distribution of enzymes occurs. Thus LDH-M₄ predominates in the white muscle fibres and LDH-H₄ in the red muscle fibres. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase occur in red muscle fibres but not in white muscle fibres. These facts, together with the present findings of a developmental increase in the M-sub-unit content but no changes in that of H-sub-units and in the activities of the two enzymes of the hexose monophosphate shunt, suggest that the changes found are solely localized in the white muscle fibres. This is supported by Chinory & George (1965), who found histologically that the white muscle fibres show increases in diameter and glycogen content in the foetal period. These changes were not detected in the red muscle fibres.

REFERENCES

- Cahn, R. D., Kaplan, N. O., Levine, L. & Zwilling, E. (1962). *Science*, **136**, 962.
- Chinory, N. J. & George, J. C. (1965). *J. Anim. Morph. Physiol.* **12**, 57.
- Clausen, J. & Øvlsen, B. (1965). *Biochem. J.* **97**, 513.
- Dawson, D. M., Goodfriend, T. L. & Kaplan, N. O. (1964). *Science*, **143**, 929.
- Dubowitz, V. (1963). *Nature, Lond.*, **197**, 1215.
- Emery, A. E. H. (1967). *Biochem. J.* **105**, 509.
- Fine, I. H., Kaplan, N. O. & Kuftinec, D. (1963). *Biochemistry*, **2**, 116.
- Greenberg, J. J. & Glick, D. (1960). *J. biol. Chem.* **235**, 3028.
- Hald, A. (1952). *Statistical Theory with Engineering Applications*, p. 522. New York: J. Wiley and Sons Inc.
- Horecker, B. L. (1965). *J. chem. Educ.* **42**, 244.
- Johnston, T. B. & Whillis, J. (Eds.) (1950). *Gray's Anatomy*, 30th ed., pp. 530-675. London: Longmans, Green and Co.
- Kendrick-Jones, J. & Perry, S. V. (1965). *Nature, Lond.*, **208**, 1068.
- Latner, A. L. & Skillen, A. W. (1964). *J. Embryol. exp. Morph.* **12**, 501.
- Lous, P., Plum, C.-M. & Schou, M. (1956). *Nord. Med.* **55**, 693.
- Markert, C. L. & Apella, E. (1961). *Ann. N.Y. Acad. Sci.* **94**, 675.
- Master, C. J. (1964). *Biochim. biophys. Acta*, **89**, 161.
- Nene, R. V. & George, J. C. (1965). *J. Anim. Morph. Physiol.* **12**, 90.
- Pfleiderer, G. & Wachsmuth, E. (1961). *Biochem. Z.* **334**, 185.
- Philip, J. & Vesell, E. S. (1962). *Proc. Soc. exp. Biol., N.Y.*, **110**, 582.
- Regnier, P. & Wieme, R. J. (1962). *Arch. int. Pharmacodyn.* **140**, 409.
- Shaw, C. R. & Barto, E. (1963). *Proc. nat. Acad. Sci., Wash.*, **50**, 211.
- Shepard, T. H., Andersen, H. J. & Andersen, H. (1964). *Anat. Rec.* **148**, 123.
- Vander Helm, H. J., Zonndag, H. A. & Harlog, H. A. Ph. (1962). *Clin. chim. Acta*, **7**, 540.
- Van Wijhe, M., Blanchaer, M. C. & St George-Stubbs, S. (1964). *J. Histochem. Cytochem.* **12**, 608.
- Vesell, E. S. & Philip, J. (1963). *Ann. N.Y. Acad. Sci.*, **111**, 243.
- Wiisen, C. & Larsson, K. S. (1964). *J. Embryol. exp. Morph.* **12**, 759.
- Wilkinson, J. H. (1965). *Isoenzymes*, pp. 47-51. London: E. and F. N. Spon Ltd.