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Control of respiration and of motility in ejaculated bull spermatozoa

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The relations between motility and respiration were studied in ejaculated bull spermatozoa respiring with lactate. Motility was quantitatively evaluated by a turbidimetric procedure as percentage of cells moving per minute from the bottom of the cuvette into the light path. For selective inhibition of ATP-consuming reactions including motility or of mitochondrial respiration, vanadate or cyanide, respectively, were used. Both inhibitors were found to produce proportional changes in motility and respiration. The simultaneous changes in motility and respiration were linked to shifts in the cellular ATP/ADP ratio. Partial uncoupling of respiration in vanadate-inhibited cells gave similar relations between respiration and ATP/ADP ratios as stepwise inhibition of ATP-utilizing reactions by vanadate. Presuming saturation kinetics with respect to the ATP/ADP ratio, half maximum constants of 1.7 and 4.7 for the ATP/ADP ratio and maximum values of about 130% and 300% (in comparison to untreated cells) were estimated for motility and respiration, respectively. Respiration showed a much steeper dependence on the ATP/ADP ratio than motility resulting in an apparent cooperativity coefficient of 2.9. From these dependences on the ATP/ADP ratio, the shares in the control of ATP turnover in untreated cells were estimated. At sufficient supply with substrate, more than 80% of control were excerted by motility and other ATP-utilizing reactions, the rest by mitochondrial ATP production, i.e., the reactions of oxidative phosphorylation.

Introduction

Motility is considered to be the most important process of ATP turnover in spermatozoa. But only few quantitative data have been reported on the share of motility in the cellular energy metabolism. This is linked with the problem to get a quantitative measure of motility in a sperm population. For sea urchin spermatozoa made permeable to extracellular ATP, about 70% of the total ATPase activity were found to be directly coupled with cell motion [1]. A similar investigation was reported for rabbit spermatozoa [2].

Recently, we have found that the simple turbidimetric procedure of Sokoloski et al. [3] allows to derive a relative measure of motility which is correlated to the oxygen uptake of intact bull spermatozoa [4]. In the present study we applied this method to follow the simultaneous effects on motility and respiration produced by specific inhibitors. The experiments were performed with ejaculated bull spermatozoa in the presence of lactate in order to allow high rates of aerobic ATP formation [5]. Since inhibition of motility or respiration is linked with changes in the cellular adenine nucleotide pattern [6] we could derive kinetic parameters of the dependence on the cellular ATP/ADP ratio for motility and respiration. With these data, the shares of motility and respiration in the control of ATP turnover were estimated

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

using the theoretical concepts of metabolic control developed by Kacser and Burns [7] and Heinrich and Rapoport [8].

Materials and Methods

Materials

Sodium monovanadate was obtained from Merck (Darmstadt, F.R.G.) and lactic acid was from Sigma Chemical Co. (St. Louis, U.S.A.). Enzymes and other biochemicals were from Boehringer-Mannheim GmbH (Mannheim, F.R.G.). All other chemicals were purchased in analytical grade from Laborchemie (Apolda, G.D.R.).

Experimental procedure

For each experiment, washed bull spermatozoa were obtained from 3 or 4 fresh ejaculates [6]. The portion of intact cells was more than 80% on the basis of succinate exclusion [9]. The incubation medium contained 10 mM lactate/140 mM NaCl/6 mM KCl/1.5 mM $MgCl_2$/20 mM Tris/10 mM Hepes, adjusted to pH 7.4 with NaOH, and the additions given in the figures. Cells were incubated in Erlenmeyer flasks under gentle shaking at 37°C. After 5 min of incubation, samples were taken for determination of adenine nucleotides, of sperm motility and of respiration. It was checked in separate experiments that the pattern of adenine nucleotides were constant between 2 and at least 10 min of incubation.

Respiration was measured in an oxygraphic vessel with a Clark-type electrode. Motility was determined by a turbidimetric method [3] as a percentage of total number of cells moving per min in the light path of an optical cuvette, as described in detail in Ref. 4. The adenine nucleotides were fluorimetrically assayed by enzymic standard procedures [10] in neutralized extracts after quenching with perchloric acid. The disturbance in the determination of ADP with pyruvate kinase and lactate dehydrogenase in vanadate containing samples [6,11] was found to be caused by a rapid nonenzymic removal of a part of NADH. This interference could be prevented by a sufficient excess of NADH. It was checked by internal standards that the full amounts of ADP and AMP were recovered under this condition. The concentration of cells in the incubation mixtures was 14.4–21.7 µl packed cells/ml, determined by high-speed centrifugation (about $1.5 \cdot 10^7$ cells/µl [9]).

Estimation of kinetic parameters

Mitochondrial respiration (v_{resp}) and motility (v_{mot}) were assumed to depend on the cellular ATP/ADP ratio according to the following empiric equations:

$$v_{resp} = \frac{V_{resp}}{1 + (X/K_{resp})^n} \quad (1)$$

$$v_{mot} = \frac{V_{mot}}{1 + K_{mot}/X} \quad (2)$$

where X is the cellular ATP/ADP ratio; V_{resp} and V_{mot} are the maximum velocities; K_{resp} and K_{mot} are the ATP/ADP ratios for half-maximum rates; and n is an apparent cooperativity coefficient. Whereas the motility was directly measured by the turbidimetric method, the respiratory rate of intact cells was taken as a difference of the measured rate of total oxygen uptake and that part insensitive to inhibition by vanadate (cf. Results). The parameters in Eqns. 1 and 2 were estimated by a least-square fit to the experimental data [12].

Calculation of control coefficients

As shown in Results, the motility v_{mot} (percent motile cells/min) was found to be proportional to the vanadate-sensitive respiration

$$v_{resp} = m v_{mot} \quad (3)$$

where m is the oxygen equivalent of motility (nmol O_2 per µl cells per percent motile cells). Therefore, both rates v_{resp} and v_{mot} were considered to be proportional to the ATP turnover in the cells. The shares of mitochondrial ATP production and of cellular ATP consumption in the control of ATP turnover is then given by the connectivity theorem of Kacser and Burns [7] as

$$C_{prod}\varepsilon^{resp} + C_{cons}\varepsilon^{mot} = 0 \quad (4)$$

Here are C_{prod} and C_{cons} the overall flux control coefficients (for the nomenclature, cf. Ref. 13) of mitochondrial ATP production and of ATP-con-

suming extramitochondrial enzymes; ε^{resp} and ε^{mot} are the elasticity coefficients of respiration and of motility with respect to the cellular ATP/ADP ratio. If respiration or motility is partially inhibited by specific inhibitors, not only the ATP/ADP ratio in the cell is changed, but also other metabolites, particularly AMP and inorganic phosphate. Such changes may influence the rates of respiration and motility, too. Since these changes are correlated with the ATP/ADP ratio, they are implicitly covered by the rate equations (Eqns. 1 and 2) and also included in the elasticity coefficients derived from these equations. It was proven that the connectivity theorem is also valid in that case, where the elasticity coefficients refer to a ratio of concentrations instead of the concentration alone [14,15]. Taking into account the summation theorem [7,8]

$$C_{prod} + C_{cons} = 1 \tag{5}$$

it follows

$$C_{cons} = \frac{-\varepsilon^{resp}}{\varepsilon^{mot} - \varepsilon^{resp}} \tag{6}$$

and

$$C_{prod} = \frac{\varepsilon^{mot}}{\varepsilon^{mot} - \varepsilon^{resp}} \tag{7}$$

The elasticity coefficients ε^{mot} and ε^{resp} were obtained from the rate laws, Eqns. 1 and 2, by numeric differentiation according to their definition

$$\varepsilon^{i} = \frac{d \ln v_i}{d \ln X} \tag{8}$$

Results

Vanadate was found to be an inhibitor for motility of intact bull spermatozoa [6]. But also other ATP-utilizing reactions must be inhibited by vanadate, since the total inhibition of ATP turnover by oligomycin diminished respiration to the same extent as vanadate [6]. The simultaneous effects of increasing concentrations of vanadate on motility and respiration are shown in Fig. 1.

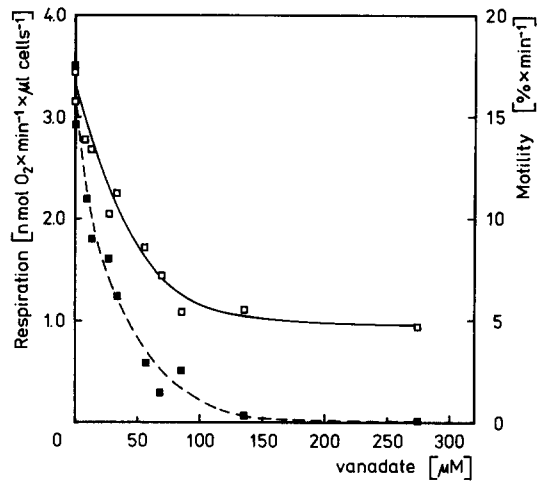

Fig. 1. Effect of vanadate on respiration (open symbols) and motility (filled symbols) of ejaculated bull spermatozoa. Respiration and motility were simultaneously measured after 5 min incubation in standard medium containing the indicated concentrations of vanadate.

The inhibition seems to be parallel for both processes, only for high concentrations of vanadate, where motility was already zero, a slight further decrease of respiration was observed. A similar picture was found if respiration was inhibited by cyanide, as demonstrated in Fig. 2. But the portion of oxygen uptake insensitive to the inhibitor was much smaller than with vanadate.

In order to test the relation between respiration and motility more sensitively, they were plotted versus each other. As seen from Fig. 3, there is a linear correlation within the accuracy of the measurements. The points for complete inhibition of motility were omitted from the calculation of the regression lines. The corresponding respiratory rates are given in Table I together with the parameters of the regression lines from several experiments. For vanadate, the rates measured with excess of the inhibitor were significant smaller than the rates obtained from the intercepts of the regression lines, the mean difference was about 10% of the total oxygen uptake in the absence of inhibitors. This indicates a different sensitivity to vanadate of motility and other ATP-utilizing processes. The slope of the regression lines for vanadate gives the oxygen equivalent consumed by oxidative phosphorylation to support motility

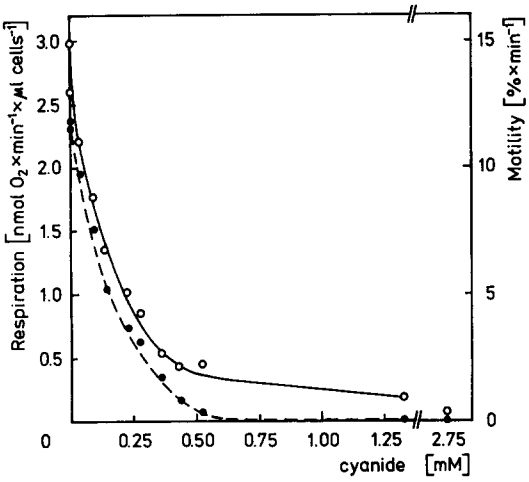

Fig. 2. Effect of cyanide on respiration (open symbols) and motility (filled symbols) of ejaculated bull spermatozoa. The experiment was performed as in Fig. 1 with cyanide instead of vanadate.

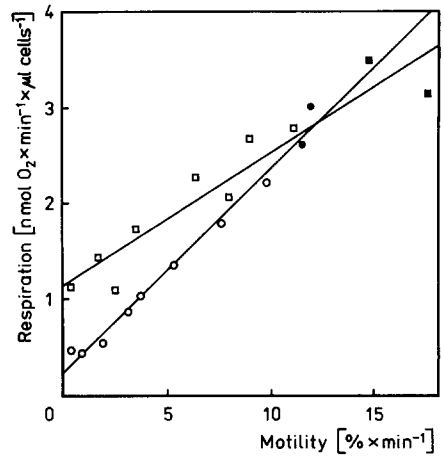

Fig. 3. Linear correlation of respiration and motility. The data points were from Figs. 1 (□, ■) and 2 (○, ●); the open symbols refer to incubations with the inhibitors cyanide or vanadate, respectively, the filled symbols to inhibitor-free incubations. The data of the calculated regression lines are given in Table I as Expts. 1 and 6 for both inhibitors.

(and probably additional ATP-utilizing reactions with a similar sensitivity to vanadate). From the steeper slope of the regression lines for cyanide follows that additional energy-utilizing reactions were inhibited in parallel to motility. A significantly different sensitivity to cyanide inhibition of motility and other energy-dependent reactions had to produce systematic deviations from the straight line and could not be detected.

The inhibition of motility by cyanide must be caused by the decreased rate of oxidative phosphorylation. Therefore, diminished cellular ATP/ADP ratios should be expected to be the immediate reason for the reduced motility. Fig. 4 presents the relations found with two different sperm preparations. Presuming simple saturation kinetics with respect to the ATP/ADP ratio (cf. Eqn. 2), a half-maximum constant of 1.7 for the ATP/ADP ratio and a maximum motility corresponding to about 130% of the value in absence of cyanide were estimated from the data. The maximum value is somewhat uncertain, since the measured points were far from the saturation plateau. But a comparable value could be estimated from experiments where the ATP/ADP ratio was varied by incubation with different concentrations of lactate (cf. Fig. 4 in Ref. 6).

The stepwise inhibition of ATP consumption by vanadate should allow to measure the dependence of oxidative phosphorylation on the cellular ATP/ADP ratio. But the respiration of cells with lactate in absence of any further additions is much

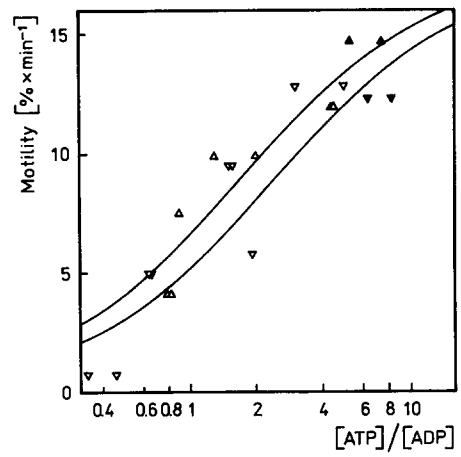

Fig. 4. Dependence of motility on the cellular ATP/ADP ratio. The ATP/ADP ratio was varied by partial inhibition of respiration with cyanide (open symbols, the filled symbols refer to incubations without inhibitor) as in Fig. 2. ATP and ADP were measured in samples taken at the same time as the sample for determination of motility. The curves were computed from Eqn. 2 for the best fit to the points, the obtained parameter values were $V_{mot} = 18$ and 16% per min, $K_{mot} = 1.7$ and 1.7 for both experiments (△, ▲ and ▽, ▼, respectively).

TABLE I

CORRELATION BETWEEN RESPIRATION AND MOTILITY OF SPERMATOZOA INHIBITED BY CYANIDE OR VANADATE

Data are given for regression lines as in Fig. 3 from different sperm preparations. For comparison, the respiratory rate measured with an excess of inhibitor at completely inhibited motility (at least 1.4 mM cyanide or 0.28 mM vanadate, respectively) is also presented; these points were not included in the regression line

Experiment	Respiration with excess of inhibitor (nmol O_2 per min per μl cells)	Regression line			
		intercept (nmol O_2 per min per μl cells)	slope (nmol O_2 per % per μl cells)	regression coefficient	data points
Inhibitor cyanide					
1	0.08	0.23	0.213	0.993	10
2	0.27	0.26	0.196	0.966	5
3	0.43	0.35	0.270	0.960	6
4	0.26	0.49	0.170	0.985	6
5	0.25	−0.23	0.182	0.951	5
Mean ± S.D.	0.26 ± 0.12	0.22 ± 0.22	0.206 ± 0.039		
Inhibitor vanadate					
6	0.92	1.13	0.139	0.948	10
7	0.81	0.81	0.135	0.979	11
8	1.09	1.37	0.131	0.886	6
9	1.30	1.74	0.165	0.926	6
10	0.84	1.05	0.143	0.997	6
11	0.43	1.06	0.132	0.945	6
Mean ± S.D.	0.90 ± 0.29 [a]	1.19 ± 0.32 [a]	0.141 ± 0.013		

[a] Difference significant in paired t-test ($p < 0.025$).

smaller than the maximum rate found with uncouplers [5], and also the further stimulation of motility with caffeine led only to a slight increase in respiration [6]. In order to vary the respiration over a larger range, the cells completely inhibited with vanadate were titrated with the uncoupler FCCP. The comparison of the points in Fig. 5 demonstrates that the drain of energy by partial uncoupling instead of ATP turnover does not produce systematic deviations in the relation between the rate of respiration and the cellular ATP/ADP ratio. The curve in Fig. 5 of the best fit gave a maximum velocity of respiration which is more than the threefold of the portion of vanadate-sensitive respiration of untreated cells. The dependence on the ATP/ADP ratio was much steeper than found for motility and corresponded to an apparent cooperativity coefficient of 2.9. Also the half-maximum constant of 4.7 differed from the value of 1.7 obtained for motility. In order to allow a direct comparison to motility, the respiratory rates in Fig. 5 were corrected by the vanadate-insensitive part (assuming the latter is independent of the ATP/ADP ratio). Therefore, the full line in Fig. 5 is considered to be proportional to mitochondrial ATP production. The oxygen equivalent of ATP consumption which is proportional to motility is given by the slope of the regression line found with vanadate (cf. Fig. 3). This value was used to express the ATP consumption in the scale of oxygen equivalents (the dashed line in Fig. 5). The intersection of both curves gives the ATP/ADP ratio for which in absence of inhibitors the rates become equal, i.e., in untreated cells.

The data collected in Fig. 5. allowed to estimate the shares in the control of ATP turnover exerted by oxidative phosphorylation and by ATP-utilizing reactions. Kacser and Burns [7] and Heinrich and Rapoport [8] have shown that in a metabolic

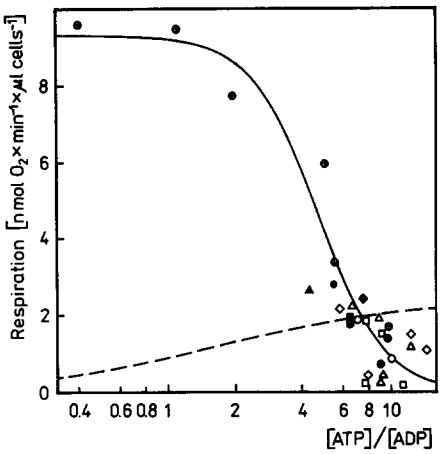

Fig. 5. Dependence of vanadate-sensitive respiration on the cellular ATP/ADP ratio. The experiments were performed as in Fig. 4 with the following additions to the incubation medium: filled symbols, no; open symbols, 0.014–0.14 mM vanadate; ⊕ 0.34 mM vanadate + 0.01–0.34 µM FCCP. The respiratory rates were corrected by the rate measured in presence of at least 0.28 mM vanadate. The figure was compiled from several experiments distinguished by the shape of the symbols. The circles (●, ○, ⊕) were from the same experiment. Only these points were used to construct the full line for the best fit to Eqn. 1, the resulting parameter values were $V_{resp} = 9.3$ nmol O_2 per min per µl cells, $K_{resp} = 4.7$ and $n = 2.9$. The dashed line indicates the motility-linked oxygen consumption, it was calculated from Eqns. 2 and 3 for the values $m = 0.14$ nmol O_2 per % per µl cells (cf. Table I) and $V_{mot} = 17\%$ per min, $K_{mot} = 1.7$ (cf. Fig. 4).

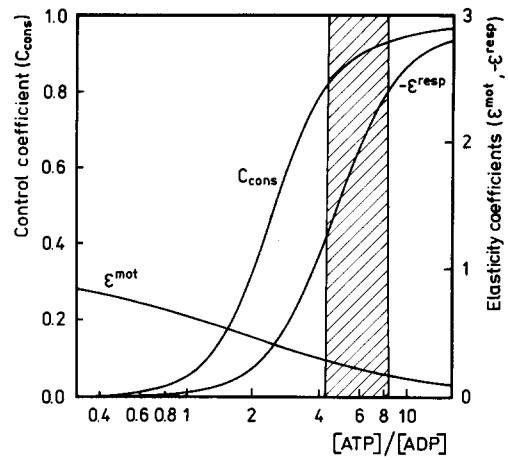

Fig. 6. Elasticity and control coefficients in dependence on the cellular ATP/ADP ratio. According to Eqn. 8, the elasticity coefficients ε^{mot} and ε^{resp} were obtained by numerical differentiation of the rate equations, Eqns. 1 and 2, with the parameter values in Fig. 5. The overall control coefficient C_{cons} of the ATP-consuming enzymes was calculated from the elasticity coefficients as given in Eqn. 6. It should be noted that because of the relation $C_{prod} = 1 - C_{cons}$ also the overall control coefficient C_{prod} of mitochondrial ATP production can be read from the figure. The shaded region markes the ATP/ADP ratios found in cells without inhibitors.

pathway the control of the flux is distributed among the various steps in it. The contribution of each step is given by its control coefficient (cf. the recently proposed nomenclature [13]) which is defined as the fractional change in the flux caused by a fractional change in the capacity of that step (e.g., a change in the amount of an active enzyme). The control coefficients can be calculated if the elasticity coefficients of the various steps are known (see Methods). The elasticity coefficients describe the dependence of the velocity of the single steps on the metabolic intermediates by which they are linked in the pathway. For the respiration coupled to ATP production and for motility, the elasticity coefficients with respect to the cellular ATP/ADP ratio are available from the curves in Fig. 5 (cf. Eqn. 8). As shown in Fig. 6 the absolute values of these elasticity coefficients change in dependence on the ATP/ADP ratio in opposite directions. Because of the apparent cooperativity found for respiration its negative elasticity coefficient grows to much higher absolute values than that of motility. This has consequences for the share in control of the ATP flux between ATP production and ATP consumption, which is indicated by the curve of the control coefficient. In absence of inhibitors, a cellular ATP/ADP ratio results where the (negative) elasticity coefficient of respiration considerably exceeds that of motility. This produces a large share of the ATP-consuming reactions in the control of more than 80%. If the substrate concentration is reduced, much smaller ATP/ADP ratios were observed [6]. Providing the elasticity coefficient of respiration is not markedly changed under this condition, the effect on the shares in control can be read for any cellular ATP/ADP from the curve in Fig. 6. With decreasing ATP/ADP ratios the control exerted by mitochondrial ATP production grows at the cost of ATP-utilizing reactions.

Discussion

The mutual changes in respiration and motility produced by vanadate and cyanide give informations about the reactions which contribute to the oxygen turnover of the bull spermatozoa. The 5–10% that were insensitive to cyanide must be caused by extramitochondrial processes of unknown nature, although an extramitochondrial generation of hydrogen peroxide is described for rabbit spermatozoa [16]. From the inhibition by vanadate follows that about 70% of oxygen are consumed by oxidative phosphorylation of ADP, since oligomycin inhibits to the same extent [6]. At least 10% of the total oxygen uptake were caused by other ATP-utilizing reactions than motility because of the lower sensitivity to vanadate. We found earlier that the inhibition of the $(Na^+ + K^+)$ ATPase by ouabain reduced the respiration by 17% [6]. The remaining 20% of respiration which could be inhibited by cyanide, but not by vanadate, must result from uncoupling of mitochondria. If this would be mainly caused by the proton leak in coupled mitochondria of motile cells, motility must be more sensitive than respiration to cyanide. This was not found; therefore, it is more likely that the degree of coupling is high in motile cells and the different extent of inhibition results mainly from the 10–15% of damaged cells present in the ejaculates.

The proportional changes in respiration and motility caused by specific inhibition of the one or the other process were here described in terms of the cellular ATP/ADP ratio, which is generally considered to be the most important signal in the coordination of ATP turnover. Of course, changes in the ATP/ADP ratio are linked to those in other metabolites, in particular AMP and inorganic phosphate. Therefore, the rate equations (Eqns. 1 and 2) are only apparent ones. Also the scatter of the measured ATP/ADP ratios have to be taken into consideration. So we cannot decide whether the drain of energy by partial uncoupling or by ATP turnover produce deviations in the relation between respiration and the ATP/ADP ratio as it was demonstrated with rat liver mitochondria [17]. Despite these limitations, the data indicate that the respiration is much more sensitive than motility to changes in the metabolite pattern indicated by the ATP/ADP ratio.

The shares in the control of energy metabolism were only given for the ATP flux without distinguishing between motility and other ATP-utilizing reactions. A theoretical analysis showed that the control coefficients of competing reactions are proportional to the flux rates through these reactions independent of their elasticity coefficients [18]. If 75% of the ATP turnover in the spermatozoa are linked to cell motion [1,6], its control coefficient is also 75% of the overall control coefficient of ATP-utilizing reactions. For the large control coefficient of ATP utilization a high cellular ATP/ADP ratio is necessary; otherwise, the elasticity coefficients are not different enough in their absolute values. Therefore, a substrate is required which can be oxidized with a sufficient rate; with other substrates much smaller ATP/ADP ratios result [5]. Besides for lactate, great capacities of respiration were found in bull spermatozoa also for pyruvate, acetate and glycerol [5]. Then the reactions of oxidative phosphorylation exert only a small control on the ATP turnover, so that the motility is nearly independent of small fluctuations in the substrate supply. This indicates the perfect adaptation of the metabolism in spermatozoa to be an active carrier of the male genome.

Acknowledgements

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