THE EFFECTS OF CRYPTORCHISM IN THE GUINEA PIG ON THE ISOENZYMES OF TESTICULAR LACTATE DEHYDROGENASE

By A. W. Blackshaw* and J. I. Samisoni*

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Summary

Unilateral cryptorchism was produced in guinea pigs by returning one testis to the abdominal cavity and by preventing its re-entry into the inguinal canal and scrotal sac. The retained testes were examined after 1–2, 3–4, or 5–7 weeks and estimates made of lactate dehydrogenase activity.

Disk electrophoresis in 7·5% acrylamide gel was used to separate the isoenzymes of lactate dehydrogenase in the scrotal and cryptorchid testes. Scrotal testis contained two isoenzymes not found in heart or muscle. Cryptorchism produced a rapid decrease in the amounts of these isoenzymes.

The activities of lactate dehydrogenase subunits were calculated. Heart-type subunit activity did not change significantly during cryptorchism but muscle-type subunit activity rose sharply within 3–4 weeks, falling slightly at 5–7 weeks. Testis-type subunit activity fell rapidly and at 5–7 weeks had disappeared. A rise in lactate dehydrogenase activity occurred in the scrotal testis during the experimental period.

I. Introduction

The normal scrotal testis is maintained at a lower temperature than that of the abdominal cavity. An increase in testicular temperature, produced by scrotal insulation, fever, or the return of the testes to the abdominal cavity leads to a rapid degeneration of the seminiferous tubules and a failure of sperm production and maturation (Moore and Oslund 1924; Gunn, Sanders, and Granger 1942; Nelson 1951).

Retention of the testes in the abdomen (cryptorchism) gives a convenient method for studying the effects of raised temperatures on testicular function. Most observations have been confined to histological studies of spermatogenesis; biochemical or histochemical investigations of cryptorchism are relatively few. Tepperman, Tepperman, and Dick (1949) found little difference in glycolytic activity between the scrotal and cryptorchid testis, but Blackshaw and Samisoni (1966) showed that in the mature, natural, cryptorchid ram anaerobic glycolysis was greater in the cryptorchid testis.

Histochemical studies by Kormano, Harkonen, and Kontinen (1964) have indicated that considerable changes occur in the distribution and activities of a number of enzymes, including lactate dehydrogenase, in the cryptorchid rat testis.

* Department of Physiology, University of Queensland, St. Lucia, Brisbane.

Lactate dehydrogenase has been shown to exist in various molecular forms or isoenzymes (Appella and Markert 1961; Cahn *et al.* 1962). In most tissues, five separate forms can be distinguished but Blanco and Zinkham (1963) and Goldberg (1963) have demonstrated the presence of additional isoenzymes in the adult testis. These isoenzymes are believed to play a significant part in spermatogenesis and sperm function (Zinkham, Blanco, and Clowry 1964).

This paper is concerned with changes in the isoenzymes of testicular lactate dehydrogenase after cryptorchism in the guinea pig.

II. Materials and Methods

Mature guinea pigs weighing between 500 and 800 g were used. The animals were given free access to water and a diet of commercial pellets. Throughout the experimental periods the environmental temperature was kept at 23±2°C. The animals were anaesthetized with Evipan (40 mg/kg); the scrotal sac was opened on one side and the testis displaced into the abdominal cavity. The inguinal canal was closed by a single stainless steel suture. Animals were killed at weekly intervals from 1 to 7 weeks after unilateral cryptorchism.

The testes were dissected free from the fat and epididymis, weighed, and homogenized in 0·15M NaCl at 4°C. The homogenate (10%) was centrifuged at 20,000 *g* for 30 min and the clear supernatant used for enzyme assays and also for electrophoresis. Protein was determined by the biuret method (Layne 1957). Lactate dehydrogenase activity was measured by the method of Wroblewski and La Due (1955) in which the rate of oxidation of reduced nicotinamide adenine dinucleotide ($NADH_2$) was measured at 340 mμ with pyruvate as substrate.

The isoenzymes of lactate dehydrogenase were separated by disk electrophoresis in acrylamide gel supported in 100 by 5 mm glass tubes. The method was modified from that described by Ornstein and Davis (1962). The acrylamide used was AM-9 Chemical Grout (American Cyanamid Company) containing 95% acrylamide and 5% of the cross-linking co-monomer *N,N'*-methylenebisacrylamide.

A 7½% (w/v) small-pore (separation) gel was used and the enzyme sample was incorporated into a large-pore gel (3% w/v acrylamide). The gels were prepared as follows:

Stock Solutions	Volumes (ml)	
	7½% Gel	3% Gel
30% Acrylamide	25·0	10·0
3·0M Tris.HCl (pH 8·9)	12·5	—
0·47M Tris phosphate (pH 6·9)	—	12·5
Water	11·5	54·5
Tissue extract	—	10·0
β-Dimethylaminopropionitrile	1·0	0·5
Ammonium persulphate (0·14% w/v)	50·0	12·5

The reagents were mixed in the order given and polymerization took place within 10–15 min. Electrophoresis was conducted at 4°C with a constant current of 2·5 mA per gel. Higher temperatures and currents were found to inactivate the muscle

type isoenzymes. The Tris–glycine buffer of Ornstein and Davis (1962) was used, and the progress of electrophoresis was followed by adding bromophenol blue to the cathodic buffer, the dye migrating ahead of the fastest lactate dehydrogenase isoenzyme. Electrophoresis was continued until the dye band had run about 60–70 mm.

Lactate dehydrogenase activity was localized by staining with a medium containing nitroblue tetrazolium as dye, *N*-methylphenazonium methosulphate as electron acceptor, NAD as coenzyme, and sodium lactate as substrate (Helm *et al.* 1962). The relative mobility of each isoenzyme was estimated by comparison with the bromophenol blue marker band and expressed as a percentage. The relative proportions of the isoenzymes were estimated by scanning the stained gels in an integrating densitometer.

TABLE 1

EFFECT OF CRYPTORCHISM ON TESTIS WEIGHT IN THE GUINEA PIG

Experimental Period (weeks)	No. of Animals	Testis Weight (mg/100 g body wt.)	
		Scrotal	Cryptorchid
1–2	6	289	214
3–4	6	257	93
5–7	8	262	44

Summary Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Square	Variance Ratio
Periods	2	35,512	30·78**
Linear	1	69,736	60·44**
Quadratic	1	2,289	1·98
Within periods (error)	17	77,023	

** $P<0·01$.

Insufficient numbers of animals survived in some of the groups, and for the purpose of statistical analysis survivors were grouped into three treatment periods, covering weeks 1 and 2, weeks 3 and 4, and weeks 5, 6, and 7, respectively.

Differences in testis weights and lactate dehydrogenase activities between the scrotal and cryptorchid testes were compared by an analysis of variance. Mean values for the relative mobility and proportion of each isoenzyme were calculated for each period. The mobility estimates for all the cryptorchid testes were pooled for comparison with those from all the scrotal testes and with muscle prepared from an homogenate of guinea pig diaphragm.

III. Results

The mean weights of the scrotal and cryptorchid testes are given in Table 1. A summary of the analysis of variance of the difference in weight between the two types of testis is also given in Table 1. This difference was used as the variate and the analysis shows that there was a highly significant linear response to cryptorchism.

Table 2

RELATIVE MOBILITIES OF THE LACTATE DEHYDROGENASE ISOENZYMES OF GUINEA PIG TESTIS AND DIAPHRAGM

Values given are the ratio of the migration distance of the isoenzyme to that of the bromophenol blue marker band expressed as a percentage

Isoenzyme	Scrotal Testis	Cryptorchid Testis	Diaphragm
1	38·2	38·2	37·7
2	31·3	30·9	31·2
3	23·6	23·0	23·5
X_1	20·5	19·9	—
4	15·1	14·7	15·4
X_2	12·2	9·8	—
5	—	3·7	3·8

Following electrophoresis of the testis extracts and the histochemical detection of lactate dehydrogenase activity in the acrylamide gel, the relative mobilities of the isoenzymes of normal and cryptorchid testes were compared with those of guinea pig diaphragm which is known to possess five isoenzymes of lactate dehydrogenase. These are called LD 1 to LD 5, in that order, starting from the anode.

Table 3

EFFECT OF INCREASING PERIODS OF CRYPTORCHISM ON THE PROPORTIONS OF LACTATE DEHYDROGENASE ISOENZYMES IN THE GUINEA PIG TESTIS

Values are given as percentages

Period (weeks)	Testis	Isoenzyme Bands						
		LD 1	LD 2	LD 3	LD X_1	LD 4	LD X_2	LD 5
1–2	Scrotal	43	23	12	15	5	2	0
	Cryptorchid	42	22	14	12	5	0	3
3–4	Scrotal	45	24	14	10	4	3	0
	Cryptorchid	28	26	22	3	14	0	7
5–7	Scrotal	33	24	15	13	4	11	0
	Cryptorchid	28	29	28	0	14	0	0

The calculated relative mobilities for each isoenzyme were based on variable numbers of observations. The mean values showed close correspondence between similar bands in different tissues with no overlap of the 99% confidence limits between adjacent bands in a single tissue (Table 2). In only one case did the 99%

confidence limits exceed $\pm$ 1 unit and this was in the estimates of LD X_2 in the early cryptorchid testis where the limits were $\pm 2 \cdot 3$ units. The scrotal testis contained isoenzymes not seen in the diaphragm; these were designated LD X_1 (lying between LD 3 and LD 4) and LD X_2 (lying between LD 4 and LD 5). LD 4 and LD X_2 were not always present in the normal testis and LD X_2 always disappeared after the normal testis had been retained in the abdomen for a short period.

TABLE 4

EFFECT OF CRYPTORCHISM ON THE ACTIVITY OF LACTATE DEHYDROGENASE IN THE GUINEA PIG TESTIS

Percentage of activity is given in parentheses

Period (weeks)	Subunits	Lactate Dehydrogenase Activity per 1 mg Protein			
		Scrotal Testis		Cryptorchid Testis	
1–2	Muscle-type	16·9	(17)	17·9	(15)
	Heart-type	65·3	(65)	81·5	(70)
	Testis-type	17·5	(18)	17·2	(15)
Total		99·7	(100)	116·6	(100)
3–4	Muscle-type	19·6	(16)	52·1	(35)
	Heart-type	83·4	(70)	90·2	(62)
	Testis-type	16·6	(14)	4·1	(3)
Total		119·6	(100)	146·4	(100)
5–7	Muscle-type	27·2	(16)	44·3	(30)
	Heart-type	100·6	(61)	102·4	(70)
	Testis-type	37·7	(23)	0·0	(0)
Total		165·5	(100)	146·7	(100)

Summary Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios			
		Total Activity	Muscle-type Subunits	Heart-type Subunits	Testis-type Subunits
Periods	2	0·38	6·06**	0·97	12·27**
Linear	1	0·12	2·80	1·90	23·62**
Quadratic	1	0·65	9·31**	0·04	0·92
Within periods (error mean square)	17	631	246	362	210

** $P < 0 \cdot 01$.

Stained gels were evaluated by an integrating densitometer and the relative proportions of each band calculated for the three experimental periods. The mean values for the scrotal and cryptorchid testes are given in Table 3. For the calculation

of confidence limits (99%) the percentages were converted to angles; in no case did the limits exceed $\pm 2\%$. The proportion of LD X_2 in the scrotal testes increased during the third experimental period. Cryptorchid testes, however, showed an early loss of LD X_2 (1–2 weeks) and in 3–4 weeks only a small amount of LD X_1 remained, disappearing completely during the third experimental period (5–7 weeks).

The overall activity of lactate dehydrogenase was estimated by the rate of oxidation of $NADH_2$ (Table 4). Assuming a tetrameric structure for each isoenzyme, the activities of the muscle-, heart-, and testis-type subunits were also calculated (Table 4). The difference between the scrotal (control) and cryptorchid testes was used as the variate for testing the effect of cryptorchism over the three periods. The analyses of variance are also given in Table 4 for the total and subunit activities of lactate dehydrogenase. There were no significant changes in heart-type subunit activity at any period of cryptorchism. However, there was an increase in the activity of the muscle-type subunits in the cryptorchid testes. The response was curved, falling away in the third experimental period. The activity of the testis-type subunits fell rapidly and was absent in the third experimental period.

Scrotal testes also showed increases in enzyme activity over the three experimental periods. There were significant linear effects for all the subunit types, the variance ratios and probability levels for the muscle-, heart-, and testis-type subunits being 5·68 ($P<0{\cdot}05$), 12·65 ($P<0{\cdot}01$), and 9·5 ($P<0{\cdot}01$) respectively (degrees of freedom = 1 and 17).

IV. Discussion

The cryptorchid guinea pig testis lost weight rapidly after only a short period in the abdomen. This gross evidence of atrophy was paralleled by histological evidence of tubular degeneration and shrinkage (Moore and Oslund 1924; Moore 1951). Proliferation of Leydig cells was visible at 21 days and marked by 56 days (Samisoni, unpublished data). Histochemical studies of normal guinea pig testes have shown strong lactate dehydrogenase activity in the tubules and interstitial cells. Cryptorchism has produced increased interstitial activity but has had little effect on the tubular enzyme (Samisoni, unpublished data).

There was a gradual rise in the overall lactate dehydrogenase activity of the scrotal testis during the experimental period and the relative activities of cryptorchid and scrotal testes were eventually reversed. Clegg (1965*a*, 1965*b*) has demonstrated a compensatory response of the scrotal rat testis in unilateral cryptorchism and some similar effect may be present in the guinea pig.

The observations of Blanco and Zinkham (1963), Zinkham, Blanco, and Kupchyk (1964), and Zinkham, Blanco, and Clowry (1964) showed that in a variety of animal species there occurred one or more isoenzymes of lactate dehydrogenase which were unique to the testis ("band X"). Detailed studies by Blanco, Zinkham, and Kupchyk (1964) on pigeon testes suggested that the band X isoenzymes were under the control of a genetic locus *C* distinct from the loci *A* and *B* which control the muscle- and heart-type isoenzymes.

Zinkham, Blanco, and Clowry (1964), found two band X (LD X) isoenzymes in homogenates of guinea pig testis and also noted the occasional absence of LD 4. We have observed this as well as the absence of the second LD X isoenzyme from some apparently normal testes. The same workers have further shown that aspermatogenesis, induced in the guinea pig by injecting homogenates of homologous testes combined with Freund's adjuvant, leads to a loss of band X without an alteration in the activity of the other isoenzymes.

Cryptorchism also produced a loss of LD X material in the guinea pig testis. Complete disappearance took 3–4 weeks and during this period the testis decreased to less than 40% of the scrotal testis weight and showed the marked histological and histochemical changes discussed earlier. Cryptorchism did not appear to affect the activity of heart-type subunits but there was a rapid increase and then a slight fall in the activity of muscle-type subunits as the testis-type subunit activity fell to zero. In the cryptorchid testes the ratios of heart-type to (muscle+testis)-type subunits were relatively constant being 2·32 (1–2 weeks), 1·65 (3–4 weeks), and 2·02 (5–7 weeks).

Goldberg (1964) and Wilkinson and Withycombe (1965) have examined some of the properties of band X of human spermatozoa, which in its sensitivity to pyruvate inhibition resembles LD 1 or heart isoenzyme. Blackshaw (unpublished data) has shown that the lactate dehydrogenase of bull spermatozoa is closer to the muscle-type enzyme (LD 5) in pyruvate sensitivity but its heat lability is similar to that of the heart-type enzyme. The enzyme from guinea pig spermatozoa has a relatively low K_m but is not very sensitive to fairly high pyruvate levels. This ability to operate in the presence of high levels of pyruvate is an advantage to spermatozoa but its importance in spermatogenic function is unknown. However, the findings of Blackshaw and Samisoni (1966) on the cryptorchid ram testis and of Ewing, Green, and Stabler (1965) on the cotton rat testis during the non-breeding season indicate a preference towards glycolysis by the inactive testis which may be related to an increase in the relative amounts of muscle-type lactate dehydrogenase in the degenerate testis.

Ressler, Olivero, and Joseph (1965) have suggested that LD X may not be genetically distinct from the normal lactate dehydrogenase isoenzymes. These may be complexed with some substance produced during or associated with spermatogenesis, thus altering their migratory and perhaps chemical properties.

Although there appears to be a close association between muscle-type and testis-type subunits in the cryptorchid guinea pig testis, this association may be fortuitous, as histologically there is a loss of the cells which would be expected to contain testis-type subunits.

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