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Light Microscopical Observation of the Centromere Region in the Urodelan Chromosomes

Takeshi SETO*

Abstract : Structure of the centromere region in urodelan chromosomes was studied by light microscopy. The centromere region can not be seen as a particular structure in routine cytological preparations. Published works employing the modified cytological technique for light microscopy presented a quadruple structure or a spotted structure. However, there are still problems on the results since the figures have not given persuaded visualization in comparable to the electron microscopy.

Large sized chromosomes in urodelan cells were favorable to observe more detail structure than the mammalian chromosomes. In squashed preparation of uncolchicinized chromosomes of *Cynops pyrrhogaster*, the majority of chromosomes in the cell showed the stretched-out centromere at metapahse. The centromere region was raised to outside toward the poles on opposite side of sister chromatid. The structure was quite unlike with the previous studies which were prepared by rather violent treatment for the chromosome details. The result indicated that the possibility of destructive effects by the cytological treatments, such as colchicine and hypotonic solution, have disturbed the illustration of a real feature of the delicate centromeres.

Introduction

The term centromere is synonymous with kinetochore as the region on the chromosome that becomes attached to the spindle microtubules (Ris and Witt 1981). This specialized region is usually narrower than the chromosome arm. Then term "primary constriction" is used in case of morphological identification of each chromosome in karyoanalysis.

Centromere structure has been actively studied by electron microscopy on plant and animal cells. Evidences obtained from mammalian metaphase chromosomes demonstrated the fine structure of centromere region as a trilaminar disk (Jokelainen 1967, Brinkley and Stubblefield 1970, Comings and Okada 1971, Roos 1973, Ris and Witt 1981). By contrast the light microscopical observation of centromere has been poorly established since the centromere region can not be seen as a particular structure at the site of primary constriction in routine cytological preparations.

There are some reports in the literature from workers who described on structural patterns of the centromere under a light microscope employing the modified technique involving a hypotonic treatment (Ohnuki 1968, Khan 1969, Seto 1972) or a fixation method (Clapham and Östergren 1978), or by application of a silver staining technique (Brown and Loughman 1980). These works did not give persuaded visualization in comparable to the evidence by electron microscopy. The present paper describes more detailed observation of centromere region without suffering violent treatment. The large size of the urodelan cell species makes it ideal for morphological details of chromosomes using light optics. The

* Department of Biology, Faculty of Education, Shimane University, Matsue, Japan 690.

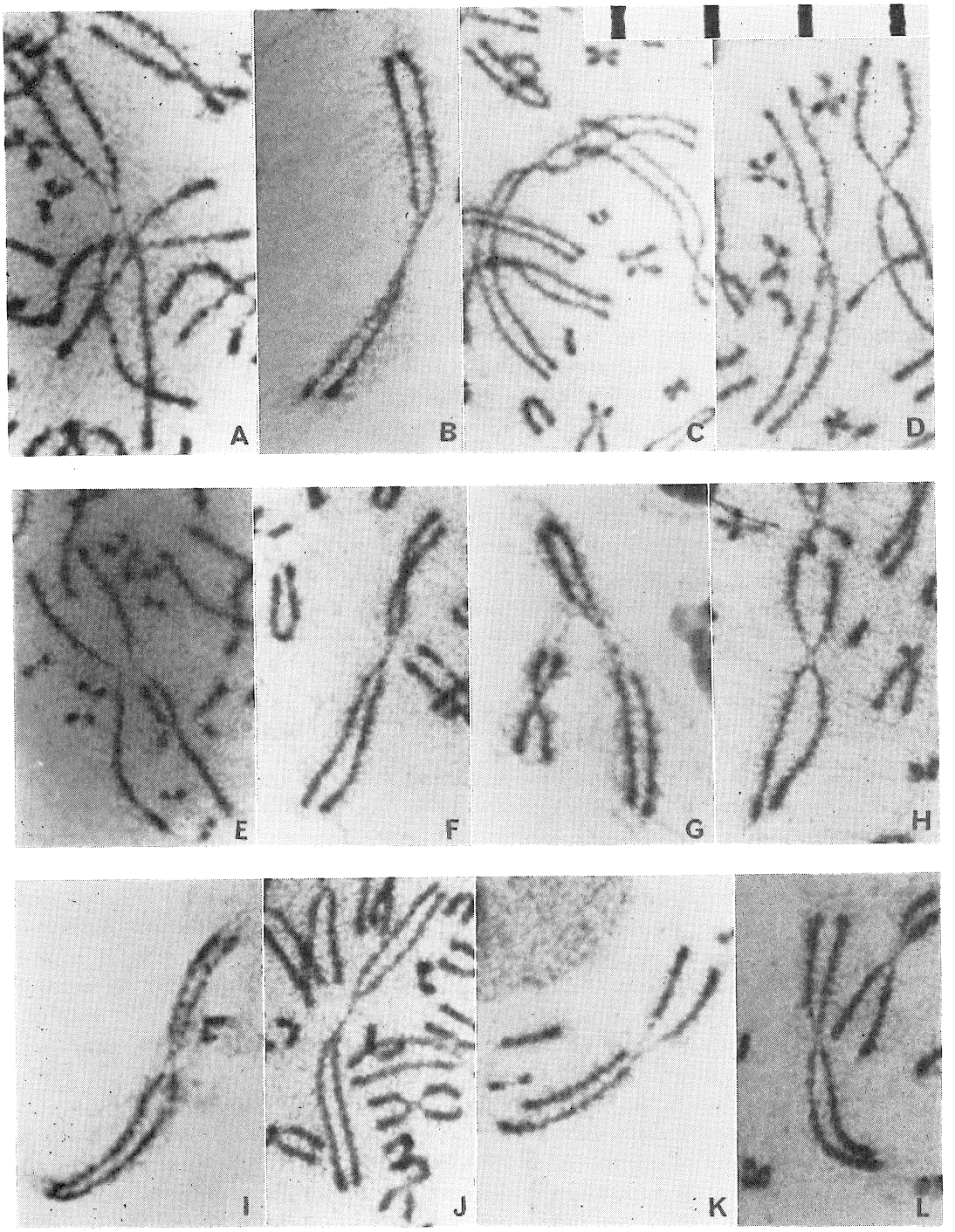

Figure 1. The largest chromosomes in the karyotype of *Hynobius n. nebulosus* (♀), selected from gut epithelial cells. These were treated with colchicine for 48 hours and the modified hypotonic solution. Several types of the centromeric chromomeres can be seen. Scale : 1 division=10 μm.

influence of such cytological treatments as colchicine, hypotonic solution, fixation, air-dry preparation and staining procedures on centromere morphology were comparatively studied. The consideration is made concerning the reliability of published techniques for the centromere detection.

Materials and Methods

Materials used in the present observations were urodelan somatic cells *in vivo* and *in vitro* derived from adult newts, *Cynops pyrrhogaster*, and adult hynobiid salamanders, *Hynobius nebulosus nebulosus*.

In vivo study of metaphase chromosomes was made thoroughly by a technique of Kezer and Sessions (1979) using intestinal tract. Gut epithelial cells were treated *in vivo* with colchicine (SIGMA) at 0.2 mg per gram of body weight for 48 hours before fixation. Metaphase-arrested chromsomes were stained in most cases with a conventional Giemsa stain. The improved staining procedures for identification of specific chromosome region were also attempted as well as the differential staining by Giemsa. These were the Cd banding by Eiberg (1974), the silver staining by Brat *et al.* (1975) and Brown and Loughman (1980).

For the morphological study of non-pretreated chromosomes in the cytological preparations, cultured newt lung cells *in vitro* were used. These were grown in the Rose culture chamber by a method of Seto and Rounds (1968). Dividing cells were fixed at metaphase without colchicine and hypotonic pretreatments. The chromosomes were observed both before and after staining for comparing the visualization of centromeres. A carbol fuchsin stain (Carr and Walker 1961) was applied in the case. Chromosomes from gut epithelial cells of newts were also observed in the non-pretreated, squashed preparation.

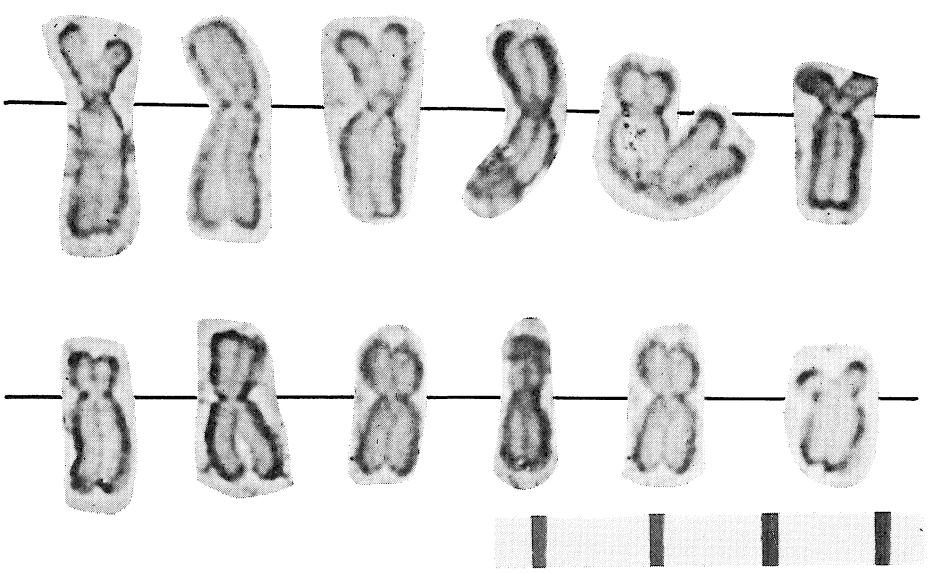

Figure 2, Selected chromosomes from newt somatic cells treated with silver nitrate after making the squash preparation. Majority of chromosomes appeared two identical dots on the lateral edges of the centromere region, but periphery of chromosome arms was also stained to a certain extent. Scale : 1 division=10 μm.

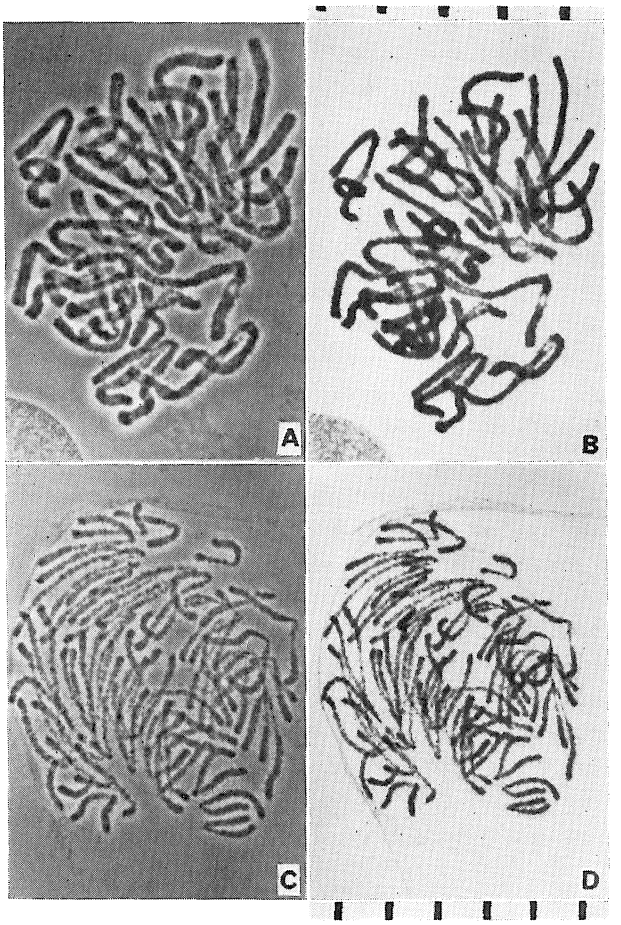

Figure 3. Chromosomes at metaphase and anapahse of mitotic division, showing that comparative features of centromeres in unstained and stained preparations. *A* & *C*, unstained, phase-contrast. *B* & *D*, stained with a carbol fuchsin stain. Scale : 1 division=10 μm.

Observations

Centromere Region of Colchicine Treated Chromosomes

Chromosome preparations which were processed by colchicine treatment and the conventional staining revealed that the primary constriction have no specific structure in any chromosome. The centromeres in large and extended chromosomes displayed as achromatic or less stainable features in the region. No other characteristic feature than the constriction or concaved appearance has observed in the centromere region.

In the preparation made by the modified technique involving the hypotonic treatment (Seto 1972) the centromere region appeared as an uncoiled strand of chromonemata. As shown in figure 1, the largest pair of *Hynobius* somatic chromosomes demonstrated there were a variety of centromere features ; some were condensed centromeric body appeared in the middle of fibrilar zone (Figs. 1D, 1F), and some were a pair of chromomere showing parallel arrangement (Figs. 1A, 1E) or linear arrangement on the centromere region (Figs. 1B, 1C, 1J). A quadruple structure composed of four centromeric chromomeres infrequently seen (Figs. 1H, 1E). However the structure was not invariable even in the same chromosome of different cells.

A silver staining technique for locating the centromere also applied to the newt intestine cells using a method of Brown and Loughman (1980). Majority of chromosomes in individual cell appeared two identical dots on the lateral edges of the centromere region (Fig. 2). Density of the spot was variable and the staining pattern in each chromosome varied in size from an ovoid dot to an minute spot. The periphery of chromosome arms was darkly stained in most cells indicating that the silver dye tended to remain on the edges.

Centromere Region in the Uncolchicinized Chromosomes

The chromosomes of both unstained and stained cells which were not exposed to colchicine and hypotonic solution were examined at both stages of metaphase and anaphase of newt lung cell division *in vitro*. A characteristic feature was that the primary constriction of the chromosomes was not very distinct and conversely, centromere region was raised to

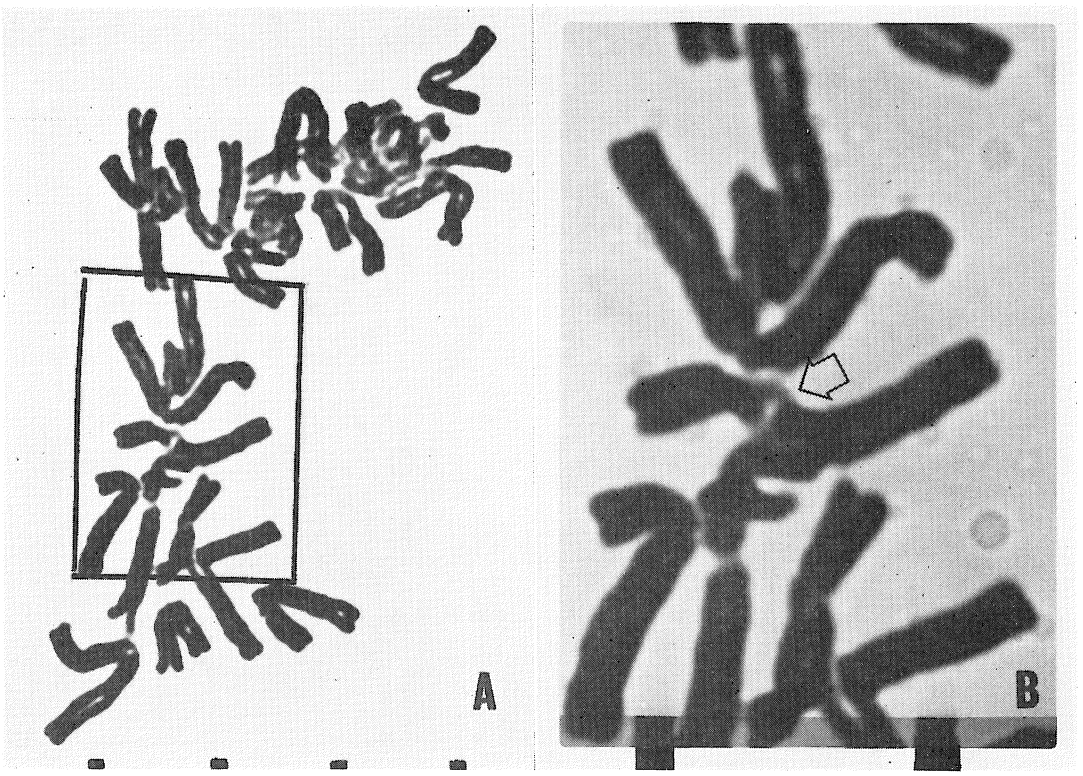

Figure 4. Particular feature of centromere region was observed in a cultured newt cell *in vitro*. An unsquashed cell was treated neither with colchicine nor hypotonic solution before the cell fixation. **A**, metaphase chromosomes in the cell. **B**, enlarged figure of a part of **4A**, note the stretched-out shape of the centromere (*arrow*). Scale : 1 division=10 μm.

outside toward the poles on opposite side of sister chromatids. Figures 3 and 4 demonstrated examples of the stretched-out centromere located on opposite side of sister chromatids at metaphase. Stainability of the region to Giemsa stain was not likely to well-stained chromosome arms. Most chromatids were not splitted along the chromosome arm, but the hole between the chromatid in the middle of the centromere region was of distinct (Fig. 4B).

In the squashed preparation of uncolchicinized chromosomes from newt gut epithelium, the centromere region showed the stretched-out shape which have more fine tip than the unsquashed preparation (Fig. 5). Such stretched-out figure was not seen in colchicinized chromosomes. The results indicated that the centromere region of uncolchicinized chromosomes were unlikely with colchicinized metaphase chromosomes. Then, less cytological procedures such as uncolchicinized, non-hypotonic treatment, and non-squashed preparation will represent more actual figures of the centromere.

Discussion

There is general agreement on the major aspects of centromere ultrastructure, although there is significant variation on some detailes (Jokelainen 1967, Brinkley and Stubblefield

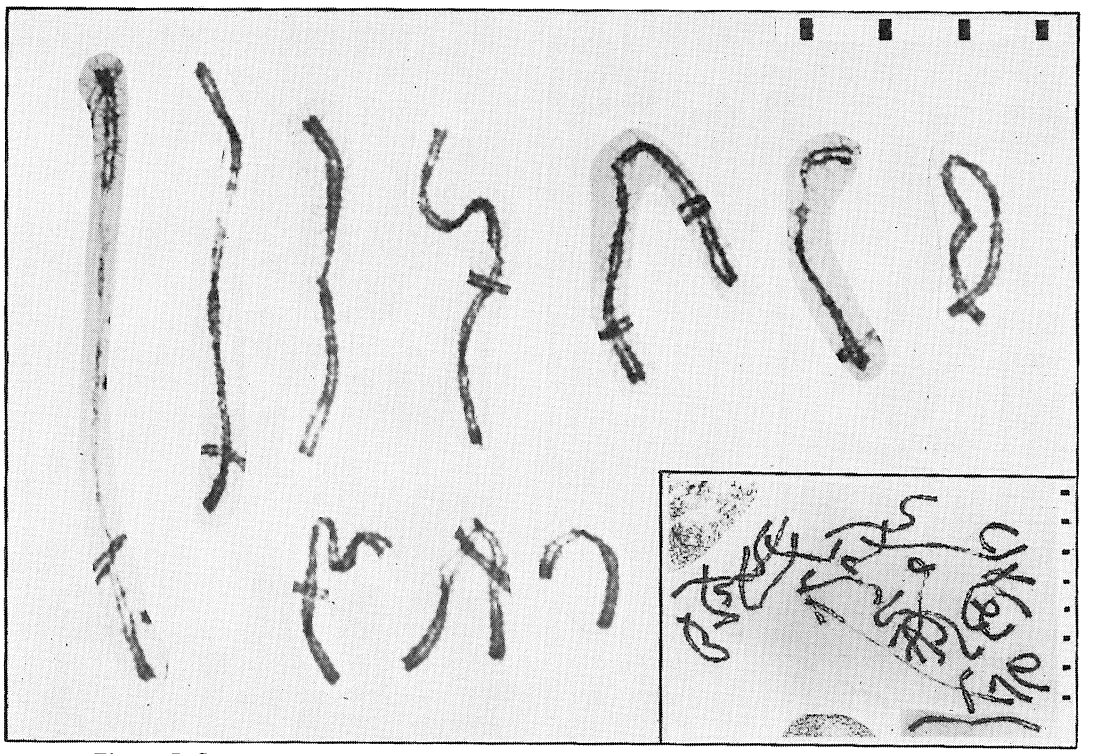

Figure 5. Selected chromosomes from a newt mitotic cell without colchicine treatment, followed by conventional fixation and staining methods. Centromeres appeared as more fine stretched-out shape in the squashed preparation. Scale : 1 division=10 μm.

1970, Comings and Okada 1971, Roos 1973, Ris and Witt 1981). Based on the studies by electron microscopy the centromere structure is explained that they can be divided into two distinct classes ; the "ball and cup" characteristic of higher plant and some species of animals, and the "trilaminar disk" typical of many mammalian cells. However, it is perhaps worth noting to the Rieder's hypothesis that both classes of centromeres many be found in the same cell depending on the stage of division (Rieder 1979) ; prophase centromeres in PtK_1 cells may resemble the ball-and-cup variety but then differentiate during prometaphase into the trilaminar structure.

On the other hand, the investigation at the level of a light microscope has not been probed and no definitive structural patterns of centromere has been displayed. In addition, the centromere is very minute in general and less conspicuous than the chromomeres, and they are rather delicate and labile organelles. This could be the main reason that the centromere visualization by the light microscope was not as fully established as the ultrastrucure research.

Centromere region usually appeared as a unstainable gap or a constriction in metaphase chromosome of colchicine-treated cells under a light microscope. In human chromosomes a dark staining chromomere or a "well-demarcated circle" has observed in the middle of the unstained region and it was indicated as the centromere or kinetochore (Lubs and Blitman 1967, Chen and Palek 1969, Khan 1969). Khan (1969) reported a morphological pattern of the centromere region to be characterized by a quadruple structure having four distinct centromeric chromomeres. He insisted the centromere was formed by fibrous

connections present between the four centromeric chromomeres.

In contradiction to Khan's work, Ohnuki (1968) with a fine preparation of the spiral structure of human chromosomes, demonstrated that the centromere displayed no specifically differentiated configuration other than uncoiled threads ; there was neither existence of a defined special body or so-called spherule as a component of centromere, nor any differentially stained body such as knobbed thread, as described in certain plants. Our results with urodelan chromosomes which were obtained by a preparation with and without colchicine-and hypotonic-pretreatment supported the Ohnuki's conclusion. It is probable that "labile" centromere is affected drastically by the treatment used in the chromosome preparations and then lost its detectable structure. Therefore we do not agree the Khan's description that a quadruple structure composed of centromeric chromomeres and fibrous connections is centromere itself, he may have simply illustrated chromatid fiber and pericentric chromomeres.

Silver stainings were also regarded as a centromere visualization technique by several authors (Brat *et al.* 1979, Brown and Loughman 1980). Present observation, however, did not confirm these methods were effective to observe centromeres at the optical level. The reasons were, first, both techniques have difficulty to reproduce centromere-spcific dots, second, the silver positive dots appeared on the centromere region were improbably larger size than that of actual kinetochore size, and third, silver stain appeared not specific to centromere alone but also positive to the periphery of chromosome arms.

Eiberg (1974) and Evans and Ross (1974) described improved Giemsa staining technique which revealed specific paired dots in the centromere region of human chromosomes. They hypothesized that these dots may represent the centromeres and particularly their associated proteins. According to Roos (1975) with his optical and electron microscopic evidences, however, the centromeric dots were not kinetochores but a specific DNA-protein composition of the centromeric chromatin. He also found that a two hour exposure of rat kangaroo cells to 0.05 μg per ml colcemide destroyed all microtubules and altered the fine structure of the centromere. Thus he proved that possibility of destructive effects by cytological treatment such as colchicine and hypotonic solution to delicate and labile centromeres. Our results coincide the Roos' evidence and so far as we know the stretched-out structure at metaphase could be a real figure of centromeres at the light microscope level.

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