Genet. Res., Camb. (1965), **6**, *pp.* 411–418
With 1 *text-figure*
Printed in Great Britain

Maintenance of kappa particles in cells recently deprived of gene K (stock 51, syngen 4) of *Paramecium aurelia**

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(*Received* 14 *September* 1964)

Sonneborn (1943) showed that kappa particles in stock 51 (syngen 4) of *Paramecium aurelia* required the dominant gene K for their maintenance, and Chao (1953) later found that replacement of this gene by its recessive allele (k) resulted in the loss of kappa particles only after a period of between eight and fifteen fissions. Analogous results were obtained by Gibson and Beale (1962) for maintenance of mu particles in stock 540, syngen 1 of *P. aurelia*, controlled by genes M_1 and M_2, and as a result of a detailed study of the latter system the metagon hypothesis was put forward.

The work to be described here was begun with the object of investigating whether the metagon hypothesis could be applied to the mechanism whereby gene K supports the maintenance of kappa particles in stock 51, and if possible of obtaining further information about metagons, since in some respects this material would appear to have technical advantages over the mate-killers previously studied. For example, kappa-bearing paramecia can be identified simply by their ability to kill sensitive cells, or by resistance to killing by other killer cells, and these are processes which do not involve death of the cells under study. However, as will be made clear in the following account, this system has serious technical disadvantages for a metagon-type study, owing to the liability of animals of genotype kk to lose all kappa particles prematurely and irreversibly under certain conditions, especially starvation. This starvation effect will be described here, and its significance discussed.

1. MATERIALS AND METHODS

The stock of *P. aurelia* (all kindly supplied by Dr T. M. Sonneborn) were as follows:

syngen 4: stock 51 (killer), genotype KK, containing kappa; stock d4-57 (sensitive), genotype kk, with serotype marker genes a^{29}, d^{32} but otherwise isogenic with stock 51;

syngen 8: stock 31 (sensitive).

The ability of paramecia to act as killers was tested by a modification of Sonneborn's (1939) and Austin's (1948) methods. This modification will be referred to here as the 'drop method'. A drop (ca. 0·01 ml.) containing 15–30 animals of sensitive stock 31, serotype H (fed for one fission 24 hours before use) was placed on a

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glass slide within a paraffin wax ring just large enough to contain the drop, and the animal to be tested for killing ability was then pipetted into the drop. The slide was inverted over a depression cavity containing a little culture fluid to prevent drying up of the drop, and the contacts sealed with vaseline. Killing of one or more sensitive animals could be observed by microscopic examination through the slide, between three and eight hours after the start of the test (at 25°–28°C.). The surplus unaffected sensitive animals were then immobilized by addition of a drop of anti-31H serum, and the test animal (or two if it had divided in the drop) recovered for further experiments. This procedure was found to give more killed sensitive animals than would be expected from the data of Austin (1948). Sometimes as many as 10 humped sensitives were produced in 12 hours by one killer animal, and this was presumed to be due to the closer proximity of animals in the small drop used here, permitting more rapid uptake by the sensitive paramecia of the killing agents (P particles).

Staining for kappa particles was carried out as follows. Up to 32 animals were placed in a small drop on a cover-slip, the surplus fluid removed, and, just before it became dry, the cover-slip was dropped into fixative (5% acetic acid in N/10 HCl) for 5–30 min. (the exact time being unimportant). With care the complete sample of paramecia was retained on the cover-slip. After fixation the material was rinsed in N HCl, hydrolysed for 6 min. in N HCl at 60°C., rinsed again in cold N HCl, stained in thionin-SO_2 complex for $1\frac{1}{2}$ hours, dehydrated and cleared. (This procedure of hydrolysis and staining was basically the same as that described by De Lamater (1951)).

2. RESULTS

(*i*) *Classification by the 'drop method' of animals recently deprived of gene K*

Heterozygous (K/k) killer paramecia were prepared by crossing stock 51 (killer) with stock d4-57 (sensitive), and then passed through autogamy. Confirmation that the animals had in fact undergone autogamy was obtained by observing segregation of the antigenic marker genes (a^{29} and a^{51}). At various numbers of fissions following autogamy, samples of animals were tested individually for ability to act as killers, using the 'drop method'. About half the clones thus tested yielded killer animals at all stages, and these clones were assumed to be homozygotes of the type KK and discarded. The remaining clones (assumed to be kk) changed to non-killers at various stages and were studied more carefully.

In these tests the paramecia, after autogamy, were grown continuously in surplus culture fluid at 16°–18°C. Considerable variations were found in the stages at which non-killers arose from killers, but it can be stated that all animals tested were killers at the 7th fission stage, and nearly all were non-killers at the 12th fission stage. At intermediate stages various proportions of killers and non-killers were found, as indicated in Table 1.

In Fig. 1, the distribution of killers in certain pedigreed lines is shown. Here the samples for testing were selected by taking, from the 8th fission onwards, the

Table 1. *Proportion of killers among animals 7–12 fissions after loss of gene* K *(Drop Method)*

	No. of fissions following autogamy					
	7	8	9	10	11	12
Clone No.	K U Nk	K U Nk	K U Nk	K U Nk	K U Nk	K U Nk
1	—	55:1:3	44:0:20	21:0:41	4:1:52	0:0:60
2	—	54:2:3	31:0:33	11:0:53	2:1:60	0:0:60
3	58:3:0	—	—	—	—	—
4	60:0:0	—	—	—	—	—
5	—	56:1:3	42:0:9	—	—	—
6	—	58:0:5	46:0:9	—	—	—
7	—	—	20:0:24	—	23:0:26	—
8	—	—	39:0:29	—	—	—
9	—	—	—	2:0:60	—	—
10	—	—	—	59:0:0	—	—
11	—	—	—	—	8:0:16	—
Total	118:3:0	223:4:14	222:0:114	93:0:154	37:2:154	0:0:120
% Killer	100	94·2	65·8	37·7	21·3	0

K = killer; U = classification uncertain due to non-typical killing, taken as killer in calculating percentage; Nk = non-killer.

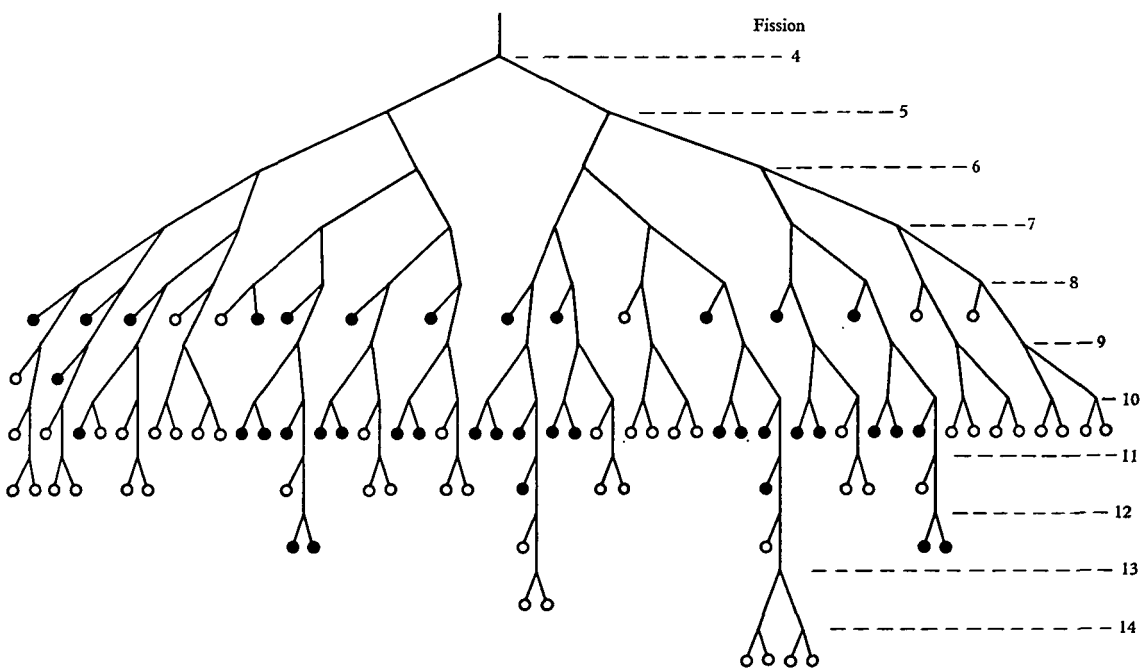

Fig. 1. Pedigree of killer and non-killer paramecia (genotype *kk*) derived from a *Kk* animal in autogamy.

One animal was taken after the 4th fission following autogamy, producing sixteen animals after the 8th fission.

Circles indicate tested animals: black—with kappa particles; white—without particles. Untested animals were allowed to divide further.

https://doi.org/10.1017/S0016672300004298 Published online by Cambridge University Press

progeny of animals whose sisters were themselves killers. In such a selected sample it was found that a few killer paramecia were still present after twelve fissions. These results also show that the distribution of killers within a clone is non-random, i.e. some sub-lines contain many more killers than would be expected from the overall frequency at a given fission stage.

(ii) Classification of kk animals by staining kappa particles

Clones of *kk* animals derived from heterozygous killers were obtained by autogamy as in the previous section and samples of these animals were stained after various numbers of fissions. All 8th fission animals (out of 139 examined) were found to contain stained particles, and from the 9th fission onwards proportions of animals without stained particles were found. However, owing to the effect of starvation on kappa particles in *kk* animals (to be described below), it was considered undesirable to starve the animals for longer than $1\frac{1}{2}$ hours at 28°C. before fixing and staining. Consequently some bacteria always remained and, where the number of kappa particles was small, it could not always be excluded that the stained particles recorded were bacteria rather than kappa particles. Thus at later stages the classification of animals by this method was uncertain. A continuous range of number of particles per animal was observed, but by the 12th fission stage only one animal out of 85 examined contained an appreciable number (> 100) of kappa particles.

(iii) Effect of re-testing kk animals for killing ability after recovery from the drops

It was originally planned to use the drop method to test *kk* animals for killing ability at a given fission stage, recover the tested animals from the drops by eliminating the sensitive animals (of stock 31 H) with antiserum, allow the recovered animals to pass through one or more further fissions, and then test the daughter animals for killing ability again by the drop method. However, it was unexpectedly found that once having been subjected to the conditions in the drops, killer animals of genotype *kk* nearly always transformed to non-killers, as shown by their behaviour when mixed with sensitive animals in a second drop.

Such loss of killing ability invariably occurred when the animals were first tested at the 3rd fission or later stages, and sometimes after only one or two post-autogamous fissions. No exact data on this latter point are available, however, The effect depends on the length of time in the drop and other conditions.

Once killing ability of *kk* animals was lost, it was impossible to restore it by any of the following treatments, each given at 20° and 27°C.: (1) further starvation; (2) growth in excess bacterized medium for three further fissions; (3) feeding in bacterized medium diluted with Dryl's solution (Dryl, 1959) in the proportions 1:1 and 1:3, for three further fissions.

(iv) Staining of animals recovered from drops

Experiments were carried out to determine the effect of placing paramecia in drops at various stages following autogamy of *Kk* animals, on presence of stainable particles in the cytoplasm.

The earliest stage tested was that before the first post-autogamous fission ('zero fission' animals). Animals at this stage, after remaining in drops for four days, were found to retain the kappa particles, (though the macronuclear fragments were lost as has been found by others [Sonneborn, 1947; Gibson and Beale, 1962]).

To show the effect of the drop treatment on 1st fission animals, *Kk* killers in autogamy were fed for 9 hours on bacterized medium and were then placed, before dividing, in drops with sensitives. All acted as killers. After 16 hours at 28°C., the remaining sensitives were killed with serum, leaving two animals per drop, due to one fission of the original killer animals. These pairs of first fission animals were recovered and starved for 3 hours in exhausted culture medium. One member of each pair was stained and examined for the presence of particles and macronuclear fragments; the other member was allowed to undergo further fissions and used to determine the genotype (*KK* or *kk*), and also the serotype, as a useful check that autogamy had in fact occurred. The results are shown in Table 2A and show that kappa particles had disappeared from all *kk* animals, but not from any of genotype *KK*.

Similar experiments were carried out with the 2nd fission animals. Here first fission animals were placed in the drops, found to be killers, allowed to divide once

Table 2. *Presence of kappa particles in 1st and 2nd fission animals obtained following autogamy of* Kk *paramecia (killers) and subjected to starvation (see Text)*

A. 1st fission animals stained

Clone	Kappa	Genotype (of sisters)	Serotype (of sisters)	No. of macronuclear fragments in stained animals
1	*	*KK*	29A	6
2	*	*KK*	51A	4
3	*	*KK*	51A	6
4	*	*KK*	51A	8
5	*	*KK*	51A	6
6	*	*KK*	29A	3
7	†	*kk*	51A	5
8	†	*kk*	29A	5

B. 2nd fission animals stained

9	*	*KK*	29A	3
10	*	*KK*	29A	1
11	*	*KK*	51A	0 or 1
12	*	*KK*	29A	3
13	*	*KK*	51A	4
14	ca. 150	*kk*	51A	3
15	†	*kk*	51A	3
16	†	*kk*	51A	0
17	ca. 50	*kk*	29A	1
18	†	*kk*	51A	1

* More than 200 kappa particles.
† No particles.

and then treated as above. The results are shown in Table 2B. It will be seen that two out of five *kk* animals did in fact retain some kappa particles, though fewer than in any of the *KK* animals; the remaining three *kk* animals did not have any particles. There was no correlation between presence or absence of kappa particles, and number of macronuclear fragments. (Thus, even though the latter presumably contained the *K* gene, this did not affect the ability to maintain kappa).

A few tests were also done on animals at later stages. Some 3rd fission and 5th fission *kk* animals which had acted as killers by the drop method were recovered, washed in exhausted culture fluid for 1½ hours, stained and examined microscopically. They were found to be devoid of particles, except that if stained immediately on recovery from the drops, a few particles could sometimes be seen. The latter may have been either kappa particles or bacteria.

It is therefore clear that placing *kk* killer animals in drops with sensitive animals for 8–12 hours at 25°–28°C. results not only in loss of killing ability, but also in partial or complete loss of kappa particles. This effect takes place with animals at any stage after the first post-autogamous fission of *Kk* animals, but not before. By contrast *KK* animals, at the corresponding fission stage, and treated similarly to the above-described *kk* animals, were always found to contain large numbers of particles.

(*v*) *Further experiments on the effect of starvation on kappa particles in kk animals*

The effect of placing *kk* killer animals in the drops for 8–12 hours would be expected to involve a moderate degree of starvation. The animals would be competing with the large excess of partially starved stock 31 sensitive animals for the small amount of nutrient available in the drops. It therefore seemed likely that the cause of the loss killing ability in the drops was starvation.

Presence of the sensitive animals in the drops was shown to be not directly relevant to this phenomenon, for when 1st-fission *kk* killer animlas were allowed to exhaust the nutrient by leaving them alone for 4–5 days in a drop of bacterized medium sufficient for three fissions, thus exposing the animals to starvation conditions at the 4th fission stage, all transformed to non-killers. Again, when 3rd-fission *kk* animals were placed in exhausted culture medium for 2 hours or more at 28°. at pH 7–8, and then stained, no kappa particles could be seen. *KK* animals at the corresponding fission stage, and subjected to the same treatment, were seen to contain large numbers of kappa particles.

3. DISCUSSION

The results described above show that kappa-bearing paramecia, if deprived of the gene *K* at autogamy and thereafter grown at the maximum rate (i.e. with excess food) at about 18°C., continue to maintain kappa particles in all animals for at least seven post-autogamous fissions; but after twelve fissions few animals contain an

appreciable number of particles, sufficient to make the paramecia act as killers. These results are at variance both with the earlier data of Chao (1953) on the same material, and also with the results of Gibson and Beale (1962) working with mu particles from stock 540, syngen 1. In both these two latter investigations it was shown that the particles (kappa or mu) were maintained in some cells for as long as fifteen fissions following loss of the appropriate dominent gene (*K* or *M*). At present it is not possible to account satisfactorily for these discrepancies.

The fact that rather mild starvation of *kk* killer animals (e.g. 2 hours at 28°C. in exhausted medium, for 7th fission animals), results in an irreversible and complete elimination of kappa particles, introduces a serious technical difficulty into experiments designed to study exactly the rate of disappearance of kappa particles following replacement of gene *K* by *k*. Especially where the number of kappa particles is small their exact observation requires elimination of all bacteria by starvation, yet the latter condition itself modifies the numbers of the very particles being counted.

Nevertheless, the starvation effect, though exceedingly inconvenient from a technical point of view, is of considerable theoretical interest. Assuming metagons are present in this material and behave similarly to those derived from stock 540, syngen 1, two possibilities may be suggested: either (1) the metagons are destroyed by mild starvation, or (2) the metagons are not affected but kappa particles are destroyed because of the absence of some other factor (possibly a nutritional one); and the latter must presumably emanate from gene *K*, since mild starvation of *KK* animals does not eliminate kappa particles.

The starvation effect on animals which have passed through only one fission following loss of gene *K* by autogamy implies that such a gene is active in the young macronuclei even at this stage, for similar starvation of animals whose macronuclei contain the gene *K* does not eliminate the kappa particles. Such a result is unexpected in view of the considerable delay usually found between introduction of a gene by conjugation, and development of the new phenotype. For example antigenic characters apparently require five fissions for their expression (see Beale, 1954). On the other hand Gibson and Beale (1962) found that re-introduction of the gene M_2 in *P. aurelia*, syngen 1, resulted in a renewed ability to maintain mu particles after only one fission. A further conclusion from the starvation effect on 1st fission animals concerns the possible function of the macronuclear fragments. Since the starvation effect was found in some cells which contained macronuclear fragments derived from a macronucleus containing the gene *K*, it has to be concluded that the latter gene is inactive in the fragments.

SUMMARY

1. Heterozygous (*K*/*k*) killer paramecia containing kappa particles from stock 51, syngen 4 of *Paramecium aurelia* were passed through autogamy, and the *kk* offspring tested for killing ability and for presence of stainable kappa particles at various fission stages.

2. After eight fissions, a small proportion of the paramecia were non-killers, and a few lacked stainable particles. After the 12th fission nearly all cells lacked kappa.

3. Mild starvation of the *kk* kappa-bearing paramecia resulted in complete loss of kappa and conversion to non-killers, even when applied to animals only one fission after the loss of gene *K* at autogamy.

4. The results obtained here show some discrepancy with data obtained earlier, both with kappa, and with mu particles in stock 540, syngen 1. The reasons for this discrepancy are at present not clear.

REFERENCES

AUSTIN, M. L. (1948). The killing action and rate of production of single particles of paramecin 51. *Physiol. Zoöl.* **21**, 69–86.

CHAO, P. K. (1953). Kappa concentration per cell in relation to the life cycle, genotype, and mating type in *Paramecium aurelia*, variety 4. *Proc. natn. Acad. Sci. U.S.A.* **39**, 103–12.

DELAMATER, E. C. (1951). A staining and dehydrating procedure for the handling of microorganisms. *Stain Technol.* **26**, 199.

DRYL, S. (1959). Antigenic transformation in *Paramecium aurelia* after homologous antiserum treatment during autogamy and conjugation. *J. Protozool.* **6** Suppl. 25.

GIBSON, I. & BEALE, G. H. (1962). The mechanism whereby the genes M_1 and M_2 in *Paramecium aurelia*, stock 540, control growth of the mate-killer (mu) particles. *Genet. Res.* **3**, 24–50.

SONNEBORN, T. M. (1939). *Paramecium aurelia:* mating types and groups; lethal interactions; determination and inheritance. *Am. Nat.* **73**, 390–413.

SONNEBORN, T. M. (1943). Gene and cytoplasm. I. The determination and inheritance of the killer character in variety 4 of *Paramecium aurelia*. II. The bearing of determination and inheritance of characters in *P. aurelia* on problems of cytoplasmic inheritance, pneumococcus transformations, mutations and development. *Proc. natn. Acad. Sci. U.S.A.* **29**, 329–43.

SONNEBORN, T. M. (1947). Recent advances in the genetics of *Paramecium* and *Euplotes*. *Adv. Genet.* **1**, 264.