# THE MECHANISM OF ACTION OF COLCHICINE

## Binding of Colchincine-$^3$H to Cellular Protein

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ABSTRACT

The majority of the colchicine-$^3$H bound by tissue culture cells (KB or Hela) was found to be present as a noncovalent complex with a macromolecule which appears in the soluble fraction after homogenization. Similar binding was demonstrated in vitro and was confined to a component of the soluble fraction. The binding-equilibrium constant and the kinetic constants were essentially the same in vivo and in vitro. Bound radioactivity was reisolated and shown to be present in a molecule with the same chromatographic behavior and specific antimitotic activity as colchicine. In vitro assay of binding activity of a variety of cells and tissues showed a correlation with the presence of microtubules. High binding activity was given by dividing cells, mitotic apparatus, cilia, sperm tails, and brain tissue. Binding to extracts of slime mold or to purified muscle proteins was very low or undetectable. The binding site had a sedimentation constant of 6S and it is suggested that the protein is a subunit of microtubules.

## INTRODUCTION

Colchicine is known to inhibit mitosis in a wide variety of plant and animal cells by interfering with the structure of the mitotic spindle (1). Other noteworthy effects of colchicine include the following: (*a*) reversibly inducing the retraction of the axopods of the Heliozoan, *Actinosphaerium* (2); (*b*) disrupting the formation of striations and myotubes in embryonic chick muscle (3, 4); (*c*) interfering with the orientation of newly deposited cellulose fibrils in plant cell walls (5); (*d*) suppressing saltatory movement in cultured mammalian cells (6); (*e*) causing acute poisoning in warm-blooded animals (7); and (*f*) relieving the symptomatic distress of gout (8). An understanding of the mechanism of action of colchicine should provide an explanation of these striking but apparently diverse phenomena.

In a previous report (9) it was shown that colchicine in concentrations as low as $5 \times 10^{-8}$ M can arrest mitosis in human carcinoma cells without inhibiting DNA, RNA, or protein synthesis or the progress of cells around the cell cycle and that colchicine-$^3$H was reversibly bound to some cellular constituent(s). The kinetics of inhibition of mitosis and binding of colchicine could be quantitatively described by a simple binding mechanism involving a single class of binding sites. These results suggest a specific mode of action and thus the diverse effects of colchicine imply that a target site is found in a variety of cell types.

The present report[1] is concerned with studies on

[1] Preliminary notes covering parts of the work reported here have appeared in the Abstracts of the papers presented at the 1963 meeting of the American Society for Cell Biology, (1963. *J. Cell Biol.* **19**:70A) and in Abstracts of Biophysical Society Meeting, WF-12, 1966.

the nature of colchicine binding. The primary objectives were to (*a*) demonstrate a colchicine-macromolecule complex in colchicine-treated cells, (*b*) study conditions for the colchicine-binding reaction in vitro, (*c*) determine whether the bound colchicine is chemically unchanged, and (*d*) survey colchicine-binding activity in various cell types and model systems. The results are consistent with the hypothesis that the binding site is the subunit protein of microtubules.

## MATERIALS AND METHODS

### *Culture Methods and Tissue Preparations*

The culturing of human cells, strain KB, the preparation of tritium-labeled colchicine, and the assay of colchicine binding to KB cells have been described in detail previously (9). Some experiments were also performed with HeLa cell cultures, grown in a similar manner. Homogenates were prepared from suspension cultures using a motor driven, stainless steel–nylon homogenizer at 0°C (10). This homogenizer was used in the various procedures subsequently described unless otherwise noted. All operations following homogenization were carried out at 0°–4°C. Cells were washed three times by centrifugation in isotonic phosphate-buffered saline and homogenized in 0.24 M sucrose, $10^{-3}$ M $MgCl_2$, 0.01 M tris buffer, pH 7 (SMT), or in 0.24 M sucrose, $2 \times 10^{-2}$ M $MgCl_2$, $2 \times 10^{-3}$ M ATP, 0.01 M tris buffer, pH 7 (SMAT).

Sea urchins (*Lytechinus pictus* or *Strongylocentrotus purpuratus*) were obtained from Pacific Biomarine Supply Co., Venice, Calif. Gametes were obtained by injection of 0.5 ml of 0.53 M KCl. *Arbacia punctulata* were collected at Woods Hole, Mass. and were spawned by application of 10-v ac current across the test. Protein extracts were prepared from eggs washed three times in isotonic 0.53 M KCl by suspending in four volumes of 0.01 M Sodium phosphate buffer, 0.01 M $MgCl_2$, pH 6.5 (phosphate–magnesium solution) and homogenized in a Potter type teflon–glass homogenizer.

*Tetrahymena pyriformis* was grown axenically in 2% proteose peptone (Difco Laboratories, Detroit, Mich.) and cilia were isolated according to the method of Watson and Hopkins (11).

Extracts of ciliary axonemes were prepared according to a modification of the procedure of Gibbons (12). Sea-urchin sperm tails were detached from the heads by sonication in a Branson Model S-75 Sonifier for 2 min at 0°C and were isolated by several cycles of sedimentation. Protein was extracted from the intact tails by dialysis against 0.05 M tris-thioglycollate buffer, pH 7.5 as described by Gibbons (13).

Slime mold (*Physarum polycephalum*) was grown in Petri dishes on Quaker Oats and allowed to migrate off the oatmeal onto a bed of 2% corn-meal agar (Baltimore Biological, Baltimore, Md.) according to the method of Camp (14) as modified by M. Adelman in this laboratory. Mold was harvested from the agar with a glass rod, washed three times with distilled water, resuspended in buffer, and homogenized. Homogenates were prepared from fresh or frozen mold under varying conditions of ionic strength and pH by using 0.01 M tris or 0.01 M phosphate buffers and various concentrations of KCl, divalent cations and ATP.

Homogenates of various tissues were prepared from rabbits, fasted 1 day prior to sacrifice. Fresh tissue was dissected from animals anesthetized with Nembutal or $MgSO_4$, and the tissue was cooled in isotonic phosphate-buffered saline, was minced with scissors, washed twice with saline and once with SMT (or SMAT), and was homogenized in three volumes of the same solution. The homogenates were centrifuged for 1 hr at 35,000 rpm in a Spinco 40 rotor at 3°C.

Brain tissue was obtained from freshly sacrificed rats, rabbits, or hogs. White and gray matter were obtained by dissection of the corpus callosum and outer layer of the cerebral cortex, respectively. The tissue was homogenized by the procedure described for other tissues. A sample of lyophilized squid axoplasm was generously provided by Dr. F. O. Schmitt of the Massachusetts Institute of Technology, and by Dr. S. Huneas of the University of Chile. The material was dissolved in 0.2 M KCl, in phosphate–magnesium solution.

Myosin was prepared according to the method of Holtzer and Lowey (15). Actin was prepared according to the method of Carsten and Mommaerts (16).

G-100 Sephadex was obtained from Pharmacia Fine Chemicals Inc., Piscataway, N. J., and allowed to swell in buffer for at least 24 hr. Fines were removed by repeated suspension and decantation. The Sephadex slurry was degassed by aspiration just prior to use. Blue dextran was used in 1% solution to determine the column-void volume.

### *Zone Sedimentation*

Linear sucrose gradients (5–20% sucrose) were prepared in 0.01 M phosphate, 0.01 M $MgCl_2$, 0.1 M KCl, pH 6.5, and were run in Spinco SW 39 or SW 65 rotors. A 0.15-ml sample was layered onto 5 ml of sucrose solution and 36 nine-drop fractions were collected.

### *Assay for Colchicine Binding to Macromolecules*

Material from the various cell and tissue preparations was clarified by centrifugation for at least 15 min at 100,000 *g*. In some cases centrifugation was

continued for 2.5 hr. A 1-ml aliquot of the supernatant solution was incubated at 37°C with 0.1 ml of stock colchicine, final concentration $2.5 \times 10^{-6}$ M, $10^6$ cpm. For routine determinations of binding the sample was subjected to gel filtration on a 1 × 15-cm Sephadex G-100 column and eluted with 0.01 M phosphate or 0.01 M tris buffer, pH 7. Fractions of 1.5 ml were collected and 0.2 ml was added to 10 ml diotol scintillation fluid (17), and counted in a Packard Tri-Carb Scintillation Spectrometer.

Protein content was estimated by using the Lowry method (18) and is expressed as milligram of protein based on a bovine-serum albumin standard (Armour Industrial Chemical Co., Chicago, Ill.).

## RESULTS

### *Colchicine Binding to Intact Mammalian Cells*

Suspension cultures of KB cells were incubated for 12–18 hr with colchicine-$^3$H at concentrations of $10^{-6}$–$10^{-7}$ M. Under these conditions the colchicine-binding sites are saturated (9). Cells were harvested and washed three times in phosphate-buffered saline by centrifugation to remove free colchicine. Previously it was shown that radioactivity taken up by the cells could be only slowly removed by washing, and the activity remaining after three washes was designated as bound colchicine. The cells were homogenized in 0.24 M sucrose (SMT solution) and the homogenate was fractionated by differential centrifugation. The results of a typical experiment are shown in Table I. Of the total radioactivity, 90–95% was found in the 100,000 *g* supernatant. That the supernatant radioactivity was slowly dialyzable compared to free colchicine indicated the presence of macromolecularly bound radioactivity.

In order that the amount of bound colchicine could be determined quantitatively, aliquots of the supernatant were subjected to gel filtration on G-100 Sephadex columns. A molecule will be eluted from a column in a volume $V_e$, which depends on its partition coefficient, $K_d$, between the interior and exterior gel spaces: $V_e = V_o + K_d V_i$. The void volume, $V_o$, is the volume external to the gel beads and $V_i$ is the internal volume. For low molecular weight molecules such as colchicine, $K_d$ is close to one while for macromolecules, it is a small number approaching zero. Therefore, the elution profile of a sample containing free colchicine and colchicine bound to a macromolecule should show two peaks. The ratio of radioactivity in the two peaks is a measure of the degree of binding while the volume ratio, $V_e/V_o$, gives information concerning molecular size (19). The results of a typical experiment are shown in Fig. 1 *a*. A 0.5-ml aliquot of the soluble fraction was applied to a 1 × 15-cm Sephadex column. A radioactivity peak migrating with the protein is clearly resolved from a second peak appearing at the bed volume of the column.

TABLE I

*Distribution of Bound Colchicine in Homogenates of KB Cells**

| Fraction | Centrifugation conditions | Total fraction radioactivity | Total radioactivity |
|---|---|---|---|
| | | *cpm* × $10^{-3}$ | % |
| Particulate I (nuclear) | 10 min, 700 g | 4 | 2.5 |
| Particulate II (mitochondrial) | 1 hour 10,000 *g* | 4 | 2.5 |
| Particulate III (microsomal) | 1½ hr, 100,000 *g* | 5 | 3 |
| Supernatant (soluble) | | 150 | 92 |
| G-100 Sephadex (macromolecular) | | 85 | 57 of soluble radioactivity |

* A culture of K.B. cells was grown overnight in $10^{-7}$ M colchicine-$^3$H, harvested by centrifugation and washed three times with isotonic phosphate-buffered saline, and homogenized in SMT solution.

In a number of experiments the ratio of bound to total radioactivity ranged from 30 to 60%. The bound radioactivity immediately reapplied to a second column yielded about 80% binding. Since the time necessary to rerun the eluate is about 1 hr the decrease in binding is consistent with a slow dissociation of the colchicine-site complex. The rate of dissociation is also comparable to the loss of radioactivity from cells resuspended on a colchicine-free medium (9).

It can be concluded that the majority of the colchicine in washed cells is bound to a soluble macromolecule. The value of 60% binding is an underestimate of the actual binding in whole cells since about 2.5 hr is required from the time of homogenization to completion of the Sephadex-filtration procedure.

Treatment of the colchicine complex with 8 M urea for 30 min reduced the binding to less than 0.5% of the initial value. Precipitation of the pro-

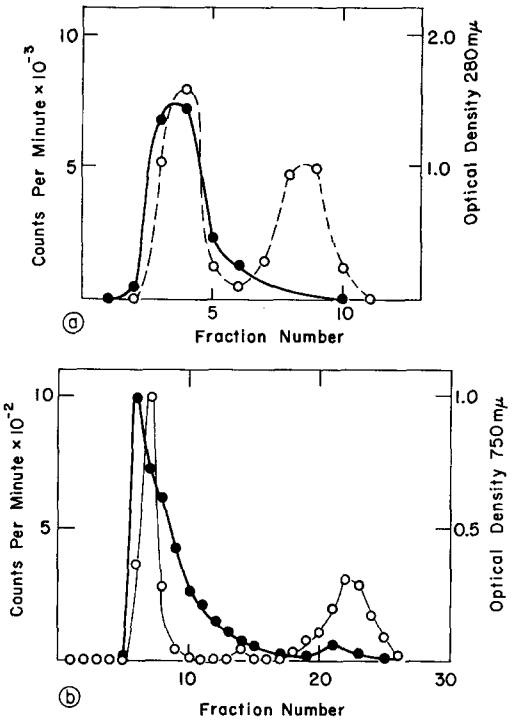


FIGURE 1 Gel filtration on G-100 Sephadex of soluble fraction from K.B. cells. *a* Colchicine binding in vivo; cells grown overnight in $10^{-7}$ M colchicine-$^3$H medium, ●——● protein (OD at 280 mμ), O-----O radioactivity. *b* Colchicine binding in vitro; soluble supernatant incubated with $2.5 \times 10^{-6}$ M colchicine-$^3$H for 1 hr at 37°C, ●——● protein (optical density at 750 mμ), O——O radioactivity. For further details see text.

tein with cold 50% ethanol, acetone, or 0.5 N perchloric acid also caused a complete release of the bound radioactivity. Thus it is probable that binding requires the integrity of the protein structure.

## *Colchicine Binding to Extracts of Mammalian Cells*

The kinetics of colchicine binding to intact cells was described quantitatively by a simple second-order binding mechanism (9) and we therefore might expect to obtain similar binding with cell extracts. Cell homogenates prepared in 0.24 M sucrose (SMT solution) were incubated with $2.5 \times 10^{-6}$ M colchicine for 1 hr at 37°C. Under these conditions, the sites were nearly saturated in the in vivo experiments. Material was centrifuged at low speed to remove nuclei, and at 100,000 *g* for 2 hr to sediment mitochondria and microsomes. The supernatant showed high binding activity as assayed by gel filtration (30,000–60,000 cpm per milligram protein) which was of the same magnitude as that obtained with supernatants prepared from cells labeled in vivo. The elution profile for a typical in vitro binding experiment is shown in Fig. 1 *b*.

The presence of a colchicine-macromolecule complex can also be demonstrated by zone sedimentation, by applying an aliquot of the bound radioactivity fractions from a Sephadex column to a 5–20% sucrose density-gradient. Most of the radioactivity migrates as a 6S peak. In some experiments there appeared to be a second peak at about 1.5S but it is difficult to distinguish radioactivity in this region from free colchicine. Zone sedimentation was performed with material from a variety of sources including KB cells, sea urchin eggs, sea urchin mitotic apparatus, cilia proteins, and colchicine-binding protein isolated from whole brain homogenates. The results were essentially the same with all systems and a figure illustrating the pattern from a typical experiment with sea urchin eggs is included on page 540 in the accompanying paper (Fig. 5).

The KB cell homogenates were fractionated by differential centrifugation before incubation with colchicine. There was some variability in different preparations but the following general results were obtained. Successive removal of nuclei and mitochondrial fractions did not reduce the binding compared with the total homogenate. Further sedimentation for 2–5 hr to remove microsomes generally produced a decrease in binding which in some cases was greater than 50%. The loss of activity was not due to a requirement for a particulate fraction since allowing the nuclei- and mitochondria-free material to stand for the same period of time at 4°C led to a similar loss in binding activity. A number of compounds were tested for their ability to preserve the binding activity. Mercaptoethanol ($10^{-3}$ M), ascorbic acid ($10^{-3}$ M), EDTA ($10^{-3}$ M), $CaCl_2$ ($10^{-3}$–$10^{-2}$ M), and KCl (0.5 M) added to the homogenization medium did not prevent the loss of activity. The combination of $MgCl_2$ ($2 \times 10^{-2}$ M) and ATP ($2 \times 10^{-3}$ M) generally reduced the loss of activity to 10–20% in 3 hr. These reagents were not necessary for the binding step itself since their removal by gel filtration just prior to incubation with colchicine did not reduce the specific binding activity.

The activity could best be stabilized by excess colchicine itself. If the 100,000 *g* supernatant was incubated with $2.5 \times 10^{-6}$ M colchicine-$^3$H for 1.5 hr at 37°C and then was allowed to stand at 0°C, more than 70% of the activity was still present even after 24 hr. There was no loss of activity in 4 hr compared to a 70% decrease in the control (Fig. 2). In addition it was found that up to 80% of the binding activity could be recovered from redissolved lyophilized powders of the high speed supernatant after several months of storage at −20°C.

The factors responsible for the loss of binding activity are not understood as yet and the variable loss of activity in different experiments made a systematic study of the optimal conditions difficult to interpret.

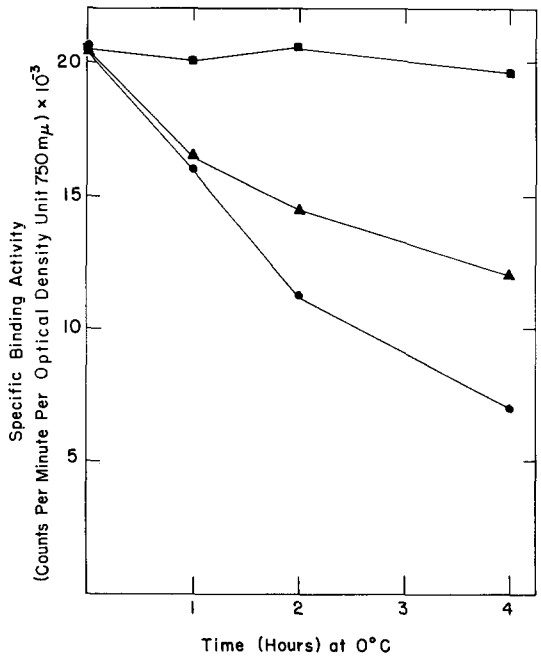


FIGURE 2 Loss of colchicine-binding activity of soluble fraction of HeLa cells at 0°C. Cells were homogenized in 0.24 M sucrose, 0.01 M phosphate buffer and centrifuged 1 hr. The supernatant was allowed to stand for indicated times at 0°C then incubated with $2.5 \times 10^{-6}$ M colchicine-$^3$H for 1 hr at 37°C and bound radioactivity determined by Sephadex column assay. ●——● stored in 0.01 M phosphate buffer; ▼——▼ stored in 0.01 M phosphate buffer plus $10^{-2}$ M $MgCl_2$ and $2 \times 10^{-3}$ M ATP; ■——■ incubated at zero time with colchicine-$^6$H for 1.5 hr at 37°C in 0.01 M phosphate buffer, then allowed to stand at 0°C with colchicine present.

## *Kinetics of Colchicine Binding In Vitro*

In the previous study (9) binding to intact cells was quantitatively described by the simple mechanism $C + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CS$ where $k_1$ and $k_{-1}$ are the forward and backward rate constants, and $(C)$, $(S)$, and $(CS)$ are the concentrations of colchicine, free binding-sites and bound colchicine respectively. The equilibrium constant is $K = \frac{k_1}{k_{-1}}$. The kinetics of colchicine binding to the soluble supernatant from mammalian cells was investigated to determine whether the process could be accounted for by the same mechanism and whether the values of the kinetic constants were similar to those of whole cells.

Binding to intact cells was terminated by centrifugation and washing, a procedure which required about 5–10 min. To follow the reaction in solution it was necessary to terminate binding before applying the sample to the Sephadex column. Loading and elution of the column required about 30 min. Fortunately the rate of binding was found to depend on temperature, and at 0°C the extent of binding was about 5% of the value at 37°C after 1 hr of incubation.

Measurements of binding kinetics were made with cell homogenates centrifuged for 1 hr at 100,000 *g*. The supernatant was divided into 1-ml aliquots which were incubated with various concentrations of colchicine for various intervals. The reaction was stopped by cooling the test tube in an ice bath for several minutes before applying the sample to a 1 × 10 cm G-100 Sephadex column. The additional binding due to the sample standing at 0°C was negligible. The very small apparent binding obtained by mixing a sample with colchicine at 0°C and running over Sephadex immediately was subtracted from all values as a zero time control.

The kinetics of binding was analyzed in the manner described previously for whole cells (9). (*a*) The rate of binding, $R$, should be given by $R = k_1(C)(S) = k_1(C)(S_0)$ at early times, where $S_0$ is the initial concentration of binding sites. The initial rate of binding was found to be proportional to colchicine concentration from $10^{-7}$ M to $5 \times 10^{-6}$ M. (*b*) At equilibrium, $1/(CS) = 1/S_0 + (1/KS_0)1/(C)$. Since $(CS)$ is the concentration of found colchicine at equilibrium a plot of $1/(CS)$ versus $1/(C)$ should be linear.

In the previous experiments in vivo the binding reached a maximum value which was maintained for several hours and the interpretation of the data as an equilibrium process was unequivocal. However in solution there is a loss of binding activity with time and binding reached a maximum and decreased slowly after about 1.5 hr of incubation at 37°C. A plot of (maximum binding)$^{-1}$ versus (colchicine concentration)$^{-1}$ was linear for concentrations greater than $2 \times 10^{-7}$ M which allowed a rough estimation to be made of the apparent equilibrium constant and the concentration of sites. By combining with initial rate data, values of the constants were calculated and are given in Table II together with the values previously found for intact cells. The agreement is reasonably good but interpretation of the data is complicated by the lability of the binding sites.

TABLE II

*Values of Colchicine-Binding Parameters for KB Cells and Cell Extracts at 37°C*

| | *In vitro* | *In vivo* |
|---|---|---|
| $k_1$ liters moles$^{-1}$ hours$^{-1}$ | $0.7 \times 10^6$ | $0.5 \times 10^6$ |
| $k_l$ hours$^{-1}$ | 0.6 | 0.13 |
| $K$ liters moles$^{-1}$ | $1.1 \times 10^6$ | $4 \times 10^6$ |

* The in vivo results are taken from Taylor (9).

Analysis of the problem of the maximum binding in the presence of a reaction which leads to loss of free sites showed that approximately linear reciprocal plots would still be obtained but the apparent equilibrium constant becomes $K = k_1/(k_{-1} + k_d)$ where $k_d$ is the rate constant for site denaturation (20). Based on a value of $k_d$ estimated from measurements of the loss of binding activity on standing, the apparent value of $K$ was estimated to be about five times too small which is to be compared with the factor of three found experimentally. The concentration of binding sites in the extracts expressed as a per cent of total cell protein was 1–2% compared with 4–8% obtained with intact cells, but this difference is compatible with the rate of denaturation of the sites in vitro.

Although some reservations must be held because of the lability of the sites in solution, the results support the conclusion that the same binding reaction occurs in vivo and in vitro.

### *Colchicine Binding by a Variety of Cells and Tissues*

A survey of colchicine binding activities of various cells, tissues, and model systems was made to determine the distribution of the binding sites and to test for a correlation with mitotic activity or other parameters. The results are summarized in Table III. Activity is expressed as counts per minute of bound radioactivity per milligram of protein in soluble extracts of tissues homogenized in SMT solution. Some variation in the extractability of proteins of different tissues must be expected so the results can only be compared approximately. Among six tissues with the exception of brain, those with a higher mitotic rate, e.g. spleen and bone marrow, showed higher binding activity but considerably less than cultures of a mammalian carcinoma (KB or HeLa) or sea urchin eggs. However, the high activity of brain established that binding is not confined to a protein found only in dividing cells. Although some division may occur in glial cells, mitotic processes can be ruled out since the binding was equally high in extracts of white and gray matter. In addition a sample of lyophilized axoplasm of the giant nerve of the squid showed an even higher binding activity than whole mammalian brain.

Binding activity was also tested in systems associated with motility. Although extracts of muscle had a low activity comparable to liver or kidney, the purified muscle proteins, G actin, F actin, or myosin, showed essentially no binding. Zero binding is taken to mean an activity less than 10 cpm/mg protein which is almost four orders of magnitude less than the value routinely obtained with KB cells.

Extracts of slime mold, containing actomyosin-like proteins, showed essentially no binding. A number of different extracts were prepared with a range of temperatures, times, pH's, and ionic strengths with or without added ATP and Mg, and all showed negligible binding. However moderate binding activity was consistently obtained with soluble proteins from *Tetrahymena* cilia which could not be attributed to contamination with cell body proteins, and a high activity was demonstrable with the proteins obtained from sperm tails. In addition, significant binding was obtained from the proteins of the isolated mitotic apparatus (20).

TABLE III

*Survey of Colchicine Binding Activity in Extracts of a Variety of Cells, Tissues, and Model Systems*

| System | Specific binding* activity |
|---|---|
| | *cpm/mg protein* $\times 10^{-3}$ |
| Cells | |
| KB cells | 48.0 |
| HeLa cells | 54.0 |
| *A. punctulata* eggs unfertilized or 15 min after fertilization | 23.0 |
| *L. pictus* eggs, unfertilized | 25.0 |
| *Tetrahymena pyriformis* cells | 0.1 |
| *Physarum polycephalum* | 0.1 |
| Tissues | |
| Rabbit liver | 3.0 |
| Rabbit muscle | 4.8 |
| Rabbit kidney | 5.1 |
| Rabbit spleen | 9.0 |
| Rabbit bone marrow | 9.6 |
| Rabbit lung | 18.0 |
| Rabbit, pig, or rat, whole brain | 50.0 |
| Pig, corpus callosum (white matter) | 61.0 |
| Pig, cerebral cortex (grey matter) | 63.0 |
| Squid axoplasm | 180.0 |
| Organelles | |
| *Tetrahymena pyriformis* cilia | 16 |
| *S. purpuratus* or *A. punctulata* sperm tail | 60–90 |
| *A. punctulata* mitotic apparatus | 13 |
| *S. purpuratus* mitotic apparatus | 15–30 |
| Model systems | |
| Myosin | <.01 |
| G-actin | <.01 |
| F-actin | <.01 |
| Hemoglobin | <.01 |
| Bovine serum albumin | <.01 |

* 1 ml of tissue extract (or protein solution) was incubated with $2.5 \times 10^{-6}$ M colchicine-$^3$H for 1 hr at 37°C, quickly cooled to 0°C and subjected to gel filtration over G-100 Sephadex to determine bound count. Sample protein was determined according to Lowry (17). Each datum given is the averages of at least three determinations.

## *Isolation of Bound Colchicine*

The mechanism proposed for the binding step requires the formation of a noncovalent complex of colchicine with some macromolecules(s). Therefore it was necessary to show that the radioactivity bound to whole cells or supernatant extracts was attributable to chemically unchanged colchicine.

A direct determination of physical properties, such as absorption spectrum or optical rotation of the radioactive material recovered from cells, could not be made because of the small quantities involved. However, a number of indirect experiments provided fairly convincing evidence that the molecule was unchanged by the binding process.

(*a*) Large-scale binding experiments with cells and cell extracts ($2.5 \times 10^9$ cells) were performed and bound radioactivity was obtained by Sephadex filtration. The protein was precipitated with cold 0.5 N and was washed with 0.2 N perchloric acid. The combined supernatants and washings were extracted exhaustively with chloroform. The chloroform was evaporated, and the residue was dissolved in methanol. At this step the yield was about 75% of the initial bound radioactivity. Aliquots of the methanol solution were chromatographed on thin layer silica gel plates in butanol–ammonia–water 2:1:2 or 10% methanol–chloroform with unlabeled colchicine present. The bulk of the radioactivity had an $R_f$ equal to that of the carrier colchicine. A second faster peak containing from 10 to 30% of the radioactivity was also present. This compound was found previously as a contaminant of the radioactive colchicine and appears to be a colchicine degradation product since it is formed during storage of preparations of colchicine-$^3$H which have been purified by thin layer chromatography (9). (*b*) The colchicine is labeled in the tropolone methyl-ether group which can be hydrolyzed in 1 hr at 100°C in 0.1 N HCl. Hydrolyses of the material purified by thin layer chromatography released more than 90% of the radioactivity in a volatile form. (*c*) The purified material was tested for its ability to inhibit mitosis. Concentration was estimated on the assumption that the specific radioactivity of the isolated material is equal to that of the colchicine-$^3$H sample. The material produced the characteristic colchicine block of mitosis at a concentration of less than $10^{-7}$ M.

On the basis of these three criteria it is reasonable to conclude that the bound radioactivity is due to colchicine which has not been chemically modified.

## CONCLUSIONS

Colchicine is bound to a macromolecule in intact cells which appears in the soluble fraction after

homogenization. A similar binding takes place when soluble extracts from both dividing and nondividing cells are incubated with colchicine. The binding is reversible, appears to depend on the macromolecule being in its native state, and does not involve chemical modification of colchicine. The kinetics of the reaction both in intact cells and soluble extracts can be described as the simple formation of a noncovalent complex with a single class of binding sites.

Colchicine-binding activity was found to correlate strictly with neither mitotic activity nor motility, but rather with those sources abundant in microtubules. Microtubules form the framework of the mitotic spindle (21, 22); they make up the 9 + 2 array of filaments in cilia and sperm tails (23); and they are present in large numbers in neuronal processes (24). They are the only structures known to be common to these four sources which are all high in binding activity. In addition, colchicine can reversibly cause the disassembly of the densely packed, double-coil array of microtubules which are the predominant structure of the axopods of the Heliozoan, *Actinosphaerium* (2). Colchicine is also reported to cause the disappearance of microtubules in interphase HeLa cells (25). These observations, taken together, suggest that the binding sites are the subunit proteins of microtubules. A direct test of this hypothesis can be made using sea-urchin sperm tails which provide an essentially pure source of microtubules. Preliminary experiments (Table III) showed very high binding to this source and the results of a detailed study will be reported elsewhere.

Porter, in a recent review (26), has suggested that microtubules have primarily a cytoskeletal role in the development and maintenance of asymmetric cell forms. Colchicine, by disrupting the microtubule organization could interfere with any cell function dependent on these form-determining elements. On this basis explanations can be offered for the effect of colchicine on other systems. For example, embryonic chick muscle (contrary to adult muscle) is rich in microtubules which are located at the periphery of the cell, parallel to the myotube and the developing myofibrils (27). After exposure to $10^{-6}$ M colchicine, well formed myotubes containing striated myofibrils become fragmented, undergo fission, and lose the parallel array of myofibrils (4).

The cellulose microfibrils which run circumferentially around the wall of many plant cells are parallel to an underlying array of microtubules (28). Colchicine interferes with the orientation of newly deposited cellulose fibrils presumably by preventing the formation of the underlying array of microtubules.

Even the effect of colchicine in relieving the symptoms of gout may be explainable in terms of its action on microtubules. It has been reported that the phagocytic action of leukocytes upon the urate crystals deposited in the joints of gouty patients is the primary cause of the associated inflammation, and it has been suggested that colchicine provides relief by suppressing this action (29). The phagocytic activity may be dependent on an intact microtubule structure, since the related phenomena of extension of filamentous processes from cell surfaces is reported to be sensitive to colchicine (30) and apparently involves microtubules (31).

The binding of colchicine to a protein present in neurons and axoplasm is of interest since the acute physiological disturbances characteristic of colchicine poisoning result primarily from a toxic action on the central nervous system (7). Complete absence of binding to muscle proteins is in agreement with the general results of a number of investigators that the proteins of muscle are not related to the mitotic apparatus (32). Microtubules have not been found associated with streaming in slime mold (33) and *Nitella* (34), and available evidence so far has not supported a close relationship of the mechanism of streaming in these species to the mitotic spindle and ciliary mechanisms. The failure to detect any binding activity in extracts of slime mold prepared under a variety of conditions is consistent with this view.

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