Lactate Dehydrogenases in Human Testes

Abstract. *A unique form of lactate dehydrogenase was observed in the starch-gel electrophoretic patterns of adult human testes. It was present in sperm, but absent in prepubertal testes. Its electrophoretic mobility, heat stability, kinetic behavior with pyridine nucleotide analogs, and chromatographic characteristics on diethylaminoethyl cellulose were intermediate to those observed for lactate dehydrogenase isozymes 3 and 4.*

Lactate dehydrogenase (LDH) of mammalian tissues can be separated into five distinct forms by starch-gel electrophoresis (*1*). The most rapidly migrating component has been designated LDH-1, and the slowest, LDH-5. The type and amount of the molecular forms (isozymes) of LDH vary in different tissues from the same animal. In adult human heart, for example, the dominant bands are LDH-1, LDH-2, and LDH-3, whereas in adult human liver the dominant band is LDH-5. Isozyme patterns also change during development (*1*), and the question has been raised whether these variations are related to the development of new cell types. To study this problem the complement of LDH isozymes in human testes and sperm was determined.

Human testes were obtained at the time of operation in two patients, and within 24 hours after death in 25 patients. One part of tissue was homogenized in two parts of water in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 25,000*g* for 20 minutes, and the clear supernatant was fractionated by starch-gel electrophoresis in the vertical device of Smithies (*2*) with the discontinuous buffer system of Poulik (*3*) at *p*H 8.6; the voltage gradient, 6/cm, was applied for 12 hours at 4°C. After electrophoresis the starch slab was sliced horizontally and overlayered with 135 ml of 0.19*M* tris buffer solution, *p*H 8.0, containing sodium DL-lactate, nicotinamide adenine dinucleotide (NAD), nitro-blue tetrazolium, and phenazine methosulfate.

The preparations were placed in a dark room at 37°C for 1 hour, and then washed with distilled water. A purple band of formazan appeared wherever there was LDH activity. In some experiments areas of starch in the unstained strip corresponding to regions of activity in the stained preparation were cut out, frozen, and homogenized to elute the isozymes for further studies.

Five main groups of LDH activity were present in 11 prepubertal testes (Fig. 1). Isozyme 1 always appeared as a single band. A minor band usually trailed isozymes 2 and 3 and preceded isozyme 4. Isozyme 3 sometimes appeared as a double band. Isozyme 5 presented a faint trail of activity in the cathodal direction. The supernatant fraction of homogenates from 16 postpubertal testes also exhibited five regions of LDH activity. In addition a major area of LDH activity appeared between isozymes 3 and 4 in 13 of the patients whose ages ranged between 31 and 88 years. Six were Negroes and seven were Caucasians. The new band ("band X") was absent in homogenates from three patients aged 24, 54, and 72 years. Two were Negroes and one was Caucasian. Microscopic sections of testes from two of these patients revealed a marked decrease in spermatogenesis. Sections were not available on the other patient.

The electrophoretic pattern of LDH isozymes from many other human tissues has been determined in our laboratory: diaphragm, mature ovary, skeletal muscle, heart, stomach, spleen, adrenal, kidney, liver, lung, colon, spinal cord, lymph node, thymus, lens, blood, and cerebrospinal fluid. The designated LDH "band X" was seen only in the postpubertal testis.

Some of the testicular homogenates were subjected to electrophoresis with the starch gel in a horizontal position and the continuous tris buffer system at *p*H 8.0. The rates of migration of the isozymes corresponded to those observed in the vertical system. "Band X" always migrated between isozymes 3 and 4. Minor bands and double bands were never observed in the horizontal gels, suggesting that their presence in vertical gels might be the result of some physicochemical interaction between the homogenate and the medium of electrophoresis.

Seminal fluid was obtained from three normal Caucasian adults. The sperm were washed with 0.14*M* sodium chloride, frozen, and thawed three times, and centrifuged at 25,000*g* for 20 minutes. Electrophoresis of the supernatant fractions showed that most of the LDH was "band X" (Fig. 1). A faint band was sometimes seen in the isozyme-3 and -4 regions. The supernatant fraction of freshly obtained seminal fluid demonstrated activity in the isozymic regions, 1, 2, 3, and 4.

After this report was submitted we learned that Goldberg has found five

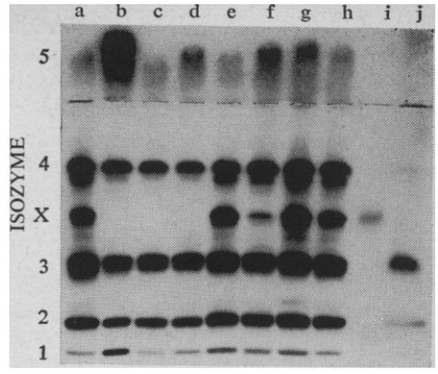

Fig. 1. Starch gel electrophoretic pattern of LDH in homogenates from (*a*) postpubertal testis; (*b*) diaphragm; (*c*) mature ovary; (*d*) prepubertal testis; (*e, f, g, h*) postpubertal testes; (*i*) washed sperm; (*j*) supernatant fraction seminal fluid. Origin is the line just below the isozyme 5 area.

LDH isozymes in human sperm (*4*). The sperm used by Goldberg were disrupted by sonification, whereas we used freezing and thawing. To compare our results, thoroughly washed human sperm was subjected to sonification, and the solutions obtained were fractionated by starch-gel electrophoresis. The predominating LDH was still "band X," and slight activity was present in the LDH 3 and 4 regions. Perhaps the polyacrylamide-gel method is more sensitive than the starch-gel method for detecting small amounts of LDH. A difference in sensitivity of this degree, however, does not alter our original recognition of the fact that the predominant type of LDH in human sperm is the new isozyme which we have designated "band X."

To reveal differences in the enzymatic specificity of the isozymes from testes, each was eluted from the starch and the activity with cofactor analogs was measured (*5*). No consistent difference was seen in the ratio of thionicotinamide adenine dinucleotide to nico-

Table 1. Ratios of reaction rates of NAD analogs to NAD for six types of LDH isozymes obtained from postpubertal testis. The concentration of lactate was $2 \times 10^{-1}M$, and of NAD and the analogs, $9 \times 10^{-4}M$.

Type of isozymes from LDH					
1	2	3	"X"	4	5
Nicotinamide-hypoxanthine dinucleotide/NAD					
1.2	1.2	1.0	1.0	0.9	0.8
Pyridine-3-aldehyde adenine dinucleotide/NAD					
0.5	0.6	0.7	0.6	0.5	0.5
Acetyl pyridine adenine dinucleotide/NAD					
0.02	0.05	0.2	1.2	0.5	0.02
Thionicotinamide adenine dinucleotide/NAD					
1.4	1.6	1.3	1.8	1.3	1.0

tinamide adenine dinucleotide (NAD) or the ratio of pyridine-3-aldehyde adenine dinucleotide to NAD (Table 1). The ratio of nicotinamide hypoxanthine dinucleotide to NAD decreased gradually from isozyme 1 to isozyme 5. Quite striking was the high ratio of acetylpyridine adenine dinucleotide to NAD observed for "band X."

The heat stability of the isozymic fractions was determined by heating eluates from the starch at 40, 50, and 60°C for 15 minutes. The rates of inactivation for "band X" were between those observed for isozymes 3 and 4.

Diethylaminoethyl cellulose column chromatography was performed on homogenates of testes containing "band X." The enzymes were eluted by the addition of increasing concentrations of sodium chloride in 0.01*M* sodium phosphate buffer, *p*H 7.0. Major peaks of enzymatic activity occurred at salt concentrations of 0.001, 0.075, 0.125, and 0.20*M*. The type of LDH in each of these effluents was identified by starch-gel electrophoresis. The material from the 0.001*M* concentrations gave a faint band in the isozyme-4 region, that from the 0.075*M* exhibited faint bands in the "band X" and isozyme-3 area, and the 0.125*M* and 0.20*M* developed single bands in the region of isozymes 2 and 1 respectively. Isozyme-5 activity was never detected in the effluents.

Appella and Markert (*6*) dissociated LDH into four polypeptides which could be separated into two classes on the basis of charge. From this and other evidence these investigators (*7*), and more recently Cahn, Kaplan, Levine, and Zwilling (*8*) have advanced the hypothesis that each LDH isozyme is comprised of four polypeptide subunits which are assembled from two different kinds of polypeptides (A and B) synthesized under the control of two different genes. By this hypothesis, isozymes 1 through 5 would have the following polypeptide structure: AAAA, AAAB, AABB, ABBB, and BBBB. The presence of a sixth LDH isozyme in sperm suggests that one, or possibly two different genes become active in spermatogonia at the time of puberty.

Damaged cells may release their enzymes into the circulation. Usually changes in the serum LDH isozyme pattern may be correlated with the repertory of LDH isozymes in the damaged organ (*9*). Since the new isozyme in postpubertal testis is shared by no other tissue, its presence in serum would indicate testicular pathology.

The functional and metabolic significance of the "band X" material in sperm is unknown. That it might be an index of male fertility is an interesting possibility (*10*).

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References and Notes

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Lactic and Malic Dehydrogenases in Human Spermatozoa

Abstract. *Human spermatozoa, representing a homogeneous population of postmitotic cells, contain five electrophoretically distinct lactic dehydrogenases and two malic dehydrogenases. This indicates that molecular heterogeneity of enzymes is characteristic of the individual cell and is not a reflection of heterogeneity of cell types within a tissue.*

Lactic dehydrogenase (LDH) and malic dehydrogenase (MDH) from various species of animals and within different tissues of the same animal exist in multiple molecular forms (*1*, *2*). The term *isozyme* has been suggested to designate this heterogeneity of molecular types for a single enzyme (*2*). Since these dehydrogenase isozymes appeared in a wide variety of tissues, usually including a multiplicity of cell types, the isozyme complement of a particular tissue might be a reflection of cytologically heterogeneous material, and any particular cell type might possess only a single form of the enzyme.

Spermatozoa, postmitotic cells of known and controllable history and obtainable as a homogeneous population, were selected as the ideal material with which to attempt to resolve this question. Spermatozoa produce considerable quantities of lactic acid under aerobic as well as anaerobic conditions (*3*); this suggests, therefore, that an active LDH is present in high concentration in these cells.

This paper reports the extraction and characterization of LDH and MDH from human spermatozoa with particular reference to the occurrence of multiple molecular forms of these enzymes in these cells.

Ejaculated human spermatozoa were washed free of seminal plasma by centrifugation (2000*g* for 20 minutes) in 0.1*M* sodium phosphate buffer at *p*H 7.4. The cells in suspension were placed in a small beaker in an ice bath and disrupted by sonification for 10 minutes with a Branson model S-75 Sonifier. The suspension of disrupted cells was centrifuged at 10,000*g* for 30 minutes at 4°C, and the supernatant fluid was used for enzyme assay.

The activity of LDH was measured essentially as described by Kornberg (*4*), by following spectrophotometrically the oxidation of $NADH_2$ (reduced nicotine adenine dinucleotide) with pyruvate as substrate. Whenever possible, protein concentrations were estimated from the absorbancy values of appropriate solutions at 260 and 280 mμ (*4*).

Disc electrophoresis in a polyacrylamide gel, as described by Ornstein and Davis (*5*), was used to separate the proteins, liberated from the cells by sonification, which moved toward the anode. With this method about 20 μg of protein was resolved in 45 minutes from a 0.1 ml volume of cell suspension. Eight columns of gel, each conducting approximately 5 ma, were subjected to electrophoresis simultaneously. The columns of gel were removed from the glass supporting cylinders immersed in a dish of cold tap water by rimming with a blunt-edged dissecting needle. The columns were then fixed and stained for protein in naphthol-blue black dissolved in a 5:5:1 solution of water, methanol, and acetic acid. Excess stain was removed from the sample electrophoretically with 7½ percent acetic acid. Unfixed gels were rinsed with cold tris buffer, 0.1*M*, *p*H 8.3, and incubated at 37°C for from ½ to 1 hour in a reaction mixture comparable to that described