J. Reprod. Fert. (1981) **62**, 483–492 *Printed in Great Britain*

The roles of lipid and protein in the protection of ram spermatozoa at 5°C by egg-yolk lipoprotein

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Summary. Both the low-density lipoprotein fraction of egg yolk (LDF) and sonicated lecithin liposomes provided an equal measure of protection for ram spermatozoa during cold shock, but LDF was superior to lecithin during cold storage. The protective activity of LDF during storage at 5°C was not altered by a subfractionation procedure which did not alter the molecular organization. Removal of the protein from the surface of LDF particles gave preparations with altered lipid:protein ratios. Neither the low-lipid fraction nor bovine plasma albumin protected spermatozoa but the high-lipid fraction was as protective as LDF. Survival of spermatozoa decreased as the lipid:protein ratio fell below 1·67 compared with a ratio of 4·76 for LDF. The absolute lipid content was more important than the ratio except at low ratios. Lipid-depleted preparations bound more effectively to the plasma membrane than did LDF whereas the lipid-enhanced preparation showed little binding capability.

It is concluded that the phospholipid of LDF provides the protection to the sperm cell membrane. The protein of LDF serves to solubilize the lipid and bind it to the cell membrane. The importance of the role of the protein during cold storage is discussed.

Introduction

The precise mechanism by which egg yolk protects spermatozoa subjected to cold stresses is unknown. The low-density lipoprotein fraction (LDF) is now well-established as the active constituent (Pace & Graham, 1974; Watson, 1976; Foulkes, 1977), but there is little information on the nature of its interaction with the sperm cell membrane. A clarification of the roles of the lipid and protein components of LDF in membrane protection might indirectly shed some light on the nature of the membrane changes occurring in response to cooling and freezing.

Exogenous phospholipids have been demonstrated to exert a protective action on spermatozoa during cold shock (Kampschmidt, Mayer & Herman, 1953; Blackshaw, 1954; Quinn, Chow & White, 1980), cold storage (Lardy & Phillips, 1941) and freezing (Martin, 1963; Lanz, Pickett & Komarek, 1965; Gebauer, Pickett, Komarek & Gaunya, 1970). However, egg-yolk lipoproteins were more effective than phospholipids in storage studies (Kampschmidt *et al.*, 1953) and during freezing and thawing (Gebauer *et al.*, 1970). These observations have led to suggestions that egg yolk contributes two distinct factors, one of which protects against cold shock (a 'resistance' factor) and a second which maintains viability (a 'storage' factor) (Kampschmidt *et al.*, 1953). As part of a continuing study of LDF, this paper considers the actions of lipid and protein in the protection of ram spermatozoa by LDF during cold storage.

0022-4251/81/040483-10$02.00/0

Materials and Methods

To test their protective activity for spermatozoa, all lipid and protein preparations were dispersed at the concentrations stated in the text in a basic diluent containing 185 mM-glucose, 17 mM-fructose, 31 mM-NaCl, 10 mM-Na_2HPO_4 and 5 mM each of NaH_2PO_4 and KH_2PO_4 with 500 i.u./ml each of penicillin and dihydrostreptomycin; the pH was 6·98.

LDF was prepared from fresh hen egg yolks as described by Watson (1976). In outline, the method entails separation of the low density lipoproteins by centrifugation in 4 M-NaCl at 90 000 *g*, having first separated and discarded the yolk granules. Remaining high-density lipoproteins were removed by suspension in 4 M-NaCl followed by a second centrifugation stage. LDF was finally resuspended in distilled water to the original volume of yolk and dialysed for 24 h against distilled water. For Exp. 2 the preparative method of Martin, Augustyniak & Cook (1964) was followed yielding 'crude LDF' (essentially similar to LDF referred to above) which was then subfractionated by repeated centrifugation into the two subfractions characterized by Martin *et al.* (1964).

For Exp. 1, lecithin (egg grade 1, BDH, Poole, Dorset, U.K.) (20 mg) in chloroform was dried under N_2 and then dispersed by sonication into 20 ml distilled water in a N_2 atmosphere as described by Quinn *et al.* (1980). Selective solubilization of LDF apoprotein (Exps 3, 5 and 6) was performed using 6 M-guanidinium chloride as described by Kurisaki & Yamauchi (1977).

Protein concentrations were measured by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine plasma albumin standards. Lipid turbidity was eliminated before spectrophotometry by extraction with ether after full colour development had occurred. Total lipids were estimated by the method of Bragdon (1951) using palmitic acid standards, and results were expressed as mg equivalents of palmitic acid. Lipid phosphorus was estimated by the method of Raheja, Kaur, Singh & Bhatia (1973).

Semen was collected from adult Finnish Landrace rams by electroejaculation with a Ruakura electroejaculator. For Exp. 1, 0·5 ml aliquots of diluted semen (1 in 20) in glass tubes at 30°C were cold-shocked by transferring to ice-water at 0°C for 2 min. The samples were warmed rapidly to 37°C for scoring. In experiments in which the effects of slow cooling and storage were studied (Exps 1–5), semen was diluted 20-fold at 30°C, cooled to below 5°C over 2 h in a domestic refrigerator and stored for 72 h. The diluent was then removed and the sedimented spermatozoa were revived by addition of an equal volume of basic diluent at 37°C. Motility scores (Emmens, 1947) and percentage motile scores (to the nearest 10%) were accorded to all samples arranged in random sequence. Means of three estimates of each sample were used for analysis.

The ability of preparations to bind to sperm cell membranes (Exp. 6) was assessed by interference with binding of 1-anilinonaphthalene-8-sulphonate (ANS), a fluorescent membrane probe, as described by Watson (1979), except that all preparations were in basic diluent and not phosphate-buffered saline. Results are expressed as the mean film density of developed negative micrographs of fluorescent sperm heads; a lower density reflects poorer fluorescence.

Computer analyses of variance were carried out on all data; percentage data were subjected to arcsin transformation before analysis (Snedecor & Cochran, 1967). Individual degree-of-freedom contrasts were computed for treatments to isolate the components of variance. The nomenclature 'L' and 'Q' are taken from Cochran & Cox (1957) and denote the linear regression and deviation from linearity, or quadratic curvature, respectively.

Results

Experiment 1

The protective effect of pure phospholipid, lecithin (phosphatidylcholine), was compared with LDF under conditions of both cold shock and cold storage. Equal amounts of phospholipid (lipid P × 25) were present in both diluents. The results of factorial experiments (2 × 3 × 4) involving 2

treatments, each of 3 concentrations investigated on a split-ejaculate basis in 4 ejaculates, are shown in Table 1 together with overall means. The analyses of variance for the cold-storage results are also shown but similar analyses of the cold-shock data were not performed since the large number of 'zero' motility observations would have given falsely inflated variance ratios. It can be seen that while both additives provided a measure of protection during cold shock and cold storage, LDF was apparently more beneficial than lecithin during cold storage owing to a failure of the highest concentration of lecithin to maintain motility. This accounts for the significant A × $B_{(1)}$ interaction term in the analyses. Substantial differences were observed between ejaculates, particularly in their responses to cold shock.

Table 1. Mean motility scores (means of ejaculates from 4 rams) of ram spermatozoa cold-shocked or cooled and stored for 72 h at 5°C in diluents containing lecithin or LDF

Treatment	Phospholipid concentration (mg/ml)	Cold-shocked		Cooled and stored	
		% motile	Motility	% motile	Motility
Basic diluent					
Mean ± s.e.m.	—	<0·1	0·21 ± 0·158	24·2 ± 5·98	1·58 ± 0·493
Basic diluent + lecithin	0·13	10·2	0·71	34·2	1·92
	0·65	12·1	0·83	41·7	2·25
	3·25	9·4	0·92	20·0	0·92
Mean ± s.e.m.	—	10·6 ± 5·20	0·82 ± 0·183	32·0 ± 5·20	1·70 ± 0·267
Basic diluent + LDF	0·13	10·4	0·67	34·2	1·92
	0·65	12·9	0·95	43·3	2·38
	3·25	13·5	1·04	51·7	2·75
Mean ± s.e.m.	—	12·3 ± 5·65	0·89 ± 0·190	43·0 ± 4·25	2·35 ± 0·216

Summary of analyses of variance of storage data

Source of variation	d.f.	Variance ratio: Angles motile	Variance ratio: Motility
A. Treatments	1	8·53*	15·15**
B. Concentrations	2		
(i) L	1	0·17	0·16
(ii) Q	1	3·86	6·07*
C. Ejaculates	3	8·40*	15·58**
A × $B_{(i)}$	1	12·19*	20·04**
A × $B_{(ii)}$	1	3·27	4·95
Pooled 1st order Ejaculate interactions	9	1·65	0·93
A × B × C (error) variance	6	*48·68*	*0·1682*

* $P < 0·05$; ** $P < 0·01$.

Experiment 2

Subfractionation of LDF resulted in two fractions, one of which was more opaque than, and the other similar to, the parent LDF. The freeze-dried and reconstituted subfractions were both opaque suggesting some alteration of the structure of the particles in solution. The survival of ram spermatozoa after cooling and storage in diluents containing these preparations is shown in Table 2. A 5 × 2 × 4 factorial experimental design was used with 5 treatments each at 2 concentrations replicated over 4 ejaculates. There was no significant difference in response between concentrations or between the two subfractions. The percentage of motile cells was significantly lower in diluents containing freeze-dried subfractions compared with fresh subfractions (% motile, $P < 0·05$), which did not differ from the parent LDF in protective

activity. Fresh unfractionated LDF was therefore used as a control in all subsequent experiments. As in all subsequent storage experiments, differences between ejaculates were significant ($P < 0.05$).

Table 2. The survival of ram spermatozoa (means of ejaculates from 4 rams) after 72 h at 5°C in diluents containing preparations of egg-yolk low-density fraction (LDF)

	Protein concentration (mg/ml)					
	0·4		2·0		Mean	
Treatment	% motile	Motility	% motile	Motility	% motile	Motility
1. Crude LDF	41·7	2·63	42·5	2·71	42·1	2·67
2. LDF Subfraction 1	40·0	2·75	40·0	2·71	40·0	2·73
3. LDF Subfraction 2	38·3	2·75	45·8	2·71	42·1	2·73
4. Freeze-dried LDF 1	35·8	2·75	32·5	2·29	34·2	2·52
5. Freeze-dried LDF 2	38·3	2·83	37·5	2·63	37·9	2·73
Mean	38·8	2·74	39·7	2·61		

Summary of analyses of variance

		Variance ratio	
Source of variation	d.f.	Angles motile	Motility
A. Treatments	4		
1 versus 2–5	1	2·15	0·01
2 and 3 versus 4 and 5	1	6·42*	0·81
2 and 4 versus 3 and 5	1	2·41	0·79
2 and 5 versus 3 and 4	1	0·29	0·81
B. Protein concentrations	1	0·16	1·64
C. Ejaculates	3	36·00***	20·37***
Pooled 1st order interactions	19	0·90	0·73
A × B × C (error) variance	12	*12·25*	*0·1071*

* $P < 0.05$; *** $P < 0.001$.

Experiment 3

Using a technique for selectively solubilizing protein from the surface of LDF particles it was possible to produce preparations with enhanced or depleted lipid:protein ratios as compared to LDF. The experiment had a 3 × 3 × 4 factorial design. LDF and the 2 subfractions were compared for protective activity at 3 concentrations equated on the basis of protein content, with 4 ejaculate replicates. The results and analyses of variance (Table 3) showed that the lipid-depleted fraction was significantly less protective than the lipid-enriched fraction and LDF ($P < 0.001$); the latter two preparations did not differ significantly. The lipid-depleted fraction provided little more protection than the basic diluent and, in contrast to LDF and the lipid-enriched fraction, no benefit was evident from increasing its concentration. This finding was responsible for the significant interaction in the percentage motile score, signifying deviation from parallelism in dose-response ($P < 0.05$). However, the interpretation of this experiment was complicated by a tendency for the lipid-depleted preparation to flocculate on standing, indicating that it was not truly in solution. The absence of protection could have been due to the lack of lipid or to poor solubility.

Experiment 4

To investigate this question, LDF was compared with a soluble protein, bovine plasma albumin (BPA). The results of a 2 × 3 × 4 factorial experiment of 3 concentrations of LDF and BPA compared on an equivalent protein basis are shown in Table 4, together with summaries of

Table 3. The survival of ram spermatozoa (means of ejaculates from 4 rams) after 72 h at 5°C in diluents containing LDF and lipid preparations derived from it

	Protein concentration (mg/ml)							
	0·08		0·4		2·0		Mean	
Treatment	% motile	Motility	% motile	Motility	% motile	Motility	% motile	Motility
1. LDF	31·7	2·04	47·0	2·26	56·7	2·84	45·0	2·33
2. Lipid-enriched	42·5	2·42	52·5	2·33	54·2	2·54	49·7	2·43
3. Lipid-depleted	27·5	1·75	25·8	1·75	27·5	1·88	26·9	1·79
Mean	33·9	2·07	41·8	2·11	46·1	2·42		
Basic diluent			25·0	1·58				

Summary of analyses of variance

Source of variation	d.f.	Variance ratio: Angles motile	Variance ratio: Motility
A. Treatments	2		
(i) 1 versus 2	1	2·60	0·12
(ii) 1 and 2 versus 3	1	71·03***	23·06***
B. Concentrations	2		
(i) L	1	18·26**	5·53*
(ii) Q	1	0·43	1·02
C. Ejaculates	3	7·30**	24·07***
$A_{(ll)} \times B_{(l)}$	1	8·25*	1·16
Remainder of 1st order interactions	15	1·08	2·38
A × B × C (error) variance	11†	*17·50*	*0·1303*

† 12 − 1 d.f. for missing datum.
* $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$.

Table 4. The survival of ram spermatozoa (means of ejaculates from 4 rams) after 72 h at 5°C in diluents containing LDF or bovine plasma albumin (BPA)

	Nominal protein concentration (mg/ml)							
	0·1		0·5		2·5		Mean	
Treatment	% motile	Motility	% motile	Motility	% motile	Motility	% motile	Motility
LDF	31·7	2·29	46·7	2·71	43·3	2·67	40·6	2·56
BPA	21·7	1·46	19·2	1·38	17·5	1·21	19·5	1·35
Basic diluent			20·8	1·28				

Summary of analyses of variance

Source of variation	d.f.	Variance ratio: Angles motile	Variance ratio: Motility
A. Treatments	1	90·62***	61·05***
B. Concentrations	2		
(i) L	1	1·21	0·11
(ii) Q	1	3·27	0·68
C. Ejaculates	3	6·04*	0·11
$A \times B_{(l)}$	1	8·71*	2·74
Remainder of 1st order interactions	10	2·78	1·44
A × B × C (error) variance	6	*11·92*	*0·1435*

* $P < 0·05$; *** $P < 0·001$.

the analyses of variance. BPA was totally without protective activity compared with LDF ($P < 0{\cdot}001$). Although LDF exhibited a dose-related response, the common linear response (L) was not significant because of the absence of dose–response with BPA. The deviation from parallelism of these dose–responses accounts for the significant interaction term in the percentage motile score ($P < 0{\cdot}05$).

Experiment 5

Experiments 3 and 4 suggest that lipid is responsible for protection and this was investigated in Exp. 5, in which various proportions of lipid-enriched and lipid-depleted fractions were recombined to produce 6 solutions containing dispersed lipoprotein with lipid:protein ratios ranging from 0·2 to 8·63. These solutions showed a decreasing tendency to flocculate on standing as the ratio increased from 0·2 to 4·23, indicating that the protein of the lipid-depleted fraction had not denatured and retained its capacity to recombine with lipid. A factorial experiment (7 × 3 × 4) was performed involving 6 treatments plus LDF, each at 3 comparable

Table 5. The survival of ram spermatozoa (mean of ejaculates from 4 rams) after 72 h at 5°C in diluents containing preparations of LDF with various lipid : protein ratios

Treatment (lipid : protein) ratio)	Protein concentration (mg/ml)							
	0·07		0·42		2·5		Mean	
	% motile	Motility	% motile	Motility	% motile	Motility	% motile	Motility
1. 0·20	20·0	1·34	20·8	1·25	**42·5**	**2·13**	27·8	1·57
2. 0·93	19·2	1·42	**33·3**	**1·95**	**49·2**	**2·58**	33·9	1·97
3. 1·67	21·7	1·46	**46·7**	**2·50**	**50·8**	**2·50**	39·7	2·15
4. 2·80	27·5	1·80	**38·3**	**2·46**	**46·7**	**2·38**	37·5	2·21
5. 4·23	30·0	1·96	**45·0**	**2·54**	**40·8**	**2·38**	38·6	2·29
6. 8·63	**39·2**	**2·33**	**47·5**	**2·58**	**42·5**	**2·13**	43·1	2·35
7. 4·76 (LDF)	**33·3**	**1·96**	**50·8**	**2·71**	**54·2**	**2·71**	46·1	2·46
Mean	27·3	1·75	40·4	2·28	46·7	2·40		
Basic diluent			21·7	1·71				

Absolute lipid concentration (protein conc. × lipid : protein ratio) > 0·3 mg/ml is indicated by bold numerals.

Summary of analyses of variance

Source of variation	d.f.	Variance ratio	
		Angles motile	Motility
Treatments	6		
1 versus 2–7	1	27·84***	41·27***
2 versus 3–7	1	9·53**	9·13**
3 versus 4–7	1	0·66	2·59
4 versus 5–7	1	3·94	1·96
5 versus 6 and 7	1	4·48*	0·90
6 versus 7	1	0·94	0·66
Protein concentrations	2		
L	1	91·96***	52·94***
Q	1	3·97	7·05*
Ejaculates	3	16·68***	26·83***
Treatment × Conc. interactions	12	3·30*	3·38*
Remainder of 1st order interactions	24	1·46	1·21
Pooled 2nd order interactions (error) variance	36	*22·17*	*0·1112*

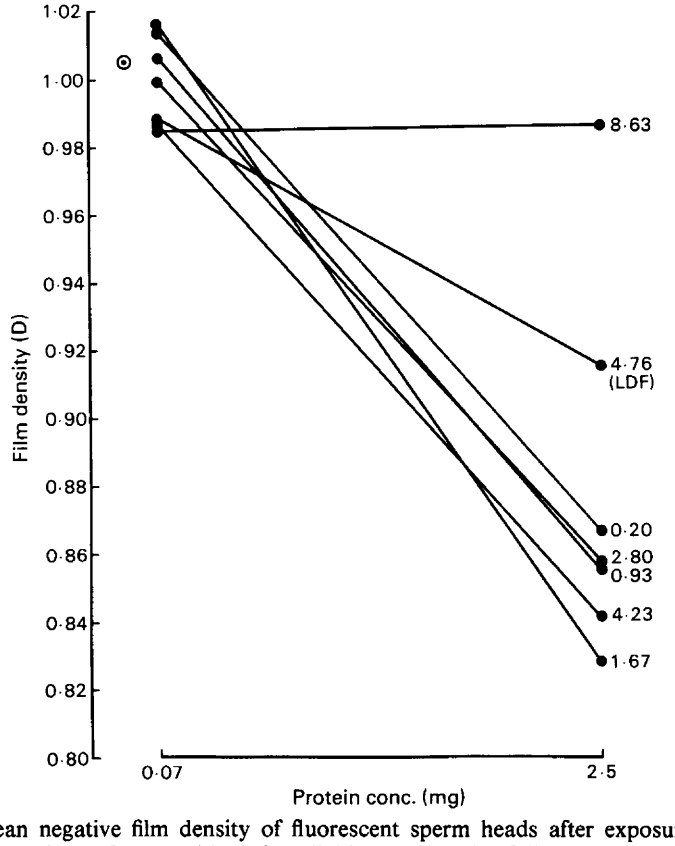
* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

protein concentrations replicated over 4 ejaculates. The results of sperm survival after 72 h at 5°C is shown in Table 5 together with summaries of the analyses of variance. Individual degree-of-freedom contrasts between treatments were computed using an orthogonal matrix which made no assumptions about the relationships between treatments.

There was a significant increase in sperm survival in diluents containing the range of lipid:protein ratios 0·2–1·67. Higher ratios provided little further benefit, except that LDF and the preparation with the ratio 8·63, which did not differ significantly from each other, were just significantly better than the lower ratios for the percentage motile score ($P < 0·05$). In diluents containing an absolute lipid concentration <0·15 mg/ml, the percentage of spermatozoa motile after 72 h was no greater than that in the basic diluent, while motility scores were poorer. More than 30% of spermatozoa were motile when >0·3 mg lipid/ml was present irrespective of the lipid:protein ratio (see Table 5). The highly significant linear response to increasing protein concentration was probably also attributable largely to the lipid content because as the protein content increased so did the absolute lipid content. The lipid:protein ratio became more important at lower absolute lipid and protein concentrations, accounting for the significant Treatment × Protein concentration interaction term. Even at the highest ratios, however, the response was still depressed if only 0·07 mg protein/ml was present.

Experiment 6

The attachment of these lipoprotein preparations to the sperm cell membrane at concentrations of 0·07 and 2·5 mg protein/ml was investigated by their action in preventing



Text-fig. 1. Mean negative film density of fluorescent sperm heads after exposure to diluents containing preparations of LDF with various lipid:protein ratios followed by diluent containing 1 mM-ANS (see text). ⊙ Basic diluent control. Figures against the lines are lipid:protein ratios.

ANS binding. The results (Text-fig. 1) show that at the low concentration no attachment took place since there were no significant differences in fluorescence between any of the diluents. At the higher concentration, however, all lipoprotein solutions except that with a lipid:protein ratio of 8·63 prevented subsequent binding of ANS compared with the basic diluent (basic diluent V^s all lipoprotein solutions—$P < 0{\cdot}001$). The preparation with the highest ratio (8·63) differed from lipoprotein preparations with lower ratios ($P < 0{\cdot}001$), while LDF (lipid:protein ratio 4·76) was less effective in preventing ANS binding than were preparations with ratios of 4·23–0·2 ($P < 0{\cdot}05$). There were no significant differences between these latter preparations. Ram differences were also non-significant.

Discussion

LDF consists of spheres of lipoprotein containing neutral lipid cores of variable size surrounded by a layer of lipovitellenin composed principally of glycoprotein and phospholipid with hydrophobic groups orientated to the interior and hydrophilic groups on the surface (Evans, Bandemer, Davidson, Heinlein & Vaghefi, 1968; Evans, Bauer, Bandemer, Vaghefi & Flegal, 1973). Subfractionation of LDF by ultracentrifugation, a procedure which is not destructive of the molecular organization, did not enhance the degree of protection to ram spermatozoa at 5°C by LDF (Exp. 2), which is in agreement with the findings of Pace & Graham (1974), who studied cryoprotection of bull spermatozoa. Freeze-drying caused some loss of activity, as previously reported (Watson, 1976). This was probably due to denaturation resulting from the removal of water which is essential for the molecular organization of very low density lipoproteins such as LDF.

The results of Exp. 1 add support to an earlier suggestion that while lecithin and egg-yolk lipoprotein provide equal protection to spermatozoa during the brief but severe stress of cold shock, the lipoprotein fraction is superior to lecithin during cooling and cold storage (Kampschmidt *et al.*, 1953). In this experiment the protective substances were compared at equal phospholipid concentrations and it therefore comprised a more rigorous test of relative protection than has previously been presented. A similar quadratic response to lecithin concentration and linear response to lipoprotein concentration was observed with bull spermatozoa before and after freezing in a concentration range comparable to that used in this experiment (Gebauer *et al.*, 1970).

The superiority of lipoprotein over phospholipid preparations of egg yolk for the protection of spermatozoa during certain cold stresses has been interpreted as evidence for the presence of two distinct factors (Kampschmidt *et al.*, 1953). The 'resistance' factor (presumed to be lecithin) was considered to be present in both phospholipid and lipoprotein preparations. Certainly, lecithin has been shown to be protective for spermatozoa in a variety of cold stresses (Kampschmidt *et al.*, 1953; Blackshaw, 1954, 1958; Martin, 1963; Gebauer *et al.*, 1970; Quinn *et al.*, 1980). The 'storage' factor was envisaged as being related to the protein alone or the lipoprotein complex (Kampschmidt *et al.*, 1953).

The selective removal of protein from LDF produced a lipid-depleted (or protein-rich) fraction which tended to flocculate on standing. The apoprotein, vitellenin, is not very soluble (Burley, 1975) especially in solutions compatible with sperm survival. The inability of this fraction to protect spermatozoa (Exp. 3) may, therefore, have been due either to its being unavailable to the cells on account of poor solubility or to its lack of lipid. However, BPA, a soluble protein which has little lipid associated with it, afforded no protection to spermatozoa (Exp. 4), which suggests that lipid is involved in protection.

Experiment 5 showed that the absolute concentration of lipid is of prime importance but at the lowest protein concentration, even when sufficient lipid was present, the response (39·2%

motile, motility 2·33) was marginally lower than that obtained in the presence of greater concentrations of protein. This could be due to changes in the relative proportions of phospholipid and neutral lipids in the lipoprotein preparations. Since guanidinium chloride selectively solubilizes the surface coat of LDF particles (Kurisaki & Yamauchi, 1977), it is probable that the remaining lipid-rich fraction (ratio 8·63) was relatively poorer in phospholipid; di- and tri-glycerides are ineffective in protecting spermatozoa (Lardy & Phillips, 1941; Lanz *et al.*, 1965). Thus, although the total lipid concentration was adequate, there may have been insufficient phospholipid to provide maximum protection. Lipid phosphorus estimations were not made on these preparations.

It has been demonstrated that egg-yolk low-density lipoproteins form a strong attachment to the sperm cell membrane which cannot be disrupted by serial washing (Foulkes, 1977; Watson, 1979). Since phospholipids fail to bind to spermatozoa (Quinn *et al.*, 1980) it is likely that the binding sites for the membrane are located in the protein component of LDF. In Exp. 6, it was shown that removal of a protein-rich component from the surface of LDF significantly reduced its ability to associate with the cell. Steer, Martin & Cook (1968) concluded that LDF contains a portion of its protein buried within the molecule and unavailable to enzymic digestion. It is probable that the protein remaining in the lipoprotein with a ratio of 8·63 was mostly this inaccessible protein, which thus could not interact with the cell membrane. Conversely, the protein-rich product of the guanidinium solubilization was more effective than LDF in attaching to the membrane, suggesting that binding sites on the apoprotein had been exposed.

This interpretation of the fluorescence data has been criticized on the grounds that no account is taken of the partitioning of the fluophore between the cell and the exogenous lipoprotein (Quinn *et al.*, 1980; MacDonald & Foulkes, 1981). However, the fluorescence measured is solely that associated with the sperm head after removal of free lipoprotein, and serial washing did not significantly alter this fluorescence (Watson, 1979). Therefore, even if the fluophore does partition onto the exogenous lipoprotein, it is still a measure of the attachment of the lipoprotein to the cell. Nevertheless, the observation that ANS fluorescence of bull spermatozoa measured by spectrofluorometry is increased by the presence of lipoprotein (MacDonald & Foulkes, 1981) is puzzling, and one can only surmise that differences in the methods used may account for the conflicting results.

While the phospholipid component of LDF is of primary importance in the protection of spermatozoa, the suggestion that the presence of protein enhances this protection particularly at high concentrations of phospholipid during slow cooling and storage (Exp. 1) requires some explanation. Lecithin dispersions, even after sonication, are not true solutions (Finer, Flook & Hauser, 1972). It may be that at high concentration the lecithin liposomes reaggregate during storage at 5°C, thus effectively reducing the surface area available for interaction with the cells. LDF, on the other hand, forms a true solution, the protein solubilizing the lipid, which would account for the observation that the high-ratio lipoprotein (8·63) was indeed protective. Although the role of the membrane-binding action of LDF protein (Exp. 6) in the protection of spermatozoa is not proven, a subsidiary explanation is that the protein of LDF may enable the spermatozoa, inactivated by the low temperature, to retain a sufficient concentration of phospholipid in close association with the plasma membranes when sedimented in the tube during storage. The nature of the membrane modification induced by exogenous phospholipid with or without associated protein remains open to investigation. The view presented in this paper obviates the need to invoke the concept of separate and distinct 'resistance' and 'storage' factors in egg yolk.

I am indebted to Mr W. J. Anderson for expert technical assistance and to Dr J. A. Foulkes for helpful comments on the manuscript. The analyses of variance were carried out by computer using a program kindly supplied by Dr I. C. A. Martin, University of Sydney, Australia. The work was supported by a grant from the Wellcome Trust.

References

Blackshaw, A.W. (1954) The prevention of temperature shock of bull and ram semen. *Aust. J. biol. Sci.* **7**, 573–582.

Blackshaw, A.W. (1958) The effects of incubation temperature and cold shock on the metabolism of ram spermatozoa. *Aust. J. biol. Sci.* **11**, 581–588.

Bragdon, J.H. (1951) Colorimetric determination of blood lipides. *J. biol. Chem.* **190**, 513–517.

Burley, R.W. (1975) Studies on the apoproteins of the major lipoprotein of the yolk of hen's eggs. I. Isolation and properties of the low molecular weight apoproteins. *Aust. J. biol. Sci.* **28**, 121–132.

Cochran, W.G. & Cox, G.M. (1957) *Experimental Designs*, 2nd edn. John Wiley & Sons, New York.

Emmens, C.W. (1947) The motility and viability of rabbit spermatozoa at different hydrogen-ion concentrations. *J. Physiol., Lond.* **106**, 471–481.

Evans, R.J., Bandemer, S.L., Davidson, J.A., Heinlein, K. & Vaghefi, S.S. (1968) Binding of lipid to protein in low density lipoprotein from egg yolk. *Biochim. Biophys. Acta* **164**, 566–574.

Evans, R.J., Bauer, D.H., Bandemer, S.L., Vaghefi, S.B. & Flegal, C.J. (1973) Structure of egg yolk very low density lipoprotein. Polydispersity of the very low density lipoprotein and the role of lipovitellenin in the structure. *Archs Biochem. Biophys.* **154**, 493–500.

Finer, E.G., Flook, A.G. & Hauser, H. (1972) Mechanism of sonication of aqueous egg yolk lecithin dispersions and nature of the resultant particles. *Biochim. Biophys. Acta* **260**, 49–58.

Foulkes, J.A. (1977) The separation of lipoproteins from egg yolk and their effect on the motility and the integrity of bovine spermatozoa. *J. Reprod. Fert.* **49**, 277–284.

Gebauer, M.R., Pickett, B.W., Komarek, R.J. & Gaunya, W.S. (1970) Motility of bovine spermatozoa extended in "defined" diluents. *J. Dairy Sci.* **53**, 817–823.

Kampschmidt, R.F., Mayer, D.T. & Herman, H.A. (1953) Lipid and lipoprotein constituents of egg yolk in the resistance and storage of bull spermatozoa. *J. Dairy Sci.* **36**, 733–742.

Kurisaki, J. & Yamauchi, K. (1977) Selective solubilization of apolipoproteins from hen's egg yolk very low density lipoprotein with guanidine hydrochloride and urea. *J. Biochem.* **81**, 443–449.

Lanz, R.N., Pickett, B.W. & Komarek, R.J. (1965) Effect of lipid additives on pre- and post-freeze survival of bovine spermatozoa. *J. Dairy Sci.* **48**, 1692–1697.

Lardy, H.A. & Phillips, P.H. (1941) Phospholipids as a source of energy for motility of bull spermatozoa. *Am. J. Physiol.* **134**, 542–548.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275

MacDonald, B.J. & Foulkes, J. A. (1981) A spectrofluorometric investigation, using 1-anilino naphthalene-8-sulphonate, of the interaction between washed bovine spermatozoa and seminal plasma or egg-yolk lipoprotein. *J. Reprod. Fert.* (in press).

Martin, I.C.A. (1963) Effects of lecithin, egg yolk, fructose and period of storage at 5°C on bull spermatozoa deep-frozen to −79°C. *J. Reprod. Fert.* **6**, 441–449.

Martin, W.G., Augustyniak, J. & Cook, W.H. (1964) Fractionation and characterization of the low-density lipoproteins of hen's egg yolk. *Biochim. Biophys. Acta* **84**, 714–720.

Pace, M.M. & Graham, E.F. (1974) Components in egg yolk which protect bovine spermatozoa during freezing. *J. Anim. Sci.* **39**, 1144–1149.

Quinn, P.J., Chow, P.Y.W. & White, I.G. (1980) Evidence that phospholipid protects ram spermatozoa from cold shock at a plasma membrane site. *J. Reprod. Fert.* **60**, 403–407.

Raheja, R.K., Kaur, C., Singh, A. & Bhatia, I.S. (1973) New colorimetric method for the quantitative estimation of phospholipids without acid digestion. *J. Lipid Res.* **14**, 695–697.

Snedecor, G.W. & Cochran, W.G. (1967) *Statistical Methods*, 6th edn. Iowa State University Press, Ames.

Steer, D.C., Martin, W.G. & Cook, W.H. (1968) Structural investigation of the low-density lipoprotein of hen's egg yolk using proteolysis. *Biochemistry, N.Y.* **7**, 3309–3315.

Watson, P.F. (1976) The protection of ram and bull spermatozoa by the low density lipoprotein fraction of egg yolk during storage at 5°C and deep-freezing. *J. Thermal Biol.* **1**, 137–141.

Watson, P.F. (1979) An objective method for measuring fluorescence of individual sperm cells labelled with 1-anilino naphthalene-8-sulphonate (ANS) by means of photomicrography and densitometry. *J. Microsc.* **117**, 425–429.

Received 4 October 1980