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The relation between virus-like particles and R bodies of *Paramecium aurelia*

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1. INTRODUCTION

Kappa is a bacterium-like cytoplasmic element which is responsible for the toxic activity of certain killer strains of *Paramecium aurelia* (reviews in Sonneborn, 1959; Preer, 1968). When a killer paramecium is crushed between a coverslip and a slide and examined under the phase microscope it is found that up to 40% of the kappa particles contain a refractile structure called the R body (Preer & Stark, 1953). Kappa particles which contain an R body are called 'brights' because of their appearance in the phase microscope, and kappa particles which lack R bodies are called 'non-brights'. Some years ago it was established that non-brights are capable of giving rise to populations which contain brights, but that brights are probably non-reproductive (Preer, Siegel & Stark, 1953; Sonneborn, 1959; Smith, 1961; Mueller, 1963). R bodies have a number of remarkable features. First, each is a ribbon of protein which is normally wound in about ten turns into a roll of about 0·5 μ in diameter, but which has the capacity to unroll suddenly into a long twisted ribbon or tube-like structure some 15 μ long (Mueller, 1962; Anderson, Preer, Preer & Bray, 1964; Preer, Hufnagel & Preer, 1966). Secondly, the R body is somehow related to the toxic activity of killers, for the toxic agent itself is normally the bright; in one strain of killers, the isolated R body is toxic (Preer & Preer, 1964). Finally, if R bodies are freed from kappa particles by lysing them with suitable treatments and then inducing them to unroll by lowering the pH or treating with phosphotungstic acid, numerous virus-like particles (so-called because they look like viruses in the electron microscope) and capsomere-like elements are found sticking onto the original inner end of the unrolled tapes (Preer & Preer, 1967).

It has been suggested that the virus-like particles are the toxin and that the R body functions as a vehicle for delivering the toxin to its site of action within sensitive paramecia (Anderson *et al.* 1964; Mueller, 1965; Preer & Preer, 1967). It has also been suggested that the virus-like particles are found within non-brights in a latent form and that their induction brings about the formation both of mature virus-like particles and of the R body as well (Preer & Preer, 1967). It has been pointed out that if this last suggestion is correct, then the virus-like particles should be present in brights and absent in non-brights.

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In this paper we report a number of observations on virus-like particles and R bodies in sectioned killers. We confirm the prediction that the virus-like particles are associated primarily with the bright kappa particle. Furthermore, they are usually found in close proximity to (often within) the R bodies themselves. These findings establish a close relationship between the virus-like particles, R bodies, and toxic activity.

2. MATERIAL AND METHODS

The work was carried out with stocks 7 and 562 of syngen 2 of *P. aurelia*. Stock 7 was originally collected by T. M. Sonneborn from Pinehurst, N.C. It causes sensitives to spin on their longitudinal axes before dying. Stock 562 was collected by G. H. Beale from Milan, Italy. The original 562 contained not only kappa in its cytoplasm but also another symbiont in its macronucleus. A strain lacking the macronuclear symbiont, but containing kappa, was derived by L. B. Preer (unpublished) and used here. 562 kappa causes sensitives of syngen 1 to form large vacuoles before they die, but no spinning is induced. The paramecia were cultured in an infusion of grass (0·15 g/l., boiled, filtered and autoclaved), buffered with 1·0 g of $Na_2HPO_4 . 12H_20$ per l., and inoculated the day before use with *Klebsiella aerogenes*. Cultures were doubled in volume twice a week, using uninoculated infusion. Stock 7 was cultured at room temperature (about 17 °C) and stock 562 kappa was cultured at 25 °C. The cultures were filtered through cheese-cloth and centrifuged to concentrate them. Paramecia were fixed by adding 0·3 ml of 30% OsO_4 in CCl_4 to about 10^6 paramecia in 0·6 ml of their own culture medium (Afzelius, 1962; Jurand & Preer, 1968). Fixation was at room temperature for 20 min. with agitation every few minutes. The fixed paramecia were dehydrated by passing through 70%, 95% and three changes of absolute alcohol. Before embedding, absolute alcohol was replaced by 1,2-epoxypropane. Embedding was in Araldite, using a rotary mixer as described by Jurand & Ireland (1965). Thin sections were cut using glass knives in a Porter-Blum Sorvall microtome. Staining was for 20 min with a 1% potassium permanganate solution containing 2·5% uranyl acetate. Although a number of other methods of fixation and staining were tried, the above procedure gave the best results.

Kappa particles (of stock 7) were isolated by first homogenizing (in a stainless steel milk homogenizer, A. H. Thomas Co., Philadelphia) up to 4 ml of packed paramecia in 10 vol. of Dryl (1959) solution at 4 °C. A volume of 0·01 M phosphate buffer, pH 7·0, equal to the volume of Dryl solution used was then added. Twelve tablets (26 g) of filter-paper pulp (Whatman) were placed into 200 ml of distilled water and poured into a column 2·2 cm in diameter and washed with the phosphate buffer until the wash was clear. With the fluid level about a centimetre above the top of the column, the homogenate was pipetted onto the column. Additional buffer was added to the column and wash fractions were collected. The wash contained mitochondria, cilia and bacteria; kappa, trichocysts and body wall fragments were retained in the column. After the buffer wash was complete, as

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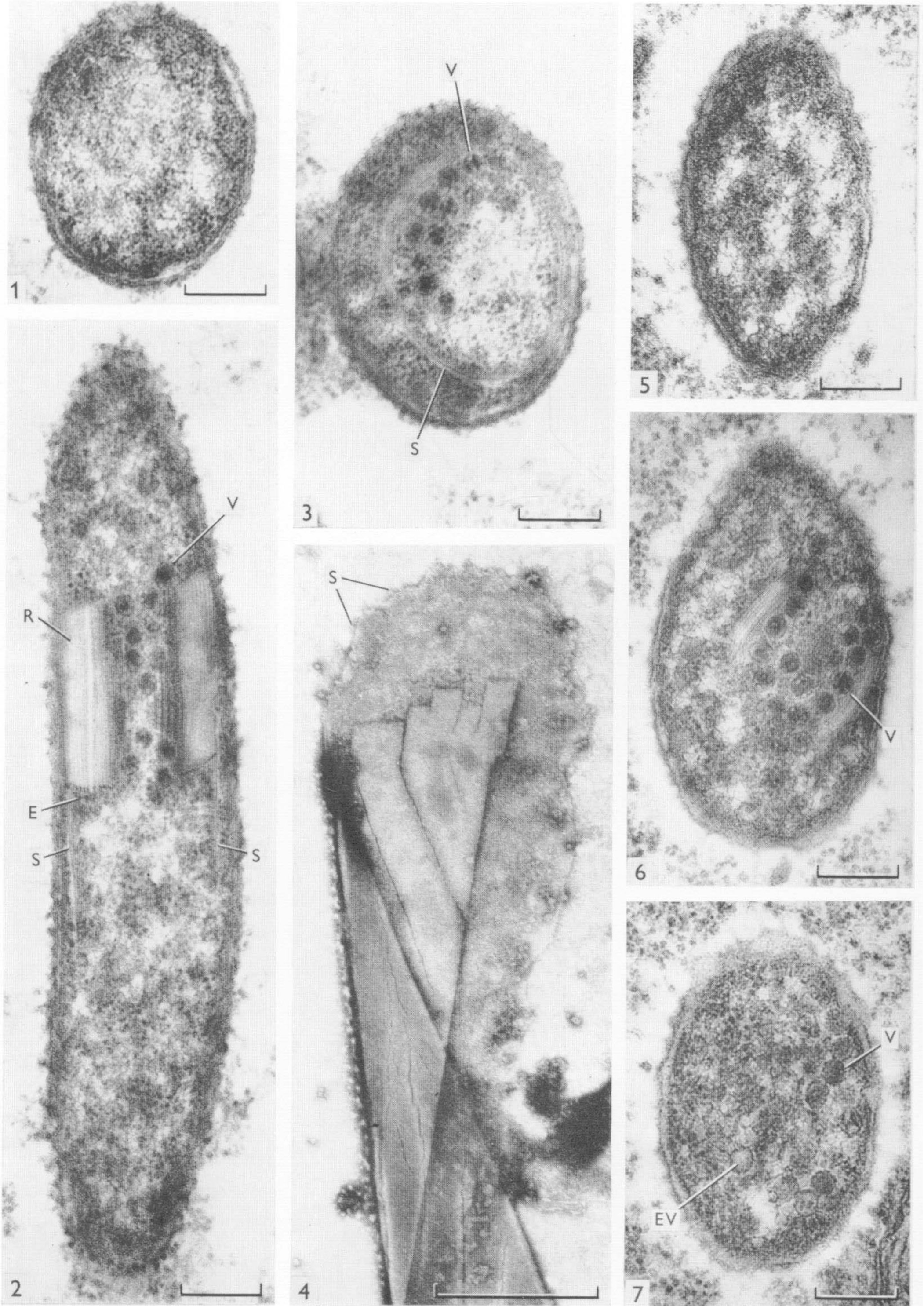

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judged by lack of turbidity in the fractions, the kappa particles were eluted by adding 300 ml of 0·5 M-NaCl in 0·005M-Na_2HPO_4 (pH 8·0). The turbid eluate was collected and concentrated by centrifugation. Free R bodies were obtained by lysing kappa particles with deoxycholate and paramecium extract as described earlier (Preer & Preer, 1967).

An American Optical Co. 'Bright Medium' oil-immersion phase objective was used for phase microscopy. Negative staining was carried out by adding a droplet of suspension to a grid which had been previously coated with Formvar and carbon. Another droplet of 5% phosphotungstic acid (previously adjusted to pH 7·3) was added to the grid and mixed. Excess fluid was then removed by touching a piece of filter paper to the mixed droplets. Electron microscopes were the Philips 200, AEI 6, and AEI 6B.

A number of preparations were studied in the electron microscope. For statistical purposes approximately 100 sections of kappa were chosen at random from each of three different preparations.

3. RESULTS

(i) *The presence of virus-like particles in sections*

In most of the sections lacking R bodies, no virus-like particles could be seen in either stock 7 (Pl. 1, fig. 1) or stock 562 (Pl. 1, fig. 5). In most of the sections in which R bodies could be seen, from 1 to 14 structures up to 900 Å in diameter were found both in stock 7 (Pl. 1, figs. 2 and 3) and in stock 562 (Pl. 1, fig. 6). The structures in stock 7 appeared to be identical to the virus-like particles described earlier (Preer & Preer, 1967) in negatively stained material. Negatively stained preparations of stock 562 revealed similar structures. The virus-like particles were concentrated inside the core of the R body and in the areas immediately adjacent to the core. A section with virus-like particles but without R bodies (stock 562) is shown in Pl. 1, fig. 7.

DESCRIPTION OF PLATE

All bars represent 0·2 μ. R = R body, V = virus-like particle, EV = empty virus-like particle, S = sheath, E = dark staining edges of R body.

Fig. 1. Section of kappa in stock 7 with neither virus-like particles nor R body. × 60000

Fig. 2. Longitudinal section of kappa in stock 7 showing virus-like particles, R body, and sheath. × 60000

Fig. 3. Cross-section of kappa in stock 7 through sheath, one corner of R body and virus-like particles. × 60000

Fig. 4. Outer end of an unrolled R body of stock 7 showing portions of broken sheath. Negative staining. × 120000

Fig. 5. Section of kappa in stock 562 with neither virus-like particles nor R body. × 60000

Fig. 6. Longitudinal section of kappa in stock 562 showing virus-like particles and R body. × 60000

Fig. 7. Section of kappa in stock 562 showing no R body, but virus-like particles, both filled and empty. × 60000

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(ii) *The distribution of virus-like particles among brights and non-brights*

Not in every instance could individual virus-like particles in the sections be identified with certainty. Nevertheless, different observers agreed sufficiently well to justify tabulating the estimates of numbers in randomly selected sections of three preparations (Table 1). The first two preparations were both of stock 7 and were identical except that they were taken at different times. It is noted that in preparation A, 12 of 13 sections which had a portion of an R body contained virus-like particles, most containing more than one. In the same preparation only 12 of 88 sections in which an R body was absent contained virus-like particles, most of the 12 sections having only one virus-like particle. The mean number of virus-like particles per section was accordingly much higher among the sections containing an R body (5·2 in preparation A) than among the sections lacking an R body (0·2 in the same preparation). It is noted that in preparation A, although only 13 % of the sections contained R bodies, an independent determination of the fraction of brights made from crushed animals using the phase-contrast microscope gave 25 % on the same preparation. As would be expected, some sections through brights evidently fail to include the R body. These same general relations were found in the other two preparations. Thus the correlation between the presence of R bodies and virus-like particles was very high in sections.

Although the correlation between *sections* which contained part of the R body and the presence of virus-like particles was high, it was not perfect. Nevertheless, the data are entirely consistent with an absolute correlation between *whole kappa particles* containing R bodies (brights) and virus-like bodies, as will now be shown.

If all brights contain virus-like particles, an occasional section containing an R body but lacking virus-like particles would be expected, for the number of virus-like particles is low and the section thickness is only about one-seventh the diameter of the bright. Virus-like particles were observed in all but three of 26 R body-containing sections. Therefore most, and probably all, brights contain virus-like particles.

If non-brights never have virus-like particles, sections lacking an R body but containing virus-like particles would still be expected because sections through brights may fail to include the R body. The expected frequency of sections which pass through brights but fail to include the R body may be estimated from Table 1 as the difference between the percentage of whole kappas with R bodies and the percentage of sections with R bodies, or 12 % for preparation A, 5 % for B and 17 % for C. When these percentages are multiplied by the total number of sections in each preparation one obtains the expected numbers of 12 for A, 5 for B and 17 for C. The observed numbers of sections without R bodies, but with virus-like bodies, were 12 for A, 2 for B and 11 for C. These numbers agree well with the expected, especially since it is likely that sections through brights which miss the R body also sometimes fail to include virus-like bodies. The data provide no evidence that kappas without R bodies have virus-like particles. If such kappas exist they must be rare. This conclusion is substantiated by observations of around

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Table 1. *The distribution of virus-like particles in random sections of kappa*

Preparation	R body in section	No. of virus-like particles in each section	No. of virus-like particles per section	Sections with virus-like particles	Totals: Sections with virus-like particles	Totals: Sections with R bodies	Totals: Whole kappa with R bodies
A. Stock 7	Present	0, 1, 2, 2, 2, 3, 4, 5, 8, 8, 9, 10, 14	68/13 = 5·2	12/13	24/101 = 24 %	13/101 = 13 %	24/97 = 25 %
	Absent	76 with 0, 1, 1, 1, 1, 1, 1, 1, 1, 2, 2, 2, 2	16/88 = 0·2	12/88			
B. Stock 7	Present	0, 0, 2, 3, 5, 5, 7, 11	33/8 = 4·1	6/8	8/95 = 8 %	8/95 = 8 %	67/500 = 13 %
	Absent	85 with 0, 1, 1	2/87 = 0·02	2/87			
C. Stock 562	Present	2, 5, 7, 8, 12	34/5 = 6·8	5/5	16/97 = 16 %	5/97 = 5 %	66/301 = 22 %
	Absent	81 with 0, 1, 2, 2, 2, 2, 2, 3, 5, 6, 11	39/92 = 0·4	11/92			

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20 sections of dividing kappa particles in preparation C. Although the sections passed near the centres of the kappa particles and should have revealed R bodies had they been there, no R bodies were seen; furthermore, no definite evidence of virus-like bodies was found in any of them.

(iii) *The frequency of 'empty' virus-like particles in intact brights*

It was possible to make a rough estimate of the number of virus-like particles in the whole kappa particles from the numbers in the sections. First, the total number of virus-like particles in the 101 random sections of preparation A was taken from Table 1 as 84. Since sectioning should often cut virus-like particles into more than one part, the counts should be biased by being too high. Therefore, it is convenient to think of, and estimate, the number of *centres* of virus-like particles in the 101 sections. The diameter of a virus-like particle and the section thickness are about the same (about 800 Å), so that a single virus-like particle could be sectioned into no more than two parts; therefore, the number of centres must lie between 84 and 42. A fairly good estimate can probably be obtained if it is assumed that a virus-like particle is divided into *two* recognizable parts only if a cut passes through the middle one-half of the diameter. Two recognizable parts should therefore be produced by about one-half the cuts and one recognizable part by the other cuts. It is easily, seen, then that one-third of the recognizable sections would not have centres and 84 should be decreased by one-third to yield 56 centres. We should like to divide the number of centres, 56, by the number of volumes of brights in the sections to obtain the number of virus-like particles per bright, assuming that all the virus-like particles are present in the brights. The total volume of kappa in the 101 sections was determined by multiplying the section thickness of 800 Å by the area (estimated from photographs); a value of $7{\cdot}56\,\mu^3$ was obtained. The fraction of the $7{\cdot}56\,\mu^3$ which was derived from brights is

$$\frac{0{\cdot}37\times 0{\cdot}25}{(0{\cdot}37\times 0{\cdot}25)+(0{\cdot}19\times 0{\cdot}75)} = 0{\cdot}39,$$

i.e. the average volume of brights estimated from phase microscopy at $0{\cdot}37\,\mu^3$ times the fraction of brights (found by observations on whole kappa particles of preparation A to be 24/97 or 25%) divided by the sum of the same product plus the product obtained by multiplying the average volume of a non-bright ($0{\cdot}19\,\mu^3$) times the fraction of non-brights (75%). The fraction 0·39 multiplied by the total volume of sections of kappa ($7{\cdot}56\mu^3$) gave $2{\cdot}95\mu^3$, the total volume of sections of brights. This volume divided by the volume of one bright represents 2·95/0·37 or eight brights, yielding 56/8 or seven virus-like particles per bright. A similar calculation for preparation B gave five virus-like particles per bright.

Negative staining of unrolled, isolated R bodies has revealed a small number of 'filled' virus-like particles and a larger number of 'empty' virus-like particles, along with numerous free capsomere-like structures. It has been suggested (Preer & Preer, 1967) that the filled particles consist of a thin envelope closely surrounding a protein coat made of the capsomere-like elements (and, if the structures are

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viruses, an inside core of nucleic acid). The empty forms appear to consist only of the envelope. Since the virus-like particles seen in the sections stain much darker than the surrounding areas of kappa it is reasonable that they represent the filled virus-like particles. This conclusion is supported by the finding of very pale, apparently empty, virus-like particles in certain sections (Pl. 1, fig. 7). If these are the empty particles, then it is evident that they are so difficult to see in sections that they can only be observed under unusually favourable circumstances.

When 34 unrolled R bodies from a portion of the culture used for preparation B were examined in negative staining it was found that the number of filled virus-like particles per R body ranged from 0 to 9 (mean 1·0) while the number of empty virus-like particles ranged from 0 to 32 (mean 10·9). The low number of filled virus-like particles found in negative staining (mean of 1·0) compared with the number of filled particles in sections of preparation B (computed above to be about 5) might be explained by the fact that during isolation and unrolling of R bodies many virus-like particles (both full and empty) fall free of the R bodies. It may also be that some of the filled particles became empty during isolation. Nevertheless, it is unlikely that most of the numerous unfilled particles found in negative staining (10·9) were derived by the emptying of full particles, for the estimated number of filled particles in sections was only 5. These relations suggest that most of the virus-like elements are never assembled in stock 7, being present only in the form of unfilled envelopes (which are difficult to see in sections), free capsomere-like structures, and, if they are viruses, as free nucleic acid.

(iv) *The sheath and the asymmetry of the R body*

Attention should also be drawn to the sheath-like extension found on one end of the R body of stock 7 (but not stock 562) (Pl. 1, figs. 2, 3). Once observed in sections, it proved easy to find in micrographs of negatively stained material (Pl. 1, fig. 4). It is visualized as a cylindrical jacket enclosing the R body and extending outward from one end.

A hitherto unknown characteristic of the R body of stock 7 is seen in longitudinal sections and reveals an apparent asymmetry in the edges of the R body on the end from which the sheath extends. The cut ends of the ribbons stain much darker on the end adjacent to the sheath (Pl. 1, fig. 2).

4. DISCUSSION

The data show the presence of the virus-like particles in most if not all kappa particles with R bodies (brights) and their absence in most if not all kappa particles without R bodies. Since the R body has already been shown to be uniquely correlated with the toxic activity of killers, the virus-like particles also are now seen to be correlated with killing activity.

The relation between brights and the virus-like particles is in agreement with the theory that the virus-like particles are viruses which are present in non-brights in a latent form. There presumably they often become spontaneously induced,

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producing viruses and resulting in the R body protein as well. Destruction of the reproductive capacity of the kappa particles in which the viruses develop must also occur, thereby explaining the fact that kappa particles containing R bodies cannot reproduce.

It is likely that many of the 'empty' virus-like particles on unrolled R bodies seen in negative staining are present in the intact kappa particles and do not arise by the emptying of filled virus-like particles during isolation of R bodies. Unfilled particles must exist in intact brights, for they can sometimes be seen in sections. Furthermore, the data suggest that the number of filled particles in sections is too low to be the source of the numerous unfilled particles seen in negative staining. If the virus-like particles are really viruses, then their developmental processes must be highly inefficient, numerous envelopes (empty virus-like particles) and capsomeres being formed but only a few ever being assembled into complete viruses. Such inefficiency may well mean degeneration and reflect a loss of the need for the viruses to retain their ability to infect, being transferred entirely by heredity. Induction may retain a selective advantage, however, in being necessary for the manifestation of the toxic activity, even though few fully mature forms are produced. In this respect the virus-like particles are suggestive of the bacteriocins (see discussion in Sonneborn, 1959) except that the latter are toxic to other bacteria rather than to cells of the same species in which the bacteria live, as in the case of the bacteria-like kappa particles and their toxin. The recent work on the toxin and inheritance in killer yeast by Woods & Bevan (1968) is also suggestive of similar elements.

The sheath which appears to enclose the R body of stock 7 is interesting from the point of view of the mechanism of the unrolling of the R body. If the sheath completely encloses the R body, as it appears to do, then it must be broken when unrolling occurs. It may be that the R body of stock 7 is always under tension, but is restrained from unrolling by the sheath. Spontaneous unrolling, which is frequently observed, would be due to spontaneous breakage of the sheath. The action of sodium dodecyl sulphate, which is known to unroll the R bodies of stock 7 (Preer & Preer, 1964), would operate by dissolving the sheath. The unrolling observed in the food vacuoles of sensitives (Preer & Preer, 1967) could be due to the action of digestive enzymes on the sheath. Unrolling of the R body of stock 7 should therefore not be reversed by removal of the conditions inducing unrolling, just as observed. The R bodies of stock 51, on the other hand, have no sheath as judged from published micrographs (Dippell, 1958; Rudenberg, 1962). It would appear that the tension conducive to unrolling in stock 51 is present only when the R bodies are at low pH, and results from a pH-dependent change in the molecular conformation of the protein of which the R body is made. As might be expected, it is reversible and independent of the agents inducing unrolling in stock 7. Nothing is known of the phenomenon of unrolling of the R bodies of stock 562 except that they do not seem to respond in the same way as the R bodies of stock 7.

The parallel between the R bodies and inclusions formed in association with cells infected with adenovirus (Morgan, Rose & Moore, 1957) has already been

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pointed out (Sonneborn, 1959; Preer & Preer, 1967). Recent observations on inclusions in plant cells infected with viruses are also interesting in this regard (see, for example, Wrischer, 1968). However, the pinwheel-like structures observed in plants are markedly different in form from the R body.

SUMMARY

Virus-like particles can be seen in sections of kappa of stocks 7 and 562. The virus-like particles are specifically associated with R bodies and are rare or absent in kappa particles lacking R bodies. This observation clearly links the virus-like particles with kappa's toxic activity; it also supports the suggestion that kappa is infected with a lysogenic virus whose induction results in the production of the R body and viruses. Many 'unfilled' virus-like particles are present in whole kappa particles. Wound R bodies of stock 7 are surrounded by a sheath, whose breakage may provide the mechanism which causes the unrolling of the R body. One edge of the R body ribbon stains more intensely than the other.

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