STRUCTURE OF THE INCOMPATIBILITY GENE

I. SPONTANEOUS MUTATION RATE

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INTRODUCTION

ALL branches of genetics, formal mendelism, evolution, selection and gene physiology are concerned with differences arising ultimately from gene mutations. A study of the mutation process is therefore of prime importance in genetics and is probably the only method available for attacking the problem of gene evolution. But spontaneous mutations occur so infrequently that, even with special techniques in *Drosophila*, much labour must be expended for little result. Thus, despite its fundamental importance, our knowledge of spontaneous mutation is still very limited. More is known about artificially induced mutations and their relationship to chromosomal changes. This is due partly to the greater attention that has been given to this type of experiment and partly to the greater return of data for the output of work. If an efficient method for detecting rare mutations can be found it should be used to study not only induced but also, and indeed more particularly, spontaneous changes.

Any new method must fulfil certain exacting needs, and since these have been clearly stated by Timoféeff-Ressovsky (1934) only a brief summary need be given here, as follows :—

(i) The stocks must be genetically pure or, if this is impossible, as in the case of a permanent heterozygote, the stocks must be of known constitution in respect of the genes under examination.
(ii) An exact analysis of any changes must be made.
(iii) The number of mutations examined must be large enough to give significant results.
(iv) The method must be suitable for the detection of small, as well as large, changes.

The first two requirements can be satisfied by the usual genetical techniques in many animals and plants, but the last two have been a great obstacle to mutation experiments. In *Drosophila*, Muller's method for detecting sex-linked lethal mutations and his attached-X method for sex-linked recessive " visible " mutations have reduced enormously the labour of detecting mutations, but even then large numbers of individuals have to be tested.

In *Zea Mays* the advantages of endosperm characters for the detection of mutations have been described by Stadler (1946). In this species the requirements are met as follows :—

(i) Genetic purity is obtained by long continued selfing.
(ii) A detailed analysis of the kind of change is fulfilled because the one operative gene has four alleles with recognisably different effects on anthocyanin production.
(iii) Two of these alleles control endosperm colour, which is expressed in the seed, and large numbers can be examined quickly for the mutant character without the necessity of growing the plants.
(iv) These mutants when subjected to a viability test in the haploid gametophyte in the next generation reveal small differences between themselves even when they are phenotypically similar.

As a rule, the result of any genetic change in the highly organised genotype is less efficient than the normal, hence only the normal genes will tend to survive. The ideal test for mutation should not only overcome this low survival but should also be a natural detector or sieve which allows only gametes or cells with the mutant genes to pass the test. Furthermore, the method should make it possible to determine the exact stage at which the mutation occurred in the developmental history of the gamete or cell tested. In a recent series of papers Delbrück and Luria (Delbrück, 1942 ; Luria and Delbrück, 1943) and Demerec and Fano (1945), working with bacteria, have utilised such a sieve test for mutation studies by culturing the bacteria in a medium which is charged with lethal bacteriophage. Only resistant mutants can survive.

It is against this background that the method to be described here for detecting mutations of the incompatibility gene in the higher plants should be considered.

BASIS OF THE INCOMPATIBILITY SIEVE FOR MUTANTS

The locus which has been selected for the study of mutations is the incompatibility or S gene in the higher plants. It is a gene which has been intensively studied and has been found to conform in many different species to the original system found by East and Mangelsdorf (1925) in *Nicotiana*. In brief, this system is controlled by a gene S which exists in a very large number of different alleles. Each allele confers a highly specific mating reaction on pollen grains and styles, such that, where pollen and styles carry the same allele, pollen-tube growth is stopped short. This type of mating is said to be *incompatible*. When the pollen parent differs from the female parent in one or both of its S alleles growth is normal and mating is said to be *compatible*.

Now with incompatible pollination an occasional pollen grain,

as a result of mutation, carries an allele different from those in the style, and this will produce a long tube and effect fertilisation. Thus the S gene in the female tissue can act as a gametic sieve allowing only gametes with S alleles different from the two alleles in the style to pass and fertilise an egg. It is this sieve which is the core of the method I have used for selecting and testing mutations. One seed produced in 200 flowers incompatibility pollinated can represent one mutation at the S locus, during the differentiation of the anthers, in as many as a million gametes.

Mutations occurring at the S locus in plants have been recorded as single examples in *Antirrhinum glutinosum* (Gruber, 1932), *Trifolium pratense* (Williams and Silow, 1933), *Beta vulgaris* (Owen, 1942) and *Papaver nudicaule* (Fabergé, 1942). But, it must be remembered, a search for mutations was not being made in any of these cases and the mutant plants usually appeared as extreme rarities among the progeny from compatible pollination. Under such conditions only the most obvious effects of mutation would be observed, for example, a mutation to another self-incompatible allele would usually escape notice or if found would be discarded as an irregularity due to contamination. But a mutation to a self-compatible allele would be detected without making test crosses. It is not surprising, therefore, that the three mutations recorded were, in fact, self-compatibility alleles.

Other features of the S gene recommend it as a subject for mutation experiments :—

1. *The large number of alleles.* In species which have been tested, approximately 80 per cent. of the total number of alleles tested were found to be different (Williams, 1947 ; Atwood, 1944). For example, Williams in his work with red clover found 78 different alleles out of a possible maximum of 88 in 44 tested plants. Thus there appears to be no limit to the number of alleles.

The species used in these studies are *Oenothera organensis* and *Prunus avium.* In *Oenothera* 40 different alleles have been found in natural populations (Emerson, 1939), and in *Prunus* a minimum of 9 alleles in 66 cultivated varieties (Crane and Brown, 1937). The figure of 9 in *Prunus* under-estimates greatly the number of alleles which exist in this species as a whole. First, because it is a minimum number necessary to explain the 26 incompatibility groups found—the actual number in this material could be as high as 48. Secondly, the material tested is much inbred and cannot, therefore, be a random sample of the species.

Such a large number of alleles means that not only are there many possibilities for different mutations, but also the test for detecting them, as pointed out by Atwood (1944), will be extremely sensitive, and mutations with small effects will not escape observation.

2. *The pleiotropic action of the* S *gene.* An S allele produces in the pollen grain a substance that is not identical with the substance produced in the style by the same allele but is a complement to it.

These two substances react as do an antigen and its corresponding antibody. Furthermore, in the pollen, an **S** allele operates singly; in the style, alleles operate in heterogenic pairs, each producing its specific substances. This pleiotropic nature, with different haploid gametic, and diploid stylar, actions, greatly facilitates the detection and analysis of mutant alleles. For example, a mutation giving a small difference, such as would be masked in the style by any dominant or *supporting* action of the other allele, can be detected in the haploid pollen grain.

The choice of species for experimental work with mutations depends upon a number of factors :—

(i) The incompatibility must be rigorous, in other words there should be no environmental pseudo-fertility as found in *Nicotiana*, *Raphanus*, etc. This would complicate the detection of mutations.
(ii) The genetics of incompatibility in the species chosen must conform without reservation to the **S** system.
(iii) Incompatibility should be easily detectable by examination of the pollen-tubes in the style.
(iv) A large stigma allowing a large number of pollen grains to be applied to each flower is useful.
(v) The number of ovules in the ovary. This last factor requires detailed consideration.

The advantage of the many-seeded ovary is that the number of seeds set in each ovary tells us how many pollen grains contain a mutant **S** allele (2, 4, 8, etc.) and hence how many cell-divisions back in development mutation occurred.

The disadvantage with a many-seeded ovary is that it usually fails to develop when only one or two ovules are fertilised, therefore the most frequent class of mutation, *i.e.* those occurring in the pollen mother cells, cannot be recovered by the seed method. Recently, however, I have overcome this difficulty by the use of auxin and, as I shall show later, the pollen-tube and seed-set methods can be used and compared in the same plant.

Since the advantage with many-seeded and single-seeded species are complementary, a representative of each has been used in these studies. *Oenothera organensis* has been used as the many-seeded species because it satisfies all the requirements and has been studied intensively for other purposes (Emerson, 1939; Lewis, 1942, 1943). *Prunus avium*,* the single-seeded species used, has all the essentials of a good experimental plant, but it is not possible to utilise pollen-tube observations.

The only deliberate attempt to find mutations at the **S** locus that I am aware of was made in 1938 (but not published) by my

* In *Prunus avium* there are potentially two seeds, but it is rare for more than one to develop.

colleague Dr G. H. Beale, who treated *Oenothera*, *Antirrhinum* and *Nicotiana* with X-radiation. The results were discouraging. The technique of using a high temperature to give a more rigorous differentiation of pollen-tube growth (Lewis, 1942) and an improved method of pollen-tube staining had not then been found. It is only by these methods that single mutant pollen tubes—the most frequent class—can be observed.

METHODS

Pollination

The number of gametes tested for mutation cannot be known exactly. They must be estimated by determining the mean number of pollen grains placed on a stigma and it is therefore essential to standardise the method of pollination. The aim was to get an even distribution of pollen over the whole of the stigmatic surface—in *Oenothera* two anthers, in *Prunus* three or four were used for each stigma.

Pollen-tube examination

Flowers of *Oenothera*, required for pollen-tube examination, were cut from the plants, pollinated and placed in a dark incubator at 30° C. for 24 hours. Under these conditions the majority of incompatible tubes do not grow more than 2 mm., a small proportion grow a little longer but never more than 20 mm. (Lewis, 1942). Compatible tubes, however, grow 160 mm., *i.e.* near to the base of the style. Since it is necessary to count the compatible tubes only, the lower 60 mm. of the style was fixed in an alcoholic solution of iodine in potassium iodide (iodine, 1 gm. ; Kl, 15 gm. ; 30 per cent. alcohol, 100 c.c.). The material was kept in this solution until required and was examined by squashing between two glass slides, six styles on each slide. The slides were examined under a binocular microscope at a magnification of 20.

It was possible by this method to examine a large number of styles very quickly (see plate, fig. 1). When a pollen-tube was present, a conspicuous black line could be seen ; such sections were examined more closely under a magnification of 100 to determine the exact number of tubes. The sickle-shaped starch grains were unmistakable at this magnification.

When there were more than about ten tubes in a style it was difficult to make an exact count because the starch grains do not occupy the whole length of the tube ; in any one view of the style some of the tubes which have no starch grains in that part may escape observation. With such styles the iodine stain can be followed by cotton blue ; this stains the cytoplasm in parts of the tube that lack starch grains (see plate, fig. 2).

Recovery of single-seeded capsules

The most commonly observed spontaneous mutation should be at the latest stage of development, thus they should be found in isolated pollen grains. Tests were therefore made to find the minimum number of fertilised ovules that are necessary to stimulate capsule swelling in *Oenothera*.* Stigmas were pollinated with one, two, four and eight compatible pollen grains; some pollinated in this way received no further treatment, others were sprayed with β naphthoxyacetic acid (160 parts per million of water) 24 hours after pollination. This was used because I had found that growth substances stimulate capsule swelling in *Oenothera* (Lewis, 1946*a*). The results are given in table 1 and illustrated in the plate, fig. 3.

TABLE 1

Results of tests to find the minimum number of fertilised ovules necessary to stimulate capsule development, and the use of β naphthoxyacetic acid to enable seeds to develop which would otherwise fail owing to inadequate ovary stimulation.

No. to each stigma	No. of stigmas pollinated	No. of swollen capsules	Seeds in each capsule
Without β.N.A. 1 .	10	0	...
,, ,, 2 .	10	1	2
,, ,, 4 .	10	2	2, 2
,, ,, 8 .	10	6	8, 8, 6, 6, 3, 2
With β.N.A. 1 .	8	8	1, 1, 1, 1, 1, 1, 0, 0
,, ,, 2 .	2	2	2, 2
,, ,, 4 .	8	6	4, 3, 3, 2, 1, 0

Thus the minimum number of seeds for untreated capsules is two, and even with two seeds only 10 per cent. of the capsules develop. Spraying with β.N.A., however, enabled a 75 per cent. recovery of capsules with one seed to be obtained; this method was adopted, therefore, in the estimation of mutation rates by seed setting.

* The number of pollen grains necessary to produce a seed is an old problem, not for the present reason—the requirements of ovary stimulation—but for the requirements of the fertilisation of a single ovule. Darwin (1868) quotes Naudin as having pollinated *Mirabilis jalapa*, a plant with a single-seeded ovary, with one, two and three pollen grains. From seventeen flowers with a single pollen grain one flower alone perfected its seed, and the plant produced never attained its "proper dimension and bore flowers of remarkably small size." Darwin (*loc. cit.*) states that when Gartner pollinated *Malva*, a plant with many seeds in the ovary, with thirty pollen grains, no seeds ripened and with forty grains there were a "few seeds of small size."

As these observations were used by Darwin in support of his theory of pangenesis, it is not surprising that they were repeated. Mendel (letter 1870, pub. 1905) and Correns (1900) did so and both found that single pollen grains on the stigmas of *Mirabilis* would produce fully normal seedlings as frequently as would be expected from the proportion of viable pollen.

FIG. 1.—Iodine-squash preparation of the lower part of a style in *Oenothera* showing a single mutant pollen tube. × 50.

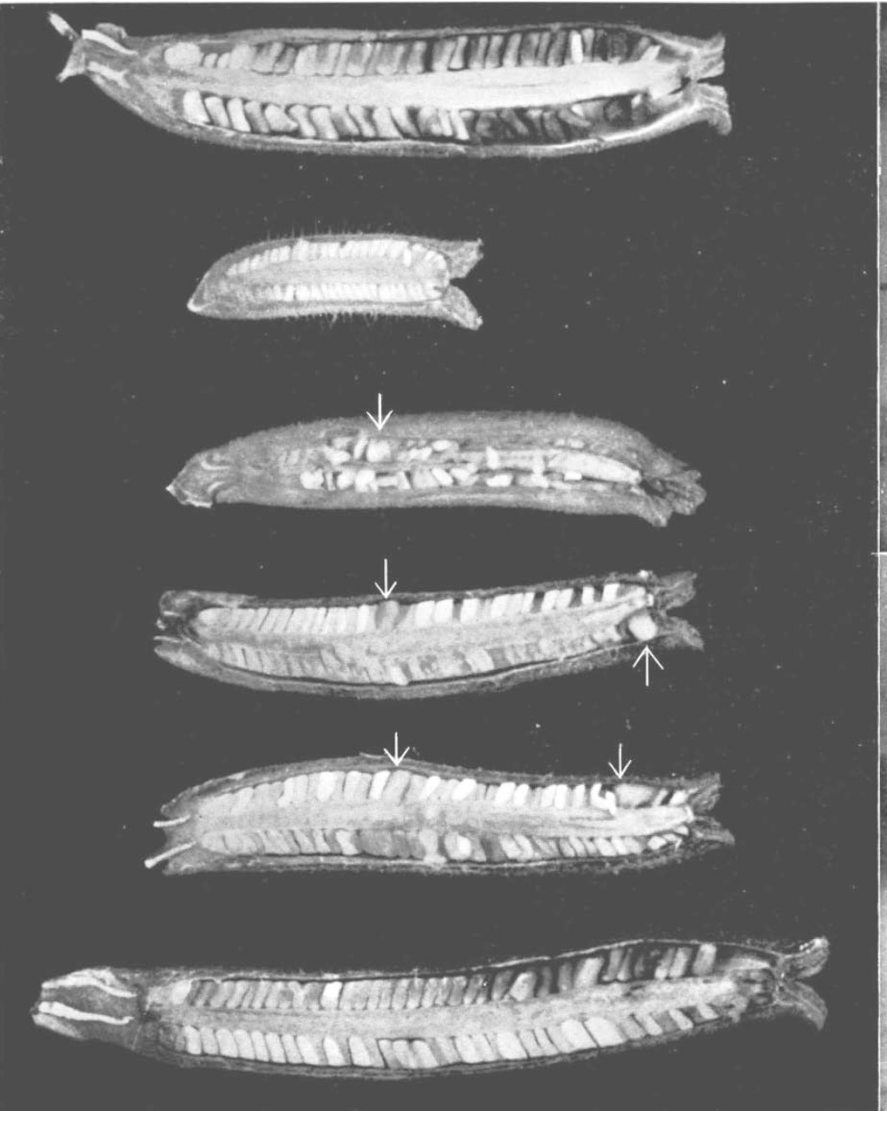
FIG. 2.—Iodine cotton-blue squash preparation to show sickle-shaped starch grains. This style contained several pollen tubes two of which are shown; the one on the right is near the tip of the tube and contains dense cytoplasm and no starch grains, the one on the left is further from the tip and has starch grains. × 300.

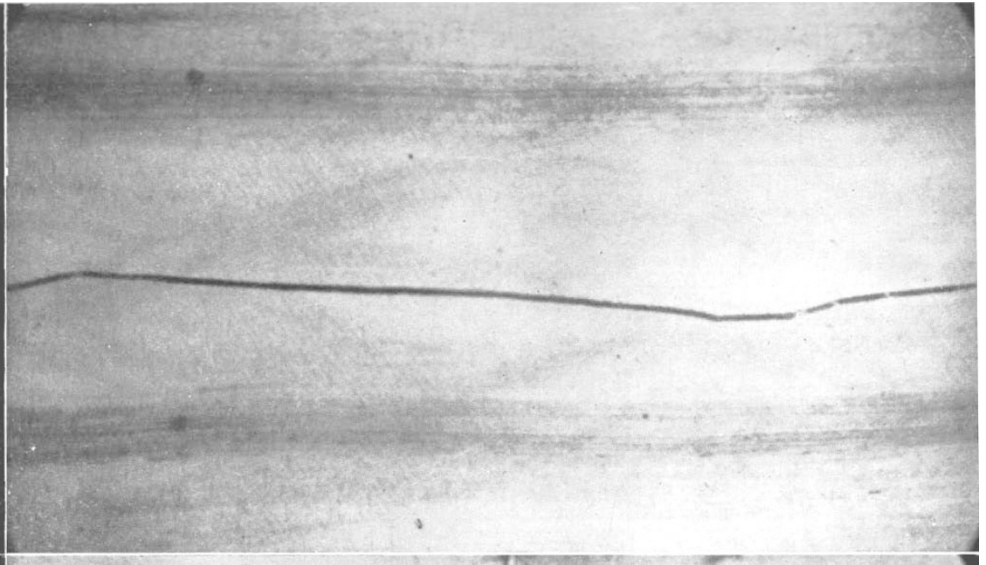
FIG. 3.—Capsules of *Oenothera organensis*.

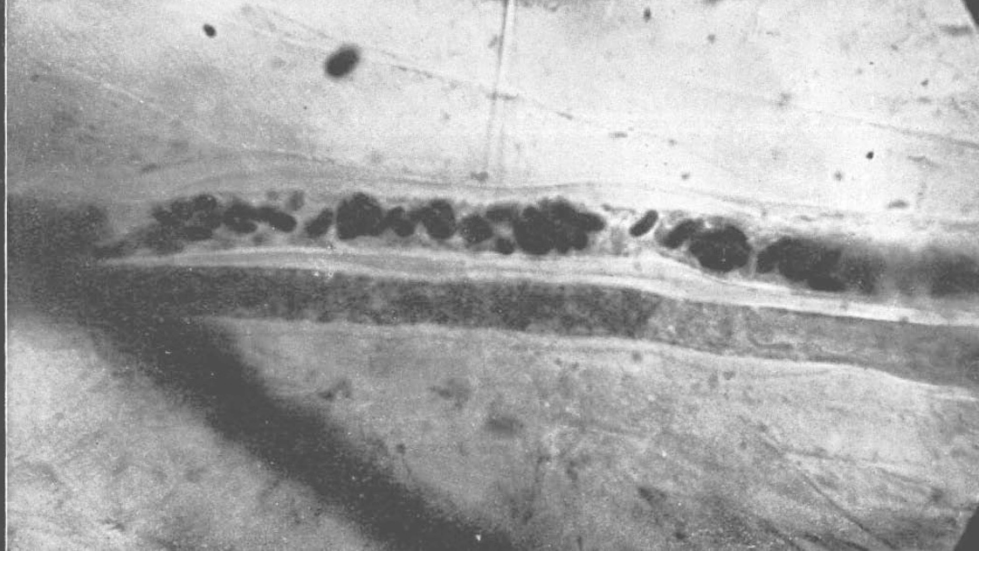
1. Compatibly pollinated—full of good seeds.
2. Incompatibly pollinated—not swollen, no seeds.
3. Pollinated with one compatible pollen grain and sprayed with β Naphthoxyacetic acid—one good seed (indicated by arrow).

4 and 5. Pollinated with two compatible pollen grains and sprayed with β.N.A.—two good seeds.

6. Incompatibly pollinated and sprayed with β.N.A.—no good seeds. Larger size of capsule associated probably with total absence of seeds.







Estimation of pollen grains on the stigma

Comparative mutation rates can be found by observing the number of mutants per flower pollinated, but to determine the actual rate it is necessary to know the number of pollen grains that are being tested. To do this the mean number of grains per stigma pollinated must be known. Two methods of estimation are available.

1. *Dilution method.* For *Prunus avium* stigmas were pollinated by a standard technique, and ten pollinated stigmas were placed in each of five flasks containing 25 c.c. of a mixture containing 3·5 parts by volume of absolute alcohol to one part of chloroform. The density of this mixture was such that the pollen grains remain suspended ; its fat solvent action allowed the sticky pollen grains to become evenly dispersed. After the flask had been shaken vigorously ten samples of 0·1 c.c. were withdrawn by an Ostwald 0·1 c.c. pipette and poured on to a special counting cell marked off in lines 1 mm. apart. The liquid was allowed to evaporate and the pollen grains counted under a binocular microscope.

Oenothera pollen is strung together on viscin threads. For an even dispersion in the liquid it was therefore found necessary to collect and thoroughly dry the pollen in a desiccator. It was then drawn through a muslin filter under pressure and applied to the stigma with a brush so that the whole surface was covered with a thin layer of pollen. Two pollinated stigmas were placed in each of five flasks containing 50 c.c. of the alcohol-chloroform mixture. Fourteen samples of 1·0 c.c. were withdrawn from each flask, evaporated and counted.

2. *Stigmatic area method.* A second method based on estimating the area of the receptive surface of the stigma and the number of pollen grains that could lie in a single layer in unit area was also tried in *Oenothera.* The length and diameter of the four lobes and the diameter if the central surface of the stigmas of 20 flowers were measured under the microscope. From these figures the area of the stigma was calculated. To determine the number of grains per unit area, pollen mounted in water was spread on a slide and arranged one layer thick by moving a cover-glass over them. In this way it was possible to get an entirely independent estimate of the number on a stigma.

These two methods of estimation, as the results given later will show, agree remarkably well. There is, however, another source of error in *Oenothera* ; it is not known exactly how the standard pollination of using two anthers for the scoring of mutations compares with the result of brushing dried pollen on to the stigma for the estimation of the mean number of pollen grains. This error, however, is not likely to be large in comparison with the sampling error of the mutation rate.

Isolation

In any work on mutation, isolation and the possibility of contamination from other stocks may be an acute problem. It was

found, however, to give little trouble in *Oenothera*. Thus two clones, both with the same S alleles, were isolated at Ockley, Surrey, 21 miles from the nearest plants of *Oenothera organensis*. Clones 2^{17} and 1^{2} were isolated in separate insect-proof houses, while clones 1^{14} and 1^{1} were isolated by 400 yards from the nearest plants. Even these last two clones, which had the least rigorous isolation, were found to be free from foreign pollen by two simple observations. First, out of the thousands of flowers that have opened during four years and have not been artificially pollinated none have set seeds spontaneously. Secondly, under the conditions of the experiment the majority of seeds or pollen tubes produced are singles : yet if insects had carried pollen to these clones it is unlikely that only one pollen grain would be carried each time.

RESULTS

Prunus avium

The analysis of variance of pollen counts by the dilution method on stigmas of *Prunus* is given in table 2. The mean number of pollen grains per stigma varies slightly with the variety according to the size of the stigma.

TABLE 2

Analysis of variance of data obtained from estimates of the number of pollen grains on stigmas of Prunus avium

	$S(x-\bar{x})^2$	D.F.	M.S.
Between 10 style samples	2070·9	45	46·02
Within 10 style samples	126·6	4	31·65
Total	2197·5	49	44·80
Mean pollen grains per stigma		1135±4·0	

The mutation rates, which are given in table 3, are calculated from the number of seeds produced after self- or cross-incompatible pollination. The data have been obtained from two sources : the published results of pollination work that has been carried out over a number of years (Crane and Brown, 1937), and from a large tree of *Prunus avium* Seedling No. 42 (Bigarreau de Schrecken × Governor Wood) of known genetical constitution growing in the open ground. The tree was covered with a pollen-proof cage ; a hive of bees was introduced into the cage before any cherry trees were in flower. The bees self-pollinated the tree very efficiently and foreign pollen was completely excluded. Thirty seeds were produced in 1946 and 26 in 1947 from an estimated number of 42,000 flowers each year ; this gives a rate for all mutations fo 0·6 per million pollen grains.

The mutation rates, as shown in table 3, are significantly different

between the S groups. Furthermore, the different clones within a group show a remarkably similar mutation rate; this is seen particularly well in the S3.4 group.

In analysing the published data it was found that in certain incompatibility groups there was a greater tendency to set a few seeds after selfing than in others. Two of the groups, S3.4 and S3.5 given in table 3 produced more selfed seeds than most others; the third group S1.2 produced very few.

TABLE 3

Mutation rate of the S *gene in different genotypes of* Prunus avium

Variety	Genotype	Flowers pollinated	Pollen grains tested ($\times 10^6$)	Seeds	Mutation rate ($\times 10^{-6}$ pollen grains)	
					Mean	Fiducial limits
Group V						
Bohemian Black . .	S3.4	1,948		5		
Late Black Big. . .	S3.4	2,078		5		
Turkey Heart . .	S3.4	2,226		6		
Total . . .	S3.4	6,252	7·0±·02	16	2·3	1·7-2·8
Group III						
Emperor Francis . .	S3.5	3,459		6		
Big. Napoleon . .	S3.5	2,969		2		
Total . . .	S3.5	6,428	7·3±·02	8	1·1	0·5-1·9
Group I						
Total of seven varieties	S1.2	17,157	19·4±·06	6	0·3	0·1-0·6
Seedling No. 42 . .	S3.6	84,000	95·3±·3	56	0·5	0·5-0·6

This difference in illegitimate setting between groups is evidence that specific mutation and not general contamination is responsible.

Oenothera organensis

The analysis of variance of the data obtained from the dilution method of estimating the mean number of pollen grains on a stigma of *Oenothera* is given in table 4. Two clones with stigmas of different sizes were selected for estimation, and in consequence the number of grains is a little different, being approximately 4800 in one clone and 5600 in the other.

The results of the stigmatic area method gave an upper and lower limit of 3686-5280 pollen grains in clone 1[14]. This compares very well with the figure obtained by the dilution method.

Mutation data obtained from three clones with both the seed set and the pollen-tube methods are given in tables 5 and 6. Table 5 gives the number of *capsules* containing 0, 1, 2, etc., seeds and the

number of *styles* containing 0, 1, 2, etc., compatible pollen tubes after self-pollination.

TABLE 4

Analysis of variance on data obtained from estimates of the number of pollen grains on stigmas of Oenothera organensis. *Pollen from 5 batches of 2 pollinated stigmas was removed into 50 c.c. of solvent and counts were made in fourteen 1 c.c. samples.*

	Clone 1^{14}			Clone 1^{2}		
	$S(x-\bar{x})^2$	D.F.	M.S.	$S(x-\bar{x})^2$	D.F.	M.S.
Between 2 style samples	7,082·24	4	1,770·56	74,615·68	4	18,653·92
Within 2 style samples .	55,024·29	65	733·65	58,212·59	64	909·57
Total . . .	62,106·53	69	900·09	132,828·27	68	1,953·27
Mean number of pollen grains per stigma	4,823·5±89·6			5,625·7±134·0		

TABLE 5

The number of capsules with their seed content and styles with their content of compatible pollen tubes after self-pollination in Oenothera. *The number of seeds per capsule and pollen tubes per style are grouped into a geometric series, each term of which represents the number of pollen grains arising from a single cell in a corresponding stage of development of the germ track.*

Seeds per capsule or pollen tubes per style	Clone 2^{17} (S4.6)		Clone 1^{18} (S3.6)		Clone 1^{14} (S3.6)		Total
	No. of capsules	No. of styles	No. of capsules	No. of styles	No. of capsules	No. of styles	
0	1519	713	932	587	5877	1926	11,554
1	22	13	14	5	38	14	106
2	4	2	1	0	4	1	12
3-4	3	0	1	1	8	0	13
5-8	2	1	4	1	2	0	10
9-16	2	0	0	0	2	0	4
17-32	1	0	0	1	0	0	2

THE TIME OF MUTATION

How do these capsules with two or more seeds arise? Only a small proportion, about 8 per cent., can be the result of two or more independent mutations because two such mutations occurring together would arise once in approximately 10^{11} pollen grains.

How are we to explain it? General evidence shows that the definitive gamete chromosome is formed at the time of crossing-over, *i.e.* at the end of the pachytene stage of meiosis, and that the groups of two pollen tubes are the result of mutations that have occurred before pachytene.

Experimental evidence for this is found from the results of X-radiation treatments. Treatments given to successively earlier

stages in development of the pollen have given progressively higher multiple mutants (Lewis, 1946*b*, and unpub.).

Assuming a constant mutation rate per division in the germ track up to and including the pollen mother cells, the number of mutations that are attributable to any particular cell generation will double as the number of cells doubles in each generation. The number of cells at the various stages falls into a geometric series ; the single mutants represent the last term while the total of the multiple mutants represents the total of all the preceding terms. Thus the number of mutants occurring in cells two mitotic divisions before meiosis, *i.e.* recorded as 5-8 seeds or tubes, should be twice as frequent as those occurring in cells that are three divisions before meiosis, *i.e.* recorded as 9-16 seeds or tubes.

The single mutation must, however, have originated at meiosis, and it is therefore possible that in their frequency they will not obey the same laws as those applying to the earlier and multiple mutations coming from the premeiotic mitosis. Table 6 suggests that this is, in fact, the case. The data, expressed as mutation rates per million pollen grains per division, have been grouped into two stages of development :—(1) *single mutants*, those occurring after pachytene of meiosis and (2) *multiple mutants*, those occurring up to this stage.

TABLE 6

Mutation rates calculated from the data on Oenothera *given in table 3, on the basis of 5000 pollen grains per stigma*

Stage of development	Method	Mutation rate ($\times 10^{-6}$ S genes per division)			
		Clone 2^{17}	Clone 1^{18}	Clone 1^{14}	Total
P.M.C. *	SEED	2·83	2·94	1·28	**1·75** 1·37-2·21†
Post-pachytene (*single mutants*)	P.T.	3·57	1·68	1·44	**1·96** 1·33-2·78
P.M.C. *	SEED	1·54	1·26	0·54	**0·80** 0·55-1·13
Pre-pachytene and mitotically dividing cells (*multiple mutants*)	P.T.	0·82	1·01	0·10	**0·24** 0·12-1·05

* A mutation occurring after gene reproduction in meiosis would give one mutant pollen grain ; thus mutations at this stage are observed as a single seed or compatible pollen tube. Mutations at the earlier stage are recorded as multiple seeds or pollen tubes.

† The fiducial limits based upon Steven's (1942) method.

The single or meiotic mutations seem to be much more common than the multiple mutations. There is, however, one difficulty about this simple test. It assumes that all mutant pollen grains are observed, whereas it might be that in a proportion of cases they fail to produce pollen tubes and to give rise to seed.

Clearly if some of the pollen grains fail to germinate, some of the higher multiple mutations will be contributing to the lower groups,

for example, some doubles will be recorded as singles and some singles will be unrecorded.

The *recovery* factor p, which is the proportion of compatible pollen grains that produce pollen tubes or seeds, has been estimated emperically from the number of seeds or tubes recovered from counted numbers of pollen grains, and the value obtained was 0·76.

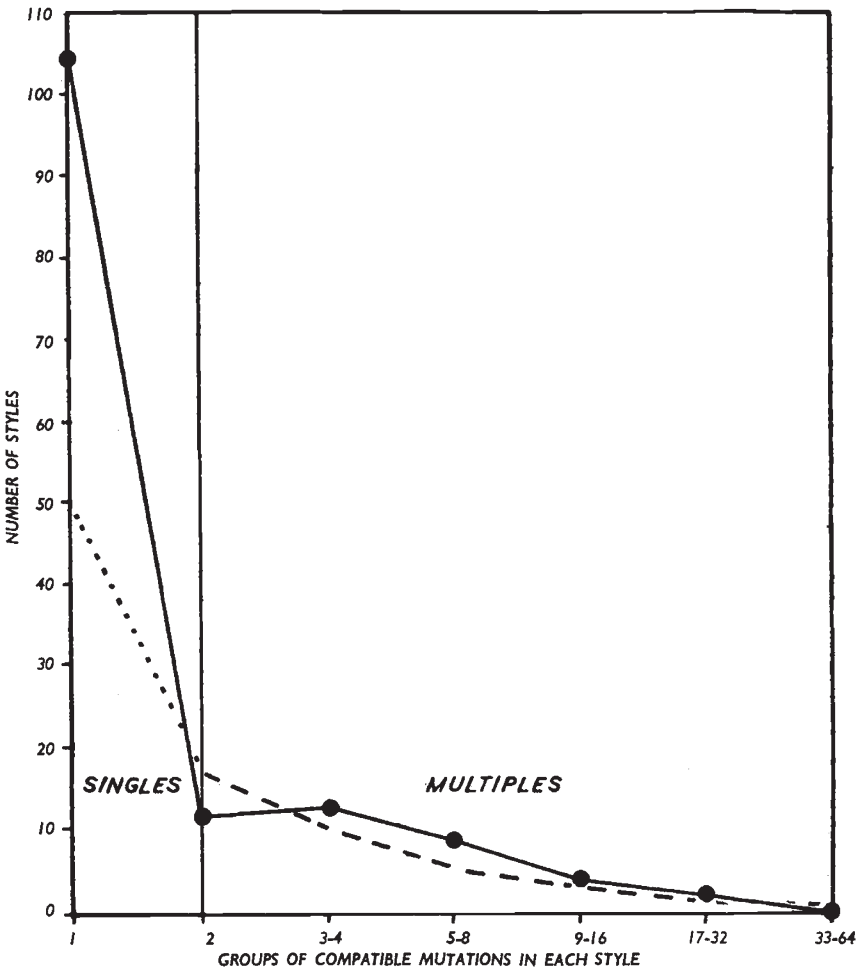


FIG. 1.—Observed frequency of mutant pollen grains grouped in the geometric series 1, 2, 4, etc., corresponding to the cell stage at which the mutation occurred. (Broken line, expected frequency based on the multiple groups, when the proportion of germinating pollen, p, is $\frac{3}{4}$; dotted line, extrapolation.)

I am indebted to my colleague, Dr K. Mather, who has calculated the expected frequencies of the terms in the series using a value of $p = \frac{3}{4}$, and these values have been used to give the expected curve in fig. 1.

The agreement, or lack of it, between the meiotic and pre-meiotic mutation rates is to be tested by the agreement between the frequency

of single mutations on the one hand and multiples as a whole on the other. The latter class must, however, first be tested for internal agreement. In all, there were 40 multiple mutations and when the expected distribution amongst the various groups, 2, 3-4, 5-8, etc., was calculated with $p = \frac{3}{4}$, it was found that the values observed fitted satisfactorily with $\chi^2_{[3]} = 4{\cdot}229$, $p = 0{\cdot}30$-$0{\cdot}20$. The multiples may therefore be pooled for comparison with the singles. With $p = \frac{3}{4}$ we expect that 55 per cent. of all mutations will be singles and 46 per cent. multiples. The total observed was 146, so that 80·7 should be single and 65·3 multiple. The numbers of observed were 106 single and 40 multiples, which differs significantly from expectation, $\chi^2_{[1]} = 17{\cdot}759$; p, very small. Thus we must assume that mutation at meiosis is more common than mutation at any single pre-meiotic division. In fact on the basis of 40 observed multiples only 49·4 singles would be expected, so that the observed frequency is 2·1 times higher than the expected value.

The value of p seemed, however, to be critical for the expectation, so that it was thought advisable to retest the data using $p = \frac{1}{2}$. Again the multiple classes were homogeneous among themselves ($\chi^2_{[3]} = 3{\cdot}786$, $p = 0{\cdot}30$-$0{\cdot}20$), and again the singles were significantly higher than the frequency expected from the multiples ($\chi^2_{[1]} = 9{\cdot}546$, $p = {\cdot}01$-$0{\cdot}001$). The ratio of observed to expected, however, is much less at 1·7, so that if p were really much below $\frac{1}{2}$ a fair agreement would be obtained.

The data for the external, emperical value of p were, however, clearly against such a low value, and a further internal test supported this. The frequency of the three and four compatible groups were observed to be five and eight respectively. The expected with $p = \frac{3}{4}$ and $p = \frac{1}{2}$ are given in table 7.

TABLE 7

The expected frequencies of the 3 and 4 groups of mutants with values of the recovery factor p *of* $\frac{3}{4}$ *and* $\frac{1}{2}$

Mutant group	Found	Expected $p = \frac{3}{4}$	Expected $p = \frac{1}{2}$
3	5	7·104	8·279
4	8	5·895	4·721
		$\chi^2_{[1]} = 1{\cdot}375$	$\chi^2_{[1]} = 3{\cdot}576$
		$p = 0{\cdot}30$-$0{\cdot}20$	$p = 0{\cdot}06$

From this test we see that $p = \frac{3}{4}$ gives a much better fit although it does not exclude the value of $\frac{1}{2}$. It does, however, and this is important, exclude values much below $\frac{1}{2}$.

The excess of single mutants cannot be due to a low value of the recovery factor, and it is impossible to escape the conclusion that there is some factor at work during meiosis which raises the frequency of mutation over that found in the pre-meiotic divisions.

Three important conclusions arise from the results given in table 6 and fig. 1.

In the first place the two methods of determining mutation rate agree satisfactorily. Close agreement is found in the single mutants, and even with the multiple mutants, although in all the clones there are more multiple mutants observed by the seed method; the two methods do not differ significantly.

In the second place, the clones differ. Clone 2^{17}, S4.6, has a higher mutation rate than clone 1^{14}, S3.6; the mutation rate from the pooled results of all clones is therefore only an average. From all the data, including those from four clones not shown in table 6, the final figure, which is based on more than 13,000 flowers and approximately 65,000,000 pollen grains is :—

$1{\cdot}87 \pm {\cdot}2$ ($\times 10^6$ S genes per division) post-pachytene stage.
$0{\cdot}60 \pm {\cdot}1$ ($\times 10^6$ S genes per division) pre-pachytene and mitotically dividing germ cells.

Finally, the mutation rate during and after gene reproduction in meiosis is about twice as high as that before this stage. If much of spontaneous mutation occurs in the process of gene reproduction, the special conditions—pairing and crossing-over—at meiosis can account for the higher rate of mutation at this time than when the gene is reproducing in mitosis.

Evidence, of a different kind, that spontaneous mutations can be caused by crossing-over has been obtained for chromosome II in *Drosophila melanogaster* by Wigan and Misro (1948, and unpub.). They find that significantly more lethal mutations occur in heterozygous females which undergo crossing-over and recombination, than in heterozygous males or homozygous females which do not.

MUTATION RATES

The mutation rates of the S gene estimated in *Prunus avium* and *Oenothera organensis* include, apart from true mutations of the S gene, two other types of change.

1. Mutations of modifying genes. These can be estimated only after progeny tests of the spontaneous mutants have been made; these are awaiting the flowering stage. All the X-ray induced changes that have been tested (Lewis, 1946) are S mutants; this indicates that mutations of modifiers are probably only a small proportion of the whole.

2. Unreduced chromosome complement in pollen grains. I have found that in certain S genotypes of *Oenothera* unreduced pollen grains with two different S alleles, through competition between the alleles, can produce long pollen tubes in styles carrying the same alleles (Lewis, 1943). Such pollen tubes would be scored as mutants. It is clear that this source of error does not apply to the seed-set method since triploid progeny would be distinguished from diploids by their

general appearance and bad pollen. Even when observing pollen tubes this source of error does not exist in *Oenothera* genotypes S4.6 and S2.4, since in these types unreduced pollen grains do not give long tubes. In genotypes S2.6 and S3.6 this error is probably small, for the number of mutant tubes found in these types is not higher than in the other genotypes.

It remains, however, that the mutation rates for *Oenothera* and *Prunus* are maximum estimates. An exact comparison is given in table 8 with the figures reduced to the same terms.

TABLE 8

Mutation rate per million pollen grains in four incompatibility groups of Prunus *and three clones of* Oenothera, *from tables 3 and 6*

Prunus avium	*Oenothera organensis*
2·3 ± ·5	4·3 ± ·3
1·1 ± ·3	3·6 ± ·6
0·5 ± ·07	1·7 ± ·2
0·3 ± ·2	...

Despite the possible sources of error, the figures for *Oenothera* based upon both the pollen tube and seed-set methods and for *Prunus* based upon the seed-set method are in remarkable agreement. The rate is slightly higher in *Oenothera* but they are of the same order of magnitude.

In view of the fact that the S gene can exist in a very large number, possibly thousands, of different detectable alleles, it is surprising that the mutation rate is of a low order. The rate is, in fact, very similar to that found in many of the most stable genes in maize where only two alleles are known (Stadler, 1942).

THE POPULATION GENETICS OF INCOMPATIBILITY

It has been shown (Wright, 1939) that the number of S alleles in natural populations of *Oenothera organensis* is dependent upon the mutation rate. On theoretical grounds he found that to maintain the 40-50 alleles that are present in the wild population of about 500 plants it was necessary to assume an abnormally high mutation rate. A solution of the problem was obtained, however, by assuming a mutation rate of 10^{-5} or 10^{-6} per generation, with a division of the population into small and localised inter-breeding groups, such that 98 per cent. or more of the pollination is by pollen from immediate neighbours. This solution for *Oenothera*, as Sewell Wright points out, is not entirely satisfactory, because the degree of division invoked appears to be excessive. Furthermore, the difficulty of reconciling a low mutation rate with a large number of alleles in natural populations

may be even more acute in *Trifolium* species where still larger numbers of alleles exist.

It is normal to measure mutation rates as the proportion of mutant plants among the progeny, and this is the rate used by Sewell Wright. To distinguish this from the *actual* rate, which is the proportion of mutants in the gametes, we may call it the *effective* rate. In the following discussion we shall see that these are not the same and this can be a large factor in the calculations.

The high degree of local interbreeding invoked in these theoretical considerations of wild populations does, in fact, increase the difference between these rates to a magnitude which will affect the calculations. If the flowers are being pollinated by their neighbours there will be a high proportion of self-pollination and incompatible cross-pollination. Thus some stigmas will receive a lower number of compatible grains than the number of ovules in the ovary, and at the same time there may be on these stigmas a large number of incompatible grains from selfing. For example, 300 or 3000 self-pollen grains on such a stigma would give an effective mutation rate in this flower of 300 or 3000 times the actual rate, as the case may be. If 10 per cent. of the flowers were pollinated in this way the mutation rate would be multiplied by 30 or 300 times. This we can call the *self-pollinating factor.*

Clearly the magnitude of this factor depends upon the proportion of incompatible to compatible pollination occurring in the population. This proportion in turn depends directly upon the amount of local pollination and indirectly on the effective mutation rate. More localised pollination will lead to an increase in the self-pollination factor, and this to an increase in the effective mutation rate. Effects working in the opposite direction are :—(i) a less localised pollination, and (ii) a high effective mutation rate, because it will reduce the amount of incompatible pollination in the next generation. This would constitute a balanced system.

It would be interesting to know the proportion of incompatible pollination occurring in natural populations of *Oenothera*. With large trees such as *Prunus* or *Pyrus* species, the amount of self-pollination can in some years be near unity ; hence the effective mutation rate, *i.e.* the proportion of mutant plants in the progeny must bear little direct relation to the actual rate.

SUMMARY

1. Two methods of determining the mutation rate of the incompatibility gene are described :—(i) by the number of compatible tubes found in styles and (ii) by the number of seeds set in capsules, after both self- and cross-incompatible pollination.

2. In *Oenothera organensis* the two methods are satisfactory, and on comparable material give estimates of the mutation rate that agree closely. In *Prunus avium* only the seed-set method can be used.

3. The mutation rate in *Oenothera* based on 65,000,000 pollen grains is :—

(i) $1 \cdot 87 \pm \cdot 2$ per 10^6 **S** gene divisions in the post-pachytene stages of meiosis.

(ii) $0 \cdot 60 \pm \cdot 1$ in the pre-pachytene stages of meiosis and in the mitotically dividing germ cell precursors.

It is suggested that the higher rate at post-pachytene may be due to crossing-over.

4. In *Prunus avium* a difference in mutation rate between two of the tested genotypes is highly significant, the highest being $2 \cdot 3 \pm \cdot 5$ and the lowest $0 \cdot 3 \pm \cdot 2$ per 10^6 pollen grains for pre-+post-pachytene.

5. The *effective* mutation rate of the **S** gene, as measured by the number of mutant plants in the progeny of a generation depends upon the ratio of compatible to incompatible pollination in wild populations. The lower the ratio the higher will be the effective rate. This is an important factor in calculating the number of different **S** alleles that can be maintained in a population.

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