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Lipid Peroxidation and the Reactions of Superoxide and Hydrogen Peroxide in Mouse Spermatozoa

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ABSTRACT

Mouse spermatozoa released from the cauda epididymidis underwent spontaneous lipid peroxidation during aerobic incubation at 37°C in medium containing 113 mM NaCl, 0.4 mM EDTA, and 15 mM sodium phosphate (NTPC). The rate of lipid peroxidation, as measured by malonaldehyde production, was 0.045 nmol malonaldehyde/h per 10^8 cells. The motility of these cells declined with time in medium NTPC; the percent spermatozoa showing no motility increased linearly with production of malonaldehyde. All flagellar activity stopped at 0.80 nmol malonaldehyde/10^8 cells, independent of the malonaldehyde production rate. Spermatozoa suspended in NTPC at 24°C produced $O_2^{\cdot-}$, with an intrinsic rate of 1.96 nmol/min per 10^8 cells; this increased to 3.80 nmol/ min per 10^8 cells in 10 mM cyanide. Mouse sperm contain 3.5 U/10^8 cells of superoxide dismutase activity, 91% of which is sensitive, and 9% of which is insensitive, to cyanide inhibition. Mouse sperm also produce H_2O_2, all of which can be attributed to the action of superoxide dismutase on $O_2^{\cdot-}$ produced. Mouse sperm contain high levels of glutathione and of glutathione reductase and peroxidase activities, implicating the glutathione system as the major protective enzyme system against cell damage by autoxidation. This is in contrast to rabbit spermatozoa, which have little endogenous glutathione and rely on superoxide dismutase as protective enzyme against peroxidative damage.

INTRODUCTION

While studies of promoter-induced lipid peroxidation in mammalian spermatozoa have been quite extensive and have covered a number of species (Jones and Mann, 1973, 1976, 1977; Jones et al., 1978, 1979, Mann et al., 1980; Mennella and Jones, 1980; Mann and Lutwak-Mann, 1981), studies of spontaneous lipid peroxidation have thus far been confined primarily to rabbit spermatozoa (Alvarez and Storey, 1982, 1983a,b). We have presented eivdence that superoxide (SO) produced intracellularly is the principal inducer of lipid peroxidation (Alvarez and Storey, 1983a), with the perhydroxyl form $HO_2^{\cdot}$ being 300-fold more effective than its conjugate base, the anion form $O_2^{\cdot-}$ (Alvarez et al., 1983). Superoxide dismutase (SOD) is the primary enzymatic defense against lipid peroxidation in rabbit spermatozoa (Storey and Alvarez, 1983a), which have no detectable catalase activity and produce H_2O_2 (Holland and Storey, 1981). They have low detectable endogenous glutathione, although they possess glutathione peroxidase and glutathione reductase activities (Li, 1975; Holland and Storey, 1981). The low endogenous glutathione seems peculiar to the rabbit and the boar. Spermatozoa from other species have been reported to have total glutathione contents of the order of 0.5–1.3 nmol/10^8 cells while the figure for rabbit has been given as 0.1 and for boar as 0.3 (Li, 1975). Rabbit spermatozoa have also been reported to be relatively resistant to damage by exogenous H_2O_2, while mouse spermatozoa were found to be relatively sensitive (Wales et al., 1959). Since we have some previous metabolic characterization of mouse spermatozoa (Carey et al., 1981; Heffner and Storey, 1981), these cells seemed to offer a useful comparison to rabbit spermatozoa with regard to spontaneous lipid peroxidation, loss of motility, production of SO and H_2O_2, and the activities of the enzymes which are considered to protect against O_2 toxicity (Chance et al., 1979): SOD, catalase, and glutathione peroxidase/

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reductase. In this paper, we report the results of an investigation of these aspects of O_2 reactions in epididymal mouse spermatozoa.

MATERIALS AND METHODS

Media

Four different media were used for sperm suspensions in this study. Once was a modification of NTP, the high Na^+ medium of Alvarez and Storey (1982), which was designated NTPC, with the composition: 113 mM NaCl, 12.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4, 1.7 mM $CaCl_2$, 20 mM Tris, 1.5 mM D-glucose, 0.4 mM EDTA, 0.6% penicillin-streptomycin adjusted with HCl to pH 7.4 ± 0.05. For studies in which the concentrations of K^+ and Na^+ were changed in NTPC, the KCl concentration was varied from 0–100 mM so that (NaCl) + (KCl)=130 mM. The second medium was a complete culture medium (CM) for in vitro fertilization (Wolf and Inoue, 1976), which is a modified Krebs'-Ringer bicarbonate buffer containing bovine serum albumin (BSA), glucose, pyruvate and lactate (Toyoda et al., 1971). The third medium used was a Tris buffer (TNC; Heffner and Storey, 1981), with the composition: 126 mM NaCl, 20 mM Tris, 1.7 mM $CaCl_2$, adjusted with HCl to pH 7.4. The fourth medium was a modification of the latter, designated TNCPE, with the composition: 20 mM Tris, 126 mM NaCl, 1.7 mM $CaCl_2$, 12.5 mM NaH_2PO_4, 2.5 mM Na_2HPO_4, 0.4 mM EDTA, 0.6% penicillin-streptomycin, adjusted with HCl to pH 7.4–7.5. Where designated, BSA at 10 mg/ml was added to NTPC (NTPCA) and TNC (TNCA).

Reagents

Xanthine oxidase, xanthine, superoxide dismutase (Type I) from bovine erthrocytes, cytochrome *c* (Type VI) from horse heart, oligomycin, rotenone, catalase (Type C-40) from bovine liver, glutathione reductase (Type IV) from yeast, oxidized and reduced glutathione, aminotriazole, sodium pyruvate, sodium lactate, BSA, thiobarbituric acid (TBA), adenine nucleotides and N,N′-ethylenediamino tetraacetic acid, disodium salt (EDTA) were from Sigma Chemical Co. (St. Louis, MO). Glutathione peroxidase was from Boehringer Mannheim (Indianapolis, IN). Yeast cytochrome *c* peroxidase was the gift of Professor C. P. Lee, Wayne State University. Malonaldehyde-bis (dimethylacetal) was from Aldrich Chemical Co. (Milwaukee, WI). Trichloroacetic acid and inorganic salts were from J. T. Baker (Phillisburg, NJ). Filipin was obtained from Polysciences, Inc. (Warrington, PA). Penicillin-streptomycin was from Gibco Labs. (Grand Island, NY).

Preparation of Spermatozoa

Epididymal mouse spermatozoa suspensions were prepared from mature Swiss Webster mice. Excised caudae epididymides of the designated number of mice (8 to 11) were minced into 2 ml of specified medium and the sperm were allowed to disperse for 15 min at 25°C. Particulate matter was then removed. Duplicate aliquots of 5 μl of sperm suspension were removed, diluted to 1 ml with distilled water and the number of sperm determined by hemocytometer. The final cell concentration in the stock suspension was $0.6–3 \times 10^8$ cells/ml. For experiments in which sperm were to be incubated in media of different ionic compositions, the sperm were recovered by centrifugation at 300 × *g* for 10 min and washed once by a duplicate centrifugation and resuspension in the experimental medium. Spermatozoa with permeabilized plasma membranes were obtained by treatment with filipin (Carey et al., 1981). The cells were washed twice by centrifugation at 300 × *g* for 10 min in NTPC and resuspended in that medium at about 1×10^8 cells/ml. Filipin was added as a 20-mM solution in dimethylformamide (DMF) to 0.15 ml of sperm suspension at a final concentration of 0.3 mM. After 10 min incubation at room temperature, the sperm suspension was diluted by addition of 0.85 ml NTPC. The cells were recovered and washed once with NTPC by centrifugation as described above. The final sperm suspension was in 0.10 ml of ice-chilled NTPC; the sperm were kept at 0°C in ice until used. These cells are designated filipin-treated mouse epididymal sperm (FTMES).

Aerobic Incubation of Spermatozoa

Spontaneous lipid peroxidation was induced by exposure of the spermatozoa to O_2 during aerobic incubation. The stock solution of spermatozoa was diluted 3-fold to give a sperm suspension containing $0.2–1.0 \times 10^8$ cells/ml; 0.3 ml of suspension was placed in widemouthed specimen bottles (55 × 28 mm) held in a shaking water bath at 37°C. The caps of the bottles had liners made of Teflon which prevented contamination of the samples and provided a seal tight enough to prevent loss of malonaldehyde by volatilization from the suspension.

Determination of Lipid Peroxidation by Malonaldehyde Production

The determination of malonaldehyde was carried out by using a slight modification of the method used previously (Alvarez and Storey, 1982). To 0.3 ml of sperm suspension was added 0.15 ml 40% trichloroacetic acid (TCA) and 0.15 ml of the chosen medium, after incubation was terminated by chilling in ice. The diluted suspension was centrifuged at 2500 × *g* for 10 min; the supernatant was added to 0.15 ml 2% (w/v) (0.14 M) thiobarbituric acid (TBA) in distilled water followed by dropwise addition of SN NaOH to give a clear solution. The rest of the procedure was carried out exactly as described by Alvarez and Storey (1982).

Motility Assay

Sperm motility was estimated by the method used in previous studies (Alvarez and Storey, 1982, 1983a) in which the percentage motility in duplicate aliquots of the sperm suspension was estimated by microscopic examination and averaged.

Determination of Rate of H_2O_2 Generation

The formation of H_2O_2 in the sperm suspension was followed by measuring the oxidation of acetylated ferrocytochrome *c* catalyzed by cytochrome *c* peroxidase at room temperature (21–23°C) with a DW-2A dual wavelength spectrophotometer, as described by Holland and Storey (1981).

Determination of Rate of $O_2^{\cdot-}$ Generation and Superoxidase Dismutase Activity

The formation of $O_2^{\cdot-}$ in the sperm suspension was monitored by the reduction of acetylated ferricytochrome *c* (Azzi et al., 1975) as described by Holland et al. (1982). The activity of superoxide dismutase (SOD) was determined by inhibition of the above reduction reaction (McCord and Fridovich, 1969) by added sperm, using the xanthine/xanthine oxidase system as $O_2^{\cdot-}$ generator (Holland et al., 1982).

Glutathione Peroxidase and Reductase Activities, and Glutathione Content

Both enzymatic activities were determined in mouse epididymal spermatozoa permeabilized with filipin (FTMES). In this series of determinations, the cells were *not* recovered by centrifugation and washed after dilution of the suspension to terminate the 10-min incubation. In this way, loss of glutathione from the suspension was prevented. Glutathione peroxidase was assayed by the coupled enzyme method utilizing excess glutathione reductase, which couples oxidation of NADPH to the reaction of the peroxidase with hydroperoxide and reduced glutathione (Paglia and Valentine, 1967). The reaction conditions used were those described for assay of this enzyme in bovine semen by Smith et al. (1979), using exogenous (60 μg protein/ml) reductase and 1 mM cumene hydroperoxide to elicit maximal activity of the enzyme (Lawrence and Burk, 1976). The oxidation of NADPH was monitored by the absorbance change at 365–395 nm using the DW-2A dual wavelength spectrophotometer. Glutathione reductase was assayed in the same system using 5 mM glutathione disulfide as substrate. The activities of the two enzymes were linear with cell concentration over the range 0.7 to 1.7 × 10^7 cells/ml. Total endogenous glutathione was estimated by pretreating FTMES with exogenous glutathione peroxidase (2.5 μg protein/ml) and 1 mM cumene hydroperoxide for 5 min to convert any reduced glutathione to its disulfide. The peroxidase was then inhibited by addition of 0.05 mM $ZnCl_2$ (Splittberger and Tappel, 1979). The glutathione disulfide was determined by addition of 0.2 mM NADPH, followed by glutathione reductase (60 μg protein/ml). The absorbance change due to NADPH oxidation on addition of the reductase was monitored at 365–395 nm as described above. Addition of NADPH and glutathione reductase in the absence of peroxidase pretreatment gave the intrinisic glutathione disulfide. Glutathione was calculated by difference.

RESULTS

When incubated aerobically in medium NTPC at 37°C, intact mouse epididymal spermatozoa produced malonaldehyde linearly with incubation time over a period of 20 h (Fig. 1a), with a net rate of 0.045 nmol/h per 10^8 cells calculated from the initial 20 h of aerobic incubation. The maximal accumulation in the medium was 1.2 nmol/10^8 cells. The linear increase of malonaldehyde in the medium represented a balance between its production and its slow removal through further reaction, as was also shown for rabbit sperm (Alvarez and Storey, 1982). The nature of the malonaldehyde-consuming reactions was not examined. Mouse

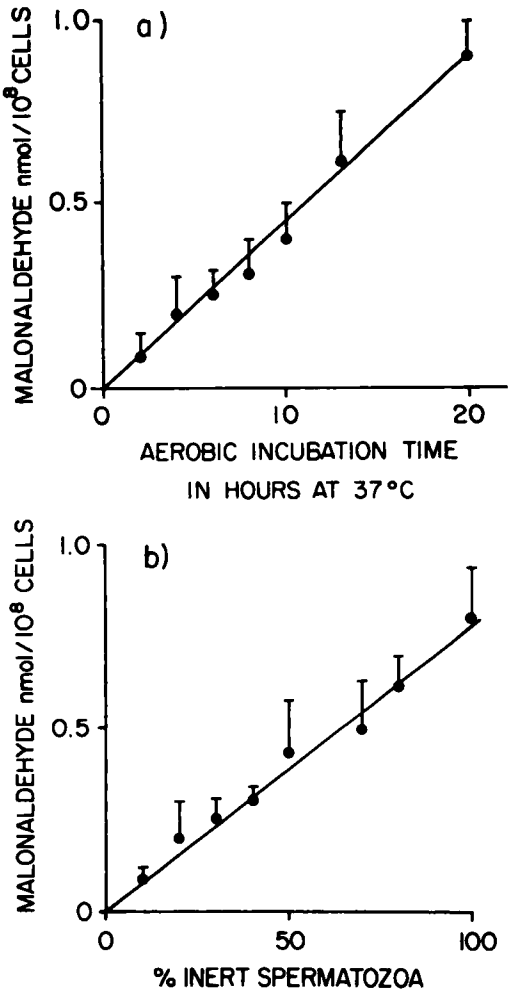

FIG. 1. Malonaldehyde production and mouse sperm motility. Malonaldehyde production by mouse epididymal spermatozoa as a function of aerobic incubation time at 37°C in medium NTPC. The composition of the medium, the sampling procedure,and the spectrophotometric method for determination of malonaldehyde are described in *Materials and Methods*. Sperm concentration ranged between 0.6 and 3 × 10^7 cells/ml. Each point represents the mean of 5 experiments; error bars are the standard deviations. The increase in malonaldehyde from 0–20 h can be represented by a linear regression equation through the origin of the form y=0.0453x (r=0.993). *b*) Correlation between malonaldehyde production and percent inert spermatozoa during aerobic incubation at 37°C in medium NTPC. The experimental conditions were those described for Fig. 1a. Each point represents the mean of 5 experiments; error bars are the standard deviations. The linear regression equation calculated through the origin has the form y=0.0078x (r=0.996).

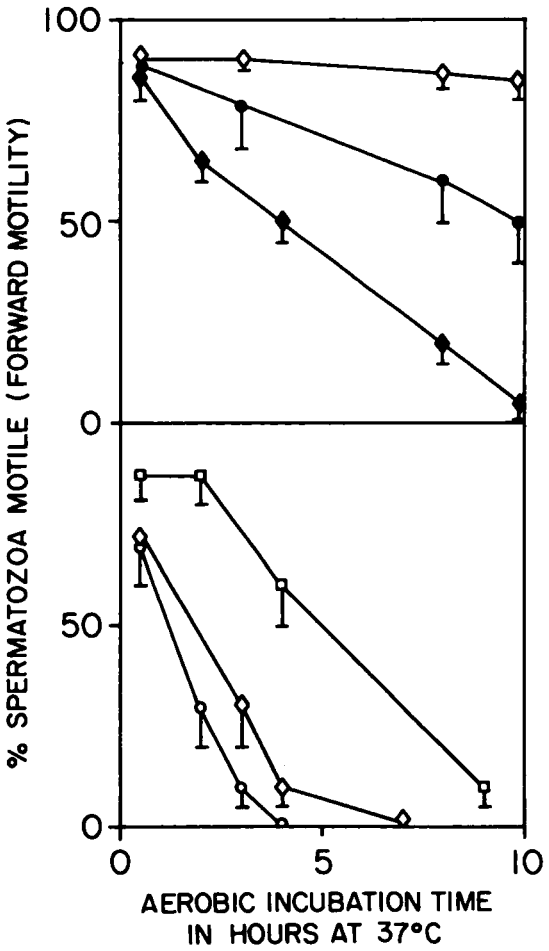

FIG. 2. Sperm motility as a function of aerobic incubation time at 37°C in media NTPC (●--●) NTPCA (◇--◇), and CM (◆--◆) (*upper panel*); TNC (○--○), TNCA (◇--◇) and TNCPE (□--□) (*lower panel*). The compositions of the media and the assay of motility are described in *Materials and Methods*. The experimental conditions were those given for Fig. 1a. Each point represents the mean of 5 experiments; error bars are the standard deviations.

epididymal spermatozoa in medium NTPC showed an initial 90% forward motility which decreased to 50% over a period of 10 h (Fig. 2). When net malonaldehyde production was assayed during this period and compared to the percent inert sperm in a given time, a linear correlation was obtained (Fig. 1b). This correlation yields the figure of 0.8 nmol malonaldehyde/10^8 cells as the index of lipid peroxidation corresponding to a completely inert population of sperm: the lipoperoxidative lethal end point.

The effect of medium composition on the decline of mouse sperm forward motility with time was further examined. Motility in TNC declined to zero in a period of 4 h with an initial value of 70% forward motility (Fig. 2). On the other hand, addition of 15 mM sodium phosphate and 0.4 mM EDTA to TNC (TNCPE) resulted in a significant enhancement in sperm motility, with an initial value of 90% which declined to 10% over a period of 9 h (Fig. 2). When cells were incubated in medium CM, which contained 2% BSA, 90% of the cell population showed forward motility initially, which declined to 5% over a period of 10 h (Fig. 2). When BSA was added to NTPC (NTPCA), a marked reduction in loss of motility was observed (Fig. 2), consistent with the effect of BSA on rabbit sperm in this respect (Alvarez and Storey, 1983b). BSA, however, had little effect when added to TNC to give TNCA.

The linear correlation between malonaldehyde production and cessation of motility remained valid for all the media tested, despite the difference in time required for the sperm to become inert in the different media (Fig. 3). The figure of 0.8 nmol malonaldehyde/10^8 cells was found for this correlation, which was the same as that obtained in NTPC. Rates of

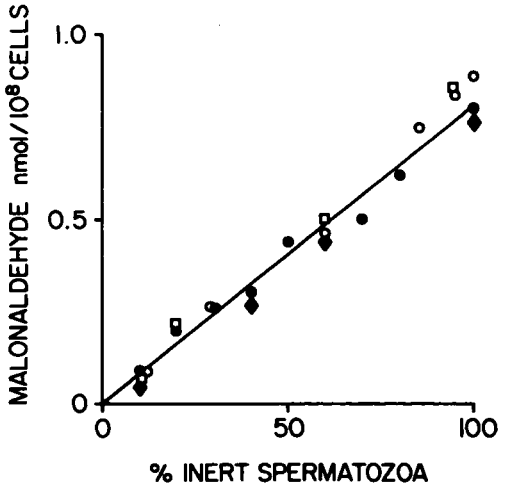

FIG. 3. Correlation between malonaldehyde production and percent inert spermatozoa in media NTPC (●--●), TNC (○--○), CM (◆--◆) and TNCPE (□--□) during aerobic incubation at 37°C. The experimental conditions were those given for Figs. 1 and 2. Each point represents the mean of 5 experiments. The linear regression equation calculated through the origin has the form $y=0.00815x$ ($r=0.985$).

malonaldehyde production in the six media tested are shown in Table 1. The maximum difference in these rates was nearly 20-fold (NTPCA compared to TNC), but the points obtained in all media fell on the linear correlation of Fig. 3. The loss of motility in mouse sperm depended on the extent of lipid peroxidation and was independent of the rate, just as was found with rabbit sperm (Alvarez and Storey, 1982).

As we have previously reported, high K^+ concentrations in the extracellular medium produced a rapid loss in motility which correlated with an increased rate of lipid peroxidation compared to the loss seen in media with high Na^+ (Alvarez and Storey, 1982); this was not due to a specific effect of Na^+ since choline could be substituted for Na^+ (Storey and Alvarez, 1983b). When mouse epididymal spermatozoa were exposed to progressively higher K^+ concentrations in the extracellular medium, the result was a steep decrease of mouse sperm motility and increase in lipid peroxidation within the same time period. Mouse sperm require a low K^+ concentration of 2–5 mM for optimal maintenance of motility in high Na^+ media (Fig. 4). The dependence of motility of K^+ concentration in the medium, shown in Fig. 4, was very similar to that observed with rabbit sperm (Alvarez and Storey, 1982).

Intact mouse epididymal spermatozoa produced $O_2^{\cdot-}$ at a rate that was linear with time (data not shown). The identity of $O_2^{\cdot-}$ as the reductant of acetylated ferricytochrome *c* was confirmed by adding exogenous SOD which completely abolished the reduction. The assayed rate of $O_2^{\cdot-}$ production did not increase linearly with cell concentration in intact epididymal mouse spermatozoa. Instead, the concentration dependence was hyperbolic, as was previously observed with rabbit epididymal spermatozoa (Holland et al., 1982). Analysis of this concentration dependence by means of a double reciprocal plot (Fig. 5a) yielded the intrinsic rate of $O_2^{\cdot-}$ production, v_{int}, and the rate constant K_s for the reaction of $O_2^{\cdot-}$ with the cells. From the slope of the double reciprocal plot (Fig. 5a), v_{int} was calculated to be 1.96 nmol/min per 10^8 cells for mouse spermatozoa. The intercept of the plot yielded a K_s value of 19.1×10^{-8} (cells/ml)$^{-1}$ min^{-1}. In the presence of 10 mM cyanide, a concentration inhibitory to rabbit sperm Cu-Zn SOD activity (Holland et al., 1982), the rate of production of $O_2^{\cdot-}$ in mouse epididymal spermatozoa increased, while retaining its hyperbolic dependence on cell concentration. The double reciprocal plot obtained in the presence of cyanide (not shown) yielded values of 3.80 nmol/min per 10^8 cells and 19.7×10^{-8} (cells/ml^{-1}) min^{-1} for v_{int} and K_s, respectively. The increase in v_{int} observed in the presence of cyanide should correspond to the loss of

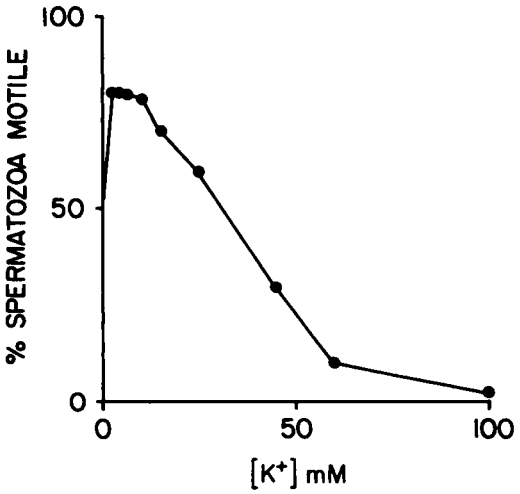

FIG. 4. Sperm motility after 3 h aerobic incubation time at 37°C as a function of K^+ concentration in media corresponding to NTPC, from 0 mM K^+, 130 mM Na^+ to 100 mM K^+, 30 mM Na^+. The experimental conditions were those described for Figs. 1, 2 and 3. Each point represents the mean of 2 experiments.

TABLE 1. Rate of lipid peroxidation as measured by malonaldehyde production in epididymal mouse spermatozoa under aerobic incubation at 37°C in various media.[a]

Medium[b]	Malonaldehyde production rate (nmol/h per 10^8 cells)
CM	0.0810 ± 0.020
NTPC	0.0390 ± 0.010
NTPCA	0.0156 ± 0.006
TNC	0.2800 ± 0.040
TNCA	0.2200 ± 0.030
TNCPE	0.0750 ± 0.015

[a] Malonaldehyde was determined by the thiobarbituric assay described in *Materials and Methods*. Values are the means ± SD of three determinations.

[b] For compositions see *Materials and Methods*. NTPCA and TNCA contained BSA at 10 mg/ml.

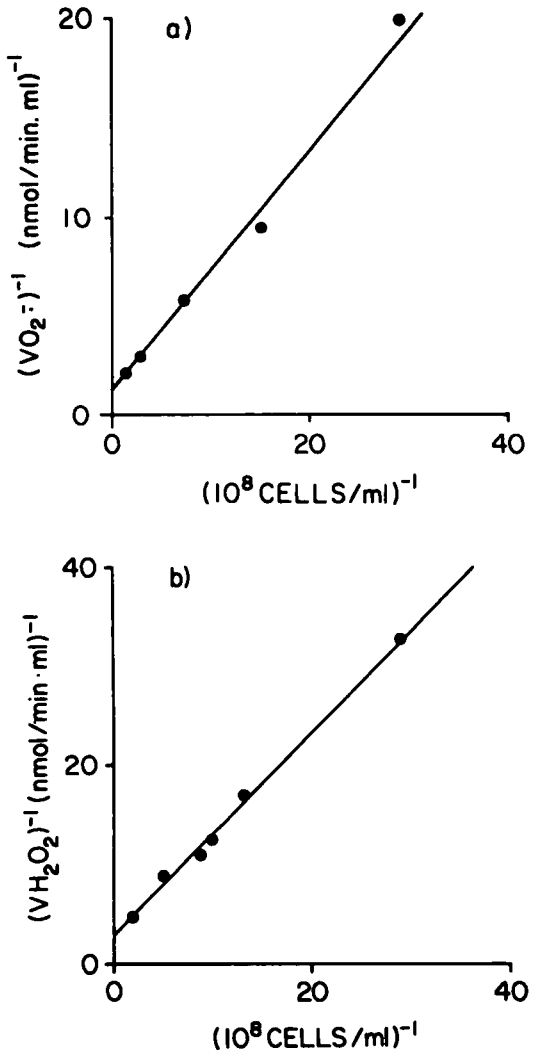

FIG. 5. Rate of $O_2^{\cdot-}$ and H_2O_2 production as a function of cell concentration by intact mouse epididymal spermatozoa presented in the form of a double reciprocal plot. *a*) For $O_2^{\cdot-}$, the assay was carried out in medium NTPC containing 80–90 μmol of acetylated ferricytochrome *c*. The linear regression equation calculated for the double reciprocal plot has the form y=0.51x + 1.4 (*r*=0.994). *b*) For H_2O_2, the assay was carried out in medium NTPC containing 90–95 μM of acetylated ferrocytochrome *c* and 0.25 μM cytochrome *c* peroxidase. The linear regression equation calculated for the double reciprocal plot was the form y=1.05x + 2.5 (*r*=0.997).

activity of the Cu-Zn SOD in mouse sperm sensitive to cyanide inhibition. In order to test this directly, the cyanide-sensitive and cyanide-insensitive SOD activities were assayed by the indirect method of McCord and Fridovich (1969) as applied to rabbit sperm by Holland et al. (1982). The cyanide-sensitive superoxide dismutase activity observed was 3.2 U/10^8 cells, with a remaining 0.34 U/10^8 cells not sensitive to cyanide inhibition. The latter corresponds to the Mn-containing enzyme located in the mitochondria (Weisiger and Fridovich, 1973).

Mouse epididymal spermatozoa produced H_2O_2 at a rate that was linear with time. Exogenous catalase abolished H_2O_2 production, confirming the identity of H_2O_2 as the oxidant of acetylated ferrocytochrome *c* in the presence of cytochrome *c* peroxidase. As in the case of $O_2^{\cdot-}$ generation, the rate of H_2O_2 production had a hyperbolic dependence on cell concentration. This dependence would be expected if the cells were to react with H_2O_2 in a second order reaction at a rate significant compared to the peroxidase-catalyzed reaction of H_2O_2 with the acetylated ferrocytochrome *c* used to assay H_2O_2 production, as was found to be the case with $O_2^{\cdot-}$ and acetylated ferricytochrome *c* (Holland et al., 1982). Analysis of this dependence by means of the double-reciprocal plot relation derived for the data obtained with $O_2^{\cdot-}$ (Holland et al., 1982) yielded the plot shown in Fig. 5b. From the slope of the plot (Fig. 5b), the v_{int} calculated for H_2O_2 production was 0.95 nmol/min per 10^8 cells. The intercept of the slope yielded a K_S value of 12.1×10^{-8} $(\text{cells/ml})^{-1}$ min^{-1}. In the presence of aminotriazole at concentrations inhibitory to catalase (Margoliash et al., 1960), the rate of production of H_2O_2 in intact spermatozoa as measured by the oxidation of acetylated ferrocytochrome *c* did not show any appreciable increase, suggesting that these cells lack intracellular catalase activity, as do other mammalian spermatozoa (Mann, 1964).

The glutathione peroxidase and glutathione reductase activities of mouse spermatozoa are given in Table 2. The peroxidase activity given is the maximal one assayed with cumene hydroperoxide. Also given in Table 2 are the contents of glutathione and glutathione disulfide. Glutathione peroxidase is quite specific for glutathione and the reductase is equally specific for the glutathione disulfide (Chance et al., 1979). The use of these two enzymes in determining the total glutathione, GSH plus GSSG, of the cell should give the content free of interferences intrinsic to the chemical methods (Li, 1975).

TABLE 2. Activities of glutathione reductase, glutathione peroxidase and glutathione content of epididymal mouse sperm treated with filipin (FTMES).[a]

Enzyme	Enzyme activity (μmol/min per 10^8 cells)
Glutathione reductase	
NADPH	0.120 ± 0.090 (4)
NADH	0.047 ± 0.004 (3)
Glutathione peroxidase	0.200 ± 0.100 (3)
Glutathione	**Content (nmol/10^8 cells)**
Total glutathione	96.0 ± 7.2 (3)
Glutathione disulfide as GSH[b]	37.0 ± 2.6 (3)
Glutathione	59.0[c]

[a]Values are the mean of the number of experiments shown in parenthesis ± SD.

[b]Disulfide content given as GSH equivalents which is twice the molar GSSG content.

[c]Calculated by difference.

DISCUSSION

Mouse and rabbit spermatozoa share one trait in common: both are susceptible to spontaneous lipid peroxidation and the degree of peroxidation gives a linear correlation with motility loss. Spermatozoa from the two species also lack catalase activity, have SOD activity, and produce both H_2O_2 and $O_2^{\cdot-}$ in the medium. The H_2O_2 produced by the sperm can be attributed entirely to the action of SOD: v_{int} for H_2O_2 production by mouse sperm was 0.95 nmol/min per 10^8 cells, which would correspond to a v_{int} for $O_2^{\cdot-}$ of 1.90 nmol/min per 10^8 cells in the absence of SOD activity. This is in good agreement with the calculated difference between v_{int} for $O_2^{\cdot-}$ of 3.80 nmol/min per 10^8 cells in the presence of cyanide and 1.96 nmol/min per 10^8 cells in its absence: 1.84 nmol/min per 10^8 cells. The same source of H_2O_2 was identified in rabbit sperm (Holland et al., 1982). But a marked quantitative difference was found between the rates of sperm $O_2^{\cdot-}$ and H_2O_2 production in the two species: mouse sperm are far more active in this regard. Since $O_2^{\cdot-}$ is the source of H_2O_2, it would be useful to compare the rates of $O_2^{\cdot-}$ production, expressed as v_{int}, as done in Table 3. In the absence of cyanide, the value

TABLE 3. Comparison of values of v_{int} for production of $O_2^{\cdot-}$ and of K_S for reaction of $O_2^{\cdot-}$ with the cells for rabbit and mouse spermatozoa in the presence and absence of cyanide.[a]

	(CN^-)	v_{int} (nmol/min per 10^8 cells)	K_S (cells/ml)$^{-1}$ min^{-1}
Rabbit	0	0.17	1.2×10^{-8}
(NTP)	10 mM	1.61	19.9×10^{-8}
Mouse	0	1.96	19.1×10^{-8}
(NTPC)	10 mM	3.80	19.7×10^{-8}

[a]Values for rabbit spermatozoa are from Alvarez and Storey (1983a). The medium is shown in parentheses for each species. For rabbit, the difference between v_{int} in the presence and absence of CN^- is 1.44 nmol/min per 10^8 cells, corresponding to 0.72 nmol/per 10^8 cells for H_2O_2 production, in agreement with the value of 0.7–0.8 found (Holland and Storey, 1981). For mouse, the difference is 1.84 nmol/min per 10^8 cells, corresponding to 0.92 nmol/min per 10^8 cells for H_2O_2 production also in agreement with the value of 0.95 found (this paper).

of v_{int} for mouse sperm is 10-fold that for rabbit sperm; in the presence of cyanide, the values of v_{int}, which are nearly maximal, still differ by more than 2-fold. In rabbit sperm, the SOD activity and intrinsic $O_2^{\cdot-}$ production rate are quite closely matched. But in mouse sperm, the intrinsic $O_2^{\cdot-}$ production rate runs far ahead of the cells SOD capacity to dismutate it.

Comparison of the dismutation capacity in terms of enzyme units (McCord and Fridovich, 1969) of the cyanide-sensitive SOD activity yields similar values for the sperm from the two species. Rabbit sperm were found to have 2.7 $U/10^8$ cells of this activity (Holland and Storey, 1981), from which one calculates 1 $U/10^8$ cells=0.53 nmol $O_2^{\cdot-}$ (min per 10^8 cells). For mouse sperm, the corresponding figure is 3.2 $U/10^8$ cells, from which one calculates 1 $U/10^8$ cells=0.58 nmol $O_2^{\cdot-}$/min per 10^8 cells. Mouse sperm have 0.34 $U/10^8$ cells of cyanide-insensitive SOD activity, compared to 0.14 $U/10^8$ cells for rabbit sperm; this amount of activity difference would not seem to have much physiological impact.

The values of K_S, the second order rate constant for reaction of $O_2^{\cdot-}$ with the cells, are compared in Table 3. A clear difference between the two species is that the K_S value for mouse sperm is the same in the absence as in the presence of cyanide, while K_S is 16-fold lower in rabbit sperm in the absence of cyanide. We have interpreted the difference in K_S values found with rabbit sperm as reflecting the reactions of $O_2^{\cdot-}$ with the plasma membrane alone in the absence of cyanide, and the reaction of $O_2^{\cdot-}$ with the plasma plus intracellular components in its presence when 95% of the intracellular SOD is inhibited (Alvarez and Storey, 1983a). The near identity of K_S for rabbit sperm in the presence of cyanide and of K_S for mouse sperm (Table 3), independent of cyanide, suggests that the reaction of $O_2^{\cdot-}$ with mouse sperm occurs both with plasma membrane and intracellular components, with little interference from intracellular SOD. This would be observed if the SOD in mouse sperm were already saturated by internally generated $O_2^{\cdot-}$. The observation that rabbit and mouse sperm have about the same intracellular SOD activity means that the considerably larger mouse sperm have less protective enzyme relative to their size than do rabbit sperm. We conclude that, while SOD provides some protection to mouse spermatozoa against lipid peroxidation induced by SO, its capacity is overwhelmed by the high intrinsic rate of SO production and so this enzyme plays a minor role as an enzyme protecting against oxidative damage.

The major protective enzyme in mouse sperm appears to be the glutathione/glutathione reductase/glutathione peroxidase system which functions in most somatic cells (Chance et al., 1979). It is in this system that sperm from rabbit and mouse show the most striking difference. Rabbit sperm have little or no detectable glutathione (Li, 1975; Holland and Storey, 1981) so that, even though they have low levels of the appropriate enzymatic activities, they still lack the substrate to use them. Mouse sperm have GSH plus GSSG contents much higher than those reported for other mammalian sperm which contain glutathione. Li (1975) reported contents of glutathione, as measured by the reductase reaction alone, ranging from 0.5 to 1.3 nmol/10^8 cells for sperm from dog, ram and man. The reductase reaction measures the content of glutathione disulfide; comparison with Table 2 shows that the mouse spermatozoa have a glutathione disulfide content more than an order of magnitude greater than that observed in sperm from these other mammalian species. The activities of the reductase and the peroxidase are also an order of magnitude or more greater than those observed in the sperm of the other species (Li, 1975; Holland and Storey, 1981). It seems likely that mouse sperm depend on the glutathione system for major protection against oxidative damage. This suggests that there may be a wide variation in the contributions of the glutathione and SOD protective systems in sperm from different species. To the best of our knowledge, a systematic examination of this variation has not yet been carried out.

Mouse sperm produce H_2O_2 and also react with H_2O_2, as shown by the hyperbolic dependence of measured H_2O_2 production on sperm cell concentration (Fig. 5b). This dependence implies that the reaction between H_2O_2 and the cells is second order, so that the same form of the equation yielding the double reciprocal plot (Holland et al., 1982) in Fig. 5a for $O_2^{\cdot-}$ could be used for H_2O_2 in Fig. 5b. The situation found with rabbit sperm in regard to the apparent dependence of H_2O_2 production on sperm cell concentration is quite different. With these cells, the reaction of H_2O_2 with the cells seems to have saturation kinetics with regard to H_2O_2, becoming zero order with respect to H_2O_2 and first order with respect to cell

concentration above 0.2 μM H_2O_2 and 2×10^7 cells/ml (Holland and Storey, 1981). It is possible that these observed differences with regard to cell reaction with H_2O_2 reflect the difference in sensitivity to H_2O_2-induced cell damage in sperm from rabbit and mouse (Wales et al., 1959), but this was not assessed. Further characterization of the reaction of H_2O_2 with spermatozoa of the two species was deemed beyond the scope of this study; it is currently under investigation in our laboratory.

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