Oxygen metabolism of mammalian spermatozoa

Generation of hydrogen peroxide by rabbit epididymal spermatozoa

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Rabbit spermatozoa from the cauda epididymis produced 0.7–0.8 nmol of H_2O_2 /min per 10^8 cells at cell concentrations below 10^7 cells/ml with linear dependence on cell concentration. Above 2×10^7 cells/ml, the rate again became linear with cell concentration but decreased to 0.1–0.2 nmol/min per 10^8 cells. Spermatozoa treated with amphotericin B, which makes the plasma membrane highly permeable to low-molecular-weight compounds, showed a similar dependence of H_2O_2 production rate on cell concentration; below 10^7 cells/ml the rate was 0.3–0.4 nmol/min per 10^8 cells; above 2×10^7 cells/ml, the rate was 0.1–0.2 nmol/min per 10^8 cells. Hypo-osmotically treated rabbit epididymal spermatozoa, a preparation useful for studying mitochondrial function in sperm [Keyhani & Storey (1973) *Biochim. Biophys. Acta* **305**, 557–565] produced 0.1–0.2 nmol/min per 10^8 cells in the absence of added substrates. The dependence of rate on cell concentration was linear from 10^7 to 2.2×10^8 cells/ml. This endogenous rate was unaffected by rotenone, but stimulated 4-fold by antimycin A. Addition of the mitochondrial substrates lactate plus malate increased the rate of H_2O_2 production to 0.3 nmol/min per 10^8 cells. The decreased rate of H_2O_2 production observed with intact sperm at high cell concentrations is attributed to reaction of H_2O_2 with the cells, possibly with the plasma membrane, which is lost after hypo-osmotic treatment. Rabbit spermatozoa have glutathione peroxidase and glutathione reductase activities, but these seem to play little role in removal of H_2O_2 generated. The rate at low cell concentration is taken to be the unperturbed rate. The sources of H_2O_2 production in rabbit spermatozoa have been tentatively resolved into a low-molecular-weight component, lost after amphotericin treatment, a mitochondrial component and a rotenone-insensitive component that has not been identified.

Whereas intracellular O_2 consumption not connected to mitochondrial cytochrome oxidase has been known for many years, the physiological significance of partially reduced intermediates of O_2 , namely $\text{O}_2^{\cdot -}$ and H_2O_2 , has become clear only during the last decade (Chance *et al.*, 1979). The intracellular sources of H_2O_2 formation include the mitochondria, the endoplasmic reticulum, peroxisomes and soluble enzymes. As H_2O_2 is toxic to cell constituents, those cells producing H_2O_2 are faced with the problem of removing it rapidly and safely. This may be accomplished through the action of intracellular catalase in liver and kidney or, in tissues that lack intracellular catalase such as brain, lung and heart, by exporting H_2O_2 into the bloodstream

where erythrocyte catalase completes the task. Another system that protects cells against H_2O_2 is the glutathione peroxidase/glutathione reductase system. Since glutathione peroxidase has higher activity with organic hydroperoxides, this system may be more functional in removing potentially harmful lipid hydroperoxides rather than H_2O_2 (Chance *et al.*, 1979).

Although H_2O_2 production in most mammalian organs and tissues has been examined in some detail (Chance *et al.*, 1979), this reaction in sperm cells has been almost totally ignored since the early work of McLeod (1943) and Tosic & Walton (1945). These reports suggest that H_2O_2 produced as a consequence of spermatozoal metabolism of components of their seminal plasma is involved in limiting the viability of sperm *in vitro* (McLeod,

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1943; Tosic & Walton, 1946). These studies were performed with whole or diluted semen incubated for prolonged periods and relied on a colorimetric assay that was not very sensitive nor completely specific for H_2O_2 (Tosic & Walton, 1945, 1946). Availability of a new, highly specific and sensitive assay for H_2O_2 utilizing the enzyme cytochrome *c* peroxidase (EC 1.11.1.5) (Boveris *et al.*, 1972) prompted us to re-investigate H_2O_2 production in spermatozoa as part of a long-term study of the factors controlling viability of spermatozoa *in vitro*. Initial questions that should be answered include the following. (a) Do mammalian spermatozoa produce H_2O_2 ? (b) Do they dispose of H_2O_2 produced endogenously or exogenously? (c) Where in the cell is H_2O_2 generated? (d) What factors modulate this generation? It is to those questions that the present study is addressed, using rabbit spermatozoa as the model.

Materials and methods

Reagents

Yeast cytochrome *c* peroxidase, prepared by the method of Yonetani & Ray (1965), was the gift of Professor T. Yonetani, Johnson Research Foundation, University of Pennsylvania. Glucose oxidase from *Aspergillus niger* (type II), catalase (type C40) from bovine liver, glutathione reductase (type IV) from yeast, oxidized and reduced glutathione, cytochrome *c* (type VI) from horse heart, D- and L-alanine, aminotriazole and amphotericin B were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Cumene hydroperoxide (73%) was purchased from Polysciences Inc., Warrington, PA, U.S.A. All other chemicals were of analytical reagent quality and purchased from a variety of sources.

Preparation of spermatozoa

Spermatozoa were obtained from excised caudae epididymides of New Zealand White rabbits by reverse flushing of the vasa deferentia with approx. 10 ml of buffer (113 mM-KCl/15 mM-potassium phosphate/3 mM-MgCl₂/0.4 mM-EDTA/20 mM-Tris/HCl, pH 7.4) and allowing the spermatozoa to exude from a nick in the base of the cauda epididymis directly into tubes held in ice. The intact spermatozoa were then sedimented at 750 *g* for 10 min at 4°C. The supernatant was discarded and the pellet gently resuspended in 10 ml of buffer and the washing process was repeated twice more. The

spermatozoa were then resuspended in a minimal quantity of buffer. Triplicate portions of 5 μl were removed, diluted to 1 ml with water, and the number of spermatozoa was estimated by haemocytometer. The concentration of spermatozoa was adjusted to vary in the range $(1.0 \pm 0.2) \times 10^9$ spermatozoa/ml.

Spermatozoa with plasma membranes made permeable were prepared by treatment with the polyene antibiotic amphotericin. This antibiotic has been shown to remove sterols from physiological (Bittman, 1978) and model lipid membranes (Van Hoogevest & De Kruijff, 1978), thus making the membranes freely permeable to low-molecular-weight compounds, but not to higher-molecular-weight substances. Intact spermatozoa washed and suspended at $(1.0 \pm 0.2) \times 10^9$ spermatozoa/ml were exposed to 200 μM -amphotericin for 15 min on ice. They were then used directly in experiments with no attempt being made to remove any excess amphotericin to avoid further damage to the plasma membrane. Spermatozoa with the plasma membrane removed were prepared from intact spermatozoa using the hypo-osmotic treatment of Keyhani & Storey (1973). The suspending medium for the different preparations of rabbit spermatozoa was the Tris/phosphate described above.

Determination of O_2 uptake

O_2 consumption by the various spermatozoal preparations was measured using the miniaturized polarographic oxygen electrode assembly described previously (Storey *et al.*, 1978) with a sample volume of 0.13 ml. The concentration of spermatozoa was in the range $(0.5\text{--}2.0) \times 10^9$ cells/ml for these determinations, and the medium used was the Tris/phosphate buffer described by Keyhani & Storey (1973). Only suspensions of spermatozoa in which the oxidation of substrate was tightly coupled to generation of ATP and which responded to oligomycin and uncoupler of oxidative phosphorylation were used in these studies. The intactness of spermatozoa was verified by the inability of exogenous ADP at 0.5 mM to stimulate O_2 uptake by more than 10% in the presence of lactate plus malate.

Procedure for determination of rate of H_2O_2 generation

The formation of H_2O_2 can be assayed with cytochrome *c* peroxidase directly, as in reaction (1), or by catalytic oxidation of ferrocytochrome *c* by H_2O_2 as in the sum of reactions (1) and (2):

Boveris *et al.* (1972) have previously monitored directly the appearance of the H₂O₂·enzyme complex to estimate H₂O₂ generation, but because of interference at the wavelength pair used (419/407nm) owing to light scattering from the highly anisotropic spermatozoal suspensions, this approach could not be extended to spermatozoa. Accordingly, the enzyme was used in catalytic quantities. Preliminary experiments showed that, after addition of spermatozoa, there was an appreciable rate of endogenous oxidation of the ferrocytochrome *c* before addition of enzyme. This decreased the sensitivity of the assay due to baseline drift. Acetylation of ferrocytochrome *c* by the method of Azzi *et al.* (1975), followed by reduction with dithionite, and purification on a Sephadex G-25 column in a Pasteur pipette, provided a derivative of ferrocytochrome *c* that remained a substrate for cytochrome *c* peroxidase, yet was not oxidized by spermatozoa in the absence of this enzyme. The concentration of spermatozoa used in this assay was too low for any significant optical interference from changes in the redox state of the spermatozoal cytochromes. The optimum concentration of acetylated ferrocytochrome *c* for this assay was found to be in the range 80–120 μM ; that for cytochrome *c* peroxidase was in the range 0.2–0.3 μM . The concentrations routinely used were approximately 100 μM for the acetylated ferrocytochrome *c* and 0.23 μM for the peroxidase, unless otherwise indicated. The oxidation of acetylated ferrocytochrome *c* was measured at room temperature (21–23°C) with a DW-2A dual-wavelength spectrophotometer (American Instrument Co.) using the wavelength pair 550/540nm and the difference absorption coefficient $\Delta\epsilon = 19.1 \text{ mm}^{-1} \cdot \text{cm}^{-1}$. When H₂O₂ was generated by the glucose/glucose oxidase couple, the assay gave a linear relationship between the amount of glucose oxidase added in the range 0.08–0.8 $\mu\text{g/ml}$ and H₂O₂ production in the range 0.1–1.5 $\mu\text{M/min}$, in excellent agreement with the results of Boveris *et al.* (1972), who used formation of the peroxidase·H₂O₂ complex to assay H₂O₂. At lower glucose oxidase concentrations, the curve flattened out, whereas beyond the linear range the rate of H₂O₂ generation was high enough that non-enzymic oxidation of the acetylated ferrocytochrome *c* occurred. Spermatozoa employed in these studies were maintained at concentrations such that H₂O₂ production fell as close as possible to the middle of the linear range of the assay. The specificity of oxidation of acetylated ferrocytochrome *c* was assessed by addition of catalase to the assay mixture either before or subsequent to the initiation of H₂O₂ production through addition of glucose oxidase. With 0.23 μM -cytochrome *c* peroxidase, 2 μM -catalase decreased the apparent rate of H₂O₂ production by 85% and 10 μM eliminated it

entirely. These results are in good agreement with those of Boveris *et al.* (1972), who calculated that a catalase/cytochrome *c* peroxidase ratio of 5:1 halved the detectable rate of H₂O₂ rate production using the cytochrome *c* peroxidase·H₂O₂ complex assay.

Glutathione peroxidase and glutathione reductase

Glutathione peroxidase and glutathione reductase activities were determined on three preparations: suspensions of intact sperm that had been sonicated; hypo-osmotically-treated sperm; and the supernatant obtained from the hypo-osmotic treatment. Intact sperm were sonicated with an Artek Sonic Dismembrator (model 150) with a titanium microtip as described by Storey & Kayne (1977) for a total of 3 min in 1 min intervals separated by a cooling period of 1 min. The concentration of spermatozoa before sonication was taken as the cell concentration of the sonicated suspension. The sonicated suspension was used without further treatment: this preparation was designated whole sperm, sonicated. Glutathione peroxidase was assayed by the coupled enzyme method utilizing excess glutathione reductase that couples oxidation of NADPH to the rate of reaction of the peroxidase with the hydroperoxide and reduced glutathione (Paglia & Valentine, 1967). The reaction conditions used were those described for assay of this enzyme in bovine semen by Smith *et al.* (1979), using cumene hydroperoxide to elicit maximal activity of the enzyme (Lawrence & Burk, 1976). Cumene hydroperoxide was used at 0.48 mM; H₂O₂ as substrate was used at 0.12 mM (Smith *et al.*, 1979). The oxidation of NAPH was monitored by the change $A_{340-375}$, using the DW-2A dual-wavelength spectrophotometer. Glutathione reductase was assayed in the same system using oxidized glutathione as substrate. The activities of the two enzymes were linear with cell concentration over the range $(0.5-3.0) \times 10^8$ cells/ml.

Results

Production of H₂O₂ by spermatozoa

Both intact (Fig. 1a) and hypo-osmotically-treated spermatozoa (Fig. 1c) produce H₂O₂ at a rate that is linear with time. This linearity is maintained for at least 25 min (results not shown). Catalase added before addition of cytochrome *c* peroxidase (Figs. 1b and 1d) decreased the detectable rate of H₂O₂ production and eliminated the initial rapid change in absorbance seen on addition of cytochrome *c* peroxidase; the rapid change reflects reaction with a preformed pool of H₂O₂ generated by the spermatozoa.

The rate of H₂O₂ production from hypo-osmotically-treated spermatozoa was linear with cell

Fig. 1. Production by H_2O_2 by intact and hypo-osmotically-treated rabbit epididymal spermatozoa and its sensitivity to added catalase

The reaction medium contained, in a final volume of 0.5 ml, 113 mM-KCl, 15 mM-potassium phosphate, 3 mM-MgCl₂, 0.4 mM-EDTA, 20 mM-Tris/HCl, pH 7.4. For traces (a) and (b), the reaction mixture also contained 98 μ M-acetylated ferrocytochrome c and intact spermatozoa at 0.60×10^8 cells/ml. Shown are the spectrophotometric traces obtained with the wavelength pair 540/550 nm; upward deflection of the trace corresponds to oxidation of acetylated ferrocytochrome c. Catalase (2 μ M-haem) and cytochrome c peroxidase (0.24 μ M) were added at the points indicated on the traces. In trace (d), the H_2O_2 production rate decreases from 0.113 nmol/min per 10^8 cells to 0.031 nmol/min per 10^8 cells after catalase addition. In trace (b), prior addition of catalase results in a rate of 0.061 nmol/min per 10^8 cells. In trace (c), the two rates are 0.114 and 0.047 nmol/min per 10^8 cells respectively. In trace (d), the rate is 0.065 nmol/min per 10^8 cells.

concentration over the range tested (Fig. 2c) at 0.15 nmol/min per 10^8 cells. The rate at high cell concentrations was markedly lower with intact (Fig. 2a) and amphotericin-treated (Fig. 2b) spermatozoa than at low cell concentrations. Below about 10^7 cells/ml, the rate was 0.76 nmol/min per 10^8 cells for intact spermatozoa (Fig. 2a, inset) and 0.35 nmol/

Fig. 2. Rate of H_2O_2 production as a function of cell concentration

The reaction mixture was that described in the legend of Fig. 1. The concentration of acetylated ferrocytochrome c was 92–102 μ M. Regression equations were calculated for intact spermatozoa (a) as $y = 0.76x + 0.019$ for the concentration range $(0.01-0.1) \times 10^8$ cells/ml ($r = 0.998$) and as $y = 0.12x + 0.086$ ($r = 0.986$) in the range $(0.2-2.5) \times 10^8$ cells/ml. For amphotericin (b), the equations were: $y = 0.35x + 0.012$ ($r = 0.988$) in the range $(0.01-0.2) \times 10^8$ cells/ml, and $y = 0.13x + 0.058$ ($r = 0.996$) in the range $(0.2-2.4) \times 10^8$ cells/ml. For hypo-osmotically-treated spermatozoa (c), one regression equation was calculated for the range $(0.1-2.2) \times 10^8$ cells/ml: $y = 0.15x + 0.008$ ($r = 0.982$). At the very-low cell concentrations, the rate of H_2O_2 production is sufficiently low that it nears the sensitivity limit of the assay. The calculated intersection of the regression lines at these low concentrations (a, inset; b, inset, and c) indicates the sensitivity limit of the assay.

min per 10⁸ cells for amphotericin-treated spermatozoa (Fig. 2b, inset) and was linear with increasing cell concentration. Above 2×10^7 cells/ml, the rate again became linear in cell concentration with both preparations, but decreased in magnitude to 0.12 nmol/min per 10⁸ cells for intact and to 0.13 nmol/min per 10⁸ cells for amphotericin-treated spermatozoa. This result implies that H₂O₂ produced and exported by intact and amphotericin-treated rabbit spermatozoa can also be consumed by spermatozoa as the concentration of cells in suspension increases. To check this implication, intact and amphotericin-treated sperm were incubated with the glucose/glucose oxidase H₂O₂-generating system (Table 1). In the two experiments, both types of spermatozoa inhibited H₂O₂ detection in a manner dependent on spermatozoal concentration. Exogenous catalase caused a complete inhibition of H₂O₂ detection in the presence of

intact spermatozoa and an 83–85% inhibition with amphotericin-treated spermatozoa. These results indicate that the H₂O₂ made by intact spermatozoa is first exported to the suspending medium and then reacts with some component of the sperm cell. One likely candidate for this component is the plasma membrane, since hypo-osmotically-treated spermatozoa, from which the plasma membrane has been largely removed, do not appear to react with exogenous H₂O₂.

Two enzyme systems that are the major means of intracellular hydroperoxide removal are catalase and glutathione peroxidase/glutathione reductase (Chance *et al.*, 1979). The presence of catalase activity was examined by means of the effect of aminotriazole, a specific inhibitor of catalase (Margoliash *et al.*, 1960), on the production of H₂O₂ by spermatozoa. No effect of aminotriazole on H₂O₂ production by either intact or amphotericin-treated spermatozoa was seen at concentrations up to 400 μ M, a concentration that should be sufficient to diminish markedly the activity of micromolar concentrations of catalase in the presence of the micromolar concentrations of H₂O₂ that accumulate in the absence of cytochrome *c* peroxidase (Figs. 1a and 1c) (Margoliash *et al.*, 1960). Rabbit spermatozoa apparently do not contain significant quantities of catalase, in agreement with the reported absence of catalase in other mammalian spermatozoa (Mann, 1964). The glutathione peroxidase and glutathione reductase activities are given in Table 2. The peroxidase activity is maximal, having been assayed with cumene hydroperoxide as a substrate; the activity measured with H₂O₂ was 22% that obtained with the organic hydroperoxide. Although these activities are comparable with those of H₂O₂ production from the two types of sperm preparation, it is doubtful that they contribute significantly to H₂O₂ removal. The production rate of H₂O₂ from amphotericin-treated cells, which would have lost any endogenous glutathione and NADPH through the membrane made permeable, is lower than that of the intact cells. Attempts to assay endogenous glutathione in these cells were unsuccessful at a sensitivity of 0.1 nmol/10⁸ cells.

Source of production of H₂O₂ by spermatozoa

Perturbation of the electron-transport and energy-conservation capabilities of mitochondria from a number of tissues has been shown to alter H₂O₂ production; these organelles are known to be an intracellular source of H₂O₂ (Boveris & Chance, 1973). Such perturbations were examined in hypo-osmotically-treated spermatozoa, which are particularly useful for examining mitochondrial reactions in spermatozoa (Keyhani & Storey, 1973). Rotenone, at concentrations up to 3-fold above those that maximally decreased O₂ consumption by

Table 1. Recovery of H₂O₂ generated by glucose/glucose oxidase in the presence of intact and amphotericin-treated spermatozoa

		H ₂ O ₂ production (nmol/min)
The reaction mixture contained, in a final volume of 0.5 ml, 113 mM-KCl, 15 mM-potassium phosphate, 3 mM-MgCl ₂ , 0.4 mM-EDTA and 20 mM-Tris/HCl, pH 7.4. The concentration of acetylated ferrocyanochrome <i>c</i> was 102 μ M in Expt. 1 and 112 μ M in Expt. 2. The assay was initiated by addition of 0.23 μ M-cytochrome <i>c</i> peroxidase.		
Expt. 1	Additions	
Intact sperm	Glucose oxidase (0.25 μ g/ml)	0.495
	+ 2.1×10^7 sperm	0.432
	+ 10.7×10^7 sperm	0.322
	+ Aminotriazole (0.5 mM)	0.353
	+ Catalase (20 μ M)	0
Amphotericin-treated sperm	Glucose oxidase (0.25 μ g/ml)	0.550
	+ 2.5×10^7 sperm	0.456
	+ 12.5×10^7 sperm	0.346
	+ Aminotriazole (0.5 mM)	0.377
	+ Catalase (20 μ M)	0.079
Expt. 2		
Intact sperm	Glucose oxidase (0.25 μ g/ml)	0.562
	+ 2.0×10^7 sperm	0.446
	+ 14.7×10^7 sperm	0.271
	+ Aminotriazole (0.5 mM)	0.298
	+ Catalase (20 μ M)	0
Amphotericin-treated sperm	Glucose oxidase (0.25 μ g/ml)	0.475
	+ 3.2×10^7 sperm	0.428
	+ 12.6×10^7 sperm	0.286
	+ Aminotriazole (0.5 mM)	0.310
	+ Catalase (20 μ M)	0.090

Table 2. *Activities of glutathione peroxidase and glutathione reductase in rabbit epididymal spermatozoa*

For assay of peroxidase activity, the reaction mixture contained, in a final volume of 0.5 ml, 113 mM-KCl, 15 mM-potassium phosphate, 3 mM-MgCl₂, 0.4 mM-EDTA, 0.2 mM-NADPH, 1 mM-reduced glutathione, glutathione reductase (1 unit/ml) and 20 mM-Tris/HCl, pH 7.4. Effective sperm concentrations were between 1×10^8 and 3×10^8 cells/ml. For assay of reductase activity, the reduced glutathione and glutathione reductase were omitted. The peroxidase assay was initiated by addition of 0.48 mM-cumene hydroperoxide, the reductase activity by addition of 0.5 mM-oxidized glutathione. Further details of procedure are given in the Materials and methods section. Values are means $\pm$ S.D. for four determinations.

	NADPH (nmol/min per 10^8 cells)
Glutathione peroxidase	
Whole sperm, sonicated	4.88 ± 0.61
Hypo-osmotically-treated spermatozoa	3.53 ± 0.29
Supernatant from hypo-osmotically-treated spermatozoa	1.59 ± 0.29
Glutathione reductase	
Whole sperm, sonicated	0.81 ± 0.20
Hypo-osmotically-treated spermatozoa	0.56 ± 0.12
Supernatant from hypo-osmotically-treated spermatozoa	0.23 ± 0.09

spermatozoa metabolizing endogenous substrate, had no significant effect on H₂O₂ production by hypo-osmotically-treated spermatozoa. Under these conditions, the O₂ uptake and H₂O₂ production rates were nearly matched. Antimycin A, however, stimulated H₂O₂ production by hypo-osmotically-treated spermatozoa 3.6-fold. Smaller stimulations were found with both intact (2.4-fold) and amphotericin-treated spermatozoa (1.6-fold). Catalase added either before (Fig. 1) or subsequent to (Table 3) antimycin A stimulation decreased the ability of the assay to detect H₂O₂. Catalase was most effective with intact spermatozoa and least effective with amphotericin-treated spermatozoa. The finding with intact cells is expected, since the H₂O₂ is exported and neither enzyme has access to an intracellular compartment. The finding with amphotericin-treated spermatozoa is surprising, since it implies that the peroxidase has access to a compartment that produces H₂O₂ from which catalase is excluded. Such differential accessibility is in line with the relative sizes of the two enzymes, catalase (mol.wt. 240000) being a much larger enzyme than cytochrome c peroxidase (mol.wt. 32000). Differences in affinity of the respective enzymes for H₂O₂ could not explain this effect (Boveris *et al.*, 1972).

Inhibition of oxidative phosphorylation by ad-

Table 3. *Effect of antimycin A on the rate of H₂O₂ production by intact, hypo-osmotically- and amphotericin-treated spermatozoa and its vulnerability to catalase*

The reaction mixture was as described in Table 1, except that the concentrations of acetylated ferrocytochrome c varied between 84 μ M and 107 μ M for amphotericin-treated spermatozoa. Values are means $\pm$ S.D. for the numbers of experiments shown in parentheses.

	H ₂ O ₂ production (nmol/min per 10^8 cells)
Intact spermatozoa	0.135 ± 0.008 (4)
+Antimycin A (4 μ g)	0.332 ± 0.028 (4)
+Catalase (10 μ M)	0.086 ± 0.016 (4)
Amphotericin A-treated spermatozoa	0.230 ± 0.040 (3)
+Antimycin A (4 μ g)	0.325 ± 0.022 (3)
+Catalase (10 μ M)	0.224 ± 0.029 (3)
Hypo-osmotically-treated spermatozoa	0.013 ± 0.022 (5)
+Antimycin A (4 μ g)	0.371 ± 0.033 (5)
+Catalase (10 μ M)	0.136 ± 0.022 (5)

dition of oligomycin had no effect on H₂O₂ production. Similarly, uncoupling of electron transport and phosphorylation through the use of the uncoupler 1,3-bis(hexafluoroacetyl)acetone, designated compound 1779 (Heytler, 1979), did not affect spermatozoal H₂O₂ production. Addition of antimycin A after uncoupler did not result in a stimulation in H₂O₂ production about that obtained with antimycin alone.

In addition to the mitochondria, Chance *et al.* (1979) list a variety of subcellular sources of H₂O₂, notably the enzymes of the endoplasmic reticulum, peroxisomes and cytoplasm. To check this, the activity of another potential source of H₂O₂, D-amino acid oxidase, an enzyme usually considered present in the peroxisome, was assessed in amphotericin- and hypo-osmotically-treated spermatozoa, using the D- and L-isomers of alanine. Both isomers of alanine cause an apparent increase in H₂O₂ production of less than 20% with both amphotericin- and hypo-osmotically-treated spermatozoa; the D-isomer was no more effective than the L-isomer.

Effect of mitochondrial substrates on H₂O₂ production

The effect of substrates on mitochondrial H₂O₂ production was examined in greater detail, using hypo-osmotically-treated spermatozoa in suspension. This is summarized in Table 4. Addition of ADP increased the production of H₂O₂ by 1.8-fold compared with that with endogenous substrate. Addition of ATP was without effect on H₂O₂ production. Antimycin A still caused a substantial

Table 4. *Effect of ATP and ADP and added substrates on H₂O₂ generation by hypo-osmotically-treated spermatozoa*

The reaction mixture was as described in Table 1. The concentration of hypo-osmotically-treated spermatozoa ranged from 0.63×10^8 – 0.97×10^8 cells/ml and the acetylated ferrocytochrome *c* concentration was between 95 μ M and 115 μ M. Values are means $\pm$ S.D. for the numbers of experiments shown in parentheses.

Additions	H ₂ O ₂ production (nmol/min per 10 ⁸ cells)
Endogenous	0.144 $\pm$ 0.027 (6)
+ADP (0.5 mM)	0.238 $\pm$ 0.043 (6)
+Antimycin A (4 μ g)	0.404 $\pm$ 0.064 (6)
Endogenous	0.163 $\pm$ 0.011 (3)
+ATP (0.5 mM)	0.176 $\pm$ 0.024 (3)
+Antimycin A (4 μ g)	0.354 $\pm$ 0.026 (3)
Endogenous	0.109 $\pm$ 0.008 (3)
+Lactate (3 mM)	0.156 $\pm$ 0.019 (3)
+Malate (2 mM)	0.232 $\pm$ 0.022 (3)
+ADP (0.2 mM)	0.245 $\pm$ 0.024 (3)
+Antimycin A (4 μ g)	0.384 $\pm$ 0.006 (3)
Endogenous	0.104 $\pm$ 0.014 (3)
+Succinate (2 mM)	0.141 $\pm$ 0.011 (3)
+ADP (0.2 mM)	0.182 $\pm$ 0.008 (3)
+Antimycin A (4 μ g)	0.361 $\pm$ 0.035 (3)

rise in H₂O₂ production added after either ADP or ATP. Spermatozoa metabolizing lactate and malate also show an increased rate of H₂O₂ production. This combination of substrates has previously been shown (Storey & Kayne, 1978) to result in maximal O₂ consumption and maximal reduction of the mitochondrial nicotinamide nucleotide pool. Addition of 0.5 mM-ADP after these substrates caused a further rise in H₂O₂ production, but the effect was small compared with that observed with spermatozoa metabolizing endogenous substrates. Addition of oligomycin followed by the uncoupler of oxidative phosphorylation, compound 1779, had no further effect on the rate of H₂O₂ production elicited by ADP addition, although these compounds had the expected effect on the rate of O₂ consumption by the spermatozoa (Storey, 1978). Although the energy state of the mitochondria appears to have little effect on the rate of H₂O₂ production, this rate does vary with available substrates, be they substrates for electron transport or for oxidative phosphorylation. The mitochondrial source of H₂O₂ production would therefore seem to be the one subject to modulation by factors such as energy status of the cell, as determined by the ATP and ADP concentrations, and substrate availability.

Discussion

The questions that the present study addressed itself to can be answered as follows. (a) Rabbit

spermatozoa produce and export H₂O₂ to the suspending medium. (b) Rabbit spermatozoa lack intracellular catalase, as do other mammalian spermatozoa (Mann, 1964). (c) Part of the H₂O₂ is of mitochondrial origin, and part comes from other cellular components. (d) The mitochondrial H₂O₂ production varies with substrate availability and appears to be the source of cellular H₂O₂ production subject to modulation, although the range of modulation is not substantial. Rabbit spermatozoa, which we assume are characteristic of other mammalian spermatozoa with regard to hydroperoxide metabolism, produce H₂O₂ as a result of their being in an environment containing O₂ rather than as part of their metabolic strategy (Storey, 1980).

The dependence of H₂O₂ production in a suspension of intact rabbit sperm on the concentration of cells was unexpected and provides evidence that these cells react with exogenous H₂O₂. Support for this postulate comes from the results presented in Table 1; intact sperm decrease the amount of H₂O₂ produced by glucose oxidase seen by the ferrocytochrome *c*/cytochrome *c* peroxidase assay system. The maximal rate of H₂O₂ production by intact sperm at cell concentrations less than 10⁷ cells/ml is about 0.8 nmol/min per 10⁸ cells. This is interpreted to be the unperturbed rate. As the concentration of cells in the suspending medium increases, the rate of reaction of H₂O₂ in the medium with the sperm cells increases until a lower net rate is attained, representing the balance between H₂O₂ production and export by the cells, and the rate of removal of exported H₂O₂ by reaction with the cells. The latter rate becomes independent of H₂O₂ concentration in the medium above 0.2 μ M and becomes linearly dependent only on cell concentration above 2×10^7 cells/ml, implying a reaction with substrate saturation kinetics. The most likely candidate for the cell component reacting with H₂O₂ is the plasma membrane, or some region thereof, since this is the component that is largely removed by hypo-osmotic treatment (Keyhani & Storey, 1973). Another possible, but less likely, candidate would be the acrosomal component, which is also damaged and removed by this treatment.

The unperturbed rate of H₂O₂ production by intact spermatozoa of 0.7–0.8 nmol/min per 10⁸ cells is about twice the rate observed with amphotericin-treated cells. As amphotericin treatment greatly increases the permeability of cell plasma membranes to compounds of low molecular weight (Bittman, 1978), the decrease in rate after amphotericin treatment is most reasonably interpreted as loss of low-molecular-weight components. This in turn suggests a tentative resolution of sources of H₂O₂ production into the following components. One is a low-molecular-weight component, which is lost on treatment with amphotericin and accounts

for 0.3–0.4 nmol of H_2O_2 /min per 10^8 cells. The mitochondrial component accounts for another 0.3 nmol of H_2O_2 /min per 10^8 cells, under conditions of normal metabolism in rabbit spermatozoa (Storey & Keyhani, 1974), whereas the balance of 0.1–0.2 nmol/min per 10^8 cells is the component produced by hypo-osmotically-treated spermatozoa in the absence of substrate, which is unaffected by rotenone.

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