Perry, L. J., & Wetzel, R. (1984) *Science (Washington, D.C.) 226*, 555–557.
Perry, L. J., Heyneker, H. L., & Wetzel, R. (1985) *Gene 38*, 259–264.
Remington, S. J., Anderson, W. F., Owen, J., Ten Eyck, L. F., Grainger, C. T., & Matthews, B. W. (1978) *J. Mol. Biol. 118*, 81–98.
Richardson, J. (1981) *Adv. Protein Chem. 34*, 167–339.
Saxena, V. P., & Wetlaufer, D. B. (1970) *Biochemistry 9*, 5015–5022.
Sekiguchi, M., & Iida, S. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 2315–2320.
Sogami, M., Petersen, H. A., & Foster, J. F. (1969) *Biochemistry 8*, 49–58.
Thornton, J. M. (1981) *J. Mol. Biol. 151*, 261–287.
Tsugita, A. (1971) in *The Enzymes* (Boyer, P. D., Ed.) pp 334–411, Academic Press, New York.
Tsugita, A., Inouye, M., Terzaghi, E., & Streisinger, G. (1968) *J. Biol. Chem. 243*, 391–397.
Villafranca, J. E., Howell, E. E., Voet, D. H., Strobel, M. S., Ogden, R. C., Abelsen, J. N., & Kraut, J. (1983) *Science (Washington, D.C.) 222*, 782–788.
Wallace, R. B., Johnson, M. J., Hirose, T., Miyake, T., Kawashima, E. H., & Itakura, K. (1981) *Nucleic Acids Res. 9*, 879–893.
Wang, A., Lu, S. D., & Mark, D. F. (1984) *Science (Washington, D.C.) 224*, 1431–1433.
Wetzel, R. (1986) in *Protein Engineering* (Inouye, M., & Sarma, R., Eds.) Academic Press, New York (in press).
Wetzel, R., & Goeddel, D. V. (1983) in *The Peptides—Analysis, Synthesis, Biology* (Gross, E., & Meienhofer, J., Eds.) Vol. 5, pp 1–64, Academic Press, New York.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468–500.

Bis(8-anilinonaphthalene-1-sulfonate) as a Probe for Tubulin Decay†

A. R. S. Prasad, Richard F. Luduena, and Paul M. Horowitz*
Department of Biochemistry, The University of Texas Health Science Center, San Antonio, Texas 78284
Received July 25, 1985

ABSTRACT: The fluorescent apolar probe bis(8-anilinonaphthalene-1-sulfonate) (Bis-ANS) has been used to detect structural correlates of the well-known but poorly understood decay of tubulin function, by which tubulin loses its ability to polymerize and bind drugs in a complex, time-dependent way. The present results indicate that the decay of tubulin is accompanied by the appearance of hydrophobic areas, which bind a total of six Bis-ANS molecules with a dissociation constant of 19 μM. This binding seems to be a result of localized structural changes that are taking place in the tubulin molecule and can be used as a probe for these changes. In particular, circular dichroism measurements revealed no significant changes in the average secondary structure of the protein during the time required for complete binding of the Bis-ANS molecules. Preincubation of tubulin with the antimitotic drugs colchicine, podophyllotoxin, and vinblastine slows the rate of appearance of the hydrophobic region. Vinblastine has the maximal effect followed by colchicine and podophyllotoxin. In contrast, preincubation with maytansine has no effect. In addition, lowering the temperature decreases the rate of appearance of this region. These results correlate with the effect of drugs on the alkylation of tubulin sulfhydryl groups by iodoacetamide [Luduena, R. F., & Roach, M. C. (1981) *Biochemistry 20*, 4444–4450] and with the ability of inhibitors of microtubule assembly to permit the polymerization of tubulin into nonmicrotubule structures.

Horowitz et al. (1984) have recently shown that the hydrophobic probe bis(8-anilinonaphthalene-1-sulfonate) (Bis-ANS)[1] binds to tubulin, inhibiting the formation of microtubules. They have shown this to be due to the strong binding at a single site on tubulin with a K_d of 2 μM. The monomer of Bis-ANS, 1,8-ANS, also binds to tubulin at a single site but with no effect on microtubule assembly and a K_d of 25 μM (Horowitz et al., 1984; Bhattacharyya & Wolff, 1975). Most of these studies were done at a fixed time and temperature. It is well-known that tubulin shows time- and temperature-dependent structural changes and is known to have multiple binding sites for several drugs (Luduena, 1979). Bis-ANS has been shown to bind at more than one site in several proteins (Daniel & Weber, 1966; Anderson, 1971; Takashi et al., 1977; Bohnert et al., 1982). The experiments described herein demonstrate the presence of more than one site for the binding of Bis-ANS to tubulin and that additional binding sites appear as a function of time and temperature. Maximum binding of Bis-ANS is attained in 2 h at 37 °C. Colchicine, podophyllotoxin, and vinblastine prevent changes that are taking place in the tubulin molecule. These results may indicate that the decay of tubulin involves the appearance of hydrophobic areas on the surface of the molecule and that low temperature and certain drugs can stabilize the tubulin molecule and slow down decay.

† Supported by National Institutes of Health Grants GM23476 and CA26376 to R.F.L. and GM25177 to P.M.H. and by Robert A. Welch Foundation Grants AQ723 to P.M.H. and AQ726 to R.F.L.
* Address correspondence to this author.

[1] Abbreviations: MAPs, microtubule-associated proteins; 1,8-ANS, 8-anilinonaphthalene-1-sulfonate; Bis-ANS, bis(8-anilinonaphthalene-1-sulfonate); EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-0739$01.50/0

Materials and Methods

Materials

Bis-ANS was obtained from Molecular Probes, Inc. (Junction City, OR). Colchicine and GTP were from Sigma Chemical Co. (St. Louis, MO). Podophyllotoxin was from Aldrich Chemical Co. (Milwaukee, WI). Vinblastine sulfate was a kind gift from the Eli Lilly Co. (Indianapolis, IN). Maytansine (NSC 153858) was the kind gift of Dr. Matthew Suffness of the Developmental Therapeutics Program, Chemotherapy, National Cancer Institute. All other reagents were of analytical grade.

Methods

Tubulin Preparation. Cow brains were obtained locally from the Roegelein Co. Microtubule protein was prepared from the cerebrums of cow brains and resolved into tubulin by chromatography on phosphocellulose (Whatman P11). (Fellous et al., 1977). The integrity of the purified tubulin was assayed by the ability of a sample of it to polymerize into microtubules when reconstituted with τ (0.15 mg/mL) and MAP 2 (0.3 mg/mL). Turbidity measurements at 350 nm were used to assay microtubule formation (Gaskin et al., 1974). Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

All studies were done using a buffer consisting of 100 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.4, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 1 mM GTP.

Heat-stable MAPs were purified from brain microtubules by the method of Fellous et al. (1977).

The temperature dependence of the rate of decay of tubulin's ability to form microtubules was tested by incubating samples of phosphocellulose-purified tubulin (6.31 mg/mL) in buffer either at 35 or at 0 °C in the presence or absence of 2 mM reduced glutathione. At various times up to 250 min, aliquots were removed and mixed with buffer and heat-stable MAPs at 1.0 and 0.4 mg/mL, respectively, in a total volume of 500 μL. These diluted samples were placed in a Gilford 250 spectrophotometer and incubated at 37 °C. Polymerization was monitored by turbidimetry at 350 nm. The polymerization rate, expressed as A_{350}/min, was measured at the point of inflection of each curve.

Fluorescence Measurements. Fluorescence measurements were made with a Perkin-Elmer MPF-44A spectrofluorometer used in the ratio mode and equipped with a temperature-controlled cell holder. Slit widths on the excitation and emission monochromators were 6 nm. To measure the fluorescence intensity of bound Bis-ANS, the samples were exicted at 385 nm, and emission was measured at 490 nm in a cuvette with a 1-cm path length. The fluorescence intensity was monitored by using a strip chart recorder in order to follow the time course.

Determination of the binding constant for Bis-ANS was made by using a double titration method based on a derivation of Wang & Edelman (1971), as described by Horowitz & Criscimagna (1985). Briefly, Bis-ANS at several fixed concentrations was mixed with tubulin at various concentrations, and the fluorescence intensities were measured. In a second titration, tubulin at several fixed concentrations was mixed with Bis-ANS at various concentrations, and the fluorescence intensities were measured. The common intersection points on plots of the inverse of the fluorescence intensities vs. the inverse of the concentration of the varied components gave the values of K_d/n and K_d, respectively, where n is the number of Bis-ANS binding sites and K_d is the dissociation constant for the Bis-ANS/site complex. All measurements were made at 37 °C and at the end of 1.5, 3, and 24 h. Excitation was at 385 nm, and emission was at 490 nm.

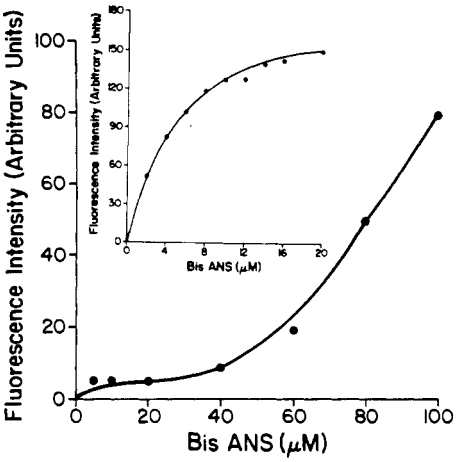

FIGURE 1: Binding of Bis-ANS to tubulin as a function of Bis-ANS concentration. Phosphocellulose-purified tubulin at 0.2 mg/mL in buffer was mixed with 0–100 μM Bis-ANS, and the fluorescence intensity was measured after 1 min at 37 °C. The inset in the figure shows the curve obtained with 0–20 μM Bis-ANS. Excitation was at 385 nm, and emission was at 490 nm.

The quantum yield for Bis-ANS bound to tubulin at primary and secondary sites was measured by comparing the area under the fluorescence emission spectra for 2 and 10 μM Bis-ANS in 1-propanol with the areas under emission spectra obtained with a sample of Bis-ANS bound to tubulin as described by Horowitz et al. (1984). For the primary site, the spectra were measured immediately after addition of Bis-ANS. For the secondary sites, the spectra were measured after incubation of tubulin with Bis-ANS for 2 h at 37 °C.

Circular Dichroism. Measurements were made with a Jasco J-500 C recording spectropolarimeter. Spectra were scanned from 260 to 205 nm at 37 °C using a cell with a path length of 0.01 cm. The protein concentration was 0.224 mg/mL.

Results and Discussion

Figure 1 illustrates the effect of adding increasing concentrations of Bis-ANS to 2 μM tubulin at pH 6.4 at 37 °C. Binding of Bis-ANS resulted in an increase in fluorescence according to a pattern which was apparently hyperbolic up to approximately 20 μM Bis-ANS (Figure 1, inset) and which became sigmoidal at higher concentrations. This result suggested interactions at more than one site and that these sites differed in their affinity for Bis-ANS. A Scatchard analysis of the data which generated the hyperbolic portion of the curve shown in Figure 1 (inset) showed that there was a single high-affinity binding site, presumably identical with the single site found by Horowitz et al. (1984) which apparently mediates Bis-ANS inhibition of microtubule assembly (Horowitz et al., 1984). Figure 1 also demonstrates the presence of lower affinity binding sites.

A determination of the binding parameters was done according to the procedure described under Methods. The results are shown in Figure 2. Analysis of the binding data gave a binding constant of 19 μM and n = 6. Binding behavior was measured as a function of time up to 24 h, and there was very little variation in the binding parameters. For example, at 24 h, the K_d was 17 μM and n was 7. The data at the end of 1.5 h are shown in Figure 2. Thus, tubulin apparently has two types of Bis-ANS binding sites: a primary site responsible for the inhibition of microtubule assembly with a K_d of 2 μM and

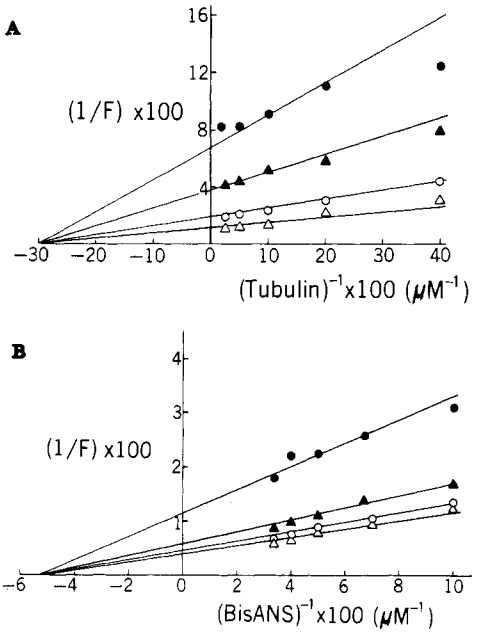

FIGURE 2: Double-reciprocal plot for the binding of Bis-ANS to tubulin. (A) Plot of the inverse of the fluorescence intensity as a function of the inverse of the protein concentration at various fixed concentrations of Bis-ANS. The tubulin concentration was varied from 2.5 to 30 μM. Each line corresponds to a fixed concentration of Bis-ANS: (●) 1 μM; (▲) 2 μM; (○) 5 μM; (△) 10 μM. (B) Plot of the inverse of the fluorescence intensity as a function of the inverse of the Bis-ANS concentration at various fixed concentrations of tubulin. The Bis-ANS concentration was varied from 10 to 30 μM at fixed concentrations of 2 (●), 4 (▲), 6 (○), and 8 (△) μM tubulin. All measurements were done at 37 °C. Excitation was at 385 nm, and emission was at 490 nm.

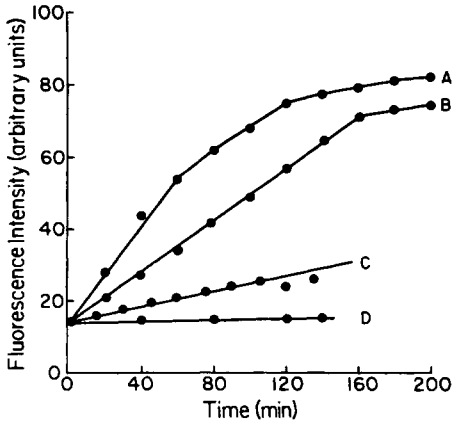

FIGURE 3: Effect of time and temperature on the binding of Bis-ANS to tubulin. (A) Phosphocellulose-purified tubulin at 0.2 mg/mL was mixed with 10 μM Bis-ANS and incubated at 37 °C. Fluorescence intensity was measured at specified intervals of time as described under Methods. (B) Phosphocellulose-purified tubulin at 0.2 mg/mL was incubated at 37 °C. At specified intervals, an aliquot was withdrawn and mixed with 10 μM Bis-ANS, and the fluorescence intensity was measured. (C) As in (B) except the temperature of incubation was at 0 °C. (D) As in (A) except the temperature of incubation was at 0 °C. Excitation was at 385 nm, and emission was at 490 nm.

six secondary sites with a K_d of 19 μM.

To compare the primary and secondary Bis-ANS binding sites, we examined the effect of incubation time and temperature on the binding of Bis-ANS to tubulin. The results of such an experiment are shown in Figure 3. This experiment was done in two ways. In the first experiment, tubulin (2 μM) was mixed with Bis-ANS (10 μM) and incubated for a specified length of time at 37 °C. In the second experiment, tubulin was preincubated alone at 37 °C. At various intervals,

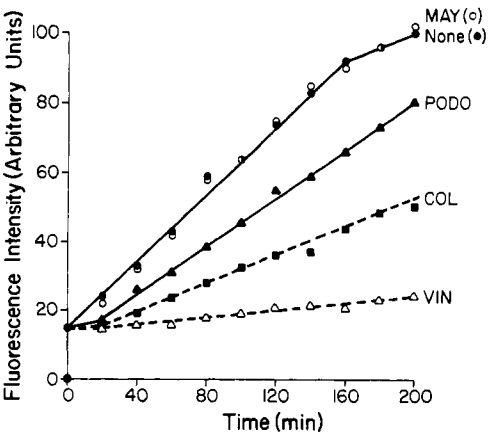

FIGURE 4: Effect of drugs on the binding of Bis-ANS to tubulin as a function of time and temperature. Phosphocellulose-purified tubulin at 0.2 mg/mL was preincubated with the indicated drugs at a concentration of 20 μM at 37 °C. At different time intervals, an aliquot was withdrawn and mixed with 10 μM Bis-ANS, and fluorescence intensity was measured. Excitation was at 385 nm, and emission was at 490 nm.

aliquots were withdrawn and made 10 μM in Bis-ANS, and the fluorescence intensity was measured immediately. As shown in Figure 3, the fluorescence intensity reached a maximum in about 2 h. The maximum fluorescence intensity obtained was approximately the same in both experiments and differed only in the rate of increase of fluorescence. As indicated earlier, this increase in fluorescence intensity was due to increased binding of Bis-ANS to tubulin. We observed that circular dichroism spectra of tubulin were essentially identical after incubation for various times up to 1 h at 37 °C, suggesting only minor changes in the secondary structure of protein. The presence of GTP might have stabilized the protein against decay which has been observed in the previous circular dichroism studies (Ventilla et al., 1972). Bhattacharya & Wolff (1975) did not observe any change in the ultraviolet spectrum even after 3 h at 37 °C. Hence, the increased binding of Bis-ANS to tubulin must be due to localized structural changes that are taking place in the protein. We found that the quantum yield for Bis-ANS bound to the primary site was 0.45, in agreement with the earlier report (Horowitz et al., 1984), while the quantum yield of Bis-ANS bound to the weak sites was 0.38. In addition, the emission maximum for bound Bis-ANS was the same at both sites.

It is well-known that the presence of various antimitotic drugs stabilizes the tubulin molecule against decay (Wilson, 1970; Ventilla et al., 1972; Tan & Lagnado, 1975; Lee et al., 1975; Bhattacharyya & Wolff, 1976; David-Pfeuty et al., 1977). Hence, we have looked at the effect of preincubation of tubulin with 20 μM concentrations of various drugs on the binding of Bis-ANS to tubulin. Figure 4 shows the time course of Bis-ANS binding obtained when tubulin (2 μM) was preincubated with various drugs at 37 °C. The effect of all but one of the drugs was to bring about a decreased binding of Bis-ANS to tubulin. Vinblastine had the maximal effect followed by colchicine and podophyllotoxin. Maytansine had no apparent effect. It must be noted that the initial jump in the fluorescence associated with the binding of Bis-ANS that prevents microtubule formation is the same with all the drugs. This suggests that in contrast to binding at the secondary sites, the presence of drugs does not affect the binding of Bis-ANS to the primary site. For the secondary sites, the variations in the effectiveness of various drugs must be due to the differences in their binding characteristics. It is interesting that the effects of the three drugs on the binding of Bis-ANS to tubulin are

exactly paralleled by their effects on alkylation of tubulin by iodo[^{14}C]acetamide (Luduena & Roach, 1981a,b); 20 μM concentrations of vinblastine, colchicine, and podophyllotoxin inhibit alkylation by 51%, 35%, and 16%, respectively (Luduena & Roach, 1981a), while maytansine, even at 100 μM, has no effect (Luduena & Roach, 1981b). At a concentration of 20 μM, the same one used in Figure 4, the inhibitory effects of the first three drugs reach plateau values. Although it is tempting to conclude that the effects of the drugs reflect the magnitude of each drug's effect on the tubulin molecule to which it binds, we cannot discount the possibility that the different effects are due in part to different binding constants or binding rates. There also appears to be a correlation between the effect of a drug on the binding of Bis-ANS to tubulin and its ability to induce tubulin to polymerize into nonmicrotubule structures. Vinblastine, which readily causes tubulin to polymerize into spirals (Bryan, 1972; Erickson, 1975), had the strongest inhibition of Bis-ANS binding; colchicine, which under certain conditions can induce tubulin to aggregate (Andrew et al., 1983), had an intermediate effect, while maytansine, which appears to inhibit all forms of tubulin polymerization (Bhattacharyya & Wolff, 1977), had no effect.

In order to see if the rate of appearance of the low-affinity Bis-ANS binding sites correlated with any other parameter of tubulin decay, we examined the time and temperature dependence of the ability of tubulin to polymerize to microtubules. This effect was measured in both the presence and absence of reduced glutathione, in order to correct for any effects on decay due to possible oxidation of assembly-critical sulfhydryls. As can be seen, the glutathione had little effect, but the time and temperature did. At 0 °C, the ability to polymerize was lost very slowly with a $t_{1/2}$ estimated to range from 211 to 330 min. At 35 °C, however, the polymerization ability decayed with a $t_{1/2}$ of 53–58 min, which is within the range for the half-times of appearance of the low-affinity Bis-ANS binding sites estimated from Figure 3 to range from 50 min (curve A) to 85 min (curve B). This $t_{1/2}$ is somewhat greater than the value of 25 min obtained by Wiche et al. (1977) for the $t_{1/2}$ of decay of polymerization of glial cell tubulin at 37 °C. The difference between our value and that of Wiche et al. (1977) could be due to a different cell source, different MAPs, different isolation procedures, and different buffer.

The results of the present work indicate that the decay of tubulin's ability to form microtubules is associated with the exposure of hydrophobic areas on the tubulin molecule. Thus, if conditions do not favor assembly, and in the absence of specific ligands, the tubulin–tubulin interaction sites lose their specificity but increase their hydrophobicity, leading to irreversible slow aggregation. The presence of certain ligands will arrest this process to an extent related to their ability to induce tubulin to polymerize into nonmicrotubule structures. Thus, it is tempting to speculate that the fundamental difference between polymerization and aggregation is related to the orderly control of hydrophobic exposure.

ACKNOWLEDGMENTS

We gratefully acknowledge the skillful technical assistance of Phyllis T. Smith in preparing microtubule protein.

Registry No. PODO, 518-28-5; COL, 64-86-8; VIN, 865-21-4; Bis-ANS, 25551-04-6.

REFERENCES

Anderson, S. R. (1971) *Biochemistry 10*, 4162–4168.
Andreu, J. M., Wagenknecht, T., & Timasheff, S. N. (1983) *Biochemistry 22*, 1556–1566.
Bhattacharyya, B., & Wolff, J. (1975) *Arch. Biochem. Biophys. 167*, 204–209.
Bhattacharyya, B., & Wolff, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2375–2378.
Bhattacharyya, B., & Wolff, J. (1977) *FEBS Lett. 75*, 159–162.
Bohnert, J. L., Malenick, D. A., Anderson, S. R., Teller, D., & Fischer, E. H. (1982) *Biochemistry 21*, 5570–5576.
Bryan, J. (1972) *Biochemistry 11*, 2611–2616.
Daniel, E., & Weber, G. (1966) *Biochemistry 5*, 1893–1900.
David-Pfeuty, T., Erickson, H. P., & Pantaloni, D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5372–5376.
Erickson, H. P. (1975) *Ann. N.Y. Acad. Sci. 253*, 51–52.
Fellous, A., Francon, J., Lennon, A. M., & Nunez, J. (1977) *Eur. J. Biochem. 78*, 167–174.
Gaskin, F., Cantor, C. R., & Shelanski, M. L. (1974) *J. Mol. Biol. 89*, 737–758.
Horowitz, P. M., & Criscimagna, N. L. (1985) *Biochemistry 24*, 2587–2593.
Horowitz, P., Prasad, V., & Luduena, R. F. (1984) *J. Biol. Chem. 259*, 14647–14650.
Lee, J. C., Harrison, D., & Timasheff, S. N. (1975) *J. Biol. Chem. 250*, 9276–9282.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Luduena, R. F. (1979) in *Microtubules* (Roberts, K., & Hyams, J., Eds.) pp 65–116, Academic Press, London.
Luduena, R. F., & Roach, M. C. (1981a) *Biochemistry 20*, 4444–4450.
Luduena, R. F., & Roach, M. C. (1981b) *Arch. Biochem. Biophys. 210*, 498–504.
Takashi, R., Tonomura, Y., & Morales, M. F. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2334–2338.
Ventilla, M. L., Cantor, C. R., & Shelanski, M. (1972) *Biochemistry 11*, 1554–1561.
Wang, J. L., & Edelman, G. M. (1971) *J. Biol. Chem. 246*, 1185–1191.
Wiche, G., Honig, L. S., & Cole, R. D. (1977) *Nature (London) 269*, 435–436.
Wilson, L. (1970) *Biochemistry 9*, 4999–5007.