THE ENZYMATIC ACTIVITY OF BULL TESTIS AND EPIDIDYMAL SPERMATOZOA

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Summary

The reduction of the tetrazolium salt, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT), has been used to study the enzymatic activity of bull testis cells and epididymal spermatozoa. Significant reduction of INT occurred when the substrates were succinate, glucose 6-phosphate, lactate, sorbitol, malate, and glyceraldehyde 3-phosphate. When β-hydroxybutyrate and glutamate were substrates significant reduction of INT was only found in epididymal spermatozoa.

Freezing reduced β-hydroxybutyrate and glutamate dehydrogenase activity of epididymal spermatozoa but increased the activity of succinate dehydrogenase in both cell types.

K_m values for succinate, glucose 6-phosphate, and lactate dehydrogenases were obtained, which are close to those recorded elsewhere, although confidence limits are wide. Optimum pH levels for succinate dehydrogenase were 8·42 (testis) and 8·81 (spermatozoa), with corresponding values for lactate dehydrogenase of 7·96 and 7·93. The activity of glucose 6-phosphate dehydrogenase in both cell types was little affected by pH and activity fell only when the pH was below 7·5 and above 8·5. Phosphate (0·014–0·056M) stimulated succinate and glucose 6-phosphate dehydrogenase activity of testis cells but had little effect on the dehydrogenases of epididymal spermatozoa.

I. Introduction

The reducing activity of spermatozoa and mouse testis cells has been studied by Blackshaw (1963*a*, 1963*b*).

These observations have been made by the use of the tetrazolium salt, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT), which is readily reduced by tissues and isolated cells in the presence of suitable substrates. INT is able to compete with molecular oxygen as an electron acceptor (Pearson and Defendi 1954) and anaerobic conditions are not necessary.

It is convenient to dispense cells as a suspension but it has been shown that cells isolated from solid organs are not metabolically normal (Laws and Stickland 1956; Kalant and Young 1957; Berry 1962). In the preparation of cell suspensions from mouse testis, Blackshaw (1963*b*) incubated teased tubules in a medium containing hyaluronidase and trypsin. However, the possible deleterious effects of enzymatic methods of cell isolation led Jacob and Bhargava (1962) to use a direct mechanical disruption of solid tissues to yield cell suspensions. This method has been adapted to the isolation of testis cells from the bull testis.

In the results presented below, isolated bull testis cells and epididymal spermatozoa were tested for the presence of various dehydrogenase activities. More

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detailed studies were also made on succinate, lactate, and glucose 6-phosphate dehydrogenases to determine approximate K_m values, and the effects of pH and phosphate levels on activity.

II. Materials and Methods

Bull testes were obtained from abattoirs and only normal organs were used. As soon after slaughter as possible the testes, contained in the scrotum, were packed in ice until used. The time between slaughter and the preparation of the cells was usually under 3 hr, but storage periods as long as 24 hr did not appear to change enzyme activities appreciably.

Equal samples, taken from each testis of a pair, were combined and finely cut up with scissors in a small amount of saline. In preliminary rests the chopped testis was incubated for 15 min at 37°C in 0·15M NaCl containing 2 mg/ml trypsin and 150 units/ml hyaluronidase (testicular). The amount of medium used was 1 ml/100 mg of tissue. To aid the dispersion of the cells a stirring device, consisting of a perforated stainless steel plate mounted on a long handle, was moved vertically through the suspension at intervals during incubation. The cell suspension was then filtered through several layers of gauze to remove large cell clumps and fibrous tissue. Following centrifugation for 10 min at 3000 r.p.m. the supernatant was removed. The cells were washed once in 0·15M NaCl and before resuspension the compact cell mass was weighed. Fresh saline was then added to give a suspension containing 100 mg (wet weight) per millilitre. On microscopic examination the cells were found to be well dispersed, with only occasional small clumps.

In later experiments the chopped testis was placed in a test tube containing saline (1 ml/100 mg). A loose-fitting rubber pestle, as described by Jacob and Bhargava (1962), was used to disperse the cells; 12–18 up and down strokes served to break down the testis. The suspension was then treated as before and made up to give 100 mg of cells in 1 ml of 0·15M NaCl.

Slightly more cell debris was obtained with this method than with the enzymatic preparation, but the ease and speed of the mechanical dispersion made it the method of choice.

Epididymal spermatozoa were obtained by chopping the tails of two epididymides with scissors in 25 ml of 0·15M NaCl. After filtering, the suspension was centrifuged at 3500 r.p.m. for 10 min. The spermatozoa were then washed once in 10 ml of saline and finally made up to about 5 ml. A cell count was made and the suspension adjusted to 10^9 spermatozoa per millilitre.

Both cell suspensions were added in 0·1 ml amounts giving 10 mg of testis cells and 10^8 spermatozoa in final volumes of 1·0 ml.

The basic media used were 0·15M NaCl and an isotonic medium 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 most experiments the buffer level was kept constant, and additions were made at the expense of the saline.

All substrates, except glyceraldehyde 3-phosphate were prepared as 0·15M solutions, although further dilution was necessary for the experiments to determine

reaction rates. Glyceraldehyde 3-phosphate was available only as the sparingly soluble barium salt; this was dissolved in a small amount of 0·1N HCl and the barium precipitated with Na_2SO_4. The clear solution obtained after centrifugation was then adjusted with distilled water to 0·075M.

In all cases, apart from the estimation of reaction rates, the substrates were used at 0·015M concentration which ensured a high degree of saturation of the various enzymes.

For examination of the effects of pH, the simple phosphate buffer was replaced by one prepared from 0·17M $NaH_2PO_4.2H_2O$, 0·1M sodium borate, and 0·1M NaOH. This buffer was relatively stable at high pH.

To investigate the effects of phosphate on enzyme activity, 0·1M Tris–HCl buffer at pH 7·8 was used. Phosphate buffer was also prepared at pH 7·8 and appropriate amounts added to give the desired phosphate level in the final volume of 1·0 ml. The tetrazolium salt (INT) was prepared as 0·2% (w/v) in 0·15M NaCl and added to the medium to give a level of 0·02% (w/v). The cofactor nicotinamide adenine dinucleotide phosphate (NADP) was used in the estimations of glucose 6-phosphate dehydrogenase activity, and nicotinamide adenine dinucleotide (NAD) was used for lactate, malate, sorbitol, β-hydroxybutyrate, and glyceraldehyde 3-phosphate dehydrogenases. Both cofactors were added to give a final level of 0·1 mg/ml.

For glucose 6-phosphate dehydrogenase estimations, 0·02M sodium fluoride and 0·002M iodoacetic acid were included to block glycolysis. It was found in preliminary experiments that 0·015M KCN was necessary to enhance the activity of malate and glyceraldehyde 3-phosphate dehydrogenases.

In all experiments the final volume was 1·0 ml. The cofactors NAD and NADP (0·1 mg/ml) were added immediately before the cells, and the INT immediately after. An incubation time of 20 min was used at a temperature of 37°C and the reaction stopped by the addition of 4 and 6 ml of acetone to the testis and epididymal cells respectively.

The cells were extracted with the acetone, centrifuged, and the optical density (O.D.) of the clear supernatant read at 490 mμ in a Unicam SP 600 spectrophotometer. The results were expressed as O.D./10 mg wet weight of testis or O.D./10^8 spermatozoa.

Statistical analyses were performed in which either the t-test or the analysis of variance was used. Initially, K_m values were determined by an unweighted least squares method in which the double reciprocals $1/v$ and $1/s$ introduced by Lineweaver and Burk (1934) were used. However, the estimation of error is considerably biased as the fit of the regression is based too much on the lower velocities. The weighted regression technique of Wilkinson (1961) was therefore used in which the least squares fitting of the relationship $v = V(1+K_m/s)$ gives the best estimate of K_m and its standard error.

Provisional estimates of K_m were made from a weighted fit of a linear regression. These estimates were then adjusted by fitting a bilinear regression of v on the corresponding values of the provisionally fitted Michaelis–Menten function. The details of the procedure, which is rather time consuming, are best obtained from Wilkinson's (1961) paper.

III. Results

The two methods of testis cell dispersion were compared in terms of the yields of cells obtained. The mean amount of packed wet cells after mechanical dispersion of 1·000 g of testis was 0·317±0·0237 g, while the yield after enzymatic treatment was 0·215±0·0212 g. The mean difference was highly significant ($t = 3·211$; d.f. $= 46$; $P<0·01$). No difference in enzymatic activity of the two preparations was observed.

The activities of a variety of dehydrogenases in bull testis cells and epididymal spermatozoa were studied. The mean optical densities for the difference between substrate and no substrate are given in Table 1 for the testis and spermatozoa. Standard errors are included as well as the significance of the differences observed.

Table 1

Reduction of INT by bull testis cells and epididymal spermatozoa in the presence of various substrates

The mean differences between control and substrate tubes are given in optical density (O.D.) units, together with the standard error and the number of replications in parenthesis

Substrate	Mean Difference for Testis Cells (O.D. units/10 mg wet wt.)	Mean Difference for Epididymal Spermatozoa (O.D. units/10^8 spermatozoa)
Experiment 1		
Succinate	0·071±0·021** (10)	0·152±0·030** (9)
Glucose 6-phosphate	0·074±0·028** (9)	0·138±0·046* (9)
Lactate	0·281±0·074** (9)	0·237±0·046** (8)
Sorbitol	0·089±0·025** (10)	0·161±0·029** (9)
β-Hydroxybutyrate	0·008±0·004 (10)	0·066±0·015** (9)
Glutamate	0·000±0·007 (10)	0·034±0·012* (9)
Malate	0·137±0·023** (21)	0·036±0·018 (8)
Glyceraldehyde 3-phosphate	0·140±0·019** (17)	0·081±0·039* (8)
Experiment 2		
Malate	—	0·119±0·023** (7)†
Glyceraldehyde 3-phosphate	—	0·073±0·017** (7)†

* $P<0·05$. ** $P<0·01$. † Washed spermatozoa.

No significant activity of β-hydroxybutyrate and glutamate dehydrogenases was evident in the testis, but both enzymes were demonstrated in epididymal spermatozoa.

The measurement of malate and glyceraldehyde 3-phosphate dehydrogenases proved difficult in epididymal spermatozoa, as the inclusion of potassium cyanide in the medium greatly increased the non-specific reduction of tetrazolium. In these tests the presence of neither enzyme could be shown unequivocally.

In order to decrease non-specific reduction in the presence of cyanide, the epididymal spermatozoa were washed five times by repeated centrifugation and resuspension in fresh diluent. The results (experiment 2, Table 1) show considerable

and significant reduction of INT in the presence of malate and glyceraldehyde 3-phosphate.

The failure to demonstrate significant amounts of β-hydroxybutyrate and glutamate dehydrogenase activity in testis cells prompted experiments to test the

TABLE 2

EFFECT OF FREEZING ON THE REDUCTION OF INT BY TESTIS AND EPIDIDYMAL SPERMATOZOA

Mean optical densities are given for seven replications for all testis cells substrates, and for five replications for all epididymal spermatozoa substrates except β-hydroxybutyrate and glutamate, where there were six replications respectively

Substrate	Concn. (M)	Activity of Testis Dehydrogenases in:		Activity of Epididymal Spermatozoa Dehydrogenases in:	
		Control Cells	Frozen Cells	Control Cells	Frozen Cells
Succinate	0	0·044	0·014	0·096	0·024
	0·015	0·113	0·141	0·258	0·467
Glucose 6-phosphate	0	0·041	0·045	0·091	0·064
	0·015	0·215	0·260	0·386	0·441
Lactate	0	0·054	0·044	0·124	0·062
	0·015	0·467	0·489	0·547	0·555
β-Hydroxybutyrate	0	0·061	0·045	0·126	0·052
	0·015	0·073	0·062	0·228	0·164
Glutamate	0	0·061	0·045	0·126	0·052
	0·015	0·064	0·047	0·170	0·091

Analysis of Variance

Substrate	Variance Ratios for Testis Dehydrogenases				Variance Ratios for Epididymal Spermatozoa Dehydrogenases			
	Effect of Substrate	Effect of Freezing	Interaction	Error Variance† $\times 10^6$	Effect of Substrate	Effect of Freezing	Interaction	Error Variance‡ $\times 10^6$
Succinate	578·3**	0·1	50·1**	115	41·1**	2·1	8·8*	11,115
Glucose 6-phosphate	118·7**	1·9	1·9	2234	45·5**	0·1	0·7	12,371
Lactate	283·9**	0·1	0·4	4529	145·9**	0·5	0·9	7,176
β-Hydroxybutyrate	14·7**	11·8**	0·5	106	156·9**	65·5**	0·4	437
Glutamate	0·5	15·6**	0	124	87·4**	297·2**	0·3	119

* $P<0·05$. ** $P<0·01$.

† No. of degrees of freedom = 18 for all substrates.

‡ No. of degrees of freedom = 12 for first three substrates, 15 for β-hydroxybutyrate, and 10 for glutamate.

effect of freezing cell suspensions, as this is known to activate these enzymes in some mitochondrial preparations. For comparison, epididymal spermatozoa were used, and also substrates for succinate, glucose 6-phosphate, and lactate dehydrogenases, which enzymes are readily demonstrated by tetrazolium reduction in both cell types.

The results and extracts of the analyses of variance are given in Table 2. Freezing had no significant overall effect except for the β-hydroxybutyrate and glutamate tests where it produced a loss of reducing activity. Although not high, there was significant β-hydroxybutyrate, but no glutamate, activity shown in the testis cells. However, the interactions showed that freezing increased the activity of succinic dehydrogenase in both testis and spermatozoa.

A more detailed examination of three representative enzymes was made. The reaction rates of succinate and lactate dehydrogenases were measured over a substrate range of $2{\cdot}00\times10^{-2}$M–$6{\cdot}25\times10^{-4}$M, while the rate of glucose 6-phosphate dehydrogenase was measured over the range $1{\cdot}56\times10^{-4}$M–$4{\cdot}98\times10^{-6}$M.

K_m values were calculated by the method of Wilkinson (1961). These values for testis cells and epididymal spermatozoa are given in Table 3 with 95% confidence limits. In further experiments the level used for all substrates was 0·015M to ensure adequate saturation of the enzyme.

TABLE 3

K_m VALUES FOR ENZYMES OF BULL TESTIS CELLS AND EPIDIDYMAL SPERMATOZOA

Cells	Substrate	No. of Replications	$10^4\times K_m$ (M)	95% Confidence Limits
Testis	Succinate	5	8·356	1·137–15·575
	Glucose 6-phosphate	5	0·078	0·029– 0·126
	Lactate	7	13·162	3·978–22·346
Epididymal spermatozoa	Succinate	7	4·900	0·239– 9·561
	Glucose 6-phosphate	5	0·162	0·044– 0·280
	Lactate	7	11·156	6·065–16·247

The effects of pH changes on succinate, lactate, and glucose 6-phosphate dehydrogenases are shown in Figure 1 (testis) and Figure 2 (epididymal spermatozoa). Although previous experiments using mouse testis had indicated that inorganic phosphate slightly inhibited the activity of glucose 6-phosphate dehydrogenase, it was decided to use the same phosphate-containing buffer for the three enzymes. It is seen that the optimum pH for succinate dehydrogenase was 8·42 for the testis and 8·81 for spermatozoa, while for lactate dehydrogenase the corresponding pH levels were 7·96 and 7·93.

It is apparent that the activity of glucose 6-phosphate dehydrogenase in both types of cells was little changed over a fairly wide pH range, with activity falling only below about pH 7·5 and above pH 8·5.

The influence of inorganic phosphate on these enzymes was also studied. A Tris–HCl buffer (pH 7·8) was used, and added phosphate replaced 0·15M NaCl in the medium. It is seen from Table 4 that phosphate stimulated the activity of testis succinate and glucose 6-phosphate dehydrogenases but had no significant effects on testis lactate dehydrogenase, or the enzymes of epididymal spermatozoa.

IV. Discussion

The preparation of cell suspensions from solid tissues has been achieved by a variety of methods (Rinaldini 1958) and it is important when preparing a suspension by mechanical or chemical treatments to incur a minimum of damage to the cells.

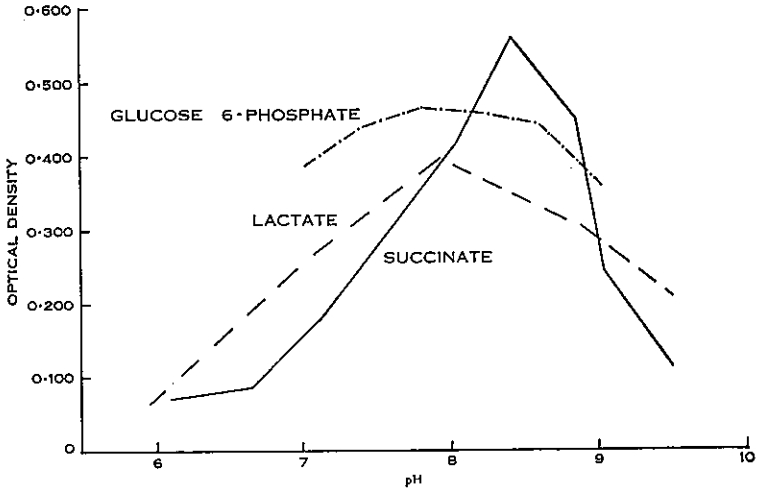

Fig. 1.—Effect of pH on the activities of succinate, glucose 6-phosphate, and lactate dehydrogenases of bull testis.

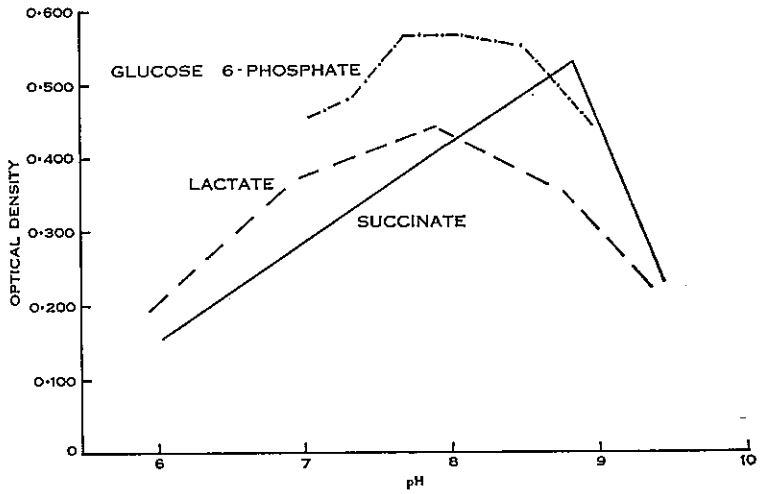

Fig. 2.—Effect of pH on the activities of succinate, glucose 6-phosphate, and lactate dehydrogenases of bull epididymal spermatozoa.

Incubation of the tissue in various proteolytic enzymes or hyaluronidase has the disadvantage of subjecting the cells to an unfavourable environment for some time before the experimental procedures are applied. Mechanical breakdown of tissue may

be carried out at low temperatures, and little time is needed to prepare a washed cell suspension. In the present experiments mechanical breakdown of the testis also gave a greater yield of cells, a factor which may be important when available amounts of tissue are small.

The occurrence of various dehydrogenases was found to be similar in testis cells and epididymal spermatozoa. The main differences observed were the absence of glutamate and low β-hydroxybutyrate activity in the testis.

TABLE 4

EFFECT OF PHOSPHATE ON THE REDUCTION OF INT BY BULL TESTIS CELLS AND EPIDIDYMAL SPERMATOZOA IN THE PRESENCE OF SUCCINATE, GLUCOSE 6-PHOSPHATE, AND LACTATE

Values are given as optical densities (O.D.) and the number of replications for each substrate are also given in parenthesis

Phosphate Concn. (M)	Mean Difference (O.D.) between Control and Substrate Tubes					
	Testis Cells			Epididymal Spermatozoa		
	Succinate (8)	Glucose 6-Phosphate (7)	Lactate (8)	Succinate (5)	Glucose 6-Phosphate (7)	Lactate (5)
0	0·126	0·188	0·379	0·337	0·337	0·429
0·014	0·171	0·195	0·419	0·333	0·351	0·420
0·028	0·177	0·226	0·434	0·325	0·335	0·418
0·056	0·208	0·221	0·380	0·341	0·369	0·433

Analysis of Variance

Source of Variation	Variance Ratios					
Substrate	215·7**	523·9**	451·5**	727·4**	245·8**	1011·0**
Phosphate	—	—	—	0·2	0·2	0·3
Linear	16·6**	7·9**	1·0	—	—	—
Quadratic	0·4	0·1	1·9	—	—	—
Cubic	0·1	0·8	0	—	—	—
Pooled error variance $\times 10^6$	2145(d.f.=49)	1153(d.f.=42)	5753(d.f.=42)	1532(d.f.=28)	6948(d.f.=42)	1785(d.f.=28)

** $P<0·01$.

Although cyanide was successfully used to demonstrate the presence of malate and glyceraldehyde 3-phosphate dehydrogenases, there was initially a high level of non-specific reduction of INT by epididymal spermatozoa. This was reduced to acceptable levels by washing the cells five times in saline, when the addition of both substrates led to the reduction of significant amounts of INT. These observations on bull testis and epididymal spermatozoa agree well with the occurrence of dehydrogenases in ejaculated ram and bull spermatozoa and in mouse testis (Blackshaw,

unpublished data), except that malate activity was not demonstrated in ejaculated spermatozoa.

The failure to demonstrate glutamate or marked β-hydroxybutyrate dehydrogenase activity in testis cells does not necessarily imply the absence or relative lack of these enzymes. Cooper and Lehninger (1956) have shown that the oxidation of β-hydroxybutyrate by particles of mitochondria is greater than by whole mitochondria, while Lehninger, Sudduth, and Wise (1960) found that the enzyme was bound to particulate material. The bound enzyme was inactive and sonic disruption of mitochondria was necessary for activity. Glutamate dehydrogenase of mitochondria is also in a latent form which can be released by freezing or osmotic action (Bendall and de Duve 1960). On the other hand, the mitochondria of spermatozoa are organized in a helix in the mid-piece, and are separated from the medium by only a cell membrane. In this position they may be able to metabolize β-hydroxybutyrate and glutamate more readily.

Although washed and isolated testis cells show many enzymatic activities, these cells are not normal. Lactic acid production from glucose proceeds at a very low rate, and there is evidence that cytochrome *c* is lost from the cells. On the other hand, washed spermatozoa show marked glycolytic activity (the extent depending on the degree of washing) and retain some cytochrome *c* (Blackshaw, unpublished data).

Mammalian spermatozoa possess both the Embden–Meyerhoff pathway and the Krebs cycle, and some of the dehydrogenase enzymes involved in these have been demonstrated, as well as one enzyme (glucose 6-phosphate dehydrogenase) of the pentose phosphate pathway. The significance of this enzyme, which has one role in the production of NADPH for synthetic purposes, is seen in testis cells, but not in spermatozoa, where synthesis is not important.

The estimates of the K_m values for bull testis enzymes are of much the same order as for mouse testis (Blackshaw 1963*b*). However, the confidence limits are wider in the present study, this being due mainly to the method of calculation used which gives a greater but more reliable estimate of error (Wilkinson 1961). Similar comments hold for the comparison of K_m values for ejaculated and epididymal spermatozoa. The wide confidence limits also reflect the difficulty of measuring K_m values in whole cells where the apparent values may be influenced by the ease of access of the substrates to the enzymes within the cell.

The K_m values for glucose 6-phosphate dehydrogenase are quite comparable with the value of $1{\cdot}3\times10^{-5}$M found by Glock and McLean (1953) but are much lower than that of $3\text{–}5\times10^{-4}$M given by McNair Scott and Cohen (1953) for partially purified *Escherichia coli* enzyme.

The effects of pH on enzymatic activity show patterns similar to those of mouse testis and ejaculated spermatozoa, except that the optimum pH for succinate dehydrogenase is shifted to the alkaline side (8·42) for bull testis cells, lying between that for spermatozoa (near pH 9·0) and mouse testis (near pH 8·0) (Blackshaw 1963*a*, 1963*b*).

The curves for glucose 6-phosphate dehydrogenase were rather different, there being a fairly flat response between about pH 7·5 and 8·5, with activity falling off beyond these values. This response is similar to that obtained by Glock and McLean (1953) for rat liver enzyme in glycylglycine buffer.

Although it has been shown that phosphate stimulates glycolysis and inhibits oxygen uptake by spermatozoa and testis (Blackshaw 1960, 1962), it has only slight effects on succinate and lactate dehydrogenases of ejaculated spermatozoa and mouse testis (Blackshaw 1963*a*, 1963*b*). Similar observations are reported here for these enzymes in bull testis and epididymal spermatozoa.

Phosphate has been shown to have a slight inhibitory effect on glucose 6-phosphate dehydrogenase of mouse testis, but this was not observed in the present experiments on bull testis and spermatozoa, where there was evidence of a slight stimulation of the testis enzyme although the activity of the spermatozoal enzyme was unaffected. In other tissues, Kravitz and Guarino (1958) have recorded inhibition of glucose 6-phosphate dehydrogenase by 0·05M phosphate. Glaser and Brown (1955) also found that phosphate inhibited the activity of this enzyme, but the levels used were very high (0·150M and 0·217M).

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