Substrate specificity of the lactate dehydrogenase isoenzyme C₄ from human spermatozoa and a possible selective assay

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Summary. The activity of lactate dehydrogenase (EC 1.1.1.27) in normal human sperm lysates and in human heart and liver homogenates was determined by using a variety of 2-oxoacids as substrates. Sperm preparations were active with pyruvate, 2-oxobutanoate, 2-oxopentanoate and 2-oxohexanoate, while heart and liver extracts utilized only pyruvate and 2-oxobutanoate. Selective staining after gel electrophoresis indicated that the fraction corresponding to lactate dehydrogenase C₄, the sperm-specific isoenzyme, was responsible for the utilization of substrates with a linear chain of 3 to 6 carbon atoms. The use of 5 mM 2-oxohexanoate allowed the selective determination of isoenzyme C₄ in preparations containing different lactate dehydrogenase molecular forms.

Introduction

The lactate dehydrogenase (EC 1.1.1.27) isoenzyme which is specific to the testis and spermatozoa (LDH-X or C₄) from many species (Blanco & Zinkham, 1963; Goldberg, 1963; Zinkham, Blanco & Clowry, 1964) presents unique catalytic properties (Blanco, Zinkham & Walker, 1975; Blanco, Burgos, Gerez de Burgos & Montamat, 1976). It is a very specialized enzyme which must be related to metabolic processes supplying energy for sperm motility and survival (Storey & Kayne, 1977; Gerez de Burgos, Burgos, Montamat, Moreno & Blanco, 1978), and the determination of its activity in spermatozoa could therefore be useful for assessing the quality of a semen sample.

Because spermatozoa contain a mixture of different lactate dehydrogenase isoenzymes, the assay for total activity does not provide a direct estimation of LDH C₄. It is necessary, in addition, to obtain an electrophoretic zymogram and to determine, by densitometry, the relative amount of each isoenzyme. This procedure is complicated and time consuming for clinical purposes and its sensitivity is poor. The ideal would be a direct selective assay for LDH C₄.

One of the distinctive features of LDH C₄ is its affinity for 2-oxoacids of carbon chain longer than that of pyruvate. This wider substrate specificity allowed the selective determination of the isoenzyme C₄ present in rat and mouse testicular homogenates which contained a mixture of all the lactate dehydrogenase isoenzymes (Blanco et al., 1976). However, the substrate spectrum of LDH C₄ is not identical for all species and previous study of substrate specificity is required before a selective assay can be designed in a particular species.

This paper presents the results of such a study on human spermatozoa and indicates the possibility of developing a selective assay for LDH C₄.
Materials and Methods

Tissue homogenates

Heart and liver tissue were obtained within 12 h after death, at autopsy of normal people killed in accidents. One part of the tissue was suspended in four parts of distilled water (w/v) and homogenized in an all-glass Potter-Elvejhem grinder. The preparation was centrifuged at 20,000 g for 20 min at 4°C. The supernatants were used for electrophoresis and enzyme assays.

Sperm lysates

Semen was obtained from normal donors by masturbation. All the samples used contained more than 70 × 10^6 spermatozoa and more than 40 × 10^6 motile spermatozoa/ml. Ejaculates were centrifuged at 3000 g for 20 min at 4°C and the pellets of spermatozoa were washed with about 20 parts of 0.9% (w/v) NaCl solution. The washed sediment was suspended in 2 parts of distilled water, kept at −20°C for 2 h and then thawed at room temperature. After thawing, it was submitted to three 30-sec bursts of sonication (Faetron: Argentina) at 100 W. The lysates were finally centrifuged at 4°C and 20,000 g for 20 min, and the supernatants were used for analyses.

Electrophoresis

Tissue and sperm extracts were separated by means of starch gel electrophoresis and lactate dehydrogenase activity was revealed on the gel as indicated by Zinkham et al. (1964), except that when 2-hydroxypentanoate was used as substrate the final concentration was 150 mM and the staining time was 2 or more hours.

Enzyme assays

Lactate dehydrogenase activity was determined at 37°C by recording optical density change at 340 nm as described by Blanco et al. (1976). Concentrations of substrates are given in the 'Results' section. One Unit of enzyme was considered to be the amount producing an optical density change of 2.07/min at 340 nm, which corresponds to oxidation of 1 µmol NADH in the conditions of the assay.

NAD, NADH and all the 2-oxo- and 2-hydroxyacids used were purchased from Sigma Chemical Co. (St Louis, Missouri, U.S.A.).

Results

Activity of human tissues and sperm homogenates against pyruvate

Curves obtained by plotting the percentage of maximal activity of the extract against concentration of pyruvate are represented in Text-fig. 1. Heart homogenates showed maximal activity at lower concentrations than did liver extracts. Inhibition by substrate was more marked for heart than for liver homogenates. As expected, the curve for crude heart preparations resembled that described for pure lactate dehydrogenase isoenzyme 1 (B4), while the curve for liver homogenates reproduced that known for isoenzyme 5 (A4). Sperm lysates gave a curve very similar to that of heart extracts.

Electrophoretic patterns of heart, liver and sperm extracts stained by using lactate as substrate showed the distribution of isoenzymes characteristic for each of these tissues. Human heart homogenates were rich in isoenzymes 1 (B4) and 2 (A1/B2), those of liver contained predominantly isoenzyme 5 (A4) and sperm lysates presented most of their activity in the fraction corresponding to isoenzyme C4 (Pl. 1, Fig. 1).
Electrophoretic patterns of lactate dehydrogenases in human tissue extracts: (a) heart homogenate, (b) sperm lysate, (c) liver homogenate. The substrates used were lactate (Fig. 1) 2-hydroxybutanoate (Fig. 2), 2-hydroxypentanoate (Fig. 3) and 2-hydroxyhexanoate (Fig. 4). Numbers at the top and bottom indicate the position of the common LDH isoenzymes, X indicates the position of LDH C4. All extracts were electrophoresed simultaneously in the same starch block.

(Facing p. 102)
**Text-fig. 1.** Lactate dehydrogenase activity of human tissue extracts. Initial velocity, expressed as a percentage of the maximal activity, is plotted against pyruvate concentration (0.02–10.0 mM). ●, Sperm lysate; △, heart homogenate; ○, liver homogenate. Each point represents the average of determinations on 4 different preparations for heart and liver, and on 4 pools of 4 sperm samples each. The vertical bars represent ± 1 s.d.

**Activity of human preparations against different 2-oxoacids**

Human sperm lysates catalysed the NAD-linked reduction of 2-oxobutanoate, 2-oxopentanoate and 2-oxohexanoate. Activity with 2-oxoglutarate was negligible and 2-oxooctanoate was not utilized. No activity was recorded with branched-chain 2-oxoacids such as 2-oxo-3-methylbutanoate, 2-oxo-3-methylpentanoate and 2-oxo-4-methylpentanoate, all substrates utilized by LDH C4 from other species (Blanco et al., 1976).

As shown in Text-fig. 2, maximal activity of sperm lysates was recorded at 0.5 mM for pyruvate and there was a marked inhibition at higher concentrations of this substrate. The curves for 2-oxobutanoate, 2-oxopentanoate and 2-oxohexanoate were of the Michaelian type and did not show inhibition even at concentrations of substrate as high as 10 mM. The values of $K_m$ determined by double reciprocal plots of these data were 0.114 mM for pyruvate, 0.33 mM for 2-oxobutanoate, 5.0 mM for 2-oxopentanoate and 0.8 mM for 2-oxohexanoate.

After electrophoretic separation of extracts on starch gel and staining for enzyme activity by using lactate, 2-hydroxybutanoate, 2-hydroxypentanoate and 2-hydroxyhexanoate as substrates, it was demonstrated that the only fraction showing significant activity against all the substrates was LDH C4 (Pl. 1, Figs 1–4). Isoenzymes 1 and 2 were stained when lactate and 2-hydroxybutanoate were used as substrates and isoenzyme 5 was active only with lactate (Plate 1).

Ratios of analogue:pyruvate activities for heart and liver homogenates and sperm lysates (Table 1) were obtained by assaying at two concentrations of the analogue (5.0 and 10.0 mM).
Text-fig. 2. Catalytic activity of human sperm lysates against different 2-oxoacids. Initial velocity, expressed as Units/ml is plotted against concentration of 2-oxoacid (0.02–10.0 mM). ●, Pyruvate; ○, 2-oxobutanoate; △, 2-oxopentanoate; ▲, 2-oxohexanoate. Each point represents the average of determinations on 4 pools of 4 sperm samples each. Sperm lysate pools were diluted to give about the same maximal activity per ml with pyruvate as substrate and an identical amount of each pool was used for the assays with the four 2-oxoacids. The vertical bars represent ± 1 s.d.

and at the concentration of pyruvate giving maximal activity with each preparation (0.5 mM for sperm and heart extracts and 2.0 mM for liver homogenates). Heart, a tissue rich in LDH B, was active against 2-oxobutanoate but showed negligible activity with 2-oxopentanoate and 2-oxohexanoate. Liver homogenates, which contain predominantly LDH A, exhibited low activity with 2-oxobutanoate and no activity at all with 2-oxopentanoate and 2-oxohexanoate. Sperm lysates showed high relative activity with 2-oxopentanoate and 2-oxohexanoate.

Table 2 presents the results of determinations of enzymic activity on extracts and on mixtures of extracts by using 2-oxohexanoate and pyruvate as substrates. The activity of mixtures of equal volumes of sperm extract and heart or liver homogenate, when determined with 2-oxohexanoate, was about half the activity of the original sperm lysate. The substrate 2-oxohexanoate was significantly utilized only by sperm extracts and the activity did not change when crude homogenates of tissues containing quite different isoenzymic complements were added to the sperm lysates.

Discussion

The hypothesis of Storey & Kayne (1977), which postulates the participation of LDH C in a pyruvate–lactate shuttle system transferring reducing equivalents from cytosol to mitochondria,
### Table 1. Ratios (±s.d.) of analogue:pyruvate activities for human heart, liver and sperm extracts

<table>
<thead>
<tr>
<th></th>
<th>Heart</th>
<th></th>
<th>Liver</th>
<th></th>
<th>Sperm</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mm</td>
<td>10 mm</td>
<td>5 mm</td>
<td>10 mm</td>
<td>5 mm</td>
<td>10 mm</td>
</tr>
<tr>
<td>2-Oxobutanoate</td>
<td>0.55 ± 0.04</td>
<td>0.89 ± 0.03</td>
<td>0.17 ± 0.03</td>
<td>0.38 ± 0.03</td>
<td>0.45 ± 0.03</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>2-Oxopentanoate</td>
<td>0.07 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.38 ± 0.02</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>2-Oxohexanoate</td>
<td>0.05 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.62 ± 0.03</td>
<td>0.67 ± 0.03</td>
</tr>
</tbody>
</table>

Analogue concentrations were 5 or 10 mm as indicated; the concentration of pyruvate was 0.5 mm for heart and sperm extracts and 2.0 mm for liver homogenates, because these gave maximum activity. The activities for liver extract with 5 mm-pyruvate are given in parentheses.

Values for heart and liver are from determinations on 4 different homogenates; values for sperm are from 4 pools of 4 different samples each. All sperm preparations contained LDH C₄ in a proportion higher than 80% of total lactate dehydrogenase activity, determined by electrophoresis and densitometry.

### Table 2. Lactate dehydrogenase activity (U/ml) of human tissue extracts tested with 0.5 mm-pyruvate (Pyruv.) and 5.0 mm-2-oxohexanoate (2-oxoh.)

<table>
<thead>
<tr>
<th></th>
<th>Spermatozoa</th>
<th>Liver</th>
<th>Heart</th>
<th>Spermatozoa + liver (1:1)*</th>
<th>Spermatozoa + heart (1:1)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
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<td>2-oxoh.</td>
<td>Pyruv.</td>
<td>2-oxoh.</td>
<td>Pyruv.</td>
</tr>
<tr>
<td>1</td>
<td>1.88</td>
<td>1.18</td>
<td>1.47</td>
<td>0.015</td>
<td>1.50</td>
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<tr>
<td>2</td>
<td>1.80</td>
<td>1.15</td>
<td>1.50</td>
<td>0.012</td>
<td>1.42</td>
</tr>
<tr>
<td>3</td>
<td>1.95</td>
<td>1.22</td>
<td>1.57</td>
<td>0.016</td>
<td>1.42</td>
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<td>4</td>
<td>1.72</td>
<td>1.05</td>
<td>1.42</td>
<td>0.010</td>
<td>1.40</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
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</tr>
</tbody>
</table>

Values represent the mean of duplicate determinations on each sample. Numbers in parentheses represent percentage of the activity in the mixture, taking as 100% that of the sperm lysate.

* The volume used was the same as that for the single extracts.
is compatible with previous observations on catalytic properties of the isoenzyme C₄ (Battellino, Ramos Jaime & Blanco, 1968) and subcellular localization of LDH C₄ (Machado de Domenech, Domenech, Aoki & Blanco, 1972; Montamat & Blanco, 1976), and seems applicable to the sperm isoenzyme from most species. The present results indicate that human LDH C₄, which markedly predominates in sperm lysates, shows a behaviour with increasing concentrations of pyruvate similar to that of LDH 1 (B₄). The same finding has been reported for LDH C₄ from other species (Blanco et al., 1975).

Early studies of catalytic properties of human LDH C₄ (Blanco & Zinkham, 1963; Zinkham et al., 1964; Clausen & Øvliisen, 1965; Wilkinson & Withycombe, 1965) indicated some peculiar characteristics of this isoenzyme, but its activity with a variety of substrates had not been assessed. The present results demonstrate that human sperm lysates catalyse the NAD-linked reduction of 2-oxoacids with a linear chain of up to 6 carbons. Staining of electrophoretically separated preparations indicated that this activity can be ascribed to the sperm-specific lactate dehydrogenase isoenzyme C₄. Because the affinity of LDH C₄ for substrates is not the same for the enzyme from all species studied, it is difficult to assign general physiological significance to activity with 2-oxoacids higher than pyruvate. Activity of human LDH C₄ with 2-oxopentanoate and 2-oxohexanoate cannot be referred to any known metabolic pathway and appears, at the moment, to be without functional meaning. Nevertheless, the affinity of the sperm isoenzyme for these substrates is of interest because it allows the assay of LDH C₄ in mixtures containing several molecular forms of lactate dehydrogenase. The isoenzymic complement of sperm lysates and the activity of the different lactate dehydrogenase molecular forms with 2-oxohexanoate indicate that the assay of sperm extracts with this substrate can give a selective measurement of LDH C₄ activity and add a functional dimension to analyses of sperm samples in clinical laboratories.

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References


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