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Mammalian sperm metabolism: oxygen and sugar, friend and foe

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ABSTRACT Mammalian spermatozoa expend energy, generated as intracellular ATP, largely on motility. If the sperm cell cannot swim by use of its flagellar motion, it cannot fertilize the egg. Studies of the means by which this energy is generated span a period of six decades. This review gives an overview of these studies, which demonstrate that both mitochondrial oxidative phosphorylation, for which oxygen is friend, and glycolysis, for which sugar is friend, can provide the energy, independent of one another. In mouse sperm, glycolysis appears to be the dominant pathway; in bull sperm, oxidative phosphorylation is the predominant pathway. In the case of bull sperm, the high activity of the glycolytic pathway would maintain the intracellular pH too low to allow sperm capacitation; here sugar is enemy. The cow's oviduct has very low glucose concentration, thus allowing capacitation to go forward. The choice of the pathway of energy generation *in vivo* is set by the conditions in the oviduct of the conspecific female. The phospholipids of the sperm plasma membrane have a high content of polyunsaturated fatty acids represented in their acyl moieties, rendering them highly susceptible to lipid peroxidation; in this case oxygen is enemy. But the susceptibility of the sperm membrane to lethal damage by lipid peroxidation allows the female oviduct to dispose of sperm that have overstayed their welcome, and so keep in balance sperm access to the egg and sperm removal once this has occurred.

KEY WORDS: *mitochondria, glycolysis, motility, mouse gapds-/- knockout, lipid peroxidation*

Introduction

Some 330 years have passed since the observation of Antonius Leeuwenhoek in 1677 of spermatozoa in human seminal fluid – "little animals", which could be seen with his microscope and which swam in the fluid. The fascination with motility of sperm has a long history, with spurts of advances in understanding, punctuated by long periods of inattention. From the last quarter of the 19th through the first two decades of the 20th century, most of the studies of this motility were centered on sperm from invertebrates whose fertilization strategy was external: clouds of sperm encountering clouds of eggs in sea or pond water. In his classic book, *Problems of Fertilization*, Lillie (1919) expressed the following view of sperm motility requirements:

"Spermatozoa are probably incapable of receiving nourishment outside of the gonad after they are fully differentiated; certainly in the case of external insemination there is no opportunity for the restitution of substance. We must therefore regard these cells as charged with their full available store of energy in the testis and their capacity for locomotion as thus determined and limited."

This was a reasonable assessment of the energy metabolism of spermatozoa based on the studies carried out on these cells up to that time. In the years that followed the publication of Lillie's book, serious investigations into the physiology of sperm from domestic mammals began to catch up with, and eventually overtake, the already well-established studies of the physiology of the sperm of marine animals. This review attempts to trace the path of inquiry into mammalian sperm metabolism: omissions have been committed in the hope of coherence, for which the author apologizes in advance.

Early studies: respiration; glycolysis, aerobic and anaerobic

Respiration of mammalian sperm was recognized during the two decades following the publication of Lillie's book (see Mann (1964) for review), but it was the studies of Redenz (1933) that first

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identified the need for 'outside nourishment' of bovine sperm in the absence of O_2. These sperm maintained motility in the presence of O_2, even if washed free of seminal plasma, but the washed sperm required glucose or other sugar capable of fermentation to lactate, in order to survive anaerobically. Bull sperm were available in quantities sufficient for analysis by the then available biochemical techniques and so figured prominently in studies at the Universities of Wisconsin and Pennsylvania in the early years of the 1940's. Early studies at the University of Wisconsin carried out by Lardy and Phillips (1941a, b, c) showed that bull sperm could exhibit both oxidative and glycolytic metabolism, The sperm maintained motility in the absence of exogenous substrate in the presence of air: the endogenous substrate was inferred to be intracellular phospholipid from the loss of this component over the incubation time utilized (Lardy and Phillips, 1941c).

Studies at the University of Pennsylvania were carried out during this same period by Zittle and coworkers in the Department of Bacteriology of the School of Medicine – possibly an indication that motile sperm were still mentally classified with flagella-bearing bacteria at that time. The cells utilized were sperm flushed from epididymides obtained from the local abattoir. The use of epididymal sperm was dictated by the urban location of the University of Pennsylvania as compared with the more rural setting of the University of Wisconsin, where live cattle herds were readily accessible. In Philadelphia, abattoirs were sited in designated areas of the city, but live bulls were not allowed on campus. Zittle and Zitin (1942) demonstrated activity of cytochrome oxidase in epididymal bull sperm, both in suspensions of cells ground in a glass homogenizer and disintegrated by sonication. The suspensions were separated by centrifugation into head, midpiece and tail fractions; little oxidase activity appeared in the head fraction; the bulk of the activity was in the midpiece and some in tail fractions. Mitochondria had been located in the sperm midpiece (Lillie, 1919), but their possible role in sperm respiration does not appear to have been appreciated at the time. Studies by Henle and Zittle (1942) with sperm, flushed from the epididymides with isotonic Ringer medium, showed that the cells metabolized glucose added at 5-10 mM. Motility was checked: there was no close correlation between O_2 consumption and motility, but in the one sample that showed no O_2 consumption, there was no motility. The salient finding of this study was quantitation of glycolysis in the absence and presence of O_2; at 5 mM glucose, aerobic glycolysis, measured as conversion of glucose to lactate, was 40-60% of the rate of anaerobic glycolysis.

Bovine sperm show little motility immediately upon removal from the epididymis, but aeration of the resulting sperm suspension initiated motility; cyanide inhibited this effect (Lardy *et al.*, 1945a). The aeration also produced adenosine triphosphate (ATP) in the cells, showing active oxidative phosphorylation. As isolated in this study, the epididymal sperm utilized pyruvate, *l*-lactate (but not *d*-lactate), succinate, acetoacetate, acetate and added egg phospholipids to maintain respiration and motility (Lardy and Phillips, 1944). Respiration was also stimulated by added bicarbonate. The implication of these results is that the plasma membrane over the midpiece may have been permeabilized. A comprehensive survey of metabolite oxidation in ejaculated sperm (Lardy and Phillips, 1945) provided further evidence that these cells utilized the Krebs cycle and supported oxidative phosphorylation. This study reinforced the validity of the proposal, based on this and earlier studies (Lardy and Phillips (1941a,b,c; 1943a,b,c), that two separate metabolic processes, oxidative and glycolytic, could each furnish adequate energy for motility of bull spermatozoa.

A systematic investigation of the metabolism of ram spermatozoa, patterned on that of bull spermatozoa, by Lardy *et al.* (1945b) demonstrated that washed sperm from this species had robust oxidative metabolism and could maintain motility for several hours in the absence of added glucose. The oxidative metabolism and motility was fully inhibited by cyanide under these conditions, but motility was restored upon addition of glucose. These observations were confirmed and extended by White and Wales (1961), who showed that there was little difference in respiratory and glycolytic metabolism between epididymal and ejaculated ram sperm. Ram sperm thus can also maintain motility by the two parallel modes of energy generation.

During this same period, the properties of human sperm metabolism were investigated by McLeod (1941, 1943a). Compared to the ample quantities of bull sperm available, the amounts of human sperm were small, and the variation between individual donors was considerable. Despite these difficulties, McLeod (1941) showed that the respiration rates of human spermatozoa were so small that the their metabolism could be inferred to be exclusively glycolytic. Because of the small size of the available samples, no evidence for the respiratory cytochrome enzymes were found, but further biochemical studies showed activity of these through O_2 uptake in the presence of p-phenylenediamine, which was strongly inhibited by addition of azide (Mcleod, 1943a). Reduction of methylene blue by succinate was also noted, but succinate was not able to sustain the motility of human sperm. The studies were extended to examination of loss of human sperm motility in the presence of O_2 under an atmosphere of 95% O_2/5% CO_2. While about half the cells remained motile under 95% N_2/5% CO_2 at 38°C for 8 hours, all motility was lost during this period under the O_2 atmosphere. This loss was prevented by addition of catalase to the incubation medium. These experiments were among the first to demonstrate the toxicity of H_2O_2 towards human sperm. The results of these studies and earlier studies on human sperm, the results of the studies on bull sperm described above, and the results of other studies on sperm from various mammalian species up to 1943 were summarized in a comprehensive and critical review by McLeod (1943b).

The inability of McLeod to observe the cytochromes, indicative of the oxidative pathway of sperm metabolism, was shown to be due to the low sperm density in most human samples as well as small sample size, by Mann (1951), who was able to observe the characteristic absorption bands of the respiratory pigments in samples frozen in liquid air. This method, developed by Keilin and Hartree (1949, 1950), provided enhancement of the absorption bands up to 20-fold (see detailed account by Estabrook, 1956), and so allowed the cytochromes in human sperm to be readily revealed. The bands were seen only in the sperm, not in the seminal plasma. Human sperm could thus join their other mammalian brethren as sperm cells capable of oxidative metabolism, albeit in a rather wan manner.

Availability of semen from ram, bull, and boar from the Animal Research Station, Cambridge, UK, enabled Mann (1945a,b) to undertake an extended study of enzymatic processes involved in

the energy metabolism of sperm from these species. The presence of cytochromes *a*, *b*, and *c* was confirmed by the presence of the characteristic absorbance bands under reducing conditions, using the microspectroscope method of Keilin (1925) as refined by Keilin and Hartree (1939). No trace of cytochrome was found in the seminal plasma. Washed sperm readily metabolized glucose to lactate with the production of ATP under both aerobic and anaerobic conditions. But the question remained as to the nature of the metabolizable carbohydrate available to sperm in the seminal plasma. Mann (1946) showed that fructose, rather than glucose, was that carbohydrate in the three species, and that it was synthesized in the reproductive tract accessory glands. Boar sperm, in contrast to bull and ram sperm, convert fructose to lactate anaerobically at a low rate and show poor motility under these conditions. Under aerobic conditions, these sperm show robust respiration with fructose as well as with lactate and pyruvate (Aalbers *et al.*, 1961). While the sperm from these domestic species showed quantitative differences in rates of respiration and glycolysis/fructolysis, all had the capability of generating metabolic energy from both sources. The structure of the mammalian spermatozoan offers a key to this dual capability.

Later studies: compartmentalization of respiration and glycolysis

The studies of sperm energy metabolism described above dealt primarily with the whole sperm cell as the biochemical and physiological entity. The remarkable construction of the mature mammalian sperm cell had been well worked out and described by numerous investigators in the last decades of the 19th and first decades of the 20th centuries, using light microscopy (see Lillie, 1919, for review and citations), but the partition of metabolic functions between head, midpiece, and principal piece had yet to be established. The advent of electron microscopy, with appropriate fixation methods and thin sectioning techniques, moved sperm morphology forward by a quantum leap (Fawcett, 1957). The flagellar structure of the sperm axoneme was fully revealed, the position of the nucleus in the head, with ancillary structures were visualized, and the acrosome, with variations in its structure among sperm from different species, could be seen clearly (Fawcett, 1957). Mitochondria are localized in a helical structure wrapped around the axoneme in the midpiece, which gives this element its characteristic structure; mitochondria are absent from principal piece.

Mammalian spermatozoa, unlike rat livers, are not readily homogenized to a point where subcellular organelles may be isolated for study. The early attempts at homogenization by Zittle and Zitin (1942) to attempt this were not particularly successful, as described above. A much more elaborate scheme of sonication and differential centrifugation was applied to bull sperm by Mohri *et al.* (1965), that enabled a clean midpiece preparation to be isolated. The basic structure of the midpiece: mitochondria wrapped around the axoneme, was maintained in this preparation. Mitochondrial substrates were readily oxidized. Oxidative phosphorylation efficiency, assessed as P:O ratios from O_2 consumption and ATP formation rates approached that observed in preparations of isolated mitochondria from tissue sources. The sturdiness of the midpiece was thus demonstrated. The most active substrate was glycerol-1-phosphate, which had an observed P:O ratio up to 1.7, as compared to a theoretical value of 2.0. The importance of this substrate to oxidative phosphorylation in bull sperm was the identification of glycerol kinase in these cells (Mohri and Masaki, 1967); this enzyme was found to be unique to bull sperm. This enzyme catalyzes formation of glycerol-1-phosphate from glycerol and ATP; bull sperm can thus utilize glycerol to provide one net ATP: one ATP used in formation, two ATP recovered from oxidative phosphorylation. The report of White *et al.*, 1963) glycerolphosphorylcholine diesterase is active in the cow oviduct suggests that the product of its activity, glycerol, is available to the sperm from oviductal fluid.

Discrete regions of the respiratory chain were identified as contributing to the overall process of oxidative phosphorylation (Copenhaver and Lardy, 1952). Mitochondria were clearly established as the seat of respiration and oxidative phosphorylation in the cell, and so the midpiece of mammalian sperm was assigned this specialized role. The head and the principal piece lack any respiratory enzymes and so were assigned the site of glycolytic activity. The two metabolic pathways that had been shown to proceed rather independently of each other could thus be shown to proceed in the different subcellular compartments of sperm. The consequences of this compartmentalization, resulting from maturation of the mammalian spermatozoon into the highly differentiated structure destined to fertilize the egg, have been lucidly evaluated by Travis and Kopf (2002) in a recent review.

Mitochondrial activities: oxygen as friend

Mitochondria had been earlier isolated as discrete organelles from somatic tissues, in particular, rat liver (Schneider and Hogeboom, 1950). Studies of intracellular distribution of different enzyme activities localized the components of the respiratory chain to the mitochondria (Schneider and Hogeboom, 1950, 1952; de Duve *et al.*, 1955). Control of mitochondrial respiration by inorganic phosphate (P_i) acceptors, e.g. ADP as acceptor to form ATP, led to the concept of respiratory control, as demonstrated by Lardy and Wellman (1952). Respiratory control was defined as the ratio of the O_2 uptake rate in the presence of ADP plus P_i to the rate in the presence of ATP. The former was maximal, the latter minimal; a high value for the respiratory control ratio (RCR) characterized efficient oxidative phosphorylation in “coupled” mitochondria (Chance and Williams, 1956). The RCR value can be lowered by the presence of an external ATPase activity. If this is the case, an alternate RCR can be determined from the rate in the presence of an uncoupler of oxidative phosphorylation and the rate in the presence of the ATP synthase inhibitor, oligomycin. This inhibitor allows maximal energization of the mitochondria, as no free energy is expended on ATP synthesis, which results in the slowest O_2 uptake rate exhibited by coupled mitochondria. The most rapid rate is observed in the presence of an uncoupler, in which all free energy is dissipated as heat. The uncoupler to oligomycin RCR should thus be a maximal value (Slater, 1967).

The experiments described above utilized Warburg manometers to measure O_2 uptake by the sperm cells. Development of the polarographic O_2 electrode by Clark (Clark *et al.*, 1953) provided a means to measure uptake of O_2 in a rapid, continuous, and readily quantifiable manner; its adaption to the study of mitochondrial respiration is described in detail by Estabrook

(1967). Mitochondrial O_2 uptake studies described in the following sections utilized this electrode.

Separation of mitochondrial activity from glycolytic activity was achieved in rabbit epididymal sperm by hypotonic treatment, which lysed and effectively stripped off the plasma membrane, leaving the sperm structure itself intact (Keyhani and Storey, 1973). The mitochondria remained in the midpiece region, arranged in the helical sheath formation characteristic of the intact sperm, and they did not swell. The mitochondrial ultrastructure remained unchanged in the hypotonically treated cells, and mitochondrial activities remained fully intact. Cytosolic factors, e.g. pyridine nucleotide, that mediate glycolysis were lost, so that mitochondrial activities alone could be assessed. Rabbit epididymal sperm, in contrast to rabbit ejaculated sperm, are obtained as a clean suspension, free of somatic cells, so that hypotonic treatment does not introduce artefacts due to lysed somatic cells. As measured with the O_2 electrode, rabbit sperm mitochondria utilized malate and pyruvate as substrates with oxidative phosphorylation of ADP to ATP as shown by RCR of 1.9. The low value of RCR is due to the activity of the flagellar ATPase; the RCR measured as the ratio of the rate of O_2 uptake with uncoupler to the rate in the presence of oligomycin was 7.7, indicative of well coupled mitochondria. Assay for respiratory chain cytochromes, using low temperature spectrophotometry (Estabrook, 1956) showed the presence of cytochrome oxidase (cytochromes *a* and a_3), cytochromes b_{561} and b_{566}, and cytochromes *c* and c_1.

The remarkable stability of the mitochondrial sheath making up the midpiece of rabbit sperm and sperm from other mammals, including human, derives from the mitochondrial capsule selenoprotein, which is a transformed version of phospholipid hydroperoxide glutathione peroxidase (Calvin *et al.*, 1981; Ursini *et al.*, 1999; Foresta *et al.*, 2002; Maiorino and Ursini, 2002). This protein, which in spermatogenesis provides enzymatic protection against lipid peroxidation, becomes the major structural protein in providing the rigid structure, resistant to osmotic swelling in hypotonic media, of mitochondria of the midpiece of mature sperm. Additional stability derives from the large degree of disulfide bond formation that occurs during epididymal transit for mammalian sperm, with most of the structural stabilization being seen in the principal piece (Bedford and Calvin, 1974). The structure of the mammalian sperm mitochondrial capsule stabilizes the inner and outer mitochondrial membranes and so provides an intermembrane space that is, in effect, sperm specific. The genetic response to the milieu of this space has been expression of a sperm-specific isoform of cytochrome *c*, designated cytochrome c_t to differentiate it from the somatic form, cytochrome c_s(Goldberg *et al.*, 1977; Hake and Hecht, 1993). The subunit VIb of cytochrome oxidase is the one subunit of this multiunit enzyme that accesses the intermembrane space; it too has a sperm-specific isoform (Hüttermann *et al.*, 2003). The implication of these findings is that the sperm-specific subunit VIb provides the interaction site with cytochrome c_t in the midpiece mitochondrial intermembrane space, but experimental support for this implication is thin. That cytochrome c_t is superior to cytochrome c_s in maintaining oxidative phosphorylation in mouse sperm comes from the study of Narisawa *et.al.* (2002), which showed that mice null for cytochrome c_t, while fertile and capable of oxidative phosphorylation with cytochrome c_s as substitute, had lower activity of oxidative phosphorylation and underwent premature loss of germ cells due to apoptosis.

The technique of hypotonic plasma membrane lysis was extended to boar sperm by Calvin and Tubbs (1978), to epididymal bull sperm by Storey (1980), and to mouse sperm by Carey *et al.* (1981). Mitochondrial activities, assessed as O_2 uptake with various mitochondrial substrates, were observed in all cases, but with considerable variation between species. Respiratory control was exhibited by the sperm mitochondria so exposed. Hypotonically treated boar sperm showed robust mitochondrial oxidation of glycerol-1-phosphate through the flavoprotein-linked dehydrogenase in the inner mitochondrial membrane [mitoDH]. Sufficient of the nominally cytosolic NAD-linked glycerol-1-phosphate dehydrogenase [cytoDH] activity was left on the sperm structure that exogenous NADH oxidation through the glycerol-1-phosphate shunt could be demonstrated (Calvin and Tubbs, 1978). The shunt reaction operates by NADH reduction of the dihydroxyacetone-1-phosphate (DHAP) generated by mitochondrial oxidation of glycerol-1-phosphate (G1P):

$$G1P + (O_2)\ [\text{mitoDH}] \Rightarrow DHAP;$$
$$DHAP + NADH \Rightarrow NAD^+ + G1P\ [\text{cytoDH}]$$

This reaction provides one of the pathways for re-oxidation of NADH produced in the cytosol through the action of the glycolytic pathway enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAP-DH). In boar sperm, the oxidation of G1P to DHAP provides an entrée for this substrate into the glycolytic pathway: the DHAP is rapidly interconverted to glyceraldehyde-3-phopsphate (GAP) by the triose phosphate isomerase present in the sperm. The GAP enters the ATP-producing stage of the glycolytic pathway through the action of GAP-DH (Jones and Gillan, 1996). Mouse and bull sperm mitochondria exhibit active mitochondrial oxidation of G1P (Carey *et al.*, 1981; Storey, 1980). Mouse sperm can remain motile in the absence of added substrate (Heffner *et al.*, 1980), presumably by utilizing G1P derived from phospholipid in this manner. This substrate is the most active for O_2 uptake in bull sperm. Rabbit sperm mitochondria, by contrast, show minimal activity of mitochondrial G1P oxidation (Storey and Keyhani, 1974).

Lactate metabolism in mammalian sperm is mediated by a lactate dehydrogenase (LDH) isoform that is sperm-specific (Blanco and Zinkham, 1963; Goldberg, 1963) and was designated at the time as LDH-X. In modern nomenclature, it is designated LDH-C4. Extensive studies on the effects of respiratory inhibitors and uncouplers of oxidative phosphorylation by the Lardy group (Van Dop *et al.*, 1977; Hutson *et al.*, 1977; Van Dop *et al.*, 1978) led to the conclusion that intramitochondrial LDH was involved in sperm pyruvate and lactate metabolism. LDH in mammalian somatic cells is not found in the mitochondria; intramitochondrial localization appears to be confined to sperm and its sperm-specific isoform. Intramitochondrial LDH activity was demonstrated in hypotonically treated rabbit epididymal sperm by fluorimetric monitoring of the redox state of mitochondrial pyridine nucleotide, presumed to be NAD (Storey and Kayne, 1977, 1978). Lactate produced a marked reduction of intramitochondrial NAD^+, as shown by increased NADH fluorescence. Of significance was the observation that the extensive reduction of NAD^+, produced by lactate in the presence of the respiratory chain inhibitor rotenone which blocks oxidation of

intramitochondrial NADH, could be reversed by addition of an equimolar amount of pyruvate. This reaction could only occur if there were intramitochondrial LDH activity.

The mitochondrial matrix carries but a small percentage of the sperm LDH activity. In rabbit sperm, the LDH activity that remains bound to the sperm structure after hypotonic treatment is substrate-accessible and readily assayed by measuring the rate of exogenous NADH oxidation by pyruvate catalyzed by the enzyme. Distribution of LDH activities showed that some LDH activity was on the surface of the plasma membrane, presumably deposited there during separation of the residual body in the last stages of spermiogenesis. The distribution of activities was determined as: 85% bound to the hypotonically de-membranated sperm structure, hence the cytosolically active enzyme; 10% stuck to the outside of the plasma membrane; 4.4 % intramitochondrial (Alvarez and Storey, 1984a).

Glycolysis: sugar as friend

As the mitochondrial activities of sperm were being sorted out, studies of sperm glycolysis proceeded by two basic routes: flux and product distribution measurements using ^{14}C-labelled glucose as substrate and determination of activities of the enzymes making up the Embden-Meyerhof pathway of glycolysis. An example of the ^{14}C-labelled glucose method is the study of Voglmayr and Amann (1973) on bovine sperm that compared aerobic and anaerobic glycolysis, using uniformly ^{14}C-labelled glucose ([U-^{14}C]glucose), to assess the effects of various added steroids on this aspect of bull sperm energy metabolism. Of the steroids examined, androstenedione, testosterone, and 5-β-androstanedione, stimulated aerobic glycolysis by less than 50%; the mechanism remains unknown. Product analysis provided a quantitative distribution of the fate of glucose between oxidation to CO_2 and production of lactate. Both epididymal and ejaculated sperm were tested; little difference in rates of glucose utilization between sperm from the two sources was noted. From these values, the percentage distribution of utilized glucose was calculated by Storey and Kayne (1975). For ejaculated sperm under aerobic conditions, 16% of the glucose was oxidized to CO_2, 66% was catabolized to lactate; the balance of 18% converted to other metabolites, with < 1% incorporated into sperm lipid. Epididymal sperm yielded 18% oxidized to CO_2, 63% catabolized to lactate, and 10% converted to other metabolites under these conditions. Under anaerobic conditions, 84% of the glucose went to lactate with ejaculated sperm, 78% went to lactate with epididymal sperm.

Leakage of glycolytic enzymes into the suspending medium from bull, boar, and ram sperm, washed rather too vigourously or subjected to cold shock, prompted the investigation by Harrison and White (1972) of whence came these activities. The three enzymes assayed were hexokinase (HK), 6-glucose phosphate isomerase (GPI), and LDH. Sperm from all three species were examined. All three enzymatic activities were found in cytoplasmic droplets and were—'soluble' in the sense intrinsic to biochemists. Even if classifiable as 'soluble', varying amounts of the three enzymes were found bound to the sperm recovered after hypotonic shock. Of the three, LDH was bound in the highest amount in sperm from all three species. GPI and HK were more readily lost. Bull sperm were found to be the most tenacious with regard to retention of GPI, ram sperm the least.

Hypotonically treated rabbit epididymal sperm also proved to be tenacious in retaining glycolytic enzymes: LDH, pyruvate kinase (PK), enolase (EN), 3-phosphoglyceromutase (PGM), GAP-DH, Triosephosphate isomerase (TPI), and fructose 1,6-bisphosphate aldolase (ALD) were all present on the sperm structure recovered from hypotonic treatment (Storey and Kayne, 1975). Retention was by association rather than covalent binding: three washes of the recovered sperm in KCl medium reduced activity by 10% for LDH and PK; maximal reduction was 57% for EN. Storey and Kayne (1975) proposed that the enzymes of the Embden-Meyerhof pathway were organized on the sperm structure to maximize glycolytic flux, in analogy to the proposal of Arnold and Pette (1968) that the same enzymes in skeletal muscle fibers were bound to muscle fibers in what could be considered a multi-enzyme complex. The binding in skeletal muscle appeared to be to F-actin, as indicated by binding determinations with purified actin (Arnold and Pette, 1970).

With the advent of genetic analysis and gene manipulation in the mouse, this mammalian model took center stage for elucidation of glycolysis in mammalian sperm. Among the enzymes of the Emben-Meyerhof pathway, GAP-DH plays a central role. The reactions upstream from GAP-DH, starting with glucose, consume two molecules of ATP per mole of glucose, while the reactions downstream to lactate generate four molecules of ATP per mole of glucose, for a net production of two ATP per mole of glucose catabolized to lactate. Analysis of cDNA from a mouse spermatogenic library revealed a spermatogenic cell-specific sequence for GAP-DH, which was then shown to be a sperm-specific isoform of that enzyme expressed by its own gene (Welch *et al.*, 1992). The enzyme was designated GAPD-S, the gene *gapds*. Further characterization of this isoform revealed that it had a higher molecular weight than the somatic isoform due to a proline-rich extension at the N-terminus; otherwise, it was identical to the somatic isoform (Bunch *et al.*, 1998). Antibodies to the sperm isoform allowed immunolocalization of GAPD-S to the entire length of the sperm principal piece and also showed absence from the sperm head. The metabolic implication of this observation is that glycolytic activity is restricted to the principal piece. This leads to the prediction that the principal piece acts as scaffold for the sequence of glycolytic enzymes of the Embden-Meyerhof pathway, a prediction validated by Krisfalusi *et al.* (2006), who demonstrated the presence of LDH, ALD, and PK on this scaffold. The analogy with the organization of these enzymes with that in skeletal muscle for maximal flux was thus supported. Glycolysis can therefore serve as supplier of ATP to the flagellar dynein ATPase for motility in mouse sperm; evidence that this is the case is presented below. The concept that the sperm-specific enzyme isoforms of the Embden-Meyerhof pathway are so constructed as to bind to this scaffold remains to be fully validated, but the number of these isoforms is impressive: phosphoglycerate kinase-2 (PGK2), GPI, phosphofructose kinase-1 (PFK1), and EN (see Vemuganti *et al.*, 2007, for listing and references), and aldolase. The recent finding of Vemuganti *et al.* (2007) that three sperm-specific isoforms of aldolase are localized in the mouse sperm flagellum, of which two have N-terminal extensions not found in the others. These N-terminal extensions are tempting candidates for anchor sequences to the fibrous sheath of the flagellum, but have not yet won experimental confirmation.

Hexokinase-1 offers a more complicated picture with regard to germ cell-specific expression: three mRNAs are expressed for an isoform, all of which lack the coding region for the porin binding domain found in the somatic isoform, but which have coding regions for different N-terminal regions (Mori *et al.*, 1993). Only one protein isoform is found in mouse sperm, HK1-SC (Travis *et al.*, 1998). In contrast to the somatic isoform, HK1-SC acts as a membrane-bound protein and is localized throughout the sperm cell: in the fibrous sheath of the principal piece; associated with the mitochondria of the midpiece; and bound to the plasma and acrosomal membranes of the head. Further analysis of the N-terminal domains of the different putative isoforms showed that these acted as address sequences to target the different domains of the sperm cell, and that they were cleaved to yield the single isoform HK1-SC (Travis *et al.*, 1998, 1999).

Further studies in human and rat provided evidence that GAPD-S is not a mouse monopoly. The sperm-specific enzyme isoform and its gene have been found in rat (Welch *et al.*, 2006) and in human sperm (Welch *et al.*, 2000); in the latter sperm it is designated GAPD2. Given the conservation of the sperm-specific enzyme over the evolutionary divergence between these three mammalian species, one may reasonably assume that GAPD-S is characteristic of the enzyme equipment of sperm from most, if not all, mammals.

Glycolysis and motility: sugar as friend

The extent to which glycolysis provides the ATP necessary for motility in mouse sperm in particular, and mammalian sperm in general, became of interest as a result of the findings described above. The mouse sperm principal piece is an ideal compartment for high glycolytic flux: it is long and thin, with a high surface-to-volume ratio, favoring unimpeded transport of glucose into and lactate out of the compartment, and with the glycolytic pathway enzymes arranged as a large unit favoring maximal flux on the fibrous sheath scaffold. Indication that mitochondrial oxidative phosphorylation might not be necessary for flagellar function came from the observation of Travis *et al.* (2001) that sperm maintained normal motility in the presence of uncouplers of oxidative phosphorylation, suggesting that ATP derived from oxidative phosphorylation contributed little if any energy to the flagellum. Impressive evidence for glycolysis as sole driving force for mouse sperm motility was provided by generation of mice in which the *gapds* gene had been "knocked out"; these were designated *gapds*$^{-/-}$ (Miki *et al.*, 2004). The beauty of the experiment derives from the fact that, since this gene is expressed only in male germ cells, females were totally unaffected, and the males were healthy, but sterile. Examination of the sperm revealed at best a twitchy flagellar motion; the sperm were effectively immotile. The intracellular ATP content was but 10% that of the wild type sperm, but the O_2 uptake with pyruvate plus lactate in the absence of added glucose by the midpiece mitochondria was the same as that of the wild type. The mouse sperm principal piece is long, some 87 μm (Cummins and Woodall, 1985), so that diffusion of ATP from midpiece to the end piece could be a bit daunting. But the glycolytic enzyme complex spread along the sperm tail can mount a flux sufficient to provide for all the ATP needed for motility. The efficacy of glucose in maintaining mouse sperm motility was also documented by Mukai and Okuno (2004). But these investigators also found that the sperm could remain motile with pyruvate or lactate in the absence of glucose; motility ceased in the presence of inhibitors of mitochondrial oxidative phosphorylation. The implication of this observation is that mitochondrially generated ATP can diffuse far enough into the flagellum to power normal motility. The absence of GAPD-S in the head implies that supply of ATP to the head cannot come from glycolysis and so must rely on ATP generated by the midpiece mitochondria (Carey *et al.*, 1981) with subsequent diffusion into the head.

The ATP requirements of the sperm flagellum for motility may be estimated from the activity of the flagellar ATPase with access to ATP. Determination of this activity in hypotonically treated rabbit epididymal sperm preparations showed considerable variation – activities ranged between 0.5 and 5 μmol/min-10^9 cells, with the higher activities being found in sperm from the more mature rabbits (Storey and Kayne, 1980). Pyruvate kinase (PK) remains bound to the sperm structure in these preparations, and the activities determined for this enzyme were close to those of the ATPase in any given preparation. The match between activity of these two enzymes, and the observation that the activities were always an order of magnitude greater than those of mitochondrial O_2 uptake, provided further evidence that glycolysis can maintain motility in rabbit sperm. Assay of the ATPase utilized an enzyme-linked method, in which phosphoenolpyruvate in the presence of added PK converted the ADP formed in the ATPase reaction back to ATP. The pyruvate thus formed was reduced to lactate in the presence of added NADH and LDH; the rate was obtained from the rate of loss of NADH fluorescence as it was oxidized to NAD^+. This assay allowed one to assess the possible activity of adenylate kinase in the flagellum. The reaction:

ATP + AMP => 2 ADP

produces ADP above and beyond that produced in the ATPase reaction in proportion to the activity of the adenylate kinase. From the observed increase rate in ADP production, one could estimate an adenylate kinase rate about 1.3 times greater than the ATPase rate. A robust adenylate kinase can provide a separate source of ATP to the flagellum and make up in part for the perils of diffusion. Schoff *et al.* (1989) found adenylate kinase in bull sperm flagella as well as in bull sperm mitochondria. One can extrapolate from these findings that this enzyme activity is a part of the energy metabolism equipment of most, if not all, mammalian sperm.

The concept that the motility of mammalian sperm is driven solely by glycolysis in the organized pathway of the principal piece was reinforced by the findings in the *gapds*$^{-/-}$ knockout mouse. But the question remained: is this the case or all mammalian sperm? In a comprehensive and critical review, Ford (2006) examined this question in detail. Considerable effort, outlined in the review, has been expended over the years on the action of α-chlorhydrin in inducing male infertility. This compound itself is not the culprit: it is metabolized to β-chlorolactaldehyde, which is a potent, and apparently irreversible, inhibitor of GAPD-S. Another precursor to β-chlorolactaldehyde is 6-chloro-6-deoxyglucose. Rat sperm treated with this compound remained motile and maintained normal ATP content with pyruvate plus lactate as energy substrate in the absence of glucose (Ford and Harrison, 1981). Epididymal ram sperm and ejaculated boar sperm, treated with chlorhydrin, in a like manner maintained motility for 60 min with pyruvate plus lactate as energy substrates in the absence of glucose, but addition of glucose rapidly diminished motility in the

presence of these substrates, and did not sustain motility as sole substrate (Ford and Harrison, 1985). The implication of these experiments, of the experiments of Mukai and Okuno (2004), and of the experiments with *gapds*$^{-/-}$ mice (Miki *et al.*, 2004) is that glucose acts as a metabolic poison, akin to 2-deoxyglucose, when GAPD-S in sperm is knocked out or its activity blocked by inhibitor. The mechanism is effectively the same: 2-deoxyglucose is phosphorylated by ATP with hexokinase as enzyme catalyst; since the product, 2-deoxyglucose-6-phosphate cannot be further metabolized, the intracellular ATP gets consumed by the reaction. GAPD-DH/GAPD-S is the gateway to the second stage of the Embden Meyerhof pathway of glycolysis that provides a net synthesis of ATP; the first stage, upstream from GAP-DH consumes ATP by phosphorylation of glucose and fructose 6-phosphate. Blockage of the activity of GAP-DH prevents net synthesis of ATP but allows its consumption. Blockage in the case of the *gapds*$^{-/-}$ knockout is the truly clean experiment, as there is no problem of inhibitor side effects, but the effects with inhibitor or knockout of the enzyme are essentially the same. As expected from these considerations, Miki *et al.* (2004) found that GAP content of the sperm from the knockout mice was fourfold higher with glucose as substrate as compared to the wild type mice. The further implication of these experiments is that, in sperm from a number of species, either glycolysis or oxidative phosphorylation, independently of each other, can maintain motility. Diffusion of ATP from the midpiece mitochondria to the nether region of the midpiece is not the problem it has been presumed to be. Which pathway fuels the motility to speed the fertilizing sperm to the egg in these species therefore must depend on the substrates provided in the oviduct. Humans appear to be one mammalian species whose sperm do rely substantially, if not entirely, on glycolysis for motility (Ford and Rees, 1990; Williams and Ford, 2001), hardly surprising in view of the weak oxidative activity of their mitochondria (Storey, 1978).

Glycolysis and capacitation: in bull sperm, sugar as foe

In sperm from many mammalian species (see Williams and Ford, 2001, for references), glucose is needed as energy substrate for the various processes subsumed under capacitation (see Gadella, this issue, for review of capacitation). But, in the case of bull sperm, it is inhibitory. Capacitation of bull sperm *in vitro* appears to be unique among mammalian sperm, in that is strongly enhanced by the inclusion of heparin in the capacitating medium (Parrish *et al.*, 1988). Addition of 5 mM glucose to the medium blocks the heparin-enhanced capacitation (Parrish *et al.*, 1989). Two characteristics of bull sperm capacitation are the requirement for controlled Ca^{2+} intracellular influx from medium Ca^{2+} (Parrish *et al.*, 1999) and a rise in intracellular pH mediated by heparin (Vredenburgh-Wilberg and Parrish, 1995). A separate pathway leading to capacitation, driven by an intracellular rise in cAMP through the action of protein kinase A (PK-A) and inhibited by the cAMP antagonist Rp-adenosine-3', 5's-cyclic monophosphorothioate, was shown not to involve the rise in intracellular pH (Uguz *et al.*, 1994). These observations suggested that the inhibitory effect of glucose could operate by two possible mechanisms: enhancement of the activity of the enzyme phosphodiesterase to lower the intracellular content of cAMP, or inhibition of the rise in intracellular pH. The recent study of Galantino-Homer *et al.*, 2004) revealed that the first of these mechanisms did not apply: glucose had no effect on the activity of bull sperm phosphodiesterase. Analysis of the consequences of the kinetics of glycolysis by bull sperm in the presence of 5 mM glucose demonstrated that the second of these mechanisms did apply: the rate of aerobic glycolysis in bull sperm is sufficiently high at this glucose concentration that production of lactate would decrease intracellular pH, rather than allow it to increase. Fructose in the semen would have the same effect, thus inhibiting capacitation until the fructose had been diluted out in the cow's reproductive tract. Glucose inhibition of capacitation does not occur in the cow's oviduct because the glucose concentration in the oviductal lumen is only 50-100 μM (Carlson *et al.*, 1970), a concentration at which the glycolysis rate is negligible. This example further supports the concept that it is the oviductal lumen of the female whose contents determine the metabolic pathway driving motility of mammalian sperm *in vivo*. Still to be resolved is the mechanism by which heparin induces the rise in intracellular pH in these sperm.

Reactive oxygen species (ROS) and lipid peroxidation: oxygen as foe

The early observation by McLeod (1943) that H_2O_2, produced during incubation of human sperm in an atmosphere of 95% O_2, was toxic to these cells as monitored by loss of motility, was extended to bovine sperm by Tosic and Walton (1950). They noted that bovine and porcine sperm had little catalatic activity; lack of endogenous catalase in mammalian sperm from most mammalian species became evident in subsequent studies (Mann, 1964). A survey of H_2O_2 toxicity to sperm from bull, ram, dog, mouse, rabbit, and human, as assessed by loss of motility, demonstrated a remarkable difference in resistance to motility loss between species (Wales *et al.*, 1959). Rabbit sperm were by far the most resistant; they could swim through 10 mM H_2O_2 with seeming indifference.

Elucidation of the damaging effects of ROS on mammalian sperm first came from the elegant studies of Jones and Mann (1973, 1976, 1977a,b) on peroxidation of the membrane lipids of boar and ram sperm. The study was extended to human sperm in collaboration with Sherins (Jones *et al.*, 1978, 1979). Loss of motility and metabolic activity – e.g. fructolysis– was shown to be caused by loss of plasma membrane permeability barriers due to peroxidative degradation of the membrane phospholipids. The prime targets for this degradation were the most unsaturated of the polyunsaturated fatty acid (PUFA) moieties of the phospholipids: arachidonic (AA) and dicosahexaenoic (DHA) acids, present as acyl groups at the 2-position of the glycerol backbone of the phospholipid. DHA is a major PUFA constituent of mammalian sperm plasma membrane phospholipids, rendering these cells rather more sensitive to peroxidative degradation than somatic cells. Lipid peroxidation in these studies was shown to require O_2; none occurred under an atmosphere of N_2. The experiments were carried out under conditions of active induction of peroxidation: Fe^{2+} and ascorbate in the incubation medium. These conditions provided reaction rates much more rapid that the spontaneous peroxidation rates, and so allowed an extensive survey of reaction parameters. Jones and Mann (1977b) showed that the probable initiators of peroxidation were superoxide radi-

cal, $O_2^{\cdot -}$ and the hydroxyl radical, HO·; the 'usual suspect', H_2O_2, did not play a role, consistent with the observations of Wales *et al.* (1959). They further showed that peroxidative damage was mediated by endogenous lipid peroxides, as well as damage inflicted by exogenous lipid peroxides (Jones and Mann, 1977a). A comprehensive review of sperm plasma membrane lipid peroxidation induced by Fe^{2+} and ancillary agents has recently been provided by Aitken *et al.* (1993); the reader is referred to this most useful account for an overview of the chemistry involved.

While the use of the Fe^{2+}/ascorbate system allowed a far ranging series of experiments to be carried out on lipid peroxidation, the question remained as to whether this sort of damage could occur in the absence of inducers. The reactions are far slower, but spontaneous lipid peroxidation of sperm plasma membrane does occur under essentially physiological conditions in the presence of O_2 with the same degradative effects on the sperm plasma membrane as observed with peroxidation inducers (Alvarez and Storey, 1984a). The rates of spontaneous phospholipid peroxidation in sperm from various mammalian species have been determined: human (Aitken and Clarkson, 1987; Alvarez *et al.*, 1987; Alvarez and Storey, 1995); rabbit (Alvarez and Storey, 1982, 1983); mouse (Alvarez and Storey, 1984b). The rate of lipid peroxidation was followed in these studies by the rate of generation of malondialdehyde (MA) as monitored by formation of a the highly absorbing and highly fluorescent adduct with thiobarbituric acid (TBA)(Barber and Bernheim, (1967)). Despite limitations due to the relatively low yield of MA from lipid peroxide breakdown, this method is highly sensitive and, if carried out under carefully controlled conditions (Aitken *et al.*, 1993), gives excellent correlation with other lipid peroxidation assays: (Jones and Mann, 1976; Smith *et al.*, 1982).

Spontaneous lipid peroxidation as monitored by MA production is linear with time over a period of hours, during which sperm motility progressively ceases. The rate is linear in cell number. The rate is also linear with O_2 concentration in the suspending medium, up to medium saturated with air. The rate constant for the loss of fatty acids due to peroxidative degradation is first order and increases with the number of double bonds in the fatty acid moieties of the phospholipids (Bielski *et al.*, 1983). The most striking characteristic of spontaneous lipid peroxidation as observed in these cells is the high activation energy (E_A) of 79 kcal/mol (330 kJ/mol). This value is close to that of 80 kcal/mol determined for the bond energy of the C-H bond of an allylic methylene CH_2 group (Korcek *et al.*, 1972), a group characteristic of the PUFA acyl moieties. The high value of E_A confers an advantage in storage stability on sperm in the epididymis of mammals with scrota: the rate of spontaneous lipid peroxidation at a scrotal temperature of 30°C should be some sixfold lower than the rate at 37°C.

The spontaneous lipid peroxidation pathways in mammalian sperm have been described in detail by Alvarez and Storey (1995) and Storey (1997). Briefly, the pathways are the following. The initiator of lipid peroxidation is the ROS, superoxide anion, $O_2^{\cdot -}$, which is produced mainly as a side reaction of midpiece mitochondrial electron transport to O_2. Apparent production of $O_2^{\cdot -}$ via a sperm-specific NADPH oxidase (Aitken and Clarkson, 1987) has recently been shown to reflect the chemistry of the lucigenin detection system rather than actual $O_2^{\cdot -}$ generation (Richer and Ford, 2001; Ford, 2004; Baker *at al.*, 2004; Baker *et al.*, 2005). The $O_2^{\cdot -}$ anion is itself a reductant rather than an oxidant, but its conjugate acid, $HOO^{\cdot}$, is a potent oxidant and acts as initiator of peroxidative chain reactions. The two are in rapid equilibrium. The peroxidative chain reactions produce phospholipid hydroperoxides, ROOH. Upon attack of these phospholipid moieties by O_2, the acyl chains of the phospholipids fragment, and membrane damage, with loss of membrane permeability barriers, ensues.

Activities of ROS in useful regulation pathways in sperm have also been studied extensively, but are deemed beyond the scope of this review.

Enzyme defenses against lipid peroxidation

Mammalian sperm share the two main defense systems against the ravages of ROS with somatic cells. One is the enzyme, superoxide dismutase (SOD), which catalyzes removal of the $O_2^{\cdot -}$anion: $2\ O_2^{\cdot -} + 2\ H^+ \Rightarrow O_2 + H_2O_2$ and so inhibits initiation of peroxidation. In rabbit and human sperm, the primary SOD activity has been shown to be due to the CN^- sensitive Cu/Zn; the mitochondrial SOD activity is detectable but essentially negligible (Holland and Storey, 1981; Alvarez *et al.*, 1987).

The other defense system consists of the enzyme, glutathione peroxidase (GPX), which requires intracellular reduced glutathione, GSH, as co-substrate with lipid hydroperoxide, ROOH. The reaction produces the relatively innocuous hydroxylipid, ROH, with resulting oxidation of glutathione to glutathione disulfide, GSSG. GSH is regenerated through the action of the enzyme glutathione reductase (GRD), which utilizes NADPH as co-substrate to reduce GSSG to GSH. This system removes the initial product of lipid peroxidation, ROOH, which inhibits further lipid breakdown caused by fragmentation of the lipid hydroperoxide by attack of O_2 (Alvarez and Storey, 1995).

Human sperm have both systems, and both are key to inhibition of lipid peroxidation driven by production of $O_2^{\cdot -}$ (Aitken and Clarkson, 1987; Alvarez *et al.*, 1987; Aitken *et al.*, 1993; Alvarez and Storey, 1989, 1995). Production of the NADPH needed for regeneration of GSH has been shown to occur through the combined actions of hexokinase and glucose-6-phosphate dehydrogenase, with the activity of the dehydrogenase being rate-limiting (Storey *et al.*, 1998; Williams and Ford, 2004). Mouse sperm are more heavily dependent on the GPX system for inhibition of lipid peroxidation (Alvarez and Storey, 1984a, 1989). As pointed out above, the mouse sperm head lacks GAPD-S but has hexokinase, so that glycolysis is precluded, but glucose-6-phosphate can be formed as substrate for its dehydrogenase to generate NADPH. Mouse sperm GRD can also utilize NADH as co-substrate reductant to regenerate GSH (Alvarez and Storey, 1984a), so that protection against lipid peroxidation is afforded the length of the principal piece. By way of contrast, rabbit sperm lack the GPX system and so rely on a robust activity of SOD for antilipoperoxidative action (Holland and Storey, 1981; Alvarez and Storey, 1989). The toxic effect of 1-5 mM H_2O_2 on human and mouse sperm, not observed in rabbit sperm, was shown to result from com-

plete oxidation of intracellular GSH to GSSG in a chemical reaction, thus depriving GPX of its co-substrate reductant and so shutting down the defense system.

Energy metabolism, motility, lipid peroxidation: in balance

Mammalian sperm may attain access to their conspecific egg by mobilizing metabolic energy production in the form of ATP to drive motility. The metabolic energy production may be fueled by the Embden-Meyerhof pathway of glycolysis exclusively, by mitochondrial oxidative phosphorylation exclusively, or a combination of both pathways. The particular pathway is species specific and appears to be set by the demands and conditions of the female oviduct. The oviductal lumen contains O_2, which the egg needs to survive and to proceed with fertilization. The sperm are thus at risk for lipid peroxidation, but this pathway can be considered essential to prevent them from overstaying their welcome in the oviduct. Once the egg is fertilized, sperm become targets for elimination, and the female oviduct acts to maintain the correct balance of sperm availability and sperm degradation.

Envoi

Dave Garbers was a valued friend and colleague. His unexpected early loss is thus a blow to the field of reproductive biology and cell biology as well as to his friends and colleagues. It was therefore all the more satisfactory in constructing this review to revisit the pioneering work in the field of sperm metabolism by Henry Lardy, who was Dave Garbers' PhD mentor. It represents a scientific lineage for which we all may be grateful.

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