

THE MICHAELIS–MENTEN CONSTANT FOR FRUCTOSE AND FOR GLUCOSE OF HEXOKINASE IN BULL SPERMATOZOA

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Summary. The Michaelis–Menten constant (K_M) of fructose and of glucose as substrate for bull spermatozoa has been measured: fructose $K_M = 1.04$ mM, glucose $K_M = 0.06$ mM. The saturation rate of fructose utilization was found to be 1.8 times that of glucose utilization. This is in general agreement with characteristics of hexokinases extracted from different sources.

INTRODUCTION

Spermatozoa of the higher mammals (e.g. bull, dog and human) are known to possess the complete glycolytic–fructolytic pathway. Glucose and fructose as well as the intermediates of the Meyerhof–Emden cycle can be utilized as exogenous substrate. The extensive work done on this subject has been recently reviewed by Salisbury & Lodge (1962) and by Mann (1964).

The rate-limiting factor in glycolysis or fructolysis is generally accepted to be the enzyme hexokinase, which regulates the first phosphorylating step of the hexose. Most experimental material presented leads to the conclusion that at high substrate concentration (>5 mM) the rate of utilization of glucose and fructose by spermatozoa is approximately the same (e.g. Mann & Lutwak-Mann, 1948).

It has been observed (Van Tienhoven, Salisbury, VanDemark & Hansen, 1952; Van Tienhoven, 1960) that, in the presence of glucose, the rate of fructolysis is repressed. Mann (1964) has suggested that this is due to competitive inhibition of hexokinase in spermatozoa.

In the course of experiments aimed at relating metabolic rates to the motility of spermatozoa (Rikmenspoel, 1965), the problem of controlling the rates of fructolysis and glycolysis arose. It was decided to measure the Michaelis–Menten constant (K_M) for fructose and for glucose of bull spermatozoa. A knowledge of these K_M values will show whether it is possible to introduce kinetic controls on the rate of fructolysis (by means of competitive inhibition with suitable analogues) in spermatozoa.

This paper presents the results of experiments which show that the hexokinase from bull spermatozoa has kinetic properties which resemble very closely those of hexokinases from other sources.

MATERIALS AND METHODS

Source of semen

The semen obtained from Holstein bulls was generously provided by the New York Artificial Breeders Cooperative in Ithaca, New York. After collection, the semen was diluted 5 times in a modified Krebs–Ringer phosphate–egg-yolk diluent, described below, and cooled to 4° C. The diluted semen was flown to the laboratory in New York City while being cooled. Experiments were always performed on the same day the spermatozoa had been collected.

Diluent fluid

Egg yolk was fractionated and dialysed against 0.9% NaCl to remove all molecules that could serve for substrate, as previously described (Rikmenspoel, 1965).

The dialysed egg-yolk solution was then diluted with appropriate amounts of stock solutions, such that the final diluent contained: 0.145 M-NaCl, 0.005 M-KCl, 0.002 M-MgSO₄, 0.001 M-CaCl₂, 10% 0.100 M-Na phosphate buffer (pH 7.2), and 10% egg-yolk equivalent.

Analysis of the diluent showed that the content of glucose retained after the dialysis was <3 µg/ml.

Washing and incubation

The spermatozoa were washed twice in the egg-yolk diluent by centrifugation at 400 g for 8 min. It has been reported (Rikmenspoel, 1965) that this washing procedure did not impair the motility of the spermatozoa and presumably enzyme activity remained intact. The washed spermatozoa were diluted in the egg-yolk diluent to contain 10⁸ motile spermatozoa/ml. Glucose or fructose were added in the appropriate amount for each experiment. This entire process was carried out at 4° C.

Incubations were done in a 35° C waterbath, each tube containing 1.0 ml of diluted semen. For each incubated tube, an identical tube was kept at 4° C; the rates of fructolysis–glycolysis were considered negligible at this temperature. After 15 min, the reaction was stopped by precipitation with ZnSO₄ and Ba(OH)₂. Utilization was taken as the difference in substrate content between the 35° C and 4° C incubated tubes.

In the experiments at higher fructose concentration (>300 µg/ml), the errors in the sugar determinations become rather large (see below). For this reason, the incubations at 35° C were done for 45 min, in order to get a bigger utilization of substrate.

Sugar determinations

Fructose and glucose were determined according to Nelson (1944) and Somogyi (1945).

Precipitation of the egg-yolk medium with Zn and Ba salts was often not complete. So all samples were, after the centrifugation which followed the precipitation, heated to 100° C for 20 min and then filtered through millipore filters. The fluid so obtained remained clear throughout the analysis.

The accuracy obtained with the Nelson–Somogyi method was $\pm 3 \mu\text{g}$ (S.D.) of oxidizable sugar. The linear range was found to extend to slightly above $200 \mu\text{g/ml}$. Samples with sugar additions of 200 and $300 \mu\text{g/ml}$ were diluted 2 times, those containing $400 \mu\text{g/ml}$ and more were diluted 4 times with distilled water after the precipitation with Zn and Ba salts. As a consequence, the relative accuracy of the sugar determination was proportionally reduced.

RESULTS

Fructose

On a large number of samples, 15-min incubations were made with additions of 0, 50, 100, 200 and $300 \mu\text{g/ml}$ fructose, and 45-min incubations with 0, 300, 400 and $600 \mu\text{g/ml}$ fructose.

A small amount of fructose from the original seminal plasma always remained, even after washing the spermatozoa twice. The amount of residual fructose in each experiment was taken as the difference between the tubes with no additions before and after incubation. It was judged that this would give a more reliable figure than the difference between the washed semen and the washing fluid only. The amount of residual fructose varied between 4 and $13 \mu\text{g/ml}$, with an average value of $5 \mu\text{g/ml}$.

For presentation of the results, the utilization per 15 min was plotted against the average concentration of fructose present during the incubation. This average concentration was taken to be the amount of the addition plus the residual fructose minus half of the utilization.

The results of all determinations (Text-fig. 1) show considerable spread, but it is clear that on the average the utilization increases with the fructose concentration. The values for the 45-min incubation fall lower than those for 15-min incubation, due to ageing of the spermatozoa during the experiments. This is in agreement with the data of Freund, Mixner & Mather (1957, 1959), who reported that fructolysis decreases by $1.2\%/min$ during incubation. For evaluation of kinetic constants, the averages of the utilization as shown in Text-fig. 1 were corrected by a factor such that at $300 \mu\text{g/ml}$ the two types of experiments were normalized.

Text-fig. 2 shows a Lineweaver & Burk (1934) plot of the averages from Text-fig. 1. The line drawn through the points is a least squares fit in which each point was weighted inversely proportional to the length of the error bar in Text-fig. 2.

The values obtained in this way are: maximum saturated rate (V_{\max}) of fructolysis, $0.029 \mu\text{g mol}$ ($5.2 \mu\text{g}$)/ $\text{min}/10^8$ spermatozoa and Michaelis–Menten constant, 1.04 mM .

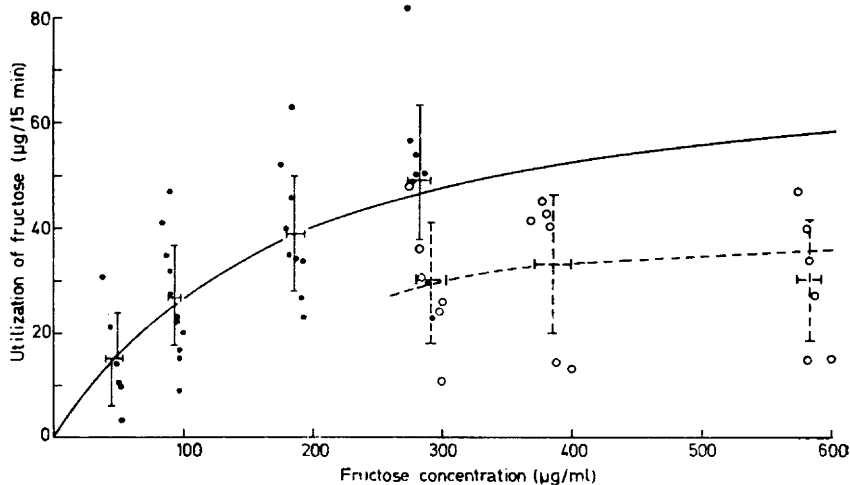
Glucose

Incubations of the washed semen were done for 15 min with addition of 0, 25, 50, 100 and $200 \mu\text{g/ml}$ glucose.

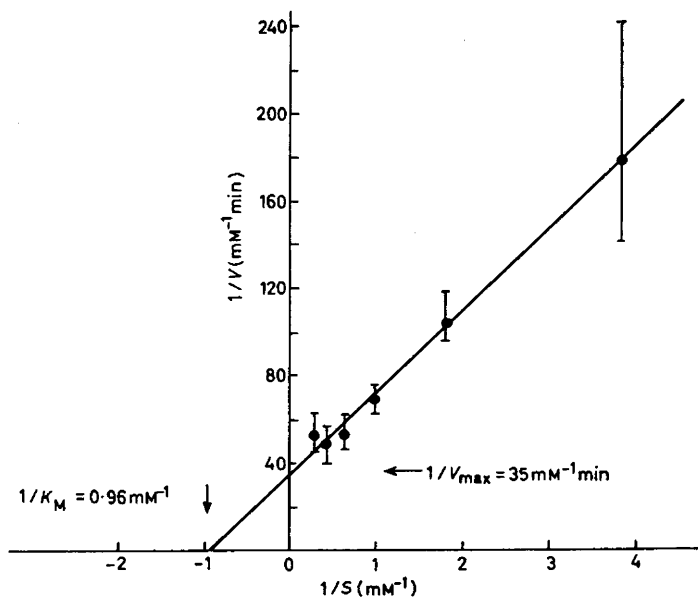
Since residual fructose, left from the seminal plasma after the washing procedure, is, in the present series of experiments, to be considered an artifact, special care was taken in washing the semen. Utilization of residual fructose is shown by

the sample on which no glucose was added. For the six series of incubations this fructose utilization varied from 4 to 9 μg , with an average of 7 μg .

For each experiment it was assumed that in the tubes with various additions



TEXT-FIG. 1. Utilization of fructose/15 min as a function of the fructose concentration. The vertical bars show the standard deviation of the points grouped together as indicated. Sperm concentration $10^8/\text{ml}$; incubation time: ●, 15 min; ○, 45 min; temperature 35°C .



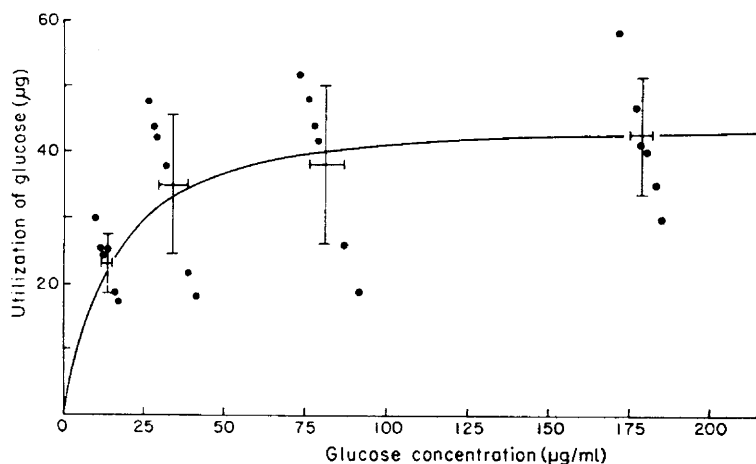
TEXT-FIG. 2. Lineweaver & Burk plot of the averages of the points, grouped as shown in Text-fig. 1. The vertical bars represent the standard errors.

of glucose, the utilization of residual fructose was the same as in the tube with no addition. The total hexose disappearance was accordingly corrected in each case to obtain the glucose utilization. For the higher glucose concentrations, the

correction thus applied is probably too big, due to the repression of fructolysis by glucose. The error introduced by this possibly slight over-correction is not important however, in view of the other errors present in the experiment.

Text-fig. 3 shows the results of the six experiments. The glucose utilization is plotted against the average glucose concentration; this latter was taken as the amount of the addition minus half the utilization.

Close inspection of Text-fig. 3 shows that the samples with 25 μg addition have an average utilization close to this amount. This means that the figure for the utilization at that concentration may be on the low side. Shorter incubation is not well possible, however, in view of the error in the determinations. Text-fig. 4 shows a Lineweaver & Burk plot of the averages of Text-fig. 3. As a good



TEXT-FIG. 3. Glucose utilization as a function of the glucose concentration. The vertical bars are the standard deviations of the points grouped together as indicated. Sperm concentration $10^8/\text{ml}$, incubation time 15 min, temperature 35°C .

linear relation appears in Text-fig. 4, the error in the point for 25 μg addition mentioned above is probably not serious,

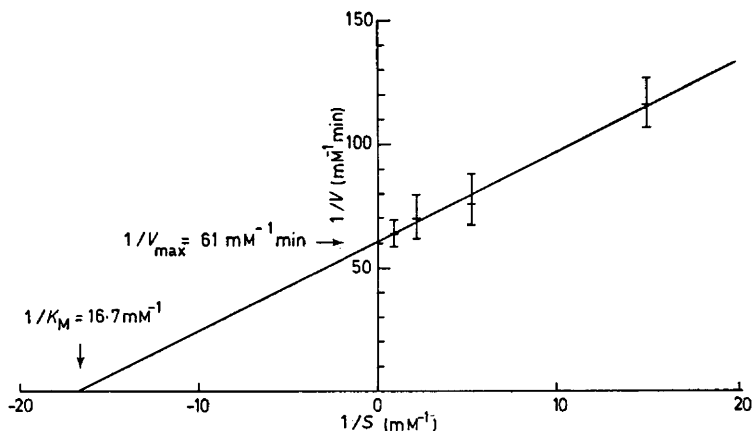
The least squares fit shown in Text-fig. 4, gives for the kinetic data on glycolysis: $V_{\text{max}} = 0.016 \mu\text{mol}$ ($2.9 \mu\text{g}$)/min/ 10^8 spermatozoa; $K_M = 0.06 \text{ mM}$.

Effect of respiration

In ejaculated bull spermatozoa, the respiration is supposed to have little or no repressive effect on the rate of the Meyerhof Emden cycle (Lardy & Phillips, 1941, 1943; Schmidt, Lodge & Salisbury, 1959). However, the experiments which are referred to were performed with the concentrations of substrate which are high compared to the K_M values found in the present paper. For this reason, the rate of fructolysis at a fructose concentration of 50 and 100 $\mu\text{g}/\text{ml}$ was compared with that in the same conditions, but with 10 mM amytal sodium added. It is known (Gonse, 1962; Rikmenspoel, 1965) that this concentration causes about 80 to 90% reduction of the respiration of bull spermatozoa.

The results of six experiments are summarized in Table 1. It is clearly shown

that, at substrate concentrations below the K_M value, the influence of respiration on the rate of fructolysis is also small. The validity of the kinetic data reported is therefore ascertained.



TEXT-FIG. 4. Lineweaver & Burk plot of the averages of the data of Text-fig. 3. The vertical bars are the standard errors.

TABLE 1

UTILIZATION OF FRUCTOSE WITH AND WITHOUT INHIBITION OF THE RESPIRATION BY 10 mM AMYTAL SODIUM, AVERAGED OVER SIX EXPERIMENTS

Fructose addition ($\mu\text{g/ml}$)	Utilization and standard deviation ($\mu\text{g}/15 \text{ min}$)	
	No amytal	10 mM amytal
50	16 ± 5	19 ± 5
100	27 ± 9	25 ± 7

DISCUSSION

The results presented in the previous section strongly suggest the existence of a hexokinase in bull spermatozoa with properties which are analogous to those of other hexokinases. Table 2 shows a comparison of the present kinetic data with figures reported in the literature on hexokinases extracted from different sources by Crane (1962). It can be seen that the present K_M values are well in the range of those found before. The ratio of the maximum rates for fructolysis to glycolysis of 1.8 reported here, is in agreement with the figures of 1.5 to 2.4 for other hexokinases.

Our present data show a maximum rate of fructolysis of about 3×10^6 molecules of fructose/sec/spermatozoon. The ATP yield of this fructose turnover is, at a net gain of 2 ATP/fructose, 6×10^6 ATP/sec/spermatozoon.

Earlier experiments (Rikmenspoel, 1965), showed that normally motile spermatozoa, in which fructolysis was excluded by washing out the substrate, had a turnover of oxygen of approximately 3.3×10^6 molecules O_2 /sec/spermatozoon. The maximum ATP yield in this case is about 20×10^6 ATP/sec/spermatozoon. It was found that the work performed by the motility of the spermatozoa accounted for approximately 20% of the energy which could become available from hydrolysis of the formed ATP.

It has been reported by many authors (compare Mann, 1964, and Bishop,

TABLE 2

KINETIC DATA FOR HEXOKINASES FROM DIFFERENT SOURCES (CRANE, 1962), AND PRESENT RESULTS ON BULL SPERMATOZOEA

	Source of material				
	Calf brain	Baker's yeast	Ascites tumour	Sea urchin eggs	Bull sperm. (this paper)
K_M for fructose (mM)	1.6	0.7	2.5	0.75	1.04
K_M for glucose (mM)	0.008	0.1	0.028	0.03	0.06
$\frac{\text{Max. rate fructose}}{\text{Max. rate glucose}}$	1.5	1.8	2.4	1.8	1.8

1962) that fructolysis alone, under anaerobic conditions, can maintain the full motility of bull spermatozoa. The figures reported in this paper would, in that case, mean that either the 'efficiency' of fructolysis is three times higher than that of respiration, or that the coupling of the oxidative phosphorylation is much less than optimum. Quantitative data on rates of fructolysis, respiration and motility taken under various conditions should be of great interest in clarifying this situation.

ACKNOWLEDGMENT

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