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Ultrastructural investigation of fetal rat brain hemisphere tissue in nonadherent stationary organ culture

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Summary. Fetal rat brain fragments grown in nonadherent stationary organ culture for 50 days have been investigated ultrastructurally. Synaptogenesis and myelin formation occurred at the same time as the corresponding time-dependent events in the developing brain in vivo. Intermediate junctions were observed between cellular processes lining a central cavity in the fragments and later associated with astrocytes at the surface. Gap junctions and tight junctions were also present. In some fragments cilia were observed in the central cavity. Subependymal basement membrane labyrinths were observed in all fragments after 10 days in culture. The ultrastructural characteristics and the tissue-like structure in general were preserved for at least 50 days in this tissue culture system. The brain fragments may therefore be a valuable supplement to existing culture methods for nervous tissue.

Key words. Nonadherent organ culture; rat brain tissue; ultrastructure.

Introduction

In the developing brain in vivo there is a differentation of primitive neuroectodermal cells into neurons and glial cells which together make up the nervous tissue. In recent years several tissue culture models have been elaborated to study this evolution in vitro. In these models it has been possible to investigate both morphological, biochemical and electrophysiological differentiation[3, 6, 8, 9, 11, 12, 14–16, 20, 21, 24, 27, 29–33, 37, 38]. An objection against these cultures as models for nervous tissue has been that the original histiotypic structure of the tissue is destroyed, either by dissociation or by reorganization due to adherence to a substratum. In order to avoid these problems we have recently described a method for the maintenance of fetal rat brain fragments in nonadherent stationary organ culture for 50 days[35]. Light microscopic and immunohistochemical investigations demonstrated good correlation between cellular differentiation and migration in the fragments and in vivo. The aim of the present report was to study development and organization of cells and ultrastructural characteristics of nervous tissue in the brain fragments as revealed by transmission electron microscopy.

Materials and methods

Animals. Rats of the inbred BD IX- strain were used[10].
Nonadherent organ culture. Whole brains were obtained from fetal rats at the 18th day of gestation. The brain lobes were dissected free, the meninges carefully removed and the cortical tissue cut into approximate cubes, measuring about 800 µm in all directions. Dulbecco's modification of Eagle's medium (DMEM, Flow Laboratories, Glasgow, Scotland) supplemented with 10% heat-inactivated newborn calf serum, four times the prescribed concentration of 100 × non-essential amino acids, L-glutamine (200 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml) was used. The fragments were grown in multiwell dishes (Nuncleon, Nunc Plastic, Denmark) base-coated with 0.5 ml 0.75% semisolid agar (Agar Noble, Difco Laboratories, Detroit, U.S.A.). One fragment was transferred to each well and 1 ml medium was added. The fragments were maintained at 37 °C in 5% CO_2 in air with 100% relative humidity. The medium was changed every second day. The culture period was 50 days and the fixation times were days 0, 1, 2 and 5 and every 5th day thereafter until day 50.
Transmission electron microscopy (TEM). Fragments were fixed for 24 h in 2% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose, postfixed for 1 h in 1% osmium tetraoxide in cacodylate buffer and dehydrated in graded ethanol concentrations to 100%. After 2 × 10 min in propylenoxide and 12 h in a 1:1 mixture of propylenoxide/Epon 812, the fragments were embedded in pure Epon 812. The final polymerization was carried out at 60 °C for 3 days.

Two representative fragments from each observation point were cut on a ultramicrotome (Reichert ultramicrotome, 'Om U3'). The thin sections were mounted on copper grids and stained with uranyl acetate 30 min and lead citrate 8 min. Sections of the fragments were systematically mapped under a transmission electron microscope (Philips EM 300), at a magnification of 6200. Overlapping photographs were taken from one surface to the other of the cubic fragments and along the largest diameter of the spheric ones. A part of the surface was photographed in the same manner. By mounting the pictures on large paper sheets the fragments were reconstructed at approximately the same magnification as used for the microscopy. These reconstructions were also used in the investigation of the fragments.

Under the microscope and in the reconstructions myelinated axons and junctions were counted in both explants from each observation point. This was done to obtain a quantitative estimate of the changes in their relative incidence during the culture period.

Identificaton of tissue elements. The identification was done according to Booz and Desaga[2], Caley and Maxwell[5], Gray[13], Mori and Leblond[22,23] and Peters et al.[26].

Results

Cellular composition. At explantation the brain fragments were histiotypic structures with the 5 zones described by The Boulder Committee[36]. During the first 20 days in culture cell differentiation and cell migration took place. The result was the development of spheric structures with three different layers (fig. 1). The central zone consisted of neurons and macroglia, mainly astrocytes. It was surrounded by a layer of cellular processes. In the periphery macroglia, especially oligodendrocytes, were in the majority. During the next 20 days some neurons migrated outwards and came to be situated at or right beyond the surface of the fragments. Beyond that, the described 3-layered structure remained unaltered. During the last 10 days in culture no clear layer organization could be observed any more. Neuropil became the major constituent. The cells present, mostly neurons and astrocytes, became distributed all over the fragments. In this period astrocytes and their processes formed a 1-cell-thick, nearly continuous, layer that coated the fragments. Further details and illustrations on the general development of cultured brain tissue are given elsewhere[35].

Single cell death in the form of nuclear rupture, breaks in the cell membrane and accumulation of vesicular profiles were observed after 1 day in culture. This tendency to single cell degeneration did not appear to increase with the age of the fragments, and at the end of the culture period the cell morphology in general was well preserved.

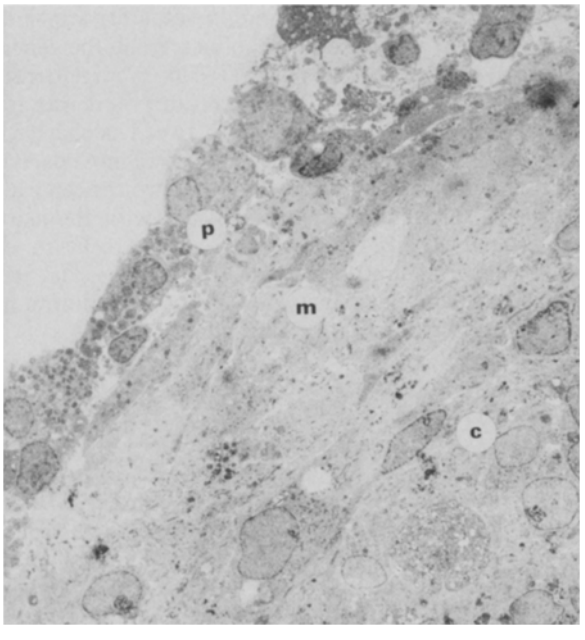

Figure 1. Fragment, 25 days old, showing the characteristic 3-layered structure. × 1500. Abbreviations are: c, central zone; m, middle zone; p, peripheral zone.

The neuropil. Neuropil was identified according to the definition in Stedman's medical dictionary as 'The complex, feltlike net of axonal, dentritic and glial arborizations that form the bulk of the central nervous system's gray matter and in which the nerve cell bodies lie embedded'[34]. At the time of explantation there was no such network. There was, however, a region just beneath the cortical plate where bundles of neuronal outgrowths ran parallel to the pial surface. Apart from that cell bodies dominated the whole structure. Along with the maturation of the cells the amount of intercellular elements increased rapidly, and at day 10 most of the space between the cell bodies was densely packed with cellular outgrowths. Nearly all of them were of neuronal origin. At the periphery, however, glial processes dominated. In the 3-layered fragments seen after approximately 20 days, characteristic neuropil was present. In the middle layer, bundles of neuronal and astrocytic processes made up a dense network. Embedded in this network, there were a few cells, both neurons and macroglia. Centrally the cell density was high and the neuropil was not as conspicuous as in the middle layer. It had, however, the same constituents and the same dense appearance as described (fig. 2). During the last 10 days in culture the neuropil became the major constituent of the fragments. Astrocytic processes, rich in microfilaments, were most numerous, but neuronal elements were also present, especially in the center.

Junctions. In this context, junctions between cells and cellular processes include intermediate junctions, tight junctions, gap junctions and synapses.

At day 0 the mean density of junctions in the fragments was $4.2(\pm 0.55) \times 10^3/mm^2$. Most of them were gap junctions and tight junctions. In the cortical plate and just beneath it, however, synapses were observed (fig. 3).

After 2 days intermediate junctions were found between cells lining a cavity formed inside the fragment (fig. 4). The average junctional density at this stage was about the same as at the time of explantation.

Towards day 10 most of the junctions were found in the central parts of the fragments. There was an increasing number of mature synapses in the same area. In spite of

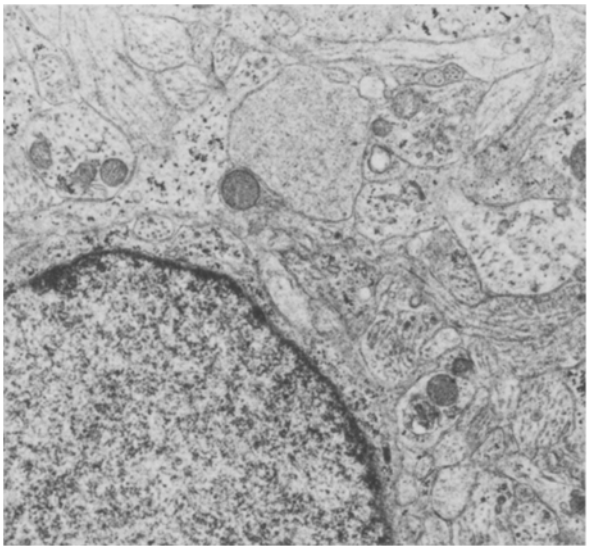

Figure 2. Neuropil in the center of a 25-day-old fragment. Densely packed, mainly neuronal processes characterized by parallel microtubuli are seen. × 19,000.

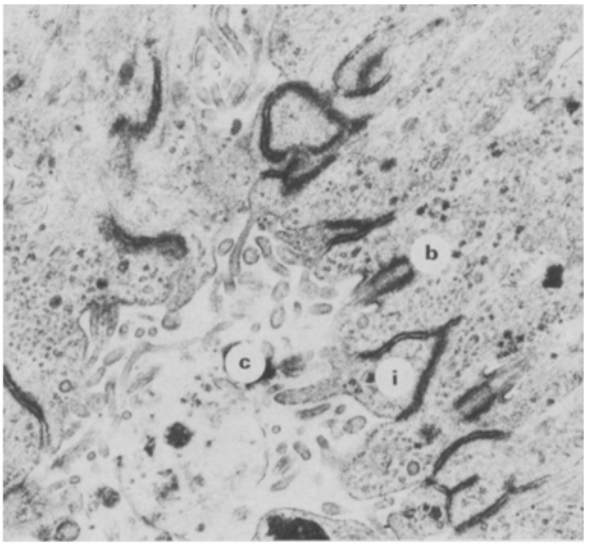

Figure 4. Intermediate junctions (i) between cellular processes lining a central cavity (c) in a 2-day-old fragment. Basal bodies (b) are present in the surrounding cells. × 15,800.

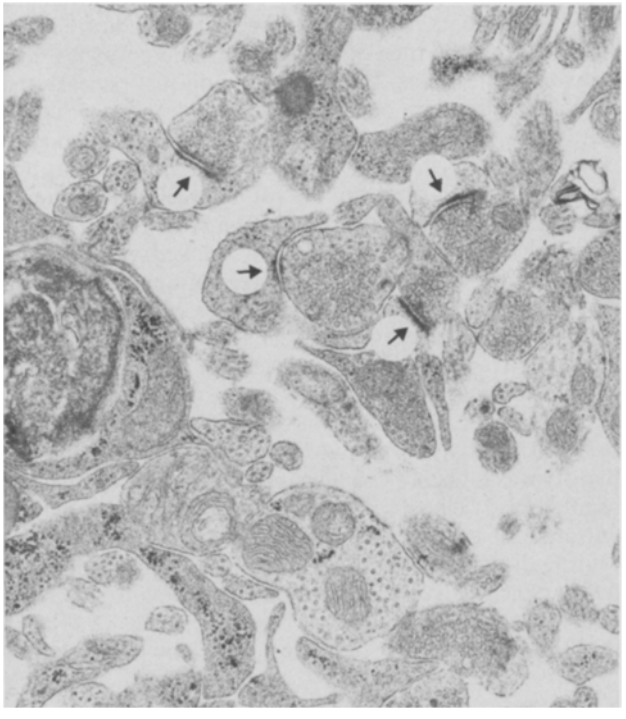

Figure 3. Synapses (arrows) in the center of a 35-day-old fragment. × 28,500.

this, the mean density of junctions in the fragments did not change compared with the first days in culture.

A major increase in the number of junctions, both absolutely and relatively took place during the next 10 days, especially between day 15 and day 20. This seemed mainly to be due to an increase in the number of synapses in the central parts of the fragments. Most of them were asymmetric and occurred between neuronal processes. There was also an increase in the number of junctions in the peripheral regions, but to a smaller extent. Most of them were gap junctions. The maximum junctional density in the center of the fragments at day 20 was $24.3 \times 10^3/mm^2$. More than 50% of them were synapses. The average density at this time was $10.9(\pm 1.4) \times 10^3/mm^2$.

Towards day 35 the average junctional density increased further to $17.1(\pm 0.5) \times 10^3/mm^2$, while the maximum density was about the same as after 20 days. In most parts of the fragments synapses were the main junctional type. Gap junctions and tight junctions were in the majority only in the periphery.

Intermediate junctions around a central cavity were observed in most fragments until day 35. After this stage they were not seen anymore.

Between days 40 and 50 the relative number of synapses decreased, while the corresponding value for gap junctions increased. The result was an increase in the general junctional density towards day 45. The average density then was $28.3 (\pm 1.3) \times 10^3/mm^2$. At day 50 it was $16.1 (\pm 1.8) \times 10^3/mm^2$. Gap junctions between astrocytes or astrocytic processes were in the majority in most parts of the fragments. Synapses were found in groups centrally. Intermediate junctions were associated with the astrocytes at the surface. They connected the cells to the underlying tissue and the astrocytic processes to each other. The junctional density in the fragments during the culture period is summarized in figure 5.

Myelin. The first stages of myelin formation were observed after 10 days in culture. They were seen as slim oligodendrocytic outgrowths partially surrounding neuronal processes. The myelination took place close to the oligodendrocytes at the surface of the fragments. Multilamellated myelin appeared after 20 days. It was found around neuronal processes in the other parts of the fragments. At day 20 the mean density of myelinated axons was $8.8(\pm 2.5) \times 10^2/mm^2$ (table). After 40 days the corresponding value had increased to $19.4 (\pm 2) \times 10^2/mm^2$ (table). The myelinated axons were often found in groups of 2 or 3. At the latest observation points only 1 or myelin

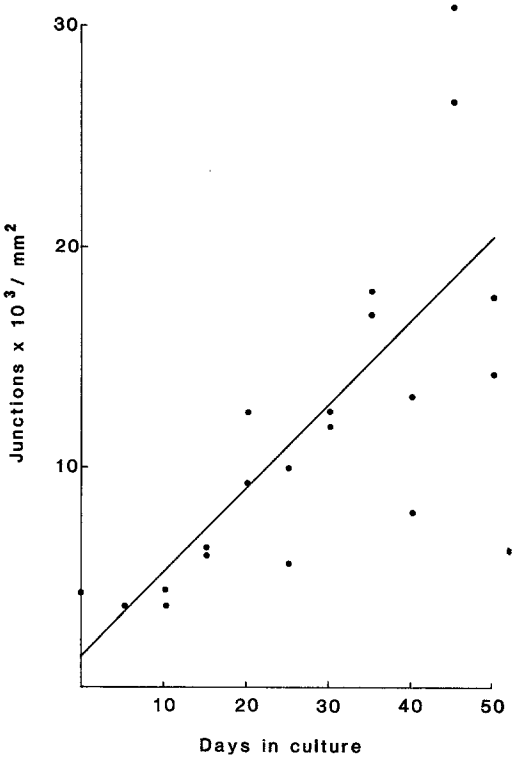

Figure 5. Correlation diagram showing the increase in the relative number of junctions during the culture period. Each point represents the average junctional density in one fragment.

Density of myelinated axons at three different stages

Day	20	40	50
Myelinated axons × 10^2/mm^2	8.8	19.4	4.4

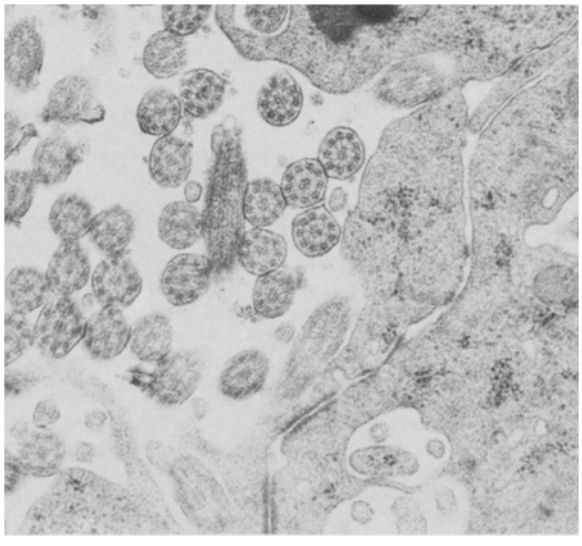

Figure 6. Cilia in a central cavity after 30 days in culture. × 35,000.

structures were found in each fragment. This gives a density of approximately 4.4 (± 1.2) × 10^2/mm^2.

Cilia. Such structures were observed after 30 days in culture, in the central cavities previously mentioned (fig. 6). Each cilium had 9 pairs of peripheral tubules

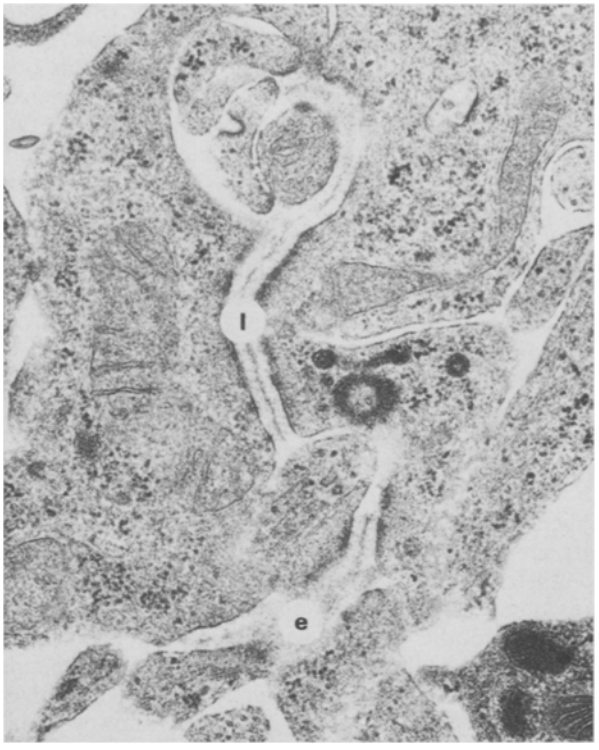

Figure 7. Subependymal basal membrane labyrinths in a 20-day-old fragment with an electron dense region (e) and a lamina containing region (l). × 70,000.

arranged in a regular circle around a central pair. Basal bodies were present in the cells lining the cavity. In similar cavities in younger fragments there were slim processes. They did not have the characteristic structure mentioned above, but basal bodies in the surrounding cells suggested a ciliary nature (fig. 4).

Subependymal basement membrane labyrinths. These structures were observed for the first time after 10 days in culture (fig. 7). Some parts of the labyrinths were filled with an electron dense substance. Other regions had a central lamina with adjacent plasmalemmas which appeared thickened. After day 10 they were present at all the observation points. They were centrally located in the fragments.

The ultrastructural differentiation of the rat brain fragments was uniform in different fragments and in different experiments.

Discussion

The study showed that the ultrastructural differentiation in the rat brain fragments to a large extent resembled the corresponding sequence of events in developing rat brains in vivo.

The first signs of myelination were observed after 10 days in culture, close to the oligodendrocytes at the periphery of the fragments. Since the brain tissue was excised approximately 3 days before birth this corresponds to the 7th postnatal day which is when myelination commences in vivo[7]. The multilamellated myelin present after 20 days seemed to be identical to myelin in normal brains.

The development of synapses resembled the corresponding event in vivo. In rat brains there is a major increase in the number of synapses when the young animals open their eyes[1]. A similar event was observed in our fragments at the corresponding time, between the 15th and the 20th day in culture. In the well-differentiated fragments most of the synapses were asymmetric, as they are in the mature rat brain cortex[17].

In vivo intermediate junctions occur between cells lining the 3rd ventricle[4,25]. In the fragments they were found in the center. This may be explained by the curving of the tissue explants around a center at the ependymal surface occurring during the first 5 days in culture[35].

The cilia observed in the fragments had the characteristic morphology of motile ependymal cilia in vivo[4]. It seems reasonable to presume that their central localization is due to the same rounding process as that mentioned above.

Subependymal basement membrane labyrinths in normal rats are characterized by an electron-dense substance completely filling the intercellular space[18,19]. In the presently described brain fragments parts of these structures had another appearance. The clefts were lined with thickened plasmalemmas and they had a central lamina. This corresponds to the morphology of such labyrinths in rabbits[18,19]. To the best of my knowledge, however, they have not been described in rats before, neither in vivo nor in vitro. The significance of this phenomenon for the general assessment of the model is not clear as yet.

In the present study, ultrastructural development of fetal rat brain fragments in nonadherent stationary organ culture has been described. Compared to other culture methods for nervous tissue, our system offers the advantage of a reduced number of external factors that may influence cellular differentiation and organization[35]. In the fragments the appearance of different ultrastructural features characteristic for nervous tissue corresponded to the same time-dependent events in vivo.

Nervous tissue grown in this way also develops subependymal basement membrane labyrinths which have not previously been described in comparable culture systems. Furthermore, the fragments remain viable and organized for a longer period of time than nervous tissue grown in other nonadherent organ cultures[8,37]. We therefore conclude that the described brain fragments may be attractive as a model for brain tissue in vitro.

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Mini-Review

Non-reciprocal fertility among species of the *Aedes (Stegomyia) scutellaris* group (Diptera: Culicidae)

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Summary. The genetic basis of non-reciprocal fertility in the *Aedes scutellaris* group has been viewed in relation to cytoplasmic incompatibility in the *Culex pipiens* complex and related to the presence or absence of rickettsia-like microorganisms *Wolbachia* spp. A crossing scheme is proposed to explore the genetic basis of non-reciprocal fertility between two species by crosses involving a third mutually compatible species.
Key words. Cytoplasmic incompatibility; *Aedes scutellaris; Wolbachia;* speciation.

There has been considerable interest in the phenomenon of non-reciprocal fertility in mosquitoes because of its potential use in population suppression programs. Also, the mechanism(s) underlying non-reciprocal fertility may be important for an understanding of speciation. This phenomenon is widespread in the family Culicidae; an increasing number of species complexes are being recognized and crossing relationships between sibling species are at present being studied. Non-reciprocal fertility in the *Aedes scutellaris* group was first reported to occur between *Ae. scutellaris scutellaris* Walker and *Ae. scutellaris katherinensis* Woodhill[1]. Fertile hybrids were obtained by crossing *Ae. s. scutellaris* females with *Ae. s. katherinensis* males; in the reciprocal cross *Ae. s. katherinensis* females were inseminated by *Ae. s. scutellaris* males but all eggs laid were inviable. Woodhill[2] further showed that the *Ae. s. katherinensis* females when crossed with hybrid males derived from the cross *scutellaris* females × *katherinensis* males laid inviable eggs; the other three backcrosses were successful. The origin and nature of such non-reciprocal fertility is of genetic interest and can be analysed using egg hatch as a marker since viable hybrids can be obtained in one direction and the hybrids can be backcrossed to parental species.

Smith-White[3] drew attention to possible genetic mechanisms underlying this phenomenon of non-reciprocal fertility and suggested a sequential backcrossing scheme which was later exploited by Laven[4] studying the *Culex pipiens* complex. Smith-White and Woodhill[5], studying the *Ae. scutellaris* complex, used this backcrossing scheme to determine the possible genetic bases for non-reciprocal fertility. The latter authors concluded that non-reciprocal fertility must depend either on anomalous meiosis in oogenesis or on nucleus-independent cytoplasmic factors. The second mechanism paralleled the findings of Laven[4,6], based on his extensive sequential backcrossing experiments with the *C. pipiens* complex. Since this phenomenon of non-reciprocal fertility or unidirectional incompatibility appears to have a cytoplasmic basis, this is often referred to as cytoplasmic incompatibility, and these terms are used interchangeably. McClelland[7] has proposed an alternative to the hypothesis of cytoplasmic inheritance to account for the phenomenon of non-reciprocal fertility, reciprocal fertility and reciprocal incompatibility, which involves cytoplasmic conditioning alleles and preferential segregation.

Yen and Barr[8] attributed this cytoplasmic incompatibility in the *C. pipiens* complex to the presence of rickettsia-like organisms, *Wolbachia* spp. Later they demonstrated that the incompatibility barrier could be broken by removing these microorganisms by a treatment of larvae with antibiotics, e.g. tetracycline hydrochloride[9]. Further, Barr[10] offered a hypothesis to account for strain differences affecting fertility and multiplicity of crossing types in *C. pipiens* based on the presence of different kinds of symbionts and their ability to evolve with their hosts. These microorganisms have also been reported in species of the *Ae. scutellaris* group[11–14]; further, incompatible crosses have been known to become compatible after heat or antibiotic treatment[15]. However, several questions raised by Trpis et al.[15], and Subbarao[16] remain unanswered and the mechanism of non-reciprocal fertility remains unsettled. Furthermore, for some crosses both antibiotic and heat treatments failed to produce compatibility[17,18]; in other crosses the presence or absence of *Wolbachia* seemed to be in conflict with the crossing