The Colchicine-Binding Protein of Mammalian Brain and Its Relation to Microtubules*

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ABSTRACT: A procedure is described which allows the isolation of relatively large quantities of a colchicine-binding protein from mammalian brain. The protein is a dimer of mol wt 120,000 and has a $s^0_{20,w} = 5.8$ S. In 6 M guanidine hydrochloride plus 0.12 M mercaptoethanol, it is converted into a 60,000 molecular weight unit. The dimer binds 1 mole of colchicine and 2 moles of guanosine triphosphate. At one of the guanosine triphosphate binding sites, the nucleotide exchanges completely with free guanosine triphosphate in less than 15 min at room temperature. At the second guanosine triphosphate site, exchange was not detected. As isolated, the protein contains 0.5–0.8 mole/120,000 g of a mixture of guanosine diphosphate and guanosine triphosphate. The native configuration of the protein as judged by colchicine or guanosine triphosphate binding activity is unstable ($t_{1/2} = 11$ hr at 0°). The binding of guanosine triphosphate or colchicine appears to stabilize the protein. In terms of molecular weight, sedimentation constant, amino acid composition, colchicine, and guanosine triphosphate binding sites, the protein is very similar to the subunit protein of cilia and flagella microtubules. On the basis of circumstantial evidence we suggest that the protein is the subunit of neurotubules.

Colchicine is best known as an inhibitor of mitosis whose action appears to be on the organization of spindle microtubules (Eigsti and Dustin, 1955; Inoue, 1952) although more recent work has revealed that it also interferes with a variety of cellular functions dependent upon microtubules (Tilney *et al.*, 1966; Green, 1962; Okazaki and Holtzer, 1965). In previous studies it was shown that colchicine forms a complex with a protein of sedimentation constant 6 S which is found in tissue culture cells, strain KB or Hela, in fertilized or unfertilized sea urchin eggs, in the mitotic spindle, and in a variety of tissues including nerve and brain (Borisy and Taylor, 1967a,b). The purest source of the colchicine binding protein is the flagellum or cilium and in the case of sea urchin sperm tails the 6S colchicine-binding protein has been identified with the subunit of the central pair of microtubules (Shelanski and Taylor, 1967, 1968).

The ever increasing volume of work describing microtubule structures in a great variety of cell types, from plants, animals, and protozoa, indicates that the microtubule is the most widely distributed type of intracellular filament. The colchicine-binding protein is also present in many cell types, and it is reasonable to make the hypothesis that colchicine binding is a specific property of the protein subunit of microtubules. Furthermore, the binding of [^{3}H]colchicine can be used as a sensitive assay for the detection and purification of the presumed microtubule protein. We have tested this hypothesis by isolating a colchicine-binding protein from brain and comparing its properties with the microtubule subunit of cilia and flagella. Mammalian brain was chosen as a starting material because extracts prepared from brain homogenates have a high-specific colchicine-binding activity and fresh brain is readily available in large quantities. In addition one can be reasonably sure that the protein which is obtained has not been derived from flagella or mitotic spindles. An isolation procedure developed solely on the basis of colchicine-binding activity yielded a pure protein whose properties are very similar to those of the subunit protein of cilia and flagella microtubules.

Materials and Methods

Reagents. $(NH_4)_2SO_4$ and sucrose were Mann Enzyme Grade. GTP, GDP, and ATP used in the study of stabilization or binding were high-purity products obtained from Pabst Laboratories. The GTP used during the purification procedure was a 95% pure product (grade II-S) obtained from Sigma Biochemical Co. Tritiated colchicine was prepared as described previously and had a specific activity of 2.5 Ci/mmole (Taylor, 1965). Unlabeled colchicine was USP grade (Fischer Scientific Co.). Tritiated GTP (Schwarz BioResearch Co.) was stated to have 95% radioactive purity, and had a specific activity of 1.12 Ci/mmole. All other chemicals used were reagent grade. The standard buffer used in most experiments

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§ This study was supported by a U. S. Public Health Service research grant (GM 10992) and also by an award from the Muscular Dystrophy Association. Recipient of a research career development award from the U. S. Public Health Service.

contained 0.01 M sodium phosphate buffer–0.01 M $MgCl_2$ (pH 6.5) P–Mg solution) and where indicated, 0.1 mM GTP (P–Mg–GTP solution).

Protein Determination. Protein was determined by the method of Lowry *et al.* (1951). The purified protein was used to make a standard curve of protein concentration *vs.* optical density based on refractometric and dry weight calibrations.

Electrophoresis and Chromatography. Gel electrophoresis was performed on 7.5% acrylamide gels made up in 8 M urea. Protein samples were dialyzed against 8 M urea and 0.12 M mercaptoethanol (pH 8) for at least 24 hr prior to use. The samples were run on a Canalco electrophoresis apparatus at a current of 5 A/gel until the tracking dye reached the end of the gel (about 1.5 hr). The running buffer was 5 mM Tris-glycine (pH 8.6). The gels were stained for 4 hr in 1% Amido-Schwartz in 7% acetic acid and destained electrophoretically.

The binding of GTP or colchicine was routinely determined by gel filtration on G-100 Sephadex columns (Pharmacia Co.) eluted with P–Mg or P–Mg–GTP buffer (Borisy and Taylor, 1967a). A sample volume of either 0.5 or 1.0 ml was incubated at 37° for 1 hr with nucleotide or colchicine, applied to a 1 × 15 cm column, and 1-ml fractions were collected.

The binding of ^3H-labeled materials was determined by counting aliquots dissolved in Bray's (1960) solution. The binding of unlabeled nucleotides or colchicine was determined from the absorption spectrum. In the case of colchicine, binding was measured by optical density at 350 mμ in the presence of the protein (ϵ 1.65 × 10^4) or on the supernatant after first precipitating the protein in 0.5% perchloric acid (ϵ 1.40 × 10^4 in perchloric acid). In the case of GTP the protein was precipitated first and the amount of GTP was determined from absorption of the supernatant at 256 mμ (ϵ 1.24 × 10^4).

In some experiments in which better resolution was required gel filtration was performed on 2.5 × 35 cm columns of G-200 Sephadex equilibrated in P–Mg or in P–Mg–GTP buffer and run at 4°, at a flow rate of about 10 ml/hr maintained by a peristaltic pump (Holter Co.). The column was calibrated from the elution volumes of Blue Dextran 200 (Pharmacia Co.) and bovine serum albumin. The method of Andrews (1964) was employed for determination of molecular weight and diffusion coefficient.

Thin-layer chromatography was used to examine the distribution of bound nucleotides. Protein was precipitated by the addition of cold 5% perchloric acid and nucleotides were isolated by the methods of Tsuboi and Price (1959). Chromatography was performed on thin-layer silica gel plates (Eastman chromatograms) in an ascending solvent system consisting of a 6:1:3 mixture of 1-propanol–ammonia–water. About 5 hr was required for separation and the spots were visualized by quenching of fluorescence excited by an ultraviolet lamp.

Filter Assay for Colchicine Binding. A rapid filter assay procedure for colchicine binding was developed to facilitate the handling of a large numbers of samples. DEAE-cellulose Chromedia paper (No. DE81, Whatman Co.) was cut into 2.5-cm circles and equilibrated in P–Mg solution. In the standard assay procedure, 1 ml of protein solution was incubated for 1 hr at 37° with 2.5 × 10^{-6} M [^3H]colchicine (approximately 10^6 cpm) and applied to the filter. Unlabeled colchicine (1 ml of 10^{-4} M) was added and the volume was made up to 10 ml with P–Mg solution. Filtration was performed using a manifold which held 12 stainless steel filter assemblies and filtration was allowed to proceed under the weight of the solution. About 8 min was required for complete filtration. The slow flow rate is necessary to allow quantitative adsorption of the protein to the filter paper. After filtration was complete, vacuum was applied, and the filters were washed rapidly, five times in cold P–Mg solution. The filters were then placed in 5 ml of Bray's (1960) solution and counted. The radioactivity was eluted from the filter by the scintillation fluid and the presence of the filter did not appear to affect counting efficiency. The filter assay was compared with the Sephadex G-100 column procedure for a 100-fold range of colchicine concentrations present in the incubation mixture. The binding measured by filtration averaged 85% of the binding determined by the column procedure.

Amino Acid Analysis. Protein samples were hydrolyzed in 6 M HCl in sealed, evacuated tubes. The samples were maintained at 110° for 24 and 48 hr and amino acid composition was analyzed with a Beckman amino acid analyzer. L-α-Amino-β-guanadinopropionic acid and L-norleucine were run with the protein as internal standards. Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton (1946). An analysis was also performed in a Phenox analyzer using a 50 × 0.9 cm Phenox resin column at pH 5.35 and 51.5°. The conditions were chosen to resolve histidine and 3-methylhistidine.

Molecular Weight Determinations. Molecular weights were determined by the high-speed equilibrium method of Yphantis (1964) using interference optics and employing a three-channel centerpiece. Each channel contained 0.01 ml of FC-3 fluorocarbon oil and 0.10 ml of either the solvent or the protein solution. Native protein was dialyzed at least 24 hr in 0.1 M KCl in P–Mg or P–Mg–GTP prior to use. Molecular weight determinations in guanidine hydrochloride were performed on protein dialyzed at least 2 days in 5 or 8 M guanidine hydrochloride–0.12 M mercaptoethanol in 0.05 M phosphate buffer (pH 8). Sedimentation was performed at 20° for 14–20 hr at 26,000 or 28,000 rpm in the case of the native protein and for 24 hr at 24,000 rpm for the guanidine-treated material. The interference optical system was aligned by the method of Gropper (1964). Fringe patterns were recorded on Kodak 11G plates and fringe displacements were measured using a Nikon Shadowgraph which was aligned to the air fringes. Only those runs were used in which the fringes were flat over at least the first 0.5 mm of the cell.

Molecular weights were obtained from the equation $M = [2RT/(1 - \bar{v}\rho)w^2]\mathrm{d} \ln C_{(r)}/\mathrm{d}t^2$, by plotting the logarithm of fringe displacement against r^2. It should be noted that in Figures 8–10 the logarithm of fringe displacement is shown plotted against r in order to give a better appreciation of the position of the fringes in terms of the cell dimensions. In all experiments the value of

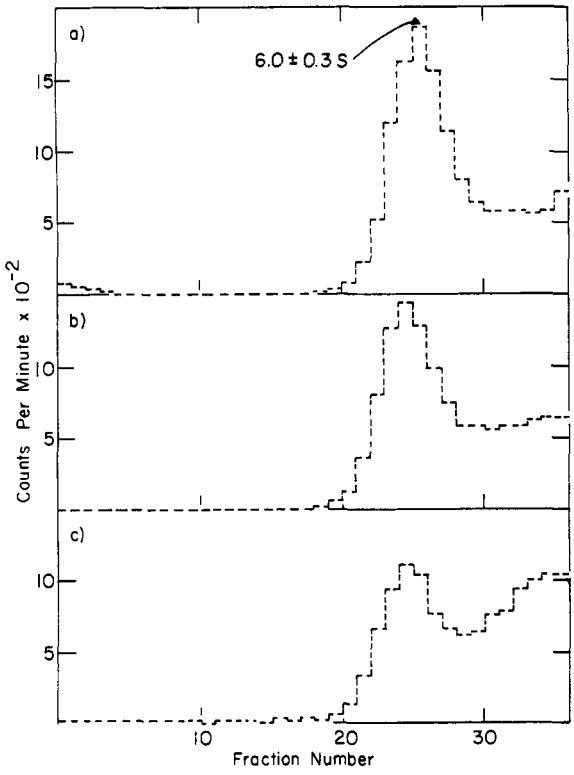


FIGURE 1: The isolation procedure. Determination of sedimentation constant of binding site at various stages of the isolation procedure. (a) Soluble supernatant obtained after centrifugation of brain homogenate for 30 min at 10,000 rpm. (b) 38–49% $(NH_4)_2SO_4$-insoluble fraction. (c) Fraction eluted from DEAE-Sephadex A-50 with 0.8 M KCl. Aliquots (1 ml) from each type of material were incubated in 2.5×10^{-6} M [^{3}H]colchicine, 1 hr, 37°; bound radioactivity was separated by gel filtration on G-100 Sephadex and a 0.2-ml aliquot was layered on a 5–20% linear sucrose gradient, containing 0.1 M KCl in P–Mg buffer; centrifugation for 16 hr at 35,000 rpm and 2°, SW-39 rotor. Fractions (9 drops) were collected. In each case the radioactivity peak occurs at 6.0 ± 0.3 S determined relative to a bovine serum albumin marker.

$\bar{v}$ calculated from the amino acid composition was used (0.73).

Purification of the Colchicine-Binding Protein. An isolation procedure was developed by using specific colchicine-binding activity as an assay. The major purification step involved gradient elution from a DEAE-Sephadex column, but because of the lability of the protein, a small column had to be used to minimize the time required to obtain separation. Following a study of the stability of the protein and its elution behavior on DEAE-Sephadex, a second procedure was developed using batch adsorption and elution from DEAE-Sephadex. Larger quantities of material could be purified in a much shorter time and although the yield was relatively low, this was not a serious limitation since about 100 mg of purified protein was obtained per preparation.

PROCEDURE 1. Porcine brain was obtained within 1-hr slaughter through the courtesy of Thompson Farms Packing Co. and placed in a beaker packed with ice. All subsequent operations were performed in a 4° cold room using ice-cold solutions. Approximately 880 g of brain was used as starting material. The superficial blood vessels and meninges were removed, and the tissue was minced with scissors, washed three times by suspension in three volumes of 0.24 M sucrose in P–Mg buffer and strained through cheesecloth. The mince was resuspended in two volumes of the same solution and homogenized in a Sorvall Omni-Mixer at setting 9 for 30 sec.

In the earlier experiments the importance of including GTP in the isolation medium was not recognized, consequently the fractionation procedure was originally developed without added nucleotide. In later experiments and in all samples prepared by the batch DEAE method (Procedure 2), 10^{-4} M GTP was added to the resuspended mince before homogenization. The homogenate was centrifuged in a Sorval GSA rotor at 16,000*g* for 30 min and the pellet was discarded. The supernatant was brought to approximately 38% saturation at 0° by gradual addition over a 15-min period of 212 g of solid $(NH_4)_2SO_4$/l. of supernatant. The suspension was allowed to stand for 0.5 hr and was centrifuged at 10,000*g* for 20 min in a Sorvall GSA rotor. To the resulting supernatant 71 g of $(NH_4)_2SO_4$/l. was added bringing the saturation to about 49%. The suspension was allowed to stand for 0.5 hr and was then centrifuged at 10,000*g* for 20 min. The supernatant was discarded and the pellet was redissolved in approximately 50 ml of P–Mg buffer/l. of original homogenate. At this point the solution can be lyophilized and stored at −20° for months without loss of colchicine-binding activity.

A 2-ml sample (from $(NH_4)_2SO_4$ fractionation procedure) containing about 5–10 mg/ml of protein was applied to a 1 × 5 cm column of DEAE-Sephadex (A-50) equilibrated in 0.02 M sodium phosphate buffer–0.01 M MgCl (pH 6.5) and the column was developed by increasing the sodium chloride concentration at pH 6.5 and 4°. The best purification resulted from a combination of a step and an exponential gradient. The column was eluted first with P–Mg solution containing 0.1 M salt and then with 0.3 M salt followed by an exponential increase between the limits 0.3 and 0.8 M. Very little colchicine-binding activity was eluted from the column until the salt concentration exceeded approximately 0.5–0.6 M.

PROCEDURE 2. The procedure described above was modified to produce a pure protein in a minimum of time, although with some reduction in yield. The homogenate was prepared as in procedure 1 except that only one volume of buffer was used per volume of mince. For this higher protein concentration the ammonium sulfate precipitation range was reduced. Protein precipitating between 32 and 43% saturation (177 and 248 g per l., respectively) was retained. Only 10 min was allowed for precipitation to occur after addition of $(NH_4)_2SO_4$ was completed. The 43% $(NH_4)_2SO_4$ precipitate was suspended in about 200 ml of P–Mg–GTP buffer by means of gentle homogenization with a Potter-type homogenizer. The solution was distributed into eight 50-ml conical centrifuge tubes containing 15 ml of packed DEAE-Sephadex (A-50) which had been equilibrated in P–Mg buffer. The tubes were stirred intermittently for 30 min to allow the protein to adsorb and the Sephadex was pelleted by low-speed centrifugation.

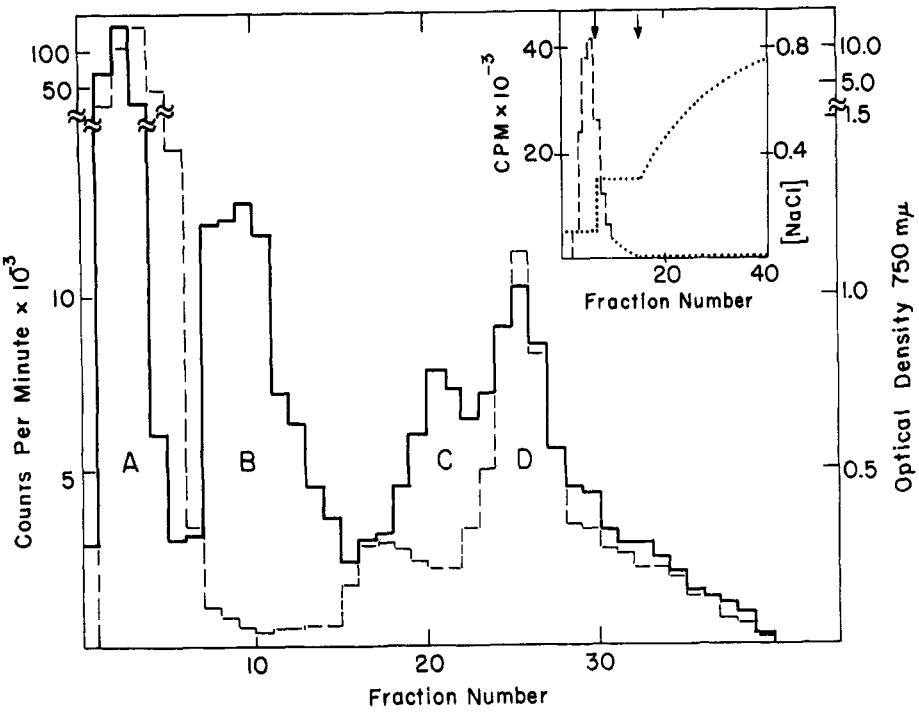


FIGURE 2: Chromatography of the 38–49% $(NH_4)_2SO_4$ fraction on DEAE-Sephadex A-50. A 8-mg/ml solution (2 ml preincubated with 2×10^{-5} M [^{3}H]colchicine, was applied to a 1×5 cm column in P-Mg buffer (pH 6.5). (Column was eluted with the NaCl concentrations shown in the inset (· · · ·) and 2-ml fractions were collected (——), optical density 750 mμ (Lowry protein determination); (- - -) radioactivity; the inset figure, dashed line, shows the elution profile of [^{3}H]colchicine alone.

The pellets were washed twice in five volumes of 0.5 M KCl in P–Mg–GTP buffer, allowing 10 min/wash with intermittent stirring. The supernatants were discarded and the protein was then eluted from the resin by washing twice in 10 ml of 0.8 M KCl in P–Mg–GTP allowing 10 min/wash. After removal of the Sephadex by centrifugation, the supernatants were pooled and 24.8 g of $(NH_4)_2SO_4$ was slowly added per 100 ml of supernatant with constant stirring. The precipitate was then collected by centrifugation at 35,000*g* in the Spinco 30 rotor. The pellets, which consisted of essentially pure colchicine-binding protein, were redissolved in P–Mg–GTP and dialyzed overnight to remove excess $(NH_4)_2SO_4$. Long exposure to 0.8 M KCl appeared to accelerate denaturation and the final $(NH_4)_2SO_4$ precipitation served both to concentrate the protein and remove it from the high KCl environment.

Results

In a previous study in which specific colchicine binding activity was surveyed in a variety of cells and tissues, it was found that extracts of mammalian brain contained relatively large amounts of this protein (Borisy and Taylor, 1967a). In all of the cases investigated the sedimentation constant of the binding site was 6 S. Therefore the isolation procedure was developed by measuring the specific activity of colchicine binding at each step and also determining the sedimentation constant of the binding site by density gradient centrifugation. Figure 1 shows that the bound radioactivity has a sedimentation constant of 6 S in the soluble supernatant obtained from whole brain homogenates, in the $(NH_4)_2SO_4$ precipitate and in the batch DEAE eluate. Assay of each tube by the DEAE filter method showed that the trail of radioactivity leading up to the meniscus was not protein bound. Its position on the gradient is presumably due to dissociation of the colchicine–protein complex during the period of centrifugation.

A typical column elution profile for the DEAE-Sephadex (A-50) step is shown in Figure 2. To follow the purification the protein sample was first incubated with 2×10^{-5} M [^{3}H]colchicine at 37° for 1 hr and then quickly chilled to 0°. The majority of the protein did not adsorb to the ion exchanger (Figure 2, fraction A). Fractions

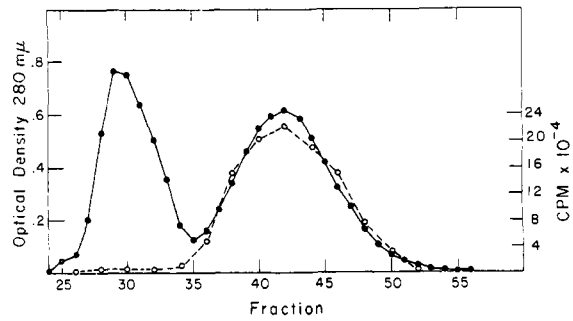


FIGURE 3: Chromatography of the purified protein on G-200 Sephadex. The protein was purified in the presence of 0.1 mM GTP and the column was equilibrated and eluted with 0.1 mM GTP in P-Mg buffer. A 2.5-ml sample was applied and 2.6-ml fractions were collected. Column size was 2.5 × 35 cm. Colchicine-binding activity was determined for the indicated fractions by incubating 0.5-ml aliquots with 2.5×10^{-5} M [^{3}H]colchicine for 1 hr at 37° and the bound radioactivity was determined by filter assay. (—●—) Optical density at 280 mμ (protein); (—○—) bound radioactivity.

TABLE I: Colchicine Binding of Fractions from DEAE Chromatography.

Fraction	Radioactivity Bound to Column Fractions[a]: Total Radioactivity (cpm × 10^{-3})	Bound Radioactivity (cpm × 10^{-3})	% Bound
4	133	0.6	0.005
18	3	0.06	0.02
26	11	3.6	33
	Binding Activity of Column Fractions[b]: Total Protein ($OD_{750\ m\mu}$ units[c])	Bound Radioactivity (cpm × 10^{-3})	Binding Act. (cpm × 10^{-3} per OD unit)
A	15	46	3
B and C	5	5	1
D	2	216	108

[a] Brain extract was incubated with 2×10^{-5} M [^{3}H]colchicine prior to application to DEAE column. Aliquots (1 ml) of fractions of protein peaks were assayed for bound radioactivity by gel filtration on G-100 Sephadex. [b] Brain extract was applied to DEAE without preincubation with colchicine. Fractions were subsequently assayed for binding activity by incubating 1-ml aliquots at 2.5×10^{-6} M [^{3}H]colchicine for 1 hr at 37° and determining the bound count by gel filtration. Specific binding activity is not directly comparable with *a* because carrier colchicine was added in the first experiment. [c] Protein was determined according to method of Lowry *et al.* (1951).

TABLE II: Purification of Colchicine Binding from Porcine Brain.

Step	Vol (ml)	Act./ml[a] ((cpm/ml) × 10^{-3})	Total Act. (cpm × 10^{-6})	Total[b] Protein (mg)	Sp Act. (cpm/mg) × 10^{-3}	Yield (%)	Purification
1. Homogenate	700	404	273	28,000	10.8	100	1
2. Soluble supernatant	430	707	304	5,700	53.1	106	5
3. $(NH_4)_2SO_4$ (38–49%)	40	4,000	160	1,270	126	59	12
4. Column DEAE[c]			48	38	1,260	20	720

[a] Activity per milliliter is the bound radioactivity determined by gel filtration of 1 ml of material incubated for 1 hr at 37° with 2.5×10^{-6} M [^{3}H]colchicine. [b] Protein concentration was determined by method of Lowrey *et al.* (1951). Values are expressed in milligrams based on the calibration curve for Folin reaction using purified colchicine-binding protein. [c] Values for DEAE step are given in terms of the total sample but in practice only a small aliquot of the $(NH_4)_2SO_4$ step was used for chromatography.

from the central regions of the radioactivity peaks were subjected to gel filtration over G-100 Sephadex to determine the macromolecularly bound radioactivity. The results are summarized in Table I which shows that only the last radioactivity peak represents colchicine bound to protein. In a separate experiment, in which the protein was not preincubated with colchicine, each of the protein peaks was assayed for its ability to bind colchicine. The results, which are also included in Table I, again show that only the last peak has any significant colchicine binding activity. The first radioactivity peak is due to free colchicine since it is eluted at the bed volume of the column. The insert in Figure 2 shows that the position of elution of free colchicine from the column does in fact correspond to the profile of the first peak. The second small radioactivity peak can be ascribed to dissociation of the colchicine–protein complex since other experiments showed that at salt concentrations above 0.3 M there is some dissociation of radioactivity. The elution of a peak rather than a gradual release of radioactivity is due to the relatively sharp increase in ionic strength at the beginning of the exponential gradient.

The specific activity in the third peak was relatively constant, indicating that the material was essentially pure at this stage. Measurements of the enrichment of colchicine-binding activity throughout the course of the purification procedure are summarized in Table II. The specific activity of the preparation was increased approximately 100 times over that of the

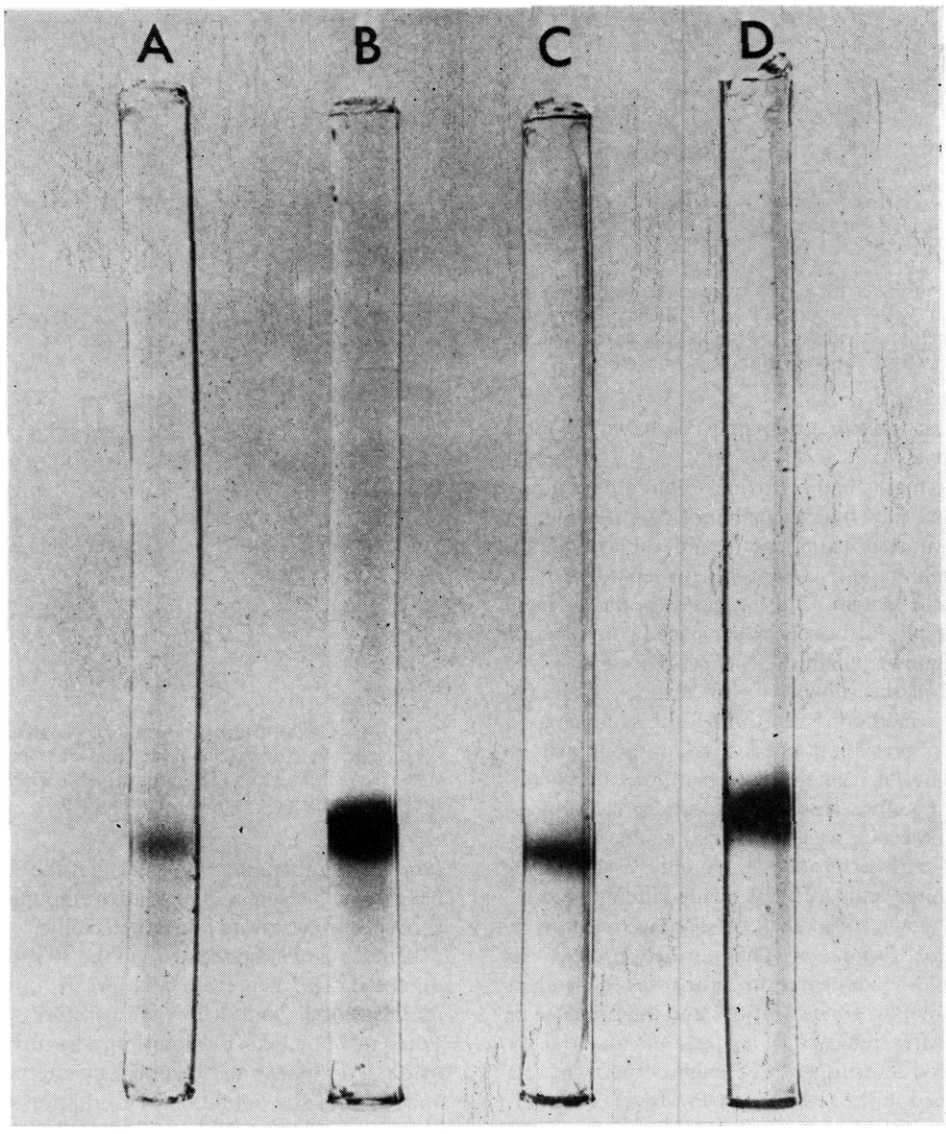


FIGURE 4: Acrylamide gel electrophoresis of purified protein denatured in 8 M urea plus 0.12 M mercaptoethanol (pH 8). For patterns A and B, 0.01 and 0.2 mg of protein were applied. Patterns C and D were obtained from a different protein preparation using 0.015 and 0.15 mg of protein. Gel length, 9 cm; R_F values approximately 0.25–0.28.

crude homogenate. Calculated from the known specific activity of the colchicine, the final product contains about 0.5 mole of colchicine bound/100,000 g of protein. This value indicates that the product is highly purified, but it is not intended as a quantitative measurement of purity since there is some dissociation of colchicine during the procedure, as well as some loss in binding activity by denaturation. It is also evident from the results shown in Table II that the high specific activity of brain extracts is in part due to the small fraction of brain protein which is soluble at low ionic strength.

A similar study of the purification obtained in the batch DEAE procedure indicated a purification of about 12-fold with respect to the activity of the soluble extract compared with about 20-fold for the gradient elution procedure. The yield, based on total binding activity, was 25–30%. The results were somewhat variable regarding yield and enrichment of binding activity because of a strong tendency of the purified protein to aggregate, which is more pronounced at the higher concentrations used in the batch procedure.

Gel filtration (on G-200 Sephadex) of the protein purified by batch procedure (Figure 3) shows the presence of two peaks, one a very high molecular weight aggregate which is eluted in the void volume of the column and a second component with a molecular weight of about 100,000 and a diffusion coefficient of about 5.2 $\times 10^{-7}$ cm^2 sec^{-1}. It can be seen in Figure 3 that colchicine-binding activity coincides with the lower molecular weight peak and that the optical density and bindind profiles are reasonably symmetric and coincident. If GTP is omitted from the purification procedure the relative size of the aggregate peak is greatly increased.

Colchicine binding was never found associated with the aggregated material.

Acrylamide gel electrophoresis of the purified protein after denaturation in 8 M urea in the presence of 0.12 M mercaptoethanol yielded a single major band (Figure 4).

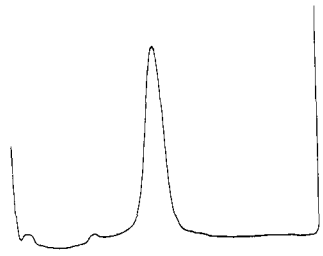


FIGURE 5: Densitometer trace of a typical gel electrophoresis pattern stained with Amido-Schwartz. Electrophoresis is from left to right. R_F about 0.29. Deflections at the far left and right are from the ends of the gel holder.

A densitometric trace of a gel pattern is shown in Figure 5 which indicates that about 90–95% of the protein is present in the major band. Bovine serum albumin, denatured in urea plus mercaptoethanol, was included in most electrophoresis experiments and the two bands from the albumin sample were widely separated from the microtubule protein. In a few cases the major band was found to split into two closely spaced components, but it is not known whether this effect represents a true heterogeneity of the subunits or an artifact of the gel electrophoresis method. Even though the samples used for gel electrophoresis contained variable amounts of the aggregated material, a single major band was always obtained. It is therefore reasonable to interpret the presence of high molecular weight material as due to aggregation of the purified protein. In summary, the purification procedure yields a 6S colchicine-binding protein, which gives a single component on disc electrophoresis.

Sedimentation Properties. The purified protein exhibited complex sedimentation properties dependent upon ionic strength, concentration, and the presence of nucleotides. After removal of aggregated material by prior high-speed centrifugation, a single component was usually obtained in 0.1 M KCl and P–Mg–GTP buffer. The extrapolated sedimentation constant, $s^0_{20,w}$, was 5.8 S but the apparent sedimentation constant increased significantly with increasing protein concentration (Figure 6), indicating strong protein–protein interactions.

Some examples of the sedimentation patterns obtained with this protein are shown in Figure 7. At high concentrations (12 mg/ml) the boundary becomes asymmetric and shows considerable spreading (Figure 7A). At lower concentrations the same sample shows a normal boundary. The patterns shown in Figure 7B were obtained at concentrations of 3 and 6 mg per ml and from these patterns the preparation would be judged to consist of essentially a single component. Figure 7D shows that the non-gaussian boundary obtained at 8 mg/ml (upper trace) takes on a more normal appearance when 10^{-3} M GTP is included in the buffer (lower trace).

Thorough dialysis of the solution against P–Mg–GTP buffer to remove salt leads to the appearance of a discrete component with a sedimentation constant of about 30 S (uncorrected). In Figure 7C, the lower trace was obtained at a concentration of 10 mg/ml. The formation of the 30S component is reversible and depends strongly upon concentration. The upper trace in Figure 7C was obtained by diluting the solution to a concentration of 5 mg/ml and it is evident that the 30S component has markedly decreased in relative amount. At still lower concentrations it is no longer detectable.

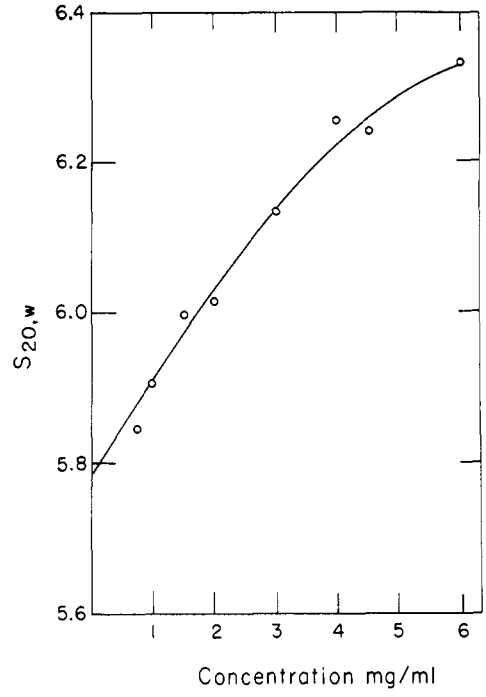
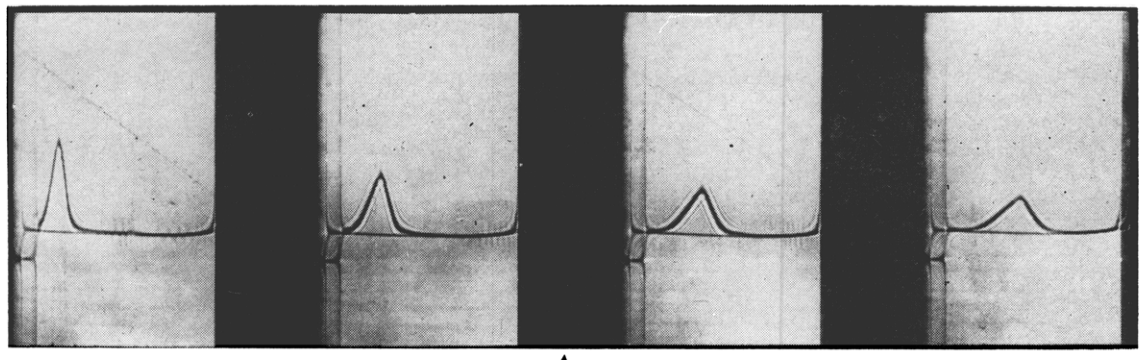


FIGURE 6: Concentration dependence of the sedimentation coefficient. Purified protein was dialyzed for 12 hr against 0.1 M KCl in P–Mg–GTP prior to experiments. Sedimentation was performed at 56,000 rpm and 20°.

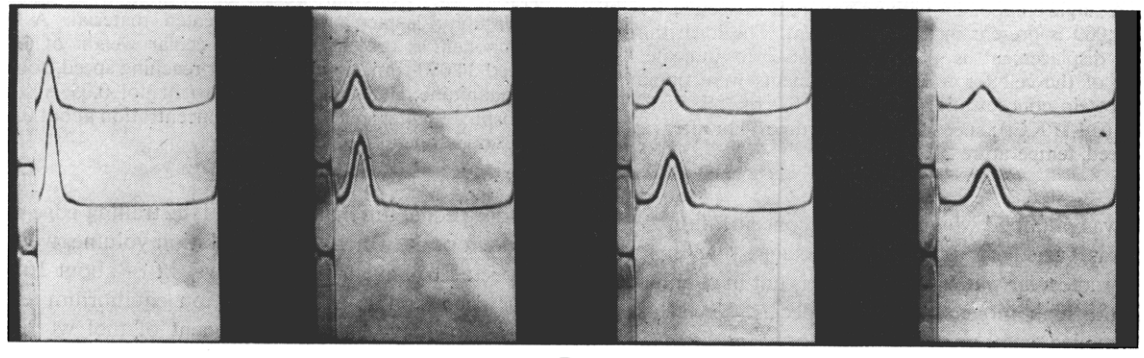
A much larger aggregate than the 30S species was also observed. This aggregate was formed optimally at low ionic strengths and at low temperatures (0–4°) and resulted in a highly turbid solution. The turbidity could be made to disappear and reappear by alternately warming and cooling the solution. The addition of 0.1 M KCl completely eliminated the turbidity. Examination of aliquots of the turbid solution under the phase microscope revealed large numbers of bundles of fibrous aggregates or tactoids which disappeared as the solution rewarmed on the microscope slide.

Molecular Weight. Molecular weight determinations on the native protein by the Yphantis high-speed equilibrium method yielded two kinds of results. In some experiments a single component with a molecular weight of about 120,000 was observed. Figure 8 shows a typical plot of the logarithm of fringe displacement *vs.* r, which is linear across the centrifuge cell indicating the presence of a single component. In several instances, a smaller component with a molecular weight of about 33,000 was observed, yielding the type of biphasic plot illustrated in Figure 9. The difference between measured fringe displacement and the contribution obtained by extrapolating the linear region to increasing r values was replotted (open circles, Figure 9). The plot was linear and yielded a molecular weight for the heavier species of about 130,000.

The amount of the 33,000 component was variable but was usually less than one-third of the total protein. No change in the relative amount of the 33,000 compo-



A



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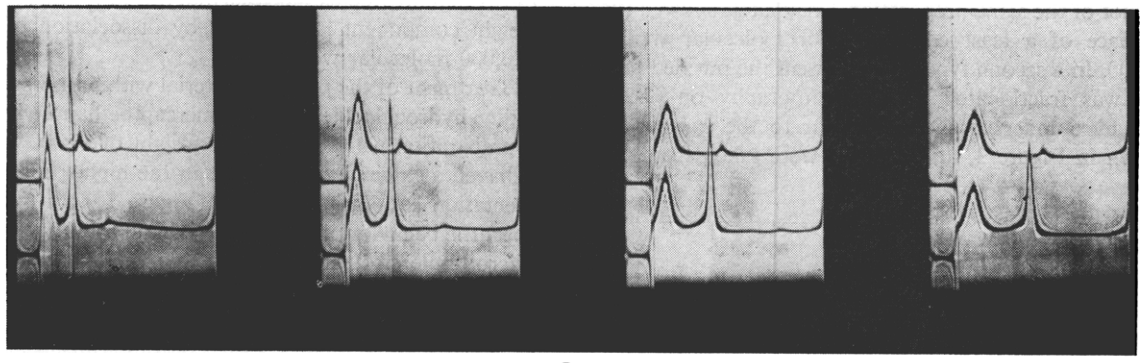
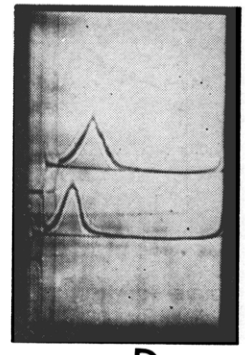


C

FIGURE 7: Sedimentation properties of the purified protein under various conditions. (A) Sedimentation at high concentration (12 mg/ml) showing rapidly spreading asymmetric boundry. The sedimentation coefficient is about 7.4 S; 0.1 M KCl–P–Mg–GTP buffer (pH 6.5); speed 48,000 rpm; temperature 23°; bar angle 60°; and times 20, 36, 42, and 58 min after reaching speed. (B) Sedimentation at lower concentrations (6 and 3 mg per ml showing a more normal boundary. The sedimentation coefficient at the higher concentration, 6.33 S, and at the lower concentration, 6.13 S. Centrifugation in 0.1 M KCl in P–Mg–GTP (pH 6.5); speed 56,000 rpm; temperature 20°; bar angle 55°; and times 14, 22, 30, and 38 min after reaching speed. (C) Sedimentation at low ionic strength showing reversible formation of a high S component. The protein was dialyzed for 12 hr against P–Mg–GTP solution. The concentration of protein in the lower trace was about 10 mg/ml and in the upper trace, 5 mg/ml. The sedimentation coefficients are 7 and 31 S at the higher concentration and 7 and 33 S at the lower concentration. Centrifugation at 44,000 rpm; temperature 20°; bar angle 55°; and times 8, 12, 16, and 20 min after reaching speed. (D) Effect of GTP on the shape of the sedimentation boundary. The protein was purified without added nucleotide and centrifuged at a concentration of about 8 mg/ml. The solvent was P–Mg buffer (pH 6.5) for the upper trace, and P–Mg buffer, plus 1 mM GTP for the lower trace. The other conditions were: speed 48,000 rpm, temperature 23°, bar angle 60°, and time 48 min after reaching speed.



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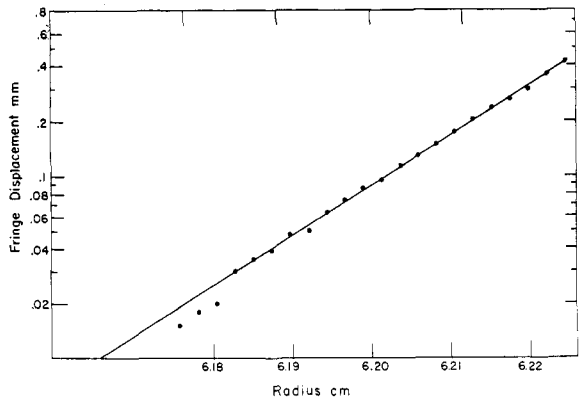

FIGURE 8: Molecular weight determination of the native protein. A single component with an apparent molecular weight of 121,000 is present in this experiment. The logarithm of fringe displacement is plotted against r to indicate the region of the cell for which measurements were obtained. The protein concentration was about 0.2 mg/ml, in P–Mg buffer (0.1 M KCl): speed 26,000 rpm, time 14 hr after reaching speed, temperature 20°.

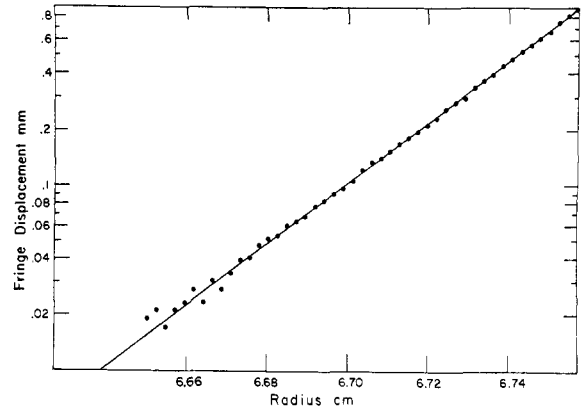

FIGURE 10: Molecular weight determination of guanidine hydrochloride–mercaptoethanol-treated material. A single component is present with a molecular weight of 62,000; speed 34,000 rpm, time 24 hr after reaching speed, solvent 5 M guanidine HCl–0.12 M mercaptoethanol–0.05 M sodium phosphate buffer (pH 8), protein concentration about 0.6 mg/ml, and temperature 20°.

nent was obtained when the initial concentration in the cell was varied by a factor of 4, suggesting that the lower molecular weight component is not in equilibrium with the high molecular weight species. Nevertheless, the smaller component appears to be a dissociation product of the higher molecular weight species. In one experiment in which a single component was observed with a molecular weight of 115,000 a second run on an aliquot of the same preparation 1 day later, showed the presence of a least component of molecular weight 30,000. In a second type of experiment the purified protein was fractionated by chromatography on G-200 Sephadex under conditions similar to the experiment shown in Figure 3, and samples were taken from the

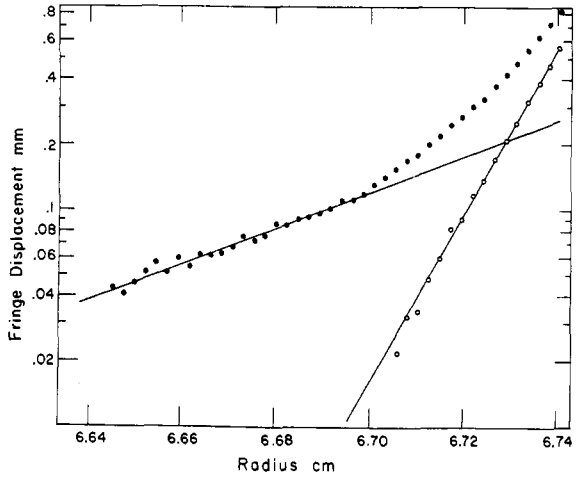

FIGURE 9: Molecular weight determination of the native protein, for the case in which two components are present. The molecular weight of the least component is 32,000 and of the heavy component, 136,000; speed 28,000 rpm, time 16 hr after reaching speed, protein concentration about 0.3 mg/ml, solvent 0.1 M KCl in P–Mg buffer, temperature 20°. (●) Measured fringe deflection; (○) difference between measured deflection and extrapolated contribution of the least component.

leading edge, the maximum, and the trailing edge of the protein peak, which, from its elution volume, would be expected to have a molecular weight of about 100,000. For each sample a sedimentation equilibrium experiment still gave a least component of mol wt 33,000. Furthermore, preparations which contained moderate amounts of the 33,000 component still yielded a single major band on disc electrophoresis. Consequently all of the available evidence suggests that this lower molecular weight component is produced by dissociation of the 120,000 molecular weight unit.

Treatment of the purified material with 5 or 8 M guanidine hydrochloride in 0.12 M mercaptoethanol yielded a preparation with a molecular weight of 57,000 which behaved as a single component in the high-speed sedimentation equilibrium method (Figure 10). In this respect the behavior of the protein is similar to that of the microtubule protein from cilia and sperm tails which exists as a 120,000 molecular weight dimer in the native form and as a 60,000 molecular weight component in the presence of guanidine hydrochloride (Shelanski and Taylor, 1968). However, we have no simple explanation to offer for the apparent dissociation of the native material from brain into a subunit with half the molecular weight of the particle obtained by guanidine hydrochloride treatment. Reduction in guanidine hydrochloride is generally expected to break proteins into their constituent polypeptide chains (Tanford *et al.*, 1967). It should be noted that a component of about 30,000 molecular weight was sometimes observed with protein from sea urchin sperm microtubules but only after long exposure to guanidine hydrochloride (Shelanski and Taylor, 1968). The molecular weight data are summarized in Table III.

Amino Acid Composition. The amino acid composition is shown in Table IV, together with data obtained previously for the central pair microtubules of sea urchin sperm tail (*S. purpuratus*; Shelanski and Taylor, 1968). It is evident that the compositions are similar. Lysine, phenylalanine, and tyrosine are the only residues which differ by more than 10%. As noted previously for the

TABLE III: Molecular Weight Determinations.[a]

Source	Deten	Molecular Weight
Native protein, single component present	7	119,000 ± 8,000
Native protein, two components present	7	33,000 ± 4,000 (least component) 133,000 ± 15,000 (heavy component)
Protein denatured in guanidine hydrochloride plus mercaptoethanol	6	57,000 ± 6,000

[a] All determinations were made using Yphantis high-speed equilibrium method at 20°. Native protein was measured in 0.1 M KCl–K–Mg buffer (pH 6.5). Denatured protein was measured in 5 or 8 M guanidine hydrochloride–0.12 M mercaptoethanol–0.05 M sodium phosphate buffer (pH 8).

sea urchin material, the composition resembles that of muscle actin. However actin is known to contain 1 mole/mole of 3-methylhistidine (Asatoor and Armstrong, 1967) and a separate determination made under conditions capable of resolving this amino acid failed to detect any 3-methylhistidine in the brain protein.

Binding of Guanosine Nucleotides and Colchicine. If the purification procedure is carried out in the absence of added GTP, the final product contains a bound nucleotide which causes a slight hump in the absorption spectrum of the protein at 268 mμ. The material remaining in solution after precipitation of the protein in 0.5 N perchloric acid has absorbtion spectra at pH 1 and 11 identical with a guanosine nucleotide (Figure 11). The amount of nucleotide present is in the range of 0.5–0.8 mole/120,000 g of protein. The nucleotide released by perchloric acid precipitation was isolated by the method of Tsuboi and Price (1959) and chromatographed on thin-layer silica gel plates. Spots migrating with R_F values identical with those of GTP, GDP, and occasionally GMP were observed.

TABLE IV: Amino Acid Compositions of Colchicine-Binding Protein of Mammalian Brain and Central Pair Microtubules of Sea Urchin Sperm.[a]

Amino Acid	Brain Protein (mole %)	Sperm Microtubule (mole %)
Lys	3.9	6.1
His	2.4	2.8
Arg	4.4	5.1
Asp	10.3	9.6
Thr	6.3	6.2
Ser	5.9	5.3
Glu	14.2	14.1
Pro	5.2	5.2
Gly	7.9	7.9
Ala	7.5	7.7
Cys-$^1/_2$	1.9	1.5
Val	6.3	6.3
Met	2.3	2.5
Ile	4.5	4.5
Leu	7.7	7.9
Tyr	3.9	3.0
Phe	4.8	3.5
Trp	0.7	0.8
CH_3-His	0	

[a] Values for brain protein are averages of 24- and 48-hr hydrolysates, except Ser and Tyr, which were corrected to zero time. Trp was determined spectrophotometrically. Data on central pair microtubules of sea urchin sperm *S. purpurates* from Shelanski and Taylor (1968).

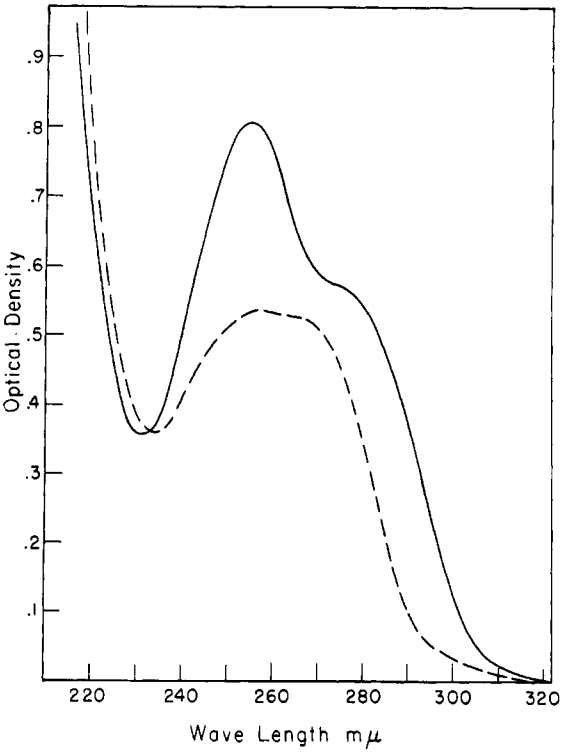

FIGURE 11: Ultraviolet spectrum of the bound nucleotide. Protein prepared in the absence of added nucleotide was precipitated with 5% perchloric acid and the absorption spectrum of supernatant was determined. (——) pH 1 and (- - -) pH 11, adjusted with NaOH and redrawn from trace obtained in Cary 14.

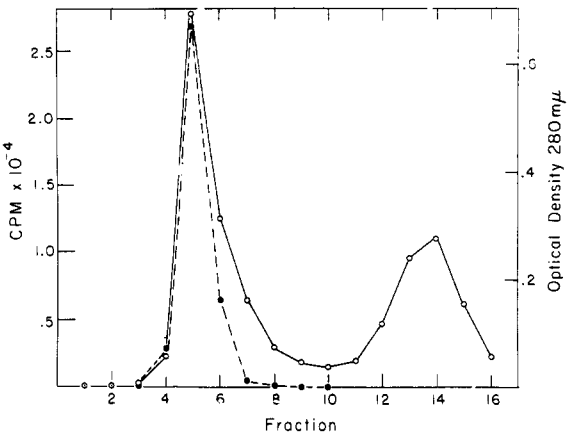

FIGURE 12: Binding of [^{3}H]GTP to protein purified in the absence of added nucleotide. A 0.5-ml aliquot, at a concentration of about 8 mg/ml, was incubated with 10^{-5} M [^{3}H]-GTP for 1 hr at 37°. Binding of [^{3}H]GTP determined by gel filtration on a 1 × 10 cm G-100 Sephadex column; fraction volume 1.0 ml, (- - -) optical density at 280 mμ (protein), sample diluted fivefold, and (——) radioactivity (0.5-ml aliquot).

The protein purified in the absence of GTP is capable of binding [^{3}H]GTP. Incubation of an aliquot with 10^{-5} M [^{3}H]GTP for 1 hr at 37° followed by gel filtration on G-100 Sephadex gave the elution profile illustrated in Figure 12. Although the peak of radioactivity moves with the protein peak there is a distinct trailing of the radioactivity behind the protein profile, indicating a relatively rapid dissoication of the complex. When [^{3}H]GTP was added to protein prepared and stored in the presence of 10^{-4} M GTP, there was a rapid binding of radioactivity as measured by gel filtration which was essentially complete in 15 min at room temperature.

Thus there appear to be two kinds of nucleotide binding sites. At one site, exchange of bound and free nucleotide occurs with a half-life of less than 15 min at room temperature.

But the presence of GTP and GDP bound to the protein after purification in the absence of nucleotide, a procedure which requires several hours, suggests a second type of binding site at which exchange proceeds very slowly, if at all.

The question of the number and type of binding sites was investigated in some detail by the gel filtration method. For all experiments the protein was isolated in the presence of 10^{-4} M GTP. In the first series the solution was incubated with 10^{-3} M GTP for 1 hr to saturate the binding sites and then subjected to gel filtration on 1 × 15 cm columns of G-100 Sephadex, and GTP binding was determined by absorption at 256 mμ after precipitation of the protein in 0.4 N perchloric acid. Approximately 1.7–1.8 moles of GTP was bound per 120,000 g of protein (Table V, series 1).

In the second series of experiments, free nucleotide was removed by gel filtration and the solution was incubated for 1 hr at 37° with 1 mM [^{3}H]GTP and 1 mM colchicine (unlabeled). The binding was measured after gel filtration as before. Colchicine was determined by absorption at 350 mμ before or after precipitation of the protein, and [^{3}H]GTP was determined by counting aliquots of the supernatant after precipitation of the protein. The results (Table V, series 2) showed that approximately 1 mole of GTP and 1 mole of colchicine were bound per mole of protein. The importance of determining the binding of both molecules in the same experiment lies in the fact that the ratio of one colchicine to one GTP binding site is obtained independent of errors arising from the presence of inactive protein or from the measurement of protein concentration. Parallel experiments showed that there was no competition between the binding of the two molecules.

In a third series of experiments, the protein was incubated for 1 hr at 37° with 1 mM [^{3}H]GTP and sub-

TABLE V: Colchicine and GTP Binding to Brain Protein (Moles/120,000 g).

Series I Total GTP[a]	Series III[c] Specific Activity of Free GTP/Specific Activity of Bound GTP
1.7	2.02
1.9	2.35
1.8	2.02

Series II[b] Total Colchicine	[^{3}H]GTP Exchange	Colchicine/ [^{3}H]GTP
0.85	1.14	0.75
1.03	0.98	1.05
0.84	0.88	0.96

[a] Protein was purified in presence of 10^{-4} M GTP. For series I, protein solution was incubated with 10^{-3} M GTP at 37° for 1 hr. Free nucleotide was removed by gel filtration on 1 × 15 cm G-100 Sephadex columns. Bound nucleotide was determined by ultraviolet spectrum following perchloric acid precipitation of protein. [b] For series II, free nucleotide was first removed by gel filtration. Protein was then incubated with 10^{-3} M colchicine and 10^{-3} M [^{3}H]GTP for 1 hr at 37°. Free colchicine and [^{3}H]GTP were removed by gel filtration. The bound colchicine was assayed using $OD_{350\,m\mu}$. [c] For series III, protein was incubated 1 hr at 37° in the presence of 10^{-3} M [^{3}H]GTP. The samples were chromatographed on a 1 × 15 cm G-100 Sephadex columns and sufficient fractions were collected to include the small molecule regions. Aliquots of all fractions were counted for [^{3}H]GTP. Perchloric acid was then added to aliquots of all fractions, samples were clarified, and the GTP content was determined by absorption spectrum. Ratio of specific activity in small molecular region (free GTP of incubation mixture) and protein-bound GTP was calculated. Appropriate controls eliminated the possibility of significant quenching by the protein. In each series the data listed are for three independent experiments.

jected to gel filtration as before except that elution was continued until the peak of small molecules came off the column. An aliquot of each fraction was counted, perchloric acid was added to the remainder, the protein precipitate was removed by centrifugation, and the concentration of GTP was determined from the absorption at 256 mμ. This procedure allowed a comparison to be made between the specific activity of the free GTP in the incubation mixture and the GTP bound by the protein. The ratio was approximately two (Table V series 3) indicating that only one-half of the protein-bound nucleotide is capable of exchange. In summary, the results indicate that per 120,000 g of protein there is one colchicine site, one rapidly exchangeable GTP site, and one GTP site which does not exchange appreciably under the conditions of the experiments. Presumably the site which did not exchange accounts for the guanine nucleotides associated with the protein prepared in the absence of added GTP.

In the course of these studies it was found that the inclusion of 10^{-4} M GTP and 10^{-2} M $MgCl_2$ in all solutions used in the isolation procedure largely prevented the loss in colchicine-binding activity during preparation of the protein. Protection of binding activity by GTP, GDP, and ATP was investigated by first removing free nucleotides by passage over a G-100 Sephadex column and storing the protein preparation at 0° in the presence or absence of nucleotide. At intervals, aliquots were incubated with [^{3}H]colchicine for 1 hr at 37° and the bound radioactivity was determined by filter assay. Figure 13 shows the decay of binding activity with time plotted on a semilogarithmic scale. In the absence of added nucleotide, colchicine-binding activity decayed with a half-life of 11 hr. In the presence of 10^{-3} M GTP, the half-life was about 90 hr; 10^{-4} M GTP and 10^{-3} M GDP were less effective while 10^{-3} M ATP afforded only slight protection. The loss of activity appeared to be irreversible since the addition of 10^{-3} M GTP to a preparation stored at 0° in the absence of nucleotide did not lead to a regain of colchicine-binding activity.

Since colchicine is bound to a specific site on the protein the loss of binding activity by a first-order reaction indicates that a process of irreversible denaturation or dissociation may be involved. Also, the decay is still first order in the presence of nucleotide, and the equilibrium constant, K, for nucleotide binding at a site which stabilizes the protein can be estimated from the slopes of the curves, if it is assumed that the protein–nucleotide complex is resistant to denaturation. It can easily be shown that $K = [N]^{-1}(\lambda_0/(\lambda - 1))$, where [N] is the concentration of free nucleotide and λ and λ_0 are the rate constants for loss of binding activity in the presence and absence of nucleotide, respectively. The equilibrium constants estimated from the curves shown in Figure 12 are 2×10^4, 2×10^3, and 5×10^2 M^{-1} for GTP, GDP, and ATP, respectively.

It was shown previously (Borisy and Taylor, 1967a,b) that the cytoplasmic protein from cultured cells and sea urchin eggs was stabilized by colchicine binding and a similar effect was demonstrated for the brain protein. It was expected that the binding of colchicine would protect against the loss of GTP-binding activity. A solution of the protein was incubated with 10^{-3} M colchicine for 1 hr at 37°, and then stored at 0° and tested for the ability to bind [^{3}H]GTP. In the absence of colchicine, GTP-binding activity decayed in a first-order reaction with a half-life of 11 hr. Since this value is the same as the rate of loss of binding activity found for colchicine, it is reasonable to conclude that colchicine and GTP, though bound to different sites, both require the native configuration of the protein. Preincubation with colchicine more than doubled the half-life of GTP-binding activity.

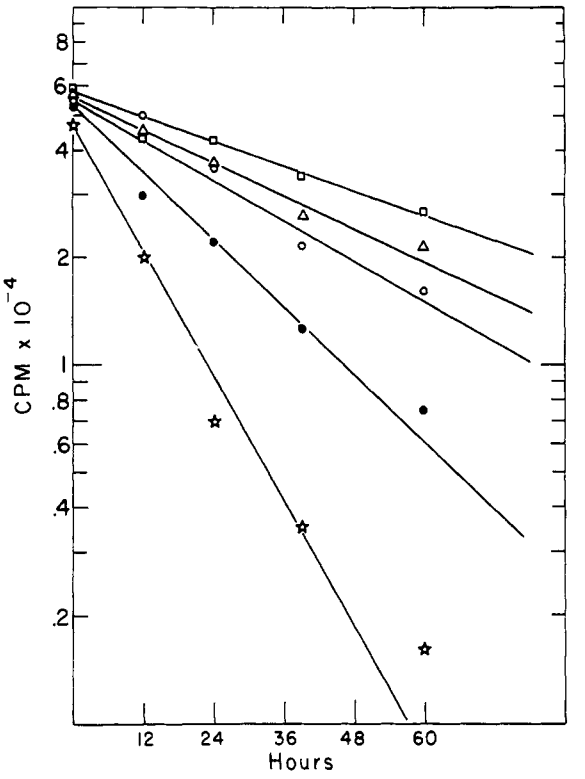

FIGURE 13: Protection of colchicine-binding activity by bound nucleotides. Protein was prepared in 10^{-4} M GTP, free nucleotides were removed by gel filtration, concentrations of nucleotides were added to 1.0-ml aliquots, and the protein was stored at 0° in P–Mg buffer. At the indicated times, 0.2-ml samples were withdrawn and incubated 1 hr at 37° with 0.5 ml of 2.5×10^{-5} M [^{3}H]colchicine. Bound radioactivity was determined by filter assay. (□) 1 mM GTP, (△) 1 mM GDP, (○) 0.1 mM GTP, (●) 1 mM ATP, and (☆) no added nucleotide.

Discussion

By using affinity for colchicine as an assay, a procedure has been developed which allows the isolation, in a highly purified form, of a colchicine-binding protein from homogenates of whole mammalian brain. On the basis of its molecular weight, sedimentation constant, amino acid composition, and number of colchicine and guanine nucleotide binding sites, the protein is very similar to the subunit protein obtained from microtubules of sea urchin sperm tails and *Tetrahymena* cilia (Shelanski and Taylor, 1968; Renaud *et al.*, 1968; Stephens, 1968a).

Therefore it is reasonable to conclude that colchicine

binding provides a specific assay for the presence of microtubule protein. The isolation procedure has been described in detail since it should be applicable to the purification of microtubule protein from a variety of cells. Brain appears to be a good source of this protein, since it makes up about 1 or 2% of the total protein and 5 or 10% of the protein soluble after homogenization in sucrose. Although ciliated cells occur in regions of the brain, such as the ependyma, their contribution to the protein content would be extremely small and in addition the protein from doublet tubules of the cilia would not be extracted by our procedure (Shelanski and Taylor, 1967). Thus it is assumed on the basis of circumstantial evidence that the protein is the subunit of neurotubules, since these are the only microtubule structures present in sufficient amounts to account for the yield of protein. The assumption still requires direct proof by first obtaining intact tubules from brain as a starting material. Also it is not known what fraction of the protein was actually present in brain in a polymerized form.

The available electron microscope evidence is certainly consistent with the identification of neurotubules with other cytoplasmic microtubules (Porter, 1966). Also, neurotubules have been shown to be derived from the centriole of the neuron in much the same way as other cytoplasmic microtubules (Robbins and Gonatas, 1964).

Proteins which appear to be very similar have now been obtained from a protozoan (*Tetrahymena* cilia), an echinoderm (sea urchin sperm tails), and a mammal. Thus there is a well-defined class of closely related proteins which are the subunits of at least some of the microtubules described by electron microscopists. The question of whether all microtubules are constructed from the same subunit, obviously cannot be answered but a 6S colchicine-binding protein has been detected in a number of systems, which are known to contain microtubules, including mitotic apparatus from sea urchin eggs and cytoplasm of mammalian and echinoderm cells (Borisy and Taylor, 1967a,b). Furthermore since colchicine binding is a specific property of the well-characterized tubule proteins, the ability of colchicine to block mitosis and disrupt microtubule structures in a large variety of plant, animal, and protozoan cells, implies that these microtubules are constructed from proteins which are related to the class described here.

All of the tubule proteins so far studied have the ability to bind guanine nucleotides. Although the binding has not been studied in detail, preliminary evidence indicates that GTP is more strongly bound than GDP and that the affinity for ATP is very small compared with GTP. The function of the bound nucleotide is not yet understood, and the available evidence does not allow speculation on its possible role in polymerization or in the generation of mechanical forces in motile systems. However the experiments do show that bound GTP appears to be necessary to maintain the native configuration of the protein as measured by colchicine-binding activity.

Since there are two binding sites per dimer only one of which exchanges readily with free GTP, the simplest interpretation of the binding data is that the dimer is formed by head-to-tail interaction of the 60,000 molecular weight subunits and one GTP site is buried in the region of contact. Other interpretations are possible, including nonidentical monomer units or a change in conformation when one GTP is bound.

GTP appears to reduce the formation of aggregates in solution, and the presence of GTP yields a more symmetric boundary for the 6S dimer in the ultracentrifuge. Both observations suggest that the nucleotide may prevent nonspecific interactions between molecules possibly by reducing denaturation or dissociation. Solutions of the protein show a complex variety of interactions including nonspecific aggregation, formation of a 30S component at low ionic strength, and formation of tactoids at low temperatures and of filaments at pH 5.5. A preliminary examination of the various kinds of aggregates in the electron microscope, negatively stained with phosphotungstate or uranyl acetate, has not revealed the presence of microtubules. However, Stephens (1968b) has reported the formation of microtubules from the subunit protein obtained from sea urchin sperm tail, when the polymerization reaction was nucleated with tubule fragments.

The evidence reported here serves to establish the general properties of the brain protein and its relation to other microtubule proteins. Large quantities of the protein are easily obtainable in a highly purified form by the isolation procedure that we have described and a systematic study of the interactions under a variety of conditions must be undertaken before any further conclusions can be drawn about the mechanism of tubule formation or the function of GTP.

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Diabetogenic Action of 5-Thio-D-glucopyranose in Rats*

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ABSTRACT: Rats intraperitoneally administered 5-thio-D-glucopyranose rapidly develop glucosuria and hyperglycemia. With a dose of 50 mg/kg the blood D-glucose rises to approximately 300 mg/100 ml within 2.5 hr, then drops to 160 mg/100 ml within 6 hr. Rats given booster doses of 5-thio-D-glucopyranose every hour maintain blood D-glucose levels of 300 mg/100 ml.

The same effect is observed in rats with ligated kidneys given a single 50-mg/kg dose of 5-thio-D-glucopyranose. Administration of insulin completely nullifies the diabetogenic effect. Livers of rats fasted for 20 hr had 50% less glycogen 2.5 hr after injection of 50 mg/kg of 5-thio-D-glucopyranose than saline-injected controls. Rats injected with 5-thio-D-glucopyranose excreted 97% of the sugar in the urine indicating little or no metabolism of 5-thio-D-glucopyranose. Investigations with rat liver slices, kidney slices, and diaphragms show a marked decrease in the uptake of D-glucose by these tissues when molarity ratios of 5-thio-D-glucopyranose/D-glucose from 0.5 to 1 are present in the initial incubation media at 37°. 5-Thio-D-glucopyranose did not inhibit the metabolism of D-glucose in kidney homogenates but did slightly inhibit glycolysis (8.3% with a molarity ratio 5-thio-D-glucopyranose/D-glucose of 0.50). After a series of three injections of 5-thio-D-glucopyranose at 2-hr intervals there is a 43% increase in the total catechol amine content of the urine. A 73% increase in blood nonesterified fatty acids of rats fasted for 16 hr is observed 0.5 hr af er a single 150-mg/kg dose of 5-thio-D-glucopyranose. The nonesterified fatty acid level then returns to a normal fasting level within 2 hr.

5-Thio-D-glucopyranose is neither a substrate nor an inhibitor of D-glucose oxidase which is used to analyze for D-glucose. 5-Thio-D-glucopyranose does not inhibit yeast hexokinase but acts as a poor substate. Kinetic measurements give a K_m of 4×10^{-3} M and a V_{max} (glucose − 100) of 1.3.

5-Thio-D-glucopyranose was first prepared by Feather and Whistler (1962). Since 5-thio-D-glucopyranose differs from D-glucopyranose by having sulfur in place of oxygen in the pyranose ring it may act as an antagonist of this ubiquitous metabolite. At the time of this writing all but one publication concerning sugars with sulfur in the ring dealt solely with their chemical properties. Shankland *et al.* (1968) found that 5-thio-D-glucopyranose in molarity ratios of 5-thio-D-glucopyranose/D-glucose as low as 0.03 effectively interferes with the utilization of D-glucose for development of *Drosophila melanogaster*. Molarity ratios of 0.31 and above prevented development from the larval to the pupal stage. This inhibition was attributed to interference of enzyme(s) and/or the transport of D-glucose across cell membranes due to the structural similarity of the two sugars. In the present paper we report effects of 5-thio-D-glucopyranose in intact rats and in several isolated systems.

Experimental Section

Materials

Crystalline 5-thio-D-glucopyranose was prepared by the method of Rowell and Whistler (1966): mp 135–136°, $[\alpha]_D^{20}$ +188° (*c* 1.56, water).

Wistar rats from our colonies, maintained on Purina Lab-Block feed, were given water *ad libitum*. Experimental rats were injected with various volumes of 5-thio-D-glucopyranose solutions. Control rats received intraperitoneal injections of equivalent volumes of isotonic saline. Blood was obtained by cardiac puncture unless otherwise stated.

Regular iletin insulin was purchased from Eli Lilly and Co., Indianapolis, Ind. Yeast hexokinase and ATP[1] were obtained from Calbiochem.

* Journal Paper No. 3250 of the Purdue Agricultural Experiment Station, Lafayette, Indiana 47907. *Received January 19, 1968.*

[1] ATP is the dipotassium salt of adenosine triphosphate.