*VIRUS-LIKE BODIES IN KILLER PARAMECIA**

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Each killer strain of *Paramecium aurelia* liberates into the medium in which it lives toxic particles which kill sensitive strains of paramecia.[1, 2] It has been suggested[1, 3] that this toxic activity might be due to viruses present within the bacteria-like symbionts (kappa particles) found in killers. Here we report the discovery of particles associated with the refractile inclusions (R bodies) within kappa. The appearance of these particles in the electron microscope suggests that they are viruses. In one strain they resemble polyhedral viruses with characteristic capsomere-like subunits, and in another they resemble naked helical viruses. We also present observations on the characteristics of R bodies and their toxic activity. Previous work[4–9] has shown that the R body is a tape about $^1/_2$ μ wide, 14 μ long, and 130 Å thick. It is normally wound in about ten turns into a short hollow roll with a length and diameter of about $^1/_2$ μ. It is capable, however, of suddenly extending ("unrolling") into a long twisted ribbon. Finally, we consider the possibilities that the virus-like elements are normally in a provirus state, that their induction results in the formation of R bodies, that the protein of the virus-like elements is the toxin, and that unrolling of R bodies delivers the toxin to its site of action in sensitives. If further evidence validates this picture, then the killer phenomenon provides an example of a remarkable and unique relationship between toxic virus-like elements, their bacteria-like hosts (kappa), a highly specialized organelle (the R body) important in delivering the toxin to its site of action within sensitives, and the cell (*Paramecium*) which contains them all.

Materials and Methods.—Strains of *Paramecium aurelia*, culture methods, assays for killing activity, and methods of counting particles have been described previously.[8–10]

Kappa of stock 51 was isolated by a modification[9] of the method of Smith.[11] Kappa of stock 7 was isolated by passing homogenates through a filter paper column; kappa was adsorbed onto the column and later eluted as described previously.[9] Kappa particles of stock 7 (not stock 51) could be stored in the deep freeze without great losses in killing activity. Most of the soluble constituents of the homogenate, along with mitochondria, cilia, and lipid droplets, passed through the column and were then lyophilized, to be used later in preparing "paramecium extract."

Lysis of kappa particles of stock 51 was obtained with $^1/_2$% sodium deoxycholate. Although sodium deoxycholate lyses kappa particles of stock 7 when whole killers are treated,[12] it does not lyse isolated kappa particles of stock 7.[9] Lysates of isolated kappa particles of stock 7 active in killing could be obtained, however, by treatment with a mixture of deoxycholate and paramecium extract. The latter was prepared by resuspending about 45 mg of the lyophilized material from the column in 1 ml of distilled water, centrifuging at 25,000 $\times$ g for 5 min, and discarding the precipitate. A mixture of a suspension of 10^7 to 10^9 stock 7 kappa particles in $^1/_3$ ml of 0.01 M sodium phosphate buffer at pH 7.0, plus $^1/_3$ ml of 5% sodium deoxycholate plus $^1/_3$ ml of paramecium extract gave lysis of 85–95% of the kappa particles in five min. Higher percentages of lysis could be obtained by immersing a 5-ml plastic centrifuge tube containing $^1/_2$ ml of suspended kappa particles into the bath of a Branson model HD-50 ultrasonic cleaner at 2°C for about five min; however, some activity was lost during this treatment. R bodies were isolated from lysates by centrifuging suspensions diluted to 10 ml with buffer at 500 $\times$ g for two min and discarding the supernatant.

In tests for the action of enzymes on killing activity, R bodies of stock 7 were suspended in 0.01 M phosphate buffer at a concentration of about 10^8/ml, and enzymes (Nutritional Biochemi-

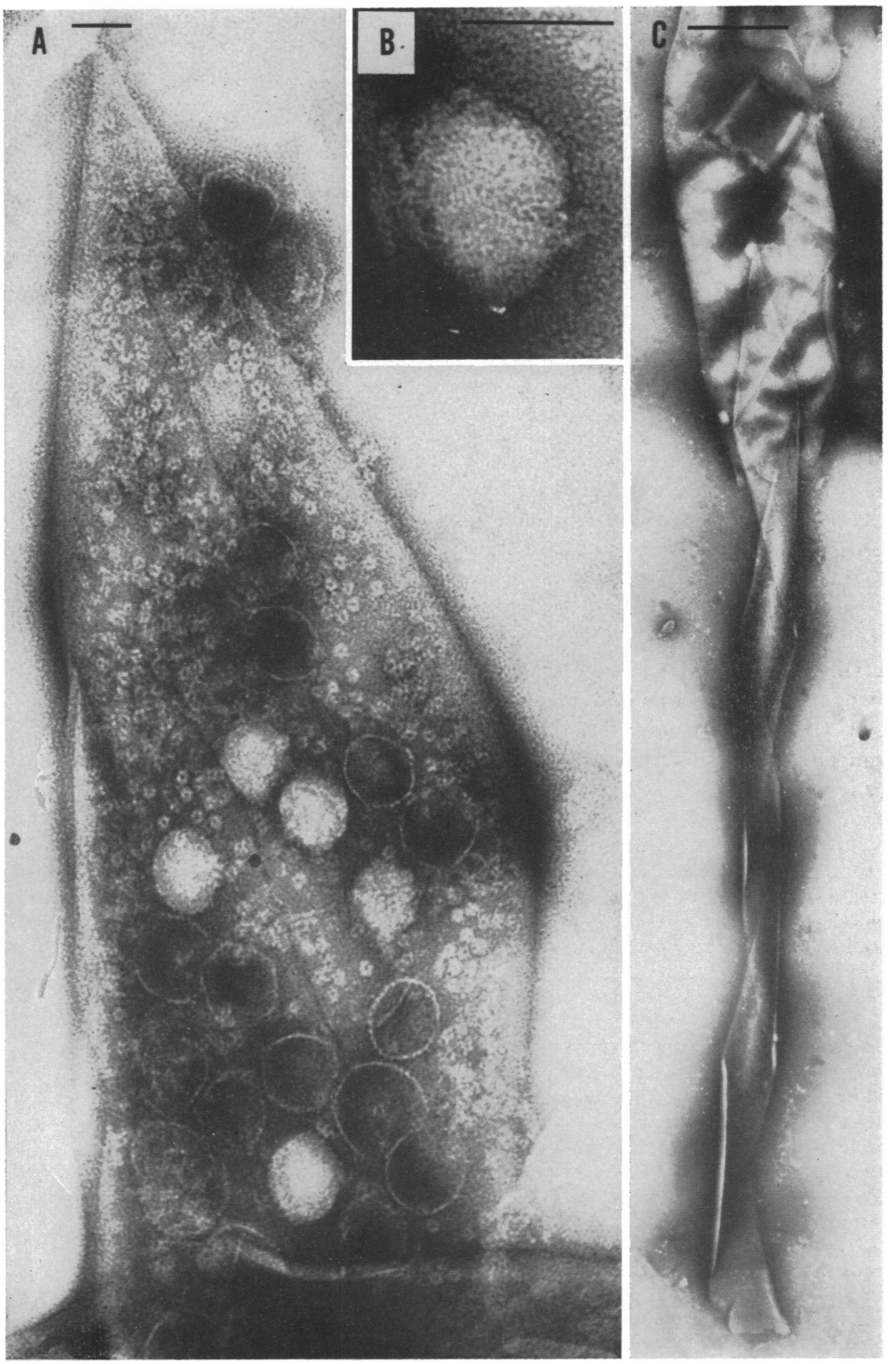

FIG. 1.—(*A*) Inside tip of unrolled R body (stock 7) with virus- and capsomere-like structures. *Bar*, 500 Å. ×176,000. (*B*) Virus-like body and capsomere-like particle near unrolled R body (stock 7). *Bar*, 500 Å. ×430,000. (*C*) Kappa particle (*upper third of figure*) containing partly unrolled R body extending through break in end of kappa (stock 7 ml). *Bar*, 0.5 μ. ×25,000.

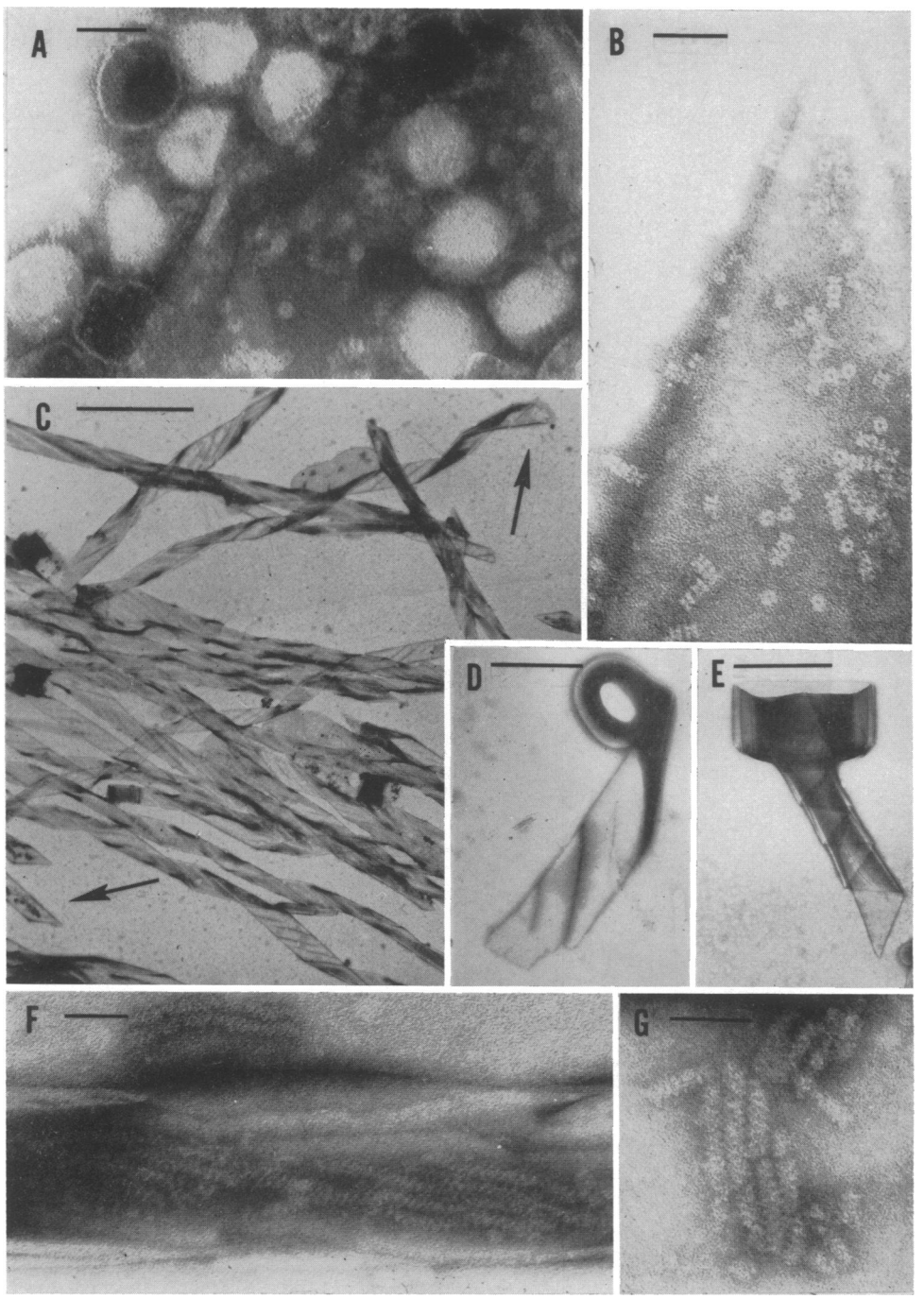

FIG. 2.—(*A* and *B*). Virus- and capsomere-like elements near tips of unrolled R bodies (stock 7). *Bar*, 500 Å. ×205,000. (*C*) Unrolling R bodies (stock 7). Arrows indicate no virus-like particles on blunt end (*upper right*), several on acute end (*lower left*). *Bar*, 2 μ. ×8,400. (*D*) R body (stock 7) unrolling from outside with blunt end free of virus-like bodies. *Bar*, 0.5 μ. ×26,500. (*E*) R body (stock 51) unrolling from inside producing a hollow tube. Virus-like elements washed free of acute end during isolation. *Bar*, 0.5 μ. ×28,000. (*F*) Unrolled tubular R body (stock 51) with helical virus-like structures inside and (*at top*) outside. *Bar*, 500 Å. ×199,000. (*G*) Helical virus-like structures (stock 51). *Bar*, 500 Å. ×239,000.

cal Corp.) were used at a concentration of 1 mg/ml. RNase, DNase (in 0.01 *M* $MgCl_2$), and chymotrypsin were all tested at pH 8.0. Trypsin was tested at pH 7.5. Lysozyme and pectinase were used at pH 7.0. β-Amylase, cellulase, pepsin, and chitinase were tested at pH 6.0. Exposure was for 3 hr at 30°C, at which time the R bodies were washed, diluted, and titered. Control suspensions were highly active with end points[10] (using sensitive stock 16) at dilutions of about 10^{-6}.

Separation of rolled and unrolled R bodies was accomplished by layering about 0.5 ml of a suspension of particles onto a linear density gradient of sucrose (10–65% w/v in 15 ml) and centrifuging at 3000 × *g* for 30 min. After centrifugation, 3/4-ml samples were removed from the top with a syringe.

Negative staining of suspensions[9] was without fixation using 5% phosphotungstic acid at pH 7.0. The specimens were examined in a Philips model 200 electron microscope.

Results.—Observations on virus-like bodies of stocks 7 and 51: Purified kappa particles of stock 7 were lysed with sodium deoxycholate and paramecium extract. When isolated and negatively stained, many of the R bodies unroll, revealing numerous virus-like bodies (Figs. 1*A*, *B*, 2*A*, *C*). They range from 500–1200 Å and are often hexagonal in outline. Many appear dark, suggesting that they are empty, the stain having diffused inside. Others appear light and are presumed to be filled. However, such interpretations must be accepted with caution in negative staining.[13] The envelope-like membrane of the empty particles is very thin, on the order of 15 Å.

The same photographs reveal much smaller capsomere-like elements about 100 Å in diameter which often appear hexagonal, pentagonal, or H-shaped (presumably when oriented differently) (Figs. 1*A*, *B*, 2*A*, *B*). There is a strong suggestion that the capsomere-like elements are present only within the "filled" virus-like bodies, perhaps making up a layer just inside the envelope.

While both ends of the ribbon making up the 51 R body terminate in an acute angle[9] (inside end can be seen in Fig. 2*E*), in R bodies of stock 7 only the inner end of the ribbon forms an acute angle (Figs. 1*A*, 2*B*, *C*). The outside end (which always unrolls first in the R bodies of stock 7[9]) is blunt and irregular (Figs. 1*C*, 2*C*, *D*). Both the virus-like and capsomere-like elements are found primarily on the inside end of unrolled R bodies of stock 7. They are also observed in the material surrounding the R body which has flowed from kappa particles lysed on the electron microscope grids. Rupture of kappa by ultrasonication revealed similar but less numerous structures.

Different virus-like elements are demonstrated in stock 51 by the use of other methods. If phosphotungstic acid at pH 4.5 is added to suspensions of kappa particles which have just been partially disrupted by freezing and thawing, then long, apparently helical, virus-like elements about 180 Å in width are observed (Figs. 2*F*, *G*). They may also be seen after the addition of phosphotungstic acid at pH 4.5 to suspensions of intact kappa particles of stock 51 on the electron microscope grids. The low pH causes some R bodies to unroll while in the kappa particles, thereby disrupting the kappa membrane. In fully unrolled R bodies the virus-like structures are found inside what was originally the innermost turns of the rolled R body, and hence are all near one end. (In the R bodies of stock 51, as shown in Figure 2*E*, unrolling occurs from the inside,[9] while in stock 7, as seen in Figure 2*D*, unrolling occurs from the outside.) The virus-like bodies are also seen in the material surrounding the R body when kappa particles lyse on the grids. They are absent in preparations of R bodies isolated after lysis by deoxycholate or

by freeze-thawing, probably because they do not stick to R bodies and hence are lost in the centrifugations used during isolation.

Some properties of isolated R bodies of stock 7: The toxic particles produced by stocks 7 and 51 are the kappa particles which contain R bodies.[10, 11, 14] Furthermore, isolated R bodies of stock 7 (but not 51) are active in killing sensitives.[12] Efforts were made to determine whether unrolled R bodies of stock 7 are active. Spontaneous unrolling at room temperature yielded mixed populations of rolled and unrolled forms. Almost complete separation was obtained on a sucrose gradient, and it was concluded that unrolled forms do not affect sensitives. Exposure of isolated R bodies to 4 per cent phosphotungstic acid at pH 7.0 for one to two minutes caused about half to unroll, and a loss of about half the activity. Separation revealed that rolled forms are active, and unrolled, inactive. Treatment of R bodies with 0.25 per cent sodium lauryl sulfate caused complete unrolling and complete loss of killing activity. These observations suggest that unrolling inactivates R bodies.

Since it is possible that R bodies must be ingested by sensitives in order to exert their toxic action,[1] we investigated the fate of isolated R bodies exposed to sensitives. Using phase microscopy, we found that both rolled and unrolled R bodies are taken into food vacuoles of sensitive stock 16. After exposing sensitives (kept for about an hour beforehand in culture fluid from which the bacteria had been centrifuged) to rolled R bodies for seven minutes and then washing them, samples were periodically crushed and examined. Few R bodies are unrolled after $^1/_2$ hour, many after 1 hour, and virtually all after $1^1/_2$ hours.

What is the chemical nature of the toxin itself? Unlike the situation in stock 7, the R body-containing kappa particles of stock 51 lose all toxic activity on lysis; and isolated R bodies from stock 51 are inactive.[12] Therefore, earlier conclusions about the chemical nature of the toxin in stock 51 based on inactivation studies[15–17] may not be valid. Inactivating agents may merely rupture the outer membrane of the kappa of stock 51 rather than act directly on the toxin. We have investigated the effect of enzymes on killing activity of R bodies isolated (using ultrasonication) from stock 7. RNase, DNase, lysozyme, pectinase, β-amylase, cellulase, chitinase, and trypsin were found to be without effect. However, after R bodies were in chymotrypsin in concentrations as low as 1 μg/ml (3 hr at 30°C), they induced paralysis rather than the usual ciliary reversal and spinning. No effect of the chymotrypsin was noted in the electron microscope. Chymotrypsin had no effect on the killing activity of intact R body-containing kappa particles of stock 7.

The virus-like bodies have not yet been isolated, nor has it been possible to obtain sufficient numbers of purified R bodies in order to analyze the nucleic acid content of the adhering virus-like bodies. However, data on the composition of the ribbons were obtained as follows. Suspensions of 10^8 to 10^9 R bodies isolated after ultrasonication were extracted with ethyl alcohol and alcohol-ether mixtures. Their appearance under the electron microscope did not change, indicating that lipids are probably not a major component. Extraction with 0.5 *M* perchloric acid for 15 minutes at 90°C[18] and spectrophotometry of the extracts revealed only traces of nucleic acid. Examination of the nucleic acid-free residue in the electron microscope revealed unrolled R bodies which were normal except that the

virus-like and capsomere-like structures could no longer be identified. Therefore R bodies probably do not contain appreciable quantities of nucleic acids or other compounds soluble in hot perchloric acid. After extraction, the α-napthol test for sugars[19] showed less than 1 μg glucose equivalents in 10^9 R bodies. Although the extracted ribbons remained intact in most solvents, they went into solution readily and completely in 70 per cent formic acid. The absorption spectrum of the resulting solution was typical of proteins with an absorption maximum at 276 mμ. The Lowry test for proteins[20] gave values almost identical to those based on the absorption of ultraviolet light. In four different experiments the amount of protein per 10^9 R bodies was estimated at 43, 18, 42, and 50 μg. Thus the only component of R body ribbons identified was protein. Lipid, nucleic acids, or carbohydrates were not found in more than traces.

Discussion.—Are kappa particles infected with virus-like agents? The size, frequent hexagonal shape, envelope-like layer, and the "hollow" and "filled" appearances of the large bodies found in kappa of stock 7 suggest the appearance of a number of negatively stained viruses.[13] The small particles appearing hexagonal or pentagonal in outline resemble viral capsomeres.[13] Likewise, the elongated, helical structures in stock 51 resemble the naked helical viruses in appearance and size.[13] Analysis of nucleic acid in the virus-like particles awaits isolation of the particles.

If mutants of kappa which lack toxic activity also lack virus-like elements, as has been suggested,[1] then it might be possible to reinfect them. However, no attempts at reinfection have been reported. If the virus-like elements are not infective, then they might be more like certain mutant phages or the episomal elements of colicins.[1]

As will be seen below, a number of otherwise puzzling attributes of kappa and its toxic activity become intelligible if the elements observed in kappa are viral in nature.

Do R bodies result from the activity of virus-like elements within kappa? Kappa particles which do not contain R bodies are capable of entering kappa-free strains and changing them into killers, while kappa particles which contain R bodies are not.[11, 14] This and other considerations have led to the conclusion that R body-containing kappa particles are probably not self-reproducing.[1] The inability of R body-containing kappa particles to reproduce is easily explained if the virus-like elements in kappa particles lacking R bodies are in a provirus state and if they mature and result in the formation of both the virus-like structures and the R body. Indeed, kappa particles containing R bodies are often seen to lyse spontaneously in homogenates,[4] just as bacteria are presumed to be lysed by lysogenic phages. If these suggestions are correct, then the mature virus-like bodies should be present in kappa particles containing R bodies and absent in kappa particles lacking R bodies. Although our observations on partially lysed kappa particles suggest such a distribution of the virus-like elements, the evidence at this time is not decisive.

Structures which resemble R bodies have been described.[1] Those found in HeLa cells infected with adenoviruses are particularly suggestive.[21] The structures have a central area surrounded by layers of about the same number and thickness. The central area as well as the surrounding areas often contain viruses.

Do virus-like elements constitute the toxic agent in R body-containing kappa par-

ticles? The effect of chymotrypsin on the killing activity of R bodies of stock 7 suggests that the specific toxin is protein. It is difficult to see how pretreating R bodies with chymotrypsin could bring about a change in the nature of the toxic action if the effect on sensitives is produced by a multiplication of the virus-like elements. Furthermore, we have been unable to find virus-like particles in centrifugal fractions of homogenates of sensitives exposed to R bodies of stock 7. It is therefore likely that killing is due to the activity of a toxic protein.

If the virus-like agents are indeed viral in origin, then both the capsomere-like structures and envelopes are presumably protein and, along with the R body itself, are possible candidates for the toxin. The fact that the toxic activity of R bodies of stock 7 is very sensitive to such agents as high temperature and pH extremes while R bodies themselves are very resistant[8, 9] suggests that the R body is not the toxin. Although the stability of the virus-like bodies has not yet been fully investigated, they appear more sensitive to chemical and physical treatments than R bodies.

Another set of facts supports the notion that the virus-like structures are the toxin. Isolated R bodies of stock 7 are active and bear virus-like elements; isolated R bodies of stock 51 are inactive and are free of virus-like elements. Assuming that the kappa particles of stock 51 also kill by means of virus-like elements, we began a search for such elements which are lost soon after kappa particles are lysed. This search proved successful and revealed the helical structures.

Does unrolling of R bodies serve to deliver the toxic agent to its site of action? Wound R bodies isolated from stock 7 are active, but unrolled R bodies are inactive, as would be expected if unrolling is important in introducing the toxin into sensitives. Nevertheless, the possibility that this inactivation is due to an observed reduction in number of adhering virus-like elements cannot be ruled out. All efforts to obtain particles smaller than R bodies capable of killing have failed, as would be expected if unrolling is essential for killing.

It is not known how the toxin enters sensitives,[1] but recent studies by Dryl[22] suggest that the food vacuole may be the site of entry. The suggestion that R bodies have a role in carrying the toxin into sensitives is supported by Mueller's observation[23] of an unrolled R body of stock 51 protruding through the wall of a food vacuole in a sensitive paramecium which had ingested stock 51 kappa. In view of the known action of low pH in unrolling R bodies of stock 51,[7, 9] it is likely that low pH within the food vacuoles[24] induces R bodies within kappa to unroll there. Unrolling of R bodies of stock 7 in food vacuoles is due to an unknown factor since low pH does not induce their unrolling.

The end of the hollow tube which first extends from the inside of the rolled portion of the unrolling R body of stock 51 is filled with virus-like elements. Since ribbons readily penetrate kappa membranes, they might penetrate the walls of food vacuoles and deliver their contents into the cytoplasm. In the case of the R bodies of stock 7, unwinding starts with the outer blunt end, the inner end with its concentration of virus-like elements unrolling last. Perhaps, therefore, in stock 7 the roll itself is pushed through the walls of the food vacuole, or else a general breakdown of the walls of the food vacuole occurs.

Summary.—Kappa particles contain virus-like bodies which may be responsible for the toxic activity of killers. The toxin and the R body, an inclusion of kappa,

appear to be proteins. The virus-like forms may arise when a provirus-like stage is "induced," yielding both the virus-like bodies and R bodies. The suggestion that "unrolling" of R bodies delivers the toxin to its site of activity within sensitives is discussed.

This paper is dedicated to our teacher and friend, RALPH E. CLELAND, on the occasion of his seventy-fifth birthday.

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