STUDIES ON THE STRUCTURE OF THE CHROMOSOMES

I. *Allium cepa*—Stain Fixatives and Their Utility

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Introduction

Haematoxylin and Feulgen squashes of acetic alcohol fixed roots of *Allium cepa* do not give strictly comparable pictures of nuclear and chromosomal details. Superposable pictures could be obtained with these stains if fresh roots are exposed to N HCl at 60° C. for an optimum period of time and then processed (Subramaniam and Subramanyam, 1961). The above procedure revealed not only the morphology but also some of the structural details of the chromosomes.

This interesting result could be traced to the omission of fixation of the tissue before hydrolysis and is reminiscent of the procedures in vogue for stain fixatives. The most popular among these are solutions of orcein in acetic acid or in mixtures of N HCl and acetic acid. But these have rarely been reported as suitable for a study of chromosome structure without specific pre-treatment of the material (Law, 1943).

Is the inability of the stain fixative to reveal the structural details of the chromosomes due to the absence of any accurate control of the staining procedures: or, is it due to the unsuitability of acetic acid as a fixative and orcein as a stain?

Solutions of orcein (1%—La Cour, 1941; Kaufmann, 1960. 2%—Axelrad and McCulloch, 1958; Sussman, 1961; Sparano, 1961) in a mixture of N HCl and 45% acetic acid in varying proportions (N HCl: 1% Aceto-orcein, 1: 10—La Cour, 1941. 1: 9—Mitra and Steward, 1961. 2% 1: 9—Sharma and Bal, 1953; Tjio and Levan, 1954; Zeilinga, 1956; Nambiar and Upadhya, 1961) have been claimed to be superior to solutions in acetic acid alone. The traditional method of gently heating the material in a drop of the dye acid mixture (La Cour, 1941) followed by most workers does not envisage any accurate control of the temperature or the duration of such exposure since the objective was only an easy separation of the cells. Peary (1955) stained

mammalian cells with aceto-orcein at 60° C. for five minutes and Singh (1961) following a similar procedure for plant material emphasizes the necessity for a control of the temperature to obtain a uniform staining of the cells.

Simultaneous fixation and hydrolysis in N HCl as a prelude to staining with leuco basic fuchsin was employed by Heitz (1931) and Fernandes (1937). Following on the above lines differing concentrations of HCl (10%—Gerstel, 1949; 20%—Li and Jackson, 1961) have been used as a medium for fixation and to facilitate an easy separation of the cells after staining with aceto-orcein. But none of these modifications have been reported as revealing the structural details of the chromosomes.

It was thought interesting, therefore, to elucidate whether the structure of the mitotic chromosomes of *Allium cepa* could be revealed with orcein prepared in N HCl and whether by controlling the time and temperature of exposure of the roots, similar pictures could be obtained with aceto-orcein and N HCl-aceto-orcein (1 : 10).

Material and Methods

The solubility of orcein (B.D.H.) in N HCl appears to be less than 0·5%. A precipitate appears on cooling when 0·5 gm. of orcein is transferred to 100 ml. of boiling N HCl. Since a sediment appears also on storage, the stain was filtered before use. Fresh roots of germinated bulbs of *Allium cepa* were clipped and transferred to vials of the stain maintained at 60° C. in a water-bath. Exposure to the stain for a period of 8 min. was sufficient to yield good optimally stained preparations. After cooling, the material was teased in a small drop of the stain on a clean slide and then squashed. The squashes ringed with paraffin retain their clarity for 3–5 days. Selected stages were photographed on 35 mm. Agfa Document film using a Leica attachment.

Observations

If the stain is fresh and the roots are squashed 15–30 min. after cooling the vials to room temperature, the peripheral cells show a deeper staining than those in the interior. When the stain ages, there is a progressive decrease in the intensity of colouration. Exposure for the same duration leaves the cells of the inner layers unstained. To obtain a comparable intensity, a longer storage of the roots in the stain at room temperature was found necessary.

Though the effect of storage from 5 min. to 5½ hrs. was investigated, a stay longer than 30 min. in the fresh stain obscures the structural details

of the chromosomes in the cells of the peripheral layers. The cells of the core develop an intensity of colouration comparable to that of optimally stained peripheral cells. Long storage renders the tissue so soft that the cells macerate when pressure is applied. Optimal staining could also be obtained by leaving the roots overnight in the stain at room temperature. Control of the time of storage eliminates the need for any destaining since the chromosomes and cytoplasm show differing gradations of intensity. In cells with lightly tinted cytoplasm, the nucleus and chromosomes stand out in sharp contrast.

The nucleus is brightly stained in the cell illustrated in Photo 1. The nucleoli do not stain with HCl-orcein and hence appear as unstained areas. The chromosomes which have a beaded appearance in early prophase (Photo 2) appear as caduceusly wound bi-partite structures in mid-prophase (Photo 3). The metaphase chromosomes (Photos 4–6) are quadri-partite as exemplified by enlargements (Photos 7 and 8) of two of them. The two chromatids are twisted round each other and each chromatid appears to be composed of two chromonemata wound round each other. Thin strands are often seen running between the chromatids (arrows in Photos 7–11: *cf.* Sharp, 1934; Mensinkai, 1939) and in favourable examples there is a strand bridging the gap between the chromatids at the tip of each chromosome (Photos 10 and 11).

In regions of a squash where the pressure has been too much, the real structure of the chromosome is lost. But it is problematical whether the tendency for fragmentation is due to the stain fixative (*cf.* Sharma and Roy, 1955) and not due to pressure. Sometimes the metaphase chromosomes were seen as paired beaded strings. Obviously this configuration has to be interpreted as an artefact. The preparations also revealed a few cells with highly contracted chromosomes whose primary constrictions were distinct. The daughter chromosomes at anaphase are bi-partite and composed of two chromonemata wound round each other (Photos 12 and 13). Rare instances of stickiness of chromosomes at metaphase and bridge formation at anaphase were also observed (Photo 14). A telophase with a stained phragmoplast showing the unstained cell plate in its middle is illustrated in Photo 15.

There is an improvement in the clarity of the preparations if fresh roots are hydrolysed in N HCl at 60° C. for 8 min. before staining and squashing in cold HCl-orcein. The quadri-partite and bi-partite structure of meta- and ana-phase chromosomes respectively were then as clear as in HCl-hæmatoxylin preparations (Subramaniam and Subramanyam, 1961).

HCl-Orcein, Aceto-Orcein and HCl-Aceto-Orcein—A Comparison.—It was thought desirable to elucidate whether similar structural details of the

chromosomes could be revealed with 1% aceto-orcein and 1% aceto-orcein-HCl (10: 1) mixture used by La Cour (1941). Fresh root-tips were exposed to these stain fixatives kept at 60° C. for 8 min. and then squashed. When lightly stained, the chromosomes are brownish red in aceto-orcein, dull red in HCl-aceto-orcein and rose red in HCl-orcein. This is not surprising since the shade of colouration is said to depend on the pH of the solution (Kornhauser, 1952). The improvement in their clarity also follows that order. Though the quadri-partite structure of the metaphase chromosomes was seen first in HCl-orcein, the same configuration could be made out in preparations lightly stained with the others also. Overstaining masks the real structure.

Staining with 1% *Aceto-Orcein.*—The details of structure of the resting and prophase nuclei were essentially similar to those in HCl-orcein preparations. The chromosomes at late prophase (Photo 16) and pro-metaphase (Photo 17) were quadri-partite. The two chromatids of the metaphase chromosomes (Photos 18 and 19) were often loosely twisted round each other (arrows in Photos 18 and 19) and the spaces between the caduceusly coiled half chromatids appeared as a linear row of vacuoles (Photos 20–22). The structural details of the daughter chromosomes at anaphase are, therefore, superposable on those of the chromatids at metaphase (Photos 23 and 24). It has to be presumed that each anaphase chromosome is composed of two chromatids. A rare instance of the precocious assumption of the quadri-partite condition by two of the chromosomes of an anaphase group is illustrated in Photos 25 and 26.

*Staining with N HCl-*1% *Aceto-Orcein* (1 : 10) *Mixture.*—The chromosomes were dull red in cells with lightly stained cytoplasm. As compared to aceto-orcein preparations there appeared to be a slight improvement in the clarity of the structural details of the chromosomes. A quadri-partite metaphase chromosome with bi-partite chromatids wound round each other (arrow in Photo 27) and bearing a bifid satellite is shown in Photos 27 and 28. The strands connecting the two chromatids are seen in Photo 28. A satellited anaphase chromosome is illustrated in Photos 29 and 30. The chromosome bridges at anaphase exhibited the same bi-partite condition (Photo 31).

Permanent Preparations of HCl-Orcein Squashes.—Several media like 40% alcohol, 5% and 10% acetic acid and N HCl were tried for the release of the coverslips. The first three destained the material to varying degrees. A long stay in 40% alcohol was necessary to loosen the coverslip. But then, the decolourization of the squash had progressed rather far. The removal of the stain was rather slow in 5% and 10% acetic acid and negligible in N HCl.

When coverslips released in the latter were quickly dehydrated with absolute alcohol or tertiary butyl alcohol the stain was leached out of the cytoplasm. Slightly overstained material was, therefore, processed to obtain the proper grade of staining in permanent preparations. In Canada balsam mounts, the chromosomes alone retained the stain and the cytoplasmic boundaries were difficult to locate. Counterstaining is imperative if cell outlines have to be made out. Eighteen months storage has not produced any serious deterioration in colour.

Staining of Fixed Material with HCl-Orcein.—One of the limitations of aceto-orcein is that it is refractory when used on tissues preserved in 70% alcohol (Darlington and La Cour, 1950). It was of interest in this context to elucidate whether HCl-orcein could be used on such material. Therefore, roots fixed in acetic alcohol and stored in 70% alcohol were brought down to water, exposed to HCl-orcein at 60° C. for 6–10 min., cooled to room temperature and then squashed.

The contrast between the chromosomes and cytoplasm was not as good as in fresh roots fixed and stained with HCl-orcein. There was a decrease in this contrast with an increase in the time of storage in 70% alcohol.

DISCUSSION

The structural details of the chromosomes revealed by HCl-orcein, aceto-orcein, and HCl-aceto-orcein are essentially similar to those obtained in fresh roots, fixed in N HCl at 60° C. and then stained with hæmatoxylin or leuco basic fuchsin (Subramaniam and Subramanyam, 1961). They are, however, inferior to HCl-hæmatoxylin preparations in clarity.

Since the same types of configuration are seen in material stained with different dyes, the question arises whether the anaphase chromosomes are only bi-partite (Mazia, 1961) or whether a further resolution is possible even at the light microscope level (Merriman, 1904; Nebel, 1939). Some recent analyses of results from auto-radiography appear to be based on the assumption that the anaphase chromosomes are generally quadri-partite (Pelc and La Cour, 1960; Steffensen, 1960). The relatively rare instance of two anaphase chromosomes showing a quadri-partite structure (Photos 25 and 26) while emphasizing that the techniques are capable of revealing such a condition, if they do exist, suggest that they are the result of a precocious non-synchronous replication of two of the chromosomes.

The chromonema and matrix, the two important constituents of a chromosome, are said to be clearly distinguishable in favourable living

material and the sausage-shaped chromosomes usually illustrated are considered to be the result of the matrix staining heavily (Cleveland, 1949). Is it then the matrix that has been removed from the chromosomes of *Allium cepa* when fresh roots were exposed to N HCl, HCl-orcein, aceto-orcein or HCl-aceto-orcein at 60° C. for an optimal length of time? Acid hydrolysis is known to remove RNA and histones (Kaufmann, Gay and McDonald, 1960) and an "RNA cycle" has been described during mitosis (Mazia, 1961, p. 303).

The inter-chromatid connections (Photos 7–11, 28) appear to be the remnants of the matrix and those bridging the free ends of pairs of chromatids cannot be considered as a region of the pellicle. Even though evidence for a pellicle is lacking in the electron micrographs published so far, a clear distinction of the matrix from the chromonema has also not been possible (Rozsa and Wycoff, 1951; Wischnitzer, 1960; Kaufmann *et al.*, 1960). Cleveland's (1949) description that "the two new membranes which surround each chromatid fuse to form a common nuclear membrane" (p. 12) suggests that a pellicle may be capable of demonstration.

The results presented (Photos 4–14, 18–26, 27–31) indicate that the chromosomes have material easily dissociable from the Feulgen positive chromonemata (Callan and MacGregor, 1958; Kaufmann *et al.*, 1960, p. 83). A removal of the matrix does not also affect the structural integrity of the chromosome. Since the satellite thread (Photos 28 and 30) appears bereft of such a matrix, a critical study of this region may be highly profitable.

Summary

The structure of the mitotic chromosomes of *Allium cepa* has been elucidated by controlling the temperature and time of exposure of fresh roots to stain fixatives. The details seen in material stained in N HCl-orcein for 8 min. at 60° C. and squashed after varying intervals of storage at room temperature were essentially similar to pictures obtained with 1% aceto-orcein and 1% aceto-orcein-N HCl (10 : 1) under identical conditions of handling. The chromosomes appear quadri-partite at metaphase and bi-partite at anaphase. A rare instance of the precocious assumption of a quadri-partite condition by two anaphase chromosomes is illustrated. Caduceus coiling of chromonemata was seen in chromosome bridges also. Chromosomes have material easily dissociable from the chromonemata and their removal does not affect the structural integrity of the chromosome.

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Description of Photographs

Plate XXVII

Photos 1, 2, 4, 6–12. *From temporary mounts of HCl-orcein squash preparations.*
Photos 3, 5 and 13. *From permanent preparations.*

Photo 1. A resting nucleus with unstained nucleoli. Note the contrast between the nucleus and the cytoplasm, × *ca.*, 1,500.

Photo 2. Early prophase showing the beaded appearance of the chromosomes, × *ca.*, 1,500.

Photo 3. Prophase chromosomes are bi-partite (arrow), × *ca.*, 2,500.

Photos 4, 5 and 6. Chromosomes at metaphase are quadri-partite. Each chromatid is composed of a pair of caduceusly coiled chromonemata. Photos 4 and 5, × *ca.*, 2,500. Photo 6, × *ca.*, 1,500.

Photos 7 and 8. The chromosomes indicated by arrows in Photo 6 enlarged to show the structure. A thin strand connects the distal ends of the chromatids, × *ca.*, 6,000.

Photo 9. Inter-chromatid connections at metaphase, × *ca.*, 1,500.

Photos 10 and 11. Two of the chromosomes from Photo 9 enlarged to illustrate the strands bridging the distal ends of the chromatids, × *ca.*, 6,000.

Photos 12 and 13. Caduceus coiling of chromonemata at anaphase, × *ca.*, 2,500.

Plate XXVIII

Photos 14 and 15. *From HCl-orcein squash preparations.*

Photo 14. Caduceus coiling of chromonemata in the chromosomes and chromosome bridges at anaphase, × *ca.*, 2,500. Permanent preparation.

Photo 15. The phragmoplast and the unstained cell plate of telophase. Temporary mount, × *ca.*, 1,500.

Photos 16–21. *From temporary mounts of Aceto-orcein squash preparations.*

Photo 16. A late prophase with quadri-partite (arrows) chromosomes, × *ca.*, 1,500.

Photo 17. A pro-metaphase with quadri-partite chromosomes (arrow), × *ca.*, 1,500.

Photos 18 and 19. Two metaphases with quadri-partite chromosomes. The relational coiling of the chromatids in the chromosomes is indicated by arrows. Photo 18. × *ca.*, 2,500. Photo 19. × *ca.*, 1,500.

PHOTO 20. Quadri-partite metaphase chromosomes, × *ca.*, 1,500.

PHOTO 21. One of the chromosomes in Photo 20 enlarged to reveal its structure, × *ca.*, 6,000.

PLATE XXIX

PHOTOS 22–26. *From temporary mounts of Aceto-orcein squash preparations.*

PHOTO 22. Enlargement of a chromosome from Photo 20 to reveal its structure, × *ca.*, 6,000.

PHOTOS 23 and 24. Two anaphases. The chromosomes are bi-partite. Photo 23. × *ca.*, 2,500. Photo 24, × *ca.*, 1,500.

PHOTO 25. An anaphase in which two of the chromosomes—indicated by arrows—are precociously quadri-partite. × *ca.*, 2,000.

PHOTO 26. One of the quadri-partite chromosomes in Photo 25 enlarged, × *ca.*, 6,000.

PHOTOS 27–31. *From temporary* 1% *Aceto-orcein-NHCl* (10 : 1) *squash preparations.*

PHOTO 27. Arrow indicates the bi-fid satellite of a quadri-partite metaphase chromosome. × *ca.*, 1,500.

PHOTO 28. Enlargement of the SAT-chromosome. Arrow indicates inter-chromatid connections. × *ca.*, 6,000.

PHOTO 29. The arrow indicates the satellited bi-partite anaphase chromosome. × *ca.*, 1,000.

PHOTO 30. Enlargement of the SAT-chromosome of Photo 29, × *ca.*, 6,000.

PHOTO 31. The caduceus coiling of the chromonemata in the chromosome bridges at anaphase. × *ca.*, 1,500.

CP, Cell Plate; CY, Cytoplasm; NU, Nucleolus; PHR, Phragmoplast.

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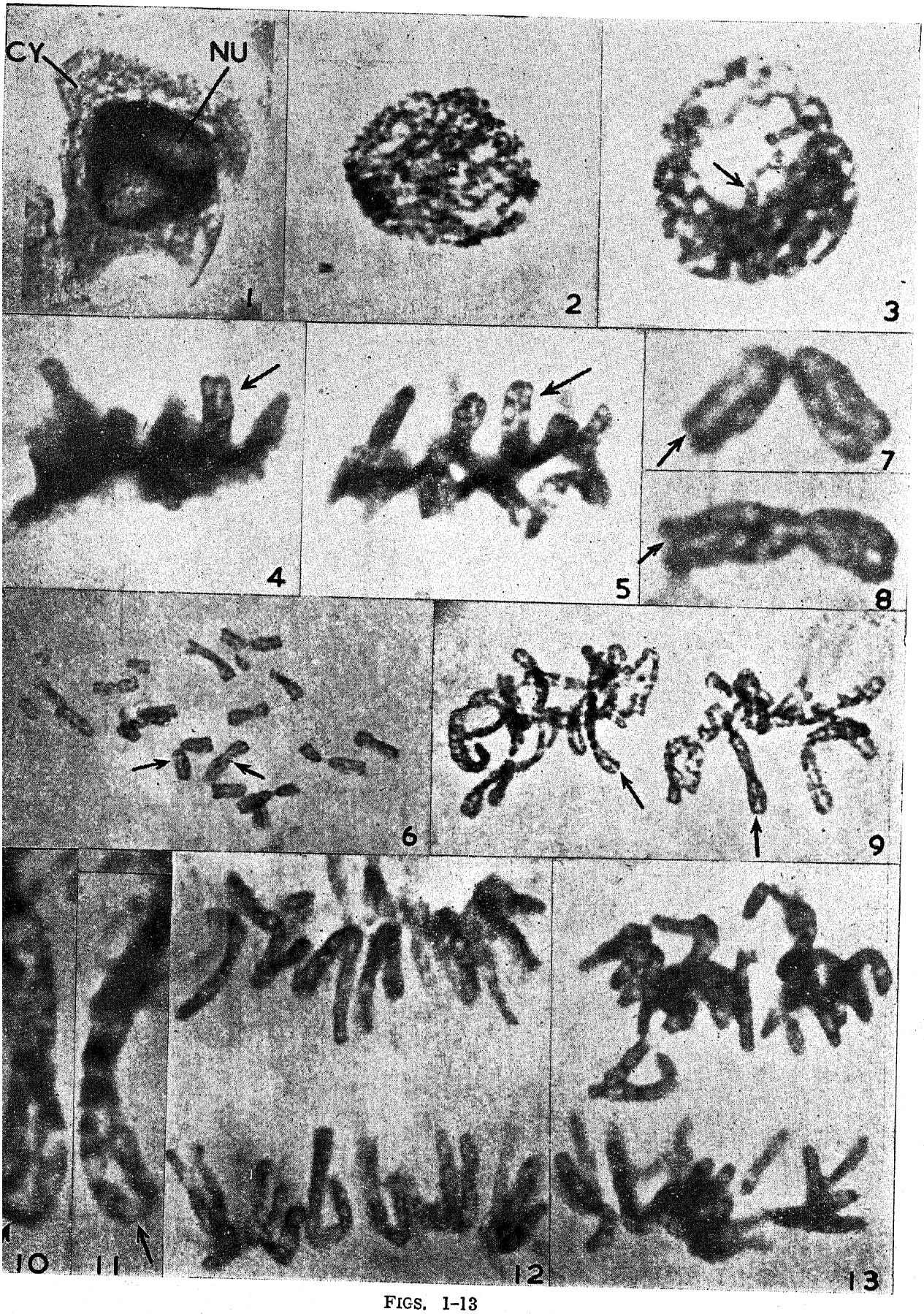
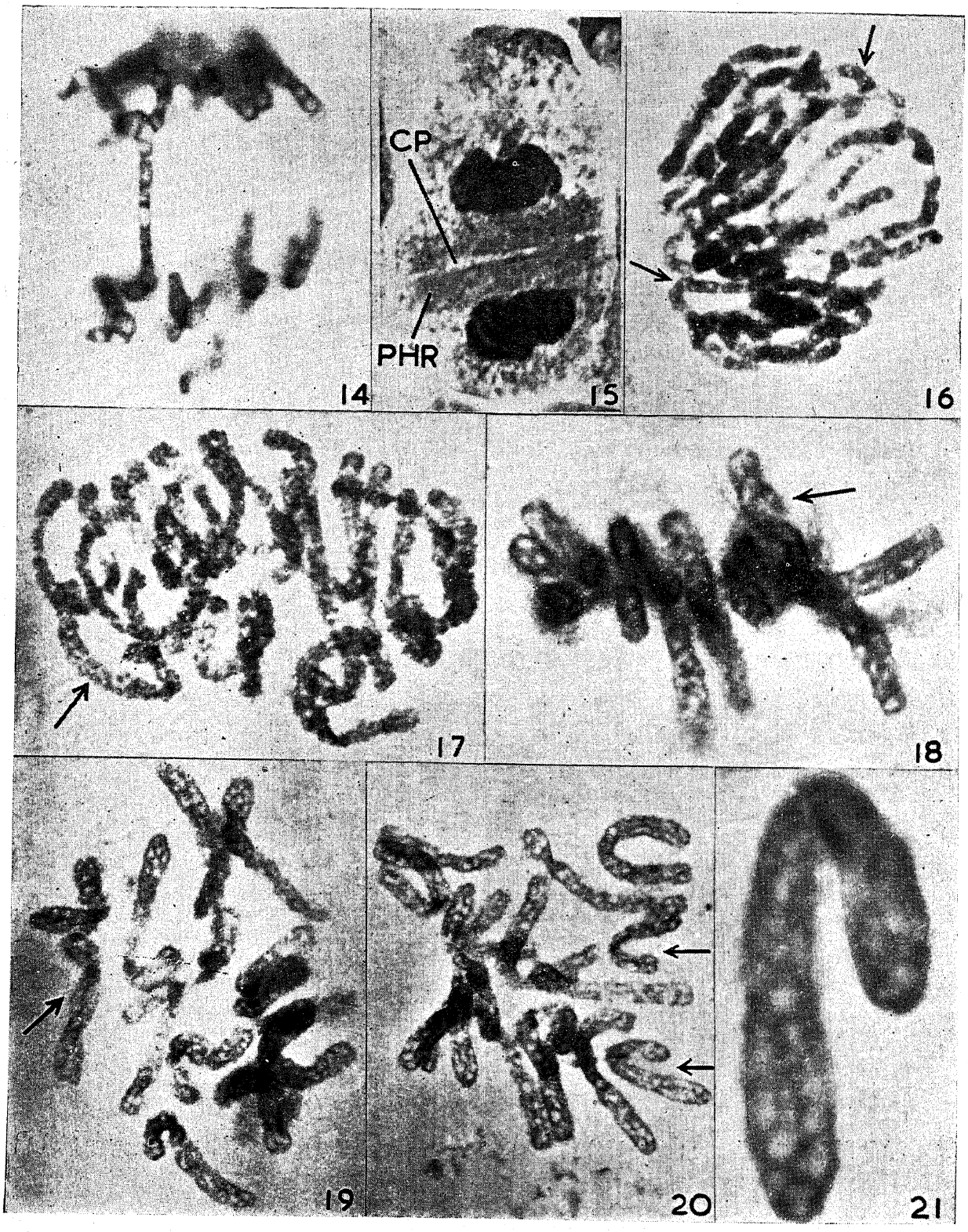
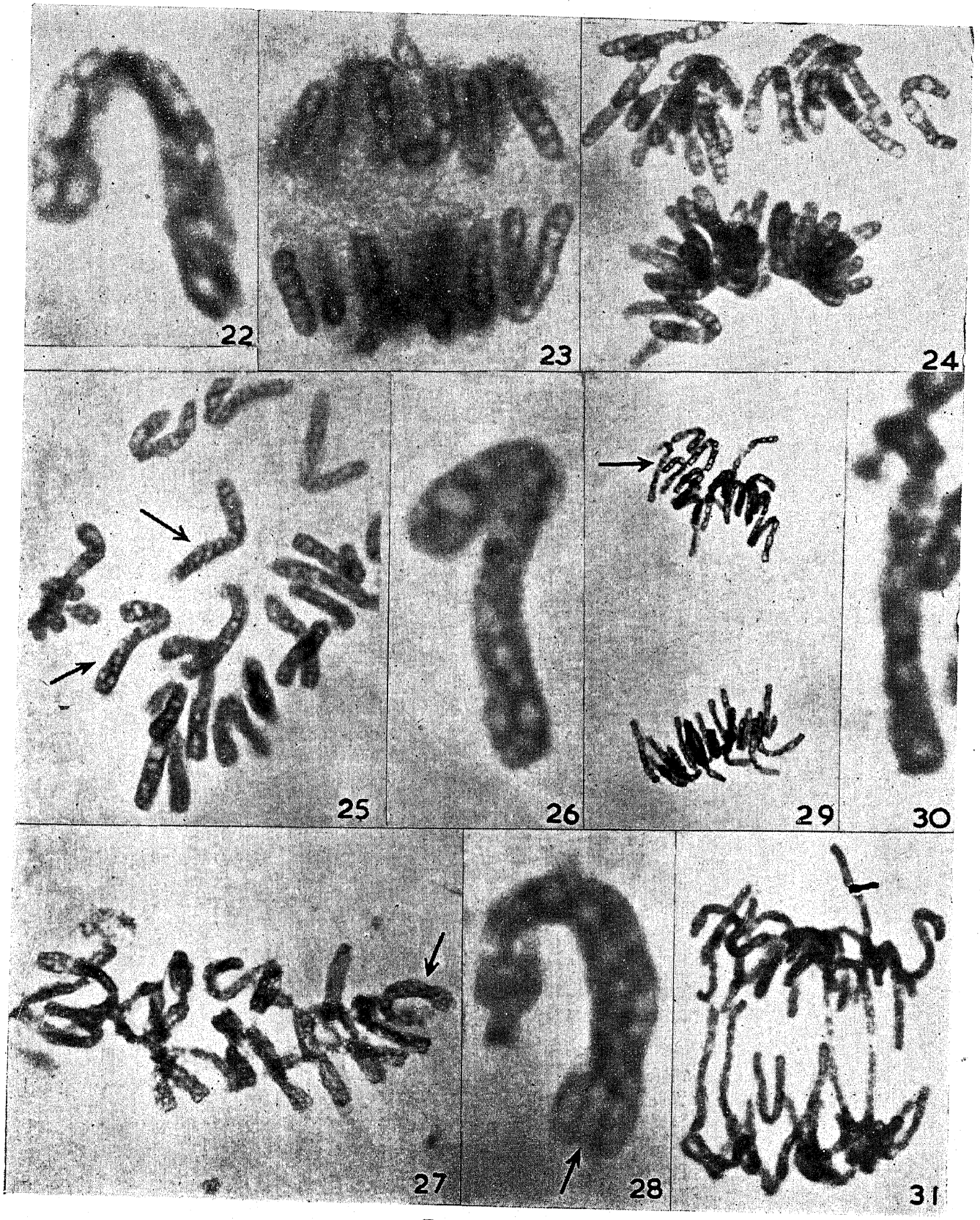


FIGS. 1–13



FIGS. 14–21



FIGS. 22-31