IONIC GRADIENTS IN SOME INVERTEBRATE SPERMATOZOA[1]

H. B. STEINBACH AND PHILIP B. DUNHAM[2]

Department of Zoology, University of Chicago, Chicago 37, Illinois, and the Marine Biological Laboratory, Woods Hole, Massachusetts

While the variety of male gametes seems almost infinite, the vast majority of animals produce a spermatozoan of fairly typical structure consisting of a head, midpiece and tail (Retzius, 1910). The indications from many different sources are that these three regions (plus other specialized areas such as the acrosome) have highly specific functions. The midpiece, with its mitochondrial structure, is implicated in general metabolism, the head is heavy with genetic information via DNA, and the tail functions as a locomotor organelle to propel the information unit towards its ultimate repository, *i.e.*, the ovum or death (*cf.* Mann, 1949).

Compartmentalized as it is in neat morphological packages, the typical spermatozoan becomes a tempting material with which to attempt to identify various bits of chemical machinery ubiquitous in all cells. A fairly extensive body of literature exists on the chemical and metabolic structure of the head and, to a lesser extent, on the midpiece. Isolated tail fractions have been shown to contain special fibrous proteins which may contribute the contractile properties (*cf.* Nelson, 1959), some of these components being arranged according to a precise numerology that awaits final elucidation (*cf.* Serra, 1960).

One of the most characteristic features of living cells, neglecting for the moment bacteria, etc., is the existence of an ionic gradient $K_i > K_o$ and, for most cells $Na_o > Na_i$ (Steinbach, 1952). These gradients are generally associated with irritability of cells and have at times been discussed as a reflection, at least, of an available free energy gradient (Fleckenstein, 1954). Ionic gradients have not been studied in any detail in sperm cells and, so far as we are aware, no previous attempt has been made to determine whether the production and/or the maintenance of an ionic gradient is a function of the whole structure or only a part thereof. The present report presents preliminary data on the distribution and exchangeability of Na and K of whole sperm and of separated head-midpiece fractions and tails. The head-midpiece complex, for the sperm of three species tested, appears capable of maintaining good ionic gradients. Tails, separated from the rest of the complex, appear to contain Na, K and Cl in free diffusion equilibrium with the environment.

Material and Methods

Mature sperm were collected as follows:

1. Male *Arbacia punctulata* were induced to shed into sea water by electrical stimulation. The suspensions were then diluted appropriately and used as described below.

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[2] Trainee in United States Public Health Service Training Program 2G-150.

2. *Mytilus edulis* sperm were collected by mincing the gonads in sea water, straining the resultant brei through cheesecloth and then subjecting to mild centrifugation to remove heavier contaminants. Microscopic examination showed these preparations to be reasonably free of tissue fragments.
3. *Phascolosoma gouldi* sperm were collected by draining body fluids of mature males into sea water and removing the large cell components by mild centrifuging. Clean suspensions of sperm could be obtained easily in this fashion.

The general pattern of treatment of sperm suspensions was as follows: The suspensions, maintained at sea-water table temperature (18–22° C.), were adjusted to the desired chemical composition. Separation of head-midpieces from tails was accomplished by 5–10 seconds at high speed in a Servall Blender. This method gave suspensions that could be separated by 10 minutes' centrifuging at ca. 2000 G into a pellet of apparently tail-less head-midpiece structures and a cloudy supernatant of filamentous tails. Especially in Mytilus, the tail suspension fibers frequently aggregated into sheaves.

Tails were collected as pellets by centrifuging the supernatant of the low speed centrifugation for 10 minutes at 15,000 G in a Servall superspeed centrifuge. This resulted in a clear supernatant apparently devoid of formed elements.

Concentration of cells was adjusted so that 10 ml. of suspension of whole sperm yielded on the order of 100–200 mg. of packed pellet as determined by weighing previously tared centrifuge tubes. Rather than attempt extensive cell counts, estimates of trapped fluid in the pellets were made by adding either C^{14} inulin to the suspension or $Na_2S^{35}O_4$ thus enabling the determination of inulin or sulphate "spaces." As noted below, there is certainly some question as to whether this is a true measure of a morphological volume of extracellular or cellular space, but the method seems more meaningful than the highly variable cell count method of determining cell volume. With fractions of such varying chemical composition it was deemed unwise to attempt to express Na or K contents per unit of nitrogen or of dry weight. Thus concentrations are expressed as millimols per 1000 grams of wet pellet or as figures derived from these on the basis of inulin spaces and/or dry weights.

Na and K were measured on extracts of pellets or on supernatant fluids with the Coleman flame photometer. Chlorides were frequently measured on the same solutions with an Aminco-Cotlove chloride meter. Extracts were prepared by heating briefly the suspended pellet in 10 ml. of water with 2 drops of glacial acetic acid added. Digestion with HNO_3 of residues from the above treatment showed almost complete removal of Na, K and Cl from the suspended material.

C^{14} was counted in the usual manner with a thin window gas flow counter.

Results

Table I summarizes the results of the first series of observations on the basic composition of the three types of sperm. Figures for bull and human sperm, uncorrected for inulin spaces, are given for comparison. Inulin spaces of the pellets are high and there are reasons to believe that the inulin is penetrating into some portions of the cell mass. However, inulin space is not greatly variable with time and is used in calculating cell water concentrations. Several points need

emphasis. K is concentrated in the sperm as it is in other cellular systems. If cell water volume could be truly represented as total pellet volume minus dry weight and inulin space, the K concentration of cell water is very high indeed for Arbacia and Phascolosoma.

Na and Cl contents are variable, as will be apparent also from subsequent tables. Table I indicates that no chloride is present in the non-inulin space. On the contrary, the fact that inulin space frequently exceeds chloride space of the pellet is an indication that inulin penetrates to some extent within the morphological boundaries of the sperm.

TABLE I

Dry weights and inulin spaces (as % of wet pellet) and concentrations as mM./1000 grams of wet pellets. Derived concentrations (mM./L. cell water) calculated assuming 97% of dry weight of pellet to be in sperm and external medium of composition Na = 410, Cl = 500, K = 10 mM. Composition of bull and human sperm from Cragle et al., 1958 and Keitel and Jones, 1956.

	% Dry (pellet)	Inulin %	% Dry (sperm)	Na		Cl		K	
				Pellet	$Cell_{H_2O}$	Pellet	$Cell_{H_2O}$	Pellet	$Cell_{H_2O}$
Arbacia (2)	24	46	45	195	23	150	—	136	440
Mytilus (4)	27	26	36	166	107	161	—	78	145
Phascolosoma (8)	18	44	32	209	76	216	—	115	300
Bull				76				62	
Human			16	101		28		39	

It is known that sperm of such forms as Arbacia and Mytilus can survive, properly diluted in sea water, for periods of several hours. Table II shows that these sperm can also maintain their ionic gradients for extended periods of time. With Mytilus there is a decrease of pellet size with time. In the absence of counts of viable cells, it is impossible to tell whether this involves an average decrease in cell size or a disintegration of some fraction of the populations. Whichever the cause, the decrease takes place within the first hour to hour and a half, pellet size being constant thereafter.

Potassium in sperm is nearly completely exchangeable (Fig. 1) at a fairly rapid rate. Half-time for exchange of cellular K with K^{42} of the medium is on the order of three hours for Mytilus, 1.5 hours for Phascolosoma, and less than an hour for Arbacia.

Figure 1 shows that the time course of exchange of K^{42} added to the medium with K of the pellet follows the usual course of an initial rapid exchange (extracellular?) followed by a second slower component. There is, however, no indication that any significant amount of K is sequestered within the sperm or otherwise made inaccessible to free exchange. The exchange of Na^{24} with Na of sperm is very rapid. Accurate results have not been obtained but the half-time for exchange is distinctly less than 30 minutes for all three types of sperm.

Table III lists average values for the analyses of head-midpiece and tail fractions as compared to whole sperm from the same species. The values listed

TABLE II

Pellet concentrations (mM./1000 gm. wet) of Na, Cl and K for samples of a suspension centrifuged at the times indicated. Sperm suspensions stored on sea water table at 20° C.

Time minutes	Weight of pellet %	Conc. mM./Kg.		
		Na	Cl	K
Mytilus				
10	100	162	126	74
30	98	159	164	75
90	91	155	151	67
210	90	155	139	64
Arbacia				
20	100	193	287	110
130	101	168	267	88
300	100	174	232	99

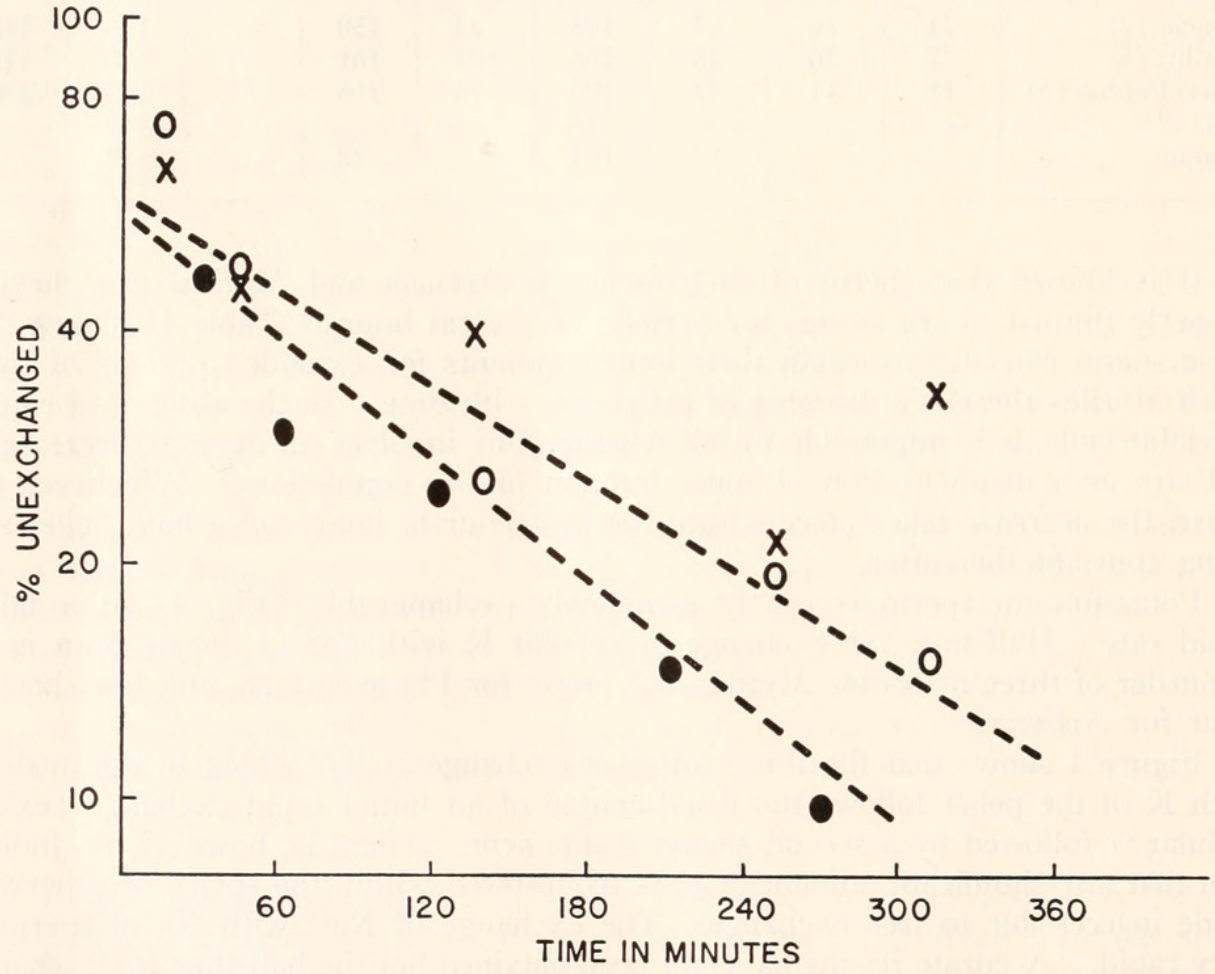

FIGURE 1. Time course of exchange of K^{42}, added to suspension fluid at zero time, with K of Mytilus sperm. Figures uncorrected for inulin space or dry weight. Dots and circles represent separate experiments; crosses are results from one run with isolated Mytilus sperm head-midpieces.

in Table III were obtained from analyses in which samples of whole sperm, head-midpiece and tail were taken from the same batches and processed during coincident time periods. Weights given are pellet weights obtained by weighing the previously tared centrifuge tubes after the supernatant fluids had been decanted, followed by inverting the tubes on filter paper for about five minutes. The gain in weight due to similar treatment of centrifuge tubes containing only pure water (or the supernatant from the centrifugation of whole sperm suspension) is in the range 10–15 milligrams (5–10% of the usual pellet weight).

With Arbacia and Mytilus sperm, head-midpieces and tail pellets contained less K and more Na and Cl than did the whole sperm, inulin spaces increasing markedly. Phascolosoma sperm heads, on the contrary, had a higher K content with lower Na and Cl, inulin spaces remaining about the same. Tail fractions of all three forms had very high inulin spaces, high Na and Cl contents and low K.

TABLE III

Relative weights, inulin spaces and Na, Cl and K concentrations of whole sperm, head-midpieces and tail pellets. Units as in Table I.

	Relative weight	Inulin space %	Na	Cl	K
Whole sperm					
Arbacia (4)	100	41	191	200	119
Mytilus (2)	100	39	244	271	44
Phascolosoma (8)	100	44	202	206	106
Heads-midpieces					
Arbacia (4)	72	56	305	329	54
Mytilus (2)	96	52	302	353	31
Phascolosoma (8)	64	44	173	192	130
Tails					
Arbacia (4)	33	85	394	401	15
Mytilus (2)	21	68	356	373	10
Phascolosoma (8)	20	92	422	384	25

Pellet weights of head and tail fractions corrected for the drainage factor (10 mg.) added together should approximate the corrected pellet weight of the whole sperm, since comparable volumes were centrifuged, starting with a standard suspension. This summation holds reasonably well for Arbacia and Phascolosoma. Mytilus sperm heads swell and hence the pellet weights of the parts add to more than the pellet weight of the whole sperm.

While head fractions undoubtedly were contaminated to a slight degree by separated tails, it is probable that the pellet weights indicate minimum values for weights of sperm tails as compared to the whole. On this basis tails make up 20–30% of the total sperm weight.

The increase in inulin space for the pellets of head fractions of Arbacia and Mytilus sperm as compared to whole sperm could represent either a general increase in inulin penetration into all units or a complete destruction of some of the units. It is impossible to select, on any rigorous basis, between these two alternatives since no method was available to us to distinguish, by independent criteria, dead heads

from live heads. However, much of the increase in Na and Cl and decrease of K could be accounted for by assuming that the increase in inulin space represented complete physiological destruction of the appropriate number of morphologically intact heads. Phascolosoma sperm would then be assumed to have tougher heads, all surviving the homogenization of the sperm suspension. Head fraction pellets of Arbacia and Mytilus sperm maintain relatively constant ionic contents over periods of several hours, thus showing that, if partial injury is responsible for the decreased K contents, the effects are not progressive. On the whole, we suggest that homogenization results in destruction of some units of the head suspension, the K content of individual intact heads remaining at at least as high a level as that of intact sperm.

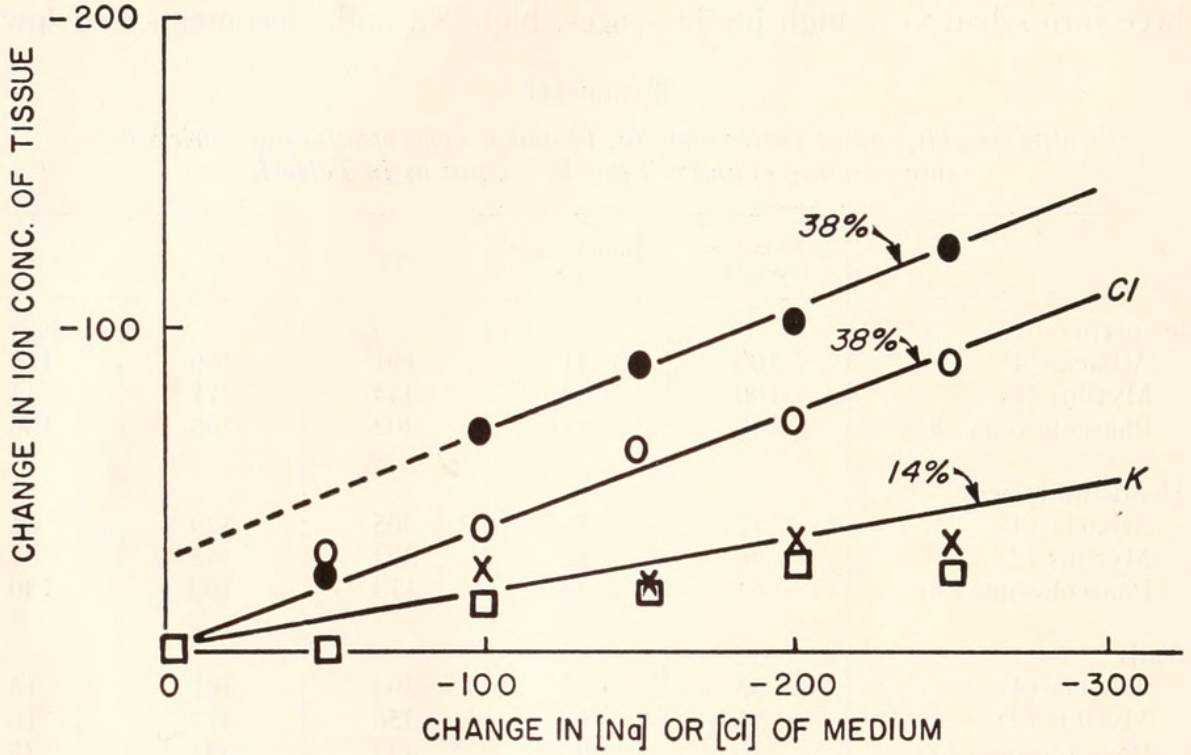

FIGURE 2. Values for Na (●), K (x), Cl (○) and pellet weights (□) of Arbacia sperm suspended 30 minutes in sea water diluted with 10 mM. KCl. Units in mM. per liter or per Kg. wet weight of pellet. The greatest dilution was a 50 : 50 mixture of sea water and 10 mM. KCl. Figures at arrows represent slopes of curves.

When Arbacia sperm are treated for 30 minutes with mixtures of sea water and 10 mM. KCl, thus varying Na_o, Cl_o and total ionic strength but holding K_o constant, the cells swell slightly as indicated by the increase in pellet weight (Fig. 2). In approximately 50% sea water the weight increase is slightly over 20%. Using the usual methods of calculation this would indicate a "non-osmotic" space (b value) of nearly 80%. Thus there is no evidence for an ordinary osmotic behavior of the sperm cells. As the medium is diluted, K_o holds constant, K_i decreases in somewhat greater degree than can be accounted for by the increase in cell volume. Pellet Na_i and Cl_i decrease with decrease in Na_o and Cl_o but in a manner indicating that the change, in the greater dilutions, is primarily in the inulin space fraction.

Arbacia treated with various concentrations of KCl in sea water (Na_o held constant, K_o and Cl_o varied) show a remarkably constant Na_i concentration (Table

TABLE IV

Arbacia sperm exposed to ionic concentrations indicated, prepared by adding KCl to sea water. Cell water concentrations calculated assuming 35% inulin space, 40% dry weight of sperm.

Na			$\frac{Na_i}{Na_o}$	Cl			$\frac{Cl_o}{Cl_i}$	K			$\frac{K_i}{K_o}$
Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$	
420	218	180	0.43	510	218	102	5.0	10	83	205	20.5
420	218	180	0.43	610	303	230	2.6	110	176	352	3.2
420	218	180	0.43	710	375	375	2.2	210	202	338	1.6
420	201	138	0.33	810	435	390	2.1	310	269	409	1.3
420	222	191	0.45	910	535	550	1.6	410	335	490	1.2
420	233	220	0.52	1010	650	760	1.3	510	405	580	1.1

IV). K_i and Cl_i increase markedly with no evidence of the reciprocal loss of Na_i noted for such systems as the frog sartorius. Based on calculated cell water concentrations, K_i/K_o is over 20 and Cl_o/Cl_i is 5 in normal sea water. Both ratios approach unity as total external ionic strength approaches two times that of sea water. There is no significant change in volume as total ionic strength is varied until very high concentrations are reached.

Mytilus sperm, treated in a similar fashion, show some increase in Na_i, Na_o being held constant (Table V). Both K_i and Cl_i increase somewhat but especially K_i is regulated to change much less than K_o, whereas K_i/K_o in normal sea water is about 15, the ratio is 0.5 in sea water made half molar with KCl.

In view of the interest in the use of glycerol as protective agent in freezing sperm, Mytilus sperm were suspended in 13% (by volume) of glycerol in sea water, controls being suspended in sea water diluted in corresponding fashion with distilled water. Little volume change was noted in 4–5 hours. K_i decreased with time in both glycerol-treated sperm and controls. Glycerol-treated sperm lost K and gained Na and Cl during the first half hour of treatment, the difference then being maintained. Motility in glycerol was maintained in an odd vibratile fashion, motility in the controls appeared normal throughout the experiment.

TABLE V

Mytilus sperm suspended 30 minutes in solutions of KCl in sea water as indicated. Calculations of cell H_2O concentrations assuming 40% dry weight of sperm and using inulin spaces indicated. K_i/K_o ratios calculated using cell water concentrations.

Relative volume pellet	% Inulin pellet	Na			$\frac{Na_i}{Na_o}$	Cl			$\frac{Cl_o}{Cl_i}$	K			$\frac{K_i}{K_o}$
		Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$		Medium	Tissue	$Cell_{H_2O}$	
100	32	420	197	155	0.37	510	197	83	6.1	10	66	153	15.3
98	33	420	205	166	0.39	610	257	163	3.7	110	127	226	2.1
98	32	420	202	166	0.39	710	313	210	3.4	210	171	255	1.2
102	34	420	232	225	0.53	810	404	325	2.5	310	225	301	0.9
109	41	420	266	265	0.63	910	483	310	2.9	410	266	274	0.7
106	42	420	275	285	0.68	1010	555	375	3.1	510	310	278	0.5

Discussion

While the results reported here must be regarded as a part of a preliminary survey, certain conclusions may be drawn. In common with all cells studied, spermatozoa of the type studied maintain high K and low Na and Cl concentrations as compared to their normal active environment. In mammals this ionic differentiation becomes pronounced only after the sperm descend to the lower portions of the reproductive tract or are released. In this case the ionic gradients are probably established due to changes in the seminal fluid, not in the sperm themselves (Salisbury, 1956). The precise environments during formation and maturation of sperm of the invertebrates used in the present studies are not known to us.

As the ionic environment is altered, there is evidence that minimum portions of Na_i and K_i are held rather firmly, K_i being considerably higher than Na_i. There is no indication of a reciprocal relationship between Na_i and K_i nor is there any equality of the Donnan Ratios K_i/K_o and Cl_o/Cl_i except under very abnormal conditions (0.5 *M* KCl in sea water).

Both K_i and Na_i are rapidly and nearly completely exchanged with the environment, thus showing that ion selection does not involve a rigid sequestering of the elements. Some evidence for active extrusion mechanisms might be obtained from more precise flux measurements but no evidence for the process can be adduced from the present data. Rather, sperm behave as though they could retain relatively fixed amounts of Na and K, meanwhile allowing exchanges freely between medium and cell. In general the electrolytes of sperm behave rather similarly to those of certain smooth muscles from invertebrates (*cf.* Steinbach, 1940).

With respect to the problem of localization of the ion distribution mechanisms, these results are suggestive but by no means conclusive. It is certain that the head-midpieces of all three types of sperm retain K and continue to exclude Na and Cl with reference to the medium. With Phascolosoma, head-midpiece fractions maintain higher respective ionic gradients than do whole sperm. With Arbacia and Mytilus the gradients of head-midpiece fractions are reduced. While there is no compelling evidence, it is tentatively assumed that mechanical damage to portions of the head-midpiece fractions of Arbacia and Mytilus is responsible for the decrease in magnitude of Na_o/Na_i, K_i/K_o and Cl_o/Cl_i in the head pellets as compared to whole sperm pellets.

Tail fractions always showed very high inulin spaces and little if any ability to maintain ion gradients. It is suggested that the contractile organelle of the spermatozoan is entirely dependent on the ion-concentrating mechanisms of the head-midpiece portion for its ionic gradients, assuming such exist *in vivo*. On the basis of the known structures and metabolic activities it would be logical to look for evidence that the midpieces play major roles in the processes. Mitochondria in general are known to be able to retain selectively various ions.

Isolated sperm tails contain enzymes usually associated with contractile processes and also show spontaneous movements on application of ATP. These spontaneous movements are best described as twitchings rather than conducted waves as seen in normal attached tails. It seems possible that normal coordinated activity of sperm may depend upon ionic gradients imposed on the contractile tails by the metabolic activity of the midpiece regions.

The sperm used in these experiments were all of the simple type presumably

possessing short midpieces and tails with the 9 + 2 arrangement of fibrils (Afzelius, 1955). It is anticipated that comparative studies with mammalian and other sperm with longer midpieces and more complicated flagellar structure, indicating a possible extension of mitochondrial components, will shed further light on the compartmentalization of ionic gradients.

Summary

1. The concentrations of Na, K and Cl of pellets of sedimented sperm of Arbacia, Mytilus and Phascolosoma have been determined. With the aid of inulin space determinations on the pellets, it can be shown that sperm resemble other cells studied in maintaining, relative to the sea water environment, high internal K concentrations and low Na and Cl.

2. Sperm of the three species were separated into head-midpieces and tail fractions. The former retain the ionic selection properties of the whole sperm. Isolated tails appear freely diffusible to all constituents including inulin.

3. Using radioactive tracers it is shown that Na and K of sperm are freely exchangeable with the environment.

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