Cellular Oxidative Phosphorylation. I. Measurement in Intact Spermatozoa and Other Cells*

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ABSTRACT: The use of 2-deoxyglucose as a phosphate acceptor and of fluoride to inhibit motility and the hydrolysis of phosphorylated compounds permits the measurement of oxidative phosphorylation in intact bovine spermatozoa. Added hexokinase, adenine nucleotide, and magnesium salts enhanced phosphate uptake. Under optimum conditions the ratio of phosphate molecules fixed to oxygen atoms consumed is from 1/3 to 2/3 that measured with isolated mammalian mitochondria.

Barriers to the diffusion of phosphorylated compounds through compartments and across the cells' membranes apparently limit the observed phosphorylation efficiency, for normal P:O ratios are obtained with spermatozoa in which the cell membrane has been altered by physical or chemical means. Intact ascites tumor cells also fix significant amounts of phosphate

In order to study the control of cellular aerobic energy metabolism, it would be useful to have a method available which would make possible the observation of oxidative phosphorylation within the cell under a wide variety of conditions. The need for such a method has been emphasized by Lehninger (1954), Bishop (1962), and Rickmenspoel (1965).

Measurement of cellular oxidative phosphorylation has been reported by Lynen and Koenigsberger (1950), Zöllner (1952), Lynen (1956), Quastel and Bickis (1959), Ibsen *et al.* (1959), Hess and Chance (1959), Chance (1959b), and Emmelot and Bos (1959). The methods used in those reports, while sometimes ingenious, are indirect and appear to be of narrow applicability. The validity of some of them has been questioned by Chance (1959a) and Morton (1965).

Here, a method is presented which enables the direct assay of cellular ATP[1] synthesis by oxidative phosphorylation over a wide range of conditions. The method depends upon the transfer of the terminal phosphate of ATP to 2-deoxyglucose, primarily by endogenous hexokinase, forming 2-deoxyglucose 6-phosphate which is not metabolized (Hess, 1961) and not inhibitory to hexokinase (Crane, 1962). The uptake of P_i for ATP formation is thus made permanent and equals glucose 6-phosphate production if glucose is used as acceptor in the presence of fluoride. ΔP_i is measured along with oxygen consumption to obtain the P:O ratio. Glycolysis and certain major ATP-utilizing pathways are eliminated by the use of 2-deoxyglucose and the presence of fluoride. Rates of cell penetration by P_i and 2-deoxyglucose can obviously place a limit on the measured rate of phosphorylation. Measures to diminish penetration barriers are dealt with in the two following papers (Morton and Lardy, 1967a,b).

Experimental Section

Cell Sources and Preparation. Bovine epididymides were obtained through the courtesy of Oscar Mayer and Co. The caudal portion of the epididymis along with about 30 cm of the connected vas deferens was removed from freshly slaughtered bulls and stored at 2° for use the same day. When needed, the cells from 2 to 15 epididymides were extruded with a 10-ml syringe containing ice-cold medium as described by Henle (1938). The sperm were then centrifuged at 1600*g* for 5 min at 2° and resuspended (Lardy and Phillips, 1943a) with a syringe pipet in sufficient ice-cold medium to yield the desired concentration when added to the flasks on ice.

Bovine-ejaculated spermatozoa, from studs of proven fertility, were kindly supplied by American Breeders Service, Inc. The cells were allowed to cool to room temperature at a rate slow enough to avoid cold shock (Gladcinova, 1937). They were then washed as indicated above except that they were resuspended at room temperature in 0.25 M sucrose and added to the incubation medium which was also at room temperature. The experiments were begun between 1 and 3 hr after the cells were ejaculated.

Ehrlich ascites tumor or Sarcoma 180, implanted in 18–22-g female mice (Webster strain) 5–7 days earlier, was harvested and washed with 0.25 M sucrose by 3-min spins at room temperature in a clinical centrifuge.

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[1] Abbreviations used in this report: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; DPN, diphosphopyridine nucleotide; DPN^+, oxidized DPN.

The mice were sacrificed by cervical dislocation. Samples containing excessive amounts of erythrocytes were discarded. Erythrocytes in the remaining samples were almost completely removed by withdrawing the red pellet from the bottom of the centrifuged cells with a syringe pipet after each of several washings. The cell concentrations were adjusted to approximately 10^8 cells/ml by the observation that the cellular pellet contains about 10^9 cells/ml.

The authors express their appreciation to Mr. Mel DePamphilis of the Department of Biochemistry, University of Wisconsin, for the gift of *Escherichia coli* B cells grown in tryptone. The *E. coli* in the late-log phase of growth were centrifuged at 1600*g* for 5 min and resuspended in sufficient 0.01 M K_2HPO_4, pH 7.0, or 0.25 M sucrose to give approximately 7×10^8 cells/ml in the incubation flasks.

Incubations were carried out in one side arm, micro-Warburg flasks, whose total volume was 5–10 ml. One-half of the 1-ml incubation volume was contributed by the added cell suspension. In the center well of the flask was 0.1 ml of 5 N KOH and a piece of fluted filter paper extending 5 mm above the lanolin-coated rim.

When P:O ratios were determined, flasks containing cells and other incubation components were placed at 30-sec intervals in the Warburg constant-temperature bath, usually set for 37°. A 10-min period was allowed for thermal equilibration for each flask after which the zero-time flasks received a final concentration of 5% perchloric acid (all concentrations stated in these reports are final concentrations unless otherwise indicated). Simultaneously, the contents of the side arms were introduced into the main flask and the flasks were placed on ice. At this time, after the first manometer readings were quickly recorded, the flasks to be incubated were also tipped to receive their side-arm contents. After the incubation period (15–90 min, depending on the time required to consume 30–100 μl of O_2), the final manometer readings were made, the reactions were stopped with perchloric acid, and the flasks were placed on ice. The solids were removed by centrifugation and the supernatant fluid was analyzed for P_i by the method of Lowry and Lopez (1946) or for glucose 6-phosphate by the method of Noltman *et al.* (1961). For each variable there were one zero-time flask and two incubation flasks. Postexperimental cell counts were made with the aid of a hemocytometer or calculated from the total nitrogen content of a known volume of the original cell suspension. The nitrogen determinations were made on a Coleman, Model 29, nitrogen analyzer.

In all experiments reported, apparent oxygen consumption was corrected for gas evolved from flasks containing 1 ml of medium without cells. An increase in gas volume, caused by warming the cold solutions to 37°, continued beyond the 10-min thermal equilibration period. The slow equilibration of gases between the aqueous and gas phases is probably caused by lack of surface agitation in the small flasks used; in flasks of 16-ml total volume, equilibration occurs much more rapidly (Lardy and Copenhaver, 1954). The volume increase was most pronounced in the early part of the experiment and reached a total of about 8 μl in most cases. In certain experiments to be presented in a later paper, failure to make this correction would raise the P:O ratios above the commonly accepted theoretical limits. Mohri *et al.* (1965) reported P:O ratios of 3.2 for several DPN-linked substrates with midpieces derived from ejaculated bull sperm in manometer flasks of the size described here. It is possible that the P:O values they reported would be reduced by about 10%, were the same corrections made.

The slow gas equilibration described above probably does not influence the accurate measurement of oxygen consumed in flasks containing rapidly respiring cells, for any oxygen deficit would be established during the thermal equilibration period. This gradient would be maintained as long as the cells continue to respire and would facilitate diffusion of oxygen from the gas to aqueous phases. All data reported are averages of duplicate experiments.

Reagents were from the sources indicated: adenine nucleotides and DPN^+, P-L Biochemicals; 2-deoxy-D-glucose, Calbiochem; KF, Baker's reagent; two-times-crystallized alcohol dehydrogenase and types II–IV yeast hexokinase, Sigma. L-(+)-Lactic acid (Tris salt) was prepared from the corresponding calcium salt by ion exchange on Dowex 50X-10 resin. The other reagents were of best quality obtainable from commercial sources. All acid substrates were neutralized with Tris.

Results

Bovine Epididymal Spermatozoa. Phosphate uptake associated with sperm respiration is absolutely dependent on the presence of hexose (Figure 1). Although glucose and fructose were found to meet this requirement, 2-deoxyglucose was chosen because it inhibits glycolysis completely in bull sperm at 18 mM (Lodge, 1963). This permitted fluoride, another component of this system which inhibits sperm glycolysis (Lardy and Phillips, 1943c), to be varied at will. The presence of yeast hexokinase at a concentration commonly used for mitochondrial systems caused a small but significant stimulation of phosphate uptake, respiration, and P:O ratio.

Figure 2 illustrates the striking effect of KF on the P:O ratio of these cells. For most sperm samples fluoride ion must be present for net phosphate uptake. Sensitivity of respiration to 40 mM KF also varies with the cell sample from moderate inhibition as in Figure 2 to slight stimulation. Slight inhibition is most commonly observed. Fluoride inhibits several ATP-requiring processes including sperm motility (Lardy and Phillips, 1943b) and mitochondrial adenosine triphosphatase (Lardy and Wellman, 1953). It inhibits phosphatases (*cf.* Hewitt and Nicholas, 1963) and myokinase (Siekevitz and Potter, 1953) in other systems. The net effect of these inhibitions by fluoride appears to be that more ATP successfully traverses from mitochondria to the cell membrane hexokinase without being utilized by ATP-requiring enzymes.

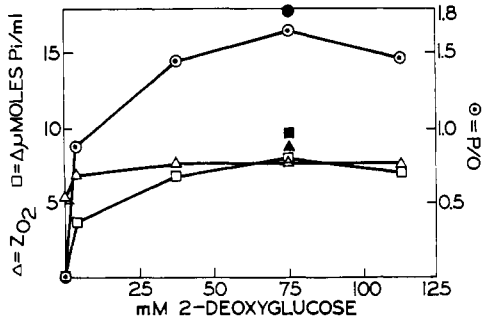

FIGURE 1: The response of intact bovine epididymal spermatozoa to 2-deoxyglucose. $\Delta = Z_{O_2} = \Delta = \mu l$ of $O_2/10^8$ cells per hr = 0.4 Q_{O_2} (Lardy and Phillips, 1941), □ = Δ = micromoles of Pi, and ○ = P:O ratio. Solid data points indicate the presence of 0.1 mg/ml of Sigma type IV yeast hexokinase. A 1.0-ml final volume was incubated at 37° and pH 7.0. The medium contained 40 mM KF, 16 mM phosphate (Tris salt), 10 mM $MgSO_4$, and 0.5 mM Na_2ATP (Tris neutralized). The substrate was 10 mM L-(+)-lactic acid (Tris salt). The 9.3 × 10^8 cells/flask were isolated in 0.25 M mannitol.

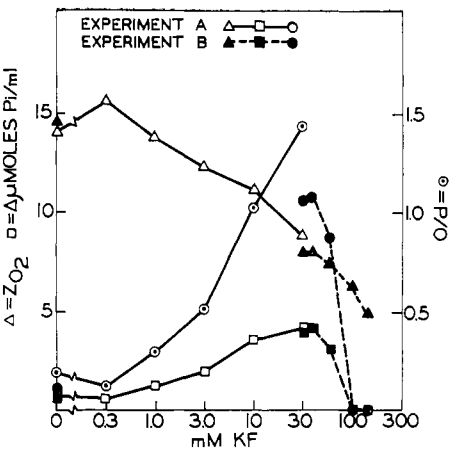

FIGURE 2: The response of intact bovine epididymal spermatozoa to KF. Dark data points = expt B. Conditions were as in Figure 1 except that 0.1 mg/ml of Sigma type IV yeast hexokinase and 75 mM 2-deoxyglucose were present. The same sperm sample (7.2 × 10^8 cells/ml in 0.25 M sucrose) was used in both experiments. Experiment B was performed 3.5 hr after expt A.

Added ATP, ADP, and AMP stimulated respiration (20–30%) and phosphate uptake (40–50%) with 2-deoxyglucose in the medium. Guanosine and inosine di- and triphosphates caused a lesser but measurable response. Uridine and cytosine di- and triphosphates did not elevate the Z_{O_2} values or observed P:O ratios. The optimum concentration of ATP was between 0.5 and 2 mM. Higher concentrations to 6 mM depressed phosphate uptake slightly but did not alter respiration. Magnesium sulfate or chloride added at 1–10 mM did not affect respiration, but caused a 100% increase in net phosphate uptake and resultant P:O ratio; 20 mM $MgSO_4$ was higher than optimal.

Henle and Zittle (1942) observed that, for bovine epididymal spermatozoa, optimal rates of respiration per cell were obtained with from 4 to 8 × 10^8 cells/ml. We obtained maximal P:O ratios with from 4 to 12 × 10^8 cells/ml. Alteration of the sperm cell membrane does not shift the sperm concentration range for maximal respiration (Morton and Lardy, 1967b).

Results to be presented in the following paper indicate pH 7.0 to be most favorable for sperm ATP synthesis. This pH was used in most experiments reported here.

Inorganic phosphate was usually added to provide a concentration of 15 mM, but in some experiments only 5 mM was employed to make changes in P_i concentration more accurately measurable. In these cases the experiments were stopped before P_i concentration became limiting.

For intact bovine epididymal spermatozoa the P:O ratio was found to be relatively constant over the temperature range examined (20–37°). For most experiments 37° was chosen to make comparison with data in the literature more direct, because it is near *in vivo* temperatures, and because a high rate of metabolism was desirable experimentally.

It was found that sperm isolated in 0.25 M sucrose, 0.25 M mannitol, 0.25 M lactose, or 0.15 M KCl had essentially the same metabolic rates and phosphorylation efficiency. However, if 0.15 M NaCl was used (77 mM after dilution into the flasks), P:O ratios were depressed. In the presence of 40 mM KF, additions of 50–200 mM NaCl were increasingly deleterious to respiration and P:O ratio observed; however, these cells could tolerate 50 or 100 mM KCl quite well. The presence of varying concentrations of ouabain, the inhibitor of cell membrane sodium transport ATPase, had no effect on net P_i uptake or respiration, either in the presence or absence of fluoride over the 0–150-mM range of Na^+ ion tested. Unless otherwise noted, sodium ion was not added to the medium except that contributed by the disodium salt of ATP.

The addition of oxidizable substrate strongly enhanced rates of respiration above the endogenous and increased phosphate esterification. With L-(+)-lactate maximal rates were observed with 10–38 mM concentrations and 2 mM was near maximal. For all experiments reported, substrates were added at 10 mM except succinate which was 20 mM. The results obtained with intact bovine epididymal spermatozoa oxidizing various substrates are presented in Table I. P:O ratios varied from 1/3 to 2/3 of the maximum values usually obtained with mammalian mitochondria (Copenhaver and Lardy, 1952). Rutamycin (Lardy *et al.*, 1965), valinomycin (McMurray and Begg, 1959), and 2,4-dinitrophenol inhibited phosphate uptake completely.

The catalytic function of L-malate in the oxidation of endogenous substrate is shown in Table II. In this

TABLE I: Apparent Phosphorylation Efficiency of Intact Bovine Epididymal Spermatozoa.[a]

Expt	Substrate	Z_{O_2}	Obsd P:O Ratio
A	Endogenous	3.4	0.9
	Lactate	8.5	1.8
	α-Ketoglutarate	8.4	1.8
	Malate	6.5	1.4
	Succinate	9.8	1.4
	α-Glycerophosphate	10.8	0.7
B	Lactate	9.1	2.0
C	Lactate	6.0	1.3
	Lactate + malate	10.9	2.0
D	Endogenous	3.1	0.3
	Pyruvate	5.4	0.9
	Pyruvate + malate	8.2	1.6
	Citrate	4.5	0.8
	Fumarate	4.5	1.0
	β-Hydroxybutyrate	4.9	0.8
	Glutamate	3.9	0.4
E	Lactate	8.1	1.7
	Lactate + 10^{-4} M DNP	17.4	0.0
F	Lactate	8.6	0.9
	Lactate + 3 μg/ml of rutamycin	3.7	0.0
G	Lactate	6.5	1.6
	Lactate + 0.01 μg/ml of valinomycin	6.3	0.0

[a] Conditions were similar to those in Figure 2 except that fluoride was 40 mM. Cells/ml × 10^8: A, 6.8; B, 9.7; C, 6.3; D, 8.0; E, 5.3; F, 9.2; and G, 7.6. When malate was added in addition to another exogenous substrate it was 1 mM.

TABLE II: Response of Intact Bovine Epididymal Spermatozoa to D- and L-Malate.[a]

Substrate	Addn (mM)	Z_{O_2}	P:O
Endogenous		4.4	0.4
Endogenous	L-Malate (0.1)	7.4	1.2
Endogenous	L-Malate (1.0)	7.0	1.2
Endogenous	D-Malate (1.0)	4.5	0.3
Endogenous	L-Malate (10)	8.7	1.5
10 mM pyruvate		7.5	0.7
10 mM pyruvate	L-Malate (1)	10.1	1.2
10 mM pyruvate	D-Malate (1)	7.5	0.8

[a] Incubation conditions the same as Figure 2. KF (40 mM) was present. 10^9 sperm/ml.

50-min experiment, 0.1 μmole of L-malate elevated oxygen consumption from 3.3 to 5.5 μatoms, in addition to any stimulation it may have exerted during the 10-min thermal equilibration period. L-Malate also enhanced oxygen consumption in the presence of pyruvate. D-Malate was not oxidized and did not stimulate the oxidation of endogenous substrate nor pyruvate. Thus malate did not function by enhancing transport of other organic substrates as it may in some systems (Ferguson and Williams, 1966; Chappell, 1966).

Bovine-Ejaculated Spermatozoa. If these cells were sedimented from the seminal plasma and resuspended in 0.25 M sucrose, net phosphate uptake was rarely observed. However, if epididymal cells were suspended in 10% seminal plasma no P_i uptake occurred either, unless the plasma had been previously deproteinized with perchloric acid and neutralized with KOH. It is tentatively concluded that the powerful phosphatases in seminal plasma (Mann, 1964) hydrolyze the 2-deoxyglucose 6-phosphate formed from ATP and release the bound phosphate to the medium. When the ejaculated cells were centrifuged from the plasma and suspended in a volume of 0.25 M sucrose and 10^{-3} M EDTA equal to three times that of the original semen and then sedimented and resuspended in 0.25 M sucrose of the original semen volume, high P:O ratios were observed.

Ejaculated sperm were similar to epididymal sperm in their response to 2-deoxyglucose and KF except that KF at 1–3 mM depressed respiration of ejaculated cells 25–50% (*cf.* Lardy and Phillips, 1943b), whereas significant respiratory inhibition in this fluoride concentration range is rare for epididymal sperm. This subject will be treated elsewhere.

With ejaculated spermatozoa, maximal P:O ratios

TABLE III: Apparent Phosphorylation Efficiency of Intact Bovine-Ejaculated Spermatozoa.[a]

Expt	Substrate	Z_{O_2}	Obsd P:O Ratio
A	Endogenous	7.2	0.8
	Lactate	6.8	1.7
	α-Ketoglutarate	8.9	1.7
	Malate	8.0	1.8
	Succinate	12.2	1.7
	α-Glycerophosphate	9.2	1.3
B	Lactate	4.6	2.1
C	Lactate	7.3	2.5
D	Lactate	5.5	2.1
E	Lactate	4.1	2.3

[a] Conditions were similar to those of Figure 1 except that 0.25 mg/ml of Sigma type II yeast hexokinase and 75 mM 2-deoxyglucose were present. Cells were suspended in 0.25 M sucrose. The experiment and cells/ml × 10^8 were: A, 6.2; B, 6.8; C, 5.1; D, 7.2; and E, 11.5.

TABLE IV: P:O Ratios Observed for Ehrlich Ascites Tumor Cells.[a]

Expt	Substrate	Inhibitor	Z_{O_2}	Obsd P:O
A	β-Hydroxybutyrate		190	0.8
B	Succinate		350	0.9
	Pyruvate		210	0.6
	L-(+)-Lactic acid (K^+ salt)		250	0.6
	D-(−)-Lactic acid (K^+ salt)		160	0.4
C	Succinate		260	0.5
	Succinate	Actinomycin D	260	0.5
	Succinate	Puromycin	260	0.5
	Succinate	Actinomycin and puromycin	250	0.6
	Succinate	Iodoacetate	290	0.5

[a] Conditions as in Figure 3 except that 10 mM P_i and 20 mM KF were present. Experiment A, 0.69 × 10^8 cells/ml; expt B, 0.56 × 10^8 cells/ml; and expt C, 0.63 × 10^8 cells/ml. The cells were suspended in 0.25 M sucrose. Actinomycin was 3.5 μg/ml; puromycin, 10^{-4} M; and iodoacetate, 10^{-3} M.

were observed with from 3 to 10 × 10^8 cells/ml. In this system the Z_{O_2} and P:O ratio of ejaculated spermatozoa were even more sensitive to added NaCl than these processes were in epididymal sperm; therefore, the addition of sodium ion was again avoided as much as possible. Potassium ion at 40 mM was slightly stimulatory to respiration and Z_{O_2}, and was not toxic at 150 mM.

Table III indicates the P:O ratios observed for bovine ejaculated spermatozoa. Endogenous respiration of these cells is higher than for epididymal spermatozoa (Lardy *et al.*, 1949) and this difference is apparent even in the presence of fluoride. Added substrates did not greatly enhance rates of oxygen consumption but did improve the P:O ratios. With lactate as substrate, P:O ratios of 1.7 to 2.5 were obtained repeatedly. As

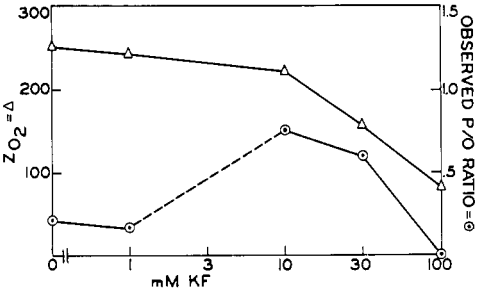

FIGURE 3: The response of intact Ehrlich ascites tumor cells to KF. Δ = Z_{O_2}, ○ = P:O ratio. The pH 7.4 incubation medium contained 5 mM potassium phosphate, 10 mM magnesium sulfate, 2 mM ATP, 12.5 mM glucose, and 0.17 mg/ml of Sigma type III yeast hexokinase. The substrate was succinate and the cells 0.98 × 10^8/ml were isolated in 0.25 M sucrose.

TABLE V: P:O Ratios Observed for Sarcoma 180 Ascites Cells.[a]

Substrate	Z_{O_2}	Obsd P:O Ratio
Endogenous	113	0.6
L-(+)-Lactic acid (Tris salt)	128	0.6
α-Ketoglutarate	143	0.5
Succinate	167	0.5

[a] Conditions as in Figure 2, except KF was 10 mM, Sigma type II yeast hexokinase was present at 0.25 mg/ml. The 1.3 × 10^8 cells/ml were suspended in 0.25 M sucrose.

with epididymal spermatozoa, ejaculated cells oxidizing α-ketoglutarate yielded P:O ratios no higher than the values for other DPN-linked substrates. However, the occurrence of the substrate-level phosphorylation step associated with α-ketoglutarate oxidation is indicated by the fact that omission of P_i in the presence of dinitrophenol (DNP) inhibits α-ketoglutarate oxidation but enhances the rate of lactate oxidation.

Ehrlich and Sarcoma 180 Ascites Tumor Cells. Ehrlich ascites cells exhibited a relatively low P:O ratio that was elevated by 10 mM fluoride (Figure 3). The phosphorylation efficiency of these cells was found to be independent of pH from 6.2 to 8.6; however, the respiratory rate was 40% greater at pH 8.6 than at pH 6.2; the increase was linear with increasing pH. The

addition of increasing amounts of glucose as phosphate acceptor produced a Crabtree effect with maximal P:O ratios occurring when oxygen consumption was depressed maximally (50%). Tables IV and V present the results observed thus far with these cells.

E. coli cells at a concentration of about 7×10^8/ml were capable of oxidizing lactic acid (Tris salt) and L-serine rapidly under the conditions used for epididymal sperm. Fluoride at 40 mM inhibited respiration 20% when lactate was substrate. In these experiments only small net phosphate uptake was observed, giving observed P:O ratios of 0.1.

Discussion

As will be demonstrated in the next two papers of this series, the P:O ratios observed with intact spermatozoa are not so great as those occurring within the mitochondria of the cells studied. Since this method depends upon the reaction of mitochondria-derived ATP with extramitochondrial or extracellular hexokinase, physical barriers and enzymatic hazards probably limit the amount of ATP reaching the phosphate acceptor system. Thus, assuming a constant efficiency of ATP synthesis, the observed P:O ratio for a cell-like ascites tumor whose mitochondria are surrounded by an abundance of cytoplasmic enzymes including the endoplasmic reticulum would predictably be lower than that observed for spermatozoa with relatively little cytoplasm and whose cell membrane hexokinase is very near the mitochondrial helix.

The stimulation of respiration and phosphate uptake by certain nucleotides implies that these compounds may enter the cell and participate in phosphate transfer. It has been found that spermatozoa diluted in calcium-free Ringer–phosphate lose compounds that absorb at 260 mμ. The presence of added nucleotide may be beneficial by reducing the net loss of nucleotides from the cells. Maskin *et al.* (1964) were unable to interpret some of their data regarding the effects of added insulin and hexokinase upon sperm glycolysis partly because they could find no report in the literature demonstrating that ATP leaves the cell. The observations presented here, that respiration and net phosphate uptake were stimulated by the addition of yeast hexokinase, suggests that ATP may be among the 260-mμ absorbing molecules leaking from the cell. While this may seem intuitively unacceptable, diffusion of ATP from the mitochondrion was taken for granted for a long time before unequivocal proof for such movement was obtained (Pressman, 1958; Klingenberg and Pfaff, 1966). In intact tissues a small permeability of neighboring cells to adenosine triphosphate would appear to be of little consequence.

Diffusion of nucleotides from spermatozoa under the conditions described here and in paper II (Morton and Lardy, 1967a) occurred in the absence of 2-deoxyglucose. The loss is therefore not dependent on the specific nucleotide-depleting effect of 2-deoxyglucose observed in Ehrlich ascites cells by Letnausky (1964). The latter effects undoubtedly result from the failure of 2-deoxyglucose 6-phosphate to inhibit hexokinase and thereby prevent depletion of high-energy phosphate reserves.

The stimulation of respiration by DNP in the presence of hexokinase shown in Table I does not occur in cells with altered cellular membranes (Morton and Lardy, 1967a,b) or in isolated rat liver mitochondria (Lardy and Wellman, 1952). It may be explained by a short circuit produced by preventing the formation of high-energy phosphoryl compounds, thus by-passing the apparently rate-limiting transit of ATP to the cell membrane hexokinase and ADP back to the mitochondria.

It is likely that if optimal concentrations of each component of the system were carefully determined for each cell type studied, higher P:O ratios would be obtained. Here this was done only for bovine epididymal spermatozoa. It is expected that a modification of this method may be applied to suspensions of many single cell types.

A diminished P:O ratio is a sacrifice that for some purposes can be tolerated in a method that directly measures ATP synthesized by cellular oxidative phosphorylation. The net phosphate uptake in this method is reasonably high and factors altering the efficiency of cellular oxidative phosphorylation can readily be detected by a decrease in this uptake.

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