

## ASSOCIATION OF THE LACTIC DEHYDROGENASE X<sub>4</sub> ISOZYME WITH MALE-PRODUCING RABBIT SPERMATOZOA

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Lactic dehydrogenase (LDH), the enzyme which catalyses the final step in anaerobic glycolysis, exists in numerous molecular forms whose molecular nature and physiological significance are still the subject of controversy. Using paper electrophoresis or other fractionation methods with a limited capacity for resolving proteins, a maximum of five isozymes of LDH are found in most tissues (Appella & Markert, 1961; Cahn, Kaplan, Levine & Zwilling, 1962; Fritz & Jacobson, 1965; Vesell, 1961). These isozymes have been considered to be tetramers formed from two kinds of subunits; one called the H subunit, since this type predominates in heart tissue, and the other called the M subunit, since this type predominates in striated muscle (Cahn *et al.*, 1962). Sorting these two subunits into all possible groups of four yields five different molecular forms of LDH: the M<sub>4</sub>, M<sub>3</sub>H, M<sub>2</sub>H<sub>2</sub>, MH<sub>3</sub>, and H<sub>4</sub> isozymes. However, the identification of a new isozyme in spermatozoa and testes, called the X<sub>4</sub> isozyme, and the resolution of the other five isozymes into numerous sub-bands by more refined electrophoretic techniques indicate that the molecular nature of LDH is considerably more complex than was previously realized (Stambaugh & Buckley, 1967; Blanco & Zinkham, 1963; Zinkham, Blanco & Kupchyk, 1963; Wilkinson & Withycombe, 1965).

The presence of large quantities of the X<sub>4</sub> isozyme in spermatozoa presents something of a paradox, since the X subunit possesses essentially the same enzymatic properties as the H subunit (Stambaugh & Buckley, 1967). Recent evidence indicates that each subunit may consist of two, instead of one polypeptide chain (Schatz & Segal, 1969; Millar, Frattali & Willick, 1969; Appella & Zito, 1968). In 1967, we presented evidence that the X<sub>4</sub> isozyme and other LDH sub-bands are formed from hybrid subunits consisting of the polypeptides normally found in the H and M subunits (Stambaugh & Buckley, 1967). According to this hypothesis, the H subunit consists of two polypeptide chains; the h polypeptide, which imparts heart-type enzymatic properties to the subunit, and an A or anionic polypeptide, which has little effect on the enzymatic properties. The M subunit consists of the m polypeptide, which imparts the muscle type of enzymatic properties, and a C or cationic polypeptide, which also has little effect on the enzymatic properties. The X subunit, then, is a

hybrid formed from these same h and C polypeptides found in the M and H subunits.

A survey of various rabbit tissues revealed that the X<sub>4</sub> isozyme also occurs in tissues, such as kidney and heart, which are characterized by their deficiency of the m kinetic type (Stambaugh & Buckley, 1967). This distribution suggested to us that large quantities of the X subunit might only be formed in cells completely devoid of the m polypeptide. The intriguing part of this relationship is that large quantities of the X subunit occur only in the spermatozoa, which are haploid, or in mature testes (Goldberg & Hawtrey, 1967), which contain haploid spermatids and secondary spermatocytes. This raised the question whether the gene for synthesis or the control of synthesis of the m polypeptide might be sex-linked. Since the female lacks a Y chromosome and still makes the m polypeptide, the only alternative is that the gene locus for the m polypeptide might be located on the X chromosome. That is, the X<sub>4</sub> isozyme might be characteristic of male-producing spermatozoa, due to RNA synthesis during the early haploid stages of spermatogenesis, or to segregation of

TABLE 1  
LDH KINETIC ASSAY OF RABBIT, RHESUS MONKEY,  
AND HUMAN SPERM LAYERS

Fraction	Ratio h/m		
	Rabbit	Rhesus monkey	Human
Supernatant	1.05	1.48	1.15
No. 1	0.549	1.39	0.907
No. 2	0.397	0.747	0.233
No. 3	0.283	0.725	0.103
No. 4 (bottom layer)	0.259	0.208	0.065

messenger RNA from the sex chromosomes in the final diploid stages. This is the hypothesis which we attempted to test by fractionating rabbit spermatozoa on discontinuous dextran density gradients (4→24%). After centrifugation at 100 g, fractions of equal volume were removed sequentially from the top of the tube. Approximately 0.3% of the total number of spermatozoa were contained in the supernatant fraction, which was the fraction used for intrauterine insemination. Fifty per cent of the spermatozoa in the supernatant fraction were washed in isotonic saline and used for an immediate kinetic assay of LDH (Stambaugh & Post, 1966). Only those supernatants with spermatozoa containing a definite predominance of the h kinetic type were used for insemination (i.e. h/m > 1.0). The other fractions were also washed and their LDH content analysed. The results of the kinetic assays (Table 1) demonstrated that the supernatant layer of spermatozoa contained predominantly the h kinetic type, which represents primarily the hC or X subunit. Below this layer, there was a gradual progression to the m kinetic type, with the bottom fraction containing about 75% m and 25% h. Similar LDH distributions were obtained for rhesus monkey and human spermatozoa. The electrophoretic patterns in

polyacrylamide gels confirmed the progression of LDH type from the predominantly hC<sub>4</sub> or X<sub>4</sub> isozyme in the supernatant layer to the predominantly mC<sub>4</sub> isozyme in the bottom fractions of the gradients. Intrauterine inseminations were performed on adult New Zealand White female rabbits under anaesthesia by laparotomy and injection of the remaining half of the supernatant fraction into the two uterine horns at 0 time. A control group, using unfractionated washed spermatozoa, were also run simultaneously. Human chorionic gonadotrophin was used to induce ovulation, and the rabbits were killed 27 days later. The sex of the foetuses was determined both anatomically and histologically.

TABLE 2  
INTRAUTERINE INSEMINATION WITH FRACTIONATED AND UNFRACTIONATED EJACULATED RABBIT SPERMATOZOA

	Males	Females	Ratio M/F
Unfractionated spermatozoa	48	46	1.04
Supernatant spermatozoa from density gradient centrifugations	68	32	2.12

The results (Table 2) demonstrated an altered sex ratio of the foetuses obtained using fractionated spermatozoa from the supernatant layer, as compared with the normal ratio using unfractionated spermatozoa. These results support the hypothesis that LDH may be an octamer and the X subunit a hybrid one, involving the same genes and polypeptides as the H and M subunits. If the association of the X subunit with male-producing spermatozoa proves to be valid and not coincidental, the exciting possibility exists that the X<sub>4</sub> isozyme can be used as an assay for determining the percentage of male- and female-producing spermatozoa in fractionated semen specimens.

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