Cleistogamy in Viola Riviniana with Especial Reference to its Cytological Aspects.

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With Plates XI and XII and four Figures in the Text.

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INTRODUCTION.

It has been shown (16) that seed production in the cleistogamous flowers of Viola odorata depends upon a true fertilization. The present study of V. Riviniana was undertaken to determine the conditions in another Viola species and so help towards a generalization for the genus. Now the chasmogamous flowers of V. odorata are invariably sterile and only the cleistogamous ones set seed; whereas in V. Riviniana some seed is set by the open flowers as well. Thus, there is the corresponding problem of seed production in those chasmogamous flowers which set seed, but owing to the infrequency of seed production by these flowers, and the consequent scarcity of material, it has not been possible to investigate it. Fertilization is, therefore, described for the cleistogamous flowers only.

MATERIAL.

All the material used in the investigation was obtained from plants growing either in the grounds or in the botanical garden at Royal Holloway College. Three groups of plants were kept under observation, viz.:

1. Plants of V. Riviniana Reichb., blue and white forms, sent by courtesy of Mr. Hales from the Chelsea Physic Garden, which were grown in sun and shade plots in the botanical garden.

2. Two patches of wild plants, which may be described as follows:
   (a) V. Riviniana Reichb. growing among ivy and grass, well shaded by cedar trees above, receiving sun from south-west and west.
   (b) V. Riviniana var. nemerosa (N.W. and H.), also growing among ivy and wild arum, shaded by cedar and hawthorn trees, receiving sun from south-east and south.

These two groups of wild plants were not interfered with in any way, so that it was possible to study fresh material of the various types of flower as they actually occur in nature. Observations were begun on the plants from the Chelsea Physic Garden in the spring of 1926, and on the wild plants in the spring of 1927, and were continued until July 1928. These groups of plants proved very good sources of material for morphological and for cytological work. Occasional attacks of 'smut' caused by Urocystis violae seemed to have no effect whatever upon the life cycle.
Especial Reference to its Cytological Aspects.

METHODS.

The general form of the types of flower produced by the three groups of plants of *V. Riviniana* during the flowering season was studied as far as possible in fresh material. A calendar was kept recording the periods during which the chasmogamous, semi-cleistogamous, and cleistogamous flowers appeared. For the cytological investigation the buds and flowers were always fixed as soon as possible so that never more than ten or fifteen minutes elapsed between gathering and fixing; sometimes fixing was done in the field.

The following fixing fluids gave the best results:

1. Allen's modification of Bouin's fluid.
2. Flemming's fluid, weak solution.
3. Medium chrom-acetic.
4. Acetic alcohol (2:3).

Hermann's and Merkel's fluids were also used to a very small extent. Chloroform proved to be a very satisfactory and rapid clearing agent, the material being less brittle than when cleared in xylol. Sections were cut 8 μ in thickness.

The following stains were used most successfully:

1. Heidenhain's iron-alum-haematoxylin, usually counter-stained with orange G, following Allen's Bouin fixative.
2. Gram's iodine, gentian-violet method.
   (a) Clausen's modification (8) after Allen's Bouin fixative.
   (b) Newton's modification (7) after Flemming's fluid and medium chrom-acetic.

Pollen smears. Smear preparations of the pollen mother-cells and very young pollen-grains of chasmogamous flowers of *V. Riviniana* were tried. Although the stamens are not favourable material for this method, being so small, some good preparations were obtained. Excellent fixation and staining were given by Flemming's fluid, weak solution, followed by Newton's iodine gentian-violet stain.

MORPHOLOGY.

Chasmogamous flowers. The general morphological features of the open flowers of *V. Riviniana* are represented in Text-fig. 1. The flowers show the grooved spur which distinguishes the species from *V. sylvestris*, with which it is sometimes confused. The spur of the blue flowers of *V. Riviniana* Reichb. is blunt and of dull whitish blue, whereas that of *V. Riviniana* var. *nemorosa* is quite a deep purplish blue, and this difference in colour is noted in the expanded petals.
West.—Cleistogamy in *Viola Riviniana* with Cleistogamous flowers. The morphology of the permanently closed flowers of *V. Riviniana* is particularly interesting. Cleistogamous flowers appear when the chasmogamous season has finished, and may therefore be called the summer flowers. The reduction of the floral members of these flowers is carried to what is almost the extreme limit possible: only those parts essential for the protection of the reproductive organs and for seed production are fully developed. The other parts are either absent or represented merely by rudimentary structures. A cleistogamous flower of *V. Riviniana* shows the following features.

**Calyx.** The five normal sepals enclose the flower very tightly and show no sign of opening until fruit formation.

**Corolla.** The petals are represented merely by two to five small membranous strap-shaped structures almost entirely unpigmented. In extreme cases the corolla is absent.

**Androecium.** Considerable variation is found in the extent of fertility of the five stamens. They are all present, though very much reduced. The greater part of the tissue below the connective is sterile and appears as a long stalk-like structure. The anterior stamens have two to four pollen-sacs each, which, although less than one-third of the size of the pollen-sacs in

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**Text-fig. 1.** Chasmogamous flower. 1. Flower partly dissected. 2a. Anterior stamen. 2b. Lateral or posterior stamen. 2c. Style.
a chasmogamous flower, are the largest in the cleistogamous flower and are
certainly the ones functional in self-pollination. The nectaries typical of
the anterior stamens in open flowers are absent. The lateral stamens have
either two or three pollen-sacs each, or there is only one; occasionally one

lateral stamen is sterile. The posterior stamen is invariably sterile. The
orange coloured connectives of the stamens are fully developed and arch
like a roof over the curved style and stigma, providing excellent protection
for the reproductive organs (Text-fig. 2). The cells of the epidermal
layer of the pollen-sac wall show the characteristic thickenings on the inner
and radial walls even in cleistogamous flowers in which dehiscence does not
take place. The stamens are connected to one another by peg-like outgrowths
developed on the contiguous edges of the outer pollen-sacs. These inter-
lock in such a way that often, when a flower is dissected, all the five stamens
come away together.

Gynaeceum: The ovary of a cleistogamous flower is normal, but the style
and stigma are much modified. The style is bent over in the form of a
hook, and by this curvature the stigmatic surface is brought over and
down towards the pollen-sacs of the two anterior stamens. The stigma, as
found in open flowers, is absent and the stigmatic surface is represented
merely by the flattened end of the tubular style and is in close contact with
the upper surface of the anterior pollen-sacs; the stigmatic aperture is much
larger than that in an open flower.

Semi-cleistogamous flowers. Between the full periods of chasmogamous
and of cleistogamous flowers, a few flowers, designated semi-cleistogamous,
are produced, which, besides linking the open and closed flowers in regard
to time of flowering, are found to illustrate, in a series of forms, the assumed
stages in reduction from chasmogamy to cleistogamy. The fact that this
process of reduction can be found occurring during one flowering season is
evidence for the origin of the cleistogamous flower from the chasmogamous

in such a way. The only distinction between the earliest semi-cleistogamous
flowers and the chasmogamous flowers is merely that the petals of the
former are considerably smaller than those of the latter. As the season
progresses the semi-cleistogamous flowers show more and more indications
of cleistogamy in the following features: reduction of the petals and of the
number and size of the fertile pollen-sacs, and the curvature of the style and
stigma. The latest of these flowers differ from a cleistogamous flower
merely in that they show one or two small, slightly pigmented petals
extending beyond the sepals. Text-fig. 3 illustrates three semi-cleisto-
gamous flowers which show the gradual reduction of the petals and the
closing of the flower. The change in the relative positions of the stigma
and pollen-sacs, brought about by the curving of the style, may be followed
by reference to Text-figs. 1 (Fig. 1), 2 (Fig. 2), 3 (Fig. 4). The following
annual cycle of flower production may be deduced for *V. Riviniana* from
the records kept from 1926 to 1928:

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<th>Type of Flower</th>
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<tr>
<td>Chasmogamous flowers</td>
<td>March to middle of May.</td>
</tr>
<tr>
<td>Semi-cleistogamous flowers</td>
<td>May.</td>
</tr>
<tr>
<td>Cleistogamous flowers</td>
<td>Middle of May to September.</td>
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**Pollination and Seed Production.**

Two kinds of pollen-grain are produced in the pollen-sacs of the
chasmogamous flowers, and in addition there are many shrivelled pollen-
grain walls. Of the good grains the majority are large, either tetrahedral
or oval, thin walled, and contain finely granular protoplasm. The smaller grains have thicker lobed walls, and the protoplasm is dense and lumpy. A small proportion of these small grains germinate within the anthers: the large grains are shed in the binucleate condition. The pollen-grains of cleistogamous flowers, many of which degenerate, are like the smaller grains of the open flowers; the good grains germinate within the anthers. It is an interesting fact that these pollen-grains which germinate within the anthers of chasmogamous flowers are similar in form to those found in cleistogamous, and that the pollen-grains functional in cross pollination are morphologically, and probably physiologically, different from those which germinate within the anthers.

Cross pollination in the open flowers of *V. Riviniana* has not been made a part of the present study: the general features of the pollination mechanism in *Viola* are well known. The neat and direct method of self-pollination in the cleistogamous flowers has been described by other workers, and only the essential features are here recalled. The pollen-grains germinate within the pollen-sacs; the pollen-tubes so produced penetrate the anther wall at the apex of the sac, pass directly into the stigmatic orifice, thence through the stylar canal into the ovary. Pollen-tubes from the anterior stamens only are functional in pollination. The observations made on pollination in semi-cleistogamous flowers are scanty, but enough to indicate that the pollen germinates within the anthers, and the pollen-tubes traverse a considerable distance towards the stigma. The greater number of the pollen-tubes, however, fail to reach the stigma, and this probably accounts for the noticeable failure of seed production by semi-cleistogamous flowers.

Good seed is set by cleistogamous flowers, except towards August and September, when a considerable number of flowers die off without producing seed. The open flowers of the cultivated *V. Riviniana* set seed, but many open flowers of the wild plants fail to do so: the causes of this failure are not known. Throughout this work it was noticed that no capsules ever produced by the flowers of *V. Riviniana var. nemorosa*. Dissection of mature and even faded flowers showed that the pollen was retained with the anthers in pollinium-like masses, one 'pollinium' in each

![Text-fig. 4. Pollen-grains of the chasmogamous flower. 1-3. Forms of pollen-grains which are shed. 4. Form of pollen-grain which germinates within the anther.](https://academic.oup.com/aob/article-abstract/os-44/1/87/127718)
Cleistogamy in *Viola Riviniana* with pollen-sac. The good grains, several of which were seen to have germinated, were bound together by shrivelled pollen-grains, and although the anthers dehisced normally the pollen-masses were not dispersed. A few pollinating experiments were carried out with these pollen-masses and one positive result was obtained. A pollen-mass from an anterior stamen of one of these flowers was transferred to the stigma of another. The flower was tied up in a pollinating bag, and after twenty days a good capsule had developed. This is considered to show that the pollen was not sterile, and that seed production failed because the pollen was not dispersed. Apparently pollen was never brought to these flowers from other *Viola* flowers.

**CYTOLOGY OF THE POLLEN.**

The stages described in the following account of the meiotic division in the pollen mother-cells of *V. Riviniana* were studied in microtome sections of chasmogamous buds. Some stages were observed in cleistogamous material, and as far as can be concluded from these the pollen development is similar in both kinds of flower. Those stages in pollen meiosis shown by smear preparations confirm the conclusions drawn from microtome sections and include resting nuclei, synizesis showing the variation in nucleolar form and the connexion of the thread to the nucleolus, the linking of the bivalent chromosomes prior to the heterotype division, as well as some stages between heterotype telophase, homotype telophase, and pollen-tetrad formation.

It was often found that the pollen mother-cells at the apex of a pollen-sac lagged behind the pollen mother-cells at the base as regards the stage reached in development. For instance, in one particular pollen-sac the stages from diakinesis to heterotype metaphase, or from interkinesis to homotype telophase could be followed from the apex to the base. This gradual progression of the stages throughout one pollen-sac enabled one to visualize more clearly the phases passed through in living cells. More prolonged phases, such as diakinesis, were often found throughout a whole pollen-sac. Again, the stages shown by the various pollen-sacs in one bud differed widely. In one case the anterior stamens showed first contraction to open spireme, the laterals diakinesis and heterotype division stages, and the posterior stamen homotype division and tetrad formation. Thus the anterior stamens are considerably later in development than the others. The chasmogamous flowers are decidedly protandrous, but in cleistogamous flowers the pollen and ovules must of necessity be mature at the same time. Since only the anterior stamens are functional in the pollination of cleistogamous flowers, the retardation of development in the corresponding stamens of chasmogamous flowers seems to be a definite adaptive tendency towards cleistogamy.
Especial Reference to the Cytological Aspects.

The following account of meiosis in the pollen mother-cells is practically confined to a description of those features which are apparently peculiarly characteristic of *V. Riviniana*. It may, therefore, be concluded that those details not described are quite typical of a meiotic division in the prophase of which two contractions are passed through.

**Meiosis. Heterotype division.** The large nucleoli of the resting pollen mother-cells are usually vacuolate and occasionally contain a small crystal body (Pl. XI, Fig. 1). Many of the nucleoli in the newly differentiated tapetal cells contain a similar but larger crystal body; in some nucleoli two or three smaller crystal bodies are present. During synizesis or first contraction, the nucleolus is flattened against the nuclear membrane, either close to the contraction knot or on the opposite side of the nucleus (Pl. XI, Fig. 2). Nucleoli of much altered and very peculiar shapes occur in many of the pollen mother-cells at this stage. The change in form seems to be brought about by a spreading of the nucleolar material against the nuclear membrane, accompanied by vacuolation, so that the nucleolus becomes flattened and much attenuated; it is almost plate-like in form. The outline of many such nucleoli is irregularly lobed. A similar change and variation in shape of the pollen mother-cell nucleoli during synizesis is recorded for *Lathyrus odoratus* (14). Throughout first contraction connexion is maintained between the nucleolus and the synizetic knot by a delicate thread or threads passing from the knot to the nucleolus. The point of connexion is usually marked by a small bulge on the surface of the nucleolus. In good preparations, especially when Newton’s staining method is used, this bulge is deeply stained while the rest of the nucleolus is quite unstained (Pl. XI, Fig. 3). Since it is probable that this bulge is similar in position, form, and function to the ‘nucleolar body’ described by Latter for *Lathyrus* the same term is adopted here, although its origin and function are not so clearly defined as they appear to be in *Lathyrus*.

At diakinesis twenty bivalent chromosomes of various shapes are scattered within the nucleus. The univalent halves of the early bivalent chromosomes are quite separate, and delicate unstainable extensions are seen, probably of linin material, at the free ends of the univalents (Pl. XI, Fig. 4). In some nuclei similar threads appear to connect some of the bivalent chromosomes to one another (Pl. XI, Figs. 5, 6). The univalent chromosomes close together later forming compact bivalents with little variety in shape. At this stage a peculiar formation is noticeable in the nuclei: the bivalents are connected or linked together in groups, and it seems that they are connected by the linin threads mentioned above (Pl. XI, Fig. 7). In some nuclei this linking is very marked, and indeed the bivalents seem to be fused to one another, so that the limits of some chromosomes are indistinguishable. Much variation is found in the degree of this linking and in the number of groups of linked chromosomes formed in different nuclei. It is, therefore,
difficult to offer an interpretation of this unusual phase, since, although it seems to be a constant feature of pollen development immediately preceding the heterotype division, there is apparently no definite rule according to which the chromosome groups are formed, nor any constancy in the number of chromosomes in the groups in different pollen mother-cell nuclei. The gradual disappearance of the nucleolus during diakinesis may be correlated with the appearance of chromatin areas in the cytoplasm during heterotype telophase. In those nuclei which show clearly the stages of spindle formation a thickening of the nuclear membrane is evident during late diakinesis, possibly owing to the development of spindle fibres within the nuclear membrane (Pl. XI, Fig. 8). This disappears later and a tripolar spindle is formed, which afterwards becomes bipolar.

In some pollen mother-cells at metaphase, the chromosomes, still in their linked groups, form a ring at the equator of a ‘hollow’ spindle: in others the chromosomes form a plate at the equator of a ‘solid’ spindle. It is thought that the ‘solid’ spindle is not an alternative structure to the ‘hollow’ spindle, but rather that it is merely a later development. The two are, however, distinctly recognizable.

The period of interkinetal rest is quite long. The heterotype spindle disappears; two or three nucleoli are formed within each resting nucleus and about twenty minute beads of chromatin appear upon the linin reticulum; these may represent the heterotype chromosomes. The chromatin beads form the basis of chromosome formation during the homotype prophase.

Pollen-grain wall formation. At the beginning of the delimitation of the four microspores a narrow groove appears along each of four lines equidistant on the surface of the pollen mother-cell cytoplasm, and midway between the nuclei. This cytoplasmic furrowing is accompanied by an ingrowth of material from the thick pollen mother-cell wall along these lines, and a wedge of wall material fills each groove (Pl. XI, Figs. 9, 10). The furrowing and accompanying ingrowth of the wall continue until the four walls meet in the centre of the cell, and thus the four members of the tetrad are formed. A similar process of pollen tetrad-wall formation has been described for Lathraea spp. (9) and in Lathyrus odoratus (14). In pollen mother-cells in which furrowing has begun, delicate cell-plates are seen at the equators of the spindles (Pl. XI, Fig. 9). These possibly assist in the formation of the tetrad wall, although they appear to be merely evanescent structures. A thin wall is secreted around each microspore, and the thick tetrad wall disappears; it becomes spongy in appearance, and seems merely to dissolve in the contents of the pollen-sac (Pl. XI, Figs. 11, 12). The walls of the young free pollen-grains become thickened, and clearly show four tetrahedral lobes.

Development of the binucleate pollen-grain. A large central vacuole is
MANN — SHOOT DEVELOPMENT OF STRAWBERRY.
formed within the young uninucleate pollen-grain and the nucleus is pushed to the periphery. At the beginning of the nuclear division within the pollen-grain twenty long rod-shaped, or slightly curved chromosomes are formed within the resting nucleus. The daughter chromosomes which separate on the short spindle are V-shaped, and quite separate from one another. A cell-plate is formed during telophase, and although this soon disappears it may participate in the differentiation of the protoplasm around the generative nucleus. The lenticular generative cell is at first close to the wall of the pollen-grain; its nucleus is smaller than the vegetative nucleus, and when first formed each nucleus is composed almost entirely of a single nucleolus. In the mature pollen-grains, especially of open flowers, the generative cell is no longer flattened against the pollen-grain wall, but has moved into the cytoplasm of the pollen-grain. It is long, narrow and spindle-shaped, and contains an oval nucleus with a stainable reticulum and small nucleolus. A small stainable body is sometimes observed in the protoplasm at one end of the generative nucleus (Pl. XII, Fig. 16).

Spermatogenesis and germination of the pollen. Spermatogenesis proceeds similarly in both chasmogamous and cleistogamous pollen-grains. The male-cells formed in the two kinds of flower of *V. Riviniana* are exactly alike, and very similar to those described by Madge (16) for *Viola odorata*: it may, therefore, be that this form of the male gametes is common to the genus. During the division of the generative nucleus of *V. Riviniana*, particularly during the later phases, several small stainable granular bodies appear on the spindle. At telophase they occur at the equator, and are distributed in approximately equal numbers between the two male cells (Pl. XII, Fig. 18). These granules undoubtedly give rise to or persist as the stainable ‘dots’ in the cytoplasm of each male gamete. Although it is not yet proved, it seems very probable that these granules originate, possibly by fragmentation, from the one larger stainable body observed at one end of the resting generative nucleus. Two pear-shaped male cells are formed at spermatogenesis, each containing a spherical or oval nucleus in the round end, and the stainable ‘dots’ in the cytoplasm of the tapering end (Pl. XII, Fig. 19). In cleistogamous flowers the generative cell may divide within the pollen-grain, or in the pollen-tube after germination has begun. The male gametes, with their pointed ends adjacent, are always preceded by the tube nucleus during the growth of the pollen-tube (Pl. XII, Figs. 20, 21). Parts of pollen tubes containing the male gametes and tube nucleus were seen in the stylar canal, and also within the ovary chamber. These preparations show that each male nucleus is still within a cytoplasmic sheath when the pollen-tube is about to enter the micropyle of an ovule. In many angiosperms, male cells, not merely male nuclei, are formed at spermatogenesis, and among these *V. Riviniana* may now be included. It is not yet known whether the whole male cell takes part in fertilization, but some
West.—Cleistogamy in Viola Riviniana

Evidence for this is found in *V. Riviniana*. The later development of the pollen-tube is described in the account of fertilization.

Degeneration of the pollen. A considerable amount of the pollen degenerates in both cleistogamous and chasmogamous flowers. In the former degeneration may set in at almost any stage of development whether in the uninucleate or binucleate pollen-grains, or even after germination has begun.

Cytology of the embryo-sac.

A study was made of the early development of the embryo-sac from the megaspore mother-cell, both for its intrinsic interest and for comparison with the development of the pollen mother-cell. Comparisons of these critical stages in the development of the male and female gametophytes in any one plant are surely of great interest and of value to both cytologist and geneticist, but so far the attention of investigators has been focused more particularly upon the pollen development. The present comparison in *Viola Riviniana* cannot claim to be exhaustive, yet it brings out some striking points of similarity in the development of the pollen and megaspore mother-cells.

Resting megaspore mother-cell. The single archesporial cell, differentiated in the third layer of cells of the nucellus becomes the megaspore mother-cell direct, and is recognized by its size and comparatively dense granular protoplasm. The large spherical nucleus, containing a large nucleolus and scanty reticulum is very like the nucleus of a resting pollen mother-cell.

Meiosis. Heterotype division. During the first contraction phase, or synizesis, the reticulum becomes collected into a tight knot on one side of the nucleus: no continuous thread is distinguishable within it. The nucleolus is flattened against the nuclear membrane, either close to the knot, or on the opposite side of the nucleus. Some variation in the form of the nucleolus occurs, but not to such an extent as is noticeable in the pollen mother-cells. Careful examination of the nuclei at this stage shows that the knot is connected to the nucleolus by a fine thread or threads. The deep stain often taken by that part of the nucleolus to which the thread passes suggests that it may have a function similar to that suggested for the nucleolar body in the pollen mother-cell, although no well-defined nucleolar body is seen (Pl. XI, Figs. 13, 14).

In early diakinesis the twenty bivalent chromosomes are connected to one another by linin threads; these connexions later break down, and the newly-formed bivalents are somewhat spindle-shaped (Pl. XI, Fig. 15). The tapering ends are unstainable, and probably of linin material, while the central part is quite distinct and stainable. It seems just possible, from a comparison with the process of spindle formation described by Hughes-
Especial Reference to its Cytological Aspects. 99.

Schrader (12) in the first maturation division of the egg of Acroschismus that these unstainable extensions of the bivalent chromosomes may be incipient spindle fibres. The bipolar heterotype spindle would then be formed by a later orientation and amalgamation of the twenty small spindles. However, these continuations certainly seem to disappear during diakinesis, and since spindle formation was not studied in detail, this is left as a suggestion only. The bivalent chromosomes are all about equal in size and more or less diamond-shaped. Linking of the chromosomes occurs in some megaspore mother-cells at this stage, but it does not seem to be a constant feature.

These, then, are the most important features in meiosis, where there is striking resemblance between the development of the megaspore mother-cell and the pollen mother-cell:

1. The connexion of the spireme to the nucleolus during the early prophases.
2. The variation of nucleolar form during first contraction.
3. The form of the bivalent chromosomes during early diakinesis, showing the unstainable continuations.
4. The linking of the bivalent chromosomes to one another during the heterotype division.

Synergidae. The chief interest in the eight-nucleate embryo-sac centres round the synergidae, and, in particular, their filiform apparatus (Pl. XII, Figs. 22–26). The synergidae are very similar in form to those described by Ishikawa (13) in the embryo-sac of Oenothera. Each is a large pyriform cell tapering towards the micropylar end of the sac. A distinct and well-developed filiform apparatus forms an apical cap in each of the synergidae; the base of the cell is occupied by a large vacuole, and the nucleus is found in the cytoplasm above this vacuole. The visor-like notch appears as a lateral indentation in the cytoplasm.

The Filiform Apparatus. The presence of a highly refractive cap at the vertex of the synergidae, giving a fibrillar appearance under the microscope, was recorded as early as 1856 by Schacht in his observations on Gladiolus segetum. This specialized part of the cell he described as a bundle of threads running longitudinally over the exterior of the vertex of the synergid, and he called it the filiform apparatus. It is a structure confined to angiosperms, and is recorded in the embryo-sacs of many monocotyledons and dicotyledons. Although little is known about it, considerable advance in the study of the filiform apparatus was made by Strasburger, and later, by Habermann (11). These workers found that it is a cap of cellulose nature, pierced by canals, in the apical cytoplasm of the synergid. Habermann observed that sugars and possibly proteins are present in the synergid vacuole, and suggested that these substances are secreted by the filiform apparatus into the micropyle, where they provide a chemical stimulus.
for the pollen-tube. In his account of the synergidæ of Oenothera, Ishikawa (13) states that the filiform apparatus is a solid mass of conical shape perforated by a number of minute canals which converge from the base to the apex.

The synergidæ in the embryo-sac of Viola Riviniana seem particularly favourable for a more detailed study of the filiform apparatus, which is equally well developed in the embryo-sacs of cleistogamous and of chasmo-gamous flowers. As a result of this study some evidence is brought forward which suggests that the filiform apparatus is composed of a plexus of minute pores or tubules in a stainable matrix rather than merely canals in the protoplasm.

The early development of the filiform apparatus was not followed, but since the apical protoplasm of the young synergidæ contains numerous minute round vacuoles, having almost a honeycomb appearance, it seems that the filiform apparatus is vacuolar in origin. In fully formed synergidæ the filiform apparatus is stained by orange G, congo red and lichtgrün, but these transparent stains are of no use in elucidating the structural details. With Clausen's iodine gentian violet stain the filiform apparatus appears as a bright purple cap in each synergid, and presents quite a striking appearance under magnification. The use of this stain made it possible to study its structure in some detail. Considerable variation is observed in the appearance of the filiform apparatus in different preparations. Usually it appears to be a solid cap, stained purple, perforated by numerous small unstained pores wider at the base, and converging towards the apex; sections in different planes show the same structure (Pl. XII, Fig. 22). Quite frequently the filiform apparatus is seen as a rather ragged cap from the base of which long irregular prolongations extend into the cytoplasm. This appearance may be due to faulty fixation or to degeneration (Pl. XII, Fig. 23). In one preparation of the ovary of a cleistogamous flower the pores of the filiform apparatus appear as well-defined tubules, each one conical in form, and wider at the open basal end. These are much larger than the pores described above, and much fewer in number. The walls of these tubules are stained and the contents unstained. Since only one, out of the numerous preparations examined, shows this structure of the filiform apparatus, it may be a special case (Pl. XII, Fig. 24). Although chlor-zinc-iodine stains it blue, and it is dissolved by zinc chloride in hydrochloric acid, when treated with freshly prepared cuprammonia for twenty-four to forty-eight hours, or even longer, the filiform apparatus was never dissolved: it either remained apparently unchanged or else became altered in some way into a mass of minute stainable rods of a granular appearance, each of which, from their number and size, probably represented one of the original pores (Pl. XII, Fig. 25). This effect was not observed frequently. Thus, although the first two tests suggest that the filiform apparatus is composed
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of cellulose, the cuprammonia reaction certainly seems to throw some doubt upon this conclusion. The filiform apparatus is a very special part of the synergid: this is obvious from the part it plays during the entry of the pollen-tube, described later. The suggestion made by Habermann that substances are secreted through the filiform apparatus into the micropyle is supported by this investigation. Specially stainable granular and globular bodies often occur in the synergid cytoplasm; these may represent the substances secreted (Pl. XII, Fig. 26). It would seem that the tubular structure suggested in this paper would bring the form and function of the filiform apparatus well into line.

Occurrence of more than one embryo-sac. The development of more than one megaspore of the tetrad was observed in the ovules of some cleistogamous flowers fixed in the spring of 1927. In every case one functional eight-nucleate sac had developed from the chalazal megaspore. In some ovules one extra megaspore had reached the binucleate or the tetranucleate stage, and there were three instances in which two extra megaspores had developed as far as the tetranucleate stage.

Fertilization.

Entry of the pollen-tube. When the pollen-tube reaches the end of the stylar canal it either grows down against the inner surface of the ovary wall or grows out into and traverses the ovary chamber. The entry of the pollen-tube into the embryo-sac is similar to that described for V. odorata. In V. Riviniana the pollen-tube grows right through the filiform apparatus of one synergid, there becoming very much constricted, and it seems to continue for some distance into the synergid cell, but does not apparently grow right through it. The contents of the synergid are disorganized by this invasion, and appear merely as a deeply stainable mass. The other synergid remains intact until after fertilization.

In the case of some cleistogamous flowers whose embryo-sacs had not been entered by a pollen-tube, the nucleus in one synergid had degenerated, while that of the other synergid was quite normal. This degeneration is interesting in view of the part played by one synergid in the reception of the pollen-tube. It suggests that there may be a physiological difference between the two synergids: that the pollen-tube enters a particular one, and if no pollen-tube comes, the degeneration nevertheless occurs as a matter of course.

Sexual and triple fusion. It is probable that the end of the pollen-tube dissolves, and the contents flow out through the base of the synergid into the embryo-sac. Much deeply stainable material is carried into the embryo-sac, and this surrounds the ovum; it disappears quite soon. In spite of repeated efforts to demonstrate the first stages in fertilization, the earliest stage observed was that at which nuclear fusion had already begun. The
passage of the male gametes within the embryo-sac must therefore be very rapid. No changes in the form of the male nuclei after liberation from the pollen-tube such as those described for *V. odorata* were observed in this species. The male nuclei in the embryo-sac do not contain any stainable reticulum, and are apparently in a resting condition. They are quite different in structure from the reticulate male nuclei observed in the pollen-tube within the ovary, but it is not known when the change takes place. The first male nucleus is found flattened against the ovum nucleus on the side opposite to the destroyed synergid. The larger nucleolus of the ovum and the nucleolus of the male nucleus remain distinct for some time after the nuclear membranes have fused. The polar nuclei are still separate, and the second male nucleus fuses with one of these. This male nucleus is represented almost entirely by its nucleolus, which is at first distinct from the nucleolus of the polar nucleus, but later forms one large oval fusion nucleolus with it. It was not observed whether a single definitive endosperm nucleus is formed.

The above account rather suggests that the male gametes are represented only by their nuclei at fertilization, and so it is of interest to note that in a few cases a small stainable body appears close to the first male nucleus at sexual fusion. This body recalls the stainable 'dot' in the 'tail' of the male cell, and although the cytoplasmic sheath was not seen within the embryo-sac, it suggests that the first male nucleus is accompanied by some of the cytoplasm, and there is also the possibility that this takes part in the fusion.

A cellulose membrane is secreted around the fertilized egg, which does not divide until after four or eight free endosperm nuclei are formed. The later development within the embryo-sac shows no particular points of interest.

**DISCUSSION.**

*The bivalent chromosomes.* The linking between the bivalent chromosomes during the meiotic divisions in *V. Riviniana* is a feature not previously recorded in plant cytology, and a consideration of the possibilities offered by this linking, though necessarily tentative, may not be without value. The only reference, of which the writer is aware, to such a linking of bivalent chromosomes, is made by Clausen (2) in describing an illustration of a heterotype metaphase figure in the pollen mother-cells of *V. arvensis*. The chromosomes are scattered over the spindle and are connected by threads, and Clausen says that 'the chromosomes seem to have difficulty in separating'. In a later paper (4) he states that the twenty bivalent chromosomes of *V. Riviniana* lie far apart at heterotype metaphase and are easily counted. The formation of groups of linked chromosomes during the heterotype division is recorded for *Oenothera* spp. by Cleland (5, 6) and by Sheffield (19). In these species of *Oenothera*, however, there is no true
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Diakinesis stage during which pairing of homologous maternal and paternal chromosomes takes place. The chromosomes formed from the univalent spireme which emerges from synapsis do not all form pairs, but all or some are linked by fine threads in a chain or a ring of fourteen or of a smaller number. The arrangement of the chromosomes seems to be constant for any one species. The chromosomes are still linked at heterotype metaphase, and in anaphase adjacent chromosomes pass to different poles and thus the separation of the homologous univalents is brought about. In *V. Riviniana*, however, the linking is of the bivalent chromosomes and does not replace diakinesis, so that it is essentially different from the linking in *Oenothera*. Three possible explanations suggest themselves. Firstly, the occurrence of linking in early diakinesis suggests that the chromosomes are not separated in the last premeiotic resting nucleus: that they, or their representatives are already arranged in a certain order in the spireme and that by this linking the particular arrangement is maintained throughout meiosis. The linking in *Oenothera* shows that the univalent chromosomes are already in order in the post-synaptic spireme. Secondly, if the bivalent chromosomes are linked together as soon as they are formed, or possibly before, and the linking persists throughout the meiotic division, the independent assortment of the chromosomes, giving all the possible combinations in the pollen-grains and megaspores, is necessarily very much limited. Such an effect of the linking would be comparable with, but probably much more comprehensive than the linking of the genes, since presumably, entire chromosomes pass linked together into the megaspores and microspores. Thirdly, in some cases the linking is so complete as to amount almost to a fusion between some of the bivalent chromosomes, and here there certainly seems to be an opportunity for interchange of material, possibly genetical, between the ends of adjacent chromosomes. A process analogous to 'crossing over' between homologous chromosomes is suggested here. In view of all the evidence now supporting the individuality of the chromosomes, and in particular the construction of chromosome 'maps', this suggestion is a little bold; nevertheless, the possibility exists. It must always be remembered that in *V. Riviniana* much variability is seen in the degree of this linking, and this would seem to impose serious limitations upon any theoretical application which it suggests. Further evidence for the existence of this linking phase would be welcome, and it would be of considerable interest to investigate other species of Viola with this end in view.

The male gametes. The male nuclei in *V. Riviniana* never become vermiciform, and there is therefore nothing to suggest that they are capable of movement. At the same time, the form of the whole male cell certainly indicates motility and the appearance of a small deeply stainable body at one pole of the resting generative nucleus suggests, superficially at least, a
West.—Cleistogamy in Viola Riviniana with comparison with the blepharoplast of antherozoids. A similar deeply staining granule is described in the generative cell of Lilium auratum, and it is suggested by Welsford (20) that this granule may be a centrosome, but no spindle fibres were seen near it. Other granules occur in the cytoplasm of the generative cell of Lilium auratum between the nucleus and the wall. These are extruded from the nucleus, sometimes forming a band-like structure, and are considered to be vestiges of a blepharoplast. The later history of the body in the generative cell of V. Riviniana is not known. So far it has not been seen at the pole of the spindle, so that it may not function as a centrosome. Nevertheless, it is probable that this body fragments and gives rise to the granules which appear on the spindle at telophase and are included in the cytoplasm. The presence of a deeply stainable body near the first male nucleus at sexual fusion is noted in the account of fertilization.

Crystal bodies in the nucleolus. The presence of crystal bodies in the nucleolus is recorded in different parts of plants and at various stages of development. For instance, crystalline structures are present in the nuclei of the root tip-cells of Galtonia candicans (8); these are not within the nucleolus, but are thought to originate from this. In Allium cepa (18) regularly shaped, highly refractive bodies occur in the resting phase nucleoli: they are apparently absorbed during spireme formation. The nucleolus of the resting nuclei of Anacyclus Pyrethrum (17) frequently contain a refractive body, crystalline in structure. Similar crystals are present regularly in the premeiotic pollen mother and tapetal cells of Lathyrus odoratus (19), and with varying frequency in the pollen mother-cells of Oenothera spp. (13), of Lathraea clandestina and L. squamaria (10), and are recorded in the endosperm nuclei of Macrozamia Fraseri before free nuclear division (15). To these instances the premeiotic pollen mother-cells, tapetal-cells, and nuclei of the developing and mature embryo-sac of V. Riviniana may now be added, although the crystals are not always observed. It is suggested by Gates and Latter (10) that the constant occurrence of these crystals in cells entering upon an increased period of activity indicates that they are associated in some way with the metabolism of the cells. This is supported by the fact that crystals occur in those cells of V. Riviniana which are entering upon either of the two cytological crises in the life history of the plant, viz., reduction division and nuclear fusion.

The nucleolar body. The close association of the spireme with the nucleolus during the meiotic prophases in which the spireme is formed is now recognized in several plants, and the conclusion is very naturally drawn that the nucleolus holds a source of chromatin material which is supplied to the spireme. The presence of a special nucleolar body with which the connexion is maintained was first described by Latter in the pollen mother-cells of L. odoratus, who suggests that this body elaborates chromatin
from prochromatin material within the nucleolus and then transfers the chromatin to the spireme. The term 'nucleolar body' is adopted in the present account of the pollen mother- and megaspore mother-cells of *V. Riviniana* since, from its position, staining properties and apparent function, it is quite similar to that in *Lathyrus*. The origin of this body in *V. Riviniana* is, however, unknown. Since crystal bodies are not frequently seen in the resting pollen mother-cell nucleoli it seems doubtful whether it originates from one of these, as it probably does in *Lathyrus*.

**Summary.**

**Morphology.**

1. The general morphological features of the chasmogamous, semi-cleistogamous, and cleistogamous flowers of *Viola Riviniana* are described, and the annual cycle of flower production is given.

2. The semi-cleistogamous flowers seem to show, in a series of forms, a gradual reduction from chasmogamy to cleistogamy. These flowers exhibit the following features: (a) reduction of petals and closure of flower, (b) reduction of pollen-sacs, (c) germination of the pollen within the anther, and (d) the curvature of the style over towards the anterior stamens.

3. The cleistogamous flowers show extreme reduction of pollen-sacs, and the style is curved so that the stigma is almost in contact with the anterior stamen sacs.

4. Two kinds of pollen-grain are found in the anthers of chasmogamous flowers: many large grains, which are shed, and a few smaller grains which germinate within the anther. The latter are similar in size and form to the grains of cleistogamous flowers.

5. The chasmogamous flowers often fail to set seed; the cleistogamous flowers set good seed, except perhaps towards the end of the season.

**Cytology.**

6. The haploid number of chromosomes of *V. Riviniana* is 20.

7. Pollen meiosis. During first contraction, open spireme and second contraction, the spireme is connected to the nucleolus by a thread or threads, passing to the nucleolar body.

8. In early diakinesis and throughout the later stages of the meiotic division the bivalent chromosomes are linked into groups.

9. Pollen tetrad formation takes place by the grooving of the pollen mother-cell cytoplasm and accompanying ingrowth of wall material from the thick pollen mother-cell wall. This disappears later, and a wall is secreted around each pollen-grain.

10. Two male cells are formed at spermatogenesis. Each contains a spherical reticulate nucleus and one or two chromatin bodies in the cytoplasm of the 'tail'.
West.—Cleistogamy in Viola Riviniana with

11. Crystal bodies frequently occur in the nucleoli of the newly differentiated, uninucleate tapetal cells. During pollen development the tapetal cells become multinucleate.

12. Degeneration of the pollen in cleistogamous flowers may set in at any stage of development.

13. The eight-nucleate embryo-sac is normal in development and in construction. The synergids have a well-developed filiform apparatus. The polars retain their individuality until after fertilization.

14. The pollen-tube enters one synergid through the filiform apparatus. The two male gametes and some stainable material from the pollen-tube are liberated through the base of the synergid into the embryo-sac.

15. The second male nucleus fuses with one polar nucleus, and this fusion nucleus remains distinct from the second polar nucleus for some time.

16. Cases of the development of extra immature embryo-sacs in some ovules are recorded.

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DESCRIPTION OF PLATES XI AND XII.
Illustrating Miss G. West's paper on Cleistogamy in *P. Riviniana*, with special reference to its Cytological Aspects.


PLATE XI.
Fig. 1. A resting pollen mother-cell; the nucleolus contains a small crystal body and a small vacuole; the reticulum is very slight. A.B.; I.G.V.
Fig. 2. First contraction (synizesis). The contraction knot is connected to the nucleolus by delicate threads; the nucleolar body appears as a small deeply stainable projection on the surface of the nucleolus. A.B.; H.
Fig. 3. Open spireme. The spireme is scattered throughout the nucleus, and is connected by one, or possibly more, delicate threads to the nucleolar body. F.W.; G.V.
Fig. 4. Part only of a nucleus showing an early stage in the formation of bivalent chromosomes. The paired chromosome initialis appear as small stainable areas in a rather thick granular thread. The spireme is much attenuated, except where chromosome formation is taking place, and the connecting threads are scarcely seen. F.W.; G.V.
Fig. 5. Early diakinesis. The univalent halves of the twenty bivalent chromosomes are quite distinct; faint linear continuations of the chromosomes are seen, and delicate threads apparently still connecting some of the bivalents. F.W.; G.V.
Fig. 6. Later diakinesis. Faint links threads still appear between the bivalent chromosomes, which are somewhat larger than in Fig. 5. The nucleolus, much smaller than in Figs. 4 and 5, is beginning to disappear. F.W.; G.V.
Fig. 7. Late diakinesis. The nucleolus appears merely as a small faintly stainable area in the nucleus. Many of the bivalents are now compact, although the double nature is still seen in a few. The twenty chromosomes are seen, and some are already linked. F.W.; G.V.
Fig. 8. Intranuclear spindle formation. The spindle fibres diverge from one point within the nucleus; the nuclear membrane is still present on the opposite side of the nucleus. A.B.; H.
Figs. 9-12. Pollen grain development. Pollen-grain wall formation.
Fig. 9. Beginning of grooving of cytoplasm and ingrowth of thick pollen mother cell-wall. The
spindle fibres have almost disappeared, and indications of evanescent cell-plates are seen in the cytoplasm. A.B.; H.

Fig. 10. The ingrowing walls have almost reached the centre. A.B.; H.

Fig. 11. Tetrad formation completed; each microspore has a delicate wall of its own; the thick tetrad wall is dissolving. A.B.; I.G.V.

Fig. 12. Three pollen-grains of a tetrad still connected by a few traces of the old pollen mother-cell wall. A.B.; I.G.V.


Fig. 13. Early open spireme. The nucleus has become spherical, and the spireme is apparently connected to it. Chromatin transference seems to be in progress, and the chromatin appears to have accumulated at some points on the thread. F.W.; G.V.

Fig. 14. The nucleus of a megaspore mother-cell showing the connexion of threads to the deeply stained area which is probably of the same nature as the nucleolar body in the pollen mother-cell. M.C.; G.V.

Fig. 15. Diakinesis showing the twenty bivalent chromosomes. The halves of the chromosomes are very clear and show the un stainable continuations, which are probably of lim. A.B.; H.

PLATE XII.

Figs. 16-21. Spermatogenesis.

Fig. 16. Generative cell before division. The chromatin body is seen at one pole of the nucleus. A.B.; I.G.V.

Fig. 17. Metaphase, profile view, of division of generative nucleus. A.B.; I.G.V.

Fig. 18. Telophase of division of generative nucleus; the division figure occupies almost the whole length of the cell. The nuclei show a stainable reticulum, and a cell-plate is formed at the equator. The chromatin bodies, possibly formed by the fragmentation of the large one seen in Fig. 16, are seen upon the spindle. A.B.; I.G.V.

Fig. 19. Formation of the male cells by constriction of the protoplasm of the generative cell on either side of the cell-plate. The spindle fibres have disappeared; the bending of the cell shown is not usual; it may be due in this case to the limits imposed by the pollen-grain wall upon the elongation of the cell. The chromatin bodies are only faintly shown in this pollen-grain. A.B.; I.G.V.

Fig. 20. Part of a pollen-tube in the anther sac—showing the two male cells and the tube-nucleus. F.S.; H.

Fig. 21. Part of a pollen-tube in the ovary, showing the two male cells and the tube-nucleus. This preparation is much de-stained, which may account for the apparent absence of chromatin bodies. F.W.; H.


Fig. 22. Surface view of the vertices of the synergids in tangential longitudinal section, showing the well developed filiform apparatus. The ends of the pores or canals appear as small clear areas in the stained matrix. A.B.; I.G.V.

Fig. 23. Filiform apparatus showing stringy prolongations extending into the synergid cytoplasm. F.W.; I.G.V.

Fig. 24. Longitudinal section of the filiform apparatus. The preparation suggests that the apparatus is composed of definite tubes. A.B.; I.G.V.

Fig. 25. Filiform apparatus after treatment with cuprammonia, and subsequently stained; it seems to have been changed into a mass of rods which are stainable. A superficial view is drawn, the filiform apparatus of one synergid only appears in the section. A.B.; I.G.V.

Fig. 26. Synergids showing cytoplasmic inclusions, some of which are of a highly refractive nature. F.W.; H.

Figs. 27-32. Fertilization.

Fig. 27. Composite drawing from sections through one embryo-sac. The sac has been entered by a pollen-tube, and the destroyed synergid is drawn merely in outline; the other is quite healthy. Some deeply stained material from the pollen-tube surrounds the egg-cell. The first male nucleus appears near the egg-nucleus, and the second male nucleus is pressed against one polar nucleus. A small chromatin body is seen near the second male nucleus, and a more faintly stained one near the first male nucleus. A.B.; H.
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Fig. 28. An active male nucleus with a reticulum appears within the egg-cell: the nucleolus of the egg-nucleus contains a crystal. A.B.; I.G.V.

Fig. 29. Fusion of the first male and egg nuclei; the male is flattened against the female nucleus. The preparation shows much stainable material around the egg-cell. A.B.; H.

Fig. 30. After nuclear fusion; the male nucleolus is seen within the nuclear area of the egg-cell. A.B.; H.

Fig. 31. The two polars and the nucleolus of the second male before fusion of nucleoli; the male nucleolus is within one polar nucleus. Figs. 29 and 31 are taken from sections through the same embryo-sac. A.B.; H.

Fig. 32. The lower larger nucleus is the one formed by the fusion of one polar and a male nucleus; the other polar nucleus is still distinct. The nucleoli are very vacuolate. A.A.; H.