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The Effects of Sperm Extracts and Energy Sources on the Motility and Acrosome Reaction of Hamster Spermatozoa in vitro

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ABSTRACT

Extracts of washed spermatozoa from hamster and guinea pig cauda epididymidis and of human ejaculated sperm, were all found to stimulate the motility of unwashed hamster epididymal spermatozoa *in vitro*, and to support development of fertilizing ability. The motility of hamster spermatozoa was only sustained in the presence of both sperm extracts and appropriate energy substrates, indicating a synergistic effect of the motility-stimulating component of the sperm extracts with energy sources. Albumin was also required for the development of fertilizing ability by hamster sperm. Comparison of different combinations of energy substrates indicated that pyruvate was the most important energy source for sperm motility and the acrosome reaction, but glucose and lactate played supporting roles. In all three species examined, the component of the sperm extracts that was responsible for stimulating hamster sperm motility was found to be heat-stable and to have the same elution volume on a Sephadex G-10 column, with an estimated molecular weight of about 200. These properties are identical to those of the sperm motility-stimulating factor found in blood serum and adrenal gland, suggesting that the active components may be similar if not the same.

INTRODUCTION

Hamster spermatozoa are extremely sensitive to dilution in culture media, which rapidly depresses or even abolishes their motility (Bavister, 1974). This phenomenon, which is referred to as the 'sperm dilution effect,' limits studies of the behavior of hamster sperm *in vitro*, particularly those aimed at defining precisely the conditions that favor the occurrence of capacitation and the acrosome reaction. Such studies could be useful in establishing the nature and mechanism of these important prefertilization changes in spermatozoa. The sperm dilution effect can be greatly reduced by incubating hamster sperm with egg cumulus-cell masses (Bavister, 1971), or with the dialyzable fraction of follicular fluid or blood serum (Yanagimachi, 1969a, 1970a). It was recently demonstrated that extracts of washed frozen-thawed hamster sperm can also diminish the sperm dilution effect; when incubated with such extracts, epididymal hamster sperm not only remain motile for several hours even when greatly diluted (Bavister, 1974), but they can also develop the 'activated' type of movement that is characteristic of capacitated sperm (described by Yanagimachi, 1970b).

These observations suggest that components important for motility are lost from the sperm in highly dilute suspensions, but can be replaced if they are incorporated into the suspending medium. These sperm components could be important for sperm motility *in vivo*; the idea that they have some physiological role would be strengthened if it were demonstrated that they can support the acquisition of fertilizing ability by spermatozoa. The analysis of the chemical structure of the motility-stimulating substance, and consequently the investigation of its mode of action, would be considerably facilitated if we were able to prepare it in large amounts from, for example, blood serum or the adrenal gland, both of which contain a sperm motility-stimulating factor which has been partially characterized (Morton and Bavister, 1974; Bavister, 1975; Bavister et al., 1976); however, for these purposes it is necessary first to establish, or at least to provide a strong indication, that the active substances from these sources and from sperm are the same.

The mode of action of the motility-stimulating substance is presently unknown; some

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insight into the way in which it stimulates sperm motility would be gained by determining whether or not it provides an essential energy substrate to the sperm. Hamster sperm extracts are almost ineffective in supporting sperm motility when glucose is the only energy source provided in the culture medium (Bavister, unpublished data). By contrast, the sperm motility-stimulating activity of such extracts is very pronounced in media containing pyruvate in addition to glucose (Bavister, 1974). This discrepancy suggested a possible role for oxidative metabolism in stimulating high levels of sperm activity, and perhaps consequently in the development of fertilizing ability. For this reason, we felt it would be informative to define the optimal energy substrate requirements for hamster sperm *in vitro*. It is also important to determine whether the sperm motility-stimulating activity is found in sperm of species other than the hamster, in which case it could be a general feature of spermatozoa, and thus perhaps of fundamental importance for mammalian sperm physiology.

The present investigation was carried out in order to answer the following questions: 1) Is the sperm motility-stimulating component of sperm extracts itself an essential energy source for the performance of spermatozoa *in vitro*?; 2) What are the optimum energy sources for maintaining motility and for supporting capacitation and the acrosome reaction of hamster spermatozoa in the presence of sperm extracts *in vitro*?; 3) Do the spermatozoa of species other than the hamster contain motility-stimulating activity?; 4) Do hamster spermatozoa acquire the ability to fertilize eggs during incubation with sperm extracts?; 5) Are there similarities in the properties of the motility-stimulating components derived from spermatozoa and those from other sources? In this communication, the term "acrosome reaction" is used to refer to loss of acrosomal components as discerned by phase contrast microscopy; this change may not be identical to that occurring in the fertilizing sperm *in vivo*.

MATERIALS AND METHODS

Preparation of Sperm Extracts

The caudae epididymides were removed from 3- to 4-month old hamsters weighing 130–150 g and the distal tubules were punctured under mineral oil (Squibb) in 10 to 20 places with a sterile hypodermic needle. The epididymides were gently squeezed with forceps to assist removal of their contents. Approximately 0.2 ml of viscous epididymal sperm suspension was obtained in this way from each pair of epididymides. This material was suspended in 10 ml of washing medium, consisting of equal parts of Dulbecco's phosphate-buffered saline (PBS) and isotonic (290 mM) sucrose solution. Disperson of sperm into this medium was aided by gently sucking the epididymal contents in and out of a wide-bore Pasteur pipette. The sperm suspension was centrifuged at 800 × g for 5 min at 23°C; the supernatant was discarded, and the packed sperm were washed once more in the same way. This washing procedure was carried out in order to remove the epididymal plasma, which has been reported to contain a sperm motility-stimulating substance (Morton and Chang, 1973) and thus would obscure the sperm-derived motility-stimulating activity. The packed sperm were carefully resuspended in 0.5 ml of PBS, and recentrifuged; the supernatant from this step ('sperm washing') was saved for assessment of its motility-stimulating activity. Finally, the packed sperm were resuspended in another 0.5 ml of PBS and frozen in liquid nitrogen or in a −20°C freezer, then thawed (23°C) and centrifuged at 1500 × g for 10 min at 23°C. The supernatant from this step, containing an extract of washed hamster sperm, is referred to as the 'sperm extract.' Examination of the frozen-thawed sperm by phase-contrast microscopy revealed that acrosomal disruption invariably occurred as a result of freezing, but no other gross structural changes were apparent.

Guinea pig cauda epididymal spermatozoa were treated in the same way as described for hamster spermatozoa, except that 0.9 percent NaCl solution was used instead of the washing medium. Human ejaculates were allowed to liquify at 23°C for ½ to 1 h, then the sperm were washed twice in 10 ml of washing medium, following the procedure given for hamster sperm. In preparing hamster sperm extracts, the use of 0.9 percent NaCl solution instead of the washing medium, or failure to exercise sufficient care during sperm resuspension, almost invariably resulted in very low sperm motility-stimulating activities in the sperm extracts. This degree of care was unnecessary with guinea pig or human spermatozoa, since even when quite vigorous resuspension of the sperm was carried out during washing, potent sperm extracts were still obtained. All 'sperm washings' and 'sperm extracts' were sterilized by Millipore filtration. The sperm concentrations in the final sperm suspensions used to prepare the sperm washings and sperm extracts varied between 2×10^8/ml to 5×10^8/ml (hamster), 1×10^7/ml to 1×10^8/ml (guinea pig) and 1×10^7/ml to 4×10^7/ml (human).

Procedure for Testing Sperm Extracts and Energy Sources

The culture medium used for testing sperm motility and penetrating ability was Tyrode's solution modified as follows. Extra sodium bicarbonate was added to give a final concentration of 25 mM (with appropriate reduction of NaCl). The concentration of glucose was 5.0 mM. Bovine serum albumin (Fraction V fatty acid-free, Pentex Biochemicals; 3 mg/ml), phenol red (10 ug/ml) and penicillin (100 i.u./ml) were added. This basic medium (modified Tyrode with

albumin: designated TA) was further modified in a variety of ways by the addition of different amounts of sodium pyruvate (Na salt, crystalline Type II dimer-free, Sigma Biochemicals) and sodium lactate (DL-lactate, grade DL-V, 50 percent L-isomer, Sigma Biochemicals). The initial concentrations of these substrates in the culture medium were: 0.1, 0.33 or 1.0 mM (pyruvate) and 4, 10 or 25 mM (lactate). In some cases, glucose was omitted (see Table 1). The concentrations of bicarbonate, phenol red, penicillin, albumin and all energy substrates as just given were reduced by 10 percent in the final test drops due to the addition of sperm extracts in PBS, or PBS controls (see below). Sodium pyruvate was added from a 10 mM or 100 mM solution (freshly prepared) in 0.9 percent NaCl; sodium lactate and/or glucose were incorporated into the various culture media (during their preparation) from isotonic solutions (150 mM and 295 mM, respectively), with appropriate reductions in the amounts of sodium chloride used.

The pH of the stock solution of sodium lactate was adjusted with 2N NaOH to approximately 7.4 in order to prevent changes in the pH of those culture media in which lactate was included. Once the optimal concentrations of lactate and pyruvate were determined (see Results), these energy substrates were routinely included in the culture medium, which was then designated TALP. The osmotic pressure of all variants of the culture medium, as determined by freezing-point depression, was between 285 and 295 mosmols. All culture media were sterilized by Millipore filtration.

Drops of culture media (45 μl) were pipetted into a plastic petri dish (Falcon Plastics, 60 × 15 mm, no. 1007) containing 10 ml of mineral oil (Squibb). These drops had to be encouraged to adhere to the surface of the dishes with a round-tipped sterile glass needle drawn from a Pasteur pipette. The dishes were incubated for 2 to 3 h at 37°C in 5 percent CO_2 in air to equilibrate the culture media; the pH of all media was approximately 7.6 following equilibration. Five ul of whole or fractionated sperm extracts in PBS, or 5 μl of PBS (controls), was added to each drop of culture medium. The drops were then inseminated with 0.25 μl of a freshly-prepared hamster sperm suspension (cauda epididymal contents diluted 1/50, usually in TA) to yield a final sperm concentration of approximately 1 to 2 × 10^5/ml. In the experiments with glucose-free media (Table 1), glucose was also omitted from the media used to prepare the sperm suspensions. Thus, extracts of washed, frozen-thawed hamster sperm were tested for their ability to sustain the motility of unwashed epididymal hamster spermatozoa. It was considered that the minute amounts of epididymal plasma (estimated final dilution 1/10,000) introduced into the test drops along with these unwashed spermatozoa would not significantly interfere with the results.

After insemination, the dishes containing the sperm suspensions were returned to the incubator. At intervals, dishes were placed on a warm (38°C) stage under a dissecting microscope and the percentages of motile spermatozoa were estimated; in some instances, the percentages of motile sperm showing activated motility were also estimated. In order to estimate the proportion of spermatozoa showing an acrosome reaction, aliquots of some sperm suspensions were transferred to a microscope slide, covered with a cover-glass supported on vaseline spots and 50 to 100 motile spermatozoa were examined in each sample. In some experiments, a sperm motility index, combining the percentage of motile sperm and the degree of motility (Bavister, 1974) was calculated.

In our preliminary experiments, different combinations of sodium pyruvate (0.09, 0.3 and 0.9 mM, final concentrations) with sodium lactate (3.6, 9.0 and 22.5 mM, final concentrations) were tested in medium TA (i.e., with glucose 4.5 mM) containing hamster sperm extract (diluted 1/10 in each culture drop). Four tests were conducted with each of these 9 combinations; in addition, the highest level of pyruvate was tested in the absence of lactate, and vice versa, glucose being present in both cases.

Bioassay for Testing Sperm Capacitation

After preincubating hamster epididymal spermatozoa for 4½ to 5 h with sperm extracts, their ability to penetrate washed, cumulus-free eggs was tested. The procedure used was a modification of that described by Bavister and Morton (1974). The cumulus-free eggs were washed twice in PB1-dextran, then rinsed in a drop of culture medium (with or without albumin, as appropriate: see Table 2) before being distributed among the different preincubated sperm suspensions. After incubating the gametes for 1 or 1½ h, the eggs were compressed between a slide and cover glass and examined by phase-contrast microscopy for evidence of sperm penetration. Penetrating spermatozoa were recorded as either showing or not showing signs of nuclear decondensation (see legend to Table 2). In two experiments with guinea pig sperm extracts (Table 2), albumin was omitted from the culture medium used to preincubate the hamster spermatozoa and subsequently for egg penetration testing; the purpose was to check that all soluble materials, including protein, that might contribute to the development of fertilizing ability of the sperm had been removed from the eggs.

Fractionation of Sperm Extracts

Because preparations are inevitably diluted during fractionation on gel columns, with consequent reduction of potency, sperm extracts were combined, lyphilized and concentrated before applying them to the column. Five hamster sperm extracts, totalling 1.35 ml, were combined, lyphilized and resuspended in 0.3 ml of distilled water; some undissolved material was removed by centrifugation at 10,000 × g, 10°C for 15 min before applying the preparation to a Sephadex G-10 column (72 × 0.95 cm) equilibrated with PBS at 23°C; 0.5 ml fractions were collected while eluting in PBS at 20 ml/h. Four guinea pig sperm extracts (2 ml total volume) were concentrated in a similar way to 0.25 ml and fractionated on Sephadex G-10 in the same manner. Similarly, 5 human sperm extracts were combined (total volume 1.5 ml), reduced to 0.3 ml and fractionated. Aliquots of the Sephadex G-10 fractions of these sperm extracts were tested in the same way as described above for whole sperm extract preparations.

RESULTS

Effects of Energy Sources on Sperm Motility

In our preliminary experiments, we aimed to find the most suitable combination of energy sources for sustaining sperm motility, in the presence of hamster sperm extracts. With glucose as the sole energy source, sperm motility declined steadily over the whole 6 h incubation period. With 0.9 mM pyruvate, high levels of motility were observed during the first 2 h, but motility diminished rapidly after this time. Sperm motility was almost completely suppressed in the presence of 22.5 mM lactate for the first 2 h, but by 4 h many sperm became very motile. It seemed that a combination of these substrates might produce a more consistent motility profile (change in motility index with time). In the presence of 4.5 mM glucose, the motility profile fluctuated markedly depending on the concentration of pyruvate used, but was much less influenced by the lactate concentration. The best overall response was obtained with 9.0 mM lactate and 0.09 mM pyruvate, in the presence of 4.5 mM glucose. After 5 h incubation in TA medium containing these levels of the three energy substrates, about 70 percent of the sperm were motile and approximately half of these showed an acrosome reaction. Accordingly, these concentrations of lactate and pyruvate were used in all subsequent experiments; the culture medium containing 9.0 mM lactate and 0.09 mM pyruvate is referred to as TALP. This medium also contained 4.5 mM glucose unless otherwise indicated (see Table 1).

In the next series of experiments, the interaction between energy sources and sperm extracts was studied. In the absence of sperm extract, sperm motility was not maintained for the duration of the incubation period (5 to 6 h) even when all three energy substrates were provided, as shown in Table 1; in fact, all movement ceased within 2 h under these conditions. Conversely, the sperm extracts failed to support motility in the absence of substrates. Only when the culture medium contained both sperm extract and an energy substrate was motility sustained throughout the experiment. However, when glucose was the sole energy source, the percentage of motile sperm was low, and very few sperm showed

TABLE 1. Effect of energy sources and sperm extract on motility of hamster epididymal spermatozoa.

Energy source in culture medium	Sperm extract[a]	Percentage of motile sperm[b]	Percentage of activated sperm[c]	No. of acrosome-reacted sperm[d]
Glucose	−	0	0	0
Glucose Lactate Pyruvate	−	0	0	0
None	+	0	0	0
Glucose	+	20–30	0–5	24/400 (6%)
Lactate Pyruvate	+	30–50	10–25	189/612 (31%)
Glucose Lactate Pyruvate	+	50–80	50–70	254/508 (50%)

Spermatozoa (approx. 2 × 10^5/ml) incubated 5 to 6 h with hamster sperm extract in medium TA or TALP (see Materials and Methods) with or without 4.5 mM glucose; lactate and pyruvate (where present) 9.0 mM and 0.09 mM, respectively (all concentrations of energy substrates given are final levels). Data from 6 duplicate experiments, each with a different sperm extract preparation.

[a]Presence (+) or absence (−) of hamster sperm extract.

[b]Estimated percentage at end of experiment: range of 6 experiments.

[c]Estimated percentage of motile sperm showing activation at end of experiment: range of 6 experiments.

[d]Number of motile sperm showing acrosome reaction/number of motile sperm: total of 6 experiments. Percentage in parentheses.

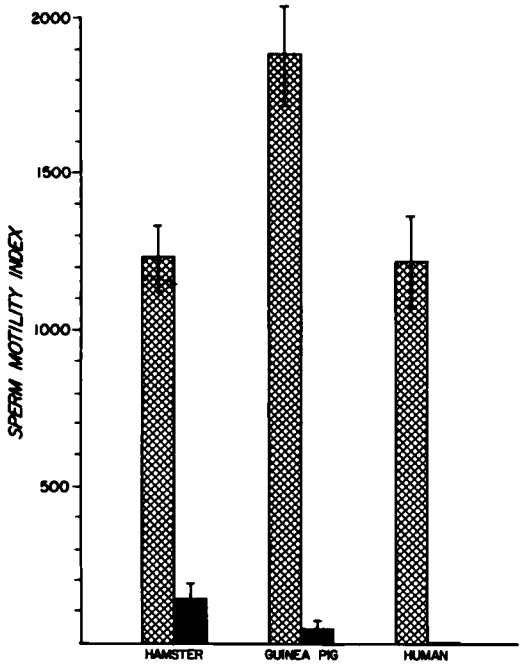

FIG. 1. Effect of sperm extracts from different species on the motility of hamster epididymal spermatozoa *in vitro*. Sperm concentration approx. 2 X 10^5/ml. Culture medium: TALP (see Materials and Methods) with glucose 4.5 mM, lactate 9.0 mM and pyruvate 0.09 mM (final concentrations). Cross-hatched columns: sperm motility index obtained in the presence of sperm extracts; stippled columns: sperm motility index obtained in the presence of 'sperm washings.' Sperm motility index (= percent motile sperm X (grade of motility)^2; Bavister, 1974) calculated from estimated motility after incubation for 4 h. Height of column gives mean motility index from 6 determinations with extracts prepared from spermatozoa obtained from 6 different animals (hamster); 4 determinations with sperm extracts from 4 animals (guinea pig); 7 determinations with sperm extracts from 4 subjects (human). Vertical bars give SEM. All sperm extracts 1/10 in culture medium.

activation or an acrosome reaction (Table 1). By contrast, the combination of lactate and pyruvate, and especially all three substrates together, sustained motility for 5 to 6 h in a high proportion of spermatozoa, and many of these underwent an acrosome reaction.

Motility-Stimulating Activity of Extracts of Sperm from Different Species

Extracts prepared from hamster, guinea pig and human spermatozoa clearly possessed sperm motility-stimulating activity (Fig. 1). The guinea pig sperm extracts were particularly effective in maintaining a high level of hamster sperm motility. By contrast, the sperm washings were virtually or completely unable to support hamster sperm motility for 4 h (Fig. 1).

Capacitation of Hamster Sperm Incubated with Sperm Extracts

After incubation of hamster spermatozoa for 5 h in TALP containing hamster, guinea pig or human sperm extracts, 121/370 (33 percent), 90/200 (45 percent) and 132/180 (73 percent), respectively, of the motile spermatozoa (60 to 80 percent motile) showed an acrosome reaction. Hamster cauda epididymal spermatozoa that had been preincubated for 4½ to 5 h in TALP containing hamster, guinea pig or human sperm extracts were able to penetrate washed, cumulus-free hamster eggs within 1 h after adding the eggs to the spermatozoa (Table 2). The guinea pig and human sperm extracts were particularly effective in supporting hamster sperm capacitation, as judged by the proportion of eggs containing spermatozoa with decondensing nuclei in the vitellus at 1 h after introduction of the eggs into the sperm suspensions. By 1½ h, decondensing sperm nuclei were seen in most of the eggs that were inseminated with spermatozoa preincubated with guinea pig or human sperm extracts (Table 2). In two experiments in which hamster spermatozoa were preincubated with guinea pig sperm extract in medium without albumin, no penetration subsequently took place when cumulus-free eggs were added to the sperm suspensions (Table 2).

Properties of the Motility-Stimulating Component in Sperm Extract

These experiments were aimed at comparing some of the properties of the sperm motility-stimulating component of sperm extracts with those of the 'sperm motility factor' (SMF) derived from human blood serum (Morton and Bavister, 1974; Bavister, 1975) and from hamster adrenal gland (Bavister et al., 1976). Samples of hamster, guinea pig and human sperm extracts were heated at 100°C for 15 min, then cooled; when these treated preparations were tested in the sperm motility bioassay, there was no detectable reduction in their ability to support motility of hamster sperm compared with unheated controls. When extracts of sperm from all three species were fractionated on Sephadex G-10, the elution volumes of the motility-stimulating components were virtually the same (Fig. 2), and were

TABLE 2. Penetration of cumulus-free eggs by hamster epididymal spermatozoa preincubated with sperm extracts.

Sperm extract derived from:	Penetration at 1 h				Penetration at 1½ h			
	Total eggs inseminated (no. of expts.)	Eggs with vitelline spermatozoa		Total eggs penetrated/eggs inseminated (%)	Total eggs inseminated (no. of expts.)	Eggs with vitelline spermatozoa		Total eggs penetrated/eggs inseminated (%)
		Stage I[a]	Stage II[b]			Stage I[a]	Stage II[b]	
Hamster	33 (5)	20	0	20/33 (61)	69 (7)	22	26	48/69 (70)
Guinea pig	49 (6)	33	15	48/49 (98)	57 (3)	3	53	56/57 (98)
					30[c] (2)	0	0	0/30 (0)
Human	44 (5)	23	7	30/44 (68)	87 (6)	4	71	75/87 (86)

Spermatozoa (approx. 2 × 10^5/ml) preincubated with sperm extracts for 4½ to 5 h in medium TALP (see Materials and Methods); energy substrate concentrations as in legend to Table 1.

[a]Stage I penetration: sperm head fused to vitelline membrane but no discernable nuclear decondensation.

[b]Stage II: decondensing sperm head in the vitellus.

[c]Albumin omitted from culture medium in these 2 experiments.

comparable to the elution volumes of the amino acids used as molecular weight markers.

In order to demonstrate that the ability of the sperm extracts to support capacitation and permit spermatozoa to acquire fertilizing ability was not abolished by eliminating the high molecular weight components with gel filtration, hamster spermatozoa were preincubated for 4 to 5 h in TALP together with the most active G-10 fractions (58 and 59 combined: Fig. 2) of guinea pig sperm extracts, then washed cumulus-free eggs were added. In a total of six such experiments, 71 out of 75 eggs were penetrated within 1½ h after adding the eggs to the sperm suspensions (48 eggs were at stage I of penetration and 23 at stage II).

DISCUSSION

Effect of Energy Substrates and Sperm Extracts on Sperm Motility

Our results show that the nature of the energy substrates provided in the culture system strongly influences the motility of hamster sperm, in the presence of sperm extracts (Table 1). The combination of pyruvate and lactate was far more effective in sustaining sperm motility than glucose alone, as demonstrated both in preliminary studies and in the experiments described in Table 1. The requirement for pyruvate indicates the importance of oxidative metabolism for sperm motility. Lactate may well play a supporting role by maintaining sufficient levels of pyruvate, either in the culture system or in the sperm themselves; the effect of lactate could perhaps be exerted by inhibiting reduction of pyruvate and/or by providing a substrate pool from which pyruvate could be continuously generated. The virtual failure of glucose as the sole energy source to support sperm motility, in the presence of sperm extracts (Table 1), may derive from an inability of the sperm under our experimental conditions to generate, or utilize, sufficient pyruvate from glucose for optimal performance. It seems likely that pyruvate provides the principal, if not the sole, driving force for the development of activated motility and the acrosome reaction in spermatozoa incubated with sperm extracts.

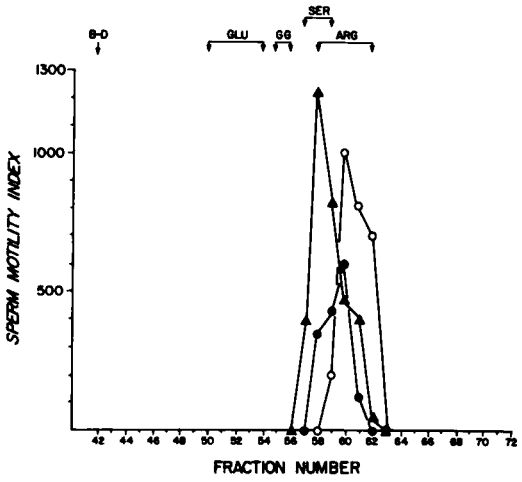

FIG. 2. Fractionation of sperm motility-stimulating activity of sperm extracts on Sephadex G-10. Sperm concentration approx. 2 × 10^5/ml. Culture medium: TALP (as in legend to Fig. 1). Sperm motility index at 6 h with hamster epididymal spermatozoa incubated with fractions of sperm extracts from hamster (●), guinea pig (▲) or human (○). Motility index zero outside peaks. All fractions 0.5 ml, diluted 1/10 in TALP. Markers (from separate calibration runs): blue dextran, mol. wt. 2 × 10^6 (B–D): glycylglycine, 132 (GG): glutamic acid, 147 (GLU): serine, 105 (SER): arginine, 174 (ARG).

The potentiating action of glucose on sperm motility, in the presence of pyruvate, lactate and sperm extracts (Table 1) cannot be explained at present. Possibly a particular ratio of pyruvate to glucose may be important for optimal sperm motility *in vitro*. Rogers and Yanagimachi (1975) found that guinea pig spermatozoa underwent an acrosome reaction much more rapidly in culture media containing lactate and pyruvate than in media containing glucose as the sole energy source, which again stresses the importance of oxidative metabolism in the development of the acrosome reaction. In the guinea pig experiments, however, the presence of glucose in addition to lactate and pyruvate actually slowed the development of the acrosome reaction (Rogers and Yanagimachi, 1975), which may reflect a difference in the metabolism of hamster and guinea pig spermatozoa, or might have resulted from differences in the culture conditions employed.

Our finding that glucose by itself is not an adequate energy substrate for the performance of hamster spermatozoa *in vitro*, apparently is in contrast to the results of several studies (cited below) in which good motility of hamster sperm was maintained, and (in most of these cases) their fertilizing ability was developed, in Tyrode's solution, which provides glucose as the only energy source. However, it

is probable that in these studies, other components that were included in the culture systems, i.e., cumulus masses and associated tubal fluid (Yanagimachi and Chang, 1964), follicular fluid or blood serum (Yanagimachi, 1969a, b; 1970a, b) provided additional energy sources for the spermatozoa. Blood serum is known to contain lactate and pyruvate, while cumulus cells can produce substantial amounts of pyruvate *in vitro* (Donahue and Stern, 1968). The provision of oxidizable substrates (pyruvate and/or lactate) in these ways probably explains the ability of hamster spermatozoa to remain motile and undergo an acrosome reaction when incubated with cumulus masses or blood sera diluted only with 0.15M NaCl solution (Barros, 1968; Barros and Garavagno, 1970; Talbot et al., 1974). In a study by Miyamoto and Chang (1973) the incidence of acrosome-reacted mouse spermatozoa *in vitro* was greatly increased when the basic culture medium containing glucose was supplemented with pyruvate or lactate, while the percentage of eggs undergoing fertilization rose from 0 percent in the basic medium to 95 percent in medium supplemented with pyruvate, lactate and albumin. The results of this study suggest that oxidative metabolism is important for optimal development of the fertilizing ability of mouse sperm *in vitro*. This inference is consistent with the conclusions that we have reached in our present study with hamster spermatozoa.

Although the energy substrates used in the present study clearly played an important part in determining the extent to which hamster sperm motility was maintained *in vitro*, sperm rapidly became immotile in the absence of the sperm extracts; this observation is in agreement with the results of a previous study (Bavister, 1974) which indicated that some component of hamster spermatozoa is essential for sustained sperm motility. Our present experiments demonstrate that, conversely, sperm extracts cannot support sperm motility in the absence of some energy substrate. We conclude that sperm extracts do not exert their remarkable action on sperm by providing a usable source of energy. Their mode of action thus remains to be established.

Homologous and Heterologous Sperm Extracts

Our results show that extracts of spermatozoa from guinea-pig and human, as well as those from the hamster, are able to stimulate the motility of hamster sperm *in vitro* (Fig. 1). For the purpose of discussion, we shall refer to the active components, whatever they may be, as 'sperm motility factor' (SMF). It is clear that the SMF that we have studied is associated with the sperm themselves, and was not derived from residual epididymal plasma or seminal plasma contaminants, since not only were the sperm thoroughly washed, but also the 'sperm washings' (supernatants of washed sperm suspensions before freeze-thawing) were virtually or completely devoid of SMF (Fig. 1). This does not rule out the possibilities that SMF might also be present in epididymal or seminal plasma, or that some of the SMF we studied might have been derived from residual free cytoplasmic droplets associated with the washed spermatozoa. The absence, or virtual absence, of SMF in the 'sperm washings' (Fig. 1) shows that SMF was not released from the sperm, at least in substantial amounts, until they were disrupted by freeze-thawing; we do not yet know where the SMF is localized in the sperm.

When hamster spermatozoa in the present study were washed in 0.9 percent NaCl, or were resuspended too vigorously during the washing procedure, the motility-stimulating potency of the sperm extracts was very low, suggesting that the SMF readily leaks out of hamster spermatozoa when they are damaged. This was also apparent in a previous study (Bavister, 1974), in which it was concluded that SMF leaked from hamster spermatozoa that were damaged by incubation in hypotonic culture media. The demonstration of the presence of SMF in hamster spermatozoa, the apparent ease with which it leaks out of the spermatozoa during washing (and probably during dilution), and the ability of sperm extracts to support the motility of low concentrations of hamster spermatozoa in culture media, provide additional support for the hypothesis previously advanced (Bavister, 1974) that the sperm dilution effect in the hamster may result from progressive loss of SMF from the spermatozoa with increasing dilution. We conclude that hamster spermatozoa recovered from the cauda epididymidis contain one or more components (SMF) that are essential for sperm motility *in vitro*, at least under the culture conditions used in the present study.

Not only do extracts of guinea pig and human spermatozoa also stimulate hamster sperm motility *in vitro*, but the chemical substance that is responsible for their activity

appears to be the same as that derived from washed hamster sperm (see below for discussion of physicochemical properties of SMF). The extracts of hamster, guinea pig and human spermatozoa also supported development of the acrosome reaction and allowed spermatozoa to penetrate eggs. Hamster spermatozoa that had been preincubated for several hours with extracts of sperm from any of these 3 species were able to penetrate washed, cumulus-free eggs within 1 h (Table 2). Sperm of other species may also contain SMF. Hanada and Chang (1976) reported that the inclusion of living or dead spermatozoa from the guinea pig, rat, mouse or hamster, or extracts of frozen-thawed guinea pig sperm, in the culture medium enabled hamster epididymal spermatozoa to survive in the medium and to penetrate denuded (cumulus-free) hamster eggs. These authors used the percentage of eggs penetrated as the index of the efficiency of capacitation of hamster sperm by homologous and heterologous sperm, and did not supply quantitative or comparative data on sperm motility or the acrosome reaction. In view of our results, however (see Fig. 1), there can be little doubt that stimulation of sperm motility would have accompanied sperm capacitation under the conditions used by Hanada and Chang (1976), and it seems very probable that the component that was responsible for supporting sperm motility and capacitation in their conditions was the same as that involved in our experiments, i.e., SMF (see below). If this were the case, it would be intriguing that sperm from 5 species (hamster, guinea pig, human, mouse and rat) contain the same motility-stimulating component.

In the present study, no eggs were penetrated by spermatozoa that had been preincubated with sperm extracts in the absence of albumin (Table 2), indicating that protein is essential for the development of fertilizing ability of hamster spermatozoa *in vitro*. This observation is consistent with the results of previous studies (Yanagimachi, 1969a, 1970a; Morton and Bavister, 1974) which showed that, while vigorous motility of hamster spermatozoa can be sustained in culture media in the presence of the low molecular weight fraction of follicular fluid or of blood serum, development of an acrosome reaction required the presence of protein in addition. The development of the acrosome reaction and of fertilizing ability by hamster spermatozoa *in vitro* thus requires the combined action of 1) SMF, which can be derived from a variety of sources, such as from spermatozoa themselves (present study), follicular fluid (Yanagimachi, 1969a), blood serum (Yanagimachi, 1970a; Bavister and Morton, 1974) or the adrenal gland (Bavister et al., 1976), 2) protein, such as serum albumin and 3) appropriate energy substrates, i.e., pyruvate.

Properties of Sperm-Derived SMF

The sperm motility-stimulating properties of hamster, guinea pig and human sperm extracts, attributed to SMF, clearly reside in the very low molecular weight fraction (Fig. 2). Although the low molecular weight fraction of the sperm extracts could efficiently sustain the motility of hamster spermatozoa in culture media, the possibility remained that some higher molecular weight components of the sperm extracts might be required for the development of fertilizing ability. This was not the case: hamster spermatozoa that were preincubated in culture medium with the low molecular weight fractions of guinea pig sperm extracts were subsequently able to penetrate a high proportion of washed, cumulus-free hamster eggs.

It seems likely that the molecular weight of the sperm-derived SMF is close to that of amino acids used as markers (Fig. 2), i.e., between 100 and 200. The Sephadex G-10 elution volumes of SMF derived from blood serum, adrenal gland and spermatozoa are virtually identical, and the SMF from all of these sources is heat-stable (Morton and Bavister, 1974; Bavister, 1975; Bavister et al., 1976; present study). Thus, it is very likely that the same substance is involved, and it may even be that the SMF component of spermatozoa is derived indirectly from the blood circulation, but we cannot test these notions until the chemical structure of SMF has been elucidated.

Some fundamental questions concerning SMF remain to be answered. What is the chemical nature of SMF? Is the same substance, or group of substances, involved in all the different locations in which sperm motility-stimulating activity has been detected? What is the mode of action of SMF? Is SMF important for the motility of human spermatozoa and sperm of other species? Does SMF have a role in sustaining sperm motility and the development of fertilizing ability *in vivo*? The answers to these questions may provide considerable

insight into the physiology and the biochemistry of mammalian spermatozoa.

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