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Kappa and Other Endosymbionts in *Paramecium aurelia*

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INTRODUCTION

Nature and Significance

The evidence that the endosymbionts of *Paramecium aurelia* are gram-negative bacteria is now overwelming. There are many kinds found within paramecia, and at least two rather different bacterial groups appear to be represented. The size and shape of the endosymbionts is typical of bacteria. They lack mitochondria and

a nuclear membrane. Most are seen by electron microscopy to be surrounded by two double membranes. The inner is a typical plasma or unit membrane and the outer is thought to be the cell wall. They contain deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, and lipid in the expected proportions. The DNA has a unique base ratio different from that of the paramecium's nuclear and mitochondrial DNA. They contain ribosomal RNAs with sedimentation coefficients typical of bacteria and not of paramecia. Their ribosomal RNAs hybridize with the DNA of *Echerichia coli*, but not with the DNA of paramecia. The endosymbiont, mu, has been shown to possess a DNA-dependent RNA polymerase which is sensitive to rifampin and actinomycin D. Isolated kappa endosymbionts respire, can utilize glucose, and contain most of the enzymes of the glycolytic pathway; the pentose phosphate shunt and the citric acid cycle have been demonstrated. Kappa and mu have an electron transport system with cytochromes quite different from those of paramecia, yet virtually identical to those of certain bacteria. It has been reported that the endosymbionts lambda and mu can be cultured free of living paramecia, although at a very low growth rate and population density. Mu contains diaminopimelic acid and probably muramic acid as well. Typical bacterial flagella are found on the endosymbionts lambda, sigma, and delta. And finally, the most recent evidence indicates that kappa is infected with defective, lysogenic bacteriophages, at least one of which is inducible by ultraviolet light.

Most of the endosymbionts may also be recognized by the fact that they produce toxins which kill sensitive strains of paramecia. Paramecia bearing such endosymbionts are therefore called killers. The presence of the symbiont renders its host resistant to the toxin which it produces. The endosymbionts obviously profit from their association with their hosts, acquiring from them all necessary nutrients, a place of abode, and a buffer between them and the external environment. Although the ability to kill confers an advantage on paramecia in the laboratory and generally no harmful effects are observed, it is not certain whether the effects of the bacteria on the paramecia in nature are beneficial or not. Accordingly, the term "endosymbiont" is used here in its broadest sense: it indicates only that the two live together.

Had our knowledge of the endosymbionts started with cytological observations, it is likely that they would have been considered from the outset to be diverse species of bacteria, fastidious and difficult to culture free of their hosts. Surely all would have quickly been given binomial names as in other similar cases amongst various organisms. Instead they were postulated by Sonneborn in the early 1940s (123) as the genetic basis for the killer character. Because they were transmitted according to the rules of cytoplasmic inheritance, but originally not visualized cytologically, Sonneborn gave them Greek letters for names, kappa, mu, etc., as is the custom for genetic cytoplasmic factors. Even at this time, however, Altenburg (2a) suggests that kappa might, in fact, be a symbiont. It was not until the late 1940s that kappa was finally observed cytologically by Preer (85). There followed a long period of confusion concerning its nature and significance. In Sonneborn's review (130) in 1959 he concluded that kappa probably represents an intermediate between viruses and bacteria. Only recently have the many pieces of evidence cited above indicating the bacterial nature of kappa and the other endosymbionts become available.

Nevertheless, the corollary that the endosymbionts are "only bacteria" and therefore have nothing to do with cytoplasmic inheritance does not follow. Indeed, the impossibility of drawing a firm line between heredity and infection was seen and discussed by Darlington as early as 1944 (29). The early work on kappa must be credited with a major role in stimulating the discussions which led to our understanding of the matter. Later the work on lysogenic phages reinforced our view of the close relationship between heredity and infection. The matter was thoroughly discussed by Lederberg in 1952 (60). More recently it has become creditable that such indisputable examples of cell organelles as mitochondria and chloroplasts may also have originated as ancient free-living prokaryotes. In a recent review of cytoplasmic inheritance, Preer (90) concluded that a very large percentage of the known cases of cytoplasmic inheritance may, and in numerous cases in fact do, stem from symbiotic viruses and bacteria.

The endosymbionts of *P. aurelia* are easy to observe. Paramecia are collected from ponds and streams and brought into the laboratory. A few cells are placed on a microscope slide, fixed for a few seconds in osmic acid vapor, and treated, if needed, with a drop of acetone to remove lipid, and a drop of lacto-orcein stain and a Vaseline-ringed cover slip are added (16, 99). The preparation is then observed with a dark phase-contrast microscope. Many of the paramecia are found to contain dark intracellular symbionts the size and shape of bacteria which are located in the cytoplasm outside the

food vacuoles (Fig. 1). They number in the hundreds and sometimes even in the thousands per paramecium. At least one-half of the collections from many geographical locations contain high proportions of paramecia with such forms (99, 128). Laboratory conditions do not favor the maintenance of the endosymbionts in some stocks, and after paramecia have been cultured for a time, many strains lose their endosymbionts. In many others, however, the symbionts flourish and are maintained indefinitely. One of the most common reasons for loss is too rapid multiplication of the paramecia, resulting in a dilution of the number of endosymbionts and their eventual loss from many lines. But other factors as well are of importance. It should not be concluded that lines which lose their endosymbionts easily regain them, with endosymbionts readily shuffling in and out of different lines in a state of equilibrium. Although we have little information about what occurs in nature, the acquisition of new endosymbionts in laboratories generally occurs with great rarity and under highly artificial conditions. Moreover, the specific strains of paramecia are very restricted with respect to the specific strains of endosymbionts which they may harbor.

The endosymbionts of *P. aurelia* constitute well-integrated intracellular organisms producing phenotypic changes in their hosts. Most are transmitted only by cytoplasmic heredity and are normally capable of multiplication only within the cytoplasm of their hosts. It may be that the association between the endosymbionts and their hosts is only relatively recent. On the other hand, the numberous adaptations which will be reviewed in this paper make it entirely possible that the association is very ancient indeed, perhaps going all the way back to pre-Cambrian times. Kappa and the other endosymbionts of *P. aurelia* should certainly be considered bacteria, but they also function as elements of cytoplasmic inheritance.

Background

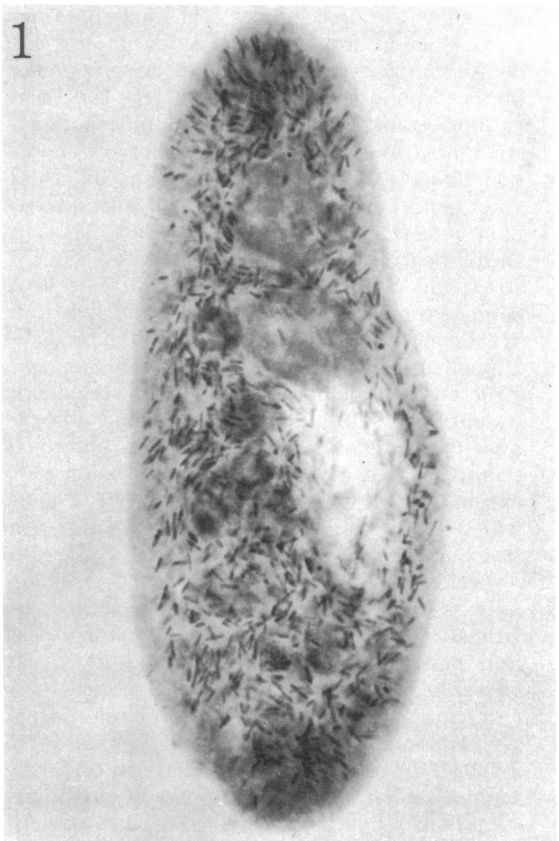
P. aurelia consists of 14 varieties or syngens, defined on the basis of the mating system. Mating types were discovered in *P. aurelia* by Sonneborn in 1938. Mating type I mates with type II and constitutes syngen 1; mating type III mates with type IV and constitutes syngen 2, and so on. Mating types and cytological processes in *P. aurelia* have been reviewed many times. See, for example, the reviews by J. Preer (88) and Beale (14).

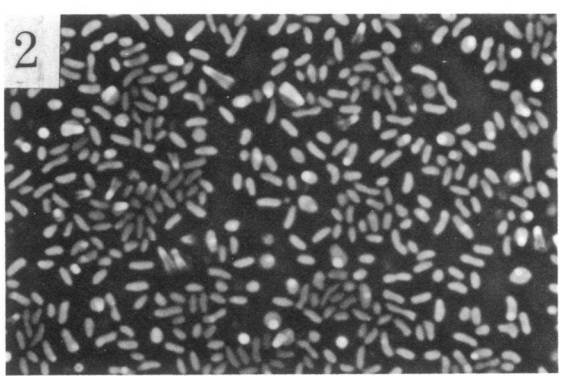
Most of the endosymbionts were discovered in naturally occurring stocks of *P. aurelia*. A stock represents the descendants of a single paramecium isolated from nature. A few of the endoysmbionts were discovered after some years in the laboratory as mutants. Here the term "strain" is used in a general way to designate a stock or a mutant, hybrid, or otherwise modified line of *P. aurelia*. ("Line" has its usual "dictionary" meaning, individuals with a common ancestor.) To illustrate, stock 51 is a kappa-bearing killer which originated from a stream in Spencer, Indiana. Strain 51.s is a kappa-free stain derived from stock 51. Strain 51m1 contains a mutant kappa isolated by R. V. Dippell from stock 51. Strain d4-186 was constructed in the laboratory of T. M. Sonneborn by inducing suitable conjugations and autogamies involving stocks 51 and 32; it is a derived strain of syngen 4 and is isogenic with stock 51, except that it is homozygous for the *k* gene which does not allow the maintenance of kappa (see below).

Because of the regular occurrence of autogamy, all stocks and strains are generally assumed to be homozygous. Data on the stocks and many of the strains considered here appear in Table 1.

Because a very extensive review of the endosymbionts was published by Sonneborn in 1959 (130), only work since that time will be emphasized in this review. Some of the points established by the early studies will now be summarized.

Killer paramecia liberate toxic particles into the medium in which they live. The toxic particles are generally harmless to their hosts, but cause the death of certain sensitive strains of paramecia. Killers and sensitives were discovered in 1938 by Sonneborn (122), at about the time of his discovery of mating types. The first killers were found in syngen 2, but the lack of suitable gene differences made it difficult to interpret the results of crosses designed to reveal the basis of inheritance of the killer trait. In 1943, however, Sonneborn discovered that strain 51 of syngen 4 was a killer. Furthermore, the necessary genic variability was found in syngen 4. Crosses between strain 51 killer and sensitives of different genotypes in syngen 4 showed clearly that the basis for the killing trait was a cytoplasmic factor (123). He called the cytoplasmic factor kappa. Kappa was perpetuated only in paramecia of genotyes *KK* and *Kk*, eventually being lost when *Kk* changed to *kk* as a result of the nuclear processes occurring during conjugation or autogamy. Thus the cross of *KK* with kappa $\times$ *kk* without kappa yields from each pair one F1 exconjugant clone *Kk* with kappa and the other exconjugant *Kk*





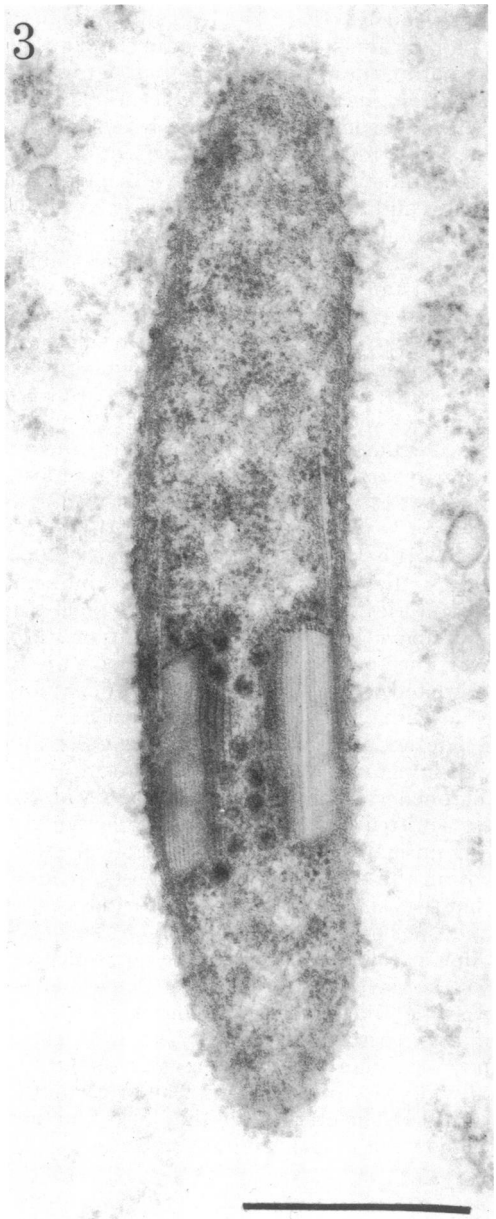


FIG. 1. *Osmium-lacto-orcein preparation of stock 239 (syngen 4), whole Paramecium containing lambda symbionts, the numerous dark-stained rods filling the cell; dark phase-contrast. ×500.*

FIG. 2. *Unfixed, purified preparation of stock 51 (syngen 4) kappa. The rods with uniform color are nonbright kappas; those containing a light spherical refractile body are bright kappas. Note in the upper center and middle left that some of the refractile bodies are very slightly unrolled due to cover slip pressure, taking a characteristic V shape; bright phase-contrast. ×1,650; photograph provided by C. Kung.*

FIG. 3. *Electron micrograph of stock 7 (syngen 2) Paramecium showing longitudinal section through a bright kappa. Note dark-staining spherical phage-like structures inside the coiled refractile body. Surrounding the refractile body and extending beyond it on either side is a fine membrane, the sheath. ×60,000, scale 0.5 μm; after Preer and Jurand (92) with the permission of Genet. Res.*

TABLE 1. *Some strains of Paramecium aurelia*[a]

Syngen	Stock or strain	Symbiont	Killing	Origin	Ref.
1	16	None	Standard sens for spin killing	Woodstock, Md.	53
	540	Mu	MK	Mexico	17
	548	Mu	MK	Los Angeles, Calif.	17
	551	Mu	MK	San Francisco, Calif.	17
	555	Mu	MK	Monterey, Calif.	17
	561	Delta	None	Pisa, Italy	17
2	7	Kappa	Spin	Pinehurst, N.C.	84
	7m1	Kappa	Par	7	84
	7m2	Kappa	Par	7m1	13
	8	Kappa	Vac	Halethorpe, Md.	84
	11	None	None	Baltimore, Md.	86
	36	Kappa	Spin, vac	Hamden, Conn.	84
	50	Kappa	Spin, vac	Oregon	84
	114	Sigma	Rapid lysis	Bloomington, Ind.	17
	197	Kappa	Spin	Germany	87
	249	Kappa	Spin	Florida	99
	308-2	Kappa	Vac	Jacksonville, Fla.	86
	310	Kappa	Vac, par	New Zealand	99
	511	Kappa	Vac	Figate, Edinburgh	99
	517	Kappa	Vac	Gif, France	99
	526	Kappa	Par	Genova, Italy	98
	527	Kappa	Par	Genova, Italy	98
	537	Kappa	Spin	Genova, Italy	98
	562	Kappa	Vac	Milan, Italy	17
		Alpha	None		98
	563	Kappa	Spin	Milan, Italy	98
	564	Kappa	Spin	Wexford, Ireland	98
	570	Mu	MK	Georgia, U.S.S.R.	17
	576	Kappa	Spin	Norwich, England	99
	1010	Nu	None	Tennessee	17
	1035	Delta	None	Sakhalin Island, U.S.S.R.	Collected by D.V. Ossipov
	1038	Kappa	Spin	Syktykar, U.S.S.R.	99
	1039	Kappa	Vac	Lomonosov, U.S.S.R.	99
	1041	Kappa	Vac, par	Leningrad, U.S.S.R.	99
	Bl 166-1	Kappa	Spin	Edinburgh, Scotland	99
	Hu35-1	Nu	None	Edinburgh, Scotland	17
	SG		Par	Pisa, Italy	78
3	92	None	Standard sens for rapid lysis killing	Philadelphia, Pa.	118
	152	None	General sens	New Haven, Conn.	99
4	32	None	None	Towson, Md.	122
	47	Kappa	Vac	Berkeley, Calif.	31
	47	Kappa	Hump	Mutant of 47 vac	31
	47	Kappa	Spin	Mutant of 47 hump killer	31
	51	Kappa	Hump	Spencer, Ind.	17
	51m1	Kappa	Spin	51	31
	51m5	Kappa	Weak hump	51	31
	51m42	Kappa	Weak hump	51	150
	51m7	Kappa	Hump, slow kappa growth	51	130, 48
	51m43	Kappa	Res non-killer	51m42	150
	51A2	Kappa	Res non-killer	51	71, 73
	51 pi	Pi	Sens non-killer	51, 51m1, 51m5	45, 96
	51r	Kappa	Hump (aureomycin resistant)	51	35

TABLE 1—*Continued*

Syngen	Stock or strain	Symbiont	Killing	Origin	Ref.
	116	Kappa	Hump	Bloomington, Ind.	99
	139	Kappa	Hump	Florida	45
	139 pi	Pi	Sens non-killer	Florida	45
	169	Kappa	Hump	Morioka City, Japan	62
	239	Lambda	Rapid lysis	Florida	17
	277	Kappa	Hump	Florida	130
	298	Kappa	Hump	Panama	99
	A1	Kappa Delta	Hump	Australia	137
	A2	Kappa Delta	Hump	Australia	137
	A4	Delta	Par	Australia	137
	d4-186	None	None	Iosgenic with 51, *kk* from 32	31
5	87	Nu	None	Philadelphia, Pa.	17
	314	Nu	None	Illinois	17
6	225	Delta	Par	Florida	17
8	31	None	Standard sens for hump killing	Baltimore, Md.	62
	130	Mu	MK	Ft. Lauderdale, Fla.	108
	131	Mu Delta	MK	Ft. Lauderdale, Fla.	108
	138	Mu	MK	Florida	108
	214	Gamma	Vac	Florida	17
	216	Lambda	Rapid lysis	Florida	17
	229	Lambda	Rapid lysis	Florida	17
	299	Lambda	Rapid lysis	Panama	17
	327	Lambda	Rapid lysis	DeLand, Fla.	Unpublished
	565	Gamma	Vac	Uganda	17
	Or11	Delta Mu or Nu		Orlando, Fla.	Unpublished
Unidentified	A7	Delta	Par	Australia	137
	A13	Delta	Par	Australia	137
	A18	Tau	Par	Australia	137
	A30	Kappa	Par hump	Australia	137
	A35	Kappa	Par hump	Australia	137
	A37	?	Par vac	Australia	137

[a] Hump, aboral hump-killing; MK, mate-killing; par, paralysis-killing; res, resistant to killing; sens, sensitive to killing; spin, spinning-killing; vac, vacuolization-killing.

without kappa. If autogamy is now induced in the first exconjugant clone, one-half of the resulting clones become *KK* with kappa and one-half become *kk* without kappa. For a detailed account of conjugation and autogamy see Beale (14). Sonneborn (123) found that the resistance of killers to the toxin which they produced is due to kappa, for kappa-free paramecia of gentoypes *KK*, *Kk*, and *kk* are all nonkiller and sensitive.

After these discoveries, genetic techniques continued to yield major advances in our understanding of the hypothetical, invisible kappa particle, the genetic basis for the killer trait. In 1946, Preer (84) found that rapid feeding made it possible to cause paramecia of syngen 2 to duplicate more rapidly than kappa, leading to its elimination in increasing proportions of lines of descent. A quantitative treatment of the kinetics of loss led to the estimates that the kappa of stock 7 of syngen 2 can multiply at a maximal rate of 2.0 duplications per day (whereas the paramecia undergo 3.3 fissions per day) and that the initial number of kappas in a strong killer is about 450. In 1948 Dippell showed that kappa is mutable (31). In 1950 Siegel discovered mate-killers which contain mu rather than kappa (108). Mate-killers cause their sensitive partners to die after conjugation, but unlike kappa liberate no toxin into the medium in which they live. In 1948 (127) Sonneborn showed that if kappa-free sensitives

of stock 51 are exposed to a concentrated homogenate of 51 killers, the sensitives can become infected with kappa and after a period of slow growth become killers.

Cytological investigations of kappa began with the finding by Preer in 1948 that kappa could be seen with a light microscope as a Feulgen positive cytoplasmic particle (86). Use of phase microscopy in 1953 (97) revealed that some kappas in every population contain a refractile or R body. Because the R body appears bright by bright phase-contrast microscopy, kappas bearing R bodies were called bright or B particles. Similarly kappas without R bodies became known as nonbright or N particles. In 1953 Preer et al. (96) showed that the toxic particles liberated into the medium are whole, bright kappa particles. Visualization of kappa by microscope led to cytological observations on mu and the discovery that mu lacks R bodies (96). Early work by electron microscope in 1958 by Hamilton and Gettner (44) and Dippell (32) revealed that the fine structure of kappa is clearly that of a prokaryote. R bodies were found to be lamellar in structure.

Studies on the biochemistry of killing and of kappa also began early (142, 147) and have since contributed much to our view of the bacterial nature of the endosymbionts.

Summaries of these matters appeared in a review of the genetics of *Paramecium* and *Euplotes* by Sonneborn in 1947 (125). In 1954 they were again reviewed, this time by Beale in his book on the genetics of *P. aurelia* (14). By far the most extensive review of kappa was by Sonneborn in 1959 (130). It consisted of 126 pages and, as indicated above, will be taken as the point of departure for this review. G. H. Ball in 1969 (10a) reviewed the endosymbionts of Protozoa. Three other reviews by the authors should also be cited, one on the genetics of Protozoa in 1968 (88), one on the morphology of *P. aurelia* in 1969 (54), and another in a review of cytoplasmic inheritance in 1971 (90).

GENERAL CHARACTERISTICS

Kappa and Pi

Kappa, the first symbiont discovered in *P. aurelia*, was recognized initially only by its killing action on sensitives. Kappa now is distinguished (17) from other particles which cause killing by the presence of minute inclusions about 0.5 μm in diameter. They are called refractile or R bodies. R bodies are easily observed by crushing a paramecium on a slide with a cover slip and by observing with a bright phase-contrast microscope (97). A purified preparation of 51 kappas seen in bright phase is shown in Fig. 2. Bright phase is preferable to dark phase, for the higher contrast generally obtained with dark phase leads to a phase reversal in the case of the highly refractile R body and results in a spurious image. Some ambiguity is associated with using the R body as the distinguishing criterion for kappa, for only a fraction (normally less than 50%) of the kappa in any kappa population are brights (i.e., have R bodies). Furthermore, the nonkiller pi endosymbionts, which very likely descended from kappa by mutation, have no R bodies. And, finally, a recently discovered mate-killing endosymbiont (the endosymbionts in mate-killers discussed below are generally called mu) in syngen 2 has R bodies (99). Nevertheless, the definition of kappa as those endosymbionts which either contain R bodies, or can produce endoysmbionts with R bodies, or were descended from endosymbionts with R bodies proves useful.

Sections of bright and nonbright kappas as seen by electron microscope are shown in Fig. 3 and 4. The nonbright kappa looks like a section of a gram-negative bacterium (17). An inner unit membrane is present and an outer cell wall serves to distinguish kappa from the mycoplasmas. No nuclear regions are seen, but difficulty in observing them is common among bacteria. A survey of the many kinds of kappa is given in a subsequent section.

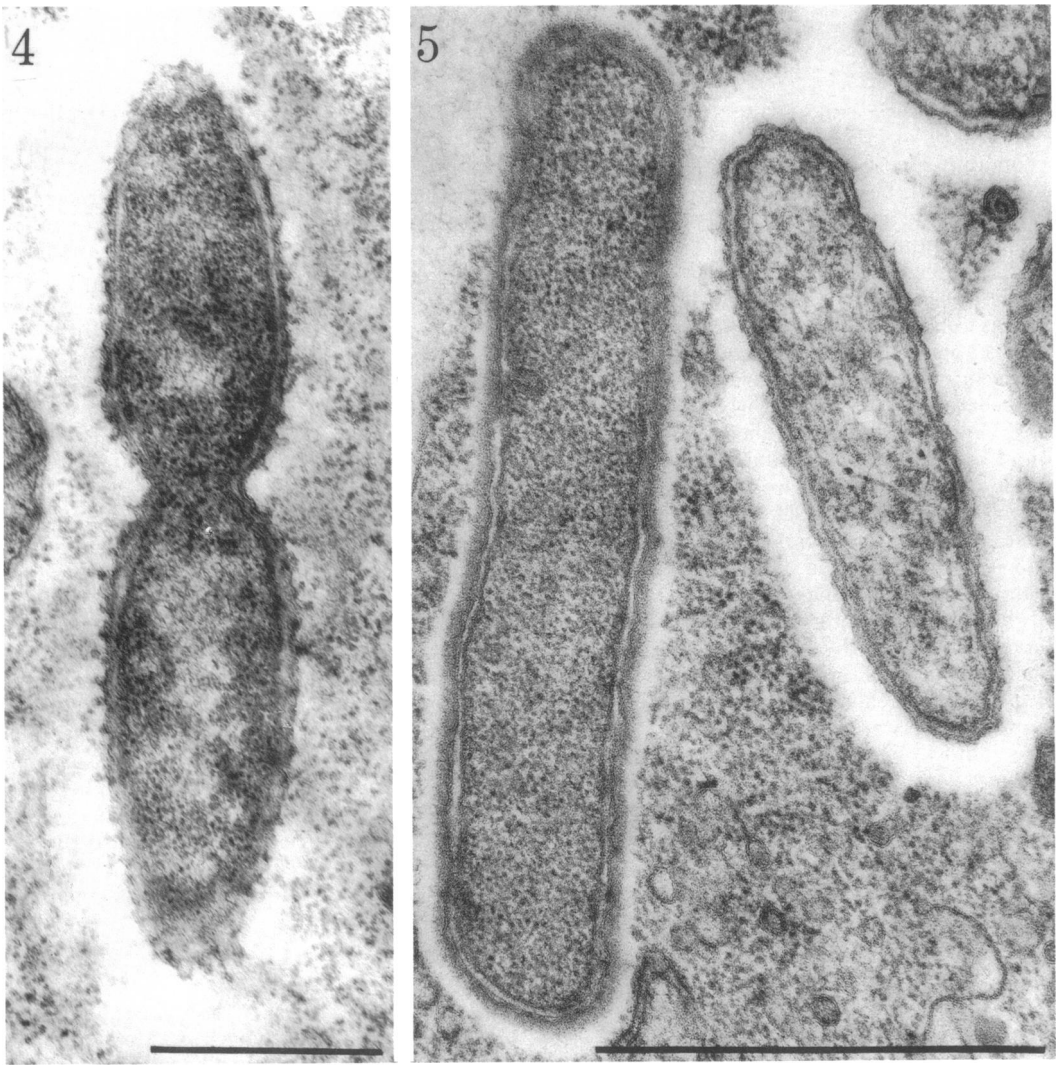
Mu

Mate-killers were discovered by Siegel in 1950 (108) in stock 138 of syngen 8. After conjugation, sensitive mates die or produce a limited number of progeny which die. No toxin can be detected in the medium in which mate-killers live, nor do homogenates of mate-killers kill. Cell-to-cell contact, but not nuclear transfer, is necessary for mate-killing. (Yet see the recent report of Williams [154] discussed in the section on host-free culture.)

Mate-killing is due to mu, which looks much like kappa without R bodies (15) (Fig. 5). R bodies are not associated with mate-killing, except in the syngen 2 mate-killer stock 570 (99) (Fig. 6). Mate-killers in other stocks of syngen 8 were studied by Levine (61). Several mate-killer stocks have been found by Beale and Jurand in syngen 1 (15, 16).

Lambda and Sigma

Strains of paramecia bearing lambda (Fig. 7) are rapid-lysis killers (134). Killing is generally observed within 30 min after exposure of sensitives to a killer culture, in contrast to an hour or even days for the other killers under similar



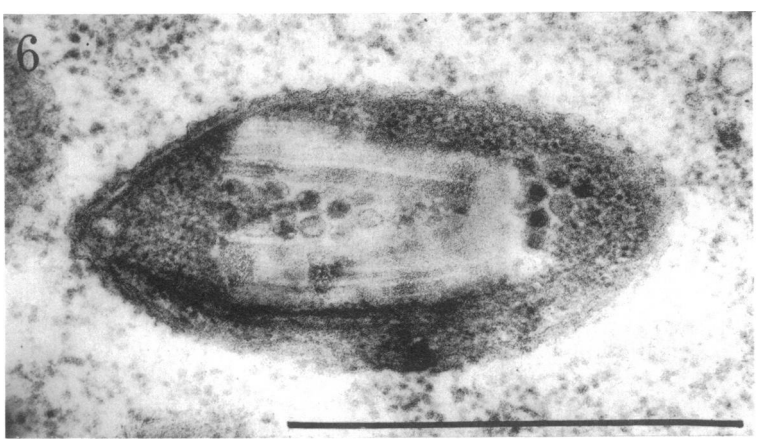
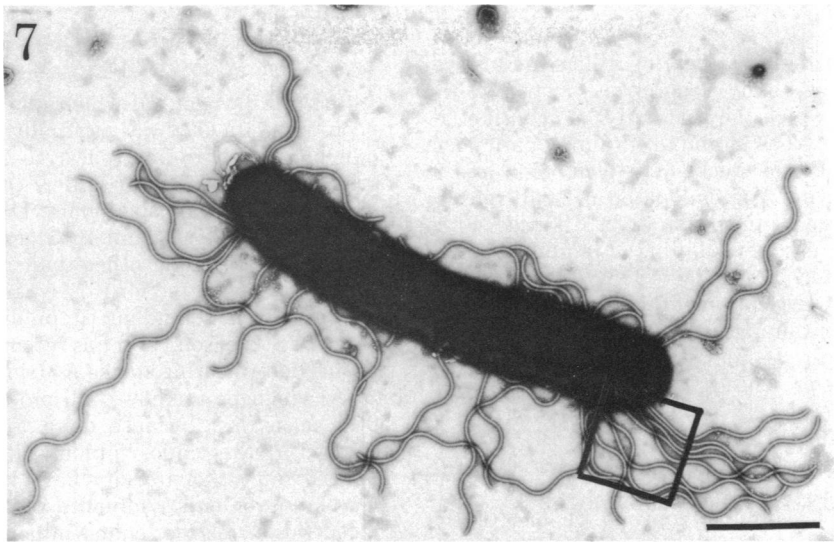


FIG. 4. *Electron micrograph of section of stock 7 (syngen 2) Paramecium showing nonbright kappa apparently dividing. ×60,000, scale 0.5 μm; after Beale et al. (17) with the permission of J. Cell Sci.*

FIG. 5. *Electron micrograph of section of stock 131 (syngen 8) Paramecium showing two endosymbionts in cytoplasm: left, delta; right, mu. Note electron-dense material surrounding outer membrane of delta. ×60,000, scale 1 μm.*

FIG. 6. *Electron micrograph of section of stock 570 (syngen 2) Paramecium, exceptional mate-killer bearing symbionts which contain a refractile body (in longitudinal section here) and spherical dark-stained phage-like structures. ×60,000, scale 1 μm; after Preer et al. (99) with permission of J. Cell Sci.*



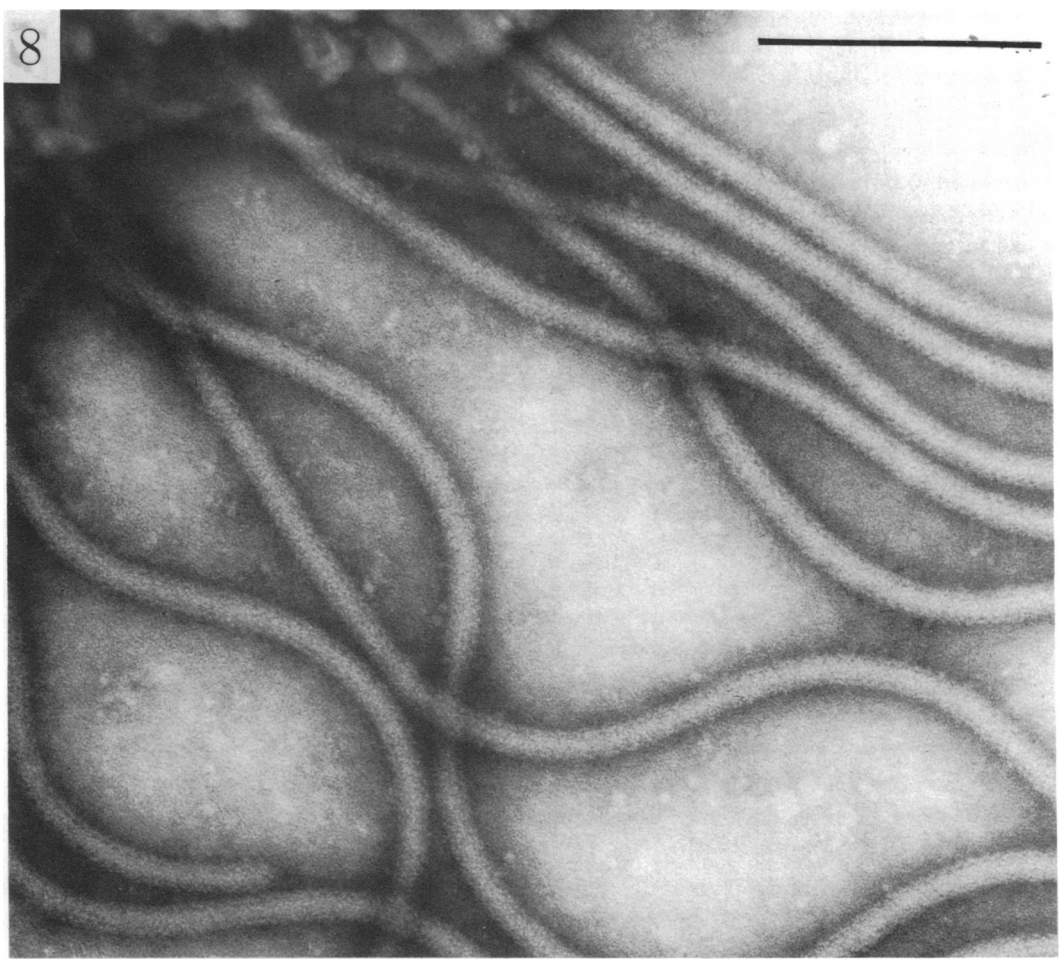


FIG. 7. *Electron micrograph of purified stock 327 lambda (syngen 8) with peritrichous flagella; negative staining was with phosphotungstic acid. ×15,000, scale 1 μm.*

FIG. 8. *Enlargement of flagella in area outlined in Fig. 7. Note periodicity in ultrastructure. ×150,000, scale 0.25 μm.*

conditions. Lambda-bearing killers are also reported to be highly specific with respect to the sensitives which they can kill. Sonneborn et al. (134) report that lambda-containing paramecia kill only certain stocks of syngens 3, 5, and 9. However, van Wagtendonk et al. (143) refer to lambda-free strains of stock 299 (syngen 8) as sensitives, and weak killing of lambda-free 299 was reported by Williams (154). Nevertheless, there is no description of the effect of lambda on lambda-containing controls which would indicate that the killing is specific. Until decisive evidence is presented, it must be concluded that lambda-free strains of lambda-bearing stocks are resistant.

Lambda is found only in syngens 4 and 8. Lambda is characterized by the fact that it is seen by phase microscopy to be one of the largest endosymbionts (commly 0.7 by 5 μm). Jurand and Preer (52) observed it by electron microscopy and found it to be covered in long, peritrichous flagella (Fig. 7 and 8).

Sigma (134) is similar to lambda in causing rapid-lysis killing, in the range of sensitives it affects, in its large size, and in being heavily flagellated (52). It has been found only in stock 114 of syngen 2. Unlike lambda, which is a straight rod, sigma (Fig. 9 and 10) is a long, broad, curved, or spiral rod and achieves the largest size (15 μm) of any of the endosymbionts.

Gamma

Killers containing gamma (Fig. 11 and 12) cause vacuolization of sensitives and are very strong killers (17). The endosymbionts are small, about 0.7 μm long, and often double. Note the contrast in size between gamma (Fig. 11) and sigma (Fig. 10). Sections under an electron microscope (Fig. 12) reveal that gamma is enclosed in an extra set of membranes, which appear to be continuous with the endoplasmic recticulum.

The two known strains both belong to syngen 8, one from Uganda and one from Florida.

Tau

The tau killer, stock A18 of unknown syngen, has been described by Stevenson (137). Stock A18 is characterized by him as a moderately effective paralysis killer. The culture of A18 in our laboratory shows numerous endosymbionts, but we have been unable to detect any killing activity. The endosymbionts show no marked distinguishing features with the exception of zones of lower electron density. Tau thus appears to be much like nu or mu.

Delta

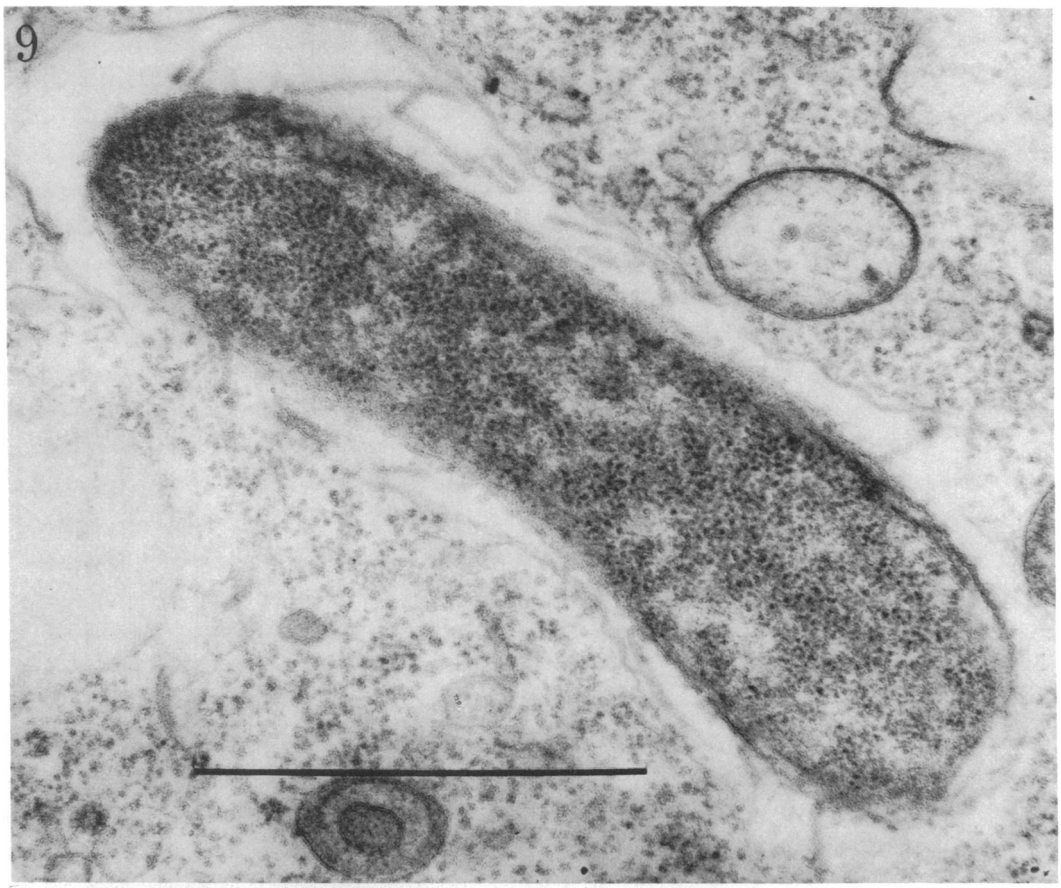
Stock 225 (syngen 6), which was later found to contain delta (17), was originally reported by Sonneborn (128) to be a paralysis killer. Presently stock 225 is not a killer, but contains numerous delta endosymbionts. Delta-bearing stocks 561 (syngen 1) and 1035 (syngen 2) are not killers. No killing other than mate-killing has ever been reported for 131 (syngen 8), which contains both delta and mu (unpublished data). However, Stevenson (137) has recently reported that his delta-bearing stocks A4 (syngen 4), A7, and A13 (syngen unknown) all produce paralysis of sensitives. Because delta is sometimes found with other endosymbionts, it is possible that the reports of toxicity have been due to other kinds of endosymbionts present in low concentration. A toxic endosymbiont originally present with delta in 225 could easily have been lost in the 10 years between the time it was first tested for toxicity and the time delta was identified in it. In any event, unless further evidence is obtained it must be concluded that there is considerable doubt as to whether delta itself produces any toxic effects. Again the necessity for proper controls must be emphasized, for any bacterium in sufficiently high concentration can cause the death of paramecia.

Delta (Fig. 5 and 13) is easily identified in sectioned material in an electron microscope by its thick cell wall (17), and is similar in appearance to gram-positive bacteria. However, delta, like all of the other endosymbionts, is gram negative. Delta is also sparsely flagellated, and a careful search of sectioned material always reveals a few peritrichous flagella. The flagella have not been found in negatively stained preparations. It was also found (17) that for a short period after paramecia containing delta of stock 225 are crushed onto a slide, the deltas could be seen by phase microscopy to swim about quite actively. Similar swimming has been observed within the cytoplasm of paramecia. It is interesting that the heavily flagellated lambda and sigma are never seen to swim.

Delta is the most widely distributed endosymbiont, having been found in all of the syngens known to bear symbionts (syngens 1, 2, 4, 5, 6, and 8), except for syngen 5. As indicated above, it is present in stock 131 along with mu. Note the two particles lying side by side in the cytoplasm in Fig. 5.

Nu

Nu (17, 49, 134) has no distinctive morphological features and appears by both light and



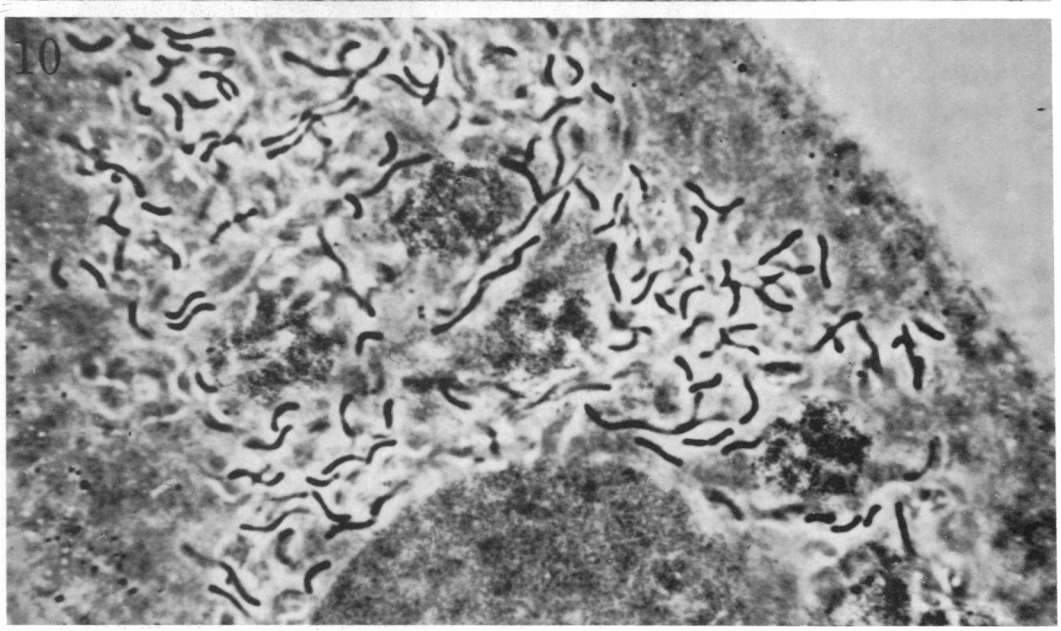


FIG. 9. *Electron micrograph of stock 114 (syngen 2) Paramecium showing section of sigma with peritrichous flagella. ×60,000, scale 1 μn ; after Beale et al. (17) with permission of J. Cell Sci.*

FIG. 10. *Osmium-lacto-orcein preparation of stock 114 (syngen 2) Paramecium containing sigma, numerous large, dark-stained helical forms; dark phase-contrast. ×1,200; after Beale et al. (17) with permission of J. Cell Sci.*

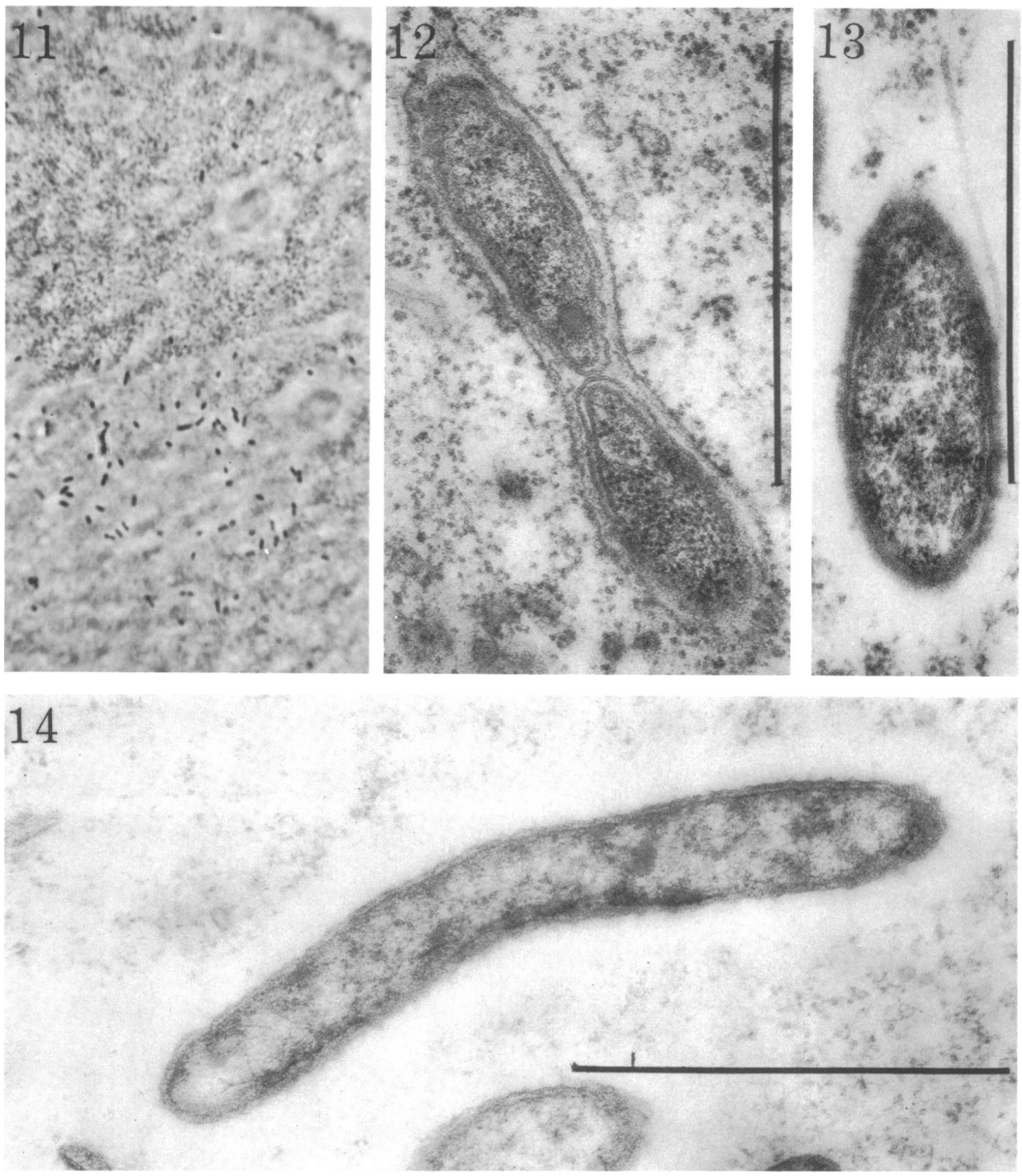


FIG. 11. *Osmium-lacto-orcein preparation of stock 214 (syngen 8) Paramecium bearing very small dark-staining symbionts, gamma; dark phase-contrast. ×1,200; after Beale et al. (17) with permission of J. Cell Sci.*

FIG. 12. *Electron micrograph of section of stock 565 (syngen 8) Paramecium showing gamma, apparently dividing. Note presence of additional outer membrane. ×60,000, scale 1 μm; after Beale et al. (17) with permission of J. Cell Sci.*

FIG. 13. *Electron micrograph of stock Or 11 (syngen 8) Paramecium showing delta with characteristic electron dense material surrounding it and to the right, a single flagellum. ×60,000, scale 1 μm.*

FIG. 14. *Electron micrograph of section of stock 1010 (syngen 2) Paramecium showing nu symbiont. ×60,000, scale 1 μm.*

electron microscopy to be much like mu (Fig. 14). Paramecia bearing nu, however, kill neither their mates nor other strains of paramecia placed into culture with them. They resemble pi, but differ from pi in not having been descended from kappa, in so far as we know. It is possible, however, that they represent evolutionary progenitors or descendants of kappa.

Nu was first reported in stocks 87 and 314 of syngen 5. Stock 87 was reported (49) to be more resistant to lambda-bearing rapid-lysis killers of stocks 214 and 299 than the corresponding strains which have been freed of nu. No such effects of nu on their hosts have been investigated for the nus in stocks 314 (syngen 5), 1010, and Hu 35-1 (syngen 2).

Alpha

Alpha is the only endosymbiont in *P. aurelia* which is found in the macronucleus (98). It occurs in great numbers there (Fig. 15), rarely in the cytoplasm, and is totally absent from the micronucleus. The one naturally occurring stock in which it is found (stock 562 of syngen 2) has alpha in the macronucleus and kappa in the cytoplasm and is a killer. Artifically produced, kappa-free, alpha-bearing strains, however, are not killers, and no effects of alpha on its host are known. Alpha occurs as long spirals approximately 5 μm long, or as much shorter, curved, and often double forms. The long, spiral forms of alpha are highly infective to a few alpha-free strains of syngen 2 of *P. aurelia*. Paramecia of such strains readily acquire alpha when cultured in the same vessel with alpha bearers. The smaller forms of alpha predominate when the paramecia are multiplying rapidly.

Alpha has a special problem in accommodating to the life cycle of *Paramecium*. During autogamy and conjugation the old macronucleus containing alpha breaks into fragments which are destined to be lost. New macronuclei are formed from the micronuclei, all of which are alpha-free. During conjugation spiral forms of alpha find their way from the old fragments into the newly developing macronuclear anlagen (Fig. 16). Somewhat later many of the smaller reproductive forms are seen in macronuclei (Fig. 17). It has been shown that many lines of paramecia may be freed of alpha by isolating paramecia from rapidly multiplying clones which have just undergone conjugation.

A micronuclear endosymbiont of *Paramecium caudatum* designated omega by Ossipov et al. (81a, 81b, 81c) has recently been described. It is very similar in appearance to alpha.

R BODIES AND PHAGE-LIKE STRUCTURES

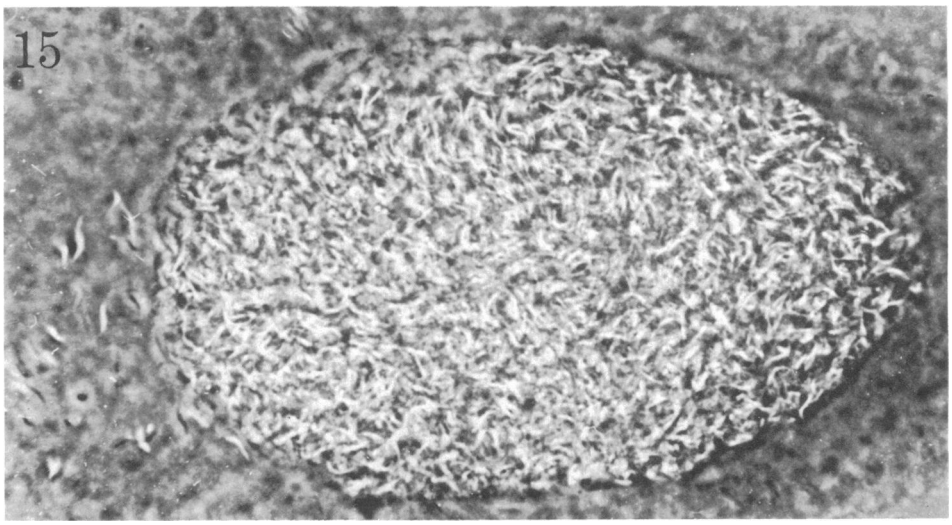
The proportion of endosymbionts bearing R bodies in each population of kappa varies with environmental conditions. Thus, Preer and Stark (97) reported that when stock 51 was cultured at a maximal fission rate, the brights (R body-containing kappas) dropped to about 1%, but when cultured at only 0.1 fission per day, the frequency rose to 21%. The frequency also varies with stock and also strain within stock. For example, high and low bright strains of 51 killers now being carried in our laboratory at about 0.5 fission per day show about 35 and 2% brights, respectively.

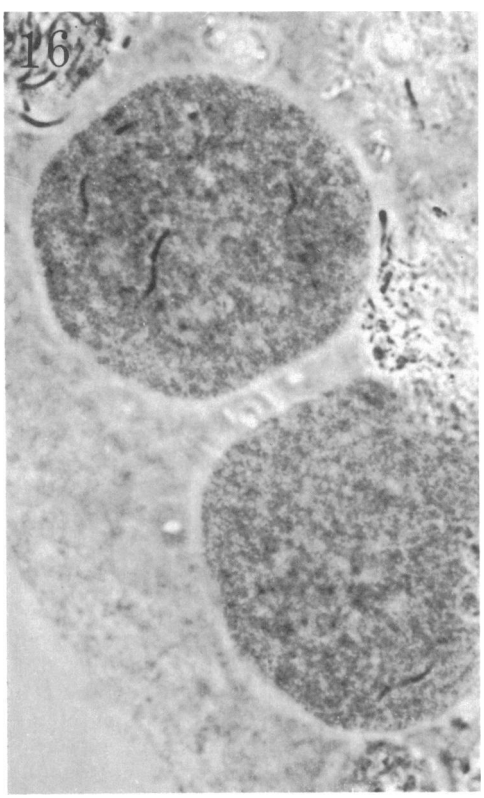
As already noted, the ability of a strain of particles to produce refractile or R bodies is used as a diagnostic criterion for kappa. This ability is generally held by all members of the kappa population, for when kappa is diluted to one endosymbiont per paramecium by rapid multiplication of paramecia and then is restored to the full complement of kappa by slow multiplication of paramecia, the kappa population in each paramecium again consists of both brights and nonbrights (97).

It has also been concluded that nonbrights can reproduce and brights cannot. Sonneborn (130) cites the following two lines of evidence. First, nonbrights are infective and brights are not. Second, double (presumably dividing) forms of nonbrights are often observed, whereas double forms of brights are very rare. Therefore, in a population of kappa, nonbrights multiply producing other nonbrights: occasionally a nonbright transforms into a bright with the production of an R body. Brights, however, are unable to reproduce.

The preceding considerations, taken with the fact that toxic activity is associated entirely with brights, led Sonneborn (130) and DeLamater (personal communication) independently to suggest many years ago that kappa is infected with a virus whose induction leads to production of R bodies and toxin. This conjecture, as we will now see, has been dramatically supported by a new line of investigation which stemmed from a remarkable observation by Mueller.

Mueller (69) observed that upon lowering the pH, isolated 51 R bodies suddenly transform into long filamentous structures, with a diameter just visible by phase contrast microscopy and a length of about 10 to 15 μm. Although R bodies had been observed to be laminate in structure in the early work by electron microscopy, it was not until later that a clear picture of its structure emerged. Anderson et al. (3) found that the R body of stock 7 is a ribbon about 13 nm thick, 0.5 μm wide and 10 to 15 μm long. In its normal compact form, it is wound into a tight roll of ribbon consisting of about 10 turns. A compact R body of stock 511 (which is similar to stock 7) is shown in Fig. 18. The roll is about 0.5 μm in diameter. The inner end of the ribbon forms an acute angle, and the outer end is irregular or blunt in form (91), as seen in the unrolled R body of stock 1039 (also similar to 7)





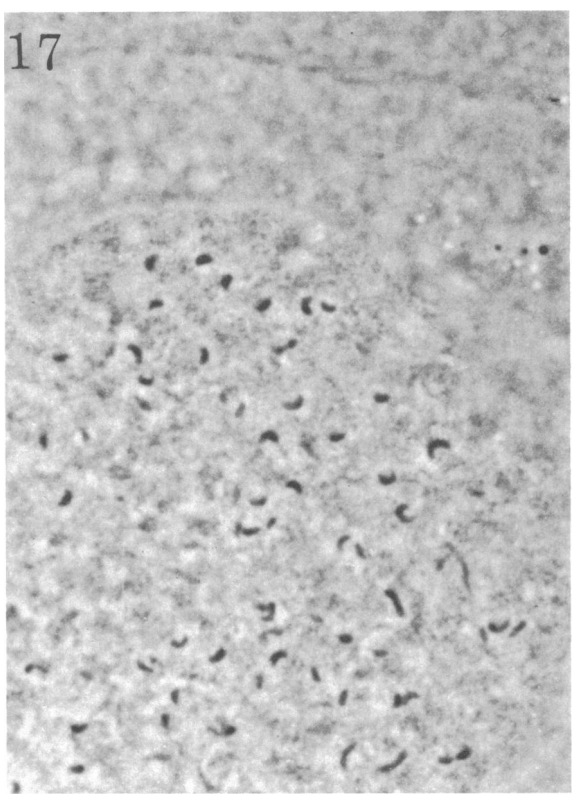


FIG. 15. *Osmium-lacto-orcein preparation of vegetative macronucleus of stock 562 (syngen 2) Paramecium containing alpha and appearing as abundant bright spiral rods; bright phase-contrast. ×1,000; after Preer (98) with permission of J. Protozool.*

FIG. 16. and 17. *Osmium-lacto-orcein preparation of stock 562 (syngen 2) Paramecium. Figure 16, the two large macronuclear anlagen at autogamy containing a few dark-stained spiral forms of alpha. Figure 17, vegetative macronucleus of fast-growing paramecium containing dark-stained short, rod and crescent forms of alpha; dark phase-contrast. ×1,500; after Preer (98) with permission of J. Protozool.*

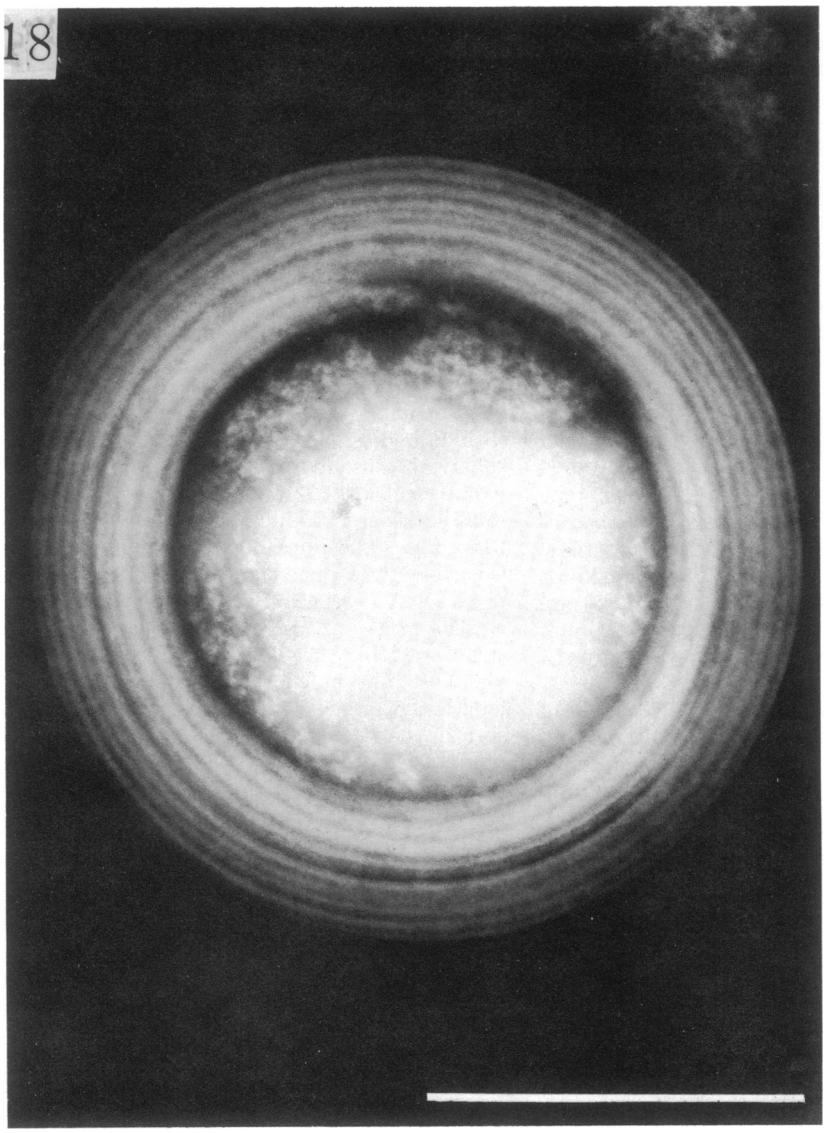
in Fig. 19. Upon heating the rolled ribbon to about 60 C or treating it with sodium dodecyl sulfate or phosphotungstate, it suddenly and irreversibly unrolls into a long, twisted filament. Unrolling is always from the outside end (Fig. 20). Stock 51 R bodies were found to be similar, but with a few conspicuous differences (91). Both inner and outer ends of the ribbon of 51 R bodies form an acute angle. The ribbon unrolls only in response to lowering the pH below 6 and does so reversibly. When the pH is raised above 7, rerolling occurs. Unrolling, unlike in stock 7, is from the inside (Fig. 21) and produces a very tightly twisted ribbon formed into a longer hollow tube (Fig. 22). When the coils are just beginning to loosen in R bodies which unroll from the inside, the characteristic V form shown in Fig. 23 is seen. These two kinds of R bodies exemplified by stocks 7 and 51 are characteristic of numerous other stocks, and variations in their structure are discussed in the next section.

Preer and Preer (94) isolated the R bodies of stock 7 in a relatively pure form and analyzed them. They found protein in their preparations, but only traces of lipid, carbohydrate, and nucleic acid. They estimated about 4×10^{-14} g of protein per R body. R bodies are extremely insoluble and could only be solubilized in 70% formic acid. In agreement with the conclusion that they contain mainly protein, their density was estimated by density gradient centrifugation to be about 1.3 (95). The rather uniform and geometrical shape of their ends and thinness (around 13 nm) suggest that they may be made up of only one, or at most a few, kind(s) of subunits.

The R body is a curious and almost unique biological structure. The most nearly comparable structures seem to be the trichocysts of the green algal genus *Pyramimonas* (66), which bear a remarkable resemblance to R bodies. Somewhat similar structures have been found in flagellated Protozoa such as *Chilomonas paramecium*, where they are also called trichocysts. (See review by Hovasse et al. [50].) In *Pyramimonas* each cell contains up to four trichocysts. Each consists of a rolled ribbon about 0.5 μm wide, 35 μm long, and 5 nm thick, wound into a compact roll of 15 to 25 turns. Discharge or unrolling also occurs and is like that of the 51 R body, producing, by unwinding from the inside, a long hollow tube with the angular tip of the ribbon on the end. The difficulty in imagining how convergent evolution could have produced such bizarre and similar structures in widely different organisms is only matched by the problem of imagining how an apparently virus-induced bacterial structure (see below) can be phylogenetically related to an algal structure. No vestiges of bacterial or virus structures are found in *Pyramimonas;* its trichocysts appear to be normal cell organelles. R bodies have also been compared with certain structures found in cells infected with viruses (67, 156).

Preer and Preer (94) also reported the presence of virus-like or phage-like structures visible in an electron microscope and associated with R bodies. Stocks like 7 and 562 contain spherical (icosohedral) phages, often hexagonal in outline, 50 to 120 nm in diameter (Fig. 24). In some killer stocks, such as 562, tail-like structures are sometimes observed (99) (Fig. 25). Occasionally a fine strand of material thought to be DNA is seen issuing from the tail region, as seen in Fig. 26. Stock 51 shows no spherical structures, but has instead helical structures about 18 nm in width and of indeterminate length. The stock 51-type helical structures are seen in the micrographs of a negatively stained preparation of stock 298 (Fig. 27). They are seen rarely in sectioned material (Fig. 28). Originally (94), they were postulated to be helical viruses, but it is now felt that the structure of their rather loose helices is just as consistent with the view that they represent incomplete or abortive assemblies of phage-like elements whose original form might even have been spherical.

Evidence that the spherical forms are phages has been considerably strengthened by their isolation from 562 kappa (Fig. 29) by Preer et al. (95) using isopycnic centrifugation in CsCl gradients. The structures were found to have a density of about 1.45 and to contain about 1.6×10^{-16} g of DNA and 2.0×10^{-16} g of protein per particle. The density of their DNA is estimated in CsCl gradient centrifugation to be 1.700, whereas that of whole 562 kappa DNA is 1.702. (These revised values are based on new work and are slightly less than those originally reported.) The small difference (0.0015) was found to be reproducible. This fact plus asymmetry in the peak of the whole kappa DNA which can be accounted for by the presence of the lower density of the DNA of the phage-like particles suggests that the small difference is significant. The amount of DNA per particle is large and easily enough for a large phage. T2, for example, has about 2.0×10^{-16} g. Because all efforts at infection of the phage-like forms has failed and because only "empty" structures are found almost exclusively in stock 1039 (see Table 2), it is concluded that the phage-like



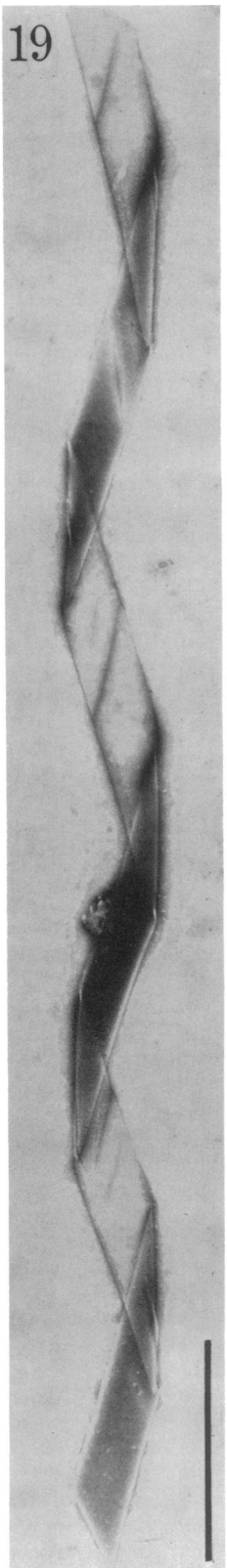


FIG. 18. *Electron micrograph of stock 511 (syngen 2) intact, coiled refractile body of kappa; phosphotungstic acid.* ×*200,000, scale 0.25 μm.*

FIG. 19. *Electron micrograph of stock 1039 (syngen 2) unrolled refractile body of kappa. Note that end at bottom terminates in an acute angle, whereas end at top is blunt and irregular; phosphotungstic acid.* ×*30,000, scale 1 μm.*

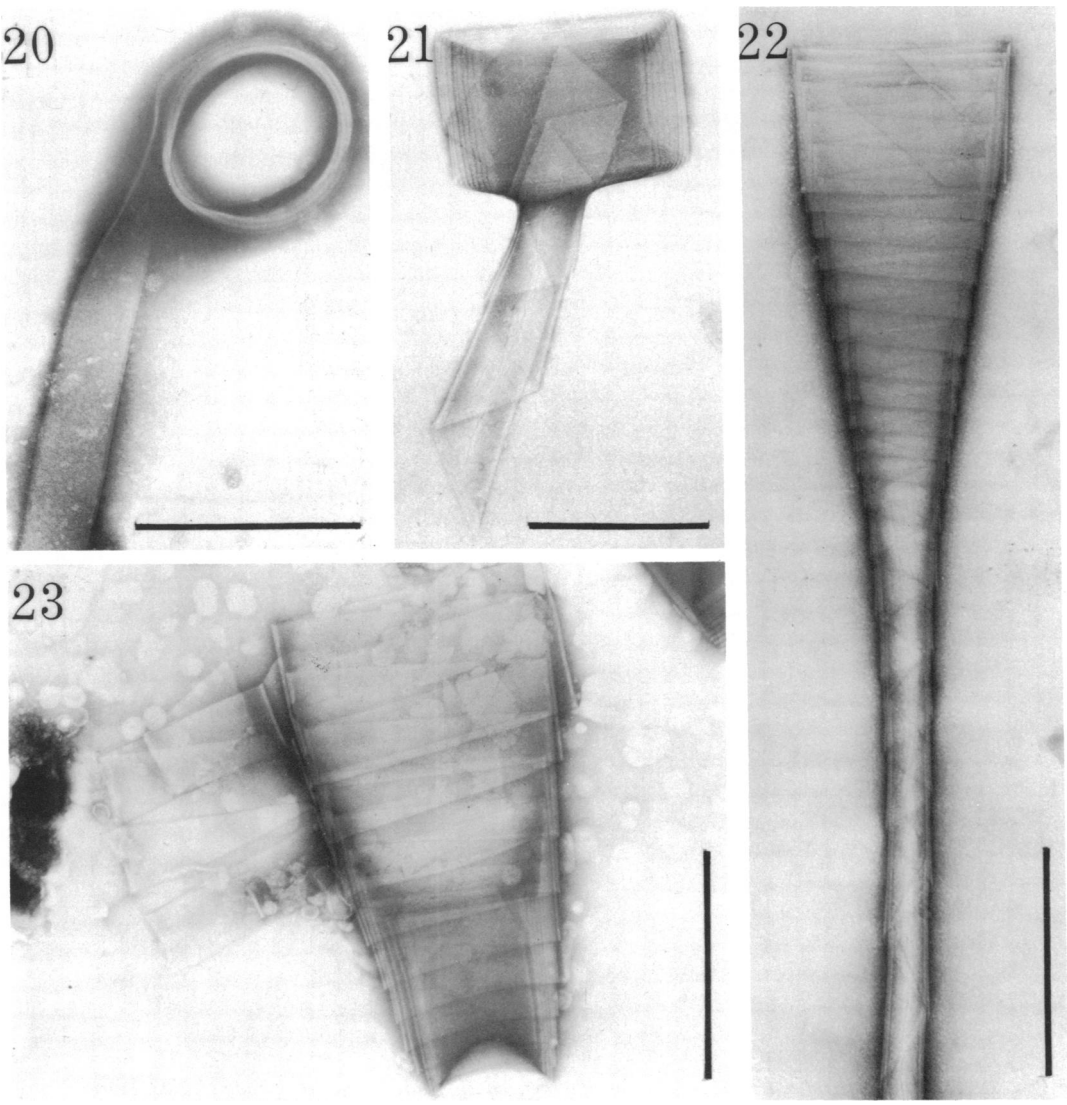


FIG. 20. *Electron micrograph of stock 51ml (syngen 4) isolated refractile body showing 7 type refractile body ribbon unrolling from the outside; phosphotungstic acid. ×60,000, scale 0.5 μm; after Preer et al. (99) with permission of J. Cell Sci.*

FIG. 21, 22, and 23. *Electron micrographs of stock 51 (syngen 4) isolated refractile bodies. Figure 21, ribbon unrolling from the inside; phosphotungstic acid ×48,000, scale 0.5 μm; after Preer et al. (99) with permission of J. Cell Sci. Figure 22, ribbon almost completely unrolled from the inside, forming a straight hollow tube. Figure 23, ribbon as it begins to unroll from the inside, and a loosening of the outer coils occurs. Figures 22 and 23, phosphotungstic acid, ×30,000, scale 1 μm; after Preer et al. (91) with permission of J. Ultrastruct. Res.*

structures are defective phages.

The first observations of the defective phages were made on unrolled R bodies (94). It was noted at that time that one could account for all of the known facts if it were assumed that kappa is infected with phage which is present in a prophage form in nonbrights and that occasionally it spontaneously undergoes induction and begins its lytic cycle, during which defective phages and also the R body are synthesized. With these syntheses, toxicity for sensitive paramecia is also produced. Preer and Jurand (92) then set out to study the correlation between the defective phages and R bodies. They found that the defective phages of stocks 7 and 562 could be observed in sections with an electron microscope. A statistical study showed that the defective phages are specifically associ-

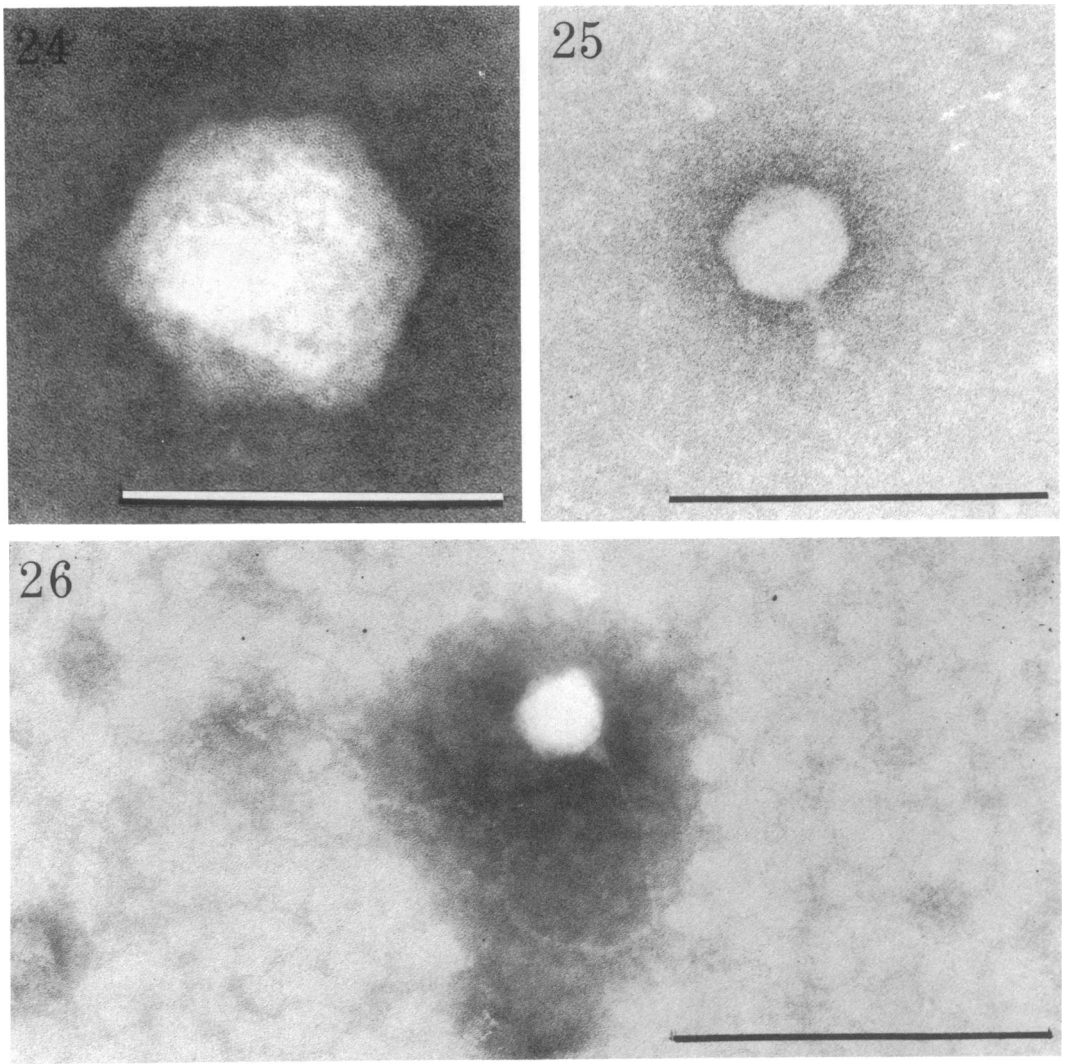


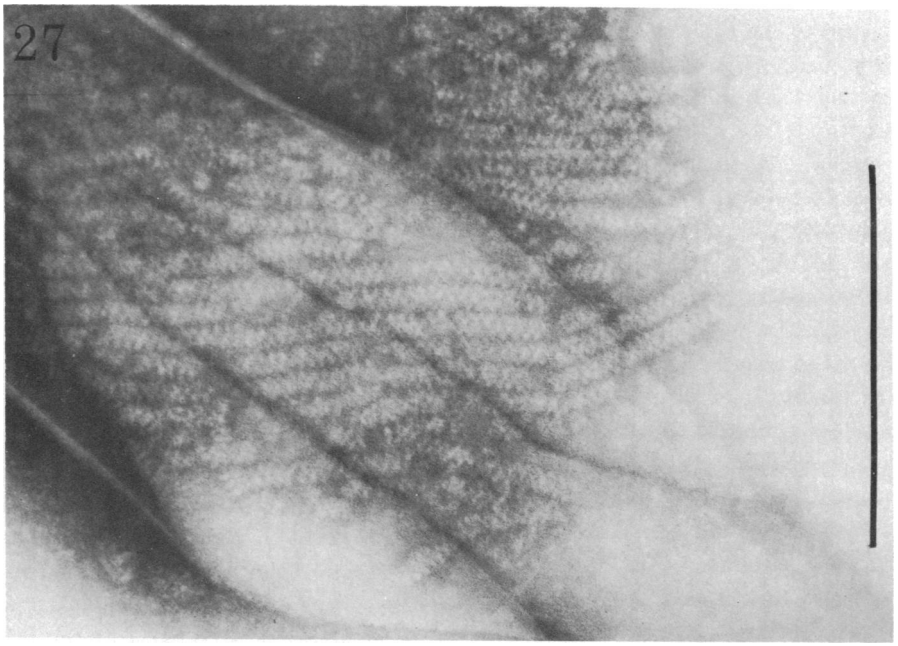
FIG. 24 and 25. *Electron micrograph of stock 562 (syngen 2) isolated spherical phage-like structure. Figure 24, hexagonal outline; phosphotungstic acid. ×500,000, scale 0.1 μm. Figure 25, tail-like appendage, phosphotungstic acid. ×200,000, scale 0.25 μm. After Preer et al. (99) with permission of J. Cell Sci.*

FIG. 26. *Electron micrograph of stock 1041 (syngen 2) phage-like structure appearing in medium showing fine strand of material, presumably DNA, issuing from tail region; phosphotungstic acid. ×200,000, scale 0.25 μm; after Preer et al. (99) with permission of J. Cell Sci.*

ated with brights and are rare or absent in nonbrights. These observations were later confirmed by observations with an electron microscope by Grimes and Preer (42) by using serial sections of 562 kappa. All kappa particles with R bodies had defective phages, whereas only 3 of 78 kappas without R bodies had defective phages. Whether the three exceptions represented relatively early stages in the developmental processes or whether the formation of defective phages and R bodies may be occasionally uncoupled is unknown.

The close relationship between the presence of the spherical defective phages and the R body is further suggested by their topographical proximity. Although the defective phages are often found scattered about the kappa, they are much more frequently found localized in or near the central core of the R body (92).

One other group of structures seen on the R bodies of some stocks may represent elements of incompletely assembled phages. They are capsomere-like elements about 10 nm in diameter which often appear to be hexagonal, pentagon-



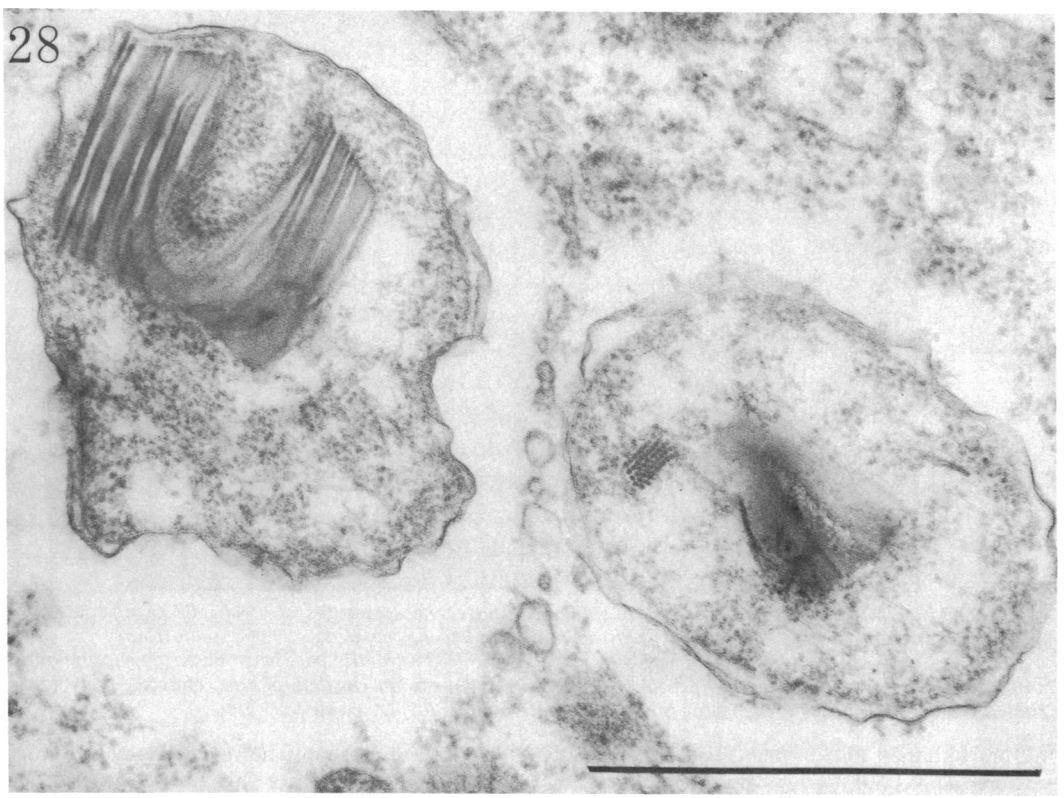
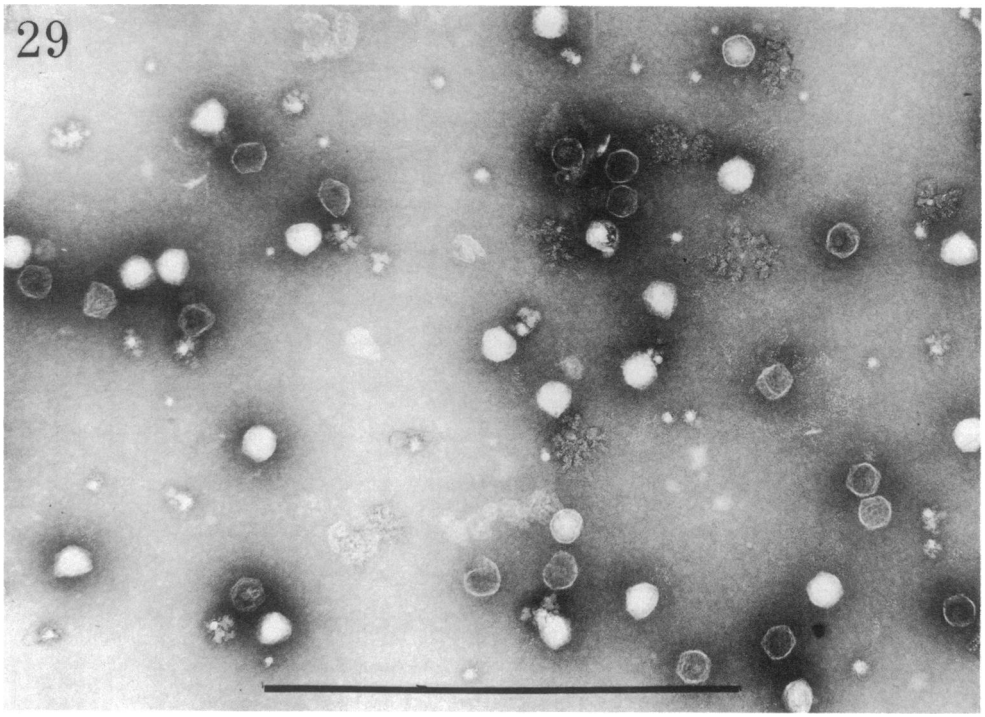


FIG. 27. *Electron micrograph of masses of stock 298 (syngen 4) helical phage-like structures associated with the unrolled refractile body; phosphotungstic acid. ×200,000, scale 0.25 μm; after Preer et al. (99) with permission of J. Cell Sci.*

FIG. 28. *Electron micrograph of section of stock 116 (syngen 4) Paramecium showing two kappas. Note several rows of small spherical structures near grazing section of refractible body in kappa on right, probably helical phage-like structures seen on end. ×60,000, scale 1 μm; after Preer et al. (99) with permission of J. Cell Sci.*



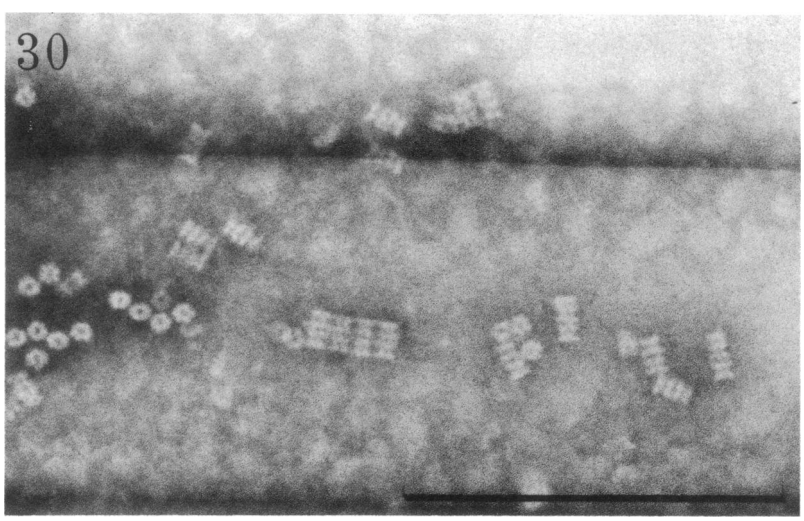


FIG. 29. *Electron micrograph of isolated spherical phage-like structures of stock 562 (syngen 2) kappa purified by cesium chloride isopycnic centrifugation; phosphotungstic acid. ×63,000, scale 1 μm.*

FIG. 30. *Electron micrograph of stock 570 (syngen 2) unrolled refractile body with adhering capsomere-like structures oriented to appear as hexagonal or pentagonal units on the left of the refractile body and as H-shaped, stacked units on the right; phosphotungstic acid. ×200,000, scale 0.25 μm.*

al, or H-shaped (94) in negative-stained preparations (Fig. 30). They are often extremely numerous and are found to cover the inside ends of the R bodies. It was originally thought that the defective phages might consist of two layers, an outer smooth envelope and an inner coat assembled from the capsomeres. Subsequent electron microscopy, however, has not produced additional support for this view. It may be that the outer envelope itself when unassembled may appear as capsomere-like elements. It may also be that they represent incompletely assembled subunits of tails or R body ribbons, or even perhaps only large protein molecules important

in the synthesis of R bodies or phages. The notion that the R body protein itself is related to the protein of the defective phages, or at least specified by their DNA, is an interesting possibility, but only speculation at the present time.

The helical forms found in stock 51 kappa have not been isolated. However, Dilts (30) has recently studied the DNA of stock 51 kappa. Electron microscopy reveals that most of the DNA isolated from stock 51 kappa and separated on ethidium bromide-cesium chloride gradients consists of linear fragments of variable length. A significant fraction, however, consists of covalently linked closed circles of DNA approximately 20 μm in length (about 0.6×10^{-16} g per circle). Dilts estimates the DNA to be about 0.002 less than that of 51 kappa (see Table 4). Preliminary evidence shows an association between this circular DNA and R bodies. This DNA, then, presumably is the counterpart of the DNA of the spherical defective phages.

Recently, additional support for the view that kappa contains a defective phage has been found with the discovery that R bodies may be induced in 51 kappa by ultraviolet light (101). A few hours after exposure to ultraviolet light, the frequency of brights goes up and the frequency of nonbrights goes down. Eventually the frequency of brights also goes down, and all the kappas are lysed. The sequence of events is much like the induction of certain colicins or temperate phages by ultraviolet light.

Thus, it is now certain that the bacterium kappa contains DNA which can reproduce within kappa, possibly integrated into the bacterial chromosome, possibly not, without killing kappa. Furthermore, it can become induced and in a lethal synthesis kill its host, kappa, and produce microscopically observable forms which appear to be defective phages. An association between these elements and R bodies and toxin production is well established for the spherical phage-like forms and likely for the helical forms. It has been proposed by Dilts (personal communication) that the spherical defective phages in stocks 7, 562, etc. be designated Sp 7, Sp 562, etc. He 51, He 1038, etc. designate the helical structures and their determinants in stocks 51, 1038, etc.

KINDS OF KAPPA

Kappas are the commonest of all the endosymbionts, and collections of hundreds of

TABLE 2. *Classes of kappa*[a]

Class	Strain	R body			Phage-like structures		Capsomeres	Killing	Activity isolated R body	Isolation technique
		Syngen	Type	Sheath	Kind	Sticky				
51	51	4	51[b]	–	Hel	–	–	Hump	–	ECTEOLA wash[c]
	116	4	51	–	Hel	–	–	Hump	–	ECTEOLA wash
	298	4	51	–	Hel	–	–	Hump	–	ECTEOLA wash
7	7	2	7	+	Sph	+	++	Spin	+++	Filter-paper eluate
	576	2	7	+	Sph	+	++	Spin	+++	Filter-paper eluate
	Bl166-1	2	7	+	Sph	+	++	Spin	++	Filter-paper eluate
	249	2	7	+	Sph	+	++	Spin	++	Filter-paper eluate
	1041	2	7	+	Sph	+	+++	Vac, par		Filter-paper eluate
	310	2	7	+	Sph	+	+	Vac, par	++	Filter-paper eluate
	1039[d]	2	7	+	Sph	+	+++	Vac	+++	Filter-paper eluate
562	562	2	7	–	Sph	–	–	Vac	+	ECTEOLA wash
	517	2	7	–	Sph	–	–	Vac	–	ECTEOLA wash
	511	2	7	–	Sph	–	–	Vac	–	ECTEOLA wash
1038	1038	2	7	+	Hel	+	+	Spin	+	Filter-paper eluate
51m1	51m1	4	7	–	Sph	+	+++	Spin	–	ECTEOLA wash
570	570	2	7	–	Sph	+	–	MK	–	ECTEOLA eluate

[a] From Preer et al. (99), with permission of the Journal of Cell Science. Hel, helical; hump, aboral humps; MK, mate-killer; par, paralysis; sph, spherical; spin, spinning; vac, vacuolization. The + and – indicate presence or absence, and the number of + signs indicate a rough measure of quantity.

[b] 51-Type R bodies unwind from inside, are induced to unroll and reroll by lowering and raising pH, form a tight tube when unrolled, and the outside end of the roll forms an acute angle. 7-type R bodies unwind from outside, are unaffected by pH, form a loose twisted ribbon when unrolled, and outer end of roll is blunt or irregular in shape. Some R of 116 and 298 appear to be of the 7 type and have spherical phages and no capsomeres.

[c] Some symbionts can be isolated by the fact that they, unlike all other cellular organelles, fail to stock to ECTEOLA. Others stick to filter paper or ECTEOLA and can be eluted at high salt concentration.

[d] Very few phage-like structures and almost all empty.

stocks of kappa-bearing paramecia throughout the world have produced numerous variants. They are distinguished by the characteristic, prelethal effects they produce in sensitives, by the form and properties of their R bodies, and by their phage-like and other inclusions.

It can be seen from Table 1 that kappa has been found only in syngens 2 and 4. It is common in these two syngens, especially in syngen 2.

The kinds of kappa were recently surveyed by Preer et al. (99). Table 2 shows the three major (7, 51, and 562) and three minor (51 ml, 570, and 1038) classes of kappa which they found, along with attributes of representative strains. The three minor classes are represented only by single strains.

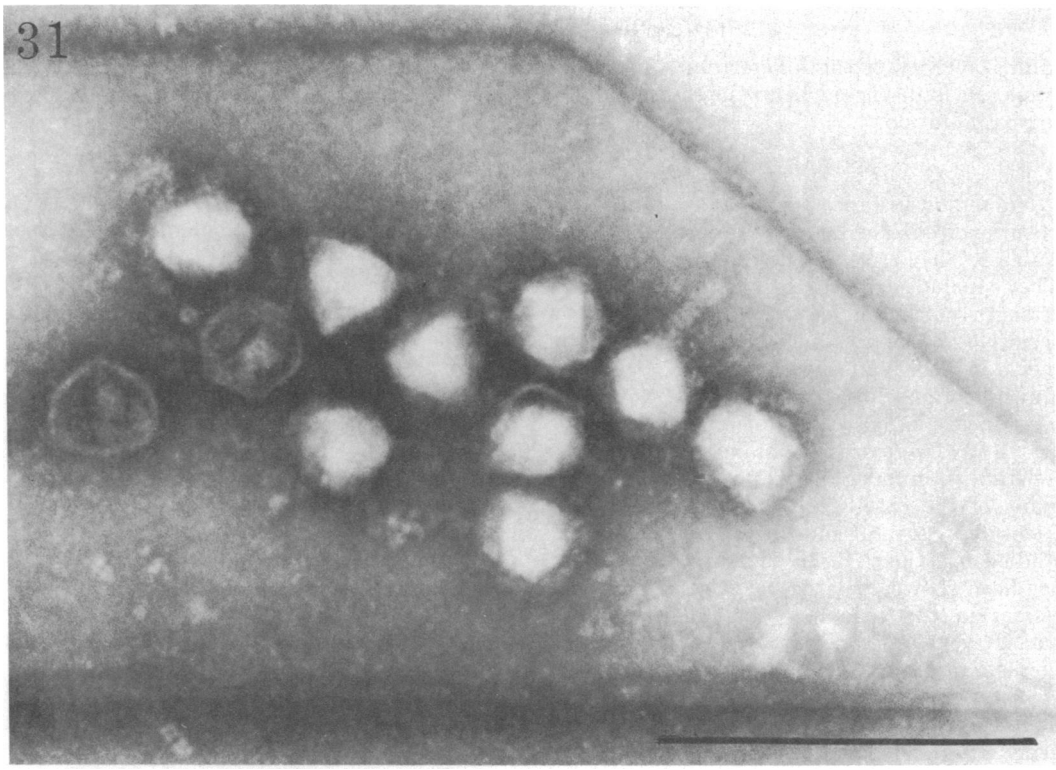
The 51 class forms a homogeneous group of stocks, all within syngen 4, all with 51-type R bodies. They are distributed like syngen 4, usually in the warmer regions of the world. Collections have been made in Indiana, Florida, Japan, and Australia. Their R bodies are highly distinctive, unrolling from the inside of the roll to form a tight narrow tube (Fig. 22). They unroll below pH 6.5 and reroll when the pH is raised above pH 6.5 (91). Both ends of the ribbon end in an acute angle. The rolled R bodies contain no enclosing membranous sheath like that found in the 7-type R bodies described below. No spherical defective phages are found, but helical structures (Fig. 27 and 28) about 18 nm wide and of varying length are seen. The known killers all induce postoral humps in sensitives prior to death of the sensitives. The 51-type kappas do not adsorb to epichlorohydrin triethanolamine cellulose (ECTEOLA) column. Because other large particles in a homogenate of paramecium do adsorb (trichocysts, mitochondria, etc.), a ready method of isolating this class of kappa is provided (112). The isolated kappas produce the normal killing action exhibited by the whole paramecia on sensitive strains of paramecia. Disruption of the kappas with ultrasonic treatment leaves free R bodies, but all toxic action is lost (100).

The 7 group of kappas is by far the commonest and most varied. It is generally found like its host, syngen 2, in the somewhat cooler regions of the world, collections coming from North Carolina, Scotland, France, Germany, and northern and central Russia. R bodies are of the 7 type, i.e., they unroll irreversibly with heat and certain chemical treatments (see section on R bodies) from the outside (see Fig. 20); pH does not affect their unrolling. They form a loose, twisted ribbon; the outer end of the roll is blunt or irregular, and the inner is acute, as shown in Fig. 19. Spherical, defective phages stick to the isolated unrolled ribbons (Fig. 31) and capsomere-like structures are also found to be associated with the ribbons (Fig. 30). A membranous sheath, shown in Fig. 3, encloses the rolled R body. Several kinds of prelethal effects are produced in sensitives by different strains of killers: spinning, vacuolization, paralysis, and combinations of these. Isolated R bodies are moderately to very strongly active in producing killing in sensitives. The kappas adsorb irreversibly to ECTEOLA, but can be isolated by adsorbing them to a filter paper column at low ionic strength and eluting them at high ionic strength.

The 562 class of kappas are represented by single stocks from Edinburgh, Scotland, France, and Italy. They have 7-type R bodies, but no sheath. Their defective phages are spherical. They are numerous and do not stick to the R body ribbon, facilitating isolation of the defective phages (see Fig. 32). No capsomeres are found. They are all vacuolizers. Disruption of kappa by ultrasonic treatment leads to almost complete inactivation of the toxic activity. They fail to stick to ECTEOLA and thus can be purified like kappas of the 51 group.

The remaining three strains show marked differences from each other and from the other groups. Strain 1038 of syngen 2 is from Siberia. It is remarkable in having all the properties of 7, except that it has helical rather than spherical defective phages. Strain 51 ml is a syngen 4 killer, regarded for many years as a mutant of 51 (31). It has many of the properties of the 7 group (7-type R bodies, spherical phage-like structures, capsomeres, and spinning action on sensitives), yet others of the 51 group (inactive isolated R bodies, and failure to adsorb to ECTEOLA). Its origin will be considered in the section on kappa mutation. Finally, 570, from Georgia in southern Russia, is a syngen 2 matekiller with endosymbionts containing R bodies. These endosymbionts with R bodies are the only ones known which, when present in the medium, have no effect on sensitive strains. Killing occurs only at conjugation. The endosymbionts are much like those of the 562 group, with spherical phage-like structures but no capsomeres. Unlike all of the other kappas, it adsorbs to ECTEOLA but can be purified by elution with high-ionic-strength buffer.

Pi has arisen in several stocks with concomitant loss of R bodies and the ability to kill. Paramecia with pi are also sensitive to the



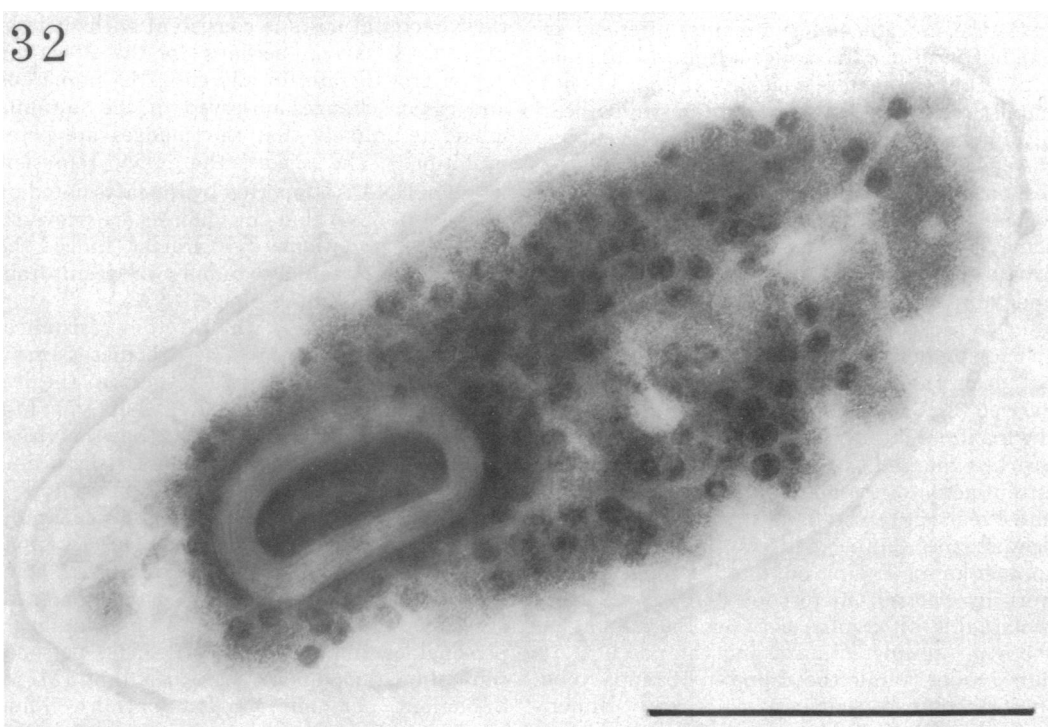


FIG. 31. *Electron micrograph of stock 249 (syngen 2) unrolled refractile body with spherical phage-like structures adhering to it; phosphotungstic acid. ×200,000, scale 0.25 μm; after Preer et al. (99) with permission of J. Cell Sci.*

FIG. 32. *Electron micrograph of stock 562 isolated whole kappa pretreated with 2% sodium deoxycholate before staining with phosphotungstic acid. Note spherical phage-like structures outside refractile body coil; phosphotungstic acid. ×51,000, scale 1 μm; after Preer et al. (99) with permission of J. Cell Sci.*

killing activity of the killers from which they come. Pi is discussed below in the section on kappa mutation.

MUTATION

Numerous instances of killer mutants have been reported. The known cases are recorded in Table 1. They are only known for kappa killers. They include changes in the nature and strength of the prelethal effects produced in sensitives, the resistance imparted to paramecia by the endosymbionts they bear, the multiplication rate of kappa, and antibiotic resistance of kappa. All are spontaneous, except that X rays were used in obtaining the mutants resistant to aureomycin. Dippell (31) analyzed many of the cases genetically, and in subsequent years several additional cases have been studied by others. In all such cases but one it has been shown that the basis for the changes resides in the genome of kappa, not in the nuclear genes of *Paramecium*. The one exception is a mutation of *K* to *k* studied by Hanson in 1956 (48). Sonneborn (130) concludes that all such changes affecting the properties of kappa likely represent mutations of kappa.

It has long been known that in several of the cases the mutant endosymbionts differ not in one but in numerous respects from the original type. Thus, pi bearers no longer kill, pi does not impart resistance to the paramecia which bear it, pi is longer and more slender than 51 kappa, the capacity to produce R bodies is lost, and the response to temperature, starvation, and serological properties may be modified (45, 48). Still some properties do not change when pi is produced. Some sera still agglutinate both pi and kappa (110), and pi and kappa respond alike to the *K* and *k* alleles.

The spinner mutants of 51 also differ markedly from the hump killers from which they are derived (31). The two killers kill each other, and the maximal multiplication rate of the kappa in 51m1 is markedly reduced (31). Furthermore, an antigenic difference was reported by Siegel and Preer (110) who found that a serum made against the spinner killer would agglutinate spinner kappa and pi, but not 51 kappa. Recent work by electron microscopy (99) has revealed remarkable differences between the kappas in the 51m1 spinner mutant and the parental 51 hump killer. While the kappa in 51 tends to be short and broad, that in 51m1 is more slender, much like 51 pi. Furthermore, the R bodies in 51m1 are of the 7 type, totally unlike those of 51. Most surprising, however, is the fact that 51m1 kappa has spherical, defective phages associated with its R bodies, rather than the helical elements found in the kappa of 51 hump killers. The major point of similarity still remaining is the fact that both kinds of kappa disappear in the presence of the *kk* genotype and survive in the presence of *KK* (31). But work on DNA hybridization (see below) indicates a very close relationship.

Widmayer (152) studied mutant 51m42, which is a weaker killer than 51. She concludes that the change was accompanied by a reduction in the size and capacity of the R body to unroll. The paralysis mutant 7m1 also appears to have smaller R bodies (97).

What can be concluded about the nature of the killer mutants? It has generally been thought that they represent mutations in the genome of kappa. Now that we know that kappa contains phage-like DNA, we must consider the possibility that the differences may lie within the DNA of the defective phages. If the mutations do represent changes in the defective phages, then major changes in toxicity, paramecium resistance, R body, phage, kappa morphology, antigenic properties, and growth rate of kappa are influenced by the phages. Our knowledge of the effects of temperate phages on their bacterial hosts is consistent with such an hypothesis, except, perhaps, for the effects on kappa growth rate. In any case, the numerous and major changes observed in the mutants makes it unlikely that the changes are point mutations. The theory that they represent losses in DNA is supported by the facts noted by Sonneborn (130) that the changes are irreversible and degradational in character. Indeed the mutation from kappa to pi may well result from a complete loss of the phage DNA.

The mutant killers which retain resistance, but no longer kill or produce R bodies (51m43, 51A2), are interesting with respect to the hypothesis that mutation usually results from loss of phage DNA. If it is assumed that the virus-like DNA is completely missing in these mutants, resulting in a loss of the capacity to kill and produce R bodies, then resistance would have to be imparted by the genome of kappa. This assumption is probably not required, however, for it now appears that 51m43 can on rare occasions still produce R bodies (D. Widmayer, personal communication; S. Pollack, personal communication) and even weak killing (M. V. Schneller, personal communication). Thus 51m43 and 51A2 could represent changes in the DNA of the phage-like elements which led to loss of R body and diminished toxin production rather than complete elimination of the DNA.

Still another possible explanation for the origin of at least some of the mutant killers seems to have been largely overlooked, namely that killers may have contained originally more than one kind of kappa. For example, could it not be that the original 51 killer contained mostly 51 hump kappa, but contained also a few particles of spinner kappa and, in fact, all of the other mutant types as well? It might be argued that one would have to postulate the initial presence of an implausibly large number of types of kappa in both the original 51 and 47 kappa stocks. At any rate, it has been observed that stocks 116 and 298 (hump killers with 51-type R bodies) contain small numbers of kappas with 7-type R bodies (99). On the other hand, since Dippell (31) found that mixed populations of hump and spinner kappa imparted to their hosts the ability to produce and resist both killing actions, the initial populations of spinner kappa would have to have been very low in order to have escaped detection. Also, the finding of extremely close similarity in DNA hybridization (see below) does not favor the view. The response of all the mutant types of 51 kappa to *K* and *k*, in view of the considerable specificity of maintenance genes, also argues against the hypothesis. Nevertheless, it must be admitted that at least some of the rather diverse mutant types such as 51 hump and 51 spinner kappas may indeed have both been present in the original stocks of 51. The possibility simply cannot be rigorously excluded. It is, of course, entirely possible that different ones of the killer "mutations" have different bases.

INFECTION

A number of studies have been done on artificial infections, originally discovered by Sonneborn (127) and studied by Tallan (139), and the older papers have been reviewed in some detail by Sonneborn (130). When 51 sensitives are exposed to concentrated homogenates of 51 killers or to isolated 51 kappa, the sensitives pick up kappa and become killers. Extracts of paramecium (139, 140) and calcium ions (68) both increase the frequency of infection. It has further been shown that isolated 51 kappa endosymbionts may be separated on sucrose gradients into a population consisting primarily of particles with R bodies and a population primarily free of R bodies (70, 112). Tests of infection on these fractions lead to the conclusion that R body-containing kappa particles are not infective and that R body-free kappa particles are.

We (unpublished data) have shown in similar experiments that stock 7 sensitives may be infected with isolated 7 kappa and 562 sensitives with isolated 562 kappa (89). Lambda in homogenates is infective according to Williams (154). In vitro cultured lambda is infective according to van Wagtendonk et al. (143), but not infective according to Williams (154).

Alpha is exceptional among the symbionts in being highly infectious in a few strains and stocks of syngen 2 of *P. aurelia* (98). Simply culturing alpha-bearing and alpha-free paramecia together results in infection of the alpha-free paramecia. A survey of numerous strains and stocks for infection revealed that only a few, all in syngen 2, were infective. Infection of syngens 1, 4, 6, and 8 failed.

Infection by microinjection of cytoplasm was first reported by Koizumi and Preer (56) who established strain 7 (syngen 2) kappa in the cytoplasm of strain 51 sensitive (syngen 4). In its new host, 7 kappa multiplied at a maximal rate somewhat lower than it normally attains in syngen 2. This result is interesting in view of the fact that 51 kappa has a maximal multiplication rate in its normal host which is much greater than 7 kappa in its normal host. Transfer of kappa between 7 and 51 has been confirmed independently by Gibson (37) by using microinjection.

Gibson in the same paper reported upon an extensive series of transfers by using microinjection. Many of the transfers were between syngens. See Table 3. He reported that (i) the mus of syngens 1 and 8 are interchangeable, (ii) lambda of syngen 8 will persist in syngens 2 and 4, (iii) kappa of syngen 2 will grow in syngen 8, and (iv) alpha of syngen 2 will grow in syngen 4. He reported failure of transfers of mus of syngens 1 and 8 into syngens 2 and 4 and kappa of syngens 1 and 8. It should be pointed out that his account is contradictory in respect to the

TABLE 3. *Successful intersyngenic transfer of symbionts*[a]

Recipient cells (syngen)	Symbionts injected (syngen)
540 (1)	138 mu (8)
7 (2)	51 kappa (4)
562 (2)	299 lambda (8)
51 (4)	7 kappa (2)
51 (4)	299 lambda (8)
51 (4)	562 alpha (2)
138 (8)	7 kappa (2)
299 (8)	7 kappa (2)
299 (8)	A_1 kappa (2)

[a] From I. Gibson (ref. 37); unsuccessful transfers are listed in the text.

success of transfer of kappa into syngen 8. Gibson also reported upon a number of transfers within syngens, and a review of these cases will be found in the section below on genic control. Koizumi (personal communication), unlike Gibson, finds that most intra- and intersyngenic transfers are unsuccessful. Lambda, for example, has never become established in a lambda-free line, even though on the order of a thousand transfers of cytoplasm have been made. The 299 lambda could not even be transferred to lambda-free 299. The reason for the disagreement is unknown. (see also the section on nongenic factors affecting maintenance.)

It has been reported (131, 132) that certain stocks of *Didinium nasutum* and *Dileptus* may be infected with mu, kappa, or sigma by feeding them with killers. The report that successful infection depends upon a prior exposure of *Didinium* to paramecia bearing maintenance genes for kappa and mu, however, has not been confirmed (25).

Infection into strains lacking maintenance genes is discussed below.

CHEMISTRY AND PHYSIOLOGY

Gross Analysis

The results of analyses of isolated 51 kappa (114), 138 mu (120), 540 mu (136), 139 pi (121), and 299 lambda (121, 145) indicate the presence of RNA, DNA, carbohydrate, and lipid. The amounts fall within the range of values reported for bacteria. However, the quantitative data are not very diagnostic because the values for bacteria vary tremendously, depending upon nutritive state and kind of bacteria (64).

One of the major variables in the analyses of the endosymbionts appears to be the estimation of the dry weight of a single particle. One of us (J.R.P., unpublished data) has measured 20 to 30 51 kappa, 540 mu, and 299 lambda by phase microscopy and computed the following mean dimensions (length by width) in μm: kappa, 1.67×0.6; mu, 1.54×0.4; lambda, 4.5×0.7. These values agree well with the values for pi, mu, and lambda given by Soldo et al. (121). Taking the shape as a cylinder, the density as 1.15, and the content of water and other volatile substances as 80%, the dry weights of single particles are calculated to be as follows: kappa, 0.10×10^{-12} g; mu, 0.044×10^{-12} g; and lambda, 0.40×10^{-12} g. With the same assumptions, a cell of *Escherichia coli*, 2.0×1.0 μm, would have a dry weight of 0.18×10^{-12} g, within the accepted range (64). Smith-Sonneborn and van Wagtendonk (114), however, give data which lead to a dry weight of 0.77×10^{-12} g for 51 kappa, a value which must be in error. Kung's (57) measurement of 0.13×10^{-12} g seems more likely. Van Wagtendonk and Tanguay (145) gave values of protein, RNA, DNA, and carbohydrate which alone add to 4.6×10^{-12} g per lambda particle, impossibly high. The values for protein, RNA, and DNA, however, were revised downward rather drastically by Soldo et al. in 1970 (121).

Electron Transport System

Kung (59) studied and compared the cytochromes of kappa-free paramecia of stock 51 with the cytochromes of isolated kappa from stock 51. The paramecia contained cytochromes *a*, a_1, *c*, and an unknown. Kappa contained cytochromes a_1, a_2, b_1, and o. Kung stated that the cytochromes of kappa are very different from those of their paramecium hosts and are virtually identical to those of bacteria of the families *Enterobacteriaceae* and *Brucellaceae*. He also noted that the cytochromes of 51 kappa are rather different from those of the bacterial families *Azotobacteriaceae*, *Lactobacteriaceae*, *Pseudomonadaceae*, *Bacillaceae*, and *Micrococcaceae*. Kung showed that kappa has nicotinamide adenine dinucleotide oxidase activity which is inhibited by antimycin A, 2-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO), and KCN. Although both 51 paramecia and 51 kappa contain cytochrome oxidases, Kung finds that none can oxidize the typical cytochrome *c* of mammals. Earlier reports of cytochrome oxidase in *Paramecium* (21, 93, 111) were not based on direct observation of the oxidation of reduced cytochrome *c*, but on increases in oxygen uptake after addition of reducing agents such as ascorbate.

The fact that cytochromes of kappa are virtually identical to those of *Klebsiella aerogenes*, the organism on which Kung's paramecia were cultured, raises the possibility of bacterial contamination. This possibility, however, was completely ruled out by Kung's demonstration that his purified preparations of kappa contained from 1:300 to 1:3,000 as many bacteria as kappa, amounts which he showed were far too small to give readings within the range of sensitivity of his instruments. Bacterial concentrations were determined both by plating for viable bacteria and by making microscopic counts. In counting, Kung utilized the fact that 51 kappa is easily lysed by dilute (0.01%) solutions of sodium deoxycholate, whereas *Klebsiella* and other free-living bacterial contaminants in his cultures were not. Kung also showed that concentrated

bacteria from the media in which the paramecia were cultured had absorption spectra which were qualitatively unlike those of kappa.

Williams (153) has also demonstrated cytochromes in mu and found that the cytochromes of 540 mu and 138 mu differ from each other as well as from the host *Paramecium*.

Respiration

Kappa has the capacity for glycolysis or the pentose-phosphate shunt, or both, and can also carry out the tricarboxylic acid cycle by utilizing cytochromes. No known cell organelles, only organisms, can carry out all these functions.

Kung (57) used oxygen electrodes to show that isolated 51 kappa respires. The first determinations were made at about 2.5 h after the paramecia were homogenized and purification was begun. At that time the QO_2 was about 17 μliters of O_2 per mg (dry weight) per h. By using Kung's estimate of dry weight of one kappa particle as 1.3×10^{-13} g, the rate becomes 2.2×10^{-9} μliters per kappa per h. The rate decreased with time, dropping to one-half in about 5 h. Many years ago Simonsen and van Wagtendonk (111) reported that 51 killers respired at almost twice the rate of 51 sensitives. The difference in rate of respiration between 51 killers and kappa-free 51 was found to vary from 80 to 390 μliters per paramecium per h. Because a 51 killer contains on the order of 1,000 kappas, the total respiration due to kappa in each killer would amount to about 0.0022 ($1{,}000 \times 2.2 \times 10^{-9}$) μliters per kappa per h. Thus, each kappa in a killer would have to respire at about 35 to 175 (80:0.0022 to 390:0.0022) times the rate observed by Kung if the increased respiration were to be accounted for directly by kappa itself. Although Kung found the rate of respiration of isolated kappa does decrease rapidly with time, as noted above, the loss in the first minutes after homogenization would have to be at a truly astounding rate to account for the observed difference. Thus, it is likely that the original differences in respiratory rate between killers and sensitives (111) must be explained in some other way. This conclusion was reached by Sonneborn in his 1959 review (130), and the interested reader is referred to that paper for a detailed account of the earlier studies of Simonsen and van Wagtendonk on this and related matters.

Kung found that the respiratory rate of isolated 51 kappa was stimulated by glucose and sucrose, the respiratory rate rising in many experiments to about twice its normal value. Stimulation was also obtained with intermediates of the tricarboxylic acid cycle, succinate, fumarate, pyruvate, etc. No increases were observed with the addition of numerous other sugars. Respiration was inhibited by KCN, CO, and HOQNO. Malonate, antimycin A, and 2,4-dinitrophenol had no inhibitory effect on the respiration of intact kappa.

Enzymes in the Metabolic Pathways

Kung (58) demonstrated several glycolytic enzymes in both kappa-free stock 51 and in isolated kappa: hexokinase, phosphofructose kinase, aldolase, glyceraldehyde-3-*P* dehydrogenase, enolase, and pyruvate kinase. Alcohol dehydrogenase was found in paramecia, but not kappa. Interestingly, enzymes of the pentose-phosphate shunt were found in kappa, but not paramecia: glucose-6-*P* dehydrogenase, 6-phosphogluconate dehydrogenase, and transaldolase. It should be noted, however, that the existence of the shunt in paramecia is debatable. It was also not found by Levine (63), but evidence for its presence was provided by Vloedman et al. (148) and by Tait (quoted in reference 58). Kung notes that in eukaryotic cells the glycolytic enzymes and enzymes of the pentose-phosphate shunt are found in the cytoplasm outside of cell organelles. Their presence in kappa implies that kappa is a cell by itself. Isocitrate dehydrogenase of the citric acid cycle was found in both kappa and paramecia.

The 138 mu has been found to have glutamate dehydrogenase, malate dehydrogenase, and isocitrate dehydrogenase, all with different Michaelis constants from those present in the host paramecia (41).

The 540 mu has been shown to have a rifampin and actinomycin D-sensitive, DNA dependent, RNA polymerase (136, 153).

Constituents of the Bacterial Cell Wall

Stevenson (135) showed by chromatography that isolated stock 540 mu contains diaminopimelic acid and probably muramic acid as well. The amount of diaminopimelic acid was 0.07% of the dry weight, within the range of values known for bacteria (155). Stevenson points out that these compounds are typical of the bacterial cell wall. Diaminopimelic acid is unknown in other organisms.

Ribosomal RNA

Baker (6) found that 540 mu contains two high-molecular-weight species of RNA corresponding to 16*S* and 23*S*. The RNAs were indistinguishable by electrophoresis in poly-

acrylamide gels from 16S and 23S ribosomal RNA of *E. coli*. The major RNAs from paramecia of stock 540 without mu were found to be 18S and 25S, the values for paramecia without mu agreeing with the values reported earlier by Reisner et al. (102). Paramecia with mu showed all four RNAs. These findings, of course, help to establish the bacterial nature of mu.

Gibson et al. (41) state that the ribosomal RNAs of 138 mu and 562 alpha are similar to those of 540 mu.

DNA Densities

Soon after the isolation of 51 kappa by Smith-Sonneborn came the first determinations of buoyant density of endosymbiont DNA by using CsCl centrifugation (113). It was reported that the density of the DNA of 51 kappa was 1.696, whereas the density of the DNA from 51 paramecia was 1.689. Numerous determinations have since been carried out, and the results are summarized in Table 4. Density determinations of the endosymbiont DNAs vary from 1.694 to 1.708, whereas values for their *Paramecium* hosts vary from 1.685 to 1.693.

The densities of the DNAs of kappa, mu, gamma, alpha, and nu are rather similar, estimates ranging from 1.694 to 1.702. Densities of phage DNAs in the two cases studied (562 and 51) are about 0.002 less than the DNA of the kappa in which they are found.

Lambda and sigma form a group with similar morphological and physiological characteristics. According to Behme (20) their densities are also similar (239 and 299 lambda being 1.708 and 114 sigma being 1.704). The values for the lambdas are only slightly less than the value of his reference *Aerobacter aerogenes* DNA (1.715). An astonishingly different value, however, was recently published by Soldo and Godoy (117) who reported a density of 1.686, slightly less but very close to the density of 299 *Paramecium* DNA. If Soldo and Godoy's preparations had a DNA of density 1.708, it might well not have been distinguishable from that of their reference *E. coli* DNA (1.710). However, data on melting (117, 120) confirm their determinations of density. The answer to these conflicting results may be that there is a difference in strains, for Allen et al. (1) report that the Miami strain of 299 (used by Soldo and Godoy) has a difference in esterases which distinguishes it from the Bloomington strain of 299 used by Behme. Nevertheless, the Miami strain originally came from Bloomington.

Nucleic Acid Hybridization

Behme (20) has isolated DNA from the different particles and studied their relationships by DNA hybridization experiments. The experiments were carried out with the membrane filter technique. The percentage of hybridization obtained in different combinations is given in Table 5. Kappa of stock 51 (syngen 4) (hump killer), 51m1 (spinner killer), and 51m43 (resistant non-killer) show maximal hybridization (99–110%). They provide strong evidence that 51m1 and 51m43 are indeed mutants rather than selected products of mixed initial infections of different particles. The 7 kappa (syngen 2) and 139 pi (syngen 4) are closely related to

TABLE 4. *DNA densities*

	Endosymbionts	
Kappa	7	1.697 (20), 1.698 (88)
Kappa	51	1.695 (41), 1.696 (113), 1.697 (20), 1.700 (30)[a]
Kappa	51m43	1.696 (20, 113)
Kappa	51m1	1.695 (20)
Kappa	562	1.702 (95)[b]
Pi	139	1.694 (20)
Mu	138	1.696 (20)[c]
Mu	540	1.694 (136, 41), 1.696 (20)[c]
Mu	551	1.695 (41)
Lambda	299	1.686 (117), 1.708 (20)
Lambda	239	1.708 (20, 19)
Sigma	114	1.704 (20, 19)
Gamma	214	1.697 (20)
Alpha	562	1.695 (41)
Nu	87	1.695 (20)
	Paramecia	
	7	1.688 (2)
	51	1.685 (41), 1.687 (28), 1.688 (28), 1.689 (2)[d], 1.689 (113, 138), 1.691 (51)
	562	1.686 (41), 1.688[d] (2)
	138	1.690 (2)[d], 1.691 (41)
	540	1.686 (41), 1.688 (2)[d], 1.689 (2), 1.692 (2), 1.693 (136)
	551	1.686 (41), 1.688 (2)[d]
	299	1.688 (117), 1.689 (2)[d], 1.689 (41)
	114	1.686 (41), 1.688 (2, 20)[d]

[a] Several workers, S. Pollack (unpublished data), and D. Widmayer (unpublished data) have found that the DNA from 51 kappa is often resolvable into two major peaks. J. A. Dilts (30) believes that one represents kappa DNA, the other circular phage DNA. She gives values of 1.698 for the phage and 1.700 for kappa.

[b] The original paper (95) reported a density of 1.705. More recent work in our laboratory indicates that this estimate is high and that a better value is 1.702 for 562 kappa and a slightly lower value, 1.700, for 562 phage DNA.

[c] R. J. Behme (19) reported a density of 1.700 for 138 mu and 1.701 for 540 mu, but he later (20) revised the values and gave the ones listed above.

[d] Axenic.

the 51 particles, the range of hybridization among the five strains being from 72 to 86%. The 540 (syngen 1) and 138 (syngen 8) mus are related to each other (75 to 79%). The kappas, mus, 214 gamma (syngen 6), and 87 nu (syngen 5) are all related, showing hybridization values with each other of 40 to 84%.

This whole group, however, is quite distinct from the remaining endosymbionts, lambda and sigma, giving hybridization values of only 3 to 28% with them. The 239 lambda (syngen 4) and 299 lambda (syngen 8) are closely related to each other (82 to 85%) and form a relatively close group with 114 sigma (syngen 2), the three stocks varying from 66% upwards. It should be remembered, however, that Behme (28 C) performed his experiments on 299 lambda DNA of density 1.708, whereas Soldo and Godoy (117) report that 299 lambda DNA has a density of 1.686.

Gibson et al. (41) isolated DNA from 562 alpha, 540 mu, 551 mu, 299 lambda, and particle-free 540 paramecia. They used the DNA as a template to synthesize RNA by using RNA polymerase from *E. coli*. The resulting complementary or cRNAs were then hybridized with the DNAs. The degree of hybridization was expressed as percentages of the homologous combinations. The two mus showed the greatest degree of similarity (58 to 62%). Alpha was also fairly similar to the two mus, the three combining fairly strongly in all combinations (32 to 62%). Even 299 lambda showed weak reactions with the other three (10 to 16%). Reactions with *Paramecium* nucleic acid was very low (1 to 5%). The results appear to be in complete agreement with the results of Behme described above. Because of the rather unusual morphology of alpha, one might have guessed it to be unrelated to mu, so the homology between the two reported here is somewhat surprising. This apparent relationship between alpha and the other symbionts has been ignored in the taxonomic system proposed in the conclusions of this review because of other traits discussed there and pitfalls inherent in the use of cRNAs to establish phylogenetic relationships.

Baker (7) finds that the ribosomal RNA of 540 mu hybridizes with the DNA of *E. coli*, but not the DNA of the host paramecia, further emphasizing the bacterial nature of mu.

DNA Kinetic Complexity

Soldo and Godoy (117) estimate that a single 299 (Miami strain) lambda contains 0.0124×10^{-12} g of DNA, equivalent to a molecular size of about 7.5×10^9. They studied the kinetics of renaturation and reported the molecular size to be 0.71×10^9. If one corrects for G+C content, the value is lowered to 0.39×10^9 daltons. They conclude that each lambda has 10 to 20 copies of each DNA sequence. The value for the genome size is particularly interesting, for it indicates that the genome of lambda is closer to that of the mycoplasmas (107), around 0.8×10^9, than the more complex bacteria such as *E. coli* (149), 2.5×10^9. Of special interest also is the presence of a multicopy genome in lambda. Single-copy genomes have been found in free living bacteria, whereas multicopy genomes are the rule for mitochondria. Nevertheless, it should be remembered that the lambda DNA used in these determinations had a density of 1.686, whereas Behme (19, 20) reports that 299 lambda DNA has a density of 1.708.

TABLE 5. *DNA reassociation reactions*[a]

DNA filters	DNA fragments											
	Gamma	Kappa					Mu		Nu	Sigma	Lambda	
	214	7	51	51m1	51m43	139	138	540	87	114	239	299
Gamma 214	100	64	74	79	69	71	61	69	66	28	11	27
Kappa 7	63	100	72	73	72	73	50	49	62	27	12	19
Kappa 51	74	72	100	101	99	81	58	48	70	27	26	19
Kappa 51m1	75	69	99	100	103	80	60	44	74	27	27	15
Kappa 51m43	78	67	110	107	100	81	61	53	70	20	23	20
Pi 139	84	72	86	79	84	100	62	40	65	26	27	15
Mu 138	63	51	61	56	54	61	100	79	47	27	15	28
Mu 540	59	57	66	55	60	47	75	100	62	24	14	16
Nu 87	62	64	68	68	69	69	44	57	100	27	18	10
Sigma 114	16	17	18	16	18	17	11	16	13	100	66	69
Lambda 239	6	3	15	15	13	15	13	10	10	70	100	82
Lambda 299	19	9	17	10	8	8	20	11	7	78	85	100

[a] Modified from Behme (20); the values indicate the percentage of filter-DNA sequences similar to the DNA fragments added.

The genome size of the DNA of 540 mu and 551 mu has been measured by Gibson et al. (41) from renaturation kinetics. Values of 3.4×10^9 daltons for 540 mu and 3.3×10^9 daltons for 551 were obtained. Analysis of the amount of DNA per mu indicates only one genome per mu. These values are, of course, enormously larger than the values obtained above for lambda. In any event, the results of Gibson et al. (41) suggest that in number and size of genomes, mu closely resembles bacteria.

Host-Free Culture

In 1963 van Wagtendonk et al. (143) reported that 299 lambda symbionts could grow free of paramecium in a complex medium. The medium was identical to the medium developed by van Wagtendonk and his group for the axenic cultivation of *P. aurelia*. In 1970, Gibson (36) reported that attempts in his laboratory to obtain growth of lambda, free of paramecium, in the same medium used by van Wagtendonk et al. (143) were unsuccessful. But in 1971 Williams (154) in Gibson's laboratory reported success in the culture of both 299 lambda and 138 mu in similar media. She reported that growth was slow (one fission per day) and that high population levels were never observed (normal upper limits being around 20,000 endosymbionts per ml for both lambda and mu). Perhaps some of the conflicting results, as well as the failure of van Wagtendonk and his group to exploit their discovery, was due to a change in strains (see discussion above under DNA density).

Because of the possibility of confusion of lambda and mu with bacterial contaminants, special evidence that the cultured forms are, in fact, lambda and mu is of particular importance. Van Wagtendonk et al. (143) and Williams (154), too, reported that their cultured lambda was able to kill sensitive strains of paramecia. Williams found strong killing of the sensitive stock 92 (syngen 3) and weak killing of lambda-free 299 (syngen 8). Mu-free 540 (syngen 1) was unaffected. Williams also cites the results of killing tests with her in vitro-cultured 138 mu. She found the 138 mu killed lambda-free 299, but did not affect stocks 92 and 540. No other controls to demonstrate specificity were provided (e.g., it would have been of interest to compare the sensitivity of symbiont-free and symbiont-containing 299 and 138). It is remarkable, however, that, in spite of very extensive tests, mate-killing action by mu in any way other than cell-to-cell contact at conjugation has never been found before. Isolated particles of the 570 syngen 2 mate-killer produce no *specific* killing even in very high concentrations (J. R. Preer, unpublished data).

Van Wagtendonk et al. also reported that their cultured lambda could reinfect lambda-free 299 and convert it back to killer. Schneller (personal communication), on the other hand, was not able to get infection with lambda from homogenates of paramecia. However, Williams in contradiction to both Schneller and van Wagtendonk et al. was able to get infection of 299 lambda isolated from paramecia, but failed to get infection with in vitro-cultured lambda. Her in vitro-cultured mu was also uninfective. She concludes that in vitro culture results in a change in the properties of the symbionts.

The evidence for in vitro growth will be stronger when these contradictions are resolved and better evidence is obtained that the cultured bacteria were indeed derived from the cytoplasm of killer paramecia.

ANTIGENS AND SURFACE PROPERTIES

In 1957 Siegel and Preer (110) obtained antiserum against paramecia bearing kappa, mu, and pi. A drop of antiserum was added to a microscope slide, endosymbiont-bearing paramecia were added, and the cells were then crushed with a cover slip. Examination with a phase-contrast microscope revealed that when homologous antiserum was used, the endosymbionts were agglutinated into clumps. Further tests revealed that the syngen 2 kappas (stocks 7, 7m1, and 50) formed a cross-reacting group. The syngen 8 mus (130, 131, and 138), kappas (51, 51m1, and 169), and pi (51) formed a second cross-reacting group. One exception was the failure of 51m1 antiserum to agglutinate 51 kappa; however, the reciprocal mixture did yield agglutination. These results suggest a serological difference between the kappas of 51 and 51m1. Furthermore, Hanson (45, 47) found sera which distinguished between 51 kappa and pi. (See discussion by Sonneborn [130].)

The rather surprising first conclusion from these facts is that kappa of syngen 4 is more closely related to mu of syngen 8 than to kappa of syngen 2. Because the paramecia of syngens 4 and 8 are rather closely related to each other and those of syngen 2 are relatively less close, it is seen that the antigenic differences parallel host relationship. This conclusion is not what is concluded from the experiments on hybridization of DNA. They indicate that kappas form one homogeneous group and mus another. The surface agglutinogens probably do not have

great pertinence to phylogeny, for they may well represent only single gene products. Furthermore, the question might be raised as to whether the surface antigens are derived from their hosts rather than the endosymbionts. However, this possibility is rendered rather unlikely by the fact that sera prepared by injecting sensitive paramecia do not agglutinate the endosymbionts. The agglutinating antigens of the outer surface of kappa are synthesized only in killers.

There seems to be little or no relationship between the serological properties of the surfaces of the endosymbionts and their properties of adsorption on ion-exchange columns (89, 99). The members of one group (the class 7 kappas and stock 1038; see Table 2) are very "sticky," adsorbing to glass slides and cover slips and reversibly to filter paper at low ionic strength. Stock 7 itself (the others have not been tried) adsorbs to ECTEOLA at low ionic strength and can be eluted at high ionic strength. A third group (the class 51 kappas, class 562 kappas, 51m1, and 299 lambda) does not adsorb to ECTEOLA. It is not known whether the adsorptive properties of the endosymbionts is determined by the host or by the endosymbionts themselves. An observation which suggests that the host determines the attribute is that, although stock 7 kappa ordinarily sticks to glass, it does not do so after it has been transferred to a kappa-free strain of stock 51 and become established there (J. R. Preer, unpublished data). A genetic analysis of the difference between 562 and 7 kappas (both of syngen 2) would be interesting in this respect.

In 1966 Mueller (72) demonstrated by precipitation in gel an antigen present in 51 kappa and in homogenates of 51 killers which had kappa removed by centrifugation. The antigen was lacking in 51 sensitives.

EFFECTS OF THE ENDOSYMBIONTS ON THEIR HOSTS

Killer paramecia liberate toxic particles into the medium in which they live and also are resistant to the toxin. When kappa is lost from a strain of killers the paramecia lose both characteristics. The reason for the loss of the capacity to produce toxin is obvious, for the toxic particles are the kappa particles containing R bodies. The reason for the loss of resistance, however, is not so obvious. Somehow the presence of kappa causes paramecia to become resistant. This phenomenon constitutes one of the more interesting unsolved problems about kappa.

A striking fact about the resistance induced by kappa is its specificity. Although all kappa-bearing strains are resistant to their own toxin and become sensitive to it when freed of kappa, most killers kill each other. Thus the hump killer 51 and the spinner killer 51m1 (derived from 51) kill each other. The same is true of the vacuolizer 8 and spinner 7. There are exceptions, however, for the spinner 7 kills the paralysis mutant 7m1, but 7m1 does not affect 7. The resistant nonkiller mutant 51m43 found by Widmayer (150) is particularly interesting because it demonstrates that R body formation and toxicity are not necessary for resistance.

Widmayer and Preer (unpublished data) have found that the resistance induced by kappa is not absolute and can, in fact, be overcome by large doses of toxin. Paramecia of stock 7 were cultured at different fission rates (84) for a week and then tested against concentrated suspensions of isolated (10) stock 7 kappa. It was found that some of the more rapidly growing cultures, while still containing sufficient kappa to be killers, were nevertheless susceptible to the high concentrations of isolated kappa. That the effect was specific was shown by the fact that somewhat more slowly growing cultures which contained still more kappa were completely resistant to the toxic effects of the same concentrations of isolated kappa of their own kind. The same results were found with stock 51 killers.

It is interesting to inquire whether the endosymbionts always bestow resistance on their hosts as in the case of kappa. Conferred resistance has been shown for mu in syngens 1 and 8 (38, 108, 109), but no information is available in respect to delta or gamma. Lambda and sigma were reported originally by their discoverers to affect only syngens 3, 5, and 9 (131a, 134). However, an effect of in vitro-cultured 299 lambda particles on lambda-free 299 was reported by Williams (154). More recently Schneller (personal communication) has found a weak sensitivity of lambda-free 299 to 299 lambda killers.

Soldo, van Wagtendonk, and co-workers (115, 118, 144) and also Burbanck and Martin (22) have shown that when bearers of endosymbionts are placed into axenic media, one of three things may occur: the paramecia may die, the paramecia may live and the endosymbionts disappear, or both may survive. If paramecia in the first case are examined just before death it is found that the endosymbionts have increased to exceedingly high numbers (118). The suspicion that the endosymbionts are the cause of death rather than the result of poor medium is rein-

forced by the fact that endosymbiont-free strains multiply perfectly well in the same medium. The conclusion that unusual environmental conditions can upset the balance between endosymbiont and host, leading in some cases to loss of the endosymbiont and in others to death of the host, seems inescapable (118).

J. R. Preer (unpublished data) has seen lines of paramecia containing 114 sigma and 561 delta in bacterized medium which contained extraordinarily high numbers of endosymbionts coupled with a much-reduced fission rate of the paramecia. Occasionally from such cultures a line of paramecia is obtained which can multiply at the usual rapid rate, and in such cases the number of endosymbionts per paramecium is found to have dropped to a much lower level. Although one cannot be certain of cause and effect in such cases, one suspects that the balance between endosymbiont and paramecium has gotten out of balance to the detriment of the paramecium.

Nobili (77) has shown that amacronucleate killer paramecia, which are regularly formed in lines homozygous for the gene *am*, do not live as long as amacronucleate paramecia lacking kappa.

The most striking fact, however, is the truly remarkably small number of such cases. Generally, there is no evidence that the particles have any effect on their hosts whatsoever, aside from converting them into resistant killers, even when present in remarkably high concentrations.

In 1963, work by Soldo (116) suggested that the apparent inertness of the endosymbionts may be due primarily to our lack of adequate means for identifying such effects. His work suggested that endosymbionts can provide a positive benefit to their host in ways other than the obvious advantage of being a killer rather than a sensitive in mixed cultures in the laboratory. He reported that 299 lambda-bearing paramecia in axenic medium do not require folic acid, whereas 299 lambda-free strains do. Soldo's work, however, has recently been questioned by Williams (154) who reported that she could culture both lambda-bearing and lambda-free strains of 299 axenically in the absence of folic acid. It is suggested by Soldo (personal communication) that the proteose peptone used by Williams contained folic acid. More recent work by Soldo and Godoy (117a) has confirmed and extended Soldo's original finding.

It has been argued that the selective advantage enjoyed by killers over sensitives in the laboratory does not extend to nature because concentrations of paramecia there are probably very dilute. Indeed, the coexistence of killers and sensitives in collections from the same pond has been cited in support of this argument. One might well note, however, that the endosymbionts are easily lost and, with the exception of alpha, infection in the laboratory almost certainly only occurs under conditions never occurring in nature. If infection in nature is rare, then one must assume some positive selective force in favor of endosymbiont-bearers to account for their abundance.

An interesting case of an effect of an endosymbiont on its host is 87 nu of syngen 5. In spite of the fact that nu does not produce any toxic effects, Holtzman (49) reported that nu makes its host more resistant to 299 lambda killers.

MIXED POPULATIONS

It would be expected that if two kinds of endosymbionts occupy exactly the same ecological niche within a cell, one kind should eventually be lost due either to selection or drift. In fact, mixed populations do seem rather rare. Stock 562 of syngen 2 contains both kappa and alpha, but one inhabits the macronucleus and the other the cytoplasm, differing niches possibly explaining the association.

Stock 131 of syngen 8 has long been known to contain mu (61), and recently it was found also to contain delta (See Fig. 5). Likewise several additional collections of syngen 8 stocks from Orlando, Florida, have been found to contain both delta and another symbiont resembling either mu or nu (unpublished data). Stevenson (137) also reports the presence of delta with a 51-type kappa in stocks A1 and A2 and the present authors confirm this observation. Perhaps the endosymbionts can act synergistically in some cases.

Of course, all of the mutants of stocks 51, 139, 47, and 7 arose in mixed populations. The authors also have noted that strains 116 and 298, which are killers similar to 51, contain a small percentage of R body-containing endosymbionts very unlike 51 and morphologically much like the mutant 51m1. Hanson (46) studied a line containing the slow hump kappa mutant 51m7 and pi. Specifically he looked for wild-type recombinants, but found none.

Artificially produced mixed populations have also been reported. Balsley (11, 12, 13) found populations of 7 kappa and 114 sigma and also 7m1 kappa and 114 sigma produced after cytoplasmic exchange during conjugation in syngen 2. By using microinjection, Gibson (37) reported that 562 alpha-bearing lines were successfully

infected with 299 lambda, 51 kappa, and 7 kappa. The 51 kappa-bearing lines were infected with 299 lambda, 562 alpha, and A1 kappa.

Gibson (37) also has made the remarkable observation that when mu-bearing 138 (syngen 8) paramecia are injected with lambda-bearing cytoplasm from 299 (syngen 8), both endosymbionts disappear. Also surprising are three cases where only the injected endosymbionts persisted, the originals disappearing: paramecia bearing 138 mu (syngen 8) injected with cytoplasm containing 7 kappa (syngen 2), stock 299 lambda bearers (syngen 8) injected with cytoplasm containing 7 kappa, and stock 299 lambda bearers injected with cytoplasm containing A1 kappa. A formal explanation consistent with these results is that the cytoplasms of 299 and 138 are toxic to the symbionts borne by each other, that 7 cytoplasm is toxic to 138 mu and 299 lambda and that A1 cytoplasm is toxic to 299 lambda. One is reminded of the recent work of Poulson and co-workers in which certain of the spirochaetes responsible for the trait, sex ratio, in *Drosophila* bear viruses which are able to infect and kill the spirochaetes of different strains (81, 104, 105).

NONGENIC FACTORS AFFECTING MAINTENANCE

The endosymbionts often are lost soon after newly collected strains are brought into the laboratory. Spontaneous loss is common at later times too in many stocks, such as, for example, 114 sigma or 562 kappa. Some endosymbionts such as 1010 nu are very stable.

Most of the endosymbionts have a maximal multiplication rate less than the maximal multiplication rate of the paramecia. Thus, when the paramecia are fed an abundance of food in many stocks, the number of kappa per paramecium steadily declines (even though the absolute number of kappa and paramecia in a culture are steadily increasing). Continuation of rapid feeding leads finally to the production of paramecia completely free of kappa. As rapid feeding continues, the fraction of the paramecia in a culture which has been completely freed of kappa increases. After a period of rapid feeding, restriction of the food supply allows the kappa to increase in lines of cells in which kappa has not been completely eliminated. It is easy to take advantage of these facts in designing experiments which reveal the time at which kappa is completely lost. The time depends upon the multiplication rates of the kappa and the paramecia and also on the initial concentration of kappa. The proportion of paramecia free of kappa at different times then may be used to calculate the multiplication rate of the kappa and the initial number of kappa per paramecium. Studies of this kind were carried out in the late 1940s by J. R. Preer (84) and were important in establishing our view of the nature of kappa. Sonneborn showed that the relative multiplication rate of kappa and *Paramecium* may be altered by modifying the temperature (124). These studies were reviewed in detail by Sonneborn (130).

Spontaneous loss of 51 kappa at macronuclear regeneration has been reported (79, 130). The endosymbiont of killer stock SG (syngen 2) has been reported by Nobili (79) to undergo occasional loss at autogamy. Furthermore, Koizumi (54a) and Koizumi and Kobayashi (55) have recently found that when 51 kappa is microinjected into 51 sensitives, infection is much less likely if the recipients are undergoing autogamy or conjugation. There is, therefore, no doubt that in some stocks at certain stages, nuclear reorganization is a critical period for the survival of endosymbionts. As will be seen presently in the next section on genic control, this conclusion may be of especial importance. Whether this effect is due to nuclear reorganization per se or whether it is due to the mild starvation used to induce conjugation and autogamy is an open question.

Numerous agents are more harmful to the endosymbionts than to *Paramecium* and have been used to effect "cures" by a number of investigators. These include high temperature, low temperature, X rays, ultraviolet radiation, streptomycin, nitrogen mustard, chloromycetin, terramycin, aureomycin, penicillin, and 2,6-diaminopurine (reviewed by Sonneborn [130]). A more recent study has considered the effects of 8-azaguanine on mu (40). Soldo et al. (119) have studied the effect of penicillin on lambda and note abnormalities in the cell wall as found for bacteria.

The effects of X rays on kappa were of particular interest because they provided estimates of X ray-sensitive volume at a time when kappa was known only as a genetic, not a cytological entity. The estimates indicated that kappa was very large and led eventually to its discovery in the microscope (86).

GENIC CONTROL

Genes

In 1943 (123) Sonneborn crossed 51 killers with stock 32. After inducing autogamy in the F1 he found that kappa was influenced by nuclear genes. Kappa persisted in clones which

were *KK* or *Kk*, but when a killer clone which was *Kk* became *kk* after autogamy, kappa was lost. Killer stock 51 is *KK*, whereas stock 32 is *kk*. Other stocks contain no kappa, are sensitive, but are *KK*. Therefore, kappa is necessary for the killer phenotype, *K* is an allele for the maintenance of kappa, and *k* is an allele for the absence of kappa.

Chao (26) showed that in paramecia of genotype *Kk*, syngen 4 kappas of strain 51 are found in about one-half the usual number found in paramecia of genotype *KK*. A similar halving of the number of kappas in the syngen 2 stock 7 was reported in heterozygotes by Balsley (12). Thus *K* is not dominant over *k* as was originally thought.

S alleles at the two loci S_1 and S_2 have been discovered in stock 29. *S* alleles increase the probability of loss of kappa (8, 9, 126, 130). Killer stock 51 has the genotype $KKs_1s_1s_2s_2$ and under normal conditions kappa is never lost. In the genotypes $KKS_1S_1s_2s_2$ and $KKs_1s_1S_2S_2$, kappa is often lost but can be maintained indefinitely. In $kks_1s_1s_2s_2$ and in $KKS_1S_1S_2S_2$, kappa is always lost.

Aside from the loci in stocks 29 and 32, which affect the maintenance of 51 kappa, a number of genes have been found which affect the maintenance of the different endosymbionts. They are listed in Table 6. Generally, the genes affecting rather different endosymbionts, such as kappa and lambda, are specific. Thus, lambda is maintained as well in *kk* as in *KK*. On the other hand, the alleles affecting similar endosymbionts were originally thought to be the same, thus 51 (hump killer) and its mutants, 51m1 (spinner killer), 51m43 (resistant nonkiller), and 51π (sensitive) strains all appear to have the same original *K* allele (31, 45, 151), and disappear in the presence of *kk* from stock 32.

Recently Gibson (37) has studied the hump killers 51 and 116. They both were collected from near Bloomington, Indiana, and are indistinguishable in their killing activity. Remarkably, however, Gibson reports that 116 has a gene which will not support 51 kappa. He cites as evidence data from a cross of 51 killer by 116 sensitive, which he states gave in the F-2 by autogamy from the killer exconjugant the expected 1:1 segregation indicative of a single-gene difference. A. Rosenthal (unpublished data) in our laboratory more recently has crossed 116 killer by a strain isogenic with 51 killer, but carrying a mutant gene marker. After autogamy, the lines derived from both exconjugant clones of each pair gave all killers. The marker gene showed normal segregation. Thus, the conclusion that 51 and 116 carry different maintenance genes is not confirmed.

The mate-killers of syngen 8 (stocks, 130, 131, and 138) form a peck-order in crosses with each other; 138 kills 130 and 131, whereas 130 kills only 131. Levine (61) showed that in respect to maintenance genes the three are alike and concluded their differences lie within mu. Recently it has been reported by Gibson (37) that contrary to the finding of Levine there is a genic difference between stocks 131 and 138 in respect to maintenance genes. Although not clearly stated by Gibson, it is inferred that 131 lacks

TABLE 6. *Genes affecting symbiont maintenance*

Syngen	Stock	Genotype	Symbiont excluded	Ref.
1	513	$m_1m_1m_2m_2$	540 mu	38
	540		548 mu	16
	551		551 mu	16
2	1010	*kk*	7 kappa	12
	1010	single gene	7 m1 kappa	13
	114	single gene	562 alpha	98
	576	single gene	562 alpha	37
	11	single gene	114 sigma	Balsley, unpublished data
4	32	*kk*	51 kappa	123
	32	*kk*	51 m1	31
	32	*kk*	51 pi	45
	32	*kk*	51 m43	151
	29	$S_1S_1S_2S_2$	51 kappa	8
8	214	single gene	229 lambda	106
	137	*mm*	138 mu	108
	131		138 mu	37[a]

[a] This report of Gibson does not agree with earlier findings of Levine (61).

maintenance alleles for 138 mu.

Beale and Jurand (16) reported that the three mate-killers of syngen (540, 548, and 551), unlike those of syngen 8, kill each other in all possible crosses (540 × 548, 540 × 551, 548 × 551). In all crosses both exconjugants die, aside from an occasional failure of 551 to kill its mate. The crosses 548 × mu-free 540 and 551 × mu-free 540 yield F-2 results, which suggest that 540 has genes which support neither 548 nor 551. These results imply a high degree of specificity between gene and symbiont. Nevertheless, repetition of these crosses at later times has not always yielded consistent results, and Beale (personal communication) states that further work is obviously needed.

These contradictions considered above in regard to the 51 and 116 killers and the genic control of the mus of both syngens 8 and 1, as we will now see, but scratch the surface of numerous contradictions in regard to these phenomena. Perhaps one key to some of the contradictions is the finding that some endosymbionts appear much more likely to be lost at nuclear reorganization than at other times. (See section above on nongenic factors affecting maintenance.) Great care and, especially, adequate control matings are needed.

Metagon Hypothesis

Gibson and Beale (39) reported a rather regular kinetics of loss for 540 mu in syngen 1 after change of genotype from one which supports mu to one which does not. Gibson and Beale explained their results by hypothesizing stable messenger RNA molecules, which they called metagons, produced by the supporting gene. The metagons were assumed to be progressively diluted out by fission after change of genotype. Reduction in number of mu and loss of mu was assumed to occur only when the last messenger molecule was lost from the cell. In still later experiments, however, variations in the kinetics of dilution similar to those described below for kappa were encountered (I. Gibson, personal communication; 18, 25). Thus, the basic experiments which led to the theory have not proved repeatable.

Gibson and collaborators also produced an extensive series of experiments which supported and extended the hypothesis. A detailed review of these experiments can be found in Preer (90). A recent study by Byrne (25) has revealed that many of the experiments could not be confirmed. Consequently, no further description of the metagon hypothesis will be given here.

Loss of Kappa after Change from Kk to kk

When autogamy is induced in killers of genotype *Kk*, one-half of the resulting clones become *KK* and one-half become *kk* and lose kappa. Chao in 1953 (26) reported that although 51 kappa is lost at different times in different lines, when loss does come it is very sudden, the number of kappas dropping from a high level to zero in each paramecium, usually in only one fission cycle. This remarkable fact has never been contradicted by any of the numerous later workers.

It is also clear that under conditions of maximal fission rate, following the change of genotype to *kk*, different lines lose 51 kappa at markedly different times. Chao (26) reported loss occurred between fissions 8 to 15, Yeung in 1965 (157) reported 8 to 12, Beale and McPhail in 1967 (18) found loss was later, for after 15 to 16 fissions around one-half of the clones tested still had kappa, and one clone still retained kappa at 27 fissions. The data of Byrne in 1969 (25) indicate that kappa is lost from most lines prior to fission 20. After a rapid loss during the first 20 fissions, a new and much slower rate of loss was maintained up to at least 60 fissions. Poon (83) reported a range of 9 to 85 fissions for loss of 51 kappa, the mean time of loss usually being over 15 to 20 fissions. Ng and Yeung (75) reported the mean time of loss to be 19.5 to 24.5 fissions. It would appear that different conditions or different populations of 51 kappa vary markedly from time to time in different laboratories and that the time of loss is very sensitive to these unknown and uncontrolled factors.

Beale and McPhail (18) concluded that great variation between sublines within clones occurred, but that there was no association between specific autogamous clones and time of loss. Poon (83), however, varied the age (time since last autogamy) of both the parental and F-1 lines used to obtain the F-2 clones, and found that clonal age did affect the time of loss of kappa; the greater the age in both generations, the more rapid the loss. Nevertheless, he also found great intraclonal variation.

Beale and McPhail (18) noted that for unexplained reasons some lines tend to multiply somewhat more slowly than normal and that such lines always lose kappa sooner than normal. Widmayer (151) reported that paramecia containing the resistant nonkiller mutant of 51 kappa, 51m43, when multiplying at the rather slow rate of three fissions per day, lost kappa in all cases by the fourth fission. Yeung (157) and Poon (83) found that starvation led to the rapid

elimination of kappa (sometimes within one fission after autogamy), but Byrne (25) found no effect of starvation in her studies. In a detailed study of the effect of starvation Ng and Yeung (75) reduced fission rate by adding culture medium which had been inoculated with bacteria and aged several months to fresh medium. It was found that the average number of fissions before total loss of kappa in isolation lines was 1.8 at one fission per day, 3.4 at two fissions per day, 8.1 at three fissions per day, and 24.5 at the maximum rate of five fissions per day. It was alo found that short periods of starvation (for example, starvation for 6 h in paramecia which had undergone only one fission after autogamy), while apparently producing no immediate observable effect on the numbers or rate of multiplication of kappa, reduced by about one-half the number of fissions until loss occurred. The results show that the effect of starvation is not directly on kappa's ability to multiply, but on a secondary system which appears to exhibit a threshold in controlling kappa's maintenance. The nature of that system remains obscure.

In summary, although some of the numerous factors which can markedly affect the time of loss of 51 kappa after change of genotype from *Kk* to *kk* are known, most are probably still unknown. Perhaps insufficient attention has been given to the specific strain of 51 kappa being studied, for it is well known that numbers and character of R bodies and killing potency may vary markedly. Not only can the environment vary from one study to the next, but so may also the genetic constitutions of the populations of kappa and their infecting phages. The maintenance of kappa at high levels until its sudden disappearance in individual lines remains an enigma. Perhaps the possibility that loss is brought about by the sudden induction of phage should be investigated.

Loss of Endosymbionts after Injection into Paramecia Lacking Maintenance Genes

As noted above, Gibson reported that strain 116 is homozygous for a gene which cannot support 51 kappa. Nevertheless, he reported in the same paper (37) that when 51 kappa is microinjected into the cytoplasm of a symbiont-free strain of 116, kappa persists indefinitely. A similar observation was made for 138 mu, which Gibson said can be maintained in stock 131 which he claims lacks maintenance genes. However, our interpretation of the significance of these reports is drastically altered when it is noted that Rosenthal and Levine (see section above on the genes) concluded that maintenance genes were present in the stocks injected by Gibson.

Maintenance of symbionts after microinjection has been reported by Gibson (37) in the presence of genes for nonmaintenance in three other cases, but all under more restrictive conditions. In these cases, retention of the injected symbionts was permanent only if the injections were made at not more than 20 to 30 fissions after heterozygotes had undergone autogamy and become homozygous for genes for nonmaintenance. If the injections were made later, the symbionts were not retained. The three cases were 540 mu injected after autogamy into symbiont-free segregants obtained from a cross of 540 $\times$ 513, 299 lambda injected into symbiont-free segregants obtained from a cross of 299 $\times$ 131, and 562 alpha injected into symbiont-free segregants obtained from a cross of 562 $\times$ 576.

The meaning of these findings, if substantiated, is not clear. Gibson suggests that the genes controlling maintenance are not equally active at all stages of the life cycle. Perhaps a more thorough investigation will show that certain of the strains Gibson believes to lack maintenance genes actually have them. The tendency for symbionts to be lost during nuclear reorganization even in the presence of maintenance genes was discussed above in the section on nongenic factors affecting maintenance. If this explanation is not correct, then maintenance genes may have to be redefined.

Which is Active, K or k?

The question of whether *K* produces a factor for the maintenance of kappa or whether *k* produces an inhibitor has been considered in some detail, but has never been resolved. Sonneborn (130) favors the view that *K* produces a factor necessary for kappa. He cites as evidence (i) the reduced number of kappas in heterozygotes, (ii) the evidence of Chao (27) which shows a positive correlation between macronuclear size (and therefore number of *K* genes) and kappa number in *KK* clones which have macronuclei of different sizes, and (iii) the transitory rise in kappa number after conjugation in cells of genotype *KK* when additional nuclear material in the form of macronuclear fragments is present (26). Nevertheless, it appears that the reduced number of kappas in heterozygotes could just as well be due to an inhibitor produced by *k*. Although it is true that a cell with more nuclear material should have more *K* genes, it does not follow that more kappa stems from the specific increase in *K* genes in cells with more nuclear material. The symbiotic

relationship between kappa and *Paramecium* may very well represent a delicately balanced state in which a change in any one of numerous factors may cause a change in the population level of kappa. Thus, cultures of paramecia containing 51 kappa often lost their kappa in our laboratory under conditions of slow growth. Furthermore, it is well known that when killers are placed in axenic media of various sorts, kappa may sometimes rise to very high levels, or may disappear altogether (118). Very high numbers of kappa are found in certain slow-growing mutant paramecia (130). In our opinion, the question of whether *K* or *k* (or perhaps both) are active is still unanswered.

KILLING

Specific Endosymbiont-Induced Resistance

The sensitivity of paramecia to the toxin produced by killers is affected by numerous factors. Most striking is the fact that all killers are resistant to their own toxin, whereas the corresponding kappa-free and mu-free strains are sensitive. Lambda-free paramecia are probably resistant to lambda killing (see above discussion on host-free culture). The resistance of symbiont-free lines of gamma, sigma, tau, and delta killers has never been investigated. Most killers exhibit little or no cross-resistance. For example, the spinner and hump kappa killers kill each other. The spinner killer 7 kills its paralysis mutant 7ml, yet the converse is not true, for 7 is unaffected by 7ml. Even in this case the possibility cannot be excluded that resistance by 7 to 7ml may be due to the small number of 7ml kappas regularly found in 7. Both kill kappa-free stock 7.

Nevertheless, unpublished results of Preer and Widmayer suggest that the resistance induced by kappa is not complete, for when killers of stock 7 are cultured at a sufficiently rapid rate to reduce the kappa level and intensity of killing only slightly, the killers can be killed by high concentrations of isolated kappa of stock 7. The fact that the killing is true kappa killing is confirmed by the fact that stronger killers produced by slower growth are resistant to the same concentrations of isolated kappa. Yet even the stronger killers succumb when the kappa concentration is made still greater (see section on effects of the endosymbionts on their hosts).

The specific resistance conferred by endosymbionts such as kappa remains as one of the most intriguing unsolved problems about kappa. Resistant, nonkiller mutants of the kind studied by Widmayer are informative. They show that R bodies and killing activity are not essential in producing resistance. The presence of prophage, however, may be. It may be that certain genes of paramecia are turned off or on by the presence of the prophage in kappa, resulting in resistance.

Other Factors Influencing Resistance

Various environmental and genetic factors determining resistance to kappa-bearing killers have been studied. Well-fed paramecia are more resistant than starved paramecia (130). Certain serotypes are more resistant than others (5). Stock 53 cultured at 31 C is more resistant to stock 7 killer than when it is cultured at a lower temperature (J. R. Preer, unpublished data). The specific stock is also important, for sensitive stock 53 is more resistant to killer stock 7 than is sensitive stock 30. Yet both of these sensitive syngen 2 stocks along with all stocks of syngen 2 are uniformly more resistant to the 7 killer than are the other syngens of *P. aurelia* (129). The effect is so marked that it may be used as an easy and reliable method of identifying syngen 2. Other species of *Paramecium* may also be sensitive, whereas still others are resistant (J. R. Preer, unpublished data). Numerous strains of *P. caudatum* have been tested and all have proven sensitive to 7 killer. The only two stocks of *Paramecium calkinsi* tested have both proved resistant to 7. Paramecia must have a functional ingestatory apparatus to be killed by stock 51, even though kappa has already been taken into a sensitive (76, 130) (see discussion in section on route of entrance of the toxin into sensitives below). Butzel and Pagliara (24) believe that this effect is caused by cessation of protein synthesis during starvation, for chloramphenicol inhibits the action of the toxin in treated sensitives. A similar inhibition is produced by azide and dinitrophenol, which prevent protein synthesis indirectly by inhibiting the terminal electron transport system (24).

Mueller and Sonneborn in 1959 (74) tested several killers with a number of different ciliated protozoa; *Tetrahymena*, *Colpidium*, *Colpoda*, and *Stentor*. They (74) also tested numerous algae, rotifers, and *Cladocera*. All were resistant except one, namely stock 51 killed *Colpidium colpidium*, the only example of a cell other than *Paramecium* being killed by a killer.

Little is known in regard to sensitivity to the killing produced by the non-kappa-bearing killers such as gamma, lambda, etc. Sensitivity to the rapid lysis, lambda-, and sigma-bearing killers is known to be very restrictive. Syngens 3, 5, and 9 of *P. aurelia* are most sensitive to the rapid lysis killers. The syngen 3 sensitive stock

152 is often used as a general sensitivity tester for all kinds of killers because it is sensitive to all known killers whatever their basis (M. V. Schneller, personal communication).

Nature of the Toxin Produced by Kappa

It has been known for many years that the toxic activity of kappa-bearing killers is normally associated only with bright kappas (70, 96, 112) and that only one bright is required to kill a sensitive (4, 78).

In 1964 Preer and Preer (100) showed that isolated R bodies of stock 7 contain most of the killing activity of the whole bright after lysis of kappa with sodium dodecyl sulfate. Ultrasonication was also found to be effective (100). When active, isolated, stock 7 R bodies are unrolled by heat, sodium dodecyl sulfate, or phosphotungstic acid, most activity is lost, and any residual activity can be ascribed to still-rolled R bodies. When 7 R bodies are broken down by extensive ultrasonic treatment, by grinding with glass beads in a Mickle disintegrator, or by use of a French press, all activity is lost. Even when very highly concentrated suspensions of R bodies, capable of killing in dilutions on the order of 10^{-10} are used, all residual activity is ascribable to remaining unbroken R bodies. Highly concentrated phage-like structures isolated from stock 562 kappa with cesium chloride gradients are also incapable of killing.

What can be concluded from these facts? Either the toxin is very labile and is destroyed or otherwise made inactive by all the treatments described above, or one of the elements necessary for killing is the unwinding of the R body. This latter possibility seems more likely, and further evidence in its favor will be discussed in the section on the role of the R body below.

One clue to the nature of the toxin is the fact that treatment of isolated R bodies from the spinner killer stock 7 with chymotrypsin changes the toxic action of the R bodies so that they cause paralysis rather than spinning in sensitives (94). This fact suggests that the toxin is protein. There is considerable evidence that the toxic effects are not due to the multiplication of the phage within sensitives. First, the initial effects of the killing action of stock 7 are extremely fast. Rapid swimming begins only 7 to 9 min after exposure at 23 C (53). The effect would appear too rapid, even for T phages in *E. coli*, if the effect is to be ascribed to phage multiplication. Second, careful examination of affected sensitives by electron microscopy has revealed no evidence of the production of phage-like structures. Third, the effect of chymotrypsin just described argues against the view that the phages enter sensitive paramecia and kill by multiplying. It is difficult to see how modification by chymotrypsin of proteins initially present in an infecting virus particle could change the subsequent process of multiplication of the virus so that it would produce a different type of toxic activity. On the other hand, if a protein associated with the R body acts as the toxin, its modification by chymotrypsin might easily lead to a modification of its effect on sensitives. Fourth, the highly active isolated R bodies of stock 1039 have only a few adhering defective phages, and virtually all appear to be empty.

Thus, these arguments make it unlikely that the toxic effects are due to phage multiplication. The possibility that the toxin is the coat protein itself deserves consideration. Suppose, for example, that the phage coat protein actually constitutes the toxin, while the unrolling of the R body serves as a necessary factor in killing. Inactive isolated R bodies might then lack adhering phages (empty or full), and active R bodies should surely retain them. Inspection of Table 2 shows that only the R bodies of the 7 class R bodies show appreciable activity when freed from brights by ultrasonic treatment. Numerous adhering phages are found in all but one case, stock 1039, which has only very few adhering phages, virtually all of which are empty, but which yields highly active isolated R bodies. Thus, it is impossible that either the intact phages or the empty phage-like structures are the toxin in stock 1039.

Are there possibilities for the toxin other than the phages? The capsomeres perhaps represent a more likely candidate, for they are found abundantly on all of the toxic isolated R bodies of class 7 kappa (see Fig. 30) and on very few others. There is, however, one glaring exception, and that is the case of 51m1 R bodies which are inactive, but bear numerous capsomeres. If the capsomeres are the toxin, they must be easily inactivated in the case of 51m1. Another difficulty with the assumption that the capsomeres are the toxin, is the fact that they are not found in the brights of the 51 and 562 classes. One would have to assume that in those two classes proteins analogous to capsomeres take on a different molecular conformation. They could, for example, be represented by the helices of the 51 group and by molecules of less regular appearance by electron microscopy in the 562 group.

Finally, it should be noted that there are no facts which rule out the possibility that the R body ribbon itself is the toxin. Such a possibil-

ity might, however, be surprising, for although the toxins appear to be rather labile substances, the R body proteins appear to be very stable.

Maturation of Toxic Particles

In 1959 (130) Sonneborn observed that in homogenates of stock 51 killers the number of toxic particles is on the order of 1% of the bright kappa particles. He suggested that after their formation brights are normally inactive and that a small proportion later acquire toxicity through a process of maturation. Such an explanation is, of course, not the only one possible. It could be that all bright kappas are toxic, but that there is only a small probability that any particular bright will be taken up and that the toxin will reach its site of action in an active form and then by its action result in the death of a sensitive. Stronger, but still not decisive, evidence for the maturation hypothesis is provided by the fact that killer homogenates increase in potency at 20 C for an hour or so after being prepared (73, 130, 133, 146, 158). Sonneborn cites a number of other peculiarities in experiments on the inactivation of the toxin which can be resolved by the maturation hypothesis. The reader is referred to Sonneborn's review (130, p. 256–262) for a discussion of these early experiments. Some later experiments are described below.

Butzel and Pagliara (24) found that treatment of homogenates with sodium azide or dinitrophenol reduces activity. The inhibition is not found if adenosine 5'-triphosphate and dinitrophenol are used together. They suggested that maturation requires an active terminal electron transport system. They found that killing is enhanced if homogenates are treated with sodium malonate or sodium fluoride and explain this finding by suggesting that maturation is enhanced by suppression of the tricarboxylic acid cycle.

Nobili (77) made a study of the kinetics of the increase of killing activity in homogenates of amacronucleate killers appearing in lines homozygous for the gene *am*. He suggested that the increases in killing indicated maturation of brights rather than an actual increase in number of brights, but no data on counts of numbers of brights were given.

More recently Preer et al. (101) suggested that the reason that ultraviolet light induces the production of R bodies in 51 kappa, but does not enhance killing activity of homogenates, may be explained by an interference of ultraviolet light with maturation of brights. They also point out, however, that ultraviolet induction under the conditions used by Preer et al. may produce brights which are defective in some way.

The hypothesis has certainly proved useful and can explain many results. Nevertheless, it is not clear to the reviewers that the hypothesis has been established. Alternative explanations may be possible in all cases.

Route of Exit of Toxin from Killers

Nobili (76) has shown that amacronucleate paramecia formed in killer lines homozygous for the gene *am* liberate toxin only during the first 3 h after their formation. If such paramecia are homogenized after 3 h, however, it can be shown that numerous toxic particles are not only present, but still increasing in number. Such paramecia do not feed and food vacuoles disappear from the cytoplasm by the end of the 3-h period. The close correlation between cessation of production of toxin and disappearance of food vacuoles is interpreted to mean that kappa is normally released to the outside through the cytopyge.

Route of Entrance of Toxin into Sensitives

The route by which the toxin gains entrance into sensitives has been debated for many years. In Sonneborn's 1959 review (130) he pointed out the difficulties in conceiving how entrance could be gained through a barrier of beating cilia and several membranes of the cortex of sensitives. In considering the possibility that the food vacuoles might be the site of entry, he noted that gulletless paramecia which are sometimes produced, as well as conjugating paramecia which have nonfunctioning gullets for a certain period, are totally resistant. Nevertheless, he discounted the importance of these facts because he had shown that sensitives exposed to the toxin prior to conjugation do not die later, provided they are allowed to conjugate soon after exposure. He also pointed out that mate killers clearly do not deliver their toxin by way of the food vacuoles of sensitives. Furthermore, he cited the conclusions from unpublished data which he said showed that sensitives remove toxin from the surrounding medium much more rapidly than killers, even when he had attempted to allow for the amount of toxin produced by killers. This experiment, he concluded, agrees better with a route of entrance other than the gullet and food vacuole. Noting the difficulty in measuring the number of toxic particles absorbed by killers which are simultaneously producing toxin, Mueller (73) turned to 51A2, a resistant nonkiller mutant. She gave clear evidence that it removes toxin from the medium at essentially the same rate as sensitives.

In 1961 Nobili (76) showed that the amacronucleate paramecia produced in sensitive lines homozygous for the gene *am*, although having gullets, do not feed and are totally resistant to the killing of stock 51 kappa. But he also showed that when sensitives containing macronuclei are exposed to toxin and then allowed to divide, the amacronucleate progeny are never killed, whereas the progeny containing nuclei often succumb. This experiment shows once again the necessity of a functional ingestatory apparatus if toxin which has already been absorbed is to kill (see discussion above under other factors influencing resistance).

In 1960 Butzel et al. (23, 34) showed that treatment of sensitives with the phospholipids lecithin and cephalin, after or during exposure to 51 toxin, makes the sensitives more resistant. They, like Sonneborn and Nobili, concluded that their experiments are in better accord with the view that the food vacuoles are not the site of entry of the toxin.

In 1967 Dryl and Preer (34) demonstrated a very precise correlation between the times of complete resistance and cessation of feeding which occur during conjugation, autogamy, and during a 25- to 27-min period at fission. They concluded that the toxin enters by way of the food vacuole. In 1971 Jurand et al. (53) made a detailed study of sensitives exposed to killer toxin and found, as already noted, that penetration of food vacuole membranes by unrolling R bodies and degeneration of food vacuole membranes are among the first visible signs of toxic activity. Extensive observations by light and electron microscopy yielded no evidence for even momentary union of bright kappas and the surface of paramecia, except in the process of food vacuole formation. In a detailed discussion of the problem, they pointed out that specialized methods of surface injection of toxin would require specialized surface molecules which would not likely be found both on the surface of brights and on the surface of free R bodies. (R bodies were first found to be toxic in 1964 [100]).

The present reviewers do not find the evidence for entrance of the toxin by routes other than the food vacuoles compelling, and conclude that the newer facts make it most likely that the food vacuole is the site of entry.

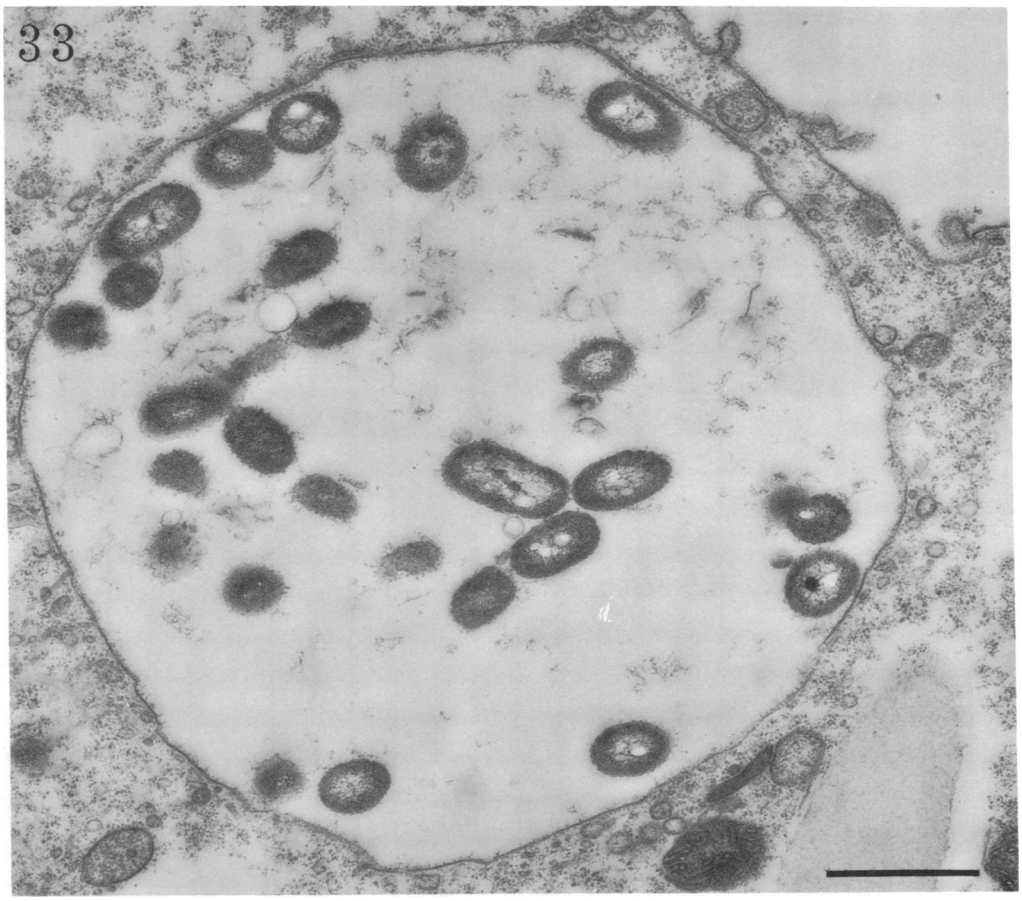
The fact that free R bodies and the bearers of R bodies, the bright kappas, are the sole possessors of toxic activity suggests an important role of the R body in killing. As already noted, Jurand et al. (53) have used the electron microscope to study the effect of isolated 7 kappa on sensitives of stock 16. Kappa is found to enter the food vacuoles where it quickly breaks down. The gullet is affected and formation of new vacuoles has virtually ceased 25 min after initial exposure. After 37 to 60 min from the time of first exposure to kappa, unrolling occurs, and many R bodies are seen in section to be penetrating the food vacuole membrane and entering the cytoplasm (see Fig. 34). In the same sections large numbers of food vacuoles show breakdown in their membranes, resulting in a mixing of the contents of the vacuoles with the cytoplasm (see Fig. 35). Such breakdown never occurs in untreated sensitives (see Fig. 33). Breakdown occurs only rarely in vacuoles formed after paramecia are removed from the kappa. These results suggest that the primary target of the toxin is the food vacuole membrane, and also suggest that unrolling of R bodies and penetration of the vacuole membranes are important in the process, but precisely how is not known.

CONCLUSIONS

Taxonomy

The data which bear on the nature of the endosymbionts of *P. aurelia* have been summarized in the first paragraph of the introduction and considered in detail in the body of this review. As already noted, the evidence that the endosymbionts are bacteria is now overwhelming. Two objections noted earlier (130) are now viewed as unimportant. The first, that there is a paucity of enzymes in kappa, has been clearly shown not to be the case (see the section on chemistry and physiology above). The second, that no well-defined nuclear regions are observed, remains. Nevertheless, this fact is not considered to be of particular importance in view of the variability and difficulty in fixing and staining the bacterial nuclear regions of numerous other bacterial species cultured under varying conditions (103).

Alpha with its long helical form and tapered ends closely resembles the gliding bacteria of the genus *Cytophaga*. This conclusion is in agreement with the unpublished work of C. Wiblin who also studied the possible bacterial relationships of the other endosymbionts. Although alpha has never been seen to move, its ability to become situated selectively in the nucleus a short time after infecting alpha-free strains of paramecia (98) implies very strongly that it is motile. Since flagella are not present, movement by gliding is likely. The DNA of alpha is estimated from its buoyant density to be 36 mol percent G+C (36% G+C) (Table 4 and reference 65), that of the genus *Cytophaga* 33 to 42% G+C (80); no closely related genera have similar G+C ratios. Alpha is so similar to



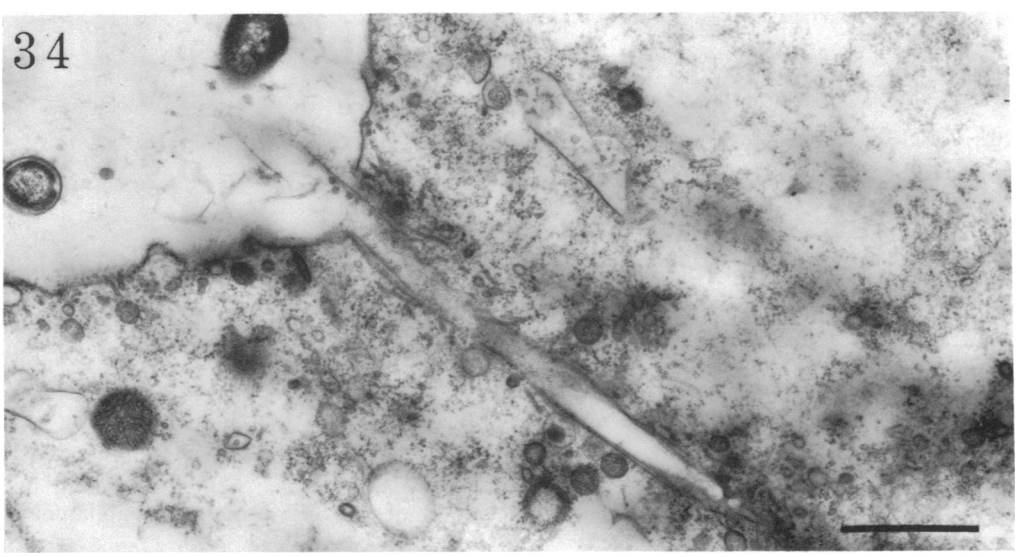


FIG. 33. *Electron micrograph of section of food vacuole of stock 16 (syngen 1) control. ×20,000, scale 1 μm.*

FIG. 34. *Electron micrograph of section of food vacuole of stock 16 after treatment with isolated kappa of stock 7. Note unwound refractile body entering the cytoplasm from food vacuole (large clear area in upper left) and the few phage-like structures adhering to the inner end of the refractile body. ×18,000, scale 1 μm; after Jurand et al. (53) with permission of J. Exp. Zool.*

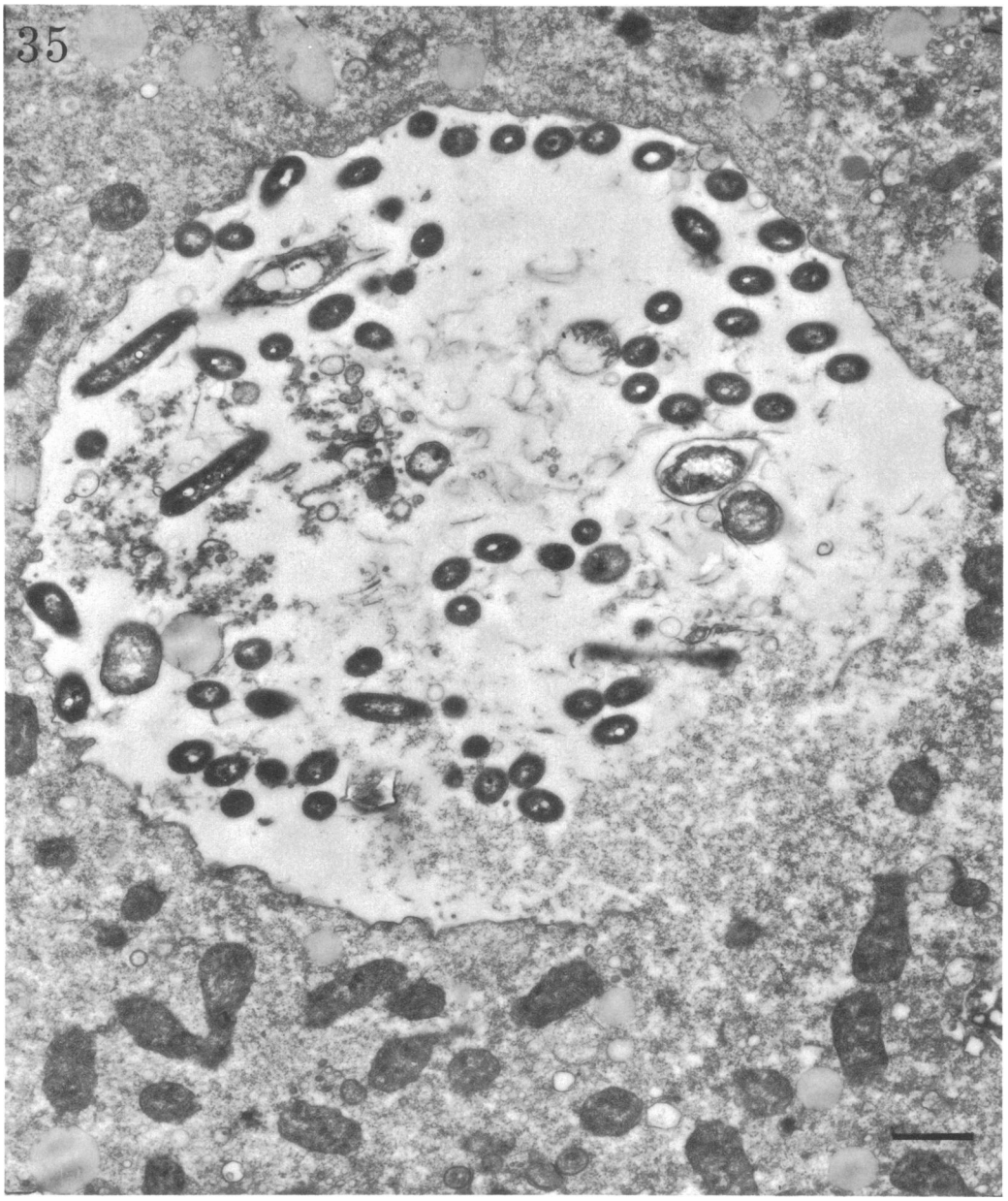


FIG. 35. *Electron micrograph of section of food vacuole of stock 16 (syngen 1) after treatment with isolated kappa of stock 7. Note extensive break in the membrane in lower right and the entrance of cytoplasm into the vacuole. ×11,000, scale 1 μm; after Jurand et al. (53) with permission of J. Exp. Zool.*

the known members of the genus that it is being designated *Cytophaga caryophila* n. sp. (see addendum). The other previously described nuclear bacterial parasites of *Paramecium* (all apparently in *P. caudatum*) are *Drepanospira muelleri* (82), *Holospora undulata*, *H. elegans*, and *H. obtusa* (43). Their form is rather similar to alpha; however, all differ from alpha in forming spores. In addition *Holospora* often forms buds in reproduction. The recently described omega (81a, 81b, 81c), found in the micronucleus of *P. caudatum*, apparently resembles alpha very closely.

The remaining forms are clearly different from all other known bacteria and are being placed into three new genera (see addendum). They are all gram-negative, nonspore-forming rods, difficult or impossible to culture outside of

living paramecia. The first genus consists of the nonflagellated forms: kappa (35 to 43% G+C) with its ribbon-like coiled R bodies, *Caedobacter taeniospiralis;* mu (33 to 37% G+C), the mate-killer endosymbionts, *Caedobacter conjugatus;* gamma (38% G+C), the small killer endosymbiont enclosed in a secondary membrane, *Caedobacter minutus*, and nu (36% G+C), the nonkilling forms; *Caedobacter falsus*. The second genus contains the large, heavily peritrichously flagellated forms which show rapid-lysis killing: lambda (49% G+C) with straight rods is designated *Lyticum flagellatum* and sigma (45% G+C) with helical or curved rods is *Lyticum sinuosum*. Delta with its sparse peritrichous flagella, thick outer layer about its cell wall, and wide distribution among the syngens of *P. aurelia* is designated *Tectobacter vulgaris*. No determinations of DNA base ratios have been made for delta.

The work on DNA hybridization by Behme (20) justifies these groupings. The strong reassociation between the kappas of different stocks of paramecia (72 to 86%) supports their status as a single species. The reduced, but marked, hybridization between kappa, mu, gamma, and nu (40 to 84%) affirm their designation as different species within a single genus, *Caedobacter*. Strong associations between the lambdas (82 to 85%) and between sigma and lambda (66 to 78%) are consistent with their designation as two species within a single genus, *Lyticum*. Finally the weak cross-reactions between *Caedobacter* and *Lyticum* (3 to 28%) suggest a weak relationship between the two. More data are needed before anything can be said about the relationship between delta and these other forms. The only data at variance with these proposed taxonomic relationships are the similarity between alpha and mu indicated by the hybridizations using cRNA above in the section on nucleic acid hybridization. Taking into consideration all factors, however, it seems reasonable to adopt the present proposals.

The endosymbionts are surely chemotrophic and presumably they derive their energy from organic compounds. The fact that kappa respires shows that it is either aerobic or facultatively anaerobic. The endosymbionts are negative to the Gram stain, and this characteristic is confirmed by electron microscopy of thin sections which shows typical gram-negative cell walls. They are generally shaped as rods and occasionally as coccobacilli. The spiral shape of sigma is not thought to be of major taxonomic significance because of the otherwise obviously close relationship between sigma and the rod-shaped lambda. The fact that the three new genera (with the possible exception of *Lyticum*) cannot be cultured free from *Paramecium* not only sets them apart from all free-living bacteria, but means that many diagnostic characteristics are unavailable.

Nevertheless, enough is known to look for relatives among known bacteria. The gram-negative aerobic rods (8th ed., Bergey's Manual) have DNA base ratios of 52 to 70% G+C and are all ruled out by the low base ratios of *Caedobacter* (35 to 43% G+C) and also *Lyticum* (45 to 49% G+C). See reference 80 for base ratios of bacteria. However, some of the gram-negative facultatively anaerobic rods have similar G+C ratios. Of these, some are ruled out on grounds other than base ratio. Thus, *Flavobacterium* (30 to 42% and 62 to 70% G+C) is colored, but the endosymbionts of *P. aurelia* when isolated in masses are all colorless. *Pasteurella* (37 to 43% G+C), unlike the endosymbionts, shows bipolar staining. *Haemophilus* (38 to 43% G+C), however, cannot be strictly ruled out. The highly motile genus *Proteus* also contains species which are rather close (36 to 42% and 51 to 53% G+C) to *Caedobacter* and *Lyticum*. *Proteus* also is virtually identical to kappa in its cytochromes, both containing a_1, a_2, b_1, and *o*. The gram-negative aerobic coccobacilli (the moraxellas) also have similar G+C contents, but most, unlike kappa, have cytochrome *c* (13a). The oxidase-negative moraxellas are placed into the genus *Acinetobacter* (37-46% GC) (13b, 141) and, except for their somewhat more coccoid form, resemble the endosymbionts in many ways.

The *Rickettsia* and relatives are of special interest because they are intracellular forms found in vertebrates or arthropods, or both. *Lyticum*, however, is very large and flagellated. It is therefore rather different from the nonflagellated, small, pleomorphic, and sometimes coccoid forms of the *Rickettsia*-like organisms. The rod-shaped *Caedobacter* is much less pleomorphic than most *Rickettsia*. G+C ratios in the *Rickettsia* vary from 30 to 45%, some being rather close to *Caedobacter* (141a): *Coxiella burneti* is 42 to 45% G+C; *Rickettsia prowazekii* and related species are 30 to 32% G+C; *Wolbachia persica* is 30% G+C, and *Rochalimaea quintana* is 39% G+C.

Summary and Concluding Remarks

It is concluded that the endosymbionts of *P. aurelia* are bacteria, that alpha is a new species of the genus *Cytophaga*, and that the remaining symbionts fall into three new genera. They are designated *Caedobacter* (kappa, mu, gamma, and nu), *Lyticum* (lambda and sigma), and *Tectobacter* (delta). Furthermore, these three genera are related to each other and possibly to

certain of the gram-negative, facultatively anaerobic rods, such as *Proteus* or *Haemophilus*, or to the aerobic, oxidase-negative, coccobacillus *Acinetobacter*. Failure to culture them is assumed to be due to evolutionary modifications brought about by their intracellular habitat. More information about their enzymatic constitution and additional studies on DNA hybridization offer means for obtaining more precise information about the possible relationships between other bacteria and the endosymbionts of *P. aurelia*.

The endosymbionts are dependent on specific nuclear genes of *Paramecium* for their maintenance. These genes appear to be highly specific in respect to the particular endosymbiont whose maintenance they control. Since stocks of paramecia often lose their symbionts spontaneously and since this loss is particularly likely to occur during nuclear reorganization in some stocks, proper controls and great care must be used in studying the action of the controlling genes. The mechanism of the action of the genes is completely unknown at this time.

All strains of kappa have phage-like structures. They do not appear to be infective and are often incomplete morphologically, indicating that they are defective phages. They are carried by all kappas, generally in a relatively inert prophage form (whether integrated with the bacterial chromosome or not is unknown) and in a lethal synthesis (which prevents kappa's further reproduction) are occasionally spontaneously induced and form large numbers of microscopically visible forms. In one strain they are ultraviolet inducible. Some, like Sp 562, the defective phage of stock 562, are spherical (icosohedral); others like He 51, the defective phage of stock 51, are helical. They resemble plasmids like those colicinogenic factors which produce microscopically visible colicins with a phage-like morphology; however, the colicins are toxic to other bacteria, whereas the defective phages of *Paramecium* kill protozoans. The DNA of He 51 consists of covalently closed circles about 20 μm in length. Induction, spontaneous or ultraviolet induced, results in the simultaneous formation of phage and the R body, a unique and remarkable structure in the form of a compact roll of ribbon consisting of protein. R bodies have the capacity to unroll suddenly (some reversibly) into long filaments. It may be that the R body is specified by the DNA of the defective phages, for R bodies only appear in the presence of the phage-like structures.

Lambda, sigma, kappa, mu, and gamma all produce toxins highly specific for *Paramecium*. The toxins of the various kappas are dependent upon the defective phages and are thought to be proteins. Each strain of kappa renders the paramecia in which it lives specifically resistant to its own toxin. The mechanism of this remarkable phenomenon is unknown. The R body itself is necessary for the killing of sensitive paramecia, possibly by unrolling in the food vacuoles of sensitives and by helping deliver the toxin to its site of action.

The defective phages, the bacterium kappa, and the protozoan *Paramecium* thus constitute a highly integrated three-element system. The selective forces involved in maintaining this system are unknown, but in the laboratory, at least, killer paramecia bearing kappa have an advantage over nonkiller paramecia lacking kappa.

The endosymbionts of *P. aurelia* are but one example of a very large and common class of biological entities which are generally transmitted by inheritance; they include intracellular parasites, symbionts, and cases of infective heredity. Numerous examples of such phenomena involving viruses, bacteria, protozoa, fungi, and the multicellular plants and animals have been reviewed elsewhere (88, 90, 130) and will not be discussed here.

ADDENDUM: DESCRIPTION OF NEW TAXONOMIC GROUPS

All type strains are present in the collections of J. R. Preer and T. M. Sonneborn in Bloomington, Ind., G. H. Beale in Edinburgh, Scotland, and I. Gibson in Norwich, England.

Cytophaga caryophila n. sp. Gr. n. *caryum*, nut or nucleus: Gr. adj. *philus*, loving, M.L. fem. adj. *caryophila*, nucleus loving. The type strain is *C. caryophila*, 562 (present in stock 562 of *Paramecium aurelia*).

Rods 0.3 to 0.5 μm in width. Nondividing, presumably mature and motile forms are 5 to 6 μm long. Dividing forms 1 to 3 μm in length. Ends tapered and rounded. Gram-negative. G+C content is 36% (buoyant density). Found in the macronucleus, but not micronucleus of *Paramecium aurelia*, stock 562, syngen 2. It has been known as alpha. Is highly infective to a few other stocks of syngen 2 of *P. aurelia*. At least one of the many stocks unable to become infected with alpha has been found to lack a suitable maintenance gene. At nuclear reorganization in *Paramecium*, it migrates from fragments of old macronucleus to newly forming macronuclear anlagen.

Caedobacteriaceae n. fam. M.L. fem.n. *Caedobacter* type genus of the family; *-aceae* ending to denote a family; M.L. fem.pl.n. *Caedobacteriaceae* the *Caedobacter* family. Rod-shaped, rarely spiral-shaped cells endo-

symbiotic within the cytoplasm of the ciliated *Protozoa*. Many stocks of *Paramecium* lacking the endosymbionts have been shown to lack suitable maintenance genes. They are gram-negative, nonspore-forming rods. Some are peritrichously flagellated. Others have no flagella. They are chemotrophic, organotrophic, and either aerobic or facultatively anaerobic. Culture free of paramecia impossible or exceedingly difficult. Relationship between most has been shown by DNA hybridization studies. DNA base ratios are 35 to 49% G+C (buoyant density).

Caedobacter n. gen. L. n *caedes*, act of killing. M.L. mas.n. *bacter*, the masculine equivalent of the Gr. neut.n. *bactrum*—a rod; mas.n. *Caedobacter*, the bacterium which kills. Small gram-negative, nonflagellated rods, endosymbiotic on *Paramecium*. Culture free of *Paramecium* impossible or exceedingly difficult. Most produce toxins capable of killing paramecia which lack the symbionts. 35 to 43% G+C (buoyant density). The type species is *Caedobacter taeniospiralis*.

Caedobacter taeniospiralis n. sp. L. n. *taenia*, ribbon, L. adj. *spiralis*, coiled. M.L. mas.adj. *taeniospiralis*, coiled ribbon. The type strain is 51 (present in stock 51 of *Paramecium aurelia*). A number of different strains with special characteristics exist. Rods or coccobacilli 0.4 to 0.7 μm wide and 1.0 to 2.5 μm long. They are distinguished from other species of the genus by the fact that 1 to 50% of all populations contain a single (rarely two) refractile or "R" body consisting of a proteinaceous tape 0.2 to 0.5 μm wide, 2 to 15 μm long, and 13 nm thick wound into a roll consisting of 2 to 15 turns. All known strains bear DNA which can, at least at some stage, become extrachromosomal and increase in quantity concomitant with the production of R bodies, phage-like structures, and a toxin which is capable of killing sensitive strains of paramecia when ingested. The phage-like structures may be either icosohedral or helical. The R bodies unroll when taken into the food vacuoles of sensitive paramecia. Long known as kappa. Oxygen uptake of strain 51 stimulated by glucose, sucrose, and intermediates of the tricarboxylic acid cycle. Strain 51 contains cytochromes a_1, a_2, b_1, and o.

Very common among the stocks of syngens 2 and 4 of *P. aurelia* where it inhabits the cytoplasm. Certain of the stocks lacking kappa do not have a suitable genotype for its maintenance. Cultivation free of the cytoplasm of *Paramecium* has not been possible. Gram negative, 35 to 43% G+C (buoyant density).

Caedobacter conjugatus n. sp. L. part. *conjugatus*, conjugated, M.L. mas.adj. *conjugatus*, conjugating. The type strain is 540 (found in stock 540 of *P. aurelia*). Rods 0.3 to 0.5 μm wide and 1.0 to 4.0 μm long. Originally called mu. They are distinguished from other species of the genus by the fact that they produce a toxin which is capable of killing sensitive strains of *Paramecium* only after cell-to-cell contact between killer and sensitive at conjugation.

The cell wall has been shown to contain diaminopicolinic acid and probably muramic acid. Cultivation free of the cytoplasm of *Paramecium* has been reported to occur on a very complex medium, but only very slow growth rates and low population densities have been obtained. Gram negative. 35 to 37% G+C (buoyant density). Found in the cytoplasm of syngens 1, 2, and 8 of *P. aurelia*.

Caedobacter minutus n. sp. L. adj. *minutus*. M.L. mas.adj. *minutus*, small. The type strain is 214 (found in stock 214 of *P. aurelia*). Rods often double, 0.25 to 0.35 μm wide and 0.5 to 1.0 μm long (singles). This very small cell is unique in being surrounded by an extra set of membranes, apparently continuous with the endoplasmic reticulum of its host. Although never rising to high concentrations in the cytoplasm, the paramecia which bear them are nevertheless very strong killers, killing sensitives of many or all of the other syngens. Gram negative, G+C content is 38% (buoyant density). Found only in the cytoplasm of syngen 8 of *P. aurelia*. Has never been cultured free of *Paramecium*. It has been known as gamma.

Caedobacter falsus n. sp. L. adj. *falsus*, false. M.L. mas.adj. *falsus*. The type strain is 225 (found in stock 225 of *P. aurelia*). Rods 0.4 to 0.7 μm wide and 1.0 to 1.5 μm long. Called "nu" originally. The only members of the genus, aside from mutants of kappa, which are not known to produce toxins. However, the type strain is said to increase the resistance of its host to the toxin produced by *Lyticum flagellatum*. Gram negative. G+C content is 36% (buoyant density). Found within the cytoplasm of *P. aurelia*, syngens 2 and 5.

Lyticum n. gen. L. adj. *lyticus*, dissolving. M.L. neut.n. *Lyticum*. Large gram-negative, flagellated rods, straight, curved, or spiral. Width 0.6 to 0.8 μm; length of single forms 3.0 to 5.0 μm. Numerous peritrichous flagella, with little or no mobility. Culture free of *Paramecium* impossible or exceedingly difficult. Produce very labile toxins which kill very quickly by lysis. Sensitives affected by lysis are all within syngens 3, 5, and 9 of *P. aurelia*. G+C is 45 to 49% (buoyant density). The type species is *Lyticum flagellatum*. Gram negative.

Lyticum flagellatum n. sp. Gr. adj. *flagel-*

lum, flagella. M.L. neut.adj. *flagellatum*, flagellated. The type strain is 299 (found in stock 299 of *P. aurelia* of T. M. Sonneborn, (Bloomington), not van Wagtendonk (Miami) collection. Straight rods 0.6 to 0.8 μm wide and 2.0 to 4.0 μm long. Originally called "lambda." Numerous peritrichous flagella. Culture free of *Paramecium* has been reported to occur in a very complex medium, but very slow growth rates and low population densities have been obtained. Produces a labile toxin which quickly lyses sensitive cells of syngens 3, 5, and 9 of *P. aurelia*. Gram negative. G+C 49% (buoyant density). Found within the cytoplasm of *P. aurelia* of syngens 4 and 8.

Lyticum sinuosum n. sp. L. adj. *sinuosus*, curved. M.L. neut.adj. *sinuosum*, curved. The type strain is 114 (found in stock 114 of *P. aurelia*). Curved or spiral rods 0.7 to 0.9 μm wide and 2.0 to 10.0 μm long, sometimes forming chains of two to three cells. Originally called "sigma." Numerous peritrichous flagella. Produces a toxin which kills sensitive cells of syngens 3, 5, and 9 of *P. aurelia*. Gram negative. G+C 45% (buoyant density). Found within the cytoplasm of *P. aurelia* of syngen 2.

Tectobacter n. gen. L. mas.n. *tectum*, covering. M.L. mas.n. *bacter*, the masculine equivalent of the Gr. neut.n. *bactrum*, a rod. M.L. mas.n. *Tectobacter*, the bacterium with a covering. Gram negative, sparsely peritrichously flagellated, sometimes motile rods. Culture free of the cytoplasm of *Paramecium* unknown. Some reports that a slow acting toxin is produced which paralyzes sensitive *Paramecium*, but no strains presently available show toxicity. G+C content unknown. Only the type species *Tectobacter vulgaris* is known.

Tectobacter vulgaris n. sp. L. adj. *vulgaris*, common. M.L. mas.adj. *vulgaris*, common. The type strain is 225 (found in stock 225 of *P. aurelia*). Straight rods 0.4 to 0.7 μm wide and 1.0 to 2.0 μm long. Originally called "delta." Distinguished by outer covering around its cell wall in sections by electron microscopy. Gram negative. G+C unknown. Found in syngens 1, 2, 4, 6, and 8 of *P. aurelia*, often with other symbionts.

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