15. Reproductive Structures of Vascular Plants

The preparation of vegetative organs of the highly diverse members of the phylum Tracheophyta, the vascular plants, is discussed in a separate chapter because such organs require similar techniques (Chap. 13). Similarly, the reproductive organs of the Tracheophyta present common problems of processing and staining and are therefore brought together in the present chapter. *Orders* as well as *Classes* ar used as major headings.

Lycopodiales

The organization of the strobilus of the club mosses should certainly be studied by dissection, and there is no point in embedding entire strobili. Ascertain the stage of sporogenesis in each cone by dissecting out a sporangium and crushing out the contents. Mature sporangia containing dry, hard, brittle spores should not be embedded unless a cytological study is to be made. Subdivide the cone transversely, and kill in *FAA,* medium chrome-acetic, or Craf III. Sections should be stained in safranin-fast green or iron hematoxylin.

The gametophytes of these plants are exceedingly rare, although they are said to occur in abundance in localized areas. Gametophytes may be purchased preserved in *FAA*. The soft thallus is easily sectioned in paraffin and stained.

Selaginellales

There is very little justification for making sections of the strobili of these plants because dissections under a binocular reveal so much more of the orderly organization of the cone. Dissected and crushed sporangia likewise present a three-dimensional picture that is lacking in sections. The study of nuclear details of sporogenesis and the development of gametophytes within the spores is a task for the experienced investigator.

Isoetales

Young sporangia of *Isoetes* arise on small sporophylls closely appressed to the rhizophore. Dissect away the sporophylls under a binocular, trim off much of the sporophyll, and kill the sporangia in medium chrome-acetic or *FAA*.

The large sporophylls and mature sporangia are inadequately represented by sections. To section mature spores, cut the sporangia away from the sporophylls, drop the ruptured sporangia into a centrifuging tube of *FAA*. Process in butyl alcohol, centrifuging the mass after each change. Much tearing of the spore wall can be expected during sectioning.

The preparation of gametophytes should be undertaken only after a study of research literature in which methods are given for germinating and processing the material.

Equisetales

Equisetum cones differentiate underground during the late summer and contain mature spores when they emerge from the ground the following spring. Young strobili should be dissected away from the rhizome, thoroughly washed, divided into several pieces, and killed in medium chrome-acetic or *FAA*. Both transverse and longitudinal sections should be made. There is little excuse for sectioning strobili containing mature spores. Compared with a dissection under a binocular, a section presents an utterly inadequate picture of the interesting organization of the cone. Mature spores should be studied in a wet mount, which is subsequently uncovered and permitted to dry, bringing about the uncoiling of the elaters.

Gametophytes can be grown by sowing newly shed spores on sterilized sphagnum. Excellent preserved gametophytes also can be purchased. Embedding and sectioning are carried out as with other soft, delicate subjects.

Ophioglossales

Botrychium is the easiest member of this order to use for the study of reproduction. The sporophylls can be teased apart and crushed to determine the stage of sporogenesis. Subdivide the fertile frond into small pieces, and kill in medium chrome-acetic or Craf II. A wide variety of stains will produce brilliant preparations.

Collectors find gametophytes to be extremely abundant in localized areas during favorable seasons. Preserved gametophytes can be purchased and are easy to process.

Filicales

The position and construction of the sporogenous area or sorus and the character of the sporophyll differ in the numerous genera. *Asplenium nidus-avis,* the bird's nest fern, bears sori on the large, leathery, entire vegetative leaves, whereas *Onoclea struthiopteris,* the ostrich fern, bears the sporangia in the tightly infolded, pod-like pinnules of special fertile fronds.

The preparation of the diverse subjects is practically identical. Select young sori, and examine a dissected portion of a sorus, using stages up to and including young thin-walled spores. Excise small portions of leaf tissue bearing sori, and kill in medium chrome-acetic or Craf II. Species having soft leaves are more economically dehydrated in alcohol or acetone, but butyl alcohol is advisable for the tougher types. Stain in iron hematoxylin to obtain the best nuclear details and in safranin-fast green for general use. Do not waste time embedding mature sporangia. The contents of the sporangium, the construction of the annulus, and the character of the wall of the mature spore are shown far better in a wet mount of fresh or preserved material. Some of the cultivated ferns have a high ratio of shriveled, undeveloped spores in the mature sporangium; sections of such material are disappointing.

Gametophytes of native ferns can be found in great abundance by an experienced collector. Such materials are useful for gross study, but the presence of soil particles among the rhizoids makes sectioning difficult and unsatisfactory. Gametophytes can be grown on nutrient agar cultures, or on porous clay flowerpots in a moist chamber. Remove a few gametophytes for examination at intervals, kill desirable specimens in medium chrome-acetic or Craf I, and prepare whole mounts (Chap. 10) or embed very carefully for microtome sections. Iron hematoxylin and safranin-fast green yield beautiful preparations. There is no need to section thalli bearing sporophytes, and permanent whole mounts are not so desirable as wet mounts that can be handled and viewed from all angles.

Gymnospermae

Members of the common genera of the Coniferales are well-known trees of great economic importance, and abundant material is easily available. The life history of the pine is probably the most widely used subject, therefore, the present discussion will be centered around reproduction in the pine. The reader should consult Chamberlain

(1935) for the morphology and seasonal sequence of the reproductive cycle in other genera and orders, and adapt the methods described here to other subjects.

Staminate cones of *Pinus* are differentiated during the season prior to the shedding of pollen. Cones can be dissected from buds and the stage of microsporogenesis ascertained by means of acetocarmine smears. Several species of *Pinus* undergo meiosis early in May, in the Chicago region. Killing fluids do not penetrate readily into large masses of highly resinous tissues. It is therefore necessary to subdivide all but the very smallest cones. Kill in *FAA* for general morphological studies and in a Nawaschin type, such as Craf II, for more critical details. Nuclei of microspores and mature pollen grains are stained adequately in hemalum-erythrosin. For the first gametophytic somatic mitosis, which takes place in the microspores before they are shed, use iron hematoxylin or safranin-fast green.

Preparations of the ovule history are much more difficult and time-consuming to make than the pollen history. The time of occurrence of interesting and important states varies with the species, the locality, and probably in a given locality in accordance with the weather conditions. In the Chicago area the megasporocyte of *Pinus laricio* is evident when the cones emerge from the bud. Fertilization has been found toward the end of June, while early embryo stages are obtainable during July (Chamberlain 1935).

The deep-seated megasporocyte is not reached readily by killing fluids, necessitating the use of vigorous fluids that produce distortion. The very young cones may be fixed entire in strong chrome-acetic, *FAA*, or *FAA*–bichloride of mercury. Such preparations are of interest principally to the student of developmental morphology. It may be preferable to cut away the young ovules from the sporophyll and strive to preserve the sporogenous and gametophytic features. Strong chrome-acetic seems to have given the best results for most students of this group. The Nawaschin modifications and Allen-Bouin modifications deserve further study.

Staining of ovulate structures is particularly difficult. Resinous materials in the cells tend to make the preparations unsightly, although the essential nuclei may be clearly differentiated. Safranin-fast green meets the requirements for all but research needs.

After the first few divisions of the zygote, microtome sections are no longer adequate for the study of embryology. The development of dissection methods has facilitated great progress in such studies.

A detailed discussion of the morphology and techniques applicable to other orders of gymnosperms is given by Johansen (1940).

Angiospermae

The angiosperms are usually the central feature of the study of reproduction in plants, representing the climax in the development of reproductive organs. Floral types and features of floral organs are studied best by dissection and whole mounts of fresh or preserved material.

THE FLOWER

Microtome sections are indispensable for the study of vasculation and histogenesis of floral organs. Each species is virtually a problem in itself; therefore, this discussion will be limited to the methods used for the successful preparation of a few useful subjects. Buds of lily and tulip are among the most satisfactory subjects for entire flower buds. The very young buds are large and easy to handle. Embedded buds can be accurately oriented for sectioning, and the parts are so large that elementary students can locate and recognize the parts on the slide. Lily buds are available over a considerable period, beginning with *Lilium umbellatum* and *L. elegans* in May, to *L. tigrinum* in August. Well-developed floral parts are shown in buds that are less than 5 mm. long (Fig. 15.4). Cut off at the base of the perianth, and remove successive slices from the tip until the tips of the anthers have been cut off. Drop into the killing fluid and pump vigorously. Fair fixation is obtained in *FAA,* but superior results are obtainable with Allen-Bouin II. Sectioning and staining are delightfully easy. Begin sectioning at the base of the flower, discard the ribbon until the sections include anthers and ovary, and discard the block when ovules are no longer present in the apical portion of the ovary.

Buds of tulip for entire sections of young flower buds are obtained from bulbs during late fall. Many varieties of Darwin tulips are in a suitable stage from mid-October to early November. Meiosis was found to occur in several Darwin varieties in October. Kill in Allen-Bouin II and carry through an alcohol-xylene, dioxan, or butanol series for entire young flower buds; for an older ovary follow the recommendations for the lily. Cut open the bulb, and dissect out the complete flower bud. Trim and kill as with lily.

Matthiola, the common garden stock or gillyflower, furnishes a suitable dicotyledonous flower for complete sections. Remove indi-

vidual flowers, trim the end of the closed perianth, and kill in *FAA* for gross study or in the fluids recommended for lily. Flowers of tomato also are excellent for advanced workers.

THE ANTHER AND OVARY

Microsporogenesis can be studied satisfactorily in the lily. The structure of the anther and sporogenous tissues are also shown well (Fig. 15.2). Whether meiosis in the anther is demonstrated with microtome sections or smears depends on facilities for the production of enough slides for class use. Slides of adequate quality for elementary classes can be produced in quantity by sectioning (Fig. 15.3 *a*), but smears are far superior for critical details (Fig. 15.3 *b*). For elementary work, the essential and more obvious features of meiosis can be demonstrated with paraffin sections from a series of anthers beginning with anthers 2 mm. long up to anthers that are just beginning to show color. Ascertain the stage by means of acetocarmine smears and handle the successive age classes in separate bottles. This saves much time in locating desired stages for sectioning. Subdivide young premeiotic anthers transversely into pieces not over 2 mm. long (Fig. 15.1 *A, B*). The excellence of fixation is influenced by the degree of subdivision. Good fixation can be obtained by slicing anthers into disks less than 1 mm. thick while holding them under the killing fluid, Allen-Bouin II. This fluid preserves the sporocytes and meiotic chromosomes well enough for elementary teaching (Figs. 15.2, 15.3 *a*). The anther pieces cannot be cut much shorter than 2 to 3 mm. because the sporocytes are loose in the anther at this stage. The chromosomes are superbly stained by iron hematoxylin, gentian violet-iodine, and safranin-fast green.

The advanced worker who wishes to demonstrate the intimate structure of the chromosome during meiosis should explore the rapidly expanding literature on smear methods, select a species on which to work, and strive to perfect his technique until he can demonstrate the structures described by investigators of the subject (Fig. 15.3 *b*).

The dyad condition and second or equational division are of very short duration in lily, and will be found in material selected and prepared by the foregoing methods. The quartet (tetrad) and microspore stages are of long duration, present during the long period of expansion of the flower bud, until the anthers begin to color. For general purposes it is adequate to kill the entire anther; *FAA* yields surprisingly good results. Test each species by means of whole mounts before making a collection for this stage. Many cultivated lilies,

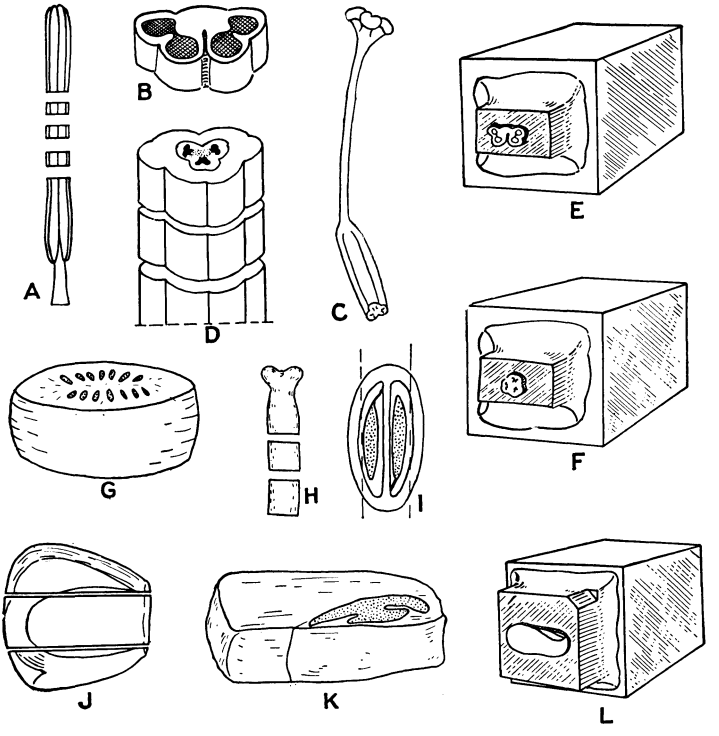

FIG. 15.1—Subdividing of reproductive organs: *A* and *B*, anther of lily; *C* and *D*, ovary of lily; *E* and *F*, mounted embedded blocks of anther and ovary, respectively; *G*, transverse disk sliced from young fruit of small-fruited variety of tomato; *H* and *I*, silique of *Matthiola; J*, kernel of corn sliced longitudinally; *K*, center piece of kernel containing essential parts of embryo; *L*, embedded kernel mounted for sectioning longitudinally. Trimmed edge of paraffin block produces a notched ribbon as in Fig. 6.4*A*.

especially the Easter lilies as well as *L. speciosum* and *L. umbellatum,* have extremely high pollen sterility, and the finished preparations show both nicely preserved pollen grains and shriveled microspores. However, such preparations are useful for illustrating pollen abortion. *Lilium regale, L. tenuifolium,* and *L. tigrinum* are particularly recommended for the study of pollen formation. The first two species have a high ratio of normal pollen, whereas only some strains of the last species are satisfactory.

For more critical fixation of microspore and pollen nuclei than is afforded by *FAA,* use the methods recommended for prophases. The somatic division of the microspore nucleus occurs over a brief

period and is seldom encountered. The monoploid (haploid) chromosome complement is interesting and deserves careful staining when found.

Lily ovary is by far the most commonly used subject for teaching the development of the ovule and female gametophyte. The objection to lily is that the nuclear history of the embryo sac differs from the condition in corn, the legumes, and other common crop plants. However, lily ovary and its parts are large, the parallel seriation of the numerous ovules makes sectioning productive, and slides of the earlier stages, up to quartet formation, can be made economically in quantities (Figs. 11.4, 15.4, 15.5). Chrome-acetic has long been a favorite fluid for this subject, and formulas 0.5–0.5 and 0.3–0.70 are excellent for the smaller sporocytes (Fig. 11.3), but the results are rather uncertain with fully expanded sporocytes and subsequent stages. Bouin's solution has been used extensively, but the results are extremely variable. Figure 11.4 *b* shows a typical Bouin image that is all too common. The rims of the integuments often show a highly wrinkled and collapsed condition. The condition of the sporocyte and integuments after embedding can be determined accurately in a melted strip of paraffin ribbon. The proportions of ingredients in the original Bouin formula have been rather rigidly accepted by most users, but it is not improbable that superior results could be obtained with carefully determined variants of the formula. The author has obtained some excellent results by using propionic instead of acetic acid as suggested by Johansen (1940). The quality of the fixation is improved if the perfectly fresh ovaries are cut into thin disks.

The most consistent results for all stages have been obtained with the Allen-Bouin modifications, especially II and III (Table 3.2). A closely graded alcohol-xylene or acetone-xylene series can produce excellent results (Fig. 11.4 *d*), but failures are frequent. The glycerin-evaporation method, the dioxan series or *TBA* are much more reliable. The contents of the mature embryo sac are apparently highly fluid and particularly difficult to preserve without excessive plasmolysis, but Allen-Bouin II usually yields adequate fixation.

Staining sections of young ovaries prior to meiosis is one of the easiest tasks. A simple hemalum stain with or without erythrosin is adequate for elementary classwork. Iron hematoxylin and safranin-fast green yield brilliant preparations. The meiotic and gametophytic division figures and nuclei should be stained with iron hematoxylin, safranin-fast green or safranin-gentian violet. The last combination and the triple stain show the spindle fibers exceptionally well.

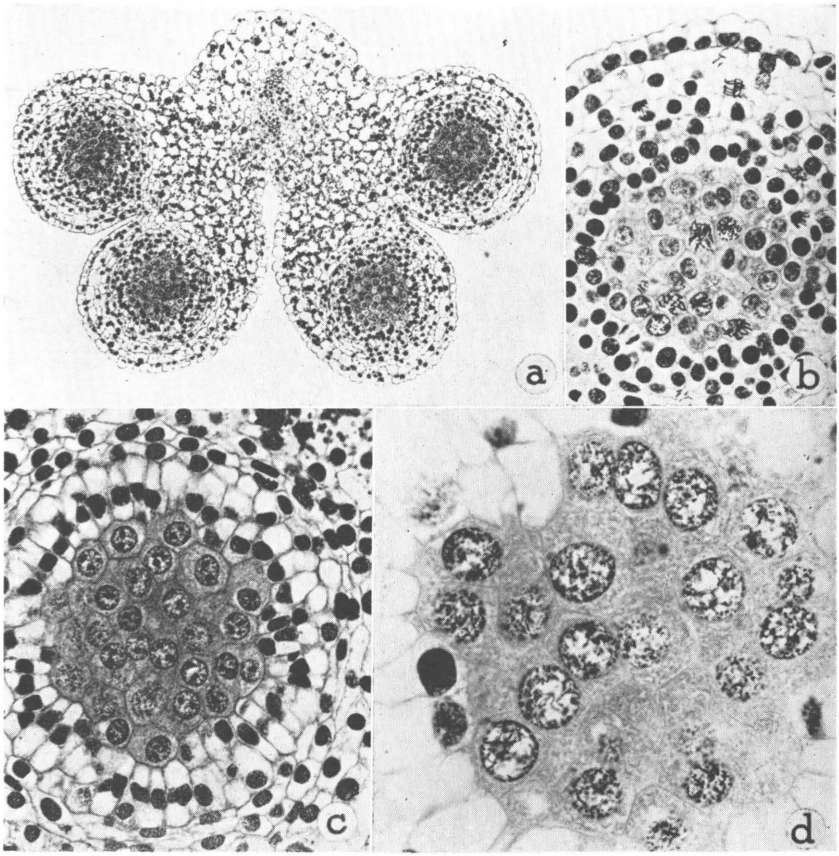

FIG. 15.2—*a,* Transverse section of anther of *Lilium regale; b,* somatic divisions in developing archesporium; *c,* archesporium, surrounded by differentiating tapetum; *d,* sporocytes in pre-leptotene phase.

The manufacture of lily ovary slides showing the seven-to-eight-nucleate stage is unproductive and expensive. Most of the slides obtained from a ribbon show incomplete embryo sacs. Cutting an ovule longitudinally through the center and having all the nuclei in one section is a matter of chance. Commercial manufacturers have a sales outlet for slides having incomplete sacs and can therefore sell the few choice slides having complete sacs at reasonable cost. For routine teaching, with its attendant breakage of slides, it may be more satisfactory to purchase slides of the seven-to-eight-nucleate stage than to make them. Good fixation has been obtained with fair regularity with Allen-Bouin II and *n*-butyl alcohol dehydration.

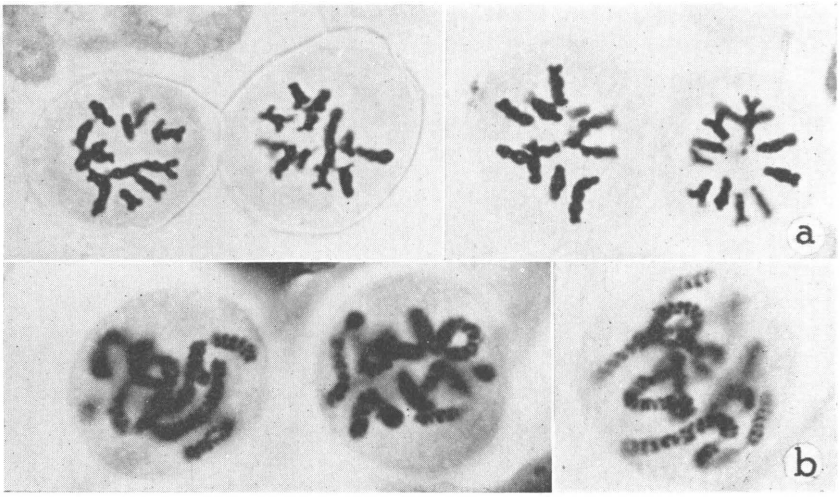

FIG. 15.3—*a, Lilium regale,* sectioned in paraffin, first division of meiosis in microsporocytes; *b,* smeared microsporocytes of *Tradescantia bracteata,* Sax-Humphrey method, iron hematoxylin.

Lilium represents a type of embryo-sac history that differs from the type found in many of our important crop plants. Slides of lily ovary are relatively easy and inexpensive to prepare. This plant should be used to show the transverse floral diagram of the flower bud (Fig. 17.4 *a*); the carpellary organization of the ovary (Fig.

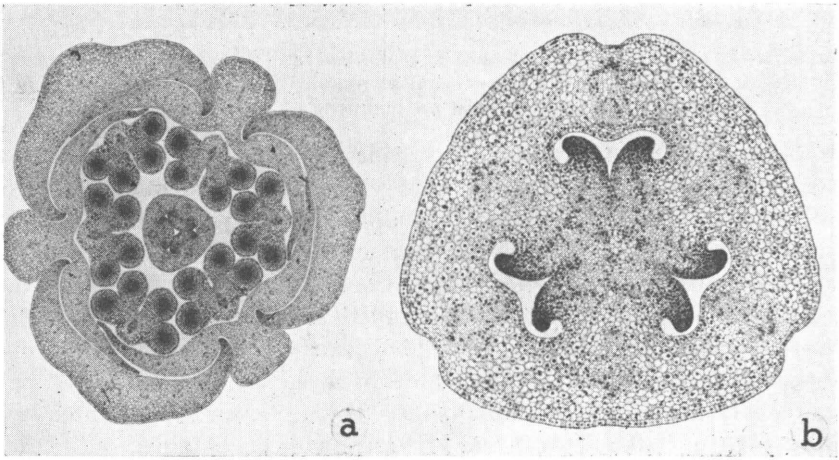

FIG. 15.4—*a,* Transverse section of flower bud of *Lilium regale; b,* ovary of same.

15.4 *b*); the origin and development of the ovule and integuments (Fig. 11.3); the origin and enlargement of the megasporocyte (Fig. 11.3); meiosis, (Fig. 11.4) and the four megaspores in the unpartitioned embryo sac (Fig. 15.5). It is of interest that the embryo sac of *Lilium pardalinum* is narrow and the megaspores are in linear order, whereas *L. umbellatum* has a broad embryo sac and a cruciate quartet (Fig. 15.5). The so-called normal type, which might better be named the common type, involves the formation of a quartet of megaspores, three of which degenerate, the fourth giving rise to the female gametophyte. This type occurs in maize, the legumes, tomato, and many other economic plants. The preparation of each of these is virtually a research task, and the reader who wishes to work on any of these plants should survey the literature on the desired plant.

Lilium is a good subject for making preparations showing fertilization (Fig. 15.6). Begin collecting 48 hr. after pollination and make collections every 12 hr. Use the killing fluids and methods recommended for the embryo sac. A series of collections will show stages from unfertilized mature embryo sac to young embryos (Fig. 15.6 *a*).

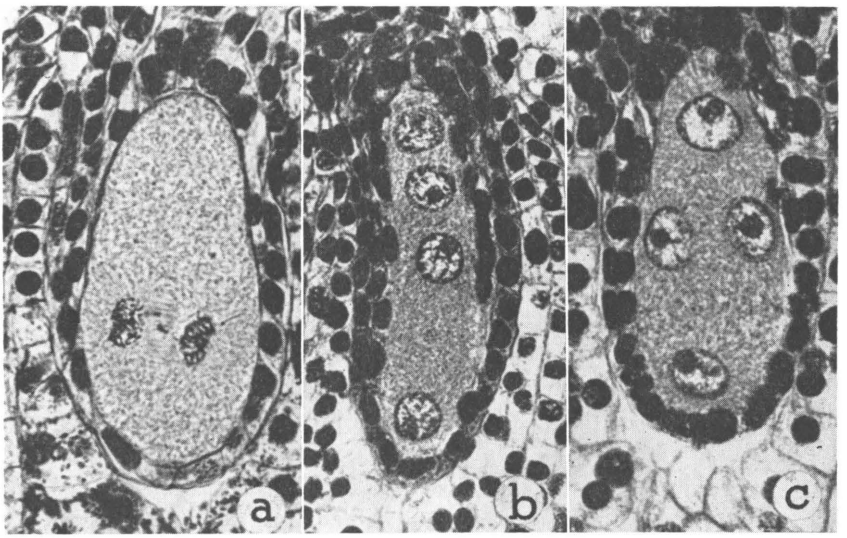

FIG. 15.5—*a*, First binucleate stage in embryo sac of *Lilium tigrinum*. Note the lagging chromosomes; *b*, linear megaspores of *L. pardalinum*; *c*, cruciate arrangement of megaspores of *L. umbellatum*.

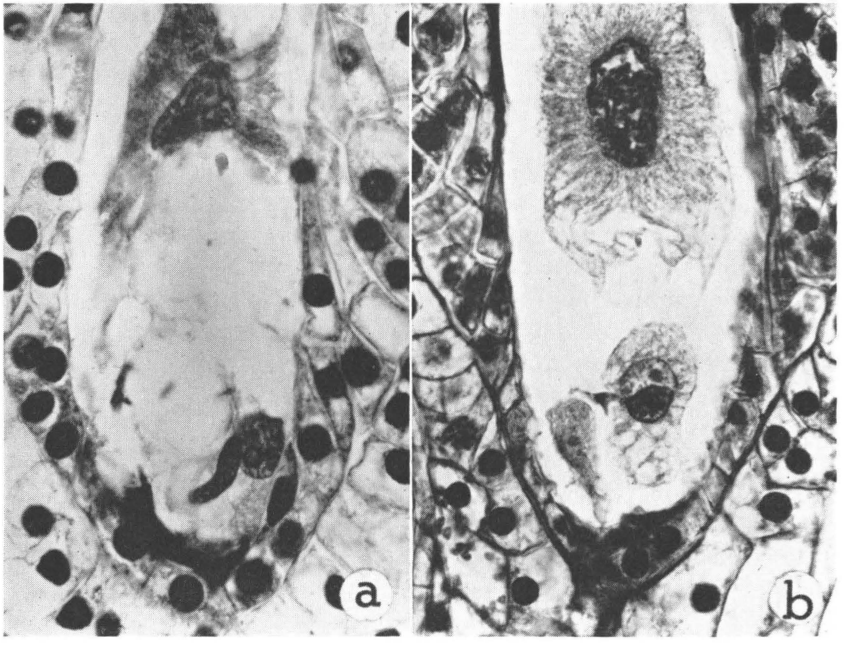

FIG. 15.6—Fertilization in *Lilium regale; a,* sperm and egg in contact; *b,* sperm appressed to egg, zygote wall evident.

THE EMBRYO, SEED, AND FRUIT

Embryology is usually neglected in elementary courses, in part because of the high cost of an adequate series of slides. Slides of early stages of embryo development are comparatively expensive to make, whereas the manufacture of slides of nearly mature embryos is more productive. The embryo of lily is large and not difficult to prepare. Use species that produce seed, such as *Lilium regale* or *L. tenuifolium.* Cut the ovaries into disks not over 2 mm. thick and divide longitudinally into three sectors, each sector containing one locule. Good fixation of the embryo can be obtained consistently with Allen-Bouin II, having the formaldehyde solution reduced to 5%. Section transversely at 15 to 18 μ. The flat developing seeds are in long tiers, and a block yields many good sections, all cut longitudinally with the axis of the embryo (Fig. 15.7). If six or eight sections are mounted on each slide, most of the slides will contain at least one accurately cut embryo. The most satisfactory stain is iron hematoxylin with a very light counterstain of fast green, which stains the cell walls of the embryo.

Slides of the caryopsis and embryo of maize are not difficult to make if the fundamentals outlined in the earlier chapters are observed. Consult the bulletins of agricultural colleges for the methods of making hand pollinations. Collect the ears at the desired intervals after pollination. Remove the husks carefully and trim away two rows of kernels without damaging the adjacent rows. With a thin, sharp scalpel cut off the intact kernels close to the cob and drop them into a Petri dish of water. Lay a kernel, with the germ upward, on a sheet of wet paper, remove chaff from the base and trim a longitudinal slice from each side of the kernel (Fig. 15.1 *J, K*). Also prepare some kernels for transverse sectioning by removing the basal and stylar portions of the kernel, saving only the portion from the tip of the coleoptile to the tip of the radicle. After the embryo is 25 days old, better infiltration of pieces for transverse sections can be obtained by transversely bisecting the embryo at the scutellar node, as well as removing the basal and stylar regions as above.

The essential morphological structures of the kernel are well developed in 25 to 30 days, and the pericarp becomes hard and brittle in 30 to 40 days. It is usually unnecessary to section the entire caryopsis after these dates. The embryo can be extracted easily between the 15th and 40th day, or until the kernel becomes so hard that the embryo is fractured if an attempt is made to dissect it out.

After the kernel has undergone maximum natural drying, or even if the kernel has been artificially dried for storage, the embryo can

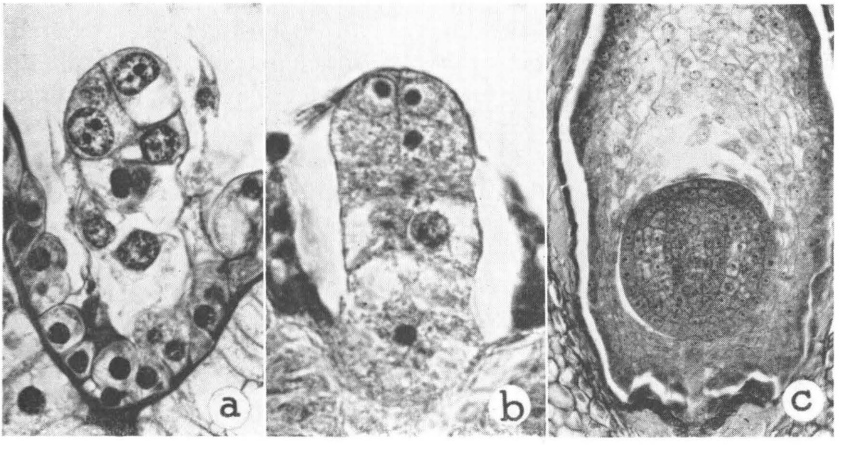

FIG. 15.7—*a,* Embryo of *Lilium regale; b,* embryo of *Lotus corniculatus* (courtesy of Dr. Harold W. Hansen); *c,* embryo of *Lycopersicum esculentum.*

be extracted. Soak the kernel in a solution that is based on the steeping liquor of corn processing plants. The solution used at present contains 2% sodium sulfite and 2% lactic acid. It is necessary to test the time and temperature factors with each lot of grain. Try 20° and 35°C., and intervals of 1 to 3 days. When the germ can be loosened easily, trim away unessential parts of the germ, subdivide if desired, and drop into the fixing fluid.

Small kernels, up to 10 days after pollination, are well fixed in Craf I or II. Older kernels and extracted embryos are penetrated better by Craf III. Xylene is the poorest solvent for infiltration, and chloroform is satisfactory only to the 15th day. Thereafter, a dioxan-normal or dioxan-tertiary butyl alcohol series makes possible the sectioning of 40-day kernels in paraffin (Sass 1945). (See frontispiece).

Embedded kernels must be soaked in warm water before sectioning. Adhesion of sections to the slide requires careful flattening of the ribbon, without overheating. For some research problems, hemalum alone permits adequate diagnostic observation. A safranin-fast green stain is attractive, and for exhibition purposes it is possible to make gaudy multiple stain preparations. Ages of kernels are given in the legends of figures 15.8 and 15.9.

Capsella bursa-pastoris is a favorite subject for embryology. The siliques are soft and easy to section. Although the seeds lie in the locules at various angles, seeds are so abundant that almost every section has complete embryos. Remove the fruits from the inflorescence, and classify them roughly into age groups in accordance with their distance from full-blown flowers. Process each class in a separate bottle. A sequence of stages in embryo development can be built up by sectioning fruits from the several lots. Trim two sides of the silique to promote penetration. The long silique of *Matthiola* also may be used. Divide transversely into 2- to 3-mm. lengths for killing, and cut microtome sections longitudinally. Use Craf I for either of these crucifers.

Lycopersicum esculentum, the tomato, is an excellent subject for dicot embryology. Use the small currant tomato, *L. pimpinellifolium,* seeds of which are obtainable from seed dealers. Slides of fertilization and the very young embryo are difficult and time-consuming

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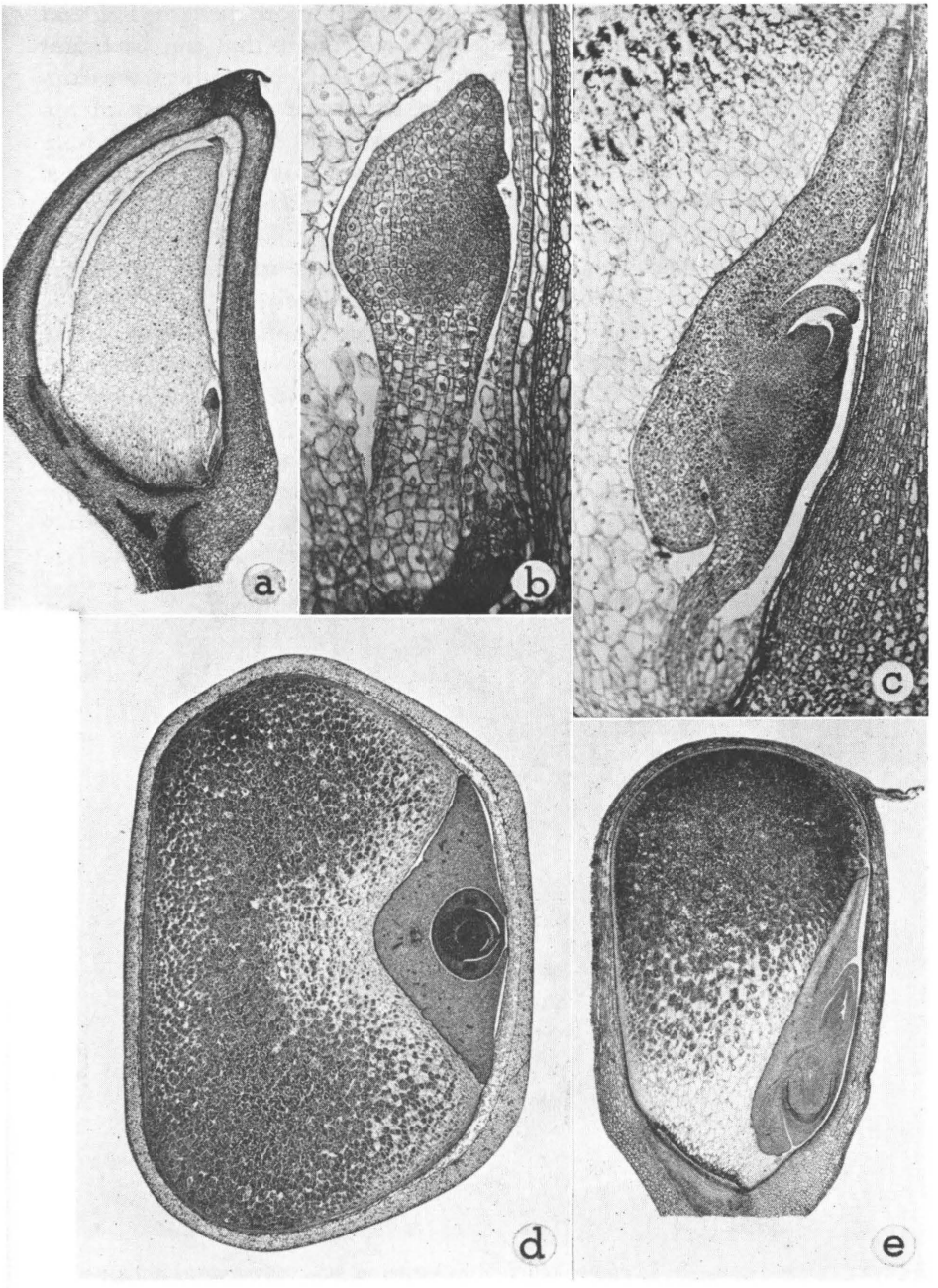
FIG. 15.8—*Zea mays*: *a,* kernel of pop corn 10 days after pollination; *b,* embryo of dent corn, 10 days; *c,* embryo, 15 days; *d,* transverse section of kernel of dent corn, 25 days; *e,* kernel of pop corn, 20 days.


a
b
c
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e

to obtain in quantity. Ten days after pollination the growing end of the embryo has developed into a large sphere that can be found in sections with adequate frequency (Fig. 15.7 *c*). Collect developing fruits of various sizes, slice out a transverse median disk of approximately one-third depth of the fruit, and kill in Craf I. Dehydrate and infiltrate with care. Section transversely, 10 μ for the earlier stages, 15 to 18 μ for larger embryos. As the seed coat of the maturing seed hardens, sectioning becomes increasingly difficult; however, the most important features of organogeny and the initiation of histogens are evident before the seed coat becomes excessively hard.

The legumes may be tempting subjects for embryology, but the best known large-seeded species, as well as many small-seeded species yield very few slides for the time expended. *Lotus corniculatus* is one of the most promising legumes. The long, straight ovary contains many ovules in straight alignment, and sectioning is fairly productive (Fig. 15.7 *b*).

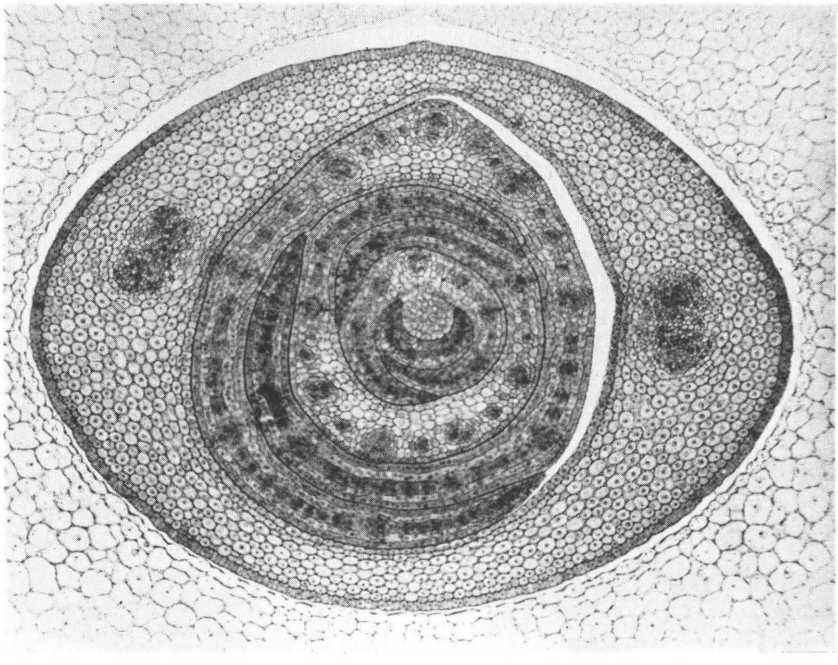

FIG. 15.9—Transverse section of plumule in kernel of yellow dent corn, 30 days after pollination (courtesy of Dr. Robert S. Fairchild).

Young fruits are processed in accordance with the methods given above for older ovaries. The currant tomato and many siliques are small enough even when nearly mature to have complete sections on a slide. Fruits that are more than 1 cm. in diameter should be subdivided and suitable pieces selected from the regions that are to be studied. The developing drupe of *Prunus virginiana* is an excellent subject. To prepare small cherry fruits for killing, remove a thin vertical slice from each side of some fruits and from the top and bottom of others, thus furnishing material for transverse as well as longitudinal sections. Kill in *FAA* for vascular study and in Allen-Bouin II or Craf III for better fixation of the embryo. The presence of brown pigmentation in many fruits produces poor contrast with the hematoxylins, but safranin-fast green is usually satisfactory.

The great array of fruits available to the technician presents a wide range of size, texture, and other properties, from the juicy berry to the flinty caryopsis. It is, therefore, quite impossible to offer generalized recommendations. The worker who ventures to prepare fruits and seeds has probably gained sufficient experience with easier subjects to adapt the fundamental methods given in this manual.