the *ij* gene, the mutated plastid, like a Frankenstein monster, is no longer under the control of its maker.

The data given here are of direct interest in connection with the nucleus-plasma problem, but they may have some significance in the field of both normal and abnormal growth and differentiation. The basis of cellular differentiation is one of the great problems of biology. All nuclei of an organism presumably have the same genic constitution, and yet morphological and physiological differences arise. That the maintenance of these differences is not entirely determined by the differing local conditions is shown by the persistence of certain specificities when cell multiplication of isolated differentiated cells occurs in tissue culture. The view that cellular differentiation is cytoplasmic seems to require that the cytoplasm contains elements of a hypothetical nature which are modified by interaction with nuclear products. In the case reported here a known constituent of the cytoplasm, the plastid, has been modified by a nuclear factor and is transmitted thereafter by cytoplasmic heredity.

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GENE AND CYTOPLASM. I. THE DETERMINATION AND INHERITANCE OF THE KILLER CHARACTER IN VARIETY 4 OF PARAMECIUM AURELIA[1]

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The present paper reports a previously unknown system of relations between a gene and a cytoplasmic substance, both of which are required for the development of an hereditary character. When some of the cytoplasmic substance is present, the gene controls its continued production; but when the cytoplasmic substance is absent, the gene cannot initiate its production. Addition of the cytoplasmic substance to an organism, lacking the character dependent on it, but containing the required gene, results in the continued production of the substance, in the development of the character determined by the combined presence of gene and cytoplasmic substance, and in the hereditary maintenance of the character in successive generations.

The pair of characters whose determination and inheritance are to be analyzed are designated "killer" and "sensitive." They are found in diverse races of variety 4 of *Paramecium aurelia*. Of the four available races of this variety, only race 51 is a killer; the other three races (29, 32 and 47) are sensitive. Fluid in which the killer race has lived kills individuals of the sensitive races. The killing of sensitive animals is preceded by characteristic morphological aberrations, particularly by shifting of the posterior part of the body to the aboral side. The killer and sensitive characters are alternatives in inheritance and never exist together in the same individual. In practice, the character of an unknown clone is tested by mixing samples of it (1) with the killer race 51, and (2) with a sensitive race. If the characteristic abnormalities and corpses are produced in mixture (1), the unknown is sensitive; if produced in mixture (2), it is a killer. Sensitive animals begin to show abnormalities only after at least four hours of subjection to the killer fluid. It is possible to cross killers with sensitives if they begin to mate soon after they are brought together, and this is readily accomplished by bringing together opposite mating types when in sexually reactive condition. If the conjugant pairs are removed to fresh culture fluid soon after they unite and the two members of each pair are put into separate culture vessels soon after conjugation has been completed, the sensitive member of the pair is never injured by the contact with a killer during mating.

In the original races, the characters killer and sensitive are invariably inherited. All the vegetative and sexual progeny within race 51 are killers. All the vegetative and sexual progeny within the sensitive races are sensitive. Only the sensitive race 32 is reported upon in this paper; results with other sensitive races bring out certain further important points which will be set forth in the next paper of this series.

When the pure killer race 51 is crossed to the pure sensitive race 32, the two exconjugants of each pair produce phenotypically different clones: one is a killer and the other is sensitive. By marking the parents, it is readily demonstrated that the F_1 killer clones are those that derive their cytoplasm from the killer parent and the F_1 sensitive clones are those with cytoplasm from the sensitive parent. This result is totally unexpected and requires explanation because, as is well known, the nuclear processes during conjugation are such that the two mates, after reciprocal fertilization, have the same genotype and should produce clones alike in their hereditary characters. The following experiments were designed to provide the required explanation.

Experiment 1: Autogamy in F_1 Killers.—Ultimate genic control of the alternative characters is indicated by observations on the results of autogamy in one of the two classes of F_1 clones, the killers. Autog-

amy[3, 4, 5] is a process in which identical, haploid, gamete nuclei in a single unmated cell unite to produce a diploid syncaryon from which all subsequent nuclei of the clone are derived. Hence all clones derived from autogamous individuals are necessarily homozygous and, when heterozygotes undergo autogamy, half become homozygous for one allele and half for the other. Of 306 F_1 killers that went through autogamy, 167 produced clones of killers and 139 produced clones of sensitives. The segregation ratio is reasonably close to the theoretical 1:1 ratio (deviation 14, standard error 8.7) and so indicates that the F_1 killers were heterozygous for a pair of alleles determining the alternative characters.

Experiments 2, 3, 4 and 5: Further Fertilizations within Each of the Two Classes Obtained in Experiment 1.—If the F_1 killers were heterozygotes, the two classes that segregated from them at autogamy (expt. 1) should be the two possible classes of homozygotes. Further breeding tests confirmed this: the sensitive F_2 clones yielded only sensitive progeny after further autogamies (expt. 2) and after conjugation with each other (expt. 3); the killer F_2 clones yielded only killer progeny after further autogamies (expt. 4) and after conjugation with each other (expt. 5). Hence the two classes of F_2 clones obtained in approximately equal numbers at autogamy in F_1 killers (expt. 1) are pure breeding or homozygous for the alternative characters. The genic determination of these characters was further tested in experiment 6.

Experiment 6: Crosses between Different Clones of F_1 Killers.—If the F_1 killers are heterozygotes, conjugation between two such clones should yield the usual F_2 ratio of 3:1 and should show which allele is dominant. From 443 pairs of conjugants the 652 clones from the two members of 326 pairs were killers and the 234 clones from the two members of 117 pairs were sensitives. This agreement with the theoretical 3:1 ratio (deviation $6^1/_4$, standard error 9) confirms the heterozygosity of the F_1 killers and shows that the killer gene (K) is dominant over its sensitive allele (k).

Since F_1 killers are heterozygous (K/k), their pure breeding parent races must have the two possible homozygous combinations: the killer race is K/K and the sensitive race is k/k. As the killer gene is dominant (expt. 6), *all* the F_1 should be heterozygous killers; but this condition has been demonstrated only for one of the two F_1 clones from each pair of hybrid exconjugants. The other is not a killer, but sensitive (see p. 330). Is the disagreement here merely phenotypic or genotypic also? Experiments 7 and 8 answer this question.

Experiment 7: Cross of F_1 Sensitives to F_1 Killers.—In order to discover the genotype of the F_1 sensitives, they were crossed to F_1 killers known (expts. 1–6) to be heterozygotes. From this cross were obtained 294 conjugant pairs yielding a killer clone from one member and a sensitive

clone from the other member of each pair: and 126 conjugant pairs yielding sensitive clones from both members of each pair. The meaning of this result is best brought out by considering first those clones that derived their cytoplasm from the F_1 killers, i.e., one member of each pair of exconjugant clones.

These cytoplasmic descendants of the F_1 killers included 294 killer clones and 126 sensitive clones. This is closer to a theoretical 3:1 ratio (deviation 21, standard error 8.9) than to any other simple genetic ratio. In the detailed paper to be published later it will be shown that the small discrepancy not due to sampling error is due to the occurrence, in a small percentage of the united pairs, of a process differing from normal conjugation. This process is essentially double autogamy or, as it has been called,[6] cytogamy. In most crosses it can readily be detected and the data can be corrected for it; but, in crosses of the type under discussion, its detection is so laborious as to be quite impracticable.

The 3:1 ratio in this group shows that both parents were heterozygotes, as in crosses between F_1 killers (expt. 6). One parent was a clone of F_1 killers (known to be heterozygotic) and the other parent was a clone of F_1 sensitives. Hence, the F_1 sensitives must also have been heterozygotic (K/k) and this agrees with expectations from the breeding experiments 1 to 6. The disagreement with expectation is merely in their sensitive phenotype. The killer gene K, dominant in race 51 cytoplasm (expt. 6), is certainly not dominant in race 32 cytoplasm.

Further information on the behavior of gene K in race 32 cytoplasm is provided by consideration of the phenotypes of the other member of each pair of exconjugant clones produced in experiment 7, namely, those deriving their cytoplasm from the F_1 sensitives. *All* of the 420 clones of this group were sensitive. From the now known genotypes of their parents, $K/k \times K/k$, one-fourth of these 420 clones should be k/k and sensitive, one-half should be K/k and sensitive (because, as shown above, K is not dominant in race 32 cytoplasm) and one-fourth should be K/K. The fact that no killer clones were obtained in this group indicates that even the K/K clones were sensitive. In other words, the killer gene K is completely unable to produce the killer phenotype in race 32 cytoplasm. Experiments 8, 9 and 10 confirm this conclusion.

Experiment 8: Autogamy in F_1 Sensitive Clones.—As in the case of autogamy in heterozygous F_1 killer clones (expt. 1), autogamy in heterozygotic F_1 sensitive clones should yield K/K and k/k clones in a ratio of 1:1. But as the F_1 sensitive clones and their autogamous progeny derive their cytoplasm from race 32 and as the killer gene K is unable to produce the killer phenotype in this cytoplasm (expt. 7), both classes of exautogamous clones should be sensitive. In agreement with this, all of the 148 exautogamous clones from F_1 sensitives were sensitive.

Experiment 9: Crosses of F_2 Sensitive Clones (Obtained by Autogamy from F_1 Sensitives) to F_1 Killers.—It is practically impossible to test whether the 1:1 ratio predicted in experiment 8 is actually obtained because each clone would have to be separately tested for genotype by elaborate breeding experiments. More important than the ratio is the question of whether there are in fact produced, as predicted, sensitive clones homozygous for the killer gene. Therefore, only a few of the exautogamous sensitive clones from experiment 8 were fully tested to see if any had the predicted K/K genotype. The test consisted in crossing them to heterozygous F_1 killers. In this cross, any tested clone that contains the recessive gene k either in homozygous or heterozygous condition would yield some pairs in which both members produce sensitive clones; but if the tested clone is homozygous for the killer gene K, all the pairs of conjugants obtained in the cross to F_1 killers would yield a killer clone from one member of the pair and a sensitive clone from the other. Among the few clones tested, three gave the latter result. For example, 36 pairs were obtained from the cross of one clone to F_1 killers and from each pair there arose one clone of killers and one clone of sensitives. The other two were tested on an even larger scale and gave the same result. Hence, some of the exautogamous clones obtained from F_1 sensitive parents are homozygous for the killer gene though phenotypically sensitive. This confirms the conclusion drawn from experiment 7 that the killer gene is unable to determine the killer phenotype in race 32 cytoplasm even when it is present in homozygous condition.

Experiment 10: Sensitive Clones Homozygous for the Killer Gene Retested after the Passage of Several Months.—In order to exclude the possibility that the sensitivity of the K/K clones with race 32 cytoplasm was due either to the delayed action of the K gene or to the mutation of K to k in race 32 cytoplasm, these clones were retested at intervals of 2, 4 and 6 months. The tests showed that the sensitive phenotype and the K/K genotype were maintained. (The tests were the same as the one employed in experiment 9.) During the six months that these clones were cultured, many successive sexual generations must have occurred, for autogamies recur at intervals of 3 to 7 days in mass cultures of variety 4. Hence the gene K remains constant and is not only temporarily but permanently incapable of determining the killer phenotype in race 32 cytoplasm.

These results raise the question of cytoplasmic inheritance. In cytoplasm of race 51, the gene K determines the killer character; in cytoplasm of race 32, the same gene does not determine the killer character. This difference in the effect of gene K in different cytoplasms persists through many sexual generations, presumably without limit. Does this warrant the conclusion that the cytoplasms of the two races possess hereditary

differences that are independent of the genes? Experiment 11 answers this question.

Experiment 11: Cross of F_2 Sensitive Clones (Derived by Autogamy from F_1 Killers) to the Killer Race 51.—Experiments 1, 4 and 5 showed that half of the clones obtained by autogamy from F_1 killers were sensitive because they were homozygous for the sensitive gene k. The cytoplasm of these clones is derived from race 51. If the property of race 51 cytoplasm which permits the killer gene K to determine the killer character is inherited independently of the genes, reintroduction of gene K into these sensitive clones should result in the restoration of the killer character. The gene K was put back into them by mating them to the homozygous killer race 51; but the killer character failed to develop. In all of the 96 pairs of conjugants, the clone produced by the exconjugant deriving its cytoplasm *directly* from the killer parent remained a killer; but the clone produced by the exconjugant deriving its cytoplasm *indirectly* from race 51, through a sensitive F_2 clone, remained sensitive. Hence, when the gene K is replaced by its sensitive allele, k, the cytoplasm of race 51 becomes, like the cytoplasm of race 32, incapable of developing the killer phenotype when gene K is reintroduced into it. The property of race 51 cytoplasm which permits the killer gene to determine the killer character is thus not inherited independently of the genes, but is dependent on the uninterrupted presence of the gene K.

This experiment shows that the killer character depends on the combined presence of the killer gene K and something else. The other factor, when present, is reproduced under the influence of gene K and ceases to be reproduced when gene K is absent. Moreover, the failure of the killer character to develop in sensitive clones into which gene K has been introduced shows that gene K is unable to initiate the production of this other essential factor and that the latter is not carried over from one mate to the other during conjugation. In other words, it is not present in the "male," migratory, gamete nucleus at the time of fertilization. Experiments 12 and 13 were designed to throw light on the location within the cell of the essential factor other than gene K.

Experiment 12: Transfer of the Other Factor from one Cell to Another.—Under certain conditions, not yet entirely known,[7] pairs of conjugants in variety 4 either remain united unduly long or permanently. At the normal time for separation of the mates, they separate everywhere except in the region of the paroral cones where a thin connecting band of cytoplasm appears. After prolonged union in this way, in some pairs the mates separate completely; but in others the band of union increases in width and the mates remain permanently united, though normal single animals are given off from the separated regions at the first few fissions. Such connections between mates provide opportunity for the transfer

of material from one to the other, especially in those pairs that develop a broad connecting band. In crosses between killers and sensitive clones with the killer gene *K*, when a cytoplasmic connection was established between the mates, the normal single animals produced from *both* of them yielded killer clones. Hence the formerly sensitive mate must have produced a clone with the killer phenotype and this result is correlated with the possession of the gene *K* plus temporary cytoplasmic continuity with a killer animal. In the same crosses, when separation of the mates occurred at the normal time, each mate remained phenotypically unchanged: one produced a clone of killers and the other a clone of sensitives. Hence the transformation of the sensitive into a killer clone in the cytoplasmically united pairs must have been due to the transfer from the killer to the sensitive mate of the material required in addition to gene *K* for the development of the killer phenotype. Moreover, of the gamete nuclei, syncaryon and derivatives of the syncaryon, only the migratory gamete nucleus goes across from one mate to the other in the cytoplasmically united pairs, and previous experiments have shown that the migratory nucleus does not carry the essential material with it; therefore this material at this time must be outside of these nuclei. The material could be in either the cytoplasm or the many pieces into which the old, disintegrating macronucleus has broken down, for these are both free to migrate from mate to mate across the broad cytoplasmic connecting band. There is, however, no known direct connection between the pieces of the old macronucleus and the new nuclei formed from the products of the syncaryon; and the old macronuclear pieces soon disappear, while the killer character is permanent and hereditary. Hence the essential material must be at least for a time outside the nuclei in the cytoplasm. There is as yet no evidence concerning the question as to whether it is ever in the nuclei. The substance whose continued production is controlled by gene *K* and whose presence is required for the development of the killer phenotype may therefore be designated as the killer cytoplasmic factor or substance. Whether this is the same as the substance that produces the killing action on sensitive cells or a precursor of it remains to be discovered.

Experiment 13: Demonstration of the Killer Cytoplasmic Factor in Cells That Have Just Lost the Killer Gene.—The killer cytoplasmic factor was shown in experiment 11 to disappear from cells that lose the killer gene *K*. However, only if this factor did not exist in the cytoplasm but was always indissolubly connected with gene *K* would its disappearance be expected to coincide exactly with the loss of gene *K;* otherwise, its disappearance should follow loss of gene *K* by an appreciable amount of time. If it could be detected in the cell for a considerable period after gene *K*

is replaced by k, this would provide further evidence for its cytoplasmic localization.

The method of detection employed was essentially the same as the one used in experiment 11: F_2 sensitives (k/k) that had arisen from F_1 killers (K/k) at autogamy were mated to the killer race 51 (K/K). In experiment 11 this cross was made several days after the genotype had changed at autogamy from K/k to k/k. At that time there was no evidence of the presence of the cytoplasmic factor, for return to the K/k genotype did not result in the development of the killer phenotype. In the present experiment, the same cross was made at intervals of only two to five fissions (one to two days) after the F_1 killer (K/k) had changed to an F_2 sensitive (k/k) at autogamy. Description of the laborious technique involved in bringing about conjugation so early in the history of a clone of known constitution will be reserved for the full paper to appear later.

Altogether 21 such F_2 sensitive clones were induced to conjugate with the killer race 51 within five fissions (2 days) after their origin at autogamy. Three of the four that conjugated two or three fissions after autogamy, four of the ten that conjugated three or four fissions after autogamy, and one of the seven that conjugated five fissions after autogamy, making a total of eight crosses, yielded clones of killers from *both* members of a pair of conjugants. Hence the sensitive parent, as well as the killer parent, must have yielded a clone of killers in these eight cases; and the cytoplasmic factor must still have been present. The data also indicate that the cytoplasmic factor is present in fewer and fewer cells with increase of time and number of fissions since loss of gene K, until, as shown in experiment 11, it has completely disappeared after a few days. The period during which the cytoplasmic factor remains after the gene for its production is removed corresponds closely to the period previously found[8,9] for the "cytoplasmic lag" in change of phenotype following other changes in hereditary constitution in *P. aurelia*. Comparable gradual loss of a gene-controlled cytoplasmic factor may be involved in many or all such situations.

Both experiments 12 and 13 show: (1) that the factor required in addition to gene K for development of the killer phenotype is present in the cytoplasm of killer cells; (2) that gene K determines the continued production of this cytoplasmic factor, for sensitive cells converted into killers became permanently and hereditarily so; and (3) that this can be accomplished when the cytoplasmic factor is at least initially outside the nucleus in the cytoplasm. Both experiments also show (4) that there was present in one cell at least twice as much of the cytoplasmic factor as required by gene K to enable it to produce more. This was shown in experiment 12 by the fact that the killer member of the cyto-

plasmically united pairs provided enough of the cytoplasmic factor to enable genes in both cells of the pair to produce more; and in experiment 13 by the fact that in each of three different crosses of exautogamous clones to race 51, there were obtained *two* pairs of conjugants yielding killer clones from both members of the pair. Hence, in each of these three clones there was present after gene K was lost enough of the cytoplasmic factor so that it could be distributed to two daughter cells and still have in each enough to bring about the production of more cytoplasmic factor when gene K entered the cell.

The preceding 13 experiments provide the basic information required for discovery of the system of determination and inheritance of the killer and sensitive characters. Many other breeding experiments have been performed to test further the validity of the conclusions drawn in the preceding pages. Space does not permit a detailed account, but the crosses and observed results in some of the more important experiments are listed below.

Experiment 14.—Cross of F_1 killers (K/k) to race 32 (k/k); 210 pairs of conjugants. Result: 116 pairs yielded sensitive clones from both members of the pair; 94 pairs yielded a killer clone from one member and a sensitive clone from the other.

Experiment 15.—Cross of F_1 killers (K/k) to race 51 (K/K); 104 pairs of conjugants. Result: killer clone from each member of every pair.

Experiment 16.—Cross of F_1 sensitive (K/k) by F_1 sensitive (K/k); 431 pairs of conjugants. Result: all 862 clones sensitive.

Experiment 17.—Cross of F_1 sensitive (K/k) to race 32 (k/k); 200 pairs of conjugants. Result: all 400 clones sensitive.

Experiment 18.—Cross of F_1 sensitive (K/k) to race 51 (K/K); 107 pairs of conjugants. Result: in every pair, one member produced a sensitive clone and the other produced a killer clone.

In every experiment the observed results are those required by the conclusions drawn from the first 13 experiments.

The determination and inheritance of the alternative characters killer and sensitive in races 51 and 32 appear therefore to involve the following system. Killer depends upon the combined presence of the dominant gene K and a cytoplasmic substance. The continued production of this substance depends upon gene K; but gene K is unable to initiate its production when none is present. The alternative character sensitive invariably develops regardless of genic constitution when the cytoplasmic substance is absent. A recessive allele of K is unable to determine the continued production of the cytoplasmic substance even when some of it is present. All of the nine pairs of characters examined in five varieties of *P. aurelia* show the same peculiar division of the F_1 into two classes. This suggests that a comparable system of determination and inheritance

is widespread in this species. The significance of the system in relation to a number of problems of biology will be discussed in the next paper of this series.

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GENE AND CYTOPLASM. II. THE BEARING OF THE DETERMINATION AND INHERITANCE OF CHARACTERS IN PARAMECIUM AURELIA ON THE PROBLEMS OF CYTOPLASMIC INHERITANCE, PNEUMOCOCCUS TRANSFORMATIONS, MUTATIONS AND DEVELOPMENT[1]

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The first paper[3] of this series demonstrated that the character "killer" in variety 4 of *Paramecium aurelia* is dependent upon a cytoplasmic substance which normally fails to accompany the "male" gamete nucleus as it passes from one mate to the other during conjugation. The continued production of this determining cytoplasmic substance depends, however, on a dominant gene, K; replacement of K by its recessive allele, k, results in the disappearance of the active cytoplasmic substance. Nevertheless, the gene K is unable to initiate production of the cytoplasmic substance; introduction of K into a sensitive cell is not followed by development of the killer character. But if a non-killer (sensitive) cell containing gene K is supplied with some cytoplasm from a killer cell, or if a genotypically sensitive cell containing the cytoplasmic factor is supplied with gene K, the gene K controls the continued production of the killer cytoplasmic substance. This system of determination and inheritance appears to be typical for all characters in most varieties of *P. aurelia*. The preceding facts may have important applications to other fields of biology. The present paper attempts to point out some of these.

1. Cytoplasmic Inheritance.—Inheritance through the cytoplasm (aside from plastid inheritance) has been reported by a number of investigators