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Spontaneous Lipid Peroxidation in Rabbit Epididymal Spermatozoa: Its Effect on Sperm Motility

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ABSTRACT

Rabbit spermatozoa released from the cauda epididymidis into Tris phosphate medium containing KCl or NaCl and 0.4 mM EDTA underwent spontaneous lipid peroxidation during aerobic incubation at 37°C. In the medium containing 130 mM K^+ and 0 mM Na^+ (KTP), the rate of lipid peroxidation, as measured by malonaldehyde production, proceeded at a linear rate of 0.045 nmol malonaldehyde/h per 10^8 cells for 22 h. The motility of these spermatozoa declined with time in medium KTP, with 40% initial forward motility decreasing to zero in 4 h and initial 60% flagellar beating ceasing after 12 h. The percent inert spermatozoa showing no flagellar motion in KTP increased linearly with production of malonaldehyde; all flagellar activity stopped at 0.5 nmol malonaldehyde/10^8 cells. In the Tris phosphate medium containing 120 mM Na^+ and 10 mM K^+ (NTP), the percentage of sperm showing forward motility was close to 100% and this declined to 60% after 16 h aerobic incubation. Flagellar beating was not observed. In medium NTP, the rate of lipid peroxidation was 0.0056 nmol malonaldehyde/h per 10^8 cells, eightfold lower than that observed in KTP. The same linear correlation between malonaldehyde production and percent inert sperm was found as for KTP: 0.5 nmol malonaldehyde/10^8 cells also corresponded to cessation of flagellar motion. The dependence of motility maintenance on K^+ concentration in Tris phosphate medium containing (Na^+ + K^+)=130 mM showed maximal maintenance at 10 mM K^+, with a decline at 0 mM K^+ and steep decline at K^+ concentrations greater than 30 mM. This strong dependence of rabbit sperm peroxidation on ionic composition of the medium is suggested to involve perturbation of the equilibrium between $O_2^{\cdot-}$ and its conjugate acid $HO_2\cdot$, the latter species being the agent of peroxidation.

INTRODUCTION

That O_2 may be toxic to spermatozoa was reported nearly 40 years ago by McLeod (1943), who showed that high O_2 tensions were deleterious to the motility of human sperm. Since most mammalian sperm contain little or no catalase (Mann, 1964), the toxic effect has usually been attributed to H_2O_2 (Tosic, 1947; Tosic and Walton, 1950; Wales et al., 1959; Bar-Sagie et al., 1981). The observation that bull and ram spermatozoa lost motility on storage at 4°C in concert with loss of phospholipid, particularly plasmalogen, suggested that lipid peroxidation might be the pathway of O_2 toxicity (Jones and Mann, 1973; Mann and Lutwak-Mann, 1981). An extensive study of lipid peroxidation in mammalian spermatozoa by Jones and Mann (1976), showed that these cells are highly susceptible to this process, and that the peroxidation products are potent spermicides (Jones and Mann, 1977a,b; Jones et al., 1978, 1979; Mann et al., 1980). In this work, lipid peroxidation was promoted by means of Fe^{2+} plus ascorbate, which has the advantage of markedly accelerating the process. Diezel et al. (1980) showed a good correlation between loss of motility and formation of lipid peroxidation products in human spermatozoa, also using Fe^{2+} plus ascorbate.

The rabbit oviduct has a mean intraluminal PO_2 of 60 Torr (Mastroianni and Jones, 1965), which should be of a magnitude sufficient to maintain production of H_2O_2 and $O_2^{\cdot-}$ and lipid peroxidation in rabbit spermatozoa, if these resemble other mammalian cells in this regard (Chance et al., 1979). We have demonstrated that rabbit epididymal spermatozoa

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produce H_2O_2 and, as expected, lack detectable catalase (Holland and Storey, 1981). These cells also produce $O_2^{\cdot-}$, but have both cytosolic and mitochondrial superoxide dismutase activities (Holland et al., 1981). Those observations suggested that lipid peroxidation could occur spontaneously in rabbit spermatozoa in the absence of promoters and that this reaction might limit the "lifetime" of these cells. In this paper, we report the occurrence in rabbit spermatozoa of spontaneous lipid peroxidation in the absence of added promoters and the consequences of this process for sperm motility.

MATERIALS AND METHODS

Reagents

Malonaldehyde-bis(dimethylacetal) was from Aldrich Chemical Co. (Milwaukee, WI); trichloracetic acid and inorganic salts were from J. T. Baker (Phillipsburg, NJ) and of the highest purity available. Cytochrome *c* (horse heart, type VI), xanthine (sodium salt, grade III), xanthine oxidase (from buttermilk, grade III), D-glucose, thiobarbituric acid (TBA), and N-N'ethylenediaminetetraacetic acid, disodium salt (EDTA) were from Sigma Chemical Co. (St. Louis, MO).

Suspending Media

Four different media were used for sperm suspensions in this study. One was a modification of the high potassium medium of Keyhani and Storey (1973), designated KTP, with the composition: 113 mm KCl, 12.5 mM KH_2PO_4, 2.5 mM K_2HPO_4, 3 mM $MgCl_2$, 20 mM Tris, 1.5 mM D-glucose, 0.4 mM EDTA, 0.6% penicillin/streptomycin, adjusted with HCl to pH 7.4. The bicarbonate variant of KTP, designated KTB, had 15 mM $KHCO_3$ replacing the 15 mM KH_2PO_4 + $KHPO_4$ in KTP, but was otherwise identical in composition. It was treated with 5% CO_2 in air to bring the pH to 7.4. The high sodium medium, designated NTP, contained 10 mM KCl, 103 mM NaCl, and 15 mM NaH_2PO_4, but was otherwise identical in composition to KTP. The fourth medium was made up to simulate the ionic composition of the rabbit oviduct (David et al., 1969) and was designated OSM (oviduct-stimulating medium). Its composition was: 108 mM NaCl, 10 mM KCl, 0.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 40 mM $NaHCO_3$, 0.5 mM $NaHPO_4$, 1.5 mM glucose, 0.4 mM EDTA, and 0.6% penicillin/streptomycin; it was treated with 5% CO_2 in air to bring the pH to the range 7.6–7.8. All media contained 0.4 mM EDTA in order to chelate heavy metal cations which might act as promoters of lipid peroxidation or accelerate the otherwise negligibly slow Haber-Weiss reaction (Weinstein and Bielski, 1979).

Preparation of Spermatozoa

Spermatozoa were obtained from the caudae of excised epididymides of mature male New Zealand White rabbits by retrograde flushing through the vas deferens with the appropriate medium. The spermatozoa were sedimented at 750 × *g* for 10 min, the supernatant discarded, and the sperm were resuspended in 4 ml medium. Duplicate aliquots of 5 μl sperm suspension were removed, diluted to 1 ml with distilled water, and the number of sperm per aliquot determined by hemocytometer. The final cell concentration in the stock suspension ranged from 1–5 × 10^8 cells/ml. During the preparation of spermatozoa, special care was taken to prevent contamination of the sperm sample with hemoglobin from epididymal blood vessels; this pigment interferes with the spectrophotometric procedure used to determine the extent of lipid peroxidation (Barber and Berheim, 1967) which is described below.

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 10-fold to give a sperm suspension containing 1–5 × 10^7 cells/ml. One ml of this suspension was placed in wide mouth specimen bottles (55 × 28 mm), which were placed in a shaking water bath at 37°C to insure good contact between gas and liquid phases. Incubations in bicarbonate media (KTB and OSM) were carried out under 5% CO_2 in air. 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

Production of malonaldehyde was used as an index of spontaneous lipid peroxidation by spermatozoa in suspension (Jones and Mann, 1973, 1976), using a modification of the thiobarbituric acid (TBA) assay described by Barber and Bernheim (1967). Aerobic incubations of sperm suspensions in the chosen media (see above) were terminated at selected time intervals by immersing the incubation bottles in crushed ice and chilling the sperm suspensions to 0°C. The sperm suspensions were left in ice for 15 min and then added to a mixture of 0.5 ml 40% aqueous trichloroacetic acid (TCA) plus 0.5 ml of the chosen medium in ice-chilled test tubes, to give 2 ml of diluted suspension. The diluted sperm suspension was centrifuged at 2500 × *g* for 10 min, and 1 ml of the supernatant added to 0.25 ml 2% (w/v) thiobarbituric acid (0.14 M) in distilled water to which sufficient 1 M NaOH had been added to give a clear solution. The stock TBA solution was kept in 85 × 13 mm test tubes covered with aluminum foil to guard against photo-induced damage of the TBA anion. After addition of the 1 ml supernatant to the TBA solution with shaking, the test tubes, equipped with agate marble vapor condensers (Placer et al., 1966), were placed in a boiling water bath for precisely 10 min and then allowed to cool for 30 min at 20-22°C. The absorbance of the solution was determined with a DW-2A dual wavelength spectrophotometer (Travenol Laboratories Instrument Division, Savage, MD) using the wavelength pair 534–570 nm. Malonaldehyde, obtained by acid hydrolysis of the bis(dimethylacetal) in the TCA solution was used as standard. The difference extinction coefficient of the malonaldehyde-TBA reaction product was calculated to be 190 ± 12 mM^{-1} cm^{-1} in

all four media used (SD of 6 determinations). This value is in reasonable agreement with that of 152 mM^{-1} at 548 nm given by Placer et al. (1966) and that of 156 mM^{-1} cm^{-1} at 530 nm determined by Wills (1969) and recommended for use at 535 nm by Beuge and Aust (1978). The use of a final TBA concentration of 0.4% (0.028 M) instead of 0.125% (Beuge and Aust, 1978) or 0.2% (Barber and Bernheim, 1967), the lack of hemoglobin contamination due to careful removal of blood vessels, and the presence of EDTA at a final concentration of 0.32 mM in the color development reaction reduced interference from glucose (Barber and Bernheim, 1967) and from iron-induced chromogenic reactions (Gutteridge, 1981) to practically nil. Blank corrections were therefore small. This method gives high sensitivity and good linearity with malonaldehyde concentration: 0.01 μM is readily detectable; linearity extends to 10 μM.

Motility Assay

Sperm motility was estimated by a modification of the method of Heffner and Storey (1982), in which three replicate random samples of 20 sperm each in a 50-μl aliquot of suspension were scored under the dissecting microscope for the number of sperm actively moving forward. The percentage of sperm with forward motility was calculated from the average of the three samples. In this study, two samples were scored for forward motility; variation in the percentage forward motility was within ± 5% for duplicate samples. The assay was extended to include another category of sperm activity, flagellar beating. Sperm in this category showed flagellar motion but did not move to any perceptible extent. The third category included in the assay was inert spermatozoa, those cells which showed no motion of any kind. This assay proved to be more rapid than the more elaborate one developed by Heffner and Storey (1981) for a study of motility in mouse sperm, with little loss in accuracy, and so was well suited to the rapid assay of motility required at the selected time points of aerobic incubation. The aliquots of the sperm suspensions being incubated aerobically were taken for motility assay just before chilling the incubation mixture in preparation for determination of lipid peroxidation by the TBA reaction procedure (see above).

RESULTS

When incubated aerobically in medium KTP at 37°C, intact rabbit epididymal spermatozoa produced malonaldehyde linearly with incubation time over a period of 22 h (Fig. 1), with a net rate of 0.045 nmol/h per 10^8 cells. The maximal accumulation in the medium was 1.0 nmol/10^8 cells. At the end of this time, net production ceased; a slight linear decline in medium content of malonaldehyde was observed and may represent the slow removal of this by-product of lipid peroxidation through further reaction. The linear increase of malonaldehyde in the medium therefore represents a balance between production and consumption by further reaction; no attempt to dissect this process into the two component reactions was undertaken.

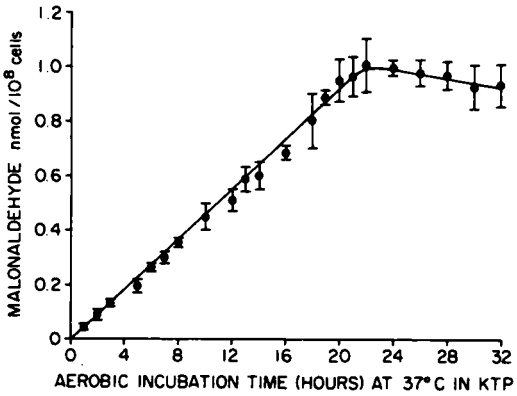

FIG. 1. Malonaldehyde production by rabbit epididymal spermatozoa as a function of aerobic incubation time at 37°C in medium KTP. The composition of the medium, the sampling procedure, and the spectrophotometric method for determination of malonaldehyde are described in *Materials and Methods.* Sperm concentration ranged between 0.1 and 0.5 X 10^8 cells/ml. Each point represents the mean of 5–10 experiments; error bars are the standard deviations. The linear increase in malonaldehyde from 0–22 h can be represented by a linear regression equation through the origin of the form: y=0.45 x (r=0.997).

Rabbit spermatozoa in medium KTP initially showed 40% forward motility and 60% flagellar beating. Both forward motility and the flagellar beating decreased with time; the sperm population became inert after about 12 h (Fig. 2). When net malonaldehyde production was assayed during this period and compared to the percent inert sperm at a given time, a linear correlation was obtained (Fig. 3.). This correlation yields the figure of 0.5 nmol malonaldehyde/10^8 cells as the index of lipid peroxidation corresponding to a completely inert population of sperm.

The effect of medium composition on the decline of rabbit sperm forward motility and flagellar beating with time was examined further. Replacement of the phosphate in medium KTP by bicarbonate to give medium KTB exacerbated this decline (Fig. 2). On the other hand, if most (but not all) of the K^+ in medium KTP was replaced by Na^+ to give medium NTP, the sperm initially all showed forward motility, and this motility declined only slowly over 16 h (Fig. 4). The rate of malonaldehyde production in NTP was 0.0056 nmol/h per 10^8 cells, eightfold lower than that observed in KTP. There was no perceptible

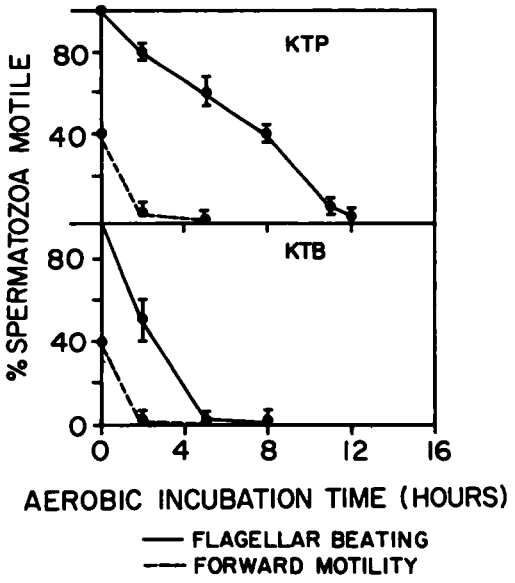

FIG. 2. Sperm motility and flagellar beating as a function of aerobic incubation time at 37°C in media KTP and KTB. The composition of the media and the assay of motility and flagellar beating are described in *Materials and Methods*. The experimental conditions were identical to those for Fig. 1. Each point represents the mean of 10 experiments in KTP and 3 in KTB; error bars are the standard deviations.

flagellar beating in medium NTP; the sperm either had forward motility or were inert. Forward motility was maintained in medium NTP as well as in medium OSM (Fig. 4). The latter medium was made up to approximate the ionic composition of rabbit oviduct lumen (David et al., 1969) and resembles the medium in which in vitro fertilization of rabbit eggs occurs (Brackett and Oliphant, 1975).

The linear correlation between malonaldehyde production and cessation of flagellar activity remained valid in all four media tested, despite the large difference in times required for the sperm to become inert in the different media (Fig. 5). The figure of 0.5 nmol malonaldehyde/10^8 cells, obtained in medium KTP, was also obtained for the other three media as the index of lipid peroxidation at which the entire sperm population became inert.

Since the greatest effect on maintenance of sperm motility was caused by substituting Na^+ for K^+, the effect of varying K^+ concentration on motility was examined. The medium had the same composition as KTP and NTP with regard to the other ions present, but the alkali metal ion concentration was varied from 0 mM K^+, 130 mM Na^+ to 118 mM K^+, 12 mM Na^+. Aerobic incubations were carried out for 11 h,

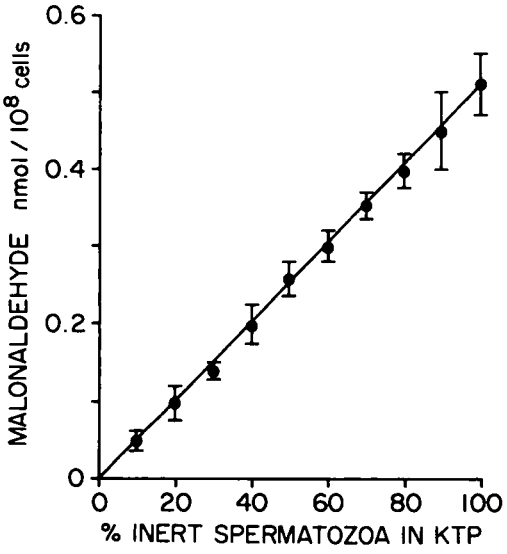

FIG. 3. Correlation between malonaldehyde production and percent inert spermatozoa during aerobic incubation at 37°C in medium KTP. The experimental conditions were those obtaining for Fig. 1. Each point represents the mean of 10 experiments; error bars are the standard deviations. The linear regression line calculated through the origin has the form: y=0.0052 x (r=0.997). Note that at x=100%, y=0.5 nmol/10^8 cells.

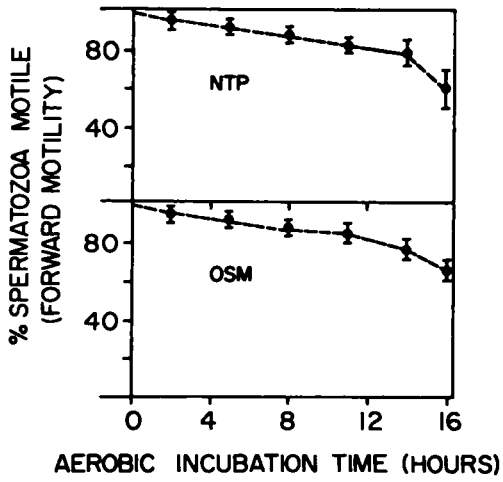

FIG. 4. Sperm motility as a function of aerobic incubation time at 37°C in media NTP and OSM. The composition of the two media and the assay of motility are described in *Materials and Methods*. Flagellar beating was not observed in these media. The experimental conditions were those obtaining for Fig. 1. Each point represents the mean of 3 experiments; error bars are the standard deviations.

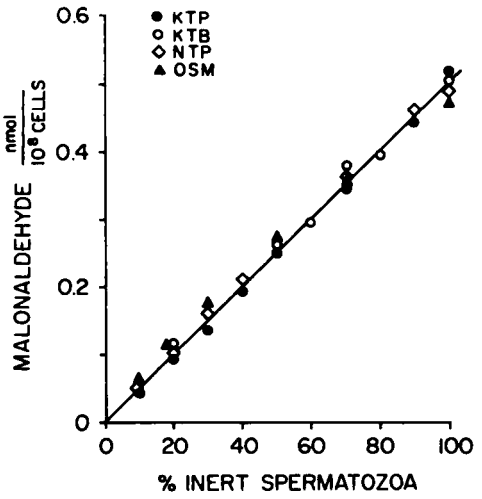

FIG. 5. Correlation between malonaldehyde production and percent inert spermatozoa in media KTP, KTB, NTB, and OSM during aerobic incubation at 37°C. The experimental conditions were those obtaining for Figs. 1, 2 and 4. Each point represents the mean of 10 experiments in medium KTP and 3 experiments in KTB, NTP, and OSM. The linear regression lines calculated through the origin has the form: $y=0.0046\ x$ ($r=0.957$). Note that at $x=100\%$, $y=0.5$ nmol/10^8 cells as in Fig. 3.

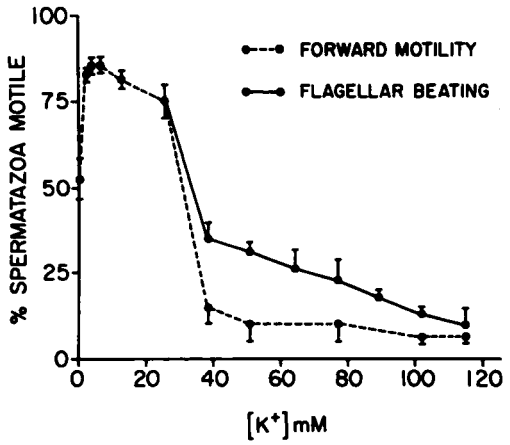

FIG. 6. Sperm motility after 11 h aerobic incubation time at 37°C as a function of K^+ concentration in media corresponding to NTP, from 0 mM K^+, 130 mM Na^+ to 118 mM K^+, 12 mM Na^+. The experimental conditions were those obtaining for Figs. 1 and 4. Each point in this set of experiments represents the mean of 3 experiments; error bars are the standard deviations. Note that in medium KTP (0 mM Na^+, 130 mM K^+) the forward motility had dropped to 0% and the flagellar beating to 8% during this time of incubation (Fig. 2).

after which the sperm were assessed for forward motility and flagellar beating. The results are shown in Fig. 6. Below 30 mM K^+, only forward motility was observeed, and maintenance of this motility over the 11-h period was optimal at 10 mM K^+. Surprisingly, omission of K^+ from the medium had a deleterious effect on motility maintenance. As K^+ concentrations were increased above 30 mM, a sharp decrease in the maintenance of forward motility and flagellar beating and an increase in inert spermatozoa were observed. Since an increase in inert sperm correlates with net malonaldehyde production (Figs. 2 and 5), the solid curve in Fig. 6 also represents the effect of Na^+/K^+ composition of the medium on the rate of lipid peroxidation.

DISCUSSION

The determination of lipid peroxidation in this study utilized the highly sensitive and convenient method based on the reaction of TBA with the malonaldehyde produced during the process (Barber and Bernheim, 1967; Wills, 1969; Buege and Aust, 1978). It should be emphasized that, as pointed out by Galanopoulou et al. (1982), this method is an indirect measure of the peroxidation of polyunsaturated fatty acyl moieties. The substances which produce the chromophore may actually be precursors to malonaldehyde, a product which forms by decomposition of cyclic peroxidase and is but a relatively minor product of the autoxidation reaction (Buege and Aust, 1978; Porter et al., 1981; Galanopoulou et al., 1982). Despite these possible limitations, malonaldehyde production, as measured by the TBA reaction, has been shown to give an excellent correlation with four other indices of lipid peroxidation in hepatocytes: chemiluminescence, formation of fluorescent products, formation of ethane, and formation of pentane (Smith et al., 1982). With ram sperm, there was a good correlation between ascorbate-Fe^{2+}-induced peroxide formation measured iodometrically and by malonaldehyde formation; the correlation was less good with formation of fluorescent products (Jones and Mann, 1976). In view of the extensive use of malonaldehyde production as an index of lipid peroxidation in mammalian spermatozoa (Mann, 1980), it is our opinion that this index is still the most useful one available for use in assessing damage to sperm by formation and breakdown of lipid peroxides.

The mechanism by which lipid peroxidation produces inert spermatozoa has been shown by Jones and Mann (1977b) to be damage to the sperm membrane, which results in leakage in intracellular constituents, including adenine and pyridine nucleotides, and enzymes. Without intracellular ATP, there can be no motility (see review by Bishop, 1962). It would therefore be expected that the percent inert sperm produced by this mechanism would correlate with the extent of lipid peroxidation and not with the time of exposure to aerobic incubation. This is precisely what was observed (Fig. 5). Despite an eightfold lower peroxidation rate observed in medium NTP as compared to medium KTP, all sperm became inert at the same point of malonaldehyde production: 0.5 nmol/10^8 cells. This figure provides a convenient index for lethal damage to rabbit spermatozoa by lipid peroxidation. The question of what percent of the lipid in the rabbit sperm plasma membrane has undergone peroxidation at this index of peroxidative lethality was not addressed in this study. From the studies of Jones and Mann (1976) and Mann et al. (1980) it is clear that the phospholipids of spermatozoa are prime targets for peroxidation, and that certain of the polyunsaturated acyl moeities of the phospholipids are particularly vulnerable. An answer to this question would therefore require an extensive analysis of plasma membrane phospholipid as a function of net malonaldehyde formation and so was deemed outside the scope of the present investigation.

It is important to note the distinction between the effect of the ionic composition of the medium on lipid peroxidation, which irreversibly converts motile sperm to inert sperm, and on patterns of motility, including reversible loss of motility. The latter does not involve sperm damage. It is of interest in this regard that Na^+ has a beneficial effect on the motility of rat spermatozoa (Wong et al., 1981a) and that Na^+ can restore motility to rat spermatozoa which have been rendered immotile by suspension in choline choride (Wong et al., 1981b). The divalent ion Ca^{2+} is also required to maintain motility in rat (Morton et al., 1978) and mouse (Heffner and Storey, 1981) sperm, but not in rabbit sperm (Morton et al., 1978; Storey, 1975). These ionic effects require an intact plasma membrane for their manifestation. The ionic effects considered in this study are on the membrane-damaging effects of peroxidation.

The strong dependence of the rates of lipid peroxidation in these spermatozoa on the ionic composition of the medium was unexpected. The lowest rate of peroxidation occurs in media which have their Na^+ and K^+ composition close to that of oviductal or extracellular fluid. While this rate dependence on Na^+/K^+ concentration might seem reasonable on teleological grounds, it ultimately must find an explanation on chemical grounds. Our working hypothesis for the explanation of this result derives from the important observation of Gebicki and Bielski (1981) that superoxide anion, $O_2^{\cdot-}$ is inert with regard to initiation of chain oxidation of linoleic acid by O_2, whereas its conjugate acid, the perhydroxyl radical $HO_2\cdot$, is very potent in this regard. The pK_a of the perhydroxyl radical is 4.7 (Bielski, 1978). Ionic effects induced by K^+ or Na^+ at membrane surfaces or with intracellular phospholipids (as well as decreased pH), which would stabilize $HO_2\cdot$ relative to $O_2^{\cdot-}$, should accelerate lipid peroxidation. A corollary to this hypothesis is that the superoxide species produced by the spermatozoa play a key role in spontaneous lipoperoxidation. This hypothesis is currently under experimental testing.

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