THE ENZYMATIC ACTIVITY OF MOUSE TESTIS

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Summary

The reduction of a tetrazolium salt, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride has been used to estimate the enzymatic activity of mouse testis.

K_m values of succinate and lactate dehydrogenases have been measured and are $4 \cdot 14 \times 10^{-4}$M and $7 \cdot 45 \times 10^{-4}$M respectively. Optimum pH levels were found to be 7·99 for succinate and 7·93 for lactate dehydrogenase.

Inorganic phosphate produced a slight stimulation of succinate dehydrogenase but had no significant effect on the activity of lactate dehydrogenase. Measurements were made of the activity of glucose 6-phosphate dehydrogenase. This was slightly inhibited by inorganic phosphate.

Comparisons of enzyme activities showed that lactate dehydrogenase was most active and that both succinate and glucose 6-phosphate dehydrogenase had lower but similar activities.

I. Introduction

The metabolic activity of the testis has not been extensively studied, and even less attention has been given to the localization and measurement of its enzymatic activities.

Dickens and Greville (1932) showed that the testis rapidly metabolized glucose but not fructose. These observations were confirmed and extended by Blackshaw (1962), who also showed that inorganic phosphate inhibited respiration and stimulated glycolysis.

The localization of enzymes by histochemical methods has developed considerably in recent years. Pearse (1960), and Niemi and Ikonen (1962) have used the reduction of tetrazolium salts to determine the sites of enzyme activity in the testis of the rat. Earlier, very brief reports of succinic dehydrogenase activity in the testis were given by Nachlas *et al.* (1957) and Walker and Seligman (1961).

It has been shown by Nachlas, Margulies, and Seligman (1960), and Blackshaw (1963) that the reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) offers a convenient method for the estimation of enzyme activity in liver and spermatozoa.

In the present paper the activities of succinate, lactate, and glucose 6-phosphate dehydrogenases have been measured under different conditions.

II. Materials and Methods

Adult male mice were used in all experiments (mean weight of 34·8 g) and were kept at a temperature of between 70 and 75°F. The mice were killed by a blow on the head, and the testes quickly dissected out. After weighing, each testis was

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bisected and the tubules teased out with fine forceps. The tubule mass was incubated for 15 min at 37°C in 0·15M NaCl containing 2 mg/ml trypsin and 150 units/ml testicular hyaluronidase. During incubation the tissue was repeatedly sucked up and down a wide-bore pasteur pipette to aid in dispersion. In this way the tubules were reduced to isolated cells, with only a few larger pieces and the fibrous capsule remaining intact. The cell suspensions from both testes were combined, centrifuged for 10 min at 2500 r.p.m., and the supernatant replaced by 4·0 ml 0·15M NaCl. Centrifugation was repeated, and the cells dispersed in either 1·0 or 2·0 ml of saline.

The basic media used in the tests were 0·15M NaCl, and an isotonic solution containing 36 vols. 0·13M $Na_2HPO_4.12H_2O$, 14 vols. 0·17M $NaH_2PO_4.2H_2O$, and 50 vols. 0·15M NaCl. In all experiments the buffer level was kept constant, and additions made at the expense of the sodium chloride medium. In most cases additions were made by including appropriate volumes of 0·15M sodium succinate, sodium lactate, or glucose 6-phosphate in the diluent.

TABLE I

K_m VALUES OF MOUSE TESTIS ENZYMES

Enzyme	Replications	$10^4 \times K_m$ (M)
Succinate dehydrogenase	7	4·14±0·76*
Lactate dehydrogenase	5	7·45±0·92

* Standard error.

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 total volume was 0·9 ml and to this was added 0·1 ml of the testis suspension.

After an incubation period of 30 min at 37°C, the cells were extracted with 4·0 ml acetone and the optical density measured at 490 mμ.

The effects of pH variation were studied, and in these tests the usual phosphate buffer was replaced by one prepared from 0·17M $NaH_2PO_4.2H_2O$, 0·10M sodium borate, and 0·10M NaOH. This mixture enabled a high pH to be maintained. In other experiments the effects of inorganic phosphate were tested, and here the phosphate buffer was replaced by 0·1M Tris–HCl.

The activity of glucose 6-phosphate dehydrogenase was initially determined using 0·15M NaCl, this being replaced as required by suitable amounts of phosphate buffer (pH 7·2). Additions of 0·02M NaF and 0·002M sodium iodoacetate were also made to block the action of glycolytic enzymes.

The cofactors di- and triphosphopyridine nucleotides (DPN and TPN) were used in the study of lactate and glucose 6-phosphate dehydrogenases (0·1 mg/ml). In the final experiments the activities of succinate, lactate, and glucose 6-phosphate

dehydrogenases were measured in samples from the same testis. Phosphate buffer was replaced by 0·1M Tris–HCl, and 0·02M NaF and 0·0002M sodium iodoacetate were included in the glucose 6-phosphate medium.

In all experiments the reduction of INT was measured in optical density units, and the results examined by the analysis of variance.

TABLE 2

EFFECTS OF pH ON THE ACTIVITIES OF SUCCINATE AND LACTATE DEHYDROGENASES IN THE PRESENCE OF 0·015M SUBSTRATE

Mean optical densities for five replications

Nominal pH	Succinate Dehydrogenase		Lactate Dehydrogenase	
	Final pH	Optical Density	Final pH	Optical Density
6·00	6·06	0·093	6·09	0·113
7·00	6·99	0·154	6·90	0·245
8·00	7·99	0·271	7·93	0·399
9·00	8·80	0·106	8·63	0·329
10·00	9·35	0·075	9·27	0·276

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios	
		Succinate	Lactate
Replications	4	11·4**	35·6**
pH:	(4)		
Linear	1	2·5	54·0**
Quadratic	1	53·3**	80·4**
Cubic	1	2·1	0
Quartic	1	28·2**	10·6**
Residual (error)	16	0·0014†	0·0016

* $P < 0·05$. ** $P < 0·01$.

† Error mean square.

III. RESULTS

The reaction rates of succinate and lactate dehydrogenases of mouse testis were measured over a substrate concentration range of $2·00 \times 10^{-2}$M–$6·25 \times 10^{-4}$M. The K_m value for each enzyme was calculated by using the double reciprocal plot method of Lineweaver and Burk (1934), and is given in Table 1. For both enzymes

it was estimated that, at 0·015M substrate level, the reaction rate would be about 90–95% of the maximum. This level was therefore used in further experiments.

The effects of pH variations on the reduction of INT with succinate and lactate as substrates were also measured. A nominal pH range of 6·0–10·0 was used but as the actual values were variable the mean final pH levels are given with the optical densities in Table 2 and Figure 1. In both instances the optimum pH was close to 8·0, being 7·99 for succinate and 7·93 for lactate dehydrogenase.

Phosphate has been shown to stimulate the glycolytic activity of the testis, and its effects on succinate and lactate dehydrogenases were examined. It is clear from Table 3 that, although there is a slight stimulating effect on succinate dehydrogenase activity, phosphate has no significant influence on lactate dehydrogenase activity.

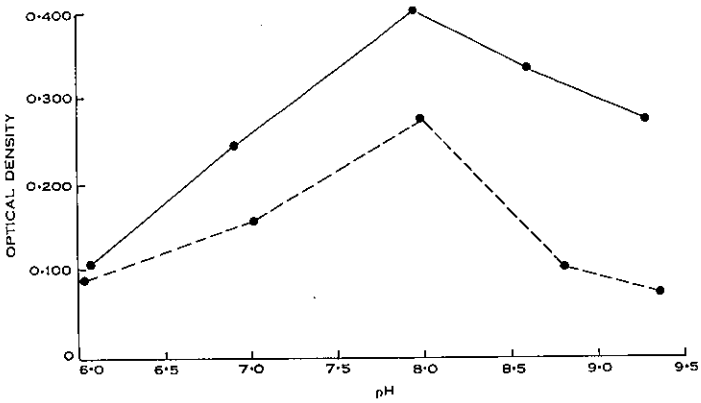

Fig. 1.—The effect of pH on the activities of succinate and lactate dehydrogenases of mouse testis. ●— — —● Succinate dehydrogenase. ●————● Lactate dehydrogenase.

The activity of glucose 6-phosphate dehydrogenase appeared to be less than that of the other enzymes, and in these tests the cells from two testes were suspended in 1·0 ml 0·15M NaCl. A factorial plan was used to study the effects of glucose 6-phosphate, TPN, and inorganic phosphate. The results (Table 4) show that glucose 6-phosphate stimulated the reduction of INT by testis tissue. An effect of TPN was evident only in the presence of substrate, and, although phosphate caused an overall stimulation, the interactions showed that in the presence of glucose 6-phosphate it produced a decrease in the reduction of INT.

The relative activities of the three enzymes were compared by using the same basic medium (50 vols. 0·15M NaCl and 50 vols. 0·1M Tris–HCl at pH 7·5). The appropriate coenzymes, DPN and TPN, were added as required, and to the medium for glucose 6-phosphate dehydrogenase were also added 0·02M NaF and 0·0002M sodium iodoacetate. The substrates were included at the same concentration level (0·015M).

The results (Table 5) show that, under these conditions, lactate dehydrogenase was most active, and that both succinate and glucose 6-phosphate dehydrogenase

were of lower but equal activity. ($F_{1,15} = 368 \cdot 0$, $P < 0 \cdot 001$ for the comparison between lactate and the other enzymes, and $F_{1,15} = 2 \cdot 3$, $P > 0 \cdot 05$ for the comparison between succinate and glucose 6-phosphate dehydrogenase.)

TABLE 3

EFFECT OF PHOSPHATE ON THE ACTIVITIES OF SUCCINATE AND LACTATE DEHYDROGENASES OF MOUSE TESTIS

Mean optical densities for five replications

Phosphate Concn. (M)	Succinate Dehydrogenase		Lactate Dehydrogenase	
	No Succinate	0·015M Succinate	No Lactate	0·015M Lactate
0	0·033	0·175	0·093	0·404
0·014	0·036	0·193	0·098	0·410
0·028	0·043	0·214	0·099	0·389
0·056	0·061	0·240	0·106	0·416

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratios	
		Succinate	Lactate
Replications	5	4·02	4·42
Effect of phosphate:	(3)	—	—
No phosphate *v.* phosphate	1	7·80*	0·19
Linear phosphate effect	1	9·19**	0·12
Quadratic phosphate effect	1	0·15	0·62
Substrate	1	371·08**	469·72**
Interactions:			
All replicate interactions	20	0·77	1·27
Phosphate/lactate	3	0·94	0·12
Residual (error)	15	0·00086†	0·00224

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

† Residual (error) mean square.

IV. DISCUSSION

The reaction rates of both succinate and lactate dehydrogenases of testis are of the same order as those for ram and bull spermatozoa (Blackshaw 1963). Similar values for succinate dehydrogenase have been obtained for heart muscle preparations by Wang, Tsou, and Wang (1956), and Keilin and King (1960) who used variations of the ferricyanide assay method, and by Singer, Kearney, and Massey (1957) who developed the phenazine methosulphate method.

The reduction of INT by mouse testis proceeded satisfactorily in the absence of phenazine methosulphate as an electron carrier, although Nachlas *et al.* (1960) found that its use increased the sensitivity of their technique for the measurement of rat liver succinate dehydrogenase.

TABLE 4

ACTIVITY OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE OF MOUSE TESTIS AS INFLUENCED BY TRIPHOSPHOPYRIDINE NUCLEOTIDE (TPN) AND PHOSPHATE LEVELS

Mean optical densities for five replications

Phosphate Concn. (M)	TPN Concn. (mg/ml)	Glucose 6-Phosphate Dehydrogenase Activity	
		No Glucose 6-Phosphate	0·028M Glucose 6-Phosphate
0	0	0·019	0·068
0	0·1	0·009	0·112
0·028	0	0·054	0·072
0·028	0·1	0·054	0·093

Analysis of Variance

Source of Variation	Degrees of Freedom	Variance Ratio
(1) Replications	5	12·1**
(2) Glucose 6-phosphate	1	215·0**
(3) TPN	1	21·3**
(4) Phosphate	1	17·4**
Interactions		
All replicate interactions	15	3·2*
(2) × (3)	1	23·3**
(2) × (4)	1	39·9**
(3) × (4)	1	2·6
(2) × (3) × (4)	1	2·9
Error	20	0·00014

* $P < 0·05$. ** $P < 0·01$.

The activities of both succinate and lactate dehydrogenases were greatest at pH near 8·0 (7·99 and 7·93 respectively). The optimum pH for succinate dehydrogenase is close to that of 7·8 found by Wang, Tsou, and Wang (1956), and 7·6 found by Singer, Kearney, and Massey (1957) for the heart muscle preparation. It is

considerably lower than the values of 8·90 and 8·69 found for ram and bull spermatozoa by Blackshaw (1963). However, the optimum pH for lactate dehydrogenase lay in the same range as for spermatozoa and other tissues (Blackshaw 1963).

Although phosphate has been shown to stimulate the glycolytic activity and inhibit the oxygen consumption of mouse testis (Blackshaw 1962), there was little evidence of any important effect on either succinate or lactate dehydrogenase.

TABLE 5

COMPARATIVE ACTIVITIES OF SUCCINATE, LACTATE, AND GLUCOSE 6-PHOSPHATE DEHYDROGENASES OF MOUSE TESTIS

Mean optical densities for four replications

Substrate Concn. (M)	Activities		
	Succinate Dehydrogenase	Lactate Dehydrogenase	Glucose 6-Phosphate Dehydrogenase
0	0·096	0·124	0·108
0·015	0·346	0·782	0·289

The increased rate of reduction of INT by the testis when both TPN and glucose 6-phosphate are added indicates the presence of an active dehydrogenase. In support of the findings of Kravitz and Guarino (1958), phosphate did slightly inhibit glucose 6-phosphate dehydrogenase. The overall stimulation obtained suggests that other enzyme systems were operating despite the use of inhibitors for glycolysis, and glyceraldehyde 3-phosphate, which requires phosphate for activity, may be involved.

The comparisons of enzyme activity show a predominance of lactate dehydrogenase activity, which supports the histochemical report of Niemi and Ikonen (1962). These authors also found that succinate dehydrogenase was mainly located in the cells of the seminiferous tubules, while lactate and other dehydrogenases were more restricted to the Leydig cells.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

BLACKSHAW, A. W. (1962).—*Aust. J. Biol. Sci.* **15**: 207.

BLACKSHAW, A. W. (1963).—*Aust. J. Biol. Sci.* **16**: 201.

DICKENS, F., and GREVILLE, G. D. (1932).—*Biochem. J.* **26**: 1546.

KEILIN, D., and KING, T. E. (1960).—*Proc. Roy. Soc.* B **152**: 163.

KRAVITZ, E. A., and GUARINO, A. J. (1958).—*Science* **128**: 1140.

LINEWEAVER, H., and BURK, D. (1934).—*J. Amer. Chem. Soc.* **56**: 658.

NACHLAS, M. M., MARGULIES, S. I., and SELIGMAN, A. M. (1960).—*J. Biol. Chem.* **235**: 499.

NACHLAS, M. M., TSOU, K. C., DE SOUZA, E., CHENG, C. S., and SELIGMAN, A. M. (1957).—*J. Histochem. Cytochem.* **5**: 420.

NIEMI, M., and IKONEN, M. (1962).—*Endocrinology* **70**: 167.

PEARSE, A. G. E. (1960).—"Histochemistry." 2nd Ed. (J. and A. Churchill Ltd.: London.)

SINGER, T. P., KEARNEY, E. B., and MASSEY, V. (1957).—*Advanc. Enzymol.* **18**: 65.

WALKER, D. G., and SELIGMAN, A. M. (1961).—*J. Biophys. Biochem. Cytol.* **9**: 415.

WANG, T. Y., TSOU, C. I., and WANG, T. L. (1956).—*Scientia Sinica* **5**: 73.