Properties of Colchicine Binding Protein from Chick Embryo Brain. Interactions with Vinca Alkaloids and Podophyllotoxin*

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ABSTRACT: The 120,000 molecular weight colchicine binding protein found in the 100,000*g* supernatant fraction of chick embryo brain homogenates is considered to be a dimer subunit of brain microtubules. The colchicine binding activity of the protein was markedly influenced by the experimental conditions, and by other chemical agents which disrupt microtubules. Maximum colchicine binding activity was obtained at pH 6.7–6.8. A shift in the pH from this region exerted no effect on the protein–colchicine association reaction, but greatly influenced the binding activity of the protein indirectly, by increasing the inactivation constant of the protein. Ionic strength also affected the inactivation process. Binding activity was least stable at low ionic strength. Stability was increased by the addition of sodium salts; optimal stability was obtained with approximately 200 mM sodium glutamate. As in the case of pH, changes in ionic strength did not influence the protein–colchicine association reaction. The vinca alkaloids, vinblastine and vincristine, stabilized colchicine binding activity, but did not appear to influence the colchicine–protein association reaction. Stabilization was proportional to the logarithm of the vinca alkaloid concentration and occurred in a concentration range in which these agents produce their disruptive effects on microtubules. Vinblastine stabilized the binding activity of the protein to the same degree, whether or not colchicine was bound to the protein. With high concentrations of vincristine, nearly complete stabilization of colchicine binding activity was observed. In contrast to the vinca alkaloids, podophyllotoxin inhibited the binding of colchicine to the protein. Podophyllotoxin exerted no inhibitory effect when tested on preformed protein–colchicine complex. Podophyllotoxin did not inhibit the binding of colchicine by increasing the inactivation constant of the binding activity. Rather, an increase in stability of the binding activity was observed, even when podophyllotoxin was added to protein which already had the colchicine binding site(s) occupied. Therefore, the inhibitory effects of podophyllotoxin on the binding of colchicine may not be brought about by a simple competitive mechanism.

Colchicine exerts its antimitotic action through an effect on spindle microtubules, and there is increasing evidence which supports the hypothesis that many of the other biological actions of colchicine are also caused by a disruptive action on microtubules (Malawista, 1965; Wilson and Friedkin, 1967; Borisy and Taylor, 1967). Results of several studies have indicated that colchicine binds to a protein subunit of microtubules. Borisy and Taylor (1967) found a direct correlation between the amount of colchicine binding obtained in a tissue or cell type with the observable occurrence of microtubules, and Shelanski and Taylor (1967) demonstrated that the time-dependent appearance of a 6S colchicine binding protein in solution was associated with the disappearance of the central pair microtubules in suspensions of isolated sea urchin sperm tails. Furthermore, the binding of colchicine *in vivo* and *in vitro* to a 105,000 molecular weight protein in grasshopper embryos considered to be a microtubule subunit was shown to be specifically related to the antimitotic activity of the colchicine (Wilson and Friedkin, 1967).

This communication describes some of the biochemical properties and colchicine binding characteristics of a microtubule subunit found in the soluble fraction of chick embryo brain homogenates. Evidence which demonstrates the existence of several binding sites and the effects of interactions of other spindle poisons with these sites is presented. The possible significance of these results with regard to the role of the colchicine binding subunit in microtubule formation is discussed.

Materials

Colchicine-acetyl-t. Pure colchicine *acetyl-t* was prepared and rechromatographed as described in a previous paper (Wilson and Friedkin, 1966). Stock solutions at 7.6×10^{-4} M (based on absorbancy at 350 mμ, ε 16,740) were stored at −20° under nitrogen gas. The specific activity was 240 mCi/mmole.

Miscellaneous Chemicals. Podophyllotoxin was obtained from Aldrich Chemical Co. (mp 112–114, λ_{max} 290 mμ in 95% ethanol, ε 3750). Vinblastine sulfate (99.8% pure) and vincristine sulfate (97.2% pure) were gifts from Eli Lilly and Co. The preparation of lumicolchicine-*acetyl-t* by irradiation of colchicine-*acetyl-t* with ultraviolet light was described in an earlier paper (Wilson and Friedkin, 1966). Sephadex G-100 and Blue Dextran 2000 were obtained from Pharmacia, Inc. Trypsin (twice crystallized) was obtained from Mann Research Laboratories. Disks of Whatman DE81 Chromedia paper (2.5 cm diameter) were obtained from the Reeve Angel Co.

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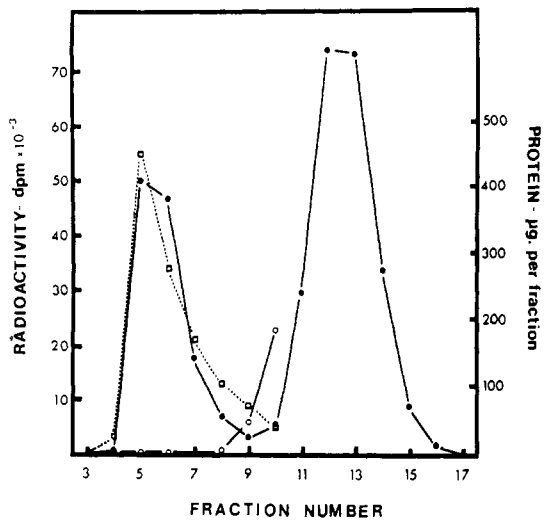

FIGURE 1: Gel filtration of colchicine complex. An aliquot of a 100,000*g* supernatant extract of chick embryo brain containing 1.74 mg of protein in phosphate-glutamate buffer (pH 6.8) was incubated for 1.5 hr at 37° with 1.2×10^{-6} M colchicine-*acetyl-t* in a final volume of 640 µl. A second identical aliquot of the extract was incubated with colchicine at 0°; a third aliquot was incubated with 1.2×10^{-6} M lumicolchicine-*acetyl-t* at 37°. Samples were filtered through columns of Sephadex G-100 as described in Methods section and 1-ml fractions were collected. Closed circles = radioactivity, 37° colchicine experiment. The radioactivity profiles for the lumicolchicine and the 0° colchicine experiments (open circles) were the same. The protein profile (squares) is that of the 37° colchicine experiment.

Methods

Colchicine-acetyl-t Assay Procedures

Gel Filtration Assay. Bound colchicine-*acetyl-t* was determined by gel filtration on 1 × 15 cm columns of Sephadex G-100. Aliquots of incubation mixture (1.0 ml) containing bound and free colchicine-*acetyl-t* were applied to columns which were previously equilibrated with 20 mM sodium phosphate buffer containing 100 mM sodium glutamate at pH 6.8. The columns were eluted at a flow rate of 6 ml/hr with the same buffer. Protein-bound colchicine was quantitatively separated from unbound colchicine (see Figure 1) and the entire bound colchicine peak was collected, usually in a single volume. Aliquots of bound colchicine peaks were dissolved in 15 ml of Bray's solution (1960) for determination of radioactivity. This method yielded the most accurate binding results, and column assays could be performed either simultaneously utilizing several columns, or at different times with the same column with excellent precision. The standard error with this system was less than 2.5%.

DE81 Paper Disk Assay. Aliquots of incubation mixture (usually 100 µl) containing free and bound colchicine were applied directly to slightly moistened 2.5 cm disks of Whatman DE81 Chromedia paper at 0°. After 10 min the paper disks were washed by immersion in five successive 30–40-ml changes of 10 mM sodium phosphate buffer (pH 6.8) (5 min/wash, 0°) to remove all unbound colchicine. The paper disks with adhering protein-bound colchicine-*acetyl-t* were then counted directly in a scintillation vial containing 5 ml of Bray's solution. This method, which is a modification of a method developed by Weisenberg *et al.* (1968), depends upon the adherence of colchicine receptor protein to filter paper which is impregnated with DEAE-cellulose. An aliquot of reaction mixture is placed directly on the paper, and a series of washes is used to remove unbound colchicine rather than the filtration techniques employed previously. As many as 20 paper disks were easily processed at one time, and all of the disks were washed together. Negligible binding of unbound colchicine to the paper disks occurred in controls, either in the presence or absence of protein (less than 1% of the amount obtained with protein-bound colchicine). Unbound colchicine was almost completely removed from the disks after the fourth wash. The binding results of duplicate experiments always agreed to within 6%. Greater variation was obtained in duplicate experiments assayed at different times (10–15%); therefore, all colchicine binding assays in an experiment were usually assayed together. In duplicate experiments, binding values obtained with the paper disk method were approximately 55–65% of those obtained with the gel filtration method. Lower binding with the paper disk assay may be due to the use of low ionic strength conditions which results in the inactivation of the protein and loss of protein from the paper during the washing procedure.

General Procedures. Homogenization of chick embyro brains was accomplished with a motor-driven Teflon–glass tissue homogenizer. The freshly dissected brains obtained from 12- to 18-day-old chick embyros were homogenized for 30 sec at 0° in 2–3 ml of 20 mM sodium phosphate buffer containing 100 mM sodium glutamate (pH 6.8) (referred to as phosphate glutamate buffer in this paper).

Tritium was determined with a Tri-Carb scintillation counter. Internal standards of tritiated water were used to determine counting efficiency. All Sephadex column chromatography, centrifugation, and paper disk binding assays were performed at 0–4°. High-speed centrifugation was performed in the Spinco Model L preparative ultracentrifuge with No. 40 rotor. Protein was determined by the method of Lowry *et al.* (1951).

Results

Binding of Colchicine to Microtubule Subunit Protein. Colchicine binding activity was found in 100,000*g* supernatant extracts of chick embryo brain homogenates after incubation of the extracts with colchicine-*acetyl-t* at 37°. Colchicine, bound to a macromolecule, was separated from unbound colchicine by filtration of the incubation mixture through a 1 × 15 cm column of Sephadex G-100 (Figure 1). In contrast with results obtained in other tissues (Borisy and Taylor, 1967; Shelanski and Taylor, 1967; Wilson and Friedkin, 1967), significant colchicine binding activity was also found associated with the particulate fraction of brain (approximately 50% of that found in the soluble fraction). Similar results in brain have been reported by Feit *et al.* (1970). The particulate activity was not used in this study. Some of the characteristics of the colchicine binding macromolecule and of the colchicine binding reaction were in agreement with results of similar experiments in other systems. No binding was observed in the macromolecular fraction after incubation of the supernatant extract with lumicolchicine-*acetyl-t* (Figure 1). Lumicolchicine is an isomer of colchicine which is devoid of any antimitotic activity (Linskens and Wulf, 1953) and

does not bind to the 105,000 molecular weight colchicine receptor protein found in grasshopper embryos (Wilson and Friedkin, 1967).

The molecular weight of the colchicine complex was determined by the method of Andrews (1964). A 2.5 × 65 cm column of Sephadex G-100 was equilibrated with phosphate-glutamate buffer, and calibrated with the following protein standards: chymotrypsinogen A (beef pancreas), molecular weight = 25,000; ovalbumin, molecular weight = 45,000; serum albumin, molecular weight = 68,000; γ-globulins (human), molecular weight = 160,000. A 100,000*g* supernatant extract of chick embyro brain (1.5 ml, containing 4.5 mg of protein) prepared in phosphate-glutamate buffer was incubated with 2.4×10^{-6} M colchicine-*acetyl-t* for 1.5 hr at 37°, cooled to 0°, and filtered through the column. Bound colchicine appeared as a single sharp peak corresponding to a molecular weight of 120,000. This is similar to the molecular weight of the colchicine binding microtubule protein reported in other systems (Adelman *et al.*, 1968; Wilson and Friedkin, 1967; Weisenberg *et al.*, 1968). At a concentration of 2.4×10^{-6} M colchicine, approximately 0.15 molecule of colchicine was bound per molecule of total protein found in the 120,000 molecular weight fraction. Incubation of the colchicine complex with 0.005% trypsin for 30 min at 37° destroyed 96% of the bound complex.

The colchicine binding reaction was both temperature and time dependent (Wilson and Friedkin, 1967; Borisy and Taylor, 1967). The amount of colchicine binding obtained after incubation of brain supernatant extracts with colchicine at 0° was less than 1% of the value obtained at 37° (Figure 1). With 2×10^{-6} M colchicine, binding at 37° increased rapidly for the first 90 min, then leveled off at about 2–2.5 hr. Colchicine binding activity was also influenced in a characteristic manner by other agents which disrupt microtubules (described below), and the colchicine-binding macromolecule is considered to be a 120,000 molecular weight protein dimer subunit of brain microtubules.

Factors Which Influence Colchicine Binding Activity. The binding activity of the colchicine receptor protein was unstable. This property of colchicine binding protein may reflect a change in the protein that normally occurs in the cell, and which is related to the ability of the protein to participate in microtubule structure (see Discussion). Because of the instability, the experimental conditions for quantitative determination of colchicine binding activity, both during the binding reaction with colchicine and in the binding assay, were critical. By trial and error, a standard set of optimal incubation and assay conditions was developed. When these conditions were employed, the amount of colchicine binding activity obtained in soluble extracts of brain was proportional to the amount of total protein present in the extract. The amount of supernatant protein present during incubation with colchicine could be varied from 50 to 880 μg without any deviation from linearity of the binding values.

First-Order Loss of Colchicine Binding Activity of Brain Microtubule Protein. In soluble extracts of chick embryo brain, colchicine binding protein undergoes an aging process whereby the ability to bind colchicine decays according to first-order kinetics (Figure 2). A similar loss in colchicine binding activity was seen in microtubule protein isolated from porcine brain (Weisenberg *et al.*, 1968) and HeLa cells (Borisy and Taylor, 1967). Under the conditions of the experiment described in Figure 2 (pH 6.8, 100 mM sodium glutamate, 37°), the half-time for inactivation was 275 min which corresponds to an inactivation constant of 2.5×10^{-3} min^{-1}. Since the colchicine binding reaction was carried out for 120 min at the same temperature, extrapolation of the line back 120 min from the zero-time colchicine binding point (no preincubation) yielded the colchicine binding capacity of the protein at the beginning of the experiment (480 dpm/μg of protein).

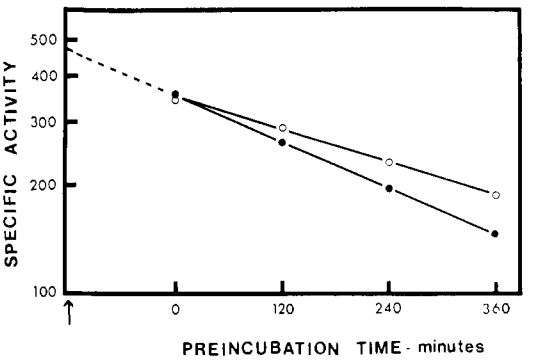

FIGURE 2: Loss of binding ability by colchicine receptor protein and loss of colchicine from the bound complex upon aging at 37°. A 100,000*g* supernatant extract of chick embryo brain containing 380 μg/ml of protein was prepared in phosphate-glutamate buffer (pH 6.8). Aging in the absence of colchicine (closed circles): tubes containing aliquots of the extract (190 μg of protein each) were preincubated in the absence of colchicine for the times indicated above at 37°, and then were incubated with 2.5×10^{-6} M colchicine-*acetyl-t* for 2 hr at 37° (final volume = 620 μl). Bound colchicine was assayed by the gel filtration method (see Methods) and is expressed as specific activity (disintegrations per minute of colchicine bound per microgram of protein in the incubation mixture). Extrapolation of the line back to include the time of the colchicine incubation (arrow) yielded a specific activity of 480 dpm/μg of protein as the initial binding capacity of the extract. Aging of bound colchicine complex (open circles): bound colchicine complex was prepared as described above by incubating tubes containing the supernatant extract with 2.5×10^{-6} M colchicine for 2 hr at 37° (no preincubation). Unlabeled colchicine (final concentration = 1.1×10^{-3} M) was added to each assay mixture, and tubes containing the preformed complex were incubated for the times indicated at 37°. Bound colchicine was determined as described above.

The rate of release of colchicine from the bound complex was very similar to the rate of loss of the colchicine binding activity of the protein (Figure 2). A large excess of unlabeled colchicine was added to the brain extract which contained preformed radioactive colchicine complex in order to prevent any further labeled complex formation, and the rate of dissociation of the colchicine complex was determined at 37°. The colchicine–protein complex dissociated according to first-order kinetics, with a half-time of 410 min ($k = 1.7 \times 10^{-3}$ min^{-1}). The rate of loss of colchicine from the bound complex was only slightly slower than was the rate of loss of the binding activity of the receptor protein. These results, in agreement with those of Borisy and Taylor in HeLa cells (1967), suggest that the bound colchicine stabilized the protein. However, the marked increase in stability of the protein as seen in HeLa cells after the binding of colchicine was not seen after the binding of colchicine by microtubule protein of chick embryo brain. In other experiments, the addition of an excess of unlabeled colchicine to preformed colchicine com-

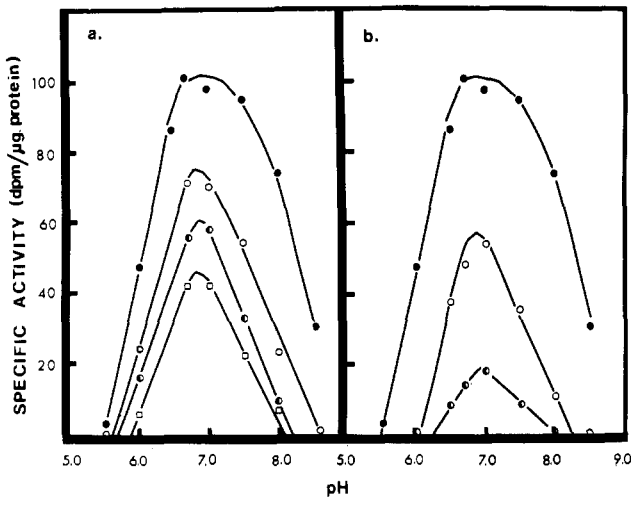

FIGURE 3: Colchicine binding *vs.* pH; 0 and 37°. Solutions of 40 mM sodium phosphate buffer containing 200 mM sodium glutamate were prepared at various pH values. Aliquots of a 100,000*g* supernatant extract of chick embryo brain prepared in distilled water were added in equal volumes to the buffer solutions, yielding solutions of brain extract in 20 mM sodium phosphate and 100 mM sodium glutamate at various pH values. At zero time, and after the preincubation times indicated, mixtures were tested for colchicine binding ability by incubating 600-μl volumes of the mixtures (each containing 282 μg of protein) with 2.0×10^{-6} M colchicine-*acetyl-t* at 37° for 1.5 hr. Bound colchicine was assayed by the paper disk method (see Methods) and is expressed as disintegrations per minute bound per microgram of protein present in each assay. (a) Preincubation at 37°: closed circles = no preincubation; open circles = after 90-min preincubation; half-open circles = 180 min; squares = 300 min. (b) Preincubation at 0°: closed circles = no preincubation (same curve as in part a); open circles = 480 min; half-open circles = 1350 min.

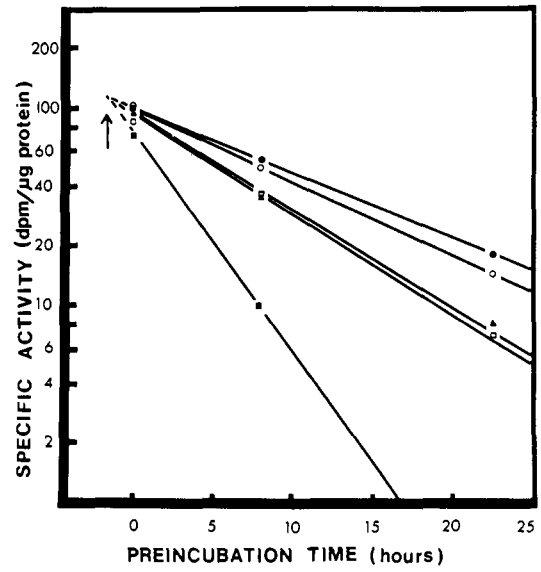

FIGURE 4: Loss of colchicine binding ability as a function of pH at 0°. The experimental method is described in the legend of Figure 3. Closed circles = pH 7.0; open circles = pH 6.7; triangles = pH 7.5; open squares = pH 6.5; closed squares = pH 8.0. The initial binding capacity, which is the same for each pH, was determined by extrapolating each line back to include the incubation time for the colchicine binding reaction.

plex had no effect on the loss of the bound labeled colchicine, up to a concentration of 10^{-3} M colchicine. However, above this concentration, excess colchicine greatly increased the rate of release of the bound labeled colchicine. Therefore, high concentrations of colchicine can destroy the colchicine complex, apparently through a nonspecific interaction with the protein.

Effect of pH on Colchicine Binding Activity. Colchicine binding activity was strongly influenced by pH. The colchicine binding profile obtained after incubating aliquots of a brain supernatant extract with colchicine over a pH range between 5.5 and 8.5 for 2 hr at 37° appears in Figure 3 (closed circles). Maximum binding occurred at pH 6.7–6.8. Binding activity decreased sharply with a lowering of the pH, and fell more gradually at higher pH values. Figure 3 also shows the pH profiles for the colchicine binding activity remaining in the same supernatant extracts after preincubation of the extracts at 37° for 90, 180, and 300 min (part a) and after preincubation at 0° for 480 and 1350 min (part b). The loss in colchicine binding activity during preincubation at 0° was first order with time for each pH tested, and the inactivation constants were different (Figure 4). All of the lines extrapolated back to the same point, which indicates that the initial colchicine binding capacity of the protein at each pH was the same. Identical results were obtained in the 37° experiment. Here also, the initial colchicine binding capacity for each pH was the same; only the inactivation constants were different. The inactivation constants for loss of colchicine binding activity, expressed as half-times, were calculated for each pH from the data in Figure 3 and are shown in relation to pH in Figure 5. It can be seen that the pH profile for the loss of colchicine binding activity during preincubation at 37° was identical with the zero time (no preincubation) profile of Figure 3. A slight shift in the pH stability is apparent when the 0 and 37° preincubation patterns are compared (Figure 5). The optimal pH for maintenance of colchicine binding activity at 0° may be slightly higher than at 37°. In other experiments, exposure of supernatant extracts to more extreme pH such as pH 4.5 and 10.5 for less than 10 sec at 0° irreversibly destroyed all of the colchicine binding activity of the protein. Therefore, the colchicine binding activity of microtubule protein is stable only over a narrow pH range. Similar effects of pH on the colchicine binding activity of microtubule protein(s) were observed in extracts of isolated sperm tails of *Strongylocentrotus purpuratus* (L. Wilson, unpublished data). These results suggest that changes in pH influence the binding of colchicine through an indirect effect on the protein, and do not directly influence the formation of the colchicine complex, nor the molecular forces involved in the protein–colchicine interaction.

Influence of Temperature on Colchicine Binding Activity. The effects of temperature on the colchicine–protein association reaction with brain microtubule protein were similar to those reported in grasshopper embryos (Wilson and Friedkin, 1967). In addition, the stability of the colchicine binding activity of the protein was influenced by temperature. Binding activity was approximately twice as stable at 0° than at 37° between pH 6.7 and 7.5 (Figure 5). Higher temperatures rapidly destroyed activity. Warming a brain extract containing colchicine binding protein to 58° for 90 sec at pH 6.8 de-

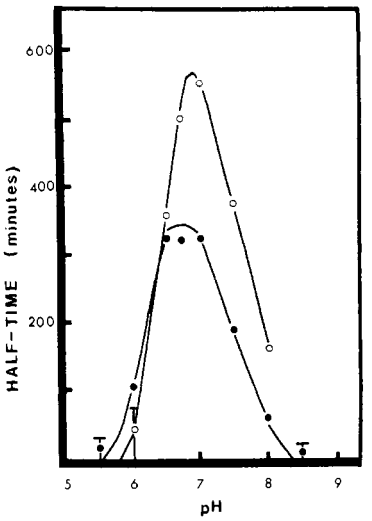

FIGURE 5: Stability of colchicine binding activity as a function of pH and temperature. Half-times for the loss of colchicine binding activity at each pH tested were calculated from the data of Figure 3 both for 37° and 0°, and are shown above. Closed circles = 37°; open circles = 0°.

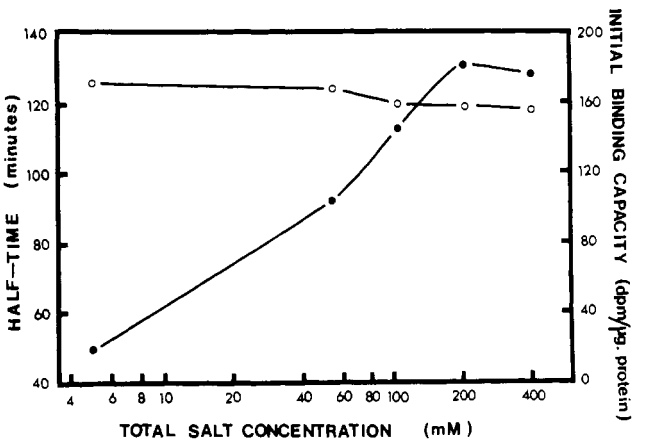

FIGURE 6: Effect of NaCl on colchicine binding activity. A 100,000*g* supernatant extract of chick embryo brain was prepared in phosphate-glutamate buffer (pH 6.8) and filtered through a 1 × 15 cm column of Sephadex G-100 which was previously equilibrated with 5 mM sodium phosphate buffer (pH 6.8). The macromolecular peak (fractions 3–8; see Figure 1) was collected and contained 1.07 mg of protein/ml in 5 mM sodium phsophate (pH 6.8). Aging experiments of the type shown in Figure 4 were performed with increasing concentrations of NaCl. Samples were incubated with colchicine (2.9×10^{-6} M) for 90 min at 37° at zero time, and after 3- and 6-hr preincubation at 37°. Binding was assayed by the gel filtration method. Half-times for each NaCl concentration were determined and are shown above (closed circles). Extrapolation of the straight lines back to include the colchicine incubation time yielded the initial binding capacity of extracts at each NaCl concentration (open circles). Specific activity = disintegrations per minute of bound colchicine per microgram of protein in each incubation mixture (428 μg). Total salt concentration = 5 mM sodium phosphate plus the conentration of NaCl added (therefore, 5 mM salt = no addition of NaCl).

stroyed over 75% of the colchicine binding activity of the protein. Freezing brain extracts in phosphate-glutamate buffer for 2–3 days, either with or without bound colchicine, resulted in no loss of binding activity.

Ionic Strength. The ionic strength and type of salt also markedly influenced colchicine binding activity. As in the case of pH, changes in colchicine binding activity with different concentrations of salt were not due to a direct effect on formation of the colchicine–protein complex, but resulted from a change in the half-time of the inactivation process. A 100,000-*g* supernatant extract of chick embryo brain was passed through a column of Sephadex G-100 which was previously equilibrated with 5 mM sodium phosphate buffer (pH 6.8) and the macromolecular volume was collected. This procedure yielded colchicine binding protein in 5 mM sodium phosphate at pH 6.8, with all of the small molecules present in the original extract removed. The effects of increasing NaCl concentrations on colchicine binding activity were tested at 37° exactly as described previously for pH. First-order loss of binding activity was obtained at all NaCl concentrations. Increasing concentrations of NaCl stabilized the colchicine binding activity of the protein in a concentration-dependent manner (Figure 6). Maximum stability occurred at approximately 200 mM NaCl.

When the straight lines were extrapolated to initial binding capacity, the values were approximately the same for all NaCl concentrations (Figure 6). Therefore, increasing concentrations of NaCl exert no direct effect on the formation of the colchicine–protein complex. Similar effects were observed with sodium acetate and sodium glutamate, but with increased stabilization. At a concentration of 100 mM, the stabilizing ability of sodium glutamate was approximately 20% greater than sodium acetate,and 50% greater than sodium chloride.

Preliminary experiments have shown that the ionic strength and type of salt also influence the colchicine binding assay on Sephadex G-100 columns. Bound colchicine complex was most stable on columns equilibrated with sodium glutamate, and in general, the use of 100–200 mM sodium glutamate provided the best overall conditions for colchicine binding. The effects of ionic strength and pH on preformed bound colchicine complex were not investigated systematically, but preliminary evidence indicates that the effects of both on the stability of the protein are the same whether or not colchicine is bound to the protein.

Influence of Other Agents Which Disrupt Microtubules on Colchicine Binding Activity. VINCA ALKALOIDS: VINBLASTINE SULFATE AND VINCRISTINE SULFATE. The vinca alkaloids vinblastine and vincristine can disrupt a number of microtubule systems (George *et al.*, 1965; Wisniewski *et al.*, 1968; Malawista *et al.*, 1968), and earlier work has shown that vinblastine sulfate can influence the binding of colchicine in grasshopper embryos (Wilson and Friedkin, 1967). The addition of vinblastine sulfate to a brain extract containing colchicine binding protein markedly stabilized the colchicine binding activity of the protein (Figure 7). In the presence of 3.1×10^{-4} M vinblastine at 37°, the protein lost its ability to bind colchicine according to first-order kinetics with a half-time of 880 min ($k = 0.8 \times 10^{-3}$ min^{-1}) while the half-time for the control in the absence of vinblastine was 280 min ($k = 2.5 \times 10^{-3}$ min^{-1}). This represented a threefold increase in stabilization of the binding activity by vinblastine. Vinblastine had little or no effect on the initial colchicine binding capacity of the protein; therefore, vinblastine does not appear to directly influence the association reaction between colchicine and the protein. Similar stabilization with vinblastine also

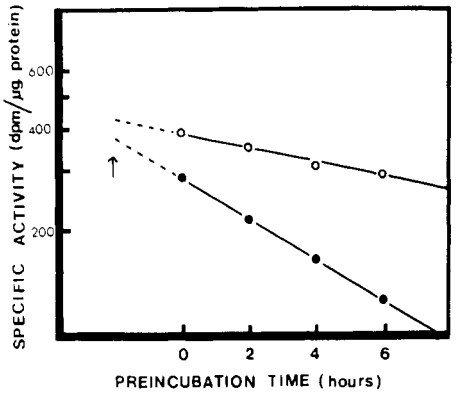

FIGURE 7: Loss of colchicine binding activity of microtubule protein upon aging at 37° in the presence and absence of vinblastine sulfate. Tubes containing aliquots of a 100,000*g* supernatant extract of chick embryo brain in phosphate-glutamate buffer (pH 6.7; 400 μg of protein each) were preincubated in the presence and absence of 3.1×10^{-4} M vinblastine sulfate for the times indicated at 37°. After preincubation, mixtures were incubated with 2.6×10^{-6} M colchicine-*acetyl-t* for 2 hr at 37°. Colchicine binding was assayed by the gel filtration method and is expressed as disintegrations per minute of colchicine bound per microgram of protein in each incubation mixture. Closed circles = no vinblastine; open circles = with vinblastine.

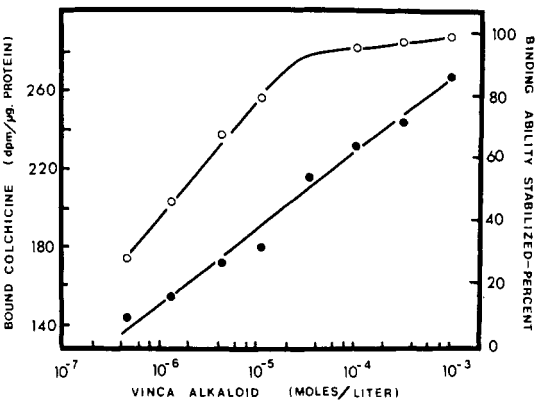

FIGURE 8: Stabilization of colchicine binding activity by vinca alkaloids. Tubes containing 250-μl aliquots of a 100,000*g* supernatant extract of chick embryo brain in phosphate-glutamate buffer (pH 6.7; 355 μg of protein per tube) were preincubated for 3 hr at 37° with increasing concentrations of vinblastine or vincristine in a final volume of 520 μl. The colchicine binding activity in each tube was determined after incubation with 3.0×10^{-6} M colchicine-*acetyl-t* for 2 hr at 37°. A control aging experiment was also performed without any vinca alkaloid (as described in the legend of Figure 2) to determine the initial colchicine binding capacity of the extract and the inactivation constant in the absence of vinca alkaloid. The per cent of binding ability stabilized represents the amount of binding activity remaining after 3-hr preincubation as compared with the unstabilized control (130 dpm/μg of protein) and the initial binding capacity of the extract (290 dpm/μg of protein). Bound colchicine was assayed by the paper disk method and is expressed as dpm bound per μg of protein present in the supernatant extract. Open circles = vincristine; closed circles = vinblastine.

occurred on preformed protein–colchicine complex. Protein–colchicine complex was prepared from the same brain extract used in the experiment of Figure 7. A 500-fold excess of unlabeled colchicine was added to prevent any further binding of labeled colchicine and the rate of release of labeled colchicine from the bound complex was determined at 37° in the presence and absence of 3.1×10^{-4} M vinblastine. In the absence of vinblastine, the protein lost previously bound colchicine with a half-time for release of the colchicine of 420 min ($k = 1.7 \times 10^{-3}$ min^{-1}), while in the presence of vinblastine, the half-time was 1260 min ($k = 0.6 \times 10^{-3}$ min^{-1}). This also represents a threefold stabilization by vinblastine. The stabilization of binding activity by a given concentration of vinblastine was the same whether or not colchicine was bound to the protein. These experiments suggest that release of colchicine from the bound complex primarily reflects the instability of the binding activity (see Discussion). The stabilization of colchicine binding activity at 37° by vinblastine was proportional to the logarithm of the vinblastine concentration (Figure 8). At 1×10^{-3} M vinblastine, the colchicine binding activity was nearly ten times more stable than it was in the absence of vinblastine (Table I). Colchicine binding protein precipitates at vinblastine concentrations greater than 10^{-3} M (Marantz *et al.*, 1969; Olmsted *et al.*, 1970; Wilson *et al.*, 1970); therefore, higher concentrations of vinblastine were not tested. Stabilization of colchicine binding activity also occurred with vincristine. Stabilization was proportional to the logarithm of the vincristine concentration to approximately 5×10^{-5} M vincristine (Figure 8), where stabilization approached 100%. Vincristine was about ten times more potent than vinblastine since the concentration of vinblastine necessary to attain 50% stabilization of colchicine binding activity was 3×10^{-5} M, while a concentration of 2×10^{-6} M vincristine produced the same effect. Maximum stability of colchicine binding activity in the presence of 1×10^{-3} M vincristine was over 100 times greater than in the absence of vincristine. The slopes for vinblastine and vincristine stabilization were reasonably similar, suggesting that stabilization by both compounds was mediated *via* a common mechanism. In contrast with vinblastine, 2×10^{-3} M vincristine did not cause precipitation of colchicine binding protein.

Other Agents Which Stabilize Colchicine Binding Activity. Several compounds in addition to the vinca alkaloids stabilize colchicine binding activity. The addition of 1.1×10^{-3} M GTP (Sigma, Grade II) and 10^{-3} M Mg^{2+} to a 100,000*g* supernatant extract of chick embryo brain increased the half-time at 37° from 252 min ($k = 2.8 \times 10^{-3}$ min^{-1}) to 630 min ($k = 1.1 \times 10^{-3}$ min^{-1}), a 2.5-fold increase in stability. These results are in agreement with those of Weisenberg *et al.* (1968). Vinblastine was more potent than GTP, since addition of only 3.4×10^{-4} M vinblastine to another aliquot of the same protein extract resulted in a half-time of 1500 min ($k = 0.46 \times 10^{-3}$ min^{-1}). This was more than twice the stability seen with GTP. High concentrations of glucose also stabilized colchicine binding activity. In one experiment, the addition of 20% w/v glucose to a 100,000*g* supernatant brain extract increased the stability of the colchicine binding activity at 37° 4.5-fold.

Inhibition of Colchicine Binding by Podophyllotoxin. Podophyllotoxin, the major active principle of podophyllum resin, is a potent spindle poison whose cytological effects appear to be very similar to those of colchicine (Cornman and Cornman,

TABLE I: Stabilizing Effects of Vinblastine and Vincristine on the Loss of Colchicine Binding Activity of Microtubule Protein at 37°.

Concn (moles/l.)	Half-Time (min)	
	Vinblastine	Vincristine
4.6×10^{-7}	294	410
1.4×10^{-6}	396	600
4.1×10^{-6}	462	1050
1.2×10^{-5}	515	1700
3.7×10^{-5}	790	
1.1×10^{-4}	965	8450
3.3×10^{-4}	1130	15000
1.0×10^{-3}	2260	~56000

[a] Half-times for each concentration of vinblastine and vincristine were calculated from the data of Figure 8. The half-time for one control was 255 min.

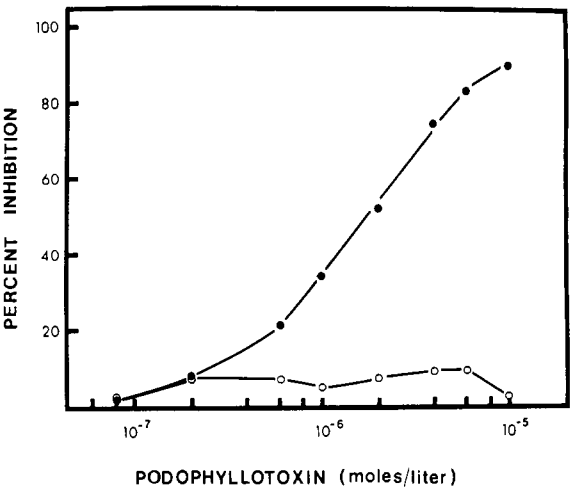

FIGURE 9: Inhibition of colchicine binding by podophyllotoxin. A 100,000*g* supernatant extract of chick embryo brain was prepared in phosphate-glutamate buffer (pH 6.8; see Methods). Preincubation with podophyllotoxin (closed circles): tubes containing aliquots of the brain extract (1.43 mg of protein/tube) were preinucbated with increasing concentrations of podophyllotoxin for 1 hr at 37°, and then incubated for an additional 2 hr with 1.9×10^{-6} M colchicine-*acetyl-t* at 37° (final volume = 600 μl). Bound colchicine was assayed by the paper disk method. The specific activity of bound colchicine in the absence of any podophyllotoxin (0% inhibition) was 103 dpm/μg of total protein present in the incubation mixture. Incubation of preformed colchicine complex with podophyllotoxin (open circles): bound colchicine complex was prepared by incubating tubes containing the supernatant extract (1.43 mg of protein/tube) with 1.9×10^{-6} M colchicine-*acetyl-t* for 2 hr at 37°. Excess unlabeled colchicine was then added to each incubation mixture (final concentration of unlabeled colchicine = 1.0×10^{-3} M; ratio of unlabeled to labeled colchicine = 530:1). Tubes containing the preformed complex were then incubated with increasing concentrations of podophyllotoxin for 2 hr at 37° (total volume = 1.0 ml). Bound colchicine was determined as described above. The specific activity in the absence of any podophyllotoxin (0% inhibition) was 78 dpm/μg of total protein present in the incubation mixture.

1951; Kelly and Hartwell, 1954; Wisniewski *et al.*, 1968). In contrast to the effects of vinblastine and vincristine, podophyllotoxin prevents the binding of colchicine to microtubule subunit protein (Wilson and Friedkin, 1967). A typical S-shaped log concentration *vs.* per cent inhibition curve was obtained when labeled colchicine was incubated with a 100,000*g* supernatant extract of brain to which podophyllotoxin had been added previously (Figure 9). Fifty per cent inhibition was obtained with a molar ratio of podophyllotoxin to colchicine of 1:1. In a second experiment with the same protein extract, the colchicine–protein complex was made prior to the addition of podophyllotoxin. In this case, the podophyllotoxin had no effect on the preformed colchicine–protein complex. These results suggest that podophyllotoxin may prevent the binding of colchicine by interacting with the protein at the colchicine binding site. When the data for the inhibition curve (closed circles) were plotted on a Hill plot according to the method of Loftfield and Eigner (1969), a straight line was obtained with a slope of 1.74. This suggests that more than one, and probably two, molecules of podophyllotoxin are required to inhibit the binding of one molecule of colchicine. Evidence that the inhibition of colchicine binding by podophyllotoxin is not due to a decrease in the stability of the protein was obtained in preincubation experiments similar to those described earlier for vinblastine sulfate (Figure 7). Rather than a decrease, a slight increase in the stability of the colchicine binding activity was observed. In one experiment, the half-time at 37° in the absence of podophyllotoxin was 306 min, while in the presence of 2.1×10^{-6} M podophyllotoxin, the half-time for loss of colchicine binding activity was 516 min. It is possible that podophyllotoxin was reversibly bound at the colchicine binding site, and as seen in the case of colchicine, the binding of podophyllotoxin to the site stabilized the protein. However, it is also conceivable that podophyllotoxin inhibits colchicine binding by indirectly affecting the association reaction between colchicine and the protein. The association reaction is both time and temperature dependent, and the possibility of conformational changes occurring either before or during the association reaction has been discussed previously (Wilson and Friedkin, 1967). In a preliminary experiment, the effects of podophyllotoxin were studied on the preformed colchicine complex where, if podophyllotoxin acts only at the occupied colchicine binding site, no stabilization of the complex should be observed. In the absence of podophyllotoxin, the inactivation half-time for the control at 37° was 420 min, while in the presence of 1.1×10^{-5} M podophyllotoxin, the inactivation half-time was 960 min. Since podophyllotoxin stabilized the colchicine binding activity when the colchicine site was occupied, it is likely that stabilization by podophyllotoxin was mediated *via* an effect at a site other than the colchicine binding site, and the possibility remains that podophyllotoxin inhibits colchicine complex formation through an allosteric mechanism.

Discussion

The 120,000 molecular weight colchicine binding protein found in the soluble fraction of chick embryo brain homogenates is considered to be a subunit of microtubules. The results of this investigation have shown that the amount of colchicine binding activity obtained during incubation of an

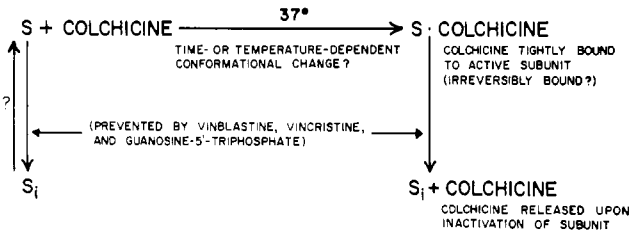

FIGURE 10: Inactivation of colchicine binding activity. S = active microtubule subunit (molecular weight = 120,000 ± 5000). The subunit is in a state whereby under the proper conditions of time and temperature, it can bind colchicine. S_i = inactivated subunit. The protein subunit undergoes inactivation whether or not colchicine is bound to it. Inactive subunit cannot bind colchicine, and active subunit which contains bound colchicine releases the colchicine immediately upon inactivation.

extract containing microtubule protein with labeled colchicine depends upon a number of factors. The experimental conditions do not directly influence the formation of the protein–colchicine complex, but affect the colchicine binding reaction indirectly by altering the stability of the colchicine binding activity. One important factor which affected this process was the pH of the system. At all pH values, the initial colchicine binding capacity of the microtubule protein was unchanged. Therefore, the association reaction between colchicine and the subunit was not affected by pH. However, the rate of loss of colchicine binding activity was markedly influenced by a slight shift in the pH. A second important factor, the ionic strength, also influenced only the inactivation process. Binding activity rapidly decayed at low ionic strength. High concentrations of NaCl stabilized colchicine binding activity without affecting the association reaction between colchicine and the protein.

A schematic description of the inactivation process is shown in Figure 10. Colchicine is shown strongly combining with an active form of microtubule subunit protein. The complexity of the association reaction, a slow reaction requiring elevated temperatures, has been discussed previously (Wilson and Friedkin, 1967). The inactivation process occurs whether or not colchicine is bound to the protein. Inactive subunit cannot bind colchicine, and active subunit which contains bound colchicine releases the colchicine immediately upon inactivation. Inactivation can be prevented by vincristine, vinblastine, and guanosine 5′-triphosphate.

The rate of release of colchicine from the bound complex must reflect two processes. One process, the inactivation process, results in the release of colchicine from the protein due to a change in the protein such that it can no longer bind colchicine. The second process is the normal reversibility of the complex which occurs directly at the active colchicine binding site. The present data argue that once the colchicine complex has formed, loss of bound colchicine must primarily be due to the inactivation process. The degree of stabilization obtained with a given concentration of vinblastine was always the same, whether or not the protein was complexed with colchicine. Almost complete stabilization of preformed colchicine–protein complex occurred with high concentrations of vinblastine. In these experiments, a large excess of unlabeled colchicine had been added to prevent any further binding of labeled colchicine. The loss of colchicine from bound complex in unstabilized preparations must have been due primarily to the inactivation process, and not the reversibility of the active protein–colchicine complex. Therefore, the interaction of colchicine with its binding site(s) on an active microtubule subunit must be, if at all, only slightly reversible.

The biochemical nature, both of the colchicine–protein association reaction, and of the inactivation process which the colchicine binding subunit undergoes, is not understood. It is conceivable that one or both of these processes may be related to the ability of the protein to participate in microtubule structure. For example, slight environmental changes within the cell, such as a change in local concentration of a critical ion, the binding of a normal chemical regulator, or a drug to the protein, could result in conformational changes in the protein which influence the ability of the subunit to polymerize or depolymerize. Certain microtubules exhibit rather delicate stability, such as those found in the mitotic spindle of dividing cells (Marsland, 1966; Marsland and Asterita, 1966), or those found in the axopods of *Actinosphaerium nucleofilum* (Tilney, 1965; Tilney and Porter, 1967). In both cases, the microtubules are destroyed by increasing hydrostatic pressure, lowering temperature, or application of colchicine. Removal of the colchicine, lowering the pressure, or increasing the temperature results in re-formation of microtubule structure.

Evidence in support of the hypothesis that the inactivation process which results in the loss of colchicine binding ability reflects some aspect of the biochemical role of the subunit in microtubule formation is derived from a consideration of the stabilization experiments with vinblastine, vincristine, and GTP. Stabilization of colchicine binding activity in grasshopper embryos by vinblastine (originally reported as a stimulation of colchicine binding) occurred not only in cell-free extracts, but also in the intact embryos, suggesting that the process occurred normally in living tissue (Wilson and Friedkin, 1967). Stabilization of colchicine binding activity in brain extracts with low concentrations of vinblastine and vincristine suggests that a selective site(s) on the microtubule subunit is involved. The disruptive actions of vinblastine and vincristine on microtubules of the mitotic spindle may be due to the inability of the protein to undergo a conformational change necessary for normal participation of the subunit in the microtubule structure. The fact that guanine nucleotides are normally bound to the subunit and that addition of GTP to a solution of the protein also stabilizes the colchicine binding activity of the protein (Weisenberg *et al.*, 1968) suggests GTP may play an important role in such a process.

Increasing evidence suggests that the variety of chemical agents which disrupt microtubules do so by at least several different mechanisms. Vinblastine and colchicine interact with the mictotubule subunit at different sites. Possibly the action of podophyllotoxin is brought about through an interaction at still another site. Several other chemical agents also disrupt microtubules. Cupric ions, which cause the dissolution of microtubules in the axopods of *Actinosphaerium nucleofilum* (Roth *et al.*, 1968), destroy the colchicine binding activity of microtubule protein through an interaction at a site other than the colchicine binding site (Wilson and Bryan, 1970). With other antimitotic agents whose action appears to involve microtubules such as griseofulvin (Malawista *et al.*, 1968), we have not been able to demonstrate any effect on the protein subunit which alters the colchicine–protein association re-

action (L. Wilson and M. Friedkin, unpublished data), or influences the inactivation process (S. Mizel, J. Bryan, and L. Wilson, unpublished data). Therefore, the mechanism of action of griseofulvin may not involve any of these processes.

It is conceivable that the colchicine binding dimer subunit is the basic building block of microtubules. It should possess vertical sites to allow association of the subunits to form protofilament fibers, and lateral sites to allow association into the microtubule structure. There could be additional sites which play a role in the regulation of the stability or function of the microtubule. The picture emerges, albeit a speculative one, that the dimer subunit of microtubules is a protein having a number of binding sites. Interaction with these sites, either by normal chemical regulators, drugs, or the influence of local environmental factors, could result in conformational changes in the subunit which modify its ability to function in the microtubule structure.

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References

Adelman, M. R., Borisy, G. G., Shelanski, M. L., Weisenberg, R. C., and Taylor, E. W. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 1186.

Andrews, P. (1964), *Biochem. J. 91*, 222.

Borisy, G. G., and Taylor, E. W. (1967), *J. Cell Biol. 34*, 525, 535.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Cornman, I., and Cornman, M. E. (1951), *Ann. N. Y. Acad. Sci. 51*, 1443.

Feit, H., Barondes, S. H., Dutton, G., and Shelanski, M. L. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 252.

George, P., Journey, L. J., and Goldstein, N. M. (1965), *J. Nat. Cancer Inst. 35*, 355.

Kelly, M. G., and Hartwell, J. L. (1954), *J. Nat. Cancer Inst. 14*, 967.

Linskens, H. F., and Wulf, N. (1953), *Naturwissenschaften 40*, 487.

Loftfield, R. B., and Eigner, E. A. (1969), *Science 164*, 305.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Malawista, S. E. (1965), *J. Exp. Med. 122*, 361.

Malawista, S. E., Sato, H., and Bensch, K. G. (1968), *Science 160*, 770.

Marantz, R., Ventilla, M., and Shelanski, M. (1969), *Science 165*, 498.

Marsland, D. (1966), *J. Cell Physiol. 67*, 333.

Marsland, D., and Asterita, H. (1966), *Exptl. Cell Res. 42*, 316.

Olmsted, J. B., Carlson, K., Klebe, R., Ruddle, F., and Rosenbaum, J. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 129.

Roth, L. E., Pihlaja, D. J., and Shigenaka, Y. (1968), *J. Cell Biol. 39*, 115-A.

Shelanski, M. L., and Taylor, E. W. (1967), *J. Cell Biol. 34*, 549.

Tilney, L. G. (1965), *Anat. Record 151*, 426.

Tilney, L. G., and Porter, K. R. (1967), *J. Cell Biol. 34*, 327.

Weisenberg, R. C., Borisy, G. G., and Taylor, E. W. (1968), *Biochemistry 7*, 4466.

Wilson, L., and Bryan, J. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 941.

Wilson, L., Bryan, J., Ruby, A., and Mazia, D. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 807.

Wilson, L., and Friedkin, M. (1966), *Biochemistry 5*, 2463.

Wilson, L., and Friedkin, M. (1967), *Biochemistry 6*, 3126.

Wisniewski, H., Shelanski, M. L., and Terry, R. D. (1968), *J. Cell Biol. 38*, 224.