METABOLISM OF SEMEN AFTER FREEZING

I. EFFECTS OF WASHING, SKIM-MILK SOLIDS, AND POTASSIUM AND MAGNESIUM IONS ON THE METABOLISM OF THAWED RAM AND BULL SPERMATOZOA

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Summary

Ram spermatozoa after storage at −79°C were damaged when washed twice in 10 volumes of diluent, the procedure used for recently ejaculated cells. After freezing, the oxygen uptake of spermatozoa washed once in 10 volumes of diluent was the same as that of unwashed cells. Using this degree of washing it was not possible to obtain thawed cells free of fructose and lactate.

Spermatozoa after freezing in a milk diluent had a higher rate of metabolism at 37°C than cells frozen in a synthetic medium. Freezing decreased the oxygen uptake and the amounts of fructose oxidized and lactate accumulated by ram and bull spermatozoa during subsequent incubation at 37°C.

Addition of magnesium to incubation diluents had only small and variable effects. The presence of 5–15 mM potassium chloride stimulated the metabolism of both recently ejaculated and thawed spermatozoa. However, in the presence of Tris buffer, but not of phosphate buffer, the increase in metabolism on potassium addition was relatively smaller with recently ejaculated than with thawed spermatozoa.

I. Introduction

Although the deep freezing of bull spermatozoa is now important in artificial breeding practice, the use of deep-frozen ram semen has met with limited success, as fertility has been poor despite satisfactory revival (Emmens and Robinson 1962; Sadleir 1966).

Despite the many studies of freezing techniques, the effects of freezing on the metabolism of ram and bull spermatozoa have received little attention. White, Blackshaw, and Emmens (1954) observed a large decrease in oxygen uptake and lactate accumulation by cells from both species. With bull spermatozoa Leidl and Rüsse (1956) observed that the oxygen consumption of thawed samples was less than that of controls and it decreased more rapidly with time. O'Dell and Almquist (1958), as well as observing decreased post-thawing production of lactic acid, found no evidence of respiratory activity by bull spermatozoa during storage for 6 months at −79°C. Although the fertility of frozen bull spermatozoa does not decrease greatly with time (Melrose 1962), Sullivan and Mixner (1963) found that the sugar utilization of thawed semen declined linearly with increasing time of storage, with the decline being less for semen stored at −196 than at −79°C. The reduced accumu-

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lation of lactic acid in thawed bull semen could not be entirely attributed to the reduction of numbers of motile spermatozoa by freezing (O'Dell and Almquist 1958; Mizuho, Niwa, and Soejima 1963), indicating that the metabolism of the surviving spermatozoa was impaired.

To measure the indices of metabolism, other than oxygen uptake, especially of thawed spermatozoa, the incubation flask must only contain small amounts of substrates. For this reason, in the present report the spermatozoa were washed before incubation at 37°C, and incubation diluents such as those of Jones and Martin (1965) could not be used, although some experiments were carried out to see if the presence of milk solids in the incubation medium had marked effects on the metabolism of thawed cells. As skim-milk preparations can be removed by washing and are suitable for freezing ram spermatozoa (Emmens and Robinson 1962; Jones and Martin 1965) they were used for freezing the semen. Thus the metabolic effects of differences in milk diluent preparation and of techniques of handling the semen after thawing were investigated. As potassium and magnesium are important in the metabolism and motility of spermatozoa (Blackshaw 1953; White 1956; Mann 1964; Wales and O'Shea 1966), their effect on frozen spermatozoa was examined.

II. Materials and Methods

(*a*) *Semen*

Semen was collected from rams after electrical stimulation and from bulls by means of an artificial vagina.

(*b*) *Diluents*

The two basic freezing diluents used were:

(1) Synthetic diluent—5 mM mono- and disodium phosphate buffer (pH 7·0), 247 mM glucose, 5 mM potassium chloride, 49 mM sodium chloride, and 3 g/100 ml of a lyophylized preparation of non-dialysable solids from heated (92°C for 10 min) skim cow milk. The portion of this diluent added at 5°C also contained 15 ml/100 ml glycerol. This diluent was based on one described by Lapwood and Martin (personal communication).

(2) Milk diluent—9 g/100 ml skim-milk powder (Bonlac non-fat milk). The portion of the latter diluent added at 37°C also contained 17 mM fructose while the diluent added at 5°C contained 140 mM fructose and 15 ml/100 ml glycerol (Jones and Martin 1965).

For incubation of spermatozoa at 37°C the following two diluents were used:

(1) Phosphate diluent—20 mM mono- and disodium phosphate buffer (pH 7·2) and 127 mM sodium chloride.

(2) Tris diluent—20 mM Tris–HCl buffer (pH 7·2) and 134 mM sodium chloride.

All diluents contained 30 mg/100 ml penicillin and 50 mg/100 ml dihydrostreptomycin. Diluents containing milk powders were stored overnight at 5°C (Jones 1965) and centrifuged before use. Where potassium chloride, magnesium chloride, or fructose were included in the incubation diluents, isotonicity was maintained by adjusting the sodium chloride content.

(*c*) *Freezing, Storage, and Thawing*

The semen was diluted with four parts of diluent at 25–30°C and cooled to 5°C over approximately 2 hr. All manipulation of the cooled semen was carried out in a refrigerated room with precooled materials and apparatus. About 2 hr after reaching 5°C the diluted semen was mixed with an equal volume of glycerol-containing diluent added as three aliquots over 30 min. The spermatozoa were maintained at 5°C for a total of 5 hr and then 1·5-ml samples were frozen in a device described by Polge and Lovelock (1952) and modified by Jones and Martin (1965).

Frozen semen was stored for 18 hr at −79°C and thawed in a water-bath at 37°C and kept at 25°C until a sufficient volume of semen could be thawed.

(*d*) *Washing of Spermatozoa*

Recently ejaculated semen was washed twice in 10 volumes of basic incubation diluent (O'Shea and Wales 1965).

After freezing, spermatozoa were washed by centrifuging the thawed semen at 2500 r.p.m. (*c.* 1000 *g*) for 10 min at room temperature. The supernatant was replaced with an equal volume of diluent and the spermatozoa resuspended and centrifuged for 8 min. The spermatozoa were diluted to the required volume for incubation.

Several preliminary experiments were carried out before this washing technique was adopted. The metabolism of thawed spermatozoa washed as above in phosphate diluent containing potassium and magnesium was compared with that of aliquots centrifuged once only and resuspended in diluent. The results for four ejaculates showed a large fall in oxygen uptake, substrate oxidized, and percentage spermatozoa unstained due to freezing, but there was no effect of washing after freezing.

To measure the effects of centrifuging, aliquots of thawed semen were washed using 650, 1000, or 1700 *g* centrifugal force. The percentages of the total number of spermatozoa recovered in the plugs (49, 64, and 71 respectively) rose as the centrifugal force was increased ($P < 0{\cdot}05$). The first supernatants were recentrifuged at 1700 *g* but further recovery of cells was limited (15, 10, and 4% respectively). There was no effect of changes in centrifugal force on the percentage of spermatozoa unstained and the same percentages were obtained in the plugs and supernatants.

(*e*) *Incubation of Spermatozoa*

0·4 ml of the suspension of washed spermatozoa was incubated for 3 hr at 37°C with 0·5 ml of diluent containing substrate and 0·1 ml of carrier-free labelled substrate in 0·9 g/100 ml sodium chloride, in 5 ml Warburg flasks containing CO_2-free potassium hydroxide (20 g/100 ml) in the centre well.

(*f*) *Analytical Methods*

The percentage of spermatozoa unstained before incubation was estimated using Congo red–nigrosin in phosphate buffer (Entwistle, personal communication). The techniques used for the assay of radioactivity, the preparation of protein-free extracts of incubated suspensions, and the assay of lactate and fructose have been described previously (O'Shea and Wales 1965).

(*g*) *Statistical Methods*

In the experiments to estimate the effects of various milk solids, Duncan's multiple range test (Duncan 1955) was used to rank the treatments. Data expressed as percentages were converted to angles before analyses of variance were performed. Where metabolic data from fresh and thawed spermatozoa were compared, heterogeneity of variance in the raw data was removed by converting to logarithms before analyses of variance were carried out.

III. Results

(*a*) *Experiment 1: Comparison of Milk and Synthetic Diluents*

The metabolism of ram spermatozoa frozen in synthetic diluents containing dialysed skim-milk solids from two sources was compared with that of cells frozen in milk diluent. After washing in phosphate diluent the spermatozoa (1–6 $\times 10^8$ cells per flask) were incubated in the presence of 10 mM fructose, 1 mM potassium chloride, 2 mM magnesium chloride, and 3 g/100 ml dialysed milk powder. The results and standard errors of the means for six ejaculates (Table 1) show that freezing in the milk diluent gave a higher level of metabolism and a higher percentage of unstained

spermatozoa. There were no differences between the batches of dialysed skim-milk solids.

(b) Experiments 2 and 3: Effect of Degree of Washing

Ram spermatozoa, after freezing in milk diluent and washing, were diluted with washing diluent to 2·5 times the original semen volume. The suspensions were immediately deproteinized and analysed for fructose and lactate. Mean values ± standard errors for twelve ejaculates were 0·76±0·16 μmoles fructose per millilitre and 0·056±0·017 μmoles lactic acid per millilitre.

TABLE 1

CHARACTERISTICS OF RAM SPERMATOZOA AFTER STORAGE AT −79°C IN MILK DILUENT OR IN SYNTHETIC DILUENTS

Values are the means for six ejaculates and the metabolic data are expressed as μmoles per 10^8 cells over the experimental period (3 hr)

Diluent	Oxygen Uptake	Fructose Oxidized	Lactate Accumulated	Spermatozoa Unstained (%)
A. Milk	0·73	0·051	0·26	31
B. Synthetic (1)	0·35	0·024	0·17	21
C. Synthetic (2)	0·27	0·020	0·13	20
Standard error of mean	0·052*	0·0045*	0·037*	1·63*
Significance of differences†	A > B = C	A > B = C	A > C, A = B, B = C	A > B = C

* Ten degrees of freedom.

† Duncan's multiple range test (Duncan 1955). $P < 0·05$.

Because of the residual substrate left by the washing method more severe washing was investigated, as well as the effect of centrifuging diluents before use. Aliquots of ram semen were frozen in milk diluent which had either been centrifuged and the precipitate discarded or not centrifuged (control). After thawing, all aliquots were centrifuged and then washed in the phosphate diluent containing 2 mM magnesium chloride and 10 mM potassium chloride. One aliquot of the spermatozoa was washed a second time. All samples were incubated in phosphate diluent with 3 mM fructose, 2 mM magnesium chloride, and 10 mM potassium chloride. The results for four ejaculates (1–5 $\times 10^8$ cells per flask) show that the second washing depressed the oxygen uptake (Table 2), although there was no significant effect on fructose oxidation.

(c) Experiments 4, 5, and 6: Effect of the Addition of Potassium, Magnesium, and Milk Solids to the Incubation Diluent

Ram spermatozoa, after freezing in milk diluent, were washed in phosphate diluent containing 1 mM potassium chloride and 2 mM magnesium chloride. Aliquots of the three ejaculates were incubated in phosphate diluent containing 3 mM fructose, 1 mM potassium chloride, and 2 mM magnesium chloride, and with the addition of

0·5, 1·5, or 4·5 g/100 ml of skim-milk powder, or non-dialysable skim-milk solids, or casein. A similar experiment was carried out with four ejaculates in Tris diluent. The spermatozoa incubated in the presence of the higher levels of skim-milk powder had an oxygen uptake 25–100% greater than those in the other treatment groups, but more fructose oxidation occurred with the dialysed milk solids, presumably due to competition by substrates in the skim-milk powder.

TABLE 2

EFFECTS OF CENTRIFUGING THE FREEZING DILUENTS AND OF THE DEGREE OF WASHING AFTER FREEZING ON THE OXIDATIVE METABOLISM OF FROZEN RAM SPERMATOZOA

Values are the means for four ejaculates expressed as μmoles per 10^8 spermatozoa over the incubation time (3 hr)

Freezing Diluent	No. of Washes	Oxygen Uptake	Fructose Oxidized
A. Control	1	0·52	0·021
B. Centrifuged	1	0·48	0·019
C. Centrifuged	2	0·34	0·032
Standard error of mean		0·033*	0·0057*
Significance of differences†		A = B > C	A = B = C

* Six degrees of freedom.

† Duncan's multiple range test (Duncan 1955). $P < 0·05$.

Thus, in experiment 6 ram semen was frozen in milk diluent, washed in phosphate or Tris diluent, and incubated in phosphate or Tris diluent with 3 mM fructose and in the presence or absence of 10 mM potassium chloride, 2 mM magnesium chloride, and 4·5 g/100 ml dialysed skim-milk solids. The mean results (Table 3) for three ejaculates (1–3 $\times 10^8$ cells per flask) show that potassium stimulated both oxygen uptake ($P < 0·01$) and fructose oxidation ($P < 0·01$). In Tris buffer there was a greater oxidation of fructose than in phosphate buffer ($P < 0·01$) although the oxygen uptake was similar. Addition of non-dialysable skim-milk solids and of magnesium had no effect.

(d) Experiment 7: Effect of Addition of Ions after Washing with Saline

After storage in milk diluent at −79°C ram and bull semen were washed in isotonic saline. Aliquots of the spermatozoal suspension (1×10^8 cells per flask) were incubated in phosphate or Tris diluent in the presence of 3 mM fructose, 0, 1, or 10 mM potassium chloride, and 0, 2, or 10 mM magnesium chloride. The oxygen uptake could not be measured with two of the three ejaculates of ram semen and the amount of fructose oxidized was very low. The results for three ejaculates of bull semen (Table 4) showed that addition of potassium gave a linear increase in oxygen uptake ($P < 0·01$) with both buffers and a linear increase in fructose oxidized in Tris diluent ($P < 0·05$). Addition of magnesium gave a small linear decrease in amount of fructose oxidized ($P < 0·05$).

TABLE 3

EFFECTS OF NON-DIALYSABLE SKIM-MILK SOLIDS (4·5 g/100 ml), POTASSIUM (10 mM), AND MAGNESIUM (2 mM) ON THE METABOLISM AT 37°C IN PHOSPHATE OR TRIS BUFFER OF RAM SPERMATOZOA AFTER STORAGE AT −79°C

Values are the means for three ejaculates expressed as μmoles per 10^8 spermatozoa over the experimental period (3 hr)

Milk Solids	Magnesium	Potassium	Oxygen Uptake in:		Fructose Oxidized in:	
			Phosphate Buffer	Tris Buffer	Phosphate Buffer	Tris Buffer
−	−	−	0·73	0·73	0·035	0·048
−	−	+	0·84	0·94	0·055	0·079
−	+	−	0·64	0·70	0·035	0·048
−	+	+	0·93	1·01	0·056	0·078
+	−	−	0·73	0·72	0·043	0·057
+	−	+	0·91	0·88	0·068	0·068
+	+	−	0·76	0·75	0·045	0·059
+	+	+	0·92	0·98	0·062	0·070
Standard error of mean			0·076*		0·0065*	

* 30 degrees of freedom.

TABLE 4

EFFECT OF POTASSIUM AND MAGNESIUM ON THE OXIDATIVE METABOLISM OF THAWED BULL SPERMATOZOA AFTER WASHING IN ISOTONIC SALINE

Values are the means for three ejaculates expressed as μmoles per 10^8 spermatozoa over the experimental period (3 hr)

Potassium (mM)	Magnesium (mM)	Oxygen Uptake in:		Fructose Oxidized in:	
		Phosphate Buffer	Tris Buffer	Phosphate Buffer	Tris Buffer
0	0	0·36	0·27	0·028	0·023
0	2	0·41	0·36	0·024	0·022
0	10	0·44	0·38	0·018	0·019
1	0	0·49	0·45	0·027	0·029
1	2	0·46	0·43	0·025	0·029
1	10	0·45	0·49	0·022	0·026
10	0	0·54	0·58	0·028	0·036
10	2	0·59	0·58	0·027	0·035
10	10	0·52	0·50	0·027	0·032
Standard error of mean		0·044*		0·0029*	

* 34 degrees of freedom.

(*e*) *Experiments 8–11: Effects of the Addition of Potassium and Magnesium During Incubation at 37°C on the Metabolism of Cells Before and After Storage*

Because of the results with ram semen in experiment 7, the appropriate buffers were used in the washing diluents.

In experiment 8, aliquots of ram spermatozoa, washed in phosphate or Tris diluent before incubation, were incubated at 37°C in the same diluents containing 6 mM fructose and in the presence or absence of 2 mM magnesium chloride and 5 mM potassium chloride alone or in combination. The metabolism of spermatozoa after storage in milk diluent at −79°C was compared with that of aliquots incubated shortly after collection. The mean results for three ejaculates (1–3 $\times 10^8$ cells per flask) are presented in Table 5. Oxygen uptake, fructose oxidation, and lactate accumulation were decreased by freezing ($P<0\cdot01$) and increased by the addition of potassium ($P<0\cdot01$). The addition of magnesium increased the oxygen uptake ($P<0\cdot05$). Although the oxygen uptake was similar with the two buffers, the amount of fructose oxidized was greater ($P<0\cdot01$) and that of lactate accumulated was smaller ($P<0\cdot01$) in the presence of Tris.

A similar experiment was carried out with bull spermatozoa (1–2 $\times 10^8$ cells per flask). In general freezing decreased ($P<0\cdot01$) and addition of potassium increased ($P<0\cdot01$) cell metabolism (Table 5). There were interactions such that the increase in lactate accumulation on addition of potassium was greater in phosphate buffer than in Tris buffer with control but not with stored spermatozoa ($P<0\cdot05$), and addition of potassium increased fructose oxidation in all treatments except the control spermatozoa in Tris diluent ($P<0\cdot05$).

In experiment 10 ram spermatozoa were treated in the same manner as in experiment 8 and incubated in diluents containing 0, 5, 10, or 20 mM potassium. The mean results for four ejaculates (1–3 $\times 10^8$ cells per flask) are presented in Table 6. Freezing depressed cell metabolism ($P<0\cdot01$). There were no differences in the effects of the three levels of potassium and in general potassium addition increased cell metabolism ($P<0\cdot01$). However, there was a significant interaction such that lactate accumulation by control spermatozoa was not increased by addition of potassium in Tris buffer but was increased in the other treatments ($P<0\cdot05$).

The effects on bull spermatozoa of increasing potassium in the absence of magnesium were also examined (Table 6). The mean results for four ejaculates (1–2·5 $\times 10^8$ cells per flask) show that freezing depressed cell metabolism. Addition of potassium resulted in the same effects on fructose oxidation and lactate accumulation as in experiment 9. In addition there were several treatment interactions in oxygen uptake. The increase in oxygen uptake on addition of potassium was relatively greater with stored cells than with fresh cells ($P<0\cdot05$) and was greater in the presence of phosphate buffer ($P<0\cdot05$). Oxygen uptake was greater in phosphate buffer than in Tris buffer with control but not with stored spermatozoa ($P<0\cdot01$).

In experiments 8–11 fructose utilization by the control samples was greatly increased by the addition of potassium in the presence of phosphate but was only slightly increased (ram) or unaltered (bull) by potassium in the presence of Tris buffer.

IV. Discussion

After freezing, ram spermatozoa are susceptible to damage from washing. As most suitable freezing diluents contain sugars (Emmens and Robinson 1962) this means that, with a limited degree of washing, residual fructose and lactate remain. Thus, in contrast to recently ejaculated semen where virtually all exogenous substrates can be removed (O'Shea and Wales 1965), substrate oxidation by thawed

TABLE 5

EFFECTS OF POTASSIUM AND MAGNESIUM IONS ON THE METABOLISM AT 37°C IN TRIS- OR PHOSPHATE-BUFFERED DILUENTS OF RAM AND BULL SPERMATOZOA BEFORE AND AFTER STORAGE AT −79°C

Values are expressed as μmoles per 10^8 spermatozoa over the experimental period (3 hr) and are the means for three ejaculates

Treatment	Buffer	Mg^{2+} Concn. (mM)	K^+ Concn. (mM)	Ram Spermatozoa				Bull Spermatozoa			
				Oxygen Uptake	Fructose Oxidized	Lactate Accumulated	Fructose Utilized	Oxygen Uptake	Fructose Oxidized	Lactate Accumulated	Fructose Utilized
Control	Phosphate	0	0	1·65	0·229	0·58	0·60	0·63	0·091	1·16	0·81
		0	5	2·68	0·370	2·50	2·10	1·03	0·142	2·35	1·47
		2	0	2·94	0·364	1·80	1·68	0·67	0·089	1·22	0·82
		2	5	2·91	0·395	2·64	2·13	1·00	0·142	2·36	1·46
	Tris	0	0	2·22	0·308	0·72	0·56	0·64	0·108	0·74	0·59
		0	5	2·66	0·381	0·93	1·28	0·67	0·109	0·87	0·72
		2	0	2·65	0·375	0·83	1·03	0·71	0·120	0·71	0·48
		2	5	2·75	0·416	0·85	1·27	0·69	0·112	0·84	0·55
Stored at −79°C	Phosphate	0	0	0·33	0·018	0·11		0·25	0·014	0·33	
		0	5	0·45	0·026	0·18		0·42	0·021	0·46	
		2	0	0·32	0·015	0·09		0·29	0·018	0·39	
		2	5	0·51	0·030	0·23		0·35	0·019	0·42	
	Tris	0	0	0·31	0·020	0·08		0·21	0·014	0·27	
		0	5	0·51	0·037	0·17		0·33	0·017	0·33	
		2	0	0·47	0·029	0·10		0·29	0·016	0·24	
		2	5	0·54	0·033	0·17		0·35	0·017	0·38	

TABLE 6

EFFECTS OF POTASSIUM ON THE METABOLISM AT 37°C IN TRIS- OR PHOSPHATE-BUFFERED DILUENTS OF RAM AND BULL SPERMATOZOA BEFORE AND AFTER STORAGE AT −79°C

Values are expressed as μmoles per 10^8 spermatozoa over the experimental period (3 hr) and are the means for four ejaculates

Treatment	Buffer	K^+ Concn. (mM)	Ram Spermatozoa				Bull Spermatozoa			
			Oxygen Uptake	Fructose Oxidized	Lactate Accumulated	Fructose Utilized	Oxygen Uptake	Fructose Oxidized	Lactate Accumulated	Fructose Utilized
Control	Phosphate	0	1·95	0·219	0·93	0·84	1·50	0·154	0·97	0·82
		5	2·74	0·307	3·20	2·24	2·62	0·255	2·47	1·89
		10	2·67	0·304	3·24	2·18	2·29	0·237	2·58	1·99
		20	2·76	0·306	3·23	2·24	2·40	0·263	2·77	2·13
	Tris	0	2·23	0·265	0·89	0·92	1·46	0·160	0·51	0·67
		5	2·57	0·298	1·06	1·26	1·57	0·182	0·63	0·73
		10	2·57	0·323	1·09	1·21	1·59	0·182	0·64	0·59
		20	2·48	0·306	1·07	1·11	1·57	0·177	0·61	0·66
Stored at −79°C	Phosphate	0	0·23	0·011	0·06		0·40	0·012	0·41	
		5	0·33	0·017	0·16		0·91	0·022	0·52	
		10	0·35	0·017	0·18		0·69	0·019	0·52	
		20	0·37	0·018	0·19		0·77	0·023	0·52	
	Tris	0	0·26	0·014	0·05		0·41	0·013	0·27	
		5	0·36	0·022	0·11		0·71	0·026	0·39	
		10	0·38	0·022	0·12		0·70	0·028	0·43	
		20	0·38	0·025	0·15		0·70	0·031	0·48	

semen cannot be studied in the presence of a single added substrate. For example, the removal of residual lactate plus cell damage would explain the non-significant increase in fructose oxidation, coupled with a decreased oxygen uptake, with more severe washing (Table 2). In addition, the decrease in metabolism due to storage makes the measurement of metabolic activity, especially glycolysis, more difficult. These factors emphasize the necessity of using isotopic tracers when more than the oxygen uptake of thawed spermatozoa is required. From the preliminary experiments it was concluded that there is no preferential precipitation of dead or alive spermatozoa during centrifuging and the method of washing described in Section II was chosen as giving reasonable recovery of spermatozoa with minimal risk of damage. Although recently ejaculated semen may be washed using a lower centrifugal force, washing of all semen was standardized.

Addition of various complex substances or macromolecules to the incubation diluent could be expected to increase the metabolism of thawed spermatozoa, as has been observed with recently ejaculated (Mann 1964) or cooled semen (O'Shea and Wales 1966), but no increase was obtained with dialysed skim-milk solids. Additives such as skim-milk powder introduce exogenous substrates, and to keep the metabolic conditions as simple as possible they were not used in subsequent incubation, despite the increased oxygen uptake of thawed cells with skim-milk powder (experiment 4).

Spermatozoa frozen to $-79°C$ in milk diluent had a higher subsequent rate of metabolism at 37°C than did cells after storage in the synthetic diluent. These results (Table 1) are not strictly comparable with those reported by Jones and Martin (1965), as their synthetic incubation diluents contained large amounts of hexoses. However, it does appear that ram spermatozoa after storage in milk diluent are more tolerant of incubation conditions than are cells stored in synthetic diluents.

As has been reported with recently ejaculated semen (Salisbury and Lodge 1962; Murdoch and White 1966; Wales and O'Shea 1966), addition of magnesium has somewhat variable and small effects on the metabolism of thawed spermatozoa. Addition of potassium greatly increases the metabolism of thawed ram and bull semen. The increased metabolism of recently collected bull spermatozoa on addition of potassium has a tendency to be smaller in the presence of Tris buffer than with phosphate buffer (Tables 5 and 6). A similar picture is seen only in the glycolytic metabolism of ram semen. With ram spermatozoa, Wallace and Wales (1964) found no difference in effects of potassium addition with Tris or veronal diluents, but they washed the spermatozoa more lightly. The relatively larger response by thawed cells, as compared to controls, to potassium addition in the presence of Tris may be related to the large loss of potassium during cooling (O'Shea and Wales 1967) and freezing (Quinn and White 1966).

Despite this large loss of ions by frozen cells there were no differences in the response of thawed cells to addition of 5, 10, or 20 mM potassium. Thawed bull spermatozoa showed higher metabolism in 10 mM than in 1 mM potassium (Table 4), but this may have been related to the use of unbuffered saline for washing.

In general, addition of potassium and magnesium gave similar effects with thawed spermatozoa as with recently collected semen. As well the metabolic responses of bull and ram spermatozoa to freezing and to addition of ions were similar. It is apparent that neither the fall in glycolytic and oxidative metabolism nor the loss of

potassium can explain the difference between the fertility of frozen bull and ram semen.

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