GENETIC STUDIES ON CYTOPLASMIC PARTICLES IN PARAMECIUM

By

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ABSTRACT OF THESIS

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Title of Thesis GENETIC STUDIES ON CYTOPLASMIC PARTICLES IN PARAMECIUM

The object of the investigation was to determine whether the metagon hypothesis applies to the system of genes and kappa particles in stock 51 killers, syngen 4, of Paramecium aurelia.

Clones of genotype kk were derived from Kk (heterozygote) killers and samples of animals were studied at precisely known fissions (1-15 fissions after the change of genotype) with respect to ability to act as killers, resistance to killing, and presence of kappa particles by microscopic examination.

While in general, the findings were not in conflict with the metagon hypothesis, evidence in direct support was lacking. Some discrepancies with previous findings by Gibson and Beale, Chao, and others, were observed, and were discussed.

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I. INTRODUCTION

Sonneborn (1938) discovered that some stocks of Paramecium aurelia when mixed with other stocks were able to cause characteristic abnormalities eventually resulting in death of the latter. By appropriate crosses, some involving cytoplasmic exchange during conjugation, he was able to show that the phenotype of these stocks, called 'killers', was dependent on a cytoplasmic factor, called 'kappa', and that the maintenance of kappa was dependent on the presence of a dominant gene K, (Sonneborn, 1943, 1946a). After the replacement of gene K by the allele k, kappa was maintained only for a limited number of fissions (Sonneborn, 1946).

This so-called 'phenotypic lag' was studied for stock 51, syngen 4, kappa by Chao (1953a, 1954a) who made use of the discovery by Preer (1948b, 1948c, 1950b) that kappa particles were microscopically visible, either by staining or by phase-contrast observation of crushed animals. Chao stained samples of some clones of animals at successive fissions after the autogamy of heterozygous (Kk) killers yielding equal numbers of clones of the two homozygotes KK and kk. He found that the number of kappa particles in genotypes KK and kk were both at the level of the genotype Kk up to the 6th fission. From the 8th fission onwards, kk animals either contained kappa at the Kk level, or less, or none at all. At later fissions up to the 14th, he found an increase in the proportion of animals without kappa, and a fall in mean kappa

count in those still retaining any. At the 15th fission, all lacked kappa. The phenotypic lag was explained as the result of physiological activity of fragments of the macronucleus of the previous sexual generation, one or more of which may persist in the cytoplasm for as late as the 6th fission, (Chao, 1954a; Sonneborn, 1959). This explanation could give no basis for the maintenance of kappa up to the 14th fission, however.

In other syngens (1 & 8) of P. aurelia, some killers contained another kind of cytoplasmic particle, called 'mu'. These paramecia acted as killers of their mates at conjugation, but did not kill other paramecia swimming about in the same fluid, (Siegel, 1952). Exhaustive studies were made on the phenotypic lag of presence of mu particles after replacement of gene M by m in stock T7, syngen 1, by Gibson & Beale (1962). They found that mu particles were present in all mm animals up to the 7th fission, and thereafter a decreasing proportion of animals with later fissions retained mu as with the situation of kappa in stock 51 described above. However, Gibson & Beale found that when animals of the genotype mm retained any mu particles between the 7th and 14th fissions after the loss of gene M, a full complement of mu particles was present.

Evidence was obtained that mu particles in mm animals were inherited unilaterally at late fissions, i.e., when such mate-killers were allowed to pass through three further fissions (up to the 18th fission), one or more of the progeny contained mu particles. Further, when mu particles were distributed to daughter

cells at the 15th or later fissions, they increased in one sister but disappeared in the other within six hours. The 'metagon hypothesis' proposed by Gibson & Beale (1962) to explain these results is briefly as follows: (1) in the presence of gene M, a number (ca. 1000) of metagons are produced in the cytoplasm; (2) metagons are stable after the loss of gene M and are distributed at random without further production or loss to daughter cells at fission; (3) the presence or absence of mu particles is exclusively determined by the presence or absence of one or more metagons.

The object of the present investigation was to find out whether the metagon hypothesis applies to stock 51 kappa, and if so, to obtain further information concerning the operation of this system of interacting cellular factors.

II MATERIAL AND METHODS

(1) Stocks. The following stocks of P. aurelia were used:-

(a) Stock 51, syngen 4, killer;

(b) stock d4-57, syngen 4, isogenic with stock 51 except for the serotype marker genes a^{29} d^{32}, and k, sensitive;

(c) stock d4-186, syngen 4, containing gene k from stock 29, isogenic or nearly so with stock 51, sensitive, (Dippell, 1950);

(d) stock 31, syngen 8, sensitive;

(e) Doublet killers, serotype 32D, isolated from the progeny of a cross stock 51 killer x d4-57 sensitive where exconjugants failed to separate.

All except the last mentioned stock were kindly supplied by Prof. T.M. Sonneborn.

(2) Anti-sera.

Flask cultures of animals pure for the particular serotype (identified with anti-sera kindly supplied by Prof. Sonneborn) were concentrated, broken, and injected into rabbits for antisera as described by Sonneborn & LeSuer (1948) and elsewhere.

(3) General Methods of Culture

Lettuce infusion used as culture medium was prepared by boiling 1.5 - 2.7 g. baked lettuce powder, with or without addition of one pellet of sodium hydroxide, in a conical flask with 1.5 litres of distilled water for 8 minutes with continuous shaking. This was then filtered through two layers of filter paper, dispensed into 500 ml. quantities and autoclaved at 15 lbs. pressure

for 20 minutes. The quality of some lettuce infusion was found to be improved by washing the dried lettuce powder with one or more changes of cold distilled water before boiling.

Aerobacter aerogenes was used as food for the animals. Bacteria from agar slants were inoculated directly one slant each into 500 mls. lettuce infusion medium and incubated for two days or more at 28 - 33°C. Where necessary the pH was adjusted to 7 - 8 using sodium hydroxide.

Exhausted culture medium was autoclaved Berkefeld filtrate from old flask cultures of paramecia. pH adjustments were made with either hydrochloric acid or sodium hydroxide before autoclaving. This was dispensed into sterile test-tubes and boiled again before use.

Maintenance of cultures, daily isolation, and induction of mating and autogamy followed the methods of Sonneborn (1950b).

(4) Identification of kappa-bearing animals

(a) Drop Method. This method involved placing the paramecia to be identified in the same drop as a group of sensitive animals and observing whether any of the latter were killed.

Paraffin wax rings were made with a piece of glass tubing placed on a glass slide. A drop of culture of stock 31, serotype H, ~~culture~~, containing 15-30 animals was placed in the ring by micropipetting. Animals to be identified were then introduced individually into each drop. The glass slide was inverted over water or culture fluid. To prevent dessication, the capillary space between the surfaces in contact was sealed with water, culture fluid or vaseline. When

water or culture fluid was used, regular repetition of sealing was necessary depending on how plane were the surfaces in contact. When smaller numbers of animals were to be identified, the drops were arranged as described above but on the bottom of a ~~petri~~ Petri dish instead of on a glass slide. This was inverted over the ~~petri~~ Petri dish cover containing a layer of culture fluid.

To recover the identified animal inside the drop, anti-31H serum was added. Usually one large or two small animals remained unaffected. In the latter case, it was assumed that the animal introduced had divided once during the test. Occasionally, numbers recovered were not as expected. Screening with anti-31D serum was sufficient to remove 'suspects' which were probably present in the 31 H cultures but undetected in samples tested before experiments.

Killing took place initially between 3 - 8 hours at 25 - 28°C, longer at lower temperatures, depending on other conditions. Good results were obtained with stock 31H cultures fed for one fission (see Preer, 1948c) 18 - 28 hours before use. Depleted and acid cultures were found less suitable because lysis was sometimes found. Given time, affected sensitive animals developed the characteristic hump as described in Dippell (1950). Any form of drying was found undesirable, the most troublesome being fluid and animals leaking out between the wax ring and the glass surface. Otherwise, control killers always kill and control sensitives never do. The method is therefore superior to that described by Austin (1948a).

Under optimal conditions, large numbers of sensitive animals were found affected within a few hours after killing had begun by both KK animals and kk F_2 animals at early fission stages. The numbers humped far exceeded the prediction given by Austin (1948) that one sensitive was affected per killer per 5 hours. However, experimental conditions were not the same.

By using a smaller number of doublet killers instead of stock 31 animals, animals to be tested were identified as sensitives by the same method (see Results III).

(b) Treatment of experimental animals with kappa preparations

Preparations of kappa, as described by Smith (1961), was used to identify large numbers of sensitives. The method of preparing kappa was as follows: ca. 200 mls. of killer culture were concentrated by a Berkefeld filter and then by low speed centrifuge to 1 ml. Berkefeld filtrate was used to resuspend the animals. This was then concentrated by low speed centrifuge to about 2 mls. The animals were broken by repeated freeze-thawing, suspended in M/10 potassium phosphate buffer at pH 7, and then centrifuged into a pellet at 15,000 r.p.m. The preparation was passed through a column of ecteola suspended in the same buffer. The eluate was left at room temperature for 2 hours and then diluted with Berkefeld filtrate to a concentration of approximately, 1,000 broken killers per ml.

(c) Direct cytological examination. (i) Removal of bacteria. Samples of animals were rinsed through 3 or more depressions of exhausted sterile culture medium and then incubated for half the

total interval at 25 - 28°C, i.e. ~~3/4 - 1 1/2~~ 0.75 – 1.5 hours. The procedure was repeated. (ii) Permanent stained preparations. Animals were made to adhere to coverslips by using albumin (Chen, 1944 as described in Wichterman, 1953). Loss occurred during and after hydrolysis. Later, it was found that there was sufficient coagulable material in exhausted sterile culture medium to bind the animals to the coverslip either by drying or by acid fixatives. (See below). In later work, up to 32 animals were placed on to a clean coverslip and as much as possible of the culture medium was carefully pipetted off. The margin of the drop receded during this procedure while the animals collected at the centre. The coverslip was then observed under the microscope, face down, and dropped into the fixative at the degree of 'dryness' required. This resulted in a usually circular single layer of animals pressed tightly together capable of resisting considerable mechanical disturbance such as vigorous rinsing. With care, the complete sample could be retained. The additional advantage was that scoring could be done for all the animals under oil immersion without moving the field of observations.

Reagents: N-HCl was prepared by diluting 1/9-11 HCl (sp. gr. 1.18). Thionin solution was prepared by saturation of Gurr's thionin at room temperature or higher 18 - 28°C. (cf. De Lamater 1951a). Thionin-SO_2 complex was prepared by adding one drop of thionyl chloride per 10 mls. stain 15 - 30 minutes before use as described by De Lamater (1951a).

Fixation was by one of the following: N/3 to N-HCl

5 - 30 minutes, last 3 mins. in N-HCl; 5% acetic acid in N/10 HCl 5 - 30 minutes, then rinsed through 2 changes of N-HCl; air-dry, and then as above.

Hydrolysis was by N-HCl for 6 minutes at 60°C after which the preparations were placed in N-HCl at room temperature for 2-3 minutes, followed by thorough rinsing.

Preparations were then stained in thionin-SO_2 complex for ~~1 1/2~~ 1.5 hours, and again rinsed thoroughly to remove excess acid.

Dehydration and clearing was done in absolute alcohol-xylol or acetone-xylol without intermediate grades, and finally the preparations were mounted in neutral Canada Balsam.

The procedure after fixation was based on the method evolved by De Lamater 1951a. Results agreed with previous observations that with few exceptions, any fixative is suitable for the Feulgen Method for detecting DNA (Wichterman, 1953) and that the intensity of staining depends on optimal conditions (Preer, 1948a). Critical conditions appeared to concern first, the quality and concentration of the stain, and second, fixation. Duration of fixation and of staining did not seem important.

(5) Identification of genotypes KK and kk

(a) By the drop method. Animals at any fission stage between the 1st and the 7th of the F_2 were first identified as killers, recovered as described above, and tested again for ability to act as killers. Animals of the kk genotype rarely killed again in the second test, and certainly would not do so if they were later than

the 3rd fission. (N.B. This technique makes use of the effect of starvation on kappa in kk animals to be described in Results IV).

(b) A slower but more convenient method was to identify in the first instance one or more animals per clone between the 2nd and the 7th fission either by the drop method or by incubation with a small drop of sensitives on a depression slide. (For unknown reasons, more than one animal was found necessary if the identification was made using the much larger drop of sensitives on depression slides). The remainder of the clone was grown rapidly and allowed to starve down mildly in the depression in which they were grown. To this a drop of sensitive culture was added. Clones of the genotype kk would not show killing even if the culture was less than 8 fissions old. They were in fact sensitive to killing by other killer animals.

The validity of the methods was confirmed by growing clones identified as KK to the F_3 to show there was no segregation of killer and sensitive clones. Clones identified as kk were crossed to Stock 51 killers. The killer exconjugants of these crosses were then passed through autogamy and segregation of killer and sensitive clones was detected. Identification was by method (b) described above and also be testing samples known to have reached 15 or more fissions.

(6) Sampling

Kk killers in autogamy were allowed to divide once at 25 - 28°C. One of these 1st fission animals was grown in excess food at 18°C (except where stated otherwise) while its genotype

was identified by testing its sister as described above. Isolations were made every 2 or 3 fissions (12 - 18 hours) until exactly 64 7th fission animals were obtained as described by Gibson & Beale (1962). Sampling was as follows:

(a) 32 of the 64 7th fission animals were discarded and the remainder isolated into 32 lines initially. Thereafter one from each line per fission stage was selected until the penultimate fission required. These were allowed to divide once and as many as possible of the 64 animals were tested. The method was one selecting against sisters except the last fission, and may not be the same as the sampling method given by Gibson & Beale (1962).

(b) One from each of the 64 7th fission lines was sampled at a subsequent fission.

(c) 32 of the 64 7th fission animals were allowed to divide once and used for the 8th fission. The remainder were grown at 25 - 28°C for one further fission yielding 64 animals at the 8th fission. Of these, 32 were grown at 25 - 28°C for one fission to be used for the 9th fission, and the others at 18°C for 10th and later fissions the next day.

Fission was not synchronous for all lines. Clones for experiments were therefore selected for synchrony of fission. Temperature adjustments were made where necessary. Slow growing animals were isolated into fresh medium and incubated at 28°C while the remainder of the sample were kept at room temperature. Those that failed to divide in 3 hours at 28°C (i.e. nearly one fission slower than the rest) were regarded as abnormal and discarded. For

the Feulgen Method, isolation for samples was done soon after fission in the first instance in bacterized medium at room temperature while waiting to collect the remainder at 28°C for the next three hours. In order to avoid fission, those growing at room temperature for more than ~~1 1/2~~ 1.5 hours were transferred without rinsing into sterile culture fluid at room temperature. The final collection was starved for ~~1 1/4 - 1 1/2~~ 1.25 - 1.5 hours at 25 - 28°C. For the drop method, there was no necessity to collect the whole sample all at once. Animals were introduced into the drops of sensitive cultures continuously, as they divided for the fission required.

III RESULTS

Three methods were used to attempt to classify the phenotype of animals after the loss of gene K. The first method involved testing whether the animals acted as killers or not; the second involved direct microscopic examination in stained preparations for presence or absence of kappa; and the third involved testing whether the animals were resistant or sensitive to killing by killer animals or by a kappa preparation from the brei of killer animals.

I. Proportion of killers in kk animals 7-13 fissions after the loss of gene K (Drop Method).

Table 1 gives the results obtained by studying samples from 11 clones of genotype kk, derived by autogamy of F_1 Kk killers and identified by method (b). All animals were killers at the 7th fission and all were non-killers at the 12th. For intermediate fissions, (8th - 11th) varying proportions of killers and non-killers were found, and the latter increased with later fissions in general. (See, however, clone 7). Considerable heterogeneity was found between clones at a given fission, except the 8th.

Diagrams (1) and (2) represent an effort to study the distribution of killers in pedigreed lines, particularly the progeny of sisters of non-killers. As far as possible, if an animal was found to be a non-killer, then both fission products of its sister after one fission, or all four fission products of its sister after two fissions were tested; and if an animal was found to be a killer then only one fission product of its sister after one fission or

T A B L E I.

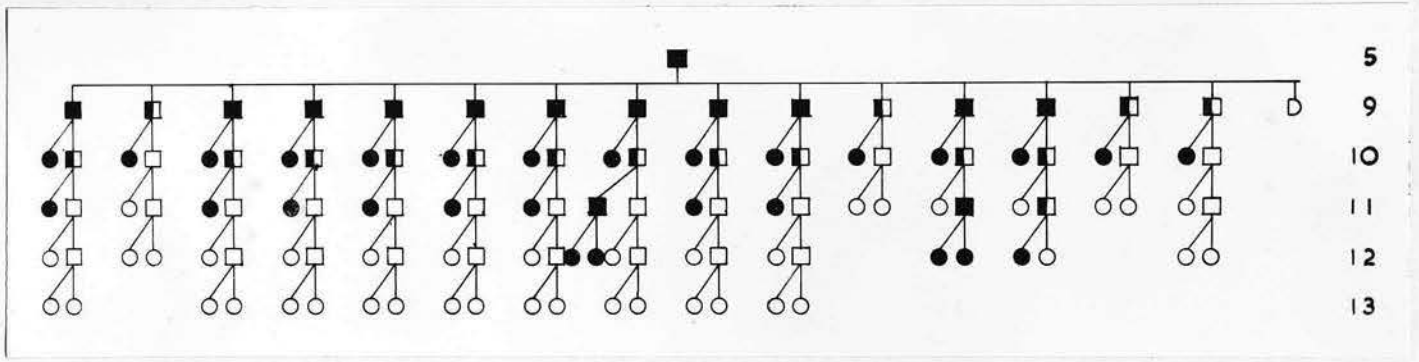
Proportion of killers in animals 7-12 fissions after loss of gene K (Drop Method).

Clone No.	No. of fissions after substitution of Kk by kk	7 K.U.Nk	8	9	10	11	12	Temperature of culture. °C	Sampling Method
1		-	55:1:3	44:0:20	21:0:41	4:1:52	0:0:60	18	(b)
2		-	54:2:3	31:0:33	11:0:53	2:1:60	0:0:60	18	(b)
3		58:3:0	-	-	-	-	-	18	-
4		60:0:0	-	-	-	-	-	18	-
5		-	56:1:3	42:0:9	-	-	-	16 or 18	(b)
6		-	58:0:5	46:0:9*	-	-	-	16 or 18	(b)
7		-	-	20:0:24	-	23:0:26	-	16 ±	(b)
8		-	-	39:0:19	-	-	-	16 ±	(a)
9		-	-	~~2:0:60~~	2:0:60	-	-	16 ±	(a)
10		-	-	~~59:0:0~~	59:0:0	-	-	16 ±	(a)
11		-	-	-	-	8:0:16	-	16 ±	(a)
TOTAL		118:3:0	223:4:14	222:0:114	93:0:154	37:2:~~114~~ 154	0:0:120		
% KILLER		100	94.2	65.8	37.7	21.3	0		
CONTINGENCY X^2		-	0.98	95.05	-	-	-		
DEGREES FREEDOM		-	3	5	-	-	-		
P		-	0.8-0.9	0.001	-	-	-		

Note K = killer
U = uncertain (taken as killer in calculating percentage)
Nk = Non-killer
* = some dividing when introduced into drops

DIAGRAM 1

Distribution of Killers in Pedigreed lines



Lowest Estimate of 'kappa-plus' animals

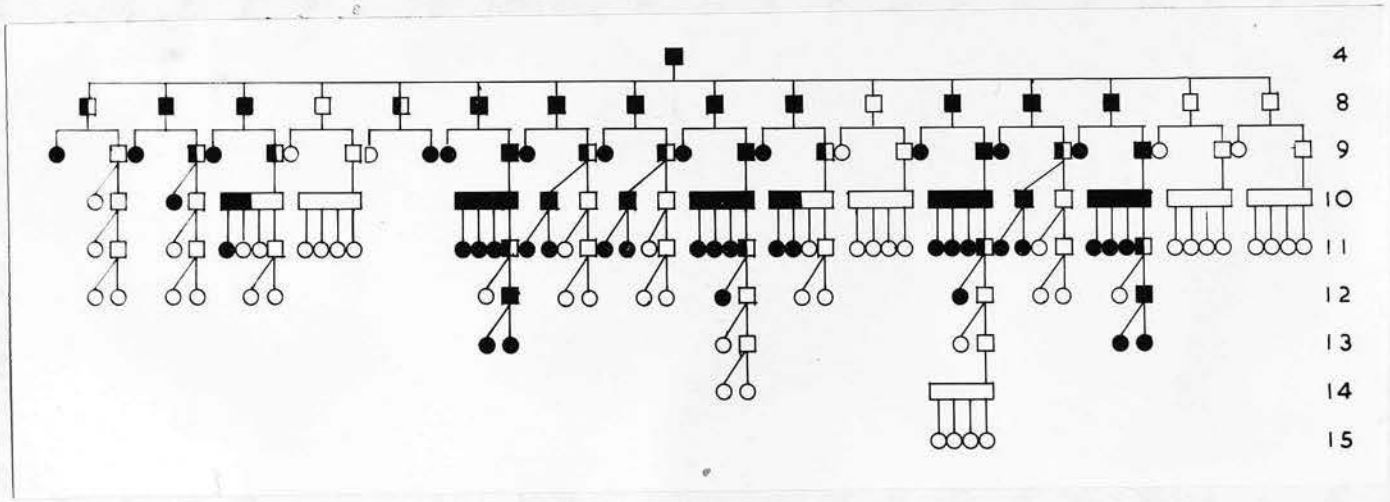
	9	10	11	12
Kappa-plus	15	26	11	5
Unknown or Kappa ~~present~~ absent	1	4	19	23
TOTAL	16	30	30	28

Explanatory Note:

Square or rectangle	- not tested
Circle	- tested
Shaded	- kappa present, or known to be present in more than half of progeny
Half shaded	- kappa known to be present in not more than half of progeny
Empty	- kappa absent or not known
D	- died, counted as 'empty'

DIAGRAM 2.

Distribution of killers in pedigreed lines



Explanatory note: See Diagram (1)

	Lowest estimate of 'kappa-plus' animals					
Fission No.	8	9	10	11	12	13
Kappa-plus	12	22	14	25	4	4
Unknown or kappa absent	4	10	16	31	18	4
TOTAL	16	32	30	56	22	8

three of four fission product of its sister after two fissions were tested. The killer trait was found to persist in some lines some fissions later than in others, and was not the same for the two clones studied.

An attempt was made to find out whether killers could be found in late fissions by testing whole clones instead of samples. This was done as follows: One kk clone was grown at 18°C in 64 separate depressions to beyond 12th fission and all animals were distributed into 16 depressions containing 31 H animals. Another kk clone was grown under the same conditions to 11th - 12th fission and tested with 31 H sensitives by the drop method using 8 - 16 of these animals per drop instead of testing singly. No killing was found. Thus in some clones, killers were not present even at the 11th fissions.

II Proportion of animals with kappa particles 8 - 15 fissions after the loss of gene K. (Microscopic examination of stained preparations).

As in Section I, F_1 Kk killers were passed through autogamy and the F_2 kk clones, identified by method (b), were examined in successive fissions, but here the animals were stained and the presence of kappa particles was observed directly. All 8th fission animals were found to contain stained particles. From the 9th fission onwards, proportions of animals without stained particles were found. Because it was impractical to starve the animals too long (see Section IV) it was not possible to exclude the possibility that some of the stained particles were bacteria. Therefore it

TABLE II

Proportion of animals bearing kappa 8-15 fissions after loss of gene K.

Clone No. \ No. of fissions after substitution of Kk by kk	8 L.S.O.	9	10	11	12	13	15	Sampling
1	32:0:0	23:2:2	9:4:17	10:0:21*	0:3:29	0:19:8 ⑨	-	(c)
2	37:9:0	19:11:4	32:5:7	-	0:14:0	-	-	(c)
3	-	~~29~~ 25:5:3	5:12:15	-	1:4:34	-	0:6:32 ⑨	(c)
4	61:0:0	-	-	-	-	-	-	(a)
5	-	~~0:5:42~~	0:5:42	-	-	-	-	(a)
~~6~~	-	-	-	-	-	-	-	(a)
~~7~~ 6	-	-	-	0:4:30	-	-	-	(a)
TOTAL	130:9:0	67:18:9	46:26:81	10:4:51	1:21:63	-	-	
% kappa +	100	90.4	47.1	21.5	35.9			
% large No.	92.1	71.3	30.1	15.4	1.2			

Note L = 'large No.' of particles
S = 'Small No.' of particles
0 = No particles (See text)

* In this preparation, 10 animals had approximately the same number of particles, and 21 had none. There were no intermediate numbers of particles unlike other preparations.

⑨ In these two preparations, presence of bacteria was strongly suspected.

was not possible to decide whether kappa particles could be totally absent in all animals at a particular late fission.

A continuous range of numbers of particles per animal was observed in all but one preparation. The classification of 'large' and 'small' numbers was arbitrary. 'Small' numbers referred to less than 100 by cursory inspection. Heterogeneity with regard to the proportion of animals with stained particles at a given fission was again observed. The increase in proportion of animals lacking particles at later fissions was less regular than the increase in proportion of non-killers found in experiments described in Section I.

III Preliminary studies on resistance to killing by homologous kappa in animals after the loss of gene K

(a) Resistance of single animals 4 - 8th fissions after the loss of gene K to doublet killers produced.

As before, three clones of kk animals were derived from autogamy of Kk killers and their genotype identified by method (b). Four lines of 4th or 5th fission animals were sampled from each clone and placed singly in drops of doublet killers for 8 hours at 27°C. Survivors were removed from the drops by being morphologically distinct from doublets and allowed to divide once in bacterized medium where necessary. All lines were lost within three fissions by humping, lysis or disappearance.

(b) Resistance of mass cultures 8th - 12th or more fissions after the loss of gene K to homologous kappa from brei of killers.

In this experiment kk clones were obtained from autogamy of Kk killers and their genotype was identified by method (b). Each kk

clone was derived from a 1st fission animal and was cultured at 18°C in bacterized medium up to experimental treatment which was at 27°C.

Kappa preparations from broken killers (see Methods) containing ten times the amount of paramecia capable of humping all animals were added drop by drop to the following cultures:

(i) One kk clone grown and allowed to starve down in a depression, and containing about 200 animals (corresponding to 8th/9th fission).

(ii) One kk clone grown to 12th/13th fission, unstarved, but rinsed and suspended in several depressions of Berkefeld filtrate.

(iii) Two kk clones grown and starved down each in 16 separate depressions (estimated to be at the 12th or more fissions).

(iv) Controls stock 51 killers and d4-57 sensitives from test tube cultures fed for one fission the day before.

The results of these tests were as follows. Control killers were unaffected. Both control sensitive and all experimental animals showed blistering ca. 3 hours after addition of the kappa preparation. In 5 hours nearly all were humped or blistered. The treated cultures were left overnight (more than 12 hours after addition of kappa preparation), and survivors were found in both control sensitives and in experimentals (not more than 20). They were transferred to fresh depressions of kappa preparation. All were humped eventually in the second test. Thus it appeared that between the 4th - 8th fission after the loss of gene K all animals may have been sensitive (or became sensitive during the test), but

this is not absolutely certain because lysis was observed. From the 8th fission on, all kk animals were found sensitive to killing.

The significance of the results in Sections I, II and III should be considered only in conjunction with the effect of starvation on kk animals described in Section IV below.

IV The effect of various conditions on the maintenance of kappa particles in animals after the change of genotype from Kk to kk and KK

(a) Loss of killing ability by starvation. At an early stage of this work, Kk killers were allowed to pass through autogamy, giving rise to F_2 clones of genotype KK or kk after one division (first fission). One of the 1st fission sisters ~~for~~ of each clone was used for the identification of genotype by method (b), and the other sister allowed to divide for a precisely known number of fissions (4th - 6th).

Samples of animals were then tested with stock 31 sensitives by the drop method, and all animals acted as killers (irrespective of genotype kk or KK). However, when these animals were recovered from the drops (using antiserum against the 31 sensitive survivors to eliminate the latter) and then retesting the rinsed kk animals with sensitives (again by the drop method) either immediately or after one further fission from the first test, all animals of the genotype KK acted as killers, but all animals of the genotype kk did not. It should be pointed out here, however, that in some later experiments, 1st and 2nd fission kk animals did occasionally act as killers a second time, but 3rd or later fission animals were never found to do so.

TABLE ~~III.~~

Effect of pH & Length of Starvation on the loss of Kappa ~~in kk Animals~~ at a temperature of 28°C

pH	Duration (Hours)	KK (F_2)	kk (F_2)	d4-57 kk	Temperature
6.8	1.5 ~~1 1/2~~	++	++	+	28°
7.4	~~1 1/4 - 1 1/2~~ 1.25 - 1.5	++	++	±	28°
8.0	1.25 ~~1 1/4~~	++	-	-	28°
8.0	2	++	-	-	28°
7-8 (uncertain)	2 or more	++	-	-	28°

(1) pH was measured using pH papers range 6.8 - 8.3

(2) F_2 animals KK or kk were grown at 18°C and were at 7th or 8th fission after autogamy of Kk killers.

(3) ++ refers to large numbers of stained particles in all cells.
+ refers to small numbers of stained particles, not in all cells.
± refers to near absence of stained particles in some preparations, none in others

(4) Results refer to sterile Berkefeld filtrate, but apply to 2% sterile yeast extract except in the latter, removal of bacteria (stained particles) in d4-57 is less certain for any condition.

It was also observed that if the F_2 clones of genotypes KK and kk were cultured in a hanging drop of bacterized medium, grown for 4 - 5 days at the end of which they were starved down, only animals of the KK genotype acted as killers when subsequently tested with sensitives by the drop method.

To confirm that loss of killing ability in kk animals was not due to conditions associated with the drop method, F_2 clones were cultured in depression slides instead of in hanging drops and subsequently tested for killing ability when they were starved. Again, only KK animals were found to act as killers. kk animals were not only non-killers, but were in fact sensitive to homologous kappa (see Section III).

Once killing ability was lost in kk animals it was found impossible for this character to be restored. The following treatments were given to kk killer animals recovered from the drops:-

(i) Further starvation for 1 hour

(ii) Feeding in bacterized medium for 1 hour

(iii) Feeding in bacterized medium for 3 further fissions.

(iv) Feeding in mixtures of Dryl's medium (Dryl, 1959) and bacterized medium in proportions of ca 1:1 and 3:1 for 3 further fissions.

Duplicates were set at room temperature ca. 20°C and 27°C. None of these treatments restored killing ability to kk animals which had previously lost it. By comparison, KK animals acted as killers up to four successive recoveries if they were fed in bacterized medium for one hour after each recovery.

To summarize, animals of the kk genotype lost their ability to act as killers if they were (1) placed with sensitives in the drops for a certain period (8 - 12 hours), (2) allowed to grow and then exhaust the food available in the drop or, (3) allowed to grow and exhaust the food available in depression slides. Attempts to restore killing ability were not successful. These findings suggested that the loss of ability to act as killers by kk animals under the conditions described was due to the loss of kappa particles as a result of starvation. Confirmatory evidence is given in section (b) below.

(b) Loss of kappa by starvation as shown by microscopic examination of stained preparations.

Some 3rd and some 5th fission kk animals which had acted as killers by the drop method were recovered, stained and then examined under the microscope and were found not to contain any kappa particles. Sometimes, however, kk animals recovered from the drop (3rd - 6th fission), had small numbers of particles which may have been kappa but possibly were bacteria. Thus it is clear that placing kk animals in the drops with sensitives for 8 - 12 hours leads to elimination of kappa. By contrast KK animals at the corresponding fission stage were found to contain large numbers of particles, readily distinguishable from kk animals where particles were found. Macronuclear fragments were absent in these preparations, as would be expected if starvation had taken place (see Sonneborn, 1947a).

To show that it was starvation which had caused the

loss of kappa particles, some kk animals at the 3rd - 8th fissions were starved in sterile culture medium (see Methods) for 2 or more hours at 28°C at pH 7 - 8, after which no kappa particles could be seen by microscopic examination of stained preparations. KK animals at the corresponding stage were found to contain large numbers of kappa particles under these conditions.

In view of the effect of starvation on kk animals described above, it was found necessary to make further studies to determine how long a period of starvation kk animals could tolerate without losing kappa particles. The effect of pH of the medium on the elimination of kappa particles by starvation was also studied. This was however, complicated by the finding that when the pH was 6.8 or below, bacteria were not removed so effectively from the animals, thus making it difficult to establish with certainty that kappa particles had been removed. The results summarized in Table ǂ3ǂ suggested that the effect of pH may be secondary, causing loss of kappa in kk animals in a shorter time because of its effect on elimination of bacteria from animals.

Because loss of kappa in kk animals took place after ~~1 1/4~~ 1.25 hours and before 2 hours of starvation it was found useful to study whether kk animals at different stages of the interfission cycle would be more or less susceptible to a given condition of starvation (and as described in 'Methods' section, animals used in experiments did not divide at precisely the same time). This was done as follows. One clone of kk animals was grown from the 1st to the 10th fission at 18°C and then sampled at ~~1/2~~ 0.5 hour or less, ~~1 1/2~~ 1.5 hours and ~~2 1/2~~ 2.5

after the 10th fission when they were then grown at 28°C. The samples were starved for ~~1 1/4 - 1 1/2~~ 1.25 - 1.5 hours at 28°C at pH ca. 7.4. The ~~proportions~~ numbers of animals containing kappa particles were 4:6, 5:5, 5:6 respectively. Thus there is no evidence that kk animals at different stages of the interfission cycle behave in a different way towards this condition of starvation.

The findings in this section were applied to developing methods such as identification of genotype, conditions of starvation before staining etc.

V The Relation between loss of kappa and loss of macronuclear fragments

(a) Preliminary studies on starvation of kk and KK animals one fission after autogamy of ~~KK~~ Kk killers

Twenty-four animals in autogamy (genotype Kk) were placed in a small drop of bacterized medium in a depression slide, and the depression was made air-tight using a coverslip. The surfaces in contact were smeared with vaseline. After 36 hours at 28°C, 54 animals were recovered, rinsed in sterile culture medium and then stained. Approximately half of the 54 animals were found to lack kappa while the remainder were found to contain large numbers of kappa particles. Macronuclear fragments were found in all of the animals. Thus it appears that (1) loss of kappa by starvation can take place at the 1st fission after the loss of gene K (2) loss of kappa ~~has no relationship~~ is not directly related to the presence of macronuclear fragments and (3) since no killing was found, 1st and 2nd fission kk animals may be resistant even after kappa particles ~~were~~ are lost.

TABLE ~~IV.~~ 4

Loss of Kappa by Starvation in 1st & 2nd fission kk animals

	Clone No.	Kappa Particles	No. of Macronuclear Fragments	Genotype of sister(s)	Genotype of sister(s)
A (1st fission)	1	+	6	KK	29 A*
	2	+	4	KK	51 A*
	3	-	5	kk	51 A*
	4	+	6	KK	51 A*
	5	+	8	KK	51 A*
	6	-	5	kk	29 A
	7	+	6	KK	51 A*
	8	+	3	KK	29 A
B (2nd fission)	1	100 - 200	3	kk	51 A*
	2	-	3	kk	51 A*
	3	-	0	kk	51 A*
	4	50	1	kk	29 A
	5	-	1	kk	51 A*
	6	+	3	KK	29 A*
	7	+	1	KK	29 A*
	8	+	0 or 1	KK	51 A
	9	+	3	KK	29 A
	10	+	4	KK	51 A

K or k / serotype	KK	kk
29 A	5	2
51 A	6	5

Note: (1) No. of kappa particles + refers to ca. ~200, - to none.
(2) For identification of genotype of sister, see 'Methods'.
(3) * genotype checked by further breeding
(4) No. of macronuclear fragments ca. 20 per animal in autogamy.
(5) There was no change in mating type (VIII) where studied
(6) Genotype and serotype of sister refer to that sister of the stained animal (see text). For 2nd fission animals in B, the genotype & serotype of the 1st fission sister, which was not placed in the drop were the same as that sister of the stained animal.

To show that the loss of kappa in animals (assumed here to be of the genotype kk, see below) had not taken place before the 1st fission, Kk killers in autogamy (zero fission) were allowed to starve for four days at 28°C. Each day, a sample of animals were removed, and starved further in depressions of sterile culture medium for 2 - 3 hours at 28°C at pH ca. 7.4 to remove bacteria which might be present, stained and were examined under the microscope. All animals in all samples were found to contain large numbers of kappa particles. Further, by the fourth day, the majority of the animals (still at 'zero fission') were found to contain no macronuclear fragments. The macronuclear anlagen (KK or kk and 2 per animal) were intensely stained. Samples of animals were then fed and allowed to pass through autogamy. Segregation of KK and kk and serotypes 29A and 51A were found. This experiment shows that loss of kappa did not take place before the 1st fission. It also shows that kappa particles were still present after the macronuclear fragments (Kk) disappeared regardless of the genes in the developing new macronuclei (KK or kk).

(b) Demonstration of loss of kappa particles at the 1st and 2nd fissions in animals of the genotype kk.

Table 4 B gives the result on the loss of kappa particles in 2nd fission animals. Here Kk killer animals were allowed to divide once after autogamy. One of the 1st fission sister animals was used for identification of genotype KK or kk by method (b), and the other was tested with sensitives by the drop method. Killing was found in all the drops, but all the 1st animals were found to have divided once while in the drops (after 16 hours)

The pairs of 2nd fission animals were recovered and then starved for ~~2 1/2~~ 2.5 hours in sterile culture medium ~~for 2 1/2 hours~~ at pH 7.4 28°C. After starvation, one of the 2nd fission sisters was stained and the other grown on to confirm the genotype identified already. This was done by the same method as before, and also by further breeding analysis for some clones. (See Methods section).

Table 4A gives the results on the loss of kappa particles in 1st fission animals. Here Kk killers in autogamy were fed for 9 hours in bacterized medium, and were then tested (at 'zero fission') with sensitives by the drop method. Two 1st fission animals were recovered after 16 hours at 28°C per drop. These were treated in exactly the same way as the 2nd fission animals described above.

VI Relation of genotype to the loss of kappa in kk animals by starvation.

(a) Antigenic markers. It can be seen in Table 4 that loss of kappa in kk animals by starvation has no relation with the segregation of the antigenic markers at the A locus.

(b) Gene k from stock 29 in d4-186. All the experiments described in the previous sections (I - V) were performed using stock d4-57 as the sensitive parent (isogenic with stock 51 except for serotype markers a^{29}, d^{32} and k of unknown origin but probably from stock 29 or stock 32). Since Chao (1953) did not report on any effect of starvation on kk animals in the F_2 by using d4-186 instead of d4-57, (see, however, Chao, 1955), a preliminary attempt was made here to find out whether the k allele in d4-57 and d4-186 differ with respect to the loss of kappa by starvation in kk animals.

It was found that method (b) for the identification of genotype kk and KK applies when d4-186 was used as the sensitive parent. Furthermore, by feeding Kk killers in autogamy for one fission and starving the 1st fission animals as described in Section Va, again approximately half the number of these animals were found not to contain kappa while the remainder had large numbers. Thus there is no evidence that the gene k in d4-186 can be different from that in d4-57.

D I S C U S S I O N

I. Review of previous work on kappa

Sonneborn (1938) first showed that some stocks of Paramecium aurelia were able to liberate into the external medium a substance called 'paramecin' to which some other stocks were sensitive and eventually killed in a characteristic way. He also showed that each of these stocks, called 'killers', was resistant to the paramecin it liberated, and also to paramecin producing the same kind of killing effect as its own but liberated by other stocks of killers. By appropriate crosses, some involving cytoplasmic exchange, it was shown that the killer phenotype was caused by a cytoplasmic factor called 'kappa' which was determined by a dominant gene called K(Sonneborn, 1943, 1946a). Many other killer paramecia have been found since and are called by the letter of the Greek alphabet describing their action, e.g. lambda (lysis) (Schneller 1958 cited in Sonneborn, 1959). It was proposed that each kind of particle required the presence of certain genes and that different kinds required different genes. (Sonneborn, 1959; Schneller, Sonneborn and Mueller, 1959).

Another kind of killer was described and studied by Siegel (1952, 1953, 1954), differing from those described above in that no extracellular killing substance could be detected. These however acted as killers to their mates during conjugation if the latter were sensitive. Stock T7 studied by Gibson and Beale (1962)

was derived from this kind of killer called 'mate-killers' containing the cytoplasmic factor called 'mu'.

Austin (1948a, 1951) showed that paramecin was in the form of discrete particles (now called 'Pn' and 'P' respectively (Sonneborn, 1959)). She showed that one stock 51 killer animal was able to liberate enough Pn or one P particle to kill one sensitive animal within 5 hours at 27°C on the average. Pn was not affected by dilution with culture fluid up to 1000 fold and may be liberated at any time within the interfission cycle. It was further shown that the fluid in which killers had previously lived lost killing activity when passed through fine bacteriological filters.

Up to now, little is known about how sensitive animals are affected by P. Various grades of resistance were found with both environmental and genetic factors, among these being: Prior exposure of the sensitive animals to low temperature (17 hours at 10°C), excess food, conjugation or autogamy, and serotype (Sonneborn, et al., 1946, 1959; Austin, 1951). The currently accepted view proposed by Preer (1957) and extended by Sonneborn (1959) considers P as a 'capsule of toxin' injected into sensitives at adsorptive sites.

Preer (1948b) demonstrated that the cytoplasmic factor kappa consisted of DNA-containing particles microscopically visible by suitable staining, and his discovery has been confirmed in studies of all other killers. (See Sonneborn, 1959). However, not all sensitive stocks lack particles, e.g. animals bearing pi, which was shown to be a mutant of 51 kappa (Hanson, [illegible], 1954a) were sensitive to 51 P. Furthermore, killer stocks with the number of kappa

reduced to a low level were sensitive (Preer, ~~1948~~ 1948a, Chao, unpublished, cited in Hanson, 1957, ~~See~~ see below).

By phase-contrast microscopy, Preer and Stark (1953 found that there were two kinds of kappa particles. B or 'brights' were distinguishable from N by having one or two refractile bodies called R which was once thought to be the bearer of Pn, because R was found in all stocks which liberated Pn but not in others which did not, such as pi and mu, and because the sedimentation rate of Pn was not distinguishable from that of B. B particles were held to arise from N and were themselves unable to divide; this interrelation was found from studies of various forms of particles thought to be developmental stages of B as well as by reason of the association with Pn (Preer, Siegel and Stark, 1953; Sonneborn, 1959; Mueller, 1963).

The growth rate of kappa was not always the same as that of the animals bearing them, and the distribution of kappa to daughter cells at fission was not precise. By growing certain stocks of syngen 2 at maximum rate, Preer (1948a) found the change of phenotype of these cultures from strong killer to weak killer, resistant non-killer and finally sensitive. When growth rate was reduced by limiting the food supply, he found a reverse change to the strong killer phenotype in some of these sensitive lines, while others remained permanently sensitive. Stock 51, syngen 4, cannot outgrow its kappa (Sonneborn, 1945a, 1945b, 1946a). However, conditions were found under which this type of kappa also could be removed or reduced in number, resulting in changes of phenotype similar to that found by Preer in syngen 2 killers.

Loss of kappa in stock 51 was achieved by growth at extremes of temperatures e.g. 38.5°C for 12 - 36 hours (Sonneborn, 1946a) or at 10°C, when the reproduction of kappa was slower than that of the animals (Sonneborn, Dippell and Jacobson, 1947); by treatment with X-rays (Nanney, 1954a), or by treatment with various antibiotics or metabolic inhibitors (summarised in Sonneborn, 1959). Sudden loss in some lines were found after macronuclear regeneration (Sonneborn, 1945a, b). Reduction in kappa number was achieved by Sonneborn (1946a) by controlled cytoplasmic exchange in a cross stock 51 killer x 47 which had lost kappa. He obtained lines with a kappa level so low that the killer phenotype was not expressed at maximal fission rate. These however became killers when the fission rate declined with clonal age. Chao (unpublished, cited in Hanson 1957) was able to obtain stock 51 animals with reduced kappa number. His description of phenotypic changes with kappa is tabulated below.

No. of kappa	Phenotype
140	Can act as killer, resistant
20 - 65	Non-killer, resistant
0 - 20	Non-killer, sensitive

The 'strong killer' phenotype appears when the number of kappa increases. Thus Sonneborn and Chao (unpublished, cited in Sonneborn, 1959) found some senescent lines which contained 1000 or more kappa particles and were exceptionally potent killers. Similarly Hanson (1957) found that conditions which resulted in the highest number of kappa per cell also yielded the strongest killers. On the other hand, Nobili (1960) found an increase in killing activity in amacronucleate animals where

the number of kappa particles remained unchanged.

Once kappa is lost, it cannot be produced de novo. Kappa can however be re-introduced either by cytoplasmic transfer during conjugation (Sonneborn, 1946a) or by direct infection with preparations from broken killers (Sonneborn, 1948b; Tallan, 1959, 1961; Smith, 1961). Success of infection was identified by the establishment of the killer phenotype. It was found to depend on several factors: first, the genotype of the recipient; second, the use of N particles with or without B; third, an unspecific co-factor found in the supernatant after centrifuging broken killers at 25,000 g for 5 minutes, which can be replaced by salt solutions e.g. sub-lethal concentrations of $CaCl_2$. (Mueller, 1961; Smith, 1961; Tallan, 1961).

Extensive studies were made on the number of kappa per cell under different environmental conditions, resulting in the conclusion that the mean number of kappa per cell remained constant under a particular set of conditions (Chao, 1953a, 1954a; Hanson, 1957). It was found that kappa was halved approximately at fission followed by an increase with the maximum rate in the first third of the inter-fission cycle by growth in excess food at 27°C (Chao, 1954a). Kappa counts made by Chao and Hanson for 51 kappa were tabulated below (for mating type VIII).

Conditions	No. of Kappa		Observer
Excess food 5 fissions/day	371	(240-637)	Chao
Limited food 2 1/2 fissions/day	627	(493-749)	Hanson
Limited food 1/2 fission/day	444	(353-546)	Hanson
Starvation for 2 days (previous conditions unknown)	237	(124-345)	Hanson
Cerophyll medium 5 fissions/day	769		Chao

Enormous variability in kappa number per cell is known (summarized in Sonneborn, 1959), even under a particular set of conditions e.g. Chao's mean count of 371 spread over a range from 240 - 637. He also found other conditions could affect the mean count, e.g. growth in cerophyll medium instead of lettuce infusion.

Genes in the macronucleus determine in syngen 4 (1) whether kappa can be maintained (2) whether the stocks can be infected with kappa. Some are known e.g. K, S loci; others are uncertain (Tallan, 1959; Sonneborn, 1959).

Earlier, it was mentioned that kappa is determined by the presence of the dominant gene K. Sonneborn (1943, 1945a) crossed 51 killers to 32 sensitives and obtained in the F_2 derived by autogamy of the F_1 killer (Kk) segregation of killer clones (Kk) to sensitive clones (kk) in the ratio of 1:1. He made backcrosses of the F_2 kk animals to KK animals and found that to the F_3 (Kk) some clones were killers if the F_2 kk animals were at a stage two to five fissions after autogamy, but no clone was a killer if they were six or more fissions after autogamy. He concluded that kappa was lost 2 - 5 fissions after the loss of gene K. Chao (1953a, 1954a) studied the relationship of the dosage of gene K to the number of kappa particles as well as the changes following genic substitution. His findings showing the mean counts of visible kappa in stained preparations in genotypes KK and Kk, and in genotypes KK and kk in successive fissions after autogamy of Kk killers from a cross 51 killer x d4 - 186 sensitive, are tabulated below. (All refer to mating VIII).

TABLE

Kappa counts for the F_2 obtained by autogamy from Kk Killers of Mating Type VIII

Fission Genotypes	1 KK kk	2 KK kk	3 KK kk	4 KK kk	5 KK kk	6 KK kk	7 KK	7 kk	8 KK	8 kk	9 KK	9 kk	10 KK	10 kk	11 KK	11 kk	12 KK	12 kk	13 kk	14 kk	15 kk
Kappa counts	397	429	276	306	241	162	141	157	177	0	308	0	267	0	247	0	266	0	0	0	0
	408	444	308	328	272	189	164	171	199	125	310	4	340	27	299	0	272	0	0	0	0
	427	490	382	339	307	209	183	178	212	180	311	109	343	28	339	16	306	0	0	0	0
	427	522	413	341	328	218	185	179	214	185	314	112	362	86	339	46	316	0	0	0	0
	429	526	421	346	331	222	214	184	223	188	333	152	372	138	357	58	345	0	0	0	0
	449	543	448	349	332	234	219	195	242	201	336	152	409	157	361	59	348	4	5	4	0
	451	563	461	367	334	266	243	224	247	230	342	200	489	169	388	73	369	49	32	15	0
	466	568	487	393	357	293	244	266	263	235	355	222	489	194	452	117	379	94	63	17	0
	609	579	517	433	362	338	325	269	273	256	378	224	495	196	458	156	396	108	78	27	0
	619	600	531	464	430	355	339	305	280	284	409	241	519	216	496	164	406	146	98	79	0
MEAN	478	523	424	366	323	249	225	212	223	188	339	141	398	121	374	69	341	40	27	14	0

Sonneborn (1947b) found that the maintenance of kappa in KK animals was also affected by genes at other loci in some kk stocks e.g. 29 and 32. These, called 'suppressors' are dominant and designated by S. More than one was known and was studied in detail by Balbinder (1957, cited in Sonneborn, 1959). He found that the kappa level remained normal except in those animals which suddenly lost kappa completely often without a fission. Conditions which resulted in reducing the number of kappa particles in ss animals increased the probability of ~~loss~~ loss in SS animals. Starvation for a few days was found highly effective. The probability of loss was also higher when the genotype was Kk instead of KK. Sonneborn (1959) however did not accept the contention by Preer and Balbinder that S genes acted like k.

So far infection of kk stocks by kappa has not been attempted but infection of sensitive stocks in syngen 1 and 8 by kappa from syngen 4 was unsuccessful (Tallan, 1957, cited in Sonneborn, 1959). Other genes were found to affect infectivity however, e.g. Balbinder (1957, cited in Sonneborn, 1959) found that the establishment of kappa introduced by cytoplasmic transfer was rendered more difficult by the presence of gene S. In his studies on infectivity of 51, 169 d4 - 3, d4 - 101 (F_2 from d4 - 186 x 169 homozygous for 169 K). Tallan (1959) obtained evidence that the establishment of kappa in the infected animals ~~were~~ was not related to mating type, serotype, or the gene K but to causes yet unknown, which he thought to be activity of other genic loci.

As for mate-killers, mu particles were described as

rapidly lost between 3rd - 6th fission when the gene M in stock 138 (syngen 8) was replaced by m in 130 (Siegel 1952, 1953, 1954) and loss of mu after the loss of gene M was confirmed by Levine (1952, 1953) for stock 131 and Beale (1957) for 540.

Later studies by Gibson (1961) and Gibson and Beale (1962) revealed however that a full complement of mu particles were found in some animals as late as 18 fissions after replacement of M by m in stock T7 (isogenic with 513 except for gene M_2 from 540). These workers found mu particles were maintained in all mm cells derived from autogamy of Mm mate-killers up to the 7th fission. Evidence was obtained that at later fissions (7th - 18th), mu particles were inherited unilaterally at fission in some lines. They found that each octet derived by three fissions of a mate-killer always contained one or more mu-bearers. Indirect evidence was also obtained for some lines that when a mate-killer divided, mu particles increased in one sister, while in the other they decreased and finally disappeared within six hours. The 'metagon hypothesis' proposed to explain these results is summarised as follows:- (1) A number of particles called 'metagons' were produced in the presence of gene M into the cytoplasm (ca. 1000); (2) After the loss of gene M, metagons were distributed at random with cell division, without further production or loss (3) metagons were stable to some environmental conditions studied: 18-25° , starvation, replacement of bacteria by yeast as food, but not to RNA-se (4) the presence of mu depended on whether one or more metagons was present. Loss of metagons at fission resulted in the destruction of mu particles. (For further evidence see Gibson and Beale, 1963, 1964; Beale, 1964).

DISCUSSION

II. Present Results

Experimental results obtained here have revealed that kappa particles are present in all animals for at least 7 fissions after the loss of gene K, under the conditions described. However, by applying an appropriate degree of starvation kappa can be removed from all kk animals, including those having undergone only one fission following autogamy. The loss of kappa when it occurs is rapid, presumably without an intermediate stage, and within 3/4 0.75 hour or less under certain conditions. The observations are consistent with the hypothesis that products of gene K maintain a 'favourable' cytoplasmic state by preventing destruction of kappa (Beale, 1954).

It is the purpose of the present investigation to find out how such 'favourable' cytoplasmic states may persist in successive fissions after the loss of gene K, making use of the presence of kappa as an 'indicator' (See Gibson and Beale, 1962), to answer whether the metagon hypothesis applies, and to find out what further information may be obtained. Three methods were used to detect the presence of kappa: (1) Whether kk animals can act as killers. (2) Whether they are resistant to killing and (3) whether they can be seen to contain kappa when stained and examined under the microscope.

The drop method ascertains the presence of kappa in an animal, if it acted as a killer, perhaps not less than a certain number (which must be more than 65 according to Chao unpublished,

cited by Hanson, 1957). When killing was not found there is no way to asses whether the non-killer had any kappa (up to 140) or none at all (See page 28).

It is not known whether a killer can have a smaller number of kappa particles than a non-killer because 'B' particles are present in the former but not in the latter, although evidence in variety 2 (Preer, 1948) on increasing kappa level seems to require that a certain number of kappa particles must be present before the killer phenotype can be detected.

Of 121 7th fission animals (2 clones), 118 were killers, 3 were less certain, suggesting large numbers of kappa were present in nearly all cells. From the 8th to the 11th fission inclusive, a general decrease in proportion of killers with later fissions was found, suggesting some killers had divided to yield non-killers, i.e. animals with probably a smaller number or none. At the 12th fission, killers were not found (see below, however).

In the study of pedigreed lines, it was found that sisters, or their progeny, of non-killers rarely contained killers, whereas progeny of sisters of killers contained more killers than would be expected from random distribution. This suggests that most killers divided to yield daughter cells which were either both killers or both non-killers. Some killers were however found at the 13th fission suggesting that some killers which could have yielded killers later than the 12th fission were not sampled in the experiments described in the paragraph above. Against this view is the finding in another

experiment where no killing activity was found in two whole clones (4000 or more) of kk animals at 11th or more fissions by testing in groups.

If they are not different from KK animals, kk animals may be expected to be resistant to killing when 20 or more kappa particles are present (see page 28). It was ~~however~~ however found that kk animals between 4th - 8th fission could be sensitive to killing by doublet killers by the drop method, and from 8th fission or after, all animals could be sensitive to kappa preparations. However, these animals were presumably starved for too long a period, so that kappa particles might have been lost during the test. It needs to be pointed out here that animals have to be starved partially to show sensitivity to killing (see Sonneborn, Dippell and Jacobson, 1946; Sonneborn, 1946a). No attempt was made to stain a sample of the animals for kappa particles before the addition of kappa preparations, because it was intended that whole clones should be tested.

In direct examination of stained animals, any number of kappa particles should be recognized (Chao, 1953a, 1954a), unless they happened to lie over or under other stained constituents of the cell e.g. macronucleus, micronuclei, and macronuclear fragments if present. The judgment of whether bacteria may be present is entirely subjective, and the meagre data presented indicates that the period of starvation for removal of bacteria is pH dependent. Difficulty was encountered in selective removal of bacteria without causing loss of kappa (cf. Chao, 1953a, 1954).

By cursory inspection of stained kk animals, when kappa was present, the number appeared to vary from cell to cell in a continuous distribution. From the 9th fission onwards, some animals were found to lack kappa, and the numbers of kappa were in general fewer with later fissions.

Apart from experimental error, there may be further reasons for inaccuracy in the methods of assessing the presence of kappa described. First starvation causes the loss of kappa in kk animals, but unfortunately cannot be avoided ~~unfortunately.~~ For the drop method, it is known that loss of kappa took place but the exact time at which this may occur is ~~not known~~ unknown, and may not be the same for every drop. Austin (1948, 1951) found that the liberation of the killing P particles by killers may take place at any time within the interfission cycle. If this does not take place before starvation commences, kk animals may be wrongly identified with respect to killing ability. In preparing samples of kk animals for staining, the degree of starvation is further complicated by environmental influences such as pH, although the period of starvation under a particular set of conditions must be limited to within a certain period. There is also no way to show that each animal in a sample had been given the amount of starvation which would not result in loss of kappa. Second, sampling was on a random basis for most experiments but there is slight evidence that at least killers are not distributed at random.

None of the methods therefore can give an accurate assessment of the presence of kappa. The persistence of the

presence of kappa in some lines of a clone but not in others of the same clone suggests a particulate mechanism of maintenance. However, owing to error and vague indication of differences between clones, such results can only be viewed as inconclusive.

Some of the results obtained here for the maintenance of kappa after the loss of gene K are at variance with the data obtained by Gibson and Beale (1962) for the maintenance of mu after the loss of gene M. First, starvation causes the loss of kappa in ~~KK~~ kk animals. With mu particles in mm animals, by contrast, starvation was found to result in an increase in the number of mu particles per cell. Second, even in the absence of starvation, no kk animal ~~contain~~ contained a 'full complement' of kappa particles beyond a certain number of fissions (11th or more) in some clones. Third, the distribution of killers i.e. animals with 'large' numbers of kappa is non-random. It is therefore concluded that the metagon hypothesis in its original form does not apply to stock 51 kappa.

Here the maintenance of kappa in kk animals appears to depend on more than the products of the K gene alone. A dilution of this product by 100-fold or more, by growth in excess food at 18°C for 7 fissions, does not result in the loss of dominance of the killer phenotype. On the other hand, starvation can cause the loss of all kappa particles when this product is only halved, (i.e. at the 1st fission). Thus, the presence of kappa may not be an 'indicator' of products of K under some conditions, or else the products of K are unstable, but are capable of catalysing further production in the presence of gene K. At least, the observed

effect of starvation on loss of kappa in kk animals suggests that the control of cytoplasmic entities over the phenotype is never as stable as macronuclear genes are.

The data presented for the effect of further 'dilution' beyond the 7th fission is too irregular and confusing for any explanation at present. There are some general features: (1) a sharp drop in the proportion of animals supposedly with a 'large' number of kappa; (2) a decrease in number of kappa when present, and (3) a tendency of sisters to be alike.

It is not possible to find out whether a fall in kappa number is due to (1) reduction without fission or (2) inhibition of multiplication in various degree followed by halving at fission. A continuous distribution in number of kappa can arise by either, or both, plus perhaps unprecise distribution of kappa at fission. Here it should be pointed out that a range in number of kappa particles is also found in stock 51 killers under supposedly identical conditions (Chao, 1953a, 1954a; Hanson, 1957). That sisters tend to be alike (killers or non-killers) again suggests that maintenance of kappa may be dependent on unequal distribution of other components than products of K at fission (see Kimball, Vogt-Köhne and Caspersson 1958; Kimball, Caspersson, Svenson and Carlson, 1958; Kimball and Vogt-Köhne, 1960).

If such an interpretation is correct, it will mean that in the absence of starvation, a dilution of the products of K beyond a certain threshold (ca. 100 fold) results in their inability to counter inhibitory effects of various degree which may arise

from unprecise fission. The apparent particulate control of maintenance may then be unreal. However, the two processes are not mutually exclusive~~ly~~: total loss of kappa at early fissions (9th) together with persistence of large numbers at later ones (11th) suggests both processes may be in operation.

That no killers were detected beyond a certain number of fissions (11th or more) in some clones, and that killers usually divided into daughter cells either both killers or both non-killers suggests that even if the products of gene K are particulate, a single particle (or metagon) cannot support a large number of kappa. This view is consistent with Chao's hypothesis (1953a) that the number of kappa particles corresponds to the dosage of gene K, but appears to contradict the view that 100-fold dilution does not result in a fall in number of kappa particles.

All such tentative explanations should be tested experimentally, by studying (1) what does the presence of kappa, as well as the number, in a kk cell actually signify? (2) Is there evidence of a correspondence between the number of particles of products of K to the dosage of K before autogamy? (3) How may a distinction be made between actual loss of products of K or a reversible inactivation under some conditions?

There is indirect evidence that a 'favourable' cytoplasmic state may be found which is unrelated to the products of K. First in T7, ribonuclease removed the metagons (products of M) but did not cause loss of mu until one fission later (Gibson and Beale, 1963). Second, in Sonneborn's experiment on the reintroduction of K to kk

animals between 2nd and 5th fission, some exconjugant clones (F_3 genotype Kk) were killers. Mating in Paramecium aurelia requires 6 - 7 hours at 28°C, during which the conjugants cannot feed. Such a period of starvation is judged here sufficient to cause loss of kappa in animals of the genotype kk at corresponding fission stages but in the vegetative part of the life-cycle.

Another possible means of altering 'unfavourable' cytoplasmic states may be achieved by genic selection. First, suppressors of K are known (Sonneborn, 1947b; Balbinder, 1956, 1957 , cited in Sonneborn, 1959), but enhancers ~~has~~ have yet to be found. There is again some evidence that gene K in other genomes may not function in exactly the same way. Hanson (1957) found that when stock 51 was crossed to kk stocks not isogenic with it, the F_2 examined at some stage from autogamy showed a higher proportion of killer clones than 1:1. On the other hand, Tallan (1959) found that the infectivity of stock 51, and stock 169 is similar but not the same as d4 - 101 which bears 169 K but other genes from 169 and d4 - 186 (nearly isogenic with 51, Dippell, 1950). His criterion of infectivity is the proportion of animals infected being able to yield killer clones. Thus, lower infectivity in d4 - 101 seems to contradict more 'favourable' maintenance of kappa when stocks are not isogenic as found in Hanson's experiment. Whether genic selection may succeed depends on whether the view given above is correct. Here one needs to point out that in syngen 2, all stocks were able to maintain kappa (Preer, 1948).

Returning to the problem of dosage of gene K, the present results predict that gene K must be present in the macronucleus for maintenance of ~~Kappa~~ kappa during starvation. However, the peculiarity of the life cycle of Paramecium aurelia requires one fission (after autogamy) before the new genotype can be considered constituted i.e. by maturation of the anlagen. Such a view may be compared with the prediction of the metagon hypothesis by varying the dosage of K initially. Thus if the F_1 Kk killers are subjected to macronuclear regeneration giving KK macronuclei and Kk micronuclei, the F_2 kk animals at the first fission will consist of a cytoplasm with the dosage K equal to that of Kk. On the other hand by fusing the F_1 into doublets giving rise to Kk/Kk, backcross to two kk animals should yield Kk/kk giving a dosage of K relative to other cell components half that of Kk, and equal to that in 'lst fission' kk animals derived from autogamy of Kk.

That the view chosen here may be correct is substantiated by Chao's studies on backcrosses of Kk killers to kk sensitives with cytoplasmic exchange. He found that kappa was 'rapidly lost' if the genotype was kk but gave no information as to the exact fission stages at which kappa was lost, or how long he had starved these exconjugants, or their progeny, before cytological examination (Chao unpublished, cited in Sonneborn, 1959). If Kk/kk doublets are able to maintain kappa it will be interesting to know what numbers of kappa will be found, and how long kappa may persist by substitution of the genotype by kk/kk. This may be compared with

F_2 derived from autogamy of animals with double the initial dose of K. This may, if successful, permit a better interpretation of the effect of dilution of products of K beyond the 7th fission in the present investigation.

As to the question of whether K products are lost, reversibly inactivated, or irreversibly inactivated, more information as to the nature of these products and their possible role should be obtained first, as described above. Here one can point out that stability or reversible inactivation may be proved by successful infection of kk animals which had lost kappa by starvation. However, Tallan (1959) was unable to infect kk animals with killer brei which presumably contained products of gene K. Identity of product of K with the 'messenger' can also be proved by successful construction of a DNA-RNA hybrid using the DNA from KK animals with RNA from kk animals which had lost kappa by starvation compared with other combinations. (See Nygaard and Hall, 1964).

To recapitulate the discussion, evidence in direct support of the metagon hypothesis is lacking, but no finding is in direct conflict with it. Discrepancies with the data for maintenance of mu in T7 were found. A choice between a modified form of the metagon hypothesis and one which requires the maintenance of kappa as inheritance of the metabolic patterns of whole cells cannot be made at present. It is hoped that further information for the maintenance of kappa in stock 51 by comparison with that of mu in T7 may bridge the gap in these two theories which are not mutually exclusive.

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