THE REDUCING ACTIVITY OF RAM AND BULL SPERMATOZOA

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Summary

Succinic and lactic dehydrogenase activities of ram and bull spermatozoa were measured by the reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride. The K_m values obtained were of the same order as those for other tissues.

The succinic dehydrogenase had an optimum pH of between 8·5 and 9·0 while the optimum pH of lactic dehydrogenase was between 7·5 and 8·0. Phosphate slightly inhibited the succinic dehydrogenase but the effect on lactic dehydrogenase is more complex. Glucose 6-phosphate dehydrogenase activity was obtained in washed ram and bull spermatozoa.

I. Introduction

The activities of some enzymes associated with spermatozoa have been investigated by Smith, Mayer, and Merilan (1957*a*, 1957*b*) who measured the effects of washing, and the season of the year, on the activity of succinic and malic dehydrogenases. Mohri (1957) also examined the dehydrogenases of sea urchin spermatozoa, and recently King and Mann (1959) described some of the properties of sorbitol dehydrogenase prepared from bull spermatozoa.

The location of succinic dehydrogenase in the midpiece and tail of spermatozoa has been shown cytochemically by Nachlas *et al.* (1957) and Nelson (1959). Adenosine triphosphatase activity has also been located in this region (Nelson 1958).

The activity of succinic dehydrogenase was determined by Kun and Aboud (1949) who used the reduction of 2,3,5-triphenyl tetrazolium chloride to measure enzyme activity. Subsequently Seligman and Rutenberg (1951) used tetrazolium salts for the histochemical localization of specific enzyme systems. For the optimum development of colour, some tetrazolium salts require anaerobic conditions, but Pearson and Defendi (1954) introduced 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) which was able to compete with molecular oxygen as an electron acceptor.

Although the metabolic activities of spermatozoa have been extensively studied, investigations of enzyme systems are few in number. In this paper, estimates of the activities of some dehydrogenases of ram and bull spermatozoa have been made, the reduction of INT being used as an indicator of their activity.

II. Materials and Methods

Ram semen was collected by electrical stimulation of ejaculation (Blackshaw 1954) and bull semen with an artificial vagina.

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In most experiments washed cells were used, but in some tests an extract was made by grinding the spermatozoa with aluminium oxide.

The fresh semen was washed by diluting it five times in either isotonic sodium chloride solution or phosphate–chloride buffer. The suspension was centrifuged for 10 min at 2500 r.p.m. and the supernatant removed. Resuspension in fresh diluent and centrifugation were repeated a further four times and the spermatozoal suspension made up to its original volume. The washed spermatozoa were then incubated at 37°C for 1 hr to remove as much endogenous substrate as possible.

The basic media used in most of the tests were 0·15M NaCl and an isotonic solution containing 36 volumes of 0·13M $Na_2HPO_4.12H_2O$, 14 volumes of 0·17M $NaH_2PO_4.2H_2O$, and 50 volumes of 0·15M NaCl. In all experiments the buffer level was kept constant, and any additions were made at the expense of NaCl. In most cases, additions were made by adding appropriate volumes of 0·15M sodium succinate or lactate to the medium.

INT was prepared as a 0·2% (w/v) solution in 0·15M NaCl and added to the incubation medium to give a concentration of 0·02% (w/v). The media were placed in tubes in a water-bath at 37°C, and allowed to equilibrate at that temperature for 10 min. The washed spermatozoal suspension (0·1 ml) was then added to give a total volume of 1·0 ml. After an incubation period of 20 min (ram) or 30 min (bull) the coloured formazan was extracted with acetone and the optical density measured at 490 mμ.

In some experiments, extracts of spermatozoa were prepared from cells washed twice in 0·15M NaCl. These were ground with washed aluminium oxide and the extract made up to the original semen volume. Some extracts were tested for reducing activity with lactate as a substrate, and others with glucose 6-phosphate as substrate. Under these conditions the reducing activity was much lower than when using whole spermatozoa, and to get good colour production the incubation period was extended to 1 hr.

The effects of pH on the activities of succinic and lactic dehydrogenases were studied. In these tests it was necessary to replace the usual phosphate buffer with one prepared from mixtures of 0·17M $NaH_2PO_4.2H_2O$; 0·10M sodium borate and 0·10M NaOH in order to maintain a high pH. Where complete replacement of phosphate was required 0·10M Tris–HCl at pH 7·2 was found satisfactory.

When whole spermatozoa were used to determine lactic dehydrogenase activity, the cofactor diphosphopyridine nucleotide (DPN) was not necessary; when cell extracts were used, reduction of INT only occurred in the presence of DPN (0·1 mg/ml).

Measurements of the reduction of INT by washed spermatozoa and cell extracts were made with glucose 6-phosphate as the substrate (0·028M), and added triphosphopyridine nucleotide (TPN), at a level of 0·1 mg/ml, as cofactor. In order to block glycolysis, 0·02M NaF and 0·0002M sodium iodoacetate were added to the incubation medium, which, in both series, was 0·15M NaCl.

The results of all experiments were recorded in optical density units and an analysis of variance was made to assess their significance. In the tables of summary analyses of variance, the residual error mean square is given at the foot of the appropriate variance ratio column.

III. Results

Preliminary tests showed that INT was readily and rapidly reduced by washed spermatozoa in the presence of several substrates. An incubation period of either

Table 1

K_m values for succinic and lactic dehydrogenases of ram and bull spermatozoa

Species	No. of Replications	Preparation	Substrate	$K_m \times 10^{-4}$ (M)
Ram	4	Whole sperm	Succinate	4·05±0·44*
	8	Whole sperm	Lactate	2·62±0·48
	8	Sperm extract	Lactate	5·16±0·49
Bull	6	Whole sperm	Succinate	8·05±3·10
	6	Whole sperm	Lactate	3·33±1·38
	5	Sperm extract	Lactate	11·16±4·64

* Standard error.

20 min (ram) or 30 min (bull) was found adequate for good colour development when whole cells were used, but a period of 1 hr was necessary when cell extracts were tested.

The effects of different levels of succinate and lactate on the rate of reduction of INT by ram and bull spermatozoa were measured. In both cases seven substrate

Table 2

Effect of pH on the reduction of INT by bull and ram spermatozoa with succinate and lactate as substrates

Mean optical densities are shown and the number of replications are given in brackets

Nominal pH	Succinate				Lactate			
	Ram Spermatozoa (6)		Bull Spermatozoa (9)		Ram Spermatozoa (6)		Bull Spermatozoa (7)	
	Final pH	Optical Density	Final pH	Optical Density	Final pH	Optical Density	Final pH	Optical Density
7·0	6·97	0·192	6·79	0·426	6·93	0·182	6·77	0·482
8·0	8·12	0·266	7·79	0·598	7·98	0·207	7·77	0·620
9·0	8·90	0·291	8·69	0·671	8·77	0·158	8·75	0·533
10·0	9·52	0·181	9·27	0·298	9·19	0·104	9·31	0·196
11·0	10·12	0·102	9·81	0·185	9·84	0·066	9·89	0·160

levels ranging from 0 to 0·02M were used. The method of Lineweaver and Burk (1934) was employed to estimate K_m values which are given in Table 1 together with their standard errors.

The metabolic rate and motility of spermatozoa is influenced by the pH of the suspending medium; therefore measurements were made of the effects of pH on the succinic and lactic dehydrogenase activities of ram and bull spermatozoa. A

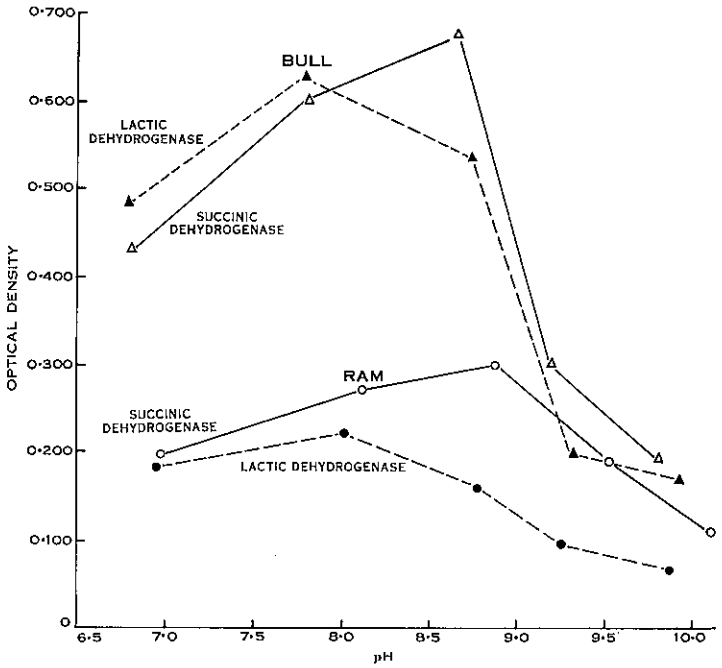

Fig. 1.—Effect of pH on the activities of succinic and lactic dehydrogenases of ram and bull spermatozoa.

pH range of 7·0–11·0 (nominal) was used, and at the end of the incubation period the actual pH was measured with a glass-electrode.

TABLE 3

EFFECT OF PHOSPHATE ON THE ACTIVITY OF SUCCINIC DEHYDROGENASE AS MEASURED BY THE REDUCTION OF INT

Mean optical densities for 9 ram and 6 bull ejaculates given

Phosphate Concn. (M)	Ram Spermatozoa	Bull Spermatozoa
0	0·219	0·383
0·014	0·215	0·359
0·028	0·199	0·333
0·056	0·184	0·326

The mean final pH and optical densities are shown in Table 2 and in Figure 1. Both ram and bull spermatozoa showed similar optimum pH levels for each enzyme,

the greatest activity for succinic dehydrogenase was found at pH 8·90 (ram) and 8·69 (bull) while for lactic dehydrogenase the optimum pH levels were 7·98 and 7·77 respectively.

The effects of inorganic phosphate on the activity of succinic and lactic dehydrogenases were examined in spermatozoa washed five times in 0·15M NaCl. The activity of succinic dehydrogenase (Table 3) was only very slightly reduced by high

TABLE 4

EFFECT OF PHOSPHATE ON THE REDUCTION OF INT WITH 0·015M LACTATE AS SUBSTRATE

Mean optical densities for 6 ram and 6 bull ejaculates given

Phosphate Concn. (M)	Ram Spermatozoa		Bull Spermatozoa	
	No Lactate	0·015M Lactate	No Lactate	0·015M Lactate
0	0·252	0·732	0·177	0·545
0·014	0·260	0·902	0·211	0·644
0·028	0·226	0·786	0·163	0·567
0·056	0·292	0·851	0·198	0·597

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio	
		Ram	Bull
Ejaculate differences	5	53·8**	115·7**
Effect of lactate	1	1441·3**	1032·5**
Effect of phosphate			
No phosphate *v.* phosphate	1	12·8**	6·1*
Linear phosphate effect	1	0·2	3·0
Quadratic phosphate effect	1	14·9**	9·6**
Interactions			
Ejaculate × lactate	5	32·1**	17·1**
Ejaculate × phosphate	15	1·4	1·6
Lactate × phosphate	3	5·1*	1·1
Residual (error)	15	0·0026	0·0019

* $P < 0·05$. ** $P < 0·01$.

levels of phosphate (0·056M) in both ram and bull spermatozoa ($F_{(1,24)} = 6·8$ and $F_{(1,15)} = 4·9$; $P < 0·05$ for the linear component in each species). However, the effect of increasing the level of phosphate on lactic dehydrogenase showed a highly significant quadratic component in both species (Table 4), indicating two phases in the response.

The possible occurrence of glucose 6-phosphate dehydrogenase was also tested in two series of experiments. In the first tests, cells washed in 0·15M NaCl were used,

and in order to inhibit glycolysis, 0·02M NaF was added to the incubation medium. Sodium iodoacetate (0·0002M) was also added to half the tubes to inhibit glyceraldehyde 3-phosphate dehydrogenase. As required 0·028M glucose 6-phosphate was also included.

The results (Table 5) show a considerable reduction of INT by spermatozoa in the presence of glucose 6-phosphate. The addition of TPN did not significantly

TABLE 5

EFFECT OF GLUCOSE 6-PHOSPHATE ON THE REDUCTION OF INT BY RAM AND BULL SPERMATOZOA

Mean optical densities of 7 ram and 7 bull ejaculates given

Iodoacetate Concn. (M)	Triphosphopyridine Nucleotide Concn. (mg/ml)	Ram Spermatozoa		Bull Spermatozoa	
		No Glucose 6-Phosphate	0·028M Glucose 6-Phosphate	No Glucose 6-Phosphate	0·028M Glucose 6-Phosphate
0	0	0·059	0·279	0·083	0·251
0	0·1	0·093	0·325	0·052	0·241
0·0002	0	0·043	0·235	0·067	0·224
0·0002	0·1	0·064	0·229	0·058	0·266

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios	
		Ram	Bull
Ejaculates	6	37·2**	3·8*
Glucose 6-phosphate	1	134·4**	104·4**
Iodoacetate	1	6·9*	0·2
Triphosphopyridine nucleotide	1	1·9	0·0
Interactions			
All ejaculate interactions	18	9·2**	1·8
All treatment interactions	4	1·4	1·0
Residual	24	0·0043	0·0035

* $P < 0·05$. ** $P < 0·01$.

influence the reduction of INT and only the activity of ram spermatozoa was significantly lessened by iodoacetate indicating that some reducing activity may have been due to glyceraldehyde 3-phosphate dehydrogenase.

However, when extracts of spermatozoa were used (Table 6) significant reduction of INT by ram semen extracts only took place in the presence of both glucose 6-phosphate and TPN. When bull semen extracts were tested, both TPN and glucose 6-phosphate significantly increased reduction of INT.

IV. Discussion

The rates of reaction of succinic dehydrogenase obtained in the present experiments fall into the same range as those reported for other tissues. Singer, Kearney, and Massey (1957) found a K_m value for heart muscle succinic dehydrogenase of $1{\cdot}3\times10^{-3}$M while Wang, Tsou, and Wang (1956) reported a value of $5{\cdot}8\times10^{-4}$M at 38°C. Working at a lower temperature (23°C) Keilin and King (1960) obtained a

Table 6

Effects of glucose 6-phosphate and triphosphopyridine nucleotide on the reduction of INT by extracts of ram and bull spermatozoa

Mean optical densities given for 7 ram and 6 bull ejaculates

Triphosphopyridine Nucleotide Concn. (mg/ml)	Ram Spermatozoa		Bull Spermatozoa	
	No Glucose 6-Phosphate	0·028M Glucose 6-Phosphate	No Glucose 6-Phosphate	0·028M Glucose 6-Phosphate
0	0·035	0·039	0·051	0·072
0·1	0·031	0·119	0·061	0·106

Analysis of Variance

Source of Variation	Ram		Bull	
	Degrees of Freedom	Variance Ratio	Degrees of Freedom	Variance Ratio
Ejaculates	6	7·2*	5	50·7**
Effect of glucose 6-phosphate	1	23·8**	1	35·2**
Effect of triphosphopyridine nucleotide	1	15·7**	1	15·9**
Interactions				
Ejaculate interactions	12	2·7	10	5·0
Glucose 6-phosphate × triphosphopyridine nucleotide	1	21·0**	1	4·7
Residual	6	0·0006	5	0·0002

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$.

K_m of $1{\cdot}2\times10^{-3}$M for heart muscle preparation. The methods of assay employed have differed widely: Wang, Tsou, and Wang (1956) and Keilin and King (1960) used variations of the ferricyanide assay while Singer, Kearn, and Massey (1957) developed the phenazine methosulphate method. In this study the reduction of INT has proceeded successfully without the use of phenazine methosulphate as an electron acceptor although Nachlas, Margulies, and Seligman (1960) found that its use increased the sensitivity of their technique for measuring succinic dehydrogenase.

The K_m values for lactic dehydrogenase are higher for the spermatozoal extracts than for whole cells. This is possibly due to damage of the enzyme during extraction with a resulting lower affinity for the substrate. Comparisons of K_m values for spermatozoa with those of other tissues show considerable variations. Beck (1958) records values in the range of $2 \cdot 5$–$4 \cdot 9 \times 10^{-5}$M for leucocytes while Boxer and Shonk (1960) give a value of $5 \cdot 3 \times 10^{-4}$M for tumour lactic dehydrogenase. It is evident that K_m values vary with enzymes from different sources and with different substrates.

Succinic dehydrogenase activity was found to be greatest between pH 8 and 9. This is considerably higher than that of 7·6 recorded by Singer, Kearney, and Massey (1957) and 7·8 recorded by Wang, Tsou, and Wang (1956).

Nachlas, Margulies, and Seligman (1960) measured the succinic dehydrogenase activity of rat liver homogenate by INT reduction and showed that the optimum pH varied with the buffer system. In buffers containing phosphate (0·03M) the optimum pH was about 7·7 but in Tris buffer it was 8·3. However, phosphate was included in the pH tests of spermatozoal succinic dehydrogenase.

The optimum pH for lactic dehydrogenase activity of spermatozoa was lower than for succinic dehydrogenase and lay between 7·5 and 8·0. The enzyme from red cells and sera appears to be heterogeneous and Vessell and Bearn (1958) have found three peaks of activity in red cell haemolysates with optimum pH requirements of 8·0, 8·2, and 8·5. Tsao (1960) has shown that lactic dehydrogenase from cytologically homogeneous tissue may also be heterogeneous on electrophoresis, and it is possible that the enzyme from spermatozoa may be similar.

Although the optimum pH for both enzymes is relatively high, the motility of ram and bull spermatozoa is greatest near pH 7·0 (Blackshaw and Emmens 1951). On the other hand Cragle and Salisbury (1959) have shown, with bull spermatozoa, that the optimum pH for oxygen uptake is 6·2. This contrasts with the effect on fructose utilization, where, over a pH range of 4–8, there was a linear increase in fructolysis.

Phosphate was reported to be necessary for the activation of purified succinic dehydrogenase (Kearney, Singer, and Zastrow 1955; Kearney 1957), but Wang, Tsou, and Wang (1958) suggested that any action of phosphate was non-specific and possibly could be due to chelation of heavy metal ions. Nachlas, Margulies, and Seligman (1960) have also shown that the enzyme activity is not dependent on phosphate particularly at higher pH. However, at the lower pH used for spermatozoa (pH 7·4) phosphate apparently has a slightly depressing effect on succinic dehydrogenase.

The effect of phosphate on lactic dehydrogenase suggests that there may be two actions. Firstly on lactic dehydrogenase itself, producing stimulation and then depression, and secondly at higher levels the activation of another system which has sufficient substrate for reaction. At higher concentrations (0·08M) Mann and White (1957) have shown that phosphate inhibited the oxidation of lactate by ram spermatozoa.

The presence of glucose 6-phosphate dehydrogenase in ram and bull spermatozoa seems likely, as the initial tests on washed spermatozoa showed that the inclusion of

glucose 6-phosphate in the medium produced a rise in tetrazolium reduction. Failure of TPN in this test to increase reduction suggests that the cofactor may not be easily lost from the cells by repeated washing and pre-incubation; but when cell extracts were used, reduction of INT only occurred in the presence of TPN and glucose 6-phosphate. Although Salisbury and Nakabayashi (1957) could not demonstrate glucose 6-phosphate dehydrogenase activity in bull semen extracts, Wu *et al.* (1959) have recorded its presence in testicular and epididymal spermatozoa. In cells immobilized by washing, Flipse and Almquist (1955) also found evidence for this pathway. In the present tests the cells were repeatedly washed and motility was greatly depressed.

Microscopic examination of spermatozoal suspensions shows that most of the reduced tetrazolium is in the midpiece of the cell and little or none is visible in the tail region, but Nelson (1959) reports that dye reduction by succinic dehydrogenase occurs in the tail as well, when examined by electron microscopy. Although Bishop and Mathews (1952) showed that 2,3,5-triphenyl tetrazolium chloride inhibited motility, tetrazolium salts have proved useful in showing enzyme activity in spermatozoa. Similar techniques could be used for the colorimetric estimation of enzyme activity in tissues such as the testis and seminal vesicles.

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