Genet. Res., Camb. (1963), **4**, *pp.* 446–456
With 1 *text-figure*
Printed in Great Britain

Heterogeneity among spermatogonia of *Drosophila melanogaster* in sensitivity to X-rays

By H. Slizynska
Institute of Animal Genetics, Edinburgh, 9

(*Received* 31 *May* 1963)

1. INTRODUCTION

In many experiments, both in *Drosophila* (Ward & Alexander, 1957; Alexander, 1960; Chandley & Bateman, 1960; McCarthy & Nafei, 1963), and mice (Hertwig, 1940; Russell, 1954), no translocations were found in irradiated spermatogonia. Contrary findings are, however, not infrequent; Catsch & Radu (1943) and Savhagen (1960) in *Drosophila*, and Griffen (1958) in mice, produced some translocations by irradiation of spermatogonia.

The absence of translocations in premeiotic cells is often attributed to the losses during meiosis. It should be remembered, however, that although about half of the translocations are lost in meiosis, the chances for forming a translocation are about twice as high in diploid spermatogonial cells as in haploid post-meiotic germ-cells; this should to a large degree compensate for the losses occurring during meiosis. The rarity or absence of translocations in premeiotic stages remains, therefore, unexplained. Muller (1954) discusses the possible causes for it.

In all previous work, translocations were scored by genetical methods. It was thought that a cytological analysis might throw some light on the question. Such an analysis, covering all stages from spermatozoa to spermatogonia and scoring all types of large structural changes, is presented below.

As a basis for comparison with genetically scored translocations, a repetition of the older work on a large scale, with the now available refined brood-techniques and covering sex-chromosomes as well as the large autosomes, was considered necessary. Such a test was carried out by Mr McCarthy and Mr Nafei in this Institute, and its results are presented in this volume (McCarthy & Nafei, 1963).

The cytological analysis of structural changes induced by X-rays in early stages of spermatogenesis in *Drosophila melanogaster* has so far been limited to mutations and genetically detected translocations. The present paper gives the results of a salivary gland chromosome analysis of successive broods produced by irradiated fathers. Part of these results, dealing mainly with the proportion of different types of change, with mosaicism, and with the proportion of breaks in the heterochromatic regions of the chromosomes, is being published separately in a paper comparing the effects of formaldehyde food and X-rays (Slizynska, 1963*b*). The present paper is concerned with the response to X-rays of different types of germ-cells.

2. MATERIAL AND METHODS

Two-days old *y w* males of *Drosophila melanogaster* were irradiated with 2500 r. On the next day, single males were mated to 3 *Ore-K/M-5* females. Every 4 days each male was supplied with 3 fresh females of the same type; in this way four broods were obtained, covering the period from 2 to 17 days after irradiation. F_1 females of the constitution *y w/M-5*, recognizable as larvae by the white Malpighian tubules, were used for sex-linked lethal tests; the cytological analysis was done on *y w/Ore-K* F_1 female larvae. Parental males that failed to produce progeny in any one brood were discarded in order to avoid, as far as possible, the storing of spermatozoa and to minimize the overlapping of broods. The number of examined F_1 individuals from treated males ranged from 1 to 14.

In each brood 500–600 treated X-chromosomes were tested for sex-linked lethals. In the cytological analysis the whole complement of the chromosomes was examined, except the Y-chromosome. The following types of change were scored: translocations (T), inversions (In), deficiencies (Df), repeats (Rp), duplications (Dp) and loss of chromosome 4.

3. RESULTS

For an evaluation of data on brood-patterns it is necessary to correlate each brood with a treated germ-cell stage. This is always difficult. Auerbach (1954), using induced crossing-over in irradiated males for the identification of treated cell stages, considers samples of spermatozoa collected 10–12 days after irradiation (3 days broods, 3 females per male) as representing spermatogonial stages. She emphasized, however, that this conclusion applies only to the strains and breeding methods used by her in these experiments, and that the rate of sperm utilization, and with it the brood pattern, may vary strongly between experiments. Other authors (Sobels & Van Steenis, 1957; Chandley & Bateman, 1960), applying similar methods, reached the same conclusion. Savhagen (1961*a*, *b*), who identified cell stages with the aid of both crossing-over and non-disjunction, concluded that spermatozoa used 7–8 days after irradiation represent cells treated in early meiosis, while later broods (12–14 and 14–17 days) originate mainly from spermatogonial cells. Though these authors did not all use the same brood technique or the same strains, their results agree fairly well.

With the slow breeding method (3 females per male every 4 days) used in the present experiment cells treated as spermatogonia are probably represented mainly by brood *d* (14–17 days after treatment). This agrees with the fact that lethal frequency in brood *d* was below that found in spermatozoa (Table 1, item 3).

Additional support for attributing brood *d* to treated spermatogonia comes from one particular case which, because of its bearing on this crucial point, will be described in some detail. One male in brood *d* had 5 normal daughters and 2 daughters which carried the same Rp of the type abcd/cdef in the left arm of the

third chromosome. Now, there are good reasons for attributing the origin of a Rp to a sister chromatid exchange, giving rise to a Rp in one chromatid and a Df in the other (Slizynska, 1963*a*). If such an exchange occurs in a spermatocyte, it will yield one spermatozoon with a Rp and one with a Df. The presence of the same Rp in two spermatozoa requires an additional division. This puts the origin of the observed case in brood *d* into the last spermatogonial division or even earlier. Consequently, it may be accepted that broods *a* and *d* derive mainly from treated spermatozoa and spermatogonia respectively, and broods *b* and *c* from intermediate stages, namely spermatids and spermatocytes.

The data will be presented and analysed at three different levels.

(i) The proportion, in each brood, of males yielding at least one structural change in their tested progeny. These males will be called 'positive', while males without any change in their progeny will be called 'negative'.

(ii) The proportion of 'sensitive' germ-cells per positive male, the term 'sensitive' being applied to any germ-cell that developed into a spermatozoon with at least one structural change. It is true that the implied 1:1 correlation between treated cell and tested spermatozoon will be lowered by the formation of bunches from treated spermatogonia, but the amount of bunching in the present experiment was very low.

(iii) The overall genetic damage as measured by break frequency, and the distribution of breaks in the sensitive cells.

(i) *The brood pattern of positive males* (*Table 1, items 6 and 7*)

It will be realized, that the significance of this distribution rests on the number of offspring, which in the present case varied from 1 to 14.

The proportion of positive males in broods *a* and *b* is high and depends directly on the number of progeny tested (Table 1, items 6 and 7). When males which had a small number of tested offspring (less than 4) are omitted, the proportion of positive males increases from about 54% to over 80%. It seems reasonable to conclude that, with sufficiently large samples of tested progeny, all males would behave as positive in these broods. The situation is different in brood *d*: the proportion of positive males is much smaller (about 20%) and does not increase when males with small progeny samples are omitted. Brood *c* occupies an intermediate position between broods *b* and *d*.

These results indicate that, in regard to spermatozoa and spermatids, most or all males are sensitive to X-rays; the population of treated males is homogeneous in this respect. In regard to spermatogonia on the other hand, the males represent a heterogeneous population consisting of positive and negative males; the latter do not have chromosomal changes even among larger numbers of tested spermatogonia.

The heterogeneity between males was tested by Dr B. Woolf (see Appendix). For the purpose of clarity, the relevant data are collected in Table 2.

Comparison of proportions of changed cells with proportions of positive males in

the four broods suggests the possibility that in brood *d*, males may be heterogeneous in respect to the response to X-ray treatment.

Table 1. *Results and analysis*

	a	*b*	*c*	*d*
1. Brood				
2. Days after treatment	2–5	6–9	10–13	14–17
OVERALL DATA				
3. Sex-linked lethals (per cent)	8·2	11·5	8·6	5·5
4. Cells tested (no.)	200	200	200	400
5. Cells changed: no.	51	57	43	27
per cent	25·5	28·5	21·5	6·8
ANALYSIS OF DATA				
6. All males analysed: total no.	46	53	44	73
positive*: no.	25	28	22	16
per cent	54·3	52·8	50·0	21·9
7. Males with more than 3 germ-cells examined: total no.	22	23	22	63
positive: no.	18	20	16	13
per cent	81·8	86·9	72·7	20·6
8. Sensitive† cells in positive males (per cent)	33·8	39·9	31·2	33·3
9. Total number of breaks	120	164	114	88
10. Breaks per germ-cell	0·63	0·85	0·57	0·22
11. Cells: no. with 2 breaks	40	35	32	17
no. with > 2 breaks	10	22	10	9
12. Breaks in cells with > 2 breaks (per cent)	33·3	57·3	43·9	60·9
13. Breaks per cell with > 2 breaks	4·0	4·3	5·0	5·9

* Males yielding at least one structural change among their progeny.
† Germ cells carrying at least one structural change.

The alternatives to be tested for are: (1) That the probability, p, that a larve will show changes does not vary from one male to another, or (2) that p does vary from male to male.

In the latter case p will have a probability distribution, with a mean value P, and a variance V about this mean. In the former case, V will be zero. Robertson (1951) described a method for estimating V from a set of binomial sampling data, and of testing the significance of the estimate by comparing it with its standard

Table 2. *Data for a test of heterogeneity between males*

Brood	No. of males	No. of germ-cells tested	Mean cells per male	Cells changed		Positive males	
				no.	%	no.	%
a	46	200	4·35	51	25·5	25	54·0
b	53	200	3·77	57	28·5	28	53·0
c	44	200	4·55	43	21·5	22	50·0
d	73	400	5·48	27	6·75	16	22·0

https://doi.org/10.1017/S0016672300003839 Published online by Cambridge University Press

error. This method was applied to all four broods. The values of t (estimate of V)/(S.E. of V) are given in Table 3.

In the first three cases, t falls well short of statistical significance, and the null hypothesis of a constant value of p is not disproved But in the fourth case, V does differ very significantly from zero. It seems therefore, that p in this brood does vary between males.

Table 3. *Test of heterogeneity between males*

Brood	t for heterogeneity
a	−0·76
b	0·73
c	1·47
d	6·08

The actual estimate of V in this brood is 0·0120–0·0020. Although of course the test does not demonstrate that there are two sharply contrasted kinds of males, some with completely insensitive germ-cells, and some with germ-cells of the same sensitivity as that shown in earlier broods, it is interesting to calculate how the observed value of V could have arisen if this hypothesis were in fact true. It turns out that V would be about 0·012 if 27% of males had 25% germ-cells able to show changes, and 73% of males had no sensitive germ-cells.

(ii) *The brood pattern of sensitive germ-cells*

The third item of Table 1 presents the brood pattern of sex-linked lethals. It shows the usual shape, with a peak in brood *b* and a drop from *c* to *d*. The differences between the first three broods are small, probably because the long brood interval gave rise to considerable overlap. The drop in lethal frequency from *c* to *d* is, however, quite clear. The overall frequencies of germ-cells with structural changes (Table 1, item 5) run roughly parallel to the lethal frequencies, with an even more pronounced drop from *c* to *d*.

An analysis of sensitive cells of positive males (Table 1, item 8) reveals the interesting fact that the positive males in all broods (except *b*) have approximately the same proportion of sensitive germ-cells. Thus, while brood *d* had only 20·6% positive males as compared with 72·7% in brood *c*, in all these males about one-third of the germ-cells carried structural changes. The drop in the overall frequency of sensitive cells from *c* to *d* is, therefore, wholly due to the drop in the proportion of positive males.

(iii) *The distribution of breaks in sensitive cells* (*Table* 1, *items* 9 *to* 13)

The overall radiation sensitivity of the treated germ-cell samples may be expressed as the mean frequency of chromosome breaks per treated cell (Table 1, item 10). Since rearrangements were scored, the lowest frequency is 2. Measured in this way, sensitivity parallels lethal frequency (Table 1, item 3) in being highest in brood *b* and lowest in brood *d*. This picture changes completely when sensitive

germ-cells only are considered (Table 1, items 11 to 13). The ratio of cells carrying multiple breaks (cells with more than two breaks, Table 1, item 11) to cells with only two breaks in spermatogonia is almost as high as in spermatids and much higher than in spermatozoa or spermatocytes.

On the other hand, the average number of breaks per cell carrying multiple breaks increases from spermatozoa, over spermatids and spermatocytes, to spermatogonia (Table 1, item 13). These two factors: the number of cells with more than two breaks and the accumulation of breaks in these cells, determine what proportion of all breaks takes part in multiple break structural changes (Table 1, item 12).

It seems probable that the observed differences between sensitive cells in the accumulation of breaks are underestimates. In salivary gland chromosomes only viable new rejoining can be scored. Since one unviable reunion is sufficient to kill a cell, and since the chance of such reunions increases with the number of breaks in a nucleus, broods *b* and *d*, with more breaks per sensitive cell, must have sustained more losses than broods *a* and *c*. In addition some translocations produced in *d* and probably in *c* will have been eliminated in meiosis.

4. DISCUSSION

The results summarized in Fig. 1 show that different criteria may be used for assessing sensitivity to chromosome breakage by X-rays, and that these result in different rank orders of sensitivity between germ-cell stages.

The results presented in the foregoing chapter show that the overall sensitivity of germ-cells to irradiation is caused by: (1) the proportion of males which responded to X-rays, (ii) the proportion of sensitive cells in those males, and (iii) the degree of sensitivity of cells which respond to treatment. The salient points are illustrated in Fig. 1.

Curve 1 shows the pattern of overall sensitivity, with a slight peak in brood *b* and a marked drop from brood *c* to brood *d*. Comparison with the remaining curves (2 and 3) shows that the drop in brood *d* is wholly due to a drop in the frequency of positive males (curve 2). The peak in *b*, on the other hand, is only partly due to a slightly higher proportion of positive males; increase in the frequencies of sensitive cells is an additional cause. Curve 3 shows that the percentage of sensitive cells is not lower in brood *d* than in *c*, so that the drop in the overall frequency of rearrangements in spermatogonia is not caused by a shortage of sensitive cells in those males that show any response at all (positive males). Curves 4 and 5 show that, on the contrary, the frequency of breaks in sensitive spermatogonia is higher than in any other stage, and that breaks tend to cluster together in some of the spermatogonia.

There is thus no correlation between (*a*) the percentage of males that respond to treatment and the proportion of their germ-cells that show structural changes, and (*b*) the proportion of germ-cells with structural changes per male and the average number of chromosome breaks in these cells.

This is clearly illustrated by a comparison of irradiated spermatozoa (brood *a*) and spermatogonia (brood *d*). Only 21% of the males yielded structural changes in spermatogonia, as compared with 82% that yielded changes in spermatozoa. But in all these males, structural changes were found in about one-third of the sampled germ-cells. Moreover, while in spermatozoa 67% of the chromosome breaks occurred

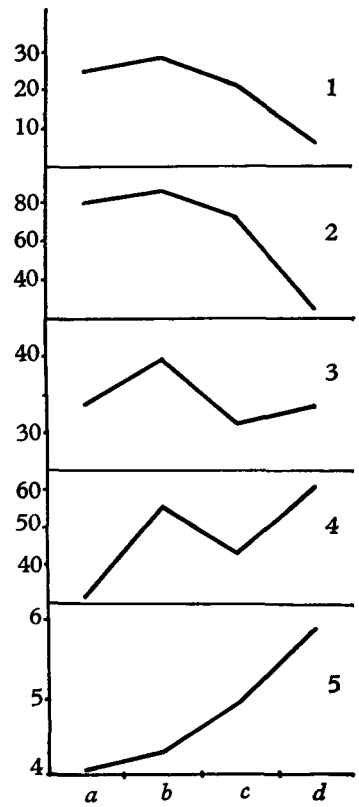

Fig. 1. Diagram of cytological analysis. 1—Overall proportions of sensitive germ-cells (Table 1, item 5). 2—Proportions of positive males (Table 1, items 7 and 8). 3—Proportions of sensitive cells in positive males (Table 1, item 8). 4—Proportions of breaks found in cells with more than two breaks (Table 1, item 12). 5—Average number of breaks per cell with more than two breaks (Table 1, item 13).

in cells with two breaks only, in spermatogonia over 60% of all breaks were found in cells with more than two breaks. Thus in brood *d* there was a pronounced heterogeneity between males as well as between the germ-cells of individual males.

It is suggested that both heterogeneities are due to the long-known fact that X-rays induce many more structural changes in highly condensed chromosomes than in extended ones. In the adult male, most of the spermatogonial cells are in interphase when the chromosomes are uncoiled and widely separated from each other. This accounts for the low overall sensitivity of spermatogonia. Some

spermatogonia, however, will be undergoing mitoses at the time of treatment, and these will show high sensitivity to irradiation, comparable in degree to the sensitivity of spermatozoa and spermatids with condensed chromosomes. Heterogeneity between the males would arise if at the time of irradiation spermatogonial mitoses are more numerous in some males than in others. A preliminary cytological examination of the testes from 2–3 days old *y w* males suggested that this may be true (Slizynski, unpubl.).

Why the number of chromosome breaks is higher in sensitive spermatogonia than in sensitive spermatozoa cannot be explained from the present data. It might be attributed to a greater breakability of spermatogonial chromosomes or to conditions in spermatogonial cells that favour the formation of structural changes.

The finding of a high number of chromosome breaks in spermatogonial cells appears to stand in contrast to reports on the absence of structural changes in these cells (Ward & Alexander, 1957; Alexander, 1960; Chandley & Bateman, 1960; McCarthy & Nafei, 1963). The results reported in the present paper are, however, not unique. Both in *Drosophila* (Catsch & Radu, 1943; Savhagen, 1960) and in mice (Griffen, 1958) translocations had been found in spermatogonial cells. Many possible causes for this discrepancy between experiments come to mind. The brood pattern may be changed by conditions such as length of brood period, number of females per male, type of stock used for irradiation, etc. (Auerbach & Moser, 1953; Traut, 1960). The heterogeneity between males increases the statistical error of changes scored in late broods. Steffensen (1962) has reported that dose rate, temperature and metabolic activity can affect the rejoining of broken chromosomes in such a way that the ratio of interchromosomal to intrachromosomal changes is diminished. All these factors, in conjunction with the low overall frequency of structural changes in spermatogonia, may well result in scarcity or complete absence of structural changes in experiments in which only translocations are scored or only lethal changes are examined cytologically.

SUMMARY

1. Sensitivity of *Drosophila melanogaster* male germ-cells to chromosome breakage by X-rays has been measured by the structural changes found in the salivary gland chromosomes of larvae from irradiated fathers and untreated mothers. The genetical effectiveness of irradiation on the same males was measured by the frequency of sex-linked lethals.

2. Assessed by the overall percentage of germ-cells carrying structural changes, sensitivity follows the well-known pattern: it is highest in spermatids and decreases over spermatozoa and spermatocytes to spermatogonia.

3. This overall sensitivity has been analysed on three levels:

(*a*) The proportion in successive broods of 'positive' males, *i.e.* males which respond to irradiation. The results indicate that spermatozoa and spermatids in most or all males are equally sensitive to X-rays, while in regard to spermatogonia the males represent a heterogeneous population consisting of positive and negative

males. This is fully confirmed by statistical analysis which was carried out by Dr B. Woolf.

(*b*) The proportion of 'sensitive' germ-cells per positive male, *i.e.* germ-cells carrying at least one structural change. While the proportion of sensitive cells per positive male is about 1 : 3 for spermatozoa, spermatocytes and spermatogonia, it is higher for spermatids. This is the main reason for the observed increase in overall sensitivity from spermatozoa to spermatids and for its subsequent drop in spermatocytes. On the contrary, the drastic drop in overall sensitivity from spermatocytes to spermatogonia results entirely from a drop in the proportion of positive males.

(*c*) The distribution of breaks in sensitive cells. The clustering of breaks in sensitive cells is highest in spermatogonia, somewhat lower in spermatids, and lowest in spermatocytes and spermatozoa. It indicates that spermatogonial cells are a heterogeneous population, consisting of cells that are either highly sensitive to X-rays or not sensitive at all.

4. The fact that the sensitivity of spermatogonia varies both between and within individual males is tentatively attributed to the presence or absence of spermatogonial mitoses.

I am greatly indebted to Dr C. Auerbach for her constant interest and help during the preparation of the manuscript, and to Dr B. Woolf for the paragraphs on the statistical analysis of the heterogeneity between males.

REFERENCES

ALEXANDER, M. L. (1960). Radiosensitivity at specific autosomal loci in mature sperm and spermatogonial cells of *Drosophila melanogaster*. *Genetics*, **45**, 1019–1022.

AUERBACH, C. & MOSER, H. (1953). Analysis of mutagenic action of formaldehyde food. I. Sensitivity of *Drosophila* germ cells. *Z. indukt. Abstamm.-u. VererbLehre*, **85**, 479–504.

AUERBACH, C. (1954). Sensitivity of *Drosophila* testis to the mutagenic action of X-rays. *Z. indukt. Abstamm.-u. VererbLehre*, **86**, 113–125.

CATSCH, A. & RADU, G. (1943). Über die Abhängigkeit der röntgeninduzierten Translokationsrate von Reifezustand der bestrahlten Gameten bei *Drosophila melanogaster*. *Naturwissenschaften*, **31**, 318–319.

CHANDLEY, A. C. & BATEMAN, A. J. (1960). Mutagenic sensitivity of sperm, spermatids, spermatocytes and spermatogonia in *Drosophila melanogaster*. *Heredity*, **15**, 363–375.

GRIFFEN, A. B. (1958). Occurence of chromosomal aberrations in pre-spermatocytic cells of irradiated mice. *Proc. nat. acad. Sci., Wash.*, **44**, 691–694.

HERTWIG, P. (1940). Verbbare Semisterilität bei Mausen nach Röntgenbestrahlung, verursacht durch reziproke Chromosomentranslokationen. *Z. indukt. Abstamm.-u. VererbLehre*, **79**, 1–27.

MCCARTHY, J. C. & NAFEI, H. (1963). A test for X-ray induced translocations in spermatogonia of *Drosophila melanogaster*. *Genet. Res.* **4**, 485–486.

MULLER, H. J. (1954). The nature of the genetic effects produced by irradiation. *Radiation Biology*, Vol. 1, Chap. 7, Section 10.

ROBERTSON, A. (1951). The analysis of heterogeneity in the binomial distribution. *Ann. Eugen., Lond.*, **16**, 1–15.

RUSSELL, W. L. (1954). Genetic effects of radiation on mammals. *Radiation Biology*, Vol. 1, Chap. 12.

SAVHAGEN, R. (1960). The relation between the rate of induced translocations and treated cell stages in males of *Drosophila melanogaster*. *Hereditas*, **46**, 651–667.

SAVHAGEN, R. (1961 *a*). The frequency of XO males and induced autosomal crossovers after irradiation of *Drosophila melanogaster* males in air and commercial nitrogen. *Hereditas*, **47**, 23–42.

SAVHAGEN, R. (1961*b*). The relation between X-ray sensitivity and stages of development of treated cells in spermato- and spermiogenesis of *Drosophila melanogaster*. *Hereditas*, **47**, 43–68.

SLIZYNSKA, H. (1963*a*). Origin of repeats in *Drosophila* chromosomes. *Genet. Res.* **4**, 154–157.

SLIZYNSKA, H. (1963*b*). Mutagenic effects of X-rays and formaldehyde food in spermatogenesis of *Drosophila melanogaster*. *Genet. Res.* **4**, 42–51.

SOBELS, F. H. & VAN STEENIS, H. (1957). Chemical induction of crossing-over in *Drosophila* males. *Nature, Lond.*, **179**, 29–31.

STEFFENSEN, D. M. (1962). A variable distance for chromosome exchange dependent on cellular activity, temperature and other conditions. *Radiat. Res.* **16**, 590.

TRAUT, H. (1960). Uber die Abhangigkeit der Rate strahleninduzierten Translokationen und recessiv geschlechtgebundener Letalfaktoren vom Stadium der Spermatogenese bei *Drosophila melanogaster*. *Z. indukt. Abstamm.-u. VererbLehre*, **91**, 201–205.

WARD, C. L. & ALEXANDER, M. L. (1957). Cytological analysis of X-ray induced mutations in eight specific loci in the third chromosome of *Drosophila melanogaster*. *Genetics*, **42**, 42–54.

APPENDIX: TEST FOR HETEROGENEITY IN BINOMIAL PROPORTIONS

BY BARNET WOOLF

Institute of Animal Genetics, Edinburgh 9

The purpose of this note is to give computational details for the test for heterogeneity in binomial sampling, derived by Robertson (1951) and used in the preceding paper by Slizynska, in the hope that it may be found useful by other workers. The rather formidable formulae given by Robertson can be reduced to very simple calculations. If there are k samples and the data are set out as a $2 \times k$ table, where each row specifies the numbers, a and b, of two species of individuals and the total $n = a+b$ in that particular sample, the only quantities to be calculated from the data are

$$\sum a = A \qquad \sum b = B \qquad \sum n = N$$

$$\sum a^2 = C \qquad \sum b^2 = D \qquad \sum n^2 = S$$

If the values of $p = a/n$ in the different samples vary too greatly to be reasonably compatible with a common population value for p, there will be a significantly positive value for t, where

$$t = \frac{N(AD+BC) - AB(N+S)}{AB[2(S-N)]^{1/2}}$$

If t is adjudged significant, the value, V, of the variance of the individual population values of p around their common mean P may to a first approximation be estimated by

$$V = \frac{N(AD+BC) - AB(N+S)}{N^2(S-N)}$$

https://doi.org/10.1017/S0016672300003839 Published online by Cambridge University Press

and the standard error of V may be taken as V/t. These formulae are very convenient for machine calculation, as, except for the square root in the first one, they involve only whole numbers.

The exact significance level for t is rather uncertain, as the distributions of the relevant parameters are unknown. Also, as Robertson explains, the procedure is biased, both because P is estimated as A/N, which gives undue weight to the larger samples, and for other reasons. It is therefore wise to make a generous allowance over the normal distribution 5% point of 1·96 for t. But the value of $t = 6{\cdot}08$, obtained by Slizynska for brood d, is clearly highly significant, whereas the other three values clearly are not.