Biochem. J. (1967) **105**, 599
Printed in Great Britain

The Determination of Lactate Dehydrogenase Isoenzymes in Normal Human Muscle and Other Tissues

By A. E. H. EMERY
University Department of Medical Genetics, The Royal Infirmary, Manchester

(*Received* 31 *March* 1967)

1. A technique has been developed, based on preferential inhibition by urea, for determining the amounts and proportions of the M and H sub-units of lactate dehydrogenase (referred to as LDH-M and LDH-H respectively) in human tissues, including muscle. 2. There was good agreement between the results obtained with urea inhibition and those obtained with starch-gel electrophoresis. 3. With increasing age there was a significant decrease in the total amount of lactate dehydrogenase and the amount of LDH-M in skeletal muscle. This could not be accounted for by the replacement of functioning muscle tissue by fibrous connective tissue. 4. The proportion of LDH-M was less in certain muscles (e.g. soleus and extra-ocular) than in other muscles (e.g. gastrocnemius and rectus abdominis). 5. The proportions of LDH-M and LDH-H did not differ significantly in different superficial limb muscles and were not significantly affected by either age or sex. 6. Specimens of muscle from 86 different individuals (all Europeans) have been subjected to electrophoresis, but no variants of lactate dehydrogenase isoenzymes have been found.

The enzyme LDH* is composed of two types of sub-units referred to as A and B by Markert (1962) and as M and H by Cahn, Kaplan, Levine & Zwilling (1962), since M sub-units predominate in skeletal muscle and H sub-units predominate in heart muscle. These types of sub-units are believed to combine in various tetrameric associations to produce five isoenzymes such that LDH-1, the most rapidly migrating isoenzyme, has the composition HHHH; LDH-2, HHHM; LDH-3, HHMM; LDH-4, HMMM; and LDH-5, MMMM. Evidence in support of this hypothesis has come from several sources (Vesell, 1965). The M and H sub-units of LDH are referred to below as LDH-M and LDH-H respectively.

Several investigators have demonstrated that both LDH-5 and LDH-1 are inhibited by urea, though the former is inhibited more than the latter (Plummer, Wilkinson & Withycombe, 1963; Richterich & Burger, 1963; Brody, 1964; Brody & Engel, 1964; Emerson & Wilkinson, 1965; Hardy, 1965; Konttinen & Lindy, 1965; Withycombe, Plummer & Wilkinson, 1965; Lindy & Konttinen, 1966). The present work shows that under certain experimental conditions in the presence of urea there is an increase in the activity of LDH-1 and a decrease in the activity of LDH-5. These findings have led to the development of a technique for determining the relative proportions of LDH-H and LDH-M in human muscle and other tissues.

* Abbreviation: LDH, lactate dehydrogenase.

No previous study appears to have been made of LDH isoenzymes in human tissues by this technique, and no systematic study has been made of the effects of age and sex on the amounts and proportions of LDH-M and LDH-H in fresh biopsy specimens of normal human skeletal muscle.

EXPERIMENTAL

Tissues. All specimens of muscle (300–500mg.) were removed at operations conducted under general anaesthesia from individuals with no history of any neuromuscular disorder. Some specimens of tissue other than muscle were obtained at autopsy. After excision any adherent fat or connective tissue was removed and the specimens were then immediately washed in 0·9% NaCl, to remove any contaminating erythrocytes, blotted dry, weighed (wet weight) and then homogenized in about 5vol. of water in a Potter–Elvehjem homogenizer, the tube being kept cold by surrounding it with ice. Enzyme assays, nitrogen determinations and electrophoresis were carried out on the whole suspension suitably diluted with water. In none of the specimens of muscle did the blood content, estimated by the method described by Holzer, Sedlmayr & Kiese (1956), exceed 5% and in most cases the blood content was less than 2%, which agrees well with the findings of others (Gitlin & Janeway, 1954; Pennington, 1963). Differences in enzyme content in different specimens of muscle cannot therefore be due to differences in the amount of blood present in these specimens.

Chemicals. NAD, *p*-nitro-blue tetrazolium and phenazine methosulphate were obtained from Sigma Chemical Co. (London) Ltd., London, S.W. 6. Starch was supplied by Connaught Medical Research Laboratories, Toronto,

Canada. Purified LDH-1 (pig heart) and purified LDH-5 (rabbit muscle) were obtained from C. F. Boehringer Corp. (London) Ltd., London, W. 5. All other chemicals were supplied by British Drug Houses Ltd., Poole, Dorset.

LDH estimation. LDH activity was determined spectrophotometrically with lactate as substrate. The method used is based on that of Wacker, Ulmer & Vallee (1956) with certain modifications (Boutwell, 1961). The reaction mixture consisted of 1·5ml. of 50mM-tris–HCl buffer, pH 9·0, 1·0ml. of 0·25M-sodium lactate, pH 9·0, 0·3ml. of 50mM-NAD and 0·2ml. of suitably diluted homogenate. The NAD solution was freshly prepared before each experiment. Activity was calculated from the rate of increase of $E_{340}^{1\,cm.}$ during the 5 min. period after the addition of the homogenate, at 25°. The concentration of each homogenate was so adjusted (usually 1:330, w/v, dilution in water) that the change in extinction was approx. 0·020–0·040/min. Under these conditions the reaction rate is linear and directly proportional to the amount of enzyme present. The effects of urea were studied by incorporating urea in the tris–HCl buffer; in this case, after the addition of the homogenate to the reaction mixture, time (1–2min.) was allowed for equilibration, and then the rate of increase in $E_{340}^{1\,cm.}$ was determined over the next 5min. All enzyme determinations were carried out within 0·5–1hr. after the tissues had been excised. Enzyme activites are expressed as μmoles of substrate converted/min. at 25° and pH 9·0.

Non-collagen nitrogen estimation. The amount of non-collagen nitrogen in muscle homogenates was determined by digesting the homogenate (1:33, w/v, dilution in water) overnight at 20° with 9vol. of 0·05N-NaOH (Lilienthal, Zierler, Folk, Buka & Riley, 1950), centrifuging the digest and then determining the amount of nitrogen in the supernatant fluid by a micro-Kjeldahl procedure.

Electrophoresis. Homogenates were subjected to vertical starch-gel electrophoresis (Smithies, 1959) at 4° and 4·0v/cm. for approx. 18hr. (Emery, Sherbourne & Pusch, 1965). The starch gels were buffered at pH 8·5 with mM-EDTA–25mM-boric acid–45mM-tris (final concns.) as described by Boyer, Fainer & Naughton (1963). The concentration of each homogenate was so adjusted that the volume (30μl.) of material subjected to electrophoresis always contained about the same amount of LDH activity (85–95mμmoles/min.). After electrophoresis, LDH activity was located by the method of Dewey & Conklin (1960) with certain modifications (Blanco & Zinkham, 1963). The proportions of the individual isoenzymes were determined with a Chromoscan recording and integrating densitometer (Joyce–Loebl Co. Ltd.). Electrophoresis was carried out the same day that a biopsy specimen was excised and only on freshly prepared homogenates.

For each specimen of tissue the amount of enzyme activity, the amount of non-collagen nitrogen and the percentages of the various isoenzymes on electrophoresis are the mean values of duplicate determinations on the same homogenate.

RESULTS

The effects of different concentrations of urea on the activities of purified LDH-1 (from pig heart) and purified LDH-5 (from rabbit muscle) were found to be quite different (Fig. 1). With 2M-urea (final concentration in the reaction mixture) there

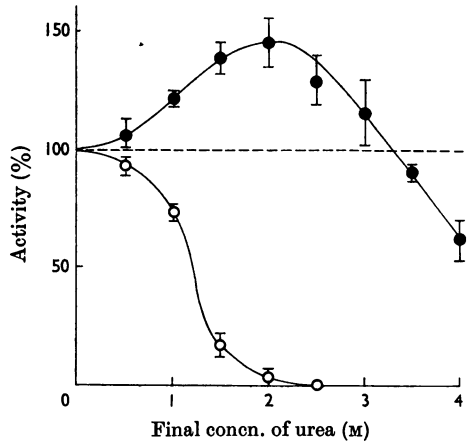

Fig. 1. Percentage activity of purified LDH-1 (●) and LDH-5 (○) in the presence of urea with lactate (final concn. 83mM) as substrate at pH 9·0 and 25°. Each point is the mean of three experiments, and the vertical lines represent 1 S.E.M. on either side of the mean.

was almost complete inhibition of LDH-5, whereas the activity of LDH-1 was approx. 1·5 times the activity in the absence of urea. Very similar results were obtained when homogenates of human heart and liver were used.

Previous investigators have shown that, with lactate as substrate, LDH-1 is inhibited after it has been incubated with 2M-urea alone for about 15min. before the reaction rate is determined (Withycombe *et al.* 1965). This finding has been confirmed in the present investigation. However, when the enzyme is added to urea in the presence of NAD and substrate there is increased enzyme activity. Under these conditions the percentage increase in activity with 2M-urea gradually decreases from about 150% after 5–6min., to about 145% after 10min. and 140% after 20min.

Since in the presence of 2M-urea the activity of LDH-1, which is composed entirely of H sub-units, is increased 1·5 times, and the activity of LDH-5, which is composed entirely of M sub-units, is almost completely inhibited, it is presumed that in any mixture of isoenzymes with 2M-urea the activity of all the H sub-units would be increased 1·5 times and the activity of all the M sub-units would be almost completely inhibited. It should therefore be possible to measure the amounts and proportions of H and M sub-units in any particular tissue by determining the enzyme activity in the presence and absence of 2M-urea:

If activity in the absence of urea = T
and activity in the presence of 2M-urea = U

then activity due to LDH-H only in the absence of urea $= U/1{\cdot}5$

and the percentage of LDH-M $= \frac{T-U/1{\cdot}5}{T} \times 100$

In this way the amounts and proportions of LDH-M and LDH-H have been determined in aqueous homogenates of various tissues removed at autopsy (Table 1). The results agree in general with those obtained by other methods such as densitometry of isoenzyme bands after electrophoresis (Wróblewski & Gregory, 1961), elution from the supporting medium after electrophoresis (Vesell & Bearn, 1961), column chromatography on DEAE-Sephadex (Richterich, Schafroth & Aebi, 1963) or the use of coenzyme analogues (Dawson, Goodfriend & Kaplan, 1964).

Further evidence that the method of urea inhibition, as used in this investigation, is probably a reliable means of assessing the relative proportions of M and H sub-units in human tissues comes from the close agreement between the observed and predicted relationships between the percentage of LDH-5 on electrophoresis and that of M sub-unit as determined by urea inhibition. Assuming that the various isoenzymes of LDH are tetramers formed by random combinations of the two types of sub-units (M and H), and that the proportions of the various isoenzymes depend on the relative proportions of the two sub-units, it is possible to calculate from the binomial expansion the percentage of LDH-5 for different proportions of M sub-units. The relative proportions of the five isoenzymes are given by the terms of the expansion:

$$(H+M)^n = n!H^rM^{n-r}/(n-r)!r!$$

where M and H represent the relative proportions of M and H sub-units (e.g. 1/10 and 9/10), r is the number of H sub-units contributing to any particular isoenzyme (e.g. in LDH-1, r is 4) and n is the total number of sub-units in each isoenzyme (i.e. 4). The predicted relationship between the percentage of LDH-5 and the percentage of M sub-units (Table 2) is in fairly close agreement with the observed relationship (Fig. 2). The observed relationship was obtained by determining the percentage of LDH-5 from the results of electrophoresis and that of LDH-M from the results of urea inhibition.

The activities and proportions of LDH-M and LDH-H in various skeletal muscles from individuals with no neuromuscular disease are given in Table 3. There was considerable variation in the total activity of LDH (LDH-M and LDH-H) even within the same muscle, though there was clearly less enzyme in soleus and extra-ocular muscles than in the other skeletal muscles that have been studied. There was a slight but significant regression on age for the total amount of LDH and for the amount of LDH-M (Table 4). The regressions on age for males and females were both significant, but the difference between them was not significant. There was slightly more enzyme in muscles from

Table 1. *Activities and proportions of LDH-M and LDH-H in various tissues*

For details see the text.

Tissue		Enzyme activity (μmoles/min./g.)			Enzyme activity (% of total)	
		Total	H	M	H	M
Liver	I	21·6	1·6	20·0	7·4	92·6
	II	26·3	1·3	25·0	5·0	95·0
Spleen	I	13·5	3·1	10·4	23·0	77·0
	II	17·3	4·0	13·3	23·1	76·9
Lung	I	17·4	7·5	9·9	43·1	56·9
	II	8·4	2·6	5·8	31·0	69·0
	III	3·6	1·9	1·7	52·8	47·2
	IV	6·3	2·1	4·2	33·3	66·7
Thyroid	I	13·4	5·9	7·5	44·1	55·9
	II	12·1	4·9	7·2	40·5	59·5
Lymph node	I	13·8	5·3	8·5	38·4	61·6
	II	10·8	5·2	5·6	48·2	51·8
Kidney medulla	I	17·4	7·2	10·2	41·4	58·6
	II	38·0	20·7	17·3	54·5	45·5
Kidney cortex	I	15·1	13·2	1·9	87·4	12·6
	II	20·6	18·0	2·6	87·4	12·6
Brain	I	9·8	8·4	1·4	85·7	14·3
	II	10·3	9·6	0·7	93·2	6·8

Table 2. *Predicted percentage of LDH-5 for different proportions of H and M sub-units*

For details of the calculation see the text.

Proportion of sub-units (%)		
H	M	LDH-5 (%)
100	0	0·00
70	30	0·81
50	50	6·25
40	60	12·96
30	70	24·01
20	80	40·96
10	90	65·61
0	100	100·00

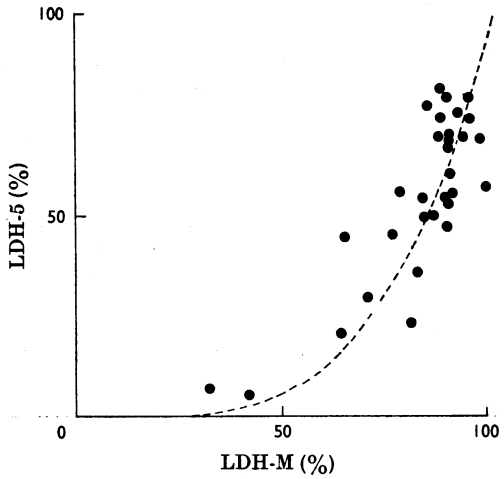

Fig. 2. Observed (●) and predicted (---) relationships between the proportion of LDH-5 and the proportion of LDH-M in various skeletal muscles.

males compared with females but the difference was not significant.

The proportion of LDH-M was greater in certain muscles (e.g. gastrocnemius and rectus abdominis) than in other muscles (e.g. soleus and extra-ocular), but did not differ significantly in different superficial limb muscles. There was no significant correlation between age and the proportions of LDH-M (correlation coefficient 0·17) and LDH-H (correlation coefficient 0·16). The proportion of LDH-M was slightly greater in males (89·5 ± 3·8%) than in females (86·8 ± 4·5%), and that of LDH-H was slightly greater in females (13·2 ± 4·5%) than in males (10·5 ± 3·8%), but the differences were not significant (these values are means ± S.D.). Specimens of skeletal muscle from 86 individuals (all Europeans) with no neuromuscular disease have been subjected to electrophoresis, but no variants of LDH isoenzymes have been found.

DISCUSSION

In the present investigation LDH activity was determined with lactate as substrate. The rate of reaction is greater with pyruvate as substrate, but there are several theoretical as well as practical reasons for preferring the reverse reaction (Snodgrass, Wacker, Eppinger & Vallee, 1959; Fawcett, Ciotti & Kaplan, 1961; Amador, Dorfman & Wacker, 1963; Amador, Reinstein & Benotti, 1965).

Under the experimental conditions used in this investigation it has been found that with 2M-urea there was almost complete inhibition of LDH-5 whereas the activity of LDH-1 was approx. 1·5 times the activity in the absence of urea. The shapes of the curves for the effects of various concentrations of urea on LDH-1 and LDH-5 are comparable with those obtained by Fritz (1965) for the effect of oxaloacetate on the activities of these isoenzymes. Fritz (1965) reported that with low concentrations of oxaloacetate LDH-1 was inhibited, but the activity of LDH-5 was increased to about 120% of the activity in the absence of oxaloacetate. This effect was ascribed to allostery; oxaloacetate probably brings about a molecular alteration in LDH-5 that leads to the exposure of new substrate sites and therefore to increased enzyme activity. Possibly urea in low concentrations has a similar effect on LDH-1.

Several previous investigators have studied the LDH content of human skeletal muscle, but without exception all have used pyruvate as substrate (Ronzoni, Berg & Landau, 1960; Schmidt & Schmidt, 1960; Tada, Watanabe & Chikaoka, 1961; Heyck, Laudahn & Lüders, 1963; Kar & Pearson, 1963; Richterich *et al.* 1963; Dawson & Kaplan, 1965; Monckton & Nihei, 1965; Johnston, Wilkinson, Withycombe & Raymond, 1966; Kleine & Chlond, 1967). Those investigators who have studied the enzyme content of different skeletal muscles and have used fresh biopsy material rather than autopsy material, in which there is some loss of enzyme activity, have failed to demonstrate any significant difference in the amount of LDH activity in different superficial limb muscles (Laudahn & Heyck, 1963). However, some recent findings suggest that the enzyme content of red muscle may be less than that of white muscle (Kleine & Chlond, 1967), and in the present investigation less enzyme was found in soleus (red) than in adjacent gastrocnemius (white) muscle. Monckton & Nihei (1965) have found that the LDH content of limb muscles is significantly

Table 3. *Activities and proportions of LDH-M and LDH-H in various skeletal muscles*

For details see the text.

Muscle	Sex	Age (years)	Enzyme activity (μmoles/min./g.)			Enzyme activity (μmoles/min./100 mg. of non-collagen N)			Enzyme activity (%)	
			H	M	Total	H	M	Total	H	M
Rectus abdominis	F	78	1·5	12·6	14·1	4·6	39·0	43·6	10·6	89·4
	M	58	3·2	20·7	23·9	8·2	53·8	62·0	13·2	86·8
	M	61	4·2	32·6	36·8	14·1	108·3	122·4	11·5	88·5
	M	60	5·5	28·2	33·7	16·8	86·2	103·0	16·3	83·7
	M	69	3·7	23·9	27·6	20·4	131·9	152·3	13·4	86·6
Gastrocnemius	M	35	3·7	31·9	35·6	11·3	97·8	109·1	10·4	89·6
	M	44	4·8	29·3	34·1	15·2	93·4	108·6	14·0	86·0
	F	25	4·3	23·6	27·9	17·9	97·8	115·7	15·5	84·5
Deltoid	M	23	1·4	22·0	23·4	8·7	138·8	147·5	5·9	94·1
	M	31	1·6	53·8	55·4	7·0	236·4	243·4	2·9	97·1
Pectoralis major	F	50	4·8	16·2	21·0	21·2	72·3	93·5	22·7	77·3
	F	40	3·6	28·2	31·8	15·8	124·0	139·8	11·3	88·7
	M	33	3·5	32·1	35·6	17·0	157·2	174·2	9·8	90·2
	F	45	2·6	22·3	24·9	13·8	116·2	130·0	10·6	89·4
	F	39	3·1	29·1	32·2	16·8	160·4	177·2	9·5	90·5
	M	51	3·2	32·8	36·0	12·3	127·7	140·0	8·8	91·2
Quadriceps	F	18	3·6	31·5	35·1	18·7	161·5	180·2	10·4	89·6
Sterno-mastoid	F	20	5·4	25·2	30·6	27·0	126·1	153·1	17·6	82·4
Sartorius	M	52	1·4	16·4	17·8	6·3	74·9	81·2	7·8	92·2
Diaphragm	F	17	3·4	28·0	31·4	12·5	103·7	116·2	10·8	89·2
Latissimus dorsi	M	70	3·5	24·0	27·5	13·4	92·7	106·1	12·6	87·4
Soleus	M	81	6·4	3·6	10·0	—	—	—	36·0	64·0
	M	70	7·3	3·1	10·4	—	—	—	29·8	70·2
Ocular muscles*	—	—	3·0	1·4	4·4	—	—	—	68·2	31·8
	—	—	1·7	1·2	2·9	—	—	—	58·6	41·4

* In each experiment the material from three patients was combined.

Table 4. *Relationship between age* (X) *and amount of enzyme activity* (Y) *in various skeletal muscles*

Total LDH (1) is based on the 21 results given in Table 2. Total LDH (2) is based on the results of 50 fresh biopsy specimens of various skeletal muscles in which the amounts of LDH-H and LDH-M were not determined. LDH activities are given as means ± S.D. The results on ocular and soleus muscles have been excluded. N.S., Not significant.

	No. of results	LDH activity (μmoles/min./100 mg. of non-collagen N)	Regression line	Significance of regression coefficient
LDH-H	21	14·2 ± 5·5	$Y = 17{\cdot}8 - 0{\cdot}08X$	N.S.
LDH-M	21	114·3 ± 43·3	$Y = 168{\cdot}9 - 1{\cdot}24X$	$P < 0{\cdot}02$
Total LDH (1)	21	128·5 ± 44·4	$Y = 186{\cdot}7 - 1{\cdot}33X$	$P < 0{\cdot}02$
Total LDH (2)	50	121·5 ± 50·2	$Y = 199{\cdot}1 - 1{\cdot}67X$	$P < 0{\cdot}001$
Males	24	124·0 ± 46·3	$Y = 200{\cdot}3 - 1{\cdot}58X$	$P < 0{\cdot}05$
Females	26	119·1 ± 54·4	$Y = 198{\cdot}7 - 1{\cdot}77X$	$P < 0{\cdot}01$

greater than that of trunk muscles. Dawson & Kaplan (1965) considered the possible effects of age and sex on the LDH content of skeletal muscle. They noted that '. . . younger individuals, particularly men, tended to have . . . higher levels of total LDH' in their muscles, but results at different ages were not given and their observations were confined to autopsy material. In the present study it was found that there is a small but significant decrease in LDH content with increasing age. Since this effect is seen when the amount of LDH is expressed in terms of the amount of non-collagen nitrogen,

the effect of age cannot be due merely to gradual replacement of functioning muscle tissue by fibrous connective tissue. No significant difference was found in the LDH content of muscles from males and females.

By using the technique of urea inhibition it was found that certain muscles (e.g. soleus and extra-ocular) have a greater proportion of LDH-H than other muscles (e.g. gastrocnemius and rectus abdominis). These findings are in agreement with those of Kar & Pearson (1963), who used agar-gel electrophoresis, and Dawson & Kaplan (1965), who used coenzyme analogues. Muscles situated deeper in the limb (e.g. soleus) and concerned more with support than with phasic contraction have a greater proportion of the more rapidly migrating iso-enzymes than neighbouring superficial muscles such as the gastrocnemius. These observations suggest that the isoenzyme pattern of a particular skeletal muscle may be determined by the proportion of red and white fibres in that muscle. By an ingenious technique involving the determination of iso-enzyme patterns in isolated muscle fibres, van Wijhe, Blanchaer & St George-Stubbs (1964) have presented evidence that supports this idea.

Grateful thanks are due to Dr A. H. Gowenlock for much helpful advice and to the Muscular Dystrophy Group of Great Britain for a research grant. Miss V. Hodson and Miss C. Mold gave valuable technical assistance.

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