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Properties of spermatozoal superoxide dismutase and lack of involvement of superoxides in metal-ion-catalysed lipid-peroxidation reactions in semen

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1. The distribution and properties of superoxide dismutase were examined in mammalian semen, and the enzyme was used to investigate the role of superoxides in metal-ion-catalysed lipid-peroxidation reactions in spermatozoa. 2. Superoxide dismutase activity was detected in seminal plasma and spermatozoa from all species studied, exceptionally high activity being found in donkey semen. The enzyme is easily solubilized from spermatozoa, as 85–90% of the total activity is released by cold shock, a relatively mild form of cellular damage. 3. Purification and characterization of the enzyme from supernatant fractions prepared from cold-shocked boar spermatozoa showed it to be cyanide-sensitive, to have a mol.wt. of 31000, a pI of 5.9 and to contain 1.85 g-atoms of copper and 1.91 g-atoms of zinc per mol of protein. However, extensive sonication of spermatozoa released a small amount of a cyanide-insensitive enzyme, presumably a mangano superoxide dismutase, from the mitochondrial matrix. 4. The presence of superoxide dismutase in spermatozoa, either intracellularly or extracellularly, did not inhibit ascorbate/Fe^{2+}-catalysed lipid-peroxidation reactions, suggesting that superoxides are not essential intermediates in this system.

Superoxide dismutase is widely distributed in cells and micro-organisms that posses an aerobic form of metabolism (reviewed by Fridovich, 1975). Three different forms of the enzyme have been described: a cupro–zinc enzyme, which is primarily cytoplasmic, a mangano enzyme, found in the mitochondrial matrix, and an iron-containing protein, isolated from bacteria. It is thought that the physiological role of superoxide dismutase is to protect cells from the toxic effects of superoxide anions (O_2^-) generated during enzymic oxidation of NADPH in microsomal and mitochondrial electron-transfer reactions. It catalyses a dismutation reaction leading to the formation of H_2O_2 and O_2:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The H_2O_2 is then destroyed by tissue catalases and peroxidases.

Mammalian spermatozoa are well known to be highly susceptible to damage by O_2 (MacLeod, 1943; VanDemark *et al.*, 1949), and it has been suggested that this is a consequence of the accumulation of H_2O_2 due to their low content of catalase and glutathione peroxidase (Mann, 1964; Li, 1975; Abu-Erreish *et al.*, 1978). The toxicity of H_2O_2 towards spermatozoa is well documented (VanDemark *et al.*, 1949; Wales *et al.*, 1958), but is unlikely to be the sole factor involved in damage by O_2. H_2O_2 has been positively identified in spermatozoa only in connection with the deamination of certain amino acids (Tosic & Walton, 1946), and the amounts of catalase that need to be added to produce a significant protective effect on spermatozoa are many times in excess of those required to destroy H_2O_2. However, a second route of O_2 toxicity is through peroxidation of unsaturated lipids in cell membranes, mediated by free radicals much as superoxides or hydroxyl radicals or by singlet oxygen (Barber & Bernheim, 1967; McCay *et al.*, 1976). Defence against the harmful effects of free radicals *in vivo* is normally provided by a variety of scavenging agents such as α-tocopherol, glutathione peroxidase and superoxide dismutase. It has been shown that spermatozoa are very sensitive to lipid peroxidation catalysed by sodium ascorbate and trace elements such as Fe^{2+} (Jones & Mann, 1976,

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1977; Jones *et al.*, 1979). The production of superoxides during the autoxidation of Fe^{2+} in solution was first proposed by Weiss (1935) and has been studied in detail by Michelson (1977), who has suggested that superoxides generated from contaminating trace elements are the prime initiation step in the peroxidation of lipids in foodstuffs. The protective effect of superoxide dismutase in a variety of experimental situations suggests a major role for O_2^- in the peroxidation process (Fee & Teitelbaum, 1972; Pederson & Aust, 1973; Zimmermann *et al.*, 1973; Kellogg & Fridovich, 1975; Michelson, 1977).

The objectives of the work described below were to investigate the nature and properties of the superoxide dismutase enzyme present in spermatozoa and to examine the role of superoxides in metal-ion-catalysed lipid-peroxidation reactions with spermatozoa as a model system.

Materials and methods

Reagents

Chemicals were of A.R. grade whenever possible, and aqueous solutions were freshly prepared in glass-distilled deionized water. Sigma Chemical Co. (Poole, Dorset, U.K.) supplied adrenaline, cytochrome *c* (type III), xanthine, xanthine oxidase, α-oxoglutarate (sodium salt), NADH, molecular-weight markers (Dalton mark VI vial), β-mercaptoethanol and Tris hydrochloride. Sephadex G-75 and DEAE-Sephadex A-50 were purchased from Pharmacia (London W5, U.K.), CM-cellulose (CM-23) was from Whatman Biochemicals (Maidstone, Kent, U.K.). Other reagents were supplied by BDH Chemicals (Poole, Dorset, U.K.). Organic solvents were redistilled before use.

Semen

Semen was collected from rams, bulls, stallions and donkeys by using an artificial vagina. Boar and human semen was obtained by massage. Seminal gel, where present, was removed by filtering through two layers of surgical gauze. The semen was then centrifuged at 1000*g* for 10min, seminal plasma removed, and the sperm pellet resuspended in phosphate-buffered saline (138mM-NaCl/7mM-Na_2HPO_4/3mM-NaH_2PO_4, pH7.2) to twice the original volume of semen. After centrifuging at 1000*g* for 10min, the supernatant (first wash fluid) was removed and the spermatozoa were resuspended in phosphate-buffered saline to give a final concentration of between 10^8 and 10^9cells/ml. In some experiments (see the Results section) the washing procedure was repeated another two times. Seminal plasma and wash fluids were re-centrifuged at 5000*g* (4°C) for 15min to ensure removal of all spermatozoa and cellular debris and stored frozen at −30°C. The concentration of spermatozoa was determined by using a haemocytometer.

Preparation of supernatant fractions from cold-shocked and sonicated spermatozoa

Spermatozoa were cold-shocked by cooling them rapidly from 25°C to 0°C (Mann & Lutwak-Mann, 1955), re-warming to 37°C and repeating the process once more. After centrifugation at 1000*g* (4°C) for 15min the supernatant (cold-shocked supernatant) was stored at −30°C. Complete disruption of spermatozoa was achieved by sonicating them for 2min (Kerry's Ultrasonics model KT100 sonicator, power setting 2), and a 1000*g* supernatant (sonicated supernatant) was collected as described above. Sonication was performed at 4°C in 20s bursts with 20s intervals to avoid localized heating.

Enzyme assays

The activity of superoxide dismutase was measured either by the procedure of McCord & Fridovich (1969) or by that of Misra & Fridovich (1972). The latter assay method was preferred because of its greater sensitivity and ease for monitoring large numbers of fractions from column effluents. The method depends on the inhibition by superoxide dismutase of the spontaneous autoxidation of adrenaline to adrenochrome at pH10.2. The reaction was performed at 30°C in 1ml of 50mM-sodium carbonate buffer, pH10.2, containing 0.3mM-adrenaline and 0.1mM-EDTA. One unit of activity is defined as the amount of enzyme required to inhibit the change in absorption at 480nm by 50%. Glutamate dehydrogenase and malate dehydrogenase were assayed spectrophotometrically at 340nm (Schmidt, 1974; Bergmeyer & Bernt, 1974).

Purification of superoxide dismutase

The enzyme was purified to apparent homogeneity from supernatant fractions prepared from cold-shocked boar spermatozoa. Pooled supernatants were concentrated by ultrafiltration on Amicon UM-10 filters, washed twice with 50mM-Tris/HCl buffer, pH7.4, containing 100mM-KCl and chromatographed on a Sephadex G-75 column (30cm × 2cm²) with the same eluting buffer. Fractions containing enzyme activity were dialysed against 2.5mM-potassium phosphate buffer, pH7.4, concentrated on UM-10 filters and chromatographed on a DEAE-Sephadex A-50 column (14cm × 2cm²) equilibrated with the same buffer. The enzyme was eluted with a gradient of 2.5–200mM-potassium phosphate buffer, pH7.4. Further purification was achieved on a CM-cellulose (CM-23) column (7cm × 1.3cm²) equilibrated with

2mM-potassium acetate, buffer, pH5.1, and application of a gradient of 50–200mM-KCl in the acetate buffer. The molecular weight of the purified enzyme was determined by gel filtration on a Sephadex G-100 column (90cm × 2cm²) previously calibrated with bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000), seminal ribonuclease (mol.wt. 23000), ribonuclease (mol.wt. 13500) and cytochrome *c* (mol.wt. 11000). The u.v.-absorption spectrum of the protein was measured in 5mM-acetate buffer, pH5.1.

Electrophoresis

Purity of the enzyme preparation was checked by electrophoresis on cylindrical 7.5% (w/v) polyacrylamide gels (10cm × 0.5cm) containing 1% (w/v) sodium dodecyl sulphate in 50mM-Tris/acetate buffer, pH7.4 (Fairbanks *et al.*, 1971), or on non-denaturing 7% (w/v) polyacrylamide gels at pH8.9 (Davis, 1964). The current in both systems was set at 2mA/gel, and protein was stained with 0.05% Coomassie Brilliant Blue in 40% (v/v) methanol/7% (v/v) acetic acid followed by destaining in the same solvent without dye. Enzyme activity was detected on non-denaturing gels by the method of Beauchamp & Fridovich (1971) in the presence and absence of 1mM-KCN.

Isoelectric focusing

Isoelectric focusing was performed on a 5% (w/v) polyacrylamide slab gel (10cm × 16cm × 0.2cm) in an LKB Multiphor 2117 apparatus with a 2% Ampholine gradient between pH3.5 and 10.0. The current was set at 20mA for 4–5h.

Experiments on lipid peroxidation

Lipid peroxidation was initiated by the addition of 0.5mM-sodium ascorbate and 0.05mM-$FeSO_4$ either to a 4ml suspension of washed motile boar spermatozoa (approx. 0.5×10^9 cells/ml) or to a 4ml emulsion of a lipid extract (final concn. 25–30μg of lipid P/ml) from spermatozoa. Incubation was in air at 37°C in a shaking water bath. At different time intervals 0.5ml portions were withdrawn, deproteinized with 0.5ml of 20% (w/v) trichloroacetic acid and centrifuged at 5000*g* for 10min. The concentration of malonaldehyde, indicating the extent of lipid peroxidation, was measured in the supernatant fraction by the thiobarbituric acid reaction as described previously (Jones & Mann, 1976).

The system for generating superoxide radicals was identical with that described by McCord & Fridovich (1969), and consisted of 0.1mM-EDTA, 0.05mM-xanthine and approx. 100pM-xanthine oxidase in 3ml of 50mM-potassium phosphate buffer, pH7.8. The concentration of spermatozoa was adjusted to approx. 10^8 cells/ml, and suspensions were incubated for 60min at 25°C. Peroxidation was measured by the thiobarbituric acid reaction.

Other procedures

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as standard. Lipids were extracted according to the Bligh & Dyer (1959) procedure and stored under N_2 at −30°C in chloroform/methanol (4:1, v/v). Phosphorus was determined by the Fiske & SubbaRow (1925) assay method after digestion of the lipid in 70% (v/v) $HClO_4$. Copper and zinc were determined by atomic-absorption spectroscopy on a Pye Unicam SP.90 spectrometer in accordance with the manufacturer's recommended procedure.

Results

Distribution of superoxide dismutase in mammalian semen

Superoxide dismutase activity was detected in spermatozoa and seminal plasma from all species investigated (Table 1). The highest activity was recorded in donkey and stallion semen and the least amount in cock semen. In 1ml of boar sperm-rich semen (10×10^8–15×10^8 cells/ml) 66% of the total activity was associated with spermatozoa and the remainder with the seminal plasma and cytoplasmic droplets. Successive wash fluids from spermatozoa contained progressively less superoxide dismutase activity, reflecting removal of the seminal plasma. Thus, in a typical experiment, 55units/ml (3.66units/mg of protein) were found in the seminal plasma, 25.7units/ml (11.52units/mg of protein) in the first wash fluid, 5.4units/ml (10.8units/mg of protein) in the second wash fluid and 0.6unit/ml (80.9units/mg of protein) in the third. However, it is possible that washing also removed some intracellular superoxide dismutase, as the enzyme leaks out of spermatozoa very readily; cold shock, a relatively mild form of damage, released 85–91% of the activity solubilized by extensive sonication. After centrifugation of a sonicated supernatant at 100000*g* for 60min, less than 8% of the dismutase activity was found in the pellet. Sonication followed by hypo-osmotic shock (10-fold dilution with distilled water) did not release significantly more activity than did sonication alone.

Purification and properties of superoxide dismutase from boar spermatozoa

The enzyme was purified to a state approaching homogeneity from supernatant fractions of cold-shocked spermatozoa as starting material. After ultrafiltration and chromatography on Sephadex G-75, fractions showing enzyme activity were

Table 1. *Survey of superoxide dismutase activity in mammalian semen*

Whole semen was centrifuged at 1000*g* for 10min, and seminal plasma was removed and re-centrifuged at 5000*g* for 10min. The sperm pellet was washed once in phosphate-buffered saline, resuspended to twice the original volume of semen and sonicated for 2min. A 1000*g* supernatant was prepared as described above and superoxide dismutase activity was measured by the adrenaline assay method (Misra & Fridovich, 1972). Values are means ± S.D. for the numbers of samples indicated. Each sample was assayed in duplicate.

		Superoxide dismutase activity		
		Seminal plasma		
	No. of samples	(units/ml)	(units/mg)	Spermatozoa (units/10^9 cells)
Boar	4	31.7 ± 11.1	5.6 ± 4.5	120 ± 46
Rabbit	3	417 ± 125	9.9 ± 3.4	33.6 ± 9.2
Stallion	3	1600 ± 260	117 ± 17	266 ± 9
Donkey	3	5260 ± 1830	380 ± 165	1430 ± 390
Cock	3	9.6 ± 5.4	0.7 ± 0.4	15.5 ± 8.3
Ram	5	161 ± 26	2.9 ± 0.2	105 ± 20
Bull	4	128 ± 540	1.9 ± 1.3	26.9 ± 2.1
Man	4	149 ± 99	3.3 ± 1.0	147 ± 30

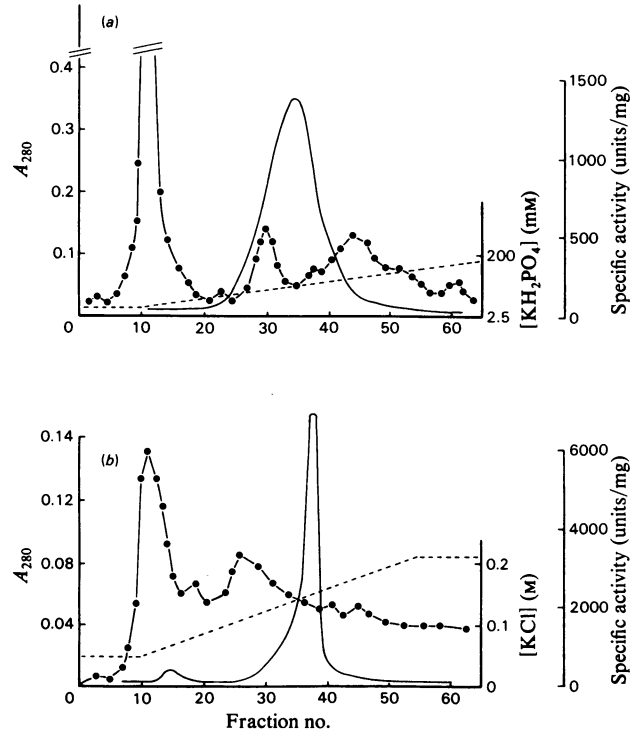

Fig. 1. *Purification of spermatozoal superoxide dismutase*

Supernatant fractions from cold-shocked boar spermatozoa (specific activity 33.0 units/mg of protein) were concentrated on Amicon UM-10 filters and chromatographed on Sephadex G-75 in 50mM-Tris/HCl buffer, pH 7.4, containing 100mM-KCl. Fractions showing enzyme activity were pooled, applied to a DEAE-Sephadex A-50 column (*a*) and eluted with a 2.5–200mM-potassium phosphate gradient at pH 7.4. Final purification was achieved on a CM-cellulose column (*b*) in 2mM-potassium acetate buffer, pH 5.1, by applying a gradient of 50–200mM-KCl. Fractions 35–40, showing only one band on polyacrylamide gels containing sodium dodecyl sulphate, were pooled to give a final specific activity of 4985 units/mg of protein. ●—●, A_{280} (protein); ——, enzyme specific activity; – – – –, salt gradient.

pooled and re-chromatographed on DEAE-Sephadex A-50 at pH 7.4 (Fig. 1). The enzyme was eluted along with other contaminating proteins, and final purification was achieved by chromatography on CM-cellulose to a specific activity of 4985 units/mg of protein. A single protein band corresponding to a mol.wt. of 16000 was obtained on sodium dodecyl sulphate/polyacrylamide gels in the presence of 1% β-mercaptoethanol (Fig. 2); in the absence of β-mercaptoethanol the mol.wt. was

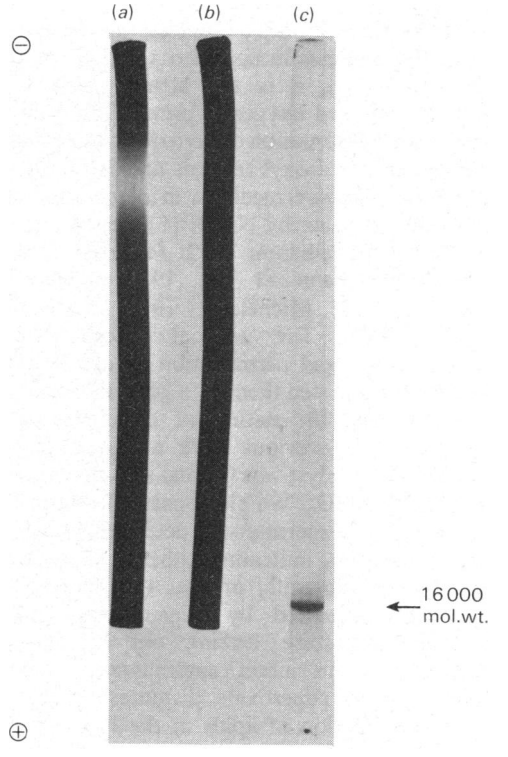

Fig. 2. *Polyacrylamide-gel electrophoresis of purified spermatozoal superoxide dismutase*

Activity of the purified enzyme was detected on 7% polyacrylamide gels by its ability to inhibit the photoreduction of Nitro Blue Tetrazolium (Beauchamp & Fridovich, 1971). After electrophoresis gels were immersed in 2.45 mM-Nitro Blue Tetrazolium for 20 min and then transferred to a solution containing 28 mM-tetramethylethylenediamine and 0.03 mM-riboflavin in 36 mM-potassium phosphate buffer, pH 7.8, for 15 min. All the above steps were performed in the dark. The gels were then placed in dry test tubes and illuminated for 10 min. Areas showing superoxide dismutase activity remained achromatic, whereas the remainder of the gel stained blue. Activity was developed in the absence (*a*) and presence (*b*) of 1 mM-KCN. A single band with a mol.wt. of approx. 16000 was obtained on 7.5% polyacrylamide gels containing sodium dodecyl sulphate (*c*). Each gel received 15 μg of protein.

equivalent to 31000. Chromatography on Sephadex G-100 gave a value of 30000, indicating that the enzyme is a dimeric protein composed of two equal subunits. Isoelectric focusing in a pH gradient ranging from 3.5 to 10.0 revealed several minor bands, with the main component localized at pH 5.9. The activity of the enzyme on polyacrylamide gels was completely inhibited by 1 mM-KCN, suggesting a cupro–zinc superoxide dismutase (Fig. 2), a conclusion that was confirmed by metal analysis, which yielded 1.85 g-atoms of copper and 1.91 g-atoms of zinc per mol of protein. The metal-free apoenzyme prepared by the method of Fee (1973) exhibited less than 4% of the activity of the holoenzyme, but could be restored to 62% of its original value by the addition of 2 equiv. of copper and zinc per mol of protein. The u.v.-absorption spectrum of the protein in acetate buffer, pH 5.1, was that of a typical cupro–zinc superoxide dismutase, with a peak at 272–278 nm and a molar absorption coefficient of $1.8 \times 10^4\ M^{-1} \cdot cm^{-1}$ at 276 nm.

Effect of cyanide and intracellular localization of spermatozoal superoxide dismutase

Cyanide is a potent inhibitor of the cupro–zinc superoxide dismutase (Rotilio *et al.*, 1972), but has no effect on the mangano enzyme. By measuring dismutase activity in the presence and absence of cyanide it is possible to distinguish between the two forms of the enzyme in a mixture. In mammalian cells the cyanide-sensitive dismutase is localized in the cytoplasm and intermembrane space of mitochondria, whereas the cyanide-insensitive form is confined to the mitochondrial matrix (Weisiger & Fridovich, 1973; Tyler, 1975). Compared with other cells, spermatozoa contain relatively little cytoplasm, but have a substantial amount of mitochondrial material in the midpiece region. Therefore it was decided to determine (*a*) the percentage of superoxide dismutase activity in sonicated supernatants inhibited by KCN, and (*b*) the relative amounts of each type of dismutase released from spermatozoa after cold shock and sonication. The effects of increasing concentrations of KCN on superoxide dismutase activity in supernatants prepared from cold-shocked and sonicated boar spermatozoa are shown in Fig. 3. For comparison, values are included for the erythrocyte enzyme (commercial preparation from bovine blood; Sigma). In our hands, the erythrocyte cupro–zinc superoxide dismutase was inhibited by about 92% in the presence of 1 mM-KCN. Increasing the concentration of KCN had no further inhibitory effect, and hence we have taken this value as the base-line for calculating relative inhibition of the spermatozoal enzyme. For example, if x units of dismutase activity/ml were found in the absence of KCN and y units/ml in the presenc eof KCN, then the activity

of cupro–zinc superoxide dismutase was $(x - y)/0.92$ units/ml. 0.1 mM-KCN inhibited dismutase activity in cold-shocked and sonicated supernatants by 88 and 79% respectively. Essentially similar results were obtained with the cytochrome *c* assay method of McCord & Fridovich (1969), the only difference being that about 10-fold greater concentrations of KCN were required to achieve the same extent of inhibition. This may be due to the higher pH (10.2) of the adrenaline assay method. Assuming that sonication caused maximum disruption of spermatozoa, it can be seen from Table 2 that, whereas cold shock released 85% of dismutase activity, it solubilized only 3 and 26% of glutamate dehydrogenase and malate dehydrogenase respectively (mitochondrial matrix markers). Thus the decreased inhibition by KCN of superoxide dismutase activity in supernatants from sonicated spermatozoa probably reflects the release of the cyanide-insensitive enzyme from the mitochondrial matrix. Because of its low activity, no further attempt was made to purify the cyanide-insensitive enzyme.

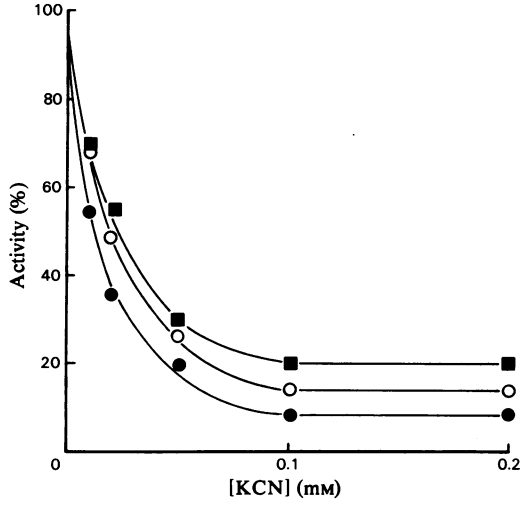

Fig. 3. *Inhibition of superoxide dismutase activity by KCN*

Supernatant fractions from cold-shocked (●) or sonicated (■) boar spermatozoa were assayed for dismutase activity (Misra & Fridovich, 1972) in the presence of 0–0.2 mM-KCN. The behaviour of the bovine erythrocyte superoxide dismutase (●) is shown for comparison.

Role of superoxides in lipid-peroxidation reactions in spermatozoa

Although there is ample literature on the involvement of free radicals in lipid-peroxidation reactions (Fridovich, 1975), it is not always clear which species of activated oxygen is primarily responsible for initiating the formation of peroxides. Superoxides, singlet oxygen, hydroxyl radicals and H_2O_2 have all been reported as intermediates in peroxidatic reactions catalysed either by NADPH or ascorbate plus Fe^{2+} (Fee & Teitelbaum, 1972; Pederson & Aust, 1973; Zimmermann *et al.*, 1973; Kellogg & Fridovich, 1975; Michelson, 1977; Nakano & Noguchi, 1977). The unusual susceptibility of spermatozoa to lipid peroxidation (Jones & Mann, 1976, 1977) suggested them as a good model system for investigating the nature of these free-radical intermediates. In previous work we found that the most effective catalyst was 0.5 mM-sodium ascorbate plus 0.05 mM-$FeSO_4$. We also observed that the rate of peroxidation in spermatozoa declined significantly during incubation, indicating either that there was exhaustion of substrate, or that the reaction was progressively inhibited by constituents such as superoxide dismutase leaking out of peroxide-damaged cells. In intact spermatozoa compartmentalization of superoxide dismutase would not prevent peroxidation of lipids in the outer layer of the plasma membrane. Since cold shock released over 85% of the intracellular dismutase into the surrounding medium, it was decided to compare peroxidation in normal and cold-shocked sper-

Table 2. *Release of cyanide-sensitive and cyanide-insensitive forms of superoxide dismutase from spermatozoa*

Boar sperm-rich semen was centrifuged at 1000*g* for 10 min, the seminal plasma was removed and the sperm pellet was washed twice in phosphate-buffered saline. Washed cells were resuspended to about 0.8×10^9 spermatozoa/ml, and then used directly or cold-shocked or sonicated as described in the Materials and methods section. The centrifuged supernatant fractions were analysed for enzyme activity. Abbreviation: N.D., non-detectable.

		Control supernatant	Cold-shocked supernatant	Sonicated supernatant
Superoxide dismutase				
Total	(units/ml)	1.8	45.8	53.5
Cyanide-sensitive	(units/ml)	1.8	43.9	45.6
Cyanide-insensitive	(units/ml)	N.D.	1.9	7.9
Glutamate dehydrogenase	(units/l)	N.D.	1.5	29.8
Malate dehydrogenase	(units/l)	N.D.	860	3210
Protein	(mg/ml)	0.1	0.9	2.2

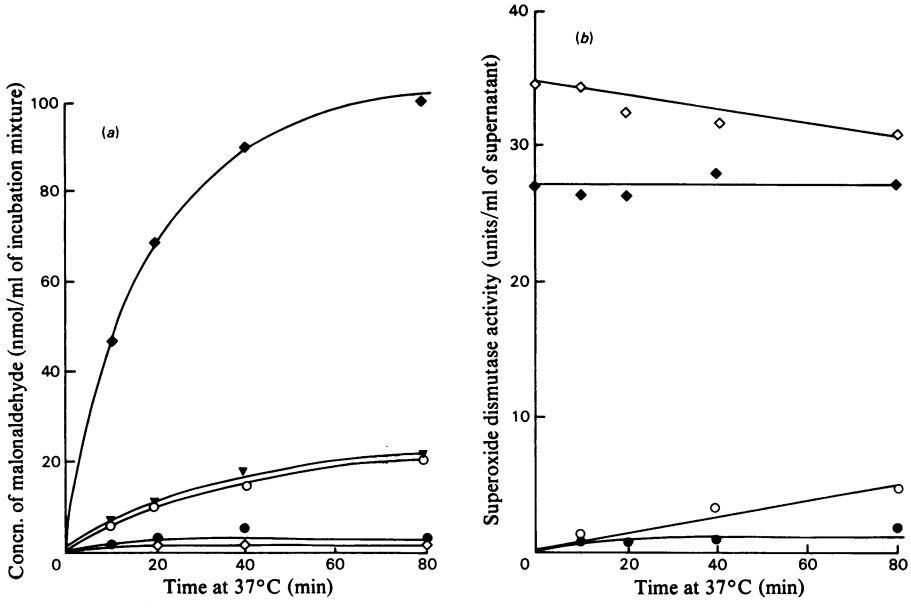

Fig. 4. *Lipid peroxidation and superoxide dismutase activity in boar spermatozoa*

Suspensions (4 ml) of washed normal or cold-shocked boar spermatozoa (approx. 0.5×10^9/ml) were incubated at 37°C with or without 0.5 mM-sodium ascorbate plus 0.05 mM-$FeSO_4$. At different time intervals portions were withdrawn for assay of malonaldehyde or superoxide dismutase activity as described in the Materials and methods section. (*a*) Concentrations of malonaldehyde in normal spermatozoa (●), normal spermatozoa + ascorbate + $FeSO_4$ (○), cold-shocked spermatozoa (◇), cold-shocked spermatozoa + ascorbate + $FeSO_4$ (◆) and normal spermatozoa + ascorbate + $FeSO_4$ + 0.5 ml of supernatant from cold-shocked spermatozoa (▼). (*b*) Superoxide dismutase activity in a supernatant fraction from normal spermatozoa (●), normal spermatozoa + ascorbate + $FeSO_4$ (○), cold-shocked spermatozoa (◇) and cold-shocked spermatozoa + ascorbate + $FeSO_4$ (◆).

matozoa. The results are shown in Fig. 4(*a*). With normal spermatozoa there was a linear increase in malonaldehyde concentrations during the first 40 min of incubation, reaching a maximum after 60 min. The activity of superoxide dismutase in the incubation medium also increased during this period, reflecting progressive leakage of intracellular constituents due to membrane damage (Fig. 4*b*). However, the rate of accumulation of malonaldehyde by cold-shocked spermatozoa was 5 times that in normal intact cells, despite the presence of high superoxide dismutase activity in the extracellular medium for the duration of the incubation period. Furthermore, when a total lipid extract from normal spermatozoa was incubated with ascorbate and Fe^{2+} in the presence of a cold-shocked supernatant (final dismutase activity 81 units/ml or a purified preparation of spermatozoal superoxide dismutase (1700 units/ml), peroxidation was not inhibited. The addition of catalase (2500 units/ml) also failed to inhibit peroxidation. Incubation of intact spermatozoa in a superoxide-generating system utilizing xanthine and xanthine oxidase produced negligible concentrations of thiobarbituric acid-reactive material.

Discussion

Superoxide dismutase in semen

Superoxide dismutase activity was present in spermatozoa and seminal plasma from all species investigated, with donkey semen being the richest source of the enzyme. The presence of superoxide dismutase in seminal plasma may seem unusual, since it is primarily an intracellular enzyme and not a normal component of extracellular fluid. However, seminal plasma is produced by an apocrine or merocrine type of secretory activity in accessory glands (Mann, 1964) and hence contains a wide variety of intracellular constituents not usually found in blood or lymph. The high activity in stallion and donkey plasma is probably derived from the secretions of the ampullary glands, which are large and well developed in these species.

Evidence was obtained for two forms of superoxide dismutase in boar spermatozoa: a cyanide-sensitive enzyme that is cytoplasmic and leaks out of spermatozoa very readily if the plasma membrane is damaged, and a cyanide-insensitive enzyme that is released after extensive sonication to solubilize mitochondrial-matrix enzymes. Because of the

relative ease with which the cyanide-sensitive enzyme is solubilized after damage to the spermatozoal plasma membrane, it was not possible to investigate its intracellular distribution, but in all probability the bulk of the activity resides in the midpiece/flagellum region, where mitochondria and residual cytoplasm are found. The spermatozoon head and acrosome (a modified lysosome) are unlikely to contain much superoxide dismutase; in rat liver cells less than 9% of the total activity is associated with the nuclear and lysosomal fractions (Tyler, 1975). The metal content, behaviour on Sephadex G-100 and mobility on polyacrylamide gels containing sodium dodecyl sulphate indicate that the cyanide-sensitive enzyme isolated from spermatozoa has many properties in common with the cupro–zinc superoxide dismutase isolated from other sources (Fridovich, 1975).

O_2 toxicity, superoxides and lipid-peroxidation reactions

Hyperbaric O_2 has long been known to have toxic effects on cells and to enhance radiation-lethality (Gerschman *et al.*, 1954; Oshino & Chance, 1977). The primary causes of this O_2 toxicity are due to a variety of free radicals that are produced in a chain reaction beginning with the univalent reduction of molecular O_2 to a superoxide (Fridovich, 1977; Oshino & Chance, 1977). These free radicals, particularly hydroxyl radicals, are powerful oxidizing agents, and they amplify the potential dangers of superoxide formation. Henry & Michelson (1977) reported that 'pulses' of superoxides are formed in O_2/Fe^{2+}/phosphate mixtures, and Gutteridge *et al.* (1979) have shown that hydroxyl radicals produced from superoxides are involved in lipid-peroxidation reactions in brain liposomes. Therefore we considered the possibility that superoxides were involved in the ascorbate/Fe^{2+}-catalysed peroxidation of phospholipids in spermatozoa (Jones & Mann, 1976), and that this may form the basis for their susceptibility to O_2 toxicity. However, since superoxide dismutase and/or catalase in the extracellular medium did not inhibit peroxidation, as monitored by malonaldehyde formation, the participation of superoxides and H_2O_2 in the reaction seems unlikely. The high malonaldehyde concentrations produced in suspensions of cold-shocked spermatozoa can be explained on the grounds that more intracellular lipid was available for peroxidation after damage to the plasma membrane. The species of active oxygen involved in our system remains to be identified, but preliminary experiments with free-radical scavengers indicate that singlet oxygen may be one intermediate (R. Jones, unpublished work).

Inhibition of peroxidatic reactions in spermatozoa may be important for the long-term storage of semen, as high concentrations of organic peroxides have been found in spermatozoa stored aerobically at −20°C for periods longer than 1 year (R. Jones, unpublished work). The addition of free-radical-scavenging agents to semen before freezing therefore may have beneficial effects on the survival of spermatozoa *in vitro*.

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