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Diploid Spermatozoa in Rabbit Semen and Their Experimental Separation from Haploid Spermatozoa

R. A. BEATTY[1] AND N. S. FECHHEIMER[2]

Department of Genetics, University of Edinburgh, U. K.

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In rabbits, 1112 diploid (2N) spermatozoa were recognized by their large heads which were found to have an area in optical projection of 1.532 (± 0.034 SE) times the area of those deemed to be normal haploids (1N). This ratio is of the same order as the theoretical figure of 1.587 for a ratio of quadratic dimensions involving objects expected to differ twofold in volume. 2N spermatozoan heads are stockier in shape and more pear-shaped than those of haploids. A frequent partial or complete doubling of the tail (37% of 2N spermatozoa) indicates some prior cytological doubling event consistent with diploid status. Partial tail doubling is nearly always proximal, in the midpiece segment, and arguments relating to centriolar function can be developed. The heads of 2N spermatozoa can bear one, two, or (very exceptionally) three separate tails. Motile specimens have been observed of all types of 2N spermatozoa with partial or complete tail doubling, or with single tails. Twenty-seven percent of 2N spermatozoa are "live" (unstained by nigrosin–eosin) and bear one tail. A further 19% are "live" but with partial or complete tail doubling. A survey of eight potential sources of variation in AS-strain rabbits aged 29 weeks showed that the incidence of 2N spermatozoa (mean 0.31%) varied between brothers but otherwise exhibited little variation in relation to kinship, nor between duplicate ejaculates or duplicate miscroscope preparations, and the heritability estimate in this partially inbred strain was zero. In a search for material with a higher incidence of 2N spermatozoa, three strains (AS, AD and R) were sampled from 26 to 192 weeks of age and marked strain and age effects were apparent, with a prominent strain/age interaction such that young males of AD-strain had the unusually high incidence of ca. 1.5% 2N spermatozoa. These young AD-strain males provide the highest known natural incidence of 2N spermatozoa. Colchicine injection failed to increase the incidence. When whole semen of young AD-strain males (average incidence 1.6% 2N spermatozoa in controls) was centrifuged in a dextran-based density gradient, an upper fraction with 0.4% 2N spermatozoa was recovered from the tubes, and a lower fraction with 2.9%. The two figures differ significantly from each other and from the control figure for whole semen. The technique was highly repeatable and constitutes a success in physical separation of living spermatozoa in accordance with their genetic content. Improvement in the degree of separation is under investigation. The fraction with a high incidence provides a new source of 2N spermatozoa for further experiments. In the fraction with a low incidence, genetically deleterious spermatozoa have been removed from mammalian semen by artificial means, and the results can be viewed as a pilot experiment of interest in a medical context.

Mammalian spermatozoa with large heads are commonly thought to be "diploid." The parentheses, often used because the evidence is indirect, will be omitted in what follows. There is a general opinion that these spermatozoa constitute a discrete size class and are not merely an arbitrary selection from the tail end of a unimodal frequency distribution. On formal distributional grounds, one large and possibly diploid rabbit spermatozoon was considered to fall outside the main frequency distribution of head length

[1] Agricultural Research Council Unit of Animal Genetics.

[2] Present address: Department of Dairy Science, The Ohio State University, Columbus, Ohio 43210.

(Beatty, 1961). The combined evidence of several workers shows clearly that DNA content, protein content and dry mass in the head of diploid spermatozoa of bulls is close to twice the normal (Salisbury, Birge, de la Torre and Lodge, 1961; Gledhill, 1964, 1965; Salisbury and Baker, 1966a; Esnault and Ortavant, 1967). Another form of evidence in bulls is a frequent partial or complete doubling of the tail that indicates some cytological doubling process (Esnault and Ortavant, 1967): this is supported in the present work with rabbits. In the bull testis, Esnault and Ortavant found that cells with double the normal DNA content occurred among spermatids and spermatozoa but not in primary and secondary spermatocytes. They concluded that diploid spermatozoa arose by a direct transformation of secondary spermatocytes into spermatids and spermatozoa, without a division of the cytoplasm at the second meiotic division. The sex chromosome content of such spermatozoa should be XX or YY, but not XY. In human spermatozoa whose number of Y chromosomes could be judged from the number of fluorescent spots after quinacrin staining, Pearson and Bobrow (1970) mention giant spermatozoa with two spots (YY?), and Sumner, Robinson and Evans (1971) recorded three spermatozoa with about twice the normal DNA content, one having two spots (YY?), and two having no spots (XX?). There seems little doubt from the various forms of evidence that these large-headed spermatozoa are indeed diploid.

Our main objective in the present work was to attempt a physical separation of living diploid and haploid spermatozoa, since this might give a precedent for controlling the process of transmission of genetic information by separating spermatozoa according to their individual genetic content. Since the average incidence of diploid spermatozoa is low in mammals, a preliminary objective was to produce better experimental material by seeking for any environmental or genetic circumstances that would permit one to locate or bring into being males with an unduly low and particularly with an unduly high incidence of diploid spermatozoa. This would eliminate the extremely laborious process of surveying a large assemblage of males until suitable ones are found. We wished also to extend knowledge of the morphology and viability of diploid spermatozoa.

MATERIALS AND METHODS

The AD, AS and R strains of rabbit maintained in this Department were used. The animals within a strain are genetically fairly uniform, because the inbreeding coefficient is high, and because the breeding methods prevent the formation of sublines. The only constant genetic nonuniformity is at the albino locus of the AS-strain, where a forced segregation (matings only between heterozygotes) yields in each generation homozygous albinos, heterozygotes and homozygous light chinchillas in approximately 1:2:1 ratio. Semen was collected with an artificial vagina. Permanent nigrosin–eosin smears were made by the method of Beatty (1957). These preparations display the spermatozoa well and have the advantage that the degree of staining of the head is generally accepted as a measure of viability, the convention being that spermatozoa with unstained heads are termed "live," those with stained heads "dead." Large head size was the sole criterion of diploidy used. Doubling of the tail axis was noted but not used as a criterion. In all, 1112 diploid spermatozoa were scored.

The incidence of diploid spermatozoa was determined at a magnification of ×625, each apparently diploid spermatozoon being rechecked at ×1250. Slides were coded and examined in a randomized order.

For size and shape comparisons of diploid and haploid spermatozoa in AD-strain, one nigrosin–eosin preparation was made from each of nine ejaculates. (A balanced subsampling of three ejaculates from each of three males was incorporated but preliminary analyses of variance showed no significant individual male component and the identity of the three males was thereafter ignored.) The dimensions were measured from drawings made with a camera lucida at linear magnification ×3919 (Wild M-20 microscope with drawing tube, ×100 oil immersion and ×2.5 intermediate magnification). Only "live" spermatozoa bearing tails were scored (three diploids and six haploids per preparation). Appropriate coding and randomization procedures were used, to avoid

bias. The following dimensional characteristics were recorded for each spermatozoon: head length, maximum head breadth, head area in optical projection, Shape Index 1 = (head length)/(head breadth), Shape Index 2 = (distance from head base to point of intersection of principal axes of spermatozoon)/(head length). Thus, for each characteristic, readings were recorded for 27 diploid and 54 haploid spermatozoa. The overall means are given in Table 2, together with their ratios, and standard errors on 8 *df* based on the discrepancy in ratio between preparations. A significant departure of the ratio from unity meant a significant difference between the diploid and haploid means.

The rationale of the centrifugation experiments was that diploid spermatozoa are so distinctive in size and DNA content that they would be unlikely to centrifuge at the same rate as haploids. The levels of the centrifuge tubes containing maximal and minimal proportions of diploid spermatozoa were determined by preliminary experiments not recorded here. A density gradient was used in order to minimize convection currents and vertical mixing. Enough sodium fluoride was incorporated in the medium to prevent translatory swimming. We used a highly alkaline pH (9.4) to minimize autoagglutination (Branham, 1970).

The following stock solutions for density gradients were prepared (Beatty and Burgoyne, unpublished); the barbitone sodium (sodium barbiturate) is a laboratory reagent with 98.5% purity. A direct formula for Solution C can be obtained by averaging those for A and B. A and B are recorded because, if they are mixed in appropriate ratio, the sodium fluoride content can be adjusted if required without changing pH, osmotic pressure or specific gravity.

Solution A. Glucose 3.8393 g, sodium chloride 0.1824 g, barbitone sodium 0.3652 g, N/10 hydrochloric acid 0.473 ml, deionized water to 100 ml.

Solution B. Glucose 3.7612 g, sodium chloride 0.0570 g, barbitone sodium 0.3836 g, N/10 hydrochloric acid 0.497 ml, sodium fluoride 0.1000 g, deionized water to 100 ml.

Solution C. Mixture of equal volumes of A and B.

Solution D. Solution C diluted to 0.663 strength with deionized water.

Solution E. 16.375 g/"Ficoll" (Pharmacia, Lot To. 6374) dissolved in 84.625 g of Solution D.

Solution F. Mixture of equal volumes of Solutions C and E.

Ten-milliliter density gradients were prepared by a double-chamber continuous mixing technique from 5 ml of Solution E (sp gr ca. 1.07) and 5 ml of Solution F (sp gr ca. 1.04). pH was ca. 9.4 throughout the gradient, osmotic pressure 1.00 expressed as a sodium chloride equivalent % w/v. The gradients were formed in graduated conical glass centrifuge tubes with internal dimensions 87 mm from the base to the 10-ml mark, 13.3 mm (maximum) diameter.

Semen layered on the gradient was centrifuged at 1000 rev/min (240*g*) in a Mistral Refrigerated Centrifuge for 40 min at 20°C. The top 1 ml was removed with a finely drawn aspiration tube and discarded. The second 1 ml (Fraction 2) was recovered and mixed with 9 ml of Fluid A. The next 3.5 ml in the gradient were discarded. The following 1 ml fraction (Fraction 6.5) was recovered and handled as for Fraction 2. The remaining fluid in the gradient was discarded. The two tubes containing Fractions 2 and 6.5 were centrifuged at 2000 rev/min for 20 min at 20°C, the supernatant removed, and the cells resuspended in 1 ml of Fluid A. Nigrosin–eosin slides were now made from a drop of each suspension, and had already been prepared from a drop of each fresh semen sample. For each diploid spermatozoon a record was kept of whether or not the head was stained, and how many tails it bore.

RESULTS

(1) *Morphology and Viability of Diploid Spermatozoa*

Examples of diploid and haploid spermatozoa are shown in Fig. 1.

In Table 1, 161 diploid spermatozoa are classified by the degree of staining of the head and by the properties of the tail. They came from the nigrosin–eosin preparations of strains AD, AS and R in the experiment described in (3) of Results. The relative numbers of diploid spermatozoa in the categories defined by the properties of the tail are of the same order in "live" as in "dead" spermatozoa, except that tailless diploid spermatozoa are always "dead." Thirty-seven percent of all diploid spermatozoa display partial or complete doubling of the tail: in none of these was a double distal end associated with a single proximal end, though rare exceptions were observed in other material. The original point of insertion of the tail or tails is particularly clear in the heads of tailless spermatozoa (see Fig. 1). Twenty-seven percent of all diploid spermatozoa are "live" and show no ab-

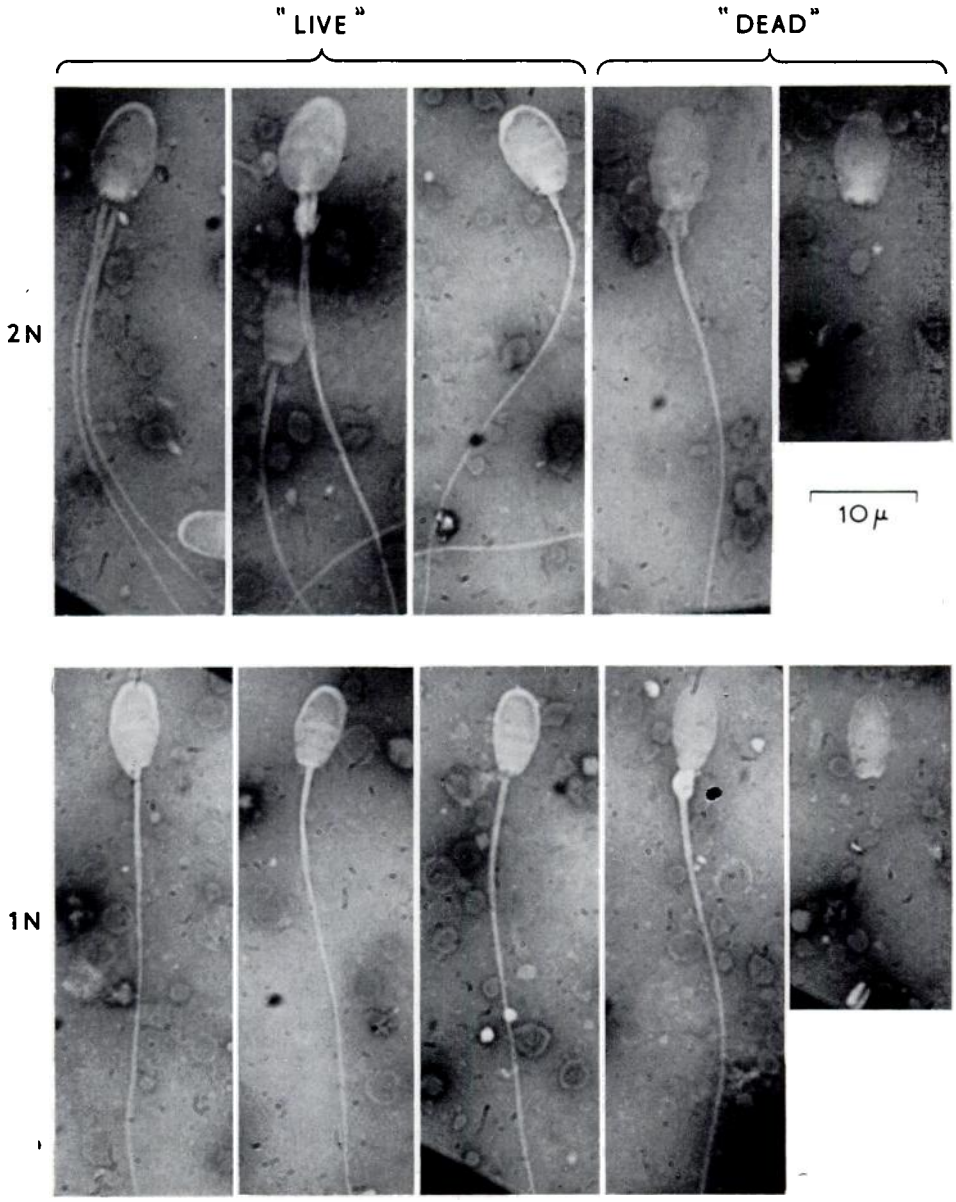

FIG. 1. Diploid and haploid spermatozoa from nigrosin–eosin preparations. Magnification ×1400. Unstained spermatozoa are marked "live," stained ones "dead."

normality other than the large size of the head (cf. the 78% "live" haploid spermatozoa from the same slides). A further 19% of diploid spermatozoa are also "live" but show partial or complete doubling of the tail. In slides of fresh semen, motile specimens of all the categories of tailed diploid spermatozoa listed in Table 1 have been seen. Three-tailed 2N spermatozoa were not seen in this survey, but one was seen in other material. It had three clearly defined "insertion points" on the head and two of the tails were probably confluent along a segment of the midpiece.

TABLE 1

DIPLOID SPERMATOZOA IN POOLED DATA FROM STRAINS AS, AD AND R[a]

	Diploid spermatozoa			Haploid spermatozoa from the same slides
	%-Age unstained ("live")	%-Age stained ("dead")	Total	
One normal tail	27	14	41	Usual
Incompletely doubled tail				
Midpiece all or part double, principal piece single	8	7	15	Very rare
All of midpiece double, part of principal piece double	4	7	11	
Midpiece single, principal piece all or part double	0	0	0	
Two separate tails	7	4	11	
No tail	0	22	22	No data
All spermatozoa	46	54	100	78% "live"

[a] From nigrosin–eosin preparations classed by properties of the tail and by the staining of the head, as percentages of the total number of 161.

TABLE 2

SIZE AND SHAPE OF DIPLOID AND HAPLOID SPERMATOZOAN HEADS FROM NIGROSIN–EOSIN PREPARATIONS[a]

	Mean of diploids	Mean of haploids	Ratio of diploid/haploid means ± $SEM_{[s]}$	Interpretation
Dimensions				
Head area, μm^2	43.1	28.2	1.532 ± 0.034[c]	Diploid spermatozoan heads are larger than haploids in all dimensions measured
Head length, μm	9.20	7.77	1.184 ± 0.010[c]	
Head breadth, μm	5.73	4.46	1.284 ± 0.017[c]	
Shape indices				
Index 1	1.606	1.741	0.923 ± 0.012[c]	Relative to haploids, diploid heads are "stockier" (lower head length/breadth ratio) and more pear-shaped (line of maximum breadth is nearer the base of the spermatozoan head)
Index 2	0.471	0.508	0.928 ± 0.028[b]	

[a] Each diploid mean is based on 27 spermatozoa, each haploid mean on 54. The Shape Indices and the source of standard errors (SEM) are described under Materials and Methods.

[b] Significance level (P) from the analysis of variance: 0.05–0.025.

[c] Significance level (P) from the analysis of variance: $\ll 0.001$.

Measurements of size and shape of diploid and haploid spermatozoan heads are given in Table 2. In relation to haploids, diploid heads are larger and "stockier" in shape. They are also more pear-shaped (maximum breadth nearer the base of the head) (as in the bull, Esnault and Ortavant, 1967).

(2) *Survey of Factors Affecting the Incidence of Diploid Spermatozoa in AS-Strain*

From 1958–1964 a first semen collection had been taken routinely from every male offspring of AS-strain at the age of ca. 29 weeks. Nigrosin–eosin slides were prepared from these and from a second ejaculate 1 week later. From this material 138 slides

TABLE 3

PERCENTAGE COMPONENTS OF VARIANCE FROM AN ANALYSIS OF TRANSFORMED ($\sqrt{X + 3/8}$) NUMBERS OF DIPLOID SPERMATOZOA PER 1000 SPERMATOZOA, SCORED ON 138 MICROSCOPE SLIDES PREPARED FROM 69 AS-STRAIN MALES AGED CA. 29 WEEKS

Item		*df*		%-Age variance component
Year groups		2		14[b]
Sires within year groups	Litters within year groups	22		0
Dams within sires		9	46	0 9[NS]
Litters within dams		15		—
Male offspring within litters		20		31[c]
Ejaculates within male offspring		33		0
Microscope slides within ejaculates		36		0
Theoretical error variance		(∞)		46
Total		137		100 (actual, 0.54)

Significance levels (*P*):
[a] NS > 0.05 (not significant).
[b] Ca. 0.025–0.01.
[c] 0.05–0.025.

from 69 male offspring were selected in accordance with the sampling structure of Table 3. The selection was so arranged that there were three arbitrary year groups, no animal was listed both as a sire and as a sampled male offspring, no dam was mated to more than one sire, and no male offspring, dam or sire appeared in more than one year group. Appropriate randomization procedures were used during the selection of slides. The number of diploid spermatozoa among 1000 spermatozoa scored was recorded for each slide. The counts of diploid spermatozoa were judged to fall into a Poisson distribution and were transformed into $\sqrt{X + 3/8}$ before analysis (Rao, 1952). A Poisson distribution transformed in this way has a theoretical sampling variance of 0.25.

The sampling structure is defined by the hierarchical list of items in Table 3, each item being treated as a "random variate" and contributing its variance component to the mean squares of each item above it. Mean squares were calculated without difficulty. But the numbers of observations were balanced at two levels only (1000 spermatozoa per preparation, 2000 spermatozoa per male) and a formal evaluation of all components and their coefficients would have been impossibly laborious. The following simplification was therefore introduced. It was clear that four items had variance components best estimated as zero in value, this being obvious for two of the items, and assessed by exact procedures for the other two. Assuming that the components of these four items were truly zero in value, the data could be set out in the following simplified form: year groups; litters within year groups; male offspring within litters; theoretical variance. Exact procedures applied to the simplified hierarchical arrangement led to %-age variance components, to conventional significance tests for male offspring within litters and for litters within year groups, and to an approximate significance test for year groups.

The results are shown in Table 3 as percentage components of variance that illustrate the relative importance of the specific effect of each source of variation. The major source of variation is the theoretical sampling error. Good repeatability of observation is shown by the zero components for duplicate microscope slides and duplicate ejaculates. Significant differences exist between the males of a litter. The nonsignificant component for litters indicates that there is no collective effect of parity, season, or the individuality of the litter. The zero components for dams within sires and sires within years mean that heritability must be estimated as

zero. Small and possibly significant differences between year groups suggest genetic drift or a change in environmental imponderables. A preliminary examination had shown no effect of the three genotypes of animal (see Materials and Methods) segregating in the strain.

This experiment on males of a particular age from one somewhat inbred strain shows that none of the sources of variation can be utilized to locate, as experimental material for further work, individual males with a particularly high or low incidence of diploid spermatozoa. Differences between males seem to be real but a further calculation indicated that only about one male in 20 would have a true incidence of diploid spermatozoa greater than double or less than half the strain average of 0.31%. A prohibitive amount of work would be needed to survey a stock with the intention of detecting such males. The zero heritability prevents all thought of starting a genetic selection program on AS-strain designed to produce substrains with a high or low incidence.

(3) *Age and Strain Effects on the Incidence of Diploid Spermatozoa*

Nigrosin–eosin preparations were made from semen collected over a short period of time from 46 rabbits representing three strains AD, AS and R and varying in age from ½–4 years. The percentages of diploid spermatozoa are shown in Fig. 2, where Expt. B confirms a part of a trend in Expt. A, using different males.

Minor fluctuations in the curves are primarily an expression of error variance. However, the plots for AD-strain show an initial fall to about 65 weeks, a rise to a peak at about 110 weeks and a final fall to about 4 years. AS- and R-strains show, perhaps, traces of a similar trend. The reality of this complex trend in AD-strain was supported by the results of a multiple regression of the

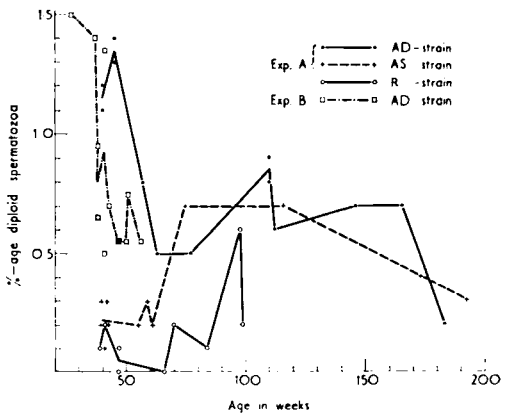

FIG. 2. Percentage of diploid spermatozoa in 46 males of three strains at different ages. Each plot represents a different male. The percentages in Expt. A came from 1000 spermatozoa scored on one nigrosin–eosin slide per male, and in Expt. B from 1000 spermatozoa scored on each of two slides per male.

transformed ($\sqrt{X + \frac{3}{8}}$) counts on the first four powers of the age. This was done by the automatic elimination facility of the MULTREG program devised by R. E. Day and R. L. Middleton of the Edinburgh Regional Computing Centre, as presented in the BISRA PEGASUS program OR/CA/39/65. From the independent variates offered to it the program selects the best set of variates that give collectively a significant regression, and that individually have significant partial regression coefficients. The remaining variates, whose inclusion would not significantly increase the predictive power of the regression equation, are automatically eliminated. The regression retained negative linear and cubic items and a positive quadratic item, the fitted curve having one minimum and one maximum.

The main conclusion is that both age and strain affect the incidence of diploid spermatozoa. The most prominent expression of this is an age/strain interaction: in the age range of ca. ½–1 year, a particularly high initial incidence of diploid spermatozoa occurs in AD-strain and falls rapidly with increasing age. From the empirical point of view of

locating individual males with a high incidence, it is clear that the best material is to be found by the simple procedure of selecting the youngest possible males of AD-strain, where an incidence of up to about 1.5% can be expected. The lowest incidence (ca. 0.1%) occurs in R-strain males up to about 80 weeks old. The trend in AS-strain is consistent with the incidence of 0.31% recorded for 29-week males in (2) above.

(4) *Lack of Effect of Colchicine Injection on the Incidence of Diploid Spermatozoa*

An attempt was made to increase the incidence of diploid spermatozoa beyond the maximum of 1.5% recorded in the previous experiment. Three AD-strain males were given bilateral intratesticular injections of 0.1 mg of colchicine (in 0.1 ml Hanks' Salt Solution) twice weekly for 9 weeks. Two control animals were given the same treatment but without colchicine. Semen samples were collected before the treatment, and weekly from the third week of treatment for 13 weeks. A nigrosin–eosin preparation was made from each sample and 500 spermatozoa were scored. The incidence of diploid spermatozoa showed no trend with time. From the seventh week of treatment, the overall sperm count in samples from treated males (but not from controls) began to decline and the animals began to refuse the artificial vagina.

TABLE 4

INCIDENCE OF DIPLOID SPERMATOZOA IN NIGROSIN–EOSIN PREPARATIONS OF CENTRIFUGED FRACTIONS 2 AND 6.5 AND OF FRESH SEMEN BEFORE CENTRIFUGATION

	Number and percentage of diploid spermatozoa	Total spermatozoa scored
Fraction 2	19 (0.4%)	4700
Fraction 6.5	139 (2.9%)	4800
Unseparated control	65 (1.6%)	4000
Totals	223	13,500

(5) *A Part-Separation of Diploid from Haploid Spermatozoa*

This experiment utilized young AD-strain males expected to have an incidence of about 1.5% diploid spermatozoa [see (3)]. Twenty-four separate runs were made, 18 with semen collected from one or two pooled ejaculates from individual males. On two occasions semen from four males was pooled and the result split among three centrifuge tubes handled thereafter as independent runs. The centrifugation procedure is described under Materials and Methods and it may be recalled that Fraction 2 was isolated from near the top of the tube, Fraction 6.5 from lower down. Nigrosin–eosin slides were prepared from each fraction, as well as from the fresh semen of 20 runs before centrifugation. A total of 200 spermatozoa per slide was scored, except for one slide with 100.

Good repeatability of the results in different runs allowed the figures to be pooled over all runs (Table 4). The percentages of diploid spermatozoa in Fraction 2, Fraction 6.5 and in the unseparated fresh control were, respectively, 0.4%, 2.9% and 1.6%. The three percentages all differ significantly from one another. Evidently, the incidence of diploid spermatozoa was increased to about twice the control level in Fraction 6.5 and decreased to about one-fourth the control level in Fraction 2. As judged by these two fractions, diploid spermatozoa sediment more rapidly than haploid ones.

From the data in Table 5 it can be shown that there are no significant differences between Fractions 2 and 6.5 or between either of these and the control in the proportion of "live" and "dead" diploid spermatozoa, nor in the relative numbers of diploid spermatozoa bearing none, one or two tails. Further, at all points where a comparison

TABLE 5

NUMBERS OF DIPLOID SPERMATOZOA IN AD-STRAIN; FROM NIGROSIN-EOSIN PREPARATIONS OF CENTRIFUGED FRACTIONS 2 AND 6.5, AND OF FRESH SEMEN BEFORE CENTRIFUGATION, CLASSIFIED AS UNSTAINED OR STAINED, AND BY THE NUMBER OF TAILS PER SPERMATOZOON

	Unstained ("live")	Stained ("dead")	Number of tails			Totals
			0	1	2[a]	
Fraction 2	6	13	2	10	7	19
Fraction 6.5	64	75	29	78	32	139
Unseparated control	28	37	14	39	12	65
Totals	98 (44%)	125 (56%)	45 (20%)	127 (57%)	51 (23%)	223 (100%)

[a] Includes partial doubling of the tail.

can be made, these relative numbers for AD-strain are of the same order as the figures for all three strains combined in Table 1.

DISCUSSION

Our results appear to constitute a successful physical separation of two classes of spermatozoa that differ in genetic content. The degree of separation is modest, but there is no doubt that from fresh rabbit semen containing 1.6% diploid spermatozoa, centrifuged fractions can be obtained that show regularly incidences of either 2.9% or 0.4%. The fraction with a high incidence may be useful for further experimental work. The fraction with a low incidence has potential relevance to medical research, since we have removed from "normal" semen a proportion of deleterious spermatozoa, which are either infertile or, if fertile, would be expected to give triploid embryos. The difference in genetic content between diploid and haploid spermatozoa is a somewhat specialized one, and the material had been chosen because the great phenotypic difference in the head size of the two classes gave the best available material for a test case. The technique would require refinement (now under investigation) before there could be any thought of separating spermatozoa that differ by only one chromosome, e.g., by removing disomic spermatozoa that would give trisomic embryos. The prospect of controlling sex ratio by separating X- and Y-bearing spermatozoa in this way seems more remote.

A genetic control of the incidence of diploid rabbit spermatozoa is implicit in the strain differences. In bulls Salisbury and Baker (1966b) have detected a genetic control evidenced by differences between sire lines. Our estimate of a zero heritability *within* one strain (AS) is perhaps to be expected in view of the high inbreeding coefficient. The strain differences in the incidence of diploid spermatozoa interact with age of male, and useful experimental material is provided by young males of AD-strain, which have an incidence of up to about 1.6%.

Negative findings are that the incidence of diploid spermatozoa is not demonstrably affected in our material by possible sources of variation associated with technical and observational differences between duplicate microscope preparations, by environmental and biological differences between duplicate ejaculates, by the identity of the litter within year groups (which includes such factors as parity and change in time of year), nor by differences in genetic constitution at the albino locus. In the centrifugation experi-

ments, diploid spermatozoa in the two fractions did not differ in the proportion of "live" (unstained) heads, nor in the proportion of spermatozoa with none, one or two tails: the success of the separation technique was not, therefore, mediated by these characteristics.

Of the spermatozoa showing partial doubling of the tail, only the proximal end is doubled, the distal end being single (apart from rare exceptions). If the hypothesis of Esnault and Ortavant (1967) is accepted, that diploid spermatozoa arise by chromosomal division accompanied by failure of cytoplasmic division at the second meiosis, if it is assumed that the centrioles have usually divided, and since it is known that centrioles tend to repel rather than attract each other, the following interpretative arguments may be developed. Centrioles that fail to move apart during the formation of a flagellum will act synergistically and produce a single tail. (The same consequence would result from an undivided centriole.) Centrioles initially close together will produce a single distal end to the flagellum since this is the first part that forms but as they move apart they will split the axis and give a double proximal end. Centrioles that have already moved apart before flagellum formation will produce two separate flagella. These interpretative hypotheses fit the data, but can be tested only by direct study of differentiation of spermatozoa.

There is some dimensional evidence that the diploid spermatozoa are truly diploid. They would be expected to have twice the volume of haploids. Assuming that the unknown thickness of the diploid spermatozoan head (a linear dimension) in a direction perpendicular to the plane of the microscope preparation is $2^{1/3} = 1.260$ times that of a haploid, then for a quadratic dimension (projected head area) the diploid should be $2^{2/3} = 1.587$ times the haploid. Our figure of 1.532 ± 0.034 for head area is in reasonable agreement with expectation. Because of the known difference in the head length/head breadth ratio between diploids and haploids, the linear dimensions of length and breadth are less suitable for comparing with expectation. In fact, our 2N/1N head breadth ratio of 1.284 ± 0.017 does agree well with the linear expectation, but the 2N/1N head length ratio of 1.184 ± 0.010, though of the right order, differs significantly from expectation.

We can present no direct evidence on the fertility of diploid spermatozoa. Their incidence is normally so low that they would not in themselves be expected to have any appreciable effect on the fertility of semen, though there seems to be a connection between a high incidence of diploid spermatozoa and infertility of the ordinary haploid spermatozoa in the sample (Salisbury and Baker, 1966b). In the present work the nigrosin–eosin staining technique indicates that the proportion of "dead" spermatozoa is higher in diploid than in haploid spermatozoa. Nevertheless, a substantial proportion are "live" on this criterion, and motile specimens have been observed in fresh semen. On these criteria, there is no bar to a possible fertility of some diploid spermatozoa.

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REFERENCES

BEATTY, R. A. (1957). Nigrosin–eosin staining of rabbit spermatozoa and the fertility of semen. *Proc. Roy. Soc. Edin.* (*B*), **67**, 1–31.

BEATTY, R. A. (1961). Genetics of gametes. V. The frequency distribution of the head length of rabbit spermatozoa, and a search for dimorphism. *Proc. Roy. Soc. Edin.* (*B*), **68**, 72–82.

BRANHAM, J. M. (1970). Separation of rabbit semen into two populations of spermatozoa by centrifugation. *J. Reprod. Fertil.* **22,** 469–482.

ESNAULT, O. AND ORTAVANT, R. (1967). Origine des spermatozoïdes diploïdes présents dans l'ejaculat d'un taureau *Charolais*. *Ann. Biol. Anim. Biochim. Biophys.* **7,** 25–28.

GLEDHILL, B. L. (1964). Quantitative ultramicrospectrophotometry of presumed diploid bovine spermatozoa. *5th Int. Congr. Anim. Repr. & A.I. Trento (Italy)* **3,** 489–494.

GLEDHILL, B. L. (1965). Cytophotometry of presumed diploid bull spermatozoa. *Nord. Vet. Med.* **17,** 328–335.

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

RAO, C. R. (1952). "Advanced Statistical Methods in Biological Research." Wiley, New York.

SALISBURY, G. W., BIRGE, W. J., DE LA TORRE, L AND LODGE, J. R. (1961). Decrease in nuclear Feulgen-positive material (DNA) upon aging in *in vitro* storage of bovine spermatozoa. *J. Biophys. Biochem. Cytol.* **10,** 353–359.

SALISBURY, G. W. AND BAKER, F. N. (1966a). Frequency of occurrence of diploid bovine spermatozoa. (Brief Communication). *J. Reprod. Fertil.* **11,** 477–480.

SALISBURY, G. W. AND BAKER, F. N. (1966b). Nuclear morphology of spermatozoa from inbred and linecross Hereford bulls. *J. Anim. Sci.* **25,** 476–479.

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