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The Influence of some Fractions of Egg Yolk on the Survival of Ram Spermatozoa at 5°C

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Abstract

Ram spermatozoa were stored at 5°C in diluents containing various fractions of egg yolk prepared by dialysis, ultrafiltration and ion-exchange chromatography. They survived storage best in the presence of components of egg yolk which were non-dialysable and were not filtered through membranes which retained substances of molecular weight greater than 100 000. The substances isolated in peak B of the ion-exchange chromatogram of whole egg yolk described by Seideman *et al.* (1969) gave greater protection than those from other fractions from this chromatographic system. These data indicate that the low-density lipoprotein fraction of egg yolk is the most likely source of protection to ram spermatozoa against the effects of storage at 5°C.

Introduction

In an earlier paper (Watson and Martin 1973), evidence was presented which suggested that proteins in egg yolk protect ram spermatozoa against the detrimental effects of prolonged storage at 5°C. This paper presents further data on the molecular size of the active substances and their chromatographic behaviour.

Materials and Methods

The method of collection of semen, the composition of diluents and preparative procedures used in these experiments have been reported (Watson and Martin 1973). Semen was diluted 20-fold in all experiments. In these experiments the supernatant solution from a diluent containing 3·75% (v/v) egg yolk, centrifuged at 35 000 *g* for 30 min, was used as the reference diluent with which all treatments were compared. Samples were cooled from 30 to 5°C at a constant rate of 0·28°C per minute.

Dialysis was carried out in cellulose dialysis tubing of 10 mm flat width (Visking–Union Carbide). The non-dialysable portion of the egg yolk diluent was obtained by dialysing 10 ml of diluent containing 7·5% egg yolk against 90 ml of diluent containing no egg yolk for 48 h at 5°C. The dialysable portion was obtained by dialysing 10 ml of diluent containing no egg yolk against 90 ml of 7·5% egg yolk diluent. The theoretical reduction in concentration of the dialysable substances of egg yolk if equilibrium was attained was therefore 90% in the solution containing the non-dialysable substances and 10% in the one containing the dialysable substances. All diluents were adjusted to concentrations equivalent to those in the 3·75% centrifuged egg yolk diluent before the addition of semen.

Ultrafiltration (expts 2–4) was performed with 'Diaflo' equipment (Amicon–Membrane Filtration Industries) at room temperature, at filtration pressures recommended for each membrane. The retentate after filtration was rediluted to the original volume with diluent containing no egg yolk.

The chromatographic procedure of Seideman *et al.* (1969) with carboxymethylcellulose (CMC) was used to fractionate egg yolk proteins, and the protein concentration of the effluent from the column was estimated by absorption of light at 280 nm. Protein estimations in experiment 6 were performed by the method of Lowry *et al.* (1951).

Lower concentrations of egg yolk or its fractions were derived by dilution of portions of the solution with diluent containing no egg yolk, just prior to the addition of semen.

Analyses of variance were computed for each set of observations in the six experiments. In experiments 2, 3 and 4 the variance of the first-order interaction of treatments (i.e. A × B) was significantly greater than the higher-order interaction (A × B × C) used as the error term to calculate the variance ratios (F_1). In these cases the A × B variance was used as divisor [e.g. A/(A × B)] to test the significance of the treatment. Main effects and variance ratios are listed in Table 3 under F_2.

Results

The response of ram spermatozoa to the non-dialysable and dialysable portions of egg yolk was tested in experiment 1. The mean scores of activity of spermatozoa stored for 72 h in these fractions, used separately and recombined, are shown in Table 1, together with the analyses of variance.

Table 1. Experiment 1: Mean survival of ram spermatozoa in dialysable and non-dialysable portions of egg yolk diluent

All treatments stored for 72 h at 5°C

Treatment	0·15% egg yolk		0·75% egg yolk		3·75% egg yolk		Mean	
	Motility	% motile	Motility	% motile	Motility	% motile	Motility	% motile
1. Undialysed diluent	1·61	24·44	2·39	40·00	2·56	44·44	2·19	36·29
2. Non-dialysable portion	2·17	26·67	2·11	31·11	2·28	34·44	2·19	30·74
3. Dialysable portion	1·44	20·00	1·50	18·89	1·89	23·33	1·61	20·74
4. Recombination of 2 and 3	1·83	27·78	2·11	33·33	2·83	48·89	2·26	36·67
Mean	1·76	24·72	2·03	30·83	2·39	37·78		
Diluent without egg yolk							1·67	22·22

Analyses of variance

Source of variation	D.F.	Motility		Percentage motile	
		Variance	*F*	Variance	*F*
A. Treatments	3	7·36	5·53*	4466·67	10·48**
1 *v.* 4	1	0·22	0·17	5·56	0·01
1 and 4 *v.* 2 and 3	1	8·51	6·40*	9344·44	21·92***
2 v. 3	1	13·35	10·04**	4050·00	9·50**
B. Levels of egg yolk	2	10·63	7·99**	4608·33	10·81**
Linear	1	21·09	15·86**	9204·17	21·59***
Quadratic	1	0·17	0·13	12·50	0·03
C. Ejaculates	2	12·01	9·03**	12 508·33	29·34***
A × B	6	1·73	1·30	719·44	1·69
A × C	6	0·91	0·68	219·44	0·51
B × C	4	0·84	0·63	204·17	0·48
A × B × C (error)	12	1·33		426·39	

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

Those diluents containing either dialysable or non-dialysable preparations had significantly lower protective activity than the undialysed or recombined diluents

[orthogonal contrast, treatments 1 and 4 *v.* 2 and 3 (Table 2), $P < 0{\cdot}05$ (motility score); $P < 0{\cdot}001$ (percentage motile)]. Although the non-dialysable fraction had some activity, the dialysable fraction possessed none [contrast 2 *v.* 3 (Table 1), $P < 0{\cdot}01$ (both scores)].

Table 2. Mean survival of ram spermatozoa in diluents containing egg yolk separated by ultrafiltration

All treatments stored for 72 h at 5°C. Each experiment replicated six times with ejaculates from different rams

Treatment	0·15% egg yolk		0·75% egg yolk		3·75% egg yolk		Mean	
	Motility	% motile	Motility	% motile	Motility	% motile	Motility	% motile
Experiment 2: exclusion limit of membrane—molecular weight 10 000								
Unfiltered diluent	1·75	27·22	2·61	46·11	2·94	58·33	2·43	43·89
Retentate	1·97	27·67	2·39	41·67	2·69	54·44	2·35	41·26
Filtrate	1·30	17·22	1·11	15·00	0·94	15·00	1·12	15·74
Mean	1·67	24·04	2·04	34·26	2·19	42·59		
Diluent without egg yolk							1·22	17·78
Experiment 3: exclusion limit of membrane—molecular weight 30 000								
Unfiltered diluent	1·47	22·78	2·19	38·33	2·58	51·11	2·08	37·41
Retentate	1·69	28·89	2·08	36·11	2·47	42·78	2·08	35·93
Filtrate	1·11	16·11	1·06	17·78	1·22	21·11	1·13	18·33
Recombination	1·39	24·44	1·94	33·89	2·53	49·44	1·95	35·92
Mean	1·42	23·06	1·82	31·53	2·20	41·11		
Diluent without egg yolk							1·17	18·33

Ultrafiltration techniques were used to ascertain more precisely the molecular size of the protective substances in egg yolk. Scores of the survival of spermatozoa in the retained and filtered portions of diluents separated by membranes which nominally retained substances of molecular weight greater than 10 000 and 30 000 are shown in Table 2. Active substances were retained during filtration and the filtrates were devoid of protective activity. In analyses of variance following the same pattern as that given for experiment 1, all contrasts of filtrate *v.* retentate were significant even when the more stringent F_2 criterion was used. However, when a filter was used which retained substances of molecular weight greater than 100 000, there was a slight reduction in activity of the retained portion compared with the unfiltered diluent, and the filtrate did not possess any significant activity (expt 4, Table 3). Recombination of the filtrate and retentate restored the protective activity to a level comparable with the unfiltered diluent. A significant treatment × level interaction was seen ($P < 0{\cdot}001$), which reflected the differences in slope of the responses to the various treatments. When the variance ratios for main effects were recalculated with this interaction variance (F_2), their significance was substantially reduced or lost. Hence, this interaction is of prime importance in the interpretation of the results of this experiment. The significant treatment × ejaculate interaction ($P < 0{\cdot}001$) indicated the variability of the response of spermatozoa from individual rams to the various treatments.

The egg yolk proteins were fractionated on a column of CMC and three major components were isolated corresponding to peaks A, B, and C of Seideman *et al.*

(1969). Each of these fractions was reconcentrated by ultrafiltration to a final protein concentration of approximately 2 mg/ml by use of a membrane which retained substances of molecular weight greater than 30000. Because the solvent solutions used to elute these fractions from the column differed in composition from the standard diluent used for spermatozoa, semen was also suspended in portions of the solvent

Table 3. Experiment 4: Mean survival of ram spermatozoa in diluents containing egg yolk separated by ultrafiltration

Ultrafiltration through a membrane with an exclusion limit of molecular weight 100000. All treatments stored for 72 h at 5°C

Treatment	0·15% egg yolk		0·75% egg yolk		3·75% egg yolk		Mean	
	Motility	% motile	Motility	% motile	Motility	% motile	Motility	% motile
1. Unfiltered diluent	1·67	23·89	2·25	35·00	0·58	49·44	2·17	36·11
2. Retentate	1·56	22·22	2·08	32·22	0·39	37·78	2·01	30·74
3. Filtrate	1·53	21·67	1·33	18·33	1·25	18·89	1·37	19·63
4. Recombination	1·72	25·00	2·28	35·00	2·64	44·44	2·21	34·81
Mean	1·62	23·20	1·99	30·14	2·22	37·64		
Diluent without egg yolk							1·38	20·00

Analyses of variance

Source of variation	D.F.	Motility			Percentage motile		
		Variance	F_1 [A]	F_2 [B]	Variance	F_1	F_2
A. Treatments	3	24·58	41·66***	5·18*	9082·87	42·39***	4·38
1 *v.* 4	1	0·17	0·29	0·04	136·11	0·64	0·07
1 and 4 *v.* 2 and 3	1	40·50	68·64***	8·54	17112·50	79·87***	8·24*
2 *v.* 3	1	33·06	56·03***	6·97*	10000·00	46·67***	4·82
B. Levels of egg yolk	2	19·61	33·24***	4·14	11272·22	52·61***	5·43*
Linear	1	38·52	65·29***	8·13	22533·33	105·17***	10·85*
Quadratic	1	0·69	1·17	0·15	11·11	0·05	0·01
C. Ejaculates	5	11·05	18·73***		8621·39	40·24***	
A × B	6	4·74	8·03***		2075·93	9·69***	
A × C	15	3·26	5·53***		996·20	4·65***	
B × C	10	0·65	1·10		250·56	1·17	
A × B × C (error)	30	0·59			214·26		

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.
[A] Divisor for F_1 is A × B × C.
[B] Divisor for F_2 is A × B.

effluent corresponding to each egg yolk fraction. All solutions were adjusted to pH 7·0 and an osmolarity of 310 m-osmol by addition of appropriate amounts of solid sodium hydroxide and glucose before semen was added. As with all previous experiments, three levels of egg yolk and a diluent containing no egg yolk were also included for comparison. Table 4 shows the mean survival of spermatozoa after storage for 72 h at 5°C (expt 5) and the mean improvement in survival as a result of the presence of egg yolk or its fractions. Peak A was of little benefit to spermatozoa, while substances from peaks B and C gave a considerable protection. Since peak B was most effective in keeping spermatozoa viable during storage, this fraction was chosen for the estimate of potency relative to the diluent containing egg yolk.

For the estimate of relative potency (expt 6) peak B was obtained as before and the solution was adjusted to pH 7·0 and 310 m-osmol. A centrifuged egg yolk diluent was prepared from the same yolk. An estimate of the total protein concentration was made of the egg yolk diluent and the fraction, and the volume of the fraction

Table 4. Experiment 5: Survival of ram spermatozoa in diluents containing egg yolk fractions separated by ion-exchange chromatography

Fractions A, B and C all contained 2 mg protein per millilitre. All treatments stored for 72 h at 5°C. Three ejaculates were used as replicates in this experiment

Nature of additive	Additive present		No additive		Mean improvement[A]	
	Motility	% motile	Motility	% motile	Motility	% motile
0·15% egg yolk	2·28	30·00			0·84	14·44
0·75% egg yolk	2·50	40·00	1·44	15·56	1·06	24·44
3·75% egg yolk	2·89	64·44			1·45	48·88
Fraction A	2·06	23·33	1·78	18·89	0·28	4·44
Fraction B	2·28	40·00	1·28	16·67	1·00	23·33
Fraction C	2·28	31·11	0·94	12·22	1·34	18·98

[A] Score with additive minus score without additive.

Table 5. Experiment 6: Mean survival of ram spermatozoa in diluents containing egg yolk proteins and fraction 2 protein

All treatments stored for 72 h at 5°C

Treatment	0·14 mg/ml protein		0·70 mg/ml protein		3·50 mg/ml protein		Mean	
	Motility	% motile	Motility	% motile	Motility	% motile	Motility	% motile
Soluble egg yolk	1·81	27·92	2·25	37·92	2·88	55·00	2·31	40·28
Peak B	1·15	20·42	1·83	28·75	2·08	39·17	1·69	29·45
Mean	9·48	24·17	2·04	33·34	2·48	47·09		
Diluent without egg yolk							1·42	22·50

Summary of analyses of variance

Source of variation	D.F.	*F*(six-point assay) Motility	*F*(six-point assay) % motile	D.F.	*F*(five-point assay) Motility	*F*(five-point assay) % motile
Differences between substances	1	53·41***	47·96***	1	13·72**	13·06**
Common linear regression	1	91·11***	143·07***	1	51·38***	113·62***
Departure from parallelism	1	0·35	4·73*	1	2·87	1·06
Deviation from regression	2	1·72	1·24	1	0·54	2·29
Ejaculates	7	13·81***	26·12***			
Interactions	23	1·32	1·86			
Residual (error) variance	14	0·79	264·29			
Estimate of potency of peak B relative to egg yolk					0·135	0·204
95% fiducial limits					0·052–0·274	0·119–0·326

* $P < 0·05$. ** $P < 0·01$. *** $P < 0·001$.

was reduced by ultrafiltration to yield a total protein concentration equal to that of the egg yolk diluent. A six-point assay was set up using the response of ram spermatozoa after storage at 5°C for 72 h in these diluents; no controls other than diluent without egg yolk were incorporated in the experiment, since the adjusted solvent of peak B gave a survival of spermatozoa comparable with that of the diluent with no egg yolk.

The results and analyses of data (Table 5) show significant differences between treatments ($P < 0{\cdot}001$ on six-point assay; $P < 0{\cdot}01$ on five-point assay), and a significant common linear regression for levels of treatments ($P < 0{\cdot}001$). In the analysis of the six-point assay a significant departure from parallelism of the response to the treatments was seen for the percentage of motile spermatozoa ($P < 0{\cdot}05$). However, the lowest level of peak B was inactive, and when this was deleted from the analysis (five-point assay) the preparations showed similar parallel responses. The estimate of relative potency was thus made with the values for the five-point assay. Peak B protein had only 13·5 and 20·4% of the activity of total soluble egg yolk protein, in preserving the motility of spermatozoa and the percentage motile, respectively.

Discussion

In contrast to the findings of Tosic and Walton (1947) and Yassen and Foote (1967), dialysis of the diluent did not increase its beneficial effects on spermatozoa. In fact, dialysis reduced activity in both the non-dialysable and the dialysable fractions (expt 1). This experiment, together with those employing ultrafiltration, indicated that substances of high molecular weight in egg yolk are protective and, taken in conjunction with data previously reported (Watson and Martin 1973), constitute strong evidence that the proteins of egg yolk are involved.

Part of the effect of any protein added to a diluent could be due to its buffering action. The diluent used for chilling in this series of experiments contained a high proportion of metabolizable sugar (180 mM glucose, 17 mM fructose) and a low level of phosphate buffer (20 mM). Thus the metabolism of the spermatozoa could have caused the development of acid conditions and a depression of survival of spermatozoa. It was, however, unlikely that the effects of egg yolk or its fractions were due to their influence on pH in these experiments, for the following reasons:

(1) The dilution rate of 20-fold gave an average of about 100 million spermatozoa per millilitre. The metabolic activity of this concentration of spermatozoa at 5°C has not caused significant shifts in pH in other experiments (Martin and Watson, unpublished data);

(2) All samples were resuspended in diluent free of egg yolk after storage at 5°C and incubated at 37°C whilst scores of survival were made (Watson and Martin 1973). Thus pH was in the range 6·8–7·0 when the samples were being examined;

(3) Significant protective activity was observed at low protein levels of between 0·7 and 3·5 mg/ml. Accordingly, we consider that the egg yolk fractions tested have shown specific value in protecting living cells against the effects of lowered temperature.

The proteins of egg yolk have been divided into high- and low-density fractions by ultracentrifugation. The high-density fraction contains six proteins: phosvitin,

α-, β-, and γ-livetin, and α- and β-lipovitellin (Bernardi and Cook 1960). The low-density fraction, composed of lipoproteins, has not been finally characterized, but two components have been isolated (Martin *et al.* 1964; Saari *et al.* 1964).

Centrifugation of the egg yolk diluent precipitated an inactive fraction comprising approximately 25% of the total yolk solids (Watson and Martin 1973). This estimate is similar to the value of 23% for the egg yolk granules, which contain 70% lipovitellins, 16% phosvitin, and 12% low-density lipoprotein (Burley and Cook 1961). These authors concluded that one or more low-density lipoproteins form an integral part of the granules. Peak B from the chromatographic separation of egg yolk, which contained the greatest protective activity for spermatozoa (expt 5), did not contain the lipovitellins (Seideman *et al.* 1969). Thus the lipovitellins are probably not responsible for the protective activity of egg yolk.

Seideman *et al.* (1969) concluded that peaks B and C contained some phosvitin. Although these were the fractions containing protective activity (experiment 5), it is unlikely that phosvitin is the active component since most of this protein was present in the granule fraction (Burley and Cook 1961) which was shown to be inactive (Watson and Martin 1973). Peak A, composed primarily of the mixed α-, β-, and γ-livetins (Seideman *et al.* 1969), was shown to be inactive in protecting spermatozoa (expt 5). These proteins may therefore also be discounted as active substances.

The protein fraction not excluded by any of these considerations is the free low-density lipoprotein fraction. The activity remained in solution after centrifugation at 35000 *g* (Watson and Martin 1973), which is consistent with the behaviour of low-density lipoproteins during centrifugation. Moreover peak B, containing the greatest protective activity, also contained the highest lipid content of all the fractions (Seideman *et al.* 1969). These authors inferred that this fraction contained the low-density lipoproteins, which have a lipid content of about 89% (Cook and Martin 1969).

However, the results of experiment 1 (dialysis) and experiment 4 (ultrafiltration: 100000 molecular weight filter) indicated that the low-density lipoprotein fraction alone might not be responsible for the protection. The low-density lipoproteins have molecular weights ranging from 3×10^6 to 10×10^6 (Cook and Martin 1969) and are present as hydrated spherical micelles with diameters ranging from 11·7 to 48·0 nm (Powrie 1968). Particles of this size should not pass through a dialysis sac or ultrafiltration membrane, but some activity was lost under these conditions. A possible explanation is that some smaller component becomes dissociated in these circumstances, resulting in loss of activity in the parent molecule. Reassociation, with restoration of activity, apparently occurs when the components are recombined.

The proteins of peak B, when assayed relative to the total soluble proteins in the egg yolk diluent, were shown to have only 20% or less of the potency (expt 6). Peak B therefore contains proportionally less of the active proteins than does the centrifuged egg yolk diluent, and the chromatographic procedure used in these experiments is unsuitable for preparing a fraction of egg yolk high in activity. This may be due either to a reduction in potency of the active proteins during fractionation or to the inclusion in peak B of a large proportion of inactive protein. Seideman *et al.* (1969) have indicated that peak B contained proteins other than the low-density fraction. From the experiments presented it is concluded that egg yolk contains (lipo-)protein(s) active in preserving spermatozoa, and potent at low concentration. Although no

firm conclusions can be drawn at this stage regarding the identity of this substance, the low-density lipoprotein fraction warrants further investigation. Future studies will be directed towards isolation of this fraction using ultracentrifugation.

Acknowledgments

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