GENETIC STUDIES ON SPERMATOZOA OF INBRED STRAINS OF MICE

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KALI NATH SHARMA
B. Sc. (Calcutta University),
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Submitted to the University of Edinburgh as
a Thesis in fulfilment of the requirements
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GENERAL INTRODUCTION

In animals, the expression of genetic factors has been studied almost exclusively among the attributes of the embryo or the adult. The study of the effect of genetic factors on the gametes themselves has been termed the genetics of gametes. Genetic effects on gametes are of particular interest, because they may cause, or indeed, be expressed directly as differences in the fertility of the gametes, and this in turn might have repercussions on both fundamental and applied genetics, where it is generally assumed that the chances of a particular ovum being fertilised by a particular spermatozoon are not affected by their genic content. The relation of the genetics of gametes to mammalian biology has been presented by Beatty (1956, 1957a, 1957c, 1958a, 1958b, 1959), Beatty and Napier (1959a, 1959b), and Braden (1956, 1957a, 1957b, 1958a, 1958b, 1959).

The present study has been conducted with the visible, as distinct from the behavioural, characteristics of spermatozoa of the laboratory mouse, _Mus musculus_. This species was chosen for the following reasons. First, the chief long-term application of this kind of work is likely to be in mammals. Secondly, the genetics of the mouse has been studied intensively (Grüneberg 1952). Thirdly, internationally known inbred strains are available to all workers, and results on particular strains or crosses can readily be confirmed or extended. Fourthly, mice are an inexpensive source of spermatozoa throughout the year, and breed quickly. Finally, an eventual possibility in this kind of work is to attempt the artificial separation of spermatozoa according to their genetic content and thus, after artificial insemination, to attain some measure of control over genetic segregation ratios; in the mouse, a technique for artificial insemination is already available (Snell, Hummel and Abelmann 1944).

The spermatozoan characteristics chosen for study were mainly the dimensions of the head and midpiece, though two other characteristics presumptively related to spermatozoan fertility and three more characteristics associated with physiological maturation of spermatozoa have also been studied. There is little precedent in mice for quantitative studies of this kind, and almost no information about the relative importance of biological and technical sources of variation was available before the beginning of the experiments. It was felt desirable, therefore, to arrange somewhat complex sampling structures in such a way that the various sources of error variation would not bias the main results. The sampling structure also permitted estimation of the magnitudes of these various sources of error variation after the experiments were completed, and this formed an important subsidiary objective, since it aided the interpretation of the analyses in the present work, and yielded information that could be of service in designing future experiments.

All the observations (apart from the measurements of live spermatozoa) were conducted with nigrosin-eosin preparations where the staining mixture consists of 30 g. nigrosin (Gurr), 5 g. eosin (Gurr) and 300 cc distilled water (Hancock 1951). This mode of preparation was adopted for the following reasons.

The main object of the work was to study the dimensional characteristics of spermatozoa. The measurement of such characteristics is present results supplement those of Braden (1957b) on strain differences in the behaviour of mouse gametes close to the time of fertilization. Other work is that of Snell and Poucher (1943), Snell (1944) and Clayton and Edwards (1957) on spermatozoan antigens specific to inbred strains of mice; of Hancock (1953) on a spermatozoan abnormality characterising a related group of bulls; and of Edwards (1955) on the unequal fertility of spermatozoa from inbred strains of mice, as judged from the numbers of offspring recorded after heterospermic insemination.

With the ovum, examples are known in this first category. Beatty and Fischberg (1951) attributed a high incidence of triploid embryos in a particular stock of mice to a tendency in the strain towards submersion of the second polar body of the egg. This was supported by Braden (1957b), whose paper also brought to light numerous differences between strains of mice in the incidence of abnormalities of egg maturation and fertilization, conditioned by the strain to which the female belonged. Cytogenetical anomalies in the mammalian egg with particular reference to parthenogenesis and polyploidy are reviewed by Beatty (1957c).

A second category of observation arises when single hereditary factors are known to affect the phenotype of gametes, and when it is unknown whether gene action on the gamete is mediated by the somatic or the gametic gene complements, or by both. A major example is the identification of the antigens of blood in the spermatozoa of mammals (e.g. Landsteiner and Levine 1926). A probable connection between the melanising power of rabbit spermatozoa _in vitro_, and the genetic constitution of the rabbit at the albino locus, has been reported by Beatty (1956). The present work suggests that spermatozoan characteristics of the mouse are unaffected by an agouti-locus allele, but are possibly affected by alleles at the albino locus.

A third and particularly interesting category of observation is when gene action on gametes is attributable to single genetic factors (genes, or chromosomal sex factors segregating as units) acting in the individual gamete. Basically, this type of observation demands the demonstration of two or more different phenotypic classes of gamete segregating from a heterozygous soma. There was some slight evidence for such segregation in the work on melanizing activity of rabbit spermatozoa mentioned above. Braden (1958a) has produced strong evidence that T locus alleles may have a direct effect on mouse spermatozoa bearing them. Reports that the X- and Y-bearing spermatozoa of mammals differ in size have been discussed by Beatty (1959) who finds no crucial evidence for dimorphism. The control of sex ratio by separation of X- and Y-bearing spermatozoa after counter-streaming centrifugation (Lindahl, 1956 1958) or by electrophoresis (Gordon 1958) or after gravitational fall (Bhattacharya 1958) would come into the present category.

The present studies are concerned chiefly with direct comparisons of the spermatozoan characteristics of particular inbred strains. In the second chapter, F_1 crosses between certain of these strains have also been studied and have yielded what is believed to be the first demonstration of heterosis in the spermatozoan phenotype.

CHAPTER 1

STRAIN DIFFERENCES IN SPERMATOZOA FROM EIGHT INBRED STRAINS OF MICE

1. INTRODUCTION

Until recently, the expression of genetic factors in animals has been studied almost exclusively in the zygote. Genetic effects on the phenotype of gametes form a field of study, the genetics of gametes.

The main objectives in the present section were to acquire information on the following points. What are the visible ways in which the phenotype of the spermatozoa varies? What controls this variation, and in particular, how much of it is determined genetically? Braden (1956) had reported strain differences in mice in the shape of the spermatozoan head, and possibly in its length and breadth. The present investigation brings to light numerous genetic differences, as judged by differences between strains, in the characteristics of spermatozoa from eight inbred strains of mice. Sources of variation within strains have also been evaluated, some of the information being vital for correct appreciation of the differences between strains.

2. MATERIALS AND METHODS

a) The inbred strains

The history and relationships of the standard inbred strains of mice are given by Heston (1949) and by Carter, Dunn, Falconer, Grüneberg, Heston and Snell (1952). The relationships of the strains used in this work, with the approximate time of their origin, are shown in Fig. 1.

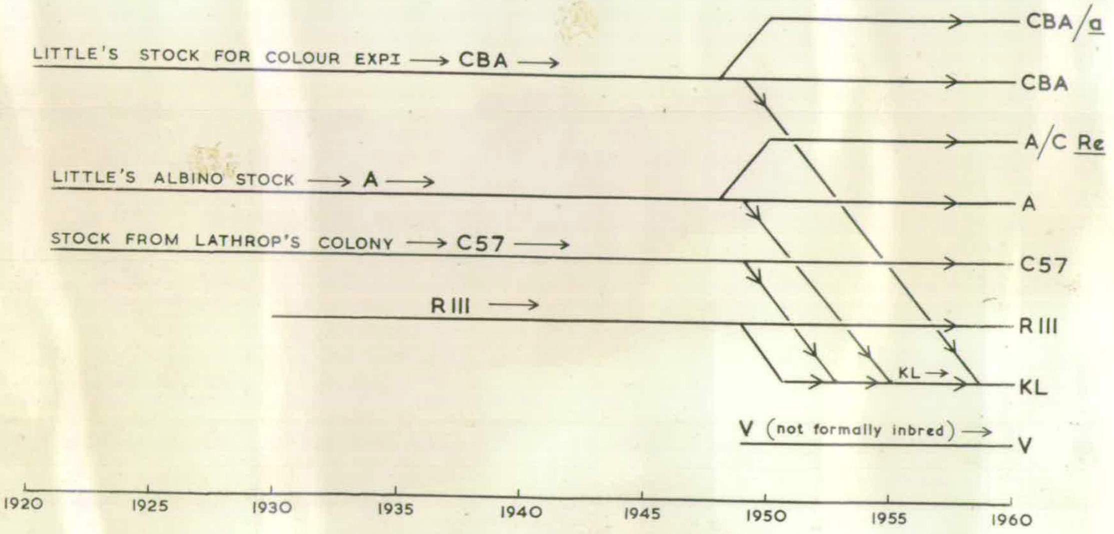

FIGURE 1. HISTORY AND RELATIONSHIP OF INBRED STRAINS OF MICE.

KL originated (circa 1949) by a 4-way cross between A/Fa, C57BL/Fa, CBA/Fa, and RIII/Fa ; the intersections between the horizontal KL line and each of the 3 arrowed diagonal lines do not represent a sequence in crossing and are not to be read against the time scale.

The symbols used to designate the strains, followed in brackets by the accepted international symbol, if any, are given below. All strains have been inbred brother to sister for more than twenty generations, except for V strain, which is highly though not formally inbred, and would have been studied separately if any outstanding peculiarities had become evident in variance within the strain.

The strains are as follows:

V (no international symbol):- originally "E-stock" (Beatty and Fischberg 1949), or "silver stock" (Beatty and Fischberg 1951); selected for high incidence of heteroploid embryos (Beatty 1954); maintained chiefly by sib-matings with periodic culling of sub-lines; genotype, *sisi bb*, segregating for *A* and *a* and perhaps for *d*, $\underline{sb}_2$.

A (A/Fa):- genotype, *aa bb cc*.

A/(CRe) (no international symbol):- originated *circa* 1948 from A/Fa by addition of *C* and *Re* through five generations of back-crossing to A/Fa; then full inbreeding with forced segregation at the *c* locus; local symbol REB; genotype, *aa bb ReRe* and either *cc* or *+c*.

CBA (CBA/Fa):- genotype, wild type.

CBA/a (CBA/Be-*a*):- arose *circa* 1948 after mutation from *A* to *a* in the CBA/Ca stock (at that time the same as CBA/Fa); local symbol MB; genotype, *aa*.

KL (no international symbol):- originated *circa* 1949 from a four-way cross between A/Fa, C57BL/Fa, CBA/Fa and RIII/Fa; then several generations of selection for large body size; then full inbreeding (stock of Falconer and Latyszewski); genotype, *aa*.

RIII (RIII/Fa):- genotype, *cc*.

C57 (C57BL/Fa):- genotype, *aa*.

b) Sampling of the strains

The stocks of mice have been kept for many generations under standard conditions of maintenance at the Institute. Throughout the present investigation, the cages were in no particular sequence on the racks, and their order was frequently changed. The sampling is summarised in Table 1, in which it will be seen that the strain means of age (140 - 210 days) range over a restricted period of what is commonly regarded as the useful breeding life of male mice (about 50 - 280 days). For reasons of time, it was not possible to arrange for the average age of the strains to be identical and, as mentioned later (p. 15), there is, in any case, little evidence that age of male affects any spermatozoan characteristic.

The choice of the eight strains, and the number of thirteen males per strain was dictated by availability. Litters older than 330 days or younger than 60 days were rejected. The sampling was designed to give as many litters as possible per strain. When more than thirteen litters per strain were available, thirteen were selected at random; when exactly thirteen litters were available, all were chosen. One male was selected at random from each litter. When less than thirteen litters existed per strain, one male was chosen at random from each, and the process repeated until thirteen males were available for that strain. The 104 males (thirteen for each of the eight strains) thus chosen were killed in randomised order over a period of four days, and their spermatozoa mounted as nigrosin-eosin preparations that were inspected at once. Four males giving unsatisfactory preparations were replaced by litter mates, chosen at random, and recorded in random position on the list of males yet to be killed.

c) Preparations

All dissections and the making of preparations (slides) were carried out in a constant temperature room at 19 ± 2°C. The mice were killed by stunning and breaking the neck. The *vasa deferentia* were dissected out and cleaned externally; the contents of both the *vasa* were stripped immediately into a single drop of 0.85% NaCl with the aid of two forceps. After one minute, the spermatozoa were mixed with the saline by gently pipetting up and down, and the suspension allowed to stand another minute. One drop of nigrosin-eosin stain (made to the formula of Hancock 1951) was mixed into the suspension and left for two minutes. Two slides were smeared thinly with the stained suspension, using the edge of another slide, and allowed to dry on the bench; drying took about one minute, and was always complete between four to five minutes after the mouse was killed. Coverslips (1½" x 7/8") were mounted with DePeX. Each of the 208 preparations was coded by a second party, and the code was withheld until all observations had been completed.

d) Apparatus and measuring procedure

For the study of the dimensional characteristics of the spermatozoa, the projection microscope described by Beatty and Napier (1959a), giving a magnification of x 6950, was used. The nominal resolving power of the microscope was *c.* 0.22μ for white light. Measurements were made on drawings of projected images of spermatozoa. The unusually high magnification was employed in order to minimise the effect of such technical factors as the thickness of the pencil lines or the coarseness of the scales on the measuring instruments. Straight lines were measured with a steel rule graduated to 0.5 mm., and curved lines (those along the main axis of the midpiece) were measured with a rotameter graduated to 1/16" (= 1.6 mm). For measuring areas, a planimeter graduated to 0.1 cm^2. was used. Observations recorded as percentages were made by direct vision through an ordinary microscope with oil-immersion objective and x 10 ocular, and without a filter. The whole of the present investigation is two-dimensional in the plane of optical projection of the spermatozoa which lie flat against the slide.

e) Description and sampling of spermatozoan characteristics

All the preparations of the experiment were placed in a randomised order before examination. Except for restrictions described below, spermatozoa were sampled by selecting each isolated spermatozoon that came into the field of view during a systematic search of a preparation. This was considered to be the equivalent of formal random sampling, since there was little reason to expect any relationship between the attributes of a spermatozoon and its position on the preparation. Groups of agglutinated spermatozoa were ignored.

It had been intended to measure only randomly chosen unstained spermatozoa with normal acrosomal caps. This was achieved, except for occasions (9.9% of all spermatozoa, distributed fairly equally over the strains) when there was a scarcity of unstained spermatozoa. The head and midpiece of five spermatozoa per slide were outlined on paper, with one paper for each of the 1040 spermatozoa. The acrosomal and post-nuclear caps were included in the sketch of the head. The papers were placed in random order and the following characteristics measured: head breadth, head area, midpiece length (called midpiece length A) and midpiece area.

The breadth of the sickle-shaped head is defined as follows. The half of the incurved edge of the sickle nearest to the base of the head is moderately straight, though closer inspection shows that it resembles two cycles of a flattened sinusoidal curve. A base line was drawn through this curve. Head breadth was measured as the maximum breadth of the spermatozoan head at right angles to and measured from the base line. (See Fig. 2).

The midpiece breadth was determined by dividing the area by the length. This was thought to give a better estimate than would direct measurement, since the actual breadth is only about 0.8µ; in effect, the breadth has been measured as an average along the length of the more or less rectangular midpiece.

The 1040 sheets of paper were then classified by the subjective criterion of whether the head appeared to be "thick" or "thin", and the % "thick" spermatozoa was listed for each slide. This characteristic was studied in order to see if a quick method of measuring head breadth would, when analysed, give results comparable with those from detailed measurement. Full analysis of the % "thick" spermatozoa was carried out but, because of the transitory interest of this characteristic, only the conclusions are reported (p. 26).

While each slide was in position for the observations described above, an additional five spermatozoa were selected at random, with no attention to the qualities of the head, and with the sole restriction that each bore a kinetoplasmic globule on the midpiece. The length of the midpiece, and the point of attachment of the kinetoplasmic globule, were marked on paper. From these drawings, midpiece length (called midpiece length B) was measured. There is, therefore, a slight difference in category between midpiece lengths A and B; the former are from spermatozoa that do not necessarily bear a kinetoplasmic globule and whose heads are of a certain class, while the latter are all from spermatozoa bearing a globule and with unclassified heads.

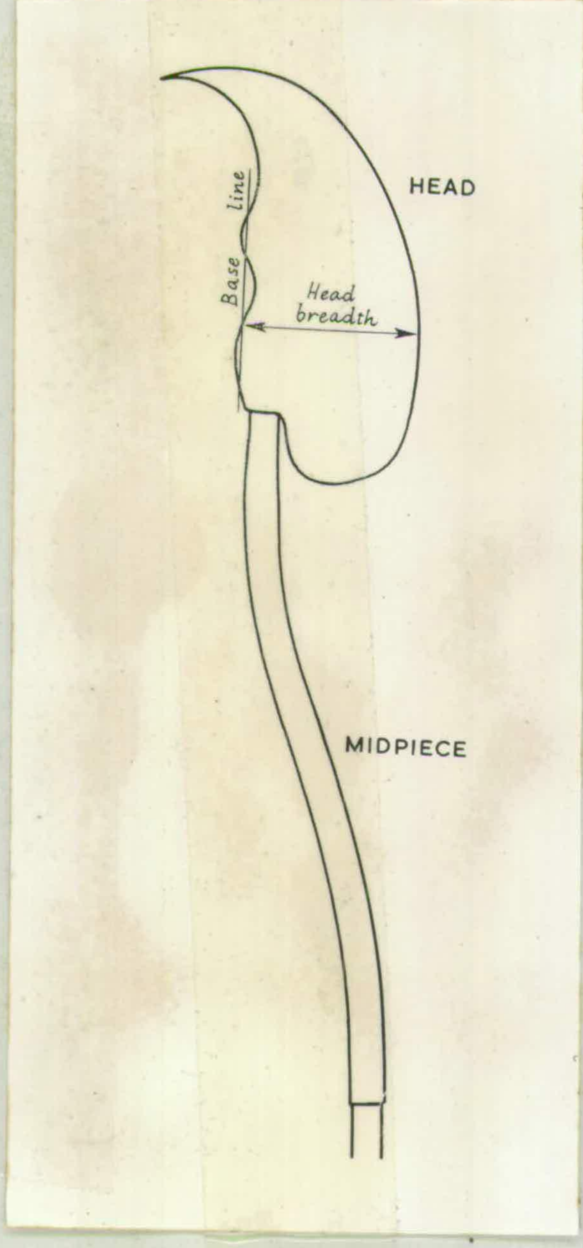

FIGURE 2 : DIAGRAM OF THE SPERMATOZOAN HEAD AND THE MIDPIECE SHOWING THE MEASUREMENT OF HEAD BREADTH.

The base line is drawn through the two cycles of the sinusoidal curve. The head breadth is indicated by the straight line with arrow heads drawn at right angles to the base line.

The <u>globule end position</u> was measured as the distance between the point of attachment of the kinetoplasmic globule and the rear end of the midpiece. The <u>globule migration position</u>, or the relative distance travelled by the kinetoplasmic globule during its migration from the front to the rear of the midpiece, was determined as the ratio of (a) the distance between the attachment of the globule and the front end of the midpiece, and (b) the total length of the midpiece.

The characteristic of "head length" of the spermatozoa was not measured. The peculiar shape of the head does not allow for any easily definable single measurement that can be called the natural length of the head. The curved beak of the acrosomal cap is very delicate and presents varying degrees of curvature. In some mammalian species, the point of attachment of the midpiece is at one end of the longitudinal axis of the head and provides a fixed point for measuring the length; in mice, this is not so.

All the slides were then placed in a new random order and examined with an ordinary microscope. The following characteristics, studied concurrently, were listed from 50 spermatozoa per slide; the percentage of spermatozoa with unstained heads (called <u>% unstained</u>) and the percentage of spermatozoa with normal acrosomal caps (called <u>% normal caps</u> B). Unstained spermatozoa were those whose heads were lighter in colour than the immediate background. Spermatozoa with normal caps were those in which the caps were neither loose, missing nor irregular. All spermatozoa with abnormal caps were stained, but the converse did not hold. With the slides in a new random order, the following characteristics, scored concurrently, were listed from 50 spermatozoa per slide; the percentage of spermatozoa bearing a kinetoplasmic globule (called <u>% with globule</u>), and the percentage of spermatozoa with normal acrosomal caps (called <u>% normal</u>

caps A). There is no essential difference between the A and B series for % normal caps; the two series were drawn from the same slides but on different occasions, and permit a check on repeatability of observation and analysis.

For purposes of analysis, the characteristics of spermatozoa fall naturally into two groups - the mensuration data and the percentage data. The latter were analysed after the angular (arcsin) transformation (Snedecor 1956), the standard error of a transformed percentage being independent of the actual value of that percentage.

f) Factors and their effects: biometrical methods

An extensive series of graphs, and a preliminary experiment not recorded here, gave no compelling evidence that any spermatozoan characteristic was related to the age and weight of the male over the range studied in this work. Further, for dimensional characteristics no real differences between litter means within strains could be demonstrated (p. 18), in spite of the varying ages and weights of the litters. Strain means of spermatozoan characteristics were not significantly correlated with strain means of age or weight (p. 23). Finally, there were no marked differences among strains in their average age or weight. It was therefore thought reasonable to ignore age and weight of the males in the present investigation.

The factors studied are perhaps best stated in conjunction with the statistical model used for analysis. The characteristics of a spermatozoon are affected by various factors (= sources of variation; for example, males in litters), that are listed in the subsequent analysis of variance. Each factor has an effect (e.g. the males-in-litters effect). The factor and its effect are given the same symbol (e.g. M(L)). Random effects (e.g. the differences between random males of the same litter) are measured quantitatively as <u>variance components</u> (e.g. $\sigma_{M(L)}^2$) that are isolable from the hierarchical analyses of variance; the significance of effects is tested by comparing mean squares in the analyses. Fixed effects (differences among particular strains of mice) are, in the strict sense, measured by <u>constants</u> that are not true variance components but are computed as if they were. For convenience, and in the absence of any general term denoting the measures of both fixed and random effects, these constants will be referred to as variance components. The following effects were studied, the first being of primary interest in this work; the inbred strain effect, I; the males-in-strains effect, M(I); the preparations-in-males effect, P(M); the spermatozoa-in-preparations effect, S(P). There were equal numbers of observations at each level of the factors listed above. Provision had been made for orthogonal analysis of a possible difference between litter means in the presence of two levels of unbalance in numbers (varying numbers of males per litter and of litters per strain). If litters are taken into account, the males-in-strains effect, M(I), is partitioned into a litters-in-strains effect, L(I), and a males-in-litters effect, M(L). It was assumed that there was no significant families-in-strains environmental effect, and, within an inbred strain, family means would be unlikely to differ for any genetic reason. Two special sources of variation, E1 and E2, are described on p. 32 .

Most of the computations are of standard form (e.g. Anderson and Bancroft 1952; Snedecor 1956). Some examples of detailed application of biometrical methods to spermatozoa of the rabbit are given by Beatty and Napier (1959a, 1959b). Normality of distribution and homogeneity of variance have been assumed; unpublished work by R.A. Beatty has demonstrated the validity of these assumptions at several levels of analysis.

A few small and non-significant components of variance, irrationally "negative", have been treated as real figures throughout computation (Beatty and Napier 1959a). An approximate method was used for assessing the significance of "all individual comparisons" among several means with unequal numbers of observations per mean.

Abbreviations additional to the symbols for factors and strains include: d.f. (degrees of freedom); μ (0.001 mms.); P (probability of null hypothesis); V.R. (variance ratio); S.E. (standard error); S.D. (standard deviation); *t* ('Student's' *t*); MS (mean square); *r* (correlation coefficient); σ^2 (variance component of a random factor, and also the analogous constant for a fixed factor). Differences between strains, differences among strains, and strain differences, are synonymous phrases, all meaning "differences between means of strains".

3. RESULTS

a) Presentation

The original data are summarized in Table 5 as all strain means of all the spermatozoan characteristics studied, and elsewhere as comparisons, mean squares and variance components.

The significance of the sources of variation affecting each spermatozoan characteristic is tested (p. 18) from the analyses of variance. The analyses, and the component structure (p. 16 , Table 3) lead to the isolation of components of variance (p. 20 , Table 4). The strain averages for each spermatozoan characteristic, and the average standard error per strain mean, are given in Table 5; from this arises a detailed statement of the significance of all differences among strain means of all characteristics (p. 21). Outstanding strain differences are emphasised (p. 23), and observations on living spermatozoa described (p. 24).

Experimental conclusions are recalled in the more general context of the Discussion, with some unavoidable repetition, but freed from the minutae of statistical statement and argument.

b) Analyses of variance

The individual basis of the analyses in Table 2 was one spermatozoon in the mensuration data, while in the percentage data it was one angularly transformed percentage derived from 100 spermatozoa per male. Angular percentages were obtained by pooling the data for the two preparations of each male, expressing the result as an actual percentage, and then angularly transforming it.

For each mensuration characteristic, 832 degrees of freedom were available for the spermatozoa-in-preparations source of variation. This would have given a mean square unnecessarily well-determined in relation to the rest of the analysis. To avoid computational labour the information was reduced to 208 degrees of freedom by selecting at random from the record sheets, data for only two spermatozoa per slide. The data from all five spermatozoa per slide were used for calculating mean squares at all higher levels of analysis.

In interpreting the analyses of variance shown in Table 2, it is necessary at the outset to consider whether or not there is a real litters-in-strains effect, L(I). If it is not real, then the M(I) mean square is an appropriate error term for assessing the significance of the strain effect, I. If the L(I) effect is real, then the M(I) term in the analysis must be split up into L(I) and M(L), and the L(I) term would form the basis of an error term for testing the significance of the strain effect. Arguments against the reality of the L(I) effect are, firstly, that it is non-significant, in eleven of the twelve analyses, and only in one analysis does it attain a low level of significance; secondly, in six of the analyses, apparent differences in magnitude between the L(I) and M(L) mean squares are partly a reflection of what appears to be a small subnormality of the M(L) mean squares, probably due to chance fluctuations. An argument supporting the reality of the L(I) effect is that its mean square is slightly (though non-significantly) greater than the M(L) mean square in eleven of the analyses, and significantly greater in one analysis, though at a low level of significance. An argument for allowing the use of the M(I) mean square as the error term for testing the strain effect, even in the presence of a possibly real L(I) effect, is that the M(I) and L(I) mean squares do not differ greatly in magnitude. On the whole, the litters-in-strains effect, even if it should be real, is likely to be small, and the results have been analysed and presented in the first place on the assumption of no real L(I) effect. But modificatory notes have been added to the legends of the Tables and in the text, so that the results can be re-assessed, if subsequent work should point to a real L(I) effect; the underlying principles are explained but the computations are not presented in detail.

In the mensuration data, the results appear to be clear-cut. On the assumption of no real litters-in-strains effect, there are significant differences among strain means for head breadth and area, midpiece length A and B, midpiece breadth, globule end position and globule migration position. (If a real litters-in-strains effect is assumed, the strain differences for midpiece breadth and globule end position do not quite attain significance). In the lower levels of the analyses of mensuration data in Table 2 (columns b - f) there are no significant effects of litters in strains, males in litters, or preparations in males, except for significant differences between duplicate preparations for midpiece area and breadth, and for globule migration position. The strain differences in the size of the midpiece can be further interpreted. First, the virtual constancy of midpiece area among strains is as striking in the analysis of variance as in the means of Table 5; from the latter, it may be calculated that the grand average of midpiece area (18.23 μ^2) has a S.E., based on differences between strain means, of only ± 0.185 μ^2 (7 d.f.). Second, and by contrast, there are highly significant strain differences in midpiece length and breadth. Strain means of the length and breadth of the midpiece must, therefore, be negatively correlated; this was confirmed directly by correlating the strain means of midpiece breadth in Table 5 with the midpiece length A or B; both correlations were significant. These two correlations do not appear on p. 23, where a particularly high level of significance was used.

In the percentage data, there are highly significant differences between strains for % unstained and for % normal caps A and B, but the % with globule is not significant. There is a low level of significance attached to the L(I) mean square for % normal caps A, but the L(I) mean squares for % normal caps B and for the other characteristics are not significant. The M(I) and M(L) mean squares are all highly significant in comparison with the theoretical variance of an angularly transformed percentage; this may indicate true M(I) and M(L) effects but, alternatively, may reflect differences between duplicate preparations (see Legend to Table 4).

It will be seen that the general patterns of the analyses of midpiece length A and B are similar, as might be expected, since these two categories of length differed but little. The analyses of % normal caps A and B are also similar.

The relative contributions of the various sources of variation are more easily visualised from variance components (Table 4), which are derived from the material ~~from the material~~ in Tables 2 and 3. In the mensuration data, outstanding points are the large magnitude of the strain components for head breadth and head area, and for midpiece length, while the strain component for midpiece area is zero. All L(I) and M(L) components for all mensuration characteristics are non-significant and small. The largest component in any analysis is on the right hand side of the Table: S(P) in the mensuration data, M(L) in the percentage data. In the percentage data, the L(I) effect is evidently not well-determined, since large estimated variance components cannot be shown to be significant.

c) Comparisons of all strain means of all characteristics

Several points are immediately evident from Table 5, where all strain means are shown for all characteristics. For instance, CBA/*g* and its parent strain CBA are closely alike; A/C*Rg* and its parent strain A are alike except in head breadth; the C57 head is small. Detailed interpretation of the Table demands appreciation of the standard error per strain mean, as described below.

For any particular spermatozoan characteristic, the difference between two strain means would be deemed significant if it exceeded ($\sqrt{2}$) x (S.E. per strain mean in Table 5) x (*t*), provided that only *one* such comparison is made, planned in advance of the experiment, or stated before consulting the results. But 28 comparisons are possible among 8 strain means, and one or more "significances" may be found that are, in fact, chance fluctuations appearing out of a large number of tests. This difficulty was overcome by using the method of Duncan (1955) to assess the significance of all comparisons among all means for each characteristic, the resulting interpretation of Table 5 being presented in the main body of Table 6.

The A and B series of midpiece length were treated as duplicates; on the rare occasions when they gave different significance levels for a particular comparison, the lower significance was chosen. The A and B series of % normal caps were treated similarly.

Table 6 also bears a modificatory foot-note, prepared as follows, for amending the body of the Table if a real L(I) effect is to be assumed. A table similar to Table 5, but showing strain means of litter means, was first compiled, and the differences among means calculated. But, all the available methods for testing "all differences among means" seem to demand equal numbers of observations in the subclasses, whereas inequalities existed in the present data. An approximate method was, therefore, used which was considered adequate, since the inequalities were not large. Duncan's 1955 method was not adapted, because the treatment of his "Rule 2" could not be visualised. Instead, an adaptation was made of the earlier Tukey method (as described, with modifications, by Snedecor 1956). Least significant differences (L.S.D.) were computed; differences between strain means of litter means exceeding the L.S.D. were deemed significant, while those not exceeding the L.S.D. were considered non-significant. The L.S.D. was computed as $\sqrt{MS \text{ x } Q/n_1n_2}$, where MS is the L(I) mean square in the analysis of variance (Table 2), n_1 is the harmonic mean of the numbers of observations per litter in the whole experiment, n_2 is the harmonic mean of the numbers of litters in the two particular strains under comparison, and Q is a factor from the 0.05 P Table of May (1952), entered for 78 d.f. and 8 groups. A Table similar to Table 6 was set up, listing all significant differences between strain means of litter means; comparison of this Table with the body of Table 6 led at once to the modificatory note at the foot of Table 6. The A and B series for midpiece length and for % normal caps were treated as in the previous paragraph.

Table 6 is, therefore, a complete statement of all the significant differences between strains and, by exclusion, of all differences that are not significant. CBA/g and CBA do not differ significantly from one another in any characteristic, A and A/CRg differ only in head breadth, while each of the remaining strains differs significantly from each other strain in at least three spermatozoan characteristics.

d) Correlation of spermatozoan characteristics

The columns of strain means in Tables 1 and 5 form fourteen sets of data among which ninety-one correlation coefficients were calculated. Several "significances" due only to chance fluctuation would be expected from so many unplanned comparisons. As a safeguard, therefore, only those correlations greater than 0.83 were marked significant, the significance level being $P < 0.01$. The significant correlations were: between the A and B series for midpiece length, $r = +0.97$; between A and B series for spermatozoa with normal caps, $r = +0.96$; between head breadth and head area, $r = +0.90$; and between the migration position and end position of the kinetoplasmic globule, $r = +0.99$. The last correlation has little interest, being a necessary consequence of the definition of the two correlates. It will be noted that no spermatozoan characteristic was significantly correlated with age or weight.

e) Summary of outstanding spermatozoan characteristics of particular strains

Table 7 summarizes the more striking characteristics of the different strains, chosen on the following principles. The characteristics were those showing an overall significance of $P < 0.005$ for the differences among strain means in the analyses of variance in Table 2. For each such characteristic, the two strains with the highest and the two with the lowest mean value of that characteristic were selected from Table 5, but were retained only if they differed significantly from at least three other strains. In the original data sheets, some of these differences were obvious. For instance, the mean head breadth and head area of each of the thirteen C57 males were smaller than the corresponding means for each of the thirteen A strain males.

f) Measurements on living spermatozoa

C57 and CBA males were selected in pairs of approximately equal age, but with the age differing between pairs. The twenty mice of ten pairs were killed in randomised order, and the head breadths of their living spermatozoa determined in the usual way (though without coding), the semen being mounted in saline under a coverslip ringed with vaseline. Ten spermatozoa, temporarily at rest, but showing motility after measurement, were measured per male. An analysis of variance, with an individual basis of one spermatozoan head measured in μ, yielded the following mean squares: strains, ~~1804~~ 18·04 (1 d.f.); pairs, 0.249 (9 d.f.); interaction, 0.230 (9 d.f.); spermatozoa in preparation, 0.113 (180 d.f.). The interaction is barely significant. The mean square for pairs is not significant, thus suggesting once again that age has little effect on head breadth. The difference between strains is highly significant. Mean head breadths for C57 and CBA spermatozoa were 3.00μ and 3.61μ respectively.

In comparison with spermatozoa in nigrosin-eosin preparations (Tables 2 and 5) it would appear, therefore, that living spermatozoa have smaller heads, and an increased variance within preparations; all this, however, might be due to living spermatozoa not lying flat against the slide, and being viewed at slightly varying angles. The change in breadth might also be due to a possible swelling of the spermatozoan head in nigrosin-eosin preparations. The only conclusion drawn from the present section is that an observation made with nigrosin-eosin preparations is confirmed from living spermatozoa; the spermatozoa of C57 have a significantly narrower head than those of CBA.

4. DISCUSSION

a) Strain differences in spermatozoan characteristics

The main finding in these permanent nigrosin-eosin preparations is that several characteristics of spermatozoa vary significantly among the eight inbred strains. Some differences are not inconsiderable; for instance, the head breadth of A strain spermatozoa is about 10% greater than that of the C57 strain, and in V strain there are about twice as many unstained spermatozoa as in KL; differences were sometimes strikingly clear by inspection of the original data. In the mensuration characteristics, the inbred strain effects fall into two clear groups according to the characteristic studied; the effects are either highly significant and large (variance components between 18% and 24%), or else they are not significant or at a moderate level of significance, and are small (variance components between 0% and 3%). (See Table 4).

Even in comparison with what may be an over-stringent error term, based on an assumed reality of the litters-in-strains effect, strain means are found to differ significantly in the breadth and area of the spermatozoan head, in midpiece length, in the incidence of spermatozoa with an unstained head, and in the incidence of those with normal acrosomal caps. Strain differences in head breadth and area are highly correlated. The differences in head breadth are so marked that independent analyses, from measured breadths, or from a crude classification of spermatozoan heads as "thick" or "thin" (p. 12), gave an almost identical rank order of strain means, and almost identical lists of significant differences among these means. With a less stringent but, nevertheless, probably valid estimate of error (assuming no real litters-in-strains effect), three further characteristics show significant differences among strains; the midpiece breadth, the globule end position, and the globule migration position.

The variation in the midpiece is of interest. Within small and stated limits of error, no variation exists between strains in the area of the midpiece. As judged from these eight strains, the average midpiece area per strain can be regarded as a constant typical of the species. But there are significant (and negatively correlated) strain differences in the length and breadth of the midpiece. The essential attribute of the midpiece susceptible to strain variation must, therefore, be its shape, and the shape typical of a strain is not apparently subject to any litters-in-strains or males-in-litters effect. Between duplicate preparations, however, the area and breadth are found to differ significantly, but not the length; the preparation technique probably causes small deformations of the midpiece, with the longer axis the more resistant to deformation. All observations were two-dimensional, and nothing can be said about the volume of the midpiece.

Whether significant or not, the variance components for the strain effects on the kinetoplasmic globule are of little interest, being extremely small for each observed attribute of the globule. Strain variations in the incidence of unstained heads, and of those with normal acrosomal caps, are alike; this was to be expected, since most of the spermatozoa with normal acrosomal caps are also unstained.

The spermatozoan characteristics fall into three main groups relative to the biology of spermatozoa. Most of the characteristics are dimensions. The incidences of unstained spermatozoa and of those with a normal acrosomal cap probably bear a positive relationship towards the fertility of semen; there is evidence for this in the rabbit (Beatty 1957b); and the incidence of stained spermatozoa is related to fertility in the bull (Bishop 1955) and to spermatozoan viability in several mammals (references in Beatty 1957b). It may well be that most of the abnormal caps are artefacts representing the response of the spermatozoa to the challenge of the method of preparation, but their incidence is, nevertheless, connected with semen quality as mentioned above. The incidence of spermatozoa with a kinetoplasmic globule, and the two measures of the position of the globule on the midpiece, are related to the physiological maturation or "ripening" of spermatozoa in the genital tract of the male (Mann 1954). In each of these biological contexts, therefore, at least two characteristics differ significantly among strains of mice.

In the face of the manifold variation among strain means (Table 6), it is noteworthy that CBA and CBA/a do not differ in any characteristics of their spermatozoa whatsoever; as may be seen from Table 5, the two sets of means are almost the same. Since CBA/a arose as a mutation from A to a in the CBA strain, (see Fig. 1) and the two inbred strains must be genetically identical, apart from invisible mutation and genetic drift, it is inferred that the non-agouti mutation a does not affect spermatozoan characteristics in the mouse, in the sense that AA and aa mice have virtually identical spermatozoan characteristics. The heterozygote Aa has not, however, been examined. Also, the near-identity of the results from these two closely related strains gives an empirical check on the validity of the sampling, measurement and analysis in this experiment. Two other strains, A and A/CRe, related to one another, (see Fig. 1), though less closely than are CBA/g and CBA, were indistinguishable in all characteristics except head breadth. Since A strain is homozygous for albino, whereas some of the A/CRe individuals are heterozygous, the possibility exists that albino locus alleles may have some effect on head breadth of the spermatozoa. Other pairs of strains are not closely related enough for arguments about specific genes to have much validity.

b) Variation within strains.

With most biological characteristics (for instance, the body weight), one would expect to find demonstrable litter and male effects in an experiment of this size, betraying the influence of age, number in the litter, number in a cage, maternal effects, behavioural relationships in the cage, and various imponderables connected with maintenance, health, and so on. By contrast, the mensuration characteristics of spermatozoa in a strain seem to be quite free of demonstrable variation associated with the identity of the litter or of the male. Even the technical variation between duplicate preparations is almost negligible (and is non-significant) for head breadth, head area, and for midpiece length. It is concluded that the mensuration characteristics of spermatozoa are extraordinarily independent of many environmental, biological and technical factors. However, as discussed below, some significant variation has been detected between duplicate slides for midpiece area and breadth, and for the globule position; it is also well known that spermatozoa are affected by gross changes in their environment. It will be noted in mensuration data that the main component of variance is that expressing differences between spermatozoa on a slide; part of this variation must be attributed to observational uncertainty associated with the limits of optical resolution of the microscope.

c) Genetic and non-genetic variation

Under the sampling conditions of this work with inbred mice, it is assumed that strain components of variance reflect genetic effects, and that all the variation within a strain is non-genetic. The possibility of heterozygosity or fresh mutation in an inbred strain can not be absolutely excluded, but was assumed to be minor on degree. For the present purposes, V strain will be treated as a formally inbred strain. Variation between litters in strains would reflect environmental or maternal effects, while the males-in-litters variation would show environmental effects. Variation between duplicate preparations is attributed to technique, and variation between spermatozoa in preparations is attributed to a combination of technique, observation, and of natural variation between spermatozoa. Age and weight seem to play a negligible role in spermatozoan variation (p.15) and will not be considered further.

For head breadth, head area, and midpiece length, genetic variation is represented by marked differences among strains, while the only non-genetic variation occurs in the differences between spermatozoa in preparations. The midpiece area has no genetic variation, but there is a moderate non-genetic variation attributable to technique and a large non-genetic component for differences between spermatozoa in preparations. The midpiece breadth, the globule end position, and the globule migration position, each has a very small though significant genetic component, a moderate component attributable to technique, and a large component for non-genetic variation between spermatozoa in preparations. Among the percentage data, where interest was concentrated at the upper levels of the analysis, the main conclusions are that there is a moderately large genetic component for the incidence of spermatozoa with unstained heads and with normal acrosomal caps; the genetic component for the incidence of spermatozoa bearing a globule is small and non-significant.

As a guide to work on the quantitative genetics of spermatozoa, it will be noted that the head breadth in particular, and also the head area and the midpiece length, are "good" characteristics, with a large genetic component and no demonstrable non-genetic components other than the variation between spermatozoa on a preparation. These characteristics should have a considerable potential for response to selection.

d) Biometrical analysis of spermatozoan characteristics

In the mensuration data, there is a detailed resemblance between the two independent analyses of the midpiece length, and the rank order of strain means from the one analysis is almost identical with that from the other; analyses of closely related strains are in good agreement; no means squares are more than slightly subnormal, and no variance components more than slightly 'negative'. But, in the percentage data, analyses of the two sets of observations on the incidence of normal acrosomal caps are in fair rather than good agreement, and there is obviously much intangible variation within strains. This contrast between mensuration data and percentage data also applies to rabbit (p.32).

The demonstration of the reality of differences between means of measurements made on two or more populations of small objects is not necessarily limited to differences of greater magnitude than the nominal resolving power of the microscope. The coarser the resolution, however, the larger is the number of individuals that may have to be measured to establish a difference. For a valid assessment of such small mean differences, the following conditions must hold, though experience may show that some can be relaxed:- (1) physical and observational sources of error must be randomised, as well as the order of presentation of the objects to the observer; (2) the material must be coded, so that the observer does not know the class of object before him; (3) the observer must not, by inspection, be able to recognise that two or more classes of object are being presented to him; and (4) the comparisons of means must emerge from a single experiment designed to reveal them. The present work, which largely concerns mean differences, was designed to meet these conditions. Absolute magnitudes of single observations, or means of such observations, are of a different status; within the fringe of uncertainty of the optical resolution, any given observer might consistently over- or under-estimate the true value, and this bias might differ from that of another observer, or from that of the same observer on a different occasion. But, when mean differences are being assessed under the conditions described above, most of this bias would be expected to cancel out.

e) Measurements of living and dead spermatozoa

An excellent account of difficulties involved in making unbiassed measurements of spermatozoa was given nearly half a century ago by Zeleny and Faust (1915). In planning the present work, a high priority was given to eliminating possible sources of subjective variation attributable to the observer and to technique. In consequence, spermatozoa were examined in dried smears (permanent nigrosin-eosin preparations) where they are no longer alive. This aided measurement, because the spermatozoa lie flat in the field of optical projection, and are not moving. In addition, all the preparations of an experiment can be examined in a coded and randomized order, and remain available for further study. One comparison, of head breadth, has been checked with living spermatozoa; the narrowness of the spermatozoan head of C57 in comparison with that of CBA was first suggested by the preliminary data of Braden (1956) in living spermatozoa, has been confirmed in the present work with nigrosin-eosin preparations (Table 7) and again confirmed with living spermatozoa (p. 24).

f) Comparison with spermatozoan characteristics of the rabbit.

Strain differences are also found in the rabbit (Beatty and Napier 1959a). The effects of sources of variation on rabbit spermatozoa and on those of mice are similar. In both species, the breadth and area of the spermatozoan head are independent of numerous technical, environmental and observational sources of variation, and there are no significant differences between males within litters. In the rabbit, differences between "litters within weeks of the experimental period" were, admittedly, significant for head breadth and head area, but might have been confounded with a genetic component not present in the inbred mice; in mice, there was no significant litters-in-strains effect for these characteristics. Two special forms of error variation, E1 and E2, were described and considered by Beatty and Napier (1959a). In the rabbit, the potential "individual semen sample error" effect (E1), attributable to the particular circumstances under which each semen sample was handled, was found not to exist. In the present work, where the E1 effect would be confounded with males-in-litters effect, their collective effect was found to be negligible; hence, there is no evidence in either species for a real E1 effect on any mensuration characteristic studied. An "individual slide error" (E2), attributable to the particular observational circumstances under which each slide was examined, was found to be non-existent for head breadth in the rabbit; in the present work, it would be confounded with the preparations-in-males effect, but their collective effect was found to be negligible for head breadth, head area, midpiece length and globule end position. Hence, there is no evidence that E2 affects these characteristics of the mouse. But, in the mouse, E2 may well account for the significant differences between duplicate slides for midpiece area, midpiece breadth, and globule migration position. The deformations of the midpiece mentioned on p. 26 would be a part explanation of E2.

Significant strain differences in the mouse for the incidence of unstained heads (a measure of fertility), were also demonstrable in the rabbit (Beatty and Napier 1959b).

5. SUMMARY

1. Genetic effects on the visible characteristics of spermatozoa, as judged by significant and often striking strain differences, have been studied in the inbred strains of mice, A, A/GRe, CBA, CBA/g, KL, RIII and C57, and in the partially inbred strain V.

2. The spermatozoan characteristics, observed in permanent nigrosin-eosin preparations of spermatozoa from the *vas deferens*, were:- (a) *dimensions, in optical projection*; head breadth and area; midpiece length, breadth and area; relative and actual positions of the kinetoplasmic globule on the midpiece; (b) *percentages* of spermatozoa with unstained heads, or with normal acrosomal caps, or of those bearing a kinetoplasmic globule. The incidence of unstained heads, and of heads with normal caps, relates to semen fertility; observations on the

kinetoplasmic globule are measures of the physiological maturation of spermatozoa.

3. The effects of the following factors on spermatozoan characteristics were studied: strains, litters-in-strains, males-in-strains, males-in-litters, preparations-in-males, spermatozoa-in-preparations, special errors E1 and E2. Age and weight of the male, also studied, did not seem to affect any spermatozoan characteristic.

4. Numerous significant strain differences were found for all spermatozoan characteristics except midpiece area and incidence of spermatozoa bearing a globule. The magnitude of the strain differences was up to some 10-50% of the mean value of the characteristic. Strain variation in the midpiece is essentially in shape, with negatively correlated strain differences for length and breadth, the area being virtually identical for all strains. Strain variation in the attributes of the kinetoplasmic globule is unimportant.

5. Comparison of two closely related strains, indistinguishable in all spermatozoan characteristics, suggested that the *a* and *A* alleles have no differential effect on the spermatozoan phenotype. A difference in breadth of the spermatozoan head between two less closely related strains suggested, on the other hand, an effect of albino-locus genes on spermatozoa.

6. There were no demonstrable effects of litters in strains, or of males in litters, on any mensuration characteristic of spermatozoa. The dimensions of spermatozoa are extraordinarily independent of many biological, environmental and technical sources of variation. The characteristics recorded as percentages are less independent, and much intangible variation exists. The factors found to have significant effects on the characteristics of head breadth and area, or of midpiece length,

were the genetic differences between strains, and the non-genetic variation between spermatozoa in preparation; these characteristics appear particularly suitable for quantitative studies on the genetics of the phenotype of gametes.

7. The narrowness of the spermatozoan head of C57 in comparison with that of CBA has been checked with living spermatozoa.

8. The spermatozoa of mice and rabbits have analogous patterns of variation.

9. The present status of the genetics of mammalian gametes is reviewed.

CHAPTER 2

THE PHENOTYPE OF MOUSE SPERMATOZOA IN FOUR INBRED STRAINS AND THEIR F_1 CROSSES

1. INTRODUCTION

In the foregoing pages, evidence of genetic effects on the spermatozoan phenotype of mice has been presented in the form of highly significant differences among eight inbred strains. The characteristics studied were head area, head breadth, midpiece area, midpiece length, midpiece breadth, end position and migration position of the kinetoplasmic globule; and incidence of unstained spermatozoan heads, indidence of heads with normal acrosomal caps, and incidence of spermatozoa with a kinetoplasmic globule.

In this chapter, studies have been extended, in the first place to F_1 crosses between inbred strains of mice, and evidence for heterosis in the spermatozoan phenotype has been sought for in those of the above mentioned characteristics that were found to be particularly suitable for genetic studies. A second objective was to confirm the existence of differences in the spermatozoan phenotype among four of the inbred strains used in the previous work. A virtually complete confirmation was achieved, with respect to the characteristics studied. The two objectives were pursued in a single planned experiment.

Observations on the spermatozoan phenotype in F_1 crosses of mice had been made by Braden (1956).

2. MATERIALS AND METHODS

The following have already been described in detail in Section 2 of Chapter 1: the inbred strains; the making of nigrosin-eosin preparations of spermatozoa; measuring equipment; the definitions of the spermatozoan characteristics; general remarks on biometrical methods. The projection microscope described by Beatty and Napier (1959a) was again used for dimensional characteristics. The steel rule used before for measuring straight lines was replaced by vernier calipers reading up to 0.1 mm.

The four inbred strains, CBA, A/CRe, RIII, and C57, were taken for the present investigation. The six possible crosses were made. The object of study was the variation in the spermatozoan phenotype among the ten genetic groups (four pure lines and six crosses). For reasons of time and space, reciprocal crosses were not studied. Each cross was, however, made in a stated direction with respect to the sex of the parents. (Table 8).

The stocks of the ten genetic groups were built up over the same period of time. The ages of the available males ranged from 50 to 159 days; from these males, five litters per genetic group were selected in such a way that the age distribution was roughly comparable from one group to another. Two males per litter were selected at random.

The 100 males were killed in randomised order over a period of four days. The contents of the two *vasa deferentia* of each male were mixed and made into nigrosin-eosin preparations in a constant temperature room at 19 ± 2° C, with two preparations (slides) per male. The 200 slides were coded by a colleague who withheld the code until all observations on them had been completed.

The coded preparations were placed in a randomized order, and two spermatozoa per slide (with unstained heads and normal acrosomal caps) were drawn under the projection microscope at a magnification of x 6950, with a separate sheet of paper for each spermatozoon. The drawings were placed in a randomized order, and measurements made of the head area, head breadth, midpiece area, and midpiece length.

With the preparations in a new randomized order, the following characteristics were scored from twenty spermatozoa per slide, using an oil immersion objective with an ordinary microscope, and a green filter similar to the Wratten 58; % unstained (the percentage of spermatozoa whose heads were, on the whole, less deeply stained than the immediate background of the preparation), and % normal caps (the percentage spermatozoa with normal acrosomal caps).

In the analysis of the results, the ages of the males were not taken into account for the following reasons. The average age in the ten genetic groups was more or less balanced. Graphical representation in this and the previous work (Chapter I) showed no clear relationship within genetic groups between spermatozoan characteristics and age.

Analyses were carried out by standard methods (e.g. Snedecor 1956). Percentages were studied after the appropriate angular transformation.

3. RESULTS

a) Presentation

The group averages of each spermatozoan characteristic are summarized in Table 9. Differences between litter means within groups are given in the form of the average standard error per group mean; these standard errors are the basis of all the subsequent tests of significance.

The average figures for pure strains combined and crosses combined are also shown.

The course of analysis of differences among the ten group means is conveniently referenced to the following tables, and the text associated with each. In Table 10, the variation among groups is analysed in terms of the four genotypes involved and of an average comparison between pure strains and crosses. Differences among the pure strains alone are then given (Table 11), while Table 12 shows explicitly the agreement between the present results and earlier ones shown in Table 6. Heterosis in spermatozoan characteristics is assessed (Table 13). Analyses of variance of variance are described.

The term heterosis is defined as the extent to which a cross deviates from the mid-parent (i.e. from the average of the two parental types), using the figures shown in Table 9 without transformation other than the angular transformation employed for the percentage data.

b) General analysis of the differences among ten genetic groups

The first step in analysis was to deal with the question; for each spermatozoan characteristic, are the differences among the ten means in Table 9 attributable only to sampling error? This was tested by a simple partition of the variance among 50 litter means into between groups (on 9 d.f.) and between litter means within groups (on 40 d.f.). The mean square on 40 d.f. was a measure of sampling error against which the significance of the between groups mean squares could be tested. The results are shown in the top and bottom lines of Table 10. Differences among the ten genetic groups are significant for all characteristics except midpiece area, though the latter approaches the conventional significance level of P = 0.05. It was concluded that real differences had been demonstrated among the group means of all characteristics except midpiece area.

The second step in analysis was to find whether the variation between group means could be interpreted in terms of the genetic relationships between the groups. This was carried out by regression analysis on the following model. The group means were taken as the dependent variate, Y. Independent variates X_1, X_2, and X_3 were set up, representing the A/C<u>Re</u>, RIII, and C57 genomes. The values entered under X_1, X_2, X_3 are conveniently explained by example: in the genetic group A/C<u>Re</u> each male contains one A/C<u>Re</u> genome and no contribution from any other genome, and X_1, X_2, and X_3 are entered as 1, 0 and 0 respectively; in the A/C<u>Re</u> x C57 cross, each male contains a half genome of A/C<u>Re</u> and a half genome of C57, and X_1, X_2, X_3 are accordingly entered as 0.5, 0, 0.5. No independent variate was needed for the CBA genome, since all males were fully identified by specifying three of the four genomes. A further independent variate X_4 was also set up, bearing values of 0 for a pure strain and 1 for a cross. A multiple regression of Y on the X was then carried out, on 4 d.f.. Because of the structure of the sampling, X_4 was uncorrelated with the other X, thus simplifying the computation of the inverse matrix, and permitting the effect of X_4 to be shown as a separate line in Table 10. Normality of distribution and parallelism of regression lines were assumed. Homogeneity of variance was also assumed; the assumption was supported at two levels of the sampling structure by the results of an analysis described on p.46.

These computations led to the partitioning of the variance among genetic groups (on 9 d.f.) into the three categories shown in Table 10. The collective effect of the four genomes, on 3 d.f., is listed as the "genomes" source of variation in the Table. The regression on X_4 measured the average deviation of crosses from mid-parents, and is entered as the "heterosis" source of variation. The residual variation, on 5 d.f., is the "remainder".

It will be seen from Table 10 that the "genomes" and "heterosis" effects account, collectively, for much of the demonstrable variation between the ten genetic groups. The remainder mean square is non-significant, and scarcely larger than the error mean square, for head area, midpiece area, midpiece length, and % unstained, and is barely significant for head breadth. However, in one analysis, that of % normal caps, the remainder is compellingly significant. The general result is, therefore, that the analysis appears to have been reasonably successful in partitioning the variation among group means in terms of tangible sources of variation. Most of the variation between genetic groups can be interpreted in terms of the average effects of the four genomes, and the average effect of heterosis. This general statement must be qualified by pointing out certain discrepancies; these are the occasions when the remainder mean square is significant or nearly so (see p. 45) and also the situation for midpiece area where a "significant" genomes effect is somewhat doubtful because there are no compellingly significant differences among the ten groups as a whole.

c) Differences between the pure strains

In the present experiment, four of the strains examined in the previous work (Chapter 1) were re-investigated. From the values of the spermatozoan characteristics in Table 9, it is possible to write down the rank order of the strains for each characteristic, and also to compare the strains individually with one another. The significance of these comparisons is set out in full in Table 11. The strain averages and a list of significant differences are available for the same four strains of the previous experiment in Tables 5 and 6.

A comparison of the two experiments is summarised in Table 12, where the strains are placed in rank order according to the average values of their spermatozoan characteristics. It will be seen that the rank orders for head area, head breadth, and midpiece length are identical. For the % unstained the two right hand strains (CBA and C57), and for the % normal caps the two left hand strains (CBA and RIII) in the first experiment have become interchanged in the present experiment; this is, however, scarcely surprising because there is little difference between the two strains concerned in the two characteristics and, therefore, a slight sampling error may place the strains in an altered rank order. Even the midpiece area which does not differ significantly between genetic groups show good agreement in rank order in the two experiments. This indicates a possible existence of small but real differences between genetic groups in their midpiece area.

Because the characteristics of head area, head breadth, and midpiece length agree in rank order, the signs (positive or negative) of the differences between individual means must also agree, six comparisons being possible among four strain means. The significance level of each individual comparison, classed either as $P > 0.05$ or $P < 0.05$, was identical in both experiments. A substantial degree of agreement was also found for significance levels of individual comparisons among the other characteristics.

The repeatability of results can also be visualised from correlations between the strain means in the two experiments, as shown in the right hand column of Table 12. These correlations are high and two of them attain significance: the significance tests are, however, over-stringent, since the correlation coefficient is based on only 2 d.f.

Finally, another estimate of strain differences can be obtained in the present experiment by using the multiple regression equations calculable from the computations explained on p. 40. Estimated differences among pure strains can then be calculated, using the whole weight of the experiment, i.e. utilising the indirect information in the crosses as well as the direct information from the pure strains alone. The results of this analysis are, however, valid only if the assumptions inherent in the model are fully obeyed, and only if the remainder mean square is nearly equal to the error mean square, and also if differences between the reciprocal crosses, not studied in this experiment, are negligible. Because of these complexities, the alternative estimates do not seem worth setting out in detail, but it may be recorded that there was substantial agreement between estimates and significance tests obtained in this way, and those obtained from the pure strains alone in the present as well as the previous experiments.

The use of a green filter did not noticeably affect the classification of spermatozoa as stained or unstained. In the present experiment, the average percentage of unstained spermatozoa over all pure strains was 33.7%; in the previous work without a filter, the average over the same strains was 30.3%.

The conclusions are that the differences among strain means reported in the previous experiment have been confirmed to a remarkable degree in the present one. For the well-differentiated characteristics, in which the sampling error is relatively small, the agreement was virtually complete.

d) <u>Heterosis in the spermatozoan phenotype</u>

The first objective of this experiment included a search for evidence of heterosis in the spermatozoan phenotype; i.e., of significant deviation of crosses from their mid-parent. The average heterosis of all crosses in comparison with all pure strains can be visualised from the averages at the bottom of Table 9. The significance of this average heterosis is shown by the significance of the "heterosis" mean square in Table 10, but the meaning of average heterosis and of its significance are affected when the "remainder" mean square in the Table is significant. Each cross may also be compared individually with its two parental types, the direction and significance of these individual comparisons being summarised in Table 13. The following picture emerges from these various comparisons. (It should be borne in mind below that the classification of parental types as "larger" or "smaller" is in terms of their spermatozoan characteristics, and not their body weight).

The general analysis of <u>head area</u> appeared to have been particularly successful, in that the highly significant "genome" and "heterosis" effects take out a large proportion of the total variance, and leave a remainder mean square that is scarcely larger than the error mean square. The conclusion is that heterosis in head area is a real phenomenon and is shown to about the same extent by all crosses. Comparison of individual crosses with their parental types is in good agreement with this general conclusion, as might be expected. The head area of the cross is always larger than that of the smaller parental type, and significantly so for five of the crosses. It is larger than the larger parent in five crosses (significantly so for one cross), and smaller only in one cross. All crosses exceed the mid-parental value, and significantly so in four crosses. The C57 x A/C<u>Rg</u> cross seemed to be a particularly clear example of heterosis, since the head area was significantly greater than that of the mid-parent or either parent separately.

For midpiece length also, the general analysis of variance appears to be clear-cut, but the average heterosis, though significant, is not at a high level of significance. The individual comparisons of crosses with their parental types, in Table 13, show that all crosses have a longer midpiece than either the smaller parent or the mid-parent, and three of the crosses have a longer midpiece than the larger parent. The only individual comparison involving any degree of significance is CBA x A/CRe, which is significantly greater than the smaller parent or the mid-parent.

In the general analysis of % normal caps, the significant average heterosis is accompanied by a significant "remainder" mean square, which indicates that individual comparisons might be more interesting. In fact, two of the crosses show a slight non-significant negative heterosis, two a non-significant positive heterosis, while two show a significant positive heterosis, the more striking example being the CBA x C57 cross.

In the general analysis of head breadth, the average heterosis effect is marked as non-significant, but the significant "remainder" mean square indicates once again that the average effect may not be very meaningful, and that individual comparisons of crosses with their parental types might be more valuable. In fact, one cross does show heterosis; the CBA x RIII cross is significantly greater than the smaller parent or the mid-parent, and greater than the larger parent, though not significantly so. The other five crosses show a slight non-significant negative heterosis.

Finally, the general analyses of midpiece area and of % unstained show no significant average heterosis and the non-significant "remainder" mean square indicates that this absence of demonstrable heterosis is consistent. It was concluded that no heterosis in these characteristics could be shown, and individual comparisons of crosses with parents were not carried out.

In summary, the head area provides the main example of heterosis in the spermatozoan phenotype; there is a highly significant average heterosis that is more or less uniform for each cross. The same interpretation, though at a lower level of significance, can be placed on heterosis in midpiece length. In % normal caps, and head breadth, the concept of an average heterosis over the whole experiment has little meaning; the demonstrable heterosis is confined to a few particular crosses. No heterosis could be shown in midpiece area and in % unstained.

e) Analysis of variance of variance

In an attempt to investigate the variance of variance, the pooled within-preparation variance for each litter was treated as a normal variate and subjected to an analysis of variance, with partition into "between ten genetic groups" and "between litters within groups (error)". A similar analysis was performed on the pooled "between male" variance for each litter. These approximate analyses showed no trace of any significant differences in variance among the ten genetic groups. It was felt that further analysis was unlikely to be profitable, and that detailed examination of the variance of variance would require an experiment of different design. However, the negative results did suggest that variance was more or less homogeneous between the genetic groups at two levels of the sampling structure, thus supporting an earlier assumption. (p. 40).

4. DISCUSSION

The main advances derived from the present study are as follows. The previous work (Chapter 1), was conducted with inbred strains only, whereas the present work brings in F_1 crosses as well. Differences between the males from the various strains and crosses can largely be accounted for in terms of the genetic relationships between the males. Heterosis in the visible spermatozoan phenotype has been demonstrated for the first time.

a) Average genetic effects

The overall differences among the ten group means (four pure strains and six crosses) are highly significant for all spermatozoan characteristics except midpiece area. When this significant variation between group means is partitioned according to the genetic relationships between the ten groups, all or nearly all of it can be accounted for in terms of two factors: firstly, by a "general combining ability" for each of the four parental genomes involved, measured by the "genomes effect", and secondly, by a "heterosis effect" differentiating the spermatozoa of crosses from those of pure strains. The "remainder" variance after the partition is often little greater than the sampling error that underlies the whole experiment, and is estimated by differences among litter means within groups. This remainder is small and non-significant for four spermatozoan characteristics (head area, midpiece area and length, and percentage of unstained spermatozoa). It is small (though just formally significant) for head breadth, and only for the percentage of spermatozoa with normal caps does it attain a higher level of significance.

A general picture of the results is, therefore, that the inheritance of genetic factors affecting the spermatozoan phenotype can be explained fairly successfully in terms of average effects of the parental genotype and an average effect of heterosis. There is undoubtedly a large element of "general combining ability" among the four parental types. This general picture is subject to discrepancies associated with particular crosses and particular spermatozoan characteristics, described below.

b) Differences in the spermatozoan characteristics of pure strains only

In Chapter I, differences in the spermatozoa of eight inbred strains were reported. The re-investigation of four of these strains in the present work showed virtually complete repeatability of the previous results. For the characteristics of head area, head breadth, and midpiece length, in which sampling error is relatively small, it was found that all differences between means of strains in the first experiment were of the same sign (positive or negative) as in the present experiment, and the levels of significance testing the reality of these differences were identical. Even with characteristics subject to a higher degree of sampling error (midpiece area, % unstained, and % normal caps) the agreement was good.

This agreement between the two experiments is of importance because, however carefully sampling and analysis are carried out, the real test of the validity of the conclusions lies in confirmation by repetition. The agreement also supports the argument (p. 31) that average differences can be shown to have a real existence even if the magnitude of the difference is of the same order as, or even smaller than the nominal resolving power of the microscope.

c) Heterosis

Heterosis is defined in this work as the deviation of the cross from their mid-parent. The overall analysis of differences among the ten genetic groups shows a highly significant and consistent heterosis displayed by all crosses for head area, and also significant and apparently consistent heterosis in midpiece length. For head breadth and % normal caps, the average heterosis over all crosses had little meaning; certain crosses displayed heterosis, others did not. Midpiece area and % unstained showed no heterosis.

The main interest of the findings is that heterosis in the spermatozoan phenotype has been demonstrated for the first time. Detailed figures are available from the data in Table 9; for instance, the % normal caps in the CBA x C57 cross is 73.4 and in the mid-parent is 64.9, while the average head area over all crosses is about 3% greater than the average of the four pure strains. There is at present no objective basis for saying whether heterosis of this order of magnitude is "large" or "small". To take an example from domestic animals, where the main criterion is economic, a 3% rise in the weight of fowl eggs might be of no advantage, whereas a 3% rise in the number of eggs might be of considerable importance.

It must be pointed out that the heterosis reported in the present study refers to untransformed dimensions, and angularly transformed percentages. The magnitude of heterosis, and even its sign, vary according to the scale of measurement. In the most extreme example, however, the C57 x A/CRe cross, the mean head area was significantly larger than in either parent, and no rational scale transformation could possibly make it smaller than the larger parent, or the mid-parent.

As it happened, all significant heterosis in the present work was positive in sign. The concept of hybrid vigour, however, attaches a positive sign to deviations in the direction of increased fitness. With spermatozoa, the fitness is presumably summed up by the potentiality of the spermatozoa to reach and fertilize an egg normally. The dimensional characteristics of spermatozoa have no known relation to fertility (though some real relationship may well exist), and one can not yet say if the increase in dimensions encountered in the crosses represents a positive or negative hybrid vigour; in short, one does not know at present if a spermatozoon is the better for being large or for being small. But one can speak of positive hybrid vigour in the % normal caps, because semen containing a high percentage of spermatozoa with normal caps must presumably be more fertile than semen with a smaller percentage.

Table 8 shows that there is an obvious heterosis in body weight (except in one cross) in the same direction as the heterosis in spermatozoan characteristics. The point, however, was not pursued further in view of the fact that the body weight is subject to the influence of several factors such as age, litter-size and a number of other pre-natal and post-natal conditions. The effects of these factors could not be conveniently assessed in the present experiment. It may, however, be recalled that extensive observations by Robertson (1958) on Drosophila melanogaster have shown that inbreeding reduces body size mainly by reduction in cell size and the present work perhaps provides a parallel, in so far as the smaller body size in the inbred strains, as indicated by the lower weight, is accompanied by smaller cell (spermatozoan) size.

d) The constancy of the midpiece area

Attention was drawn earlier (p.19) to the fact that the midpiece length and breadth varied between inbred strains with a high degree of significance, whereas the midpiece area did not, and the variance component for strain differences in area was zero. This was supported by the present work, which again showed significant strain differences in midpiece length (the breadth was not studied), while there was no firm evidence for strain differences in midpiece area, though the possibility of small real differences can not be wholly excluded. The present work also showed no heterosis in midpiece area.

The conclusion is that the midpiece area seems to be relatively resistant to genetic variation, and in this respect is unlike all the other characteristics. Genetic variation in the material so far studied affects only the length and/or breadth, and it is, therefore, the shape rather than the size of the midpiece that is subject to genetic variation. It will be realised that all this work is two-dimensional, with size and shape judged from the spermatozoa as seen in optical projection. No data are available concerning variation in the dimension at right angles to the plane of projection.

e) <u>The mode of gene action on spermatozoa</u>

The variance has so far been used merely as a part of the analytical process in order to assess comparison of <u>means</u>. Variation in the variance itself is, however, of interest. <u>A priori</u>, two opposed tendencies might be expected to affect the variance of variance. First, the well-known genetical thesis that F_1 hybrids are more uniform in their characteristics than inbred strains might lead one to expect a decreased variance in the crosses. But, on the other hand, the spermatozoa of an inbred strain are alike genetically, whereas those of crosses are diverse as a result of segregation of genes at the male meiosis. Hence, if gene action were exerted on the developing spermatozoa in its haploid state after the reduction division, the variance in the crosses might be increased.

Analysis of the pooled within-preparation variance showed no trace of significant differences among the ten genetic groups. However, the conclusion was that the experiment was inadequate for studying this particular problem.

f) Living spermatozoa

For reasons stated in Chapter 1 (General Introduction and p. 31), most of the work on the genetics of spermatozoa has been conducted with permanent nigrosin-eosin preparations in which the spermatozoa are no longer alive. It is important to know whether or not comparisons made from these preparations agree with those made from observations on living spermatozoa. The available results are encouraging. The narrowness of the C57 spermatozoan head in comparison with that of CBA was suggested by the preliminary results of Braden (1956), has been confirmed in the first part of the present work with both living and dead spermatozoa (p.24, and Table 7), and is again confirmed with dead spermatozoa in the second part (p. 42).

A new paper by Braden (1959) contains information about the morphology of the spermatozoa of mice. The data for dimensions of spermatozoa can not be analysed formally, because the sampling is not fully specified, but suggest that C57 spermatozoan heads are narrower not only than those of CBA but also of RIII and A. These three comparisons agree with the results obtained in the first experiment (Table 6), and two of them are again confirmed from nigrosin-eosin preparations in the present work (Table 11). Other comparisons of pure strains available from Braden's data seem to be overshadowed by sampling error and would require neither confirmation nor denial.

5. SUMMARY

1. A quantitative study has been made of the characteristics of mouse spermatozoa as seen in optical projection in permanent nigrosin-eosin preparations. There were ten genetic groups, comprising four inbred strains and the six possible crosses (reciprocal crosses were not studied).

2. The characteristics studied were the area and breadth of the spermatozoan head, the area and length of the midpiece, the percentage of unstained spermatozoa, and the percentage of heads with normal acrosomal caps. Significant differences were found among the ten groups for all characteristics except midpiece area.

3. Significant variation among the ten groups could, in general, be accounted for largely in terms of the genetic relationships between the groups, on the simple quantitative model that there is a "genomes effect" attributable to the four types of parental genome involved, and an average "heterosis effect" differentiating crosses from pure strains.

4. The clearest evidence of average heterosis was found in the head area and, to a lesser extent, in the midpiece length. With the percentage of spermatozoan heads bearing normal acrosomal caps, and with the head breadth, the heterosis was exhibited only by particular crosses. There was no demonstrable heterosis in the midpiece area or in the percentage of unstained spermatozoa. All significant heterosis was positive, in the sense that F_1 crosses had larger spermatozoan dimensions, and a higher percentage of spermatozoa with normal acrosomal caps.

5. The results of two independent series of observations on the spermatozoan characteristics of four inbred strains were in good agreement.

6. Comparisons of the spermatozoan characteristics from living spermatozoa and from spermatozoa in nigrosin-eosin preparations are so far in agreement.

GENERAL SUMMARY AND CONCLUSIONS

The study of genetic effects on the characteristics of gametes has been called the genetics of gametes. The genetics of gametes can be regarded as the study of the genetics of the carriers of genes from one generation to the next; this has relevance to quantitative genetics in general, and is particularly relevant to experiments for controlling the transmission of hereditary factors from the parent to the offspring.

As evidence of such genetic effects, numerous differences have been found in the characteristics of spermatozoa from eight inbred strains of mice, and have been confirmed in a separate experiment with four of the strains. The spermatozoan characteristics studied are mainly dimensional, but others relate to semen fertility and to the physiological maturation of spermatozoa. The dimensional characteristics of spermatozoa are insulated to an unusual degree from the effects of environmental and other factors. The characteristics of head breadth and area, and of midpiece length, which show highly significant strain differences, appear to be particularly well suited to quantitative study of the genetics of the phenotype of gametes. Comparison of related strains suggests that the spermatozoan characteristics of mice are not affected by an agouti-locus allele but are possibly affected by alleles at the albino-locus. The variation in the spermatozoan phenotype between first generation crosses made from the pure strains can largely be accounted for in terms of an average of the values of the characteristics of the parental types, together with an effect of heterosis. The phenomenon of heterosis in spermatozoa, which is nearly but not quite the same as "hybrid vigour", appears to have been demonstrated for the first time.

Maternal and cytoplasmic effects on spermatozoa have not been studied. They require future examination not only to amplify understanding of the genetics of spermatozoa, but because spermatozoa are in some ways peculiarly adapted to quantitative genetic study, and might form useful material for the investigation of such effects <u>per se</u>.

As a field of study, the genetics of gametes is at present in a preliminary phase concerned largely with building up a body of fact, and with developing the necessary techniques for establishing those facts. Until this phase is further advanced, it would seem inappropriate to consider in too great detail the possible implications for general genetics. However, if it should be demonstrated that more than one or two genes affect the phenotype of gametes bearing them, it might become necessary to reconsider a basic postulate of quantitative genetics, that the chances of a given ovum being fertilised by a given spermatozoon are unaffected by their genic content. Further, if spermatozoa should be affected by their own genes, it might become possible to separate spermatozoa according to their genic content, and thus control the process of transmission of hereditary characteristics from one generation to the next. Possibilities of this kind are long-term aims that form stimuli for undertaking research on the genetics of gametes.

Some general conclusions in the field, with the contributions of other workers taken into account, as well as the particular results of the present work, are as follows. Firstly, there is now a good deal of evidence of genetic effects on the morphological and also the behavioural phenotype of gametes, and a beginning has been made to distinguishing effects due to the genome of the male soma from those attributable to the genic content of the gametes themselves. Secondly, there is evidence of heritable variation affecting the viability of spermatozoa, and relevant, therefore, to the general subject of male fertility. Thirdly, experiments purporting to control sex ratio by separating X- and Y- bearing spermatozoa have already been reported; if these can be confirmed, they will have shown that the two kinds of spermatozoa differ phenotypically in accordance with their chromosomal content.

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TABLE 1.

Sampling of the eight strains.

Strain	No. of litters	Means of 13 males per strain.	
		Age, days	Weight, gms.
V	13	142	33.2
A	11	182	26.7
A/C*Re*	12	156	27.8
CBA	10	153	29.8
CBA/*a*	13	139	32.2
KL	4	173	30.1
RIII	13	160	27.9
C57	10	213	26.8
Grand means		165	29.3

Means of litter means in place of means of male means gave only negligible changes in the ages and weights.

(Legend to Table 2 : Analyses of variance.)

Individual bases of each analysis (described on p. 18) are (a) 1 spermatozoon in the mensuration data, and (b) 1 angularly transformed percentage derived from 100 spermatozoa per male.

Symbols (sources of variation) stand for (see pp. 15 & 16) :-
I inbred strains; L(I) litters-in-strains; M(I) males-in-strains;
M(L) males-in-litters; P(M) preparations-in-males;
S(P) spermatozoa-in-preparations.

Significance levels are :
* $0.05 > P > 0.025$
** $0.025 > P > 0.01$
*** $0.01 > P > 0.005$
**** $0.005 > P$

The indicated levels of significance of the inbred strains effect (I) are based on comparison with the M(I) effect, assuming that no real L(I) effect exists. If the L(I) effect is assumed to be real, the test of significance for the I effect involves unequal numbers in subclasses, and combinations of the I, L(I) and M(L) mean squares (method of Satterthwaite and Cochran, as in Snedecor 1956). When this test is used, the significance marked in the Table for the I sources of variation is modified as follows : for midpiece breadth and globule end and migration positions, the I effect is reduced to non-significance ; the remaining tests of the I effect have unaltered significance levels, except for a reduction in the significance level of the I effects among percentage data.

In percentage data, the error term for testing the significance of the M(I) and M(L) effects is the theoretical variance of the angular percentage (8.21).

Table 2: Analyses of Variance.

Column reference	a	b	c	d	e	f
Source of variation	I	L(I)	M(I)	M(L)	P(M)	S(P)
d.f.	7	78	96	18	104	208
Key to mean squares for variance ratios testing significance of source of variation.	a,c1)	b,d	c,e2)	d,e2)	e,f	–
MENSURATION DATA						
Head breadth, μ	1.4094****	0.0500	0.0459	0.0280	0.0341	0.0310
Head area, μ^2	89.54****	3.73	3.56	2.84	2.97	2.60
Midpiece length A, μ	14.160****	0.637	0.586	0.363	0.485	0.380
Midpiece length B, μ	11.915****	0.519	0.471	0.262	0.378	0.385
Midpiece area, μ^2	4.38	4.41	4.49	4.86	3.96****	2.10
Midpiece breadth, μ	0.02686***	0.00967	0.00931	0.00776	0.00707****	0.00358
Globule end position, μ	26.52**	10.21	9.99	9.07	10.32*	7.43
Globule migration position	0.0504**	0.0207	0.0202	0.0182	0.0209**	0.0146
PERCENTAGE DATA AS ANGULAR PERCENTAGES						
Unstained	306.0****	76.4	70.7****	46.2****	–	–
Normal cap A	315.8****	101.0*	91.1****	48.0****	–	–
Normal cap B	198.7****	67.0	62.2****	41.1****	–	–
With a globule	84.7	47.7	44.0****	28.1****	–	–

TABLE 3.

Variance component structure of analyses of variance.

Effect	Coefficients of variance components in expectations of mean squares.				
	$\sigma_{S(P)}^2$	$\sigma_{P(M)}^2$	$\sigma_{M(L)}^2$	$\sigma_{L(I)}^2$	σ_I^2
I	1	5	10	14.808	130
L(I)	1	5	10	11.815	
M(L)	1	5	10		
P(M)	1	5			
S(P)	1				

For mensuration data : as in the table. For percentage data : delete all reference to effects and components for P(M) and S(P) , and divide remaining coefficients by 10. The status of $\sigma_{M(L)}^2$ in percentage data is not the same as in mensuration data (see legend to Table 4).

(For explanation of the symbols see pp. 16 and 17.)

TABLE 4 : Components of variance.

Spermatozoon characteristic	Total computed variance	Components as %-age of total computed variance				
		σ_{I}^{2}	$\sigma_{L(I)}^{2}$	$\sigma_{M(L)}^{2}$	$\sigma_{P(M)}^{2}$	$\sigma_{S(P)}^{2}$
<u>Mensuration data</u>						
Head breadth, μ	0.0433	24^{****}	4	-1	1	72
Head area, μ^{2}	3.39	19^{****}	2	0	2	77
Midpiece length A, μ	0.515	20^{****}	5	-2	4	74
Midpiece length B, μ	0.481	18^{****}	5	-2	0	80
Midpiece area, μ^{2}	2.52	0	-1	4	15^{****}	83
Midpiece breadth, μ	0.00463	3^{***}	3	1	15^{****}	77
Globule end position, μ	8.10	2^{**}	1	-2	7^{*}	92
Globule migration position	0.0161	1^{**}	1	-2	8^{**}	91
<u>Percentage data, as angular percentages</u>						
Unstained heads	88.8	19^{****}	29	52^{****}	-	-
Normal caps A	108.4	14^{****}	41^{*}	44^{****}	-	-
Normal caps B	72.7	13^{****}	30	57^{****}	-	-
With globule	47.2	5	35	60^{****}	-	-

Significance levels are repeated from Table 2. Total computed variance is the sum of the actual variance components for five effects in the mensuration data and for three effects in the percentage data. $\sigma_{M(L)}^{2}$ is not of the same status in the percentage data as in the mensuration data ; in percentage data, no P(M) variance was estimated, and $\sigma_{M(L)}^{2}$ therefore contains some variance due to the P(M) effect and to the theoretical variance of an angular percentage. The Table is calculable from the information in Tables 2 and 3.

Table 5: Strain averages of all spermatozoon characteristics

	Mensuration data								Percentage data, in angular transformation			
Strain	Head breadth in μ	Head area in μ^2	Midpiece length A, in μ	Midpiece length B, in μ	Midpiece area in μ^2	Midpiece breadth in μ	Globule end position in μ	Globule migration position	Unstained	Normal Cap A	Normal Cap B	With globu
V	3.70	22.7	22.5	22.6	18.0	0.798	4.82	0.788	37.6	59.5	58.8	29.4
A	3.96	24.5	22.3	22.4	18.4	0.827	3.64	0.838	31.8	48.7	50.9	26.2
A/C<u>Re</u>	3.81	24.1	22.5	22.4	18.4	0.822	3.72	0.835	30.7	50.8	52.9	25.2
CBA	3.80	24.3	21.7	21.8	18.0	0.832	3.55	0.837	28.7	58.6	58.3	31.2
CBA/<u>a</u>	3.83	24.3	21.6	21.9	18.3	0.849	4.26	0.806	27.0	53.9	56.8	28.1
KL	3.84	24.1	22.0	22.1	18.2	0.830	4.52	0.796	22.6	48.1	49.4	25.9
RIII	3.78	23.3	22.1	22.2	18.1	0.818	4.10	0.815	35.2	53.2	56.4	23.0
C57	3.60	22.4	22.3	22.5	18.3	0.819	3.84	0.829	26.7	45.7	49.6	26.7
S.E. for each strain mean	± 0.0188	± 0.166	± 0.0671	± 0.0602	± 0.186	± 0.00846	± 0.277	± 0.0125	± 2.33	± 2.65	± 2.19	± 1.8
Significance of differences among the 8 strain means	****	****	****	****	N.S.	***	**	**	****	****	****	N.S

The averages are means of male means. Standard errors, each for 96 d.f., are based on the M(I) mean squares in Table 2. Significance levels are repeated from column <u>a</u> of Table 2. Strain averages based on means of litter means varied only negligibly from those shown above.

(Legend to TABLE 6.)

The Table, constructed from the data of Table 5, compares strain means of male means by the procedure described on p. 21. All significant comparisons are listed, all non-significant comparisons omitted. The characteristics " % with a globule " and " midpiece area " do not appear, since no overall significant differences among means of strains were found in the analysis of variance (Table 2).

Modification if the L(I) effect is assumed real. An approximation is to regard comparisons between columns (a) and (c) as non-significant, and to judge comparisons between columns (a) and (b) as significant at the < 0.05 instead of the < 0.01 level. A more exact modification is : (1) all comparisons between columns (a) and (c), and all comparisons involving midpiece breadth, globule end position, and globule migration position, are to be considered non-significant ; (2) in what remains of columns (a) and (b) consider the following comparisons non-significant :

head breadth, RIII > V .
head area KL > RIII , RIII > V .
midpiece length KL > CBA , A > KL .
% unstained V > C57 , RIII > KL .
% normal caps V > KL .

(3) all comparisons now remaining in columns (a) and (b) are significant at the $P = < 0.05$ level.

TABLE 6 : List of all significant comparisons among means of strains.

Spermatozoan characteristic	Strain with means of spermatozoan characceristics each larger than and significantly different from those in columns (b) and (c)	Strains with means of spermatozoan characterisitics each smaller than and significantly different from those in column (a), classified by significance level of the difference	
		P = <0.01	P= 0.05-0.01
	(a)	(b)	(c)
Mensuration data			
Head breadth	A	V A/CRe CBA CBA/a KL RIII C57	-
	A/CRe CBA CBA/a KL RIII	V C57	-
	V	C57	-
Head area	A A/CRe CBA CBA/a KL	V RIII C57	-
	RIII	V C57	-
Midpiece length	V A/CRe	CBA CBA/a KL RIII	-
	V	-	A
	C57	-	RIII
	A C57	CBA CBA/a KL	-
	RIII	CBA CBA/a	-
	KL	CBA	-
Midpiece breadth	CBA/a	V	A/CRe RIII C57
	A CBA KL	-	V
Globule end position	V	A CBA	A/CRe C57
	KL	-	CBA
Globule migration position	A CBA	-	V KL
	A/CRe C57	-	V
Percentage data, in angular transformation			
Unstained	V	CBA/a KL C57	CBA
	RIII	KL	CBA/a C57
	A A/CRe	-	KL
Normal cap	V	KL C57	A
	CBA	-	A KL C57

TABLE 7.

Summary of outstanding characteristics of spermatozoa from different strains.

Strain	Head breadth	Head area	Midpiece length	percentage of unstained heads	percentage with normal caps
V	narrow	small	long	high	high
A	wide	large			
CBA		Large	Short		High
CBA/<u>a</u>			Short		
RIII				High	
C57	Narrow	Small			

Strains A/C<u>Re</u> and KL have no demonstrably outstanding characteristics. The Table, based on strain means of male means, is derived from Tables 5 and 6. If the litters-in-strains effect is assumed to be real, the two right hand columns are to be deleted.

TABLE 8.

Sampling of the ten genetic groups.

Group	Mean weight, gms.	Mean age, days.
Pure strains		
CBA	25.8	116
A/C*Re*	26.9	132
RIII	25.8	112
C57	23.3	113
F_1 crosses		
CBA x RIII	30.0	127
CBA x C57	32.2	113
CBA x A/C*Re*	29.0	105
RIII x C57	30.5	114
RIII x A/C*Re*	25.6	92
C57 x A/C*Re*	29.9	112
Grand mean	27.9	114

There are 5 litters per genetic group, and 2 males per litter. In each F_1 cross, the first strain symbol is that of the female parent.

TABLE 9 : Group averages of all spermatozoan characteristics.

Standard errors, each on 40 d.f., were calculated from the differences between litter means within groups.

Groups	Mensuration data				Percentage data in angular transformation	
	Head area^{2}	Head breadth	Midpiece area^{2}	Midpiece length	% Unstained	% Normal cap
CBA	23.8	3.66	18.1	21.8	26.3	70.0
A/CRe	23.2	3.68	18.6	22.2	34.5	66.6
RIII	22.9	3.58	18.1	22.2	41.2	72.6
C57	22.2	3.43	18.5	22.4	32.8	59.8
CBA x RIII	23.9	3.71	18.1	22.1	28.3	71.0
CBA x C57	23.7	3.53	18.5	22.2	26.3	73.4
CBA x A/CRe	24.2	3.66	18.7	22.4	28.2	71.8
RIII x C57	23.1	3.49	18.6	22.4	29.8	69.6
RIII x A/CRe	23.4	3.62	18.1	22.2	37.9	67.2
C57 x A/CRe	23.9	3.49	19.3	22.6	29.9	69.2
S.E. for each group mean	± 0.1999	±0.02752	±0.2720	±0.1374	±2.845	±1.868
Means of all pure strains	23.0	3.59	18.3	22.1	33.7	67.2
Means of all crosses	23.7	3.58	18.5	22.3	30.1	70.4

TABLE 10: Analyses of Variance

The individual basis of the analyses is the group mean, as in Table 9.
The error mean square is the square of the corresponding S.E. given in Table 9.

Sources of variation	d.f.	Spermatozoan characteristics, mean squares, and significance levels.					
		Head area in ^{2}	Head breadth in	Midpiece area in ^{2}	Midpiece length in	Angular % unstained	Angular % Normal cap
Genetic groups	9	.3643****	.009052****	.1468	.0531**	25.19****	15.45****
Genomes	3	.8648****	.023895****	.2570**	.0946****	56.76****	20.84**
Heterosis	1	.3804****	.000084	.1103	.0934*	31.39	23.31**
Remainder	5	.0610	.001942*	.0887	.0202	5.02	10.64**
Litters within genetic groups (error)	40	.0400	.000758	.0740	.0189	8.09	3.49

Key to significance levels - as in Table 2.

TABLE 11.

List of all significant comparisons among the means of the pure strains.

Spermatozoan characteristics	Strains with means of spermatozoan characteristics each larger than and significantly different ($p < .05$) from those in (b). (a)	Strains with means of spermatozoan characteristics each smaller than and significantly different ($P < .05$) from those in (a). (b)
Head area	CBA	A/CRe, RIII, C57
	A/CRe	C57
Head breadth	A/CRe	RIII, C57
	CBA, RIII	C57
Midpiece length	C57, A/CRe, RIII	CBA
% Unstained	RIII	CBA
% Normal caps	RIII	A/CRe, C57
	CBA, A/CRe	C57

All significant comparisons are included, all non-significant comparisons omitted. Midpiece area is omitted, since no over-all significant differences among group means were found in the analysis of variance (Table 10).

Method: As in Duncan (1955), with the means of pure strains and the standard error per mean (on 40 d.f. from the whole experiment) taken from Table 9.

TABLE 12.

Comarison of RANK ORDER of pure strains in the first experiment and the present one, and the CORRELATION between the two experiments.

Spermatozoan characteristics	First experiment	Present experiment	Correlation co-efficient on 2 d.f.
Head area	CBA A/CRe RIII C57	CBA A/CRe RIII C57	+ .937
Head breadth	A/CRe CBA RIII C57	A/CRe CBA RIII C57	+ .971*
Midpiece area	A/CRe C57 RIII CBA	A/CRe C57 CBA RIII	+ .950(*)
Midpiece length	C57 A/CRe RIII CBA	C57 A/CRe RIII CBA	+ .946
% Unstained	RIII A/CRe CBA C57	RIII A/CRe C57 CBA	+ .784
% Normal caps	CBA RIII A/CRe C57	RIII CBA A/CRe C57	+ .860

The strains with larger values of spermatozoan characteristics are shown on the left of each rank.

The rank order in the first experiment is from Table 5 and was based upon means of male means ; means of litter means from the original data of that experiment were found to give the same rank order. The 'A' and 'B' series for midpiece length and for % normal caps in the previous experiment were averaged.

The correlations are between the strain means in Table 9 of the present experiment and the corresponding means in Table 5 of the previous one.

Significance levels : (*) $P = 0.05$

* $0.05 > P > 0.025$

TABLE 13 : Comparisons of Crosses with Parents and Mid-parents.

Midpiece area and % unstained are omitted because of the absence of overall significance for the "heterosis" items in Table 10. The Table is constructed from the data in Table 9 using the following standard errors each on 40 d.f.

S.E. for 'a' and 'b' comparisons = $(\sqrt{2})$ (S.E. in Table 9)
S.E. for 'c' comparison = (0.8666) $(\sqrt{2})$ (S.E. in Table 9),
where 0.8666 is the reciprocal of the square root of the harmonic mean of 1 and 2 (because each cross is 1 genetic group whereas the mid-parent value is the mean of 2 genetic groups.).

Cross	Comparison (i)	Head area	Head breadth	Midpiece length	% Normal caps
CBA x RIII	a	+ < .001	+ .005-.001	+	+
	b	+	+	-	-
	c	+ .05-.025	+ .01- .005	+	-
CBA x C57	a	+ ≪ .001	+ .025-.01	+	+ ≪ .001
	b	-	- .005-.001	-	+
	c	+ .025-.01	-	+	+ < .001
CBA x A/C<u>Re</u>	a	+ < .001	-	+.005-.001	+
	b	+	-	+	+
	c	+ .005	-	+ .025-.01	+
RIII x C57	a	+ .025-.01	+	+	+ < .001
	b	+	- .025-.01	+	-
	c	+	-	+	+
RIIIx A/C<u>Re</u>	a	+	+	+	+
	b	+	-	-	- .05-.025
	c	+	-	+	-
C57 x A/C<u>Re</u>	a	+ ≪ .001	+	+	+ .001
	b	+ .025-.01	- ≪ .001	+	+
	c	+ ≪ .001	-	+	+ .025-.01

(i) a = (cross) - (smaller parent)
b = (cross) - (larger parent)
c = (cross) - (mid-parent).
By 'smaller' and 'larger' parents are meant the parents with the smaller or larger value of the spermatozoan characteristic in question.

In the body of the Table the '+' and '-' signs show if the comparison is positive or negative, while the figures (values of P) indicate that the comparison is significant and state the level of significance.