Cellular Oxidative Phosphorylation. II. Measurement in Physically Modified Spermatozoa*

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ABSTRACT: Mild agitation of bovine epididymal spermatozoa in the presence of 75- or 85-μ diameter glass beads alters the cells to permit the observation of a large increase of net P_i uptake and respiration with a concomitant increase in P:O ratios to near-theoretical values. The uptake of organic dye, loss of nucleotides, protein, the acrosome, and motility, with no gross anatomical change in sperm morphology, suggest that only the cell membranes were modified by the bead treatment.

The cofactor requirements and other environmental parameters affecting oxidative phosphorylation were defined. Bead treatment of bovine-ejaculated spermatozoa depressed respiration and only occasionally enhanced P:O ratios. The cell membranes of bovine epididymal spermatozoa were also modified by exposure to hypotonic solutions for brief periods. This produced a slight depression in respiration but a significant elevation in the observed efficiency of oxidative phosphorylation. The validity of the methods developed in these reports to measure oxidative phosphorylation is discussed.

The mitochondria of the sperm cell are firmly attached around the proximal portion of the contractile elements in the sperm flagella. In some organisms, such as certain insects and mollusks (André, 1962), the mitochondria of this midpiece region appear to be fused, while in others, for example, the rat or bat (André, 1962; Fawcett, 1962), they are discrete. As yet, functional mitochondria have not been isolated from spermatozoa, although Mohri *et al.* (1965) have isolated entire midpieces from bull sperm which retain a capacity for oxidative phosphorylation. In the first paper of this series Morton and Lardy (1967a) reported a method for the observation of oxidative phosphorylation occurring within the intact cell. Here a way is presented to modify physically a portion of the cellular membrane of bovine spermatozoa while leaving the remainder of these cells essentially unaltered. This method enables the modifying effect of the cellular membrane and cytosol upon sperm oxidative phosphorylation to be investigated.

Materials and Methods

The cells were obtained and prepared as in the previous paper (Morton and Lardy, 1967a) with the exception that occasionally the epididymal sperm were removed from the epididymis up to 3 days after this rgan was placed in the cold. Metabolically active sperm were obtained from these organs 9 days after the bull was killed. Fertilization in the rabbit has been achieved after insemination with sperm stored in the excised epididymis for as long as 40 days (Hammond and Asdell as cited in Lasley and Mayer, 1944).

The experimental methods also include those of the previous paper. Lactic acid was determined by the method of Barker and Summerson (1941), and protein by the biuret method (Gornall *et al.*, 1949). The method of vital staining was that of Mayer *et al.* (1951). Giemsa stain was used as in Iype *et al.* (1963). Glucose 6-phosphate was measured with the aid of glucose 6-phosphate dehydrogenase (Noltmann *et al.*, 1961).

In addition to the reagents already described in the previous paper, horse heart cytochrome *c* (Sigma type III) was used. Bovine serum albumin fraction V, Calbiochem C grade, was utilized both for direct additions and as the starting material for the preparation of lipid-extracted bovine serum albumin by the personally communicated method of Professor P. Randle (Morton, 1965). Superbrite glass beads of selected diameters, obtained from Minnesota Mining and Manufacturing, were placed overnight in concentrated H_2SO_4 and then rinsed in double-distilled, deionized water until the washings were the pH of the water. A small quantity of KOH was then added and the beads rinsed back to neutrality.

The direct addition of 5 mM DPNH[1] proved toxic in the test system, therefore, DPNH was generated by adding 0.02 mg of yeast alcohol dehydrogenase (Sigma, twice crystallized) from the side arm to 5 mM DPN^+ and 3.1 mM ethanol in the main chamber of the War-

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[1] Abbreviations used in this report: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; DPN^+, oxidized DPN; DPNH, reduced DPN.

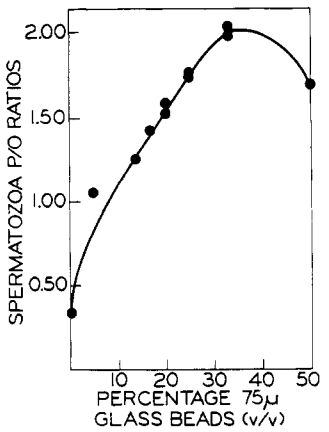

FIGURE 1: The response of bovine epididymal spermatozoa to 75-μ glass beads. The data are from several experiments run at 30° and pH 7.4. The treated sperm suspended in 0.25 M sucrose varied from 5 to 13 $\times$ 10^8 cells/ml. The medium contained 10 mM succinate, 20 mM KF, 5–10 mM Pi, 15 mM Tris, 2 mM ATP, 5 mM $MgSO_4$, 0.17 mg/ml of Sigma type III yeast hexokinase, and 12.5 mM glucose.

burg flasks. The addition of 5 mM semicarbazide as an acetaldehyde trap decreased the observed P:O ratio about 30%. When glass beads (1.25 g) were added to Warburg flasks, the manometer constants were appropriately corrected for the decreased total volume.

Results

Bovine epididymal spermatozoa suspended in 0.25 M sucrose were shaken at 0° with one-third volume of glass beads 75 μ in diameter for 15 min at 160 strokes of 3.0 cm/min. The beads were removed from the cells by about 30 sec of low centrifugation and the resulting supernatant cell suspension was pipetted into the Warburg flasks. Such cells consumed oxygen more rapidly and fixed much more phosphate in the test system (Table I, expt A) than did untreated cells.

When further experiments demonstrated that phosphorylation efficiency could be enhanced by even milder shaking with beads, the effect of beads added directly to the Warburg flasks was examined (expt B–D in Table I). The beads were resuspended when the flasks were placed in the Warburg bath and again at the end of thermal equilibration. The increase in phosphorylation efficiency was about the same as that following the shaking treatment described above.

The response of bovine epididymal spermatozoa to increasing concentrations of 75-μ diameter glass beads is shown in Figure 1. In the work that follows, the optimal quantity (33%, v/v) was employed in the Warburg flasks. Although bead diameters from 30 to 100 μ gave similar results, beads of 75 or 85 μ were used in the following experiments.

The nature of the effect of glass beads in causing increased P:O ratios and Z_{O_2} values was next evaluated.

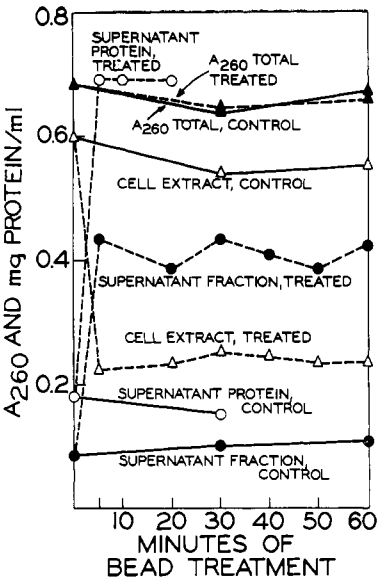

FIGURE 2: Loss of cell components into the supernatant fluid caused by treatment with glass beads. Experimental conditions: 4.8 $\times$ 10^8 cells/ml were added to sufficient 75-μ diameter glass beads to make the bead volume 33% and the mixture was shaken at 160 strokes of 3.0 cm/min at 0°. The beads were removed at the time interval indicated by 30 sec of low-speed centrifugation. The remaining cell suspension was accelerated to 30,000g and decelerated. The cell-free supernatant fraction was assayed for protein and an aliquot was deproteinized with 5% perchloric acid, neutralized with KOH, centrifuged, and measured for 260-mμ absorbance. The cellular pellet was resuspended in 5% perchloric acid at 2° for 20 min and recentrifuged. The perchlorate was removed by neutralization with KOH and centrifugation. The resulting supernatant fraction was measured for 260-mμ absorbance. Open circles = protein; closed circles and triangles = 260-mμ absorbance.

Bead-treated spermatozoa no longer excluded the organic dye erythrosin B of the vital stain method of Mayer *et al.* (1951). Figure 2 shows that less than 5 min of mild agitation of the spermatozoa in the presence of glass beads was sufficient to cause the loss of soluble protein and 260-mμ absorbing components from the cell. The spectrum of the supernatant fraction of bead-treated cells which had been deproteinized with 5% perchloric acid, and neutralized to pH 7.0 with KOH to precipitate perchlorate, contained a symmetrical peak at 259 mμ and a trough at 235 mμ and was of such character as to agree with the report of Abraham and Bhargava (1962) that sperm contain 60% adenosine, 22% guanosine, and 18% cytosine nucleotides.

Present among the soluble proteins leaking from bead-treated cells were the glycolytic enzymes enabling the conversion of glucose 6-phosphate to lactate as indicated by Figure 3 (note that time is plotted on a log scale). It is assumed the lag phase results from the slow accumulation of intermediates between glucose 6-

TABLE I: Oxidative Phosphorylation in Bovine Epididymal Spermatozoa Treated with Glass Beads by Two Methods.[a]

Expt	Substrate	Treatment	P:O	Z_{O_2}
A	Succinate	None	1.0	6.1
	Succinate	Pretreated with beads	1.7	8.6
	Fumarate	None	0.6	6.2
	Fumarate	Pretreated with beads	1.6	10.3
B	α-Ketoglutarate	Beads in flask	2.3	7.6
	α-Ketoglutarate	Pretreated with beads	2.1	14.6
C	α-Ketoglutarate	None	1.4	5.8
	α-Ketoglutarate	Beads in flask	2.2	7.2
D	Lactate	None	0.7	11.4
	Lactate	Beads in flask	2.3	12.5

[a] Incubation conditions, except for experiment A which were like Figure 1, were as follows: 37°, pH 7.0, 40 mM KF, 15 mM H_3PO_4 (Tris salt), 0.5 mM ATP, 10 mM $MgSO_4$, 37.5 mM 2-deoxyglucose, and 0.1 mg of Sigma type IV yeast hexokinase in a final volume of 1 ml. Experiment and cells/ml × 10^8: A, 8.8 in both cases; B, 9.5 and 6.5; C, 13.8; and D, 2.8.

phosphate and lactate. In another experiment glycolytic intermediates above triosphosphate dehydrogenase were found to be converted to lactate by this soluble enzyme fraction while 3-phosphoglycerate was converted primarily to pyruvate. This is consistent with the DPNH requirement of lactate dehydrogenase. Glucose was not converted to lactate in significant quantities by the soluble extract because hexokinase is bound to the sperm cell membrane (B. E. Morton and H. A. Lardy, manuscript in preparation). Glycolytic intermediates beyond phosphofructokinase were converted more rapidly to pyruvate or lactate than those before. This is consistent with the rate-limiting behavior of this enzyme in other systems (Wu and Racker, 1959; Lardy, 1961).

Microscopic examination of the bead-treated spermatozoa revealed no clues as to the modification in this cell produced by beads until Giemsa stain was applied and it was found that the acrosome was missing. Hathaway and Hartree (1963) used 150-μ diameter glass beads to damage or remove this delicate structure which covers the proximal third of sperm heads (Hancock, 1952). The acrosome is associated with egg penetration (Mann, 1964) and possibly protein biosynthesis (Iype *et al.*, 1963). It appears to be the most fragile portion of the sperm cell and its loss could damage the integrity of the external membrane system of this cell and account for the observations presented above.

The components of the system used in the measurement of cellular oxidative phosphorylation, including some of those presented for intact sperm in the previous paper of this series, were next evaluated. Figure 4 demonstrates the absolute requirement of a hexose acceptor for the measurement of ATP synthesis in bead-treated cells. In other experiments 50 or 100 mM 2-deoxyglucose gave the same result as 75 mM. The addition of yeast hexokinase had no influence on the measured cellular oxidative phosphorylation. This was confirmed in several experiments using 75 mM 2-deoxyglucose.

The addition of fluoride enhanced P:O ratios in physically modified bovine epididymal spermatozoa as effectively as it did with intact cells. The addition of 40 mM fluoride to bead-treated sperm was found to stimulate respiration slightly but consistently as opposed to the mild inhibition usually produced in intact spermatozoa at this concentration. The response of bead-treated sperm to added adenine nucleotides was essentially the same as that of intact spermatozoa (Morton and Lardy, 1967a).

The addition of 5–14 mM magnesium sulfate (or chloride) caused a sixfold stimulation of P_i uptake and a 30% stimulation of respiration in physically modified sperm, while in the intact cell, P_i uptake was stimulated less than twofold and respiration not at all. This suggests that the sperm cell membrane is capable of retaining magnesium ion within the cell in a magnesium-free environment.

With bead-treated epididymal spermatozoa both respiration and phosphate uptake were maximal at pH 6.9–7.0 and P:O ratios were relatively constant over a slightly wider range. These results, in duplicate, from three experiments may be compared with the optimum of 7.5–8.0 for respiration of epididymal bull sperm reported by Henle and Zittle (1942). An inspection of their data suggests they interpreted it incorrectly and that pH 7.0 was optimal in their studies as well. Lardy and Phillips (1943b) found pH 7.0 to be optimal for respiration in bovine-ejaculated spermatozoa while Mohri *et al.* (1965) for unstated reasons utilized pH 7.5 in their studies of ejaculated bull sperm midpieces. In our studies the pH at the completion of incubation was found to have fallen from 7.0 to 6.8.

The addition of 0–3% bovine serum albumin had little consistent beneficial effect while concentrations of lipid-extracted bovine serum albumin above 0.3%

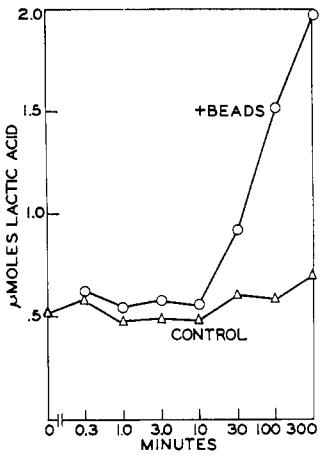

FIGURE 3: Conversion of glucose 6-phosphate to lactate by the supernatant fraction of control and bead-treated bovine epididymal spermatozoa. Experimental conditions: spermatozoa were suspended in 0.25 M sucrose (9.7×10^8 cells/ml) and one-half of the suspension was shaken with beads for 30 min as described in the text. The two suspensions were centrifuged at 105,000*g* for 1 hr. Volumes (0.2 ml) of the supernatant fractions which contained 1.8 and 3.7 mg of protein/ml for control and bead treated, respectively, were diluted to 1.0 ml with the pH 7.4 incubation medium to give final concentrations of 10 mM glucose 6-phosphate, 2 mM ATP, 2 mM DPN^+, 10 mM P_i, and 5 mM $MgSO_4$. The reaction mixtures were incubated at 37° for the times indicated, and stopped with a final concentration of 5% perchloric acid. O = bead-treated sperm supernatant fraction. Δ = control sperm supernatant fraction.

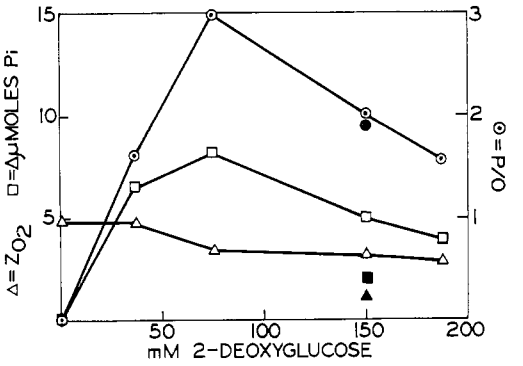

FIGURE 4: The response of bovine epididymal spermatozoa to 2-deoxyglucose. Experimental conditions: 37°, pH 7.0, 0.5 mM Na_2ATP, 10 mM α-ketoglutarate, 15 mM H_3PO_4 (all Tris neutralized), 10 mM $MgSO_4$, 40 mM KF, 0.2 mg/ml of Sigma type IV yeast hexokinase, and 1.25 g/ml, 75-μ diameter glass beads. Cells (9.3×10^8) were prepared in 0.25 M mannitol.

strongly inhibited respiration and P:O ratios. Cytochrome *c* additions in concentrations up to 10^{-4} M had no effect upon this system when DPNH-linked substrates were oxidized. Calcium chloride, manganese sulfate, and EDTA (tested individually) had no effect upon this system until their concentrations exceeded 10^{-3} M beyond which point all depressed respiration and P:O ratios.

As in the case of intact sperm, 0.25 M sucrose, 0.25 M lactose, and 0.154 M KCl were acceptable media for the isolation and preparation of the cells while 0.154 M NaCl depressed P:O ratio and Z_{O_2} values. NaCl in the incubation medium was found to be inhibitory to respiration and P:O ratio at all concentrations tested (50–300 mM). The addition of varying concentrations of ouabain had no effect upon the net P_i uptake either in the presence or absence of fluoride.

The addition of the substrate α-ketoglutarate caused an increase in ΔP_i, Z_{O_2}, and P:O ratios that reached a plateau at 10–30 mM. Concentrations much above 30 mM were inhibitory. At 10 mM α-ketoglutarate, 3×10^{-4} M malonate caused a slight increase in P:O ratio by inhibiting succinic dehydrogenase (Quastel and Whetham, 1924). However, at 20 mM α-ketoglutarate, malonate was without effect. This was probably because α-ketoglutarate had reached a saturating concentration.

Occasionally, P:O ratios observed in this system with 10 mM succinate oxidation slightly exceeded 2.0. Possibly this concentration was not sufficient completely to exclude electron transport resulting from the oxida-

TABLE II: Phosphorylation Efficiency of Physically Modified Bovine Epididymal Spermatozoa with Various Substrates.[a]

Substrate	P:O	Z_{O_2}
Endogenous	2.5	3.8
β-Hydroxybutyrate	2.7	5.1
α-Glycerophosphate	1.9	11.8
L-(+)-Lactic acid	2.8	8.8
Pyruvate	2.3	3.5
Acetate	1.8	5.8
Citrate	2.5	6.2
Isocitrate	1.9	8.3
α-Ketoglutarate	2.9	8.1
Glutamate	1.6	3.7
Succinate	2.0	10.7
Fumarate	2.6	5.8
Malate	2.7	6.1
DPNH-generating system	3.0	5.2

[a] These results were obtained under optimal conditions from many experiments. Other experiments gave similar data but owing to the nature of the epididymal sperm source considerable variation is often observed. Incubation conditions are those of Table I. Sperm concentrations ranged from 7.2 to 12.7×10^8 cells/ml.

TABLE III: The Response of Bovine-Ejaculated Spermatozoa to Glass Beads and Fluoride.

Substrate	Addn (mM)	P:O	Z_{O_2}
L-(+)-Lactic acid (Tris salt)		0.2	9.4
L-(+)-Lactic acid (Tris salt)	F^- (10)	1.1	7.7
L-(+)-Lactic acid (Tris salt)	F^- (40)	1.5	6.2
L-(+)-Lactic acid (Tris salt)	Beads[a]	0.8	3.2
L-(+)-Lactic acid (Tris salt)	F^- (10) + beads	1.9	7.4
L-(+)-Lactic acid (Tris salt)	F^- (40) + beads	1.9	5.2
α-Ketoglutarate		0.1	7.5
α-Ketoglutarate	F^- (40)	1.6	4.6
α-Ketoglutarate	Beads	0.0	2.0
α-Ketoglutarate	F^- (40) + beads	1.5	3.9

[a] Glass beads (33% v/v, 75-μ diameter) were present in the Warburg flask. Conditions were similar to those of Table I except that 75 mM 2-deoxyglucose and 0.5 mg/ml of Sigma type II yeast hexokinase were present. 3.9×10^8 cells/ml.

tion of the DPN-linked endogenous substrate. The ratios did not exceed 2.0 when 20 mM succinate was present. Table II lists representative P:O ratios from 20 experiments.

Physically Modified Bovine-Ejaculated Spermatozoa. These cells were washed with sucrose–EDTA as described in paper I of this series (Morton and Lardy, 1967a). Mild agitation with glass beads during incubation depressed respiration (Table III) but only occasionally was this treatment found to enhance phosphorylation efficiency. This result is difficult to explain in view of the fact that bead treatment improves phosphorylation efficiency of epididymal sperm and certain chemical agents that damage cell membranes enhance P:O values of ejaculated sperm (Morton and Lardy, 1967b). The depression of respiration caused by shaking with beads is not observed with epididymal spermatozoa and could not be reversed by the addition of 5 mM DPN, 10^{-3} M cytochrome *c*, or both.

Table III also shows ejaculated sperm to be similar to epididymal cells in that the respiration of the intact cell is depressed by fluoride but that of physically modified cells is stimulated by this agent. Potassium ion at 100 mM increased the P:O ratio of physically modified ejaculated sperm but 150 mM depressed this parameter. Intact ejaculated cells in the same experiment could tolerate 150 mM K^+, undoubtedly because of the modifying influence of their intact cellular membrane. Table IV gives the typical P:O ratios observed for physically modified bovine-ejaculated spermatozoa.

Bovine Epididymal Spermatozoa Subjected to Hypotonicity. The addition of a large volume of water (40 volumes) to a pellet of centrifuged sperm which had been resuspended in the small amount of 0.25 M sucrose remaining after decantation, caused damage that was not seen by the light microscope but could be detected by the observation that all cells were permeable to the vital stain of Mayer *et al.* (1951). After the cells were quickly centrifuged, resuspended in 0.25 M sucrose, and assayed for net P_i uptake and respiration, the preliminary results of Table V were obtained. This brief exposure of epididymal spermatozoa to hypotonic conditions must have damaged the sperm cell wall sufficiently to render it permeable to erythrocin B and adenine nucleotides but it apparently did not alter the mitochondrial structure severely. The acrosome was not detached in these water-lysed cells.

Uncouplers of Oxidative Phosphorylation. Figure 5 describes the response of epididymal sperm in the presence and absence of beads and fluoride, to valinomycin, an antibiotic that uncouples oxidative phosphorylation by diverting energy to transport of potassium (Moore and Pressman, 1964). Nearly identical

TABLE IV: P:O Ratios Observed for Physically Modified Bovine-Ejaculated Spermatozoa.[a]

Expt	Substrate	Other Variable	P:O	Z_{O_2}
A	Lactate	No glass beads	1.9	5.2
	Lactate	+glass beads	1.6	4.1
	α-Ketoglutarate	+glass beads	1.3	4.7
	Malate	+glass beads	1.5	3.8
	Endogenous	+glass beads	0.0	1.0
	α-Glycerophosphate	+glass beads	0.6	4.4
B	α-Ketoglutarate	No glass beads	1.6	6.2
		+glass beads	1.5	3.9
C (av of 3 expt)	Lactate	No glass beads	1.9	6.1
		+glass beads	1.8	4.9

[a] Conditions as in Table III. Experiment and sperm/ml × 10^8: A, 6.1; B, 3.9; C, 5.4 (average).

TABLE V: P:O Ratios and Z_{O_2} Values Observed for Water-Lysed Bovine Epididymal Spermatozoa.[a]

Expt	Conditions	Substrate	P:O Ratio	Z_{O_2}
A	Control	Lactate	0.5	5.3
	H_2O treated	Lactate	1.8	4.3
B	Control	α-Ketoglutarate	1.6	5.1
	H_2O treated	α-Ketoglutarate	2.0	3.3
	H_2O treated	Lactate	2.0	3.2
	H_2O treated	Succinate	1.0	3.8

[a] Conditions were similar to those in Table III but no glass beads were present. Cells/ml × 10^8: expt A, control 4.2; lysed 5.8; expt B, control 5.8; lysed, 4.5.

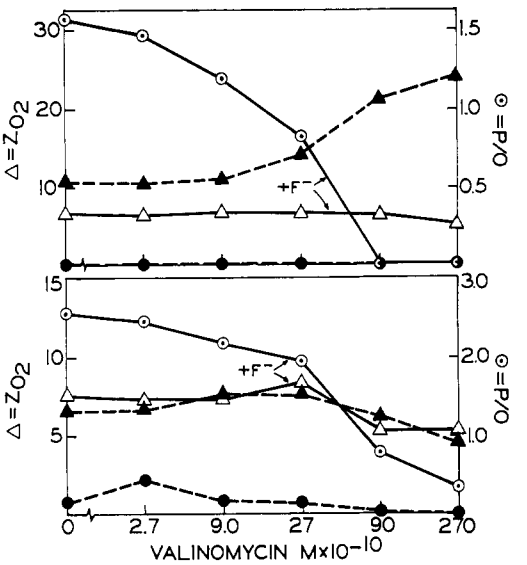

FIGURE 5: The multiple response of bovine epididymal spermatozoa to valinomycin. Experimental conditions: same as Figure 4 except the substrate was 40 mM Tris–lactate. KCl was present when KF was omitted, 2-deoxyglucose was 75 mM, type II hexokinase was used at 0.5 mg/ml, and the cells were prepared in sucrose. There were 7.6 × 10^8 sperm/ml but no beads in the upper experiment while 9.4 × 10^8 sperm/ml and 1.25 g/ml, 75-μ diameter beads were present in the lower experiment.

results were observed with dinactin, an antibiotic that functions like valinomycin but shows broader ion specificity (Graven *et al.*, 1966). DNP, an uncoupler not known to be associated with ion transport, caused similar responses with the exception that in the absence of beads but presence of fluoride this agent caused a small stimulation in Z_{O_2} which peaked at 3 × 10^{-5} M. Besides illustrating the complete inhibition of net P_i uptake by these uncouplers, Figure 5 also illustrates that not only do both glass beads and fluoride separately increase the observed P:O ratio, but their separate presence also blocks the respiratory stimulation of the uncouplers. In addition, fluoride inhibits respiration in the absence of both uncoupler and beads, but stimulates or protects respiration in the absence of uncoupler when beads are present.

Discussion

The following evidence is presented to demonstrate that the cellular method presented in this series actually measures ATP synthesized by oxidative phosphorylation. (1) The addition of hexose as an ATP acceptor in the presence of endogenous or exogenous hexokinase is required for net P_i uptake. (2) ΔP_i was found to be essentially equivalent to glucose 6-phosphate production. In an early experiment with epididymal sperm pretreated with 75-μ glass beads, the average P:O ratios calculated on the basis of decrease in extracellular P_i were 1.5 with succinate and 2.0 with β-hydroxybutyrate. When the P:O ratios were calculated on the basis of increase in extracellular glucose 6-phosphate, assayed with glucose 6-phosphate dehydrogenase, the values were: succinate, 1.5 and β-hydroxybutyrate, 1.9. (3) The rate of P_i uptake could be increased by adding adenine nucleotide, one of the required components in oxidative phosphorylation. The response in ΔP_i was much greater than the quantity of adenine nucleotide added. (4) The addition of fluoride, an inhibitor of several ATP-requiring enzymes, markedly increased the uptake of P_i observed.

Only two processes for the formation of large quantities of ATP are known to exist in spermatozoa, namely, glycolysis and oxidative phosphorylation (Lardy and Phillips, 1941; Mann, 1964). That glycolysis is not contributing to the net P_i uptake is indicated by the following. (1) Fluoride, a known inhibitor of glycolysis, is present in high concentrations. (2) The substitution of 2-deoxyglucose for glucose caused no alteration in ΔP_i. Glycolysis in ejaculated bull sperm is completely inhibited by 18 mM 2-deoxyglucose (Lodge, 1963). Much higher concentrations were present here. (3) The equivalency of ΔP_i with Δglucose 6-phosphate would not exist if glucose 6-phosphate were actively glycolyzed.

Further evidence that the orthophosphate is fixed by oxidative phosphorylation is that ΔP_i is proportional to ΔO over a wide range. Similar P:O ratios have been observed with from 1 to 13 μmoles of P_i uptake. No P:O ratios above the traditional theoretical values were observed except in the case of succinate, ascorbate, and α-glycerophosphate when these substrates were present in concentrations too low to compete effectively with the oxidation of the DPN-linked endogenous substrates. Specific inhibitors of oxidative phosphorylation such as rutamycin and 2,4-dinitrophenol abolish net phosphate uptake.

Among the differences resulting from the physical modification of the cellular membrane of sperm probably the most interesting is the increase in the observed

P:O ratio and the elimination of the stimulating effect of added hexokinase. The combined evidence suggests that these phenomena reflect the release of sperm hexokinase from inhibition by glucose 6-phosphate. Both oxidation (Lardy and Phillips, 1943a,b) and glycolysis (Lardy and Phillips, 1943c) of glucose by bovine spermatozoa are strongly and irreversibly inhibited by DL-glyceraldehyde. This substance exerts its inhibitory effect by forming L-sorbose 1-phosphate which inhibits mammalian hexokinases (Lardy *et al.*, 1950) in the same manner as its analog, D-glucose 6-phosphate (Crane and Sols, 1954).

When the utilization of ATP in intact spermatozoa is blocked by fluoride, the accumulated glucose 6-phosphate probably blocks hexokinase sufficiently to make it rate limiting for respiration and phosphate uptake. The slight but consistent increase of respiration and phosphate uptake on addition of yeast hexokinase to intact spermatozoa (Morton and Lardy, 1967a) indicates that some ATP may diffuse from the intact cells to react with external hexokinase or that yeast hexokinase can penetrate the sperm cell, albiet to only a limited extent. Because the K_i of yeast hexokinase for glucose 6-phosphate is relatively high (Hammes and Kochavi, 1962), this enzyme can function at concentrations of glucose 6-phosphate that would be much more inhibitory to mammalian hexokinases. Alteration of sperm cell membrane permeability by bead treatment would permit glucose 6-phosphate to diffuse into the entire reaction volume and thus diminish inhibition of the sperm cell hexokinase. This could account for the failure of added yeast hexokinase to increase respiration or phosphate uptake in bead-treated cells. Ascites tumor cells were discomposed by the bead treatment.

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