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ENERGY CONSERVATION CAPACITY AND MORPHOLOGICAL INTEGRITY OF MITOCHONDRIA IN HYPOTONICALLY TREATED RABBIT EPIDIDYMAL SPERMATOZOA

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SUMMARY

Hypotonic treatment of rabbit epididymal spermatozoa in 10 mM phosphate buffer disrupts the plasma membrane and removes the cytoplasmic droplet from those cells to which it is still attached. There is, however, no effect on the mitochondria, which retain their helical configuration around the axial filament complex, have intact inner and outer membranes, and show the same cristal morphology as do the mitochondria in untreated cells. Hypotonically treated spermatozoa respire with added malate–pyruvate, succinate, and ascorbate *plus* *N,N'*-tetramethyl-*p*-phenylenediamine, but not with added fructose or NADH. Respiration is inhibited by oligomycin and stimulated by uncoupler, showing that the mitochondria have retained their capacity for energy conservation. The uncoupled respiration rate is not further stimulated by added cytochrome *c*. Reduced-*minus*-oxidized difference spectra obtained at −196 °C of the hypotonically treated cells show a full complement of cytochromes, including cytochrome *c*. This result implies that the cytochrome *c* lost from mammalian spermatozoa on storage or chilling [Mann, T. (1951) *Biochem. J.* 48, 386–388] is cytoplasmic cytochrome *c* not yet incorporated into the mitochondria. The mitochondrial cytochrome *c* remains firmly held inside the intact outer membrane.

INTRODUCTION

Despite the evident importance of the spermatozoan in the propagation of metazoan life, the mitochondria of these cells have received remarkably little study. The mitochondria are arranged in a helical array around the axoneme in the midpiece of spermatozoa, and no method is presently available for the isolation of these organelles in intact form. Two methods to circumvent this problem have been devised: one is isolation of a purified midpiece fraction; the other is alteration of the sperm cell plasma membrane to make it permeable to substrates and inhibitors. The first approach was used by Zittle and O'Dell[1] who subjected bovine epididymal spermatozoa to sonic disintegration and differential centrifugation and obtained relatively pure headpiece and midpiece fractions. Mann[2,3] reported that shaking with glass

Abbreviations: TMPD, *N,N'*-tetramethyl-*p*-phenylenediamine; 1799, bis(hexafluoroacetonyl) acetone.

beads was effective in breaking sperm cells into headpieces, midpieces and tailpieces; he also demonstrated that succinate oxidase activity and the cytochromes, as revealed by their absorbance bands, were in the supernatant fraction consisting of further disrupted midpieces *plus* some tailpieces. A detailed study of mitochondrial oxidative activity was carried out by Mohri *et al.*[4] using a purified midpiece fraction from bovine spermatozoa prepared by sonication. Stambaugh and Buckley[5] have devised a method for obtaining a highly purified midpiece fraction from rabbit sperm, but the amount available is of necessity very small because of limited amounts of starting material and losses during cell breakage and purification by density gradient. Morton and Lardy[6,7] have taken the second approach and have devised two methods for modifying the cell membrane of bull sperm to increase its permeability: shaking the cell suspension with glass beads very gently to avoid cell breakage and treating the cells with filipin, a polyene antibiotic. None of these methods, however, provides a means for preparing an intact, tightly coupled mitochondrial preparation in high yield which can serve as a basis for examining the energy metabolism of spermatozoa.

In this paper, we report that simple hypotonic treatment of rabbit epididymal spermatozoa provides a preparation in which the mitochondria remain attached to the midpiece, but may be studied directly by the techniques developed for the study of isolated mitochondria. The rabbit is a particularly useful source of spermatozoa, since *in vivo* fertilization techniques have become available for the rabbit largely through the work of Brackett, Seitz, Stambaugh, and co-workers[8–11] and thus the role of spermatozoan energy metabolism in the fertilization process can be studied.

MATERIALS AND METHODS

Reagents

All reagents were of the best grade available commercially and were used without further purification. Adenine nucleotides and NADH were obtained from Boehringer Mannheim Corp.; oligomycin was obtained from Sigma Chemical Co.; rotenone was obtained from K and K Laboratories. The uncoupler bis(hexafluoroacetonyl) acetone (designated 1799) was generously provided by Dr Peter G. Heytler of E. I. duPont de Nemours Co.

Preparation of sperm suspensions

Epididymal spermatozoa were flushed from the excised epididymides of mature male white New Zealand rabbits with a salt medium of the following composition[12]: 113 mM KCl, 12.5 mM KH_2PO_4, 2.5 mM K_2HPO_4, 3 mM $MgCl_2$, 0.4 mM EDTA, 20 mM Tris, adjusted to pH 7.4 with HCl. Each epididymis was flushed with 10 ml of medium. The spermatozoa were collected by centrifugation at $800 \times g$ for 10 min and washed twice by resuspension in the KCl medium, 10 ml per epididymis, and centrifugation. If intact epididymal spermatozoa were to be used directly, the washed cells were suspended in a minimal quantity of KCl medium. These operations were carried out at room temperature.

Hypotonically treated rabbit epididymal spermatozoa (HTRES) were prepared by resuspending the final pellet from the washing procedure described above in 10 ml ice-chilled 10 mM potassium phosphate buffer, pH 7.4, for each epididymis. The suspension was mixed gently with shaking, and the treated cells were collected by

centrifugation at $800 \times g$ for 10 min. The cells were washed twice by the procedure described above with ice-chilled KCl medium. The final pellet was then resuspended in a minimal quantity of cold KCl medium. The suspension was kept ice-chilled until use.

Ejaculate was obtained by means of the artificial vagina (Holborn Surgical Instrument Co., Ltd, London). The spermatozoa were washed free from the gel with KCl medium, suspended in 10 ml medium per ejaculate, and washed as described for epididymal spermatozoa.

Electron microscopy

Epididymal spermatozoa were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h, washed with phosphate buffer containing 10% sucrose and post-fixed with 1% OsO_4 in the same buffer for 2 h, dehydrated in alcohol, and embedded in epoxy resin according to Luft[13]. The sections were stained for 10 min with uranyl acetate and then with lead citrate according to Reynolds[14]. The grids were examined with a JEOL (JEM T8) electron microscope operating at 60 kV with a 50-μm objective aperature at a magnification of 5000 to 30000.

Polarigraphic assay of respiratory activity

The respiratory activity of sperm cell suspensions was assayed with a polarographic oxygen electrode essentially as described by Estabrook[15]. In order to conserve material, the reaction vessel was reduced in size to 0.22 ml. The oxygen electrode was made from a silver tube 2 mm outside diameter and 1 mm inside diameter, with a platinum wire 20 μm in diameter embedded in epoxy resin filling the center. The tip was covered with a Teflon membrane 0.025 mm thick. This electrode is a miniature version of the Clark electrode described in Estabrook's procedure. It was constructed by Mr K. Olofsson of the Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania. All assays were run in the KCl medium described in the previous section.

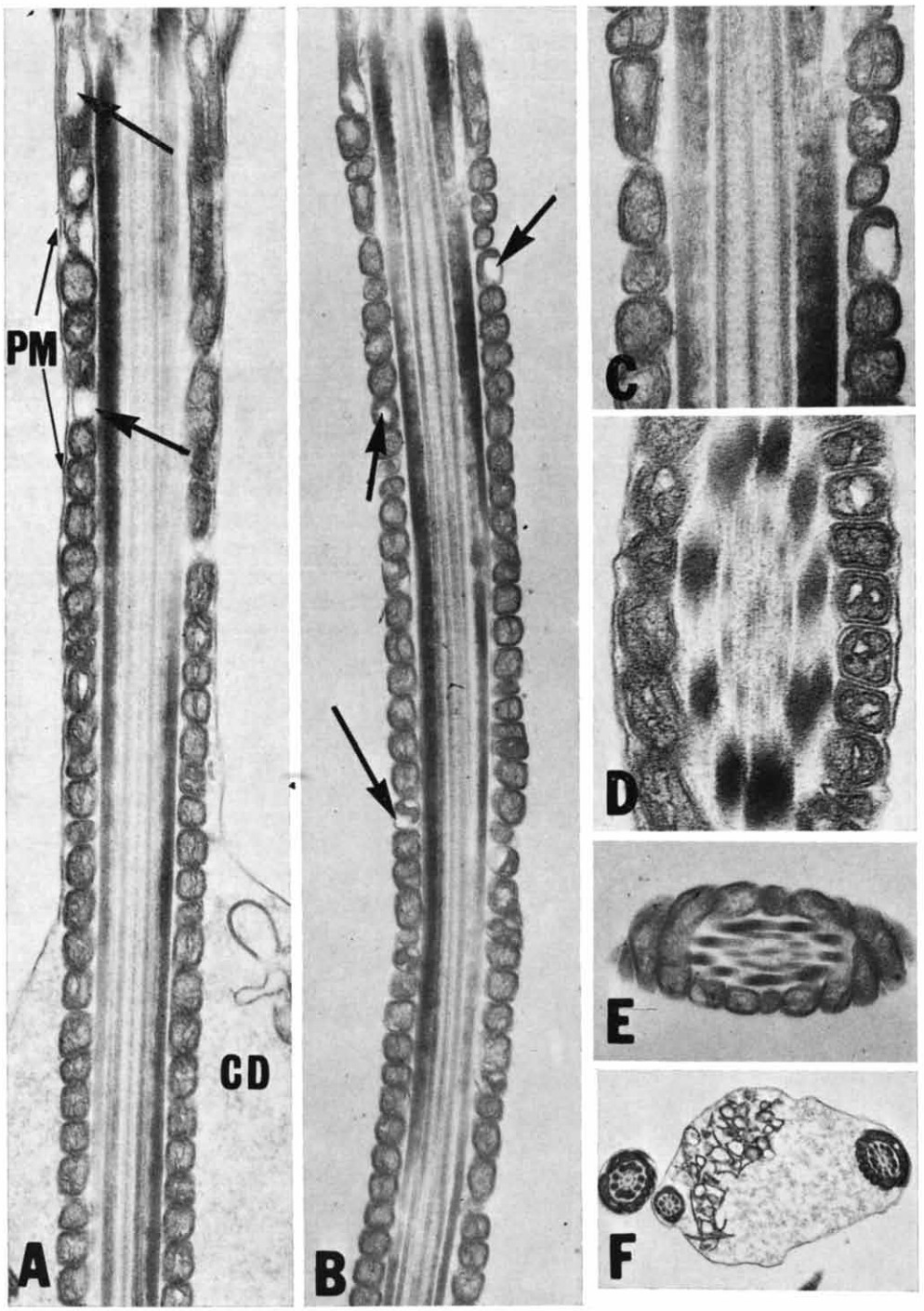
Spectrophotometry

Difference spectra of sperm cell suspensions were obtained at the temperature of liquid N_2 (-196 °C) using the differential split beam spectrophotometer described by Chance[16] equipped for the low temperature technique developed by Estabrook[17] as modified by Bonner[18]. In these experiments, KCl medium was also used.

RESULTS

Morphology of mitochondria in hypotonically treated spermatozoa

A most surprising result of this study is the finding that the mitochondria of rabbit epididymal spermatozoa are completely resistant to damage by hypotonic treatment. This is evident from comparison of the electron micrographs presented in Figs 1A and 1B. These show a longitudinal section through the midpiece of intact, untreated epididymal spermatozoan washed with KCl medium (Fig. 1A) and a similar section through the midpiece of an epididymal spermatozoan treated with hypotonic 10 mM phosphate buffer, followed by washing with KCl medium (Fig. 1B). The plasma membrane and part of the cytoplasmic droplet seen in Fig. 1A are missing


PM
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A
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in Fig. 1B. The mitochondria retain their shape, size, and relative position after the hypotonic treatment. An enlargement of this section in Fig. 1C shows that both the inner and outer mitochondrial membranes are completely intact: the cristal pattern is essentially unchanged compared to the pattern in mitochondria of the intact, untreated spermatozoa (Fig. 1D). The helical arrangement of the mitochondria around the axial filament complex[19] remains unaffected by hypotonic treatment, as shown in the transverse section depicted in Fig. 1E. A number of membrane structures are also apparent in the cytoplasmic droplet (Fig. 1F). These structures are very much like those seen in the cytoplasmic droplets attached to the midpiece of bull and ram epididymal spermatozoa in the electron micrographs presented by Bloom and Nicander[20]. Their work, which also included studies on rabbit spermatozoa, indicates that these membrane structures are residual pieces of Golgi apparatus and endoplasmic reticulum. The spermatozoa treated hypotonically retain their gross morphology, with head-, mid-, and tail pieces still connected as in the untreated cell. Other contaminating cells are lysed and their debris removed during the washing procedure; the resulting suspension of hypotonically treated spermatozoa is free of contamination by other cells.

The washed epididymal spermatozoa suspended in the KCl medium are motile, the percent motility being approximately 80%. The hypotonically treated spermatozoa are immotile; a maximum of 1–2% show slow tail movement. Motility is restored to approximately 50% of the cells suspended in the KCl medium by addition of 10 mM dithiothreitol, 3 mM ATP, 10 mM each pyruvate and malate, and 50 mM phosphoenolpyruvate. The motions of the flagella are slower and not as well coordinated as in the untreated spermatozoa. The conditions used for restoration of motility are essentially those of Gibbons and Gibbons[21] who obtained motility in sea urchin spermatozoa which had been treated with dilute Triton X-100 to remove the cell membrane.

Oxidative activity of mitochondria in hypotonically treated spermatozoa

The mitochondria of rabbit spermatozoa oxidize pyruvate *plus* malate with tight coupling of energy conservation to substrate oxidation, as shown in Fig. 2. The calculated ADP/O ratio is 2.8 for the first addition of ADP; the respiratory control ratio[22] is poor, however, being only 1.8. This is due to hydrolysis of the formed ATP by an intracellular ATPase, most probably the flagellar ATPase, since oligomycin gives substantial inhibition of the respiratory rate. This inhibition is reversed by

Fig. 1. (A) Longitudinal section through the midpiece of an untreated rabbit epididymal spermatozoan. PM indicates the plasma membrane, CD the cytoplasmic droplet. The arrows show two mitochondrial sections which have low electron density; similar sections are also seen in ejaculated rabbit sperm[41]. 28000×. (B) Longitudinal section through the midpiece of a hypotonically treated spermatozoan. Note the absence of the plasma membrane. The arrows show mitochondrial sections of low electron density similar to those in A. 28000×. (C) Section as in B of hypotonically treated spermatozoan at higher magnification, showing intact inner and outer membranes and cristae of the mitochondria. 52000×. (D) Oblique section of untreated spermatozoan showing plasma membrane outside of the mitochondrial sheath, and the inner and outer membranes of the mitochondria. 50000×. (E) Transverse section of hypotonically treated spermatozoan, showing helical arrangement of mitochondrial sheath and lack of plasma membrane. 26000×. (F) Transverse section of untreated spermatozoan through the cytoplasmic droplet, showing pieces of membrane between mitochondrial sheath and plasma membrane. 9000×.

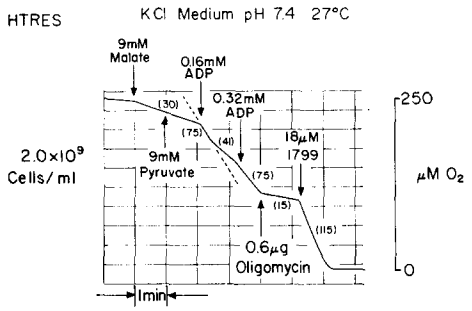

Fig. 2. Record of oxygen consumption by hypotonically treated rabbit epididymal sperm (HTRES) in KCl medium, obtained with the oxygen electrode as described in the text. Number in parentheses are rates of oxygen uptake expressed as ngatoms O/10^9 cells per min. The substrate is malate *plus* pyruvate.

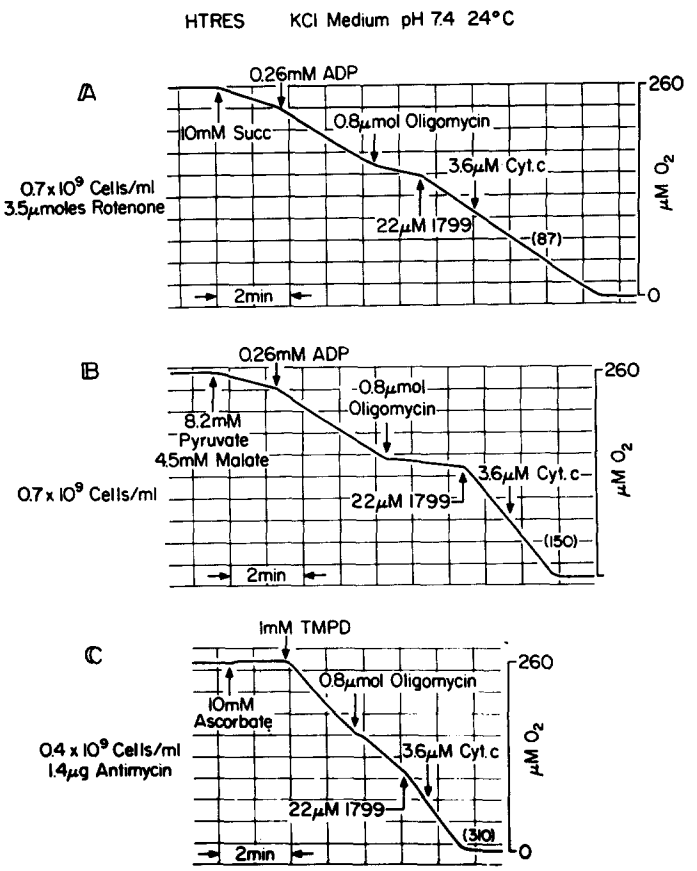

Fig. 3. Record of oxygen uptake, as measured with the oxygen electrode, of hypotonically treated rabbit epididymal sperm (HTRES) suspended in KCl medium utilizing as substrate: succinate (A); pyruvate *plus* malate (B); and ascorbate *plus* TMPD (C). The number in parentheses on each record gives the rate of oxygen consumption in ngatoms O/10^9 cells per min. The respiratory control ratios, calculated as the ratio of uncoupled rate to oligomycin-inhibited rate is 3 for succinate (A), 8 for malate *plus* pyruvate (B), and 1.5 for ascorbate *plus* TMPD (C).

addition of the uncoupler 1799. The respiratory control ratio calculated from the uncoupled and oligomycin inhibited rates is 7.7.

Mann[23] reported some twenty years ago that spermatozoa from bull, ram, and man lost cytochrome *c* quite readily if stored for 24 h at 10 °C, or if treated in such a way (*e.g.*, shaking, or freezing and thawing, or suspension in pure water) that the cell was damaged, even if slightly; this has since been shown for spermatozoa from other sources[24]. The question of whether cytochrome *c* is removed from the mitochondria under hypotonic conditions so as to affect their oxidative activity is answered in the negative by the experiments shown in Fig. 3. Neither with succinate (Fig. 3A), nor with pyruvate *plus* malate (Fig. 3B), nor with ascorbate *plus* TMPD (Fig. 3C) as substrates is the respiratory activity of the hypotonically treated spermatozoa stimulated by added cytochrome *c* in the uncoupled state, which corresponds to the maximum rate of oxygen consumption. In the first two cases (Figs 3A and 3B) the initial rate is stimulated by ADP; this State 3 rate with ADP is inhibited by oligomycin and this inhibition is overcome by uncoupler to give a maximal rate. In the third case (Fig. 3C) TMPD is added at a concentration high enough not to limit the oxidation rate. Under these conditions, where there is some uncoupling by TMPD, inhibition by oligomycin and subsequent stimulation by uncoupler are still observed.

The response to various substrates and inhibitors of mitochondrial oxidative activities is shown in Fig. 4. Complete inhibition by rotenone of pyruvate *plus* malate oxidation is evident from the record of Fig. 4A. Succinate oxidation is not inhibited by rotenone, but is slower relative to the oxidation of pyruvate *plus* malate. Oxidation of L-1-glycerol phosphate (not shown) is slower than that of succinate. Ascorbate *plus* TMPD is, by contrast, oxidized very rapidly. The existence of ATPase activity is clear from examination of the record of Fig. 4B; addition of ATP to the respiratory cell suspension causes a marked increase in the rate of oxygen consumption which is little affected by further addition of ADP. This respiration with pyruvate *plus* malate is sensitive to inhibition by antimycin A (as is respiration with succinate), while the rate of oxygen consumption with ascorbate *plus* TMPD is unaffected by this inhibitor, as expected[25].

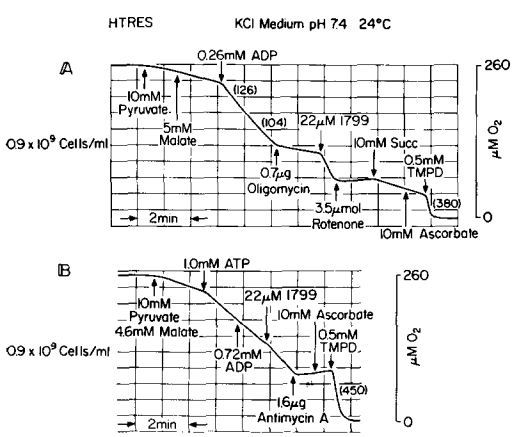

Fig. 4. Response of oxygen uptake by hypotonically treated rabbit epididymal sperm (HTRES) in KCl medium to respiratory inhibitors. Numbers in parentheses give oxygen uptake rates in ngatoms $O/10^9$ cells per min. (A) Effect of rotenone addition. (B) Effect of antimycin A addition.

Oxygen consumption by the hypotonically treated sperm with added fructose or NADH as substrate is negligible.

For purposes of comparison with the oxidative activity of hypotonically treated spermatozoa, the oxidative activity of washed, untreated epididymal sperm was examined. Since the major contaminant of a suspension of these cells is erythrocytes which have no oxidative activity, the observed oxygen uptake may be safely attributed to the sperm cells alone. These oxidize fructose readily with some slight stimulation of rate by ADP and uncoupler (Fig. 5A); fructose 6-phosphate and pyruvate also stimulate the rate of this oxidation which is completely sensitive to inhibition by rotenone. Pyruvate is oxidized by the untreated cells directly, but without stimulation of the oxidation rate by malate or ADP (Fig. 5B), in contrast to the hypotonically treated spermatozoa. Pyruvate oxidation is tightly coupled to energy conservation, as shown by the near zero rate upon addition of oligomycin and rapid rate on subsequent addition of uncoupler. In the presence of rotenone, there is no oxidation of either L-1-glycerol phosphate (Fig. 5A) or of succinate (Fig. 5B) in contrast to the oxidation observed with hypotonically treated spermatozoa. TMPD is readily permeable to the cell membrane and is readily oxidized (Fig. 5C); ascorbate, however, is not permeable. The product of the one-electron oxidation of TMPD, namely Wurster's Blue[26], must diffuse back out of the cell before it can be rereduced to TMPD

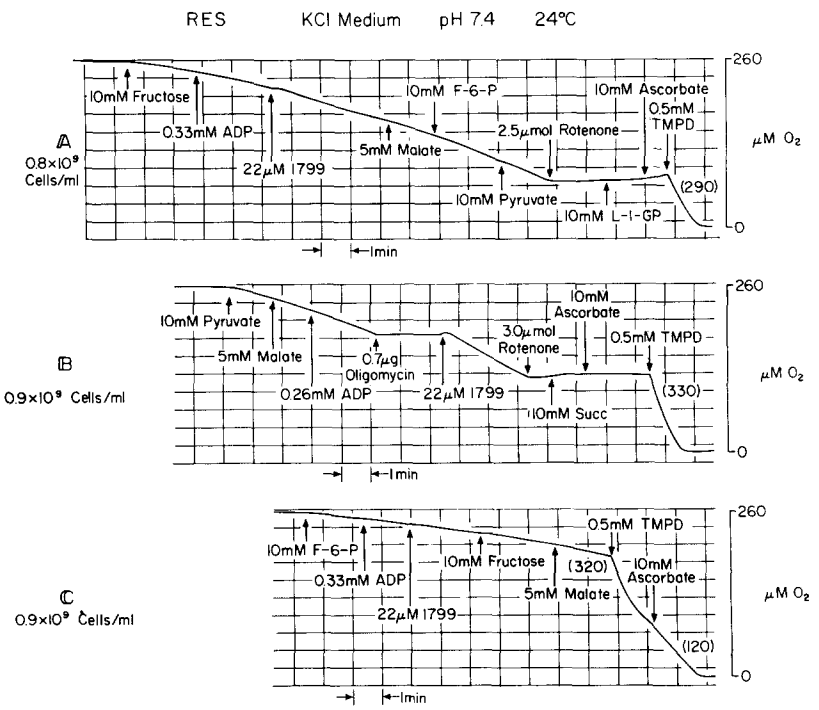

Fig. 5. Record of oxygen uptake by untreated rabbit epididymal sperm (RES) suspended in KCl medium utilizing as substrate: fructose (A); pyruvate (B); fructose 6-phosphate (F-6-P) (C). The oxidation of fructose (A) and of pyruvate (B) is completely sensitive to inhibition by rotenone. The strong inhibition by oligomycin (B) implies that the internal ATPase effectively keeps the ADP content of these cells high: they are normally in State 3.

by ascorbate. As a result, a suspension of epididymal spermatozoa oxidizing TMPD in the presence of ascorbate appears bluish purple to the eye, wheras a suspension of hypotonically treated cells remains colorless under the same conditions. The experiment of Fig. 5C also shows that Wurster's Blue can be itself oxidized to a compound no longer reducible to TMPD by ascorbate: the initial rate of oxidation observed on addition of TMPD in the absence of ascorbate decreases steadily and cannot be restored by subsequent addition of ascorbate.

Mitochondrial respiratory chain carriers in hypotonically treated spermatozoa

The reduced-*minus*-oxidized difference spectrum obtained at −196 °C of a suspension of hypotonically treated rabbit epididymal spermatozoa is shown in Fig. 6B; comparable spectra for suspensions of washed spermatozoa from rabbit ejaculate and of yeast cells are shown in Figs 6C and 6A, respectively, as controls. Difference absorbance maxima are observed at 432 and 444 nm in the Soret region, corresponding to cytochromes *b* and cytochromes $a+a_3$ respectively, in the hypotonically treated spermatozoa (Fig. 6B). The α-band of reduced cytochrome *c* is also evident at 549 nm. The two bands at 555 nm and 559 nm may be assigned, in the light of recent findings, to cytochrome c_1 *plus* cytochrome b_{566} and to cytochrome b_{561} *plus* cytochrome b_{566}, respectively. Cytochrome c_1 has an absorbance maximum in the reduced state at 551–552 nm at −196 °C[27]; cytochrome b_{566}[28–31], also known as cytochrome b_T[32,33], has two maxima, the smaller one of which is at 555 nm[28,34]. The summation of the

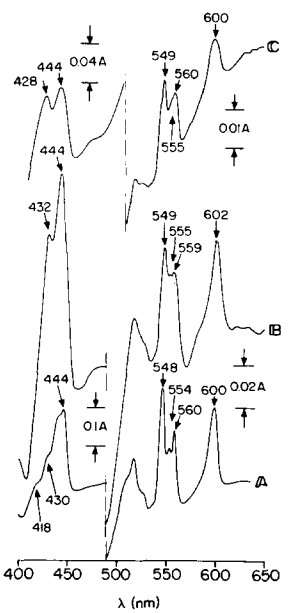

Fig. 6. Reduced-*minus*-oxidized difference spectra of cell suspensions obtained at −196 °C. Peak positions are noted in nm. The reductant is dithionite in all cases; oxidized cells are suspended as is in KCl medium. (A) *Candida utilis* cells suspended at $5 \cdot 10^9$ cells/ml. (B) Hypotonically treated rabbit epididymal sperm (HTRES) suspended at $1.36 \cdot 10^9$ cells/ml. (C) Washed spermatozoa from ejaculate suspended at $0.74 \cdot 10^9$ cells/ml. Note that the recording sensitivity for B and C is the same, and twice that for A.

two give the maximum at 555 nm. The maximum at 559 is a combination of the peaks of reduced cytochrome b_{561} with its low temperature absorbance maximum at 558 nm and reduced cytochrome b_{566} with its larger low temperature maximum at 562 nm[34]. The maximum at 602 nm is attributable to reduced cytochromes $a+a_3$[35]. The difference absorbance minimum in the region 460–470 nm is attributable to the flavoprotein components and is of the magnitude observed in mitochondria isolated from other tissues. The ratio of the cytochrome *c* difference extinction at 549 *minus* 540 nm to that of cytochromes $a+a_3$ at 602 *minus* 630 nm is about 1.6 in the hypotonically treated cells, as compared to 1.7 in intact yeast cells, and is within the range observed in isolated mitochondria. This ratio is about 2 in spermatozoa from ejaculate, showing that about 20% of the total cytochrome *c* in the cell was removed during the hypotonic treatment.

The cytochromes $c+c_1$ and $a+a_3$ in hypotonically treated spermatozoa are reduced by ascorbate *plus* TMPD, but little cytochrome *b* is reduced under those conditions, as shown in Fig. 7A. The cytochrome *b* which is partially reduced is cytochrome b_{561}. The cytochrome c_1 peak is not resolved in this spectrum; the same lack of resolution is also observed with isolated pigeon heart mitochondria under comparable experimental conditions[33]. The cytochromes *b* are reduced by succinate in the presence of antimycin A, while cytochromes $c+c_1$ and $a+a_3$ remain fully oxidized, as shown in the difference spectrum of Fig. 7B. The shoulder of b_{566} at 556 nm is well resolved, but the major α-bands of the two cytochromes are not,

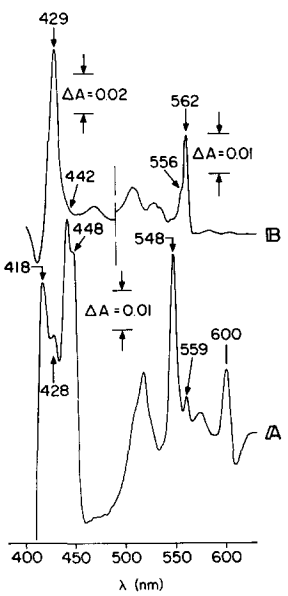

Fig. 7. Reduced-*minus*-oxidized difference spectra of hypotonically treated rabbit epididymal sperm (HTRES) suspended in KCl medium obtained at −196 °C. (A) Substrate is 25 mM ascorbate *plus* 0.6 mM TMPD and the reduced cells are anaerobic; the oxidized cells have been treated with rotenone at 25 μmoles/10^9 cells and aerated. The cells are suspended at $1.2 \cdot 10^9$ cells/ml. (B) Substrate is 25 mM succinate; the cells have been treated with 21 μg antimycin A/10^9 cells and are aerobic. The oxidized cells used as reference were treated with rotenone as in A. The cells are suspended at $2.4 \cdot 10^9$ cells/ml.

because of the red shift of some 1 to 2 nm induced in cytochrome b_{561} by antimycin A[31,33]. There is no evidence of a component corresponding to cytochrome b_d reported by Gonse[36] in bull spermatozoa.

DISCUSSION

The remarkable resistance to damage by hypotonic treatment of the mitochondria of rabbit spermatozoa is a most unexpected finding in this study. Rat liver mitochondria have long been known to behave as osmometers: the matrix space enclosed by the inner membrane swells in media of low osmolarity and, if the swelling is of sufficient amplitude, the outer membrane bursts. In fact, an effective method for separating the outer membrane of liver mitochondria from the inner membrane is to swell the mitochondria in 20 mM phosphate buffer, which ruptures the outer membrane and allows the two membranes to be isolated separately by differential centrifugation[37]. This process also produces swollen inner membrane ghosts which show no cristae. Even the outer membranes of plant mitochondria, which are far more resistant to osmotic shock than are those of animal mitochondria, succumb to rupture at osmolarities below 80 mosM[38]. The outer membranes of the mitochondria in the rabbit epididymal spermatozoa treated with 10 mM phosphate are completely intact, however; the mitochondria are not swollen and the cristal structure of the inner membrane is retained. The mechanism by which the stabilization of these membranes is achieved remains for the moment a mystery. The fact that the mitochondria also retain their helical configuration around the axial filament after hypotonic treatment suggests that the mechanism by which the mitochondria are assembled in this helical sheath is somehow connected to the means by which the mitochondria aquire the ability to resist swelling; just how the two are connected is unclear.

A consequence of the resistance of the outer mitochondrial membrane to rupture by hypotonic shock is that cytochrome *c* is retained in the mitochondria. The impermeability of the mitochondrial outer membrane to cytochrome *c* in rat liver mitochondria has been demonstrated by Wojtczak and Zaluska[39], and in plant mitochondria by Douce *et al.*[38]. The results reported here demonstrate impermeability in spermatozoa mitochondria; two washes with KCl medium after disrupting the plasma membrane in hypotonic buffer fail to remove the cytochrome *c* from these mitochondria with their intact outer membrane, as is evident from the reduced-*minus*-oxidized difference spectra (Figs 5B and 6A). In constrast to this, treatment of rat liver mitochondria with 15 mM KCl to break the outer membrane followed by two washes with 0.15 M KCl, removes over 95% of the cytochrome *c*[40,41].

The ratio of cytochrome *c* to cytochrome *a* absorbance is 2.0 in intact spermatozoa compared to 1.6 in hypotonically treated spermatozoa, showing that about 20% of the total cellular cytochrome *c* is removed by hypotonic treatment. The stubborn retention of the remaining 80% of the cytochrome *c* by the mitochondria during KCl washing strongly suggests that the cytochrome *c* which is readily removed is cytoplasmic. There is convincing evidence that the cytochrome *c* molecule is synthesized *in toto* on the cytoplasmic ribosomes[42–45]. Part of this cytochrome *c* is tightly bound and part more loosely bound to a microsomal fraction from which it can be recovered[43]. Transport of cytochrome *c* through the impermeable outer mitochondrial membrane proceeds by a mechanism as yet not understood, but one which may

involve slightly modified forms of the cytochrome which are found in the cytoplasm[45,46]. It is most probably this cytochrome *c*, whose synthesis is complete but whose transport into the mitochondria has not yet occurred before final maturation of the spermatozoan from the spermatid stage, which is readily lost when the cell membrane is damaged or altered during storage or cold shock[23,47,48].

While the mitochondrial membranes are resistant to hypotonic treatment, they are permeable to dehydrogenase substrates, to inhibitors, to uncouplers, and to the adenine nucleotides. The reactions of these mitochondria can be as readily studied as those isolated from other tissues, even though they remain attached to the structures of the sperm cell. The mitochondria are tightly coupled, and thus provide the basis for examining the energy metabolism of spermatozoa. The question of which non-mitochondrial enzymes are retained by the hypotonically treated spermatozoa, and the extent to which this preparation may be useful as a model for studying mitochondrial–extramitochondrial interactions in whole cells is currently also under investigation.

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