Biochimica et Biophysica Acta 808 (1985) 316–322
Elsevier

BBA 41793

Interdependence of mitochondrial ATP production and extramitochondrial ATP utilization in intact spermatozoa

Walter Halangk, Ralf Bohnensack and Wolfgang Kunz

Institut für Physiologische Chemie, Medizinische Akademie Magdeburg, Leipziger Strasse 44, 3090 Magdeburg (G.D.R.)

(Received December 20th, 1984)
(Revised manuscript received April 10th, 1985)

Key words: Respiration; Vanadate; Oxidative phosphorylation; Dynein ATPase; Caffeine; (Bovine spermatozoa)

The dependence of both respiration and total activity of ATP-consuming reactions on the cellular adenine nucleotide pattern was investigated in intact bovine spermatozoa. ATP consumption was manipulated by inhibition with vanadate and activation with caffeine, leading to a decrease or increase in the rate of respiration up to 70% or 20%, respectively. Oligomycin blocked the respiration to the same extent as did vanadate, suggesting that the total extramitochondrial ATP-consuming activity is vanadate-sensitive. The major part of ATP utilization must be linked to dynein ATPase, since inhibition of (Na^+, K^+) ATPase by ouabain showed only a small effect on respiration (−17%). Being a potent inhibitor of dynein ATPase, vanadate drastically reduced the amount of motile cells, whereas caffeine tended to increase the intensity of motion. The effects of vanadate or caffeine on respiration were paralleled by changes in cellular ATP, reflecting the response of mitochondrial respiration on the cellular ATP/ADP ratio. Respiration was found to depend on changes in the ATP/ADP ratio in the range from about 3 (+caffeine) to 9 (+vanadate). The range of response of ATP consumption to the ATP/ADP ratio was determined by varying the mitochondrial ATP production via the concentration of lactate which was used as substrate. The measured effects on both respiratory rate and ATP/ADP ratio suggested that ATP consumption was markedly dependent on ATP/ADP ratios below 5. It is concluded that lactate concentrations above 1 mM sufficiently supply bovine spermatozoa with substrate and the energy turnover is mainly limited by the activity of dynein ATPase rather than by the capacity of mitochondrial oxidative phosphorylation.

Introduction

For isolated mitochondria, the dependence of respiration on the extramitochondrial ATP/ADP ratio was determined by varying extramitochondrial ATP utilization [1–3]. The results of these studies imply that the activity of mitochondrial respiration in the absence of any intramitochondrial ATP requirement is controlled by the extramitochondrial adenine nucleotide pattern resulting from the activity of ATP-utilizing enzymes. This concept should be true of the situation within intact cells, too. Because of their simple energy metabolism, spermatozoa appear to be a suitable object for investigating this problem. ATP turnover is mainly determined by only one enzyme, dynein ATPase, as derived from studies on sperm tail models [4]. Variations in the activity of this enzyme should be a suitable approach to ascertain the response of respiration to changes in the extramitochondrial adenine nucleotide pattern in intact cells.

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

In addition to cellular respiration, the rate of ATP utilization is also expected to depend on the ATP/ADP ratio. Recently, it was demonstrated that high-degree motility requires substrates which can be rapidly oxidised so that a high cellular ATP/ADP ratio results [5].

In the present paper, investigations are described which analyze the interdependence of aerobic ATP production and ATP utilization, especially the dependence of both processes upon the adenine nucleotide pattern. To this end, ATP-utilizing reactions were varied by incubations of cells with vanadate, ouabain, or caffeine. Vanadate is known to inhibit dynein ATPase [6], yet other ATP-linked enzymes are influenced as well (for review see Refs. 7 and 8). Ouabain was used to block (Na^+, K^+) ATPase. Caffeine has been described as activating sperm motility [9–11]. The rate of mitochondrial ATP production was manipulated via the concentration of the substrate oxidised.

The present findings demonstrate that even under saturating supply of substrate the rate of respiration and the activity of ATP-consuming processes are related to each other by the cellular ATP/ADP ratio.

Materials and Methods

Spermatozoa of fresh bull ejaculates were separated from seminal plasma by centrifugation at $600 \times g$ for 6 min within 1 h after ejaculation. Additionally, the cells were washed three times in a medium containing 120 mmol NaCl/50 mmol Hepes per litre, pH 7.4 (37°C). For experiments in which the energetic situation of the cells was studied in the complete absence of exogenous substrates, spermatozoa were washed five times. Only sperm suspensions containing more than 80% intact cells and at least 60–70% motile cells were used. Viability was estimated by an oxygraphic method based on the restricted permeability of the cellular membrane for externally added succinate [12]. Sperm concentrations were determined as volume of cells after high-speed centrifugation, where 1 μl cells corresponds to about $1.5 \cdot 10^7$ spermatozoa [12].

For metabolic studies, a basic medium was used containing 140 mmol NaCl/6 mmol KCl/1.5 mmol $MgCl_2$/20 mmol Tris/10 mmol Hepes per litre (pH 7.4). Incubations were performed in Erlenmeyer flasks under gentle shaking at 37°C. Oxygen consumption was determined by a polarographic method using a Clark-type electrode. From the incubation mixtures, samples were taken and quenched by perchloric acid to determine total cellular adenine nucleotides by fluorometric standard procedures [13]. Percentage motile cells were estimated by counting under the microscope. To this end, spermatozoa were preincubated for 5 min in the presence of substrate and effectors. Cells were then diluted with medium of the same composition. Immotile cells in this sample were counted in a chamber for erythrocyte counting. Total cell counts were determined in the same way after dilution with distilled water. The mode of motion was not further characterized.

Chemicals and enzymes

Yeast hexokinase (EC 2.7.1.1.), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), lactate dehydrogenase (EC 1.1.1.27), adenylate kinase (EC 2.7.4.3.), pyruvate kinase (EC 2.7.1.40), phosphoenolpyruvate, ATP, ADP, $NADP^+$, NADH and FCCP were purchased from Boehringer (Mannheim). Rotenone and L(+)lactic acid were obtained from Sigma (St. Louis), oligomycin from Calbiochem (San Diego), and antimycin A from Serva (Heidelberg). Ouabain (*g*-strophanthin) and caffeine were products from Arzneimittelwerk (Dresden). Vanadate was purchased as anhydrous sodium monovanadate from Merck (Darmstadt) and, while being heated, dissolved to a stock solution (0.1 M) in the basic medium. All other chemicals were analytical grades.

Results

Effect of ATP utilization on sperm motility and respiration

Washed ejaculated spermatozoa incubated in the presence of 10 mM lactate exhibited a high percentage of motile cells [5]. Table I shows that percentage motile cells drastically decreased in the presence of 0.22 mM vanadate. A similar inhibition was observed if ATP was exclusively produced by fructolysis and respiration was blocked by antimycin A. Caffeine was tested to stimulate

TABLE I

EFFECT OF VANADATE, OUABAIN AND CAFFEINE ON SPERM MOTILITY

Motility is given as percentage of cells which showed some motion after 5 min preincubation (14.3–19.4 μl cells/ml) with substrate and effectors at 37°C. Data as $\bar{X} \pm$S.D. from (n) ejaculates.

Substrate	Effector	Motility (%)
Lactate (10 mM)		
	–	72 ± 11 (5)
	Vanadate (0.22 mM)	5 ± 6 (4)
	Ouabain (1 mM)	67 ± 3 (3)
	Caffeine (10 mM)	78 ± 9 (3)
Fructose (10 mM) + Antimycin A (5 μM)		
	–	77 ± 10 (3)
	Vanadate (0.22 mM)	7 ± 2 (2)

TABLE II

SPERM RESPIRATION IN THE PRESENCE OF EFFECTORS OF ATP TURNOVER

Spermatozoa (13.1–21.9 μl cells/ml) were preincubated for 5 min in the presence of 10 mM lactate plus effectors indicated. Percentage of effects is calculated in relation to the unaffected sample of the same experiment. Given are $\bar{X} \pm$S.D. of (n) ejaculates.

Effector	Rate of respiration (nmol $O_2 \cdot min^{-1} \cdot [\mu l\ cells]^{-1}$)	Inhibition (–) or activation (+) (%)
Without	3.17 ± 0.51 (14)	
Vanadate (0.22 mM)	0.87 ± 0.21 (11)	–73 ± 6
Ouabain (1 mM)	2.89 ± 0.75 (7)	–17 ± 12
Caffeine (10 mM)	4.34 ± 0.61 (5)	+19 ± 8
Rotenone (5.6 μM)	0.21 ± 0.01 (4)	–93 ± 1
Oligomycin (0.88 μM)	0.78 ± 0.01 (3)	–73 ± 0.5

the spermatozoal motility. Only a small effect on percent motile cells was noted, while enhanced intensity of motion was found under the microscope. These findings are in good agreement with observations by Garbers et al. [9,10] who reported marked stimulations by methylxanthines only on spermatozoa with reduced motility and on epididymal spermatozoa containing a smaller amount of motile cells. Being an inhibitor of (Na^+, K^+) ATPase, ouabain failed to increase the motility. In fact, a minor inhibition of motility was observed, apparently produced by secondary effects, e.g., by changes in the intracellular ionic composition.

Since, with lactate as substrate, the ATP required for motion is produced by oxidative phosphorylation the effects observed to be exerted on motility should parallel those on respiration. Data in Table II clearly reveal the marked inhibitory effect of vanadate on the respiratory rate. The remaining, vanadate-insensitive part of oxygen consumption must be mainly attributed to mitochondrial respiration, since rotenone gave a nearly complete inhibition. Oligomyin-induced blocking of mitochondrial ATP formation yielded the same degree of inhibition as did vanadate. Therefore, it is reasonable to conclude that also vanadate completely blocks the cellular ATP turnover. The remaining amount of some 25% respiration was also observed by Rikmenspoel et al. [14] and may be caused by uncoupled mitochondria in damaged cells naturally occurring in ejaculates [12], as well as by ion movements across the mitochondrial inner membrane. Specific inhibition of (Na^+, K^+) ATPase by ouabain revealed only a small effect on spermatozoal respiration. Apparently, the contribution of (Na^+, K^+) ATPase to the total cellular energy turnover is less important. Caffeine producing merely a small enhancement of percent motile cells markedly stimulated respiration, probably via increasing the intensity of motion.

In order to rule out additional inhibitory effects of vanadate or ouabain on the respiratory chain, it was checked whether cells pretreated with these inhibitors have the full capacity of the respiratory chain. Therefore, the respiration was titrated with the uncoupler FCCP. The polarographic traces in Fig. 1 show that the electron transport of vanadate- or ouabain-treated cells was not impaired.

Relation between respiration and cellular energy state at varied ATP turnover

Since vanadate, ouabain and caffeine directly influence the rate of ATP utilization, their effects on respiration must be mediated via the resulting adenine nucleotide patterns. Because vanadate disturbs the enzymatic determination of ADP and AMP [15], only ATP could be measured in this case. Therefore, Fig. 2 plots the respiration versus the total cellular ATP content. Associated ATP/ADP ratios and the total adenine nucleotide con-

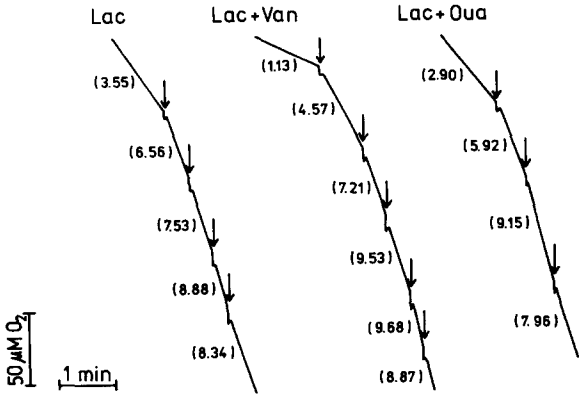

Fig. 1. Uncoupler-stimulated respiration in the presence of inhibitors of extramitochondrial ATPase. Spermatozoa (20 µl cells/ml) were preincubated in the presence of 10 mM lactate and 0.22 mM vanadate or 1 mM ouabain for 5 min, and then transferred into the oxygraphic chamber. Uncoupling was performed by stepwise additions of small amounts of FCCP (each arrow indicates addition of about 70 nmol FCCP to 1 ml sample). Numbers in parentheses represent respiration rates (nmol $O_2 \cdot min^{-1} \cdot [\mu l\ cells]^{-1}$).

tent of samples free of vanadate are given in the legend. A rough estimation of the ATP/ADP ratio in the presence of vanadate yields a value of

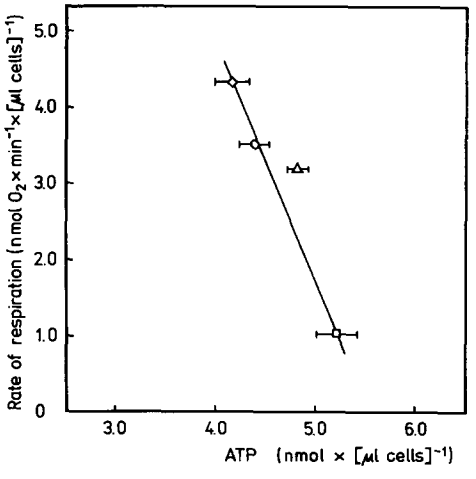

Fig. 2. Rate of respiration vs. cellular ATP level. Spermatozoa were preincubated as described in Table II. Samples were then taken to determine respiration and adenine nucleotides. The latter were transferred into ice-chilled perchloric acid (final concentration, 0.55 M) and after 10 min neutralised by 0.4 M KOH + 0.1 M Tris. Symbols: ○, without effector; □, vanadate; △, ouabain; ◇, caffeine. ATP content is given as $\bar{x} \pm$ S.E.M. ($n = 4$). Respiration was measured for two samples. Adenine nucleotide pattern of samples without effectors (nmol·[µl cells]$^{-1}$): ATP, 4.33±0.21; ADP, 0.88±0.13; AMP, 0.37±0.04; ATP/ADP ratios: ○ 5.08; ◇ 4.38.

at least 10 compared to a value of 5 measured with untreated cells. The enhanced respiration produced by caffeine was paralleled by a decrease in ATP content and ATP/ADP ratio; on the other hand, the ouabain-induced inhibition of respiration corresponds to an elevated ATP level.

So far, only situations have been considered which were produced by the full action of different effectors. Since caffeine and vanadate produced extreme states of maximum stimulation and total inhibition, respectively, of ATP turnover, titration with vanadate of spermatozoa pretreated with caffeine was supposed to result in some intermediate state of ATP turnover. Results of a typical experiment are given in Fig. 3, where the insert depicts the response of respiration to vanadate. It can be seen that about 200 µmol vanadate/l were necessary to induce 50% inhibition of caffeine-stimulated respiration, whereas such a concentration was sufficient for an almost total inhibition of untreated cells (Cf. Tables I and II). The sensitive response of respiration to cellular ATP is demonstrated by the decrease to only 80% of maximum ATP level, which provoked at 6-fold enhancement of respiration. ATP/ADP ratios were at least 9 in the presence of plentiful vanadate as estimated from the total content of adenine nucleotides, and 3.2 as measured in the presence of caffeine alone.

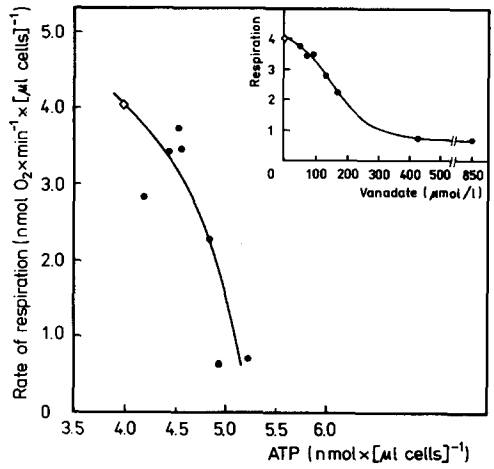

Fig. 3. Rate of respiration vs. ATP level at various vanadate concentrations. Spermatozoa (18.7 µl cells/ml) were preincubated for 5 min with 10 mM caffeine plus various vanadate concentrations as indicated in the insert. ◇, without vanadate (AXN [nmol·µl cells^{-1}]: ATP, 3.99; ADP, 1.23; AMP, 0.59; ATP/ADP, 3.24), ●, plus vanadate.

Influence of substrate supply on the relation between respiration and cellular energy state

Previous work has shown spermatozoal respiration to depend markedly on the substrate oxidised [5]. Lactate was found to be the most powerful substrate tested. In order to investigate the influence of hydrogen supply on the energy state, spermatozoa were incubated in the presence of various lactate concentrations. Fig. 4a shows respiration vs. lactate concentration. Maximal respiration was reached at about 1 mM lactate. Fig. 4b was obtained from ATP/ADP ratios of the same sperm sample. Low lactate concentrations gave small ATP/ADP ratios because of decreased mitochondrial ATP production. The curve in Fig. 4b indicates how cellular ATP utilization shown here as the rate of respiration, depends on the ATP/ADP ratio. The rate of mitochondrial ATP production equals that of extramitochondrial utilization; therefore, the rate of respiration is also indicative of the latter. Since in this experiment, only the lactate concentration was varied, the effect on the rate of ATP utilization must be exclusively mediated by changes in the ATP/ADP ratio.

Data depicted in Figs. 2 and 3 on the one hand and in Fig. 4b on the other hand demonstrate that the cellular ATP level is a function of both the activity of ATP consumers and the activity of ATP production. The interdependence of ATP utilization, ATP production and actual adenine nucleotide pattern is shown in Fig. 5. Here, experiments are summarized, in which ATP production via oxidative phosphorylation was limited by the supply of lactate (cf. Fig. 4), or ATP consumption by dynein ATPase was blocked by vanadate (cf. Fig. 3). The slight variations in the response of respiration to ATP detected between the tested cell preparations may be caused by the differences in the total cellular content of adenine nucleotides [12]. It is clear that such effect must grow in parallel to the actual ATP level. On principle, the data show that identical rates of energy turnover as indicated by the respiratory rate can result from inhibition of ATP consumption as well as of ATP production, but quite different ATP levels occurred. In addition, one experiment is given to demonstrate that the cells were capable of building up a high ATP level even in case of diminished ATP production when the ATP consumption was inhibited by vanadate. Here, the ATP production was limited by a partial inhibition of respiration with rotenone. Obviously the reduced redox energy was sufficient to supply the vanadate-insensitive energy consuming processes.

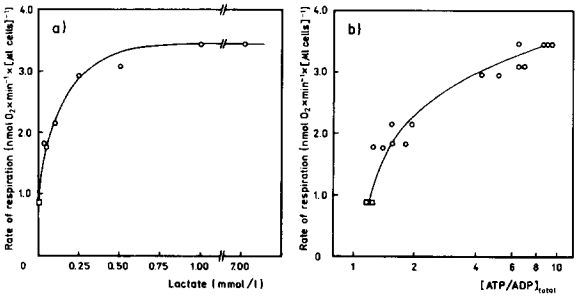

Fig. 4. Effect of lactate concentration on respiration and cellular ATP/ADP. Spermatozoa (14.4 μl cells/ml) were preincubated for 5 min with the initial concentrations of lactate as indicated in (a). After that two samples were taken for determination of ATP and ADP, and the respiration was measured. The respiration was plotted vs. the initial concentration of lactate (a) and vs. the ATP/ADP ratio (b). The utilization of lactate during the preincubation period was not taken into account.

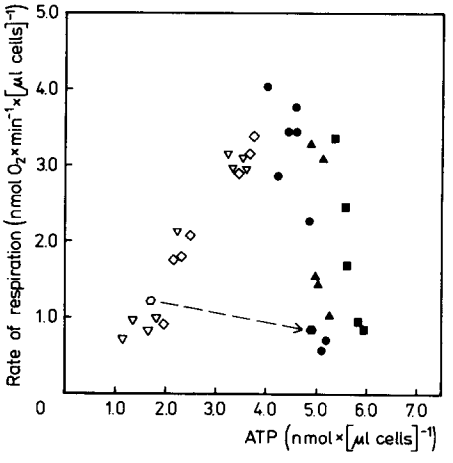

Fig. 5. Interdependence of respiration, ATP utilization and cellular ATP content. Sperm preparations (14.4–23.7 μl cells/ml) were preincubated for 5 min with: various concentrations of lactate (0 to 14.3 mM) (◇, ▽), or 10 mM lactate plus various concentrations of vanadate (0 to 846 μM) (●, ▲, ■). The various symbols represent independent experiments. Hexagons correspond to samples containing 10 mM lactate + 83 nM rotenone in absence (○) or presence of 1.5 mM vanadate (●).

Discussion

The present findings demonstrate that bovine spermatozoa constitute an appropriate model to study the regulation of energy metabolism in intact mammalian cells. They can be obtained without laborious preparation procedures. Furthermore, the simple structure of their energy metabolism is of advantage. Cellular energy transformations are directed only to the transport of genetic material, and 70–80% of the ATP produced were estimated to be utilized by dynein ATPase of the flagellum [4] which is responsible for the tail motion. We found vanadate to be a suitable tool to inhibit dynein ATPase in intact bull spermatozoa as well. To our knowledge, this has not been demonstrated to date. Although vanadate was evidenced to inhibit dynein ATPase as an isolated enzyme in micromolar concentrations, no inhibition by 10 mM vanadate was seen with intact sea urchin spermatozoa [6]. On the other hand, an uptake of vanadate by various mammalian cells was observed at concentrations as used in the present work [7]. The use of vanadate and other effectors permitted ATP-consuming reactions of the cell to be manipulated without affecting the mitochondrial reactions of ATP production. In this way it was possible to study the response of mitochondrial respiration to variations in the cellular adenine nucleotide pattern as produced by effectors of ATP-consuming enzymes. On the other hand variations on the substrate side, e.g., in substrate concentration or application of inhibitors, allowed the activity of ATP production to be influenced by mitochondrial oxidative phosphorylation without immediate effects on ATP-utilizing enzymes. The latter were altered only indirectly by changes in the adenine nucleotide pattern caused by the varied activity of the ATP producer. This approach to influence separately the producer or consumer side of ATP metabolism is believed to provide more information about interdependence in cell metabolism than do methods such as pH changes applied by Christen et al. [16]. Even though the latter workers noted marked effects on the respiratory rate they had to admit that both sides, mitochondrial ATP production and cellular ATP consumption, were altered.

The dependence on the cellular adenine nucleotide pattern found for mitochondrial ATP production (by changed activities of ATP consumption) as well as for ATP consumption (by changed activities of ATP production) indicates that in untreated cells supplied with efficient substrate, both processes are sensitive to changes in the adenine nucleotide pattern. The most pronounced dependence on the ATP/ADP ratio was found in regions which differ for both processes. ATP production was efficiently suppressed when the ATP/ADP rose to above a value of about 5, where the influence on ATP consumption was weak; and vice versa, the ATP/ADP ratio must be markedly decreased to below 5 to slow down the rate of ATP utilization. The response of the mitochondrial ATP producer could not be traced into this region, because we were unable to stimulate ATP utilization beyond the extent observed with caffeine. Since, in the presence of efficient substrates, respiration was nearly doubled by addition of uncouplers [5], it appears that respiration in untreated bovine spermatozoa is mainly controlled by the activity of dynein ATPase. The range from 4 to 10 for the cellular ATP/ADP ratio where the response of mitochondrial respiration was marked, agrees with results obtained for other mammalian cells such as in liver [17–20] and heart [21]. For isolated sperm mitochondria such data are not known, but to the extent this problem has been investigated, isolated mitochondria from many sources showed the respiratory rate to markedly depend on ATP/ADP ratios as high as 10 to 100 [2,3]. The same discrepancy becomes evident when the response region of cellular ATP utilization (ATP/ADP ratio from about 1 to 5) is compared to kinetic data of the main consumer, dynein ATPase. The well-characterized enzyme of sea urchin spermatozoa has a K_m value of 0.3 mM for ATP [6] which corresponds to an ATP content of less than 0.3 nmol ATP/μl cells. This is at least one order of magnitude smaller than the ATP content in untreated cells. There may be several causative factors for these discrepancies. It must be considered that only the total cellular ATP and ADP levels could be measured, while their distribution among mitochondria and cytosol as well as the exact volume of cytosolic compartment were unknown. Furthermore, the particular morphological structure of spermatozoa may produce con-

centration gradients of adenine nucleotides from the midpiece where mitochondria are located, along the thin tail with dynein ATPase, although it was estimated that the diffusion should be sufficient throughout the length of the tail [14]. In addition to these considerations connected with the particular structure of spermatozoa, there is possible binding of adenine nucleotides which was also discussed for other mammalian cells to explain the different ranges found for the response of respiration to the ATP/ADP ratio with isolated mitochondria and intact cells [21–23].

Acknowledgements

The skilful technical assistance of Miss U. Rietz is gratefully acknowledged. The authors thank Mrs. K. Frank for counting of motile spermatozoa.

References

1 Davis, E.J. and Lumeng, L. (1975) J. Biol. Chem. 250, 2275–2282
2 Küster, U., Bohnensack, R. and Kunz, W. (1976) Biochim. Biophys. Acta 440, 391–402
3 Kunz, W., Bohnensack, R., Böhme, G., Küster, U., Letko, G. and Schönfeld, P. (1981) Arch. Biochem. Biophys. 209, 219–229
4 Gibbons, B.H. and Gibbons, I.R. (1972) J. Cell. Biol. 54, 75–94
5 Halangk, W., Bohnensack, R., Frank, K. and Kunz, W. (1985) Biomed. Biochim. Acta 44, 411–420
6 Gibbons, I.R., Cosson, M.P., Evans, J.A., Gibbons, B.H., Houck, B., Martinson, K.H., Sale, W.S. and Tang, W.-J.Y. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2220–2224
7 Macara, I.G. (1980) Trends Biochem. Sci. 4, 92–94
8 Ramasarma, T. and Crane, F.L. (1981) Curr. Topics Cell. Reg. 20, 247–301
9 Garbers, D.L., First, N.L. and Lardy, H.A. (1973) Biol. Reprod. 8, 589–598
10 Garbers, D.L., First, N.L., Sullivan, J.J. and Lardy, H.A. (1971) Biol. Reprod. 5, 336–339
11 Schoenfeld, C., Amelar, R.D. and Dubin, L. (1973) Fert. Steril. 24, 772–775
12 Halangk, W., Bohnensack, R. and Kunz, W. (1980) Acta Biol. Med. Germ. 39, 791–808
13 Bergmeyer, H.U. (1970) Methoden der Enzymatischen Analyse, Vol. 3, pp. 2024–2033 and 2051–2055, Akademie-Verlag, Berlin, 2nd Edn.
14 Rikmenspoel, R., Sinton, S. and Janick, J.J. (1969) J. Gen. Physiol. 54, 782–805
15 Gankema, H.S., Groen, A.K., Wanders, R. and Tager, J.M. (1983) Eur. J. Biochem. 131, 447–451
16 Christen, R., Schackmann, R.W. and Shapiro, B.M. (1983) J. Biol. Chem. 258, 5392–5399
17 Siess, E.A. and Wieland, O.H. (1976) Biochem. J. 156, 91–102
18 Akerboom, T.P.M., Bokelman, H., Zuurendonk, P.F., Van der Meer, R. and Tager, J.M. (1978) Eur. J. Biochem. 84, 413–420
19 Soboll, S., Akerboom, T.P.M., Schwenke, W.D., Haase, R. and Sies, H. (1980) Biochem. J. 192, 951–954
20 Letko, G., Küster, U. and Pohl, K. (1983) Biomed. Biochim. Acta 42, 313–333
21 Soboll, S. and Bünger, R. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 125–132
22 Soboll, S., Werdan, K., Boszik, M., Müller, M., Erdmann, E. and Heldt, H.W. (1979) FEBS Lett. 100, 125–128
23 Veech, R.L., Lawson, J.W.R., Cornell, N.W. and Krebs, H.A. (1979) J. Biol. Chem. 254, 6538–6547