Lactate Dehydrogenase Isoenzymes of Human Semen

By JØRGEN CLAUSEN AND BJARNI ØVLISEN
University Department of Biochemistry and University Department of Obstetrics and Gynaecology,
Rigshospitalet, Copenhagen, Denmark

1. A lactate dehydrogenase isoenzyme present in human spermatozoa and semen was isolated and characterized biochemically in term 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_3M, 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_x) 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°. Only samples free of leucocytes and bacteria were used.

Each sample was treated singly as follows: the spermatozoa were isolated by centrifuging for 15min. at 2000g and 4° followed by five successive resuspensions of the cells in an equal volume of 0·9% NaCl. The isolated spermatozoa were mixed with 0·5vol. of 0·05M-KH_2PO_4–Na_2HPO_4 buffer, pH7·5, 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° by rotating the piston for 5min. at 40rev./min. Under these conditions 70% of the spermatozoa were destroyed. After centrifugation at 10000g for 20min. the supernatant fluid was stored at 4° 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 & Riishede (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 100μl. of suitably diluted sperm extract, 1·0mM-pyruvate, 0·33mM-NADH and 50mM-phosphate buffer, pH7·5, in a final volume of 3ml. (Bergmeyer, Bernt & Hess, 1962; Clausen & Gerhardt, 1963). Changes in extinction at 366mμ were measured in silica cells of 1cm. path length at 25° 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°, the dilution factor of the sample, and a molar extinction coefficient of NADH at 366mμ of 3·3cm.^{2}/μmole (Hohorst, 1956), the number of LDH enzyme units present in 1ml. 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 0·20M-L-lactate and 8·3mM-coenzyme were used. These values are higher than the optimum ones estimated as described below. Buffers used in lactate systems of pH > 8·0 were made with 50mM-glycine and NaOH.

---

* Abbreviation: LDH, lactate dehydrogenase.

Protein content. Protein of sperm extracts and sperm plasma was determined by the Lowry method as modified by Lous, 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 microscope 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 10000*g* the supernatant solution was examined for enzymic homogeneity by agar-gel electrophoresis. By this procedure the predominant isoenzyme of human heart tissue (H_4), of human liver tissue (M_4) and of human spermatozoa (LDH_x) were isolated. The yields were 48·6% for the H_4-form, 21·7% for the M_4-type and 53·0% for the LDH_x. 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 H_4 and from 2·06 units/mg. of protein to 7·90 units/mg. of protein for M_4.

Reactions of the isoenzymes with NAD analogues

The activities of the isoenzymes were determined in the presence of 3-thionicotinamide–adenine dinucleotide, 3-pyridinealdehyde–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 mM). The following solutions were mixed in the cuvette: 2·85 ml. of 0·05 M-glycine–NaOH buffer (pH 9·00) containing 0·20 M lithium lactate, 50 μl. of glycine buffer containing 0·5 M-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 mM). 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 M-lithium lactate. K_m values were calculated by the method of Lineweaver & Burk (1934). K_m values for lactate are results of duplicate analyses. The deviations from the means were at most ±10%. K_m 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 Terapeutisk) 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(NO_3)$_2$ + 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-CaCl$_2$ 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: 3·68). 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 H_2M_2 and H_1M_3. 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_x 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 0·0067–0·148 unit/μg. of DNA phosphorus or 0·00159–0·255 unit/mg. of protein.

Properties of the LDH_x isoenzyme from spermatozoa

Stability of the enzyme. When the isolated LDH_x 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 H_4, but not significantly so from those of M_4 (Table 1).

Relationship of activity to substrate concentration. The optimum concentration of pyruvate in the forward reaction of LDH_x, with NADH as coenzyme, is 0·9 mM. This value is higher than those for H_4 and M_4. 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 LDH_x from human spermatozoa, M_4 from human liver or H_4 from human heart, 2·850 ml. of 50 mM-phosphate buffer (pH 7·5), containing 1·0 mM-pyruvate, and 0·33 mM-NADH in a total volume 3·0 ml. In the standard assay of the back reaction the enzyme was mixed with 2·850 ml. of 50 mM-glycine buffer (pH 9·0) containing 0·2 M-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) $K_{m \text{ (nucleotide)}}$ was determined in glycine–NaOH buffer (pH 9·0) and 0·2 M-lactate. (C) $K_{m \text{ (lactate)}}$ was determined in glycine–NaOH buffer (pH 9·0) and 8·3 mM-coenzyme.

(A)	H_4	M_4	LDH_x
Optimum pH for lactate oxidation (back reaction)	10·75	9·75	8·75–9·00
Optimum pH for pyruvate reduction (forward reaction)	8·00	7·25	7·50
Saturating concn. of lactate in the assay method	0·20 M	0·16 M	0·16 M
Saturating concn. of pyruvate in the assay method	0·25 mM	0·5 mM	0·9 mM
	Nucleotide saturation limits (mM)		
NAD^+ (back reaction)	0·83	1·25	0·83
3-Thionicotinamide–adenine dinucleotide (back reaction)	0·83	0·83	0·83
3-Pyridinealdehyde–adenine dinucleotide (back reaction)	0·83*	0·83	0·83*
Nicotinamide–hypoxanthine dinucleotide (back reaction)	1·67	1·25	0·83
NADH (forward reaction)	0·83*	0·17†	0·33*
	$K_{m \text{ (nucleotide)}}$ (mM)		
(B)			
NAD^+ (back reaction)	0·50	0·29	0·109
3-Thionicotinamide–adenine dinucleotide (back reaction)	0·21	0·28	0·125
3-Pyridinealdehyde–adenine dinucleotide (back reaction)	0·66	1·18	0·106
Nicotinamide–hypoxanthine dinucleotide (back reaction)	0·50	0·34	0·33
	$K_{m \text{ (lactate)}}$ (mM)		
(C)			
Nucleotide: NAD^+ (back reaction)	2·9	18	25
3-Thionicotinamide–adenine dinucleotide (back reaction)	4·1	56	11
3-Pyridinealdehyde–adenine dinucleotide (back reaction)	15	130	40

* Broad maximum.
† Narrow maximum.

as coenzyme, identical with that of M_4 but lower than the value for H_4 (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 0.16M-lactate.

Relationship of activity to nucleotide concentration. The saturation limits of the different nucleotides were identical (0.83mM) in the back reaction of LDH_x. The value is also identical with those found for H_4 and M_4 with the exception of higher values found for nicotinamide–hypoxanthine dinucleotide and NAD^+ as coenzymes for M_4 (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 $K_{m \text{ (nucleotide)}}$ of LDH_x in its reaction with NAD^+, 3-thionicotinamide–adenine dinucleotide and 3-pyridinealdehyde–adenine dinucleotide are lower than those of H_4 and of M_4 in contrast with the value for nicotinamide–hypoxanthine dinucleotide$^+$, which is similar to that of M_4. The $K_{m \text{ (lactate)}}$ values of LDH_x are higher than those of H_4 and of M_4 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 $t_{\frac{1}{2}}$ values for the digestion of LDH_x, H_4 and M_4 LDH isoenzymes by the proteolytic enzymes are given in Table 2. The rate of inactivation of LDH_x in the trypsin, carboxypeptidase and papain experiments is intermediate between the rates for the H_4- and M_4-isoenzymes. On the other hand α-chymotrypsin inactivates LDH_x more rapidly than either H_4 or M_4.

DISCUSSION

The LDH_x isoenzyme is present in seminal plasma and in extracts of spermatozoa. However, this isoenzyme could not be detected in semen from

Table 2. *Inactivation of isoenzymes H_4, M_4, LDH_x by proteolytic enzymes*

All reactions were first-order and rates of proteolysis are expressed as the time for 50% inactivation. Experimental conditions are given in the text.

	Time (min.)		
	H_4	M_4	LDH_x
Trypsin	30	14	22
Carboxypeptidase	104	149	114
α-Chymotrypsin	457	17	13
Papain	332	85	69

10 subjects showing aspermia although the other five LDH isoenzymes were invariably present (B. Øvlisen, unpublished work). It is thus probable that LDH_x originates from sperm cells. Zinkham *et al.* (1963) found that LDH_x 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 LDH_x type. We are not able to say whether the high LDH_x content of seminal plasma is due to outward diffusion of the isoenzyme from sperm cells or to spontaneous destruction of the cells.

The LDH_x 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 LDH_x 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, LDH_x seems more related to M_4 than to H_4.

The saturation limits and the values of $K_{m \text{ (nucleotide)}}$ that we find show significant differences in the kinetic properties of H_4, M_4 and LDH_x. Values for $K_{m \text{ (nucleotide)}}$ published by Nisselbaum, Packer & Bodansky (1964) are not comparable with ours because these authors used different pH, substrate and temperature. Values for $K_{m \text{ (pyruvate)}}$ 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 H_4 and M_4 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

& Jacobson, 1963). 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 LDH_x is structurally distinct from H_4 and M_4.

REFERENCES

Bergmeyer, H.-U., Bernt, E. & Hess, B. (1962). In *Methoden der enzymatischen Analyse*, p. 736. Ed. by Bergmeyer, H.-U. Weinheim: Verlag Chemie.

Blanco, A. & Zinkham, W. H. (1963). *Science*, 139, 601.

Cahn, R. D., Kaplan, N. O., Levine, L. & Zwilling, E. (1962). *Science*, 136, 962.

Ceriotti, G. (1952). *J. biol. Chem.* 198, 297.

Clausen, J. & Gerhardt, W. (1963). *Acta neurol. scand.* 39, 305.

Dawson, D. M., Goodfriend, T. L. & Kaplan, N. O. (1964). *Science*, 143, 929.

Dewey, M. M. & Conklin, J. L. (1960). *Proc. Soc. exp. Biol., N.Y.*, 105, 492.

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* 66, 375.

Fritz, P. J. & Jacobson, K. B. (1963). *Science*, 140, 64.

Gerhardt, W., Clausen, J., Christensen, E. & Riishede, J. (1963). *Acta neurol. scand.* 39, 85.

Glick, D. (1963). *Quantitative Chemical Techniques of Histo- and Cytochemistry*, vol. 2, p. 222. New York: Interscience Publishers–J. Wiley and Sons Inc.

Goldberg, E. (1963). *Science*, 139, 602.

Hohorst, H. J. (1956). *Biochem. Z.* 328, 509.

Kaplan, N. O. & Ciotti, M. M. (1961). *Ann N.Y. Acad. Sci.* 94, 701.

Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* 56, 658.

Lous, P., Plum, C.-M. & Schou, M. (1956). *Nord. Med.* 55, 693.

Markert, C. L. & Møller, F. (1959). *Proc. nat. Acad. Sci., Wash.*, 45, 753.

Nisselbaum, J. S., Packer, D. E. & Bodansky, O. (1964). *J. biol. Chem.* 239, 2830.

Pesco, A., McKay, R. H., Stolzenbach, F., Cahn, R. D. & Kaplan, N. O. (1964). *J. biol. Chem.* 239, 1753.

Wieland, T. & Pfleiderer, G. (1962). *Angew. Chem.* 74, 261.

Wieme, R. J. (1959). *Agar-Gel Electrophoresis (Thesis)*. Brussels: Arscia Uitgaven N.V.

Wüst, H. (1962a). In *Methoden der enzymatischen Analyse*, p. 824. Ed. by Bergmeyer, H.-U. Weinheim: Verlag Chemie.

Wüst, H. (1962b). In *Methoden der enzymatischen Analyse*, p. 828. Ed. by Bergmeyer, H.-U. Weinheim: Verlag Chemie.

Zinkham, W. H., Blanco, A, & Kupchyk, L. (1963). *Science*, 142, 1303.