Lactate Dehydrogenase Isoenzymes of Human Semen

BY JORGEN CLAUSEN AND BJARNI ØVLISEN
University Department of Biochemistry and University Department of Obstetrics and Gynaecology, Rigshospitalet, Copenhagen, Denmark

(Received 16 October 1964)

1. A lactate dehydrogenase isoenzyme present in human spermatozoa and semen was isolated and characterized biochemically in terms of its pH for optimum activity and by means of $K_m$ values for lactate, NAD$^+$ and NAD analogues. The results were compared with those obtained with the human heart-type and the liver-type lactate dehydrogenase isoenzymes. 2. The enzyme was characterized by its resistance to digestion with different proteolytic enzymes. The time for 50% digestion in terms of residual dehydrogenase activity was compared with times obtained for the $H_4$- and $M_4$-types.

All human and other animal tissue cells contain lactate dehydrogenase isoenzymes, which are tetramers of two polypeptide chains: the H- and the M-chain. Thus five different isoenzymes with different electrophoretic mobilities and different composition occur, corresponding to the tetramers $H_4$, $H_2M$, $H_2M_2$, $HM_3$ and $M_4$ (Dewey & Conklin, 1960; Wieland & Pfleiderer, 1962). Further, in semen and extracts of testes from different mammalian species a sixth lactate dehydrogenase isoenzyme has been described with a mobility between the isoenzymes $H_2M_2$ and $HM_3$ (Zinkham, Blanco & Kupchyk, 1963; Blanco & Zinkham, 1963; Goldberg, 1963). This isoenzyme (LDH.) has not been characterized in detail, and it is therefore the aim of this study to provide data on its enzymological properties.

MATERIALS

Human semen was obtained from the Laboratory of The Copenhagen Health Insurance Society. The samples were collected in a glass tube during coitus interruptus or by masturbation. The samples were stored until use at 4°C. Only samples free of leucocytes and bacteria were used.

Each sample was treated singly as follows: the spermatozoa were isolated by centrifuging for 15 min. at 2000g and 4°C followed by five successive resuspensions of the cells in an equal volume of 0.9% NaCl. The isolated spermatozoa were mixed with 0.5 vol. of 0.05 M-KH$_2$PO$_4$-Na$_2$HPO$_4$ buffer, pH 7.5, containing 5% of Triton X-100 (alkylphenoxypolyethoxyethanol; Rohm and Haas Co. lot no. 5330) in a plastic centrifuge tube fitted with a closely fitting Teflon piston. Disintegration was carried out at 0°C by rotating the piston for 5 min. at 40 rev./min. Under these conditions 70% of the spermatozoa were destroyed. After centrifugation at 10,000g for 20 min. the supernatant fluid was stored at 4°C until required. When extracts were required for micro-electrophoresis Triton X-100 was not used because it interferes with the formazan staining.

Extracts of human liver and human heart tissue were prepared as described by Gerhardt, Clausen, Christensen & Riischede (1963). These were used for isolation of LDH* isoenzymes $H_4$ and $M_4$ as described below.

Unless otherwise stated the chemicals used were those of highest purity obtained from British Drug Houses Ltd.

METHODS

Estimation of LDH activity. For routine assay the reaction mixture contained 100 µl. of suitably diluted sperm extract, 1.0 mm-pyruvate, 0.33 mm-NADH and 50 mm-phosphate buffer, pH 7.5, in a final volume of 3 ml. (Bergmeyer, Bernt & Hess, 1962; Clausen & Gerhardt, 1963). Changes in extinction at 366 nm were measured in silica cells of 1 cm. path length at 25°C in an Eppendorf or a Vitatron photometer equipped with a mercury lamp as light-source. The concentration of enzyme was adjusted to give a change in extinction of about 0.030/sec. On the basis of the extinction change at 25°C, the dilution factor of the sample, and a molar extinction coefficient of NADH at 366 nm of 3.3 cm.$^2$/µmole (Hohorst, 1956), the number of LDH enzyme units present in 1 ml. of the original sample was expressed as µmoles of substrate transformed/min. From the total protein content or total DNA P content of the sample the specific LDH activity was expressed as µmoles of substrate transformed/min./mg. of protein or per µg. of DNA P.

The effect on LDH activity of variations in pH and in concentrations of substrate and of NAD$^+$ (NADH) were estimated in the above system or by dehydrogenation of lactate in the presence of NAD$^+$. The lactate reduction system was also used for estimation of the $K_m$ values of the individual isoenzymes. For determination of $K_m$ values 0.20 M L-lactate and 8-3 M-coenzyme were used. These values are higher than the optimum ones estimated as described below. Buffers used in lactate systems of pH > 9.0 were made with 50 mm-glycine and NaOH.

* Abbreviation: LDH, lactate dehydrogenase.

Bioch. 1965, 97
Protein content. Protein of sperm extracts and sperm plasma was determined by the Lowry method as modified by Louis, Plum & Schou (1956), with tyrosine used as standard.

Determination of DNA. Sperm extracts and sperm plasmas were analysed as described by Ceriotti (1952) and modified by Glick (1963). The phosphorus content of the DNA standard was determined by the method of Fiske & Subbarow (1925).

Microelectrophoresis on agar gel

Preparation of agar-gel slides. The microscopic slides were first coated with a 1 mm. layer of 1% agar (Difco Special Noble Agar) dissolved in water. This layer was dried before the electrophoretic agar-gel medium was applied, as a 1 mm. homogeneous layer, as described by Wieme (1959). The agar coat on the glass surface prevented migration of the protein solution between the glass surface and the agar gel. Distortion of the agar layer during the electrophoretic run was prevented by sealing the trough with a layer of melted electrophoretic medium.

Electrophoretic procedure. Electrophoresis of seminal plasma and of extracts was performed as described by Wieme (1959) at pH 8.6 in 0.05 M barbitone buffer containing 1% of Difco Special Noble Agar. The running time was 28 min. The separated LDH isoenzymes were made visible by formazan formation (Gerhardt et al. 1963). The relative proportions of enzyme units in the different isoenzyme bands were estimated by scanning the slides at 546 mμ in the Vitatron photometer equipped with a scanning device, an automatic recorder and an integrator. Beer's law was valid in the scanning procedure. Thus for each isoenzyme in an extract a straight-line relationship was observed between the area of the peak in the recorder tracing and the reciprocal of the dilution, provided that the number of enzyme units applied to the electrophoretic slide exceeded 0.02 unit. The number of enzyme units in an individual isoenzyme band was calculated by multiplying the fractional activity in the band by the total units applied to the slide.

Separation of LDH isoenzymes. A 1 mm.-broad trough across the coated slide was made by forcing a 1 mm. thick filter paper (Whatman no. 17) through the agar. The extract (50 μl.) was then placed in the trough. Eight to ten slides were run simultaneously and one slide was stained for LDH activity. On the basis of the distribution of the LDH bands in this slide the remaining agar strips were cut into pieces each containing only one LDH band. The separated isoenzymes were eluted from the agar gel with 0.05 M-phosphate buffer (pH 7.5) containing 5% of Triton X-100. Buffer (1 vol.) was added to 2 vol. of pooled agar pieces and the mixture was homogenized as described above. After centrifugation for 20 min. at 10,000g the supernatant solution was examined for enzyme homogeneity by agar-gel electrophoresis. By this procedure the predominant isoenzyme of human heart tissue (H₄), of human liver tissue (M₄) and of human spermatozoa (LDH₄) were isolated. The yields were 48-6% for the H₄-form, 21-7% for the M₄-type and 53-0% for the LDH₄. By this electrophoretic isolation the specific LDH activities were increased from 1-33 units/mg. of protein to 7-18 units/mg. of protein for H₄ and from 2-06 units/mg. of protein to 7-90 units/mg. of protein for M₄.

Reactions of the isoenzymes with NAD analogues

The activities of the isoenzymes were determined in the presence of 3-thionicotinamide–adenine dinucleotide, 3-pyridinealddehyde–adenine dinucleotide and nicotinamide–hypoxanthine dinucleotide as described by Cahn, Kaplan, Levine & Zwilling (1962) and Kaplan & Ciotti (1961). These analogues, which were all used in the oxidized forms, were obtained from Pabst Laboratories, Milwaukee 5, Wisconsin, U.S.A. They were used at the same concentration as that of NAD⁺ (8-3 mμ). The following solutions were mixed in the cuvette: 2-85 ml. of 0-05 M-glycine–NaOH buffer (pH 9-00) containing 0-20 mμ lithium lactate, 50 μl. of glycine buffer containing 0-5 μmol NAD analogues and 100 μl. of electrophoretically purified isoenzyme solution.

Michaelis constants

The constants were determined for lactate in the presence of about 10 times the saturation limit for NAD⁺ and NAD analogues (8-3 mμ). The saturation limits were estimated by measuring the minimum concentration of coenzyme (or substrate), which, under the experimental conditions used, was required for maximum LDH activity. Further, constants for the NAD⁺ and NAD analogues were determined in the presence of 0-20 mμ lithium lactate. Kₘ values were calculated by the method of Lineweaver & Burk (1934). Kₘ values for lactate are results of duplicate analyses. The deviations from the means were at most ± 10%. Kₘ values for the nucleotides are results of single determinations.

Treatment with proteolytic enzymes

To 1 vol. of isolated enzyme fraction was added an equal volume of a solution of proteolytic enzyme. The mixtures were incubated at 37° and the LDH activity was estimated at zero time and at every 10 to 15 min. as long as activity was present. Proteolytic digestion was performed with trypsin, papain, peptidase, carboxypeptidase and α-chymotrypsin.

Digestion with trypsin (Novo Terapeutikum) was performed with a solution containing 250 mg. of trypsin dissolved in 10 ml. of 0-05 M-phosphate buffer (pH 7.5) containing 5% of Triton X-100.

Digestion with papain was performed by using a solution of the proteolytic enzyme containing 7-88 mg. of cysteine–HCl, 18-6 mg. of EDTA and 25 mg. of papain (Merck 1:350)/10 ml. of phosphate buffer (0-05 M; pH 7-5).

Digestion with a peptidase from hog intestinal mucosa (Sigma lot no. 93 B-1960) was performed with a solution containing 7-25 ml. of 0-05 M-veronal buffer (pH 8-6) + 2-5 ml. of 0-01 M-Co(NO₃)₂ + 0-500 ml. of peptidase in veronal buffer as described by Wüst (1962a). Carboxypeptidase was used as a solution containing 4-8 ml. of 0-05 M-tris buffer, pH 7.8, 0-100 ml. of 0-25 M-CaCl₂ solution and 0-100 ml. of carboxypeptidase from bovine pancreas (Sigma lot no. 23 B-1590; 50 mg. of protein/ml.) (Wüst, 1962b). Digestion with α-chymotrypsin was carried out at pH 7.25 as a compromise between the pH optimum of 9-0 and the optimum of stability at pH 4-0. α-Chymotrypsin (50 mg., Sigma lot no. 113 B-0310), with an activity of 9-0 units/mg., was dissolved in 2 ml. of 0-05 M-phosphate buffer, pH 7.25, containing 5% of Triton X-100.
RESULTS

LDH activities of human semen, seminal plasma and of extracts of isolated spermatozoa

The LDH activity of semen is in the range 0.97–14.55 units/ml. (mean of 25 samples: 3.88). The LDH activity of 1 ml of total extract of isolated spermatozoa from 1 ml of total sperm varied from 0.22 to 1.82 units/ml. in 35 samples. In another series of experiments (30 samples) the corresponding values for seminal plasma were in the range 0.97–4.37 units/ml. Electrophoresis of semen, seminal plasma and spermatozoal extracts revealed in all cases the intermediate LDH isoenzyme situated between H2M2 and H1M3. This fraction accounts for 6–8–57% of total activity of semen (mean 34 ± 1%), but 80–100% of activity of spermatozoal extract. The specific LDH activities of semen were in the range 0.0036–0.566 unit/μg of DNA phosphorus or 0.034–1.18 units/mg of protein. The corresponding values of extracts were 0.011–0.132 unit/μg of DNA phosphorus or 0.061–3.15 units/mg of protein.

The values for seminal plasma were 0.0087–0.148 unit/μg of DNA phosphorus or 0.00159–0.255 unit/mg of protein.

Properties of the LDHx isoenzyme from spermatozoa

Stability of the enzyme. When the isolated LDHx fraction was stored at –20° enzyme activity disappeared within 10 days. At 4°, however, the activity decreased only 40% in 10 days.

Effect of pH on activity. The pH for maximum activity was 7.5 for the forward reaction and 8.75–9.00 for the back reaction. The pH optima are distinctly different from those of isoenzyme H4, but not significantly so from those of M4 (Table 1).

Relationship of activity to substrate concentration. The optimum concentration of pyruvate in the forward reaction of LDHx, with NADH as coenzyme, is 0.9 mM. This value is higher than those for H4 and M4. The optimum concentration of lactate for the back reaction is 0.16 M with NAD+

---

Table 1. Kinetic data obtained at 25° on lactate dehydrogenase isoenzymes from human tissues

(A) Standard conditions for forward reaction: 100 μl of suitably diluted LDHx from human spermatozoa, M4 from human liver or H4 from human heart, 2–850 ml of 50mM-phosphate buffer (pH 7.5), containing 1–0 mM-pyruvate, and 0–33 mM-NADH in a total volume of 3 ml. In the standard assays of the back reaction the enzyme was mixed with 2–850 ml of 50 mM-glycine buffer (pH 9.0) containing 0.2 mM-lithium lactate and 0.83 mM-oxidized nucleotide. The saturation limit is defined as the minimum concentration of substrate or coenzyme, which, under the present experimental conditions, is required for maximum LDH activity. (B) Km (nucleotide) was determined in glycine–NaOH buffer (pH 9.0) and 0.2 mM-lactate. (C) Km (lactate) was determined in glycine–NaOH buffer (pH 9.0) and 8.3 mM-coenzyme.

<table>
<thead>
<tr>
<th></th>
<th>H4</th>
<th>M4</th>
<th>LDHx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum pH</td>
<td>10.75</td>
<td>9.75</td>
<td>8.75–9.00</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>8.00</td>
<td>7.25</td>
<td>7.60</td>
</tr>
<tr>
<td>Saturating conc. of lactate in assay method</td>
<td>0.20 M</td>
<td>0.16 M</td>
<td>0.16 M</td>
</tr>
<tr>
<td>Saturating conc. of pyruvate in the assay method</td>
<td>0.25 mM</td>
<td>0.5 mM</td>
<td>0.9 mM</td>
</tr>
<tr>
<td>Nucleotide saturation limits (mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD+ (back reaction)</td>
<td>0.83</td>
<td>1.25</td>
<td>0.83</td>
</tr>
<tr>
<td>3-Thionicotinamide–adenine dinucleotide (back reaction)</td>
<td>0.83</td>
<td>0.83</td>
<td>0.83</td>
</tr>
<tr>
<td>3-Pyridinealdehyde–adenine dinucleotide (back reaction)</td>
<td>0.83</td>
<td>0.83</td>
<td>0.83</td>
</tr>
<tr>
<td>Niotinamide–hypoxanthine dinucleotide (back reaction)</td>
<td>1.67</td>
<td>1.25</td>
<td>0.83</td>
</tr>
<tr>
<td>NADH (forward reaction)</td>
<td>0.83</td>
<td>0.17†</td>
<td>0.33*</td>
</tr>
<tr>
<td>Km (nucleotide) (mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD+ (back reaction)</td>
<td>0.50</td>
<td>0.29</td>
<td>0.109</td>
</tr>
<tr>
<td>3-Thionicotinamide–adenine dinucleotide (back reaction)</td>
<td>0.21</td>
<td>0.28</td>
<td>0.125</td>
</tr>
<tr>
<td>3-Pyridinealdehyde–adenine dinucleotide (back reaction)</td>
<td>0.66</td>
<td>1.18</td>
<td>0.106</td>
</tr>
<tr>
<td>Niotinamide–hypoxanthine dinucleotide (back reaction)</td>
<td>0.50</td>
<td>0.34</td>
<td>0.33</td>
</tr>
<tr>
<td>Km (lactate) (mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleotide: NAD+ (back reaction)</td>
<td>2.9</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>3-Thionicotinamide–adenine dinucleotide (back reaction)</td>
<td>4.1</td>
<td>56</td>
<td>11</td>
</tr>
<tr>
<td>3-Pyridinealdehyde–adenine dinucleotide (back reaction)</td>
<td>15</td>
<td>130</td>
<td>40</td>
</tr>
</tbody>
</table>

* Broad maximum.
† Narrow maximum.
as coenzyme, identical with that of M₄ but lower than the value for H₄ (Table 1). A distinct substrate inhibition was found in the forward, but not in the back, reaction. With the NAD analogues as coenzymes for the back reactions the saturation limits were in all cases below 0.16 m-lactate.

**Relationship of activity to nucleotide concentration.**
The saturation limits of the different nucleotides were identical (0.83 mm) in the back reaction of LDH₄. The value is also identical with those found for H₄ and M₄ with the exception of higher values found for nicotinamide–hypoxanthine dinucleotide and NAD⁺ as coenzymes for M₄ (Table 1). The 3-pyridinealdehyde–adenine dinucleotide saturation curve was characterized by a broad maximum because higher concentrations of the analogue gave rise to inhibition of the back reaction. This phenomenon was not observed in the back reactions catalysed by the other nucleotides, but was even more pronounced for NADH in the forward reaction (Table 1).

**Michaelis constants of nucleotides and of lactate in the back reaction.** These are given in Table 1. The values of \( K_m \) (nucleotide) of LDH₄ in its reaction with NAD⁺, 3-thionicotinamide–adenine dinucleotide and 3-pyridinealdehyde–adenine dinucleotide are lower than those of H₄ and of M₄ in contrast with the value for nicotinamide–hypoxanthine dinucleotide⁺, which is similar to that of M₄. The \( K_m \) (lactate) values of LDH₄ are higher than those of H₄ and of M₄ when NAD⁺ is coenzyme, but intermediate between the values of the two other isoenzymes when 3-thionicotinamide–adenine dinucleotide or 3-pyridinealdehyde–adenine dinucleotide are coenzymes in the back reaction.

**Relationship of activity to proteolytic treatment.**
The inactivation of LDH activity by proteolytic digestion proceeds logarithmically. With the exception of the peptidase, which did not inactivate any of the isoenzymes, the inactivation proceeded until enzyme activity was eliminated. It was not possible by means of electrophoresis during the proteolysis to detect any active sub-units with mobilities different from the isoenzymes undergoing digestion. The \( t_4 \) values for the digestion of LDHₓ, H₄ and M₄ LDH isoenzymes by the proteolytic enzymes are given in Table 2. The rate of inactivation of LDHₓ in the trypsin, carboxypeptidase and papain experiments, is intermediate between the rates for the H₄- and M₄-isoenzymes. On the other hand \( \alpha \)-chymotrypsin inactivates LDHₓ more rapidly than either H₄ or M₄.

### DISCUSSION

The LDHₓ isoenzyme is present in seminal plasma and in extracts of spermatozoa. However, this isoenzyme could not be detected in semen from 10 subjects showing aspermia although the other five LDH isoenzymes were invariably present (B. Øvlsen, unpublished work). It is thus probable that LDHₓ originates from sperm cells. Zinkham et al. (1963) found that LDHₓ was the predominant LDH isoenzyme in human spermatozoa, and this is confirmed by the present finding that 80–100% of LDH activity of sperm cells is of the LDHₓ type. We are not able to say whether the high LDHₓ content of seminal plasma is due to outward diffusion of the isoenzyme from sperm cells or to spontaneous destruction of the cells.

The LDHₓ isoenzyme from semen or testes consists of sub-units different from the H- and M-chains present in LDH isoenzymes from other mammalian tissues (Markert & Møller, 1959; Zinkham et al. 1963). The lactate oxidation rates catalysed by LDHₓ in the presence of different nucleotides differ from those obtainable with LDH fractions made up from H- or M-sub-units or both (Zinkham et al. 1963). Our results confirm and extend these reports. In terms of pH optima, and of the lactate concentration required to saturate the enzyme, LDHₓ seems more related to M₄ than to H₄.

The saturation limits and the values of \( K_m \) (nucleotide) that we find show significant differences in the kinetic properties of H₄, M₄ and LDHₓ. Values for \( K_m \) (nucleotide) published by Nisselbaum, Packer & Bodansky (1964) are not comparable with ours because these authors used different pH, substrate and temperature. Values for \( K_m \) (pyruvate) are not given in this paper because of the inhibition obtained with higher pyruvate concentrations and because of the depression of LDH activity at higher NADH concentrations. However, such \( K_m \) values for H₄ and M₄ have been presented by Dawson, Goodfriend & Kaplan (1964) and by Nisselbaum et al. (1964).

We, like Pesco, McKay, Stolzenbach, Cahn & Kaplan (1964), have been unable to split the isoenzymes into sub-units either by freezing solutions containing high concentrations of salts (Zinkham et al. 1963) or by means of β-mercaptoethanol (Fritz

### Table 2. Inactivation of isoenzymes H₄, M₄, LDHₓ by proteolytic enzymes

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>H₄</th>
<th>M₄</th>
<th>LDHₓ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>30</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td>Carboxypeptidase</td>
<td>104</td>
<td>149</td>
<td>114</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>457</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>Papain</td>
<td>332</td>
<td>85</td>
<td>69</td>
</tr>
</tbody>
</table>

All reactions were first-order and rates of proteolysis are expressed as the time for 50% inactivation. Experimental conditions are given in the text.
We attempted to obtain enzymically active sub-units from isolated isoenzymes by proteolytic digestion, but electrophoresis of isoenzymes during digestion revealed no such active fragments. The different rates of inactivation of isoenzymes by trypsin, carboxypeptidase, α-chymotrypsin and papain suggest that LDH₂ is structurally distinct from H₄ and M₄.

These investigations have been supported by King Christian X's Fund, The Fund for Promotion of Medical Science and from The Technical Research Fund, Copenhagen, Denmark. We are grateful to Dr. R. Hammen, of the Laboratory of The Copenhagen Health Insurance Society, for obtaining supplies of semen.

REFERENCES


