Van Hell, H., Matthijsen, R., and Overbeek, G. A. (1964), *Acta Endocrinol. 47*, 409.
Vincent, J. P., Lazdunski, M., and DeLeage, M. (1970), *Eur. J. Biochem. 12*, 250.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Williams, J., and Lowe, J. M. (1971), *Biochem. J.121*, 203.

Thyroid Tubulin: Purification and Properties†

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ABSTRACT: Colchicine-binding protein was purified from beef thyroid glands with a procedure which allows isolation of this protein from a source low in tubulin. The protein dimer has a molecular weight of 114,000. Sodium dodecyl sulfate–acrylamide gel electrophoresis indicates that the subunits have molecular weights of 55,000 ± 2000. Two monomeric subunits were identified by urea–acrylamide gel electrophoresis. Amino acid analysis shows differences from other tubulins in lysine, aspartic, and glutamic acids contents. The protein binds ~0.85 mol of colchicine and contains 0.8 mol of alkali-labile phosphate/mol of dimer. Combination of colchicine with the tubulin dimer induces marked fluorescence at 430 nm. This constitutes a convenient method for studying colchicine binding. On the basis of molecular weight, subunits, sedimentation constant, amino acid composition, and colchicine-binding activity, the protein is very similar to the subunit protein of cilia, flagella, and brain tubules.

Ample evidence has accumulated which indicates that tubulin, or the colchicine-binding protein of many tissues, is the subunit of the microtubule (Olmsted and Borisy, 1973). At the same time it has become apparent that colchicine interferes with the secretion of hormones and other cellular products and granule movement (Lacy *et al.*, 1968; Malawista, 1965; Williams and Wolff, 1970; Wolff and Williams, 1973). In the thyroid, colchicine and other agents that disaggregate microtubules inhibit TSH[1] and dibutyryl cyclic AMP-stimulated colloid droplet formation and ^{131}I release from the thyroid gland (Williams and Wolff, 1970, 1972; Nève *et al.*, 1972). The effect was specific in the sense that the hormone biosynthetic pathway and glucose-metabolizing reactions were undisturbed by colchicine concentrations that totally abolished secretion (Wolff and Williams, 1973). By correlating the antisecretory potency of various drugs with antimitotic potency and their ability to displace [^{3}H]colchicine from the binding sites, it was established that this inhibition of secretion produced by colchicine is most probably due to an effect on microtubules and is connected with binding of this drug to a specific site in the subunit tubulin. The mechanics of the participation of microtubules in the secretory process are difficult to specify. Unlike the microtubules of cilia, basal bodies, mitotic spindles, axons and even the pancreatic β cells, the microtubules of the thyroid gland show no obvious organization into structures that might participate in mechanical events (Wolff and Williams, 1973). The question arose whether or not the "unorganized" tubules from thyroid tissue consist of tubulin that is identical with the well-characterized tubulins of brain or cilia. We therefore purified and characterized this protein from beef thyroid glands.

Materials and Methods

Materials. $(NH_4)_2SO_4$ was Mann Enzyme grade. GTP (Grade II-S) was obtained from Sigma. Tritiated colchicine (ring C, methoxy-^{3}H), a product of New England Nuclear Corp., had a specific activity of 2.5 Ci/mmol. All other chemicals used were of reagent grade. The standard buffer used in most experiments contained 0.01 M sodium phosphate–0.01 M $MgCl_2$ (pH 6.5) and 0.1 mM GTP and is called PMG. When 0.25 M sucrose was present in the same buffer for homogenization it is called SPMG.

Methods. Protein was determined by the method of Lowry *et al.* (1951). Crystalline bovine albumin was used as standard. For colchicine-binding assays the DEAE-cellulose filter disk method of Weisenberg *et al.* (1968) was used as modified by Williams and Wolff (1972).

Disc gel electrophoresis was carried out in three different systems. (1) Sodium dodecyl sulfate–polyacrylamide gels were run as described by Weber and Osborn (1969) with phosphate buffer (pH 7.1). The gel contained 5% acrylamide and 0.2% bisacrylamide. The samples were run on a Büchler electrophoresis apparatus at a current of 4 mA/tube at 23° until the tracking dye reached the end of the gel. (2) Urea–polyacrylamide gels were made according to Everhart (1971) with the inclusion of 8 M urea in 5% polyacrylamide gels. Electrophoresis was performed at 400 V in 5 mM Tris–glycine buffer (pH 8.4) at 23°. (3) Discontinuous buffer systems at different gel concentrations (5, 5.5, 6, and 6.5%) were run according to Davis (1964), with 0.05 M Tris–glycine (pH 8.7) in the upper chamber, and 0.06 M Tris–0.05 M HCl (pH 7.4) in the lower and 2.5 mA/tube at 23°. In all gels, polymerization was initiated by the addition of Temed (*N,N,N′,N′*-tetramethylethylenediamine) and ammonium persulfate. The gels were stained with 0.02% Coomassie Brilliant Blue in 50% methanol and 9% acetic acid and destained in 5% methanol and 7.5% acetic acid; they were scanned with a Gilford spectrophotometer equipped with a linear transport attachment.

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[1] Abbreviations used are: TSH, thyroid-stimulating hormone; dibutyryl cyclic AMP, N^6,2-*O*-dibutyryladenosine 3′:5′-monophosphate; DEAE, diethylaminoethyl.

For amino acid analysis protein samples were hydrolyzed in 6 M HCl in sealed, evacuated tubes. The samples were maintained at 110° for 24, 48, and 72 hr and amino acid composition was determined with a Beckman Model 120C instrument by the methods of Moore and Stein (1963) or Spackman *et al.* (1958); norleucine was used as internal standard.

Total phosphate analysis of purified protein samples was carried out following the procedure of Ames and Dubin (1960). The alkali-labile phosphate content of purified protein was determined as described by Reddington *et al.* (1973).

Fluorescence spectra were measured on a Perkin-Elmer fluorescence spectrophotometer, MPF-3, equipped with a Hitachi recorder.

Purification of the Colchicine-Binding Protein. Bovine thyroid glands were kept on ice, trimmed free of fat and connective tissue, and chopped with a razor blade. Chopped material was then prehomogenized in SPMG solution (15 ml/gland) in a Servall Omnimixer for 30 sec at full speed and then homogenized with Polytron homogenizer for 15 sec at one-third full speed. The homogenate was centrifuged at 100,000*g* for 60 min, and the pellet was discarded. The supernatant solution was brought to 30 mM Ca^{2+} with $CaCl_2$. After 10 min the solution was made 20 mM with respect to phosphate by addition of 0.2 M phosphate buffer (pH 6.5). The mixture was then adjusted to pH 7.4 by the dropwise addition of 0.2 M NaOH. All these operations were performed at 0° with precooled solutions. The suspension was allowed to stand for 30 min and was centrifuged at 20,000*g* for 10 min at 4°. The supernatant solution was discarded and the pellet was dissolved in PMG buffer containing 10 mM EGTA. The solution was brought to approximately 35% saturation at 0° by gradual addition over a 15-min period of 209 g of solid $(NH_4)_2SO_4$/l. of solution. The suspension was allowed to stand for 30 min and was centrifuged at 10,000*g* for 20 min. To the resulting supernatant solution 94 g of $(NH_4)_2SO_4$/l. was added to bring saturation to 50%. The suspension was allowed to stand for 30 min and was then centrifuged at 10,000*g* for 20 min. The supernatant was discarded and the pellet was redissolved in 105 ml of PMG buffer/l. of original homogenate. This solution was dialyzed three times at 4° and 1 hr against about 1.3 l. of PMG buffer solution.

The dialyzed solution was then applied to a DEAE-cellulose (Whatman DE-52) column (2.4 × 12 cm) which had been previously equilibrated with PMG buffer (pH 7.0). The column was eluted with PMG buffer and then with 0.2 M NaCl in the same buffer until the bulk of the protein had been eluted. The colchicine-binding protein was then eluted with a linear (0.2–0.8 M) NaCl gradient. The peak of microtubule protein appeared at approximately 0.54 M NaCl.

Before being applied to a G-200 Sephadex column, the DEAE-purified protein was concentrated to $^1/_{30}$th of its original volume by ultrafiltration at 30 psi of N_2 with a UM-10 Amicon membrane. The concentrated protein was then purified on a Sephadex G-200 column (1.4 × 50 cm) in PMG + 0.1 M KCl + 0.1 mM dithiothreitol (pH 7.0).

Results

Because of the high concentration of thyroglobulin in beef thyroids (~80% of the soluble protein) and the low concentration of tubulin, additional means of separating tubulin were sought. Since Wilson *et al.* (1970) had shown that Ca^{2+} ion, although not specific, tended to precipitate tubulin, the precipitation of thyroid proteins by Ca^{2+} was investigated in some detail. The addition of Ca^{2+} to a 100,000*g* supernatant extract of thyroid gland caused precipitation of the protein in a concentration- and pH-dependent manner. The yield could be markedly increased by the subsequent addition of phosphate. Precipitation of colchicine-binding protein started above Ca^{2+} concentrations of 10 mM (Figure 1A). About 50–55% of the total colchicine-binding protein precipitated at Ca^{2+} concentrations above 25 mM at pH 7.4. Complete precipitation took 35–45 min at 0°.

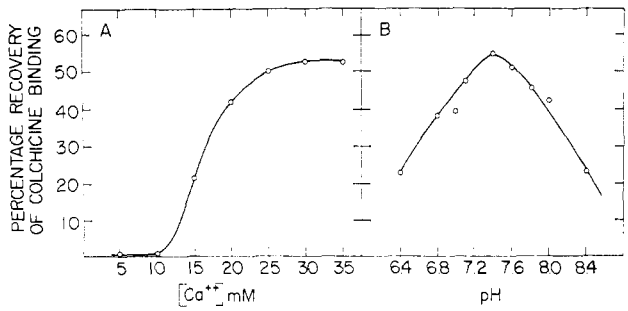

FIGURE 1: Calcium phosphate precipitation of colchicine-binding protein. (A) Increasing concentrations of $CaCl_2$ were added to 10 ml of 100,000*g* supernatant solution of beef thyroid gland in SPMG buffer (pH 6.5). After 10 min, 0.2 M phosphate buffer (pH 6.5) was added to make the phosphate concentration 20 mM (the pH of the solution was adjusted to 7.4 at 0° with 0.2 M NaOH). The samples were allowed to stand for 30 min and were centrifuged at 20,000*g* for 10 min. The precipitates were dissolved in PMG buffer containing 10 mM EGTA. (B) 10 ml of 100,000*g* supernatant was brought to 30 mM with respect to $CaCl_2$ and 20 mM with respect to phosphate at different pH values. They were allowed to stand for 30 min and were then centrifuged at 20,000*g* for 10 min. The precipitates were dissolved in PMG buffer containing 10 mM EGTA. Colchicine-binding activity was determined for 1 hr at 37° with 2×10^{-6} M [^{3}H]colchicine.

Maximum precipitation occurred near pH 7.4 (Figure 1B) at 30 mM calcium and decreased markedly as the pH was lowered. As seen in Figure 1A,B, only 55% of the colchicine-binding protein originally present in the 100,000*g* supernatant extract was recovered in the redissolved precipitate. At higher pH the yield decreased and the specific activity of the precipitated protein decreased. The calcium-precipitated material slowly redissolved when resuspended in 10 mM EGTA in PMG buffer at pH 7.0. Resuspension required large volumes of buffer to avoid turbidity. The purification of protein was approximately 7-fold at this stage with respect to the 100,000*g* supernatant. The $(NH_4)_2SO_4$ step led to a 2-fold additional purification. The ammonium sulfate precipitated material was dissolved in a minimum amount of PMG and was further purified on a DEAE-cellulose column (Figure 2). The bulk of the protein was eluted with 0.2 M NaCl. Tubulin was eluted with gradient of 0.2–0.8 M NaCl in PMG buffer. Very little colchicine-binding activity was eluted from the column until the salt concentration reached approximately 0.5 M.

The colchicine binding fractions were pooled and concentrated by ultrafiltration. Gel filtration of this material on Sephadex G-200 shows the presence of two peaks (Figure 3). The remaining thyroglobulin eluted in the void volume and the second component was identified as colchicine-binding protein. A summary of yields and purification of thyroid tubulin for the various steps is given in Table I.

Disc gel electrophoresis of the Sephadex-purified material was performed in a discontinuous system. A single band only was detected even in overloaded gels. By use of different gel concentrations (Davis, 1964) the molecular weight of this material was calculated to be 114,000. This is in good agreement with the molecular weight determined for tubulin from

TABLE I: Purification of Colchicine-Binding Protein from Beef Thyroid Gland.

Step	Vol (ml)	Act./ml[a] (cpm/ml)	Total Act. (cpm × 10^{-4})	Total Protein (mg)	Sp Act. (cpm/mg)	Yield (%)
1. Homogenate	740	1900	141	178 × 10^3	7.9	100
2. Soluble supernatant	400	3510	141	40 × 10^3	35.1	99.9
3. Calcium precipitate	315	2370	74	3030	246.0	53.0
4. $(NH_4)_2SO_4$ (35–50%)	80	7650	61	1220	500.0	43.5
5. DEAE-cellulose column	91	3240	29	12.0	24,500	21.0
6. Sephadex G-200	26	5300	14	8.0	17,500	10.0

[a] Incubated for 15 min at 37° with 2.0 × 10^{-6} M [^{3}H]colchicine. For step 6 activity was measured on eluates of a 0.9 × 35 cm column of Sephadex G-200 to increase elution rate and decrease loss of binding.

other tissues and is also consistent with a sedimentation coefficient of 5.8–6.0 S previously reported for the thyroid protein in sucrose density gradients (Williams and Wolff, 1970). The subunit structure of thyroid tubulin was investigated in sodium dodecyl sulfate and in urea gels. In sodium dodecyl sulfate, the microtubule protein ran as a single component (Figure 4a) with an apparent molecular weight of 55,000 ± 2000 as determined from a plot of the log of the molecular weight *vs.* mobility (Weber and Osborn, 1969). It was found that no reduction is necessary to obtain subunits in the sodium dodecyl sulfate system, suggesting that subunits are not linked by disulfide bonds.

Reduced and carboxymethylated (Crestfield *et al.*, 1963) thyroid microtubule protein shows two closely spaced bands in 8 M urea–acrylamide gels in Tris–glycine buffer (Figure 4b). Electrophoretically distinguishable subunits have previously been observed in microtubule protein isolated from chick embryo brain (Bryan and Wilson, 1971), pig brain (Feit *et al.*, 1971; Olmsted *et al.*, 1971), and rat brain (Eipper, 1972). As in these studies, the thyroid subunits will be referred to as α and β, where β is the more rapidly (anodally) migrating of the two components. The bands do not stain with equal intensity; an $\alpha:\beta$ ratio of 1.20 was obtained when scanned after staining with Coomassie Brilliant Blue.

Colchicine Binding. Because Sephadex chromatography entailed considerable losses in colchicine binding, it was necessary to determine the stoichiometry of the protein–colchicine complex on the DEAE-purified material. When this was incubated at 37° with 5 × 10^{-6} M [^{3}H]colchicine until completely equilibrated (Figure 5), 0.52 mol of colchicine was bound per 114,000 g of protein. The figure shows that the decay rate of the colchicine-binding site was negligible, hence corrections to zero time as employed by Wilson (1970) were not required. Higher concentrations of colchicine did not increase the amount of colchicine bound per dimer. To obtain the true colchicine:tubulin stoichiometry, the above value of 0.52 mol of colchicine/114,000 g was corrected for the residual thyroglobulin contamination (as determined by Sephadex G-200). By this method, we calculate that 0.85 mol of colchicine is bound per 114,000 g of thyroid tubulin. This value agrees with those obtained for brain tubulin (Weisenberg *et al.*, 1968; Bryan, 1972).

Phosphate Analysis. For phosphate estimation protein samples were freed of buffer phosphate and GTP by the method of Eipper (1972). The purified microtubule protein from thyroid tissue contained 0.81 mol of phosphate/dimer, in agreement with the findings on brain protein (Eipper, 1972). These values are greater than those of Reddington and Lagnado (1973). Although not identified as phosphoserine, 0.69 mol of phosphate/mol of dimer could be liberated from the thyroid protein by treatment with 1.0 M NaOH at 37° for 18 hr. Whether or not this phosphate was incorporated by any of the known protein kinases remains to be determined. It is clear, however, from the *in vitro* polymerization experiments (Weisenberg, 1972; Borisy and Olmsted, 1972; Shelanski and Shelanski, 1973) that the phosphorylation reaction *per se* is

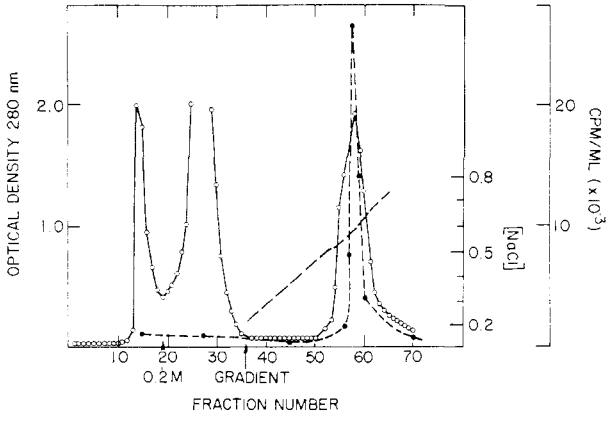

FIGURE 2: Chromatography of 35–50% $(NH_4)_2SO_4$ fraction on DEAE-cellulose. 10 ml of solution containing 14 mg of protein/ml was applied to 2.4 × 12 cm column in PMG buffer (pH 7.0). The column was first eluted with PMG buffer, then 0.2 M NaCl, and finally with a 0.2–0.8 M NaCl gradient (- - - - -). The NaCl concentration was calculated from conductivity measurements. Colchicine-binding (●- -●) activity was determined with 2.0 × 10^{-6} M [^{3}H]colchicine for 15 min at 37°.

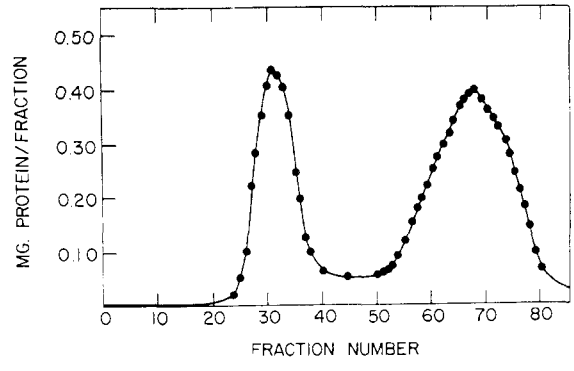

FIGURE 3: Gel filtration of the DEAE-purified protein on G-200 Sephadex. The column (1.4 × 50 cm) was equilibrated and eluted with 0.1 M KCl containing 0.1 mM dithiothreitol in PMG buffer (pH 7.0). A 2.5-ml sample was applied and 0.75-ml fractions were collected.

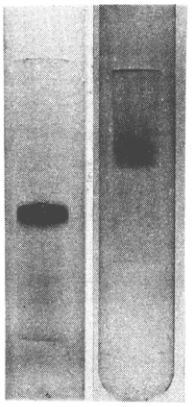

FIGURE 4: Disc gel electrophoresis of purified thyroid tubulin. (A, left) Sodium dodecyl sulfate–acrylamide gel electrophoresis of purified colchicine binding protein. Conditions as described in the text. (B, right) Urea–acrylamide gel electrophoresis of reduced and carboxymethylated subunits. Conditions as described in the text.

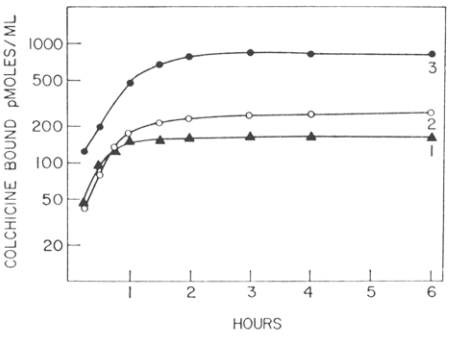

FIGURE 5: [^{3}H]Colchicine binding to tubulin at different stages of purification as a function of time. 5 ml of each protein solution in PMG buffer (pH 7.0) was incubated with 5×10^{-6} M [^{3}H]colchicine; at different intervals 0.5 ml were taken out from each solution and bound colchicine was measured by the filter disk method of Weisenberg *et al.* (1968). 1 = crude 100,000*g* supernatant, 2 = DEAE-purified protein, and 3 = (35–50%) $(NH_4)_2SO_4$ fraction.

not necessary for tubulin aggregation or disaggregation. Whether or not the presence of covalently bound phosphate in tubulin is a precondition for its ability to aggregate must still be determined.

Amino Acid Composition. The amino acid composition (mean of two separate tubulin preparations) is shown in II, together with data obtained previously from different sources. Duplicate or triplicate analysis of the same protein and from two different lots of protein agreed within ±2%, with a like error in the amino acid standards. The serine and threonine contents were corrected for hydrolytic loss. It is evident that significant differences from other tubulins were found in lysine, aspartic, and glutamic acid. These cannot be ascribed to thyroglobulin contamination since the arginine content of that protein is high, but is low in tubulin, etc. Furthermore, gel filtration removed the remaining traces of thyroglobulin (Figure 4a). Finally iodine analysis of the purified preparation yielded an iodine content of <0.02 μg/ml of protein. Even with an assumed iodine content of only 0.1% for thyroglobulin, this would amount to no more than 2% contamination. These data also indicate that tubulin is not significantly iodinated in the thyroid gland. With the same analytical conditions used for thyroid tubulin, brain tubulin from the rat, prepared according to Weisenberg *et al.* (1968), yielded an amino acid analysis identical with that reported by these authors.

It thus seems highly unlikely that contamination can account for the amino acid differences and we are forced to conclude that the composition of thyroid tubulin has distinct amino acid sequences and is apparently more polar than other tubulins. The interesting question why tubulins from the same species but from different organs (see Table II, column d) exhibit differences in amino acid composition remains to be clarified.

Fluorescence Spectrum. Fluorescence of the tubulin–colchicine complex was first noted by Arai and Okuyama (1973). Free colchicine has no fluorescence in H_2O when excited at 350 nm, but the protein–colchicine complex has high fluorescence with a maximum at 430 nm. Figure 6A shows the effect of protein concentration on fluorescence at 430 nm. A linear relationship is obtained between protein concentration and fluorescence intensity at 430 nm. The pH optimum of fluorescence is found to be 7.0 in phosphate

TABLE II: Amino Acid Compositions of Various Tubulins (mol %).

Amino Acids	From Thyroid	*a*	*b*	*c*	*d*	*e*
Lys	8.2	3.9	6.2	4.6	4.3	3.9
His	2.3	2.4	2.3	2.7	2.4	2.2
Arg	3.2	4.4	5.3	4.9	5.1	4.7
Asp	13.3	10.3	9.8	10.6	11.0	11.1
Thr	6.8	6.3	6.1	5.4	5.4	6.8
Ser	5.3	5.9	5.2	6.8	4.4	7.3
Glu	17.5	14.2	13.2	13.0	13.0	13.5
Pro	5.2	5.2	5.0	5.3	4.5	4.7
Gly	7.2	7.9	7.9	8.4	8.4	9.1
Ala	6.2	7.5	7.8	7.3	7.5	7.6
$^1/_2$-Cys	1.5	1.9	1.6	2.3	1.5	2.4
Val	5.1	6.3	6.5	6.7	7.3	7.9
Met	2.3	2.3	2.2	2.8	3.2	1.6
Ile	3.9	4.5	4.9	4.5	4.9	4.4
Leu	6.9	7.7	8.5	7.3	7.5	7.2
Tyr	3.4	3.9	2.9	3.4	3.7	3.6
Phe	4.0	4.8	3.9	4.1	4.0	4.8
Trp		0.7			0.8	

[a] From pig brain (Weisenberg *et al.*, 1968). [b] From mouse neuroblastoma (Olmsted *et al.*, 1970). [c] From squid axone (Davison and Huneeus, 1970). [d] From bovine brain (Arai and Okuyama, 1973). [e] From chick embryo brain (Bryan and Wilson, 1971).

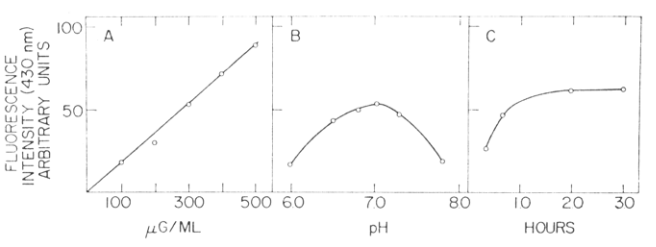

FIGURE 6: Fluorescence properties of the tubulin–colchicine complex. Fluorescence intensity was measured at 430 nm, with the activating wavelength set at 350 nm. (A) Tubes containing increasing quantities of protein were incubated with 1.23×10^{-5} M colchicine (pH 7.0) for 1 hr at 37°. (B) Tubes containing 300 μg of protein at different pH were incubated with 1.23×10^{-5} M colchicine for 1 hr at 37°. (C) Tubes containing 300 μg of protein at pH 7.0 were incubated with 1.23×10^{-5} M colchicine for different times at 37°.

buffer (Figure 6B). This pH optimum is characteristic of the tubulin–colchicine complex of brain and thyroid tubulins measured by binding of [^{3}H]colchicine (Wilson, 1970; Williams and Wolff, 1973). Figure 6C shows the binding of colchicine as a funtion of time as studied by fluorescence. The binding is slow at 37° and takes several hours for equilibration. This result agrees with those of Williams and Wolff (1972) in the case of intact thyroid tissue, Wilson (1970) in chick embryo brain, and Owellen *et al.* (1972) in porcine brain tubulin.

Discussion

With the addition of several purification steps (calcium phosphate precipitation and Sephadex chromatography), it was possible to adapt the purification procedure (no. 1) of Weisenberg *et al.* (1968) to thyroid tissue. A protein was obtained that was pure by disc gel electrophoretic criteria under several running conditions. On the basis of molecular weight of dimer and subunits, amino acid composition, fluorescence of the protein–colchicine complex, [^{3}H]colchicine binding, and phosphate content, the protein is very similar to the protein obtained from other sources (Shelanski and Taylor, 1968; Weisenberg *et al.*, 1968). The molecular weight of all tubulins so far purified ranges from 100,000 to 120,000 as analyzed by a variety of methods. The thyroid protein has a sedimentation coefficient of 5.8–6.0 S in sucrose gradients (Williams and Wolff, 1970) and a molecular weight of 114,000 as determined by disc gel electrophoresis, and thus falls into the above weight range. Similarly, the subunit molecular weight of 55,000 ± 2000 is in agreement with values reported by others (Bryan and Wilson, 1971; Feit *et al.*, 1971). Like the brain and cilia systems, the subunits can be distinguished in urea gels as a more anodally migrating species (β subunit) and a second, slower subunit (α subunit). Recent findings of Eipper (1972) indicated that the native microtubule protein of rat brain is phosphorylated specifically on the β subunit which may contribute to its higher mobility. Whether or not these subunits also exhibit small differences in molecular weights cannot be ascertained from the present data.

In addition to these chemical similarities to other tubulins, many functional similarities of thyroid tubulin exist (Wolff and Williams, 1973): (1) slow equilibration with colchicine and slow onset of the inhibitory effect; (2) pH optimum for colchicine binding near 6.7–7.0; (3) high lability of the binding site and protection by Mg^{2+}, GTP, and colchicine; (4) similar affinity of colchicine analogs and congeners for the binding site. It seems reasonable to conclude, therefore, that the biochemical properties that pertain to microtubules in a variety of tissues may be applicable to the secretory system of the thyroid gland. A satisfactory description of the role of tubulin or microtubules in the secretory process cannot, however, be drawn from other tissues because of major differences in microtubule distribution and because an understanding of microtubule function in such tissues is equally vague at present.

The present method has permitted determination of the colchicine:tubulin dimer stoichiometry as approaching 1:1. This is in agreement with reports for several other tubulins (Weisenberg *et al.*, 1968; Bryan, 1972; Owellen *et al.*, 1972).

The binding of colchicine to tubulin can be assessed by two general methods: binding of [^{3}H]colchicine (Weisenberg *et al.*, 1968) and fluorescence enhancement. It has been found that free colchicine exhibits no fluorescence when activated at its absorption maximum (λ_{max} 350 nm), but when combined with tubulin it shows fluorescence in the visible region, characteristic of the tropolone ring of colchicine (λ_{max} 430 nm). Therefore, separation of free and bound colchicine is unnecessary. Moreover, at 350 nm there is no absorption from the protein, and the fluorescence measured at 430 nm reflects the colchicine complex alone and is thus specifically useful in studying the binding interaction. So far, the fluorescent and [^{3}H]colchicine assessment of binding are in excellent agreement.

Acknowledgments

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References

Ames, B. N., and Dubin, D. T. (1960), *J. Biol. Chem. 235*, 769.
Arai, T., and Okuyama, T. (1973), *Seikagaku 45*, 19.
Borisy, G. G., and Olmsted, J. B. (1972), *Science 177*, 1196.
Bryan, J. (1972), *Biochemistry 11*, 2611.
Bryan, J., and Wilson, L. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1762.
Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Davison, P. E., and Huneeus, F. C. (1970), *J. Mol. Biol. 52*, 429.
Eipper, B. A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2283.
Everhart, L. P. (1971), *J. Mol. Biol. 61*, 745.
Feit, H., Slusarek, L., and Shelanski, M. L. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2028.
Lacy, P. E., Howell, S. L., Young, D. A., and Fink, C. J. (1968), *Nature (London) 219*, 1177.
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.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.
Nève, P., Ketelbant-Balasse, P., Willems, C., and Dumont, J. E. (1972), *Exp. Cell Res. 74*, 227.
Olmsted, J. B., and Borisy, G. G. (1973), *Annu. Rev. Biochem. 42*, 507.
Olmsted, J. B., Carlson, K., Klebe, R., Ruddle, F., and Rosenbaum, J. L. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 129.
Olmsted, J. B., Witman, G. B., Carlson, K., and Rosenbaum, J. L. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2273.
Owellen, R. J., Owens, A. H., and Donigian, D. W. (1972), *Biochem. Biophys. Res. Commun. 97*, 685.
Reddington, M., and Lagnado, J. R. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 30*, 188.
Reddington, M., Rodnight, R., and Williams, M. (1973), *Biochem. J. 132*, 475.
Shelanski, M. L., and Shelanski, V. B. (1973), *Proc. 4th Int. Congr. Endocrinol., Washington, D. C., 1972.*
Shelanski, M. L., and Taylor, E. W. (1968), *J. Cell Biol. 38*, 304.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Weisenberg, R. C. (1972), *Science 177*, 1104.
Weisenberg, R. C., Borisy, G. G., and Taylor, E. W. (1968), *Biochemistry 7*, 4466.

Williams, J. A., and Wolff, J. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1901.
Williams, J. A., and Wolff, J. (1972), *J. Cell Biol. 54*, 157.
Wilson, L. (1970), *Biochemistry 9*, 4999.
Wilson, L., Bryan, J., Ruby, A., and Mazia, D. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 807.
Wolff, J., and Williams, J. A. (1973), *Recent Progr. Horm. Res. 29*, 229.

Molecular Characterization of Proteins in Detergent Solutions†

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ABSTRACT: The molecular weight and Stokes radius of a protein in a detergent solution can be determined unambiguously by measurement of sedimentation equilibrium and sedimentation velocity, with analysis of the data by equations appropriate for multicomponent systems. The procedure can be simplified without much loss of accuracy by use of a calculated buoyant density factor, and detergent partial specific volumes required for this calculation have been measured. The Stokes radius can be measured independently by gel exclusion chromatography and the molecular weight can then be determined by use of sedimentation velocity alone, which is of considerable advantage since gel chromatography and sedimentation velocity do not require as high a degree of protein purity as sedimentation equilibrium. A simplified procedure for calibration of gel chromatography columns is described, and the significance and interpretation of the Stokes radius are discussed. The most important procedures have been verified by experimental measurements using the AI apoprotein of human high density serum lipoprotein in several detergent solutions.

This paper describes procedures suitable for the determination of the molecular weight and Stokes radius of proteins in detergent solutions. The procedures are intended primarily for the study of proteins that are only sparingly soluble in simple aqueous media, such as membrane proteins, and for detergent solutions in which these proteins are not grossly denatured. They are of course equally applicable to strong denaturing detergents, such as sodium dodecyl sulfate (SDS),[1] and can be used, for example, for the determination of the molecular weight of glycopolypeptides, which behave anomalously in SDS polyacrylamide gel electrophoresis.

With application to membrane proteins in mind, allowance is made for possible inclusion of bound lipids with detergent-extracted proteins, and methods for obtaining the desired information for incompletely purified proteins are considered. All of the procedures are adapted to minimize the amount of protein required: considerably less than 1 mg is needed for most of the measurements, and the same sample can often be used for more than one measurement. The emphasis on economy of material dictates a modest goal as regards accuracy, and we consider an accuracy of $\pm 5\%$ in molecular weight and Stokes radius as satisfactory.

Materials and Methods

Materials. SDS used for density measurements and for chromatography was synthesized by the method of Emerson and Holtzer (1967). "Lauryl sodium sulfate" (Schwarz/Mann, Orangeburg, N. Y.) was used for sedimentation studies, and it has been our experience that this product is generally similar to pure synthetic SDS. However, the same manufacturer provides a product named "sodium lauryl sulfate," which we have found to be of inferior quality and unsuitable for use in molecular characterization.

Tetradecyltrimethylammonium chloride (TTAC) was purchased from Lachat Chemicals Inc., Chicago Heights, Ill., and twice recrystallized from methanol–ether; sodium deoxycholate and Triton X-100 were the same preparations used previously (Makino *et al.*, 1973); Triton N-101 was provided by Rohm and Haas Co.; other nonionic detergents were purchased from Sigma Chemical Corp., St. Louis, Mo. The commercial detergents were dried in a vacuum desiccator over P_2O_5, and used without further purification.

The preparation of the AI polypeptide chain of human high density lipoprotein is described elsewhere (Reynolds and Simon, 1974).

Methods. Density measurements were carried out using an Anton Paar precision densimeter, Model DMA 02C, at 25.00°, maintained constant to within ±0.006°. Density measurements with this instrument have a precision of 2×10^{-6} g/cm^3, and this is essential if measurements are to be made at sufficiently low concentrations to permit an accurate evaluation of $\bar{v}$ for detergents both below and above the cmc.

Sedimentation equilibrium and velocity measurements were made using a Beckman Model E analytical ultracentrifuge, equipped with photoelectric scanner. A wavelength of 280 nm was employed. Use of the photoelectric scanner makes it possible to use very low protein concentrations: initial protein concentrations for the experiments of Table II were about 0.2 mg/ml. Sedimentation equilibrium measurements are routinely made at several different rotor speeds, and the results are discarded if self-consistent data are not obtained.

Gel exclusion chromatography measurements were made at

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; TTAC, tetradecyltrimethylammonium chloride; cmc critical micelle concentration; DOC, sodium deoxycholate.