J. Reprod. Fert. (1976) **48**, 9–15

A difference in dry mass between the heads of X- and Y-bearing human spermatozoa

A. T. Sumner and J. A. Robinson

Medical Research Council, Clinical & Population Cytogenetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, U.K.

Summary. An integrating microinterferometer was used to measure the dry mass of sperm heads. The dry mass was found to be proportional to DNA content, and thus provides a useful method of estimating sperm DNA content.

Using this technique we have confirmed that human spermatozoa which show none and one quinacrine-fluorescent spot are X- and Y-bearing respectively. However, the measurements suggest that many of the spermatozoa with two quinacrine-fluorescent spots are not YY-bearing, as previously thought, but might be incompletely condensed Y-bearing spermatozoa.

Introduction

The use of quinacrine fluorescence techniques has made possible the identification of the human Y chromosome in non-dividing nuclei, including spermatozoa. In somatic cells, it is possible to confirm the identification of the Y chromosome by comparison with metaphase chromosomes from the same individual. With spermatozoa this is not possible, but we have shown, by Feulgen microdensitometry, that spermatozoa with a fluorescent spot had significantly less DNA than those without a fluorescent spot, and that the difference corresponded to that expected for haploid genomes containing an X or a Y chromosome (Sumner, Robinson & Evans, 1971). Nevertheless, these results were unsatisfactory in several ways. The problems involved in using the Feulgen reaction with spermatozoa are well known (Bouters, Esnault, Salisbury & Ortavant, 1967; Gledhill, Gledhill, Rigler & Ringertz, 1966; Esnault, 1973), and although we were able to obtain a satisfactory result, the variability of our measurement was undesirably great. In addition, it has been found that irradiation of quinacrine-stained chromosomes damages the DNA so as to cause an excessively weak Feulgen reaction. Evidently, if the genome of spermatozoa were to be measured with the precision needed to detect rather small differences, techniques of measurement requiring the minimum of pretreatment would be desirable.

Such a technique is integrating microinterferometry whereby the dry mass of microscopical objects can be measured at the touch of a button. Equipment for this technique recently became available to us, and this paper describes our experiments to find the best methods for preparing sperm heads for dry mass measurements, and also shows that there is a difference in dry mass between the heads of X- and Y-bearing spermatozoa.

Methods and Results

The instrument used was the Vickers M86 Integrating Microinterferometer (Vickers Instruments, York, England). The principles and use of this instrument have been described by Goldstein & Hartmann-Goldstein (1974). When the instrument is operated, it produces a result which represents the integrated optical path difference (o.p.d.) (i.e. refractive index × thickness) in arbitrary units and, if required, the area of the object as well. The area of the specimen to be measured is selected using an electronic masking system. All measurements were made using the ×75, n.a. 1·1, water-

immersion objective, and all specimens were measured while immersed in distilled water. This not only produced the best optical conditions for the objective, but also reduced the greatest o.p.d. to less than about 0·95 of a wavelength, which is the maximum the instrument can measure.

Methods of preparation

All semen samples, produced by masturbation, were washed three times with 0·9% sodium chloride solution before further processing (which will be described below).

Preparation methods for sperm slides were assessed by two criteria; suitability for quinacrine fluorescence of Y bodies and consistency of dry mass measurements.

Effect on quinacrine fluorescence. Unfixed spermatozoa were spread on slides using the Cytospin centrifuge (Shandon Scientific Instruments). These preparations were stained with quinacrine according to our usual method (Sumner *et al.*, 1971), either without prior fixation, or following fixation in methanol–acetic acid (3:1 v/v) for 30 min. When fresh, both types of preparations showed good quinacrine fluorescence, about 50% of the spermatozoa showing Y bodies, but the fixed preparations were clearer. In unfixed spermatozoa only, the mid-piece showed strong quinacrine fluorescence.

The unfixed spermatozoa aged quickly; after a day or more the quinacrine fluorescence was less good, and a lower proportion of Y bodies was found. Fixation of these aged preparations shortly before staining produced no improvement; unlike the spermatozoa which were fixed when fresh, the mid-piece continued to show bright quinacrine fluorescence.

Spermatozoa fixed in suspension with methanol–acetic acid gave the most satisfactory results (Sumner, 1971) as the quinacrine fluorescence of these preparations was not noticeably affected by ageing. However, fixed spermatozoa which had been immersed in water for dry mass measurement could not be used for subsequent quinacrine fluorescence; the spermatozoa appeared fuzzy and damaged, and the Y body count was low.

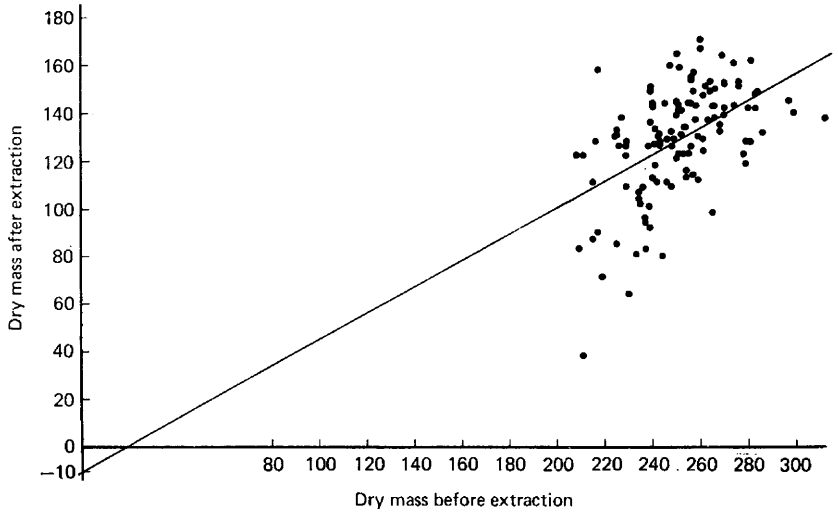
Effect on dry mass. The effects of various treatments on dry mass measurements of the sperm heads showed that each treatment appeared to lower the dry mass, and at the same time greatly reduced the variability of the sample. The difference in mean (±S.D.) dry mass (arbitrary units) between fixed spermatozoa which were unstained ($258{\cdot}25 \pm 39{\cdot}57$, $n = 154$) and those stained with quinacrine ($252{\cdot}77 \pm 21{\cdot}62$, $n = 142$) was not significant ($t_{294} = 1{\cdot}46$; $P > 0{\cdot}10$) but the difference in variance was ($F = 3{\cdot}35$; $P < 0{\cdot}001$). Fixed spermatozoa had a significantly lower mean (±S.D.) dry mass ($t_{381} = 4{\cdot}62$, $P < 0{\cdot}001$) and were less variable ($F = 5{\cdot}12$, $P < 0{\cdot}001$) than unfixed spermatozoa ($293{\cdot}68 \pm 89{\cdot}52$, $n = 229$). It thus appears that fixation and subsequent immersion in aqueous liquids (as for quinacrine staining) remove material from the sperm heads, mainly from the acrosome.

The quinacrine fluorescence and dry mass measurements indicate that fixation is essential, and washing in water desirable, for best results. The following procedure was therefore adopted for all subsequent work described here. (1) The semen sample was washed three times in 0·9% (w/v) sodium chloride. (2) The sample was fixed in methanol–acetic acid (3:1 v/v), for 3 periods of 10 min each. (3) The spermatozoa were spread on a slide by dropping a small portion of the suspension into fresh fixative. (4) The slide was stained with 0·5% quinacrine for 3 min, rinsed briefly in deionized water, mounted in a minimum of deionized water and sealed with rubber solution. (5) Selected fields were photographed on Polaroid film with a ×40 phase-contrast objective and condenser and visible light (to reduce exposure to u.v. light). (6) The photographed spermatozoa were re-located by quinacrine fluorescence and the position of the spermatozoa and number of Y bodies were recorded. (7) The coverslip was removed and the slide washed for several hours in running tap water to remove quinacrine and other extraneous materials. (8) The dry mass of the spermatozoa, immersed in distilled water, was measured with the ×75 water-immersion objective (no coverslip).

Proportion of DNA in sperm heads

If dry mass measurements are to be used to obtain an estimate of the amount of DNA in sperm heads, it is essential that the amount of DNA be proportional to total dry mass (i.e. DNA + protein).

To test this, a slide which had already been measured to obtain the dry mass of the sperm heads was treated with 5% trichloracetic acid (90°C for 15 min) to extract DNA, and the same 124 spermatozoa were measured again. Regression coefficients of dry mass of extracted on unextracted sperm heads were calculated. The slope of the regression line (*b*) (representing the proportion of the integrated o.p.d. due to protein) was 0·554 ± 0·059 (standard error) and its intercept −9·874 ± 19·680 (standard error). The fact that the intercept is not significantly different from zero is strong evidence of a similar ratio of DNA to protein in all the sperm heads measured (Text-fig. 1).



Text-fig. 1. Scatter diagram of the dry mass of human sperm heads extracted with 5% trichloracetic acid (90°C for 15 min) compared with the same sperm heads before extraction. The calculated regression line of extracted on unextracted spermatozoa is: $y = -9{\cdot}874 + 0{\cdot}554x$.

Dry mass and area of different classes of spermatozoa

Spermatozoa from a fertile 47,XYY man were processed as described earlier, and those of normal morphology classified according to whether they had 0, 1 or 2 fluorescent bodies (F or Y bodies). Spermatozoa were only classified as possessing 2 fluorescent bodies when they were clearly separated. Observations on 1600 spermatozoa from four separate slides showed that the proportions in each class were 49·25%, 48·38% and 2·38% respectively. Results of dry mass and area measurements on sperm heads from this man are listed in Table 1; a few of the selected spermatozoa proved to be diploid, and are listed as a separate class, regardless of the number of fluorescent bodies they contained.

The results in Table 1 show that the heads of haploid spermatozoa with no fluorescent body are 2·13% larger in dry mass than spermatozoa with one fluorescent body, and that this difference is highly significant. However, the difference in dry mass between spermatozoa with one and two fluorescent bodies is so small as to be almost negligible; the result of the *t* test ($P > 0{\cdot}95$) in fact gives a strong indication that the two classes are not distinguishable. Nevertheless, the figures are not adequate to show that sperm heads with two fluorescent bodies have significantly *lower* dry mass than sperm heads with none.

None of the differences in area between the classes of haploid sperm is statistically significant. The dry mass of diploid sperm heads is 1·992 times that of haploid sperm heads, remarkably close to the expected value considering the small number of diploid spermatozoa measured. However, the area of diploid sperm heads was only 1·389 times that of haploid sperm heads, compared with the expected value of 1·587 ($2^{\frac{3}{4}}$) (Sumner, 1971). This discrepancy may be because all the diploid

Table 1. Dry mass and area measurements (in arbitrary units) of human haploid sperm heads with different numbers of fluorescent bodies, and of diploid sperm heads

		Dry mass		Area	
	No. measured	Mean	S.D.	Mean	S.D.
Haploid spermatozoa					
No fluorescent body (0)	590	248·88	20·54	226·95	30·75
One fluorescent body (1)	481	243·62	20·36	224·30	30·52
Two fluorescent bodies (2)	26	243·85	16·68	234·11	36·71
Total	1097	246·46	—	225·96	—
Diploid spermatozoa	4	490·92	22·58	313·83	19·05

Paired comparisons:

Comparison	Difference (%)	Standard error	*t*	d.f.	Significance
Dry mass					
0/1	5·26 (2·13)	1·257	4·18	1069	$P < 0·0001$
2/1	0·22 (0·09)	4·066	0·05	505	$P > 0·95$
0/2	5·03 (2·04)	4·087	1·23	614	$P \sim 0·22$
Area					
0/1	2·65 (1·17)	1·883	1·41	1069	$0·1 < P < 0·2$
2/1	9·81 (4·34)	6·213	1·58	505	$0·11 < P < 0·12$
0/2	−7·16 (3·17)	6·216	1·15	614	$P > 0·2$

spermatozoa measured had well condensed heads, while many of the haploid spermatozoa, although morphologically normal, were not fully condensed.

Since the results with spermatozoa containing two fluorescent bodies differed from earlier work (Sumner *et al.*, 1971), further measurements were made in an attempt to establish the status of such spermatozoa. More spermatozoa with two fluorescent bodies were measured, bringing the total to 51 (the maximum number of such spermatozoa on the slides which were measurable), and their measurements were compared with those of the closest spermatozoa without a spot and with one fluorescent spot. Mean values for these sperm heads and results of paired comparison *t* tests are given in Table 2. These show that sperm heads with two fluorescent spots did have a significantly lower dry mass than those with no fluorescent spots, but did not differ significantly from those with one fluorescent spot. The converse is true of area, sperm heads with two spots being significantly larger than those with none.

Table 2. Dry mass and area measurements (in arbitrary units) of 51 human haploid sperm heads with two fluorescent bodies, and of adjacent sperm heads with none and one fluorescent body

No. of fluorescent bodies	Dry mass		Area	
	Mean	S.D.	Mean	S.D.
2	238·28	20·71	235·10	42·16
1	241·13	24·55	227·28	35·67
0	246·21	21·46	222·96	29·13

Comparison	Difference (%)	Standard error	*t*	Significance
Dry mass				
2/1	−3·41 (−1·41)	3·643	0·94	$P > 0·3$
2/0	−8·31 (−3·44)	3·192	2·13	$P < 0·05$
0/1	5·08 (2·10)	3·901	1·30	$P \sim 0·2$
Area				
2/1	7·32 (3·21)	6·922	1·06	$P \sim 0·3$
2/0	12·19 (5·33)	6·159	1·98	$P \sim 0·05$
0/1	−4·32 (−1·89)	5·814	0·74	$P > 0·4$

Size of the Y chromosome in the sperm donor

The spermatozoa used in this study were obtained from a 47,XYY man with large Y chromosomes. To obtain the expected values for differences between X-, Y- and YY-bearing spermatozoa, chromosomes from metaphase preparations of his lymphocytes were measured. The chromosomes were stained with methylene blue (Deitch, 1966) or the Feulgen reaction. Using the Vickers M86 microinterferometer in the densitometric mode, two Y chromosomes, two number 2 chromosomes, and four F-group chromosomes were measured in each cell. The ratios of integrated optical density for the different types of chromosomes were similar with the different staining reactions:

	Y/2	*Y/F*	*F/2*
Mean	0·302	1·148	0·264
Coefficient of variation	6·19%	5·96%	6·12%
Standard error	0·004	0·015	0·004

By comparison with values for DNA content and dry mass of chromosomes identified by banding techniques, the expected values for the different classes of spermatozoa were calculated (Table 3).

Table 3. Published sizes of chromosomes (% of haploid autosomes) and expected values for DNA content of different classes of human sperm heads

Parameter	Chromosome 2	Mean of F group	X chromosome	Reference
DNA content	8·44%	2·28%	5·40%	Mendelsohn *et al.* (1973)
Dry mass	8·40%	2·34%	5·10%	Bahr & Larsen (1974)
Mean of above	8·42%	2·31%	5·25%	

Size of Y chromosomes

Compared with chromosome 2: $8{\cdot}42 \times 0{\cdot}302 = 2{\cdot}543\%$
Compared with F group chromosomes: $2{\cdot}31 \times 1{\cdot}148 = 2{\cdot}650\%$
Mean value of Y chromosomes: 2·597% of haploid autosomes

Expected DNA content of different sperm classes

Y-bearing (Autosomes (A) + Y) = 102·597
X-bearing (A + X) = 105·25
YY-bearing (A + 2Y) = 105·194
Difference between X- and Y-bearing spermatozoa (as % of their mean value) = 2·499%

Comparison of measured and expected values for X- and Y-bearing spermatozoa

Measured difference between spermatozoa with none and one fluorescent spot (Table 2): $5{\cdot}26 \pm 1{\cdot}257$ (standard error) $= 2{\cdot}13 \pm 0{\cdot}510\%$
Expected difference is 2·499% (above) which is 0·723 of a standard error from the measured value

It is clear that the difference found between dry mass of sperm heads with none and one fluorescent spot is not significantly different from the expected difference in DNA content between X- and Y-bearing spermatozoa. However, YY-bearing spermatozoa should have a DNA content virtually indistinguishable from that of X-bearing sperm. Since spermatozoa with two fluorescent spots have significantly lower dry mass than those with no spots (Table 3), the results do not support their identity with YY-bearing spermatozoa.

Discussion

As shown in this paper it is possible to use integrating microinterferometry to estimate the DNA content of human spermatozoa. This technique, in which the dry mass of the sperm head is actually measured, reduces the preparative procedures to a minimum, although in our experiments fixation was required so that we could classify the spermatozoa by quinacrine fluorescence. Our dry mass measurements show a coefficient of variation of 8·3%, compared with 15·2% for our earlier Feulgen measurements (Sumner *et al.*, 1971), a notable improvement.

Validation of this technique requires that there should be a linear relationship between DNA and dry mass (or, strictly, integrated optical path difference) of the sperm heads. This was confirmed by the linear regression of integrated o.p.d. of spermatozoa from which DNA had been extracted on that of intact spermatozoa. The intercept of this line at the origin indicates that sperm heads of all sizes contain a similar proportion of DNA. However, the slope of the regression line, 0·55, cannot be taken as the proportion of protein in the sperm head. Although early work indicated that the specific o.p.d. was similar for DNA and protein, more recent work suggests that the value for DNA may be as low as two-thirds of that for protein (Lindstrom, Zetterberg & Carlson, 1966; Carlson & Gledhill, 1966; A. T. Sumner, unpublished results). In this case DNA would constitute something like 55% of the mass of the sperm head.

We have used measurements of dry mass of human sperm heads to confirm our earlier results (Sumner *et al.*, 1971) that there is a difference in DNA content between spermatozoa showing a quinacrine-fluorescent body, and spermatozoa with no such body. In the present work we found a difference of 2·13% between these two classes; the difference was extremely significant, and corresponded closely to that expected for the difference in DNA content between X- and Y-bearing sperm in this individual, an XYY man with large Y chromosomes. We feel that the evidence is now irrefutable that human spermatozoa with one and no quinacrine-fluorescent spots represent Y- and X-bearing spermatozoa respectively.

The proportion of spermatozoa with two Y bodies in this man (2·38%) is higher than that reported elsewhere (1·3%) (Pawlowitzki & Pearson, 1972). Initially it was thought that this might represent a higher than normal proportion of YY spermatozoa, because the donor is a 47,XYY male, although previous meiotic studies (Thompson, Melnyk & Hecht, 1967) had indicated that one of the Y chromosomes is usually lost in the germinal tissue. It has been assumed that spermatozoa with two fluorescent spots have two Y chromosomes (Pearson & Bobrow, 1970; Pawlowitzki & Pearson, 1972), and our earlier DNA measurements supported this, although the sample available to us was too small for the results to reach statistical significance. In the present study with a larger Y chromosome, the difference between spermatozoa with one and two Y chromosomes was greater (and therefore easier to measure), and a spermatozoon with two Y chromosomes would have virtually the same DNA content as one with an X chromosome. However, the dry mass of the spermatozoa with two spots proved to be significantly lower than that of X-bearing spermatozoa, and as low as or lower than that of Y-bearing spermatozoa. This seems to be incompatible with them being YY spermatozoa, but should not, perhaps, be regarded as conclusive proof of their status.

If the spermatozoa with two spots are not YY spermatozoa what is their nature? Measurements of area indicate that they are larger than other spermatozoa, suggesting that they are not fully condensed. This is supported by the phase-contrast and fluorescence images of some of them in which the usual division into a thin anterior part and a thicker posterior part was not seen. This reduced condensation may itself be sufficient to account for the presence of two fluorescent spots; interphase nuclei, which are much less condensed than sperm nuclei, show a higher proportion with two spots (duplexes) (Robinson, 1971). It has also been shown that males with a large Y chromosome have a higher proportion of duplexes than those with a Y chromosome of average size (Robinson & Buckton, 1971). It may be that the higher proportion of spermatozoa with two Y bodies found in our sperm donor reflects the size of his Y chromosome rather than his XYY status. The brilliantly fluorescent region may appear subdivided into two or more zones, especially in large Y chromosomes (Buhler, Müller, Müller & Stalder, 1971; Knuutila & Gripenberg, 1972; Jalal, Pfeiffer, Pathak & Hsu, 1974). Separation of such zones by stretching the intermediate dim zone would, of course, produce two fluorescent spots from a single Y chromosome. On the other hand, reduced condensation might lead to some loss of material from the sperm head during processing, producing an artificially low value for dry mass. With the small numbers of such spermatozoa available, present techniques do not seem to be capable of resolving the issue.

Some YY spermatozoa must of course be present to account for the production of XYY zygotes; but if the proportion of true YY spermatozoa is less than that showing two spots by quinacrine fluorescence, it may be unnecessary to postulate reduced fertilizing efficiency by YY spermatozoa (Sumner *et al.*, 1971).

References

BAHR, G.F. & LARSEN, P.M. (1974) Structural "bands" in human chromosomes. *Adv. cell. molec. Biol.* **3**, 191–212.

BOUTERS, R., ESNAULT, C., SALISBURY, G.W. & ORTAVANT, R. (1967) Discrepancies in analysis of deoxyribonucleic acid in rabbit spermatozoa, involving Feulgen staining (Feulgen-DNA) and ultraviolet light absorption (UV-DNA) measurements. *J. Reprod. Fert.* **14**, 355–363.

BUHLER, E.M., MÜLLER, H., MÜLLER, J. & STALDER, G.R. (1971) Variant of the fluorescence pattern in an abnormal human Y chromosome. *Nature, Lond.* **234**, 348.

CARLSON, L. & GLEDHILL, B.L. (1966) Studies on the dry mass of bull spermatozoal heads. *Expl Cell Res.* **41**, 376–384.

DEITCH, A.D. (1966) Cytophotometry of nucleic acids. In *Introduction to Quantitative Cytochemistry*, pp. 327–354. Ed. G. L. Wied. Academic Press, New York.

ESNAULT, C. (1973) Reactivation of the Feulgen reaction of ram spermatozoa by dithiothreitol. *J. Reprod. Fert.* **32**, 153–157.

GLEDHILL, B.L., GLEDHILL, M.P., RIGLER, R. & RINGERTZ, N.R. (1966) Changes in deoxyribonucleoprotein during spermiogenesis in the bull. *Expl Cell Res.* **41**, 652–665.

GOLDSTEIN, D.J. & HARTMANN-GOLDSTEIN, I.J. (1974) Accuracy and precision of a scanning and integrating microinterferometer. *J. Microscopy* **102**, 143–164.

JALAL, S.M., PFEIFFER, R.A., PATHAK, S. & HSU, T.C. (1974) Subdivision of the human Y chromosome. *Humangenetik* **24**, 59–65.

KNUUTILA, S. & GRIPENBURG, U. (1972) The fluorescence pattern of a human Yq^+ chromosome. *Hereditas* **70**, 307–308.

LINDSTROM, M., ZETTERBERG, A. & CARLSON, L. (1966) Quantitative microspectrophotometric analysis of nucleic acids in concentrated solutions, in solid droplets and in lymphocytes. *Expl Cell Res.* **43**, 537–545.

MENDELSOHN, M.L., MAYALL, B.H., BOGART, E., MOORE, D.H. & PERRY, B.H. (1973) DNA content and DNA based centromeric index of the 24 human chromosomes. *Science, N.Y.* **179**, 1126–1129.

PAWLOWITZKI, I.H. & PEARSON, P.L. (1972) Chromosomal aneuploids in human spermatozoa. *Humangenetik* **16**, 119–122.

PEARSON, P.L. & BOBROW, M. (1970) Fluorescent staining of the Y chromosome in meiotic stages of the human male. *J. Reprod. Fert.* **22**, 177–179.

ROBINSON, J. (1971) Y chromosome fluorescence in buccal mucosa cells: a study on a newborn population. *Annls Hum. Genet.* **35**, 61–65.

ROBINSON, J.A. & BUCKTON, K.E. (1971) Quinacrine fluorescence of variant and abnormal human Y chromosomes. *Chromosoma* **35**, 342–352.

SUMNER, A.T. (1971) Frequency of polyploid spermatozoa in man. *Nature, Lond.* **231**, 49.

SUMNER, A.T., ROBINSON, J.A. & EVANS, H.J. (1971) Distinguishing between X, Y and YY-bearing human spermatozoa by fluorescence and DNA content. *Nature, New Biol.* **229**, 231–233.

THOMPSON, H., MELNYK, J. & HECHT, F. (1967) Reproduction and meiosis in XYY men. *Lancet* **ii**, 831.

Received 4 December 1975