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# Respiratory and Phosphorylative Activity of Amphibian Spermatozoa

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*Bufo arenarum* or *Leptodactylus chaquensis* spermatozoa have an endogenous respiration ($Z_{O_2}$ = 1.4 – 4.2) that was not significantly enhanced by glucose or fructose. Succinate was oxidized by these spermatozoa at higher rates ($Z_{O_2}$ = 5 – 14) while endogenous respiration was only slightly stimulated by glutamate or citrate.

Both the endogenous and exogenous respiration were sensitive to respiratory chain inhibitors (rotenone, antimycin or cyanide).

The respiration of frog and toad sperm was coupled to the synthesis of ATP. P/O ratios of 0.54 were determined in the presence of potassium fluoride and exogenous hexokinase. Oligomycin inhibited phosphorylation completely and respiration partially. The latter inhibition was released by uncouplers like dinitrophenol.

It is concluded that frog and toad sperm have an active oxidative metabolism coupled to the synthesis of ATP.

The sperm metabolism of some vertebrates, especially mammals (including man) and some invertebrates (molluscs and sea urchin), has been extensively studied (Mann, 1964). On the other hand nearly nothing is known about the energy metabolism of spermatozoa of Amphibia except a preliminary report from Bernstein (1954) that the frog sperm obtains energy for motility from aerobic metabolism. The present paper deals with frog and toad oxidative and phosphorylative metabolism. It was thought that such a study might be useful for a better understanding of the biology of reproduction in Amphibia.

## MATERIAL AND METHODS

Spermatozoa were obtained from the testes of toads (*Bufo arenarum*) or frogs (*Leptodactylus chaquensis*) collected in the vicinity of San Miguel de Tucumán (Argentina). Toad testes were homogenized in a Potter-Elvehjem homogenizer with a loose-fitting pestle, in 250 mM sucrose and filtered through glass-wool. Sperm cells were collected by centrifugation at 400 × g for 5 min and resuspended in the same medium. All procedures were done at room temperature. Spermatozoa of frogs were obtained by simply extruding the testes into 250 mM sucrose, 10 mM Tris-HCl (pH = 7.4). Cell suspensions, stored in the cold, were used without further manipulation. Frog testes gave a better yield of spermatozoa than toad testes ($10^9$ and $10^8$ cells per testis respectively) but mature male frogs were available only during the summer (Cei, 1949; Barbieri, 1956). Sperm counts were made in a hemocytometer.

### *Determination of respiratory activity*

The respiratory activity of sperm cells was determined polarographically with a Teflon covered Clark electrode (Gilson Oxygraph or Yellow Spring Instrument Co.) in 125 mM sucrose, 5 mM Tris-HCl (pH = 7.4) at 30°.

### *Determination of P/O ratios*

P/O ratios were determined, after Morton and Lardy (1967) in small vessels (7 ml) of a Gilson differential respirometer. The reaction medium (1.5 ml) was 125 mM sucrose, 5 mM Tris-HCl (pH = 7.4), 5 mM $MgSO_4$, 1 mM ATP, 75 mM 2-deoxyglucose, 10 mM potassium phosphate (pH = 7.4) and 1 × $10^6$ cpm carrier-free $^{32}P$. The reaction was started by adding the latter five components from the side arm after 10 min preincubation at 30°. The reaction was stopped after 60 min with 0.15 ml of 50% (w/v) trichloroacetic acid. The organic $^{32}P$ was determined by an isobutanol-benzene extraction

method (Nielsen and Lehninger, 1955) and counted in a Beckman LS-233 liquid scintillation counter (Gould *et al.*, 1972).

## RESULTS

Figure 1 shows that toad spermatozoa had an endogenous respiration which was not significantly increased by addition of glucose or fructose, was slightly stimulated by glutamate or citrate and was strongly enhanced by succinate. Similar results were obtained with frog sperm. The $Z_{O_2}$ of the endogenous respiration was between 1.4–4.2 for both species. It increased to 5–14 with succinate.

The endogenous respiration was sensitive to mitochondrial respiratory chain inhibitors such as cyanide, antimycin or rotenone. Fig. 2A shows an oxygen electrode tracing of the oxidation of endogenous substrates by frog spermatozoa. Rotenone (4 nmoles/ml) blocked this respiration. As expected, addition of succinate restarted oxygen uptake since rotenone does not inhibit succinate oxidation (Eraster *et al.*, 1963). The uncoupler 2-4 dinitrophenol further stimulated respiration which was finally inhibited by antimycin.

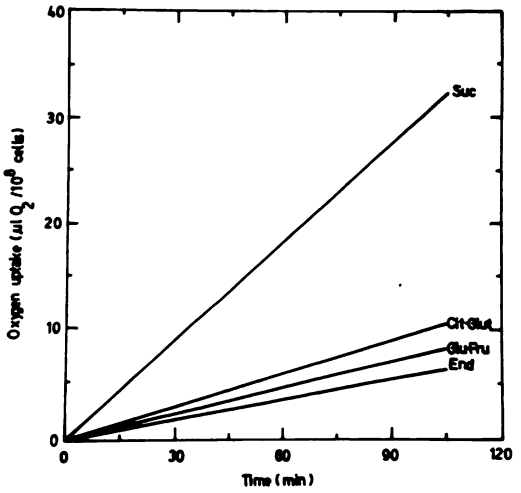


FIG. 1. The oxygen consumption of toad spermatozoa with different substrates was measured in a Gilson Respirometer at 30°. The reaction medium (1.5 ml) was Ringer-1 mM phosphate (pH 7.4) with or without 5 mM substrate and 8 × $10^8$ cells per vessel. *End:* endogenous respiration, no substrate added; *Glu:* glucose; *Fru:* fructose; *Cit:* citrate; *Glut:* glutamate and *Suc:* succinate.

The oxidation of succinate was also inhibited by the energy transfer inhibitor oligomycin (Fig. 2B). This inhibition was completely released by dinitrophenol. The resulting respiratory control (uncoupled/oligomycin inhibited respiration rates) was 4.2. The respiratory inhibition of frog sperm cells by oligomycin suggests that this respiration was coupled to the synthesis of ATP. Table 1, expt. 1, shows that indeed this was the case. Oligomycin inhibited the oxygen uptake by 44% and nearly completely the P/O ratios. The observed P/O ratios depended on the presence of potassium fluoride and exogenous

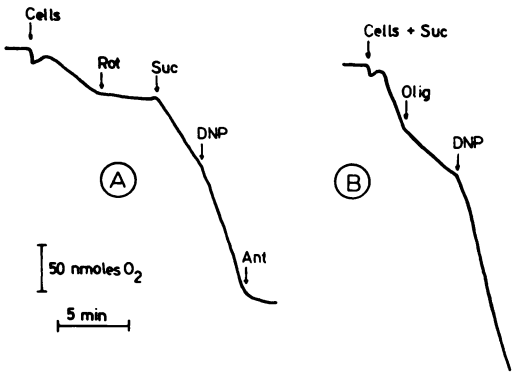


FIG. 2. The oxygen uptake of frog spermatozoa was followed in a Gilson Oxygraph as described in Methods. 5 × $10^8$ cells were used per run. The additions were succinate (Suc) 10 mM; rotenone (Rot), 4 µM; 2-4 dinitrophenol (DNP), 100 µM; antimycin (Ant), 2 µg and oligomycin (Olig), 5 µg. The reaction was carried out at 30° in a total volume of 1.6 ml.

TABLE 1

OXIDATIVE PHOSPHORYLATION IN TOAD SPERMATOZOA

P/O ratios of toad spermatozoa were determined as described in the text; 2 × $10^8$ and 4 × $10^8$ cells/vessel were used in expt. 1 and 2 respectively. The substrate was 20 mM succinate.

| Expt. | Additions | $\Delta P_i$ (µmol) | $\Delta O_2$ (µat) | P/O |
|---|---|---|---|---|
| 1 | KF, 40 mM | 0.11 | 0.69 | 0.16 |
| | KF, 40 mM; oligomycin 10 µg. | 0.01 | 0.39 | 0.02 |
| 2 | none | 0.29 | 5.02 | 0.06 |
| | KF, 10 mM | 0.60 | 4.97 | 0.12 |
| | hexokinase 5 I.U. | 1.07 | 4.97 | 0.20 |
| | KF, 10 mM; hexokinase 5 I.U. | 2.68 | 4.77 | 0.54 |

hexokinase (Table 1, expt. 2). The former raised the P/O ratios of toad sperm cells from 0.06 to 0.12 and the latter to 0.20. Added together a P/O ratio of 0.54 was observed. Higher concentrations of potassium fluoride strongly diminished respiration, slightly affecting phosphorylation and resulting in higher P/O ratios (Fig. 3). The presence of hexokinase in the reaction medium gave higher phosphorylation rates especially with low potassium fluoride concentrations.

## DISCUSSION

The rate of endogenous respiration of sperm cells of *Bufo arenarum* or *Leptodactylus chaquensis* was increased by succinate but not significantly by glucose or fructose and only slightly by glutamate or citrate. These observations are similar (including the observed $Z_{O_2}$) with those carried out with mammals and human sperm (Mann, 1964; Morton and Lardy, 1967). The sensitivity of the oxygen uptake to respiratory chain inhibitors and to oligomycin and the enhancement of succinate oxidation by dinitrophenol (Fig. 2) prove that frog and toad sperm have an active oxidative metabolism coupled to the synthesis of ATP. The observed P/O ratios are low compared with the theoretical P/O ratios of isolated mammalian mitochondria. However, the determinations of P/O ratios in whole cells is a difficult task (Morton and Lardy, 1967). The $Z_{O_2}$ of bovine spermatozoa (Morton and Lardy, 1967) were similar to those reported here for frog and toad sperm but the P/O ratios were higher.

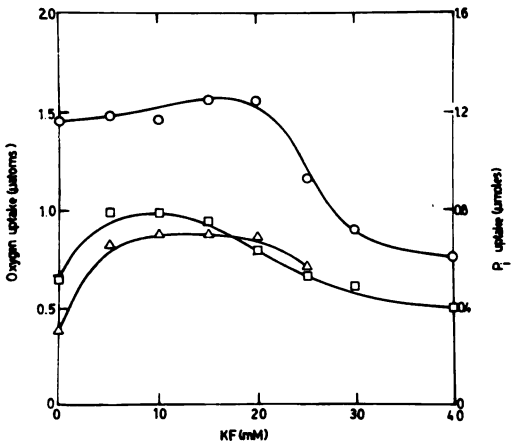


FIG. 3. Effect of KF and hexokinase on oxygen uptake and phosphorylation of frog spermatozoa. Experimental conditions were as described in the text for P/O ratio determinations. $5 \times 10^8$ cells were used per vessel. 20 mM succinate (plus 4 $\mu$M rotenone) were used as substrate. (●——●) oxygen uptake; (▲——▲) phosphorylation rate in the absence, or in the presence (■——■), of hexokinase.

However, the P/O ratios of ascites cells determined with the identical method by the same authors were similar to those of frog and toad sperm cells. The method is based on the use of 2-deoxyglucose as phosphate acceptor from the synthesized ATP and on the addition of fluoride to inhibit ATP-requiring processes. As described for bovine spermatozoa (Morton and Lardy, 1967) fluoride enhanced phosphate uptake by frog sperm cells (Fig. 3) and therefore P/O ratios. However, concentrations of fluoride higher than 20 mM strongly inhibited respiration (50% with 40 mM KF) but phosphorylation was less inhibited. Addition of yeast hexokinase in the absence or in the presence of low concentrations of fluoride increased the P/O ratios (Table 1 and Fig. 3). This stimulation may be due to a low endogenous hexokinase activity.

The low P/O ratios observed may be the consequence of a low efficiency of the ATP trapping system (2-deoxyglucose and exogenous hexokinase) because of diffusion barriers.

The results presented in this paper show that aerobic metabolism exists in amphibian spermatozoa. Species from both genera *Leptodactylus* and *Bufo* gave similar results. This oxidative metabolism coupled to the synthesis of ATP may be, as earlier suggested by Bernstein (1954), the energy source for motility in aerobiosis.

## ACKNOWLEDGMENT

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