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Evidence for Involvement of Microtubules in the Action of Vasopressin in Toad Urinary Bladder

II. Colchicine Binding Properties of Toad Bladder Epithelial Cell Tubulin

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Summary. Colchicine, podophyllotoxin, and vinblastine have been found to inhibit the action of vasopressin on water movement in the toad urinary bladder. Tubulin is the major colchicine binding component of toad bladder epithelial cells, accounting for approximately 3.3% of the total cell protein. More than 99% of the tubulin is found in the soluble fraction after sonication, the remainder is in the particulate fraction. Similar to the characteristics of the binding of colchicine to tubulins from other sources, the binding of colchicine to toad bladder tubulin is temperature- and time-dependent, is inhibited competitively by podophyllotoxin ($K_i = 5.5 \times 10^{-7}$ M), and has a binding constant of 1×10^6 liters/mole at 37°. Binding activity decays according to first-order kinetics and is stabilized by vinblastine. The characteristics of the interactions of colchicine and podophyllotoxin with epithelial cell tubulin *in vitro* closely parallel the ability of these drugs to inhibit the response to vasopressin *in vivo*. These results, coupled with those of functional and morphological studies, support the view that the ability of these drugs to affect vasopressin-induced water movement across toad bladder epithelial cells is related to the depolymerization of cytoplasmic microtubules.

Colchicine, podophyllotoxin, and vinblastine have become useful tools to probe whether microtubules are involved in a particular biological process. Although many specific details concerning how these drugs act still remain to be elucidated, their actions on microtubules are now sufficiently well understood to permit formulation of an overview mechanism [9, 10, 18, 20]. All three drugs bind to tubulin, the fundamental unit of microtubules, and prevent microtubule polymerization. This occurs through a "substoichiometric poisoning" mechanism, in which the drug

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concentrations required to inhibit assembly are considerably below the concentrations of tubulin which are present. Pre-existing labile microtubules ultimately depolymerize in the presence of the drugs as a result of inhibition of the assembly reaction.

In the present series of studies on the action of vasopressin (*see* [13, 17]), a combined physiological, biochemical, and morphological approach has been utilized which provides evidence that the inhibition of vasopressin-induced water movement in toad urinary bladder by colchicine, podophyllotoxin, and vinblastine is due to the interaction of these agents with tubulin and consequent interference with microtubule function. Our findings suggest that the inhibition of the vasopressin response induced by these drugs is due to depolymerization of previously existing microtubules, rather than to inhibition of formation of new microtubules. In this paper, we report on the colchicine binding properties and quantitation of toad bladder epithelial cell tubulin.

Materials and Methods

Chemicals and Biological Materials

Colchicine (Ring C, methoxy-^{3}H) was obtained from New England Nuclear Corporation, Boston, Massachusetts, and diluted to a specific activity of either 2.5, 3.0, or 5.0 Ci/mmole by addition of purified unlabeled colchicine. Podophyllotoxin was obtained from Aldrich Chemical Co. (Alfred Bader Library of Rare Chemicals) (mp, 112–114° $\max_{290\,nm}$ in 95% ethanol, $\varepsilon = 3750$). Vinblastine sulfate was a generous gift from The Eli Lilly Company, Indianapolis, Indiana. Bio-Gel P 10 was obtained from Bio-Rad Laboratories. Lumicolchicine was prepared by ultraviolet irradiation of colchicine, as described previously [23].

Colombian toads, *Bufo marinus*, were obtained from Tarpon Zoo, Tarpon Springs, Florida. They were stored in tap water for 1–4 weeks unfed, and were sacrificed by pithing immediately before use. Fertilized chick embryos were obtained from Kimber Farms, Fremont, California, and maintained through 13 days of development at 38° in a humidified Jamesway incubator.

Methods

Preparation of toad bladder epithelial cell extracts. Bladders were excised immediately prior to use, and the epithelial cell layer removed by scraping the inside surface of the bladders with the edge of a glass coverslip. Scraped epithelial cells were suspended in 20 mM sodium phosphate buffer containing 100 mM sodium glutamate, pH 6.75 (phosphate-glutamate buffer) at 0°, and sonicated with a Biosonik II sonifier (power setting 100 W; three bursts of 5–10 sec). Usually, cells from 2–4 bladders were sonicated in a total volume of 5 ml. Sonicates were centrifuged at 39,000 × *g* for 45 min in a Sorvall RC 5 preparative centrifuge with a SS-34 rotor. Particulate and soluble fractions thus

obtained were utilized for determination of colchicine binding activity as described below.

Preparation of chick brain supernatant fractions. Usually 2 chick brains (200–250 mg wet wt) from 13-day-old embryos were removed and sonicated as above in 5.0 ml phosphate-glutamate buffer, and supernatant fractions were prepared by centrifugation at $39{,}000 \times g$ for 45 min at 0–4°. Forty-two percent of the protein in these extracts is tubulin [1]. Supernatant fractions were diluted to a tubulin concentration of 0.5 mg/ml for use in colchicine binding experiments.

Colchicine binding assay procedures. The colchicine binding activity of the tubulin contained in toad bladder epithelial cell fractions and embryonic chick brain supernatant fractions was measured as described in detail previously for chick brain tubulin [1]. Supernatant fractions were incubated with labeled colchicine under the desired conditions, always in phosphate-glutamate buffer, and bound colchicine was determined after separation of bound and free colchicine by gel filtration on 1×18 cm columns of Bio-Gel P 10. The amount of colchicine binding activity in the particulate fractions of toad bladder sonicates was determined by suspending the particulate fractions in phosphate-glutamate buffer, then incubating with colchicine under the desired conditions. The quantity of bound colchicine was determined after washing the particulate material 3 times by centrifugation and resuspension in phosphate-glutamate buffer. Unless otherwise stated, all procedures were carried out at 0–4°.

Protein. Protein concentrations were determined by the method of Lowry *et al.* [8], with bovine serum albumin as a standard.

Results

Properties of the Colchicine Binding Activity in the Soluble and Particulate Fractions of Sonicated Toad Bladder Epithelial Cells

Colchicine binding specificity of epithelial cell fractions. The binding of colchicine to tubulin exhibits a unique set of properties (*see* [21] for a review) which can be used to determine whether the binding of colchicine in an extract of a particular cell or tissue in question is to tubulin. Thus, our initial question in toad bladder epithelial cell fractions was to determine whether the colchicine binding activity present in the fractions was due to tubulin, or to another colchicine binding component by comparing a number of characteristics of the binding activity in toad bladder fractions with those of purified tubulin.

The results of these studies are shown in Table 1. Identical to results obtained with the binding of colchicine to purified embryonic chick brain tubulin, the colchicine binding activity in the supernatant fraction of toad bladder epithelial cell extracts was markedly reduced by incubation at 0 °C as compared with a control performed at 37 °C. Addition of 1×10^{-5} M podophyllotoxin, which competes with colchicine for the same site, inhibited the binding by 85 % in this fraction. The binding

Table 1. Properties of colchicine binding activity in soluble and particulate fractions of sonicated toad bladder epithelial cells

Incubation conditions (colchicine, 2.6×10^{-6} M)	Percent of control binding activity	
	Soluble fraction	Particulate fraction
37° (control)	100	100
0°	14	54
37° plus podophyllotoxin (1×10^{-5} M)	15	78
37° plus vinblastine (5×10^{-5} M)	144	129
37° plus lumicolchicine (1.6×10^{-4} M)	88	–

Soluble and particulate fractions of toad bladder epithelial cell sonicates were prepared, and aliquots of the soluble fraction (0.5 ml) and resuspended particulate fractions (total volume, 0.5 ml) were incubated with labeled colchicine for 2 hr under the desired conditions. Bound colchicine was determined for each fraction as described in *Methods*. Control specific activity of the soluble fraction was 178 dpm/μg total protein; and of the particulate fraction, 19 dpm/μg total protein.

activity was stabilized, and thus apparently increased, in the presence of vinblastine, which binds to tubulin at separate sites [4, 12, 22]. The binding of colchicine was not appreciably altered by the presence of lumicolchicine (molar excess of lumicolchicine to colchicine of 60:1), an isomer of colchicine which does not bind to tubulin [21, 23]. It is clear from these results (as well as those to be described) that tubulin is solely responsible for the colchicine binding activity in the soluble fraction of the bladder cells.

By contrast, the situation in the particulate fraction was more complex. Binding of colchicine was reduced by 46% at 0°C as compared with a reduction of 86% in the soluble fraction. Inhibition by podophyllotoxin was also considerably less in the particulate fraction than in the soluble fraction. These findings indicate that, although some of the particulate binding activity is due to tubulin, much of it clearly is not. We estimate that greater than 50% of the particulate colchicine binding activity is to a colchicine binding component which is not tubulin.

Time-dependence of colchicine binding activity soluble fraction. The binding of colchicine at 23° increased slowly with time of incubation [16]. Maximum binding still had not been attained after 4 hr of incubation. This temperature was chosen because it is the same temperature at which the functional studies were performed [17]. It is clear that the rate of binding of the drug to tubulin in the soluble fraction of the epithelial cells is very slow, which is similar to the binding of colchicine to tubulins from other sources [11, 21].

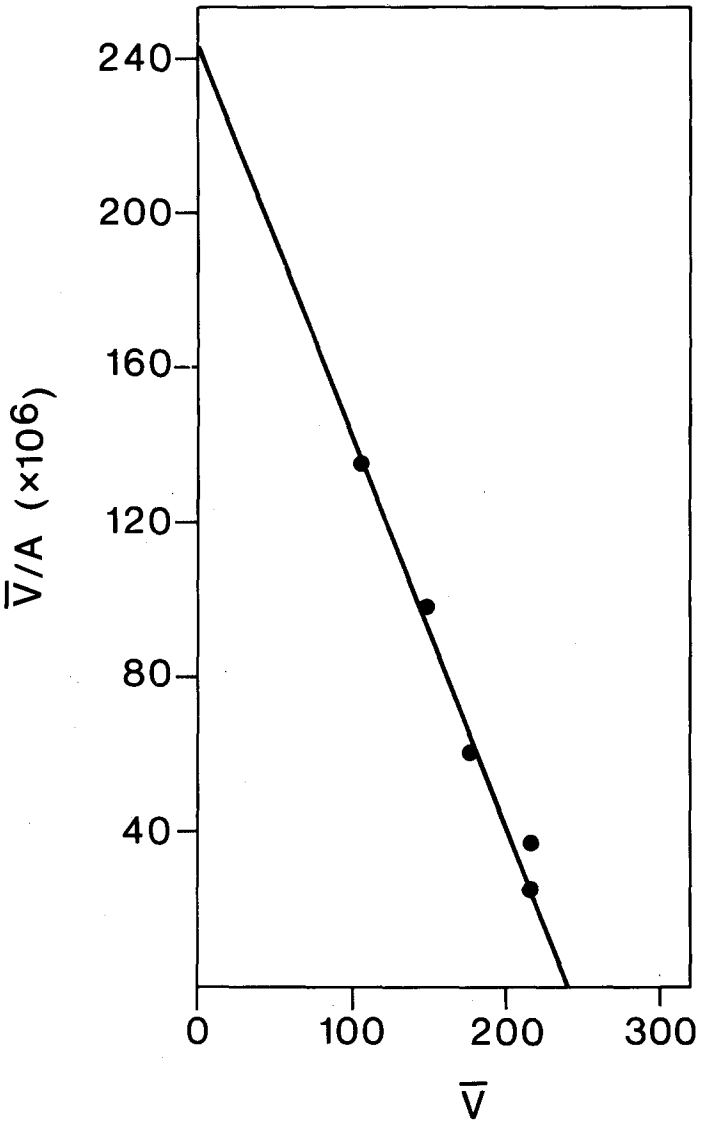

Fig. 1. Binding of colchicine to toad bladder epithelial cell tubulin (Scatchard Plot, [14]). Supernatant fractions of epithelial cell sonicates (0.5 ml, 1.64 mg protein/ml) containing 5.7×10^{-4} M vinblastine (to stabilize colchicine binding activity) were incubated with various concentrations of [*methoxy*-^{3}H] colchicine for 6 hr at 37 °C. Bound colchicine was determined as described in the *Methods* section. Binding values were not corrected for loss of colchicine binding activity during incubation. $\bar{V}$ = dpm/μg total protein. A = free colchicine concentration. The binding constant (K_A), 1.0×10^6 liters/mole, was calculated from the slope of the line (slope = $-K_A$)

Affinity of toad bladder epithelial cell tubulin for colchicine. The affinity of colchicine for tubulin in the soluble fraction of bladder epithelial cells at 37 °C was determined with the use of a Scatchard analysis (Fig. 1) [14]. A straight line was obtained, indicating a single affinity class of binding sites in the supernatant. The binding constant, calculated from the slope of the line, was 1.0×10^6 liter/mol. The reciprocal of this value, 1×10^{-6} M, is the concentration of colchicine that would saturate exactly half of the tubulin molecules present in the fraction. This binding constant is virtually identical to that obtained for the binding of colchicine to purified chick embryo brain tubulin at 37° [21]. This permits the use of chick brain tubulin as a colchicine binding standard for the quantitation of tubulin in the toad bladder epithelial cell supernatant fractions, discussed subsequently.

Affinity of toad bladder tubulin for podophyllotoxin. Since podophyllotoxin competitively inhibits the binding of colchicine to tubulin, it is possible to obtain an estimate of the binding constant for podophyllotoxin which is equivalent to the inhibition constant for the ability of podophyllotoxin to prevent the binding of colchicine. The results of an experiment showing the competitive nature of the inhibition and permitting calculation of an inhibition constant for podophyllotoxin is shown in Fig. 2. The inhibition constant for podophyllotoxin in this experiment was 5.5×10^{-7} M. This is the concentration of podophyllotoxin which would saturate half of the tubulin molecules present in the epithelial cell supernatant fractions.

Quantitation of Tubulin in Toad Bladder Epithelial Cell Sonicates

The minumum quantity of tubulin present in the epithelial cell extracts can be determined with the use of a time-decay colchicine binding assay as described previously [1, 2, 21], by comparing the initial colchicine binding activity in the epithelial cell supernatant and particulate fractions with the value obtained with a tissue extract containing a known quantity of tubulin. The analysis requires that the binding constants for the reaction be similar for both sources of tubulin, and that no nonspecific binding occurs. We have performed a series of time-decay assays of colchicine binding activity in the soluble and particulate fractions of epithelial cell sonicates, and have utilized a $100{,}000 \times g$ supernatant fraction of 13-day-old embryonic chick brain [1] as a standard. This chick brain fraction contains 42% tubulin. The colchicine

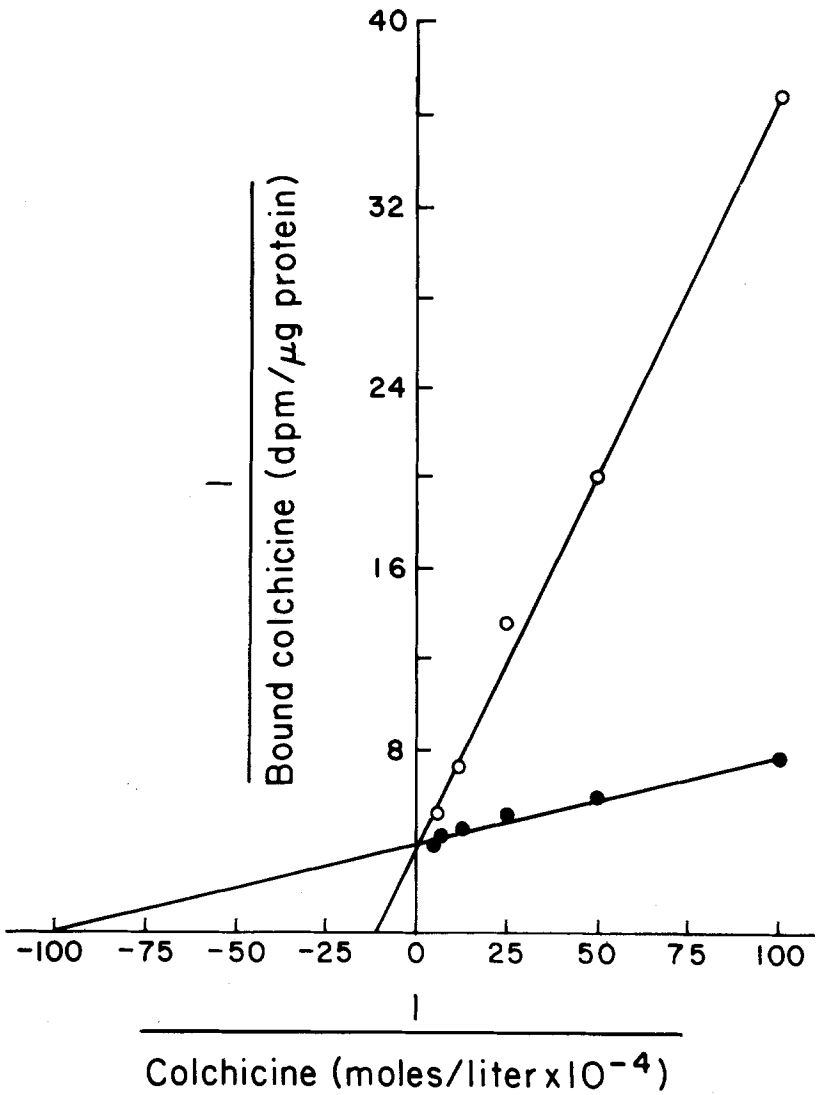

Fig. 2. Inhibition of colchicine binding activity by podophyllotoxin. Supernatant fractions of epithelial cell sonicates (1.97 mg protein/ml) containing 5.7×10^{-4} M vinblastine (to stabilize colchicine binding activity) were incubated with various concentrations of [*methoxy*-^{3}H] colchicine for 6 hr at 37 °C in the presence (open circles) or absence (closed circles) of 4.0×10^{-6} M podophyllotoxin. Bound colchicine was determined as described in *Methods*. Binding values are uncorrected for loss of colchicine binding activity during incubation. The inhibition constant (K_i) for podophyllotoxin was 5.5×10^{-7} M

binding activity is completely specific at 2×10^{-6} M colchicine, and the reaction has a binding constant of between 1 and 2×10^6 liters/mole at 37 °C [1, 21].

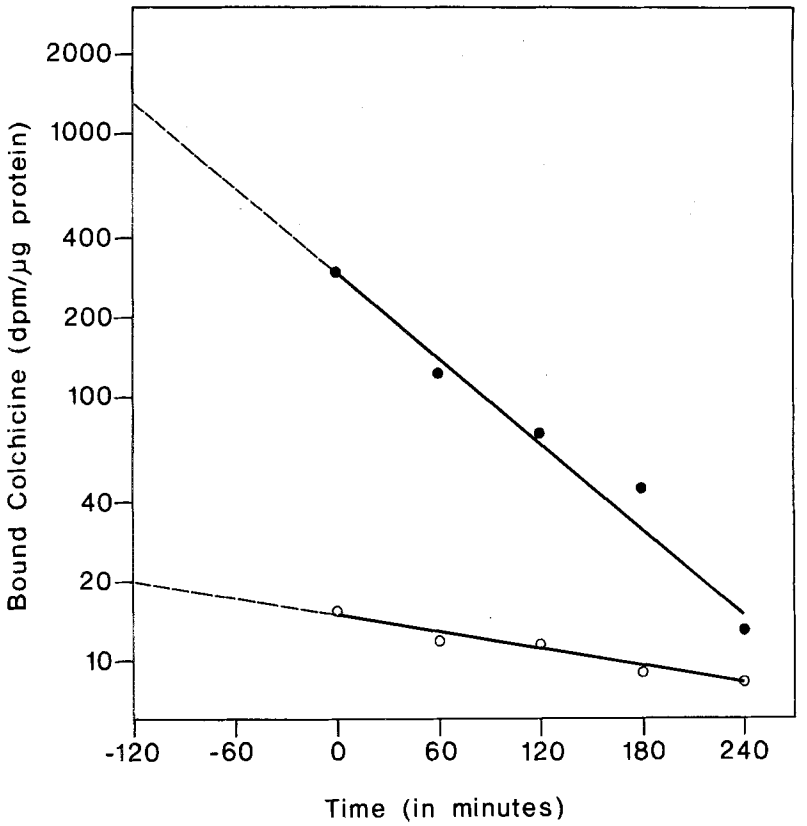

Fig. 3. Decay of colchicine binding activity in soluble and particulate fractions of sonicated bladder epithelial cells. A whole toad bladder sonicate containing 2.67 mg protein/ml was incubated at 37 °C. At the times indicated, 0.5 ml aliquots were incubated with 2.0×10^{-6} M *methoxy*-^{3}H colchicine for 2 hr at 37 °C, after which they were separated into particulate and soluble fractions as described in *Methods*. Bound colchicine was determined in each fraction as described in *Methods*. Particulate protein was 32.7% of the total protein. Extrapolation of the lines to include the time of the colchicine incubation (120 min) yielded the *initial binding capacity* of the fraction. Soluble fraction = closed circles; particulate fraction = open circles. Half-times were calculated from the slopes of the lines. Soluble fraction = 57 min; particulate fraction = 288 min

Time-decay assay: Toad bladder epithelial cell supernatant and particulate fractions. The colchicine binding activity of tubulin in the soluble fraction of toad bladder epithelial cells decayed according to first-order kinetics, with half-times at 37 °C between 54 and 62 min (Fig. 3). Initial colchicine binding capacity as determined from extrapolation of the line to zero-time of incubation in the experiment described in Fig. 3 was 1200 dpm/μg of total protein. By contrast, the colchicine binding activity in the particulate fraction of the epithelial cell sonicates decayed much

Table 2. Minimum percentage of tubulin in supernatant fractions of sonicated toad bladder epithelial cells

Experiment	Initial colchicine binding capacity (dpm/μg protein)		Percentage of tubulin in epithelial cell supernatant fractions
	Toad bladder	Chick brain	
1	740	6,100	5.1
2	740	6,400	4.9
3	1,200	11,000	4.6

Time-decay colchicine binding assays exactly like that described in the legend to Fig. 3 for the supernatant fraction of epithelial cell sonicates were carried out, and the *initial binding capacities* were determined. Identical time-decay assays were carried out simultaneously with supernatant fractions of 13-day-old embryonic chick brain sonicates [1] which contain 42% tubulin. The specific activity of the colchicine used in experiments 1 and 2 was 2.5 Ci/mmole, and in experiment 3, 5 Ci/mmole. The tubulin concentration in the toad bladder epithelial cell supernatant fraction was determined from the following relationship [2, 21]:

$$\text{Percentage tubulin in epithelial cell supernatant} = \frac{\text{IBC epithelial cell supernatant}}{\text{IBC chick brain supernatant}} \times 42$$

where IBC = initial binding capacity (dpm/μg protein) and 42 = the percentage of tubulin in the chick brain supernatant fraction.

more slowly (Fig. 3, half-time = 288 min at 37 °C). The initial colchicine binding capacity in the particulate fraction was 20 dpm/μg total protein. Thus, if all of the colchicine binding activity in the particulate fraction represented tubulin, the amount of tubulin in the particulate fraction could be no greater than 1.7% of the total tubulin in the soluble fraction. Since a considerable proportion of the binding of colchicine in the particulate fraction is nonspecific (i.e., not to tubulin), the amount of tubulin in the particulate fraction is undoubtedly much less than 1.7% of that found in the soluble fraction, and, therefore, can be considered to be a negligible proportion of the total epithelial cell tubulin.

Determination of the tubulin concentration in toad bladder epithelial cells. The quantity of tubulin present in the epithelial cell supernatant fraction was determined by carrying out separate time-decay colchicine binding assays in epithelial cell supernatant fractions, and under identical conditions, in 100,000 × g supernatant fractions of embryonic chick brain. In each case the percentage of tubulin in the epithelial cell supernatant fraction was calculated (Table 2). The mean value for the percentage of tubulin as a function of total soluble protein in the bladder epithelial cells was 4.9.

Discussion

The colchicine binding properties of toad bladder epithelial cell tubulin are similar to those of vertebrate brain tubulin in that the binding is time- and temperature-dependent, it is inhibited competitively by podophyllotoxin ($K_i = 5.5 \times 10^{-7}$ M), and has a binding constant of approximately 1×10^6 liters/mole at 37° [1, 21][1]. Similar to most other tubulins, the colchicine binding activity decays according to apparent first-order kinetics and is stabilized by vinblastine [21].

It is clear that tubulin is the major high affinity colchicine binding component of the toad bladder epithelial cells. Specificity studies on the binding of colchicine (Table 1) indicated that all of the high affinity colchicine binding activity in the soluble fraction of the epithelial cell sonicates was due to the binding of colchicine to tubulin. No other colchicine receptor was present. Tubulin constituted 4.9% of the total soluble protein in this fraction, as determined with the time-decay colchicine binding procedure and comparison with a chick brain supernatant fraction having known tubulin content and a similar binding constant. This value should be considered a minimal one, as no correction was applied for loss of any colchicine binding activity which might have occurred during preparation of the fraction.

The situation in the particulate fraction is more complex. It is evident that the colchicine binding activity in the particulate fraction is no greater than 1.7% of that found in the soluble fraction (Fig. 3). If all of this activity reflected the binding of colchicine to tubulin, the tubulin content of the particulate fraction would be 0.08% of the particulate protein, and since the total particulate protein is 32.7% of the total epithelial cell protein, the percent tubulin as a function of total bladder epithelial cell protein would be 3.33% (with the soluble fraction contributing 3.3%; the particulate fraction, 0.03%). However, as indicated from the results shown in Table 1, the particulate colchicine binding activity is due to a mixture of tubulin and a nontubulin colchicine binding component. We estimate that somewhat less than 50% of the particulate binding activity is to tubulin; the remainder is to the nontubulin component. Since the quantity of colchicine binding activity in the particulate fraction is so small compared with the amount in the soluble

1 As determined by equilibrium methods. Studies in which binding constants for colchicine have been determined by kinetic analysis have generally yielded values 10 to 20-fold greater than those obtained by equilibrium methods [7, 15]. This curious difference remains unexplained.

fraction, a correction for percentage of tubulin as a function of total epithelial cell protein would be insignificant.

The small amount of tubulin associated with the particulate fraction of the bladder epithelial cells is similar to that found in many cells (e.g., [5, 6, 23]) but considerably lower than that found in brain and thyroid [3]. The possibility that colchicine exerts its effect in the toad bladder through interaction with tubulin associated with the particulate fraction cannot be completely excluded; however, it seems improbable on purely quantitative grounds. The issue of whether the effect of colchicine on vasopressin-induced water movement might be brought about through an interaction with the particulate nontubulin component deserves further comment. We think it highly unlikely, since the characteristics of the inhibition of water movement by colchicine and other agents so strongly mirror the characteristics of the interaction of these agents with tubulin.

A number of important similarities are apparent when the action of colchicine, podophyllotoxin, and vinblastine on vasopressin-induced water movement and the ability of these drugs to bind to tubulin and disrupt microtubule function in cells are compared. For example, the concentration of colchicine required to inhibit the vasopressin response half-maximally is 1.8×10^{-6} M [17], while the concentration of colchicine required to half-maximally bind to epithelial cell tubulin and to decrease the content of microtubules in the epithelial cells half-maximally are 1×10^{-6} M and 1.4×10^{-6} M, respectively [13] (*see* further discussion below). The observation that the ability of colchicine to inhibit transcellular water movement decreases with decreasing temperature correlates remarkably well with the ability of colchicine to bind to toad bladder epithelial cell tubulin and other tubulins [5, 6, 11, 21, 23]. The lack of effect of lumicolchicine on the response to vasopressin [17] correlates with its inability to bind to tubulin [21]. Taken together, these correlations leave little doubt that the ability of the antimitotic drugs to affect vasopressin-induced water movement in toad bladder epithelial cells is due to an interference with tubulin or microtubule function in these cells.

Recent studies on the assembly and disassembly of bovine brain microtubules *in vitro* under steady-state conditions indicate that these microtubules assemble and disassemble at their opposite ends [10]. Thus, the microtubule assembly-disassembly "equilibrium" [11] is in reality a summation of two different reactions which occur at opposite ends of the microtubule, with the assembly reaction heavily favored at

one end, and the disassembly reaction heavily favored at the other end [10]. It seems probable that a similar situation exists within cells.

Colchicine, podophyllotoxin, and vinblastine all affect microtubule function in cells by inhibiting the polymerization of microtubules [9–11, 18, 20]. With colchicine, inhibition is brought about through addition of colchicine-tubulin complex(es) to the assembling microtubule end. Half-maximal inhibition of assembly occurs when only 2–4% of the soluble tubulin is complexed with colchicine [9]. With podophyllotoxin, the value is approximately 3.6% (C. Rauch and L. Wilson, *unpublished data*). Inhibition of the assembly reaction prevents any new microtubule formation and, in addition, results in the eventual depolymerization of previously existing microtubules, with the rate of depolymerization of the microtubules determined by the rate of tubulin loss from the disassembly end.

It appears likely that the inhibition of vasopressin-induced water movement induced by the antimitotic drugs is due to the depolymerization of previously existing cytoplasmic microtubules. In time-dependence studies reported in the accompanying paper [17], no inhibition of the vasopressin response was observed with colchicine unless the colchicine was added prior to the addition of vasopressin, but the degree of inhibition then increased as the period of preincubation with the drug was increased from 1 to 6 hr ([17], Fig. 5). With colchicine, this time lag could be due to a slow rate of binding of colchicine to tubulin, to a slow rate of depolymerization of existing microtubules, or to a combination of both. In contrast with colchicine, both podophyllotoxin and vinblastine produced inhibitory effects when added simultaneously with vasopressin, but like colchicine, the ability of these drugs to inhibit water movement increased substantially as the period of preincubation with the alkaloids was increased from 0 to 4 hr ([17], Fig. 5). With both podophyllotoxin and vinblastine, the time-dependence of their inhibitory effects must be due to the slow rate of depolymerization of existing microtubules, since these drugs bind rapidly to tubulin [11, 19, 21].

It is interesting that the concentrations of colchicine and podophyllotoxin which inhibit vasopressin-induced water movement half-maximally (1.8×10^{-6} M and 5×10^{-7} M, respectively [17]) are very similar to the concentrations of these drugs which half-maximally saturate bladder epithelial cell tubulin (1×10^{-6} M and 5.5×10^{-7} M, respectively). It could be argued that, since these drugs inhibit microtubule polymerization substoichiometrically, half-maximal inhibition of water movement should occur at much lower drug concentrations. Further, these results

could be interpreted as indicating that the effect of these agents is due to a stoichiometric binding to cellular tubulin, and not to microtubule disruption.

However, half-maximal depolymerization of previously existing microtubules in the epithelial cells by colchicine occurs at a colchicine concentration which is identical to that which causes half-maximal inhibition of hormone-induced water movement [13, 17]. These results support the conclusion that the effect of colchicine is due to the depolymerization of previously existing microtubules. It is possible that the concentration of colchicine attained inside the cells is somewhat lower than the extracellular concentration. However, a more likely explanation is that the tubulin concentration in the epithelial cells is much greater than 1.8×10^{-6} M (i.e., the effective colchicine concentration) and perhaps is in the range of 5×10^{-5} M (5.5 mg/ml). In this situation, half-maximal inhibition of microtubule polymerization would be occurring with 3.5% of the total cellular tubulin complexed with the drug.

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