Toxic Effect and Action of Dead Sperm on Diluted Bovine Semen

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Abstract

The toxic effect of dead sperm on diluted bovine semen and the modifying effect of certain compounds on this toxicity were investigated.

Toxicity is associated with an amino acid oxidase which becomes active only after death of sperm. A metabolic product of this enzyme, peroxide, is responsible for the toxic effect of dead sperm. The enzyme is active against aromatic amino acids only. Its toxic effect is enhanced by increasing egg yolk, inhibited by ethylenediaminetetra-acetate (EDTA), and eliminated by catalase. Inhibition of toxicity by EDTA is by protection of sperm against deleterious effects of peroxide and not by inhibition of the enzyme. The enzyme is inhibited by ethyl maleimide indicating active sulphydryl groups. It is labile to heat being inactivated by heating to 90 C for 2 minutes. It is also labile to pH 3 and pH 9. An active fraction was obtained from dead sperm by precipitation with 30% acetone. Activity of ejaculated dead sperm is only slightly less than freshly killed sperm. Practical implications are discussed.

Introduction

Shannon (12) reported that freshly killed dead sperm reduced livability of sperm in diluted bovine semen. The present study was initiated to define more clearly this effect and mode of action and reports four series of experiments. These were designed to define modifying effects of various compounds on the toxicity of dead sperm, effect of treatments on toxicity, toxicity of extracts from dead sperm, and the mode of action of the toxic factor.

Materials and Methods

Effects of three compounds in two concentrations on killed sperm toxicity was tested in three separate experiments. Compounds and proportions were a) egg yolk (5 and 20%), b) catalase (0 and 4.5 μg/ml) and c) EDTA (0 and 2 mg/ml.) Diluents were further subdivided so that within each proportion of compound one-half contained 0 and the other half 5 million killed sperm per milliliter. The diluent was Caprogen (12) and contained 20% egg yolk with exception of the 5% egg yolk. EDTA was in the form of the tetrasodium salt. Catalase was prepared from beef liver by the method outlined by Sumner and Myrback (16).

Killed sperm were obtained by freezing freshly ejaculated sperm at −79 C. Ejaculates were of good quality from bulls of known high conception rates with a minimum concentration of 1,200 $\times$ 10^6 sperm per milliliter. Split ejaculates were diluted in the experimental diluents to contain 10 million total ejaculated sperm per milliliter. Diluents which contained killed sperm, therefore, contained 10 million ejaculated sperm per milliliter + 5 million dead sperm.

Livability was measured by hours survival at 37 C. The method was an adaption of one previously described (12). Instead of the end point being fixed at 10% motile sperm, it was redefined as the time at which only one million sperm were motile. Numbers of motile sperm were determined every 4 hr except where stated.

Effect of Sperm Treatment

Killed sperm were subjected to three treatments.

Ethyl maleimide. Direct addition of ethyl maleimide was not possible because of its toxic action on sperm. However, ethyl maleimide combines with sulphydryl groups (1, 9) in which form it is apparently stable and not toxic (15). Ethyl maleimide (.03%) was added to samples of killed sperm. A sample was left for 1 hr at 25 C and then to overcome the toxic effect of the compound, was dialyzed against several changes of 14G (11)[1] at 2 C to remove excess ethyl maleimide. A sample of the original untreated killed sperm was dialyzed in a similar manner.

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[1] 2.0% Na citrate, 1.0% glycine, 1.0% glyceral, .3% glucose.

Heat. Killed sperm were heated at 90 C for 2 min.

Hydrogen ion concentration. Killed sperm were suspended in 2.9% Na citrate pH adjusted to 6.8, 6.1, 5.4, 4.0, and 3.0. After incubation for 12 hr at 25 C, they were dialyzed against 14G for 18 hr at 2 C. In a subsequent experiment samples were incubated at pH 6.8 or 9 for 12 hr and then dialyzed as stated.

The basic diluent was Caprogen plus 20% egg yolk. For a) and b) the experimental diluents were Caprogen with no additions, Caprogen plus 5 million killed treated sperm, and Caprogen plus 5 million killed untreated sperm. In the pH treatment experiment the diluents were Caprogen with no additions, and Caprogen plus 5 million killed sperm treated at the various pH. Livability was as above except that the interval between determinations was 2 hr.

Extracts of dead sperm. Extracts of dead sperm were obtained by centrifuging freshly collected semen at 475 × g for 20 min. Seminal plasma was then discarded, and the sperm were resuspended to original volume in 14G (11). Sperm were then recentrifuged as aforementioned, and the supernatant was discarded. Sperm were resuspended to half the original volume and killed by freezing at −79 C. After thawing the suspension stood overnight at 2 C and centrifuged at 38,000 × g for 15 min. The supernatant was filtered through a .8 μ Millipore filter to remove any extraneous sperm, and dialyzed against two changes of 100 volumes of distilled water for 24 hr at 2 C.

During dialysis a water insoluble precipitate developed which was separated from the supernatant water soluble fraction by centrifuging at 10,000 × g for 15 min. The precipitate remaining after decanting the water soluble fraction was dried with several washes of acetone at 0 C.

The water soluble fraction was filtered and precipitated with 6 volumes of acetone at 0 C. It was then centrifuged at 10,000 × g for 10 min, the acetone was decanted, and the remaining precipitate was dried with several changes of acetone at 0 C. Yields of water soluble and insoluble extracts were .6 and .5 g per liter of freshly collected material.

The water insoluble fraction was further fractionated. One gram of water insoluble material was dissolved in 20 ml of 14G (11) and precipitated sequentially with 25, 30, and 35% acetone. Each acetone precipitate was dialyzed against water at 2 C. The water insoluble precipitate was recovered and dried as indicated.

Two experiments compared a) toxicity of water soluble and insoluble components and b) toxicity of the three acetone fractions. The experimental diluents were Caprogen plus 20% egg yolk, and Caprogen plus 20% egg yolk with .02% of the various extracts.

Split ejaculates were diluted to contain 10 million total sperm per milliliter. Livability was measured in hours at 37 C (12).

Mode of Action

Amino acid oxidase activity was estimated by incubating 2 ml of test sample (sperm or acetone fractions) with 2 ml of a solution of amino acid dissolved in 2.9% Na citrate pH adjusted to 6.72. Because sperm have a low capacity to remove peroxide (19), oxygen uptake due to amino acid degradation was measured by adding catalase into the test system after 15 min. The O_2 evolved by catalase equals half the total O_2 consumed in amino acid degradation. Oxygen consumption was measured on a Yellowsprings 55 oxygen monitor.

Oxygen consumption is expressed as microliters per hour consumed by 50 × 10^6 sperm, due to amino acid degradation, at 37 C. The percentage of dead sperm was determined by staining with nigrosin eosin.[2] To obtain samples with 100% dead sperm, freshly ejaculated samples were killed as aforementioned.

Results

Egg yolk percentage. The modifying effect of egg yolk on killed sperm toxicity is summarized in Table 1. The mean livability of diluted semen was reduced by 20 hr at 5% egg yolk and 28 hr at 20% egg yolk (egg yolk × killed sperm interaction $P<.01$). The toxic effect of killed sperm can be reduced by lowering the egg yolk.

[2] .5% Nigrosin .8% eosin 4.5% Na citrate dihydrate.

TABLE 1. Effect of egg yolk in Caprogen diluent on livability of bull sperm at 37 C in the presence or absence of additional killed sperm.[a]

Additional killed sperm	Egg yolk 5%	Egg yolk 20%	Mean
(no.)			
None	68	67	67
5 × 10^6 ml	48	39	43
Mean	58	53	55

[a] Mean livability in hours for one ejaculate from each of 16 bulls.

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TABLE 2. Effect of catalase on mean livability of bull sperm at 37 C[a] in the presence or absence of additional killed sperm.

Addition killed sperm	Catalase		
	Nil	+ Catalase[b]	Mean
(no.)			
None	64	96	80
5 × 10⁶ ml	36	92	64
Mean	50	94	72

[a] Mean livability in hours for one ejaculate from each of 8 bulls.
[b] 4.5 μg per milliliter.

Catalase. Two significant features are apparent from the catalase experiment in Table 2. Catalase increased mean livability of diluted semen by 44 hr (P<.01) and eliminated toxicity of killed sperm (catalase × killed sperm interaction P<.01). Killed sperm reduced livability by 28 hr (P<.01) in the absence but only 4 hr (P>.05) in the presence of catalase.

Ethylenediaminetetraacetate. This complexing salt was included in diluents to test the hypothesis that toxicity was dependent on divalent metal ions. Results in Table 3 closely resemble those with catalase. EDTA increased the life of diluted samples by 42 hr (P<.01) and reduced the difference between the control and killed sperm addition from 36 hr to 13 hr. (Interaction EDTA × killed sperm (P<.01).

Ethyl maleimide. The association between toxicity and sulphydryl groups was tested by treatment of killed sperm with ethyl maleimide. Livability of 12 bulls, split ejaculates diluted in three experimental diluents was, Caprogen 66 hr, Caprogen + 5 million killed ethyl maleimide treated sperm 60 hr, and Caprogen + 5 million killed untreated sperm 40 hr. Treatment of killed sperm with ethyl maleimide increased livability by 20 hr (P<.01) but did not entirely abolish toxicity (difference between control and ethyl maleimide treatment was 6 hr P<.025.)

TABLE 3. Effect of ethylenediaminetetraacetate on mean livability of bull sperm at 37 C in the presence or absence of additional killed sperm.[a]

Additional killed sperm	EDTA		
	Nil	+ EDTA[b]	Mean
(no.)			
None	57	87	72
5 × 10⁶ ml	21	74	47
Mean	39	81	60

[a] Mean livability in hours for one ejaculate from each of 8 bulls.
[b] 2 mg per milliliter.

Heat treatment. Split ejaculates of eight bulls were diluted in three experimental diluents. The diluents and livabilities were Caprogen 63 hr, Caprogen + 5 million killed sperm heated to 90 C 61 hr, and Caprogen + 5 million untreated killed sperm 31 hr. Heating dead sperm increased livability by 30 hr (P<.01). The difference between the control and heat treated sperm was not significant.

Hydrogen ion concentration. Split ejaculates of 12 bulls were diluted in Caprogen with no additions or 5 million killed sperm treated at various pH's. Livabilities of the samples were, Control 43 hr, pH 6.8 21 hr, pH 6.1 13 hr, pH 5.4 9 hr, pH 4.0 12 hr and pH 3.0 44 hr. Livability of semen diluted in samples treated at pH's 6.8 to 4 was less than the control and pH 3.0 (P<.01). The difference between pH 5.4 and pH 6.8 of 12 hr was also significant (P<.025). No other differences were significant. Treatment at pH 3.0 for 12 hr completely detoxified dead sperm. There is also some evidence that the toxic effect was enhanced by treatment for 12 hr at pH 5.4.

Killed sperm were also detoxified by treatment at pH 9.0. Split ejaculates of 4 bulls were diluted in Caprogen with no additions or plus 5 million killed sperm treated at either pH 6.8 or 9. Livability of sperm in the control diluent was 66 hr, pH 6.8 34 hr, and pH 9 64 hr. Livability of semen incubated with killed sperm treated at pH 6.8 was less than semen incubated in either the control diluent, (difference 32 hr (P<.01)) or with killed sperm treated at pH 9 (difference 30 hr (P<.01)).

Fractionation by acetone. Split ejaculates from 6 bulls were diluted in Caprogens with no addition, or .02% water insoluble fraction, or .02% water soluble fraction. Livabilities were, no addition 58 hr, .02% water insoluble fraction 26 hr, .02% water soluble fraction 60 hr. The differences of 32 hr between the water insoluble fraction and the control and 34 hr between the water insoluble and the water soluble fractions were significant (P<.01). The difference of 2 hr in favor of the water soluble fraction was not significant.

As all the toxicity was recovered in the water insoluble fraction, the three acetone fractions of this precipitate were tested. The livability of 16 bulls' semen split between the control diluent of Caprogen, and Caprogen + .02% of the three acetone precipitates was control

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diluent 43 hr, 25% acetone precipitate 18 hr, 30% acetone precipitate 12 hr, 35% acetone precipitate 40 hr. The 25% and 30% acetone precipitates reduced livability compared to either the control or the 35% precipitate ($P<.01$). There was no significant difference between the control diluent and the 35% acetone precipitate.

The toxic fractions behaved in the same manner as killed sperm. That is, their toxicity was increased by increasing egg yolk, significantly reduced by both EDTA and ethyl maleimide, eliminated by heat treatment and catalase, and was labile to pH 3.0 and pH 9.0.

Mode of Action

The experiments are consistent with the hypothesis that the toxic factor is a peroxide producing enzyme. The protective action of catalase and the lability of the factor to certain treatments, especially the binding of SH groups support this view.

A series of investigations determined the substrate(s) for peroxide formation and activity of acetone extracts, the effect of various treatments on peroxide formation by killed sperm, and the relative activity of live and killed sperm.

Substrates and activity of acetone extracts. Tosic (13) and Tosic and Walton (14, 15) demonstrated an amino acid oxidase in sperm which was active against aromatic amino acids only. The amino acid oxidase activity of killed sperm was determined as it seemed likely that their toxicity could be due to this enzyme.

Killed sperm were active against phenylalanine and tryptophan, but not against glycine, alanine, or serine. Because of the low solubility of tyrosine, it was not possible to test the activity of the enzyme against this amino acid.

Amino acid oxidase activity was also determined for acetone fractions prepared from killed sperm. The 25% and 30% fractions were active, whereas the 35% fraction and water soluble fraction were inactive against phenylalanine.

Effect of treatments on peroxide formation. Ethyl maleimide significantly reduced the amino acid oxidase activity of freshly killed sperm. Sperm from four split ejaculates were killed by freezing. Killed sperm 50×10^6 per ml were incubated in either a 5% saturated phenylalanine solution or the same test solution + .03% ethyl maleimide. Oxygen consumption due to phenylalanine degradation was 7.2 μliters/hr for the control sample and 2.5 μliters/hr for the sample incubated in the presence of ethyl maleimide (difference 4.7 μliters/hr $P<.01$). Heating to 90 C for 2 min and treatment at pH 3.0 or 9.0 abolished oxidase activity. EDTA had no effect on amino acid oxidase activity of killed sperm but did appear to maintain livability of ejaculated sperm incubated in the presence of phenylalanine.

The inability of EDTA to inhibit activity of the enzyme excluded the possibility that this activity was dependent on divalent metal ions. Failing inhibition of the enzyme EDTA seemed likely to have protected sperm from the adverse effects of peroxide.

To test this hypothesis split ejaculates of 6 bulls were diluted in a citrate-glucose buffer with varying EDTA and H_2O_2. Results in Table 4 show that while H_2O_2 depressed O_2 consumption by 3.3 μliters/hr ($P<.01$) its effect was much reduced in the presence of EDTA. Peroxide depressed O_2 consumption by 1.2 μliters/hr in the presence of EDTA and by 5.3 μliters/hr in its absence. (Interaction EDTA $\times$ H_2O_2 $P<.01$).

Relative activity of live and dead sperm. Oxygen consumption due to phenylalanine degradation by 50 million killed sperm was 10 μliters/hr compared to 2.4 μliters/hr for 50 million ejaculated sperm (4 split ejaculates, average percentage live of ejaculated samples = 79%). The difference between killed and ejaculated sperm in O_2 consumption, due to oxidase activity was highly significant ($P<.01$). The O_2 consumption of the ejaculated samples would be consistent with the premise that amino acid oxidase activity was restricted to dead sperm in the ejaculated material. The difference in activity between live and dead sperm was further investigated.

Oxygen consumption due to amino acid oxidase activity of 17 samples was determined. Eight samples were fresh ejaculates with a range of from 15% to 58% dead, five were freshly collected samples mixed with varying amounts of freeze killed sperm to obtain samples with a

TABLE 4. Oxygen consumption of sperm suspended in 2.9% Na citrate + .3% glucose with different proportions of ethylenediaminetetraacetate and hydrogen peroxide.[a,b]

EDTA	H_2O_2 Nil	H_2O_2 .0003%	Mean
	(μliters O_2/50 $\times$ 10^6 sperm/ml)		
0	10.0	4.7	7.4
.2%	10.9	9.7	10.3
Mean	10.5	7.2	8.9

[a] All diluents pH adjusted to pH 6.8.

[b] All suspensions stood for 4 hr at 20 C before estimation of oxygen consumption.

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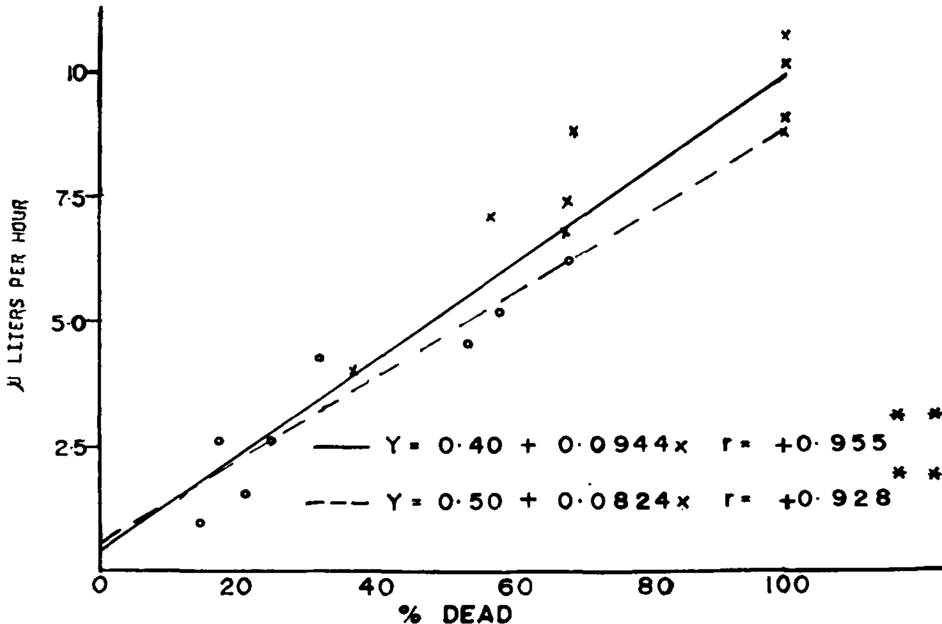

FIG. 1. Oxygen consumption due to phenylalanine degradation by samples of sperm with varying amounts of killed and dead sperm in 50% saturated phenylalanine solution. O, Samples with ejaculated dead sperm only. ×, Samples with a mixture of ejaculated dead sperm and freshly killed sperm. ——, Regression on all samples. - - -, Regression on ejaculated samples. ++, $P < .01$.

range of from 36% to 68% killed and dead sperm, and 4 were samples killed by freezing. Samples were incubated in a 50% saturated phenylalanine solution. Results are in Fig. 1.

There was a highly significant correlation of + .955 ($P<.01$) between the percentage of dead (killed + ejaculated dead) sperm and amino acid oxidase activity. Estimates of O_2 consumption due to amino acid oxidase activity derived from the regression were .4 μliters/hr for 50×10^6 live sperm and 9.84 μliters/hr for 50×10^6 dead sperm. When only the eight untreated ejaculated samples, with varying percentages of dead sperm, were considered the comparable estimates were 0.50 μliters/hr and 8.74 μliters/hr ($Y = .5 + .0824x$. $r = .96$ $P<.01$).

Oxygen consumptions of samples consisting of a mixture of ejaculated and freeze killed dead were adjusted for the percentage of ejaculated dead sperm by the aforementioned regression. The regression of the adjusted figures (nine samples) was $Y = .74 + .0894x$ ($r = .942$ $P<.01$). The estimate of O_2 consumption from the regression, for 100% freshly killed sperm was 9.68 μliters/hr. The estimated O_2 consumption due to amino acid oxidase activity of ejaculated dead sperm (8.74 μliters/hr) was 90% of that of freshly killed dead sperm.

High phenylalanine in the test solution reduced motility of live sperm during the test. In a further experiment therefore, phenylalanine was reduced from 50% saturation to 5% saturation. Six samples were tested, five normal ejaculates with a range of 9% to 37% dead and one sample killed by freezing. The results are in Figure 2.

Regressions were highly significant ($P<.01$). All the amino acid oxidase activity was associated with dead sperm. The O_2 consumption of ejaculated dead sperm due to phenylalanine degradation (5.96 μliters/hr) was 83% of the freshly killed sample (7.22 μliters/hr).

Discussion

Tosic (17) and Tosic and Walton (18, 19) demonstrated that bovine sperm were capable of oxidizing aromatic amino acids. Our study shows that this activity is either confined to, or very much greater in, dead than in live sperm.

The enzyme is labile to heat and pH 3.0 and pH 9.0. It is dependent on SH groups for full activity as shown by the reduction both in toxicity and phenylalanine degradation of killed sperm treated with ethyl maleimide. Regressions on amino acid oxidase activity indicate that ejaculated dead sperm have a slightly lower oxidase activity than freshly killed sperm. How-

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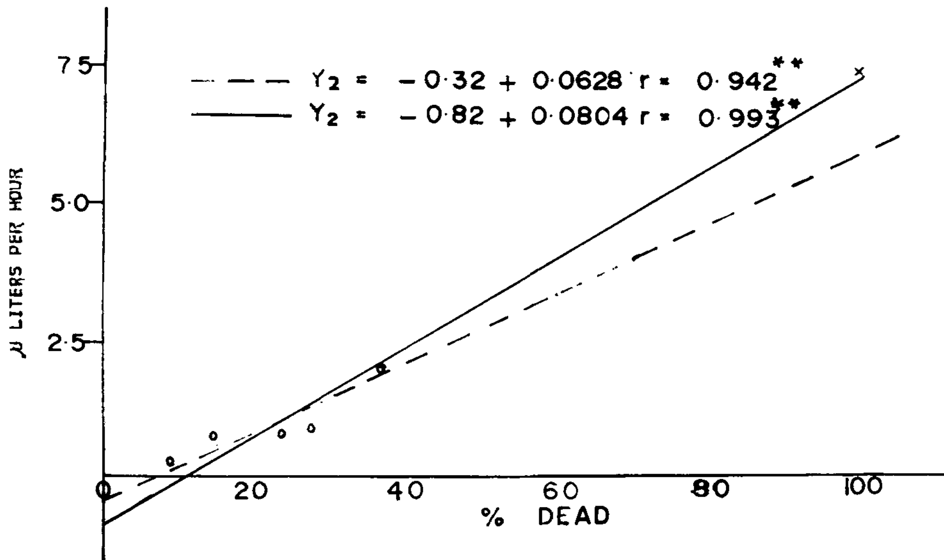

FIG. 2. Oxygen consumption due to phenylalanine degradation by samples of sperm with varying amounts of killed and dead sperm in 10% saturated phenylalanine solution. ○, Samples with ejaculated dead sperm only. ×, Samples with mixture of ejaculated dead sperm and freshly killed sperm. ——, Regression based on all samples. – – –, Regression based on ejaculated samples. ++, $P < .01$.

ever, as ejaculated dead sperm have been subjected to elevated temperatures before ejaculation, the activity is remarkably high. Studies on the stability of the enzyme at body temperatures may yield a worthwhile biochemical test to determine the time of death of sperm in the male genital tract. Preliminary investigations in this laboratory indicate that death probably occurs a few days prior to ejaculation.

Clearly the toxic effect of dead sperm is due to their amino acid oxidase activity. Treatments which destroy or inhibit oxidase activity (e.g. ethyl maleimide, heat treatment, acid or alkali treatment) also eliminate or inhibit toxicity. The action is indirect being mediated through peroxide, a degradation product of aromatic amino acid oxidation. Substances which protect sperm from peroxide, such as catalase or EDTA also protect them from harmful effects of dead sperm.

The enhanced toxicity associated with increased egg yolk is probably due to the elevated substrate available for peroxide formation (10). While catalase is present in egg yolk (3) and could be expected to mask the effect of increased substrate, the amount is very small. Even storage at 5 C, significant responses in survival of sperm in egg yolk diluents have been reported (2, 4, 5). The very marked response in our study could be related to incubation at 37 C which would stimulate enzyme activity. The effect of temperature on response is probably of some importance for while workers have failed to demonstrate a significant fertility response to added catalase in egg yolk diluents stored at 5 C (5, 6), we have obtained significant responses in fertility with the addition of catalase to diluted semen used at ambient temperatures (7).

Knowledge of the mode of action of the enzyme has considerable practical application. Deep frozen semen has a limited life when thawed. Indeed, addition of thawed frozen semen to a freshly diluted sample reduces livability similar to dead sperm (13). Presumably the effect is due to the large number of sperm killed in the freezing process.

We have been interested in developing rediluted deep frozen semen for use at ambient temperatures. However, toxicity of the enzyme at this storage temperature was a serious limiting factor in this program.

By appropriate measures to reduce the toxicity of the enzyme, by reducing egg yolk from 20% to 5%, by saturating the diluent with N_2 to reduce the available O_2 for its activity, and by including catalase in the diluent, thawed rediluted deep frozen semen has been successfully used on a large scale in New Zealand for 3 years (7, 8, 14). Successful results have also been obtained with exports of this material to Australia.

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