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Kappa, mu and the metagon hypothesis in *Paramecium aurelia**

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1. INTRODUCTION

The metagon hypothesis was proposed by Gibson & Beale (1961, 1962) to account for the kinetics of loss of the bacterial symbiont, mu 540, following the replacement in its host, *Paramecium*, of genes M_1 and M_2 by their alleles m_1 and m_2. The hypothesis held that loss of mu is due to loss of a non-replicating entity, the metagon, which is produced and maintained as a large population in paramecia possessing one or more M genes, but which ceases to be further produced and is nearly randomly distributed to the progeny cells after the genotype changes to $m_1m_1m_2m_2$. Metagons were further characterized as being destroyed by RNase (Gibson & Beale 1963), remarkably stable *in vivo* (Gibson & Beale, 1962) and *in vitro* (Gibson & Beale, 1964) and infectious (Gibson & Beale, 1964). Gibson & Sonneborn (1964) reported that RNA extracted from $M_1M_1M_2M_2$ paramecia which specifically hybridized with DNA from the same paramecia has high metagon activity and that the metagon replicates rapidly like a virus when introduced into the ciliate *Didinium nasutum*.

The obvious theoretical importance of a link between mRNA and viruses led to the attempts reported here to try to confirm the above observations and extend them to other paramecium-symbiont systems. Comparable results have not been consistently achieved with mu 540, with mu 138 and mu 130 of syngen 8 or with kappa 51 of syngen 4, and many results are not compatible with the metagon hypothesis as it was originally developed.

2. MATERIALS

The syngen 1 stocks, 540, 513, and T_7 have been described by Gibson & Beale (1961). The syngen 8 stocks are from the Sonneborn collection: stock 138 is of genotype MM and is a mate-killer by reason of carrying mu 138; stock 130 is also of genotype MM and a mate-killer, but with a different mu, mu 130. Stock 151 is of genotype mm, lacks mu, and is therefore sensitive. Of the syngen 4 stocks, three

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are from the Sonneborn collection: stock 51 is a killer bearing kappa 51 and is of genotype *KK*; 51S is identical except it has no kappa and is therefore sensitive; d4-2 is largely isogenic with these two stocks but has the *k* allele from stock 29, lacks kappa 51, and is therefore also sensitive. Two stocks were kindly supplied by Dr Janine Beisson: stock 401 originated from a cross between stock 51 and a branch of stock d4-2 which bears a u.v.-induced recessive temperature-sensitive mutation, ts_{401} and is of genotype $KKts_{401}ts_{401}$ with kappa 51; stock 111 is a *kk*-sensitive derived from d4-2 which carries a recessive temperature-sensitive mutation, ts_{111}, which is unlinked to either ts_{401} or *K*. Stock d4-79 is a *kk*-sensitive homozygote for both ts_{111} and ts_{401} derived for these experiments by a cross of stock 401 to stock 111 and a backcross to stock 111.

Stock 21 of *Didinium nasutum*, the only stock used in this work, was collected from Twin Lakes, near Bloomington, Indiana.

3. METHODS

(i) *Culture methods*

During the course of these experiments three culture media—baked lettuce, cerophyl and Scotch grass—and two buffer systems—$CaCO_3$-$Ca(OH)_2$ and Na_2HPO_4 (0·6 g/l.)—were used in various combinations. No consistent differences in results were found with the different combinations of medium and buffer, all of which were inoculated with *Aerobacter aerogenes*. Most of the clones were maintained at room temperature with excess food in daily reisolation lines, the number of fissions since conjugation or autogamy being recorded daily.

(ii) *Methods used in studying the kinetics of loss of symbionts*

In all experiments designed to study symbiont loss, heterozygous killers were either backcrossed to homozygous recessive sensitives or made to go through autogamy. In either case, equal proportions of two classes of clones are expected. One class should be heterozygous (following a backcross) or homozygous dominant (following autogamy) and maintain kappa or mu; the other class should be homozygous recessive and therefore unable to maintain the killer organisms. These classes could not be immediately distinguished in daily reisolation cultures in excess food so all clones were carried until they produced sensitives or until a 1:1 segregation became apparent and no new sensitive lines were observed for several days. At this point clones which still had mu or kappa were classified as carrying a dominant allele and some of the kappa-bearers were subjected to further genotypic analysis. This laborious method was used instead of the usual easier and more rapid method which identifies *KK* or *Kk* clones by their retention of killing activity for 10–15 cell generations after fertilization and through one or two periods of mild starvation because the latter method—although it yields statistically good 1:1 segregations—involves the risk of overlooking rare clones of special interest: *kk* clones that maintain kappa unusually long. A small experiment indeed showed this risk to be real since a few clones, not sensitive by the rapid method after

13–14 cell generations, did produce sensitives by the other method after 20–45 generations.

Mu-bearing cells were identified microscopically by the methods of Gibson & Beale (1961) or of Beale & Jurand (1966). Tests for kappa were made by adding to the cells to be tested (usually 3–30 cells in 0·1–0·2 ml. of culture fluid) a drop of homogenate of stock 51 killers. The homogenate was made by forcing a killer culture, concentrated to about 10^4/ml, through an injection syringe onto a glass surface. All living cells were removed from this homogenate before it was used. After at least 12 h at 27 °C the mixtures of animals and killer homogenate were examined for cells exhibiting stages of the hump-killing specific to the action of stock 51 killers on sensitive paramecia. The clones were then classified as sensitive if all of the animals were affected, mixed if both sensitive and resistant cells were present, and resistant if no killing occurred. When, as happened occasionally, the homogenate of stock 51 failed to kill all of the cells in control tests on equal numbers of sensitive cells (stock d4-2 or d4-79) grown under the same condition as the experimental cells, the results with that homogenate were discarded.

In several experiments with syngen 4 animals the temperature-sensitive mutations present in the stocks were used as marker genes to confirm that normal cross-fertilization had taken place in both the original cross of killer by sensitive and the backcross. To test for temperature-sensitivity, one animal from each clone was isolated, put at 37 °C, and observed after 24 and 48 h. Cells homozygous for either or both ts genes produce usually 4–16 cells then die at the high temperature whereas non-temperature-sensitive cells produce at least 64–128 viable cells, depending upon the amount of food available. In cases where the result was questionable, several cells were tested before classifying a clone. In all experiments the observed segregation was in good aggrement with that expected.

(iii) *Exposure of killers to RNase*

Animals to be treated with RNase came from tube cultures that had been grown for one fission per day by limiting the amount of food supplied (method of Preer, 1948) and had not yet exhausted their food supply when they were used. A mixture of equal volumes of the culture and of RNase in Dryl's (1959) salt solution, pH 6·8–7·1, was incubated at 27 °C overnight. Survivors were washed and isolated into fresh culture fluid, allowed to divide several times, and the progeny examined for mu or tested for kappa. All RNase preparations were tested for activity on RNA (reagent grade from Nutritional Biochemical) by the method of Dickman, Aroskar & Kropf (1956).

(iv) *Purification of kappa*

Purified preparations of kappa were obtained by the ECTEOLA column technique as described by Smith-Sonneborn & Van Wagtendonk (1964) except that the initial homogenate was added directly to a slurry of 1·5 g ECTEOLA in 50 ml of sodium phosphate buffer, another 50 ml of buffer were used to wash this column, and the combined effluent was passed through a second column prepared with

0·5 g of ECTEOLA. The effluent from the second column was used to obtain the kappa pellet. In some of the experiments the purified preparation was exposed for 1 h at 27 °C to various concentrations of RNase (Worthington) dissolved in phosphate buffer. Bentonite was then added at a final concentration of 1 mg/ml to adsorb the RNase, and most of the Bentonite was removed by centrifuging at 1500 ***g*** for 5 min. This also removed an undetermined proportion of the kappa, but enough was left to form a pellet when the supernatant was centrifuged at 10000 ***g*** for 15–20 min. This pellet was resuspended in 2–3 ml of phosphate buffer.

(v) *Infection of didinia with purified kappa 51*

The introduction of kappa into didinia was achieved by incubating at 27 °C overnight a mixture of about 0·1 ml bacterized cerophyl medium, 0·1 ml cerophyl medium containing approximately 100–200 paramecia, one or more didinia, and a drop of purified kappa suspension. If, as sometimes happened, the didinia in this mixture were quickly killed, another mixture was prepared with the same components, except that the drop of kappa suspension was taken from a more dilute suspension—1 part of the original suspension to 9 parts of phosphate buffer. After overnight incubation, the surviving didinia were either left in the infection mixture and fed additional paramecia or were transferred to standard cerophyl medium containing paramecia. They were subsequently fed whenever they had cleared their culture of paramecia.

(vi) *Tests for maintenance of kappa 51 by didinia*

A modification of the method of Sonneborn (1965) was employed. Didinia were disrupted by forcing approximately 0·5 ml of Didinium culture (approximately 500 cells) through a hypodermic syringe fitted with a Swiny hypodermic adapter (Millipore Filter Corporation) and a 25-gauge needle. One drop of this homogenate was added to about 200 sensitive paramecia (detectors) in 0·2 ml medium, another to about the same number and concentration of killer paramecia (controls, resistant to killing action). These mixtures were kept overnight at room temperature and observed the next day for paramecia exhibiting stages of hump-killing. If hump-killing was observed only in the mixture containing sensitive paramecia, the didinia were classified as killers bearing kappa 51; if in neither mixture, the didinia were classified as non-killers lacking kappa 51. Non-killers were retested several times since it was found that some cultures which were non-killers on the first test later became killers. After the third test no new killer clones appeared.

4. RESULTS

(i) *Kinetics of loss of killer organisms following loss by the host paramecia (at conjugation or autogamy) of the gene necessary for their maintenance*

(*a*) *Mu 540.* Gibson & Beale (1962) report loss of mu 540 by an increasing percentage of cells from 0 in generation 7 to 4–8 in generation 8, about 50 in generation 11, and more than 90 in generation 15, and they initially based the metagon hypothesis

on such figures. In four experiments we failed to obtain similar results. In three of the experiments *no* cells with mu were found at the earliest observations (generation 5 or 6). In the 4th experiment on 12 lines from nine clones, four lines were

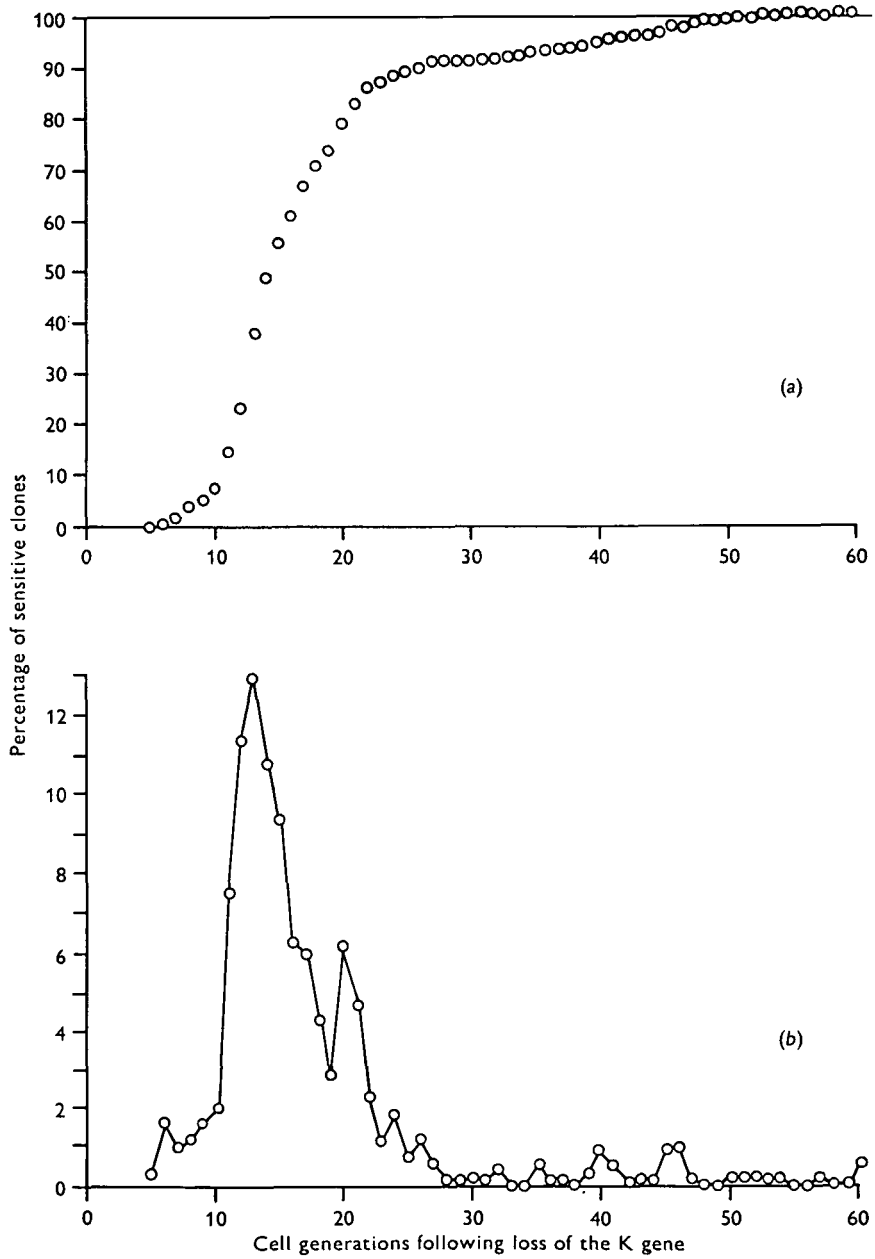

Fig. 1. The percentage of sensitive clones at each cell generation following the change of genotype from *KK* to *kk*. (*a*) The cumulative percentage of *kk* clones which had produced some sensitive cells at each generation. (*b*) The same data plotted as the percentage of clones which *first* began producing sensitives at each generation. The relatively constant, low rate of loss after about 30 generations is obvious in this plot.

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partially and six completely mu-free before generation 8; three lines contained some cells with mu at generation 8 or 9, one at generation 12, and none at later observations.

(*b*) *Mu 138.* Four comparable experiments were run on the mate-killer, mu 138 of syngen 8. As with mu 540, in three of the experiments no cells with mu were found at the earliest observations (generations 4, 5 and 7 respectively); but in one experiment a pattern resembling that reported for mu 540 by Gibson and Beale was found: at generations 7, 9, 10, 13 and 17 the precentage of mu-free cells in the only surviving mm clone was 9, 28, 20, 75 and 90, respectively.

(*c*) *Kappa 51.* Because mu 540 and mu 138 are technically much more difficult to work with than kappa 51, and because Chao (1953) had reported an 8–15 fission lag in kappa 51 loss comparable to the lag reported for mu 540, this system was investigated more extensively. Figure 1 includes data on 523 sensitive segregant clones from the total of 1025 clones obtained in five experiments, the remaining 502 being resistant. Figure 1*a* shows the cumulative percentage of *kk* clones which had produced sensitives at each fission; Fig. 1*b* shows the same data plotted as the percentage of *kk* clones first producing sensitives at each fission. Although the five experiments did not give exactly comparable patterns of loss, no consistent correlation between these differences and any known differences in the experimental conditions was found.

In order to compare the results in Fig. 1 with those of Gibson and Beale, the different method of scoring and its effects on the curve need to be noted. Whereas Gibson & Beale (1962) scored the percentage of cells free of mu using different clones for each generation examined, we scored the percentage of clones with at least one sensitive cell using samples of the same clone repeatedly until some sensitives were found. After each reisolation from the single line of descent of a clone, the remaining animals (usually 7–31) were tested for resistance to hump-killing by killer homogenates and the generation at which any sensitive cells were first observed in the clone was recorded. These data would be exactly comparable to those of Gibson and Beale, on their implied assumption that all clones show the same kinetics, if only one cell of a clone were examined at each test. However, since 7–31 cells per clone were examined, and usually some resistant cells were also present when sensitives were first observed, the percentage of sensitive *clones* is higher than the percentage of sensitive cells. Thus, for our data to agree with those of Gibson and Beale, the points on our curves should be consistently higher than theirs. This expectation is realized only for generations 5–7 when nearly 3 % of our *clones* had begun to produce sensitives, while Gibson and Beale found no *cells* lacking mu during this period. After generation 8 our points are invariably and significantly lower than theirs. For example, in generation 11, when half of Gibson and Beale's *cells* lacked *mu*, only 15 % of our *clones* were found to contain sensitive cells. With their results and their implied assumption that all clones show the same kinetics of loss, nearly all clones they examined at this stage would have been scored as containing at least one mu-free cell even if as few as 7 cells per clone had been examined.

The original metagon hypothesis predicts extremely low probabilities of finding mu 540 in *all* cells of even small samples of clones at generation 12 or later. Yet no cells lacking kappa 51, as measured by sensitivity to killing, were detected in 75 % of the clones examined at generation 12, 44 % at generation 15, 20 % at generation 20, 7·5 % at generation 30, 5 % at generation 40, and 1·5 % at generation 60. Several later experiments, although designed specifically to study other problems, gave comparable results. The data of Fig. 1 are thus obviously at variance with the kinetics of loss of mu 540 on which the metagon hypothesis was originally based. In some of the experiments the genotypes of clones that remained resistant after about 50 % of the clones had produced sensitives, i.e. after the expected 1 : 1 ratio had been approximated were studied by subcloning, permitting the subclone to undergo autogamy (by starvation), isolating nine autogamous cells, and testing the resulting exautogamous clones for resistant (or killer) *v.* sensitive phenotypes. The parent clone was diagnosed as *KK* if all nine clones tested as resistant, as *Kk* if some were of each type, and as *kk* if all were sensitive. When a group of nine included only resistant or some sensitive and some resistant lines on the first test (clonal age 8–10 generations), the resistant ones were cultured for 8–10 more generations and retested; and this procedure was repeated until no further sensitive lines were found. Clones which were diagnosed as *KK* or *Kk* by the autogamy test never produced sensitives in the main experiment. In all cases but one, clones diagnosed as *kk* by the autogamy test eventually produced sensitives in the main experiment. In fact, the autogamous progeny of the still resistant *kk* clones were all sensitive on the first test in agreement with Chao's (1954*a*, *b*, 1955) report that *kk* clones which have maintained kappa unusually long lose it promptly after autogamy. The one exception referred to above was a clone that produced at autogamy nine sensitive clones but never produced sensitives in the main experiment. A second and larger group of autogamous progeny from this clone consisted of 18 resistant and 27 sensitive lines. The total (18 : 36) deviates significantly from the 1 : 1 expected of a heterozygote. Perhaps this clone was *kk*, but capable of maintaining kappa even after autogamy.

An attempt was made to test whether resistance in *kk* clones is due to the presence of kappa and not to some unknown factor. Several *kk* clones which had produced no sensitives by 25 fissions were later tested for the ability to kill. In three such lines, one after 27 fissions, one after 36 fissions, and one after 45 fissions, some typical hump-killing of sensitive tester-cells was observed. The genotype of these lines was almost certainly *kk* since all three, following autogamy, gave rise to only sensitive clones.

(ii) *Trails of inheritance*

The most convincing evidence that one metagon was necessary and sufficient to maintain many mu 540 in a cell and that metagons were nearly randomly distributed at cell division with little or no loss or replication was the trail of mu 540 in only one or a few descendants of a 15th generation $m_1m_1m_2m_2$ mu-bearing cell. At this generation any cell that contains mu must, on the metagon hypothesis, contain

at least one metagon and in any reasonable sample none will contain more than two or three. If such a cell goes through three more divisions, usually only one, rarely two or three, of its eight progeny should have mu, and this is exactly what was reported to occur by Gibson & Beale (1962).

It was impossible to try to confirm this prediction with either mu 540 or mu 138 since both were usually lost from *all* cells before generation 15. Generation 15 cells in the kappa system could not be used because a very high percentage of these cells still were resistant. In fact, no suitable generation could be found. However, it was possible to use lines which were in the process of producing sensitives. Fifty-six resistant cells from six lines that contained both resistant and sensitive cells were isolated and their progeny tested after 2–4 further generations. Of these 56, three produced only resistant progeny, three produced only sensitives, and the remainder produced both resistant and sensitive progeny. Eighty-nine resistant cells from the 100% resistant and the mixed groups were reisolated, but 79 of them died in typical hump-killer fashion before dividing. (If these 79 were reclassified as sensitives, the above results would be shifted to one 100% resistant, 22 100% sensitive, and 33 mixed groups of progeny.) The progeny of the ten survivors after 2–4 more generations were again tested. Nine produced exclusively sensitives and one produced one resistant and 15 sensitive cells. This single resistant cell was isolated and after four more generations, it produced six resistant and ten sensitive progeny.

Clearly resistance shows no trail of inheritance. Whether or not this excludes a trail of inheritance of metagons depends upon the significance attached to the results of tests for resistance and sensitivity. In these experiments resistance signifies the presence of kappa 51 and could not be due to a weak or inactive killing homogenate since the homogenate used killed 100% of the tester-sensitive cells. The interpretation of sensitivity is more open to question. The real problem arises from Chao's (unpublished) report (cited by Hanson, 1957) that animals with fewer than about 25 kappa are sensitive. If a sensitive cell has kappa, it should have at least one metagon, so the important question is whether our sensitives did or did not have kappa. Two observations are relevant to this point. First, when cells from a line which had produced only sensitives in the tested samples were examined by phase-contrast microscopy kappa could never be seen in any cells. Secondly, when samples of such lines were carried for several fissions after they had ceased producing resistant cells, no new resistant cells were produced as would be expected if the level of kappa were fluctuating but some was always present. These observations are consistent with and point to the conclusion that these sensitives did indeed lack kappa. This indicates, on the metagon hypothesis, that they also lacked metagons. Therefore the data presented in the preceding paragraph justify the tentative conclusion that trails of inheritance of metagons do not regularly occur in the kappa system.

Other observations also suggest that a trail of inheritance does not occur in this system. Several lines of descent were followed many generations after they first began producing sensitives to ascertain whether the first production of sensitives is indicative of the approach to 100% sensitivity in a line. In some cases it is not. Of

four such lines followed, one first produced some sensitives on two successive tests (generations 10 and 14), none on the next two tests (generations 18 and 22), and only sensitives at the next test (generation 25). The second line produced some sensitives on successive tests at generations 8, 12 and 16, and then none until generation 61. The third line first produced some sensitives at generations 13 and 16 but no more until generation 40. The fourth line produced some sensitives in the tests at generations 11 and 15, then only resistants until the tests at generations 22 and 26 when some sensitives were present, and again produced only resistant cells until the test at generation 38. Apparently some sensitive cells may arise early in a clone in which the progeny of sister cells are 100 % resistant for many generations. Again, this is at variance with the metagon hypothesis *if* sensitivity in the cells which are produced early is due to the loss of metagons. If this were true, the sister cells of these sensitives should have only a few metagons to be distributed to their progeny, and the chance of continually reisolating a metagon-bearing cell would be negligible. Such results would weigh even more against trails if resistance is assumed to require the presence of more than one metagon. Only if the decline of kappa to the level of sensitivity is assumed to be independent of low levels of metagons, are these observations irrelevant to the question of trails and of the metagon hypothesis in general. This point cannot be tested in the absence of a critical test for metagons, so it must remain an open question.

(iii) *RNase effect*

Gibson & Beale (1963) reported that, while RNase does not directly destroy mu 540, it causes the loss of the symbionts after one fission of $M_1M_1M_2M_2$ mate-killers which have survived a 12–24 h exposure to a concentration sufficient to kill 90 % or more of the cells. This was interpreted as meaning that mu 540 is protected from destruction by an RNase-sensitive substance identified as the metagon. This RNase effect was the first indication of the RNA nature of the metagon itself or of an essential component of it.

Kappa 51 is also apparently unaffected by RNase since exposure to concentrations as high as 100 mg/ml did not destroy the ability of purified kappa to infect and reproduce in cells which were genically capable of maintaining it. However, attempts to confirm the loss of mu 540, mu 138, mu 130 and kappa 51 following RNase treatment have been completely unsuccessful (Table 1). Such results raise questions about the dependence of these killer organisms on an RNA entity.

(iv) *Attempts to demonstrate metagons in* Didinium-*kappa system*

Certain stocks of *Didinium nasutum*, a ciliate that feeds almost exclusively on living paramecia, can maintain mu or kappa which are introduced into them via the paramecia they eat even if the didinia are thereafter fed only paramecia that lack mu, kappa and metagons (Sonneborn, 1965; Gibson & Sonneborn, 1964). Gibson & Sonneborn (1964) and Gibson (1965) reported that mu 540 persists only in didinia which have eaten metagon-carrying paramecia. According to Gibson (1965) didinia which have been supplied with metagons can maintain them in-

definitely without being resupplied, but lose them upon encystment or following exposure to RNase. Experiments were designed to extend this work using purified preparations of symbionts, but it could not be done with mu because the stocks of didinia that maintained mu 540 have died and attempts to isolate stocks which maintain mu 540, mu 138, or mu 130 have been unsuccessful. On the other hand,

Table 1. *Maintenance of mu by* MM *cells and kappa by* KK *cells following RNase treatment*

(In all experiments a series of concentrations of RNase was used but only the one or two concentrations giving the highest percentage of death with any survivors are reported.)

Stock no.	Source of RNase	No. animals per ml before treatment	RNase (mg/ml)	Approx. % death	No. survivors isolated	No. fiss. following RNase treatment when examined	Cells with mu or kappa (%)*
540	Worth.	60	0·5	97	6	5–7	100
540	Worth.	10	1·0	97	1	3	100
540	Worth.	10	0·5	77	3	4	100
540	Sigma Nutri.	450	0·5	97·5	10	4–6	100
138	Biochem.	100	0·5	60	40	2	100
138	Worth. Nutri.	120	0·5	87·5	9†	4	100
138	Biochem. Nutri.	500	1·0	98·5	6	3	100
130	Biochem. Nutri.	500	0·6	90	25	3	100
130	Biochem.	500	0·4	66	15†	3	100
401	Sigma	50	0·125	94	3	9	100
(51 kappa)	Sigma	50	0·06	90	5	5	100

* All progeny of the survivors of stocks 540, 138 and 130 were examined for mu by phase-contrast microscopy. The groups of progeny from the stock 401 survivors were tested for the ability to kill sensitive tester cell in the typical kappa 51 hump-killed fashion.

† In these two cases there were more survivors but they were not isolated.

stock 21 of *Didinium* maintains kappa 51, so this system was used. Didinia of stock 21 were exposed to purified kappa preparations and different samples fed various stocks (51, 51S or d4-2) of paramecia for about 12 h. Subsequently the didinia of all three sets were fed only stock d4-2 for at least a week and then were tested for killing activity. The results in Table 2 show that, regardless of which paramecia were used as food, the didinia acquired and maintained kappa although, by hypothesis, stock d4-2 has no metagons. The experiment was repeated using kappa preparations which had been treated with various concentrations of RNase to destroy any possible contaminating metagons. The results were the same: didinia acquired and maintained kappa just as well when fed stock d4-2, genetically incapable of producing metagons, as when fed stock 51S which should by hypothesis, contain metagons (Table 2). Finally, the experiment was repeated once more

using didinia which had been encysted for at least 6 months and then exposed to RNase to avoid the possibility that they were carrying metagons from a previous unknown infection. The results were the same (Table 2).

These results demonstrated that, at least in this case, didinia do not require RNase-sensitive metagons from paramecia to acquire and maintain kappa 51, as they reportedly do for mu 540.

Table 2. *Acquisition of purified 51 kappa by stock 21 didinia*

(All RNase treatments of the purified kappa preparations were carried out at 27 °C for 1 h. The didinia were exposed to RNase at 27 °C overnight. Each sample of didinia tested consisted of the progeny of one to about ten didinia exposed to the infection mixture.)

Treatment of didinia	Initially fed on *Paramecium* stock	Concentration of RNase with which kappa treated (mg/ml)	No. samples tested for killing	Samples of didinia showing killing (%)
None	(*kk*; no kappa) d4–2	0	14	85·7
None	(*KK*; no kappa) 51S	0	13	85·4
	(*KK*; kappa) 51	0	10	40·0
None	d4-2	0	9	0
	d4-2	25	9	66·7
	d4-2	50	8	82·5
	d4-2	100	8	87·5
None	51S	0	8	50
	51S	25	8	37·5
	51S	50	8	87·5
	51S	100	4	50
Six months encystment and 0·5 mg/ml RNase treatment of encysted didinia	d4-2	0	16	75
	d4-2	100	17	59
	51S	0	17	82
	51S	100	18	78

5. DISCUSSION

Repeated efforts by a number of workers to obtain consistently the kinetics of loss of mu 540 from $m_1m_1m_2m_2$ paramecia on which the metagon hypothesis was first based have failed. I. Gibson (personal communication), G. H. Beale (personal communication), and Beale & McPhail (1967) all have reported variation comparable to that reported here in the kinetics of loss, and they have been unable to find any conditions which will regularly yield the type of kinetics originally reported although this is still occasionally observed. Likewise several investigators have reported considerable variation in the loss of kappa from *kk* cells. Yeung (1965) found that kappa 51 was retained by all *kk* cells for as long as seven generations under some conditions but never found more than a very small fraction of cells with kappa by the 12th generation. Under other conditions he reported loss by

all cells after the first division. Widmayer (1966 and personal communication) found that both kappa 51 and one of its mutants, kappa 51m43, persisted for only 4–7 generations. Beale & McPhail (1967) reported loss as early as the 8th generation (the earliest examined) but also found retention of kappa by some lines for as long as 26 or 27 generations.

Only two factors—growth rate and food supply—have been convincingly shown to affect kappa maintenance, and these effects apparently vary under different conditions and in different experiments. Beale & McPhail (1967) gave evidence that *kk* lines which, for unknown reasons, reproduced even slightly slower than sister lines lost kappa earlier. In the five experiments reported here, no such correlation between growth rate and time of loss could be established. Widmayer's (1966 and personal communication) rapid loss of kappa 51 and kappa 51m43 could be due, at least in part, to restricting the reproductive rate to 1 fission per day by limiting the food supply. Yeung (1965) reported that even moderate starvation (2 h or more at 28 °C in exhausted culture fluid) of *kk* cells led to rapid loss of kappa 51 at any time after the first fission following the change of genotype. Experiments designed to confirm this result have shown no detectable effect during at least the first ten generations of even rather severe starvation (12–24 h at 27 °C in non-nutritive salt solution or exhausted cerophyl or baked lettuce media). Such severe starvation at generation 12 or later may hasten the loss of kappa. The impression left by all the reports is that, while starvation and growth rate probably influence the maintenance of kappa 51 in *kk* clones, other as yet undetermined factors are also important.

Are all these results reconcilable with the metagon hypothesis as it was originally proposed? Do they exclude the possibility of any sort of metagon system? Beale & McPhail (1967) note that 'the agent supporting kappa particles may be unstable, especially under conditions producing sub-optimal growth, and secondly, the long continued maintenance of kappa in some branches of a clone, supports the notion of a replicating determinant. Thus these results certainly do not support the metagon theory as previously proposed. If this theory is to be maintained any longer, we shall have to postulate the existence of accessory factors which (*a*) cause the metagon to become unstable, and (*b*) cause it to replicate.' Our kinetic data agree that, *if* there is actually a discrete entity produced by $K(M)$ and not $k(m)$ cells, it must be conditionally unstable and able to replicate. They further suggest that a modified metagon hypothesis, to be workable, should probably include two other postulates. First, the rate of metagon replication should be inversely proportional to the number of metagons per cell, varying from zero or near zero when many metagons are present to slightly more than one duplication per paramecium generation at very low metagon concentrations. Secondly, the adjustment of the rate of metagon replication to metagon concentration is not immediate but follows a lag. With these assumptions, the metagon concentration in *kk* cells could decrease markedly in the early cell generations so that, during generations 8–25, kappa-free cells are produced with a high frequency. Then the metagon level, in cells still having any, could rise to a statistical equilibrium value low enough to yield, by random

distribution at cell division, a steady low probability of metagon-free cells. In such a case, the long tail of the kinetic curve (Fig. 1) beyond generation 25 would be expected.

Such a modified metagon hypothesis could also formally explain the failure to find trails in many cases. Yeung (1965), Beale & McPhail (1967) and Widmayer (1966 and personal communication) have all observed, as we have, the frequent failure of a *kk* kappa-bearing cell to produce any kappa-bearing progeny. Conditional instability of metagons could account for this. The production of unexpectedly high proportions of kappa-bearing progeny from cells most of whose close relatives had lost kappa, could be explained by an inverse proportionality of metagon replication and concentration. Even the production of some sensitive cells in a clone in which sister cells produce only resistant progeny for many more generations could conceivably be explained by these assumptions.

The failures reported here and by others (D. Widmayer, personal communication; I. Gibson, personal communication) torid cells of kappa 51, mu 540, mu 138, or mu 130 by RNase treatment suggest another necessary modification of the metagon hypothesis. Either the metagon has no essential RNA component, the successes of Gibson & Beale (1963) in eliminating mu 540 being due perhaps to a contaminating substance in the RNase, or metagons are conditionally protected from the action of RNase.

Finally, it seems clear that no RNase-sensitive metagon is required for the acquisition and maintenance of kappa 51 by didinia. This, of course, does not bear on the question of whether metagons could replicate in didinia; that question cannot be answered by such a system. However, the claim of metagon replication in didinia loses its uniqueness in suggesting the origin of RNA viruses from mRNA if replication of metagons in paramecia must also be invoked (cf. Beale & McPhail above; see also Reeve & Ross, 1962).

In sum, the observations reported here and by others cannot be accounted for by the original metagon hypothesis, and a number of *ad hoc* assumptions such as conditional instability, replication in recessive cells, and correlation of replication rate with metagon level, must be made to reconcile it with recent data. Because these assumptions cannot be verified in the absence of any definitive test for metagons it seems more reasonable to seek other explanations that would account for all the results so far reported than to continue making *ad hoc* assumptions in order to save the metagon hypothesis.

SUMMARY

1. The loss of mu 540, mu 138, and kappa 51 following the loss of the dominant genes required for their maintenance was studied. With mu 540 and mu 138 the loss usually occurred within 4–7 cell generations, but occasional cells with mu were found as late as generation 12 with mu 540 and generation 17 with mu 138. Kappa 51 was lost between 5 and 60 fissions in different clones. The variability in time of loss remains unexplained.

2. No trail of inheritance of resistance to specific kappa killing comparable to the trail of inheritance reported for mu 540 by Gibson & Beale (1962) was found.

3. No loss of kappa 51, mu 540, mu 138, or mu 130 was found following treatment of paramecia with RNase.

4. *Didinium nasutum* was shown to be able to acquire and maintain kappa 51 when it could not possibly have acquired any RNase-sensitive metagons from paramecia.

5. These results show that the metagon hypothesis cannot be accepted without a number of *ad hoc* and at present untestable assumptions.

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