Lactate Dehydrogenase Isoenzymes of Sperm Cells and Testes

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1. The kinetic and metabolic properties of lactate dehydrogenase isoenzyme LDH_x from human sperm cells and rat testes were studied. 2. LDH_x shows a sensitivity to inhibition by stilboestrol diphosphate, urea and guanidinium chloride different from that of the LDH-H₄ and LDH-M₄ isoenzymes. 3. About 10 and 20% of the total lactate dehydrogenase activity of testes and sperm cells respectively were associated with particulate fractions. In sperm cells 11% was localized in the middle piece and 18.8% in the head fraction. LDH_x was found in all particulate fractions of sperm cells. The middle piece contained 41.0% of total LDH_x activity and showed high succinate dehydrogenase activity. 5. The pH-dependence of lactate/pyruvate and NAD⁺/NADH concentration ratios were estimated. Lactate dehydrogenase in sperm cells has maximal activity with NADH as coenzyme at pH 7.5 and with NADPH as coenzyme at pH 6.0. At pH 6.0 a 10% greater oxidation of NADPH than of NADH was found. At acid pH lactate hydrogenase may function as an enzyme bringing about transhydrogenation from NADPH to NAD⁺. 6. In agreement with the stoichiometry of the lactate dehydrogenase reaction, the lactate/pyruvate concentration ratio decreased with increasing pH. 7. The lactate/pyruvate and NAD⁺/NADH concentration ratios were estimated with glucose, fructose and sorbitol as substrates and as a function of time after addition of these substrates. During a 20 min. period after the addition of the substrates, changes in lactate/pyruvate and NAD⁺/NADH concentration ratios were noticed. Increasing concentration of the substrates mentioned gave rise to asymptotic increases in lactate and pyruvate. 8. Sorbitol did not act as a substrate for LDH_x. 9. The findings described are consistent with the idea that LDH_x is different from other known lactate dehydrogenase isoenzymes, but that it has a metabolic function similar to that of the isoenzymes of other tissues.

In most mammalian tissues there are two primary sub-units of LDH* combined together in groups of four to give the active enzymic structure. These two primary proteins have been designated H and M, as they are individually prominent in heart and skeletal muscle respectively (see the survey by Wilkinson, 1965). In sperm cells and testes a further LDH isoenzyme (LDH_x) of unique sub-unit composition occurs (Zinkham, Blanco & Kupchik, 1963; Blanco & Zinkham, 1963; Goldberg, 1963). Although some aspects of the biological function and enzymic properties of LDH_x have been elucidated (Clausen & Øvlisen, 1965; Wilkinson & Withycombe, 1965; Eliasson, Häggman & Wiklund, 1967), systematic studies of the compartmentalization, similarities and differences between this isoenzyme and LDH isoenzymes of other tissues are lacking. In continuation of

previous studies (Clausen & Øvlisen, 1965) the present paper gives some data on the catalytic and biological properties of this enzyme compared with those of the pure LDH-H₄ and LDH-M₄ isoenzymes.

MATERIALS AND METHODS

Materials

Human sperm. This was obtained from the Laboratory of the Copenhagen Health Insurance Society. For routine assay of LDH isoenzyme, the human sperm cells were isolated and extracted with 0.15M-NaCl as described by Clausen & Øvlisen (1965).

Rats. Adult Wistar rats (200–300 g.) were used for studies of compartmentalization of LDH isoenzymes in particulate fractions of testes. The testes were isolated by ligation and cutting off of epididymis and associated vessels during light ether anaesthesia.

Human liver and heart tissue. These tissues, used for pH studies and for isolation of LDH-M₄ and LDH-H₄, were

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

obtained as post-mortem specimens about 16 hr. after death. Only macroscopically normal tissue was used.

Chemicals. Unless otherwise stated the chemicals used were those of highest purity obtainable from British Drug Houses Ltd. (Poole, Dorset).

Methods

Assay of LDH activity. This was performed on 100 μ l. samples of suitable dilution (Clausen & Øvlsen, 1965). All results of total LDH determinations are mean values of double determinations.

The number of LDH enzyme units present in 1.000 ml. of the original sample is expressed as μ moles of NADH oxidized/min. at pH 7.5 (25°), with a reaction mixture containing 1.0 mm-sodium pyruvate and 0.33 mm-NADH in 50 mm-sodium phosphate buffer (Clausen & Øvlsen, 1965). The LDH specific activity was expressed as units/mg. of protein in the sample.

Assay of succinate dehydrogenase activity. This was used as an arbitrary measure of mitochondrial content of particulate fractions. The succinate dehydrogenase [succinate-(acceptor) oxidoreductase, EC 1.3.99.1] was assayed as described by Green, Mii & Kohout (1955) with 2,6-dichlorophenol-indophenol as electron acceptor. The reaction mixture (2.000 ml.) for routine assay consisted of 40 μ l. of particulate fraction and 1960 μ l. of 20 mm-sodium succinate, 35 μ M-2,6-dichlorophenol-indophenol, 2.0 mm-KCN, 50 mm-EDTA (disodium salt) and 0.05% bovine albumin (Behringwerke A.-G., Marburg, Germany). The succinate dehydrogenase activity was assayed by reading the change in E_{600} at 30 sec. intervals for 5 min. against a blank containing all components except succinate (temperature 25°). These assay conditions were established on the basis of systematic variations in pH, concentration of succinate and 2,6-dichlorophenol-indophenol as suggested by Singer & Kearney (1965). The concentrations used were those giving saturation with succinate and maximum of activity with 2,6-dichlorophenol-indophenol. Higher concentrations of this redox indicator gave rise to inhibition of succinate dehydrogenase activity. On the basis of the change in E_{600} at 25° and the molar extinction coefficient of 2,6-dichlorophenol-indophenol at 600 nm. of 1.91×10^7 cm.²/mole (Basford & Huennekens, 1955) the number of enzyme units present in 1.000 ml. of the original sample was expressed as μ moles of 2,6-dichlorophenol-indophenol transformed/min. The succinate dehydrogenase specific activity was expressed as units/mg. of protein in the sample.

Determination of protein content. Protein was determined by the Lowry method as modified by Lous, Plum & Schou (1956). Tyrosine was used as standard and the protein content was calculated on the basis of the tyrosine content of bovine serum albumin (Lous *et al.* 1956). Before the protein determination 50 μ l. of particulate suspension was solubilized at 37° in 200 μ l. of 2 N-NaOH for 16 hr.

Determination of LDH isoenzymes. Microelectrophoresis on agar-gel slides was performed as described by Wieme (1959) at pH 8.6 in 50.0 mm-sodium diethylbarbiturate buffer containing 1% Difco Special Noble Agar. For routine assay the separated isoenzymes were detected by staining by the formazan method (Van der Helm, 1962). The relative proportions of the different isoenzyme bands were estimated by scanning the slides at 546 nm. in the

Vitatron photometer equipped with scanning device, automatic recorder and integrator.

Previous systematic investigations have revealed that Beer's law is valid as long as the integrator can be used (Clausen & Øvlsen, 1965). From the results of the scanning and on the basis of the sub-unit composition of the LDH isoenzymes containing H- or M-sub-units or both, the H/M sub-unit ratio was determined.

Assay of pH-dependence of LDH isoenzyme pattern. The isoenzymes of total liver, sperm and heart extracts were electrophoretically separated at pH 8.5 in agar gel. The isoenzymes were developed with lithium lactate and NAD⁺ as described above except that the substrate solutions were in 50.0 mm-sodium diethylbarbiturate buffers at pH 5.0, 6.0, 7.0 and 8.0.

Assay of coenzyme-dependence of LDH pattern. The possible function of NADPH as coenzyme for LDH at different pH values was tested by assay of total LDH activity of the middle piece and total sperm-cell extract with sodium pyruvate as substrate. The results were compared with those obtained with NADH as coenzyme. The coenzyme concentration used was 0.33 mm, as the saturation limit for NADPH was found to be 0.27 mm and previous studies revealed 0.33 mm to be the saturation limit for NADH (Clausen & Øvlsen, 1965).

Finally, the LDH isoenzyme patterns of total sperm, liver and heart tissue extract were developed after electrophoresis on agar gel by using the LDH reagent at different pH values with NAD⁺ (8.3 mm) and NADP⁺ (8.3 mm) as coenzymes.

Determination of substrates in metabolic experiments. (a) Lactate and pyruvate. These were determined during metabolic experiments (see below) in the medium of suspended sperm cells by the LDH methods of Hohorst (1962) and Bücher, Czok, Lamprecht & Latzko (1962) respectively. In routine studies the standard procedures and reagents of C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany) were used.

(b) Coenzymes. NAD⁺ and NADH were determined fluorimetrically by the method of Lowry, Roberts & Kappahn (1957). The fluorimetric determination of coenzymes was performed in a Photovolt fluorimeter equipped with a mercury lamp. The coenzymes were determined in samples of a volume twice that indicated by Lowry *et al.* (1957). These volumes were pipetted by means of Hamilton syringes standardized by weighing samples of distilled water. Repeated pipettings of 2 μ l. and 50 μ l. samples showed it possible to deliver these volumes with standard deviations of $\pm 4\%$ and $\pm 0.4\%$ respectively.

Each series of coenzyme determination was performed together with standard samples: 0.25 mm-NAD⁺ (85.5% of the β -form) and 0.25 mm-NADH (83.2% of the β -form) (C. F. Boehringer und Soehne G.m.b.H.).

Systematic studies revealed linearity between percentage fluorescence and concentration of NAD⁺ (NADH) below the concentrations indicated above. NAD⁺ and NADH were determined with standard deviations of about $\pm 8\%$ and about $\pm 7\%$ respectively.

Isolation of head, middle piece and tail of human sperm cells. The sperm cells of pooled samples of fresh human sperm (6–10 ml.) were collected by centrifugation at 2000g for 10 min. The cells were washed three times with Ca²⁺-free Ringer solution for spermatozoa (Mann, 1946). The pellet of sperm cells was suspended in 10 vol. of 0.25 M-sucrose

containing 1.0 mM-EDTA (disodium salt) and disintegrated in a Potter-Elvehjem homogenizer equipped with a loose-fitting glass pestle at 250 rev./min. for 2 min. The homogenate was fractionated by centrifugation in a Sorvall RC-2 centrifuge (Mohri, Mohri & Ernster, 1965). The heads and middle pieces were obtained as the sediment of centrifugation at 900 g_{max} for 10 min. at 4°. Two distinct layers of the sediment appeared (Mohri *et al.* 1965), namely an upper pink layer of middle pieces and a white bottom layer of heads. The upper middle-piece pellet was separated from the more tightly packed head fraction by gently shaking up in the sucrose-EDTA medium (see above). From the 900 g supernatant of the crude homogenate the tails were isolated by centrifuging at 100 000 g_{max} for 60 min. at 4° in a Spinco L-2 ultracentrifuge (Spinco rotor no. 40). The fractions were monitored by electron microscopy (see below).

Preparation of testes suspension for ultracentrifugation. A 6 g. portion of rat testes was cut into pieces rinsed, with 0.15 M-NaCl to remove blood and homogenized in 18.0 ml. of 0.32 M-sucrose (250 rev./min.) for 2 min. in a loose-fitting Potter-Elvehjem homogenizer with glass pestle at 4°. About 0.5 ml. of the homogenate was removed for measurement of total LDH activity, LDH isoenzymes, succinate dehydrogenase activity and protein.

Preparation of subcellular fractions of testes. The homogenates were diluted tenfold with 0.32 M-sucrose. The nuclear fraction was isolated by centrifugation at 900 g_{max} for 10 min. at 4° in a Sorvall RC-2 centrifuge. The collected supernatants were then centrifuged at 11 500 g_{max} for 20 min. at 4° in a Spinco L-2 ultracentrifuge (Spinco rotor no. 40) to obtain the crude mitochondrial sediment. The mitochondrial sediment was resuspended in 10 ml. of 0.32 M-sucrose and layered on a 40 ml. sucrose gradient of equal volumes of 0.8 M-, 1.0 M-, 1.2 M- and 1.4 M-sucrose (De Robertis, Pellegrino de Iraldi, Rodriguez de Lores Arnaiz & Salganicoff, 1962). The gradient, made by successive application of 10 ml. of each of the sucrose solutions, was heated for 70 min. at 37° in a water bath and then cooled to 0° before use. Centrifugation at 50 000 g_{max} for 2 hr. at 0° in a swinging-bucket Spinco 25-2 rotor produced in the gradient layers the corresponding mitochondrial subfractions: A, B, C, D and E respectively. The middle layers were removed by vacuum aspiration and suspended in 0.15 M-NaCl. After centrifugation at 18 000 g_{max} for 20 min., each pellet was washed three times with 0.15 M-NaCl, suspension of the pellet being avoided. The microsomal sediment was isolated from the 11 500 g supernatant by centrifugation at 100 000 g_{max} for 2 hr.

Enzyme extraction of particulate fractions. All the particulate fractions thus collected were suspended in 0.5 or 1.0 ml. of 0.15 M-NaCl, homogenized at approx. 500 rev./min. for 5 min. at 0° in an all-glass homogenizer and centrifuged at 18 000 g_{max} for 20 min. at 4°. In some experiments the mitochondrial and microsomal pellets after homogenization were further extracted with 1.0 ml. of 1.0% (v/v) Triton X-100 in 0.15 M-NaCl, rehomogenized and centrifuged as above. Initial studies showed that 2.0% Triton X-100 did not change the specific LDH activity of soluble samples. The supernatants of the Triton extracts were tested for total LDH activity, total protein and isoenzyme pattern. Succinate dehydrogenase activity was determined directly in the suspensions.

Electron microscopy. Samples were fixed in 1% osmium

tetroxide in iso-osmotic sodium diethylbarbiturate buffer, pH 7.4, for 30–60 min., dehydrated in acetone and embedded in Vestopal. Sections were cut with glass knives on an LKB Ultratome and examined by a Philips 100B electron microscope. Photographs were taken at magnifications of 1300–7500.

Partial purification of LDH isoenzymes. The LDH-H₄ and LDH-M₄ isoenzymes were isolated from human brain and liver homogenate respectively by preparative agar-gel microelectrophoresis (Clausen & Øvliisen, 1965). The fractions were isolated from the agar gel by cutting out the appropriate areas of the gel and liberating the protein by freezing and thawing. The isolated fractions (specific activity about 7.2 units/mg. of protein, as indicated by Clausen & Øvliisen, 1965), were used for all studies in the present paper except for the denaturation experiments. These were performed with crystalline LDH-H₄ from ox heart (Sigma Chemical Co., St Louis, Mo., U.S.A.) with a specific activity of 39.6 units/mg. of protein. Agar-gel electrophoresis of this preparation revealed, by the formazan staining reaction, 97.4% of LDH-H₄ and 2.6% of LDH-H₃M. In the denaturation experiments the LDH-M₄ was obtained from rabbit muscle (Sigma Chemical Co.) with a specific activity of 34.0 units/mg. of protein. Agar-gel electrophoresis revealed 79.5% of LDH-M₄, 10.3% of LDH-HM₃, 5.1% of LDH-H₂M₂ and 5.1% of LDH-H₃M.

Purification of LDH_x. Isolation of pure LDH_x from whole sperm was attempted by using a method similar to that described by Schwert, Millar & Takenaka (1962) and the separation of LDH-H₄ and LDH-M₄ as suggested by Plummer, Elliott, Cooke & Wilkinson (1963). The difficulty of limited amount of material was overcome by the daily collection and pooling of sperm samples. Immediately after pooling, the sperms were homogenized in a Potter-Elvehjem disintegrator (250 rev./min. for 2 min.). The supernatant from centrifugation at 2000 g for 10 min. at 4° was precipitated at pH 6.8 at 3.0 M-(NH₄)₂SO₄. The precipitate obtained was isolated by centrifugation at 2000 g for 10 min. at 4° and suspended in 3.0 M-(NH₄)₂SO₄ and stored at 4° until sufficient amounts were collected. The 3.0 M-(NH₄)₂SO₄ precipitate originating from 200 ml. of sperm was treated as for the isolation of LDH-H₄. However, no fractions could be obtained with specific activity above 0.0965 unit/mg. of protein. This procedure was therefore unsuitable for obtaining larger quantities of LDH_x.

To obtain larger quantities of prepurified LDH_x not contaminated with LDH isoenzymes containing H- and M-sub-units, Triton X-100 extracts (1% Triton X-100 in 0.15 M-NaCl) of the middle pieces were used (see below). The middle pieces contain, as shown below, only LDH_x, and Triton X-100 extraction liberated enzyme with high specific activities (see Table 2).

Denaturation and inhibition experiments. (a) Denaturation. The activities of the three LDH isoenzymes were measured in the presence of increasing steps of 0.5 M-urea (three times recrystallized from ethanol) and of guanidinium chloride adjusted to pH 7.5 at 23°. The LDH activities were determined after incubation for 2 hr. For 1.0 M-urea and guanidinium chloride the changes in LDH activity were followed for 24 hr.

(b) Inhibition with stilboestrol diphosphate (Honvan). The absorption spectra of 12 mM-stilboestrol diphosphate in water and of 12 mM-stilboestrol in propylene glycol showed maxima at 300 nm. but no absorption peak between

320 and 500 nm. The absorption peaks of 0.33 mM-NAD⁺ and of 0.33 mM-NADH were unaltered after mixing with equal volumes of the components mentioned above.

Previous studies (Clausen & Gerhardt, 1963) revealed the LDH isoenzymes containing M sub-units to be more sensitive to inhibition with stilboestrol diphosphate in the presence of acid phosphatase than is LDH-H₄. To characterize the enzymes further, purified LDH-H₄, LDH-M₄ and LDH_x in a suitable dilution were mixed with equal parts of acid phosphatase from potatoes (Sigma Chemical Co. batch no. 124B-8630; specific activity 0.4 μ mole of *p*-nitrophenyl phosphate hydrolysed/min./mg. of protein at pH 4.8 at 37°) and 1800 μ l. of this solution was mixed with 200 μ l. of 0.117 M-stilboestrol diphosphate in 0.15 M-NaCl. Samples were assayed for LDH activity over a 24 hr. period.

After exactly 20 min. of treatment with stilboestrol diphosphate the LDH activity was assayed, with 0.1 mM-, 0.2 mM- and 0.3 mM-sodium pyruvate, in samples containing increasing concentrations of stilboestrol diphosphate from 4.7 mM to 18.8 mM in the presence of 0.33 mM-NADH. On the basis of the Dixon (1953) plot the type of inhibition and the inhibitor constants were calculated.

The stilboestrol diphosphate inhibition was further studied by using increasing concentrations of NADH (from 0.0 to 4.5 mM) in the presence of a constant concentration of stilboestrol diphosphate (5.56 mM) and a fixed reaction time of 20 min. In one series of experiments the NADH was added simultaneously with the stilboestrol diphosphate. In another series of experiments the NADH was not added until the stilboestrol diphosphate had been allowed to react for 20 min. Finally the direct action of free stilboestrol on LDH isoenzymes was followed by adding 10 mM-stilboestrol in propylene glycol to the reaction mixture to give final concentrations of 30, 12 and 6 μ M before assay of LDH activity.

Metabolic experiments. Whole fresh sperm, maximally 2 hr. after collection, or sperm cells were used for metabolic studies of changes in lactate, pyruvate, NAD⁺ and NADH concentrations.

The lactate/pyruvate and oxidized coenzyme/reduced coenzyme concentration ratios were measured at pH values occurring in the vaginal secretion. The pH was varied from 5.5 to 8.0 in increments of 0.25 pH unit. The buffers used were in one series of experiments 50.0 mM-sodium phosphate and in another 50.0 mM-tris-HCl buffer containing NaCl (0.15 M). In three other experiments the 50.0 mM-sodium phosphate buffer contained fructose (8.5 mM), glucose

(13.8 mM) or sorbitol (1.1 mM). The samples were mixed with the buffer solution about $\frac{1}{2}$ hr. before the determination of the respective substrates (concentration of sperm cells about 3×10^8 /ml.). The above-mentioned ratios were also determined at pH 7.5 [50 mM-sodium phosphate buffer containing NaCl (0.15 M)] where the concentration of glucose was varied from 2.78 to 44.5 mM, that of sorbitol from 0.55 to 8.24 mM and that of fructose from 2.78 to 27.8 mM.

The sorbitol used was recrystallized three times from ethanol. At the pH values used no spontaneous alterations in concentrations of lactate, pyruvate and the substrates were noticed within 30 min.

RESULTS

LDH activity influenced by urea and guanidinium chloride

Addition of increasing amounts of urea to purified LDH-H₄ caused total inhibition at 2.5 M-urea and at 2.0 M-guanidinium chloride after a reaction time of 2 hr. at pH 7.5. LDH-M₄ was inhibited totally at 1.5 M-guanidinium chloride and 1.5 M-urea. In contrast with the greater sensitivity of LDH-M₄ to urea and guanidinium chloride, LDH_x was first inactivated at 3.5 M-urea and 2.0 M-guanidinium chloride.

LDH-H₄ was inactivated by about 50% by treatment with 1.0 M-urea at pH 7.5 for 24 hr. Guanidinium chloride inactivated LDH-H₄ by 50% in 8 hr. LDH-M₄ was inactivated by 50% after about 18 hr. with 0.5 M-urea and in 40 min. by 0.5 M-guanidinium chloride at pH 7.5. LDH_x was inactivated by 50% after 28 hr. with 0.5 M-urea and after 8 hr. with 0.5 M-guanidinium chloride.

Action of stilboestrol diphosphate (Honvan) on LDH activity

Previous studies (Clausen & Gerhardt, 1963) showed LDH-M₄ of crude homogenates to be sensitive, and the purified enzyme to be insensitive, to the action of stilboestrol diphosphate. This led to the conclusion that the acid phosphatase present

Table 1. *Kinetic data for the inhibition of LDH isoenzymes LDH-H₄, LDH-M₄ and LDH_x by stilboestrol diphosphate in the presence of acid phosphatase*

Experimental conditions are given in the text.

	LDH-H ₄	LDH-M ₄	LDH _x
Type of stilboestrol diphosphate inhibition	Uncompetitive	Competitive	Competitive
<i>K_i</i> (pyruvate)* (stilboestrol diphosphate inhibition)	2.92×10^{-6} M	4.20×10^{-5} M	2.81×10^{-4} M
<i>K_m</i> (pyruvate)*	1.34×10^{-4} M	1.02×10^{-3} M	2.84×10^{-4} M
Time for 50% decrease in activity at 11.7 mM-stilboestrol diphosphate	2 hr.	20 min.	2 hr.

* S.D. $\pm$ 3–5%.

Fig. 1. Dixon (1953) plot of the inhibition of LDH_x by stilboestrol diphosphate in the presence of the following concentrations of sodium lactate: $\blacktriangle$, 0.05 mM; $\blacktriangledown$, 0.10 mM; $\bullet$, 0.20 mM. Experimental conditions are given in the text.

Fig. 2. Reactivation by addition of NADH of LDH-M_4 inhibited by stilboestrol diphosphate. Purified LDH-M_4 (200 μl .; activity 16 units) containing 1.0 unit of potato acid phosphatase was mixed with 20 μl . of 117 mM-stilboestrol diphosphate and 200 μl . of NADH (various concentrations). After 20 min. samples were assayed for LDH activity. Experimental conditions are given in the text.

led to the formation of free (nascent) stilboestrol (see below). Therefore the action of stilboestrol diphosphate on purified preparations of LDH-H_4 , LDH-M_4 and LDH_x was studied in the presence of potato acid phosphatase. All three enzymes were inactivated (Table 1 and Fig. 1). Constants from kinetic studies of the inhibition by stilboestrol

diphosphate at 0.1 mM-, 0.2 mM- and 0.3 mM-pyruvate (below the saturation limit of this substrate) are also shown in Table 1. At higher inhibitor concentrations proportionally higher inhibition at low substrate concentration occurred. It was shown that increasing the concentration of NADH in the reaction mixture effected a partial reversal of the inhibitory action of stilboestrol diphosphate. Thus, under the experimental conditions in Fig. 2, where after a reaction time of 20 min. stilboestrol diphosphate decreased LDH activity to 17.5% of the original value, the presence of 4.0 mM-NADH partially restored the activity to 21.3% of the original value. Free stilboestrol (30 μM) gave a 10% decrease in LDH-M_4 activity; however, a more detailed study of the effect of free stilboestrol on LDH isoenzymes could not be performed because of the poor solubility of this component.

Distribution of LDH in particulate fractions of sperm cells and rat testis homogenates

Topographical studies of sperm cells. Tables 2 and 3 show that LDH specific activities were roughly similar among the fractions obtained by ultracentrifugation. The supernatant from the differential centrifugation contained the highest LDH specific activity: six to 14 times that of the particulate fractions (Table 3). The particulate fractions contained roughly similar LDH specific activities. However, Table 2 indicates the middle piece to contain 11% of the total LDH activity and 21% of the total LDH_x activity of total sperm whole homogenate. The succinate dehydrogenase specific activity was four times as high in the middle-piece fraction as in the head fraction. No succinate dehydrogenase activity was found in the tail fraction. The head fraction contains a higher ratio of total LDH specific activity to total succinate dehydrogenase specific activity than the middle piece.

Agar-gel microelectrophoresis revealed the presence of LDH isoenzymes containing H- and M-subunits in the supernatants from ultracentrifugation and in extracts of the head and tail fractions. However, the middle-piece fraction contains only the LDH_x fraction (Table 3); therefore the specific activity of the middle-piece fraction refers exclusively to the LDH_x fraction. The LDH_x specific activity seems higher in the head fraction and in the supernatant (microsomal fraction) than in the middle-piece fraction.

Electron microscopy of the particulate fractions obtained by ultracentrifugation revealed the presence of intact sperm cells besides pure head fragments in the head fraction. The middle-piece fraction contained mainly small vesicles of variable size, sometimes equipped with internal cristae or spiral structures. The tail fraction contained

Table 2. *Total LDH and succinate dehydrogenase activities of particulate fractions of human sperm cells*

The results of two separate experiments are shown: the determination of LDH and succinate dehydrogenase (SDH) specific activities are based on a centrifugation schedule different from that used for determination of yields of enzymic activities.

Fraction	LDH specific activity (μ moles/min./mg. of protein)	SDH specific activity (μ moles/min./mg. of protein)	Yield of LDH units (% of total LDH units of total sperm-cell homogenate)	Yield of LDH _x units (% of total LDH _x units of total sperm-cell homogenate)	LDH extractable with Triton X-100 (% of LDH extractable with 0.15 M-NaCl)
Head					
0.15 M-NaCl extract	1.12		18.8	25.8	
0.15 M-NaCl-Triton X-100 extract	1.16	0.31			6.6
Middle piece					
0.15 M-NaCl extract	1.21		11.3	21.0	
0.15 M-NaCl-Triton X-100 extract	1.97	1.18			41.0
Tail					
0.15 M-NaCl extract	1.75			9.7	
0.15 M-NaCl-Triton X-100 extract	1.08	0.00	8.0		9.0
Supernatant	*	0.00	20.8	30.0	

* Lost, but cf. Table 3.

Table 3. *Total LDH activity and percentage distribution of LDH isoenzymes in particulate fractions of human sperm cells*

Fraction	Total LDH activity (μ moles/min./mg. of protein)	Distribution of LDH isoenzymes (%)					
		LDH-H ₄	LDH-H ₃ M	LDH-H ₂ M ₂	LDH-HM ₃	LDH _x	LDH-M ₄
Head	0.73	0.0	0.0	21.0	5.3	73.7	0.0
Middle piece	0.29	0.0	0.0	0.0	0.0	100.0	0.0
Tail	0.31	0.0	0.0	26.1	6.5	65.2	2.2
Supernatant	4.26	0.0	0.0	19.4	3.1	77.5	0.0

elongated tail-piece fragments. These experiments suggest that the middle-piece fraction containing the particulate succinate dehydrogenase activity also contains the LDH_x fraction. The presence of LDH_x in the head fraction may be explained by contamination.

After disintegration and extraction of the particulate fractions with 0.15 M-sodium chloride the remnants isolated by centrifugation at 20000g_{max} for 30 min. were extracted with 0.15 M-sodium chloride containing Triton X-100 (1.0%) at 23°. By this means it was still possible to extract LDH of high specific activity from the remainder of all particulate fractions (Table 2). As shown in Table 2 specific activities roughly similar to those of the sodium chloride extracts were obtained by re-extraction with Triton X-100, but proportionally more LDH activity was extractable from the middle-piece fraction than from any other particulate fraction.

Distribution of LDH_x in particulate fractions of rat testes. Table 4 shows that the highest succinate

dehydrogenase specific activity occurred in mitochondrial subfraction *D* obtained by sucrose-density-gradient ultracentrifugation of rat testis whole homogenate. These findings agree well with the electron-microscopical investigations, which showed the mitochondrial subfraction *D* to be rich in morphological structures characteristic of mitochondria, e.g. ellipsoid structures with internal cristae. Tables 4 and 5 further show that the highest LDH specific activity is also localized in mitochondrial subfraction *D*. However, the nuclear fractions also contain a high LDH specific activity. After extraction of the particulate fractions with 0.15 M-sodium chloride, the residue (washed three times) obtained by centrifugation at 20000g for 30 min. at 4° was re-extracted with 0.15 M-sodium chloride containing Triton X-100 (1.0%). As shown in Table 4 the re-extraction with Triton X-100 leads to a further liberation of LDH activity. This liberation was especially pronounced in mitochondrial subfraction *D*.

Agar-gel microelectrophoresis revealed an uneven

Table 4. *Total LDH and succinate dehydrogenase activities of particulate fractions of rat testes*

The particulate fractions were separated by sucrose-density-gradient ultracentrifugation (see the text). To evaluate clearly the possible enzymic differences among the individual particulate fractions, only the central portion of each fraction was isolated by suction from the centrifuge tube. Therefore the yields of total LDH units in the fractions could only be evaluated roughly. The lowest yield encountered was about 1% of total LDH units of crude homogenate in mitochondrial subfraction *A*. The highest yield, 10.4%, was found in mitochondrial subfraction *D*. SDH, Succinate dehydrogenase.

Fraction	LDH specific activity (μ moles/min./mg. of protein)	Ratio between LDH units in extracts with and without Triton X-100	SDH specific activity (μ moles/min./mg. of protein)
Nuclear fraction			
0.15 M-NaCl extract	0.75	1:2	0.00
0.15 M-NaCl-Triton X-100 extract	0.06		
Mitochondrial subfraction <i>A</i>			
0.15 M-NaCl extract	0.16	1:1	0.032
0.15 M-NaCl-Triton X-100 extract	0.01		
Mitochondrial subfraction <i>B</i>			
0.15 M-NaCl extract	0.92	1:2	0.0091
0.15 M-NaCl-Triton X-100 extract	0.06		
Mitochondrial subfraction <i>C</i>			
0.15 M-NaCl extract	1.79	1:2	0.087
0.15 M-NaCl-Triton X-100 extract	0.06		
Mitochondrial subfraction <i>D</i>			
0.15 M-NaCl extract	4.19	1:4	1.83
0.15 M-NaCl-Triton X-100 extract	1.13		
Mitochondrial subfraction <i>E</i>			
0.15 M-NaCl extract	2.52	1:3	0.64
0.15 M-NaCl-Triton X-100 extract	0.30		
Microsomal fraction			
0.15 M-NaCl extract	0.27	1:2	0.034
0.15 M-NaCl-Triton X-100 extract	0.46		

distribution of LDH isoenzymes (Table 5) in the particulate fractions. Saline extraction of the particulate fraction liberated mainly LDH isoenzymes containing H- and M-sub-units. Thus LDH-H₄ predominated in the nuclear fraction and in mitochondrial subfractions *A* and *B*. However, the mitochondrial subfractions *C*, *D* and *E*, and the microsomal fraction as well, seem to have a larger content of LDH isoenzymes containing M sub-units. LDH_x was found only in saline extracts of the nuclear fraction and mitochondrial subfraction *E*.

Variation of total LDH activity and LDH isoenzyme pattern with pH

The pH variation of total LDH_x activity of total sperm cells was identical with that of the middle piece. The pH variation was dependent on the coenzyme used. Thus with NADH (0.33 mM) as coenzyme pyruvate reduction was maximum at pH 7.5, whereas with NADPH (0.33 mM) the optimum was at pH 6.0. At this pH the LDH_x activity was 10% higher with NADPH than with NADH as coenzyme (Fig. 3).

Table 6 shows that the total sperm exhibits an increasing relative activity of LDH_x with decreasing pH down to pH 6.0 with NAD⁺ as substrate. On the other hand, it was not possible to detect LDH_x at pH 5.0, 6.0 and 8.0 with NADP⁺ as coenzyme.

Sorbitol and LDH_x activity

By replacing lactate by 1.1 mM-sorbitol it was possible to trace in total sperm both LDH_x and also LDH-H₂M₂ and LDH-M₃ by enzyme-electrophoresis. However, these fractions were not detected in the agar-gel electrophoresis when the total sperm was dialysed for more than 3 hr. before electrophoresis. Thus it may be the endogenous lactate in the total sperm that leads to the development of the three above-mentioned LDH isoenzyme fractions. With sorbitol as substrate, only the application slit exhibits an intensively stained area in agar-gel electrophoresis.

Metabolic studies

Lactate/pyruvate and oxidized coenzyme/reduced coenzyme concentration ratios of total sperm and sperm

Table 5. Total LDH activity and percentage distribution of LDH isoenzymes in particulate fractions of normal rat testes

	Total LDH activity (μ mole/min./mg. of protein)		Distribution of LDH isoenzymes in 0.15 M-NaCl extract (% of total LDH recovered from gel)						LDH isoenzyme activity in 0.15 M-NaCl extract (μ mole/min./mg. of protein)					
	0.15 M-NaCl extract	Triton X-100 extract	LDH-H ₄	LDH-H ₃ M	LDH-H ₂ M ₂	LDH-HM ₃	LDH-LDH _x	M ₄	LDH-H ₄	LDH-H ₃ M	LDH-H ₂ M ₂	LDH-HM ₃	LDH-LDH _x	M ₄
Nuclear fraction	0.32	0.71	41.9	34.5	3.6	9.1	5.4	5.4	0.13	0.11	0.011	0.029	0.017	0.017
Mitochondrial subfraction A	0.12	0.41	100.0	0.90	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00
Mitochondrial subfraction B	0.20	0.40	78.6	21.4	0.00	0.00	0.00	0.00	0.00	0.15	0.04	0.00	0.00	0.00
Mitochondrial subfraction C	0.60	0.24	46.6	53.4	0.00	0.00	0.00	0.00	0.06	0.28	0.32	0.00	0.00	0.00
Mitochondrial subfraction D	1.07	0.33	36.0	40.0	8.0	16.0	0.00	0.00	0.38	0.43	0.08	0.17	0.00	0.00
Mitochondrial subfraction E	0.54	0.41	16.7	10.4	6.3	29.2	18.7	18.7	0.09	0.06	0.03	0.16	0.10	0.10
Microsomal fraction	0.43	0.44	41.6	58.4	0.00	0.00	0.00	0.00	0.15	0.25	0.00	0.00	0.00	0.00
Supernatant of microsomal fraction	0.39	0.83	34.6	46.2	11.5	7.7	0.00	0.00	0.13	0.18	0.04	0.03	0.00	0.00

Fig. 3. pH optima of the LDH_x-catalysed reduction of pyruvate with NADH (●) and with NADPH (▼) as coenzyme. Experimental conditions are given in the text.

Table 6. Effect of pH on LDH_x activity expressed as a percentage of total LDH activity of total sperm at each pH with NAD⁺ or NADP⁺ as coenzyme in enzyme-electrophoresis

Coenzyme	LDH _x activity (% of total sperm LDH activity)			
	pH 5.0	pH 6.0	pH 7.0	pH 8.0
NAD ⁺	0.0	49.1	43.5	40.3
NADP ⁺	0.0	0.0	26.6	0.0

cells as functions of time, pH and substrate. Fig. 4 shows that, in the first 20 min. after the addition of 1 vol. of 13.9 mM-glucose in 50.0 mM-sodium phosphate buffer, pH 7.5, containing sodium chloride (0.15 M) with or without ATP (66 μ M) to 1 vol. of normal total sperm, variation in the lactate/pyruvate and oxidized coenzyme/reduced coenzyme concentration ratios occurs. Similar results were obtained with the addition of 1.1 mM-sorbitol or 8.32 mM-fructose. The variations in the concentration ratios were similar for the three substrates, with a peak for each of the ratios in the first 5 min. After about 20 min., however, both con-

Fig. 4. Variation in (a) lactate concentration (▼) and pyruvate concentration (▲), (b) lactate/pyruvate concentration ratio (●) and (c) NAD^+/NADH concentration ratio (■) after addition of glucose (13.9 mm) to the substrate. Experimental conditions are given in the text.

centration ratios seemed to reach a stabilized level. It was therefore decided to estimate the above-mentioned concentration ratios at various pH values with different substrates after an initial stabilization period of exactly 30 min. About 60% of the total NAD pool was in the oxidized form (Fig. 4).

pH-dependence of lactate/pyruvate and oxidized coenzyme/reduced coenzyme concentration ratios with different substrates. Fig. 5 shows the pH-dependence of lactate concentration, pyruvate concentration, lactate/pyruvate concentration ratio and NAD^+/NADH concentration ratio with glucose (13.9 mm) as substrate for total sperm (see above). Identical results were obtained with 1.1 mm-sorbitol and 8.32 mm-fructose. In all cases the lactate concentra-

tion steadily decreased as a function of pH from pH 5.5 to pH 7.5. Above pH 7.5, however, the lactate/pyruvate concentration ratio remained constant or slightly increased, depending on the presence or lack of ATP (66 μM) (Fig. 5). However, the addition of ATP did not alter the pH optimum for the production of lactate or pyruvate. Identical results were obtained with sperm cells, and they did not depend on the type of buffer used (sodium phosphate and tris buffers).

Lactate/pyruvate and oxidized coenzyme/reduced coenzyme concentration ratios as functions of concentrations of different substrates. Addition of increasing amounts of glucose at pH 7.5 to the substrate of the total sperm as described above led to increasing lactate and pyruvate production until a glucose concentration of about 20 mm (Fig. 6), above which lactate and pyruvate production decreased slightly. Similarly lactate and pyruvate production increased slightly during addition of sorbitol up to 2.78 mm and during the addition of fructose up to 11.0 mm. Similar values were obtained when 66 μM -ATP was added with the substrates. Neither the NAD^+/NADH nor the lactate/pyruvate concentration ratio changed very much during these experiments. [In preliminary experiments it was found that lactate production was heavily depressed (maximally 50%) by addition of increasing amounts of sorbitol from 0.28 to 2.8 mm; however, this was not caused by sorbitol itself because recrystallized sorbitol did not give this pronounced inhibition.]

DISCUSSION

The LDH_x isoenzyme present in testes and sperm cells has been found in various animal species (Goldberg, 1965; Zinkham, Blanco & Clowry, 1964; Goldberg & Hawtrey, 1967). The present paper extends previous studies (Wilkinson & Withycombe, 1965; Wilkinson, 1965; Clausen & Øvlsen, 1965) by showing LDH_x to be more closely related to LDH-H_4 than to LDH-M_4 in its sensitivity towards stilboestrol diphosphate, although the types of inhibition of LDH-H_4 and LDH_x differed. The K_i values indicate a sevenfold higher affinity of the inhibitor for LDH-M_4 than for LDH_x . The results of urea and guanidinium chloride denaturation are in agreement with the studies of Wilkinson & Withycombe (1965). Thus LDH_x seems more resistant to inactivation caused by urea and guanidinium chloride than are LDH-H_4 and LDH-M_4 .

The cytochrome system of the sperm cell is located in the middle piece (and tail) rather than in the sperm head (Zittle & Zitin, 1942; Hrudka, 1964; Mann, 1964), and electron microscopy shows a spiral mitochondrial apparatus localized in the

Fig. 5. (a) and (c) Lactate concentration (▼) and pyruvate concentration (▲) in the reaction mixture and (b) and (d) lactate/pyruvate concentration ratio (●) as functions of pH (phosphate buffer) in media of sperm cells containing (a) and (b) glucose (13.9 mM) with ATP (66 μ M) or (c) and (d) glucose (13.9 mM) without ATP. Experimental conditions are given in the text.

middle piece (see the survey by Mann, 1964). These facts, together with the present findings, which indicate the middle piece to contain the highest succinate dehydrogenase specific activity and LDH_x but no other LDH isoenzymes, argue for a special compartmentalization of these two enzymes. The LDH activity of the sperm head may be explained by the presence of fragments of the middle piece or of broken sperm cells in this fraction. Re-extraction of particulate fractions with Triton X-100 led to the liberation of LDH of a specific activity as high as that already extracted with 0.15 M-sodium chloride from head and tail. From the middle piece LDH of even higher specific activity could be liberated, exclusively of the LDH_x type.

The compartmentalization may, however, be an artifact caused by an affinity of LDH_x for mitochondrial constituents. This is improbable because during fractionation of rat testes the LDH_x was not preferentially localized in mitochondrial sub-

fraction *D* of rat testes obtained by sucrose-density-gradient centrifugation, although this fraction exhibited succinate dehydrogenase specific activity higher than that of mitochondrial subfraction *E* containing LDH_x.

The present findings are in partial agreement with the results of Güttler & Clausen (1967), indicating an affinity of kidney mitochondria for LDH-H₄ (cf. Table 5). However, in contrast with this, rat testis nuclei exhibited no preferential affinity for LDH-M₄. Although LDH_x shows high enzymic affinity for 2-hydroxybutyrate (Wilkinson & Withycombe, 1965) the fact that LDH_x and succinate dehydrogenase are localized in the middle piece, where the mitochondrial apparatus is found (see above), may indicate a functional relationship. Studies of erythrocytes, lacking both mitochondria and the nucleus, or of malignant cells, containing only few mitochondria, have revealed an LDH reaction giving rise to a lactate/pyruvate concentra-

Fig. 6. Lactate concentration (▼) and pyruvate concentration (▲) as functions of increasing concentration of glucose in the medium of sperm cells. Experimental conditions are given in the text.

tion ratio increasing with increasing pH, opposite to that determined by the stoichiometric implication of H^+ in the LDH reaction (Rappoport, Ababei, Wagenknecht & Sarkur, 1965). No such paradoxical pH effect was found in sperm cells. The lactate/pyruvate concentration ratio of these decreases with increasing pH, in accordance with the stoichiometry of the LDH reaction. From the equilibrium constant of LDH the lactate/pyruvate concentration ratio decreased only threefold from pH 6.0 to pH 8.0. However, the $NAD^+/NADH$ concentration ratio decreased about 30-fold when calculated stoichiometrically. The variation in lactate/pyruvate concentration ratio with pH of sperm cells found in the present work is similar to that described by Thieden (1965) in liver slices. However, in contrast with liver slices, sperm-cell lactate and pyruvate concentrations exhibited different pH maxima (see above). Studies on tissue-culture cells (Paul, 1959), however, show a relatively constant, or only slight increase in, lactate/pyruvate concentration ratio with increasing pH.

Comparison of the content of NAD^+ as a percentage of the total content of $NAD^+ + NADH$ in total sperm with that of other organs (see the survey by Mahler & Cordes, 1966) shows similarity to that of liver, kidney and heart tissue, with 60% of the total NAD content in the oxidized state. Thus it must be concluded that use of the lactate/pyruvate concentration ratio as a cytoplasmic marker of the redox state of the cytoplasm (Holzer, Schultz & Lynen, 1956) is limited by the fact that LDH isoenzymes may be compartmentalized differently. The presence or lack of mitochondria especially may give rise to different lactate/pyruvate concentration ratios in different tissues. The mitochondria may affect the lactate/pyruvate concentration ratio at a different pH.

To evaluate the possibility that LDH_x had enzymic capacities other than the catalysis of lactate oxidation, other naturally occurring substrates for sperm cells were investigated for their possible function as substrates in the LDH reaction. Pure sorbitol did not give rise to a fraction stainable in the formazan reaction and located at the site of LDH_x . Similarly agar-gel electrophoresis revealed no detectible LDH_x activity at pH 6.0–8.0 with $NADP^+$ as coenzyme. With NAD^+ as coenzyme a slight percentage increase in the LDH_x component of the LDH activity of total sperm was noticed from pH 8.0 to 6.0. From these findings it was concluded that LDH_x catalyses lactate oxidation with NAD^+ as coenzyme preferentially at acid pH. The affinity of LDH_x for $NADP^+$ at acid pH is nil. This, correlated to the demonstration that pyruvate reduction by the middle piece in the presence of NADPH exhibited a maximum at pH 6.0, may indicate a biological transhydrogenation through the pathway:

At pH 6.0, a pH that may occur in the female reproductive tract, the reduction of pyruvate by NADPH may account for the 10% higher activity than the reduction with NADH. The significance of this transhydrogenation pathway cannot be evaluated because NADH exhibits product inhibition (Clausen & Øvliisen, 1965). Therefore determination of K_m (nucleotide) for NADH oxidation is meaningless. Further, studies by Holzer & Witt (1964) indicate NADPH oxidation through pyruvate-lactate transformation to be inhibited by NAD^+ . The increase in lactate and pyruvate concentrations of the sperm-cell medium with increasing amounts of glucose, fructose or sorbitol up to a certain limit may be explained by a feedback control, i.e. the inhibiting action of glucose 6-phosphate on hexokinase (Morton & Lardy, 1967) or the substrate inhibition of phosphofructokinase (Chance, Higgins, Holmes & Connelly, 1958).

In anaerobically glycolysing *Saccharomyces carlsbergensis* and in the perfused heart, sustained oscillations of nicotinamide nucleotide concentrations have been described. The present studies indicate that these cells also exhibit variations in concentrations of components of the glycolytic pathway. The oscillatory response is probably a universal property of the glycolytic system (Chance, Ghosh, Higgins & Maitva, 1964; Ghosh & Chance, 1964; Williamson, 1965).

The present study indicates that LDH_x is compartmentalized in sperm cells. Although LDH_x

seems different from other isoenzymes, the relationship of the catalytic mechanism to the glycolytic pathway and probably to the function of the mitochondrion seems to indicate that LDH_x functions similarly to LDH isoenzymes in other tissues.

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