Perspectives

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Sonneborn and the Cytoplasm

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IN the late 1930s when Tracy Sonneborn was in graduate school and just starting his work on the genetics of protozoa, classical studies on genetics were almost finished. The second edition of *Principles of Genetics* by E. W. SINNOTT and L. C. DUNN (1932) that appeared in that period was quite sophisticated, containing a good account of chromosome theory, segregation ratios produced by complex gene interactions, chromosome and genetic maps, polyploidy, aneuploidy, multiple factor inheritance, sex determination, and evolution. The authors argued strongly that virtually all inheritance was ascribable to nuclear genes. Only Correns's work on plastid inheritance was cited as an example of cytoplasmic inheritance, and it was pointed out that this should be considered only a minor exception to the general rule that chromosomal genes determine the hereditary characters of the organism. Other biologists, particularly cell physiologists such as L. V. Heilbrunn at the University of Pennsylvania, were not so sure. They thought that classical genetics dealt only with superficial characters and that the fundamental characters of organisms such as membrane permeability, metabolism, etc., were controlled by the cytoplasm. It is not clear what Sonneborn thought of this controversy, but from what I know about Sonneborn, he must, at least, have had an open mind about the matter.

All classical genetics was based on multicellular organisms, in which characters were seen after a complex developmental process. Once Sonneborn had discovered mating types in the protozoa it became possible to carry out classical genetic studies with the protozoa and study their inheritance without an intervening period of somatic development. Perhaps the genetics of single-cell organisms, the protozoa, would prove to be a bit different from the classical picture.

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The inheritance of mating types: SONNEBORN (1937) made mixtures of numerous isolations of different lines of Paramecium and found that certain lines mated with each other, but never with themselves. See PREER (1997) for a *Perspectives* on much of the work of Sonneborn. A careful look at the pattern of matings revealed that there were a number of different mating types, I mating with II, III mating with IV, etc. Each pair of mating types determined a different mating group or species as they are now called. After discovering mating types, the first character that Sonneborn investigated was mating type itself. His findings were summarized later (SONNEBORN 1975). Early on he found a single segregating locus that controlled mating type, but its effect was layered over what he called *caryonidal* inheritance, a phenomenon clearly at odds with the classical picture of genetics. In caryonidal inheritance, mating type is fixed when the two macronuclei formed in each cell after autogamy or conjugation are each determined for one or the other of the two segregating mating types. These two independently determined nuclei are later segregated into clones that he called caryonides. Since cytological studies showed that the nuclei of these two caryonides are derived from one homozygous nucleus, an extraordinarily high mutation rate at a particular stage in the life cycle would be required to explain the results according to classical genetics. Surprisingly, Sonneborn found that while mating types in some species of the *Paramecium aurelia* group were inherited caryonidally, in other species mating type followed the cytoplasm of each mating partner. He even found that in one species mating type was controlled by a simple Mendelian factor. Simple Mendelism clearly was not the whole explanation.

Life-cycle changes: A look at the life cycle turned up more deviations from Mendelism. It had been known for many years that, after conjugation, Paramecium undergoes a period of immaturity for a variable number of fissions when cells are unable to mate (JENNINGS

1929). See Crow (1987) for a *Perspectives* on Jennings. This period is followed by a period of maturity when mating can occur. Finally, many fissions later, cells become senescent, grow slowly, and die. Sonneborn's mentor H. S. Jennings pointed out that, in cellular inheritance, the different stages of the life cycle should be considered hereditary differences, for they can last a very long time, depending upon the strain. Their inheritance, however, is clearly not Mendelian. At both autogamy and conjugation, meiosis occurs in the micronuclei and the old macronucleus is replaced with a new macronucleus derived from the micronucleus. Both autogamy and conjugation occur in the *P. aurelia* group of species, and autogamy can substitute for conjugation in the life cycle, except a period of immaturity does not follow from autogamy. Autogamy is induced by starvation if sufficient time has elapsed since the last autogamy or conjugation and can be suppressed by supplying an excess of food. The ability of cells to undergo autogamy when starved is also part of the life cycle. None of these aspects of the life cycle fit the expectations of classical Mendelian genetics. See Sonneborn (1975) for a summary of these and related findings.

Surface antigens: Sonneborn decided to look at the surface antigens of Paramecium by injecting cells into rabbits and obtaining antibodies able to immobilize the injected strain. He immediately was plunged back into cytoplasmic inheritance, for numerous stable serotypes emerged, each distinct from the others and all cytoplasmically inherited. But he also found that genes were involved in the differences between different genetic strains Sonneborn (1948).

Cortical inheritance: When they studied the cortex of Paramecium, Sonneborn and Beisson (1965) found that deviations again followed the cytoplasmic parent. Experiments showed that the determinants of the cortex lay not in genes, and not in the fluid cytoplasm, but in the cortical structure of the surface of the ciliates. Mendelism again failed to explain the inheritance of surface features of Paramecium.

κ and its relatives: The one example, however, that appeared most significant to Sonneborn, and was easily explored, was the inheritance of the cytoplasmic factor κ, responsible for the killer trait (reviewed in Sonneborn 1975). The presence of κ caused cells to produce toxins and to become resistant to the toxin that they produced. There were different kinds of killers as evidenced by the prelethal effects on sensitive strains of paramecia. Some killers caused sensitives to spin vigorously on their longitudinal axes before dying, others were simply paralyzed, still others developed large vacuoles, etc. In most strains the toxins are liberated into the medium in which killers live and are taken up by sensitives there. In other cases the toxins are transmitted only when sensitives conjugate with killers, and these killers, called mate killers, kill their partners. Each kind of killer required specific maintenance genes, yet a cytoplasmic factor also proved to be at the basis of each character. Sonneborn pointed out that these genes could allow κ's maintenance but could not initiate it.

The plasmagene hypothesis: Faced with an almost overwhelming number of cases of cytoplasmic involvement in the late 1940s, Sonneborn could not resist the temptation to present a theory, the plasmagene theory, to explain all these results (Sonneborn 1946, 1950). Although classical Mendelian genes were found, it was equally clear that virtually every trait also showed a non-Mendelian cytoplasmic pattern of inheritance. According to the plasmagene theory, there was a gene for each trait and each gene produced a cytoplasmic self-reproducing copy of a part of itself. The plasmagene theory was reinforced by the results of Spiegelman (1946) who was working on adaptive enzymes in yeast. He found that adapted strains remained adapted to a specific substrate even after the substrate had been removed and, when crossed to nonadapted strains, cytoplasmic inheritance was seen.

The analysis of κ and its relatives: Sonneborn did most of his work on species 4 of the *P. aurelia* group of species, while I was working on species 2 (see Sonneborn (1975) for an account of the different species). In species 2, I found that if I grew the cells slowly by supplying limited amounts of new culture medium, the cells always remained strong killers (Preer 1948). However, if I fed the cells enough culture medium to maintain maximum growth, they lost their ability to kill. Starvation after a period of rapid reproduction resulted in recovery of strong killing. If, however, I supplied an excess of food for a long period, cells permanently lost their ability to kill and were maintained as sensitives, even when growth was slowed. This suggested that κ-particles could not grow as fast as the paramecia and that rapid multiplication was diluting them out. By calculating the mean number of particles from the percentage of cells that had none, I could plot the number of κ-particles during the experiment and determine the starting number in the strong killer used to begin the experiment—a few hundred. At first I assumed that the distribution of κ-particles in the cells was random and followed the Poisson distribution. When I presented my work for the first time at Indiana University, H. J. Muller was in the audience. He had just joined the faculty of Indiana University. He immediately pointed out that the distribution could not be strictly random if the particles were multiplying as they were diluted out. I accepted this and was able to get a student in the mathematics department at Indiana University, R. R. Otter, to work on the problem. He solved the problem, providing estimates of all the numbers of particles at any time during the experiment. Thereafter, I fondly called the deviation between his correct distribution and the Poisson distribution, "Muller's error." When this work was published in Genetics (Preer 1948), it seemed unwise to include all the esoteric mathematics used by

Otter to produce his solution to the complete distribution. Therefore I included only the result of Otter's calculations for the distribution of the cells with zero particles, which was all that I used in the 1948 article. When the article was reviewed for Genetics by Sewall Wright, he decided that the article was incomplete without a demonstration of the validity of the distribution of the zero class. Whereupon, he developed an algorithm to compute this class, and his algorithm and its derivation were included in the article.

Herman Muller, who came to Indiana University in 1945, was much involved in producing the classical theory of genetics. In a discussion with Muller when the plasmagene theory had just been proposed, I found him very interested in the theory and most sympathetic to it. In 1946, E. Altenburg visited Indiana University to see his old friend, Muller. Altenburg had a new theory of κ. He proposed that κ was only a symbiont that had landed in the cytoplasm of Paramecium in evolutionary times, much like the algae of *Paramecium bursaria* (Altenburg 1946a,b, 1948). This theory was, of course, in absolute conflict with the plasmagene theory. During this visit, although Altenburg talked to Sonneborn, he did not divulge his theory to him. Muller thought that Altenburg should have talked to Sonneborn about the theory and told Altenburg so (Carlson 1981). Muller was worried that Sonneborn, having invested so much time and attention to the theory, would be upset when he heard of Altenburg's alternative view. Altenburg published his theory at about this time, to Sonneborn's great discomfort. In fact, Altenburg, joined by Lindegren, attacked Sonneborn and the plasmagene theory at the Cold Spring Harbor Symposium in the summer of 1946 (see the discussion section in Sonneborn 1946) and Sonneborn responded vigorously. In the months that followed, Muller became more and more interested in the experiments that Tracy's students David Nanney and Richard Siegel were carrying out on κ and κ-like particles. Muller became a frequent visitor to the Sonneborn laboratory, discussing the results of Sonneborn's students with them. Sonneborn interpreted these visits as an interference with the work of his students and asked Muller to stop. There may have been some tension between Muller and Sonneborn. It was clear, nevertheless, that the two men had great respect for each other. This is confirmed by James Crow, who visited Indiana University in 1959 and gave a course attended by both Sonneborn and Muller and saw a great deal of each of them.

In the meantime I was continuing my work on κ at the University of Pennsylvania and found that κ could be eliminated from the cell by X rays. From the data that we obtained we could calculate the target size. Surprisingly, the target size indicated that κ was large enough to be seen in the microscope! A really hard look under the microscope indeed produced images of small Feulgen positive particles (Preer 1950). Once these had been spotted, the way was open for much better images with the electron microscope. Particles of the size and shape of bacteria, many with flagella and phage-like particles within the κ's, were seen. Some of the κ's even had circular DNA like plasmids, as shown somewhat later by Dilts (1976). In the meantime, work on respiration and other biochemical properties of isolated κ's by Kung in my laboratory proved without question that κ was originally a free-living organism that had invaded paramecia a long time ago and had now become a symbiont, completely dependent on the host for its survival (Kung 1970, 1971). Indeed, the same explanation was also applied to mitochondria when mitochondrial genetics was studied in Paramecium. Sonneborn told me that he was finally convinced by the work of Kung that κ was a symbiont and that the plasmagene theory was dead. Much later, in a letter to me dated July 28, 1976, Sonneborn affirmed that the plasmagene theory was dead. He wrote "it was awful of me to be so attached to a pet idea. That was an ordeal between my mind and my heart and it took a while for the mind to win and the heart to accept. Impersonal scientific objectivity is a goal to be sought by hard self-discipline; we are not born with it."

The end of the plasmagene theory: So what did become of plasmagenes? Many of the phenomena on which the plasmagene theory was based turned out to involve special mechanisms. κ turned out to be a symbiont. Cortical inheritance resulted from preexisting cell surface structures, providing a template for new structures in the surface of paramecia. Further work on Sonneborn's serotypes led to the isolation of the antigenic proteins (Preer 1986) and eventually to the molecular biology of their genes. Apparently cytoplasmic inheritance of serotypes was due to a special case of competing reactions in the synthesis of the proteins, which led to stability and cytoplasmic inheritance, much as had been proposed by Delbrück (1948). The work by Spiegelman on adaptive enzymes in yeast met the same fate (Novick and Weiner 1957; Spiegelman 1958). These findings were sufficient to lead to the demise of the plasmagene theory.

Although this was the death of the plasmagene theory, there were many unexplained exceptions to Mendelism found in Paramecium. Not only were life-cycle changes unexplained, but also a myriad of additional traits showing cytoplasmic inheritance were unexplained.

Homology-dependent maternal inheritance: The work that began unraveling a new mechanism for cytoplasmic transmission of traits occurred shortly after Tracy's death. This mechanism was to prove the most common and most general of all. It began with the finding of a mutant by Epstein and Forney (1984) called d48, which, unlike wild type, could not produce serotype A. Moreover, crosses to wild type revealed that d48 was inherited cytoplasmically. Since the *A* gene had been discovered by molecular means, it was now possible to do its molecular biology. Blots capable of revealing the sequences of the *A* gene

showed that the mutant d48 has the whole gene for the A protein deleted from its macronucleus. Surprisingly, genetic evidence indicated that the whole gene is present in the micronucleus of d48. Moreover, after micronuclei had been isolated, the genetic evidence was confirmed by blots of d48 DNA probed with radioactive micronuclear DNA (Preer *et al.* 1992). How does such a mutant produce an apparent cytoplasmic inheritance? Additional work involving nuclear transfers has shown (Koizumi and Kobayashi 1989) that, when a new macronucleus is formed, the new macronucleus will receive the *A* gene only if the *A* gene is present in the old macronucleus. Since d48 lacks the gene in its macronucleus, it cannot pass it on from the micronucleus to the newly forming macronucleus. How can an old macronucleus impart such highly specific information to the newly forming macronucleus? It must be through the cytoplasm and take the form of fragments of RNA or DNA. These fragments do not have to be self-reproducing themselves; they only have to determine the character of the DNA in the newly forming macronucleus. Continuation of this process at successive autogamies leads to the "cytoplasmic" pattern of inheritance. Thus d48 is best described as having "homology-dependent maternal inheritance." See Meyer and Garnier (2002) and Garnier *et al.* (2004). In summary, the wild-type *A* gene normally is passed to the new macronucleus only if it receives information by way of the cytoplasm from the gene in the old macronucleus. If that gene is missing in the old macronucleus, then a deletion occurs in the new macronucleus and homology-dependent maternal inheritance results.

While d48 languished as a peculiarity for a time, it was soon to be shown that it was a very general phenomenon. This demonstration started with gene silencing. Gene silencing occurs when a gene sequence is artificially introduced into Paramecium (Ruiz *et al.* 1998); it is highly specific for the gene introduced. The newly introduced DNA is transcribed and its RNA interferes with gene expression. This process seems to work for all genes. The effect is seen before the next autogamy. Meyer, on the other hand, introduced DNA and looked at the cells after autogamy (reviewed in Meyer and Garnier 2002). He made the surprising observation that major macronuclear deletions are often produced in the same sequences originally injected! He showed that this effect, like gene silencing, was found for virtually any sequence injected. He was able to create mutants like d48 by this means, although many deletions were not as stable as d48. Such mutants then persisted through additional autogamies. Crosses between the mutant and wild type produced a cytoplasmic pattern of inheritance. Moreover, injection of sections of DNA into mutants with a deleted section of a chromosome caused the specific reversion of the mutant back to wild type. Evidently these are cases of homology-dependent maternal inheritance. Conditions that maximize induction and conditions that maximize rescue of the mutants have been investigated by Meyer. Major factors are the nature of the strain injected and the amount of DNA injected. It should be emphasized that these effects can be duplicated for virtually any nonessential sequence of DNA injected! The importance of these findings for Paramecium genetics can hardly be overemphasized. One has only to imagine that if these changes can be introduced in this way, then they may occasionally occur spontaneously for most nonessential genes in Paramecium. The growing list of unstable, cytoplasmically inherited cases in Paramecium agrees with the conclusions that such changes do often occur spontaneously, resulting in homology-dependent maternal inheritance. Examples that may be explained in this way are mutants like d48 affecting serotypes, mating types, trichocyst discharge (Sonneborn and Schneller 1979), the behavioral mutant paranoiac (Rudman and Preer 1996), and the *Dauermodificationen* of Jollos. These cases and many others from earlier years (see Preer 1968 review for many examples) also appear to be cases of homology-dependent maternal inheritance. A similar phenomenon seems to occur in Tetrahymena as shown by Chalker and Yao (1996, 2001).

CONCLUSIONS

So what can be concluded? First, that Paramecium does have an astonishingly high frequency of diverse phenomena with persistent influences on the cytoplasm: these phenomena include symbiont inheritance, cortical inheritance, stable states of gene expression, and, finally, cases of homology-dependent maternal inheritance. The latter is of special significance because it is so common and affects so many traits. We need to know the precise molecular mechanisms of homology-dependent maternal inheritance to assay its significance to biology in general. It is unfortunate that Sonneborn did not live quite long enough to know about d48. He was right about Paramecium. The cytoplasm does play a major role in its life, affecting an astonishing number and variety of genes in the genome.

Recent work on Arabidopsis by a series of researchers (Lolle *et al.* 2005) has provided evidence that copies of Mendelian genes, supposedly RNA, are often retained in that organism where they multiply independently and later are reverse copied to DNA. Did Sonneborn give up the plasmagene theory too soon?

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