BIOPHYSICAL CHARACTERIZATION OF FOWL SPERMATOOZOA

II. INTERRELATIONSHIPS BETWEEN INTRINSIC MOTILITY AND ELECTROPHORETIC MOBILITY

D. A. PISTENMA, H. C. MEL AND N. SNAPIR*†

*Division of Medical Physics and Donner Laboratory, and
*Department of Poultry Husbandry, University of California,
Berkeley, California 94720, U.S.A.*

(Received 7th July 1969, revised 29th July 1970)

Summary. The interrelationships between the intrinsic motility and electrophoretic properties of fowl spermatozoa were studied in glycine-citrate buffer at temperatures of 3 to 5.5° C using Staflor electrophoresis. Migration patterns obtained with sperm concentrations of 25×10^6 and 250×10^6 /ml were similar and gave average electrophoretic mobilities of $1.65 \pm 0.10 \times 10^{-4}$ cm/sec/V/cm, corrected to conditions of 1% sucrose at 3° C. At 4° C, there was no significant difference between collection fractions with regard to viable (eosin-negative) spermatozoa. At 5.5° C, however, the lower collection fraction was enriched relative to the upper fraction with regard to motility, viability, fertility and hatchability. For these properties, the lower fraction did not differ from the high quality control. With lower quality starting samples, absolute enrichment may be expected.

INTRODUCTION

In contrast to the abundance of information concerning the electrophoretic properties of mammalian spermatozoa, there have been few such reports on fowl spermatozoa. Undoubtedly, the greater interest in mammalian spermatozoa is based on the economic and sociological consequences of finding a way to separate genetically differing, e.g. X- and Y-chromosome-bearing, spermatozoa. Despite the work of Schröder (1941) and of Gordon (1957), it is uncertain that apparent differences in the electrophoretic properties of rabbit spermatozoa can be attributed to different chromosomal composition (Vesselinovitch, 1960). Because the sex-determining germ cell in fowl is the egg, the sex control incentive is lacking in the study of spermatozoa of this species. However, in addition to basic knowledge about the properties of fowl spermatozoa from cocks of fertile and infertile lines, electrophoretic studies are motivated by the availability of birds with a translocation heterozygote producing spermatozoa

† Present address: Department of Poultry Science, Faculty of Agriculture, Rehovoth, Israel.

with differing chromosomal composition and fertilizing capacity (Inouye, 1952).

The stable-flow free-boundary (Staflö) method has been used to characterize cell populations such as rat bone marrow (Mel, Mitchell & Thorell, 1965) and rabbit spermatozoa (Pistenma, 1970). The flexibility of this method provides an opportunity for analytical or preparative studies using either sedimentation or electrophoresis, or a combination of the two. In addition, a fractionation of sperm populations can be achieved using differing degrees of intrinsic motility at specified controlled temperatures.

This paper reports a series of experiments which (1) confirm the conclusion from Part I (Pistenma, Snapir & Mel, 1971), that the fertilizing capacity of spermatozoa could be maintained during Staflö electrophoretic experiments, (2) characterize the sperm populations with regard to average and extreme values of electrophoretic mobility, and (3) partially fractionate and relatively enrich the sperm populations by utilizing a combination of electrophoresis and intrinsic motility.

MATERIALS AND METHODS

The characteristics of the ejaculates, sperm handling and evaluation procedures and details of the various solutions are presented in Part I (Pistenma *et al.*, 1971) and, therefore, are not repeated here.

Two groups of electrophoretic experiments were performed. The first group was conducted at 4° C, or lower, to minimize the effect of intrinsic motility on migration. The second group was conducted at 5.5° C in a deliberate attempt to achieve enrichment of one collection fraction with motile spermatozoa.

Sample preparation

Ejaculates were collected from mature White Leghorn fowls by abdominal massage (Burrows & Quinn, 1939) and pooled until 2.0 ml or more of undiluted semen was obtained. A 0.5-ml aliquot of pooled semen was diluted 1:2 with modified Krebs' solution for a control specimen and the remainder was diluted 1:2 with glycine-citrate buffer at pH 6.94. A 0.1-ml aliquot was diluted 1:100 in a solution of 1% formalin in normal saline for counting in a haemocytometer. Both samples were cooled from room temperature to 2° C in 45 min. The glycine-citrate sample was then diluted to a concentration of approximately 250 to 300 × 10⁶ spermatozoa/ml and subjected to Staflö electrophoresis.

Staflö apparatus

The essential features of the Staflö apparatus employed in these experiments are a 3 × 16 channel flow-cell, designated No. XI (Tippetts, Mel & Nichols, 1967), duplicate 48-channel collection systems and a 16-channel, multi-syringe pumping system. Sixteen 50-ml plastic syringes on the pump rack contain the density gradient solutions which are delivered by polyethylene tubing to the respective inlets on the flow-cell. A variable speed drive allows a wide range of accurately controlled solution flow rates. A 20-ml syringe is often used for the sperm sample suspension in order to achieve an initial sample stream thickness

approximately half that of other fluid layers with resulting greater resolution of the migrating species ('thin sample technique'; Tippetts *et al.*, 1967). The sperm suspension is stirred magnetically to maintain a uniform concentration throughout the experiment.

TEXT-FIG. 1. Schematic diagram of Staflo flow-cell No. 11 illustrating fowl spermatozoa upward-electrophoretic pattern.

The inlets to the flow-chamber are arranged vertically, as shown in Text-fig. 1, thereby forming flowing layers each 0.125 cm high, 0.80 cm wide and 30 cm long. The multiple, horizontally flowing layers are stabilized by hydrodynamic feedback principles, rather than by use of any solid supporting medium (Mel, 1964a; Tippetts *et al.*, 1967). The fluid in each of the sixteen layers leaves the flow-cell through three horizontal channels, thereby allowing separation of the faster moving fluid in the central region from the slower moving fluid near each side-wall. In this study, only the collection fractions from the central channels of the flow-cell were evaluated.

TEXT-FIG. 2. Schematic diagram showing ideal three-dimensional Staflo upward-electrophoretic migration pattern of fowl spermatozoa.

In these experiments, only four of the six electrode segments were activated giving an electric field across the downstream two-thirds (20 cm) of the flow-cell as shown schematically in Text-figs. 1 and 2.

Buffer solution and density gradient

Using the low-ionic-strength glycine-citrate buffer described in Part I (Pistenma *et al.*, 1971), a sucrose density gradient was established by weight/volume percentage in the flowing layers as follows: No. 4, 1.0%; No. 5, 1.5%; No. 6, 2.0%; No. 7, 2.5%; No. 8, 3.0%; No. 9, 4.5%; No. 10, 4.8%; No. 11, 5.1%; No. 12, 5.4% and No. 13, 5.7%. Layers 1 to 3 contained a higher conductivity medium resulting from a glycine-citrate content approximately three times that of Layers 4 to 12. Layers 14 to 16 contained the same high conductivity medium with 6% sucrose. The resulting top and bottom 'conductivity barriers' buffer the intermediate layers against changes in pH during the experiment and confine the migrating species to the intermediate layers of the flow-cell (Mel, 1964b). Initial discontinuities between layers are smoothed by gradient-stabilizing diffusion of sucrose during the first one-third (10 cm) of the flow-chamber before reaching the region of the 'programmed electric field' (Tippetts *et al.*, 1967). The sperm suspension was introduced into Layer 8 and was initially supported by the density shelf provided by the sucrose increment from Layers 8 to 9.

Temperature control

All experiments were conducted in a refrigerated room maintained at the temperatures already indicated. Temperature measurements inside the flow-chamber during electrophoresis have shown the average temperature rise to be less than 0.5° C under the operating conditions used in these experiments.

Observation and evaluation of spermatozoa

The gross electrophoretic pattern was observed by eye, using the 'slit lamp technique' (Tippetts *et al.*, 1967). Individual spermatozoa and the microscopic steady-state pattern were observed with a special microscopic optical system developed for use with the Staflo apparatus (Pistenma, 1970). Immediately after each run, the sperm concentration in each collection container was determined with a haemocytometer. The spermatozoa in each fraction were re-concentrated by centrifugation at the specified cold-room temperature and were subsequently evaluated at room temperature (21 to 24° C) for motility (see Part I, Pistenma *et al.*, 1971) and viability (nigrosin-eosin staining; Hancock, 1951). Spermatozoa were categorized as viable (eosin-negative) or non-viable (eosin-positive). The spermatozoa from each fraction and from the control sample were inseminated into White Leghorn hens of separate groups between 13.00 and 15.00 hours on the day of the experiment.

RESULTS

Text-figure 1 illustrates the migration pattern (overall side-view appearance of the continuously flowing sample stream in the flow-chamber) observed in both sets of experiments. The paraboloid migration profile (cross-section of the migration pattern) observed with the slit lamp technique is illustrated in Text-fig. 2. Vertical migration distances measured in the longitudinal centre line by this technique were used to calculate the electrophoretic mobility values.

Electrophoretic experiments

General observations during the series of experiments at temperatures of 4° C or lower indicated that (1) the migration patterns determined by the slit lamp technique were essentially the same at sperm concentrations of 25×10^6 and 250×10^6 /ml, (2) in all experiments, the height of the migration profile at the outlet in the centre line of the flow-chamber was less than 2.0 mm (cf. height of a flow layer, 1.25 mm), thereby limiting the fractionation of the sample to two layers, (3) at pH 7.0 and with the top electrode positive, all spermatozoa were vertically orientated with their heads downwards, and (4) at a flow-chamber temperature of 4° C, a high percentage of individual spermatozoa showed a moderate degree of tail movement.

Despite an attempt to effect an even division of the sample between the two layers at the outlet, the bottom collection fraction generally had a higher sperm concentration, and in all experiments, a higher percentage and degree of motility (though less than in the control sample). In one experiment, the upper fraction which contained only 10% of the spermatozoa at a concentration of 10×10^6 /ml had only 1 to 2% motile spermatozoa as compared to about 70%

TABLE 1
APPARENT ELECTROPHORETIC MOBILITIES OF
FOWL SPERMATOZOA AT 4° C

Sperm concentration	Apparent electrophoretic mobility (units of 10^{-4} cm/sec/V/cm)	
	Average	Range
25×10^6 /ml	1.56	1.50 to 1.61
250×10^6 /ml	1.69	1.59 to 1.79

motility in the bottom fraction. When comparable numbers of spermatozoa were collected in the two fractions, for experiments at 4° C or lower, there were no significant differences in viability between the two fractions and the control spermatozoa. Even though the sperm fractions in several experiments had Grade II or III motility, insemination of 150 to 200×10^6 spermatozoa in 0.15 ml failed to give any fertile eggs. Control inseminations with Grade III or IV spermatozoa did give fertile eggs. Possible reasons for the loss of fertilizing capacity of spermatozoa in the collection fraction in this first group of experiments are considered in the Discussion.

The average and ranges of apparent electrophoretic mobilities for one pair of electrophoretic runs at 4° C with the same Stafllo operating conditions but differing sperm concentrations are shown in Table 1.

Electrophoresis-enrichment experiments

Two enrichment experiments were conducted using upward electrophoresis at 5.5° C. The steady-state migration pattern during these experiments was similar to that illustrated in Text-fig. 2. Again, because the pattern spread was small, only two fractions were obtained (containers 5 and 6). The spermatozoa

in the fractions were re-concentrated by centrifugation at 1400 *g* for 5 min at 5° C before evaluation at room temperature, and artificial insemination. The six hens in each of the same three groups were inseminated on each occasion with the respective collection fractions or control spermatozoa. Eggs were collected from the day after the first insemination until 4 days after the second insemination (a total of 8 days). The fractionation results with respect to sperm distribution, motility, viability, fertility and hatchability are summarized in Table 2.

In Table 2, it is seen that Fraction 6 has both a higher concentration and a better quality of spermatozoa than Fraction 5, judged by motility and viability. In addition, the percentages of fertility and hatchability for Fraction 6 are

TABLE 2
AVERAGE ENRICHMENT-FRACTIONATION AND ARTIFICIAL INSEMINATION
RESULTS OF TWO ELECTROPHORETIC EXPERIMENTS WITH FOWL SPERM-
ATOZOA AT 5.5° C

	<i>Fraction 5</i>	<i>Fraction 6</i>	<i>Control</i>
Sperm conc. (units of 10 ⁶ /ml)	87	140	1500
% spermatozoa collected:			
Average	38.3	61.7	—
Range	36.2 to 39.8	60.2 to 63.8	—
Motility (grade)	II	IV	IV
Viability (eosin-negative) (%)	77	90.5	91.8
Artificial insemination			
Volume (ml)	0.15	0.15	0.10
No. of spermatozoa (units of 10 ⁶)	145	170	150
Evaluation of eggs:			
Total no.	39	46	46
No. fertile	5	26	25
% fertile	12.8	56.6	54.4
No. hatchable	3	19	21
% hatchable	7.7	41.3	45.7

significantly higher than those for Fraction 5 ($P < 0.01$ in a one-tail test; Goldstein, 1964). There was no difference between Fraction 6 and the control with regard to motility, viability, fertility or hatchability.

Following one of these experiments which used a concentration of 250×10^6 spermatozoa/ml, a comparative run was made using one tenth the initial concentration. Although the pattern spread was slightly greater for the higher concentration, the average migration distances were the same. The mobility, calculated from the average migration distance, was slightly greater ($\approx 2 \times 10^{-4}$ cm/sec/V/cm at 5.5° C) than in Table 1. However, the measurements in Table 1 are considered more reliable because the migration distances involved were greater.

DISCUSSION

Technical considerations

Despite the additional procedures associated with the electrophoretic experiments, the final fertilization results reported herein are as good as those in the

dilution studies alone (Part I, Pistenma *et al.*, 1971). Two improvements in sperm handling may have contributed to these results: A shorter centrifugation time (by 25 to 45 min) was used, and cooling from room temperature to 3° C was accomplished in 45 min (as compared to 2 hr in Part I) following a similar successful procedure reported by Proudfoot & Stewart (1967).

It was noted that, for several of the earlier electrophoretic experiments, no fertile eggs were obtained following insemination of adequate numbers of re-concentrated spermatozoa of Grade II or III motility. Control inseminations (Grade III or IV), however, gave fertile eggs. Furthermore, in the subsequent electrophoresis-enrichment experiments, a fertilization rate of 56.6% was achieved from Staffo collection Fraction 6 as compared with the 54.5% control value. The loss of fertilizing capacity (but not motility) in the early experiments was most likely due to trace contamination of the apparatus either by Roccal (benzalkonium chloride) used in cleaning or by a toxic substance associated with new polyethylene tubing.

Electrophoretic mobility measurements

Even though there was good agreement between the average values of electrophoretic mobility for the similar pairs of high and low concentration runs, the range of mobilities was somewhat greater for both high concentration experiments, possibly reflecting a small degree of density gradient instability. Alternatively, this may be a consequence of the relative difficulty in defining the limits of the low concentration migration pattern. Only having slight instability at a concentration as high as 250×10^6 spermatozoa/ml is remarkable in view of the threshold for instability of 25×10^6 /ml for rabbit spermatozoa in other density gradients (Pistenma, 1970). The increased stability in the experiments with fowl spermatozoa may be attributed to a larger increment in the density gradient between layers (0.5% sucrose/layer versus 0.10 to 0.15% sucrose/layer for experiments with rabbit spermatozoa) and a lower sedimentation rate for fowl spermatozoa (Bhattacharya, 1962).

In addition to the apparent electrophoretic mobility of 1.69×10^{-4} cm/sec/V/cm for the 250×10^6 /ml sperm sample shown in Table 1, an average value of 1.52×10^{-4} cm/sec/V/cm was obtained in another experiment under similar conditions at 4° C. When these values are corrected to conditions of a 1% sucrose solution at 3° C, they become 1.75×10^{-4} and 1.57×10^{-4} cm/sec/V/cm, respectively. These latter values compare favourably with the 1.68×10^{-4} cm/sec/V/cm observed in a downward electrophoretic experiment using the glycine-phosphate buffer (see Part I, Pistenma *et al.*, 1971) corrected to 1% sucrose at 3° C (Pistenma, 1970). They are also comparable to the value of $1.545 \pm 0.020 \times 10^{-4}$ cm/sec/V/cm reported by Bey (1965), using a low-ionic-strength buffer.

The relative contribution of intrinsic motility to net migration at 4° C can be estimated from the experiment in which the upper collection fraction contained 99% immotile spermatozoa at a concentration of 10×10^6 /ml. (Because it was shown in Part I (Pistenma *et al.*, 1971) that concentrations as low as 8×10^6 /ml could be tolerated for up to 6 hr at 3° C, it is probable that the spermatozoa were immotile at the beginning of the experiment.) The average

electrophoretic mobility of these immotile spermatozoa was 1.67×10^{-4} cm/sec/V/cm compared to average and minimum apparent mobility values of 1.52×10^{-4} and 1.36×10^{-4} cm/sec/V/cm for the whole sample. Theoretically, this distribution represents both a range of electrophoretic mobilities and a differential contribution of intrinsic motility of opposite direction, amounting to perhaps as much as 19% of the true electrophoretic mobility. In order to define the true contribution of motility to the net migration, experiments should be conducted at temperatures ranging from less than 1° C to about 15° C, and with lower sperm concentrations, to ensure that the migration is not influenced by any kind of local density gradient instability.

Relative enrichment

The viability discrimination achieved in these Staflo experiments is illustrated by the difference in the percentage of viable spermatozoa between the two collection fractions at 4° C (none) and at 5.5° C (significant, as shown above). This is attributed to a significant increase in the magnitude of the intrinsic motility vector of the spermatozoa in going from 4° C to 5.5° C. This downward orientated motility vector is also believed to explain the depletion in Fraction 5 and the relative enrichment in Fraction 6 with regard to all of the criteria enumerated in Table 2. With an initial sample of about 50% motile spermatozoa (rather than 90%), absolute enrichment would be expected. This would be even greater if a slightly higher temperature and/or an increased migration distance were used. Such enrichment has been obtained in experiments with rabbit spermatozoa (Pistenma, 1970). It is significant that, despite the additional stresses of dilution, electrophoresis and centrifugation, the spermatozoa in Fraction 6 were as good as in the control sample by all of the criteria in Table 2.

CONCLUSIONS

A natural extension of the present fractionation studies is the evaluation of the fertilizing ability of sub-populations of spermatozoa characterized by differing degrees of intrinsic motility, obtained by differential fractionation in Staflo-electrophoresis-motility or sedimentation-motility experiments. With regard to fowl spermatozoa, this extension would be of particular interest in studying the translocation heterozygote spermatozoa. For mammalian spermatozoa, there is the additional possibility of obtaining information on the relationship of motility to the sex-ratio of offspring.

ACKNOWLEDGMENTS

The support of the U.S. Atomic Energy Commission, Lawrence Radiation Laboratory (W 7405-eng-48), and the Department of Poultry Husbandry, University of California, Berkeley, is gratefully acknowledged.

REFERENCES

- BEY, E. (1965) *The electrophoretic mobility of sperm cells*. In: Cell Electrophoresis. Ed. E. J. Ambrose. Little, Brown, Boston.

- BHATTACHARYA, B. CH. (1962) Die verschiedene Sedimentationsgeschwindigkeit der X- und Y-Spermien und die Frage der willkürlichen Geschlechtsbestimmung. *Z. wiss. Zool.* **166**, 203.
- BURROWS, W. H. & QUINN, J. P. (1939) Artificial insemination of chickens and turkeys. *Circ. U.S. Dep. Agric.* 525.
- GOLDSTEIN, A. (1964) *Biostatistics: an introductory text*. MacMillan, New York.
- GORDON, M. J. (1957) Control of sex ratio in rabbits by electrophoresis of spermatozoa. *Proc. natn. Acad. Sci. U.S.A.* **43**, 913.
- HANCOCK, J. L. (1951) A staining technique for the study of temperature-shock in semen. *Nature, Lond.* **167**, 323.
- INOUE, N. (1952) *Two X-ray induced translocations in the domestic fowl*. M.S. thesis, University of California, Berkeley.
- MEL, H. C. (1964a) Stable-flow free-boundary migration and fractionation of cell mixtures. I. Apparatus and hydrodynamic feedback principles. *Jnl theor. Biol.* **6**, 159.
- MEL, H. C. (1964b) Stable-flow free-boundary migration and fractionation of cell mixtures. III. Migration principles—sedimentation and electrophoresis. *Jnl theor. Biol.* **6**, 307.
- MEL, H. C., MITCHELL, L. T. & THORELL, B. (1965) Continuous free-flow fractionation of cellular constituents in rat bone marrow. *Blood*, **25**, 63.
- PISTENMA, D. A. (1970) *Biophysical studies of spermatozoa*. Ph.D. thesis, University of California, Berkeley.
- PISTENMA, D. A., SNAPIR, N. & MEL, H. C. (1971) Biophysical characterization of fowl spermatozoa. I. Preservation of motility and fertilizing capacity under conditions of low temperature and low sperm concentrations. *J. Reprod. Fert.* **24**, 153.
- PROUDFOOT, F. G. & STEWART, D. K. R. (1967) The effect of oxygen and types of container on the retention of the fertilizing capacity of fowl spermatozoa stored *in vitro*. *J. Reprod. Fert.* **13**, 251.
- SCHRÖDER, V. (1941) Künstliche Geschlechtsregulation der Nachkommenschaft der Säugetiere und ihre biologische Kontrolle. *Z. Tierzücht. ZüchtsBiol.* **50**, 1.
- TIPPETTS, R. D., MEL, H. C. & NICHOLS, A. V. (1967) *Stable-flow free-boundary (Staflow) electrophoresis: three dimensional fluid flow properties and applications to lipoprotein studies*. In: Chemical Engineering in Medicine and Biology. Ed. D. Hershey. Plenum Press, New York.
- VESSELINOVITCH, S. D. (1960) Electrophoresis of spermatozoa and sex control. *Cornell Vet.* **50**, 326.