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Killing activity from lysed kappa particles of *Paramecium**

By LOUISE B. PREER and JOHN R. PREER, Jr.
Biology Department, University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.

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The fluid in which certain strains of *Paramecia*, called 'killers', have lived is able to kill other strains of *Paramecia* called 'sensitives'. Assuming that this effect was due to an antibiotic-like toxin, Sonneborn, Jacobson & Dippell (1946) called the killing substance paramecin (Pn). Subsequently it was found that the killing activity is associated with relatively large, complex particles (Austin, 1948; Preer, Siegel & Stark, 1953). Sonneborn (1959) has referred to these particles as P or P particles.

Sonneborn (1943) discovered that the genetic basis for the killer character involved a self-reproducing cytoplasmic component which he called 'kappa'. Preer (1946, 1948*a*) postulated the particulate nature of kappa and estimated that there are several hundred particles in a killer animal. X-ray inactivation data (Preer, 1948*b*, *c*) indicated that kappa should be visible with a microscope, and DNA-containing kappa particles were subsequently demonstrated (Preer, 1948*b*, *c*, 1950).

Two kinds of kappa particles were described by Preer and Stark (1953): 'brights', referred to as 'B particles' by Sonneborn (1959), containing a bright (under the bright phase microscope) refractile body known as the 'R' or 'R body', and 'non-brights' known as 'N'. It was inferred that non-brights are self-reproducing and give rise to brights. Preer, Siegel & Stark (1953) presented evidence indicating that the killing or P particle is identical with the bright or B particle. Recent studies by Smith (1961) and Mueller (1963) on isolated brights and non-brights have demonstrated directly that bright particles have killing activity and that non-brights are able to infect sensitives, transforming them into killers. Electron micrographs of bright particles of *P. aurelia*, syngen 4, stock 51 killers made by Hamilton & Gettner (1958) and by Dippell (1958) show the refractile body in the bright particle as a number of double membraned lamellae (arranged concentrically or in the form of a coil) about a central core of granular material. The granular material may be continuous with the granular material throughout the bright particle outside of the refractile body. For an exhaustive review of kappa, see Sonneborn (1959).

There have been speculations that the refractile body within the bright kappa

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particles actually represents the poison. However, lysis of stock 51 kappa particles by sodium lauryl sulfate yielded free refractile bodies but a complete loss of killer activity (Mueller, 1963). In this study we have used sodium deoxycholate to lyse stock 7 kappa particles in order to free the refractile bodies. Evidence is presented that a large portion, if not all, of the killing activity is associated with the refractile bodies.

1. MATERIAL AND METHODS

Killer animals of *P. aurelia*, syngen 2, stock 7 were used. Stock 7 killer is known as a spinner killer and causes affected sensitive animals of certain stocks to swim forward with a distinctive clockwise rotation, spiralling in wide right-handed helices. This is described in detail by Sonneborn (1938*a*, *b*, 1939) and Preer (1948*a*). *P. caudatum* stock 141 was used as a sensitive tester.

The standard procedures for culturing paramecia were followed (see Sonneborn, 1950). Killer cultures were grown in $2\frac{1}{2}$ gal. jugs containing a 0·15% infusion of Scotch grass (M. McIntyre Co., Edinburgh, Scotland) buffered with 1 g. of $Na_2HPO_4 \cdot 12H_2O$ per litre and inoculated one day before use with *Aerobacter aerogenes*. Jugs were fed every other day for approximately half a fission and kept at room temperature. Sensitive cultures were needed in lesser volume and were grown in two-litre flasks kept at 27° C.

Collection of paramecia, homogenization, and centrifugation were carried out as described earlier (Preer & Preer, 1959). All pipettes and centrifuge tubes (except plastic tubes which are rendered opaque) used in purification procedures were coated with silicone to minimize loss of material.

Partial purification of refractile bodies for use in a number of experiments was accomplished using the following procedure: 0·5 ml. of packed stock 7 killer paramecia are obtained in a pear-shaped oil testing centrifuge tube. Fifteen times the volume of $\frac{1}{2}$% sodium deoxycholate pH 7·7 is added, and the paramecia are resuspended and allowed to stand for 15 min. This preparation is centrifuged at $25{,}000 \times G$ for 10 min. in the International refrigerated centrifuge and the supernatant is discarded. Care is taken to remove the fat droplets from the top and sides of the tube by wiping with cheesecloth. The precipitate is washed in Dryl solution (Dryl, 1959), centrifuged again at $25{,}000 \times G$ for 10 min. and resuspended in 1 c.c. of Dryl solution and homogenized by forcing through a No. 26 gauge syringe needle several times. Portions consisting of 0·25 c.c. of this preparation are placed in each of three centrifuge tubes containing 5 c.c. of 2·4 M sucrose and centrifuged in the SW 39 head of the Spinco model L centrifuge at 35,000 r.p.m. for 1 hour. The supernatants of these three tubes are combined, and 60 c.c. of Dryl solution are added slowly while stirring. This is centrifuged at $25{,}000 \times G$ for 30 min. and the precipitate is resuspended in 1 c.c. Dryl solution or 0·002% sodium deoxycholate in Dryl solution and homogenized with a syringe needle. This preparation which possesses most of the killing activity, contains many refractile bodies, an occasional bright kappa particle, many trichocysts and trichocyst fragments and some bacteria.

In some experiments crude preparations of refractile bodies were used. These were made by treating killer animals with ½% sodium deoxycholate, centrifuging at 25,000 × *G*, washing, and resuspending the precipitate in Dryl solution as described above. A final low speed 100 × *G* centrifugation for 10 min., retaining the supernatant, completes this procedure.

Assays for killing activity were performed as follows. Depression slides were used to make serial dilutions (number one, full strength; number two, ½ strength; number three, ¼, etc.) of the preparation to be tested. Dryl solution was used as diluent. 0·33 c.c. of the preparation to be tested was placed in each depression and 0·33 c.c. of a culture of sensitive animals containing approximately 200 animals was added. Tests were incubated in moist chambers at 27° C overnight. The number of affected animals which could be counted during a two-minute interval was then determined for each mixture. The activity in arbitrary units was finally computed as 2*S*, where *S* represents the number of the dilution in which twenty-five affected animals were counted (interpolations to the nearest tenth dilution were made when necessary). To determine the killing activity sedimented in centrifugation experiments the number of units of activity in the precipitate was divided by the number in the supernatant plus precipitate.

Counts of refractile bodies were made by adding a small volume of a standard yeast cell suspension to the preparation to be counted, and then scoring the number of refractile bodies and yeast cells under a microscope equipped with a bright phase oil immersion lens. For each determination twelve fields were usually counted. The number of refractile bodies and yeast cells counted varied; the average number for these experiments was approximately ninety refractile bodies and 130 yeast cells. After the counts of refractile bodies were made, the supernatant and precipitate fractions were each diluted with 10 c.c. of Dryl solution. Killing tests were then set on serial dilutions of the dilute supernatant and precipitate.

2. RESULTS

(*i*) *Killing activity in suspensions of lysed kappa particles*

It was found that exposure of killers of stock 7 to ½% sodium deoxycholate at pH 7·7 for 15 min. lysed the paramecia as well as most of the bright and non-bright kappa particles. Nevertheless such preparations generally had about half of the killing activity of simple homogenates. When sodium deoxycholate lysates were centrifuged at 25,000 × *G* for 3 min., the killing activity was recovered in the precipitate and not in the supernatant, indicating that the killing substance was not in solution and that a particle was still responsible for killing.

When a lysate prepared with ½% sodium deoxycholate was examined under phase, it was found that virtually all of the bright kappa particles were lysed and that their only identifiable remnants were the free refractile bodies. These were readily detected in the bright phase microscope by their size (approximately three-fourths of a micron), brightness, and their distinctive shape when viewed from different angles. Particles often appear doughnut-shaped. As they move about

in Brownian movement doughnuts often turn so that they appear as squarish particles (see Fig. 1).

Experiments were performed to determine the rate of sedimentation of the particles responsible for killing activity in the material treated with sodium

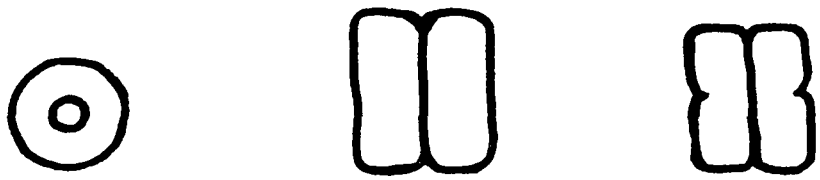

Fig. 1. Sketches of refractile bodies freed from bright kappa particles after lysis with ½% sodium deoxycholate, showing distinctive appearances when viewed under bright phase contrast.

deoxycholate. In these experiments one portion of a suspension of killers was homogenized and a second portion was treated with ½% sodium deoxycholate. Each was then diluted appropriately so that it was centrifuged in the same negligibly low concentration (0·002%) of sodium deoxycholate. (Such concentration was

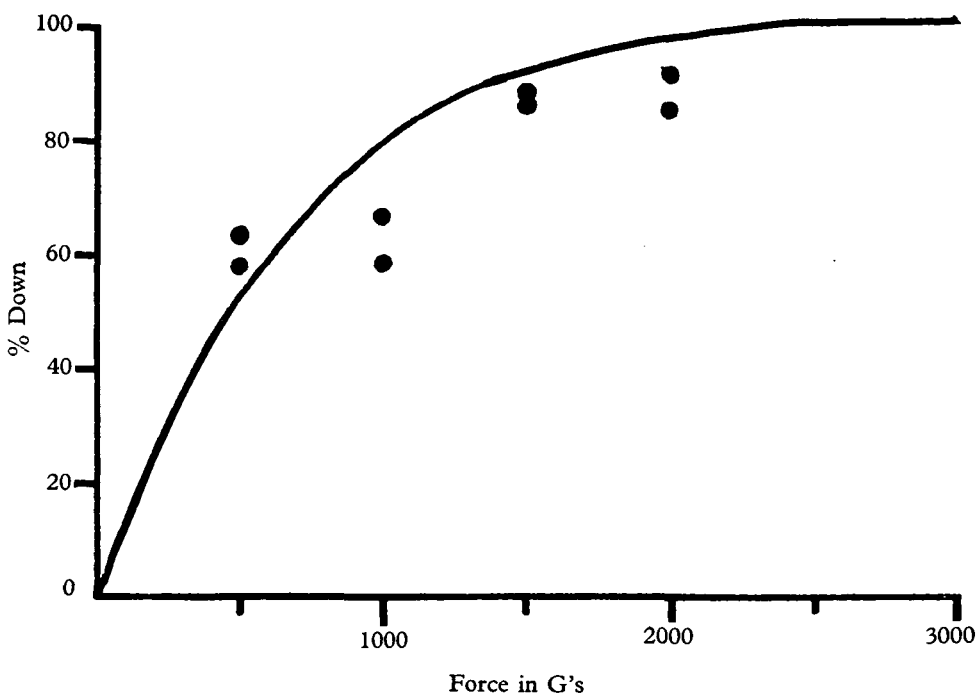

Fig. 2. Sedimentation of killing activity in an homogenate of stock 7 killer animals. Percentage killing activity centrifuged down at different centrifugal speeds is plotted. The smooth curve was fitted by Preer, Siegel & Stark (1953) to points obtained in similar experiments. It is superimposed on the points we have obtained in these experiments.

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found to have no observable effect on killer activity or kappa morphology.) In each run 1 ml. of material was placed into a tube about 0·5 cm. in inside diameter and centrifuged in a horizontal centrifuge for 10 min. Several different speeds were used. Supernatant and precipitate fractions of the control and treated preparations were assayed for killing activity as described above, in order to determine the percentage of killing substance spun down at each centrifugal speed. The results are given in figures 2 and 3. Superimposed on the points is the activity curve of killing particles from Preer, Siegel & Stark (1953) showing the percentage killing particles centrifuged down in similar experiments they performed using killer homogenates.

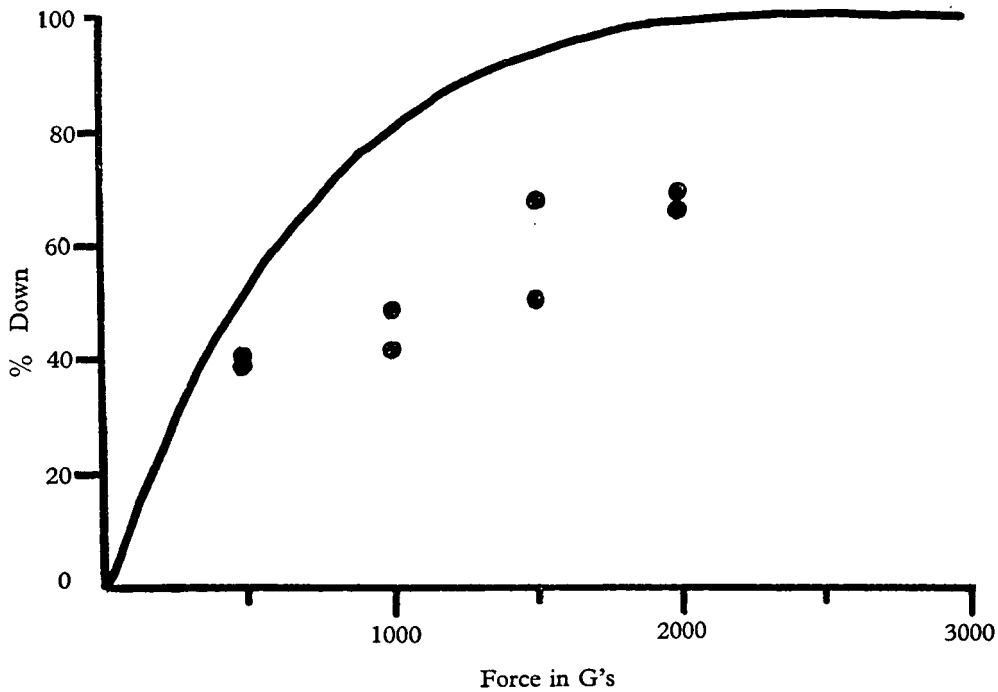

Fig. 3. Sedimentation of killing activity from a ½% sodium deoxycholate lysate of stock 7 killer animals. Percentage killing activity centrifuged down at different centrifugal speeds is plotted. Comparison of this graph with the superimposed activity curve of killing particles from Preer, Siegel & Stark, 1953, shows killing activity in treated preparation is associated with a more slowly sedimenting particle.

The control in the present experiment (Fig. 2) shows good agreement with their results. It is evident that the killing activity in the treated preparations (Fig. 3) is associated with a more slowly sedimenting particle. It is calculated from the data that the sedimentation coefficient of killing activity in the treated preparations is only about 0·4 as great. Assuming similar densities and shapes, the volume of the killing particle in the treated preparations is calculated to be about one-fourth as great as that of the particles in the untreated preparations. Since the normal bright killer particles are several times larger than the refractile bodies, and the refractile bodies are the only particles of this magnitude visible after sodium

deoxycholate treatment, the results suggest that the killing particles are the refractile bodies.

These experiments also provide data on how much killing activity is retained after lysis with sodium deoxycholate relative to that found after homogenization in Dryl solution. In one experiment four successive determinations gave 65% retained, 56%, 44% and 45%. In a second experiment, four determinations yielded 70%, 55%, 50% and 55%. One determination in a third experiment showed 40% retained.

(ii) *Refractile body counts and killing activity of centrifugal fractions*

To test the possibility that refractile bodies are responsible for killing activity, counts of refractile bodies were made on centrifugal fractions which were sub-

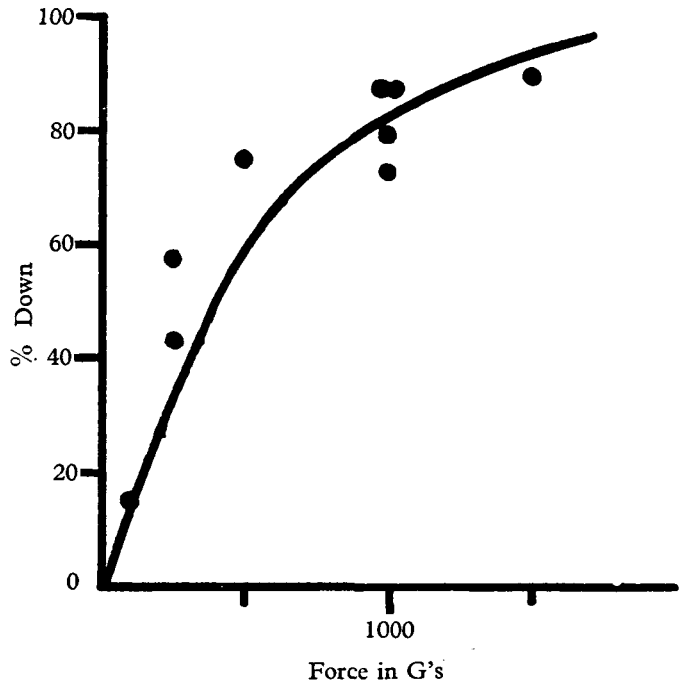

Fig. 4. Sedimentation of killing activity in partially pure preparations of refractile bodies. Percentage killing activity centrifuged down at different centrifugal speeds is plotted. The smooth curve is of the form $Y = 1\text{-}e^{-ax}$ and was fitted to the points by least squares.

sequently tested for killing activity. It was necessary to obtain partial purification of the refractile bodies in order to count them under the phase microscope. The cilia, body wall, and mitochondria disintegrate in sodium deoxycholate. Fat droplets and soluble components are easily removed by centrifugation. The chief remaining contaminants are crystals, bacteria, and trichocysts. Some further purification was achieved by centrifugation at high speed in a high molar sucrose solution, as described in the section on material and methods.

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Nine experiments were performed using partially purified preparations of refractile bodies in which corresponding counts of refractile bodies and assays of killing activity were made on supernatants and precipitates from various low speed centrifugations, each of 10 min. duration. Figure 4 gives the percentage killing activity centrifuged down at various speeds. The smooth curve is of the form $Y = 1 - e^{-ax}$ and was fitted to the points in Fig. 4 by least squares. The points in Fig. 5 give the percentage of refractile bodies centrifuged down at various speeds. The curve of Fig. 4 is transposed directly to Fig. 5. Figure 5 agrees well with the curve

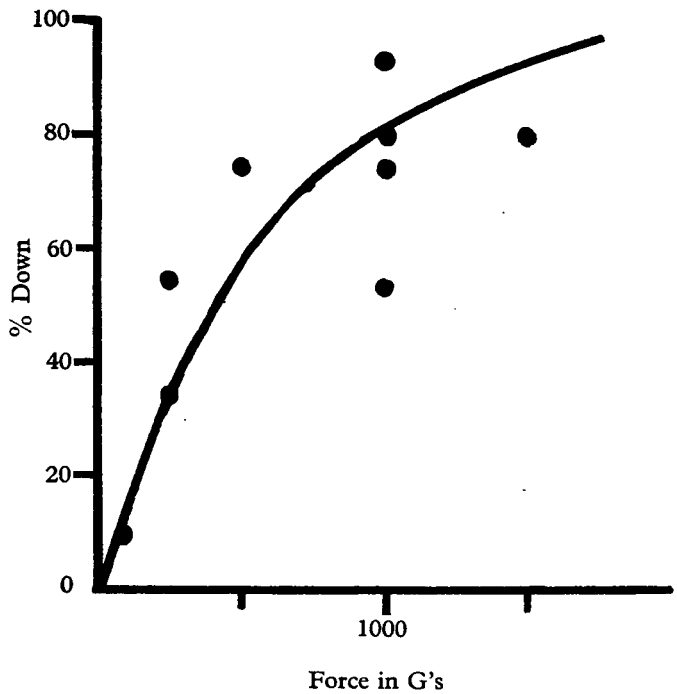

Fig. 5. Sedimentation of refractile bodies in partially pure preparations of refractile bodies from ½% sodium desoxycholate lysates. Percentage refractile bodies centrifuged down at different centrifugal speeds is plotted. The smooth curve represents killing activity and is the same as the one shown in Fig. 4.

based on killing activity in Fig. 4, supporting the conclusion that the refractile bodies are responsible for killing activity. It is noted that after partial purification, killing particles are found to centrifuge down at a faster rate than they did previously (Figs. 3 and 5). The reason for this is unknown. Perhaps in the more concentrated suspensions used in these experiments the particles aggregate to a greater degree. No difference in appearance of refractile bodies could be observed.

If the refractile bodies are the killing particles then their number should not exceed the number of affected animals in a killing test. Owing to the large experimental errors in determination of the total number of affected sensitives and also of refractile bodies, accurate comparisons were not possible. However, the two were certainly of the same order of magnitude.

(iii) *Changes in activity during storage of refractile bodies*

When partially purified refractile bodies are stored over a period of days their number decreases slowly. Assays for killing activity show that it also declines, but more rapidly than the number of refractile bodies. Thus, at 0° C. there is a sharp drop in the concentration of refractile bodies and killing activity in the first 24 hours, followed by a levelling off on successive days until the sixth day when 45% of the refractile bodies but only 18% of the killing activity remained. At 27° C. virtually all of the killing activity was lost in the first 24 hours, whereas 50% of the refractile bodies were still present as late as the second day.

Suspensions of partially purified killing particles undergo a peculiar change in the type of killing they produce when stored at 27° C. for 24 hours. Fresh suspensions, if not too concentrated, cause stock 141 animals to spin rapidly on their longitudinal axes; if more concentrated, sensitives are paralysed. At intermediate concentrations a combination of the two effects causes sensitives to spin, but very slowly. Aged killing particles, on the other hand, completely lose their ability to cause spinning while retaining their ability to paralyse apparently undiminished. The aged killing substance thus produces an effect very similar to that of the 7ml paralysis mutant (Preer, 1948*a*) and similar mutants studied by Dippel (1950). One explanation for this result might be that the stock 7 killers contain a mixture of spinning and mutant paralysis-inducing particles with the latter more resistant to ageing. If so, then it should be possible to isolate lines pure for each type of killing. However, some twenty clones of stock 7, derived from single kappa particles seventeen fissions previously (produced by rapid multiplication—see Preer, 1948*a*, *b*), each showed both kinds of killing, exactly like the parental culture.

Crude preparations of refractile bodies, made as described above, when placed at a high temperature also showed a marked decrease in spinning killing activity, although paralysis killing persisted. Thus in one experiment 75% of the spinning killing activity was lost in a crude preparation of refractile bodies placed at 31° C. for 2 hours as compared with an aliquot kept at 0° C. It was also noted that this loss of spinning killing could be retarded by adding homogenates or homogenate fractions before placing the refractile body preparation at 31°. The addition of a $25{,}000 \times G$ supernatant or precipitate of a 35% suspension of stock C141 animals in Dryl solution, or of the $25{,}000 \times G$ supernatant of a homogenate of the same concentration of stock 7 animals served to protect the killing activity of refractile body preparations placed at 31° C. for 2 hours. The treated preparation had approximately fifty times as much activity as the suspensions to which only Dryl solution had been added.

3. DISCUSSION

The fact that sodium deoxycholate lyses the bright kappa particle yet leaves approximately half of the killing activity demonstrates conclusively that it does not take an intact bright particle to kill a sensitive.

Since all of the remaining activity in a lysate produced with sodium deoxycholate

sediments at relatively low centrifugal forces (but somewhat greater than the force required to sediment the intact bright) it is clear that the activity in such lysates is associated with some fragment of the dissociated bright particle. The only fragment microscopically visible is the refractile body, strongly suggesting that it is the killing particle. A direct determination of the rate of sedimentation of concentrated, partially purified refractile bodies and of killing particles from lysates show that both do sediment at approximately the same rate, even though under these somewhat different conditions, the killer particles sediment faster than normal. Although one cannot completely rule out the possibility that the killing activity in lysates is associated with some other unrecognized fragment of the bright particle with a similar sedimentation coefficient, this possibility is unlikely, and we must conclude that the refractile body is the killing particle.

Is the refractile body the seat of killing activity in the normal bright particle? Since the refractile body represents much less than half the volume of the bright particle, yet in lysates has fully half of the activity, then if the refractile body is not the sole seat of killing activity, it must at least be more potent, volume for volume, than the remainder of the bright. The reduced activity of lysates relative to homogenates with intact brights might have several explanations. First, the sodium deoxycholate might cause some inactivation of particles. The experiments on aged refractile bodies show that morphologically intact, but inactive, particles may exist. In fact, Mueller (1963) has been able to prepare relatively pure but inactive suspensions of refractile bodies from stock 51 killers using sodium lauryl sulphate. We have found that sodium lauryl sulphate also inactivates the killing substance of stock 7, and Sonneborn (personal communication) reports (and we have confirmed) that sodium deoxycholate completely inactivates the killing substance of stock 51. Second, a portion of the killing activity might be associated with a part of the bright other than the refractile body. And, third, lysis may prevent a maturation of killing activity within bright particles which has been postulated to occur after homogenization (see Sonneborn, 1959).

The nature of the loss of spinning action and retention of paralysis killing in aged killer preparations is not clear. The phenomenon does not seem explicable in terms of mutant kappa particles with different activities and stabilities, but could be explained on the basis of two non-genetic kinds of killing particles. The transformation of one kind of particle into another as a result of storage seems just as likely, however.

SUMMARY

Lysis of stock 7 killers with $\frac{1}{2}\%$ sodium deoxycholate, yields preparations with one half as much killing activity as homogenates, while lysing virtually all of the bright particles known to have killing activity in homogenates. The killing activity which persists is associated with particles which sediment at the same rate as the refractile bodies, the only visible remnant of the bright particles. It is concluded that killing activity is probably associated primarily, if not exclusively, with the refractile bodies of the bright kappa particles. Partially purified killer particles

when stored at 27° C. for more than one day, progressively lose their ability to produce spinning of sensitives but retain their ability to produce paralysis. Loss of activity in crude preparations of refractile bodies kept 2 hours at 31° C. can be retarded by homogenates of either killers or sensitives.

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