A STUDY OF SOME PROPERTIES OF THE CYTOPLASMIC FACTOR, "KAPPA," IN PARAMECIUM AURELIA, VARIETY 2[1]

JOHN R. PREER, JR.[2]

Zoology Department, Indiana University, Bloomington, Indiana

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[2] The author was a NATIONAL RESEARCH COUNCIL PREDOCTORAL FELLOW while part of the work was done, and a NATIONAL INSTITUTE OF HEALTH FELLOW during the remaining part of the work. Present address: Zoological Laboratory, University of Pennsylvania, Philadelphia.

I. INTRODUCTION

IN a preliminary paper (PREER 1946) it was shown that certain animals of variety 2 of *P. aurelia* may increase in number faster than the particles of a self-reproducing cytoplasmic factor which they carry. This cytoplasmic factor, termed "kappa," is concerned with the character known as "killer." Animals which contain a sufficient quantity of kappa are killers and liberate one of a group of closely allied antibiotics called "paramecins" into the fluid in which they live, making it poisonous to certain "sensitive" stocks. When animals increase faster than kappa, the number of particles of kappa per animal decreases until finally it reaches zero in some individuals. When animals with a decreased number of particles, but not completely lacking them, reproduce slowly, the number of particles of kappa increases faster than the animals until the original concentration is restored. It was shown that these changes in number of particles of kappa per animal are accompanied by both quantitative and qualitative changes in the killer character of the animals.

These changes in kappa concentration brought about by variation in reproductive rate, together with the accompanying changes in the character of the animals, have suggested explanations for numerous problems associated with self-reproducing bodies within the cytoplasm. Attention has centered on these problems recently as a result of the work of SONNEBORN on Paramecium (1943 and 1945a, b), LINDEGREN (1945) and SPIEGELMAN (1945) on yeast, L'HÉRITIER and SIGOT (1944–45) on Drosophila, BILLINGHAM and MEDAWAR (1947) on guinea pigs, and numerous workers on cancer. SONNEBORN (1947a, b) has interpreted certain of his data on the cytoplasmic factor, kappa, in variety 4 of *P. aurelia* as involving similar variations in particle concentration due to fission rate. As he points out, changes in the concentration of particles of self-reproducing cytoplasmic components, however brought about, may be of fundamental importance to an understanding of many biological problems,

such as developmental differentiation, "Dauermodifikationen," senescence and protozoan life cycles, and heredity in bacteria and other vegetatively reproducing forms.

Our knowledge of kappa in *P. aurelia* began with SONNEBORN'S (1943) investigation of the determination and inheritance of the killer character. The full genetic analysis has been carried out only for killers of variety 4 of this species. The production of paramecin depends upon the presence of the cytoplasmic factor kappa which is maintained and increased only in the proper genetic background, namely in the presence of a dominant gene, *K*. If *K* is replaced by its recessive allele, *k*, kappa disappears in the course of a few fissions and the resulting clone no longer produces paramecin; in fact, it becomes sensitive to the action of paramecin. If the gene *K* is reintroduced after kappa has been lost, kappa is not reformed and the production of paramecin is not resumed. Thus, gene *K* cannot initiate the production of kappa, but in its presence kappa is maintained and increased providing some kappa is already present. The ability to produce paramecin can be restored to sensitives with gene *K* by reintroducing some cytoplasm from a killer during conjugation; both kappa and the restored killer character are thereafter maintained permanently during reproduction.

The possibility of controlling the concentration of kappa in the variety 2 killers of *P. aurelia* by taking advantage of the relation between the rate of multiplication of the animals and the rate of increase of kappa has provided a technique for investigating certain properties of kappa. This technique has led to the discovery of three characteristics of kappa (PREER 1946): (1) the number of particles of kappa per animal; (2) the rate of increase of kappa; and (3) the capacity of a single particle of kappa to restore, under appropriate conditions, the normal concentration.

The present paper provides in full the data upon which the preliminary paper was based, together with the results of new work on the same and related problems. Data are given which bear on the rate of increase of kappa under different conditions of temperature, fission rate of the paramecia, and concentration of kappa. Much new material is presented for the determination of particle number and rate of increase of the particles, and all of the pertinent data are treated here by mathematical relations recently derived by R. R. OTTER of PRINCETON UNIVERSITY (private communication) which are a great advance over previously employed mathematical techniques. These relations which OTTER has derived provide the theoretical starting point for any quantitative study involving the distribution of self-reproducing cytoplasmic particles.

II. THE STOCKS AND THEIR CHARACTERS

A. *The stocks*

Eight of SONNEBORN'S stocks of *P. aurelia* have been used in this study. Each stock consists of the descendants of a single individual isolated from a stream or pond. A list of the stocks and their sources follows (see SONNEBORN

1938): stock E from Elkridge, Md.; stock G from near Pinehurst, N. C.; stock H from Halethorpe, Md.; stock K from Baltimore, Md.; stock 36 from Hamden, Conn.; stock 50 from an unknown locality in Oregon; stock 53 from Bloomington, Ind.; stock P from Woodstock, Md.

Seven of the stocks (E, G, H, K, 36, 50, 53) belong to variety 2 of *P. aurelia* and one stock (P) belongs to variety 1. (See SONNEBORN and DIPPELL 1946, for an account of the varieties of *P. aurelia*.) Variety 2, which has formed the main basis for this work, is a separate genetic species, for its sexual isolation from the other varieties is complete.

B. *Killers and non-killers*

Four of the variety 2 stocks (G, H, 36, 50) and one mutant (Gm1), derived from stock G in the course of the investigation are called "killers," for they liberate the antibiotic, paramecin, into the fluid in which they live, making it poisonous to certain other stocks. The paramecin produced by any one stock is not poisonous to the animals which produce it, but is poisonous to the animals of certain other killer and non-killer stocks. For example, the paramecin produced by stock G kills all the other seven stocks except stock 53; and the paramecin produced by stock 50 kills all the stocks except 36, K, and possibly H. When animals are killed by the paramecin produced by a particular stock, they are said to be "sensitive" to that paramecin; when they are not affected by a particular paramecin, they are said to be "resistant" to that paramecin. The terms "sensitive" and "resistant" only have meaning relative to the paramecin produced by a particular stock. For instance, the killer stock H is resistant to its own paramecin, yet sensitive to the paramecin produced by stock G. Early in the course of the investigation the killer stock G was singled out for most intensive study. Consequently, there will be given more detailed accounts of this stock than the others.

The remaining four stocks (E, K, 53, P) were non-killers and were used as detectors of paramecin. As has been pointed out, these stocks differ in their susceptibility to the different killers. The variety 1 stock, P, was found to be sensitive to all the killers, and was adopted as a standard for the detection of paramecin. Stock E was originally described by SONNEBORN (1939) as a killer similar to stock G. At present, however, this stock is no longer a killer. Repeated tests carried out by the author under a variety of conditions show that not only does it fail to kill, but it is now sensitive to the paramecin produced by stock G, paramecin to which it was formerly resistant. Loss of the killer character was reported for a stock of variety 4 by SONNEBORN (1943). Such losses are understandable in view of the observations presently to be set forth.

Permanent non-killer cultures of each of the four killer stocks (G, H, 36, 50), in addition to the four normally non-killer stocks, have been produced in the course of this investigation by means which are described in Section V. It will be shown there that these cultures have lost the killer character because they have lost the cytoplasmic factor, kappa. The non-killer culture of each of the killer stocks is sensitive to the paramecin produced by certain of the killers and resistant to that produced by others, just as in the case of the non-killer stocks.

C. *The paramecins*

Following the method of SONNEBORN, JACOBSON, and DIPPELL (1946) who named the paramecin produced by the variety 4 stock 51, Pn51, we may designate the variety 2 paramecins as PnG, PnH, Pn36, Pn50, and PnGm1. The letters Pn signify paramecin, and the following letter or number indicates the stock or mutant which produces the paramecin. For instance, PnG means the paramecin produced by stock G; and PnGm1 means the paramecin produced by stock G, mutant strain number one.

All paramecins ultimately kill sensitives, but they differ in the way they affect sensitives prior to killing. Further, any one paramecin produces characteristically different prelethal stages on different sensitive stocks. These differences are, however, minor in comparison with the diversities between the actions of different paramecins on the same stocks.

The characteristic prelethal effects of the different paramecins are as follows: PnG causes spinning of sensitives about their longitudinal axes in a direction the reverse of the normal rotation of swimming. PnH causes vacuolation and swelling. Pn36 and Pn50 are identical in action; they combine the effects of PnG and PnH, producing spinning as well as vacuolation and swelling. PnGm1 produces paralysis. The effects of these paramecins will now be given in more detail.

D. *The action of PnG*

1. Previous observations on PnG

There have been previous observations on the action of PnG. SONNEBORN (1939) noted that PnG caused animals to "stop feeding, develop many crystals in the cytoplasm, and spin vigorously on their longitudinal axes. Within a few days all die." SONNEBORN (unpublished) further noted that PnG induced, in certain sensitive stocks of variety 1, clumping somewhat resembling the agglutinative sex reaction; but conjugation never occurred. The "agglutinated" animals also spun while paired.

2. The response of stock P to PnG

a. *General features*

The following observations confirm and extend those of SONNEBORN. The effects of PnG may be illustrated by the sequence of responses of stock P at 27°C. The first observable response appears after about two hours, when the animals swim in wide spirals, alternately rotating for a few seconds on their longitudinal axes in a direction the reverse of normal, and then rotating normally. The reverse rotation then becomes faster and more frequent; forward motion slows down; and by the end of three or four hours the animals spin vigorously and continuously on their longitudinal axes, often in the absence of forward locomotion. The direction of spinning is invariably in a reverse direction to the rotation of normal swimming. After four to six hours, when spinning has reached its peak, the pseudo-conjugation referred to above may be observed. At this time the clear appearance of the animals indicates that they

have stopped feeding. After seven to nine hours, there appear macronuclear changes typical of those occurring during normal fertilization processes (autogamy and conjugation). Subsequently the spinning becomes much slower and many animals merely swim in wide spirals with reversed rotation; partial paralysis may result; crystals develop in the cytoplasm. During the next five or six days the animals also assume a pear-shaped form, become thinner, and finally die.

b. *Induction of macronuclear breakdown*

At 27°C in one mixture of the sensitive stock P plus fluid from a culture of stock G filtered free of animals, periodic staining showed that the early skein stage of macronuclear breakdown occurred during the seventh to ninth hours after mixture. This was quickly followed by more advanced stages of macronuclear changes. All stock P animals show vigorous spinning. Controls, consisting of animals from the same culture of stock P plus fluid from a culture of the non-killer stock K filtered free of animals, underwent no nuclear reorganization. This shows that the effect was due to some substance produced by stock G, presumably paramecin. This induction of pseudo-conjugation and nuclear reorganization is similar to the action of the killer stock Ru22 of *P. bursaria* described by CHEN (1945).

c. *Spinning*

(1) The normal rotation of swimming.—In swimming, paramecium describes a spiral path. Spirals are of two types, "right" and "left." The coils of a dextrally coiled snail and the threads of a right-handed screw are examples of right spirals. It is easy to determine the direction of the spiral path described by paramecium by visualizing the spiral oriented so that it is vertical. If the portions of the coils on the side nearest the observer extend between lower left and upper right, the spiral is a right spiral. If the portions of the coils on the side nearest the observer extend between lower right and upper left, the spiral is a left spiral.

Since the aboral surface of a paramecium is always directed to the outside of the spiral, the animal rotates on its longitudinal axis once for each complete spiral. Just as there are two types of spirals there are also two types of rotation, "left" and "right." It is accepted usage to speak of rotation which is counterclockwise with respect to the observer as left rotation and rotation which is clockwise as right rotation. It is customary in the literature dealing with the behavior of paramecium arbitrarily to designate the direction of rotation as the direction when the anterior end of the animal is directed away from the observer. Thus, if a paramecium is visualized with its anterior end directed away from the observer and it rotates in a clockwise direction, it is said to rotate to the right. If it rotates in a counterclockwise direction it is said to be rotating to the left. In swimming, the direction of rotation is determined by three factors: (1) the direction of the spiral, (2) whether the animal moves forward or backward, and (3) the *invariable* orientation of the animal so that its aboral surface is directed toward the outside of the spiral.

The direction of spiralling and rotation has been studied in detail by JENNINGS (1906) and by BULLINGTON (1930). BULLINGTON has pointed out that, although *P. aurelia* in forward motion normally describes a left spiral (and thus rotates to the left), occasionally animals spiral to the right (and consequently rotate to the right). In backward swimming, without exception, animals describe a right spiral (and thus rotate to the left).

The author's observations confirm BULLINGTON. In certain stocks (not described in this paper) and under certain cultural conditions, right spiralling was common; but under the ordinary conditions of culture which were used, the stocks rarely showed right spiralling.

(2) The effects of PnG on rotation and spiralling.—PnG causes sensitives to rotate and spiral to the right instead of to the left. The effect is exhibited in all degrees of intensity, from very rapid continuous right rotation with suspended locomotion, to slow intermittent right spiralling. The backward swimming of avoiding reactions is unaffected, rotation being to the left in the characteristic manner.

There are various factors which affect the character of this reversal of rotation; the concentration of paramecin, the temperature, the length of exposure, and the particular sensitive stock. When the concentration of paramecin is low, fewer animals are affected, spinning is less vigorous, and animals may alternate brief periods of right spiralling with periods of normal swimming in which spiralling is to the left. Spinning is slower and appears after a longer exposure at lower temperatures than at higher. At 27°C spinning is most vigorous after approximately four to six hours' exposure, and subsequently is reduced in speed. The character of the reversal of rotation also depends on the particular sensitive stock (see below).

(3) Reversal of rotation as a diagnostic character for detecting the presence of PnG.—When right rotation is slow and only a few individuals are observed rotating to the right at any time, because of one or more of the reasons just discussed, the other effects of PnG are usually not apparent. In such cases the direction of rotation becomes a highly diagnostic character for the detection of PnG. It has been pointed out, however, that there are other factors (but not common in the culture conditions employed here) which may cause right spiralling. Therefore, it was necessary to make sure that none of these factors were present when observations were made. Absence of such factors was assured by ascertaining that the controls of the unmixed killers and sensitives showed no reverse rotation. The detection of such weak reactions to PnG based on direction of rotation is difficult until considerable experience has been gained, but then it becomes highly reliable. Numerous experimental cultures, some permanent non-killers, others turning from non-killer to killer have been examined, and hundreds of such cultures have been identified as containing PnG by the presence of single affected animals, to be later confirmed by strong reactions.

3. The response of other stocks to PnG

The response of animals of stocks other than stock P is somewhat different. SONNEBORN (1939) pointed out that the variety 2 stocks seem to be more re-

sistant to the effects of PnG than do stocks of the other varieties. Cultures of the variety 2 stock G which have become sensitive to PnG because they lost kappa never show the vigorous spinning induced in stock P. They usually exhibit only a slow right rotation, intermittently, and in a small proportion of the animals. The more striking effects observed in mixtures of PnG plus stock G sensitives are partial paralysis and typical avoiding reactions with normal left rotation. Death occurs after one to two weeks of continuous exposure to PnG. Different stocks exhibit different degrees of susceptibility. One variety 2 stock, stock 53, seems to be completely resistant.

E. *The action of PnH*

PnH was reported by SONNEBORN (1939) to make animals of susceptible stocks "stop feeding, become sluggish and badly vacuolated and die within 36 hours." Effects are first noticed after about eight hours at 27°C, and most sensitives are dead after 18 hours. SONNEBORN showed that certain variety 2 stocks are resistant and some are sensitive to this paramecin. PnH never induces right rotation.

The intensity of the vacuolation caused by PnH seems to have decreased somewhat since the observations by SONNEBORN (1939). Although PnH still causes vacuolation, the animals which become vacuolated are less numerous and the vacuoles smaller. The tendency to form vacuoles has thus become much weaker than it was in 1939 when originally described by SONNEBORN. PnH caused much more severe vacuolation of sensitives in 1940 when I first observed its action than it does now. A similar change in the character of a killer was reported by SONNEBORN (1943). Strong killers which acted quickly on sensitive animals were said to have changed to weak killers which acted slowly.

F. *The action of Pn36 and Pn50*

Pn36 and Pn50 are practically indistinguishable in their action. They closely resemble PnH in type of abnormalities produced, but act somewhat more quickly. In addition, some stocks (for example stock P) respond to these paramecins with fairly rapid intermittent right spinning in a small proportion of the affected animals. Stocks vary in their sensitivity, and some seem completely resistant.

G. *The action of PnGm1*

PnGm1 causes paralysis of sensitives. They settle to the bottom, stop feeding, and become almost completely immobile except for occasional avoiding reactions. With continuous exposure to PnGm1 they become thinner and more completely paralyzed. It is difficult to observe when they actually die. With stock P (one of the most sensitive stocks to the action of PnGm1) partial paralysis is noted after about 12 hours and a strong paralysis after 24 to 48 hours. Approximately 15 days pass before the animals die. Different stocks differ in their sensitivity, some being completely resistant. Sensitive cultures of stock G (those lacking kappa) are more resistant than stock P to the action of this paramecin; about three weeks of exposure are required to kill them.

III. METHODS

A. *Method of culture*

The method for culturing the paramecia was the same as the one described in detail by SONNEBORN and DIPPELL (1946).

B. *Methods of testing for the killer character*

The usual method of ascertaining whether the animals in a particular culture were killers, resistant non-killers, sensitives, or a mixture of these types was as follows: three samples of the culture were placed into separate concavities on a Pyrex spot plate. To one sample was added an approximately equal sample of a culture of stock P, known to be strongly sensitive to the action of all five killers of variety 2. To the second sample was added an approximately equal sample of a known strong killer. This killer culture was a member of the same stock as the stock under examination. The third sample served as a control; no other culture was added to it. Two other controls, unmixed samples of the standard sensitive stock P and of the standard strong killer stock, completed the test. The five depression cultures, unless otherwise indicated, were kept at approximately 22°C, and observations were made on them after 18 to 24 hours. (A second observation after 48 hours was made when looking for the action of PnGm1.) The different reactions and the manner of recording them are discussed in the following paragraphs.

If killing action was observed only in the mixture with the standard sensitive stock P, the tested culture was a killer. In tests on different cultures which reacted as killer, marked differences in the intensity of the reaction appeared. When nearly 100 percent of the animals of stock P were affected strongly (as evidenced by about half the animals in the mixture showing characteristic abnormalities or death) the reaction was recorded (see tables)as +++, and the tested culture was called a strong killer. When considerably fewer stock P animals were affected and the abnormalities were less severe, the tested culture was a moderate killer and the reaction was recorded as ++. When very few stock P animals were affected and the reaction was just recognizable with certainty, the tested culture was a weak killer and the reaction was recorded as +.

If killing action appeared only in the mixture with the standard killer culture, the tested culture was a sensitive. The reaction occurred with the same gradations in effect, and these were recorded as −, −−, −−−, signifying that the tested clone was a weak, moderate, or strong sensitive, respectively.

When no effect appeared either in the mixture with the standard sensitive or in the mixture with the standard killer, the tested culture was classified as a resistant non-killer, and recorded as 0.

Occasionally cultures gave double reactions; that is, killing appeared both in the mixture with the standard sensitive and in the mixture with the standard killer. In these cases, killing usually appeared in the unmixed control of the tested culture, indicating that both killers and sensitives were present in the same culture. The relative strength of the reactions in the mixtures with

the standard sensitive and in the mixture with the standard killer was recorded with the appropriate number of pluses and minuses. For example, a ++− reaction means that the culture is both a moderate killer and a weak sensitive, and indicates that both killers and sensitives are present in the culture. In such cases, however, there are also probably present some animals which are recognizable neither as killers nor as sensitives; for as mentioned above, occasionally entire cultures consist of such resistant, non-killer animals.

It was necessary to use another method of testing when it was required, as in the experiments described in Sections VI and VII, to determine whether *any* of the animals in a culture was a killer. Standard sensitives were added directly to the culture to be tested. If killing action appeared, it was recorded with the appropriate number of pluses to indicate the strength of killing. If no killing appeared, the culture was recorded as non-killer.

C. *The test tube method of controlling fission rate*

1. General procedure

Control of fission rate proved to be one of the major necessities of the work. Various special techniques were devised and will be described in the accounts of the experiments. One such technique, however, was used commonly and may be described at this point.

Cultures were maintained in test tubes. Daily the contents of each tube were well mixed and a definite volume of fluid and animals was removed. This volume was then replaced with an equal volume of fresh culture fluid. It is evident that the larger the volume discarded and replaced by fresh fluid, the faster the animals will multiply, providing the population density is restored in the course of a day. For instance, if half the volume, and consequently half the number of animals is removed each day and replaced with fresh fluid, then the volume remaining after discarding the portion is increased by a factor of two, or doubled once each day. If the animals multiply sufficiently to restore their original number by the end of each day, then they must double in number. Thus they will increase at a rate of one fission a day (assuming the same fission rate for all individuals). If they increase more slowly than this, then the population density will decrease day by day and the animals will eventually disappear from the tube. If they increase more rapidly, then the culture will become progressively more concentrated. Similarly, when the volume of culture retained is increased daily (with fresh culture medium) by any constant factor, that factor expressed in doublings is equal to the fission rate of the animals.

The validity of this method, as already pointed out, depends upon two conditions: (1) there must be no progressive increase or decrease in population density over a period of days, and (2) all animals must multiply at the same rate. These conditions will now be considered.

2. The validity of the method

a. *The condition of constancy of population density*

If there is a *progressive* increase or decrease in the concentration of animals,

a discrepancy between the true and the calculated fission rates will result, although such changes in concentration must usually be very large to introduce an appreciable error. For instance, let us suppose a culture was maintained by this method at a calculated rate of one fission per day for 20 days, and the animals actually went through only 18 fissions for an average rate of 0.9 fissions per day. The final concentration of animals would lack two doublings (19th and 20th fissions), which would have given a four-fold increase in concentration and thus restore the original concentration. Hence, a discrepancy of only 0.1 fission per day would result in a final observed concentration of animals equal to one fourth of the original value—a difference so great as to be readily detected. In practice, population counts, to ascertain whether large variations are occurring, were used to increase the accuracy of the technique. (Population counts are estimates of the population density, made by counting the number of individuals in a known volume of a sample of the culture.) To illustrate, in the example just given, the calculated rate of 1.0 fission per day would be reported as 0.9 fission per day if the final concentration of animals were one fourth the original concentration.

b. *The condition of equality of fission rate of all animals*

The second condition upon which the validity of the method depends is that all animals multiply at the same rate. This does not mean that all animals must undergo the same number of fissions each day; the conditions will be fulfilled if the *average* number of fissions in each line of descent is the same for all lines in any given period. This is evident from the following considerations. Suppose that on one day some animals undergo too few fissions (that is, less than the calculated number for a day), and that others undergo too many fissions (more than the calculated number). Assume that these fast and slow rates on this day so balance each other that the first condition on which the test tube method is based is fulfilled, namely, that there is no progressive change in population density. If the rate of fission on subsequent days is sufficiently fast in the lines which underwent too few fissions, and sufficiently slow in the lines which underwent too many fissions, then the average rate would be the same in all lines. Furthermore, if all lines have the same average rate, then over any extended period they all undergo the same number of fissions. And since the population remains constant, the true rate must equal the calculated rate.

There is, however, a situation in which the first condition of population constancy holds, but in which the second condition is not met. This results in a discrepancy between true and calculated rates of fission. This situation occurs when animals in certain lines of descent consistently reproduce more slowly than others or cease to reproduce and die. Since the population density can only be maintained by a higher rate of fission of the remaining animals, the true fission rate tends to be greater than calculated.

c. *The limits of accuracy*

It will be shown subsequently in this paper (p. 387) that the combined discrepancies between calculated and true fission rates are not greater than 0.2

fission per day in most experiments. The failure of cultures to meet exactly these two conditions of constancy of population density and uniformity of fission rate means that the "test tube method" of controlling fission rate should be considered a convenient approximate method of controlling fission rate of mass cultures, but not to be used when great accuracy (cf. p. 388) is desired.

3. *Specific procedure for calculating fission rates*

Calculations of the amount of a culture to be discarded and replaced with fresh culture fluid in order to obtain a desired rate of fission were made as follows. Let the volume of culture maintained daily be equal to x. Let the amount of this culture discarded daily be $x-y$, so that y is the volume retained. If now the volume is restored to x (by addition of $x-y$ fresh culture fluid), let s be defined by $x=2^s y$; that is, s is the number of doublings of the residue, y, required to restore the original volume, x. As pointed out above, s is also the fission rate of the animals (when the conditions set forth in the preceding paragraphs are fulfilled). Now the fraction of the total volume, x, retained each day (at the time part of the culture is discarded) is y/x; and from the definition of x, $y/x=2^{-s}$. Hence to maintain any desired fission rate, s, it is required only to retain daily a fraction of the culture (y/x) equal to 2^{-s}. For example, to maintain a culture at a reproductive rate of three fissions per day, it is required to retain daily 2^{-3} or one eighth of the culture and restore the original volume with fresh culture fluid.

IV. THE INHERITANCE OF KILLER CHARACTERS

The determination and inheritance of the killer characters in variety 2 has not been reported except for SONNEBORN's (1943) remark that the observations thus far made agree with the findings in variety 4 and indicate the presence of cytoplasmic factors. This remark (SONNEBORN personal communication) was based simply on the observation that in the F_1 from a cross of stock G killer by stock H killer, the two members of each conjugant pair produced clones with killer characters like the parent from which they derived their cytoplasm. This result is comparable to SONNEBORN's (1943) results on variety 4 killer by sensitive crosses, in which the killer conjugant produced a killer clone, the sensitive conjugant, a sensitive clone. The author has observed, similarly, that no change in character occurs in the F_1 of crosses of G killer by the sensitive stock K, H killer by stock K, or G killer by H non-killer. In view of what is known further in variety 4, this makes it seem likely that cytoplasmic factors are involved in production of the killer character in variety 2. Finally, the evidence to be set forth presently shows that numerous self-reproducing particles which segregate amitotically at fission of the animals are necessary for the determination of the killer character in variety 2, making the conclusion that they represent kappa inescapable. The word "kappa" is used here as a general term to refer to the various cytoplasmic factors determining the killer character (but not any other characters) in variety 2 as well as variety 4. It is not meant to imply that these factors are identical in the various killer stocks.

TABLE I

Stocks G, H, and 50. The character of initially slowly dividing strong killer cultures after reproduction for various periods at different fission rates. This is shown by the results of tests which indicate whether animals are killers (indicated by +), resistant non-killers (indicated by 0), or sensitives (indicated by −); the number of + and − symbols indicates strength of reaction.

STOCK	MEAN DAILY FISSION RATE	KILLER CHARACTER AFTER										
		1 DAY	2 DAYS	3 DAYS	4 DAYS	5 DAYS	6 DAYS	7 DAYS	8 DAYS	9 DAYS	10 DAYS	15 DAYS
G	0.1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	0.6	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	1.0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2.0	+++	+++	+++	++−	+	0	−	−	−	−−	−
	3.0	+++	++	+−	+−	−−	−−−	−−−	0	−−−	−−−	−−−
H	0.1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	0.6	+++	+++	+++	+++	+++	+++	+++	++	++	+++	+++
	1.0	+++	+++	++	++	+	++	+	+	0	+	+++
	2.0	+++	+++	+	0	0	0		−	0	0	0
	3.0	+++	+	0	0	−	−	0	−	0	−	−
50	0.1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	0.6	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	1.0	+++	+++	++	++	++	++	+	0	+	+	0
	2.0	+++	+++	+	+	0	0	−	0	0	0	0
	3.0	+++	+	0	0	−	0	0	−	0	0	−

V. THE CONTROL OF THE KILLER CHARACTER IN VARIETY 2

Soon after this study of the variety 2 killers began, a striking difference between them and the variety 4 killers became apparent. Killer stocks in variety 2, in contrast to those in variety 4, commonly gave rise to non-killer, sensitive progeny. Analysis of the factor or factors responsible for the sporadic loss of the killer character was made possible by a remarkable observation which proved to be of great significance. Slowly reproducing cultures of killers seemed to remain consistently strong killers, but more rapidly reproducing cultures lost the killer character. Experiment No. 1 was designed to give pertinent information concerning this observation.

Experiment No. 1: The experiment was started with three slowly reproducing, strong killer cultures of stocks G, H and 50. Five subcultures were made from each of these. By use of the "test tube technique" (see p. 358), the five subcultures of each stock were cultured respectively at rates of 0.1, 0.6, 1.0, 2.0, and 3.0 fissions per day at 27°C. Every day a sample was taken from each tube. A portion of each sample was then tested for the killer character. (The cultures maintained at three fissions per day were held for a day until the food was exhausted, allowing one or two more fissions, before tests were made; this was done so that these animals would be in more nearly the same nutritive condition as the more slowly reproducing cultures.) On various days, when samples were taken from the tubes, portions of the sample from each tube were transferred to separate spot plate depressions and fed only enough to allow about 0.1 fission per day. These were tested subsequently as explained below. Results are summarized in tables 1 and 2.

TABLE 2

Stocks G, H, and 50. Time (in days) for return of killer character (during multiplication at 0.1 fission per day) after loss of the killer character as a result of reproduction for various periods at two and three fissions per day.

STOCK	NUMBER OF DAYS FOR RETURN OF KILLER CHARACTER							
	AFTER REPRODUCTION AT 2 FISSIONS PER DAY FOR				AFTER REPRODUCTION AT 3 FISSIONS PER DAY FOR			
	4 DAYS	6 DAYS	10 DAYS	15 DAYS	4 DAYS	6 DAYS	10 DAYS	15 DAYS
G		4	4	6		7	9	Never
H	7	8	9	12	7		19	Never
50		11	13	19	13	17	19	Never

Table 1 shows that animals multiplying 0.5 fission per day and less retained full killing strength. Animals cultured at one fission per day remained strong killers in the case of stock G, but became weak killers or resistant non-killers in the case of the other two stocks. Animals cultured at two fissions per day or

more rapidly lost their killing ability and finally became sensitive or resistant non-killer. At these rates stock G finally became strongly sensitive; stocks H and 50 acquired the weak and sporadic sensitivity characteristic of all non-killer cultures of these stocks. It should be noted that in changing from killer to sensitive a series of quantitative as well as qualitative changes were shown: strong killer, weak killer, resistant, weak sensitive, and finally (in the case of stock G) strong sensitive. Furthermore, the faster the animals reproduced, the sooner these changes began to take place and the faster they progressed.

The samples mentioned above, which were removed from the rapidly reproducing cultures at different times after they had become non-killers or sensitives, were allowed to increase at approximately 0.1 fission per day. A series of tests was made every day or two on the killing action of these cultures, using subcultures of them for testing. The first several tests in the series showed that the cultures remained sensitive for a period; but later tests usually proved they developed a weak killing action, followed on still later tests by stronger killing action. Often, however, killing was strong at its first appearance.

Table 2 gives the number of days of slow reproduction which passed before tests showed definite killing. The table shows in general that the longer fast reproduction continued, the longer was required for the killer character to return. This was evidenced even after the killing tests (recorded in table 1), made on the subcultures at the time they were first removed from the tubes, showed no further change toward sensitive. For instance, reference to table 1 shows that stock 50 multiplying at two fissions per day had lost all killing and gave a "O" reaction on tests subsequent to the fourth day. Yet table 2 shows that after six days at two fissions per day, 11 days were required for killing to return; after ten days, 13 days were required; and after 15 days, 19 days were required.

Table 2 also shows that for any given time of rapid reproduction, the more rapid the reproductive rate, the longer the time required for return of killing activity. For example, stock G, cultured at two fissions per day for ten days, required *four* days at 0.1 fission per day for killing to return; whereas, when cultured at three fissions per day for this same length of time (ten days), *nine* days at 0.1 fission per day were required. In general, the return to killing was more rapid in stock G than in H or 50.

Finally, cultures of all three stocks cultured at three fissions per day for 15 days did not become killers again when cultured slowly, though these particular cultures were periodically tested for three months. In other experiments, cultures of stocks G, H, 36, and 50, made non-killer or sensitive by similar long periods of rapid reproduction, have subsequently been cultured slowly under a variety of conditions for a year; but they still lack the killer character. The loss thus seems to be permanent.

* * *

From this experiment the following conclusions may be drawn: (1) When animals are fed only a small amount of food so that a slow rate of fission is

maintained, the cultures remain pure killer indefinitely. On the other hand, when more food is made available and the rate of fission is increased, cultures lose their ability to kill and become non-killers or sensitive. (2) Non-killer and sensitive cultures produced in this manner will revert to killer after a few days when their reproductive rate is slowed or stopped, unless the fast reproduction has been maintained beyond a certain critical point. (3) The longer and the faster the rapid fissions are maintained, the longer it takes the cultures to revert to strong killers. (4) If rapid fissions are maintained long enough, animals are produced which permanently lack the killer character.

Only one reasonable explanation for these facts has occurred to the writer. The rate of increase of the hereditary basis for killing, kappa, fails to keep pace with the rate of increase of the animals; the amount of kappa per animal is progressively reduced in quantity by successive fissions, and finally animals are produced which have no kappa at all. Animals with many particles of kappa are strong killers, but as the number is progressively reduced there appear weak killers, resistant non-killers, weak sensitives, and finally, in the case of stock G, strong sensitives.

VI. DEMONSTRATION THAT ONE PARTICLE OF KAPPA IS SUFFICIENT TO ENABLE AN ANIMAL TO PRODUCE KILLER PROGENY

The relation between the rate of increase of kappa and the fission rate of the animals described in the previous section makes it possible to determine whether a single particle of kappa in an animal is sufficient to enable that animal to produce progeny with the normal particle number when reproduction is slowed. Experiments performed to test this point were designed as follows:

From an originally killer culture, which had been reproducing rapidly (and, hence, had been experiencing a decline in its kappa concentration), a number of animals were isolated into separate containers. Each of these isolated animals was allowed to continue its rapid multiplication for a limited period of time, thus further reducing the kappa concentration. As all of the progeny produced from any one isolated animal were kept together in one container, if the isolated animal possessed a particle of kappa, then some of its progeny in the container must also possess kappa, providing no kappa is destroyed. Whether killer animals can arise among the progeny (when their reproduction is slowed or stopped, so that kappa concentration can again rise), depends on the minimum number of particles of kappa required for regeneration of the original kappa concentration.

If one particle is enough, then every animal that contained at least one particle of kappa when isolated will yield some killers among its progeny. The percentage of isolated animals whose progeny could experience regeneration of kappa would be completely independent of the duration of the rapid fissions after isolation. The progressive lowering of mean kappa concentration subsequent to isolation, though it occurs, is here irrelevant; the decisive fact is

simply whether an animal in the culture contains at least one particle of kappa.

On the other hand, if regeneration of kappa is blocked unless *more* than one particle is present in an animal, then some animals which had only the minimum (or somewhat more than the minimum) number of particles of kappa when isolated would yield progeny none of which contained the minimum number of particles. This is true because, as set forth above, the mean kappa concentration is further reduced during the period of rapid reproduction following isolation. Under such conditions, therefore, the percentage of isolated animals whose progeny could experience regeneration of kappa would decrease as the period of rapid fissions is prolonged, for more and more would have their kappa concentration brought below the assumed minimum for regeneration.

Therefore, if one particle is sufficient, the percentage of isolated animals producing killer progeny would be independent of the number of fissions following isolation; and if more than one particle is required, the percentage of animals producing no killer progeny should increase with the number of rapid fissions subsequent to isolation. The following experiments were performed to test this point.

Experiment No. 2. In one experiment a culture was started by isolating a single killer of stock G. The culture was maintained at a fission rate of 3.3 fissions per day for 16 fissions at 27°C in order to reduce the number of particles per animal. A number of animals was then removed from this culture and isolated into separate containers. The isolations were separated into two series. The first series was allowed to undergo eight rapid fissions at a rate of three fissions per day, and the second series was allowed to undergo 15 rapid fissions at three fissions per day. Then multiplication was stopped in each series and tests were made to discover whether any of the progeny of each of the original animals became killers. It was found that 10.9 percent (14 out of 128) of the isolations of the first series which underwent eight rapid fissions gave rise to cultures consisting of all permanent non-killer progeny. And 10.2 percent (13 out of 127) of the isolations of the second series which underwent 15 rapid fissions gave rise to all permanent non-killer progeny.

Since approximately ten percent of the animals failed to produce any killer progeny, the kappa concentration must have been reduced to a level low enough to yield significant differences as a test for the hypothesis. For if ten percent of the cultures gave rise to *no* killers after eight rapid fissions, many of the other cultures must have had the minimum (or slightly greater) number of particles per animal necessary for regeneration of normal particle concentrations. The fact that the percentage was not greater following 15 rapid fissions than following eight rapid fissions indicates that one particle is sufficient for regeneration of the original concentration of kappa.

Experiment No. 3. This experiment was performed merely to supplement the preceding one under somewhat different conditions. The main difference in the two experiments was that in this experiment, instead of allowing both sets of animals to multiply rapidly after isolation, the first series of isolates

was allowed to undergo no rapid fissions, and the second series was allowed to undergo ten rapid fissions.

In this experiment also a culture was started by isolating a single killer of stock G. The culture was maintained at 27°C at a fission rate of 3.4 fissions per day for 17 fissions in order to reduce the number of particles per animal. A number of animals was then removed from this culture and isolated into separate containers. The isolations were separated into two series. The first series consisted of a number of these original animals which were isolated into "exhausted fluid"—fluid filtered from a culture of *P. caudatum* which had exhausted most of the food. Fresh culture fluid was subsequently furnished the animals in such quantities that the animals reproduced at about one half fission per day and thus allowed animals capable of becoming killers to do so. The second series of isolations was made into fresh culture fluid of a quantity such that the animals underwent ten more rapid fissions at a rate of 3.4 fissions per day. Reproduction was then slowed to about 0.1 fission per day to allow killing to develop. Tests showed that 54.6 percent (53 out of 97) of the original animals in the first group and 55.6 percent (55 out of 99) of the original animals in the second group had lost the ability to give any killer progeny under these conditions.

Since 54.6 percent of the animals had less than the minimum number of particles for particle regeneration when isolated, the level of kappa concentration must have been sufficiently low to provide significant differences as a test for the hypothesis. Thus again the percentage in the two groups was essentially the same, showing that one particle of kappa is sufficient for reversion.

* * *

The question might be raised as to what would be expected in such experiments if there is destruction of kappa constantly taking place along with the production of more kappa. If such is the case, then the rate of increase of kappa can be considered a *net* rate of increase, for it would represent a balance between destruction of kappa and production of more kappa.

If it is assumed that more than one particle is necessary for reversion it is clear that the general relations in the experiment will be the same whether there is destruction or not. For the percentage of isolated animals producing no progeny capable of becoming killer must again increase as the increasing number of rapid fissions progressively reduces the particle concentration in *all* the progeny below the number necessary for reversion.

If it is assumed that one particle is sufficient for reversion the expectations can be made clear by the following consideration. At any time, the particles of kappa may be classified into two categories: (1) particles which will give rise to new particles at a certain net rate of increase, and (2) particles which will themselves be destroyed, or whose descendants will all be destroyed. The particles of the second category may be ignored, for they give no permanent lines of descent and hence cannot cause any animal to become killer again. The particles of the first category are comparable to particles in the case in which it

was assumed that there is no destruction, for both give rise to increasing numbers of particles when reproduction is slowed. There is no reason to believe that the number of particles of this category in the original isolations would decrease as the animals undergo successive rapid fissions. Consequently, in the series of isolations which underwent more rapid fissions there should be no increase in the percentage of isolations giving exclusively permanent sensitive progeny. And since the data showed no increasing percentages, one particle must be sufficient for reversion, no matter whether destruction occurs or not.

VII. ESTIMATION OF THE NUMBER OF PARTICLES OF KAPPA AND THEIR RATE OF INCREASE

It has been shown that some of the progeny of killers may be freed of kappa by letting them undergo a series of rapid fissions. It is possible to measure the proportion of the progeny of a strong killer which has been freed of kappa after different numbers of rapid fissions. These data may be used to estimate the number of particles of kappa in the strong killer and their rate of increase. The proportion of the progeny lacking kappa is the proportion of the progeny incapable of producing killers when isolated and cultured at a slow fission rate. It was shown in Section VI that this is true, for a single particle is sufficient to enable an animal to produce killers when cultured slowly; therefore, only animals with no particles produce exclusively permanent non-killer progeny.

First will be given the details of three experiments performed to determine the percentage of animals with no particles of kappa after different numbers of rapid fissions. The account of these experiments will be followed by calculations of the number and rate of increase of the particles, making use of the experimental data.

A. The experiments

Experiment No. 4. This experiment was started by isolating 192 of the progeny of a single killer of stock G which had undergone eight rapid fissions at 27°C. The 192 isolations underwent four more rapid fissions, giving 16 animals in each culture. One of the 16 from each culture was then isolated to start another series of 192 cultures. The remaining 15 animals in each culture, the "left-overs," were retained and treated as described in the next paragraph. When the newly isolated animals had undergone four or five rapid fissions, the process of isolation was repeated giving a new set of 192 isolations and a second set of left-over cultures. The process was repeated each time the newly isolated animals underwent four or five more fissions. This procedure of repeated reisolation at short intervals is known as "maintaining animals in isolation."

The animals in each set of left-over cultures continued multiplying rapidly for about four more fissions until their food was exhausted. At this time reproduction was suppressed to allow animals with kappa to produce killers. It was then determined what proportion of each set of left-over cultures contained no killers. The proportion obtained for each set represents the proportion of isolated individuals with no particles of kappa.

The results of the tests are given in the following tabulation:

MEAN NUMBER OF FISSIONS (n)	NUMBER OF LINES OF DESCENT		PROPORTION LACKING KAPPA (W_n)
	TOTAL	LACKING KAPPA	
12	192	2	0.01
16	192	47	0.24
21	191	141	0.74
26	191	178	0.93
31	191	186	0.97
36	191	189	0.99

The average fission rate in the isolation lines was 3.4 fissions per day.

Experiment No. 5. This experiment was started by isolating 120 of the progeny of a single killer of stock G which had undergone seven rapid fissions at 27°C. These 120 animals were also "maintained in isolation" as described in Experiment No. 4. The proportion of the animals with no particles of kappa after different numbers of fissions was not determined from the left-over cultures as in Experiment No. 4. These left-overs were discarded. Instead, the proportion of animals with no particles was determined from a series of isolations made as follows. Each time the animals underwent three or four fissions, *two* animals instead of *one* animal were removed. One was isolated in fresh culture fluid to give the new series of isolations in the usual manner, but the other was isolated into "exhausted fluid" (see p. 366) and cultured slowly to allow killers to appear among its progeny if it had contained particles of kappa when isolated. These slowly multiplying series of isolations were then tested and it was determined what proportion of the animals had produced all permanently sensitive progeny. As already pointed out, this represents the proportion with no particles of kappa. Because of culturing difficulties, certain of the animals in the rapidly multiplying isolation lines did not maintain a uniform high fission rate. These cultures were discarded, and this resulted in a reduction in the number of cultures tested as the experiment progressed.

The results of the tests are given in the following tabulation:

MEAN NUMBER OF FISSIONS (n)	NUMBER OF LINES OF DESCENT		PROPORTION LACKING KAPPA (W_n)
	TOTAL	LACKING KAPPA	
10	120	0	0
14	112	12	0.11
18	80	37	0.46
21	78	56	0.72

The average fission rate was 3.4 fissions per day.

Experiment No. 6. Instead of beginning with a single animal as in Experiments No. 4 and No. 5, 40 strong killers, previously multiplying at a rate of 0.4

fission per day (test tube method) at 27°C, were used to start this experiment. A temperature of 27°C was maintained during the experiment. Each initial animal was allowed to undergo two fissions, giving a total of 160 animals. One hundred twenty of these animals were isolated, and the experiment was continued just as described for Experiment No. 5. The animals were maintained by the isolation technique. The proportion of the animals with no kappa after different numbers of fissions was obtained from series of animals isolated into "exhausted fluid" and grown slowly as in the preceding experiment.

The results of the tests are given in the following tabulation:

MEAN NUMBER OF FISSIONS (n)	NUMBER OF LINES OF DESCENT		PROPORTION LACKING KAPPA (W_n)
	TOTAL	LACKING KAPPA	
13	120	5	0.04
16	120	37	0.31
20	117	78	0.67
23	111	98	0.88
27	88	85	0.97

The rate of fission was 3.4 fissions per day.

B. *Calculation of the number of particles and their rate of increase*

Two methods have been described (PREER 1946) by which the rate of increase of kappa and the number of particles of kappa in a killer have been computed. It was pointed out that the first method, based on the Poisson distribution, was not strictly applicable. Furthermore, the second method consisted of empirical "pencil and paper" trials which were very laborious. It does not now seem worthwhile to give a detailed presentation of either of these methods in view of a mathematical solution recently developed by OTTER (personal communication, to be published later). OTTER's method is discussed and applied to the data in the following sections.

1. The general formula

In developing and applying general mathematical formulas it was necessary to make certain assumptions. These assumptions were: (1) the kappa concentration among the strong killers used to start Experiments No. 4, No. 5, and No. 6 was considered uniform. (2) it was necessary to assume how much, if any, particle destruction or loss occurred; (3) it was necessary to specify whether kappa particles multiply by duplication, triplication, or reproduce by higher multiples; (4) it was assumed that particles are segregated at random to the two animals produced at each fission. (5) it was assumed that the probability for a particle to reproduce, disintegrate, or remain intact in an interfission interval is the same for each particle and for each interfission interval. This would mean that kappa increased at a constant exponential rate during the experiments. These particular assumptions were made because of their sim-

plicity; qualifications and a general discussion of the assumptions will be taken up later.

OTTER starts with a single animal which has just arisen as one of the two products of a fission. At this time the animal contains m particles. Now the animal multiplies by fission. In each interfission interval some of the particles may disintegrate; others may remain unchanged, duplicate, triplicate, or reproduce by higher multiples. p_k represents the probability that any one particle will increase by a multiple of k during an interfission interval. For example, during each interfission interval p_0 represents the probability of a particle increasing by a multiple of 0, in other words, of disintegrating; p_1 is the probability of a particle duplicating, etc. Since the sum of all the probabilities for any particle must be one, we may write:

$$\sum_{k=0}^{\infty} p_k = 1. \tag{1}$$

It can be shown how the values of p_k and k are a measure of the rate of increase of the particles. In order to do this, let us first denote the total number of particles present at the beginning of an interfission interval as x, and then determine how many particles will be present at the end of the interval. As has already been pointed out, the probability of any one of the particles giving rise to k particles is p_k. Therefore, p_k approximates the proportion of the x particles which gives rise to k particles. The number of the x particles giving rise to k particles is therefore xp_k. This number of particles (xp_k) gives rise to kxp_k particles during the interfission interval. The total number of particles produced from all the original x particles is then

$$\sum_{k=0}^{\infty} kxp_k \quad \text{or} \quad x\sum_{k=0}^{\infty} kp_k.$$

The multiple by which the x particles increased in the interfission interval is therefore

$$\sum_{k=0}^{\infty} kp_k.$$

This multiple is designated as $\bar{k}$.

$$\bar{k} = \sum_{k=0}^{\infty} kp_k \tag{2}$$

$\bar{k}$ represents the average rate of increase of kappa and is expressed as the average multiple by which all the particles are increased in each interfission interval.

Previously the rate of increase of kappa has been expressed as doublings per day. Doublings per day may be related to $\bar{k}$ as follows. Let f represent the number of kappa doublings per day and s the number of fissions per day. Then f/s represents the number of doublings of kappa in each interfission interval

and $2^{f/s}$ is the multiple by which kappa increases in this period. So we may write:

$$\bar{k} = 2^{f/s}. \tag{3}$$

At each fission the particles are distributed at random to the two new individuals. The probability that any animal after n fissions will have i particles is designated as $W_{n,i}$. In this paper we shall only be concerned with $W_{n,0}$, which is the probability of any animal after n fissions having zero particles. Therefore, $W_{n,0}$ will be designated by W_n for simplicity. W_n is approximately equal to the proportion of a large group of individuals having no particles. OTTER has shown these quantities to be related according to the following iteration formula.

$$W_n^{1/m} = \sum_{k=0}^{\infty} p_k \left(\frac{1 + W_{n-1}^{1/m}}{2}\right)^k. \tag{4}$$

A demonstration of this formula will be given presently, but first several other relations necessary for the calculation of $\bar{k}$ and m will be considered.

In the special case when $m=1$, we may designate W_n by Q_n.

$$Q_n = \sum_{k=0}^{\infty} p_k \left(\frac{1 + Q_{n-1}}{2}\right)^k. \tag{5}$$

OTTER points out that if values of Q_n calculated by iteration from this formula are raised to the mth power one obtains the corresponding values of W_n calculated by iteration from (4).

$$Q_n^m = W_n. \tag{6}$$

OTTER demonstrates as follows that the relation

$$\frac{\log W_n}{\log W_{n-1}} = \frac{\bar{k}}{2} \tag{7}$$

is approximately true. Starting with (5)

$$Q_n = \sum_{k=0}^{\infty} p_k(1 + u)^k$$

where u is defined to be $\dfrac{Q_{n-1} - 1}{2}$. Expanding by the binomial

$$Q_n = \sum_{k=0}^{\infty} p_k + \sum_{k=0}^{\infty} k p_k u + \sum_{k=0}^{\infty} \frac{k(k-1)}{2} p_k u^2 + \cdots.$$

If $\bar{k} \leqq 2$ it can be shown that $\lim_{n\to\infty} Q_n = 1$; hence if n is sufficiently large, Q_{n-1} is close to 1, and u is small. Therefore, we may neglect all except the first two terms.

$$Q_n = 1 + \sum_{k=0}^{\infty} kp_k u = 1 + \bar{k}u$$

and

$$\log Q_n = \log (1 + \bar{k}u). \tag{8}$$

The following approximation

$$\log (1 + \bar{k}u) = \frac{\bar{k}}{2} \log (1 + 2u) \tag{9}$$

can be shown, for,

$$\log (1 + \bar{k}u) - \frac{\bar{k}}{2} \log (1 + 2u) = \frac{\bar{k}}{2} \log \frac{(1 + \bar{k}u)^{2/\bar{k}}}{1 + 2u}.$$

Expanding, and remembering u is small

$$\frac{\bar{k}}{2} \log \frac{(1 + \bar{k}u)^{2/\bar{k}}}{1 + 2u} \approx \frac{\bar{k}}{2} \log \frac{1 + 2u + \frac{2/\bar{k}(2/\bar{k} - 1)}{2} \bar{k}^2u^2}{1 + 2u} \approx 0.$$

From (8) and (9)

$$\frac{\log Q_n}{\log Q_{n-1}} = \frac{\bar{k}}{2}.$$

And from (6)

$$\frac{\log W_n}{\log W_{n-1}} = \frac{\bar{k}}{2}.$$

Thus, equations (5), (6), and (7) can all be developed from (4). The development of (4) by OTTER, making possible the calculation of the proportion of animals with no particles ($W_{n,0}$), is part of a more general consideration which includes a treatment of the values of $W_{n,i}$. OTTER's demonstration of (4) is consequently somewhat lengthy and will not be given here. SEWALL WRIGHT (personal communication) has pointed out a simple demonstration of equation (4). "An animal with one kappa particle has by definition the probability Q_0 of losing it before the first fission, and probabilities Q_1, Q_2, etc. of having the number indicated by the subscript. The chance of no kappa in one of the progeny is one if none was present before fission, 1/2 if one was present, $(1/2)^2$ if two were present, $(1/2)^k$ if k were present, assuming random segregation. Thus,

$$Q_1 = p_0 + p_1(1/2) + p_2(1/2)^2 + \cdots + p_k(1/2)^k + \cdots.$$

Turning now to an animal of the nth generation, this may trace to any of the above cases before the first fission. If kappa was lost then, it stays lost [contribution to Q_n is $p_0(1)$]. If kappa remained single, it was either lost at

the first fission [contribution $p_1(1/2)$] or it was transmitted to the latter, in which case it has by definition a chance Q_{n-1} of loss in the following $(n-1)$ generations [contribution $p_1(1/2)Q_{n-1}$]. Total contribution for the case of single kappa particle is

$$p_1\left(\frac{1 + Q_{n-1}}{2}\right).$$

If kappa doubled in the first animal (chance p_2), each particle had the same chance of ultimate loss as above, giving

$$p_2\left(\frac{1 + Q_{n-1}}{2}\right)^2$$

as the contribution of this case to the total probability of loss. Similarly if kappa tripled in the founder the contribution to Q_n is

$$p_3\left(\frac{1 + Q_{n-1}}{2}\right)^3."$$

This gives equation (5)

$$Q_n = p_0 + p_1\left(\frac{1 + Q_{n-1}}{2}\right) + p_2\left(\frac{1 + Q_{n-1}}{2}\right)^2 + \cdots + p_k\left(\frac{1 + Q_{n-1}}{2}\right)^k + \cdots$$

$$Q_n = \sum_{k=0}^{\infty} p_k\left(\frac{1 + Q_{n-1}}{2}\right)^k.$$

Equation (6) may be demonstrated independently of (4), for it "involves merely the hypothesis that the fates of the original m particles are independent and that, therefore, the probabilities of loss (Q_n) can be multiplied to find the probability of loss for all." And substituting (6) into (5) we get (4):

$$W_n^{1/m} = \sum_{k=0}^{\infty} p_k\left(\frac{1 + W_{n-1}^{1/m}}{2}\right)^k$$

2. The method of calculating the value of $\bar{k}$

The method of calculation of $\bar{k}$ from the experimental determination of W_n can be obtained from the following equation derived from equation (7):

$$\log(-\log W_n) - \log(-\log W_{n-1}) = \log \bar{k} - \log 2.$$

Thus if log (−log) of the experimental values of W_n is plotted against the number of fissions, n, a straight line should result with slope [log $(-\log W_n)$ $-\log(-\log W_{n-1})$] equal to log $(\bar{k}/2)$. By plotting the data in this manner and measuring the slope of the resulting line one can thus calculate $\bar{k}$.

3. The method for calculating the value of m

After $\bar{k}$ has been computed by the method just outlined, it is possible to calculate m, the original number of particles. The exact method and results of this calculation will vary with the particular assumptions which are made about the behavior of the particles. m can be calculated assuming particles increase by duplication or triplication or by higher multiples. Also it can be assumed that there is destruction of particles as well as multiplication of particles. Any set of assumptions is possible provided that the average rate of increase, $\bar{k}$, is made equal to the value of $\bar{k}$ calculated from the experimental values of W_n. After it has been decided what assumptions are to be made regarding destruction of particles and their manner of reproduction, appropriate values of p_k must be assigned, remembering that $\sum_{k=0}^{\infty} p_k$ must equal one (equation 1), and that $\bar{k}$ must equal $\sum_{k=0}^{\infty} kp_k$ (equation 2).

For example, suppose that $\bar{k}$ has been calculated to have a value of 1.5. If we assume that there is no destruction of particles, p_0 must equal zero. If we assume that the method of particle reproduction is by duplication, then all values of p_k greater than p_2 will equal zero. Thus, in any given interfission interval a certain proportion (p_2) of the particles will duplicate and the remaining proportion (p_1) will remain intact. By means of equations (1) and (2) we find $p_1=0.5$ and $p_2=0.5$. Now use is made of equation (5). Substituting these values into (5),

$$Q_n = 0.5\left(\frac{1 + Q_{n-1}}{2}\right) + 0.5\left(\frac{1 + Q_{n-1}}{2}\right)^2. \qquad (10)$$

This equation, or a similar equation derived by making a different set of assumptions may be utilized to calculate m as follows.

Considering equation (10), when $n=0$, there is one animal with one particle of kappa. Therefore the probability of the animal having no particles is zero ($Q_0=0$). Substitution into equation (10) shows $Q_1=0.3750$. Using this value for Q_1, we can substitute into (10) and obtain $Q_2=0.5801$. Similarly Q_3 is found to be 0.7071. By proceeding in this manner it is possible to determine the value of Q_n for any value of n. m may now be determined by substituting into equation (6) an experimental value of W_n and the corresponding calculated value of Q_n. A better average value for m will be obtained if the value of W_n used lies on the straight line which has been fitted to the log (−log) of the experimental values of W_n plotted against n in the manner already outlined.

4. Application of OTTER'S method to the data of the experiments

The data of experiments No. 4, No. 5, and No. 6 have been used to solve for $\bar{k}$ and m by the methods already outlined. The log (−log) of the experimental values of W_n are plotted against n in figure 1. The straight line which seemed best to fit the points was drawn. That the deviation of none of these points from the straight line is statistically significant was shown by the following consideration.

It will be recalled that W_n, the probability that an animal has no particles of

kappa, is determined by ascertaining what proportion of the animals in a sample have no particles. But it is obvious that, by chance, the proportion of the animals with no kappa in a given sample may not represent W_n precisely. Some idea of the magnitude of the expected chance deviation of the determinations of the proportion of animals with no kappa from the true value of W_n

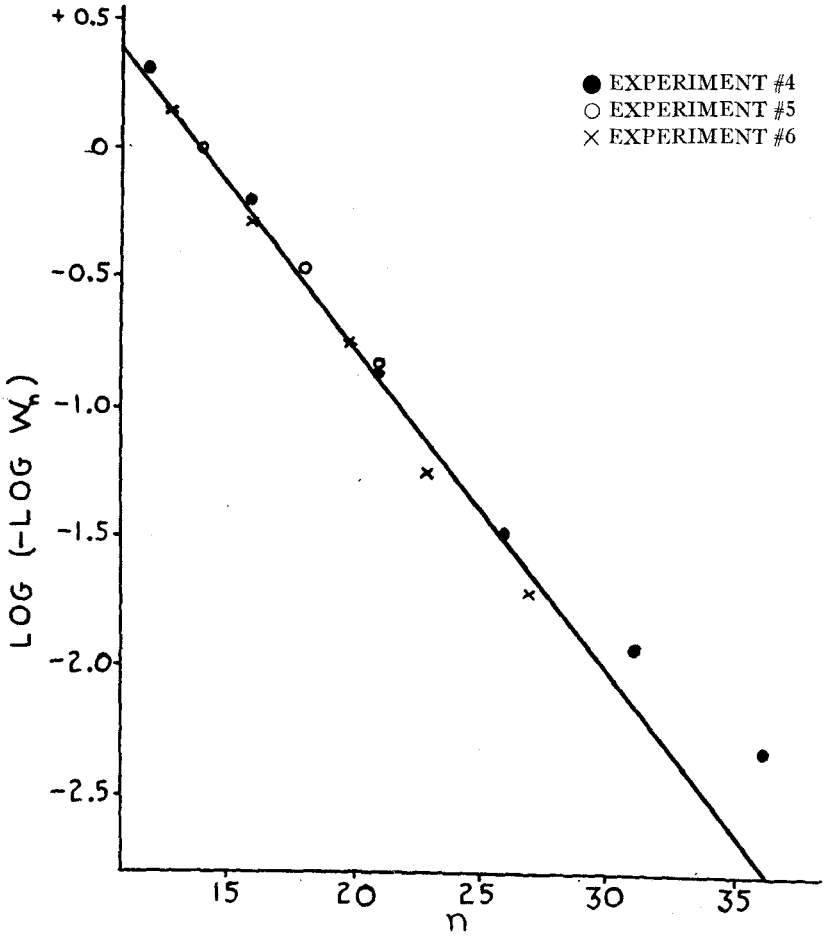

FIGURE 1. *Log (−log) W_n (the proportion of animals with no kappa) plotted against n (number of fissions) from the data of Experiments 4, 5, 6.* Fission rate was 3.4 fissions per day.

can be obtained by computing the 95 percent confidence interval for each experimental value of W_n. This has been done. Use has been made of tables and charts of confidence intervals given by RICKER (1937) and CLOPPER and PEARSON (1934). The meaning of the 95 percent confidence interval may be illustrated by the experimental value of 0.97 (186 animals without kappa out of a total of 191) for W_n found in Experiment No. 4 when $n=31$. The confidence interval in this case is found to be 0.94 to 0.99. This means that the probability is 0.95 that the true proportion of the animals with no particles, in

the population from which the sample of 191 animals was taken, lies within the limits 0.94 to 0.99. We may find whether the line drawn in figure 1 passes within the confidence interval by reading the value of log $(-\log W_n)$ from the line at $n=31$ and computing W_n by taking antilogs. W_n is found to be 0.98—and this figure lies within the 95 percent confidence interval (0.94–0.99), showing that the line does not deviate more from the experimentally determined point on the curve than might be expected by chance. The line drawn in figure 1 passes well within the 95 percent confidence interval for the experimental values of W_n on which all points are based, indicating that the line is within the probable limits of chance variation of all the experimentally determined points.

The value of $\bar{k}$ was determined by measuring the slope of the line plotted in figure 1. The slope of this line was found to be -0.126. $\bar{k}$ was computed as 1.50. The rate of increase of kappa expressed by $\bar{k}=1.50$ may be converted into doublings per day of kappa, f, by taking into account the average fission rate of the animals, s, which was 3.4 fissions per day. Utilizing equation (3) the value of f is computed to be 2.0.

The value of m was determined on the basis of several assumptions. First assume no destruction and assume particles increase by duplication. In this case, as already explained, since $\bar{k}=1.5$, $p_1=0.5$, and $p_2=0.5$, and the other terms of p_k equal zero. Computations based on equation (10) as already outlined, show $Q_{18}=0.99673$. Reference to figure 1 shows $W_{18}=0.483$. Substituting into equation (6), $m=220$.

Second, assume destruction of particles takes place. Since any amount of destruction can be assumed, let the rate of destruction in each interfission interval be equal to 0.1 of the particles present at the beginning of each interval. In other words $p_0=0.1$. Let particle reproduction be by doublings. All p_k greater than p_2 will therefore equal zero; $p_1=0.3$; $p_2=0.6$. Q_{18} is found to equal 0.99701 and m equals 240.

Third, assume a greater rate of destruction. Let $p_0=0.25$. Assuming particles reproduce by duplication, all p_k greater than p_2 will equal zero; $p_1=0$; $p_2=0.75$. Q_{18} is found to be 0.99736 and m equals 270.

Fourth, assume no destruction of particles, and assume particle reproduction is by triplication. In this case all terms of p_k are equal to zero except p_1 and p_3; $p_1=0.75$; $p_3=0.25$. Q_{18} is found to be 0.99721 and m equals 260.

Fifth, assume no destruction of particles, and assume particles reproduce by forming octets. All terms of p_k except p_1 and p_8 are zero; $p_1=0.92857$; $p_8=0.071429$. Q_{18} is found to be 0.99823 and m equals 410.

So, on the basis of the combined data of Experiments No. 4, No. 5, and No. 6 when the animals grew 3.4 fissions per day, the particles increased at a rate of of 2.0 duplications per day. If particles reproduced by doubling and none were destroyed, the initial particle concentration was about 220 per animal. If destruction of particles occurred or if particles reproduced by higher multiples than by doubling, the particle number is calculated as greater than 220. Just how much greater than 220 depends upon how much destruction is assumed, and upon how large one assumes the multiple by which the particles reproduce.

5. The assumptions and their validity

The assumptions which were made in the development of OTTER's equations have already been outlined. It has just been shown by these equations that if $\log(-\log W_n)$ is plotted against n a straight line should result. When the experimental values of W_n were plotted in this manner (figure 1), a straight line was obtained as predicted. Experiment No. 7 (see p. 379), which is comparable to the preceding experiments, gave data which show a straight line relationship even more clearly (see figure 2). The fact that the data are in agreement with this prediction based on the assumptions provides an indication of the validity of certain of those assumptions. The assumptions will now be discussed individually.

The first assumption was that the kappa concentration among the strong killers used to start Experiments No. 4, No. 5, and No. 6 was uniform. This assumption is supported by the fact that although Experiments No. 4 and No. 5 were started from single animals and Experiment No. 6 was started from 40 different animals, the data all fall together on the same curve.

Second, assumptions were made concerning the destruction of particles of kappa. Since a straight line would be obtained whether destruction occurred or not, no evidence is obtained from the data. Later, p. 396, it is shown that destruction does take place at 30.4°C and higher. It seems possible, therefore, that some destruction occurred in these experiments which were run at 27°C. Consequently, the figure of 220 particles assuming no destruction, may be too low. The figure of 240 particles assuming 0.1 of the particles destroyed in each interfission interval, or 270 assuming 0.25 destroyed in each interval, are possibly better estimates. If the rate of destruction exceeded 0.25, even higher particle numbers would be indicated.

Third, assumptions were made concerning the manner of reproduction of kappa. Here also the data provide no test for deciding how reproduction occurs for a straight line would be obtained under any circumstances. Multiplication by duplication provides the simplest hypothesis and yields a calculated particle number of 220. But it has been shown that if reproduction proceeds by higher multiples, greater values of m will be computed (assuming no destruction, for triplication, 260; for reproduction by octet formation, 410).

Fourth, it was assumed that particles segregate at random to the two products of each fission. Agreement of the predicted straight line with the data support the assumption.

Fifth, OTTER assumes that the probability for a particle to reproduce, disintegrate, or remain intact in an interfission interval is the same for each particle and for each interfission interval. Two different aspects of particle behavior arise from this assumption, and each will be considered separately. The first aspect is that the reproductive rate in all lines of particle descent will not be exactly the same; the rate in some lines will, by chance, be greater than in others. Whether kappa particles actually exhibit such a variation in rate is unknown, but if the rate of increase in different lines of particle descent were somewhat more or somewhat less uniform than assumed, it seems probable that the resulting distribution of particles among the animals in the

population would be closely approximated by the distribution calculated by OTTER.

The second aspect of this assumption is that the average rate of particle increase must be exponentially constant throughout the whole of each experiment. In considering the validity of this point it is necessary to regard the first part of each experiment when the kappa concentration was relatively high as distinct from the second part when the kappa concentration was low. During the first part no measurable proportion of animals lacked kappa; during the second, the measurements of the proportions of animals lacking kappa were made. The agreement of the data with the predicted straight line provides strong evidence for exponential increase, but only for the second part of the experiment when the measurements were made. These data obtained during the second part of the experiment provide no measure for the rate of kappa increase during the first part. Furthermore, in view of work reported in the next section of this paper it seems likely that kappa increased more slowly during the first part of the experiment when the concentration was high. The following considerations seem to support these conclusions. In general, processes which require some end product in order for more to be formed are autocatalytic. Such autocatalytic processes are characterized by exponential increase as long as the concentration of the end product is low in relation to substrate or "food supply." As kappa production is dependent on the presence of some kappa, it seems likely that it is also an autocatalytic process and should therefore, while in low concentration show exponential increase. In view of the fact that autocatalytic processes proceed at a lower rate while the end product is in high concentration in relation to the substrate, it might be expected that the rate of increase of kappa was slower early in the experiments when the particle concentration was high. If the assumption of a constant exponential rate of increase of kappa did not hold throughout the whole of each experiment, and such a low rate of increase early in the experiment occurred, then the calculated number of particles would be too low. A quantitative approach to this situation is made possible by the data of experiment No. 7 appearing in the next section of this paper. Calculations given there, which are based on the assumption that particle increase is slower at higher than at lower particle concentrations, indicate that the particle numbers calculated in this section should all be doubled.

VIII. EFFECTS OF VARIOUS CONDITIONS ON THE RATE OF INCREASE OF KAPPA

The results of the preceding section showed that while the animals of stock G multiplied at their maximum rate of 3.4 fissions per day at 27°C, the rate of increase of kappa could be estimated at 2.0 doublings per day. As was pointed out, this rate was determined while kappa was present in relatively low concentration in the animals. The experiments in the present section are concerned with the effect of fission rate, of the concentration of kappa, and of temperature on the rate of increase of kappa. Comparisons have also been

made, with respect to the rate of kappa increase, between different killer stocks.

A. *The effect of the fission rate of the animals and the concentration of kappa on the rate of increase of kappa*

The effect of fission rate and kappa concentration on the rate of increase of kappa are considered together in this section because data which have provided information on the effect of the one have also given information on the effect of the other. The experiments are considered under three headings: (1) the rate of increase of kappa while animals multiply at a rate of 2.6 fissions per day, (2) the maximum rate at which animals can reproduce and still retain killing activity (which is called the critical fission rate for killing) and the maximum rate at which animals can reproduce and still retain kappa (the critical fission rate for kappa), and (3) the effect of low fission rates on the rate of increase of kappa while kappa is in intermediate concentration. The significance of these subjects in relation to the rate of growth of kappa will become apparent in the discussion of each which follows.

1. The rate of increase of kappa while animals multiply at a rate of 2.6 fissions per day.

Experiment No. 7. This experiment is similar to Experiment No. 6 except for the lower rate of fission (2.6 per day) maintained here. As in Experiment No. 6, the proportion of animals with no kappa after different numbers of fissions was determined. These data were then used to calculate the rate of increase of kappa.

Reduction of fission rate from the normal 3.4 fissions per day to 2.6 fissions per day was obtained without altering the standard temperature of 27°C, by culturing the animals in a solution composed of one part of the standard lettuce culture fluid (see SONNEBORN and DIPPELL 1946) diluted with seven parts of a balanced salt solution used by TAYLOR and VAN WAGTENDONK (1941) for culturing Colpoda.

The experiment was started by isolating into the lettuce-salt solution 20 killers of stock G which had been multiplying at 27°C at a rate of 0.4 fission per day (test tube method). After these 20 animals had been through three fissions, 120 of the resulting 160 animals were isolated. These 120 animals were cultured by the isolation technique (see p. 367). The new isolations, instead of being made every four or five fissions, as described on p. 367, were made every day. The fission rate in the lettuce-salt medium was more variable than in the standard lettuce medium. Consequently, on each day it was found that the total number of fissions undergone since the start of the experiment was greater in some lines than in others. In order to have a close approximation of W_n based on an average value for n, it was necessary that the number of fissions n, undergone in different lines be not too divergent. For this reason, lines were discontinued when the total number of fissions undergone in any line of descent since the beginning of the experiment deviated more than two fissions from

the average number of fissions of all lines retained up to that time. Daily, on the sixth through the 17th days, a series of animals was isolated into "exhausted fluid" (see p. 366) and cultured at a low rate of fission to allow killing ability to develop if kappa were present. The resulting cultures were tested for the presence of killer animals in order to determine what proportion of the isolations had no kappa. The procedure was exactly the one set forth on p. 368 for Experiment No. 5. The occasional discarding of lines and the occasional death of animals resulted in less than 120 animals tested for kappa in each series.

The results of the tests are given in the following tabulation:

MEAN NUMBER OF FISSIONS (n)	NUMBER OF LINES OF DESCENT		PROPORTION LACKING KAPPA (W_n)
	TOTAL	LACKING KAPPA	
14.6	104	0	0
16.6	90	1	0.01
19.2	67	3	0.04
22.0	69	8	0.12
24.4	77	12	0.16
27.2	47	15	0.32
29.6	81	38	0.47
32.3	64	35	0.55
35.1	60	39	0.65
37.6	58	42	0.72
40.1	57	45	0.79
42.8	63	52	0.83

In order to determine the rate of increase of kappa, the log ($-\log W_n$) was plotted against n. (See figure 2.) The data fall on a straight line, showing they fit the OTTER equations. The slope of the line was measured and found to be

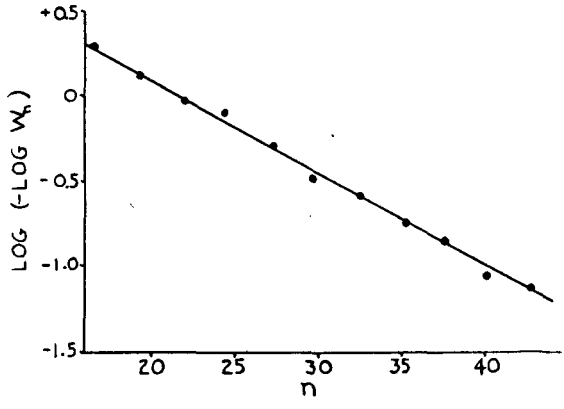

FIGURE 2. *Log (−log) W_n (proportion of animals with no kappa) plotted against n (number of fissions) from the data of Experiment 7.* Fission rate was 2.6 fissions per day.

-0.054. Using equations already developed it was determined from this slope that the rate of increase of kappa was 2.1 duplications per day, ($\bar{k}=1.76$). Comparison of this rate, while the animals were undergoing 2.6 fissions per day, with the rate of 2.0 duplications per day of kappa when the fission rate was 3.4 shows that this difference in fission rate has little or no effect on the rate of increase of kappa. It should be emphasized that in both determinations kappa was present in low concentration, for the rate is calculated from the proportion of the animals with no particles; and these proportions, of course, are only large enough to be measured when kappa is present in low concentration.

Additional information on the rate of increase of kappa can be obtained by calculating the number of particles in the original animals from these data and then comparing this number with the number of 220 found in the preceding experiments. This calculation may be made by the method already indicated. If it is assumed that there is no destruction and that the particles increase by duplication, the values of p_k can be calculated from equations (1) and (2), remembering that in this case $\bar{k}=1.76$. It is found that $p_1=0.24, p_2=0.76$; all other values of p_k are zero. Substituting into equation (5) and solving for some value of Q_n, it is found $Q_{31}=0.99361$. Reference to figure 2 shows that when $n=31$, $W_n=0.483$. m is found to be 110.

What is the meaning of the difference between the previous determination of 220 particles and the present determination of 110 particles? That the two calculated values represent a real difference in kappa number in the animals seems unlikely. As the animals used in the two experiments were from the same culture, in which the animals maintained a constant rate of 0.4 fission per day (test tube method) at 27°C, they would be expected to have approximately the same particle number. We must therefore look elsewhere for possibilities in explaining the discrepancy. One possibility is suggested by the results of Experiments No. 9 and No. 10 which indicate that at fission rates between 0.4 and 1.6 fissions per day while kappa is in high and intermediate concentrations its rate of increase is slower at lower fission rates than at higher. It is possible that this same relation holds at higher fission rates (2.6 to 2.4). In the first part of this experiment (No. 7) in which the animals multiplied at 2.6 fissions per day, and in the first part of the experiments (No. 4, No. 5 and No. 6) in which the animals multiplied at 3.4 fissions per day, kappa was in high and intermediate concentrations. If kappa increased more slowly while the animals were increasing at 2.6 fissions per day than it did when the animals were increasing at 3.4 fissions per day, the calculated number of kappa particles would be lower for the case in which the fission rate was 2.6 than the case in which it was 3.4. It follows that the estimate of 220 particles of kappa, made on the basis of data obtained from animals reproducing at 2.4 fissions per day, is more reliable than the estimate of 110. Yet if high and intermediate concentrations of kappa do lower the rate of increase of kappa, then this effect may also be present in some degree at 3.4 fissions per day, and even the estimate of 220 may be too low.

The author is indebted to SEWALL WRIGHT (personal communication) for a quantitative treatment of the problem based on the assumption that particles increase more slowly when in high concentration than when in low. This treatment indicates higher particle numbers than is indicated by calculations based on a constant rate of kappa increase, and has the advantage of eliminating the discrepancy between the estimates of 220 and 110 made when animals reproduced at 3.4 and 2.6 fissions per day respectively. WRIGHT's treatment is as follows:

Assume "that the percentage rate of increase of kappa during interfission varies inversely with the approach to a certain upper limit L. In symbols

$$\frac{dx}{xdt} = a\left(1 - \frac{x}{L}\right).$$

This is the differential equation of the logistic or Pearl-Verhulst growth curve.

$$x = \frac{Lx_0e^{at}}{L - x_0 + x_0e^{at}}.$$

"In the previous discussion in which a constant rate was assumed $dx/xdt = a$, $x = x_0e^{at}$, time in days was given by n/s where s is number of fissions per day. The multiplication of kappa per day could be expressed in any of the ways 2^f, k^{-s}, or e^a. Thus $a = f \log_e 2$ or $s \log_e \bar{k}$, and $e^{at} = \bar{k}^n = (2r)^n$, (where $2r = \bar{k}$). Since the logistic approaches the simple exponential curve when kappa is depleted, we may write

$$x = \frac{Lx_0(2r)^n}{L - x_0 + x_0(2r)^n}.$$

"An animal that starts with x_0 particles will have

$$\frac{2rx_0}{1 + (2r - 1)\frac{x_0}{L}}$$

at the end of interfission on the average (n = 1). This number is halved on the average at fission. After another multiplication and halving it becomes

$$\frac{rx_1}{1 + (2r - 1)\frac{x_1}{L}}$$

or

$$\frac{r^2x_0}{1 + (2r - 1)(1 + r)\frac{x_0}{L}}.$$

After the third fission it becomes

$$\frac{r^3 x_0}{1 + (2r - 1)(1 + r + r^2)\frac{x_0}{L}}$$

and after n fissions

$$\bar{x}_n = \frac{r^n x_0}{1 + (2r - 1)\left(\frac{1 - r^n}{1 - r}\right)\frac{x_0}{L}}.$$

"With sufficiently slow fission, $\bar{x}$ reaches equilibrium at a number a little less than L by the end of interfission and thus a little less than L/2 just after fission. Thus, x_0/L should be approximately $\frac{1}{2}$, giving the following:

$$\bar{x}_n = 2(r^n)(1 - r)x_0,$$

where $2r = \bar{k}$ is the rate of multiplication of kappa when near depletion.

$$x_0 = m = \frac{\bar{x}_n}{2(r^n)(1 - r)}.$$

The estimate for x_0 on the assumption of a constant rate r is $\bar{x}_n/r^n$. Thus an estimate of the number of particles in a strong killer immediately after fission is obtainable by merely dividing the estimates made by using OTTER's method by $2(1-r)$." The estimate of 220 particles based on 3.4 fissions per day and assuming a constant rate of kappa increase becomes 440 assuming logistic increase. The estimate of 110 particles assuming a constant rate becomes 460 assuming logistic increase.

"The estimates for the logistic curve took no cognizance of the reduced variance to be expected if duplication of kappa rises as the number of particles decreases and the reverse. However the average percentage rate of multiplication is

$$\frac{dx}{xdt} = a\left(1 - \frac{\bar{x}}{L}\right)$$

in a population with mean number $\bar{x}$, so that the curve of depletion for $\bar{x}$ may be taken as typical. The percentage of animals wholly lacking kappa in a given generation depends on the average number and the variance when depletion is extreme. In the later generations, multiplication is essentially exponential and the variance at this time is affected only slightly by that in the early generations. Thus the error in ignoring variance in the treatment of logistic growth should not be great."

To summarize, while animals of stock G containing low concentrations of kappa undergo 2.6 fissions per day at 27°C, kappa undergoes approximately 2.1 doublings per day. There is an indication, however, that kappa increases more slowly than this when it is present in higher concentration. Computations

based on this assumption and on the assumptions that particles increase by duplication and that no destruction occurs, indicate that the particle number is approximately 450 in strong killers.

2. The critical fission rate for killing and the critical fission rate for kappa

The critical fission rate for killing is defined as the maximum rate that animals can reproduce and retain killing activity. In a similar manner, the critical fission rate for kappa is defined as the maximum rate that animals can reproduce and still permanently retain kappa. The significance of these rates in providing a measure of the rate of increase of kappa is made clear by the following considerations.

When killers reproduce slowly, the net rate of increase of kappa must exactly equal the rate of increase of the animals. If kappa increased more slowly, it would decrease in concentration and be lost from the animals, but this does not happen at low fission rates. If kappa increased more rapidly than the animals, then it would steadily increase in concentration until the animals consisted of nothing but kappa. This, of course, is an absurdity. Therefore, the rate of increase of kappa and the rate of fission must be exactly equal.

When killers reproduce somewhat more rapidly, the net rate of increase of kappa must still exactly equal the rate of fission of the animals for the same reasons just outlined. But if the fission rate is increased to a value beyond the critical fission rate for kappa, then the concentration of kappa gradually decreases and increasing proportions of animals come to lack kappa completely.

It will be shown below under *b*. that the critical fission rate for kappa in stock G at 27° C is approximately two fissions per day. Therefore, when animals multiply at two fissions per day, kappa must also increase at a rate of two doublings per day. But it has already been shown that kappa increases approximately two doublings per day in stock G at 27°C when animals undergo 2.6 fissions per day and 3.4 fissions per day. This means that as the fission rate is raised from low to high values, the rate of kappa increase rises only until the critical rate of two doublings per day is reached. Further increases in fission rate result in no further rise in the rate of increase of kappa. The rate of increase of kappa has reached its maximum value at the critical fission rate for kappa. We may thus speak of a "maximum rate of increase" of kappa under any given set of environmental conditions. The "maximum rate of increase" of kappa at different temperatures is referred to in this sense later in this paper.

It will be shown below in *b*. that, for stock G the critical fission rate for kappa is approximately equal to the critical fission rate for killing. Consequently, the measurement of either of these critical fission rates gives a measure of the maximum rate of growth of kappa for the particular set of conditions under which the measurement is made.

To determine the critical fission rate for killing it is necessary to culture killers at a series of graded fission rates and test periodically for the presence of killing action. Strength of killing will, as shown in Experiment No. 1, steadily decline in the most rapidly reproducing cultures of the series. When

no further decrease in killing action is noted, then the most rapidly multiplying culture showing any killing action is reproducing at the critical fission rate for killing. The critical fission rate for kappa can be determined in a similar manner; instead of testing for killing action, tests are made periodically to determine whether animals have kappa.

This section is composed of two experiments dealing with (a) the accuracy of the test tube technique for the determination of critical fission rates, and (b) the relation of the critical fission rate for kappa to the critical fission rate for killing.

a. *The accuracy of the test tube technique for the determination of critical fission rates*

The simplest and most convenient method for obtaining a graded series of fission rates is by the use of the test tube method. However, in order for fission rates calculated by this method to equal true fission rates, two conditions had to be met (see p. 358): there must be no progressive changes in population density, and the fission rate of the animals in all lines of descent must have the same average value. It was shown how changes in population density can be measured and how calculated fission rates can be adjusted when the first condition is not met. But whether all the animals in test tube cultures maintain the same average fission rate is unknown. Therefore, the accuracy of the method had to be determined experimentally. This was done by ascertaining the critical fission rate for killing at 27°C in stocks G and 50 by use of the test tube technique, and comparing the results with a simultaneous determination of the critical fission rate for killing made by the accurate but laborious method of controlling the fission rate in isolation cultures (for the isolation technique see p. 368). In the isolation cultures it was not necessary to *assume* that animals in all lines of descent reproduce at the same rate, because one can *count* all animals and thus compute exactly the rate of fission. The details of the experiment follow.

Experiment No. 8. This experiment was started with slowly dividing killers of stock G and stock 50. The experiment consisted of two parts. In both parts, determinations of critical fission rate for killing were made by testing killing activity in cultures maintained at different fission rates. The two parts differed only in the methods used for controlling fission rate. This was controlled by the test tube method in the first part of the experiment, and by a special method which involved the use of the isolation technique in the second part of the experiment.

Part 1: This first part included two series of test tube cultures, identical, except that one consisted of animals of stock G and the other consisted of animals of stock 50. Each series was started from slowly multiplying killers, and consisted of 12 cultures maintained respectively at 0.4, 0.6, 0.8, 1.0, etc. fissions per day, up to 2.6 fissions per day. Tests for killing action were begun on the fourth day of the experiment and were made every two days until it seemed clear that the strength of killing showed no progressive decrease in

any of the cultures. The experiment was terminated after the cultures had been maintained for 12 days.

The results showed that there was no progressive decrease in killing strength in any culture subsequent to the sixth day. The culture containing the most rapidly multiplying animals which still retained killing ability at the end of the 12 days was the 1.8 fission per day culture of stock G and the 1.0 fission per day culture of stock 50. These two fission rates, therefore, represent the critical rate of fission for killing for these two stocks at 27°C. The detailed data on strength of killing in these cultures on successive tests are not given because the results are very similar to data already tabulated for Experiment No. 1 (see table 1) and to the more complete data to be given presently for Experiment No. 9 (see table 3).

Part 2: This second part of the experiment involved 120 lines of descent cultured by the isolation technique—60 lines of stock G and 60 of stock 50. The isolations were grown at different rates of fission by the methods outlined in the next paragraph. Instead of making isolations every four or five fissions as described in the experiment on p. 367 isolations were made every six (rarely seven) fissions. From the number of days required for the six fissions, the fission rate for each line could be accurately computed. Strength of killing of the animals growing at the different rates was obtained by testing the leftover cultures. These leftover cultures (each containing 64 animals produced by the six fissions, minus the one animal used to make the new isolation) were tested immediately for killing activity by adding sensitive stock P testers to the cultures. Killing activity in each test was recorded after 24 hours at 22°C.

Fission rate was varied in two ways. The first method involved the use of culture fluid prepared from desiccated, baked lettuce powder prepared by DIFCO LABORATORIES. Certain batches of their "mass production" product yielded a culture medium in which, for unknown reasons, the paramecia reproduced more slowly than in media prepared from the standard lettuce powder made in our own laboratory. The second method involved the use of exhausted fluid (see p. 366), which contained little food material, plus small quantities of fresh standard culture fluid. By varying the quantities of the latter the desired fission rates could be obtained. The rule which was found to hold roughly was that 0.001 ml of culture fluid allows one animal to undergo one fission. To illustrate the application of the rule, consider that one fission per day is desired. In this case cultures are fed 0.001 ml of fluid per animal per day. If two fissions per day are desired, animals are fed 0.003 ml of fluid per animal per day (0.001 ml per animal for the first fission plus 2×0.001 ml to enable the two products of the first fission to undergo one more fission. The proper amount of fluid per animal per culture to give other fission rates was calculated in a similar manner.

Twenty-four of the isolations of stock G and 24 of the isolations of stock 50 were grown in the Difco medium to reduce the fission rate; the other 36 isolations of each series were grown at different rates by the second method just described. Since the control of fission rate by these two methods proved to be

imperfect, it was not possible to culture a planned number of lines at a given rate. It was possible only to attempt a graded series of rates, measure the rates obtained, and select for use those lines which did give the desired rate. Animals grown in the Difco medium averaged between 1.5 and 2.5 fissions per day. The fission rate in certain lines was variable. These lines were discarded. Animals maintained in exhausted culture fluid plus standard culture fluid reproduced at fission rates ranging from 0.4 to 3.0 fissions per day.

Killing tests on leftovers from lines grown by use of the Difco medium and by use of the exhausted fluid plus standard culture fluid gave comparable results at the same fission rates. Consequently, the results on all lines are considered together. In general, no progressive decrease in killing strength subsequent to the sixth day was evidenced.

First, let us consider the results on Stock G. Of the 18 lines in which the animals multiplied at rates of 0.7 to 1.6 fissions per day, tests on the leftovers showed that all remained strong killer on all tests during days six to nine after beginning the experiment. (Although the experiment was continued for 12 days, leftovers were only available for testing after every six or seven fissions as pointed out above. In many instances this resulted in the last test occurring several days before the 12th day.) Of two lines in which the animals multiplied 1.8 fissions per day, one was non-killer on all tests during days six to ten; the other was sometimes weak killer and sometimes non-killer on days six to 12 but was weak killer on the last test on the 12th day. Of the eight lines in which the animals multiplied 2.0 fissions per day, five were non-killer on all tests during days six to twelve; the remaining three varied, but only one showed killing activity on the last test on the 12th day. Of the 12 lines in which the animals multiplied 2.7 to 3.0 fissions per day, all had become non-killer by the fourth day. Although the animals of none of the lines multiplied at rates between 2.0 and 2.7 fissions per day, the data indicate that 2.0 fissions per day is probably the critical fission rate for killing, for only one of eight lines multiplying at this rate showed any killing activity on the 12th and last day of the experiment.

Next, we may consider the results on stock 50. Of the 14 lines in which the animals multiplied at rates of 0.4 to 0.75 fission per day, all were strong killer when leftovers were tested on the ninth day. The 13 lines in which the animals multiplied at a rate of 1.0 fission per day varied when leftovers were tested on the sixth and 12th days; some lines which were non-killer on the sixth day were killer on the 12th day and vice versa; six were non-killer on the 12th day, and six were weak killer. Of the 21 lines in which the animals maintained rates of 1.2 to 2.0 fissions per day, all were non-killer by the fourth day. These results show clearly that the critical fission rate for killing in stock 50 is very close to 1.0 fission per day.

* * *

In summary, the critical fission rate for killing at 27°C, as determined by the test tube method, is 1.8 fissions per day for stock G and 1.0 fissions per day for stock 50. By the more accurate isolation method, the critical fission rate

for killing in stock G was determined to be 2.0, and for stock 50 it was determined to be 1.0. The close agreement between the results obtained with the two different methods indicates the correctness of the assumptions on which the test tube method was based. As was predicted in discussing those assumptions (p. 359), the difference between the calculated fission rate and the true fission rate must be small and in a direction tending to make the calculated rate too low.

Attempts were made to perform experiments (similar to Experiments No. 4 to No. 7) to determine the proportion of animals with no kappa after different numbers of fissions by using the test tube method to control fission rate. Although the results approximated those obtained in the previously reported experiments at comparable fission rates, they were not sufficiently reproducible to be considered reliable for use in calculating particle number and rate of kappa increase.

b. *The relation of the critical fission rate for killing to the critical fission rate for kappa*

In the following experiment five parts of a culture of stock G were cultivated at different fission rates by the test tube method. Tests were made for killing strength and the presence of kappa in order to determine both the critical fission rate for killing and the critical fission rate for kappa.

Experiment No. 9. Five subcultures from a culture of slowly multiplying G killers were maintained at 27°C by the test tube method, one at each of the following fission rates: 1.2, 1.4, 1.6, 1.8, and 2.0 fissions per day. Two types of tests were made on the animals in these test tubes. The first type, made every four or five days, was for killing action. These tests differed from the usual type described on p. 357 in that they did not include the mixture of the unknown culture with known strong killer. Therefore, no information on sensitivity of cultures was obtained; only killing action was determined. The second type of tests, made every week or ten days, was carried out to determine whether the animals still retained kappa. Each test was performed by isolating 21 individuals from a tube, allowing them to reproduce slowly to enable animals with kappa to produce killer progeny, and then testing for killing action. It was thus determined how many of the 21 animals contained no kappa. This provided a rough estimate of the proportion of the animals with no kappa in the tube.

The results of the tests for killing are recorded in table 3. As a rule, the animals cultured at 1.8 fissions per day and less retained killing activity, but animals cultured at 2.0 fissions per day lost killing activity. In the cultures which did not lose killing ability, killing strength was, during most of the experiment weaker in the faster multiplying cultures. Finally, cultures which did not lose killing strength showed increase in killing strength at some time subsequent to the 13th day.

The results of the tests for the presence of kappa are recorded in table 4. When animals were cultured at 1.2 and 1.4 fissions per day, no animals lacking

TABLE 3

Stock G. Strength of killing of initially slowly dividing strong killer cultures after reproduction for various periods at different fission rates. Number of + symbols indicates strength of killing. The symbol NK indicates no killing activity.

MEAN DAILY FISSION RATE	STRENGTH OF KILLING AFTER											
	0 DAYS	7 DAYS	10 DAYS	13 DAYS	15 DAYS	20 DAYS	24 DAYS	27 DAYS	34 DAYS	41 DAYS	51 DAYS	58 DAYS
1.2	+++	++	++	++	++	++	+++	+++	+++	+++	+++	+++
1.4	+++	+	++	+	++	++	++	++	++	+++	+++	+++
1.6	+++	+	+	+	+	+	++	+	+	++	++	+++
1.8	+++	+	+	+	NK	NK	+	+	+	+	+	++
2.0	+++	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK	NK

kappa were found. In the tube in which animals multiplied at 1.6 fissions per day, one animal lacking kappa was found in the group of 21 animals removed on the 15th day, but all tested animals had kappa on subsequent days. In the group cultured at 1.8 fissions per day, several animals lacking kappa were occasionally found. Kappa finally disappeared from all the animals in the tested groups from the tube in which the animals were cultured at 2.0 fissions per day.

The following conclusions can be drawn from these results. First, the data give the critical fission rate for kappa. Kappa clearly can increase at a rate of 1.8 doublings per day when the animals multiply at this same rate. If kappa could not increase at this rate, then in the culture growing 1.8 fissions per day, increasing proportions of the animals would have lost kappa. Although some animals in the culture did lose kappa, the proportion of these animals without kappa did not increase consistently. Since kappa disappeared slowly in the 2.0 fission per day culture, it is evident that kappa cannot quite maintain this rate. In the previous experiment (No. 8), when the fission rate was controlled by the test tube method, the true rate in the case of stock G was shown (p. 387) to be somewhat higher than the calculated rate of 1.8 fissions per day. Therefore, we may conclude that kappa increased at

TABLE 4

Stock G. Number of animals without kappa out of samples of 21 from initially slowly dividing strong killer cultures after the cultures had reproduced for various periods at different fission rates.

MEAN DAILY FISSION RATE	NUMBER OF ANIMALS WITHOUT KAPPA OUT OF SAMPLES OF 21 AFTER						
	11 DAYS	15 DAYS	24 DAYS	34 DAYS	44 DAYS	51 DAYS	58 DAYS
1.2	—	0	0	0	—	—	—
1.4	—	0	0	0	—	—	—
1.6	—	1	0	0	0	0	0
1.8	1	3	4	3	0	0	1
2.0	2	13	13	17	21	20	21

least 1.8 duplications per day and probably slightly faster when the fission rate had the same value. The rate of increase of kappa at this fission rate corresponds fairly closely to the determinations of the rate of increase of kappa at higher fission rates—2.1 doublings per day at a fission rate of 2.6 and 2.0 doublings per day at a rate of 3.4. This seems to indicate that kappa can increase roughly two doublings per day at a fission rate of two fissions per day, and that increase in the fission rate above this level has little or no effect on the maximum rate of increase of kappa.

Second, there are indications that kappa cannot increase as fast in high concentration as it can in low concentration. This was suggested by the results of Experiment No. 7 (p. 379). How the data indicate such an effect of kappa concentration on the rate of increase of kappa can be best brought out by considering what would be the expected consequences of such an effect. If kappa does not increase as fast in high concentration as in low, then the concentration must be reduced when kappa is maintaining its maximum rate at the critical fission rate for kappa. Hence, when animals are taken from a slowly dividing culture of strong killers, with presumably the maximal concentration of 220 or more particles of kappa per animal, and are allowed to multiply at the critical fission rate for kappa (1.8 fissions per day), the kappa will at first multiply at a submaximal rate until the concentration per animal is reduced to the level at which the maximal rate for kappa (1.8 doublings per day) can occur. The kappa concentration would then be maintained at this level.

Two effects of this drop in kappa concentration should appear in this experiment. First, if the concentration of kappa drops to a very low level, occasionally there should be produced by chance animals which lack kappa completely. Such animals were in fact found in the cultures reproducing at 1.6 and 1.8 fissions per day. (There is the alternative possibility, however, that they are animals descended from lines in which the animals reproduced somewhat faster than others in the cultures at rates exceeding the rate of increase of kappa.) The second effect expected, if the concentrations of kappa are lower when the fission rate is near but not above the critical rate, is that the killling reactions should be weaker at these fission rates. The data of table 3 show clearly that this does occur. It might be argued, however, that this reduction in killing potency is not necessarily indicative of lower kappa concentrations, and that there is possibly some other factor intrinsic in rapid growth which inhibits killing action. These alternate explanations of both of the expected consequences of a reduced kappa concentration near the critical fission rate make it possible to conclude only that the data provide an indication of a slower rate of kappa increase when kappa is in high concentration.

Third, it may be concluded from experiments of this type on stock G that cultures of animals reproducing at the critical fission rate for kappa usually exhibit weak killing action, and hence that the critical fission rates for kappa and for killing are identical. An examination of tables 3 and 4 shows, in general, that the 2.0 fission per day culture, from which kappa disappeared, also lost killing, and that the 1.8 fission per day culture, which never lost kappa, re-

tained killing. In practice, however, tests for killing activity performed on three or four days would be required to establish this identity of the two critical rates, for as appears in table 3 the 2.0 fission per day culture, which eventually lost kappa, gave a "+" reaction on the 41st day, and the 1.8 fission per day culture, which never lost kappa, gave no killing action on the 16th and 20th days.

Increase in strength of killing subsequent to the 13th day occurred in all cultures which did not lose kappa. This is a regular occurrence in experiments of this type. The cause and meaning of this apparently adaptive change is unknown. Such changes would occur, however, if kappa particles differ hereditarily in their maximum rate of increase. The initial decrease in killing strength, according to this explanation, would be caused by loss of the more slowly increasing particles; the subsequent increase in killing strength would be due to a gradual raise in concentration of the more rapidly increasing particles. If such diverse kappa particles exist in an animal with much kappa, it should be possible to "isolate" them by reducing the kappa concentration to a single particle by the differential reproductive rate method used in the previous experiments. This was attempted, and the isolated particles were allowed to increase to yield killers in which all the particles, being recently derived from one, should have been characterized by the same hereditary multiplication rate. The test tube method was then employed to determine the critical fission rates for the kappas descended from different isolated particles. Not only were there no differences in critical rate, but all showed the same "adaptive" phenomenon. This means, therefore, that the particles of kappa in the original killer did not differ hereditarily in multiplication rate. However, the possibility remains that, in the experiments showing "adaptive" changes, such differences arose by mutation during the course of the long period preceding the appearance of the adaptation. This has not yet been tested.

In summary, this experiment has shown (1) that the critical fission rate for kappa is at least 1.8 fissions per day and probably slightly greater, indicating that kappa can increase at least 1.8 doublings per day while animals are multiplying at the same rate, (2) that the critical fission rate for killing is approximately equal to the critical fission rate for kappa in stock G, and (3) that kappa can probably increase faster in low concentration than it can in high.

3. The effect of low fission rates on the rate of increase of kappa while kappa is in intermediate concentration

In the preceding experiment a study was made of the effect of low fission rates on the rate of increase of kappa while kappa was in *high* concentration. In the experiment now to be reported a study was made of the effect of low fission rates while kappa is in *intermediate* concentration.

Kappa was reduced to a low level by a series of rapid fissions. Then it was possible to measure the time and number of fissions required for animals to become killer again while reproducing at different slow rates of fission. From

these data it was possible to compare the rate of increase of kappa at the different fission rates. The account of this experiment follows.

Experiment No. 10. A subculture from a slowly reproducing culture of stock G was maintained at 27°C by the test tube method at a calculated rate of three fissions per day for four days. Estimates of the population density (see p. 359) made at the beginning and the end of this period showed that the animals had actually undergone eleven fissions during the four days. Therefore, the true fission rate $11/4 = 2.75$ fissions per day. Three things were now done. First, a sample of the animals was tested for killing. This test proved the animals to be weakly sensitive non killers. Second, 30 animals were isolated and cultured at a low rate of fission; later, their progeny were tested for killing ability to determine what proportion of the original isolates had lacked kappa. These tests showed that *all* of these 30 isolates had contained kappa, indicating that most, or all, of the animals in the culture from which the isolations were taken also contained kappa. Third, the remaining animals were separated into nine cultures and maintained by the test tube method, feeding amounts designed to yield the following rates: culture No. 1, 0 fissions per day; No. 2, 0.1; No. 3, 0.2; No. 4, 0.4; No. 5, 0.6; No. 6, 0.8; No. 7, 1.0; No. 8, 1.2; No. 9, 1.4. Every day a sample of 0.1 ml of fluid and animals were removed from each of these nine cultures and an equal number (about 150) of stock P sensitive testers was added to each sample. The number of affected stock P sensitives in each sample after 24 hours at 22°C was recorded.

Before discussing these results, a discrepancy in the fission rates of the test tube cultures needs to be mentioned. Estimates of the population density in these nine cultures made at the time they were started and also several days later showed that each underwent approximately two more fissions than calculated on the basis of the above listed fission rates. However, it was possible to estimate the *true* average fission rate in each of the test tubes *during the period* required for the animals in each to reach the strength of killing specified in the next paragraph. This was done by adding the two additional fissions undergone in each tube to the calculated number of fissions for the period, and dividing by the number of days in the period in question.

The results obtained from the daily tests made on the animals in the nine cultures are given in table 5, which shows the number of sensitive animals affected in the daily test on each culture. At first, the cultures remained nonkillers; then they gradually became killer. In general, after killing once appeared, more stock P animals were affected on each subsequent test. The number of days and the number of fissions from the beginning of the slow fission rate until a test showed ten stock P animals affected have been computed for each tube culture. Since only rarely were exactly ten animals affected, a rough interpolation was usually made in determining this number of days. For instance, table 5 shows that no animals of culture No. 1 were affected in the tests until the fifth day when four were affected, and that on the sixth day, 17 were affected. A rough interpolation was made, and the number of days until ten animals were affected was estimated at 5.5. Dividing the number of fissions

TABLE 5

Stock G. The number of animals of sensitive stock P affected on successive days in tests of samples from nine slowly multiplying cultures initially rendered non-killer by 2.75 fissions per day for four days. The nine cultures were multiplying at a graded series of rates ranging from approximately one-half fission per day for the first culture to one and one-half fissions per day for the ninth culture.
(See text p. 392 for details)

CULTURE NUMBER	NUMBER OF ANIMALS AFFECTED AFTER										
	1 DAY	2 DAYS	3 DAYS	4 DAYS	5 DAYS	6 DAYS	7 DAYS	8 DAYS	9 DAYS	10 DAYS	11 DAYS
1	0	0	0	0	4	17	27	11	23	—	—
2	0	0	1	0	7	23	12	21	37	—	—
3	0	0	0	1	6	31	33	44	—	—	—
4	0	0	0	0	1	11	23	44	—	—	—
5	0	0	0	0	0	11	30	46	—	—	—
6	0	0	0	0	1	7	21	32	—	—	—
7	0	0	0	0	0	3	2	10	26	—	—
8	0	0	0	0	0	2	0	4	17	24	34
9	0	0	0	0	0	0	0	0	0	5	13

that occurred during this period by the number of days in the period, the true fission rate can be computed. In the present example, culture No. 1, though not fed, went through two fissions (see above, p. 392). Hence, the true fission rate was $2/5.5 = 0.4$. In like manner, the data of table 5 may be used to calculate, for each of the nine cultures, the true fission rates for the period until ten animals of stock P were affected. These calculations follow:

CULTURE	NUMBER OF FISSIONS	NUMBER OF DAYS	TRUE FISSION RATE
1	2	5.5	0.4
2	2.5	5.2	0.5
3	3	5.2	0.6
4	4.4	6	0.7
5	5.6	6	0.9
6	7	6.2	1.1
7	10	8	1.2
8	12.2	8.5	1.4
9	17.1	10.8	1.6

In order to draw conclusions concerning the rate of increase of kappa from these data, it is necessary to assume that all cultures have approximately the same concentration of kappa particles per animal when a 0.1 ml sample affects ten sensitives in a test. Although this may not be strictly true, the strength of killing was shown in Experiment No. 1 to be closely correlated with the kappa concentration. Therefore, all samples affecting ten sensitives must have approximately the same number of particles per animal.

If we knew how many doublings in amount kappa underwent from the time of the beginning of the slow reproduction until ten animals were affected, we should be able to calculate the rate of increase of kappa. Although this is not known, the general relations can be made clear by making calculations on the basis of arbitrary values for number of doublings of kappa. First, assume that the kappa concentration per animal doubled only once during the period of slow reproduction until ten animals were affected. In the case of culture No. 1, reference to p. 393 shows that the animals underwent two fissions or doubled in number twice during the period. Hence, kappa must have doubled three times during the period 5.5 days in order to bring about one doubling in concentration per animal. This gives a rate of 3/5.5, or about 0.5 doubling per day. If one proceeds in this manner for all the cultures, the rate of kappa increase, assuming one doubling in kappa concentration, may be calculated for each. In like manner, one may calculate for each culture the rate of kappa increase assuming that 4.5 doublings in kappa concentration occurred during the period of slow reproduction before ten sensitive animals could be affected in a test. The calculated rate of kappa increase expressed in number of doublings per day on the basis of these two arbitrary values for the number of doublings of kappa concentration, are as follows:

CULTURE	MEAN DAILY FISSION RATE	CALCULATED NUMBER OF DOUBLINGS OF KAPPA PER DAY	
		ASSUMING ONE DOUBLING IN KAPPA CONCENTRATIONS	ASSUMING 4.5 DOUBLINGS IN KAPPA CONCENTRATION
1	0.4	0.5	1.2
2	0.5	0.7	1.3
3	0.6	0.8	1.4
4	0.7	0.9	1.5
5	0.9	1.1	1.7
6	1.1	1.3	1.9
7	1.2	1.4	1.8
8	1.4	1.6	2.0
9	1.6	1.7	2.0

Obviously, kappa increases faster when the fission rate is faster, regardless of which of the two arbitrary values is assumed as a basis of calculation. The assumed value of 4.5 is clearly a limiting upper value, for it leads to kappa rates of two doublings per day, which previous experiments have shown to be at or near the upper limit. While the assumed value of one is not a limiting lower value, the preceding tabulation shows that the increase of kappa with increasing fission rates is more marked when the assumed value is lower, hence still lower assumed values would only bring out the point more strongly. While the true value probably lies somewhere between the two assumed values, determination of the true value is of little consequence. The

main point, already clearly shown, is that at intermediate kappa concentrations, the rate of kappa increase rises as the fission rate rises.

B. *The effect of temperature on the rate of increase of kappa*

Before this part of the work was undertaken, it was shown by SONNEBORN (1947a, b) that the extremes of high and low temperatures are both capable of inactivating kappa in the variety 4 killer stock 51. The following experiment indicated that high temperature may also inactivate kappa in variety 2. Furthermore, it has been possible to estimate the rate of increase of kappa at different temperatures.

It was shown in Experiment No. 9 that the critical fission rate for kappa is essentially the same as the critical fission rate for killing. It was further indicated that the numerical value of this rate is close to the maximum rate of increase of kappa (see p. 384). Assuming that the equality of these critical fission rates in stock G holds at different temperatures, it follows that the determination of the critical fission rate for killing at some particular temperature will give a measure of the maximum rate of increase of kappa at that temperature. The purpose of the following experiment was to make such determinations.

Experiment No. 11. The experiment was started with a slowly reproducing killer culture of stock G. The culture was split into six series of cultures, one series to be cultivated at each of the following Centigrade temperatures: 11°, 18°, 22°, 27°, 29.4°, 30.4°. One of the tube cultures in each series was maintained by the test tube method at 0 fissions per day, another at 0.2 fission

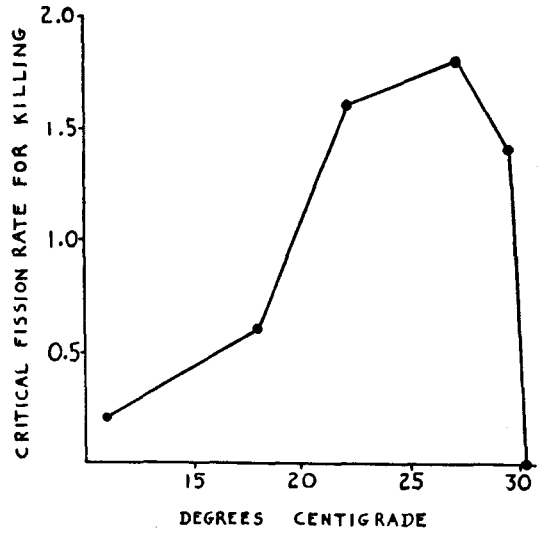

FIGURE 3. *The effect of temperature on the critical fission rate for killing based on the data of Experiment 11.* The critical fission rate for killing is the maximum number of fissions per day animals can maintain and still retain killing activity (therefore equal to the maximum rate of increase of kappa, see p. 396).

per day, another at 0.4, another at 0.6, and so on, to give a graded series of tubes at each temperature up to the maximum fission rate at each temperature. Killing tests were made every few days until it was evident that killing activity had ceased to grow weaker in any of the tubes. Cultures were retained for the following periods before the final determination of killing strength was made: 60 days at 11°, 21 days at 18°, 12 days at 22°, 12 days at 27°, 19 days at 29.4°, 19 days at 30.4°.

The fission rates of the most rapidly reproducing cultures at each temperature which retained killing activity (criticial fission rates for killing) are shown graphically in figure 3.

Killing strength seemed weaker at all temperatures at fission rates near the critical fission rate for killing. At higher temperatures killing strength was also weaker in the cultures maintained at the slowest rates (0, 0.2, 0.4 fission per day). This effect was very marked at 29.4°C, weakly expressed at 27°C, and not present at all at lower temperatures. In general, the most intense killing activity occurred in cultures maintained at 18°C, the weakest killing activity at 29.4°C. (Other experiments have shown that this effect of temperature on killing strength is even more strongly marked in stock H. Stock H cultured at 27°C shows fairly strong killing activity; but after ten days at 18°C, it becomes much stronger.)

As pointed out above the critical fission rate for killing at each temperature where killing activity is retained is approximately the maximum rate of increase of kappa at that temperature. At all temperatures, the critical fission rate for killing is less than the maximum fission rate of the animals. The maximum rate of kappa increase occurred between 22° and 29.4°C. The rate dropped off rapidly above 27°C. As killing activity disappeared from animals reproducing at all rates at 30.4°C, kappa must be inactivated at this temperature. There is no evidence that kappa was destroyed at a temperature of 11°C in the case of stock G.

C. *Comparison of the critical fission rate for killing in the different killer stocks.*

The critical fission rate for killing at 27°C has been determined for the different killer stocks in several experiments. If the critical fission rate for killing is equivalent to the critical fission rate for kappa in the other killer stocks, as it is in stock G, then the determinations represent the maximum growth rate of kappa in these stocks. Since the determinations were made in several different experiments, all of which gave essentially the same results, they will all be considered together.

The experiments were performed just as described in the preceding experiment, utilizing the test tube method of controlling fission rate at 27°C. The determination on each stock was made by starting with a strong killer culture which was split into a series of test tube cultures. These test tube cultures were maintained at different fission rates to give a graded series of rates from zero to three fissions per day: 0, 0.2, 0.4, 0.6, and so on. Killing tests were made

every few days until it was evident that killing had ceased to grow weaker in any of the tubes (usually ten to 16 days). The most rapidly reproducing culture in each series which still showed killing activity was then noted.

It was found that the fastest reproducing culture which retained killing activity varied with the different stocks and sometimes was slightly different in different experiments on the same stock. For stock G the critical fission rate for killing was determined as 1.8 fissions per day, rarely 1.6 or 2.0; for stock H, 1.0 or rarely 0.8; stock 36, 1.4; stock 50, 1.0 or rarely 1.2. The mutant Gm 1 was not tested.

It was found that killing was usually weak and occasionally lacking at the critical fission rate for killing, often making an exact determination somewhat difficult. This was brought out by the data given in table 3 for Experiment No. 9. It seems likely that the difficulty in precisely measuring the critical fission rate for killing accounts for much of the variation in the values obtained for the critical rates in different experiments, rather than variation in the rate of increase of kappa.

If the critical fission rate for killing in these stocks approximated the critical fission rate for kappa, then the determinations of critical fission rates for killing must represent approximately the maximum rates of increase of the different kappas. These are: for stock G. 1.8; stock H, 1.0; stock 36, 1.4; stock 50, 1.0. These rates are probably somewhat too low, for it has been pointed out (see p. 387) that the maximum rate of increase for kappa in stock G is near 2.0 rather than 1.8. The low rates of increase for stocks H and 50 would explain the relatively long time required for sensitive cultures of these stocks to become killer again in Experiment No. 1 (see p. 362). Much less time was required for stock G.

There is a possibility, of course, that equality of the critical fission rate for kappa and the critical fission rate for killing does not hold for the other stocks. This would be true if a higher kappa concentration is required for animals of these stocks to evidence killing. Such an explanation would also account for the relatively long time required for sensitives to become killer in stocks H and 50. The final answer to the question must await determination of the critical fission rate for kappa in the different killer stocks.

IX. DISCUSSION

A. *The combination of kappa with gene K*

The present work makes it possible to eliminate one of the two alternative hypotheses proposed by SONNEBORN (1945a, b) to account for some of his observations on kappa in variety 4. His two hypotheses were the concentration hypothesis and the hypothesis of the union of kappa with gene *K* in the macronucleus. According to the concentration hypothesis, animals are sensitive if kappa is present in the cytoplasm in low concentration. According to the alternative hypothesis, which SONNEBORN preferred, animals with small amounts of kappa are sensitive because the kappa under these conditions is bound in the macronucleus to the gene *K*. On this hypothesis, it was required

that kappa be unable to pass from the intact macronucleus to the cytoplasm where it had to be in order to control the killer character.

One of the main results of the present investigation on variety 2 kappa is the demonstration that kappa may be lost completely from killers by a series of rapid fissions in the absence of macronuclear disintegration. As pointed out earlier (PREER 1946), this is inconsistent with SONNEBORN's preferred hypothesis. SONNEBORN (1947a, b) consequently rejected this hypothesis in favor of the alternative concentration hypothesis and showed how the concentration hypothesis, which may be considered established by the investigations reported in the present paper, can also be reconciled with all of his observations.

B. *Is kappa a symbiont?*

ALTENBURG (1946), referring to the work of the author, notes the parallel between the number of particles of kappa and the number of symbiotic green algae in *P. bursaria*. Relative to this, it should be noted that these algae also can be reduced in number and removed entirely from animals by a series of rapid fissions (JENNINGS 1938) as has been done with kappa in variety 2. Because of the parallel in kappa and algal number in *P. bursaria* and because of the lack of complete coordination of rates of kappa and animal increase, ALTENBURG has made the suggestion that kappa is a derivative of symbiotic algae such as are present in *P. bursaria*.

A comparable suggestion has been made by LINDEGREN (see SONNEBORN 1947b) who holds that kappa is a virus. SONNEBORN (1947b) has given a detailed critique of the view that kappa may be a symbiont. His argument is based essentially on his observation that the killer character is not a unique case in the group B varieties of *P. aurelia*, but on the contrary is representative of all characters thus far studied in this material. Each appears to depend on a distinct cytoplasmic factor comparable to kappa. A system of cytoplasmic factors appears to constitute the normal mechanism of genetic control.

C. *Variation in the concentration of cellular constituents as a consequence of differential multiplication rates*

The experiments reported in this paper have shown how differences between the rate of multiplication of kappa and the rate of multiplication of the paramecia lead to variations in the concentration of kappa. This discovery is in agreement with a few remarkably comparable cases in the literature. Reference has already been made to JENNINGS' (1938) observation that the concentration of symbiotic algae in *P. bursaria* can be reduced by allowing the paramecia to multiply rapidly.

Similarly LWOFF and DUSI (1935) have reported a case of reduction of the number of plastids in *Euglena mesnili* by differential multiplication rates. The organisms have normally about 100 plastids. When *E. mesnili* is cultured in the dark, the plastids retain their chlorophyll and normal morphological appearance, but their multiplication rate is inhibited. This lowered multiplication rate, over a period of several months, results in a gradual reduction in plastid

number. Finally, individuals are produced which completely lack plastids. But, since such individuals have a low viability, it has not been possible to establish pure cultures of *E. mesnili* lacking plastids.

Finally, there remains the case of the "genoid" or cytoplasmic factor determining sensitivity to CO_2 in Drosophila (L'HÉRITIER and TEISSIER 1938 and 1944). L'HÉRITIER and SIGOT (1944–45) have shown that when small amounts of the cytoplasmic factor are introduced along with the sperm into the egg at fertilization, the inhibitory effect of extreme temperature reduces the rate of increase of the cytoplasmic factor to less than the division rate of the embryonic cells. They have shown that this results in the production of many cells lacking the cytoplasmic factor. This situation is remarkably similar to the reduction of the number of particles of the cytoplasmic factor in the variety 2 killers, which was also accomplished by differential rates of multiplication.

D. *Comparison of the present results on number of particles and rate of increase of kappa in variety 2 with those in variety 4*

The author's calculations of particle number in variety 2 have been confirmed for variety 4 by SONNEBORN (1947a, b). Analysis of his data on the progeny of animals without kappa into which small amounts of kappa had been introduced at conjugation led to an estimate of approximately 256 particles of kappa in normal killers. This is in complete agreement with the author's first estimate for variety 2 killers (PREER 1946).

On the other hand, there are marked differences between the rate of increase of kappa in these two varieties of *P. aurelia*. SONNEBORN (1945b) early obtained evidence that kappa in variety 4 can increase at a rate of six doublings per day, a rate equal to the maximum rate of fission at 27°C. This is in marked contrast to the situation in variety 2 where kappa cannot keep pace with the maximum fission rate of only 3.4 fissions per day.

In another respect, however, there is agreement between the two varieties in the behavior of kappa. SONNEBORN (1947b) pointed out that in variety 4 slowly multiplying killers are stronger killers than more rapidly multiplying animals. He suggested that this may be explained by assuming that kappa rises to higher concentrations when animals reproduce more slowly. This, of course, implies an effect of concentration on the rate of increase of kappa as pointed out on p. 390. The work of the author on variety 2 confirms this observation made by SONNEBORN.

E. *The rules of amitotic distribution of autocatalytic particles at cell division*

Just as there are mathematical rules governing the distribution of genes at mitosis, meiosis, and fertilization, there is also a mathematics of amitotically distributed autocatalytic bodies at cell division. The relations given in this paper are fundamental to any study of the distributional behavior of such bodies. Even though the segregation to the two products of each division may

not be completely random (as has been assumed), the relations lead the way to a proper analysis. Starting with an animal (or animals) containing any given number of particles, and for any relative rate of particle and animal increase, it can be calculated what percentage of animals at any time have no particles. Methods developed by OTTER (see p. 372) also enable one to calculate the percentage with one particle, two particles, three particles, etc., somewhat in the manner of the Poisson distribution.

Certain ideas emerge from a consideration of OTTER's relations which are not readily apparent otherwise. For instance, OTTER has investigated the limit of the proportion of animals with no particles (W_n) as the number of fissions (n) approaches infinity in equation (4) (see p. 371). He has found that when the average multiple by which the particles increase in each interfission period, $\bar{k}$, is equal to or less than two, then W_n approaches one. This means that the proportion of animals with no particles after n fissions, W_n, gradually increases. In the case when particles and animals increase at the same rate ($\bar{k}=2.0$) this fact leads to interesting speculations. For example, consider the hypothetical case of an animal (or cell) with ten particles of randomly segregating autocatalytic bodies which double in number every time the animals double in number. If it is assumed that there is no destruction of particles and that particles reproduce by duplication, equation (4) may be applied as outlined on page 374. It is found that 5 percent of the progeny of such an animal after 10 fissions will lack particles; 20 percent, after 20 fissions; 30 percent, after 30 fissions; and 40 percent, after 40 fissions. If the starting number of particles were 20 instead of 10, then after 10 fissions the number lacking kappa would be negligible; after 20 fissions, 4 percent; after 30 fissions, 10 percent; after 40 fissions, 17 percent; and so on. Thus when particle and animal rates are exactly the same, the distribution of particles becomes extremely nonrandom after a small number of fissions, and the percentage of animals lacking the particles increases steadily. If higher particle numbers are taken, then the process requires longer. But when dealing with time from an evolutionary point of view, even large particle numbers show the same effect.

We are forced to conclude that if segregation of particles to the two products of each fission is random, and if particle numbers remain uniform, then what might seem the natural process of exactly correlated particle and animal reproductive rates cannot exist. When, by chance, particle numbers drop too low, the rate of particle production must increase; and when they rise too high, the rate of particle production must drop. An alternative would be that some selective process might maintain uniformity by removing animals with extreme particle concentrations, but this lowered survival value for the species would be costly from the standpoint of evolution.

F. *The mutation of cytoplasmic factors*

In order to study effectively the mutation of cytoplasmic factors, it must be possible to obtain animals, or cells, which contain only the mutant particles of

the cytoplasmic factor. This segregation of particles may be accomplished for kappa by culturing animals at a rapid fission rate until the kappa concentration is reduced to the point that a large percentage of the animals contain only single particles of kappa. If animals are now isolated and cultured at a low fission rate, individuals will be obtained which contain kappa particles which are all "descended from" single particles. Animals containing single particles may be produced on a large enough scale to make mutation studies feasible. SONNEBORN (1947a, b) has pointed out that the same thing may be accomplished if the particle concentration is reduced by heat inactivation.

In this connection, it is interesting to note that the mutant killer, Gm1, arose under just such conditions. Gm1 arose in Experiment No. 3 in which a series of isolations was made from a culture derived by 17 rapid fissions from a single typical stock G killer. The animals after isolation were cultured at a slow rate of fission to allow kappa to increase faster than the animals and produce killers. The fact that approximately 50 percent of these isolations produced no killers and therefore had no kappa indicated that the particle number per animal was so low that an appreciable proportion of the isolations which contained kappa must have had only one particle. All except one of the isolations which gave rise to killers produced normal stock G killers. This exceptional isolation gave rise to killers all of which were of the mutant type. The circumstances of the origin of Gm1 make it seem very likely that the mutation involved a change in a single particle of kappa, and that this particle or a "descendant" of this particle was the only particle in the single animal which gave rise to the mutant killer. During the period of slow multiplication following isolation of the animal, new particles of the same mutant type were produced and animals became killers when the particle concentration had increased sufficiently. Genetic tests have not yet been performed to determine definitely whether the mutation involved kappa or the gene.

It is interesting to compare the inactivation of kappa at high temperature with gene mutation. SONNEBORN (1947b) reports 100 percent inactivation of kappa in the variety 4 stock 51 in 36 hours at 38.5°C; inactivation also occurred slowly at low temperatures near 10°C. Similar heat inactivation of kappa has been shown for kappa in the variety 2 killers (see p. 396). The "inactivation" of a gene would be manifested as a gene mutation. Although increase in temperature does accelerate the rate of gene mutation, there is no known case of gene mutation in which the mutation rate even remotely approaches the inactivation rate for kappa at high temperature. Likewise *low* temperature does not increase the frequency of gene mutation. The stability of the gene is markedly different, then, from the stability of kappa. Whether this instability is a peculiarity of kappa, or whether it is a characteristic of other autocatalytic cytoplasmic factors is at present unknown. In the case of kappa, we are forced to conclude that either (1) the nature of kappa is very different from the nature of the gene, at least in respect to the qualities which make for stability, or (2) that the location of kappa in the cytoplasm makes it much more vulnerable to inactivations than the location of the gene in the chromosome.

X. SUMMARY

Descriptions of the different types of killers in variety 2 of *P. aurelia* are given. Each different killer produces one of a series of antibiotics known as paramecins which have the property of killing certain sensitive strains of paramecia. Descriptions of the action of these different paramecins on different sensitive strains of paramecia are given.

A new technique for controlling fission rate is described.

Killers of variety 2 may be cultured at a fission rate exceeding the duplication rate of the cytoplasmic factor, kappa, which is necessary for the killer character. This results in a progressive decrease in the number of particles of kappa, until finally increasing proportions of the animals come to lack kappa completely.

The progressive decrease in particle concentration is accompanied by a series of quantitative and qualitative changes in the characters associated with kappa. The presence of kappa in high concentration enables animals to produce paramecin and renders them resistant to the action of the paramecin they produce. As the particle concentration is lowered, first, animals produce less paramecin; then paramecin is no longer produced but the property of resistance is retained; and finally animals lose their resistance and become sensitive to paramecin.

It has been shown that if animals have at least one particle of kappa, then the original concentration of kappa can be restored by culturing the animals at low fission rates.

The proportion of the progeny of a strong killer freed of kappa after different numbers of rapid fissions was measured. From these data it was possible to calculate the particle concentration in the strong killer and the rate of increase of kappa while the paramecia were reproducing at a higher rate. It was estimated that a strong killer contains approximately 450 particles of kappa. Uncertainties involving some of the assumptions on which the method of calculation was based, make it necessary to conclude that the estimate of 450 particles may be somewhat too low.

The relation of fission rate to the rate of increase of kappa is as follows. When killers reproduce slowly, the net rate of increase of kappa just equals the fission rate. If the fission rate increases to a somewhat higher rate, the rate of increase of kappa rises until it equals the fission rate. But if the fission rate is increased beyond a certain critical rate, then the concentration of kappa gradually decreases. At this critical fission rate kappa is increasing at its maximum rate, for its rate of increase remains essentially constant as the fission rate is increased beyond the critical rate. This maximum rate of increase is approximately two doublings per day for stock G at 27°C.

When animals which have had their kappa concentration reduced to a low level by a series of rapid fissions are now cultured at a lower fission rate, kappa increases somewhat faster than the animals and the original kappa concentration is gradually restored. As the kappa concentration rises, the rate of increase of kappa although greater than the fission rate, is not independent of it

but tends to approximate it. When a sufficiently high concentration is attained, the rate of kappa increase levels off and comes to equal the fission rate.

The concentration of kappa also affects the rate of increase of kappa, for kappa increases more slowly while in high concentration than while in low.

Temperature affects the rate of increase of kappa. The rate of two doublings per day of stock G kappa occurs only at about 27°C. At somewhat lower and at higher temperatures its rate is less. Between 29.4°C and 30.4°C and at higher temperatures stock G kappa is inactivated.

It is pointed out that the results of these studies are inconsistent with the theory of the combination of kappa with the gene *K*. The relation of the present work to the question of whether kappa is a symbiont is discussed. A comparison is made between the results on variety 2 of *P. aurelia* and those of SONNEBORN on variety 4 with respect to the number of particles and rate of increase of kappa. Other work which has demonstrated a variation in the concentration of cellular constituents as a consequence of differential multiplication rates is compared with the present work. The significance of the mathematical relations developed by Otter which are concerned with the amitotic distribution of autocatalytic particles at cell division is considered. Finally, certain aspects of the problem of the mutation of cytoplasmic factors are discussed.

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