Fertilization and Sexuality in Ciliates

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Hausmann/Bradbury, Ciliates: Cells as Organisms

1 Introduction

Fertilization is the fusion of two nuclei at the beginning of the diploid phase of the life cycle. Certain processes requisite for the nuclear fusion, such as approaching of two nuclei, fusion of gametic cells, and interactions between them, are also dealt with under the heading of fertilization.

Sexuality is the diversification of biological units, such as the cell and the individual, into different types which are complementary for the occurrence of fertilization. The diversification is usually into sexes (hence the term sexuality), but sexuality so defined may include the diversification into other kinds of mating classes. Many investigators do not regard mating types in ciliates as sexes, but the diversification into mating types is included in sexuality because mating types are often complementary for the occurrence of fertilization. It is proposed below to group sexes, mating types and all other kind of classes of biological units, which are complementary for the occurrence of fertilization, as «gamotypes» (gamos = marriage, union in Greek). Sexuality is then the diversification of biological units into gamotypes.

Fertilization and sexuality in ciliates are essentially the same as in other eukaryotes, but they have some remarkable features. For example, in conjugation of many ciliates each partner fertilizes the other and is fertilized in turn, thus behaving as a hermaphrodite. Another example is that the diversification into mating types is often multiple in contrast to the binary female-male diversification.

2 Conjugation, autogamy and their variations

Fertilization in ciliates occurs during conjugation, autogamy and some of their variations. In conjugation, two cells pair and begin a series of developmental processes: 1) meiosis of the micronucleus (germinal nucleus); 2) production of one or more haploid pronuclei in each conjugating cell (conjugant); 3) transfer of the pronucleus from one conjugant to the other; 4) fusion of two pronuclei into a zygote nucleus (synkaryon) (Fig. 1); 5) one or more rounds of mitosis of the zygote nucleus; 6) differentiation of the products of these mitoses into the new micronucleus and the new macronucleus (somatic nucleus); 7) removal of the old macronucleus; 8) separation or complete fusion of the united cells; 9) reorganization of cortical structures. By these processes, old individuals are replaced by new ones (Figs. 2–4).

Preconjugants are cells having the capacity to undergo preconjugant interaction by which cells gain the capacity to unite in conjugant pairs. After the pair separates into two cells or fuses into a single cell, they are called exconjugants. The terminological inconsistency that exconjugants are formed during conjugation has been generally tolerated. Conjugation in a narrower sense can indicate only the stages during which cells are paired (Miyake 1981a); however, this usage of the term is not adopted here, partly because conjugation so defined ends at various stages of nuclear develop-

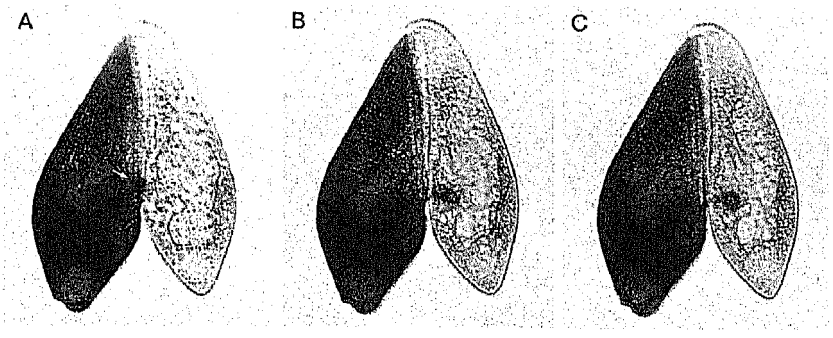

Fig. 1: Fertilization in conjugation of *Blepharisma japonicum*. Three successive stages of the same pair consisting of a wild-type cell containing red pigment granules (left) and an albino cell lacking the pigment (right). Albino cell, mating type I; red cell, mating type II. At these stages of conjugation, some of the pigment granules accumulate at the vicinity of pronuclei, making them visible without staining. **(A)** The migratory pronucleus in the red cell (arrow) is at the site of nuclear transfer. Pronuclei in the albino cell are invisible because of the lack of pigment. **(B)** The same pair as in (A), 31 min later. The migratory pronucleus of the red cell has entered the albino cell. This nucleus has brought in pigment granules, which also surround the stationary pronucleus of the albino cell making it visible. **(C)** The same pair as in (B), 6 min later. The two pronuclei have fused to form a zygote nucleus. Photographs of living, unstained cells. × 210 (Miyake 1978).

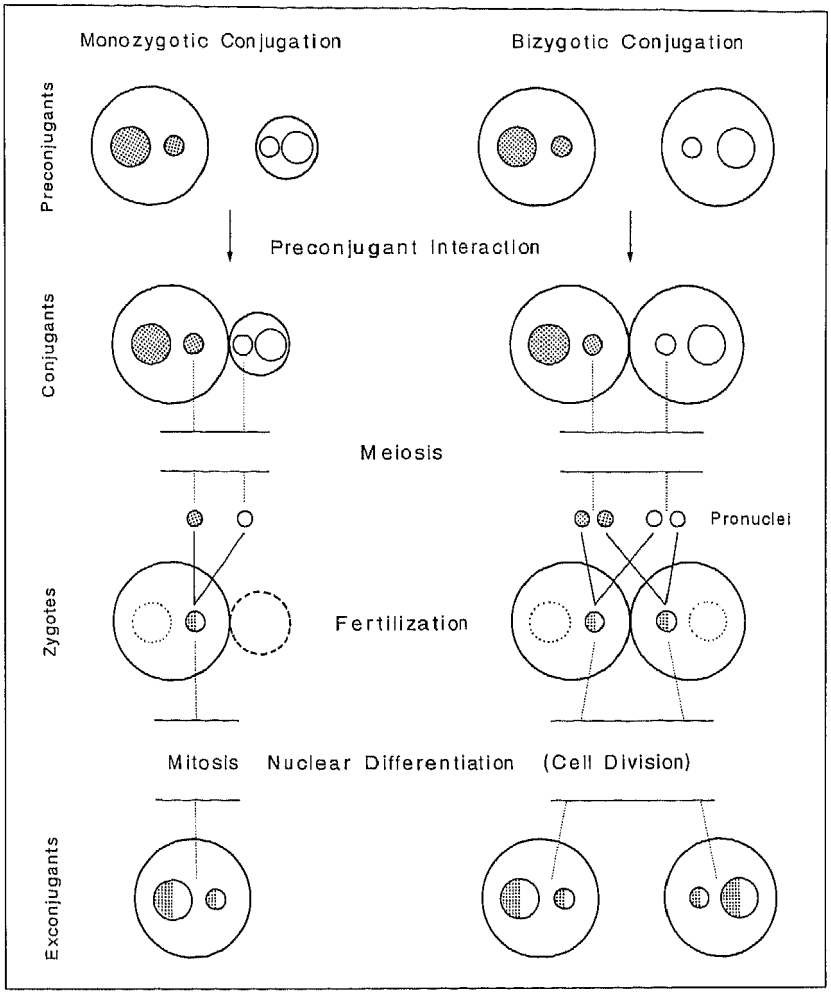

Fig. 2: A simplified diagram of conjugation in ciliates. Circles with inner circles represent cells. A circle made of a broken line is a degenerating cell. The larger and the smaller inner circles are the macronucleus and the micronucleus, respectively. Half-filled circles are hybrid nuclei. Circles made of a dotted line are degenerating macronuclei. Monozygotic conjugation, in which a conjugant pair produces only one zygote, is often anisomorphous. Preconjugant interaction then occurs between the macropreconjugant (left) and the micropreconjugant (right). Bizygotic conjugation, in which a conjugant pair produces two zygotes, is usually isomorphous, and preconjugant interaction occurs between complementary mating types. A zygote may be a conjugant or an exconjugant.

ment, depending the species of ciliate. Preconjugants and conjugants are also called gamonts (Grell 1967, 1973).

In many ciliates, morphologically similar cells conjugate with each other (isomorphous conjugation). In others, two cells of distinctly different structure and/or size conjugate (anisomorphous conjugation). Conjugation can also be classified according to whether a conjugant pair produces two zygotes (bizygotic or temporary or partial conjugation) or a single zygote (monozygotic conjugation). In bizygotic conjugation, the two conjugants in a pair separate into two exconjugants (Fig. 2). In monozygotic conjugation, the two conjugants fuse into a single exconjugant (total conjugation) or separate into a large exconjugant which continues development and a small exconjugant which dies (Fig. 2). «Partial» and «total» concern only cell fusion, not whether the whole process of conjugation is partial or total.

Isomorphous bizygotic conjugation (Figs. 2, 3) is the most common type. Each conjugant produces two or more haploid pronuclei. Of these pronuclei, those which enter the other conjugant are called migratory pronuclei, while those which remain in the same conjugant are called stationary pronuclei. In most species, two pronuclei are formed: one migratory and the other stationary. A migratory pronucleus of one conjugant fertilizes a stationary pronucleus of the other. Thus a conjugant pair produces two zygotes by cross fertilization.

Anisomorphous monozygotic conjugation (Figs. 2, 4) occurs in peritrichs and some suctorians. Each conjugant produces one pronucleus. The smaller conjugant (microconjugant) is absorbed by the larger one (macroconjugant) or it degenerates after transferring its pronucleus along with some of its cytoplasm into the large conjugant. A conjugant pair thus produces only a single zygote by cross fertilization.

Isomorphous monozygotic conjugation has been reported in various groups of ciliates but only in a few species, e.g., *Ephelota gemmipara* (Grell 1953), *Metopus sigmoides* (Noland 1927), *Urosty-*

la hologama (Heckmann 1965). This type of conjugation also occurs accidentally in species undergoing isomorphous bizygotic conjugation, when conjugants fail to separate. Anisomorphous bizygotic conjugation has been reported (Wenrich 1954 for review), but its regular occurrence is still to be confirmed.

Conjugation is divided into the pre- and the post-

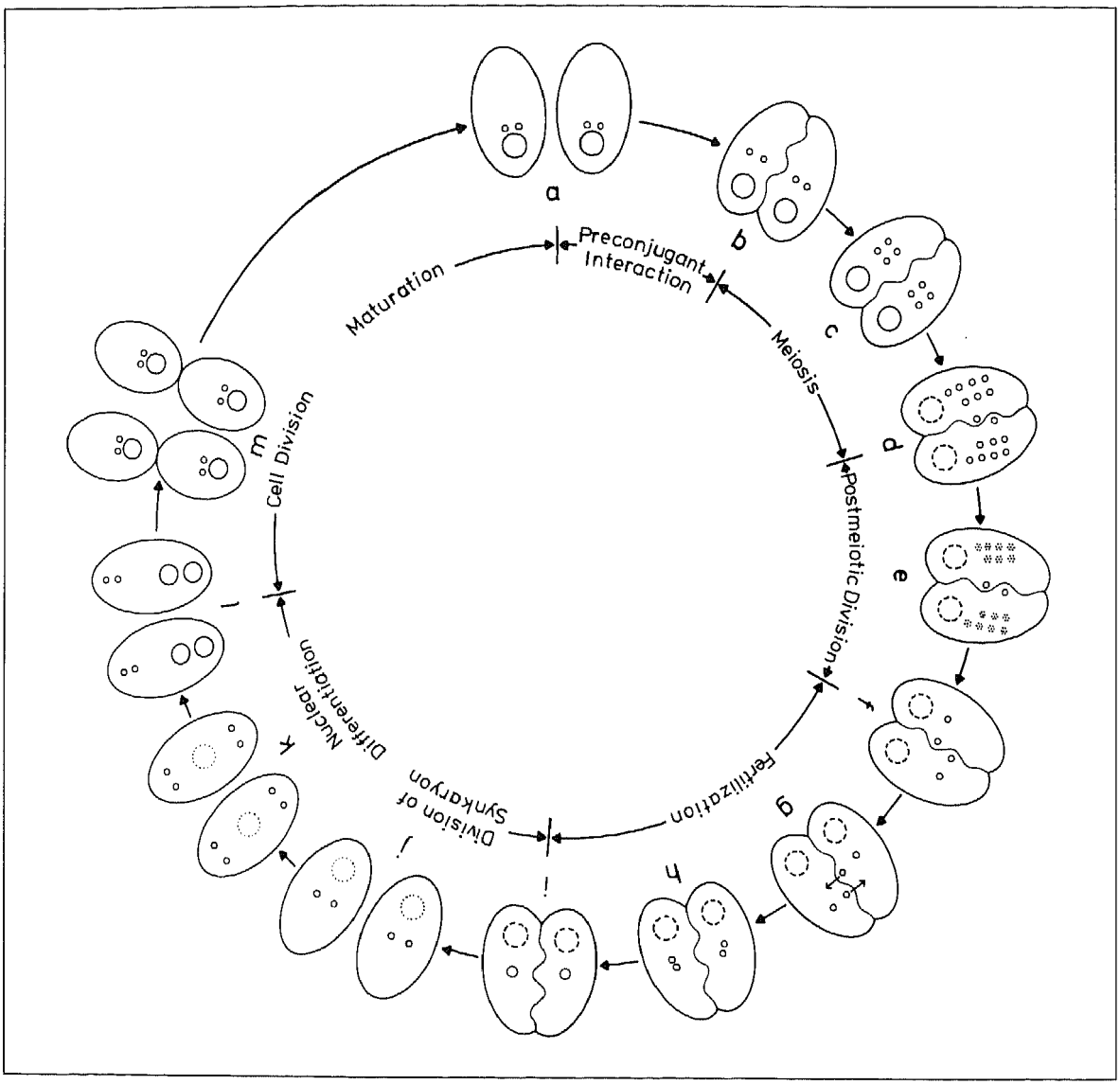

Fig. 3: Diagram of conjugation and conjugation cycle in species of *Paramecium aurelia* complex (isomorphous bizygotic conjugation). Each cell has one polygenomic macronucleus (large circle) and two diploid micronuclei (small circles) **(a).** Cells of complementary mating types undergo preconjugant interaction and unite in pairs **(a–b).** Both micronuclei in each conjugant then undergo meiosis to produce 8 haploid nuclei one of which enters the paroral region **(b–d).** The nucleus in the paroral region undergoes mitosis to produce two haploid pronuclei, while all the other 7 nuclei degenerate **(d–f).** The two conjugants of a pair exchange one of the two pronuclei **(g).** In each conjugant, two pronuclei fuse to form a zygote nucleus (synkaryon) **(h–i).** Thus two zygotes of identical genotype are formed. It takes about 6 h at 25°C from the onset of pairing to the nuclear fusion. The zygote nucleus produces 4 nuclei by two successive mitoses **(i–k).** The two posteriorly located nuclei become macronuclear anlagen, while the two anteriorly located nuclei become micronuclei **(k–l).** Meanwhile conjugants separate into two exconjugants **(i–j).** In the first cell division of exconjugants, micronuclei undergo mitosis, while developing macronuclei are segregated into daughter cells **(m).** The old macronucleus disappears after fragmentation (symbolically shown by the fading out of the macronucleus). In this way the original nuclear condition is restored to complete conjugation. Cells after conjugation gain the capacity to conjugate at maturation which, is usually achieved by a number of cell divisions. When such cells conjugate, the conjugation cycle is completed (Miyake 1974).

karyogamic phases. The prekaryogamic phase extends up to karyogamy, i.e., the formation of zygotes. The postkaryogamic phase begins with zygote formation and ends at the restoration of the original nuclear conditions. Often restoring the numbers of nuclei requires cell division e.g., when two or more macronuclear anlagen are formed in species having one macronucleus per cell, they are segregated in daughter cells by cytokinesis until each cell receives one macronucleus (Fig. 3).

The conjugation cycle is closed when exconjugants or their descendants conjugate again. Like the division cycle, which is from one division to the next, the conjugation cycle spans from one conjugation to the next (Fig. 3).

Compared with sexual processes in other unicellular organisms, ciliate conjugation has two characteristics; 1) reversibility of the conjugant union and 2) replacement of the somatic nucleus. In other unicellular organisms, paired cells either completely fuse to form a zygote, or they produce gametes which then fuse (Grell 1967, 1973). In most ciliates, however, the two cells in a conjugant pair do not completely fuse. Intercellular bridges are formed between them, but each cell retains its identity until the pair separates into single cells. In other unicellular organisms, nuclei are not differentiated into germinal and somatic nuclei (except for the heterokaryotic Foraminifera [Grell 1973]). Both of these characteristics of ciliate conjugation, which have no parallels among other unicellular organisms, do have parallels in metazoa. The reversible union of two individuals in conjugation resembles copulation in metazoa, particularly that of hermaphrodites in which two individuals temporarily unite to exchange male gametes, and it resembles the reversible cell unions that are common in metazoa, particularly during embryonic development (Miyake 1982). The replacement of the somatic nucleus parallels the life cycle of sexually reproducing metazoa. Somatic cells in such metazoa die with the death of individual, but their germinal cells can combine to produce new individuals with different somatic cells.

Austin (1965) noted another curious similarity between ciliate conjugation and metazoan fertilization in that often an egg is diploid when it unites with a sperm. Meiosis begins (or resumes) only after a sperm enters it, as, similarly, meiosis begins in ciliates only after they pair. These striking similarities between ciliate conjugation and metazoan sexual reproduction may be viewed as support of the hypothesis that metazoa have evolved from an ancestral form of ciliates (Hadzi 1963; Hanson 1958).

Irrespective of the validity of the hypothesis, ciliate conjugation encompasses important features of fertilization in metazoa, such as specific cell union, karyogamy, and activation (initiation of development). Therefore, ciliate conjugation has been con-

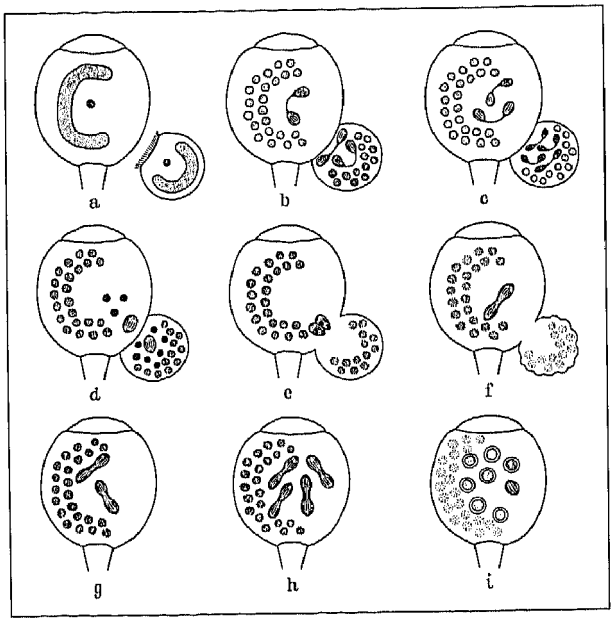

Fig. 4: Diagram of conjugation in peritrichs (anisomorphous monozygotic conjugation). Conjugation occurs between a sessile macropreconjugant and a free-swimming micropreconjugant, each of which has a long polygenomic macronucleus and a round diploid micronucleus **(a).** A conjugant pair consists of a macro- and a microconjugant derived from the macro- and the micropreconjugant, respectively **(b).** The micronucleus in the macroconjugant undergoes meiosis producing 4 haploid nuclei, of which three degenerate leaving one as a pronucleus **(b–d).** The micronucleus in the microconjugant undergoes one round of mitosis and meiosis producing 8 haploid nuclei, of which seven degenerate leaving one as a pronucleus **(b–d).** The two pronuclei fuse into a zygote nucleus (synkaryon) in the macroconjugant **(e).** The microconjugant completely fuses with the macroconjugant or separates from it to degenerate, leaving only one exconjugant. The synkaryon produces 8 nuclei by three successive mitoses **(f–i).** Seven of them become macronuclear anlagen which develop into new macronuclei, while the remaining one becomes the new micronucleus **(i).** In subsequent divisions of the exconjugant, the micronucleus undergoes mitosis, while developing macronuclei are segregated in daughter cells until each cell receives one macronucleus. The old macronucleus disappears after fragmentation. The pattern of prekaryogamic micronuclear divisions varies among peritrichs (Raikov 1972 for review). The pattern shown here (meiosis in the macroconjugant, one preliminary mitosis and meiosis in the microconjugant) occurs in *Vorticella microstoma* (Finley 1943) (Grell 1973).

3 Mating types

Mating types are types of individuals identified by physiological characters that mark them off into mating classes. Conjugation does not ordinarily occur between individuals of the same mating type; it occurs between individuals of complementary mating types (Sonneborn 1957a).

To identify mating types so defined, the occurrence or non-occurrence of conjugation between individuals must be tested. The test is usually carried out by mixing two groups of individuals rather than two individuals, since ciliates are usually handled as groups of individuals, and since no reliable method has been developed to induce conjugation between only two individuals. This practice introduces two problems. 1) The test requires groups consisting of individuals of the same mating type. This requirement can be met by using clones, since mating types are often inherited unchanged during asexual reproduction. 2) If only one of the two mixed groups stimulates the other, conjugation may occur between individuals of the stimulated group, but when single individuals of the two groups are mixed, conjugation does not occur. Therefore, the occurrence of conjugation in the mixture of two groups of individuals indicates that they belong to different mating types, but it does not necessarily indicate that they belong to complementary mating types. To show complementarity, pairs between different mating types must be observed.

The most common obstacle to valid mating-type tests is spontaneous selfing (referred to as selfing below) that is conjugation occurring in a group of individuals without the participation of any other groups. Selfing may occur in groups of individuals of two or more mating types, but other causes may induce it (Section 8.2). In species where intraclonal selfing is common, it is difficult to identify mating types. For example, most clones of *Fabrea salina* studied by Demar-Gervais (1971) underwent selfing and mating types were not clearly established in spite of extensive efforts. *Blepharisma* behaved similarly (Woodruff 1935; Wilfert 1972; Isquith IR, personal communication). However, the tendency to selfing in *Blepharisma* differs among cell lines and strains. When clones that do not undergo selfing for months or years are examined, complementary mating types I and II were found in *B. intermedium* (now *B. japonicum*) (Miyake and Beyer 1973) and *B. stoltei* (Rivola and Miyake 1985).

When such non-selfers are used as mating-type standards, it was found that *B. japonicum* can change mating type during asexual reproduction, resulting in selfing (Miyake and Beyer 1973; Bleyman 1975). Mating-type change is sporadic in many clonal cultures, cells changing mating type only after tens or hundreds of fissions. In Hf-selfers, however, many cells, often nearly all cells, conjugate whenever clonal cultures are deprived of nutrients. Recently, it was found that cell division of Hf-selfers results in daughter cells which differ in mating-type expression: the anterior daughter cell is mating type I; the posterior daughter cell is mating type II at first and then changes to mating type I after about 24 h (Miyake and Harumoto 1990) (Fig. 5). The phenomenon is similar to the mating-type interconversion in homothallic strains of yeasts in which cell division produces daughter cells with and without the capacity to change mating type at the next cell division (Klar 1987; Wolpert 1988). The old puzzling observation by Calkins (1912) that in *B. undulans* conjugation occurs between two daughter cells recently derived from a single cell can now be understood as conjugation between complementary mating types produced by such asymmetrical cell division. Thus in *B. japonicum*, and probably also in other species of *Blepharisma*, the occurrence of conjugation is based on the interaction between distinct mating types.

Mating types have been found in dozens of species, representing all three classes of ciliates (Corliss 1979): *Dileptus anser* (Vinnikova and Tavrovskaja 1973) and *Tokophrya lemnarum* (Colgin-Bukovsan 1976) in Kinetofragminophora; *Paramecium primaurelia* (then *P. aurelia*) (Sonneborn 1937) and *Tetrahymena thermophila* (then *T. pyriformis* variety 1) (Nanney and Caughey 1953) in Oligohymenophora; *Euplotes vannus* (Heckmann 1963)

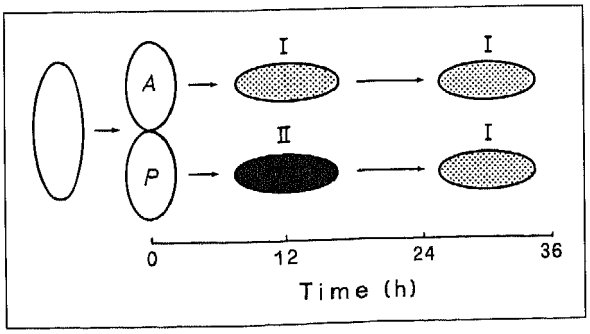

Fig. 5: Asymmetrical mating-type expression in anterior **(A)** and posterior **(P)** daughter cells after cell division of Hf-selfers in *Blepharisma japonicum*. I: Mating type I. II: Mating type II (Miyake and Harumoto 1990).

sidered as a model system for the study of fertilization (Metz 1954; Hiwatashi 1969; Miyake 1978).

Autogamy, found in *P. aurelia* by Diller (1936), is a fertilization-including process taking place in single cells. The nuclear events in autogamy are the same as those in conjugation except that each cell undergoes self-fertilization. Figure 3 serves as a diagram of autogamy and the autogamy cycle in species of the *P. aurelia* complex, if preconjugant interaction, cell union, and nuclear transfer (g in Fig. 3) are disregarded. In *Paramecium*, the two pronuclei are sister nuclei produced by mitosis of a haploid nucleus. Since they fuse to form a zygote nucleus, autogamy in *Paramecium* produces zygotes which are homozygous for all nuclear genes.

Autogamy occurs only in some species of a genus or in some stocks of a species. For example, in *Paramecium*, autogamy occurs in *P. tetraurelia* and *P. trichium*, but not in *P. caudatum* and *P. bursaria*, while conjugation occurs in all these species (Wichterman 1985). Only some stocks undergo autogamy in *Euplotes minuta* (Nobili 1966; Siegel and Heckmann 1966) and *E. crassus* (Luporini 1974). The capacity is genically controlled (Siegel and Heckmann 1966; Dini and Luporini 1980). In the genus *Tetrahymena*, autogamy has been described in the species *T. rostrata* as occurring in a cystic stage of the life cycle (Corliss 1965).

The following four processes can be regarded as variations of conjugation and autogamy.

1. In macronuclear regeneration (MR) in *Paramecium aurelia*, variety 1 (now *P. primaurelia*) (Sonneborn 1940) and macronuclear retention (also MR) in *Tetrahymena pyriformis*, syngen 1 (now *T. thermophila*) (Allen 1967), the old macroucleus is not removed as in normal conjugation or autogamy. Instead it produces the macronucleus of exconjugant and exautogamous clones. Since new micronuclei are derived from the synkaryon, MR produces heterokaryons. Regeneration or retention of the macronucleus is of importance in genetics as well as developmental biology of ciliates and is treated more in detail in the chapters by Bleyman and Schmidt.

2. Endomixis, first described by Woodruff and Erdmann (1914) in *P. aurelia*, begins in single cells, like autogamy, but, neither meiosis nor karyogamy occur in endomixis. Micronuclei divide mitotically; one of the mitotic products continues dividing; some of the division products develop into macronuclei, while others remain micronuclei; and the old macronucleus degenerates. Diller (1936), who made extensive cytological studies on autogamy and hemixis in *P. aurelia*, questioned whether endomixis actually occurred at all, and Sonneborn (1947) after extensive genetic and cytological studies of *P. aurelia* was unable to confirm the existence of this process. Apomictic processes similar or even identical to endomixis have been reported in some other ciliates as variations of conjugation and autogamy (Raikov 1972 for review).

3. Cytogamy, found in *P. caudatum* by Wichterman (1940), is normal bizygotic conjugation except that each conjugant undergoes self-fertilization. The nuclear events in cytogamy are, therefore, those of autogamy. Cytogamy, which occurs spontaneously in some conjugants, can be experimentally induced in *Paramecium* (Sonneborn 1970 for review) and *Tetrahymena* (Orias 1986 for review).

4. Hemisexual conjugation was discovered in *Blepharisma japonicum* (Miyake et al. 1991). In conjugation of this multimicronucleate heterotrich, some micronuclei (somatomicronuclei) do not divide at all and start differentiating into macronuclei at an early stage of conjugation. Other micronuclei in the same cell undergo conventional nuclear processes, including meiosis, karyogamy, and mitoses, to produce zygote-derived macronuclei and micronuclei. Thus there are two paths of macronucleus differentiation, one asexual, the other sexual. The two paths proceed side by side in each cell, but eventually one path dominates the other. If the sexual path dominates, conjugation is normal in the sense that both macro- and micronuclei are derived from the same synkaryon. If the asexual path dominates, conjugation is hemisexual in the sense that only the micronucleus originates from the synkaryon, the macronucleus being formed from the somatomicronucleus without meiosis and karyogamy. Hemisexual conjugation regularly occurs in cultures of high-frequency (Hf) selfers described in the next section. In other cultures conjugation is usually normal.

Raikov (1972) and Vivier (1984) have reviewed various types of nuclear processes in conjugation, autogamy, and their variations. Time tables of the nuclear processes in conjugation using statistical methods have been constructed for three species, *Blepharisma japonicum* (Miyake et al. 1979a, 1991), *Tetrahymena thermophila* (Martindale et al. 1982) and *Euplotes octocarinatus* (Kuhlmann and Heckmann 1991).

and *Stentor coeruleus* (Webb and Francis 1969) in Polyhymenophora.

The mating-type system of a species or a syngen (sibling species) is either binary or multiple, depending on whether the number of mating types is two or more than two. The multiple systems are further divided here into homopolar and heteropolar systems. In homopolar systems each mating type is complementary with all of the other mating types in the system, e.g., *P. bursaria*, syngen 1 (4 mating types) (Jennings 1938), *T. thermophila* (7 mating types) (Nanney and Caughey 1953) (Table 1); in heteropolar systems complementarity is lacking in some combinations of mating types, e.g. *Uronychia transfuga* (10 mating types) (Reiff 1968) (Table 2).

The mating-type system of *E. crassus* was first described as a homopolar multiple system consisting of 5 and possibly more mating types (Heckmann 1964), but Magagnini and Santangelo (1977) found that some combinations of mating types lakked complementarity. They called it the «block phenomenon» (Table 3). When 38 mating types of this species derived from various places were studied, many more cases of the block phenomenon were found (285 out of 703 two-by-two mixtures of different mating types) (Valbonesi et al. 1992). Therefore, the mating-type system of *E. crassus* appears to be a heteropolar multiple system with a complicated network of complementary and non-complementary relationships. On the other hand, *E. crassus* might consist of syngens, as suggested by an attempt to divide the «*E. vannus* complex» (*E. vannus*, *E. crassus*, and *E. mutabilis*) into five and possibly more syngens (Machelon 1982; Génermont et al. 1985).

The block phenomenon was also found in *Stylonychia mytilus*; some clones do not conjugate with two or more mating types, thus behaving as if they are these two or more mating types (Ammermann 1982) (Table 4). Therefore, heteropolarity is perhaps common in multiple systems consisting of large numbers of mating types (see 4.3, 8.2). These examples of heteropolar multiple systems indicate that conjugation does not result from a mere difference in mating type, but from complementarity between mating types, as noted by Sonneborn (1957a).

In some cases, a system is heteropolar in spite of the fact that conjugation occurs in all combinations of populations of mating types. For example, in *E. octocarinatus* conjugation occurs in all two-by-two combinations of ten mating types I–X (Heckmann and Kuhlmann 1986). In some mixtures, however, only homotypic pairs of one of the two mating types are formed, while in others both homotypic

Table 1: Homopolar multiple mating-type system of *Tetrahymena thermophila* consisting of 7 mating types. +: Occurrence of conjugation. –: No conjugation (Nanney and Caughey 1953).

Mating types	I	II	III	IV	V	VI	VII
I	–	+	+	+	+	+	+
II		–	+	+	+	+	+
III			–	+	+	+	+
IV				–	+	+	+
V					–	+	+
VI						–	+
VII							–

Table 2: Heteropolar multiple mating-type system of *Uronychia transfuga* consisting of 10 mating types. +: Occurrence of conjugation. –: No conjugation (based on data of Reiff, 1968).

Mating types (Genotypes)		I	II	III	IV	V	VI	VII	VIII	IX	X
I	(mt^1mt^2)	–	+	+	+	+	+	–	–	+	+
II	(mt^3mt^4)		–	+	+	+	+	+	+	–	–
III	(mt^1mt^3)			–	+	+	+	–	+	–	+
IV	(mt^2mt^4)				–	+	+	+	–	+	–
V	(mt^2mt^3)					–	+	+	–	–	+
VI	(mt^1mt^4)						–	–	+	+	–
VII	(mt^1mt^1)							–	+	+	+
VIII	(mt^2mt^2)								–	+	+
IX	(mt^3mt^3)									–	+
X	(mt^4mt^4)										–

Table 3: Block phenomenon in *Euplotes crassus*. +: Occurrence of conjugation. –: No conjugation. S 12 behaves as if it belongs to the same mating type as S 21 and S 23; because conjugation does not occur in the mixtures S 12 × S 21 and S 12 × S 23. However, S 21 and S 23 are different mating types because, conjugation occurs when they are mixed together. Therefore, the mating type of S 12 cannot be the same as the mating type of S 21 or S 23. S 12 belongs to a distinct mating type lacking complementarity to S 21 and S 23. For further details see text (3, 4.3.3, 4.3.4) (based on data of Magagnini and Santangelo 1977).

Stocks	Mt formulas[a]	S 21	S 22	S 23	S 12
S 21	$G_{21}R_{22}R_{23}$	–	+	+	–
S 22	$G_{22}R_{12}R_{21}R_{23}$		–	+	+
S 23	$G_{23}R_{21}R_{22}$			–	–
S 12	$G_{12}R_{21}R_{22}R_{23}$				–

[a] See 4.3.3

and heterotypic pairs are formed (Table 5). This is due to the fact that each mating type excretes one or two gamones (signals for conjugation [Section 4.1]) which can induce conjugation in only some of the mating types (Table 6). Thus complementarity does not occur in all combinations of mating types. For more details of this system see Section 4.2.5.

Mating types of the same species are morphologically similar. It has long been known in peritrichs and some suctorians that conjugation regularly occurs between cells of different sizes (Maupas 1889; Finley 1939, 1952; Mügge 1957; for reviews see Wenrich 1954; Grell 1973; Raikov 1972; Vivier 1984). These two classes of individuals, large and small, are female and male, respectively (Finley 1939; Grell 1973). Mating types have not yet been found in these ciliates. Mating types and sexes in ciliates are discussed in Section 8.1.

Mating-types are determined genotypically and epigenetically. Which kind of determination prevails depends on species. In genotypic determination, the mating type of a cell is determined by the genotype of the zygote from which it is asexually derived. Genes participating in this determination are mating-type genes *(mt)*. In some binary systems, a pair of alleles *Mt* (dominant) and *mt* (recessive) determine the two mating types in the system, *Mt/Mt* and *Mt/mt* corresponding to one mating type, *mt/mt* to the other, e.g., syngens 3 and 12 in *P. caudatum* (Hiwatashi 1968), *P. aurelia* species

Table 4: Mating behavior of clones belonging to more than one mating type in *Stylonychia mytilus*. +: Occurrence of conjugation. –: No conjugation. The block phenomenon, shown in Table 3, is seen. For details see text 3, 4.3.3, 4.3.4 (based on data of Ammermann 1982).

Mating types	Mt-formulas[a]	S 1/25	S 3/25	S 5/25	S 23/24/25	S 4/22/25	S 4/22
S 1/25	$G_1R_3R_4R_5R_{22}R_{23}R_{24}$	–	–	–	–	–	+
S 3/25	$G_{25}R_1R_4R_5R_{22}R_{23}R_{24}$		–	–	–	–	+
S 5/25	$G_{25}R_1R_3R_4R_{22}R_{23}R_{24}$			–	–	–	+
S 23/24/25	$G_{25}R_1R_3R_4R_5R_{22}$				–	–	+
S 4/22/25	$G_{25}R_1R_3R_5R_{23}R_{24}$					–	–
S 4/22	$G_4R_1R_3R_5R_{23}R_{24}R_{25}$						–
S 1	$G_1R_3R_4R_5R_{22}R_{23}R_{24}R_{25}$	–	+	+	+	+	+
S 3	$G_3R_1R_4R_5R_{22}R_{23}R_{24}R_{25}$	+	–	+	+	+	+
S 4	$G_4R_1R_3R_5R_{22}R_{23}R_{24}R_{25}$	+	+	+	+	–	–
S 5	$G_5R_1R_3R_4R_{22}R_{23}R_{24}R_{25}$	+	+	–	+	+	+
S 22	$G_{22}R_1R_3R_4R_5R_{23}R_{24}R_{25}$	+	+	+	+	–	–
S 23	$G_{23}R_1R_3R_4R_5R_{22}R_{24}R_{25}$	+	+	+	–	+	+
S 24	$G_{24}R_1R_3R_4R_5R_{22}R_{23}R_{25}$	+	+	+	–	+	+
S 25	$G_{25}R_1R_3R_4R_5R_{22}R_{23}R_{24}$	–	–	–	–	–	+

[a] See 4.3.3

Table 5: Heteropolar multiple mating-type system of *Euplotes octocarinatus* consisting of 10 mating types. +: Occurrence of heterotypic conjugation. (+): Occurrence of only homotypic conjugation of one mating type. –: No conjugation. For details see text 4.2.5 (based on data of Heckmann and Kuhlmann 1986).

Mating types (genotypes)			I	II	III	IV	V	VI	VII	VIII	IX	X
		Excreted gamones	1,2	3,4	1,3	2,4	1,4	2,3	1	2	3	4
I	(mt^1mt^2)	1,2	–	+	+	+	+	+	(+)	(+)	+	+
II	(mt^3mt^4)	3,4		–	+	+	+	+	+	+	(+)	(+)
III	(mt^1mt^3)	1,3			–	+	+	+	(+)	+	(+)	+
IV	(mt^2mt^4)	2,4				–	+	+	+	(+)	+	(+)
V	(mt^1mt^4)	1,4					–	+	(+)	+	+	(+)
VI	(mt^2mt^3)	2,3						–	+	(+)	(+)	+
VII	(mt^1mt^1)	1							–	+	+	+
VIII	(mt^2mt^2)	2								–	+	+
IX	(mt^3mt^3)	3									–	+
X	(mt^4mt^4)	4										–

Table 6: Induction of conjugation in each mating type by cell-free fluid of other mating types in *Euplotes octocarinatus*. +: Occurrence of conjugation. –: No conjugation. For details see text 4.2.5 (based on data of Heckmann and Kuhlmann 1986).

Sources of cell-free fluid			Cells									
Mating types (genotypes)			I	II	III	IV	V	VI	VII	VIII	IX	X
		Excreted gamones	1,2	3,4	1,3	2,4	1,4	2,3	1	2	3	4
I	(mt^1mt^2)	1,2	–	+	+	+	+	+	+	+	+	+
II	(mt^3mt^4)	3,4	+	–	+	+	+	+	+	+	+	+
III	(mt^1mt^3)	1,3	+	+	–	+	+	+	+	+	+	+
IV	(mt^2mt^4)	2,4	+	+	+	–	+	+	+	+	+	+
V	(mt^1mt^4)	1,4	+	+	+	+	–	+	+	+	+	+
VI	(mt^2mt^3)	2,3	+	+	+	+	+	–	+	+	+	+
VII	(mt^1mt^1)	1	–	+	–	+	–	+	–	+	+	+
VIII	(mt^2mt^2)	2	–	+	+	–	+	–	+	–	+	+
IX	(mt^3mt^3)	3	+	–	–	+	+	–	+	+	–	+
X	(mt^4mt^4)	4	+	–	+	–	–	+	+	+	+	–

13 (now *P. tredecaurelia*) (Sonneborn 1974a for review). In some multiple systems, mating types are determined by two or three pairs of alleles at different loci, each allele either dominant or recessive. For example, mating types I, II, III and IV in *P. bursaria*, syngen 1, are determined by combinations of two pairs of alleles, A-B-, aaB-, aabb and A-bb, respectively (Siegel and Larison 1960). In other multiple systems, mating-types are determined by multiple alleles with serial dominance ($mt_1 < mt_2 < mt_3 < --$), e.g., *E. vannus* (Heckmann 1963), *E. crassus* (Heckmann 1964), *E. minuta* (Nobili 1966). In other multiple systems, mating types are determined by co-dominant multiple alleles (mt_1, mt_2, mt_3, $--$), e.g., *E. patella* (Kimball 1939a; Akada 1985a) (Tables 7, 8), *Uronychia transfuga* (Reiff 1968) (Table 2), *E. octocarinatus* (Heckmann and Kuhlmann 1986) (Tables 5, 6), *E. raikovi* (Luporini and Miceli 1984, 1986). Genetics of mating types is treated in detail in the chapter by Bleyman.

In epigenetic determination, factors other than the genotype of the zygote participate in determination of mating type. In «caryonidal determination», each caryonide (the clone whose macronuclei are derived from a single macronuclear anlage) is independently determined for mating type, producing different mating types among clones of the same genotype, e.g., *P. primaurelia* (then *P. aurelia*, species 1) (Sonneborn 1974a for review), *T. thermophila* (then *T. pyriformis*, variety 1) (Nanney and Caughey 1953; Nanney 1956). The mating-type ratio among caryonides is influenced by environmental conditions. In «unstable caryonides», very often individuals of different mating types appear within a caryonide, e.g., *P. pentaurelia* (then *P. aurelia*, syngen 5) (Bleyman 1967), *P. septaurelia* (then *P. aurelia*, syngen 7) (Taub 1966a). In *T. thermophila* unstable caryonides can be stabilized after many fissions. This phenomenon is explained by the assortment of macronuclear allelic genes during asexual reproduction (Allen and Nanney 1958).

In «cytoplasmic» or «clonal» determination, each individual in an exconjugant clone inherits the mating type of its cytoplasmic parent at conjugation. Therefore, individuals in an exconjugant clone are usually of the same mating type, e.g., *P. tetraurelia* (then *P. aurelia*, species 4) (Sonneborn 1974a for review).

In some cases of epigenetic determination, mating types change frequently and reversibly under the control of internal and external factors. In most stocks of *P. multimicronucleatum*, syngen 2, each individual oscillates between mating types III and IV responding to the light-dark cycle (Sonneborn 1957b). The oscillation is circadian; once the rhythm of oscillation is set by a 24 h light-dark cycle, the mating type continues oscillating under constant light or dark conditions maintaining the rhythm of about 24 h (Barnett 1966). The cycling character is determined by a pair of alleles *C* and *c*; homozygotes for the recessive allele *c* have no cycle, providing reference clones of mating types III and IV (Barnett 1966). As described above (Fig. 5), Hf-selfers of *B. japonicum* produce daughter cells

of mating types I and II by asymmetrical cell division. A similar phenomenon has long been known in sexual differentiation in peritrichs in which the macro- and the micropreconjugant are produced by asymmetrical cell division (Finley 1939, 1952; for review see Wenrich 1954; Raikov 1972; Vivier 1984).

It should be noted that genotypic determination also occurs in ciliates whose mating types are usually determined epigenetically. For example, homozygotes for the recessive gene *mtI* in *P. primaurelia* can express only mating type I (Sonneborn 1939). On the other hand, epigenetic determination also occurs in ciliates whose mating types are usually determined genotypically. For example, heterozygotes for *mt* alleles in *E. crassus* may express the mating type controlled by the less dominant allele when clones get old (Heckmann 1967). In *P. caudatum* syngen 12, a lower fission rate promotes expression of the recessive *mt* allele in heterozygotes (Hiwatashi 1960).

In many ciliates, the expression of mating types occurs in two steps, maturation and preconjugant formation. Maturation is an irreversible process occurring in the clonal life history. Cells after conjugation are usually immature, lacking the ability to conjugate. They become mature after certain numbers of cell division (Bleyman 1971; Nanney 1974; Takagi 1988). Immaturity occurs after autogamy in *E. minuta* (Luporini 1970), but not in species of *P. aurelia* complex (Sonneborn 1957a).

Miwa et al. (1975) showed, by a series of cytoplasmic transfer experiments in *P. caudatum*, that the immaturity is caused by a cytoplasmic factor. The factor was isolated, identified as a protein of about 10 kD, and named immaturin (Haga and Hiwatashi 1981).

Maturation can be a stepwise process. In *P. bursaria*, syngen 1, an exconjugant clone first gains the ability to conjugate with two of the four mating types in the syngen. The ability to conjugate with the third mating type appears later when maturation is complete. Jennings (1939), who found this phenomenon, called the intermediate stage of maturation the stage of adolescence. The stepwise maturation in this ciliate has been explained by assuming a sequential expression of the two *mt* loci described above (Siegel and Cohen 1963). In *P. multimicronucleatum*, syngen 2, first the ability to undergo the mating reaction, then the ability to form the holdfast-union, and finally the ability to form the paroral-union appear in the clonal history (Takagi 1971). In *D. anser*, the ability to excrete gamone (Section 4.1) appears first, followed by the ability to react to gamones of other mating types (Tavrovskaja 1979) (Section 4.2.8). The same phenomenon was found also in *E. raikovi* (Miceli and Luporini 1983) (Section 4.2.6) and *E. octocarinatus* (Kuhlmann and Heckmann 1989) (Section 4.2.5).

Preconjugant formation is the transformation of non-preconjugants into preconjugants (cells with the capacity to undergo preconjugant interaction [Section 4.1]) in mature cells. It is usually induced by depriving cells of nutrients. The process is reversible; if preconjugants are fed, they return to mature but non-preconjugant cells (e.g. Wellnitz and Bruns 1982). However, in some ciliates, e.g., *E. charon* (Valbonesi et al. 1987), conjugation occurs when cells are well fed.

Previous reviews on mating types in ciliates include Sonneborn (1957a, 1978), Preer (1969), Heckmann (1970), Grell (1973), Nanney (1977) and Nobili et al. (1987). Those in *Paramecium* include Butzel (1974), Sonneborn (1974a), Tsukii (1988) and Wichterman (1985). Those in *Tetrahymena* include Sonneborn (1974b).

4 Preconjugant interaction

4.1 Introduction

When cells of complementary mating types are mixed together under appropriate conditions, they start forming conjugant pairs after a time lag which usually lasts for 0.5–2 h. During this «preconjugative lag» the two types of cells undergo preconjugant interaction which consists of a series of processes including cell agglutination. Although agglutinating cells are often paired (preconjugant pairs), they are only loosely united by ciliary adhesion and are distinguishable from conjugant pairs in which cells are more tightly united by direct pellicular contact. This section mainly deals with signals and receptors of preconjugant interaction. Conjugant union, which is the most important consequence of preconjugant interaction, is dealt with in the next section.

Some ciliates excrete signal substances for preconjugant interaction into the external medium. Others retain these signals on the cell surface and do not excrete them in detectable amounts. These

signals are all called gamones. Those ciliates which excrete gamones are called gamone excreters, while those which retain them on the cell surface are called gamone carriers.

The term gamone was adopted to indicate any specific substances participating in preconjugant interaction, irrespective of whether they are excreted or not, in an attempt to construct a unifying view of preconjugant interaction applicable to all ciliates (Miyake 1974). Later, the usage was modified so that it indicates signal substances, not receptors, in preconjugant interaction (Miyake 1981a, 1983).

Luporini and Miceli (1986) questioned the adoption of this term based on: 1) The term gamone is an abbreviation of «Gametenhormon» (Hartmann and Schartau 1939), but ciliates interacting for conjugation are not gametes; 2) Gamones are said to be signals for interactions between cells complementary for fertilization (Miyake 1983), but ciliates interacting for mating are not complementary cells for fertilization. These arguments, however, are not strictly relevant to the issue. As Hartmann and Schartau (1939) note, gamones can indicate, in addition to gamete hormones, any specific substances participating in mating and fertilization in general, because the term comes from the Greek «gamos» (marriage, union). Therefore, the term gamones can be used also for intercellular signals in the conjugation of unicellular organisms.

Luporini and Miceli (1986) suggested that diffusible mating-type substances in ciliates should be called «mating pheromones», following the practice in other fields of microbiology. Excreted gamones are indeed pheromones (Miyake 1981b), however, «gamone» is not only shorter than «mating pheromone», but also more versatile. It can indicate both excreted and cell-bound signals and thus is useful for a general concept of preconjugant interaction. The term also can be used for signals conveying sexual interactions between individuals as well as between cells, and making the term particularly suitable for conjugation signals in unicellular organisms, which are individuals and cells at the same time.

Preconjugant interaction can be classified according to whether it is mediated by excreted gamones (fluid-mediated) or by cell-bound gamones (cell-mediated). It can also be classified by the presence or absence of the «waiting period», which is the time between the mixing of different mating types and the onset of cell agglutination. In all species of *Paramecium* so far studied, cells of complementary mating types start sticking together within a few seconds after they are mixed together. In other ciliates, distinct agglutination begins only after a waiting period which often occupies a large fraction of the preconjugative lag. Therefore, four classes of preconjugant interaction are conceivable: 1) cell-mediated, without waiting period; 2) cell-mediated, with waiting period; 3) fluid-mediated, without waiting period; and 4) fluid-mediated, with waiting period. Of these, the third class has not yet been found. The other three classes of preconjugant interaction are described below using one species as an example for each class. It should be noted, however, that it is often difficult to draw a sharp line between the three classes: some ciliates, which usually have a waiting period, may lack it under certain conditions (e.g., *Euplotes crassus*, see below); some ciliates may have both excreted and cell-bound gamone as suggested in Sections 4.2.6, 4.2.7, 4.2.9.

Paramecium caudatum (cell-mediated, without waiting period): Preconjugant interaction begins with the sticking together of cells (mating reaction), which often produces clumps of agglutinating cells. The mating reaction, which is caused by ciliary adhesion, not only holds potential mates together, but also switches on the cellular mechanisms of conjugation. The ability to undergo the mating reaction is mainly on the oral side of the cell (Hiwatashi 1961). After about 1 h of the mating reaction, cells start forming conjugant pairs joining more tightly by their pellicles, first at the anterior tip (holdfast union), then over a broader area that includes the paroral region. Prior to the holdfast union, cilia degenerate on the oral side of the cell and the pellicular union is formed between deciliated surfaces. For review see Hiwatashi (1969, 1981), Hiwatashi and Kitamura (1985), Miyake (1974), Fujishima (1988) and Watanabe (1990).

Euplotes crassus (cell-mediated, with waiting period): When two complementary mating types are mixed, cells usually start agglutinating after about 1 h of the waiting period. They form preconjugant pairs in which two cells loosely attached first vis-à-vis and then along their lengths (Heckmann and Siegel 1964). In these pairs, cells are held together by a specific adhesion between the adoral zone of membranelles (AZM) of one cell and the ribbed wall (RW), which is on the right side of the peristomial floor, of the other (Luporini and Dallai 1980). Shortly later, conjugant pairs are formed.

Heckmann and Siegel (1964) showed that cells do interact during the waiting period and explained it as a time of weak mating reaction. According to their hypothesis, an initial weak mating reaction, which is hardly visible, induces, by positive feed-

back, a stronger mating reaction which is visible as agglutination. Indeed, under certain conditions, *E. crassus* undergoes a weak but distinct agglutination immediately after cells of complementary mating types are mixed together (Miyake and Nobili 1974). Heckmann and Siegel's hypothesis was also supported by Dini and Miyake (1982) who showed that cells of *E. crassus* have a protein for preconjugant interaction in a state of equilibrium of turnover and synthesis and that the protein increases after mixing the two mating types. Heckmann (1963) assumed that during the waiting period of *E. vannus*, whose preconjugant interaction is similar to that of *E. crassus* (Heckmann 1964), cell-bound substances are transferred by cell-cell contact to carry out preconjugant interaction. This assumption is supported by the demonstration that proteins are exchanged between cells during preconjugant interaction in *E. crassus* (Luporini et al. 1979; Luporini and Gabrielli 1981).

Blepharisma japonicum (fluid-mediated, with waiting period): When mating types I and II are mixed, cells usually start agglutinating after 1–2 h of the waiting period by a specific adhesion between the adoral zone of membranelles (AZM) of one cell and a row of cilia anterior to the undulating membrane (antUMC) of the other. First the AZM and then antUMC gain the capacity to unite. Since the AZM and the antUMC are respectively on the left and right side of the peristomial floor, cells in a preconjugant pair are held together by two AZM-antUMC linkages in such a way that they face each other by the peristomial floor where conjugant union is formed 1–2 h later (Honda and Miyake 1976). For other aspects of preconjugant agglutination, see Miyake (1981b) and Miyake and Beyer (1973).

Preconjugant interaction in this species is described in seven steps as follows (Fig. 6). Mating type I autonomously excretes gamone 1 (step 1 in Fig. 6). Gamone 1 specifically acts on mating type II (step 2) to transform it so that it can unite (step 3) and at the same time to induce or enhance its production and excretion of gamone 2 (step 4). Gamone 2 specifically acts on mating type I (step 5) to transform it so that it can unite (step 6) and also to enhance its production and excretion of gamone 1. Transformed cells unite in pairs upon their contact (step 7). Thus the two mating types stimulate each other through a positive feedback loop (steps 1, 2, 4, and 5) (Miyake and Beyer 1973).

Transformed cells can unite in any combinations of mating types. In a mixture of mating types I and II, both homotypic (type I – type I, type II – type II) and heterotypic (type I – type II) pairs are formed. They look alike at least at the beginning (Bedini et al. 1978), but meiosis and other nuclear processes of conjugation occur only in heterotypic pairs (Miyake 1968a, 1975). Homotypic pairs eventually separate into single cells, returning to the asexual stage. This «homotypic arrest», an effective device to prevent inbreeding, is characteristic of *Blepharisma*; in other ciliates, at least some of the homotypic pairs can undergo the nuclear processes of conjugation.

In all classes of preconjugant interaction, cell agglutination is the most common and conspicuous process. The agglutination, which is mediated by a specific adhesion between differentiated cell surfaces (e.g., cilia on the oral side in *P. caudatum*, CM and LM in *E. crassus*, AZM and antUMC in *B. japonicum*), aids the formation of conjugant pairs, not only by holding potential mates together but also by making them face with each other at their presumptive sites of conjugant union (Miyake 1981a).

Other processes known to occur in preconjugant interaction are 1) retardation in swimming, e.g., *P. caudatum* (Kitamura and Hiwatashi 1984a), *Euplotes patella* (Kimball 1939a); 2) courtship dancing, e.g., *Stylonychia mytilus* (Grell 1951) and *E. vannus* (Heckmann 1963); 3) chemoattraction, e.g., *B. japonicum* (Honda and Miyake 1975) (Section 4.2.3) (Fig. 8), *Dileptus anser* (Afon'kin and Yudin 1987) (Section 4.2.8), *Tokophrya infusionum* (Sonneborn 1978) and probably *E. woodruffi* (Kosaka 1991) (Section 4.2.7); 4) nuclear activation, e.g., *P. tetraurelia* (Metz and Foley 1949), and

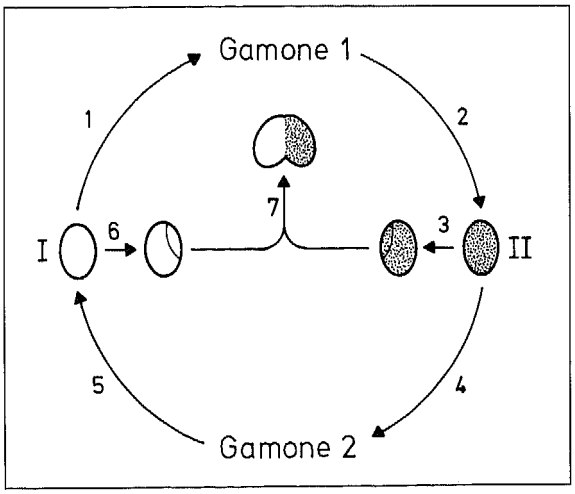

Fig. 6: Diagram of preconjugant interaction in *Blepharisma japonicum*. I: Mating type I. II: Mating type II (Miyake 1978).

5) cell division, e.g., *D. anser* (Tavrovskaja 1974) (Section 4.2.8) and *T. thermophila* (Wolfe 1974).
Depending on whether ciliates are regarded as individuals or cells, preconjugants and conjugants interacting at conjugation can be regarded as mating individuals or fusing gametes (see Section 2, 8.1). It is this dual nature of preconjugants and conjugants that gives their interaction the characteristics of mating as well as those of gamete-gamete interaction. Among the former are 1) relatively long (1–2 h) period of interaction, 2) positive feedback in mutual stimulation, and 3) separation of conjugants after an exchange of pronuclei. Among the latter are 1) specific agglutinative reaction, 2) triggering of developmental processes which leads to zygote formation and development of new individuals, and 3) fusion of conjugants.
In addition to the induction of preconjugant interaction, mating types have another important function: the recognition of potential mates. Mating types enable cells to distinguish other cells of the same species from hundreds of different kinds of unicellular species in their natural environment. In both of these mating-type functions, gamones play the central role.

4.2 Gamones and gamone receptors

Gamones are usually detected and assayed by their capacity to induce conjugant pairs in target cells. Other cellular responses, such as the shortening of the preconjugative lag or the waiting period, preconjugant agglutination, chemoattraction, and induction of cell division, can also be used for detection and assay. So far gamones have been isolated only from gamone excretors. Gamone excretion in ciliates was first discovered by Kimball (1939a) in *Euplotes patella*, but gamones were first isolated and characterized in the second excretor to be discovered, *Blepharisma japonicum* (Miyake 1968a) (see below).

4.2.1 *Paramecium*

Experimental studies on gamones were first carried out in several species of *Paramecium*. Although they do not excrete gamones, killed cells and isolated cilia have gamone activity because they specifically stick to living cells of the complementary mating type, inducing them to conjugate. Studies on such systems showed that gamones are mainly on the cell membrane of the cilia located on the oral side of the cell and that they are probably proteins. For review see Metz (1954), Hiwatashi (1969, 1981), Hiwatashi and Kitamura (1985), Miyake (1974, 1981a), Kitamura (1988), and Watanabe (1990). Kitamura and Hiwatashi (1976) obtained membrane vesicles retaining gamone activity by treating isolated cilia of *P. caudatum* with urea and EDTA, but the gamones have not yet been isolated.
In *P. tetraurelia*, a protein of 31 kD, pI 6.8 in the ciliary membrane is virtually absent in the log phase. It increases in the early stationary phase and decreases in starved cells, suggesting that this protein might participate in preconjugant interaction (Adoutte et al. 1980).

4.2.2 *Tetrahymena thermophila*

Synthesis of some proteins are stimulated during preconjugant formation (Suhr-Jessen 1984) and preconjugant interaction (Garfinkel and Wolfe 1981; Ron and Suhr-Jessen 1981; Van Bell 1983; Suhr-Jessen 1984). Gamones and receptors might be found among these proteins. Love and Rotheim (1984) attempted to isolate membrane vesicles with gamone activity. If there is a positive feedback in gamone production, as suggested by Katz et al. (1987), more convincing results will be obtained by preparing membrane vesicles from cells pretreated by membrane vesicles of the other mating type. Endocytosis appears to play a role in preconjugant interaction, suggesting that gamone-receptor complexes are internalized to function (Rotheim and Love 1982).
Concanavalin A (Con A) inhibits conjugation in *T. thermophila* (Ofer et al. 1976; Frisch and Loyter 1977). The inhibition appears to be due to the binding of Con A to the surface rather than the uptake of Con A (Frisch and Loyter 1977; Casci and Hufnagel 1988). Pagliaro and Wolfe (1987) found that the Con A-receptor complex associates with the cytoskeleton and suggested that Con A inhibits conjugation by binding to gamone receptors.

4.2.3 *Blepharisma*

Gamone 1 of *B. japonicum* (blepharmone) was isolated, identified as a glycoprotein of about 20 kD with a pI of 7.5. It can induce pairing in type II cells at a concentration of 3 pM (Miyake and Beyer 1974). Gamone 1 contains about 175 amino acids

(nearest integers; Lys[7], His[1], Arg[4], Asp[26], Thr[17], Ser[19], Glu[7], Pro[8], Gly[13], Ala[13], Cys[4], Val[11], Met[6], Ile[8], Leu[12], Tyr[13], Phe[6]) and 6 sugars (ibid; glucosamine[3], mannose[3]) (Braun and Miyake 1975). It is heat sensitive and can be kept frozen in the presence of 0.01% bovine serum albumin. Gamone 1 has at least two functions: in type II cells it induces 1) pair formation and 2) production of gamone 2 (Figs. 6, 9).

Well fed cells do not excrete gamone 1. If such cells are suspended in non-nutrient medium, excretion of gamone 1 begins after several hours, reaching a peak after about 1 day (0.08 U/h/cell). One unit (U) is the smallest amount of gamone activity that can induce at least one pair in 500–1000 cells that are suspended in 1 ml of a non-nutrient salt medium. When the excretion rate drops later, it can be enhanced by gamone 2 (Miyake and Beyer 1973).

Gamone 2 of *B. japonicum* (blepharismone) was isolated and identified as 3-(2'-formylamino-5'-hydroxybenzoyl)lactate (Fig. 7). The molecular formula of crystallized gamone 2 is $(C_{11}H_{10}NO_6)_2Ca\ xH_2O$ (x, about 7 in dried condition). Taking this formula, gamone 2 can induce cell union in type I cells at a concentration of about 1.3 nM (Kubota et al. 1973). The calcium salt of gamone 2 is stable (Miyake 1981a). Gamone 2 is the only non-peptide gamone known in ciliates. It resembles the neurotransmitter serotonin both in structure (a tryptophan derivative) and in function (a specific intercellular signal) (Miyake 1978).

Racemic gamone 2 has been chemically synthesized in various ways (Tokoroyama et al. 1973, 1978; Entzeroth and Jaenicke 1981; Entzeroth et al. 1983). It is about half as active as the natural compound, which was explained by assuming that one of the enantiomorphs (D-) is less biologically active than the other (L-) (Tokoroyama et al. 1973). The assumption was confirmed by Entzeroth (dissertation 1983 cited in Jaenicke 1984) who synthesized D-blepharismone, which was scarcely active. Therefore, natural gamone 2 is L-blepharismone.

The chemical structure of gamone 2 suggests that tryptophan is the precursor of gamone 2 (Kubota et al. 1973). Indeed, if type II cells are incubated with [^{14}C]tryptophan, gamone 2 is labelled by ^{14}C (Miyake 1974, 1981b). Jaenicke (1984) proposed the following pathway of gamone 2 biosynthesis: L-tryptophan → 5-hydroxy-L-tryptophan → 5-hydroxy-N-formyl-L-kynurenine → L-blepharismone.

Entzeroth and Jaenicke (1981, 1982) examined the relative importance of various parts of the gamone 2 molecule for gamone activity by comparing the activity of synthesized gamone 2 derivatives (Fig. 7). The 2-hydroxy group of the side chain appears to be essential: N-formyl-5-hydroxy-kynurenine has no activity (Fig. 7). The formylamino group appears to be more important than the hydroxy group at position 5: 2-deformylamino-blepharismone has about 1/1200 of blepharismone activity, but 2-acetamido-blepharismone has no activity; 5-

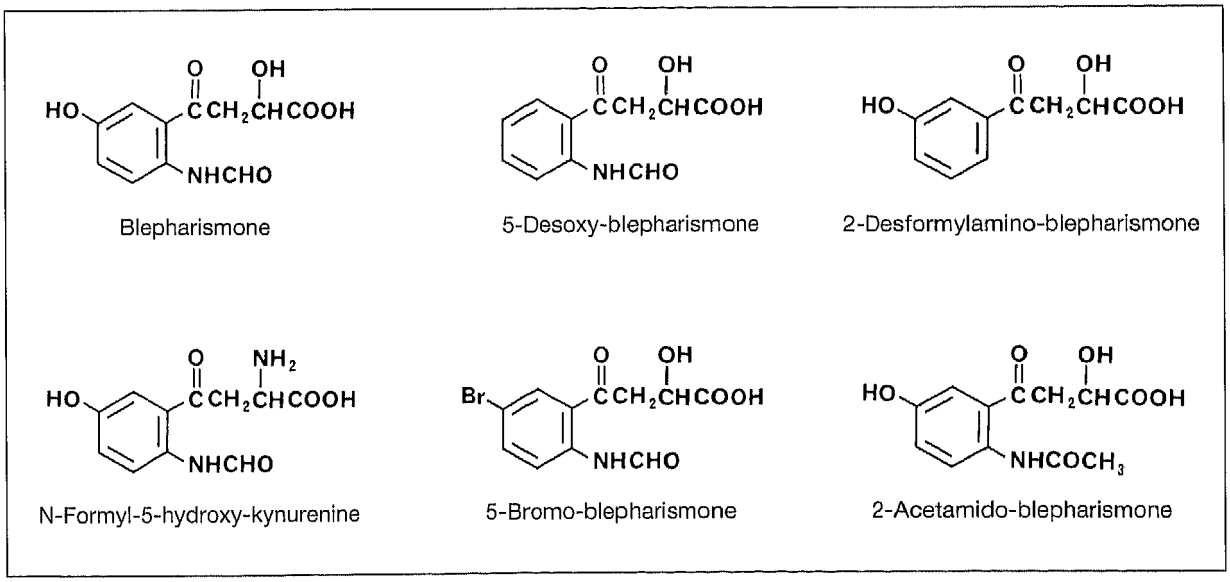

Fig. 7: Blepharismone (gamone 2 of *Blepharisma japonicum*) and five of synthetic derivatives with or without the gamone activity of blepharismone. Those in parentheses lack activity (Kubota et al. 1973, for blepharismone; Entzeroth and Jaenicke 1981, 1982, for blepharismone derivatives).

desoxy-blepharismone has about 1/40 of blepharismone activity, but 5-bromo-blepharismone has no activity (Fig. 7). They found, among analogues with different substitutions at position 5, a reverse correlation between the gamone activity and the lipophilicity of the substituted part, and suggested that the hydroxy group at position 5 binds to a hydrophilic area of the hypothetical receptor.

Three amino acids, 5-hydroxytryptophan, tryptophan, and leucine, competitively inhibit gamone 2 in this order (leucine being least inhibitive); the L-isomers are stronger inhibitors than the D-isomers (Miyake 1974, 1982). They do not inhibit the conjugation-inducing effect of gamone 1 (Miyake, unpublished results). Three compounds which are structurally closer to gamone 2, N-formyl-5-hydroxy-kynurenine (Fig. 7), N-formyl-kynurenine, and 5-hydroxy-kynurenine, competitively inhibit gamone 2 in this order (5-hydroxy-kynurenine least inhibitive), all more strongly than any of the above amino acids (Entzeroth and Jaenicke 1982). None of these inhibitors had gamone 2 activity. These results suggest that gamone 2 acts on type I cells by reversibly uniting to the receptor on type I cells.

Revoltella et al. (1976) showed that ^{125}I-labelled gamone 2 unites to type I cells fixed with formaldehyde and glutaraldehyde with a relative binding-affinity constant of 10^{-7} l/M and that the union is inhibited by unlabelled gamone 2. About 3×10^6 gamone 2 molecules united to a single cell under the conditions used.

Gamone 2 attracts type I cells at 10^{-3} U/ml (1.3 pM) or at even lower concentrations (Honda and Miyake 1975). This implies that type II cells attract type I cells. That this is really the case was demonstrated by using a chamber with a lattice window (Miyake and Rivola 1989) (Fig. 8). Type I cells also attract type II cells, but to a much lesser extent.

The chemoattraction by gamone 2 is due to kinesis (Miyake and Rivola 1989); type I cells moving toward a lower concentration of gamone 2 perform avoiding reactions more often. To sense the rapid change of gamone 2 concentration, the affinity between gamone 2 and receptor should not be too high. Results obtained by Honda and Miyake (1975) and Honda (1979) suggest that the chemoattraction and the induction of cell union are mediated by the same receptor.

In addition to inducing pair formation and chemoattraction, gamone 2 can promote the excretion of gamone 1 in type I cells. Thus, like gamone 1, gamone 2 is a pleiotropic signal.

If type II cells are moderately starved, gamone 2 is autonomously excreted in some cultures (*augex* [autonomously gamone excreting] form), but not in others (non-*augex* form). The latter can excrete gamone 2, if they are treated by gamone 1 (Miyake and Beyer 1973). This gamone 2 induction by gamone 1 takes about 2 h. In the example shown in Fig. 9, both intra- and extracellular gamone 2 appeared between 100–120 min after gamone 1 was added to non-*augex* type II cells, indicating that the produced gamone 2 was quickly excreted.

Gamone 2 production by non-*augex* type II cells is induced, even if the gamone 1 treatment is discontinued after 1, 1.5, or 2 h by washing cells free of gamone 1. The induced gamone 2 production continues for 3–4 h at the same rate as in the control which is continuously treated by gamone 1. Gamone 2 production then drops below the level of the control, but it continues for many more hours (Miyake, 1978). Therefore, the mechanism of gamone 2 production, once induced by gamone 1, continues functioning without gamone 1 for hours before it starts decaying. This suggests that gamone 1 induces an enzyme or enzymes for gamone 2 biosynthesis, possibly one or more of the enzymes participating in the hypothetical pathway of gamone 2 biosynthesis mentioned above.

Gamone excretion has been found also in *B. stoltei*, *B. americanum*, *B. musculus* (Miyake and Bleyman 1976), *B. tropicum* (Miyake and Mancini 1978), and a marine species, *Blepharisma* sp. (Ricci and Esposito 1981). In the first four of these species and in *B. japonicum*, the gamone excreted by mating type I appears to be species specific, while the gamone excreted by mating type II appears to be the same molecule, blepharismone (Miyake and Bleyman 1976; Miyake 1981a, b).

4.2.4 *Euplotes patella*

Kimball (1939a, 1942) found six mating types (I–VI) in *E. patella* and showed that they are determined by three codominant alleles, mt^1, mt^2, and mt^3. He tentatively concluded that each of these alleles controls the excretion of one of the three gamones 1, 2, and 3, each of which induces conjugation in mating types which do not excrete the same gamone (Table 7). Although conjugation occurred when any two mating types are mixed together, Sonneborn (1947) concluded from the data of Kimball (1939a, 1942) and Powers (1943) that heterotypic pairs are formed only in the mixtures in which gamone(s) of each mating type can induce conjugation in the other mating type; in other mixtures only homotypic pairs of one of the two

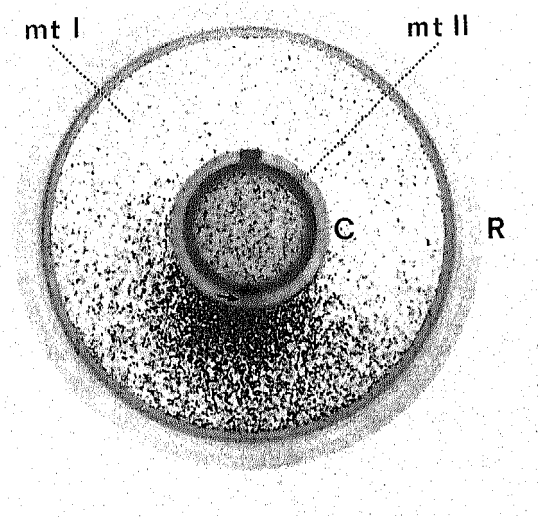

Fig. 8: Chemoattraction of mating type I (mt I) by mating type II (mt II) in *Blepharisma japonicum*. Photograph taken 20 h after mt I and mt II (non-*augex*) were respectively placed outside and inside the chamber **(C)** which has a window (arrow). The window is covered with a sheet of plastic net (pore size, about 12 × 12 μm) which can not pass blepharismas. The chamber is made of a plastic cylinder 5 cm high having, at 5 mm from the bottom, a hole 2 mm in diameter (window). The cylinder and an outer plastic ring 1 cm high **(R)** are both pressed on a sheet of Parafilm placed on a glass plate. Cells were kept at 24 ± 1°C under dim light except during observations. At the beginning, all cells were single and distributed homogeneously inside and outside the chamber. Outside the chamber, mt I cells started accumulating at the window after 2 h. Since non-*augex* mt II cells begin excreting gamone 2 about 2 h after they are exposed to gamone 1 (Fig. 9), mt I cells must have started accumulating at the window soon after mt II cells began excreting gamone 2. After 20 h, many homotypic pairs were found both inside and outside the chamber. In this photograph, single cells (about 100 × 300 μm) and pairs are seen as small dots. mt I: 7 ml of a 3000 cells/ml suspension of clone R107. mt II: 0.65 ml of a 3000 cells/ml suspension of clone R18. × 0.8. Culture and handling of cells were as described in Miyake et al. (1991).

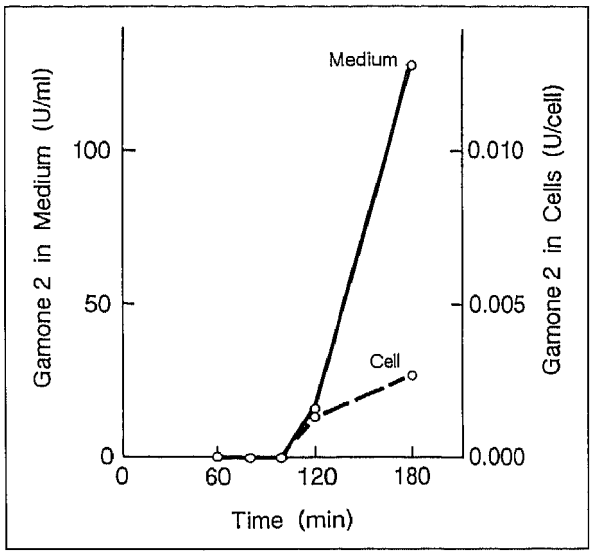

Fig. 9: Induction of production and excretion of gamone 2 by gamone 1 in non-*augex* mating type II in *Blepharisma japonicum*. Gamone 1 (10^4 units/ml) was added at time 0 to mating type II (5000 cells/ml) (modified from Miyake and Beyer 1973).

Table 7: Induction of conjugation in each mating type by cell-free fluid of other mating types in *Euplotes patella*. +: Occurrence of conjugation. –: No conjugation (based on data of Kimball 1942).

Mating types (genotypes)			I	II	III	IV	V	VI
		Excreted gamones[a]	1,2	1,3	3	1	2,3	2
I	(mt^1mt^2)	1,2	–	+	+	+	+	+
II	(mt^1mt^3)	1,3	+	–	+	+	+	+
III	(mt^3mt^3)	3	+	–	–	+	–	+
IV	(mt^1mt^1)	1	–	–	+	–	+	+
V	(mt^2mt^2)	2,3	+	+	+	+	–	+
VI	(mt^2mt^2)	2	–	+	+	+	–	–

[a] See 4.2.4

Table 8: Results of two-by-two mixing of the six mating types of *Euplotes patella*. +: Occurrence of heterotypic conjugation. (+): Occurrence of only homotypic conjugation of one mating type. –: No conjugation (based on data of Kimball 1942 and rationalization of Sonneborn 1947).

Mating types (genotypes)			I	II	III	IV	V	VI
		Mt-formulas[a]						
I	(mt^1mt^2)	$G_1G_2R_3$	–	+	+	(+)	+	(+)
II	(mt^1mt^3)	$G_1G_3R_2$		–	(+)	(+)	+	+
III	(mt^3mt^3)	$G_3R_1R_2$			–	+	(+)	+
IV	(mt^1mt^1)	$G_1R_2R_3$				–	+	+
V	(mt^2mt^3)	$G_2G_3R_1$					–	(+)
VI	(mt^2mt^2)	$G_2R_1R_3$						–

[a] See 4.3.3

mating types are formed (Table 8). However, intraclonal selfing and other kinds of «exceptional conjugation» were common in their materials and there were cases that did not fit Sonneborn's rationalization.

Sato (1983) found that *E. patella* syngen 2 (Katashima 1961) excretes gamones. Ten mating types (I–X) were found in this syngen (Katashima 1961; Akada 1985a). Akada (1985a) showed that these mating types are determined by four codominant

alleles, mt^1, mt^2, mt^3, and mt^4, each of which appears to control the excretion of one of the four gamones 1, 2, 3, and 4. Although intraclonal selfing occurred, experimental results were considered valid only when pairs were not found in the control. The heterozygous mating type VI (mt^2mt^4) excretes gamones 2 and 4, which are chromatographically separable (Akada 1986a).

Autonomous gamone excretion was not always high enough to make gamones in a cell-free fluid detectable, but the treatment by non-self gamone enhanced the excretion (Akada 1985a, b). Gamones are heat resistant (Akada 1985b, 1986a). Gamones 2 and 4 appear to be small proteins with little or no carbohydrate moiety (Akada 1986a).

As in Kimball's strain (no longer available), conjugation occurs when any two mating types are mixed together. Also gamone induces conjugation in the mating type which does not excrete the same gamone in accordance with Kimball's scheme (Table 7). The results, however, do not fit Sonneborn's scheme (Table 8): heterotypic pairs appear in combinations of mating types in which they are not expected to occur from the scheme (Akada 1985a).

4.2.5 *Euplotes octocarinatus*

Heckmann and Kuhlmann (1982, 1986) found ten mating types I–X in *E. octocarinatus*, a species morphologically similar to *E. patella*, and showed that they are determined by four codominant alleles, mt^1, mt^2, mt^3 and mt^4, each controlling the excretion of gamones 1, 2, 3, and 4, respectively. Each gamone induces conjugation in mating types which do not excrete the same gamone (Table 6). Heterozygous mating type I (mt^1mt^2) excretes gamones 1 and 2 which are electrophoretically separable (Weischer 1985). Conjugation occurs when any two mating types are mixed together, but heterotypic pairs were formed only in the mixtures in which gamone(s) of each mating type can induce conjugation in the other; in other mixtures, only homotypic pairs are formed (Table 5).

Thus Kimball's scheme (Table 7) and Sonneborn's rationalization in *E. patella* (Table 8), both of which were plagued by conflicting data, are perfectly applicable to *E. octocarinatus*, suggesting that these schemes are basically correct. Unlike cultures of *E. patella* mentioned above, those of *E. octocarinatus* were virtually free from selfing and «exceptional conjugation».

Gamones 1, 2 (Weischer 1985; Weischer et al. 1985; Weischer and Heckmann, personal communication), 3, and 4 (Schulze Dieckhoff et al. 1987) were all isolated and identified as acidic polypeptides of about 20 kD. They are only slightly glycosylated, if at all. For gamones 3 and 4, the pI value is 3.2.

The amino acid sequence of gamone 3, based on the cDNA sequence (Meyer et al. 1991) is as follows: Tyr – Tyr – Cys – Trp – Glu – Glu – Pro – Tyr – Thr – Ser – Ser – Ile – Thr – Gly – Cys – Ser – Thr – Ser – Leu – Ala – Cys – Tyr – Glu – Ala – Ser – Asp – Cys – Ser – Val – Thr – Gly – Asn – Asp – Gln – Asp – Lys – Cys – Asn – Asn – Val – Gly – Gln – Asn – Met – Ile – Asp – Lys – Phe – Phe – Glu – Leu – Trp – Gly – Val – Cys – Ile – Asn – Asp – Tyr – Glu – Thr – Cys – Leu – Gln – Tyr – Val – Asp – Arg – Ala – Trp – Ile – His – Tyr – Ser – Asp – Ser – Glu – Phe – Cys – Gly – Cys – Thr – Asn – Pro – Glu – Gln – Glu – Ser – Ala – Phe – Arg – Asp – Ala – Met – Asp – Cys – Leu – Gln – Phe.

Gamone 1 is stable at 4 °C and heat resistant (Weischer et al. 1985). Other gamones are also similarly stable (K. Heckmann personal communication). Each of these gamones induces pair formation in target cells at a concentration of 1 pM or even lower. These gamones resemble gamones in *E. raikovi* (Section 4.2.6) in the low pI value, the stability, and a high cystine content, while they resemble gamone 1 of *B. japonicum* in a high tyrosine content.

Kusch and Heckmann (1988a) obtained a polyclonal antibody against purified gamone 1. The antibody reacted with gamones 1 and 2 but not with gamones 3 and 4. Using the protein A-gold labelled antibody, they found gamone 1 in cytoplasmic cisternae, small and large cytoplasmic vesicles, and cortical ampoules in cells of mating type VII (mt^1/mt^1) and suggested that gamone 1 stored in vesicles is excreted via the cortical ampoules.

This scheme of gamone excretion is consistent with the characteristic activity of cortical ampoules during preconjugant interaction in *E. crassus* observed by Verni et al. (1978). In *E. crassus*, however, the excreted content of cortical ampoules does not necessarily contain gamones, because in this species excreted gamones were not detected by a rigorous test (Miyake and Nobili 1974).

Kusch and Heckmann (1988b) purified gamone 1 excreted by mating type VII cells of *E. octocarinatus* as well as gamone 1 contained in these cells. These two gamone samples were indistinguishable. Well fed cells of mating type VII do not excrete gamone 1. When slightly starved cells are suspended in a non-nutrient medium (10^4 cells/ml), the ga-

mone activity in the medium rises and then levels off after about 24 h; however, if cells are resuspended in a fresh medium, gamone activity in the medium starts rising. The result can be explained in two ways: 1) Cells may stop gamone excretion when gamone concentration in the medium reaches a certain level; 2) cells may continue gamone excretion, but may also remove the gamone from the medium by destruction, uptake, or both (Kusch and Heckmann 1988b). The same phenomenon had been found in *E. raikovi* by Luporini and Miceli (1986) (Section 4.2.6).

Kuhlmann and Heckmann (1989) observed in detail the process of maturation in *E. octocarinatus*. The capacity to excrete gamone(s) appears first, followed by the capacity to respond to gamones of other mating types, as in *D. anser* (Tavrovskaja 1979) (Section 4.2.8) and *E. raikovi* (Miceli and Luporini 1983) (Section 4.2.6). Mature exconjugant clones heterozygous at the *mt* locus excrete two of the four gamones, G 1, G 2, G 3, G 4, and respond to two other gamones. Initially, such clones neither excrete any of these gamones nor respond to any of them. They start excreting one gamone after about 15 fissions and the other gamone five to ten fissions later. Then they develop the capacity to respond to two other gamones, first for one gamone and five to ten fissions later for the other gamone (Fig. 10). Mature exconjugant clones homozygous at the *mt* locus excrete one of the four gamones and respond to three other gamones. In the process of maturation such clones often acquire the capacity to respond to a particular gamone one by one in three steps. The order of the acquisition of the two or three capacities to respond to different gamones appears to be at random. Thus during maturation, not only gamones but also the capacity to respond to a particular gamone behaves as separable units, suggesting the presence of a specific receptor for each gamone.

4.2.6 *Euplotes raikovi*

Miceli et al. (1981) found twelve mating types (I–XII) in *E. raikovi*, a marine species. Conjugation occurred when any two of them were mixed together. In the eight strains studied, each belonging to a different mating type, all but one excreted gamone which induced conjugation in many other strains (Luporini et al. 1982, 1983). Mating types are determined by codominant multiple alleles, mt^1, mt^2, ---, each controlling the excretion of a specific gamone (Luporini and Miceli 1984, 1986). Mating type designations were changed (Luporini and Miceli 1984, 1986) so that the designation of a mating type can directly indicate genotype at the *mt* locus and vice versa. For example, those mating types determined by genotypes, mt^1mt^1, mt^2mt^2, and mt^1mt^2, are now mating types I, II, and I–II, respectively.

The mating-type system of *E. raikovi* is similar to that of *E. patella*. Kimball's scheme for excreted gamones in *E. patella* (Table 7) is not applicable to *E. raikovi*; however, according to Luporini and Miceli (1984, 1986), this does not indicate any differences between the mating-type systems of the two species, because Kimball's scheme was established disregarding «exceptional conjugation».

Gamone of strain 13 was isolated, identified as a glycoprotein of about 12 kD with a pI of 4, and named as euplomone *r*-13 (Er-13) (Miceli et al. 1983). It is heat resistant and stable at 4°C. It induced conjugation in target cells at a concentration of 3 pM. Later strain 13 was discovered to be a heterozygote (mt^1mt^2) excreting two similar gamones, Er-1 and Er-2 (Luporini and Miceli 1984, Luporini et al. 1986). Thus Er-13 was a mixture of two isogamones. Concetti et al. (1986) purified Er-1 and identified it as a protein of about 12 kD with a pI of 3.7. Carbohydrate and a yellow-colored compound, both associated with the original Er-13 sample, were not detected in purified Er-1.

Four more gamones, Er-2, Er-3, Er-11 (Raffioni et

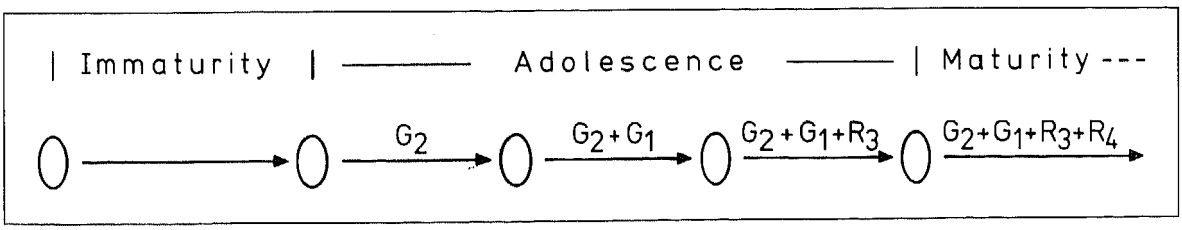

Fig. 10: Successive acquisitions of mating-type characters of mating type I during clonal development from immaturity to maturity in *Euplotes octocarinatus*. G_1: Excretion of gamone 1. G_2: Excretion of gamone 2. R_3: Capacity to respond to gamone 3. R_4: Capacity to respond to gamone 4 (Kuhlmann and Heckmann 1989).

al. 1987), and Er-10 (Raffioni et al. 1989), have been isolated from homozygous mating types, II (mt^2mt^2), III (mt^3mt^3), XI ($mt^{11}mt^{11}$), and X ($mt^{10}mt^{10}$), respectively. They are effective in inducing homotypic pairs in target cells at concentrations of 0.25–12.0 pM. The amino acid sequence of their subunits was determined for Er-1 (Raffioni et al. 1988), Er-10 (Raffioni et al. 1989), and Er-2 (Raffioni S, Luporini P, Miceli C, Bradshaw RA, cited in Bradshaw et al. 1990) (Fig. 11). Excreted gamones are assumed to be dimers (Bradshaw et al. 1990), but they may be tetramers as Er-1 appears to be a tetramer in the crystalline state (Anderson et al. 1990).

Properties of these isogamones are summarized in Table 9. They are characterized by a high content of cystine, which probably contributes to their stability, and by low pI values. In sea water (pH = about 8.2) they should be highly charged negatively and hence should unite ionically with various positively charged molecules, such as amino sugars and amine compounds. The association mentioned above of carbohydrate and yellowish compound to gamones may be due to this ionic association; however, the yellowish compound may be a cofactor of gamones (Concetti et al. 1986; Luporini and Miceli 1986).

Fiori et al. (1990) obtained monoclonal antibodies against Er-1 and Er-2. Some of them recognized only Er-1 or Er-2, while others recognized both gamones, indicating that Er-1 and Er-2 have specific as well as common epitopes.

Miceli et al. (1989) isolated and sequenced cDNA clones encoding Er-1. Analyzing their base sequences, they proposed that cells first synthesize a

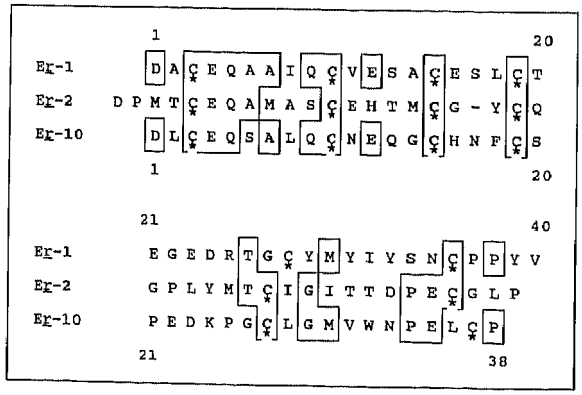

Fig. 11: Comparison of the amino acid sequences of the three isogamones of *Euplotes raikovi*, Er-1, Er-2, and Er-10. A two residue-shift and one gap are introduced in Er-2 to improve the alignment with the other two gamones. The half-cystine residues are emphasized by asterisks (Bradshaw et al. 1990).

Table 9: Properties of euplomones-*r*, isogamones in *Euplotes raikovi* (from Bradshaw et al. 1990).

Subunit molecular weight	4000–5000
Subunit structure	Non-covalent homodimer
Amino acid content	38–40 residues 15–16% cystine 2.5–5% basic residues 12.5–17.5% acidic residues
Isoelectric point	3.7–4.0
N-terminal	Aspartic acid (free)
CHO	None

precursor peptide, Pre-Pro-Er1, consisting of 75 amino acids. Then Pro-Er1 is formed by removing 19 amino acid residues at the amino terminus, which is probably a signal peptide. When another 16 amino acid residues are removed, Er-1, consisting of 40 amino acids, is formed and excreted. Similar precursors containing pre and pro regions were found also for Er-2 and Er-10 in the same way (Miceli et al. 1991). The amino acid sequence of the pre region is identical in Er-1, Er-2 and Er-10. The pro region is also highly conserved.

Miceli et al. (1992) obtained evidence that mating type I cells produce membrane-bound Er-1 (Er-1memb), in addition to soluble Er-1. They assume that Er-1memb may be a binding site for soluble Er-1 thus functioning as the receptor for the self gamone postulated by the self-recognition hypothesis (see below and Section 4.3.5). However, both Er-1 and Er-1memb may function as gamone, excreted and cell-bound gamone, respectively, as discussed in Section 4.1.

Cells suspended in nutrient-free sea water autonomously excrete gamone(s). The gamone activity in the medium rises and then levels off at about 10^4 U/ml. If cells are resuspended in the sea water, gamone activity in the medium increases again; however, if cells are suspended in a sea water to which 10^4 U/ml of the gamone is added, an increase in gamone activity is not detected. Based on these results, Luporini and Miceli (1986) concluded that cells can sense the environmental level of their own gamone.

They also found that the conjugation-inducing effect of foreign gamone is competitively inhibited by the gamone excreted by its target cells (Luporini and Miceli 1986). Based on these two findings and other pieces of evidence, Luporini and Miceli (1986) thought that recognition of a self gamone

might participate in conjugation. For more details of this self-recognition hypothesis see Section 4.3.5. To examine this hypothesis, Ortenzi et al. (1990) studied the binding of E*r*-1 to cells of mating type I, which excrete E*r*-1, and found that ^{125}I-E*r*-1 binds specifically to high affinity binding sites of these cells (apparent K_d = [4.63 ± 0.12] × 10^{-9} M). The binding was inhibited by unlabelled E*r*-1, E*r*-2, E*r*-10, antibodies specific for E*r*-1, and human IL-2. They also found that the membrane of mating type I cells contains a protein of 28 kD which specifically binds to E*r*-1.

Exconjugant clones start excreting gamone(s) very early in their life cycle, although the capacity to respond to foreign gamones is acquired much later, after 30–40 fissions (Miceli and Luporini 1983). To test the hypothesis that the presence of a gamone influences the acquisition of the capacity to respond to the gamone, Luporini and Miceli (1984) cultured 26 exconjugant clones of mt^2mt^2 (genotypically destined to produce E*r*-2 and to respond to other gamones by conjugation) under the presence of E*r*-1 (1 μg/ml). At maturity, 16 clones did not respond to E*r*-2 and even to E*r*-1. This result indicates that the presence of a gamone during the immature period can prevent cells from developing the capacity to respond to the gamone.

4.2.7 *Euplotes woodruffi*

In *E. woodruffi* syngen 3, one mating type excretes a gamone that can induce homotypic conjugation in cells of complementary mating types (Kosaka 1990). Frozen, thawed, and washed cells retained the gamone activity, suggesting that the gamone is also cell-bound. Kosaka (1991) found that frozen and thawed mature cells of one mating type specifically attract mature cells of complementary mating type.

4.2.8 *Dileptus anser*

Vinnikova and Tavrovskaja (1973) found in *D. anser* three mating types (I–III) which are complementary to each other. These mating types appear to be determined by three alleles showing serial dominance (Yudin and Afon'kin 1987). Each mating type excretes a gamone or gamones that induce conjugation (Vinnikova and Tavrovskaja 1973; Tavrovskaja 1979), cell division (Tavrovskaja 1974), and chemoattraction (Afon'kin and Yudin 1987) in other mating types. In the process of maturation of exconjugant clones, the ability of gamone excretion appeared first, followed by the ability to react to gamones of other mating types (Tavrovskaja 1979).

The gamone of mating type II, which is heat resistant (Tavrovskaja 1974), has been partially purified and tentatively identified as a polypeptide of about 3 kD (Parfenova et al. 1989). The purified sample was effective at a concentration of 1 ng/ml in inducing cell division in mating types I and III. Assuming that a single gamone induces both conjugation and cell division, induction of conjugation required a higher concentration of gamone than induction of cell division (Tavrovskaja 1979).

A striking feature of gamones in *D. anser* is the cell-division-inducing effect, which has not been reported for any other purified gamones. In *T. thermophila* (then *T. pyriformis* syngen 1), preconjugant interaction induces cell division under certain conditions (Wolfe 1974) suggesting that cell-bound gamones can also induce cell division. *Dileptus* is one of the ciliates in which the occurrence of preconjugation division (cell division which regularly precedes conjugation) has been reported (Raikov 1972 for review). More cases of gamone-induced cell division may be found among those ciliates in which the occurrence of preconjugation division is known. It should be noted, however, that in some ciliates preconjugation division produces two types of cells complementary for conjugation, e.g., some peritrichs and Hf-selfers in *B. japonicum* (3). Therefore, the phenomenon described as preconjugation division may include at least two distinctly different kinds of cell division: one is induced by preconjugant interaction, the other is to produce two types of cells which can undergo preconjugant interaction. In both cases, the striking association between cell division and conjugation provides a problem worthy of further study as pointed out elsewhere (e.g. Cleffmann and Miyake 1986; Ng 1990).

4.2.9 *Oxytricha bifaria*

Excreted gamones of *O. bifaria* were detected by Ricci et al. (1975 a) and studied using diffusion chambers described by Esposito et al. (1976). There are three mating types which are complementary to each other. The gamone of one mating type can shorten or eliminate the waiting period in the other two mating types, but cannot induce conjugant pairs in any of them. For the formation of conjugant pairs, «visible reaction», which occurs

between complementary mating types mediated by direct cell-cell contact, is needed. It appears therefore that cell-bound gamones also participate in preconjugant interaction (Ricci et al. 1980b). For review see Ricci (1981).

4.2.10 Suctoria

In sessile ciliates, *Ephelota gemmipara* (Grell 1973) and *Tokophrya infusionum* and *T. lemnarum* (Sonneborn 1978), excretion of gamones has been inferred based on observations of interacting preconjugants. In *Tokophrya*, complementary mating types placed in a certain distance orient and stretch toward each other, even if they are too far apart to reach each other.

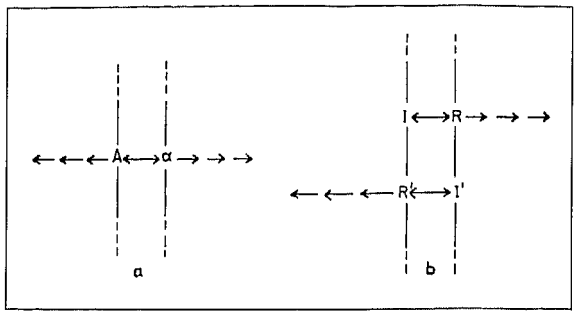

Fig. 12: Two possible schemes of preconjugant interaction postulated by Metz (1948, 1954) for *Paramecium* species with the binary mating-type system. Each series of arrows represents the activation process of the mechanism of conjugation in one conjugant. **a.** Simultaneous activation of conjugants by interaction of a single pair of surface substances, A and α. **b.** Simultaneous activation of conjugants by interaction of two pairs of surface substances, I and R, R' and I' (Metz 1948).

4.3 Hypothetical schemes

Several hypotheses on reacting substances in preconjugant interaction have been put forward. They will be presented in chronological order.

4.3.1 Metz's schemes

Metz (1948, 1954) presented two possible schemes for the mating reaction in *Paramecium*: 1) the mating reaction is due to the reaction between a pair of substances A and α, each of which is on the cell surface of one of the complementary mating types (Fig. 12a); 2) the mating reaction consists of two reactions each occurring between a pair of inducing and reacting substances, $I - R$ and $I' - R'$, on the cell surface. I of one mating type reacts with R of the other mating type, while I' of the latter reacts with R' of the former (Fig. 12b). In both schemes, the reaction between these substances switches on the cellular mechanism of conjugation. Since there was no evidence for the second scheme, Metz adopted the first one, and this scheme has been widely used in the study of conjugation in *Paramecium* (e.g., Tsukii's model of mating-type substances [Tsukii 1988]).

The scheme can be applied to multiple mating-type systems by postulating two or more pairs of substances. For example, in the homogeneous multiple system of *P. bursaria*, syngen 1, consisting of four mating types, is supposed to have two pairs of mating-type substances, A, α and B, β. Each mating type contains one substance from each pair in four different combinations, A, B; A, β; α, B; and α, β (Metz 1954). Then each mating type can undergoes mating reaction (A-α, B-β or both) with all other three mating types. This application was also experimentally supported (Cohen and Siegel 1963).

However, Metz applied neither scheme to gamone excretors. Thus interaction among six mating types of *E. patella* studied by Kimball (1939a, 1942) (4.2.4) was left unexplained.

4.3.2 Reiff's scheme

Reiff (1968) adopted Metz' second scheme (Fig. 12b) to explain interaction among 10 mating types in *Uronychia transfuga* (Table 2), a gamone carrier. Thus the four codominant alleles, mt^1, mt^2, mt^3, and mt^4, control the production of inducing substances I_1, I_2, I_3, and I_4, respectively. Each cell then has two or three of the four reacting substances, R_1, R_2, R_3, and R_4, excluding those which react with its own inducing substance(s). For example, genotypes, and inducing and reacting substances of mating types I, III, and VII are as follows:

Type I	mt^1mt^2	$I_1I_2R_3R_4$
Type III	mt^1mt^3	$I_1I_3R_2R_4$
Type VII	mt^1mt^1	$I_1R_2R_3R_4$

He postulated that the reaction of the reacting substance *(R)* of a cell with the inducing substance *(I)* stimulates the cell for conjugation and that conjugation occurs only in the combination of mating types in which each mating type stimulates the

other. For example, conjugation occurs in the mixture, type I × type III, because I_2 of type I reacts with R_2 of type III and I_3 of type III reacts with R_3 of type I. Conjugation does not occur in type I × type VII, because only type I has the inducing substance (I_2) that can react with the reacting substance of the other mating type.

Why stimulated type VII cells in type I × type VII do not conjugate between themselves was later explained by Miyake (1981a) by postulating a positive feedback in the $I-R$ reaction as follows. The amount of I on the cell surface is usually too small to induce conjugation in other mating types; however, the amount increases once cells are stimulated by the $I-R$ reaction. If two mating types stimulate each other, as in type I × type III, the amount of I on both mating types increases through a positive feedback loop. This cannot occur in type I × type VII, because only type I can stimulate type VII. That is, I_2 of type I can stimulate type VII to produce more I_1. But since I_1 cannot stimulate type I, the amount of I_2 in type I remains at the initial low level which is insufficent to induce conjugation in type VII.

4.3.3 Gamone-receptor hypothesis

In *Blepharisma japonicum*, each of the two complementary mating types I and II excretes a gamone which specifically induces homotypic pairing in the other mating type (Fig. 6) (Sections 4.1, 4.2.3). Miyake and Beyer (1973) assumed that those cells which respond to a gamone have, on their surface, a receptor specific for the gamone. Thus mating type I excretes gamone 1 (G1) and has receptor 2 (R2) which specifically recognizes gamone 2 (G2). The mating-type formula (Mt-formula) is then G_1R_2. Mating type II excretes G2 and has receptor 1 (R1) which specifically recognizes G1. Mt-formula is then G_2R_1. Receptors were assumed to switch on the cellular mechanism of conjugation when they unite with their gamones. This scheme is the same as Metz's second scheme (Fig. 12b), which Reiff adopted to explain his results on *U. transfuga* (Section 4.3.2), except that G1 and G2 are excreted signals.

Miyake (1981a) generalized this scheme to make it applicable to all ciliates. In this gamone-receptor (GR) hypothesis, mating types are produced by differential distribution of gamones and receptors among individuals. Each mating type has 0, 1 or more of different kinds of gamones and 0, 1 or more of different kinds of receptors except the one(s) for its own gamone(s). When a receptor on the cell surface unites with a gamone specific for the receptor, the cellular mechanism of conjugation is switched on. Gamones may be excreted or cell-bound.

The GR-hypothesis was applied (Miyake 1974, 1981a) to the multiple mating-type system of *E. patella* studied by Kimball (1939a, 1942) (Section 4.2.4; Tables 7–8). Because the system has three gamones, G1, G2, and G3, the GR-hypothesis assumes that the system has three receptors, R1, R2, and R3, each specific for G1, G2, and G3, respectively. Since mating type I produces G1 and G2, it can have only one receptor, R3. The tentative Mt-formula of mating type I is, therefore, $G_1G_2R_3$. Since mating type IV produces only one gamone, G1, it can have two receptors, R2 and R3, and its tentative Mt-formula is $G_1R_2R_3$. Using this reasoning, tentative Mt-formulas of all the six mating types of the system are given as shown in Table 8. With these formulas, all the results in Tables 7 and 8 can be explained. For example, in type I ($G_1G_2R_3$) × type II ($G_1G_3R_2$) G2 of type I reacts with R2 of type II, while G3 of type II reacts with R3 of type I. Thus each type is stimulated by the other, resulting in heterotypic as well as homotypic conjugation. In type I ($G_1G_2R_3$) × type IV ($G_1R_2R_3$), G2 of type I reacts with R2 of type IV, but type IV has no gamone to react with receptor of type I. Thus only type IV cells are stimulated and only homotypic pairs of type IV are formed.

Heckmann and Kuhlmann (1986) fully explained all their results on 10 mating types in *E. octocarinatus* (Section 4.2.5) shown in Tables 5 and 6 by adopting the GR-hypothesis. Kuhlmann and Heckmann (1989) also found clones which do not excrete any of the four known gamones (G1, G2, G3, and G4. These clones responded to all these gamones as predicted by the GR-hypothesis. In addition, their finding that in the process of maturation the capacity to respond to a particular gamone behaves as a separable unit (Section 4.2.5) (Fig. 10) supports the GR-hypothesis, which postulates a specific receptor for each gamone (Kuhlmann and Heckmann 1989).

As described in (Section 4.3.2), the apparently complicated complementarity relationships among 10 mating types in *U. transfuga* (Table 2) can be fully explained by Reiff's scheme, which is essentially the same as the GR-hypothesis, if supplemented by the assumption of a positive feedback in the gamone-receptor reaction (Section 4.3.2). Thus the GR-hypothesis can be applied not only to gamone excretors but also to gamone carriers.

In the homogeneous multiple mating-type system consisting of five mating types (I–V), e.g., *E. vannus* (Heckmann 1963), proposed Mt-formulas for mating types I, II, III, IV, and V, are $G_1R_2R_3R_4R_5$, $G_2R_1R_3R_4R_5$, $G_3R_1R_2R_4R_5$, $G_4R_1R_2R_3R_5$, and $G_5R_1R_2R_3R_4$, respectively (Miyake 1981a). These formulas explain why any mating type can conjugate with any other mating types.

Experimental support for this scheme was obtained by Lueken et al. (1983) using four mating types, A, B, C, and D, of *E. vannus*, which are complementary with each other. Mixtures of two mating types (e.g., C and D) were continuously shaken, thus allowing them to interact while preventing them from forming conjugant pairs. When the shaking stopped after a few days, they could no longer conjugate with each other; however, they retained the capacity to conjugate with other two mating types (A and B). Thus the capacity to interact with a mating type is selectively lost by interacting with this mating type for an extended period. Since *E. vannus* is a gamone carrier (Heckmann 1963), the effect of cell-free fluid cannot be used as an evidence for the presence of gamones and receptors, but these experiments indicate that each mating type possesses operationally separable mating-type-specific factors for preconjugant interaction, such as the gamones and receptors postulated by the GR-hypothesis.

Lueken et al. (1983) explained their result using the GR-hypothesis as follows. In the mixture, type C ($G_cR_aR_bR_d$) × type D ($G_dR_aR_bR_c$), Gc in type C reacts with Rc in type D while Gd in type D reacts with Rd in type C. If preconjugant interaction is prolonged by the shaking which prevents formation of conjugant pairs, Rd in type C and Rc in type D are selectively lost by «exhaustion». As a result, types C and D, which have been shaken for two days, can no longer conjugate with each other; however, they can conjugate with type A ($G_aR_bR_cR_d$) and type B ($G_bR_aR_cR_d$), because they still have R_a and R_b. The «exhaustion» may be interpreted as follows. Receptors pick up gamones when cells collide and separate. The Gc-Rc and Gd-Rd complexes thus formed are then lost, possibly by internalization. After a few days of shaking, cells are so starved that they can hardly replenish lost receptors resulting in the selective loss of Rd in type C and Rc in type D.

The block phenomenon found in *E. crassus* (Table 3) and in *Stylonychia mytilus* (Table 4) provides an opportunity to test the GR-hypothesis. The phenomenon can be explained by the GR-hypothesis by postulating that a mating type not reacting with a certain mating type lacks the receptor for the gamone of the latter mating type. Proposed Mt-formulas in the two mating-type systems with the block phenomenon are shown in Tables 3 and 4, together with the results of mixing of mating types. All results of mixing can be explained by using these Mt-formulas, if it is assumed that a positive feedback in the gamone-receptor reaction is needed for the occurrence of conjugation. This same assumption supplemented the GR-hypothesis to explain fully the complementarity relations in 10 mating types of *U. transfuga* (Section 4.3.2). More critical tests of the GR-hypothesis may be carried out by making new mixtures of different mating types, the results of which can be predicted by the hypothesis.

One of the basic assumptions of the GR-hypothesis is that ciliate cells do not have the receptor for their own gamone in the functional form. How this mutual exclusion between a gamone and its receptor is achieved can be explained in various ways. Probably the most straightforward explanation is that the very presence of a gamone inhibits its receptor. Indeed, Reiff (1968) assumed that each mating type has the genetic potential to produce all the reacting substances (receptors in the GR-hypothesis) but that the presence of an inducing substance (gamone in the GR-hypothesis) somehow inhibits the production of its reacting substance. Kuhlmann and Heckmann (1989) also assumed that each mating type produces all the receptors including the one for its own gamone but that the receptor is somehow prevented from becoming functional by the presence of its gamone, possibly by downregulation. This exclusion mechanism may function all the time whenever gamones and receptors are being produced, but it may operate only for a short time in the immaturity period, shutting down permanently the synthesis of a particular receptor. The latter assumption is supported by the finding that exconjugant clones of *E. raikovi* grown in the presence of an experimentally added gamone failed to respond to this gamone at maturity (Luporini and Miceli 1984) (Section 4.2.6).

The other basic assumption of the GR-hypothesis, that the gamone-receptor reaction switches on the mechanism of conjugation, was adopted, because it appears to be simpler than other alternatives. It is still to be proved, however.

The GR-hypothesis may have difficulty explaining the large number of mating types found or expected in some ciliates without postulating unrealistically large number of different kinds of receptors on the surface of a single cell. Possible solutions of

this problem are presented below (Section 4.3.4) in connection with the hypothesis of Ammermann (1982) who pointed out the problem for the first time.

4.3.4 Ammermann's inhibitor-receptor hypothesis

A radically different scheme was proposed by Ammermann (1982) for *Stylonychia mytilus*, a gamone carrier, in which dozens of mating types have been reported (Ammermann 1965, 1982). In this scheme, each cell has on the surface a mating-type-specific mating-inhibiting substance (J) and a receptor (R) for this inhibitor. If two cells of the same mating type collide, the inhibitor of one cell reacts with the receptor of the other cell resulting in no conjugation (Fig. 13 A). If cells belonging to different mating types collide, no inhibitor-receptor reaction occurs resulting in conjugation (Fig. 13 B). Although not explicitly stated, the hypothesis implies that each cell has a potential capacity to conjugate with any other cell, since the absence of the J–R reaction results in conjugation. Yet cells must also recognize their own species, because, as a rule, conjugation occurs between cells of the same species. No explanation for this mechanism of species recognition was offered.

Ammermann (1982) found that some mating types behave as if they belong to two or three mating types (Table 4). The phenomenon is the same as the block phenomenon (Table 3), first described by Magagnini and Santangelo (1977) in *E. crassus*. Ammermann explained the phenomenon by assuming two or three pairs of inhibitors and receptors (Fig. 13 C).

This hypothesis can explain large number of mating types by assuming only one or a few pairs of inhibiting substances and receptors on the cell surface. Ammermann (1982), who estimates the number of mating types in *S. mytilus* to be higher than one hundred, emphasizes this as an advantage of his hypothesis over the GR-hypothesis. This is not necessarily the case, however, because of the block phenomenon.

Unlike the inhibitor-receptor hypothesis, which explains the block phenomenon by increasing the number of inhibitor-receptor pairs on the cell surface, the GR-hypothesis explains the phenomenon by reducing the number of receptors (Section 4.3.3). For example Ammermann's mating type S 1/25, which belongs to mating types 1 and 25, are assumed to lack receptors for gamones of mating types S 1 and S 25. The tentative Mt-formula for S 1/25 is, therefore, $G_1R_3R_4R_5R_{22}R_{23}R_{24}$, as shown in Table 4 together with tentative Mt-formulas for some other mating types. Then S 1/25 cannot conjugate with S 1 ($G_1R_3R_4R_5R_{22}R_{23}R_{24}R_{25}$) and S 25 ($G_{25}R_1R_3R_4R_5R_{22}R_{23}R_{24}$) because of the lack of mutual stimulation which is a requisite for the positive feedback in the gamone-receptor reaction (for the need of the positive feedback for the occurrence of conjugation, see Sections 4.3.2, 4.3.3). For example, in the mixture S 1/25 × S 25, G1 of S 1/25 reacts with R1 of S 25, but G25 of S 25 cannot react with any receptors in S 1/25. Similarly, mating type S 23/24/25 lacks receptors for gamones of mating types S 23, S 24 and S 25 (Mt-formula for S 23/24/25: $G_{25}R_1R_3R_4R_5R_{22}$).

Thus, the increase in the number of mating types by the block phenomena does not require any additional receptor on the cell surface. That is, in a mating-type system with the block phenomenon, the number of different receptors on the cell postulated by the GR-hypothesis can be much less than the total number of mating types in the system. The

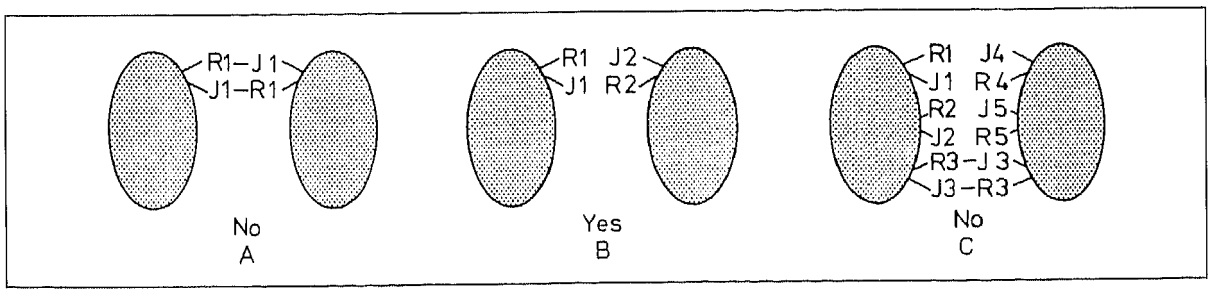

Fig. 13: Diagram for the scheme of preconjugant interaction in *Stylonychia mytilus* postulated by Ammermann (1982). **(A)** Encounter of cells of the same mating type. **(B)** Encounter of cells of complementary mating types. **(C)** Encounter of cells of different but non-complementary mating types each belonging to three mating types. Yes: Occurrence of conjugation. No: No occurrence of conjugation. J: Mating inhibiting substance (inhibitor). R: Receptor of J. (Ammermann 1982).

great heteropolarity of the multiple mating-type system of *E. crassus* studied by Valbonesi et al. (1992) (3) suggests an extensive occurrence of the block phenomenon also in *S. mytilus*.

Potentially more important for solving the problem of many mating types is the possibility that one gamone reacts with two or more similar but different receptors in cells of other natural populations. In species having many mating types, two populations, which have been isolated from each other, may have similar but slightly different arrays of gamones and receptors. In such populations, each gamone of one population might react with two or more receptors of the other population. If so, each mating type of one population reacts with all mating types in the other population and, therefore, is judged as different from all these mating types. This increases the total number of mating types in the species. As more populations are studied, the number of mating types in the species continues to increase, giving an impression that the total number of mating types in a species is virtually infinite. Yet each cell of a population has only a set of different gamones and receptors available in the population which is probably less than a few dozens. The validity of this assumption can be tested by examining mating types in progenies after crossing two geographically isolated populations. One of the predictable results is the appearance of the block phenomenon due to the mutual exclusion between a gamone and some receptors with which this gamone reacts.

In the above discussion, it was tentatively postulated that each cell of *S. mytilus* expresses only one gamone at a time. However, the mode of mating-type determination in this species is poorly understood. It appears to be epigenetic, possibly caryonidal like in *T. thermophila* (Ammermann 1965, 1982), but available data cannot exclude the possibility that two or more gamones are regularly expressed on the same cell at a time. If this is the case, different schemes, in which each cell has many less receptors, can be constructed.

4.3.5 Self-recognition hypothesis

Luporini and Miceli (1984, 1986) proposed another scheme for *E. raikovi*. They assumed that each cell has the receptor for the gamone excreted by the same cell (self gamone) and that the reaction between the receptor and self gamone produces an inhibiting effect on conjugation. Therefore, conjugation between cells of the same mating type is inhibited through this self-recognition process. Gamones of other mating types (foreign gamones) can also react with the same receptor. The competitive binding by foreign gamones reduces the inhibiting effect by the self gamone, resulting in conjugation. Thus in this self-recognition hypothesis, a mating type, which excretes a single kind of gamone, has only one kind of receptor, which can bind to all kinds of gamones in the same species. The binding with self gamone inhibits conjugation, while the binding with foreign gamone induces conjugation by lifting this inhibition.

The findings that self gamone competitively inhibits the conjugation-inducing effect of a foreign gamone (Luporini and Miceli 1986) (Section 4.2.6) and that a gamone binds to the cell which produces it (Ortenzi et al. 1990) (Section 4.2.6) are consistent with the hypothesis. However, these results are not contradictory to alternative hypotheses such as the GR-hypothesis. For example, since gamones in *E. raikovi* are chemically similar isogamones, a self gamone may bind to receptors for foreign gamones, competitively inhibiting the conjugation-inducing effect of foreign gamones just as chemical analogues of gamone 2 competitively inhibit gamone 2 in *B. japonicum* (Section 4.2.3).

Luporini and Miceli (1986) found that gamone activity of a cell suspension of *E. raikovi* stops increasing when the activity reaches a certain level. They assume that this phenomenon is due to the cell's capacity to recognize the concentration of self gamone and is thus supporting evidence for the self recognition hypothesis.

Heterozygous mating types, such as mt^1mt^2, in which each cell produces two different gamones, require further amplification of the self-recognition hypothesis. According to the hypothesis, each gamone of a heterozygous mating type inhibits conjugation by binding to its receptor, but at the same time it induces conjugation by preventing the inhibiting effect of the other gamone excreted by the same mating type. However, selfing in such heterozygotes occurs only in exceptional cases. Luporini and Miceli (1986) base their explanation on the finding that most heterzygotes excrete two gamones in different amounts. They tentatively assumed that 3/4 of the receptors of a cell must be heterotypically occupied to provide the cell with the capacity to conjugate and that this is not achieved if two gamones are excreted, for example, in a 7:3 ratio. Whether this assumption is consistent with the fact that foreign gamones can induce conjugation at very low concentrations of the pM order remains to be examined.

The fundamental assumption of this hypothesis is that self recognition inhibits the occurrence of conjugation. In this respect it is similar to Ammermann's inhibitor-receptor hypothesis. In both cases, however, the occurrence of inhibition of conjugation by self gamone (or inhibitor) is still to be demonstrated by showing, for example, that conjugation can be induced by inactivating or removing receptors, and by inactivating or removing self gamone (or inhibitor).

Each of the five schemes described above has its origin in an effort to explain the experimental results in a particular ciliate. Although serious attempts to apply the scheme to ciliates in general has been made only for the GR-hypothesis, other schemes are also potentially applicable to ciliates other than the one for which the scheme was originally presented. These working hypotheses make it possible to investigate preconjugant interaction from various angles.

Reviews of preconjugant interaction in ciliates include Metz (1954), Grell (1973), Miyake (1974, 1978, 1981a), Nanney (1977, 1980), Sonneborn (1978), Esposito and Nobili (1982), Vivier (1984), Luporini and Miceli (1986), Orias (1986), Nobili et al. (1987). Preconjugant interaction of individual ciliates has been reviewed for *Blepharisma* (Miyake 1981b), *Blepharisma* and *Euplotes* (Luporini and Miceli 1986), *Euplotes* and *Paramecium* (Metz 1954), *Oxytricha* (Ricci 1981), *Paramecium* (Hiwatashi 1969, 1981; Hiwatashi and Kitamura 1985), and *Tetrahymena* (Goodenough 1980, Orias 1986).

5 Conjugant union

Preconjugant interaction transforms the participating cells so that they can form a conjugant union. Transformed cells tightly unite into pairs (conjugant pairs) at a particular area of the cell surface by the direct contact of pellicles, not by a ciliary adhesion. Thus, the conjugant union is formed at a nonciliated area of the cell surfaces, as in *Blepharisma* (Bedini et al. 1978), or cilia degenerate first and the union is formed between deciliated surfaces as in *Paramecium* (Watanabe 1990 for review).

In bizygotic conjugation (Figs. 2, 3), two conjugants of a pair remain united until they separate into exconjugants. The conjugant union is morphologically different according to the species and stage of conjugation, but it generally consists of one or more of three components: 1) juxtaposed cell membranes with a distance of 20–50 nm between them; 2) cytoplasmic bridges; and 3) vacuoles (Figs. 14, 18) (Miyake 1981a for brief review; Wolfe 1982 for details in *Tetrahymena*).

The cell fusion caused by the cytoplasmic bridge is reversible in bizygotic conjugation, but it is irreversible in monozygotic conjugation. Irreversible or reversible, cell fusion is necessary for the occurrence of cross fertilization and hence is an essential component of conjugation. It is also one of the features that ciliate conjugation shares with fertilization, but not with mating, in metazoa.

Earlier studies, carried out mainly on *Blepharisma, Euplotes, Paramecium*, and *Tetrahymena*, have shown that the formation of conjugant union requires; 1) protein synthesis, and 2) the accumulated effect of preconjugant interaction (Miyake 1974, 1978, 1981a, 1982 for review).

In gamone-induced homotypic union in *B. japonicum*, the formation of a conjugant union is «isolated» from many other phenomena in conjugation because of the homotypic arrest (Section 4.1). Yet the homotypic union is morphologically identical with the heterotypic union at least in the early stages (Bedini et al. 1978). Working on this system, Miyake and Honda (1976) determined the following: 1) Gamone-induced protein synthesis is particularly high during the first 1–2 h of gamone treatment. 2) Formation of the conjugant union requires another 1–2 h of the gamone treatment during which induced protein synthesis is low. 3) A large portion of the proteins synthesized during these periods is associated with low density particles larger than 10^3 kD, suggesting that most of the gamone-induced proteins are associated with membranes.

In *T. thermophila*, Elliott and Zieg (1968) observed swelling, elongation, and pinching off of cytoplasmic saccules at the oral region during preconjugant interaction and suggested that these saccules participate in conjugation. This characteristic saccule activity is inhibited by cycloheximide which also inhibits pair formation (Flickinger and Murray 1974). In *B. japonicum*, Bedini et al. (1978) observed in homotypic and heterotypic pairs that saccule-like structures are abundant near the newly-formed conjugant union and that long microtubules are associated perpendicularly and obliquely to the cell surface at the united region (Fig. 14). These characteristic features were observed also at the

Fig. 14: Cell union of gamone-induced homotypic pairs in *Blepharisma japonicum*. A transverse cross section of a homotypic pair of albino clone A1 (mating type I) of the Bangalore strain, 10 h after beginning of gamone 2 treatment (1.6×10^2 U/ml). The cell union starts at the vicinity of the antUMC (a row of cilia anterior to the undulating membrane), which can be located by its kinetosomes (k), and extends to the left on the peristomial floor towards the adoral zone of membranelles. No kinetosomes are seen in the peristomial floor. The regular juxtaposition of cell membranes is interrupted by one large vacuole (v) and a cytoplasmic bridge in the process of formation. Arrows: Microtubules characteristic of conjugant union, which are perpendicularly associated to the cell membrane. p3 and p4: Type 3 and type 4 pigment granules of albino cell (Kurita 1968). m: Mitochondrion. Bar: 1 μm. For materials and methods see Bedini et al. (1978) (Bedini C, Lanfranchi R, Nobili R, Miyake A, cited in Miyake 1978).

presumptive site of conjugant union in gamone-treated cells (Fig. 15).

Based on these and other results available at the time (Miyake 1974, 1978, 1981a, 1982 for review), the following working hypothesis on the mechanism of conjugant-union formation was constructed (Miyake 1982) slightly modifying those proposed previously (Miyake and Honda 1976; Miyake 1978, 1981a, 1981b). 1) Gamone-receptor binding induces protein synthesis. 2) Most of the induced proteins are incorporated in the membrane of cytoplasmic saccule-like structures increasing their number. 3) Gamone-receptor complexes form clusters at the presumptive site of cell union. 4) Microtubules grow from these clusters towards the interior cytoplasm. 5) These microtubules transport saccule-like structures to the cell surface. 6) Saccules are incorporated into the cell surface enabling it to unite with a similarly altered surface (Fig. 16).

Although this scheme was primarily constructed for conjugation in *Blepharisma*, it may serve as a working hypothesis for the study of conjugant union in other ciliates. Recent studies presented below are generally consistent with this scheme.

Inhibitors of microtubule assembly prevent pair formation in *Oxytricha bifaria* (Banchetti et al. 1982) and *T. thermophila* (Kaczanowski et al. 1985) suggesting the participation of microtubules in conjugant-union formation. Synthesis of a number of proteins is stimulated during preconjugant interaction in *T. thermophila* (Garfinkel and Wolfe 1981; Ron and Suhr-Jessen 1981; Van Bell 1983; Suhr-Jessen 1984; Suhr-Jessen et al. 1986). Some of these proteins may participate in the formation of the conjugant union.

In *T. thermophila*, the anterior part of the cell, where the conjugant union is formed, undergoes «tip transformation» during preconjugant interaction (Wolfe and Grimes 1979). This process includes 1) deformation of the tip of the cell from pointed to truncated and 2) appearance of a smooth, cilia-free surface. Wolfe (1982) and Suganuma et al. (1984) confirmed not only these changes but also the earlier observation of Elliot and Zieg (1968) that saccule-like structures are abundant at or near the presumptive site of conjugant union. They suggested that these saccules contribute to the formation of new cell surface. Wolfe (1985) showed how cytoskeletal elements participate in conjugant union in *T. thermophila*.

In *E. crassus*, Verni and Rosati (1987) detected a rapid activation of adenylate cyclase in preconjugant interaction and suggested that it could represent the appearance of a non-protein factor postulated by Dini and Miyake (1982) to occur at the beginning of preconjugant interaction in this species. Possible participation of the adenylate cyclase – cyclic AMP system in preconjugant interaction is reviewed by Rosati and Verni (1991).

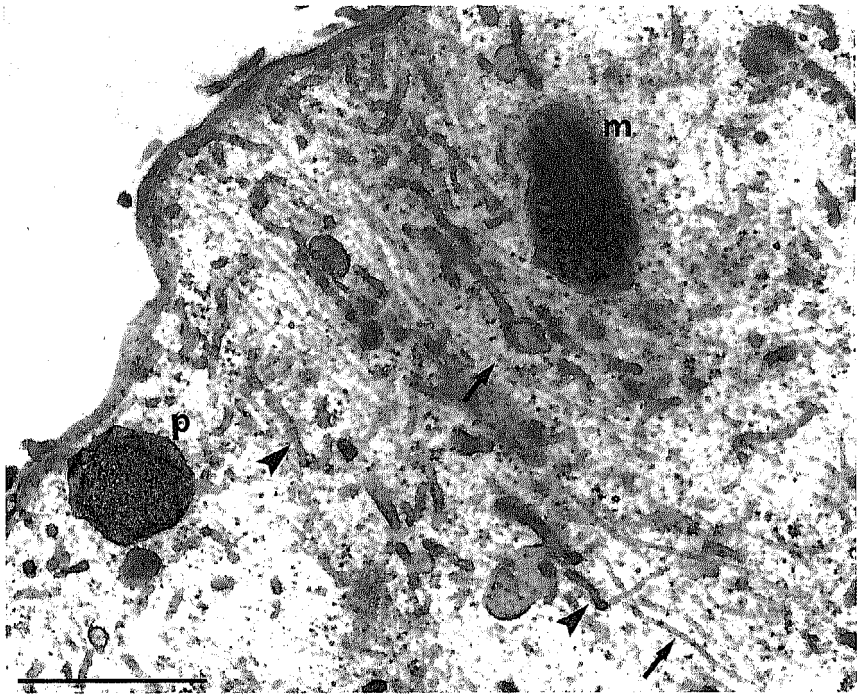

Fig. 15: Presumptive site of conjugant union ready to unite in *Blepharisma japonicum*. A transverse cross section of a doublet cell (a morphological variant having two sites for conjugant union) of mating type II of the Bangalore strain at the anterior part of the peristomial floor. The cell is at the terminus of a homotypic chain of four doublet cells, induced by a 7 h treatment by gamone 1 (10^4 U/ml), thus having one free peristomial floor. Arrows: Microtubules characteristic of conjugant union (see Fig. 14). Arrowheads: Saccules supposedly carried by microtubules to the presumptive site of cell union to provide it the capacity to unite. p: Pigment granule. m: Mitochondrion. Bar: 1 µm. (From Miyake A, Nobili R, Lanfranchi A, Bedini C, cited in Miyake 1978).

In *T. pyriformis* syngen 1 (now *T. thermophila*), Con A binds to the margin of the conjugant union (Frisch and Loyter 1977). This localization of Con A receptors occurs in the absence of Con A and is inhibited by cycloheximide (Watanabe et al. 1981). The Con A binding during preconjugant interaction occurs at the presumptive site of the conjugant union, and the binding increases in parallel with the progress of the tip transformation (Wolfe et al. 1986).

A similar phenomenon was observed in *Euplotes vannus* (Lueken et al. 1981; Lueken and Oelgemöl-

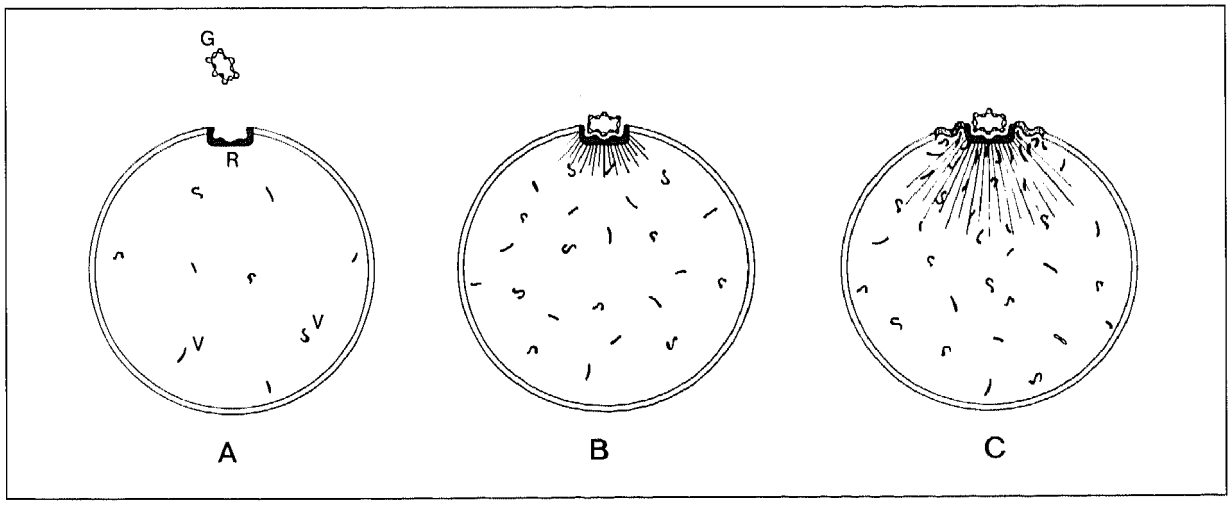

Fig. 16: Diagrammatic illustration of a hypothesis on the mechanism of gamone-induced cell union. **A:** Approaching of gamone (G) to its surface receptor (R). **B:** Gamone-receptor binding induces, 1) formation of microtubules (straight lines) emanating from the cell surface at the presumptive site of cell union towards the interior of the cell, and 2) increase of membrane vesicles (V) (saccules). **C:** Membrane vesicles are transported by means of microtubules to the cell surface near the gamone-receptor binding and alter the surface so that it can unite with a similarly altered surface. Receptors are postulated to be in many clusters at the presumptive site of cell union (Miyake 1982).

ler 1985, 1986). In *E. vannus*, the Con A binding site appeared at the presumptive site of the cell union as a distinct oval field, as early as 20 min after mixing complementary mating types (waiting period, 60 min; preconjugative lag, 100 min) (Lueken and Oelgemöller 1985). The intensity of preconjugant interaction is positively correlated with development of the Con A binding site (Lueken and Oelgemöller 1986).

Thus Con A binding can be used as an index of the transformation of cell surface during preconjugant interaction. Con A might bind selectively to the newly formed membrane, which is assumed to play an essential role in the formation of conjugant union in the hypothetical scheme shown in Fig. 16.

6 Micronuclear changes up to fertilization in conjugation

6.1 Outline

The micronucleus in the stationary phase usually lies close to the macronucleus. In conjugation, it separates from the macronucleus, e.g., *P. caudatum* (Wichterman 1940). Fujishima and Hiwatashi (1977) found that in *P. caudatum* this phenomenon occurs during preconjugant interaction well before conjugant pairs are formed. They called the phenomenon «early micronuclear migration» (EMM). It occurs in preconjugants, but not in immature cells or in well-fed mature cells. Cronkite (1977, 1979) found that calcium ionophore A23187 induces EMM in mature, but not in immature, cells and suggested that changes in internal Ca^{++} concentration play a role in EMM.

The micronucleus in preconjugants is diploid. In conjugants, it undergoes prekaryogamic divisions to produce one or more (usually two) haploid pronuclei which then undergo fertilization. In some ciliates, these divisions consist of only two meiotic divisions, e.g., *Blepharisma japonicum* (Miyake et al. 1979a, 1991); *Cycloposthium bipalmatum* (Dogiel 1925). In many others, they consist of three divisions, two meiotic and one postmeiotic division. Still in others, meiosis is preceded by mitosis (preliminary micronuclear division), e.g., *Euplotes octocarinatus* (Kuhlmann and Heckmann 1991); *Vorticella campanula* (Mügge 1957).

Various patterns of prekaryogamic micronuclear behavior have been capably reviewed by Raikov (1972). The most frequently reported pattern is «no preliminary division + two meiotic divisions + one postmeiotic mitosis». This 0 + 2 + 1 pattern was considered standard (Raikov 1972).

In the sexual life cycle of eukaryotes, the diploid and the haploid phase alternate by means of fertilization and meiosis. The haploid phase in ciliates is extremely brief. In many ciliates, meiosis is followed by only one mitosis before fertilization and even this mitosis is lacking in some ciliates. A comparable situation is found in metazoa. In other protists such a brief haploid phase is found in Heliozoa and some Polymastigina (Grell 1973).

Postmeiotic mitosis might be a relic of the haploid phase of the life cycle in the ciliate ancestor, but it occurs in many contemporary ciliates, suggesting that it might have a function such as to secure the production of pronuclei as postulated below. In many ciliates, only one selected nucleus, which is localized at the particular region near the site of pronuclear exchange, can divide to produce pronuclei. If a ciliate with only one micronucleus takes the 0 + 2 + 0 pattern of prekaryogamic divisions, there are only two micronuclei at the time when one of the nuclei must take the correct position for the next stage (pronuclei-producing division). Unless a very reliable mechanism has been developed for this positioning, having more nuclei at this particular time may make it less likely that any conjugants are left without pronuclei. The occurrence of a preliminary division in some ciliates may be similarly considered. If so, the 0 + 2 + 0 pattern of prekaryogamic division would be found more commonly in ciliates with many micronuclei than in ciliates with one or two micronuclei. This is still to be tested, because the study of nuclear phenomena in conjugation of multimicronucleate ciliates has been lagging. In *Blepharisma*, a multimicronucleated heterotrich, the 0 + 2 + 1 pattern was reported in four species including *B. japonicum* (Raikov 1972 for review), but more recent studies showed that the pattern is 0 + 2 + 0 in *B. japonicum* (Miyake et al. 1979a, 1991) and *B. stoltei* (Rivola and Miyake 1985).

In all patterns of prekaryogamic divisions, at least once some micronuclei degenerate, leaving others to continue the germ line (Figs. 3, 4). Little is known about the mechanism which controls the survival versus the degeneration of micronuclei. Observations suggest that the cytoplasm at a par-

ticular region, such as the paroral region, plays an important role in the survival of micronuclei (Raikov 1972 for review). Results of nuclear transplantation experiments in *Paramecium caudatum* (Yanagi 1987) are consistent with this hypothesis. In *B. japonicum*, a particular kind of cytoplasm, which has a high affinity for pigment granules, associates with germ line nuclei during the period from prometaphase of meiosis II to the second mitosis of the synkaryon (Fig. 1), suggesting that this cytoplasm protects the germ line nuclei from degeneration (Miyake 1981a). A similar observation was made in *T. thermophila* (Numata et al. 1985); an intermediate filament protein of 49 kD, which is found in vegetative cells in some cortical structures, including mucocysts and the oral apparatus (Numata et al. 1983), associates with germ line nuclei during the comparable period.

Meiosis in ciliates was once confused by reports on cytological peculiarities (Sonneborn 1949; Raikov 1972 for discussion), but it is now generally believed that meiosis in ciliates essentially conforms to the classic scheme of meiosis (Miyake 1981a; Raikov 1972, 1982 for review). The synaptonemal complex has been observed in some ciliates, e.g., *B. americanum* (Jenkins 1973), *B. japonicum* (E. Schulte, A. Miyake, K. Heckmann personal communication), *Didinium nasutum*, *Dileptus anser*, *Paramecium* species of the *aurelia* group, and *Stentor coeruleus* (Raikov 1982 for review).

6.2 Initiation and progression of meiosis

If conjugant pairs of *B. japonicum* (Miyake et al. 1979b) and *P. caudatum* (Fujishima 1988) are separated into single cells soon after the formation of conjugant pairs, they may return to the asexual condition. But there is a point of no return at about 1 h. If conjugants are separated shortly after this time, each cell undergoes all the nuclear changes of conjugation except the exchange of pronuclei between cells. Such cells can be considered «activated» as in activated metazoan eggs. In *P. tetraurelia*, and possibly also in *P. calkinsi*, preconjugant interaction alone can induce activation without participation of conjugant union, but this is not the case in other ciliates including, *P. caudatum*, *P. multimicronucleatum*, and *B. japonicum*, in which activation requires formation of a conjugant union (Miyake 1981a for review).

In *B. japonicum*, activation occurs in heterotypic pairs but not in homotypic pairs (homotypic arrest) (4.1). Using this characteristic, a unique experimental system was designed for the study of activation. If doublet cells (a morphological variant with two sites for conjugant union) are treated by the gamone of the other mating type, they unite side by side to form chains of homotypically united cells. As in homotypic pairs, activation does not occur in any of these cells. If, however, a cell of a complementary mating type forms a conjugant union at one end of such a chain, meiosis and other nuclear changes of conjugation begin there and propagate through the chain (Miyake 1975). If such a chain is separated into single cells, meiosis occurs later only in those cells already activated at the time of separation. Working on this system, Miyake et al. (1977) found that activation propagates more slowly in longer chains, that the propagation of activation slows down as it proceeds in the chain, and that it seldom reaches the sixth cell in the chain. They concluded that the signal for activation produced by the heterotypic union diffuses along the chain, and hence that the signal is a substance transferable through united cells and effective above a specific concentration.

Activation requires protein synthesis (Miyake et al. 1979b) as does the progression of meiosis. Friedl et al. (1983) found that cycloheximide arrests meiosis at the following six stages: I, between the pairing of cells and the swelling of micronuclei; II, leptotene; III, zygotene; IV, pachytene; XI, interkinesis; and XII, prometaphase II. Five of these arrests were reversible. They concluded that the progression of meiosis requires at least six proteins, each specific to stages I, II, III, IV, XI and XII, and each synthesized in this order during conjugation. A similar result was obtained in *Stylonychia pustulata* (Yano and Suhama 1990).

Based on these and other results available at the time (reviewed by Miyake 1978, 1981a, 1982; Miyake and Heckmann 1986), the following scheme of initiation and progression of meiosis was constructed as a working hypothesis (Miyake 1982) (Fig. 17). 1) Heterotypic cell union induces the production of a «master factor» through protein synthesis. 2) This factor switches on two cellular components, X and Y, of which X requires a lower concentration of the master factor. 3) Once switched on, X produces, through protein synthesis, meiosis factor 1, which induces the swelling of the micronucleus. 4) Similarly, Y produces meiosis factors 2, 3, 4, – – in a sequence, each of which induces the progression of meiosis at a particular step.

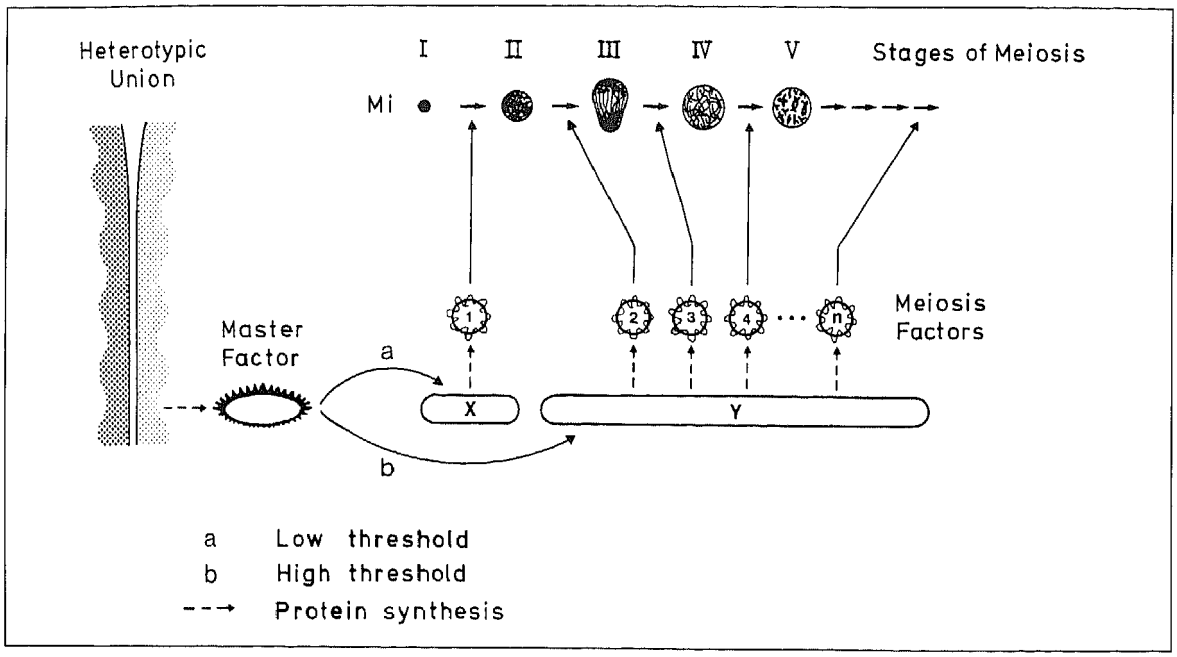

Fig. 17: Hypothetical scheme of the initiation and progression of meiosis in *Blepharisma japonicum*. Mi: Micronucleus. X and Y: Hypothetical cellular components. Stages of meiosis I, II, III, IV and V roughly correspond to the resting stage, leptotene, zygotene, pachytene and diplotene, respectively (Miyake 1982).

If only X is switched on because of a low concentration of master factor, the nuclear events of conjugation stop at the stage of micronuclear swelling resulting in partial activation.

In *Oxytricha bifaria*, in which conjugant pairs are asymmetrical, the right conjugant is activated earlier than the left one (Ricci et al. 1975 b; Ricci et al. 1980 a). In *O. hymenostoma*, cells of complementary «mating types» paired and fused into single cells without being activated (Esposito et al. 1978). However, if paired cells were experimentally separated in early stages of pairing, activation occurred in one of the mating types, suggesting that the other mating type has an activation-inhibiting factor (Ricci et al. 1980 a). In *Paraurostyla levis*, syngen 1, when certain «mating types» participate in cell pairing, paired cells fuse into single cells without being activated (Takahashi 1973, 1977); micronuclei undergo mitosis but none of meiosis, karyogamy, and macronuclear formation (Takahashi 1974).

In *Paramecium*, the micronucleus in preconjugants is in the G1 phase. It undergoes premeiotic DNA synthesis soon after conjugant pairs are formed (Fujishima 1988 for review). Transplantation experiments of micronuclei indicate that meiosis is controlled by cytoplasmic factor(s) (Fujishima and Hiwatashi 1981).

Fujishima and Hori (1989) induced germinal vesicle breakdown (GVBD) in oocytes of a toad *Bufo bufo* by injecting extracts of conjugating *P. caudatum* cells. They called the hypothetical factor for this induction «meiosis-reinitiation-inducing factor» (MRIF). The amount of MRIF, measured by GVBD-inducing activity, rose from zero to the highest level within 1 h after mixing complementary mating types (preconjugative lag, about 3/4 h), dropped at the crescent stage (roughly corresponding to pachytene), and rose again at metaphase I. *Paramecium* in the log phase also contains MRIF, suggesting that the factor controls both meiosis and mitosis. It appears to be a heat-labile soluble protein (Fujishima and Hori 1989). This factor is not identical with MPF (Maturation promoting factor) (Masui and Clarke 1979 for review), because cycloheximide inhibits the GVBD-inducing effect of MRIF but not that of MPF. In a synchronously dividing culture of *T. pyriformis*, MRIF appears only at the M phase (Fujishima et al. 1991).

In *T. thermophila* some proteins are specifically synthesized during conjugation, particularly during meiotic prophase (Van Bell 1983; Suhr-Jessen 1984; Martindale et al. 1985), suggesting that they participate in meiosis. Martindale and Bruns (1983) found three conjugation-specific genes, pC1, pC2, pC7, and a conjugation-induced gene pC3 by making cDNA of mRNA in conjugating cells. Their transcripts are particularly abundant

during meiotic prophase (Martindale et al. 1985). Uridine incorporation by micronuclei at meiotic prophase has been reported (Sugai and Hiwatashi 1974; Martindale et al. 1985). Although the micronucleus is generally thought to be transcriptionally inactive, these results suggest that some of the conjugation-specific genes might be transcribed in the meiotic micronucleus. Martindale et al. (1986) tested the possibility that some such genes might be among the 10–20% of the sequences not included in the macronucleus (Yao and Gorovsky 1974), but pC1, pC2, and pC7 were found in both the micro- and the macronucleus.

6.3 Pronuclear transfer and karyogamy

In most ciliates, each conjugant produces one or two pronuclei, largely depending on whether conjugation is monozygotic or bizygotic. However, in Trachelocercidae (Karyorelictida), whose conjugation is bizygotic, more than two pronuclei are produced in each conjugant, possibly representing a phylogenetic rudiment of gametogenesis (Raikov 1972).

Earlier studies on the transfer of pronuclei have been reviewed by Raikov (1972). As he notes, the movement of the pronucleus to the other conjugant is not always through a broad bridge. The pronucleus may have to break through a barrier or squeeze through a narrow cytoplasmic bridge. The latter case was observed in *P. multimicronucleatum* (Inaba et al. 1966) (Fig. 18). André and Vivier (1962) think that the transfer of pronuclei in *P. caudatum* occurs in a similar way by means of amoeboid movement of pronuclei.

In *Heliophrya erhardi*, Lanners (1980) found that the migratory nucleus is characteristically associated with coated microtubules and suggested that these microtubules play a role in the migration of pronuclei. In *T. thermophila*, the migratory pronucleus at the conjugant union is associated with a hemispherical meshwork of microtubules (fertilization basket) in such a way that the pronucleus appears to be pressed to the boundary between conjugants by the basket (Orias et al. 1983). Considering that the transfer of pronuclei is blocked by inhibitors of microtubule assembly (Hamilton and Suhr-Jessen 1980), Orias et al. (1983) suggested that the fertilization basket pushes the migratory pronucleus through the boundary into the other conjugant. Orias and Orias (1983) think that the membrane barrier at the boundary turns into a curtain of tubular structures and that the pronucleus can pass through this curtain without tearing off the membrane (Orias 1986 for review).

In *Euplotes vannus*, conjugants fuse over the whole

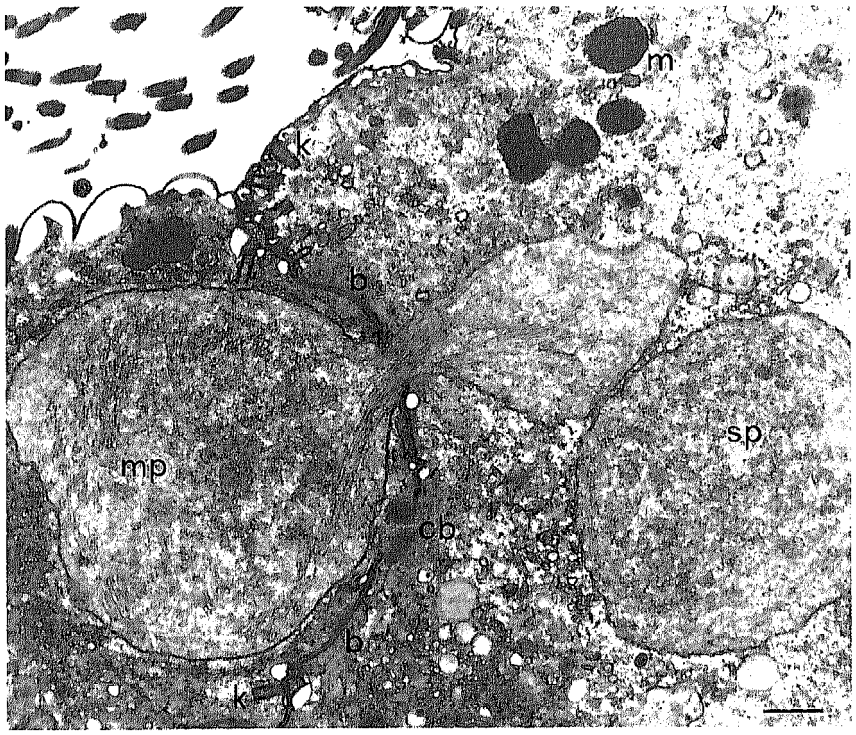

Fig. 18: Cell-to-cell transfer of a migratory pronucleus in *Paramecium multimicronucleatum* syngen 2. Electron micrograph of a transverse section at the united region of a conjugant pair. A migratory pronucleus (mp) in the left conjugant is migrating into the right conjugant through a narrow cytoplasmic bridge. sp: Stationary pronucleus. b: Boundary between the two conjugants of the pair where two cell membranes are juxtaposed. cb: Cytoplasmic bridge. k: Kinetosome. m: Mitochondrion. Bar: 1 μm (Inaba et al. 1966).

united area at an early stage of conjugation, but two sheets of microtubules persist as barriers preventing free cytoplasmic mixing between conjugants. At the time of pronuclear transfer, these microtubular sheets are replaced by incompletely formed «pseudomembranous structures» through which pronuclei are transferred (Nobili 1967). Fusion of the whole united area occurs also in *H. erhardi* (Lanners 1980). In this case, free cytoplasmic mixing is prevented by a thick epiplasmic layer.

Karyogamy often takes place near the site of pronuclear transfer (Fig. 1). In *T. thermophila*, karyogamy is prevented by inhibitors of microtubule assembly (Hamilton and Suhr-Jessen 1980; Kaczanowski et al. 1985), suggesting that microtubules play a role in this process too.

The synkaryon is already 4C when it is formed in *T. thermophila* (then *T. pyriformis* syngen 1) (Doerder and DeBault 1975) and *P. caudatum* (Harumoto and Hiwatashi 1982). The synkaryon immediately starts dividing to produce macro- and micronuclei of new individuals (see chapter by Raikov).

If a synkaryon of *P. caudatum* is transplanted into a cell in the stationary phase, it becomes a micronucleus in the resting stage (Harumoto and Hiwatashi 1982). Therefore, the occurrence of mitosis of the synkaryon is controlled by the cytoplasmic condition of the zygote. This condition is probably one of a series of changing conditions triggered by activation at the beginning of conjugation. Orias (1986), however, thinks that the conjugant pair faces the fundamental decision, whether to develop or not, at the time of karyogamy. He suggests that the fertilization basket participates in the production of the «go» signal for development.

7 Chemical induction of conjugation and autogamy

In some ciliates, conjugation can be induced between cells of the same mating type by chemical treatments. This «chemical induction of conjugation» (referred to chemical induction below) was first carried out in *P. caudatum* (Miyake 1956, 1958) and was successfully applied to several species of *Paramecium* (Miyake 1968b). Endo (1987) found that chemical induction is applicable to six out of 36 natural stocks of *P. bursaria*, a species which was once reported as non-responsive to chemical induction.

Various agents effective for chemical induction can be grouped in two classes: 1) inorganic cations, and 2) DNA-binding compounds. The first group includes K^+, Rb^+, Cs^+ Mg^{++}, Mn^{++}, etc. Their effective concentration greatly depends on the concentration of Ca^{++} in the medium. In the induction of the holdfast union by K^+ in *P. caudatum*, the most effective K^+/Ca^{++} ratio is given by the formula, $[K^+] / \sqrt{[Ca^{++}]} = 1$, when $[Ca^{++}]$ is 0.05–0.30 mM (Kitamura and Hiwatashi 1984b). In the induction of the whole process of conjugation by K^+ in *P. caudatum*, the concentration of Ca^{++} must be lower than 0.1 mM (Miyake 1958). A series of experiments dealing with the problem of how Ca^{++} participates in the induction of conjugation has been reviewed by Hiwatashi and Kitamura (1985).

In induction by DNA-binding compounds, acridine dyes such as acriflavine and acridine yellow have been used (Miyake 1958, 1968b; Nakamura 1984). Recently, Harumoto and Miyake (1990, 1991) reported that DAPI (4', 6-diamidino-2-phenylindole), safranin O (an azine dye) and Hoechst 33258 are highly effective in chemical induction. They suggest that those agents which have a high affinity for AT-rich DNA are particularly effective. When DNA-binding compounds are used, the low concentration of Ca^{++} is less important for induction. The two kinds of induction, one by ionic imbalance, the other by DNA-binding compounds, are synergistic, indicating that they have at least one common step.

The genetic background of *P. tetraurelia*, *P. octaurelia* (Cronkite 1979 for review), and *P. bursaria* (Endo 1987) affects their responsiveness to chemical induction. These genetic backgrounds do not affect their capacity to undergo a mating reaction. On the other hand, cells with genetic backgrounds which abolish the capacity to undergo mating reactions are responsive to chemical induction (Tsukii, 1988).

Trypsin treatment, which virtually eliminates the capacity of cells to undergo the mating reaction, does not affect the chemical induction of conjugation. On the other hand, addition of $CaCl_2$, which completely inhibits chemical induction, does not affect the induction of conjugation by the mating reaction (Miyake 1969, 1974, 1981a). Therefore, the two inductions of conjugation, one by natural

gamones and the other by chemical agents, have different steps.

On the other hand, chemical induction and induction of conjugation by gamones (isolated cilia prepared from preconjugants) are synergistic (Miyake 1969, 1974, 1978). These results, together with the fact that chemical induction is possible only in preconjugants (cells ready to undergo preconjugant interaction), indicate that these two inductions have at least one common step. For discussions on these common and different steps, see Miyake (1974, 1981a).

Chemical induction can produce viable exconjugant clones after reciprocal fertilization at least in some pairs (Miyake 1968b). Genetic analyses were carried out using chemical induction in *P. caudatum* (Hiwatashi 1967) and *P. tetraurelia* (Miyake 1968b). For review on chemical induction see Hiwatashi (1969), Miyake (1974, 1978, 1981a), and Hiwatashi and Kitamura (1985). For methods see Sonneborn (1970).

Zweibaum (1912) made an extensive study of the effect of inorganic salts on conjugation and reported that 25 salts, particularly those of Na, Al, Fe, and Au, were effective in inducing conjugation in *P. caudatum*. In his experiment, selfing occurred in the control, making the interpretation of his results difficult. Hopkins (1921) confirmed Zweibaum's experiment in one race in which selfing occurred in the control, but not in other 11 races tested. Although these results resemble to some extent the chemical induction of conjugation by inorganic cations, the inhibitory effect of Ca^{++}, a characteristic feature of chemical induction, is not shown in these works. Because of this and other differences, Miyake (1958) concluded that the promotion of conjugation by inorganic salts reported in these earlier works is a quite different phenomenon from the chemical induction of conjugation by ionic imbalance.

In *Euplotes vannus*, a marine hypotrich, some clones undergo selfing when exposed to a low concentration of K^+ (Lueken et al. 1987). The effective ionic imbalance (low K^+) is different from the one for chemical induction of conjugation in *Paramecium* (low Ca^{++}), but the result suggests that chemical induction of conjugation is possible in ciliates other than *Paramecium*.

Autogamy can be induced by chemical treatments in some species and syngens which do not undergo natural autogamy (Miyake 1981a, Watanabe 1990 for review; Sonneborn 1970 for methods). This chemical induction of autogamy was first carried out in *P. multimicronucleatum* syngen 2 by adding ficin (a proteinase) to conjugation-inducing chemical agents (KCl + acriflavine + Ca-poor condition) (Miyake 1968c, d). Ficin was thought not only to inhibit pair formation but also to play an essential role in inducing nuclear changes in single cells. Both in *P. caudatum* (Ito, 1969) and *P. multimicronucleatum* syngen 2 (Shimomura and Takagi 1984), however, it was found that conjugation-inducing chemical agents can induce nuclear changes of conjugation in isolated single cells in the absence of ficin or any other proteinases. Natural autogamy is not known in either of them. Tsukii and Hiwatashi (1979) genetically confirmed in *P. caudatum* that the nuclear changes induced in single cells by chemical agents are indeed those of autogamy. Therefore, chemical induction of conjugation also induces autogamy. In *P. multimicronucleatum*, cells are committed to autogamy after about 2 h of treatment by autogamy-inducing chemical agents (Shimomura and Takagi 1985).

Chemical induction of conjugation and autogamy has demonstrated that ciliates can conjugate without preconjugant interaction and that autogamy can be induced in ciliates not undergoing natural autogamy. Any working hypotheses for the mechanism of conjugation and autogamy must take into account the fact that the same chemical treatment can induce conjugation and autogamy (Miyake 1981a for discussion). Research possibilities opened up by these discoveries are still mostly to be exploited.

8 General discussion

8.1 Mating types, sexes, and gamotypes

When Sonneborn (1937) discovered two classes of individuals complementary for conjugation in *Paramecium*, he called them sexes. But he soon coined a new term «mating types» for these classes of individuals (Sonneborn 1938). The reasoning was as follows. In conjugation of *Paramecium*, each conjugant produces a migratory and a stationary pronucleus. The migratory pronucleus enters the other conjugant and fuses there with the stationary pronucleus to form the synkaryon. Because of their

functional similarity with the sperm and the egg nucleus, the migratory and the stationary pronucleus can be regarded as the male and the female gametic nucleus, respectively. If each conjugant produces both the male and the female pronucleus, it is hermaphroditic. A conjugant cannot be considered as a female or a male, if it is already bisexual.

In addition, there are more than two mating types in many ciliates, conflicting with the notion of bipolarity of sexes (Hartmann 1956). Therefore, mating types in ciliates are not considered as sexes (Grell 1967, 1973; Raikov 1972; Nanney 1980).

Nevertheless, mating types in ciliates are functionally similar to sexes in other organisms; both are classes of biological units identified based on their complementarity for the occurrence of fertilization. If one is not constrained by the bipolarity of sexes, mating types may be regarded as sexes (Vivier 1965, 1984; Hiwatashi 1986). Although this unifying view is attractive, Sonneborn's argument described above makes it hardly acceptable. The assumption in his argument that the two pronuclei produced by a conjugant are functionally differentiated has not yet been experimentally verified, but this does not basically change the argument as discussed below.

The term sexes was originally used to indicate female and male of humans and some animals. Later the original meaning was extended so that the term can be applied to individuals of other organisms. Female is the producer of female gametes (eggs) and male is the producer of male gametes (sperm). If an individual behaves as a female and a male producing both female and male gametes, it is hermaphroditic. The female and the male gamete are respectively the recipient and the donor of a haploid nucleus for fertilization. The male gamete is smaller and is absorbed by the female gamete. If not completely absorbed, the unabsorbed part of the male gamete (e.g., sperm tail) degenerates. The female gamete is larger and absorbs the male gamete. Gametes are not necessarily haploid, since eggs in many metazoa are diploid when they receive sperm (2). If the fusion between the male and the female gamete fails to occur, the male gamete dies, while the female gamete can develop by parthenogenesis.

In ciliates with anisomorphous monozygotic conjugation, the macro- and the microconjugant respectively behave as the recipient and the donor of a haploid nucleus (Figs. 2, 4). The microconjugant is totally absorbed by the macroconjugant or a part of the former detaches from the latter only to degenerate. If the micro- and the macropreconjugant do not unite, the micropreconjugant dies, while the macropreconjugant can undergo asexual reproduction. Therefore, the macro- and the microconjugant, and hence also the macro- and the micropreconjugants, can be regarded as the female and the male gamete, respectively. Ciliates are individuals as well as cells. An individual which functions as a female gamete is female. Therefore, the macropreconjugant can be regarded as female. Similarly, the micropreconjugant can be regarded as male.

In ciliates with isomorphous bizygotic conjugation, each conjugant is a recipient as well as a donor of a haploid nucleus (Figs. 2, 3), thus behaving as a macroconjugant as well as a microconjugant. If the macro- and the micropreconjugant are female and male, respectively, preconjugants of those ciliates which undergo isomorphous bizygotic conjugation are hermaphroditic.

Since the majority of ciliates undergo isomorphous bizygotic conjugation, hermaphroditism prevails in ciliates. Dioecism is found only in a few higher taxa (e.g., peritrichs, suctorians). Studies on nuclear phenomena at conjugation indicate that monozygotic conjugation in Peritricha is derived from bizygotic conjugation (Grell 1973). It appears, therefore, that hermaphroditism is more ancient than dioecism in evolution of ciliates. A parallel is found in metazoa in which hermaphroditism prevails in lower taxa (e.g., cnidarians, flatworms), suggesting that it is more ancient in evolution of metazoa. In mating of many hermaphroditic metazoa, partners exchange male gametes, making it impossible to identify an individual as a female or a male. If two or more classes of individuals complementary for mating are discovered in such a metazoan species, these mating classes, which consist of bisexual individuals, will not be called sexes. This is the case in mating types in ciliates.

Therefore, if mating types are considered as sexes, the term sexes must be used in two senses, one narrower and the other broader. In the narrower sense, it indicates female and male in metazoa, in some ciliates, and in other organisms, while in the broader sense it indicates at least two different kinds of mating classes, sexes in the narrower sense and mating types in ciliates.

It may be less confusing to introduce a new term to comprise all kind of classes of biological units which are complementary for the occurrence of fertilization, such as sexes, mating types and mating classes in the gametophytic self-incompatibility system in flowering plants. Sexes and all these mating classes may be grouped as «gamotypes» (gamos = marriage, union in Greek).

During evolution gamotypes appeared whenever

biological units differentiated into different types complementary for the occurrence of fertilization. Gamotypes may occur at different levels of organization, such as the nucleus, the cell, the individual, and the population. However, the occurrence of fertilization does not necessarily imply the presence of gamotypes. Therefore, problems such as whether the two pronuclei in autogamy are gamotypically different or not remain open until the complementarity between the two biological units under consideration is experimentally examined.

8.2 Gamotype dogma

Before the discovery of mating types (Sonneborn 1937), conjugation was studied in cultures which undergo selfing, including intraclonal selfing. At that time it was generally believed that in isomorphous conjugation each cell can conjugate with any other cell in the same species (for history see Sonneborn 1941; Wichterman 1953; Vivier 1960). However, after the discovery of mating types, it has been shown in one case after another that selfing in a clone is due to the presence of different mating types in the clone or to the capacity of the clone to produce different mating types (Kimball 1939b; Sonneborn 1957b; Allen and Nanney 1958; Hiwatashi 1960; Barnett 1966; Taub 1966a, b; Bleyman 1967; Heckmann 1967).

Thus the pendulum shifted to the other direction fostering the view that there must be different mating types for conjugation to occur. Including anisomorphous conjugation in peritrichs and some suctorians, the view may be expressed in a more general form: «For the occurrence of conjugation in ciliates there must be individuals of different gamotypes». This view, which is called gamotype dogma here, has supplanted opposing views.

Sonneborn (1941) discussed the gamotype dogma in detail and supported it. However, the wide occurrence of intraclonal selfing led to speculation that there might also be conjugation that does not depend on different cell types (Vivier 1960; Miyake 1968b; Grell 1973). Although this has never been proved in natural selfing, it is the case in the chemical induction of conjugation in which certain chemical agents induce selfing in cells of one and the same mating type (6). Induction of conjugation by some «killers» (Miyake 1981a for review) may be similar to chemical induction. However, since these inductions require conditions rarely met in nature, e.g., low Ca^{++} concentrations, DNA-binding agents, it is doubtful that such conjugation plays any role in the life cycle of ciliates in nature.

Some of the hypotheses on preconjugant interaction (Section 4.3) presuppose the occurrence of conjugation without gamotype differences. For example, in the self-recognition hypothesis (Section 4.3.5), a spontaneous decrease of the self-recognition process may lead to conjugation between cells of the same mating type. In the GR-hypothesis (Section 4.3.3), malfunctions of the mutual exclusion between gamones and their receptors may also lead to the same result. These possibilities are still to be tested experimentally, however.

Two works on selfing questioned the gamotype dogma. In intraclonal selfing of *Euplotes eurystomus*, it appeared as if cells of identical mating type conjugate (Machelon 1986). In selfing of *E. patella*, syngen 2, Akada (1986b) detected only one kind of gamone in the medium surrounding conjugating cells and suggested that conjugation occurs between cells of identical mating type.

On the other hand, new experimental support for the gamotype dogma was provided by the finding that Hf-selfers of *B. japonicum* produce daughter cells of complementary mating types by cell division (Fig. 5). *Blepharisma* has often been cited as a possible example of hypothetical ciliates in which conjugation occurs without mating-type differences (Sonneborn 1939; Miyake 1968b; Isquith and Hirshfield 1968; Wilfert 1972). Selfing is particularly common in Hf-selfers in which conjugation regularly occurs when clonal cultures are deprived of nutrients. Probably such a phenomenon was generalized to question the presence of mating types in *Blepharisma*. Luporini and Miceli (1986), based on their interpretation of previous studies on *Blepharisma*, believed that the mating-type system has not yet evolved in this genus, each species virtually consisting of a single mating type. Since the presence of complementary mating types has been established in *B. japonicum* and *B. stoltei* and since selfing in Hf-selfers has been shown to be due to the presence of complementary mating types produced by asymmetrical cell division, it is no longer likely that conjugation occurs in *Blepharisma* without mating-type differences.

Cell differentiation by means of asymmetrical cell division is a phenomenon of wide occurrence, e.g., mating-type interconversion in homothallic strains of yeasts (Klar 1987), stem cells in metazoa (Wolpert 1988). In ciliates, some peritrichs produce complementary gamotypes, the macro- and the micropreconjugant, by asymmetrical cell division.

Whether this phenomenon plays a role in intraclonal selfing in other ciliates, including the selfing in *E. eurystomus* and *E. patella* described above, is worth testing.

8.3 Evolution of mating types

The majority of contemporary ciliates are hermaphroditic (Section 8.1). Among this vast array of hermaphroditic groups, various kinds of mating types and mating-type systems have evolved. Since most of them remain to be investigated, the whole picture of mating types in ciliates is obscure. Nevertheless, available evidence permits the discussion of a few general problems on evolution of their mating types.

Mating-type systems in ciliates are divided into the binary and the multiple systems. They are also divided by whether cells excrete gamones or carry them on the cell surface. Thus there are four classes of mating-type system: 1) binary and gamone-excreting, 2) binary and gamone-carrying, 3) multiple and gamone-excreting, and 4) multiple and gamone-carrying. The problems of which of these four classes is the ancestral form and how other classes have evolved from this ancestral form are discussed below based on the GR-hypothesis described in Section 4.3.3.

A comparative study on five species of *Blepharisma* (Section 4.2.3) suggests how a mating type system could have evolved from an ancestral form. Mating-type systems of these species are binary and gamone-excreting, that is, mating types I and II excrete gamones 1 and 2, respectively. Gamone 1 is more or less species-specific, while gamone 2 is not. It appears that mating type II of all the five species excrete the same molecule, blepharismone, as gamone 2, which suggests that gamone 2 is evolutionally older than gamone 1. One can, therefore, postulate an ancestral ciliate which had gamone 2, but no gamone 1 (Miyake and Bleyman 1976).

In this hypothetical species with gamone 2 (G2) and its receptor (R2), three Mt-formulae, G_2, R_2, and G_2R_2, can be postulated. Since the existence of G_2R_2 contradicts one of the basic assumptions of the GR-hypothesis, mutual exclusion of gamone and its receptor in the same cell, mating types of this species are tentatively G_2 and R_2. In this system, G_2 can stimulate R_2 for conjugation, but, having no receptor, it cannot be stimulated. R_2 can be stimulated by G_2, but, having no gamone, it cannot stimulate G_2. Then only homotypic pairs of R_2 individuals can be formed. If two individuals, one G_2 the other R_2, meet, conjugation does not occur. Therefore, G_2 and R_2 are not complementary mating types (3), although they are different mating types. Even such a primitive and inefficient mating-type system can control the occurrence of conjugation by regulating the synthesis and expression of gamone and receptor; however, if the mutual exclusion between gamone and its receptor did not operate in this ancestral species, it had G_2R_2 which can stimulate itself for conjugation as postulated by Miyake and Bleyman (1975).

These mating-type systems do not work, if the homotypic arrest (Section 4.1) occurs as in contemporary *Blepharisma*. The homotypic arrest is so far unique to *Blepharisma*, suggesting that it developed only after the genus *Blepharisma* had been formed, and so it can be assumed that homotypic arrest was not operating in ancestral ciliates.

If the hypothetical ancestral ciliates with one gamone receptor pair (GR pair) acquires one more GR pair, they now have two gamones, G1 and G2, and two receptors, R1 and R2. Such a ciliate can produce eight Mt-formulas which do not conflict with the basic assumption of the GR-hypothesis; G_1, G_2, R_1, R_2, G_1G_2, R_1R_2, G_1R_2, G_2R_1. Of these, only G_1R_2 and G_2R_1 can stimulate each other and hence probably they were preserved at the expense of others. They are complementary mating types like those found in contemporary species of *Blepharisma*.

It is also possible that ancestral *Blepharisma* species, and even ancestral ciliate species, already possessed two GR pairs with two complementary mating types inherited from their predecessors, most probably flagellates. If so, the problem of mutual exclusion between the gamone and its receptors mentioned above can be shifted far back to the past, before the appearance of ciliates.

If gamones are proteins, the acquisition of a new GR pair is most probably achieved through duplication of gamone genes and receptor genes. Once duplication occurs, one of the genes can freely mutate without affecting the mating function of the cell.

Acquisition of the third and more GR pairs can increase the number of mating types to establish the multiple mating-type system. In the multiple system, the chance of mating is larger than in the binary system. If combined with a long immature period, the multiple system may fit outbreeding (Sonneborn 1957a) which is generally favored by organisms. Therefore, the multiple system is likely to

have selective advantage over the binary system, and evolved to the highly multiple mating-type systems by accumulating more gamones and receptors. The optimum number of mating types for a species depends on various factors related to the mode of reproduction of the species (Iwasa and Sasaki 1987), but these details are disregarded here mainly because not much is known about the mode of reproduction of ciliates in their natural habitats.

Gamones and receptors may also be lost. In a species with many GR pairs, the loss of a gamone or a receptor may not be a serious defect for a cell, because it can still conjugate with many mating types. The adverse effect of their loss is smaller when there are more of them. Thus the net increase of GR pairs slows down until an equilibrium is reached where the increase is cancelled by the decrease. Since any loss of gamone or receptor produces new mating types, the total number of mating types in a natural population can greatly increase once it accumulates a considerable number of gamones and receptors.

Therefore, one of the possible general tendencies in evolution of mating types is the increase of the number of mating types. If so, contemporary ciliates which have only two mating types must have a particular reason to maintain the binary mating-type system. This problem will be discussed later.

Gamones can be small molecules like blepharismone, a tryptophan derivative, or macromolecules like blepharmone, a glycoprotein. A comparable situation is found in the endocrine system of metazoa, in which small molecules such as thyroxin and adrenaline are used as signals along with proteins such as thyroid-stimulating hormone and insulin. As ciliate species and mating types increase in number, more variations and higher specificities are required for gamones. To meet this requirement, gamones probably increased their molecular complexity. Thus gamones of low molecular weight would have been replaced by more complex gamones.

Excreted gamones have more constraints on their size than cell-bound gamones. Excreted gamones must be stable enough to travel through the extracellular space to reach the target. They should also be produced in large numbers to have a reasonably high probability of hitting the target. These constraints prevent gamones from increasing their size and from assuming certain configurations which compromise their stability.

From the point of view of economy, small molecules like amino acid derivatives appear suited to be excreted gamones, but their variation is limited. Probably, therefore, relatively stable, small proteins are best for excreted gamones in contemporary ciliates.

Cell-bound gamones are less constrained. Since they are carried on the cell, smaller numbers may suffice. In addition, they are better protected by the cellular environment. Thus cell-bound gamones are freer in increasing their size as well as their complexity to exploit larger variability and specificity. Therefore, the second general tendency in evolution of mating types may be the faster increase in the number of mating types in gamone carriers than in gamone excreters.

Once gamones become cell-bound, they will specialize in this new environment making it more difficult to return to excreted gamones, that is, the transition from gamone excreters to gamone carriers tends to be irreversible. Therefore, the third general tendency in evolution of mating types may be the transition from gamone excretion to gamone carrying.

Intermediates between gamone excretors and gamone carriers are conceivable. In such ciliates, gamones are loosely bound to cells, being liberated slowly into the medium, or gamones are in two forms, one excreted, the other cell bound. Examples of such intermediate forms might be found in *Euplotes raikovi* (Section 4.2.6), *E. woodruffi* (Section4.2.7), and *Oxytricha bifaria* (Section 4.2.9).

It may be concluded that the most primitive of the four classes of mating-type systems mentioned above is the binary and gamone excreting system. Gamones of ancestral ciliates were probably small molecules such as blepharismone.

How is the binary mating-type system maintained in some contemporary ciliates, such as *Blepharisma* and some species of *Paramecium*, against the presumed advantages of the multiple mating-type system? In *Paramecium*, although neither gamones nor receptors have been isolated, preconjugant interaction in species with the binary mating-type system, such as *P. tetraurelia* and *P. caudatum*, traditionally has been explained as the reaction between a pair of mating-type substances, A and α (Section 4.3.1). In order to make the GR-hypothesis compatible with this scheme, each of these mating-type substances is considered as a complex molecule consisting of a gamone and a receptor (Miyake 1974). Then each mating type has one of the two gamone-receptor molecules (GR-molecules), G1-R2 and G2-R1, which correspond to A and α, respectively. In such ciliates, formation of the multiple mating-type system can be achieved by adding new pairs of complementary substances, B and β, C and γ, and so on. The number of mating

types are then 2, 4, 8, ---, or 2^n. Indeed, syngens in *P. bursaria* have 2, 4, and 8 mating types (Wichterman 1985 for review), a characteristic not found in other genera so far. That is, in order to form a multiple mating-type system, the number of mating types must jump from two to four. This is achieved by adding a new pair of GR-molecules, G3-R4 and G4-R3, but this would be much more difficult than obtaining a new GR-pair G3 and R3. Probably only a few species of *Paramecium* have managed to clear this high barrier to gain a homopolar multiple mating-type system consisting of 4 mating types, while others produced new syngens maintaining the binary system.

The assumption of GR-molecules can also explain the unique characteristic of *Paramecium*, the occurrence of agglutinative mating reaction throughout preconjugant interaction (Section 4.1). In most gamone carriers other than *Paramecium*, gamone and receptor in each mating type are probably not physically linked. During preconjugant interaction, gamones may be exchanged between complementary mating types upon their contacts through a specific binding between gamone and receptor. However, this cannot occur in *Paramecium* in which gamone and receptor form the GR-molecule, because receptors must stay on the cell surface. If preconjugant interaction is to continue in *Paramecium*, cells of complementary mating types must remain attached to each other, probably by means of a specific binding between complementary GR-molecules, resulting in cell-cell agglutination.

In *Blepharisma*, the formation of the GR-molecule is impossible, because this ciliate is a gamone excretor: the gamone is excreted while the receptor must stay on the cell surface. The binary system of *Blepharisma* can be explained, however, by considering a characteristic feature of this genus, the homotypic arrest (Section 4.1). One of the simplest way to explain the homotypic arrest is to assume that activation in *Blepharisma* requires a complete set of gamones and receptors (G1, G2, R1, R2) in the cell. If so, only heterotypic pairs can achieve activation (initiation of nuclear changes of conjugation [Section 6.2]). For example, if homotypic pairs are induced in mating type I (G_1R_2) by gamone 2, only G1, G2 and R2 are available. Since R1 is lacking, activation does not occur resulting in the homotypic arrest.

When *Blepharisma* acquires a new gamone, G3, it is most likely to be a protein gamone, because a new gamone may be formed more easily by mutation of one of the duplicated genes of G1 (a glycoprotein) than by producing a new enzyme to synthesize a new small-molecule gamone like G2 (a tryptophan derivative). If G3 is a protein derived from G1 by mutation, its receptor R3 should be more like R1 than R2. Therefore, G3 and R3 are likely to replace G1 and R1, but not G2 and R2, in the function for activation. The acquisition of a new GR pair, G3 and R3, cannot produce a homopolar multiple mating-type system. For example, three mating types, I ($G_1R_2R_3$), II ($G_2R_1R_3$), and III ($G_3R_1R_2$), form a heteropolar triple mating-type system. Type I is complementary with type II but not with type III; in I × III, pairs are formed but activation does not occur because of the lack of G2. In this system, G2 and R2 cannot be lost, because their function in activation cannot be substituted; however, G1 and R1, or G3 and R3 can be lost resulting in a binary system consisting of complementary mating types, G_1R_2 and G_2R_1, or G_3R_2 and G_2R_3. Under such a condition, the system may break up in two binary mating-type systems G_1R_2 and G_2R_1, and G_3R_2 and G_2R_3, which may eventually become different species. This can explain the present condition of the genus *Blepharisma* in which several species, each consisting of two complementary mating types, have species-specific protein gamones (G1 family) and a common non-protein gamone, G2.

Thus by assuming that the homotypic arrest is due to the requirement of all of the two GR-pairs for the occurrence of activation and that *Blepharisma* acquired the homotypic arrest soon after the origin of the genus, the persistence of a binary mating-type system in *Blepharisma* can be understood as well as why *Blepharisma* maintains a small, supposedly ancient gamone, blepharismone, as the generic gamone.

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