Kinetics of the aromatic L-amino acid oxidase from dead bovine spermatozoa and the effect of catalase on fertility of diluted bovine semen stored at 5°C and ambient temperatures

P. Shannon and B. Curson

New Zealand Dairy Board Artificial Breeding Centre, Newstead, R.D.4, Hamilton, New Zealand

Summary. At limiting velocity 50×10^6 dead spermatozoa consumed $13.6 \mu\text{l O}_2/\text{h}$ and degraded 6.1×10^{-7} mol L-phenylalanine/h. Substrate concentration in egg yolk was equivalent to 6.1×10^{-3} M-L-phenylalanine. At a substrate concentration of 3×10^{-2} M-L-phenylalanine, oxidase activity was significantly affected by oxygen tension ($r = 0.947$, $P < 0.01$) and temperature ($r = 0.995$, $P < 0.01$). Non-return rates were not affected when semen was stored at 5°C in 20% egg yolk diluents with catalase but were significantly increased with semen stored at ambient temperatures of 15–23°C.

Introduction

Tosic & Walton (1950) reported that spermatozoa contain an aromatic L-amino acid oxidase (no EC number). One of the end products of L-phenylalanine degradation, H_2O_2 , is toxic to spermatozoa (Tosic & Walton, 1950; Mann, 1964: pp. 384–385; Shannon & Curson, 1972). Tosic & Walton (1946) also demonstrated that egg yolk, a common constituent of bovine semen diluents, contained dialysable substrates for the enzyme. Although peroxide was produced from egg yolk by bovine spermatozoa, neither reducing the substrate concentration by dialysis of egg yolk (Tosic & Walton, 1946) nor eliminating peroxide by the inclusion of catalase in semen diluents (Hafs 1961; Foote & Dunn, 1962) significantly affected fertility when semen was stored in egg-yolk diluents at 5°C. However, Shannon & Curson (1972) reported that the enzyme is active only after death of spermatozoa which has enabled a more critical evaluation of the enzyme's properties. The objectives of the present study were to determine (1) activity of the enzyme at various substrate concentrations, oxygen tensions and temperatures; (2) substrate concentrations in egg yolk; and (3) the fertility of diluted bovine semen with and without catalase at 5°C and ambient temperatures.

Materials and Methods

Oxidase activity

Dead spermatozoa were obtained by freezing freshly ejaculated spermatozoa at approximately –80°C. Sperm concentrations were estimated using an absorbtimeter which had been previously calibrated with haemocytometer counts. (Spectronic 20, Bausch & Lomb Inc; Rochester N.Y. U.S.A.)

Oxidase activity was measured by the method of Shannon & Curson (1972). Except when otherwise stated, 50×10^6 dead spermatozoa/ml were incubated in 0.1 M-sodium citrate, pH 7, with various amounts of L-phenylalanine. Oxygen uptake due to amino acid degradation was measured by adding catalase to the test system when oxygen tension was 70% of air saturation. The oxygen evolved due to addition of catalase equals half the total oxygen consumed in amino acid degradation. Oxygen consumption was measured by an oxygen probe electrode with the test solution being sealed from air contact (model 55 oxygen monitor, Yellow Springs Inst. Co., Yellow Springs, Ohio, U.S.A.).

Effect of substrate concentration. Oxidase activity was measured at 37°C with 50×10^6 dead spermatozoa/ml at L-phenylalanine concentrations of 3×10^{-4} , 1.5×10^{-3} , 7.5×10^{-3} , 1.5×10^{-2} and 3×10^{-2} M.

Substrate concentration in egg yolk. Egg-yolk substrates were prepared by dialysing 1 volume of egg yolk against 3 volumes of H₂O for 48 h at 5°C. The diffusate was adjusted to contain 0.1 M-sodium citrate and the oxidase activity of dead spermatozoa suspended in the diffusate was determined at 37°C.

Oxygen tension. Oxygen tension was modified by gassing diluents with nitrogen for various periods and was measured against air-saturated buffer at 37°C and recorded as a percentage of 100% air saturation. The scale on the oxygen meter was then expanded so that the reading was 100 at the start of the test. Substrate concentration was 3×10^{-2} M.

Temperature. The oxidase activity in 50×10^6 dead spermatozoa/ml was determined at 5°C intervals from 15 to 50°C. Oxygen consumption could not be detected below 15°C by the assay method and because of low activity at 15°C the sperm concentration was increased to 200×10^6 spermatozoa/ml. Substrate concentration was 3×10^{-2} M.

Fertility trials. All semen was diluted in Caprogen (Shannon, 1965) plus 20% egg yolk. Catalase, when added, was at 4.5 µg/ml. Catalase (EC 1.11.1.6) was prepared in crystalline form in this laboratory (see Shannon & Curson, 1972). Oxygen tension in diluted semen issued to technicians was approximately 40% of air saturation. Four trials were conducted. In all trials semen was diluted to contain 5×10^6 spermatozoa/ml: 0.5 ml was used per insemination so that the insemination dose was 2.5×10^6 spermatozoa.

In Trial 1 ejaculates from 10 bulls were divided and diluted in Caprogen or Caprogen + catalase. Semen from one bull was used on each day of the experiment. All technicians received both diluents on each day of the experiment. Practical considerations dictated that technicians used semen issued on an experimental day at one storage temperature only. Thus half the technicians used semen issued on one day at 5°C and the other half at ambient temperatures. On the next day of the experiment, temperature of use by technicians was reversed. The experiment ran for 10 days, during which time each technician received equal amounts of semen in both diluents, which on 5 occasions they used at 5°C, and the remaining 5 at ambient temperatures.

The trial was conducted in May and June (autumn) and ambient temperatures varied between 10 and 15°C. The age of the semen at time of use was 30–54 h. Conception rate was considered to be the percentage of cows not returning to oestrus by 24 days after the first service.

In Trial 2, ejaculates from 15 bulls were divided and diluted in Caprogen or Caprogen + catalase, and the semen was 30 h old when used.

In Trial 3, catalase was incorporated in all diluted semen on one day of each week during 9 weeks in October, November and December. Semen was routinely collected from each bull (N = 19) every 3 days so that in each 3-week period semen was diluted 6 times in diluents without and once in diluent with catalase.

The protocol of Trial 4 was the same as that for Trial 3 except that catalase was incorporated in diluents on 6 days of the week and not on the seventh. A total of 23 bulls was used. In Trials 2, 3 and 4 ambient temperatures were between 15 and 23°C. Estimates of fertility were 49-day non-return rates to first service. Data were analysed by analysis of variance techniques outlined in Snedecor & Cochran (1972).

Results

Oxidase activity

Substrate concentration. There was no detectable oxygen uptake when dead spermatozoa were incubated in the buffer without L-phenylalanine. The apparent Michaelis–Menton constant was therefore derived by the method given by Mahler & Cordes (1966). The correlation between V and s/v where V = reaction velocity and s = molar substrate concentration was -0.976 ($P < 0.01$). The Michaelis–Menton constant was 3.28×10^{-3} M and at limiting velocity 50×10^6 dead spermatozoa consumed $13.6 \mu\text{l O}_2$ and degraded 6.1×10^{-7} mol L-phenylalanine/h. Oxidase activity determined by the Briggs–Haldane equation, $Vs/(s+K_m)$, where V = limiting velocity, s = substrate concentration, and K_m = Michaelis–Menton constant, was significantly correlated ($r = 0.996$, $P < 0.01$) with actual activity (see Table 1).

Table 1. The effect of L-phenylalanine concentration on O_2 consumption by dead bovine spermatozoa

Conc. L-phenylalanine (M)	O_2 consumption ($\mu\text{l/h}/50 \times 10^6$ dead spermatozoa)
3×10^{-2}	12.1 ± 0.2
1.5×10^{-2}	11.3 ± 0.2
7.5×10^{-3}	9.3 ± 0.2
3×10^{-3}	7.0 ± 0.2
1.5×10^{-3}	3.8 ± 0.1
3×10^{-4}	1.2 ± 0.1

Values are the mean $\pm$ s.e.m. of 12 replicates.

Substrate concentration in egg yolk. Oxygen consumption due to aromatic amino acid degradation by 50×10^6 dead spermatozoa in egg yolk diffusates was 2.9, 4.2, 4.9 and $5.1 \mu\text{l O}_2/\text{h}$ in 4 experiments. The estimated L-phenylalanine equivalent was between 8.9×10^{-4} and 2.0×10^{-3} M with a mean of 1.5×10^{-3} M. The diffusate was equivalent to 25% egg yolk, and the estimated L-phenylalanine equivalent in whole egg yolk is 6.1×10^{-3} M.

Oxygen tension. The effect of oxygen tension on oxidase activity is shown in Table 2. The relationship between oxidase activity and oxygen tension is asymptotic ($y = -16.75 + 9.41x^{0.25}$) ($r = 0.947$, $P < 0.01$) where y = O_2 consumption in $\mu\text{l/h}$ and x = % air saturation. Predicted activity at 20 and 40% air saturation is 30% and 55% of activity at 100% air saturation.

Table 2. The effect of O_2 tension on O_2 consumption ($\mu\text{l/h}/50 \times 10^6$ dead spermatozoa) due to L-phenylalanine degradation

O_2 tension	O_2 consumption	O_2 tension	O_2 consumption	O_2 tension	O_2 consumption
100%	12.1	60%	9.7	43%	6.7
100%	11.4	58%	8.8	43%	6.3
85%	12.1	54%	10.4	41%	8.0
81%	12.0	52%	8.5	31%	5.6
79%	11.8	45%	7.8	31%	4.6
69%	12.0	44%	7.9	23%	3.5

Temperature. Enzyme dependence on temperature is shown in Table 3. The effect of temperature on oxidase activity is given by $\log \hat{y} = -4.1007 + 3.2914 \log t$ ($r = 0.995$, $P < 0.01$) where t = $^\circ\text{C}$ and $\hat{y}$ = $\mu\text{l O}_2/\text{h}$. Activity was very dependent on temperature.

Table 3. The effect of temperature on O₂ consumption due to L-phenylalanine degradation

Temperature (°C)	O ₂ consumption (µl/h 50 × 10 ⁶ dead spermatozoa)
50	31.1 ± 0.4
45	20.5 ± 0.5
40	13.7 ± 0.2
35	10.2 ± 0.3
30	5.3 ± 0.3
25	4.1 ± 0.2
20	1.6 ± 0.1
15	0.3 ± 0.1

Values are the mean ± s.e.m. of 6 replicates.

Fertility trials

There was an interaction between temperature of storage and catalase addition ($P < 0.05$), with catalase depressing the non-return rates of semen used at 5°C (−3.1%) and increasing them with semen used at ambient temperatures (+0.4%) (Table 4). When semen was used at ambient temperatures of 15–23°C (Table 5) catalase significantly increased non-return rates with semen used at a mean age of 9 h (0.93 ± 0.25 , $P < 0.01$, combined results of Trials 3 and 4) and 30 h (1.3 ± 0.58 , $P < 0.025$, Trial 2, and 1.73 ± 0.20 , $P < 0.01$, combined results Trials 3 and 4). Although the results indicate a greater response to catalase inclusion for semen used at a mean age of 30 h at time of use rather than at a mean age of 9 h, the results cannot be strictly compared because semen of different ages was used by different technicians. However, Trial 2 was conducted with technicians who normally used semen aged 9 h.

Table 4. Percentage non-return rates at 24 days after inseminations with semen stored at 5°C or ambient temperatures with or without catalase (Trial 1)

Catalase	Temperature of storage					
	5°C		Ambient (10–15°C)		Mean	
	No. of cows inseminated	Non-return rate %	No. of cows inseminated	Non-return rate %	No. of cows inseminated	Non-return rate %
0	2088	82.5	2096	83.3	4184	82.9
4.5 µg/ml	2007	79.4	2047	83.7	4054	81.6

Difference between diluents N.S. Difference between storage temperatures N.S. Interaction of storage temperature and diluent, $P < 0.05$.

Table 5. The effect of catalase on percentage non-return rates at 49 days after insemination (no. in parentheses) with diluted semen stored at ambient temperatures (15–23°C)

Trial	Semen aged 9 h			Semen aged 30 h		
	No catalase	+Catalase	Difference ± s.e.d.	No catalase	+Catalase	Difference ± s.e.d.
2	—	—	—	64.0 (13 682)	65.3 (14 169)	+1.3 ± 0.58 ^b
3	63.9 (94 671)	64.7 (16 117)	+0.8 ± 0.41 ^a	68.1 (148 286)	69.7 (27 854)	+1.6 ± 0.28 ^c
4	63.5 (30 233)	64.5 (146 337)	+1.0 ± 0.3 ^c	66.2 (44 126)	68.0 (23 010)	+1.8 ± 0.25 ^c

^a $P < 0.05$; ^b $P < 0.025$; ^c $P < 0.01$.

Discussion

Some general characteristics of the bovine aromatic L-amino acid oxidase have been reported before, namely that the enzyme is active only in dead spermatozoa, is heat, acid and alkali labile and depends on free sulphhydryl groups for activity (Shannon & Curson, 1972). The present kinetic studies show that, at full air saturation of the buffer and at limiting velocity, 50×10^6 dead spermatozoa produce 6×10^{-7} mol H_2O_2 /h. The rate of substrate degradation is similar to that of fructose (see Mann, 1964: p. 294). Unlike respiration of live spermatozoa, which appears not to be affected by oxygen tension until very low levels of oxygen tension are reached (see Mann 1964: pp. 272–273), the activity of the oxidase is affected by oxygen tension. Activity at 40 and 20% of air saturation was reduced by 45% and 70% respectively.

The rate of H_2O_2 evolution is governed by the number of dead spermatozoa, substrate concentration, oxygen tension and temperature. In a closed system such as a tube filled and sealed for transport prior to artificial insemination the maximum possible concentration of H_2O_2 will also depend on the proportion of dead spermatozoa, because both live and dead spermatozoa compete for available oxygen. At a particular temperature the oxygen tension will be reduced at a constant rate by live spermatozoa (virtually independent of oxygen tension) and by the activity of the aromatic L-amino acid oxidase which is reduced as the oxygen tension decreases but which is increased as the H_2O_2 kills more spermatozoa. The time taken to reach the maximum possible concentration of H_2O_2 will be markedly affected by temperature of storage, the estimated rate of H_2O_2 evolution at 20°C being 95 times the rate at 5°C.

It is probably this effect of temperature which accounts for the difference in response to catalase at different temperatures, i.e. that catalase has no effect or depresses non-return rates at 5°C and increases them at ambient temperatures. Because of the small size of the present trials and the low significance reached, such a conclusion can only be tentative, but this postulate is supported by (1) the results of other workers who have shown that catalase does not increase fertility when semen is stored at 5°C (Hafs, 1961; Foote & Dunn, 1962), except possibly after prolonged storage (Foote & Dunn, 1962); and (2) the consistently positive fertility response we have obtained to catalase inclusion in diluents when semen is stored at ambient temperatures. It follows that catalase is unlikely to be of benefit with deep-frozen semen which is stored at 5°C for only a short period before freezing, and is inseminated immediately after thawing (see Foote, 1976). Conversely, catalase inclusion may be valuable when frozen semen is thawed, further diluted and stored at ambient temperature before use (Shannon, 1972), because of the high proportion of dead spermatozoa present after thawing.

References

- Foote, R.H. (1976) Fertility of bull semen frozen with β -amylase, β -glucuronidase and catalase. *J. Dairy Sci.* **59**, 2014–2017.
- Foote, R.H. & Dunn, H.O. (1962) Motility and fertility of bull semen extended at high rates in yolk extender containing catalase. *J. Dairy Sci.* **45**, 1237–1241.
- Hafs, H.D. (1961) Fertility of bull semen with added catalase. *J. Dairy Sci.* **44**, 1529–1536.
- Mahler, H.R. & Cordes, E.H. (1966) *Biological Chemistry*, pp. 228–229. Harper and Row, New York.
- Mann, T. (1964) *The Biochemistry of Semen and of the Male Reproductive Tract*. Methuen, London.
- Shannon, P. (1965) Contribution of seminal plasma, sperm numbers and gas phase to dilution effects of bovine spermatozoa. *J. Dairy Sci.* **48**, 1357–1361.
- Shannon, P. (1972) The diatube method of freezing semen for use as rediluted deep freeze. *Proc. 7th Int. Congr. Anim. Reprod. & A.I., Munich* **4**, 1363–1367.
- Shannon, P. & Curson, B. (1972) Toxic effect and mode of action of dead sperm on diluted bovine semen. *J. Dairy Sci.* **55**, 614–620.
- Snedecor, G.W. & Cochran, W.G. (1972) *Statistical Methods*, pp. 258–298. Iowa State University Press, Ames.
- Tosic, J. & Walton, A. (1946) Effect of egg yolk and its constituents on the respiration and fertilising capacity of spermatozoa. *J. agric. Sci., Camb.* **37**, 69–76.
- Tosic, J. & Walton, A. (1950) Metabolism of spermatozoa. The formation and elimination of hydrogen peroxide by spermatozoa and effects on motility and survival. *Biochem. J.* **47**, 199–212.

Received 2 June 1981