COMPARISON OF EPIDIDYMAL AND EJACULATED SEMEN OF THE RAM

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Summary. A comparison has been made of the physiological and chemical properties of ejaculated and epididymal semen collected from rams with a surgical fistula into one vas deferens.

The most striking difference between the spermatozoa was the resistance of epididymal cells to cold shock, which was correlated with the attachment of kinoplasmic droplets.

Other intrinsic differences between the spermatozoa were small and there was little evidence of a difference in metabolic pattern.

The two types of seminal plasma differed considerably in chemical composition, although the pH was about the same and glycerylphosphorylcholine was present in high concentration in both.

Evidence was obtained that the fructose in ejaculated ram semen enables the motility of the spermatozoa to be better maintained under anaerobic conditions than is the case with epididymal spermatozoa.

INTRODUCTION

The literature suggests there may be important differences in the properties of epididymal and ejaculated spermatozoa. Perhaps the most conspicuous change is the loss of the kinoplasmic droplet; as spermatozoa move through the epididymis, the kinoplasmic droplet migrates towards the tail of the spermatozoon and is seldom found attached to the cell after ejaculation (Gresson & Zlotnik, 1945; Branton & Salisbury, 1947; Mukherjee & Bhattacharya, 1949; Rao & Berry, 1949; Bialy & Smith, 1958). An increase in specific gravity also occurs, suggesting a general shrinkage and dehydration of the protoplasm during the ripening process (Lindahl & Kihlström, 1952; Lindahl, Kihlström & Strom, 1952). In addition, the fertilizing capacity of spermatozoa increases as they move along the epididymis (Young & Simeone, 1930; Munro, 1938) and in some species, at least, the cells acquire the potential to move (Mukherjee & Bhattacharya, 1949; Rao & Berry, 1949; Salisbury, 1956).

Epididymal spermatozoa from the bull and boar have been shown to be more resistant than ejaculated cells to a sudden fall in temperature; the so-called 'cold shock' (Lasley & Mayer, 1944; Lasley & Bogart, 1944). According to Lardy, Hansen & Phillips (1945), there are also important differences in the metabolic pattern of epididymal and ejaculated bull spermatozoa, which they attribute to a 'metabolic regulator' released in an active form after ejaculation (Lardy, Ghosh & Plaut, 1949).

The fact that spermatozoa survive for longer periods in the epididymis than they do at the same temperature after ejaculation again suggests there might be intrinsic differences between the two types of cells (Hammond & Asdell, 1926; Asdell & Salisbury, 1941). Particularly favourable chemical and physical conditions in the epididymis might, of course, also account for this. Detailed information on the chemical composition of epididymal fluid is, however, lacking.

One of the difficulties in the study of epididymal spermatozoa, hitherto, has been the necessity to kill animals in order to collect material by the methods of Czarnetzky & Herle (1938) or Lasley & Bogart (1944). Thus, it has been impossible to make repeated observations on the epididymal spermatozoa of an individual and difficult to compare epididymal and ejaculated spermatozoa from the one animal.

The problem has been approached by establishing a fistula into the vas deferens of one testis close to the tail of the epididymis so that on electrical stimulation ejaculated spermatozoa could be obtained from the penis, and epididymal spermatozoa from the fistula (White, Larsen & Wales, 1959). It is the purpose of this paper to report the results of a comparison of epididymal and ejaculated spermatozoa obtained from a number of rams in this way.

MATERIAL AND METHODS

The operation for establishing epididymal fistulae in the ram and the method of collecting epididymal semen have been previously described (White, Larsen & Wales, 1959).

The volume of the ejaculated semen was measured in a graduated centrifuge tube and that of the epididymal semen in an opsonic pipette. The pH of the secretions was determined electrometrically with micro-electrodes.

Motility was estimated by visual examination under a low-power microscope by the system of Emmens (1947). Full motility was rated as 4 and complete immotility as 0. Since there was evidence that the motility of epididymal spermatozoa declined in the absence of a glycolyzable sugar, the semen was examined routinely after mixing with a small volume of diluent (0.132 M-NaCl, 0.005 M-KCl, 0.001 M-KH₂PO₄, 0.001 M-MgSO₄ 7H₂O, 0.01 M-Na₂HPO₄ 12H₂O) containing 100 mg/100 ml fructose, or after it had been well aerated.

Total sperm counts were made with a haemocytometer, the semen being diluted with 3% sodium-chloride solution.

A mixture of 2% congo red and 5% nigrosin (Gurr) in 2.6% sodium-citrate dihydrate was used to assess the percentage of live spermatozoa. One drop of semen was mixed with two drops of stain and duplicate smears made after 5 min. One hundred spermatozoa were counted on each slide and the mean percentage of unstained spermatozoa was used as unit observation in the analyses of variance. In the cold-shock experiments, the semen was held at 30°C and the stain at the required temperature — i.e., 30°, 25°, 20°, 15°, 10°, 5° and 0°C, as described by Hancock (1951).

The percentage of attached kinoplasmic droplets was determined from a

mixture of a drop of semen with 5 ml of diluent containing a drop of neutral formalin. This suspension was mounted on a slide under a coverslip and examined with the phase-contrast microscope. The percentage of spermatozoa with attached kinoplasmic droplets was estimated by examining 100 spermatozoa.

All semen samples used for chemical analyses were frozen in a thermos of dry ice immediately after ejaculation and maintained at -79°C until required for the analyses. Total reducing sugar was estimated by Somogyi's (1952) modification of the Nelson (1944) colorimetric method. Fructose was estimated by the method of Mann (1948) with an incubation period of 20 min for colour development (White, 1959). Lactic acid was estimated by the method of Barker & Summerson (1941). Glycerylphosphorylcholine was measured by the technique of White (1959), which is essentially an adaptation of Ryley's (1955) method of estimating glycerol after removing any sugars by copper-lime treatment.

Before all motility and metabolic experiments, fructose-free diluent was added to the epididymal semen to bring it to about the same cell concentration as the ejaculated semen. For some studies, the spermatozoa were first washed; this was done by diluting the semen 1:10, and centrifuging at 1500 rev/min (about 300g) for 10 min. The supernatant was replaced with an equal volume of diluent and the spermatozoa dispersed and recentrifuged. The second supernatant was removed and the volume adjusted to that of the original semen. The washed suspension was then treated in the same way as the unwashed semen.

For motility studies, ejaculated and epididymal semen (adjusted to the same cell concentration) was usually diluted 1:10 or 1:20. As quarter grades were frequently used in the scoring of motility, the actual score over an experimental period has been multiplied by 4 to avoid fractions. These values are referred to subsequently as the motility index (Emmens, 1948). In some experiments, visual estimates of the percentage of motile spermatozoa were made to the nearest 5%. This estimate, after angular transformation, was used as unit observation in the analyses of variance.

Collections from two to four fistulated rams had to be pooled for metabolic experiments, but where possible different combinations of animals were used for each replication. In pooling ejaculates, the relative contribution of the ejaculated semen of each ram was kept the same as that of the epididymal semen; the final concentration of spermatozoa in suspensions was adjusted to 100 to 200 million/ml by the addition of appropriate diluent. Oxygen uptake was measured over 3 hr by the direct Warburg technique (Umbreit, Burris & Stauffer, 1949). The shaking rate was 114 strokes/min and the gas phase air. For anaerobic experiments, 1 ml of diluted sperm suspension was pipetted into a narrow tube, covered with 2 cm of oxygen-free paraffin and stoppered. Fructose and lactic acid were estimated at the start and end of experiments. After the metabolic experiments, total counts of spermatozoa were made in duplicate. Oxygen uptake, fructose utilization and lactic acid production (per 10^8 cells) over the experimental period was then calculated and used as unit observation in the analyses of variance.

RESULTS

PHYSIOLOGICAL AND CHEMICAL CHARACTERISTICS

Table 1 shows some of the physiological and biochemical characteristics of epididymal and ejaculated semen collected during several months from thirteen rams with fistulae into one vas deferens. Both types of semen were of high quality as judged by motility, total sperm counts and the percentage of unstained cells (see White, Larsen & Wales, 1959). The volume of epididymal semen obtained was small, but very concentrated, and had a higher percentage of attached kinoplasmic droplets than the ejaculated semen. Simultaneous observations were made on ejaculated and epididymal semen from the same animal, but owing to the small volume of fluid obtained from the fistula, only one biochemical estimation could be performed at each collection.

TABLE 1

OBSERVATIONS ON EPIDIDYMAL AND EJACULATED SEMEN COLLECTED FROM THIRTEEN RAMS WITH FISTULA INTO ONE VAS DEFERENS

	No. paired observa- tions	Ejaculated		Epididymal	
		Mean	S.D.	Mean	S.D.
Volume (ml)	103	1.3	0.49	0.17	0.12
pH	3	7.3	0.2	7.5	0.2
Reducing sugar (mg/100 ml)	3	699	153	36	13
Fructose (mg/100 ml)	7	300	101	10	10
Lactic acid (mg/100 ml)	3	31	9	37	10
Glycerylphosphorylcholine (mg/100 ml)	10	319	142	567	338

The paired observations were made on various animals at different times.

The pH of both fluids was about the same as that of blood, and the concentration of total reducing sugar and fructose was much higher in the ejaculated semen than in the epididymal fluid, which contained only traces of these substances. Glycerylphosphorylcholine was present in high concentrations in both secretions.

MOTILITY IN PRESENCE AND ABSENCE OF FRUCTOSE

Since epididymal fluid itself is devoid of fructose and the spermatozoa are mixed with the fructose-containing secretions of the other accessory glands only on ejaculation, the effect of the sugar on the motility of ejaculated and epididymal spermatozoa was studied.

The sperm suspensions were sealed in capillary tubing (i.e., under anaerobic conditions) and also shaken in open tubes (i.e., aerobic conditions). Under aerobic conditions, there was little difference in motility score between the two types of cells over 4 hr, whether the diluent contained 100 to 200 mg/100 ml fructose or not (Table 2). Under anaerobic conditions, the motility of the two types of spermatozoa was similar when fructose was present. Without fructose, motility was low, but the motility of the epididymal spermatozoa was more depressed than that of the ejaculated cells.

EFFECTS OF DILUTION

Since ejaculated spermatozoa of the ram and other mammalian species are known to be adversely affected by high dilution (Emmens & Swyer, 1948; Blackshaw, 1953; White, 1954), a comparison of the motility of epididymal and ejaculated ram spermatozoa has been made at 40, 4.0 and 0.4 millions/ml (Table 3). Motility was scored immediately after dilution and at hourly intervals for 4 hr. At the highest cell concentration ($40 \times 10^6/\text{ml}$), the viability of the epididymal and ejaculated spermatozoa was equally good. Both types of

TABLE 2

EFFECT OF FRUCTOSE ON THE MOTILITY OF EJACULATED AND EPIDIDYMAL RAM SPERMATOZOA.
VALUES ARE THE MEAN OF SEVEN REPLICATIONS

Spermatozoa	Fructose	Motility indices	
		Aerobic	Anaerobic
Ejaculated	—	75.6	35.9
	+	76.3	64.7
Epididymal	—	59.9	17.9
	+	63.3	63.7

Summary of the analysis of variance of the anaerobic results

The residual mean square is shown at the base of the variance ratio column.

Source of variation	d.f.	Variance ratio
Sperm differences	1	10.4*
Effect of fructose	1	100.2†
Ejaculate differences	6	3.5
Interactions:		
Sperm/fructose	1	8.2*
Sperm/ejaculate	6	2.1
Fructose/ejaculate	6	3.5
Residual	6	61

* $P < 0.05$

† $P < 0.01$

cell are clearly severely affected by high dilution, and at very low cell concentrations ($0.4 \times 10^6/\text{ml}$) most spermatozoa were immotile. At the intermediate cell concentration, however, the motility index of the ejaculated cells was significantly greater than that of the epididymal spermatozoa, indicating that they are somewhat less susceptible to the effects of dilution.

RESISTANCE TO COLD SHOCK AND HIGH TEMPERATURES

The proportion of cells staining with supra-vital dyes (Easley, Mayer & Bogart, 1942; Lasley, Easley & McKenzie, 1942; Hancock, 1951) has been used as a criterion to study the susceptibility of ejaculated and epididymal spermatozoa to cold shock. Four rams with fistulae into the vas deferens were used and three comparisons of the epididymal and ejaculated spermatozoa made with each animal (Table 4).

Two of the rams (Nos. 1 and 2) gave epididymal spermatozoa with a very high percentage of attached kinoplasmic droplets, and on all occasions these cells were much more resistant to cold shock than were the ejaculated spermatozoa. In the other two rams (Nos. 3 and 4), the percentage of epididymal spermatozoa with attached kinoplasmic droplets was not much higher than in the ejaculated spermatozoa and the epididymal cells were only slightly more resistant to cold shock. Table 4 shows the percentage of kinoplasmic droplets attached to the spermatozoa and the percentage of unstained cells after cold shocking to 0°C. A significant correlation of 0.8 was found on analysis (d.f. = 11, $P < 0.01$).

TABLE 3
EFFECT OF DILUTION ON THE VIABILITY OF
EJACULATED AND EPIDIDYMAL RAM SPERMATOZOA.
MEAN VALUES FOR TEN REPLICATIONS ARE GIVEN

Sperm concentration ($\times 10^6/\text{ml}$)	Motility indices of sperms	
	Ejaculated	Epididymal
40.0	64.0	60.3
4.0	45.9	31.4
0.4	7.6	3.3

Summary of the analysis of variance

The residual mean square is shown at the base of the variance ratio column.

Source of variation	d.f.	Variance ratio
Ejaculate differences	9	1.9
Effect of dilution		
Linear	1	544.9†
Quadratic	1	5.3*
Sperm differences	1	14.3†
Interactions:		
Ejaculates/dilution	18	1.0
Ejaculates/sperm types	9	1.9
Dilution/sperm types		
Linear	1	0.0
Quadratic	1	6.2*
Residual	18	59

* $P < 0.05$

† $P < 0.01$

The effect of seminal plasma on the susceptibility of six samples of epididymal ram spermatozoa to cold shock was also studied, and comparisons made with ejaculated cells from the same animal on each occasion. The epididymal spermatozoa, which had a high proportion of attached kinoplasmic droplets, were again much less affected by cold shocking at 0° and 5°C than were the ejaculated cells. Seminal plasma, however, had no significant effect on the percentage of unstained epididymal spermatozoa after cold shocking, although it caused a slight decrease in motility score and the percentage of motile cells.

The effects of exposing ejaculated and epididymal ram spermatozoa to temperatures of 45° and 50°C for 5 min are shown in Table 5. The epididymal cells were more resistant to high temperatures.

KINOPLASMIC DROPLETS

Most of the fistulated rams gave epididymal spermatozoa with a high percentage of attached kinoplasmic droplets. The ejaculated semen collected from these rams had a low percentage of attached kinoplasmic droplets and, presumably, the droplets are removed as the spermatozoa pass down the tract from the epididymis. Various treatments were tried in an attempt to shed some light on the mechanism of their removal.

TABLE 4

RELATION BETWEEN ATTACHED KINOPLASMIC DROPLETS (A.K.D.) AND SUSCEPTIBILITY OF RAM SPERMATOZOA TO COLD SHOCKING AT 0°C. THE MEAN OF THREE EJACULATES IS GIVEN FOR EACH RAM

Ram No.	Ejaculated spermatozoa		Epididymal spermatozoa	
	% A.K.D.	% Unstained	% A.K.D.	% Unstained
1	2	17	55	48
2	10	24	42	36
3	0	12	4	21
4	1	13	9	17

Summary of the analyses of variance

The residual mean squares are shown at the base of the variance ratio columns.

Source of variation	d.f.	Variance ratios	
		% A.K.D.	% Unstained
Sperm differences	1	14.6*	12.9*
Ejaculate differences			
Between animals	3	5.5*	5.4*
Within animals	8	0.4	0.8
Interaction (Error)	11	222	92

* $P < 0.05$

Incubating epididymal spermatozoa with seminal plasma for 2 hr at 37°C did not influence the percentage of attached kinoplasmic droplets. Allowing the epididymal spermatozoa to stand for 24 hr at room temperature had no effect, nor did alterations in pH (6 to 8) or tonicity (50 to 150). Epididymal spermatozoa were also incubated for 2 hr at 37°C with 1% trypsin, chymotrypsin, papain, ribonuclease, amylase and hyaluronidase. Of these enzymes, only hyaluronidase caused a significant decrease in the percentage of spermatozoa with attached kinoplasmic droplets (55% compared with a control of 79%; $t_{(3)} = 2.6$; $P < 0.05$).

METABOLISM OF EJACULATED AND EPIDIDYMAL SPERMATOZOA

Lardy, Ghosh & Plaut (1949) claim that washed epididymal spermatozoa of the bull have a lower endogenous respiration than ejaculated cells, but if a glycolyzable sugar is added then the oxygen uptake is higher. Moreover, on addition of sugar the epididymal spermatozoa are said to produce lactic acid much more rapidly under anaerobic than under aerobic conditions, whereas with ejaculated spermatozoa the rate of glycolysis is not much higher in the

TABLE 5

COMPARISON OF THE SUSCEPTIBILITY OF EJACULATED AND EPIDIDYMAL RAM SPERMATOZOA TO HIGH TEMPERATURE WHEN SUSPENDED IN RINGER'S SOLUTION. MEAN VALUES FOR SIX REPLICATIONS ARE GIVEN

	<i>Temp. °C</i>	<i>Motility</i>	<i>% Motile</i>
Ejaculated	30	4.0	72
	45	4.0	61
	50	0.8	24
Epididymal	30	3.9	63
	45	3.8	68
	50	2.1	43

Summary of the analysis of variance

The residual mean square is shown at the base of the variance ratio columns.

<i>Source of variation</i>	<i>d.f.</i>	<i>Variance ratios</i>	
		<i>Motility</i>	<i>% Motile</i>
Sperm differences	1	4.7	2.5
Temperature differences	2	104.5†	22.3†
Ejaculate differences	5	1.7	2.3
Interactions:			
Sperm/temperature	2	9.2†	4.2*
Sperm/ejaculate	5	2.3	1.5
Temperature/ejaculate	10	0.5	2.0
Residual	10	3.8	94

* $P < 0.05$

† $P < 0.01$

absence of oxygen (see Table 6). These differences, according to Lardy, Ghosh & Plaut (1949), are due to the presence in bull spermatozoa of a metabolic regulator which occurs in the epididymal spermatozoa in a bound form, but is released in an active form after ejaculation.

The oxygen uptake, aerobic and anaerobic fructose utilization and lactic acid production of six samples of ejaculated and epididymal ram spermatozoa are shown in Table 7. Twice-washed spermatozoa, with and without the addition of 100 mg/100 ml of fructose, were compared with unwashed controls. Since fructolysis and lactic acid production did not occur in the absence of fructose, only the results where this sugar has been added to the diluent have been included in the statistical analyses.

TABLE 6

METABOLISM OF EJACULATED AND EPIDIDYMAL BULL SPERMATOZOA
(CHANGE/10⁸ CELLS)

Spermatozoa	Oxygen uptake (μ l)		Lactic acid production (μ g)	
	Endogenous	Glucose	Aerobic	Anaerobic
Ejaculated	20	16	80	100
Epididymal	6	10	52	224

Data from Lardy, Ghosh & Plaut (1944) *Science*, 109, 365.

TABLE 7

METABOLISM OF EJACULATED AND EPIDIDYMAL SPERMATOZOA OF THE RAM (CHANGE/10⁸ CELLS). MEAN VALUES FOR SIX REPLICATIONS ARE GIVEN

Spermatozoa	Fructose	Oxygen uptake (μ l)	Fructose utilization (μ g)		Lactic acid production (μ g)	
			Aerobic	Anaerobic	Aerobic	Anaerobic
Ejaculated	—	52	78	76	8	65
	+	51	202	307	212	330
Washed ejaculated	—	11	—14	—15	10	18
	+	53	263	324	262	381
Epididymal	—	28	—7	13	5	1
	+	68	347	420	322	382
Washed epididymal	—	12	0	11	7	10
	+	58	360	393	270	368

Summary of the analyses of variance

The residual mean square is shown at the base of the variance ratio columns.

Source of variation	d.f.	Oxygen uptake	Variance ratios	
			Fructose utilization	Lactic acid production
Ejac. v. epidid. sperm.	1	0	17.1†	3.7
Effect of washing	1	28.4†	0.1	0.2
Effect of fructose	1	108.2†		
Ejaculate differences	5	1.7	15.6†	5.2
Aerobic v. anaerobic	1		6.0*	24.0†
Interactions:				
Sperm./washing	1	1.0	0.9	1.2
Sperm./fructose	1	14.4†		
Sperm./ejaculate	5	3.1*	6.2†	3.5*
Washing/fructose	1	16.3†		
Washing/ejaculate	5	2.6	1.0	1.8
Fructose/ejaculate	5	0.7		
Conditions/sperm.	1		0.2	1.0
Conditions/washing	1		0.3	0.2
Conditions/ejaculate	5		0.6	4.4†
Residual	21	111	741	487

* $P < 0.05$

† $P < 0.01$

Because of the presence of fructose in the accessory secretions, the endogenous respiration of unwashed ejaculated spermatozoa was higher than that of epididymal cells. The endogenous respiration of washed epididymal and ejaculated spermatozoa, however, was similar and the addition of fructose increased the oxygen uptake to the same extent.

The fructose utilization of epididymal spermatozoa tended to be higher than that of ejaculated cells, but this varied from ejaculate to ejaculate and, in general, no striking difference was found in the metabolic pattern of ejaculated and epididymal spermatozoa. Both types of cell showed a slight Pasteur effect, i.e., an increase in fructose breakdown and lactic acid production under anaerobic conditions.

DISCUSSION

It seems reasonable, in view of the proximity of the fistula to the tail of the epididymis, to expect that chemical analysis of the fistula fluid would, in general, reflect conditions prevailing within the epididymis. The fluid analyzed might, of course, contain some secretions from the vas deferens as well as the epididymis and fairly constant leakage from the fistula could possibly cause some quantitative variation in composition. The pH values recorded for epididymal fluid in this paper agree with those of Bishop & Matthews (1952), who found that the pH in the rabbit vas with an intact blood supply was about 7.4. It seems unlikely, therefore, that pH is an important factor in limiting the activity of spermatozoa in the epididymis as has been suggested in the past.

The virtual absence of fructose in epididymal fluid, and its high concentration in ejaculated semen, confirms the finding of Mann (1946) that the seminal vesicles are chiefly responsible for the fructose of ram semen. At least some of the material estimated as reducing sugar in epididymal secretions appears to be ascorbic acid (White & Wales, 1960, unpublished data). In the ejaculated semen, fructose constitutes the bulk of total reducing sugar (Mann, 1954); the discrepancy between these values in Table 1 is due to the fact that it was not possible to perform both analyses on the same sample.

The values for glycerylphosphorylcholine, although high, are rather less than previously found by Dawson, Mann & White (1957) using the choline method of Appleton, LaDu, Levy, Steel & Brodie (1953). Ram spermatozoa are incapable of metabolizing glycerylphosphorylcholine and, despite the high concentration in the epididymis, glycerylphosphorylcholine cannot act as a source of energy in this organ (Dawson, Mann & White, 1957). There does, however, appear to be an enzyme in fluids of the ewe's genital tract that can break down glycerylphosphorylcholine (White & Wallace, 1961), so that free glycerol or phosphoglycerol may be made available to the spermatozoa after copulation.

The question of what constitutes the substrate for the metabolism of spermatozoa stored in the epididymis is an intriguing one. It must be remembered that there may be constant interchange between the blood and epididymal fluid, so that despite the low concentration of reducing sugar in the epididymal fluid at any one instance, it, and perhaps also acetate (Scott, White & Annison, 1961), could still be important in the economy of the spermatozoa.

The finding that the motility of epididymal spermatozoa falls off *in vitro* if they are deprived of both oxygen and a glycolyzable sugar has been adequately explained (see Mann, 1954) on the basis that spermatozoa contain intracellular material that can serve as an energy source for motility under aerobic conditions. Under anaerobic conditions, intracellular reserves cannot be utilized and the motility of epididymal spermatozoa will only be maintained in the presence of added sugar, since epididymal semen itself contains no fructose.

The reason that ejaculated spermatozoa withstand dilution better than epididymal cells is probably the presence of protective proteins in the seminal plasma. White (1954) found that none of a large number of low-molecular-weight constituents of seminal plasma protected ram spermatozoa against the harmful effects of high dilution. Protection was, however, given by 10 % seminal plasma and a variety of high molecular weight substances such as protein and starch (Blackshaw, 1953).

The present studies suggest that there are only slight physiological differences between ejaculated spermatozoa and spermatozoa in the cauda epididymis. The most notable difference observed was the resistance of epididymal spermatozoa to cold shock, particularly those with attached kinoplasmic droplets. The kinoplasmic droplet apparently contains lipid, since it stains with Sudan 111 and osmium tetroxide (Mukherjee & Bhattacharya, 1949). The phospholipid, lecithin, protects spermatozoa against cold shock (Phillips & Lardy, 1940; Kampschmidt, Mayer & Herman, 1953; Blackshaw, 1954) and, according to Lasley & Bogart (1944), the high resistance of spermatozoa from the epididymis of the stallion and boar can be maintained after ejaculation by the addition of this substance to the medium. It seems unlikely, however, that the ability of epididymal spermatozoa to withstand cold shock is due to the lipid content of the kinoplasmic droplet. The presence of an attached kinoplasmic droplet is usually regarded as evidence of an immature cell and this rather than the droplet *per se* may be the important factor in determining the susceptibility of ram spermatozoa to cold shock.

A high percentage of spermatozoa still possess an attached kinoplasmic droplet as they enter the vas deferens. Few ejaculated spermatozoa, however, have attached droplets and, presumably, they are removed as the spermatozoa pass down the reproductive tract from the epididymis. The mechanism of removal, however, remains obscure. The effect of hyaluronidase is not sufficiently great to account for the very rapid detachment of the droplets which must often occur on ejaculation. Since hyaluronidase has some action, however, a polysaccharide such as hyaluronic acid may be involved in the attachment of the droplet to the spermatozoa. Bialy & Smith (1958) claim that the kinoplasmic droplets of bull spermatozoa are, to some extent, detached by seminal plasma.

The fact that ejaculated and epididymal spermatozoa collected from the fistulated rams showed no difference in metabolic pattern is difficult to reconcile with the results of Lardy, Hansen & Phillips (1945) and would seem to call into question the existence of a metabolic regulator in ram spermatozoa.

It must, of course, be remembered that these workers used bull spermatozoa and there may be a species difference. However, caution is needed in inter-

preting metabolic experiments with slaughter-house material. Apart from the delay between killing bulls and starting the experiments, epididymal and ejaculated spermatozoa from the one animal were not compared and the results could be confused by variation between animals, which can be considerable. There might also be differences in washing technique. Unless ejaculated ram and bull spermatozoa are thoroughly washed, sufficient fructose may be left in the suspension to confuse the issue. This may account for the claim that washed epididymal spermatozoa have a lower endogenous oxygen uptake than the ejaculated cells and a relatively greater response to added sugar.

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