THE GENOTYPIC CONTROL OF KAPPA IN *PARAMECIUM AURELIA*, SYNGEN 4, STOCK 51¹

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THE killer trait in all stocks of *Paramecium aurelia* which have been investigated has been found to depend upon the presence of a cytoplasmic factor, kappa, carried by killers but absent from sensitive animals (SONNEBORN 1943). Kappa is a DNA containing particle of about one micron in size (PREER 1950a; PREER and STARK 1953). When kappa disappears from the cytoplasm of a killer, the animal loses the ability to produce *P* particles (responsible for the killing action) and at the same time becomes sensitive to them (SONNEBORN 1946; PREER 1948).

The understanding of the relation of kappa to the nuclear genes of *Paramecium* has undergone changes with increasing knowledge and is further modified by the results to be presented in this paper. SONNEBORN (1943) early demonstrated the presence of a pair of alleles, *K* and *k*, which play a decisive role in the control of the maintenance of kappa particles. Under standard conditions of culture, kappa and the killer trait are maintained by animals of genotype *KK* and *Kk*, but they are lost by animals of genotype *kk*. When this was the only known locus related to the maintenance of kappa, the possibility of an intimate and direct relation between the allele *K* and kappa was entertained (SONNEBORN 1945). This possibility, first rejected on other grounds (SONNEBORN 1946), was soon rendered untenable by the discovery of a second locus (*S-s*) affecting the maintenance of kappa (SONNEBORN 1947b). Animals of genotype *KK SS* give rise to a "mixed" clone, that is a clone characterized by the presence of sensitive as well as killer lines of descent. Since the sensitives are killed by the action of *P* particles secreted by the killer animals, mixed clones are characterized by the occurrence within them of a specific type of autolethality. A clone of genotype *KK ss* behaves as a pure killer and produces no sensitives under standard conditions of culture. Mixed clones were obtained following crosses between the killer stock 51 and either of the sensitive stocks 29 or 32 in syngen 4 of *Paramecium aurelia*. Stock 51 carries the allele *s*, stock 29—and presumably stock 32—carry the allele *S*. As will appear, two such *S-s* loci have now been found and more are suspected of existing.

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The present paper is devoted mainly to one aspect of the problem of nuclear control of kappa: the number of loci involved and their interactions, both in their effect upon the maintenance of the particles in killers, and their establishment in previously sensitive animals. The nature of the effects of these genes on kappa is dealt with more directly elsewhere (BALBINDER 1957 and in preparation).

MATERIALS AND METHODS

The following stocks of *Paramecium aurelia* from SONNEBORN's collection were employed: stock 31 of syngen 8; stocks 29, 51, d4-186, and d4-22 of syngen 4. All are sensitive except the killer stock 51. Stock d4-186 (referred to as stock 186 by DIPPELL 1950) carries gene *k* (from stock 29) but is otherwise virtually isogenic with stock 51; stock d4-22 carries the same *k* gene and also genes H^{29} (for antigen H) and D^{32} (for antigen D) from stocks 29 and 32, respectively, but is otherwise essentially isogenic with stock 51. Stocks 51 and 29 were the sources of the genes affecting kappa. Stock 31 was used as the detector of killing action. The stocks d4-186 and d4-22 were used mainly for control crosses, the latter when serotypic markers were desired. Serotypic markers were used to establish that true crosses were obtained and also to determine the cytoplasmic ancestry of the clones derived from each of the members of a conjugating pair. The antigens selected, 51H and 29H in some cases, and 51D and 32D in others can be readily recognized serologically. Heterozygotes for either the H or the D types can be easily distinguished from the respective homozygotes (SONNEBORN and BALBINDER 1953, for the H types; SONNEBORN *et al.*, unpublished, for the D types). When these markers were not available, nonallelic antigen markers, such as 51A and 51B (SONNEBORN 1951) were employed. True crosses were distinguished from selfing in some cases by the segregation of known markers (such as *K-k*) in the autogamous F_2 .

The culture medium used for most of the work reported in this paper was a baked lettuce infusion inoculated with *Aerobacter aerogenes*, incubated at 27°C for 24 hrs and finally adjusted to a pH of approximately 6.5 before use. In some cases, a dehydrated lettuce infusion (Difco) and in a few others a commercial rye infusion (Cerophyll) were used as culture media. In all cases *A. aerogenes* was used as food organism. The differences in culture media used had no detectable effect on the results reported in this paper.

The methods employed for making crosses, avoiding and inducing autogamy, testing for killing ability, mating type and serotype, and detecting cytoplasmic exchange were those described by SONNEBORN (1950).

Experimental method

Following a cross, each of the exconjugants was isolated separately into fresh culture fluid and allowed to divide once. One of the daughter cells was tested for serotype, to make sure each pair of exconjugants had yielded one line of each of the serotypically diverse parents. The remaining animal was allowed to develop

into a clone. By a series of daily reisolations, clones derived from exconjugants (F_1 clones) were allowed to undergo about 20 rapid fissions. After the 20th fission, an F_2 generation by autogamy was obtained, each ex-autogamous individual being isolated into a separate culture slide and allowed to produce a clone. After autogamy, only homozygotes are obtained (SONNEBORN 1939, 1947a). Both the F_1 and F_2 clones were tested for killing ability and serotype, and observed for the presence of autolethality after completing 10–11 fissions. Clones were classified as sensitives if killing was observed only in mixtures of the unknown with killers; as killers if killing was observed only in mixtures of the unknown with sensitives; as mixed if killing was observed in both types of mixtures as well as in samples of the unknown by itself.

F_2 clones testing as sensitives at the 10th fission test were discarded, killer and mixed clones being retained for further analysis. The latter were observed under different conditions of culture, as described below, in order to differentiate various types of mixed clones from each other and from pure killers.

Criteria used for identifying the different types of F_2 clones: The characteristics of different types of mixed clones and pure killer clones under a variety of conditions of culture have been described in detail elsewhere (BALBINDER 1957). They will only be briefly summarized here.

Three types of methods were used:

1. Serial subcultures in depression slides at 27°C using ten animals to start each subculture. Generally these were carried through six successive subcultures (about 50 fissions after autogamy). Tests for killing ability and estimates of the percentage of sensitives present were carried out on each subculture.

2. Mass cultures in tubes at 27°C for long periods of time, with frequent testing for killing and autolethality.

3. Exposure to conditions favoring loss of kappa, such as high (35°C) or low (14°C) temperature. At 35°C mass cultures of each clone were allowed to starve for four days, with samples of about 100 animals being withdrawn periodically and subcultured slowly at 27°C. Kappa in killers is progressively destroyed or inactivated in the cytoplasm at temperatures above 33.8°C (SONNEBORN 1947b). However, as long as a single kappa particle remains and is able to reproduce it will restore the normal number of particles after a short period of slow growth at 27°C (PREER 1948). At 14°C the animals were grown with excess food, so that they would reproduce at their maximal rate (one fission per day at this temperature). Under these conditions, kappa multiplies at a slower rate than the paramecia and is gradually diluted out (CHAO 1954).

Three main types of clones could be distinguished by these three methods. The first two methods allow for distinction of the three types of clones on the basis of the frequency of occurrence of autolethality and the percentage of sensitives present at each occurrence, as well as the eventual fate of the killer trait: i.e., whether it is totally lost or retained. The third method differentiates among the three types of clones by the rates at which they become completely sensitive. Loss of the killer trait in mixed clones is due to the disappearance of kappa from

the cytoplasm, possibly by actual destruction of the particles (BALBINDER 1957). The three types of clones and their behavior upon culture by each of the three methods were the following:

1. MS (mixed-sensitive) clones. These showed frequent and abundant auto-lethality by methods 1 and 2; lost kappa completely, the whole clone becoming sensitive after long culture in tubes by method 2, and lost kappa rapidly at both 14°C and 35°C (method 3).

2. M clones (permanently mixed). These sporadically produced sensitives when cultured by methods 1 and 2, never lost their killing ability completely after long periods of culture in tubes by method 2, and lost kappa at intermediate rates by method 3. By cultivation with different combinations of fission rate and temperature, two subgroups of M clones, M_1 and M_2 , could be differentiated (BALBINDER 1957 and in preparation). On testing, these were shown to be genotypically different (see Table 1).

3. Pure killers. These always tested as killers by methods 1 and 2 and lost kappa slowly by method 3. The behavior of pure killer segregants in crosses involving mixed clones was paralleled by pure killer controls.

For each cross, the postautogamous F_2 clones were cultured in parallel series employing two or all three of these methods or minor variations thereof. Thus, the identification of each clone is based on at least two independent criteria. In all experiments, control crosses between the killer or killers used for the experimental crosses and either one of the sensitive stocks d4-186 or d4-22 were performed simultaneously.

RESULTS

Genetic analysis of mixed clones—Number of loci relevant to kappa distinguishing stock 51 from stock 29; their effect on kappa maintenance in homozygotes; their synergism: The homozygous F_2 segregants derived from an F_1 killer after crossing pure killers of stock 51 by pure sensitives of stock 29 gave about 50 percent pure sensitives (kk) as expected. The remaining 50 percent were KK pure killers and mixed clones never deviating significantly from one fourth MS to three fourths non-MS (i.e., M_1 plus M_2 plus pure killers) clones. In six backcrosses to stock 51 killers, selecting an MS segregant from each successive autogamous generation for backcrossing to stock 51, no pure sensitives were obtained after autogamy but the ratio of MS to non-MS clones remained 1:3. In cases where the F_2 clones were more closely observed, four phenotypic groups (MS, M_1 , M_2 , pure killer) were discernible (BALBINDER 1957) in a ratio of 1:1:1:1. These observations suggested that genes at two loci, segregating independently from each other and from the $K-k$ alleles were responsible for the production of mixed clones. Following SONNEBORN's (1947b) system of nomenclature the alleles at each one of these two loci were designated S_1 and S_2 for loss of kappa and, respectively, s_1 and s_2 for kappa maintenance. The phenotypes and genotypes of each class could then be written as follows:

Phenotype	Genotype
Pure killer	$KK\ s_1s_1\ s_2s_2$
M_1	$KK\ S_1S_1\ s_2s_2$
M_2	$KK\ s_1s_1\ S_2S_2$
MS	$KK\ S_1S_1\ S_2S_2$

This hypothesis was confirmed by a number of breeding experiments, the results of which are given in Table 1. On performing the genetic analysis of

TABLE 1

Crosses between MS clones, M clones, and pure killers. Ratios observed in the postautogamous F_2 generation following each cross. In some cases all segregating genotypic classes could be scored, in others only some. All clones involved in these crosses were homozygous KK but varied with respect to the alleles at the loci S_1 - s_1 and S_2 - s_2 . For further explanation see text

Cross		No. of exp.	Autogamous F ₂								Total	χ ²	P
Phenotype	Genotype		Observed M				Expected M						
			Killer	M ₁	M ₂	MS	Killer	M ₁	M ₂	MS			
			s_1s_1 s_2s_2	S_1S_1 s_2s_2	s_1s_1 S_2S_2	S_1S_1 S_2S_2	s_1s_1 s_2s_2	S_1S_1 s_2s_2	s_1s_1 S_2S_2	S_1S_1 S_2S_2			
Killer × MS	s_1s_1 s_2s_2 × S_1S_1 S_2S_2	2	19	14	23	19	18.75	18.75	18.75	18.75	75	2.17	0.6
		1	42	63		37	35.5	71.0		35.5	142	2.14	0.4
		6	385			116	375.75		125.25		501	0.91	0.35
Killer × M (M ₁ or M ₂)	s_1s_1 s_2s_2 × S_1S_1 s_2s_2 or s_1s_1 S_2S_2	2	45	41		0	43	43		0	86	0.18	0.65
M ₁ × M ₂	S_1S_1 s_2s_2 × s_1s_1 S_2S_2	1	113			32	108.75		36.25		145	0.66	0.4
M ₁ × M ₁ or M ₂ × M ₂	S_1S_1 s_2s_2 × S_1S_1 s_2s_2 or s_1s_1 S_2S_2 × s_1s_1 S_2S_2	1	148			0	148		0		148	...	...
M ₁ × MS or M ₂ × MS	S_1S_1 s_2s_2 × s_1s_1 S_2S_2 × S_1S_1 S_2S_2	1	51			45	48		48		96	0.38	0.55

mixed clones, the experiments could be designed so as to distinguish all segregating genotypic classes or only some of them. In general MS segregants were readily recognizable from M and killer segregants, and most experiments were therefore designed to yield a ratio of non-MS (killer and M) clones:MS clones. A few experiments were designed to distinguish all phenotypes. As can be seen, in all cases the observed results were in agreement with expectations. They agree with SONNEBORN's (1947b) observation that stock 29 carries a gene which brings about a permanently mixed condition when introduced into stock 51 killers. It is likely that SONNEBORN's S gene is identical with either S_1 or S_2 .

Tests for additional genes carried by stock 29 which might be involved in the production of mixed clones failed to reveal any (BALBINDER 1957). It is possible, however, that genes at other loci may exist if their individual effects are extremely small and a large number of them is necessary for a detectable manifestation.

No effects of mating type or cytoplasmic exchange between mates on the F_2 ratios were observed, although these were looked for.

Individually, judging by their ability to eliminate kappa on culture at extreme temperatures (method 3), S_1 acts more effectively than S_2 (BALBINDER 1957). It is clear, from the results presented, that the individual effects of each, S_1 and S_2 , are cumulative in the double homozygotes (MS clones). The question now arises as to whether synergism also exists between these alleles and the gene k . What would happen in combinations between S_1 and/or S_2 on one hand, and k on the other? Since kk animals develop into pure sensitive clones, this problem could only be studied in Kk heterozygotes. This is discussed in the next section.

Effects of alleles at the three loci connected with kappa maintenance when one or more loci are heterozygous: The relevant data are summarized in Table 2. The production of sensitive animals in F_1 clones derived from the killer exconjugants at about ten fissions past their origin at conjugation, following crosses between killers and sensitives of different genotypes, was used as a criterion to detect possible effects of various gene changes from the pure killer genotype $KK s_1 s_1 s_2 s_2$. The substitution of k for one K does not in itself accomplish this (cross 11), nor does the replacement of up to three of the four s genes for their S alleles (crosses 7 and 8). However, the substitution of both k for one K and an S for one s did yield sensitives within ten fissions in nearly all clones (15/17, see crosses 4 and 5). Hence it appears that one k in conjunction with one S can accomplish what neither alone can. It would thus seem that k and the S alleles act synergistically.

CHAO (1953) showed that for kappa maintenance the parental genotype of the animals is effective for the first eight fissions following nuclear reorganization, the F_1 genotype becoming operative after that. Although the data in Table 2 indicate that the prezygotic genotype of the killer parent has an effect on determining the proportion of clones showing sensitive animals, they make it clear that the differences observed among the various F_1 's cannot be accounted

TABLE 2

Killer and mixed clones observed at the tenth postzygotic fission among the descendants of the killer exconjugants in crosses between killers and sensitives of different genotypes

Cross no.	Prezygotic genotype of killer	F ₁ genotype	No. of experiments	Total no. F ₁ tested	No. killers	No. mixed	Percent mixed
1	KK s ₁ s ₁ s ₂ s ₂	Kk S ₁ s ₁ S ₂ s ₂	7	148	99	49	32.0
2	KK S ₁ S ₁ s ₂ s ₂	Kk S ₁ S ₁ S ₂ s ₂	1	11	0	11	100.0
3	KK s ₁ s ₁ S ₂ S ₂	Kk S ₁ s ₁ S ₂ S ₂	1	6	1	5	83.0
4	KK S ₁ S ₁ s ₂ s ₂	Kk S ₁ s ₁ s ₂ s ₂	1	7	0	7	100.0
5	KK s ₁ s ₁ S ₂ S ₂	Kk s ₁ s ₁ S ₂ s ₂	1	10	2	8	80.0
Totals			11	182	102	80	...
6	KK s ₁ s ₁ s ₂ s ₂	KK S ₁ s ₁ S ₂ s ₂	7	116	115	1	0.8
7	KK S ₁ S ₁ s ₂ s ₂	KK S ₁ S ₁ S ₂ s ₂	1	10	10	0	0.0
8	KK s ₁ s ₁ S ₂ S ₂	KK S ₁ s ₁ S ₂ S ₂	1	9	9	0	0.0
9	KK S ₁ S ₁ s ₂ s ₂	KK S ₁ s ₁ s ₂ s ₂	1	11	11	0	0.0
10	KK s ₁ s ₁ S ₂ S ₂	KK S ₁ s ₁ S ₂ s ₂	1	18	18	0	0.0
Totals			11	164	163	1	...
11	KK s ₁ s ₁ s ₂ s ₂	Kk s ₁ s ₁ s ₂ s ₂	11	160	160	0	0.0

for by the effect of the parental genotype. A comparison of crosses 1 through 5 shows that in all cases where the killer parent was homozygous for either S₁ or S₂ (crosses 2 through 5) most of the F₁ clones (31/34 or 91 percent) yielded sensitives within ten fissions. On the other hand, when the killer parent was homozygous for both s₁ and s₂, a much smaller fraction of the clones 49/148 or 32 percent did so (cross 1). This was true despite the fact that the F₁ of the latter cross contained one dose of both S₁ and S₂ while in two of the former crosses (4 and 5) the F₁ contained one dose of either S₁ or S₂, not both. However, a comparison between crosses 1, 6, and 11, where the prezygotic genotype was the same, shows that the percentage of mixed clones observed was higher for cross 1 than for the other two, and this is correlated with the F₁ genotype. Since the effects of the parental genotype were only manifested in the cases where the F₁ genotype was propitious for the production of sensitive animals (i.e., crosses 1 through 5 as compared to crosses 6 through 10), it is possible that some sort of synergism also exists between prezygotic and postzygotic genotypes, as well as between alleles at the three different loci within the same genotype. Unfortunately, the number of F₁ clones analyzed is too small to conclude that such an interaction does exist. This deserves further investigation.

The possibility of effects of diverse prezygotic killer genotype make it difficult to infer whether, in the cases analyzed, the magnitude of the effect of S genes on kappa is proportional to their total number in combination with one dose of k. To settle this point, experiments are required in which the same killers are crossed to sensitives of different genotypes with respect to the K-k and S-s loci.

Dosage dependent effects have been reported for the $K-k$ alleles. CHAO (1953) showed that Kk heterozygotes have one half as much kappa as KK homozygotes of the same mating type. Dosage dependent effects have also been observed for the S alleles. Thus, MS clones ($KK S_1 S_1 S_2 S_2$) showed the presence of some sensitives within 10–17 fissions and large numbers of sensitives in 100 percent of the cases by the 30th fission (BALBINDER 1957), while $KK S_1 s_1 S_2 s_2$ heterozygotes followed for more than 30 fissions never showed the presence of sensitive animals, with the single exception shown in Table 2 (cross 6). A single such exception is of dubious significance. These facts, together with the results presented in the preceding section and elsewhere (BALBINDER 1957) suggest that the genes controlling the maintenance of kappa constitute a set of multiple factors which lack dominance and act synergistically but unequally. The order of effect on loss of kappa is: $k > S_1 > S_2$, and $k > S_1$ plus S_2 . In homozygous condition, kk can be thought of as epistatic to all the alleles at the S_1-s_1 and S_2-s_2 loci, since $kk s_1 s_1 s_2 s_2$ animals are sensitive. According to CHAO (1953), kappa is completely eliminated in the latter between the eighth and 15th fissions following the acquisition of the kk genotype. Whether in homozygotes for kk containing at least one of the S alleles, kappa is eliminated before the eighth fission remains unknown.

It should be mentioned that the stage at which the observations were made, the tenth postzygotic fission, did have an influence on the total number of mixed clones observed. This is illustrated by the behavior of MS clones, mentioned above. However, for comparing the effects of the different genotypes on kappa maintenance, the tenth fission test proved adequate.

The differences observed between the different groups of crosses were not influenced by the amounts of cytoplasm exchanged at conjugation between the mates (BALBINDER 1957).

The establishment of kappa in previous sensitives: role of genes k , S_1 , and S_2 : CHAO (unpublished, reviewed by SONNEBORN 1959) showed that the k gene affects kappa establishment in homozygotes. He observed cytologically that in conjugating pairs of Kk killers $\times$ kk sensitives, kappa was transferred to the kk mate when sufficiently broad cytoplasmic bridges were formed, but quickly disappeared when the genotype remained kk . The fact that S_1 and S_2 , as well as k , affect kappa maintenance and act synergistically raises the question of whether the S genes may also affect kappa establishment. A suggestion that this might be the case is found in the behavior of stock 169, a syngen 4 killer isolated from nature which in some ways parallels the behavior of mixed clones. Stock 169 shows spontaneous autolethality in laboratory cultures (REISNER, unpublished). TALLAN (1956, 1957) conducting studies on the establishment of kappa in sensitive animals after exposure to killer breis, showed that kappa becomes established less readily in stock 169 sensitives than in sensitives of stock 51. He showed these differences to be independent of the type of kappa used and dependent on nuclear factors not allelic with $K-k$.

SONNEBORN (1943, 1946) has shown that kappa can be transmitted from a

killer to a sensitive animal during conjugation, if a cytoplasmic bridge forms between the mates and persists for a certain length of time. For example, in crosses between certain killers and sensitives, SONNEBORN (1946) found no transfer of kappa if the bridge lasted less than three minutes; transfer of kappa in only some pairs if the bridge lasted from 3½ to 30 minutes, and invariable transfer of kappa followed by its establishment in the sensitive mate (thus transforming it into a killer) if the cytoplasmic bridge lasted over 30 minutes. Thus, the amount of cytoplasmic exchange must be taken into account when considering the role of the genes in the control of kappa's ability to become established in sensitives.

The results of several experiments are summarized in Table 3. The number of clones derived from the sensitive mate, following several killer × sensitive crosses, in which kappa became established after ten postzygotic fissions as well as those where it did not, are given for different periods of persistence of the cytoplasmic bridge. In cases where a relatively small amount of cytoplasm was exchanged between the mates (bridges lasting 0–5 minutes, for instance), allowing for few kappa particles to enter the cytoplasm of the sensitive mate, it is possible that these were only transmitted to one of the first fission products, and that this one was sacrificed to perform a serotype test. This could have led to an underestimate of the total number of F₁ clones for each cross where kappa was capable of becoming established. However, since all F₁ clones were treated identically, comparison between them to determine the relative ability of different genotypes to influence the establishment of kappa is possible. It is also likely that in some cases the kappa level remained low for the first few fissions after conjugation, with occasional individuals within the clone receiving no kappa

TABLE 3

Relationship between genotype, cytoplasmic exchange (as measured by delay in separation at the paroral cone region following conjugation) and establishment of kappa. Number of clones, derived from the sensitive exconjugant of killer × sensitive crosses, showing no killing ability (sensitives) and killing ability (killers and mixed) after ten postzygotic fissions

Time of sep. at paroral cone (minutes)	Prezygotic genotype of sens. parent	<i>kk S₁S₁ S₂S₂</i>				<i>kk s₁s₁ s₂s₂</i>				<i>KK S₁S₁ S₂S₂</i>			
		Genotype of F ₁											
		<i>Kk S₁s₁ S₂s₂</i>				<i>Kk s₁S₁ s₂S₂</i>				<i>KK S₁s₁ S₂s₂</i>			
		S	K&M	Total	Percent K&M	S	K&M	Total	Percent K&M	S	K&M	Total	Percent K&M
0–5	..	36	1	37	2.7	70	17	87	19.5	6	8	14	57.0
6–23	..	44	6	50	12.0	11	18	29	61.6	10	18	28	64.3
>23	..	6	2	8	25.0	1	3	4	75.0	0	31	31	100.0
Totals		86	9	95	...	82	38	120	...	16	57	73	...

The genotype of the killer parent was always *KK s₁s₁ s₂s₂*, the sensitive parent's genotype being different in each case as shown in the table. Symbols: K=killers; M=mixed; S=sensitives.

at the time of cell division. These would develop into sensitive sublines within a predominantly killer clone, as fission rate decreased towards the tenth fission due to limiting food supply. Under such conditions kappa could reproduce faster than the animals and reach killer concentrations in those individuals possessing particles. Clones containing killer and sensitive animals would test as mixed, and a number of these were observed. Since in these clones kappa reproduction did take place to the extent that killer animals were present, kappa was considered to have become established in them.

As can be seen in Table 3, in all cases the genotype of the killer mate was the same, but the genotypes of the sensitives varied at the $K-k$ and $S-s$ loci. It is therefore likely that in some of the crosses (particularly those where the sensitive mate was homozygous kk), the reduced ability of kappa to become established might have been due to the influence of the parental genotype during the early stages of establishment (before the eighth fission). However, from the fact that kappa did become established in clones derived from kk sensitives after the K gene was introduced, even when the cytoplasmic bridges lasted a short time, it is clear that the prezygotic genotype is not in exclusive control of kappa establishment.

Regardless of whether the effects of the prezygotic or zygotic genotype predominate, two facts emerge clearly from a consideration of Table 3: (1) the S genes do have an effect on kappa establishment and, (2) the action of the k and S genes in affecting kappa establishment is synergistic. This can be seen by comparing the percentage of establishment in clones of all three genotypes for the same duration of the cytoplasmic bridge. Thus, for example, for bridges lasting 0.5 minutes, kappa was established in 57 percent of the $KK S_1s_1 S_2s_2$ clones, 19.5 percent of $Kk s_1s_1 s_2s_2$ clones and 2.7 percent of $Kk S_1s_1 S_2s_2$ clones. In general, independently of the duration of the bridge, establishment was significantly lower in clones heterozygous at all three loci than in those heterozygous at either the $K-k$ or $S-s$ loci. Clearly then, the S_1 and S_2 alleles inhibit kappa establishment, at least when they are in combination with k . Whether S_1 and S_2 by themselves have a detectable effect on kappa establishment cannot be determined since the data necessary for a proper comparison (establishment in animals of genotype $KK s_1s_1 s_2s_2$) are lacking thus far. It is apparent that k by itself allowed for less kappa establishment than S_1 and S_2 together. This is certainly true for those cases where very little cytoplasm was exchanged (0.5 minutes delay); the differences observed for longer delays may not be significant.

As Table 3 shows, the k and S genes do not control establishment of kappa through an effect on the duration of the cytoplasmic bridge but must act after kappa has entered the cytoplasm. This is in full agreement with CHAO's observations mentioned before. The results also point to a relation between the amount of cytoplasm exchanged and genotype with respect to establishment. Thus, for F_1 genotype $Kk S_1s_1 S_2s_2$ kappa was established in 12.0 percent of the clones derived from sensitives when the bridges persisted for 6-23 minutes, while for F_1 genotype $Kk s_1s_1 s_2s_2$ establishment took place in 19.5 percent of the clones

derived from sensitives when the bridges persisted for only 0.5 minutes. Whether establishment in these cases depended on the total number of kappa particles entering the cytoplasm or on different amounts of an essential substance such as, for instance, the cofactor described by TALLAN (1956, 1957), is not known.

We can conclude from these results that the k and S genes act synergistically in controlling kappa's ability to become established in the cytoplasm of previously sensitive animals, in a manner similar to their control of kappa's stability in the cytoplasm of killers. Their effects again appear to be unequal, with $k > S_1$ plus S_2 . Whether the magnitude of the effect is proportional to the total number of S genes in combination with k remains to be investigated.

DISCUSSION

The results presented in this paper confirm and extend SONNEBORN's (1947b) observations showing that the role of the nucleus in controlling the killer trait is not confined to the $K-k$ locus. Two more loci, S_1-s_1 and S_2-s_2 have been demonstrated, one of them probably identical with SONNEBORN's $S-s$ locus. Alleles at all three loci act synergistically both in kappa elimination and establishment. Other cases of hereditary traits controlled by multiple factors have been reported for *Paramecium aurelia* (KIMBALL 1949; PREER 1950b).

Further investigation may reveal that more than the three loci demonstrated thus far are connected with the control of kappa. Some indications have already been found. In crosses between stock 51 killers and stock 32 sensitives for example, the instability of kappa gradually increased in backcrosses with approaching isogenicity to stock 32 (SONNEBORN, unpublished). Another sensitive stock in syngen 4, stock 203, produces no sensitive F_2 clones at the tenth postautogamous fission following a cross to a killer, but 100 percent of the segregants show autolethality (SIEGEL, unpublished).

The presence, in certain stocks, of genes with small individual effects such as S_1 and S_2 , is an indication that, at least in some cases, sensitives could arise in nature from killers. Large mutations, such as $K \rightarrow k$ for example, might be eliminated after the first autogamy as they would render the homozygotes sensitive to P particles, while mutations with a smaller effect would be able to spread through the population. In such an event, changes in the environment (such as a decrease in temperature) by enhancing the expression of the mutated gene or genes, could bring about the elimination of kappa from all individuals in the population (as in the case of MS clones). Once the whole group becomes sensitive, large mutations would have a chance of becoming established. The cases of stock 169, mentioned earlier, and stock 203, are interesting in this connection since both are homozygous for K while apparently possessing other genes, with small individual effects, which are antagonistic to kappa. SONNEBORN and SCHNELLER (unpublished) have found that of 27 syngen 4 stocks isolated from nature, 17 are homozygous for K and 10 for k . Of the former, one third show autolethality in crosses to killers and may have genes similar to S_1 and S_2 . The only kk stocks which have been investigated, stocks 29 and 32 carry S -like genes.

In syngen 2, no case has been found where the killer trait is under the control of a single pair of alleles similar to $K-k$. No indications exist, either, for the presence of S -like genes. SONNEBORN (1959) points out that all tested syngen 2 stocks have a genetic constitution favorable to kappa. The syngen 2 situation needs more extensive investigation.

The relation between kappa and the genes controlling its maintenance: The results presented in this paper showing that alleles at the $K-k$ and $S-s$ loci act synergistically in controlling both kappa maintenance and establishment, as well as the fact mentioned earlier that the alleles at each locus appear to exhibit no dominance, can be interpreted as meaning that kappa is controlled by a set of multiple factors whose individual effects are unequal but whose combined effects are cumulative, each gene contributing a certain amount, the effects of alleles at the three loci being similar in nature. This interpretation is strengthened by the fact that both in the case of the S genes (BALBINDER 1957) and in the case of k (CHAO 1953), kappa is eliminated abruptly, possibly by direct destruction in the cytoplasm. BEALE (1954), on the basis that kappa loss in kk homozygotes is abrupt, has suggested that the control exercised by gene K is not through the supply of some essential substance, but through the maintenance of a cytoplasmic state favorable for kappa reproduction. Kappa is known to be destroyed by a heterogeneous array of agents (antibiotics, heat, radiation, etc.; see SONNEBORN 1959, for review), and it is not impossible that some of the alleles involved in the control of kappa are responsible for the production, or accumulation in the cytoplasm, of substances which are harmful to kappa. On the other hand, as SONNEBORN (1959) has pointed out, the striking relation between dosage of the gene K and the kappa concentration, suggests that this allele might supply an essential requirement for kappa reproduction, the allele k being essentially inactive, and the alleles at both S loci acting in a completely different fashion. At the present time, no decisive evidence is available in favor of either view. It is clear nevertheless, as PREER (1957) has pointed out, that the manner of elimination of kappa by alleles at the three loci indicates that a more complex mechanism than merely supplying or withholding of a substance required for kappa reproduction is involved in kappa control.

The action of the S loci provides some insight into the results reported by TALLAN (1956, 1957) on stock 169. He found that this stock yields fewer killers than does stock 51 when sensitives of both stocks are exposed to infection by the same preparations of kappa, this difference being due to genes at one or more loci not linked to $K-k$. These results, together with the fact mentioned earlier that killers of stock 169 show autolethality, i.e., they produce sensitive animals, can be interpreted in the light of the knowledge of the S loci. Stock 169 is known to carry K ; it may also be assumed to carry one or more S -like loci which are responsible both for the autolethality observed and the lesser success with attempted infection. This, of course, needs direct testing. TALLAN (1957 and in press) believes that the differences between stocks 51 and 169 in their susceptibility to infection are due to a decreased ability of kappa to become established in stock 169 after

penetration. Thus, the stock 169 genes influencing infection seem to act in a fashion similar to genes k , S_1 and S_2 in preventing kappa from becoming established after it has entered the cytoplasm.

TALLAN (1957) demonstrated a cofactor necessary for the establishment of kappa, but not for its uptake, in the breis of killers. On centrifugation of the brei, this cofactor could be separated from the infective unit (kappa). Whether this cofactor, the nature of which is unknown, is only necessary for the initial steps of establishment or whether it is a constant requirement for kappa maintenance is not known. The fact that it is present in killer breis may be an indication that it is always necessary. Since TALLAN reports that fresh culture fluid also enhances infection, although to a much smaller extent than the supernatant fraction of the brei (which contains the cofactor), it is possible that the cofactor is originally supplied by the medium and stored in the cytoplasm of killer animals, where it would be present in high concentration. Nevertheless, TALLAN's finding raises the question of whether any connection exists between the cofactor and the genes controlling the maintenance of kappa.

The role of the cytoplasm and the question of "sigma": SONNEBORN (1947b), in his initial work on the gene S , was led to postulate a cytoplasmic factor "sigma." Sigma was invented to account for two observations:

1. the coexistence of persistent sensitive and persistent killer sublines of descent in clones of a single genotype ($KK SS$); and
2. the transmission by these sensitive sublines, through their cytoplasm, of something which, when introduced abundantly into killers during conjugation, resulted in the production of sensitive descendants.

This something was called "sigma"; it was assumed to compete with kappa. This competition sometimes resulted in the disappearance of kappa (in pure sensitive sublines). The question of whether sigma was produced by gene S , or was merely maintained by it as kappa is by K was left open but the latter was indicated by the existence of pure killer sublines. All of SONNEBORN's comments were set forth as preliminary and tentative, and the full data have never been published.

In the present work, no attempt was made to repeat the studies of SONNEBORN on which the postulation of "sigma" was based. That is, sensitives from M clones (presumably $KK s_1 s_1 S_2 S_2$) were not crossed to killers to discover whether cytoplasmic transfer had an effect on the killer mate; i.e., the production of sensitives after ten postzygotic fissions. However, sensitives from MS clones ($KK S_1 S_1 S_2 S_2$) were studied in this way. No effect of the cytoplasm was found, the results being entirely explainable by an effect of the genotype. Similarly, the effects of cytoplasmic exchange between MS and killers on the F_2 ratios of such crosses were studied and pairs where the exconjugants had undergone no detectable exchange were compared to pairs where the mates had fused to form double animals. The results of these experiments were clear and unequivocal: no cytoplasmic effect was observed, the results being strictly accountable by the presence or absence of the S genes. This means that, whatever it is in mixed clones which is responsible

for the elimination of kappa, it must be gene initiated. Thus, while the observations do not justify the conclusion that "sigma" does not exist, they do not require its existence and can be fully explained by the action of the genes without resort to an additional cytoplasmic factor.

The cytoplasm, however, must play some role since this is where the kappa particles are located and it is difficult to see how the genes may affect them without in some way affecting the cytoplasm first. Some of the observations reported here and elsewhere (BALBINDER 1957), such as the fact that MS clones always lose kappa after long periods at 27°C, and the apparent synergism between the prezygotic and zygotic genotype on kappa elimination mentioned in connection with Table 2, may very well mean that there is an accumulation in the cytoplasm of products of the action of the *S* genes which create conditions unfavorable to kappa. These conditions may persist and become intensified when the proper genes are present, but may disappear very quickly if these are replaced by their alleles. It is possible that some of these products may be transmitted through cytoplasmic bridges at conjugation, but would be lost in the presence of the wrong genotype. The role of the cytoplasm in the production of mixed clones is still in need of thorough investigation, and may yield some important clues as to the nature of the relation between kappa and the genotype.

SUMMARY

1. In syngen 4 of *P. aurelia* the maintenance of kappa in the cytoplasm of killers is controlled by genes at three loci: *K-k*; *S₁-s₁*; and *S₂-s₂*. At each locus two alleles exist: one favoring and the other opposing the maintenance of kappa.

2. The same loci control the maintenance of kappa in killers and its ability to become established in sensitives after it is introduced through cytoplasmic bridges.

3. Both in their effects on maintenance as well as establishment, the actions of the alleles at the three loci are cumulative (synergistic) although their individual effects differ, i.e., *K* is more effective than *s₁* and *s₂* in favoring kappa, and *k* is more effective than *S₁* and *S₂* in opposing it.

The relation between kappa and the genes controlling its maintenance is discussed.

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