The Biochemical Events of Mitosis. II. The *in Vivo* and *in Vitro* Binding of Colchicine in Grasshopper Embryos and Its Possible Relation to Inhibition of Mitosis*

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ABSTRACT: Colchicine was utilized in a study of the biochemical mechanism by which colchicine inhibits cell division. Ninety-six per cent of labeled colchicine taken up by intact grasshopper embryos could be accounted for in the 100,000*g* supernatant fraction after homogenization. About 30–40% of the colchicine taken up was complexed to a high molecular weight substance (species A) as determined by equilibrium dialysis and gel filtration. The binding was temperature dependent, being completely absent at 0°, as well as time dependent. A similar temperature- and time-dependent binding to species A occurred *in vitro*. Several lines of evidence strongly indicate that binding of colchicine to species A is directly related to the effects of colchicine on cell division. Lumicolchicine, a structural isomer of colchicine that is devoid of antimitotic activity, did not bind to species A nor did it interfere with the binding of colchicine to species A. Podophyllotoxin strongly inhibited the binding of colchicine both *in vivo* and *in vitro*. Picropodophyllotoxin, a stereoisomer of podophyllotoxin having a weaker antimitotic activity, inhibited colchicine binding to a lesser degree than podophyllotoxin. Stimulation of colchicine binding rather than an inhibition was obtained with vinblastine. Species A was found to be a protein with a molecular weight of approximately 105,000. The equilibrium between colchicine and species A heavily favored complex formation since bound colchicine did not exchange with unlabeled natural colchicine added in large excess. The binding of colchicine to species A appears to be noncovalent. Chemically unaltered colchicine could be reisolated from the species A–colchicine complex upon extraction with organic solvents.

The significance of these results is discussed in terms of the possibility that colchicine interferes with cell division by directly binding to a subunit of the mitotic spindle.

Knowledge of the biochemical and biophysical aspects of cell division, especially of the structure and function of the mitotic apparatus, has been limited until recently by the lack of appropriate techniques. With the availability of tritium-labeled colchicine (Taylor, 1965; Wilson and Friedkin, 1966) and the recent advances in our knowledge of the mitotic apparatus and spindle (Inoué, 1964; Sakai, 1966; Kiefer *et al.*, 1966; Kane, 1967; Stephens, 1967), a new approach to the nature of the antimitotic activity of colchicine and further elucidation of biochemical mechanisms of mitosis are possible.

This communication describes the *in vivo* and *in vitro* binding of colchicine to a high molecular weight substance in grasshopper embryos.We believe the binding is directly related to the effects of colchicine on cell division since in a variety of situations the binding was influenced in a manner predicted by this hypothesis.

Experimental Section

Materials

Grasshopper Embryos. Encoptolophus sordidus (Burnmeister) embryos were used in all experiments. Most of the techniques developed in the laboratory of Dr. J. G. Carlson (Carlson and Gaulden, 1964) for study of neuroblast cells in *Chortophaga* were ideally suited for use with *Encoptolophus*. Mature females were reared in a terrarium and then maintained in regular egg-laying cages. Eggs were collected twice weekly from the adult cages and stored at room temperature for the first 3 or 4 days. They were next transferred to a 17° (±1°) incubator for 6 weeks. During this period, the embryos developed to a stage equivalent to 14 days at 26° (estimated ages of the embryos, based on development of *Chortophaga* embryos, are reported here as the number of days of development at 26° ± 2 days). Thereafter, they were stored at 4° (a temperature that blocks further development) for a period up to 4 months without ill effects. After 4 months, the quality of some

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of the embryos began to deteriorate and they were not used.

Isotonic Shaw's medium (Carlson and Gaulden, 1964) was employed in all experiments. It was prepared by mixing five parts of concentrated Shaw's medium with four parts of distilled water, and is referred to as isotonic medium throughout this paper.

Colchicine-acetyl-^3H. Pure colchicine-acetyl-^{3}H was prepared and rechromatographed as described in Figure 3 of a previous paper (Wilson and Friedkin, 1966). Stock solutions at two concentrations (10.5 and 525 μg/ml, based on absorbancy at 350 mμ (ϵ 16,740)) were prepared in distilled water, divided into 2-ml portions, and stored at $-20°$ under nitrogen. The specific activity was 240 mc/mmole.

Lumicolchicine. Aqueous solutions of labeled and unlabeled purified colchicine (3×10^{-5} M) were irradiated with ultraviolet light for 2–4 hr as described in an earlier paper (Wilson and Friedkin, 1966). Complete conversion of colchicine to β- and γ-lumicolchicines (referred to as lumicolchicine throughout this paper) was achieved as determined by the change in the absorption spectrum.

Podophyllotoxin and Picropodophyllotoxin. Podophyllotoxin was obtained from Aldrich Chemical Co. (mp 112–114°, λ_{max} 290 mμ in 95% ethanol (ϵ 3750)). Picropodophyllotoxin was prepared from podophyl lotoxin by mild alkaline isomerization (modified from von Wartburg *et al.*, 1957). A solution of podophyllotoxin (200 mg in 10 ml of methanol and 1 ml of 28% NH_3 in water) was refluxed for 30 min. This mixture when cooled yielded a flocculent precipitate of picropodophyllotoxin which was collected and washed twice with 10-ml portions of absolute ethanol at 0°. The picropodophyllotoxin was recrystallized from hot absolute ethanol, washed with 0° ethanol, and dried *in vacuo* at 107° (mp 221–224°, λ_{max} 287 mμ in 95% ethanol (ϵ 3800)). The melting point was determined with the Nalge melting point apparatus. Complete conversion to picropodophyllotoxin was easily demonstrated by thin layer chromatography on Eastman silica gel chromatogram sheets with methanol as the developing solvent (R_F of podophyllotoxin 0.67–0.70; R_F of picropodophyllotoxin 0). Since picropodophyllotoxin is not very soluble in water at room temperature, a stock suspension was used. This, when warmed to 60 or 70°, gave a clear solution from which different dilutions could be obtained.

Miscellaneous Chemicals. Vinblastine (97.4% pure) was a gift from Eli Lilly and Co. All grades of Sephadex and Blue Dextran 2000 for determination of void volumes were obtained from Pharmacia, Inc. Crystalline trypsin was obtained from Armour Laboratories. DNase (one-time crystallized) was obtained from Worthington, and RNase, from Sigma.

Methods

In Vivo Binding of Colchicine by Intact Grasshopper Embryos. Whole demembranated embryos (four to eight) were transferred to a small incubation tube containing labeled colchicine in 1.8 ml of isotonic medium. The usual concentration of colchicine was 2.2×10^{-6} M unless indicated otherwise. Each tube was incubated for the desired length of time at 37°. Control tubes were incubated at 0°. The incubation period was terminated by immersing the tube in an ice bath to stop incorporation. Embryos were then removed from the tube and transferred to a Syracuse watch glass containing 10 ml of colchicine-free isotonic medium for 3 min at 0° as a preliminary wash. Two washing methods were employed to remove unbound colchicine from the embryos.

Method I. Previously incubated embryos, suspended in a drop of isotonic medium, were placed onto a 2.4-cm diameter glass-fiber disk (934AH, Hurlbut Paper Co.) on a Millipore microanalysis filter holder. The embryos were then washed with 3-ml portions of 0° isotonic medium by allowing the medium to filter slowly through the disk without suction for 5 min. After the remaining medium (about 1 ml) was suctioned through, another 3-ml portion of medium was added. This was continued for a total of five washes. The disks were air dried for 15 min and excess portions surrounding the spot containing the embryos were trimmed away. The remaining piece of filter disk with adhering embryos (3×3 mm) was placed in 1 ml of 1 N NaOH and incubated at 37° for 75 min with occasional agitation to dissolve the embryos. Protein assays of the dissolved embryos were performed by the method of Lowry *et al.* (1951). Tritium was determined by placing 300 μl of the extract in 15 ml of scintillation fluid (see General Procedures). The glass-fiber disk did not retain any tritium, nor did it interfere with the protein assay.

Method II. After the preliminary wash at 0°, embryos were transferred to successive 10-ml portions of isotonic medium in Syracuse watch glasses at 0° for 5-min periods. Usually five such washes were performed. After the last wash, embryos were dissolved as previously described in method I for tritium and protein determinations.

The amount of colchicine bound was expressed as disintegrations per minute per microgram of total embryonic protein.

Pasteur-disposable pipets were used in all embryo transfers. Pipets were prewetted with medium containing some yolk to prevent sticking of the embryos in the inside glass walls.

The method for *in vivo* determination of colchicine-acetyl-^{3}H binding in intact embryos involved washing the embryos free of all unbound colchicine. The total counts removed in each wash were determined, and with both washing methods a plateau was reached after the third wash. With washing method I, less than 7% of the remaining embryo-bound counts was removed with each successive wash. With washing method II, between 2 and 4% of the counts remaining in the embryos were removed with each successive wash, and the specific activity (disintegrations per minute bound per microgram of protein) remained constant after the third wash. Binding results were reproducible with both

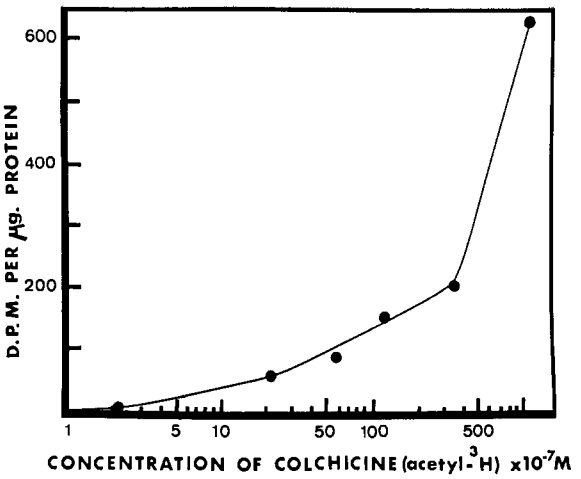


FIGURE 1: Incorporation of colchicine-acetyl-^{3}H into intact embryos *vs.* colchicine concentration. Embryos five or six at 16 ± 2 days (20–31 µg of protein/embryo) were used in each experiment. The general procedure is described in the Methods section. Embryos were incubated for 3.5 hr at 37° and washed by method I.

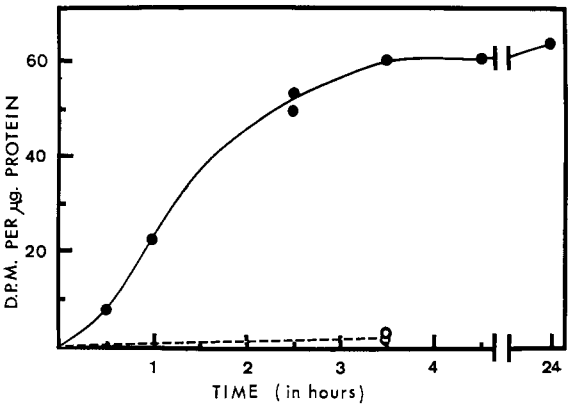


FIGURE 2: Incorporation of colchicine-acetyl-^{3}H into intact embryos *vs.* time. Embryos six to eight at 16 ± 2 days (20–32 µg of protein/embryo) were used in each experiment. The general procedure is described in the Methods section. Embryos were incubated at 37 or 0° in 2.2 × 10^{-6} M colchicine-acetyl-^{3}H and washed by method I. Closed circles, 37°; open circles, 0°.

washing methods.

General Procedures. Homogenization of embryos was accomplished with a Tri-R laboratory stirrer (S63C) and Teflon-glass tissue homogenizer (pestle = 0.308-in. diameter, clearance = 0.004–0.006 in., 2-ml capacity). Embryos were homogenized in 1–2 ml of isotonic medium at 0° for 90 sec.

Tritium was determined with a Tri-Carb scintillation counter in 1.2% 2,5-diphenyloxazole and 0.5% 2,2′-*p*-phenylenebis(5-phenyloxazole) in dioxane–anisole–dimethoxyethane (6:1:1) (Davidson and Feigelson, 1957). Internal standards of tritiated water were used to determine counting efficiency and disintegrations per minute.

Thin layer chromatography was performed with 2- or 3-in. strips of Eastman silica gel chromatogram sheets (type K301R). Development was carried out with either pure methanol or butanol–ammonia–water (2:1:2). Strips were cut into sections and tritium was determined by counting each section directly in 15 ml of scintillation fluid.

All Sephadex column chromatography and centrifugations were performed at 4°. Low-speed centrifugation was accomplished in a Servall centrifuge with an SM-25 rotor. High-speed centrifugation was performed in the Spinco Model L ultracentrifuge with either the 39L or 40 rotor.

Protein was determined by the method of Lowry *et al.* (1951).

Results

Uptake of Colchicine by Intact Grasshopper Embryos. The binding of colchicine by intact embryos is roughly proportional to the logarithm of the colchicine concentration in a concentration range that produces a maximal biological effect, *i.e.*, disruption of the mitotic apparatus at 10^{-7}–10^{-5} M (Figure 1). No saturation of the binding per log concentration is apparent as if nonspecific binding occurs at suprainhibitory levels (10^{-4} M). However, these results are consistent with those of Taylor (1965) which suggested that inhibition of mitosis occurs when only 3–5% of the target sites is complexed with colchicine. The colchicine concentration used in all subsequent *in vivo* experiments was 2.2 × 10^{-6} M, a value very close to that at which the maximum c-mitotic index was obtained (discussed later) and at which all neuroblast cells at a stage earlier than metaphase are inhibited as they enter mitosis (Gaulden and Carlson, 1951). This concentration provides enough binding so that low numbers of embryos (four or five) may be used with high counting accuracy in each experiment.

Time and Temperature Dependence of Colchicine Uptake by Intact Grasshopper Embryos. The binding of colchicine at 37° increased for the first 2.5 hr, then leveled off after 3.5 hr (Figure 2). A similar plateau was also observed by Taylor (1965) in cultures of human cells. As shown in Figure 2, colchicine binding was found to be temperature dependent, being almost completely abolished at 0°. This latter finding made possible the manipulation of embryos during the washing procedure at 0° described in Methods with some confidence in values obtained in 37° incubations.

Demonstration of Colchicine Binding in 100,000g Supernatant Fractions of Embryos Pretreated with Colchicine. Differential centrifugation of embryos containing bound colchicine in 0.3 M sucrose served as a preliminary approach to the possible isolation and characterization of the colchicine receptors. Over 96% of the tritium originally bound to the embryos *in vivo* was found in the 100,000*g* supernatant fraction.

Identical results were obtained upon differential centrifugation in isotonic medium instead of 0.34 M sucrose. Similar results were observed by Taylor (1963) who reported that more than 90% of radioactivity representing incorporated colchicine was found in a 105,000*g* supernatant fraction of human cells.

While the previous experiments demonstrated that incorporated colchicine was found only in the 100,000*g* supernatant fractions after homogenization of embryos pretreated with colchicine, they did not show whether the colchicine was present in the free or bound form. This was ascertained by the use of two methods: equilibrium dialysis and gel filtration. Results of several equilibrium dialysis experiments indicated that between 20 and 38% of the colchicine taken up by intact embryos remained in a bound form after dialysis of the 100,000*g* supernatant fractions.

A sensitive and rapid method for detection and quantitative evaluation of colchicine binding was developed by use of gel filtration. Upon passage of a 100,000*g* supernatant extract from embryos pretreated with colchicine through a Sephadex G-25 column, a bound form of colchicine was readily separated from free colchicine. A typical elution profile with isotonic medium on Sephadex G-25 is shown in Figure 3. Peak A, containing bound colchicine, emerged at the same fraction as did Blue Dextran 2000 (mol wt 2×10^6). Therefore, the colchicine complex was excluded from the gel. Since material with a molecular weight greater than 5000 is excluded from Sephadex G-25, the substance in peak A that binds colchicine must have a molecular weight above that value. The nature of the substance in peak A, called species A, is discussed later. When peak A fractions obtained from this separation were pooled and recycled through the same column, 49% of the colchicine reappeared in the peak A area. However, upon passage through a 28-cm column of Sephadex G-25, peak A could not be recovered. The instability of the species A–colchicine complex on long columns of Sephadex G-25 and not on Sephadex G-10, G-75, or G-100 cannot be explained at present.

Duplicate experiments carried out with an homogenate of the same batch of embryos gave almost identical binding results as shown in Figure 3. In one sample, 34,900 dpm was recovered in combined tubes of peak A and in the second sample, 32,500 dpm. In all other duplicate experiments, agreement within 5% was usually obtained.

Colchicine Binding to Species B. In addition to species A, another colchicine-binding substance was encountered in a variety of experiments. For example, it shows up as a small hump (labeled species B) in the elution profile displayed in Figure 3. Various aspects of the species B complex are described elsewhere (Wilson, 1967). Its nature remains obscure.

Colchicine Binding to Sephadex G-25 Fractions Obtained from an Extract of Homogenized Grasshopper Embryos. In previous experiments, the species A–colchicine complex was obtained by gel filtration of various incubation mixtures in which colchicine had been added to crude cell-free extracts. It was of interest to attempt a preliminary purification of species A before incubation with colchicine. As shown in Table I, material in fraction 5 (the fraction that would normally contain the species A–colchicine complex if supernatant extracts had been treated with the drug prior to gel filtration) still had the ability to complex

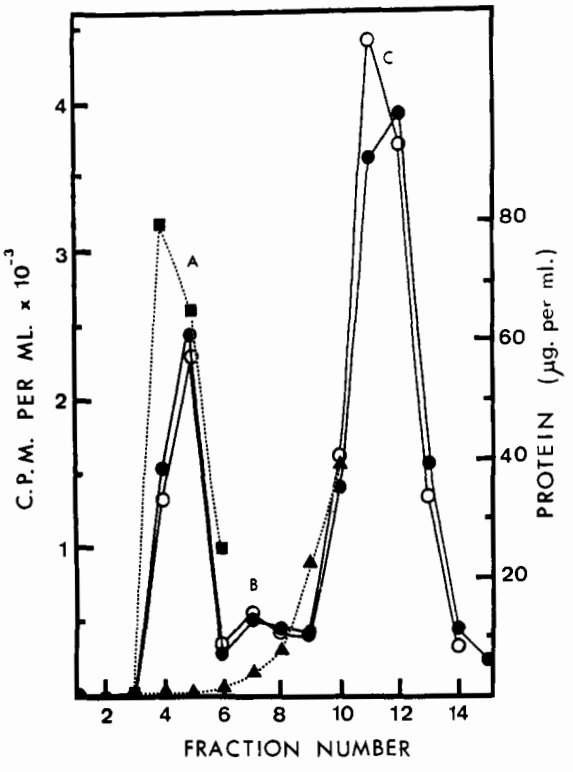


FIGURE 3: Gel filtration of bound colchicine on Sephadex G-25 with isotonic medium. A slurry of washed Sephadex G-25 in water was poured into a 1-cm column to a height of 11 cm after packing. The flow rate was adjusted to 25 ml/hr. The column was equilibrated with 100 ml of isotonic medium prior to use. Elution was also performed with this medium. Embryos (46, age 22 days) were homogenized in 1 ml of isotonic medium (see Methods). Colchicine solution (15 μl; final concentration, 8.5×10^{-7} M) was added to 450 μl of homogenate (517 μg of protein). After an incubation period of 3.5 hr at 37°, the mixture was chilled, diluted with 1.4 ml of cold isotonic medium, and centrifuged at 5000*g* for 15 min. Most of the resulting supernatant fluid (1.5 ml) was filtered through the gel column (another 100-μl portion was assayed for protein). A control containing colchicine alone (no extract) was also run. Fractions of 1.1 ml were collected. An aliquot of each fraction (0.5 ml) was assayed for radioactivity and another (0.4 ml) was assayed for protein. Closed and open circles represent duplicate runs (528 μg of protein); triangles, colchicine control; squares, protein. Peaks A and B contain bound colchicine, peak C contains free colchicine.

TABLE I: Colchicine Binding to Sephadex G-25 Fractions Obtained from a 5000*g* Supernatant Extract of Homogenized Grasshopper Embryos.[a]

Fraction	Incubation Temp (°C)	Binding to Species A (dpm/μg of supernatant protein)
3 (control)	37	0.7
5	37	19.8
5	0	1.4
8	37	1.4

[a] A 5000*g* supernatant extract (1.5 ml), prepared from an homogenate of 20 embryos (age 22 days), was subjected to gel filtration on Sephadex G-25 (see legend of Figure 3 for methods). Aliquots of the above fractions (400 μl) were incubated with labeled colchicine (9.5×10^{-7} M) for 2.5 hr at the indicated temperatures. Each incubation mixture was diluted to 1.5 ml with isotonic medium and filtered through Sephadex G-25 as above. The activity in peak A in each case was calculated on the basis of the amount of protein applied to the column.

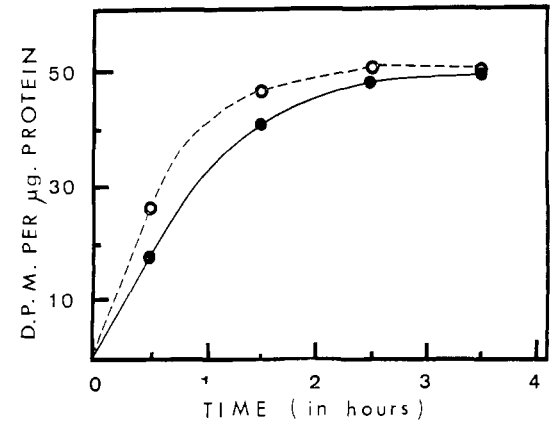


FIGURE 4: Time dependence of *in vitro* colchicine binding to species A. Binding in a whole homogenate (closed circles). Embryos (60, age 25 days) were homogenized in 1.8 ml of isotonic medium (see Methods). Aliquots of the homogenate (450 μl) were incubated with labeled colchicine (8.5×10^{-7} M) for the indicated times at 37°. Each incubation mixture was diluted, centrifuged at 5000*g*, and the resulting supernatant fraction was subjected to gel filtration on Sephadex G-25 as described in the legend of Figure 3. The radioactivity in peak A in each case was calculated on the basis of the amount of protein applied to the column. Binding in fractions 4 and 5 after Sephadex G-25 gel filtration of a 100,000*g* supernatant extract (open circles). Embryos (48, age 15–25 days) were homogenized in 1.6 ml of isotonic medium (see Methods). A 100,000*g* supernatant extract was prepared and subjected to gel filtration on Sephadex G-25 as described in the legend of Figure 3. Fractions 4 and 5 (representing the major portion of a 100,000*g* supernatant protein and containing species A) were pooled, and aliquots (400 μl) were incubated with labeled colchicine (9.5×10^{-7} M) for the indicated times at 37°. Incubation mixtures were filtered through Sephadex G-25, and activity was determined as above.

with colchicine. The binding was temperature dependent as before.

Time Dependence of Colchicine Binding to Species A. The *in vitro* binding of colchicine to species A (Figure 4) exhibited a time dependence that was quite similar to that obtained *in vivo*. Furthermore, the binding of colchicine to preisolated species A also exhibited the same time dependence.

It was conceivable that the period of time necessary to reach saturation at 37° (2.5 hr *in vitro*) reflected an activation process resulting in the conversion of species A from an inactive form to an active form which could then bind the drug. If the activation process were time dependent, species A should be completely activated in 2.5 hr and upon subsequent addition of colchicine, maximum binding should occur rapidly. When this possibility was tested, preincubation of species A did not greatly change the time requirement for binding. It is concluded that an activation process is not responsible for the time dependence. These experiments rule out two other possibilities. With intact actively dividing cells,the time-dependent binding of colchicine might reflect the uncovering of new binding sites directly related to the sequential events of mitosis. However, this cannot explain the time-dependent binding in cell-free extracts. Furthermore, the binding is not due to a rate, limiting entry of colchicine into intact cells.

If the temperature-dependent binding is due to a structural alteration in species A which occurs at 37° and not at 0° (see Discussion), it must be a highly reversible process since despite preincubation of species A at 37° for 2.5 hr, colchicine binding did not occur at 0°.

Specificity of Colchicine Binding. LACK OF BINDING OF LUMICOLCHICINE TO SPECIES A. In an attempt to demonstrate the specificity of colchicine binding and to relate this binding to the antimitotic action of colchicine, experiments were carried out with tritium-labeled lumicolchicine, a compound closely resembling colchicine but devoid of antimitotic activity (Linskens and Wulf, 1953). Lumicolchicine at 2.1×10^{-6} M had no effect on cell division in the grasshopper embryos, while a similar concentration of colchicine produced a maximal inhibitory effect on mitosis (Table II). Lumicolchicine-acetyl-^{3}H, prepared by irradiation of colchicine-acetyl-^{3}H, was poorly taken up by whole embryos; *i.e.*, less than 5% of the value obtained with the same concentration of colchicine. For example, when embryos were incubated with 2.2

TABLE II: Antimitotic Activities of Colchicine-acetyl-^{3}H and Other "Spindle Poisons."[a]

Antimitotic Agent	Molar Concn	% of Total	
		Normal Mitotic Cells	c-Mitotic Cells
None		5.1	0
Lumicolchicine-acetyl-^{3}H	2.1×10^{-6}	6.6	0
Colchicine-acetyl-^{3}H	4.8×10^{-9}	2.8	2.3
Colchicine-acetyl-^{3}H	1.8×10^{-8}	2.5	4.9
Colchicine-acetyl-^{3}H	9.8×10^{-8}	0	36.0
Colchicine-acetyl-^{3}H	5.0×10^{-7}	0	51.0
Colchicine-acetyl-^{3}H	2.5×10^{-6}	0	36.0
Colchicine-acetyl-^{3}H	1.3×10^{-5}	0	35.0
Podophyllotoxin	3.7×10^{-7}	0	42.0
Picropodophyllotoxin	3.7×10^{-7}	0	12.0
Vinblastine	2.1×10^{-4}	0	43.0

[a] Neuroblast segments were prepared from 14-day-old embryos. They were incubated for 3.5 hr at 37° in 2 ml of isotonic medium containing the desired concentration of inhibitor. After incubation, the segments were mounted in hanging drops for counting (see Carlson and Gaulden (1964) for techniques). Between 300 and 900 neuroblast cells were counted for each determination. All normal metaphase and anaphase cells were scored as normal mitotic cells. Neuroblast cells exhibiting abnormal chromosome configurations (Gaulden and Carlson, 1951) were considered to be c-mitotic cells. The relative antimitotic activities of vinblastine and podophyllotoxin were estimated to be near maximum (as compared with colchicine) when about 35% or more of the neuroblast cells were in c mitosis.

$\times 10^{-6}$ M labeled colchicine for 3.5 hr at 37° *in vivo*, approximately 60 dpm/μg of protein was incorporated (see Methods). With 2.2×10^{-6} M labeled lumicolchicine incubated under the same conditions as above, only 2.8 dpm/μg of protein was incorporated.

When labeled lumicolchicine was incubated *in vitro* with a whole homogenate of grasshopper embryos followed by gel filtration on Sephadex G-10, binding in peak A was less than 8% that of colchicine.

The possibility that lumicolchicine might interfere with colchicine binding to species A was also investigated. Unlabeled lumicolchicine (1.8×10^{-6} M) and labeled colchicine (8.0×10^{-7} M) were incubated together for 3.5 hr at 37° with a whole homogenate (352 μg of protein; total volume, 495 μl). Another incubation mixture was prepared with colchicine alone. Upon completion of the incubation, the mixtures were diluted with 1.4 ml of cold isotonic medium. Supernatant extracts (5000*g*) were prepared and applied to Sephadex G-25 columns as described in the legend of Figure 3. The binding of colchicine to species A in the presence and absence of unlabeled lumicolchicine amounted to 36.8 and 38.5 dpm/μg of protein, respectively (based on total protein applied to the column). It was concluded that lumicolchicine does not interfere with colchicine binding.

INFLUENCE OF SOME "SPINDLE POISONS" ON COLCHICINE BINDING. A variety of substances including colchicine have a profound effect on the mitotic spindle and are therefore referred to as "spindle poisons" (Dustin, 1963). It was conceivable that some of these agents might have a common mechanism of action. If so, they should compete with colchicine for the same receptor sites.

Accordingly, the relative antimitotic activities of colchicine and several other spindle poisons were determined in grasshopper neuroblast cells (Table II) and the influence of these agents on colchicine binding was investigated.

Several spindle poisons caused marked deviations in colchicine binding to species A. Tubes containing colchicine alone, colchicine plus podophyllotoxin, and colchicine plus picropodophyllotoxin were each incubated with aliquots of the same homogenate for 3.5 hr at 37° and subjected to gel filtration on Sephadex G-25. Podophyllotoxin, a potent spindle poison, caused marked inhibition of colchicine binding to species A whereas picropodophyllotoxin, a weaker antimitotic substance, had much less effect on the binding. In contrast to podophyllotoxin, vinblastine caused a definite increase of colchicine binding to species A (Table III).

The effects of the above spindle poisons on colchicine binding by intact embryos were quite similar to those obtained with cell-free extracts. These *in vivo* results are summarized in Table III along with the *in vitro* data for comparison.

Nature of the Species A Colchicine Complex. STABILITY. *In vivo*, the species A–colchicine complex was quite stable at 0° and fairly stable at 37°. In one experiment, 21 embryos were incubated in 2×10^{-6} M colchicine for 3.5 hr at 37°, washed by method II, and resuspended in colchicine-free isotonic medium at 0°. In a similar experiment, embryos were treated as above and resuspended in colchicine-free isotonic medium at 37°. At zero time and after each subsequent hour, three embryos were removed and the radioactivity bound per microgram of protein was determined. At 0°, only an 8% decrease in specific activity (disintegrations per minute per microgram of protein) was found over a 6-hr period, indicating that the complex is quite stable at this temperature. At 37°, a 40% decrease in specific activity was obtained. Since 60% of the originally incorporated colchicine was still bound after resuspension in colchicine-free medium at 37° for 6

TABLE III: Influence of Some Spindle Poisons on Colchicine Binding *in Vivo* and *in Vitro*.[a]

Experiment	Other Spindle Poison	Binding of Colchicine to Species A: Other Spindle Poison Absent	Other Spindle Poison Present	% Inhibition	% Stimulation
		Dpm/μg of Total Embryonic Protein			
In vivo colchicine (2.2×10^{-6} M)	Podophyllotoxin (9×10^{-6} M)	56.2	12.7	77	
	Picropodophyllotoxin (9×10^{-6} M)	56.2	41.2	27	
	Vinblastine (2.1×10^{-4} M)	52.3	70.2		34
		42.1	56.8		35
		Dpm/μg of Supernatant Protein			
In vitro colchicine (8.6×10^{-7} M)	Podophyllotoxin (3.9×10^{-6} M)	29.5	1.6	95	
	(7.5×10^{-7} M)	46.7	11.8	75	
	Picropodophyllotoxin (3.9×10^{-6} M)	29.5	15.3	48	
	(7.5×10^{-7} M)	46.7	38.9	17	
	Vinblastine (8.5×10^{-5} M)	50.4	75.5		50
		45.1	55.5		23

[a] *In vivo* studies were carried out as described in the Methods section. The *in vitro* experimental procedure was as follows. Embryos (41, age 18–25 days) were homogenized in 1.2 ml of isotonic medium (see Methods). Aliquots of the homogenate (425 μl) were incubated with labeled colchicine alone (8.6×10^{-7} M), labeled colchicine plus podophyllotoxin, labeled colchicine plus picropodophyllotoxin, and labeled colchicine plus vinblastine for 3.5 hr at 37° in final volumes of 460 μl. Supernatant fractions (5000*g*) were then prepared and filtered through Sephadex G-25 as described in the legend of Figure 3. The activity in peak A was calculated, based upon the amount of protein applied to the column. Binding results varied from experiment to experiment (probably due to the use of embryos stored under different conditions); however, duplicate experiments carried out with the same batch of embryos always yielded binding values that agreed to within 10%.

hr, the binding must be only partially reversible *in vivo*.

Stability experiments were also carried out *in vitro*. Unlabeled purified colchicine in large excess was added to a 5000*g* supernatant extract containing preformed colchicine complex and incubated at 37° (ratio of unlabeled to labeled colchicine was 3900:1). In two experiments (Table IV) no exchange of unlabeled colchicine with bound colchicine occurred as seen by comparison with binding values obtained with control incubations. This indicates the species A–colchicine complex, once formed, is extremely stable and that the equilibrium between colchicine and species A strongly favors complex formation.

The species A complex was found to be very stable when frozen. In one experiment, 30.2 dpm/μg of protein (based on total protein applied to the column) was bound to species A from one aliquot of a whole homogenate after incubation in colchicine and storage at 0° for 11 days prior to gel filtration. A second aliquot, filtered through the gel 6 days later, yielded 31.6 dpm/μg of protein bound to species A.

EFFECT OF VARIOUS AGENTS ON SPECIES A–COLCHICINE COMPLEXES. Results of several experiments involving treatment of colchicine complexes with various agents indicated that species A is a protein (Table IV). Both urea and trypsin almost quantitatively destroyed the complex, while ribonuclease and deoxyribonuclease exerted no effect in extremely high concentrations. Dithioerythritol (Cleland, 1964), a substance that reduces disulfide linkages, did not release the bound colchicine.

MOLECULAR WEIGHT OF SPECIES A. The molecular weight of species A was found to be approximately 105,000 as determined by the method of Andrews (1964) on a 2.5 × 50 cm column of Sephadex G-100. The following protein standards were used: bovine serum albumin, mol wt 68,000, Sigma; alkaline phosphatase, *Escherichia coli*, mol wt 80,000, Worthington Biochemical Corp.; and γ-globulin, bovine fraction II, mol wt 160,000, Armour Laboratories.

RECOVERY OF UNALTERED COLCHICINE-ACETYL-^{3}H AFTER BINDING TO SPECIES A. Several lines of evidence indicated that the tritium bound to species A was still on the *N*-acetyl group of chemically unaltered colchicine. It is clear that the label remained on a small

TABLE IV: Effect of Various Agents on the Stability of Species A Colchicine Complex.

Agent	Concentration	Dpm/μg of Supernatant Protein		
		No Added Agent	With Added Agent	% Destruction
Urea[a]	4.5 M	30.2	0.6	98
Trypsin[b,c]	0.0013%	46.7	2.0	96.6
Ribonuclease[a]	0.027%	30.2	35.5	0
Deoxyribonuclease[b,d]	0.005%	46.7	47.3	0
Dithioerythritol[a]	2.1×10^{-3} M	31.8	35.7	0
Unlabeled colchicine[a]	6.65×10^{-4} M	30.2	30.9	0
Unlabeled colchicine[a]	6.65×10^{-4} M	30.2	33.1	0

[a] A 1.5-ml homogenate, prepared in the usual manner (see Methods) from 100 embryos (age 25 days), was diluted to 3.4 ml with additional isotonic medium. Aliquots of the homogenate (450 μl) were incubated with labeled colchicine (8.5×10^{-7} M) for 3 hr at 37°, cooled, diluted to 1.6 ml with cold isotonic medium, centrifuged at 5000g to remove cell debris, and stored at −20°. To test the effect of a particular agent, two 700-μl aliquots of the above mixture were incubated (one in the presence of the agent, the other in absence of the agent) in a final volume of 1.5 ml of isotonic medium for 1 hr at 37°. After gel filtration on Sephadex G-25 as described in the legend of Figure 3, the radioactivity of peak A was determined for each incubation mixture. [b] Procedure was the same as in *a*, except that the source of colchicine complex was the 100,000g supernatant fraction from another batch of embryos. [c] Mixture containing bound complex was incubated with trypsin for 0.5 hr. [d] Mixture containing bound complex was incubated with deoxyribonuclease in presence of added bovine serum albumin (660 μg).

molecule since upon recycling of peak A fractions through columns of Sephadex G-25 (elution with water) label was only found in the free colchicine area. In addition, tritium could be quantitatively recovered from lyophilized peak A fractions by extraction in absolute ethanol or methanol at 25°. When such an extract was subjected to thin layer chromatography with added colchicine carrier and developed with methanol (see Methods) the tritium was found only in one spot coincident with colchicine at R_F 0.60. Similarly, in chromatograms developed with butanol–ammonia–water (2:1:2) tritium was again found only in one spot coincident with colchicine at R_F 0.73.

It is concluded that colchicine binding in species A must be noncovalent since labeled colchicine is so easily removed from bound form upon recycling through Sephadex G-25 and by extraction with organic solvents.

Discussion

The actions of colchicine can be attributed to a direct effect on the organization of microtubules. This broad and rapidly developing area is reviewed in the doctoral dissertation upon which this paper is based (Wilson, 1967). We must of necessity consider only a few facets of colchicine action as revealed by our studies with grasshopper embryos. The ideas developed herein have been especially influenced by the papers of Levan and Ostergren (1943), Inoué (1952), Eigsti and Dustin (1955), Sauaia and Mazia (1961), Malawista (1965), Tilney (1965a,b), Taylor (1965), Marsland (1966), Auclair and Siegel (1966), Porter (1966), and Malawista and Bensch (1967).

We propose that the binding of colchicine to a high molecular weight substance (species A) is specifically and directly related to the well-known antimitotic effects of colchicine. Justification for this thesis is based upon the following considerations.

1. Experiments with Lumicolchicine. One cogent argument in favor of the idea that the binding of colchicine demonstrated herein is specifically related to the biological effect of colchicine on cell division is the finding that lumicolchicine, having no antimitotic activity (Table II) despite its resemblance in structure to colchicine, is poorly bound and does not interfere with the binding of colchicine to species A.

2. Influence of Some Spindle Poisons on Colchicine Binding. A second argument in support of specific and biologically related binding of colchicine is the demonstration that podophyllotoxin, picropodophyllotoxin, and vinblastine influence the degree of colchicine binding to species A. Furthermore, the antimitotic activities of podophyllotoxin and picropodophyllotoxin appear to be closely correlated with the ability of these agents to inhibit colchicine binding to species A (Tables II and III).

Colchicine binding to species A was almost completely prevented both *in vivo* and *in vitro* by podophyllotoxin. Even at very low concentrations of podophyllotoxin marked inhibition of colchicine binding occurred, indicating the high specificity of the podophyllotoxin effect. Picropodophyllotoxin, an antimitotic agent less active than podophyllotoxin and colchicine, exhibited a decreased ability to block colchicine binding.

Vinblastine strongly inhibited cell division in the grasshopper neuroblast. The concentrations of vinblastine used in the binding experiments were high since a maximal effect on the colchicine binding was desired. The stimulatory effect of vinblastine on colchicine binding, while not as striking as the inhibitory effect of podophyllotoxin, was quite significant. The relation of this effect to a possible mechanism of action of various spindle poisons is discussed in section 3.

3. Temperature Dependence of Colchicine Binding. Physical factors such as temperature and pressure play an important role in the dynamic structure of the mitotic spindle and other microtubules and therefore could influence the binding of colchicine (Inoué, 1952, 1964; Tilney, 1965a,b; Marsland and Asterita, 1966).

Results of the present investigation have shown that the binding of colchicine to species A *in vivo* and *in vitro* is temperature dependent. In both cases, the binding was almost completely prevented at 0°. The temperature-dependent binding of colchicine with intact embryos might be attributable to an effect on an active process of colchicine uptake by intact cells. However, this cannot explain our results since with cell-free extracts the temperature dependence was again demonstrable. Another possibility is that the colchicine is metabolized to an active substance through a temperature-dependent process. The metabolite of colchicine could then bind to species A. However, this possibility must be ruled out because chemically unaltered colchicine could be recovered from the species A complex.

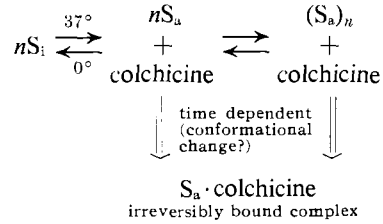
Although the species A–colchicine complex cannot be formed at 0°, when once constituted at 37°, it is stable at 0°. This indicates that the stability of the bonding forces is not reflected by the temperature dependence.

The temperature effect also rules out an adsorptive process such as the nonspecific adsorption of various aromatic substances by activated charcoal. Another possible explanation to account for the temperature-dependent colchicine binding is that an inactive form of species A exists at 0°. At 37°, it is immediately converted to an active form which then has the property to irreversibly bind colchicine, in a time-dependent manner. However, the temperature effect on binding must reflect a rapidly reversible process since species A in embryo extracts preincubated at 37° immediately loses the ability to bind colchicine upon cooling. Why then should the binding of colchicine to an activated form of species A be time dependent?

This investigation did not reveal at which level of organization colchicine might interact with the spindle. In Scheme I (a possible model), colchicine is shown combining either with a subunit (mol wt 105,000), or with a polymer with several binding sites for colchicine which then dissociates into subunits with bound colchicine. In either case, the final form of bound colchicine has a molecular weight of 105,000.

The results of Sakai (1966) and Stephens (1967) indicate that the basic subunit of the mitotic apparatus is a 2.5S protein. This may be the receptor for colchicine.

SCHEME I: Possible Interactions of Colchicine with Spindle Components.[a]



[a] S_i, inactive subunit (species A, mol wt 105,000); S_a, active subunit (species A, mol wt 105,000); and $(S_a)_n$, could represent a polymer or larger aggregate including microtubules.

The molecular weight as determined by Stephens was 105,000–120,000 (similar to the molecular weight of species A). Sakai reported a molecular weight of 34,700 for the 2.5S protein. However, this value could be low (Stephens, 1967).

It is possible that colchicine when in contact with its receptor protein causes a conformational change which is time dependent, or both time and temperature dependent, locking the complex into a form that does not dissociate and is not in equilibrium with free colchicine.

It is noteworthy that vinblastine caused a stimulation while podophyllotoxin and picropodophyllotoxin caused inhibition of colchicine binding to species A. This indicates that vinblastine and podophyllotoxin exert their antimitotic effects by different mechanisms. Podophyllotoxin may be acting by a mechanism similar to that of colchicine, preventing the polymerization process by interfering either directly or indirectly at the level of the activated unit (Scheme I). Since the inhibition of colchicine binding by podophyllotoxin is so striking at the low concentrations used, it is entirely possible that these two agents are competing for the same site. Vinblastine might act at a subsequent step, either directly or indirectly causing a depolymerization of larger aggregates. This would provide an increase in the number of smaller units available for colchicine binding. Vinblastine and colchicine exhibit striking differences in their antitumor activities. For this reason, George *et al.* (1965) suggested that the antitumor activities of these agents might be mediated *via* a mechanism unrelated to their antimitotic activities. However, differences in their mechanism of antimitotic action, as supported above, could be responsible for the variation in antitumor activity.

The possibility that colchicine accomplishes many of its actions by a direct binding to the subunits of microtubular structures remains to be determined.

We believe that experimental data on the binding of colchicine to a high molecular weight substance is highly suggestive in its possible relation to the action of colchicine on mitosis. Further elucidation of the nature of the colchicine binding is of utmost importance since it may lead to the uncovering of the mechanism

of action of colchicine, as well as providing new insights into the biochemical mechanisms involved in mitosis and cellular movement.

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Added in Proof

Two important papers have appeared (Borisy and Taylor, 1967) showing that colchicine forms a complex with protein present in isolated mitotic apparatus, cilia, and brain tissue. These authors believe that the binding of colchicine prevents the assembly of microtubule subunits.

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