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Assessment of Cell Damage Caused by Spontaneous Lipid Peroxidation in Rabbit Spermatozoa

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ABSTRACT

Damage to the plasma membrane of rabbit epididymal spermatozoa during spontaneous lipid perioxidation was examined by means of trypan blue uptake and expression of activity of the intracellular enzymes, lactate dehydrogenase and pyruvate kinase. Both the dye uptake and the expression of enzyme activity probe cell damage from lipid peroxidation as loss of integrity of the plasma membrane. A linear correlation was obtained between trypan blue staining of the cells and malondialdehyde production, a quantifiable measure of the extent of lipid peroxidation. At the point of trypan blue staining of all cells, 0.5 nmol malondialdehyde/10^8 cells was produced. This is the same amount produced at the point of complete loss of motility and superoxide dismutase activity. We have defined this as the "lipoperoxidative lethal end point." Expression of lactate dehydrogenase and pyruvate kinase activities increased with time of aerobic incubation. In the high Na^+ medium, NTP, in which lipid peroxidation is slow, there is a linear correlation between increase in expressed enzyme activities and malondialdehyde production. But in the high K^+ medium, KTP, in which lipid peroxidation is rapid, there is an initial rapid rise in expressed enzyme activity over 3 h, followed by a slower increase. Activities of rabbit sperm lactate dehydrogenase, pyruvate kinase, and flagellar ATPase were unaffected by aerobic incubations for up to 48 h, double the incubation period used for the assay of enzymatic activities for the first two. The activity of glyceraldehyde-3-phosphate dehydrogenase decreased during aerobic incubation, the time course matching the loss of motility. The subcellular distribution of lactate dehydrogenase in rabbit spermatozoa was determined: 4% in the mitochondrial matrix, 10% in the plasma membrane and 85% in the cytosolic compartment.

INTRODUCTION

One of the manifestations of O_2 toxicity to cells is lipid peroxidation (Haugaard, 1968; Tappel, 1973; Chance et al., 1979; Tappel and Dillard, 1981; Slater, 1982; Hochstein and Rice-Evans, 1982). While the effects of this reaction are complex, one result of lipid peroxidation is loss of the permeability barriers of the plasma membrane. This has been extensively documented for mammalian spermatozoa: induced lipid peroxidation leads to loss of motility due to loss of cytosolic enzymes and essential substrates such as adenine and pyridine nucleotides (Jones and Mann, 1973, 1976, 1977a,b; Jones et al., 1978; Mann et al., 1980; Mann and Lutwak-Mann, 1981). We have shown that rabbit spermatozoa undergo spontaneous lipid peroxidation in the absence of inducers with concomitant loss of motility (Alvarez and Storey, 1982). The rate of lipid peroxidation, as measured by production of malondialdehyde (MDA), is dependent on the ionic composition of the medium, but the production of MDA gives a linear correlation with motility loss, independent of the rate of production. The point where all motility ceases is 0.5 nmol MDA/10^8 cells. Lipid peroxidation in these cells appears to be mediated by superoxide: superoxide dismutase (SOD) is the primary enzymatic defense against lipid peroxidation and the activity of the enzyme declines as peroxidation increases (Holland et al., 1982; Alvarez and Storey, 1983).

While these studies have established correlations between motility loss and lipid peroxidation in rabbit spermatozoa, the nature of the cell damage was not investigated. One expected mode of cell damage is increased plasma membrane permeability to adenine and pyridine

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nucleotides. Another expected mode would be inactivation of enzymes (Haugaard, 1968) required for energy metabolism and flagellar motion. In this paper, we present a study of these two modes of cell damage as lipid peroxidation occurs in terms of changes of activity of lactate dehydrogenase (LDH), pyruvate kinase (PK), glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) and flagellar ATPase and changes of plasma membrane permeability as measured by access of exogenous adenine and pyridine nucleotides to the enzymes LDH and PK. The expression of enzyme activities due to accessibility of normally impermeable substrates represents a new, more rapid and convenient assay of membrane permeability in sperm, as compared with measure of nucleotide content (Jones and Mann, 1973). We also report quantitation of the subcellular distribution of LDH in rabbit spermatozoa.

MATERIALS AND METHODS

Reagents

Potassium oxalate and inorganic salts were obtained from J. T. Baker (Phillipsburg, NJ) and were of reagent grade purity. Resazurin was from Eastman Chemicals (Rochester, NY), acetyl-L-carnitine was from P. L. Biochemicals (Milwaukee, WI). All other reagents and enzymes were from Sigma Chemical Co. (St. Louis, MO). The uncoupler of oxidative phosphorylation bis (hexafluoroacetonyl) acetone, designated 1979 (Heytler, 1979) was generously provided by Dr. Peter G. Heytler, E. I. du Pont de Nemours (Wilmington, DE).

Suspending Media

Two media were used for sperm suspension in this study. One was the kigh K^+ medium KTP with the composition: 113 mM KCl, 12.5 mM KH_2PO_4, 2.5 mM K_2HPO_4, 3 mM $MgCl_2$, 20 mM Tris, 1.5 mM D-glucose, 0.4 mM EDTA, 0.6% penicillin/streptomycin, adjusted to pH 7.4 with HCl. The high Na^+ medium NTP contained 10 mM KCl, 103 mM NaCl, and 15 mM NaH_2PO_4, but was otherwise identical in composition to KTP. These are the two media used for earlier studies on lipid peroxidation in rabbit spermatozoa (Alvarez and Storey, 1982, 1983).

Preparation of Spermatozoa

Spermatozoa were obtained from the caudae of excised epdidimydes of mature male New Zealand White rabbits by retrograde flushing through the vas deferens with 10 ml of the appropriate medium. The spermatozoa were washed three times by centrifugations at 750 × *g* for 10 min followed by gentle resuspension in 10 ml medium. The final stock suspensions ranged from 0.4–1.5 × 10^9 cells/ml. Hypo-osmotically treated rabbit epididymal spermatozoa (HTRES) were obtained by treatment of the spermatozoa with 10 ml of 10 mM potassium phosphate (Keyhani and Storey, 1973), followed by centrifugation at 750 × *g* from 10 min and resuspension in either KTP or NTP.

Aerobic Incubation of Spermatozoa

Aerobic incubation of fresh epididymal spermatozoa was carried as described in our earlier report (Alvarez and Storey, 1982). Cells were incubated at 37°C in NTP or KTP in 1 ml suspensions containing 0.4–1.5 × 10^8 cells/ml in sealed widemouthed specimen bottles (55 × 28 mm) on a shaking water bath to ensure good contact with O_2 in the gas phase. At selected times of incubation, 100-μl aliquots were taken and assayed immediately for trypan blue staining, 5-μl aliquots were taken and assayed for the appropriate enzymatic activity, and 50-μl aliquots were taken for motility assay.

Motility Assay

Sperm motility was estimated by a modification of the method used in previous studies (Storey and Alvarez, 1982, 1983) in order to facilitate near simultaneous scoring for motility and trypan blue staining (see below). Two 50-μl aliquots taken from the sperm suspensions during aerobic incubation at the chosen time point (see above) were placed as droplets on a slide and immediately surveyed for selection of fields at 250X under bright-field illumination. A field in each quadrant of one droplet was selected and 20 cells in the field checked for forward motility, flagellar beating, or inertness. The process was immediately repeated on the second droplet. The values from the four quadrants were averaged for each droplet; variation between these duplicate values was consistently about ±5%. The final value was the average of the duplicates. The assay was readily completed within 5 min.

Trypan Blue Assay

The 100-μl aliquot taken from the sperm suspensions during aerobic incubation at the chosen time point (see above) was immediately mixed with an equal volume of 1% trypan blue in the same medium. These volumes proved convenient for rapid and complete mixing. The same procedure was employed as for assay of sperm motility: duplicate 50-μl droplets were assayed for percent trypan blue staining under bright-field illumination at 450X by the four-quadrant averaging method described above. At this concentration of trypan blue (0.5%), the dye was completely excluded by intact cells which appeared bright and colorless but was taken up by damaged cells which showed blue heads. Those cells which took up dye were always inert, which facilitated the counting. Some preferential uptake of dye in the postacrosomal region was noted in cells incubated in KTP at early incubation times, resulting in a nonuniform coloration. This assay was also readily completed within 5 min.

Lipid Peroxidation Assay

Peroxidation of sperm lipids was assayed by production of malondialdehyde (MDA), using a modification of the thiobarbituric acid (TBA) procedure of Barber and Bernheim (1967). In the modified procedure, the TBA concentration was increased to 28

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μM and the absorbance of the MDA/TBA chromogen was determined by dual wavelength spectrophotometry using the wavelength pair 534-570 nm. Full details of this modification have been described by Alvarez and Storey (1982).

Lactate Dehydrogenase Assay

The activity of rabbit sperm lactate dehydrogenase (LDH) was measured both as the reduction of pyruvate with NADH and as the oxidation of lactate with NAD+ at room temperature. Reduction of pyruvate was monitored as the decrease in absorbance of NADH on conversion to NAD+, using dual wavelength spectrophotometry instead of fluorimetry as was done previously (Storey and Kayne, 1975). The wavelength pair was 340-375 nm with a difference extinction coefficient $\Delta\epsilon$=4.21 mM^{-1} cm^{-1}. The reaction mixture contained in 1 ml was: 80–100 μm NADH and 2–6 × 10^5 cells/ml in NTP or KTP; the reaction was started by addition of 2 mM pyruvate. Oxidation of lactate was monitored with the coupled enzyme method of Guilbault (1975), in which the NADH generated by the reaction is reoxidized by diaphorase with concomitant reduction of the nonfluorescent dye resazurin to the highly fluorescent resorufin. The latter reduction is essentially irreversible, so that the steady state conconcentration of NADH remains very low. The reaction mixture contained in 1 ml was: 0.1 mM NAD+, 0.2 mM resazurin, 0.4 mU/ml diaphorase (Sigma Type II-L), and 2–10 × 10^5 cells/ml in NTP or KTP. The reaction was started by addition of 2 mM lactate. The fluorescence of the resorufin produced was monitored with an Eppendorf fluorimeter using excitation at 546 nm (Hg arc source); emission was detected at wavelengths longer than 580 nm using two thicknesses of Wratten No. 28A gelatin filter for the emission filter. The fluorescence emission of the resorufin was quantitated by addition of aliquots of a standard solution of NADH to the reaction mixture to calibrate resorufin fluorescence units as NADH concentration.

The assays described above require access of the coenzymes NADH and NAD+ to the reaction site of LDH. This is the basis of the use of this enzyme assay for determining increases in permeability of the sperm plasma membrane as lipid peroxidation proceeds. Complete permeability, and hence full access of NADH and NAD+ to the enzyme site, is defined by the hypo-osmatically treated sperm preparation (HTRES), in which the plasma membrane is disrupted (Keyhani and Storey, 1973) but in which high LDH activity is retained in the cell structure (Storey and Kayne, 1975). The LDH activity of a given preparation of sperm cells undergoing lipid peroxidation has been normalized to an aliquot of the same preparation converted to HTRES, and is expressed as LDH activity ratio. Trypan blue staining was also carried out on the same cell preparation, and the LDH activity was corrected for the 5–15% damaged cells showing dye uptake by assigning to them the activity of HTRES.

Intramitochondrial LDH in rabbit sperm (Storey and Kayne, 1977) cannot be assayed by the methods described above, since the enzyme's active site is shielded from exogenous coenzyme by the inner mitochondrial membrane. This activity was assayed indirectly, based on the finding of Storey and Kayne (1978) that lactate can be converted quantitatively to pyruvate in HTRES in the presence of O_2 by a combination of intramitochondrial LDH and the mitochondrial respiratory chain which rapidly reoxidizes the NADH as it is formed. The pyruvate produced is then assayed by oxidation of exogenous NADH catalyzed by exogenous LDH. The reaction mixture for this assay contained in 1 ml was: 80–100 μM NADH, 0.5 mM acetyl-L-carnitine, 10 μM 1799, LDH (Sigma Type XI, 850 units/mg) at 50 μg/ml, and 5–7 × 10^7 HTRES cells/ml in NTP saturated with air. The reaction was started by addition of 4 mM lactate. Oxidation of the exogenous NADH was followed by dual wavelength spectrophotometry using the wavelength pair 365-395 nm, $\Delta\epsilon$=3.18 mm^{-1} cm^{-1}. This wavelength pair minimizes light scattering artifacts at the higher sperm concentrations required for the assay.

Pyruvate Kinase Assay

The activity of pyruvate kinase (PK) was assayed by means of the enzyme-coupled system utilizing exogenous LDH and NADH; the reaction was monitored by the change in absorbance as NADH is oxidized by the pyruvate formed (Storey and Kayne, 1975). The reaction mixture contained in 1 ml was: 80–100 μM NADH, 1 mM ADP, LDH at 125 μg/ml, and 2–6 × 10^5 cells/ml in NTP or KTP. With NTP, it was necessary to add KCl to increase the K^+ concentration to 100 mM to elicit maximal activity of the enzyme (Storey and Kayne, 1980). The reaction was started by addition of 1.5 mM phosphoenolpyruvate (PEP). The absorbance change due to NADH oxidation was monitored with dual wavelength spectrophotometry using the wavelength pair of 340-375 nm. The activity of PK in fresh and aerobically incubated cells was normalized to the activity in HTRES and was corrected for damaged cells as described above for the LDH assay.

ATPase Assay

The activity of the sperm Mg^{2+}-ATPase, which is essentially all attributable to flagellar ATPase, was determined by the enzyme-coupled system described by Storey and Kayne (1980). The reaction mixture contained in 1 ml was: 80–100 μM NADH, 1 mM PEP, LDH at 120 μg/ml, PK (Sigma Type units/mg) at 150 μg/ml, and 3–5 × 10^5 cells/ml. The cells were treated hypo-osmotically to give HTRES just before assay. The reaction was started by addition of 3 mM ATP and the absorbance change due to NADH was monitored as for the PK assay.

Glyceraldehyde-3-Phosphate Dehydrogenase Assay

The activity of rabbit sperm glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) was monitored directly by the absorbance change due to NADH produced in the reaction, in the presence of inhibitors of enolase, PK and LDH to prevent pyruvate formation (Storey and Kayne, 1975). The reaction mixture contained in 1 ml was: 0.5 mM NAD+, 1 mM ADP, 5 mM KF (enolase inhibitor), 0.5 mM oxalate (PK inhibitor), 0.1 mM oxamate (LDH inhibitor), and 3–5 × 10^5 cells/ml. When added, dithioerythritol (DTE) was at 2 mM. The cells were treated hypo-osmotically to give HTRES just prior to the assay. The reaction was started by addition of 2.5 mM glyceraldehyde-3-

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phosphate and the NADH produced was monitored by the absorbance change at 340-375 nm using dual wavelength spectrophotometry.

RESULTS

Trypan Blue Staining

Rabbit epididymal spermatozoa incubated aerobically showed an increasing percentage of cells stained with trypan blue with increasing incubation time (Fig. 1). The increase corresponds to an increase in the percentage of cells with membranes made permeable to this dye by peroxidative damage. The rate of increase of staining was linear and was fourfold higher in the high K^+ medium, KTP, than in NTP. In KTP, the postacrosomal region showed a somewhat deeper blue color than the rest of the head at early incubation times. Fresh sperm samples contained about 10% cells (range: 5–15%) which took up trypan blue, as shown by the intercept at the Y axis in Fig. 1, and this was independent of suspending medium.

When the percentage of spermatozoa stained with trypan blue and malondialdehyde (MDA) production at a given time were compared, the result was a linear correlation (Fig. 2). The same correlation was obtained in both NTP and KTP. The correlation gives 0.5 nmol MDA/10^8 cells as the point where 100% of the cells become stained. This is the same end point corresponding to 100% inert spermatozoa in the correlation between MDA production and sperm motility (Alvarez and Storey, 1982).

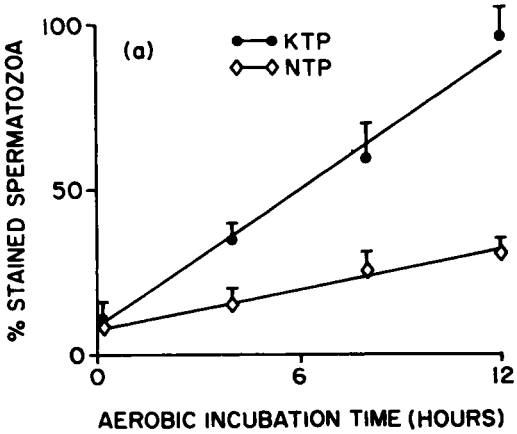

FIG. 1. Percentage of rabbit epididymal spermatozoa stained with trypan blue as a function of aerobic incubation time at 37°C in KTP (•) and NTP (◇). Sperm concentration range was 0.4–1.5 × 10^8 cells/ml. Each point is the mean of 3 experiments; error bars are standard deviations.

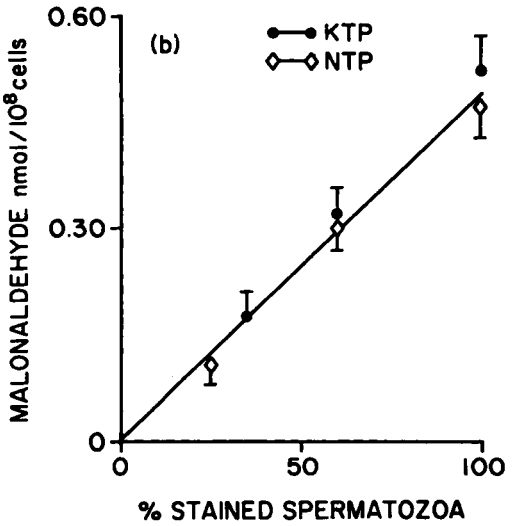

FIG. 2. Correlation between malondialdehyde (MDA) production and staining of sperm by trypan blue at fixed times of aerobic incubation at 37°C in KTP (•) and NTP (◇). Sperm concentration range was 0.4–1.2 × 10^8 cells/ml. Each point is the mean of 3 experiments; error bars are standard deviations. The regression equation calculated through the origin has the form: $y=0.0051\ X$ ($r=0.992$).

LDH and PK Activities

Increased permeability of the sperm plasma membrane due to peroxidation during aerobic incubation was assessed by expression of LDH and PK activities with increasing incubation time. The activities have been normalized to the activity determined in HTRES from the same sperm sample and are expressed as the activity ratio. The results obtained for LDH, measured as pyruvate reduction to lactate, are shown in Fig. 3 and those obtained for PK are shown in Fig. 4. In NTP, both activities increase linearly with time of aerobic incubation. But in KTP, the increase in expressed activity is rapid during the first 3 h and then decreases to a rate similar to that seen in NTP. While the activity ratios in NTP correlate linearly with trypan blue staining and MDA producton (not shown), those in KTP do not (Fig. 5). There seems to be a non-uniform increase in membrane permeability with time in KTP, leading to more rapid expression of the latent enzyme activities. In both NTP and KTP, the activity ratio attained for both PK and LDH was about 0.6 at 0.5 nmol MDA/10^8 cells, the point of MDA production

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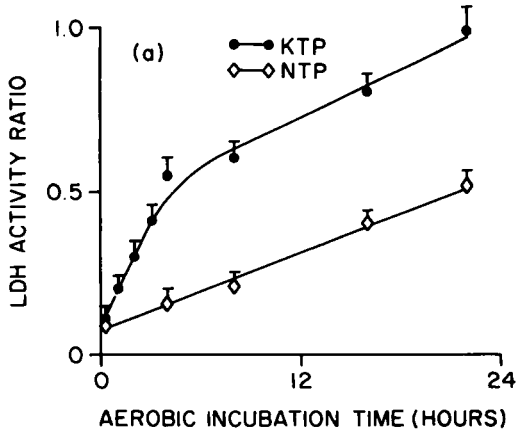

FIG. 3. Lactate dehydrogenase (LDH) activity ratio in rabbit epididymal spermatozoa as a function of aerobic incubation time at 37°C in KTP (•) and NTP (◇). The activity ratio is normalized to the activity in HTRES as described in *Materials and Methods* and has been corrected for cells staining with trypan blue at zero time. Sperm concentration range was 0.6–1.5 × 10^8 cells/ml. For the first 3 h of incubation in KTP, each point is the mean of 3 experiments; between 3 and 22, each point is the mean of 10 experiments. In NTP, all points are the means of 10 experiments. Error bars are standard deviations.

corresponding to complete staining with trypan blue (Fig. 2) and complete cessation of motility (Alvarez and Storey, 1982).

The anomalous increase in expression of LDH and PK activities in KTP with time of aerobic incubation (Figs. 3 and 4) reflects a similar anomalous decline of motility. In this medium, fresh sperm have 40–50% forward motility and 50–60% flagellar beating; the forward motility declines with aerobic incubation far more rapidly than does the flagellar beating (Alvarez and Storey, 1982). (In NTP, only forward motility was observed.) In Fig. 6 we have compared the time course of the rise in PK activity ratio with that of decline of forward motility in KTP. The time courses match fairly well, indicating that the two may be connected with peroxidation-induced membrane permeability at a particular region of the plasma membrane. At present we lack an assay for localizing membrane peroxidation, and further investigation of this point has been deferred pending development of such an assay.

"Cytosolic" LDH activity in these rabbit spermatozoa, defined as that observed in HTRES, was 530 ± 130 nmol/min per 10^8 cells ($\bar{X}$ ± SD, n=7; range 350–700). The maximal PK activity was 530 ± 250 nmol/min per 10^8 cells, ($\bar{X}$ ± SD, n=7; range 310–800). These values are in reasonable agreement with those of 430 ± 280 for LDH and 400 ± 290 for PK activity in the same units reported previously (Storey and Kayne, 1975). The PK activity in fresh intact cells is nil within experimental error, if correction is made for cells staining with trypan blue in the fresh samples (Fig. 4). The minimal LDH activity after correction for cells staining with trypan blue is 53 ± 18 nmol/min per 10^8 cells ($\bar{X}$ ± SD, n=15; range 30–67), which is about 10% of the maximal activity. The sperm plasma membrane is impermeable to both NADH and ADP and, in fresh cells, the PK activity inside the plasma membrane is not expressed because the substrate does not have access to the enzyme, NADH does have access to a fraction of the total LDH in fresh sperm, implying that this fraction is on the plasma membrane. A similar localization of LDH in rabbit sperm has been demonstrated by Goldberg (1973, 1974, 1977) and in mouse sperm by Erickson et al. (1975), using immunological methods. A fraction of rabbit sperm LDH is also in the mitochondrial matrix (Storey and Kayne, 1977). Quantitation of the localization of LDH activity in the three compartments of rabbit sperm is given in Table 1.

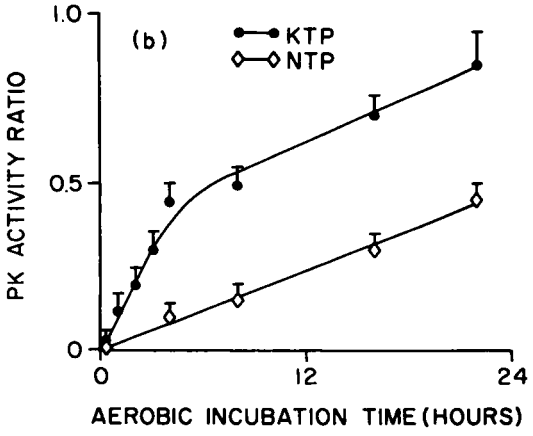

FIG. 4. Pyruvate kinase (PK) activity ratio in rabbit epididymal spermatozoa as a function of aerobic incubation time at 37°C in KTP (•) and NTP (◇). The activity ratio is the same as for LDH, defined in Fig. 3. Sperm concentration range was 0.4–1.0 × 10^8 cells/ml. For the first 3 h of incubation in KTP, each point is the mean of 3 experiments; between 3 and 22 h each point is the mean of 7 experiments. In NTP, all points are the means of 10 experiments. Error bars are standard deviations.

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Enzyme Stability

The stability of the four enzymes, LDH, PK, ATPase, and GAP-DH, was assessed with regard to aerobic incubation at 37°C to ascertain whether the long incubation times would affect their activities. After incubation, the sperm were hypo-osmotically treated to give HTRES, in which maximal enzymatic activities are expressed. Even after 48 h incubation, LDH, PK, and ATPase remained 90% or more of the activities in fresh spermatozoa. This stability ensures that the use of the expressed activities of LDH and PK as a measure of plasma membrane damage was not compromised by loss of intrinsic activity of these two enzymes. In the case of the ATPase, coupling between enzymatic activity and flagellar motion was more labile. Flagellar motion can be restored in HTRES suspended in KTP by addition of ATP and PEP (Keyhani and Storey, 1973). When this restoration of flagellar motion was attempted with sperm aerobically incubated for various times at 37°C, it was partially successful during the first 12 h of incubation. After this period, flagellar motion was irreversibly lost, despite no evident change in ATPase activity.

GAP-DH activity is considerably more labile than the activities of the other three enzymes assessed. The decline in activity after incubation in NTP and KTP is shown in Fig. 7. This enzyme has a thiol group required for activity at the active site (Harris and Waters, 1976). Treatment of the sperm cell suspension, after conversion to HTRES, with dithioerythitol (DTE) restored enzymatic activity to a minor extent after incubation in NTP and to a greater extent after incubation in KTP. During the first 8 h of incubation in KTP, restoration of enzymatic activity by DTE to the level seen in NTP alone is possible. But by 12 h, most of the activity of the enzyme is irreversibly lost. The

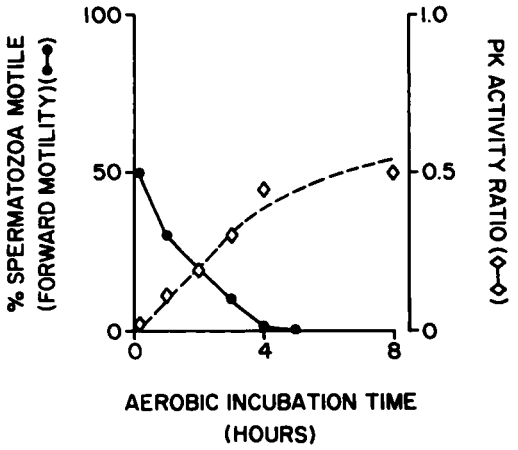

FIG. 6. Percentage of forward motility and PK activity ratio in rabbit epididymal spermatozoa as a function of aerobic incubation time at 37°C in KTP. Sperm concentration range was 0.4–1.5 × 10^8 cells/ml. Each point is the mean of 3 experiments.

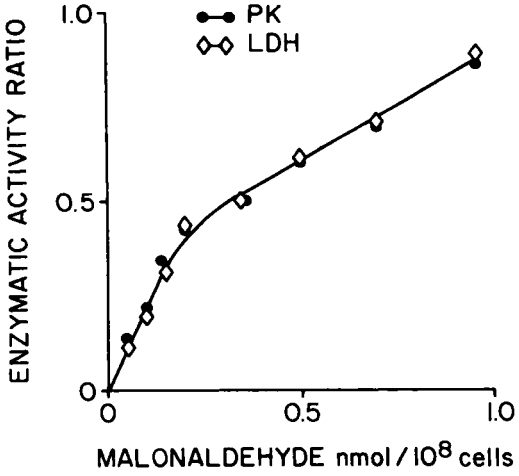

FIG. 5. LDH (◇) and PK (●) activity ratios as a function of MDA production at fixed times during aerobic incubation at 37°C in KTP. The LDH activity ratio has been corrected for plasma membrane LDH activity. Sperm concentration range was 0.4–1.5 × 10^8 cells/ml. Each point is the mean of 3 experiments.

TABLE 1. Distribution of lactate dehydrogenase activity in subcellular compartments of rabbit epididymal spermatozoa.

Compartment	LDH activity[a] (nmol/min per 10^8 cells)	Activity ratio[b]
Mitochondria	1.8 ± 0.8	0.044 ± 0.008
Plasma membrane	4.3 ± 3.0	0.100 ± 0.008
Cytosol[c]	35.0 ± 20.0	0.850 ± 0.013

[a]Activities are the oxidation of lactate to pyruvate as described in *Materials and Methods*. Values are the means ± SD for 5 different sperm samples.

[b]Activity ratios were calculated for each sperm sample from the activities measured in each compartment for that sample. Values are the means ± SD for 5 different sperm samples.

[c]LDH bound to cell structure and accessible to NAD+, as described in text.

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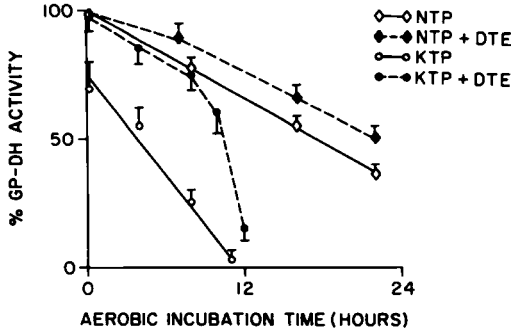

FIG. 7. Percentage of glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) activity remaining as a function of aerobic incubation time at 37°C in KTP (○), KTP plus 2 mM DTE (●), NTP (◇), and NTP plus 2 mM DTE (◆). Sperm concentration range was 0.6–1.5 × 10^8 cells/ml. Each point is the mean of 5 experiments; error bars are standard deviations.

12-h incubation point in KTP is also that at which all flagellar motion is lost, at which 0.5 nmol MDA/10^8 cells is produced (Alvarez and Storey, 1982), and at which all cells are stained with trypan blue (Fig. 2). In NTP, inactivation of the enzyme also proceeds in parallel with loss of both the motility and permeability barriers of the plasma membrane.

DISCUSSION

Damage to rabbit spermatozoa from spontaneous lipid peroxidation comes from both postulated sources. One is the loss of activity of a crucial enzyme for the energy metabolism of the cell, GAP-DH. The other is increased permeability of the cell plasma membrane to the point where adenine and pyridine nucleotides, also crucial to the cell's energy metabolism, can pass through it freely. The loss of activity of GAP-DH with increasing degree of lipid peroxidation is consistent both with the sensitivity towards oxidation of the enzyme's essential thiol group and with the loss of SOD activity as peroxidation proceeds (Harris and Waters, 1976). The latter process results in a higher intrinsic rate of superoxide production by the cells (Alvarez and Storey, 1983), with the result that the GAP-DH is exposed to ever higher concentrations of $O_2^{\cdot-}$ and $HO_2^{\cdot}$, the latter being a particularly potent oxidant (Gebicki and Bielski, 1981). The effect of DTE implies that the first reaction is oxidation of the essential thiol group to sulfenic acid, a relation readily reversed by added thiols such as DTE (Allison and Connors, 1970). Inactivation not reversible by DTE occurs when the thiol sulfur is oxidized further to higher oxidation states. The behavior of GAP-DH in KTP is anomalous in that a fraction of it is already in the inactive, DTE-reversible state even at the earliest incubation times. We have no explanation at present for this effect of high K^+.

The subcellular distribution of LDH activity (Table 1) includes 10% of this activity on the surface of the sperm plasma membrane. To the best of our knowledge, this figure represents the first quantitation by biochemical assay for plasma membrane activity of sperm LDH. This location for part of the cellular activity of this enzyme, which is the isozyme LDH-X (Goldberg, 1963; Blanco and Zinkham, 1963), was originally demonstrated by its ability to induce isozyme-specific antibodies with preparations of intact sperm as antigen (Goldberg, 1973, 1974). The plasma membrane component of the LDH activity, while small, cannot be dismissed as contamination by enzyme leaking from damaged cells in the preparation. Correction was made for such damage by assessment of trypan blue staining for both LDH and PK activities: the corrected surface PK activity was consistently nil within experimental error as expected for intact cells. Preferential leakage of LDH over PK to the extent of 10% of LDH activity in rabbit sperm is deemed highly improbable, since it is retained in HTRES and is removed only slowly by repeated washing (Storey and Kayne, 1975). The "cytosolic" LDH comprises 85% of the total LDH activity in the sperm cells and 89% of the activity directly assayable with pyruvate and NADH. This percentage is high enough that use of the LDH activity of HTRES as a maximal activity in the trypan blue correction and in the enzymatic activity ratio introduces negligible error. The location of LDH activity in rabbit sperm is most probably in the midpiece and tailpiece; this location has been established for "cytosolic" LDH in mouse, rat, and human spermatozoa (Burkhart et al., 1982). The intramitochondrial LDH complement (Table 1) found in this study is consistent with the earlier estimate of 2–6% (Storey and Kayne, 1977). In rabbit spermatozoa, all three subcellular compartments contain only the isozyme LDH-X (Storey and Kayne, 1977).

The linear correlation (Fig. 2) between trypan blue uptake and MDA production in rabbit spermatozoa undergoing spontaneous lipid peroxidation matches the linear correlations be-

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tween loss of motility, SOD activity and MDA production previously established (Alvarez and Storey, 1982, 1983). The same value of 0.5 nmol MDA/10^8 cells was obtained for trypan blue uptake by all cells as for complete loss of motility and SOD activity. This value represents the "lipoperoxidative lethal end point" for mature rabbit spermatozoa and should prove useful for comparison with the effects of spontaneous lipid peroxidation in sperm from other species.

The finding that the linear correlation between MDA production and the expressed activity of PK and LDH holds in NTP but not in KTP was unexpected. The time course of expression of these activities with aerobic incubation matches the loss of forward motility in KTP, but does not match the time course of trypan blue uptake (compare Figs. 1, 3 and 4). In the dye uptake test, the color of the sperm head is the primary criterion for scoring, since it is unequivocally either colorless or blue. The midpiece and tail have insufficient cytosol to show much color. The one difference between the color pattern shown in KTP and NTP was that the postacrosomal region had a somewhat deeper color than the rest of the head at early incubation times. This effect was not sufficiently pronounced under our dye uptake conditions to be useful quantitatively, but it does suggest that regionalization of peroxidation in KTP may be more pronounced that in NTP. But it seems to occur in both media, since the activity ratios of PK and LDH are only 0.6 at the lipoperoxidation lethal end point. At this point of aerobic incubation, all the sperm heads have become permeable to trypan blue, implying that the plasma membrane in this region has become more damaged than that which overlies the tailpiece, where much of the LDH is localized (Burkhart et al., 1982). Such regionalization is consistent with the known differences in membrane properties at different regions of the sperm plasma membrane (Friend, 1977). If peroxidation resulting in permeability of the plasma membrane to NADH, ATP and PEP were to occur more rapidly in the postacrosomal and midpiece regions, the enzymes PK and LDH would be accessible to these substrates at early incubation times before trypan blue uptake would be observed in the anterior part of the head. We have no explanation at present for such a preferential regional peroxidation in the presence of high K^+ and not in high Na^+ medium, but this differentiation may prove useful in later studies concerned with functional mapping of the sperm plasma membrane.

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