

Autoradiographic study of ^3H colchicine binding in synchronously dividing cells of antheridial filaments of *Chara vulgaris* L. during successive stages of development

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(Received: August 1, 1974)

Abstract

The intensity of ^3H colchicine binding was investigated autoradiographically as a marker of an amount of the microtubule subunits during interphase and mitosis in synchronously dividing 4-, 8-, 16- and 32-celled antheridial filaments of *Chara vulgaris*. These cells were incubated with ^3H colchicine *in vivo* or after fixation. The radioactivity of cells in the successive generations of antheridial filaments diminishes, similarly as the surface of cytoplasm and intensity of protein synthesis. During interphase the intensity of ^3H colchicine binding is proportional to the increase of cytoplasmic surface; the highest increase of radioactivity occurs in G_2 . During mitosis the increase of radioactivity continues in prophase; the highest radioactivity was found in prophase and telophase cells, the lowest in anaphase cells; a comparatively pronounced radioactivity is visible in metaphase. Radioactivity in posttelophase, as estimated per one daughter cell, is approximately one half of that of the mother cells in telophase of the previous generation suggesting the reutilization of microtubule proteins in the next mitotic cycle.

INTRODUCTION

Biochemical investigations on the mechanism of colchicine action on microtubules have revealed that this alkaloid specifically binds *in vivo* as well as *in vitro* with subunits of microtubule protein; these subunits have a sedimentation constant 6 S (Shelanski and Taylor, 1967; Borisy and Taylor, 1967). Microtubules of isolated mitotic apparatus as well as those of flagella do not bind colchicine, but after their depolymerization *in vitro* by low ionic strength or by thermal process, 1 mol. of tubulin binds approximately 1 mol. of colchicine (Borisy and

Taylor, 1967; Wilson and Meza, 1973). These experiments indicate that the binding of colchicine prevents assembly of the subunits into a microtubule. On the other hand, since colchicine specifically binds to the microtubule subunits, the level of this binding may be used to assay the presence of microtubule protein, although it cannot be excluded that colchicine may also be bound by nontubulin membrane components — endoplasmic reticulum and nuclear membranes (Stadler and Franke, 1974; cf. Bardale, 1973).

In the investigations of the cell cycle the period of synthesis of microtubule protein is the essential problem because these protein subunits are necessary for formation of the mitotic spindle and their amount could be one of the factors determining the initiation of mitosis. One of the hypotheses concerning mitosis initiation, based on experiments with the plasmodium of *Physarum polycephalum*, and the results of other authors (Sachsenmaier and coll., 1972) established the production of specific "division protein", the amount of which increases gradually during interphase; these authors have suggested that these are spindle proteins. By apply of ^3H colchicine and biochemical methods, investigations on the synthesis of microtubule protein during the cell cycle were carried out on synchronously dividing mammalian cells (synchronization obtained by selective detaching of mitotic cells). During the HeLa cell cycle, synthesis of this protein is continuous; the amount of colchicine binding protein per cell was the same at three points of interphase and proportional to the other synthesized proteins (Robbins and Shelanski, 1969). In chinese hamster cells the synthesis of microtubule protein occurred almost exclusively in G_2 phase of the aneuploid line, whereas in the diploid line the microtubule protein was synthesized in mid S phase as well as in G_2 phase; microtubule protein synthesized in G_2 phase survived into the ensuing G_1 phase, but it was catabolized in early S phase (Forrest and Klevecz, 1972).

The cells of antheridial filaments of *Chara vulgaris* showing the spontaneous synchronization of cell cycles are convenient material for autoradiographic experiments on the intensity of ^3H colchicine binding during interphase and mitosis. The mitotic spindle contains typical microtubules (Pickett-Heaps, 1968). Moreover, because with the same time of S phase duration, the time of G_2 shortens in the successive stages of antheridial filaments development (Godlewski and Olszewska, 1973), and also since limitation of cell growth, binding of ^3H actinomycin D and synthesis of RNA and protein takes place (Olszewska and Godlewski, 1972; Olszewska, 1974 a and b), it seemed probable that the relative intensity of synthesis of microtubule protein can be at least one of the factors determining earlier initiation of mitosis in successive stages of the development of antheridial filament of *Chara*. However, the results of the present experiments indicate that the in-

tensity of ^3H colchicine binding in interphase is proportional to the cytoplasmic surface and, that a more distinct increase of the microtubule protein content occurs in the early prophase, i.e. after initiation of mitosis. The microtubule proteins appear to be conserved and re-used by the next cell generation of antheridial filaments.

MATERIAL AND METHODS

Preliminary experiments with nonradioactive colchicine showed that the microtubule subunits in the cells of antheridial filaments of *Chara* are sensitive to this alkaloid, because in its presence typical c-mitosis occur.

The apical parts of *Chara vulgaris* thallus were incubated in aqueous solution of ^3H colchicine (ring-methyl ^3H , 25 $\mu\text{Ci/ml}$, 2 ci/mM for 2 hr. To protect the transformation of colchicine into nonactive luminocolchicine (cf. Bardale, 1973), the incubation was done in darkness. To make possible a comparison of the intensity of ^3H colchicine binding with radioactive amino acids incorporation in the interphase of the successive cell generations of antheridial filaments, the incubation time was the same as in our previous experiments (Olszewska and Godlewski, 1972; Olszewska, 1974 b). The material was fixed in 2% glutaraldehyde buffered to pH 7.2–7.3 with cocadylate buffer, For 1 hr at 4°C. The isolated antheridia were squeezed out onto microslides, covered with Ilford K2 liquid emulsion and exposed for 108 days. After development the autoradiograms were stained with Mayer's haematoxylin. Intensity of ^3H colchicine binding was determined on the basis of the number of grains in the autoradiograms.

To check whether the radioactivity and localization of grains in the autoradiograms during mitosis is not due to ^3H colchicine binding by the microtubule subunits at the time preceding mitosis and, therefore, their arrangement into microtubules, in analogous experimental conditions antheridial filaments fixed as above and squeezed out from antheridia were incubated with a four times higher concentration of ^3H colchicine (100 $\mu\text{Ci/ml}$) for 2 hr. Specimens were washed in excess of unlabeled colchicine (1 hr.), rinsed in tap water (0.5 hr.), covered with Ilford K2 liquid emulsion and developed after 26 days of exposition.

According to our previous statements (Olszewska and Godlewski, 1972), the length of cells determined the phases of the cell cycle. As the S phase begins as early as in telophase, the posttelophase cells were considered as the early S phase. For the other phases of interphase the approved length of cells is given in Table 1.

Measurements of the cytoplasmic surface in the early S, early and late G_2 phases were taken with a planimeter on micrographs at magnifi-

Table 1
Length of cells in μm in antheridial filaments in successive phases of interphase

Phase of development of antheridial filaments	Phase of interphase		
	late S	early G ₂	late G ₂
4-celled	14	18	22
8-celled	12	14	18
16-celled	10	12	15
32-celled	7.5	8	10

cation 10 000 \times , obtained from the same autoradiograms which were taken for counting the number of grains in emulsion.

RESULTS

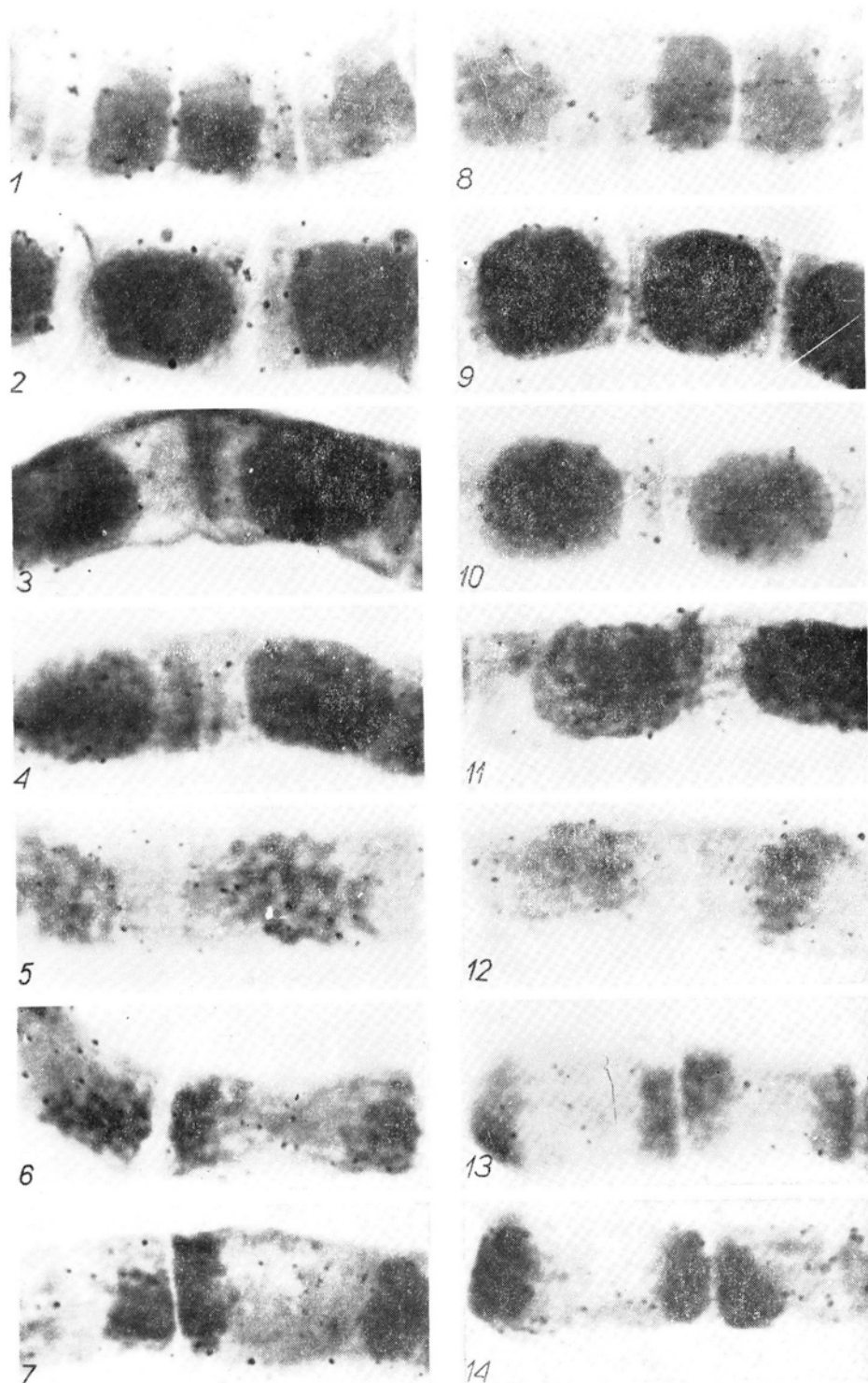
In every examined generation of antheridial filaments, i.e. in the 4-, 8-, 16- and 32-celled ones, the cytoplasmic surface indicates a slight increase during the S phase. Its main growth, gradually limited in the successive stages of antheridial filaments development occurs in G₂ (Graph 1). Since the cells of antheridial filaments in the successive stages of development reach a shorter length in prophase (Plate I and II), the mitotic spindle as well as the phragmoplast are correspondingly shorter.

Binding of ³H colchicine *in vivo*

No alterations in the course of mitosis in cells incubated *in vivo* with ³H colchicine were noticed.

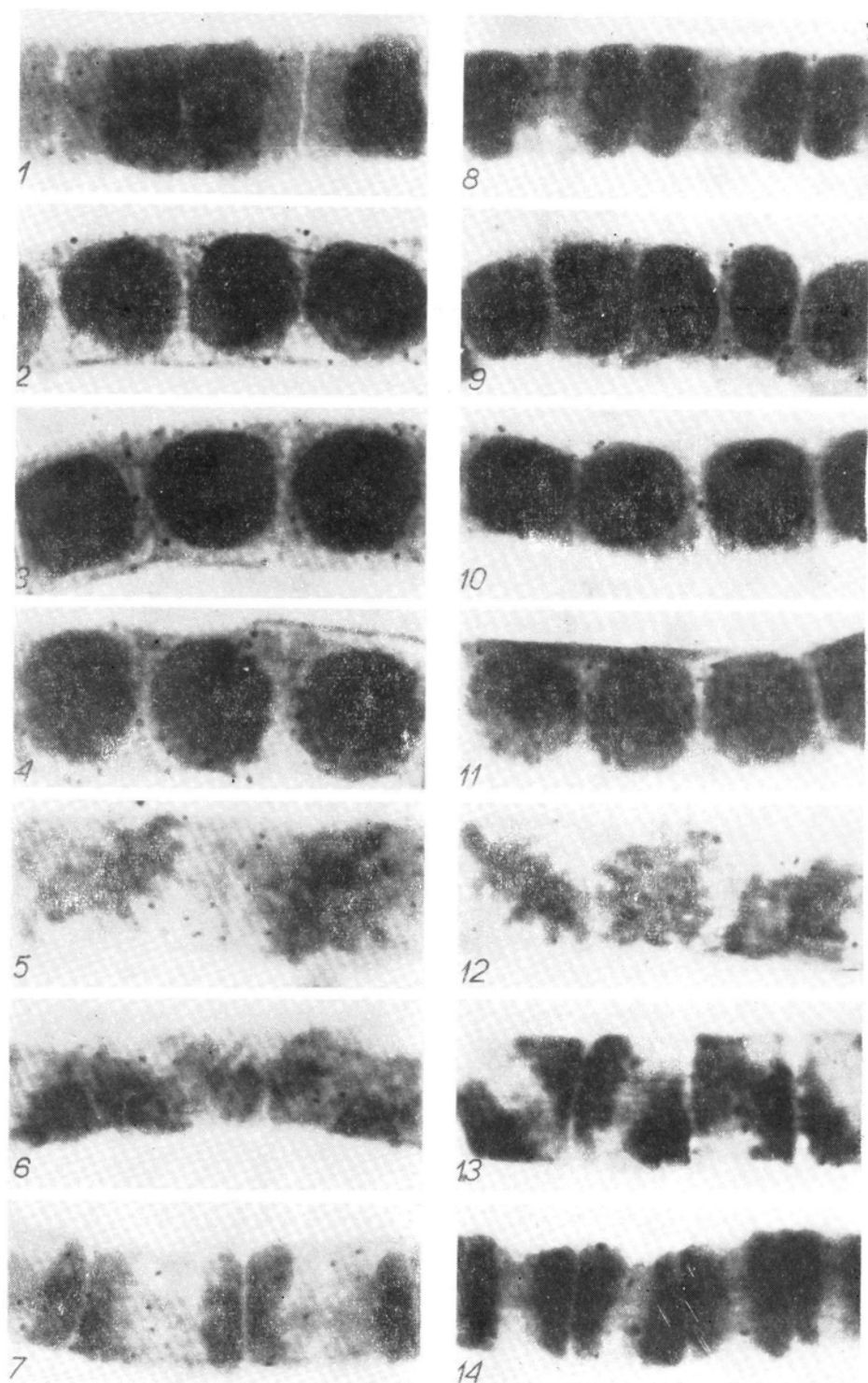
The intensity of ³H colchicine binding in interphase is proportional to the increase of the cytoplasmic surface (Graph 1). In every stage of development of antheridial filaments, ³H colchicine binding in interphase is the highest in the late G₂ (Table 2; Fig. 1; Plate I, Figs 1—3 and 8—10; Plate II, Figs. 1—3 and 8—10).

During mitosis in the successive stages of antheridial filaments development the differences in intensity of ³H colchicine binding are still visible (Table 2; Fig. 1). In 4-, 8- and 16-celled filaments the binding of ³H colchicine increases during prophase, although a part of the microtubule subunits is already built into the microtubules of the forming mitotic spindle (Pickett-Heaps, 1968). During metaphase the binding of ³H colchicine diminishes reaching a minimum level in anaphase and maximum in telophase — as calculated per one mother cell (Table 2; Graph 1; Plate I, Figs. 4—7, and 11—14; Plate II, Figs. 4—7 and 11—14).



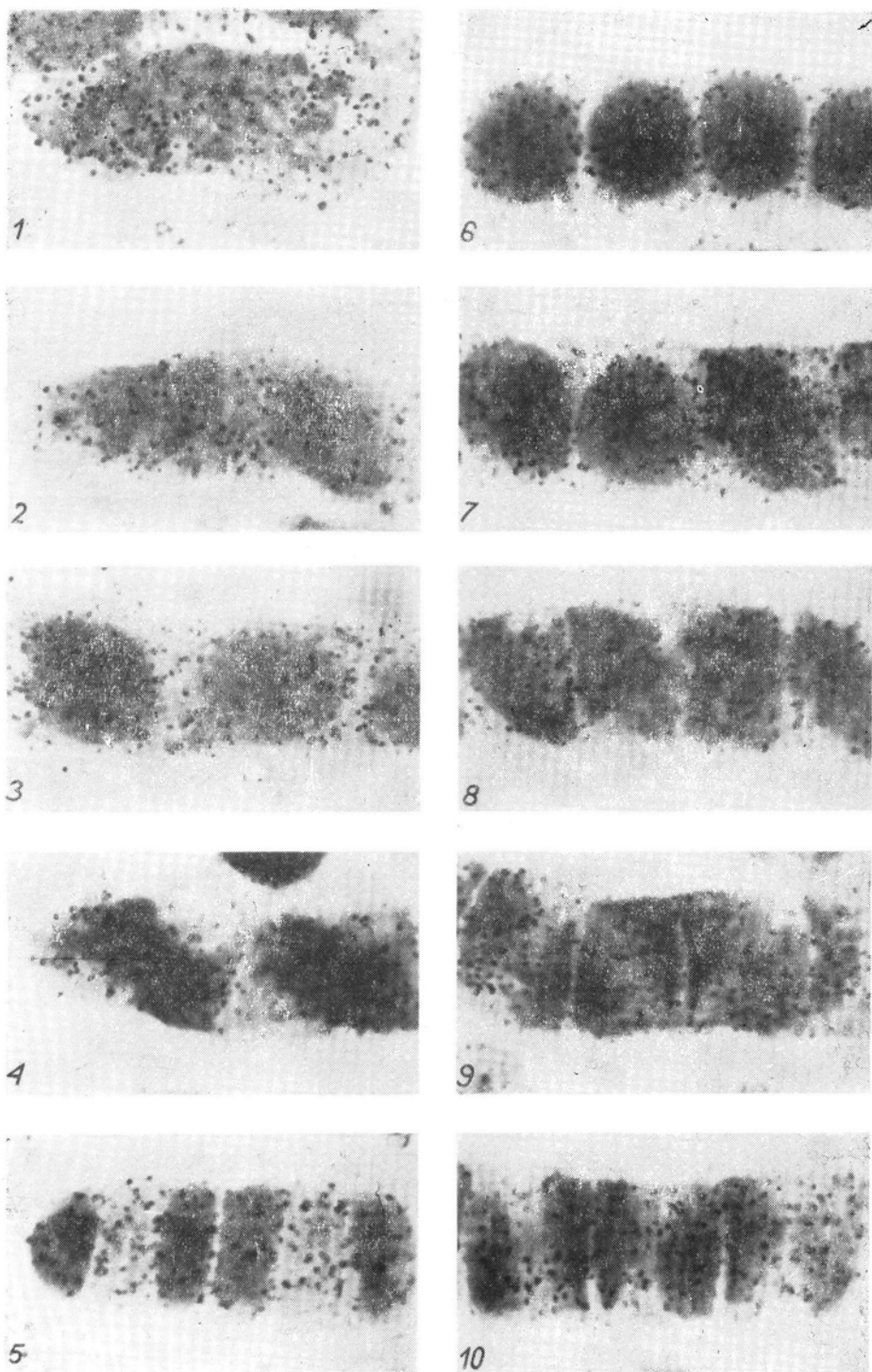
Binding of ^3H colchicine *in vivo* in 4-celled antheridial filaments (Figs. 1—7) and 8-celled antheridial filaments of *Chara vulgaris* L.; $\times 1500$

Fig. 1 and 8 — early S; Fig. 2 and 9 — early G_2 ; Fig. 3 and 10 — late G_2 ; Fig. 4 and 11 — prophase;
Fig. 5 and 12 — metaphase; Fig. 6 and 13 — anaphase; Fig. 7 and 14 — telophase



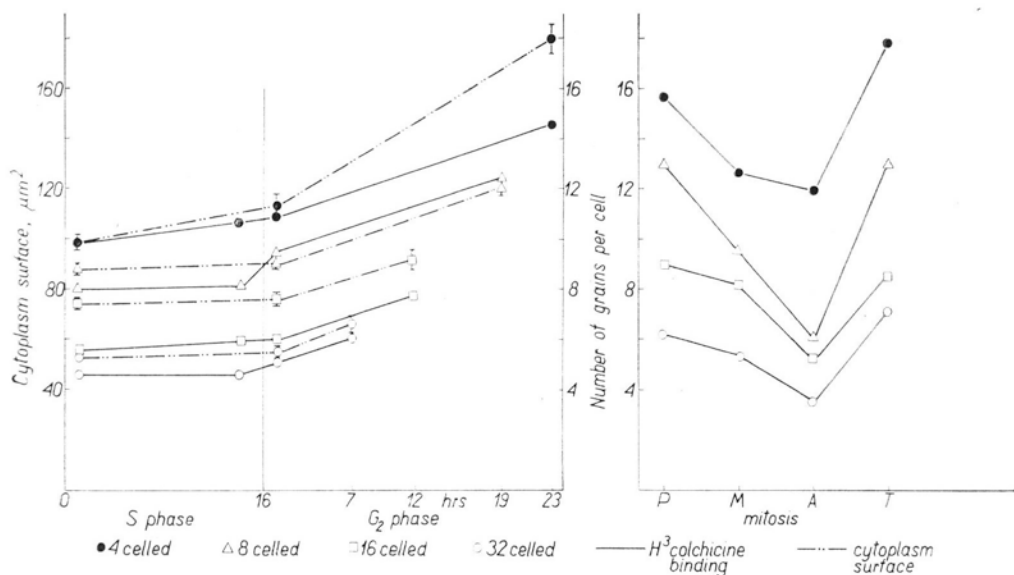
Binding of ^3H colchicine *in vivo* in 16-celled antheridial filaments (Figs. 1—7) and 32-celled antheridial filaments (Figs. 8—14) of *Chara vulgaris* L.; $\times 1500$

Fig. 1 and 8 — early S; Fig. 2 and 9 — early G_2 ; Fig. 3 and 10 — late G_2 ; Fig. 4 and 11 — prophase; Fig. 5 and 12 — metaphase; Fig. 6 and 13 — anaphase; Fig. 7 and 14 — telophase.



Binding of ^3H colchicine in fixed cells of antheridial filaments of *Chara vulgaris* L;
 × 1500

Fig. 1 — 1-celled filament, metaphase; Fig. 2 — 8-celled filament, late G₂; Fig. 3 — 16-celled filament, prophase; Fig. 4 — 16-celled filament, metaphase; Fig. 5 — 16-celled filament, late telophase; Fig. 6 — 32-celled filament, late G₂; Fig. 7 — desynchronized 32-celled filament, early prophase and metaphase; Fig. 8 — 32-celled filament, metaphase; Fig. 9 — 32-celled filament, anaphase; Fig. 10 — 32-celled filament, telophase.



Graph 1. Cytoplasm growth in interphase and ^3H colchicine binding *in vivo* during interphase and mitosis in synchronously dividing antheridial filaments of *Chara vulgaris* L.

P — prophase, M — metaphase, A — anaphase, T — telophase

As a rule in 16- and 32-celled antheridial filaments and sometimes in 8-celled ones, the axis of the mitotic spindle is in diagonal position along the diagonal line of the cell (Plate II, Fig. 5 — left cell, Fig. 12 — left cell; Plate III, Figs. 4 and 8). In the specimens in which the metaphase plate is seen from a lateral view, it may be noticed that the grains of emulsion are localized mainly in the area of the spindle poles (Plate I, Fig. 12 — right cell; Plate II, Fig. 5); correspondingly, in cells in which the metaphase plates are observed from the pole, the grains of emulsion are grouped mainly over chromosomes (Plate I, Fig. 5).

In anaphase the grains of emulsion are localized mainly in the equatorial area (Plate I, Figs. 6 and 13), but in telophase they are dispersed uniformly in the cytoplasm of both daughter cells (Plate I, Figs. 7 and 14; Plate II, Figs. 7 and 14).

The results obtained indicate that the highest amount of ^3H colchicine in every generation of antheridial filaments is bound by prophase and telophase cells (Table 2). In every following generation of antheridial filaments the cells in early S phase, i.e. posttelophase (Plate I, Figs 1 and 8; Plate II, Figs. 1 and 8) show a radioactivity (considering the standard error) of approximately one a half of that of the mother cell in telophase of the previous generation (Table 2). This fact suggests that the microtubule proteins are preserved during the successive generations and their synthesis in each stage of antheridial filaments development takes place in G_2 and prophase.

Table 2
Binding of ^3H colchicine *in vivo* in cells of antheridial filaments during interphase and mitosis

Stage of development	Number of grains							
	Interphase				Mitosis			
	early S	late S	early G ₂	late G ₂	prophase	metaphase	anaphase	telophase
4-celled	9.8 ± 0.5	10.6 ± 0.3	10.8 ± 0.5	14.6 ± 0.4	15.7 ± 0.8	12.6 ± 0.4	11.9 ± 0.6	17.8 ± 0.4
8-celled	8.0 ± 0.3	7.0 ± 0.6	9.5 ± 0.3	12.4 ± 0.3	13.0 ± 0.5	9.5 ± 0.3	6.0 ± 0.5	13.0 ± 0.7
16-celled	5.4 ± 0.2	5.4 ± 0.2	6.1 ± 0.2	7.7 ± 0.3	9.0 ± 0.1	8.2 ± 0.3	5.2 ± 0.3	8.7 ± 0.3
32-celled	4.7 ± 0.2	4.3 ± 0.2	5.2 ± 0.2	6.0 ± 0.2	6.2 ± 0.2	5.4 ± 0.1	3.6 ± 0.2	7.2 ± 0.4

Binding of ^3H colchicine in fixed cells

In spite of washing the specimens after incubation in nonradioactive colchicine and in running water, a distinct adsorption of ^3H colchicine by gelatin covering the microslides was noticed. The background formed in this way made impossible the counting of grains and also a comparison of the radioactivity in the successive stages of antheridial filaments development.

The purpose of the present experiment was the first of all to establish whether cells during mitosis, and especially in metaphase, bind ^3H colchicine. The obtained results (Plate III) reveal the presence of microtubule subunits in the mitotic cells. A comparison of the cell radioactivity during prophase and in metaphase in the same antheridial filament (Plate III, Fig. 7) shows its significant amount in metaphase. Similarly, as in experiments *in vivo*, the lowest radioactivity demonstrate cells in anaphase (Plate III, Fig. 9).

DISCUSSION

To the author's knowledge no autoradiographic experiments concerning ^3H colchicine binding during interphase and mitosis have been performed. Therefore we only can compare our data with the biochemical results for synchronized mammalian cells, or fertilized sea urchin eggs.

In cultured human amnion cells (Sisken and Wilkes, 1967) and in the diploid as well as aneuploid lines of chinese hamster cells (Forrest and Klevecz, 1972) the protein related with mitosis is synthesized in G_2 . This conclusion was made by the authors mentioned above on the basis of different experiments. The action of p-fluorophenylalanine, an analogue of phenylalanine, replacing that amino acid in protein synthesis and causing protein defectivity, is expressed in the prolongation of metaphase duration and shortening of the mitotic spindle (Sisken and Wilkes, 1967). Forrest and Klevecz (1972) have investigated the level of microtubule protein and, they compared it with the amount of protein bound with colchicine. The similar results indicating an increase of tubulin synthesis after fertilization were obtained for sea urchin eggs (Mecker and Iverson, 1971).

The results of Robbins and Shelanski (1969) revealing the same level of colchicine-binding protein as of other proteins synthesized during interphase of HeLa cells, were criticized by Forrest and Klevecz (1972), because of the very few, in their opinion, points of interphase (only 3) chosen for investigations. According to Forrest and Klevecz (1972), microtubule protein is degraded in the early S phase and synthesized in mid S phase as well as in G_2 . Our results indicate that the amount of ^3H colchicine bound *in vivo* increases during

interphase proportionally to the increase of the cytoplasmic surface and to the incorporation of ^3H phenylalanine, ^3H arginine and ^3H tryptophane into the cytoplasm of successive generations of antheridial filaments (Olszewska and Godlewski, 1972; Olszewska, 1974b). Similar results were reported by Mecker and Iverson (1971) who concluded that the rate of tubulin synthesis resembles that of total protein synthesis during the first division cycle in sea urchin zygotes. Thus, the increase of protein content binding ^3H colchicine in G_2 of all developmental stages of antheridial filaments of *Chara* is related with higher than in the S phase intensity of cytoplasmic protein synthesis.

The next phenomenon ascertained by autoradiography as well as by biochemical methods concerns reutilization of microtubule protein by daughter cells. This conclusion is based on our results as well as those of experiments with cultured human amnion cells mentioned above (Sisken and Wilkes, 1967). Those data differ from the results of Forrest and Klevecz (1972) concerning the diploid line of chinese hamster cells (but not aneuploid ones) demonstrating that microtubule protein synthesized in G_2 phase exists in G_1 , but is catabolized rapidly concomitantly with initiation of DNA replication. Moreover in our experiments a diminution of radioactivity was noted neither in early nor in late S phase; the number of grains during the period of DNA replication represents approximately one half of the telophase cells of the previous generation. Therefore we can assume that the microtubule subunits are reutilized in the next cell generations and their synthesis taking place in interphase mainly in G_2 shows the same intensity as that of other cytoplasmic proteins.

As regards the amount of microtubule subunits during mitosis, it seems likely that their rapid synthesis occurs together with the initiation of mitosis. After 2 hr. incubation the prophase and metaphase cells take up ^3H phenylalanine, but in a lower degree than in late G_2 (Godlewski, unpubl.). The radioactivity after incubation with ^3H colchicine *in vivo* increases in prophase (except in 32-celled filaments). In spite of that a certain part of subunits is already arranged into microtubules forming a spindle (Pickett-Heaps, 1968). The relatively high radioactivity of metaphase cells after incubation *in vivo* in all developmental stages of antheridial filaments could be explained as the result of binding of microtubule subunits with colchicine which took place during 2 hr. of incubation; that binding prevented the polymerization of these subunits into spindle microtubules. However, the distinct radioactivity of metaphase cells incubated with ^3H colchicine after their fixation indicates that in this mitotic phase, in spite of subunits arrangement into spindle microtubules, a large pool of monomers exists. Comparison of the radioactivity being the consequence of ^3H colchicine binding in the late G_2 , in prophase and metaphase *in vivo* as well as in fixed cells, suggests

that there occurs an increase in the amount of microtubule subunits also after initiation of mitosis. Probably this process takes place mainly in prophase, i.e. in that mitotic phase, when protein synthesis still lasts, and slightly in metaphase. In fertilized sea urchin eggs there is a pronounced depression in the rate of tubulin synthesis in metaphase (Mecker and Iverson, 1971).

In all our experiments the lowest radioactivity of cells was found in anaphase. It is a surprising result because each hypothesis interpreting the mechanism of anaphase movements on the basis of the active role of spindle microtubules (sliding hypothesis of chromosome movements — Inoué and Sato, 1967; dynamic balance between the pool of free subunits and those arranged into microtubules — Diers, cf. Bajer and Molè-Bajer, 1972, cf. Bardale, 1973; zipper hypothesis — Bajer, 1973) assumes the depolymerization of microtubules. Moreover on the basis of microtubule counts, Fuge (1974) concludes that the total amount of monomers assembled as microtubules diminishes in anaphase. On the other hand, the lowest radioactivity of cells in anaphase would be the result of very fast use of the subunits for formation of phragmoplast; this process in the endosperm cells of *Haemanthus katharinae* is preceded by breaking of microtubules of the continuous spindle fibers (Bajer and Molè-Bajer, 1972).

A high radioactivity of telophase cells is the consequence of breaking of the microtubules of phragmoplast. In antheridial filaments of *Chara*, in the same stage of telophase as in our experiments, the microtubules are not already visible (Pickett-Heaps, 1968). In a comparable situation, i.e. in fertilized sea urchin eggs, as the first mitotic division is completed, the amount of colchicine-binding material increases (Mecker and Iverson, 1971).

Added in proof: Recent experiments by M. Kwiatkowska and J. Maszewski in our laboratory indicate that at 4°C the microtubules in *Chara vulgaris* antheridial filaments disappear. Thus, in our experiments with ³H colchicine incubation of fixed cells, their high radioactivity may be due to the increase of microtubules subunits content caused by their desintegration at 4°C.

Acknowledgement

I am very indebted to Mrs. Aleksandra Wołczańska, M. Sc., for her valuable technical assistance.

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Badania autoradiograficzne wiązania ^3H kolchicyny przez synchronicznie dzielące się komórki nici spermatogenicznych Chara vulgaris L. w kolejnych stadiach ich rozwoju

Streszczenie

Autoradiograficznie badano intensywność wiązania ^3H kolchicyny jako wskaźnika zawartości podjednostek mikrotubul podczas interfazy i mitozy w synchronicznie dzielących się 4-, 8-, 16- i 32-komórkowych niciach spermatogenicznych *Chara vulgaris*. Komórki były inkubowane z ^3H kolchicyną *in vivo* lub po utrwaleniu. Radioaktywność komórek w kolejnych pokoleniach nici spermatogenicznych zmniejsza się, podobnie jak powierzchnia cytoplazmy oraz nasilenie syntezy białek. Podczas interfazy intensywność wiązania ^3H kolchicyny jest proporcjonalna do wzrostu powierzchni cytoplazmy; największy wzrost radioaktywności przypada na fazę G_2 . Podczas mitozy następuje dalsze zwiększenie radioaktywności w profazie; największą radioaktywność wykazują komórki profazowe i telofazowe, najmniejszą — komórki anafazowe; stosunkowo znaczną radioaktywność stwierdzono podczas metafazy. Radioaktywność w posttelofazie w przeliczeniu na jedną komórkę potomną stanowi w przybliżeniu połowę tej, jaką wykazywały komórki macierzyste w telofazie poprzedniego pokolenia, co sugeruje wykorzystywanie białek mikrotubul w następnym cyklu mitotycznym.

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