THE GLYCOLYSIS AND REDUCING ACTIVITY OF RAM SPERMATOZOA IN PHOSPHATE-CONTAINING MEDIA

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Summary

The reduction of 2,3,5-triphenyltetrazolium chloride by ram spermatozoa was linear in the range of cell densities from $1 \cdot 0 \times 10^8$ to $8 \cdot 0 \times 10^8$/ml.

Tetrazolium was reduced more rapidly under anaerobic conditions than aerobically unless fructose was present, when reduction was quite rapid in air.

Phosphate stimulates the reduction of tetrazolium, the breakdown of fructose, and the accumulation of lactic acid.

Both phosphate and succinate stimulate the reduction of tetrazolium by washed ram spermatozoa.

I. Introduction

The metabolism of spermatozoa is greatly influenced by the conditions imposed in their study. Thus Bishop and Salisbury (1955) have shown that phosphate ions depress the respiration of unwashed bull spermatozoa at body temperature in comparison with that occurring in 0·9 per cent. sodium chloride. Similar results have been obtained using washed ram and bull spermatozoa with fructose and glycerol as substrates (Mann and White 1956, 1957; White 1957). The reduction in respiration is accompanied by an accumulation of lactic acid (Salisbury and Nakabayashi 1957).

The reducing activity of bull semen has been studied by Smith, Mayer, and Merilan (1956, 1957*a*, 1957*b*) who used a manometric method to measure succinic dehydrogenase activity. Mohri (1957), in studies of sea-urchin spermatozoa, measured the reduction of 2,3,5-triphenyltetrazolium chloride by the succinic dehydrogenase system.

Tetrazolium salts have the property of acting as hydrogen receptors and are reduced by certain dehydrogenases to insoluble formazan pigments. Although widely applied for the histochemical localization of enzymes in many tissues, tetrazolium salts have been little used in studies of spermatozoa (Mohri 1957; Blackshaw 1958; King and Mann 1959).

The following experiments describe the use of 2,3,5-triphenyltetrazolium chloride to demonstrate the overall metabolic activity of ram spermatozoa particularly in the presence of phosphate ions.

II. Materials and Methods

Ram semen was collected by the electrical production of ejaculation (Blackshaw 1954); only ejaculates of good initial motility were used.

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The diluents employed were of varying constitution but for the preliminary tests the medium of White (1953) was found suitable. This contains 0·04M NaCl, 0·048M $Na_2HPO_4.12\ H_2O$, 0·032M $NaH_2PO_4.2\ H_2O$, and 200 mg per cent. fructose; in some tests the fructose was omitted. Otherwise the diluents were prepared from 0·154M NaCl to which was added isotonic phosphate buffer or 0·15M sodium succinate. The phosphate buffer contained 71 volumes of 0·13M $Na_2HPO_4.12\ H_2O$ and 29 volumes of 0·17M $NaH_2PO_4.2\ H_2O$ per 100 ml to give pH 7·0. The osmotic pressure of the media in some tests was varied to give relative tonicity values of 125, 100, and 75 where the tonicity of 0·154M NaCl is 100. These various levels were prepared by dilution of a 125 tonicity solution with water. Where necessary 200 mg per cent. fructose was added.

In most experiments, the incubation of the semen was carried out under anaerobic conditions in an atmosphere of nitrogen. The dilution rate of the semen was 1 in 5 and washed suspensions of spermatozoa were preparared by diluting semen 1 in 10 with 0·154M NaCl and centrifuging for 10 min at 1500 r.p.m. (approximately 300 *g*) for 10 min. The supernatant liquid was removed and 0·154M NaCl added to restore the volume. Two further centrifugations were performed with removal of the supernatants and after the last treatment the volume was adjusted to that of the original semen. In the tests of washed semen, the control samples were centrifuged under the same conditions and the packed cells resuspended each time.

Lactic acid was estimated by the method of Barker and Summerson (1941) and fructose by the method of Mann (1948). The reducing activity of the spermatozoa was determined by the reduction of 2,3,5-triphenyltetrazolium chloride to the insoluble red formazan. A suitable concentration of the tetrazolium salt was found to be 0·25 mg/ml and at the end of the incubation period 1 ml of the diluted semen was mixed with 6 ml of acetone. The formazan was readily extracted and the optical density of the coloured solution obtained was measured at 485 mμ. A series of standard solutions of 2,3,5-triphenyltetrazolium chloride were prepared containing 0·05, 0·04, 0·03, 0·02, and 0·01 mg/ml. Excess sodium hydrosulphite was added and the resulting insoluble formazan extracted with acetone. The absorption spectra of the formazan produced by the reducing activity of spermatozoa was found to be identical to that produced by the sodium hydrosulphite.

In all cases the accumulation of lactic acid, the disappearance of fructose, and the reduction of tetrazolium were measured in $\mu g/10^8$ cells/hr. The results were examined statistically by the analysis of variance.

III. Results

An initial experiment was performed to examine the effect of cell density on the reduction of tetrazolium. Semen was washed three times to remove the seminal plasma and resuspended in the isotonic buffer of White (1953) containing 200 mg per cent. fructose. Cell densities of 10^8, 2×10^8, 4×10^8, and 8×10^8 per ml were used, and the reduction of tetrazolium determined after incubation for 2 hr at 37°C.

The analysis of variance of the results for six ejaculates showed a highly significant effect for linear regression ($F_{(1,15)} = 57 \cdot 1$, $P < 0 \cdot 01$). The variance ratios for the quadratic and cubic effects were $F_{(1,15)} = 1 \cdot 4$ and 0 respectively, $P > 0 \cdot 05$. A regression line was calculated, $E = 1 \cdot 2 + 27 \cdot 6X$, where E is the amount (μg) of tetrazolium reduced and X is $\log_2$ (sperm number). The sperm number is taken as 1, 2, 4, or 8, the total sperm count being the product of the sperm number with 10^8. It is clear that over the range of spermatozoal densities likely to occur in the usual metabolic tests the cell density does not influence the rate of reduction of tetrazolium.

TABLE 1

EFFECTS OF WASHING, NITROGEN, AND FRUCTOSE ON THE ACCUMULATION OF LACTIC ACID AND THE REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE BY RAM SPERMATOZOA (MEANS OF FOUR REPLICATIONS)

Treatment	Gas Phase	Fructose (mg %)	Lactic Acid (μg/10^8 cells/hr)	Triphenyltetrazolium Chloride (μg/10^8 cells/hr)
Unwashed	Air	0	69·2	8·5
	Air	200	235·5	9·7
	Nitrogen	0	214·2	12·2
	Nitrogen	200	208·2	13·3
Washed	Air	0	1·3	6·2
	Air	200	208·2	11·7
	Nitrogen	0	1·3	12·7
	Nitrogen	200	162·5	9·7

The effect of incubation under anaerobic conditions was studied along with the effects of washing the spermatozoa free of seminal plasma and the addition of 200 mg per cent. fructose. The spermatozoal suspensions were incubated in Thunberg tubes in air or they were evacuated with a water pump and flushed with nitrogen. A factorial plan was used and, because of the limited amount of semen available, a 2^3 design with the three-factor interaction confounded with blocks was found suitable. Four replications were performed using individual ejaculates from eight rams. A summary of results for the reduction of tetrazolium and the accumulation of lactic acid is given in Table 1.

Analysis showed that anaerobic conditions favour the reduction of tetrazolium by ram spermatozoa particularly in the absence of fructose. On the other hand, the removal of seminal plasma by washing significantly reduced the accumulation of lactic acid, the effect being particularly marked under anaerobic conditions. Overall, anaerobic conditions stimulated the accumulation of lactic acid in the absence of added fructose but reduced it when fructose was present. Although the advantage of anaerobic conditions was not apparent for the reduction of tetrazolium in the presence of fructose, further tests were conducted under nitrogen as fructose was not included in all diluents.

Phosphate media have marked effects on the metabolism of bull semen and studies were made to determine the occurrence of similar possible effects with ram semen. Sodium chloride, a phosphate buffer, and a mixture of equal volumes of each were compared at relative tonicities of 75, 100, and 125 (0·154M NaCl = 100).

TABLE 2

EFFECTS OF TONICITY AND THE PRESENCE OF PHOSPHATE ON THE GLYCOLYSIS AND THE REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE BY RAM SPERMATOZOA (MEANS OF FOUR REPLICATIONS)

Relative Tonicity (0·9% NaCl = 100)	Sodium Chloride	Phosphate	Lactic Acid (μg/10^8 cells/hr)	Fructose (μg/10^8 cells/hr)	Triphenyl-tetrazolium Chloride (μg/10^8 cells/hr)
125	+	−	62·2	7·0	3·1
	+	+	79·5	91·2	19·9
	−	+	121·2	93·2	37·4
100	+	−	28·5	23·8	4·9
	+	+	184·0	133·8	24·6
	−	+	149·0	102·0	29·9
75	+	−	25·8	28·0	8·5
	+	+	169·0	79·0	21·1
	−	+	149·2	142·0	27·8

Fructose (200 mg per cent.) was included in all the media. Summary results for the reduction of tetrazolium, the accumulation of lactic acid, and the disappearance of fructose are given in Table 2. Phosphate greatly stimulated fructolysis and the

TABLE 3

EFFECT OF PHOSPHATE ON THE GLYCOLYSIS AND REDUCING ACTIVITY OF RAM SPERMATOZOA (MEANS OF SIX EJACULATES)

Phosphate (M)	Lactic Acid (μg/10^8 cells/hr)	Fructose (μg/10^8 cells/hr)	Triphenyl-tetrazolium Chloride (μg/10^8 cells/hr)
0·0000	48·7	31·5	3·0
0·0015	51·0	39·1	2·6
0·0060	61·3	40·6	3·9
0·0240	142·7	97·6	8·2
0·0960	142·7	106·6	11·3

reduction of tetrazolium but, although part replacement of chloride increased the accumulation of lactic acid, complete replacement had no further effect.

The next experiment also examined replacement of chloride by phosphate but over a phosphate range of 0·0015M, 0·006M, 0·024M, and 0·096M. Similar effects

were obtained (Table 3) and analysis showed highly significant linear effects for lactic acid, fructose, and tetrazolium.

The reduction of tetrazolium has been used to demonstrate the activity of succinic dehydrogenase in various tissues, and in the following tests the effects of phosphate and succinate were studied on washed and unwashed spermatozoa. The semen was washed three times in 0·154M NaCl and then suspended in saline containing 0·02M sodium phosphates, 0·02M sodium succinate, or the same levels of both substances. The reduction of tetrazolium after incubation for 3 hr at 37°C was estimated and the mean values for eight ejaculates are given in Table 4. It is

TABLE 4

EFFECTS OF PHOSPHATE AND SUCCINATE ON THE REDUCTION OF TRIPHENYLTETRAZOLIUM CHLORIDE BY RAM SPERMATOZOA (MEANS OF EIGHT REPLICATIONS)

Diluent	Triphenyltetrazolium Chloride Reduced (μg/10^8 cells/hr)	
	Control	Washed
Chloride	3·7	2·3
Phosphate	12·2	10·4
Succinate	10·0	10·0
Phosphate + succinate	14·4	9·4

clear that both phosphate and succinate markedly stimulated reduction of tetrazolium by unwashed cells and that a combination of the two is even better. However, after washing, although both substances stimulated reduction, a combination of the two did not further stimulate it.

IV. DISCUSSION

The reduction of some tetrazolium salts by the activity of cells or isolated enzyme systems is influenced by the gaseous phase present (Nachlas *et al.* 1957) and triphenyltetrazolium chloride has been shown by Hershey, Cruickshank, and Mullins (1958) to require relatively anaerobic conditions for reduction by skin in media containing succinate and phosphate. However, in the presence of added fructose ram spermatozoa appear to reduce tetrazolium to only a slightly less extent in air than anaerobically.

An early report by Lardy and Phillips (1943) indicated that phosphate is required for the maintenance of glycolysis by spermatozoa but has an inhibitory effect on respiration. Recent observations by Bishop and Salisbury (1955) showed that the motility and respiration of bull spermatozoa are severely inhibited by phosphate. Also Salisbury and Nakabayashi (1957) showed that the utilization of

fructose and the accumulation of lactic acid by bull spermatozoa were increased by phosphate. This increase in lactic acid appears to be partly due to the inhibitory effect of phosphate on the oxidation of the acid by spermatozoa (Mann and White 1957; White 1957). However, it is clear that phosphate greatly stimulates the reduction of tetrazolium by ram spermatozoa and also increases the rate of fructolysis.

The reduction of tetrazolium by washed spermatozoa is stimulated by succinate as well as phosphate. Both these ions have been shown by Kearney, Singer, and Zastrow (1955) and Kearney (1957) to combine with the active centre of succinic dehydrogenase and enhance its activity. It is well known that inorganic phosphate plays an essential role in the Embden–Meyerhof glycolytic scheme; in normal brain it is necessary for the oxidation of pyruvate (McIlwain, Buchel, and Cheshire 1951) and for the formation of pyruvate from fumarate (Long 1945). On the other hand, inorganic phosphate inhibits glucose 6-phosphate dehydrogenase, the enzyme catalyzing the first reaction of the hexose monophosphate shunt (Theorell 1935; Kravitz and Guarino 1958). A high phosphate level could inhibit the shunt and allow glycolysis to proceed whereas a low level could exert opposite effects. However, Salisbury and Nakabayashi (1957) have not been able to demonstrate the shunt in bull spermatozoa suspended in chloride media.

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VI. References

Barker, S. B., and Summerson, W. H. (1941).—*J. Biol. Chem.* **138**: 535.

Bishop, M. W. H., and Salisbury, G. W. (1955).—*Amer. J. Physiol.* **181**: 114.

Blackshaw, A. W. (1954).—*Aust. Vet. J.* **30**: 249.

Blackshaw, A. W. (1958).—*Aust. J. Biol. Sci.* **11**: 581.

Hershey, F. B., Cruickshank, C. N. D., and Mullins, L. I. (1958).—*J. Histochem. Cytochem.* **6**: 191.

Kearney, E. B. (1957).—*J. Biol. Chem.* **229**: 363.

Kearney, E. B., Singer, T. P., and Zastrow, N. (1955).—*Arch. Biochem. Biophys.* **55**: 574.

King, T. E., and Mann, T. (1959).—*Proc. Roy. Soc.* B **151**: 226.

Kravitz, E. A., and Guarino, A. J. (1958).—*Science* **128**: 1140.

Lardy, H. A., and Phillips, P. H. (1943).—*Amer. J. Physiol.* **138**: 741.

Long, C. (1945).—*Biochem. J.* **39**: 143.

McIlwain, H., Buchel, L., and Cheshire, J. D. (1951).—*Biochem. J.* **48**: 12.

Mann, T. (1948).—*J. Agric. Sci.* **38**: 323.

Mann, T., and White, I. G. (1956).—*Nature* **178**: 142.

Mann, T., and White, I. G. (1957).—*Biochem. J.* **65**: 634.

Mohri, H. (1957).—*J. Fac. Sci., Tokyo* (IV) **8** (1): 65.

Nachlas, M. M., Tsou, K. C., De Souza, E., Cheng, C. S., and Seligman, A. M. (1957).—*J. Histochem. Cytochem.* **5**: 420.

SALISBURY, G. W., and NAKABAYASHI, N. T. (1957).—*J. Exp. Biol.* **34**: 52.
SMITH, J. T., MAYER, D. T., and MERILAN, C. P. (1956).—*J. Dairy Sci.* **39**: 552.
SMITH, J. T., MAYER, D. T., and MERILAN, C. P. (1957*a*).—*J. Dairy Sci.* **40**: 516.
SMITH, J. T., MAYER, D. T., and MERILAN, C. P. (1957*b*).—*J. Dairy Sci.* **40**: 521.
THEORELL, H. (1935).—*Biochem. Z.* **275**: 416.
WHITE, I. G. (1953).—*J. Exp. Biol.* **30**: 200.
WHITE, I. G. (1957).—*Amer. J. Physiol.* **189**: 307.