# THE MITOTIC APPARATUS IN FUNGI, *CERATOCYSTIS FAGACEARUM* AND *FUSARIUM OXYSPORUM*

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ABSTRACT

Vegetative nuclei of fungi *Ceratocystis fagacearum* and *Fusarium oxysporum* were studied both in the living condition with phase-contrast microscopy and after fixation and staining by HCl-Giemsa, aceto-orcein, and acid fuchsin techniques. Nucleoli, chromosomes, centrioles, spindles, and nuclear envelopes were seen in living hyphae of both fungi. The entire division process occurred within an intact nuclear envelope. Spindles were produced between separating daughter centrioles. At metaphase the chromosomes became attached to the spindle at different points. In *F. oxysporum* the metaphase chromosomes were clear enough to allow counts to be made, and longitudinal splitting of the chromosomes into chromatids was observed. Anaphase was characterized in both fungi by separation of chromosomes to poles established by the centrioles, and in *F. oxysporum* anaphase separation of chromosomes was observed in vivo. Continued elongation of the spindles further separated the daughter nuclei. Maturing daughter nuclei of both fungi were quite motile; and in *C. fagacearum* the centriole preceded the bulk of the nucleus during migration. The above observations on living cells were corroborated by observations on fixed and stained material.

## INTRODUCTION

For several years there has been much controversy concerning the mechanism(s) by which fungal nuclei divide (3, 5, 25). Many recent investigations have led to a convergence of opinion concerning several aspects of nuclear division (5, 11, 21, 25). Thus, it is becoming quite common for fungal nuclear division to be described as a process occurring within an intact nuclear envelope and involving centrioles or centriole-like bodies which produce spindles. Distinct chromosomes appear and separate to opposite poles. Many investigators report a "double-bar" (3, 9, 25) or "palisade stack" (23) of chromosomes during certain stages of division; and the presence of lagging chromosomes (5, 16, 17, [1]) is common.

In spite of the similarities in nuclear division in different fungi, it must be borne in mind that individual differences occur. For instance, in *Basidiobolus ranarum* Eidam there are no centrioles, and the nuclear envelope breaks down at metaphase (24), but in *Saccharomyces* the nuclear envelope remains intact throughout nuclear division, and anaphase poles are established by centriole-like bodies (26). Such differences make it im-

[1] Robinow, C. F. 1967 and 1968. Personal communication.

possible to state at the present time that vegetative fungal nuclear division occurs in any one manner.

The cytology of the asexual nucleus of *Ceratocystis fagacearum* (Bretz) Hunt was previously studied with both phase-contrast microscopy and the HCl-Giemsa staining technique (2, 3, 5). Distinct chromosomes appeared during division and separated to opposite poles. The poles were established by the action of a centriole which split and produced a spindle between the separating daughter centrioles. Elongation of the spindle appeared to aid in the pinching off of daughter nuclei from the old nuclear envelope, which remained intact throughout division. The orientation of the spindle was consistent; the spindle was at first perpendicular to the long axis of the cell, and then, as it elongated, it rotated to become parallel. This behavior of the spindle may account for certain of the "perpendicular divisions" described in some of the earlier papers (3, 5).

Only a few other members of the genus *Ceratocystis* have been studied cytologically. Bakerspigel (9), in studying asexual nuclear division in *Ceratocystis fimbriata* Ell. et Halsted, observed chromosomal complexes, which, toward the end of division, formed into two closely appressed bars of irregular outline which then broke in the middle to complete division. He found no evidence of a spindle, but did illustrate "a fragment of chromatin lying free in the cytoplasm" between two young daughter nuclei. Other workers have interpreted chromatin fragments as evidence of lagging chromosomes (5, 16, 17). In contrast to Bakerspigel's findings, a centriole-spindle apparatus was described by Andrus and Harter (7) for crozier mitosis in a species of *Ceratostomella* (*Ceratocystis*). They observed a small "centrosome" at the periphery of the nucleus, and this centrosome divided and produced a thin spindle as division approached.

Information on asexual nuclear behavior in *Fusarium oxysporum* Schlect. is scarce, although earlier reports indicated that it is similar in many respects to that described for *C. fagacearum* (4, 18). The chromosome number in several species of *Fusarium* has been found to be n = 4 in studies of the perfect stages (15).

Recent electron micrographs have shown that nuclei of both *F. Oxysporum* and *C. fagacearum* are surrounded by a typical nuclear envelope consisting of two closely appressed membranes.[2]

[2] Aist, J. R. 1968. Unpublished data.

The purpose of the present study was to investigate further some of the earlier observations on *C. fagacearum* and *F. oxysporum* in an effort to understand them more fully. It was hoped that the additional information obtained would help clarify some of the fine points currently being disputed by fungal cytologists.

## MATERIALS AND METHODS

The cultures used in this study were a B strain of *Ceratocystis fagacearum* (Bretz) Hunt and an isolate of *Fusarium oxysporum* Schlect. They were maintained at 20–26°C on Difco Oak Wilt Agar. (Difco Laboratories, Detroit, Mich.)

### *Preparations for Staining*

Mycelium for staining was obtained by growing the fungi on coverslips as described previously (1), a liquid oak wilt medium being substituted for V-8 Juice filtrate. The liquid oak wilt medium consisted of a solution of the ingredients recommended by Barnett for perithecia production (10), but with the agar omitted. These preparations were usually suitable for staining in 1–2 days when incubated at 22°C.

### *Staining Methods*

HCl-Giemsa, aceto-orcein, and acid fuchsin staining methods were employed. The HCl-Giemsa technique used most in this study is a much shortened version of the one used in previous studies (1, 3). Coverslip cultures were fixed for 2–3 min in acetic alcohol (1:3) and transferred to 35% ethanol for 5 min. Then they were rinsed in water and hydrolyzed for 6 min in 1 N HCl at 60°C, transferred to buffer (pH 7.0 ± .02) for 10 min, and stained for about 30 min in Giemsa stain (2 drops, Giemsa stain per ml buffer). The stained coverslip cultures were then mounted in the stain, and the coverslip was ringed with immersion oil or paraffin.

This technique produced less shrinkage than the one employing dehydration in an acetone series and mounting in balsam (1); pictures of hyphae which were dehydrated in acetone will be so designated in the captions. After HCl-Giemsa staining, the nucleolus and spindle were red, the chromatin was purple, and the centriole was red or purple.

The acid fuchsin and aceto-orcein staining procedures were obtained from C. F. Robinow (26[1]). To stain the spindle fiber, coverslip cultures were fixed for 10 min in a modified Helly's fixative (26) and rinsed in 70% and then 35% ethanol. They were stained in acid fuchsin (1:60,000 in 1% acetic acid) for 15–30 sec and washed with and mounted in 1% acetic acid.

The procedure for staining the chromatin with aceto-orcein was the same as the acid fuchsin tech-

nique above, up to the point of the 35% ethanol rinse. From there, the coverslip cultures were rinsed in water, then 50% acetic acid, mounted in a drop of aceto-orcein (1% Fischer's synthetic orcein in 50% acetic acid) on a slide, and gently heated.

The acid fuchsin and aceto-orcein techniques can be combined in order to observe first the spindle, and then the chromatin. After the spindle was observed in acid fuchsin, a drop of aceto-orcein was placed at the edge of the coverslip and allowed to be drawn under the glass by capillarity.

### *Living Preparations*

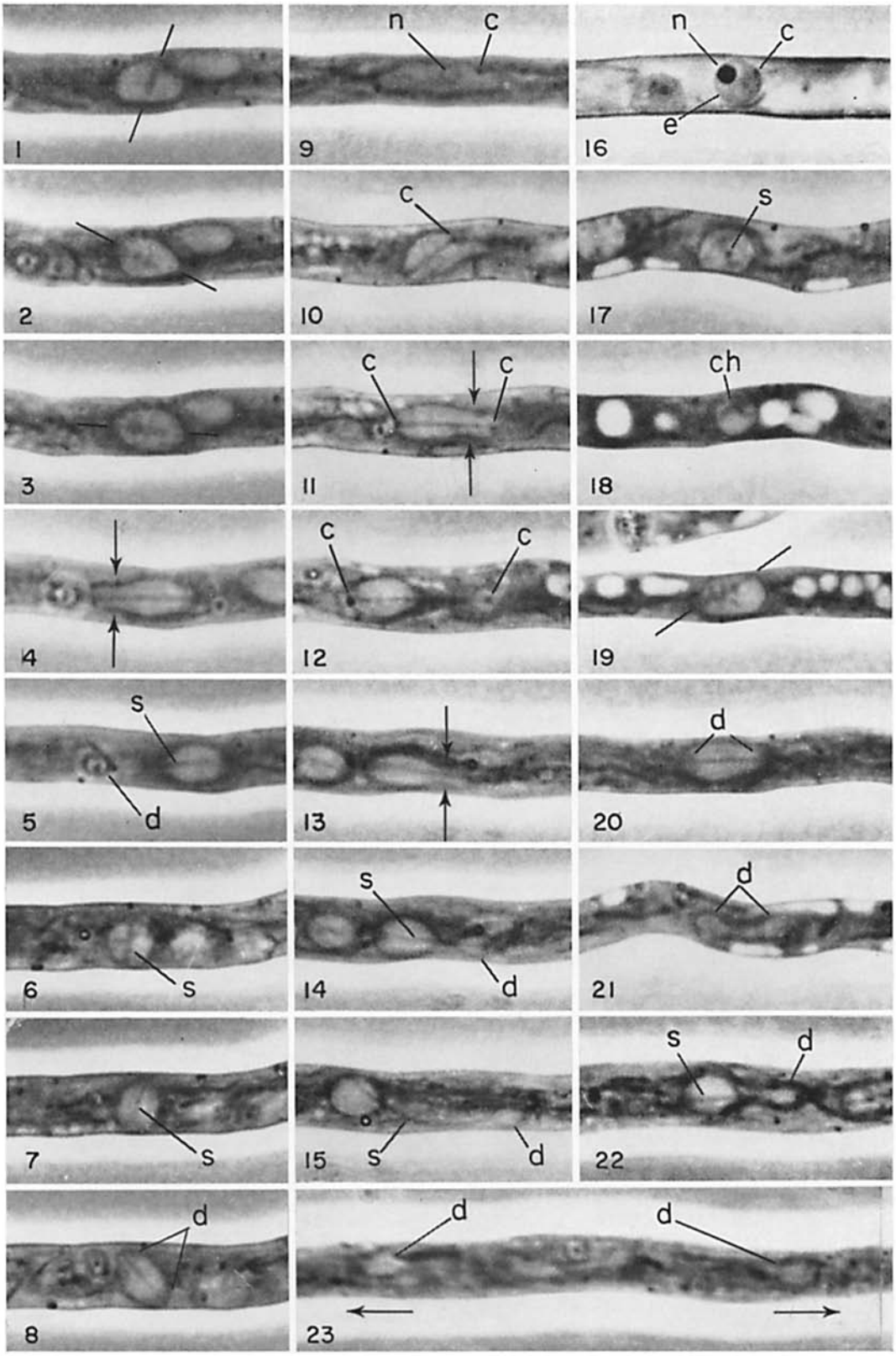
Living mycelia suitable for phase-contrast microscopy were prepared as follows: sterile microscope slides were prepared by placing two standard microscope slides on several filter papers in a Petri dish. The slides were arranged so that one lay across the other. The Petri dish with slides was autoclaved. The upper slide was then dipped in hot Difco Oak Wilt Agar and held vertically for 5 sec to drain, and then it was again placed across the slide in the Petri dish. Two agar blocks about 3 mm wide and containing hyphal tips were cut from the periphery of a rapidly growing culture and were inverted and placed on the agar slide about 2 cm apart. The inoculated agar slides were incubated in the Petri dishes at 22°C overnight or until about 5 mm of growth were obtained. Sterile water was added to the Petri dish to prevent drying of the agar. When suitable growth had occurred, the agar slide was removed from the Petri dish and a razor blade was used to trim the agar film to a thin rectangular block, 10 mm by 5 mm, on which a section of only one of the mycelia was growing. A coverslip was then applied directly to the mycelium and the edges were sealed with paraffin. Cultures prepared in this way were usually suitable for observation for at least a day, and in some preparations they grew faster than Petri dish cultures. A similar technique was used by Robinow to study *Mucor* nuclei (22).

It is very important to select the right cell for observing nuclear division when studying either living or stained cells. When a hypha is growing, nuclear divisions occur in tip cells periodically (2, 4, 18), but there are nuclear divisions in cells as far from the tip as five or six cells; in these cells most of the detail can usually be seen in phase-contrast because the cytoplasm in the older cells is not so dense as that in tip cells, and there are often fewer organelles

*Key to Abbreviations Used in Figures*

*c*, centriole
*ch*, chromosomes
*d*, daughter nucleus
*e*, nuclear envelope
*lc*, lagging chromosome
*n*, nucleolus
*r*, remnants of old nuclear envelope
*s*, spindle
*sc*, sister chromatids

FIGURES 1–23 Phase contrast photographs of mitosis in living hyphae of *C. fagacearum* Unlabeled lines indicate orientation of spindle. Figs. 1–5 Series showing various stages in division of one nucleus. In Figs. 1–3 the spindle rotates, in Fig. 4 the nuclear envelope is stretched and begins to constrict (arrows) behind daughter nucleus at left, and in Fig. 5 the envelope has recoiled and the daughter nucleus (*d*) is connected to the other daughter nucleus by the spindle (*s*). Figs. 6–8 Series of one nucleus showing perpendicular orientation of spindle (*s*) and separation of chromosomes prior to spindle rotation. Note daughter nuclei (*d*) appear as darkened ends of the envelope in Fig. 8. Fig. 9. Interphase nucleus in tip cell showing nucleolus (*n*), centriole (*c*) and nuclear envelope. Figs. 10–12 Series of one nucleus showing production of spindle between two centrioles (*c*), rotation of spindle, and pinching off (arrows) of one daughter nucleus. Figs. 13–15 Series of one nucleus showing pinching off (arrows) of one daughter nucleus (*d*) and elongation of spindle (*s*) as daughter nuclei separate. Fig. 16 Nucleus in mature cell showing nucleolus (*n*), centriole (*c*), and nuclear envelope (*e*). Fig. 17 Dividing nucleus at late prophase showing median optical cross-section of spindle (*s*). Fig. 18 Dividing nucleus showing a bundle of metaphase chromosomes (*ch*) at one side of nuclear envelope. Fig. 19 Dividing nucleus with chromosomes attached to spindle. Fig. 20 Dividing nucleus with a telophase nucleus (*d*) at each end of spindle. Fig. 21 Dividing nucleus after nuclear envelope disappeared at a stage similar to Fig. 20. Daughter nuclei (*d*) appear to separate by elongation and constriction. Figs. 22 and 23 Series of one division showing migration of one daughter nucleus (*d*) out of envelope in Fig. 22, and migration of other daughter nucleus in opposite direction in Fig. 23. Arrows show direction of migration of daughter nuclei when picture was taken. × 2400.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to interfere with observation. Another very important factor is that the nuclei in older cells divide more slowly, enabling the chromosomes to become more distinct as metaphase approaches and giving the observer more time to study each stage. Repeated observations are mandatory in order to see each stage clearly, since rarely can one see each stage of a single division in detail.

## RESULTS

### *Observations on Living Cells of C. fagacearum*

Interphase nuclei in tip cells of *C. fagacearum* are constantly changing their position and shape within the cell, but they are generally elongated. Phase-contrast microscopy reveals a more or less dense, spherical nucleolus, a large bright area surrounding the nucleolus, a nuclear envelope, and a small, dark, flattened centriole on or near the envelope (Fig. 9).

The nucleolus exhibits only slow random movements within the nucleus, but the centriole often exhibits quick movements. The centriole may move quickly back and forth along the nuclear envelope, traversing the length of the nucleus. Nuclei in older cells are distinctly spherical with a prominent nucleolus, a nuclear envelope, and a centriole (Fig. 16).

As a rule, all nuclei in a tip cell divide synchronously, but in certain older cells most of the nuclei may divide synchronously, while other nuclei in the same cell do not divide. There is a tendency for nuclei nearest the tip of a cell to be in a later stage of division than the nuclei nearest the septum.

The first sign that nuclear division is to occur is often the cessation of movement of the centriole. The nucleolus frequently begins to show marked Brownian movement and may decrease slightly in density at this time. The centriole then divides, and one-half migrates slowly toward the opposite wall of the hypha. Concomitantly, the nuclear envelope enlarges and becomes spherical to ovoid (Figs. 1–3).

As the daughter halves of the centriole move apart, a dark filamentous spindle is produced between them (Figs. 1, 6, 10, 11). Occasionally the spindle rotates slightly back and forth about its median transverse axis. As the spindle continues to elongate, it rotates until finally it is parallel to the long axis of the cell (Figs. 1–3, 6–8, 10–12). The nuclear envelope at this stage assumes roughly the shape of a football, and is traversed by the spindle which has a daughter centriole at each end (Figs. 8, 10, 20).

During the elongation of the spindle, several chromosomes appear within the nucleus (Figs. 18, 19) and attach to the spindle at different points (Fig. 19). When one views the spindle end-on, the chromosomes seem to radiate from it; and when the spindle is viewed from the side, the chromosome arms appear to extend perpendicularly from it. If the spindle rotates when the chromosomes are visible, the chromosomes rotate also, suggesting an attachment of the chromosomes to the spindle. The chromosomes eventually become separated into two groups, one at each end of the spindle. The separation into two groups occurs either when the spindle is perpendicular to the long axis of the cell (Figs. 6–8) or when it is parallel to this axis (Figs. 1–5, 20). The chromosomes fuse into a homogeneous mass at each end of the spindle, which results in darkening of the ends of the elongated nuclear envelope (Figs. 8, 20).

The process continues with further elongation of the spindle (Figs. 13–15). As this happens, the old nuclear envelope constricts just behind one of the daughter nuclei (Figs. 4–5, 11–12, 13–14). The other daughter nucleus now begins to migrate, usually in the opposite direction, and, as it does so, the old envelope becomes attenuated and fades from view (Figs. 22, 23). The spindle then breaks, with a slight recoil of the loose ends, and disappears (Figs. 22, 23). The entire process of nuclear division as described takes place in 5–10 min and has been observed in hyphae, conidiophores, and in germinating conidia.

Continuous observation of centriole behavior from prophase through maturation of daughter nuclei was carried out several times in different preparations of living material. In every case the daughter centriole first migrated toward the opposite wall of the hypha, producing a spindle oriented perpendicular to the long axis of the cell. Then as elongation continued, the spindle rotated (when its length was approximately equal to the diameter of the cell) until it was parallel to the long axis. In many other dividing nuclei the spindle was first seen oriented perpendicularly, and then it rotated until it was parallel to the cell's long axis (Figs. 1–3, 6–8, 10–12).

Continuous observation of growing hyphal tips revealed that, after a septum formed following division of the nuclei in a tip cell, a branch usually

formed 5–15 μ behind the septum. This branch generally obtained its first nucleus when a nucleus in the branch-producing cell divided and a daughter nucleus migrated into the branch. This migration often occurred before the spindle broke, in which case both daughter nuclei entered the branch. One daughter nucleus then migrated back into the main cell. A similar situation occurred when the nucleus of a germinating conidium divided; both daughter nuclei, attached by a spindle, migrated into the germ tube, and then the nucleus nearest the conidium migrated back toward the conidium after the spindle broke.

There were a number of variations of nuclear division in *C. fagacearum* which apparently resulted from differences in timing of the several events, such as nucleolar and nuclear envelope breakdown and spindle rotation, rather than from differences in the behavior of the structures involved.

The time at which the enlarged nuclear envelope disappeared was one variable. During the division of one nucleus both daughter nuclei were pinched off from the old envelope, without much change in the shape of the old envelope being observed. The daughter nuclei continued to migrate apart and the spindle broke and disappeared, leaving only the oval-shaped old nuclear envelope in the field of view. Then the old envelope slowly faded from view, without changing from its oval shape. During the division of another nucleus, in the "football" stage (such as Figs. 8 and 20), the enlarged nuclear envelope suddenly disappeared, revealing a daughter nucleus at each end of the spindle (where the darkened ends were). Each daughter nucleus was surrounded by a nuclear envelope (a small, thin, dark circle, 2–3 μ in diameter) which appeared to be attached to a centriole. After further elongation, the spindle broke and faded from view.

The behavior of the nucleolus was also quite variable during nuclear division. The nucleolus either faded from view, was lost in the cytoplasm, or was included in one of the daughter nuclei before fading from view. In certain tip cells the nucleolus was not visible between divisions.

Daughter nuclei sometimes separated so quickly that the old nuclear envelope became stretched and faded from view at the same time (Fig. 21). The separation of these daughter nuclei resembled simple elongation-construction nuclear divisions described by several writers (8, 22, 28, 30, 31).

In the nuclear divisions in which the behavior of the centrioles and spindles was specifically traced, the maturation of daughter nuclei was followed. Daughter nuclei appeared initially as small, dark spherical bodies 2–3 μ in diameter, with a dark centriole situated at the nuclear envelope. After several minutes nucleoli appeared within the nuclear envelopes, and these nucleoli were larger and had less distinct outlines than the centriole. In other instances, young daughter nuclei without nucleoli were observed to produce nucleoli a few minutes later at a site removed from the centriole. The daughter nuclei began to enlarge when they migrated out of the old nuclear envelope, and they attained full size in 10–20 min.

Maturing daughter nuclei were quite motile. They exhibited saltatory movements (6) for some time, with the centriole preceding the bulk of the nucleus during these movements. The net result was a reshuffling of the daughter nuclei in the cell before septum formation. When a septum formed, the number of nuclei sometimes differed in each of the two cells produced, indicating that both products of a nuclear division were sometimes incorporated into the same cell. The involvement of a centriole in nuclear migration in several species of fungi, including *C. fagacearum*, was reported previously (32).

Contrary to what was reported earlier in *C. fagacearum* (3), the original number of nuclei in a conidium may be more than one. The production of the first conidium by a trinucleate conidiophore was observed, and it was found that half of the daughter nuclei migrated into the conidium, resulting in a trinucleate conidium. It is not known whether the number of nuclei in this conidiophore was later reduced by unequal distribution of daughter nuclei (as occurs in growing hyphae). However, almost all conidiophores observed were uninucleate, and were producing uninucleate conidia.

### *Observations on Stained Preparations of C. fagacearum*

The chromatin in interphase nuclei of *C. fagacearum* appeared as a reticular network or a system of distinct chromatin strands (Fig. 24) when stained with HCl-Giemsa. The nucleolus may also be stained (3).

As nuclei approached division, their chromatin became increasingly dense and compact. At late

prophase, three chromatin bodies were seen in some nuclei (Fig. 25), and at metaphase these bodies occurred closely appressed to a narrow spindle (Fig. 26). The chromatids became strung out on the spindle as they separated at anaphase (Figs. 27, 28), thus momentarily obscuring the spindle from view. When the chromatids reached the poles, the spindle was again visible (Figs. 32–35), and it became quite elongated before it broke (Fig. 35). The centriole associated with maturing daughter nuclei was often conspicuous (Fig. 36).

Other important aspects of the late stages of nuclear division were observed. Lagging chromosomes were frequently seen (Figs. 29, 30), and in some cases both the lagging chromosome and the spindle were stained. The remnants of the old nuclear envelope were stained in a few cells in which daughter nuclei were separating (Figs. 33, 35).

Similar division stages were found in aceto-orcein preparations and are illustrated in Figs. 38 to 42. Aceto-orcein stained only the chromosomes and cytoplasm, but this technique allowed the behavior of the nuclear envelope to be studied (Figs. 40, 41).

### *Observations on Living Cells of F. oxysporum*

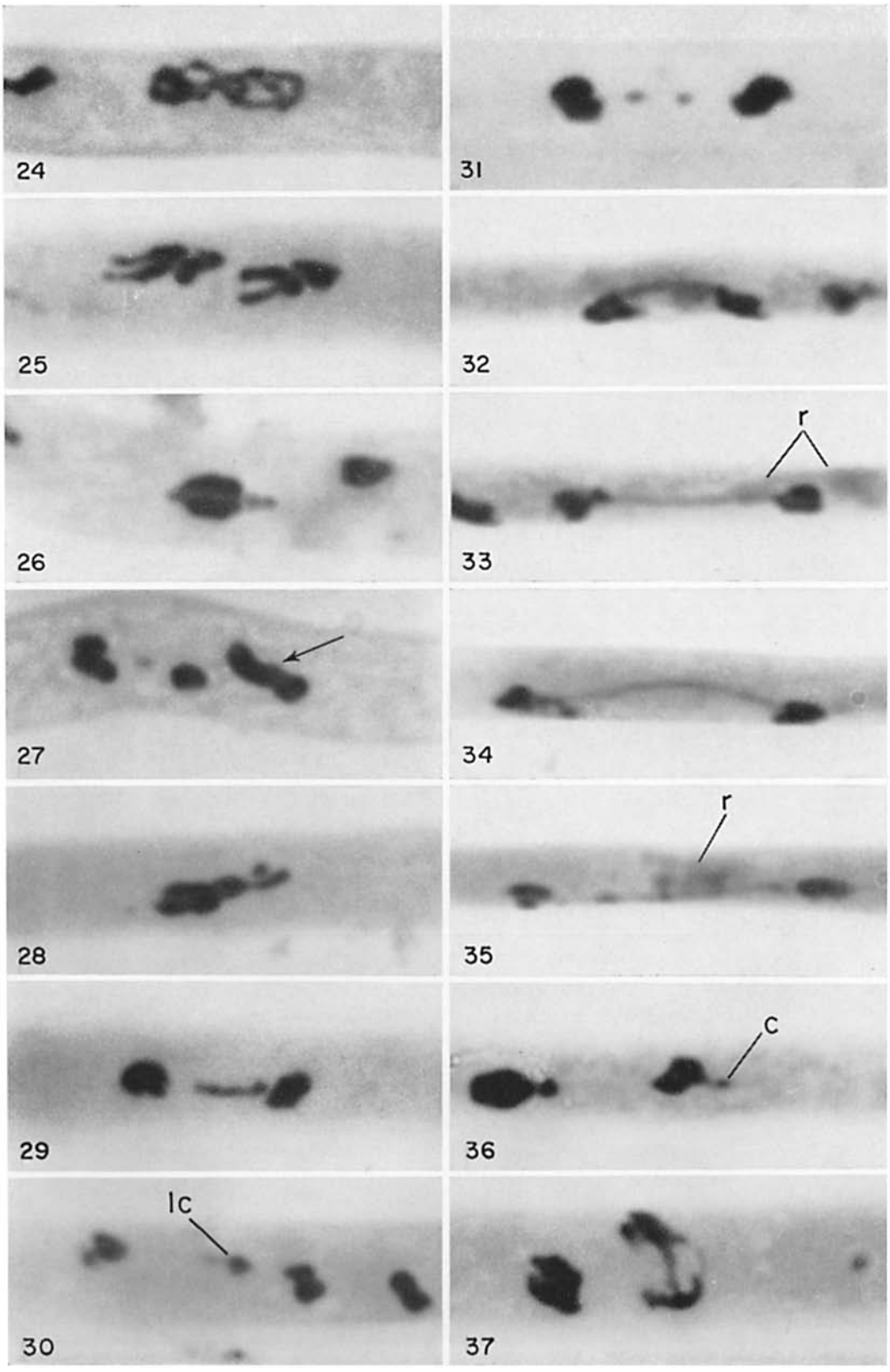
Interphase nuclei of *F. oxysporum* are similar to those of *C. fagacearum* in possessing a nucleolus, a surrounding bright area, and a nuclear envelope (Fig. 43). The nucleolus is distinct in *F. oxysporum*, but a centriole cannot be definitely recognized. Elongated nuclei are less common in *F. oxysporum* than in *C. fagacearum*.

Nuclear division is usually initiated in a hypha somewhere between the tip cell and the fifth or sixth cell from the tip. From this point of initiation, a wave of nuclear divisions travels toward or away from the tip, or in both directions. All the nuclei in a cell usually divide within a 10- to 15-min period, and each nucleus requires 5–10 min for division.

Figures 43–52 show the sequence of events in the division of one nucleus. The entire process occurs within an intact nuclear envelope. The first evidence of division is the beginning of breakdown of the nucleolus and its increased Brownian movement (Fig. 43). A bundle of prophase chromosomes then appears (Fig. 44), and the chromosomes continue to shorten and darken until they can be seen as separate bodies on a spindle (Figs. 45, 46). Metaphase chromosomes are often grouped toward one side of the nuclear envelope (Fig. 45). The centrioles and spindle can be seen occasionally at metaphase (Figs. 69, 79), and the spindle is usually visible after anaphase (Figs. 49, 68). The metaphase chromosomes divide into chromatids (Fig. 80) which separate on the spindle to opposite poles (Figs. 47, 58–62, 70). After chromatid separation is complete (Fig. 48), the spindle continues to elongate (Fig. 49), and finally the old nuclear envelope breaks down and the spindle disappears (Fig. 51). The daughter nuclei now show marked saltatory movement, and they may migrate back and forth through the cell several times before a septum is formed. During this time the daughter nuclei enlarge, reform nucleoli, and decrease in density until they are typical interphase nuclei (Fig. 52).

Although the spindle was often difficult to see from a side view, it was easily distinguished when viewed from the end (Fig. 78). In such nuclei, careful focusing showed that the spindle was almost as long as the cell diameter and was oriented perpendicular to the cell's long axis. The spindle often rotated back and forth about its median transverse axis at metaphase, and, as it did so, the chromosome arms rotated with it (Figs. 54–57). This rotation, which indicated that the chromosomes were attached to the spindle at this time, was the criterion I chose for the beginning of metaphase. Therefore, metaphase was

FIGURES 24–37 HCl-Giemsa-stained nuclei of *C. fagacearum*. Hypha in Figs. 32–36 were dehydrated in acetone-xylene. Fig. 24 Interphase nucleus with distinct chromatin strands. Fig. 25 Two prophase nuclei with three chromatin bodies each. Fig. 26 Metaphase nucleus with chromatin closely appressed to a lighter staining, thin horizontal spindle. Figs. 27 and 28 Anaphase figures (arrow in Fig. 27) in which chromosomes obscure spindles. Figs. 29 and 30 Late anaphases showing lagging chromosomes (*lc* in Fig. 30). Fig. 31 Late anaphase showing separation of ends of lagging chromosome. Figs. 32–35 Separation stages showing elongation of spindle between daughter nuclei, and light (red) staining remnants (*r*) of old nuclear envelopes. Fig. 36 Young daughter nucleus with a centriole (*c*). Fig. 37 Perpendicular division with an anaphase bridge. × 4800.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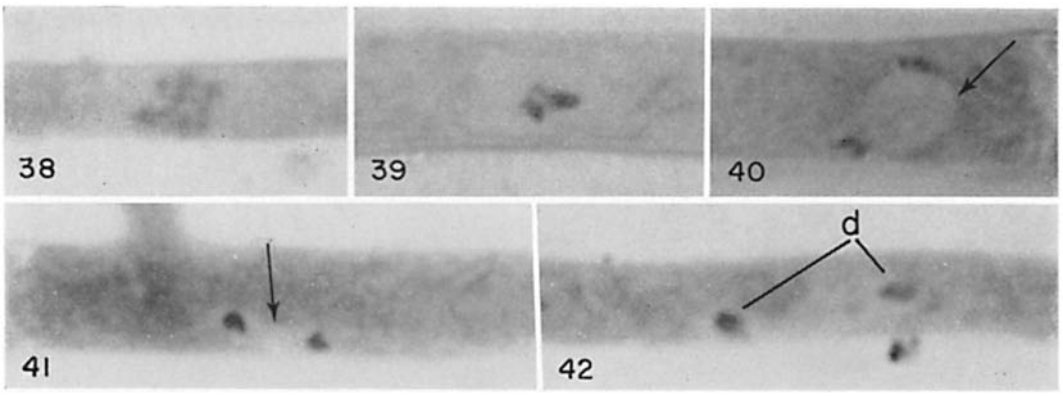



FIGURES 38–42 Nuclei of *C. fagacearum* stained with aceto-orcein. Fig. 38 Prophase. Fig. 39 Metaphase. Figs. 40 and 41 Telophase nuclei showing limits of nuclear envelope (arrows). Fig. 42 Separating daughter nuclei (*d*). × 2800.

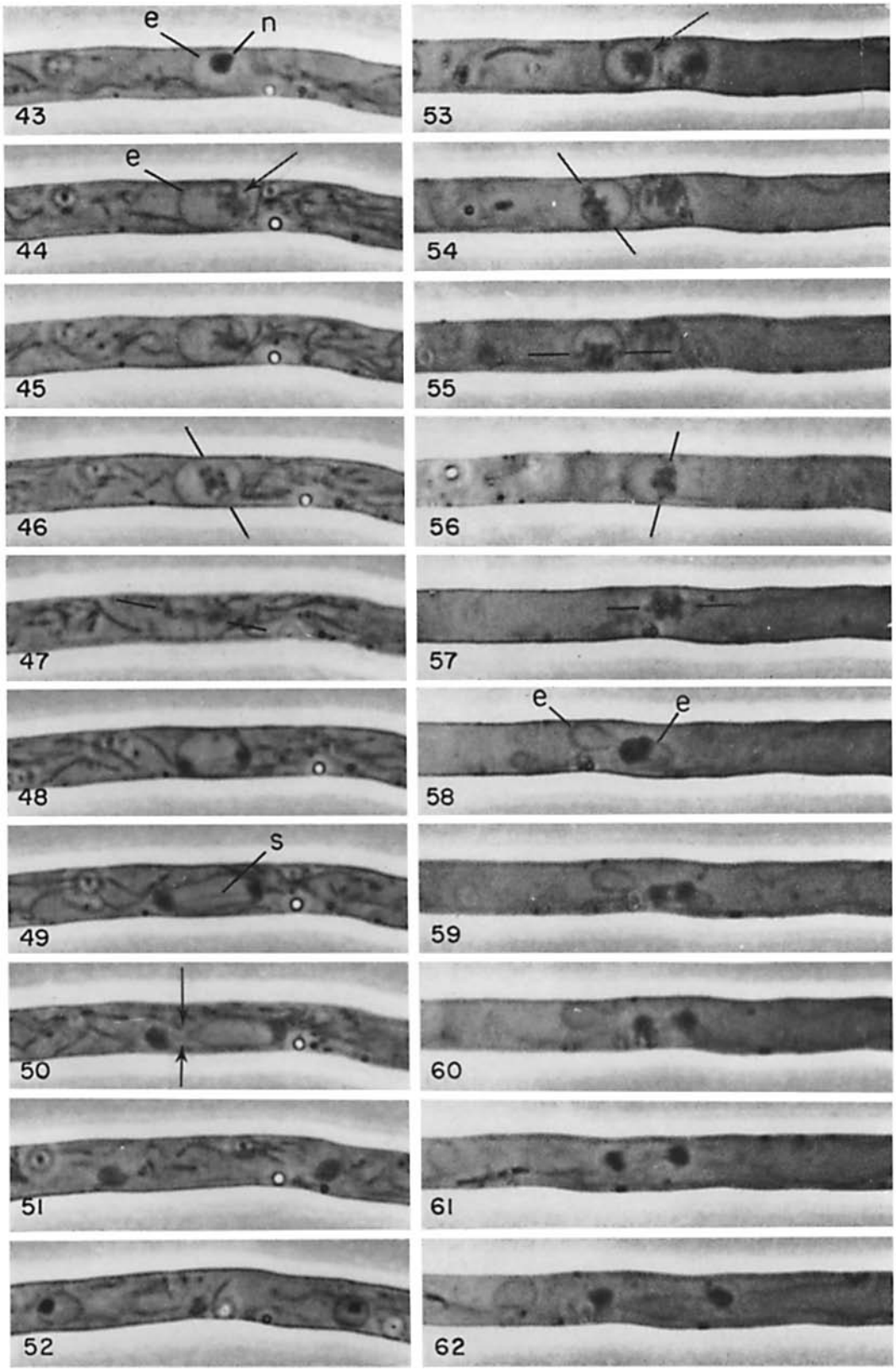
relatively long since the chromosomes became attached to the spindles 1–2 min before anaphase began, during nuclear divisions that take only 5–10 min to occur. During metaphase and anaphase, dividing nuclei often migrated in the cell, sometimes over distances up to 25 μ (Figs. 53–56).

Many variations in nuclear division were observed, and, as in *C. fagacearum*, apparently they resulted from variations in timing of the several events. Tip cell nuclei usually divided faster than nuclei in older cells; and, if the nuclear envelopes surrounding such nuclei broke down at anaphase, these divisions resembled direct nuclear divisions described by Robinow, Bakerspigel and others (8, 22, 28, 30, 31).

Differences also occurred in metaphase and anaphase configurations. Metaphase chromosomes were grouped near the center of the spindle (Fig. 79), or spread out over the middle two thirds of it (Fig. 69). Several nuclei had three chromosomes at metaphase (Fig. 80). Anaphase separation sometimes occurred when the spindle was oriented perpendicular to the cell's long axis, but it usually did not occur until the spindle was parallel (Figs. 47, 57).

Synchronous (Figs. 58–62) and asynchronous (Figs. 63–68) chromatid separation was seen. When anaphase was synchronous, chromatids could be distinguished moving on the spindle (Figs. 70, 75–77), and were usually closely appressed to it (Fig. 47). Frequently one chromatid pair had difficulty in separating, and often became stretched out between the two groups of separating chromatids (Figs. 66, 72–74), producing an anaphase bridge. This observation was further evidence that chromatid separation occurred at this stage (17).

FIGURES 43–62 Phase-contrast photographs of dividing nuclei in two living hyphae of *F. oxysporum*. Unlabeled lines indicate orientation of spindle. Figs. 43–52 A series showing the division of one nucleus in a hyphal tip. Nucleolus (*n*) is just beginning to break down in Fig. 43. A bundle of prophase chromosomes (arrow) appears at one side of nuclear envelope (*e*) in Fig. 44. Arms of metaphase chromosomes can be seen in Fig. 45; and Fig. 46 represents an optical cross-section through the chromosome arms. The chromatids are separating, closely appressed to the spindle, in Fig. 47; and in Fig. 48, anaphase is complete. In Fig. 49, the nuclear envelope has become attenuated as the spindle (*s*) elongates. One daughter nucleus is pinched off (arrows) in Fig. 50; and in Fig. 51 the nuclear envelope has broken down, leaving the dark lemon-shaped daughter nuclei lying free in the cytoplasm. In Fig. 52 the daughter nuclei have reformed nucleoli, decreased in density, and enlarged to become typical interphase nuclei. Figs. 53–62 A series showing the division of one nucleus (arrow) in an old cell. Nucleus at right in Fig. 53–55 migrated out of the field of view while dividing. Fig. 53 shows prophase nuclei, and Figs. 54–57 show spindle and metaphase chromosomes rotating back and forth prior to anaphase. Figs. 58–62 show successive stages in synchronous anaphase separation within nuclear envelope (*e*). × 2400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When anaphase separation was delayed (usually in older cells), the chromosomes usually separated as a group in which individual chromatids could not be distinguished (Figs. 53–62). Such chromatin separation closely resembled that described as direct nuclear division in *Saprolegnia* by Slifkin (29).

### *Observations on Stained Preparations of F. oxysporum*

When stained with HCl-Giemsa, chromatin in interphase nuclei of *F. oxysporum* appeared finely granular, and the nucleolus was often visible (Fig. 91). A small granule, which may be the centriole, was sometimes associated with the chromatin (Fig. 91).

The chromosomes gradually shortened and thickened during prophase (Figs. 81, 82), until at metaphase there were three visibly double chromosomes (Fig. 83). The six chromatids then separated at anaphase (Figs. 84, 85), and moved to opposite poles. As in *C. fagacearum*, lagging chromosomes which formed anaphase bridges were common (Figs. 86, 89, 90). Daughter nuclei often had a conspicuous centriole (Fig. 88).

Aceto-orcein staining revealed the same sequence of events (Figs. 93–96) and showed the persistence of the nuclear envelope through telophase. Acid fuchsin stained the spindle red (Fig. 92); when this staining was followed by aceto-orcein staining, the particular stage of nuclear division being observed was determined (Fig. 93).

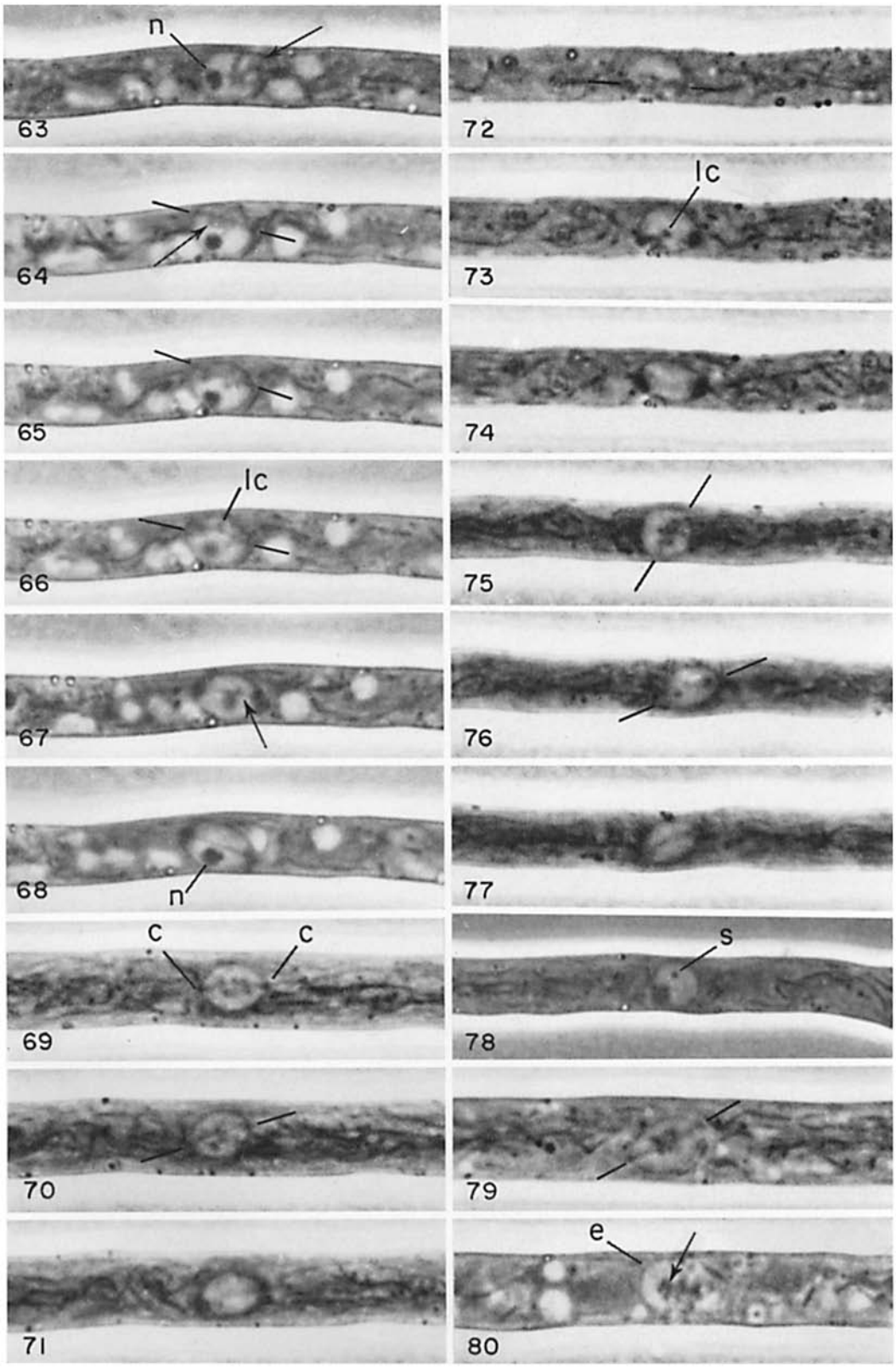
In particularly clear preparations, it was possible to determine which of the chromatin bodies were sister chromatids (Figs. 84, 85, 94).

## DISCUSSION

There are two dynamic aspects of the mitotic apparatus in both *C. fagacearum* and *F. oxysporum*. These are the behavior of the centriole-spindle complex as it develops and moves about within the cell, and the behavior of the chromosomes as they shorten and thicken and finally separate into two groups. Although both aspects involve consistent predictable events, the events are not always synchronized. Thus, one cannot always tell, merely by observing chromosome behavior, to what stage spindle production has proceeded and vice versa. Furthermore, instead of forming a metaphase plate, the chromosomes usually attach to the spindle at different points. One significant result of this behavior is the occurrence of at least three morphologically distinct anaphase configurations which can be oriented in any direction (Figs. 47, 58–62, 70). This type of behavior could account for some of the different interpretations proposed by various authors for these anaphase figures (3, 5, 9, 12, 25).

An interesting question raised by the observations on the spindle of *C. fagacearum* is why the early spindle so consistently oriented perpendicular to the cell's long axis? Olive (20) observed a consistent orientation of the meiosis 1 spindle of *Coleosporium vernoniae* B. and C., but subsequent spindles were oriented randomly. Perhaps electron microscopy can provide clues to an answer to this puzzling question.

FIGURES 63–80 Phase-contrast photographs of dividing nuclei in living hyphae of *F. oxysporum*. Unlabeled lines indicate orientation of spindle. Figs. 63–68 Series showing division of one nucleus, in which lagging chromosomes (*lc*) can be seen and nucleolus (*n*) persists through anaphase. Prophase chromosomes (arrow in Fig. 63) appear and attach to spindle at metaphase (arrow in Fig. 64). Anaphase has just begun in Fig. 65; and in Fig. 66 a lagging chromosome (*lc*) can be seen between the two groups of separating chromatids. In Fig. 67 the end of one lagging chromatid can be seen (arrow); and in Fig. 68 anaphase is complete. Figs. 69–71 Series showing division of one nucleus. In Fig. 69 the nucleus is at metaphase, and the centrioles (*c*), spindle, and chromosomes are visible. Two chromatid pairs are in focus in Fig. 70, and sister chromatids can be seen separating to opposite poles. Anaphase is complete in Fig. 71. Figs. 72–74 Series showing division of one nucleus. Anaphase has just begun in Fig. 72; and in Fig. 73 a lagging chromosome (*lc*) can be seen. Fig. 74 represents late anaphase. Figs. 75–77 Series showing division of one nucleus. Fig. 75 shows metaphase chromosomes; Fig. 76 shows two sister chromatids moving to poles at anaphase; and Fig. 77 represents telophase. Fig. 78 Dividing nucleus showing median optical cross-section through the spindle (*s*). Fig. 79 Nucleus at metaphase showing chromosomes grouped near center of a thin spindle. Fig. 80 Nucleus at metaphase showing three chromosomes aligned at different points on the spindle. Chromosome at arrow is visibly double. Only part of the nuclear envelope (*e*) is in focus. × 2400.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The present findings support Yoneda's (33) contention that nuclear division in certain fungi involves two distinct stages, division and separation. Nuclear division is complete when sister chromatids reach the poles (telophase); the remainder of the process involves separation of daughter nuclei. It is interesting that Yoneda also described a "slender body" which accomplished the separation of daughter nuclei by elongation. Robinow has recently found fiber apparatuses which are produced between two separating daughter granules in mitotic nuclei of several fungi (25, 26, [1]).

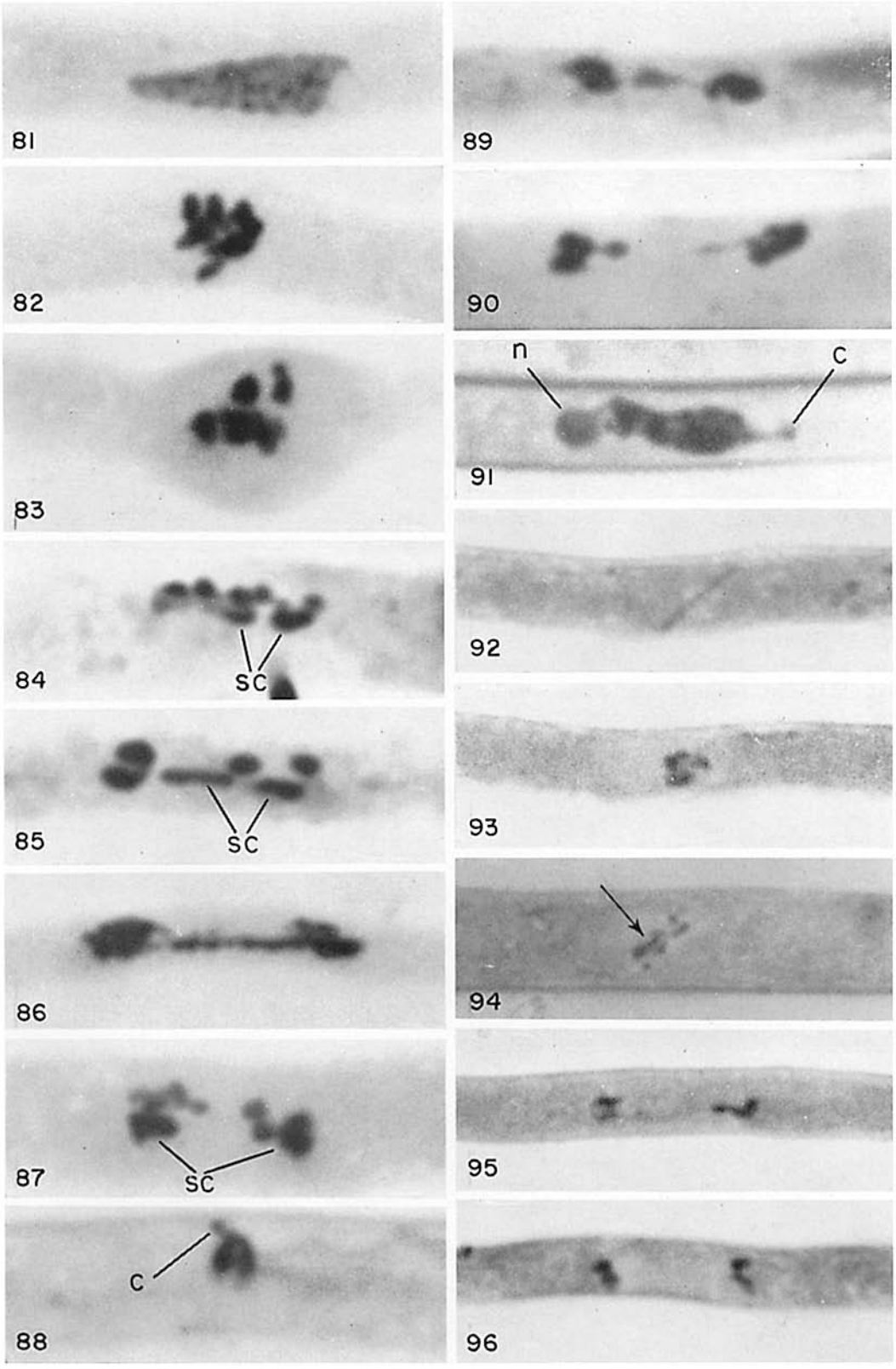
The previous interpretation of nuclear migration in fungi (32) is supported by the observations concerning maturing daughter nuclei of *C. fagacearum*. That the small dark granules involved are centrioles functionally is now established, although their fine structure is still to be determined.

The entire process of nuclear division as illustrated here is strikingly similar to Olive's observations on mitosis in living hyphae of *Itersonilia* (21), and to Girbardt's phase-contrast illustrations of nuclear division in the clamp connections of *Polystictus versicolor* (L.) Fr. (13, 14). Girbardt illustrates structures which seem to be comparable to the present illustrations of centrioles, spindles, chromosomes, and nuclear envelopes, although his interpretation of many of these structures is radically different from mine. However, more recent work by Girbardt has shown the presence of a microtubular spindle apparatus between two centrioles in a number of Basidiomycetes (25).

Knox-Davies (16, 17), studying nuclear division in developing pycnospores and hyphae of *Macrophomina phaseoli* (Maubl.) Ashby, concluded that the occurrence of lagging chromosomes and chromosome bridges is evidence of chromatid separation. The phase-contrast observations on *F. oxysporum* confirmed this. In this fungus the chromosomes became so distinct at metaphase that chromatids were recognized in the living condition (Fig. 80). Many clear observations of anaphase movement were made, and in many cases one chromatid pair separated slightly later than the other two (Figs. 66, 73), permitting the separation of this pair to be observed without optical interference from other migrating chromatids. Further evidence was that, in stained preparations, the number of separate chromatin bodies at anaphase was twice the number present at metaphase (Figs. 83–85, 93, 94). The distinct morphology of these chromatids made it possible to determine which are sister chromatids (Figs. 84, 85, 94). Furthermore, some chromatid pairs were apparently in the process of separating when the material was fixed (Figs. 84, 85, 94). It must be concluded, therefore, that chromatid separation in *F. oxysporum* occurs at the stage referred to as "anaphase" and not at some earlier stage before chromosomes become visible, as was proposed for certain other fungi (12, 22, 25). The evidence for *C. fagacearum* was not so conclusive, but the many similarities in nuclear division between this fungus and *F. oxysporum* suggested that a similar mechanism of chromatid separation occurs in both fungi.

Several investigators published division figures of meiosis in fungi which bear close resemblance to many of the mitotic figures recorded here. Olive (20), Andrus and Harter (7), and Rogers (27) all illustrate metaphase I figures in which

FIGURES 81–96 Nuclei of *F. oxysporum* stained with HCl-Giemsa (Figs. 81–91), acid fuchsin (Fig. 92) and aceto-orcein (Figs. 93–96). Nuclei in Figs. 84–86 and 89–91 were dehydrated in acetone-xylene. Fig. 81 Interphase. Fig. 82 Prophase. Fig. 83 Metaphase showing one large chromosome and two small ones. Fig. 84 Early anaphase showing separation of three chromatid pairs. Note sister chromatids (*sc*) of large chromosome. Fig. 85 Mid-anaphase showing six chromatids; one chromatid pair (*sc*) has just separated. Fig. 86 Anaphase with chromosome bridge. Fig. 87 Late anaphase showing large chromatid pair (*sc*) at poles and two smaller chromatids nearing pole at right. Fig. 88 Young daughter nucleus with centriole (*c*). Fig. 89 Anaphase with lagging chromosome. Fig. 90 Anaphase showing lagging chromatid reaching pole at left. Fig. 91 Interphase nucleus in old cell showing nucleolus (*n*), chromatin, and centriole (*c*). Fig. 92 Nucleus at metaphase showing spindle oriented diagonally. Fig. 93 Same nucleus as in Fig. 92, but stained with acetoorcein to show the three metaphase chromosomes. Fig. 94 Nucleus at anaphase showing chromatid separation (arrow). Fig. 95 Late anaphase. Fig. 96 Telophase. Figs. 81–91, × 4,800. Figs. 92–96 × 2,800.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the chromosomes are attached to the spindle at different points, rather than forming a metaphase plate. Many of their anaphase 1 and telophase 1 figures are morphologically similar to anaphase and telophase configurations in hyphae of *C. fagacearum* and *F. oxysporum*. In addition, this study lends further credibility to the contention of Andrus and Harter (7) that the nuclear envelope remains intact throughout division. These similarities between meiosis and mitosis in fungi should help to remove the discrepancies that have existed in reported mechanisms of the two types of nuclear division (25).

It should be pointed out that mitotic metaphase illustrations such as Fig. 80 are not entirely unique. Certain of Robinow's illustrations of mitosis in *Lipomyces* (23) seem comparable.

Simple elongation and constriction of nuclei, without the aid of a spindle, has been reported as the mechanism of vegetative nuclear division in several fungi studied with phase-contrast microscopy (8, 22, 28, 30, 31). Bakerspigel (8) reported that the dividing nucleus of *Neurospora crassa* Shear and Dodge was seen to rotate 90° just prior to elongation and constriction. This behavior is strikingly similar to the behavior of dividing nuclei of *C. fagacearum* which rotated 90° when the spindle became longer than the diameter of the cell. The nuclear envelopes of both *C. fagacearum* and *F. oxysporum* elongated and constricted during separation of daughter nuclei; when the envelopes broke down prematurely, the divisions then appeared as a simple elongation-constriction process (Fig. 21). Nuclear division in *F. oxysporum* appeared to be almost identical to that reported for *Saprolegnia* by Slifkin (29), if anaphase was delayed and chromatids separated synchronously (Figs. 58–62). Could it be that the inability to demonstrate a chromosome-spindle apparatus in only certain fungi reflects differences in contractibility of the chromosomes, optical properties of the spindle, and behavior and optical properties of the nuclear envelope, rather than differences in the actual mechanisms involved in nuclear division?

Phase-contrast microscopy was indispensable for this study. There was little need to guess whether a particular fixed and stained nucleus was dividing, since, if it was dividing, the other nuclei in the cell would be in various division stages which were familiar from observations on living cells. Phase-contrast not only made some structures visible which were difficult or impossible to see in stained preparations, but it allowed the time sequence of the various division configurations seen in stained material to be established with certainty. This is an advantage that should not be overlooked by those attempting to shed light on the elusive mechanisms of vegetative nuclear division in fungi.

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Note added in proof:

Since the writing of this manuscript, some preliminary observations on the fine structure of the mitotic apparatus in *C. fagacearum* and *F. oxysporum* have been made. A technique kindly demonstrated to me by C. F. Robinow has made it possible to observe living, dividing nuclei of these fungi in phase contrast, and then to fix and embed these same nuclei for study with the electron microscope. The spindles of both fungi are composed of a number of microtubules, the exact number of which has not yet been determined. *C. fagacearum* also has a centriolar plaque (26) (referred to as "centriole" in this paper) which lies outside of the nucleus in a slight depression of the nuclear envelope. Microtubules extend both into the nucleus and far out into the cytoplasm from the centriolar plaque during nuclear division.

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